A multi-scale approach investigating the physiological relevance of APC-Dia-microtubule mediated actin assembly in the Drosophila ovary

by

Olivia Molinar

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Thesis advisor: Dr. Brooke M. McCartney

Committee Members:

Dr. John L. Woolford

Dr. Jonathan S. Minden

Dr. Adam Kwiatkowski (University of Pittsburgh)

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ABSTRACT

The multifunctional Adenomatous polyposis coli (APC) proteins negatively regulate Wnt signaling, stabilize microtubules, and indirectly regulate actin through effectors such as Asef and IQGAP. We have shown that both vertebrate APC (vAPC) and Drosophila APC1 through their basic domains can bundle and nucleate actin filaments, and collaborate with the formin Diaphanous (Dia) to efficiently nucleate actin assembly *in vitro*. In addition, Drosophila APC2 (lacking a basic domain) and Dia bind directly to each other, and are required for actin furrow extension in the embryo. In contrast, APC1 does not function in actin furrow extension. While these data suggest that APC-Dia collaborations are an evolutionarily conserved mode of actin filament assembly, significant gaps exist in our understanding of their mechanism and physiological relevance. We investigated the mechanism underlying the vAPC/APC1-Dia collaboration in detail, but how APC2 affects Dia without a basic domain is not known. Here we demonstrate that APC2 interacts with Dia through its ß-catenin binding 20 amino acid repeats (20Rs), and that 20R phosphorylation by GSK3ß regulates APC2's actin furrow activity. In addition to its phosphor-regulation, we explored APC2's minimal cortical localization mechanism to further understand the role of APC2 in actin furrow extension.

Furthermore, we took advantage of the many coordinated actin-mediated processes that occur during Drosophila oogenesis to investigate the physiological role of APC1-mediated actin assembly. In stage 10B egg chambers, an array of cytoplasmic actin bundles (actin baskets) form around each nucleus in the nurse cells to secure them. Here we show that APC1, APC2 and Dia are required for the proper assembly of these actin baskets. In *APC1* or *dia* mutants, the actin baskets fail to form at stage 10B, but surprisingly they are present at stage 11. This suggests that APC1 or Dia may be able to carry out actin assembly alone, but with reduced efficiency resulting

in assembly delay. Additionally, we demonstrated that microtubules are required for proper actin cable formation and it is through the functions of microtubule-associated proteins (CLIP-190 and EB1) that this microtubule-actin interaction is mediated.

The work I performed in the McCartney Lab suggests that actin assembly is multi-faceted and tightly regulated to control for the precise spatiotemporal assembly of actin structures in the cell. More importantly, my work showed for the first time that APC1 is an actin assembly factor *in vivo*. This work suggests that different proteins can modulate actin assembly and it is possible that many novel actin assembly mechanisms remain to be discovered. Additionally, my work validated that formins are modulated *in vivo* by several different proteins that can either enhance or inhibit formin function controlling the spatiotemporal assembly of actin. In conclusion, my work focused on investigating the multifaceted process of actin polymerization, allowing us to better understand how actin structures form in the cell and how several proteins collaborate *in vivo* to form these actin structures. Additionally, we know that microtubules and actin filaments coordinate to perform many basic cellular processes but how these two cytoskeletal networks coordinate is not well understood. Through my work, we found that actin assembly is mediated by microtubule +TIP binding proteins that interact with the actin polymerization machinery demonstrating that the microtubule cytoskeletal network directly influences actin assembly.

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Chapter 1. Introduction

1.1 An overview of the cytoskeleton

Normal cellular processes such as maintenance cell shape, intracellular transport, and migration are dependent on the function of the cytoskeleton (Fletcher and Mullins 2010). Two important components of the cytoskeleton involved in these processes are microtubules and actin filaments. Microtubules regulate functions such as cellular motility, division, and organelle transport. Actin filaments are involved in endocytosis, transport of actin binding proteins on actin cables, cytokinesis, cellular motility, and maintenance cell shape. Moreover, many novel microtubule and actin-binding proteins that regulate the cytoskeleton continue to be identified. Therefore, it is important to continue to understand the function and mechanism of cytoskeletal regulatory proteins to better understand the role of the microtubules and actin filaments in basic cellular processes.

1.1.1 Actin filament assembly

Actin is polymerized from monomeric G-actin and form branched or linear filamentous actin (F-actin). F-actin is characterized by a fast growing end (plus or barbed end) and a slow growing end (minus or pointed end). Actin monomers bound to ATP are incorporated at the barbed end of the polymer, and the ATP is hydrolyzed quickly as it shifted to the pointed end (Disanza *et al* 2005). G-actin bound ADP dissociates from the minus end of the polymer. Nucleotide exchange from ADP to ATP of actin monomers can occur on its own or accelerated by Profilin and the bound ATP-actin monomer can undergo another round of polymerization.

Polymerization and depolymerization of actin filaments are important mechanisms involved in many actin-based processes. Profilin, ß-thymosin and cofilin bind to monomeric actin thus maintaining a pool of G-actin (Figure 1A). Profilin inhibits nucleation and elongation at the pointed end of the actin filament because the barbed end of the actin monomer is bound to

Profilin (Pollard and Cooper 1984). This interaction physically blocks the association of the Profilin-actin complex from associating to the pointed end (Vandekerckhove *et al* 1989 and Schutt *et al* 1993). At the barbed end, Profilin associated weakly and acts as a capping protein. Profilin binding to the barbed end prevents elongation and promotes actin filament disassembly (Hill 1983 and Courtemanche and Pollard 2014). In addition, Profilin catalyzes the nucleotide exchange of ADP to ATP in order to provide a pool of G-actin monomers that are ready for actin polymerization (Didry *et al* 1998, Pantolini *et al* 1993, and Pollard *et al* 1984). ß-thymosin blocks actin assembly by sequestering monomeric actin (Figure 1A). Cofilin is able to preferentially bind to ADP-actin monomers to inhibit ADP/ATP exchange and binds to actin filaments (Gunning *et al* 2015 and McGough *et al* 1997). In addition, Cofilin binds to the minus end of the filaments and can function in severing actin filaments (Figure 1D) (Didry *et al* 1998, Carlier et al 1997, and Condeelis 2001). The formins and the Arp2/3 complex promote actin nucleation and elongation of linear and branched polymers, respectively (Figure 1B and 1C) (Machesky *et al* 1998, Pantaloni *et al* 2001, and Svitkina *et al* 1999). Other regulatory reactions of F-actin are promoted by capping proteins that inhibit growth of polymers at the barbed end (Choe *et al* 2002, Rogers *et al* 2003, and Small *et al* 2002) and crosslinking proteins that bundle actin filaments (Figure 1D) (Pantaloni *et al* 2001, Small *et al* 2002, and Pollard *et al* 2003).

1.1.2 Formins in actin assembly

Formins are proteins that are widely expressed and conserved mechanistically throughout many species. These proteins are required for actin and microtubule assembly in many cellular processes. Twenty different formins have been identified in plants and eukaryotes (Pollard 2007 and Renault *et al* 2008). In eukaryotes, there are 15 formins that are either classified as Diaphanous related formins (DRFs) or formins (Goode *et al* 2007, Pollard 2007, and Renault *et*

al 2008). Most of our understanding of formin activity was elucidated from DRFs such as Diaphanous (Dia). The formin homology 2 (FH2) binding domain is a highly conserved sequence in formins and is required for actin nucleation (Higgs 2006, Goode *et al* 2007, Sagot *et al* 2002, and Paul *et al* 2008). Dia is auto-inhibited by interactions through the Diaphanous inhibitory domain (DID) and the Diaphanous autoregulatory domain (DAD) (Figure 1B) shown elegantly through crystal structures (Chesarone *et al* 2010). However, the autoinhibition of Dia was initially revealed by functional studies. Proteins from the Rho family bind to the GTPase binding domain (GBD) partially releasing this auto-inhibition (Higgs 2006 and Goode *et al* 2007). Dia formins dimerize through the FH2 domains generating a doughnut shape that can bind to actin monomers and nucleate filamentous actin (Chesarone *et al* 2010). In addition to FH2, an FH1 domain is present and binds to Profilin, increasing the rate of elongation (Romero *et al* 2004 and Paul *et al* 2008). Like Profilin, many other binding partners of Dia proteins have been identified. Strong evidence *in vitro* and *in vivo* suggests that APC proteins bind to Dia to promote Dia-mediated actin assembly activity (Webb *et al* 2009, Breitsprecher *et al* 2012, Jaiswal *et al* 2013, and Okada *et al* 2010).

In flies, the formins Dia, Cappuccino, FHOD, DAAM, FML, and formin3 were identified (Aspenstrom 2010). Evidence strongly suggests that formins such as Cappuccino assemble actin filaments on their own but function more effectively by interacting with other actin binding proteins. Cappuccino and Spire collaborate to efficiently form a robust actin-mesh required during streaming in late oogenesis (Dahlgaard *et al* 2007). These data are interesting because these results suggest that two actin nucleators are required to work together *in vivo*. In addition, we demonstrated that Dia and APC2 physically and genetically interact to effectively form actin furrows in the syncytial embryo (Webb *et al* 2009), although we do not know how APC2 and

Dia collaborate *in vivo.* Because Dia requires many other factors to efficiently assemble actin, we need to explore the functional role and mechanisms of Dia interactions in actin assembly. Our work specifically investigated the role of Dia-APC mediated actin assembly in Drosophila.

1.1.3 Vertebrate APC (vAPC) in actin assembly

New factors that regulate the cytoskeleton continue to be identified and in many cases the mechanisms by which these novel regulatory proteins function are not known. A novel actin assembly factor *in vitro* is vertebrate Adenomatous polyposis coli (vAPC) that can nucleate, bundle and collaborate with Dia in actin assembly (Okada *et al* 2010). APC is truncated in more than 85% of sporadic and familial incidents of colon cancer. Its role in cancer initiation is caused by the aberrant activation of Wnt signaling, but its role in cancer progression is not understood (reviewed in Nathke 2004). APC is an important microtubule and actin filament assembly protein *in vitro*. However, the physiological relevance of APC in actin assembly is not known. We also do not know if loss of cytoskeletal regulation by mutations in APC promote the progression of colorectal cancer. By understanding the function of APC, we will be able to better understand the link between APC, Wnt signaling, cytoskeletal regulation, and cancer.

1.2 APC biology

1.2.1 Vertebrate APC proteins

Among its most well studied functions, vAPC negatively regulates the Wnt pathway. The Wnt signaling pathway is responsible for regulating normal cellular processes such as cell proliferation, differentiation and patterning in development (reviews in Pinto *et al* 2003 and Peifer *et al* 2000). In this pathway, the main function of vAPC is to target the Wnt signaling effector Arm for degradation by the proteasome. Arm binding to vAPC occurs through the 15Rs and 20Rs of vAPC. Axin, a scaffolding protein, binds to vAPC through its SAMP repeats (Figure

2A). Loss of vAPC in this context disrupts these interactions and aberrantly activates the pathway by allowing ß-catenin levels to rise in the cytoplasm, followed by its translocation to the nucleus and activation Wnt induced target genes. Additionally, through its basic domain it can bind to microtubules, actin, and RNA (Barth *et al* 2008, Zumbrunn *et al* 2001, Moseley *et al* 2007, Okada *et al* 2010, Preitner *et al* 2014).

vAPC and vAPC2 are two closely-related proteins in vertebrates (van Es *et al* 1999). Unlike vAPC, vAPC2 does not contain the EB1/DLG binding site making it slightly smaller than vAPC (Figure 2B). In addition, in some contexts vAPC and vAPC2 localize to the same subcellular location but are also targeted to different places in the cell *in vivo*. For example, Axin but not Axin 2 displaces vAPC but not vAPC2 from the cytoskeleton into cytoplasmic dots (Schneikert *et al* 2014).

vAPC is expressed in most fetal tissues and adult epithelial tissues (Midgley *et al* 1997). vAPC2 is expressed ubiquitously and is highly expressed in the brain (van Es *et al* 1999). Like vertebrates, *Drosophila melanogaster* was also found to encode two APC proteins making the *in vivo* study of these proteins more tractable using this model organism (Figure 2C and 2D).

1.2.2 Drosophila APC proteins

Unlike vertebrates, Drosophila are genetically malleable, cheap to maintain, and have a short life cycle making flies a very tractable model system. Our lab began by studying APC2 because it is the smallest of all APC proteins and is highly conserved. Although APC2 is very similar to all the APC proteins, we also recognize that it does not contain the basic domain required for direct actin and microtubule interactions. It is possible that although APC2 does not contain the basic domain, it can potentially be involved in actin and microtubule interactions

through other protein domains. Therefore we need to continue understanding the mechanism of APC2 in actin assembly. On the other hand, Drosophila APC1 does contain the basic domain required for direct microtubule and actin interactions. Because Drosophila contains both APC proteins that are similar to vertebrate proteins, we can continue to study both APC1 and APC2-mediated actin assembly function in Drosophila. APC proteins in the fly will be referred as APC1 and APC2.

APC1 is expressed uniformly at low levels in the embryo and highly expressed in the central nervous system and germline (Hayashi *et al* 1997). APC1 is involved in Wnt signaling and is directly involved in Armadillo (Arm) destabilization through the 15Rs, 20Rs, and SAMP repeats. The 15-20Rs are required for binding to Arm and the SAMP repeats bind to the Axin scaffolding protein. APC1 inhibits neuronal apoptosis through the Wnt pathway in retinal development (Ahmed *et al* 1998). At the N-terminus, an APC Self-Association Domain (ASAD) is present that allows APC1 to form larger complexes with itself and with APC2 (Kunttas-Tatli *et al* 2014). Self-association of APC2 was found to be important in promoting the assembly, size, and activity of the destruction complex required for Arm destabilization in Wnt signaling. The Arm Repeats are adjacent to the ASAD domain. These repeats are highly structured and are called the Arm Repeats because of their similarity to the repeats found in Armadillo. Armadillo can bind microtubules indirectly through binding of KAP3. At the C-terminus, APC1 can bind directly to actin, microtubules, and EB1 through its basic domain (Jaiswal *et al* 2010).

For the most part, APC1 and APC2 are highly conserved proteins that share overlapping Wnt signaling roles in the brain, embryo and wing imaginal discs. (Akong *et al* 2002, Akong *et al* 2002, and Ahmed *et al* 1998). Although they all contain the Arm Repeats, 15Rs, 20R, and SAMP repeats, APC1 and APC2 are not exactly the same. APC2 is expressed ubiquitously and

expressed in many different cells, present in the cytoplasm, nucleus, associating with the actin rich cortex, and on microtubules (McCartney *et al* 1999, Hamada *et al* 2002, and reviewed Bienz 2002). Also, APC2 does contain the basic domain at the C-terminus of the protein required for direct cytoskeletal interactions. But APC2 was shown to contain a region of 30 amino acids at the C-terminal region of the protein that is involved actin assembly in the early embryo (Zhou *et al* 2011). Therefore, both of these proteins may be involved in actin assembly but it is possible that fly APC2 evolved to have a different actin assembly mechanism.

1.2.3. APC proteins in disease

The most important and well-known clinical manifestation associated with mutations in APC is colorectal cancer. This disease state affects both men and women and is the second leading cause of cancer related deaths in the United States, according the American Cancer Society. Confirmation of this correlation was also published by Moser et al 1995, demonstrating that mutations in APC cause intestinal tract tumors in mice. Most of these mutations are single nucleotide mutations that introduce a nonsense mutation within the 20Rs located in the Mutational Cluster Region (MCR). A smaller APC protein is translated that is missing some of the 20Rs, SAMP repeats, the basic domain, and an EB1 and Dlg protein binding domain. Currently, we understand that loss of APC aberrantly activates the Wnt signaling pathway and this promotes the initiation of the formation of adenomas. Additionally, APC has been postulated as an important microtubule and microfilament regulating protein but its mechanism has not been clearly delineated in these processes. By understanding the function of APC, we will be able to better understand the link between APC, Wnt signaling, cytoskeletal organization and cancer.

APC proteins are multifunctional and are expressed and localized to many different subcellular localizations. Therefore it is not surprising that loss of vAPC may result in causing other diseases such as Gardner's syndrome, Turcot syndrome, Congential hypertrophy of the retinal pigment epithelium (CHRPE) and other associated cancers (Wallis *et al* 1994 and reviewed in Jasperson *et al* 2014). Most recently, loss of APC has also been linked to intellectual disabilities and autism (Mohn *et al* 2014). Loss of APC2 has been linked to causing ovarian cancer and Sotos Syndrome (Jarrett *et al* 2001 and Almuriekhi *et al* 2015). Sotos syndrome is a disease that is characterized by intellectual disability, atypical facial features and a mutation in NSD1. NSD1 is a methyl transferase and loss of this protein causes these diseases. By studying two siblings with Sotos features but lacking the NSD1 mutation, a frameshift mutation in the APC2 gene (Figure 2B) was detected using whole-exome sequencing (Almuriekhi *et al* 2015). This nonsense mutation is located right after the last SAMP repeat deleting the C-terminal domain of APC2 that contains the basic domain and binds to EB3. The group reported that the mutation is functionally null and a downstream effector of NSD1. Additionally, loss of vAPC2 resulted in the loss of function of cytoskeletal regulation in neurons. This is not surprising because the C-terminal domain of vAPC2 also contains a basic domain (Figure 2B). It is possible that the basic domain of vAPC2 is similar to vAPC in microtubule regulation, but this continues to remain an open question. To better understand the role of APC in disease, we need to have a better understanding of the functions and mechanisms of APC at the cellular level.

1.3. Microtubules and APC

1.3.1. Indirect binding of APC to microtubules

The interaction of vAPC with the microtubule network has been investigated extensively, providing a better understanding of its functional significance. Genetic evidence suggests that knocking down APC or EB1 by RNAi treatment in mammalian cells causes chromosomal segregation defects and spindle alignment defects (Draviam *et al* 2006 and Green *et al* 2005). Indirect interactions of vAPC1 with microtubules are mediated by binding to EB1, Dia, and Kap3. Kinesin-associated protein 3 is a microtubule motor that transports APC on microtubules (Jimbo *et al* 2002). Additionally, vAPC proteins localize to the site of microtubule clusters positioned at the plus ends recruited by the +tip microtubule binding protein (EB1) (Mimori-Kiyosue *et al* 2000, Nathke 1999, and Wen *et al* 2014). Besides its other functions, APC is also known as a microtubule +tip binding protein that stabilizes microtubules with the formin Dia and the microtubule +tip binding protein, EB1 (Wen *et al* 2004). In the muscle-tendon junction of the fly, APC1 and EB1 are recruited by Shortstop (a microtubule and actin crosslinking protein) and are responsible for mediating a tendon stress response by the formation of compact microtubules at the muscle-tendon junction (Subramanian *et al* 2003). Loss of Shortstop in the larval tendon junction mislocalizes EB1 and APC1 from the microtubules ends and causes loss of microtubule polarity and attachment (Subramanian *et al* 2003). Therefore, many microtubule binding proteins can interact and recruit APC proteins to microtubules.

1.3.2. Direct binding of APC to microtubules

The C-terminal domain of vAPC is required for directional cell migration that regulates both the actin and microtubule cytoskeleton (Oshima *et al* 1997). The basic domain of vAPC binds directly to microtubules, can bundle microtubules and promote microtubule stability (Deka *et al* 1998, Mimori-Kiyosue *et al* 2000, Munemitsu *et al* 1994, and Zumbrunn *et al* 2001). Other proteins such as Tau, MAP2 and 205K MAP (MAP4 in vertebrates) also contain a highly basic

domain that binds to microtubules and actin filaments (Elie *et al* 2015, Kowalski *et al* 1993, Irminger-finger *et al* 1998)). Tau is a microtubule binding protein, highly expressed in the central nervous system (Lee *et al* 1989). Loss of Tau results in vesicular transport blockage and therefore is implicated in neurodegenerative diseases such as Alzheimers. On the other hand, MAP proteins or microtubule-associated proteins have been mostly identified and isolated by their ability to bind tubulin. Within the Tau and MAP2 sequences, an 18- amino acid tandem repeat was shown to be the domain required and sufficient for microtubule binding. MAP-2 has also been reported to bundle actin filaments reversibly *in vitro* (Sattilaro *et al* 1981). 205K MAP does not have a highly structured amino acid sequence. 205K MAP is found to be co-localized with two different populations of microtubules (in the mitotic spindle and the cytoplasm) in cultured cells and possibly influences microtubules during mitosis (Irminger-finger *et al* 1998). Lastly, APC contains a basic domain that is highly unstructured at the level of the amino acid sequences when compared to the basic domains of Tau and some MAP proteins. APC through the basic domain binds to monomeric tubulin and facilitates the *de novo* polymerization of tubulin subunits. The protein fragment is heat stable, bundles microtubules, and microtubule stabilization by the basic domain of APC is unaffected at 4°C (Dikovskaya *et al* 2010 and Zumbrunn *et al* 2010). Although Tau and APC do not have a high sequence homology, Tau and APC share these same conserved *in vitro* functions. This is a very important point to make because although they may not share sequence homology or structure conservation, it is possible that they may share the same or similar mechanisms. Therefore, continuing to understand the role of proteins with highly basic domains involved in direct actin and microtubule interactions can potentially lead us to better understand the function and evolution of these domains.

1.3.3. vAPC2 also contains a basic domain required for cytoskeletal regulation

vAPC2 or APC-L (APC-like) is a second vAPC protein that is highly expressed in the Central Nervous System (CNS) at early embryonic stages. Expression of vAPC is highly reduced in the CNS in adult stages (Yamanaka *et al* 2002). More specifically, this protein is expressed in post-mitotic neurons where it co-localizes with microtubules (Shintani *et al* 2009). At the cellular level, vAPC2 is involved in brain development through its regulation of neuronal migration and axon guidance (Shintani *et al* 2009) through its function in regulating the cytoskeleton. The N-terminal domain of vAPC and vAPC2 are highly conserved, but the C-terminal domains are only 13% identical (Nakagawa *et al* 2000). The 20Rs in vAPC2 can bind to Arm and can deplete cytoplasmic Arm in the SW480 cells (Nakagawa *et al* 1998) strongly suggesting that vAPC2 shares overlapping roles with vAPC in Wnt signaling. Lack of sequence conservation is intriguing because both C-terminal domains contain the basic domain required for actin assembly. The sequence conservation between the basic domains of vAPC and APC1 are very low as well and we thought this could be due to species differences, but sequence homology between the two vertebrate APC proteins is low as well. This brings us back to our point that although the basic domains may not be highly conserved, it is possible that their mechanisms are conserved. Evidence to support that both vAPC and vAPC2 share similar C-terminal microtubule and actin interactions comes from a yeast-2-hybrid assay. In the screen, vAPC2 was found to bind to EB3. (Nakagawa *et al* 2000). EB3 is 54% identical to EB1 that binds to and recruits vAPC to the + ends of microtubules (Su *et al* 1995) but unlike EB1, EB3 is preferentially expressed in brain tissue. In Cos-7 and SW480 cells, EB3 localizes to the cytoplasmic microtubule network where it partially localizes to the vAPC2. Taken altogether, we hypothesize that both basic domains may share similar cytoskeleton functions. Therefore, we first need to

assess the *in vitro* microtubule and actin assembly functions of vAPC2 to continue to understand the role of vAPC2 in cytoskeletal regulation.

11.4. Microtubules + TIP binding proteins

The plus ends of microtubules are very dynamic, characterized by growth or catastrophic disassembly to regulate processes in the cell such as mitosis (Brun *et al* 2009 and Kirschner *et al* 1986). Microtubule plus ends are regulated by microtubule-associated proteins that bind to and stabilize the plus ends of microtubules (Jiang *et al* 2011). One example of these proteins that tracks on microtubule +-ends is EB1/EB3. EB1 can recruit other microtubule +tip binding proteins such as vAPC and CLIP-170 (Su *et al* 1995 and *Dixit et al* 2009).

1.4.1 CLIP-170 is a cytoskeletal linker and microtubule +TIP binding protein

CLIP-170 is a cytoskeletal linker protein in vertebrates. Many homologues of CLIP-170 were identified and it is predicted that these protein variants provide redundancy in vertebrates (Lantz and Miller 1998). In Drosophila, the CLIP-190 is the only CLIP present making it an attractive model to study the function of CLIP proteins. CLIP-190 binds to myosin and binds to kinetochores in a cell cycle dependent manner (Lantz and Miller 1998, Maiato *et al* 2002, and Rogers *et al* 2004). Additionally, CLIP-190 binds to EB1 and microtubules directly through its CAP-GLY domain (Figure 6) (Pierre *et al* 1992). More relevant to our research, CLIP-170 binds to and recruits Dia at the onset of phagocytosis (Lewkowicz *et al* 2008). However, we do not know if CLIP-170 influences the actin assembly of Dia.

1.4.2 Microtubule + end binding proteins in actin assembly

Several interesting questions that remain unanswered are: how, why, and for what purpose do several microtubule + end proteins associate to the plus ends of microtubules? Recently, evidence suggests that these microtubule +TIP binding proteins are recruiting and

collaborating with formins to regulate actin assembly. For example, the yeast formin For3 is recruited by the +TIP microtubule protein Tea4 to ends of microtubules to promote the establishment of cell polarity and to localize actin assembly to new cells ends (Martin *et al* 2005). Therefore, it is possible that +TIPS recruit formins to sites that are required for rapid actin assembly. Another more recent example relevant to our work demonstrated that Dia is recruited to the sites of phagocytosis through CLIP-190 dependent recruitment and not through EB1/EB3 (Lewkowicz *et al* 2008). CLIP-170 binds to the Formin homology 2 (FH2) domain required for elongation though it is still unclear how or what they are doing when these proteins form complexes at the +TIP end of microtubules. The Goode Lab identified a Formin Enhancement Elongation Sequence (FEED) in CLIP-170 that binds to Dia and enhances its elongation rate *in vitro* (Manuscript submitted). Although we are beginning to understand the role of the Dia and CLIP-170 collaboration, there are many questions remaining: 1) Do other microtubule +TIP proteins affect the collaboration between Dia and CLIP-170, such as EB1 and APC1? 2) How do all of these interactions affect actin assembly in the cell? and 3) Is the CLIP-170 and Dia activity required *in vivo*?

1.5 Actin filaments and APC

1.5.1 Indirect interactions of APC and actin filaments

Localization of APC proteins with the actin cytoskeleton has been reported in several contexts such as embryos and epithelial cells (McCartney *et al* 1999 and reviewed by Bienz 2002). APC proteins affect actin through indirect mechanisms by binding IQGAP, ASEF and Dia. IQGAP is a GTPase activating protein (GAP) that potentially acts as a scaffold to connect the microtubule and actin cytoskeletons through the binding of several cytoskeletal proteins. For

example, evidence demonstrates that IQGAP directly binds to vAPC at the leading edge of the cell in order to promote cell migration by regulating the actin meshwork (Kawasaki et al 2003). Evidence also indicates that the interactions between Armadillo and APC2 participate in tethering mitotic spindles to cortical actin (McCartney *et al* 2001). Another example of an indirect APC-actin interaction was observed when loss of APC in neuronal cells led to excessive axon branching and microtubule de-bundling defects. These phenotypes were completely rescued by the N-terminal domain of APC that contains the oligomerization domain and armadillo repeats but not the C-terminal domain (Chen *et al* 2011.) It is possible that binding of the N-terminal half of the protein, potentially through the Arm repeats, is sufficient for the indirect binding and activity of vAPC on actin.

1.5.2. APC interacts directly with the actin cytoskeleton

More recent data implicating a direct interaction between vAPC and actin demonstrated that the basic domain binds directly to F-actin and is able to bundle actin filaments (Moseley *et al* 2007). In addition, the basic domain of vAPC is able to assemble actin *in vitro* shown in Figure 3A and 3B (Okada *et al* 2010). The basic domain was further dissected and it was shown that two Actin Nucleating Sequences (ANS1 and ANS2) are responsible for its actin nucleation activity *in vitro* (Figure 3C). ANS1 binds two actin monomers while ANS2 stabilizes the vAPC dimer. Dimerization of vAPC leads to the formation of a pre-nucleation complex with four bound actin monomers. Therefore, APC has been classified as a Tandem Monomer Binder along with Spire, Cordon Blue and Jmy (Firat-Karalar *et al* 2011). Our lab, in collaboration with the Goode Lab, found that the actin-associated processes of vAPC are conserved in fly APC1 (Jaiswal *et al* 2013) (Figure 4). These recent advances connecting APC

proteins to the actin cytoskeleton have answered many questions, yet this is only the beginning. Many questions remain about the functions of APC proteins as regulators of the actin cytoskeleton. Most importantly, what is the physiological relevance of APC1 in actin-based processes. Therefore, we need to investigate these proteins further to understand their mechanism *in vivo.* My research has attempted to address these questions using the fruit fly model organism in a coupled *in vitro- in vivo* approach to test APC function and mechanism in actin assembly.

1.5.3 Model: Actin furrow extension of APC2 and Dia

Actin based pseudocleavage furrow formation in the syncytial embryo is a great tractable genetic model to better understand actin assembly. In the Drosophila embryo, the first 13 cycles of nuclear division occur without cytokinesis. In the early nuclear divisions, the nuclei divide within the central cytoplasm of the single-celled embryo. At cycle 9, the nuclei begin to migrate to the cortex of the embryo (Figure 7A) and cycles of actin re-arrangements are observed. At interphase, actin caps form at the cortex above each nucleus when observed in cross-section. During metaphase, the actin caps disappear and are replaced with actin rings that surround the nuclei (Figure 7C). In cross-section, the actin rings appear as furrows that extend from the cortex into the cytoplasm during metaphase where they form barriers to protect the nuclei from colliding with each other during division. In turn, these cables are required to prevent nuclear damage and provide mitotic fidelity in this highly dynamic process. Our lab demonstrated that APC2 and Diaphanous (DIA) are required for actin furrow extension in the early embryo (Figure 7D, E and F). Dia recruits APC2 to the actin furrows, and Dia interacts biochemically and genetically with APC2. These results strongly suggest that an APC2-DIA complex is important in regulating these actin dynamic rearrangements (Webb *et al* 2009). Although, our data suggest

that an APC2-Dia complex is required for actin assembly in the early embryo, we currently do not know how these two proteins collaborate. Therefore, we are collaborating with the Goode Lab at Brandeis University to understand the mechanism of Dia-APC2 based actin assembly *in vitro*. On the other hand, the work of our collaborators independently and with our lab has revealed how APC1 promotes actin assembly with Dia *in vitro*, but less is understood about its *in vivo* functions. Therefore, we tested the physiological relevance of APC1 in actin furrow assembly in the early embryo. The ultimate goal of our work is to understand the role and mechanism of APC proteins in actin assembly.

1.6. Drosophila oogenesis as a model for actin assembly

1.6.1. An overview of Drosophila oogenesis

Many developmental changes occur during oogenesis such as collective cell migration, cell shape changes and intracellular transport. All these processes require a dynamic cytoskeleton. The Drosophila ovary has been a powerful system to better understand the mechanisms required for actin assembly (Reviewed from Hudson *et al* 2002 and Robinson *et al* 1998). Female Drosophila have a pair of ovaries that each contain approximately 16-18 ovarioles. Each ovariole is a strand of developing egg chambers that range from stage 1 to a fertilizable stage 14 egg chamber. 15 Nurse cells (Nc) are formed that contain all the necessary information for the oocyte to develop into a mature egg. The oocyte is located at the posterior of the egg chamber and all of these 16 germ cells are connected by actin ring canals. Each cluster of germ cells is surrounded by a layer of somatic epithelial cells called follicle cells. Together each collection of germ and somatic cells is called an egg chamber. At the anterior end of the egg chamber, a somatic group of follicle cells called the stretch cells, flatten and stretch over the

Nurse cells during late oogenesis. At stage 9, a group of follicle cells called the Border cells migrate from the anterior end of the egg chamber to the oocyte. Another group of follicular cells that migrate inward between the interface of the oocyte and the most posterior Ncs during stage 10A are called centripetal cells. These Centripetal cells and Border cells cluster together at late stage 10B to make the dorsal appendages and the operculum. Lastly, actin cables form during 10B stages around the nuclei to keep the nuclei away from the ring canals in order to prevent the ring canals from being clogged during fast streaming or dumping. All these important cellular changes occur during oogenesis and makes it an attractive model to study actin assembly.

1.6.2. APC proteins are required for Border cell migration

Border cell migration is a powerful system for studying collective cell migration (Reviewed by Rorth, 2002; Ribeiro *et al* 2003, Starz-Gaino and Montell 2004). At stage 9, two non-motile cells called polar cells (PC) cluster at the most anterior portion of the egg chamber and are surrounded by 6 to 8 outer Border Cells (oBC). This cluster of cells detaches from the stretch cells (Sc) during stage 9 and migrates between the Nc until they reach the oocyte. Functionally, Border cells are important because they contribute to the formation of the micropyle (an entrance port for the sperm).

To identify new factors in Border cell migration and delamination, De Graeve *et al* 2012, performed an RNAi screen where they found that loss of APC1 affects the delamination of the Border cells and therefore impairs Border cell migration. In addition, loss of APC1 and APC2 in oBC and adjacent follicle cells leads to defective cell migration and is rescued by over-expression of APC1 and APC2. Additionally, this group found that loss of APC1 or APC2 led to the accumulation of Armadillo and DE-Cadherin. The model suggests that APC proteins

regulate the stability of Arm and DE-Cadherin, allowing the Border Cells to detach from the stretched cells. These data were very exciting because it was the first piece of data that suggested that APC1 was important in cytoskeletal organization in the ovary.

1.6.3. Actin cable formation in late oogenesis

The oocyte is the cell that will mature into an egg but is transcriptionally inactive. Therefore, all the contents required for the oocyte to mature into a viable egg are synthesized in the Nurse cells and transported into the oocyte through stable intercellular connection called ring canals. Ring canals are formed at arrested cleavage furrows and formed by the sequential addition of proteins to assemble the robust channels composed of actin. During stages 7 through 10, selective and slow transport occurs. Cables of actin form around the ring canals from as early as stage 10B and remain until stage 10 (Riparbelli and Callaini, 1995). The transport of the components within the Nurse cells is sensitive to actin depolymerization drugs strongly suggesting that actin filaments are required for transport. For example, treating egg chambers with latrunculin B, an actin depolymerization drug, disrupts the transport of the Golgi to the oocyte. Additionally, loss of microtubules by treatment with a microtubule depolymerization drug, completely abolished the formation of the actin filaments that were coming from the inner rim of the ring canals. Loss of microtubules and the sequential loss of actin filaments through microtubule depolymerization resulted in loss of Golgi transport (Nicolas *et al* 2013) strongly suggesting microtubule-actin crosstalk. Ring canal formation has been clearly delineated but how the actin cables surrounding the ring canals are assembled is not well understood. Components of the Arp2/3 complex were knocked down but loss of this actin assembling machinery had no effect on the formation of the actin cables surrounding the ring canals.

Fast transport or dumping is characterized by the contraction of the Ncs to quickly transport or dump all of the remaining components in the Ncs into the oocyte at stage 11. At early 10A and early 10B, mini-cables form perpendicular to the actin rich cortex into the cytoplasm with their growing ends pointing to the membrane. A revised model of actin cable formation strongly suggests that the actin cables are by filopodia-like actin bundles because of their polarity in the cell (Huelmans *et al* 2013). Actin cables continue to grow and reach the nucleus. The actin cables wrap around the nucleus to push the nucleus away from ring canals (Huelmans *et al* 2013). Electron microscopy (EM) revealed that many overlapping small actin filaments are bundled together to form these actin structures. This morphology resembles the appearance of a ladder (Guild *et al* 1997) (Figure 16G). The actin bundles are hypothesized to be connected to the nuclear membrane by crosslinking proteins found in the peri-nuclear actin mesh.

1.6.4. Chickadee, Quail, and Singed are required for actin basket formation

Three genes that are required for the formation of the actin cables are *chickadee, quail*, and *singed.* Loss of any of these genes results in sterility, complete loss of actin cables, and nuclear clogging of the ring canals that leads to loss of cytoplasmic transport (reviewed in Robinson and Cooley 1998 and Hudson and Cooley 2002). Additionally, smaller and rounder egg chambers are made but not laid. Quail (Villin is the mammalian homolog) and Singed (Fascin is the mammalian homolog) are actin bundling proteins that crosslink actin filaments in the stage 10 actin cables. Quail brings actin cables loosely together while Singed packs and orders them tightly in the Ncs (reviewed in Robinson *et al* 1998). Quail overexpression can partially rescue complete loss of actin cables in the stage 10B egg chambers mutant *quail* egg

chambers. These results strongly suggest that although Quail and Singed exhibit some redundancy, and they do not possess the exact same activities in actin cable formation. On the other hand, Chickadee (Profilin is the mammalian homolog) binds to actin monomers and increases the elongation rate of the formin Dia (Goode *et al* 2007 and Higgs 2005). Chickadee most likely promotes the assembly of actin filaments which are then bundled by Singed and Quail.

1.6.5. Armadillo is required for cortical integrity and actin basket formation

Armadillo (Arm) is another protein involved in actin cable formation in stage 10B egg chambers. In *arm* germline clones, the Nc failed to assemble actin cables and exhibited the dumpless egg chamber phenotype (Peifer *et al* 1993). Other defects in *arm* mutants included defects in oocyte positioning, aberrant cell shape, and ring canals being released from the membranes. Apart from its role in the Wnt signaling pathway, Arm is part of the adherens junction (AJ). The AJ is the interface between neighboring epithelial cells and provide cell-cell adhesion, actin cytoskeleton regulation, and signaling regulation (Hartsock and Nelson 2008). It is possible that loss of Arm is causing defects in the AJ in the Ncs that leads to cortical actin instability, which could explain the actin phenotypes observed in the *arm* mutant cells.

1.6.6. Enabled (Ena) negatively regulates Dia mediated actin assembly in filopodia

Enabled (Ena/Vasp) is involved in making linear actin through its anti-capping, bundling, and anti-branching activities to promote actin assembly. It is hypothesized that elongation through Ena regulates the formation of filopodia structures (Peng *et al* 2009, Faix *et al* 2006, Kwiatkowski *et al* 2007, and Dent *et al* 2007). Ena is localized to filopodia tips where it

collaborates with Dia to form the correct type of protrusion in the cell (Gates *et al* 2009, Gates *et al* 2007, and Lebrand *et al* 2004). In addition, both Ena and Dia can recruit each other to the sites of actin assembly (Gates *et al* 2009). More recently, it was demonstrated that although Ena and Dia, promote the formation of linear actin, they have different biochemical activities (Bilancia *et al* 2014). *In vivo*, Dia and Ena contribute to different types of protrusions when compared to the protrusions they make on their own. Evidence suggests that Ena-EVH1 inhibits the Dia-FH1FH2 mediated actin assembly and therefore, Ena is acting negatively on Dia (Bilancia *et al* 2014).

1.6.7. Ena is required for actin basket formation

In *ena* mutant egg chambers, small round eggs and female fertility is reduced (Gates *et al* 2009). Defects in cortical integrity, actin cable formation, and border cell migration were observed at the cellular level in *ena* mutants. In addition, capping protein (CP) antagonizes Ena activity. Loss of capping protein promoted the early activation of actin cable assembly. Recent data suggest that Dia and Ena collaborate in dorsal closure in the Drosophila embryo. Though, Dia and Ena collaboration has not been elucidated in actin cable formation.

1.7 APC-Dia-microtubule actin mediated assembly in the Drosophila ovary

APC proteins are actin assembly factors *in vitro* and *in vivo*. APC2 is an effector of Dia mediated actin assembly but the mechanism of how APC2 influences Dia-mediated actin assembly is not understand. On the other hand, APC1 is a potent nucleator, bundling protein, and Dia collaborator in actin assembly *in vitro.* Though, we understand the mechanism of actin assembly by APC1 *in vitro*, it is not known whether these APC1 actin associated functions are physiologically relevant. Therefore, the motivation of my doctoral dissertation was to better

understand the role of APC proteins in actin filament formation *in vitro* and *in vivo* using the Drosophila ovary as model system for actin assembly. Chapters 2 and 3 are dedicated to understanding the role of APC-Dia mediated actin assembly in the early Drosophila embryo and ovary. Because of the growing literature that microtubule +-TIP binding proteins bind to and regulate formins, we explored the function of CLIP-190 and EB1 in actin assembly *in vitro* and in the Drosophila ovary.

In summary, we dissected the minimal cortical localization requirements and explored the role of APC2's in actin assembly at the cortex. We also found that APC1, APC2 and Dia are required for actin cable formation during Drosophila oogenesis. Lastly, we found through biochemical and genetic evidence that microtubules promote proper actin cable growth and morphology. Our work suggests that this actin-microtubule crosstalk is mediated by the interaction of EB1 and CLIP-190 with the APC-Dia machinery required for actin cable formation.

1.8. Figures

Figure Legends

Figure 1: Schematic of function of actin binding proteins in actin assembly

A) Binding of Profilin, Cofilin, and Thymosin-β4 to actin monomers.

B) Nucleation and elongation of linear actin filaments by formin such as Diaphanous (Dia). Nucleation is promoted by dimer formation of the FH2 domain and elongation is mediated through Profilin-actin monomer binding to the FH1 domain.

C) Formation of branched actin filaments by the Arp2/3 complex mediated by the WASP family of proteins.

D) Capping at the barbed end by capping proteins, severing and cross-linking by Cofilin and Gelsolin, and crosslinking of actin by Villin and Fascin.

Figure 2: Schematic of vertebrate and Drosophila APC proteins

A-B) Protein domains of vertebrate APC and APC2. Important mutations are highlight by the yellow thunder bolt.

C-D) Protein domains of Drosophila APC1 and APC2. Both mutations of *APC1* and *APC2* are *null* mutations generated by a missense mutation that truncates the protein.

Figure 3: vAPC is a potent actin assembly factor

A) TIRF microscopy experiments using 1μm of labeled actin and 2μm of APC1-B. The samples were imaged 2-3 minutes after polymerization initiated. (scale bar 5μm)

B) Pyrene-labeled actin activity assays. 2um of actin was incubated with 20nM of APC1-B in the presence or absence of 100nM of cytochalasin.

C) Mechanism of vAPC mediated actin nucleation. Two actin nucleation sequences (ANS) were identified. ANS1 binds to two actin monomers and ANS2 stabilizes a dimer required for actin assembly. Therefore, vAPC-basic dimerizes to create a a stables nucleation seed with a capacity of four monomers.

*Images from *Okada et al 2010*.

Figure 4: vAPC-B and Dia-C: The Rocket Launcher Mechanism

A) Single molecule 3-color TIRF microscopy of APC1-B (blue), Dia-C (red), and actin (green). APC1-B and Dia-C are able to overcome the inhibitory effect of Profilin and capping protein

Dia-C and APC-B complex together at the start of actin polymerization. APC1-B associated with the barbed end (- end) and Dia-C associated with the plus end of the actin filament. Blue arrow= barbed end and red arrow = plus end.

B) Schematic of The Rocket Launcher Mechanism.

C) Rate of Assembly increases with APC1-b and Dia-C in the presence of inhibitory proteins.

D) Actin pyrene assembly assays. APC1-B and Dia-C collaborate in actin assembly.

*Images from: Breitsprecher *et al* 2012.

Figure 5: Drosophila APC1 mediated actin assembly

A) Sequence alignment of vAPC-B and APC1-B. The Actin Nucleating Sequences (ANS1 and ANS2) are underlined.

B) Schematic of vAPC and APC1 conservation. vAPC and APC1 are highly conserved at the N-terminus but only 18% identical at the most C-terminal end of the proteins.

C) Drosophila APC1-B is a potent actin assembly factor *in vitro* tested by actin pyrene activity assays.

D) Single molecule TIRF microscopy of actin in the absence and presence of APC1-B. More actin filaments and bundling of actin filaments is observed in the presence of APC1-B.

E) Quantification of D. The number of actin filaments per field of view were counted.

* Images from Jaiswal *et al* 2013.

Figure 6: Schematic of key protein domains and protein-protein interactions potentially involved in cytoskeletal regulation

Our work will attempt to dissect the interaction between APC proteins and Dia in actin assembly. Additionally, we are interested in understanding the microtubules-actin crosstalk. We hypothesize that microtubules plus tip binding proteins interact with APC-Dia complex to mediate the formation of actin assembly.

Figure 7: APC2 and Dia are involved in actin furrow in the early embryo

A) Schematic of syncytial embryo development. Nuclear divisions without cytokinesis occur from cycles 1-13. At cycle 9, the nuclei migrate to the cortex of the cell and actin furrows are visible during this stage.

B and C) Schematic of actin furrow extension in the early embryo. Cross-section of the actin furrows form and extend with the plasma membrane during metaphase. Surface views of the sctin furrows are observed as actin caps during interphase and rings during metaphase. For analysis purposes, we focused on cycle 12 embryos.

D, D') Surface view of *WT* actin furrows.

E, E'') Surface views of $APC2^{g10}$ or APC2 null embryos.

F, F'') Surface views of *dia5 null* heterozygotes embryos.

D''', E''', F''') Cross-section views of *WT, APC2 null* and *Dia5* null heterozygotes actin furrows.

* Images from Webb *et al* 2009.

Figure 8: An Introduction to Drosophila oogenesis with an emphasis on processes in late oogenesis

A) One pair of the ovary contains about an average of 18-25 ovarioles.

B) Schematic of an ovariole. One ovariole contains 14 egg chambers at different developmental stages. The earliest stages denoted by the arrow contain the germ-stem cells. The final stage are characterize by the slow streaming of Nurse cell contents and then fast streaming into the oocytes. The oocyte continues to grow until it becomes a fertilizable embryo in stage 14.

C) Centripetal cellular migration is used to stage the Drosophila egg chambers. The centripetal cells are part of the follicular epithelium and start migrating into the egg chamber along the oocyte until the reach the Border cells in stage 10B Late. Stage 10B Late and 11 egg chambers can be distinguished by the size of the oocyte.

D) Cellular architecture of a Drosophila stage 10B-Late egg chamber. Streaming of the contents from the Nurse cells (Nc) is streamed through the ring canals to the oocyte. The nuclei prevent from clogging up the ring canals by actin cables that form in stage 10B egg chambers.

*Images from: Robinson and Cooley 1997/ Cooley and Theurkauf 1994.

Figure 1

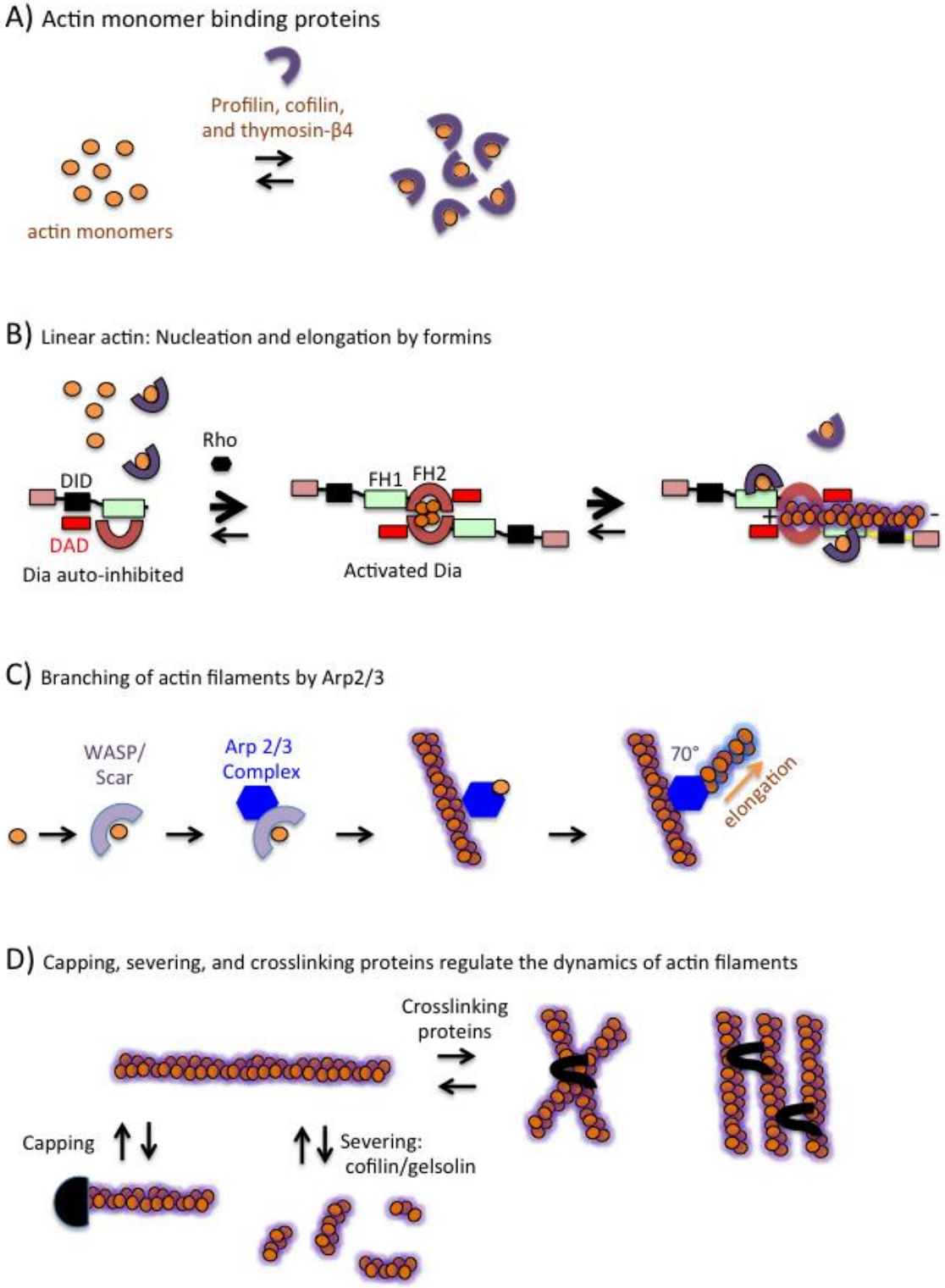

A) Actin monomer binding proteins
Profilin, cofilin,
and thymosin-β4
actin monomers
B) Linear actin: Nucleation and elongation by formins
Rho
DID
DAD
Dia auto-inhibited
FH1
FH2
Activated Dia
C) Branching of actin filaments by Arp2/3
WASP/
Scar
Arp 2/3
Complex
70°
elongation
D) Capping, severing, and crosslinking proteins regulate the dynamics of actin filaments
Crosslinking
proteins
Capping
Severing:
cofilin/gelsolin

Figure 2

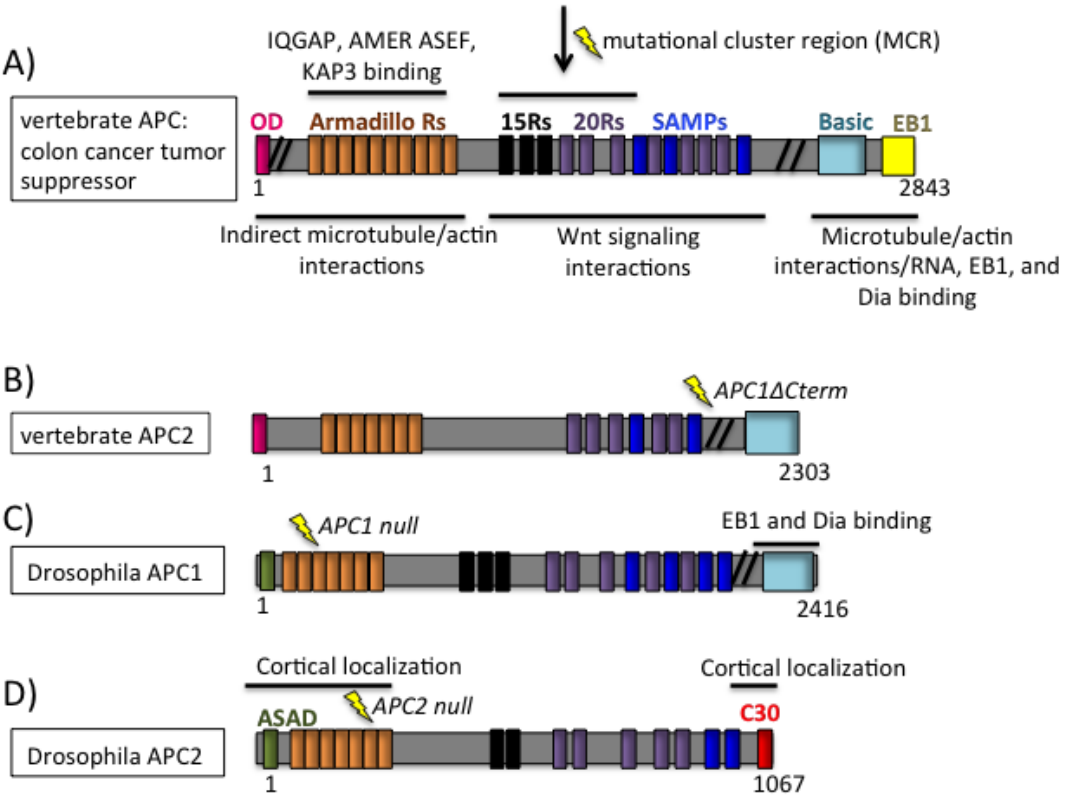

A)
IQGAP, AMER ASEF, KAP3 binding
mutational cluster region (MCR)
vertebrate APC: colon cancer tumor suppressor
OD
Armadillo Rs
15Rs
20Rs
SAMPs
Basic
EB1
1
2843
Indirect microtubule/actin interactions
Wnt signaling interactions
Microtubule/actin interactions/RNA, EB1, and Dia binding
B)
APC1ΔCterm
vertebrate APC2
1
2303
C)
APC1 null
EB1 and Dia binding
Drosophila APC1
1
2416
D)
Cortical localization
Cortical localization
APC2 null
ASAD
C30
Drosophila APC2
1
1067

Figure 3

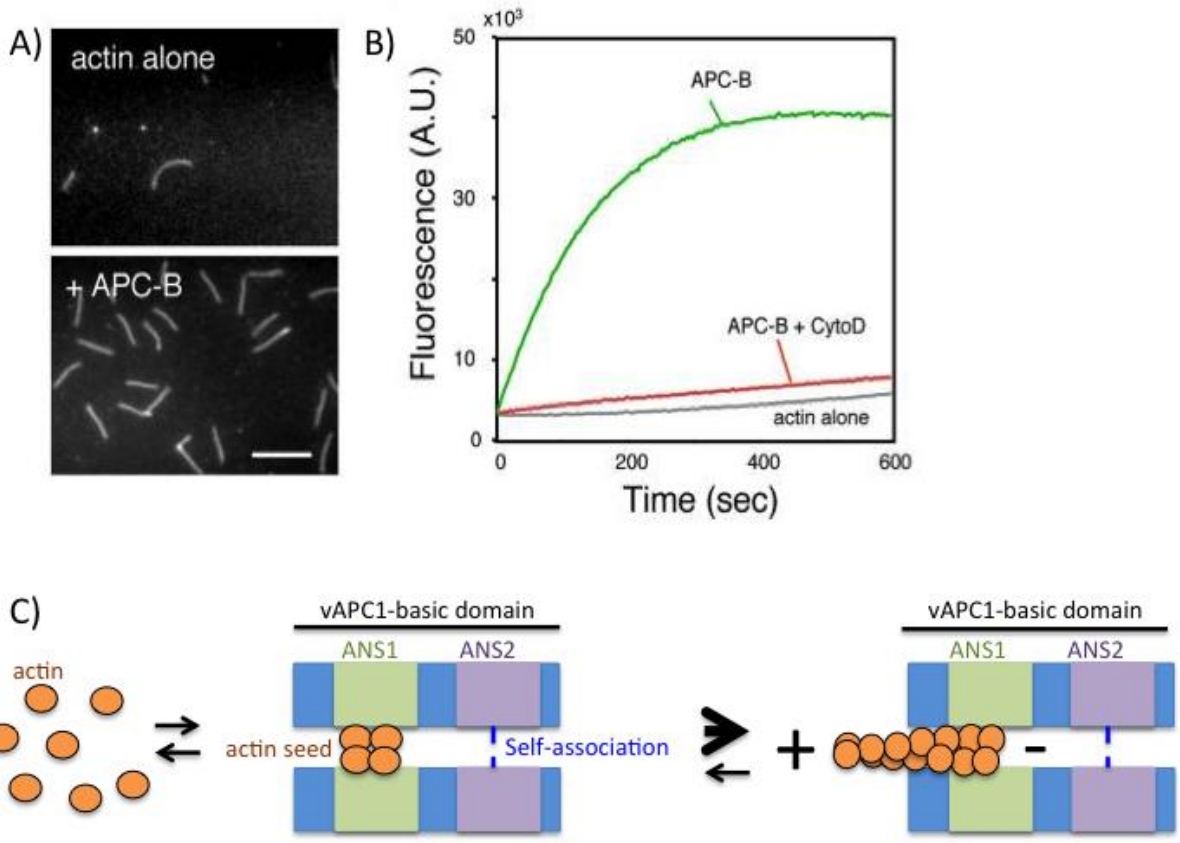
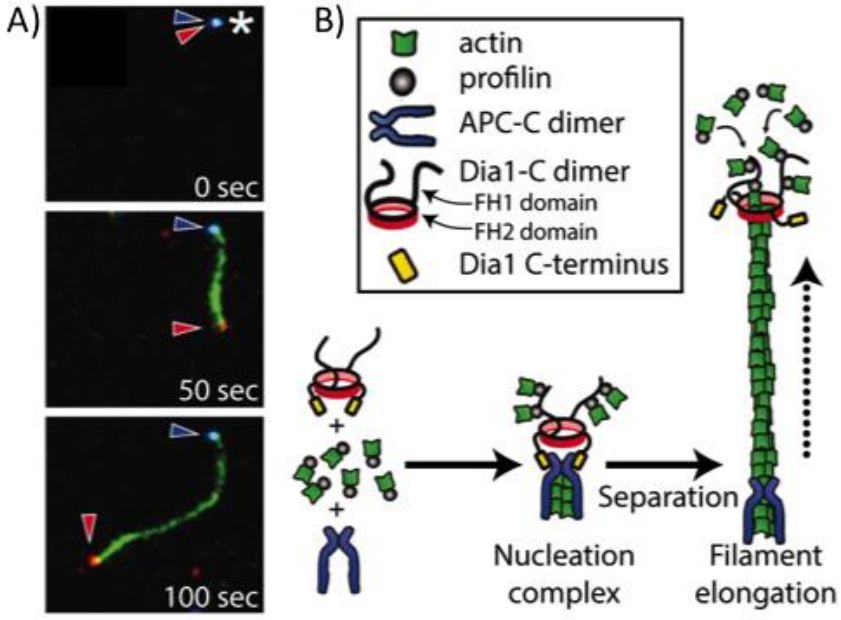

A)
actin alone
+ APC-B
B)
x10³
50
30
10
0
Fluorescence (A.U.)
APC-B
APC-B + CytoD
actin alone
0
200
400
600
Time (sec)
C)
actin
vAPC1-basic domain
ANS1
ANS2
actin seed
Self-association
vAPC1-basic domain
ANS1
ANS2

Figure 4


A)
0 sec
50 sec
100 sec
B)
actin
profilin
APC-C dimer
Dia1-C dimer
FH1 domain
FH2 domain
Dia1 C-terminus
Separation
Nucleation complex
Filament elongation

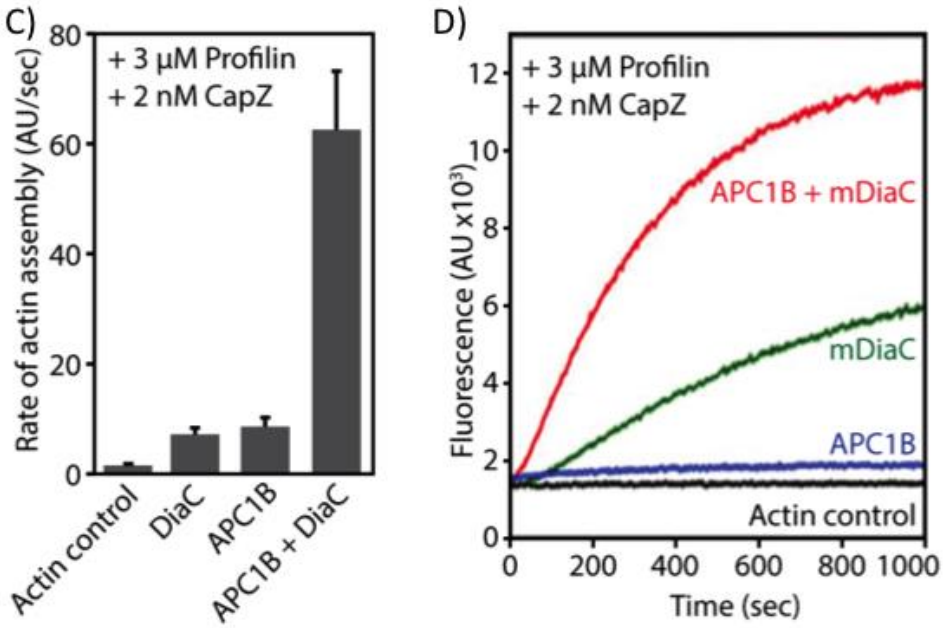

C)
+ 3 µM Profilin
+ 2 nM CapZ
Rate of actin assembly (AU/sec)
0
20
40
60
80
Actin control
DiaC
APC1B
APC1B + DiaC
D)
+ 3 µM Profilin
+ 2 nM CapZ
Fluorescence (AU x10³)
0
2
4
6
8
10
12
APC1B + mDiaC
mDiaC
APC1B
Actin control
0
200
400
600
800
1000
Time (sec)

Figure 5

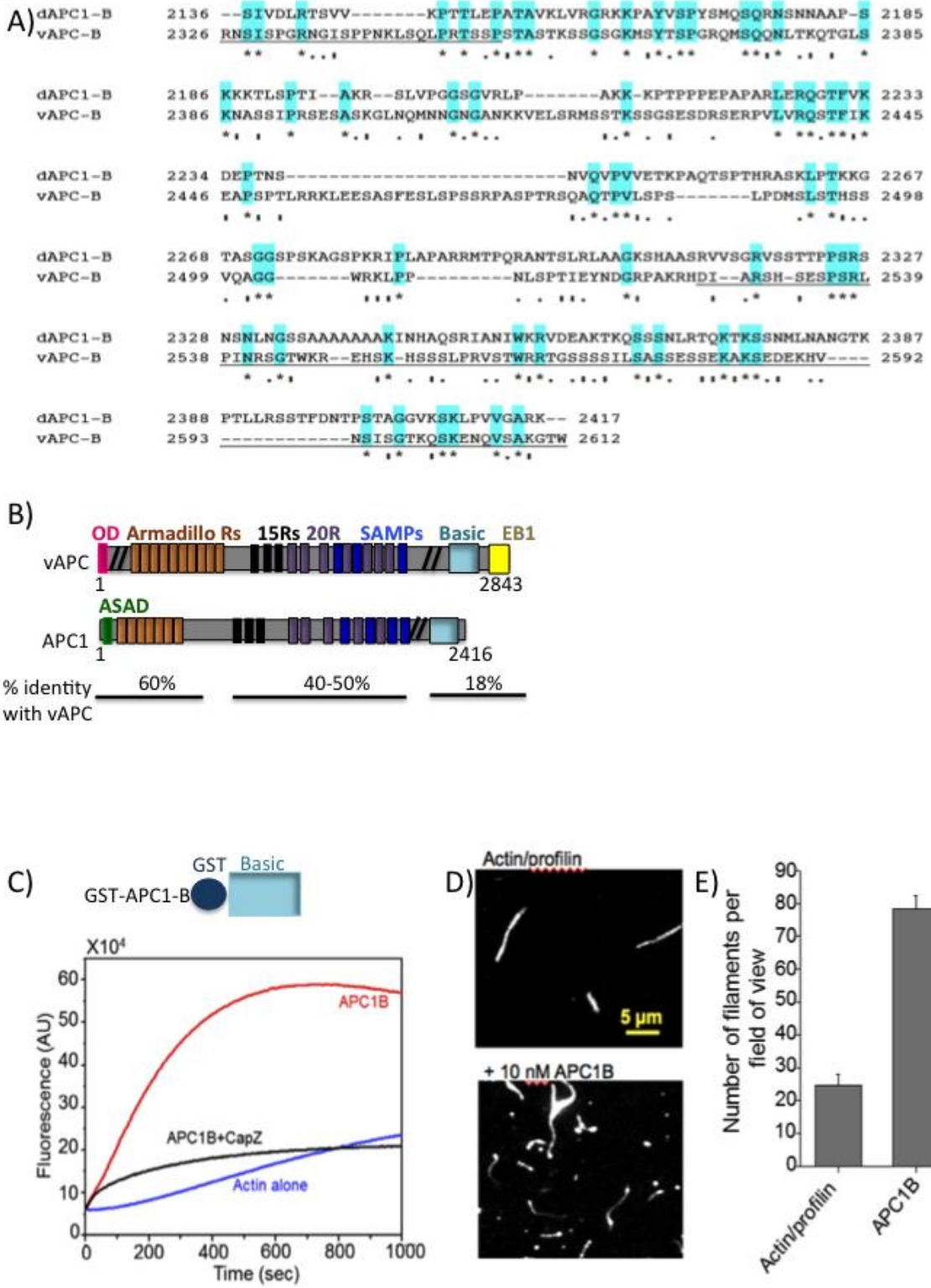

A)
dAPC1-B 2136 --SIVDLRTSVV-------KPTTLEPATAVKLVRGRKKPAYVSPYSMQSQRNSNNAAP-S 2185
vAPC-B 2326 RNSISPGRNGISPPNKLSQLPRTSSPSTASTKSSGSGKMSYTSPGRQMSQQNLTKQTGLS 2385
dAPC1-B 2186 KKKTLSPTI--AKR--SLVPGGSGVRLP-------AKK-KPTPPPEPAPARLERQGTFVK 2233
vAPC-B 2386 KNASSIPRSESASKGLNQMNNGNGANKKVELSRMSSTKSSGSESDRSERPVLVRQSTFIK 2445
dAPC1-B 2234 DEPTNS-------------------------NVQVPVVETKPAQTSPTHRASKLPTKKG 2267
vAPC-B 2446 EAPSPTLRRKLEESASFESLSPSSRPASPTRSQAQTPVLSPS-------LPDMSLSTHSS 2498
dAPC1-B 2268 TASGGSPSKAGSPKRIPLAPARRMTPQRANTSLRLAAGKSHAASRVVSGRVSSTTPPSRS 2327
vAPC-B 2499 VQAGG-------WRKLPP---------NLSPTIEYNDGRPAKRMDI--ARSH-SESPSRL 2539
dAPC1-B 2328 NSNLNGSSAAAAAAAAKINHAQSRIANIWKRVDEAKTKQSSSNLRTQKTKSSNMLNANGTK 2387
vAPC-B 2538 PINRSGTWKR--EHSK-HSSSLPRVSTWRRTGSSSSILSASSESSEKAKSEDEKHV---- 2592
dAPC1-B 2388 PTLLRSSTFDNTPSTAGGVKSKLPVVGARK-- 2417
vAPC-B 2593 ----------NSISGTKQSKENQVSAKGTW 2612
B)
OD
Armadillo Rs
15Rs
20R
SAMPs
Basic
EB1
vAPC
1
2843
ASAD
APC1
1
2416
% identity with vAPC
60%
40-50%
18%
C)
GST
Basic
GST-APC1-B
X10⁴
Fluorescence (AU)
Time (sec)
APC1B
APC1B+CapZ
Actin alone
D)
Actin/profilin
5 µm
+ 10 nM APC1B
E)
Number of filaments per field of view
Actin/profilin
APC1B

Figure 6

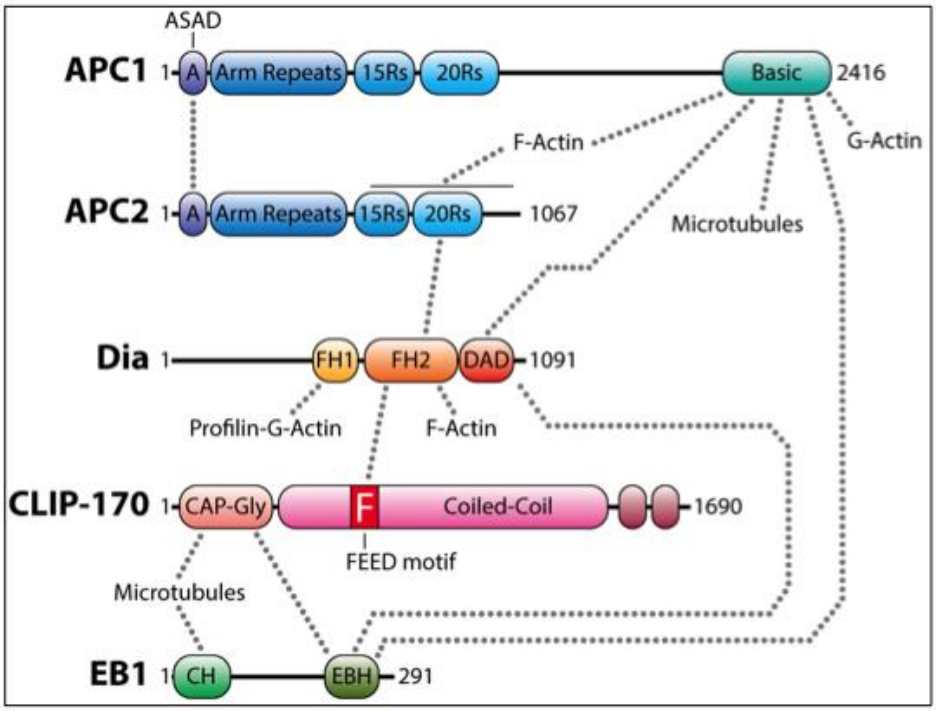
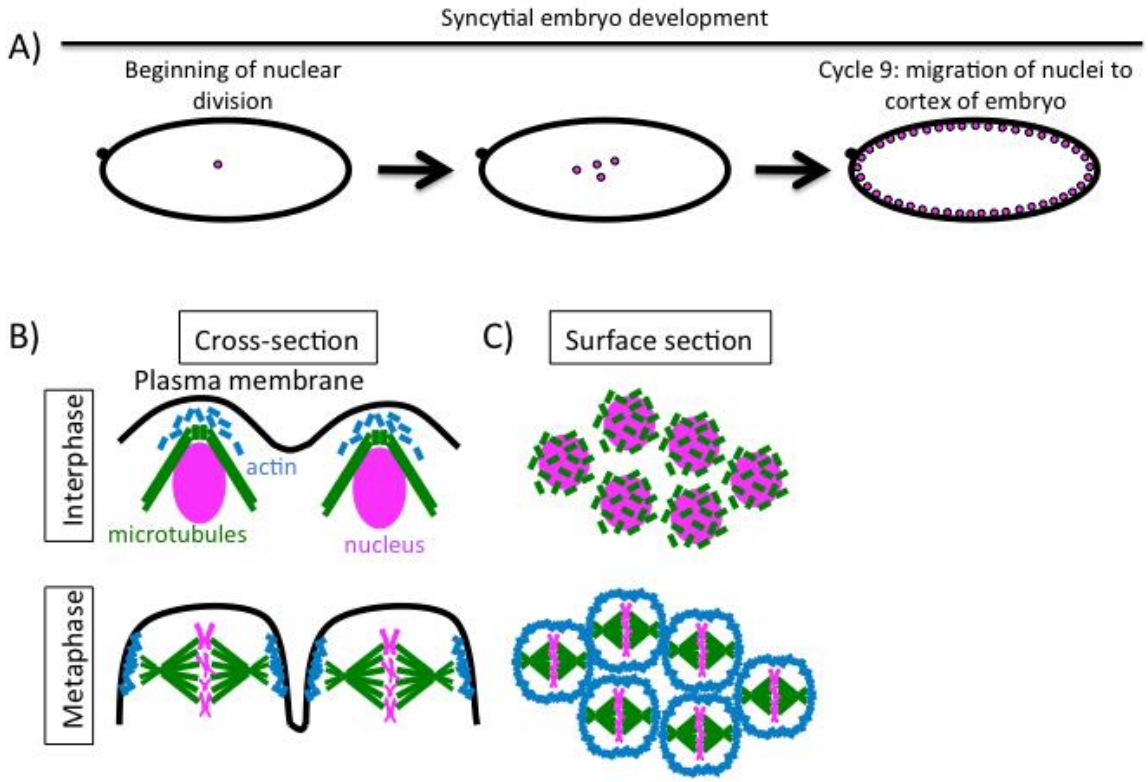

ASAD
APC1
1
A
Arm Repeats
15Rs
20Rs
Basic
2416
F-Actin
G-Actin
APC2
1
A
Arm Repeats
15Rs
20Rs
1067
Microtubules
Dia
1
FH1
FH2
DAD
1091
Profilin-G-Actin
F-Actin
CLIP-170
1
CAP-Gly
F
Coiled-Coil
1690
FEED motif
Microtubules
EB1
1
CH
EBH
291

Figure 7


A)
Syncytial embryo development
Beginning of nuclear division
Cycle 9: migration of nuclei to cortex of embryo
B)
Cross-section
C)
Surface section
Plasma membrane
Interphase
actin
microtubules
nucleus
Metaphase

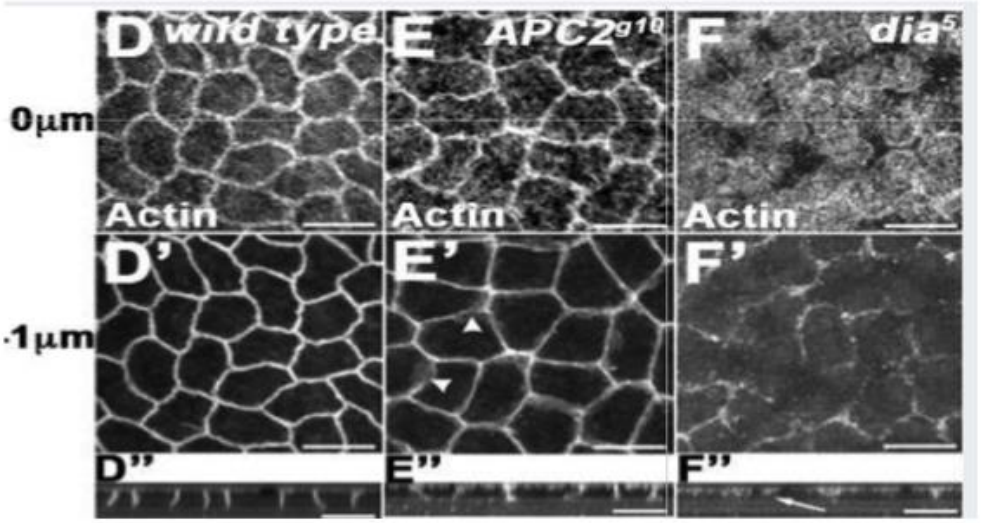
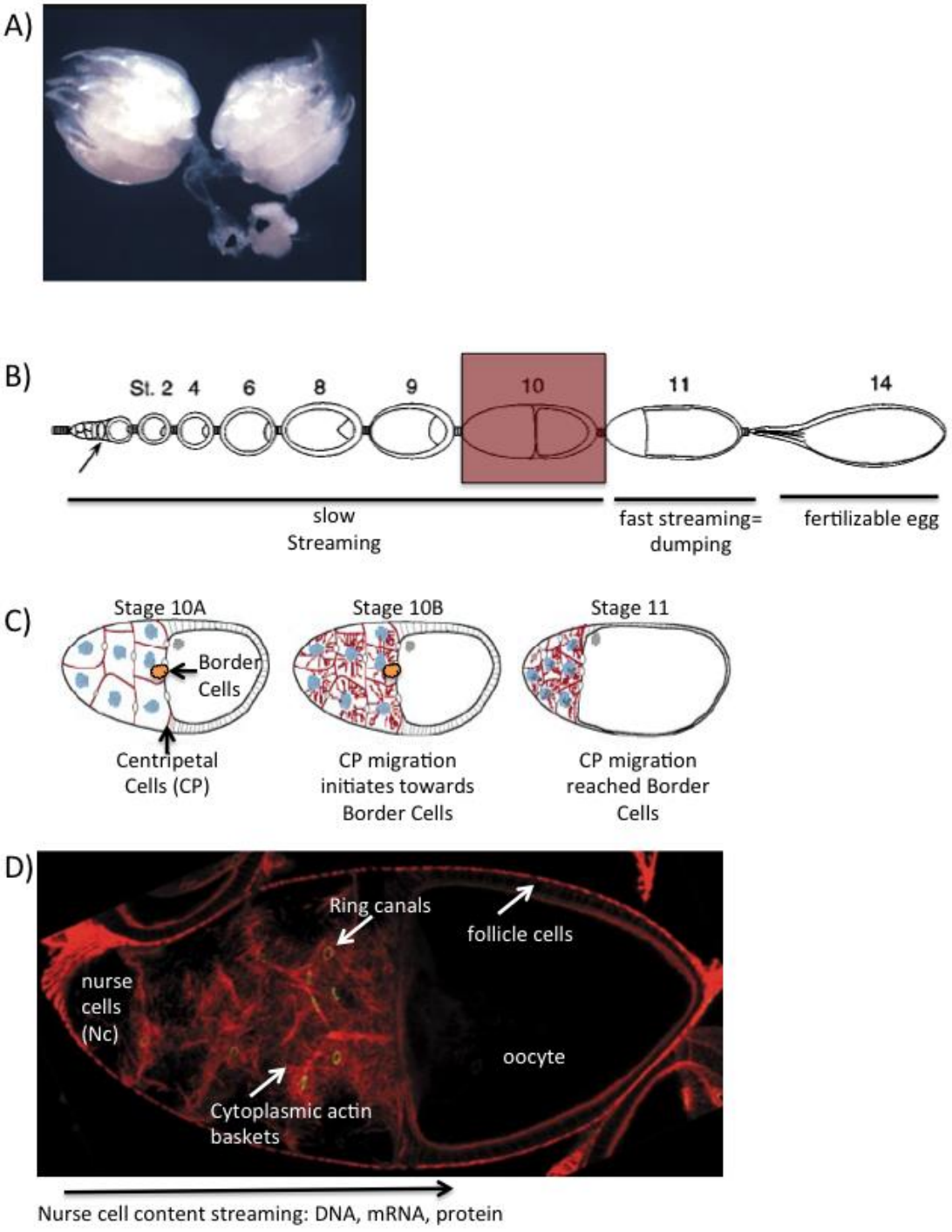

D wild type
E APC2g10
F dia5
0µm
Actin
Actin
Actin
D'
E'
F'
-1µm
D''
E''
F''

Figure 8


A)
B)
St. 2 4 6 8 9 10 11 14
slow Streaming
fast streaming= dumping
fertilizable egg
C)
Stage 10A
Border Cells
Centripetal Cells (CP)
Stage 10B
CP migration initiates towards Border Cells
Stage 11
CP migration reached Border Cells
D)
Ring canals
follicle cells
nurse cells (Nc)
oocyte
Cytoplasmic actin baskets
Nurse cell content streaming: DNA, mRNA, protein

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Chapter 2. Dissecting the cortical localization mechanism of APC2 and activity in actin assembly

2.1 Introduction

More than 80% of colon cancer incidents are linked to mutations in the colon cancer tumor suppressor Adenomatous polyposis coli (APC) (Groden *et al* 1991, Joslyn *et al* 1991, Kinzler *et al* 1991, Nishisho *et al* 1991). Most APC lesions are localized to the mutation cluster region (MCR) and truncate APC proteins. These mutations result in the loss of all the C-terminal domains involved in Wnt signaling, and microtubule and actin regulation. The multi-functional Adenomatous polyposis coli (APC) proteins regulate the actin and microtubule cytoskeletons, and negatively regulate the Wnt signaling pathway. In addition to functioning in many processes, APC also localizes to different cellular locations such as the cytoplasm, nucleus, microtubules +TIPs, actin structures, and the plasma membrane (McCartney *et al* 1999, Hamada *et al* 2002, Mimori-Kiyosue *et al* 2000, and reviewed in Bienz 2002). The distribution of APC proteins may contribute to the spatial regulation of its distinct functions. Although we are starting to uncover the mechanisms by which APC proteins are targeted to different subcellular distributions, we do not fully understand how the subcellular distributions of APC2 are controlled. Furthermore, less is known about how mutations in APC proteins affect subcellular distribution and function. Therefore, we need to better understand the mechanisms of the subcellular targeting of normal and truncated APC proteins to understand their normal and faulty roles in the cell.

2.1.1 APC in the cytoplasm

The most well studied function of APC proteins is in Wnt signaling. APC associates with a large macromolecular complex in the cytoplasm known as the destructosome. Here, together with Axin, PP2A, and the kinases GSK3β and CK1, APC targets β-catenin for

proteosomal degradation in the absence of the Wnt signal. Wnt pathway activation disrupts destructosome function leading to the accumulation of β-catenin in the cytoplasm, and its translocation into the nucleus, where it interacts with the transcription factor TCF to activate Wnt target genes. In SW480 cells, Axin localizes to the cytoplasm of the cells. Co-expressing both APC and Axin increases the length and size of the Axin puncta (Pronobis *et al* 2015 and Kunttas-Tatli *et al* 2014). In addition, APC and Axin association is regulated by phosphorylation and required for β-catenin destabilization.

2.1.2 APC in the nucleus

APC proteins were also found within the nucleus demonstrated by immunofluorescence (Anderson *et al* 2002 and Sena *et al* 2006). This leads to the hypothesis that APC functions to complement the functions of APC in Wnt signaling outside of the nucleus. To support the hypothesis that APC is localized within the nucleus, immunochemistry experiments suggest that C-terminal APC2 truncations found in colorectal cancer cell lines fail to localize APC2 to the nucleus (Galea *et al* 2001, Neufeld *et al* 1997, and Rosin-Arbesfeld *et al* 2003). Therefore, this suggested that Nuclear Localization Signals (NLS) were present at the C-terminus of APC2. In addition, treatment of cells with leptomycin B (LMB), an inhibitor of the nuclear export receptor CRM1, enriches the localization of APC in the nucleus (Galea *et al* 2001, Neufeld *et al* 1997, and Rosin-Arbesfeld *et al* 2003). Therefore, these findings further validated that APC could be shuttled in and out of the nucleus. APC is a very large protein of about 310-kDa. The large protein size of APC proteins impedes APC from crossing to the nucleus by passive diffusion. It requires a targeting sequence to enter the nucleus.

Two Nuclear Localization Sequences (NLS) were identified in the C-terminus of the protein. Loss of these signals in truncated APC proteins is a potential reason why the truncated protein is localized to the cytoplasm in cancer cell lines (Galea *et al* 2001 and Zhang *et al* 2000). Besides the two NLS targeting signals, it also contains a Nuclear Export Signal (NES) that helps it move out of the nucleus (Henderson *et al* 2000, Neufeld *et al* 1997, Tickenbrock *et al* 2002, Rosin-Arbesfeld *et al* 2002, and Rosin-Arbesfeld *et al* 2002). Evidence of APC in the nucleus suggests that it helps prevent TCF mediated transcription by interacting with the transcription complex in nucleus. Because APC binds β-catenin, it is predicted that APC2 sequesters β-catenin in the nucleus disrupting TCF mediated transcription. In addition, it was suggested that APC shuttles β-catenin to the cytoplasm where it can be destabilized by the destruction complex. Most recently, Roberts *et al* 2012 concluded that the subcellular localization of APC is not important in down-regulating β -catenin nor that shuttling APC to the nucleus is required to regulate Wnt signaling. In these experiments, different localization tags were fused to APC proteins targeting APC to different subcellular locations (Roberts *et al* 2012) and expressed them in a colorectal cell line (SW480). All of the mutants could rescue the Wnt signaling defects observed in SW480 cells where the targeted mutants down-regulated cytoplasmic β –catenin.

Because APC1 or APC2 bind to many proteins, the subcellular localization of these proteins is partially required. The interaction of APC binding proteins potentially recruits APC proteins and this in turn regulates the activity of APC proteins. For example, APC2 binding to cytoplasmic cytoskeletal proteins can localize APC2 to the cytoplasm. In turn, APC2 in the cytoplasm can function in cytoskeletal regulation. Therefore, understanding how APC proteins are localized to the different subcellular compartments and how they

interact with different APC binding proteins at these locations will be key to understand APC function.

Another vertebrate APC-like or APC2 protein that contains most of the APC1 protein domains including the basic domain required for direct microtubule and actin regulation was identified (Nakagawa *et al* 1998). APC2 is a potential tumor suppressor linked to ovarian cancer through APC2 allelic imbalance (Jarrett *et al* 2001). When over-expressed in cell culture, APC2 is localized to the Golgi apparatus, actin structures, and occasionally on microtubules (Jarrett *et al* 2001). Antibody localization of APC2 follows a similar trend, except it does not localize APC2 to microtubules. Treatment of cells with an actin disassembly drug disrupted the actin cytoskeleton, but APC2 remained associated with actin fragments, suggesting that APC2 is associated with actin (Zhou *et al* 2011). APC2 can also function in β-catenin binding and de-stabilization but is less effective than APC (Schneikert *et al* 2013). Axin but not Axin 2 displaced APC2 but not APC2 from the cytoskeleton into cytoplasmic dots (Schneikert *et al* 2014). Binding of proteins such as Wnt signaling components versus cytoskeletal proteins may regulate the sub-cellular distribution of APC proteins, which in turn regulates their function. In the chick retina, APC2 is distributed along microtubules and actin in growth cones and axon shafts. Over-expression of APC2 led to the stability of microtubules resulting in proper axonal projection (Shintani *et al* 2009). In mouse, APC2 is expressed in neurons and the brain where it potentially regulates the development of neuronal cells (Yamanaka *et al* 2002). In mouse neurons, APC2 is localized to microtubules and actin fibers (Shintani *et al* 2012) and loss of APC2 leads to impaired cell migration (Shintani *et al* 2012). Most recently, a mutation deleting the basic domain but not any of the other APC2 protein domains was

characterized in depth. Loss of the most C-terminal domains of APC2 resulted in the loss of cytoskeletal regulation in neurons (Almuriekhi *et al* 2015). In addition, through their work, this group linked APC2 as downstream effector of a histone methyltransferase (Nuclear receptor binding Set Domain containing 1 or Nsd1). Homozygous mutations of a specific point frameshift mutation at the C-terminus that deletes the basic domain of APC2 (Figure 2B) leads to Sotos syndrome that is characterized by mental retardation and abnormal facial features (Almuriekhi *et al* 2015).

2.1.3 Fly APC2 localizes to microtubules and actin

APC1 expression is restricted to gonads and central nervous system (CNS) (Hayashi *et al* 1997 and Ahmed *et al* 1998). In the CNS, APC1 is highly concentrated at the fiber actin tracks that are made by actin and microtubules (Hayashi *et al* 1997). The function of APC1 in the CNS is not known. Though, evidence strongly suggests that APC1 functions in regulating apoptosis through Wnt signaling in the Drosophila photoreceptors. On the other hand, APC2 is expressed ubiquitously throughout development and found in many different cells. APC2 is highly enriched in the actin furrows during the syncytial embryo and at high concentrations on astral microtubules (McCartney *et al* 1999 and reviewed in Bienz 2002). After cellularization, expression and localization of APC2 increases in the cytoplasm. At this time, zygotic transcription in the early embryo is turned on and it is possible that APC2 tanslocates to the cytoplasm in order to perform its function in Wnt signaling. In stem cells in the larval brain, APC2 is present as a cortical crescent where it is potentially implicated in asymmetric division through Wnt signaling. In the Drosophila ovary, APC2 promotes stem cell niche size and cap cell localization through the dual roles

of APC2 in actin assembly and Wnt signaling, respectively (Stacie Oliver, unpublished data). Although APC2 associates with actin in many contexts, APC2 does not always associate with actin. For example, APC2 is not localized with actin at the cytokenetic furrows during division (McCartney *et al* 1999). Therefore, because APC association is context specific and seems to be highly regulated, we need to further dissect how and why APC is targeted to actin.

2.1.4 Cortical APC2 is required for actin furrow extension

The fly APC2 localizes to cortical actin and is associates with actin in the pseudocleavage actin furrows in a cell-cycle dependent manner (McCartney *et al* 1999 and Yu *et al* 1999). During these stages, APC2 co-localized at the actin caps formed during interphase and localization changes to the actin furrows that form as barrier for the nuclei during metaphase to provide mitotic fidelity (Figure 7B an 7D). The association of APC2 to the cortex is actin-dependent, but we still don't know how APC2 is targeted to the actin rich cortex.

APC2 localizes to actin in the *Drosophila* syncytial embryo, where together with the formin Diaphanous, it is required for the extension of metaphase actin furrows (Webb *et al* 2009). To understand how APC2 promotes cortical actin assembly, we are investigating the mechanisms that affect the cortical localization of APC2. Previously we reported that the N-terminal domain and the C-terminal 30 amino acids (C30) together are necessary and sufficient for the cortical localization of APC2. Deletion of C30 alone resulted in loss of cortical localization in the syncytial embryo, S2 cells, and embryonic epithelia (Zhou *et al* 2011). APC2 cortical association is required for actin furrow extension but dispensable for

the role of APC2 in Wnt signaling (Zhou *et al* 2011). This further validates that subcellular localization of APC2 at the cortex is important for actin assembly. We can further use this separation of function allele (APC2ΔC30) to dissect the actin assembly function of APC2.

We identified a 15 amino acid sequence (amino acids 1048-1063) within C30 that is highly conserved within *Drosophila* species. The conserved domain is necessary and sufficient for the activity of C30 at the cortex, and disruption of a predicted coiled-coil within this sequence abolishes cortical localization. Because coiled-coils promote self-association and other protein-protein interactions, we predicted that the self-association of APC2 through the N-terminus facilitates the dimerization of the C30 coiled-coil. Consistent with this model, we showed that the N-terminal half of APC2 is required for both APC2 self association and cortical localization. Thus, we predicted that APC2 self-association through the N-terminal domain is necessary to stabilize C30 coiled-coils and promote cortical localization. To test this, we asked whether dimerization of C30 using the rapamycin inducible FRB/FKBP system can drive cortical localization in *Drosophila* S2 cells. Forced dimerization of C30 partially restored localization of these APC2 chimeric proteins, suggesting that self-association of APC2 plays a role in its cortical localization. Although APC2 binds to the actin cortex we believe that it is associated weakly with the actin cortex on its own and we do not know how APC2 is recruited to the cortex. Therefore, we predict that binding of APC2 to other proteins recruits APC2 to the cortex and stabilizes APC2 at the cortex. Our model for the cortical association of APC2 proposes that the dimerization of C30 creates a binding groove for an actin-associated protein to bind and tether APC2 to the cortex. Once at the cortex, we know that APC2 is involved in actin assembly and collaborates with Diaphanous (Dia) to form actin furrows in the early embryo. To

understand the mechanistic role of APC-Dia mediated actin assembly in the early embryo, we further explored Dia-APC2 binding. We found that the 20Rs in APC2 are required and sufficient for Dia binding. In addition we found that the 20Rs are dispensable for cortical localization but phospho-regulation of the 20Rs is required for actin assembly in the early embryo.

2.2 Materials and Methods

2.2.1 DNA constructs

Deletion fragments and point mutations were synthesized by PCR site-directed mutagenesis using mCh-APC2-N-C30 formerly cloned into the metallothionein inducible pRmHA3 plasmid (Zhou et al 2011). The following constructs were generated: mCh-APC2-N-C30-1 (1-490, 1038-48), mCh-APC2-N-C30-2 (1-490, 1049-57), mCh-APC2-N-C30-3 (1-490, 1058-67), mCh-APC2-N-C30-1 (1-490, 1038-48), mCh-APC2-N-C30-conserved (1-490, 1038-48, 1064-67), mCh-APC2-N-C30-divergent (1-490, 1049-63), mCh-APC2-N-C30-H>P (1-490, 1038-1067 [V1051P, V1055P, I1058P, L1062P]), mCh-APC2-N-C30-V>P (1-490, 1038-1067 [V1055P]), and mCh-APC2-N-C30-M>P (1-490, 1038-1067 [M1061P]).

To generate the FRB and FKBP dimerization constructs, we ligated the PCR amplified EGFP with DraIII overhangs into pMoMo 5' to the linker. pMoMo is a unique plasmid constructed by the Jarvik Lab that contains a linker and three unique sites to insert three different fragments. The plasmids containing FRB and FKBP (gifts from Dr. Jon Jarvik) fragments were digested with Sfi (NEB) and ligated to an ALwN1 (NEB) digested pMoMo-EGFP. C30 was then PCR amplified with Pflm1 restriction site overhangs and ligated to

pMoMo-EGFP-FRB or pMoMo-EGFP-FKBP. We then used the pMoMo-EGFP-FRB-C30 or pMoMo-EGFP-FKBP-C30 as PCR templates to construct the following constructs into the pRmHA3 vector through 5'-Kpn1 and 3'-BamH1 digestion and ligation: EGFP-FRB-C30 and 6XH-FKBP-C30. mCh was PCR amplified with a 5'EcoR1 and 3' Kpn1 overhangs and ligated to the 6XH-FKBP-C30 to generate mCh-6XH-FKBP-C30.

To generate a GST-APC2-N-C30-3 (1-490, 1058-67), the plasmid was PCR amplified with flanking 5'-BgLII and 3'EcoR' restrictions sites and subcloned into pLm1.

2.2.2 S2 cell transfection

S2 cells were transfected with our constructs using Effectene (Qiagen). All of our constructs were driven by the metallothionein promoter (pRmHA3) and therefore, induced after 24 hours with $CuSO_4$ at a final concentration of 40μM. Our FRB and FKBP constructs were induced with 10μM $CuSO_4$ after concentration of $CuSO_4$ was optimized for this experiment. These cells were then treated with rapamycin or without rapamycin for 6 hours.

2.2.3 Imaging and analysis

Images were acquired with a spinning-disc confocal microscope with a Yokagawa scan head (Solamere Technology Group) and a QICAM-IR camera (Qimaging) on a Zeiss Axiovert 200M using QED InVivo software. Cells were imaged live 14-16 hours post-induction. In order to quantify the cortical to cytoplasmic protein ratio, we fixed the S2 cells with 4% formaldehyde for 10 min followed by blocking in PNT (1% PBS, 1% NGS, and 0.1% Triton-X100) and treated with phal-546 (Invitrogen). By using ImageJ, we drew a line across the cells and obtained the plot profile of the pixel intensity (Zhou et al. 2011) of all the

channels. We could then use actin as marker for the cortex and calculate the cortical to cytoplasmic ratio of C30 mutant protein.

2.2.4 High-Speed Actin Co-sedimentation Assay

Full-length murine αE-catenin (aa 1-906) homodimer was purified as described (Hansen *et al*., MBoC 2013).

Chicken G-actin was incubated in polymerization buffer (20 mM HEPES [pH 7.5], 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 1 mM EGTA) for 1 hour at room temperature to polymerize filaments. Proteins were diluted to tested concentrations in reaction buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM ATP, 1 mM EGTA, 1 mM DTT) with and without 2 μM F-actin and incubated for 30 minutes at room temperature. Samples were centrifuged at 435,000g for 20 minutes in a S100-AT3 rotor (Thermo Scientific). Pellet samples were diluted in Laemmli sample buffer, separated by SDS-PAGE and stained with Coomassie blue. Gels were imaged on a LI-COR scanner, and protein bands were measured and quantified in ImageJ.

2.2.5 Immunoblotting

For FRB/FKBP expression confirmation, immunoblots were incubated with primary rabbit-α-mCherry (Clontech 1:1000) and rabbit-α-EGFP (abcam 1:5000) overnight. Secondary HRP-conjugated antibodies (α-rabbit-HRP 1:5000) were incubated for three hours.

GST-pulldowns were probed with an HRP-conjugated-anti-6XHis (Bethyl Labs 1:10,000) overnight. Immunoblots were developed using the HRP SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce).

2.2.6 Fly stocks

w^{1118}, $APC2^{g10}$ (McCartney *et al* 2006) and $APC1^{Q8}$ (Ahmed *et al* 1998) fly lines were used for our complete loss of function experiments. To perform the localization and rescue experiments, *P[endoP-EGFP-APC2-FL]* (Zhou *et al* 2010) and *P[endoP-EGFP-APC1]* (Roberts et al 2012) were crossed into $APC2^{g10}$ and $APC1^{Q8}$ backgrounds, respectively. Other transgenic flies that were used for the loss of functions analysis: *P[endoPEGFP-APC2- Δ20R], P[endoPEGFP-APC2-R1R5SA], P[endoPEGFP-APC2-R1R5SD], P[endoPEGFP-APC2-R3R5SA]*, and *P[endoPEGFP-APC2-R3R5SD]* (Zhou *et al* 2010 and Kunttas-Tatli *et al*. 2012).

2.2.7 Embryo prepping

0-2 hours embryos were collected, dechorinated by 50% bleach, and hand-peeled to remove the vitelline membrane. Embryos were fixed in 4% paraformaldehyde and blocked for one hour in PNT (1X PBS, Triton-X, and NGS). Primary antibody to stain for microtubules (DSHB E7 mouse-anti-tubulin) at a dilution of 1:500 and incubated overnight 4ºC. Secondary alexa-conjugated anti-mouse and phalloidin (Invitrogen) were incubated at room temperature for three hours. The embryos were washed, incubated for 30 minutes with DAPI, and mounted on Aqua/PolyMont.

2.2.8 Actin furrow extension analysis

To analyze the depth of the furrows, cross-sectional single images of syncytial embryos were taken at four different depths in which the most superficial surface of the embryos is 0μm, preceded by -0.8μm, - 1.6μm and -2.2μmin in order to capture complete furrows in the embryo. In order to obtain a quantitative assessment of the depth furrows, we counted the number of complete and incomplete rings at these different depths, and percentages were plotted.

2.3 Results

2.3.1 A conserved 15 amino acid motif at the C-terminus is necessary for the cortical localization of APC2

We demonstrated that the N-terminal domain of APC2 (N-term), containing the ASAD (APC self-association domain) and the Armadillo (Arm) repeats, and the C-terminal 30 amino acids (C30) are each necessary, and together sufficient, for the cortical localization of APC2 (Zhou *et al* 2011) (Figure 9B). To identify the mechanism by which C30 promotes cortical localization, we compared the sequence of C30 across Drosophila species and identified a highly conserved 15 amino acid sequence (Figure 1A)(Ezgi Kunttas-Tatli). To test for the requirement of this sequence in cortical localization, Paige Davison, Molly Bernstein, and Terrence Wong generated two constructs: one containing the N-terminus and the conserved 15 amino acids (APC2-N-C30-CV), and the other containing the N-terminus with only the non-conserved sequence in C30 (APC2-N-C30-DV; Figure 9B). When expressed in S2 cells, mCherry (mCh) tagged APC2-N-C30-CV localizes to the cortex like APC2-FL or APC2-N-C30 (Fig. 1C). Consistent with that, deletion of the conserved 15 amino acids abolished cortical enrichment (APC2-N-C30-DV; Fig. 1C). Consistent with this, other deletions disrupting these 15 amino acids prevented the enrichment of APC2 to the actin rich cortex (data not shown). Thus, these 15 amino acids are necessary and sufficient (in the context of the C-terminus) to drive cortical localization. For simplicity, we will refer to this 15 amino acid sequence as the CCES (C-terminal Cortical Enrichment Sequence).

2.3.2 The conserved motif forms a coiled-coil that is required for the function of C30 at the cortex

Two different computational predictions of secondary structure generated using COILs suggested that the CCES is a HEPTAD repeat containing an α-helix that may contribute to the formation of a coiled-coil. Coiled-coils are generated by two or more alpha-helices that associate to create a stable zipper-like conformation (Wang *et al* 2014). These often play critical roles in protein-protein interactions and protein self-association *(*Santis *et al* 2015*).* Thus, we predicted that the CCES from 2 or more APC2 molecules forms a coiled-coil that is required for the cortical localization of APC2. To test this hypothesis, Paige Davison disrupted the putative coiled-coil by changing four key hydrophobic amino acids within the HEPTAD repeat (the a and d positions) to proline. This creates kinks, disrupting the formation of α-helices and consequently coiled-coils (Santis *et al* 2015). This resulted in the complete loss of the cortical enrichment of APC2 in S2 cells, and was indistinguishable from a complete deletion of the conserved residues (Fig 10B). It is also possible that deleting the CCES domain leads to the synthesis of an unstable protein. In turn, a mutant unstable protein that is potentially not expressed at wild type levels can potentially be causing loss of APC2 cortical localization. We did not perform assays to test the stability of the protein but we did mutate other residues within the CCES domain to test whether the coiled-coil was required to drive the cortical localization of APC2. In addition, we generated a single point mutation changing a hydrophobic amino acid to a hydrophilic amino acid (valine to threonine) at the d position (Figure 10B). This too completely abolished cortical enrichment (Fig. 10B). On the other hand, we predicted that mutating a

hydrophobic amino acid that does not contribute to the formation of the coiled-coil within the CCES domain would not affect the cortical localization. As predicted, mutating the hydrophobic amino acid that was not present at the a and d positions, did not disrupt the cortical localization of APC2 (not shown).

2.3.3 Artificial dimerization of the CCES using FRB and FBKP drives APC2 to the cortex

Identification of a C-terminal coiled-coil that mediates cortical enrichment suggests that cortical APC2 is forming higher-order complexes, and that this self-association is necessary for its localization. We have previously shown that the N-terminal ASAD forms a coiled-coil that mediates association between APC2 molecules, and between APC2 and APC1 (Kunttas-Tatli *et al* 2014). Because C30 alone is not sufficient for cortical enrichment, and the required N-terminus contains the ASAD, we speculated that the role of the N-terminus is to drive efficient self-association to promote the assembly of the short CCES coiled-coil. To test this hypothesis, we asked whether dimerization of C30 containing the CCES through the rapamycin inducible dimerization of FRB and FKBP (Dixon *et al* 2015) could promote cortical enrichment. Expression of either mCh-FKBP-C30 or EGFP-FRB-C30 alone, with or without rapamycin (Figure 11A), resulted in cytoplasmic localization similar to the expression of the C-terminal half of APC2 or C30 alone (Figure 9B; Zhou *et al* 2011). Similarly, co-expression of these chimeras without rapamycin resulted in only cytoplasmic localization. In contrast, co-expression of mCh-FKBP-C30 and EGFP-FRB-C30 in the presence of rapamycin resulted in enhanced enrichment of the chimeric proteins at the cortex (Fig 11D). The reciprocal experiments using mCh-FRB-C30

and EGFP-FKBP-C30 yielded the same results. Our data suggest that dimerization of APC2 through the N-terminal ASAD is necessary for the cortical localization activity of the CCES. Consistent with this model, deletion of ASAD significantly disrupts the cortical enrichment of APC2 in S2 cells and embryonic epithelia (Kunttas-Tatli *et al* 2014).

While co-expression of the chimeras in the presence of rapamycin did drive cortical enrichment, we recognized that it did not reconstitute wild type levels of enrichment. To rule out the possibility that the fluorescent tags mCh and EGFP are interfering with FRB-FKBP binding through steric hindrance, we repeated the experiments substituting one of the fluorescent tags with a 6xHis-tag. These experiments yielded the same degree of cortical enrichment when the proteins were co-expressed in the presence of rapamycin (not shown). Two possible interpretations, that are not mutually exclusive, could potentially explain the reduced cortical enrichment induced by the chimeric APC2 FKBP-FRB protein fragments. APC2 may bind to the cortex most efficiently as oligomers, rather than the dimers produced by FRB-FKBP interactions. In addition, the N-terminal domain of APC2 may be providing two different cortical localization functions: self-association through the ASAD, and protein-protein interactions at the cortex mediated by the Arm repeats. Arm repeats are a known protein-protein interaction domain that mediates the binding of vAPC to Kap3 and Asef, for example. In these experiments, we replaced the APC Self-Association Domain with the artificial FRB-FKBP dimerization proteins, but the Arm repeats required for protein-protein interactions are still missing. Interestingly, this is consistent with our earlier work demonstrating that APC2 proteins with missense mutations in the Arm repeats exhibit reduced cortical enrichment in embryonic epithelia (McCartney *et al* 1999). Taken together, these results support a model where ASAD and the

CCES cooperate to drive APC2 localization to the actin-rich cortex. It is possible that an unknown Arm repeat binding protein(s) tethers, enhances, and/or stabilizes the interaction of APC2 with the actin at the cortex.

2.3.4 APC2 binds F-actin directly

APC2 localizes to actin and actin rich structures in many cell types and developmental contexts in Drosophila: to the cortex in S2 cells, to the adherens junctions in embryonic epithelia, and to actin cables assembled during oogenesis, for example (McCartney *et al* 1999, Roberts *et al* 2012, Hamada *et al* 2002, and Yu *et al* 1999). We and others have shown that cortical localization in both S2 cells and in embryonic epithelia is dependent on an intact actin cytoskeleton (Zhou *et al* 2011 and Hamada *et al* 2002). Clearly APC2 associates with F-actin, but we do not know whether this interaction is direct and/or indirect through other protein partners. To ask whether APC2 can bind to F-actin directly, we performed high-speed F-actin co-sedimentation assays with bacterially expressed His-tagged fragments of APC2. I cloned the APC2-N-C30 fragment and expressed APC2-Cterm and APC2-N-C30 (Figure 12A). The direct binding actin assays were done by Dr. Adam Kwiatkowski. Interestingly, both APC2-Cterm and APC2-N-C30 co-sedimented with F-actin under these conditions, like vertebrate □-Ecatenin (□Ecat), a protein with high F-actin binding affinity (Figure 12B and C).

2.3.5 The 20Rs and required for Dia binding and phosphor-regulation of the 20Rs is required for actin furrow extension

Thus far, we have shown that APC2 binds to actin. One hypothesis is that N-terminus and CCES bind to their respective binding partners at the cortex to localize APC2 to the cortex and once at the cortex, binding to other proteins at that cortex enhances the binding affinity of APC2 to the actin cortex. We also have shown that APC2 and Dia interact biochemically and genetically (Webb *et al* 2009). In the syncytial embryo, APC2 and Dia are both required for actin furrow formation and APC2 is most likely enhancing the activity of Dia at the cortex. Though, it is unclear how APC2 and Dia collaborate to form actin furrows during metaphase in order to provide the mitotic fidelity in the early embryo. Our work demonstrated that localization of APC2 to the furrows is dependent on Dia but not the other way around (Webb *et al* 2009). Therefore, Dia is required to localize APC2 to the furrows but it is not clear if APC2 binding to Dia targets APC2 to the cortex.

The C-terminus of APC2 and C-terminus of Dia interact directly through biochemical pull-down experiments (Webb *et al* 2009). To further dissect their binding, we expressed APC2 GST tagged C-terminal fragments and used GST pulldowns to test their interaction with Dia-C. The 20Rs are sufficient for binding to DiaC (Figure 13A). This was interesting because the 20Rs of APC proteins bind to β-catenin and the binding affinity of this interaction is enhanced through phosphorylation of APC2 at the 20Rs (Rubinfeld *et al* 1996). This was interesting because the same domain required for Wnt signaling is also required for actin binding. Because of this, we predicted that the 20Rs of APC2 might play a role in localizing APC2 to the cortex. Additionally, since the 20Rs were required for Dia binding *in vitro*, we predicted that the 20Rs of APC2 were required for collaboration between Dia and APC2 in actin furrow extension. Loss of the 20Rs did not mislocalize APC2 from the cortex in the syncytial embryo strongly suggesting that the 20Rs are not required

for cortical localization (Figure 13B). Though, furrow extension defects were observed when the 20Rs were deleted (Figure 13B). Therefore, these results suggest that that the 20Rs in APC2 are not involved in the cortical localization mechanism of APC2 but they are required in actin assembly in the early embryo. Taken together we hypothesize that APC2 is recruited to the cortex through the N-terminal and CCES domains (Figure 14). Once at the cortex, APC2 functions to extend the actin furrows in the early embryo. From our data, we speculate that APC2 binds to Dia through its 20Rs and collaborate in actin furrow extension (Figure 14).

The interaction of APC2-20Rs and Dia-C prompted us to ask how this interaction is regulated. Precedent indicates that phosphorylation of the 20Rs through GSK3-β enhances its binding affinity to β-catenin. Therefore, we hypothesized that is was possible that the same phospho-regulation was required to modulate the interaction between APC2 and Dia. Before we could test phosphorylation of the 20Rs on APC on Dia binding, we first wanted to assess whether GSK3β was required for actin furrow extension. McCartney *et al* 1999 demonstrated that loss of GSk3β in the early embryo displaced APC2 from the actin rich cortex, suggesting that GSK3β is required for the cortical localization mechanism of APC2. To validate these data, instead of making germline clones, we used RNAi against GSK3β. In our GSK3β knockdown analysis, we were trying to answer two questions: 1) does knockdown of GSK3β affect the cortical localization of APC2? and 2) is GSK3β required for actin furrow extension? Preliminary data suggested (not shown) that actin defects were observed in the actin furrows and loss of synchrony in nuclear division was observed. We could not quantify the actin furrow extension defects to make any definite conclusions because our preps did not yield enough embryos to analyze (data not shown). In addition,

we could not assess the cortical association of APC2 due to technical problems with our APC2 antibody.

An alternative approach toward dissecting the role of phosphorylation in APC2-Dia mediated actin assembly in the early embryo, was to we assess the actin furrow extension in APC2-20R phospho-mutants. All the phosphorylation sites were either mutated to abolish phosphorylation (mutation of S to A) or mutated to mimic phosphorylation state (S to D). These phospho-mutants were initially characterized in Kunttas-Tatli *et al* 2012. In the early embryo, phosphorylation and non-phosphorylation of the 20Rs is required for actin furrow extension (Figure 13C) (Ezgi Kunttas-Tatli unpublished data).

2.4 Discussion

APC2 is a multifunctional protein that is localized to different subcellular compartments such as the cytoplasm, nucleus, microtubule tips, and the actin rich cortex. The localization of APC2 to all these different subcellular compartments is hypothesized to help APC2 toggle between all of its different functions. A link between APC2 subcellular localization and function was established in several contexts. However it is unclear how APC2 is differentially targeted to each subcellular location. My research was focused on understanding the role of APC2 in actin assembly. Our previous work has demonstrated that the cortical localization of APC2 is required for actin furrow assembly in the Drosophila embryo. My goal was to further dissect the cortical localization mechanism of APC2 to better understand the role of APC2 in actin assembly.

2.4.1 The role of C30 in the cortical localization of APC2

We identified a 15 amino acid conserved coiled-coil in the C30 domain of APC2 necessary for cortical localization. Although, our work demonstrated that the CCES domain is required and sufficient for the activity of C30 at the cortex, it is unclear how this coiled-coil is functioning at the cortex. Coiled-coils are common motifs that are required for protein-protein interactions (Hu 2000). APC2 can potentially use this motif to bind directly to actin by creating a binding groove for actin when the C30 coil self-associates. Our work in collaboration with the Kwiatkowski lab suggests that the C-terminal domain of APC2 does bind to actin directly but that this interaction is weak. I am hypothesizing this based on the actin pelleting assays and comparing binding to either APC fragments to the known actin binding protein, α-catenin. More experiments need to be performed to test the interaction of APC2 with the actin cytoskeleton to conclusively state whether APC2 can directly to actin. In a different set of APC2-C terminal *in vitro* activity experiments, APC2 does not bundle actin filaments or stabilize F-actin (unpublished data in collaboration with the Goode Lab). All these experiments suggest that APC2-C terminal binding to actin is very weak or non-existent. In addition, APC2 lacks the basic domain that allows APC1 to interact directly with actin. Based on these other observations, we favor the model that suggests that APC2 binds to the actin rich cortex through a protein-protein interaction. It is possible that the CCES is required to bind to another protein at the cortex where it can 1) recruit APC2 to the cortex and 2) stabilize the interaction between APC2 and actin or 3) both.

The C30 domain of APC2 forms a coiled-coil. In general coils can make homo or heterodimers. We predict that self-association of C30 forms to create a binding domain for another unknown protein (protein Y) (Figure 14). In turn, protein Y binds to APC2

recruiting it to the actin cortex. To identify the interacting partner of C30, we conducted a biochemical screen to find the binding partner of C30 by using purified GST-APC2-C or GST-APC-CΔC30. The purified proteins were bound to GST beads, incubated with embryo lysate pre-incubated with cytochalasin D (to disrupt the actin cortex) to assess the proteins that come down with both forms of the purified APC2 protein through GST pulldowns Unfortunately, in this experiment, no protein band differences were found.

In parallel to the biochemical screen, I was also performing a targeted screen to identify the binding partner of C30. Due to several technical difficulties and time constraint, I stopped working on this project. Although work on this project was halted, we need to continue to study APC2 protein interactions to better understand the functions of APC2. The Rolls Lab at Penn State found that loss of certain proteins at branch-points in dendrites leads to the loss of APC2 localization from these sites. To generate a comprehensive list of candidates that may be interacting with C30 at the cortex, we need to continue to compile a list of general actin binding proteins. Additionally, the Rolls lab at Penn State University identified that loss of several proteins mislocalized APC2 from branch-points in dendrites that are rich in actin. These branch point mutants could also be required for localization of APC2 to the actin rich cortex. dsRNA will be used to knockdown these candidates and assess the cortical localization of APC2 in S2 cells. In addition, because coiled-coils can form homotypic or heterotypic interactions, we can try to focus on finding the binding partner of C30 by focusing a two-hybrid screen on predicted coiled-coils (Screen focused on coiled-coil in Newman et al). For example, we can try to fuse the CCES to an unstructured protein domain that does not bind to any other protein and use this as the bait to test for proteins that bind to APC2. Additionally, the coiled-coil is very small and we can generate a longer

protein fragment of the CCES domain. Four or five tandem repeats of the CCES sequence can be fused together. A yeast two hybrid assay using C30 only was problematic because a lot of false positive hits were obtained (Roberts unpublished data). If we modify the C30 fragment to be more stable or longer, we may have a better chance of identifying proteins that bind to the CCES domain. From this screen, we can identify more proteins that bind to the C30 domain of APC2.

In addition to identifying the C30 binding partner, we also need to further characterize the *in vitro* actin binding and actin-associated functions of APC2. For example, some data indicate that APC2 binds weakly to actin. Does this interaction actually happen *in vivo* and if it does, what are the minimal requirements for the direct binding of APC2 to actin. In addition, we know that APC1 basic domain binds directly to actin to alter actin assembly positively. APC2 lacks the basic domain required for actin assembly but it may be possible that a different domain is required for actin assembly *in vitro*. Preliminary data in collaboration with the Goode Lab strongly suggests that APC2 does not stimulate actin assembly *in vitro*. Therefore, taken altogether, it seems more likely that APC2 is not a direct effector of actin assembly but an indirect effector of actin assembly.

2.4.2 The role of the N-terminal domain in the cortical localization of APC2

The N-terminal domain is required for APC self-association (Zhou *et al* 2011 and Mattie *et al* 2010). More specifically, the ASAD domain facilitates APC homotypic and APC1-APC2 heterotypic association required for the Destruction complex assembly and activity in Wnt signaling (Kunttas-Tatli *et al* 2014). We tested the role of dimerization of APC2 proteins in the cortical localization of APC2. The FRB/FKBP dimerization protein pair

replaced the N-terminal domain of APC2 and we assessed the localization of these proteins fused to C30. Dimerization of the N-terminal domain of APC2 is partially required to localize APC2 to the actin rich cortex. The N-terminus of APC2, specifically through the Arm repeats may also bind to other cytoskeletal proteins found at the actin rich cortex. Striatin, ASEF, KAP3, and IQGAP all are proteins that affect the cytoskeleton all bind to the Arm repeats of APC vertebrate proteins. Recruitment of APC2 to the actin rich cortex through binding of a cytoskeletal protein that binds to the Arm repeats is plausible. Currently, in Drosophila, KAP3 has been identified and shown to bind directly to the Arm repeats but less is known about Arm binding to actin-associated proteins (Kunttas-Tatli *et al* 2014). Therefore, future experiments will be geared to identifying actin proteins that bind directly to the Arm repeats and affect its cortical localization mechanism.

2.4.3 APC2 in actin assembly

APC2-Dia mediated actin assembly is required for actin assembly in the early embryo. In addition it was demonstrated that Dia and APC2 bind to each other through their C-terminal domains (Webb *et al* 2009). Evidence also suggests that this protein collaboration is conserved between APC1-Dia *in vitro* (Okada et al 2010, Breitsprecher *et al* 2012, and Jaiswal *et al* 2013). Although, the mechanistic details for APC1-Dia interaction are clearly delineated, *in vivo* evidence of the physiological relevance of APC1-Dia mediated actin assembly is lacking. On the other hand, our lab demonstrated that APC2-Dia mediated actin assembly is required in extension of the actin furrows, but the mechanistic details of this interaction in actin assembly are not understood. How do APC2 and Dia coordinate to assemble actin?

To begin to address the question, we tested the direct interaction of APC2-C terminal fragments with Dia-C term and found that the 20Rs of APC2 were sufficient for Dia binding. This was interesting because β-catenin binds to this domain in the Wnt signaling pathway. The β-catenin and APC interaction is phospho-regulated by GSK3-β and phosphorylation enhances the binding affinity of the interaction (Rubinfeld *et al* 1996). In the same manner that GSK3β phosphorylation regulates β-catenin and APC complex formation, we hypothesized that the interaction between Dia and APC2 could also be regulated through this mechanism.

2.4.4 APC2 in actin assembly *in vitro*

APC1 assembles actin filaments through its basic domain and collaborates with Dia *in vitro.* Unlike APC1, APC2 lacks the basic domain required for direct actin association and assembly and we predicted that APC2 would not assemble actin filaments on its own. APC2 alone did not assemble actin *in vitro* shown through pyrene actin assembly assays. Surprisingly, APC2 did not collaborate with Dia *in vitro* but it inhibited the actin assembly activity of Dia *in vitro (*data not shown*).* In addition, we found that inhibition of Dia was specific, inhibition was dose dependent, and the 15-20Rs were the domains required for the inhibition of Dia (data not shown). To better understand this interaction *in vitro*, we have taken two approaches: 1) continue to dissect the minimal requirement domains between Dia and APC2 and 2) test the phosphorylation of APC2 in regulating the APC-Dia complex in actin assembly. Our preliminary data (not shown) strongly suggests that APC2-C binds to the FH2 in Dia, which is required for actin assembly. Therefore, it is possible that APC2 works as a clutch to stop Dia mediated actin assembly. To test the role of

phosphorylation in APC-Dia mediated complex formation and its effect on actin assembly, we cloned and expressed GST-APC2-20R-S>D to mimic phosphorylation. We have not yet tested this extensively but a question that we would like to answer is whether mimicking phosphorylation increases the binding affinity of APC2 and Dia. Additionally, we want to know how phosphorylation affects actin assembly by testing this S>D mutant in actin assembly with Dia in pyrene actin assembly assays. Preliminary data from the Goode Lab strongly suggests that the phosphorylated APC2 mutant inhibits APC1-mediated actin assembly stronger than the non-phosphorylated (data not shown). If phosphorylation of the 20Rs enhances its binding affinity to Dia, then it makes sense that inhibition is maximized with phosphorylation.

2.4.5 APC2 in actin assembly in the Drosophila early embryo

APC2 is required for actin assembly *in vivo* and interacts with Dia through its 20Rs *in vitro*. Loss of the 20Rs results in actin furrow extension defects but the 20Rs are dispensable for the cortical localization of APC2. Through this data, we inferred that loss of the 20Rs disrupted the interaction between APC2-Dia and they could no longer collaborate to form the actin furrows in the early embryo. Phosphorylation and non-phosphorylation of the 20Rs of APC2 were required for the function of APC2 at the cortex. In addition, we predict that the 20Rs of APC2 are a target of GSK3β and phosphorylation of the 20Rs by GSK3β regulates the interaction between APC2 and Dia. In addition for the phospho-regulation requirement for the extension of the actin furrows in the early embryo, we also predict that GSK3β is required for the cortical mechanism of APC2. McCartney *et al* 1999 demonstrated that APC2 is mislocalized from the cortex in GSK3β mutant embryos.

Evidence suggests that the 20Rs and SAMP repeats are the only sites in APC2 that are phosphorylated and both of these domains are not required for cortical localization. Therefore, we hypothesize that APC2 is not the only target of GSK3β. GSK3β potentially phosphorylates other proteins at the cortex that in turn, influences the cortical localization of APC2 (protein X or Y) (Figure 14). It is possible that phosphorylation is mediating the interaction between proteins at that cortex and APC2. We need to better understand how APC2 binds to the actin rich cortex to better understand how GSK3β is facilitating the cortical localization of APC2.

In conclusion, our work further dissected the mechanistic pathway of the cortical localization of APC2. A conserved coiled-coil, the CCES domain, is necessary and sufficient for the activity of C30 at the actin rich cortex potentially mediating the indirect and/or direct interaction of APC2 with the cortex. We also demonstrated that dimerization through the N-terminus is partially required for the cortical localization mechanism of APC2 and we predict that dimerization is stabilizing the small C30 dimer at the cortex. Because dimerization only partially localizes to that actin cortex, we predict that the Arm Repeats in the N-terminus are also required. Evidence demonstrates that the Arm repeats can bind other proteins. Therefore, we predict that binding of an unidentified protein through the Arm repeats is required for the cortical localization of APC2.

We further dissected the binding interaction between APC2 and Dia. The 20Rs of APC2 are required and sufficient to bind to Dia. Loss of the 20Rs in the early embryo resulted in actin furrow defects but did not abolish the cortical localization of APC2. This provides some evidence that the 20Rs may be required for the direct binding and collaboration of APC2 and Dia in actin assembly. Phospho-regulation of the 20Rs in APC2 is

also required for actin furrow extension. Thus, it is possible that phosphorylation is regulating the APC2-Dia protein complex. Although, we have filled some gaps addressing APC2 mediated actin assembly, many questions remain unanswered. For example, why does APC2 collaborate with Dia *in vivo* but inhibits Dia *in vitro*?

Although, we learned a lot using the early embryo for APC2 actin assembly- we also know now that APC1 is not required for actin furrow extension in the early embryo discussed in Chapter 3. This evidence makes the embryo a limited model to study APC-Dia mediated actin assembly. In Chapter 3, I describe the characterization of APC proteins in actin assembly. Specifically, APC2 is localized to the actin rich cortex and is required to help form actin cable arrays in late oogenesis. Therefore, using this model we hope to understand the functions of APC2 in actin assembly where we will be asking other functional and mechanistic questions: 1) Does APC2 play a role in the turnover of cortical actin through FRAP experiments, 2) Does APC2 play a role in cortical strength through micro-aspiration experiments, and 3) What is the role and mechanism of APC1-APC2-Dia in actin assembly.

2.5 Figures

Figure Legends

Figure 9: A conserved 15 amino acid sequence is necessary for the activity of C30 at the cortex

A) Multiple sequence alignment of the last 30 amino acids (C30) of different Drosophila species (highlighted in yellow). The non-conserved domain will be called divergent while the 15 amino acid sequence in between the two divergent fragments is called the conserved region.

B) Schematic of mCh tagged APC2 proteins. mCh=mCherry/APC Self Association Domain=ASAD/CCES=Cortical

C) S2 Cells transfected the mCh-APC2 constructs imaged live. WT APC2 is localized to the cortex of the cell while mutants that deleted either the N-terminus, C30, or the conserved region localized throughout the cytoplasm of the cells. Scale bar =10uM.

D) Cortical to Cytoplasmic pixel intensity ratio. Ratios close to 1 indicate that the amount of protein is the same at cortex and the cytoplasm. Ratios higher than 1 indicated that the protein localized at the cortex is higher than protein localized in the cytoplasm. Analysis was done using fixed cells stained for actin as a marker for the cortex and anti-mCherry to label APC2 protein. Errors bars are standard error of the mean (SEM) from at least 15 cells.

*bio-informatics by Ezgi Kunntas-Tatli

*Constructs made by Paige Davison, Molly Bernstein, and Terrence Wong

Figure 10: Formation of a coiled-coil in C30 is necessary for the cortical localization mechanism of APC2 at the cortex

A) The conserved 15 amino aligns to the HEPTAD repeat of a coil-coil. The hydrophobic amino acids at positions A and D are in red.

B) The mCh-APC2 constructs were transfected into S2 cells and imaged live. Either all of the hydrophobic residues were mutated to proline (blue stars). The introduction of proline generates kinks into the protein and disrupts the coiled-coil. Only one hydrophobic amino acid (valine), noted in yellow, was mutated to a hydrophilic amino acid (threonine)

C) The ratio of cortical APC2 to cytoplasmic APC2 was calculated. Both mutations disrupting the coiled-coil abolish the cortical localization of APC2. Errors bars are standard error of the mean (SEM) from at least 15 cells.

*constructs were made by Paige Davison, Molly Bernstein, and Terrence Wong

Figure 11:Dimerization of C30 is necessary but not sufficient to localize APC2 to the cortex

A) Schematic of FRB and FKBP C30 constructs. Proteins where either tagged with an EGFP of mCh tagged followed by a linker, FRB or FKBP, and the C30.

B) Schematic of hypothesis for localization without or with rapamycin. Rapamycin is required for the interaction between FRB and FKBP. Our predictions suggest that artificial dimerization will drive dimerization and that dimerization is required for the dimerization of C30 at the cortex. The FRB and FBKP are replacing the function of the N-terminus, specifically ASAD that is required for APC2 dimerization.

C) S2 cell lysates were resolved through SDS-PAGE and probed with anti-mCh and anti-GFP antibodies.

D) EGFP-FRB-C30 in the absence or presence of rapamycin. Cytoplasmic localization of the protein was observed.

E) mCh-FKBP-C30 in the absence and presence of rapamcyin. The protein was localized to the cytoplasm of the S2 cells.

F) Co-expression of EGFP-FRB-C30 and mCH-FKBP-C30 in the absence or presence of rapamycin. Rapamycin treatment partially localized C30 constructs to the actin rich cortex.

G) Cortical to cytoplasmic ratio the C30 dimerization constructs with or without rapamycin. Errors bars are standard error of the mean (SEM) from at least 15 cells.

Figure 12: APC2 binds to actin

A) Schematic of GST-tagged APC2 protein fragments.

B) High-speed cosedimentation pellet fractions of 4 μM APC2-C, APC2-NC-30, mouse αE-catenin homodimer and BSA incubated with (lanes 1-4) or without (lanes 5-8) 2 μM F-

actin. Asterisks mark full-length protein in APC2 samples. *Collaboration with Dr. Adam Kwiatkowski.

C) Percentage of total protein that pelleted with F-actin. Errors bars are standard error of the mean (SEM) from at least three independent experiments.

Figure 13: Phospho-regulation of the 20Rs is required for the actin function of APC2 at the cortex of the early embryo

A) GST pulldowns of GST-APC2 fragments suggesting that the 20Rs are sufficient for Dia-C binding.

B) Localization of APC2-Δ20Rs in the actin furrows of metaphase cycle 13 embryos. Loss of the 20Rs does not interfere with the cortical localization mechanism of APC2. Experiments by Kelly Shibuya

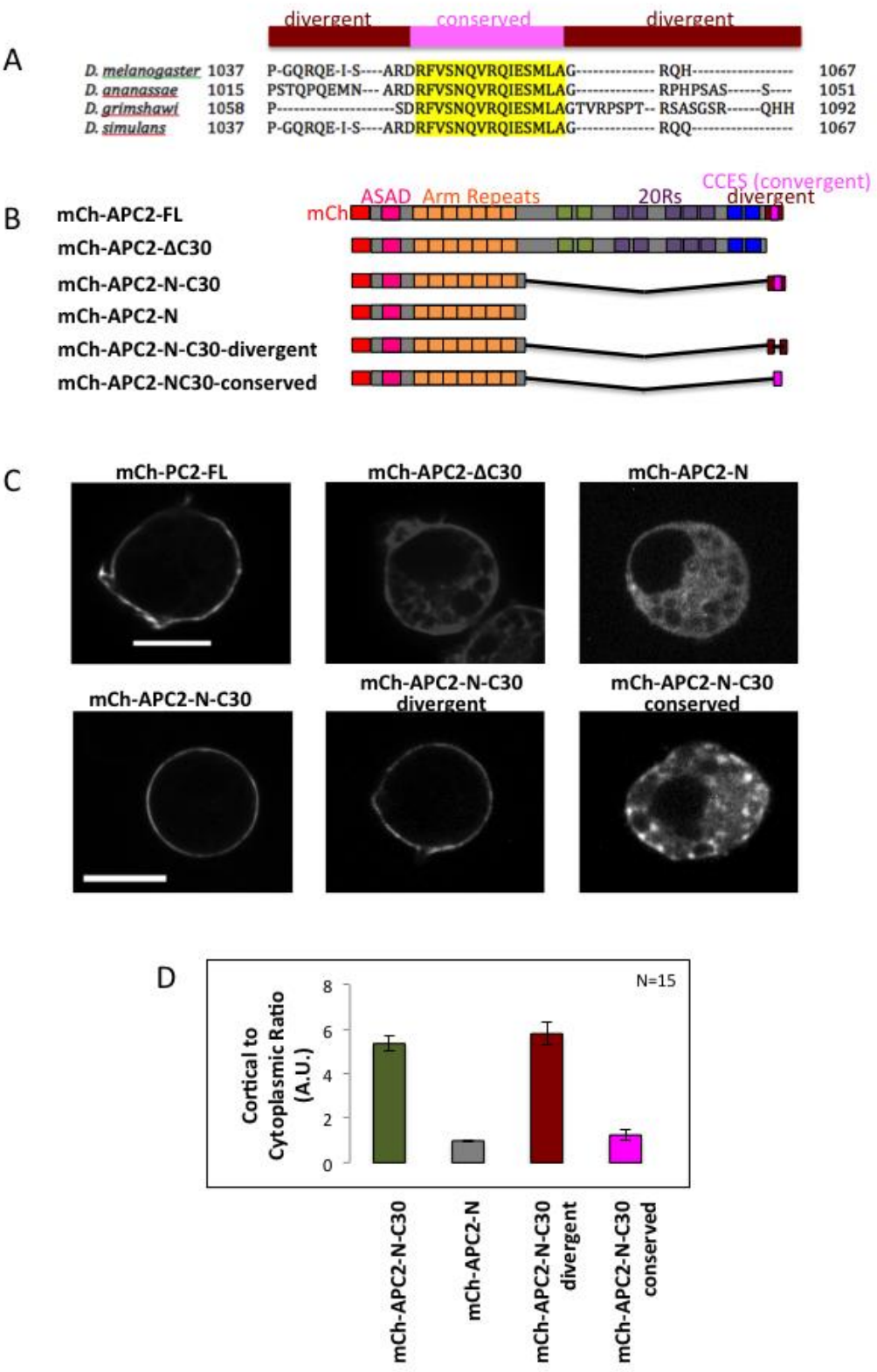
C) Quantification of actin furrow extension defects in the early embryo (N= 5 embryos for each genotype). Complete loss of APC2 and deletion of the 20Rs was tested. Additionally, APC2-20R phospho-mutants that mimicked phosphorylation (S to A) or ablated phosphorylation were also tested in actin furrow extension. Experiments done by Ezgi Kunttas-Tatli

Figure 14: The cortical localization mechanism of APC2

The CCES domain and ASAD are required for the cortical localization of APC2. The interaction between APC2 and the actin is weak and therefore, other binding proteins are required to facilitate the association of APC2 with the actin rich cortex. As to the function of APC2 at the cortex, we hypothesize that the 20Rs of APC2 are required for APC-Dia mediated actin assembly in actin furrow extension but do not are dispensable for the

cortical localization mechanism of APC2. Additionally, we believed that phosphorylation is important in regulating the interaction between APC2 and Dia potentially through GSK3β. Lastly, GSK3β is potentially involved in the cortical localization of APC2 by regulating the interaction of the other APC2 binding interactions at the cortex.

Figure 9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divergent
conserved
divergent
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D. grimshawi 1058 P----------SDRFVSNQVRQIESMLAGTVRPSPT-- RSASGSR------QHH 1092
D. simulans 1037 P-GQRQE-I-S----ARDRFVSNQVRQIESMLAG---------- RQQ---------- 1067
B
ASAD
Arm Repeats
20Rs
CCES (convergent)
divergent
mCh
mCh-APC2-FL
mCh-APC2-ΔC30
mCh-APC2-N-C30
mCh-APC2-N
mCh-APC2-N-C30-divergent
mCh-APC2-NC30-conserved
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mCh-APC2-N-C30
mCh-APC2-N-C30 divergent
mCh-APC2-N-C30 conserved
D
N=15
Cortical to Cytoplasmic Ratio (A.U.)
0
2
4
6
8
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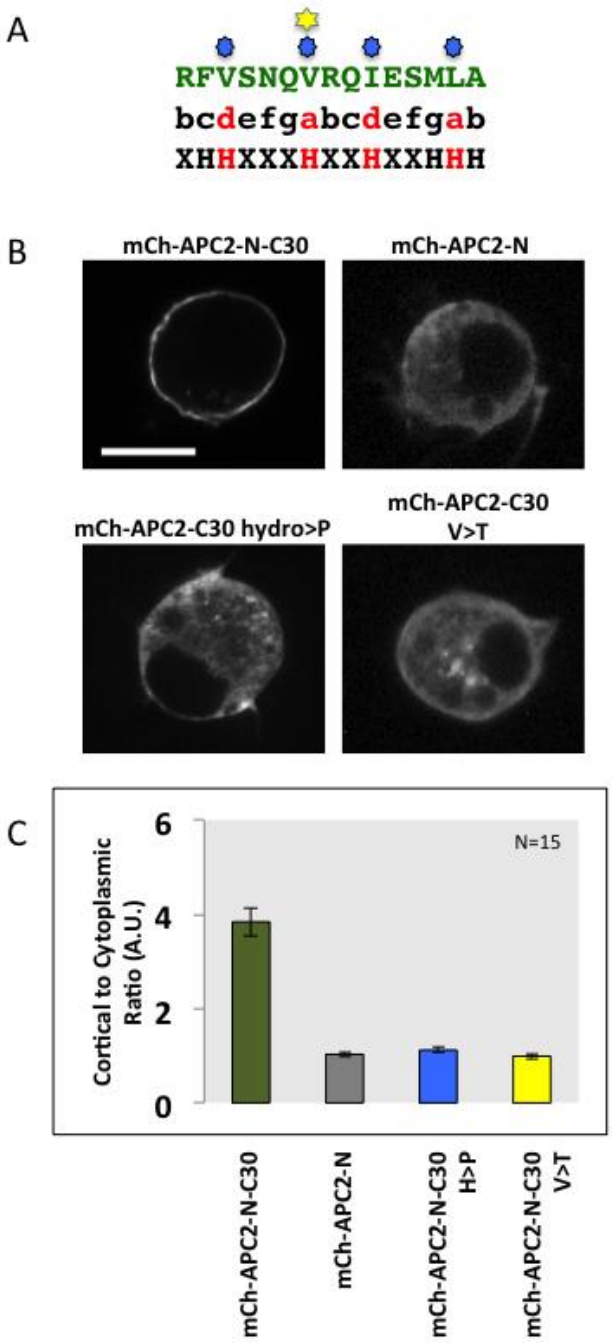
Figure 10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mCh-APC2-C30 V>T
C
Cortical to Cytoplasmic Ratio (A.U.)
6
4
2
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N=15
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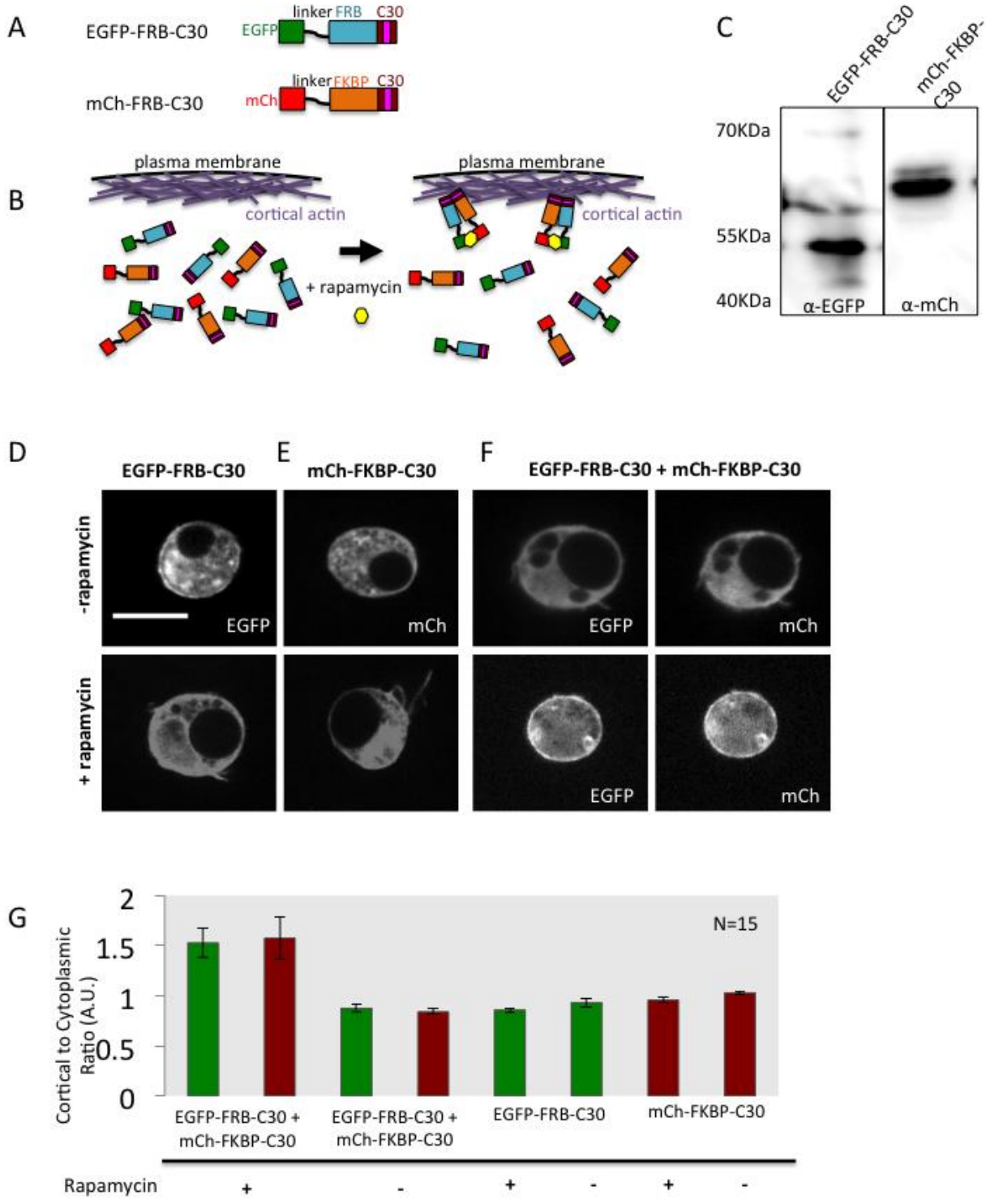
Figure 11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linker
FRB
C30
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FKBP
C30
B
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cortical actin
+ rapamycin
plasma membrane
cortical actin
C
EGFP-FRB-C30
mCh-FKBP-C30
70KDa
55KDa
40KDa
α-EGFP
α-mCh
D
EGFP-FRB-C30
E
mCh-FKBP-C30
F
EGFP-FRB-C30 + mCh-FKBP-C30
-rapamycin
+ rapamycin
EGFP
mCh
EGFP
mCh
EGFP
mCh
G
Cortical to Cytoplasmic Ratio (A.U.)
2
1.5
1
0.5
0
N=15
EGFP-FRB-C30 + mCh-FKBP-C30
EGFP-FRB-C30 + mCh-FKBP-C30
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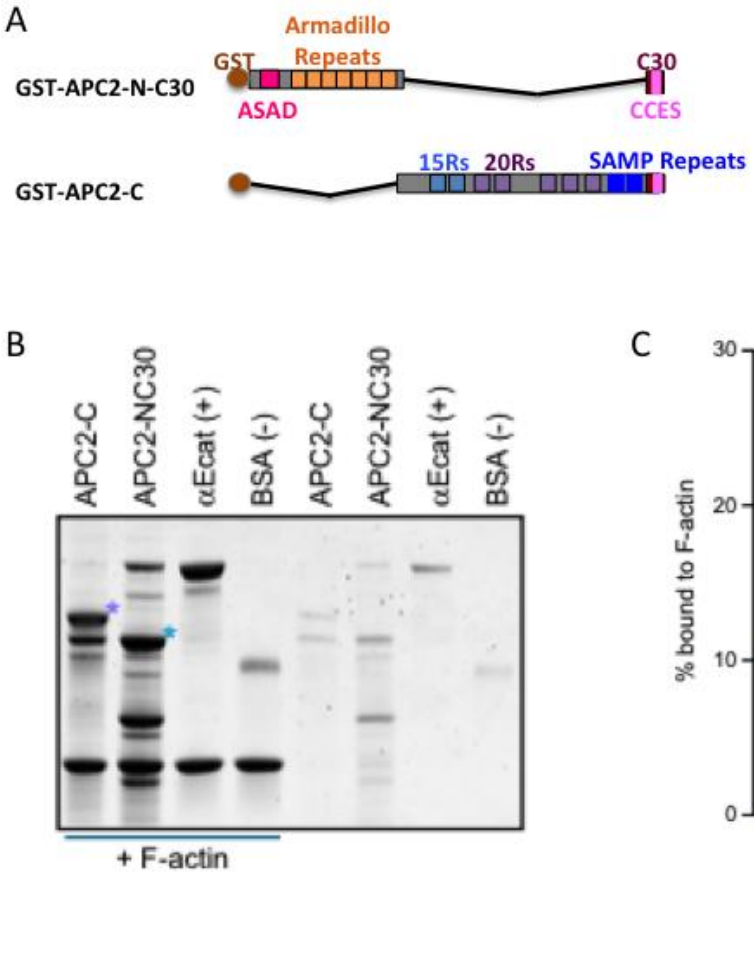
mCh-FKBP-C30
Rapamycin
+
-
+
-
+
-

Figure 12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Armadillo
Repeats
GST
C30
GST-APC2-N-C30
ASAD
CCES
15Rs 20Rs
SAMP Repeats
GST-APC2-C
B
APC2-C
APC2-NC30
αEcat (+)
BSA (-)
APC2-C
APC2-NC30
αEcat (+)
BSA (-)
+ F-actin
C
% bound to F-actin
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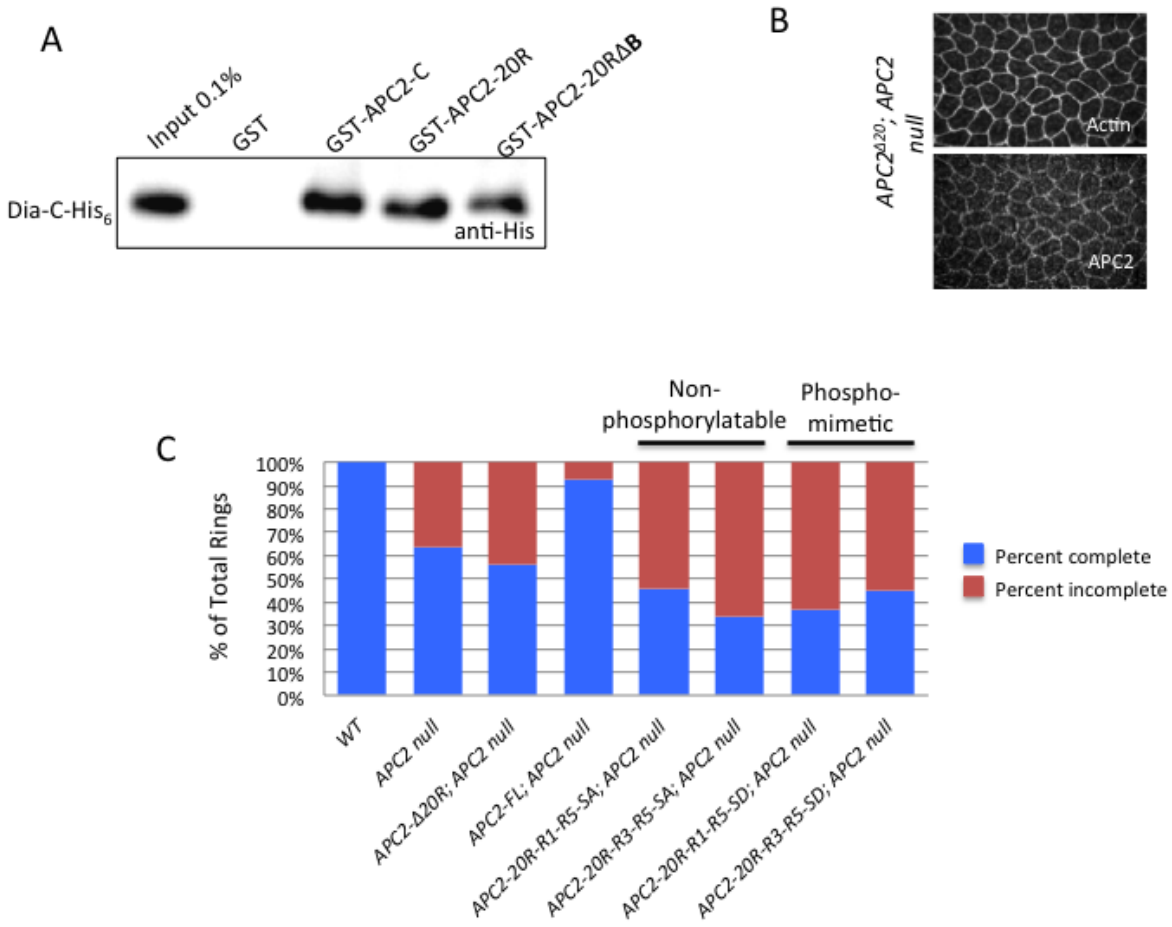
20
10
0
APC2-C
APC2-NC30
αEcat
BSA

Figure 13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Input 0.1%
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GST-APC2-20R
GST-APC2-20RΔB
Dia-C-His6
anti-His
B
APC2Δ20; APC2 null
Actin
APC2
C
Non-phosphorylatable
Phospho-mimetic
% of Total Rings
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT
APC2 null
APC2-Δ20R; APC2 null
APC2-FL; APC2 null
APC2-20R-R1-R5-SA; APC2 null
APC2-20R-R3-R5-SA; APC2 null
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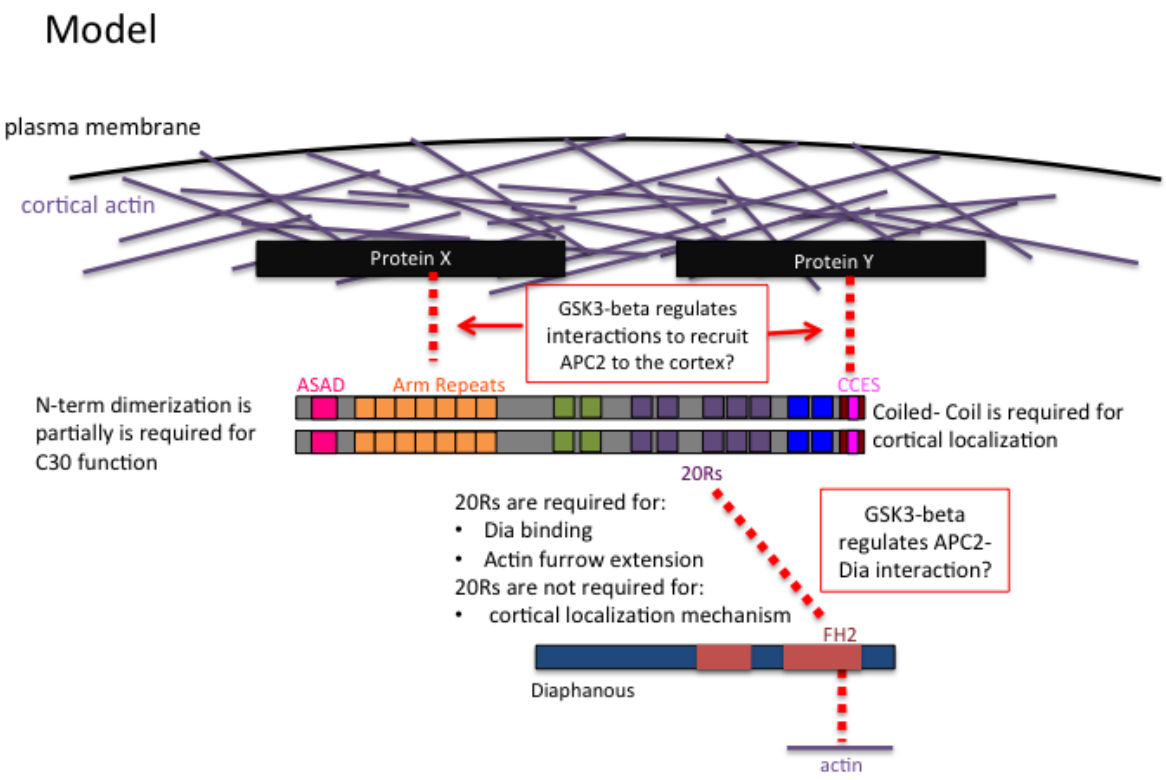
APC2-20R-R3-R5-SD; APC2 null
Percent complete
Percent incomplete

Figure 14


Model
plasma membrane
cortical actin
Protein X
Protein Y
GSK3-beta regulates interactions to recruit APC2 to the cortex?
ASAD
Arm Repeats
CCES
N-term dimerization is partially is required for C30 function
Coiled- Coil is required for cortical localization
20Rs
20Rs are required for:
• Dia binding
• Actin furrow extension
20Rs are not required for:
• cortical localization mechanism
GSK3-beta regulates APC2-Dia interaction?
FH2
Diaphanous
actin

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Chapter 3. APC-Dia mediated actin assembly in actin cable formation during oogenesis

3.1 Introduction

The organization of the microtubule and actin cytoskeletons is important for many cellular processes. Both of these networks are involved in cell migration, maintenance of the cell's shape, transport within the cell and mitosis. Many microtubule and microfilament assembly factors continue to be identified. Therefore, continuing to investigate the factors involved in microtubule and actin assembly regulation is important to understand these cellular processes *in vivo.*

The colon cancer tumor suppressor Adenomatous polyposis coli (APC) is a negative regulator of the Wnt signaling pathway and a potent regulator of the microtubule and microfilament cytoskeletal networks. APC is truncated in about 80% of familial and sporadic incidents of colon cancer. Loss of APC proteins aberrantly activates the Wnt signaling pathway. Incorrect activation of the pathway has been correlated to the initiation of colorectal cancer. Though, APC proteins are widely known for their role in Wnt signaling, less is understood about their role in the cytoskeleton or the cytoskeleton in the progression of colorectal cancer. By understanding the normal function of APC proteins *in vivo*, we will be able to understand the link between APC, the cytoskeleton and cancer.

3.1.1 vAPC in actin assembly

Vertebrate APC (vAPC) interacts indirectly with the microtubule cytoskeleton through a microtubule plus-tip binding protein, EB1, and directly binds to microtubules and bundles these polymers through its basic domain(vAPC-basic). Not only does vAPC-basic affect microtubules directly, but recent data suggest that vAPC interacts with microfilaments by binding to actin monomers and bundling actin filaments *in vitro* (Moseley et al 2007). Furthermore, vAPC-basic assembles actin filaments and synergizes with Diaphanous (Dia) to stimulate actin assembly

more effectively *in vitro* (Figure 3 and 4; Okada *et al* 2010, Breitsprecher *et al* 2013). Studies of the APC2 *Drosophila* ortholog *in vivo* implicate APC2 as a regulator of actin furrows in the syncytial embryo alongside with Dia (formin) (Webb *et al* 2009). Two *Drosophila* APC proteins we identified. We will use this tractable model organism for the *in vitro* and *in vivo* study of APC proteins in actin assembly.

3.1.2 Mechanism of vAPC mediated actin assembly

Over-expression of vAPC1-basic in NIH-3T3 cells increased the levels of actin assembly (Okada *et al* 2010) leading to the conclusion that vAPC-basic is involved in the nucleation of actin filaments. Through deletional analysis, two actin nucleating sequences (ANS1 and ANS2) within the basic domain of vAPC were identified (Figure 4; Okada *et al* 2010). Deleting both of the ANS sequences reduces the total amount of actin in the NIH3T3 cells, suggesting that these motifs are required for actin assembly in cell culture. ANS1 is required to recruit two actin monomers while ANS2 is required to stabilize a dimer formed between two vAPC-Basic proteins generating a nucleation seed. Therefore, vAPC is a tandem monomer actin binding protein that functions similarly to formins (Firat-Karalar *et al* 2011). Although these experiments are beginning to aid in understanding vAPC-mediated actin assembly, it is unclear how and where APC actin assembly is required *in vivo*. Therefore, we needed to develop a model system to test APC actin assembly.

3.1.3 APC2 and Dia in actin furrow extension

Our lab uses the Drosophila syncytial embryo as a model for actin assembly. During development of the early embryo, many dynamic cytoskeletal rearrangements occur. The early embryo undergoes 13 nuclear divisions without cytokinesis. Therefore, to protect the nuclei that

are dividing, actin pseudocleavage furrows form as dividers during metaphase to provide mitotic fidelity, prevent collisions and nuclear fallout (see Chapter 1 for detailed description of process; Figure 7). To explore the formation of these dynamic actin furrows, Rebecca Webb performed a loss of function experiment to test the role of APC2-Dia mediated actin assembly. Loss of APC2 and Dia resulted in extension defects in actin furrows. Additionally, she showed that APC2 and Dia genetically and physically interact, suggesting that both of these proteins are interacting together to positively enhance actin assembly (Webb *et al* 2009). Because APC1 is an actin assembly factor *in vitro* and a binding partner to Dia and APC2, we predicted that APC1 may be involved in actin assembly in the early embryo.

In addition, vAPC and Dia collaborate *in vitro* to assemble actin filaments (Okada *el al* 2010 and Breitsprecher *et al* 2012). vAPC-basic binds to the DAD domain of Dia-C to overcome the inhibitory effect of capping and profiling (Figure 4*) in vitro*. v-APC-basic and Dia-C complex together as overlapping puncta during the start of actin polymerization and they separate, remaining at different poles on the growing actin cable (Figure 4A and B; Breitsprecher 2012). The mechanism was named "Rocket Launcher" because Dia-basic takes off as vAPC-Basic and Dia separate. Dia remains attached to the barbed-end of the actin filament, and Dia processively elongates the actin filament. Although the Goode Lab elegantly dissected the mechanism of Dia-vAPC- mediated actin assembly *in vitro,* is it not known whether this interaction is physiologically relevant *in vivo*.

3.1.4 Late Drosophila oogenesis as a model for actin assembly

When I started working on this project, the main question that I wanted to answer was whether APC1 was physiologically relevant in actin assembly. I initially started using the Drosophila early embryo as a model for APC1, APC2, and Dia actin assembly because our lab

found that it was a great model to study APC2-Dia mediated actin assembly. It was during the beginning of my fourth year that we realized that APC1 was not involved in actin assembly in the early embryo (Figure 15 A and C). Therefore, during the rest of my doctoral research I set out to find a new actin assembly *in vivo* model to test actin-associated mechanisms of APC1 and dissect APC1-APC2-Dia-mediated actin assembly. Our motivation to test APC and Dia-mediated actin came from data that localized APC2 to the cortex of the Nurse cells in stage 10B egg chambers and present on actin cables that were extending from cortex of the Nurse cells and the ring canals into the cytoplasm (Roberts *et al* 2012 and Townsley *et al* 2000). Additionally, loss of APC1 or loss of both APC proteins (APC2 and APC1) yielded small round egg chambers indicative of dumping defects (Figure 16H). Therefore, I turned to the ovary as a model system for actin assembly.

3.1.5 Dumping in Drosophila egg chambers

During egg development in the fruit fly, the contents from the Nurse cells (Ncs) (yolk, mRNAs, proteins, and organelles) are sent slowly through the ring canals into the growing oocyte where the oocyte continues constantly to grow 90,000x (King 1970). The major events in late oogenesis are schematized in Figure 16A. During the later stages of oogenesis, stage 10B and on, actin cables grow from the cortex towards the nuclei (Figure 16C, 16E, and 16F). The actin cables form cage-like cables around the nuclei to protect the ring canals (actin structures) from being clogged (Figure 8; Guild *et al* 1997 and Huelsman *et al* 2013). Additionally, the cables orient the nuclei away from the ring canals (Guild *et al* 1997 and Huelsman *et al* 2013) during late 10B stages, where the cables continue to form around the nuclei to push it away from the ring canals. The Nc contract to push and dump the RNA, protein, and organelles that are synthesized within the Nc, through ring canals into the oocyte (Guild *et al* 1997). Loss of actin

binding proteins such as Chickadee (Profilin), Singed, Quail, Ena, and Arm (see Chapter 1 Introduction: section 1.6 for a full description of these proteins) all results in smaller and rounder eggs indicative of a "dumpless" phenotype. A dumpless phenotype is characterized by failure to dump or transfer all the contents from the Ncs into the oocyte due to the loss of the formation of actin cables or myosin-actin contractile machinery. Mini-cables (Figure 16D) are formed by actin elongating proteins such as chickadee, and bundling proteins such as Singed and Quail. Additionally, Ena and other filopodia markers were localized to the barbed end (positive end) of the actin filament (inset in Figure 16E) (Guild *et al* 1997, Gates *et al* 2009, and Huelsman *et al* 2013) where they are required to form the actin cables. Although we are continuing to understand a great deal about how these actin cables are formed, many questions remain unanswered. For example, there is not a clear model to delineate how these actin cables are being initiated, or elongated. It seems that we are only beginning to understand the role of some of the proteins involved in actin cable formation. For example, what are other factors and how are they involved in temporally and spatially regulating actin cable assembly in late oogenesis?

Preliminary data show that *APC1 null* mutant embryos qualitatively appear to be smaller and rounder (Figure 16H) suggesting that APC1 may play a role in actin assembly in the Drosophila egg chamber. We have not yet quantified this phenotype, but are planning to quantify the egg morphology phenotype in the future. Loss of both APC proteins exacerbates the phenotype suggesting that both APC proteins are involved in actin assembly in the egg chambers. This is not surprising, because APC2 is present at the cortex and on the actin cables cables in the nurse cells (Roberts *et al* 2012 and Townsley *et al* 2000). In our work, we confirmed that APC1, Dia, and APC2 were expressed and localized to the cortex and actin cables in late oogenesis. In addition, APC1, APC2, and Dia single mutants induced a delay in actin

cable formation and surprisingly, the phenotypic temporal delay in actin cable formation was rescued in stage 11 egg chambers. These results suggest that it is possible that these proteins may be redundant in actin assembly formation of the cytoplasmic actin cables in the late oogenesis. Additionally, APC1, APC2, Dia are potentially collaborating with themselves or others during elongation to rescue the actin assembly defects in stage 11 egg chambers. To continue to understand the regulation of APC2 in actin assembly and in collaboration with Dia, we assessed the phospho-regulation of the 20Rs in APC2 in actin cable formation. Phosphorylation of the 20Rs, potentially through GSK3-β, is required for the proper activation of cable formation in oogenesis. Taken together, we have demonstrated a novel *in vivo* role of APC1 mediated actin assembly and continue to understand the role of Dia-APC1-APC2 mediated actin assembly *in vivo*.

3.2 Materials and Methods

3.2.1 Fly Stocks

w^{1118}, $APC2^{g10}$ (McCartney *et al* 2006) and $APC1^{Q8}$ (Ahmed *et al* 1998) fly lines were used for our complete loss of function experiments. To perform the localization and rescue experiments, *P[endoP-EGFP-APC2-FL]* (Zhou *et al* 2010) and *P[endoP-EGFP-APC1]* (Roberts et al 2012) were crossed into $APC2^{g10}$ and $APC1^{Q8}$ backgrounds, respectively. Other transgenic flies that were used for the loss of functions analysis: *P[endoPEGFP-APC2- Δ20R], P[endoPEGFP-APC2-R1R5SA], P[endoPEGFP-APC2-R1R5SD], P[endoPEGFP-APC2-R3R5SA]*, and *P[endoPEGFP-APC2-R3R5SD]* (Zhou *et al* 2010 and Kunttas-Tatli *et al*. 2012).

The FRT-FLP-DFS technique (Chou and Perrimon, 1996) was used to generate single *APC1*^{Q8} mutants and APC2^{g10} APC1^{Q8} maternally null embryos. Crosses were heat shocked for an hour at 37˚C.

3.2.2 RNAi

The UAS/Gal system was used to express TRIP RNAi lines. Drivers used for the study are MTD-Gal4 (Dr. Lynn Cooley) and MatVP67 (Dr. Adam Martin). TRIP-RNAi lines were obtained from Bloomington: UAS-Sgg and UAS-Dia. Crosses were incubated at 27˚C.

3.2.3 Embryo prepping

0-2 hours embryos were collected, dechorinated by 50% bleach, and hand-peeled to remove the vitelline membrane. Embryos were fixed in 4% paraformaldehyde and blocked for one hour in PNT (1X PBS, Triton-X, and NGS). Primary antibody to stain for microtubules (DSHB E7 mouse-anti-tubulin) at a dilution of 1:500 and incubated overnight 4ºC. Secondary alexa-conjugated anti-mouse and phalloidin (Invitrogen) were incubated at room temperature for three hours. The embryos were washed, incubated for 30 minutes with DAPI, and mounted on Aqua/PolyMont.

3.2.4 Actin furrow extension analysis

To analyze the depth of the furrows, cross-sectional single images of syncytial embryos were taken at four different depths in which the most superficial surface of the embryos is 0μm, preceded by -0.8μm, - 1.6μm and -2.2μmin in order to capture complete furrows in the embryo. In order to obtain a quantitative assessment of the depth furrows, we counted the number of complete and incomplete rings at these different depths, and percentages were plotted.

3.2.5 Ovary prepping and staining

To examine the actin cables, ovaries were dissected in 10% formaldehyde in the presence of phalloidin at 12.5 mg/mL. Ovaries were teased out from the posterior end of the ovary to anterior end in order to remove as much of the sheath as possible. Egg chambers were washed 4 times in PBS and 0.01% of Tween-20 and mounted in Aqua/PolyMont.

3.2.6 Drosophila ovary tissue fixation for localization experiments

Female flies no more than a week old were "yeasted" overnight for either 1- 2 days. Drosophila ovaries were dissected in Schneiders media with 10% FBS and Pen/Strep. Ovaries were teased out using forceps to remove as much as the muscle sheath as possible. Ovaries were fixed with 4% paraformaldehyde, washed four times with PBS, and blocked in PNT (1xPBS, NGS, and 0.03% Titron-X100) for 1 hour. Primary antibodies were incubated at 4˚C overnight: rat-anti-tubulin Millipore (Millipore/MAB1864) 1:1000, mouse-anti-acetylated tubulin (Sigma/T7451) 1:1000, and rabbit-anti-CLIP190 (gift from Kathryn G. Miller/Washington University) 1:500. Secondary fluorescent antibodies conjugated to Alexa-488, 546, or 647 at (Invitrogen? 1:1000 and phalloidin (Invitrogen) at 1:3000 were incubated for 3 hours at room temperature. Stained with DAPI (Sigma/) for 30 minutes. Ovaries were completely separated before mounting in buffers and mounted in Aqua-Pol/Mount (PolySciences).

A second fixation method was used to optimize for microtubule and membrane associated staining slightly adapted from Dr. Kevin Edwards. 2 to 4 day old female and males flies were collected and yeasted overnight for 1 to 2 days to maximize the number of stage 10B egg chambers. Female ovaries were dissected in PBS for about 10 minutes, continuously transferring the dissected material to fresh PBS. The ovaries were stroked gently between ovarioles in order to remove as much muscle sheath as possible. Ovaries were fixed in 2% (16% EM grade

formaldehyde-Polysciences) formaldehyde for 20 minutes at room temperature without shaking. Ovaries were washed 3 times in PBT-0.2% (1xPBS, 0.2% Triton-X100) and incubated last wash for 15 minutes with wash. I transferred material to a new well, washed once with PBT-0.2%, and transferred material to an Eppendorf tube. The material was washed once with PBT-0.2% and blocked in 1 mL of PBT-0.2% with 4% NGS for one hour at room temperature while rocking. Antibodies were pre-diluted into 1mL of PBT-0.2% (NGS dropped from 4% to 1%), replaced the block with antibody solution, and incubated overnight at 4 degrees Celsius while rocking. Primary antibody solution was disposed and washed 3-4 times in a period of one hour. Pre-diluted secondary antibodys and phalloidin were incubated with ovary material for 5 hours at room temperature while rocking. I washed the samples 3 times and incubated them with DAPI for 30 minutes while rocking. Ovaries were washed 3 times, completed separated before mounting in buffers, and mounted in Aqua-Pol/Mount (PolySciences).

3.2.7 Imaging and Image Processing

Images were acquired with a spinning-disc confocal microscope with a Yokagawa scan head (Solamere Technology Group) and a QICAM-IR camera (Qimaging) on a Zeiss Axiovert 200M using QED InVivo software. All images were processed with Photoshop and quantification of pixel intensity was obtained through ImageJ analysis.

3.3 Results

3.3.1 APC1 does not a play a role in actin furrow extension

vAPC and Drosophila APC1 play parallel roles in actin assembly *in vitro*. Both of these proteins bundle and nucleate actin filaments *de novo* via the basic domain (Moseley *et al* 2007, Okada *et al* 2010 and Jaiswal *et al* 2013). Additionally, in the presence of actin assembly

inhibitors, APC proteins bind to Diaphanous. This interaction enables these proteins to form a "super seeding" nucleation complex that overcomes the inhibitory effect of actin assembly inhibitory proteins such as profilin and capping protein (Okada *et al* 2010, Breitsprecher *et al* 2012, and Jaiswal *et al* 2013). Though, we know mechanistically how vertebrate APC and Dia collaborate in actin assembly, we do not know if this interaction is physiologically relevant or whether APC1 on its own can act in actin assembly *in vivo*.

APC2 and Dia collaborate in actin assembly in the early embryo (Webb *et al* 2009) and both APC2 and Dia bind to APC1 (Wen *et al* 2004, Webb *et al* 2009, Okada *et al* 2010, Breitsprecher *et al* 2012, Jaiswal *et al* 2013, Kunttas-Tatli *et al* 2014, and Mattie *et al* 2010). This led us to predict that it was possible that APC1, which contains an actin assembly domain, could also be playing a role in actin assembly in the early embryo. Complete loss of APC1 leads to over 50% mortality of homozygous *APC1 null* adults termed semi-lethality. Therefore, to overcome *APC1 null* semi-lethality, I generated germ-line clones that were *APC1 null. APC1 null* or also known as $APC1^{Q8}$ contains a nonsense mutation that introduces a stop codon around the second arm repeat at amino acid 426 (Figure 2C) (Ahmed *et al* 1998). Embryos were fixed and labeled with phalloidin to label actin. Stacks were taken from the most surface section of the embryo to the bottom of the embryo at 0.2µm slices (Figure 15B). As a surface section, the actin furrows look like rings (Figure 7C); therefore, to quantify the phenotypic consequence of loss of APC, the number of complete and incomplete rings were counted at four different depths in the egg chamber (Figure 15B). Loss of APC1 resulted in a very mild extension defect phenotype when compared to complete loss of APC2 (Figure 15A and 15C). To confirm whether the actin furrow extension defects were due to loss of APC1, we put back APC1 into *APC1 null* embryos and asked if the APC1 transgene would rescue the APC1 actin furrow defects. The APC1 rescue

transgene did not rescue the actin furrow defects (Figure 15A and 15C). These results strongly suggest that APC1 does not play a role in actin assembly in the early embryo. These actin defects may be caused by a background mutation in the *APC1 null* flies. Another alternative interpretation could be that APC1 and APC2 are playing redundant functions in actin assembly and loss of APC1 alone is not sufficient to induce furrow extension defects. Therefore, I assessed the loss of both APC1 and APC2 embryos and assessed the depth of the furrows. Loss of function of both APC proteins did not result in an exacerbated phenotype when compared to loss of function of APC2 alone. The double mutant experiment strongly suggests that APC2 is the main effector of actin assembly during furrow extension. This was surprising because APC1 contains the basic domain required for actin assembly *in vitro* and APC2 does not. Therefore, we expected APC1 to be required in actin furrow extension but it was not. APC2 is expressed ubiquitously in the early at high levels while APC1 is expressed at high levels in the CNS (Hayashi et al 1997 and McCartney et al 1999). We didn't have a way of localizing APC1 in the early embryo to see whether APC1 was expressed and where it was localized in the early embryo. We tried to use an RFP-APC1 transgenic line (Mattie *et al* 2010), the APC1 antibody (Hayashi *et al* 1997), and most recently a GFP-tagged APC1 (Roberts *et al* 2012) with no luck of localizing APC1 to the early embryo. Taking all these results into account, it is possible that APC1 is not required for actin furrow extension because it may not be expressed in the early embryo or localized to actin furrows.

3.3.2 APC1, APC2, and Dia are all expressed and localized to the initiation sites of actin cables

APC1 is a potent actin assembly factor *in vitro* and loss of APC1 results in small round eggs (Figure 16H). APC2 is a positive regulator of actin assembly in the early embryo (Webb *et*

al 2009), and present at the actin cables in the oocyte (Roberts *et al* 2012). Thus, we hypothesized that these two proteins were strong candidates for the assembly of actin cables in late oogenesis. Additionally, because both APC1 and APC2 bind to Diaphanous and they positively influence the activity of Dia in assembly, we also hypothesized that Dia may be involved in the assembly of actin cables in late staged egg chambers. We first confirmed that APC1, APC2, and Dia were all expressed in whole ovary lysates (Figure 18E). Because APC1 and Dia collaborate in actin nucleation *in vitro,* we hypothesized that both of these proteins would be localized to the initiation site of the actin cables overlapping with Ena. APC2 is localized to the cortex of the nurse cells and throughout the length of actin cables (Roberts *et al* 2012). Currently, we do not have antibodies that work well on tissue, so we resorted to localizing APC1 and APC2 using transgenic fly lines that expressed GFP-APC1 or GFP-APC2 under the APC2 endogenous promoter. Our results indicated that all three proteins were localized to the cortex of stage 11 nurse cells where full actin cables can be observed (18A, 18B, 18D, and 18D') and (19A and C). These results were very exciting because based on the model, actin assembly initiation sites for the extension of the actin cables are at the cortex and Dia-APC1-APC2 are all present at the cortex where they may be involved in initiating actin assembly. Dia puncta does not overlap with Ena but Dia positioned adjacently. Dia appears to be directly contacting the Ena puncta, suggesting that both proteins maybe work together for form actin filaments at the initiation site of these actin cables (19C). Additionally, Dia and APC1 are localized to the actin cables in some examples (Figure 18B-C 19B). Dia and APC1 are not robustly localized to the actin cables but they do localize to actin cables in some examples. For example, APC1 is localized to the tips of the actin cables (Figure 18B) and on thick bundles of actin cables (figure 18A). Observing APC1 on thick cables was not surprising because APC1 bundles actin filaments

in vitro. Therefore, we also hypothesize that APC1 is also involved in bundling actin *in vivo*. Dia and Ena are also both present throughout the length of the actin cable (Figure 19A-B). This was harder to appreciate but can be clearly seen in Figure (19B). EM analysis of the actin cables strongly suggests that the actin cables are formed by small overlapping actin cables originating from the actin cortex with their plus end facing the cortex (Guild *et al* 1997). Therefore, it is possible that the actin nucleation sites are not only at the cortex but also throughout the length of the actin cables where barbed ends of the cable units are exposed to actin nucleating factors. In our model, we predict that APC1, APC2, Dia, and Ena, are present throughout the length of the cables where they have access to the barb ends of the mini-cable to influence actin assembly. In summary, APC1, APC2, and Dia are all expressed in the ovary. In addition, all these proteins are localized to the actin rich cortex and on actin cables. Because all these proteins were present at the cortex and on actin cables, we asked whether these proteins were involved in the formation of the actin cables.

3.3.3 Loss of APC proteins results in actin cable formation delay

Before we could test the role of APC proteins in actin assembly, we first figured how to stage the egg chambers. The Centripetal Cellular migration, the size of the oocyte, and the morphology of the follicular epithelium were used to stage the egg chambers (Figure 8 and Figure 17). In stages 10A through 10B early, small mini-cables were present (Figure 17A and 17B), longer cables are observed in 10B mid stages (Figure 17C), and long robust actin cables are observed in 10B late and 11 stages (Figure 17D and 17E). To test the functional significance of APC proteins in the formation of cables we tested the effect of *APC null* mutations. Complete loss of *APC1* induces a lethal phenotype but we do get a few *APC1 null* escapers with reduced longevity that we used for our analysis. *APC1 null* egg chambers were fewer in number, smaller

and rounder. In stage 10B-early egg chambers, the small mini-cables present in *WT* flies were absent in both *APC1* and *APC2 null* egg chambers (not shown). At stage 10B-mid, the minicables were still missing in the nurse cells (Figure 20B and 20D) and the loss of the actin cables continued during the late stage 10B egg chambers (Figure 21B and 21E). To our surprise, at stage 11, the actin cables were completely rescued (Figure 21H and 21J). Therefore, complete loss of either of APC1 or APC2 induced a delay in actin cable formation and not a complete loss of actin cable formation. We have not yet quantified the actin cable delay or the cable number, length, and morphology in these mutants to comment on the severity of these mutant actin phenotypes.

Additionally, we noticed other actin phenotypes that suggest that the cortical actin is very unstable. In *APC1 null* egg chambers, we observed small round actin accumulation in the cytoplasm (data not shown) indicative of cortical degradation (Robinson *et al* 1994, Wahlstrom *et al* 2004, and Urwyler *et al* 2012). Additionally, in APC2 egg chambers, the cortical actin between the nucleus and the oocyte is curved into the oocyte that I termed nurse cell drooping (data not shown) and is another feature of cortical instability in the egg chamber (Li *et al* 1999). To test if *APC1* and *APC2* play redundant roles in the formation of actin cables, we tested the complete loss of APC proteins in the egg chamber. Loss of both APC proteins in the Nurse cells was indistinguishable from that of the single APC1 or APC2 mutants. Therefore, from these results we can either conclude that actin cable formation is not very sensitive system to test their interaction. Another interpretation could be is that although APC1 and APC2 are both involved in actin cable formation, they may differ in mechanism and that is a possibility of why the delay or loss of actin cables in the double mutant is not exacerbated. For example, Singed and Quail are both actin bundling proteins required in 10B egg chambers. Over-expression of *quail*

partially rescues the defects observed in *singed mutants* (Cant *et al* 2008) suggesting that these proteins are partially redundant. Quail is responsible for bundling the actin cables loosely while Singed packs them tightly. Therefore, APC proteins may also assemble actin filaments but may do so slightly differently. Our results suggest that APC proteins are required temporally to form the actin cables. We hypothesize that APC1 is potentially involved in the nucleation process based on its nucleation activity *in vitro*. For the moment, we can only conclude that APC2 is involved in actin assembly of these cables but less is understood how it does this mechanism *in vitro*. Our data seems to suggest that APC2 is an effector of Dia-mediated actin assembly and cannot assemble actin on its own. On the other hand, APC1 can assemble actin on its own and collaborates with Dia. Therefore, it makes sense that loss of APC1 induces an aberrant egg morphology when compared to APC2. Loss of both proteins induces a more severe egg morphology, suggesting that both proteins are required in actin assembly.

Our results suggested that both APC proteins are involved in actin assembly in staged 10B egg chambers. To further confirm that the delay in actin assembly was due to loss of APC1 or APC2, we performed rescue experiments using APC transgenes. The phenotypic delay in actin cable formation in *APC* egg chambers was rescued in stage 10Bmid (Figure 20C and E) and 10BLate (Figure 21C and F) in APC2 and APC1 rescue experiments. Though, the delay in actin cable formation was rescued it seems that they are not completely *WT* since the cables do not appear as robust at *WT* actin cables. For example, counting the number of actin cables, their morphology, and lengths will be assessed. In addition, we are in the process of assessing the redundancy between APC1 and APC2 in actin cable formation. Preliminary data suggests that APC1 can partially rescue APC2 and vice versa suggesting that they may share overlapping roles in actin assembly in actin cable formation. We were not expecting APC1 to rescue APC2 in actin

assembly and vice versa because: 1) these rescue experiments are not consistent with the double mutant APC data and 2) APC1 and APC2 are structurally different at the C-terminus and demonstrate different biochemical functions in actin assembly *in vitro*.

3.3.4 Dia is also involved in the temporal activation of actin cable assembly

Dia is both a nucleation and elongation factor during actin assembly (Goode *et a*l 2007 and Higgs 2005). Loss of Dia using *null alleles* is lethal to the animal, therefore we decided to us dsRNA against Dia. Dia was significantly reduced by expressing RNAi with two different maternal drivers (data not shown). Loss of Dia also induced the same delay in actin cable formation in the early 10B to 10B late egg chambers (Figure 20F and 21D). Loss of Dia in both lines recapitulated the nurse cell drooping observed in *APC2 null* egg chambers. Additionally, not observed in APC mutants, the cortical actin levels in Dia dsRNA were significantly reduced strongly suggesting that Dia required for assembly of cortical actin (not shown). To further confirm the role of Dia in actin assembly, we used dia^{5} *null* heterozygotes and assessed their actin cables. dia^{5} heterozygotes demonstrated the same phenotypic consequence compared to Dia RNAi. The cortical actin in dia^{5} *null* heterozygotes was unaffected. Preliminary data demonstrated that loss of Dia through RNAi knockdown significantly reduced Dia levels below levels observed in dia^{5} heterozygotes (not shown). Therefore, it is possible that loss of cortical actin is more prominent in the Dia knockdown because less than half of Dia protein is present in the knockdown experiment when compared to a half dose reduction of Dia in the Dia^{5} *null* heterozygotes. Our results suggest that Dia is required for the formation of actin cables in stage 10B egg chambers and important in assembling cortical actin.

3.3.5 Phosphorylation of the 20Rs in APC2 are required for APC2's role in actin cable formation

APC2 collaborates with Dia in actin assembly. In Chapter 2, we found that the 20Rs of APC2 were required for actin assembly and that phosphorylation also regulates the activity of the 20Rs in actin furrow extension. Therefore, we asked whether phosphorylation of the 20Rs of APC2 is required for the formation of actin cables in late oogenesis. In these experiments, we assessed the egg chambers of transgenic flies that contained mutations that rendered all the 20Rs either to a phosphorylated state (S to D mutation) or non-phosphorylated state (S to A mutation). The phosopho-mimetic APC2-20R egg chambers induced both an early and delayed activation of actin assembly. At 10B early stages, instead of observing small actin mini-cables, long isolated actin cables were present in the cytoplasm (Figure 22A: yellow arrow). A delay in actin cable formation was also observed but not as severe as loss of *APC2 null* egg chambers. For example, in *APC2 null* egg chambers, actin cables were non-existent in stage 10B mid stages and starting to appear in 10B late stages. In the 20R phospho-mutants at 10B late stages, the actin cables were formed but resembled the actin cables in 10B mid stages. Therefore, there was a significant delay in the 20R phospho-mutants but not as severe as *APC2 null* egg chambers. Additionally, actin cables appeared in patches above the focal plane where these actin cables are normally found as ectopic actin patches (not shown). These actin cables are either ectopic patches or cables that have been mis-oriented similar to Cadherin mutants (Huelsmans *et al* 2013). Conversely, we completely blocked phosphorylation of the 20Rs by mutating all the S>A. The APC2-20R-S>A mutants exhibited the same actin defects observed when phosphorylation was constitutively turned on in late oogenesis Small round actin structures were observed in the cytoplasm indicative of cortical degradation (Spracklen *et al* 2014) in the S>A mutants similar *APC1 null* mutants (not shown). Therefore, it seems as though different phosphorylation states of the 20Rs in APC2 are required for the activity of APC2 actin assembly *in vivo*.

Phosphorylation and non-phosphorylation of the 20Rs is required for proper actin assembly during oogenesis. Mechanistically, the phosphorylation of the 20Rs of APC2 may act an inhibitory switch in the early stages of actin cable formation in the egg chamber. Phosphorylation of APC2 at the 20Rs also affects other actin assembly processes such as follicular cell organization and migration. Although this is also very exciting and has the potential to tell us a lot of about APC2 in actin assembly, this area of investigation is beyond the scope of my work.

3.3.6 GSK3β is required for the proper activation of actin cables in early and late 10B stages

Phospho-regulation of the 20Rs in APC2 is required for proper actin cable formation which led us to ask how and which kinase is required for this phospho-regulation of APC2 in this context. Evidence strongly suggests that GSK3β phosphorylates the 20Rs (Rubinfeld *et al* 1996). We hypothesized that GSK3β was required for the phospho-regulation of the 20Rs in actin cable formation. To test this prediction, we used dsRNA to knockdown GSK3-β in the germline. Loss of GSK3β also formed early thick isolated actin cables in early stage 10B egg chambers. In addition to early activation of actin cables, we observed a delay of actin cable formation in later staged egg chambers. In addition, the cables appeared to be disorganized and fewer in number (Figure 22). In *WT* egg chambers, the growth of the actin cables is constant and therefore all the actin cables are the same length. The actin cables vary in length in GSK3β mutants and are morphologically different (Figure 2B). These data strongly suggest that GSK3β is required for the proper timing of actin cable formation in early and late stage 10B egg chambers. The kinase is also required for promoting the correct number, length, organization, and morphology of the actin cables in late oogenesis.

3.4 Discussion

Vertebrate and Drosophila APC1 are potent actin assembly factors that nucleate, bundle, and collaborate with the formin Dia in actin assembly (Moseley *et al* 2007, Okada *et al* 2010, and Breitsprecher *et al* 2012). However, evidence suggesting that APC proteins are required in actin assembly *in vivo* is lacking. Understanding the individual roles of actin assembly factors is enabling us to better understand how these proteins affect many cellular processes. It was only recently that we began to appreciate that proteins with similar actin assembly activities are working together to assemble the same actin structures but how these proteins function together is not understood clearly. For example, Ena and Dia are biochemically very similar and collaborate to assemble actin filaments *in vivo (*Gates *et al* 2007 and Gates *et al* 2009). Mechanistically, Ena was shown to negatively influence Dia actin assembly by inhibiting Dia-mediated actin assembly in development (Bilancia *et al* 2014). Many protein pairs with similar functions working together and also against each other have been identified: cappu-spire, ena-Dia, and APC-Dia. Here we further investigate the role of APC-Dia mediated actin assembly in the two Drosophila actin based processes. We found that APC1 is not involved in actin assembly in the extension of actin furrows in the early embryo. There are several alternatives that can explain this result. The simplest alternative of why APC1 is not involved in actin furrow extension could be because APC1 is not expressed or localized to the sites of actin furrows. We predicted that because APC1 contained the basic domain required for actin assembly *in vitro,* APC1 would also be required in actin furrow extension. This was not the case and it was surprising to learn that APC1 is not involved in actin furrow extension. To continue to test the function of APC1 *in vivo*, we investigated the role of APC1 in actin cable formation in the ovary. My work finally demonstrated that APC1 is an actin assembly factor *in vivo* in the Drosophila

ovary and functions alongside APC2 and Dia in actin cable formation in late oogenesis. By using the ovary as a model for actin assembly, we will be able to understand the role and mechanism of APC-Dia mediated actin assembly *in vivo*.

3.4.1 APC1 in actin assembly in the early embryo

We tested the loss of APC1 in the early embryo. We found that a very mild defect was observed in actin furrow extension in the early embryo but unfortunately was not rescued by the GFP-tagged APC1 construct. This outcome was very surprising but was supported by the loss of both APC proteins. Complete loss of APC2 and APC1 did not exacerbate the APC2 phenotype. These results can be interpreted into several different ways. It is possible that the GFP-APC1 transgene is not rescuing APC1 to the levels it is actually supposed to be expressed since the expression of GFP-APC1 is being driven under the APC2 endogenous promoter. We are currently technically limited by the methods available to rescue APC1 by the two APC1 transgenes available: 1) one which over-expresses APC1 and I have not been able to express and 2) one which expresses APC1 under the endogenous levels of APC2. APC1 is a very large protein and we have not identified the endogenous promoter of APC1 to be able to make a transgenic fly driven under APC1's promoter. To overcome this problem, we can potentially use recombineering and the PACman technology to make a transgenic fly that contains the APC1 gene and all the upstream/downstream components around the APC1 locus. But because the APC1 transgene we currently have is rescuing other aspects of APC1 loss, it seems highly unlikely that generating a different APC1 rescue transgene will rescue the APC1 actin furrows.

In addition, to verify that the actin furrow defects were induced by loss of *APC1* and not another mutation, we performed a complementation assay. Several *APC1 deficiencies* were crossed to *APC1 null* flies. Although this seemed like a straightforward experiment, it was not.

Genes flanking the *APC1* DNA sequence are *sponge* and *WASP*, both of which are involved in actin assembly. Therefore, deleting large fragments either upstream or downstream could cause additional actin assembly defects because of defects in genes such as *WASP* and *sponge* not caused by *APC1* making it difficult to distinguish between *APC1* or *WASP/sponge* activity in the furrows. We performed these experiments before we realized the close proximity of these actin regulatory genes to APC1 and found that the actin furrows extension defects were more severe in *APC1 null/APC1 deficiency* embryos when compared to *APC1 null* embryos. Therefore, in this experiments, we speculate that the actin phenotypes induced by the APC1 null/APC1 deficiency experiments are not completely APC1-dependent. Taken altogether, our APC1 deficiency experiments were completely inconclusive.

Another alternative is that APC1 is not required for actin furrow extension because it is not expressed in the early embryo or localized to the actin furrows. *In situ* hybridization shows evidence that APC1 is expressed in the gonads and central nervous system (Hayashi *et al* 1997) potentially suggesting that APC1 is involved in processes in the brain or germline. Additionally, un-published evidence suggests that APC2 is highly expressed in the early embryo and decreases through development. APC1 on the other hand is expressed at low levels in the early development and increases during development. Therefore, it is more likely that APC1 is not required for actin furrow extension. To continue to test the physiological role of APC1 in actin assembly, we turned to the ovary as model for actin assembly to test APC1 in actin assembly in the ovary

3.4.2 APC-Dia model in actin assembly in the ovary

Chickadee or (Drosophila Profilin) was the first actin assembly factor identified as part of the actin machinery in actin cable assembly in stage 10B egg chambers (reviewed in Robinson

and Cooley 1997 and Hudson and Cooley 2002). In Dia mediated actin assembly, chickadee binds to the FH1 domain where it helps Dia recruit actin monomers increasing the elongation rate of Dia actin assembly (Goode *et al* 2007). Because Chickadee is involved in elongation, we predicted that other factors required for the initiation of actin cable formation were also involved. Ena is an actin assembly factor found at the plus end of actin cables (Gates *et al* 2007, Gates *et al* 2009, Huelsman *et al* 2013). Ena and Dia are also required together for actin assembly and both are also found at the cortex of the Nurse cells in stage 10B egg chambers (Figure 19). Because Dia binds to many other proteins such as APC1 and APC2, and we predicted that other factors may also be regulating Dia's activity besides Ena. In addition, Ena is required but not essential in actin cable formation, which also prompted us to look at Dia and other Dia regulatory proteins.

Loss of APC1 or complete loss of APC proteins resulted in smaller and rounder egg chambers. Small round eggs are indicative of "dumpless" phenotypes in which the contents from the Ncs failed to reach the oocyte. The dumpless phenotype is observed in proteins involved in the formation of actin cables such as Chickadee, Ena, Singed, Fascin, and Quail or in defective components of the contractile machinery. Therefore, because loss of APC proteins resulted in embryo defects that were similar to the embryonic defects caused by actin proteins required for cable formation, we predicted that APC proteins were also involved in actin cable formation.

Before we could test the function of APC proteins, we verified that they were expressed and localized to the actin cables. APC1, APC2, Dia, and Ena were highly concentrated at the cortex of the Nurse cells. Formation of the actin cables initiates at the cortex and it is most likely that APC1-Dia, APC2-Dia, and Ena-Dia complexes form here to help nucleate these actin filaments.

3.4.3 APC1- Dia localization in the Nurse cells

It is possible that Dia and Ena are potentially working together because Dia and Ena puncta although not overlapping at the TIP complex of the actin cables are in close proximity to each other. We also observed a very interesting localization of Ena and Dia. Ena puncta is localized to the tip of the actin cables and Dia is localized distally to Ena on the shaft of the actin cables. The long actin cables are formed by short parallel actin filaments that are bundled together Guild *et al* 1997) and resemble a ladder. Therefore, it is possible that Dia remains at the plus end of actin filament (as a shaft) because it is required to continuously nucleate and elongate short actin filaments from this location. Ena potentially negatively regulates the activity of Dia at the cortex to control for the length and number of actin filaments that formed by Dia.

I can envision the tip of the complex being very stable but the length of the actin cable continuing to change as it elongates resembling the "Rocket Launcher Mechanism". Dia remains at the plus end of actin cable, and APC1 tracks along the minus end of the actin filament. Instead of forming a complex with the Ena puncta, Ena extends directly from the cortex, whereas Dia is attached to the Ena puncta.

If I could localize all the proteins together, I could imagine that composition of the + end of the actin cable is as follows: Ena is at the tip of the cable, followed by a small Dia shaft that extends up to the cortex, and followed by another short APC1 fragment extending from the cortex. At times, APC1 is observed on the actin cables as puncta and at the – end of the actin bundle. These observations were exciting because Dia and Ena followed the localization of Dia-Ena on filopodia actin cables (Bilancia *et al* 2014). Therefore, because of the spatial distribution of these proteins and the direction of actin assembly, these actin cables are filopodia-like actin cables. Ena negatively regulates the formation of actin cables by decreasing the length and number of actin filaments polymerized by Dia in filopodia (Bilancia *et al* 2014). In addition, the

distribution of these proteins also resembles the rocket launcher mechanism of APC1-Dia mediated actin assembly described *in vitro*. We predict that APC1 and Dia form a complex together at the cortex where they both initiate actin assembly. Once the nucleation phase of these actin cables has been overcome, Dia remains at the plus end of the actin filaments located at cortex of the Nc where Dia continues to elongate the actin filaments. On the other hand, APC1 is localized to the minus end of the actin cables. Based on literature, and the localization experiments my model suggests that APC1-Dia-Ena potentially complex to act in actin filament initiation at the cortex of the Ncs (Figure 23). In this model, Ena associates with Dia at the plasma membrane "dimple" generated by the actin cables to inhibit Dia –mediated actin assembly controlling the number, morphology, and size of actin filaments made by Dia at the cortex. Ena is then de-activated allowing for another round of nucleation by the Dia-APC1 rocket launcher mechanism and Dia continues to elongate of the actin filaments. Then, upon activation of Ena at the cortex complex, actin cable growth is halted. Another round of Ena inhibition occurs, and new actin cable growth is initiated and elongated until Dia actin assembly is again halted by Ena. Many rounds of this inhibition and activation occur until a long actin bundle is made. This model seems consistent with the EM structure of actin cables that appear as small overlapping cables that are bundled together to form a thick actin cable. Although, to be able to confirm this model we need to be able to localize Dia and APC1 in the same sample but we are currently limited by our labeling technologies. Therefore, the Dia and APC1 shafts could be aligning with each other or they can be labeling different populations of actin cables. In addition, APC1 and Dia are both present on dense actin bundles and potentially actin to bundle actin filaments because both of these proteins can bundle actin filaments.

3.4.4 APC2-Dia localization in the Nurse cells

In stage 10B egg chambers, APC2 and Dia are localized to the cortex where they are potentially forming complexes required for actin assembly. Additionally, APC2 is localized throughout the length of the actin cables where it can potentially influence Dia actin assembly activity. Because APC2 seems to be an effector of Dia and not an actin assembly factor on its own, much more APC2 protein may be required to affect Dia activity. On the other hand, our *in vitro* work demonstrated that APC2 binds through its 20Rs to Dia and this binding inhibits Dia mediated actin assembly. Therefore, if we think about how these actin cables are being formed, we envision that new cables are nucleated and elongates at the cortex. Older cables are bundled and pushed towards the nucleus where, the actin cables contact the nucleus to push it away from the ring canals. The older cables may have their + ends exposed and can continue elongating unless a capping protein is associated with the + ends of the older actin cables. It is possible that residual Dia activity remains at those free plus ends and APC2 is localized robustly on the actin cable to inhibit Dia actin assembly to restrict Dia activity to the cortex. In this manner, APC2 acts as break to inhibit Dia mediated actin when it is not required. More work needs to be done so that we can better understand the turnover of actin assembly throughout the length of the cable. Huelsman *et al* 2013 concluded that turnover is not observed along the cable. Though, it is possible that their FRAP experiments did not capture the correct information because they were potentially FRAPing the actin cables at points that were too close to each other for Huelsman et al 2013 to visualize turnover. Therefore, by bleaching two points that are farther away from each can potentially tell us if actin cable polymerization is restricted to the cortex or whether actin cables also undergo assembly. For this, we will use live imaging and test turnover at multiple points throughout the cable. It would be ideal to use a GFP-tagged protein that is localized at the plus ends of actin cables like Dia to identify sites where actin assembly could be occurring on the

actin cable. We have tried expressing a GFP-Dia transgene live as well as GFP-APC2 and GFP-Dia, but the levels of these proteins are extremely low and cannot be visualized live. Therefore, knowing where the plus ends on the actin cables will be a major challenge to our work.

3.4.5 APC2 localization on actin cables to recruit microtubule associate proteins

Additionally, another alternative is that APC2 expressed at high concentrations on actin cables helps target APC2 binding proteins that affect actin cable assembly. For example, APC2 and APC1 form hetero complexes (Mattie *et al* 2010 and Kunttas-Tatli *et al* 2014). In addition, APC1 is microtubule +TIP binding protein that can bind to another microtubule +TIP binding protein called EB1. In turn, EB1 can bind to CLIP190 (another microtubule tip binding protein) that can in turn bind to Dia. Therefore, it is possible that the presence of APC2 on these cables recruits all the proteins to the actin cable. In chapter 4, we show that microtubules are required for proper cable growth and morphology through biochemical and genetic analysis of microtubule associated proteins. This evidence strongly suggests that the microtubule-actin cytoskeletons interact with each other to influence actin assembly but how all these proteins are targeted to actin cable is not understood well.

3.4.6 Investigating APC and Dia-mediated actin assembly

Analysis of loss of function of APC1, APC2, and Dia demonstrate that all these proteins are required for actin assembly in stage 10B egg chambers. The delay in actin cable formation induced by loss of APC1, APC2, and Dia was rescued developmentally and the actin cables appeared to be normal at stage 11. One interpretation of these results is that loss of any of these actin assembly regulatory proteins makes actin filament initiation machinery less efficient. This generates a delay and not a complete loss of actin cables. It is also possible that when the nucleation step of the actin filaments is overcome, the cables have no problem elongating

efficiently. Therefore, it is possible that other actin assembly factors that we have not yet identified are important in helping these cables elongate. For example, Ena is a factor that was recently identified to function in actin cable elongation in the egg chamber. Ena is required but not essential in actin cable formation, providing some evidence to suggest that other elongation factors are present.

Now that we have established a system to understand APC-Dia mediated actin assembly, we can begin to test the interaction of these proteins. There is a lot of work to be done in characterizing the phenotypic consequence of loss APC1, APC2, and Dia. For example, we would like to further quantify the number of cables present in our mutants and rescue experiments. It seems that the delay in actin cable formation before stage 11 is rescued in APC1 and APC2 rescue experiments. However, the length, morphology, and number of actin cables were not completely rescued. Additionally, there are a lot of actin defects in APC1, APC2, and Dia mutant egg chambers. Analysis of these defects needs to be focused to the actin cables, actin blebs, and cortex stability. Additionally, although very important but beyond the scope of my research is investigating what happens to the somatic follicular epithelium because these cells also demonstrate defects in nuclear positioning and actin defects.

Once we have characterized the actin cables and cortical stability in the single mutants, we need to investigate the relationship between all these three proteins in double and triple mutant backgrounds. We hypothesize that removing all these proteins will significantly exacerbate the loss of actin cables in the cable. Our preliminary APC double mutant data suggest that APC proteins do not genetically interact in actin cable formation but these results are not consistent with the aberrant morphology observed in the double APC mutant embryos. The double *mutant APC null* embryos are smaller and rounder when compared to APC1 null embryos

suggesting that both APC proteins are involved in actin assembly. Therefore, we need to repeat these experiments to be able to make any further conclusions about APC1 and APC2 redundancy in actin cable formation in the ovary. These experiments coupled with the analysis of the rescue of *APC1* in *APC2 null* egg chambers and vice versa will lead us to better understand whether APC proteins are redundant in actin assembly in the cables.

Now that we have established a system where all three of these proteins are required in actin assembly, we can test the physiological role of APC and Dia-mediated actin assembly. Most importantly, we can continue testing the interaction of these proteins *in vivo*, by deleting the protein domains that affect APC1-APC2-Dia binding and testing their localization and function in actin cable formation. We will be using mostly fixed tissue but transitioning to live imaging to calculate the delay of these actin cables in each of the mutants.

3.4.7 Regulation of APC2-Dia mediated actin assembly

The 20Rs of APC2 are required for β-catenin binding and phosphorylated to increase the binding affinity of APC2. In Chapter 2, we explored the binding of APC2 to Dia and find that the 20Rs of APC2 are required for the extension of the actin furrows but not APC2's cortical localization. Also, we demonstrated that APC2 and Dia bind through the 20Rs and predict that this interaction is also mediated by GSK3β phosphorylation.

To begin to address the role of the 20Rs in actin assembly in stage 10B egg chambers, we analyzed the actin cables in 20R phospho mutants. Either constitutively turning on or blocking phosphorylation in the early stages of cable formation resulted in the early activation of actin cable formation. In later stages, a delay not as severe as complete loss of APC2 was also observed. Phospho-regulation is required for the temporal control of actin assembly. This may be

do to the fact that the 20Rs are still present and continue to exhibit some residual binding to Dia. To test this, we need to test the complete loss of the 20Rs in actin cable formation.

To test whether APC2 is a target of GSK3β and whether this mechanism is required for actin cable formation, reduction of GSK3β through RNAi was performed. Early activation in stage 10B early egg chambers was also observed by the presence of long isolated cables. Though, the isolated cables did not look exactly like the 20R phospho-mutants. The cables were slightly shorter and thicker. In addition, the actin cables in late stage 10B stages demonstrated even more severe defects in actin cable morphology. For example, fewer number of cables, smaller actin cables, and the overall morphology of individual cables were observed in mutant GSK3-β egg chambers. Therefore, because similar actin assembly defects were observed in loss of GSK3β and APC2-20R phospho-mutants, we predicted APC2 is a target of GSK3β. However, it appears that the actin defects observed in GSK3-β and APC2 are not exactly the same. GSK3-β may be involved in aspects of actin cable formation independent of APC2 phospho-regulation.

We need to take into consideration that GSK3-β is involved in many processes including regulating the association of microtubule associated proteins on microtubules. Phosphorylation of MAPs such as Tau and MAP4 increases the binding of affinity of these proteins to microtubules. MAPs are required for the assembly and stability of microtubules. Our results in Chapter 4 suggest that microtubules are required for actin cable formation and if phosphorylation of MAPs is lost, it is possible that microtubules may not be formed properly. This in turn can also affect the assembly of actin cables. Therefore, to understand the role of GSK3- β on actin assembly, we need to further understand the role of microtubules in actin assembly. I did look at the microtubules in GSK3- β mutants and found that the microtubules were disrupted primarily

when the ring canals were dislodged from the cortex but further in depth analysis needs to be done. In chapter 4, we begin to address the role of microtubules in actin assembly.

In conclusion, APC1-APC2-Dia mediated actin assembly is required for actin cable formation in stage 10B egg chambers. All of these proteins localized to the cortex of the Ncs where we predict either APC2-Dia and APC1-Dia complexes form to help form actin cables. A temporal delay was observed when any of these proteins was removed, suggesting that these proteins are required for cable formation but not essential. Phospho-regulation of the 20Rs most likely by GSK3β is required for APC2's function in helping assemble actin cables in the Drosophila ovary. Mis-regulation of phosphorylation can either activate actin assembly too early or delay actin assembly. This suggests that APC1 or Dia may be able to carry out actin assembly alone, but with reduced efficiency resulting in assembly delay. On the other hand, based on the *in vitro* and *in vivo* data, we predict that APC2 is an effector of Dia and we need to further investigate their interactions to understand the mechanism of APC2-Dia mediated actin assembly. Taken together, our studies suggest that APC-Dia collaborations are evolutionarily conserved, and the Drosophila ovary is an experimentally tractable system in which to dissect both molecular mechanisms and physiological relevance.

3.5 Figures

Figure Legends

Figure 15: APC1 is not involved in actin pseudocleavage furrow extension the early embryo

A) The top surface (left) and deepest surface views (right) of the actin furrows of stage 12 metaphase embryos. These are surface views of the furrows where the furrows are observed as actin rings.

B) Schematic of a cross-section of actin furrows. Actin tracks with the plasma membrane into the embryo during metaphase, forming rings on the surface or furrows in cross-section.

C) Four different surface sections were imaged in the early embryo and the number of complete versus incomplete rings were counted. Percentages of incomplete and complete rings are shown. Complete loss of *APC1* induces a very mild actin furrow extension defect in the early embryo. Rescue with the APC1 rescue transgene did not rescue this phenotype. Additionally, complete loss of APC2 and APC1 proteins did not exacerbate actin furrow extension defects.

*Scale Bar= 10μm

Figure 16: APC1 is a potential actin assembly factor in the Drosophila ovary

A) Schematic of late Drosophila oogenesis. To assess actin assembly, we are interested in formation of the mini-cables and elongation of cables during 10A and 10B stages in Drosophila oogenesis.

B) Schematic of a whole stage 10B egg chamber. Egg chambers are composed of 15 Nurse cells (blue) and one oocyte (gray) that are all interconnected through ring canals (light blue). The follicular epithelium surrounds the oocyte (orange cells) and migrates toward the border cells (red).

C) Low magnification image of the Nurse cells in stage 10B Late egg chambers. Actin cables (white) are fully extended and surrounding the nuclei (light blue). Scale bar=25μm

D) Mini-cables are formed in 10B early staged egg chambers magnified in the inset. Scale bar=10μm

E) Actin cables are longer in stage 10B mid egg chambers. The initiation tip complex is present at the cortex and an indentation is made by the plus end of the actin cable as actin polymerization progresses at the cortex. Ena (red puncta) is part of the tip initiation complex found at the plus end of the growing actin cable observed in the inset. Scale bar=10 μm

F) Actin cables (white) surrounding and contacting the nucleus (blue) in a single Nurse cell in stage 10B late egg chambers. 10 μm

G) Ultra-structure of image of the actin cables from Guild et al 1997. Actin cables are formed by bundling small parallel actin filaments polymerized at the cortex. 30um

H) *APC1* and *APC2 APC1 null* embryos are smaller and rounder when compared to WT embryos suggesting a "dumpless phenotype".

Figure 17: Characterization of stage 10B and 11 actin cables

A-E) Centripetal cellular migration, the size of the oocyte, and the morphology of the follicular epithelium were used to stage the egg chambers.

A) WT Stage 10A: (Top) Small mini-cables present and magnified to the left. (Bottom) Centripetal cells have not invaginated and migration of the cells has not initiated.

B) WT Stage 10B early: (Top) Small mini-cables are present. Centripetal cells invaginated to align along the border of the oocyte and migration of the cells started.

C) WT Stage 10B mid: (Top) The average length of actin cables is about 10um. (Bottom) Centripetal migration has extended further but the centripetal cells have not yet reached the border cells (green circle).

D) WT Stage 10B Late: (Top) The average length of the actin cables at this stage is about 30um. (Bottom) The centripetal cells have reached the position of the border cells. The follicular epithelium is still very thick and the oocyte is about half the size of the egg chamber.

E) WT stage 11: (Top) The cables continue to grow and look very similar to stage 10B late cables in length and morphology. (Bottom) The follicular epithelium lining the oocyte gets thinner and the size of the oocyte starts increasing, taking up more than half of the size of the egg chamber.

*Scale bar= 10 μm

*Phalloidin staining to label actin.

Figure 18: APC proteins are expressed and localized in the Drosophila ovary

A) Distribution of APC1 in a stage 11 Nurse cell. *GFP-APC1; APC1 null* transgenic flies were used and probed using antibodies against GFP and Ena. APC1 is diffused in the cytoplasm and localization is highly enhanced to the cortex. At some instances, it associated with really thick actin bundles (blue) (yellow arrow).

B) APC1 is highly concentrated at the cortex and does not co-localize with Ena at the initiation sites of actin cables in stage 10B mid stages. Very small cables of 4 to 5 um are extending from the cortex and co-localized with actin cables (white arrows).

C) Few APC1 puncta decorate the actin cables and at some examples, APC1 is observed only at the tips of the actin cables in stage 10B mid Nurse cells (orange arrows) .

D) Drosophila ovary lysate probing for anti-APC1 and and anti-Dia. The APC1 antibody cross-reacts labeling both APC proteins therefore, anti-APC1 was used.

E) Distribution of APC2 in a stage 11 Nurse cell in *GFP-APC2;APC2 null* flies. APC2 is localized to the cortex and localized throughout the length of most actin cables. APC2 does not co-localize at the initiation sites of actin with cables with Ena.

E') High magnification of D. Puncta of APC2 decorate the length of the actin cables.

*Scale bars= 10 μm

*Phalloidin staining to label actin

*Because of lack of good APC antibodies, GFP-APC transgenic lines were used to localize APC proteins in Drosophila egg chambers.

Figure 19: Dia is localized to the cortex of the cell and present on actin cables

A) Nurse cell in stage 11 egg chambers probed with anti-Dia (green) and anti-Ena(red) antibodies. Ena and Dia are localized to the cortex of the cells. At times, Ena and Dia are observed on large bundles of actin bundles (blue), although this localization is very weak (yellow arrows). High magnification of Dia is observed in B (orange dotted line).

B) High magnification image of panel A. Dia co-localizes on actin cables.

C) High magnification of the cortex. Dia and Ena are concentrated at the cortex of the cell. Ena is positioned at the initiation sites to the cables of the cables and Dia localizes to the shaft of this cable in some cases.

*Scale bar= 10 μm

Figure 20: Loss of APC1, APC2, and Dia causes a temporal delay of actin able formation in mid stages of actin cables formation

A, C, and E) Actin cables all demonstrate the presence of actin cables in *WT, APC1+ APC1 null*, and *APC2+ APC2 null*. Although, actin cables are present in the rescuing transgenes, minor defects in lengths and number of actin cables are observed.

B, D, and F) Complete loss of APC1, APC2, and reduction of Dia completely abolish the formation of the small actin cables observed in stage 10B mid egg chambers.

The orange area points to areas of the cortex that were magnified to the left of the Nurse cells in each genotype.

*Scale bar= 10 μm

*phalloidin staining

Figure 21: Temporal delay of actin cable formation continues until stage 10B late egg chambers and recovers in stage 11

(A-F) are all stage 10B Late egg chambers

A) Full cables are observed in WT egg chambers.

B, D, and E). Length and number of actin cables is dramatically reduced in *APC null* and dsRNA-Dia egg chambers.

C and F) Phenotypes of *APC1 null* and *APC2 null* actin cables are rescued by APC1 and APC2 transgenes, respectively.

G-H) Stage 11 egg chambers. Actin cables recover in all genotypes. Even dsRNA-Dia(not shown).

*Scale bar 10 μm

*phalloidin staining

Figure 22: Loss of phospho-regulation of the 20Rs of APC2 potentially by GSK3-β induces early activation of actin cables in early stages and a delay in actin cable formation in late stages

A) Stage 10A or 10B early staged egg chambers. (Top) Mini-cables are present in WT egg chambers. (Middle) Long isolated actin filaments in *APC2-20R-S>A* (yellow arrow). (Bottom) Thick and short isolated cables are found in *SggRNAi* mutants (green arrow).

B) Late stage 10B egg chambers. (Top) Full actin cables in WT egg chambers. (Middle) Delay and a small decrease of actin cables in the *APC2-20R-S>A* mutants observed in a max projection. (Bottom) Decrease in length, dramatic decrease in number, and morphology defects are observed in actin cables present in Sgg RNAi mutant egg chambers.

Figure 23: APC1, Dia, and Ena in actin cable formation

Cycles of inhibition of Dia and Ena may regulate the formation of actin cable formation.

Figure 15

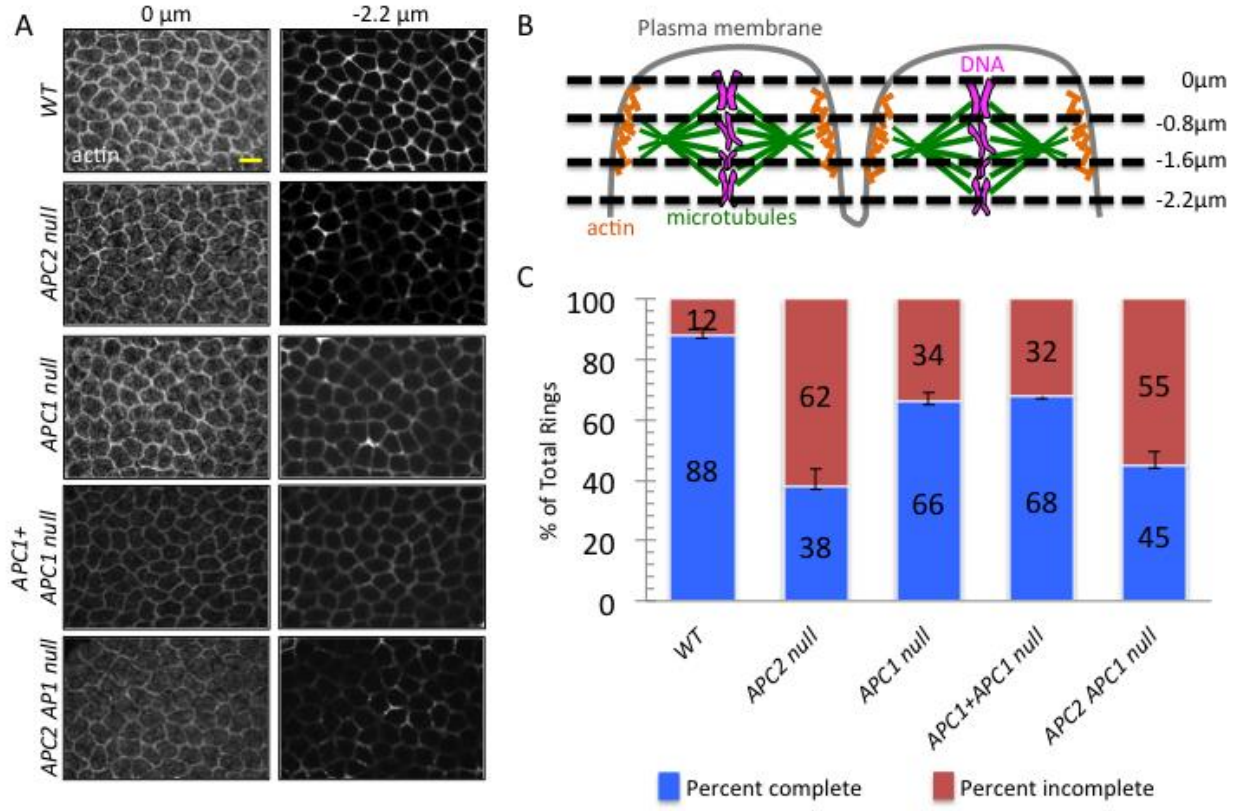

A
0 µm
-2.2 µm
WT
actin
APC2 null
APC1 null
APC1+ APC1 null
APC2 AP1 null
B
Plasma membrane
DNA
0µm
-0.8µm
-1.6µm
-2.2µm
actin
microtubules
C
% of Total Rings
100
80
60
40
20
0
12
88
62
38
34
66
32
68
55
45
WT
APC2 null
APC1 null
APC1+APC1 null
APC2 APC1 null
Percent complete
Percent incomplete

Figure 16

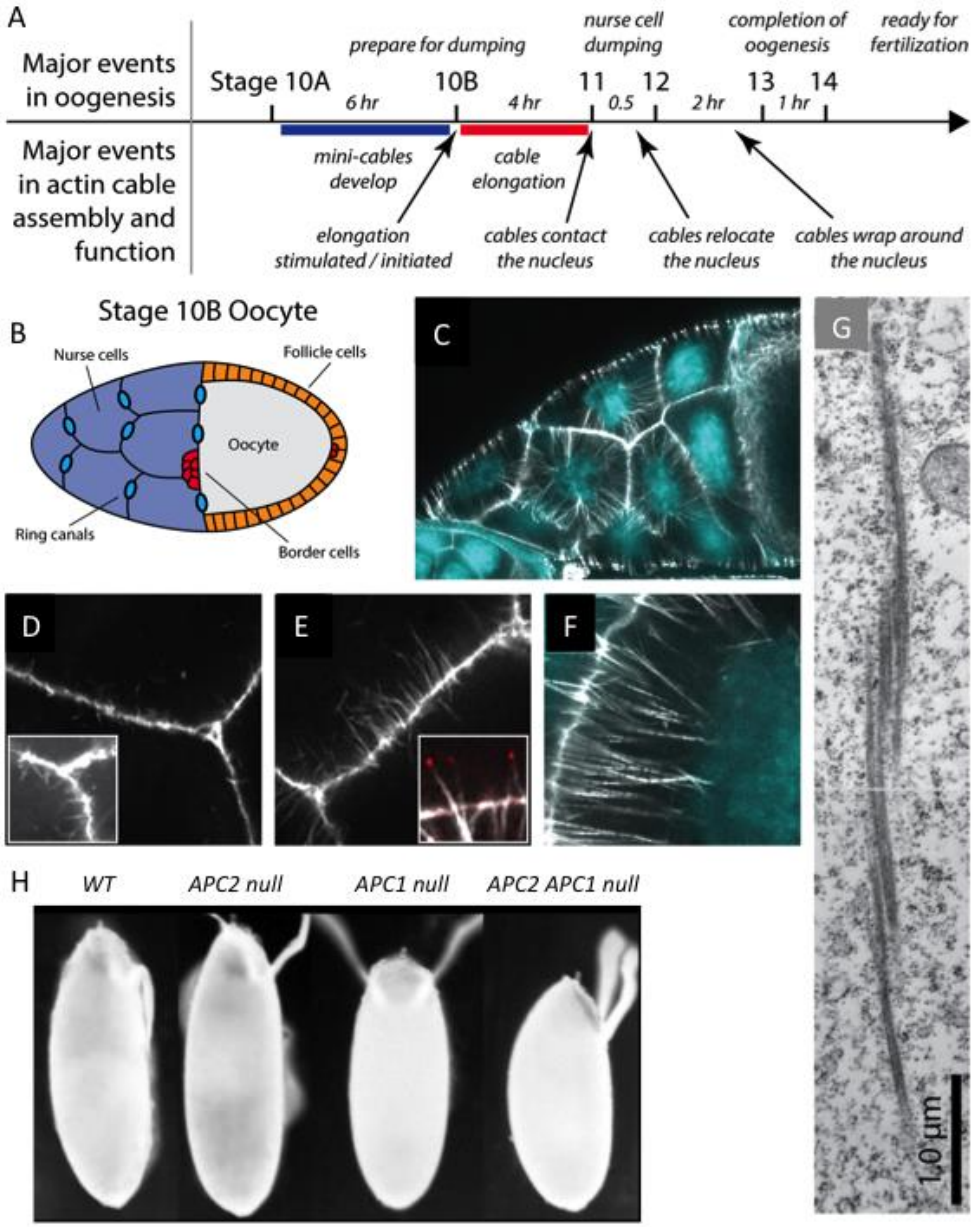

A
Major events in oogenesis
Major events in actin cable assembly and function
prepare for dumping
nurse cell dumping
completion of oogenesis
ready for fertilization
Stage 10A
10B
11
12
13
14
6 hr
4 hr
0.5
2 hr
1 hr
mini-cables develop
cable elongation
elongation stimulated / initiated
cables contact the nucleus
cables relocate the nucleus
cables wrap around the nucleus
B
Stage 10B Oocyte
Nurse cells
Follicle cells
Oocyte
Ring canals
Border cells
C
D
E
F
G
1.0 µm
H
WT
APC2 null
APC1 null
APC2 APC1 null

Figure 17

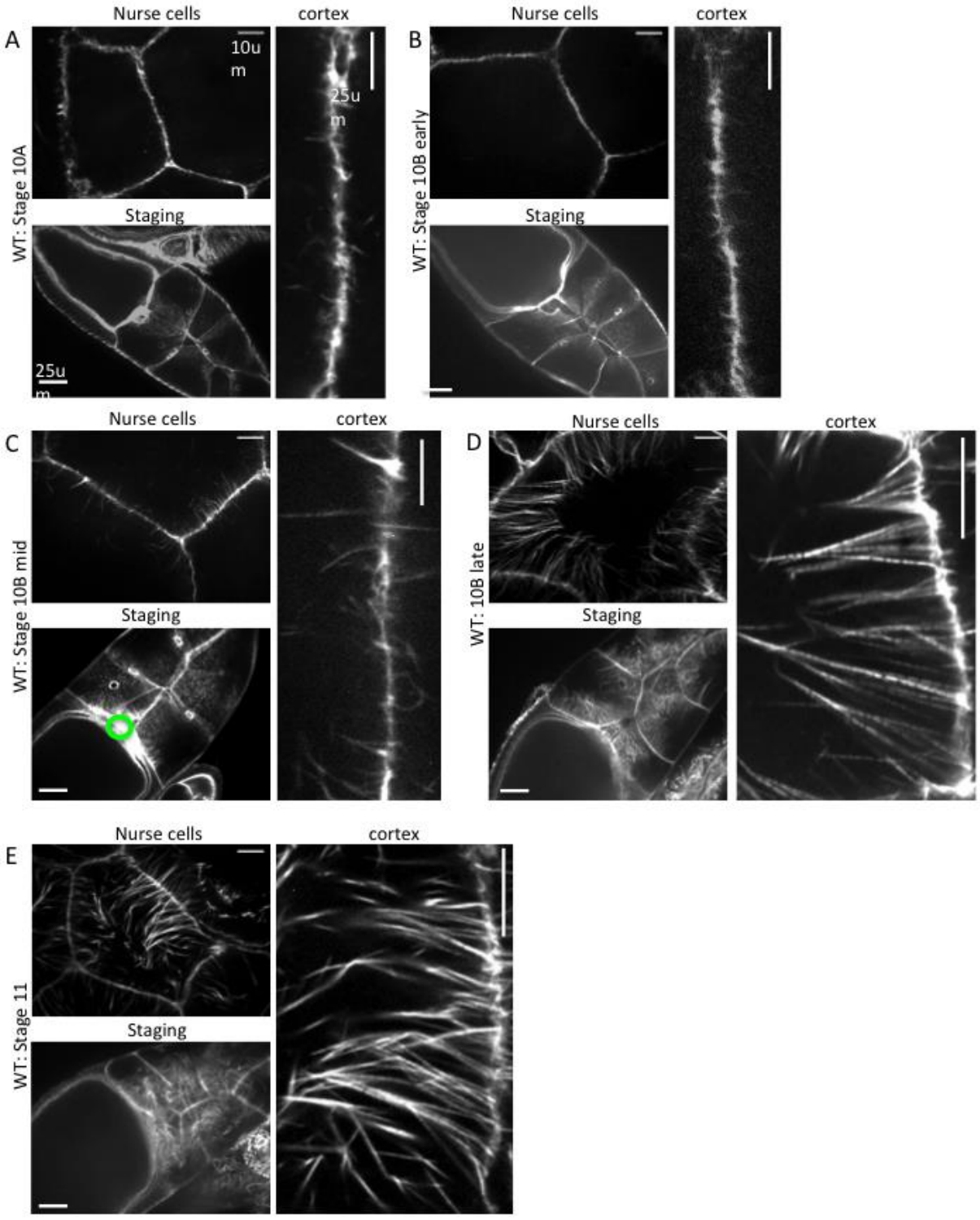

A
Nurse cells
cortex
10um
25um
WT: Stage 10A
Staging
25um
B
Nurse cells
cortex
WT: Stage 10B early
Staging
C
Nurse cells
cortex
WT: Stage 10B mid
Staging
D
Nurse cells
cortex
WT: 10B late
Staging
E
Nurse cells
cortex
WT: Stage 11
Staging

Figure 18

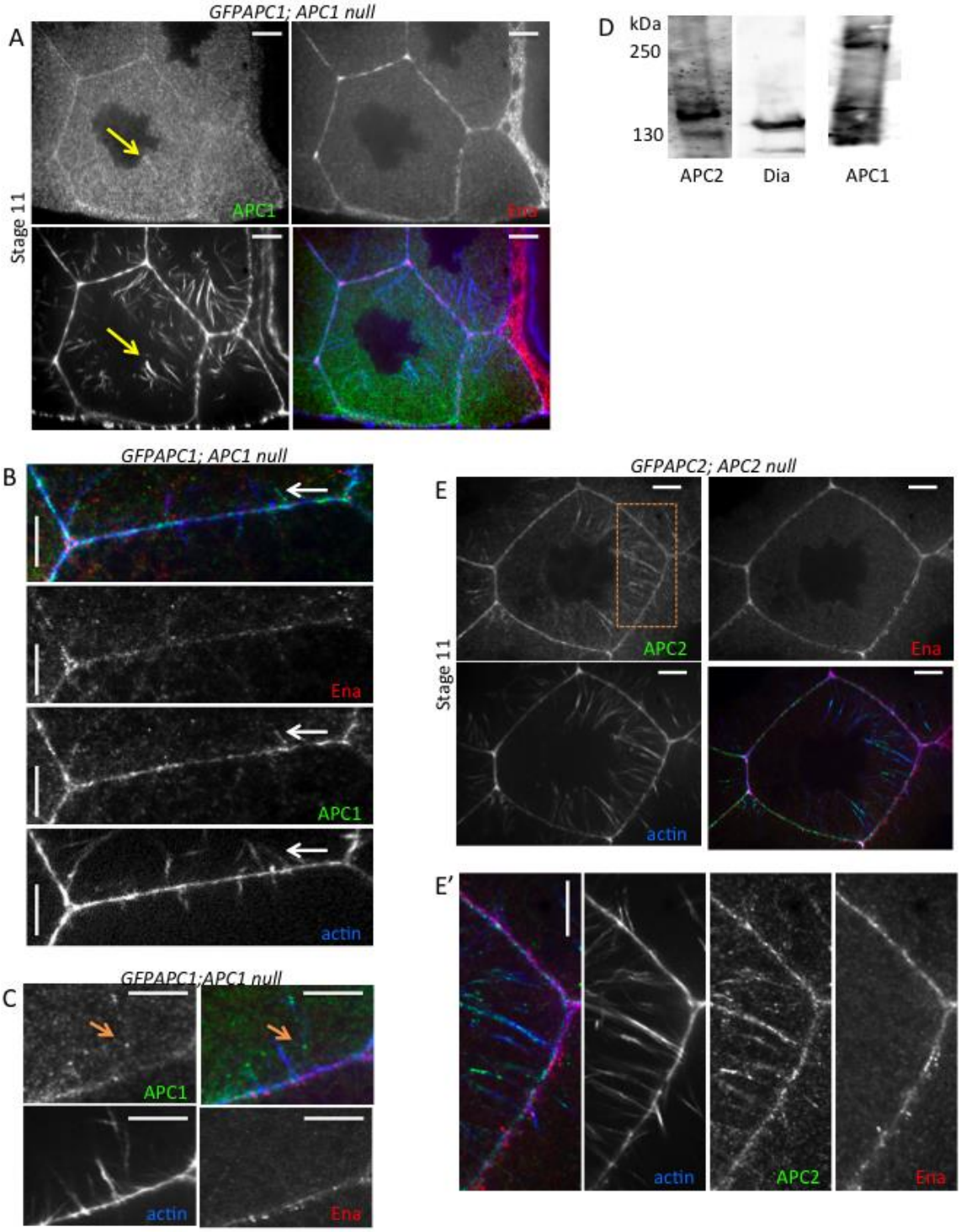

GFPAPC1; APC1 null
A
Stage 11
APC1
Ena
D
kDa
250
130
APC2
Dia
APC1
B
GFPAPC1; APC1 null
Ena
APC1
actin
C
GFPAPC1;APC1 null
APC1
actin
Ena
GFPAPC2; APC2 null
E
Stage 11
APC2
Ena
actin
E'
actin
APC2
Ena

Figure 19

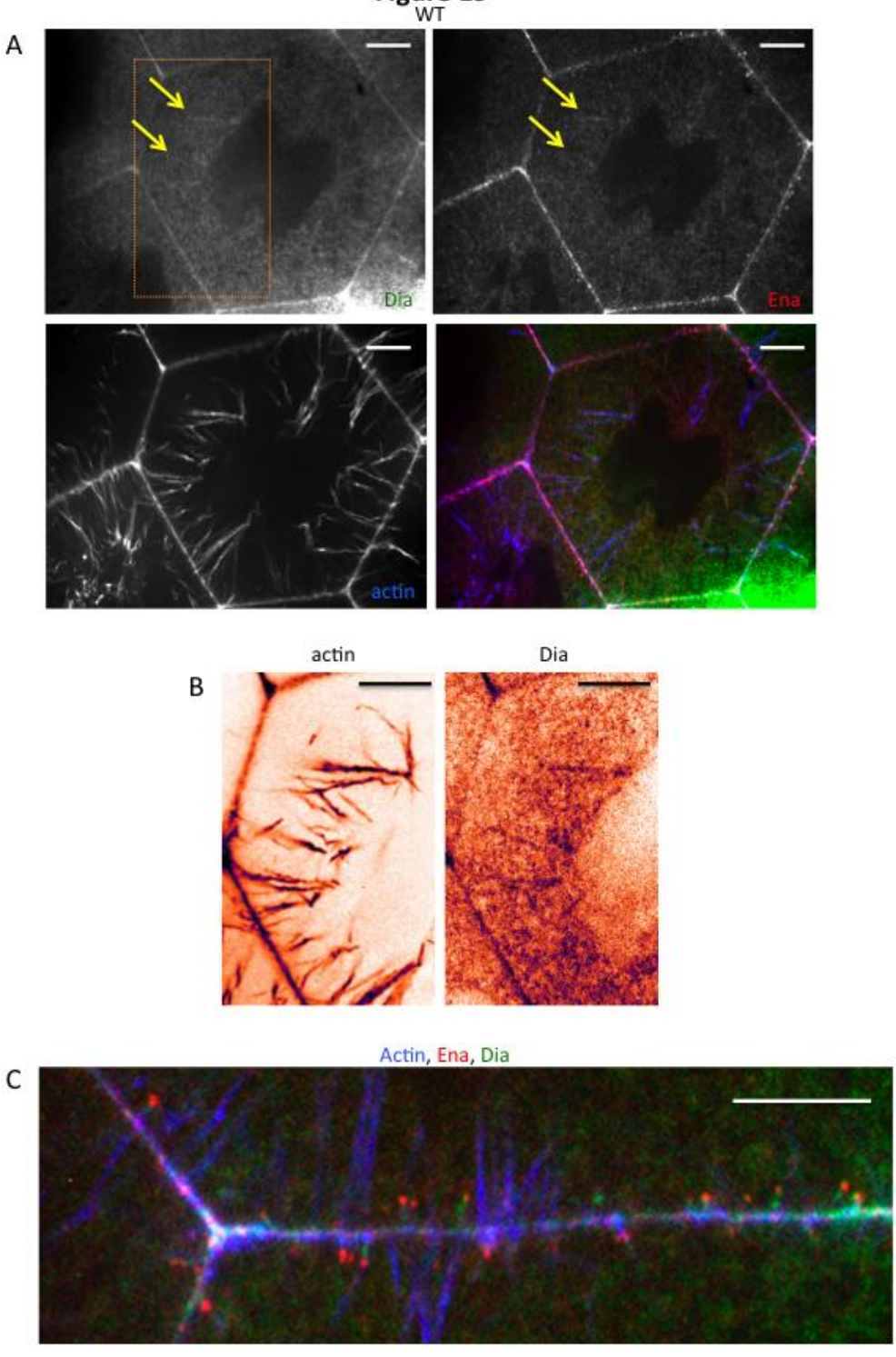


Figure 20

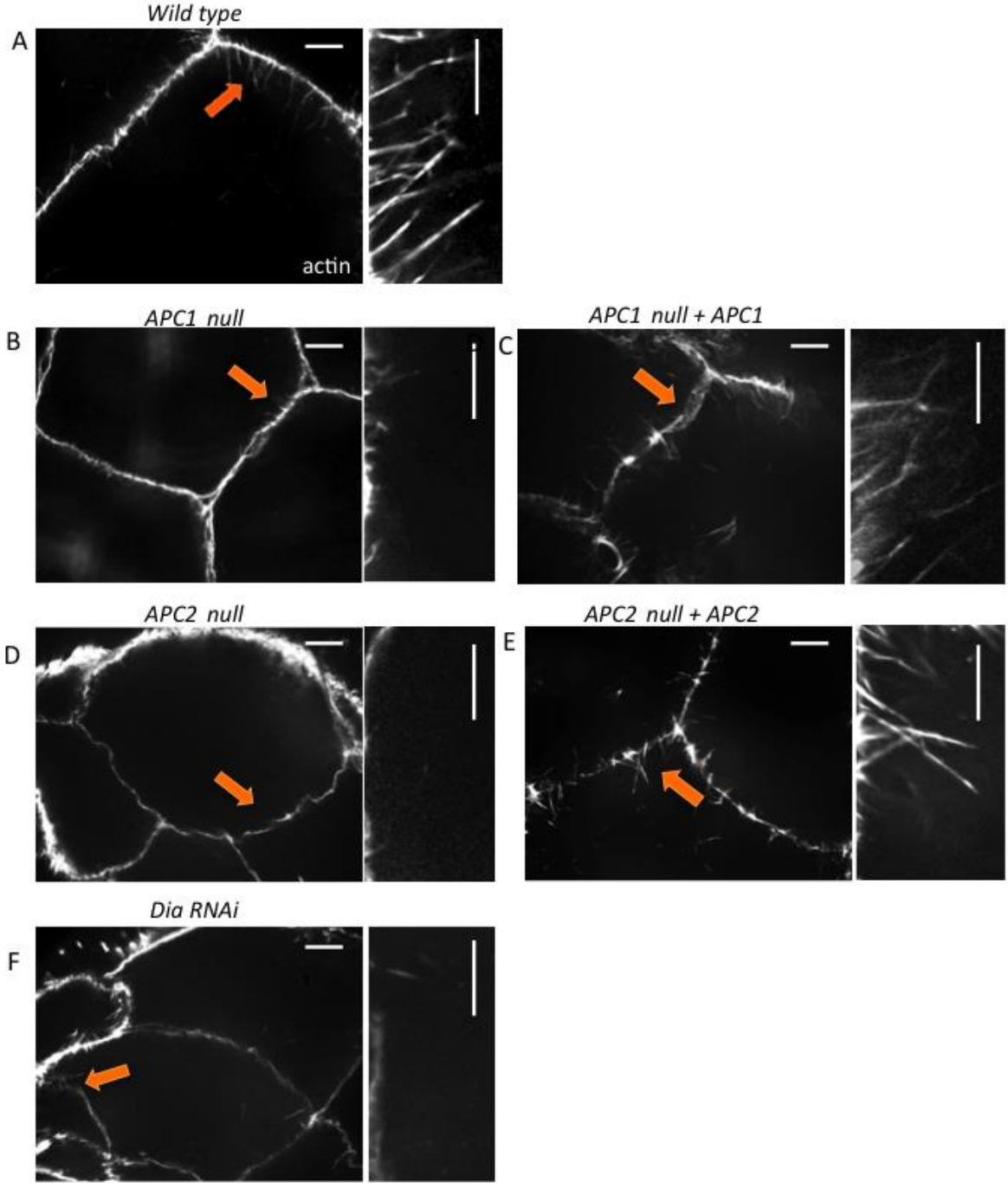

Wild type
A
actin
APC1 null
B
APC1 null + APC1
C
APC2 null
D
APC2 null + APC2
E
Dia RNAi
F

Figure 21

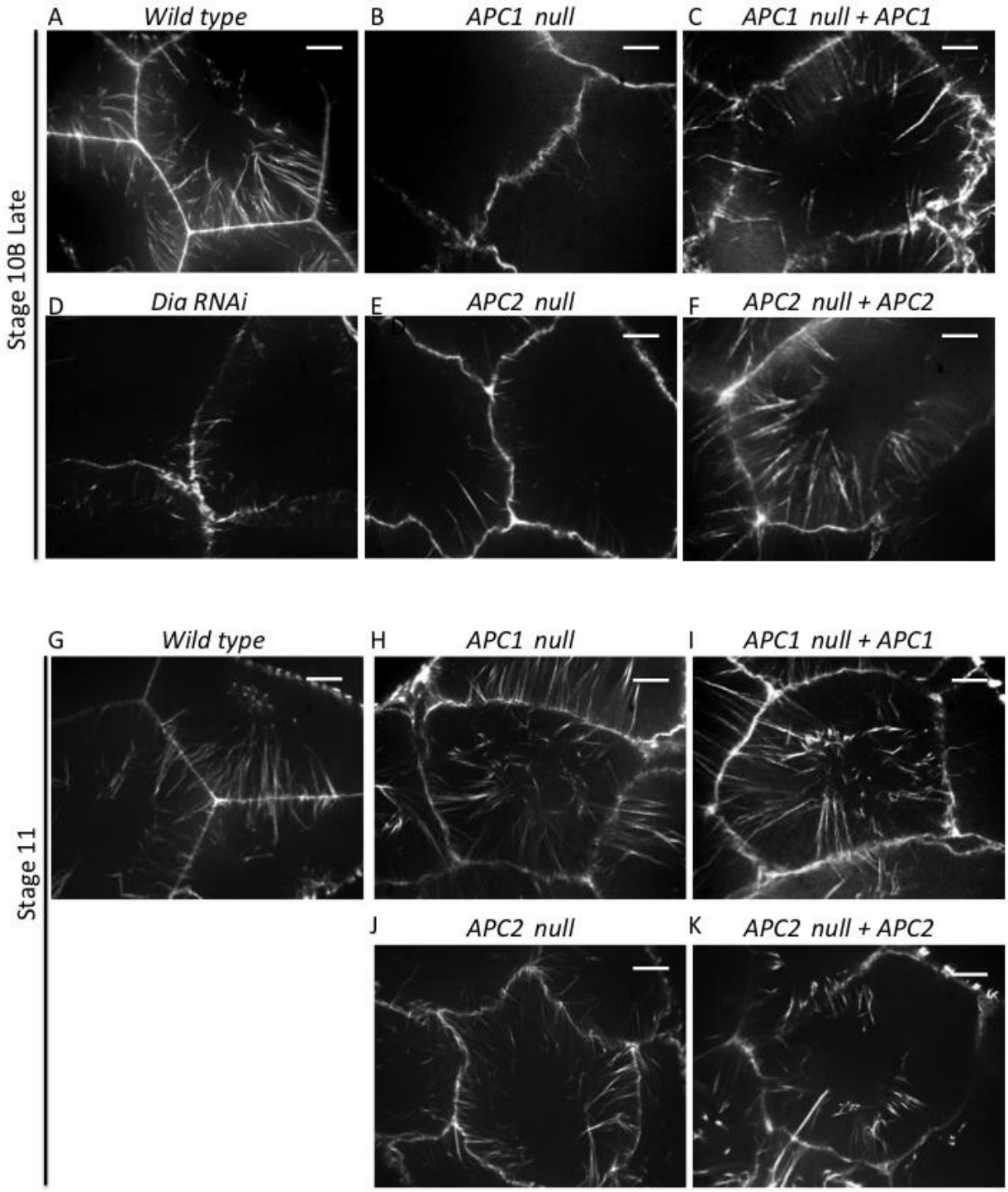

A
Wild type
B
APC1 null
C
APC1 null + APC1
Stage 10B Late
D
Dia RNAi
E
APC2 null
F
APC2 null + APC2
G
Wild type
H
APC1 null
I
APC1 null + APC1
Stage 11
J
APC2 null
K
APC2 null + APC2

Figure 22

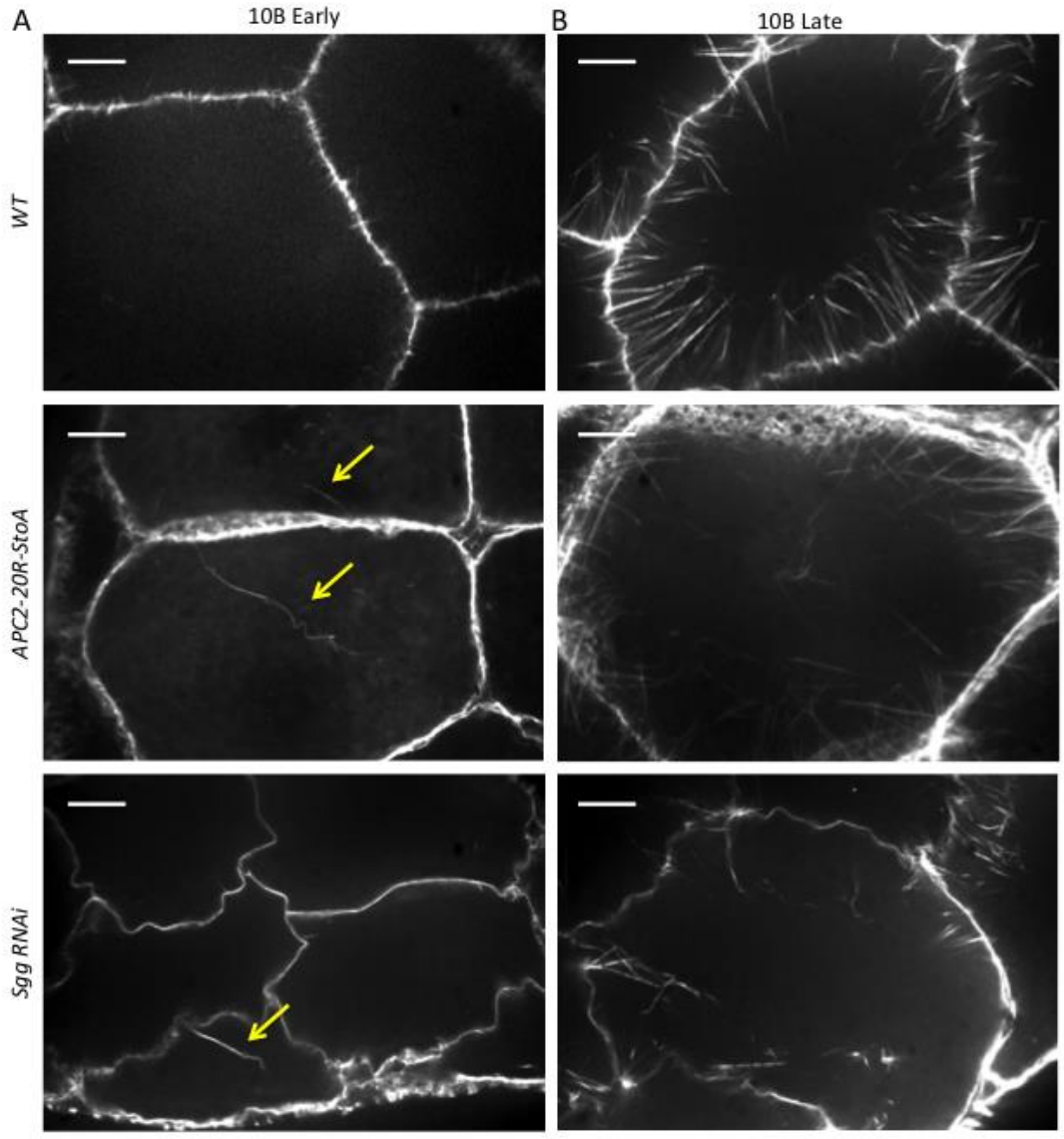
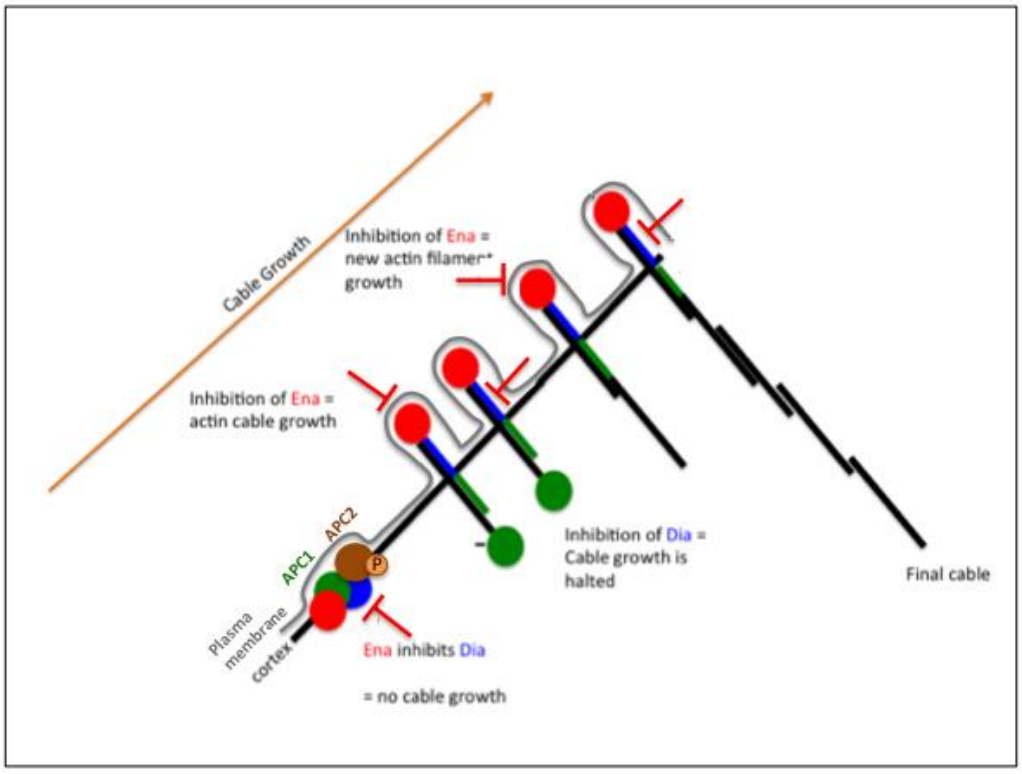


Figure 23


Cable Growth
Inhibition of Ena =
new actin filamer
growth
Inhibition of Ena =
actin cable growth
APC2
APC1
P
Plasma
membrane
cortex
Ena inhibits Dia
= no cable growth
Inhibition of Dia =
Cable growth is
halted
Final cable

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Chapter 4. Microtubule-actin crosstalk in actin cable assembly during late oogenesis

4.1 Introduction

Many cellular processes such as organelle transport, motility, shape change, cell shape maintenance and division, require both the microtubule and actin cytoskeletal networks to work in concert. The mechanism of how these networks work together is not understood clearly. Although they are distinctly involved in different processes, more recent work demonstrates that these networks function together in many other processes. Vasiliev *et al* 1970 demonstrated that the location of actin polymerization was dictated by the presence of microtubules at the leading edge of fibroblasts. In yeast, the microtubule plus end proteins such as Tea4 recruits the formin For3 (Martin *et al* 2005). The Tea1, Tea4, and For3 form a complex that is required for localized actin assembly for polarized cell growth (Martin *et al* 2005). Therefore, it is possible that microtubules and microtubule binding proteins are required to recruit actin assembly factors and spatially regulate their actin assembly activities. Although we are beginning to understand microtubule-actin crosstalk, these mechanisms are not well understood.

4.1.1 APC proteins, Dia, and Microtubule +TIP binding proteins

Microtubule plus ends are highly dynamic and regulated by microtubule +TIP binding proteins (Jiang *et al* 2011). An example of a microtubule +TIP binding protein is EB1. EB1 tracks on microtubules and can recruit APC1 and CLIP-170 to the +ends of microtubules (Su *et al* 1995 and Dixit *et al* 2009). It is intriguing that more than one +TIP binding protein is recruited to the ends of microtubules and raises the questions as to why and how are multiple +TIPs recruited. In the context of microtubules, EB1-Dia-APC1 complex to form stable microtubules (Wen *et al* 2004). But what about the role of EB1-Dia-APC1 in actin assembly?

Additionally, a Formin Homology Effector Domain (FEED) sequence was found in CLIP-170 (Goode Lab; manuscript submitted). Binding of the FEED domain in CLIP-170 to the FH2 increased the rate of actin assembly of Dia. These results were really exciting because they corroborated early data that suggested that +TIP binding proteins interacted with formins to promote localized actin assembly (Martin *et al* 2005). APC proteins can bind to EB1 (Wen et al 2004 and Sue et al 1995), and EB1 interacts with CLIP-170 (Dixit *et al* 2009) and Dia. Additionally, Dia in can bind to APC proteins (Webb et al 2009, Wen et al 2004, Okada et al 2010). Therefore, all of these proteins bind to each other directly or indirectly to potentially influence each other's behavior.

4.1.2 Microtubules in oogenesis

It was recently shown that microtubules and actin filaments are present and co-localize around and through ring canals Nicolas et al 2009). Additionally, they demonstrated that disruption of the microtubules through drug inhibition completely eliminated the array of actin filaments emanating from the ring canal. Although, the actin that forms the ring canal seems unaffected strongly suggesting that microtubules are required for the formation of actin bundles coming from the ring canals but not the ring canals themselves. From their work, they also showed that the direct crosslink between actin and microtubules is required for transport (streaming) of the contents of the nurse cells to the oocyte. What about the actin cables that are formed in the cytoplasm of the Nurse cells? Is the formation of these actin cables regulated by microtubules? In Chapter 3 we showed that APC1, APC2, and Dia are required for actin cable formation. Is Dia activity regulated by +TIPs?

In Chapter 4, our main goal was to better understand the cross-talk between the microtubule and actin cytoskeletal network taking a coupled *in vitro* and *in vivo* approach. Our research strongly suggests that actin filaments are polymerized primarily through actin assembly factors such as Dia and APC proteins but the activity of these proteins is regulated by proteins associated with the microtubules positioned adjacent to the actin cables such as EB1 and CLIP-190. Our work strongly suggests that the microtubules align with the cytoplasmic cables to influence their formation. Depolymerization of microtubules through drug treatment negatively impacted the length and morphology of the actin cables. Specifically, the microtubules parallel to the actin cables are highly acetylated, suggesting that this microtubule population is stabilized. Microtubule associated proteins such as MAPs bind to and stabilize microtubules. Because +TIPs have been found to be required for the direct interaction with formins, to collaborate in actin assembly, we hypothesized that two +TIP proteins that bind (CLIP-190 and EB1) to Dia and APC may regulate the activity of APC-Dia mediated actin assembly. We found that CLIP-190 collaborates with Dia *in vitro* and that CLIP-190 localizes to the interface of microtubule and actin intersection in the Drosophila egg chambers. Based on *in vitro* biochemical and function data that MAPs are effectors of actin assembly, we tested the role of CLIP-190 and EB1 in actin cable formation. On the other hand, EB1 inhibits APC1 and APC1-Dia mediated actin assembly. Consistent with these data, loss of EB1 in Drosophila egg chambers induces an early activation of actin cable formation. Through our genetic and biochemical data we propose that microtubule-actin cross-talk is mediated by EB1 and CLIP-190.

4.2 Materials and Methods

4.2.1 Drosophila ovary tissue fixation

Female flies no more than a week old were "yeasted" overnight for either 1- 2 days. Drosophila ovaries were dissected in Schneiders media with 10% FBS and Pen/Strep. Ovaries were teased out using forceps to remove as much as the muscle sheath as possible. Ovaries were fixed with 4% paraformaldehyde, washed four times with PBS, and blocked in PNT (1xPBS, NGS, and 0.03% Titron-X100) for 1 hour. Primary antibodies were incubated at 4˚C overnight: rat-anti-tubulin Millipore (Millipore/MAB1864) 1:1000, mouse-anti-acetylated tubulin (Sigma/T7451) 1:1000, and rabbit-anti-CLIP190 (gift from Kathryn G. Miller/Washington University) 1:500. Secondary fluorescent antibodies conjugated to Alexa-488, 546, or 647 at (Invitrogen? 1:1000 and phalloidin (Invitrogen) at 1:3000 were incubated for 3 hours at room temperature. Stained with DAPI (Sigma/) for 30 minutes. Ovaries were completely separated before mounting in buffers and mounted in Aqua-Pol/Mount (PolySciences).

A second fixation method was used to optimize for microtubule and membrane associated staining slightly adapted from Dr. Kevin Edwards. 2 to 4 day old female and males flies were collected and yeasted overnight for 1 to 2 days to maximize the number of stage 10B egg chambers. Female ovaries were dissected in PBS for about 10 minutes, continuously transferring the dissected material to fresh PBS. The ovaries were stroked gently between ovarioles in order to remove as much muscle sheath as possible. Ovaries were fixed in 2% (16% EM grade formaldehyde-Polysciences) formaldehyde for 20 minutes at room temperature without shaking. Ovaries were washed 3 times in PBT-0.2% (1xPBS, 0.2% Triton-X100) and incubated last wash for 15 minutes with wash. I transferred material to a new well, washed once with PBT-0.2%, and transferred material to an Eppendorf tube. The material was washed once with PBT-0.2% and blocked in 1 mL of PBT-0.2% with 4% NGS for one hour at room temperature while rocking.

Antibodies were pre-diluted into 1mL of PBT-0.2% (NGS dropped from 4% to 1%), replaced the block with antibody solution, and incubated overnight at 4 degrees Celsius while rocking. Primary antibody solution was disposed and washed 3-4 times in a period of one hour. Pre-diluted secondary antibodys and phalloidin were incubated with ovary material for 5 hours at room temperature while rocking. I washed the samples 3 times and incubated them with DAPI for 30 minutes while rocking. Ovaries were washed 3 times, completed separated before mounting in buffers, and mounted in Aqua-Pol/Mount (PolySciences).

4.2.2 Imaging and Image Processing

Images were acquired with a spinning-disc confocal microscope with a Yokagawa scan head (Solamere Technology Group) and a QICAM-IR camera (Qimaging) on a Zeiss Axiovert 200M using QED InVivo software. All images were processed with Photoshop and quantification of pixel intensity was obtained through ImageJ analysis.

4.2.3 Drug treatment

Drosophila ovaries were dissected in Grace's medium with 10% FBS and . Early 10B staged egg chambers were isolated and incubated in new media. To disrupt microtubules with nocodazole, egg chambers were treated with 2.5ug/mL of nocodazole in 50uL of DMSO for 3 hours. Based on previous evidence, the maximum amount of DMSO to be used in these experiments was 10uL where we would observe the least amount of defects. But based on our working stock, we had to add 50uL of DMSO. For the most part, adding 50uL of nocodazole did not affect the formation of general actin structures we continued to use 50uL of DMSO. It is worth mentioning that the cortical actin in DMSO treated nurse cells did look a little different and therefore, we cannot make out any conclusions about the surface actin. For colchicine treatment, egg chambers were treated for 3 hours with 70ug/mL. After the three hour incubation, the egg chambers were fixed

and probed with phalloidin (Invitrogen 1:1000), anti-tub (Millipore 1:1000), and DAPI. Standard secondary antibody and mounting procedures was followed.

4.2.4 Cloning of CLIP190

Alignments indicated that a functional domain for actin assembly within human-CLIP170 was also present in the fly CLIP-190 amino acid sequence. Therefore, fragment 1708bp-1629bp was PCR amplified from a cDNA obtained from Kathryn Mills using custom made primers: (F) AGATCTatgTTAGCGACGCCGAAAA

and (R) GAATTCctaCAGGGCCTCATCTTTGG. After sequencing, I found that there were two missense mutations in my clone but we decided to move forward since the mutations were outside the activity domain of CLIP190. The fragment was cloned into a pLm1(GST tagged vector) through 5'BgLII and 3'EcoR1 restriction sites.

CLIP190 (1708-1629) fragment

5'AGATCTatg(1708)TTAGCGACGCCGAAAAGCCAATTTTCCATGCAGGATCTGCTGCG
CGAGAAGCAACAACATGTGGAGAAGCTGATGGTGGAGCGCGACCTGGACCGCGAG
GATGCCCAGAACCAGGCGCTGCAGCTGCAGAAGAACATCAACGAGCTAAAAGCAA
GAATCGTTGAATTGGAGTCGGCATTGGACAATGAACGAAAGAAAACTGAAGAATTG
CAGTGCTCCATAGACGAAGCCCAGTTTTGTGGCGATGAATTGAATGCTCAGTCACAG
GTTTACAAGGAAAAAATCCATGATCTGGAGTCAAAAATCACAAAACTGGTGTCCGC
CACGCCAAGCCTACAAAGTATACTACCGCCCGATCTACCTTCAGACGATGGTGCTTT
GCAGGAGGAAATCGCCAAGCTGCAGGAAAAGATGACCATTCAGCAGAAGGAGGTT
GAATCTCGGATTGCGGAACAGCTGGAGGAGGAGCAGCGGTTGAGGGAAAATGTAA

AGTACCTTAATGAGCAAATCGCCACTCTACAGTCCGAGTTGGTGTCCAAAGATGAGG

CCCTG(1629)tagGAATTC3

4.2.5 RNAi

The UAS/Gal system was used to express TRIP RNAi lines. Drivers used for the study are MTD-Gal4 (Dr. Lynn Cooley) and MatVP67 (Dr. Adam Martin). TRIP-RNAi lines were obtained from Bloomington: UAS-Sgg and UAS-Dia. Crosses were incubated at 27˚C.

4.3 Results

4.3.1 Microtubules preferentially track along actin cables

I assessed the localization of total microtubules in stage 10B and 11 egg chambers. A clear, continuous, and organized array of microtubules is present throughout the Nurse cells (Figure 24). When co-imaged with actin at the cortex, microtubules and actin appear to not co-localize (Figure 24 A, B, and B'). Statistical analysis still needs to be carried out to conclusively state that microtubules and actin are not overlapping. It seemed that wherever the microtubule bundles were positioned, less actin was observed when compared to actin in between the gaps of microtubule bundles (Figure 24B'). Because of the clear organized cage-like distribution of microtubules, I hypothesized that microtubules are affecting the organization of actin at the surface of the nurse cells. In the cytoplasm, the microtubule network is less dense and forms around the nucleus (Figure C and C'). Additionally, some microtubules seem to originate from the cortex and co-align with some actin cables (Figure C').

4.3.2 Stabilized microtubules preferentially track along actin cables

To assess the different populations of microtubules present, we used an antibody that recognizes highly acetylated-tubulin (stabilized microtubules). Stabilized microtubules are

present at the most surface section of the nurse cells (Figure 25A). In the cytoplasm, stabilized microtubules were observed at the cortex of the cells and as cables extending from the cortex where they track along actin cables (Figure 25B and C).

4.3.3 Disassembly of microtubules affects the formation of actin cables

A different population of actin cables extending from the ring canals are completely lost when treated with colchine (Nicolas *et al* 2009). Therefore, loss of microtubules affects the formation of actin cables directly. To test whether microtubules directly affect the formation of actin cables in the Nurse cells, I disrupted the microtubule network using two different microtubule destabilizing drugs in stage 10B egg chambers. I cultured (see methods) stage 10B-early egg chambers with the microtubule inhibitor colchicine and assessed the actin cables in fixed egg chambers. The nurse cells in non-treated stage 10B egg chambers have clear and ordered microtubules at the surface (Figure 26A). Egg chambers treated with colchicine exhibit a reduction of microtubule staining throughout the surface of the egg chamber (Figure 26B). The continuity, the length, and the number of the microtubules appear to be reduced especially between nurse cells (Figure 26B). Therefore, I concluded that colchicine treatment worked effectively to reduce the overall microtubule network. The overall phenotype of the egg chambers is not disrupted in 10B egg chambers and do progress to the final stage of oogenesis consistent with other studies (Nicolas *et al* 2009). This indicated that loss of microtubules did not affect the overall development of the egg chambers. It is possible that development is unaffected because all of the microtubules were not completely lost and the remaining stabilized microtubules are sufficient to allow development to occur without any visible problems. At the cellular level, the actin at the surface has lost its overall distribution (Figure 26A). In non-treated egg chambers, the actin is concentrated towards the middle of each individual nurse cell like a

cap and is less dense throughout the circumference of the cell (Figure 26A). In egg chambers treated with colchicine, the cap morphology is lost and actin becomes uniform throughout the nurse cell surface (Figure 26A). Within the cytoplasm of the nurse cells, the actin cables appear to be fewer in number, actin cables are shorter, vary in cable length, and appear to be thinner (Figure 26A). To confirm these results, I also treated egg chambers with another microtubule inhibitor drug called nocodazole. The same trend of phenotypes was observed in both drug treatments but were exacerbated in the nocodazole treatment (Figure 26B). The actin cables in the nocodazole treatment appeared fewer in number, shorter, and looked fragmented (26B and B'). The overall actin levels were also reduced at the cortex of the cell (Figure B' and C) and at actin cables (Figure B'). Additionally, all of the egg chambers that were treated with nocodazole did not progress beyond 10B stages. It is possible that these egg chambers were either arrested at this stage and potentially exhibit a dumpless phenotype.

4.3.4 CLIP-190 collaborates with Dia in actin assembly *in vitro*

+TIP microtubule binding proteins are present at the growing end of microtubules and regulate the dynamic mechanism of microtubule assembly and disassembly (Jiang *et al* 2011). It is becoming more apparent that +TIP microtubule proteins also regulate the functions of formins promoting microtubule-actin crosstalk (Martin *et al* 2015). In our work, we are interested in understanding the role of EB1 and CLIP-190 in actin assembly, because these proteins bind to our APC proteins and Dia (see Figure 6 for a summary of protein interactions).

The Goode Lab identified a conserved sequence present in yeast and animal formin regulators. The Formin Elongation Effector Domain (FEED) was identified in CLIP-170, the vertebrate homolog of CLIP-190 (Manuscript submitted by Goode Lab). Both CLIP-170 and

CLIP-190 are proteins that are highly converved (Figure 27A). They found that the FEED domain interacted with the FH2 domain of Dia and this interaction increased the rate of Dia-mediated actin elongation. We asked whether this mechanism was conserved in the fly. To test this mechanism *in vitro* using the fly proteins, I cloned a CLIP-190 fragment that contained the conserved FEED sequences (Figure 27A). GST expression, purification, and biochemical actin assembly assays were performed by our collaborators at Brandeis University (Dr. Jessica Henty-Ridilla). Fly Dia and CLIP-190 collaborate to overcome the inhibitory of profilin (Figure 27C). More actin filaments were observed in the CLIP-190 and Dia collaboration (Figure 27D) and the rate of elongation increased as well (Figure 27E). The explosive elongation mechanisms of Fly Dia and CLIP-190 and vertebrate Dia and CLIP-170 are conserved.

4.3.4 Localization of CLIP190

Now that we know that CLIP-190 and Dia collaborate in actin assembly *in vitro*, we proceeded to examine the role of CLIP-190 in actin assembly in the ovary. To address the function of CLIP-190 in actin cable formation, we first assessed the localization of CLIP-190. Previously, CLIP-190 was found to be enriched in the developing central nervous system and the pole cells in the Drosophila embryo (Lantz et al 1998). CLIP-190 was present throughout oogenesis. CLIP-190 is present at the cortex of all the cells in early stages and diffused throughout the cytoplasm (Figure 28A-B). At stage 8, the localization of CLIP-190 switches from the cytoplasm to the nucleus but it is very difficult to appreciate whether CLIP-190 is on or in the nucleus (Figure 28B-E). At stage 10B late, the localization of CLIP-190 switches back from the nucleus to the cytoplasm (Figure 28F). During later stages of oogenesis, CLIP-190 was at the cortex, lining the follicular cells, and in the cytoplasm. In the cytoplasm, CLIP-190

localized to places where both microtubules and actin filaments intersected (Figure 29A). In high magnification images, CLIP-190 decorates microtubules and actin filaments (Figure 29B and B'). Microtubule staining was not always consistent in my experiments and therefore, I tried several different methods to localize microtubules and microtubule binding proteins. In a protocol optimized to stain cytoskeletal proteins in the ovary, microtubule staining appeared to be clear and consistent in each sample preparation. CLIP-190 staining is observed robustly in the cytoplasm, where it seems to be localize to the ends of microtubules in stage 11 egg chambers (Protocol from Edwards Lab; Figure 29C). CLIP-190 also associated with microtubules, actin filaments, and the cortex. It was unclear whether CLIP-190 associated to the peri-nuclear membrane actin mesh. This would be interesting to know because it is possible that the actin cables are binding to the nucleus through CLIP-190. In future experiments, we will use CLIP190 loss of function alleles to test the role of CLIP190 on actin cables formation in late oogenesis described in Beaven *et al* 2015.

4.3.5 EB1 inhibits APC1 and APC1-Dia mediated actin assembly *in vitro*

The levels of actin appeared lower in nocodazole or colchicine treated Nurse cells, suggesting that loss of microtubules is inhibiting actin assembly at the cortex. In contrast, in the cytoplasm, acetylated microtubules track along the actin cables and are most likely required to positively influence the formation of the actin cables based on our drug treatments. But it is unclear how microtubules restrict actin assembly at the cortex or facilitate actin assembly in the cytoplasm. Our collaborators at Brandeis University are exploring the role of microtubule dynamics on the actin cytoskeleton and have found that the +TIP microtubule binding protein,

EB1, inhibits actin assembly (unpublished). Therefore, it is possible that microtubule binding proteins bind to the actin machinery, and binding acts as a switch to enable or disable actin assembly spatially and temporally. For example, EB1-APC1-Dia are proteins required for stabilizing microtubules (Wen *et al* 2004) but if EB1 is not present in the complex, microtubule stabilization is inhibited and actin APC1-Dia mediated actin assembly resumes.

To test if the fly EB1 also inhibits actin assembly, I expressed and purified a MBP-EB1 full-length protein. Our collaborators observed that the fly EB1 completely inhibited the actin assembly activity of APC1 as effectively as vertebrate EB1 (Figure 30A) through biochemical pyrene *in vitro* actin assembly assays. Not only does EB1 inhibit APC1-mediated actin assembly, it also inhibited the collaboration between APC1 and Dia (Figure 30B). EB1 binds directly to the basic domain of APC1 and to the C-terminus of Dia and potentially disrupts APC1-Dia binding inhibiting their collaboration. Preliminary data suggest that the C-terminus of EB1 is required to inhibit the activity of APC1-basic and the collaboration between Dia-C and APC1-basic (summary of these experiments in Figure 30C). The interaction between EB1-Dia-APC1 needs to be further dissected *in vitro* so that we can better understand the mechanism of inhibition of EB1 to APC1 and APC-Dia mediated actin assembly.

4.3.6 EB1 knockdown results in early activation and disorganized actin filaments

EB1-KD promotes early activation of actin assembly. In normal development, formation of the cables in 10B egg chambers follows a gradient where the actin cables start forming at the Nurse cell/oocyte division during 10B mid stages. For simplicity, I am only showing the Nurse cell/oocyte border because the phenotype is very clear in the early stages. In Figure 31A, long abnormal actin cables are formed from the Nurse cell oocyte border. In this image, it is hard to

tell whether these cables are coming from the ring canal or the Nurse cell border. Nonetheless, these cables are long and non-existent in WT 10A egg chambers. In a 10B early egg chamber, the actin cables at the Nurse cell oocyte have not been made (Figure 31B. Full and long actin cables were observed in the EB1-KD Nurse cells that are adjacent to the oocyte. Actin assembly is coordinated and for the most part, actin cables grow at the same rate (Huelsman et al 2013). In later stages, longer isolated cables are observed (Figure 32A). In addition, actin cables in EB1-KD Nurse cells are very disorganized (Figure 32B and D). Lastly, the actin cortex in EB1-KD cells is also disrupted. At the cortex in normal cells, the cortex is smooth. In EB1-KD cells, cortical actin exhibits patches of bright spots (Figure 32C). In a close-up, these actin cables are disorganized bundles of actin cables that are normally not present in WT Nurse cells (Figure 32C'). Therefore, EB1 is required for the temporal and spatial organization of actin filaments in stage 10B egg chambers. Although, it is unclear where EB1 is present in the Nurse cells and whether the actin phenotypes we are observing are due to defects in microtubules or direct EB1 effect on actin assembly.

4.4 Discussion

4.1.1 Microtubules and actin organization at the cortex of the Nurse cells

Our work demonstrated that the 10B and later staged egg chambers in the Drosophila ovary provide a great model system to continue to dissect the mechanistic details of microtubule-actin interactions. We showed that there are distinct subcellular localizations for actin, microtubules, and stabilized microtubules. At the cortex of the cell, a highly dense microtubule network is present and is made up of stable and non-stable microtubules. Beneath the

microtubules, a layer of cortical actin was observed. A cap of actin forms centrally in the Nurse cell and the cortical intensity of this actin is decreased as it reaches the edges of the cell. When overlayed, the actin and microtubules do not overlap with one another. Instead, the microtubules are observed as highly packed tubules and the actin intensity is enhanced between the spaces where no microtubules are present. The non-overlapping organization of the microtubules and actin filaments seems as though the microtubules are restricting the polymerization of actin or *vice versa*. Treatment of colchicine did affect the distribution of actin at the surface suggesting that microtubules are required for the precise spatial polymerization of actin (Figure 26). The cap disappears and actin distribution becomes uniform throughout the surface of the cell and the overall actin levels decreased. Additionally, when treated with nocodazole, the actin levels decreased dramatically at the cortex. Therefore, it seems that microtubules are regulating localized actin polymerization at the cortex. Strong evidence suggests that the microtubule and actin filaments influence the polymerization of each other (reviewed in Waterman-Storer 1999 and Goode 2000). In addition, *in vitro*, the Goode Lab demonstrated that the vertebrate CLIP-170 and Dia collaborate in actin assembly *in vitro*. In addition to this, we know that APC1 and Dia (not shown) are localized to the surface of the Nurse cells and may function alone or together to assemble actin filaments. CLIP-190 was also localized ubiquitously to the surface of the Nurse cell. In the cytoplasm, CLIP-190 localized robustly to the interface of actin and microtubules interactions, and at the tips of microtubules ends. Our work *in vitro* demonstrated that CLIP-190 enhances Dia- mediated actin assembly. We understand a great deal about the role of the role of proteins in actin assembly, but we do not know how the actin machinery is turned off when it is not needed. Although, we don't know if EB1 is localized to the nurse cell cortex, we do know that it inhibits APC1 and APC1-Dia mediated actin assembly *in vitro*. Because, EB1

is present on microtubules, we predict that EB1 is inhibiting actin polymerization by binding to Dia and APC1 at the sites of actin cable formation. This results in a switch from APC1-Dia mediated actin assembly to stabilizing microtubules through EB1-Dia-APC1 interactions to assemble the network of stabilized microtubules. Currently, in our system, loss of EB1 aberrantly activates actin cable formation in 10B early egg chambers (Figure 31A and 31B)

4.4.2 Microtubules and actin organization in the cytoplasm of the Nurse cells

A small fraction of microtubules in the cytoplasm are present at the cortex of the Nurse cells and align with the actin cables of the actin cables (Figure 25A and C). In addition, the microtubules are present in the cytoplasm where they intersect with microtubules and actin cables that are extending from the cortex. When stained with the acetylated-microtubule antibody, we identified stabilized microtubules highly localized near the cortex of the cell aligning with microtubules and actin filaments (Figure 25C).

Microtubules are hollow helical lattices that are about 20-25nm in diameter and for the most part are stiff and resist bending forces (reviewed in Desai *et al* 1997). On the other hand, actin filaments appear as double-stranded helices of about 7nm in diameter that are flexible, relatively strong and can resist some forces (reviewed in Dominguez *et al* 2011). Based on these differences, it is possible that the parallel and intersecting microtubules are forming strong matrices that provide some stability and direction to the growing actin cables in the cytoplasm. Therefore, it is possible that the stable microtubules coming from the cortex of the cell form along with the actin to give the actin cables direction or stability. We currently do not know if these cables form before the actin cables, synchronously, or after the actin cables. We can continue to understand how these microtubules are forming using live imaging to further

understand the function of microtubules in actin cable formation. The literature in microtubule-actin crosstalk strongly suggests that each network influences the polymerization of the other.

Microtubule associated binding proteins such as Tau and MAP4 bind to microtubules to stabilize them (Drubin *et al* 1986, and Murofushi *et al* 1986). In addition to stabilizing microtubules, these MAPs are also required for microtubule assembly and bundling (Drubin *et al* 1986) *in vitro*. Not only do they affect the assembly of microtubules, but they can also assemble and bundle actin filaments. Therefore, it is possible that instead of providing the actin cables with rigidity and support, the stabilized microtubules are delivering MAPs to the sites of actin cable assembly in order to influence the growth of the cables. For example, the microtubules present at the cortex potentially generate specific sites for actin assembly. Our nocodazole and colchicine experiments seem to support this second hypothesis more. Microtubule depolymerization led to defects in actin cable formation where the cables were shorter and less in number but not completely absent. These results could be interpreted in two different ways. Acetylated microtubules are resistant to colchicine and nocodazole treatment so it is possible that the drug treatment did not completely depolymerize all the microtubules, specifically those adjacent actin cables. Therefore, residual activity of MAPs present on those non-depolymerized cables are continuing to help form few actin cables were present after drug treatment. The other alternative is that all the actin cables were not completely lost because other actin binding proteins such as Dia, Ena, and Chickadee are still present. Dia, Ena, and Chickadee are still working to assemble actin filaments in a microtubule independent mechanism. To distinguish between these two alternatives, we can cold treat our egg chambers and assess loss of microtubules and actin cables. Acetylated microtubules are resistant to colchicine and nocodazole. However, acetylated microtubules are not resistant to low temperatures such as 4˚C

and will favor disassembly (Piperno 1987). Therefore, with cold treatment we would hypothesis that all the microtubules including stabilized microtubules would be completely depolymerized and no residual activity of MAPs would remain. In addition to complete loss of actin cables, we would also observe dumping defects. In order to cold treat our cells, we would first have to incubate our egg chamber in the cold and see if development occurs. Another alternative would be to stabilize actin cables with taxol and see if this increases actin cable formation. If all the microtubules and actin cables disappeared, then that would mean that microtubules and MAPs are required and essential for cable growth, morphology, and function. Although, highly unlikely because we showed that Dia, a direct assembly factor, is required for actin assembly in the Nurse cells. Additionally, one question that we have not answered is whether CLIP-190 is required for actin assembly. CLIP-190 is the only Drosophila homolog making it easier to understand its requirement because we won't have to worry about redundancy. I also predict that Dia is required for elongation in the ovary. Complete loss of either APC1 and APC2 induces a developmental delay in actin cable extension but is rescued in stage 11. Therefore, other factors required in nucleation and elongation are present and CLIP-190 seems like a good candidate to test in actin cable formation. In addition to finding ways to completely disrupt microtubules, I also think it would be interesting to know if actin is required for microtubule assembly. Direct inhibition actin through cytochalasin inhibits microtubules assembly abolishing axon branching (Dent et al 2001). Therefore, if we treat the egg chambers with cytochalasin, does treatment also disrupt the microtubules in the Drosophila egg chamber.

4.4.3 Microtubule binding proteins directly regulating actin assembly

In Chapter 3, we showed that APC1 is required for actin assembly in staged 10B egg chambers but it does not result in a complete loss of actin cables but a delay. We predict that loss

of APC1 interferes with actin assembly directly but we have not tested this yet. We first need to identify the actin nucleating sequences within the basic domain and introduce deletions of the ANS in the fly. Therefore, it is possible that many other microtubule binding proteins are providing some redundancy to the system and maybe all MAPs work together to form the cytoplasmic actin cables. A group of proteins that are associated with microtubules are the plus tip microtubule proteins that regulate the assembly or disassembly of microtubule at the dynamic plus ends of microtubules (Jiang *et al* 2011). In our study, we focused on the role of two microtubule plus tip binding proteins: EB1 and CLIP190.

4.4.4 EB1 inhibits actin assembly *in vitro*

EB1 is a microtubule +TIP protein that tracks on microtubules and recruits APC1 and CLIP170/CLIP190 to microtubule ends (Su *et al* 1999 and Dixit *et al* 2009). Additionally, APC1 and Dia bind to EB1 to stabilize microtubules (Wen et al 2004). *In vitro*, EB1 alone inhibited APC1 mediated actin assembly. This was not surprising because the C-term of EB1 binds to the basic domain of APC1 required for actin assembly (unpublished). It is possible that amino acids required for EB1 binding are the same that are required for APC1-basic actin assembly. The basic domain can potentially bind to actin or EB1 but not both at the same time. Alternatively, the amino acids required for actin assembly in the basic domain do not overlap with the amino acids required for EB1 binding. Though, EB1 is larger than actin monomers and when EB1 binds to the basic domain it creates some steric hindrance inhibiting actin association. Also, using single molecule TIRF microscopy experiments can help to understand the inhibition mechanism of EB1 on APC-mediated actin assembly. Not only does EB1 inhibit APC1-basic actin assembly, but it also inhibits the collaboration of APC1-basic and Dia-C. The N-terminus of EB1 binds to Dia-C but we have not tested the effect of EB1 on Dia mediated actin assembly. Ideally, we

should be able to perform single molecule TIRF microscopy to further dissect the APC1-Dia-EB1 interaction on actin assembly.

4.4.5 Loss of EB1 in the Nurse cells: Early activation of actin cables and loss of microtubule organization

Our *in vivo* work demonstrates that loss of EB1 results in cortical instability because the oocyte nurse cell border protrudes into the oocyte. More striking, we observed the ectopic actin in early and late 10B stages. In later staged egg chambers, loss of cable symmetry, cortical actin organization, and actin cable organization is disrupted. Loss of actin cable symmetry can be explained by some cables forming earlier than others but it is not clear if these defects are induced by loss of proper nucleation of elongation. Loss of organization is a little more difficult to explain. I believe that the microtubule network. helps organize the actin filaments in the cytoplasm and without them, organization of actin cables may be lost. It is possible that loss of EB1 is disrupting the microtubule networks and this in turn affects the organization of cortical actin and cytoplasmic actin cables. To test this hypothesis, we will assess the microtubule networks in EB1 dsRNAi Nurse cells by immunofluorescence.

4.4.6 CLIP190 is an effector of Dia in actin assembly

CLIP190 is another microtubule +TIP binding protein that is recruited to the plus end microtubules by EB1 (Su *et al* 1995) and is a cytoplasmic linker that binds to microtubules and actin in several contexts (Lewkowicz *et al* 2008). In the context of Drosophila neurons, CLIP190 is not a dominant microtubule tracking protein but is required for assembling small linear actin dependent patches in growth cones (Beaven *et al* 2015). One of the caveats of their study was

looking only at collaboration between + TIP proteins but not the collaboration of + TIP proteins with other proteins.

In the ovary, CLIP190 localizes in the egg chambers as early as stage 2 where it outlines all the cells. This localization pattern remains localized to the lining of the cell and transitions to being localized to the nucleus in 10A. At late stage 10B, nuclear CLIP190 transitions from localizing to the nucleus to the cytoplasm. CLIP190 enrichment on sites of microtubules and actin intersections is also observed. Currently, we don't know what to make of the developmental distribution of CLIP-190, but it is intriguing. Evidence suggests that CLIP-190 localization to microtubules is cell cycle dependent (Lantz *et al* 1998). In my model system, the Nurse cells in the egg chambers are not dividing, therefore it is not possible that the change in localization of CLIP-190 is being regulated by the cell cycle. We know that CLIP190 is present on these actin cables during oogenesis but we don't know if CLIP190 is functionally capable of helping form these actin cables. To test the function of CLIP190 in actin assembly, we will be using CLIP190 alleles (Beaven *et al* 2015) alone and together with Dia to begin to dissect the role of CLIP190-Dia mediated actin assembly.

Our work in Chapter 4 assessed the cross-talk between microtubules and actin in actin cable formation in Drosophila late oogenesis. We found that stable microtubules are positioned adjacent to cytoplasmic actin cables and that microtubules are required for the proper cable growth and morphology of actin cables. We predict that actin cable formation is modulated through the interaction of microtubule binding proteins with the actin assembly machinery. CLIP190 enhances actin assembly *in vitro* and is localized to the sites of actin and microtubules in the cytoplasm of the Ncs. EB1, on the other hand, inhibits APC1-mediated actin assembly and the APC1-Dia collaboration *in vitro*. Additionally, EB1 plays a role in the in temporal and spatial

organization of actin in Drosophila egg chambers. Currently, a limitation we face in our investigation is that we are using fixed tissue to test the role of microtubules and microtubule binding proteins on actin assembly. Currently, we are trying to set up a system where we can image microtubules and actin live in the egg chambers so that we can better understand the affect of the microtubules on actin assembly. To understand actin-microtubule crosstalk, we need to answer other questions: 1) Are stabilized microtubules in the cytoplasm formed before actin cables?, 2) Do cables form faster or slower when stabilized microtubules are not present at the sites of actin cable?, 3) How exactly are microtubules being oriented by using fluorescent-tagged +TIP?, 4) Many proteins such as APC1 and APC2 are delivered through microtubules to sites of actin assembly, is this also true in our system? Taken altogether, our work demonstrated that the APC-Dia actin machinery is regulated through the interaction of microtubule +TIP proteins to control the size, morphology, and length of actin cables in Drosophila late oogenesis.

4.5 Figures

Figure Legends

Figure 24: Microtubules are present at the cortex Nurse cells and in the cytoplasm in stage 10B late egg chambers

A)Cross section of the most outer Nurse cell. (Left to right: actin/microtubules/merge) Actin is highly concentrated in the middle of the Nurse Cells (white arrow).

B) Surface cortical section of a Nurse cell. (Left to right: actin/microtubules/merge) Actin staining is enhanced in the middle of the cell. Microtubules form a dense mesh of parallel tubules. Yellow box is section magnified in B'.

B') High magnification of B. (Left to right: actin/microtubules/merge) Actin seems to be distributed as small patches throughout the surface of the cortex where bundles of microtubules are absent (white arrow).

C) Cytoplasmic view of actin cables (top) are arranged as an array of cables extending from the cortex forming a cables. Microtubules (bottom) are present in the cytoplasm as network of tubules that either localized in the cytoplasm or align with actin filaments. Orange box is magnified in C'.

C') High magnification of C. Parallel microtubules from the cortex run parallel to actin cables in some cases (white arrows). Microtubules intersect with actin cables (yellow arrow).

*Scale bar= 10μM

Figure 25: Acetylated microtubules are present at the cortex of the Nurse cells and run parallel to the actin cables in Stage 10B-Late egg chambers

A) Nurse cell (Nc) cortical section: (Top) Acetylated microtubules (red) are positioned as densely packed parallel microtubule cables.(Middle) Some non-acetylated microtubules (green) are present at the cortex but it seems that the fraction of acetylated microtubules seems to be higher (Bottom). Actin cortical view (Blue). (merge) Microtubules and acetylated microtubules sometimes co-localize together but in many cases these two networks do not overlap strongly suggesting that two populations of microtubules at the surface are present.

B) Nc cytoplasmic section: (Top) Acetylated microtubules (red) are selectively extending from to the cortex of the cell. (Middle) Total microtubules (green) are extending from the cortex aligning with actin cables but for the most part, microtubular are un-organized in the cytoplasm. (Bottom) Actin cable (blue) cables are present.

C) High magnification of stage 10B-Late egg chamber. Acetylated microtubules (green) track along actin cables (blue) noted by the white arrows while non-acetylated microtubules are intersecting the cytoplasmic actin cables noted by the yellow arrow.

*Scale bar= 10μm

Figure 26: Microtubules are required for proper actin cable length and morphology in stage 10B egg chambers

A) Colchicine treatment:(Top panel) Cortical Section View of microtubules, cortical actin, and cytoplasmic actin cables in cells treated with our water. (Bottom panel) Cortical section view of microtubules, cortical actin, and cytoplasmic cables of cells treated for 3 hours with 70ug/mL of

colchicine. Blue arrows denote the actin cables which are fewer in number, smaller, and are not as thick as normal cables.

B) Nocodazole treatment: (Top panel) Cortical section view of nurse cells (25x magnification, cortical actin, and cytoplasmic actin cables in cells treated with DMSO. All imaging and post acquisition processing parameters were identical. (Bottom panel) Cortical section view of the nurse cells (25x magnification), cortspeical actin, and cytoplasmic cables of cells treated for 3 hours with 2.5ug/mL of colchicine. Blue arrows denote the actin cables which are fewer in number, smaller, and are not as thick as normal cables. The overall actin levels at the cortex and cytoplasm is reduced.

B') High magnification of B. (Top panel) Cortical actin in DMSO and is reduced significantly in the nocodazole treatment. (Bottom panel) Cytoplasmic actin cables in DMSO extend from the cortex of the cell towards the cytoplasmic. The actin cables in the nocodazole treatment are fewer, shorter, not as continuous, and less actin dense than non-treated cables.

C) The pixel intensity throughout the length of the lines in B' were plotted. Reduced actin levels was observed in nocodazole treated Nc.

*Scale bars= 10μm

Figure 27: Fly CLIP-190 collaborate with Dia in elongation in actin assembly in biochemical actin *in vitro* polymerization

A) Top Panel: The vertebrate CLIP170 is divided into three different domains. The N-terminal contains the CAP-Gly domain, the middle of the protein contains a large coiled-coil domain and the Formin Elongation Effector Domain (FEED), and the C-terminal contains two different

motifs. CLIP170 and the Drosophila CLIP-190 are proteins that are highly conserved (Schematic Adapted from Lantz et al). Lower panel: GST-CLIP 190 protein construct.

B) Comparison of vertebrate Hs-CLIP-170 and fly Dm-CLIP-190 protein fragments in actin assembly using bulk-pyrene assembly actin assays. Fly CLIP-190 has a higher actin assembly activity when compared to vertebrate CLIP-170.

C) Fly CLIP-190 enhances Dia-dependent actin assembly viewed by labeled actin filaments in TIRF assays. The average number of actin filaments per field of view are plotted.

D) Elongation rates of individual actin filaments were tested using single molecule TIRF microscopy. Ultrafast elongation is observed when Dia, CLIP-190, and profiling are present.

*For these experiments, I cloned the CLIP-190 protein fragment with a GST tag. In addition, I expressed and purified Dia-C.

*Collaborators at Brandeis University: Dr. Jessica Henty-Ridilla expressed, purified, and test the vertebrate and fly CLIP protein fragments.

Figure 28: CLIP-190 is localized to the actin cortex, nucleus, diffused throughout the cytoplasm

A) Stage 4-7 egg chambers (40X magnification) and B) Stage 8 egg chamber demonstrate that CLIP-190 is localized to the actin cortex and is diffuse throughout the cytoplasm.

C) Stage 10A egg chamber, D) Stage 10B early, and E) Stage 10B mid (red=microtubules/green=CLIP190/blue=actin) shows that CLIP-190 is localized to the actin rich cortex but its localization pattern changed dramatically to include a large fraction of CLIP190 protein is associated with the nucleus.

F) In Stage 10B late egg chambers, CLIP-190 is localized to the actin cortex, goes back to being diffused throughout the cytoplasm and observed as puncta on MT-actin interactions. (green=microtubules/red=CLIP190/blue=actin)

*Scale Bars=10μM

Figure 29: CLIP190 associates at the intersection and tips of microtubule-actin intersections

A) CLIP190 associates at the intersection of microtubules and actin filaments in some cases in late staged egg chambers when actin cables are formed. (orange arrows). Using the first fixation method described in the materials section.

B) CLIP-190 (purple) associates on actin (cyan) and microtubules (green) in a punctate manner.

B') Higher magnification of the association of CLIP-190 on actin and microtubules.

C) CLIP-190 localization using a method for specifically looking at cytoskeletal proteins and membrane bound associated proteins in the ovary from (Kevin Edwards). CLIP -190 (red) is staining is more robust and instead of observing small puncta we see bigger puncta associated with the at the microtubule(green)-actin(blue) intersections (white arrow).

C') High magnification of C. Also, the association of CLIP-190 increases at the tips of actin cables and microtubules (orange arrow).

*Scale bar=10uM

Figure 30: EB1-Full length(FL) inhibits the APC1-basic and APC1-basic+Dia mediated actin assembly *in vitro*

A) EB1-FL inhibits the actin assembly activity of APC1-basic mediated by pyrene actin assembly assays.

B) APC1-basic and Dia-C together bind to overcome the inhibitory effect of profilin and capping proteins. This collaboration is inhibited when EB1-FL is added.

C) Table summarizing the effect of EB1 protein fragments in actin assembly tested by *in vitro* pyrene actin assembly assays.

* I expressed and purified all the MBP-EB1, APC1-B, and Dia-C fragments. The *in vitro* assays were carried our by our collaborators at Brandeis University done by Dr. Richa Jaiswal.

Figure 31: dsRNA knockdown EB1 induces early assembly of actin cables in 10A and 10B staged egg chambers

A) Stage 10A: Long actin cables are observed in the cytoplasm in EB1-KD (Right).

D) Stage 10B early: At this stage no actin cables should be observed at the nurse cell- oocyte division WT (left). In EB1-KD (right), lots of pre-mature cables are observed at the nurse cell-oocyte division.

* yellow arrow= premature actin cables

*yellow dotted line separating the Nurse cells (Nc) from the oocyte (o)

*Left Panels= WT

*Right Panel=EB1-KD

*Scale Bars=10μM

Figure 32: EB1 is required in providing actin cable symmetry and actin organization in 10Bmid-10Blate egg chambers

A and B) Stage 10B mid: EB1-KD induces loss of actin cable symmetry (red arrow) and actin cable organization (green arrow).

C and C') Stage 10B late: The cortical actin is not smooth in EB1-KD egg chambers and resolved actin filaments are observed at the surface (purple star).

D) Stage 11: In EB1-KD mutants, the actin cable organization is disrupted. For the most part, actin cables are formed parallel to each other but have lost this organization in EB1-KD chambers (green arrow).

*Left Panels= WT

*Right Panel=EB1-KD

*Scale Bars=10μM

Figure 33: Microtubules promote proper actin cable formation through EB1 and CLIP190 which potentially act in concert with actin cable machinery

Dia, APC1, APC2, and Ena all are localized at the actin rich cortex at the sites of actin cables formation. Proteins collaborate through unknown mechanisms to make short actin cables that are bundled as the cables grow towards the nucleus. The plus ends are at the cortex and generate an indentation at the cortex where actin assembly is occurring (the tip complex is localized to these indentations (Ena)). Stabilized tubules run parallel to the actin cables and microtubules associated protein such as CLIP-190 and EB1 can either enhance or inhibit actin assembly, respectively.

Figure 24

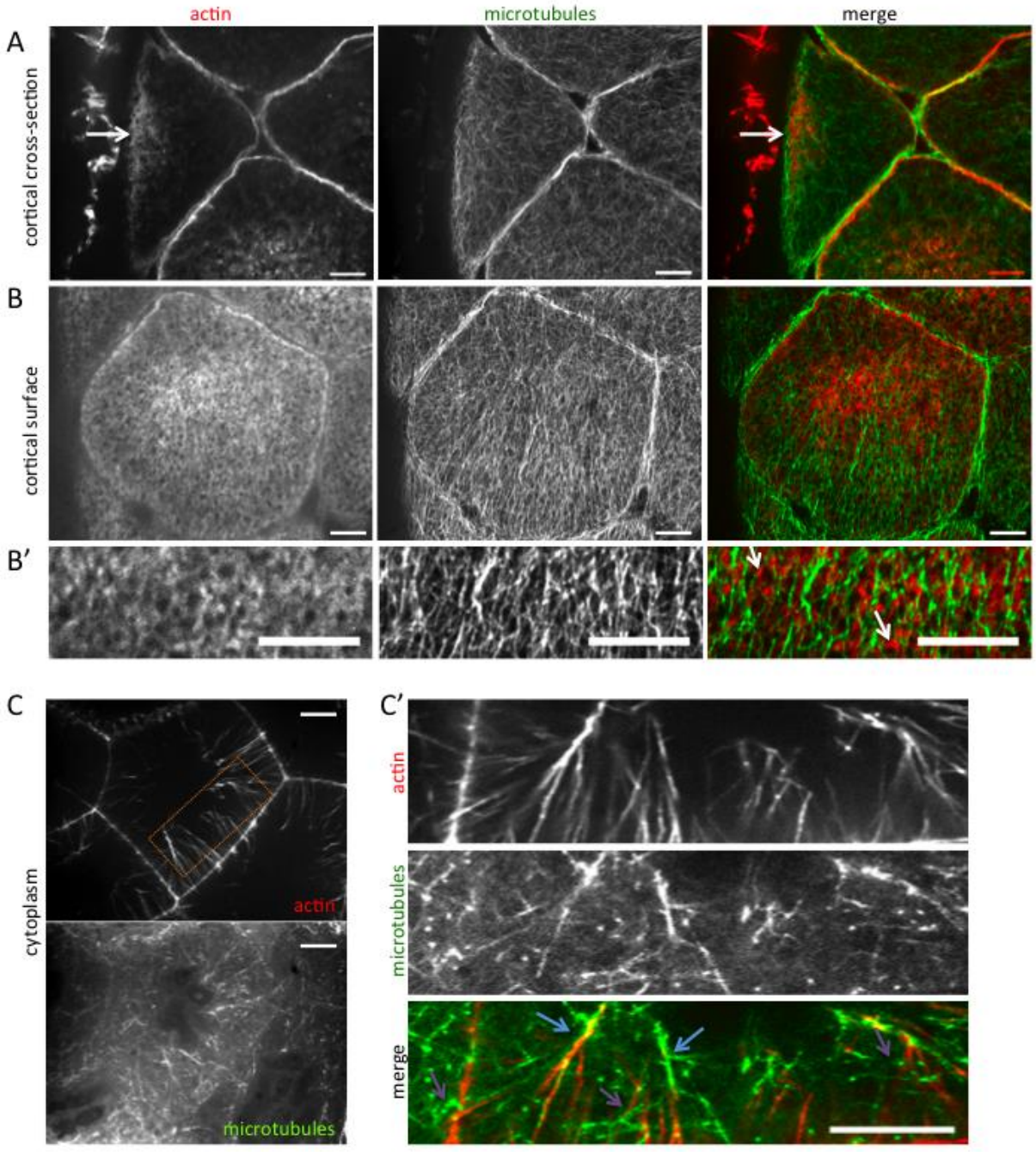

actin
microtubules
merge
A
cortical cross-section
B
cortical surface
B'
C
cytoplasm
actin
microtubules
C'
actin
microtubules
merge

Figure 25

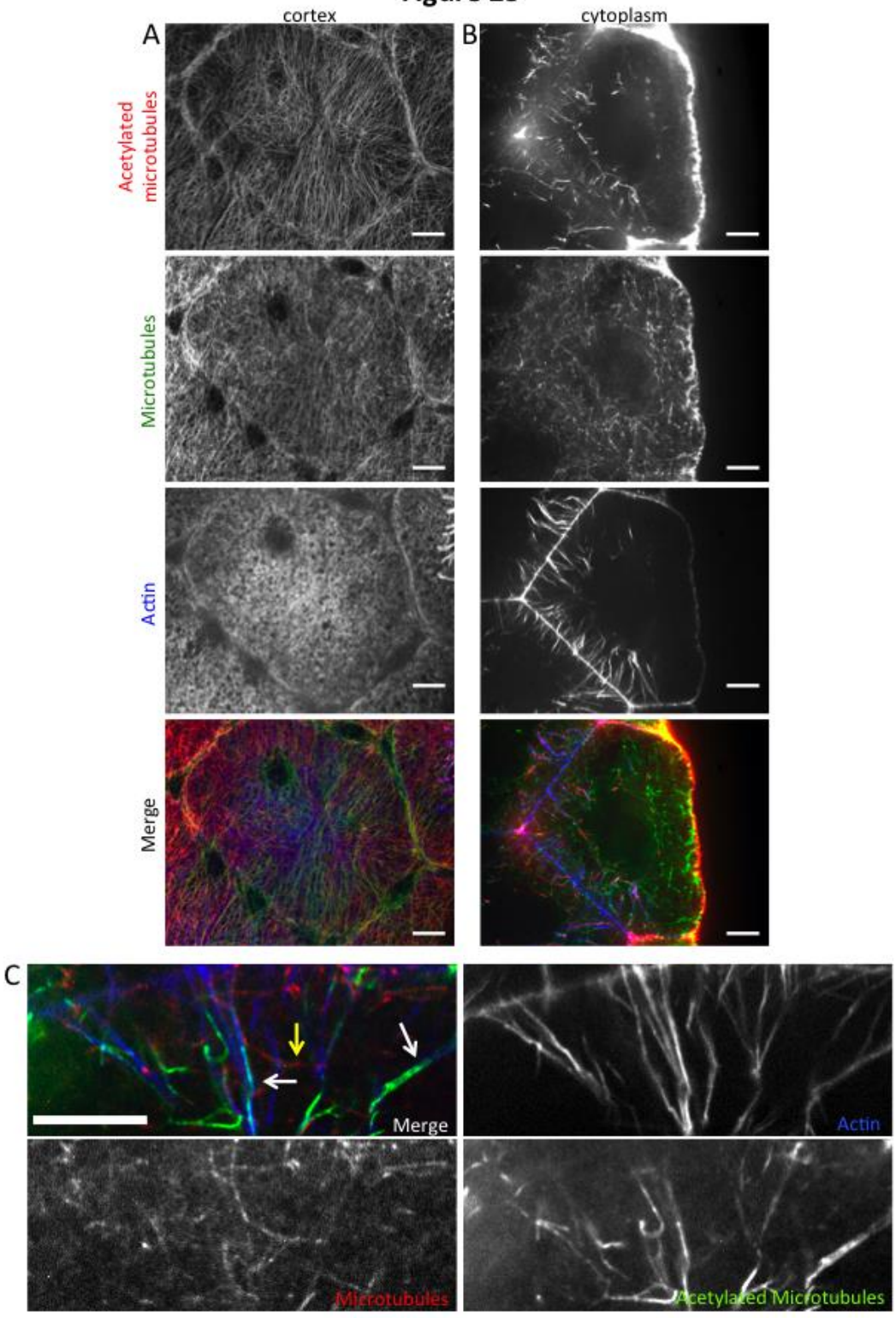

cortex
cytoplasm
A
B
Acetylated microtubules
Microtubules
Actin
Merge
C
Merge
Actin
Microtubules
Acetylated Microtubules

Figure 26

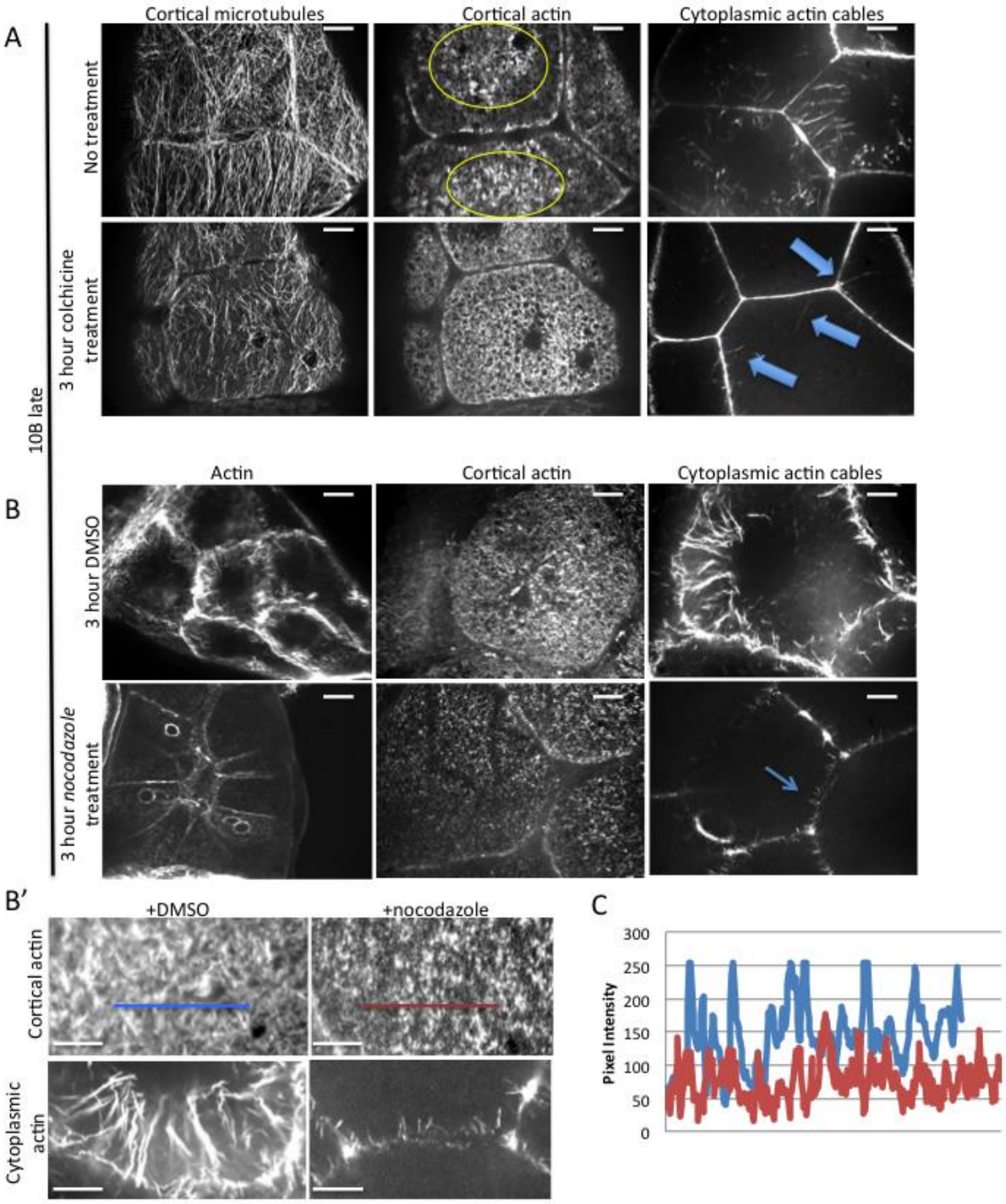

A
Cortical microtubules
Cortical actin
Cytoplasmic actin cables
No treatment
3 hour colchicine treatment
10B late
B
Actin
Cortical actin
Cytoplasmic actin cables
3 hour DMSO
3 hour nocodazole treatment
B'
+DMSO
+nocodazole
Cortical actin
Cytoplasmic actin
C
Pixel Intensity
300
250
200
150
100
50
0

Figure 27

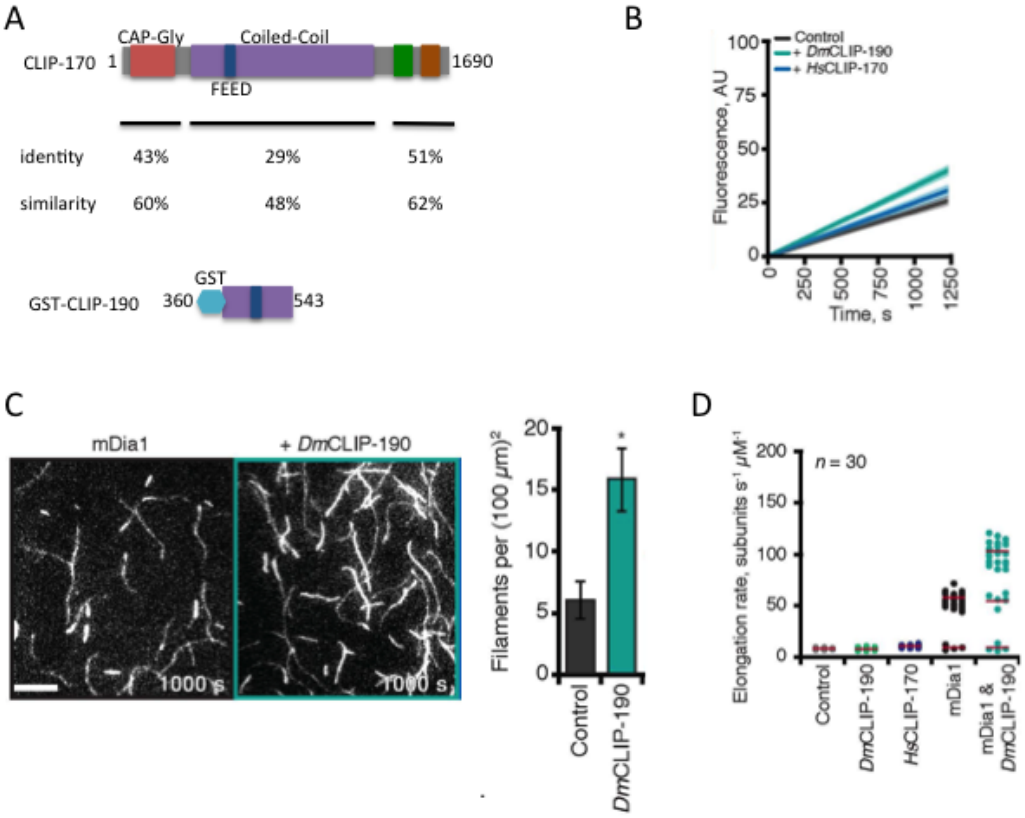

A
CAP-Gly
Coiled-Coil
CLIP-170 1
1690
FEED
identity 43% 29% 51%
similarity 60% 48% 62%
GST
GST-CLIP-190 360 543
B
Control
+ DmCLIP-190
+ HsCLIP-170
Fluorescence, AU
Time, s
C
mDia1
+ DmCLIP-190
1000 s
Filaments per (100 μm)²
Control
DmCLIP-190
D
n = 30
Elongation rate, subunits s⁻¹ μM⁻¹
Control
DmCLIP-190
HsCLIP-170
mDia1
mDia1 & DmCLIP-190

Figure 28

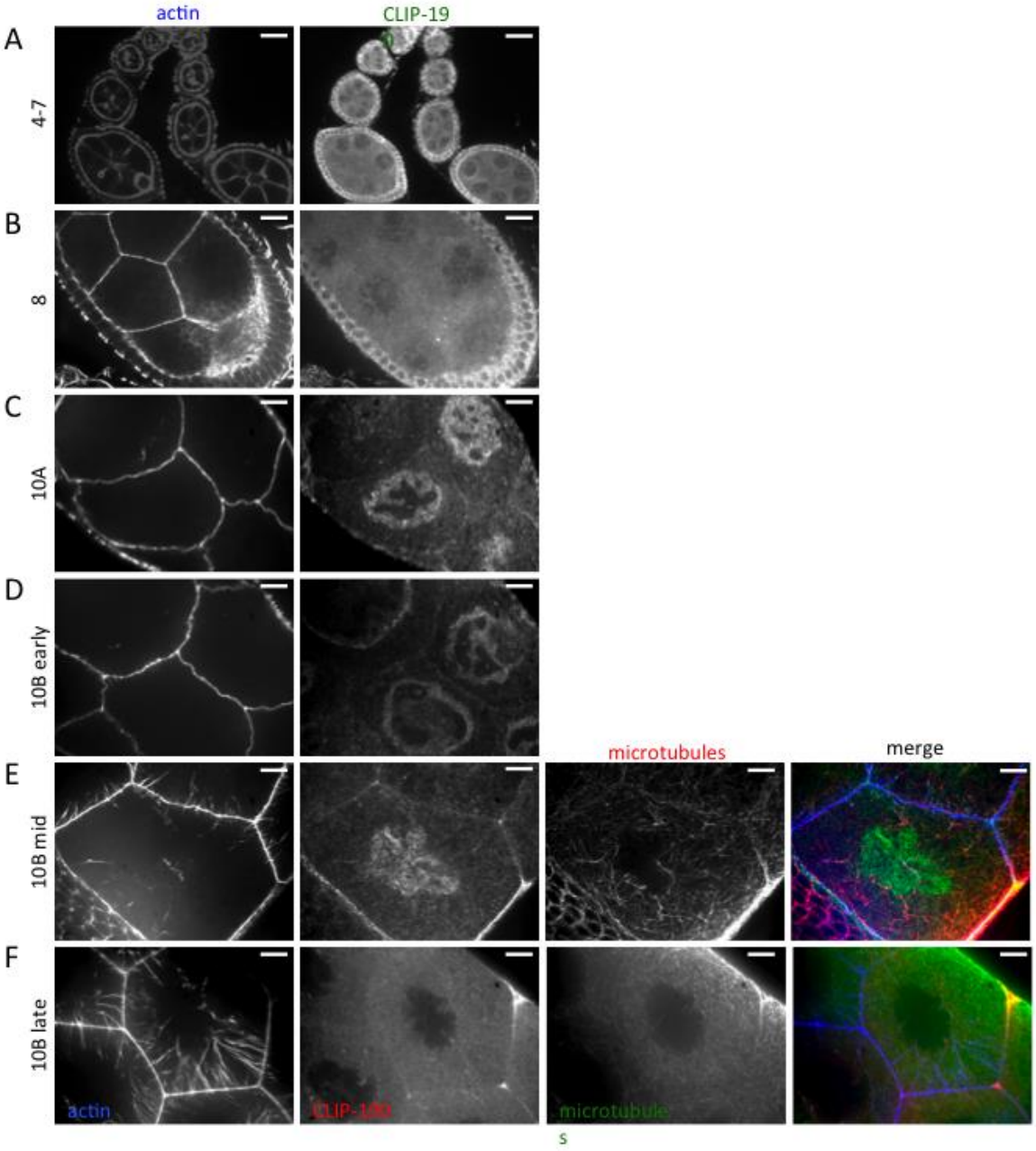

actin
CLIP-19
A
4-7
B
8
C
10A
D
10B early
microtubules
merge
E
10B mid
F
10B late
actin
microtubule
s

Figure 29

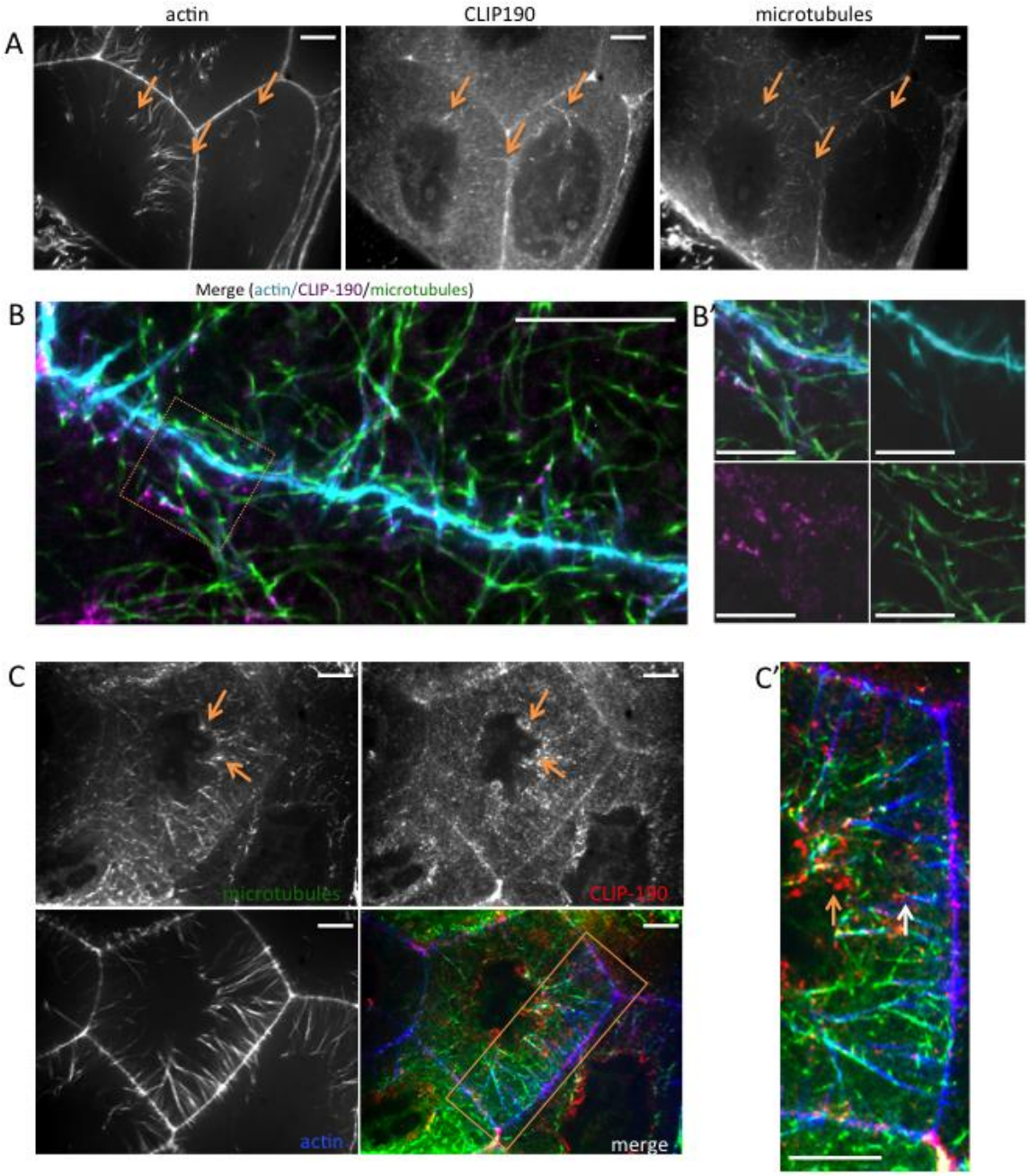
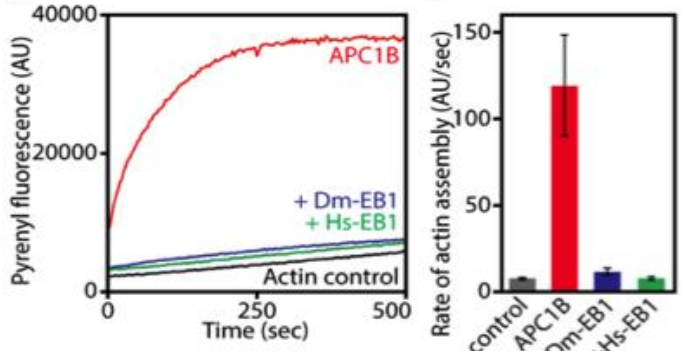
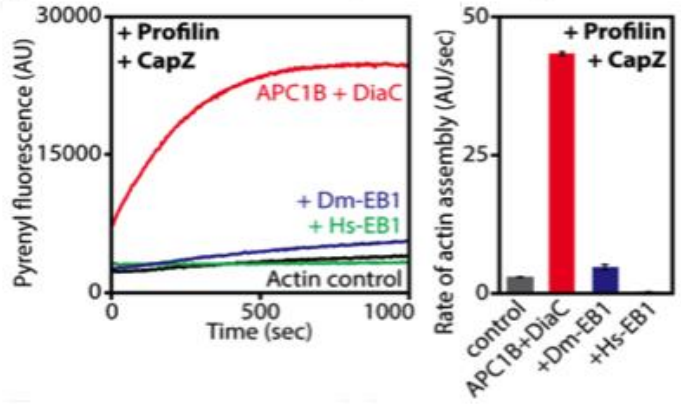

actin
CLIP190
microtubules
A
Merge (actin/CLIP-190/microtubules)
B
B'
C
C'
microtubules
CLIP-190
actin
merge

Figure 30

A



B



C

		Effect of EB1 on actin assembly		
MBP-tagged fragment	EB1 fragment bind to:	APC1-B	Dia-C	APC1-B + Dia-C
EB1-FL	APC1-B Dia-C	Inhibits	No Inhibition	Inhibits
EB1-N	Dia-C	No Inhibition	No Inhibition	N.A.
EB1-C	APC1-B	Inhibits	No Inhibition	N.A.

Figure 31

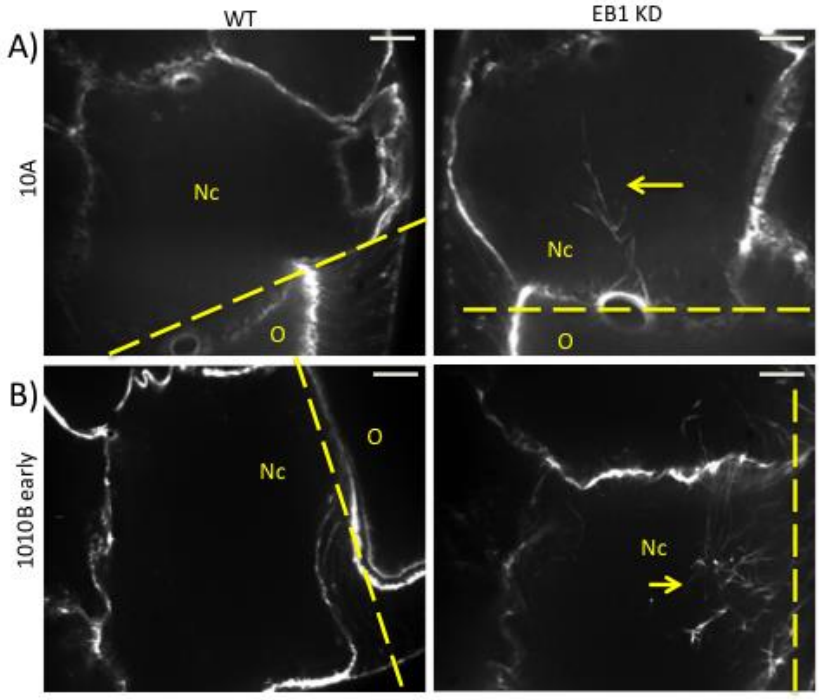


Figure 32

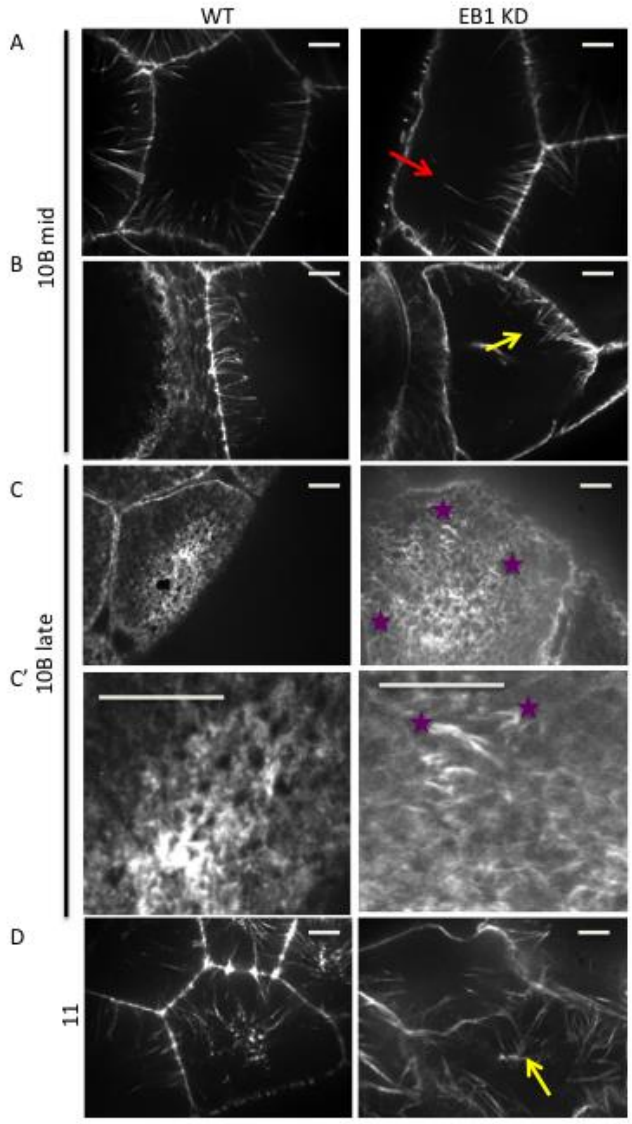
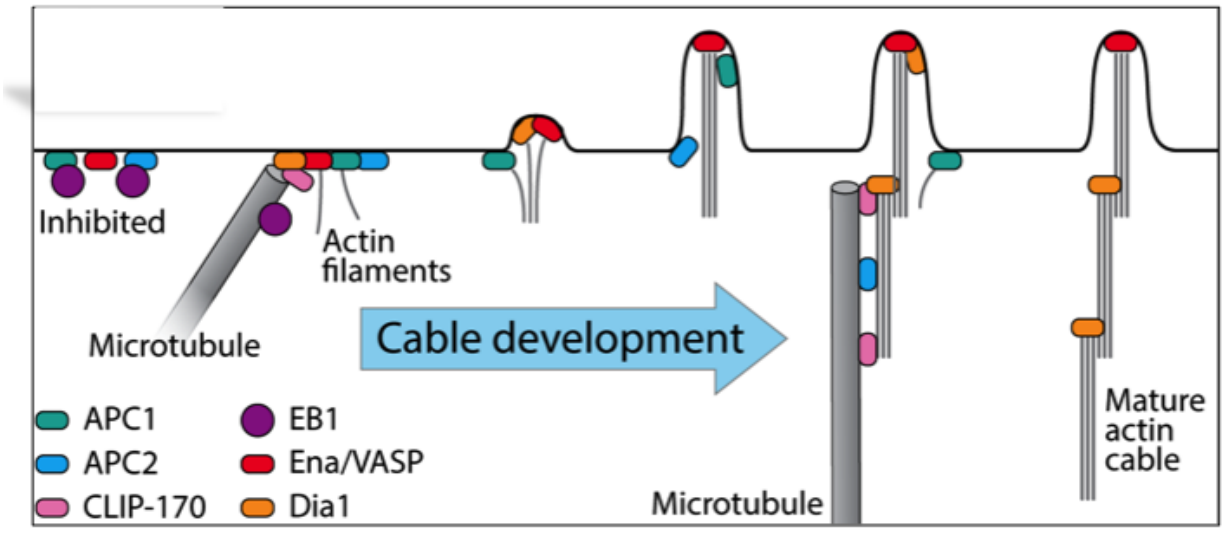


Figure 33


Inhibited
Actin filaments
Microtubule
Cable development
APC1
APC2
CLIP-170
EB1
Ena/VASP
Dia1
Microtubule
Mature actin cable

4.6 References

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Chapter 5. Discussion and Future Directions

5.1 Summary and Conclusions

The actin cytoskeleton plays important roles in cellular processes such as migration, wound healing, division, cytokinesis, intracellular transport, contraction, immune responses, and pathogen invasion (Chhabra and Higgs 2007, Barr *et al* 2007, Kaksonen *et al* 2006, Pollard *et al* 2003, Naumanen *et al* 2008, Insall *et al* 2009, and Saarikangas *et al* 2010). Currently, we understand the roles and mechanisms of some of the proteins required in actin polymerization, however we are continuing to identify more proteins and uncovering novel mechanisms involved in actin assembly. For example, formins are a family of proteins that can independently nucleate and elongate linear actin filaments (reviewed in Goode *et al* 2007, Higgs 2006, and Kirat-Karalar *et al* 2011). Formins are essential for the assembly of linear actin filaments in many contexts. For example, Diaphanous (Dia) was first characterized as an essential component of the cytokinetic furrow. Loss of Dia leads to failure of cytokinesis giving rise to multi-nucleate cells (Castrillon and Wasserman 1994). It was hypothesized that the activity of Dia is controlled by Rho-dependent Dia auto-inhibition (Seth *et al* 2006, Li and Higgs 2003, and Chesarone *et al* 2010). Dia activity in the context of actin furrow extension in the early embryo does not seem to be controlled by Rho activity (Webb *et al* 2009). In addition, Dia binds to a battery of cytoskeletal proteins such as CLIP-190 (Lewkowicz *et al* 2008), Profilin (Kursula *et al* 2008, Chang *et al* 1997, and Watanabe *et al* 1997), APC (Wen *et al* 2004, Webb *et al* 2009, Okada *et al* 2010, and Breitsprecher *et al* 2012), and EB1 (Wen *et al* 2004), We are discovering that the activity of formins is highly regulated through their interaction with proteins that either enhance or inhibit formin activity. However, there are many gaps in our understanding of formin activity and the mechanisms of how formins are regulated.

One example of formin regulation suggests that the yeast formin For3 is recruited by the +TIP microtubule protein Tea4 to ends of microtubules to promote the establishment of cell polarity and to localize actin assembly to new cells ends (Martin *et al* 2005). Dia is required to make many filopodia of

ranging sizes (reviewed in Goode *et al* 2007, Higgs 2006, and Kirat-Karalar *et al* 2011), and this activity is negatively regulated by Ena (Bilancia *et al* 2014). In addition, vertebrate APC1 (vAPC1) and Drosophila APC2 (APC2) collaborate with Dia to assemble actin *in vitro* and *in vivo*, respectively (Webb *et al* 2009, Okada *et al* 2010, and Breitsprecher *et al* 2012). However, many unanswered questions in our understanding of APC-Dia mediated actin assembly remain. We do not know if the actin associated activities of vAPC are required *in vivo* or how APC2-Dia mechanistically collaborates in actin assembly. In addition to relevance in understanding the role of cytoskeletal factors in cellular processes, loss of some cytoskeletal proteins can contribute to many diseases. Mutations and altered expression levels of microtubule and actin regulatory proteins such as EB1, APC, Dia, and CLIP-170 can lead to the loss of the spatial and temporal regulation of cytoskeletal activities and disease development (Nathke 2004, Nathke 2006, Fu *et al* 2005, Peng *et al* 2007, Sahin *et al* 2002, and Larti *et al* 2015). Aberrant cytoskeletal regulation can lead to neurodegenerative diseases, immunodeficiencies, heart and muscle defects, cancer, and developmental disorders (Mass *et al* 1990, Lynch *et al* 1997, and Zariwala *et al* 2007, and Bamburg *et al* 2010). Therefore, by understanding the role and mechanisms of actin machineries, we will be able to better understand basic cellular processes and diseases associated with loss of actin regulation.

My work in the McCartney Lab focused on understanding the mechanism of how actin structures are assembled in the cell and how proteins such as APC1, APC2, and Dia collaborate *in vivo* to assemble these actin structures. Additionally, our collaborators at Brandeis University found that two microtubule +TIP proteins, CLIP-170 and EB1, modulate that activity of Dia *in vitro*. However, it is not known if CLIP-170 and EB1 regulate the actin assembly activity of Dia *in vivo*. In order to address these questions, I investigated the formation of an array of spatiotemporally regulated actin cables during Drosophila oogenesis. In the last 20 years, we have learned that the formation of these actin cables requires actin bundling proteins (Quail/Villin (Mahajan-Miklos *et al* 1994) and Singed/Fascin; (Cant *et*

al 1994)), elongation factors (Ena and Chickadee/Profilin (Gates *et al* 2009 and Cooley *et al* 1992)), proteins required for cortical actin stability (DE-Cadherin and Armadillo/ß-catenin; (Huelsmann *et al* 2013 and Peifer *et al* 1993)), and cable orientation (DE-Cadherin; (Huelsmann *et al* 1993)), but until my work, it was not known which assembly factors initiate cables. These actin cables resemble filopodia where the growing end of the cable is oriented towards the plasma membrane. In addition to sharing the same polarity as filopodia, Ena is present at the + tip while Dia is localized at the shaft of the actin cables (Lanier *et al* 1999, Vasioukhin *et al* 2000, Nakagawa *et al* 2001, and Gates *et al* 2009). Ena is required for actin cable assembly (Gates *et al* 2009), but it was not known whether Dia is required for this process. In addition, we know that Ena is negatively regulating Dia-mediated actin assembly in some contexts (Bilancia *et al* 2014), but we do not know whether this regulation controls the assembly of actin cables. Clearly, there are many unanswered questions about Dia mediated actin assembly.

5.2 Overall impact of my work in the actin cytoskeleton field

Loss of vAPC leads to the initiation of colorectal cancer by the aberrant activation of the Wnt signaling pathway (Nathke 2004 and Nathke 2006). Although we understand a great deal about how the Wnt signaling pathway and other signaling molecules initiate colorectal cancer and drive its progression, less is known about the role of the cytoskeleton in cancer progression. Mutations in *vAPC* that lead to colorectal cancer truncate the vAPC protein, deleting many of the Wnt signaling activity domains and the domains involved in the direct binding and regulation of microtubules and actin. Is the loss of the cytoskeletal regulation of vAPC involved in promoting the progression of colorectal cancer? Before we can begin to address this question, we must first address the fundamental gaps in our understanding of the role of vAPC in actin assembly. We know that vAPC is involved in actin nucleation, bundling, and collaboration with Dia *in vitro (*Moseley *et al* 2007*,* Okada *et al* 2010*, and* Breitsprecher *et al* 2012*)*. However, we do not know if the actin-associated activities of vAPC are physiologically relevant. For the

first time, my work demonstrated that APC1 is an actin assembly factor *in vivo*. In addition, I am using an under-studied actin assembly model to dissect the mechanism of APC-Dia mediated actin assembly. In addition, my work found that microtubules are also required for the assembly of actin cables. Our work suggests that the actin cable number, morphology, and length may be controlled by the interaction of the microtubule +TIP proteins with the APC-Dia actin assembly machinery. This work is also impactful because we know that microtubules and actin coordinate in many cellular processes, but how these two cytoskeletal networks coordinate is not clearly understood. Therefore, we will be able to use actin cable formation to dissect that activity of APC and Dia mediated actin assembly, and dissect the mechanism of how actin modulators such as the microtubule +TIP proteins, CLIP-190 and EB1, influence actin assembly.

Manuscript in preparation: Molinar, O. and B.M. McCartney "APC1, APC2, and Diaphanous are required for the normal program of actin cable assembly in the Drosophila ovary."

- The basic domains of vAPC and APC1 share the same actin associated functions and collaborate with Dia *in vitro* (Jaiswal *et al* 2013). Additionally, Drosophila APC1 collaborates with vertebrate Dia further validating that the APC1-Dia collaboration is conserved between species. This makes the fruit fly a great system to study APC1 mediated actin assembly (Jaiswal *et al* 2013).
- Prior to my research, the investigation of the novel APC1 and APC1-Dia-mediated actin assembly mechanism was limited to *in vitro* work. Although, the *in vitro* work elegantly demonstrated that APC1 and APC-Dia were novel actin assembly mechanisms, *in vivo* evidence to support these findings was lacking. My research demonstrated that APC1 is an actin assembly factor. Therefore, I have established an actin assembly model where we can continue to dissect the novel mechanism of APC-Dia mediated actin assembly *in vivo*.
- I found that APC1, APC2, and Dia are all expressed and localized to the cortex of the nurse cells in Drosophila egg chambers where actin cable formation is initiated. Through loss of function

experiments, I concluded that all three proteins are required for the normal program of actin cable formation during late oogenesis.

Work to complete for the manuscript:

- I demonstrated that APC1, APC2, and Dia are all present at the cortex of the Nurse cells in stage 11 stages. In this case, we did not observe overlap of these proteins with another Ena, another factor involved in actin assembly and a Dia-mediated actin assembly regulator (Peng *et al* 2009, Faix *et al* 2006, Kwiatkowski *et al* 2007, Dent *et al* 2007, Gates *et al* 2007, and Gates *et al* 2009). There several are interpretations of why we did not observe Ena overlap with the other proteins: 1) these proteins are functioning in actin assembly differently or 2) or we capturing the wrong developmental stages. It is possible that we observed this because we cables already started assembled. Therefore, we are capturing the distribution of these proteins in mechanisms required for elongation and not initiation. What about the localization of all these proteins in earlier stages of cable development? We need to further characterize the localization of APC1, APC2, and Dia throughout stages of actin cable development. Additionally, we need to localize APC1 and APC2 against Dia to begin to address whether APC proteins may complex and work with Dia in actin assembly. Co-localization of Dia, APC1, APC2, and Ena may suggest that these proteins work together in the same complex but we need to test this directly using loss of function experiments.
- Now that we know that APC1, APC2, and Dia are required for actin cable assembly, we want to know which domains are specifically required for their activity. We know: 1) that the basic domain of APC1 is required to nucleate actin filaments (Okada *et al* 2010 and Jaiswal *et al* 2013), 2) the 20Rs, ASAD, Armadillo Repeats, and C30 are required for the function of APC2 in actin assembly (unpublished and Zhou et al 2010), and 3) the FH1, FH2, and DAD are required for the actin assembly activity of Dia (Goode et al 2007 and Higg 2005). The FH2 domain is required for nucleation of actin filaments while the FH1 domain is required for the elongation activity of Dia. We predict that loss of the FH2 domain would delay the formation of actin cables in the early 10B early and mid stages but not loss of the FH1 domain. Therefore, we need to assess the phenotypic consequence of loss of the domains required for

actin assembly of each protein. These experiments will also help us to begin to address the mechanism of APC-Dia collaboration in actin assembly, because some of the domains listed above are required for APC and Dia-mediated actin assembly. Ideally, we would like to know the minimal requirements of APC1-Dia and APC2-Dia binding so that we can only delete the amino acids required for collaboration. However, we currently know which domains are required, but we have not dissected binding of APC1-Dia and APC2-Dia further.

    - From my experience imaging the loss of function mutants, variability does exist even in the same genotype. Therefore, I need to characterize the loss of function phenotype more thoroughly: how many actin cables, length, and morphology. In addition, statistical analysis of the phenotypes will be required to conclusively comment on the severity of the phenotypes.

- To test whether this proteins complex and collaborate *in vivo*, we will use transgenic fly lines that delete the important domains for APC to Dia binding and asses actin cable formation. Lastly, we need to quantify the staining overlap of APC1, APC2, Dia, and Ena with each other, at the cortex, and at the actin cables. Staining of these proteins appears to be weak in some contexts so understanding the frequency of overlap will be required to confidently conclude that these proteins are present at specific actin structures or potentially working together in actin cable assembly.
    - The novelty of my research is that for the first time, I demonstrated that APC1 is involved in actin assembly *in vivo*. What I did not answer is whether the APC1-Dia collaboration is physiologically relevant and, whether APC1, APC2, and Dia interact to influence the formation of actin cables. To begin to address the collaboration amongst APC1-Dia and APC2-Dia double mutants will be assessed in actin cable formation. We predict that more severe delays in actin cable assembly will be exhibited in Dia-APC1 and Dia-APC2 mutants. I do not expect a complete loss of actin cables because APC1 and APC2 will still be present in the cell, respectively. present.

- Additionally, APC1 and APC2 share overlapping Wnt signaling function in some contexts. We also wanted to know if APC1 and APC2 shared overlapping roles in actin assembly. We predicted that because APC1 contains the basic domain required for actin assembly and APC2 does not, APC1 and APC2 would differ in actin assembly activities. These results appear to be corroborated by our *in vitro* data. Currently, I am working to assess whether APC1 rescues APC2 actin cable defects and *vice versa*.

Important questions that need to be answered in APC and Dia-mediation actin assembly. I will not be able to answer these questions during my time in the McCartney Lab:

- APC1, APC2, and Dia bind to each other *in vitro* and are all required for the formation of actin cables *in vivo*. Therefore, we predict that it is possible that are all three proteins are collaborating in actin cable assembly *in vivo*. To test this, we need to assess triple APC1, APC2, and Dia knockdown in actin cable formation. Ideally, this would be the way to this but due to technical difficulties- we may not be able to make a triple loss of function fly. One way that we can do this is do RNAi against all three proteins. In addition, we can generate a fly that contains deletion of all the interaction domains of APC1, APC2, and Dia by recombining alleles of the proteins that delete only the domains required for binding. We predict that loss of all three proteins will delay the formation of actin cables further because other proteins involved in actin assembly are still present. Ena an elongation factor is still present. In addition, in flies, 7 formin homologs are present that may also function in actin cable formation. It is also possible that once actin nucleation is overcome by APC-Dia collaboration, even if it is delayed, elongation factors could compensate for the delay in actin assembly. Another alternative prediction is that complete loss of all three proteins could completely abolish the formation of actin cables. This would tell us that actin assembly by APC1, APC2, and Dia is required and completely essential for actin cable initiation. This would be interesting to know because the data would suggest that the APC-Dia actin assembly machinery is sufficient and required for actin cable initiation, and regulated by other proteins such as Ena.

- In several contexts, phosphorylation of APC2 by GSK3ß controls the activity of APC2 (Kunttas-Tatli *et al* 2012). For example, phosphorylation of the 20Rs by GSK3ß increases the binding affinity of APC2 to Armadillo (Rubinfeld *et al* 1996). Through a similar mechanism, we predict that phosphorylation of the 20Rs of APC2 increases the binding affinity of APC2 to Dia. We demonstrated that the 20Rs bind to Dia and predict that this interaction may be regulated by GSK3ß phosphorylation. We will test the role of 20R phosphorylation in APC2 and Dia complex formation directly *in vitro.* In addition, GSK3ß phosphorylation affects microtubule dynamics. We need to investigate the role of GSK3ß in microtubule formation in the egg chambers. This is a very important question because we found that microtubules and microtubule binding proteins affect the formation of actin cables. Therefore, is loss of GSK3ß affecting APC2 directly to affect assembly or are the cables being affected by loss of GSK3ß independent of APC2? To test answer this question, I assessed the actin cables in mutant egg chambers where all the GSKß phosphorylation serine (S) residues were mutated to aspartic acid (D) (mimicking phosphorylation) or alanine (A) (blocking phosphorylation). Our APC2-20R-S>A and APC2-20R-S>D analysis suggests that APC2 is a target of GSK3ß *in vivo*. Additionally, both phosphorylation states are required for the actin assembly activity of APC2. These results suggest that APC2 is a target of GSK3ß. However, it is possible that GSK3ß may still be regulating microtubules. The phenotypes induced by loss of microtubules by drug treatment or genetic ablation of EB1 do not phenocopy GSK3ß or APC2-20R phosphomutants. Therefore, we can conclude that APC2 is a target of GSK3ß and this mechanism directly influences actin cable assembly.
- APC2 alone does not appear to be an actin assembly factor and inhibits APC1 and Dia mediated actin assembly *in vitro* (unpublished). On the other hand, APC2 collaborates with Dia in actin assembly *in vivo*. Therefore, we need to better understand the role of APC2 and Dia in actin assembly *in vitro* and *in vivo*. Currently, we hypothesize that APC2 does not assemble actin filaments on its own and potentially acts as a brake to turn the actin assembly of Dia off. For example, Dia is present at the plus end of an elongating filament where it is processively attached and elongates the actin filaments.

Dia could potentially continue elongating the actin cable infinitely until it runs out of free ATP-actin. But the cell does not need really long actin filaments. Sometimes it needs small and thick cables, so how does a cell control for the length and number of actin filaments nucleated and elongated by Dia. Two of possible mechanisms for this type of regulation involved APC2 and Ena. Both proteins appear to inhibit Dia-mediated actin assembly *in vitro*, and are required for actin cable assembly in Drosophila late oogenesis. The actin cables are assembled by the formation of many actin filaments that are bundled together to generate a thick cable. The cables appear as a long actin cable that is composed of smaller overlapping cables that are bundled together (similar to the layout of a ladder). Therefore, it seems possible that Ena and APC2 act as brakes on Dia to control the number and length of the actin filaments that are bundled into cables.

- Some evidence suggests that formin activity is regulated by the direct interaction of microtubule +TIP binding proteins with formins. Our *in vitro* (Collaboration with the Goode Lab) and *in vivo* work suggests that two microtubule +TIP proteins are required to modulate the actin assembly activity of Dia.
    - Quantify the phenotype of loss of microtubules in actin cable formation when treated with pharmacological agents: length, actin intensity, actin distribution, and cable morphology.
    - Quantify the overlap of acetylated microtubules and actin cables.
    - EB1, Dia, and APC1 are predicted to form a tri-partrite complex to stabilize microtubules. Does loss of any of these proteins reduce the number of acetylated microtubules?
- We found that the fly CLIP-190 enhances the nucleation and elongation activity of Dia at the plus ends of microtubules *in vitro*.
    - I discovered that CLIP-190 is expressed and localized to the cortex of the Nurse cells, and at the interface of microtubule and actin interactions in the cytoplasm. To test the activity of CLIP-190 in actin assembly, we will assess the actin cables in CLIP-190 *loss-of-function* alleles. Our *in vitro* data suggest that CLIP-190 collaborates with Dia to increase the rate of actin elongation. In addition, our *in vivo* data suggest that CLIP-190 is localized at the right

time and place to function in actin cable assembly. Therefore, we predict that CLIP-190 is required for actin cable assembly in late oogenesis. Complete loss of actin cable assembly is not expected because other elongators such as Dia and Ena are still present in the cell. Dia and Ena could still be able to elongating actin filaments but less efficiently upon loss of CLIP-190.

- We found that EB1 is an inhibitor of APC1 and APC1-Dia mediated actin assembly *in vitro*. dsRNA interference against EB1 induced premature activation of actin cable assembly in early oogenesis and the formation of abnormal ectopic actin patches at the surface of the Nurse cells in stages during 10B and older stages of egg chamber development. In addition, the actin cables seemed dis-organized. We concluded that EB1 is possibly involved in regulating the spatio-temporal assembly of actin. Our hypothesis is that EB1 may be acting to restrict the activity of APC1 and Dia to function in microtubule regulation and not actin assembly at the microtubule +TIP.
    - EB1 inhibits APC1 and APC1-Dia mediated actin assembly (unpublished data) but the mechanism of EB1 inhibition on these actin assembly factors is not known. The C-terminus of EB1 binds *in vitro* to the C-terminus of APC1 and Dia (Wen *et al* 2004, Moseley *et al* 2007, Webb *et al* 2009, Okada *et al* 2010, Breitsprecher *et al* 2012, and Jaiswal *et al* 2013). Based on this evidence, we predict that the C-terminus of EB1 specifically inhibits the domains required for the actin assembly activity of APC1 and Dia. To further dissect the minimal requirements of inhibition by EB1, we will generate C-terminal fragments of EB1, APC1, and Dia. The binding and actin assembly activity of EB1-fragments on APC1 and Dia fragments will be tested *in vitro*.
    - We are beginning to understand how and what EB1 is doing in actin assembly *in vitro* and *in vivo*. To continue to address, how EB1 affects actin cable assemble, we need to know where it is localized in the Nurse cells. To answer this question, we

need to localize EB1 in stage late oogenesis. Is it near Dia or APC1? I predict that before actin cable are assembly, EB1 is localized to the cortex of the cell, overlapping with Dia and APC1. Additionally, I also predict that EB1 is localized and expressed at higher levels than APC1 and Dia to restrict ectopic actin assembly throughout most stages of oogenesis. As the cables are being assembled, I also predict that EB1 expression or overlap with APC1 or Dia is significantly reduced to allow the normal program of actin cable assembly at the cortex occur normally.

- Use live imaging to quantify the delay of actin assembly. This will be important o know because I predict that once I start assessing the loss of the domains of APC1, APC2, and Dia that required in actin assembly, the defects in the delay of actin cables may not be as clear as when I take out the complete proteins. This may be because multiple domains of the proteins may be required in actin cable assembly. In addition, we know that actin cable assembly occurs at the interface of the Nurse cell cortical actin where polymerization occurs towards the plasma membrane. It appears as though many small actin cables of an average of 20μm in length, overlapping actin cables are bundled together (size was predicted from EM analysis from Guild et al 1997). Although, Huelsmann *et al* 2013, demonstrated that actin cables do not appear to exhibit dynamic actin turnover throughout the length of the cable, it is possible that those experiments are not telling us the complete story. For example, it is possible that the group FRAPed spots that were too close or too far away from each other and did not capture the appropriate sites of actin turnover on cables. To better assess the turnover of actin within the cables, we perform some more FRAPing analysis within the actin cables. It is possible that the plus ends of the small actin cables along the cable are free, and elongators such as Ena and CLIP-190 bind to influence the growth of the actin cables at these sites. On the other hand, it is possible that capping proteins associate to the plus ends of the actin cables, restricting actin cable assembly to the actin cortex. Lastly, we would like to better understand the relationship between the formation of microtubules and actin cables in late

oogenesis. For example, how are microtubules assembled with relationship to actin cable formation. How does loss of microtubules and microtubule binding proteins affect actin cable assembly?

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- APC2 is localized to the actin cortex through the N-term and C30 domain where it is required for the extension of actin furrows in the early embryos (Zhou *et al* 2010). My work found that a conserved Drosophila coiled-coil domain located within the C30 domain is required for the cortical localization of APC2. This 15 amino acid coiled-coil is potentially stabilized by dimerization through the ASAD domain. However, my results suggested that the N-term plays an additional role in the cortical localization of APC2.
- **Short term goals for publication completion:**
    - Demonstrate functional significance of our model *in vivo*. Our plan is to demonstrate that APC2 is required for cortical actin integrity and actin turnover.
    - Test the cortical integrity by performing micro-aspiration assays in Drosophila egg chambers mutant for the C30 and the dimerization APC Self Association Domain (ASAD) domain.
    - Assess the turnover of cortical actin in the C30 and ASAD mutants using FRAP.

5.3 References

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