Calcium and Actin Coordinate Egg Activation and the Metaphase-Anaphase Transition in *Drosophila* Oocytes

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Preface

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or is being concurrently submitted for a degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the Biology Degree committee.

Abstract

Calcium and Actin Coordinate Egg Activation and the Metaphase-Anaphase Transition in *Drosophila* Oocytes - Benjamin William Wood.

Egg activation is the process through which the mature oocyte is prepared for embryogenesis, consisting of key cellular changes including, but not limited to: i) Physical and chemical changes to the oocyte's outer covering; ii) The release of meiotic arrest, enabling the formation of a haploid oocyte; iii) Large-scale changes in the translational landscape; iv) Cytoskeletal rearrangements for regulating downstream events of egg activation and supporting further growth of the zygote. Preceding these events in *Drosophila* is a single calcium transient observed in the form of a polar wave upon hydration and swelling of the oocyte. A model outlining how the initiation of such a wave is regulated and how it then enacts these downstream effects is not yet fully outlined.

The original working model supported by previous research suggested that mechanical triggers during ovulation initiate calcium entry. How this mechanical stimulus is transduced into a calcium wave and what this then means for the source of calcium has not been explored. I first provide an in depth analysis of calcium entry dynamics at egg activation, exploring the significance of seemingly less regulated calcium events. I then investigate the source of calcium and identify an ion channel that is required for calcium entry. By utilising a combination of pharmacological and genetic analysis, I highlight the requirement of Trpm for calcium entry at egg activation. Taken together I demonstrate that calcium enters the oocyte from the peri-vitelline space (between the oolemma and vitelline membrane) through Trpm in the form of a wave.

I next ask what mechanisms regulate calcium entry through Trpm channels. I provide detailed visualisation of the actin population in the mature oocyte both before and after egg activation, focusing on the cortical actin. I reveal a clear relationship between calcium entry and the cortical actin- In particular, reduction of the cortical actin density or level of cross-linking promotes the entry of calcium. In the mature oocyte I show that the Arp2/3 machinery and tandem-actin binding domain nucleators are required for maintenance of the cortical actin. I further demonstrate that actin-binding proteins (ABPs) play a role in regulating calcium entry, likely via mediation of cross-linking and density of the cortical actin. This data therefore supports a model in which polar waves are in part a result of a reduced cortical actin density at the poles of the oocyte.

I then explore a specific downstream event of egg activation; the resumption of meiosis. I demonstrate the presence of a novel population of actin within the *Drosophila* oocyte that forms a spindle-like structure. Given this is such a recent discovery, key questions are highlighted: 1) What is the role of this spindle-like apparatus?; 2) How does the spindle-like actin regulate meiosis?; 3) Is there conservation of this population? I first highlight the requirement of Formins in production of this population. I further reveal that the spindle-like actin is required for regulating the formation and morphology of the spindle and therefore the accurate movement of chromosomes during meiosis, demonstrating remarkable conservation with mammals. Finally, I bring together concepts of calcium and actin signalling explored in the previous chapters, revealing an essential interplay between the two at the metaphase-arrested spindle.

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Abbreviations:

1-MA	1-Methyladenine
2-APB	2-aminoethoxydiphenyl borate
AI	Anaphase 1
AB	Activation Buffer
ABP	Actin Binding Protein
Act5C	Actin5C
APC	Anaphase Promoting Complex
Arp-2/3	Actin Related Protein 2/3
ATP	Adenosine Triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BAPTA-AM	BAPTA-tetrakis(acetoxymethyl ester)
cADPR	Cyclic ADP ribose
CaM	Calmodulin
CaMKII	Calmodulin dependent protein Kinase II
CaN	Calcineurin
Capu	Cappuccino
cGMP	Cyclic guanosine monophosphate
CICR	Calcium Induced Calcium Release
CRISPR	Clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
EGTA	Egtazic Acid
ER	Endoplasmic Reticulum
ERM -	Ezrin Radixin Moesin
F-actin	Filamentous Actin
FKBP	FK506 Binding Protein
Fmn-2	Formin-2
FRAP	Fluorescence Recovery after photobleaching
G-actin	Globular Actin
GDP	Guanosine Diphosphate
GECI	Genetically encoded calcium indicator
GFP	Green Fluorescent Protein
GPCR	G protein Coupled Receptor
grk	Gurken mRNA
GTP	Guanosine Triphosphate
IP3	Inositol 1,4,5-triposphate
KIND	Kinase non catalytic C-lobe domain
M1	Metaphase 1
MAPK	Mitogen activated protein kinase
Min	Minute
MPF	Metaphase promoting factor
NAADP	Nicotinic acid adenine dinucleotide phosphate
NEBD	Nuclear envelope breakdown
nos	Nanos mRNA
osk	Oscar mRNA
Osm	Osmoles
P bodies	Processing bodies
PBS	Phosphate-buffered saline
PBST	PBS + Triton X100
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PMCA	Plasma membrane calcium ATPase
RVD	Regulatory volume decrease

RYR	Ryanodine Receptor
SERCA	Sarcoendoplasmic reticulum calcium ATPase
SD	Standard deviation
SFK	Src-family protein tyrosine kinase
SH2	Src homology 2
STIM	Stromal-interacting molecule
TRP	Transient receptor potential
UAS	Upstream activating sequence
UtrCH	Utrophin Calponin-Homology domain
WASP	Wiskott-Aldrich syndrome Protein
WAVE	WASP family verprolin-homologous protein

Chapter 1

Introduction

1.1 An Introduction to Egg Activation

1.1.1 Egg Activation: A fundamental, developmental transition

The development of a multicellular organism is cyclical in nature; from single germ cells, complex three dimensional organs and systems of tissues arise, including further production of germ cells. Often, in the case of oocyte producing organisms, these germ cells are specified very early in development and eggs can be stored for years. In other oocyte producing organisms, these cells can arise when environmental conditions are most suitable for the production of offspring. In any case, germ cells develop and are stored within multicellular adults until sexual reproduction results in the fusion of germ cells and one of the first and most fundamental cellular transitions occurs: from egg to embryo.

Egg activation is the process through which the totipotent mature oocyte is prepared for embryogenesis. Often accompanied by fertilisation, egg activation consists of a plethora of key cellular changes (reviewed in Horner and Wolfner., 2008):

i) Physical and chemical changes to the oocytes outer covering to prevent polyspermy and protect the developing embryo
ii) The release of meiotic arrest, enabling the formation of a haploid nucleus inside the oocyte, whereupon fusion can occur with the sperm-derived pro-nucleus
iii) Large-scale changes in the translational landscape as some maternal mRNAs undergo degradation whilst others are translated
iv) Cytoskeletal rearrangements supporting further growth of the zygote and intimate control of key egg activation events

Unifying all of these events is an increase of intracellular of calcium in the oocyte (Stricker., 1999). The first calcium transient to be observed was visualised using aequorin in medaka fish eggs, demonstrating a propagating wave of calcium (Gilkey et al., 1978). However, a multitude of calcium transients have since been observed in the form of waves, oscillations and cortical flashes in ascidian and mammalian eggs (Stricker., 1999; Deguchi et al., 1996). These calcium transients are responsible for coordinating the above downstream events of egg activation, and despite conservation of the increase in intracellular calcium, the triggers for such are more diverse than perhaps expected.

1.1.2 Calcium Signalling in the Oocyte

Calcium signalling in the oocyte is the universal and fundamental element that coordinates downstream events in all oocytes upon egg activation. Whether this calcium is sourced from the ER or enters via plasma membrane calcium channels, the end result is an intracellular calcium transient, in the form of a flash, wave or oscillations, which triggers downstream processes such as the resumption of meiosis, changes in the translational profile of the oocyte, and global reorganisation of the actin cytoskeleton (Horner and Wolfner., 2008).

The calcium wave was first discovered in the Medaka fish egg, *Oryzias latipes*, as aequorin-injected eggs demonstrated an "explosive" wave of elevated calcium during fertilisation (Gilkey et al., 1978). The wave begins at the animal pole, the site of sperm entry, and traverses the egg to the vegetal pole as a "band". Addition of the ionophore A23187, however, triggered calcium to enter from multiple points across the oocyte, fusing upon meeting to form a spreading wave with multiple origins (Gilkey et al., 1978). In the case of a singular wave triggered by fertilisation, the wave travelled at a speed of approximately 10um/s; this speed is conserved amongst a particular category of waves referred to as "fast" calcium waves (Jaffe., 2008). Calcium waves can thus be categorised according to speed, and there exists approximately four different categories; ultraslow, slow, fast and ultrafast. Mechanisms of calcium wave propagation differ greatly between these categories:

i) In the case of ultrafast waves, these are propagated in the form of an action potential, as the calcium signal is electrically propagated between relay points. Such waves are less well understood, however, have been observed in dendritic cells of the rat embryonic brain and in calcium spike through mature jellyfish (Mackie and Meech., 1985).

ii) Fast waves, likely the most common, are triggered by calcium diffusion between relay points, such as in calcium induced calcium release mechanisms (eg. Calcium release from the ER during fertilisation). Examples of fast waves are extensive, with examples existing in fucoid algae to mouse models, from oocytes to epithelia. Most of these fast waves exist in a range of 10-30 um/s at 20 degrees Celsius, and propagated by the same fast wave mechanism; ie. a reaction-diffusion mechanisms in which the velocity of diffusion is proportional to the product of the speed if calcium-induced calcium release and the diffusion constant of free calcium (V & kD) (reviewed in Jaffe., 2002).

iii) Slow waves likely rely on mechanical propagation of the calcium signal from relay point to relay point; entry of calcium through stretch-sensitive channels resulting in intracellular calcium rises which induce contraction of acto-myosin filaments in order to open adjacent channels, for example. Examples of interest include the fertilisation wave in maize eggs and waves through post-fertilisation barnacle eggs, demonstrating that oocytes may also have the capability to produce slow waves that are not based on an IP_3/ER dependent calcium diffusion mechanism (reviewed in Jaffe., 2008).

iv) Ultraslow waves, travelling at a speed of approximately 0.2-2 um/s mainly refer to developmental waves which are far slower than slow waves; they are accompanied by local contraction and traverse excitable media. These waves have not been visualised, but rather have been inferred, as these morphogenetic processes require acto-myosin contraction, and thus in local increases in calcium; thus for instance, processes such as the inversion of the developing Volvox embryo must require a wave of calcium activity to coordinate a a wave of contraction that controls this developmental event (Viamontes et al. 1979). Similarly, the ultraslow waves of furrowing exhibited by Drosophila eye discs must similarly rely on an ultraslow wave of calcium (Jaffe., 1999). Therefore, like slow waves, the category not ultraslow waves may rely on mechanical propagation and are important for pattern generation (Jaffe., 2008).

The most utilised wave in the oocyte, therefore, is that of the fast wave, which occurs during ER mediated calcium release, however, there may be further roles played by the slow wave, as slow waves have also been demonstrated to occur in oocytes (detailed review of calcium waves by Jaffe., 1998; 2002; 2008).

1.1.3 Calcium Signal Transduction

Though calcium waves have been well characterised in many systems, how a wave of calcium is transducer into a physiological output is a broad question that remains to be fully explored. Calcium as a signalling molecule likely evolved in part due to its unique coordination chemistry; its large ionic radius, high polarisability (the metal electron cloud can be easily distorted by external electrical forces), low hydration energy (removal of shell of water molecules) and its variable bond length. All of these properties make calcium an ideal ion for interaction with coordinating ligands, far more so than magnesium, which was also abundant in the primordial seawater where life began. Additionally, the early evolution of phosphates as the energy currency of life dictated that calcium must be present at the mid to low nM range in the cytosol, as calcium phosphate salts have poor solubility, unlike magnesium phosphate salts, and thus having mM concentrations of calcium in the cytosol would severely impede on bioenergetics. Therefore, cells evolved to extrude calcium and maintain a low nM range of calcium ions within the cytosol, both in order to maintain bioenergetic processes, and also to ensure that calcium can act as a signalling molecule through coordinated increases in intracellular calcium (reviewed in Carafoli and Krebs., 2016). As such, calcium is able to act as an excellent second messenger in the cell, meaning that it not only is an ideal molecule for interaction with a variety of molecules, but it can be stored and released in great amounts, enabling amplification of much smaller signals.

Transduction of calcium flux into cellular responses occurs through the action of calcium binding proteins; these proteins contain domains such as the EF hand, the C2-domain and the annexin Ca2+ binding fold (reviewed in Carafoli and Krebs., 2016). Binding of calcium to these domains/ motifs triggers 3D changes in the shape of proteins, thus altering their dynamics, protein binding

capabilities and ultimately their function. The most conserved calcium binding domain is that of the EF hand, which has evolved even in prokaryotes (reviewed in Dominguez et al., 2015). The crystal structure of the EF hand was first characterised in parvalbumin, a calcium-binding protein found in fast-contracting muscles (Kretsinger and Nockolds., 1983). The EF-hand is a motif that consists of two alpha-helices, the “E”, a loop that binds calcium, and the “F” (Kawasaki and Kretsinger., 1994). Most EF-hands exist in pairs, forming a calcium binding lobe; however, the primary sequences of EF-hands are evolutionarily diverse and can be classified into approximately 156 subfamilies, with most having g likely evolved from a single, ancestral EF lobe (reviewed in Kawasaki and Kretsinger., 2017). EF-hand containing proteins may act as calcium sensors, such as Calmodulin, Troponin C and STIM, binding to calcium and transducing this signal, or they may act as pure calcium-buffering proteins maintaining calcium homeostasis, such as Parvalbumin and Calbindin (Carafoli and Krebs., 2016).

Calmodulin is often referred to as the primary intracellular receptor of calcium as it is essential to trigger a variety of downstream processes and is central to the highly conserved Ca2+/CaMK cascade (Marcelo et al., 2016). The Ca2+/CaM complex itself is involved in the regulation of over 120 enzymes and proteins, including ion channels, phosphatases, kinases and a multitude of others, highlighting its central importance in cell biology (Chin and Means., 2000). Ubiquitously expressed amongst eukaroytes, the structure of CaM has been solved for both calcium bound and free forms (reviewed in Marshall et al., 2015). The structure is comprised of two pairs of EF-hands in a ‘dumbell’ structure: The EF-hands exist in pairs in the terminal C- and N- lobes, connected by a long, flexible helix (Kuboniwa et al., 1995; Zhang et al., 1995; Vogel and Zhang., 1995). Each lobe is able to bind two calcium ions cooperatively, with the C-terminal lobe having a higher affinity for calcium. Each lobe undergoes a conformational change upon calcium binding, exposing hydrophobic protein binding sites which dock to target hydrophobic anchor residues (Zhang et al., 1995). Calmodulin is thus able to bind to a wide variety of downstream targets, including kinases (eg. CaMKI, CaMKII), phosphates (eg. Calcineurin), membrane receptors, channels and pumps (eg. IP_3R, PMCA, RyR). CaM can act as both a positive and negative regular, for instance inhibiting IP_3R and up-regulating PMCA (Plasma Membrane Calcium ATPase) activity (reviewed in Marshall et al., 2015). These interactions are fundamental in cell biology, and have vital roles during egg activation, such as in the resumption of meiosis. Exogenous expression of CaMKII (*CamK2g*) was able to result in activation of *CaMK2g-/-* eggs in mouse oocytes after parthenogenetic activation. Additionally, expression of a constitutively active CaMKII forced egg activation in mouse oocytes. Calcium and calmodulin regulated proteins are essential for egg activation, as CaMKII activation results in the degradation of EMI2, enabling APC/C activation, cyclin B degradation and exit from meiotic arrest.

Through the action of downstream proteins, such as CaM and CaMKII to name a few, calcium transients during egg activation are thus able to control and regulate a variety of processes, such as the release from meiotic arrest and global changes in the landscape of the cytoskeletal architecture.

1.1.4 Initiating Egg Activation: Fertilisation

Summary

Sperm-mediated calcium release and egg activation is the method employed by most mammals and marine invertebrates, and relies heavily on three different signalling pathways: 1) Inositol trisphosphate ($IP_{3)}$ mediated release from the Endoplasmic Reticulum (ER) via IP_3 Receptors (IP_3R); 2) Cyclic ADP-Ribose (cADPR) mediated release from the ER via Ryanodine Receptors (RyR); 3) Nicotinic acid adenine dinucleotide phosphate (NAADP) mediated release via NAADP receptors on the plasma membrane and acidic organelles. Mammals rely heavily on the IP_3 pathway, induced through the introduction of a soluble sperm factor, phospholipase C zeta (PLCζ), and potentially NAADP signalling. Marine invertebrates more commonly employ phospholipase C gamma (PLCγ), which is activated by a sperm receptor, in addition to the cADPR and NAADP pathways which interact with the PLC pathway through production of diacyl glycerol (DAG) by the PLC.

Fertilisation: Inositol Phosphate Signalling

In mammals and most marine invertebrates, the trigger of calcium release, and thus egg activation, is sperm entry. Interestingly, there existed three potential models for how sperm-oocyte fusion could result in intracellular calcium release (reviewed in Parrington et al., 2007). These were: 1) The sperm acts as a 'calcium-conduit', enabling flow of external calcium through the sperm and into the oocyte; 2) The sperm introduces a 'soluble factor' into the oocyte cytoplasm which instigates calcium release from internal stores; 3) The 'membrane-receptor' model in which sperm ligand interaction with a receptor on the oocyte triggers calcium release from internal stores.

The two most widely accepted models of sperm-initiated egg activation are the 'soluble-factor' and 'membrane-receptor' models. Numerous studies provided counter-evidence against the 'conduit-model', as injection of calcium ions into sea urchin, ascidian and mammalian eggs failed to initiate a calcium transient, suggesting that calcium induced calcium release (CICR) alone is not sufficient for a sustained calcium signal in the oocyte (Whitaker and Swann., 1993; Swann and Ozil., 1994). The 'membrane-receptor' model was historically the favoured model as it fits with very common somatic signalling mechanisms, however, for certain species, favour shifted upon the discovery of a soluble factor, named Phospholipase C Zeta (PLCζ) (Saunders at al., 2002; Swann et al., 2006).

First evidence that a soluble factor may be the trigger came from the study of sea urchin eggs, in which injection of a sperm extract was capable of inducing egg activation (Dale et al., 1985). This

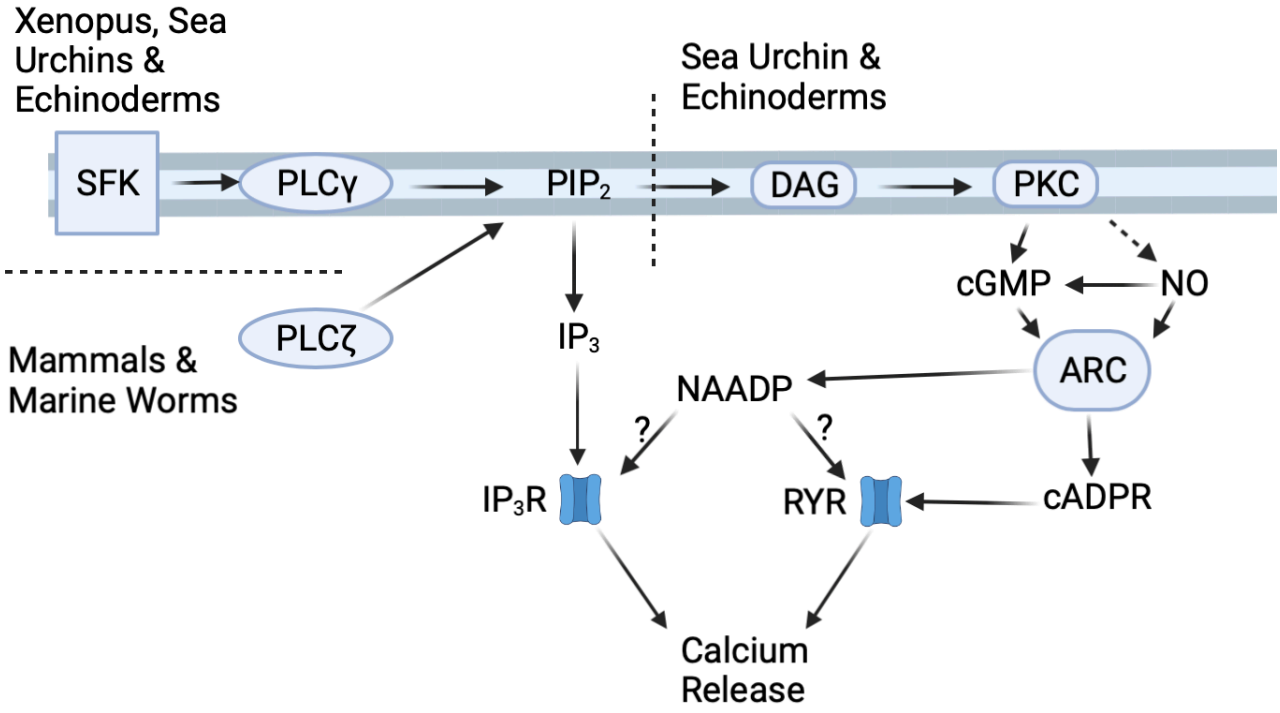

Figure 1.1: Calcium signalling pathways at fertilisation

Xenopus, sea urchins and echinoderms rely on Src Family Kinases (SFKs) and phospholipase C gamma (PLCγ) in a membrane-receptor model. Mammals and marine worms have been shown to rely on phospholipase C zeta (PLCζ) in a soluble factor model. Sea urchins and echinoderms have also been shown to rely on production of NAADP and cADPR in order to generate a calcium response. Created with BioRender.

was later repeated in a number of eggs from mammals and marine worms, demonstrating a similar induction of egg activation (Swann., 1990; Stricker, 1999; Kyozuka et al., 1998; Parrington 2001). Characterisation of this sperm-factor at a molecular level revealed initially that it was a sperm-specific PLC, and through analysis of cDNA databases, later identified the factor as PLCζ (Jones et al., 1998, 2000; Parrington et al., 1999, 2002; Saunders et al., 2002). PLCζ was confirmed to be the mouse soluble sperm-factor, and injection of the RNA and protein triggered calcium oscillations in the mouse oocyte (Saunders et al., 2002; Kouchi et al., 2005). Additionally, immuno-depletion and RNAi mediated depletion of PLCζ in mouse sperm reduced the ability of the sperm to induce calcium oscillations (Saunders et al., 2002; Knott et al., 2005).

Recent studies have verified such a model in that PLCζ knockouts were generated in mice using CRISPR/Cas9 technology (Hachem et al., 2017; Nozawa et al., 2018). They showed the males with PLCζ knockouts can still produce offspring, but with significantly reduced litter numbers (~25%). Sperm lacking the functional PLCζ were unable to initiate a calcium release when microinjected into the mouse oocytes with intracytoplasmic sperm injection (ICSI). However, IVF with such sperm was able to produce calcium oscillations, despite drastic differences in the

pattern of such oscillations. With the PLCζ knockout sperm, IVF was only able to produce 3-4 oscillations in total, whereas normal fertilisation was capable of producing 3-4 oscillations per hour over a period of 4 hours (Nozawa et al., 2018; Satouh and Ikawa., 2018). In these cases there was also reported a much higher rate of activation failure and polyspermy. It does however suggest that there may be alternative mechanisms at play to successfully trigger egg activation; with offspring still being produced and calcium oscillations still present, it is an attractive hypothesis.

Therefore, the current model for PLCζ mediated calcium entry is as follows: The entry of PLCζ into the oocyte enables it to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG) (Figure 1.1). IP_3 can then bind to its receptor (IP_3R1) on the endoplasmic reticulum membrane resulting in the release of calcium ions and an overall rise in cytosolic calcium (Wakai et al., 2011). PLCζ is especially effective due to its extreme sensitivity to calcium (Kouchi et al., 2004; Nomikos et al., 2005; Kouchi et al., 2005). At resting levels of calcium, at approximately 100uM, PLCζ is at about half capacity, and there is a steep incline in activity as the intracellular calcium concentration increases, suggesting a positive feedback loop exists, which is an important feature in the generation of calcium oscillations. These calcium oscillations are then able to regulate downstream events of egg activation such as the resumption of meiosis, cortical granule (CG) exocytosis and changes in the translational landscape (Jones., 2005; Kashir et al., 2014; Krauchunas et al., 2016).

However, the ‘soluble-factor’ model does not appear to be conserved amongst vertebrates and marine invertebrates, such as ascidians and echinoderms. A PLCζ homologue has not been discovered in these species; in fact, another member of the PLC family has been demonstrated to be the essential mediator of calcium release and egg activation, PLCγ (Runft and Jaffe, 2000; Runft et al., 2002, 2004; Giusti et al., 2003). In echinoderms and frogs, upon egg activation there is a significant increase in the activity of PLCγ (Rongish et al., 1999; Sato et al., 2000). The role of PLCγ has been largely studied through manipulation of its SH2 domains which allow the PLC to interact with tyrosine kinases (such as Src Family Kinases- SFKs); other PLC isoforms lack these domains (Reviewed in Parrington et al., 2007). Injection of isolated SH2 domains into ascidian and echinoderm eggs competes for Src binding, and as such prevents calcium release at egg activation, suggesting PLCγ is important in activating the IP_3 pathway (Carroll et al., 1997; Giusti et al., 1999; Runft et al., 2002). However, injection of these domains into *Xenopus* and Mouse oocytes had no effect on calcium release (Mehlmann et al., 1998; Runft et al., 1999, 2002). Interestingly, injection of *Xenopus* sperm extract into mouse oocytes was capable of inducing an increase in intracellular calcium, suggesting there may be a sperm-derived PLCζ in *Xenopus* (Dong et al., 2000). Though it is still unclear in cases such as amphibians, it is confirmed that there is a role for PLC enzymes in egg activation of vertebrates and marine invertebrates, but the specific of this mechanism have not been fully characterised.

The mechanism of PLCγ activation is still to be fully determined, but evidence suggests that sperm binding or fusion results in activation of Src family kinases which then phosphorylate and activate the PLCγ (reviewed in Parrington et al., 2007; Horner and Wolfner,. 2008). The activated PLCγ is then able to convert PIP_2 into IP_3 and DAG, triggering calcium release from internal stores via the IP_3R1, as previously described (Figure 1.1). Though the sperm-receptor that triggers this cascade remains un-identified, in Sea Urchin oocytes the SFK1 has been reported as the upstream kinase responsible for activating PLCγ (Giusti et al., 2003). It is likely that upstream activation of SFKs is through activation of G-proteins, as these commonly exist together in larger protein complexes, and in vertebrates, G proteins can directly stimulate SFK activity (Hall et al., 1999; Ma et al., 2000; Luttrell and Lefkowitz, 2002).

G proteins are ubiquitously expressed and conserved among species (Sprang., 1997). Such G proteins are referred to as heterotrimeric as the complex consists of three subunits: Gα, Gβ and Gγ, with distinct isoforms that carry out specific functions in a variety of tissues. Gα has GTP (guanosine triphosphate) hydrolysing activity, it is a GTPase; in its inactive state, Gα is bound to GDP (guanosine diphosphate), and exchange of the GDP for GTP results in dissociation from the βγ heterodimer, enabling the free components to interact and regulate downstream targets, such as the activation of PLC by Gq (a specific isoform of the Gα) (Figure 1.2).

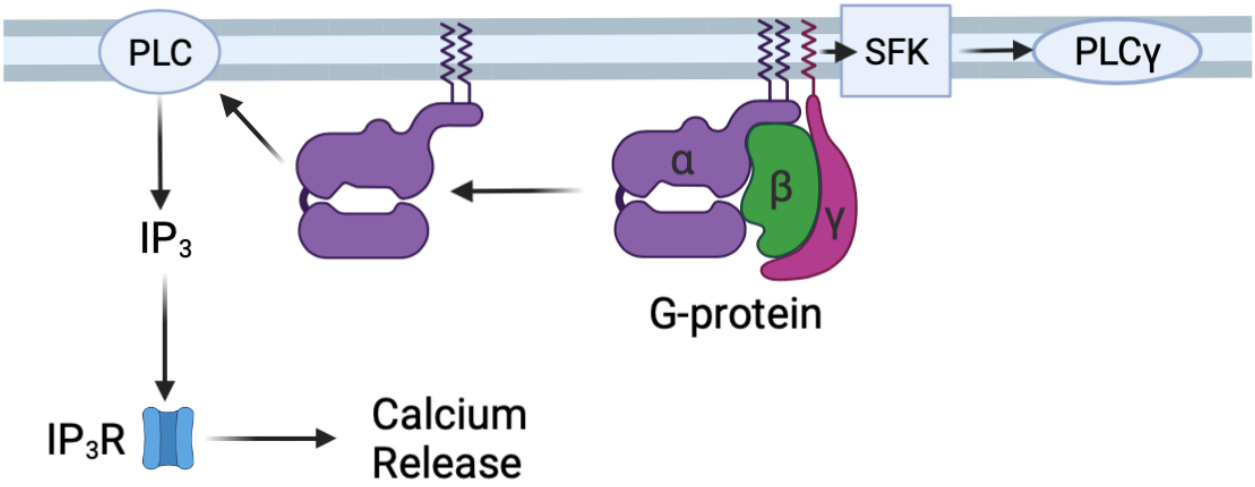

Figure 1.2: G-protein action promotes calcium release at egg activation
In sea urchins, the membrane-receptor model may also include activation of a heterotrimeric G-protein. Gα release from the complex and subsequent activation results in activation of PLC enzymes and production of IP_3, resulting in calcium release. The G-protein can also directly

Evidence suggests that G-proteins do in fact play a role in calcium release at egg activation; in sea urchin eggs, sperm activates G-proteins, stimulating production of IP_3, calcium release and cortical granule (CG) exocytosis (Turner et al., 1986). Preventing G-protein activation through the

injection of the inhibitor GDP-βS prevented calcium release in hamster and rabbit oocytes (Miyazaki 1988; Fissore and Robl, 1994). Inversely, G-proteins were ectopically stimulated through use of the non-hydrolysable GTP analog GTP-γS in mammalian, frog and echinoderm eggs, resulting in calcium release and CG exocytosis (Cran et al., 1988; Jaffe et al., 1988; Miyazaki, 1988; Crossley et al., 1991; Fissore and Robl, 1994). The specific mechanisms of G-protein involvement are poorly understood in most species; in sea urchins it has been established that Gαq and Gαs are required for calcium release, but it is the Gβγ being released that is required for Gαq and Gαs signalling rather than specific activation of the Gα subunits (Voronina and Wessel., 2004).

Ultimately, PLC enzymes are activated in mature oocytes upon fertilisation with the sperm, whether this be a sperm-derived PLC or activation of oocyte-derived PLCs, likely through G-protein and SFK interactions. This highlights the cruciality of the inositol phosphate (IP) signalling pathways in egg activation of vertebrates and marine invertebrates, relying on IP_3 mediated intracellular calcium release in order to generate calcium waves and oscillations. Of course, there are some deviations from this general mechanism, as some species rely also on external calcium, as is the case in Mollusks (Stricker, 1996; Stephano and Gould, 1997; Deguchi and Morisawa, 2003), and in one exception, the limpet *Lottia gigantea,* relies exclusively on external calcium (Deguchi., 2007).

Fertilisation: Nitric Oxide & Cyclic ADP Ribose Signalling

The role of the IP_3 pathway in fertilisation has been confirmed and corroborated amongst vertebrates and marine invertebrates, however, alternative pathways exist to generate calcium transients. In the case of fertilisation induced calcium increases, the nitric oxide (NO) and Cyclic ADP Ribose (cADPR) signalling pathways are often utilised to generate calcium release through the Ryanodine Receptor (RyR) in a convergence of calcium release pathways (Figure 1.1) (reviewed in Parrington et al., 2007).

Studies in sea urchin oocytes revealed the importance of the cADPR signalling pathway during egg activation. Inhibition of the IP_3R1 through injection of heparin, a competitive inhibitor of IP_3R did not prevent the intracellular calcium rise at egg activation, indicating that another pathway may be involved (Crossley et al., 1991). cADPR was discovered through a sea urchin egg homogenate assay, and shown to be responsible for mediating calcium release through the RyR (Galione et al., 1993). Interestingly, studies demonstrated that cADPR was produced and peaks in levels approximately 50 seconds post-fertilisation, and therefore may play a role in sustaining the calcium transient (Leckie et al., 2003). Supporting this, the duration of the calcium transient was reduced through microinjection of a cADPR antagonist, promoting the theory that cADPR is responsible for sustaining the calcium signal (Carroll et al., 1997; 2001; Thaler et al., 2004).

Evidently, both IP_3 and cADPR are playing roles in generation of the calcium transient, however, it appears that IP_3 is responsible early on for initiation of the transient, whereas cADPR is responsible for maintenance of the transient.

Whilst the mechanism for sperm-induced IP_3 signalling has been elucidated in much detail, how sperm-oocyte interactions lead to the production of cADPR is much less understood. It is thought that both Nitric Oxide (NO) and cGMP play a role in the activation of ADP ribosyl cyclase (ARC) enzymes to convert βNAD+ into cADPR, which can then act to open RyR (reviewed in Parrington et al., 2007) (Figure 1.1). A multitude of evidence exists in sea urchins which illuminates the mechanism behind this signalling pathway; Injection of cGMP and NO Synthase (NOS) was proved capable of producing calcium transients (Whalley et al., 1992; Kuo et al., 2000). It is likely that NO is activating soluble guanylyl cyclase and thus stimulate cGMP production (Willmott et al., 1996), thus contributing to the activation of ARC and thus the production of cADPR. Again, studies demonstrated that through simultaneously measuring the levels of calcium and NO that NO mediated calcium release is a late event, and thus likely responsible for sustaining the calcium transient rather than initiating it (Leckie et al., 2003).

One tempting theory for the activation of NOS proteins would place them downstream of PLC activation. When PLC enzymes are activated, the convert PIP_2 into IP_3 and DAG; it is known that IP_3 can open IP_3R on the ER to induce calcium release, however DAG also may play a role through the activation of PKC. PKC can then phosphorylate NOS proteins and thus increase NO levels to sustain the calcium wave (Matsubara et al., 2003). Furthermore, NOS are also regulated through the action of Calmodulin proteins, which are equally unregulated during the calcium transient, and binds via the highly conserved Calmodulin-Binding domains, a necessary event for NOS function (Matsubara et al., 2003). This therefore places NOS at the centre of multiple signalling pathways, highlighting that the convergence of such pathways is required for the transduction of many cellular signals, and as ever, biological events may not be regulated as simply as by one factor. How conserved the involvement of NO signalling in vertebrates is poorly understood, however, despite it playing what appears to be a vital role in marine invertebrates.

Fertilisation: NAADP Signalling

In addition to the IP_3, and cADPR mediated calcium response in Sea Urchins and other echinoderms, the NAADP signalling pathway appears to also play a role at fertilisation; microinjection of NAADP into sea urchin eggs is able to induce a calcium response, and this response is not blocked by inhibitor of IP_3 and cADPR signalling (Perez Terzic et al., 1995). Observation of the spatial dynamics of the intracellular calcium rise upon the injection of NADP demonstrated a biphasic response; an initial cortical flash of calcium followed by a global calcium increase that traverses the cytoplasm (Churchill et al., 2003). This same biphasic response was also shown in starfish oocytes (Santella et al., 2000; Lim et al., 2002).

It is not entirely clear whether external calcium is required for this calcium response, as chelation of extracellular calcium or introduction of cadmium, a calcium channel blocker, prevents the calcium transient, but does not stop the cytoplasmic calcium increase (Churchill et al., 2003). However, it has been shown previously that sea urchin eggs can activate in the absence of external calcium (McDougall et al., 1993). However, the presence of a cortical flash that is abolished in calcium free environments suggests the movement of calcium across the plasma membrane. In starfish, injection of NAADP can induce a cortical flash, possibly as the NAADP opens receptors on the membrane (Lim et al., 2002). Evidently, NAADP may be playing a role in both mobilisation of calcium across the membrane and through induction of intracellular calcium release (Patel., 2004). There has been some evidence to suggest the NAADP and IP_3 pathways may be actin in parallel to generate the calcium response, as desensitisation of the NAADP receptors can disrupt the IP_3 mediated calcium wave, and the IP_3 pathway can contribute to the NAADP-induced membrane depolarisation through activation of calcium dependent sodium channels (Moccia et al., 2006).

Interestingly, sperm can also mediate the cortical flash, and fertilisation of oocytes that have blocked NAADP receptors also prevents the cortical flash (Churchill et al., 2003). NAADP had been shown to be present in the sperm at micro molar levels, and the concentration of such increases upon contact with the egg jelly (Churchill et al., 2003). Further measuring the concentration of NAADP within the egg demonstrates a biphasic pattern; an initial increase at fertilisation followed by a second increase peaking at 4-5 minutes post-fertilisation (Churchill et al., 2003). These data suggest that the sperm delivers an initial dose of NAADP, possibly sufficient to induce the cortical flash, whereupon the egg begins to synthesise further NAADP.

Ultimately, it is clear that in sea urchins and other echinoderms, multiple pathways may be playing a role at fertilisation-induced egg activation, namely that of IP_3 signalling in order to induce calcium transients, NO and cADPR signalling to maintain such calcium transients, and additional NAADP provided by sperm that can induce calcium movements across the plasma membrane and membranes of acidic organelles. Although conservation of the PLC specific isoform is not seen, crucially, IP_3 signalling is conserved, in addition to potential roles of NAADP in regulation of calcium movements in mammals, as can be seen in insulin secretion and cardiac function, to name a few.

1.1.5 Initiating Egg Activation: Induction by the External Environment

Though fertilisation plays a large role in many species, alternative mechanisms of triggering egg activation exist, such as the change of the external environment. One example of this is the starfish, *Asterina pectinifera*; though, as explained above, starfish utilise sperm for activation, it isn't necessary for all egg activation events. In fact, the deposition of unfertilised starfish oocytes into sea water is sufficient to trigger the resumption of meiosis (Kishimoto., 1998). It was further

shown that the key component of the sea water that triggers egg activation is sodium; the completion of meiosis (as indicated by the extrusion of polar bodies), could be triggered through deposition into seawater, but not artificial seawater lacking sodium ions (Harada et al., 2003). Additionally, the presence of a IP_3 sponge (preventing IP_3 from acting on IP_3R) did not inhibit the progression of meiosis upon egg activation, indicating that this particular event of egg activation is not dependent on calcium release from intracellular stores (Iwasaki et al., 2002).

Exposure of the starfish oocytes to sea water is suggested to activate Na/H anti-porters on the plasma membrane, and therefore generating an increase in pH in the egg. This was supported by measuring the pH of eggs deposited into sodium free sea water versus regular sea water; in the case of sodium free sea water, the pH of the oocytes remained at 6.7 in comparison to those exposed to sea water which displayed an increase to 7.3 (Harada et al., 2003). How pH would regulate events of egg activation is not well understood, however it has been suggested that an increase in pH could destroy cyclin-B and thus trigger the resumption of meiosis, as this was shown in cell-free starfish egg extracts (Harada et al., 2003).

Similarly, in the marine shrimp, *Sciyonia ingentis*, the deposition of eggs into see water triggers an intracellular calcium transient. In a similar experimental set-up to the starfish, eggs were deposited into sea water and artificial sea water lacking magnesium ions, and in the absence of magnesium, the eggs failed to activate (Lindsay et al. 1992). Interestingly, this calcium wave can be triggered in both fertilised and unfertilised eggs, however, when an egg is unfertilised, it required calcium to be present in the external media, suggesting that the marine shrimp oocyte can rely on both internal and external calcium sources for egg activation.

Evidently, in the case of starfish and shrimp, as marine invertebrates, deposition of eggs in sea water can be relied on to successfully activate eggs if required, and with sea water clearly an ionic solution, this environment can be employed as such. However, other invertebrates have been shown to be affected by their external environment, triggering egg activation. In the case of the parthenogenetic sawfly *A. rosae*, almost all eggs dissected from adult females were activated simply by immersing them in a hypotonic solution or distilled water (Sawa and Oishi., 1989a). This was demonstrated to also be the case for the mosquito *A. stephensi* (Yamamoto et al., 2013). In this case, eggs oviposited into water would activate, as indicate by a darkening of the endochorion, resumption of meiosis and dephosphorylation of MEK/MAPK. Dissection of unfertilised eggs and deposition into water demonstrated the same darkening of the endochorion, whereas deposition into a control saline showed no change in pigmentation, no resumption of meiosis or no dephosphorylation of MEK/MAPK. Interestingly, holding the eggs in high sodium solutions maintains the eggs in a healthy state for up to 20 mins, whereupon transference into a low sodium solution such as distilled water triggers egg activation (Yamamoto et al., 2013). In this case it appears that a change in ionic environment is necessary to relieve the eggs from some form of inhibitory influence of sodium ions.

Though changes in the ionic environment present an obvious mechanism of regulation through membrane electrical gradients or pH, other external factors may play a role in other species. For instance, oxygen is a key regulator of egg activation in the stick insect *Carausius morosus.* It was observed that eggs that lacked micropyles would not continue development, and in the case of these micropyle-less eggs, development could be rescued by pricking them and making a small opening in the egg coverings. Further lines of evidence suggest oxygen is the key factor, as eggs layer directly in a saline solution did not develop past their arrested stage until they were removed from the solution and exposed to oxygen. Additionally, pricking normal eggs laid into the salt solution did not activate them, demonstrating the action of pricking itself did not activate the eggs. Finally, experimentation with the key components of air demonstrated oxygen rather than nitrogen was capable of removing the meiotic block and resumption of development (Went., 1982; Pijnacker., 1966a, 1966b, 1966b; Pijnacker and Ferwerda., 1976).

1.1.6 Initiating Egg Activation: Mechanical Stimulation

In many species of insects, egg activation can occur completely independently of fertilisation. This is essential in the case of parthenogenetically reproducing species- parthenogenesis is form of asexual reproduction in which an ovum can develop into an embryo and undergo development without the input of a fertilising sperm. This means that haploid offspring can be produced (ie. there is no need for fertilisation to provide two parental genomes). This is the case in Hymenoptera, such as bees and wasps, which have a haplodiploid sex-determination system in which haploid males form from unfertilised eggs, a form of parthenogenesis. Evidently, being completely devoid of fertilisation suggests that innate properties of the oocyte itself are capable of activating all of the essential processes of development without interaction with sperm in certain species, such as parthenogenetically reproducing insects.

This is in contrast to the previously discussed marine invertebrates in which fertilisation is eventually required and may play a larger part in induction of the IP_3 pathway, despite the main triggers of egg activation being, arguably, the external environment. Studies of the parasitic wasp, *Pimpla turionellae* illuminated the mechanism by which certain arthropods may trigger egg activation. In the case of the parasitic wasp, the process of egg laying occurs within a few seconds as the egg passes into the oviduct (ovulation), facultative fertilisation (meaning that the wasp is able to reproduce both sexually, through fertilisation, and asexually through parthenogenesis) may occur in the uterus, and then oviposition and injection of the eggs into host pupa. Simultaneously, meiosis resumes, oopmasmic movements occur that would fuse two pronuclei and cleavage begins. It could therefore be any of these events, ovulation, oviposition or egg injection that triggers activation of the oocyte. One observation noted that though the egg remains the same shape pre and post-ovulation, the diameter of the egg canal is approximately 1/3 the width of the oocyte, and indeed the ovipositor is constructed of solid material, unlikely to yield to a passing and more malleable egg (Went and Krause., 1973; Pampel., 1914; Smith.,

1970). It was therefore considered that passage and distortion of the egg through this narrow canal could trigger activation. Damaging the ovipositor at the base and middle demonstrated that if the egg passes through even a small part of the egg canal and emerging from the middle would activate and develop, whereas those that emerged at the base and had therefore not undergone distortion would not develop (Went and Krause., 1974a). Similarly, construction of an artificial ovipositor for explanted eggs to pass through was sufficient to squeeze the eggs and thus trigger activation and normal development in 70% of oocytes (Went and Krause 1973, 1974a, 1974b). Injection of a calcium ionophore (as the vitelline membrane may exclude passage into the oocyte) was also sufficient to trigger egg activation, suggesting a calcium dependent mechanism following mechanical stimulation (reviewed in Went., 1982). It is thus likely that mechanical stimulation of the oocyte in the case of *Pimpla turionellae* is sufficient to trigger downstream increases in intracellular calcium in order to coordinate egg activation events.

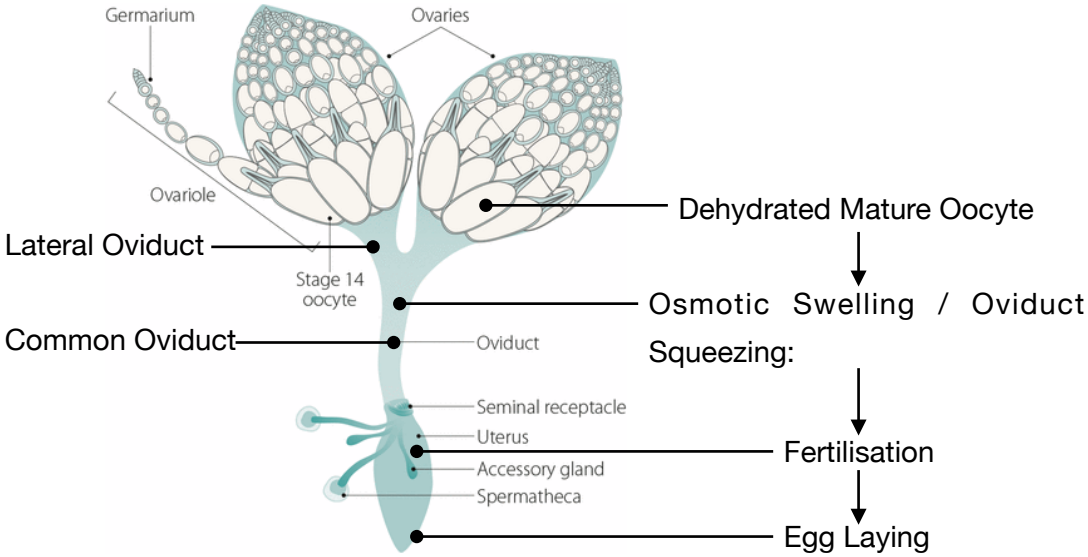

Figure 1.3: Oviposition in *Drosophila melanogaster*

Image of the Drosophila ovaries with pertinent regions during ovulation emphasised.

This method of oviposition-mediated mechanical stimulation could potentially be translated to other insects. In fact, this used to be the main theory of egg activation in *Drosophila* species, many of which can undergo parthenogenetic reproduction, as it was observed that unfertilised eggs laid by virgin female flies had still completed meiosis and undergone cross-linking of the peri-vitelline membrane (Doane., 1960; LeMosy and Hashimoto, 2000; Heifetz et al., 2001). During ovulation in Drosophila, the mature oocyte also squeezes through a small lateral oviduct into the common oviduct and eventually the uterus where it is fertilised (Figure 1.3), and so thinking originally centred around the hypothesis that this mechanical squeezing would open calcium channels and generate a calcium wave that precipitates all of the downstream events of egg activation. However, oocytes can be activated through the addition of a hypotonic buffer, and with observation that the eggs display a visible swelling upon activation, it is probable that the force

generated by osmotic pressure and swelling is able to activate the oocyte (Mahowald et al., 1983; Page and Orr-Weaver., 1997).

1.1.7 TRP Channels as Mechano-transducers

One obvious line of investigation would be to therefore test mechanically gated channels to determine whether they are responsible for the transduction of mechanical force during ovulation into a calcium signal. One such channel is the TRP channel, which was first discovered through mutational analysis and complementation in fly photoreceptors (reviewed in Montell, 2005). TRP channels are somewhat diverse and are responsible for transducing many environmental inputs such as temperature and pressure. There are seven families existing based on sequence homology, of note are TrpC, TrpV and TrpM. These different Trp channels are generally conserved in their structure, with six transmembrane domains, the channel pore formed between loops S5 and S6, enabling the formation of a non-selective cation channel pore (with exceptions of TrpV5 and TrpV6 with are calcium specific).

In fact, the recently awarded Nobel prize in physiology and medicine was shared between two researchers that illuminated the mechanisms of the receptors for temperature and touch. Trp channels were shown to be key in temperature sensation, specifically TrpV1 which opens in high temperatures (above 43 degrees Celsius) and TrpM8 which opens in cooler temperatures (around 20 degrees Celsius) (Caterina et al., 1997; 2000; Tominaga et al., 1998; McKemy et al., 2002). This research paved the way for further understanding how mechanosensitive channels function, with the discovery of Piezo 1 and Piezo 2 providing a foundation for research into mechanosensation. These proteins were shown to transduce the mechanical stimulation of poking with a micropipette into an ionic signal (Coste et al., 2010; Ranade et al., 2014; Woo et al., 2015). This research thus platformed our understanding of mechanosensation, and since we have have shown that many other ion channels act as mechano-transducers, such as the Trp family itself (Liu and Montell, 2015).

Whilst Trp channels have been well documented in other biological systems, such as in *Drosophila* photoreceptors or requirements within blood vessels in the coordination of vasoconstriction (Yue et al,. 2015), they are beginning to gain more attention in the field of egg activation. Evidence from as early as 1986 had pointed to a potential role of Trp channels in oocytes, as mechanical stimulation of *Xenopus* oocytes led to activation through opening of TrpC1 (Methfessel et al., 1986). More recent evidence has revealed the importance of Trp channels in mouse oocytes; overexpression and over-stimulation through incubation with 2-APB of TrpV3 in mouse resulted in calcium influx and egg activation (Lee et al., 2016). Furthermore, other work has suggested that TrpM7 is required in mouse oocytes; blocking this channel pharmacologically prevents calcium entry at egg activation (Carvacho et al., 2016). Finally, *c. elegans* oocytes have been shown to require the delivery of TRP3 channels from the sperm in

order to achieve a calcium rise at egg activation (Takayama and Onami., 2016). Taken together, the requirement of TRP channels in a variety of systems, and the mechanical stimulation that the *Drosophila* oocyte undergoes at egg activation suggests an enticing mechanisms whereby the force of hydration of the oocyte is transduced through TRP channels into a calcium signal that initiates the processes of egg activation.

1.2 Downstream Events of Egg Activation

1.2.1 Release from Meiotic Arrest

Overview of Meiosis

Meiosis is a fundamental and defining event of gametogenesis, essential for both the reduction of 'ploidy' of the gametes and introduction of genetic diversity. In mammals, primordial germ cells undergo a singular round of DNA replication followed by two rounds of cellular division, known as meiosis 1 (MI) and meiosis 2 (MII), thereby enabling the segregation of maternal and paternal chromosomes, followed by the separation of chromatids in order to generate four haploid germ cells (Page and Hawley., 2003; Petronczki et al., 2003, MacLennan et al., 2015).

During MI, homologous chromosomes come together during a prolonged prophase stage, which is subdivided into four further stages: 1) Leptotene: DNA has already replicated and chromatin is stretched out; 2) Zygotene: Homologous chromosomes pair side by side during synapsis, requiring the presence of the nuclear envelope and the synaptonemal complex (Takeo et al., 2011; Tsai et al., 2011; Yang and Wang., 2009); 3) Pachytene: Chromatin condenses and crossing over events take place; 4) Diplotene: Crossing over may continue and homologous chromosomes begin to separate, likely still held together by chiasmata formed during crossing over. Metaphase then begins as the nuclear envelope breaks down and diakinesis of the chromosomes occurs as they migrate to the metaphase plate. Once the chromosomes are aligned on the mitotic spindle fibres, Anaphase begins as homologous chromosomes are separated form each other independently, following which is Telophase in which the two daughter cells are formed.

MII thus follows in which the two daughter cells divide again, without a round of DNA replication. It follows that the chromosomes are thus aligned on the metaphase plate without pairing with another homologous chromosome, but rather each chromatid is pulled to opposite poles of the cell, breaking the kinetochore that usually holds them together, thus forming 4 haploid daughter cells. However, though it the case during mammalian spermatogenesis that 4 sperm cells may be formed from a singular primary spermatocyte, in the case of oogenesis, this would result in equal cellular divisions of the primary oocyte generating 4 much smaller mature oocytes. Instead, during MI and MII, cytokinesis occurs in an asymmetric fashion resulting in most of the cellular cytoplasm being passed onto one daughter cell, with the smaller one forming a polar body and usually undergoing apoptosis. Similarly, during MII, a singular daughter cell is formed and the

remaining DNA shuttled into another polar body. Therefore, eugenic meiosis is able to retain the volume of oocyte cytoplasm.

Meiotic Arrest in Vertebrates

Though oogenesis may differ between species, the process of meiosis is remarkably similar (Figure 1.4) . Oocyte development in all species relies on meiotic arrest, and in most cases, arrest at two point during meiosis (Von Stetina and Orr-Weaver., 2011). A primary arrest occurs in all species (it may be the only arrest in some cases, such as *C. elegans*) at prophase I in which the oocyte differentiates and stockpiles maternal components. For instance, in mammalian oogenesis, following synapsis and recombination of homologous chromosomes, oocytes arrest at the diplotene stage of MI. These diplotene-arrested oocytes with a surrounding layer of granulosa cells, form a primordial follicle; a unit of the ovary. These primary arrested oocytes then remain arrested until the adult oestrus/menstrual cycle begins, and a surge in luteinising hormone triggers release of meiotic arrest and thus oocyte maturation (Pan and Li., 2019).

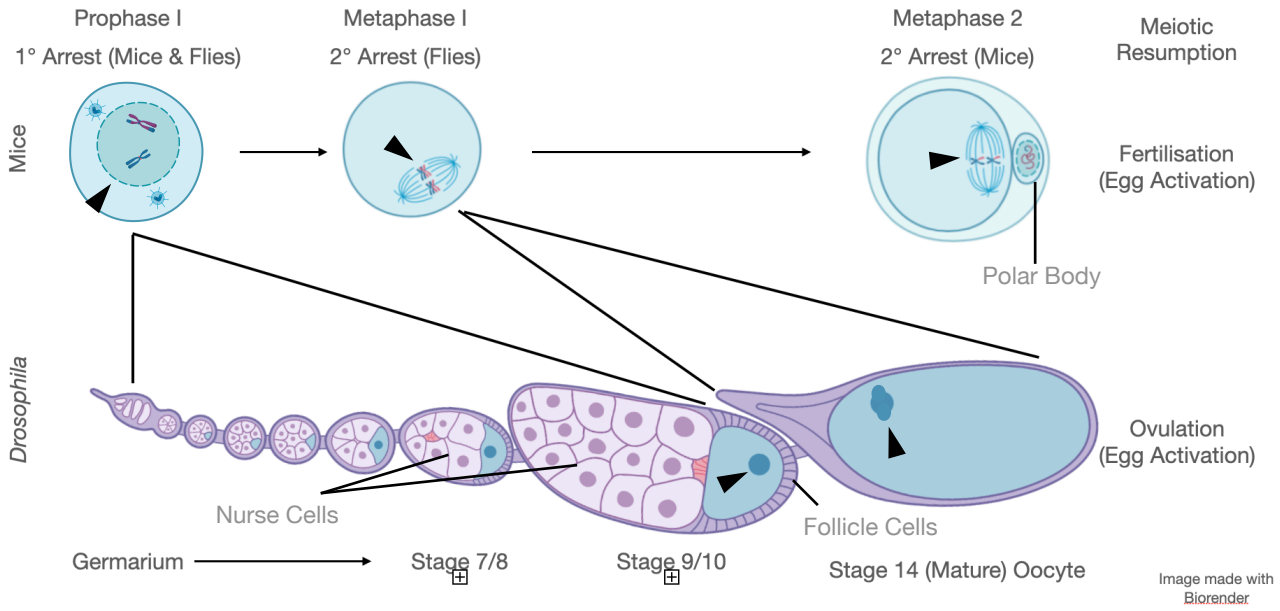

Figure 1.4: Comparison of meiosis between Mice and *Drosophila*
Mouse and Drosophila maturing oocytes undergoing meiosis. In mice, the final arrest is at Metaphase 2, whereas in Drosophila, the final arrest is at Metaphase 1. Black arrows indicate the location of the chromosomes; nuclear envelope breakdown happens during prophase I in mice and *Drosophila.* In *Drosophila,* this occurs between stage 11-12, whereupon in stage 14

Oocyte maturation is the process in which the primary arrest is released, enabling the oocyte to progress into the meiotic divisions. In most vertebrates, the oocytes progress until metaphase II, whereupon a secondary arrest occurs which has evolved to coordinate the completion of meiosis

with fertilisation. Comparatively, most insects, including *Drosophila*, continue until only metaphase I before secondary arrest occurs once again, and the trigger for oocyte maturation in these species is far less well understood (Von Stetina and Orr-Weaver., 2011). Though there is a wealth of research discussing maintenance and release of primary arrest, I will focus on secondary arrest, as this is the stage at which oocytes are held until egg activation, enabling the oocyte to resume with meiosis and become competent to progress through embryonic development (Horner and Wolfner., 2008).

As the secondary meiotic arrest occurs at metaphase, either in the first round of meiosis (*Drosophila*) or the second (most vertebrates), they key to establishment and maintenance of this arrest is through the stabilisation of cell cycle drivers, namely the driver of the G2/M transition, M-Phase Promoting Factor, MPF (Horner and Wolfner., 2008; Von Stetina and Orr-Weaver., 2011). In mammals, the MPF is a complex of Cdk1/Cyclin B; active Cdk1 is responsible for entry into M-phase by triggering the cascade that results in nuclear envelope breakdown, spindle assembly (Belenguer et al., 1990; Heald and McKeon, 1990; Peter et al., 1990a,b; Sagata 1996). The stability of Cdk1/CyclinB (MPF) ensures meiotic arrest and is regulated by the cytostatic factor (CSF).

Many proteins are potential candidates for the CSF, namely Mos, which has been shown to act as the CSF in mouse, porcine and *Xenopus* oocytes (Ohashi et al. 2003; Nishimura et al. 2009; Tunquist and Maller., 2003). In mice, oocytes lacking the *mos* gene progressed to MII and underwent spontaneous activation in the form of extrusion of the second polar body and formation of the pronucleus (Colledge et al. 1994; Hashimoto et al. 1994; Choi et al. 1996). Mos has been shown to act as an MEK (a MAPK Kinase), responsible for activating MAPK, which acts in a pathway alongside MAPK, p90rsk and Emi2 to act as a CSF (reviewed in Perry & Verlhac, 2008; Wu & Kornbluth, 2008). The main route through which these proteins prevent meiotic progression and thus metaphase arrest is through inhibition of the Anaphase promoting complex (APC). MOS is produced in response to progesterone signalling, whereupon the MOS-MAPK pathway, through the action of p90rsk, phosphorylate Emi2, activating and stabilising Emi2 (Inoeu et al., 2007; Nishiyama et al., 2007a). Emi2 is then able to inhibit the APC, most likely through preventing it associating with Cdc20, an APC activator. MOS also acts as the CSF through activation of spindle assembly checkpoint proteins, further preventing APC actin through prevention of its interaction with Cdc20 (Schwab et al., 2001; Sharp-Baker and Chen., 2001; Li et al., 1997; Tunquist et al., 2002). Therefore, through the action of the CSF, the APC is prevented from acting to degrade the MPF and promote meiotic progression.

Release from this meiotic arrest upon egg activation is essential in order to allow the completion of meiosis and formation of a pronucleus concurrently with introduction of the sperm pronucleus. In mammalian models, it has been demonstrated that the induction of a calcium transient through PLCζ triggers this metaphase release. Calcium release at egg activation inactivates the CSF via

inactivation of MAPK and MPF resulting in meiotic release and pronuclear formation (Ito et al. 2003). Elevated intracellular calcium activates CaMKII, downstream of which activates the APC (Nixon et al., 2002; Markoulaki et al., 2004; Madgwick et al., 2005). It is likely that Emi2 is degraded, as Emi2 in mouse oocytes contain motifs that would be phosphorylated through the action of CaMKII and Polo-like kinase 1 (Plx1), allowing the APC to target cyclinB for degradation and thus inactivate Cdc2 (Madgwick et al., 2006; Horner and Wolfner., 2008).

This has been demonstrated more conclusively in *Xenopus* models, as upon egg activation the rise in intracellular calcium activates CaMKII which phosphorylates Emi2, priming it for further phosphorylation by Plx, targeting the Emi2 for degradation by the SCF ubiquitin ligase, relieving the inhibition of the APC (Liu and Maller., 2005; Rauh et al., 2005) (Figure 1.5). The APC can then act in its capacity to promote anaphase through many classical pathways: 1) degradation of securin, relieving the enzyme separase of its inhibition, and thus cleaving the attachment along sister chromatids; 2) Targeting the regulatory Cyclin B of the MPF complex for degradation and thus inactivating Cdc2 (Castro et al., 2001; 2005; Sheng et al., 2002) (Figure 1.5). The calcium-regulated protein Calcineurin (CaN) has also been demonstrated to play a role in the release from meiotic arrest in *Xenopus* and *Drosophila* (Horner et al., 2006; Takeo et al., 2006; Mochida and Hunt., 2007; Nishiyama et al., 2007b). During the intracellular calcium transient in. *Xenopus*, CaN is transiently activated in a concurrent pathway to that of CaMKII. The CaN is responsible for reversing global mitotic phosphorylations and is necessary for the inactivation of MPF and exit from meiosis II (Mochida and Hunt., 2007; Nishiyama et al., 2007b). CaN dephosphorylates Cdc20 and Apc3, part of the APC, enabling the complete activation of the APC itself (Mochida and Hunt., 2007). Calcineurin, as in *Drosophila* and *Xenopus*, is likely playing a role during porcine (pig) oocyte activation. It can be seen largely localised to the cortex, as characterised through immuno-staining of Calcineurin subunits A and B (CanA and CanB), and treatment of oocytes with the Calcineurin inhibitors cyclosporin A (CsA) and hymenistatin I (HS-I) prevented parthenogenetic egg activation (Tumova et al., 2016). This suggests that Calcineurin in necessary for meiotic progression in many species. Tumova et al. also show that Calcineurin plays a role during CG exocytosis, and suggest it could be performing this role through regulation of actin filaments at the cortex.

Meiotic Arrest in *Drosophila melanogaster*

As touched upon in the previous section, there are many similarities that can be observed in *Drosophila* resumption of meiosis, and this will be discussed in greater detail in Chapter 5. To reiterate, *Drosophila* egg activation is independent of fertilisation, and occurs as the oocyte passes into the oviduct, where likely an osmotic trigger leads to swelling of the oocyte, calcium entry and resumption of the cell cycle (Doane, 1960; Heifeitz et al., 2001; York-Andersen et al., 2021). Prior to this, *Drosophila* oocytes are arrested at metaphase-I, and this process is also reliant upon elevated activity of Cdk1/CyclinB, without which one can observe disrupted meiotic maturation (Xiang et al., 2007; Von Stetina et al., 2008). Not a great deal has been examined at *Drosophila* egg activation regarding the pathways that result in meiotic resumption. Mutants for the *Drosophila* Calcipressin Sarah, a key calcium regulated signalling protein, do not show activation of the APC/C and are not able to fully proceed through meiosis (Takeo et al., 2006; Horner et al., 2006). The APC/C is activated by Cdc20 and ultimately mediates the degradation of Cyclin B (the metaphase promoting factor), enabling entry into anaphase (Swan and Schupbach, 2007; Page and Orr-Weaver, 2003).

The process of meiotic resumption has been well studied at a histological level; the metaphase arrested spindle can be observed just underneath the dorsal appendages as a small elliptical shape with focussed poles and a central mass of congressed chromosomes (Endow and Komma, 1997; Bennabi et al., 2016; Gilliland et al., 2009). The resumption of meiosis at egg activation was observed live at the spindle using the non-claret disjunctional-GFP (Ncd-GFP) transgene to label the microtubules(Endow and Komma, 1997): this is a fluorescently labelled Kinesin-14 protein, the normal function of which is to crosslink microtubules in order to generate a bipolar spindle. The metaphase-anaphase transition can be observed as a stereotypical series of events (Endow and Komma, 1997): Elongation of the meiotic spindle; 2) Contraction of the meiotic spindle; 3) Rotation of the meiotic spindle in relation to the cortex; 4) Following completion of meiosis I, the spindle is at a 90 degree angle to the cortex. The process of meiosis, specifically in the context of *Drosophila* egg activation, will be explored in more detail in Chapter 5.

1.2.4 CG Exocytosis

Physical changes to the egg occur during egg activation, namely processes that modify the egg coverings. In most vertebrates and some invertebrates the major change occurs through CG (CG) exocytosis (Shapiro et al., 1989). This process is vitally important for the prevention of polyspermy, which would result in the failure of development likely due to polyploidy or defects in meiotic segregation. Additionally, the provision of additional extracellular layers could also play a role in providing mechanical support and protection from environmental damage (Wolfner and Horner., 2008). Comparatively, *Drosophila* does not undergo CG exocytosis, possibly as polyspermy is already prevented likely through the presence of the micropyle, a specialised region

of the anterior eggshell through which the sperm can enter, and the capability of female *Drosophila* to store sperm and select a single sperm cell for fertilisation. However, the egg shell does harden in order to provide mechanical support to the developing embryo (Horner and Wolfner., 2008).

In mammals, a glycoprotein-rich extracellular matrix exists just outside of the oocyte plasma membrane, and is known as the zona pellucida (ZP). This layer is modified during CG exocytosis, enabling protection from polyspermy. CGs contain a mixture of enzymes (proteases, glycosidases and cross-linkers), which are released in a calcium dependent manner upon egg activation as the granules fuse with the oocyte plasma membrane (Wessel et al., 2001). In mouse oocytes, for instance, the granules contain ovoperoxidases that harden the extracellular matrix through cross-linking of tyrosine residues (Gulyas., 1979; Schmell and Gulyas., 1980; LaFleur et al., 1998).

CG exocytosis is highly dependent on calcium release at egg activation. In mouse oocytes, the CGs are located in the sub-cortical cytoplasm, and upon egg activation must be translocated to the oocyte plasma membrane (Tahara et al., 1996). Inhibiting the action of CaMKII prevents CG exocytosis, however, expression of constitutively active CaMKII does not fully induce CG exocyosis, indicating that CaMKII is necessary but not sufficient for this process. The full mechanism of CaMKII action on the CGs has not yet been fully elucidated, but it is likely that it functions in a similar manner to that of secretory vesicles, in which the CaMKII phosphorylates tethering proteins, releasing the vesicles and promoting their secretion (Abbott and Ducibella., 2001). Clearly, other pathways are involved in vesicle and CG secretion as the action of CaMKII alone appears to not be sufficient. Interestingly, introduction of myosin light chain kinase (MLCK) and Myosin II antagonists reduce the efficiency of CG exocytosis in mice, suggesting that motor proteins are required for CG translocation. Thus a model exists in which calcium activity at egg activation both activates the CaMKII to release CGs from tethers, and activates MLCK and thus Myosin II in order to translocate CGs to the plasma membrane in order for them to be secreted (Ducibella et al., 2006; Matson et al., 2006).

Calcium-dependent fusion with the membrane is then able to occur after CG positioning. In sea urchin oocytes, it has been suggested that fusion occurs via the SNARE complex with synaptotagmin actin as the calcium sensor (Chapman et al., 1995; Chapman., 2002; Yoshihara and Littleton., 2002). Synaptotagmin has been shown to be enriched in CGs and is required at fertilisation in sea urchins (Leguia et al., 2006). The vast majority of research into the SNARE complex has been carried out in the context of neurons, so it may be the case that similar mechanisms are at play in the oocyte. In mammalian neurons, synaptotagmin associates with the SNARE complex in a calcium independent manner (Rickman and Davletov., 2003; Shin et al., 2003). This may infer a mechanism in which synaptotagmin association with the SNARE complex prevents full SNARE assembly and blocks membrane fusion in the absence of calcium, however, upon an increase in intracellular calcium, synaptotagmin releases the SNARE complex enabling full SNARE assembly and interaction with phospholipids to promote membrane fusion (Leguia et

al., 2006). This mechanism needs to be further examined in the context of oocyte for the process of CG exocytosis to be fully understood.

1.2.5 Modifications to the eggshell

In *Drosophila melanogaster,* oocytes do not contain CGs or possess a zona pellucida, instead, the oocyte is covered by an eggshell that is essentially comprised of an inner vitelline membrane (VM; comparable to the ZP), a waxy layer and an outer chorion (for more details see Chapter 3) (Waring., 2000). This, as aforementioned, provides a barrier to polyspermy as the only gate for sperm entry is the micropyle, in addition to providing mechanical support to the oocyte and developing embryo.

It has been observed that mature oocytes held in the ovaries exist in a desiccated state as they become dehydrated during the process of oogenesis (Mahowald and Kambysellis., 1980; Spradling., 1993). However, upon ovulation and egg activation, the eggs swell, becoming hydrated and taut. This process is correlated with a decrease in VM permeability as the oocytes become impermeable to small molecules, and this is caused by cross-linking of VM proteins; the VM is already cross-linked to some extent by disulphide bridges, however, upon activation, further non-sulphide cross-linking events occur (Petri et al., 1979; Heifetz et al., 2001).

A similar process occurs in *C. elegans*, as the formation of a chitinous layer of the eggshell occurs following fertilisation, requiring EGG-3, a member of the protein tyrosine phosphatase-like (PTPL) family (Maruyama et al., 2007; Ward and Carrel., 1979). EGG-3 is associated with the plasma membrane of oocytes, and shortly after fertilisation redistributes to granules within the cytoplasm. It then regulates the action of CHS-1 (chitin synthase) and is vital for the correct localisation of CHS-1 at the plasma membrane, enabling the production of chitin to provide mechanical support for the oocyte. EGG-3 also regulates the action of the enzyme MBK-2, which is essential for targeting maternal proteins for degradation upon egg activation. It is thought that EGG-3 acts as a scaffold for the regulation of various proteins involved in egg activation, an has conclusively been shown to be vital for eggshell reinforcement (Maruyama et al., 2007; Stitzel et al., 2007).

1.3 Global changes in the actin cytoskeleton

Vast changes in the cytoskeletal network occur downstream of calcium entry upon egg activation in many species, affecting both the architecture and dynamics of microtubules and actin filaments. Here I shall focus on the array of changes that occur specifically to the networks of actin that exist in the oocyte.

1.3.1 The Actin Cytoskeletal Network at a Glance

Actin is comprised of globular, 42-kDa monomers known as G-actin, which polymerise into polar, double stranded helical filaments known as F-actin (Holmes et al., 1990; Kabsch et al., 1990;

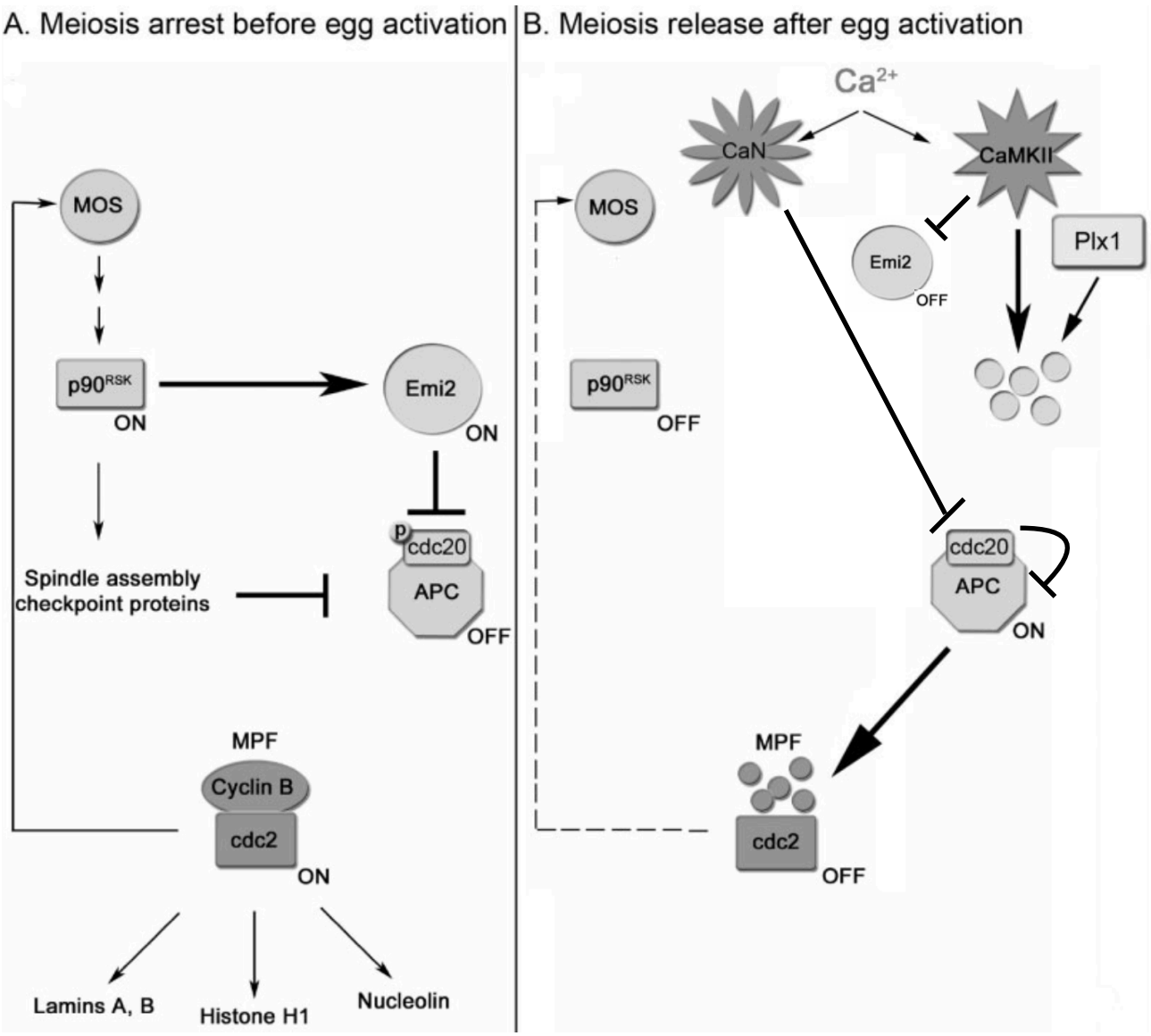

Figure 1.5: Molecular mechanisms of cytostatic factor arrest in *Xenopus* (Adapted from Horner and Wolfner. 2008). During meiosis arrest, the APC is inhibited by the action of spindle assembly checkpoint proteins and Emi2. Upon calcium release at egg activation, CaMKII promotes Plx1 activity and phosphorylates Emi2, targeting the Emi2 for degradation by the SCF ubiquitin ligase. CaN dephosphorylates Cdc20 on the APC, enabling complete activation of the APC. The APC then targets Cyclin B for degradation, deactivating cdc2, and ultimately promoting the activation of key proteins responsible for the events of anaphase.

Blanchoin et al., 2014). G-actin monomers are bound to ATP, and act as ATPases, such that after polymerisation into a filament, the bound ATP is converted into ADP and Pi (Blanchoin et al., 2002; Melki et al., 1996). G-actin may also bind divalent cations such as calcium and magnesium; with magnesium being present in the cell at high concentrations, G-actin is usually loaded with MgATP (Blanchoin et al., 2002; Pollard et al., 2000). Thus, following the rate limiting step which is nucleation, actin filament assembly produces a double stranded helix of F-actin with dynamically different ends, referred to as barbed and pointed. The barbed end elongates much faster than the

pointed end, which is usually enriched with ADP bound G-actin, which may dissociate more readily (Blanchoin et al., 2014).

1.3.2 The Actin Cortex

In oocytes, common arrangements of actin are conserved. Actin can be grouped in multiple ways, including cortical, cytoplasmic and nuclear actin. A vital component of many cells is the cortex, which can be described as a contractile shell of actin, on average between 50-200nm thick (but notably much thicker in certain oocytes), that underlies the plasma membrane (Morone et al., 2006). The cortex is a densely cross-linked network containing hundreds of ABPs, including myosin-2 motors which generate cortical tension (Chugh and Paluch., 2018). The cell cortex is responsible for the control of cellular morphogenesis, division, and polarisation (Chugh and Paluch., 2018; Tsankova et al., 2017).

Assembly of the actin cortex appears to be regulated by two key actin nucleators; formins and the Arp2/3 complex. Formins nucleate and elongate linear actin filaments, whereas the Arp2/3 complex nucleates filaments from pre-existing filaments, driving branch formation (Chugh and Paluch., 2018). The combined action of both nucleates has been demonstrated in melanoma M2 cells in which mass spectrometry and a visual screen revealed the importance of mDia1 (*DIAPH1*) and the Arp2/3 complex (Bovellan et al., 2014). The contributions of various nucleates may not necessarily be equal however, and such contributions may shift to confer specific cortical properties for cellular events; for instance, in *Drosophila* notum cells, upon entry to mitosis, the cortex shifts from being predominantly nucleated by Arp2/3 to formins (Rosa et al., 2015). Evidently, the cortex is a highly dynamic structure that requires much regulation, which may be carried out by hundreds of ABPs.

Common regulators of the actin cortex include regulators of assembly and disassembly, actin cross-linkers, actin-membrane linkers and myosin motors. Well studied regulators of assembly and disassembly are profilin (PFN1), which binds actin monomers to promote actin polymerisation, and cofilin (CFL1 and CFL2), which has actin severing and disassembling properties. Depletion of profilin (*Chickadee*) in *Drosophila* S2 cells results in abnormal cortex contractions and instability of the spindle (Dean et al., 2005), and depletion of profilin in *C. elegans* disrupts actin assembly and leads to failure of cytokinesis (Severson et al., 2002), thus demonstrating the importance of profilin in regulation of the actin cortex. Additionally, the active dephosphorylated cofilin has been shown to be essential for successful cytokinesis, likely preventing excessive F-actin accumulation during the formation of the cleavage furrow (Kaji et al., 2003).

In addition to manipulating the assembly and disassembly, the cortex may be regulated through the action of actin-crosslinkers and actin-membrane linkers. Actin-membrane linkage is largely carried out by the ezrin-radixin-moesin (ERM) family proteins and myosin-1 motors (Biro et al.,

2013; Bretscher et al., 2002). In Drosophila, only Moesin is used of the members of the ERM family, and activation of Moesin in S2 cells is required for cortex stiffening and cell rounding (Carreno et al., 2008; Kunda et al., 2008). The family of actin-crosslinking proteins is broad, including α-actinin proteins, fascin, filamin, fimbrin and spectrins (Reviewed in Chugh and Paluch). Crosslinking proteins can be categorised further, as certain proteins such as fascin and fimbrin generally bundle actin filaments into parallel or anti-parallel stacks, whereas other crosslinkers such as filament organise the actin filaments into more random arrangements. Proteins may also have the capability to create bundles and random networks, as may be the case with α-actinin (Falzone et al., 2012). α-actinin, which is ubiquitously expressed in the *Drosophila* ovaries and embryo, is a particularly interesting example, as non-muscle isoforms demonstrate calcium-dependency on their level of cross-linking; under low intracellular calcium, α-actinin is able to cross-linking multiple actin filaments, however, when the levels of intracellular calcium rise, α-actinin is unable to cross-link multiple filaments, and thus the level of cross linking in the cell decreases (Jayadev et al. 2014; Prebil et al., 2016; Sjöblom et al. 2008). This exemplifies the calcium dependency of many ABPs and cross-linkers, and thus presenting a mechanism through which calcium may directly regulate the state of the actin cortex.

In oocytes, the cortex is a much thicker structure than in most cells, and can be directly measured through optical microscopy; for example, in meiotic mouse oocytes, the cortex thickens from 1.4 to 4um through the action of Arp2/3 and the exclusion of myosin-2 (Chaigne et al., 2013). Dynamics and density of the actin cortex may be greatly regulated through the action of ABPs; cortical thickness is regulated by *CAPZB*, *DIAPH1* and *CFL1* in mitotic HeLa cells (Chugh and Paluch, 2018). Turnover of the actin cortex itself typically happens every few tens of seconds (Fritzsche et al., 2013; Mukhina et al., 2007), however, the action of myosin-2 appears to increase turnover at the contractile ring during cytokinesis (Guha et al., 2005; Murthy and Wadsworth., 2005), meanwhile cross-linking through the action of α-actinin slows turnover (Mukhina et al., 2007). Interestingly, actin-turnover at the cortex may not be equal throughout the cell, as in *Dictyostelium* cells, turnover of cortical components appears to be slower at the equator and faster at the poles (Srivastava and Robinson., 2015).

1.3.3 The Requirement of Actin for Egg Activation

Changes in the dynamics and architecture of the actin network are often required in order for egg activation to occur; in Sea Urchins actin polymerises around the sperm binding site to form a fertilisation cone, promoting fertilisation which triggers egg activation (Terasakki., 1996). What appears to be happening, as indicated through immunofluorescence and electron microscopy, is that actin polymerisation at the point of sperm entry aids in the formation of cytoplasmic bridges which mediate the transfer of the sperm nucleus into the oocyte (Tilney and Jaffe., 1980). Similarly, fertilisation cones were identified in starfish oocytes through the use of Phalloidin staining, suggesting some conservation (Puppo et al. 2008). Induction of multiple fertilisation

cones also resulted in polyspermy, suggesting that the actin cytoskeleton also provides a level of control over fertilisation, through which downstream events of egg activation can complete successfully. Interestingly, it has also been shown that accompanying this dramatic actin polymerisation event is the accumulation of α-actinin at the cortex, which may be playing a role in actin-bundling and thus formation of the cone (Hamaguchi and Mabuchi., 1986).

Actin is evidently playing a role in the successful completion of fertilisation, however, there is also evidence to suggest it plays a more direct role in the events of egg activation, which I will explore in further detail in Chapter 4. For instance, actin may be playing a role in the regulation of calcium entry during egg activation. Pharmacological manipulation of the actin cytoskeleton prevented calcium entry after artificial activation with activation buffer in *Drosophila melanogaster*, suggesting that an intact actin cytoskeleton must be required for calcium entry (York-Andersen et al., 2015). Interestingly, depolymerisation of the actin cytoskeleton in starfish oocytes has been demonstrated to result in calcium influx and the triggering of downstream events (Lim et al., 2002). Treatment of starfish oocytes with the calcium ionophore ionomycin resulted in the dispersion of the cortical actin itself (Vasilev et al., 2012), perhaps suggesting a feedback loop in which actin depolymerisation at the cortex promotes calcium entry, which acts to further depolymerise actin at the cortex.

Further work in echinoderm models corroborates these results. Hyper-polymerisation of the actin cortex through the action of the drug Jasplinakanolide (JAS) compromised cADPR-calcium release and CG exocytosis (Puppo et al., 2008). Latrunculin-A (Lat-A) treatment, resulting in the depolymerisation of the cortical actin cytoskeleton also led to a diminished calcium signal during fertilisation, but a greatly enhanced cortical flash, again reinforcing the theory that a reduced level of cortical actin may be permissive to calcium entry (Puppo et al., 2008). Again, CG exocytosis was prevented by Lat-A treatment, suggesting that regardless of hyper- or hypo-polymerisation, the deregulation of the actin cortex will have a negative impact on certain processes of egg activation (Puppo et al., 2008).

Additionally, there is a plethora evidence for the association of mechanosensitive channels with actin filaments. In mice, it has been shown that actin is required for TRPV3 mediated calcium influx (Lee et al., 2016) and further demonstrated that TRP channels may be regulated through the transduction of force through actin cytoskeleton itself (Christensen and Corey., 2007). Furthermore, it has been established that other mechanosensitive channels, such as the ENaC channel, may bind actin either directly or indirectly through actin-binding factors such as α-actinin, spectrin and filamin (Mazzochi et al., 2006; Maruoka et al., 2000; Cukovic et al., 2001). With TRP channels, it has been demonstrated that TRPN1 co-localises with actin in *Xenopus* cilia (Shin et al., 2005) and that TRPC6 and TRPL interact with regulators of the actin cytoskeleton (Dryer and Reiser., 2010; Chorna-Ornan et al., 2005). Therefore, the actin likely plays a role in transduction of an initial trigger to mediate calcium release or sustain a calcium response, and

thus large scale changes must occur both to induce a calcium response, following which actin dynamics must be reset not just to close channels, but also in preparation for embryogenesis.

1.3.4 Activation induced changes in the actin cytoskeletal networks

In addition to immediate changes at the cortex which appear to occur, such as in the depolymerisation of the actin cortex in starfish oocytes (Vasilev et al., 2012), further downstream changes in the actin cytoskeleton are induced via the intracellular calcium transient, such as broad changes in the cytoplasmic pool of actin and dynamic rearrangements of nuclear actin.

Induction of waves of F-actin appears to occur downstream of the intracellular calcium transient. This has been demonstrated in *Drosophila* oocytes, as labelling of the F-actin network using F-tractin and Lifeact indicates a wave of actin following closely behind that of the calcium wave (York-Andersen et al., 2020). Inhibiting the calcium wave prevents the actin wave, and triggering a rapid increase in calcium from all points over the cortex (cortical increase) results in a more rapid and general increase in the F-tractin signal, suggesting that the calcium wave is sufficient for dictating the polarity of the actin “wave” (York-Andersen et al., 2020). An apparent “wave” of cortical F-actin has also been shown in *Xenopus* oocytes through live visualisation of the F-actin (Bement et al., 2015). Furthermore, live imaging has also demonstrated a wave of Rho-activity, a positive regulator of F-actin polymerisation, suggesting waves of polymerisation (Bement et al., 2015). The role of such waves of actin is poorly understood, however it has been suggested that this could represent a reset in the actin architecture as many dynamic changes may occur in order for calcium to enter and coordinate other key events of egg activation.

Live imaging of the F-actin network in *C. elegans* also shows rearrangements of the actin-cytoskeleton upon egg activation. An actin cap appears in the presumptive posterior of the oocyte, which upon fertilisation disperses within the posterior half of the oocyte (Maruyama et al., 2007). In fact, the actin cytoskeleton is vital for the polarisation of the one cell stage emmbryo, dictating the anteroposterior axis of the embryo following fertilisation, indicated by the loss of polarity that occurs when the embryos are incubated with cytochalasin-D and Lat-A (Goldstein and Hird., 1996; Hill and Strome., 1988; Hill and Strome., 1990). Additionally, the actomyosin cortex of the one cell embryo was demonstrated to form a cortical lattice that asymmetrically contracts to the anterior half of the embryo, which is important for the asymmetric segregation of PAR proteins and thus the generation of the AP axis (Munro et al., 2004). Similar actomyosin dependent contractions occur in other species. Mammals and Zebrafish, for example, exhibit calcium dependent actomyosin contractions following fertilisation, which are also vital for the generation of cytoplasmic flows (Ajduk et al., 2011).

Interestingly, it has been shown that actin structures appear in the meiotic nucleus during egg activation, a key finding that I will demonstrate also demonstrate in *Drosophila* oocytes and examine in Chapter 5. Recently, a filamentous population of actin that resembles the microtubule

spindle both morphologically and functionally was demonstrated in mice and humans (Mogessie and Schuh., 2017; Roeles and Tsiavaliaris. 2019). This population of actin was shown to be generated by Formin-2 in mice, and is required for accurate chromosomal segregation, likely through regulation of the formation of the kinetochore attached microtubules (K-fibres) (Mogessie ands Schuh., 2017). This was corroborated in humans, further showing a role for the spindle-like actin in regulating the formation and structure of the microtubule spindle (Roeles and Tsiavaliaris. 2019). However, to date there is no data that contextualises this population within egg activation and the calcium transients that occur. I explore this in much greater detail in Chapter 5, providing the first evidence of this population in *Drosophila*, identification of essential factors for its production and examination of its role during the metaphase-anaphase transition, specifically providing the first evidence of a role for calcium in regulating the spindle-like actin.

1.4 Drosophila as a Model Organism

Drosophila melanogaster has been shown to be an adaptable model in contributing to our understanding of not only the field of egg activation as previously discussed, but also in our understanding of fundamental biological principles and ultimately acting as a model for human disease, such as neurodegenerative diseases and cancer.

Historically, *Drosophila* have been used as a pioneering model in the field of genetics, with Thomas Hunt Morgan truly acting as the “founder” of *Drosophila* research, utilising this model in order to refine and forefront Mendel’s theory of genetic inheritance (Kohler 1994). *Drosophila* were then used, by the protégé of Morgan himself, Muller, to show that X-rays could be used to induce mutagenesis (Muller., 1928). Not only have *Drosophila* made waves in the field of genetics, but have also been used to prove fundamentals of embryonic development, as in 1995, Christiane Nüsslein-Volhard, Eric Wieschaus, and Ed Lewis won the Nobel prize in Physiology and Medicine for demonstrating the “genetic control of embryonic development”, which has been shown to be critical for animal development universally (nobelprize.org, 2020).

Drosophila boasts one of the most extensive genetic toolkits available to a model system, whether this be the newly developed CRISPR based gene editing or more traditional means of mutagenesis, cloning and genetically driven over-expression/knock down/knock out. For instance, manipulation of gene expression within *Drosophila* is easily controllable using the upstream activating system (UAS), UAS-GAL4,, specifically UASp in the germline. The original UAS system (pUASt) drives excellent expression in somatic tissues, but very poor expression in the germline. As such, the UASp system was developed through fusing 17 copies of the UAS activator to a germline-compatible promoter that is derived from a transposon that is active in the female germline, the *P*-element (Rørth P., 1998). Using this system, a GAL4 can be expressed in a

Drosophila line that drives expression of any construct with a UASp), such as a UASp-RNAi in the germline, which would result in RNAi mediated knock down of target mRNAs. Furthermore, a plethora of imaging constructs are readily available, or at least more easily producible within *Drosophila*, many of which I will utilise throughout this thesis, such as the GCaMP3 calcium sensing construct. Therefore, with *Drosophila* genome approximately 60% homologous to that of humans, with far less redundancy in the genome and around 75% of genes responsible for human diseases having homologs in flies (Ugur et al., 2016), it is clear that biological mechanisms can be studied both *in vivo* and *in vitro* in *Drosophila,* to provide a fundamental base of knowledge that can then be extrapolated to other species.

1.5 Drosophila Oogenesis

1.5.1 Selection and Differentiation of the Oocyte in the Germline Cyst

The process of *Drosophila* oogenesis is referred to as 'meroistic oogenesis' - this term refers to the fact that the developing oocyte remains connected to sister nurse cells via cytoplasmic bridges, and these nurse cells synthesise molecules and organelles to fuel the future development of the embryo. This is the most well-studied form of oogenesis in insects, though other forms of oogenesis exist. Oogenesis is a long process, encompassing stem cell selection and differentiation, early, mid and late-oogenesis and maturation of the oocyte, all stages required in depth study and analysis to understand the myriad events occurring. I will therefore focus on the most relevant parts of oogenesis to highlight how the mature oocyte is produced and prepared for egg activation.

In *Drosophila,* the two ovaries consist of 16-20 strings of developing egg chambers (an oocyte, connected to nurses cells via cytoplasmic bridges and surrounded by a a mono-layer of epithelium, follicle cells) at different stages known as ovarioles (Figure 1.3) (Spradling., 1993). At the tip of these ovarioles exists the stem cell niche within the germarium, in which the germline stem cells (GSCs) undergo rounds of asymmetric division to produce one self-renewing GSC and the cell destined to become an egg chamber: the cystoblast (Fuller and Spradling., 2007). The cystoblast undergoes 4 rounds of incomplete mitotic divisions to produce a 16 cell cyst interconnected by cytoplasmic bridges (ring canals). Two cells have four ring canals, two have three, four have two and eight have one. The two cells with four ring canals (pro-oocytes) eventually differentiate such that one becomes the cell destined to become the oocyte, and the other joins the remaining cells to form a group of 15 nurse cells which will support the growth of the oocyte during oogenesis (Spradling., 1993; Huynh and St Johnston., 2004).

Between the two pro-oocytes there appears to be dynamic competition to decide which will become the oocyte proper (Theurkauf et al., 1993; Huynh and St Johnston., 2004), and it is likely the case that after the first incomplete division the future oocyte may already be determined (de

Cuevas and Spradling., 1998). This selection depends on the formation of a structure known as the fusome, a branching, membranous structure that connects all of the cells within the cyst through the ring canals (Lin and Spradling., 1995). The fusome is derived from the spectrosome, on organelle present in the GSC, made up of membranous vesicles and sub membranous cytoskeletal components such as ankyrins and spectrins (scaffolding proteins that recruit cytoskeleton and thus maintain the plasma membrane's structure) (Yue and Spradling.l, 1992; Lin et al., 1994; Snapp et al., 2004). The fusome orients the mitotic spindle during cytocyte divisions such that the fusome always reside in one of the two daughter cells, leaving one daughter cell without fusome until material is eventually provided through the cytoplasmic connections (Deng and Lin., 1997; McGrail and Hays., 1997; Grieder et al., 2000). Thus, with mitotic divisions inherently asymmetric, the cytocyte with four ring canals that gains the most fusome material becomes destined to be the oocyte (de Cuevas and Spradling., 1998; Grieder et al., 2000). This symmetry breaking event has just recently been illuminated further; how does the presumptive oocyte acquire the most fusome material? Recent work has shown that the microtubule minus end stabilising protein Patronin labels the future oocyte, which is recruited to the fusome via the spectraplackin (part of the spectrin superfamily), Shot (Nashchekin et al., 2016; 2021). This enables the formation of a polarised microtubule network that delivers determinants into the presumptive oocyte, thus selecting one specific cell as the oocyte through amplification of a weak anisotropy of the fusome (Nashchekin et al., 2016; 2021).

Concomitantly with the oocyte being selected, processes that will dictate oocyte differentiation are established. Primarily, the microtubule network of the cyst is polarised to direct major routes of transport towards the oocyte such that nurse-cells can provide essential proteins and mRNAs to the growing egg (Therkauf et al., 1993). Microtubules can be visualised in the cyst, extending from the oocyte through the ring canals into surrounding pro-nurse cells, with microtubule minus ends anchored within the two pro-oocytes (Grieder et al., 2000). Thus the oocyte begins to become polarised during early oogenesis due to clustering of the fusome at the presumptive anterior of the oocyte leading to accumulation of mRNAs and proteins forming a Balbiani body (Cox and Spradling., 2003). These components later dissociate and concentrate at the presumptive posterior in a tight crescent (Paré and Suter., 2000; Huynh et al., 2001), a process shown to be dependent on PAR proteins, as mutants of PAR-1 fail to generate germline cysts with an oocyte; mRNAs and proteins are still translated to the pro-oocyte, yet they fail to relocate to the posterior (Huynh et al., 2001). There is much homology with C. Elegans as PAR proteins are also essential for anterior-posterior polarity in the oocyte, PAR-2 recruiting PAR-1 to the posterior cortex (Boyd et al., 1996).

It is likely that the PAR proteins play a role in organisation of the microtubule cytoskeleton as *par* null mutants fail to properly polarise microtubules such that minus end move from the presumptive anterior to posterior upon localisation of mRNAs and proteins to the posterior (Huynh et al., 2001; Vaccari and Ephrussi., 2002). It has been further elucidated through null mutants of Bicaudal-D and Egalitarian, that these may also be targets of the PAR proteins and play a role in

early polarization of the oocyte, as in the case of mutants, further 16 nurse cell cysts are formed, with centrosomes remaining at the presumptive anterior of what would be the oocyte, rather than repolarizing (Bolivar et al., 2001).

1.5.2 Specification of the Primary Axes during Oogenesis

Drosophila oogenesis can be split into 14 morphologically distinct stages. Stage 1 begins in the germarium following the specification of the oocyte within the gremlin cyst, following this the remainder of stages represent maturing egg chambers, with the most mature moving toward the posterior end of the ovariole, towards the oviduct. Early to Mid oogenesis represents the stages up to 10b, whereupon the supporting nurse cells undergo programmed cell death and 'dump' the remainder of their contents into the oocyte (Weil., 2014).

Primary axis formation begins during early-mid oogenesis in *Drosophila* and continues through the later stages. g*urken* (*grk*) mRNA is initially localised at the posterior end of the oocyte during stages 2-6, whereupon translation of this mRNA (a TGF-alpha homolog) signals to the posterior follicle cells, causing them to adopt a posterior fate, which in turn signal back to the oocyte to trigger a drastic rearrangement of the MT cytoskeleton and movement of the nucleus to the dorso-anterior corner of the oocyte (Gonzales-Reyes et al., 1995; Neuman-Silberberg and Schüpbach., 1993; Weil., 2014). From stage 7-10a, a second round of translation of the *grk* mRNA at the torso-anterior corner generates a signalling cascade with surrounding follicle cells. Gurken binds torpedo on the adjacent follicle cells, causing them to adopt a dorsal identity and preventing pipe synthesis in only the dorsal FCs. Ventral FCs thus produce Pipe which triggers a cascade that culminates in the translocation of Dorsal protein into the ventral FCs, and ensuring ventral identity (Gonzales-Reyes et al., 1995; Zhao et al., 2012; Neuman-Silberberg and Schüpbach., 1994).

In addition to *grk* localisation, *oskar* (*osk*) mRNA is also localised to the posterior of the oocyte during early-mid oogenesis, requiring association with the RNA binding protein Stau (Micklem et al., 2000). Visualisation of the mRNA using the MS2-GFP system demonstrated the presence of fast moving particles that accumulate at the posterior, also confirmed through visualisation of GFP-stau (Zimyanin et al., 2008). Destabilisation of the MT network through injection of the drug colcemid abolished nearly all fast, directed movements of the particles, which appeared to be then moving in a simply Brownian fashion, indicating the involvement of MTs in posterior localisation of *osk.* Thus the dorsal-ventral axis is specified and the anterior-posterior axis begins to develop, which likely influences egg activation given the posterior-anterior polarity of the calcium wave.

By stage 10b, localisation of g*rk* and *osk* have defined the dorso-ventral and the presumptive pole plasm respectively. However, the A/P axis is truly defined during late oogenesis in which localisation of *bicoid* (*bcd*) *and nos* mRNAs is achieved at the anterior and posterior respectively.

In comparison to *osk*, *nos* mRNA localisation to the posterior occurs via a passive process that partially relies on cytoplasmic streaming, an anchoring mechanism in the posterior and with translational repression being vital for preventing *nos* expression in the anterior (Gavis et al., 2008; Forrest and Gavis., 2003; Weil et al., 2006). The localisation of these factors define the posterior and specifically the pole plasm. *Nos* is translated at the posterior, establishing a gradient of protein that defines the A/P axis during embryogenesis. The accurate localisation of *nos* is dependent on an mRNA localisation complex, requiring Aubergine and Rumpelstiltskin that specifically targets the 3' UTR, thus enabling posterior localisation, the mechanism of which remains to be fully investigated (Becalska et al., 2011; Jain and Gavis., 2008).

Similarly, *bcd* mRNA localisation during late oogenesis requires a complex of RNA binding proteins. Various trans-acting factors have been shown to be required through classical genetic approaches, namely Exuperantia (Exu), Swallow (Swa) and Staufen (Stau), also through interaction with the 3' UTR of *bcd* (Ferrandon et al., 1994; St Johnston et al., 1989). *bcd* mRNA localisation and its interaction with multiple factors is often used as a wider model for the mechanisms of mRNA localisation, and as such has been well studied. Swallow was initially thought to link the *bcd* mRNA with Dynein motors in order to control localisation to the anterior of the oocyte, however, super-resolution microscopy did not demonstrate any co-localisation of these factors, and in fact, it is has been shown that Swa reorganises the cytoskeleton to provide MT tracks that the mRNA can be transported to the anterior on (Weil et al., 2010). *Bcd* is thus localised to the anterior of the oocyte, however it is not translated until after egg activation where it can then generate a gradient of protein, opposed to that of Nos, and thus provide A/P positional information during embryogenesis (Gregor et al., 2007; Weil., 2014).

1.5.3 The Mature Drosophila Oocyte

The final step of Drosophila oogenesis involves the maturation of the oocyte from stage 12/13 to stage 14, resulting in the production of an egg that is capable of undergoing egg activation and thus embryogenesis.

Oocyte maturation represents the progression of the oocyte from the primary meiotic arrest, at prophase I, to the secondary meiotic arrest, at metaphase I (Aviles-Pagan and Orr-Weaver., 2018). This results in breakdown of the nuclear envelope (also known as germinal vesicle breakdown, GVBD) and the generation of a meiotic spindle, held stably together by microtubules. The specific signal that triggers the onset of maturation remains enigmatic, however, the role of cell cycle regulators has been well studied, and much conservation with vertebrate models is seen. Primarily, the action of CyclinB and CDK1 results in the breakdown of the nuclear envelope and

the assembly of the meiotic spindle, as in vertebrates (Aviles-Pagan and Orr-Weaver., 2018). However, in *Drosophila*, cyclins A and B3 also contribute to maturation of the oocyte (Bourouh et al., 2016). The action of these cyclins is dependent on the balance of Matrimony (Mtrm) and Polo-Kinase; Mtrm acts on Cdc25 phosphatase Twine to maintain prophase arrest, but it appears that upon stage 13, the levels of Polo-Kinase may exceed that of Mtrm to thus activate Twine, which can then activate cyclins A and B3 (Xiang et al., 2007; Von-Stetina et al., 2008). Additionally, maturation of the stage 13 oocyte is likely regulated by the factors Endos and Greatwall kinase, as exemplified through classical genetic experiments in which Endos mutants would result in failures of nuclear envelope breakdown and progression into metaphase I (Williams et al., 2014; Archambault et al., 2007; Vigneron et al., 2016; Von Stetina et al., 2008). Evidently, when phosphorylated by Greatwall kinase, Endos can inhibit the PP2A phosphate and this likely would result in the onset of meiosis (Williams et al., 2014.)

Thus the stages of oogenesis culminate in the formation of the mature, stage 14 Drosophila oocyte. This oocyte exists in a dehydrated state within the ovary (Figure 1.3, 1.5), whereupon it

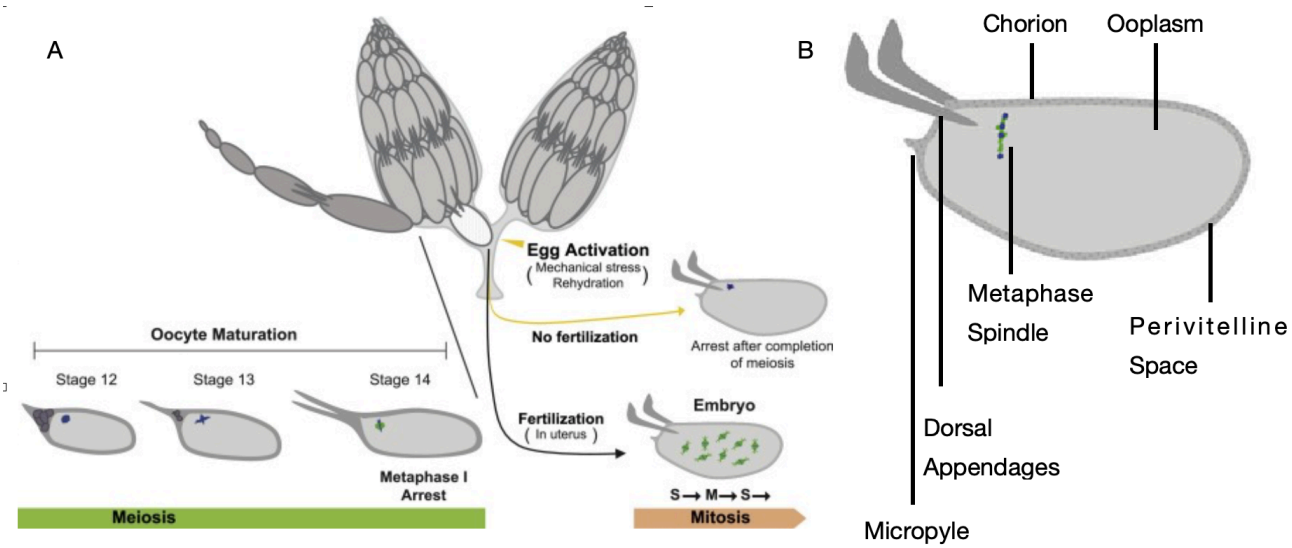

Figure 1.6: Production of a mature oocyte capable of egg activation

(A) Following maturation of the oocyte, the egg passes into the oviduct where it undergoes rehydration and egg activation. (B) A diagram of a mature oocyte with relevant features labelled.
Taken from Aviles-Pagan and Orr-Weaver., 2018

will eventually pass through the oviduct and become rehydrated (Figure 1.3, 1.5). This triggers the process of egg activation. Many questions remain surrounding this; 1) What is the source of calcium?; 2) What channels mediate calcium entry at egg activation?; 3) How is calcium entry mediated such that a polar wave occurs? These overall processes of egg activation, and specifically these questions, will be explored in detail in Chapter 3.

1.6 Focus and Aims of this PhD Thesis

In this introduction I have provided an overview of the varied mechanisms of egg activation from a number of species, focussing in particular on the roles of calcium and actin during this fundamental, developmental transition. With the vast array of genetic tools available in *Drosophila melanogaster*, I will demonstrate that this provides an invaluable tool to our understanding of egg activation more generally. *Drosophila* acts as a key model for understanding fundamental and conserved cellular processes, such as mechanisms of calcium flux (Chapter 3), regulatory functions of the cortical actin (Chapter 4), and mechanisms of actin regulated meiosis (Chapter 5), highlighting the need for further research into the interplay between calcium and actin- a theme that thoroughly permeates this thesis.

The general aims of this thesis are outlined below:

1- In Chapter 3, I aim to further investigate the mechanism of calcium entry at egg activation in mature *Drosophila* oocytes. Thus far, the field has established that a mechanical cue triggers calcium influx in *Drosophila* oocytes, which can be recapitulated by incubation of mature oocytes in a low osmolarity solution to cause oocyte swelling.

I will therefore investigate the hypothesis that the calcium entry occurs through channels in the oocyte membrane, entering the oocyte from the perivitelline space in a regulated manner.

In order to elucidate the mechanisms underpinning this hypothesis, I will use genetic and pharmacological tools, such as the well established UAS-GAL4 driven germline RNAi mediated knock down. To this effect, I aim to pinpoint which channels are required for calcium entry at egg activation and from which cellular store of calcium the influx may originate.

I will also investigate a phenotype which has, thus far, not been investigated: the cortical increase. This phenotype I hypothesise to be representative of a calcium flux event at egg activation that lacks the regulation required in order to generate a propagating wave of calcium. This phenotype can be forced through incubation with an extremely low osmolarity solution, such as water, which I aim to use in

order to demonstrate key principles of calcium entry at egg activation, such as the capacity of the oocyte to generate a calcium flux at multiple points around the cortex. I also aim to establish the dynamics of this phenotype as it appears to be a typical calcium phenotype that occurs in activating oocytes that have a disrupted actin cytoskeleton, as I will show in Chapter 4.

2- In Chapter 4, I aim to build on the work of the previous chapter by investigating more specifically what the role of the actin cytoskeleton is in regulating calcium entry at egg activation in *Drosophila* oocytes.

The interplay of actin and calcium is a field in which much is still not understood, but it is clear that they are highly interdependent. I hypothesise that the actin cytoskeleton, and in particular the cortical actin cytoskeleton, plays a role in the regulation of calcium entry at egg activation. There are two working hypotheses that may function exclusively or potentially together: i) The cortical actin prevents calcium entry, for example through providing a highly cross-linked meshwork that prevents the opening of calcium channels in the oocyte membrane; ii) The cortical actin is required for calcium entry, for example through the provision of a mechanical link between calcium channels that is able to transduce the force of the oocyte swelling into the opening of said calcium channels.

In order to investigate these hypotheses, I will firstly employ a range of tools to visualise the cortical and cytoplasmic actin networks both fixed and live. In this way I will determine the natural state of the cytoskeleton both prior to egg activation, during egg activation, and following egg activation to evaluate whether this demonstrates any key morphological indicators of its role throughout the process. I will further employ pharmacological tools, such as cytochalasin-D, in order to disrupt the actin cytoskeleton and investigate its effect thereon the calcium flux at egg activation. Finally, I will manipulate the levels of the actin cytoskeleton through genetically mediated knockout and knock down of actin nucleators, and conversely through overexpression of nucleators, in order to determine how the levels of actin in the oocyte translate into varied calcium flux phenotypes. I will further aim to establish which key actin regulators in the *Drosophila* oocyte impact on calcium flux.

3- In Chapter 5, I demonstrate a novel population of the actin cytoskeleton in *Drosophila* oocytes; a spindle-like actin pool that appears conserved to those recently demonstrated in mammals. I therefore aim to establish the role of this population of actin in *Drosophila* oocytes, and specifically to contextualise this with the major developmental event of egg activation.

I hypothesise that the population of spindle-like actin will demonstrate a great deal of conservation with mammalian systems, as the metaphase-anaphase transition in oocytes itself is conserved. It is likely that the spindle-like actin will be required for regulation of the microtubule morphology and its key role in chromosomal alignment and segregation. I further hypothesise that the spindle-like actin will interact with the major calcium signalling event that occurs at egg activation. As this occurs at the first metaphase-anaphase transition during meiosis in *Drosophila* (rather than the second which is seen in Mice), it is likely that the spindle-like actin plays a greater role at these earlier stages of meiosis in *Drosophila*, potentially being required for not only the chromosomal movements during meiosis, but also for potentiating metaphase arrest.

In order to investigate these hypotheses, I will first aim to establish which actin nucleators are required for formation of the spindle-like actin. I will use mutant lines of the actin nucleators Cappuccino and Spire to demonstrate that they are required for the accurate formation of the spindle-like actin. To this effect I will aim to demonstrate conservation of nucleation from *Drosophila* to mammals. I will further utilise imaging techniques such as Fluorescence Recovery after Photobleaching (FRAP) to evaluate the impact of the spindle-like actin on the dynamics of the microtubule spindle.

I will further use the tools established in the prior chapters, in addition to further tools such as the actin labelling agent Utrophin-GFP, to visualise live and fixed spindle populations (both microtubules and actin) in oocytes during the metaphase-anaphase transition that occurs at egg activation.

I will disrupt the actin cytoskeleton through incubation with cytochalasin-D and through knockout of key actin nucleators in order to establish the role of the spindle-actin, and actin more generally, on key elements of the spindle. To this

effect, I visualise the chromosomes and microtubules throughout the transition in both wild-type control and actin-disrupted backgrounds, therefore elucidating the role of actin in regulating the microtubule spindle and chromosomal movements.

I will further demonstrate a key relationship between this spindle-like actin and the calcium signalling pathways within the oocyte through use the established calcium indicator GCaMP3 and pharmacological agents such as BAPTA that I have established in earlier chapters. Ultimately, throughout this thesis I greatly further the understanding of the field of egg activation as a whole, and reveal new and unique mechanisms at play that should be tested in other systems.

I will therefore address the gaps in our understanding of calcium entry at egg activation in *Drosophila* in Chapter 3, highlighting unique mechanisms of calcium flux and the key channels that play a role in this. I will provide concrete evidence that indicates Trpm is required for calcium flux, yet also reveal calcium transient phenotypes that do not fit the standard model in *Drosophila* that highlight a key requirement for an intracellular regulator of calcium entry. I will therefore investigate this further in Chapter 4 by asking, “What regulates calcium flux?”. Focussing on the cortical actin, I will reveal a fascinating correlation between the state of the cortical actin and the ability of the oocyte to generate a calcium transient, pinpointing key nucleating and actin-binding factors that play a role in regulating the architecture of the cortex and thus its ability to regulate calcium entry. I will then extend this theme of calcium-actin interplay into Chapter 5 by investigating the role of actin downstream of the calcium wave- specifically I will reveal a novel population of actin within the meiotic spindle that regulates chromosomal movements and overall spindle morphology. I will further demonstrate a key relationship between this spindle-like actin and the calcium signalling pathways within the oocyte. Ultimately, throughout this thesis I greatly further the understanding of the field of egg activation as a whole, and reveal new and unique mechanisms at play that should be tested in other systems.

Chapter 2:

Materials and Methods

2.1 Fly Maintenance

Fly stocks were raised on Iberian recipe fly food at 18°C, 21°C and 25°C. The stocks were kept in vials and/or bottles and flipped every 2-4 weeks. To expand stocks in vials, flies were transferred to bottles. For dissection of mature oocytes, approximately 30 female flies with 5 male flies were transferred into a vial with Iberian recipe fly food and wet yeast for 48 hours at 25°C.

2.2 Fly Strains

Fly Line	*Genotype*	Chapter(s) used:	Reference
GCaMP3	*matα-GAL4::VP16, UASp-GCaMP3*	3-5:	Kaneuchi et al., 2015
Tubulin-Gal4	*tub-GAL4VP16*	3-5:	Siegfried Roth
Trpm RNAi	*P{TRiP.GL00173}attP2*	3:	BL35581
Trpm RNAi	*P{TRiP.HMC02889} attP2/TM3, Sb1*	3:	BL44503
Flower mutant	*fweEY08496*	3:	BL16896
Flower RNAi	*TRiP.GL01498*	3:	Ni et al., 2010
Innexin RNAi	*TRiP.HMS02481*	3:	Ni et al., 2010
STIM RNAi	*TRiP.GLC01785*	3:	Ni et al., 2010
ORAI RNAi	*TRiP.HMC03562*	3:	Ni et al., 2010
Nudel mutant	$nudel^{7}$	3:	Tearle and Nusslein-Volhard, 1987
Nudel RNAi	*TRiP.HMC03171*	3:	Perkins et al., 2009
Vm26Ab mutant	$vm26Ab^{QJ42}$	3:	Schupbach and Wieschaus, 1991
Vm26Ab RNAi	*TRiP.HMC05896*	3:	Perkins et al., 2009
Moesin::GFP	*GFP::Moesin*	4:	Edwards et al., 1997
F-Tractin.tdTomato	*UASp-F-tractin.tdTomato/Tm3*	4:	BL58989
UtrCH-GFP	*UASp-Utrophin-CH-GFP/Tm3*	5:	Lecuit et al., 2010
Lifeact-GFP	*UASp-LifeactGFP/ Tm3*	4-5:	BL58717
Ressille::GFP	*ressille::GFP/Cyo*	4:	Maik Drescher
Act5C-GFP	*UASp-Act5CGFP/Tm3*	4-5:	BL7309

Fly Line	*Genotype*	Chapter(s) used:	Reference
Jupiter-GFP	*P{PTT-GA} JupiterG00147*	3-5:	BL6836
Calmodulin::GFP	*P{PTT-un} CamP00695/cyo*	5:	BL50843
SCAR RNAi	*P{TRiP.HMC03361} attP40*	4:	BL51803
Cappuccino Overexpression	*sna/cyo;otu-capu*	4:	BL8819
Cappuccino mutant	$capu^{EY12344}$	4:	Maik Drescher
Cappuccino mutant	$capu^{EE}$*/cyo*	4-5:	BL8788
SpireB overexpression	*UASp-SpireB-RFP*	4:	Maik Drescher
SpireB RNAi	*P{TRiP.GL01503}attP2*	4:	BL43161
SpireB Mutant	$spire^{2F}$*/cyo*	5:	BL8723
Moesin RNAi	*P{TRiP.HMS00886} attP2*	4:	BL33936
Quail RNAi	*P{TRiP.GL01284}attP2*	4:	BL41856
α-actinin RNAi	*P{TRiP.HMS00193} attP2*	4:	BL34874
α-actinin mutant	$actn^{EP1193}$*/fm7a*	4:	BL10894
Supervillin RNAi	*P{TRiP.HMJ30135} attP40*	4:	BL63569
Supervillin mutant	$svil^{EY06775}$*/Tm3*	4:	BL16756
Gelsolin RNAi	*P{TRiP.HMS02269} attP2*	4:	BL41704

Table 1: Summary of fly strains used throughout this thesis

2.3 Live Sample Preparation

2.3.1 Preparing Oocytes: Ovaries were dissected, from flies that were no more than 2 weeks old and that were fattened for 48 hours on yeast, into series 95 halocarbon oil using a probe and fine tweezers (Fine Science Tools) as described previously (Weil et al., 2012). Mature oocytes were gently teased out of the ovaries and aligned parallel to each other for ease of imaging. Excess ovarian tissue was removed using a probe. Oocytes were left for 10 minutes prior to imaging to allow them to settle onto the coverslip or glass slide. The protocol described was adapted from Derrick et al., 2016.

2.3.2 Preparing *in vivo* activated eggs: Ovaries were carefully dissected from flies fattened for 48 hours on yeast, such that the oviduct was still in tact, into series 95 halocarbon oil. Ovaries were selected that displayed mature stage 14 oocytes that had begun passage through the oviduct (lateral or common). Oocytes were then gently teased out of the oviduct rather than the ovaries, carefully removing the surrounding oviduct tissues. Several ovaries were dissected at once such that multiple ovulated oocytes could be selected and aligned on a 22 by 40mm cover slip.

2.3.3 Preparing laid eggs: Flies were anaesthetised with CO_2 resulting in the relaxing of abdominal muscles and release of an egg. This egg was gently removed from the tip of the abdomen and placed in series 95 halocarbon oil. Multiple eggs were removed from multiple *Drosophila* and aligned parallel to each other on a 22 by 40mm cover slip.

2.3.4 Preparing whole flies for *in vivo* imaging:

Adult female *Drosophila* were anaesthetised on CO_2 and gently placed into a glass Pasteur pipette such that they become trapped in the narrow tip. The end of the tip is dipped into wet yeast that is as close to accessible to the fly as possible. The pipette is then sealed using cotton wool such that the fly cannot escape. The pipette is then mounted on a fluorescence scope and rotated such that imaging occurs through the dorsal side of the abdomen.

2.4 Preparation of fixed samples

This method is adapted from York-Andersen et al., 2020- in brief: 10 to 20 ovaries were dissected from flies fattened for 48 hours on yeast into Schneider's Insect Medium (SIM) (Sigma-Aldrich) in a glass bottom dish. Ovaries were splayed and oocytes gently teased out using fine forceps (11251-30 Dumont #5 forceps, Fine Science Tools) and a dissecting probe (0.25 mm straight 10140-01, Fine Science Tools). Mature oocytes were transferred into an 0.5mL eppendorf tube using a glass pipette. SIM was removed and 500 µL 4% paraformaldehyde (PFA) (Sigma Aldrich) added for 10 minutes on a rotary machine (PTR-35 360 vertical multi-function rotator, Thomas Scientific). Oocytes were then washed for 10 minutes, three times in 0.1% PBST (0.1% Triton-X 100 in PBS). Oocytes were then incubated for 2 hours in 1% PBST with the following labelling probes, before washing, staining in glycerol and DAPI and mounting on a glass slide in Vectashield with DAPI (Vector Laboratories): Alexa-Fluor Phalloidin 568, 1:500 (Molecular Probes); Alexa-Fluor Phalloidin 637, 1:500 (Molecular Probes); ChromoTek GFP-booster, 1:500 (Proteintech).

Dye/Probe:	Cell Component Labelled	Concentration	Source
Alexa-Fluor Phalloidin 637	F-actin	1:500	Sigma Aldrich
GFP-booster	Anti-GFP Nanobody enhancing signal of GFP-label.	1:500	Thermofisher Scientific

Table 2: Summary of dyes used.

2.5 Ex vivo egg activation, solutions and pharmacological treatments

2.5.1 Ex vivo egg activation

Eggs were activated *ex vivo,* adapted from Derrick et al., 2016- In brief: mature oocytes were first dissected as previously described into series 95 halocarbon oil. They were activated *ex vivo* through addition of the hypotonic, 260 mOsm, activation buffer (AB): 3.3 mM NaH_2PO_4, 16.6 mM KH_2PO_4, 10mM NaCl, 50 mM KCl, 5% polyethylene glycol (PEG) 8000, 2mM $CaCl_2$, brought to pH 6.4 with a 1:5 ratio of NaOH:KOH (Mahowald et al., 1983). AB was added either with a glass pipette, or for more fine control using a P200 Gilson Pipette, enabling displacement of the series 95 halocarbon oil. Incubation for 5 minutes is sufficient to activate oocytes, with the majority of oocytes activating within the first minute of incubation.

2.5.2 Schneider's Insect Medium, PBS and DMSO controls

All pharmacological incubations were validated with either a Schneider's Insect Medium (SIM) or PBS control and a DMSO control. Oocytes were incubated in SIM or PBS for 10 minutes whereupon samples were flooded with AB and visualised. Oocytes were incubated in DMSO diluted to a 1:1000 concentration (or an equivalent concentration based on the stock concentration of the drug) and visualised.

2.5.3 Cytochalasin-D, Colchicine and BAPTA Incubation

Stock solutions of cytochalasin-D were made in DMSO. Cytochalasin-D (Sigma Aldrich) was made to a final concentration of 2-20 μM in SIM or PBS, ensuring at least a 1000x dilution of the DMSO. Oocytes were dissected from mature female *Drosophila* and incubated for 10 to 30 minutes in this solution, within a glass bottom dish, prior to fixation. For live imaging, oocytes were dissected onto a glass coverslip in series 95 halocarbon oil and 2-3 drops of the cytochalasin D solution was added during imaging using a P200 Gilson pipette. Following 10-30 minutes incubation, activation buffer or control PBS/SIM was added using a P200 Gilson pipette in an amount to flood the sample and dilute out the cytochalasin-D. Stock solutions of colchicine were made in DMSO. Colchicine (Sigma Aldrich) was made to a final concentration of 50 μM in SIM or PBS, ensuring at least a 1000x dilution of the DMSO. Oocytes were incubated for at least 30 minutes in this solution prior to fixation as before, or flooding sample with AB for live imaging as before.

BAPTA and BAPTA-AM (Sigma Aldrich) were made to a final concentration of 33 μM in AB or PBS. Oocytes were activated immediately using BAPTA / BAPTA-AM diluted in AB, or incubated in PBS for 10 minutes to enable permeation of BAPTA-AM (or indeed no permeation of BAPTA - a control) prior to removing excess PBS and flooding with AB in order to attempt activation of the oocyte. Oocytes were imaged using confocal microscopy as described above for the imagine of whole calcium transients.

2.7 Cold-mediated depolymerisation assay

Mature oocytes expressing Jupiter-GFP were dissected into series 95 halocarbon oil on a 22 by 40mm cover slip using fine forceps and a probes as described above. The cover slip was cooled at 4 degrees in a fridge for 1 hour or -20 degrees in a freezer for 10 minutes. Oocytes were quickly transferred to the stage of the Olympus FV3000 for imaging as temperature returns to room temperature.

2.8 Fluorescent Imaging

For the *in vivo* egg activation assay, imaging was performed on a fluorescent stereoscope using the 0.25 NA, 10X objective. 488nm GFP excitation laser was set to 100%. A time-series was generated as images were acquired every 3 seconds. The GFP excitation laser intensity may be altered, but for imaging of *in vivo* egg activation the laser must be able to penetrate the cuticle of the abdomen in addition to tissued surrounding the ovaries, therefore a high excitation intensity is required.

2.9 Confocal Imaging

2.9.1 General Imaging

Fixed and live imaging was performed using either the inverted Leica SP5 or Olympus FV3000. The Leica was used for earlier whole oocyte imaging, using the 0.7 NA 20X objective. Using the Leica, settings were set such that: RFPs were excited with the 561 laser, detecting emission using HYD1 detectors set for a range of 570-700nm; GFPs were excited with the 488 laser, detecting emission using the HYD2 detectors set for a range of 500-570nm.

For live imaging of whole oocytes using the Leica SP5, Z-stacks were taken from the shallowest visible plane of the oocyte (typically just below the chorion) and images acquired every 1 or 2 μm up to a depth of 40 μm unless stated otherwise. The Olympus FV3000 was used for later whole oocyte imaging using the 1.05 NA 30X silicone objective, and imaging of intracellular components using the 1.35 NA 60X silicone objective.

2.9.2 Imaging of Calcium Waves

For high resolution imaging of calcium waves, oocytes were aligned in parallel on a 22 x 40mm coverslip in series 95 halocarbon oil. High resolution images were collected using an inverted Olympus FV3000 confocal microscope. Parameters for image collection were: 1.05 NA 60X

silicone objective, 40 µm Z-stack, 1 µm between each Z-slice, 640 x 640 resolution such that each stack was complete in approximately 30 seconds. As such, the time resolution for quantifying waves is 30 seconds. One full z-stack was allowed prior to addition of any solution, such as AB. This method is further described in York-Andersen et al., 2015; 2021.

2.9.3 Imaging of Actin and Spindle

For high resolution imaging of cortical actin, oocytes were mounted in a glass-bottomed culture dish (MatTek) in Schneider's Insect Medium (Sigma Aldrich) with a 1 mm^2 coverslip on the oocyte. For activation, Schneider's medium was gently removed and replaced with AB (Weil et al., 2008). High-resolution 3D images of the cortex were collected using an Olympus FV3000 confocal microscope, method described in detail in York-Andersen et al., 2020. Parameters for image collection were: 1.35 NA 60x silicon immersion objective, 60 µm Z-stack, 0.34 µm between each Z-slice, 2,048×2,048 pixel area of varying ROIs at the cortex, approximately 8–10 min per complete acquisition.

For high resolution imaging of the spindle, oocytes were oriented on the coverslip such that the dorsal appendages were in contact with the surface of the cover slip, therefore the dorsal side of the oocyte becomes the shallowest plane of visualisation, High-resolution images of the spindle were collected using an Olympus FV3000 confocal microscope. Parameters for image collection were: 1.35 NA 60x silicon immersion objective, 10 µm Z-stack, 0.5 µm between each Z-slice, 1024×1024 pixel area, approximately 30 seconds per stack.

2.9.4 Fluorescence Recovery After Photobleaching (FRAP) analysis

For FRAP of cortical actin or spindle components, Act5C-GFP or JupiterGFP at the cortex was bleached for 10 seconds using 100% laser intensity of the 405nm laser channel. Time lapse series of recovery was recorded every 5 seconds in single plane imaging of the cortex or every 30 seconds in Z-stack imaging of the spindle, both using the 488nm laser channel, 2 Airy unit pinhole, 1024x1024 pixels and the 1.35 NA 60x silicon immersion objective. For all FRAP series, background correction was performed by subtracting the fluorescence intensities of the unbleached cytoplasmic area from fluorescent intensities of bleached regions, with percentage fluorescence of the maximum plotted in graphs. The half life and immobile/mobile fractions were calculated graphically.

2.10 RT-PCR protocol

2.10.1 RNA Extraction

Approximately 20 ovaries were collected into a glass bottom dish containing SIM. These were transferred to a 1.5mL eppendorf, where SIM was removed and 500 uL of TRIzol (Thermofisher Scientific) was added. Ovaries were crushed using a pestle and left for 10 minutes at room temperature. 110 µL of chloroform was added (Sigma Aldrich) and the sample vortexed. The sample was spin for 10 minutes at 4 degrees celsius. The supernatant was removed and added to

500 µL of isopropanol, where the solution was mixed. This was left for 20 minutes at room temperature, or at least 2 hours at -20 degrees Celsius (but best left overnight at this temperature). This method was adapted from Hu and Wolfner., 2020 and the Invitrogen TriReagent user guide (Thermofisher Scientific).

2.10.2 cDNA synthesis

The solution of isopropanol, chloroform and RNA was spun at 5000 rpm for 15 minutes at 4 degrees celsius. The supernatant was discarded and the pellet washed with 70% EtOH in depc water. This solution was spun at 5000 rpm for 5 minutes at 4 degrees celsius. The supernatant was discarded and the pellet resuspended in 15-30 µL of depc water (using less depc water if the pellet was small). The RNA concentration (in ng/µL) was then measured using a Thermo Scientific Nanodrop 200- ND-2000 UV-Vis Spectrophotometer.

The equivalent of 2 µg of RNA was calculated based on the volume (15-30 µL) and concentration (X ng/µL), taken and added to a PCR tube. Reverse transcription was carried out using a First-Strand cDNA Synthesis Using SuperScriptTM II RT kit (Thermofisher Scientific) according to a standard protocol that can be found on the Thermofisher Scientific website (Kotewizc et al., 1985; 1989; Invitrogen). The final mix was incubated in a thermal cycler using a standard PCR thermal cycling protocol (25°C (10 min); 37°C (120 min); 85°C (5 min), after which the cDNA was then held at 4°C and, if necessary, stored at -20°C before use).

2.10.3 qPCR

10 µL of master mix from a IQ SYBR Green Supermix (Bio Rad) was added to a 96 well plate. IQ SYBR Green supermix and frozen reaction components were thawed on ice. A master mix was generated of the SYBR green Supermix (10 µL per well), 100-500 nM (per well) of forward and reverse primers of *trpm* and housekeeping genes *rpl32, α-tubulin and actin42a* (detailed in the table below) and depc water to bring the final volume in each well to 20 µL following addition of cDNA template. Mastermix was added to the 96 well plate on ice. Standardised dilutions of (10^{-1}, 10^{-2} and 10^{-3}) cDNA were added to 96 well plate (typically around 5 µL per well) bringing the final volume per well to 20 µL. Each experimental condition was performed in triplicate, such that 3 wells would correspond to one cDNA dilution and primer set. The 96 well plate was loaded into a BioRad iCycler with a standard qPCR setting (1 x 95°C (2 min); 40 or 50 cycles of 95°C (30s), 55°C (30s), 72°C (30s); 1 x 55°C (1 min)).

Gene	Forward Primer	Reverse Primer
trpm	TCACTGTGCTGGTGAAGATG	CCAGAGGTCCCAGGTATTTATTC
rpl32	CACCAGTCGGATCGATATGC	CGATCCGTAACCGATGTTG
actin42a	GCGTCGGTCAATTCAATCTT	AAGCTGCAACCTCTTCGTCA
α-tubulin	AGGATGCGGCGAATAACT	CGGTGGATAGTCGCTCAA

Table 3: Primer sequences used for qPCR analysis

The expression level of Trpm was calculated relative to the endogenous control utilising the comparative cycle threshold (CT) method (Livak & Schmittgen, 2001). Assuming consistent efficiency of amplification, one can calculate the relative expression (ER) as follows:

$$\Delta CT(S) = CT(Ginterest) - CT(Greference)$$

$$\Delta\Delta CT = \Delta CT(Stest) - \Delta CT(Scontrol)$$

$$ER = 2^{-\Delta\Delta CT}$$

S indicates a sample and CT(G) indicates the mean CT for a gene where n = 3. In each case the error bars were plotted to present the range of ER values calculated from ΔCT(S) values ± one standard error.

2.11 Quantifications and Statistical Analysis

Calcium wave phenotypic data was analysed statistically using the Fisher's exact test, enabling evaluation of the difference between proportions of two nominal variables such as "Full Calcium Wave" vs "No Calcium Wave". This test was selected due to the relatively small sample sizes (less than 50) when compared to those that can be used in a Chi-Squared test. Statistical analysis of calcium wave speeds and initiation times was performed using an unpaired students t-test on the assumption of the same variance between samples. Standard P values were employed, ie. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***). Variation, in particular standard deviations, were calculated based on the variance within a minimum three repeated sample groups from which a percentage was calculated- standard deviation therefore represents variation of the percentage of full wave phenotypes observed.

Measurements of the dimensions of the cortical actin, maximum chromosomal distances, number of aberrant chromosomes and spindle lengths, widths and angle from the cortex was performed on FiJi. Statical analysis was carried out using an unpaired student's T-test using GraphPad Prism software (Dotmatics, 2020). A paired T-test would be optimal for comparison on variable pre and post-activation, however due to technical difficulties in image acquisition,

unpaired T-tests were performed following observation of equal variance of groups through an F test using GraphPad Prism software (which is carried out by the software automatically as part of the T-test) (Dotmatics, 2020).

Proportions of oocytes with spindle-like actin in different genetic knockdowns was analysed statistically using the Fisher's exact test due to the relatively small sample size. Standard P values were employed, ie. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

Chapter 3:

Mechanisms of calcium entry during *Drosophila* egg activation

Chapter 3: Mechanisms of calcium entry during *Drosophila* egg activation

3.1 Introduction

3.1.1 Chapter Overview

Previous work has established that calcium entry into the *Drosophila* oocyte occurs via an osmolarity dependent mechanism, suggesting that swelling of the oocyte due to the uptake of fluid in the oviduct is the trigger for egg activation, opening mechanically gated channels in the oocyte membrane (York-Andersen et al., 2015; 2021).

In *Drosophila*, calcium entry occurs in the form a wave, usually with a calcium transient beginning at one of the oocyte poles and moving to the opposite. In this chapter I will provide further analysis of the standard dynamics of the calcium wave in *Drosophila*, and explore in more detail an alternative phenotype that exists; the cortical increase. This phenotype is extremely prevalent when eggs are activated in very low osmolarity conditions (eg. in distilled water) (York-Andersen et al. 2021). This phenotype was named as such as it appears that calcium entry occurs from all over the cortex, rather than from a single pole, and additionally the completion of the calcium transient occurs much quicker. As of yet, the dynamics and importance of the cortical increase phenotype have not been explored which is essential for providing a more complete understanding of a model of calcium entry in activating *Drosophila* oocytes. Furthermore, activation of oocytes in a low osmolarity solution to “force” a cortical increase could be used as a tool for investigating the regulation (or indeed mis-regulation) of calcium entry in oocytes. In this chapter and in the following chapter it therefore becomes apparent that the regulation of wave phenotypes can be mediated by the actin cytoskeleton.

In this chapter, I will provide evidence to suggest that membrane channels are involved in calcium entry upon exposure to a solution that is hyposmotic to the oocyte. At the onset of this thesis, preliminary evidence suggested the mechanosensitive Trpm may play a role in calcium entry at egg activation. I demonstrate the requirement of the channel Trpm, with increasing evidence that would suggest activation is sensitive to the concentration of Trpm protein available. I further test other candidate membrane channels and Flower calcium channel was identified as an alternative channel through which calcium entry occurs, suggesting a potential role for a membrane voltage dependent mechanism of calcium entry.

The cortical increase phenotype suggests calcium entry is possible from all points around the cortex, rather than just the poles as previously observed. Furthermore, the identification of specific membrane calcium channels indicates calcium flux across a membrane. Together these data point to a source of calcium external to the oocyte, potentially the perivitelline space. Various BAPTA treatments exclude the external environment as the source of calcium, implicating a store internal to the egg chamber, leaving the only possibility as the perivitelline space. This is further

evidenced through knock out and knock down of key vitelline membrane proteins, preventing the standard calcium wave from happening upon addition of a hyposmotic solution.

3.1.2 The Mature Oocyte

In order to understand the key processes occurring at egg activation, it is prudent to understand the current state of the oocyte as it is stored in the ovary, awaiting activation and fertilisation. The later stages of oogenesis, from stage 11 onward, are responsible for ensuring the mature oocyte is prepared to activate; part of this process is carried out by the follicle cells that encompass the maturing egg chamber. The follicle cells play a vital role in the production of numerous structures that contribute to the surrounding layers of the oocyte: the perivitelline membrane, chorion, dorsal appendages and micropyle (Figure 3.1).

The drosophila eggshell is a multilayered structure that is produced by the ovarian follicles through the secretion of essential eggshell proteins during the latter stages of oogenesis (Margaritis., 1985). Most proximal to the oocyte surface is the vitelline membrane, encapsulating a region around the oocyte known as the perivitelline space (PV Space). Not a great deal is known about the PV space, but studies in the early *Drosophila* embryo have indicated the presence of many ions including calcium (Van Der Meer and Jaffe., 1983). The next layer is the inner chorion and then a tripartite endochorion (Pascucci et al., 1996). Immuno-electron microscopy further validated this by following the distribution of eggshell proteins throughout development, confirming the vitelline membrane appears to be morphologically complete by stage 11, whereas the chorion is not fully formed until stages 13/14, whereupon activation cross-liking of chorion proteins hardens the eggshell (Pascucci et al., 1996; Horner and Wolfer., 2008).

Concomitantly with the production of the vitelline membrane and chorion, specialised follicle cells are responsible for the production of the micropyle, a region essential for sperm entry, and dorsal appendages, structures that are thought to be required for oxygenation of the oocyte (Ward and Berg., 2005). These follicle cells, roof and floor cells, become specified early in oogenesis and undergo large-scale morphogenetic movements and tubulogenesis, forming the dorsal appendages (Ward and Berg., 2005). Another population of follicle cells known as border cells undergo centripetal movements and migrate to the anterior of the egg chamber, forming the micropyle (Montell et al., 1992; Montell et al., 2012). Thus, the follicle cells play a vital role in generating the essential structures that surround the oocyte upon egg activation, providing the essential site of sperm entry and potentially generating a space (the PV space) in which calcium ions can be concentrated and stored prior to calcium entry into the oocyte.

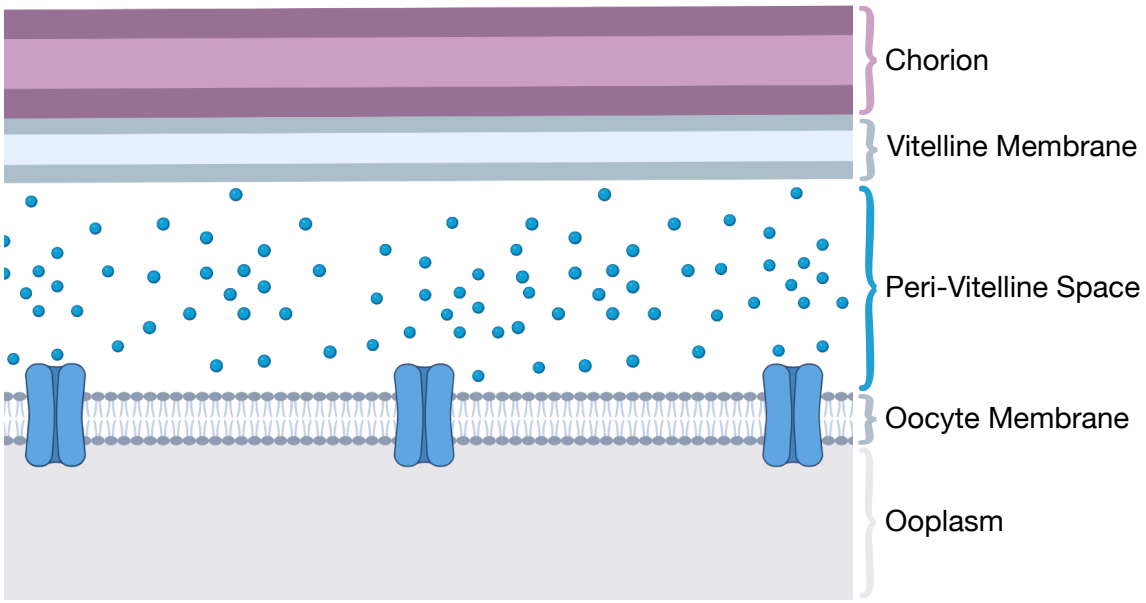

Figure 3.1: Layers of the *Drosophila* eggshell
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3.1.3 Visualising Calcium in the Oocyte

In order to understand the functional and mechanistic details of calcium signalling, both generally and in the case of *Drosophila* egg activation, fluorescent indicators are employed to visualise the live dynamics of this signalling process. Calcium dyes have been used successfully in many systems to visualise calcium; quin-2, fluo-3 and fura-2, for instance, which exist in calcium-free and calcium-bound states, altering their excitation wavelength. Therefore, calcium-bound dyes emit fluorescence at a specific wavelength and this fluorescence is directly proportional to the concentration of calcium (Mehta and Zhang., 2015). The calcium wave in the *Drosophila* mature oocyte at egg activation was first demonstrated through use of the calcium dyes Calcium Green-1 and Texas Red Dextrans. Injection of these dyes into the mature oocyte, incubation with Activation Buffer, and ratiometric imaging revealed a propagating wave from the posterior of the oocyte (York-Andersen et al., 2015).

Use of injectable calcium dyes is not always the most useful approach however, especially in the case of the *Drosophila* oocyte, as injection may lead to leaking of cytoplasm and cell rupture. As such, the employment of genetically encoded calcium indicators (GECIs) has proved an invaluable tool in the study of calcium dynamics. GECIs are available in a variety of forms that all employ a highly modular design consisting of at least a sensing unit, to detect a biochemical stimulus, and a reporting unit, usually a fluorescent marker (Newman et al., 2011; Mehta and Zhang., 2015). One such GECI is GCaMP3, a member of the GCaMP family of calcium sensors that consist of a circularly permuted enhanced GFP (cpEGFP) moiety attached to the calcium-binding protein calmodulin (CaM) and the myosin light chain kinase derived calmodulin binding peptide M13

(Nakai et al., 2001). In the presence of calcium, conformational changes are induced in CaM/M13 which result in the increase in fluorescence of the GFP.

Despite such research, the calcium increase that occurs at egg activation in *Drosophila* has not been visualised until recently. This was achieved in the most detail through *ex vivo* activation in which mature stage 14 oocytes were dissected from fattened female flies, and a hypotonic solution added, causing swelling and activation (York-Andersen at al., 2015). *In vivo* egg activation has also been visualised using GCaMP3, imaging through the cuticle of the abdomen, demonstrating a similar intracellular calcium increase in the oocyte (Kaneuchi et al., 2015). However, the use of the anaesthetic "FlyNap" and movement of the fly abdomen makes visualisation using this method much more challenging, and prevents higher resolution and magnification imaging.

3.1.4 Sources of Calcium in Oocytes

Many stores of calcium exist within cells, largely in the form of membranous organelles. A classical store of calcium is the endoplasmic reticulum, which has been studied in many systems. For instance, the analogous sarcoplasmic reticulum in myocytes acts as a calcium store; when an action potential reaches a myocyte, it results in the opening of dihydropyridine receptors in the sarcolemma, enabling the influx of calcium and subsequent calcium induced calcium release (CICR) as 4 calcium ions bind to the Ryanodine receptor (RYR) in the sarcoplasmic reticulum, opening the RYR (Reviewed in Lanner et al., 2010). RyRs have been suggested to play roles in oocytes during egg activation; use of ruthenium red, an RyR antagonist reduces the propagation velocity of the calcium transient in sea urchins, and sensitising the RyR triggered a calcium response (Galione et al., 1993; McDougall et al., 1993).

The inositol phosphate (IP_3) pathway is another classically studied pathway that has been shown to be involved in egg activation of many systems. As previously discussed, sperm binding and fusion in vertebrates results in the production of IP_3 which binds to IP_3 receptors on the endoplasmic reticulum (ER) resulting in calcium release. Both IP_3R and RyR mediated calcium release are likely involved in the generation of calcium oscillations; both show bell shaped responses to cytoplasmic calcium, enabling CICR at intermediate concentrations and inhibition of the channels at higher concentrations (Ehrlich, 1995). Oscillations of calcium are observed in many mammalian species, however in *Drosophila* oocytes there is only a single calcium transient, perhaps pointing towards alternative mechanisms of calcium entry being in place.

The IP_3 pathway has recently been implicated in the process of *Drosophila* egg activation, as CRISPR mediated knock out of IP_3 pathway components resulted in the failure of calcium wave propagation (Hu and Wolfner., 2020; Hu et al., 2020). This is, however, still disputed and alternative data in which IP_3R was knocked down using RNAi technology would suggest that the

IP_3 pathway is not necessary (but perhaps a redundant mechanism) of propagating the calcium wave (Anna York-Andersen., PhD 2018). It is still likely that the endoplasmic reticulum (ER) acts as a store of a calcium, with IP_3 receptors acting as the channels through which calcium efflux occurs, either in a calcium induced calcium release (CICR) manner, as calcium ions open IP_3 receptors, or through the action of IP_3 (Hu and Wolfner., 2020; Hu et al., 2020).

Despite controversy regarding the inositol phosphate pathway, there is much evidence to suggest that mechanically gated channels play an important role in calcium entry at egg activation (Kaneuchi et al., 2015; York-Andersen et al., 2015). Initially, broad inhibition of mechanically gated channels through application of gadolinium was achieved and demonstrably prevented calcium waves from occurring at egg activation (Kaneuchi et al., 2015). One such family of mechanically gated channels, that coincidentally is now gaining much more attention after forming part of the 2021 Nobel prize winning research, is the TRP channel. TRP channels transduce a variety of mechanical stimuli into ion fluxes (such as calcium influx), and are subdivided into seven families: TRPA, TRPC, TRPM, TRPN, TRPP, TRPV and TRPML (Montell., 2005). The *Drosophila* genome encodes 13 TRP channels, and of these, only 3 are expressed in ovarian tissues, namely *water witch (wtrw), painless and trpm* (Liu et al., 2007; Tracey et al., 2003; Hofmann et al., 2010; Drosophila FlyAtlas)

Prior to the start of this PhD, several Trp channels were investigated in *Drosophila,* namely Trpm, Wtrw (TrpA family) and Painless (TrpA family). It was shown through detailed genetic study that Wtrw and Painless were not required for the generation of calcium waves at egg activation. Preliminary evidence suggested Trpm may play a role in calcium entry, as a heterozygous mutation was sufficient to stop the generation of calcium waves (York-Andersen., PhD 2018). I will therefore aim to address the wider question of whether calcium for egg activation is sourced externally to the oocyte (ie. from a space outside the oocyte membrane, whether the external environment or a region within the eggshell), and further contribute to our understanding of such a source by providing details on which calcium channels are required to mediate the calcium flux. Specifically I will explore in detail the significance of Trpm in mediating calcium flux at egg activation.

3.1.5 Model of Drosophila Egg Activation

The model of egg activation in *Drosophila* has changed over the last few years given the evidence discussed earlier. Multiple models for the trigger of initiation itself existed. One model put forward that mechanical pressure on the surface of the oocyte as it passed into a narrow oviduct triggered egg activation, in a similar fashion to wasps (Endow and Komma, 1997; Horner and Wolfner, 2008). An alternative model suggested that the uptake of fluid and hydration of the oocyte as it passes into the oviduct provides a mechanical signal in the form of cellular swelling, triggering egg activation (Mahowald et al., 1983; York-Andersen et al., 2015; Kaneuchi et al., 2015). This final model was further corroborated by recent data, demonstrating the sufficiency of swelling, due to

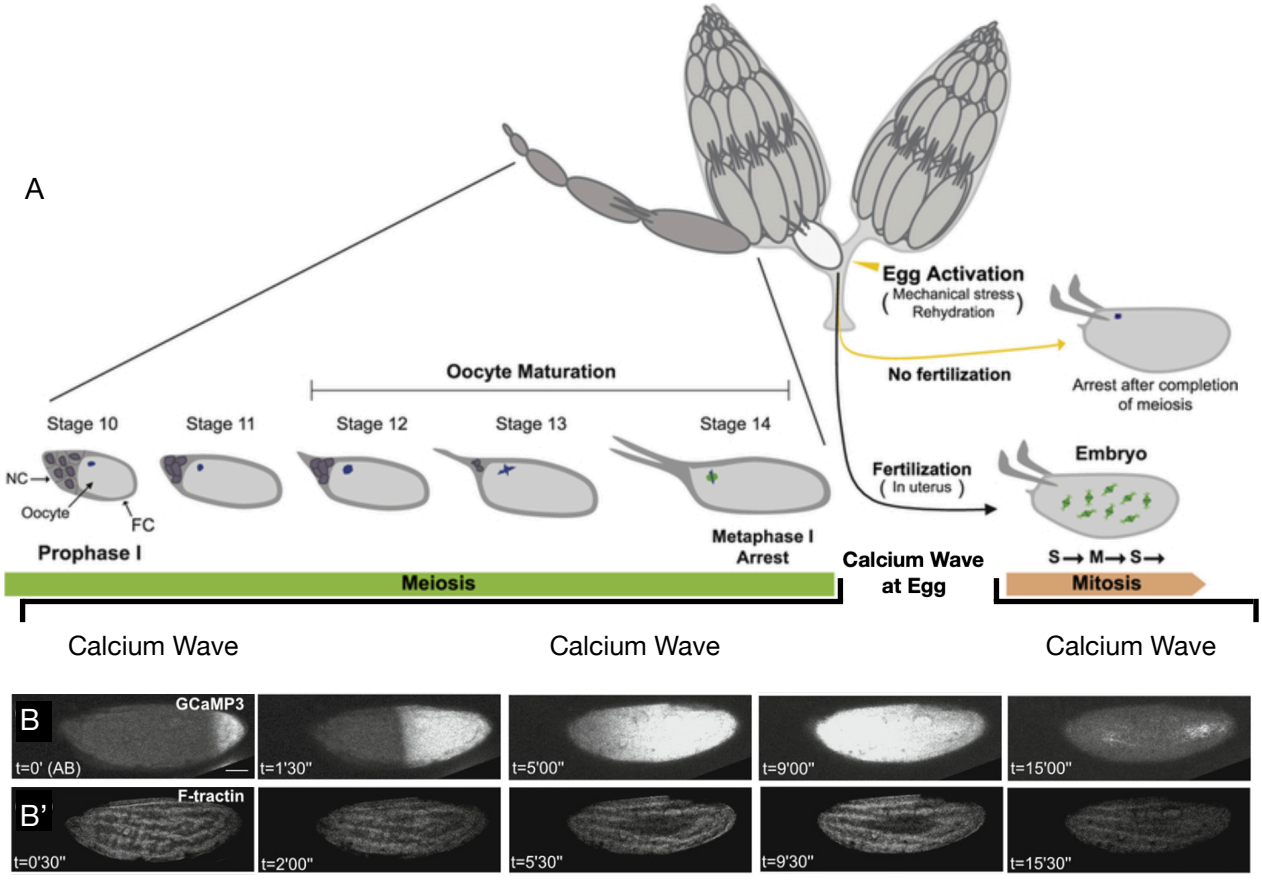

Figure 3.2: Oocyte Maturation and Egg Activation.
The mature oocyte, once ovulated, undergoes egg activation in which a global transient of calcium ions and rearranging F-actin can be observed. (Adapted from Aviles-Pagan & Orr-Weaver., 2018; York-Andersen et al., 2020). Various salient features of the calcium wave can be observed in B. At t = 0, one can observe the initiation of the calcium wave; usually this is from a single origin point, however the time taken between this initiation and the event to trigger initiation (such as addition of activation buffer) can vary. At t = 1'30", one can observe the calcium transient has traversed approximately half of the oocyte. Were the calcium transient to stop here and recede, it would be classified as a partial wave. At t = 5'00", one can observe completion of the wave, indicating that in this example it took 5 minuted for the wave to fully traverse the oocyte, which we refer to as completion. The calcium signal thus remains high until approximately t = 15'00", at which point the calcium (GCaMP3) signal returns to a state similar to pre-activation oocytes. This is referred to as recovery following the calcium wave.

application of a hypotonic buffer, to trigger the events of egg activation (York-Andersen et al., 2021). Thus I summarise the most updated model of egg activation below:

1) The mature stage 14 *Drosophila* oocyte is held in the ovary in a dehydrated state; activation is prevented through dehydration.

2) The oocyte passes into the lateral oviduct where it is rehydrated.

3) Swelling of the oocyte opens mechanosensitive calcium channels enabling calcium entry from a source external to the oocyte.

4) Calcium propagates through the oocyte as a wave, most often from posterior to anterior.

5) Calcium signalling coordinates downstream events of egg activation: i) Cytoskeletal rearrangements; ii) dispersion of P-bodies and translation of maternal mRNAs; iii) resumption of meiosis.

3.1.5 Aims of Chapter:

In this chapter, the aim is to further test the model of calcium entry at *Drosophila* egg activation and the objectives are:

1) To characterise the dynamics of the calcium wave and therefore provide a more complete analysis of the wave speeds, initiation times and origins such that meaningful comparison can be made to experimental groups, such as a cortical increase phenotype. Using GCaMP3 as a fluorescent marker of the calcium wave, I will provide a detailed analysis of wave speeds, initiation times and wave origins within the oocyte following confocal imaging which will then form a fundamental feature of analysis throughout this thesis.

2) To characterise the dynamics and physiology of the cortical increase phenotype, in particular evaluate the speed at which the cortical increase initiates and provide data on the point of entry of calcium during a cortical increase. Using GCaMP3 as a fluorescent marker during confocal imaging, I will detail the dynamics of the cortical increase, providing a detailed analysis of the cortical increase speed, initiation time and origins within the oocyte.

3) To determine the source of calcium at egg activation in order to provide a more complete model of calcium entry at egg activation in *Drosophila*. Using genetic and pharmacological manipulation, such as knockdown and knockout of perivitelline (PV) space components and chelation of internal and external calcium, I will eliminate the external environment as a source of calcium, pointing to a source within the egg chamber, likely the PV space.

4) To determine the channels required for calcium entry in order to provide a more full model of how calcium enters, likely from the pervitelline space, in order to generate either a propagating wave or a cortical increase. I will use a candidate RNAi screen of selected membrane channels with high levels of ovarian expression, in addition to mutational and pharmacological analysis. I will highlight the requirement of Trpm for calcium entry, and begin to indicate further calcium channels, such as Flower, that may also be required.

To this end, these objectives are aimed at providing a more complete model of calcium entry at egg activation. Importantly, through these objectives I seek to establish the mechanism by which calcium enters the oocyte during a typical activation process (such as the case in which a polarised, propagating wave is observed). Further to this, I aim to establish the dynamics of a cortical increase, which as I demonstrate further in chapter 4, may represent a scenario in which the oocyte is unable to effectively regulate calcium entry.

3.2 Results

3.2.1 *Ex vivo* activation of mature *Drosophila* oocytes results in a calcium wave

The calcium wave at egg activation, as aforementioned, can be recapitulated *ex vivo* through incubation of dissected, mature stage 14 oocytes in a hypnotic buffer, such as Activation Buffer (AB). Previous work has emphasised the formation of a wavefront of calcium in the oocyte at egg activation, propagating largely from the posterior pole (York-Andersen et al., 2015). In approximately 80% of cases, addition of AB results in the formation of a wave of calcium ions that propagates from one pole to the opposite pole, usually with a posterior to anterior polarity. It has been observed that the remaining 20% of cases are formed of partial waves, no waves, and cortical increases.

This is visualised through the use of the GCaMP3 (matα4-GAL-VP16 > UASp-GCaMP3), a genetically encoded calcium sensor that is constitutively expressed in the female germline. Live imaging of these dissected oocytes reveals a low background detection of intracellular calcium, with a dramatic increase occurring upon egg activation (Figure 3.3 A-A"). Time lapse imaging of a Z-stack corresponding to approximately 40uM reveals a stereotypical propagation of the calcium wave in the form of a propagating front from the posterior, anterior or both. The most prevalent phenotype exhibited by the oocyte is that of the 'full wave' (Figure 3.3 A) that initiates at the posterior pole of the oocyte and traverses to the anterior. Less common, a wave begins initiation from the posterior pole and completes through a second calcium entry event at the anterior (Figure 3.3 B). Additionally, the oocyte can undergo a complete wave through an initiation even from the anterior only (Figure 3.3 C) which, much like a posterior wave, traverses the length of the oocyte, but with an anterior to posterior polarity.

Posterior waves are the most common phenotype observed (Figure 3.3 C) and further analysis of the time taken to initiate a posterior wave indicates that they happen at a significantly quicker speed following addition of activation buffer when compared to the time taken to initiate an anterior wave (Figure 3.3 D). This data suggests that due to the polar nature of the calcium wave itself, there exists some regulatory mechanism that primes the poles to initiate the calcium wave in the majority of cases. Further to this, given the significant proportion of oocytes that initiate from the posterior alone, and the significantly quicker initiation from the posterior pole, it appears that the oocyte is intrinsically primed to initiate from the posterior. With this in mind, is it the case that calcium waves may only be initiated from the poles, and what is the regulatory mechanisms that primes these regions for calcium wave initiation? In the remainder of this results chapter, I shall explore these questions further, and extend this into Chapter 4, in which I will examine the regulatory mechanisms of calcium entry further through investigation of the role of the actin cytoskeleton.

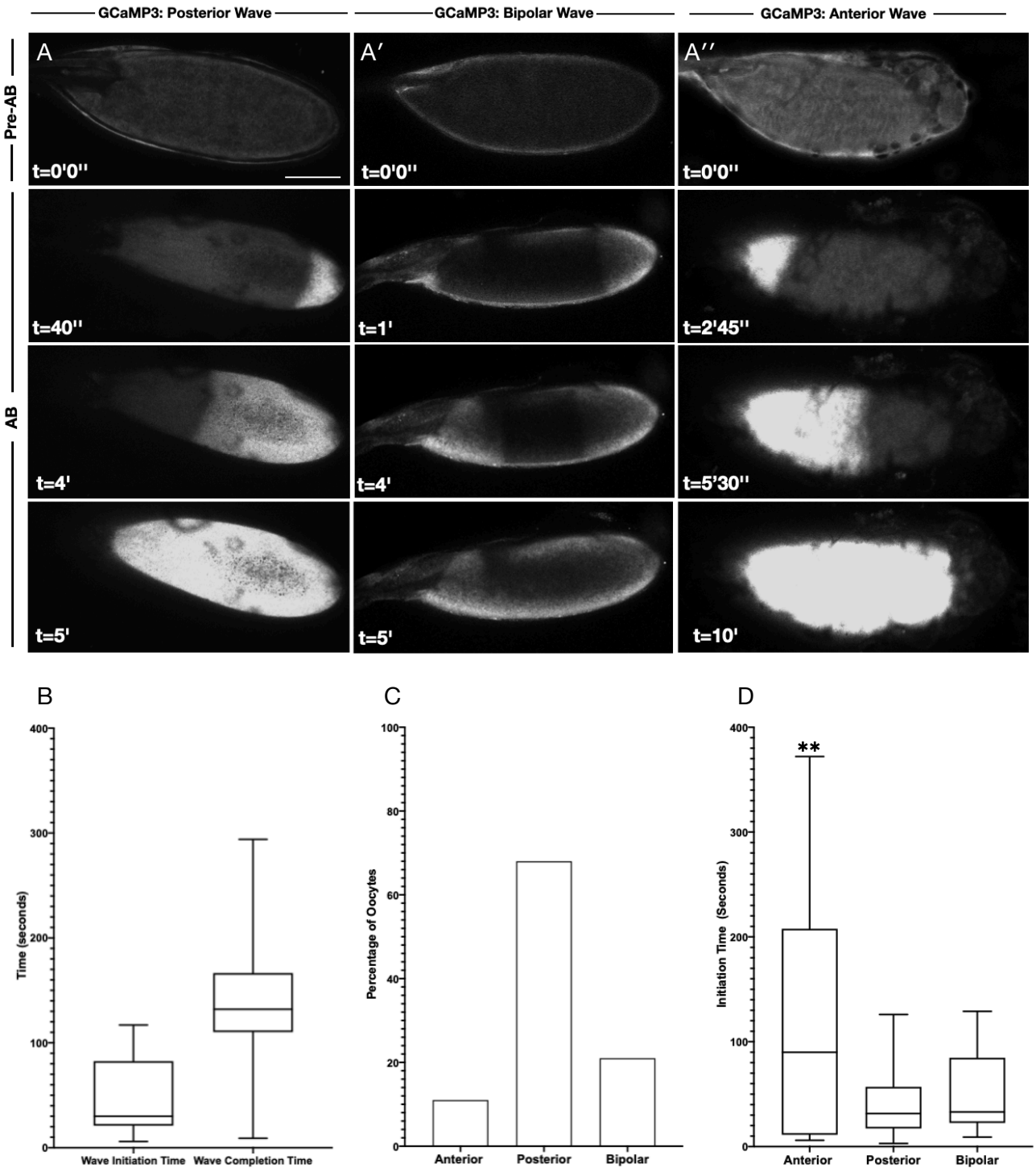

Figure 3.3: Detailed analysis of calcium waves. Mature oocytes expressing GCaMP3. **(A-A")** Wave polarities exhibited upon the addition of AB. Scale bar represents 100 μM. **(B)** Box and whisker plot identifying the speed of calcium dynamics. The average initiation time of the calcium wave was 30s and average completion time of the wave 130s (N = 64). **(C)** Bar chart indicating the proportion of waves that initiate from the anterior (11%), posterior (68%) or both poles (21%) of the oocyte (N = 64). **(D)** Box and whisker plot showing average initiation times of calcium entry depending on calcium origin point. Initiation time from the anterior is significantly slower than from the posterior pole (or indeed when there is bipolar calcium entry) (P (**)< 0.01, N = 64), students T-test.

3.2.2 A low osmolarity buffer triggers rapid calcium entry from multiple points on the cortex

The standard wave observed propagates from posterior to anterior taking on average approximately 130 seconds to complete (Figure 3.3). There are, however, a number of other phenotypes that emerge when oocytes are incubated with AB, namely that of the no wave, partial wave and cortical increase (York-Andersen et al., 2021). The cortical increase phenotype is particularly interesting, as it presents interesting implications on the mechanism of calcium entry: What channels and regulatory mechanisms could mediate calcium entry both in the form of a typical wavefront and a seemingly un-regulated calcium entry from all points over the cortex? Is this process still able to mediate the physiological events that occur at egg activation?

Generating a cortical increase phenotype ex-vivo can be triggered through incubation of oocytes with a low osmolarity buffer or distilled water. This causes a rapid swelling of the oocytes followed largely by a rapid increase in calcium. This can be observed in a small percentage of cases when an oocyte is incubated with AB, but can be forced through incubation in water, resulting in a cortical increase in 67% of cases, with the remainder of the oocytes rupturing due to the extreme swelling (Figure 3.4). The cortical increase had previously been described as rapid entry of calcium from all over the cortex, however, adjusting imaging parameters to increase the speed of imaging reveals that entry occurs from multiple points across the cortex, rather than everywhere at once. Calcium entry at these points does still appear to generate wave-like entry from these focal points. Figure 3.4 shows time lapse images in which calcium enters both from the anterior and from lateral points, and the calcium event has completed as soon as 30s following the addition of AB or distilled water (Figure 3.4). Interestingly, in both cortical increases generated by addition of AB and H2O, the regulated wavefront appears disrupted; in a standard calcium wave, there is a clearly defined straight leading edge of the wavefront (Figure 3.3), whereas in the case of the cortical increase this appears less regulated as indicated by the curvature of the wavefront (Figure 3.4). It is likely that in the case of distilled water, increased and more rapid swelling due to osmosis acts to force the calcium response through mechanically gated channels, even in regions not seemingly primed to activate these channels (anterior and posterior poles). However, given that this phenotype is occasionally observed upon the addition of AB, there is likely a level of stochasticity in this process.

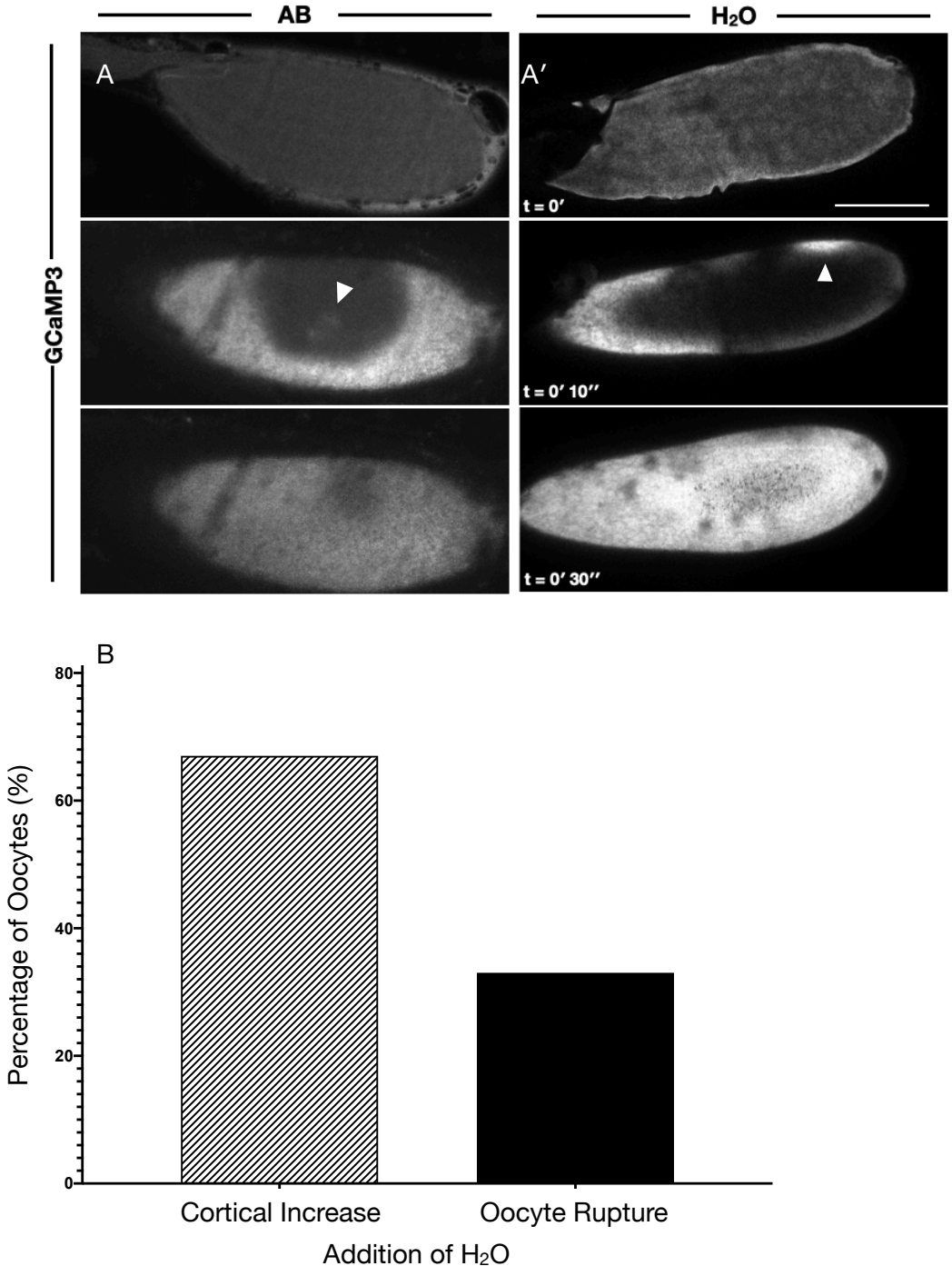

Figure 3.4: A rapid cortical increase phenotype is observable upon the addition of distilled water. Mature oocytes expressing GCaMP3. **(A)** Time series demonstrating eggs activated through incubation with AB and distilled water, in both cases completing by 30s. Scale bar = 100 μM. **(B)** Graph showing the proportion of oocytes undergoing a cortical increase (67%) or rupture (33%) when incubated with distilled water (N=27).

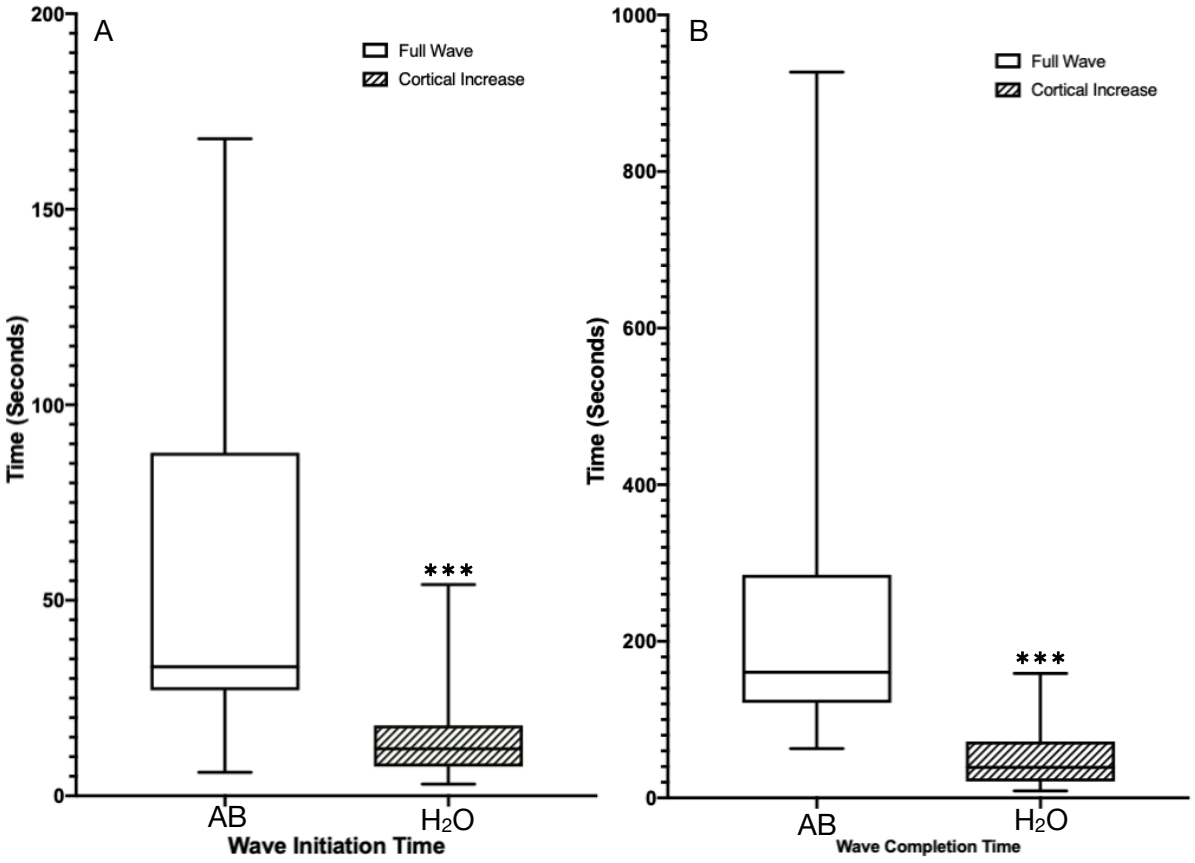

Figure 3.5: Cortical calcium entry dynamics are much faster than standard wave dynamics. Box and whisker plots showing the distribution of wave/cortical increase initiation time, the time taken for the first calcium response following addition of AB (to generate a standard wave)or distilled water (to generate a cortical increase) **(A)**, and the distribution of wave completion times, the time taken for calcium to completely encompass the oocyte **(B)**. **(A)** The median wave initiation time for observed full waves was 34s, compared to a significantly lowered 12s for observed cortical increases (P(***)<0.001, N=26), student T-test. **(B)** The median wave completion time for observed full waves was 160s, compared to a significantly lowered 40s for observed cortical increases (P(***)<0.0001, N=26).

There are key differences in the calcium dynamics between the standard wave and cortical increase phenotypes. Observation of the time taken for the oocyte to begin initiation following the addition of AB (in order to generate a full wave) or distilled water (in order to generate a cortical increase) reveals a significant decrease in the time taken to initiate in the case of the latter (Figure 3.5). When incubated in AB, the average initiation time was 35 seconds compared to when the oocyte is incubated in distilled water in which the time taken to initiate was 10 seconds. Additionally, through observation of GCaMP3 signal, the time taken for the oocyte to reach

maximum fluorescence intensity (encompassing the whole oocyte), and as such the calcium signal having propagated across the entire oocyte, was much quicker in the case of the cortical increase, with an average completion time of 40 seconds (Figure 3.5)

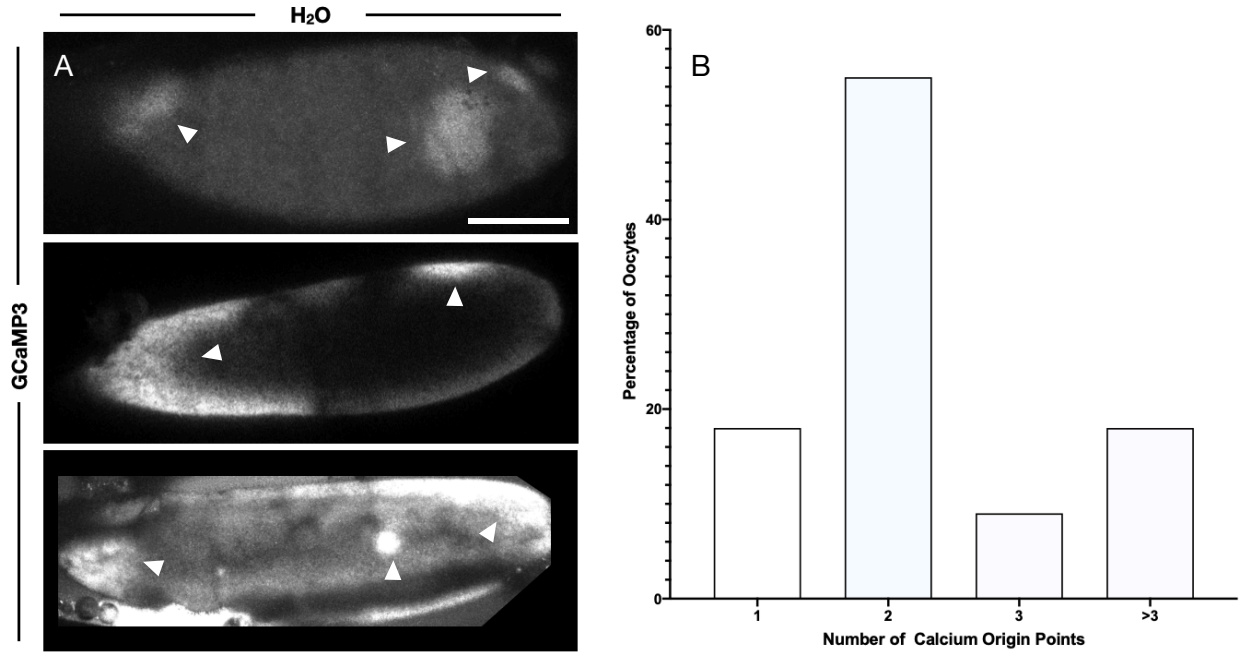

Figure 3.6: Cortical calcium entry occurs from multiple points around the cortex. Mature oocytes expressing GCaMP3. **(A)** Representative stills of mature oocyte following the addition of distilled water. In each case, multiple regions of higher fluorescence indicate origin points of calcium entry (white arrows), scale bar = 100 μM. **(B)** Graph showing the proportion of oocytes that generate a cortical increase from 1, 2, 3 or more than 3 origin points, as visualised through generation of a z-stack (n = 12 per phenotype).

Comparison of the dynamics of calcium entry between standard wave phenotypes and the cortical increase reveals some key differences. Primarily, incubation of oocytes in distilled water generates calcium entry from multiple points around the cortex (Figure 3.6), with the the most common number of origin points being 2, but a significant percentage of oocytes demonstrated 3 or more origin points. When compared with a standard wave, which only ever originates from a maximum of two origin points (the anterior and posterior pole), this indicates that a cortical increase often has more origin points. Importantly, it can be observed that calcium signals appear from regions on the cortex that are not localised to the poles, suggesting the capability of calcium entry from any point around the cortex. However, imaging of the cortical increase phenotype is challenging due to its speed, especially achieving a time resolution quick enough to capture said speeds whilst imaging. It may be the case that if one were able to image every point of entry of calcium, that it exceeds a much greater number than 3 separate origin points. This will likely not be possible due to the limitation on the imaging speed given the oocyte is approximately 500 mm in length.

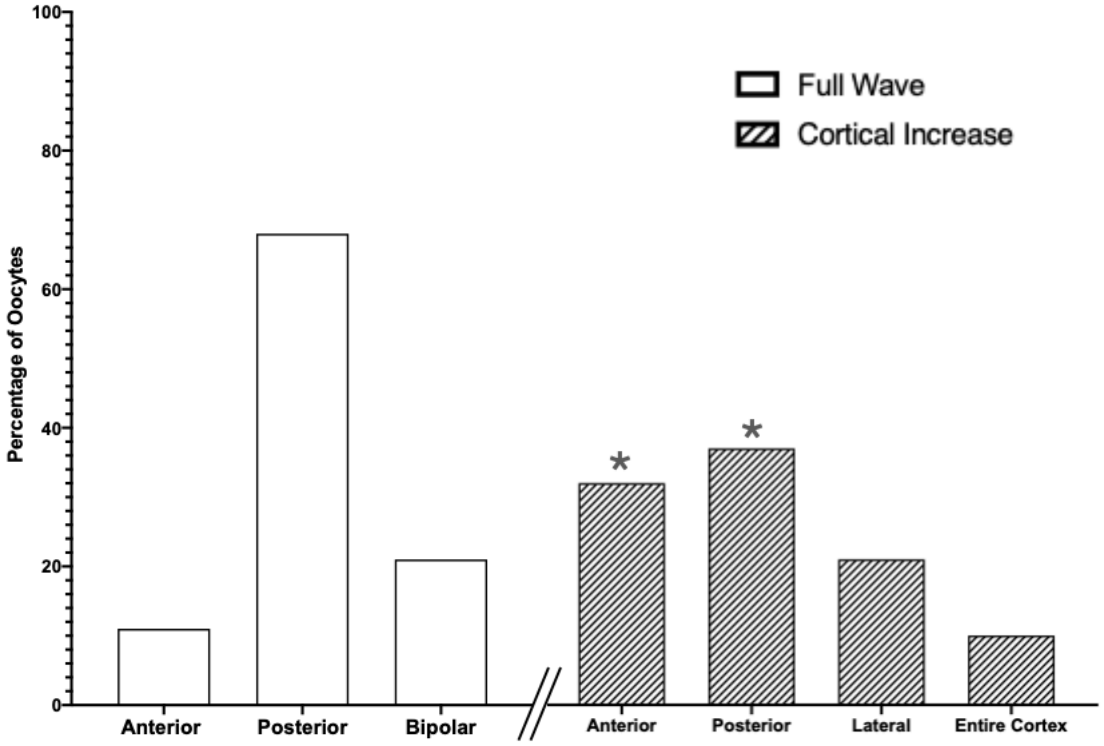

Figure 3.7: Comparison of the different origins between calcium waves (generated bv addition of AB) and cortical increases (generated by addition of H_2O). Graph showing the proportion of origin points of the calcium increase in the case of the full wave and cortical increase. In the case of a full wave, the origin point is largely restricted to the posterior of the oocyte, with a smaller percentage originating from the anterior or both poles. In the case of a full wave, there is a significant decrease in the number of posterior waves and a significant increase in the number of anterior waves ($P(*)<0.05$, N=30 full waves, N=30 Cortical Increase). Interestingly, there is also a significant percentage of calcium transients that initiate from lateral points of the cortex, where there were none in the case of the full wave. Additionally, some oocyte appeared to initiate the calcium increase from all points on the cortex simultaneously.

3.2.3: Rapid global calcium entry appears to be sufficient to mediate some downstream events of egg activation

Having established that calcium entry appears less regulated during the cortical increase phenotype, I sought to confirm that this would indeed continue to generate the same downstream events as previously visualised through the addition of activation buffer (AB, 260 mOsm). Addition of AB to mature oocytes expressing Jupiter-GFP, labelling the microtubule spindle, demonstrates the classical change in spindle morphology at egg activation as the spindle has contracted and widened by the end of activation (Figure 3.8 A,A'; Endow and Komma 1997). This can also be seen when oocytes are incubated in water, with the process occurring at a much faster rate in comparison to AB (Figure 3.8 B,B'). Additionally, the dispersion of P-bodies, a now classical event of egg activation in *Drosophila* (York-Andersen et al., 2015) still occurs upon the addition of water,

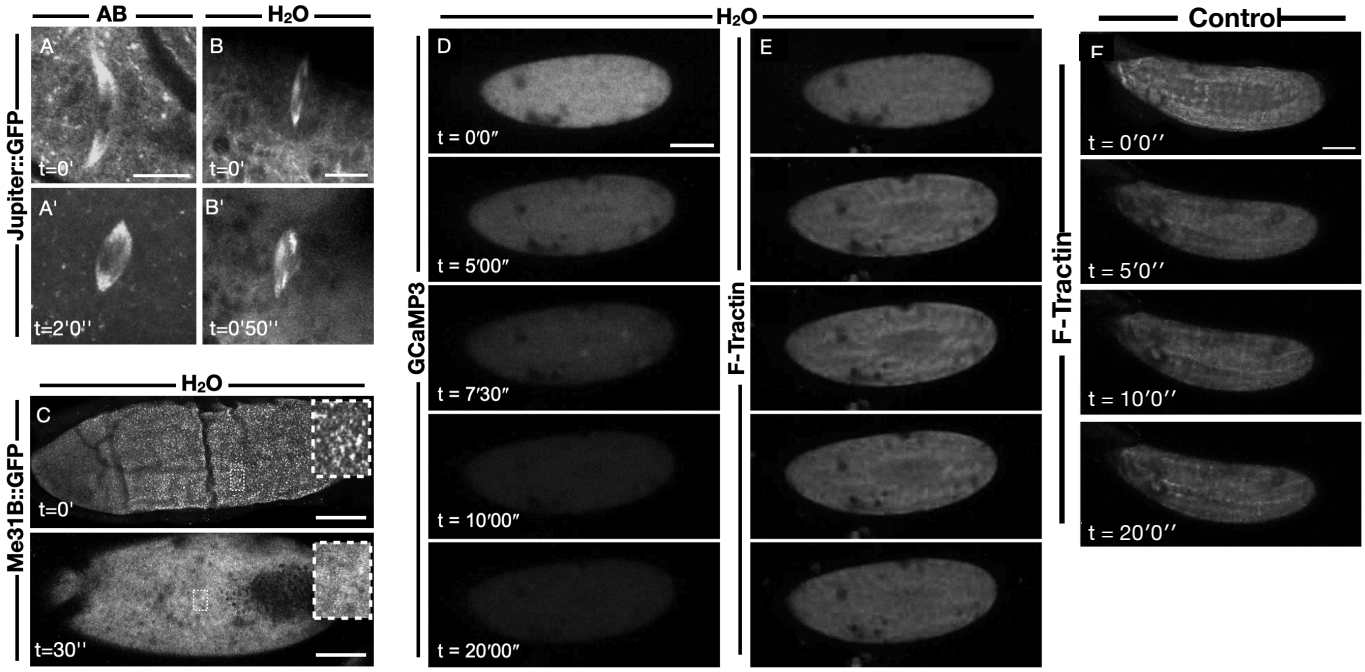

Figure 3.8: Addition of water continues to trigger downstream events of egg activation. Adult *Drosophila* expressing GCaMP3 in the germline imaged during the egg laying and ovulation process **(A-C)**. **(A-B')** Z projections (10 µm) from a live time series of mature oocytes expressing Jupiter-GFP (Microtubules). **(A-A')** Addition of AB causes contraction of the microtubule spindle that completes within 2 minutes (N=30). **(B-B')** Addition of water causes contraction of the microtubule spindle that completes within 50 seconds (N=12). **(C)** Z projections (40 µm) from a live time series of a mature oocyte expressing Me31B::GFP (P-bodies), visible as individual punctae. Addition of water causes rapid dispersion of the P-bodies. Dashed line boxes represent a 10x magnification of the original image to better visualise punctae. Scale bar = 50 µm (N = 10). **(D-E)** Z-projections from a live time series of oocytes co-expressing GCaMP3 **(D)** and F-tractin.tdTomatoe **(E)**. **(D-E)** Addition of water causes a cortical calcium increase almost instantaneously, followed by a global increase in the concentration of F-actin after approximately 5 minutes. Scale bar: 50 µm. **(F)** Control oocyte expressing F-tractin.tdTomato in which activation does not occur. The fluorescent signal does not increase throughout the time-course. Scale bar: 50 µm.

yet once more occurring at a faster rate to the usual dispersion of P-bodies (Figure 3.8 C). The implications of both a more rapid meiotic resumption and dispersion of P-bodies should be investigated further, as although it appears that they are happening similar to a wild-type scenario, it is likely there is a reduced level of regulation due to such an osmotic shock.

Finally, the reorganisation of actin that occurs following calcium entry in *Drosophila* (York-Andersen et al., 2020) still occurs upon the addition of water (Figure 3.8 D-E). The actin reorganisation is visualised as an increase in fluorescence intensity of the F-actin labelling tool F-tractin, which follows closely the increase in calcium. This usually presents as a wave of reorganising actin when AB is added, however in the case of the addition of water which generates a cortical increase, a global increase in the intensity of the F-tractin can be observed (Figure 3.8 E). Once again, the downstream implications of such an actin re-organisation have yet to be examined; there may well be a physiological consequence of having a rapid and global actin rearrangement rather than a localised and controlled one.

3.2.4 What is the source of Calcium?

The cortical increase phenotype indicates the ability of the oocyte to generate a calcium transient from all points across the cortex, in comparison to the standard wave which appears to only initiate from the poles. This poses a further question as to what is the store of calcium in the oocyte; given the apparent calcium flux at many points across the cortex, this may point to calcium entering the oocyte from an external source. It has been well established in the literature that many oocytes rely on calcium stored within the Endoplasmic Reticulum (ER) in order for egg activation to occur, and even in the case of the Drosophila oocyte, there is some evidence to suggest mechanisms involving the IP_3 pathway and the ER are required for sustained calcium signalling at egg activation (Horner and Wolfner, 2008). It has yet to be established, however, what the source of calcium is at egg activation in *Drosophila*, and in doing so this will reveal vital clues on how calcium entry is regulated upon egg activation.

To investigate this, I firstly attempted to initiate the calcium wave in a solution of AB and BAPTA (a calcium chelator), in order to ensure that the solution is absolutely calcium free. Oocytes still demonstrated the standard proportion of wave phenotypes (Figure 3.9). Incubation of the oocytes with BAPTA-AM (a membrane permeable form of BAPTA) in PBS for 10 minutes prior to activation significantly decreases the proportion of full calcium waves, with a concomitant increase in the proportion of no wave phenotypes (Figure 3.9). Incubation using BAPTA-AM in addition to PF-127, a standard solubilising agent, generates an even more dramatic increase in the no wave phenotype, almost completely abolishing the wave phenotype (Figure 3.9). This suggests that when calcium internal to the egg chamber is chelated, the calcium wave can no longer occur, pointing to an internal source. Additionally, incubation with non-membrane permeable BAPTA and addition of calcium free AB is unable to prevent a calcium wave at egg activation, suggesting external calcium is not necessary for the calcium wave. Given the literature has suggested calcium entry from the external environment through mechanically gated channels in the plasma membrane, the evidence I have provided conflicts with this model (Kaneuchi et al. 2015).

I therefore tested an alternative calcium source, that of the perivitelline space, which forms a compartment around the oocyte plasma membrane (so is external to the oocyte) but within the egg chamber itself (so is internal to the egg chamber), which would consolidate conflicting evidence from the field. This is a likely candidate as it has been shown to store calcium ions in the early embryo itself, but due to the fragile nature of the vitelline membrane in the mature oocyte, the space has not been well examined at this stage (Stein and Nusslein-Volhard, 1992). To do so I examined the role of two key proteins that are vital in the formation of the vitelline membrane itself, disrupting which would prevent the production of a the perivitelline space and as a result a compartment in which calcium ions can be effectively stored for calcium entry. Nudel is a serine protease that is best known for its role in dorso-ventral patterning of the drosophila embryo (Chasan et al., 1992; Smith and DeLotto, 1994; LeMosy et al., 1998). It has also been shown to be

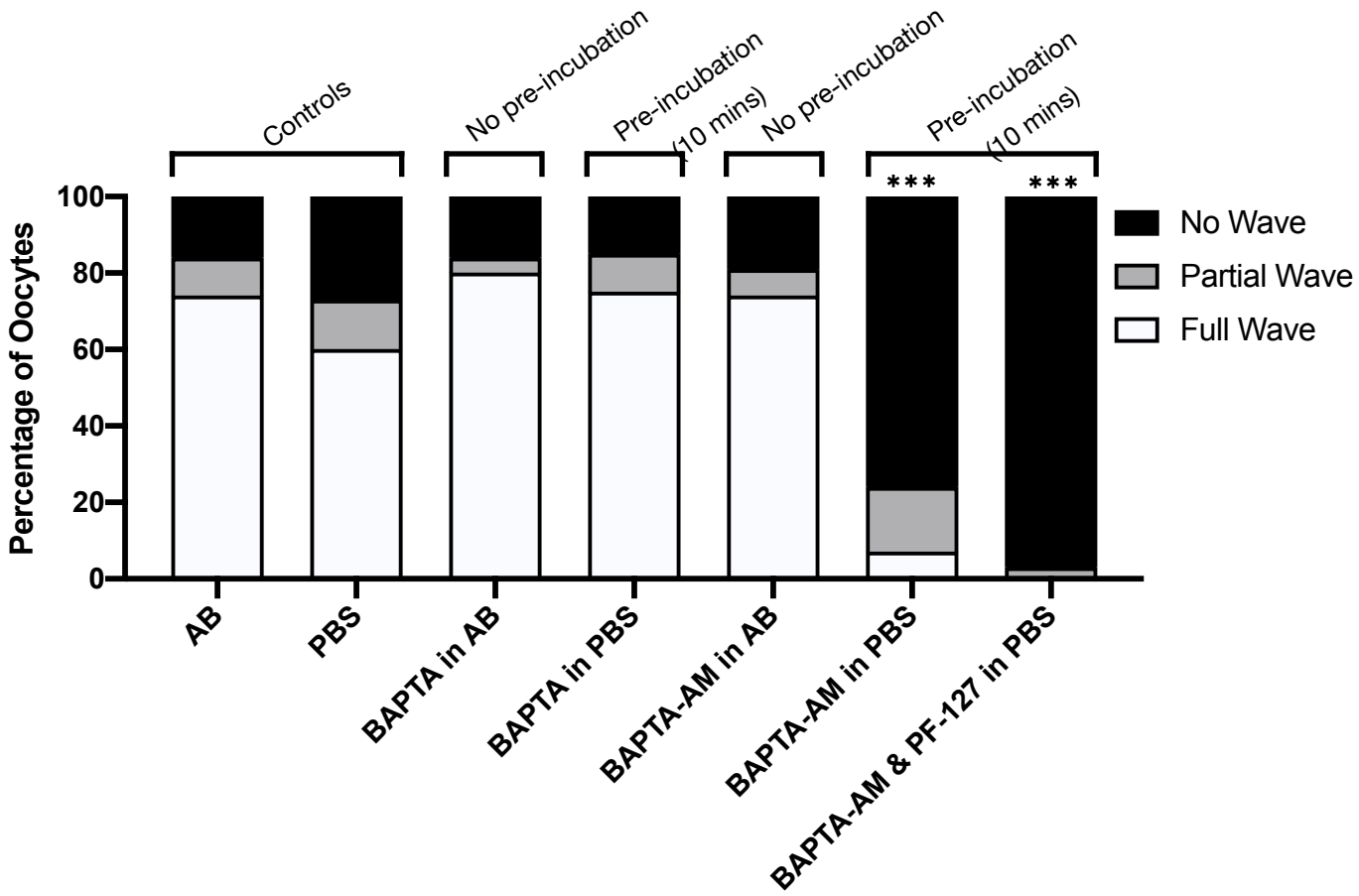

Figure 3.9: External BAPTA does not alter calcium wave phenotypes, whereas internal BAPTA abolishes the calcium wave. Wave phenotypes were scored for GCaMP3 oocytes activated under different conditions. AB: oocytes were activated with AB. BAPTA-AM in AB: oocytes were activated in AB containing BAPTA-AM at 33mM. BAPTA-AM + PF-127 in PBS: oocytes were incubated for 10 minutes with BAPTA-AM at 33mM and Pluronic F-127 in PBS before activation with AB. BAPTA-AM in PBS: oocytes were incubated with BAPTA-AM at (conc) in PBS for 10 minutes before activation with AB. PBS: oocytes were incubated with PBS for 10 minutes before activation with AB. For AB, n=76, Standard Deviation of full wave percentages (SD)=4.02%; PBS, n=65, SD=3.41%; BAPTA in AB, n=50, SD=3.60%; BAPTA in PBS, n=48, SD=3.53%; BAPTA-AM in AB, n=57, SD=4.86%; BAPTA-AM in PBS, n=30, SD=1.6%; BAPTA-AM + PF-127 in PBS, n=34, SD=0.4%. Asterisks indicate significant differences in proportions of wave phenotypes compared to BAPTA-AM + PF-127. There was a significant difference in the proportion of no waves between BAPTA-AM + P F-127 and PBS incubated oocytes (p(***)<0.001), between BAPTA-AM + P F127 and BAPTA-AM incubated oocytes (p=0.0096), and between BAPTA-AM and PBS incubated oocytes (p(***)<0.001).

required for vitelline membrane integrity during egg activation and eggshell biogenesis (LeMosy and Hashimoto, 2000). Vm26Ab is an essential vitelline membrane protein that is vital for production of the eggshell of the mature *Drosophila* oocyte, the gene of which is expressed during mid-oogenesis (Higgins et al., 1984; Cavaliere et al., 2008). Vm26AB (also known as sV23), was shown to be distributed within the vitelline membrane from stage 8 onwards, mutations of which also impact the structural integrity of the outer chorion (Pascucci et al., 1996).

Knock down of both the Nudel protease and Vm26Ab results in a significant decrease in the number of calcium waves at egg activation (Figure 3.10). The knock out of Nudel was the most successful in reducing the number of calcium waves. Complete knockout of Vm26Ab was unsuccessful however as it resulted in a loss of viability and no mature stage 14 could be obtained (Figure 3.10), which was to be somewhat expected as a mutant that generates sterile female flies.

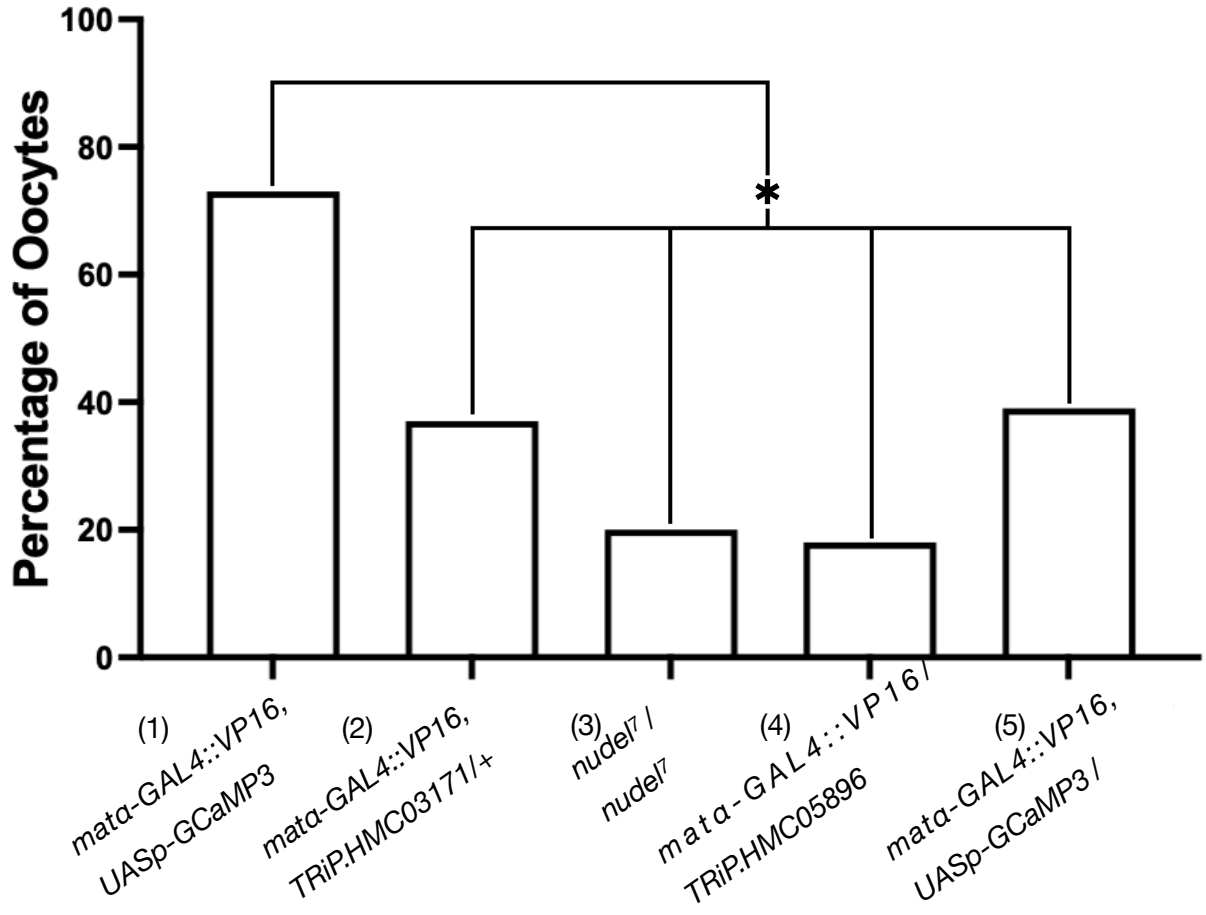

Figure 3.10: Mutation of perivitelline space components results in loss of the calcium wave. Graph showing the percentage of wave phenotypes displayed by oocytes expressing GCaMP3 in different mutant and RNAi backgrounds. Nudel RNAi, TRiP.HMC03171 (Perkins et al., 2009) results in a significant decrease in full wave phenotypes. Homozygous *nudel*^{7} mutation (Tearle and Nusslein-Volhard, 1987), results in a significant decrease in the proportion of full waves (P(*)<0.05, N=15). Vm26Ab RNAi, TRiP.HMC05896 (Perkins et al., 2009) results in a significant decrease in full wave phenotypes (P(*)<0.05, N=13). Heterozygous *vm26Ab*^{QJ42} mutation (Schupbach and Wieschaus, 1991) results in a significant decrease in full wave phenotypes P(*)<0.05, N=20, SD: (1) 5.82%, (2) 4.92%, (3) 2.42%, (4) 1.46% and (5) 2.98%).

3.2.5 Which channels mediate calcium entry at egg activation?

In order to further examine the source of calcium at egg activation, and to illuminate the mechanism of calcium entry, I also examined which channels may play a role in calcium flux at egg activation. Prior to investigation of these channels, previous work had provided first evidence that Trpm may be a key player in mediating calcium flux across the plasma membrane, both implicating the perivitelline space as a source of calcium, and indeed demonstrating the vital role that Trpm may be playing in acting as a mechanically gated channel, transducing osmotic pressure into a calcium signal (York-Andersen et al., 2021).

Previous work has focussed primarily on assessing the roles of mechanosensitive channels during egg activation, which would follow a logical model of osmotic induced pressure opening these channels. Candidate Trp channels had previously been investigated, namely Water-witch (Wtrw), Painless and Trpm (*Drosophila* FlyAtlas; York-Andersen., PhD). The only significant result to be generated from York-Andersen, PhD, was the apparent abolition of calcium events in a heterozygous Trpm mutant background. I therefore wished to confirm this through the use of germline RNAi driven with the *VP16-Matα-Gal4* to knock down the Trp channel, in addition the screening other candidates that could play a role in calcium entry or wave propagation.

For example, I tested whether the STIM/Orai components contribute to calcium entry as they act as a calcium release activated Ca^{2+} (CRAC) channel mechanism, which senses the depletion of calcium from the endoplasmic reticulum (ER), triggering the opening of channels on the plasma membrane, enabling Ca^{2+} entry from external stores (Fahrner et al., 2017; Parekh & Putney., 2005). Though there is some debate, the STIM/Orai components have been suggested to maintain the long-lasting calcium signal at fertilisation in mammals (Machaty et al., 2017). It would therefore be worth assessing whether these play a role during Drosophila egg activation, as the calcium transient is relatively long, lasting for up to 20 minutes at its longest (York-Andersen et al., 2015; Kaneuchi et al., 2015). Interestingly, STIM knock down displayed a small yet significant decrease in the number of full waves, perhaps implicating a calcium induced calcium release (CICR) mechanism of calcium entry at egg activation (Figure 3.11). However, knock down of both STIM and ORAI in a GCaMP3 background displayed no significant change in full waves.

Innexins, in particular Innexin-2, are highly expressed in the Drosophila germline, and are responsible for the formation of gap junctions between the surrounding layer of follicle cells and the oocyte. Inhibition of Innexin-2 prevents communication between follicle cells and the oocyte (Bohrmann & Zimmerman., 2008). Gap junctions have been implicated in calcium induced calcium influx mechanisms (see introduction), and blocking them has prevented the progression of calcium waves (Stavermann et al., 2015). Therefore I tested knock down of Innexin-2, but saw no significant change in the wave phenotypes (Figure 3.11). Further analysis of Innexin would be required, such as RT-PCR mediated confirmation of knock down and further mutational analysis to conclusively rule out Innexin as a candidate.

The most significant results were that of Flower (fwe) and Trpm (Figure 3.11). Fwe is a voltage-gated calcium channel that has been shown to regulate calcium entry in *Drosophila* neurons, promoting bulk endocytosis (Yao et al., 2017). It has however not been studied in the *Drosophila* germline. Knockdown of Flower resulted in a significance decrease in the number of full waves. Trpm RNAi similarly resulted in a significant decrease in the number of full waves, confirming previous work (Figure 3.11).

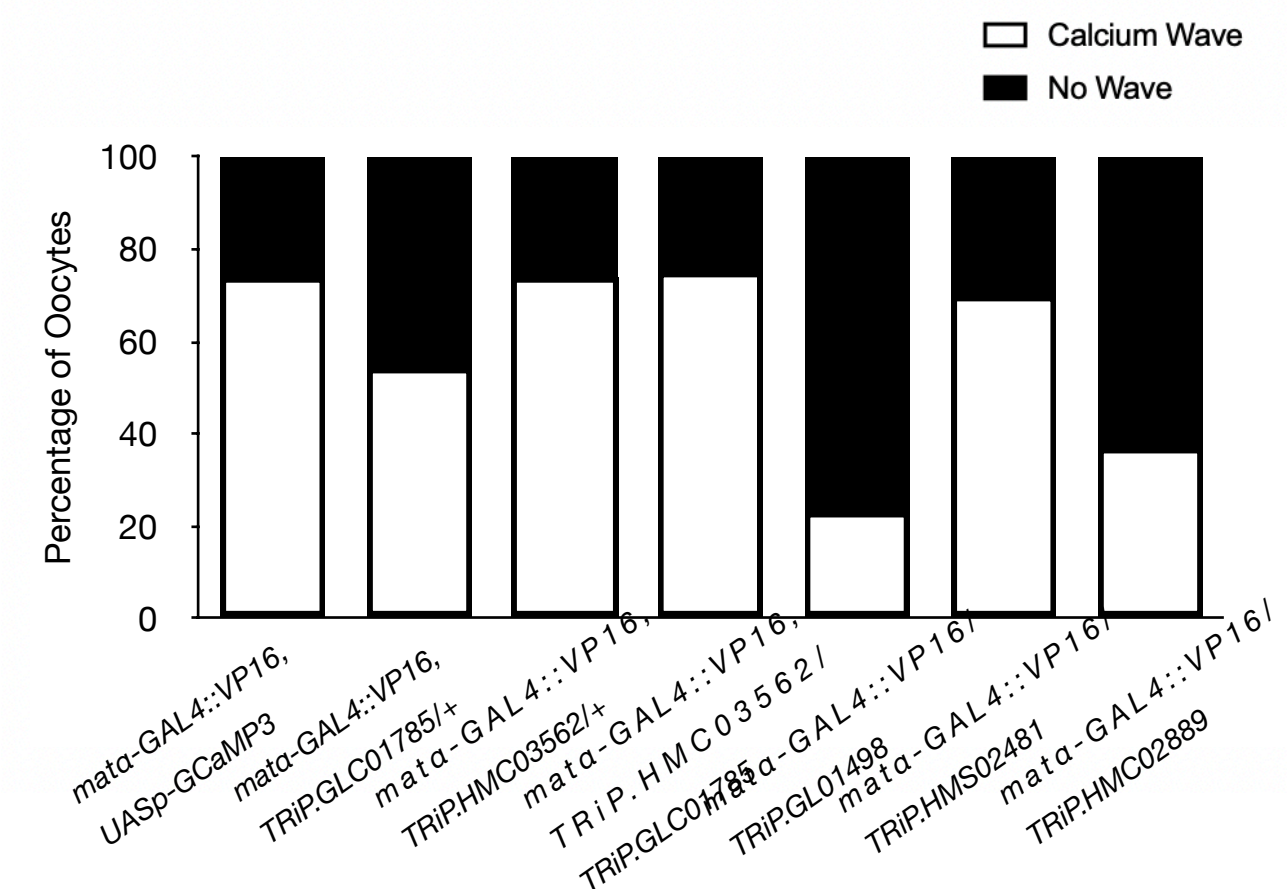

Figure 3.11: Candidate RNAi screen of potential calcium channels. Graph showing the percentages of wave phenotypes displayed by oocytes expressing GCaMP3 in different RNAi backgrounds. Stim RNAi, TRiP.GLC01785 (Ni et al., 2010), results in a small but significant drop in the number of full waves (P(*)<0.05, N=25). Orai RNAi, TRiP.HMC03562 (Ni et al., 2010), does not have an effect on the number of full waves (N=20). Interestingly, introducing both Stim RNAi, TRiP.GLC01785 and Orai RNAi, TRiP.HMC03562 had no effect on on the percentages of calcium waves (N=20). Fwe (Flower) RNAi, TRiP.GL01498 (Ni et al., 2010), results in a significant drop in the number of full waves (P(*)<0.05, N=30). Innexin RNAi, TRiP.HMS02481 (Ni et al., 2010), does not have an effect on the number of full waves. Trpm RNAi, TRiP.HMC02889 (Ni et al., 2010), results in a significant drop in the number of full waves (P(*)<0.05, N=30).

Having revealed two channels that significantly impact the calcium wave, I sought to confirm the involvement of Trpm in the calcium wave. To do this, I utilised an additional RNAi line (TRiP.GL00173) which is specific to the germline. Once more driving this with the *Matα-Gal4* provided alongside GCaMP3, I saw a significant reduction in the number of calcium waves (Figure 3.12). To confirm that this phenotype was due to a reduction in the expression of the Trpm protein, I utilised quantitative PCR to measure the relative level of gene knock down achieved in both RNAi backgrounds. In both cases, there was a significant reduction in gene expression, with TRiP.GL00173 achieving almost 50% knock down of Trpm (Figure 3.12).

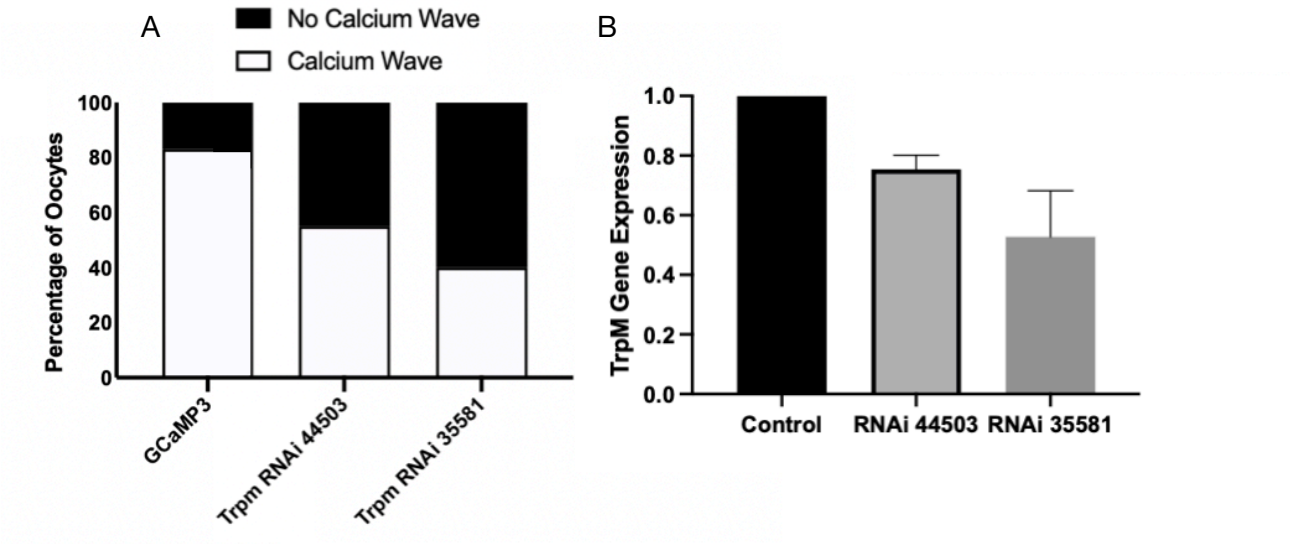

Figure 3.12: RNAi knock down of Trpm results in loss of the calcium wave at egg activation. (A) Graph showing the proportion of wave phenotypes displayed by oocytes expressing GCaMP3 in different Trpm RNAi backgrounds. Both show a significant reduction in the number of full wave phenotypes ($P(*)<0.05$, N=30). **(**B**)** Relative gene expression of Trpm following RNAi expression. Both show a small yet significant reduction in overall gene expression following RNAi treatment, as quantified by RT-qPCR.

I further confirmed the role of Trpm in the calcium wave utilising a pharmacological inhibitor of the Trpm channel known as Carvacrol. Though the precise mechanism is not known, it is likely that Carvacrol has an inhibitory effect due to its alkyl-phenol group, and has since been used as a novel inhibitor of the Trpm subfamily, in addition to other Trp channels (Parnas et al., 2009; Chen et al., 2015). Incubation of mature *Drosophila* oocytes in carvacrol prior to the addition of activation buffer resulted in a significant decrease of the number of full waves in a concentration dependent manner (Figure 3.13).

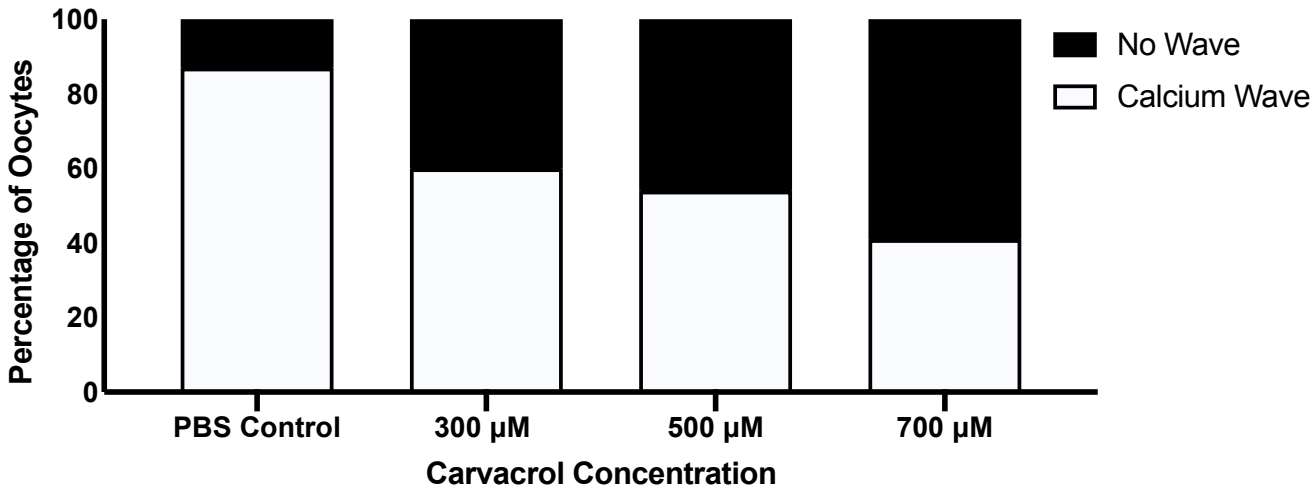

Figure 3.13: Carvacrol incubation reduces the number of calcium waves in mature oocytes. Wave phenotypes were scored for GCaMP3 oocytes activated with AB following incubation in different concentrations of carvacrol. At 300uM and 500uM, the number of calcium waves was significantly reduced ($p(*)<0.05$), N = 28 and 26 respectively. At 700uM, the number of calcium waves was reduced to a more significant degree ($P(**)<0.01$), N = 34.

Having identified a role for Trpm in calcium entry at egg activation, I sought to explore further the role of the Fwe calcium channel during egg activation. Though RNAi mediated knock down had a significant effect on the number of calcium waves, heterozygous Fwe mutants demonstrated no significant change in the number of full waves, perhaps suggesting that the system is less sensitive to the number of available Fwe calcium channels. I attempted to generate a complete knock out of the Fwe calcium channel, however this was lethal.

Whereas preliminary data had suggested that a single copy of the Trpm protein mutated was sufficient to generate a reduction in the number of full waves (York-Andersen, PhD), a single Fwe mutant has no effect on the number of calcium waves, perhaps suggesting that it requires a much higher degree of knock down to achieve an effect.

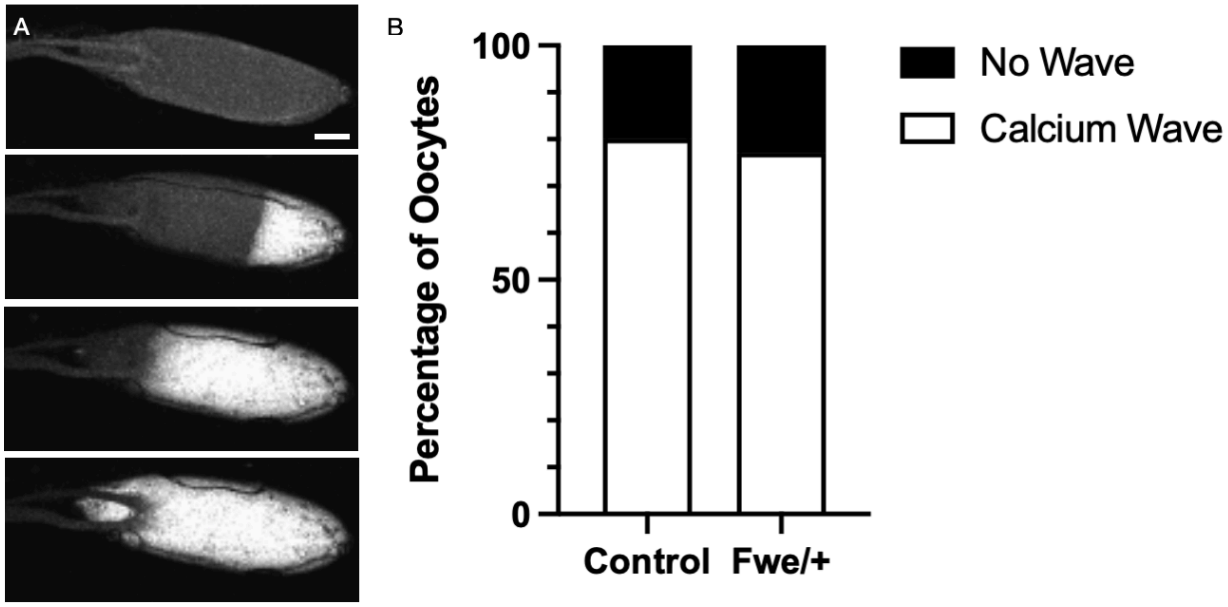

Figure 3.14: Heterozygous Fwe mutation does not significantly impact the number of calcium waves. (A) Mature oocyte expressing *VP16-Matα-Gal4* to drive expression of GCaMP3 and Fwe mutation. Upon addition of activation buffer, standard calcium waves can be observed. Scale bar: 50 μm. **(B)** Graph showing the proportion of wave phenotypes displayed by oocytes expressing GCaMP3 in control and mutants backgrounds. There is no significant change in the number of full wave phenotypes (N=24 per genotype).

3.2.6 Generating an *In vivo* egg activation assay

Throughout this chapter I have detailed and provided evidence on some aspects of the mechanism of calcium entry at egg activation *ex vivo*. To date, this is the best method of examining the plethora of physiological events within the mature oocyte, as it provides much greater resolution of all cellular details and a much higher throughput. However, *in vivo* imaging has been attempted, and may be useful in the respect of further understanding how the environment of the oviduct may impact calcium entry; I document in this chapter, for example, the presence of a cortical calcium increase. This extreme calcium wave likely occurs due to rapid swelling of the oocyte as it is submerged in a hypotonic buffer (such as water at 0 mOsm). A clear way this may be impacted *in vivo* is through the physical restraint placed on the oocyte by the walls of the oviduct, perhaps enabling a more regulated swelling and therefore an organised wave rather than a cortical increase. I therefore attempted to design a method of accurate *in vivo* wave visualisation.

Measuring *in vivo* calcium waves can be rather difficult, as imaging must be carried out through the waxy cuticle of the *Drosophila* abdomen, which provides a barrier to imaging itself. Furthermore, imaging a live adult organism is exceptionally challenging as one must somehow

immobilise the specimen such that imaging over a time-series can be achieved. Previously, this has been done by anaesthetising adult flies using the chemical FlyNap (Sartain and Wolfner., 2013), which enabled visualisation of single eggs passing into the oviduct and the observation of calcium waves. Despite this achievement, there are several issues with anaesthetising the flies, as this causes a relaxation of the ovaries and surrounding muscle tissues, which is obvious from the misshapen appearance of the abdomen.

I therefore designed a new method of imaging egg laying and ovulation live. To do this, I utilised a glass Pasteur pipette that narrows in diameter towards the tip. I filled the tip with yeast, and gently placed a *Drosophila* adult female expressing GCaMP3 in the germline into the pipette at the opposite end following anaesthetisation briefly with CO2. Gently flicking the pipette traps the fly in the narrow tip of the pipette, close to the yeast and with a narrow enough diameter to prevent large movements of the fly. It can take a while for the fly to acclimate enough to lay eggs, and it is particularly difficult to keep them in the correct orientation for visualisation, as such this method still requires optimisation.

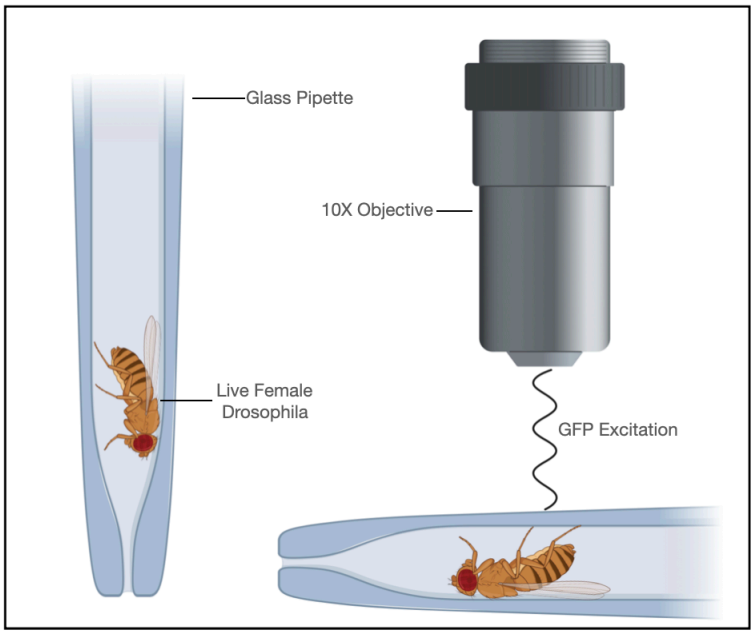

Figure 3.15: Schematic of *in vivo* imaging set-up. Female *Drosophila* are imaged upside-down through a glass pipette using a 10x objective, GFP excitation set to an intensity such that imaging can occur through the cuticle of the abdomen. Rotation of the pipette may be necessary if the *Drosophila* attempts to re-orient themselves. Eventually, *Drosophila* will acclimatise and begin ovulating and egg laying.

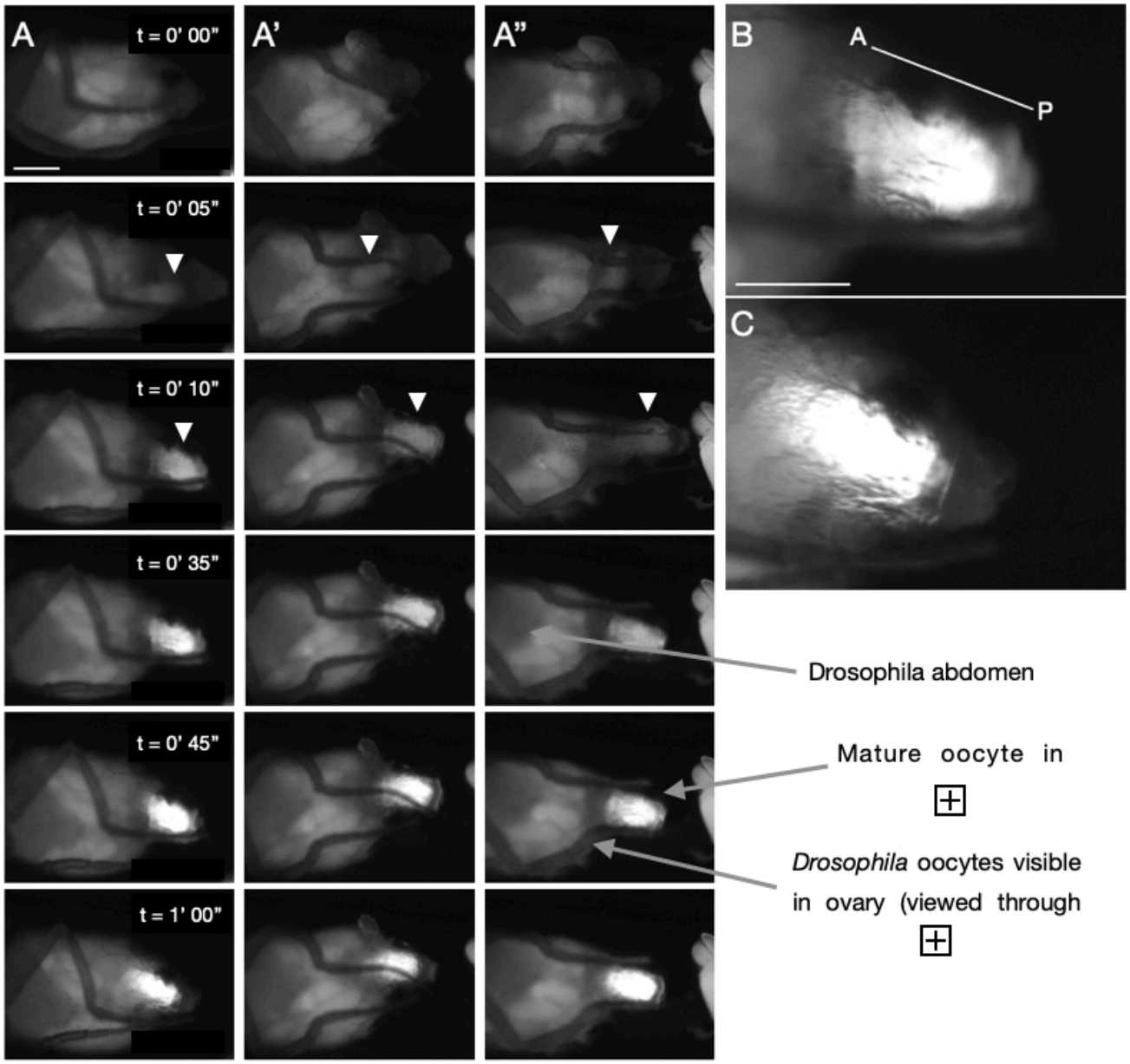

Figure 3.16: In vivo egg activation occurs as a rapid increase in calcium. Adult *Drosophila* expressing GCaMP3 in the germline imaged during the egg laying and ovulation process **(A-C)**. **(A-A")** A live time series of a single *Drosophila* female imaged over the course of ovulating three separate eggs, A represents laying of the first oocyte, A' represents the second and A" represents the third. Stills taken at the same time point during laying demonstrate that following passage of the oocyte into the oviduct (white arrowheads), the oocyte then undergoes a rapid calcium transient that completes in approximately 1 minute. Scale bar: 200 μm. **(B,C)** Magnification of this image reveals that the calcium increase still occurs in a posterior-anterior polarity. Scale bar: 200 μm. N = 18 oocytes observed.

Low magnification fluorescent imaging reveals that as the egg passes into the oviduct, a calcium transient can be observed, completing in approximately 1 minute (Figure 3.15 A'). Following deposition of this egg, the adult female continues to ovulate and lay eggs, both undergoing calcium transients during ovulation (Figure 3.15 A',A"). Enlarged images reveal that the calcium

transient may also propagate in the form of a wave, as calcium appears most concentrated initially at the posterior pole before encompassing the whole oocyte (Figure 3.15 B,C). It was difficult to control the angle of the oocyte during imaging however, so often it was difficult to distinguish whether simply the angle of the oocyte caused the posterior to appear brighter. This is consistent with other *in vivo* data in which FlyNap treated flies demonstrate a wave of calcium within the oocyte as it passes into the oviduct (Horner and Wolfner, 2008).

Although calcium transients were observed with this set-up, this still does not completely mimic a natural setting for the fly, but perhaps improved on the original use of FlyNap. This would be an interesting technique to develop, especially as a means of verifying *ex vivo* data; are there compensatory mechanisms within the oviduct itself that enables the egg to still undergo activation when certain elements are removed? It has been shown recently that *Drosophila* are still fertile in Trpm knockout flies, suggesting alternative mechanisms are at play, and it would be interesting to pursue visualisation of these lines *in vivo*.

3.3 Discussion

3.3.1 Summary of Results

In this chapter I provide an in-depth and detailed analysis on the dynamics of the calcium increase that occurs in the *Drosophila* mature oocyte at egg activation. Multiple phenotypes of 'standard' calcium entry can be observed; posterior, anterior and bipolar waves, with significantly more occurring as posterior waves. However, occasionally upon the addition of activation buffer (AB) and most frequently upon the addition of distilled water, a cortical increase phenotype can be observed. The cortical increase manifests as rapid calcium entry from multiple points around the cortex of the mature oocyte. This phenotype in-of-itself is important as it suggests that calcium entry can initiate from any point on the cortex, however the poles of the oocyte appear to be primed for initiation- to conclusively state this, further analysis should be carried out with a larger sample size.

Calcium entry occurs through channels in the oocyte plasma membrane, such as the mechanosensitive channel Trpm in addition to other channels, such as Flower, which may act in a redundant manner to ensure calcium entry occurs at egg activation. This is consistent with recently published evidence in which CRISPR mediated knock out of Trpm did not affect fertility and fertilised eggs could still be laid (Hu and Wolfner, 2020). This would perhaps suggest a level of redundancy within calcium channels such that activation can occur, and as such Flower could act in tandem with Trpm. Thus, at egg activation, I provide initial evidence that calcium may enter through such calcium channels from the perivitelline space. The perivitelline (PV) space remains a likely candidate for the store of calcium at egg activation; loss of vitelline membrane components point to this as a store (Figure 3.10). It may be the case that knock down/knockout of the vitelline membrane components causes the PV space to become leaky and the calcium is thus lost to the exterior. This would still, however, point to then PV space being required as a store of calcium during *Drosophila* egg activation.

3.3.2 Exploring the significance of different wave phenotypes

Though the calcium wave at *Drosophila* egg activation has been visualised and examined in a number of contexts (Kaneuchi et al., 2015; York-Andersen at al., 2015), it has not yet, to date, been examined in as thorough detail as provided by this thesis. I show clearly that different phenotypes of wave initiation exist in the literatures' "standard" of a full wave, and that within these different waves, there is a clear preference of initiating the wave from the posterior pole. I demonstrate the significant differences between wave phenotypes, namely the proportions of polar waves versus lateral/cortical waves between standard and cortical calcium increases. In addition, I highlight the key differences in speed of initiation and completion of these separate phenotypes, to the extent that I have established sufficient controls that will be required for the following chapters. In other words, the speed, initiation time, and multiple points of initiation of the

cortical increase is an observable phenotype when the cortical actin cytoskeleton is disrupted prior to or during egg activation, as will be discussed in Chapter 4. However, for more thorough comparison of wave phenotypes it would be prudent to generate mathematical models of the varying observable wavefronts and provide more detailed quantification than is provided in this thesis. For example, quantification of whether the wavefront appears as a straight and defined wavefront versus whether it appears with significant curvature may indicate another level of how well regulated the propagatory mechanism of the calcium wave is.

Put together, the data I have presented thus far suggests that at 260 mOsm (AB), the oocyte is capable of generating a calcium wave that initiates from the posterior pole of the oocyte. Alternative phenotypes, such as anterior waves and bipolar waves suggest that the poles of the oocyte are primed for calcium entry, but the nature of this priming has not yet been established (Figure 3.3). Visualisation of TrpM::GFP by the Wolfner group reveals a more uniform expression of the Trp channel around the oocyte, rather than enrichment of these channels at the poles, suggesting an alternative mechanism must be in place (Hu and Wolfner., 2020). One suggestion is that the curvature of the poles themselves aid in channel opening, and though experiments that attempted to round the oocyte revealed polar waves still occurred, they did not completely abolish the defined curvature at the poles, so it is still a possibility (Hu and Wolfner., 2020). Another theory is that the actin cytoskeleton may be regulating calcium entry at the poles, and in the next chapter I provide evidence toward this.

However, despite a clear preference for generating the calcium wave from the poles, the cortical increase phenotype suggests that the entire cortex has the ability to generate a calcium wave, suggesting that whatever enables calcium entry (likely a channel) can be found at all points across the cortex. This was in fact shown recently, as aforementioned (Hu and Wolfner., 2020). The cortical increase phenotype can be forced through the addition of distilled water, and displays specific calcium dynamics; it is much faster at both initiation (to reiterate, the time taken between addition of the activating solution, in this case water, and the first appearance of a calcium increase) and completion (to reiterate, the time point in which the calcium transient has completely traversed the oocyte), and disrupts the preferential initiation of the wave from the poles, significantly increasing calcium entry from many points on the cortex (Figures 3.4, 3.5, 3.6). Wave-like propagation can still be observed, but in what appears to be a mis-regulated format; well defined wavefronts can be observed when AB is used to trigger a full wave, however, the defined wavefront is far more difficult to distinguish during a cortical increase. Again, this suggests that there is some regulatory mechanism in place that distinguishes the standard wave phenotype from the cortical increase, and one suggestion for this regulation is the cortical actin cytoskeleton, which I will explore in much further detail in the next chapter.

3.3.3 The role of calcium channels at egg activation

I demonstrate in this chapter the requirement of the mechanosensitive Trpm channel for calcium entry, in addition providing first evidence of another calcium channel, Flower (Fwe) (Figures 3.11, 3.14). Fwe is an interesting candidate for a channel mediating calcium entry during egg activation, as it has primarily been studied in neuronal signalling at the synapse, acting as a regulator of endocytosis (Li et al., 2020). Fwe is an evolutionarily conserved protein from *C. elegans* to humans, with similarities in amino acid sequences of the calcium permeable pore to that of TrpV5 and TrpV6 (Kuo and Trussel, 2009). The mechanism of action of Fwe in neuronal synapses is dependent on exocytosis; at rest, Fwe is associated with synaptic vesicles (SVs), then after insertion into the membrane during fusion of SVs with the pre-synaptic membrane and release of neurotransmitters, the Fwe channels enable calcium entry and thus calcium-signalling that leads to endocytic re-uptake of vesicular membrane, including the fwe protein itself (Yao et al. 2009).

This presents an interesting question as to whether the mechanism of calcium entry remains similar to that through Trpm during *Drosophila* egg activation, or whether alternative means of regulating calcium flux in Fwe occur. In vertebrate oocytes that undergo Cortical Granule (CG) exocytosis, it presents an interesting parallel, as Fwe channel insertion into the membrane could occur during this process, adding to a positive feedback loop of calcium entry during fertilisation. CG exocytosis is not required in *Drosophila* however, as the oocyte is surrounded by a chorion that prevents polyspermy- the sperm can only enter through the micropyle. There is, however, huge amounts of endocytosis occurring prior to production of the mature oocyte, as during vitellogenesis yolk granules are made through endocytosis, this process being dependent on calcium signalling. It is possible that mechanisms exist that have not yet been investigated that utilise exocytosis as a means of further inserting calcium channels, such as Fwe, into the membrane of the oocyte to generate a positive feedback loop and calcium increase.

Unlike Fwe, I have provided more detailed evidence of the role of Trp channels in egg activation. I explore one potential means of regulation of Trpm in the next chapter, and that is through potential interactions with the cortical actin-cytoskeleton. Another, possibility that should be tested is the role of potential signalling complexes ('signalplex') surrounding the Trpm channel. In *Drosophila* it was shown that TrpL exists in a signalling complex with INAD (Inactivation no after-potential *Drosophila*) and dFKBP59 (*Drosophila* FK506 Binding Protein 59). These are proteins that have been well established as forming this signalplex in *Drosophila* photoreceptors, in which Trp channels associate with the scaffolding protein INAD. INAD contains five tandem PDZ domains which, it has been suggested, act to bind a cluster of membrane and membrane associated proteins to hold the complex in place (Tsunoda et al., 1997; Huber et al., 1996; Chevesich et al., 1997; Shieh and Niemeyer., 1995). The dFKBP59 has been shown to act as part of this complex as a novel regulator of Trpl in *Drosophila*, binding to both the channel and INAD (Goel et al., 2001).

Binding of dFKBP59 inhibits calcium entry, the action of which can be attenuated by the drug FK509 (Goel et al., 2001).

Due to conservation of the dFKBP59 binding site between primary Trp homologs, Leucine-Proline dipeptides within a region of proline-rich of the channel, it is likely that the dFKBP509 protein acts as a regulator of most Trp channels, and as such should be investigated in the context of Trpm during *Drosophila* egg activation. It is likely that dFKBP59 plays an important role in the *Drosophila* oocyte, as ovarian expression of the mRNA signal is extremely high (Drosophila FlyAtlas). It would therefore be important to also evaluate the expression of INAD within the oocyte, and through use of either co-immunoprecipitation studies or yeast-two hybrid screening evaluate whether these proteins exist in a complex with Trpm. Preliminary data (not shown) of dFKBP59 RNAi mediated knock down suggests that it may be involved in calcium entry during egg activation, however, mutational analysis of the effect of dFKBP59 and INAD would provide further evidence of their importance in regulation of Trpm channels. Given evidence provided in my second chapter, it would also be interesting to evaluate whether this signalplex has any significant interactions with the cortical actin or specifically actin-binding proteins such as α-actinin and supervillin, as it may be the case that regulation of Trpm by the actin cytoskeleton is vital for regulated calcium entry during egg activation.

3.3.4 Alternative models of calcium entry at *Drosophila* egg activation

Still to be fully investigated an elaborated is the role of potential IP_3 and store-operated calcium entry. Evidence would indicate that IP_3 mediated calcium efflux from the ER is required for the propagation of a calcium wave in *Drosophila* oocytes (Hu and Wolfner., 2019; 2020). Here, *in vivo* data demonstrated the loss of calcium wave propagation in an IP_3R RNAi background; data here was difficult to interpret however due to very low magnification images of flies anaesthetised with FlyNap. Alternative data of IP_3R mutants, however, showed no significant change in the calcium wave (York-Andersen, PhD.). Though there is some controversy within the field regarding the role of IP_3, the general direction the consensus is heading is that Trpm is required for calcium entry, and IP_3 mediated mechanisms play more of a role in ensuring a propagating wave.

Previous attempts at knocking down the IP_3R utilising RNAi mediated knock down and incubation with heparin revealed no changes in the calcium wave. However, knock down of the STIM-ORAI components that mediate store operated calcium entry (SOCE) revealed an interesting change; a small yet significant drop in the number of full waves was observed when STIM was knock down down. However, knock down of ORAI had no effect on the calcium waves. These components are known to interact with the IP_3 pathway, as mutation of the IP_3R attenuates SOCE evoked by depleting the ER of calcium (Chakraborty et al., 2016). Therefore there may be some contribution from the ER to the calcium wave, however this could still be primarily for propagation rather than initiation and should be investigated further.

3.3.5 Future Directions

There is still a great deal of unknowns with respect to *Drosophila* egg activation, especially in the regulation of calcium entry into and exit from the oocyte. As aforementioned, complete knock out of Trpm by CRISPR does not significantly impact fertility in *Drosophila* (Hu and Wolfner, 2020). This would suggest that mechanisms are in place, perhaps redundant, to ensure calcium entry at egg activation, one of which may be the calcium channel Fwe, in which my data suggests RNAi mediated knock down to significantly reduce the number of full waves (Figure 3.11). There may also be a contribution from the IP_3 pathway, though conflicting evidence exists for this, as previous data (York-Andersen, PhD Thesis), has not shown any significant change to the calcium wave when IP_3R is mutated. Therefore there is still much to be done in identification of the mechanisms of calcium entry at egg activation, as there may be further contribution from a variety of other channels. Further work should be carried out examining the impact of Fwe on calcium entry, importantly with further qPCR required to assess the levels of knock down in an RNAi background. This may provide further information on the relative levels of each channel required for calcium entry, as it appears the oocyte is more sensitive to a drop in Trpm expression in comparison to Fwe.

Additionally, there is clearly more to be investigated with regards to the similarities and differences between *ex-vivo* and *in-vivo* egg activation. Having begun to develop a new method of visualising the *in-vivo* wave, it is evident that the calcium wave at egg activation can occur rather quickly, in the order of 1 minute (Figure 3.16). Having said this, the set-up for the examining an *in-vivo* still requires modification and improvement (Figure 3.15). Though I would argue the set up used is better than previous set-ups which require the use of an anaesthetic agent, there is clearly an issue with restricting the *Drosophila* to a confined space and having to deal with movement of the *Drosophila* within the pipette. Despite these struggles, *in-vivo* waves were imaged, and as aforementioned appeared to occur in the space of around 1 minute.

I therefore examined in further detail the physiology of a cortical increase, which was still able to mediate downstream events of egg activation, just at a faster rate (Figure 3.8). However, it is likely that there were significant disruptions downstream of these events due to the rapid speed at which they occur (ie. a serious lack of regulation, such as in the rapid change in global actin), especially considering these events were forced through incubation with water which would have caused an osmotic shock to the cell. It is not entirely physiological to deposit an oocyte into a 0 mOsm solution, therefore, these results should be interpreted with care. One may expect, therefore, that the presence of the oviduct walls is essential for regulating the osmotic-mediated swelling of the oocyte (through provision of a restrictive force that prevents over-swelling), and that *ex vivo*, the most accurate recapitulation of this event is to use a buffer, such as AB, which has an osmolarity that causes swelling to occur at a speed more consistent with *in vivo* speeds.

I believe the use of *ex-vivo* activation is still the best approach as it allows a significantly higher throughput, magnification and resolution in experiments. There still appears to be a polarity associated with *in-vivo* egg activation, the calcium transient occurring as a singular propagating wave. The fundamental mechanisms of egg activation can still be highlighted and explored through *ex vivo* means, and in fact the environmental conditions better manipulated, such as osmolarity, ionic composition and temperature.

Thus far, I have only examined the mechanisms of calcium entry at egg activation. However, the mechanisms of calcium removal at egg activation are critically important yet vastly understudied as the field has focussed on understanding which channels and sources are essential for initiation. It is clear that such extrusion of calcium is vitally important, as the balance of intra versus extra-cellular calcium maintains calcium at an appropriately low level within the cell such that aberrant signalling is avoided and cells retain the potential to undergo a calcium influx which acts as a key signalling event (reviewed in Berridge et al. 2003). Now that I have provided further evidence that the perivitelline space is important as a calcium source, and that in fact this remains a store of calcium into the embryonic stages of development (Stein and Nusslein-Volhard., 1992), we need next begin dissecting the mechanisms of calcium ion removal.

A variety of protein channels mediate calcium removal in cells, such as ATPases, SERCA and PMCA, which largely pump calcium out of the cytoplasm and into their respective stores, such as the endoplasmic reticulum in the case of SERCA (Reviewed in Stafford et al., 2017)**.** In mouse oocytes, blocking SERCA through application of the drug thapsigargin resulted in significantly shorter calcium oscillations at egg activation, suggesting it plays a role in the calcium flux during this key event (Kline and Kline., 1992). In *Drosophila*, very little data exists that would point us in any one direction. Application of the ATPase inhibiting drug sodium orthovanadate prevents recovery of the calcium wave at egg activation, suggesting this is an energy-dependent process (York-Andersen et al., 2020). This would suggest that SERCA or PMCA may play a role, and given indications that calcium is sourced from the perivitelline space and is still present there in the embryonic stages of development (Stein and Nusslein-Volhard., 1992), it is likely that PMCA is required for pumping calcium back out of the oocyte. It is quite common for multiple mechanisms to be at play, however, and likely that SERCA also plays a role in removal of calcium at egg activation. Interestingly, application of thapsigargin had no effect on the timings of calcium wave recovery (York-Andersen, PhD 2018), suggesting that SERCA may play less of a role than at first expected. Furthermore, *Xenopus* oocytes were shown to utilise PMCA, as inhibition prevented calcium recovery following the calcium transient at egg activation (El-Jouni et al., 2008). It is likely, therefore, that PMCA plays a role in the removal of calcium at egg activation, which is further supported by the fact that it is highly regulated by the actin cytoskeleton, perhaps providing a physiological role for the global actin changes that occur at egg activation as arranging actin opens these channels in high calcium, therefore enabling extrusion of the calcium whereupon the

reduced calcium environment enables actin rearrangements and closing of the channels (Dalghi et al., 2013).

Therefore, initial mutational analysis should be carried out to investigate which channels are required for calcium removal at egg activation, and further analysis during the calcium transient itself as SERCA is known to help regulate oscillatory mechanisms, therefore it could well be the case that they play a role in the formation of a propagating wave. Furthermore, alternative mechanisms should be explored such as the potential role of sodium-calcium exchangers which have been shown to play a role in the calcium oscillations in mouse oocytes (Pepperell et al., 1999; Carroll, 2001). Following this, the interplay between the calcium and actin is likely crucial in regulating calcium export; as I will come to show in my next chapter, the state of the cortical actin is directly correlated to the potential for calcium influx across the oocyte membrane, which would ultimately indicate it is also key in regulating efflux.

Chapter 4:
Cortical actin cytoskeleton regulates calcium entry at egg activation

Chapter 4: Cortical actin cytoskeleton regulates calcium entry at egg activation

4.1 Introduction

4.1.1 Chapter Overview

In my previous chapter I review the variety of calcium phenotypes and channels that are required for calcium entry. One key question that remains to be answered is: How is calcium entry regulated? Many lines of evidence point to the cortical actin being a key regulator of calcium entry; 1) Localisation of the calcium channels in the membrane puts them in close proximity to the cortex; 2) Transduction of mechanical forces often relies upon an acto-myosin cortex (Kelkar et al., 2020; Vasilev et al., 2021); 3) Trp channels have been shown to make direct contact with the actin cytoskeleton (Christensen and Corey, 2007); 4) Preliminary pharmacological data suggests a requirement of actin for calcium entry (York-Andersen et al., 2015).

With this in mind, in this chapter I utilise the various genetic tools available in *Drosophila* to both visualise the changes in the various actin populations in the oocyte prior to and around egg activation. I further use mutational analysis, RNAi mediated knock down and over-expression to manipulate the cortical actin to reveal a key relationship between the cortical actin and calcium entry; over-expression or stabilisation of the cortex prevents calcium entry whereas reduction of cortical actin potentiates calcium entry. Within this key relationship, I begin to demonstrate a key role for the Arp2/3 and SpireB complexes in production of a cortical actin that enables calcium entry in the form of a regulated wave. I further demonstrate the requirement of various actin binding proteins in regulation of calcium entry, suggesting a complex relationship between calcium and regulators of the actin architecture.

4.1.2 Architecture of the Actin Cytoskeleton in Oocytes

The actin cytoskeleton is a fundamental component of all eukaryotic cells, existing as a globular 42-kDa protein, G-actin, that polymerises into polar filaments known as F-actin. Its functions are vast, being required for intra-cellular transport, organelle positioning and cellular division, movement and morphogenesis. It is so essential, in a functional context, that prokaryotic organisms possess true, yet distant, homologues of actin; MreB and ParM show high structural homology to actin, assembling into dynamic filaments (Reviewed in Carballido-López, 2006).

In broad terms, different pools of actin exist within eukaryotic cells. Cytoplasmic actin is possibly the broadest of these pools, providing actin filaments for intracellular transport and organelle positioning, yet also for the production of specialised cellular protrusions such as lamellipodia and filopodia (Reviewed in Rottner et al., 2017; Blanchoin et al., 2014).

A key component of cells is the cortex, which can be described as a contractile shell of actin associated with the plasma membrane, on average between 50-200nm thick (but notably much thicker in certain oocytes) (Morone et al., 2006). The cortex is a densely cross-linked network of F-

Actin Labelling Tool	Fixed or Live	Construct	Actin Population Labelled	Reference
F-tractin.tdTomato	Both	N-terminus of IP_3K actin-binding domain conjugated to fluorophore	All F-actin structures	Schell, 2001
Lifeact-GFP	Both	First 17 amino amino acids of the yeast actin-binding protein, Abp 140, conjugated to fluorophore	All F-actin structures	Riedl et al., 2008
Utrophin-GFP	Both (better live)	Actin binding calponis homology domain of Utrophin conjugated to fluorophore	All F-actin structures	Rauzi et al., 2010
Moesin::GFP	Both	Actin binding C-terminus Moesin tail conjugated to fluorophore	Cortical F-actin	Edwards et el., 1997
Act5C-GFP	Both (better fixed & boosted)	Monomeric actin 5C subunit conjugated to fluorophore	All cellular actin, both monomeric and polymeric	Sokol & Cooley, 2003
AlexaFluor568-Phalloidin	Fixed	Actin stabilising phallotoxin conjugated fluorophore	All F-actin structures	Cooper, 1987

Table 1: Summary of actin labelling tools used to visualise actin within the *Drosophila* germline

actin, containing hundreds of ABPs, including myosin-2 motors which generate cortical tension (Chugh and Paluch., 2018). The cell cortex is responsible for controlling cellular morphogenesis, division, and polarisation (Chugh and Palcuh., 2018; Tsankova et al., 2017).

I will utilise a number of genetic markers of actin in addition to fixation and labelling to observe the actin network in the *Drosophila* mature oocyte prior to and following egg activation.

4.1.3 Regulators of the Actin Cytoskeleton

Actin monomers are able to spontaneously polymerise into filaments- however, due to the relative instability of the filament and the action of g-actin binding and sequestering proteins, a variety of factors are required to aid the assembly and organisation of filaments. There are three major classes of actin nucleator; i) The Arp2/3 complex which is activated by a variety of factors such as SCAR (the *Drosophila* homologue of yeast Scar), related to the Wiskott–Aldrich syndrome protein (WASP), that promotes the formation of branched actin networks (Machesky et al., 1998; Mullins et al., 1998); ii) Formins, which assemble into a doughnut shaped dimer and generally promote the formation of non-branched actin networks, such as Cappuccino (Capu) (Otomo et al.., 2005b; Pruyne et al., 2002; Vavylonis et al., 2005); iii) Tandem actin-binding domain nucleators, such as Spire, which promote nucleation of actin filaments through interaction with two or more g-actin monomers in WH2 or alternative actin-binding domains (Dominquez, 2016; Quinlan et al., 2005).

The Arp2/3 complex and associated activator proteins have been shown to be required during oogenesis in *Drosophila.* A partial reduction of SCAR due to a hypomorphic allele (the *SCAR* gene is disrupted with the l(2)k13811 insertion) does not affect oogenesis (shown for heterozygous Scar mutants), however, knock out of SCAR demonstrates its necessity in the production of actin, as oogenesis then produces morphologically abnormal oocytes (Zallen et al., 2002). These phenotypes very closely resemble those caused by mutations of *Arpc1* and *Arp3* in *Drosophila* egg chambers (Hudson and Cooley, 2002). The Arp2/3 activator WASP appears to play little role, or else is dispensable, during oogenesis however, as mutations do not effect developing egg chambers (Zallen et al., 2002).

In mouse oocytes, it has been demonstrated that the actin nucleators Formin-2 (Fmn2) and Spire-1/2 are required for production of the cytoplasmic actin mesh (Montaville et al., 2014; Azoury et al., 2008). These nucleators have direct homologues in *Drosophila,* where they have been better studied; here Cappuccino (Capu, the *Drosophila* homologue of Formin-2) and Spire interact through the formin-homology 2 domain (FH2) of Capu and the kinase noncatalytic C-lobe domain (KIND) of Spire (Quinlan et al., 2007). In mice, the Fmn2-Spire complex has been demonstrated to be essential for production of a cytoplasmic mesh of actin that regulates nuclear positioning and spindle migration in the mature oocyte (Uraji et al., 2018). However, the role of Capu and Spire in *Drosophila* has been investigated prior to maturation of the oocyte, as they were demonstrated to be required for the production of an actin mesh than inhibits formation of a microtubule network required for cytoplasmic streaming in the developing oocyte, disruption of this mesh leads to premature streaming and polarity defects in the oocyte (Rosales-Nieves et al., 2006; Theurkauf, 1994; Serbus et al., 2005; Quinlan et al., 2007). Formins have also been implicated more generally in the production and turnover of the cortical actin cytoskeleton, in addition to the cytoplasmic mesh (Fritzche et al., 2013). The role of Capu and Spire in the mature oocyte have not been well investigated, however, especially the impact of these proteins upon the events of egg activation.

In addition to regulating the nucleation of actin, many factors, known as actin-binding proteins (ABPs), regulate the final architecture of the actin filaments, whether that be to promote branching, cross-linking or connecting the actin cytoskeleton to other proteins, such as channels, receptors and the microtubule cytoskeleton. For instance, membrane linkage of the actin-cytoskeleton, vital for anchorage of the cortex, is largely carried out by the ERM family of proteins, including Ezrin, Radixin and Moesin (Bretscher et al., 1999). However, in *Drosophila,* the only ERM protein is Moesin, which is required for cortex stiffening and cell rounding (Carreno et al., 2008; Kunda et al., 2008).

The gelsolin superfamily proteins are a group of calcium sensitive actin severing proteins, activated through large conformational changes in the presence of actin. Supervillin, named as it is the largest of the villin/gelsolin family of proteins, has not been shown to be calcium sensitive, but is known to have C and N termini that bind to F-actin, unlike the remainder of the villin/gelsolin proteins in which the N terminus contains F-actin severing domains (Pestonjamasp et al., 1995;1997; Janmey et al., 1998; Kwiatowski, 1999; Way et al., 1989). The N terminus domain is known to promote actin bundling, containing three actin binding sites and a binding site for myosin-II (Chen et al., 2003). As a peripheral membrane protein, it is very likely that supervillin directly regulates the levels of actin bundling and cross-linking within the cortex (Pestonjamasp et al., 1997).

Other ABPs also demonstrate calcium sensitive responses, such as in their ability to bind F-actin. α-actinin is a particularly interesting example, as non-muscle isoforms demonstrate calcium-dependency on their level of cross-linking; under low intracellular calcium, α-actinin is able to cross-linking multiple actin filaments, however, when the levels of intracellular calcium rise, α-actinin is unable to cross-link multiple filaments, and thus the level of cross linking in the cell decreases (Jayadev et al. 2014; Prebil et al. 2016; Sjöblom et al. 2008). This is likely an advantageous property for non-muscle cells, as in the asbence of calcium α-actinin is able to maintain a stable network and in the presence of calcium it releases actin filaments to enable their rapid reorganisation (Burridge and Feramisco., 1981).

I will therefore manipulate the actin architecture within the mature oocyte through modulating the levels of both actin nucleating proteins and ABPs, enabling increased and reduced levels of actin and varying levels of cross linking of the actin cytoskeleton.

4.1.4 Role of the Actin Cytoskeleton in Regulation of Calcium Flux

At egg activation in *Drosophila*, it is well known that a wave of calcium traverses the oocyte. However, recently published evidence demonstrates a wave of reorganising actin that follows the calcium wave (York-Andersen et al., 2020). Similarly, *Xenopus* eggs demonstrate a wave of cortical F-actin upon activation, utilising the F-actin markers Lifeact and Utrophin (Bement et al., 2015). Not only this, but a wave of Rho-activity was also demonstrated in both frogs and starfish; Rho is a

well-known regulator of F-actin polymerisation (Ridley and Hall., 1992; Nobes and Hall., 1995; Bement et al., 2015). In order to understand the calcium wave, it is becoming more apparent that actin dynamics at egg activation should be investigated. Although it is still unclear what the relationship between actin and calcium is, there is a clear dependence between the two: inhibiting actin polymerisation dynamics through the addition of cytochalasin-D prevents the calcium wave (York-Andersen et al., 2015). Additionally, preventing the calcium wave through the addition of 2-APB (2- Aminoethoxydiphenyl borate, a non-specific inhibit of IP3-induced calcium release and Trp channels) or a sodium solution also prevents the calcium wave. Thus the actin population is clearly necessary for calcium entry at some level, as is the case in starfish; latrunculin-A was added to oocytes which prevented calcium entry upon 1-MA stimulation, a hormone known to trigger egg activation in starfish oocytes (Kyozuka et al., 2008).

In fact, starfish oocytes are providing a model system for our understanding of the relationship between calcium and actin during egg maturation and activation. During oocyte maturation in starfish, application of the hormone 1-MA causes an increase in calcium ions, likely through activation of a yet unknown receptor, propagating from the vegetal to animal pole, and following which occurs various actin rearrangements such as the formation of surface spikes that contain bundles of f-actin (Chiba. 2020; Santella et al., 2015; Kyozuka et al., 2008). Furthermore, actin depolymerisation has been shown to result in calcium influx and triggering of downstream egg activation events (Lim et al., 2002). Treatment of starfish oocytes with 5uM ionomycin, a calcium ionophore, caused large scale dispersion of the cortical actin after 3-5 minutes. The cytoplasmic network is increasingly bundled, however (Vasilev et al., 2012). This has also been seen in MDKC cells, in which the entry of calcium causes a rapid reduction of cortical actin, but polymerisation of filaments throughout the cytoplasm (Wales et al., 2016). This suggests that calcium may have separate effects on the different populations of actin; cortical vs cytoplasmic. This may also point to a potential mechanism for calcium mediated calcium entry; calcium enters and causes cortical actin to depolymerise, potentiating further calcium entry. A mechanism for calcium induced calcium influx (CICI) has been proposed in which stretching of mechanosensitive channels enables calcium entry, and this calcium promotes filament sliding via actomyosin, thus increasing tension and opening further channels, enabling propagation of a calcium wave (Jaffe, 2007; Jaffe, 2008). This mechanism should be tested in the case of the *Drosophila* oocyte and egg activation, however with evidence also pointing to calcium induced actin polymerisation, this may point to a potential novel mechanism of calcium induced calcium influx, in which dispersion or depolymerisation of actin aids calcium entry.

Of course, there is also much evidence for the association of mechanosensitive channels with actin filaments. In mice, actin is required for TRPV3 mediated calcium influx (Lee et al., 2016), and it has been demonstrated that Trp channels may be regulated through the transduction of force through actin cytoskeleton itself (Christensen and Corey., 2007). It has been further established that other mechanosensitive channels bind actin either directly or indirectly, such as the ENaC channel which directly binds F-actin, and other channels through actin-binding factors such as α-

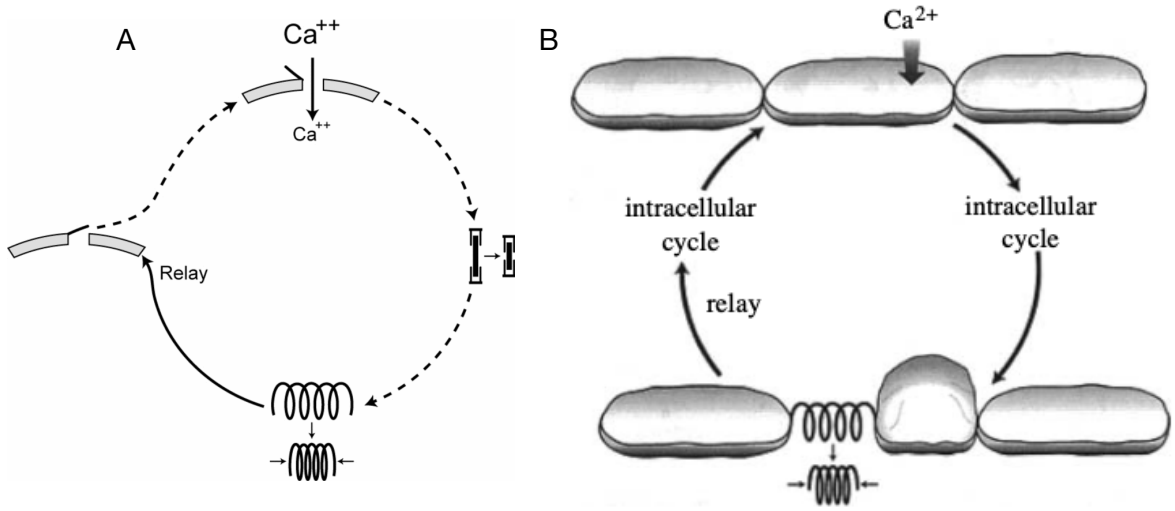

Figure 4.1 Proposed mechanisms for Calcium-Induced Calcium Influx (CICI) (Figure adapted from Jaffe 2007; 2008). (A) A schematic demonstrating the cycle of CICI. Calcium entry results in filaments (eg. Acto-myosin filaments) contracting as calcium promotes actin-myosin interaction. This increase in tension results in stretching of the cell membrane enabling nearby calcium Chanels to open and relay the signal. **(B)** A schematic contextualising CICI in relation to the plasma membrane. As above, calcium entry promotes opening of channels in the membrane to

actinic, spectrin and filamin (Mazzochi et al., 2006; Maruoka et al., 2000; Cukovic et al., 2001). As for Trp channels specifically, it has been demonstrated that TRPN1 co-localises with actin in *Xenopus* cilia (Shin et al., 2005) and that TRPC6 and TRPL interact with regulators of the actin cytoskeleton (Dryer and Reiser., 2010; Chorna-Ornan et al., 2005).

There are still many questions to be answered on the relationship between calcium and actin, and the importance of such actin dynamics. Actin has been shown to be localised with and even bound to certain membrane ion channels, such as the ENaC channel and TrpN1 (Mazzochi et al., 2006; Shin et al., 2005). Therefore, could actin be playing a role in regulating entry of calcium, perhaps through associations with mechanically gated channels? Given evidence that demonstrates the *Drosophila* oocyte undergoes swelling due to changes in external osmolarity and my now published data that indicates Trpm, a mechanically gated channel, is required for calcium entry (York-Andersen et al., 2021), it is highly likely that the cortical actin cytoskeleton plays a role in regulation of both swelling and activation of the Trpm channels, and as such should be investigated further.

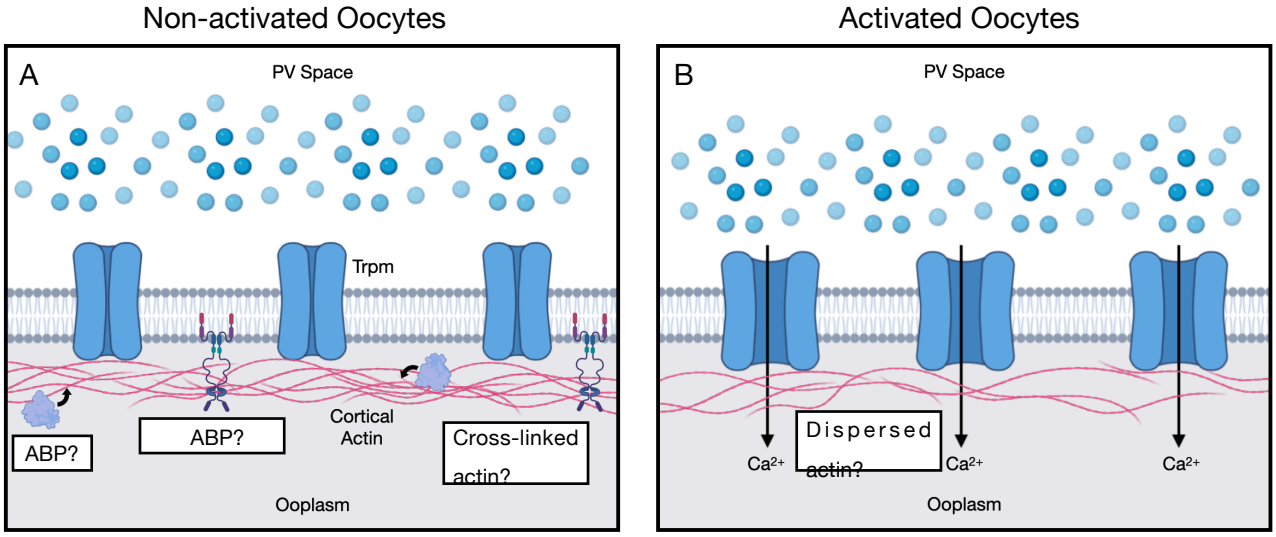

Figure 4.2 Hypothesis of actin-regulation of calcium flux. (A) In a pre-activated oocyte, I hypothesise that various actin binding proteins (ABPs) contribute to the architecture of the cortical actin, and prior to activation this results in a tightly cross-linked cortical actin that prevents premature calcium entry. **(B)** At egg activation, dispersion of the cortical actin and prevention of cross-linking enables calcium entry, meanwhile maintaining tension in the cortex such that force can be transduced to channels mediating calcium entry.

4.1.5 Aims of Chapter:

1- I will provide a detailed overview of the architecture of the actin cytoskeleton in the mature oocyte and the changes it undergoes at egg activation utilising a variety of high-resolution imaging techniques.

2- I will establish the relationship between the cortical actin cytoskeleton and calcium entry at egg activation through manipulation of the cortical actin using pharmacological and genetic approaches.

3- I will identify factors that are required to regulate the cortical actin cytoskeleton prior to and during egg activation through genetic knock down and knock out of candidate factors and observation on their impact on the calcium wave at egg activation.

4.2 Results:

Having further investigated the mechanism of calcium entry in my previous chapter, with results suggesting that swelling of the oocyte opens Trpm channels in the membrane enabling entry of calcium from the perivitelline space, the question remains how is calcium entry regulated to form a propagating wave? In this chapter I will argue that actin at the cortex plays a vital part in this regulation, and that it is likely that actin may also be playing a role in the formation of a propagating wave. Multiple models could exist, perhaps exclusively or perhaps in parallel, as to how actin at the cortex regulates calcium entry. The cortical actin could be acting as a physical barrier to calcium entry or swelling, or the actin could be acting to transduce the force of the swelling to the TrpM channels themselves. It is likely that very precise regulation of the actin cytoskeleton is required to balance such opposing forces; ie. Restraining the swelling of the oocyte whilst also acting to transduce the force of the swelling to mechanosensitive channels.

4.2.1 Distribution of the Actin Cytoskeleton in Mature Oocytes

Previous work has started to point to the requirement of actin for egg activation (York-Andersen et al., 2015), and as such a thorough characterisation of the actin distribution in the stage 14 oocyte was necessary for understanding how actin dynamics may influence calcium entry at egg activation.

Alexa-Fluor568 conjugated Phalloidin staining shows a prominent band of cortical actin that surrounds the oocyte (Figure 4.3 A). However, even at a low magnification, one can see that the distribution of cortical actin does not appear completely even. Higher magnification imaging of Phalloidin, in addition to a number of other markers of the actin cytoskeleton, reveals a reduction in the the fluorescence intensity at the poles of the oocyte (Figure 4.3 B). Comparison of this reduction of fluorescence intensity to Resille::GFP (a marker of the membranes, frequently found on the Golgi and remaining endomembrane system, and frequently used as a “cell outline marker” (confirmed in Wang et al. 2013; Jewett et al. 2017)) confirms that this reduction in the fluorescence intensity is not due to the curvature of the membrane at the poles, but likely due to a reduction in the density of the cortical actin mesh at the poles (Figure 4.3 C). This distribution was corroborated with live imaging using two different actin markers Moesin::GFP and Act5C-GFP. These were used, rather than F-tractin.tdTomato, as they produce a stronger signal that enables easier comparison between the cortex at the poles and lateral sides of the oocyte. Moesin::GFP is the actin binding C-terminus of the ERM protein Moesin, conjugated to a GFP, and has been shown to exclusively label the cortical actin (Edwards et al., 1997). Act5C-GFP consists of the actin monomer Actin5C conjugated to a GFP, and is driven through Gal4 activation of a UASp, thus driving the expression of the construct exclusively in the germline and labelling all actin.

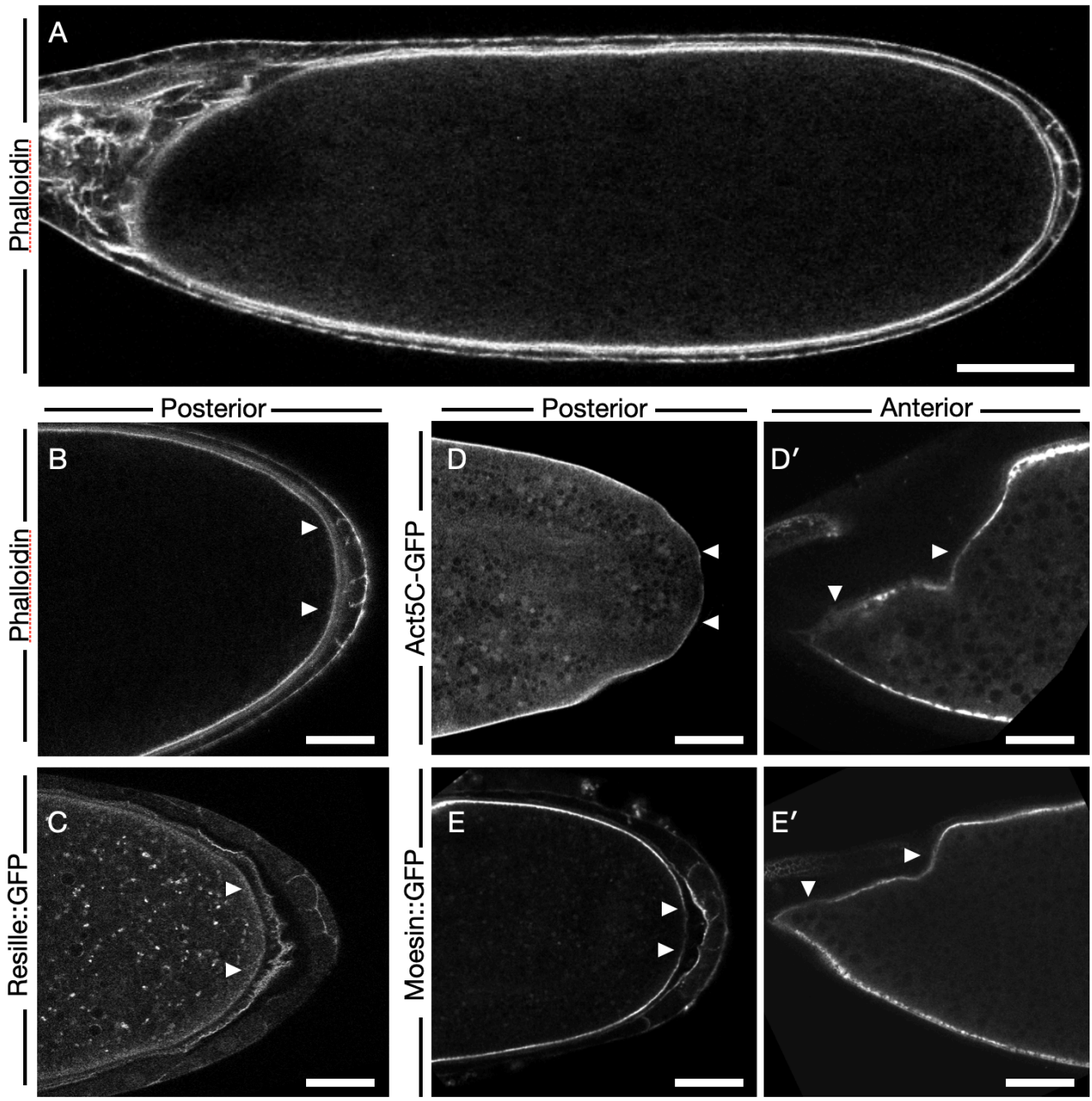

Figure 4.3: High resolution imaging of actin prior to egg activation. (A) Confocal Z-projection of a fixed mature oocyte showing actin (AlexaFluor-568 Phalloidin) demonstrating strong staining of the cortical actin in a prominent band. Scale bar: 100um. N=34. (B) Confocal Z-projection of the posterior pole of a fixed mature oocyte showing actin (AlexaFluor-568 Phalloidin) demonstrating a reduction in fluorescence at the posterior pole. Scale bar: 20 μM. N=34. (C) Confocal Z-projection of the posterior pole of a fixed mature oocyte showing membranes (Resille::GFP) demonstrating a uniform fluorescence intensity at the cortex. Scale bar: 20 μM. N=15. (D-D′) Confocal Z-projections of the posterior and anterior poles of a live mature oocyte showing all actin (Act5C-GFP). A clear reduction in fluorescence intensity is observable at the posterior (D) and anterior (D′). Scale bar: 20 μm. N=20. (E-E′) Confocal Z-projections of the posterior and anterior poles of a live mature oocyte showing cortical actin (Moesin::GFP). A clear reduction in fluorescence intensity is observable at the posterior (E) and anterior (E′). Scale bar: 20 μm. N=30.

Both Moesin::GFP and Act5C-GFP again show a marked reduction of actin at the posterior, though of course one must be careful when observing Act5C through expression of a GFP-tagged monomer as this would constitute overexpression which could lead to abnormalities in the cytoskeletal architecture. Unlike Phalloidin, which richly stains the dorsal appendages and surrounding tissues, obscuring the anterior, Moesin::GFP and Act5C-GFP are poorly expressed in the tissues surrounding the dorsal appendages. It is therefore possible to better visualise the anterior of the oocyte, which also demonstrated a marked reduction in the fluorescence intensity of these markers (Figure 4.3 D′-E′). Taken with data from my previous chapter that demonstrates the much higher frequency of calcium propagation from the poles of the oocyte, this could indicate that the reduction of actin at the poles primes these regions for calcium entry at egg activation, the potential mechanisms for which will be investigated further in this chapter.

Having examined further the architecture of the cortical actin within the mature oocyte, I sought to investigate what changes occur at egg activation; in my previous chapter I begin to show that calcium entry occurs in a regulated fashion following incubation with activation buffer, and as such I sought to investigate whether any changes in the actin cytoskeleton accompany this regulated flux of calcium. I took high resolution 3D images over the course of egg activation in oocytes expressing F-Tractin. F-tractin was generated from the rat actin-binding IP_3 Kinase A, and has been demonstrated to closely correlate with Phalloidin staining and be the least invasive actin probe in *Drosophila* oogenesis, based on fewer observable actin defects and little-to-no impact on fertility (Schell et al., 2001; Spracklen et al., 2014a). I found that F-tractin very accurately labels all F-actin, providing great detail with both cortical and cytoplasmic actin, in addition to having less background in comparison to Act5C-GFP which labels all actin. This demonstrated that prior to egg activation, the actin is distributed around well defined ridges in the dehydrated oocyte, with a clear cortical enrichment (Figure 4.4 A-A′). Upon addition of activation buffer (AB) the oocyte swells and the actin population becomes redistributed into discrete foci (Figure 4.4 B-C′). This distribution of actin is also seen in oocytes dissected from the oviduct (therefore activated *in vivo*), corroborating this change seen during *ex vivo* activation (Figure 4.4 D-D′). It is evident that a similar morphology of the actin cytoskeleton can be observed between my *ex-vivo* activated oocytes and those oocytes that were activated *in vivo*, providing an extra control for ensuring this change is physiological.

I further visualised the cortical actin utilising F-tractin.tdTomato and through taking single plane images of the cortex (Figure 4. 4). This enables better visualisation of the cortex itself, which upon the addition of AB stretches as the oocyte expands, becomes visibly thicker and again one can visualise individual punctae within the cortex (Figure 4.5 A-C). Single plane images of the cortex from laid eggs (activated in vivo) also demonstrate a thicker cortex containing individual punctae, corroborating these changes following *ex vivo* activation (Figure 4.5 D). This thickness can be measured using imageJ, forming a perpendicular line from each edge of the cortex measured as

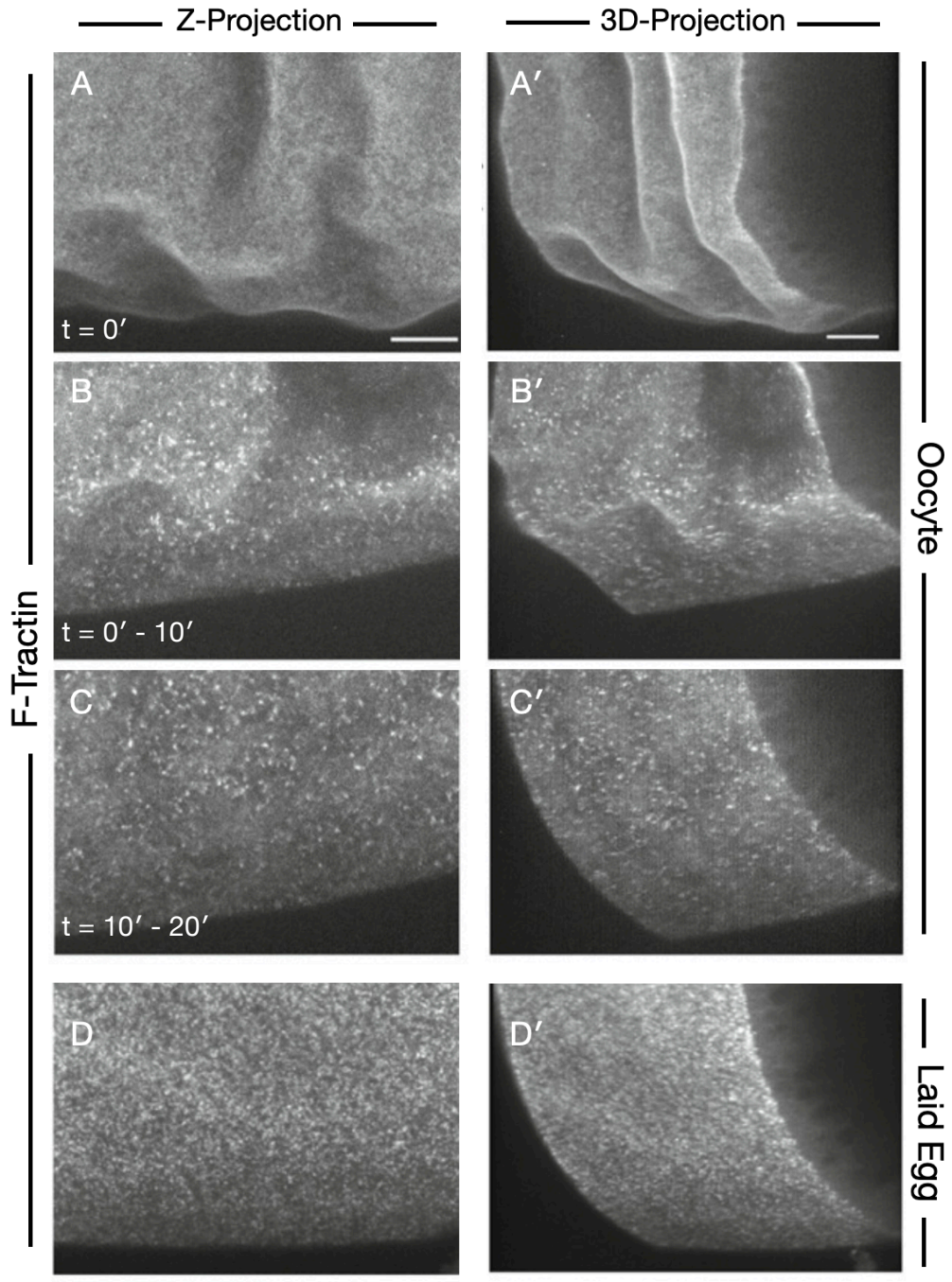

Figure 4.4: High resolution 3D imaging of actin during egg activation. (A-C) Confocal Z-projections of a live mature oocyte showing actin (F-Tractin) upon the addition of activation buffer (AB). (A′-C′) Corresponding 3D-projections of a live mature oocyte showing actin (F-Tractin) upon the addition of AB. N=5. (D) Confocal Z-projection of a live, laid egg (activated *in vivo*) showing actin (F-tractin). (D′) Corresponding 3D-projection of a live, laid egg (activated *in vivo*) showing actin (F-tractin). Scale bar: 20 μm. N=8.

the area of fluorescence significantly greater in intensity that the cytoplasmic and background fluorescence. Imaging this dispersion event at higher resolution than previously published enabled quantitative evaluation of the increase of cortical thickness (as the moesin disperses), showing that upon the addition of AB, the cortical thickness increases from on average 1.2um to 1.55 um

(Figure 4.5 E). Evidently there is a significant change in the cortical actin at egg activation, namely a significant thickening of the cortex, which likely represents a decrease in density and correlates with a reduced cortical tension, as it does in mice (Chaigne et al., 2013).

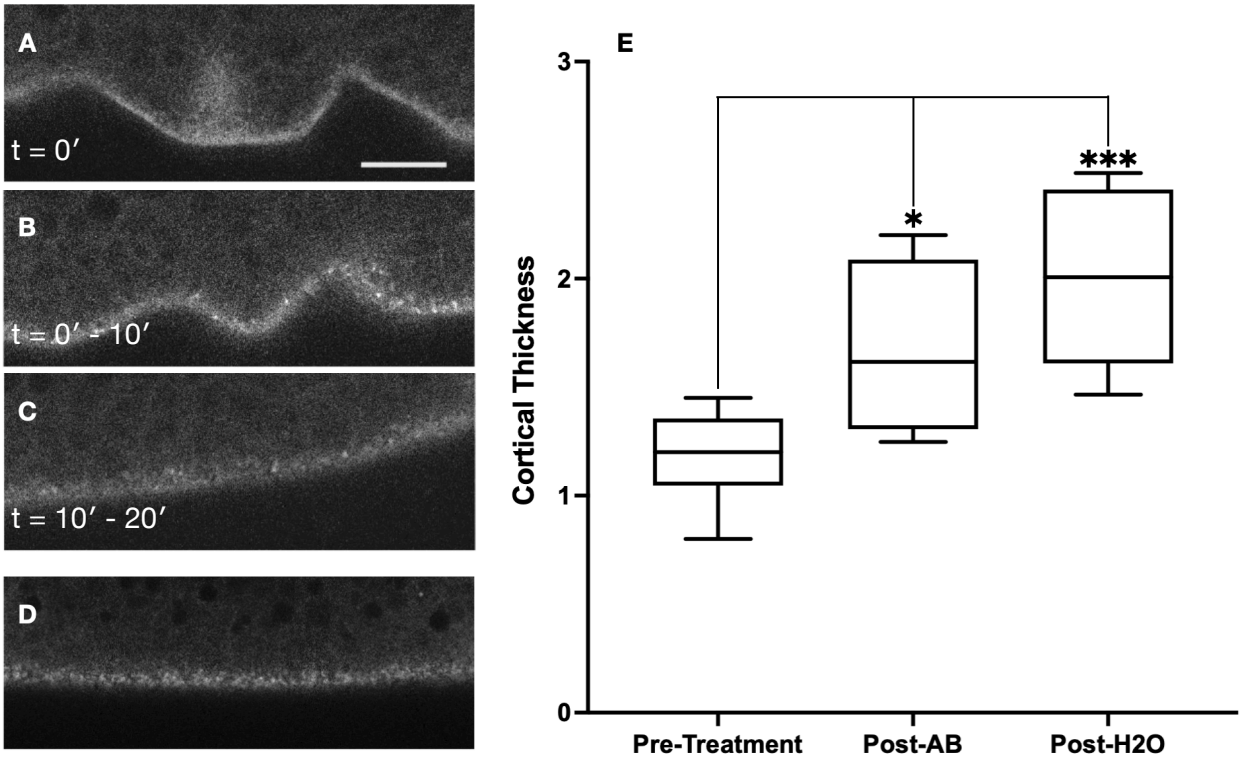

Figure 4.5: Cortical thickness increases in oocytes activated *in vivo* and through hypo-osmotic treatment. (A-C) Confocal single plane images of a live mature oocyte showing actin (F-Tractin) upon the addition of activation buffer (AB). Scale bar: 10 μM, N = 22 (D) Confocal single plane image of a live, laid egg (activated *in vivo*) showing actin (F-tractin). Scale bar: 10 μM. N = 10 (E′) Comparison of the cortical thickness (um) of non-activated (N = 44), AB treated (N=22) and H2O treated (N=13) oocytes shows a significant increase in thickness of treated oocytes.

Given evidence that suggests a reduced cortical actin may prime the poles for calcium entry, and that upon the addition of a hypotonic solution the cortex becomes more disperse, I sought to evaluate whether the addition of H_2O had a similar effect on the cortical actin. Upon the addition of water, the increase in cortical thickness increases to 2um (Figure 4.5 E). Although this a more significant increase of thickness from the pre-treated oocyte as compared to adding AB, there is no significant difference between thicknesses of AB and H_2O treated oocytes. This still may suggest that the more dramatic dispersion of cortical actin could contribute to the cortical entry phenotype of calcium at egg activation.

To assess whether the change in cortex thickness does represent a change in the dynamics of the cortical actin, Act5C-GFP was expressed in the mature oocyte under the control of tubulin-Gal4. Act5C-GFP was chosen as this labels the actin directly rather than a tool that binds to the actin- in this way I generate data that is directed to dynamics of the actin monomers themselves, rather than the dynamics of a labelling tool that binds to the actin, such as F-tractin. This labelled actin throughout the oocyte, with a prominent band corresponding to the cortex. Fluorescence recovery after photobleaching (FRAP) was carried out on the cortical actin; photo-bleaching was carried out for 5 seconds using the same setting pre- and post-activation and recovery was observed following this bleaching event (Figure 4.6 A-B). FRAP analysis of this population of actin pre and post-activation reveals different dynamics of recovery, with the halftime of recovery being significantly faster post-activation (Figure 4.6 C). This would indicate that the dynamics of the actin cortex increase post-activation as actin is recruited back to the cortex at a faster rate. This increase in dynamics at egg activation suggests that dispersion may be required for calcium entry; for example the opening of mechanosensitive TrpM channels.

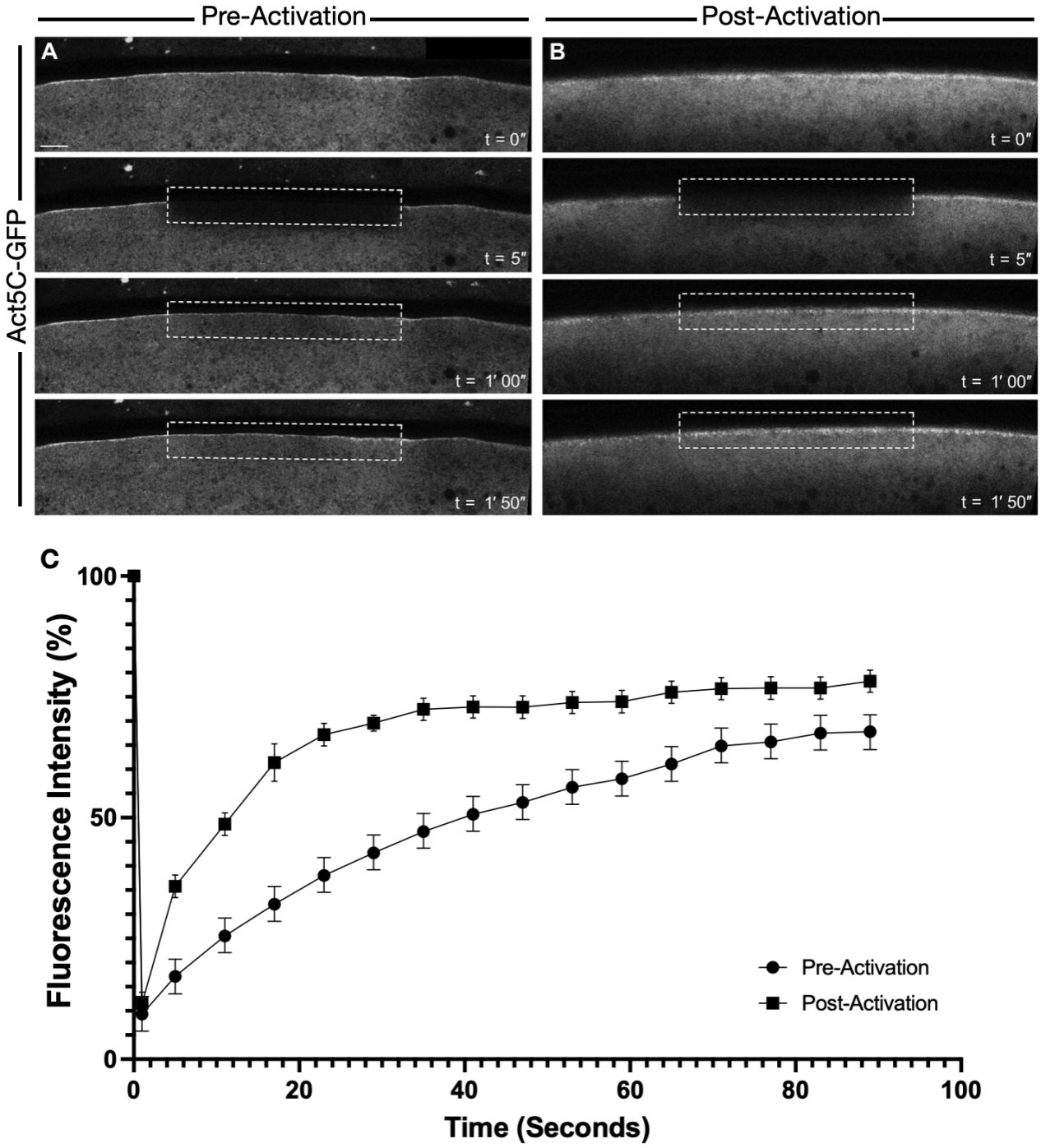

Figure 4.6: Cortical actin dynamics are faster following egg activation. (A-B) Confocal single plane images of a live mature oocyte prior to activation (A) and post-activation (B) showing actin (Act5C-GFP). Dashed boxes indicate the area bleached, the cortical band within used as a measure of fluorescence recovery. Scale bar: 20 μM. (C) Fluorescence recovery after photobleaching (FRAP) is plotted, showing intensity recovers faster and to a greater degree in activated oocytes. N = 10 per treatment.

4.2.2 Dynamic cortical actin is required is required for the calcium wave at egg activation

To summarise the data so far, the mature oocyte may have a reduced cortical actin at the poles which may prime these regions for calcium entry, and upon activation the cortical actin undergoes a significant dispersal and becomes more dynamic. Therefore, to test whether the dynamics of the cortical actin were important in regulating calcium entry at egg activation I manipulated the network of actin using pharmacological and genetics approaches.

Previous work has established that introduction of Phalloidin into mature oocytes and incubation with cytochalasin-D prevents calcium waves from occurring upon the addition of AB to mature oocytes (York-Andersen et al. 2015).

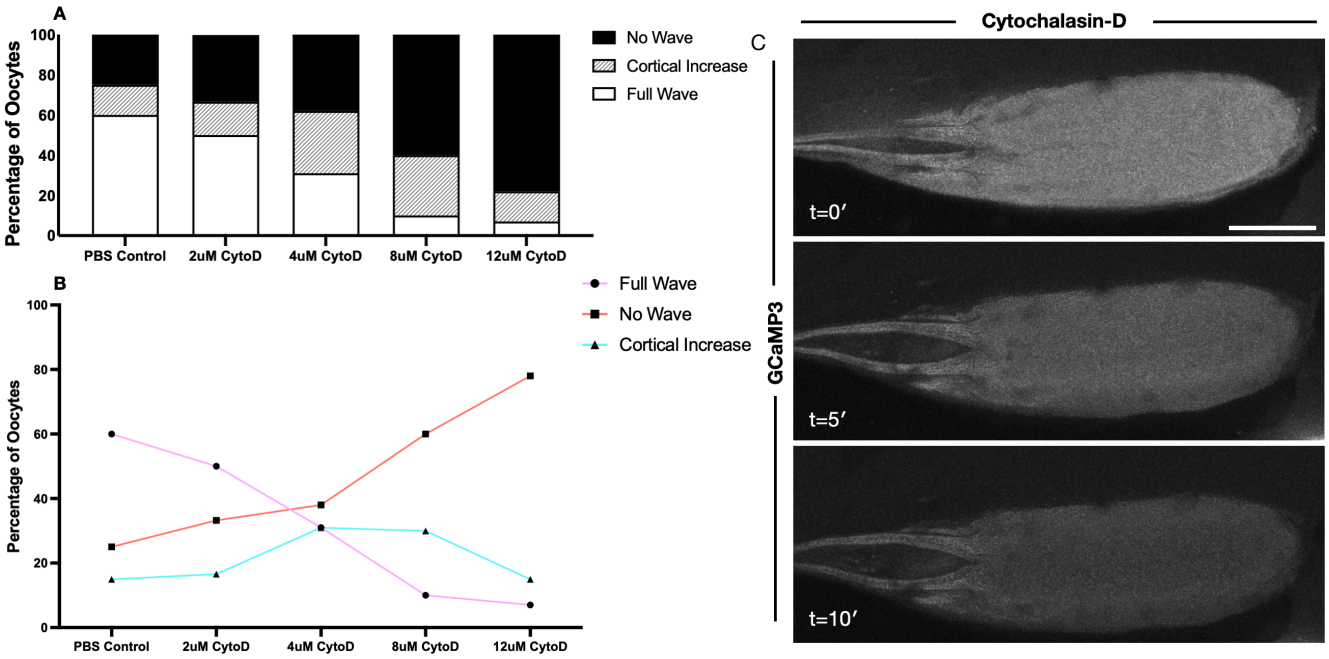

Figure 4.7: Cytochalasin-D treatment disrupts calcium entry following AB treatment. (A) Comparison of wave phenotypes following Cytochalasin-D treatment of various concentrations. Higher Cytochalasin-D concentrations prevents calcium entry at egg activation. (B) Comparison of wave phenotypes, plotted as a line graph to highlight the concentration depends effects of Cytochalasin-D treatment. Full wave phenotypes decrease and no wave phenotypes increase as concentration increases. Intermediate concentrations show an increase in cortical phenotypes. N = 20 per concentration. (C) Confocal Z-projection (40 um) of oocytes showing GCaMP3). Addition of cytochalasin-D does not cause an increase in the calcium signal. Scale bar: 100 um.

I confirmed this through repeating the incubation with cytochalasin-D. If actin is forming a barrier to calcium entry, one might hypothesise that incubation with cytochalasin-D would increase the number of calcium waves upon addition of AB. I therefore tested a range of concentrations of cytochalasin-D and found that there was a dose-dependent ratio of wave phenotypes (Figure 4.7A-B). Oocytes expressing GCaMP3 were pre-incubated in varying concentrations of cytochalasin-D in a control medium for 10 minutes, whereupon they were isolated from the medium and visualised as AB was added. At higher doses of cytochalasin-D, there was a higher

proportion of no waves, whereas at lower concentrations, the proportion of wave phenotypes was approximately the same as controls. Interestingly, at intermediate concentrations, there was a more even split of phenotypes, and where oocytes did activate, there was a larger proportion of cortical increase phenotypes (Figure 4.7 A-B). One might explain this occurrence as the higher the concentrations of cytochalasin-D result in a more complete depolymerisation of the actin network that therefore prevents transduction of any force to mechanically gated channels, whereas intermediate concentrations do not fully depolymerise the actin network.

I further confirmed that the decrease in calcium waves is not as a result of previous calcium waves (or indeed any activity) during the incubation period (Figure 4.7 C). Evidence in Lim et al. 2002 demonstrated that depolymerising the actin cytoskeleton in starfish oocytes would result in calcium release. I therefore incubated oocytes expressing GCaMP3 in a solution of PBS and cytochalasin-D. I observed no swelling of the oocyte and following 10 minutes there was no observable calcium increase (Figure 4.7 C).

This result is perhaps expected given previous work had elucidated that actin is required for calcium entry, however it does suggest that depolymerisation or reduction of the cortical actin alone is not sufficient to trigger calcium entry. It may be the case that a dense cortical actin prevents calcium entry and a reduction in this density promotes calcium entry, but the presence actin is essential for calcium entry, possibly due to connections with TrpM or other channels.

Visualisation of mature oocytes expressing both GCaMP3 (Ca^{2+}) and F-tractin (F-actin) demonstrated that upon the addition of 8uM Cytochalasin-D, there was no increase in calcium (in fact there was a drop in the background intensity of GCaMP3 fluorescence) and an apparent reduction in the level of actin, especially at the cortex (Figure 4.8 A-B'). After a 10 minute incubation period, the field of view was flooded with AB (i.e. to attempt to activate the oocyte), and this generated calcium increase from multiple point around the cortex. The wave, however, was unable to complete propagating and recedes following t=13'00''.

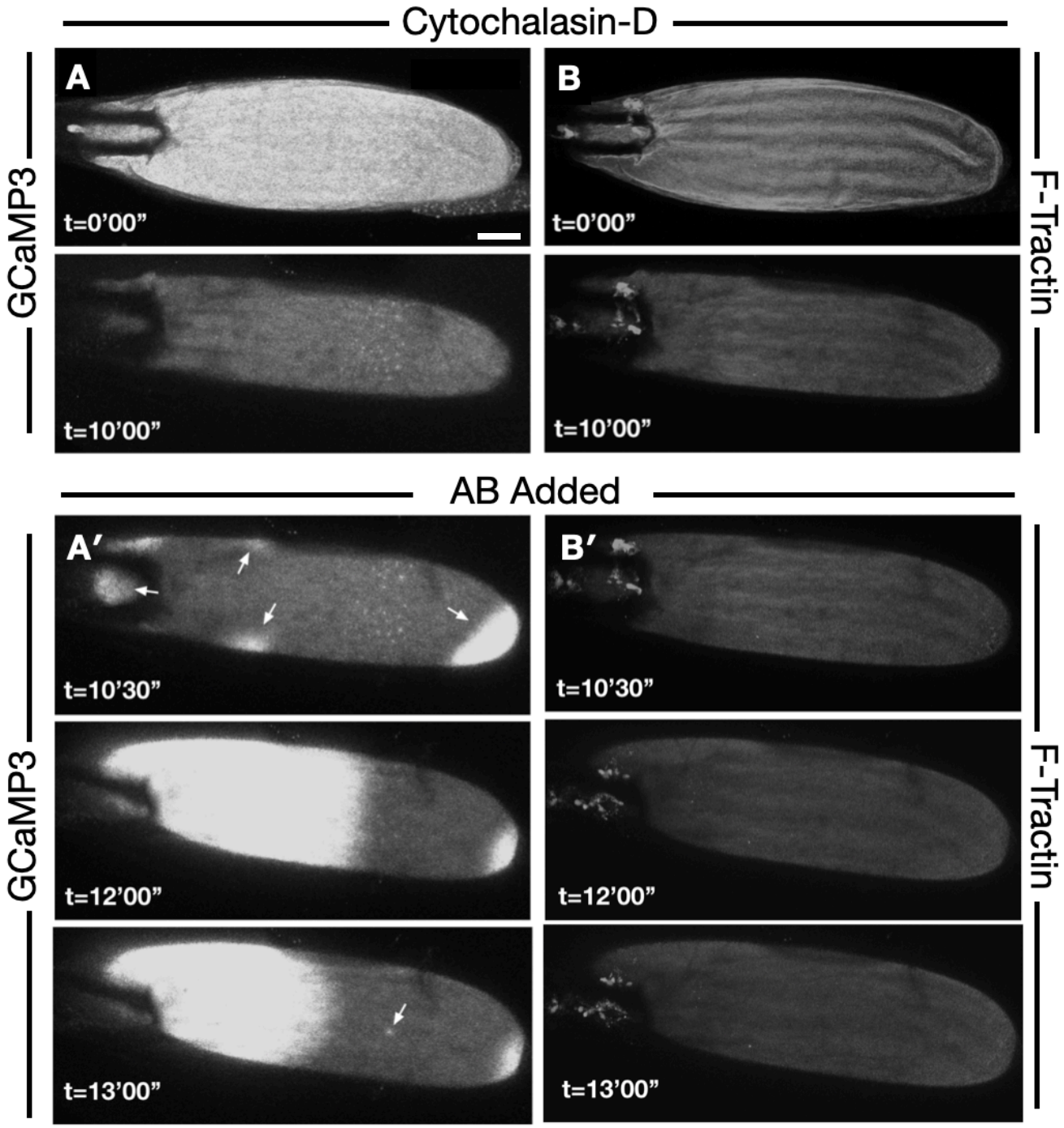

Figure 4.8: Cytochalasin-D treatment disrupts calcium entry upon AB addition. (A-B) Confocal Z-projections (40 um) of a live mature oocyte following Cytochalasin-D treatment showing calcium (GCaMP3) (A) and F-actin (F-tractin) (B). A reduction in fluorescence intensity of both GCaMP3 and F-tractin signals can be observed. (A′-B′) Confocal Z-projections (40uM) of the same live oocyte following addition of activation buffer (AB) showing calcium (GCaMP3) (A′) and F-actin (F-tractin) (B′). Calcium transients begin from multiple points across the cortex but fails to complete, whereas the F-actin signal continues to decrease. Arrows point to local regions of calcium entry. Scale bar: 50 μm.

4.2.3 Knock down of SCAR, a WASP family protein that regulates the Arp2/3 complex, speeds up calcium entry

I have established through pharmacological manipulation and high resolution visualisation that a dynamic actin cytoskeleton is important for calcium entry at egg activation. In order to further examine the role of the cortical actin at egg activation, I sought to manipulate the actin architecture through knock out/down and over-expression of the three key actin nucleating proteins: i) Arp2/3, regulated by WASP (SCAR); ii) Formins (Cappuccino); iii) Tandem actin binding domain nucleators (SpireB).

I attempted knock out of Arp2/3, however this resulted in very few healthy stage 14 oocytes (data not shown). I therefore tested the role that SCAR, a WASP family protein (Zallen et al., 2002), had in actin-regulated calcium entry through knock down using an RNAi. I confirmed that there was an efficient level of knock down through staining of oocytes expressing this RNAi driven by a Mat-Alpha Gal4 in a GCaMP3 background. These oocytes demonstrated a drop in fluorescence intensity of the actin cytoskeleton generally, suggesting a drop in actin nucleation by SCAR (Figure 4.9 A-B). However, observation of the calcium wave following addition of activation buffer revealed no significant difference in the phenotypes observed (Figure 4.9 C). I therefore measured the speed of calcium entry (ie. the time between addition of AB and first calcium entry), which

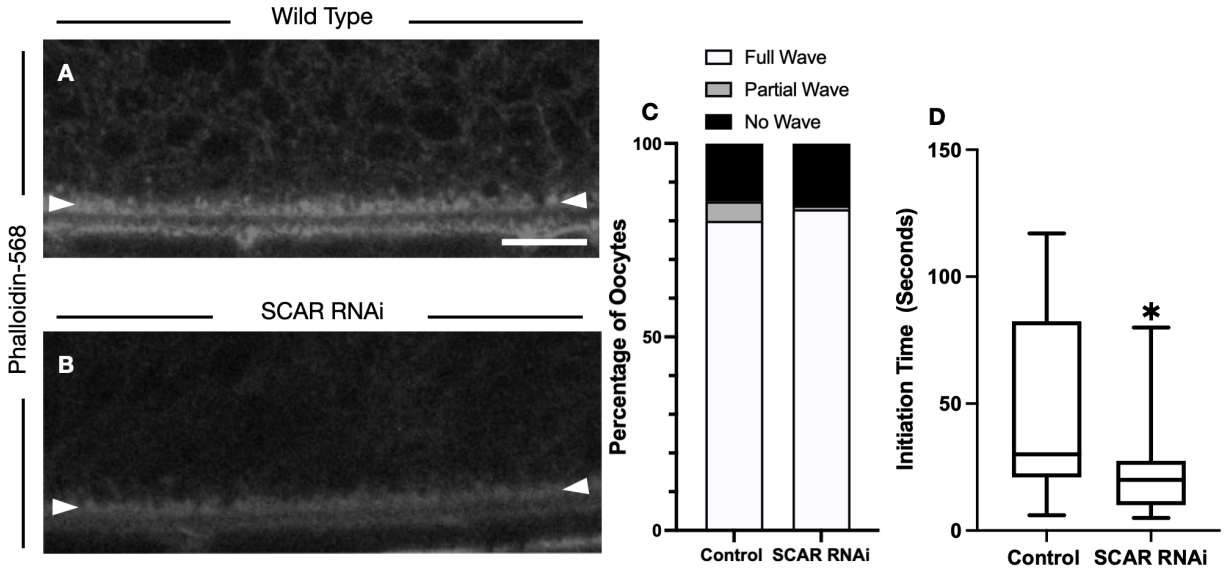

Figure 4.9: SCAR is required for regulation of calcium entry. (A-B) Confocal single plane images of the cortex of fixed mature oocytes showing F-actin (Alexa-fluor568 Phalloidin) in a wild type (A) and SCAR RNAi background (B. Scale bar: 20 μm. (C) Comparison of the wave phenotypes between control and SCAR RNAi oocytes demonstrates no significant difference. (C) Comparison of the time taken to initiate a wave following the addition of AB (Initiation Time) shows a significant reduction in this time in SCAR RNAi oocytes, indicating a faster initiation when actin is reduced. $P < 0.05$, $N = 25$ per genotype.

revealed in the case of SCAR RNAi a significant drop in the time taken for the first calcium entry (Figure 4.9 D). This suggests that the Arp2/3 pathway of actin nucleation is required for production of actin, and reduction of the amount of actin nucleation primes the oocyte for calcium entry. This

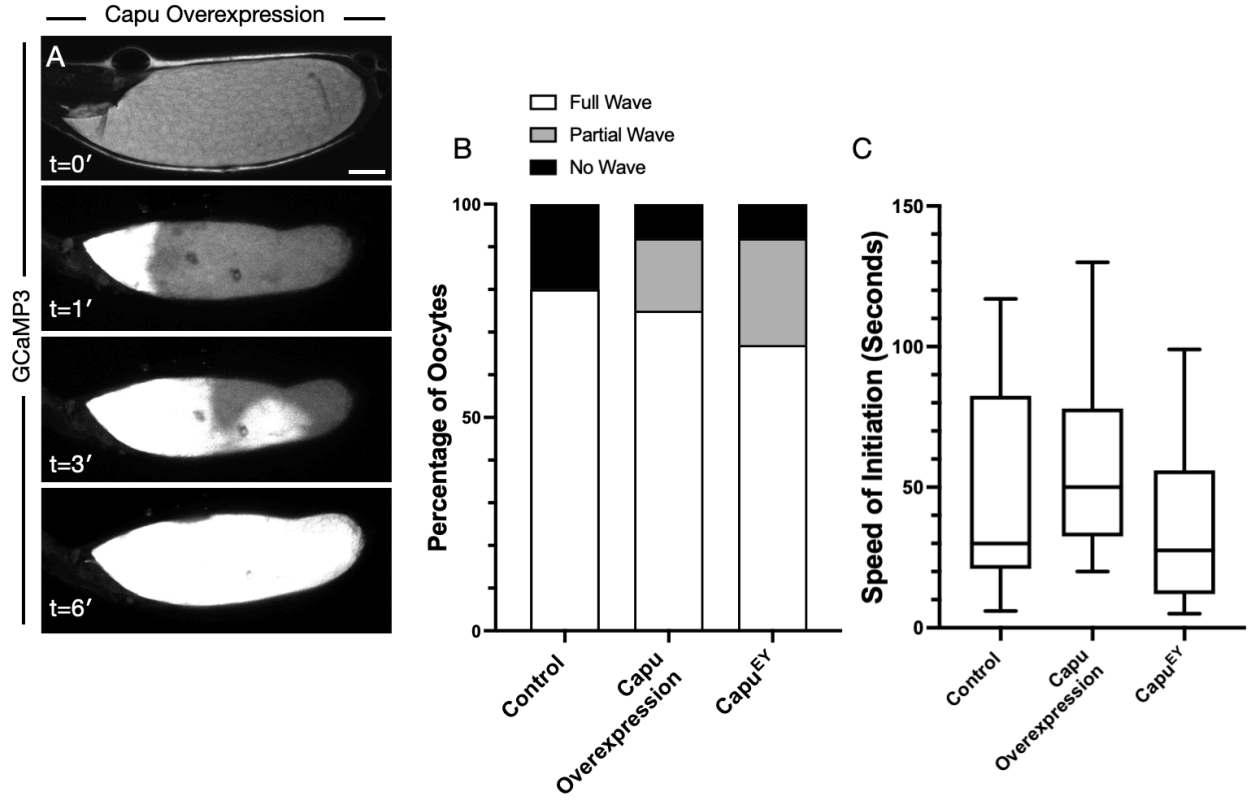

Figure 4.10: Cappuccino may be required for regulation of calcium entry. (A) Confocal Z projections (40 µM) of a live mature oocyte showing calcium (GCaMP3) in a c*apu* overexpression background (*otu-capu)*. Calcium entry is able to occur, but often with a mis-regulated wavefront. (B) Comparison of the wave phenotypes between control, c*apu* overexpression and c*apu*^{EY} mutant background oocytes demonstrates no significant difference between genotypes. (C) Comparison of the time taken to initiate a wave following the addition of AB (Initiation Time) shows no significant change in each genotype. $P > 0.05$, $N = 15$ per genotype.

result may clarify previous observations in which there is reduced cortical actin at the poles of the oocyte and that this actin population also disperses upon the addition of AB, suggesting a reduction in actin may aid calcium entry.

4.2.4 Cappuccino, a Formin protein, does not contribute to actin-regulation of calcium entry

Following manipulation of the Arp2/3 pathway, I asked whether Formins, specifically Cappuccino, played a role in generating the population of actin that regulates calcium entry. To this end, I utilised an o*tu-capu* construct to over-express Cappuccino in a GCaMP3 background and quantified the proportions of different calcium wave phenotypes (Figure 4.10 A-B). Though the number of full waves did not differ significantly from control oocytes, there did appear to be more oocytes with irregular full waves, often initiating from one pole and then completing through lateral entry (Figure 4.10 A). Despite this, there were not a significant number of these phenotypes. Furthermore, there was no significant change in the speed of calcium wave onset following addition of AB (Figure 4.10 C).

Conversely, I utilised the *cappuccino*^{EY12344} mutant line, which is a hypomorphic mutant allele meaning that it does not completely abolish the presence of Cappuccino protein (Dreschler et al., 2017). Crossing this mutation into a GCaMP3 expressing oocyte and observing the proportion of wave phenotypes revealed no significant change in the number of full waves, and additionally no change in the speed of calcium entry following addition of activation buffer (Figure 4.10 B-C). Taken together, this would suggest that Cappuccino plays little role in the production of actin that impacts calcium entry.

4.2.5 SpireB, a tandem actin binding domain nucleator, prevents calcium entry when over-expressed.

Finally, I asked whether the tandem actin binding domain nucleator, SpireB, would play a role in production of an actin population that regulates calcium entry at egg activation. SpireB works in a complex with Cappuccino, so one may expect that it would result in a similar phenotype to Cappuccino disruption (Dahlgaard et al., 2007; Quinlan et al., 2005). However, overexpression of SpireB in a GCaMP3 background, driven by the Mat-alpha Gal4, revealed a significant change in the proportion of wave phenotypes in the mature oocyte and indeed in the morphology of the oocyte itself (Figure 4.11 A-B). Oocytes appeared smaller than usual, likely due to defects earlier in oogenesis as Spire and Cappuccino are required for streaming in the developing oocyte. As such, I observed a significantly lower number of calcium waves, from 85% to 42% (Figure 4.11 B). Despite, this, the oocyte was still capable of generating calcium waves following the addition of activation buffer. Analysis of the initiation time of this wave reveals a significant delay between the addition of AB and first calcium entry when SpireB is over expressed, once more suggesting that an increased density of cortical actin prevents calcium entry. This further corroborates previous evidence which demonstrated Phalloidin stabilisation of the actin cytoskeleton prevents calcium entry (York-Andersen et al., 2015).

Further to this, I knocked down *spireB* using a mat-alpha Gal4 driven RNAi. Knock down did not significantly change the proportion of wave phenotypes (Figure 4.11 B), therefore I also analysed the calcium wave initiation time post-AB addition (Figure 4.11 C). Although this revealed a decrease in the wave initiation time, it was not significant from control speeds. Taken together, it appears that manipulation of the cortical actin through up-regulation and down-regulation of actin nucleating factors has a specific effect on calcium entry dynamics; namely increasing actin nucleation prevents calcium entry or reduces the speed of calcium wave initiation, whereas decreasing actin nucleation increases the speed of calcium wave initiation following addition of AB.

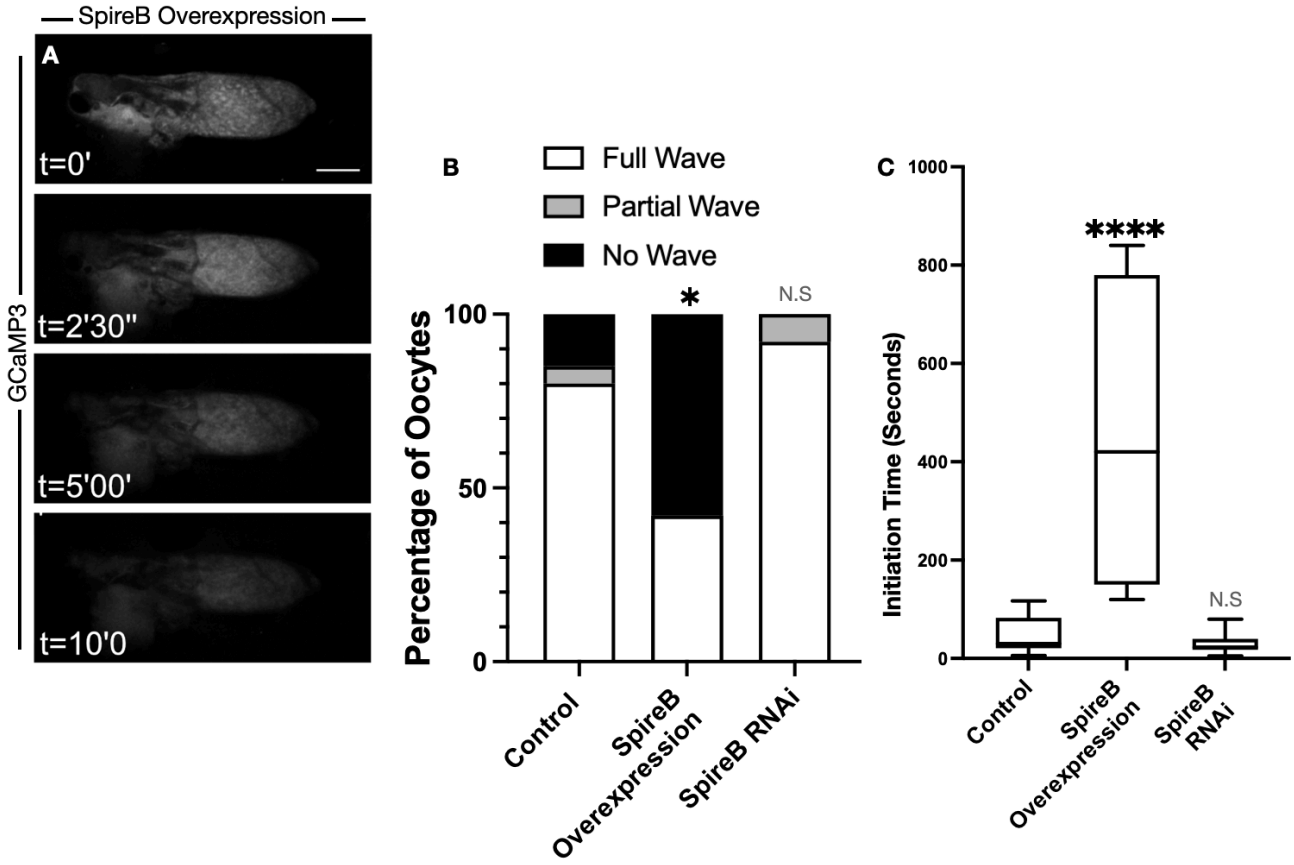

Figure 4.11: SpireB over-expression prevents calcium entry. (A) Confocal Z projections (40 μM) of a live mature oocyte showing calcium (GCaMP3) in a *spireB* overexpression background (*UASp-SpireB.tdTomato)*. (B) Comparison of the wave phenotypes between control, *spireB* overexpression and *spireB RNAi* background oocytes demonstrates a significant decrease in full waves in the overexpression oocytes but no significant change in RNAi oocytes. (C) Comparison of the time taken to initiate a wave following the addition of AB (Initiation Time) shows a significant increase in the time taken to initiate a wave in *spireB* overexpression oocytes (those that do show a wave) but no significant change in RNAi backgrounds. $P > 0.001$, $N = 20$ per genotype.

4.2.6 Knock down of actin binding proteins disrupts calcium entry at egg activation

There are various challenges that accompany the manipulation of the actin cytoskeleton; although nucleators can be over-expressed and knocked down, a broad pool of actin will be impacted, and as such one must be cautious with conclusions drawn. Having provided evidence to suggest that the stability of the actin cortex upon egg activation plays a role in calcium entry, the next stage is to dissect how calcium entry may be regulated by the actin to enable propagation of waves. The actin cytoskeleton itself is highly calcium sensitive, often regulated by calcium sensitive actin binding proteins (Blanchoin et al., 2014; Furukawa et al. 2003). For example calcium can trigger actin filament severing (Yamamota et al., 1982) and inhibit actin cross-linking by α-actinin (Fechheimer et al., 1982; Witke et al., 1993; Furukawa et al., 2003). Additional measurements of calcium ion fluxes in Dicytostelium reveals that calcium elevation corresponds with actin depolymerisation (Nebl and Fisher., 1997; Furukawa et al. 2003). Therefore, in an attempt to more subtly manipulate the actin cytoskeleton and, importantly, to begin identifying the actin binding proteins (ABPs) that may in fact be required during egg activation to regulate the actin architecture, I performed a candidate screen of specific ABPs:

1) Moesin belongs to the Ezrin/Radixin/Moesin (ERM) family of proteins which are essential organisers of the cell cortex, directly linking F-actin to membrane associated proteins (Algrain *et al.*, 1993; Turunen *et al.*, 1994; Hirao *et al.*, 1996)

2) Quail is a germline specific villin-related protein which bundles actin filaments together in a calcium independent manner (Matova et al., 1999; Cant et al., 1998; Mahajan-Miklos and Cooley, 1994).

3) α-actinin is an actin cross linking protein of the spectrin family, usually found in non-muscle tissues. It has been shown to link actin in low calcium concentrations but upon an increase in calcium this cross-linking ability is inhibited (Jayadev et al., 2014; Prebil et al., 2016; Sjöblom et al., 2008).

4) Supervillin is an F-actin binding protein in the villin family. It has been shown to bundle actin filaments, co-localises with myosin II and is important in actin recycling at the membrane (Pestonjamasp et al., 1997; Smith et al., 2010; Fang et al., 2010).

5) Gelsolin is a calcium sensitive actin severing protein; under calcium free conditions it is inactive, and when calcium is present it can rapidly depolymerise actin (Burtnick et al., 1997; Ashish et al., 2007; Garg et al., 2011; Kiselar et al., 2003).

Significant results include knock down of α-actinin, which demonstrates a much lower number of full wave phenotypes and a corresponding increase in no waves (Figure 4.12). Additionally, knock down of Supervillin results in an increase in cortical wave phenotypes, suggesting that inhibiting cross-linking of the actin network promotes calcium entry upon the addition of A (Figure 4.12).

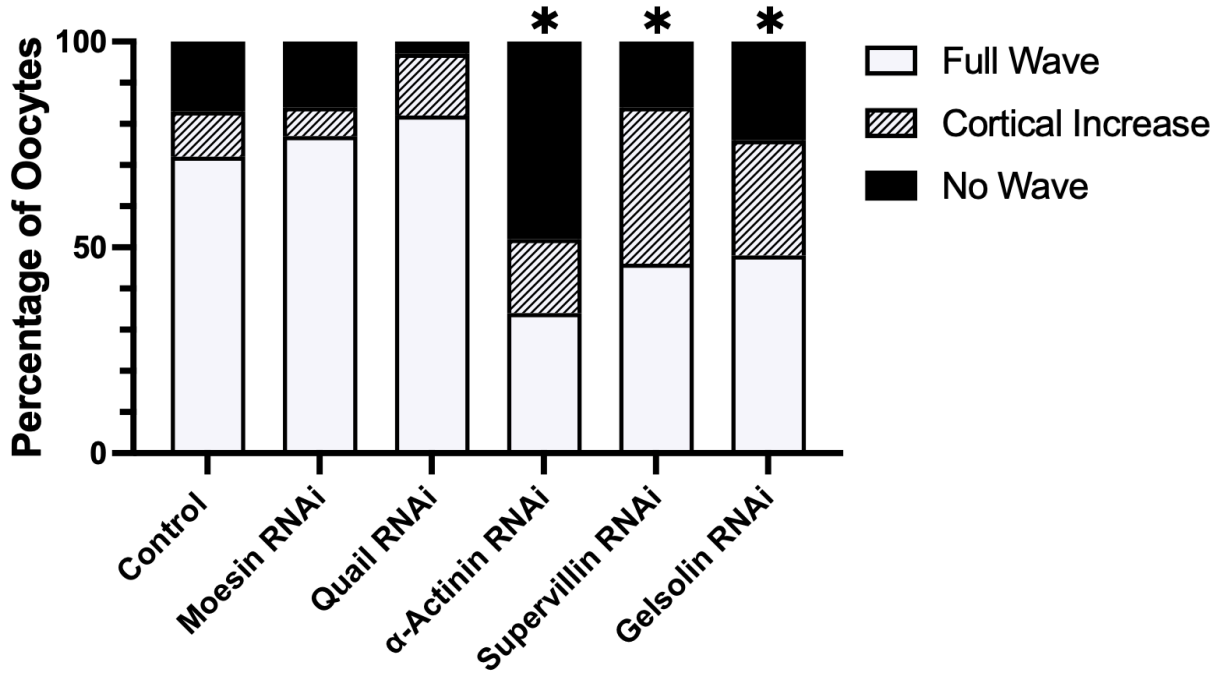

Figure 4.12: Actin-Binding Proteins (ABPs) play a role in regulating calcium entry. Comparison of wave phenotypes in oocytes of different RNAi backgrounds, showing a significant change in wave phenotypes in α-actinin, supervillin and gelsolin backgrounds. Fishers exact test: $P < 0.05$, $N = 30$ per genotype.

Similarly, knock down of Gelsolin results in a significant increase in the number of cortical increase phenotypes.

I decided to test more thoroughly the role of supervillin and α-actinin in calcium entry at egg activation through generation of homozygous mutant *Svil*^{EY} and *Actn*^{EP}. Interestingly, both revealed a different proportion of wave phenotypes depending on whether the oocytes were heterozygous or homozygous for these mutations (Figure 4.13 A-C). In the case of supervillin, heterozygous lines revealed a significant increase in the cortical wave phenotypes, corroborating previous evidence from the RNAi knock down (Figure 4.13 A,C). However, in homozygous mutants, there were significantly fewer full and cortical wave phenotypes (Figure 4.13 B, C). This follows a similar line of evidence with pharmacological evidence in which an intermediate level of knock down promotes cortical entry whereas complete knock out prevents calcium entry; this may be the case with supervillin in which an intermediate reduction in its capacity to cross-link the cortical actin promotes calcium entry, whereas a complete loss of this function is detrimental.

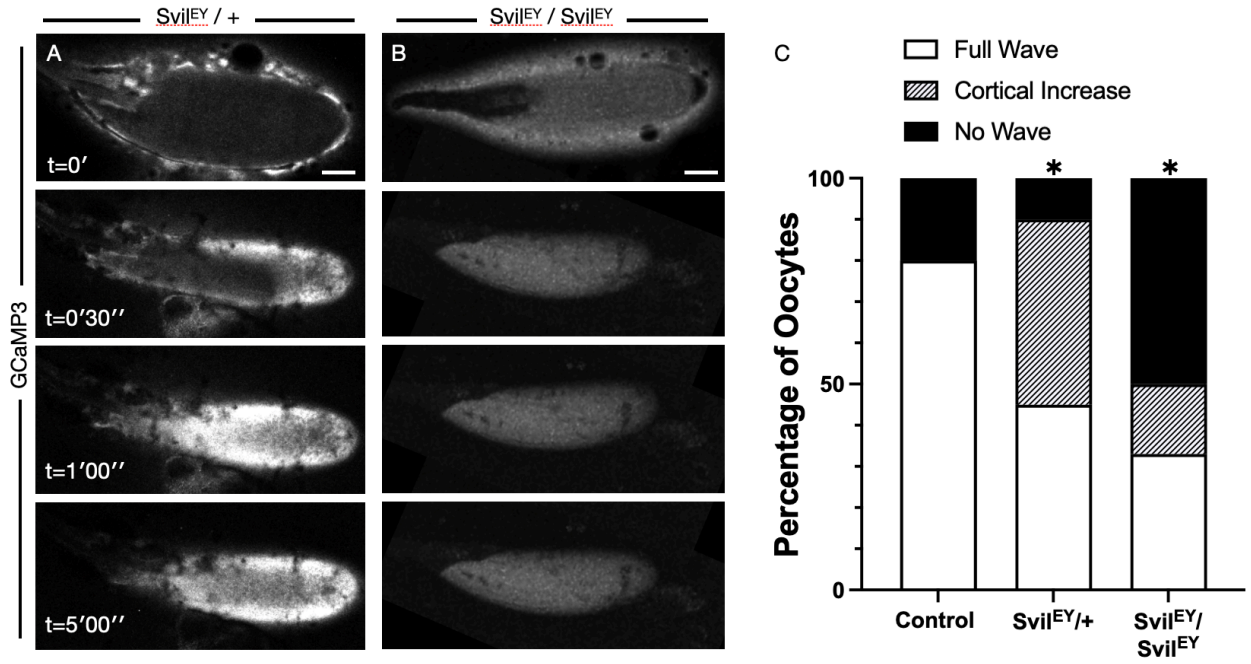

Figure 4.13: Supervillin is required for regulation of calcium entry at egg activation. (A) Confocal Z-projection (40 µm) of an oocyte showing calcium (GCaMP3) in a heterozygous *svil*^{EY} background. Calcium entry occurs in the form of a cortical increase, apparently from all points of the cortex simultaneously. (B) Confocal Z-projection (40 µm) of an oocyte showing calcium (GCaMP3) in a homozygous *svil*^{EY} background. Calcium entry is prevented. (C) Comparison of the wave phenotypes between control, hetero- and homozygous backgrounds, demonstrating a significant change in phenotypes, Fishers Exact Test: $P < 0.05$. N = 10 per genotype.

I found a similar result with knock out of α-actinin. Heterozygous lines revealed a significant decrease in the proportion of wave phenotypes, with most oocytes unable to generate a calcium wave upon addition of AB (Figure 4.14 A,C). Interestingly, in homozygous oocytes, there was a significant increase in the number of cortical wave phenotypes, with no significant change to the number of oocytes that were able to undergo a complete calcium event (Figure 4.14 B,C). This is a more difficult phenotype to explain; one hypothesis that would require further investigation is that α-actinin is required during calcium entry ahead of the calcium wave, with increased calcium inhibiting α-actinin cross-linking activity, thus enabling a dispersion of the cortical actin and further calcium entry. With a partial knock out it may be the case that compensatory mechanisms cross-link cortical actin in a calcium insensitive manner, reducing the oocytes capability to have a calcium wave. However, when completely knocked out there may be such a dramatic loss in cross-linking that calcium entry is actually promoted upon addition of AB. Clearly, there is a complex relationship between α-actinin and other ABPs and the state of the cortical actin that leads to unique and specific mechanisms of regulation of calcium entry at egg activation.

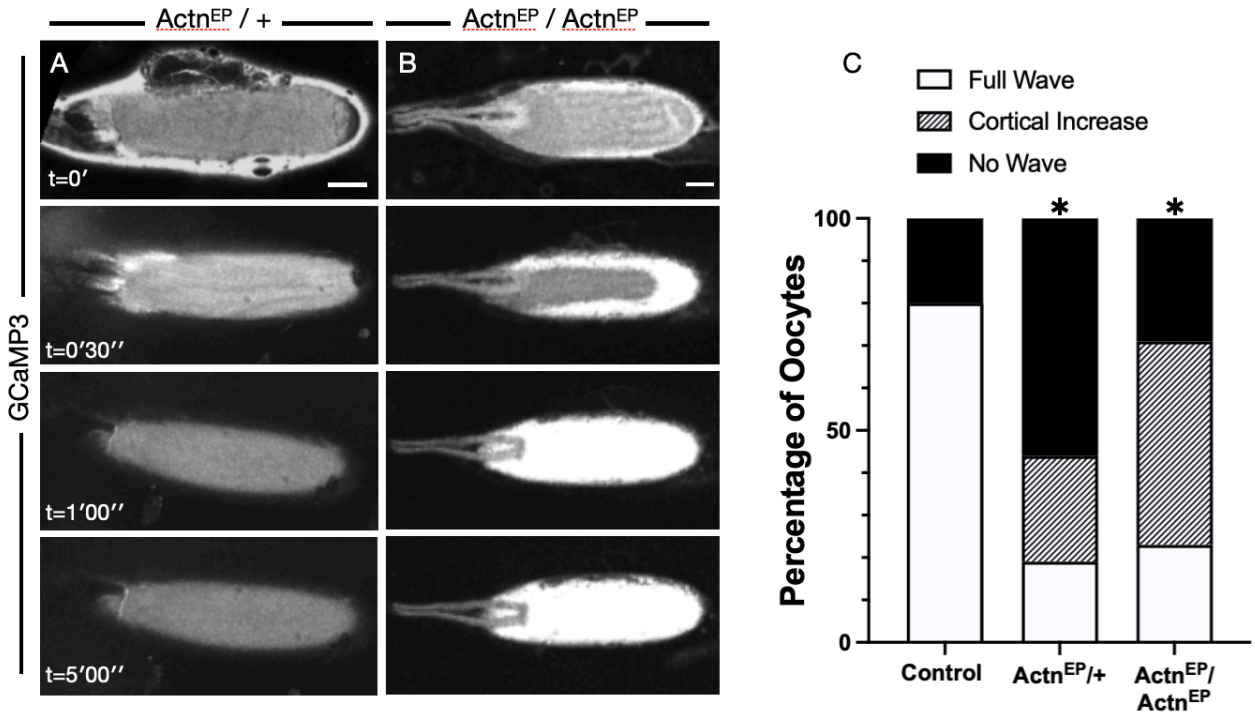

Figure 4.14: α-actinin is required for regulation of calcium entry at egg activation. (A) Confocal Z-projection (40 µm) of an oocyte showing calcium (GCaMP3) in a heterozygous *actn^{EP}* background. Calcium entry is prevented. (B) Confocal Z-projection (40 µm) of an oocyte showing calcium (GCaMP3) in a homozygous *actn^{EP}* background. Calcium entry occurs in the form of a cortical increase, apparently from all points of the cortex simultaneously. (C) Comparison of the wave phenotypes between control, hetero- and homozygous backgrounds, demonstrating a significant change in phenotypes, Fishers Exact Test: $P < 0.05$. N = 10 per genotype.

4.3 Discussion:

4.3.1 Summary and Critical Evaluation of Results:

In this chapter I demonstrate the significance of the actin cytoskeleton during egg activation, pointing to a significant contribution from the cortical actin. It has been suggested that without a dynamic actin cytoskeleton, the oocyte is unable to initiate a calcium wave (York-Andersen et al., 2015). Many model systems reveal an importance of the actin cytoskeleton in the regulation of calcium flux in oocytes, such as in starfish oocytes (Lim et al., 2002). I therefore used high-resolution imaging to characterise the cortical actin cytoskeleton prior to and during egg activation, revealing key morphological features and changes (Figure 4.3, 4.4, 4.5). Namely, prior to egg activation the cortical actin forms a dense band around the oocyte with significantly reduced cortical actin at the posterior and anterior pole (Figure 4.3). Further quantification and 3D imaging of the poles in comparison to the lateral sides of the oocyte will be necessary to confirm such a decrease in actin density. Furthermore, higher resolution and 3D imaging may be required in order to accurately quantify the density of the cortical actin, as currently the results of this thesis use thickness and qualitative fluorescence intensity as a proxy for density, however this is not a direct measurement of density itself.

During egg activation, this cortical actin becomes disperse and the mobile fraction increases, suggesting an increase in dynamism of the cortex following the addition of a hypotonic buffer (Figure 4.6). It may even be the case that upon addition of a 0 mOsm solution that the cortical actin cytoskeleton is more significantly disrupted and dispersed. These findings begin to elucidate a mechanism in which cortical actin acts as a regulatory element of calcium entry, priming the poles for initiation of a calcium wave and undergoing an apparent ‘dispersion’ or decrease in density to enable further calcium entry and therefore calcium wave propagation. Of course, to fully establish this, a causal link still needs to be identified between calcium ions, the cortical actin cytoskeleton and the channels that enable calcium entry at egg activation.

I dissect this mechanism further through the use of pharmacological and genetic tools, identifying in particular the key pathways that influence production of the cortical actin cytoskeleton in the *Drosophila* oocyte. As aforementioned, simply depolymerising the actin cytoskeleton is sufficient to produce a calcium transient in starfish oocytes (Lim et al., 2002), however I demonstrate that this is not the case in mature *Drosophila* oocytes. This is likely due to cortical actin being an essential component for the transduction of force, without which there may not be sufficient transduction to Trpm channels, which I demonstrated in my first chapter are required for calcium entry, and this has been corroborated by more recently published data (Hu and Wolfner., 2020). In fact, I show that following cytochalasin-D incubation and the addition of activation buffer (AB), the oocyte is unable to produce a calcium wave (Figure 4.7). There is, however, an intermediate concentration of cytochalasin-D that appears to increase the number of cortical increases,

perhaps pointing to partial loss of actin cytoskeleton promoting calcium entry. It is important to also note that the influence of cytochalasin D on an actin population does not selectively depolymerise the cortical actin, but all actin within the oocyte. As such, there could well be contribution from a depolymerised cytoplasmic actin to the effect of disrupting calcium waves at egg activation, though a more direct and likely candidate would still seem to be the cortical actin,

I therefore test whether the population of cortical actin can be manipulated through genetic knock down or overexpression of the three key nucleating factors: i) The Arp2/3 pathway; ii) The forming pathway; iii) The tandem-actin binding domain nucleator family. I show that SCAR, an activator of Arp2/3 does not initially appear to influence the cortical actin to a degree at which calcium entry is impacted at egg activation, however analysis of wave initiation in SCAR knockdown oocytes reveals a significantly increased speed of calcium entry, suggesting a partial knock down of actin promotes calcium entry (Figure 4.9). Both knock down and overexpression of *cappuccino*, the *Drosophila* homologue of Formin-2, has little effect on calcium entry at egg activation (Figure 4.10). Interestingly, over-expression of Spire, a tandem actin binding domain nucleator and activator of Cappuccino, does however impact calcium entry, as increased levels of actin reduce both the number of calcium waves and of those wave that do initiate, their speed of initiation (Figure 4.11). Taken together, this data reveals an important relationship between the actin, likely the cortical actin, and calcium entry; overexpression of which prevents calcium entry at egg activation, whereas reduction promotes calcium entry. This further supports previous observations in which regions of reduced cortical actin, namely the poles of the oocyte, are apparently primed to enable calcium entry and subsequent 'dispersion' of the cortical actin enables wave propagation.

I further attempt to elucidate the regulatory actin binding proteins (ABPs) that regulate architecture of the cortex to influence calcium flux during egg activation. Namely I demonstrate that the ABPs gelsolin, supervillin and α-actinin are required during egg activation for production of a standard calcium wave (Figures 4.12, 4.13, 4.14). These ABPs influence the architecture of the actin cytoskeleton, especially at the cortex. Supervillin acts to crosslink the actin at the cortex to the membrane, therefore without this, cortical actin is likely not able to efficiently regulate calcium entry. α-actinin is a particularly interesting example as it is a calcium sensitive protein- under high calcium concentrations it is less effective at binding multiple F-actin filaments, losing its cross-linking ability (Pestonjamasp et al., 1997; Smith et al., 2010; Fang et al., 2010; Burtnick et al., 2004; Kiselar et al., 2003; Jayadev et al., 2014; Prebil et al., 2016; Sjöblom et al., 2008).

Broadly speaking, though the results presented herein indicate a likely relationship between the actin cytoskeleton and calcium flux mechanisms, there are limits to how much may be interpreted. As mentioned above, manipulations of actin used in this thesis rely on knockdown of broad actin nucleators and through broad depolymerisation using pharmacological agents. Of course, multiple pools of actin will be affected from such treatments and therefore, though we can more definitively say that the actin generally is playing a significant role during egg activation,

further work and controls are necessary to prove that the cortical actin specifically regulates calcium entry. Though it does seem a likely mechanism given morphological features of the cortical actin, such a potential polar decrease in cortical actin and dispersion at egg activation, there could well be a contribution from the cytoplasmic actin.
Attempts to more specifically target the cortical actin centred on the manipulation of various actin binding proteins, that have, in the literature, been associated more specifically with the cortex, such as supervillin. However, the data presented herein remains somewhat preliminary, with far greater repeats and further genetic testing required to establish a true relationship between such ABPs, the cortical actin and calcium flux. Thus far, I have demonstrated that such ABPs are likely important in regulating calcium flux, but the precise mechanistic links remain to be investigated.

4.3.2 Actin and Calcium Interplay at Egg Activation

In this chapter I provide evidence that demonstrates the importance of communication between the actin cytoskeleton and calcium signalling. There is a clear regulatory mechanism in place in which cortical actin contributes to the control of calcium influx at egg activation, and as I shall discuss, a likely role for calcium in reorganisation of the actin architecture, which we see especially downstream of the calcium wave as a similar wave of reorganising actin is observed (York-Andersen et all., 2020). The relationship between calcium and actin is often overlooked in many developmental events, yet there is clear evidence supporting the vital importance of these interactions in many systems. For instance, an apical calcium gradient regulates the activity of many ABPs, including villins, that promote polymerisation of actin microfilaments in the growing pollen tube of angiosperms (Vidali and Hepler., 2001; Zhang et al., 2018). Similarly, maturation of starfish oocytes following treatment with 1-MA results in a transient of calcium ions that causes a reorganisation of the cortical actin that ultimately mediates microvilli retractions and alignment of CGs (Santella et al., 2015). Investigation of the relationship between calcium and actin during *Drosophila* egg activation reveals similar key relationships, both in terms of broad dynamics and specific ABPs.

Genetic manipulation of ABPs revealed intriguing dynamics between the cortical actin, calcium influx and downstream effects of the calcium wave on the actin architecture. Unlike the rest of the villin family, Supervillin is actually calcium insensitive, which simplifies the explanation of the phenotypes observed somewhat. As a protein that has been well established as an actin bundling agent with connections to Myosin-II, it appears essential for maintenance of the steady-state cortical actin (Pestonjamasp et al., 1997; Smith et al., 2010; Fang et al., 2010). Therefore, a partial knock down of this protein may increase the dynamism of the cortex due to a lower level of cross linking of F-actin filaments, explaining the observed increase in the number of calcium transients that initiate from many different points across the cortex. However, a more complete knock down may result in a loss of inter-linked actin filaments to such a degree that the force of swelling is no longer efficiently transduced to the Trpm channels, resulting in fewer calcium transients of any

kind being observed. This hypothesis could be tested further through examining what role myosin-II plays at egg activation. Spaghetti squash is the *Drosophila* myosin-II and it may be responsible more maintenance of cortical tension in the oocyte, or indeed be activated further upon calcium entry to promote further calcium entry, and as such genetic analysis would be useful in establishing this.

The role of Gelsolin and α-actinin is perhaps more difficult to dissect, as both of these ABPs are calcium sensitive. Gelsolin is an actin capping and severing protein which is stimulated by calcium; within Gelsolin, a calcium sensitive S6 helical tail, in the presence of calcium, straightens and exposes an actin binding site, enabling binding of Gelsolin to an actin filament whereupon its S1 domain can sever the actin filament or cap the barbed end (Sun et al., 1999; Burtnick et al., 2004; Kiselar et al., 2003; Yu et al., 1992). It may have been expected, therefore, that Gelsolin would act during the calcium wave, activated downstream of this transient to further sever F-actin filaments at the leading edge of the wave, thereby enabling further calcium entry. This hypothesis does not quite fit with data I show, however, that mainly presents an increase in cortical calcium entry at the expense of the standard full wave. Gelsolin should therefore be explored further to fully understand its role during egg activation and oocyte maturation. Although this data is still preliminary, it could contradict the model and hypothesis that I thus far have attempted to establish; why would an loss of an actin severing protein result in more calcium waves in a hypothesis in which a reduction of actin appears important for calcium entry?

Similarly, understanding the role that α-actinin is playing during egg activation is difficult and requires further exploration. α-actinin is a calcium sensitive actin binding protein that loses its ability to bind to multiple F-actin filaments in the presence of high intracellular calcium (Jayadev et al., 2014; Prebil et al., 2016; Sjöblom et al., 2008). I would therefore hypothesise that were it to be playing a role during egg activation, it would similarly be acting like Gelsolin, where at the leading edge of the calcium wave, α-actinin loses its ability to cross-link actin promoting a more disperse cortical actin and subsequent calcium entry. With a partial knock out (I.e. α-actinin mutant heterozygotes) of α-actinin we see a decrease in the number of calcium transients of any kind, whereas complete knockout sees an increase in the number of cortical increases (Figure 4.14). This may suggest that α-actinin acts in a more simple fashion, such as supervillin, in that loss of α-actinin results in a reduced level of cross linking and therefore promotes calcium entry from all points on the cortex. Incomplete knock out, however, may prevent the calcium wave as other calcium-insensitive ABPs may compensate for the loss of α-actinin, and as such the oocyte loses a propagatory mechanism. For both α-actinin and Gelsolin, of course, there could be an impact from loss of these ABPs earlier in oogenesis, which may also contribute to the somewhat strange phenotypes are observed at egg activation. Further analysis of the impact of these ABPs in earlier oogenesis may therefore be required to fully establish their roles at egg activation. Furthermore, it would be useful to test the role of these ABPs in a more temporally controlled fashion, for instance through the application of pharmacological inhibitors in the mature oocyte prior to egg activation.

4.3.3 The role of cortical actin as an osmotic sensor

I have revealed in this chapter a potential role of the cortical actin in regulating calcium entry; osmotic swelling of the oocyte leads to a dispersion of the cortical actin, supporting evidence in which a decrease in cortical actin primes the oocyte for calcium entry, such as naturally at the poles of the *Drosophila* oocyte or artificially when nucleators are knocked down. It appears that the cortical actin may act as an osmosensor in a broad sense in that it responds to the change in cell volume caused by hydrostatic pressure. This can be observed in many systems; the mammalian magnocellular neurosecretory cells of the nervous systems are a classic example in which hypertonicity results in cell shrinkage and the downstream activation of non-selective ion channels that trigger action potentials. Specifically, it has been suggested that cell shrinkage induces changes in the cortical F-actin which results in the activation of mechanosensitive Trpv1 channels (Prager-Khoutorsky and Bourque, 2015). Similarly, adipocytes respond to osmotic shock through remodelling of the cortical actin which results in the recruitment of GluT4 to the plasma membrane (Gual et al., 2002).

The precise pathways that lead to such effects are beginning to be explored in much more depth; recent work highlights the essential role that sub-membranous cortical actin plays as an osmotic sensor, responding to changes in both cell swelling and ionic concentration as cell volume changes (Barvitenko et al., 2021). This research highlights the collaboration of non-muscle myosin II, F-actin based protrusions and the sub-membranous F-actin as a physical sensor of the hydrostatic pressure exerted on the cell membrane, whether this be shrinkage or swelling. One such way in which this occurs is through physical linkage of F-actin to protein phosphatase 2A (PP2A), whereupon cellular swelling (hypo-osmotic driven outward movements of the plasma membrane), extending and activating the PP2A enables phosphorylation of downstream signalling proteins, often resulting in a regulatory volume decrease (RVD) (Barvitenko et al., 2021). PP2A has been shown to regulate various ionic channels in the plasma membrane, such as NKCC1 and KCC, specifically during cellular swelling (Liedtke et al., 2005; Bize et al., 2000), suggesting similar mechanisms could be at play during *Drosophila* egg activation. Oocyte swelling alone may therefore be sufficient to activate phosphorylation pathways that lead to dramatic changes in ionic flux, potentially even through regulation of Trpm.

4.3.4 Calcium and Actin interplay downstream of the calcium wave

A dynamic remodelling of the actin cytoskeleton downstream of the calcium wave was recently revealed in *Drosophila* oocytes at egg activation (York-Andersen et al. 2020). Using F-tractin and co-visualising with GCaMP3, it could be observed that a wave of remodelling actin follows the calcium wave. In particular, the wave of F-actin strictly follows behind the calcium wave, whether that be from either pole or as a cortical increase (York-Andersen et al., 2020). Interestingly, similar

“waves” of actin can be observed in other systems, such as in the Circular Dorsal Ruffle (CDR) of fibroblast cells (Bernitt et al., 2015; 2017). More significantly, a wave of cortical actin can be observed in *Xenopus* oocytes following egg activation; this wave is in part generated by Rho activity, which also occurs in the form of a wave, where subsequent F-actin polymerisation inhibits Rho activity (Bement et al., 2015).

The role of calcium in the generation of these waves has not yet been well explored- only that there appears to be a clear sequence of events in which increasing calcium activity is closely mirrored by a wave of F-actin rearrangements, suggesting calcium acts upstream (York-Andersen et al., 2020). Interestingly, when the *Drosophila* calcineurin Sarah, a key regulator of the calcium signalling pathway, is knocked out, no calcium wave is observed and as such no F-actin wave, despite oocyte swelling occurring as normal, suggesting the wave of calcium is required for this actin rearrangement (York-Andersen et al., 2015; 2020). The exact pathway that connects the calcium wave to such a rearrangement is yet to be elucidated, and likely relies upon calcium responsive ABPs and nucleators. For instance, in observable waves of actin in *Dictyostelium*, it can be observed that Arp2/3, Myosin-B and CARMIL are enriched at the leading edge of the wave (Bretschneider et al., 2009; Khamiwath et al., 2013). It remains to be investigated within *Drosophila* how this actin-calcium interplay mediates such a wave of reorganising actin. One challenge to understanding such a dynamic event is the inability to capture waves of calcium and actin in fixed samples; attempts have been made for many years but have not been successful, putting a limit on the resolution that can be obtained during imaging as the vast majority of super-resolution imaging techniques rely on fixation. I suspect with the advent of more powerful live-imaging techniques such as super resolution confocal and the provision of new labelling reagents, such as improved SiR-Actin which can be injected live, we will be able to generate a more in depth understanding of these events.

Similarly, the downstream purpose of such “waves” of actin is not well understood; one potential explanation is that this is a response to the initial depolymerisation of actin at the cortex, potentiating re-polymerisation globally. This has been shown to be the case in macrophages and Dictyostelium, in which osmotically disrupted actin and pharmacologically disrupted actin respectively result in a number of “actin waves” that represent re-polymerisation of the F-actin (Bretschneider et al., 2009). Another explanation that I touched on in my previous chapter is to regulate calcium efflux. In this chapter I show that cortical actin is likely involved in regulating calcium entry into the oocyte, so it stands to reason that it may also play a role in the removal of calcium, whereupon a wave of reorganising actin could indicate changes in the cytoskeletal landscape that enables calcium efflux, perhaps through PMCA channels (reviewed in Marshall et al., 2015). Furthermore, as I will demonstrate in my final results chapter, actin is essential for organising the meiotic spindle, both in terms of localisation and morphology. It may well be the case that such a wave of reorganising F-actin plays a role at this stage; it is well documented that rotation of the spindle is a key feature of meiosis, enabling the nuclei to be positioned in such a

way that 3 are removed as polar bodies and only one selected, ensuring maintenance of a diploid organism (Endow and Komma, 1997). Both cortical and cytoplasmic actin are likely to play a role in positioning of the spindle and its rotation during meiosis, and as such a global wave of reorganising actin is likely to have an impact here. I will therefore provide further analysis of this in my final results chapter.

4.3.5 Future Directions:

I have provided a detailed analysis that demonstrates a potential relationship between the cortical actin cytoskeleton and calcium flux; namely that the actin acts a regulatory element, dispersion of which promotes calcium entry and over-expression prevents calcium entry. An important line of investigation would be to identify a definitive link between Trpm and the actin cytoskeleton. We know that in many systems, the link between actin and Trp channels is crucial, such as in TrpV3 mediated calcium influx in mice (Lee et al., 2016). Trp channels generally have been shown to co-localise with actin, such as TrpN1 in *Xenopus* cilia (Shin et al., 2005). Connections with the actin cytoskeleton need not be direct, such as TrpC6 and TrpL which have been shown to interact with regulators of the actin cytoskeleton (Dryer and Reiser., 2010; Chorna-Ornan et al., 2005). One method of evaluating this in the *Drosophila* oocyte would be to test available antibodies and utilise STED microscopy to ascertain co-localisation with the actin cytoskeleton. Trp channels, such as the Drosophila photoreceptors Trp and Trpl, often exist in larger complexes, associating with the scaffolding protein INAD and immunophilins such as FKBP59. This has also been shown for mammalian TRP channels such as TRPC (Sinkins et al., 2004). Immunophilins are in particular associated with calcium channels and play a role in calcium flux (Hahle et al., 2019).

Again, more detailed investigation of the aforementioned ABPs should be carried out. Especially with α-actinin, Supervillin and Gelsolin, it would be illuminating to investigate their localisation and dynamics within the activating oocyte through expression of fluorescently labelled variants. Additionally, it is likely that there would be interactions with these proteins and each other, other ABPs such as Myosin-II and the Trpm channel, and as such this could be investigated through co-immunoprecipitation or yeast-two hybrid experiments to test this hypothesis. Perhaps these proteins exists in a larger complex with INAD and other immunophilins, or it may be the case that they exclusively associate with the cortical actin.

Additionally, understanding the cortical actin itself is a field that is still gaining much traction; with the advent of super-resolution techniques, we are beginning to explore in more detail the variety of components that make up the cell cortex, and those key interactions between the cortex and plasma-membrane, specifically within the nanometre gap between these two components, that is likely to be fundamental in our understanding of how changes in the plasma membrane and cortical actin are transduced between each other. It will be paramount in future experimentation, therefore, to generate fluorescent constructs of not only the cortex, but the variety of different

cortical components, such as Supervillin or the villins more generally, through which the development of further super-resolution techniques in, importantly, live tissues, will be able to resolve. This should be carried out in combination with *in vitro* studies, such as actin nucleation assays utilising nucleators conjugated to beads and manipulated with magnetic tweezers (Yu et al., 2017), which are capable of illuminating the precise nucleating force and mechanics of specific nucleators, for instance. *In vitro* studies are incredibly valuable in our understanding of the mechanics of the cortex and specific ABPs, and as such more attention should be placed on those ABPs that regulate actin architecture in addition to nucleators. However, this work should always be supported by parallel *in vivo* studies, which is where I believe the development of greater resolution live imaging techniques and indeed constructs and reagents, such as injectable SiR-actin, will provide invaluable data that contextualises a mass of *in vitro* studies, enabling actin mechanics to be placed within broader signalling pathways and contexts.

Chapter 5:

Meiosis is regulated by actin and calcium

Chapter 5: Meiosis is regulated by actin and calcium

5.1 Introduction

5.1.1 Chapter Overview

The actin cytoskeleton is a fundamental component of eukaryotic cells, and, as evidenced by my previous chapters, plays a vital role in regulating the many processes of egg activation, especially calcium entry. Roles for actin in egg activation are diverse and complex, but one largely unexplored role of actin is the direct role it plays during the meiotic segregation of chromosomes, separate to its role in regulation of spindle position. Previously thought to be primarily regulated by the microtubule cytoskeleton, chromosome alignment and segregation during meiosis has quite recently been demonstrated to be impacted significantly by a population of actin in the spindle (Mogessie and Schuh, 2017). To date, this population has only been observed in mammals, as later studies in humans demonstrated the presence of this spindle-like actin and its role in regulating the formation of the microtubule spindle (Roeles and Tsiavaliaris, 2019). I therefore extend the understanding of this population in the wider field through demonstration of a morphologically and functionally similar population of actin in *Drosophila*. I will thus provide a new model in the wider field of meiosis, highlighting *Drosophila* as an exceptionally powerful tool for our understanding of this process, having corroborated and extended our knowledge of how the spindle-actin may influence the meiotic spindle.

5.1.2 Overview of Meiosis in Vertebrates and *Drosophila melanogaster*

Meiosis is a thoroughly studied and well-documented process essential for the formation of haploid gametes during sexual reproduction. These gametes, whether egg or sperm, provide a single copy of their genomes, culminating in the production of a, commonly, diploid organism. Thus far, the general principles of meiosis have been well established, largely focusing on the formation of microtubule spindle, the interaction of these specific cytoskeletal components with a variety of spindle-proteins to regulate the congression and segregation of chromosomes.

In mammals, foetal oogonia synchronously initiate meiosis until arresting at prophase I. They are then held in this state until sexual maturity, whereupon an oocyte, or small subsets of oocytes, are released periodically, triggering resumption of meiosis as nuclear envelope breakdown ensues (Handel & Schimenti, 2010). This progression from prophase-I results in the formation of a bipolar spindle network as a variety of process nucleate the formation of microtubules, capturing chromosomes and aligning them on the metaphase plate (Bennabi et al., 2016). The major contribution of actin investigated thus far has been that of the cytoplasmic actin, which aids in the positioning of the spindle adjacent to the cortex, enabling asymmetric division to occur, leaving a single oocyte containing the necessary maternal components and ultimately three polar bodies (following completion of meiosis) in which the extra genetic material is packaged and discarded (Bennabi et al., 2016; Azoury et al., 2008). Most vertebrates then continue meiosis until metaphase II where they are arrested until fertilisation, where upon man intracellular rise in

calcium triggers the events of egg activation, including the resumption of meiosis, culminating in the formation of the maternal pronucleus which undergoes fusion with the paternal pro-nucleus, forming the diploid zygote (Bolcun-Filas and Handel, 2018).

In *Drosophila*, the process is largely conserved however there are a few key differences. Breakdown of the nuclear envelope also initiates the formation of the meiotic spindle, which can be observed just underneath the dorsal appendages as a small elliptical shape with more focussed poles than in mammals (Endow and Komma, 1997; Bennabi et al., 2016). *Drosophila* ovaries are meroistic, meaning they are continually producing oocytes from a stem cell niche in the germarium. A 16-cell cyst is produced, and the cell that is selected as the oocyte begins to undergo prophase where it arrested until stage 12 (McLaughlin and Bratu, 2015; Hughes et al., 2018). The mechanisms of oocyte maturation that initiate the prophase to metaphase transition have not yet been established, however the metaphase microtubule spindle structures and chromosomes have been observed as they progress through this transition, ultimately resulting in the formation of a bipolar spindle surrounding a central mass of congressed chromosomes (Endow and Komma, 1997; Gilliland et al., 2009). The oocytes are then arrested at metaphase until ovulation occurs, which is the the event that triggers egg activation in *Drosophila melanogaster* (Horner and Wolfner, 2008). As the oocyte passes into the oviduct, mechanical triggers result in calcium influx through Trpm channels in the plasma membrane of the oocyte (York-Andersen et al., 2021; Hu and Wolfner, 2019). This calcium transient results in the resumption of meiosis and has been observed live at the spindle using the Ncd-GFP transgene (Endow and Komma, 1997). The metaphase to anaphase transition at egg activation can be observed as a stereotypical series of events (Endow and Komma, 1997):

1) Elongation of meiotic spindle
2) Contraction of meiotic spindle
3) Rotation of meiotic spindle in relation to the cortex
4) Following completion of meiosis I, the spindle is at 90 degrees to the cortex

5.1.3 The role of the cytoplasmic and cortical actin cytoskeleton during meiosis

As mentioned previously, the role of the cytoskeleton during meiosis has largely been examined with respect to the microtubules. However, there are known roles of the actin-cytoskeleton during meiosis, such as the role it plays during asymmetric cell division. Both cytoplasmic and cortical actin have been implicated in relocation and positioning of the spindle in mice. Inhibition of myosin-light chain, the activator of Myosin-2 which is active at the poles of the spindle, slows down spindle relocation, implicating this motor-protein in this process, likely as it pulls on the cytoplasmic network of actin to bring the spindle closer to the cortex (Holubcová et al., 2013; Schuh and Ellenberg, 2008). Further to this, blocking the Myosin-5b pathway, responsible for long range vesicle transport, prevents spindle migration, further suggesting that myosin motors are required for the relocation of the spindle using the cortical actin mesh (Holubcová et al., 2013).

This model itself relies on pulling forces generated by Myosin motors within the spindle network, however another model implicates the role of pushing forces by the actin itself (Li et al., 2008; Yi et al., 2013). In this model, a cloud of actin at the lagging pole of the spindle is generated by formin-2, and the nucleation of actin itself pushes the spindle toward the cortex.

Evidently, the cytoplasmic actin may play a large role in meiosis with regards to driving the spindle relocation to the cortex. Here another population of actin is suggested to play an important role- the cortical actin. In mice, thickening of the cortex following resumption of meiosis-I is independent of Formin-2 and Spire proteins but does require Arp2/3 activity to nucleate branched actin networks (Azoury et al., 2008; Chaigne et al., 2013; Pfender et al., 2011). This thickening also represents a change in cortical tension, which was demonstrated to be essential for spindle migration, and either too high or too low tension prevents accurate spindle relocation (Chaigne et al., 2013; 2015). In *Drosophila,* a similar cortical thickening can be observed following activation and resumption of meiosis, which likely also represents a change in cortical tension as mechanically gated channels open in the membrane and calcium enters (York-Andersen et al., 2020; 2021). Though this change in the cortical actin can be observed, it is not yet clear what role the cortical actin plays in spindle positioning and whether this is indeed important.

5.1.4 The role of actin within the spindle during meiosis

The exciting discovery of a population of actin within the meiotic spindle of mouse oocytes has opened an entirely new avenue of investigation within the field of meiosis. Utilising a variety of labelling tools, Mogessie and Schuh demonstrated that a spindle-like actin permeates the meiotic spindle in mice. The tool UtrCH-GFP, consisting of the calponin-homology domain of Utrophin (that binds to actin) conjugated to an enhanced GFP, was used to label this spindle in live oocytes. Utrophin is commonly known as the non-muscle counterpart to the human protein Dystrophin (Winder et al., 1999). This result was corroborated through Phalloidin and SiR-Actin labelling of oocytes (Mogessie and Schuh, 2017). Phalloidin is derived from a fungal toxin (*Amanita phalloides*) and acts as an actin-stabilising agent, binding to actin filaments with a higher affinity than actin monomers, and can be conjugated to a variety of fluorescent proteins to act as an effective F-actin label (Cooper, 1987; Wulf et al., 1979). Similarly, SiR-Actin is partially derived from the natural toxin Jasplikanolide, an F-actin stabilising agent, conjugated to the fluorophore silicon rhodamine (SiR), enabling it to act as a fluorogenic and cell permeable actin dye (Ikeno et al., 2017; Lukinavičius et al., 2013; Lukinavičius et al., 2014). Utilising these tools, Mogessie and Schuh demonstrate clearly the presence of the actin-spindle that appears morphologically similar to that of the microtubules, in mice, human and porcine oocytes.

Further study of this population of actin in mouse oocytes revealed Formin-2 as a key nucleator of the spindle-like actin. Disruption of this population could therefore be achieved through mutation of the Formin-2 gene, or more generally through pharmacological disruption with cytochalasin-D, revealing that without the actin cytoskeleton, chromosome alignment (metaphase) and

segregation (anaphase) during meiosis is disrupted (Mogessie and Schuh., 2017). Knock out of this actin population or stabilisation through targeted expression of the calponin-homology domain to the spindle affected the population of K-fibres in the mouse spindle, indicating that the actin population was also required for production of the K-fibres, providing a possible mechanisms for how spindle-like actin regulates chromosomal movements during meiosis (Mogessie and Schuh., 2017). More recently, pharmacological manipulation of the actin population in human oocytes revealed an inter-dependence of the actin and microtubule spindle populations (Roeles and Tsiavaliaris., 2019). Co-visualisation of the actin and microtubule populations revealed concomitant changes in the actin and microtubule-spindle during meiosis and when incubated with various microtubule specific drugs. Incubation with monastrol, for instance, results in the loss of bipolarity of the microtubule spindle, which also results in a loss of bipolarity in the actin spindle. Further to this, washout procedures demonstrated that the spindle can recover bipolarity if the monastrol is removed, however, bipolarity cannot be re-established under conditions where the actin cytoskeleton is disrupted through incubation with cytochalasin-D (Roeles and Tsiavialaris., 2019). This work combined begins to suggest that the production of an actin spindle by formin proteins is required upstream of the microtubule spindle, as without the actin cytoskeleton the spindle is unable to accurately form, yet similarly changes in the microtubule spindle also have downstream effects on the spindle-like actin. There is clearly a complex relationship between these two separate cytoskeletal populations that requires further study and analysis.

5.1.5 The Requirement of Calcium in the Meiotic Spindle

As previously described in Chapter 1, the role of calcium at the meiotic spindle has been well established as essential for the release from meiotic arrest universally. For instance, the secondary meiotic arrest is established through regulation of the drivers of the G2/M transition, that being the Metaphase Promoting Factor (MPF) (Horner and Wolfner., 2008; Von Stetina and Orr-Weaver., 2011). The MPF consists of the regulatory Cyclin-B and a catalytic subunit, Cdc2 Kinase, which when active phosphorylates a variety of substrates leading to NEBD and spindle formation, which when activity is high maintains the metaphase arrested state of the oocyte (Belenguer et al., 1990; Heald and McKeon, 1990; Peter et al., 1990a,b). Upon egg activation, whether this is triggered through fertilisation or an alternative mechanism, such as ovulation in *Drosophila*, a significant release in calcium is generated (Horner and Wolfner., 2008). In mice and *Xenopus*, calcium release activates Calmodulin Kinase II (CamKII), which begins an enzymatic cascade culminating in the activation of the Anaphase Promoting Complex (APC) which targets the regulatory Cyclin of the MPF for degradation and thus promotes the resumption of meiosis (Castro et al., 2005). Additionally, it has been demonstrated in *Drosophila* and *Xenopus* that the calcium and calmodulin dependent protein Calcineurin (CaN) is required during egg activation for the resumption of meiosis (Horner et al., 2006; Takeo et al., 2006; Mochida and Hunt, 2007; Nishiyama et al., 2007b). Further calcium-sensitive proteins have been examined, and many

components of the spindle itself are sensitive to intracellular calcium, highlighting the necessary role of calcium during meiosis.

The above contribution of calcium is heavily reliant on global calcium signals occurring at egg activation, as transients are generated that traverse the whole oocyte, impacting the signalling landscape of the entire cell. However, there may be a contribution from far more localised calcium domains within the spindle itself, with possible signalling between junctions on the scale of nanometres, known as nano-domain signalling being essential for changes in the meiotic spindle at egg activation (Li et al., 2016). Utilising the genetically encoded calcium sensor GCaMP3, consisting of a permutated GFP containing the calcium sensitive domain of calmodulin and the M13 peptide of myosin light chain (Nakai et al., 2001), it was observed that an enrichment in calcium could be observed at the spindle (Li et al., 2016). Further to this, the calcium sensitive signalling protein calmodulin was shown to localise to the spindle (Li et al., 2016). In order to investigate the type of calcium signalling within the spindle, Li et al. performed incubations with the calcium chelators BAPTA and EGTA. BAPTA incubation resulted in the rapid depolymerisation of the microtubule spindle, in comparison to EGTA which did not. This is an interesting result as EGTA has a higher affinity for calcium and is able to abolish egg activation completely (Li et al., 2016). This suggests a difference in mechanism between BAPTA and EGTA; that while both are capable of preventing global calcium transients, only BAPTA is able to depolymerise the microtubule spindle. This may be explained due to the much faster kinetics of buffering with BAPTA, which enables chelation of calcium in much smaller signalling domains before the calcium is able to diffuse and bind to targets (Neher, 1998; Wang and Augustine, 2014). Certain calcium signalling units can be referred to as nano-domains, as the calcium source and sensor exist within 20nm of each other, such as the voltage-gated calcium channel and the vesicular protein synaptotagmin, which are completely insensitive to EGTA due to its slow kinetics. This could therefore suggest that such nano-domains exist within the spindle. Having demonstrated the requirement of actin in calcium entry during egg activation in Chapter 4, and the likely role that calcium sensitive actin binding proteins may play in this process, it is likely that localised or nano-domain signalling occurs to not only influence the microtubule cytoskeleton but also the actin cytoskeleton that permeates the spindle. As the presence of this population of actin has only recently been demonstrated, the potential interdependence of microtubules, actin and calcium at the spindle would be a completely novel mechanism furthering our understanding of meiosis and highlighting *Drosophila* once more as a powerful model.

5.1.6 Experimental Motivations & Approach

Previous work has highlighted the presence and potential function of a spindle-like population of actin during meiosis. It is likely that this population of actin plays a vital role during the correct segregation and alignment of chromosomes and when this process fails, aneuploidy occurs. This

could therefore be an avenue of research that helps us to understand why aneuploidy occurs and how to best prevent this during procedures such as IVF.

As a previously unexplored pool of actin and mechanism within *Drosophila*, there is not a great deal of literature surrounding this topic, and I therefore aim to use this research as a platform for further study and examination of this area, spring boarding *Drosophila* once more as a vital tool for the study of cell biology.

In this chapter I establish *Drosophila melanogaster* as a powerful model for the further study of meiosis, and reveal for the first time a novel population of actin within *Drosophila* oocytes. Using the large genetic tool-base available in *Drosophila*, I will demonstrate the use of further live markers for the visualisation of the spindle-like actin, enabling more detailed analysis of the actin spindle in real time. Additionally, I will use a combined genetic and pharmacological approach to dissect the functions of this spindle-like actin, demonstrating it to play an important role in chromosomal movements during *Drosophila* meiosis and in the formation and regulation of the microtubule spindle. I will also show that there is likely a key requirement of localised calcium at the spindle, and this calcium regulates the formation of the spindle.

5.1.7 Aims of Chapter:

1) Demonstrate the presence of a spindle-like population of actin cytoskeleton in *Drosophila*: Utilising a variety of genetic tools and fluorescent dyes, I will show that a population of actin exists that permeates the spindle and surrounds the meiotic chromosomes

2) Identify the role of the spindle-like actin in the regulation of chromosomal segregation and alignment during meiosis in the oocyte: Using a combined genetic and pharmacological approach alongside high-resolution confocal microscopy, I will show that disruption of the actin results in defects in chromosome alignment and segregation during meiosis-I.

3) Identify the mechanism of actin-mediated chromosomal regulation during meiosis: I will demonstrate utilising high-resolution microscopy and pharmacological approaches that the actin cytoskeleton acts upstream of the microtubules to organise the bipolar spindle at meiosis.

4) Investigate the role of localised calcium at the spindle during meiosis: Utilising a combination of genetic and pharmacological means, I will show that calcium is present at the spindle. I will show that it is required for both microtubule and actin spindle structures, and forms an interdependent relationship with the spindle-like actin.

5.2 Results

5.2.1 A spindle-like population of actin exists in the meiotic spindle of the *Drosophila* Oocyte

The metaphase-arrested spindle in *Drosophila melanogaster* has a very typical orientation and location in the stage 14 mature oocyte. To confirm this, I utilised the genetically-encoded microtubule labelling protein Jupiter-GFP (Bloomington). Fortunately, the fixation protocol for stage 14 oocytes is incredibly mild, utilising a short fixation time and PBS-Triton, as the oocytes are particularly delicate at this stage, meaning that microtubules can often be visualised through regular fixation protocols. Therefore, fixation of Jupiter-GFP expressing oocytes and addition of GFP-booster enables fixed visualisation of the microtubule spindle. Using this protocol, I was able to visualise the microtubule spindle, localising to the base of the dorsal appendages (Fig 5.1 A). Co-staining with the actin labelling dye Alexa-fluor Phalloidin 568 enabled visualisation of the actin cytoskeleton, which forms a tight cortical band around the oocyte, and strongly labels the surrounding follicle cell layer (Fig 5.1 A). Similarly, DAPI staining strongly labels the surrounding follicle cell nuclei of the oocyte, but importantly is able to label the metaphase arrested chromosomes that are congressed on the metaphase plate in stage 14 oocytes (Figure 5.1 A). It is evident that this spindle structure lies at the base of the dorsal appendages when compared to the bright-field image (Fig 5.1 B), which appear as long protrusions from the dorsal-anterior edge of the oocyte. Going forward, this enabled me to more easily locate the spindle apparatus in further study.

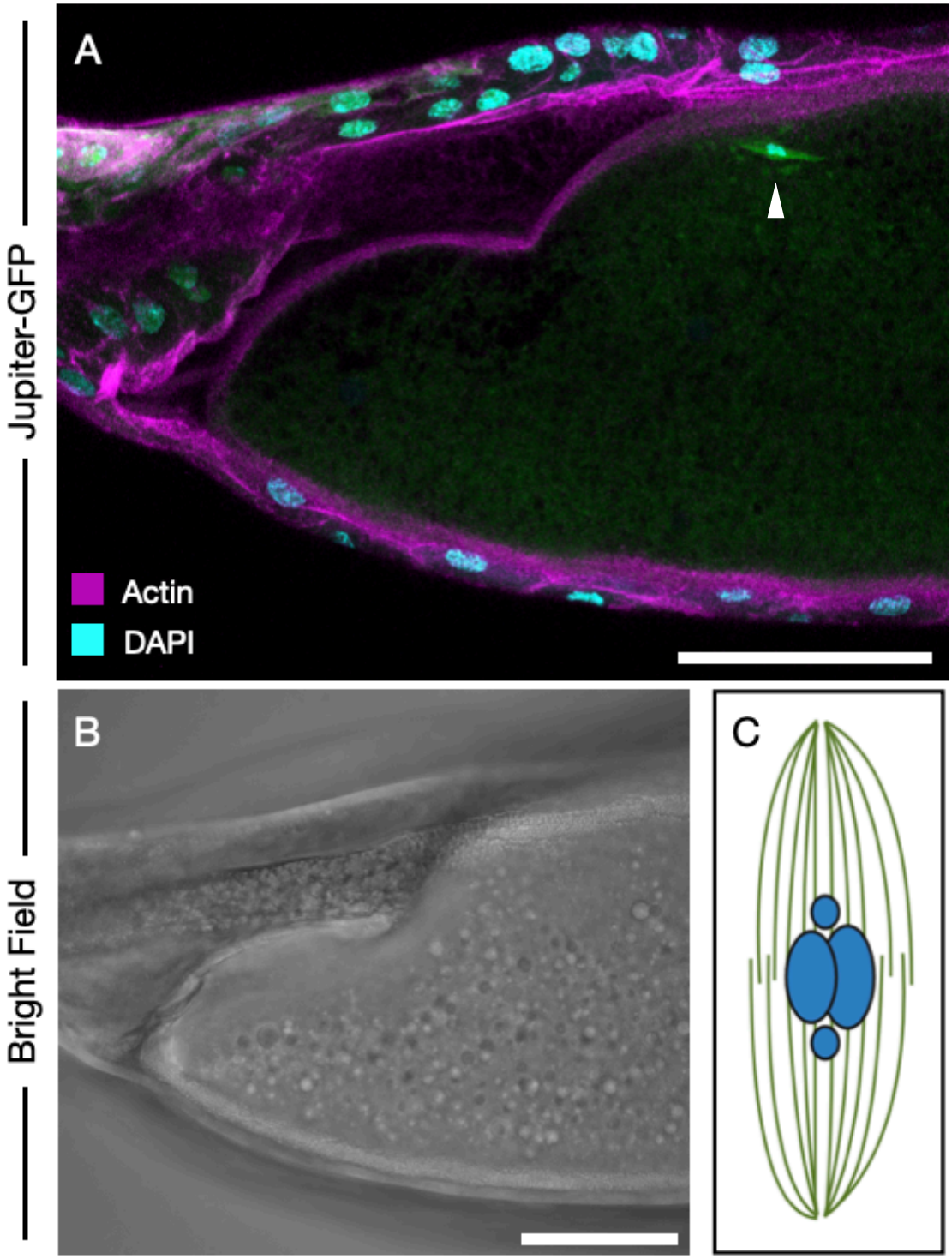

Figure 5.1: The spindle typically localises to a region below the dorsal appendages
(A) Confocal Z-projection (40 µm) of a fixed metaphase-I (MI) oocyte (anterior end only). Microtubules shown in green (Jupiter-GFP, GFP booster), Actin shown in magenta (Alexa-fluor568 Phalloidin), DNA shown in cyan (DAPI). Spindles (white arrowhead) lie parallel to the dorsal-anterior cortex of the oocyte. (**B)** Corresponding bright-field Z-projection. **C)** Schematic representing the MI arrested spindle in the *Drosophila* oocyte. Microtubules (MTs) are represented in green and the metaphase chromosomal mass is represented in blue. Spindle forms with highly focused poles with four centrally located chromosomes. Fourth non-exchange chromosomes appears as a distinct unit at the polar tips of the mass. Scale bar: 50 µM (A,B).

Having established that a population of actin cytoskeleton exists within the spindle of human, mouse and porcine oocytes (Mogessie and Schuh, 2017; Roeles and Tsiavialris, 2019), I decided to test whether a similar population of actin existed in *Drosophila* oocytes. To visualise whether there is an enrichment of actin at the spindle, I utilised the AlexaFluor 568 Phalloidin, fixing oocytes that were expressing the protein trap Histone2AS::GFP. When fixed and visualised, these oocytes displayed an obvious fluorescence signal from the H2AS::GFP, indicating the metaphase arrested chromosomes (Figure 5.2 A). Interestingly, in some oocytes, a localised enrichment of the Phalloidin could also be noted, forming an elliptical shape reminiscent of the microtubule spindle (Figure 5.2 A). The enriched actin structure I observed to have a length in the order of 10-20uM, a length also within the range of the microtubule spindle structure, which has an average length of 13uM in samples I have observed.

Co-visualisation live of Moesin::GFP, the previously described actin labelling tool that selectively labels the cortical actin, and a Histone::RFP, revealed that though the chromosomes could be visualised, once more under the dorsal appendages, the Moesin::GFP label was not enriched in the surrounding area (Figure 5.2 B). This suggests that the localised actin population at the spindle does not represent the cortical actin, but in fact a separate pool of actin within the cytoplasm.

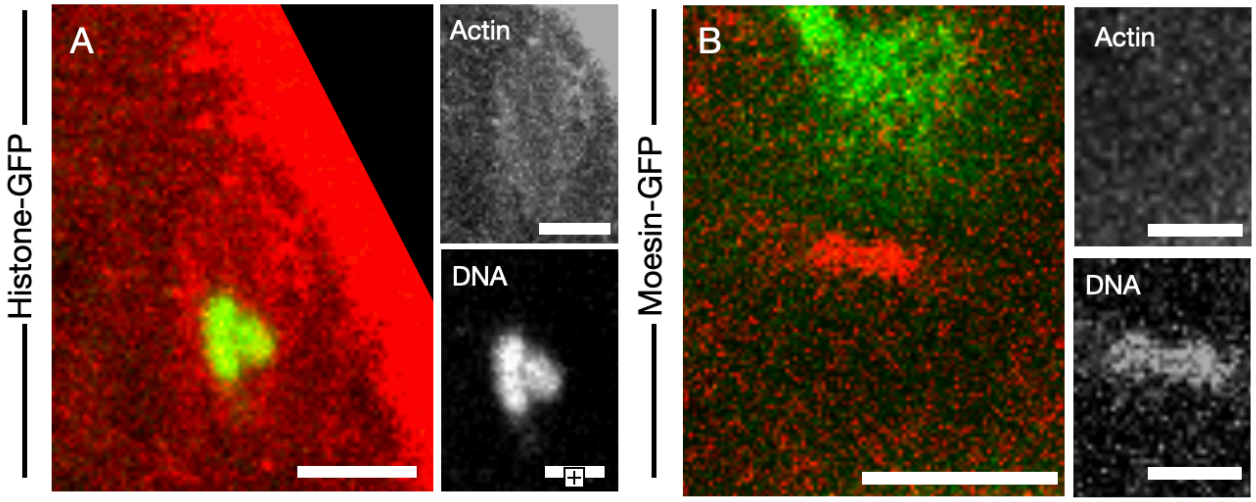

Figure 5.2: A pool of non-cortical actin is enriched at the spindle. (A) Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows actin (Alexa-Fluor568 Phalloidin) in red, DNA (Histone-GFP) in green, demonstrating a spindle-like population of actin surrounding a chromosomal mass. N=14. **B)** Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows cortical actin (Moesin-GFP) in green, DNA (Histone-RFP) in red, demonstrating a s a chromosomal mass but no enrichment of actin. Enrichment above chromosomes represents cortical actin. N=25. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

Perhaps due to poor penetration of the Phalloidin dye, though enrichment could often be observed at the spindle, there was not a great level of resolution and detail observable. In order to visualise this population of actin more effectively, I expressed the actin labelling tool UtrCH-GFP, which consists of the calponin-homology domain of Utrophin, conjugated to an enhanced GFP (Rauzi et al., 2010). This label has been used to visualise the actin live in mice, and effectively demonstrated the existence of a separate pool of actin that changes throughout meiosis (Mogessie and Schuh, 2017). High-resolution confocal microscopy of mature oocytes expressing the actin labelling tool UtrCH-GFP under the control of a tubulin-Gal4 revealed a filamentous spindle-like structure in stage 14 mature oocytes in the location of the arrested metaphase-I spindle (i.e. below the dorsal appendages) (Figure 5.3). Similarly to the labelling in mice (Mogessie and Schuh., 2017), UtrCH-GFP labels the actin-spindle at a fluorescence intensity intermediate to the high-intensity of the cortical actin and the low-intensity of the cytoplasmic actin.

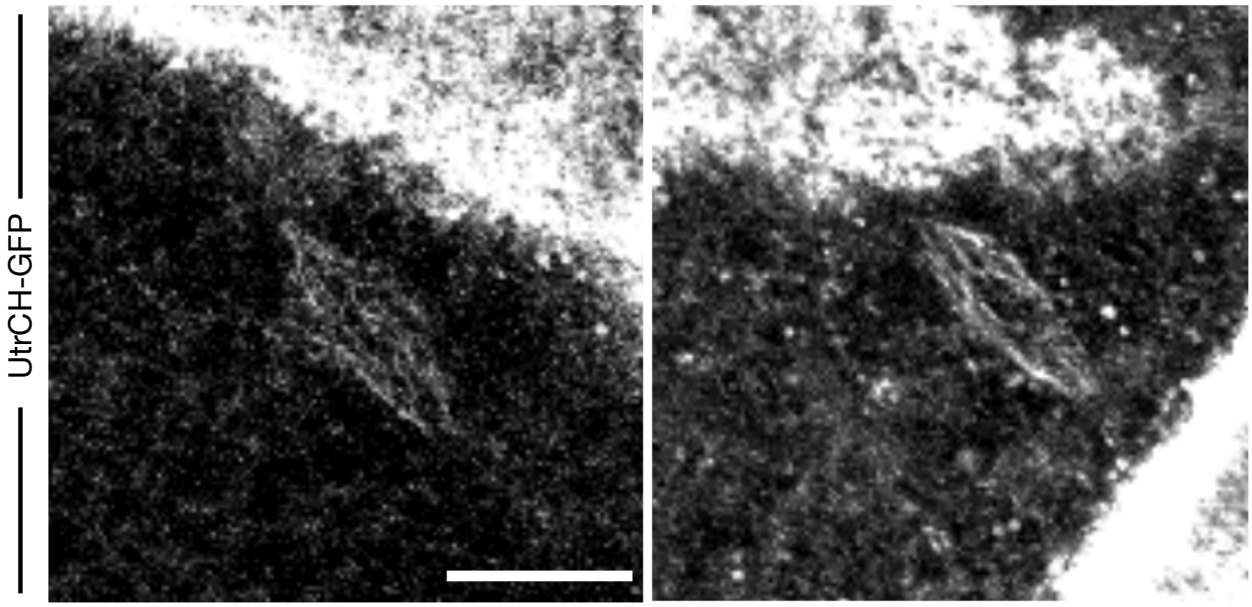

Figure 5.3: A spindle-like actin population exists in the Stage 14 oocyte. Confocal Z-projections (10 μm) of a live MI oocyte. This population of actin (UtrCH-GFP) appears as a spindle-like structure, with filaments traversing the spindle. Scale bar: 10 μm. N=45.

The next question regarding this population of actin was to investigate what machinery is involved in its production. In mice, it was shown that the Formin-2 (Fmn2) is required to generate this spindle-like actin, as when it was knocked out, the spindle-like actin no longer formed (Mogessie and Schuh, 2017). The *Drosophila* homolog of Fmn2 is the actin nucleating protein Cappuccino.

I therefore generated a line of UtrCH-GFP expressing oocytes in a heterozygous Cappuccino mutant background, utilising the *cappuccino*^{EY12344} hypomorphic mutant**.** Homozygous mutants were, however, not viable. Even with heterozygous mutants a clear phenotypic difference was visible. There was a significant increase in the number of oocytes without or with a disrupted spindle-like actin population compared to wild-type oocytes, indicating that Cappuccino is required for formation of the spindle-like actin population (Figure 5.4).

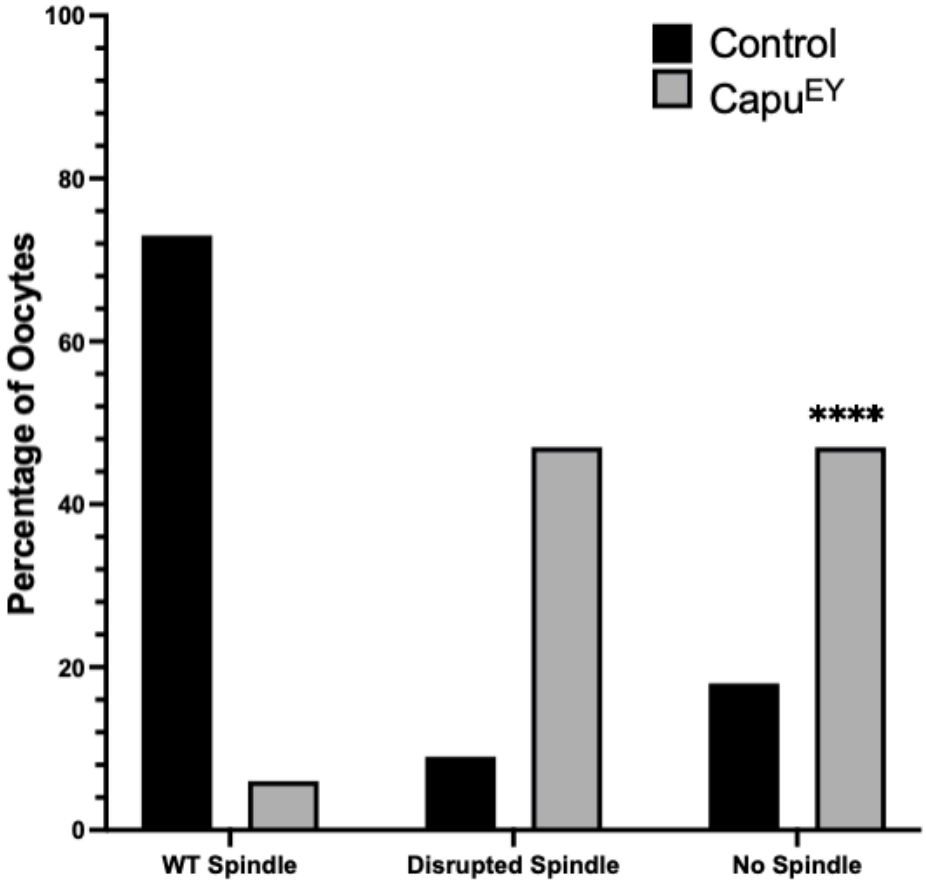

Figure 5.4: Cappuccino is required for formation of the spindle-like actin. Comparison of the number oocytes with a normal spindle-like actin population. In a *capu*^{EY12344} mutant background there are a significantly greater number of oocytes with a disrupted spindle or no spindle altogether. $P < 0.0001$, Fischers Exact Test, $N = 30$ per genotype.

5.2.2 The spindle-like population of actin undergoes a dynamic rearrangement at egg activation

The spindle undergoes a classical rearrangement at egg activation, as the spindle ultimately contracts and rotates, as demonstrated through labelling of the microtubules with Ncd-GFP (Endow and Komma., 1997). To investigate whether the population of actin observed undergoes a similar dynamic rearrangement upon egg activation, I visualised the spindle live using the UtrCH-GFP construct, and activated the eggs through the addition of activation buffer (AB). 5 minutes following egg activation, a clear rearrangement in the spindle-like actin can be observed, as the spindle begins to rotate in relation to the cortex and undergoes a clear contraction (Figure 5.5 B). Measuring the maximum width of the spindle indicates a significant increase following activation; evidently the morphological rearrangement follows a similar change to that of the microtubule spindle. This suggests that the actin spindle is required throughout the metaphase-anaphase transition, and is likely playing an active role throughout meiosis in *Drosophila* oocytes.

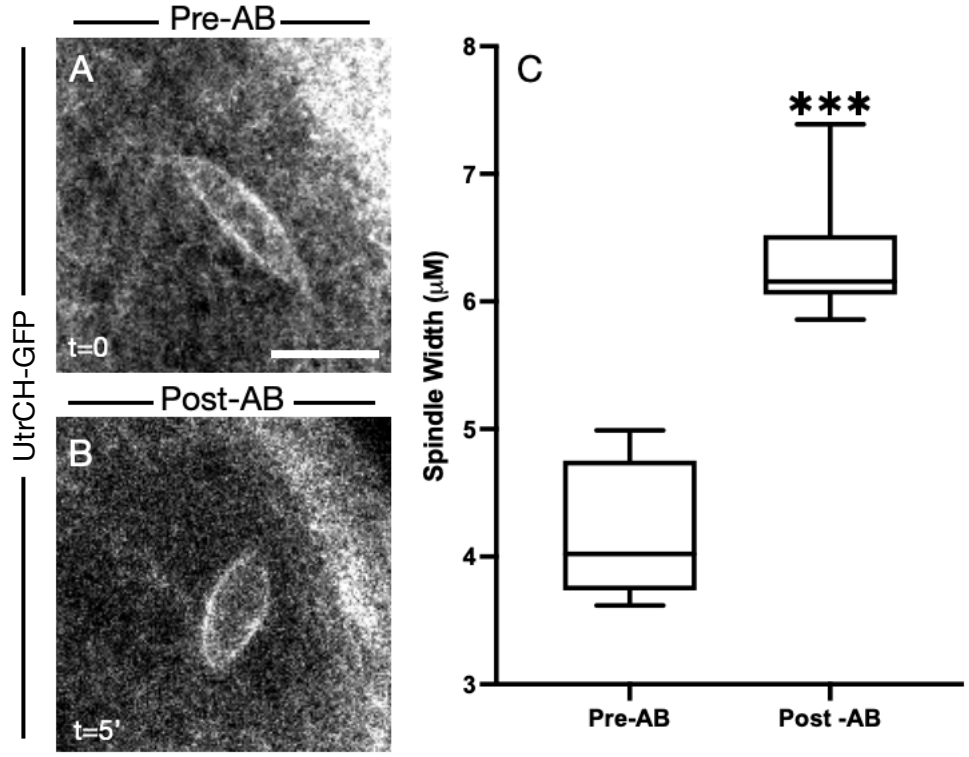

Figure 5.5: The spindle-like actin undergoes a morphological rearrangement at egg activation. (A) Confocal Z-projections (10 μm) from a live time series of an UtrCH-GFP expressing MI oocyte pre-addition of activation buffer (Pre-AB). Spindle-like actin appears narrow and long. **(B)** Confocal Z-projections (10 μm) from a live the series of an UtrCH-GFP AI oocyte post-addition of AB (Post-AB). Spindle-like actin appears contracted. **(C)** Comparison of the spindle width between pre-AB and post-AB oocytes, demonstrating a significant increase following activation. $P < 0.005$, Student's T Test. $N = 12$ for pre-AB and 8 post-AB.

In order to further verify that this population of actin corresponds to the spindle throughout egg activation, I fixed UtrCH-GFP expressing flies and stained them with DAPI and GFP-booster, pre and post-incubation with AB. Before egg activation, a region of enriched UtrCH-GFP co-localised with the DAPI stain; the chromosomes appear as a centrally congressed mass, often with the 4th non-exchange chromosomes visible either slightly separate from the mass or at the edge of the mass. This is a typical conformation of the chromosomes at metaphase-I (MI) (Gilliland et al., 2009). This mass of chromosomes appears centrally within the region of enriched UtrCH-GFP (Figure 5.6 A). Incubation in activation buffer followed by fixation of the oocytes revealed a similar enrichment of the spindle-like actin surrounding a mass of chromosomes that had increased in length (Figure 6B). This actin appears to have undergone a similar change as observed in live imaging, with an increase in the maximum spindle width, typical for anaphase-I (AI). Despite enrichment of the actin marker being obvious, it does not however have the detail observable through live imaging.

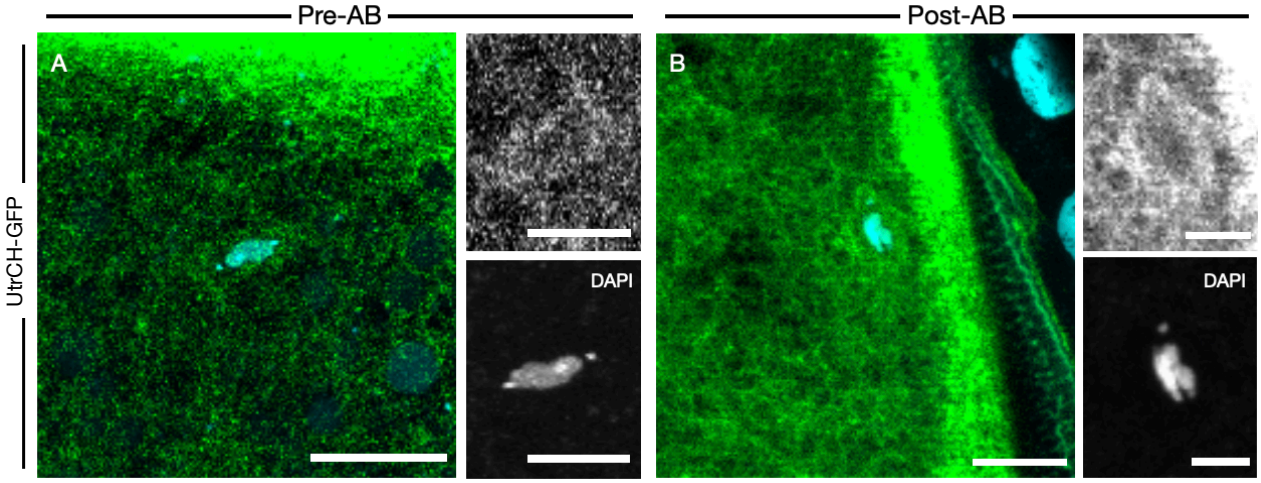

Figure 5.6: The UtrCH-GFP labelled spindle co-localises with the metaphase and anaphase chromosomes. (A) Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows actin (UtrCH-GFP) in green, DNA (DAPI) in cyan, demonstrating a spindle-like population of actin surrounding a chromosomal mass. N=22. **(B)** Confocal Z-projection (10 µm) of a fixed AI oocyte. Merge shows actin (UtrCH-GFP) in green, DNA (DAPI) in cyan, demonstrating a separating chromosomal mass and wider spindle-like actin. N=18. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

Having established that UtrCH-GFP labels a population of actin that is morphologically and dynamically similar to the metaphase and anaphase spindle, I sought to corroborate this finding utilising alternative markers of the actin cytoskeleton. I first expressed a GFP tagged actin monomer (actin 5C), which effectively labels all actin in the germline through tubulin-Gal4 mediated expression. Fixation of these oocytes and staining with DAPI revealed a similar distribution and localisation of the actin, surrounding a centrally congressed mass of chromosomes (Figure 5.7 A). Visualisation live of the Act5C-GFP was not successful (data not shown), however, fixation and enhancement of the signal using a GFP-booster enabled visualisation of the Act5C-GFP as a spindle like structure. This confirms that the fixed Alexa-Fluor 568 Phalloidin (Fig 2A) and live and fixed UtrCH-GFP (Figure 5.3 A; Figure 5.5 A) represent a unique population of actin at the spindle. Following visualisation of the metaphase spindle, I sought to to assess whether Act5C-GFP would also effectively label the anaphase spindle, which also demonstrates enrichment (Figure 5.7 B). One must be cautious with the use of Act-5C as it would constitute an over-expression of actin which could of course result in disruption of actin structures or saturation of actin signals. However, in this context it appears to corroborate evidence from UtrCH-GFP and Lifeact-GFP (Figure 5.8).

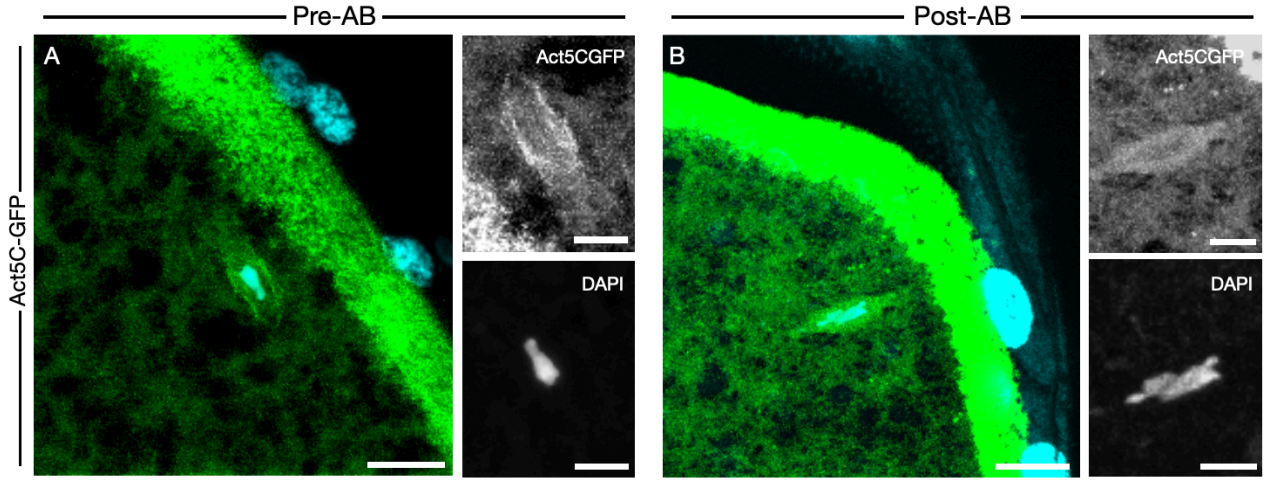

Figure 5.7: The Act5C-GFP labelled spindle co-localises with the metaphase and anaphase chromosomes. (A) Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows actin (Act5C-GFP) in green, DNA (DAPI) in cyan, demonstrating a spindle-like population of actin surrounding a chromosomal mass. N=15. **(B)** Confocal Z-projection (10 µm) of a fixed AI oocyte. Merge shows actin (Act5C-GFP) in green, DNA (DAPI) in cyan, demonstrating a separating chromosomal mass and wider spindle-like actin. N=15. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

I also expressed the actin-labelling tool Lifeact-GFP, driven by a tubulin-Gal4, in the mature *Drosophila* oocyte. It is commonly used amongst the field as it is a robust marker that effectively labels all F-actin structures within the cell (Spracklen et al., 2014). Expression of Lifeact-GFP demonstrated a clear enrichment at the metaphase spindle, once more localising to a central mass of chromosomes (Figure 5.8 A). Following incubation with AB, fixation of Lifeact-GFP expressing oocytes with DAPI also confirmed that this population of actin remains in the anaphase spindle, likely being required throughout meiosis (Figure 5.8 B). Staining reveals that this population remains filamentous and distinct from the surrounding cytoplasmic actin, which has also undergone a stereotypical change at egg activation as the cortex appears thickened and the cytoplasmic actin appears more filamentous (Figure 5.8 B).

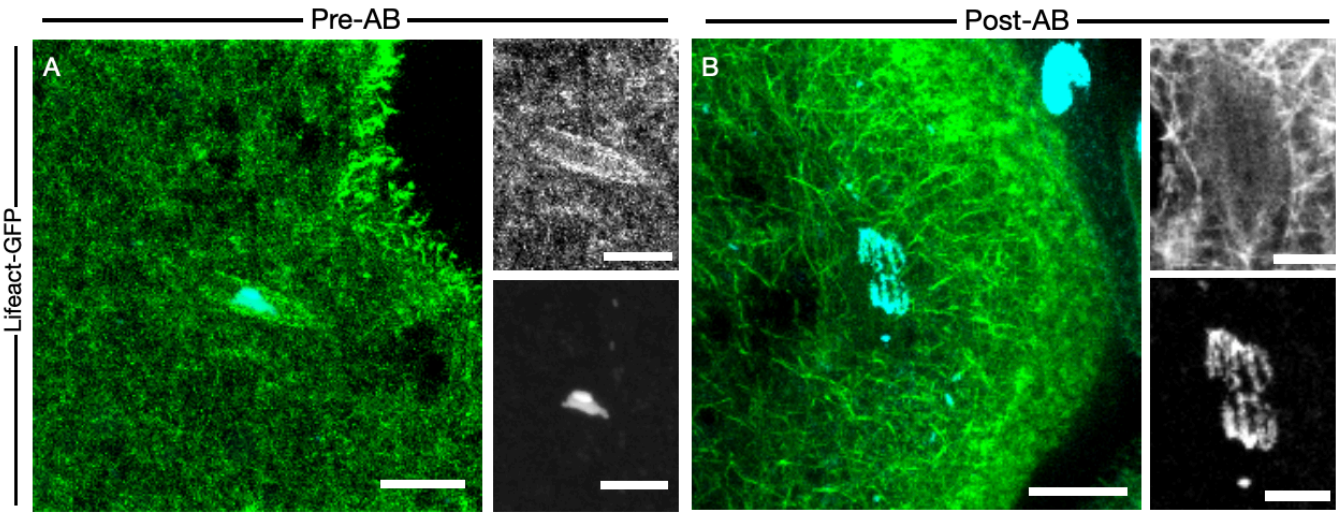

Figure 5.8: The Lifeact-GFP labelled spindle co-localises with the metaphase and anaphase chromosomes. (A) Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows actin (Lifeact-GFP) in green, DNA (DAPI) in cyan, demonstrating a spindle-like population of actin surrounding a chromosomal mass. N=17. **(B)** Confocal Z-projection (10 µm) of a fixed AI oocyte. Merge shows actin (Lifeact-GFP) in green, DNA (DAPI) in cyan, demonstrating a separating chromosomal mass and wider spindle-like actin. N=15. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

This further corroborates previous evidence that the actin visualised represents a specific spindle actin. Importantly, it also highlights the power of *Drosophila* melanogaster as a tool for study of this specific population, as a plethora of tools are available to conduct experiments with, UtrCH-GFP appearing to give the best visualisation live, whereas Act5C-GFP and Lifeact-GFP appear more successful at labelling this spindle population post-fixation and boosting using the GFP-booster.

Additionally, visualisation of Lifeact-GFP live revealed similar dynamic rearrangements at egg activation to UtrCH-GFP, mirroring the changes that occur to the microtubule spindle during the metaphase to anaphase transition at egg activation. The spindle-like actin appears long and narrow prior to the addition of AB, and contracts following addition of AB as anaphase begins (Figure 5.9 A,B). There is a clear change in morphology as the width of the spindle increases significantly following activation (Figure 5.9 C). I ultimately found that Lifeact-GFP is effective both as a live and fixed marker of the spindle like actin, UtrCH-GFP is very effective at labelling the spindle-like actin live, but less so fixed, and Act5C-GFP is most effective at labelling the spindle-like actin following fixation.

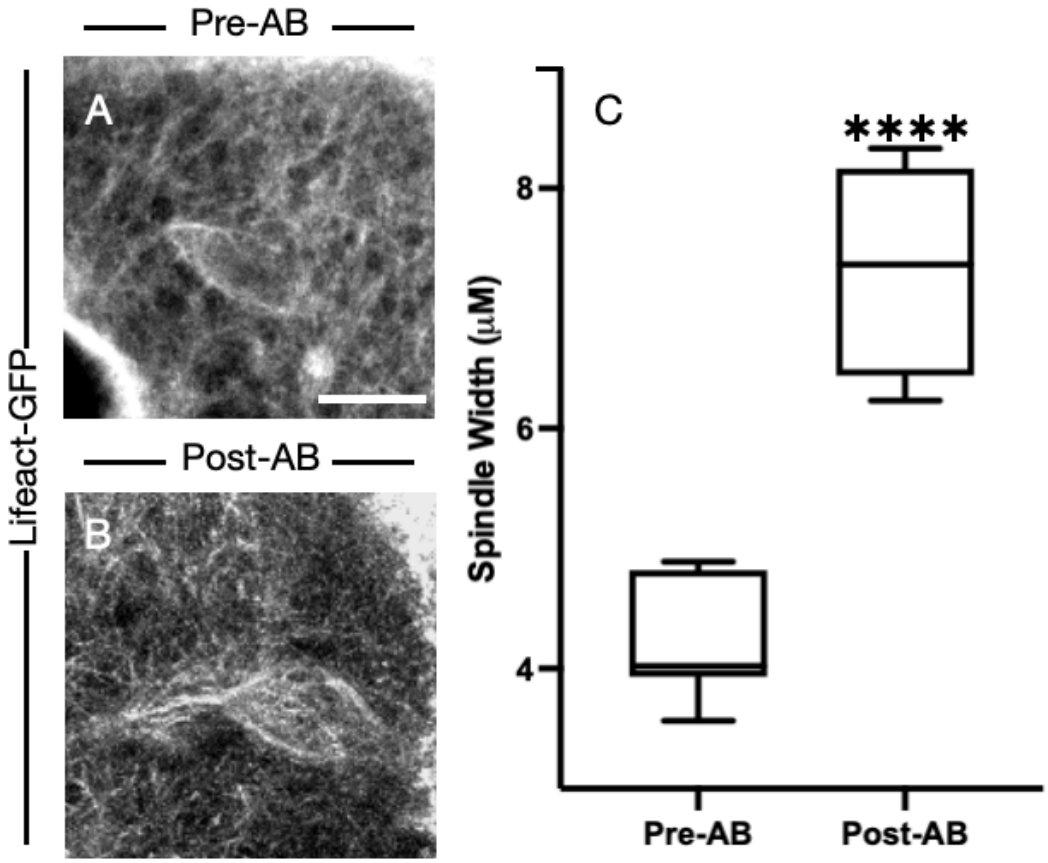

Figure 5.9: The spindle-like actin undergoes a morphological rearrangement at egg activation. (A) Confocal Z-projections (10 μm) from a live time series of an Lifeact-GFP expressing MI oocyte pre-addition of activation buffer (AB). Spindle-like actin appears narrow and long. **(B)** Confocal Z-projections (10 μm) from a live the series of an Lifeact-GFP AI oocyte post-addition of AB. Spindle-like actin appears contracted. **C)** Comparison of the spindle width between pre-AB and post-AB oocytes, demonstrating a significant increase following activation. $P < 0.005$, Student's T Test. N = 15.

5.2.3 The actin-cytoskeleton regulates the formation, morphology and dynamics of the microtubule spindle

Having discovered a novel population of actin cytoskeleton in the *Drosophila* meiotic spindle, my next step was to investigate how this population of actin was formed and what the possible function of this actin may be. Given the major role of the microtubule cytoskeleton during meiosis, I decided to test whether the microtubule cytoskeleton was required for the formation of the spindle-like actin. To do so, I incubated oocytes expressing Jupiter-GFP and UtrCH-GFP with colchicine, a microtubule depolymerising agent, and visualised the effect on the respective populations of microtubules and actin. As expected, when Jupiter-GFP expressing oocytes were incubated with colchicine, a gradual decrease in the fluorescent signal was observed, demonstrating the depolymerisation of the microtubules by the action of the colchicine (Figure 5.10 A).

However, when oocytes expressing UtrCH-GFP (labelling the actin cytoskeleton) were incubated in colchicine, no significant decrease in the fluorescent signal was observed after 30 minutes (Figure 10B). Slight changes appear in apparent proximity of the spindle to the cortical actin (the brightest signal), however this is optical and due to movement of the oocyte following addition of the colchicine containing solution. This suggests that the depolymerisation of the microtubule network does not disrupt the spindle-like actin. Further to this, it could indicate that actin acts upstream of the microtubules.

To test the role of the actin cytoskeleton on the population of spindle microtubules, I first incubated oocytes expressing Jupiter-GFP with cytochalasin-D, which acts to rapidly depolymerise actin filaments (Figure 5.11). Incubation with cytochalasin-D results in a drastic elongation of the microtubule spindle, increasing in average length. From these images, it is evident that the whole spindle structure appears stretched, including the negative space that is occupied by the chromosomes. This data suggests that the lack of the actin cytoskeleton disrupts the wild-type morphology of the metaphase spindle. Cytochalasin-D is an effective actin-depolymerising agent, and therefore while it is likely that the specific spindle-like actin population plays a role in regulating this morphology, there could also be a contribution from the cytoplasmic actin, or indeed the general gel-like properties of the cytoplasm that may be affected by significant actin disruption.

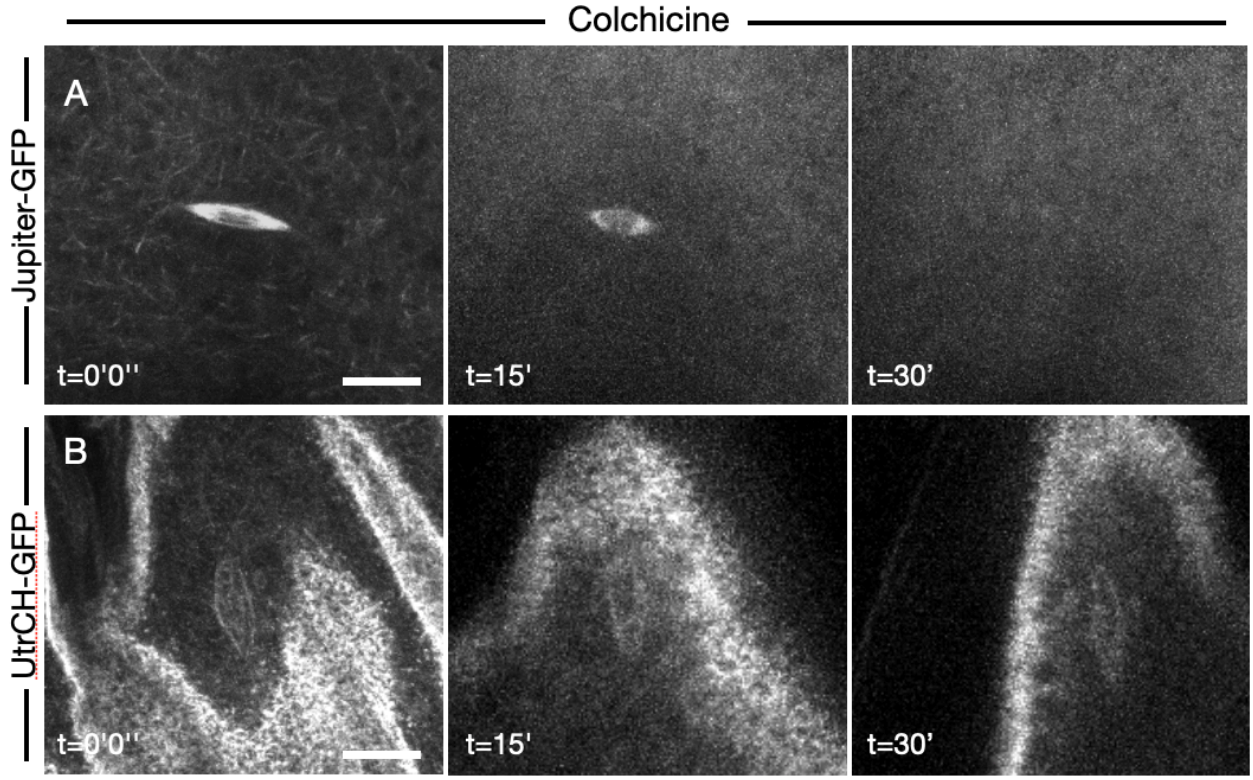

Figure 5.10: Colchicine incubation results in depolymerisation of the microtubule spindle but not the spindle-like actin. (A) Confocal Z-projections (10 µm) from a live time series of a MI oocyte treated with colchicine (t=0'). Microtubules (Jupiter-GFP) first appear as a typical spindle structure and completely depolymerise post-treatment (t=30'). N = 5. **(B)** Confocal Z-projections (10 µm) from a live time series of a MI oocyte treated with colchicine (t=0'). Actin (UtrCH-GFP) first appear as a typical spindle structure and do not depolymerise post-treatment (t=30'). N = 5.

In order to more specifically disrupt the population of actin at the spindle, I utilised mutants of Capu and Spire, the former of which is the *Drosophila* homologue of the mammalian Formin-2 which was shown to be essential for formation of the mouse spindle-like actin (Mogessie and Schuh, 2017). Spire also has homologous proteins in mammals and *Drosophila*, such as mammalian Spir-1 (Quinlan et al. 2007). I firstly tested the effect of a single Cappuccino mutant on the microtubule spindle; *cappuccino*^{EY12344} is a hypomorphic mutation generated by p element insertion of the P{EPgy2} construct (Bellen et al., 2004). Just a single copy mutation was sufficient to cause a morphological change in the microtubule spindle as labelled by Jupiter-GFP. Much like the incubation with cytochalasin-D, a single Capu mutant caused a significant increase in the length of the microtubule spindle, significantly increased from an average of approximately 13uM to 18uM (Figure 5.12 A-D).

I attempted to more completely knock out the Capu protein through generating homozygous *cappuccino*^{EY12344} flies, however the flies were unhealthy and stage 14

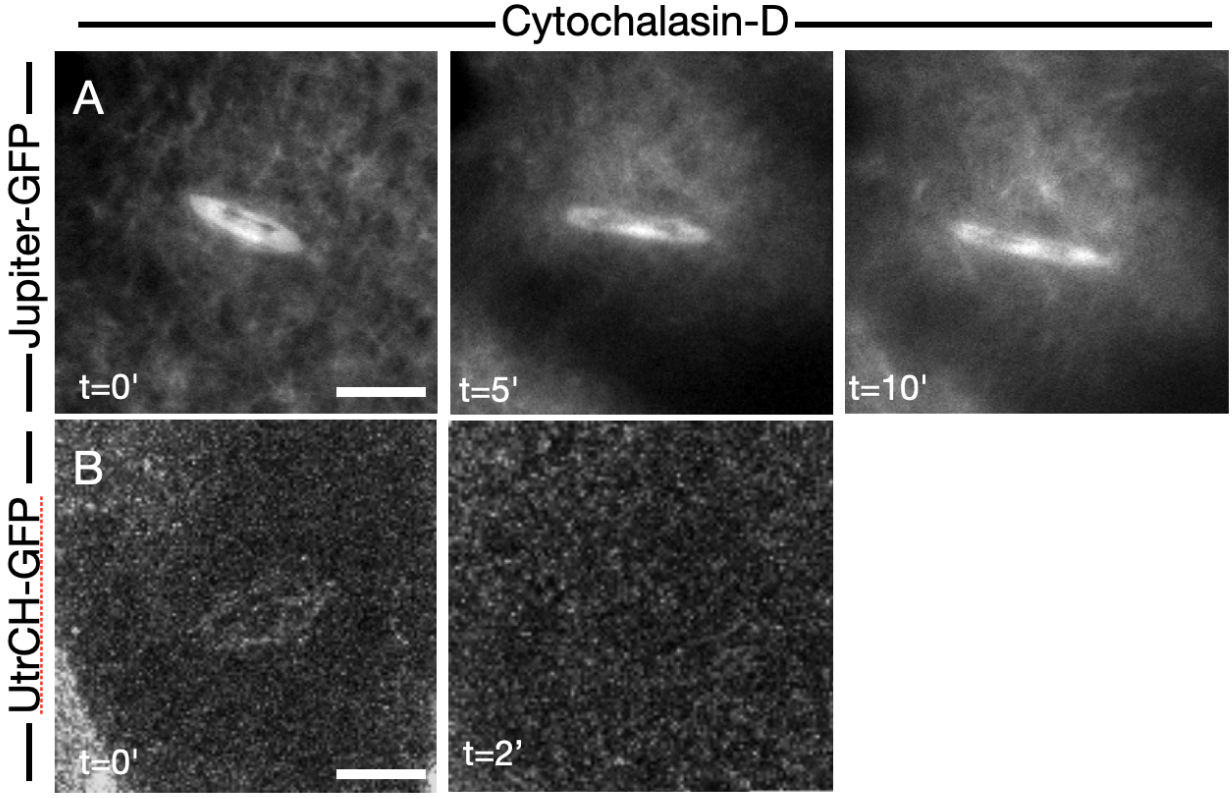

Figure 5.11: Cytochalasin-D Incubation results in elongation of the microtubule spindle at egg activation. (A) Confocal Z-projections (10 µm) from a live time series of a MI oocyte treated with cytochalasin-D (t=0'). Microtubules (Jupiter-GFP) first appear as a typical spindle structure and undergo a morphological rearrangement as the spindle elongates (t=10'). N = 5. **(B)** Confocal Z-projections (10 µm) from a live time series of a MI oocyte treated with Cytochalasin-D (t=0'). Actin (UtrCH-GFP) first appear as a spindle structure and depolymerises rapidly post-treatment (t=2'). N = 5.

oocytes were not present in the ovaries (Data not shown). Therefore, I instead generated trans-heterozygous *Drosophila* with *cappuccino*^{EY12344} and *spire*^{2F}, a method adopted in the literature (Dreschler et al., 2017; 2020), that effectively eliminates enough of the Capu/Spire complex to cause a significant impact on the capacity of the complex to nucleate actin. Generation of these trans-heterozygous *Drosophila* had a very similar impact on in the microtubule cytoskeleton, causing a significant elongation of the spindle, with the average spindle length being approximately 19um and the maximum spindle length more than double the average of wild-type oocytes (Figure 15.2 C,D). There was, however, no signifiant difference between homozygous and trans-heterozygous oocytes.

I further examined the effect of trans-heterozygous knock out of Cappuccino through the use of two different Cappuccino mutants: *cappuccino*^{EY12344} as previously described and *cappuccino*$^{EE.}$ The most obvious phenotype that manifests in this trans-heterozygous background is the complete loss of the microtubule spindle (Figure 5.13), suggesting a more complete knock out of the Cappuccino protein itself and indicating the importance of this actin nucleator in regulating the

formation of the microtubule spindle itself. Also of note is an increased number of disrupted spindles, which here refers to spindles with significant elongation as demonstrated in previous figures.

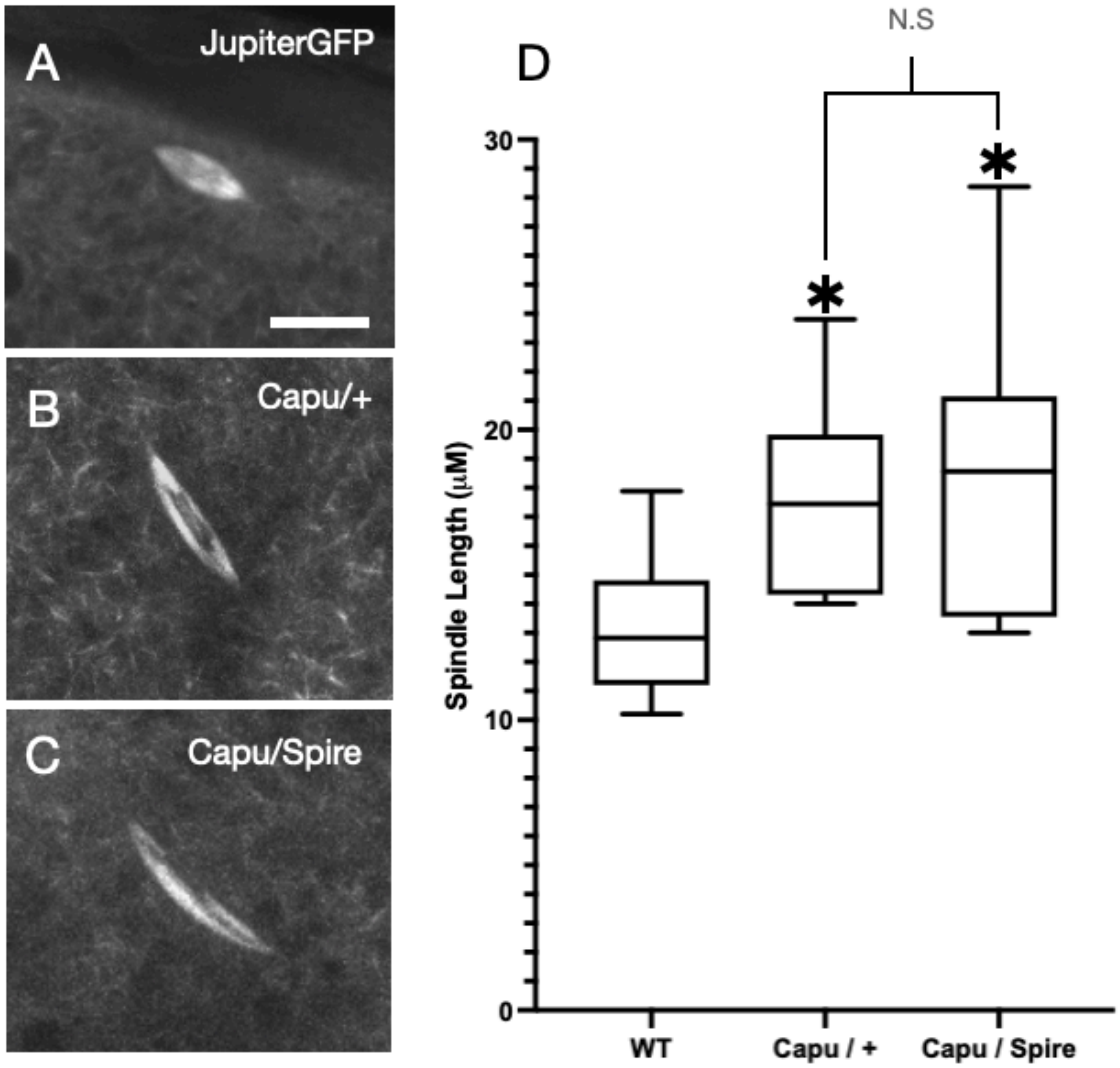

Figure 5.12: Cappuccino and Spire are required to regulate the morphology of the microtubule spindle (A) Confocal Z-projections (10 μm) from a live metaphase-I oocyte. Microtubules (Jupiter-GFP) appear as a typical spindle structure. **(B)** Confocal Z-projection (10 μm) from a live *capu*^{EY12344} heterozygous mutant MI oocyte. Microtubules (Jupiter-GFP) appear as an elongated spindle. N = 20. **(C)** Confocal Z-projection (10 μm) from a live *capu*^{EY12344}/*spire*^{2F} trans-heterozygous mutant MI oocyte. Microtubules (Jupiter-GFP) appear as an elongated spindle. N = 20. **(D)** Comparison of the microtubule spindle length indicates a significant increase in mutant background compared to wild-type. $P < 0.05$, N = 25. Scale bar: 10 μm.

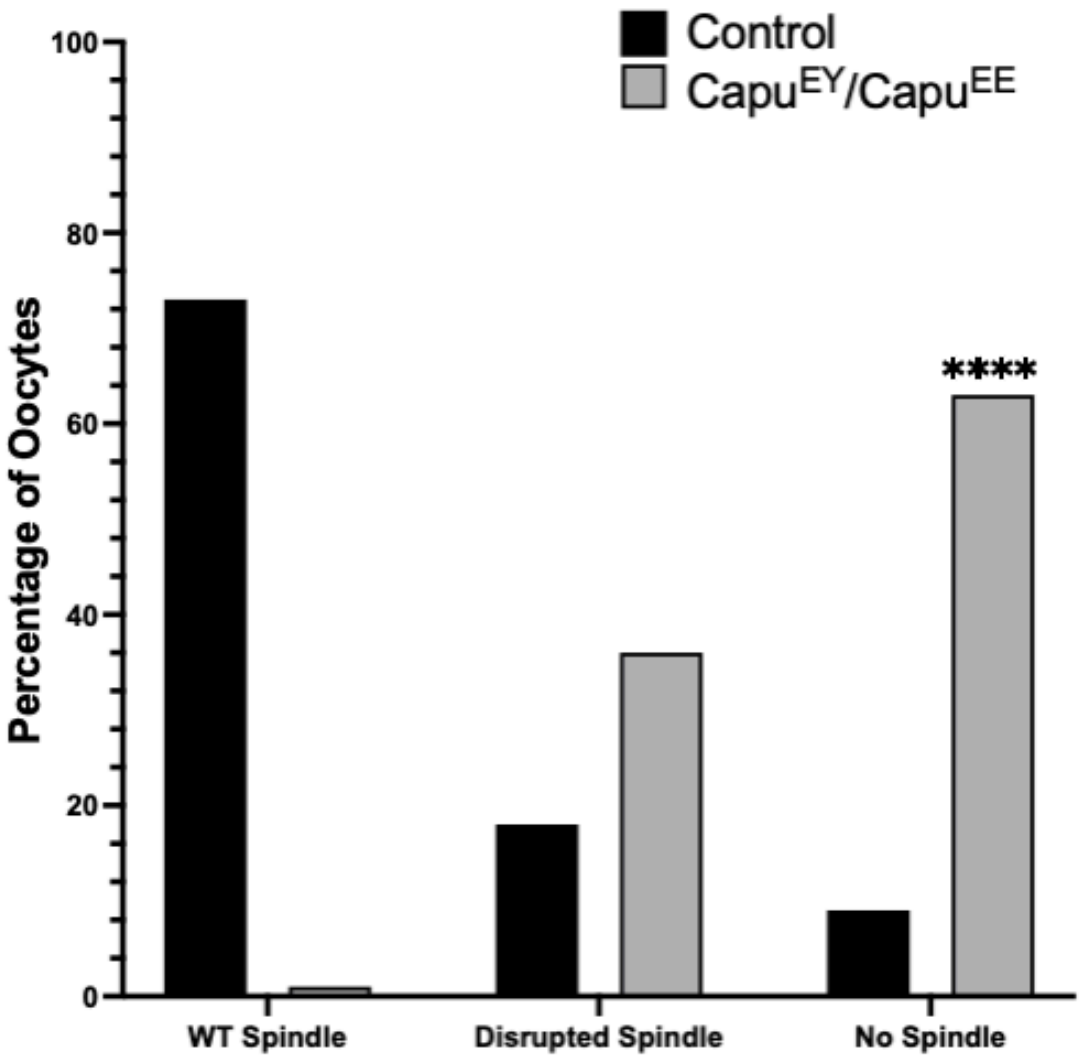

Figure 5.13: Trans-heterozygous cappuccino knockdown results in loss of the microtubule spindle. Comparison of the number of oocytes displaying a microtubule spindle structure in wild type vs trans-heterozygous c*appuccino*^{EY}/*cappuccino*^{EE}. There is a significant increase in the number of oocytes without a spindle in trans-heterozygous mutants. There are a significant number of disrupted spindle structures, generally falling under the category of 'elongated spindles'. $P < 0.001$, Fisher's Exact Test. N = 20 per genotype.

Having established that actin at the spindle may exist independently of the microtubules and that it likely acts upstream, regulating the morphology of the oocyte, I tested whether the actin cytoskeleton may be required for formation of the microtubule spindle. I first examined the dynamics of the population of microtubules at the spindle through fluorescence recovery after photobleaching analysis (FRAP). In control oocytes, expressing Jupiter-GFP in an otherwise wild-type genetic background, laser photobleaching of the entire spindle demonstrated a steady recovery of fluorescence signal post-bleach, plateauing at 85% of the original fluorescent signal (Figure 5.14). However, in a *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous genetic background,

post-bleach recovery of the Jupiter-GFP plateaus at a significantly reduced level of 71% (Figure 5.14). There is also a significantly reduced half-life in the case of the trans-heterozygous background, from 48 seconds in the control versus 28 seconds in the mutant oocytes (Figure 5.14). The likely explanation for this is that the actin cytoskeleton is required to recruit microtubules to the spindle, aiding formation, and without there is reduced recovery dynamics as less actin is able to promote turnover or formation of the spindle. However, due to technical challenges, FRAP experiments of the spindle only produced low N numbers, and as such such further repeats would be necessary for conclusiveness.

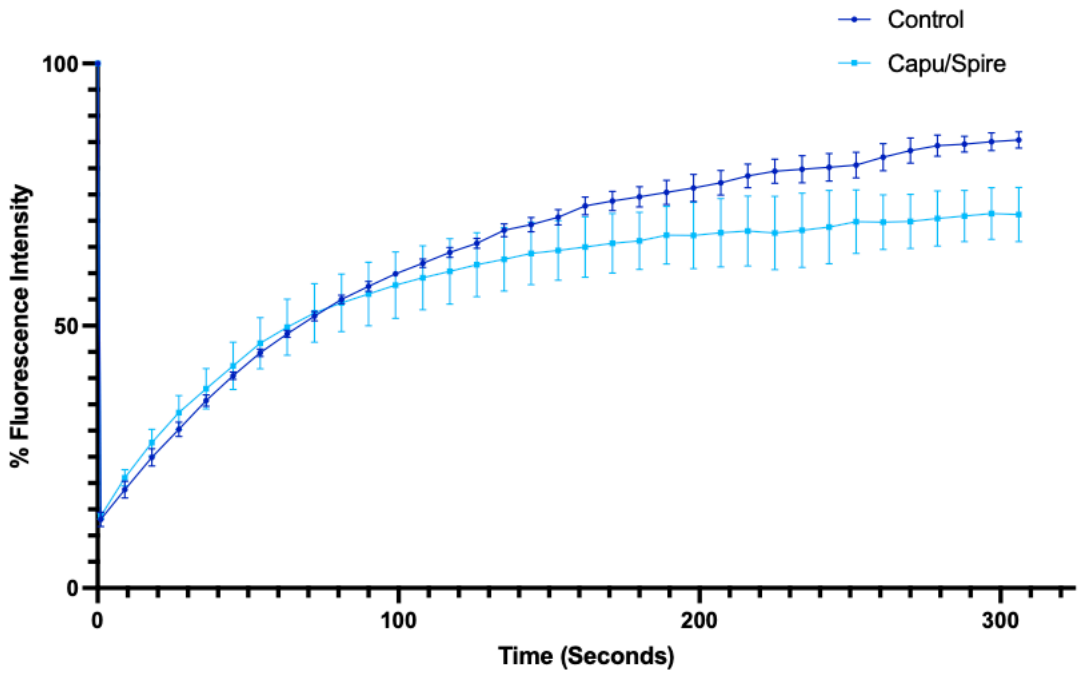

Figure 5.14: Cappuccino and Spire aid recovery of fluorescence of the microtubule spindle following photobleaching. Recovery of fluorescence intensity following photobleaching of microtubules in wild-type and *capu*^{EY12344}/*spire*^{2F} trans-heterozygous mutant backgrounds. Mutant oocytes initially show similar recovery dynamics to wild-type oocytes, but overall recovers to a lesser degree. N=5 control and N=5 *capu*^{EY12344}/*spire*^{2F}.

5.2.4 The actin cytoskeleton is required to regulate the alignment of the metaphase chromosomes and spindle

It is clear that the actin cytoskeleton is required for accurate formation of the microtubule spindle, as without it the spindle is unable to form or indeed forms with a disrupted morphology. This is consistent with results in both mice and humans, in which the actin cytoskeleton was required for formation of the K-fibres (Mogessie and Schuh, 2017) and for formation of the spindle more generally (Roeles and Tsiavialaris, 2019). In mice, this was the likely mechanism behind actin-mediated regulation of chromosome alignment and segregation during metaphase and anaphase respectively. Without the actin cytoskeleton, the K-fibres were unable to properly form, and therefore in the absence of these critical microtubules, chromosomes would not align efficiently on the metaphase plate or indeed would lag behind during segregation in anaphase (Mogessie and Schuh, 2017).

To assess the role the actin cytoskeleton may be having on chromosomal movements during meiosis, I fixed Lifeact-GFP expressing mature oocytes with DAPI to label the chromosomes. Prior to fixation, I incubated in either Schneider's Insect Medium alone, as a control medium, or Schneider's containing 10uM Cytochalasin-D. In the control, Lifeact-GFP was visible both as an enriched spindle-structure, and throughout the oocyte as a cytoplasmic mesh and enriched cortically (Figure 5.15 A). The chromosomes were also visible as the stereotypical congressed mass with the 4th non-exchange chromosomes visible at opposite ends of the mass (Figure 5.15 A). Following incubation with Cytochalasin-D, there is a clear disruption in both the actin cytoskeletal architecture and the metaphase congressed chromosomes. The Lifeact signal is still visible in the surrounding follicle cells, however in the chromosome, the cortical and cytoplasmic actin appears significantly reduced, including the spindle-like actin (Figure 15B).

Importantly, there is a dramatic change in the metaphase chromosomes as the usually congressed mass is separated and elongated, with multiple individual masses observable. The overall maximum length (measured as the maximum chromosomal distance, often the distance between the 4th chromosomes) of the chromosomes increases significantly from an average of approximately 5.3 μm to 9.1 μm.

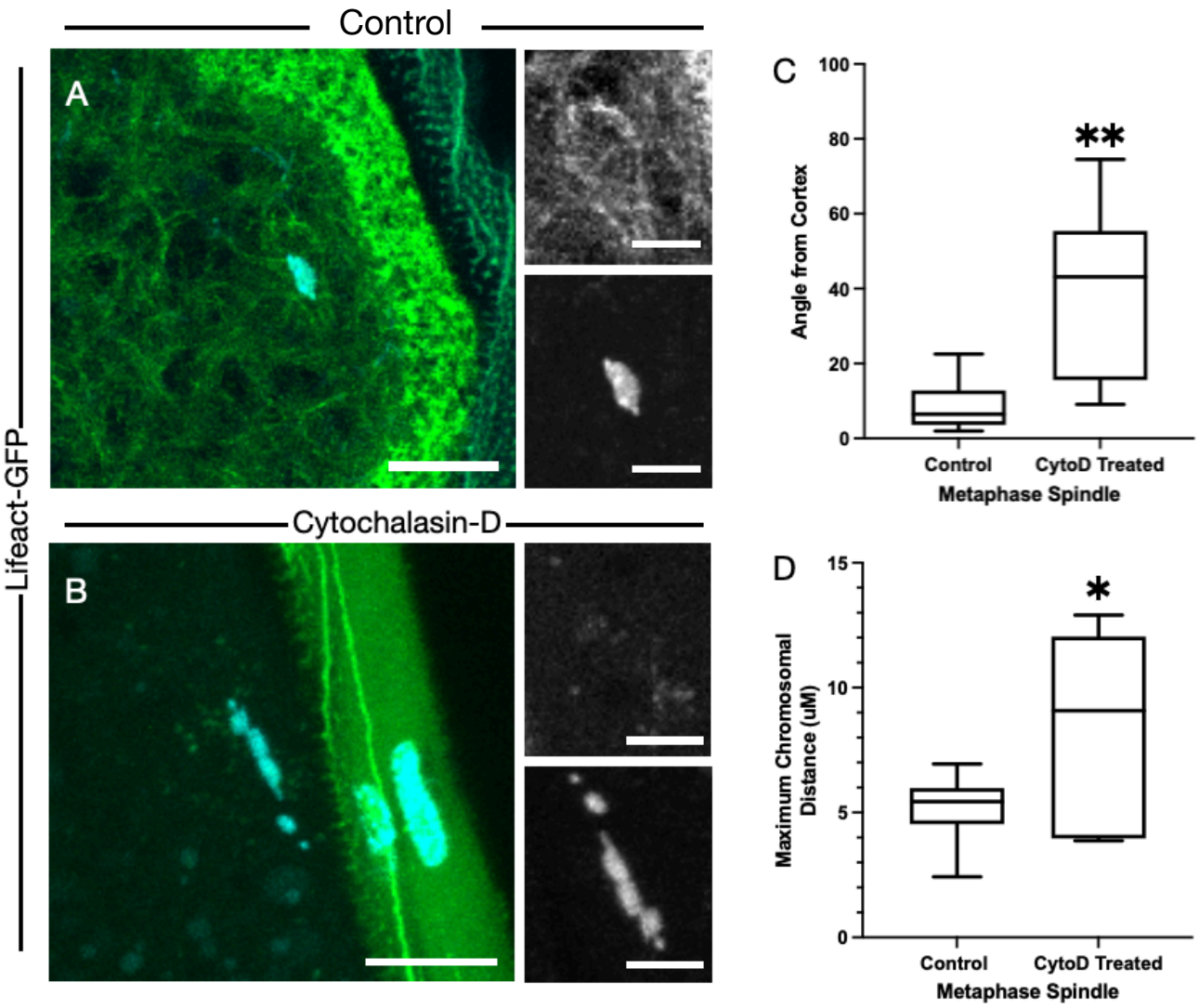

Figure 5.15: Cytochalasin-D incubation results in misalignment of the metaphase chromosomes. (A) Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows actin (Lifeact-GFP) in green, DNA (DAPI) in cyan, demonstrating a spindle-like population of actin surrounding a central chromosomal mass. Dashed-line box marks region that is magnified in single-colour images to right. **(B)** Confocal Z-projection (10 µm) of a fixed MI oocyte following incubation with the 10 µM of the actin depolymerising agent cytochalasin-D. Merge shows actin (Lifeact-GFP) in green, DNA (DAPI) in cyan, demonstrating a loss of the spindle-like population of actin and a drastic separation of the chromosomal mass into several units that elongate along the spindle axis. Cytoplasmic actin appears significantly reduced. **(C)** Comparison of the spindle-cortex angle indicates a significant increase in cytochalasin-D treated background compared to wild-type. $P<0.01$, Student's T-Test. $N = 8$. **(D)** Comparison of the maximum chromosomal distance indicates a significant increase in cytochalasin-D treated background compared to wild-type. $P< 0.05$, Student's T-Test. $N = 8$. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

Interestingly, it can also be observed that the angle made by the spindle axis and the cortex changes upon incubation with Cytochalasin-D. Usually, at metaphase, the spindle lies almost parallel to the cortex and during egg activation (anaphase), the spindle begins to reorient until it is perpendicular to the cortex (Endow and Komma, 1997). Following incubation with cytochalasin-D, there is a significant increase in the angle created by the spindle axis and the cortex (Figure 5.15 C). There is a great level of variation in this angle, perhaps suggesting that varying levels of actin depolymerisation caused by the cytochalasin-D results in a variety of angles; residual actin may be able to maintain the current spindle-cortex angle more efficiently.

Depolymerising the actin cytoskeleton utilising a broad actin-inhibitor such as cytochalasin-D evidently has a a dramatic effect of the state of the metaphase spindle. I therefore investigated whether specific knock out of Cappuccino and Spire in the mature oocyte would also result in defects in the metaphase spindle, likely through loss of the spindle-actin population. I utilised *cappuccino*^{EY12344} heterozygous and *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous mutants in Jupiter-GFP expressing backgrounds, fixing and staining them with DAPI in order to visualise both the microtubules and the chromosomes. Notably, the chromosomes appear to have undergone a far less dramatic morphological change when compared to cytochalasin-D, as the majority of oocytes still display a congressed mass of chromosomes (Figure 5.16 A). However, it is clear that this mass is no longer as condensed as the usual metaphase chromosomes, with distinct units beginning to separate to the poles of the spindle (Figure 5.16 A). This can be more accurately quantified through measurements of the maximum chromosomal distance, which shows a significant increase in the *cappuccino*^{EY12344} heterozygous background. This would suggest that Capu is required for maintaining the metaphase chromosomes at the equatorial plate of the spindle, and without the chromosomes are unable to remain as a centrally congressed mass. The likely difference between the phenotype observed in this genetic background compared to cytochalasin-D treated oocytes is likely due to more complete disruption/depolymerisation of the spindle-actin pharmacologically. With *cappuccino*^{EY12344} heterozygous lines, there is still clearly protein present able to regulate the chromosomal conformation during metaphase, yet even so this highlights the sensitivity of the system to smaller changes in the actin cytoskeleton.

Fixing and staining of *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous mutants in Jupiter-GFP expressing backgrounds demonstrated a very similar chromosomal phenotype. Again, the Jupiter-GFP labelled the spindle that encapsulates the DAPI stained chromosomes (Figure 5.16 B). It is evident, however, that the chromosomes are also undergoing a separation or drift toward the poles, with a single chromosomal mass no longer observed, but in fact what appears to be the singular 4th chromosome, and a mass representing the other 3 chromosomes drifting toward both poles of the spindle (Fig 5.14 B). Calculating the maximum chromosomal distance indicated a significant increase compared to control oocytes, much more significant in fact than both the cytochalasin-D treated and heterozygous oocytes (Figure 5.16 D). There was, however, no

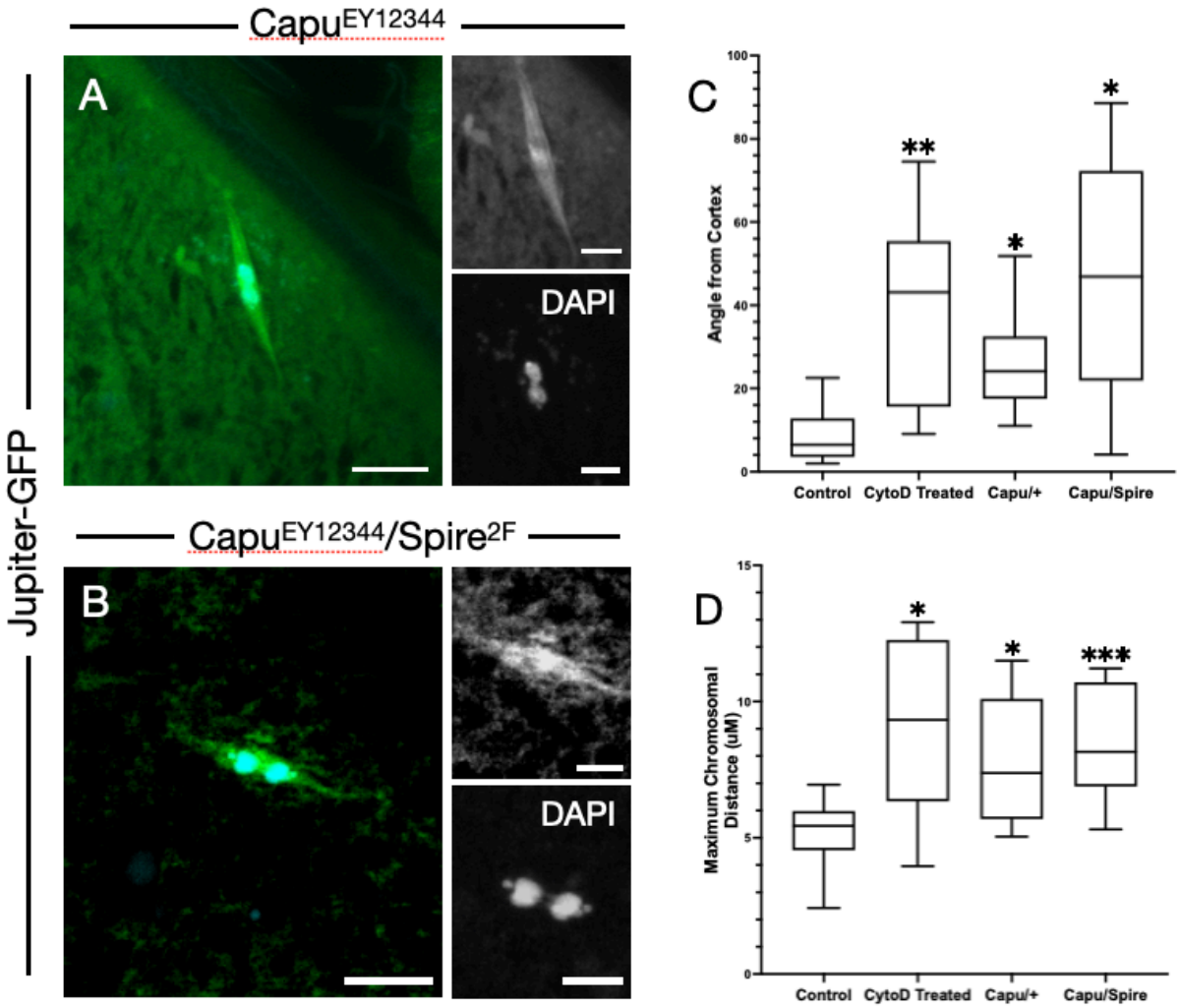

Figure 5.16: Cappuccino and Spire are required for the correct alignment of the metaphase chromosomes (A) Confocal Z-projection (10 µm) of a fixed *capu*^{EY12344} heterozygous mutant MI oocyte. Merge shows actin (Jupiter-GFP) in green, DNA (DAPI) in cyan, demonstrating a separation of the chromosomal mass into two units that begin to migrate along the spindle axis. **(B)** Confocal Z-projection (10 µm) of a fixed *capu*^{EY12344}/*spire*^{2F} trans-heterozygous mutant MI oocyte. Merge shows actin (Jupiter-GFP) in green, DNA (DAPI) in cyan, demonstrating further separation of the chromosomal mass into two distinct units that are migrating to the spindle poles. Image is contrasted to better visualise the spindle. **(C)** Comparison of the spindle-cortex angle indicates a significant increase in cytochalasin-D treated and mutant backgrounds compared to wild-type. P<0.05(*), P(**)<0.01, Student's T-Test. N = 8 per treatment group. **(D)** Comparison of the maximum chromosomal distance indicates a significant increase in cytochalasin-D treated and mutant backgrounds compared to wild-type. P(*)< 0.05, P(***)<0.005, Student's T-Test. N = 8 per treatment group. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

significant difference between cytochalasin-D treated, *cappuccino*^{EY12344} heterozygous and *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous mutant oocytes.

Additionally, measuring the angle created by the spindle axis and the cortex in *cappuccino*^{EY12344} heterozygous and *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous mutant oocytes indicated a significant increase in this angle as compared to the metaphase control (Figure 5.16 C). Again, there was no significant difference between the genetic backgrounds, despite a much greater variation (and indeed much higher values) being observed in *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous oocytes. Comparison of this angle to that caused by cytochalasin-D treatment also demonstrated no significant difference despite cytochalasin-D treatment being more significant in comparison to the control (Figure 5.16 C). This would suggest that Cappuccino and Spire are required to generate a population of actin that is important in maintaining the spindle alignment with the cortex at metaphase, and the disruption of this actin causes premature rotation.

5.2.5 The actin cytoskeleton is required to regulate the segregation of the anaphase chromosomes and alignment of the spindle

Having established that the actin cytoskeleton is required for the correct alignment of the chromosomes on the metaphase plate, with Capu and Spire playing an important role, I sought to examine whether the actin cytoskeleton was further required for the anaphase spindle.

I expressed Lifeact-GFP in oocytes to observe the actin population simultaneously with the chromosomes, stained with DAPI. In control oocytes, I simply dissected and incubated in AB for 10 minutes to ensure the vast majority of oocytes had been activated, then fixed and stained them with DAPI. In the control oocytes, the separating anaphase chromosomes can be observed, and they are sat in the midst of the anaphase spindle, which here is observed through labelling of the actin cytoskeleton (Figure 5.17 A). The chromosomes can be seen to be adopting a typical anaphase separation as they are pulled to opposite poles of the spindle, and surrounding these chromosomes a spindle-like actin can be observed; a distinct population that appears filamentous, spanning the spindle length (Figure 5.17 A). The cytoplasmic actin also appears to be heavily filamentous, and the cortical actin appears somewhat dispersed, both hallmarks of typical *Drosophila* egg activation (Figure 5.17 A).

In test oocytes, I dissected into AB containing 10 μM of cytochalasin-D. My previous work (Figure 5.11) shows that cytochalasin-D is able to disrupt the spindle-actin within 2 minutes of addition, therefore, adding the AB and cytochalasin-D simultaneously should more acutely affect the spindle at anaphase-I, ensuring any phenotypes observed are not due to a disrupted actin cytoskeleton during metaphase-I. Fixation of oocytes expressing Lifeact-GFP following a 10 minute incubation with cytochalasin-D revealed a change in the actin architecture, as the cytoplasmic actin appears less filamentous, and importantly there is a loss of the spindle-like actin as the fluorescent signal in the area surrounding the chromosomes is mostly abolished (Figure 5.17 B). Furthermore, staining with DAPI revealed a striking difference in chromosomal phenotypes; it was often observed that oocytes displayed aberrant chromosomal masses, defined as masses that were separate from the main body of dividing chromosomes (Figure 5.17 B-C). In each oocyte observed, the number of aberrant chromosomal masses was counted, and comparison of the average number between control and cytochalasin-D treated oocytes revealed a significant increase from approximately 0.25 to 1.3 aberrant chromosomal masses per oocyte (Figure 5.17 E). Comparison, however, of the maximum chromosomal distance did not reveal a significant difference between control and cytochalasin-D treated oocytes, suggesting that the spindle machinery may still be able to coordinate the segregation of the chromosomes to the poles, however this process is more prone to error (Figure 5.17 F).

Assessment of the angle of the spindle with relation to the cortex revealed that there was no significant difference between cytochalasin-D treated anaphase oocytes and control anaphase

oocytes (Figure 5.17 D). Importantly, there was also no significant difference between the anaphase spindle angle and the metaphase spindle angle following treatment with cytochalasin-D, both of which were significantly greater than metaphase control oocytes (Figure 5.17 D). This suggests that the actin cytoskeleton is able to regulate the angle of the spindle with the cortex, and disruption of this actin results in premature rotation of the spindle to an anaphase conformation. Following anaphase, there is less of a requirement of the actin spindle to maintain this spindle conformation.

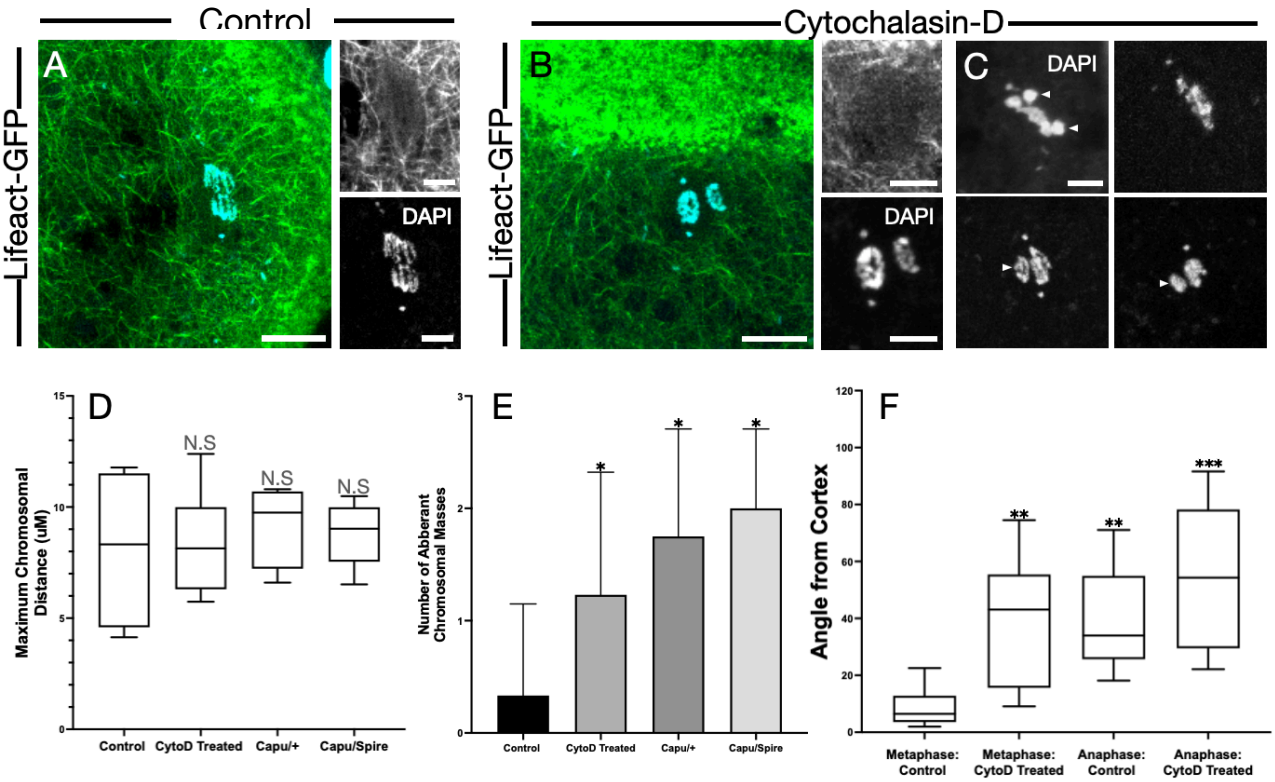

Figure 5.17: Cytochalasin-D Incubation results in chromosomal segregation errors during anaphase. (A) Confocal Z-projection (10 µm) of a fixed Anaphase-1 (AI) oocyte. Merge shows actin (Lifeact-GFP) in green, DNA (DAPI) in cyan, demonstrating a filamentous spindle-like population of actin surrounding the segregating anaphase chromosomes. N = 15. **(B)** Confocal Z-projection (10 µm) of a fixed AI oocyte following incubation with the 10 µM of the actin depolymerising agent cytochalasin-D. Merge shows actin (Lifeact-GFP) in green, DNA (DAPI) in cyan, demonstrating the loss of a spindle-like actin population and the disruption of the accurate segregation of the chromosomes. **(C)** Confocal Z-projections (10 µm) of fixed AI oocytes following incubation with the 10 µM of the actin depolymerising agent cytochalasin-D. Chromosomes (DAPI) in greyscale demonstrating further examples of the variety of disrupted chromosomal segregation phenotypes, many showing chromosomal units separated from the main mass (white arrowheads). **(D)** Comparison of the maximum chromosomal distance shows no significant difference between cytochalasin-D treated and mutant backgrounds. N = 8 per treatment group. **(E)** Comparison of the number of aberrant chromosomal masses shows a significant increase in cytochalasin-D treated and mutant backgrounds compared to the wild-type. P(*)< 0.05, Student's T Test. N = 8 per treatment group. **(F)** Comparison of the angle between the spindle and cortex shows a significant increase in cytochalasin-D treated and mutant backgrounds compared to the wild-type. There is no significant differences between cytochalasin-D and mutant oocytes. P(**)< 0.01, P(***)< 0.005, Student's T Test. N = 8 per treatment group. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

Having acutely targeted (temporally) the anaphase spindle using cytochalasin-D, I sought to target the spindle more specifically (spatially) using, again, mutants of the actin nucleators Cappuccino and Spire. Oocytes expressing Jupiter-GFP in a *cappuccino*^{EY12344} heterozygous background were dissected and incubated in AB for 10 minutes prior to fixation and staining with DAPI. Visualisation of the anaphase spindle in these oocytes revealed once more a disrupted anaphase chromosomal structure, as individual (aberrant) masses could be identified completely separate from the main body of chromosomes (Figure 5.18 A). In particular, it appears that the smallest 4th chromosomes are completely misaligned, as they usually represent each pole of the spindle, but in some cases were observed to be on the same side as the opposite 4th chromosome (Figure 18A). Significantly, the number of individual chromosomal masses once more increased significantly from approximately 0.25 masses in the control to 1.7 masses in the *cappuccino*^{EY12344} heterozygous oocytes (Figure 5.18 B-D). Again, there is no significant difference in the average maximum chromosomal distance between anaphase controls and mutants, indicating anaphase may still be occurring and chromosomes are being pulled to opposite poles (Figure 5.18 C).

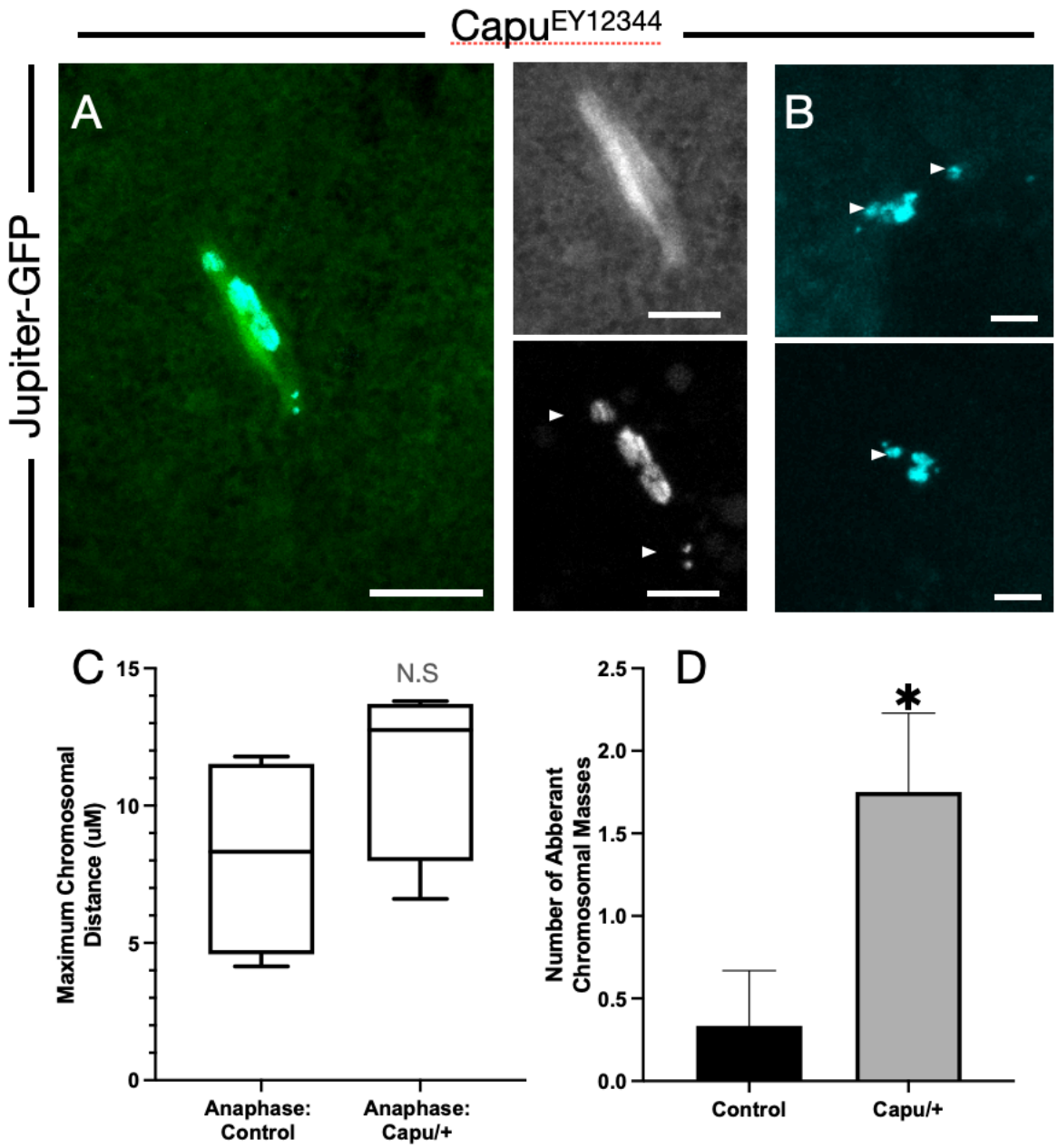

Figure 5.18: Cappuccino is required for effective chromosome segregation at anaphase. **(A)** Confocal Z-projection (10 µm) of a fixed *capu*^{EY12344} heterozygous mutant AI oocyte. Merge shows microtubules (Jupiter-GFP) in green, DNA (DAPI) in cyan, demonstrating the presence of the spindle but mis-regulation of chromosomal segregation. N = 12. **(B)** Confocal Z-projections (10 µm) of fixed *capu*^{EY12344} heterozygous mutant AI oocytes. Chromosomes (DAPI) in cyan demonstrating further examples of the variety of disrupted chromosomal segregation phenotypes, many showing chromosomal units separated from the main mass (white arrowheads). **(C)** Comparison of the maximum chromosomal distance shows no significant difference between control and mutant backgrounds. N = 20 control, N = 12 *capu*^{EY12344}/+. **(D)** Comparison of the number of aberrant chromosomal masses shows a significant increase in the heterozygous mutant background compared to the wild-type. P(*)< 0.05, Student's T Test. N = 20 control, N = 12 *capu*^{EY12344}/+. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

Furthermore, imaging *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous oocytes expressing Jupiter-GFP following activation and fixation with DAPI revealed similar disruption to the anaphase chromosomes. Aberrant chromosomal masses can be observed separate from the main chromosomal mass, and the 4th non-exchange chromosomes often appear no-longer localised to the poles of the spindle (Figure 5.19 A-B). There is once more a significant increase in the number of aberrant chromosomal masses to a value of approximately 1.95 in *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous oocytes (Figure 5.19 D). There is no significant difference between single and double mutants however (Figure 5.19 D). Like single mutants, there is no significant change in the maximum chromosomal distance, once more indicating that anaphase may still be occurring but with much less control over accurate chromosomal segregation (Figure 5.19 C). The chromosomes, though clearly disrupted, still appear to exist within the dimensions of the microtubule spindles.

Overall, there is variation in the chromosomal phenotypes observed in cytochalasin-D treated and *cappuccino*^{EY12344}/+ heterozygous and *cappuccino*^{EY12344}/*spire*^{2F} trans-heterozygous mutant oocytes. There may be differences in the mutant lines versus the cytochalasin-D treated oocytes due to more acute manipulation of the anaphase spindle using pharmacological treatment, in comparison to mutant lines in which metaphase-I is also disrupted prior to anaphase-I.

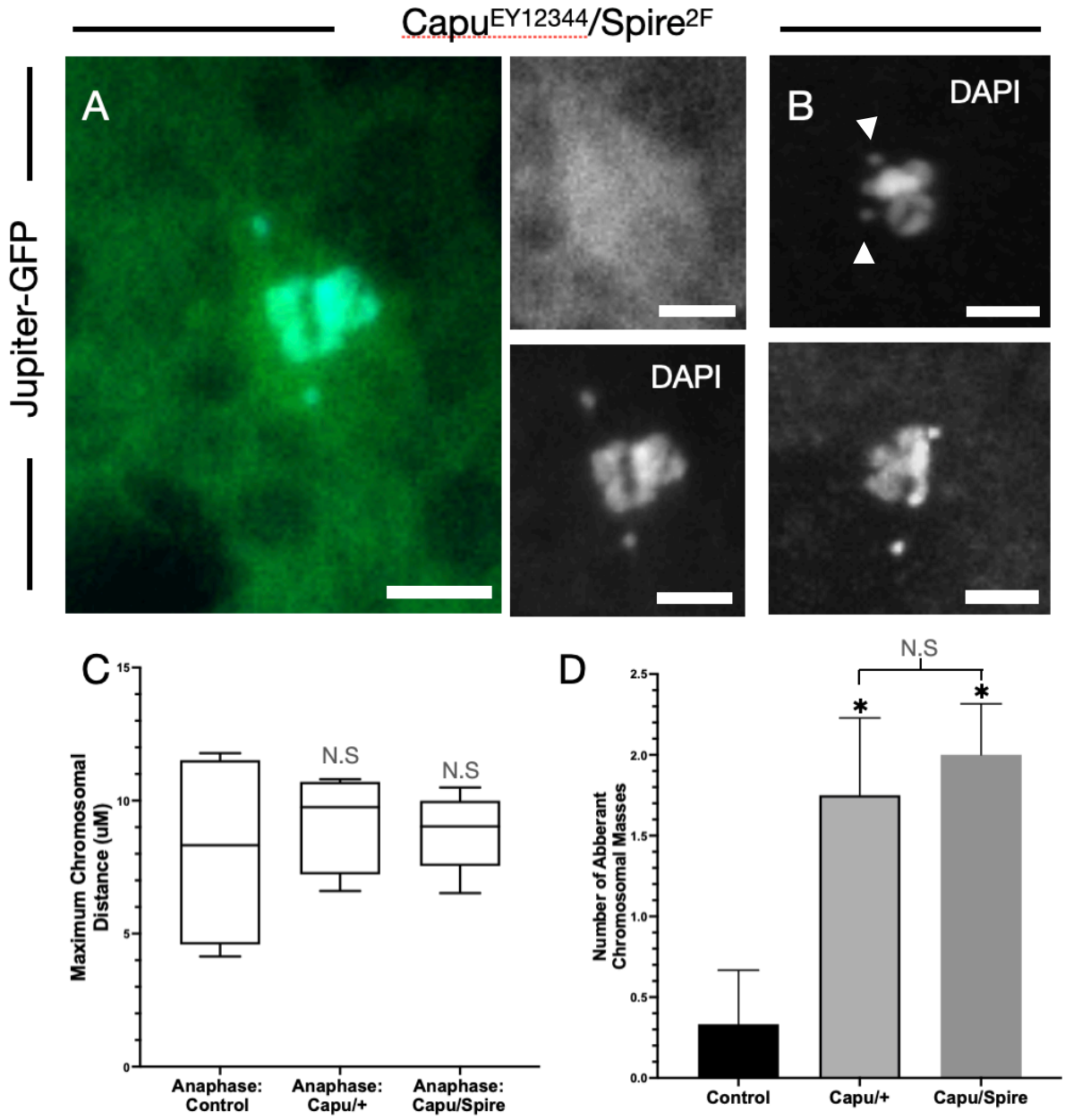

Figure 5.19: Cappuccino and Spire are required for effective chromosome segregation at anaphase. (A) Confocal Z-projection (10 µm) of a fixed *capu*^{EY12344}/*spire*^{2F} trans-heterozygous mutant AI oocyte. Merge shows microtubules (Jupiter-GFP) in green, DNA (DAPI) in cyan, demonstrating a reduced microtubule signal and mis-regulation of chromosomal segregation. N = 8. **(B)** Confocal Z-projections (10 µm) of fixed *capu*^{EY12344}/*spire*^{2F} trans-heterozygous mutant AI oocytes. Chromosomes (DAPI) in cyan demonstrating further examples of the variety of disrupted chromosomal segregation phenotypes, often showing a complete loss of polarity as the 4th non-exchange chromosomes no longer migrate to poles (white arrowheads). **(C)** Comparison of the maximum chromosomal distance shows no significant difference between mutant backgrounds or mutants and controls. N = 8 per genotype. **(D)** Comparison of the number of aberrant chromosomal masses shows a significant increase in mutant backgrounds compared to the wild-type. P(*)< 0.05, Student's T Test. N = 8 per genotype. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

5.2.6 Localised calcium signalling may be required at the spindle for effective meiosis

In my previous chapters I explore the relationship between calcium and the actin cytoskeleton, providing further data to suggest a deep interdependence between the two at egg activation. In my second chapter, data that is now published, reveals the requirement of actin prior to calcium entry; actin appears to control calcium entry, with a reduction in cortical actin at the poles of the oocyte priming these areas for a calcium wave (York-Andersen et al., 2020). Furthermore, manipulation of the cortical actin has a direct impact on the wave phenotype. Following the calcium wave, there is a corresponding wave of reorganising actin (York-Andersen et al., 2020). I have shown the impact on manipulating a variety of actin binding proteins (ABPs) on the calcium wave, suggesting that increased cross-linking of the cortical actin prevents calcium entry, and implicating various calcium sensitive ABPs such α-actinin, pointing toward a mechanism whereby calcium entry directly impacts the architecture of the cortical actin, enabling further calcium entry.

These interactions between calcium and actin are also highly likely to effect the metaphase spindle, especially given a global calcium transient occurs at egg activation that triggers the metaphase-anaphase transition and the resumption of meiosis (York-Andersen et al, 2015; Kaneuchi et al., 2015). Further to this, recent evidence highlights the presence of enriched calcium and the necessity of localised calcium signalling at the spindle for regulation of microtubules (Li et al., 2016).

I therefore utilised the GCaMP3 calcium sensor to first establish whether there may be enrichment of calcium at the spindle. Enrichment of the calcium sensor could be observed both live (data not shown) and fixed (Figure 5.20), suggesting a higher concentration of calcium is present at the spindle. Fixation and staining with DAPI confirms this calcium enrichment to be at the spindle, surrounding both the metaphase and anaphase chromosomes (Figure 5.20 A,B), Interestingly, there appears to be the greatest enrichment at the tip of each pole of the spindle in the metaphase oocyte, compared to a more uniform signal in the anaphase oocyte. There is clearly a requirement for calcium throughout the metaphase-anaphase transition as this signal remains enriched, if slightly less prominent at anaphase, likely due to a much higher background calcium signal following the calcium wave.

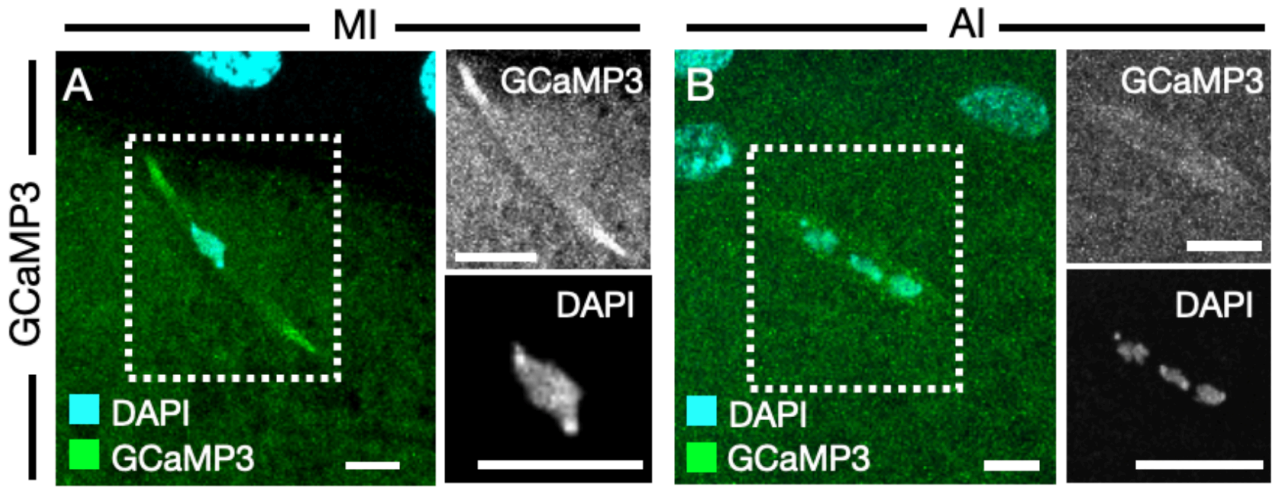

Figure 5.20: GCaMP3 has a greater fluorescent signal at the metaphase and anaphase spindle (A) Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows calcium (GCaMP3) in green, DNA (DAPI) in cyan, demonstrating a higher calcium signal in the restricted area of the spindle. N=15. **(B)** Confocal Z-projection (10 µm) of a fixed AI oocyte. Merge shows calcium (GCaMP3) in green, DNA (DAPI) in cyan, demonstrating a separating chromosomal mass and a wider spindle. N=11. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

In order to further corroborate that this enrichment does in fact represent calcium, and to begin dissecting the potential role of this calcium, I incubated oocytes in the membrane permeable calcium chelating agent BAPTA-AM. I tested the effect of BAPTA on both the population of microtubules and actin within the spindle (Figure 5.21). Incubation effectively depolymerised both populations within 15 minutes, suggesting that calcium signalling is required to maintain the spindle apparatus. This is similar to results in *Xenopus* in which BAPTA incubation caused microtubule depolymerisation in a similar fashion to Nocodazole (Li et al., 2016).

To further explore the role of calcium, I performed BAPTA-AM incubations on oocytes with labelled actin (Lifeact-GFP and Act5C-GFP) and stained with DAPI to visualise the chromosomes. Interestingly, it appears that BAPTA incubation results in the complete compaction of the chromosomes into one rounded mass (Figure 5.22). This phenotype is perhaps explained by the previous results in which BAPTA causes complete loss of the microtubules and actin within the spindle. This would suggest there are no longer any cytoskeletal elements to regulate the morphology of the chromosomes and as such they likely just adopt the most thermodynamically stable conformation.

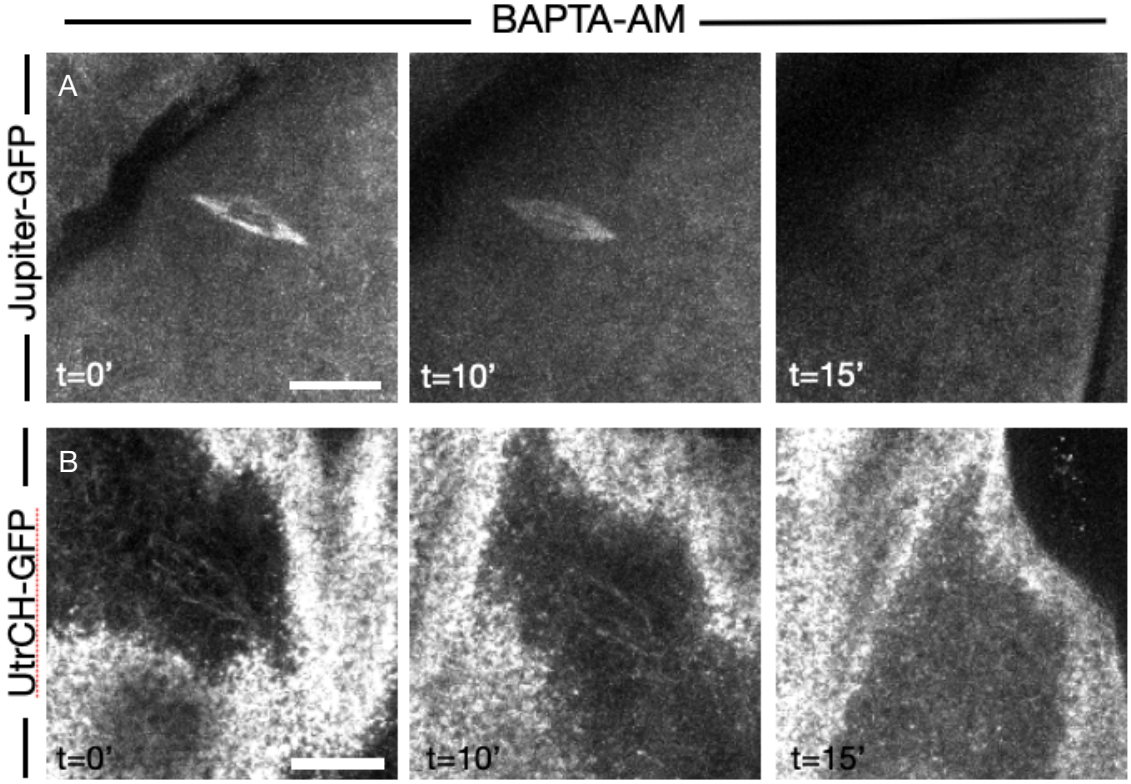

Figure 5.21: BAPTA-AM Incubation results in the depolymerisation of the microtubule and actin spindle. (A) Confocal Z-projections (10 µm) from a live time series of a MI oocyte treated with BAPTA-AM (t=0'). Microtubules (Jupiter-GFP) first appear as a typical spindle structure and completely depolymerise post-treatment (t=15'). N = 8. **(B)** Confocal Z-projections (10 µm) from a live time series of a MI oocyte treated with BAPTA-AM (t=0'). Actin (UtrCH-GFP) first appear as a typical spindle structure and completely depolymerise post-treatment (t=15'). N = 6.

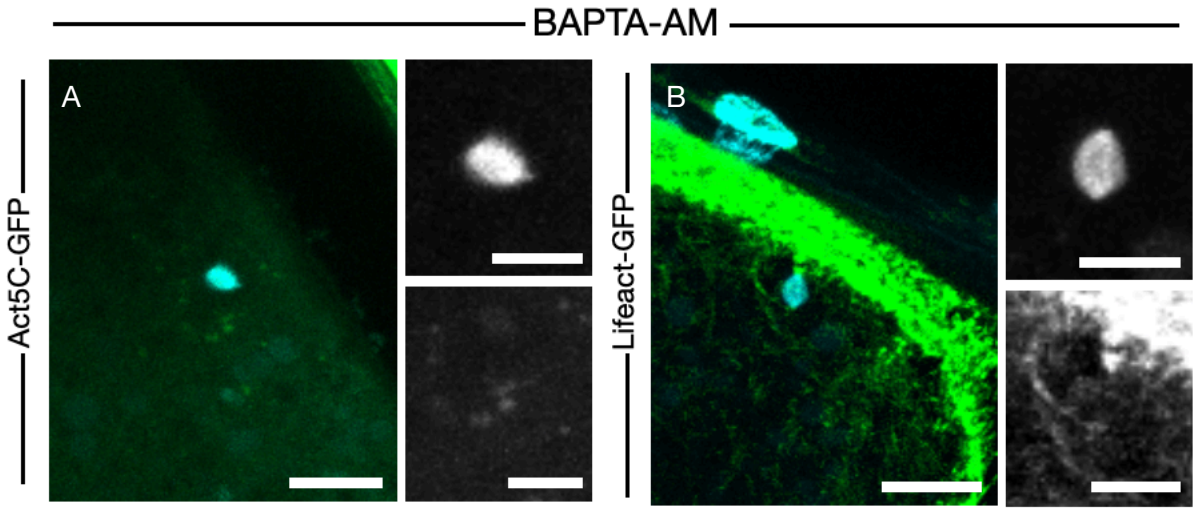

Figure 5.22: BAPTA incubation results in compaction of chromosomes into a round mass (A) Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows actin (Act5C-GFP) in green, DNA (DAPI) in cyan, demonstrating a loss of the actin spindle and round chromosomal mass. N=17. **(B)** Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows actin (Lifeact-GFP) in green, DNA (DAPI) in cyan, demonstrating a loss of the actin spindle and round chromosomal mass. N=15 per genotype. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

Finally, disruption of the spindle-like actin through introduction of the cappuccino^{EY12344} mutant resulted in a loss of enrichment of the calcium at the spindle (Figure 5.23). As expected, the chromosomes become separated into two individual masses that begin to separate toward spindle poles. However, the enrichment of the GCaMP3 can no longer be observed. This suggests that there may be enrichment of calcium in locating signalling domains surrounding the actin cytoskeleton, and loss of this population of actin results in fewer calcium signalling domains being present.

Evidently, actin is required for the enrichment of calcium at the spindle, suggesting there is an interdependent relationship between calcium and actin at the spindle. I have thus opened the door for future investigation of both the spindle-like actin and its relationship with calcium during meiosis.

This calcium data is interesting because it suggests calcium is required locally at the spindle. Complete chelation of this calcium results in depolymerisation of spindle actin and microtubules. What the GCaMP3 enrichment represents is still to be investigated; I believe that it corresponds to local signalling domains within both the microtubule and actin filaments that make up the spindle. This is supported by evidence in which loss of the spindle-like actin results in a corresponding loss of calcium enrichment. It is unlikely that actin would be actively ‘recruiting’ calcium, therefore the local calcium signal likely corresponds to enrichment of calcium in various signalling domains,

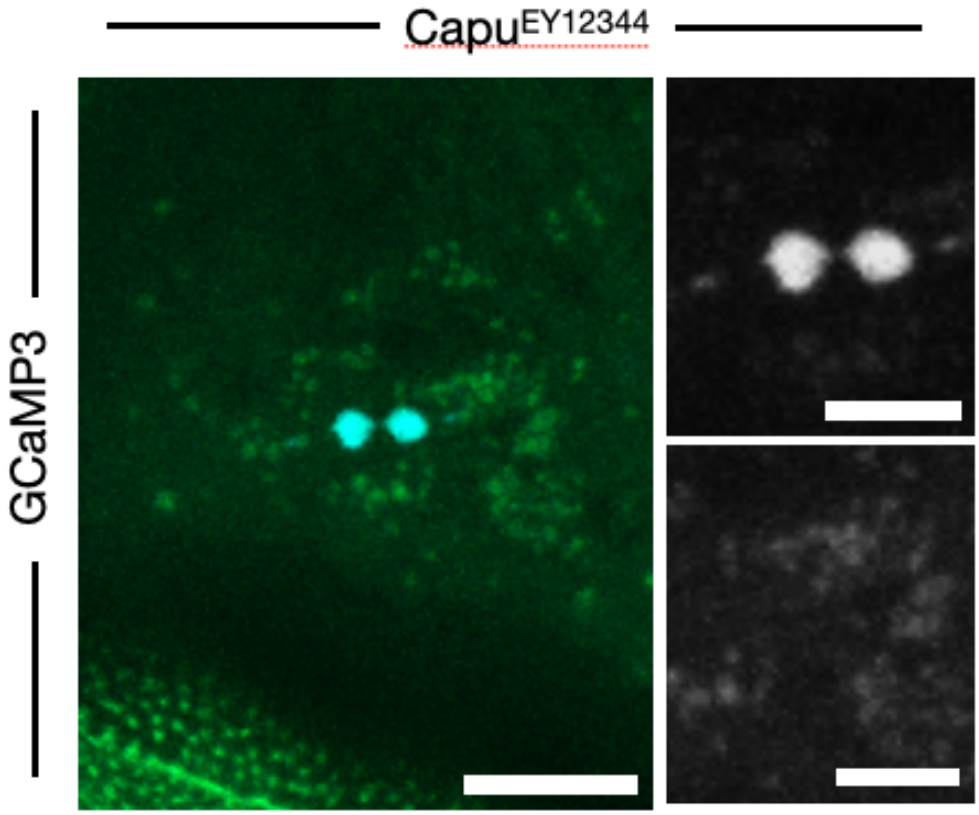

Figure 5.23: Cappuccino mutation results in the loss of the calcium enrichment. Confocal Z-projection (10 µm) of a fixed MI oocyte. Merge shows calcium (GCaMP3) in green, DNA (DAPI) in cyan, demonstrating a loss of the GCaMP3 signal in a spindle conformation and separation of the chromosomes into two separate masses. N=10. Scale bar: 10 µm (A,B), 5 µm (Magnified Panels).

whether directly between actin and microtubules, or between calcium sensitive proteins and their targets, such as Calmodulin.

As such, investigation of the localisation of Calmodulin (CaM) through expression of endogenous GFP tagged CaM reveals a remarkable enrichment at the spindle. Fixation of the CaM::GFP expressing mature oocytes results in a loss of localisation, therefore imaging was carried out live. This demonstrated enrichment of the CaM::GFP in the same region of the spindle, and interestingly, following the addition of AB and triggering the metaphase-anaphase I transition, the CaM remains associated with the spindle as it contracts and widens (Figure 5.24). This suggests that Calmodulin signalling is important throughout the transition, highlighting the need for further investigation of calcium signalling and localised calcium domains. It may be the case that the calcium signal at egg activation is transduced by CaM which then regulates changes in morphology of the actin and microtubule spindle.

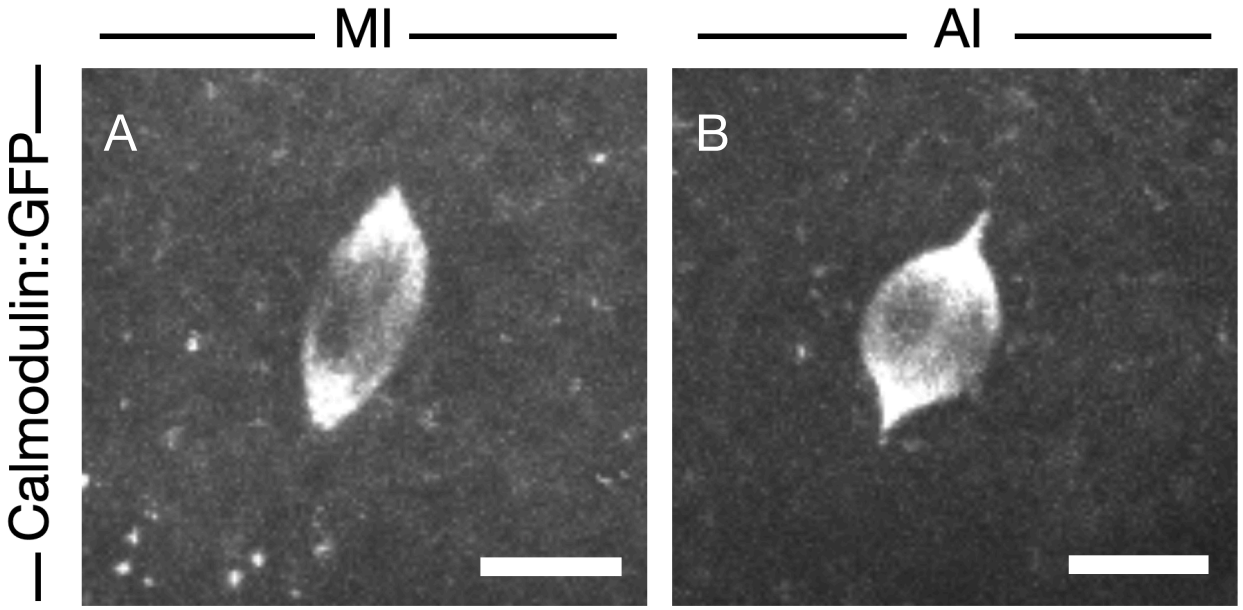

Figure 5.24: Calmodulin is enriched at the spindle throughout the metaphase-anaphase I transition. (A) Confocal Z-projection (10 µm) of a live metaphase I (MI) oocyte showing Calmodulin (Calmodulin::GFP). Calmodulin appears enriched in the MI spindle. **(B)** Confocal Z-projection (10 µm) of a live anaphase I (AI) oocyte (following addition of Activation Buffer (AB) showing Calmodulin (Calmodulin::GFP). Calmodulin appears enriched in the AI spindle, with very focussed poles. N = 10 MI and N = 10 AI. Scale bar: 10 µm (A,B).

5.3 Discussion

5.3.1 Summary of Results and Critical Evaluation

In this chapter, I reveal a completely novel population of actin cytoskeleton within the mature *Drosophila* oocyte (Figure 5.3) that plays an important role throughout meiosis, and importantly at egg activation as the oocyte undergoes the metaphase-anaphase transition. This population of actin can be labelled through a variety of tools, and manipulated through an extensive source of mutants and pharmacological agents, highlighting *Drosophila* as an important model system for the study of this particular feature of meiosis and the wider field of meiosis in general. Theis population of actin is likely required in the regulation of the microtubule spindle and chromosomal configuration during metaphase and anaphase-I. It is likely that through the regulation of the microtubule spindle that the actin has its specific effects on chromosomal configurations. When the spindle-actin is disrupted, it causes misalignment of the chromosomes during metaphase, as they begin to drift to the poles of the spindle (Figure 5.15, 5.16). At anaphase, when the actin spindle is disrupted, the chromosomes do not accurately segregate; individual chromosomal masses can be observed drifting away from the main chromosomal mass, and the alignment of the 4th chromosomes is often no-longer bipolar (Figure 5.17).

In a similar theme to the previous chapter, although plenty of resources are available for the disruption of the actin cytoskeleton more generally, it is still difficult to target specific populations of actin. In this chapter, I attempt to balance the temporal and spatial disruption of the spindle-like actin. Temporally, I utilised the actin depolymerising agent Cytochalasin-D to disrupt the actin cytoskeleton specifically in the mature oocyte either prior to or during egg activation, thus enabling visualisation of the impact the actin cytoskeleton has on the microtubule spindle and the chromosomes during metaphase-I and anaphase-I specifically. The downside of this approach, however, is that Cytochalasin-D broadly depolymerises the actin cytoskeleton, meaning cytoplasmic actin may also be disrupted and could therefore be contributing to the phenotypes observed. I therefore attempted to more specifically target the spindle-like actin through introduction of mutant *cappuccino* and *spire* nucleators. These nucleators were shown to be essential for formation of the spindle-like actin in mice (Mogessie and Schuh, 2017). I therefore demonstrated that these proteins may also be required in the accurate formation of the spindle-like actin in *Drosophila*. I therefore utilised mutant lines of said nucleators to observe the effect, hypothetically of reduced spindle-actin, on the microtubule spindle and chromosomes during the metaphase-anaphase transition. The downside of this approach, however, was that introduction of these mutant lines was not temporally controlled, meaning that *cappuccino* and *spire* could also be disrupting the actin cytoskeleton earlier in oogenesis which again may impact on the phenotypes observed. Having said this, the observable phenotypes of both Cytochalasin-D treatment and the introduction of *cappuccino* and *spire* mutants were very similar, suggesting that it is likely disruption of the spindle-like actin that results in said phenotypes.

Another great challenge on acquiring the results in this chapter was the relative difficulty of visualising the spindle and spindle components live and through a time series. Due to the relative size of the spindle to the oocyte, locating the spindle in itself can be challenging, and correct orientation of the oocyte is absolutely crucial if one is to visualise the spindle- the oocyte must be visualised directly between the dorsal appendages. However, the technique I utilise to activate oocytes or indeed to introduce many pharmacological agents involves the addition of a solution during imaging, which very often results in quite drastic movement of the oocyte and loss of the desired field of view. Steps can be taken to prevent this, such as waiting for the oocytes to fully settle on the surface of the cover-slip used for imaging and through very cautious addition of a solution. However, the technique may require further optimisation in order to be able to generate results more quickly and robustly. As such, the sample size for live imaging is often lower than desired, and had there been more time available, further repeats would have been useful to fully corroborate results.

An alternative approach was fixation, however a challenge arose during fixation as it proved that the spindle may be quite sensitive to the fixation protocol. The fixation procedure was thus optimised to be as gentle as possible, fixing oocytes for a maximum of 10 minutes before labelling, otherwise the salient features of the spindle are not easily observed or artefacts introduced. Again, a challenge arose in that although many oocytes can be fixed and dissected at once, one cannot effectively manipulate their orientation on the slide prior to being imaged, meaning that many mature oocytes are not suitable candidates for visualisation as the oocytes do not face the correct way to visualise the spindle. Again this results in the sample size being lower than desirable, and again with further time more repeats could be carried out in order to fully corroborate the results observed.

5.3.2 Conservation of the Spindle-Actin Machinery from *Drosophila* to Humans

The first study to provide a detailed description of a spindle-like actin revealed that in mice this population was essential for protecting meiotic eggs against chromosomal segregation errors (Mogessie and Schuh, 2017). Knock out of Fmn2 indicated this protein is essential for production of the spindle-like actin, without which K-fibres are unable to properly form, leading to defects in chromosomal alignment and segregation (Mogessie and Schuh, 2017). I have demonstrated a remarkable level of conservation in *Drosophila*, with this data being the first to demonstrate this population of actin in the meiotic oocyte in an invertebrate species. I have similarly shown the potential requirement of this actin population for accurate chromosomal alignment and segregation, and the probable requirement of the *Drosophila* homolog of Fmn-2 (Cappuccino) in production of this spindle-like actin. Though mostly conserved, a few differences can be observed; the spindle-actin is very apparent at metaphase-I which it is not in mice until anaphase, perhaps correlated to the fact that *Drosophila* mature oocytes are arrested at metaphase-I

compared to mice which arrest at metaphase-II; the actin could act a mechanism to aid stabilisation of the spindle until egg activation, whereupon calcium release may aid in de-crosslinking the spindle-actin network to release this metaphase arrest, though this would require much more in depth research to conclude. Furthermore, due to the unique chromosomal make-up of *Drosophila,* I can observe some unique phenotypes when actin is disrupted, such as apparent mis-regulated polarity of the spindle as the 4th non-exchange chromosomes no longer segregate accurately to opposite poles. Despite differences, which likely reflect the major biological and developmental differences between *Drosophila* and mice, there is still a remarkable level of conservation between the two.

Similarly, a population of spindle-like actin was shown to exist in humans (Mogessie and Schuh, 2017; Roeles and Tsiavaliaris, 2019). This actin followed closely the dynamics of the microtubule spindle; perturbations to the microtubule spindle resulted in downstream perturbations to the spindle-like actin, suggesting actin acts downstream of the microtubules (Roeles and Tsiavaliaris, 2019). Washout experiments with microtubule depolymerising agents in the presence and absence of actin further shows that actin is required for correct formation of the microtubule spindle, suggesting actin may also act upstream of the microtubule spindle (Roeles and Tsiavaliaris, 2019). In *Drosophila*, however, though I have shown that the actin cytoskeleton may regulate the formation and morphology of the microtubule spindle, I have not been able to demonstrate the requirement of microtubules for the formation of the spindle-like actin. In fact, my data would suggest that the spindle-like actin is somewhat resistant to changes in the microtubule cytoskeleton, as depolymerising the microtubules with colchicine had no effect on the spindle-like actin (Figure 5.10). Despite these differences, there is a clear interdependence between the actin and microtubule spindle, as morphological changes in the spindle at egg activation are closely mirrored by the spindle-like actin.

Though there is great conservation of mechanism from *Drosophila* to mammals, other systems appear to utilise specific actin populations in the spindle differently. It was originally proposed in starfish oocytes that actin plays an essential role in chromosomal capture, as a mechanism involving microtubules alone would not be efficient in larger volume cells, such as oocytes (Lenart et al., 2005). In fact, it was shown that following nuclear envelope breakdown (NEBD), that formation of a contractile actin mesh promotes delivery of the chromosomes to the microtubule spindle (Lenart et al., 2005). A more recent discovery demonstrated that formation of F-actin “patches” occurs within the starfish oocyte nucleus following NEBD (Burdynuik et al., 2018). These patches specifically form around chromosomes, and are hypothesised to sterically block interactions with the microtubules; this interaction is necessary to prevent the early capture of chromosomes, which would prevent efficient F-actin mediated chromosomal congression (Lenart et al., 2005). Therefore, though morphology of the nuclear actin networks may differ between species, it appears there is still a great conservation of its ultimate function; to coordinate chromosomal movements, preventing chromosomal loss and aneuploidy.

5.3.3 Calcium and Actin Interplay during Meiosis

My data demonstrates clearly the presence of the calcium marker GCaMP3 at the metaphase and anaphase-I spindle (Figure 5.20), in addition to the calcium-dependent signalling protein calmodulin, which associates closely with the spindle throughout egg activation (Figure 5.24). Not only this, the microtubule and spindle-like actin appear to be dependent on calcium (with disruption of these populations when incubated with BAPTA-AM (Figure 5.21)), and significant changes in the chromosomal morphology. The phenotypes observed bear remarkable similarities to *Xenopus*, in which BAPTA causes rapid depolymerisation of the microtubules in a similar fashion to nocodazole (a microtubule depolymerising agent), resulting in clustering of the oocyte chromosomes at the cortex (Li et al., 2016). In *Xenopus*, BAPTA treatment is effective at depolymerising the microtubule spindle, however EGTA is not, due to their differing speeds of action, suggesting the presence of calcium signalling nano-domains in the spindle (Wang and Augustine., 2015). Calmodulin is suggested to be a potential candidate for this nano-domain calcium signalling given its enrichment in *Xenopus* spindles, its ubiquitousness and capability to bind two calcium ions at both its C- and N-terminus (Malmendal et al., 1999; Park et al., 2008).

Whilst research focuses largely on the potential of Calmodulin, microtubules and microtubule associated proteins (MAPs) as candidates for calcium-signalling nano-domains within the spindle, my data would suggests calcium-actin nano-domains should be examined. As examined in my previous chapters, many actin-binding proteins (ABPs) are calcium sensitive, such as α-actinin and supervillin in *Drosophila*, in addition to a great variety of other ABPs. These ABPs are found in many other systems, for instance in many plants there are examples of actin as a downstream molecule of calcium signalling via ABPs such as LIM domain containing proteins and the larger family of Villins, which act as actin bundlers and promote actin turnover (Papuga et al., 2010; Qu et al., 2013). There is potential for many of these ABPs to form calcium signalling nano-domains with the spindle-like actin during meiosis and thus transduce calcium signals, such as from egg activation, into regulation of meiosis. In *Xenopus* it appears as though calcium nano-domains are required for microtubule polymerisation (Li et al, 2016), suggesting there may be a similar impact on the spindle-like actin. Given that certain ABPs are calcium sensitive and in higher calcium conditions are unable to bundle or cross-link actin, it may also be that the inverse is true, in that calcium signalling relieves actin-mediated tension on the spindle such that meiosis can occur. This would be in line with my results that suggest actin cytoskeleton keeps the spindle morphology compact; loss of actin causing significant elongation of the spindle.

5.3.4 Future Directions

My results demonstrate clearly the formation of a spindle-like actin population at the metaphase-anaphase I transition in *Drosophila*, which required the formin Cappuccino. This population of actin is important in regulating chromosomal movements during meiosis; there is much work to be

done establishing fully the mechanisms through which the actin cytoskeleton regulates microtubule-spindle formation and chromosomal alignment and segregation during metaphase and anaphase-I. It is possible that the spindle-like actin is required for promoting the formation of K-fibres, as it is in mice (Mogessie and Schuh., 2017). This could well be the case, given the significant impact the actin population has generally over the formation and regulation of the microtubule spindle, as I have demonstrated. There is, however, little literature existing in this area given it is such a recent discovery, and as such such, future work will rely heavily on multiple hypotheses generated from what we know in mammals, starfish and now *Drosophila*, and should also be influenced by mechanisms of mitosis.

I will discuss in much greater depth the future directions and paths studies in *Drosophila* could follow in Chapter 6; I highlight here that I have examined one specific stage in meiosis, that of the metaphase-anaphase I transition. This is an important context to appreciate, as it is this transition that is a key event of egg activation, not only in *Drosophila*, but also mammals (metaphase-anaphase II). Thus far, studies have only focussed on the specific changes within the nucleus whilst following the dynamics and mechanisms of the spindle-actin; however, at these key developmental transitions, global waves of calcium are present which permeate the oocyte, and in the literature thus far, have been ignored. I therefore provide the first contextual evidence of calcium-actin interplay, which should be a major focus of future work, as it is well known that calcium mediates the vast array of changes to the oocyte at egg activation, including the resumption of meiosis through a plurality of calcium-sensitive signalling molecules, including the actin-cytoskeleton itself (York-Andersen et al., 2021).

Chapter 6:

Conclusions and Perspectives

Chapter 6: Conclusions and Perspectives

Throughout this thesis I have demonstrated the unique interactions of calcium and actin throughout egg activation, both during the entry of calcium as a polarised wave or cortical increase and downstream of the calcium wave in the regulation of the meiotic spindle. I have demonstrated that as the oocyte is hydrated, osmotic swelling induces the opening of Trpm channels to mediate calcium entry at egg activation. This calcium transient is likely supported through other calcium channels. Regulation of such calcium entry is mediated by the cortical actin which appears to act initially as a barrier to calcium entry, with rearrangements of the cortical actin as the oocyte swells essential for enabling calcium entry. Furthermore, regional differences in the cortical actin, namely localised decreases of cortical actin density at the posterior and anterior prime these regions for calcium entry.

Following the calcium wave and the resumption of meiosis, I then reveal the presence of a novel and unique population of actin in *Drosophila*- the spindle-like actin. This population of actin is required for regulation of chromosomal movements and spindle morphology during meiosis. Interestingly, localised calcium signalling within the spindle is likely required for regulation of the formation of the spindle-like actin and microtubule spindle, with the enrichment of calcium signalling proteins within the spindle. Ultimately, I further our understanding of not only the process of egg activation, in particular the metaphase-anaphase transition, but also of fundamental cellular mechanisms, such as actin-mediated regulation of calcium flux.

Furthermore, there is a significant level of conservation between *Drosophila* and mammals, with parallels evident throughout this thesis: i) Trp channels are found in both the *Drosophila* and mammalian germline, promoting calcium entry at egg activation; ii) The cortical actin (and cortex generally) is a conserved cellular structure throughout eukaryotic cells, with the cortex functioning as a sensor of the osmotic environment of cells in both *Drosophila* oocytes and mammalian neuronal cells, to name just one example; iii) The spindle-like actin is conserved from mammals to *Drosophila*, with conservation of both function and morphology, suggesting this is a fundamental regulator of meiosis. I believe that this thesis springboards *Drosophila melanogaster* as a model for understanding these key cellular processes such that we can employ the powerful base of genetic tools for furthering our knowledge of fundamental cell and developmental biology which we can extrapolate into our knowledge of human biology and potential therapeutics.

6.1 Actin and Calcium Interplay at Egg activation

Actin is required at multiple stages during egg activation, both upstream, concomitantly with the calcium wave, and downstream to regulate the egg-to-embryo transition. Cortical actin is generated by a number of different nucleating factors and its architecture fine-tuned through the action of a variety of actin binding proteins (ABPs). This regulates entry of calcium in the form of a polarised wavefront, likely through transduction of cortical tension to Trpm channels as the oocyte swells. I provide a model of the generation of the cortical actin and its impact on calcium flux below:

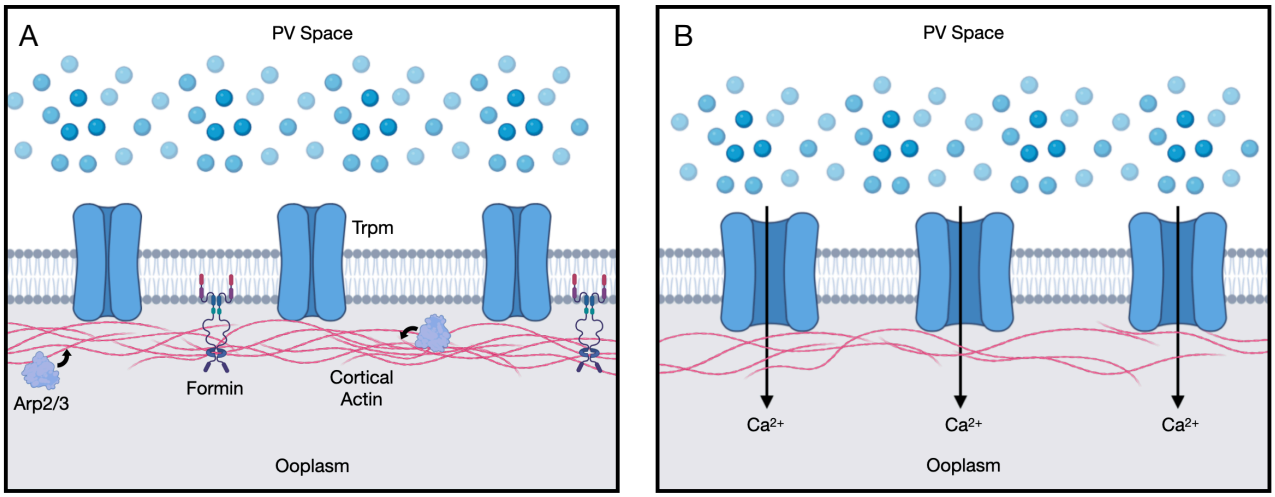

Figure 6.1: Model of cortical actin mediated regulation of calcium entry at egg activation. A) Pre-activation: Cortical actin is generated through the action of the Arp2/3 and Formin complexes. A tight cortical actin mesh prevents calcium entry through Trpm channels. B) Post-activation: Cortical actin mesh is dispersed, enabling opening of Trpm channels and calcium entry.

1- Cortical actin is nucleated by numerous factors including: i) SCAR; and ii) SpireB.

2- Cortical mesh architecture is further regulated by a variety of ABPs including: i) Gelsolin; ii) α-actinin; and iii) Supervillin.

3- This generates a specifically controlled level of cortical actin within the oocyte. Distribution of the cortical actin is not uniform as there is a reduction at the anterior and posterior of the oocyte.

4- Hydration of the oocyte results in swelling of the oocyte and consequent dispersion and increased dynamics of the cortical actin.

5- Dispersion of the cortical actin enables calcium entry, primarily from the poles which have a reduced cortical actin signal.

6- Calcium wave propagation is controlled in part by a functioning cortex; over-expression or stabilisation of cortical actin prevents the calcium wave, whereas partial reduction of the cortical actin promotes calcium entry from multiple points across the cortex.

7- Calcium entry may promote further dispersion of cortical actin at the head of the wave, enabling a calcium induced calcium influx mechanism.

8- Complete loss of the cortical actin prevents the calcium wave, likely through loss of cortical tension which is required for opening of Trpm calcium channels.

There are still elements of this model that require further investigation that are beyond the scope of this thesis due to time constraints. One of my earliest observations of the cortical actin within the oocyte was the anterior and posterior drop in density of actin, likely priming calcium entry from the poles. Having established the requirement of various actin nucleators and ABPs in the regulation of calcium entry, the next question would be how are these factors regulated such that there be such a specific density of cortical actin at the poles of the oocyte? One potential line of investigation would be to examine whether the curvature of the poles naturally decreases the density of the actin itself, perhaps through mathematical modelling of the membrane and cortex density as indicated through fluorescent labelling, or perhaps through evaluation of mutant lines that generate more uniformly rounded oocytes as was carried out to investigate Trp function (Hu and Wolfner., 2020). There is likely a molecular component regulating actin distribution also, as in the drosophila oocyte the posterior and anterior axes are determined through mutual exclusion of Par protein domains, with aPKC having been shown to be required for actin recruitment to the cortex (Barr et al., 2019). Visualisation of the cortical actin in mutant backgrounds of the Par proteins could reveal whether the reduced cortical actin signal expands or shrinks depending on which polar domain is affected. This would likely have downstream consequences on the actin wave; perhaps knock down of aPKC which promotes formation of the anterior domain would result in expansion of the posterior domain to a degree where calcium waves can be triggered more laterally.

To more thoroughly and effectively visualise the cortical actin cytoskeleton in a variety of backgrounds, the next step would be to utilise super-resolution microscopy. STED microscopy has been shown to be highly effective in visualising the nanoscale dimensions of the cortex and space between the cortex and plasma membrane, so could be used to very effectively visualise the cortex and associated proteins (Clausen et al., 2017). The difficulty with some super-resolution techniques such as STED is the requirement of extremely thin sectioning, which is challenging in the *Drosophila* oocyte which is far thicker than required. As such, this may require fixation and potentially preparing oocyte sections in a similar way to larval preparations in which the tissue is cut in half and spread such that the inside is embedded against a cover slip (filleting). This could reveal in much greater detail how the density of the cortical actin differs between the poles and

lateral sides of the oocyte, in addition to perhaps revealing more clearly what the effect of knocking down nucleators, ABPs and potentially Par proteins is upon the cortical actin.

Having identified a variety of ABPs, such as α-actinin and supervillin, that play a role in downstream regulation of calcium entry at egg activation, one interesting line of investigation to follow would be examining the part they play in calcium induced calcium influx (CICI). A mechanism for CICI has been proposed in which stretching of mechanosensitive channels enables calcium entry. This calcium then promotes filament sliding via actomyosin, thus increasing tension and opening further channels, enabling propagation of a calcium wave (Jaffe, 2008). As aforementioned, many ABPs are calcium sensitive, such as α-actinin which is unable to cross-link actin in high calcium environments (Jayadev et al., 2014; Prebil et al., 2016; Sjöblom et al., 2008). It is therefore likely that upon calcium entry at egg activation, there is a change in the activity and binding properties of ABPs, resulting in changes within the tension of the actin cortex that is transduced to downstream mechanosensitive channels such as Trpm.

Such a feedback loop of calcium entry resulting in the changing activity of ABPs and changing properties of the cortical actin could well contribute to the mechanism of slow and ultra-slow wave propagation. Slow calcium waves often rely on the mechanical propagation of the calcium signal between relay points; entry of calcium through stretch-sensitive channels resulting in intracellular calcium rises which then induce contraction of acto-myosin filaments in order to open adjacent channels (Jaffe, 2008). Furthermore, ultra slow waves may also rely on such acto-myosin contraction, primarily to produce developmental waves. For example, the inversion of the developing Volvox embryo requires a wave of calcium activity to coordinate a wave of contraction (Viamontes et al., 1977). In both slow and ultra-slow waves, mechanical propagation is required, and therefore it is likely that the variety of ABPs investigated in this thesis could contribute to these waves in other systems. Similarly, further investigation of myosin motors, such as spaghetti squash, in the *Drosophila* oocyte should be carried out; knock down of such motors may impact wave progression and therefore tell us whether such acto-myosin networks are required.

6.2 Furthering our Understanding of Spindle-Actin:

My investigation of actin within the oocyte revealed important roles for the cortical population during egg activation and for the nuclear population during the metaphase-anaphase transition. In both aspects, actin is essential for a regulated and organised egg-to-embryo transition. There is a great deal yet to investigate with regards to the spindle actin, largely due to the fact that it has only recently been discovered and there is a sheer lack of literature surrounding its role in meiosis and mitosis. Therefore, I outline below directions this project, and the field in general, could take.

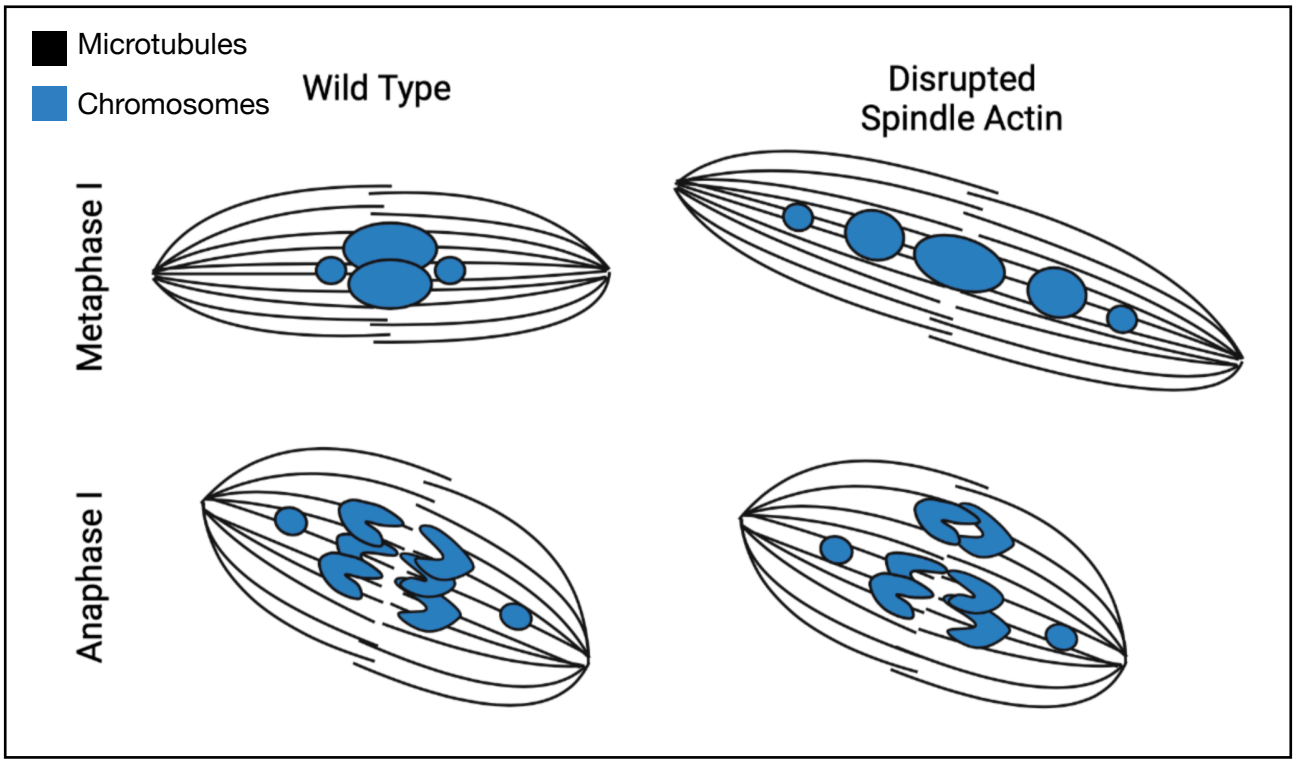

Figure 6.2: Model of the role of spindle-like actin during the metaphase-anaphase transition
Metaphase I: At metaphase, the chromosomes are congressed in a central mass; when the spindle-like actin is disrupted, the chromosomes no longer congress, the microtubule spindle is significantly elongated and premature rotation of the spindle can be observed. Anaphase I: The chromosomes begin to separate uniformly and the spindle rotates; when the spindle-like actin is disrupted, spindle rotation occurs as normal but chromosomal separation is disrupted as chromosomal masses can be observed floating away from the centrally location chromosomes.

One interesting result observed was that of the cytochalasin-D treated metaphase arrested oocytes; chromosomes from these oocytes appeared to be spread out along the spindle axis (Figure 5.15), and looked very similar to the chromosomes at pro-metaphase as they are beginning to congress. It is perhaps the case that actin is required even earlier than metaphase in *Drosophila*, despite metaphase-I being the first stage it appears to be required in during

mammalian meiosis (Mogessie and Schuh., 2017). I would therefore plan to visualise the spindle-like actin using UtrCH-GFP to determine whether a population is visible prior to metaphase-I, and indeed utilise c*apu* and s*pire* mutants to evaluate whether any defects in the chromosomes can be observed in prophase and pro-metaphase oocytes. It may be the case that there is an important role for the spindle-like actin during this maturation step, which we still know remarkably little about.

Further to this, I have elucidated mechanisms at play within the specific time frame of the metaphase-I to anaphase-I transition in *Drosophila* oocytes. It has, however, been demonstrated that the spindle-like actin is present throughout meiosis II in both mice and humans (Mogessie and Schuh, 2017; Roeles and Tsiavaliaris, 2019). Therefore it would be interesting to continue observations of the spindle-like actin following anaphase-I, studying the role of the spindle-like actin throughout the second round of meiosis. This might be particularly interesting in *Drosophila*, as unlike mammals and most vertebrates, there is no cytokinesis step as the oocyte-embryo transition will ultimately produce a syncytium. Therefore, during meiosis, 4 pronuclei are formed and are present in the fertilised oocyte, and which pro-nucleus undergoes pro-nuclear apposition (the *Drosophila* equivalent of pro-nuclear fusion) is not well understood (Callaini and Riparbelli, 1996), as such there may be a contribution from the spindle-like actin.

This should firstly be explored through expression of the actin marker UtrCH-GFP, and detailed, live dynamics of the spindle-like actin throughout metaphase-II provided. Following this, similar mutational analysis of *cappuccino* and use of pharmacological agents such as Cytochalasin-D used to reveal whether the spindle-like actin is required for the remainder of meiosis in regulating the formation of the spindle and segregation of chromosomes, which it likely is. As I have demonstrated that the actin plays a role in positioning of the spindle structure with relation to the cortex (Figure 5.15, 5.16 and 5.17), one observation of interest would be on the relative positions of the pro-nuclei. They usually align perpendicularly to the cortex, and the inner-most pronucleus is the most likely to undergo pro-nuclear apposition (Callaini and Riparbelli, 1996). I would therefore hypothesise that disruption of the spindle-like actin would affect the positioning of the pro-nuclei and therefore which nucleus is most likely to be selected for pro-nuclear apposition.

In addition to understanding the cellular role that the spindle-like actin plays during meiosis, a more thorough understanding of how the spindle-like actin regulates meiosis is needed. I have explored this aspect in two key areas: i) Interactions with the microtubule spindle and; ii) Interactions with calcium and calcium-sensitive proteins. I have demonstrated that actin functions upstream in many aspects of formation of the microtubule spindle, as without actin we see drastic changes in microtubule morphology and in extreme cases loss of the microtubule spindle altogether (Figure 5.12 and Figure 5.13). It would be prudent to investigate more precisely the reasons behind these changes; *cappuccino*, for instance, is required earlier in oogenesis in

Drosophila oocytes, so the lack of spindle structures could be due to more dramatic phenotypic effects during oogenesis (Theurkauf, 1994).
There is a clear interaction between microtubules and actin and likely great interplay between the two; it is likely the case that microtubules also regulate the formation of the spindle-like actin, forming a complex inter-regulatory network. It was shown that the tail of *cappuccino* binds with non-specific interactions to microtubules, and when bound this inhibits the nucleation of actin (Roth-Johnson et al., 2014). This provides a potential explanation as to why microtubule depolymerising agents appear to be ineffective against the spindle-like actin- the structure will have already formed prior to disruption of microtubules, and indeed the lack of microtubules may result in greater production of spindle-like actin. Having said this, it was shown that *cappuccino* does not directly bind both microtubules and actin at the same time, therefore there must be great spatio-temporal regulation of the nucleator to produce the spindle-like actin and spindle microtubules (Roth-Johnson et al., 2014), again pointing to an upstream activity of actin, perhaps being generated prior to spindle microtubules.

In mice, it was shown that actin is specifically required for the production of K-fibre microtubules, which are the filaments responsible for the alignment and segregation of chromosomes during meiosis (Mogessie and Schuh, 2017). I began analysis and investigation of this in *Drosophila*, however I was not able to effectively optimise a cold-mediated depolymerisation assay to isolate K-fibres (as these are the most stable microtubule population in the spindle and as such remain following cold shock). Use of this technique would enable us to effectively isolate this population for analysis- ideally I would visualise whether in the case of *cappuccino* mutants and cytochalasin-D that this population was being affected, as this would provide a direct mechanism for actin mediated regulation of chromosomal movements during meiosis. I would further this analysis by performing FRAP analysis to reveal whether this population's dynamics are affected by actin disruption, again to provide a causal link between spindle-like actin disruption and chromosomal segregation errors.

A key step forward in our understanding of actin within the spindle would be to push forward with investigation of mitotic spindles. To date, there has been little investigation of such, however a good model to test this would be the drosophila syncytium, as Utrophin acts as an effective marker of the actin cytoskeleton in both germline and somatic tissues, with nuclei undergoing many rounds of division, therefore lots of active mitosis to be observed. It would be very illuminating to know whether the spindle-like actin is a conserved feature within only meiotic spindles, as this would likely indicate that the actin plays a key role in mediating some of the key differences between meiosis and mitosis, such as in chromosomal alignment on the metaphase plate and perhaps even for stability during arrested periods of meiosis.

6.3 Impact and Significance:

Despite extensive research having been undertaken in the field of egg activation and fertilisation, there is still a great deal to be researched and examined. *Drosophila* provides an excellent model system for specific understanding of the events of egg activation, as this process is completely uncoupled from fertilisation itself and can be easily and effectively recapitulated *ex vivo*. The data that I provide in this thesis therefore greatly enhances and strengthens our understanding of egg activation more generally, providing key insights into novel mechanisms of calcium entry, the universal regulatory event of egg activation, and novel requirements of actin both prior to and downstream of the calcium wave. I therefore provide a strong foundation through which future research can be enacted to more deeply understand this biological system.

Understanding this system is important not only as a foundation to further investigation of molecular pathways, but also for furthering our understanding of how such systems evolved. Evolutionary and developmental biology are irrevocably linked sciences, and plenty of models of this exist. Highlighting this are the key developmental stages of egg activation and fertilisation that have become diversified throughout evolution to fine-tune control over reproduction. *Drosophila* species have shown a remarkable variation in their reproductive capabilities; parthenogenetically reproducing species (meaning oocytes are able to undergo development without input from the sperm) exist in both obligate and facultative varieties, whereas other species are reliant on fertilisation to provide a diploid genome.

In the wild, *Drosophila mangabeirai* exist as females only - the only obligate parthenogenetic species of *Drosophila* (Markow, 2013). *Drosophila parthenogenetica* virgin females are able to produce all female progeny, independent of the presence of males and indeed fertilisation, indicating its ability to facultatively select parthenogenetic means of reproduction (Stalker 1954). In fact, a single mutation within the *yemanuclein-alpha* gene is sufficient to generate parthenogenetically reproducing *Drosophila* species through changing the orientation of meiosis such that viable diploid eggs are produced without fertilisation (Meyer et al, 2010). Given my results that demonstrate the importance of the spindle-like actin for chromosomal segregation and spindle orientation, it may be that this population of actin may also have played a role evolutionarily in functional parthenogenesis. Whether the spindle-like actin would promote or prevent parthenogenesis is unclear. Given conservation of the spindle-like actin with mammalian species, which have, in an evolutionary sense, not recently utilised parthenogenetic reproduction, the spindle-like actin may have evolved as a mechanism to prevent unwanted parthenogenesis in oocytes that will be fertilised. However, in *Drosophila*, the spindle-like actin appears more significant in overall regulation and formation of the spindle compared to mammals, which may indicate in certain *Drosophila* species that extra regulatory mechanisms are in place to compensate for the lack of input from fertilisation and ensure that diploid embryos are formed. It would therefore be very illuminating to visualise spindle-like actin in parthenogenetically

reproducing *Drosophila* species and other parthenogenetic organisms more generally to cytologically examine the significance of this spindle population.

Furthermore, I have also shown that actin regulated calcium entry through Trpm channels occurs as the oocyte swells, a result that also supports the evolutionary perspective that certain *Drosophila* species are able to reproduce parthenogenetically. Alternative mechanisms to fertilisation must be in place to trigger the plethora of events, such as changes to the translational landscape and resumption of meiosis, that ultimately lead to the formation of the embryo. In some species, such as the wasp Pimpla turionella, the mechanical trigger as the oocyte passes into the oviduct is sufficient to cause displacement of the nucleus and lead to development of the embryo (Went and Krause, 1973). In *Drosophila*, passage of the oocyte into the oviduct likely results in swelling via uptake of fluid, ultimately resulting in the opening of mechanically gated Trpm channels and calcium entry. As such, egg activation is achieved prior to fertilisation; this mechanism should also be investigated in parthenogenetically reproducing *Drosophila* species, as it is highly likely to be a conserved mechanism that enables parthenogenesis. Interestingly, the requirement of mechanically gated channels is not conserved to just species of insects that utilise mechanisms alternative to fertilisation. In C.elegans, it was demonstrated that the sperm cell can deliver Trp channels to the membrane of the oocyte to potentiate calcium influx at egg activation (Takayama and Onami., 2016). Furthermore, in mice, it was demonstrated that Trp channels were required for the calcium flux at egg activation, with TrpV3 activated through over-expression and application of 2-APB and demonstrating subsequent calcium influx into the oocyte (Lee et al., 2016). TrpM7 was later shown to be required, as when blocked pharmacologically the calcium influx at egg activation was prevented (Carvacho et al., 2016). Evidently, there is a great deal to be learned from our understanding of egg activation more generally, as conserved mechanisms exist from *Drosophila* to mammals.

This further emphasises the usefulness of *Drosophila* as a model system for our understanding of biological processes at a mammalian level. CRISPR technology, to provide one example, is rapidly improving within *Drosophila,* the turnaround for such experiments being significantly shorter than in mammals. This could, and has been, used to evaluate the significance and mechanism of various biological pathways in *Drosophila*, not limited to egg activation. To focus on this key developmental stage, however, I reveal conserved pathways within the oocyte, our understanding of which can springboard potential therapeutic studies in mammals. The discovery that the spindle-like actin is important for regulating chromosomal movements during meiosis suggests that this pathway, when disturbed, can lead to aneuploidy, a key event that prevents the production of healthy eggs in humans. Furthering our understanding of this in *Drosophila* could therefore shed a great deal of light on how this process may be protected more efficiently during *in vitro* fertilisation protocols to produce healthy embryos for implantation, to suggest but one potential therapeutic application.

Ultimately, the significance of this work lies in within the entwined disciplines of evolutionary, developmental and molecular biology. Here I have presented many novel mechanisms of egg activation and a thorough platform through which the molecular and physiological links between calcium and actin should be investigated.

Chapter 7:

Bibliography

1. Abbott AL, Ducibella T. (2001) Calcium and the control of mammalian cortical granule exocytosis. *Front Biosci* 6: D792– D806.

2. Ajduk A, Ilozue T, Windsor S, Yu Y, Seres KB, Bomphrey RJ, Tom BD, Swann K, Thomas A, Graham C, Zernicka-Goetz M. (2011) Rhythmic actomyosin-driven contractions induced by sperm entry predict mammalian embryo viability. *Nat Commun*. 9;2:417. doi: 10.1038/ncomms1424.

3. Algrain M, Turunen O, Vaheri A, Louvard D, Arpin M. (1993) Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *J Cell Biol*. 129–139.

4. Amini M, Wang H, Belkacemi A, Jung M, Bertl A, Schlenstedt G, Flockerzi V, Beck A. (2019) Identification of Inhibitory Ca2+ Binding Sites in the Upper Vestibule of the Yeast Vacuolar TRP Channel. *iScience*, 11():1-12.

5. Archambault V, Zhao X, White-Cooper H, Carpenter AT, Glover DM. (2007) Mutations in Drosophila Greatwall/Scant reveal its roles in mitosis and meiosis and interdependence with Polo kinase. *PLoS Genet*. 3(11):e200. doi: 10.1371/journal.pgen.0030200.

6. Ashish, Paine MS, Perryman PB, Yang L, Yin HL, Krueger JK. (2007) Global structure changes associated with Ca2+ activation of full-length human plasma gelsolin. *J. Biol. Chem*. 282, 25884–25892

7. Aviles-Pagan EE, and Orr-Weaver TL. (2018) Activating embryonic development in Drosophila. *Semin Cell Dev Biol*, 84:100-110

8. Avilés-Pagán EE, Orr-Weaver TL. (2018) Activating embryonic development in Drosophila. *Semin Cell Dev Biol*. 84:100-110. doi: 10.1016/j.semcdb.2018.02.019.

9. Azoury J, Lee KW, Georget V, Rassinier P, Leader B, and Verlhac MH. (2008). Spindle positioning in mouse oocytes relies on a dynamic meshwork of actin filaments. *Curr. Biol.* 18, 1514-1519.

10. Barr J, Charania S, Gilmutdinov R, Yakovlev K, Shidlovskii Y, Schedl P. (2019) The CPEB translational regulator, Orb, functions together with Par proteins to polarize the Drosophila oocyte. *PLoS Genet.* 13;15(3):e1008012.

11. Barvitenko N, Aslam M, Lawen A, Saldanha C, Skverchinskaya E, Uras G, Manca A, Pantaleo A. (2021) Two Motors and One Spring: Hypothetic Roles of Non-Muscle Myosin II and Submembrane Actin-Based Cytoskeleton in Cell Volume Sensing. *International Journal of Molecular Sciences*. 22(15):7967.

12. Becalska AN, Kim YR, Belletier NG, Lerit DA, Sinsimer KS, Gavis ER. (2011) Aubergine is a component of a nanos mRNA localization complex. *Dev Biol*. 1;349(1):46-52.

13. Belenguer P, Caizergues-Ferrer M, Labbe J-C, Doree M, Amalric F. (1990) Mitosis-specific phosphorylation of nucleolin by p34cdc2 protein kinase. *Mol Cell Biol* 10: 3607–3618.

14. Bement WM, Leda M, Moe AM, Kita AM, Larson ME, Golding AE, Pfeuti, C, Su KC, Miller AL, Goryachev AB et al. (2015) Activator-inhibitor coupling between Rho signalling and actin assembly makes the cell cortex an excitable medium. *Nat Cell Biol*, 17:1471-1483

15. Bennabi I, Terret ME, Verlhac MH. (2016) Meiotic spindle assembly and chromosome segregation in oocytes. *J Cell Biol*. Dec 5;215(5):611-619.

16. Bernitt E, Döbereiner HG, Gov NS, Yochelis A. (2017) Fronts and waves of actin polymerization in a bistability-based mechanism of circular dorsal ruffles. *Nat Commun*. 19;8:15863.

17. Bernitt E, Koh CG, Gov N, Döbereiner HG. (2015) Dynamics of actin waves on patterned substrates: a quantitative analysis of circular dorsal ruffles. *PLoS One*. 9;10(1):e0115857.

18. Berridge, M.J. (2002) The endoplasmic reticulum: a multi- functional signaling organelle. *Cellular Calcium* 32, 235– 249

19. Berridge MJ, Bootman MD, Roderick HL. (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol*. 4(7):517-29

20. Bize I, Güvenç B, Buchbinder G, Brugnara C. (2000) Stimulation of Human Erythrocyte K-Cl Cotransport and Protein Phosphatase Type 2A by n-Ethylmaleimide: Role of Intracellular Mg++. *J. Membr. Biol. 177*, 159–168.

21. Blanchoin L, Boujemaa-Paterski R, Sykes C, Plastino J. (2014) Actin dynamics, architecture, and mechanics in cell motility. *Physiol Rev*. 94:235-263

22. Blanchoin L, Pollard TD. (2002) Hydrolysis of ATP by polymerized actin depends on the bound divalent cation but not profilin. *Biochemistry.* 41: 597–602.

23. Bohrmann J, and Zimmerman J. (2008) Gap junctions in the ovary of Drosophila melanogaster: localisation of innexins 1, 2, 3 and 4 and evidence for intercellular communication via innexin-2 containing channels. *BMC Dev Biol.* 27;8:111

24. Bolcun-Filas E, Handel MA. (2018) Meiosis: the chromosomal foundation of reproduction. *Biol Reprod*. Jul 1;99(1):112-126.

25. Bolivar J, Huynh JR, Lopez-Schier H, González C, St John- ston D, González-Reyes A. (2001). Centrosome migration into the Drosophila oocyte is independent of BicD, egl and of the organ- isation of the microtubule cytoskeleton. *Development* 128, 1889–1897.

26. Bosco G, Orr-Weaver TL. (2002) The cell cycle during oogenesis and early embryogenesis in Drosophila. *Advances in Developmental Biology and Biochemistry*, 12:107-154,

27. Bourouh M, Dhaliwal R, Rana K, Sinha S, Guo Z, Swan A. (2016) Distinct and Overlapping Requirements for Cyclins A, B, and B3 in *Drosophila* Female Meiosis. *G3 (Bethesda).* 8;6(11):3711-3724.

28. Bovellan M, Romeo Y, Biro M, Boden A, Chugh P, Yonis A, Vaghela M, Fritzsche M, Moulding D, Thorogate R, Jégou A, Thrasher AJ, Romet-Lemonne G, Roux PP, Paluch EK, Charras G. (2014) Cellular control of cortical actin nucleation. *Curr Biol.* Jul 21;24(14):1628-1635.

29. Boyd L, Guo S, Levitan D, Stinchcomb DT, Kemphues KJ. (1996) PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in C. elegans embryos. *Development* 122, 3075–3084.

30. Bretscher A. (1999) Regulation of cortical structure by the ezrin-radixin-moesin protein family. *Curr Opin Cell Biol*. Feb;11(1):109-16.

31. Bretschneider T, Anderson K, Ecke M, Müller-Taubenberger A, Schroth-Diez B, Ishikawa-Ankerhold HC, Gerisch G. (2009) The three-dimensional dynamics of actin waves, a model of cytoskeletal self-organization. *Biophys J*. 8;96(7):2888-900. doi:

32. Breuer M, Kolano A, Kwon M, Li CC, Tsai TF, et al. (2010) HURP permits MTOC sorting for robust meiotic spindle bipolarity, similar to extra centrosome clustering in cancer cells. *J. Cell Biol*. 191:1251–60

33. Burdyniuk M, Callegari A, Mori M, Nédélec F, Lénárt P. (2018) F-Actin nucleated on chromosomes coordinates their capture by microtubules in oocyte meiosis. *J Cell Biol.* 217(8):2661-2674.

34. Burridge K, Feramisco JR. (1981) Non-muscle alpha actinins are calcium-sensitive actin-binding proteins. *Nature*. 10;294(5841):565-7.

35. Burtnick LD, Koepf EK, Grimes J, Jones EY, Stuart DI, McLaughlin PJ, Robinson RC. (1997) The crystal structure of plasma gelsolin: implications for actin severing, capping, and nucleation. *Cell.* 90, 661–670

36. Burtnick LD, Urosev D, Irobi E, Narayan K, Robinson RC. (2004) Structure of the N-terminal half of gelsolin bound to actin: roles in severing, apoptosis and FAF. *The EMBO Journal*. 23 (14)

37. Callaini G, Riparbelli MG. (1996) Fertilization in Drosophila melanogaster: centrosome inheritance and organization of the first mitotic spindle. *Dev Biol.* 15;176(2):199-208.

38. Cant K, Knowles BA, Mahajan-Miklos S, Heintzelman M, Cooley L. (1998) Drosophila fascia mutants are rescued by over expression of the villain-like protein, quail. *J Cell Sci.* 111:213-21

39. Carafoli E, Krebs J. (2016) Why Calcium? How Calcium Became the Best Communicator. *J Biol Chem*. 291(40):20849-20857.

40. Carballido-López R. (2006) The bacterial actin-like cytoskeleton. *Microbiol Mol Biol Rev*. 70(4):888-909.

41. Carreno S, Kouranti I, Glusman ES, Fuller MT, Echard A, Payre F. (2008) Moesin and its activating kinase Slik are required for cortical stability and microtubule organization in mitotic cells. *J Cell Biol*. Feb 25;180(4):739-46

42. Carroll DJ, Ramarao CS, Mehlmann LM, Roche S, Terasaki M, Jaffe LA. (1997) Calcium release at fertilization in starfish eggs is mediated by phospholipase Cgamma. *J Cell Biol* 138:1303–1311.

43. Carroll, J. (2001). The initiation and regulation of Ca2+ signalling at fertilisation in mammals. *Semin. Cell Dev. Biol.* 12, 37-43.

44. Carvacho I, Ardestani G, Lee HC, McGarvey K, Fissore RA, Lykke-Hartmann K. (2016) TRPM7-like channels are functionally expressed in oocytes and modulate post-fertilization embryo development in mouse. *Sci Rep*. Sep 29;6:34236.

45. Castro A, Bernis C, Vigneron S, Labbe JC, Lorca T. (2005) The anaphase-promoting complex: a key factor in the regulation of cell cycle. *Oncogene.* 24:314–325.

46. Castro A, Peter M, Magnaghi-Jaulin L, Vigneron S, Galas S, Lorca T, Labbe JC. (2001) Cyclin B/cdc2 induces c-Mos stability by direct phosphorylation in Xenopus oocytes. *Mol Biol Cell.* 12:2660–2671.

47. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D. (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*. 288:306-313

48. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature.* 389:816-824.

49. Cavaliere V, Bernardi F, Romani P, Duchi S, Gargiulo G. (2008). Building up the Drosophila eggshell: First of all the eggshell genes must be transcribed. *Dev. Dyn*. 237(8): 2061-2072.

50. Cha BJ, Koppetsch BS, Theurkauf WE. (2001) In vivo analysis of Drosophila bicoid mRNA localization reveals a novel microtubule-dependent axis specification pathway. *Cell*. 13;106(1):35-46.

51. Chaigne A, Campillo C, Gov NS, Voituriez R, Azoury J, Umanã-Diaz C, Almonacid M, Queguiner I, Nassoy P, Sykes C. et al. (2013) A soft cortex is essential for asymmetric spindle positioning in mouse oocytes. *Nat. Cell Biol*. 15, 958-966.

52. Chaigne A, Campillo C, Gov NS, Voituriez R, Azoury J, Umaña-Diaz C, Almonacid M, Queguiner I, Nassoy P, Sykes C, Verlhac MH, Terret ME. (2013) A soft cortex is essential for asymmetric spindle positioning in mouse oocytes. *Nat Cell Biol*. 15(8):958-66.

53. Chaigne, A., Campillo, C., Gov, N. S., Voituriez, R., Sykes, C., Verlhac, M. H. and Terret, M. E. (2015). A narrow window of cortical tension guides asymmetric spindle positioning in the mouse oocyte. *Nat. Commun*. 6, 6027.

54. Chakraborty, S., Deb, B.K., Chorna, T., Konieczny, V., Taylor, C.W., Hasan, G. (2016) Mutant IP3 receptors attenuate store-operated Ca2+ entry by destabilizing STIM-Orai interactions in Drosophila neurons. *J. Cell Sci*. 129(20): 3903--3910.

55. Chapman ER, Hanson PI, An S, Jahn R. (1995) Ca2+ regulates the interaction between synaptotagmin and syntaxin 1. *J Biol Chem.* 270: 23667– 23671.

56. Chapman ER. (2002) Synaptotagmin: A Ca(2+) sensor that triggers exocytosis? *Nat Rev Mol Cell Biol.* 3: 498–508.

57. Chasan R, Jin Y, Anderson KV. (1992). Activation of the easter zymogen is regulated by five other genes to define dorsal–ventral polarity in the Drosophila embryo. *Development* 115,607–616.

58. Chen W, Barszczyk A, Turlova E, Deurloo M, Liu B, Yang BB, Rutka JT, Feng Z, Sun H. (2015) Inhibition of TRPM7 by carvacrol suppresses glioblastoma cell proliferation, migration and invasion. *Oncotarget*. 6: 16321-16340.

59. Chen Y, Takizawa N, Crowley JL, Oh SW, Gatto CL, Kambara T, Sato O, Li XD, Ikebe M, Luna EJ. (2003) F-actin and myosin II binding domains in supervillin. *J Biol Chem.* 14;278(46):46094-106.

60. Chevesich J, Kreuz AJ, Montell C. (1997) Requirement for the PDZ domain protein, INAD, for localization of the TRP store-operated channel to a signaling complex. *Neuron*. 18(1):95-105. doi: 10.1016/s0896-6273(01)80049-0.

61. Chiba K. (2020) Oocyte Maturation in Starfish. *Cells*. 19;9(2):476

62. Chin D, Means AR. (2000) Calmodulin: a prototypical calcium sensor. *Trends Cell Biol.* 10(8):322-8.

63. Choi T, Fukasawa K, Zhou R, Tessarollo L, Borror K, Resau J, Vande Woude GF. 1996. The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. *PNAS USA.* 93, 7032–7035.

64. Chorna-Ornan I, Tzarfaty V, Ankri-Eliahoo G, Joel-Almagor T, Meyer NE, Huber A, Payre F, Minke B. (2005) Light-regulated interaction of Dmoesin with TRP and TRPL channels is required for maintenance of photoreceptors. *J Cell Biol*. 10;171(1):143-52.

65. Christensen AP, and Corey DP. (2007) TRP channels in mechanosensation: direct or indirect activation? *Nat Rev Neuro*, 8:510-521

66. Chugh P, Paluch EK. (2018) The actin cortex at a glance. *J Cell Sci.* Jul 19;131(14):jcs186254.

67. Churchill GC, O'Neill JS, Masgrau R, Patel S, Thomas JM, Genazzani AA, Galione A. (2003) Sperm deliver a new second messenger: NAADP. *Curr Biol*. Jan 21;13(2):125-8.

68. Clausen MP, Colin-York H, Schneider F, Eggeling C, Fritzsche M. (2017) Dissecting the actin cortex density and membrane-cortex distance in living cells by super-resolution microscopy. J Phys D Appl Phys. Feb 15;50(6):064002

69. Clift D, Schuh M. (2015) A three-step MTOC fragmentation mechanism facilitates bipolar spindle assembly in mouse oocytes. *Nat. Commun*. 6:7217

70. Colledge WH, Carlton MB, Udy GB, Evans MJ. 1994. Dis- ruption of c-mos causes parthenogenetic development of unfertilized mouse eggs. *Nature.* 370, 65–68.

71. Cooper JA. (1987) Effects of cytochalasin and phalloidin on actin. *J Cell Biol*. Oct;105(4):1473-8.

72. Coste B, Mathur J, Schmidt M, Earley TJ, Ranade S, Petrus MJ, Dubin AE, Patapoutian A. (2010) Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science.* 330: 55-60

73. Cox RT, Spradling AC. (2003) A Balbiani body and the fusome mediate mitochondrial inheritance during Drosophila oogenesis. *Development*. 130(8):1579-90. doi: 10.1242/dev.00365.

74. Cran DG, Moor RM, Irvine RF. (1988) Initiation of the cortical reaction in hamster and sheep oocytes in response to inositol trisphosphate. *J Cell Sc*i 91:139–144.

75. Crossley I, Whalley T, Whitaker M. (1991) Guanosine 5-thiotriphosphate may stimulate phosphoinositide messenger production in sea urchin eggs by a different route than the fertilizing sperm. *Cell Regul* 2:121–133.

76. Cukovic D, Ehlting J, VanZiffle JA, and Douglas CJ. (2001) Structure and evolution of 4-coumarate:coenzyme A ligase (4CL) gene families. *Biol Chem.* 382:645-654 Curr Opin Neurobiol. 5(3):304-9.

77. Dahlgaard K, Raposo AA, Niccoli T, St Johnston D. (2007) Capu and Spire assemble a cytoplasmic actin mesh that maintains microtubule organization in the Drosophila oocyte. *Dev Cell*. Oct;13(4):539-53.

78. Dalghi MG, Fernández MM, Ferreira-Gomes M, et al. (2013) Plasma membrane calcium ATPase activity is regulated by actin oligomers through direct interaction. *J Biol Chem*. 288(32):23380-23393.

79. Dantas BP, Alves QL, de Assis KS, Ribeiro TP, de Almeida MM, de Vasconcelos AP, de Araujo DA, de Andrade Braga V, de Medeiros IA, Alencar JL, and Silva DF. (2015) Participation of the TRP channel in the cardiovascular effects induced by carvacrol in normotensive rat. *Vascul Pharmacol*, 67-69:48-55

80. De Cuevas M, Spradling AC. (1998) Morphogenesis of the Drosophila fusome and its implications for oocyte specification. *Development*. 125(15):2781-9.

81. Dean SO, Rogers SL, Stuurman N, Vale RD, Spudich JA. (2005) Distinct pathways control recruitment and maintenance of myosin II at the cleavage furrow during cytokinesis. *Proc Natl Acad Sci U S A*. Sep 20;102(38):13473-8.

82. Deguchi R, Morisawa M. (2003) External Ca2 is predominantly used for cytoplasmic and nuclear Ca2 increases in fertilized oocytes of the marine bivalve Mactra chinensis. *J Cell Sci* 116:367– 376.

83. Deguchi R, Osanai K, Morisawa M. (1996) Extracellular Ca2 entry and Ca2 re- lease from inositol 1,4,5-trisphosphate- sensitive stores function at fertilization in oocytes of the marine bivalve Mytilus edulis. *Development* 122:3651–3660.

84. Deguchi R. (2007) Fertilization causes a single Ca(2) increase that fully depends on Ca(2) influx in oocytes of limpets (Phylum Mollusca, Class Gastropoda). *Dev Biol* 304:652–663.

85. Deng W, Lin H. (1997) Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in Drosophila. *Dev Biol.* 1; 189(1):79-94.

86. Denis V, Cyert MS. (2002) Internal Ca(2+) release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. *J Cell Biol,* 156(1):29-34.

87. Derrick CJ, York-Andersen AH, and Weil TT. (2016) Imaging calcium in Drosophila at egg activation. *J Vis Exp* 6;(114)

88. Digan ME, Spradling AC, WaringGL, and Mahowald AP. (1979) In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*

89. Doane WW. (1960) Completion of meiosis in uninseminated eggs of Drosophila melanogaster. *Science* 132:677–678.

90. Domínguez DC, Guragain M, Patrauchan M. (2015) Calcium binding proteins and calcium signaling in prokaryotes. *Cell Calcium.* 57(3):151-65.

91. Dominguez R. (2016) The WH2 domain and actin nucleation: necessary but insufficient. *Trends Biochem. Sci.* 41, 478-490.

92. Dong JB, Tang TS, Sun FZ. (2000) Xenopus and chicken sperm contain a cytosolic soluble protein factor which can trigger calcium oscillations in mouse eggs. *Biochem Biophys Res Commun* 268:947– 951.

93. Drechsler M, Giavazzi F, Cerbino R *et al.* (2017) Active diffusion and advection in *Drosophila* oocytes result from the interplay of actin and microtubules. *Nat Commun* 8, 1520.

94. Drechsler M, Lang LF, Al-Khatib L, Dirks H, Burger M, Schönlieb CB, Palacios IM. (2020) Optical flow analysis reveals that Kinesin-mediated advection impacts the orientation of microtubules in the *Drosophila* oocyte. *Mol Biol Cell*. 1;31(12):1246-1258

95. Dryer SE, and Reiser J. (2010) TRPC6 channels and their binding partners in podocytes: role in glomerular filtration and pathophysiology. *Am J Physiol Renal Physiol*, 229:F689-F701

96. Ducibella T, Schultz RM, Ozil JP. (2006) Role of calcium signals in early development. *Semin Cell Dev Biol.* 17: 324– 332.

97. Earley S, Gonzales AL, and Garcia ZI. (2010) A dietary agonist of TRPV3 elicits endothelium dependent vasodilation. *Mol Pharmacol* 10:1124

98. Edwards KA, Demsky M, Montague RA, Weymouth N, and Kiehart DP. (1997) GFP-Moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in Drosophila. *Dev Biol*. 191:103-117

99. Ehrlich BE. (1995) Functional properties of intracellular calcium-release channels. Curr Opin Neurobiol. 5(3):304-9.

100. El-Jouni W, Haun S, Machaca K.(2008) Internalization of plasma membrane Ca2+-ATPase during Xenopus oocyte maturation. *Dev Biol*. 324(1):99-107.

101. Ellen K. LeMosy, Carl Hashimoto. (2000) The Nudel Protease of Drosophila Is Required for Eggshell Biogenesis in Addition to Embryonic Patterning. *Developmental Biology.* 217;2, 352-361.

102. Endow SA, Komma DJ. (1997) Spindle dynamics during meiosis in Drosophila oocytes. *J Cell Biol.* 16;137(6):1321-36.

103. Fahrner M, Schindl R, Muik M, Derler I, Romanin C. (2017) The STIM-Orai pathway: The interactions between STIM and Orai. *Adv Exp Med Biol.* 993:59-81.

104. Falzone TT, Lenz M, Kovar DR, Gardel ML. (2012) Assembly kinetics determine the architecture of α-actinin crosslinked F-actin networks. *Nat Commun*. May 29;3:861.

105. Fang Z, Takizawa N, Wilson KA, Smith TC, Delprato A, Davidson MW, Lambright DG, and Luna EL (2010) The membrane-associated protein, supervillin, accelerates f-actin-dependent rapid integral recycling and cell motility. *Traffic.* 11(6):782-99.

106. Fechheimer M, Brier J, Rockwell M, Luna EJ, and Taylor DL. (1982) A calcium and pH regulated actin binding protein from D. discoideum. *Cell Motil Cytoskeleton*, 2:287-308

107.Ferrandon D, Elphick L, Nüsslein-Volhard C, St Johnston D. (1994) Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. *Cell*. 30;79(7):1221-32.

108.Fievet B, Louvard D, and Arpin M. (2007) ERM proteins in epithelial cell organisation and functions. *Biochim Biophys Acta*. 1773:653-660

109. Fissore RA, Robl JM. (1994) Mechanism of calcium oscillations in fertilized rabbit eggs. *Dev Biol* 166:634–642.

110. Forrest KM, Gavis ER. (2003) Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in Drosophila. *Curr Biol.* 13(14):1159-68.

111. Fritzsche M, Lewalle A, Duke T, Kruse K, Charras G. (2013) Analysis of turnover dynamics of the submembranous actin cortex. *Mol Biol Cell* 24: 757–767.

112. Fuller MT, Spradling AC. (2007) Male and female *Drosophila* germline stem cells: Two versions of immortality. *Science.* 316:402–404

113. Furukawa R, Maselli A, Thomson SA, Lim RW, Stokes JV, and Fechheimer M. (2003) Calcium regulation of actin crosslinking is important for function of the actin cytoskeleton in Dictyostelium. *J Cell Sci* 1;116(pt 1):187-96

114. Galione A, McDougall A, Busa WB, Willmott N, Gillot I, Whitaker M. (1993) Redundant mechanisms of calcium-induced calcium release underlying calcium waves during fertilization of sea urchin eggs. *Science*. 16; 261(5119):348-52.

115. Garg R, Peddada N, Sagar A, Nihalani D, Ashish (2011) Visual insight into how low pH alone can induce actin-severing ability in gelsolin under calcium-free conditions. *J. Biol. Chem. 286*, 20387–20397

116. Gavis ER, Chatterjee S, Ford NR, Wolff LJ. (2008) Dispensability of nanos mRNA localization for abdominal patterning but not for germ cell development. *Mech Dev*. 125(1-2):81-90.

117. Gerard GF, D'Alessio JM, Kotewicz ML, Noon MC. (1986) Influence on stability in Escherichia coli of the carboxy-terminal structure of cloned Moloney murine leukemia virus reverse transcriptase. *DNA*. 5(4):271-9.

118. Gilkey JC, Jaffe LF, Ridgway EB, Reyn- olds GT. (1978) A free calcium wave traverses the activating egg of the medaka, Oryzias latipes. *J Cell Biol* 76: 448 – 466.

119. Gilliland WD, Hughes SF, Vietti DR, Hawley RS. (2009) Congression of achiasmate chromosomes to the metaphase plate in Drosophila melanogaster oocytes. *Dev Biol*. Jan 1;325(1):122-8

120. Giusti AF, Carroll DJ, Abassi YA, Terasaki M, Foltz KR, Jaffe LA. (1999) Requirement of a Src family kinase for initiating calcium release at fertilization in starfish eggs. *J Biol Chem* 274:29318 –29322.

121. Giusti AF, O'Neill FJ, Yamasu K, Foltz KR, Jaffe LA. (2003) Function of a sea urchin egg Src family kinase in initiating Ca2 release at fertilization. Dev Biol 256:367–378.

122. Goel M, Garcia R, Estacion M, Schilling WP. (2001) Regulation of Drosophila TRPL channels by immunophilin FKBP59. *J Biol Chem.* 19;276(42):38762-73. doi: 10.1074/jbc.M104125200.

123. Goldstein B, Hird SN. (1996) Specification of the anteroposterior axis in Caenorhabditis elegans. *Development*. 122:1467–1474.

124. González-Reyes A, Elliott H, St Johnston D. (1995) Polarization of both major body axes in Drosophila by gurken-torpedo signalling. *Nature*. 22; 375(6533):654-8.

125. Gregor T, Wieschaus EF, McGregor AP, Bialek W, Tank DW. (2007) Stability and nuclear dynamics of the bicoid morphogen gradient. *Cell*. 13; 130(1):141-52.

126. Grieder NC, de Cuevas M, Spradling AC. (2000) The fusome organizes the microtubule network during oocyte differentiation in Drosophila. *Development*. 127(19):4253-64.

127. Gual et al. (2002) A Crk-II/TC10 signalling pathway is required for osmotic shock-stimulated glucose transport. *J Biol Chem*. 15;277(46):43980-6.

128. Guha M, Zhou M, Wang YL. (2005) Cortical actin turnover during cytokinesis requires myosin II. *Curr Biol.* Apr 26;15(8):732-6.

129. Gulyas BJ. (1979) Cortical granules of mammalian eggs. *Int Rev Cytol* 63: 357–392.

130. Hachem A, Godwin J, Ruas M, Lee HC, Ferrer Buitrago M, Ardestani G, Bassett A, Fox S, Navarrete F, de Sutter P, Heindryckx B, Fissore R, Parrington J. (2017) PLCζ is the physiological trigger of the Ca2+ oscillations that induce embryogenesis in mammals but conception can occur in its absence. *Development*. 144(16):2914-2924.

131. Hahle A, Merz S, Meyners C, and Hausch F. (2019) The many faces of FKBP51. *Biomolecules*, 9(1):35

132. Hall RA, Premont RT, Lefkowitz RJ. (1999) Heptahelical receptor signaling: beyond the G protein paradigm. *J Cell Biol* 145: 927–932.

133. Hamaguchi Y, Mabuchi I. (1986) Alpha-actinin accumulation in the cortex of echinoderm eggs during fertilization. *Cell Motil Cytoskelet*. 6:549-559

134. Hamamoto S, Mori Y, Yabe I, Uozumi N. (2018) In vitro and in vivo characterization of modulation of the vacuolar cation channel TRPY1 from Saccharomyces cerevisiae. *FEBS J,* 285(6):1146-1161.

135. Handel MA, Schimenti JC. (2010) Genetics of mammalian meiosis: regulation, dynamics and impact on fertility. *Nat Rev Genet*. Feb;11(2):124-36.

136. Harada K, Oita E, Chiba K. (2003) Metaphase I arrest of starfish oocytes induced via the MAP kinase pathway is released by an increase of intracellular pH. *Development*. Oct;130(19):4581-6.

137. Hashimoto N, Watanabe N, Furuta Y, Tamemoto H, Sagata N, Yokoyama M, Okazaki K, Nagayoshi M, Takeda N, Ikawa Y. 1994. Parthenogenetic activation of oocytes in c-mos- deficient mice. *Nature.* 370, 68–71.

138. Heald R, McKeon F. (1990) Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell* 61:579–589.

139. Heald R, Tournebize R, Blank T, Sandaltzopoulos R, Becker P, et al. (1996) Self-organization of microtubules into bipolar spindles around artificial chromosomes in Xenopus egg extracts. *Nature.* 382:420–25

140. Heifetz Y, Yu J, Wolfner MF. (2001) Ovulation triggers activation of Drosophila oocytes. *Dev Biol* 234:416–424.

141. Higgins MJ, Walker VK, Holden JA, White BN. (1984) Isolation of two Drosophila melanogaster genes abundantly expressed in the ovary during vitelline membrane synthesis. *Dev. Biol*. 105: 155-165.

142. Hill DP, Strome S. (1988) An analysis of the role of microfilaments in the establishment and maintenance of asymmetry in Caenorhabditis elegans zygotes. Dev Biol. 125(1):75-84.

143. Hill DP, Strome S. (1990) Brief cytochalasin-induced disruption of microfilaments during a critical interval in 1-cell C. elegans embryos alters the partitioning of developmental instructions to the 2-cell embryo. *Development*. 108(1):159-72.

144. Hirao M, Sato N, Kondo T, Yonemura S, Monden M, Sasaki T, Takai Y, Tsukita S, Tsukita S. (1996) Regulation mechanism of ERM (ezrin/radixin/moesin) protein/

plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J Cell Biol*. 37–51

145. Hofmann T, Chubanov V, Chen X, Dietz AS, Gudermann T, and Montell C. (2010) Drosophila TRPM channel is essential for the control of extracellular magnesium levels. *PLoS One* 6;5(5):e10519

146. Holmes KC, Popp D, Gebhard W, Kabsch W. (1990) Atomic model of the actin filament. *Nature.* 347: 44–49.

147. Holubcová, Z., Howard, G. and Schuh, M. (2013) Vesicles modulate an actin network for asymmetric spindle positioning. *Nat. Cell Biol*. 15, 937-947.

148. Horner VL and Wolfner MF. (2008) Transitioning From Egg to Embryo: Triggers and Mechanisms of Egg Activation. *Developmental Dynamics* 237:527-544

149. Horner VL, and Wolfner MF (2008) Mechanical stimulation by osmotic and hydrostatic pressure activates Drosophila oocytes in vitro in a calcium-dependent manner. *Dev Biol*. 316:100-109

150. Horner VL, Czank A, Jang JK, Singh N, Williams BC, Puro J, Kubli E, Hanes SD, McKim KS, Wolfner MF. et al. (2006) The Drosophila calcipressin sarah is required for several aspects of egg activation. *Curr Biol.* 16:1441–1446.

151. Horner VL, Czank A, Jang JK, Singh N, Williams BC, Puro J, Kubli E, Hanes SD, Mckim KS, Wolfner MF and Goldberg ML. (2006) The Drososphila calcipressin sarah is required for several aspects of egg activation. *Curr Biol*. 16:1441-1446

152. Horner, V.L., and Wolfner, M.F. (2008a) Transitioning from egg to embryo: triggers and mechanisms of egg activation. *Dev Dyn*, 237:527-544

153. Horner, V.L., and Wolfner, M.F. (2008b) Mechanical stimulation by osmotic and hydrostatic pressure activates Drosophila oocytes in vitro in a calcium-dependent manner. *Dev Biol* 316:100-109

154. Houts GE, Miyagi M, Ellis C, Beard D, Beard JW. (1979) Reverse transcriptase from avian myeloblastosis virus. *J Virol.* 29(2):517-22.

155. Hu Q, Vélez-Avilés AN, Wolfner MF. (2020) *Drosophila* Plc21C is involved in calcium wave propagation during egg activation. *MicroPubl Biol.* 2020:10.17912/micropub.biology.000235.

156. Hu Q, Wolfner MF. (2019) The *Drosophila* Trpm channel mediates calcium influx during egg activation. *Proc Natl Acad Sci U S A*. 17;116(38):18994-19000.

157. Hu Q, Wolfner MF. (2020) Regulation of Trpm activation and calcium wave initiation during Drosophila egg activation. *Mol Reprod Dev.* 87(8):880-886.

158. Huber A, Sander P, Gobert A, Bähner M, Hermann R, Paulsen R. (1996) The transient receptor potential protein (Trp), a putative store-operated Ca2+ channel essential for phosphoinositide-mediated photoreception, forms a signaling complex with NorpA, InaC and InaD. *EMBO J*. 16;15(24):7036-45.

159. Hudson AM, Cooley L. (2002) A subset of dynamic actin rearrangements in Drosophila requires the Arp2/3 complex. *J Cell Biol.* 18; 156(4):677-87.

160. Hughes SE, Miller DE, Miller AL, Hawley RS. (2018) Female Meiosis: Synapsis, Recombination, and Segregation in *Drosophila melanogaster*. *Genetics*. 208(3):875-908.

161. Huynh JR, Shulman JM, Benton R, St Johnston D. (2001) PAR-1 is required for the maintenance of oocyte fate in Drosophila. *Development*. 128(7):1201-9.

162. Huynh JR, St Johnston D. (2004) The origin of asymmetry: early polarisation of the Drosophila germline cyst and oocyte. *Curr Biol.* 8; 14(11):R438-49.

163. Ikeno T, Nagano T, Hanaoka K. (2017) Silicon-substituted Xanthene Dyes and Their Unique Photophysical Properties for Fluorescent Probes. *Chem Asian J,* p.12.

164. Inoue D, Ohe M, Kanemori Y, Nobui T, Sagata N. (2007) A direct link of the Mos-MAPK pathway to Erp1/Emi2 in meiotic arrest of Xenopus laevis eggs. *Nature.* 446: 1100 –1104.

165. Irion U, St Johnston D. (2007) bicoid RNA localization requires specific binding of an endosomal sorting complex. *Nature*. 1;445(7127):554-8.

166. Ito J, Shimada M, Terada T. 2003. Effect of protein kinase C activator on mitogen-activated protein kinase and p34cdc2 kinase activity during parthenogenetic activation of porcine oocytes by calcium ionophore. *Biology of Reproduction* 69, 1675–1682.

167. Ito, J., and Kashiwazaki, N. (2012) Molecular mechanism of fertilisation in the pig. *Animal Science*. 83, 669-682

168. Ito, J., Yoon, S.Y., Lee, B., Vanderheyden, V., Vermassen, E., Wojcikiewicz, R., Alfandari, D., De Smedt, H., Parys, J.B., and Fissore, R.A. (2008b) Inositol 1,4,5-trisphosphate receptor 1, a wide- spread Ca^{2+} channel, is a novel substrate of polo-like kinase 1 in eggs. *Developmental Biology* 320, 402–413

169. Ito, J., Yoshida, T., Kasai, Y., Wakai, T., Parys, J.B., Fissore, R.A., and Kashiwazaki, N. (2010b) Phosphorylation of inositol 1,4,5- triphosphate receptor 1 during in vitro maturation of porcine oocytes. *Animal Science Journal* 81, 34–41

170. Iwasaki H, Chiba K, Uchiyama T, Yoshikawa F, Suzuki F, Ikeda M, Furuichi T, Mikoshiba K. (2002) Molecular characterization of the starfish inositol 1,4,5-trisphosphate receptor and its role during oocyte maturation and fertilization. *J Biol Chem.* Jan 25;277(4):2763-72.

171. Jaffe L.F. (1999) Organization of early development by calcium patterns. *BioEssays*. 21:657–667.

172. Jaffe LA, Turner PR, Kline D, Kado RT, Shilling F. (1988) G-proteins and egg activation. *Cell Differ Dev* 25(Suppl):15– 18.

173. Jaffe LF, Créton R. (1998) On the conservation of calcium wave speeds. *Cell Calcium*. 24(1):1-8.

174. Jaffe LF. (2008) Calcium waves. *Philos Trans R Soc Lond B Biol Sci*, 363:1311-1316.

175. Jaffe LF. Calcium waves. (2008) *Philos Trans R Soc Lond B Biol Sci*. 363(1495):1311-6.

176. Jain RA, Gavis ER. (2008) The Drosophila hnRNP M homolog Rumpelstiltskin regulates nanos mRNA localization. *Development*. 135(5):973-82.

177. Jang JK, Rahman T, McKim KS. (2005) The kinesinlike protein Subito contributes to central spindle assembly and organization of the meiotic spindle in Drosophila oocytes. *Mol Biol Cell.* 16(10):4684-94

178. Janmey PA, Stossel TP, Allen PG. (1998) Deconstructing gelsolin: identifying sites that mimic or alter binding to actin and phosphoinositides. *Chem Biol.* 5(4):R81-5.

179. Jayadev R, Kuk CY, Low SH, and Murata-Hori M. (2012) Calcium sensitivity of α-actinin is required for equatorial actin assembly cytokinesis. *Cell Cycle*, 15;11(10):1929-37

180. Jewett, C.E., Vanderleest, T.E., Miao, H. *et al.* (2017) Planar polarized Rab35 functions as an oscillatory ratchet during cell intercalation in the *Drosophila* epithelium. *Nat Commun.* 8, 476.

181. Jones KT, Matsuda M, Parrington J, Ka- tan M, Swann K. (2000) Different Ca2-releasing abilities of sperm extracts compared with tissue extracts and phospholipase C isoforms in sea urchin egg homogenate and mouse eggs. *Biochem J* 346:743–749.

182. Jones KT, Soeller C, Cannell MB. (1998) The passage of Ca2 and fluorescent markers between the sperm and egg after fusion in the mouse. *Developmen*t. 125:4627–4635.

183. Jones KT. (2005) Mammalian egg activation: from Ca2+ spiking to cell cycle progression. *Reproduction*. 130, pp. 813-823

184. Kabsch W, Mannherz HG, Suck D, Pai EF, Holsmes KC. (1990) Atomic structure of the actin:DNase I complex. *Nature.* 347: 37–44.

185. Kaji N, Ohashi K, Shuin M, Niwa R, Uemura T, Mizuno K. (2003) Cell cycle-associated changes in Slingshot phosphatase activity and roles in cytokinesis in animal cells. *J Biol Chem*. Aug 29;278(35):33450-5.

186. Kaneuchi T, Wolfner MF, and Aigaki T. (2015) A calcium rise occurs as activating Drosophila eggs move through the female reproductive tract. *Mol Reprod Dev* 82(7-8):501

187. Kashir J, Nomikos M, Lai FA, Swann K. (2014) Sperm-induced Ca2+ release during egg activation in mammals. *Biochem Biophys Res Commun*. 450(3):1204-11.

188. Kawasaki H, Kretsinger RH. (1994) Calcium-binding proteins. 1: EF-hands. *Protein Profile* 1:343–517.

189. Kawasaki H, Kretsinger RH. (2017) Structural and functional diversity of EF-hand proteins: Evolutionary perspectives. *Protein Sci*. 26(10):1898-1920. doi: 10.1002/pro.3233.

190. Keizer J, Li YX, Stojilkovic S, Rinzel J. 1995. InsP3-induced Ca2+ excitability of the endoplasmic reticulum. *Molecular Biology of the Cell* 6, 945–951.

191. Kelkar M, Bohec P, Charras G. (2020) Mechanics of the cellular actin cortex: From signalling to shape change. *Curr Opin Cell Biol.* 66:69-78.

192. Kelso RJ, Hudson AM, Cooley L. (2002) Drosophila Kelch regulates actin organisation via Src64-dependent tyrosine phosphorylation. *J Cell Biol.* 156:703-713

193. Khamviwath V, Hu J, Othmer HG. (2013) A continuum model of actin waves in Dictyostelium discoideum. *PLoS One.* 31;8(5):e64272

194. Kiselar JG, Janmey PA, Almo SC, Chance MR. (2003) Visualizing the Ca2+-dependent activation of gelsolin by using synchrotron footprinting. *Proc. Natl. Acad. Sci. U.S.A.* 100, 3942– 3947

195. Kiselar JG, Janmey PA, Almo SC, Chance MR. (2003) Visualizing the Ca2+-dependent activation of gelsolin by using synchrotron footprinting. *PNAS USA*. 100 (7): 3942–7.

196. Kishimoto T. (1998) Cell cycle arrest and release in starfish oocytes and eggs. *Semin Cell Dev Biol.* Oct;9(5):549-57.

197. Kline, D., and Kline, J. T. (1992a). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol*. 149, 80 – 89.

198. Kline, D., and Kline, J. T. (1992b). Thapsigargin activates a calcium influx pathway in the unfertilized mouse egg and suppresses repetitive calcium transients in the fertilized egg. *J. Biol. Chem*. 267, 17624 –17630.

199. Knott JG, Kurokawa M, Fissore RA, Schultz RM, Williams CJ. (2005) Transgenic RNA interference reveals role for mouse sperm phospholipase Czeta in triggering Ca2 oscillations during fertilization. *Biol Reprod* 72:992–996.

200. Kohler, R. E. (1994). Lords of the fly: Drosophila genetics and the experimental life. *University of Chicago Press*.

201. Kotewicz ML, D'Alessio JM, Driftmier KM, Blodgett KP, Gerard GF. (1985) Cloning and overexpression of Moloney murine leukemia virus reverse transcriptase in Escherichia coli. *Gene*. 35(3):249-58.

202. Kotewicz ML, Sampson CM, D'Alessio JM, Gerard GF. (1988) Isolation of cloned Moloney murine leukemia virus reverse transcriptase lacking ribonuclease H activity. Nucleic Acids Res. 16(1):265-77.

203. Kouchi Z, Fukami K, Shikano T, Oda S, Nakamura Y, Takenawa T, Miyazaki S. (2004) Recombinant phospholipase Czeta has high Ca2+ sensitivity and induces Ca2+ oscillations in mouse eggs. *J. Biol. Chem.*, 279, pp. 10408-10412

204. Kouchi Z, Shikano T, Nakamura Y, Shirakawa H, Fukami K, Miyazaki S. (2005) The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase Czeta. *J. Biol. Chem.*, 280, pp. 21015-21021

205. Krauchunas AR, Marcello MR, Singson A. (2016) The molecular complexity of fertilization: Introducing the concept of a fertilization synapse. *Mol Reprod Dev*. 83(5):376-86.

206. Kretsinger RH, Nockolds CE. (1973) Carp muscle calcium-binding protein II. Structure determination and general description. *J Biol Chem.* 248:3313–3326.

207. Kuboniwa H, Tjandra N, Grzesiek S, Ren H, Klee CB, Bax A. (1995) Solution structure of calcium-free calmodulin. *Nat. Struct. Biol.* 2, pp. 768-776

208. Kunda P, Pelling AE, Liu T, Baum B. (2008) Moesin controls cortical rigidity, cell rounding, and spindle morphogenesis during mitosis. *Curr Biol*. Jan 22;18(2):91-101.

209. Kuo SP, Trussell LO. (2009) A new ion channel blooms at the synapse. *Neuron*. Sep 10;63(5):566-7.

210. Kuo RC, Baxter GT, Thompson SH, Stricker SA, Patton C, Bonaventura J, Epel D. (2000) NO is necessary and sufficient for egg activation at fertilization. *Nature*. Aug 10;406(6796):633-6.

211. Kwiatkowski DJ. (1999) Functions of gelsolin: motility, signaling, apoptosis, cancer. *Curr Opin Cell Biol*. 11(1):103-8.

212. Kyozuka K, Chun JT, Puppo A, Gragnaniello G, Garante E, and Santella L. (2008) Actin cytoskeleton modulates calcium signalling during maturation of starfish oocytes. *Dev Biol* 320:426-435

213. Kyozuka K, Deguchi R, Mohri T, Miyazaki S. (1998) Injection of sperm extract mimics spatiotemporal dynamics of Ca2 responses and progression of meiosis at fertilization of ascidian oocytes. *Development* 125:4099–4105.

214. LaFleur GJ Jr, Horiuchi Y, Wessel GM. (1998) Sea urchin ovoperoxidase: oocyte-specific member of a heme-dependent peroxidase superfamily that functions in the block to polyspermy. *Mech Dev.* 70: 77– 89.

215. Lai L., Kolber-Simonds, D., Park, K.W., Cheong, H.T., Greenstein, J.L., Im, G.S., et al. (2002) Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science,* 295:1089-92

216. Lai, L., Kang, J.X., Li, R., Wang J., Witt, W.T., Yong, H.Y., et al. (2006) Generation of cloned transgenic pigs rich in omega-3 fatty acids. *Nature biotechnology,* 24:435-6.

217. Lange M, and Peiter E. (2020) Calcium Transport Proteins in Fungi: The Phylogenetic Diversity of Their Relevance for Growth, Virulence, and Stress Resistance. *Front Microbiol*, 10:3100

218. Lanner JT, Georgiou DK, Joshi AD, Hamilton SL. (2010) Ryanodine receptors: structure, expression, molecular details, and function in calcium release. *Cold Spring Harb Perspect Biol*. 2(11):a003996.

219. Leckie C, Empson R, Becchetti A, Thomas J, Galione A, Whitaker M. (2003) The NO pathway acts late during the fertilization response in sea urchin eggs. *J Biol Chem*. Apr 4;278(14):12247-54.

220. Lee HC, Yoon SY, Lykke-Hartmann K, Fissore RA, and Carvacho I. (2016) TRPV3 channels mediate Ca2+ influx induced by 2-ABP in mouse eggs. *Cell Calcium* 59:21-31

221. Lee, B., Vermassen, E., Yoon, S.Y., Vanderheyden, V., Ito, J., Alfandari, D., De Smedt, H., Parys, J.B., and Fissore, R.A. (2006) Phosphorylation of IP_3R1 and the regulation of $[Ca^{2+}]i$ responses at fertilization: a role for the MAP kinase pathway. *Development.* 133, 4355–4365

222. Lee, K., Davis, A., Zhang, L., Ryu, J., Spate, L.D., Park, K., Samuel, M.S., Walters, E.M., Murphy, C.N., Machaty, Z., and Prather, R.S. (2015) Pig oocyte activation utilising a Zn2+ chelator, TPEN. *Theriogenology*, 84(6):1024-1032

223. Leguia M, Conner S, Berg L, Wessel GM. (2006) Synaptotagmin I is involved in the regulation of cortical granule exocytosis in the sea urchin. *Mol Reprod Dev.* 73: 895– 905.

224. LeMosy EK, Hashimoto C. (2000) The nudel protease of Drosophila is required for eggshell biogenesis in addition to embryonic patterning. *Dev Biol* 217:352–361.

225. LeMosy EK, Kemler D, Hashimoto C. (1998). Role of Nudel protease activation in triggering dorsoventral polarization of the Drosophila embryo. *Development*. 125,4045– 4053.

226. Lénárt P, Bacher CP, Daigle N, Hand AR, Eils R, Terasaki M, and Ellenberg J. (2005) A contractile nuclear actin network drives chromosome congression in oocytes. *Nature*. 436:812– 818.

227. Li R, Leblanc J, He K, Liu XJ. (2016) Spindle function in Xenopus oocytes involves possible nanodomain calcium signaling. *Mol Biol Cell.* 1;27(21):3273-3283.

228. Li TN, Chen YJ, Lu TY, Wang YT, Lin HC, Yao CK. (2020). A positive feedback loop between Flower and PI(4,5)P2 at periactive zones controls bulk endocytosis in Drosophila. *eLife*, *9*, e60125.

229. Li Y, Gorbea C, Mahaffey D, Rechsteiner M, Benezra R. (1997) MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. *Proc Natl Acad Sci U S A.* 94:12431–12436.

230. Li, H., Guo, F., Rubinstein, B. and Li, R. (2008) Actin-driven chromosomal motility leads to symmetry breaking in mammalian meiotic oocytes. *Nat. Cell Biol*. 10, 1301-1308.

231. Liedtke CM, Wang X, Smallwood ND. (2005) Role for Protein Phosphatase 2A in the Regulation of Calu-3 Epithelial Na+-K+-2Cl–, Type 1 Co-transport Function. *J. Biol. Chem.* 280, 25491–25498.

232. Lim D, Lange K, Santella L. (2002) Activation of oocytes by latrunculin A. *FASEB J*. 16(9):1050-6

233. Lin H, Spradling AC. (1995) Fusome asymmetry and oocyte determination in Drosophila. *Dev Genet.* 16(1):6-12.

234. Lin H, Yue L, Spradling AC. (1994) The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development*. 120(4):947-56.

235. Lindsay LL, Hertzler PL, Clark WH Jr. (1992) Extracellular Mg2+ induces an intracellular Ca2+ wave during oocyte activation in the marine shrimp Sicyonia ingentis. *Dev Biol*. Jul;152(1):94-102.

236. Liu C, Montell C. (2015) Forcing open TRP channels: Mechanical gating as a unifying activation mechanism. *Biochem Biophys Res Commun*. 460(1):22-25.

237. Liu J, Maller JL. (2005) Calcium elevation at fertilization coordinates phosphorylation of XErp1/Emi2 by Plx1 and CaMK II to release metaphase arrest by cytostatic factor. *Curr Biol.* 15:1458–1468.

238. Liu, C., Liu, H., Luo, Y., Lu, T., Fu, X., Cui, S., Zhu, S., and Hou, Y. (2019) The extracellular calcium-sensing receptor promotes porcine egg activation via calcium/calmodulin-dependent protein kinase II. *Mol Reprod Dev,* 1002/mrd.23322.

239. Lui L, Li Y, Wang R, Yin C, Dong Q, Hing H, Kim C, and Welsh MJ. (2007) Drosophila hygrosensation requires the TRP channels water witch nanchung. *Nature.* 450(7167):294-8.

240. Lukinavičius G et al. (2013) A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nat. Chem*. 5,2:132–139.

241. Lukinavičius G et al. (2013) Fluorogenic probes for live-cell imaging of the cytoskeleton. *Nat. Methods*. 11,7:731–733, doi: 10.1038/nmeth.2972.

242. Luksza M, Queguigner I, Verlhac MH, Brunet S. (2013) Rebuilding MTOCs upon centriole loss during mouse oogenesis. *Dev. Biol*. 382:48–56

243. Luttrell LM, Lefkowitz RJ. (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled recep- tor signals. *J Cell Sci* 115:455–465.

244. Ma YC, Huang J, Ali S, Lowry W, Huang XY. (2000) Src tyrosine kinase is a novel direct effector of G proteins. *Cell* 102:635– 646.

245. Machaty Z, Wang C, Lee K, and Zhang L. (2017) Fertility: Store-operated Ca2+ entry in germ cells: Role in egg activation. *Adv Exp Med Biol.* 993:577-593

246. Machesky LM, and Insall RH. (1998) Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. *Curr. Biol.* 8, 1347-1356.

247. Macianskiene R, Martisiene I, Zablockaite D, and Gendviliene V. (2012) Characterization of Mg2+ regulated TRPM7-like current in human atrial myocytes. *J Biomed Sci*, 14;19:75

248. Mackie GO, Meech RW. (1985) Separate sodium and calcium spikes in the same axon. *Nature*. 313(6005):791-3.

249. MacLennan M, Crichton JH, Playfoot CJ, Adams IR. (2015) Oocyte development, meiosis and aneuploidy. *Semin Cell Dev Biol*. n45:68-76.

250. Madgwick S, Hansen DV, Levasseur M, Jackson PK, Jones KT. (2006) Mouse Emi2 is required to enter meiosis II by reestablishing cyclin B1 during interkinesis. *J Cell Biol.* 174:791–801.

251. Madgwick S, Levasseur M, Jones KT. (2005) Calmodulin-dependent protein kinase II, and not protein kinase C, is sufficient for triggering cell-cycle resumption in mammalian eggs. *J Cell Sci.* 118: 3849 –3859.

252. Mahajan-Miklos S, and Cooley L. (1994) The villin-like protein encoded by the Drosophila quail gene is required for actin bundle assembly during oogenesis. *Cell.* 29;78(2):291-301

253. Mahowald AP, Kambysellis M. (1980) Oogenesis. In: M Ashburner, TRF Wright, editors. *Genetics and biology of Drosophila.* Vol. 2. London: Academic Press. p 141– 224.

254. Mahowold AP, Goralski TJ, and Caulton JH. (1983) In vitro activation of Drosophila Eggs. *Dev Biol* 98:437-445

255. Mahowold AP, Goralski TJ, and Caulton JH. (1983) In vitro activation of Drosophila Eggs. *Dev Biol* 98:437-445

256. Malmendal A, Evenas J, Forsen S, Akke M (1999). Structural dynamics in the C-terminal domain of calmodulin at low calcium levels. *J Mol Biol* 293, 883-899

257. Marcelo KL, Means AR, York B. (2016) The Ca(2+)/Calmodulin/CaMKK2 Axis: Nature's Metabolic CaMshaft. *Trends Endocrinol Metab*. 27(10):706-718.

258. Margaritis LH. (1985) The egg-shell of Drosophila melanogaster III. Covalent crosslinking of the chorion proteins involves endogenous hydrogen peroxide. *Tissue Cell*. 1985;17(4):553-9.

259. Markoulaki S, Matson S, Abbott AL, Duci- bella T. (2004) Fertilization stimulates long-lasting oscillations of CaMKII activity in mouse eggs. *Dev Biol.* 272:15–25.

260. Markow TA. (2013) Parents Without Partners: Drosophila as a Model for Understanding the Mechanisms and Evolution of Parthenogenesis. *G3 (Bethesda)*. Apr 9;3(4):757-762.

261. Marshall CB, Nishikawa T, Osawa M, Stathopulos PB, Ikura M. (2015) Calmodulin and STIM proteins: Two major calcium sensors in the cytoplasm and endoplasmic reticulum. *Biochem Biophys Res Commun*. 460(1):5-21.

262. Maruoka ND, Steele DF, Au BP, Dan P, Zhang X, Moore ED, and Fedida D. (2000) α-actinin-2 couples to cardiac Kv1.5 channels, regulating current density and channel localisation in HEK cells. *FEBS* Lett, 473:188-194

263. Maruyama R, Velarde NV, Klancer R, Gordon S, Kadandale P, Parry JM, Hang JS, Rubin J, Stewart-Michaelis A, Schweinsberg P, Grant BD, Piano F, Sugimoto A, Singson A. (2007) EGG-3 regulates cell-surface and cortex rearrangements during egg activation in Caenorhabditis elegans. *Curr Biol*. 18;17(18):1555-60.

264. Maruyama R, Velarde NV, Klancer R, Gordon S, Kadandale P, Parry JM, Hang JS, Rubin J, Stewart-Michaelis A, Schweinsberg P, Grant BD, Piano F, Sugimoto A, Singson A. (2007) EGG-3 regulates cell-surface and cortex rearrangements during egg activation in Caenorhabditis elegans. *Curr Biol.* 17: 1555– 1560.

265. Matova N, Mahajan-Miklos S, Mooseker MS, and Cooley L. (1999) Drosophila quail, a villain- related protein, bundles actin filaments in apoptotic nurse cells. *Development* 126(24):5645-57

266. Matson S, Markoulaki S, Ducibella T. (2006) Antagonists of myosin light chain kinase and of myosin II inhibit specific events of egg activation in fertilized mouse eggs. *Biol Reprod.* 74: 169– 176.

267. Matsubara M, Hayashi N, Jing T, Titani K. (2003) Regulation of endothelial nitric oxide synthase by protein kinase C. *J Biochem.* Jun;133(6):773-81.

268. Matthies HJ, McDonald HB, Goldstein LS, Theurkauf WE. (1996) Anastral meiotic spindle morphogenesis: role of the non-claret disjunctional kinesin-like protein. *J Cell Biol.* 134(2):455-64.

269. Mazzochi C, Benos DJ, and Smith PR. (2006) Interaction of epithelial ion channels with the actin-based cytoskeleton. *Am J Physiol Renal Physiol.* 291:F1113-F1122

270. McDougall A, Gillot I, Whitaker M. (1993) Thimerosal reveals calcium-induced calcium release in unfertilised sea urchin eggs. *Zygote*. 1(1):35-42.

271. McGrail M, Hays TS. (1997) The microtubule motor cytoplasmic dynein is required for spindle orientation during germline cell divisions and oocyte differentiation in Drosophila. *Development*. 124(12):2409-19.

272. McKemy DD, Neuhausser WM, Julius D. (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature*. 416:52-58

273. McLaughlin J.M., Bratu D.P. (2015) Drosophila melanogaster Oogenesis: An Overview. In: Bratu D., McNeil G. (eds) Drosophila Oogenesis. *Methods in Molecular Biology, vol 1328. Humana Press, New York, NY*.

274. Mehlmann LM, Carpenter G, Rhee SG, Jaffe LA. (1998) SH2 domain-mediated activation of phospholipase Cgamma is not required to initiate Ca2 release at fertilization of mouse eggs. *Dev Biol* 203: 221–232.

275. Mehta S, Zhang J. (2015) Dynamic visualization of calcium-dependent signaling in cellular microdomains. *Cell Calcium*. 58(4):333-41. doi:

276. Melki R, Fievez S, Carlier MF. (1996) Continuous monitoring of Pi release following nucleotide hydrolysis in actin or tubulin assembly using 2-amino-6-mercapto-7-methylpurine ribonucleoside and purine-nucleoside phosphorylase as an enzyme-linked assay. *Biochemistry.* 35: 12038–12045.

277. Methfessel C, Witzemann V, Takahashi T, Mishina M, Numa S, Sakmann B. (1986) Patch clamp measurements on Xenopus laevis oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels. *Pflugers Arch*. Dec;407(6):577-88.

278. Meyer RE, Delaage M, Rosset R, Capri M, Aït-Ahmed O. (2010) A single mutation results in diploid gamete formation and parthenogenesis in a Drosophila yemanuclein-alpha meiosis I defective mutant. BMC Genet. Nov 16;11:104.

279. Micklem DR, Adams J, Grünert S, St Johnston D. (2000) Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. *EMBO J*. 19(6):1366-77.

280. Miyazaki S. (1988) Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein mediated periodic calcium rises in golden hamster eggs. J *Cell Biol* 106:345–353.

281. Moccia F, Billington RA, Santella L. (2006) Pharmacological characterization of NAADP-induced Ca2+ signals in starfish oocytes. *Biochem Biophys Res Commun*. Sep 22;348(2):329-36.

282. Mochida S, Hunt T. (2007) Calcineurin is required to release Xenopus egg extracts from meiotic M phase. *Nature.* 449:336 – 341.

283. Mogessie, B. & Schuh M. (2017) Actin protects mammalian eggs against chromosome segregation errors. *Science.* 357.

284. Montaville P, Jégou A, Pernier J, Compper C, Guichard B, Mogessie B, Schuh M, Romet-Lemonne G, Carlier MF. (2014) Spire and Formin 2 synergize and antagonize in regulating actin assembly in meiosis by a ping-pong mechanism. *PLoS Biol*. 25;12(2):e1001795.

285. Montell C. (2005) TRP channels in Drosophila photoreceptor cells. *J Physiol*. 15;567(Pt 1):45-51.

286. Montell DJ, Rorth P, Spradling AC. (1992) Slow border cells, a locus required for a developmentally regulated cell migration during oogenesis, encodes Drosophila C/EBP. *Cell*. 71(1):51-62.

287. Montell DJ, Yoon WH, Starz-Gaiano M. (2012) Group choreography: mechanisms orchestrating the collective movement of border cells. *Nat Rev Mol Cell Biol*. 13(10):631-45.

288. Morone N, Fujiwara T, Murase K, Kasai RS, Ike H, Yuasa S, Usukura J, Kusumi A. Three-dimensional reconstruction of the membrane skeleton at the plasma membrane interface by electron tomography. J Cell Biol 174: 851–862.

289. Mukhina S, Wang YL, Murata-Hori M. (2007) Alpha-actinin is required for tightly regulated remodeling of the actin cortical network during cytokinesis. *Dev Cell*. Oct;13(4):554-65.

290. Muller HJ. (1928) The Production of Mutations by X-Rays. *Proc Natl Acad Sci U S A*. 14(9):714-726.

291. Muller, E.M., Mackin, N.A., Erdman, S.E., and Cunningham, K.W. (2003) Fig1p facilitates Ca2+ influx and cell fusion during mating of Saccharomyces cervisiae. *J Biol Chem*, 3;278(40):38461-9

292. Mullins RD, Heuser JA. and Pollard TD. (1998) The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl. Acad. Sci. USA* 95, 6181-6186.

293. Munro E, Nance J, Priess JR. (2004) Cortical flows powered by asymmetrical contraction transport PAR proteins to establish and maintain anterior-posterior polarity in the early C. elegans embryo. *Dev Cell.* 7(3):413-24.

294. Murthy K, Wadsworth P. (2005) Myosin-II-dependent localization and dynamics of F-actin during cytokinesis. *Curr Biol*. Apr 26;15(8):724-31.

295. Nakai J, Ohkura M, Imoto K. (2001) A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. *Nat Biotechnol*. 19(2):137-41.

296. Nakai M, Ito J, Sato K, Noguchi J, Kaneko H, Kashiwazaki N, Kikuchi K. 2011b. Pre-treatment of sperm reduces success of ICSI in the pig. *Reproduction* 142, 285–293.

297. Nakai M, Kashiwazaki N, Ito J, Maedomari N, Ozawa M, Shino M, Noguchi J, Kaneko H, Kikuchi K. 2011a. Factors affecting fertilization and embryonic development during intracytoplasmic sperm injection in pigs. *The Journal of Reproduction and Development* 57, 183–187.

298. Nashchekin D, Busby L, Jakobs M, Squires I, St Johnston D. (2021) Symmetry breaking in the female germline cyst. *Science*. 12;374(6569):874-879.

299. Nashchekin D, Fernandes AR, St Johnston D. (2016) Patronin/Shot Cortical Foci Assemble the Noncentrosomal Microtubule Array that Specifies the Drosophila Anterior-Posterior Axis. Dev Cell. 11;38(1):61-72.

300. Nebl T, and Fisher PR. (1997) Intracellular Ca2+ signals in Dictyostelium chemotaxis are mediated exclusively by Ca2+ influx. *J. Cell Sci.* 110,2845 -2853.

301. Neher E. Vesicle pools and Ca2+ microdomains: new tools for understanding their roles in neurotransmitter release. (1998) *Neuron*. 20(3):389-99.

302. Neuman-Silberberg FS, Schüpbach T. (1993) The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell*. 75:165-74

303. Neuman-Silberberg FS, Schüpbach T. (1994) Dorsoventral axis formation in Drosophila depends on the correct dosage of the gene gurken. *Development*. 120:2457-63

304. Newman RH, Fosbrink MD, Zhang J. (2011) Genetically encodable fluorescent biosensors for tracking signaling dynamics in living cells. *Chem Rev*. 11; 111(5):3614-66.

305. Newport JW, Kirschner MW. 1984. Regulation of the cell cycle during early Xenopus development. *Cell.* 37, 731– 742.

306. Ni JQ, Zhou R, Czech B, Liu LP, Holderbaum L, Yang-Zhou D, Shim HS, Handler D, Karpowicz P, Binari R, Booker M, Brennecke J, Perkins LA, Hannon GJ, Perrimon N. (2010) A genome-scale shRNA resource for transgenic RNAi in Drosophila.

307. Ni JQ, Zhou R, Czech B, Liu LP, Holderbaum L, Yang-Zhou D, Shim HS, Handler D, Karpowicz P, Binari R, Booker M, Brennecke J, Perkins LA, Hannon GJ, Perrimon N. (2011) TRiP germline vectors pVALIUM20, pVALIUM21 and pVALIUM22.

308. Nishimura Y, Endo T, Kano K, Naito K. 2009. Porcine Aurora A accelerates Cyclin B and Mos synthesis and promotes meiotic resumption of porcine oocytes. *Animal Reproduction Science* 113, 114–124.

309. Nishiyama T, Ohsumi K, Kishimoto T. (2007a) Phosphorylation of Erp1 by p90rsk is required for cytostatic factor arrest in Xenopus laevis eggs. *Nature.* 446:1096 – 1099.

310. Nishiyama T, Yoshizaki N, Kishimoto T, Ohsumi K. (2007b0 Transient activation of calcineurin is essential to initiate em- bryonic development in Xenopus laevis. *Nature.* 449:341–346.

311. Nixon VL, Levasseur M, McDougall A, Jones KT. (2002) Ca(2) oscillations pro- mote APC/C-dependent cyclin B1 degradation during metaphase arrest and completion of meiosis in fertilizing mouse eggs. *Curr Biol.* 12:746–750.

312. Nobes CD, Hall A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*. 7;81(1):53-62

313. Nomikos M, Blayney LM, Larman MG, Campbell K, Rossbach A, Saunders CM, Swann K, Lai FA. (2005) Role of phospholipase C-zeta domains in Ca2+-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca2+ oscillations. *J. Biol. Chem*., 280, pp. 31011-31018

314. Nozaki H, Coleman AW. (2011) A new species of volvox sect. Merrillosphaera (Volvocaceae chlorophyceae) from Texas. *J Phycol*. 47(3):673-679.

315. Nozawa K, Satouh Y, Fujimoto T, Oji A, Ikawa M. (2018) Sperm-borne phospholipase C zeta-1 ensures monospermic fertilization in mice. *Sci Rep*. 8(1):1315.

316. Ohashi S, Naito K, Sugiura K, Iwamori N, Goto S, Naruoka H, Tojo H. 2003. Analyses of mitogen-activated protein kinase function in the maturation of porcine oocytes. *Biology of Reproduction* 68, 604–609.

317. Otomo T, Tomchick DR, Otomo C, Panchal SC, Machius M, and Rosen MK. (2005b) Structural basis of actin filament nucleation and processive capping by a formin homology 2 domain. *Nature.* 433, 488-494.

318. Page AW, and Orr-Weaver TL. (1997) Activation of the meiotic divisions in Drosophila oocytes. *Dev Biol* 183:195-207

319. Page AW, Orr-Weaver TL. (1996) The Dro- sophila genes grauzone and cortex are necessary for proper female meiosis. *J Cell Sci* 109:1707–1715.

320. Page AW, Orr-Weaver TL. (1997) Activation of the meiotic divisions in Drosophila oocytes. *Dev Biol* 183:195–207.

321. Page S.L., Hawley R.S. (2003) Chromosome choreography: the meiotic ballet. *Science*. 301:785–789

322. Palmer, C.P., Zhou, X.L., Lin, J., Loukin, S.H., Kung, C., and Saimi, Y. (2001) A TRP homolog in Saccharomyces cerevisiae forms an intracellular Ca(2+)-permeable

channel in the yeast vacuolar membrane. *Proc Natl Acad Sci U S A,* 98(14):7801-5.

323. Pampel W. (1914) Die weiblichen Geschlechtsorgane der Ichneumoniden. *Zeitschrift für Wissenschaftliche Zoologie,* 108:290-357

324. Pan B, Li J. (2019) The art of oocyte meiotic arrest regulation. Reprod Biol Endocrinol. 17(1):8.

325. Papuga J, Hoffmann C, Dieterle M, Moes D, Moreau F, Tholl S, Steinmetz A, Thomas C. (2010) Arabidopsis LIM proteins: a family of actin bundlers with distinct expression patterns and modes of regulation. *Plant Cell*. 22(9):3034-52.

326. Paré C, Suter B. (2000) Subcellular localization of Bic-D::GFP is linked to an asymmetric oocyte nucleus. *J Cell Sci.* 113 (Pt 12):2119-27.

327. Parekh AB, and Putney JW. (2005) Store-operated calcium channels. *Physiol Rev*, 85(2): 757-810

328. Park HY, Kim SA, Korlach J, Rhoades E, Kwok LW, Zipfel WR, Waxham MN, Webb WW, Pollack L. (2008) Conformational changes of calmodulin upon Ca2+ binding studied with a microfluidic mixer. *Proc Natl Acad Sci U S A*. 15;105(2):542-7.

329. Parnas M, Peters M, Dadon D, Lev S, Vertkinb I, Slutsky I, et al. (2009) Carvacrol is a novel inhibitor of Drosophila TRPL and mammalian TRPM7 channels. *Cell Calcium.* 45:300-309

330. Parrington J, Davis LC, Galione A, Wessel G. (2007) Flipping the Switch: How a Sperm Activates the Egg at Fertilization. *Developmental Dynamics*. 236:2027–2038, 2007

331. Parrington J, Davis LC, Galione A, Wessel G. (2007) Flipping the switch: how a sperm activates the egg at fertilization. *Dev Dyn* 236:2027–2038.

332. Parrington J, Jones KT, Lai A, Swann K. (1999) The soluble sperm factor that causes Ca2 release from sea-urchin (Lytechinus pictus) egg homogenates also triggers Ca2 oscillations after in- jection into mouse eggs. *Biochem* J 341: 1–4.

333. Parrington J, Jones KT, Lai A, Swann K. (2002) Phospholipase C isoforms in mammalian spermatozoa: potential compo- nents of the sperm factor that causes Ca2 release in eggs. *Reproduction* 123: 31–39.

334. Parrington J. (2001) Does a soluble sperm factor trigger calcium release in the egg at fertilization? *J Androl* 22:1–11.

335. Pascucci T, Perrino J, Mahowald AP, Waring GL. (1996) Eggshell assembly in Drosophila: processing and localization of vitelline membrane and chorion proteins. *Dev Biol.* 1;177(2):590-8.

336. Pascucci T, Perrino J, Mahowald AP, Waring GL. (1996) Eggshell assembly in Drosophila: processing and localization of vitelline membrane and chorion proteins. *Dev Biol*. 1;177(2):590-8.

337. Patel S. (2004) NAADP-induced Ca2+ release -- a new signalling pathway. *Biol Cell*. Feb;96(1):19-28.

338. Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A. (2002) A TRP channel that senses cold stimuli and menthol. *Cell.* 108:705-715

339. Pepperell JR, Kommineni K, Buradagunta S, Smith PJ, Keefe DL. (1999) Transmembrane regulation of intracellular calcium by a plasma membrane sodium/calcium exchanger in mouse ova. *Biol Reprod*. 60(5):1137-43.

340. Perez-Terzic CM, Chini EN, Shen SS, Dousa TP, Clapham DE. (1995) Ca2+ release triggered by nicotinate adenine dinucleotide phosphate in intact sea urchin eggs. *Biochem J*. Dec 15;312 (Pt 3)(Pt 3):955-9.

341. Perkins, L.A., Holderbaum, L., Perrimon, N. (2009). Update to TRiP stock collection.

342. Perry AC, Verlhac MH. 2008. Second meiotic arrest and exit in frogs and mice. EMBO Reports 9, 246–251.

343. Pestonjamasp K, Amieva MR, Strassel CP, Nauseef WM, Furthmayr H, Luna EJ. (1995) Moesin, ezrin, and p205 are actin-binding proteins associated with neutrophil plasma membranes. *Mol Biol Cell.* 6(3):247-59.

344. Pestonjamasp KN, Pope RK, Wilfkuhle JD, and Luna EJ. (1997) Supervillin (p205): A novel membrane-associated, F-actin-binding protein in the villin/gelsolin superfamily. *J Cell Biol* 1;139(5):1255-69

345. Pestonjamasp KN, Pope RK, Wulfkuhle JD, Luna EJ. (1997) Supervillin (p205): A novel membrane-associated, F-actin-binding protein in the villin/gelsolin superfamily. *J Cell Biol.* 1;139(5):1255-69.

346. Peter M, Nakagawa J, Doree M, Labbe JC, Nigg EA. (1990a) In vitro disassembly of the nuclear lamina and M-phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 61:591–602.

347. Peter M, Nakagawa J, Doree M, Labbe JC, Nigg EA. (1990b) Identification of major nucleolar proteins as candidate mitotic substances of cdc2 kinase. *Cell* 60:791–801.

348. Peters JM. 2006. The anaphase promoting complex/ cyclosome: a machine designed to destroy. *Nature Reviews Molecular Cell Biology.* 7, 644–656.

349. Petri W, Mindrinos M, Lombard M. (1979) Independence of vitelline membrane and chorion cross-linking in the Drosophila melanogaster eggshell. *J Cell Biol.* 83: 23a.

350. Petronczki M., Siomos M.F., Nasmyth K. (2003) Un ménage à quatre: the molecular biology of chromosome segregation in meiosis. *Cell*. 112:423–440.

351. Pfender, S., Kuznetsov, V., Pleiser, S., Kerkhoff, E. and Schuh, M. (2011). Spire-type actin nucleators cooperate with Formin-2 to drive asymmetric oocyte division. *Curr. Biol*. 21, 955-960.

352. Pijnacker LP, Ferwerda MA. (1976) Experiments on blocking and unblocking of first meiotic metaphase in eggs of the parthenogenetic stick insect Carousius morosus Br. (phasimida insecta) *Journal of embryology and experimental morphology.* 36:383-394

353. Pijnacker, L. P. (1966) Een overzicht van de ovogenese van de zich ongeslachtelijk voortplantende wandelende tak Carousius morosus Br. (Orthoptera, Phasmidae). *Genen en Phoenen* 11, 27-31.

354. Pijnacker, L. P. (1966) Effect of centrifugation of the eggs on the sex of Carousius morosus Br. *Nature.* 1184-1185.

355. Pijnacker, L. P. (1966) The maturation divisions of the parthenogenetic stick insect Carousius morosus Br. (Orthoptera, Phasmidae). *Chromosoma* 19, 99-1 I 2.

356. Pollard TD, Blanchoin L, Mullins RD. (2000) Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu Rev Biophys Biomol Struct.* 29: 545–576.

357. Prager-Khoutorsky M, Bourque CW. (2015) Mechanical basis of osmosensory transduction in magnocellular neurosecretory neurones of the rat supraoptic nucleus. *J Neuroendocrinol*. 27(6):507-15.

358. Prather, R.S., Rowland, R.R., Ewen, C., Trible, B., Kerrigan, M., Bawa, B., et al. (2013) An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. *Journal of Virology,* 87:9538-46

359. Prebil SD, Slapsak U, Pavsic M, IlC G, Puz V, Ribeiro E.D.A, Anrather D, Hartl M, Backman L, Plavec J, Lenarcic B, and Djinovic-Carugo K. (2016) structure and calcium- binding studies of calmodulin-like domain of human non-muscle α-actinin-1. *Sci Rep*, 6:27383

360. Prosser SL, Pelletier L. (2017) Mitotic spindle assembly in animal cells: a fine balancing act. *Nat. Rev. Mol. Cell Biol*. 18:187–201.

361. Pruyne D, Evangelista M, Yang C, Bi E, Zigmond S, Bretscher A, and Boone C. (2002) Role of formins in actin assembly: nucleation and barbed-end association. *Science.* 297, 612-615.

362. Puppo A, Chun JT, Gragnaniello G, Garante E, Santella L. (2008) Alteration of the cortical actin cytoskeleton deregulates Ca2+ signaling, monospermic fertilization, and sperm entry. *PLoS One*. 3(10):e3588.

363. Qu X, Zhang H, Xie Y, Wang J, Chen N, Huang S. (2013) Arabidopsis villins promote actin turnover at pollen tube tips and facilitate the construction of actin collars. *Plant Cell*. 25(5):1803-17.

364. Quinlan ME, Heuser JE, Kerkhoff E, and Mullins RD (2005) Drosophila Spire is an actin nucleation factor. *Nature.* 433, 382-388

365. Quinlan ME, Hilgert S, Bedrossian A, Mullins RD, Kerkhoff E. (2007) Regulatory interactions between two actin nucleators, Spire and Cappuccino. *J Cell Biol*. 79(1):117-128.

366. Ranade SS, Woo SH, Dubin AE, Moshourab RA, Wetzel C, Petrus M, Mathur J, Bégay V, Coste B, Mainquist J, Wilson AJ, Francisco AG, Reddy K, Qiu Z, Wood JN, Lewin GR, Patapoutian A. (2014) Piezo2 is the major transducer of mechanical forces for touch sensation in mice. *Nature.* 516:121-125

367. Rauh NR, Schmidt A, Bormann J, Nigg EA, Mayer TU. (20050 Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. *Nature.* 437:1048–1052.

368. Rauzi M, Lenne PF, Lecuit T. (2010) Planar polarized actomyosin contractile flows control epithelial junction remodelling. *Nature*. 468(7327):1110-4.

369. Rebecchi MJ, Pentyala SN. 2000. Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiological Reviews* 80, 1291–1335.

370. Rickman CM, Davletov B. (2003) Mechanism of calcium-independent synaptotagmin binding to target SNAREs. *J Biol Chem.* 278: 5501–5504.

371. Ridley AJ, Hall A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. 7;70(3):389-99

372. Riedl J, Crevenna AH, Kessenbrock K, Yu JH, Neukirchen D, Bista M, Bradke F, Jenne D, Holak TA, Werb Z, Sixt M, Wedlich-Soldner R. (2008) Lifeact: a versatile marker to visualize F-actin. *Nat Methods.* Jul;5(7):605-7.

373. Roeles J, Tsiavaliaris G. (2019) Actin-microtubule interplay coordinates spindle assembly in human oocytes. *Nat Commun*. 10(1):4651

374. Rogers, C.S., Hao, Y., Rokhlina, T., Samuel, M., Stoltz, D.A., Li, Y., et al. (2008) Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *The Journal of Clinical Investigation*, 118:1571-7

375. Rongish BJ, Wu W, Kinsey WH (1999) Fertilization-induced activation of phospholipase C in the sea urchin egg. *Dev Biol* 215:147–154.

376. Rorth P. (1998) Gal4 in the Drosophila female germline. *Mech. Dev.* 78:113–118.

377. Rosa A, Vlassaks E, Pichaud F, Baum B. (2015) Ect2/Pbl acts via Rho and polarity proteins to direct the assembly of an isotropic actomyosin cortex upon mitotic entry. *Dev Cell*. Mar 9;32(5):604-16.

378. Rosales-Nieves AE, Johndrow JE, Keller LC, Magie CR, Pinto-Santini DM, Parkhurst SM. (2006) Coordination of microtubule and microfilament dynamics by Drosophila Rho1, Spire and Cappuccino. *Nat Cell Biol.* 8(4):367-76.

379. Roth-Johnson EA, Vizcarra CL, Bois JS, Quinlan ME. (2014) Interaction between microtubules and the Drosophila formin Cappuccino and its effect on actin assembly. *J Biol Chem*. 14;289(7):4395-404.

380. Rottner K, Faix J, Bogdan S, Linder S, Kerkhoff E. (2017) Actin assembly mechanisms at a glance. *J Cell Sci*. Oct 15;130(20):3427-3435.

381. Runft LL, Jaffe LA, Mehlmann LM. (2002) Egg activation at fertilization: where it all begins. *Dev Biol* 245:237–254.

382. Runft LL, Jaffe LA. (2000) Sperm extract injection into ascidian eggs signals Ca(2) release by the same pathway as fertilization. *Development* 127:3227– 3236.

383. Runft LL, Watras J, Jaffe LA. (1999) Calcium release at fertilization of Xenopus eggs requires type I IP(3) receptors, but not SH2 domain-mediated activation of PLCgamma or G(q)-mediated activation of PLCbeta. *Dev Biol* 214:399–411.

384. Sagata N. (1996) Meiotic metaphase arrest in animal oocytes: its mechanisms and biological significance. *Trends Cell Biol.* 6(1):22-8.

385. Santella L, De Riso L, Gragnaniello G, and Kyozuka K. (1999) Cortical granule translocation during maturation of starfish oocytes requires cytoskeletal rearrangement triggered by InsP3-mediated Ca2+ release. *Exp Cell Res*. 248:567-574

386. Santella L, Limatola N, Chun JT. (2008) Calcium and actin in the saga of awakening oocytes. *Biochem and Biophys Res Com*. 460:104-113

387. Santella L, Limatola N, Chun JT. (2015) Calcium and actin in the saga of awakening oocytes. *Biochem Biophys Res Commun*. Apr 24;460(1):104-13.

388. Sartain CV, and Wolfner MF. (2013) Calcium and egg activation in Drosophila. *Cell Calcium* 53, 10-15

389. Sato K, Tokmakov AA, Iwasaki T, Fukami Y. (2000) Tyrosine kinase-dependent activation of phospholipase Cgamma is required for calcium transient in Xenopus egg fertilization. *Dev Biol* 224:453–469.

390. Satouh Y, Ikawa M. (2018) New Insights into the Molecular Events of Mammalian Fertilization. *Trends Biochem Sci.* 43(10):818-828.

391. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. 2002. PLC zeta: a sperm- specific trigger of Ca2+ oscillations in eggs and embryo development. *Development* 129, 3533–3544.

392. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. (2002) PLC zeta: a sperm-specific trigger of Ca(2) oscillations in eggs and embryo development. *Development* 129: 3533–3544.

393. Sawa M, Oishi K. (1989) Delayed sperm injection and fertilization in parthenogenetically activated insect egg (Athalia rosae, Hymenoptera). *Rouxs Arch Dev Biol*. Nov;198(4):242-244.

394. Schell MJ, Erneux C, and Irvine RF. (2001) Inositol 1,4,5-trisphosphate 3-kinase A associates with F-actin and dendritic spines via its N-terminus. *J Biol Chem*. 276:37537- 37546

395. Schmell ED, Gulyas BJ. (1980) Mammalian sperm-egg recognition and binding in vitro. I. Specificity of sperm interactions with live and fixed eggs in homologous and heterologous inseminations of hamster, mouse, and guinea pig oocytes. *Biol Reprod.* 23: 1075–1085.

396. Schuh M, Ellenberg J. (2007) Self-organization of MTOCs replaces centrosome function during acentrosomal spindle assembly in live mouse oocytes. *Cell.* 130:484–98

397. Schuh M, Ellenberg J. (2008) A new model for asymmetric spindle positioning in mouse oocytes. *Curr. Biol*. 18:1986–92

398. Schuh, M. and Ellenberg, J. (2007) Self-organization of MTOCs replaces centrosome function during acentrosomal spindle assembly in live mouse oocytes. *Cell* 130, 484-498.

399. Schuh, M. and Ellenberg, J. (2008) A new model for asymmetric spindle positioning in mouse oocytes. *Curr. Biol*. 18, 1986-1992.

400. Schupbach T, Wieschaus E. (1991) Female sterile mutations on the second chromosome of Drosophila melanogaster. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* 129: 1119--1136.

401. Schwab MS, Roberts BT, Gross SD, Tunquist BJ, Taieb FE, Lewellyn AL, Maller JL. (2001) Bub1 is activated by the pro- tein kinase p90(Rsk) during Xenopus oocyte maturation. *Curr Biol.* 11:141–150.

402. Serbus LR, Cha BJ, Theurkauf WE, Saxton WM. (2005) Dynein and the actin cytoskeleton control kinesin-driven cytoplasmic streaming in Drosophila oocytes. *Development*. 132(16):3743-52.

403. Severson AF, Bowerman B. (2002) Cytokinesis: closing in on the central spindle. *Dev Cell*. 2002 Jan;2(1):4-6.

404. Shapiro BM, Somers C, Weidman PJ. (1989) Extracellular remodeling during fertilization. In: H Schatten, G Schatten, editors. *Cell biology of fertilization*. *San Diego: Academic Press.* p 251– 276.

405. Sharp-Baker H, Chen RH. (2001) Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. *J Cell Biol.* 153:1239 –1250.

406. Sheng J, Kumagai A, Dunphy WG, Varshavsky A. (2002) Dissection of c-MOS degron. *EMBO J.* 21:6061–6071.

407. Shieh BH, Niemeyer B. (1995) A novel protein encoded by the InaD gene regulates recovery of visual transduction in Drosophila. *Neuron*. 14(1):201-10.

408. Shin JB, Adams D, Paurket M, Siba M, Sidi S, Levin M, Gillespie PG, and Grunder S. (2005) Xenopus TRPN1 (NOMPC) localizes to microtubule-based cilia in epithelial cells, including inner ear hair cells. *Proc Natl Acad Sci U.S.A.* 102:12572-12577

409. Shin OH, Rhee JS, Tang J, Sugita S, Rosenmund C, Sudhof TC. (2003) Sr+ binding to the Ca2+ binding site of the synaptotagmin 1 C2B domain triggers fast exocytosis without stimulating SNARE interactions. *Neuron.* 37: 99– 108.

410. Sinkins WG, Goel M, Estacion M, and Schilling WP. (2004) Association of immunophilins with mammalian TRPC channels. *J Biol Chem.* 13;279(33):34521-9

411. Sjoblom B, Salmazo A, and Djinovic-Carugo K. (2008) α-actinin structure and regulation. *Cell Mol Life Sci.* 65(17):2688-701

412. Smith CL, DeLotto R. (1994). Ventralizing signal determined by protease activation in Drosophila embryogenesis. *Nature.* 368,548 –551

413. Smith LD. (1970) Evolutionary morphology of the external insect genitalia. 2. Hymenoptera. *Annals of the Entomological Society of America*. 63:1-27

414. Smith TC, Fang Z, and Luna EJ. (2010) Novel interactions and a role for supervillin in early cytokinesis. *Cytoskeleton (Hoboken).* 67(6):346-64

415. Snapp EL, Iida T, Frescas D, Lippincott-Schwartz J, Lilly MA. (2004) The fusome mediates intercellular endoplasmic reticulum connectivity in Drosophila ovarian cysts. *Mol Biol Cell.* 15(10):4512-21.

416. Sokol NS, Cooley L. (2003) Drosophila filamin is required for follicle cell motility during oogenesis. *Dev Biol.* Aug 1;260(1):260-72.

417. Spracklen AJ, Fagan TN, Lovander KE, and Tootle TL. (2014a) The pros and cons of common actin labelling tools for visualising actin dynamics during Drosophila oogenesis. *Dev Biol*. 393:209-226

418. Spracklen AJ, Kelpsch DJ, Chen X, Spracklen CN, and Tootle TL. (2014b) Prostaglandins temporally regulate cytoplasmic actin bundle formation during Drosophila oogenesis. *Dev Biol*. 25:397-411

419. Spradling A. (1993) Developmental genetics of oogenesis in the development of Drosophila melanogaster. *Cold Spring Harbor Lab*. Press, NY

420. Spradling AC. (1993) Developmental genetics of oogenesis. In: M Bate, AM Arias, editors. *The development of Drosophila melanogaster*. Vol. 1. Plainview, NY: *Cold Spring Harbor Laboratory Press*. p 1– 70.

421. Sprang SR. (1997) G protein mechanisms: insights from structural analysis. *Annu Rev Biochem* 66:639–678.

422. Srivastava V, Robinson DN. (2015) Mechanical stress and network structure drive protein dynamics during cytokinesis. *Curr Biol*. Mar 2;25(5):663-70.

423. St Johnston D, Driever W, Berleth T, Richstein S, Nüsslein-Volhard C. (1989) Multiple steps in the localization of bicoid RNA to the anterior pole of the Drosophila oocyte. *Development*. 107 Suppl():13-9.

424. Stalker HD. (1954) Parthenogenesis in Drosophila. *Genetics*. 1954 Jan;39(1):4-34

425. Stafford N, Wilson C, Oceandy D, Neyses L, Cartwright EJ. (2017) The Plasma Membrane Calcium ATPases and Their Role as Major New Players in Human Disease. *Physiol Rev.* 1;97(3):1089-1125. doi: 10.1152/physrev.00028.2016.

426. Stavermann M, Meuth P, Doengi M, Thyssen A, Deitmer JW, Lohr C. (2015) Calcium-induced calcium release and gap junctions mediate large-scale calcium waves in olfactory ensheathing cells in situ. *Cell Calcium*. Aug;58(2):215-25.

427. Stein D, Nüsslein-Volhard C. (1992) Multiple extracellular activities in Drosophila egg perivitelline fluid are required for establishment of embryonic dorsal-ventral polarity. *Cell*. Feb 7;68(3):429-40.

428. Stephano JL, Gould MC. (1997) The intra-cellular calcium increase at fertilization in Urechis caupo oocytes: activation without waves. *Dev Biol* 191:53–68.

429. Stitzel ML, Cheng KC, Seydouz G. (2007) Regulation of MBK-2/Dyrk kinase by dynamic cortical anchoring during the oocyte-to-embryo transition. *Curr Biol.* 17: 1545– 1554.

430. Stricker SA. (1996) Repetitive calcium waves induced by fertilization in the nemertean worm Cerebratulus lacteus. *Dev Biol* 176:243–263.

431. Stricker SA. (1999) Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol* 211:157–176.

432. Su Z, Zhou X, Loukin SH, Saimi Y, Kung C. (2009) Mechanical force and cytoplasmic Ca(2+) activate yeast TRPY1 in parallel. *J Membr Biol,* 227(3):141-50.

433. Sun HQ, Yamamoto M, Mejillano M, Yin HL. (1999) Gelsolin, a multifunctional actin regulatory protein. *The Journal of Biological Chemistry*. 274 (47): 33179–82.

434. Swan A, Schüpbach T. (2007) The Cdc20 (Fzy)/Cdh1-related protein, Cort, cooperates with Fzy in cyclin destruction and anaphase progression in meiosis I and II in Drosophila. *Development*. Mar;134(5):891-9.

435. Swann K, Ozil JP. (1994) Dynamics of the calcium signal that triggers mammalian egg activation. Int Rev Cytol152:183– 222.

436. Swann K, Saunders CM, Rogers NT, Lai FA. (2006) PLCzeta(zeta): a sperm protein that triggers Ca2 oscillations and egg activation in mammals. *Semin Cell Dev Biol* 17:264–273.

437. Swann KA. (1990) Cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development* 110:1295–1302.

438. Tahara M, Tasaka K, Masumoto N, Mammoto A, Ikebuchi Y, Miyake A. (1996) Dynamics of cortical granule exocytosis at fertilization in living mouse eggs. *Am J Physiol.* 270: C1354– C1361.

439. Takayama J, Onami S. (2016) The Sperm TRP-3 Channel Mediates the Onset of a Ca(2+) Wave in the Fertilized C. elegans Oocyte. *Cell Rep*. Apr 19;15(3):625-637.

440. Takeo S, Lake CM, Morais-de-Sá E, Sunkel CE, Hawley RS. (2011) Synaptonemal complex-dependent centromeric clustering and the initiation of synapsis in Drosophila oocytes. *Curr Biol*. 21(21):1845-51. doi: 10.1016/j.cub.2011.09.044

441. Takeo S, Tsuda M, Akahori S, Matsuo T, Aigaki T. (2006) The calcineurin regulator sra plays an essential role in female meiosis in Drosophila. *Curr Biol.* 16:1435–1440.

442. Tartakoff A. (2015) Cell Biology of Yeast Zygotes, from Genesis to Budding. *Biochim Biopphys Acta*, 1853(7):1702-1714

443. Tearle, R.G., Nusslein-Volhard, C. (1987). Tubingen mutants and stock list. *D. I. S.* 66: 209--269.

444. Terasaki M. (1996) Actin filament translocations in sea urchin eggs. *Cell Motil Cytoskeleton.* 34(1):48-56.

445. Thaler CD, Kuo RC, Patton C, Preston CM, Yagisawa H, Epel D. (2004) Phosphoinositide metabolism at fertilization of sea urchin eggs measured with a GFP-probe. *Dev Growth Differ*. Oct;46(5):413-23.

446. The Nobel Prize in Physiology or Medicine 1995. NobelPrize.org. Nobel Media AB 2020. Mon. 18 May 2020. <https://www.nobelprize.org/prizes/medicine/1995/summary/>

447. Theurkauf WE, Alberts BM, Jan YN, Jongens TA. (1993) A central role for microtubules in the differentiation of Drosophila oocytes. *Development*. 118(4):1169-80.

448. Theurkauf WE, Alberts BM, Jan YN, Jongens TA. (1993) A central role for microtubules in the differentiation of Drosophila oocytes. *Development*. 118(4):1169-80.

449. Theurkauf WE, Hawley RS. (1992). Meiotic spindle assembly in Drosophila females: Behavior of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein. *J. Cell Biol*. 116, 1167-l 180.

450. Theurkauf WE, Hazelrigg TI. (1998) In vivo analyses of cytoplasmic transport and cytoskeletal organization during Drosophila oogenesis: characterization of a multi-step anterior localization pathway. *Development.* 125(18):3655-66.

451. Theurkauf WE. (1994) Premature microtubule-dependent cytoplasmic streaming in cappuccino and spire mutant oocytes. *Science*. 265(5181):2093-6.

452. Tilney LG, Jaffe LA. (1980) Actin, microvilli, and the fertilization cone of sea urchin eggs. *J Cell Biol*. 87(3 Pt 1):771-82.

453. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron*. 21:531-543.

454. Tracey WDJr, Wilson RI, Laurent G, and Benzer S. (2003) painless, a Drosophila gene essential for nociception. *Cell.* 113(2):261-73

455. Tsai JH, Yan R, McKee BD. (2011) Homolog pairing and sister chromatid cohesion in heterochromatin in Drosophila male meiosis I. *Chromosoma*. 120(4):335-51.

456. Tsankova A, Pham TT, Garcia DS, Otte F, Cabernard C. (2017) Cell Polarity Regulates Biased Myosin Activity and Dynamics during Asymmetric Cell Division via Drosophila Rho Kinase and Protein Kinase N. *Dev Cell.* Jul 24;42(2):143-155.e5.

457. Tsunoda S, Sierralta J, Sun Y, Bodner R, Suzuki E, Becker A, Socolich M, Zuker CS. (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. *Nature*. 17;388(6639):243-9. doi: 10.1038/40805.

458. Tumova, L., Chmelikova, E., Zalmanova, T., Kucerova-Chropva, V., Romar, R., Dvorakova, M., Hoskova, K., and Petr, J. (2016) Calcineurin role in porcine oocyte activation. *Animal,* 10:12, 1998-2007

459. Tunquist BJ, Maller JL. (2003) Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes Dev.* 17(6):683-710.

460. Tunquist BJ, Maller JL. (2003) Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes Dev.* 17:683–710.

461. Tunquist BJ, Schwab MS, Chen LG, Maller JL. (2002) The spindle checkpoint kinase bub1 and cyclin e/cdk2 both con- tribute to the establishment of meiotic metaphase arrest by cytostatic factor. *Curr Biol.* 12:1027–1033.

462. Turner PR, Jaffe LA, Fein A. (1986). Regulation of cortical vesicle exocytosis in sea urchin eggs by inositol 1,4,5-trisphosphate and GTP-binding protein. *J Cell Biol* 102:70–76.

463. Turunen O, Wahlstrom T, Vaheri A. (1994) Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. *J Cell Biol*. 1445–1453

464. Ugur B, Chen K, Bellen HJ. (2016). Drosophila tools and assays for the study of human diseases. *Dis. Model. Mech.* 9, 235–244.

465. Uraji J, Scheffler K, Schuh M. (2018) Functions of actin in mouse oocytes at a glance. *J Cell Sci*. 131(22):jcs218099.

466. Vaccari T, and Ephrussi A. (2002). The fusome and microtubules enrich Par-1 in the oocyte, where it effects polarization in conjunc- tion with Par-3, BicD, Egl, and dynein. *Curr Biol.* 12, 1524–1528.

467. Van der Meer JM, Jaffe LF. (1983) Elemental composition of the perivitelline fluid in early Drosophila embryos. *Dev Biol*. 95(1):249-52.

468. Vasilev F, Chun JT, Gragnaniello G, Garante E, Santella L. (2012) Effects of ionomycin on egg activation and early development in starfish. *PLoS One*. 7(6):e39231

469. Vasilev F, Ezhova Y, Chun JT. (2021) Signaling Enzymes and Ion Channels Being Modulated by the Actin Cytoskeleton at the Plasma Membrane. *Int J Mol Sci*. 26;22(19):10366.

470. Vavylonis D, Kovar DR, O'Shaughnessy B, and Pollard TD. (2006) Model of formin-associated actin filament elongation. *Mol. Cell.* 21, 455-466.

471. Vertii A, Hehnly H, Doxsey S. (2016) The centrosome, a multitalented renaissance organelle. *Cold Spring Harb. Perspect. Biol*. 8:a025049

472. Viamontes GI, Fochtmann LJ, Kirk DL. (1979) Morphogenesis in Volvox: analysis of critical variables. Cell. Jul;17(3):537-50.

473.Vidali L, Hepler PK. (2001) Actin and pollen tube growth. Protoplasma. 215(1-4):64-76.

474. Vigneron S, Robert P, Hached K, Sundermann L, Charrasse S, Labbé JC, Castro A, Lorca T. (2016) The master Greatwall kinase, a critical regulator of mitosis and meiosis. *Int J Dev Biol.* 60(7-8-9):245-254.

475. Vogel HJ, Zhang M. (1995) Protein engineering and NMR studies of calmodulin. *Mol. Cell. Biochem*. 149–150, pp. 3-15

476. Vogt-Eisele AK, Weber K, Sherkheli MA, Vielhaber G, Panten J, Gisselmann G, et al. (2007) Monoterpenoid agonists of TRPV3. Br J *Pharmacol.* 151:530-540.

477. Von Stetina JR, Orr-Weaver TL. (2011) Developmental control of oocyte maturation and egg activation in metazoan models. *Cold Spring Harb Perspect Biol*. 1;3(10):a005553.

478. Von Stetina JR, Tranguch S, Dey SK, Lee LA, Cha B, Drummond-Barbosa D. (2008) alpha-Endosulfine is a conserved protein required for oocyte meiotic maturation in Drosophila. *Development*. 135(22):3697-706.

479. Voronina E, Wessel GM. (2004) betagamma subunits of heterotrimeric G-proteins contribute to Ca2 release at fertilization in the sea urchin. *J Cell Sci* 117: 5995–6005.

480. Wahlstrom G, Lahti VP, Pispa J, Roos C, Heino TI. (2004) *Drosophila* non-muscle α-actinin is localized in nurse cell actin bundles and ring canals, but is not required for fertility. *Mech Dev*. 121:1377-1391

481. Wakai T, Vanderheyden V, Fissore RA. Ca2+ signaling during mammalian fertilization: requirements, players, and adaptations. (2011) *Cold Spring Harb Perspect Biol*. 3(4)

482. Wales P, Schuberth CE, Aufschnaiter R, Fels J, García-Aguilar I, Janning A, Dlugos CP, Schäfer-Herte M, Klingner C, Wälte M, Kuhlmann J, Menis E, Hockaday Kang L, Maier KC, Hou W, Russo A, Higgs HN, Pavenstädt H, Vogl T, Roth J, Qualmann B, Kessels MM, Martin DE, Mulder B, Wedlich-Söldner R. (2016) Calcium-mediated actin reset (CaAR) mediates acute cell adaptations. *Elife*. Dec 6;5:e19850.

483. Wang LY, Augustine GJ. (2015) Presynaptic nanodomains: a tale of two synapses. *Front Cell Neurosci*. 26;8:455.

484. Wang SS, Alousi AA, and Thompson SH. (1995) The lifetime of inositol 1,4,5-trisphosphate in single cells. *J Gen Physio*l 105:149-171

485. Wang YC, Khan Z, Kaschube M, Wieschaus EF. (2012) Differential positioning of adherens junctions is associated with initiation of epithelial folding. *Nature*. 28;484(7394):390-3.

486. Ward EJ, Berg CA. (2005) Juxtaposition between two cell types is necessary for dorsal appendage tube formation. *Mech Dev*. 122(2):241-55.

487. Ward S, Carrel JS. (1979) Fertilization and sperm competition in the nematode Caenorhabditis elegans. *Genes Dev.* 13: 2838– 2851.

488. Waring GL. (2000) Morphogenesis of the eggshell in Drosophila. *Int Rev Cytol.* 198: 67– 108.

489. Way M, Gooch J, Pope B, Weeds AG. (1989) Expression of human plasma gelsolin in Escherichia coli and dissection of actin binding sites by segmental deletion mutagenesis. *J Cell Biol.* 109(2):593-605.

490. Weil TT, Forrest KM, Gavis ER. (2006) Localization of bicoid mRNA in late oocytes is maintained by continual active transport. *Dev Cell.* 11(2):251-62.

491. Weil TT, Xanthakis D, Parton R, Dobbie I, Rabouille C, Gavis ER, Davis I. (2010) Distinguishing direct from indirect roles for bicoid mRNA localization factors. *Development*. 137(1):169-76.

492. Weil TT. (2014) mRNA localization in the Drosophila germline. *RNA Biol*. 11(8):1010-8.

493. Went DF, and Krause G. (1973) Normal development of mechanically activated, unlaid eggs of an endoparasitic Hymenopteran. *Nature,* 244:454-455

494. Went DF, and Krause G. (1974a) Egg activation in Pimpla turionellae (Hym.). *Naturewissenschaften* 61:407-408

495. Went DF, and Krause G. (1974b) Alteration of egg architecture and egg activation in an endoparasitic Hymenopteran as a result of natural or imitated oviposition. *Wilhelm Roux' Archiv*, 175:173-184

496. Went., D. (1982) Egg activation and parthenogenetic reproduction in insects. *Biol Rev,* 55:319-344

497. Wessel GM, Brooks JM, Green E, Haley S, Voronina E, Wong J, Zaydfudim V, Conner S. (2001) The biology of cortical granules. *Int Rev Cytol* 209: 117– 206.

498. Whalley T, McDougall A, Crossley I, Swann K, Whitaker M. (1992) Internal calcium release and activation of sea urchin eggs by cGMP are independent of the phosphoinositide signaling pathway. *Mol Biol Cell.* Mar;3(3):373-83.

499. Whitaker MJ, Swann K. (1993) Lighting the fuse at fertilization. *Development* 117:1–12.

500. Wilhelm JE, Mansfield J, Hom-Booher N, Wang S, Turck CW, Hazelrigg T, Vale RD. (2000) Isolation of a ribonucleoprotein complex involved in mRNA localization in Drosophila oocytes. *J Cell Biol*. 7;148(3):427-40.

501. Williams BC, Filter JJ, Blake-Hodek KA, Wadzinski BE, Fuda NJ, Shalloway D, Goldberg ML. (2014) Greatwall-phosphorylated Endosulfine is both an inhibitor and a substrate of PP2A-B55 heterotrimers. *Elife*. 11;3:e01695.

502. Willmott NJ, Asselin J, Galione A. (1996) Calcium store depletion potentiates a phosphodiesterase inhibitor- and dibutyryl cGMP-evoked calcium influx in rat pituitary GH3 cells. *FEBS Lett*. May 13;386(1):39-42.

503. Winder SJ, Hemmings L, Maciver SK, Bolton SJ, Tinsley JM, Davies KE, Critchley DR, Kendrick-Jones J. (1995) Utrophin actin binding domain: analysis of actin binding and cellular targeting. *J Cell Sci.* 108 (Pt 1):63-71.

504. Witke W, Hofmann A, Koppel B, Schleicher M, and Noegel AA. (1993) The Ca2+-binding domains in non-muscle type α-actinin: Biochemical and genetic analysis. *J. Cell Biol*. 121,599 -606.

505. Woo S-H, Lukacs V, de Nooij JC, Zaytseva D, Criddle CR, Francisco A, Jessell TM, Wilkinson KA, Patapoutian A.(2015) Piezo2 is the principal mechonotransduction channel for proprioception. *Nature Neuroscience.* 18:1756-1762

506. Wu JQ, Kornbluth S. 2008. Across the meiotic divide – CSF activity in the post-Emi2/XErp1 era. *Journal of Cell Science.* 121, 3509–3514.

507. Wulf E, Deboben A, Bautz FA, Faulstich H, Wieland T. (1979) Fluorescent phallotoxin, a tool for the visualization of cellular actin. *Proc Natl Acad Sci U S A*. Sep;76(9):4498-502.

508. Xiang Y, Takeo S, Florens L, Hughes SE, Huo LJ, Gilliland WD, Swanson SK, Teeter K, Schwartz JW, Washburn MP, Jaspersen SL, Hawley RS. (2007) The inhibition of polo kinase by matrimony maintains G2 arrest in the meiotic cell cycle. *PLoS Biol*. 5(12):e323. doi: 10.1371/journal.pbio.0050323.

509. Yamamoto DS, Hatakeyama M, Matsuoka H. (2013) Artificial activation of mature unfertilized eggs in the malaria vector mosquito, Anopheles stephensi (Diptera, Culicidae) J*ournal of Experimental Biology.* 216: 2960-2966; doi: 10.1242/jeb.084293

510. Yamamoto K, Pardee JD, Reidler J, Stryer L, and Spudich JA. (1982) Mechanism of interaction of Dictyostelium severin with actin filaments. J. *Cell Biol*. 95,711 -719.

511. Yang F, Wang PJ. (2009) The Mammalian synaptonemal complex: a scaffold and beyond. *Genome Dyn*. 5:69-80. doi: 10.1159/000166620.

512. Yang, M., Brand, A., Srikantha, T., Danels, K.J., Soll, D.R., and Gow, N.A. (2011) Fig1 facilitates calcium influx and localizes to membranes destined to undergo fusion during mating in Candida albicans. *Eukaryot Cell*, 10(3):435-44

513. Yao CK, Lin YQ, Ly CV, Ohyama T, Haueter CM, Moiseenkova-Bell VY, Wensel TG, Bellen HJ. (2009) A synaptic vesicle-associated Ca2+ channel promotes endocytosis and couples exocytosis to endocytosis. *Cell*. 4;138(5):947-60. doi: 10.1016/j.cell.2009.06.033.

514. Yao CK, Liu YT, Lee IC, Wang YT, Wu PY. (2017). A Ca2+ channel differentially regulates Clathrin-mediated and activity-dependent bulk endocytosis. *PLoS Biol,* 15(4): e2000931

515. Yi, K., Rubinstein, B., Unruh, J. R., Guo, F., Slaughter, B. D. and Li, R. (2013) Sequential actin-based pushing forces drive meiosis I chromosome migration and symmetry breaking in oocytes. *J. Cell Biol*. 200, 567-576.

516. Yoneda A, Kashima M, Yoshida S, Terada K, Nakagawa S, Sakamoto A, Hayakawa K, Suzuki K, Ueda J, Watanabe T. 2006. Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase Czeta. *Reproduction* 132, 393–401.

517. York-Andersen AH, Hu Q, Wood BW, Wolfner MF, Weil TT. (2020) A calcium-mediated actin redistribution at egg activation in Drosophila. *Mol Reprod Dev*. 87(2):293-304.

518. York-Andersen AH, Parton RM, Bi CJ, Bromley CL, Davis I, and Weil TT. (2015) A single and rapid calcium wave at egg activation in Drosophila. *Biol Open.* 4:553-560

519. York-Andersen AH, Wood BW, Wilby EL, Berry AS, Weil TT. (2021) Osmolarity-regulated swelling initiates egg activation in *Drosophila*. *Open Biol.* 11(8):210067.

520. York-Andersen AH. (2018) Investigating the calcium wave and actin dynamics at *Drosophila* egg activation. *University of Cambridge.*

521. Yoshihara M, Littleton JT. (2002) Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. *Neuron.* 36: 897– 908.

522. Yu FX, Sun HQ, Janmey PA, Yin HL. (1992) Identification of a polyphosphoinositide-binding sequence in an actin monomer-binding domain of gelsolin. *The Journal of Biological Chemistry*. 267 (21): 14616–21.

523. Yu, M., Yuan, X., Lu, C. *et al.* (2017) mDia1 senses both force and torque during F-actin filament polymerization. *Nat Commun.* 8, 1650.

524. Yue L, Spradling AC. (1992) A gene required for ring canal formation during Drosophila oogenesis, encodes a homolog of adducin. *Genes Dev.* 6(12B):2443-54.

525. Yue Z, Xie J, Yu AS, Stock J, Du J, Yue L. (2015) Role of TRP channels in the cardiovascular system. *Am J Physiol Heart Circ Physiol*. 308(3):H157-H182.

526. Zallen JA, Cohen Y, Hudson AM, Cooley L, Wieschaus E, Schejter ED. (2002) SCAR is a primary regulator of Arp2/3-dependent morphological events in Drosophila. *J Cell Biol*. 156(4):689-701

527. Zhang M, Tanaka T, Ikura M. (1995) Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nat. Struct. Biol.* 2, pp. 758-767

528. Zhang S, Wang C, Xie M, Liu J, Kong Z, Su H. (2018) Actin Bundles in The Pollen Tube. *Int J Mol Sci*. 22;19(12):3710.

529. Zhao T, Graham OS, Raposo A, St Johnston D. (2012) Growing microtubules push the oocyte nucleus to polarize the Drosophila dorsal-ventral axis. *Science*. 336:999-1003

530. Zhou XL, Batiza AF, Loukin SH, Palmer CP, Kung C, Saimi Y. (2003) The transient receptor potential channel on the yeast vacuole is mechanosensitive. *Proc Natl Acad Sci U S A*, 10; 100(12):7105-10.

531. Zimyanin VL, Belaya K, Pecreaux J, Gilchrist MJ, Clark A, Davis I, St Johnston D. (2008) In vivo imaging of oskar mRNA transport reveals the mechanism of posterior localization. *Cell*. 134(5):843-53.