Role of APC proteins in regulating Wnt signaling and cytoskeletal organization in Drosophila

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Ph.D. in Biological Sciences

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Thesis

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Title Role of APC proteins in regulating Wnt signaling and cytoskeletal organization in Drosophila

Presented by Ezgi Kunttas-Tatli

Accepted by the Department of Biological Sciences

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Dean Frederick Gilman Date

To my parents Veli and Kamuran for letting me chase my dream of becoming a scientist since the beginning;
and to the love of my life, my best friend, my husband Emre, who constantly supports my dream of being a scientist.
Without their love, support, inspiration and encouragement, I wouldn't be able to keep this dream alive.

Abstract

Development of an embryo is a fascinating biological process that requires effective communication between neighboring cells and coordination of movement across the entire organism. At a cellular level, this is achieved by upstream signaling pathways ultimately regulating gene expression to provide cells with cues to perform certain tasks such as cell division, migration, cell rearrangements or changes in cell shape. All of these diverse tasks ultimately rely on rearrangements of the cytoskeleton. However, it is unclear what the molecular connections are between signaling and cytoskeletal dynamics.

Adenomatous Polyposis Coli (APC) is a multifunctional protein that plays vital roles both in regulating the canonical Wnt signaling pathway and the cytoskeleton. Mutations of APC are associated with more than 80% of both familial and sporadic colorectal cancer cases. APC is one of few cytoskeletal proteins with direct links to cancer. However, as a multi-domain, multifunctional protein, a comprehensive understanding of APC biology has been difficult to achieve. While we have known for almost 20 years that APC proteins are essential negative regulators of Wnt signaling, the precise role they play both in regulating Wnt signaling and cytoskeletal have been unclear.

In order for APC proteins to perform these diverse tasks and regulate both signaling and the cytoskeleton, it needs to be highly regulated itself. In my Ph.D. project, I approached APC proteins from many different angles, in many different developmental contexts, to gain insights into the precise role they play and how they are regulated in both the Wnt signaling and the cytoskeletal context. To better understand how APC proteins are regulated, I used Drosophila as a simpler, more tractable model. The large size of the vertebrate APCs (~300 kDa) makes it difficult to perform structure/function studies in the context of a full-length protein. Similar to vertebrates, flies have two highly conserved APC proteins (APC1 and APC2). Thus, I chose to study the fly APC homologs and mostly focused on the smaller member of the family, APC2, in my studies.

To elucidate how APC proteins are regulated in the context of Wnt signaling, first we dissected the role of phosphorylation in the context of Wnt signaling. APC proteins

are highly phosphorylated, and this plays a role in APCs activity in Wnt signaling. As a part of the destruction complex, APC targets the key effector of the pathway, ß-catenin for degradation. Phosphorylation of the central 20 amino acid repeats (20Rs) has received the most attention over the last decades, and has been shown to change the affinity of β-catenin binding *in vitro*. However, many of these in vitro models lacked an *in vivo* model. To test the functional significance of 20R phosphorylation in Wnt signaling, we used Drosophila APC2 and took advantage of the awesome power of genetics in this model organism. Our studies showed for the first time in an intact animal that 20R phosphorylation played an essential role. This study also suggested functional diversity among different 20Rs as well as gave us hints about the presence of macromolecular destruction complex, which we coined the term "destructosome' (see Chapter 2). Besides the phosphorylation of the 20Rs, phosphorylation of other APC domains, such as the Axin binding SAMP repeats, had not been investigated before. Therefore, I also studied the phosphorylation of SAMP repeats and tested if it played a functionally significant role in APCs Wnt function. Similar to the 20Rs, I've shown that SAMP phosphorylation plays a previously uncharacterized role in APCs Wnt signaling function and proposed a novel idea of functional diversity among different SAMP repeats (see Chapter 3).

As mentioned above, while studying the importance of 20R phosphorylation, I got interested in the idea of higher order destruction complex structures, or destructosome. This led me to think about the role of APC proteins in the assembly of this complex. Although it has been long appreciated that human APC can self-associate, the precise role of self-association in Wnt signaling hasn't been explored in part due to the complexity of self-association in the vertebrate APC (vAPC) proteins. By using Drosophila APC2, I've identified a novel self-association domain (ASAD) and uncovered a new role for APC proteins in promoting the assembly and stability of the destructosome (see Chapter 4).

I was interested in APC phosphorylation not only in the context of Wnt signaling but also in APCs cytoskeletal roles. One of the emerging themes in APCs role in regulating the actin cytoskeleton is its interaction with the formin Diaphanous (Dia). Previous work from our laboratory suggested that Drosophila APC2 and Dia cooperated

during the formation of actin based structures during embryogenesis and this interaction was regulated. In order to understand this relationship further, I tested the role of phosphorylation as potential regulatory mechanism. My studies showed, in deed phosphorylation played a role in APCs activity in this context too (see Chapter 5). This study also revealed a potential cross talk between two pools of actin (linear and branched).

In summary, studying APC, an exciting and highly complex protein, allowed me to think about many different biological questions from signaling to cytoskeleton in various developmental contexts. The findings from my Ph.D. research uncovered new aspects of APC biology, and showed how various regulatory mechanisms weather it's phosphorylation or self-association, affect its functions, both during Wnt signaling and also in regulating the actin cytoskeleton. My studies will also help better understand the disease relevance of human APC proteins and provide novel insights.

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Acknowledgements

First and foremost, I would like to thank my graduate advisor Brooke, for being a great role model as a woman in academia. Over the last five years, I believe we went through some important times together from you getting your tenure to persevering against funding problems. I'll always remember your professionalism during this process, and the fact that you had't reflected an ounce of stress to any of us. I've learned so much from you, both personally and scientifically. Your attention to detail and your ability to see the big picture always kept me on tract. You supported all my scientific endeavors, like taking the Embryology course in Woods Hole. I know, I was away from the lab for six long weeks, but I believe those six weeks will go a long way for my career. You tolerated my recently acquired insect enthusiasm and event caught couple of bugs for me to dissect. Another thing that you endured was probably my writing, I'm forever grateful for your editorial comments and your guidance. Most importantly, you have given me the freedom to develop my own projects and follow my own curiosity. Thank you!

I would like to thank my committee members, Drs. Jon Minden, Marcel Bruchez and Lance Davidson. Jon, your expertise in biochemistry and your ability to think outside of the box always made me feel very lucky. You taught me how to run a 2D gel, and in the process you gracefully handled the frustrated/sad Ezgi, after first 'five' gels failed (due to the faulty IEF strips). You always made me feel like I could stop by your office anytime to bounce ideas off you or get your opinion on personal or scientific questions. Marcel, you definitely had great ideas throughout my thesis project and put up with my silly FRAP questions. Lance, you have been instrumental in this process. Your background in both engineering and biology makes you such a unique scientist that allowed me to learn something new every time we interacted.

I thank all the past and present members of the McCartney lab, for everything. Initially, Sandra, Becky, Megha and Mengning for showing me the ropes around the lab. I feel very lucky to spend time with Sandra every year at various meetings. I thank Becky, as a collaborator and a great example of being an excellent communicator and Mengning, to share some of your various cloning secrets and being a big part of the Genetics paper. Vince and Keith for being great lab citizens. As for the current members; I thank Olivia, Stacie and Malachi for their support, their useful suggestions and putting up with me especially the last few months. Don't forget, what happens in the fly room, stays in the fly room! There we shared personal stories, discussed scientific questions, laughed, cried, danced… So many memories, so little space. Olivia, you are a brave woman and a great inspiration to me; Stacie, I'll never forget our adventures in collecting spider egg sacs or raising the caterpillars; Malachi, it's refreshing to see someone as focused as you are. I would also like to thank many CMU and REU undergraduates

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I would also like to thank our collaborator, Dr David Roberts. You were instrumental in performing number of experiments for both the self-association and SAMP projects. In addition, I would also like to thank my MBL family, the class of 2013, for their inspiration. Especially Drs. Alejandro Sanchez and Richard Behringer. I feel privileged to be a part of the Embryology family and your progeny.

My family has been such an essential part of my success. It's difficult for me to express my gratitude in words. I thank my dad, for being an endless source of inspiration throughout my life and my mom for being such a strong women and being tolerant to why she is still waiting to have grandkids. I thank to my grandmother, for being an amazing friend all these years. Being away from you is definitely hard, but you know that this is what makes me feel content. My sister, as an architect, you always give me great perspective in life and talking to you often while commuting helped a lot. I'm also grateful to my in-laws, and other members of my family for their support and encouragement. I also want to thank my cats, Boncuk and Zeytin. You probably won't understand how important you were in this process.

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Chapter 1
Introduction

The *Adenomatous polyposis coli* (*APC*) gene encodes a large, evolutionarily conserved, multidomain protein that plays vital roles in Wnt signaling, adhesion and cytoskeletal regulation (Fig 1). Germline and somatic mutations in the *APC* gene are involved in >80% of all colon cancer cases (Polakis, 2007). Its numerous functional domains in turn allows APC to interact with numerous proteins (Fig 1B). Its multi-subcellular locations in turn means different pools of APC, playing different functional roles at the same time (Fig 1C). The fact that APC is an integral part of so many different pathways makes it a challenging but yet exciting protein to study. Over the last few decades, efforts to understand this enigmatic protein revealed important aspects of APC biology, but we are still uncovering novel roles for APC proteins.

1.1 The Structure and Evolution of APC Protein Family:

1.1.1 The Domains of the Human APC Protein

The N-terminus of the APC protein consists of an oligomerization domain and Armadillo repeats; the central portion comprises multiple 15 and 20 amino acid repeats (15Rs and 20Rs) as well as SAMP repeats; and C-terminus contains a basic domain and binding sites for EB1 and DLG (Fig. 1). The presence of these many protein-protein interaction domains reflects the numerous interaction partners and the multifunctionality of the APC proteins (Hilger and Mann, 2012). I will briefly go over the each domain in conjunction with its functional importance and disease relevance. In addition, some of these domains will be the focus of my studies and will be discussed in more detail.

The N-terminal Oligomerization Domain

APC proteins can form coiled coil based parallel dimers that fit into the classic heptad repeat motif via the N-terminal oligomerization (or more correctly dimerization) domain (Fig. 2). The precise function of the N-terminal oligomerization domain is not well understood. However, APCs ability to dimerize is thought to contribute to its disease relevance due to the possibility that the wild type APC may dimerize with both wild type and truncated mutant APC proteins (Polakis, 1997). This raises the possibility of the truncated mutant APC proteins to elicit a dominant negative effect and disrupt the tumor suppressor activity of APC (Su et al., 1993).

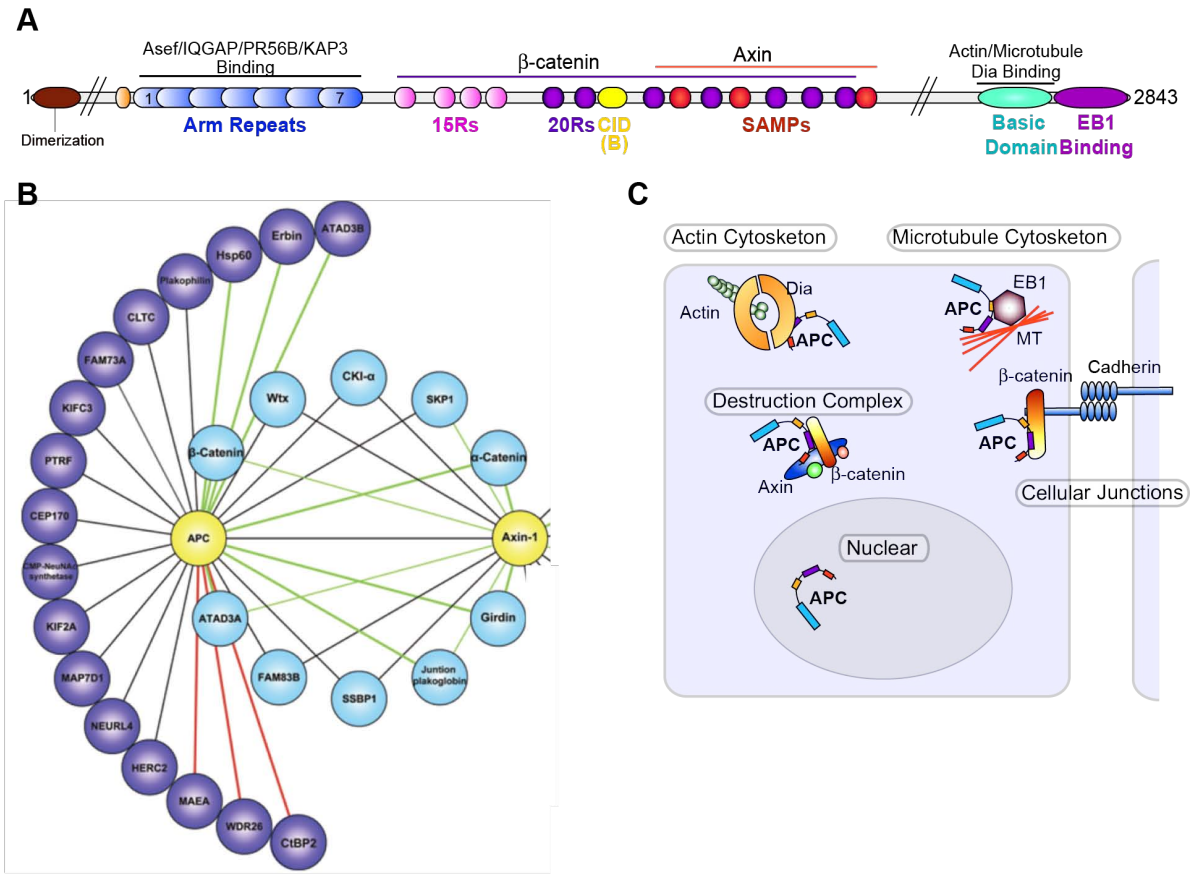


Figure 1: A) Schematic representation of human APC: APC dimerization domain (burgundy), APC self-association domain (orange), Arm repeats: Armadillo repeats (blue), 15 amino acid repeats (15Rs) (pink), 20 amino acid repeats (20Rs) (purple), β-catenin inhibitory domain (CID) (also known as region B) , SAMP repeats (red), basic domain (turquoise), EB1 binding domain (purple). The N-terminal domain mediates interactions with actin regulators Asef, IQGAP and microtubule regulator KAP3 and PP2A regulatory subunit PR56B. The central region mediates interactions with β-catenin and Axin. The C-terminal basic domain mediates interactions with both actin and microtubules as well as Dia.
B) APC interactome (modified from Hilger et al., 2011) depicts the wide range of APC binding proteins.
C) APC can be found in different subcellular locations in the cell; part of the destruction comples in the cytoplasm, associated with both actin and microtubule cytoskeleton, at adherence junctions and also nuclear.

APC Dimerization Domain

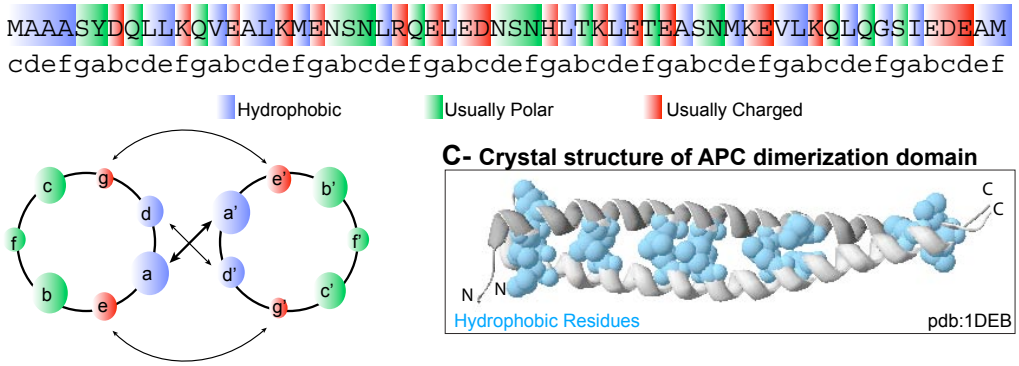


Figure 2: The N-terminal dimerization domain forms fits into the classic heptad repeat (abcdefg) motif, where a and d are hydrophobic (blue),e and g are charged (red), and b, c, f tend to be polar amino acids (green). Hydrophobic residues faceeach other and are buried in the hydrophobic core. C) The crystal structure of the APC dimerization domain forms a parallel coiled coil. pdb 1DEB was used to generate the figure.

The Armadillo Repeats

APC armadillo (Arm) repeats is a highly structured part of this large mostly unstructured protein and contains evolutionarily conserved seven armadillo repeats that also share homology to the ß-catenin (ß-cat) armadillo repeat (Fig. 2) (Zhang et al., 2011). Through these repeats, vertebrate APC interacts with a number of cytoskeletal proteins including Kinesin-associated protein 3 (KAP3), the RacGEF APC-stimulated guanine exchange factor (Asef), and the Rac1 effector Ras GTPase-activating-like protein IQGAP (Jimbo et al., 2002; Kawasaki, 2000; Watanabe et al., 2004). While APC Arm repeats are essential *in vivo*, it is unclear how they contribute to the tumor suppressor function of APC (Roberts et al., 2011).

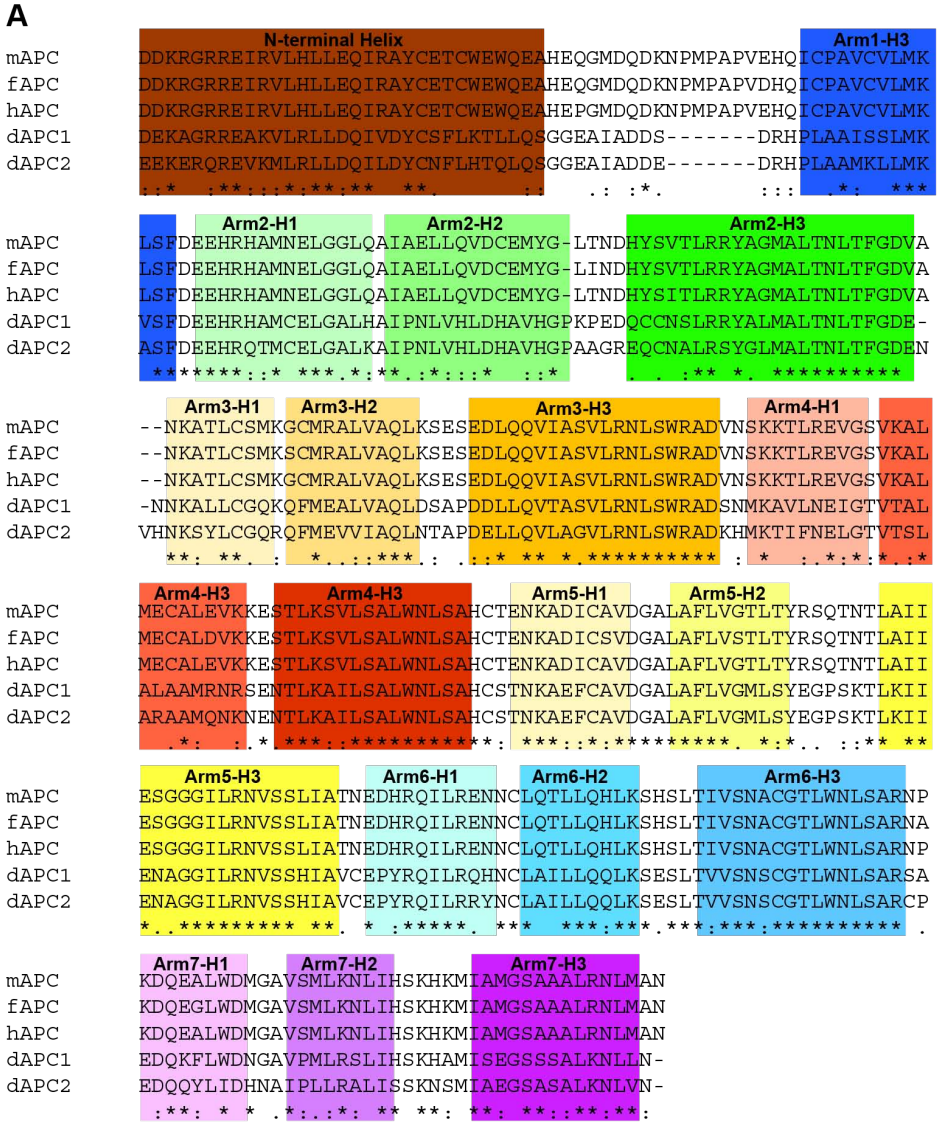
The 15 and 20 Amino Acid Repeats

Central region of APC contains four 15 amino acid repeats (15Rs), and seven 20 amino acid repeats (20Rs), both of which mediate interactions with ß-cat (Munemitsu et al., 1995) (Fig. 4). Importantly, the 20Rs contain residues that are phosphorylatable by the kinases CK1 and GSK3ß, whereas the 15Rs lack these phosphorylatable residues (Ikeda et al., 2000). *in vitro* binding assays established that the 20Rs are higher affinity binding sites for ß-cat and phosphorylation of these repeats increases the affinity up to 1500 fold (Liu et al., 2006). Because APC only binds to one molecule of ß-cat, it is not clear why there are four 15Rs and seven 20Rs (Rubinfeld et al., 1997). However, it's becoming more apparent that there is functional diversity among various 20Rs (Kunttas-Tatli et al., 2012; Roberts et al., 2011). While 20R3 has the highest affinity for ß-cat, 20R2 lacks any detectable affinity for ß-cat binding (Liu et al., 2006) and, is essential for APC function independent of its ability to bind to ß-cat (Yamulla et al., 2014). Most of the APC colorectal truncations retain all of the 15Rs and one to three 20Rs and thus able to interact with ß-cat.

The SAMP Repeats

The APC SAMP repeats bind directly to the RGS domain of Axin, another essential component of the destructosome (Fig. 5) (Behrens et al., 1998; Spink et al., 2000). These repeats are named after the conserved Ser, Ala, Met, Pro amino acid

A



B Crystal structure of APC Armadillo (Arm) Repeats

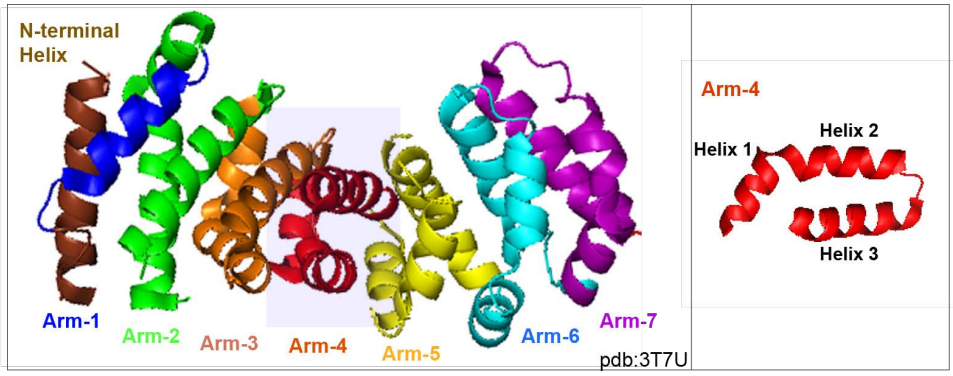
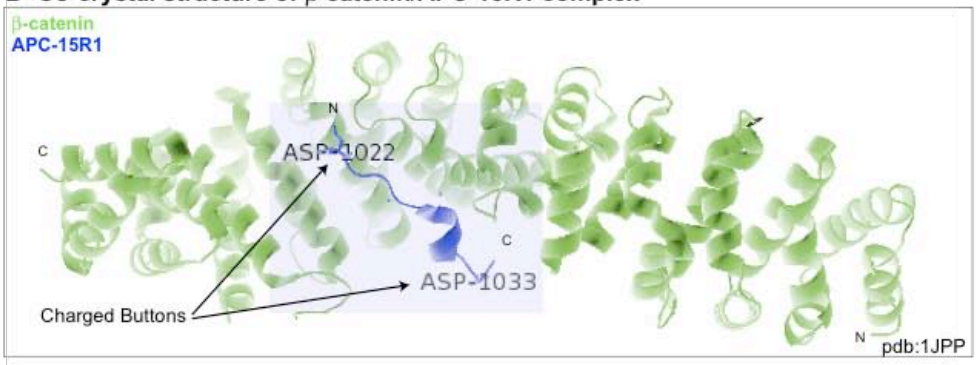


Figure 2: A, B) Sequence alignment of the Armadillo (Arm) repeats across vertebrate APCs and Drosophila APC2. There are seven Arm repeats (Arm 1 blue, Arm 2 green, Arm 3 orange, Arm 4 red, Arm 5 yellow, Arm 6 cyan, Arm 7 magenta). Each Arm repeat (except Arm 1) contains three alpha-helices (a short helix followed by two longer helices), which pack against each other in an antiparallel fashion. The repetitive packing create a binding groove. pdb 3T7U was used to generate the figure.

A- APC 15 Amino Acid Repeat 1 (15R1)

B- Co-crystal structure of β-catenin/APC-15R1 complex



C- APC 20 Amino Acid Repeat 3 (20R3)

D- Co-crystal structure of β-catenin/APC-20R3 complex

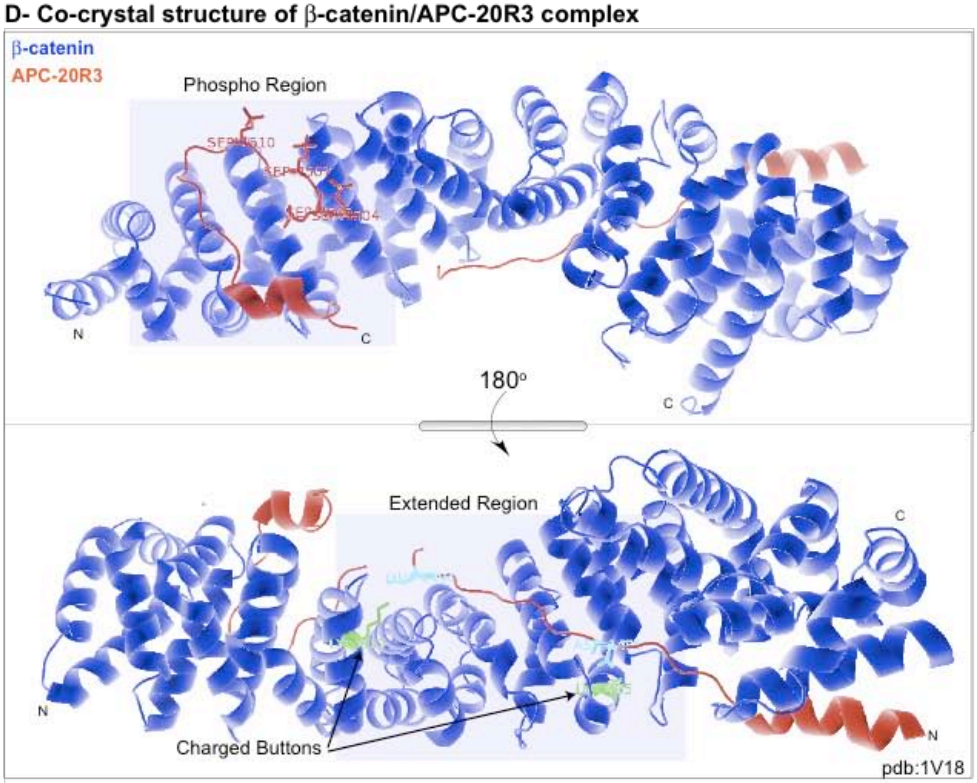


Figure 4: A) Sequence of the first 15 amino acid repeat (15R1) in human showing the charged buttons. B) Co-crystal structure between hAPC-15R1 and murine β-catenin Arm repeats, showing the where the APC-15R1s bind. C) Sequence of the third 20 amino acid repeat (20R3) in human showing the charged buttons in the Extended-region and phospho-residues in the Phospho-region. D) Co-crystal structure between hAPC-20R3 and mouse β-catenin Arm repeats, showing the where the APC-20R3 bind. pdbs 1JPP and 1V18 were used to generate the figure.

A- APC SAMP Repeat 3

B- Co-crystal structure of Axin-RGS domain and APC-SAMP3

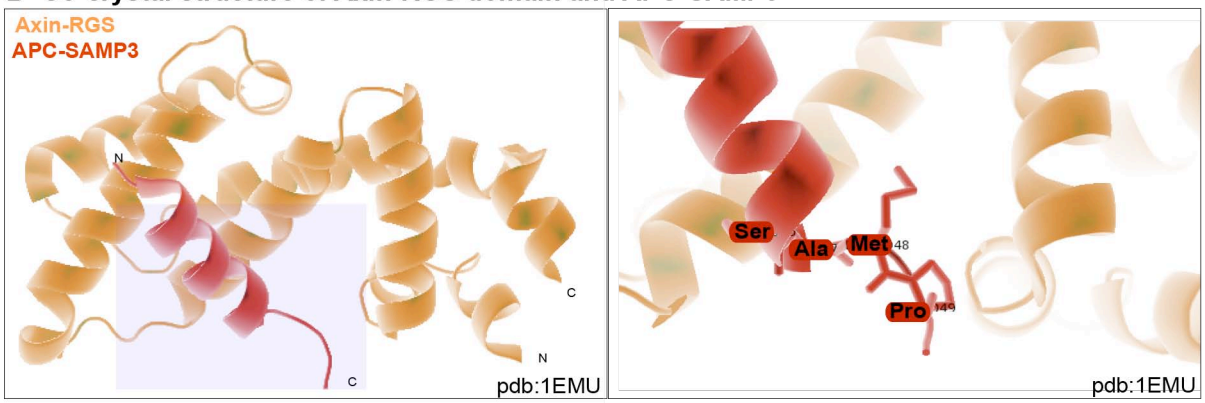


Figure 5: A) Sequence of the third SAMP repeat in human showing the conserved motifs. B) Co-crystal structure between human Axin-RGS (orange) and human APC-SAMP3 repeat (red) is shown. pdb 1EMU was used to generate the figure.

motif. In addition to this sequence motif, the co-crystal structure between Axin-RGS and APC-SAMP revealed additional upstream sequences that are important for Axin binding (Spink et al., 2000). SAMP repeats are almost always lost in the truncated mutants of APC.

The Basic Domain

The C-terminal region of APC contains a basic domain that contains a large proportion of basic arginine and lysine residues. Interestingly, the basic domain can directly interact with both microtubules and with actin monomers, and promotes microtubule assembly and stability (Munemitsu et al., 1994) as well as actin assembly *in vitro* (Okada et al., 2010). It's currently unclear how this domain is regulated to function in both of these processes. While it is probable that the basic domain is essential for some of APCs cytoskeletal functions, there is no evidence to suggest that it has any function in the negative regulation of Wnt signaling.

The EB1/HDLG Binding Domain

The C-terminal end of APC contains a binding site for the microtubule plus end binding protein EB1 and human homolog of the Drosophila discs large tumor suppressor protein. Through EB1 binding, APC regulates spindle dynamics and chromosome alignment during mitosis and truncation mutations dominantly interfere with this function (Green et al., 2005).

1.1.2 Phylogenetic Analysis of the APC Gene Family

Canonical Wnt signaling is evolutionarily conserved across all metezoans and plays essential roles during embryonic development (Logan and Nusse, 2004). Thus, most of the core components of the pathway are also highly conserved. However, there is no evolutionary study specifically on *APC* and as a part of my Ph.D. project, I wanted to take a look at how the APC gene family evolved over time to gain more insights into how it possesses its functional diversity.

Humans contain two APC homologs, *APC* and *APC2*. APC is the main APC that is expressed ubiquitously and is the most well studied APC due to its involvement in

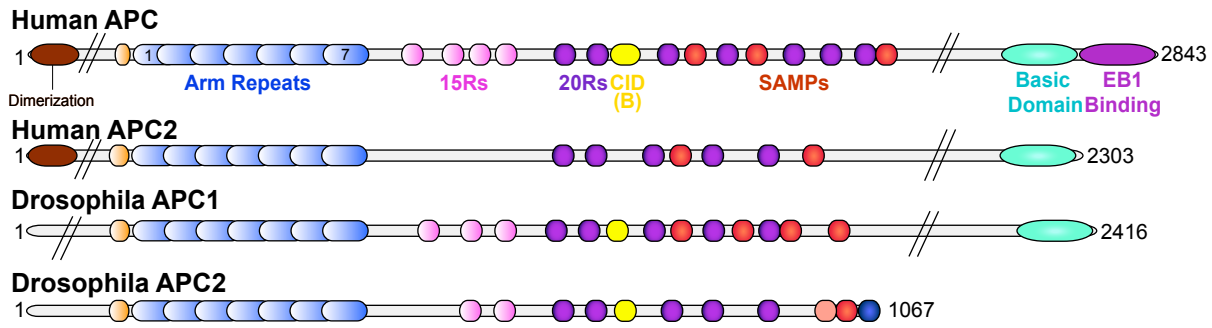


Figure 6: Schematic representation of human APCs (APC and APC2) and Drosophila APCs (APC1 and APC2). Many of the functional domains are evolutionarily highly conserved among all APCs.

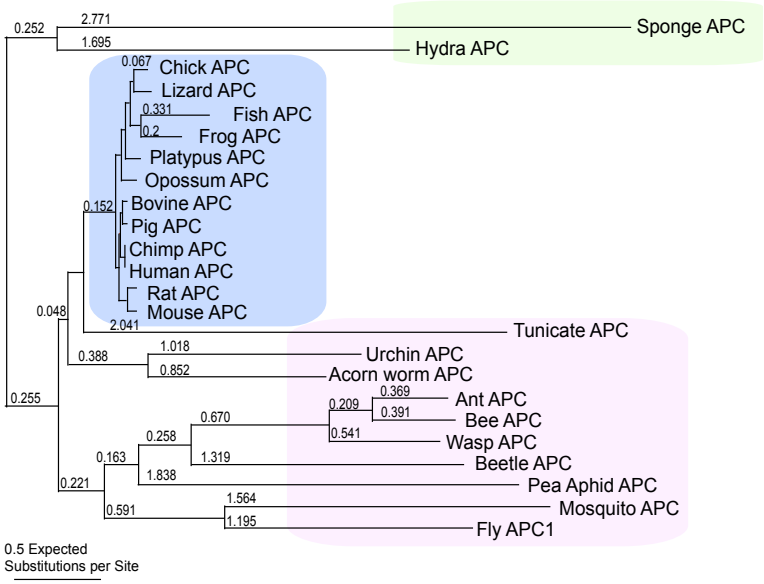
colorectal cancer. Human APC2 (hAPC2), on the other hand, is enriched in the nervous system and there is no clear connection with cancer. Both genes encode similar proteins with some differences in order and number of domains (which I will refer to as domain architecture) (Fig. 6). For instance, hAPC2 is missing the entire 15Rs and EB1/DLG binding domains. It only contains five 20Rs compared to seven in hAPC and only two SAMP repeats compared to three in hAPC.

Drosophila also has two APC homologs, APC1 and APC2. Similar to human APC proteins, most domains are functionally conserved. While dAPC2 is expressed mainly during embryogenesis, dAPC1 is enriched in the nervous system after embryogenesis (Appendix 2). Despite some differences in domain architecture, it has been thought that both proteins function redundantly in Wnt signaling (Akong et al., 2002). In order to study the evolution of the APC gene family, I analyzed a variety of sequences from different species. The human APC sequence was used as a query sequence in Protein Blast (pBLAST) across different vertebrate and invertebrate species to generate multiple sequence alignments (MSA). My analysis revealed interesting and surprising information with regards to the evolution of APC proteins.

Overall, vertebrate APC sequences are highly conserved: >80% conservation among all mammals from human to platypus, and >65% conservation among all vertebrates from human to fish (Fig. 7A, short branch lengths indicate similarity). On the other hand, vertebrate APC2's are slightly less well conserved: ~ 50% between human and fish. When we compare the APC and APC2 sequences, the level of conservation is still relatively high at the N-terminus which includes the highly structured Arm repeats (~60%), but it goes down to ~36% overall despite this the identified domains are highly conserved. Invertebrate APC sequences look quite different from hAPC (branch lengths get really long, Fig. 6A). However, this might be due the overall differences in domain architecture and failure of blast to recognize the similarities in a non-linear fashion. Regardless of the technical difficulties, presence of APC in lower metezoans is questionable (Adamska et al., 2010).

The phylogenetic tree incorporating both APC and APC2, suggests that there were two independent gene duplication events (Fig. 7B). One of these events seems to occur only in the vertebrate lineage earlier to give rise to APC and APC2. However, the

A Vertebrate and Invertebrate APCs



B Phylogenetic Tree of APC and APC2 in Metezoans

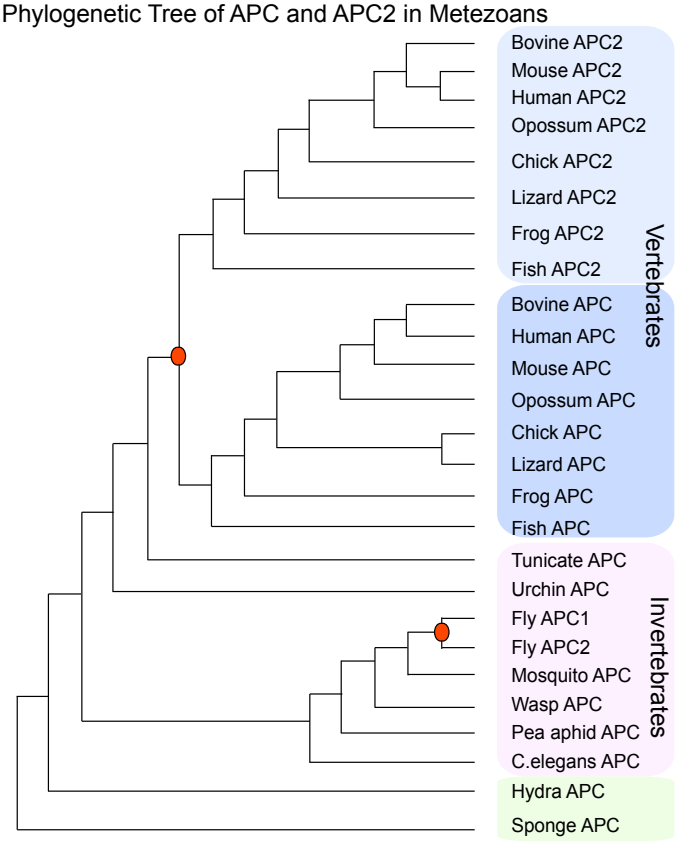


Figure 7: A) Sequence comparison of vertebrate and invertebrate APCs show the differences in sequence diversity (looger the branch length, more diverse). While the vertebrate APCs share high sequence similarity (short branch length), the invertebrate APCs are quite divergent from hAPC sequence. B) Phylogenetic analysis suggests two independent gene duplication events that resulted in the formation of two APC genes, APC and APC2 in vertebrates and APC1 and APC2 in Drosophila.

most striking finding was the fact that the second APC2 was only found in the Drosophila lineage of invertebrates that may be due to an independent duplication event that occurred very recently (Fig. 7B). Given the smaller genome size and extensive gene loss in the Drosophila lineage, it's interesting to find an opposing example (Harrison and Gerstein, 2002). Even the closest relatives to flies, mosquitos, have only one APC, while Drosophila has both APC1 and APC2. This might suggest, in other invertebrates having a single APC is sufficient.

1.2 Role of APC in the Destruction Complex:

Canonical Wnt signaling is a well-conserved pathway that regulates animal development both during embryogenesis by regulating: axis specification, cellular differentiation, proliferation and morphogenesis, as well as in the adult by maintaining certain stem cell populations (Holland et al., 2013; Logan and Nusse, 2004). Misregulation of the pathway is implicated in a vast array of human diseases like many types of cancer (colon, ovarian, breast), Alzheimer's disease, diabetes among others (Clevers and Nusse, 2012).

The canonical Wnt pathway is negatively regulated by the actions of a complex of proteins called the destruction complex that contains APC, Axin and the two kinases CK1 and GSK3ß. Destruction complex phosphorylates the effector of the pathway, ß-catenin, which leads to its ubiquitination by the ß-TrCP ubiquitin E3 ligase and subsequent degradation by the proteosome (Cadigan and Peifer, 2009) (Fig. 7). In cells receiving the Wnt signal, the Wnt ligand binds to the seven-pass transmembrane receptor Frizzled (Fz) and its co-receptor LRP5/6 This leads to the disassociation of Axin from the destructosome through the activity of Dishevelled (Dvl) and ultimately inactivates the destruction complex. Consequently, ß-cat accumulates in the cytoplasm and translocates into the nucleus. Here, together with TCF/LEF family transcription factors, ß-cat displaces the Groucho repressor and activates the transcription of Wnt target genes (Fig. 8). Therefore, loss of function mutations in components of the destruction complex, including APC, lead to ligand independent accumulation of ß-cat, and the constitutive activation of Wnt target genes that play roles in proliferation, cell

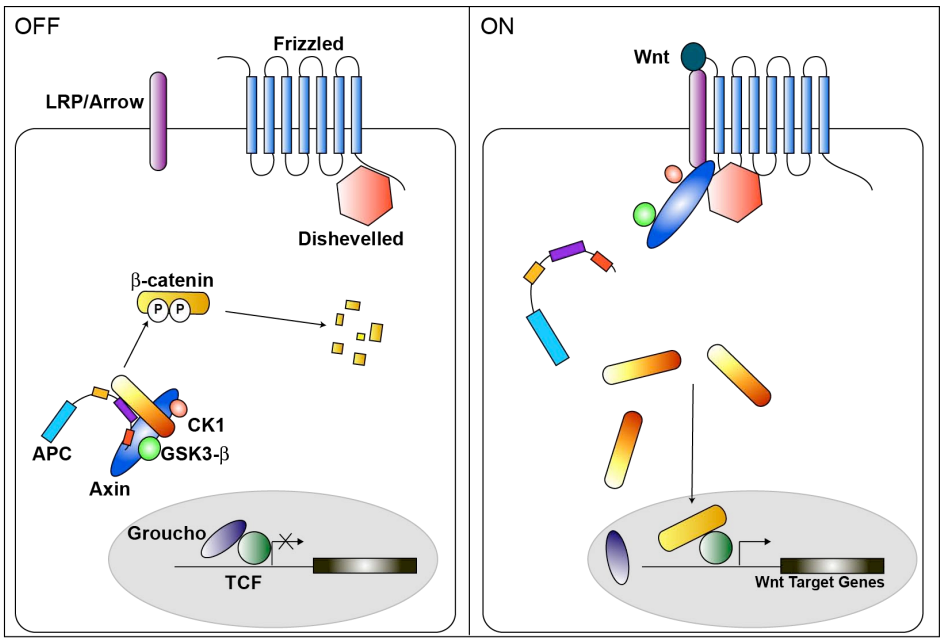


Figure 8: The canonical Wnt pathway is negatively regulated by the actions of a complex of proteins called the destruction complex that contains APC, Axin and the two kinases CK1 and GSK3ß. Destruction complex phosphorylates the effector of the pathway, ß-catenin, which leads to its ubiquitination by the ß-TrCP ubiquitin E3 ligase and subsequent degradation by the proteosome (Cadigan and Peifer, 2009). In cells receiving the Wnt signal, the Wnt ligand binds to the seven-pass transmembrane receptor Frizzled (Fz) and its co-receptor LRP5/6 This leads to the disassociation of Axin from the destructosome through the activity of Dishevelled (Dvl) and ultimately inactivates the destruction complex. Consequently, ß-cat accumulates in the cytoplasm and translocates into the nucleus. Here, together with TCF/LEF family transcription factors, ß-cat displaces the Groucho repressor and activates the transcription of Wnt target genes.

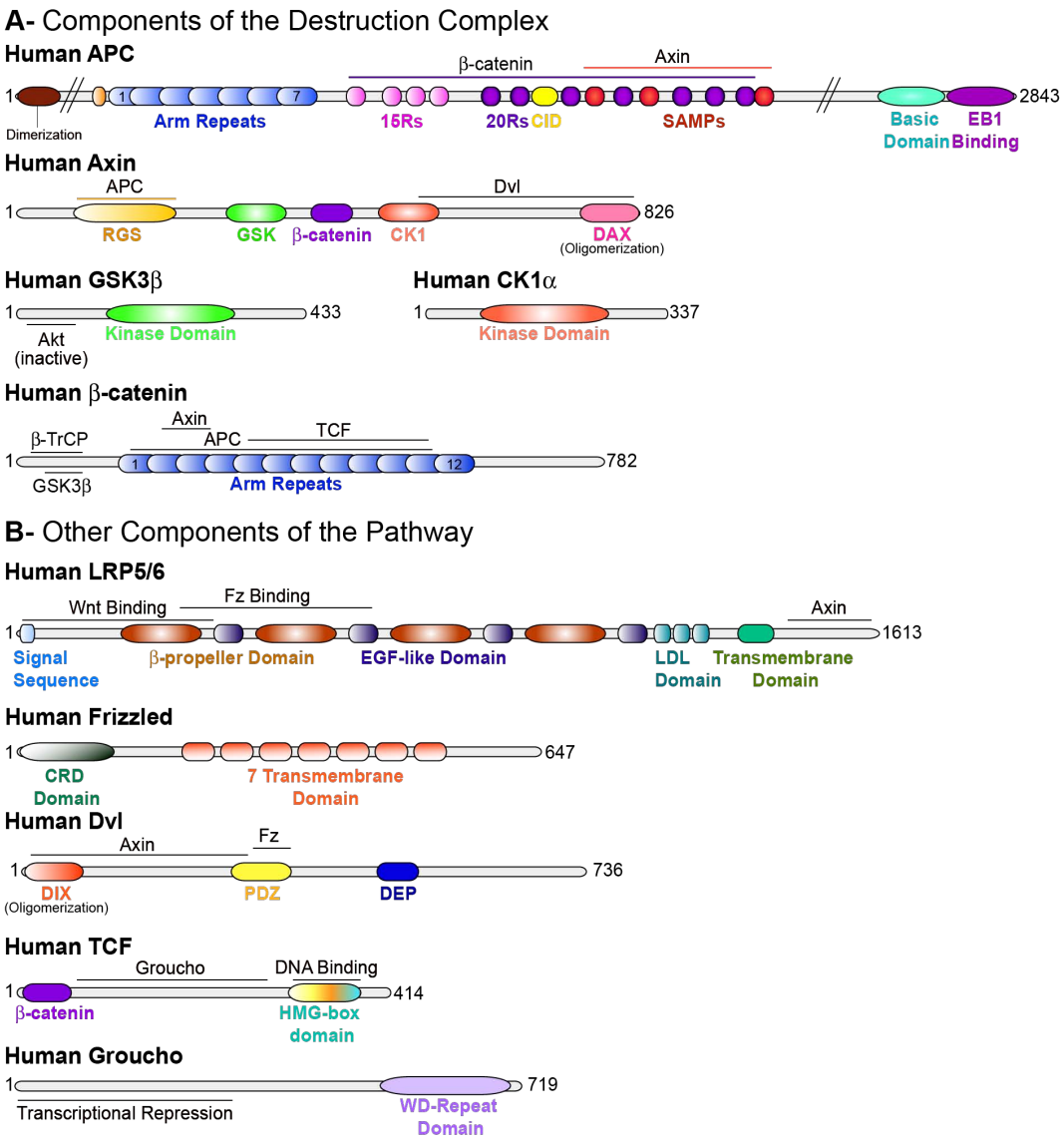


Figure 9: Schematic representation of Wnt pathway components. A) The destruction complex contains APC, Axin, and two kinases GSK3β and CK1 to target the effector of the pathway β-catenin for proteosome mediated degradation. B) Other proteins involved in Wnt signaling.

survival, and differentiation (Chen et al., 2003; Fodde, 2002; Komori et al., 2014; van de Wetering et al., 2002).

Despite its functional significance and abundant study, several fundamental questions remain as to how precisely the destruction complex works and what mechanistic roles APC plays within this complex. Several hypotheses for APC's destruction complex function have been proposed over the years. Because of its large size and the presence of many protein-protein interaction domains, APC was first thought to be the scaffold that assembled the complex. However, Axin is thought to be a better scaffold, because it can bind directly to all of the core components of the pathway including APC, ß-cat, GSK3ß and CK1, as well as Dishevelled, and the LRP5/6 co-receptor and enhance the rate of ß-cat phosphorylation (Fig. 7B) (Ha et al., 2004; Hart et al., 1998; Ikeda et al., 1998; Kishida et al., 1999; Mao et al., 2001). Later studies focused on the importance of the APC-ß-cat interactions and suggested that APC is required for ubiquitination complex to recognize the phosphorylated ß-cat (Kimelman and Xu, 2006), or to protect ß-cat from dephosphorylation by PP2A (Su et al., 2008) and, or to increase the activity of the destruction complex (Ha *et al.*, 2004). More recently, it has been suggested that APC functions downstream of ß-cat phosphorylation by mediating ß-cat's ubiquitination by ß-TRCP (Li et al., 2012; Yang et al., 2006). However, recent work questioned the importance of direct APC/ß-cat interaction and suggested a more downstream role for APC (Yamulla et al., 2014). Through my work described in this document, we discovered a novel role for APC proteins in the formation of the higher order destruction complex structures or destructosome (Kunttas-Tatli et al., 2012) (Kunttas-Tatli et al., 2014 in press). Our data support the model that APC promotes destructosome assembly by stimulating Axin polymerization via APC self-association, which I will discuss in more detail in Chapter 3.

In summary, the precise role APC proteins as a part of the destructosome in Wnt signaling is still enigmatic but it's clear that presence of APC increase the efficiency of ß-cat destruction both through its ability to self associate and also its direct connection with the degradation machinery. Optimized and efficient destructosome activity is essential for both normal development and for the prevention of Wnt signaling mediated cancers.

1.3 Role of APC in regulating the cytoskeleton:

Both actin and microtubules organize many of the dynamic cytoskeletal rearrangements that are needed for many cellular events, such as cell division, cell migration, and cell shape changes. These processes trigger drastic changes in the overall structure of a cell and are orchestrated via numerous actin and microtubule associated proteins. Some proteins function directly in polymerization/depolymerization, stabilization or bundling of actin and microtubules, while others act as regulators of these processes. Although many proteins and pathways have been identified, how these elements are orchestrated *in vivo* is still poorly understood.

Even though APCs function in regulating the Wnt pathway is an important aspect of its role in cancer, the cytoskeletal functions of APC are also thought to play a role in tumor initiation and/or progression (McCartney and Näthke, 2008). This makes understanding APCs cytoskeletal functions crucial, particularly, the role of APC proteins in regulating the actin cytoskeleton, which is the least well-understood aspect of their function and is the focus of this study.

1.3.1 Role of APC in regulating the microtubule cytoskeleton:

Because APC proteins are involved in regulating the microtubule cytoskeleton, I wanted to briefly mention some of the highlights. One of the first observations that linked APC proteins to the microtubule (MT) cytoskeleton was the fact that it localizes to the leading edge in migratory cells by interacting with MT plus ends (Näthke et al., 1996). Through these MT interactions, APC proteins can stabilize MTs both *in vivo* and *in vitro* (Zumbrunn et al., 2001). Furthermore, APC can interact with EB1 and the formin mDia1 to stabilize MTs in migratory cells and this appears to play an important role in cell migration (Gupta et al., 2014; Wen et al., 2004). In addition, APC plays a role in mitotic spindle anchoring and orientation by interacting with MTs and EB1. Loss or oncogenic APC truncations lead to chromosomal misalignments and chromosomal instability (Green et al., 2005; Näthke, 2004).

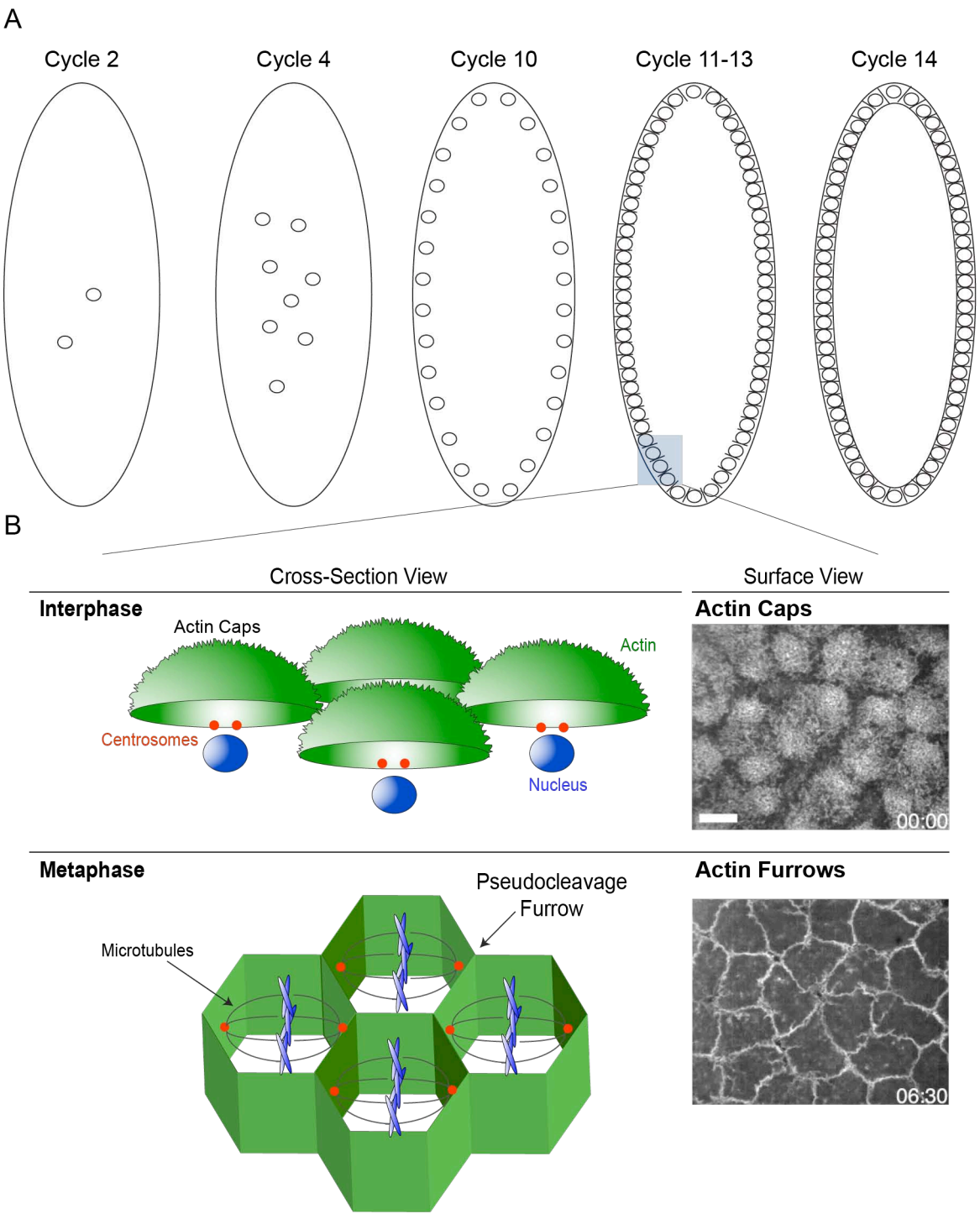


Figure 10: A) Drosophila development is syncytial with the first 13 nuclear divisions occurring without cytokinesis.The initial nuclear divisions take place in the central cytoplasm, but by nuclear cycle 10 the majority of the nuclei have migrated to the cortex.Cortical nuclei undergo four more rounds of synchronous division prior to cellularization at cycle 14. B) In concert with the nuclear divisions, cortical actin exhibits dramatic cell-cycle dependent reorganization and remodeling that coincide with specific stages of mitosis. In interphase, “actin caps” form above each nucleus that promotes centrosome separation and proper spacing of the nuclei. Actin caps expand during prophase and reorganize into actin-based extensions into the embryo called the “pseudocleavage furrows”.

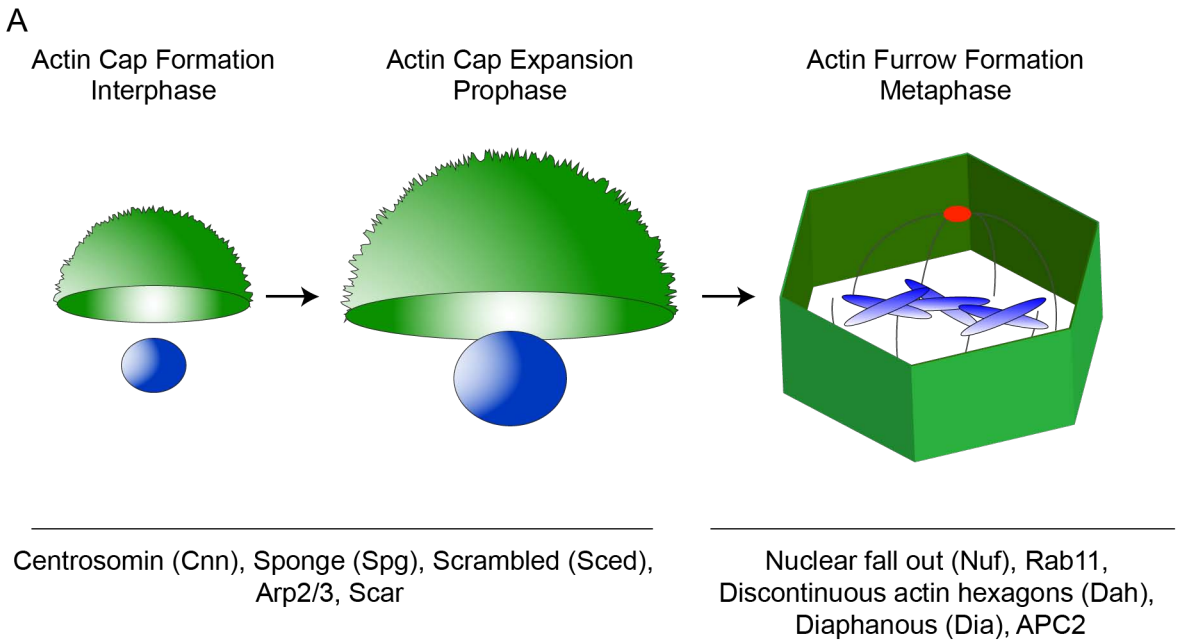


Figure 11: The actin caps form above each nucleus during interphase and expand throughout prophase. Actin isreorganized into pseudocleavage furrows during metaphase. While cap actin is mostly composed of branched actin filaments, furrows contain mostly linear. There are number of proteins involved in regulating this dynamic event (listed above).

1.3.2 Role of APC in regulating the actin cytoskeleton:

The actin cytoskeleton is essential for many dynamic cellular processes during development such as cytokinesis, cell motility and morphogenesis. This makes understanding the factors that regulate actin cytoskeleton necessary. Studies from Drosophila epithelial and syncytial cells as well as cultured mammalian cells revealed that APC localizes to cortical actin (McCartney et al., 1999; Rosin-Arbesfeld et al., 2001), and both hAPC and dAPC1 can directly interact with F-actin via its C-terminal basic domain, the same domain that directly binds MTs (Moseley et al., 2007).

More recently, our laboratory was the first to associate APC proteins (dAPC2) and the formins (Diaphanous (Dia)) during actin assembly in the whole organism (Webb 2009). This relationship was further supported by in vitro studies that dissected the underlying molecular mechanisms between the basic domain of hAPC and the DAD domain of Dia to enhance actin nucleation (Jaiswal et al., 2013; Okada et al., 2010). Once nucleation is initiated, APC stays at the pointed end while Dia tracks the barbed end to promote elongation (Breitsprecher et al., 2012). These studies also demonstrated that APC could itself nucleate actin *in vitro* via the Actin Nucleation Sequences (ANS 1 and ANS2) within the basic domain (Okada et al., 2010) (Fig 10).

While the actin related functions of APC proteins have recently received more attention *in vitro*, few studies have attempted to examine these mechanisms *in vivo*. Our lab makes use of the Drosophila syncytial embryo as a model system for studying dynamic cytoskeletal rearrangements and the relationship between APC proteins and Dia. Therefore, I will introduce our model system and discuss the behavior of the actin cytoskeleton at this time of development.

1.3.3 Drosophila APC2 and its role actin furrow extension:

The Drosophila syncytial embryo is a powerful *in vivo* system to study dynamic cytoskeletal rearrangements. Like other insects, early Drosophila development is syncytial with the first 13 nuclear divisions occurring without cytokinesis (reviewed in Sullivan and Theurkauf, 1995). The initial nuclear divisions take place in the central cytoplasm, but by nuclear cycle 10 the majority of the nuclei have migrated to the cortex (Fig. 8A). Cortical nuclei undergo four more rounds of synchronous division prior to cellularization at cycle 14. In concert with the nuclear divisions, cortical actin exhibits

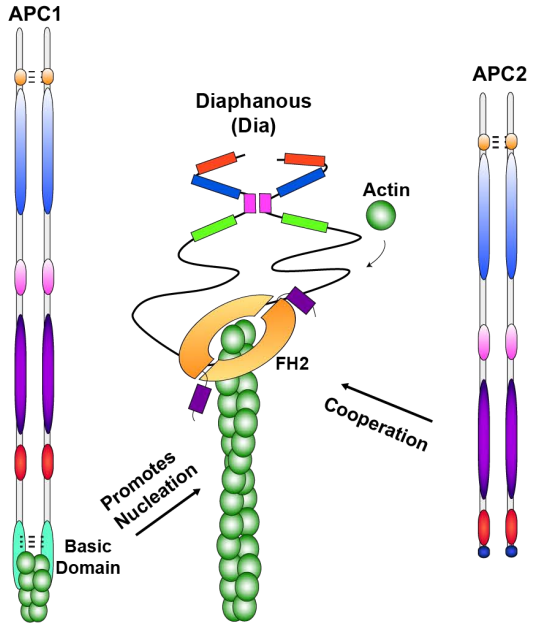


Figure 12: Both vAPC and dAPC proteins collaborate with Dia to assemble actin filaments. While vAPC and dAPC1 uses the basic domain to nucleate actin and synergize with Dia in vitro. The molecular mechansim of dAPC2 and Dia relationship is still unclear despite in vivo evidence.

dramatic cell-cycle dependent reorganization and remodeling that coincide with specific stages of mitosis. In interphase, “actin caps” form above each nucleus that promotes centrosome separation and proper spacing of the nuclei. Actin caps expand during prophase and reorganize into actin-based extensions into the embryo called the “pseudocleavage furrows” (Fig. 8B). These furrows act as physical barriers between neighboring dividing nuclei and prevent collisions to ensure mitotic fidelity. Unlike cytokinetic furrows, metaphase furrows are independent of the actomyosin-based contractile ring and Myosin II activity (Albertson et al., 2005; Royou et al., 2002). During anaphase, these furrow regress and during telophase actin is reorganized into caps above each centrosome that persist into the next interphase. Each nuclear/actin cycle happens very quickly and takes ~10-20 minutes.

Previous work from our laboratory showed that Drosophila APC2 together with the formin Dia promotes pseudocleavage furrow extension in the early embryo (Webb et al., 2009). Because the activity of Dia related formins involves a release from an autoinhibited state, we proposed that APC2 may activate Dia either directly by releasing the autoinhibition or indirectly by recruiting a Dia activator. The activity and colocalization of the APC2-Dia complex is cell cycle dependent, which suggests a potential cell cycle related regulatory mechanism. In addition to Dia and APC2, a variety of known actin regulators function in this system, which I will summarize in the following section.

Other proteins in cortical actin organization in the syncytial embryo

a) Proteins involved in actin cap formation and expansion:

Centrosome movements coincide with movements of the actin caps and failure during centrosome separation leads to defects in cap expansion (Cao et al., 2010). Centrosomes are duplicated during late anaphase and actin caps start to form above each centrosome pair during telophase. Centrosome pairs migrate along the nuclear envelope to move to the opposite poles during prophase. Proper centrosome separation is required for positioning of the mitotic spindle (Vaizel-Ohayon and Schejter, 1999).

Vast number of additional proteins plays various roles during cap formation and expansion. A centrosome protein Centrosomin (Cnn), an unconventional RacGEF Sponge (Spg), Scrambled (Sced), the branched actin nucleator Arp2/3, and its activator

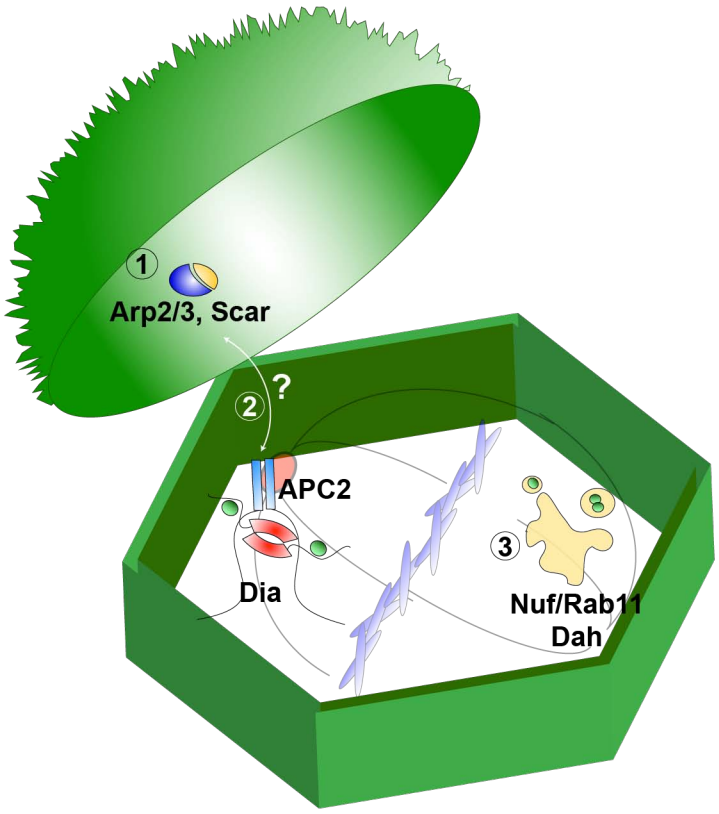


Figure 13: Various mechanisms regulate both actin cap and actin furrow formation. 1) Arp2/3 and its activator Scar is involved in cap formation and expansion. 2) APC2 Dia promotes the formation of actin furrows. 3) The recycling endosome pathway components Nuclear fall out (Nuf), Rab11 and Discontinuous actin hexagons (Dah) utilize MT-based transport of membrane and actin to the site of furrow extension. The cross-talk between these various mechanisms is not well understood.

Scar are all involved in this process and mutants display defects in cap formation and expansion as well as furrow formation, which might be an indirect consequence of the expansion defects. (Postner et al., 1992; Stevenson et al., 2002, 2001; Vaizel-Ohayon and Schejter, 1999; Zallen et al., 2002). The cap actin is mostly formed by branched actin filaments, thus, it's not surprising to find branched actin regulators in the formation and expansion of actin caps. However, little is known about the role of linear actin filaments in this process, which I will explore more in depth in Chapter 5.

b) Proteins involved in pseudocleavage furrow extension:

Actin cap expansion is followed by actin ring and furrow formation between neighboring nuclei when two caps meet each other. The best understood mechanism for pseudocleavage furrow formation is via the recycling endosome pathway and involves Nuclear fall out (Nuf), Rab11 and Discontinuous actin hexagons (Dah) (Riggs et al., 2007). These proteins utilize the MT-based transport of membrane and actin to the site of furrow extension. We predict that the delivered actin can then be used by the APC2-Dia complex to promote actin nucleation and furrow extension (Webb et al., 2009). Additional actin regulators such as Abl, RHO1, RhoGEF2 are thought to play a role in some aspects of actin activity but not directly involved in furrow formation (Grevengoed and Peifer, 2003; Grosshans et al., 2005; Webb et al., 2009).

In conclusion, during syncytial development, the actin cytoskeleton undergoes dramatic remodeling in a cell cycle dependent manner, which is mediated by numerous proteins (Appendix 4). The early Drosophila embryo provides a relatively simple system to study the functions of these proteins individually or intersections between different pathways.

1.4 Phosphorylation and Self-association to regulate APCs Activity

1.4.1 Phosphorylation as a Mechanism to Regulate APCs activity:

One of the most common post-translational modifications is phosphorylation, which is achieved by the opposing actions of protein kinases and phosphatases. One third of all cellular proteins are regulated via reversible phosphorylation, a commonly used regulatory mechanism for numerous cellular events during development of an organism (Glover, 1998). ~20-23% of APC proteins (210 out of 1067 in dAPC2 and 653

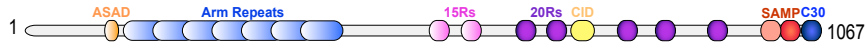
out of 2843 in hAPC) are composed of Ser, Thr or Tyr residues. Phosphorylation of APC has been shown to regulate its activity and localization in a context specific manner and thus might be regulated by the signaling status of the cell, stage of the cell cycle or different developmental stages (Ha et al., 2004; Ikeda et al., 2000; Kunttas-Tatli et al., 2012; McCartney et al., 2001). This suggests that phosphorylation acts as a key regulatory mechanism for APCs diverse functions. This raises the possibility of having different phosphorylation-profile or signature, which can modulate its diverse functions in a context specific manner. Here are some examples of phosphorylation by various kinases, and how they affect APCs activity in various contexts.

a) GSK3ß *and CK1 Phosphorylation:* APCs 20Rs contain a series of consensus sites for CK1 and GSK3ß and the significance of these sites were explored in depth both *in vitro* and *in vivo* (Ha et al., 2004; Ikeda et al., 2000; Kunttas-Tatli et al., 2012; Liu et al., 2006). These studies suggested that phosphorylation of the 20Rs plays a key role in regulating APCs activity in the destruction complex by modulating the APC's binding affinity to ß-cat and play an essential role for proper APC activity.

In addition to the importance of GSK3ß phosphorylation within the destruction complex, it is essential for regulating Drosophila APC2s localization during early development; the normal cortical localization of dAPC2 was highly disrupted in GSK3ß mutants. Furthermore, the interaction of hAPC with MTs decreases by GSK phosphorylation, suggesting a functional switch between destruction complex complex function vs MT function (Zumbrunn et al., 2001).

b) CK2 and PKA Phosphorylation: In addition to their localization to the cortex and in the cytoplasm, APC proteins can also be found in the nucleus (Henderson, 2000). Nuclear APC is thought to interact and sequester ß-cat away from the TCF-Lef transcription factors and thus provide an additional layer of negative regulation (Sharma et al., 2014). In this context, phosphorylation of sequences flanking the nuclear localization signal (NLS) by CK2 and PKA play opposing roles in APCs subcellular localization (Zhang et al., 2001). Phosphorylation of these sites by CK2 enhances NLS activity; in contrast, phosphorylation by PKA inhibits nuclear localization.

Drosophila APC2



ASAD

MTLNESDA**T**RLELTRNFLELSRNPETCTALRS**S**DCIQLLVQILHANDEGLSTAKKYASQALHNIVHNNPEEKERQREVKMLRLLDQILDYCNFLHTQLQSG

Arm Repeats

GEAIADDEDRHPLAAMKLLMKASFDEEHRQ**T**MCELGALKAIPNLVHLDHAVHGPAAGREQCNALRSYGLMALTNLTFGDENVHNKSYLCGQRQFMEVV

IAQLNTAPDELLQVLAGVLRNL**S**WRADKHMKTIFNELGTVTSLARAAMQNKNENTLKAILSALWNLSAHCSTNKAEFCAVDGALAFLVGML**S**YEGPSKTL

KIIENAGGILRNV**S**SHIAVCEPYRQILRRYNCLAILLQQLKSESLTVVSNSCGTLWNL**S**ARCPEDQQYLIDHNAIPLLRALISSKN**S**MIAEGSASALKNLVNF

RATQELMPNGDGGSLPLDKEAGHGGTLPRRF**SS**LRL**S**SNPTGSLKKVRP**ST**V**S**TTGFLNRKCE**S**RE**S**IY**S**GK**S**D**S**TK**YS**TKSEGAKNPFEIV**T**

15R1 15R2

PTEEQPIDYSMKYMEHKPN**S**SKTFEIDLDQPTDFSARYKERRSAQTAQPELKSETNEIR**S**KELQLTK**S**S**S**A**T**ELRN**S**PGLVAVSAAKQKIATE**T**ETETA

20R1 20R2 CID

ERPIN**Y**CEEG***T***PGSF***S***RFD***S***LN***S***L***T***EKPEKCMPPK**T**PTKTAVLPVHVDGNTPQNID**S**ALETPLMF**S**RR***S******S***MD***S***LVGDDETVACEDNG**S**VI**S**EYSR

20R3

MQ**S**GVI**S**PSELPD**S**PTQSMPQ**S**PRRDRKV***S***TQNNLD***T***PEQKPSTVFEDKLNRFHVEH**T**PAAF**S**CA***TS***L**S**NL**S**MMDD**S**NANAIRGQRGNDINGN

20R4 20R5

GDAPR**S**YC**T**EDT**T**AVL**S**KAP**S**N**S**DL**S**ILSIPNDLNANEAQPVPAPRADVTGMDTRMPAEDAISKMRCGGNALPSYLPVSDEMSK**Y**YVEDSPCTF**S**V

SAMP1

I**S**GL**S**HL**T**VGSAKAGPVLKLPMRTAEEAQAPKLPPRRSAVQGDAEPRLPPKK SD***S***L***SS***L**S**MD**S**DDDCNLL**S**QAIAAGSCRPQP**S**GA**S**TSSSLANA

SAMP2 C30

STSTLCRENGQSKKQVEHGDKPN***YS***SDD***S***LDDDDDDAR**S**K**S**LFEQCIL**S**GMHK**S**NDALE**S**EGEPPGQRQEISARDRFVSNQVRQIESMLAGRQH

S/T/Y Phospho-proteome Study + Computational Predictions
S/T/Y Phospho-proteome Study

Figure 14: In order to identify putative phosphorylation sites for dAPC2, I combined computational approaches with experimental evidence (phospho-proteome study). I mapped all the predicted sites on to the dAPC2 sequence. The putative domains on the schematic are also color coded onto the amino acid sequence.

c) Cdk Phosphorylation: Vertebrate APC is phosphorylated during all phases of the cell cycle, but hyper-phosphorylated during M-phase (Bhattacharjee et al., 1996). There are eleven putative Cdk sites in the C-terminal region of hAPC. *in vitro* studies demonstrated that vertebrate APC could be phosphorylated by Cdks (Trzepacz et al., 1997) and that some of these sites play a functionally significant role during mitosis. However, the precise role of Cdk phosphorylation in regulating APCs activity is still not known.

In summary, APC proteins are regulated by phosphorylation via various kinases. Some of these events direct APC to a specific subcellular location such as to the nucleus, or modulate its binding affinity towards other proteins such as ß-cat. Thus, phosphorylation is a fundamental mechanism regulating APCs and raises the possibility that APC proteins might have different phospho-signatures mediating its diverse functions. However, it is still not clear how these changes in the phosphorylation status of the protein are achieved at various times and how they are coordinated.

During my Ph.D., I was deeply interested in studying phosphorylation of as a regulatory mechanism, and used the experimentally tractable Drosophila APC2 to test how it affects APC2's role in regulating both Wnt signaling and the actin cytoskeleton. I predicted that phosphorylation of various domains of APC2 modulates its binding interactions with other proteins such as Arm, Axin within the destruction complex, or Dia in the context of cytoskeletal regulation.

1.4.1.1 Computational Analysis of APC2 Phosphorylation

To begin to identify some of these phospho-signatures, I first gathered all of the available data in the literature, as well as made use of the available computational prediction methods to create a map of Drosophila APC2 phosphorylation.

In order to identify phosphorylation sites and kinases, I used two web-based computational approaches. NetPhos uses artificial neural networks (ANN) to predict kinase-specific phosphorylation sites based on experimental evidence (Blom et al., 2004) (Fig. 12). This is advantageous because the ANN can analyze complex and nonlinear biological data and generate correlations between sequences and recognize similar but not identical sequence patterns. In contrast, other programs such as Motif Scan searches for only small sequence motifs. The Motif Scan is another web-based

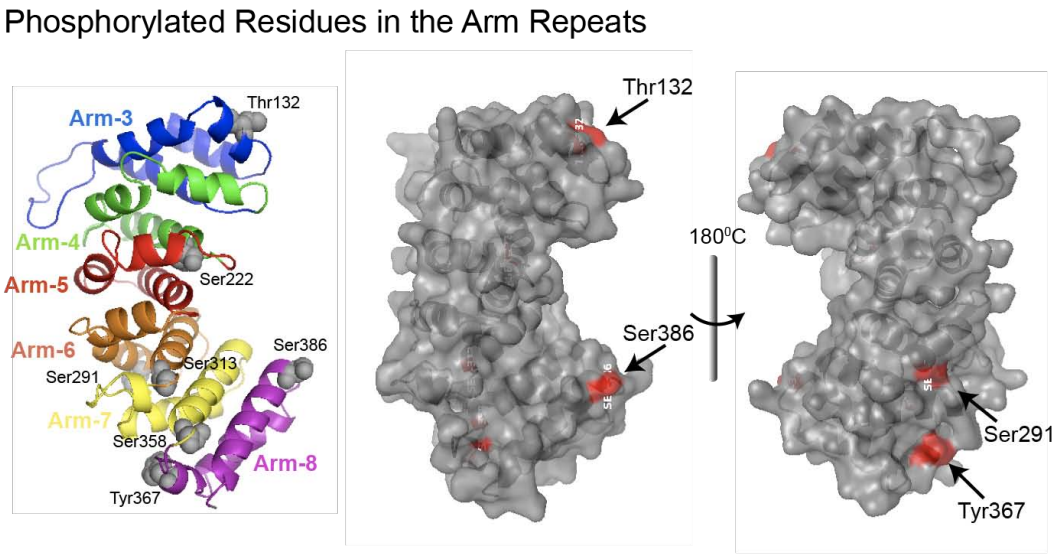


Figure 15: Few residues are predicted to be phosphorylated in the Arm repeats due to this region being tightly packed. Modeling the APC2 Arm repeats by using the hAPC crystal structure, I mapped the predicted phospho-residues (red). Many of these residues are not solvent accessible and is unlikely to get phosphorylated but some are like Tyr367.

computational program that searches for short consensus motifs (10-15 amino acid long) represented as a position-specific matrix (PSSM) for various kinases. These motifs are based on experimental binding studies, oriented peptide libraries and phage-display experiments. The computational analysis by using both of these methods revealed that only certain domains of APC2 are phosphorylated including the 20Rs (except 20R5), SAMP repeats and CID. The 20R phosphorylation has been demonstrated experimentally and thus serves as a good internal control for the accuracy of the predictions. Surprisingly, the SAMP repeats contain a number of predicted phosphorylated residues (Fig. 12). SAMP-repeat phosphorylation has not been previously reported in the literature. Lastly, CID (or region B) contains multiple residues that are predicted to be phosphorylated, which may contribute to their function. Only a few residues are predicted to get phosphorylated in the Arm repeats. This is not surprising as this is a tightly packed structural domain (Zhang et al., 2011). I carried out structural modeling analysis to identify the locations of these residues and most of them are not solvent accessible (Fig. 13).

In order to test the fidelity of these predictions, I compared these results to experimental studies including the mass spectrometry analysis for the entire Drosophila phospho-proteome (Zhai et al., 2008) (Fig. 12). This study identified 32 phosphorylation sites in dAPC2. Many of the experimentally verified sites were also identified by the computational analysis, suggesting that these predictions are indeed reliable. The lack of complete agreement is not surprising because the analyses of phosphorylation sites in Drosophila APC2 are extremely limited and may not have captured transient or stage specific events. In addition, not all experimentally predicted sites were computationally predicted, which might be due to the fact that not all kinases have a specific consensus.

1.4.1.2 2DE and DIGE Approaches to study APC2 Phosphorylation:

In addition to the computational analysis, previous studies on 1D gels also demonstrated that APC2 is a phosphoprotein (McCartney et al., 1999). The predicted molecular mass of the protein is 117 KDa. However, APC2 runs at a much higher mass, around 160 KDa, and runs as a smear on a SDS-Page gel due to multiple phosphorylation. Protein phosphatase 2A (PP2A) treatment reduces the mass of the

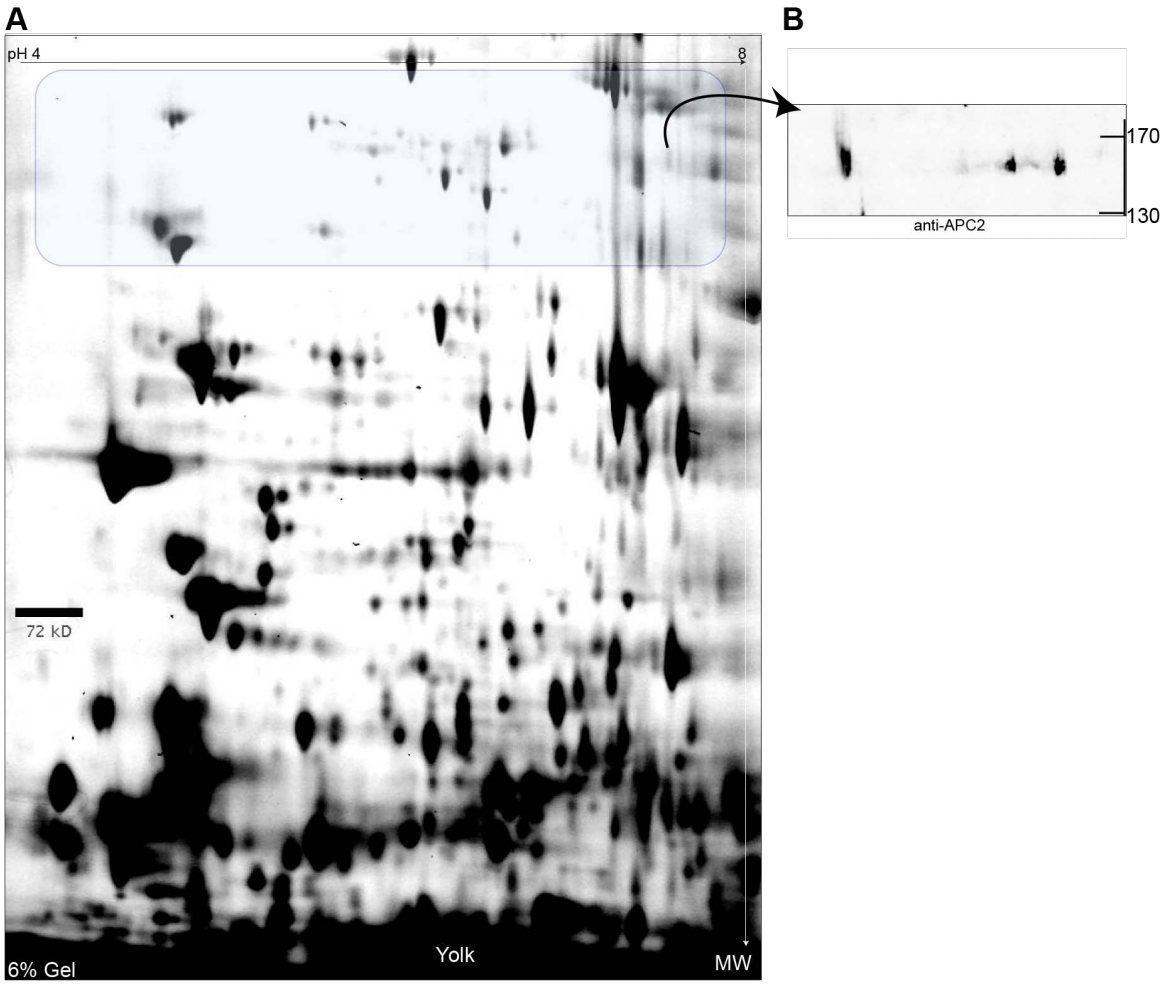


Figure 16: A) 2D gel of a 2 hour Drosophila embryo lysate, ran with a 6% gel. Top part of the gelwas cut and blotted for APC2. B) Western Blot of a region of the 2D gel probed for APC2.

protein on the gel, as well as collapses the smear into a tight band indicating that APC2 is a phosphoprotein (McCartney et al., 1999).

Both the phospho-proteome study and computational predictions informed us about some of the phosphorylation sites, but this was limited by the fact that only the prominent phosphorylated peptides were represented in the 0-24 hr. sample. Both experimental and computational studies lacked temporal information that may reveal essential aspects of APC2 regulation. I decided to map the phosphorylation sites in the blastoderm stage embryo because the syncytial embryo is a homogenous cell population with no zygotic transcription. We predicted that these features would make it easier to map the phosphorylation sites during specific stages of the cell cycle or identify important phospho-residues specific for APCs cytoskeletal functions. I performed two-dimensional gel electrophoresis (2DE) and two-dimensional difference gel electrophoresis (2D-DIGE) experiments.

One of the most effective methods for identifying protein posttranslational modifications (PTMs) such as phosphorylation is by using 2DE. Phosphorylation introduces negatively charged groups, which causes detectable changes both in the mass and charge of the protein. Even though the change in the mass is too small to detect on a 1D SDS-PAGE, the change in the pI can be detected easily on the 2DE in the horizontal plane. The 2DE gel was followed by a western blot to detect APC2 and its phospho-isoforms. I planned to couple the 2DE experiments to mass-spec (MS) analysis to map specifically the phosphorylated residues in APC2. However, I wasn't able to identify APC2 on the 2D gel, mostly due to its lower expression level and the Western blot results were inconsistent (Fig. 14). This led me to focus on DIGE experiments instead to explore overall proteomic changes in various APC mutant backgrounds. Surprisingly, there were number of changes in the *APC2* null background compared to wild type, most of which were changes in lateral plane, indication of phosphorylation changes (Fig. 15). At this point, I wasn't able to achieve my goal of identifying phospho-isoforms of APC2. Consequently, I decided to focus on the previously described computational and experimental predictions of phosphorylation. However, I'm happy to see another graduate student, Malachi Blundon, pursue this project to further elucidated the consequences of loss of APC activity on protein

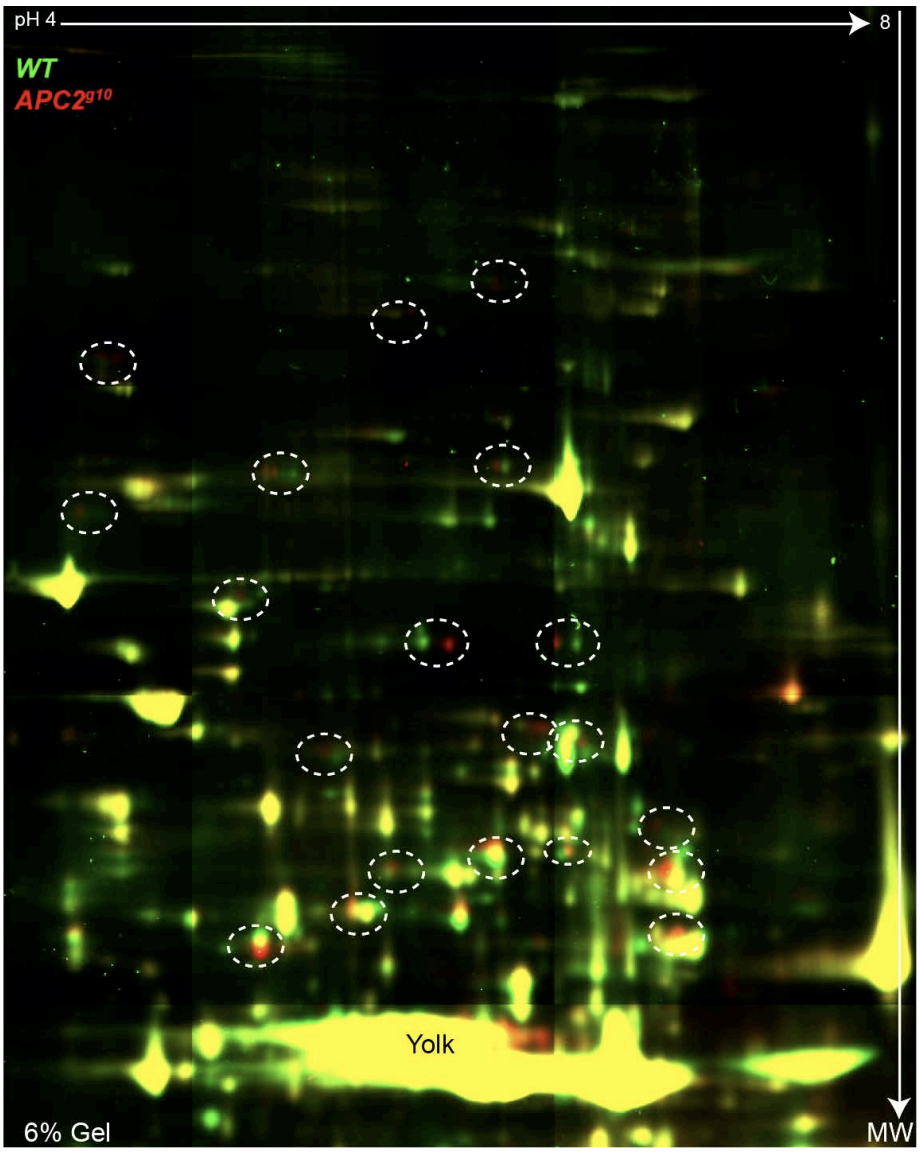


Figure 17: 2D/DIGE gel of a 2 hour lysates from wild type and APC2g10 embryos. Wild type lysate labeled with Cy3 (green) and APC2 with Cy5 (red). Surprisingly many changes in the horizontal plane were observed, indication of phosphorylation.

networks. He successfully identified a number of protein modifications in the *APC* null background, and is now exploring the molecular role of APC proteins in a global cellular context that may contribute to its role in cancer development.

1.4.2 Self-association as a Mechanism to Regulate APCs activity:

In addition to phosphorylation, self-association is another potential mechanism to regulate the activity of APC proteins both in the context of Wnt signaling or cytoskeletal regulation. However, it is not well-understood or well-studied in the field, even though it's been known for decades that Vertebrate APC (vAPC) can self-associate (Joslyn et al., 1993) and it might have an effect on cancer initiation and progression (Schneikert et al., 2011; Su et al., 1993). Studying the precise role of vertebrate APC self-association has been difficult in part due to the presence of multiple self-association domains dispersed throughout the protein.

As mentioned before, N-terminal to the Arm repeats, vAPC can form coiled-coil based parallel dimers through an N-terminal coil (oligomerization domain, OD-1) (Fig.1). Even though, this is the first dimerization domain to be discovered, the precise role of OD-1 in normal APC function is not well understood. However, APCs ability to dimerize is thought to contribute to its disease relevance. The wild type APC protein is show to dimerize with both wild type and truncated mutant APC proteins (Su et al., 1993), which raises the possibility of the truncated mutant APC proteins to act like a dominant negative and interfere with the tumor suppressor activity of APC (Su et al., 1993) (Fig 16).

In addition to the N-terminal dimerization domain, two more self-association domains were discovered. A region between the Arm repeats and the first 15R in vAPC is shown to self-associate, which has been implicated in APC's normal cytoskeletal functions during cell migration (Li et al., 2008). In addition, phosphorylation enhanced this self-association, which in turn supported the assembly of microtubule-associated APC clusters at the cell periphery (Li *et al.*, 2008). Dispersing these APC clusters by disrupting self-association reduced cell migration. Lastly, the dimerization coil ANS2 within the basic domain is essential for APC's actin nucleation function

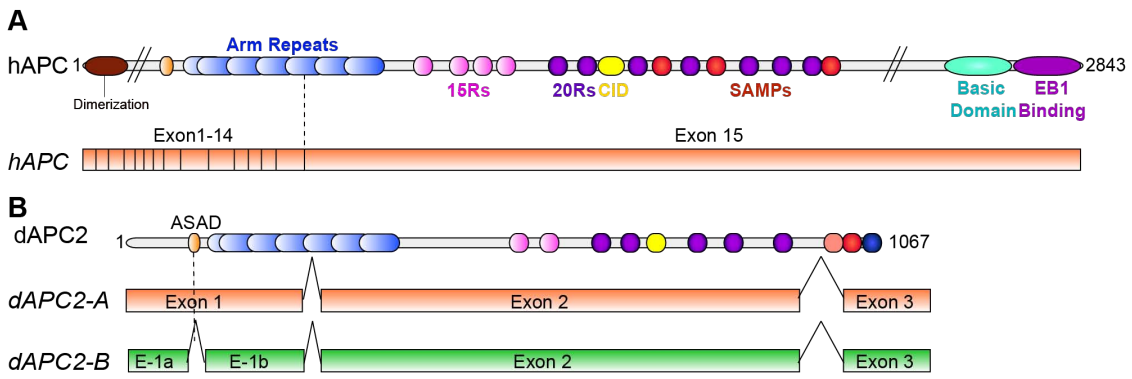


Figure 18: A) Most common *hAPC* consists of 15 exons that encode a 2843 amino acid protein. Exon 15 is the largest exon (>75% of the coding sequence) and usually the target for mutations. Alternative splicing is mostly observed in the first 14 exons that lead to the expression of various isoforms. B) dAPC2 contains only 3 exons and alternative splicing is observed in only in exon 1. Alternative splicing of exon 1 takes out a small region in the protein that contains the self-association domain (ASAD).

(Okada *et al.*, 2010). Through ANS2 based dimerization, the basic domain can recruit up to four actin monomers to form a polymerization seed.

The complexity of vAPC self-association prompted me to examine its role in the destructosome using the simpler model, Drosophila APC2 (see Chapter 4). Although Drosophila APC2 doesn't share sequence homology to the vAPC's self-association domains, previous studies from our laboratory and others have shown that Drosophila APC proteins do self-associate through an N-terminal domain (Mattie et al., 2010; Roberts et al., 2012; Zhou et al., 2011). Thus, I looked for a structural conservation and identified a region just upstream of Arm repeats (APC Self-Association Domain (ASAD)). I've shown that ASAD directly mediates self-association of dAPC2 and also association between dAPC2 and dAPC1. My studies revealed a novel role for self-association in the assembly and stability of the destructosome. These results suggest that APC proteins are required not only for the activity of the destructosome, but also for the assembly and stability of this macromolecular machine. Lastly, the presence of multiple self-association sites within vAPC suggests that the protein may have the ability to form large oligomers in addition to dimers, although it is not clear whether this occurs *in vivo*.

Alternative splicing as a mechanism to regulate APC self-association

Interestingly, alternative splicing is a potential mechanism to regulate hAPCs ability to self-associate. Splice variants of hAPC that skips exon-1 encoding the N-terminal dimerization domain have been reported. This splice variant is enriched in mouse and human brain and heart (Thliveris *et al.*, 1994). A splice variant of hAPC that deletes a portion of the ASAD has also been observed (Groden *et al.*, 1991; Joslyn *et al.*, 1991), and there are some reports of colorectal cancer associated mutations in this region that may result in increased production of the ASAD lacking isoform (van der Luijt *et al.*, 1995). However, the data are limited and the functional consequences are unclear.

Surprisingly, Drosophila also express an alternate splice form of *APC2* lacking the ASAD (Fig 16) (see chapter 4) (Appendix 1). This suggests that Drosophila may be a simpler system to examine the functional consequences of these alternative APC

isoforms. This also suggests a normal *in vivo* function for the monomeric APC (APCm). While the precise function of APCm is not known, my studies suggest that APCm could interact more efficiently with Arm repeat binding proteins such as Kap3. Consistent with this observation, the binding of the second oligomerization domain decreased Kap3 association with the Arm repeats (Li & Näthke, 2005). This suggests that APCm may exhibit enhanced binding to a broad array of Arm repeat partners including Kap3, Asef, IQGAP and the PP2A regulatory subunit (Jimbo et al., 2002; Kawasaki, 2000; Seeling et al., 1999; Watanabe et al., 2004).

1.5 APC and Human Disease

Familial adenomatous polyposis (FAP) is a colorectal cancer predisposition disease, characterized by early development of thousands of polyps. If left untreated, these polyps develop into colorectal cancer. Attenuated FAP (AFAP) is a less severe form of the disease, characterized by the presence of less than 100 polyps (Fearnhead et al., 2001).

Germline mutations in the APC gene are responsible for the inherited familial adenomatous polyposis (FAP), while somatic mutations occur in ~80% of colorectal cancers (Clevers and Nusse, 2012). The majority of these mutations are either nonsense or frameshift mutations that result in a truncated protein. Many of these mutations occur in the middle of the protein in a hot spot called the Mutation Cluster Region (MCR) and produce APC truncations missing the entire C-terminal region (Fig 17). Most patients with inherited germline APC mutations also carry additional somatic APC mutations, which can be described by Knudson's two-hit hypothesis (loss of heterozygosity) (Fearnhead et al., 2001). Loss of Wnt-associated domains and subsequent disruption of APC's function as a negative regulator is thought to be a major factor in the initiation of colon polyps (Cadigan and Peifer, 2009).

However, loss of other domains is also implicated in mitotic abnormalities and chromosomal instability due to disruption of APCs microtubule cytoskeletal roles that are thought to contribute to cancer progression (Caldwell et al., 2007). The role of APCs actin-associated functions in human disease has received less attention, which makes it difficult to understand the relationship less clear. Recent evidence points to a role for

Asef in the development of APC dependent intestinal adenomas, suggesting that disruption of at least some of APC's actin functions may contribute to cancer progression (Kawasaki et al., 2009). However, further research is required to understand the precise role of APC mutations in the initiation of colon cancer for us to generate therapeutic strategies.

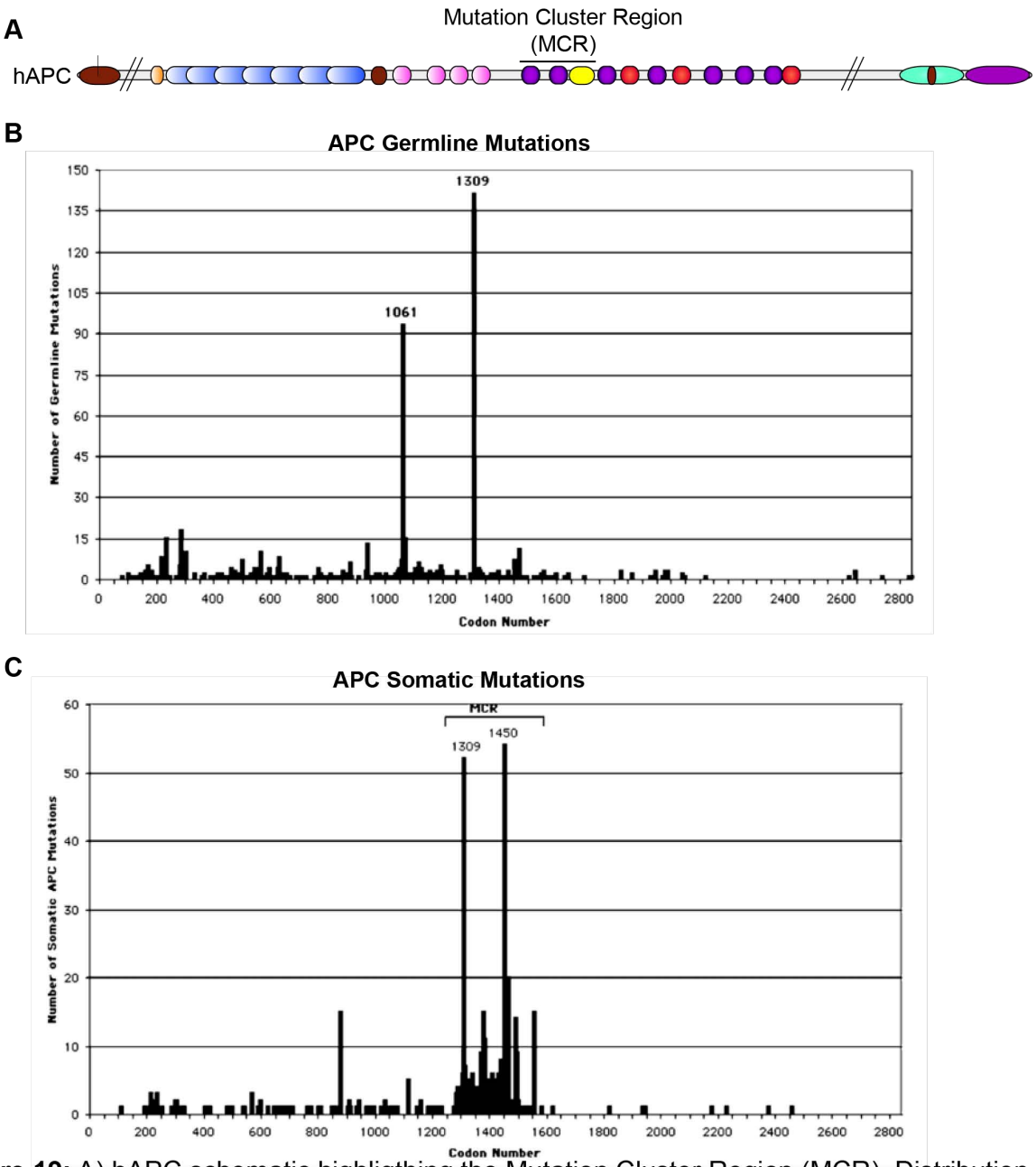


Figure 19: A) hAPC schematic highligthing the Mutation Cluster Region (MCR). Distribution of APC germline mutations (B) and somatic mutations (C) (modified from Fearnhead et al., 2001).

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Chapter 2:

The activity of Drosophila APC2 in canonical Wnt signaling requires complex interactions between the 20 amino acid repeats of APC2 and Armadillo *in vivo*

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The activity of Drosophila APC2 in canonical Wnt signaling requires complex interactions between the 20 amino acid repeats of APC2 and Armadillo *in vivo*

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Running title: APC2 mutants in Wnt signaling

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ABSTRACT

The tumor suppressor Adenomatous polyposis coli (APC) negatively regulates Wnt signaling through its activity in the destruction complex. APC binds directly to the main effector of the pathway, ßcatenin (ßcat, Drosophila Armadillo), and helps to target it for degradation. In vitro studies demonstrated that a non-phosphorylated 20 amino acid repeat (20R) of APC binds to ßcat through the N-terminal extended region of a 20R. When phosphorylated, the phospho-region of an APC 20R also binds ßcat and the affinity is significantly increased. These distinct APC-ßcat interactions suggest different models for the sequential steps of destruction complex activity. However, the *in vivo* role of 20R phosphorylation and extended region interactions has not been rigorously tested. Here we investigated the functional role of these molecular interactions by making targeted mutations in *Drosophila melanogaster* APC2 that disrupt phosphorylation and extended region interactions, and deletion mutants missing the Armadillo binding repeats. We tested the ability of these mutants to regulate Wnt signaling in *APC2* null, and in *APC2 APC1* double null embryos. Overall, our *in vivo* data support the role of phosphorylation and extended region interactions in APC2's destruction complex function, but suggest that the extended region plays a more significant functional role. Furthermore, we show that the Drosophila 20Rs with homology to the vertebrate APC repeats that have the highest affinity for ßcat are functionally dispensable, contrary to biochemical predictions. Finally, for some mutants, destruction complex function was dependent on APC1 suggesting that APC2 and APC1 may act cooperatively in the destruction complex.

INTRODUCTION

Wnt signal transduction plays essential roles in both normal embryonic development and in the maintenance of adult tissues by directing cell fates, promoting proliferation, and influencing morphogenesis (reviewed in (LOGAN and NUSSE 2004)). Consistent with its essential nature, the Wnt signaling pathway is well studied. However, many of the molecular mechanisms that regulate Wnt signaling are poorly understood. In the absence of a Wnt ligand, the pathway is negatively regulated by the activity of the degradation or destruction complex. Loss of negative regulation and constitutive activation of Wnt target genes is associated with a variety of diseases, the most well understood of which is colorectal cancer (reviewed in (POLAKIS 2007) and (LOGAN and NUSSE 2004)). The destruction complex, which includes the kinases GSK3ß, and CK1, the scaffolding protein Axin, and the colon cancer tumor suppressor Adenomatous polyposis coli (APC), acts to phosphorylate ß-catenin (ßcat), the key effector of the Wnt pathway, and target it for ubiquitination and degradation by the proteosome (reviewed in (KENNELL and CADIGAN 2009)). Loss of function of any one of these core components of the destruction complex results in the ligand independent activation of Wnt target genes. Activation of the Frizzled receptor by a Wnt ligand results in the deactivation of the destruction complex through LRP6/Arrow and the cytoplasmic protein Dishevelled. This allows for the accumulation of ßcat that enters the nucleus to promote the activation of Wnt target genes in complex with members of the TCF/LEF family of transcription factors. Thus, the destruction complex is a nexus of Wnt pathway regulation.

Within the destruction complex itself there are myriad protein-protein interactions. Axin, thought to act as a scaffolding protein in the complex, can bind directly to ßcat, APC, GSK-3ß, and Dishevelled, and is thought to promote interactions within the complex (reviewed in (CADIGAN and PEIFER 2009)). The precise role of APC in the complex is less well understood (reviewed in (MCCARTNEY and NATHKE 2008)). APC directly binds ßcat through its 15 amino acid repeats (15Rs) and 20 amino acid repeats (20Rs) (RUBINFELD *et al.* 1993; RUBINFELD *et al.* 1995; SU *et al.* 1993b). The vertebrate APC protein contains 4 15Rs and 7 20Rs (Fig. 1). The role of the 15Rs in ß-catenin degradation is controversial. Studies of mutant APC fragments in cultured cells have

yielded contradictory results, where one study concluded that the 15Rs are necessary and sufficient for ßcat degradation (Kohler), and another suggesting that they do not contribute to degradation (MUNEMITSU *et al.* 1995). Recent *in vivo* studies of 15R mutants in the context of full length APC2 in Drosophila concluded that the 15Rs are largely dispensable for ßcat degradation, but may contribute to its cytoplasmic retention (Roberts). APC also binds Axin directly through the SAMP repeats of which there are 3 in vertebrate APC (Fig. 1) (BEHRENS *et al.* 1998; HART *et al.* 1998). Conserved sequence B or CID (sequence B), that lies between 20R1 and 20R2 in both vertebrate and Drosophila APCs has recently been shown to play a significant role in ßcat degradation, although the mechanism is unknown (Kohler 2009, Roberts 2011). In cancer, most *APC* mutations are found in what is termed the mutational cluster region (MCR) that results in the production of truncated APC proteins that are missing 5 or more of the 20Rs and the SAMP repeats, retaining the N-terminal domains, the 15Rs, approximately 2 of the 20Rs, and centers over conserved sequence B (Fig 1). Such mutations disrupt destruction complex activity resulting in the constitutive activation of the Wnt pathway (reviewed in (LOGAN and NUSSE 2004)). The kinases in the complex phosphorylate ßcat to target it for degradation, but also phosphorylate Axin and APC (HART *et al.* 1998; IKEDA *et al.* 1998; RUBINFELD *et al.* 1996). In the case of APC, phosphorylation regulates the APC-ßcat interaction (LIU *et al.* 2006; RUBINFELD *et al.* 1996).

Co-crystallization of ßcat with the third 20R (20R3) of vertebrate APC revealed the specific sites of phosphorylation in the 20Rs that play a role in direct contacts with ßcat in vitro (HA *et al.* 2004; XING *et al.* 2004). While both phosphorylated and non-phosphorylated APC (P-APC and non-P-APC respectively) bind ßcat, phosphorylation of APC by CK1 and GSK3ß increases the affinity of APC for ßcat by 300-500 fold (XING *et al.* 2004). The binding of Axin to APC enhances GSK3ß and CK1 phosphorylation of APC, suggesting that the phosphorylation of APC may take place within the destruction complex (IKEDA *et al.* 2000). Mutation of 3 conserved serine residues in 20R2 significantly decreased its activity in SW480 cells, arguing that phosphorylation of APC plays a role in ßcat degradation (RUBINFELD *et al.* 1997). Interestingly, vertebrate 20R2

Figure 1

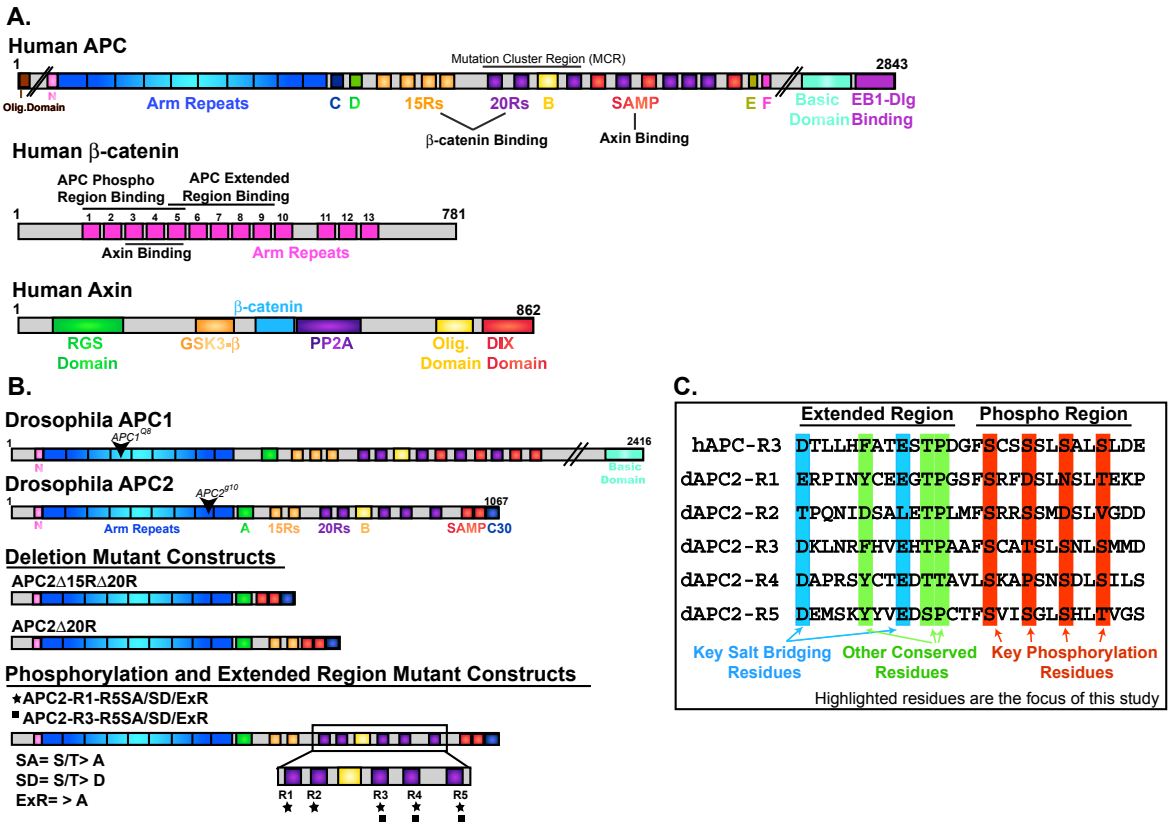


Figure 1: (A) Schematic representations of vertebrate APC, ßcat, and Axin with domains and binding partners indicated. (B) Schematic representations of Drosophila APC proteins with the sites of relevant null mutations indicated (APC1Q8 and APC2g10). Deletion mutants and targeted point mutants are shown. (C) Sequence alignment between human APC 20R3 and the 20Rs of Drosophila APC2. Designation as a phosphorylation residue or a salt bridging residue is based on Ha et al. (2004) and Xing et al. (2004).

does not bind ßcat (ref), suggesting that it may have a distinct role in Wnt signaling. Most APC 20Rs are composed of two regions referred to as the N-terminal extended region, and a phospho-region. The phospho-region of the 20R binds to Armadillo repeats 1-5 of ßcat, and the extended region of APC binds to Armadillo repeats 5-9 of ßcat (HA *et al.* 2004; XING *et al.* 2004). The interface between the 20R extended region and ßcat is primarily composed of hydrophilic interactions including two key salt bridges between APC, and two lysines in ßcat referred to as the "charged buttons". These charged buttons play a critical role in the interaction between ßcat and a number of its binding partners (FASOLINI *et al.* 2003; GRAHAM *et al.* 2000; OMER *et al.* 1999; VON KRIES *et al.* 2000). These structural findings have lead to a number of speculative models for the assembly and mechanism of the destruction complex. While we now understand at the structural level how APC and ßcat interact in vitro, the significance of these interactions to destruction complex activity *in vivo* has not been established.

To test the significance of these interactions *in vivo*, we have generated mutant forms of Drosophila APC2 that specifically either mimic or disrupt phosphorylation of the 20Rs, or disrupt residues in the extended region of the 20Rs. We have previously shown that Drosophila APC2 is essential for the negative regulation of Wnt signaling in the Drosophila embryo (MCCARTNEY *et al.* 1999; MCCARTNEY *et al.* 2006), and together with Drosophila APC1 is necessary for the negative regulation of Wnt signaling in postembryonic tissues (AHMED *et al.* 2002; AKONG *et al.* 2002). We have expressed these mutant forms of APC2 under the native *APC2* promoter in Drosophila embryos and tested their ability to negatively regulate Wnt signaling in genetic backgrounds null for either *APC2*, or *APC2* and *APC1*.

METHODS

Constructs

The specific amino acid position of the Drosophila APC2 (Flybase annotation symbol: CG6193) fragments are as follows:

APC2-Δ15RΔ20R: 1-490 plus 931-1067; **APC2-Δ20R**: 1-592 plus 931-1067; **APC2-R1-R5SA:** S610A, D613A, N616A, T619A, S660A, S663A, D666A, S752A, T755A, S758A, S761A, S799A, P802A, S805A, S808A, S877A, S880A, S883A, T886A; **APC2-**

Figure 2

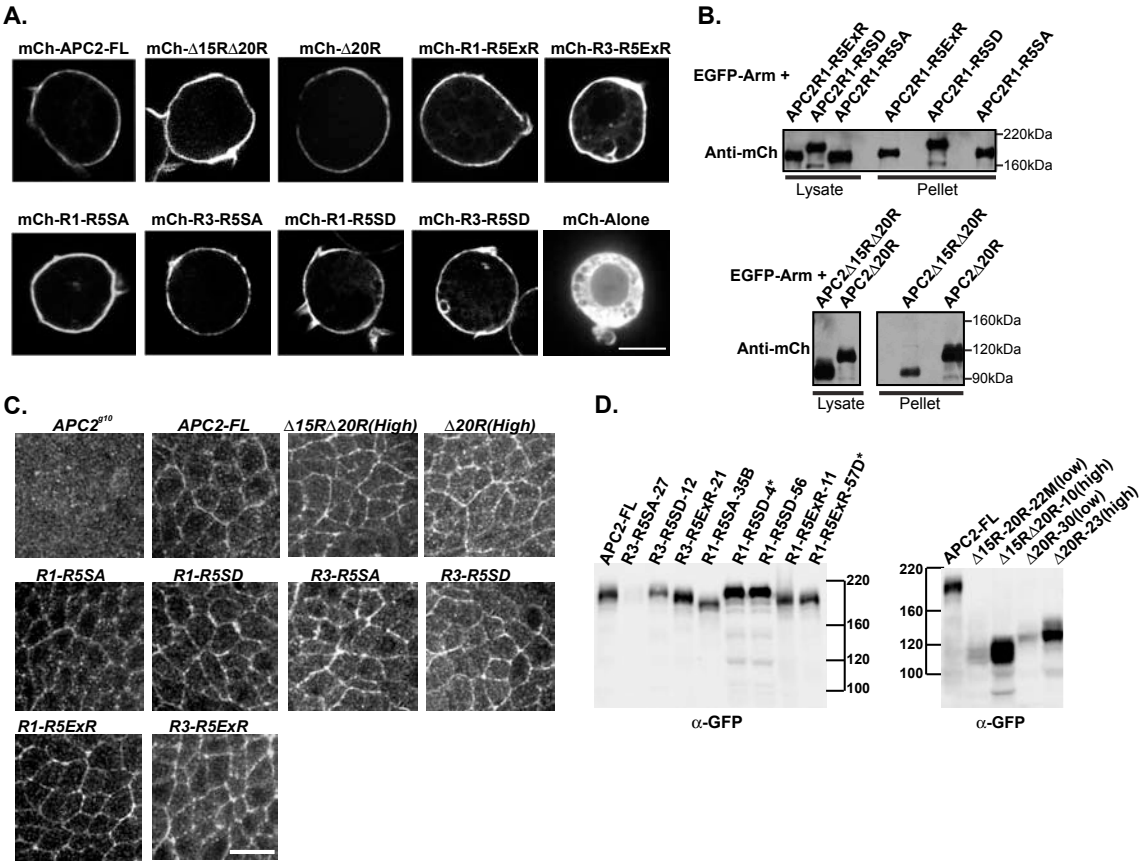


Figure 2: Characterization of APC2 mutants in S2 cells and in the Drosophila embryo. (A) Expression of mCherry (mCh) tagged versions of the targeted APC2 mutants revealed that they localize to the cortex like APC2-FL in S2 cells. (B) All of the APC2 mutants coimmunoprecipitate with EGFP-Armadillo from S2 cells suggesting that they retain the ability to interact directly or indirectly with Armadillo. (C-D) In the embryo, expression of the APC2 mutants was driven by the native APC2 promoter. All of the APC2 mutants localize to the cell cortex and to the cytoplasm in embryonic epithelia (C). (D) Immunoblot of embryonic lysates probed with anti-GFP. Equal amounts of embryonic lysate were loaded in each lane. This demonstrated that while most of the mutant transgenes are expressed at levels comparable to APC2-FL, there were some exceptions. APC2-R3-R5SA (line 27) was expressed at very low levels. In the case of deletions, we obtained lines that express at levels higher than APC2-FL and lines expressing at levels lower than APC2-FL. We have previously shown that APC2-FL expresses at levels comparable to endogenous APC2 (Zhou et al., 2011). The functional experiments shown in the following figures were conducted with all of the lines shown with the exception of those marked with an *. Scale bars = 10 μm

R1-R5SD: S610D, D613D, N616D, T619D, S660D, S663D, V669D, S752D, T755D, S758D, S761D, S799D, P802D, S805D, S808D, S877D, S880D, S883D, T886D. **APC2-R1-R5ExR:** E595Q, Y600A, E603Q, T605A, P606A, T645A, D650A, L653A, T655A, P656A, D737N, F742A, E745Q, T747A, P748A, D784N, Y789A, E792Q, T794A, T795A, D862N, Y867A, E870Q, S872A, T873A; **APC2-R3-R5SA**: S752A, T755A, S758A, S761A, S799A, P802A, S805A, S808A, S877A, S880A, S883A, T886A. **APC2-R3-R5SD:** S752D, T755D, S758D, S761D, S799D, P802D, S805D, S808D, S877D, S880D, S883D, T886D; **APC2-R3-R5ExR:** D737N, F742A, E745Q, T747A, P748A, D784N, Y789A, E792Q, T794A, T795A, D862N, Y867A, E870Q, S872A, T873A

ApaI-BamHI fragments of APC2 containing the R1 and R2 repeats were cloned into an in-house vector and subjected to multiple rounds of site-directed mutagenesis. BamHI-SalI fragments of APC2 containing the R3-R5 repeats were similarly mutagenized. These mutated fragments were used to replace the corresponding pieces in the wild type APC2. The resulting APC2 mutants were cloned into the EcoRI site in pRmHa-3 (metallothionein promoter) and pCaSpeR-2 (endogenous APC2 promoter [endoP]; (MCCARTNEY *et al.* 2006)) generating mCherry or EGFP fusions respectively.

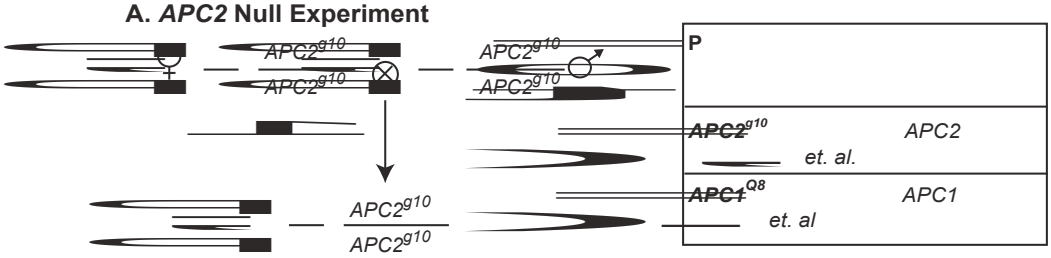
Genetics, hatch rate and cuticle preparations

Transgenic flies expressing *P[endoP-EGFP-APC2-FL], P[endoP-EGFP-APC2-Δ15Δ20R], P[endoP-EGFP-APC2-Δ20R], P[endoP-EGFP-APC2-R1-R5SA/SD/ExR] and P[endoP-EGFP-APC2-R3-R5SA/SD/ExR]* were generated using P-element mediated germline transformation (Model System Genomics of Duke University). Two independent second chromosome insertions for each transgene were crossed into the $APC2^{g10}$ (*APC2* null) and the *FRT* $APC2^{g10}$ $APC1^{Q8}$ (*APC* double null) backgrounds using standard methods. Embryos maternally and zygotically *FRT* $APC2^{g10}$ $APC1^{Q8}$ were generated using the FRT/FLP/DFS technique (CHOU and PERRIMON 1996). Relevant crosses and genotypes are shown in Figure 4.

Embryonic cuticles were prepared and hatch rate analysis was performed as previously described (WIESCHAUS and NUSSLEIN-VOLHARD 1998). The cuticle phenotype scoring criteria were previously described (MCCARTNEY *et al.* 2006). In brief, each cuticle for a given genotype was given a score between 0 (wild type) and 6 (most severe). Details for each scoring category are as follows: (0) wild type cuticle, but did

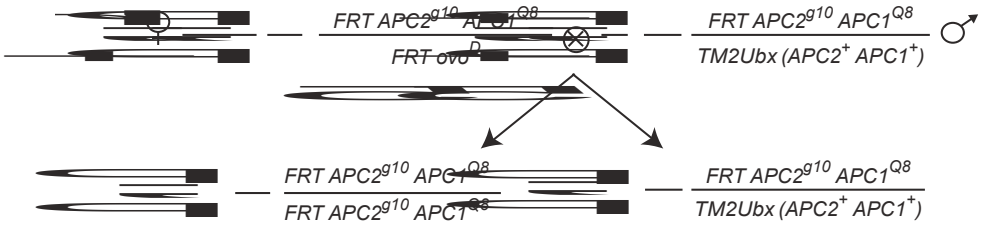
Figure 3

A. *APC2* Null Experiment



Maternally and Zygotically Null for *APC2*
(MZ)

B. *APC2 APC1* Double Null Experiment



Maternally and Zygotically Null for *APC2 APC1*
(MZ)

Maternally Null for *APC2 APC1*
Zygotically Heterozygous
(MZ+)

not hatch, (1) head defects, but no head hole/ wild type length/ 15% of denticles missing, (2) head defects or small head hole/ >70% wild type length/ most denticle bands still represented by at least one patch of denticles, (3) anterior hole/ 50-60% wild type size/ >3 patches of denticles remaining, (4) anterior hole/ 50-60% wild type size/ <2 denticle patches remaining, (5) anterior hole extends ventrally to ~30% cuticle length/ 50-60% wild type size/ no denticles, (6) anterior hole extends ventrally to ~50% cuticle length/ 25-30% wild type size/ no denticles. Cuticles with features of more than one category were designated by halves. For example, a cuticle with features of both a 2 and a 3 were designated as a 2.5. A phenotypic average (PA) was calculated from this data.

Localization to the cortex, Arm accumulation and En stripe expansion analysis:

Embryos were collected for 6 hours at 27ºC and fixed and stained as described in McCartney et al., 1999. Antibodies that are used for this analysis are as follows: anti-Armadillo (N27A1, 1:250) and anti-Engrailed (4D9, 1:50) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. anti-GFP (Abcam, 1:5000) was pre-absorbed against w^{1118} embryos before using for immunohistochemistry. anti-APC2 was used as previously described (McCartney *et al.* 2006). Secondary antibodies were conjugated with various Alexa dyes (Invitrogen, 1:1000).

Subcellular localization was determined for P[endoP-EGFP-APC2-FL] and P[endoP-EGFP-APC2-Δ15-20R], P[endoP-EGFP-APC2-Δ20R], P[endoP-EGFP-APC2-R1-R5SA/SD/ExR] and P[endoP-EGFP-APC2-R3-R5SA/SD/ExR] in the *APC2* null background. Armadillo (Arm) accumulation and Engrailed (En) expression were also assessed in these genotypes. For these analyses, two independent staining experiments were performed and five embryos from each experiment were imaged. Representative embryos are shown.

Image Analysis:

Images were acquired with a spinning disc confocal microscope with a Yokagawa scan head (Solamere Technology Group) with a QICAM-IR camera (Qimaging) on a Zeiss Axiovert 200M using QED InVivo software. For Fig 7, multiple images at 25X were

Figure 4.

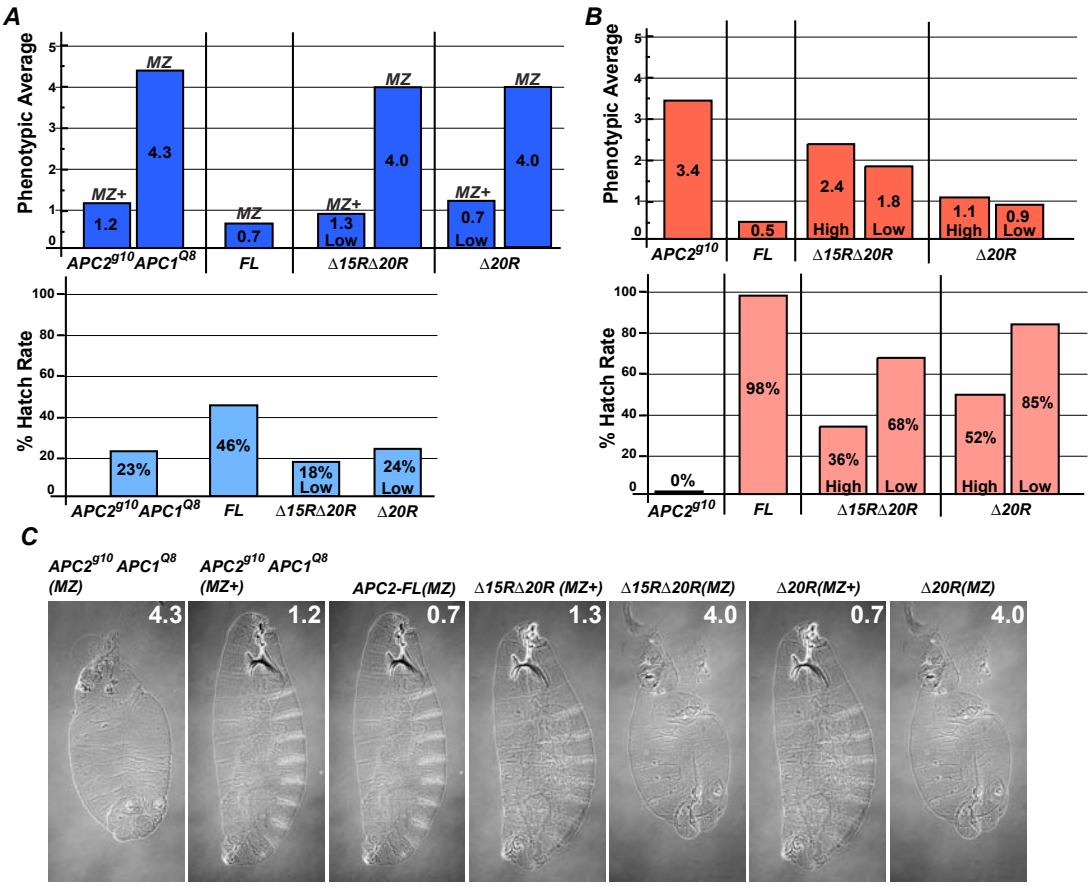


Figure 4: APC2 deletion mutants suppress APC2 null defects, but fail to rescue APC2 APC1 double null defects. (A-D) Hatch rate and embryonic cuticle analysis of APC2 null embryos and APC2 APC1 double null embryos with transgenes expressing APC2-FL, APC2-Δ15RΔ20R and APC2-Δ20R. High/Low indicates if the transgene was high or low expressing (refer to Fig. 2D). Statistical comparisons of these data are shown in Tables 1 and 2. (A) Hatch rate of APC2 null embryos alone or expressing indicated transgenes. (B) The cuticle phenotype of embryos that failed to hatch was assessed and a phenotypic average (PA) was calculated for APC2 null embryos and APC2 null embryos expressing the indicated transgenes. (C) Hatch rate of APC2 APC1 double null embryos alone or expressing indicated transgenes. (D) The cuticle phenotype of embryos that failed to hatch was assessed and a PA was calculated for APC2 APC1 double null embryos and APC2 APC1 double null embryos expressing the indicated transgenes. Cuticles were classified as either MZ+ (maternally null and zygotically heterozygous) or MZ (maternally and zygotically null). (E) Representative cuticles for indicated genotypes. Orientation: anterior is toward the top/ventral is either up or to the right. The PA and the class (MZ or MZ+) are shown for each genotype. All cuticles are shown at the same scale.

merged by using Adobe Photoshop to generate whole embryo images. Figures were prepared with Adobe Photoshop and Adobe Illustrator.

S2 cell culture, transfection, and immunoprecipitation

S2 cells were cultured and transfected with Effectene using standard protocols (Qiagen). Expression was induced at 24 hours post-transfection by adding $CuSO_4$ to a final concentration of 40 μM for 14–16 hours. Coimmunoprecipitation (co-IP) was performed as in Zhou et al. (2011). In brief, after induction cells were lysed and the lysate was preincubated with rec-G beads (Invitrogen) for 0.5 hours at 4°C. The pre-cleared lysate was incubated with anti-Armadillo antibody at 1:50 for 1 hour at 4°C. Rec-G beads were added and incubated for another 0.5 hours at 4°C prior to washing. SDS-PAGE and immunoblotting were performed using standard procedures.

RESULTS

Mutation of the extended region and phosphorylated residues in Drosophila APC2

We generated targeted mutations in Drosophila APC2 that disrupt or mimic phosphorylation, or disrupt the conserved residues in the extended region (ExR), in all five of the 20Rs (Fig. 1B,C). The selection of those residues was based on the alignment between human 20R3 that was co-crystallized with ßcat (HA *et al.* 2004; XING *et al.* 2004), and the 20Rs of APC2 (Fig. 1C). We have designated these mutants as APC2-R1-R5SA, APC2-R1-R5SD, and APC2-R1-R5ExR.

Vertebrate 20R3 has the highest affinity for ßcat, suggesting an essential role in destruction complex function (LIU *et al.* 2006). In Drosophila APC2, 20R3-20R5 share the highest homology with vertebrate 20R3 (Fig. 1C). To test the hypothesis that these repeats play a more significant role in the destruction complex function of APC2 than 20R1 and 20R2, we generated a subset of mutants where the phospho-residues and ER-residues were changed in only 20R3-20R5. We have designated these mutants as APC2-R3-R5SA, APC2-R3-R5SD, and APC2-R3-R5ExR. As a negative control, we generated two deletion mutants (Fig. 1B): one that deletes the entire 20R region and sequence B, leaving the 15Rs intact (APC2-Δ20RΔB), and one that deletes all of the Armadillo binding sites and sequence B (APC2-Δ15RΔ20RΔB). The full-length wild type form of APC2 (APC2-FL) is the positive control.

Figure 5.

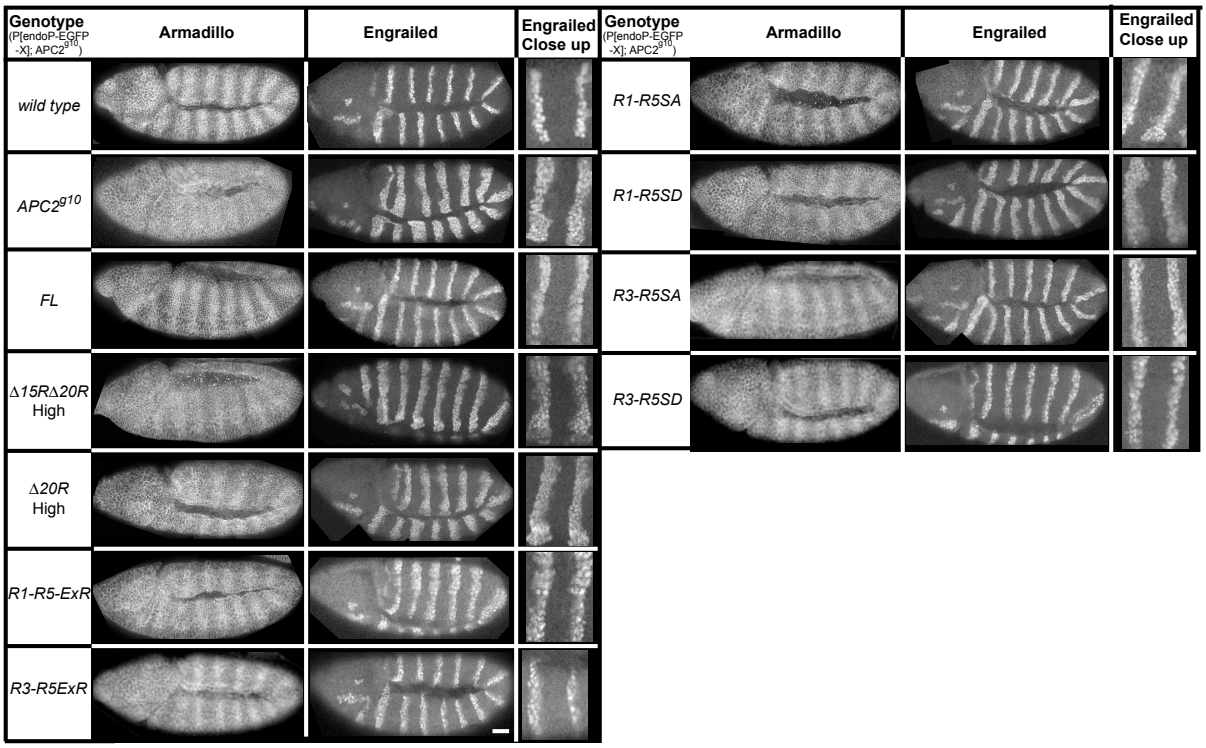


Figure 5: Armadillo and Engrailed expression in wild type and APC2 null embryos expressing APC2 wild type and mutant transgenes. Representative embryos of indicated genotypes stained for Armadillo or Engrailed. In the case of embryos expressing transgenes, the genotype is P[endoP-EGFP-X]; APC2g10 where endoP is the native APC2 promoter and X indicates a particular APC2 transgene. “High” refers to the expression level of the transgene (refer to Fig. 2D). Orientation: anterior to left and dorsal up. Scale bar = 25 μm

Targeted APC2 mutants localize properly and form complexes with Armadillo in S2 cells

To begin the characterization of these mutant proteins, we expressed them in Drosophila S2 cells. We have previously shown that APC2-FL localizes to the cortex in S2 cells, similar to its endogenous localization in embryonic cells (ZHOU *et al.* 2011). This localization requires both the Arm repeats and the C-terminal C30 domain (ZHOU *et al.* 2011), all of which are intact in our mutants. As a test of the stability and functionality of our mutants, we asked whether they localized to the S2 cell cortex. All the mutants localize to the cell cortex like APC2-FL (Fig. 2A). We further tested the ability of the deletions and the 20R1-R5 mutants to associate with Armadillo in S2 cell lysate. While we have perturbed direct APC2-Arm interactions in all of the mutants, the mutant forms of APC2 retain the ability to bind to Axin through their SAMP domain (BEHRENS *et al.* 1998; HART *et al.* 1998), and to endogenous wild type APC2 in the S2 cells through their Arm domain (ZHOU *et al.* 2011). Thus, the targeted mutants that are unable to bind Armadillo directly may still associate indirectly with Armadillo in a complex through Axin or wild type APC2. When co-expressed with EGFP-Arm, the deletion mutants and the R1-R5 mutants could be co-immunoprecipitated with Armadillo (Fig. 2B). This suggests that while we have disrupted specific residues and domains within APC2, the mutated forms of APC2 retain the ability to interact with other binding partners in the cell.

In order to test the functional significance of these domains on the destruction complex function of APC2, we generated transgenic flies expressing the EGFP tagged APC2 mutants under the native *APC2* promoter (MCCARTNEY *et al.* 2006). This has been previously shown to be sufficient to drive wild type expression of *APC2* transgenes (MCCARTNEY *et al.* 2006). We introduced all of these transgenes into an *APC2* null background (*APC2*^{g10}; Fig. 3A) or into the *APC2 APC1* double null background (*APC2*^{g10} *APC1*^{Q8}; Fig. 3B). Thus in contrast to some other structure-function types of studies with APC, in this study the mutant forms of APC2 are expressed at physiological levels in a background lacking all other APC2, or APC2 and APC1. Consistent with our observations in S2 cells, all of the APC2 mutant proteins expressed in *APC2* null embryos are enriched at the cell cortex of embryonic epithelia similar to APC2-FL or the

endogenous APC2 protein (Fig. 2C and (ZHOU *et al.* 2011)). Western blot demonstrates that expression of most of the proteins from the transgenes is comparable to endogenous APC2 (Fig. 2D). One striking difference is APC2-R3-R5 SA that expresses at very low levels (Fig. 2D and data not shown). While APC2-R3-R5 SA is low expressing in the embryo overall, but we do find some variation in expression within the epithelium. We have shown these more highly expressing cells to demonstrate the cortical localization of the protein. For the APC2-Δ15RΔ20R and APC2-Δ20R mutants, we tested two independent lines that express below the level of APC2-FL (low) or above the level of APC2-FL (high).

The role of the 15Rs and 20Rs in the destruction complex function of APC2

In Drosophila, APC2 and APC1 have redundant functions in Wnt signaling throughout development (AHMED *et al.* 2002; AKONG *et al.* 2002). In the embryo, APC2 plays a primary role in the destruction complex, presumably due to its significantly higher expression level compared to APC1 (AHMED *et al.* 1998; AHMED *et al.* 2002; MCCARTNEY *et al.* 1999). However, complete loss of APC1 enhances the *APC2* null Wnt dependent embryonic phenotypes (AHMED *et al.* 2002; AKONG *et al.* 2002). In the first set of experiments, we asked whether the APC2 deletion mutants could restore destruction complex activity in an *APC2* null background where *APC1* is wild type. We have previously shown that differences in Wnt pathway regulation can be observed for different *APC2* mutant alleles in a background wild type for *APC1* (ref).

Complete loss of Drosophila APC2 (*APC2*^{g10}) constitutively activates Wnt signaling in the embryo resulting in embryonic lethality (0% hatch rate to the larval stage; (MCCARTNEY *et al.* 2006)). These embryos exhibit embryonic cuticle defects characteristic of Wnt activation, including reduction in size due to excess apoptosis, an anterior hole due to a failure of head involution, and the production of excess smooth cuticle at the expense of denticles due to a Wnt dependent change in cell fate (MCCARTNEY *et al.* 2006). Due to phenotypic variability, we classify these phenotypes based on a 0-6 scale and calculate an average cuticle phenotype (PA) for each genotype examined (see Methods and (MCCARTNEY *et al.* 2006)). The PA for *APC2*^{g10} embryos is 3.4 (Fig. 4 and Fig. S1). In addition, complete loss of APC2 is characterized

Figure 6.

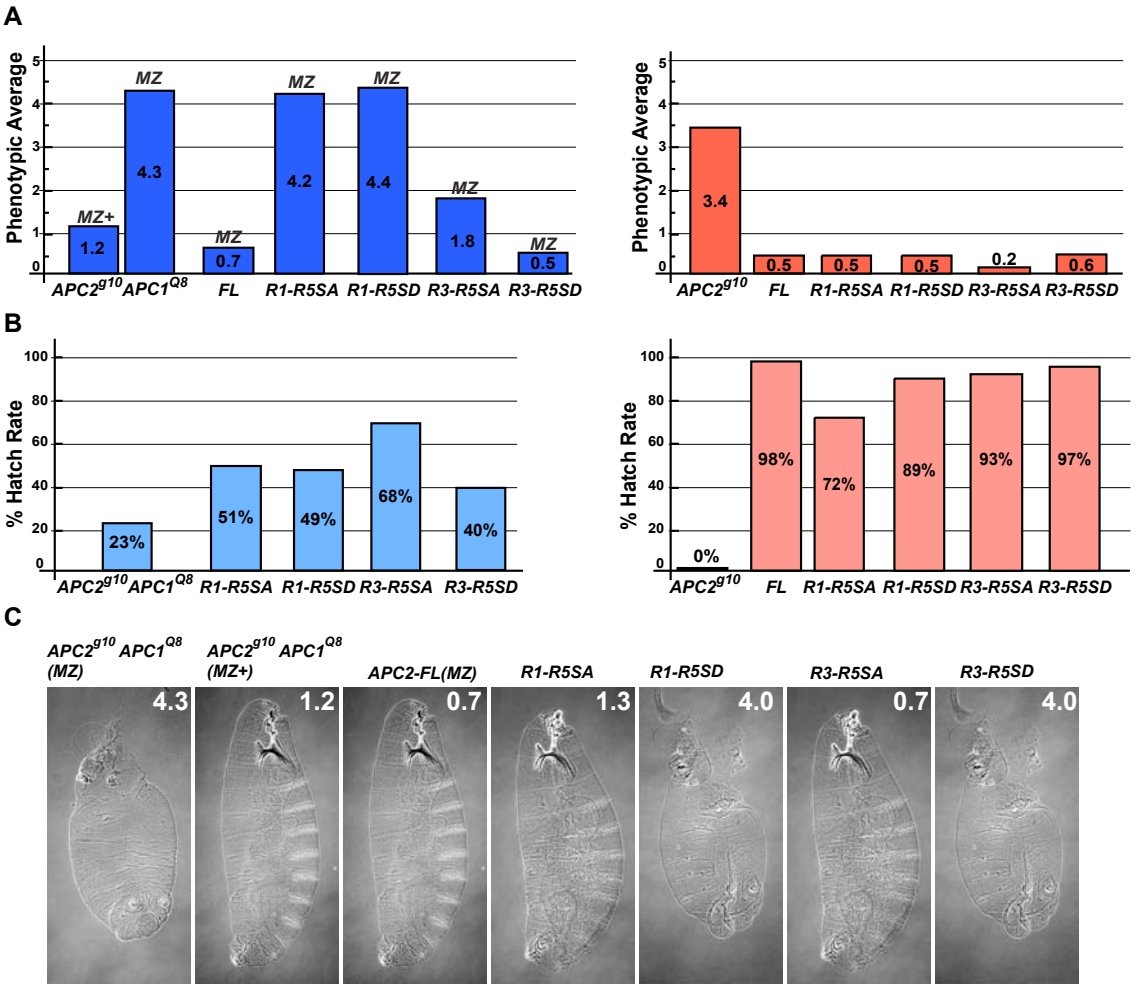


Figure 6: APC2 phospho-mutants in 20R3-R5 exhibit a strong rescue of both APC2 and APC2 APC1 null defects, while APC2 phospho-mutants in 20R1-R5 suppress APC2 null defects, but fail to rescue APC2 APC1 double null defects. (A-D) Hatch rate and embryonic cuticle analysis of APC2 null embryos and APC2 APC1 double null embryos with transgenes expressing APC2-FL, and APC2 phospho-mutants. Statistical comparisons of these data are shown in Tables 1 and 2. (A) Hatch rate of APC2 null embryos alone or expressing indicated transgenes. (B) The cuticle phenotype of embryos that failed to hatch was assessed and a phenotypic average (PA) was calculated for APC2 null embryos and APC2 null embryos expressing the indicated transgenes. (C) Hatch rate of APC2 APC1 double null embryos alone or expressing indicated transgenes. (D) The cuticle phenotype of embryos that failed to hatch was assessed and a PA was calculated for APC2 APC1 double null embryos and APC2 APC1 double null embryos expressing the indicated transgenes. Cuticles were classified as either MZ+ (maternally null and zygotically heterozygous) or MZ (maternally and zygotically null). (E) Representative cuticles for indicated genotypes. Orientation: anterior is toward the top/ventral is either up or to the right. The PA and the class (MZ or MZ+) are shown for each genotype. All cuticles are shown at the same scale.

by an accumulation of Armadillo (Arm) in the embryonic epidermis (McCartney *et al.* 2006). Due to a segmentally repeated pattern of Wingless (Wg, a Drosophila Wnt) expression in the embryo, Arm normally accumulates in stripes that correspond to cells that are activating the pathway (Fig. 5 and Peifer *et al.* 1994). All cells localize Arm to the adherens junction. In *APC2* null embryos, all cells accumulate Arm equally and no stripes are observed ((McCartney *et al.* 2006); Fig. 5). One molecular outcome of the uniform accumulation of Arm and activation of the Wnt pathway is the expansion of the expression domain of the Wg target gene Engrailed (Fig. 5). Expression of APC2-FL in the $APC2^{g10}$ embryos completely restores destruction complex activity resulting in a 98% hatch rate, very weak cuticle defects in the few embryos that fail to hatch to larvae (PA=0.2), Arm accumulation in stripes, and a wild type pattern of En expression (Fig. 4A,B and 5).

To test the hypothesis that the central repeats of APC2 are necessary for destruction complex function, we asked whether APC2-Δ15RΔ20R and APC2-Δ20R could rescue the hatch rate and cuticle defects associated with $APC2^{g10}$. We predicted that complete loss of these domains would result in a non-functional APC2 protein. Surprisingly, both deletion mutants provided enough activity to moderately rescue the hatch rate and cuticle phenotype of $APC2^{g10}$ (Fig. 4A,B, Table 1). The level of rescue was dependent on the expression levels of the transgenes (Fig. 4A,B, Table 1). Higher expression of the deletion mutants significantly reduced the degree of rescue (Fig 4A,B, Table 1). In addition, APC2-Δ20R provided more significant rescue than APC2-Δ15RΔ20R, suggesting that 15R dependent Arm binding plays a role in the Wnt signaling function of APC2. Analysis of Arm accumulation and En expression revealed a consistent trend relative to the hatch rate and cuticle phenotypes; stripes of Arm accumulation were weakly restored and the En expression domain was weakly reduced compared to $APC2^{g10}$ (Fig. 5). Taken together, these data suggest that deletion of either the 20Rs alone, or both the 15Rs and 20Rs, results in an APC2 protein that retains some function in the destruction complex in the presence of APC1.

We predicted that the surprising ability of APC2-Δ15RΔ20R and APC2-Δ20R to provide some rescue in the *APC2* null might be due to the presence of APC1. Thus, to further challenge the function of APC2-Δ20R and APC2-Δ15RΔ20R, we tested their

ability to rescue the Wnt dependent defects in the APC double null background (*APC2*^{g10} *APC1*^{Q8}). In the APC double null background, the APC2 mutants are responsible for all destruction complex activity. The APC double null embryos were generated using the FRT/FLP/DFS technique (CHOU and PERRIMON 1996). Based on this method, 50% of the embryos were maternally and zygotically double null for APC (MZ), and 50% are maternally double null but zygotically heterozygous (MZ+; Fig. 3B). In the absence of any rescuing transgene, 23% of the progeny hatched into larvae, and 25% displayed weak cuticle defects (ranging from 0-2.5; Fig. 4C-E, Table 2). These progeny represent the MZ+ class and display relatively weak phenotypes due to the paternal contribution of an *APC2*$^{+}$ *APC1*$^{+}$ chromosome (Fig. 4D,E, and data not shown). The remaining 52% of the progeny are maternally and zygotically null (the MZ class). These are embryonic lethal and display strong cuticle defects (ranging from 3-6; Fig. 4D,E, Table 2). Expression of APC2-FL in the double null background resulted in a partial rescue with an overall shift in the severity of the phenotype toward wild type: 46% of the progeny hatch and the remaining progeny exhibit a very weak cuticle phenotype (Fig. 4D,E, PA= 0.7). This suggests that all of the MZ+ embryos hatch, and the MZ embryos die with a significantly suppressed phenotype. Interestingly, neither APC2-Δ20R nor APC2-Δ15RΔ20R provided any activity in the *APC* double null background. The hatch rate and cuticle phenotype of the progeny were indistinguishable from the double null alone (Fig. 4C-E; Table 2). These results suggest that direct APC2-Arm interactions are essential for APC2's destruction complex function, consistent with previous work. Furthermore, the simplest interpretation of the ability of the APC2 deletion mutants to function only in the presence of APC1 is that APC2 and APC1 act cooperatively, rather than in independently functioning destruction complexes (see Discussion).

The role of 20R phosphorylation in the destruction complex function of APC2

To test the hypothesis that phosphorylation of the 20Rs of APC2 is essential for its destruction complex function, we tested the ability of the phospho-dead (SA) and phospho-mimetic (SD) forms of APC2 to rescue the Wnt dependent defects in *APC2* null and in APC double null embryos. Mutants that alter phosphorylation in 20R3-20R5,

Figure 7.

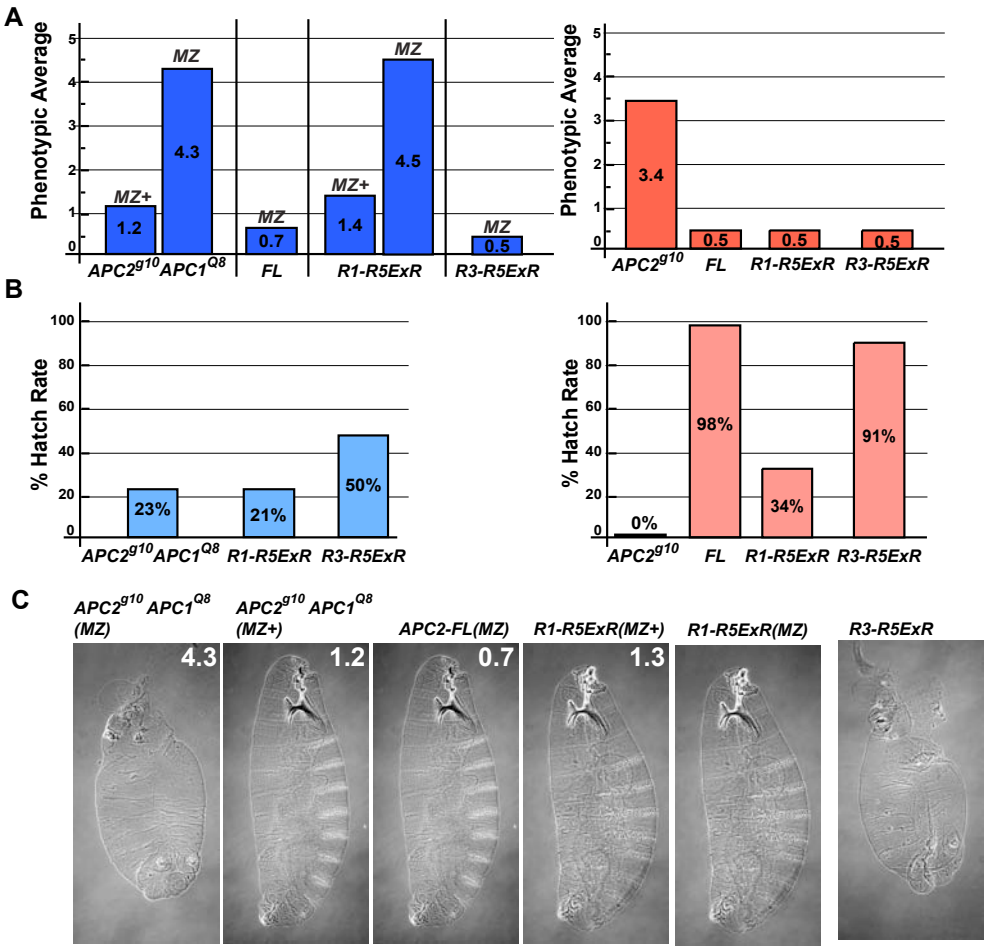


Figure 7: The APC2-R3-R5ExR mutant rescues both APC2 null and APC2 APC1 double null defects, while the APC2-R1-R5ExR mutant suppress APC2 null defects, but fails to rescue APC2 APC1 double null defects. (A-D) Hatch rate and embryonic cuticle analysis of APC2 null embryos and APC2 APC1 double null embryos with transgenes expressing APC2-FL, and APC2 ExR mutants. Statistical comparisons of these data are shown in Tables 1 and 2. (A) Hatch rate of APC2 null embryos alone or expressing indicated transgenes. (B) The cuticle phenotype of embryos that failed to hatch was assessed and a phenotypic average (PA) was calculated for APC2 null embryos and APC2 null embryos expressing the indicated transgenes. (C) Hatch rate of APC2 APC1 double null embryos alone or expressing indicated transgenes. (D) The cuticle phenotype of embryos that failed to hatch was assessed and a PA was calculated for APC2 APC1 double null embryos and APC2 APC1 double null embryos expressing the indicated transgenes. Cuticles were classified as either MZ+ (maternally null and zygotically heterozygous) or MZ (maternally and zygotically null). (E) Representative cuticles for indicated genotypes. Orientation: anterior is toward the top/ventral is either up or to the right. The PA and the class (MZ or MZ+) are shown for each genotype. All cuticles are shown at the same scale.

but leave 20R1 and 20R2 unchanged (APC2-R3-R5SA and APC2-R3-R5SD) are largely indistinguishable from APC2-FL in both the *APC2*^{g10} and *APC* double null background (Fig. 5, 6A-E; Table 1,2). The only differences we have observed are, (1) a small but statistically significant difference between APC2-R3-R5SA and APC2-FL in the *APC2* null (Table 1), and (2) in the *APC* double null background, the hatch rate for embryos expressing APC2-R3-R5SA is significantly higher than for APC2-FL (Table 2). While these differences are statistically significant, they may not represent a biologically significant difference. Interestingly, APC2-R3-R5SA is a very low expressing protein. It is not surprising that very little APC2 protein is sufficient for function. Axin is thought to be the limiting component in the destruction complex during embryogenesis (Cliffe 2003), and it is expressed at low levels in some systems (ref). The rescue data for the R3-R5 mutants suggest that while R1 and R2 do not have the sequence characteristics of the highest affinity 20Rs, they are sufficient for APC2's destruction complex function. In contrast, changes to phospho-residues in all of the 20Rs had a significant impact on APC2 function. While both APC2-R1-R5SA and APC2-R1-R5SD suppress *APC2* null defects, this rescue is weaker than that of APC2-FL or APC2-R3-R5SA or SD (Fig. 5, 6A-B; Table 1). We also observed that APC2-R1-R5 SD appeared to provide slightly more function than APC2-R1-R5 SA in this background as it exhibited a higher hatch rate (Fig. 6A, Table 1).

Examination of the activity of APC2-R1-R5SA and APC2-R1-R5SD in the *APC* double null background revealed an intriguing pattern of rescue. In both cases, the lethality was suppressed resulting in a hatch rate of approximately 50%- very close to that provided by APC2-FL (Fig. 6C; Table 2). However, when we examined the cuticle defects of the remaining embryos we found that the vast majority exhibited a very severe phenotype characteristic of the MZ class (PA=4.2-4.4; Fig. 6D,E, Table 2). This is in contrast to APC2-FL where the remaining embryos exhibit very weak cuticle defects (PA=0.7; Fig. 6D,E, Table 2). These data suggest that APC2-R1-R5SA and APC2-R1-R5SD have no function in the absence of all APC2 and APC1, as approximately 50% of the progeny in the *APC* double null experiment exhibit no rescue. However, in the presence of the paternally provided APC2 and APC1 (MZ+), APC2-R1-R5SA and APC2-R1-R5SD appear to rescue as well as APC2-FL (Table 2).

Taken together with the analysis of the deletions, we find that R3-R5 SA and SD are largely indistinguishable from APC2-FL (Fig. 4-6; Table 1-2). APC2 R1-R5 SA and SD are similar to the deletions in that they fail to rescue in the absence of all APC. However, the phospho-mutants appear to provide more function than the deletions in the *APC2* null embryos. This difference may reflect the dominant negative activity of the deletions. Furthermore, APC2 R1-R5 SA and SD are able to rescue the MZ+ class in the double null experiment to hatching, while the deletion mutants have no effect on this class (Fig. 4-6; Table 1-2).

The role of the 20R extended region in the destruction complex function of APC2

In the absence of phosphorylation, APC interacts with ßcat through the N-terminal extended region of a 20R (HA *et al.* 2004; XING *et al.* 2004). To test the role of this interaction in the destruction complex function of APC2, we disrupted the conserved residues in the extended region (Fig. 1C) of either R3-R5, leaving R1 and R2 intact (APC2-R3-R5ExR), or in all 5 repeats (APC2-R1-R5ExR). Consistent with what we observed with the phosphomutants, mutation of R3-R5 alone resulted in a protein that was largely indistinguishable from APC2-FL (Fig. 5, 7A,B; Table 1). Again, this shows that R1-R2 interactions are sufficient for APC2's destruction complex function. In contrast, disruption of the extended region in all of the repeats significantly reduced activity in the destruction complex. In the *APC2* null background, the hatch rate was only modestly rescued (34%) and the cuticle phenotype was suppressed (Fig. 7A,B, S1; Table 1). Consistent with this weak rescue, the En expression domain remained expanded and Arm accumulation was apparent (Fig. 5). This rescue profile is more similar to that of the deletions, than that of the phospho-mutants (Table 1). Consistent with that observation, in the *APC* double null background APC2-R1-R5ExR provided no rescue (Fig. 7C-E; Table 2), similar to ∆20R or ∆15R∆20R (Fig. 4C-E; Table 2). Taken together, these results suggest that the extended region interactions may play a more significant role in APC2's destruction function than phospho-based interactions *in vivo*.

Table 1: Summary of hatch rate and cuticle analysis for transgenes in the *APC2*^{g10} background

genotype	hatch rate	0-1	1.5-2.5	3-4	4.5-6	PA	category*
APC2-FL	98%	2%				0.2	1
APC2 R3-R5 SD	97%	3%	<1%			0.6	1
APC2 R3-R5 SA	93%	7%				0.2	2
APC2 R3-R5 ER	91%	8%	1%			0.3	2
APC2 R1-R5 SD	89%	11%	<1%			0.5	3
APC2 ∆20R line 30	85%	12%	2%	1%		0.9	3
APC2 R1-R5 SA	72%	18%	<1%			0.5	4
APC2 ∆15R∆20R 22M	68%	15%	8%	7%	<1%	1.8	5
APC2 ∆20R line 23	52%	31%	17%	<1%		1.1	6
APC2 R1-R5 ER	34%	50%	16%			1.0	7
APC2 ∆15R∆20R 10	36%	18%	15%	31%		2.4	8
APC2^{g10}	0%	14%	6%	74%	6%	3.4	9

*Overall, the cuticle phenotypes have a consistent, statistically significant increasing trend in severity as the hatch rate decreases [chi-square test for trend, c^2=229.24 (df=1, *n*=1033), p<0.001]. The categories were grouped so that genotypes would not differ significantly with respect to hatch rate or cuticle phenotype within each category. Categories 1-9 each have at least one genotype that differs significantly from at least one genotype in every other category, but there is some overlap between genotypes in Categories 1-3. For example, the *APC2 R3-R5 SA* hatch rate (from Category 2) differs significantly from the *APC2-FL* hatch rate (from Category 1), but not the *APC2 R3-R5 SD* hatch rate (Category 1). Hatch rate statistics were calculated using the chi-square test, c^2=2021 (df=11, *n*=6175), *p*<0.001, followed by the Tukey-type post-hoc test (df=∞, groups=12), *p*<0.05 per comparison. The statistical analysis of cuticle phenotypes was performed using the Kruskal–Wallis test, c^2=285.9 (df=11, *n*=1033), *p*<0.001, followed by the Dunn procedure for multiple comparisons using an adjusted a level of 0.0004 for a two-tailed test (*p*<0.001). The n for the hatch rate experiments was >200. For cuticle analysis, n>19.

DISCUSSION

Despite significant interest in understanding the mechanisms that govern the regulation of Wnt signaling, there are numerous outstanding questions regarding the fundamental molecular mechanisms that promote the degradation of Arm and the role that APC proteins play. We have tested the model that: (1) the 20Rs with the highest affinity for Arm are essential for APC2's destruction complex activity, (2) phosphorylation of APC2 is essential for destruction complex activity, and (3) the 20R extended region plays a role in destruction complex activity. Our findings have revealed not only that the high affinity binding sites are not essential, contrary to previous predictions, but that multiple interactions between APC2 and Arm are necessary for destruction complex function. Lastly, our data strongly suggest that APC proteins self associate in the destruction complex with implications for complex assembly and function.

APC2 20Rs with the highest affinity for ßcat are dispensable for Armadillo degradation

20R3 of vertebrate APC has the highest affinity for ßcat, suggesting that it may play the most significant role in the APC-ßcat interaction (LIU *et al.* 2006). Comparison of vertebrate 20R3 to the 20Rs of Drosophila APC2 revealed that 20R3, 20R4, and 20R5 were indistinguishable relative to their conservation with vertebrate 20R3 (Fig. 1C). Thus, we predicted that disruption of 20R3-R5 would significantly impair destruction complex function. We were surprised to find that all of our 20R3-R5 mutants (SA, SD, and ExR) have nearly wild type destruction complex activity in both the *APC2* single mutant and in the *APC2 APC1* double mutant. This indicates that 20R1 and 20R2 are sufficient for APC2's destruction complex function. 20R1 is similar to 20R3-R5 in that the key residues in the extended region are well conserved, as are the key phosphorylation residues (Fig. 1C). 20R2 has some interesting differences; while it is similar to 20R1 within the phospho-region, 20R2 is missing the key salt bridging residues found in the other repeats (Fig. 1C). Although vertebrate 20R2 does not appear to bind to ßcat (CHOI *et al.* 2006; KOHLER *et al.* 2008; LIU *et al.* 2006), Rubinfeld et al. (1997) suggested that vertebrate 20R2 does play a role in ßcat destruction in SW480 colon cancer cells. Consistent with this idea, Roberts et al (2011) recently

demonstrated that deletion of 20R2 alone produces a protein that is unable to promote ßcat degradation in either SW480 cells, or in the Drosophila embryo. Together with their other observations, the authors suggest that 20R2 and the adjacent region B (also known as CID; (KOHLER *et al.* 2009)) together form the binding site for an unknown destruction complex protein.

The field has often pondered the question of why APC has so many 20Rs. In cancer, typically 5 or more of the 20Rs are deleted leaving approximately 2 20Rs and allof the 15Rs (POLAKIS 1997). Rubinfeld et al. (1997) demonstrated that while only 1 20R of vertebrate APC was required for binding to ßcat, 3 20Rs were necessary for the downregulation of ßcat, and 4 or more were required for complete activity. The picture that has emerged from more recent studies is that not all 20Rs are created equal, and that in some cases they may have distinct roles to play in the regulation of ßcat. Our work and that of Roberts et al. (2011) suggests that that the differences in 20R function are unlikely due exclusively to differences in binding affinity to ßcat. Some may promote ßcat degradation through direct ßcat binding, or through other binding partners, and some may participate in other functions such as the cytoplasmic retention of ßcat (ROBERTS *et al.* 2011) that may negatively regulate its transcriptional function.

The role of APC2 in the cytoplasmic retention of Armadillo

Roberts et al. (2011) proposed that APC negatively regulates Wnt signaling not only by promoting ßcat destruction, but also by retaining ßcat in the cytoplasm. In this way, APC acts as a brake on Wnt signaling by preventing the transcriptional activity of nuclear ßcat. In light of this model, Roberts et al. (2011) proposed an explanation for why some APC2 mutants suppress Wnt signaling in *APC2* null embryos, but not in double null embryos as we have also observed. In the *APC2* null background, the level of cytoplasmic Arm is modestly increased. If an APC2 mutant has retention ability it will retain that excess Arm in the cytoplasm, suppress Wg signaling, and rescue the defects. In *APC* double null embryos, the level of cytoplasmic Arm is too high for cytoplasmic retention alone to prevent transcriptional activation of Wnt targets. Roberts et al. (2011) suggested that the 15Rs and 20Rs are additively responsible for the cytoplasmic retention of ßcat.

Table 2: Summary of hatch rate and cuticle analysis for transgenes in the *APC2^{g10} APC1^{Q8}* background

genotype	hatch rate	% weak (0-2.5)	% strong (3-6)	category*
APC2-FL; APC2^{g10} APC1^{Q8}	46%	52%	2%	1
APC2 R3-R5 SA; APC2^{g10} APC1^{Q8}	68%	26%	6%	1
APC2 R3-R5 SD; APC2^{g10} APC1^{Q8}	40%	56%	4%	1
APC2 R3-R5 ER; APC2^{g10} APC1^{Q8}	50%	50%	0%	1
APC2 R1-R5 SA; APC2^{g10} APC1^{Q8}	51%	3%	46%	2
APC2 R1-R5 SD; APC2^{g10} APC1^{Q8}	49%	2%	46%	2
APC2 R1-R5 ER; APC2^{g10} APC1^{Q8}	21%	31%	48%	3
APC2 ∆20R; APC2^{g10} APC1^{Q8}	24%	16%	60%	3
APC2 ∆15R∆20R; APC2^{g10} APC1^{Q8}	18%	28%	54%	3
APC2^{g10} APC1^{Q8}	23%	26%	51%	3

*Hatch rate and cuticle phenotype differences within each category are not statistically significant, with the exception of *APC2 R3-R5 SA: APC2^{g10} APC1^{Q8}*, which has a significantly higher hatch rate. The hatch rate and cuticle phenotype data are statistically significantly different between categories based on either hatch rate, cuticle phenotype, or both. Groups 1 and 2 differ significantly between each other based on cuticle phenotype, but not hatch rate (with the exception noted), whereas Groups 1 and 2 both differ significantly from Group 3 based on hatch rate and cuticle phenotype. Hatch rate statistics were calculated using the chi-square test, c^2=129.5 (df=9, *n*=3739), *p*<0.001, followed by the Tukey-type post-hoc test, q(df=∞, groups=10), *p*<0.05 per comparison. The statistical analysis of cuticle phenotypes was performed using the Kruskal–Wallis test, c^2=525.7 (df=9, *n*=1197), p<0.001, followed by the Dunn procedure for multiple comparisons using an adjusted a level of 0.0006 for a two-tailed test (*p*=0.001). The n for the hatch rate experiments was >100. For cuticle analysis, n>40.

Figure 8

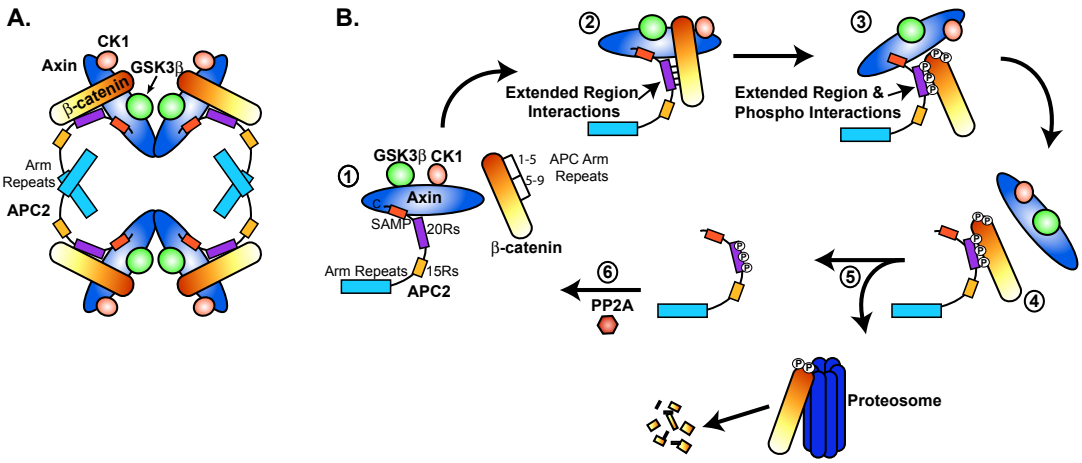


Figure 8: Models of interactions within the destruction complex (A), and the catalytic cycle of the destruction complex (B). (A) Based on the ability of Axin and APC to self associate in vivo, we predict that these interactions contribute to the assembly of the functional "destructosome". We have depicted Axin and APC here as dimers, but the formation of higher order assemblages has not been ruled out. (B) Step 1: the APC-Axin-kinase complex assembles onto the Axin scaffold. APC is in its non-P state. Step 2: ßcat enters the complex via interactions with Axin and Arm repeats 1-5 and with the extended region of a 20R of APC through Arm repeats 5-9. Step 3: GSK3ß and CKI phosphorylate both ßcat and APC. This results in the loss of the Axin-ßcat interaction as P-APC outcompetes Axin. Step 4: The P-APC-P-ßcat complex dissociates from Axin. Step 5: Proteosome associated chaperones separate APC and ßcat. ßcat proceeds to degradation by the proteosome while P-APC is released. Step 6: P-APC is dephosphorylated, perhaps via PP2A. It is now able to re-enter the Axin-kinase complex.

We considered this model as an explanation for our observations of rescue in the *APC2* null embryos. In the embryo, one row of cells in each segment expresses Wingless, a Drosophila Wnt, resulting in the activation of the pathway and the accumulation of Arm in stripes along the dorsal-ventral axis of the embryo at the germband extended stage (Fig. 5). Interstripe cells are not activating the Wnt pathway. Thus, the destruction complex is active in interstripe cells and cytoplasmic levels of Arm are low. In *APC2* null embryos that are deficient for the destruction of Arm, detectable Arm stripes are lost and all cells accumulate a uniform level of cytoplasmic Arm (Fig. 5, and (McCartney *et al.* 2006)). APC2-FL and any 20R3-R5 mutant restores destruction complex function resulting in a visible reduction of interstripe Arm (Fig. 7). Based on the model of Roberts et al. (2011), we predict that Δ20R, and 20R1-R5 SA, SD, and ExR limit Wnt signaling in the *APC2* null embryo because they retain Arm in the cytoplasm. If this is true, we predict that in the *APC2* null embryos expressing those mutants the interstripe Arm level would remain high, and simultaneously Wnt signaling would be suppressed. Instead we observe a reduction in interstripe Arm, and suppression of ectopic Wnt signaling (Fig. 5). These data are consistent with a role for these mutant proteins in Arm destruction in the Drosophila embryo. Thus, while our data do not strongly support a role for these mutants in the cytoplasmic retention of Arm in the embryo, we cannot rule out the possibility that they contribute to both Arm destruction and retention.

Self-association of APC may promote the assembly or activity of the destructosome

An alternative explanation for the single null versus double null rescue differences is the ability of APC2 and APC1 to act cooperatively in the destruction complex. Expression and genetic analysis in the embryo revealed that APC2 is the primary APC protein in the embryonic epidermis, but that nearly undetectable amounts of APC1 also contribute to destruction complex function in that tissue (Ahmed et al. 2002; Akong et al. 2002). The fact that APC2 is enriched at the cortex and is found in cytoplasmic puncta, while APC1 appears to localize to centrosomes and microtubules, suggested that APC2 and APC1 reside in independent destruction complexes localized

in distinct subcellular compartments. One caveat to this interpretation is that APC1 could only be detected when overexpressed and that overexpression could influence its subcellular distribution. Our observations of rescue in *APC2* null versus *APC2 APC1* double null embryos (this work) suggest that we re-evaluate the relationship between APC2 and APC1. The strongest APC2 mutants, including the deletions and all of the R1-R5 mutants, provide some rescue in *APC2* null embryos, but no rescue in the complete absence of *APC1*. If the activity in *APC2* null embryos was due to the combined action of APC1 based destruction complexes and independent APC2 mutant based destruction complexes, taking APC1 away would not affect the activity of the APC2 mutant destruction complexes. In the *APC2 APC1* double null background, however, the strongest APC2 mutants provide no APC dependent destruction complex activity. This suggests that APC1 and APC2 may be present in the same destruction complexes.

We propose that the basis for this cooperativity is APC self-association within the destruction complex, and that self-association is necessary for destruction complex assembly and/or activity. We have recently shown that Drosophila APC2 self-associates via the Armadillo repeats (ZHOU *et al.* 2011). Because APC1 also contains the conserved Armadillo repeats (Fig. 1B), we predict that APC2 and APC1 may have the ability to form heteroligomers (Fig. 8A). There is no precedent in the literature for direct association of Arm repeats suggesting the activity of a linker protein. Consistent with the idea of self association, overexpression of APC1 in the Drosophila embryo or larval brain redistributes APC2 from the cell cortex to the centrosome and microtubules where APC1 is localized, and overexpression of APC2 increases cortical localization of overexpressed APC1 (AKONG *et al.* 2002). Self-association may also explain why high levels of the APC2 deletion mutants have a dominant negative effect on rescue: because APC1 expression is low in the embryo, overexpressed APC2 deletions form homo-oligomers that are non-functional and compete for Axin binding in the destruction complex. If APC2 and APC1 associate in the destruction complex, they should colocalize, but we only observe this colocalization when APC1 is overexpressed. Furthermore, our studies of APC2, combined with other work on Axin strongly suggest that the destruction complex exists as cytoplasmic puncta. Endogenous APC2 localizes

to cytoplasmic puncta and given the findings presented here, we predict that endogenous APC1 is found there as well, below the level of detectability.

Vertebrate APC self-associates through at least 3 distinct domains not conserved in Drosophila APCs, including the N-terminal dimerization domain (Fig. 1A; (DAY and ALBER 2000; JOSLYN *et al.* 1993; SU *et al.* 1993a)). Furthermore, Li et al. (2008) have shown that a novel domain C-terminal to the Arm repeats (N3), and the last 300 amino acids of the protein (C3), also promote self-association. These N3-C3 and N3-N3 interactions of APC play a role in the regulation of peripheral clusters of APC in vertebrate cultured cells (LI *et al.* 2008). Within the basic domain, ANS2 promotes dimerization and is implicated in the actin nucleation function of APC in vitro (OKADA *et al.* 2010). Despite the abundant evidence that APC proteins can form dimers, a role for dimerization in APC's destruction complex function has not been demonstrated.

If APC exists as dimers or oligomers in the destruction complex, it may promote the assembly of large multi-protein complexes (Fig. 8A). Previous studies have suggested that Axin may have a similar effect on complex assembly. Axin can dimerize through 3 separable domains (LUO *et al.* 2005), and dimerization or polymerization has been implicated in destruction complex function (FIEDLER *et al.* 2011; PETERSON-NEDRY *et al.* 2008). Polymerization of Axin may increase its local concentration to promote interaction with its binding partners (FIEDLER *et al.* 2011), but the precise role of Axin self-association is not well understood. While our data are consistent with the model that APC also self-associates in the destruction complex, future experimentation will reveal precisely how self-association of Axin and APC contributes to the assembly and catalytic function of the "destructosome".

The role of phosphorylation and extended region interactions in APC's destruction complex function

Speculative models have suggested that APC with phosphorylated 20Rs (P-APC) functions in multiprotein complex assembly (HA *et al.* 2004), or as an essential step in a catalytic cycle (KIMELMAN and XU 2006; ROBERTS *et al.* 2011). Our in vivo data indicate that phosphorylation of the 20Rs is essential for the normal destruction of Arm. In the absence of all other APC, the 20R1-R5 SA mutant provides no destruction activity consistent with an essential role in complex assembly or promotion of a catalytic cycle.

To test whether P-APC is sufficient, we generated the phospho-mimetic form of APC2 where all Ser and Thr in the 20Rs were altered to aspartic acid. We found that the APC2 20R1-R5SD mutant acts as a loss of function: when APC2 20R1-R5SD is the only APC protein present, embryos express phenotypes consistent with ligand independent activation of Wnt targets**.** Taken together, these data indicate that both P-APC2 and non-P-APC2 are required for destruction complex function.

When APC is not phosphorylated, it binds ßcat through the 20R extended region. When phosphorylated, APC binds through both the extended region and through the phospho-region, increasing the binding affinity between APC and ßcat. The fact that APC2 20R1-R5SA and APC2 20R1-R5SD mutants are non-functional despite their ability to interact through the extended region, suggests that the extended region interactions are not sufficient for APC2's destruction complex function. Our data indicate that the extended region interactions are necessary for normal destruction function. In the complete absence of all extended region interactions (APC2 20R1-R5ExR), the mutant APC2 has no activity in the absence of endogenous APC2 and APC1 (Fig. 5,7). In fact, the APC2 20R1-R5ExR mutant, that still retains the ability to be phosphorylated, has less activity than either of the APC2 20R1-R5 phospho-mutants; it provides significantly less rescue in *APC2* null embryos (Table 1), and in double null embryos the APC2 20R1-R5 phospho-mutants rescue the zygotically heterozygous class (MZ+) to hatching, whereas the APC2 20R1-R5ExR mutant has no effect on either the MZ+ or the MZ class (Table 2). Taken together, our data are consistent with a model for destruction complex function that requires both P-APC and non-P-APC, and requires binding of the extended region to Arm.

The model of destruction complex assembly and action

Using other models as a foundation (HA *et al.* 2004; KIMELMAN and XU 2006; ROBERTS *et al.* 2011), we propose the following model for the assembly and activity of the destruction complex. We predict that the APC-Axin-kinase complex preassembles in part through interactions between the SAMP repeats of APC2 and the RGS domain of Axin (Fig. 8B, step 1). This is consistent with an essential role for the SAMP repeats in APC2's destruction complex function (ROBERTS *et al.* 2011), and an essential role for the APC binding RGS domain of Axin in the mouse (CHIA *et al.* 2009). Preassembly of

an APC-Axin-kinase complex is also consistent with the fact that when cytoplasmic levels of ßcat are low (in the absence of signal), the affinity between non-P-APC and ßcat is too low to promote their direct binding (HA *et al.* 2004). Here, ßcat assembles into the complex (Fig 8B, step 2) through the interaction with Axin via Arm repeats 3-5 and the extended region of the 20Rs through Arm repeats 5-9. While others have proposed that the 15Rs of APC serve in this function (KIMELMAN and XU 2006), Roberts et al (2011) have shown that the 15Rs are dispensable for APC2 function in the embryo in the presence of intact 20Rs. Further, as we have shown that the extended region is necessary, we favor a model in which the extended region of the 20Rs plays this role. Phosphorylation of the N-terminal sites in Arm, and the 20Rs of APC by CK1 and GSK3ß, displaces P-Arm from Axin and transfers it to P-APC (Fig. 8B, step 3). The notion that APC is phosphorylated within the complex is consistent with the findings that the direct binding of Axin to APC stimulates GSK phosphorylation of APC, and that the presence of ßcat further enhances APC phosphorylation (IKEDA *et al.* 2000). The displacement from Axin and disassembly of the complex (Fig. 8B, step 4) also involves the action of 20R2 specifically, and region B (ROBERTS *et al.* 2011). Because PP2A associates with the complex via Axin, it has been proposed that it may dephosphorylate APC within the complex, facilitating the turnover of ßcat to the proteosome (XING *et al.* 2003). The inability of PP1 to dephosphorylate APC in the presence of ßcat in vivo suggests that phospho-APC may not be accessible to phosphatase within the complex (HA *et al.* 2004). Instead, the interaction with the proteosome machinery may act to separate P-APC from P-Arm ((HA *et al.* 2004); Fig. 8B, step 5), sending P-Arm further down the path to proteosomal degradation, and releasing P-APC to be dephosphorylated (Fig. 8B, step 6). Non-P-APC is then available to reassemble with Axin and reinitiate the cycle.

In conclusion, the destruction complex of the Wnt signaling pathway plays a vital role in the negative regulation of Wnt signaling. Loss of this regulatory mechanism contributes to diseases such as colon cancer. As a complex molecular machine, it has been difficult to dissect its many moving parts to determine how the destruction complex assembles and how precisely these different parts contribute to ßcat phosphorylation and subsequent degradation by the proteosome. The combination of biochemical and

structural studies with rigorous in vivo testing are now beginning to reveal important factors that govern the activity of this essential molecular machine.

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Supplemental Figure 1.

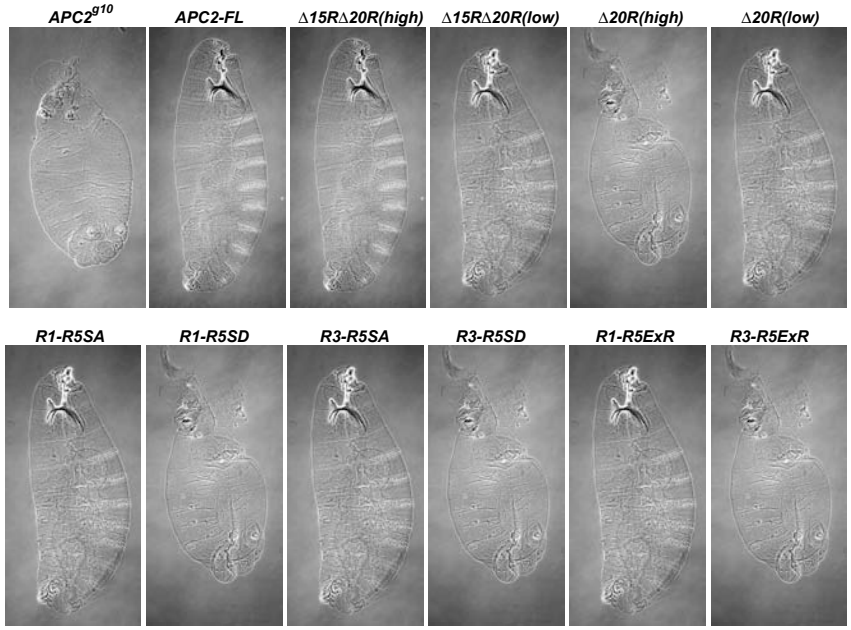

Figure S1: Representative cuticles for APC2 null embryos, and APC2 null expressing the indicated genotypes, are shown. Orientation: anterior is toward the top/ventral is either up or to the right. The phenotypic average (PA) is displayed for each genotype. All cuticles are shown at the same scale.

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Chapter 3:

The SAMP1 and SAMP2 repeats of APC2 and their phosphorylation state play distinct roles during Wnt signaling in Drosophila

* Manuscript in preparation

The SAMP1 and SAMP2 repeats of APC2 and their phosphorylation state play distinct roles during Wnt signaling in Drosophila

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Abstract

The tumor suppressor Adenomatous polyposis coli (APC) is a multifunctional protein involved in cell migration, proliferation and differentiation via its roles in regulating both Wnt signaling and the cytoskeleton. As a core component of the destruction complex together with Axin, CK1 and GSK3ß, APC targets ß-catenin/Armadillo (ß-cat/Arm) for proteosome-mediated degradation. C-terminal truncations of APC are strongly implicated in the initiation of both sporadic and familial forms of colorectal cancer. However, many questions remain as to how these mutations interfere with the APCs tumor suppressor activity. One domain that is frequently lost in these cancer-associated truncations is the SAMP repeat domain that is known to mediate interactions between APC and Axin. Both APC and APC2 proteins in vertebrates and Drosophila contain multiple SAMP repeats that don't share high sequence conservation outside of the SAMP motifs. In this study, we tested the functional redundancy between different SAMPs and how these domains are regulated by using Drosophila APC2 as our model. We first asked whether both SAMP repeats have Axin binding ability by yeast two-hybrid and showed that SAMP2 has stronger binding activity, consistent with our predictions. In addition, we tested the activity of each SAMP repeat and if phosphorylation of these repeats played an important role in regulating APC2's function during Wnt signaling by using a combination of cell culture experiments and in the intact animal. Overall, our findings support a model whereby each SAMP repeat plays a different role individually but also cooperate for efficient destruction complex function.

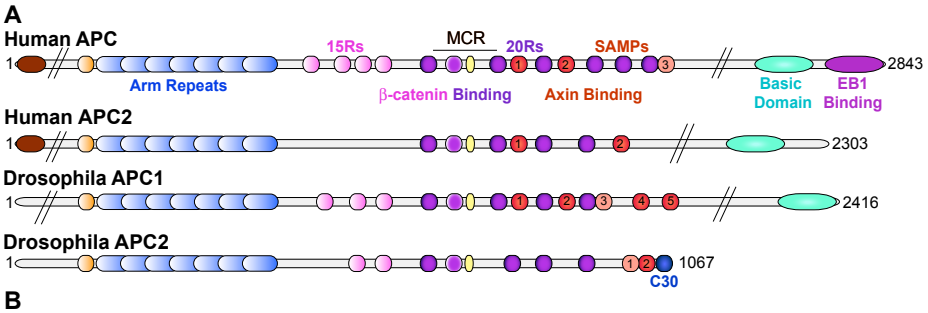
INTRODUCTION

Evolutionarily conserved canonical Wnt signaling is one of the most critical signaling pathways that regulate animal development (Logan and Nusse, 2004). Inappropriate activation or deactivation of the pathway is lethal during embryogenesis from flies to mammals, and is associated with a multitude of other diseases such as cancer (Clevers and Nusse, 2012). Thus, the tight control of Wnt signaling is essential for both normal development and adult tissue homeostasis.

The tumor suppressor Adenomatous polyposis coli (APC) is an essential negative regulator of Wnt signaling. APC is a part of a multi-protein complex called the destruction complex or destructosome that targets the key transcriptional effector of the pathway ß-catenin for phosphorylation and subsequent degradation by the proteosome (Cadigan and Peifer, 2009; Kunttas-Tatli *et al.*, 2012). When signaling is off in the absence of a Wnt ligand, cytoplasmic and nuclear levels of ß-catenin are kept low by destructosome activity. APC, Axin, and the kinases GSK3ß and CK1 are the core components of this complex, and loss of any of these leads to the unregulated accumulation of ß-catenin and expression of Wnt target genes. When signaling is on, the Wnt ligand binds to Frizzled (Fz) and LRP5/6 co-receptor complex. This in turn recruits Axin to the membrane in a process that deactivates the destructosome. Consequently, ß-catenin levels rise dramatically, leading to the expression of Wnt target genes.

APC is a highly phosphorylated, multi-domain protein that contains conserved binding sites for other components of the Wnt pathway (Fig. 1). Most notably, the 15 and 20 amino acid repeats (15Rs and 20Rs) mediate interactions with ß-catenin, while the SAMP repeats are required for Axin binding (Behrens et al., 1998; Rubinfeld et al., 1995). More than 80% of all inherited and sporadic forms of colon cancer carry mutations in the APC gene (Polakis, 2012). Most of these mutations occur in a hot spot called the Mutation Cluster Region (MCR, Fig. 1A) and produce APC truncations missing all of the SAMP repeats and many of the 20Rs (Beroud, 1996). This suggests that the SAMP repeats play an important role in APC's tumor suppressor function.

Over the years, numerous studies have addressed the importance of 15Rs and 20Rs, and how phosphorylation of 20Rs influences the functional interactions with ß-



B

hAPC-SAMP1	TIDSEKDLLDDSDDDDIEILEECIISAMPTKSSRKGKK---
hAPC-SAMP2	TSSVTIPELDDNKAEEGDILAECINSAMPKGKSHKPFR---
hAPC-SAMP3	SRNSSLSSLSIDSEDD--LLQECISSAMPKKKKPSRLK---
hAPC2-SAMP1	GPAPTGSRPRGAADQELELLRECLGAAVPARLRKVASALV
hAPC2-SAMP2	PGPGGGRDSSPSPRAAEELLQRCISSALPRRRPPVSGLRR
dAPC1-SAMP1	--DEKAPAVWTEDNEDELLLANCINMGMQRKPTEAVKSTV
dAPC1-SAMP2	-----SSNASDCGGASGHLLQQCIRDGMKKPLGEATSDPI
dAPC1-SAMP3	WQDDSLSSLSIDSEDDTNLLSQAIAAGCNRPKSNLGFSS-
dAPC1-SAMP4	---SSVDSSDSNDNQSKSLFELCILKGMYKTKEPGARAQQ
dAPC1-SAMP5	-----HHRERERERKDEKLLQECINTGISKKINAVPKN--
dAPC2-SAMP1	--SDSLSSLSMDSDDDCNLLSQAIAAGSCRPQPSGASTSS
dAPC2-SAMP2	YSSDDSLDDDDDDARSKSLFEQCILSGMHKSNDALESEGE

Figure 1: A) Schematic representation of human APC (hAPC) and APC2 (hAPC2) and Drosophila APC1 (dAPC1) and APC2 (dAPC2). Oligomerization domain: OD1 (burgundy), ASAD: APC self-association domain (orange), Arm repeats: Armadillo repeats (blue) 15Rs: 15 amino acid repeats (pink), 20Rs: 20 amino acid repeats (purple), b-catenin inhibitory domain CID (yellow), SAMP Repeats (red), Basic domain (turquoise), EB1 binding domain (magenta), C30 (dark blue). B) Sequence alignment of SAMP repeats between human and Drosophila APC proteins. SAMP signature motifs: SAMP Motif (red), upstream motif (yellow).

catenin (Kunttas-Tatli et al., 2012; Liu et al., 2006). Recent studies have revealed that there is surprising functional diversity among the 20Rs (Kunttas-Tatli et al., 2012; Roberts et al., 2011). It is not known if similar mechanisms regulate SAMP repeat activity, or if there is functional diversity amongst the SAMP repeats.

Both human APC and APC2 homologs contain multiple SAMP repeats. Human APC contains three SAMP repeats that are distributed among the 20Rs (Fig. 1A). *in vitro* studies mapped the Axin interaction domain to a 20 amino acid region containing the conserved signature motif **S**er-**A**la-**M**et-**P**ro, hence the name SAMP (Behrens *et al.*, 1998). The co-crystal structure between vertebrate Axin's RGS domain and APC's 3rd SAMP repeat led to important insights into how the two proteins interact, and uncovered additional hydrophobic residues that make important contacts for Axin binding *in vitro* (Fig. 1B, yellow) (Spink *et al.*, 2000). Both the number of SAMP repeats and their sequence identity are well conserved among vertebrate APCs (Fig. 1B and 2). However, there is greater sequence diversity in vertebrate APC2s, as well as in invertebrate APCs (Fig. 1B and 2). This might suggest the possibility of functional diversity among these repeats.

To ask whether there is functional diversity among SAMP repeats, we used the highly tractable Drosophila APC2 as our model. Drosophila APC2 contains only two SAMP repeats, dSAMP1 and dSAMP2, (Fig. 1A). dSAMP2 contains a similar sequence motif to the human homolog, while dSAMP1 is considered to be a degenerate SAMP due to lack of conservation in the SAMP signature (Fig. 1B). In addition, both SAMP repeats contain residues that are phosphorylated, suggesting a potential regulatory mechanism that has not been previously explored. Thus, first we wanted to test if dSAMP1 could interact with Axin albeit the lack of motif conservation and later we tested if phosphorylation of these repeats played an important role in APCs Wnt signaling function in cell culture and *in vivo* in Drosophila embryos. Our results show support the functional diversity in between two SAMP repeats and potential mechanism for cooperation for efficient destruction complex function

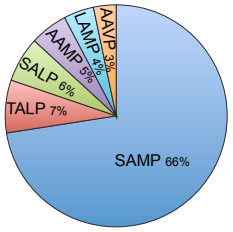
Results:

Conservation of SAMP Repeats

	SAMP Motif	SAMP	SALP	AAM/VP	Other	Total
	Additional Residues	I/LL--CI	I/LL--CI	I/LL--CI	I/LL--CI	
	Vertebrate					
Mammals	*H. sapiens APC*	3	-	-		3
	H. sapiens APC2	-	1	1		2
	M. musculus APC	3	-	-		3
	M. musculus APC2	-	-	1 (AL--CL)	LAMP	2
	B. taurus APC	3	-	-		3
	B. taurus APC2	-	2	-		2
	M. domestica APC	3	-	-		3
	M. domestica APC2	2	1 (FS--SS)	-	TALP (LK--LP)	3
Birds	*G. gallus APC*	3	-	-		3
	G. gallus APC2	2	1 (FS--SS)	1 (RK--PV)	TALP (LQ--LP)	5
Reptile	*A. carolinensis APC*	3	-	-		3
	A. carolinensis APC2	1	1 (FS--SS)	-	TALP (LK--LP)	2
Amphibian	*X. laevis APC*	3	-	-		3
	X. laevis APC2	1	2 (FS--SS) (RI--PK)	-		3
Fish	*D. rerio APC*	3	-	-		3
	D. rerio APC2	2	1 (FS--SS)	-		3
	Invertebrate					
Ascidian	*C. intestinalis APC*	-	-	-	SAIP(2) QGMP (AA--NK) KGIP (AI--EM)	2-4
	S. kowalevskii APC **Hemichordate**	2	-	1	SGIP (ST--TV)	3-4
Echinoderm	*S. purpuratus APC*	-	-	1	SAIP SGMP SGIQ (GG-KT)	3-4
	C. teleta APC **Annelid**	-	1	1	LAMP	3
	L. gigantea APC **Snail**	2	-	1		3

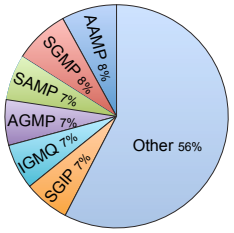
	SAMP Motif	-GM-	-GI-	Other	Total
	Additional Residues	I/LL--CI	I/LL--CI		
	Invertebrate				
Insects	*D. melanogaster APC1*	MGMQ DGMK KGMY	TGIS	AGCN (LL--AI)	5
	D. melanogaster APC2	SGMH (LF--CI)		AGSC (LL--AI)	2
	A. aegypti APC Mosquito	IGMS SGMP KGMR	AGIT AGIQ(LV--EP)		5
	T. castaneum APC Beetle	AGMP(2) IGMQ FGMA			4
	B. terrestris APC Bee	SGMP(2)	MGIQ	AAMP	4
	N. vitripennis APC Wasp		SGIS SGIP(QI--TN) SGIP(SI--LV) KGIR(KS--EV)		4
	Ant APC	IGMQ	SGIP(LK--NL)	PSIK	3

Frequency of various SAMP motifs in vertebrates



Other Trends
xAMx: 75%
xALx: 13%
S/Txxx: 88%
xxMx: 72%

Frequency of various SAMP motifs in invertebrates:



Other Trends
xGMx: 42%
xAMx: 17%
xGIx: 26%
Sxxx: 35%

Figure 2: Conservation of SAMP repeats in vertebrate and invertebrate APCs. Variations of the SAMP signature motifs are summarized across many species. Pie charts were generated based on the frequency of various SAMP motifs, other trends are also listed in %.

The conservation of SAMP repeats varies among vertebrates and invertebrates

Given the strong conservation in Wnt signaling mechanisms across animal phyla, it was not surprising to find both key negative regulators APC and Axin across the animal kingdom. In order to gain more insight into the APC-Axin interaction, we examined the level of sequence conservation of APC SAMP repeats across diverse taxa, and generated multiple sequence alignments by searching for both of the signature motifs LLxxCIxSAMP. Vertebrate APC (vAPC) SAMP repeats are invariant both in number and the sequence identity of the signature motif (Fig. 1B and 2). Interestingly, both the number and the degree of sequence conservation is weaker in vAPC2s and in invertebrate APCs.

The Ser in the SAMP motif is thought to be dispensable since it fails to make any contacts with Axin-RGS domain in the crystal structure. However, there appears to be a high selective pressure among vertebrate APCs to have a phosphorylatable residue (Ser or Thr) at this first position, which gets weaker in invertebrates (Fig. 2). Based on the crystal structure, the remaining residues in the SAMP motif make contact with Axin and play an important role in Axin binding. Having a small residue such as glycine or alanine is preferred at the second position and this trend is highly conserved in both vertebrates and invertebrates APCs (Fig. 2). Interestingly, in invertebrates a glycine is greatly preferred. As for the third position, hydrophobic Met is thought to be essential as substitutions at this position disrupt Axin binding (Fig. 2). Thus, Met is highly conserved in many vertebrate and invertebrate APC proteins. However, in vertebrate APC2s, Met is frequently replaced with Leu, and in invertebrates with Ile. Pro at the fourth position is also highly conserved in both vertebrate APCs and non-insect invertebrates and makes hydrophobic contacts with Axin-RGS. This residue is often replaced with a positively charged amino acid in insects (Fig. 2). In the case of Drosophila, there are corresponding compensatory substitutions in dAxin-RGS sequence, which allows for efficient repacking in this area and might explain the divergence from the vertebrate sequence motif.

While the conservation of the SAMP motif shows some sequence diversity, the upstream hydrophobic residues are highly conserved both in vertebrates and invertebrates (Fig. 1B and 2). The higher evolutionary pressure to retain these residues

compared with the SAMP motif itself may suggest that these residues play a more significant role in Axin binding. This might explain why mutations in these upstream sequences can be found in some cases of attenuated familial colorectal cancer patients.

Both Drosophila APC2 SAMP1 and SAMP2 can bind Axin albeit with different affinities

Both APC and APC2 proteins in vertebrates and Drosophila contain multiple SAMP repeats that don't share high sequence conservation outside of the SAMP signature motifs. To dissect the role of individual SAMP repeats in APCs Wnt signaling function, we used Drosophila APC2 as our model. Drosophila APC2 contains two SAMP repeats, dAPC2-SAMP1 and dAPC2-SAMP2. Previous studies suggested that because dAPC2-SAMP1 lacked SAMP sequence conservation, it is unlikely to bind to Drosophila Axin (dAxin). This prompted us to test the functions of these repeats individually and we generated mutants that lacked SAMP1, SAMP2 or both. To test if both dAPC2-SAMPs could interact with dAxin, we used a yeast two-hybrid (Y2H) assay.

The C-terminal half of APC2 containing the 15Rs, 20Rs, and SAMPs (APC2-Cterm), can interact with both ß-catenin and Axin-RGS (Fig. 3). As expected, removal of both 15Rs and 20Rs (APC2-Cterm-SAMP) disrupts ß-catenin binding but has little effect on Axin binding. Conversely, removal of both SAMP repeats (APC2-Cterm-15R20R) disrupts Axin binding, and has no effect on ß-catenin binding. Consistent with our hypothesis that SAMP1 is a weaker Axin binder, dAPC2-SAMP1 displayed a significantly weaker interaction with Axin compared to dAPC2-SAMP2 (Fig. 3). However, we observed the strongest binding interaction when both SAMP repeats were present, which may suggest an additive effect. Taken together, these results suggest that dSAMP2 is the primary Axin-binding SAMP that we predict will have the greater role in destructosome function.

Either SAMP is sufficient for the recruitment of APC2 to the destructosome, but the removal of SAMP2 altered Axin localization in Drosophila S2 cells

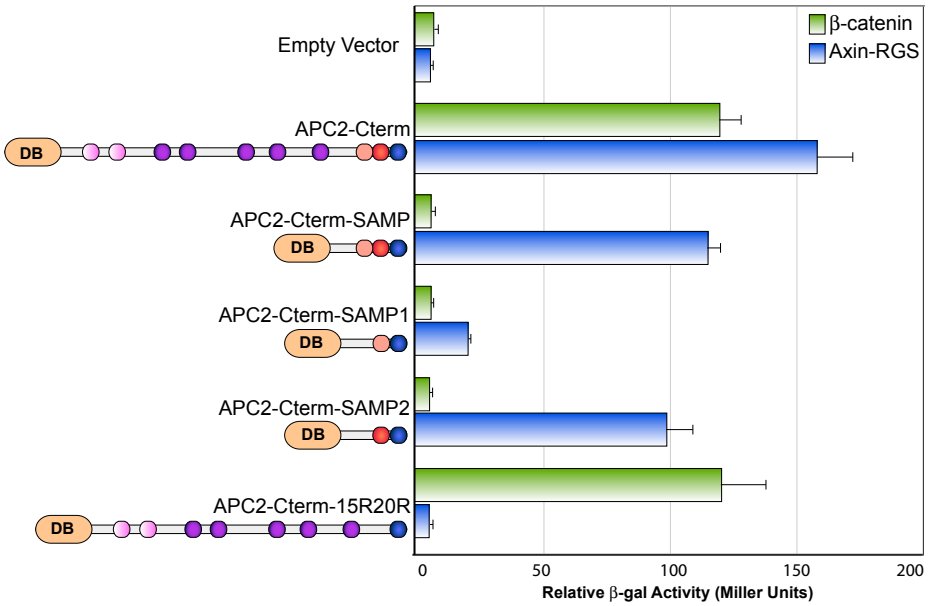


Figure 3: Both Drosophila APC2 SAMP1 and SAMP2 can bind Axin albeit with different affinities. Yeast two hybrid experiments demonstrated that Axin-RGS (blue) interacts directly with SAMP1 (weak) or SAMP2 (strong) and deletion of both SAMPs abolishes this interaction. 15Rs and 20Rs mediate interactions with β-catenin (green), which gets disrupted upon removal of these repeats.

Overexpressed or endogenous Axin in cell culture or in intact tissues forms cytoplasmic puncta due in part to its ability to form oligomers. We recently demonstrated that APC self-association is also essential for the assembly and stability of the destructosome puncta (Kunttas-Tatli et al., 2014 in press). We wanted to test, if weak vs strong ability of different SAMPs to bind to Axin based on Y2H had an affect on colocalization with Axin in cells.

To test this hypothesis, we transiently transfected Drosophila S2 cells with tagged dAxin and dAPC2. As previously reported, when expressed alone in S2 cells, Axin-GFP forms cytoplasmic puncta, while mCherry(mCh)-APC2-FL localizes to the cell cortex (Fig. 5). When co-expressed, APC2-FL localizes primarily to the cytoplasmic Axin puncta. Deletion of both Axin-binding SAMP repeats (APC2-ΔSAMP) restored the cortical localization of APC2 (Fig. 5).

To determine whether removal of APC2 SAMP1 or SAMP2 had an effect on colocalization with Axin, we co-expressed the APC2 SAMP mutants (APC2-ΔSAMP1 and APC2-ΔSAMP2) with Axin-GFP (Fig. 5). Consistent with the Y2H experiments, removal of either SAMP domain had no effect on Axin colocalization, which suggests the weaker Axin binding ability of SAMP1 had no effect on colocalization. However, removal of the stronger Axin binder, SAMP2, resulted in redistribution of Axin to the cell cortex in a proportion of the cells. Because expression levels could influence this effect, we used FACS to sort the cells and revealed that the redistribution to the cortex was greatly enhanced in cells expressing high levels of protein.

We also tested if both SAMPs were able to colocalize with Axin in SW480 human colon cancer cells, to rule out the possibility of cell type specific effects. Similar to Drosophila S2 cells, removal of either SAMP had no effect on colocalization with Axin (Fig. 6). However, unlike S2 cells, we did not observe redistribution of Axin to the cortex in dAPC2-ΔSAMP2. This is consistent with the fact that normal distribution of APC2 is not cortical in SW480 cells, possibly due to the absence of the cortical binding partner.

SAMP1, but not SAMP2, is necessary for destructosome activity in SW480 cells

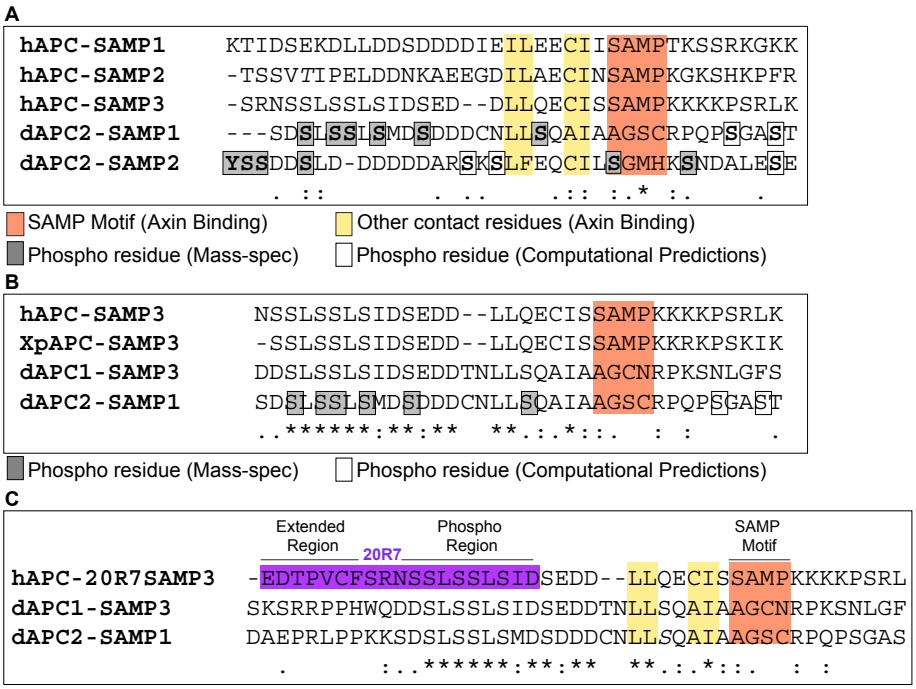


Figure 4: Sequence alignment between hAPC SAMP repeats and and Drosophila APC2 SAMP repeats. A) Phospho-residues based on mass-spec studies or computational predictions are marked with gray boxes. B) Sequence alignment of vAPC SAMP3 human and dAPC2-SAMP1 to show the high sequence conservation among this subset of SAMP repeats. C) hAPC-SAMP3 upstream sequences are part of a 20R7, and shows that this hybrid domain is conserved between humans and flies.

Because SAMP2 is a stronger Axin binder by Y2H, we predicted that it would be the primary SAMP required for destructosome activity. To test this, we asked whether SAMP2 is necessary and sufficient for APC2's destructosome function. SW480 cells endogenously express an APC MCR truncation that leads to elevated levels of ß-cat and high levels of Wnt target gene expression due to loss of destructosome activity. Previous studies showed that the expression of dAPC2 could compensate for the loss of hAPC function to suppress the elevated levels of ß-cat and the high levels of Wnt target gene expression. ß-catenin mediated transcriptional activity is measured by the well-established TOP-Flash luciferase assay (Korinek *et al.*, 1997). We predicted that the removal of SAMP2 would reduce or eliminate the ability of dAPC2 to suppress ß-cat levels and Wnt target gene activation. Surprisingly, the deletion of SAMP2 (APC2-ΔSAMP2) had no effect on APC2's destructosome function; APC2-ΔSAMP2 fully suppressed the elevated levels of Wnt target gene activity indistinguishable from APC2-FL (Fig. 6). In contrast, deletion of SAMP-1 (APC2-ΔSAMP1) significantly reduced the ability of APC2 to suppress Wnt target gene activation, similar to the deletion of both SAMP repeats (APC2-ΔSAMP), which might suggest that APC2-SAMP1 plays a functionally significant role in destructosome activity in SW480 cells and APC2-SAMP2 is dispensable.

Disrupting SAMP1 or SAMP2 phosphorylation has no effect on colocalization with Axin, but disrupting SAMP1 phosphorylation leads to Axin redistribution

APC proteins are regulated via phosphorylation in many contexts. A well-studied example is the multi-phosphorylation of the 20Rs by CK1 and GSK3ß. Phosphorylation of 20Rs regulates ß-catenin binding affinity, which play a significant role in destructosome activity *in vivo*. Mass-spectrometry studies and computational predictions suggest that multiple residues in the dAPC2-SAMP repeats are phosphorylated, suggesting that SAMP repeat phosphorylation may play a role in dAPC2's destructosome function. To test this hypothesis, we generated targeted mutations in the APC2 SAMP repeats that prevent phosphorylation. The selection of

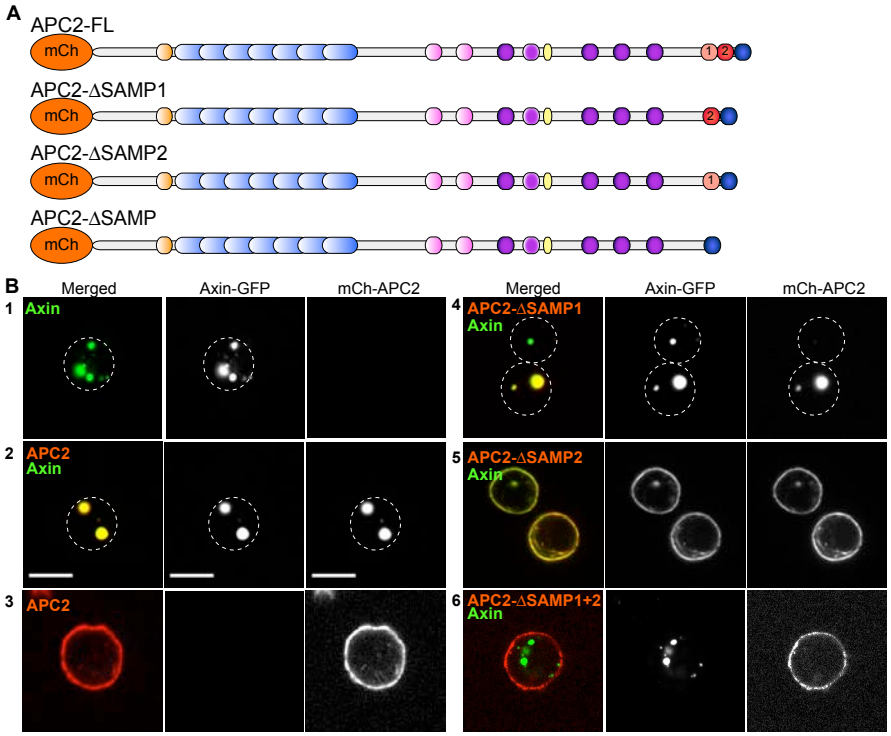


Figure 5: Either SAMP is sufficient for the recruitment of APC2 to the destructosome, but the removal of SAMP2 altered Axin localization in Drosophila S2 cells. A) Schematic representation of mCherry (mCh) tagged APC2 constructs B) Axin is GFP tagged (dotted l ines indicate cell boundaries) forms oligomers, which can be visualized as cytoplasmic puncta (1) When coexpressed, mCh-APC2-FL colocalizes in cytoplasmic puncta with Axin-GFP (2) and mCh-APC2-FL (3) is primarily cortical. Removal of APC2's either Axin interaction domains (APC2-ΔSAMP1 and APC2-ΔSAMP2) has no effect on colocalization with Axin-GFP but APC2-ΔSAMP2 alters Axin localization to cortex. Removal of both SAMPs (APC2-ΔSAMP) disrupt colocalization. Scale Bar: 10μm

residues to mutate was based on both the mass spectrometry data and our computational predictions. We have designated these mutants as APC2-SAMP1-SA, APC2-SAMP2-SA and APC2-SAMP-SA (Fig. 7).

To determine whether disruption of phosphorylation had an effect on recruitment of APC2 to Axin puncta or on puncta assembly, we co-expressed the APC2 SAMP mutants (APC2-SAMP1-SA, APC2-SAMP2-SA and APC2-SAMP-SA) with Axin-GFP. Disruption of phosphorylation had no effect on colocalization with Axin. However, similar to deletion of SAMP1 (Fig 5), blocking phosphorylation in SAMP1 (APC2-SAMP1-SA), or in both SAMPs (APC2-SAMP-SA), resulted in redistribution of Axin to the cell cortex in a proportion of the cells. To rule out the potential effect of expression levels, we FACS sorted the cells. Similar to APC2-ΔSAMP2, redistribution of Axin to the cortex was greatly enhanced in cells expressing high levels of protein (Fig. 7).

Disrupting SAMP1 phosphorylation, but not SAMP2, is necessary for destructosome activity in SW480 cells

Consistent with Drosophila S2 cells, neither disruption of SAMP1 nor SAMP2 phosphorylation had an effect on recruitment to the destructosome in SW480 cells. Similar to complete loss of SAMP1 (Fig 6), disrupting SAMP1 phosphorylation (APC2-SAMP1-SA) resulted in a protein with reduced destructosome function that only moderately suppressed the high level of Wnt target gene activation in SW480 cells (Fig. 8). In contrast, APC2-SAMP2-SA fully suppressed the elevated levels of Wnt target gene activity similar to APC2-FL (Fig. 8B). This suggests that SAMP2 phosphorylation is not necessary for the activity of the destructosome. Consistent with these observations, disruption of phosphorylation in both SAMPs (APC2-SAMP-SA) resulted in a loss of destructosome function similar to APC2-SAMP1-SA.

Upstream SAMP1 phosphorylation plays a more significant role in APC2's destructosome function

The upstream phospho-residues in dAPC2-SAMP1 are highly conserved both in vertebrate APC-SAMP3 and Drosophila APC1-SAMP3, despite the lack of sequence conservation in the SAMP motif itself (Fig 4). Therefore, we predicted that the upstream

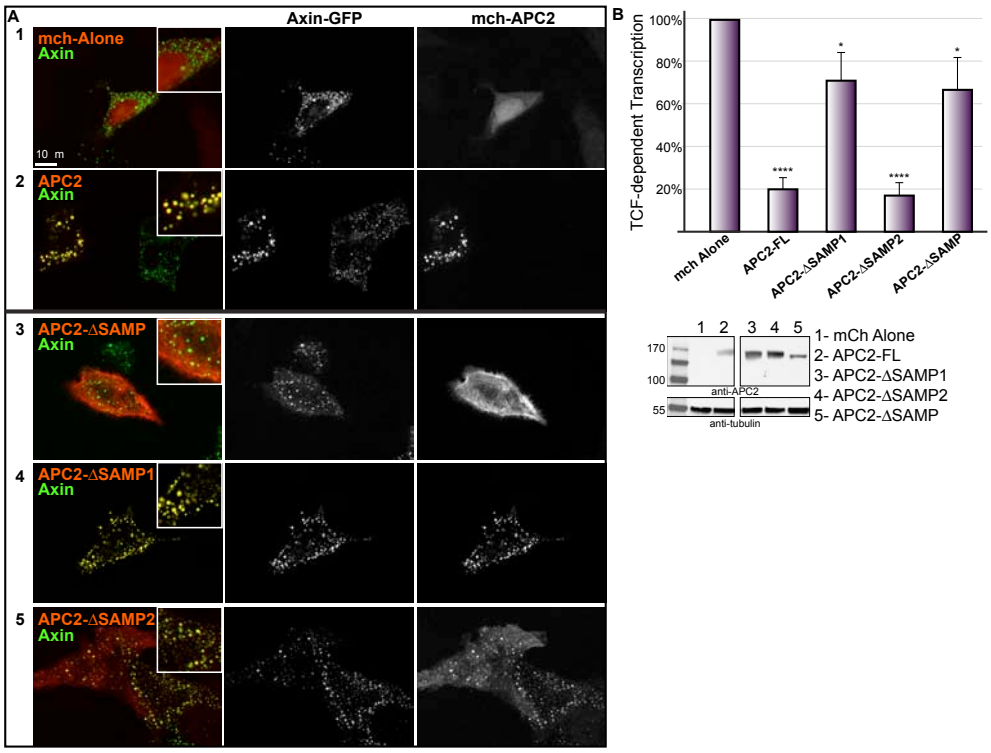


Figure 6: Either SAMP is sufficient for the recruitment of APC2 to the destructosome in SW480 cells and SAMP1, but not SAMP2, is necessary for destructosome activity in SW480 cells. A) Similar to S2 cells, Axin-GFP forms cytoplasmic puncta (1) and mCh-APC2 colocalizes with Axin-GFP in SW480 cells (2). Coexpression of ΔSAMP1 or ΔSAMP2 with Axin-GFP does not disrupt colocalization (4, 5), but removal of both domains disrupts colocalization. B) Expression of full length Drosophila APC2 was sufficient to suppress the elevated levels of ß-cat target gene activity in SW480 cells. The APC2-ΔSAMP1 and APC2-ΔSAMP mutant moderately suppressed the elevated target gene expression unlike APC2-ΔSAMP2, which looked similar to APC2-FL control. mCh-tagged APC2-FL and all the SAMP deletion constructs were expressed at comparable levels in SW480 cells used in the TOP/Flash assays. Scale Bar: 10μm

phospho-residues are the ones that play the most significant role in SAMP1's destructosome function. To test this hypothesis, we generated mutants that disrupted (APC2-SAMP1-SA$^{up)}$ or mimicked (APC2-SAMP1-SD^{up}) phosphorylation only in these residues. As a complement, we also generated a mutant that disrupts only the downstream putative phospho-residues (APC2-SAMP1-SA^{down}) (Fig. 9A).

To determine whether the phosphorylation status of the upstream sequences alone had an effect on recruitment of APC2 to Axin puncta, on puncta assembly, or on the redistribution of Axin to the cortex, we co-expressed the additional APC2 SAMP mutants (APC2-SAMP1-SA^{up}, APC2-SAMP1-SA^{down}, and APC2-SAMP1-SD^{up}) with Axin-GFP in S2 cells. Consistent with APC2-SAMP1-SA, none of these mutants affected colocalization with Axin or the formation of puncta (Fig 9B). Interestingly, none of these mutants drove Axin to the cortex as we observed with APC2-SAMP1-SA, suggesting that this effect requires both the upstream and downstream putative phospho-residues.

We also tested the ability of these mutants to suppress high levels of Wnt target gene activity in SW480 cells as a measure of their destructosome function (*This data needs to be repeated-just a preliminary result). The mutant with wild type upstream residues, but disrupted downstream residues (APC2-SAMP1-SA^{down}) had destructosome activity indistinguishable from wild type APC2, suggesting that the ability of the upstream sequences to be phosphorylated is sufficient for APC2's destructosome function and the downstream residues are not necessary. If phosphorylation of the upstream sequences alone is sufficient for destructosome activity, we predict that the phospho-mimetic APC2-SAMP1-SD^{up} will have wild type activity. Consistent with this prediction, this mutant is indistinguishable from wild type.

These results predict that disruption of the upstream sequences alone will significantly disrupt function. Surprisingly, blocking upstream SAMP1 phosphorylation (APC2-SAMP1-SA^{up}) only mildly reduced destructosome function in SW480 cells (Fig. 9C). This suggests that in the absence of upstream phosphorylation, the downstream phosphorylation is able to support some destructosome activity, albeit at significantly reduced levels. Taken together, these data suggest that the upstream phospho-

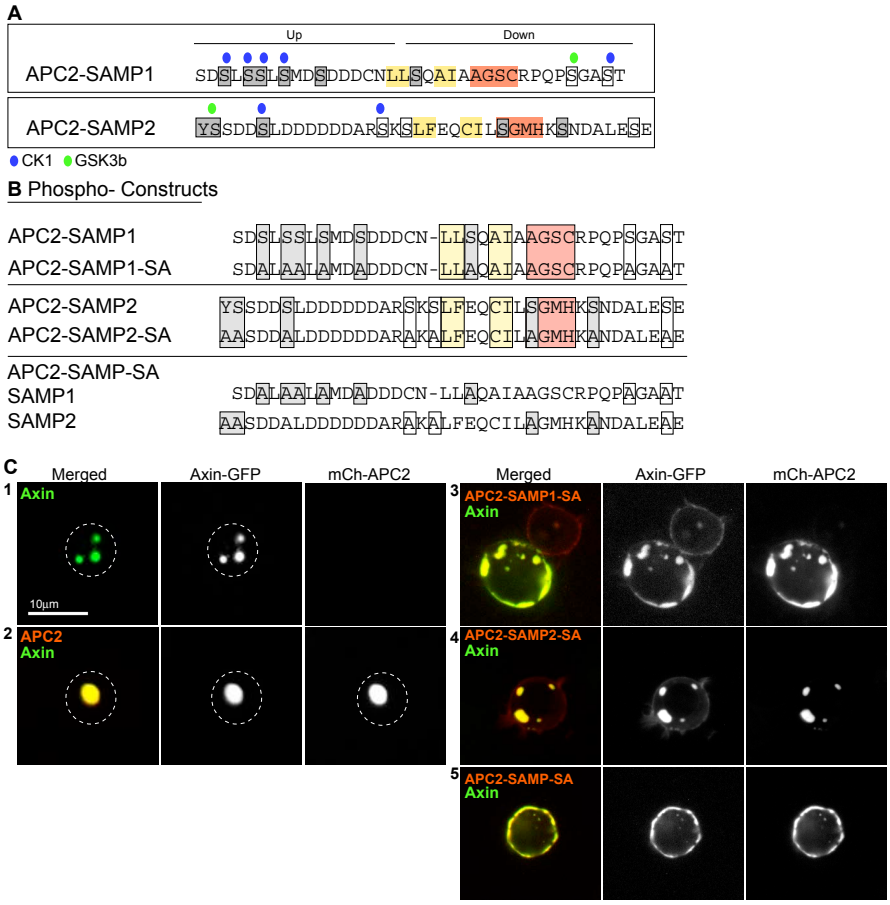


Figure 7: A) Sequences of Drosophila APC2 SAMP repeats with phospho-residues marked with grey boxes and CK1 (blue dots) and GSK3 (green dots) sites that are predicted to phosphorylate some of these residues. B) Sequences of APC2 SAMP phospho-deficient constructs. C) Axin-GFP alone forms cytoplasmic puncta and when coexpressed, mCh-APC2-FL colocalizes in cytoplasmic puncta with Axin-GFP (2). Disruption of phosphorylation in APC2's either SAMP or in both SAMPs has no effect on colocalization with Axin-GFP but APC2-SAMP1-SA and APC2-SAMP-SA alters Axin localization to cortex (similar to APC2-ΔSAMP2). Scale Bar: 10µm

residues play a more significant role in destructosome function, but that there is cooperativity between the upstream and downstream.

SAMP1 and SAMP2, and their phosphorylation, are required for destructosome activity in the Drosophila embryo

Because APC2 SAMP repeats are necessary for proper ß-cat regulation in cultured cells, we asked whether either SAMP is also necessary for destructosome activity and the negative regulation of Wnt signaling in the more physiologically relevant context in the Drosophila embryo. We expressed GFP-tagged APC2-FL, all deletion mutants (APC2-ΔSAMP1, APC2-ΔSAMP2, APC2-ΔSAMP), and the phospho-deficient mutants (APC2-SAMP1-SA, APC2-SAMP2-SA, APC2-SAMP-SA) in the embryo under the native APC2 promoter (McCartney *et al.*, 2006).

Between 4-6 hrs after egg-laying, Wnt signaling plays an essential role in the patterning of embryonic segments. While ventral cells receiving the Wnt signal make a smooth cuticle, ventral cells not receiving the signal make denticles, and this repeated pattern can be visualized in the embryonic cuticle. The same pattern can also be visualized as accumulation of ß-catenin in stripes of cells receiving the Wnt signal and in the patterned expression of the Wnt target gene *engrailed.* This pattern is disrupted in embryos with aberrant Wnt signaling such as in the null APC2 (*APC2*^{g10}*).*

In Drosophila, APC1 and APC2 play redundant roles in Wnt signaling throughout development, but APC2 is the main APC in the embryo due to its high level of expression. In *APC2* null embryos, Wnt signaling is activated uniformly throughout the ectoderm resulting in complete embryonic lethality. Embryos exhibit cuticle defects including excess smooth cuticle at the expense of denticles (Fig 10), and expansion of the *en* expression domain (Fig 10). Loss of APC1 exacerbates these *APC2* null embryonic phenotypes.

As previously shown, expression of APC2-FL in this null background is sufficient to rescue the embryonic defects. Consistent with the TopFlash analysis (Fig. 6B), and previous reports, APC2-ΔSAMP has significantly reduced destructosome function; removal of both SAMPs resulted in only a 36% hatch rate, moderate cuticle defects, and expanded *en* expression. This suggests that the SAMP repeats are necessary for

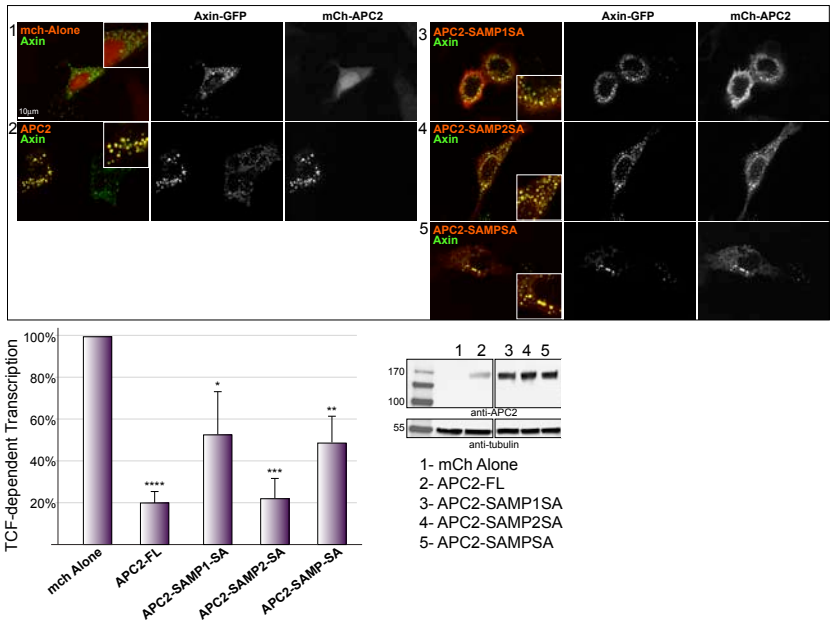


Figure 8: Disruption of SAMP phosphorylation is sufficient for the recruitment of APC2 to the destructosome in SW480 cells and phosphorylation of SAMP1 is necessary for destructosome activity in SW480 cells. A) Similar to S2 cells, Axin-GFP forms cytoplasmic puncta (1) and mCh-APC2 colocalizes with Axin-GFP in SW480 cells (2). Coexpression of SAMP1-SA, SAMP2-SA or SAMPSA with Axin-GFP does not disrupt colocalization (3, 4, 5). B) Full length Drosophila APC2 was sufficient to suppress high levels of target gene activity. The APC2-SAMP1-SA and APC2-SAMP-SA mutants moderately suppressed the elevated levels of Wnt target gene activity unlike APC2-SAMP2-SA, which looked similar to APC2-FL control. mCh-tagged APC2-FL and all the Phospho-SAMP constructs were expressed at comparable levels in SW480 cells used in the TOP/Flash assays. Scale Bar: 10µm

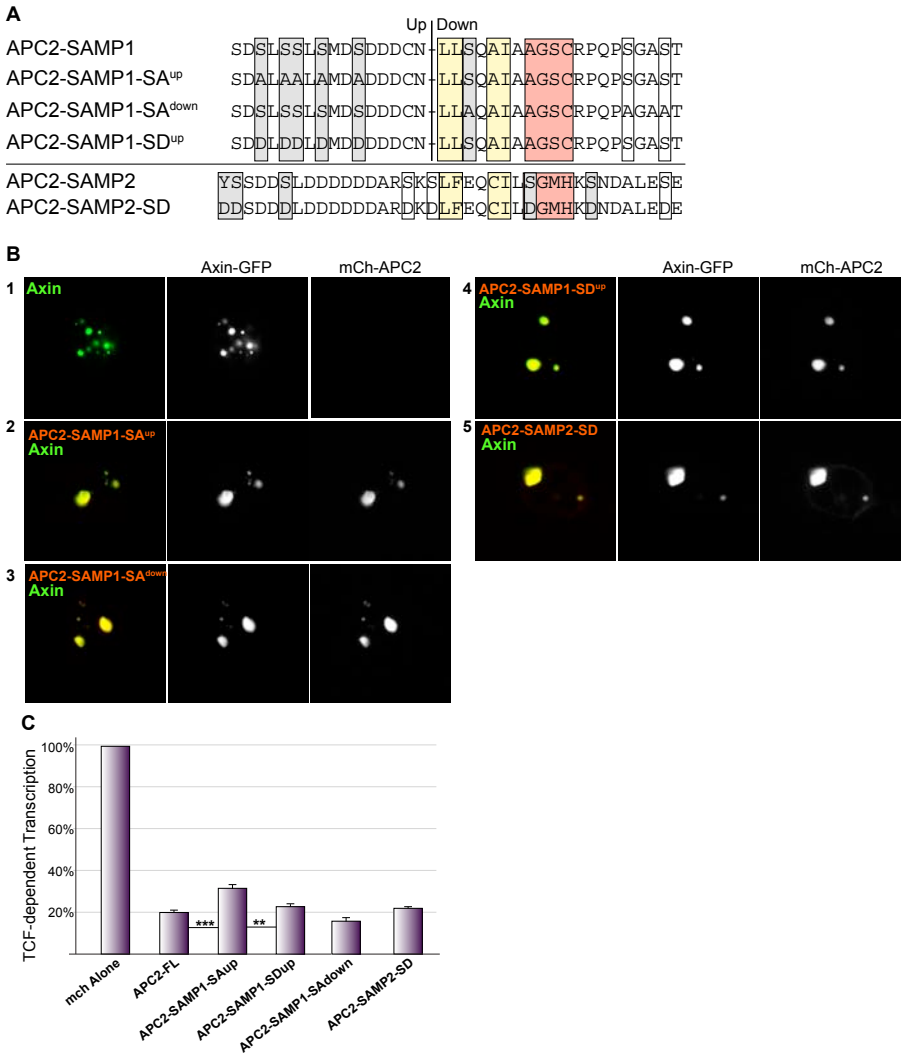


Figure 9: A) Sequence schematics of additional Phospho-SAMP mutants. SAMP1 domain was further divided into up and down and subset of phospho-residues were mutated. APC2-SAMP1-SA-up: Upstream phospho-residues were mutated to Ala, APC2-SAMP1-SA-down: Downstream phospho-residues were mutated to Ala. APC2-SAMP1-SD-up: Upstream phospho-residues were mutated to Asp to mimic phosphorylation, APC2-SAMP2-SD All phospho-residues were mutated to Asp. B) Axin-GFP in cytoplasmic puncta (1) and Axin-GFP+mCh-APC2-FL colocalizes in cytoplasmic puncta (2). In all of the additional phospho-constructs, there was no effect on colocalization with Axin-GFP. Scale Bar: 10μm

APC2's destructosome activity. Based on the TopFlash assay (Fig. 6B), we predicted that SAMP1, but not SAMP2, would be necessary and sufficient for destructosome function. Surprisingly, we found that SAMP1 and SAMP2 alone are sufficient for destructosome activity; both APC2-ΔSAMP1 and APC2-ΔSAMP2 were able to completely rescue the *APC2* null defects. Consistent with the deletion mutants, disrupting phosphorylation in either SAMP alone had no effect on APC2 function. However, disrupting phosphorylation in both SAMPs significantly reduced APC2 function, but not as severely as complete deletion of the SAMPs. This suggests that SAMP repeat phosphorylation is necessary for APC2s destructosome activity.

Discussion and Future Directions

Despite significant interest in understanding the role of APC proteins in the regulation of Wnt signaling, there are still many outstanding questions regarding the fundamental molecular mechanisms of its destructosome activity. Previous studies focusing on APC's binding to ß-cat showed that there is functional diversity among different 20Rs and phosphorylation plays an important role in APC's destructosome activity (Kunttas-Tatli et al., 2012; Roberts et al., 2011). In this study, we focused on the interaction between APC and Axin, and discovered that the SAMP repeats of APC2 appear to have distinct roles in the destructosome. In addition, we showed for the first time that the phosphorylation of SAMP repeats is necessary for APC's destructosome function.

Functional diversity amongst Drosophila SAMP repeats

APC-SAMP repeats are evolutionarily conserved among all metezoans to mediate an essential interaction between APC and Axin in the destructosome. Given the importance of this interaction in negatively regulating Wnt signaling, it's not surprising to find sequence homology between various species. The number and identity of the SAMP signature is invariant among all vertebrate APCs, while this becomes less rigid in vertebrate APC2s and invertebrate APCs. In addition, there is little sequence conservation outside of the SAMP motif among various SAMPs within same APC. However, there is conservation of these surrounding sequences among certain

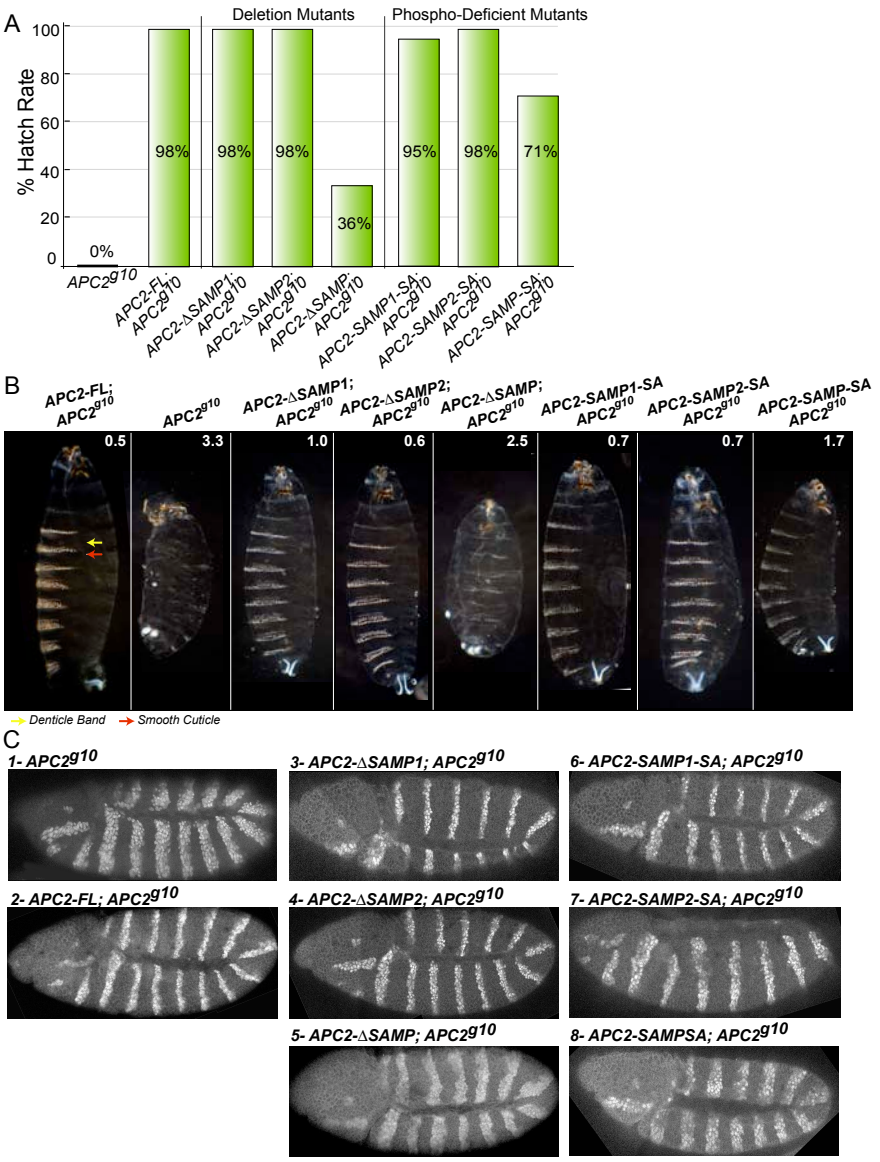


Figure 10: A) Expression of APC2-FL rescued the lethality of APC2 null (APC2g10) embryos. Removal of both SAMPs (APC2-ΔSAMP) only moderately rescued the lethality but removal of either SAMP acted like FL. Similarly, disrupting phosphorylation in both SAMPs (APC2-SAMP-SA) displayed a relatively high rescue, but both individual mutants acted like FL control. B) Expression of APC2-FL rescued the cuticle defects of APC2 null (APC2g10) embryos. The numbers indicate the phenotypic average for each genotype (scoring criteria as in McCartney et al., 2006). Cuticle images are shown at the same scale. E) Representative embryos showing Engrailed (En) protein expression in wild type (1) and APC2 null (2) embryos. APC2-FL restored the En expression domain of APC2 null (APC2g10) embryos. Similar to the hatch rate data, the En expression domain remains expanded in APC2 null embryos expressing APC2-ΔSAMP or APC2-SAMP-SA. Scale Bar: 25μm

SAMPs, such as the high conservation among human APC-SAMP3 and Drosophila APC1-SAMP3 and APC2-SAMP1. Taken together, these observations suggested that the SAMP repeats may be functionally diverse, rather than redundant.

Our findings suggest that APC2 SAMP1, despite the lack of SAMP motif conservation could weakly interact with Axin. This weak interaction has no effect on its ability to colocalize with Axin in both Drosophila S2 and human SW480 cells. However, presence of only SAMP1 caused redistribution of Axin to the cortex and disrupted normal circular puncta formation. This might suggest that the better Axin binder SAMP2 favors APC2 localization to the puncta. However, in the absence of SAMP2, somehow Axin is pulled into cortex. In the future, we would like to address the reasons why in the presence of just SAMP1, Axin is redistributed to the cortex.

We also wanted to test the ability of each domain to function in the activity of the destructosome. Deletion of SAMP1 significantly reduced the ability of APC2 to suppress the elevated level of Wnt target gene activity in SW480 cells. This suggests that SAMP1 has a more functionally significant role than SAMP2 in destructosome activity in SW480 cells, despite the fact that SAMP2 is the better Axin binder.

Our findings also indicated a potential role for SAMP phosphorylation in regulating the activity of APC's Wnt signaling function. Disrupting phosphorylation in SAMP1 elicited a functionally significant effect in SW480 cells, but was dispensable in the *APC2* null background in the intact animal. Similarly, the phosphorylation status of SAMP2 had no functional significance in either cell culture or in the animal. However, disrupting phosphorylation in both repeats caused defects both in SW480 cells and in flies. Some of the inconsistencies between the functional significance of various mutants in SW480 cells and flies have been observed before when studying APC2. For example, work in human cells has shown over-expression of internal fragments of human APC (containing at least three 20Rs) rescues ß-cat destruction and TOPFlash values (Rubinfeld et al., 1997; Roberts et al., 2011; Li et al., 2012). However, over-expression of analogous fragments of Drosophila APC2 in SW480 cells does not rescue either βcat destruction or TOPFlash (Roberts, unpublished results). This suggest even though SW480 cells are useful in testing various aspects of APC biology, it's still an

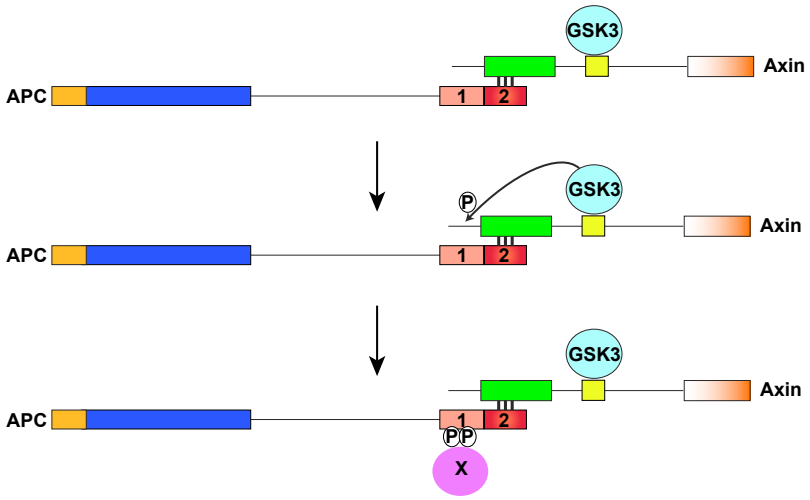


Figure 11: Model for the predicted cooperation between SAMP1 and SAMP2. Stronger Axin binder, SAMP2, binds to the RGS domain of Axin. This brings GSK3b (or CK1) to phosphorylate the phospho-residues in SAMP1. Phosphorylation of SAMP1 then recruits a currently unknown factor.

artificial environment and might not represent the true *in vivo* conditions. Completion of the analysis of the SAMP mutants in the *APC* double mutant background may alter these interpretations.

Based on our findings, we propose a model for how the SAMP repeats might cooperate in APCs destructosome function (Fig. 11). Because of its better binding, SAMP2 primarily mediates Axin binding. Recruiting APC2 into the Axin complex through SAMP2 brings APC in the proximity of GSK3ß and CK1 resulting in the phosphorylation of those target sites in SAMP1. We predict that the phosphorylation of SAMP1 may mediate an interaction between APC and another destructosome component (X) that is necessary for maximally effective destruction function. Furthermore, we predict that in the absence of SAMP2 as the primary Axin binder, the weaker SAMP signature in SAMP1 is able to compensate, and thus SAMP1 can take on the functional roles of both SAMP repeats.

Methods:

Constructs and Molecular Biology:

For generating the SAMP deletion mutants, we used standard PCR based site directed mutagenesis to delete SAMP1 or SAMP2 or both. For generating the phospho-deficient SAMP mutants, we used the GENEWIZ, Inc (NJ) gene synthesis services to synthesize a region that spanned the SAMP repeats. The piece synthesized started from the second internal 3' PstI site (Xaa) until the end of the gene (~600bp) with an addition of a 5' EcoR1 site. We then subcloned this piece into a wild type APC2 backbone with desired vectors. We subcloned desired APC2 constructs into the EcoRI site in pRmHa-3-mCherry (metallothionein promoter vector) for S2 cells, and pCS2(+)-mCherry (CMV promoter vector) for SW480 cells, and pCaSpeR-2 modified to contain the native APC2 promoter and GFP for expression in whole Drosophila (McCartney *et al.*, 2006). The mutant constructs were confirmed by sequencing.

Yeast Two-Hybrid Analysis

Matchmaker System (Clontech) was used to perform the Yeast Two-Hybrid (Y2H) analysis as described previously (Kunttas-Tatli et al., 2014 in press). APC2 constructs containing the various C-terminal domains and Axin-RGS or β-cat Arm

repeats (see Fig. 2) were cloned into the pCR8/GW/TOPO vector (Life Technologies) and gateway cloned into pGBKT7-W and pGADT7-W. β-galactosidase assays performed using the Yeast β-galactosidase Assay Kit (Thermo Scientific, Pierce) and the activity was calculated using the equation: activity = (1000 x OD420)/(T x V x OD660) where T= duration of the reaction in minutes and V=volume of the reaction in mls.

S2 cell culture, and transfection experiments:

Drosophila S2 cells were cultured in Schneider's Media (Lonza) with 10% heat-inactivated fetal bovine serum (FBS) and 1X penicillin-streptomycin at 25°C. pRmHa-3-mCherry (metallothionein promoter vector) constructs were transfected into S2 cells following the Effectene (Qiagen) protocol at a cell density of 2-3 x 10^5 cells in 6-well plates. Expression of constructs was induced 24 hrs post-transfection with $CuSO_4$ (40mM final concentration) and imaged 14-16hrs post-induction.

SW480 cell culture, transfections, and immunofluorescence

Human colon cancer SW480 cells were cultured in Dulbecco's Modified Eagle Media with High Glucose (DMEM-H) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1X Pen/Strep/Glutamine at 37°C and 5% CO_2. For transfections, SW480 cells were plated at a density of 2.5 x 10^5 cells in 6-well plates and grown overnight. Turbofect (Thermo Fisher) protocol followed to transfect pCS2(+)-APC2 DNA constructs. For immunoflourescence, cells were fixed and stained as previously described (Roberts *et al.*, 2011).

TOP/FOP Luciferase Reporter Assay

Luciferase assays were performed using the Dual Glo Luciferase System (Promega) according to the manufacturer's protocol and also as previously described (Kunttas-Tatli et al., 2014 in press). Luciferase signal was normalized to Renilla activity, and overall values normalized to the mCherry only control. The TOP/FOP Flash Luciferase constructs and the pRL Renilla transfection control were provided by Hans Clevers (Hubrecht Institute, The Netherlands). All samples were measured in triplicate per experiment, and two to three independent experiments were performed. None of the constructs displayed significant FOP Flash activity.

Fly genetics, hatch rate and cuticle analysis:

Transgenic flies expressing *P[endoP-EGFP-APC2-FL]* (Zhou *et al.*, 2011), and rest of the SAMP mutants (*P[endoPEGFP-APC2-ΔSAMP1], P[endoPEGFP-APC2-ΔSAMP2], P[endoPEGFP-APC2-ΔSAMP], P[endoPEGFP-APC2-SAMP1-SA], P[endoPEGFP-APC2-SAMP2-SA], P[endoPEGFP-APC2-SAMP-SA]* were generated using P-element–mediated germline transformation (Model System Genomics; Duke University, Durham, NC). Transgenes on the second chromosome for each construct were put into the *APC2^{g10} (APC2 null)* background using standard methods. Hatch rate analysis was performed and embryonic cuticles were prepared as previously described (Wieschaus and Nusslein-Volhard, 1998). Scoring criteria for the cuticle phenotype was previously described (McCartney *et al.*, 2006). Cuticle images were taken at the same magnification with darkfield illumination with a 20X objective.

Immunohistochemistry in the Drosophila embryo:

Embryos were collected 4-6 hrs at room temperature and fixed and stained as previously described (McCartney *et al.*, 1999). Anti-Engrailed (En; ms, 4D9, 1:50), were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Secondary antibodies were conjugated with various Alexa dyes [Invitrogen (Carlsbad, CA), 1:1000].

Imaging and image analysis:

Zeiss Axiovert 200M spinning disc confocal microscope with a Yokagawa scan head (Solamere Technology Group) and a QICAM-IR camera (Qimaging) was used to acquire the images of both cells and embryos using the QED InVivo software. For images of whole embryos stained for Arm and En, multiple fields were captured with a 25X objective and merged Adobe Photoshop post-image processing to generate whole embryo images.

Cell Sorting:

BD FacsVantage Diva option (laser 488) was used to sort the highest (top 30%) expressing S2 cells 24 hrs after induction. Cells were sorted into mattek dishes with 10XPBS and images of live cells were taken immediately.

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Chapter 4

Self-association of the APC tumor suppressor is required for the assembly, stability, and activity of the Wnt signaling destruction complex.

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Self-association of the APC tumor suppressor is required for the assembly, stability, and activity of the Wnt signaling destruction complex.

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Abstract

The tumor suppressor Adenomatous polyposis coli (APC) is an essential negative regulator of Wnt signaling through its activity in the destruction complex with Axin, GSK3β and CK1 that targets ß-catenin/Armadillo (ß-cat/Arm) for proteosomal degradation. The destruction complex forms macromolecular particles we termed the destructosome. While APC functions in the complex through its ability to bind both ß-cat and Axin, we hypothesize that APC proteins play an additional role in destructosome assembly through self-association. Here we show that a novel N-terminal coil, the APC Self-Association Domain (ASAD), found in vertebrate and invertebrate APCs, directly mediates self-association of Drosophila APC2 and plays an essential role in the assembly and stability of the destructosome that regulates ß-cat degradation in Drosophila and human cells. Consistent with this, removal of the ASAD from the Drosophila embryo results in ß-cat/Arm accumulation and aberrant Wnt pathway activation. These results suggest that APC proteins are required not only for the activity of the destructosome, but also for the assembly and stability of this macromolecular machine.

Introduction

Canonical Wnt signal transduction is an evolutionarily conserved pathway from hydra to humans, that plays essential roles in embryonic development and in adult tissue maintenance by regulating cellular differentiation, proliferation and morphogenesis (Guder et al., 2006; Logan and Nusse, 2004). Loss or constitutive activation of the pathway is lethal during embryogenesis due to defects in proliferation and differentiation (Logan and Nusse, 2004). In humans, inappropriate activation of Wnt signaling is associated not only with various types of cancer (colon, breast, and ovarian) but also with a myriad of other diseases including diabetes, Alzheimer's disease, and osteoporosis (Clevers and Nusse, 2012; Kim et al., 2013; Logan and Nusse, 2004; Oliva et al., 2013; Welters and Kulkarni, 2008). Thus, the tight control of Wnt signaling is critical for both normal development and tissue homeostasis.

In the absence of a Wnt ligand, the pathway is negatively regulated by a complex of proteins called the destruction complex, which phosphorylates the key effector of the pathway, ß-catenin (Armadillo in Drosophila) (Cadigan and Peifer, 2009). Phosphorylated ß-catenin (ß-cat) is ubiquitinated by the ß-TrCP ubiquitin E3 ligase to be degraded by the proteosome. Binding of the Wnt ligand to the co-receptor complex of Frizzled and LRP5/6 inactivates the destruction complex, allowing the accumulation and nuclear translocation of ß-cat. Together with TCF/LEF family transcription factors, ß-cat activates transcription of Wnt target genes. Loss of function mutations in components of the destruction complex lead to ligand independent accumulation of ß-cat, and the constitutive activation of Wnt target genes that play roles in proliferation, cell survival, and differentiation (Chen et al., 2003; Fodde, 2002; Komori et al., 2014; van de Wetering et al., 2002).

Adenomatous polyposis coli (APC) is a colon cancer tumor suppressor and an essential component of the destruction complex (McCartney and Näthke, 2008). Approximately 80% of all inherited and sporadic forms of colon cancer are associated with *APC* mutation (Polakis, 2012). The initiation of APC dependent colorectal cancer is primarily due to the loss of destruction complex activity and the inappropriate activation of Wnt targets (Polakis, 2007), but APC's roles in cytoskeletal regulation may also

contribute. APC is a core component of the destruction complex together with Axin, and the kinases GSK3ß and CK1. The cytoplasmic destruction complex appears to form macromolecular particles, or puncta, we termed the “destructosome” (Kunttas-Tatli *et al.*, 2012) (also known as the degradasome (Mendoza-Topaz *et al.*, 2011) or Axin complex (Li *et al.*, 2012). Despite its functional significance and abundant study, the inner-workings of the destructosome and the precise role of APC in this molecular machine are enigmatic. Several hypotheses for APC’s destruction complex function have been proposed. Due to its large size and the presence of many putative protein-protein interaction domains, APC was initially thought to act as a scaffold. However, because Axin can bind directly to all of the core components of the destruction complex including APC, ß-cat, GSK3ß and CK1, as well as Dishevelled, and the LRP5/6 co-receptor (Ha et al., 2004; Hart et al., 1998; Ikeda et al., 1998; Kishida et al., 1999; Mao et al., 2001) it is a stronger candidate for scaffolding function. Later studies proposed that APC-ß-cat interactions are required: 1) for phosphorylated ß-cat to be recognized by the ubiquitination complex as a part of a catalytic cycle (Kimelman and Xu, 2006), 2) to protect ß-cat from rapid dephosphorylation by PP2A upon ß-cat release from the destruction complex (Su *et al.*, 2008) and, 3) to increase the activity of the destruction complex when cellular levels of ß-cat are high (Ha *et al.*, 2004). However, recent work has also called into question the importance of a direct APC-ß-cat interaction for destruction complex function altogether (Yamulla *et al.*, 2014). It has also been suggested that APC functions downstream of ß-cat phosphorylation by mediating ß-cat’s ubiquitination by ß-TRCP (Li et al., 2012; Yang et al., 2006).

In addition, APC self-association may contribute to both destruction complex function and dysfunction (Kunttas-Tatli *et al.*, 2012). Vertebrate APC (vAPC) can self-associate via multiple mechanisms and domains. However, the precise role of APC self-association in normal destruction complex function, and the affects this has on cancer initiation and progression, are unclear. Two self-association domains C-terminal to the Armadillo (Arm) repeats in vAPC have been clearly implicated in APC’s normal cytoskeletal functions. The dimerization coil ANS2 (Fig. 1A) within the basic domain is required for APC’s actin nucleation function (Okada *et al.*, 2010), whereas a second

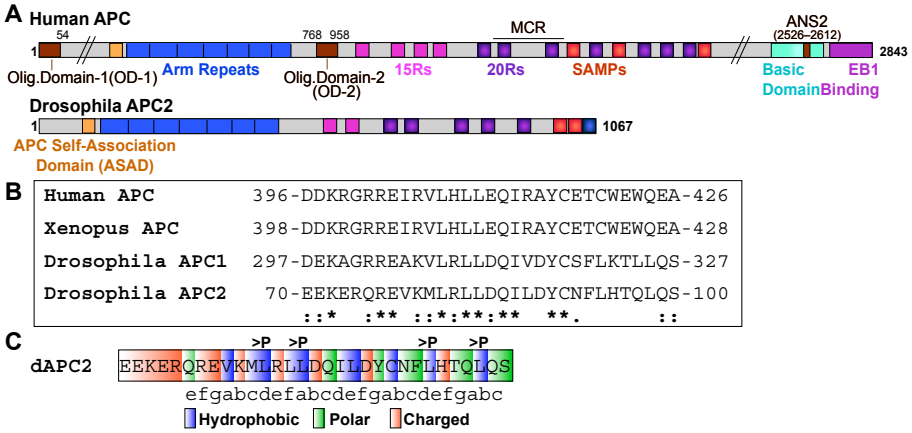

Figure 1: The APC Self-Association Domain (ASAD) is a conserved N-terminal coil. A) Schematic representation of human APC and Drosophila APC2, ASAD: APC self-association domain (orange), Arm repeats: Armadillo repeats (blue) 15Rs: 15 amino acid repeats (pink), 20Rs: 20 amino acid repeats (purple), MCR: Mutation cluster region, ANS2: Actin nucleation sequence 2 B) Sequence alignment of ASAD between human, Xenopus and Drosophila APC proteins ([*] identical, [:] conserved substitution, [.] semi-conserved substitution) C) The ASAD coil fits into the classic heptad repeat (abcdefg) motif, where a and d are hydrophobic, e and g are charged, and b, c, f tend to be polar amino acids (Gruber and Lupas, 2003). The four residues changed to proline in the APC2-ASADPro mutant are indicated.

oligomerization-domain (OD-2, Fig. 1A) can modulate the clustering of APC at microtubule plus-ends at the tips of membrane protrusions (Li *et al.*, 2008). N-terminal to the Arm repeats, vAPC can form coiled-coil based dimers through an N-terminal coil (oligomerization domain-1 (OD-1), Fig. 1A), but the precise role of OD-1 in normal APC function is not well understood. The presence of multiple self-association sites within vAPC suggests that the protein may have the ability to form large oligomers in addition to dimers, although it is not clear whether this occurs *in vivo*.

The complexity of vAPC self-association prompted us to investigate the role of APC self-association in the destructosome using the simpler and more tractable Drosophila APC2 as a model. Although neither Drosophila APC1 nor APC2 contains sequence homology to any of vAPC's self-association domains, we and others have shown that Drosophila APC proteins do self-associate through an N-terminal domain (Mattie et al., 2010; Roberts et al., 2012; Zhou et al., 2011). Consistent with this, high levels of APC2 mutants lacking the central ß-cat interaction domains (the 15 and 20 amino acid repeats) act as dominant negatives in Wnt signaling in the embryo (Kunttas-Tatli et al., 2012; Roberts et al., 2011). We predicted that this is because these mutants could associate with wild type APC2 through the N-terminal domain and compete for Axin binding through their intact SAMP repeats. Finally, our data suggested an unanticipated cooperativity between APC2 and APC1 in the destruction complex, which may be mediated through hetero-oligomerization (Kunttas-Tatli *et al.*, 2012).

To test the role of APC self-association in destruction complex function, we identified a novel N-terminal self-association domain in Drosophila APC proteins that appears to be conserved in all other APC proteins examined. Here, we demonstrate that this APC Self-Association Domain (ASAD) is necessary for the assembly and stability of the destructosome both in Drosophila S2 cells and in human SW480 colorectal cancer cells, which in turn is essential for ß-cat/Arm degradation. Furthermore, we show that loss of APC2 self-association in the Drosophila embryo leads to inappropriate activation of the Wnt signaling pathway due to loss of destructosome activity. These results suggest a novel role for APC proteins in the assembly and stability of the destructosome, in addition to their more established role in destructosome activity.

Results

An N-terminal coil mediates the self-association of Drosophila APC proteins

To dissect the role of APC self-association in destructosome structure and function, we identified a novel self-association domain in the N-terminal half of Drosophila APC2. Previously, we demonstrated that the N-terminal region of APC2 containing the Arm repeats (aa 1-490) mediates self-association (Roberts et al., 2012; Zhou et al., 2011), although it does not share sequence conservation with either OD-1 or OD-2 of human APC (Fig. 1A). OD-1 mediates the formation of homo-dimers through a parallel coiled-coil (Joslyn *et al.*, 1993). Based on this, we predicted that the Drosophila APC proteins would contain an N-terminal coil to promote self-association. We scanned the region of APC2 N-terminal to the Arm repeats (aa 1-112) using COILS to identify sequences likely to adopt a coiled-coil conformation (Lupas *et al.*, 1991). Using these predictions and the recently solved crystal structure of the region in vAPC (Morishita *et al.*, 2011; Zhang *et al.*, 2012), we identified a putative coil fitting the classic heptad repeat model (Fig.1C; (Gruber and Lupas, 2003) residing immediately N-terminal to the Arm-repeats (aa 70-100; Fig. 1A,B). Interestingly, this N-terminal coil appears to be conserved in all bilateria APC proteins examined, while OD-1 was primarily present in the deuterostome lineage (Fig. 1B; S1). Thus, we designated this novel N-terminal coil as the APC Self-Association Domain (ASAD), and hypothesized that it could mediate the self-association of Drosophila APC proteins.

To test this hypothesis, we generated a mutant version of Drosophila APC2 lacking this region (APC2-ΔASAD; Fig. 2A). In addition, we disrupted potential coiled-coil formation by changing four key hydrophobic leucine residues to proline (APC2-ASADPro; Fig. 1C, 2A). To determine if this domain is necessary to mediate APC2 self-association, we performed immunoprecipitation assays from transiently transfected Drosophila S2 cells. Previously, we showed that mCherry-tagged (mCh) APC2-FL (full length) and APC2-N (aa 1-490) could co-precipitate untagged APC2-FL, unlike mCh-APC2-C (aa 491-1067) (Zhou *et al.*, 2011) (Fig. 2B). Neither mCh-APC2-ΔASAD nor

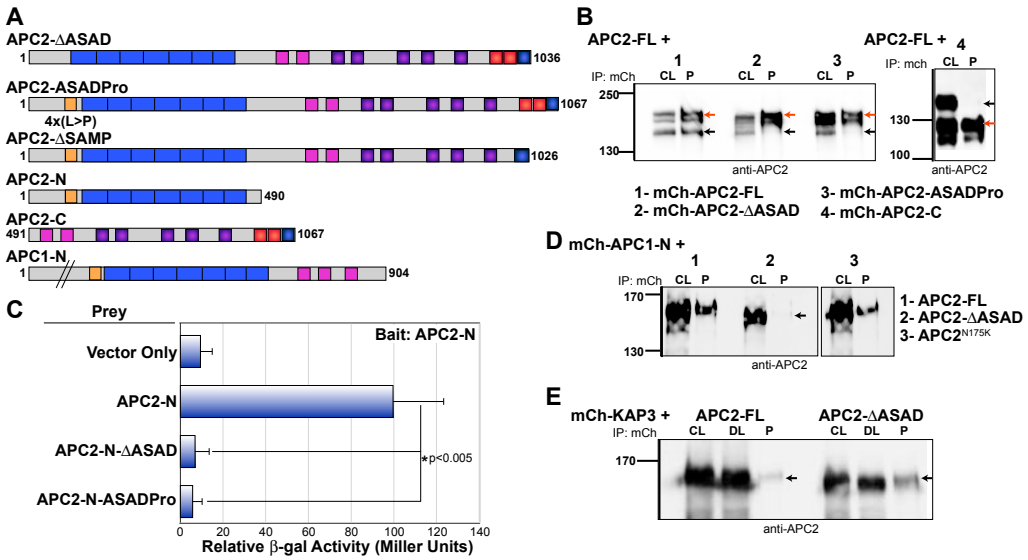

Figure 2: Removal of ASAD disrupts APC self-association. A) Schematic representation of Drosophila APC2 and APC1 constructs used in the study. B) mCherry (mCh)-tagged full length APC2 protein (red arrow) co-immunoprecipitates untagged full length protein (black arrow). mCh-APC2 ASAD mutants (both deletion and point mutant) and mCh-APC2-C (red arrows) fail to coimmunoprecipitate untagged APC2-FL (black arrows in 2-4). C) Yeast two hybrid experiments demonstrated that APC2-N can interact directly with APC2-N. Deletion of the ASAD (APC2-N-ΔASAD) or disruption of the potential coiled coil (APC2-N-ASADPro) abolishes this interaction. D) mCh-APC1-N coimmunoprecipitates untagged APC2-FL protein, but fails to coimmunoprecipitate the APC2-ΔASAD mutant (black arrow). APC2-N175K contains a mutation in the Arm repeats and retains the mCh-APC1-N interaction. E) mCh-KAP3 coimmunoprecipitates both full length APC2 and the APC2-ΔASAD mutant (black arrows). CL: Cell lysate, P: Pull down, DL: Depleted lysate

mCh-APC2-ASADPro was able to co-precipitate untagged APC2-FL, demonstrating that the N-terminal coil is necessary for self-association (Fig. 2B). Because human OD-1 mediates dimer formation through a direct protein-protein interaction (Joslyn *et al.*, 1993), we asked whether ASAD mediates direct APC2-APC2 binding. Consistent with that model, APC2-N (Fig. 2A) self-associated in a yeast two-hybrid (Y2H) assay, and this interaction was disrupted in both ASAD mutants (APC2-N-ΔASAD and APC2-N-ASADPro) (Fig. 2C).

Previous work from our lab and others indicated that both human and Drosophila APCs (APC&APCL and APC1&APC2 respectively) can hetero-associate through an N-terminal domain, and that this complex may collaborate in the destructosome (Kunttas-Tatli et al., 2012; Mattie et al., 2010; Schneikert et al., 2013). Because Drosophila APC1 also lacks OD-1, but contains the ASAD (Fig. 1B), we asked if the ASAD could mediate the association between Drosophila APC1 and APC2. Consistent with this hypothesis, mCh-APC1-N (aa 1-904) co-precipitated APC2-FL, but not APC2-ΔASAD (Fig. 2D). In contrast, a point mutation in the second Arm repeat of APC2 (N175K) that disrupts protein binding to the Arm repeats in human APC (hAPC) (Watanabe *et al.*, 2004), did not interfere with APC1-APC2 complex formation (Fig. 2D).

Given the close proximity of the ASAD to the highly structured Arm repeats (Fig. 1A), we asked if deleting the ASAD domain disrupts the folding of these repeats. The crystal structure of the human APC Arm repeats was unaffected by the absence of sequences containing the ASAD (Zhang *et al.*, 2012), suggesting that deletion of the ASAD alone is unlikely to disrupt Arm repeat binding interactions. To test this directly, we examined the interaction between the Drosophila homologue of a known human APC Arm-repeat binding protein, kinesin-associated protein 3 (KAP3; (Jimbo *et al.*, 2002)), and APC2-ΔASAD. Deletion of the ASAD did not interfere with the ability of KAP3 to co-precipitate with APC2 (Fig. 2E). In fact, KAP3 appeared to co-precipitate better in the absence of APC2 self-association, suggesting that APC2 self-association may negatively regulate Arm-repeat mediated protein-protein interactions.

Disruption of APC2 self-association leads to defects in the assembly of destructosome puncta in both Drosophila and human cells

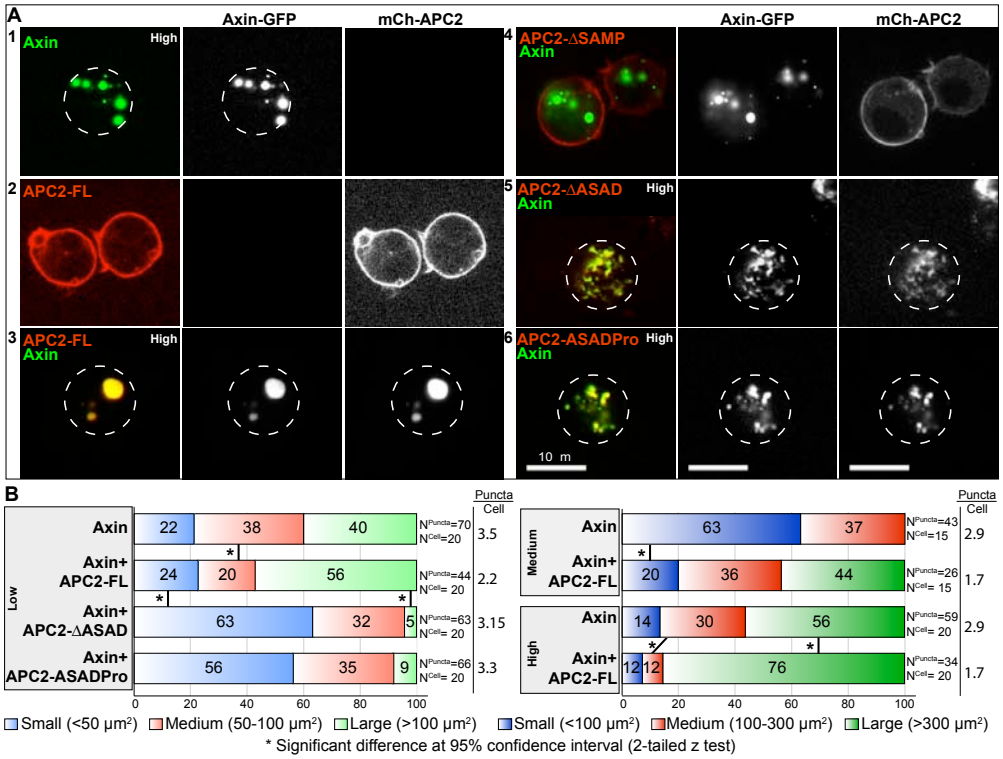

Figure 3: Disruption of APC2 self-association leads to defects in destructosome assembly in live Drosophila S2 cells. In all cases, Axin is GFP tagged and APC2 is mCherry tagged. Dotted lines indicate cell boundaries. A) When expressed alone in S2 cells, Axin-GFP oligomers can be visualized as cytoplasmic puncta (1) and mCh-APC2-FL (2) is primarily cortical. When coexpressed, mCh-APC2-FL colocalizes in cytoplasmic puncta with Axin-GFP (3). Removal of APC2's Axin interaction domains (APC2-ΔSAMP) disrupts this colocalization (4). ASAD mutants (both ΔASAD and ASADPro) colocalize with Axin-GFP, but cells coexpressing these proteins exhibit defects in puncta assembly and morphology (5, 6). B) Quantification of puncta size in S2 cells expressing Axin alone and coexpressing Axin and APC2 in cells sorted into three expression level categories (high, medium and low) by FACS using Axin-GFP. Images were taken under the same imaging conditions and puncta size was determined using Imaris. Puncta were then divided into three classes based on area (μm2). Coexpression with APC2-FL is associated with fewer, larger puncta at all three expression levels (see puncta/cell ratios). Coexpression with the ASAD mutants showed increase in the number of small puncta only in the low category. Due to the disrupted punta morphology in ASAD mutants, we were only able to asses the puncta size in this category. 2-tailed z test demonstrates significant differences between different groups. Scale Bar: 10μm

The destructosome is typically visualized as cytoplasmic Axin puncta that are observed both endogenously and when Axin is overexpressed in cell culture and in intact tissues (Fagotto et al., 1999; Faux et al., 2008; Fiedler et al., 2011; Schwarz-Romond et al., 2007b). Overexpressed Axin tagged with GFP, FLAG, RFP, myc, or HA localizes to cytoplasmic puncta in a variety of vertebrate and fly cultured cells including S2, SW480, HeLa, MDCK, and Cos-7. Over-expressed Axin has been shown to rescue ß-cat destruction in colorectal cancer cell lines (Behrens *et al.*, 1998; Hart *et al.*, 1998; Nakamura *et al.*, 1998; Roberts *et al.*, 2011), and in Drosophila embryos, cytoplasmic Axin-GFP puncta become cortical when cells activate the Wnt pathway, suggesting that these overexpression puncta are responsive to Wnt pathway activation (Mendoza-Topaz *et al.*, 2011). Axin can self-associate via its C-terminal DIX domain (also called DAX), which is essential for its function in ß-cat destruction and for its ability to form puncta (Schwarz-Romond *et al.*, 2007a, 2007b). It was recently shown that APC is essential for destructosome assembly as in the absence of APC, Axin failed to form functional destructosomes (Mendoza-Topaz *et al.*, 2011). Like Axin, we predicted that APC2 contributes to the formation of the destructosome through its ability to self-associate and form larger macromolecular assemblages (Kunttas-Tatli *et al.*, 2012). To test this hypothesis, we co-expressed Axin and APC2 in Drosophila S2 cells. When expressed alone, both Axin-GFP (Fig. 3A1) and Axin-HA (Fig. S2A) formed cytoplasmic puncta, albeit smaller in the case of Axin-HA. Thus, the GFP tag may have a slight effect on puncta size. Whereas mCh-APC2-FL localized primarily to the cell cortex (Fig. 3A2; (Zhou *et al.*, 2011)). When co-expressed with Axin-GFP, mCh-APC2-FL redistributed and localized primarily in the cytoplasmic Axin puncta (Fig. 3A3). Deletion of the Axin-binding SAMP repeats from APC2 (APC2-ΔSAMP) restored cortical localization of APC2 (Fig. 3A4), indicating that the primary mechanism for APC2's incorporation into Axin puncta is its direct association with Axin (Roberts *et al.*, 2011).

Consistent with the hypothesis that APC proteins promote the assembly of the destructosome, co-expression of Axin-GFP with mCh-APC2-FL resulted in the formation of fewer, larger Axin-GFP puncta (Fig. 3B). Because expression levels could influence this effect, we used FACS to sort the cells into three different groups based on

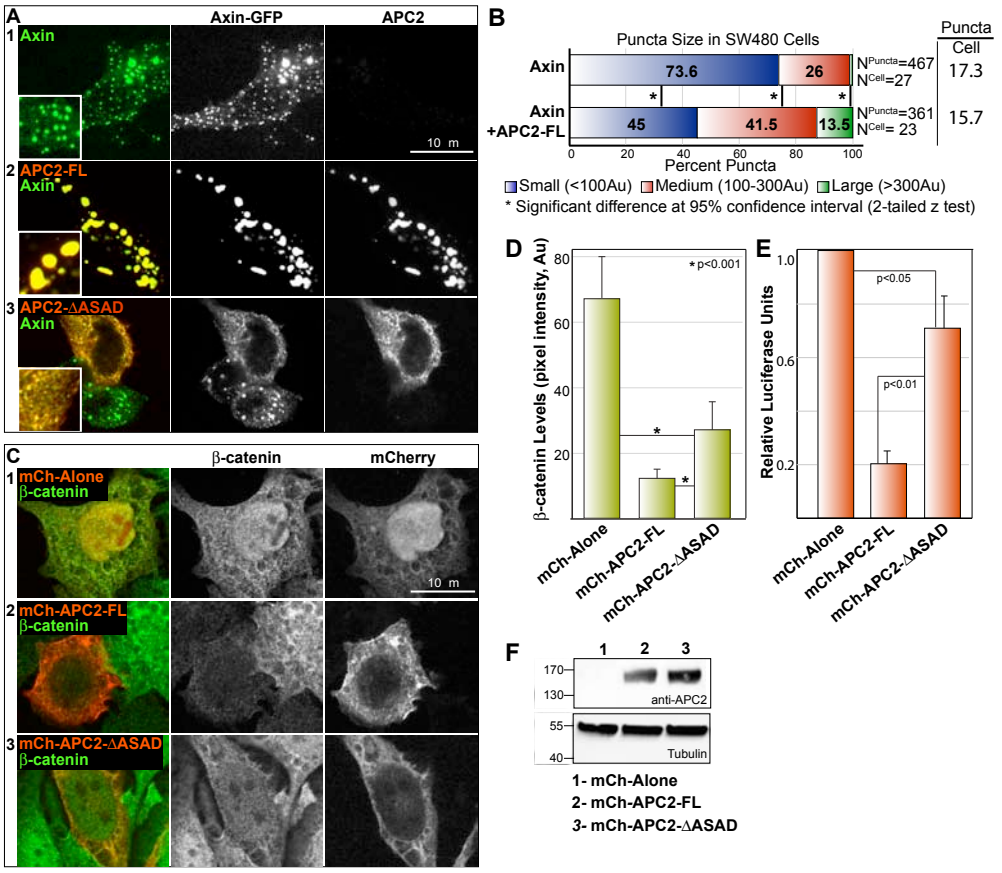

Figure 4: APC2 self-association is necessary to degrade ß-cat and regulate Wnt target gene expression in SW480 cells. A) Similar to S2 cells, Axin-GFP forms cytoplasmic puncta (1) and mCh-APC2 colocalizes with Axin-GFP in SW480 cells (2). Coexpression of APC2-ΔASAD with Axin-GFP does not disrupt colocalization (3), but is also associated with defects in puncta assembly and morphology. B) Similar to S2 cells, coexpression of Axin with APC2-FL in SW480 cells leads to fewer, larger puncta. 2-tailed z test demonstrates significant differences between the two conditions. C,D) Expression of full length Drosophila APC2 was sufficient to suppress the elevated levels of ß-cat (2) (compare to the empty vector control (1)) in SW480 cells. The APC2-ΔASAD mutant moderately suppressed the elevated ß-cat levels (3). E) In SW480 cells, expression of APC2-FL strongly suppressed activation of Wnt targets as assessed by TOP/Flash activity compared to the empty vector control. Expression of the APC2-ΔASAD mutant suppressed target gene activation compared to the empty vector control, but exhibited significantly less activity than APC2-FL Student's t-test revealed significant differences between the conditions in D and E. F) mCh-tagged APC2-FL (2) and APC2-ΔASAD (3) were expressed at equal levels in SW480 cells used in the TOP/Flash assays. Scale Bar: 10μm

expression of Axin-GFP (high, medium and low) and assessed puncta size and number. Consistent with our observations, Axin formed fewer, larger puncta in the presence of APC2-FL at all three expression levels (Fig. 3B). To test the hypothesis that APC2-FL promotes the formation of larger puncta by increasing the rate of puncta growth, we examined puncta from cells expressing Axin-GFP alone or co-expressed with APC2-FL over time (Fig. S2B). Axin-GFP expressed alone formed puncta even at the lowest detectable expression level after few hours after induction, suggesting that puncta formation is not the result of significant overexpression. Cells expressing Axin-GFP alone contained puncta that reached their maximum size by 24-48 hrs post-induction, and at 96 hrs these cells contained many smaller puncta. In contrast, cells co-expressing Axin and APC2 displayed large, and often single, misshapen puncta by 96 hrs post-induction. This suggests that APC2 primarily promotes puncta assembly, rather than accelerating their growth rate.

To determine whether the APC2 ASAD mediates puncta assembly, we co-expressed the APC2 self-association mutants (APC2-ΔASAD and APC2-ASADPro) with Axin-GFP (Fig. 3A5, 6). Expression of these self-association mutants produced a dramatic change in destructosome morphology. Puncta that incorporated the APC2 self-association mutants appeared smaller, fragmented and dispersed throughout the cytoplasm (Fig. 3A5, 6, and B). The striking alteration in destructosome morphology precluded quantification of their size at medium and high expression levels (Fig S3).

To rule out the possibility that this is a cell type specific effect, we examined the role of APC2 in destructosome assembly in SW480 human colon cancer cells. Similar to Drosophila S2 cells, expression of Axin-GFP in SW480 cells led to the formation of discrete cytoplasmic puncta (Fig. 4A1; (Fiedler *et al.*, 2011). In the presence of Drosophila APC2-FL, Axin-GFP puncta decreased in number and increased in size (Fig. 4A1, 2, and B), suggesting that the role of APC proteins in destructosome assembly is conserved in human cells. Consistent with this hypothesis, expression of APC2-ΔASAD resulted in fragmented, dispersed Axin-GFP puncta (Fig. 4A3), suggesting that APC2 self-association is also required for destructosome assembly in human cells.

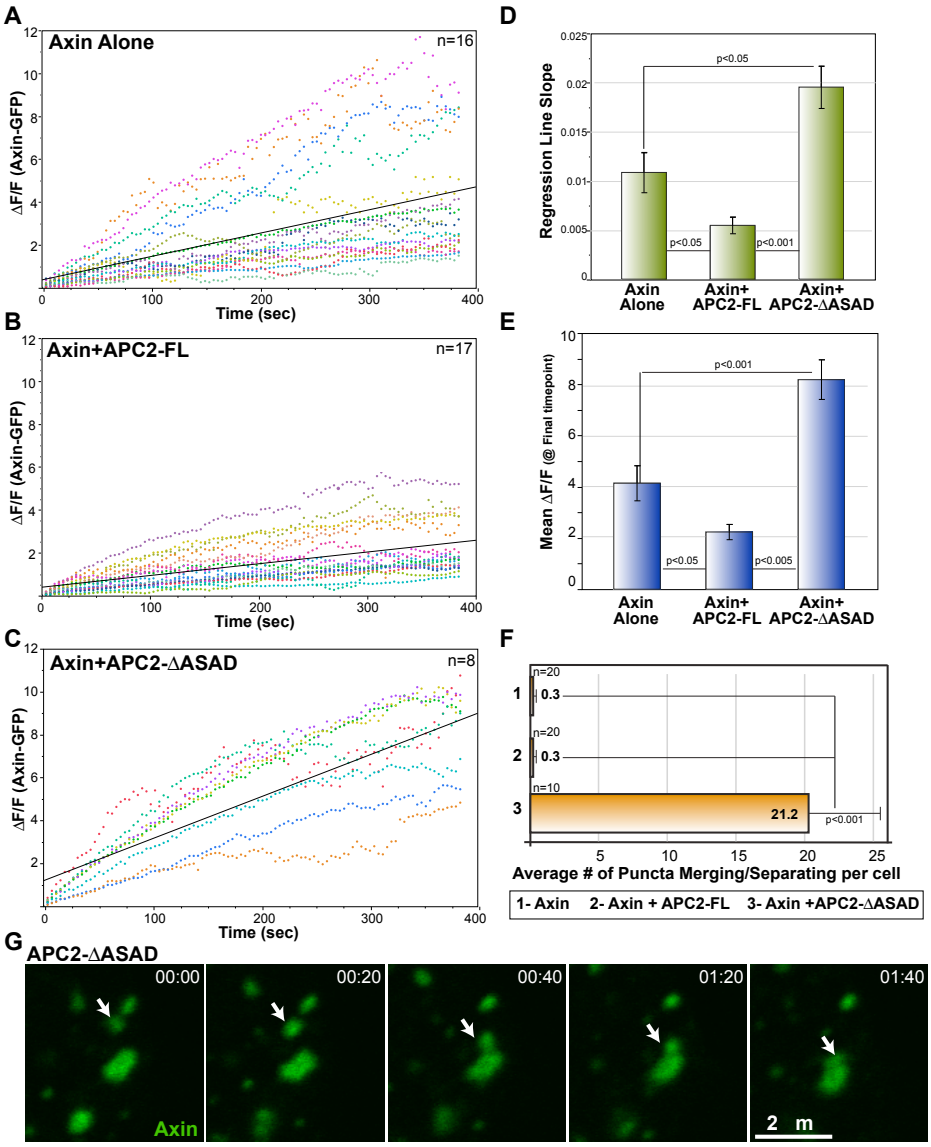

Figure 5: APC2 self-association stabilizes the destructosome A-C) Plots of ΔF/F for each condition. The recovery of individual bleached spots is shown in unique colors for each condition. Black lines are regression lines. Regression analysis indicates that the relationship between time and fluorescence varies by condition. ANOVA for regression lines p<0.0001; Tukey-Kramer HSD post-hoc test for each pair p<0.05. D) To compare the rate of recovery, we calculated the slope of the regression line for each individual sample and compared the means of these slopes for each condition. Means are plotted with SEM whiskers; Tukey-Kramer HSD post-hoc test for each pair p<0.05. E) To compare the difference in mobile and immobile fractions at the end of the experiment, time zero normalized degree of recovery at our last time point (384.12 sec) for the three conditions was determined. Means are plotted with SEM whiskers; Tukey-Kramer HSD post-hoc test for each pair p<0.05. F) We observed a significant difference in the number of puncta merging and separating events (a measure of puncta dynamics) between Axin-GFP/APC2-ΔASAD puncta compared to Axin-GFP or Axin-GFP/APC2-FL puncta. This behavior is rarely observed in Axin-GFP and Axin-GFP/APC2-FL puncta. Scale bar: 2μm. Student's t-test p<0.001 between the mutant and either of the other conditions. G) Axin-GFP/APC2-ΔASAD puncta are highly dynamic. The white arrow tracks the merging of two puncta. Time stamp in min:sec

APC2 self-association is necessary for destructosome activity in SW480 cells

Next, we asked if the defects in destructosome assembly and morphology affect destructosome function. SW480 cells express a truncated version of human APC (Nishisho *et al.*, 1991), resulting in elevated levels of ß-cat due to loss of destruction complex activity (Munemitsu *et al.*, 1995). This has made SW480 cells a useful tool to investigate the mechanisms of destructosome function. Expression of Drosophila APC2-FL can compensate for the loss of hAPC function and suppress the elevated levels of ß-cat (Fig. 4C, D; (Roberts *et al.*, 2011)). Although APC2-ΔASAD still contains all other domains required for APC's destructosome function including the ß-cat and Axin interaction domains (20Rs and SAMP repeats) (Roberts *et al.*, 2011), it only moderately suppressed the high levels of ß-cat protein (Fig. 4C3, D). This suggests that the fragmented destructosomes do not effectively target ß-cat for destruction and APC self-association is required for proper destruction complex activity. In SW480 cells expressing APC2-ΔASAD, ß-cat levels appear to decrease in both the cytoplasm and in the nucleus (Fig. 4C3). While APC2-ΔASAD mediated destruction is likely decreasing the overall level of ß-catenin protein, APC2-ΔASAD may also be reducing nuclear ß-cat by tethering it in the cytoplasm (Roberts *et al.*, 2011).

Due to elevated ß-cat levels, SW480 cells also display high levels of Wnt target gene expression, which can be detected using the well-established TOP-Flash luciferase assay (Korinek *et al.*, 1997). Expression of Drosophila APC2-FL significantly reduced the high level of reporter gene expression in SW480 cells (Fig. 4E; (Roberts *et al.*, 2011)) consistent with the strong reduction in ß-cat levels (Fig. 4C, D). APC2-ΔASAD and APC2-ASADPro were significantly impaired in their ability to suppress ß-cat mediated transcription (Fig. 4E, and data not shown). Collectively, these results suggest that APC2 self-association plays a functionally significant role in destructosome activity in SW480 cells.

APC2 self-association stabilizes the destructosome

One simple model for the role of APC self-association in destructosome assembly and morphology is that APC-APC interactions, together with APC-Axin interactions, provide

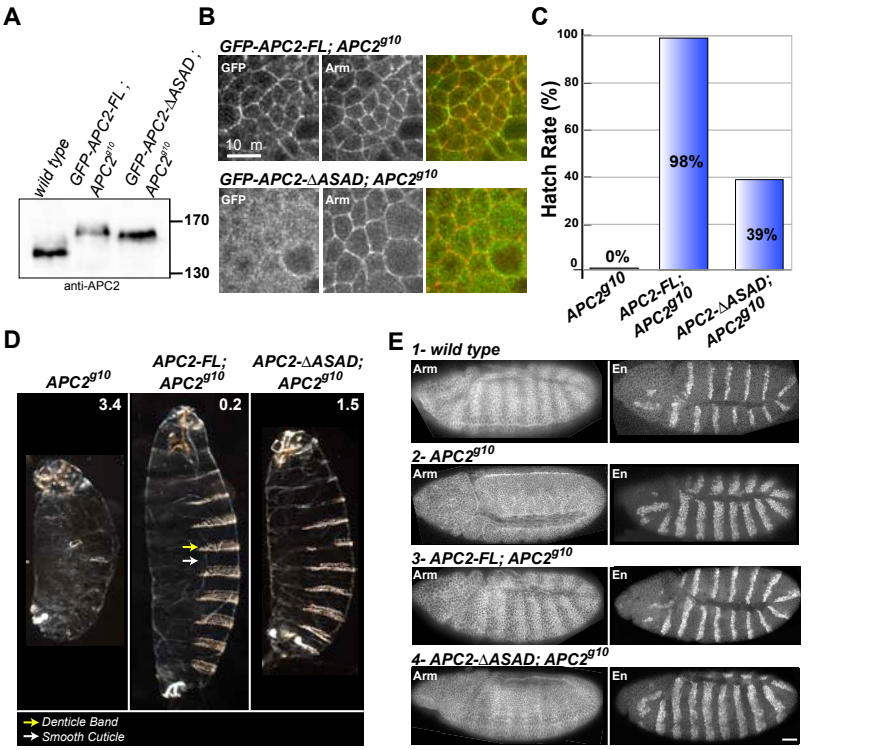

Figure 6: APC2 self-association is required to negatively regulate Wnt signaling in the Drosophila embryo. A) Immunoblot of 0-6 hr embryonic lysates demonstrates that the level of expression of GFP-APC2-FL and GFP-APC2-ΔASAD is comparable to endogenous APC2. B) GFP-APC2-FL is enriched at the cell cortex with Arm in embryonic epithelia, whereas GFP-APC2-ΔASAD is primarily cytoplasmic. Scale bar: 10μm C,D) Expression of GFP-APC2-FL rescued the lethality of APC2 null ($APC2^{g10}$) embryos and restored the wild type cuticle phenotype, whereas the APC2-ΔASAD mutant only moderately rescued the lethality and cuticle phenotype. The numbers in D. indicate the phenotypic average for each genotype (scoring criteria as in McCartney et al., 2006). Cuticle images are shown at the same scale. E) Representative embryos showing Armadillo (Arm) and Engrailed (En) protein expression in wild type (1) and APC2 null (2) embryos. APC2-FL restored wild type Arm levels and the En expression domain of APC2 null ($APC2^{g10}$) embryos. APC2-ΔASAD weakly suppressed Arm accumulation and restored a weak Arm stripe pattern in the epidermis. The En expression domain remains expanded in APC2 null embryos expressing APC2-ΔASAD. Scale Bar: 25μm

stability to the complex. When APC self-association is blocked, but APC retains its interaction with Axin and the complex, destructosome stability is reduced, leading to both fragmentation and loss of activity. To test this hypothesis, we used fluorescence recovery after photobleaching (FRAP) to assess the turn over of Axin-GFP within the puncta in S2 cells. If this hypothesis is correct, we predicted that cells expressing both Axin and APC2-FL would have a relatively large immobile fraction and a relatively small free mobile pool of Axin-GFP within the puncta. We expected that cells expressing Axin-GFP and only endogenous APC2 would exhibit Axin-GFP dynamics similar to that of cells overexpressing APC2-FL, although we may observe a larger immobile fraction of Axin-GFP in cells with additional APC2-FL. Conversely, we predicted that cells expressing Axin and APC2-ΔASAD would have a larger mobile fraction as the rate of turnover of Axin-GFP is higher with a smaller immobile fraction of Axin-GFP.

For these FRAP experiments, we chose similarly sized puncta for each condition and kept the bleached area constant. For the ΔASAD mutant where the puncta can be interconnected at higher expression levels, we chose cells with relatively low expression where we could see isolated, individual puncta. To compare the Axin-GFP fluorescence recovery between the three conditions, we first normalized for the starting post-bleach fluorescence by calculating ΔF/F for each time point in each condition and plotted this over time (Fig. 5A-C). To compare the rate of recovery, we compared the mean slope of the regression lines for each condition (Fig. 5D). Axin-GFP/APC2-ΔASAD puncta displayed a significantly greater slope than either Axin-GFP alone or Axin-GFP/APC2-FL puncta. Conversely, Axin-GFP/APC2-FL puncta exhibited a significantly reduced slope compared to the other conditions (Fig. 5D). Axin alone puncta exhibited the greatest variation in rate of recovery; some puncta displayed Axin-GFP/APC2-FL like properties, while others exhibited Axin-GFP/APC2-ΔASAD like properties (Fig. 5A). Cells expressing Axin-GFP alone are expressing significantly more Axin than the low level of endogenous APC2 in these cells (Zhou and McCartney, unpublished observations). This suggests that at a high Axin:APC2 ratio, Axin turnover rates are not well controlled and fluctuate as a consequence. When the Axin:APC2 ratio is closer to 1, as in the case of Axin-GFP/APC2-FL puncta, Axin-GFP is stabilized and the overall rates of recovery decrease significantly (Fig. 5D). On the other hand, disrupting APC2

self-association appears to drive Axin-GFP toward the opposite end of its dynamic spectrum (Fig. 5D).

After approximately 400 seconds post-bleach, Axin-GFP in Axin-GFP/APC2-ΔASAD puncta had recovered the greatest fluorescence, reflecting a relatively large mobile pool (Fig. 5E). While the degree of fluorescent recovery was more similar between Axin alone puncta and Axin-GFP/APC2-FL puncta, they were significantly different, with Axin-GFP/APC2-FL puncta displaying the weakest recovery and therefore the smallest mobile fraction. Taken together, these data suggest that APC2 promotes stability of the destructosome through its ability to self-associate.

In addition to the increased mobility of Axin-GFP in cells co-expressing APC2-ΔASAD, we observed that the fragmented puncta themselves were remarkably dynamic (Supplemental movies 1-3). Furthermore, the fragmented puncta frequently split and merged with neighboring puncta (Fig. 5F,G), a behavior rarely observed in cells expressing Axin alone, or Axin and APC2-FL (Fig. 5F).

APC2 self-association is required for destructosome activity in the Drosophila embryo

Because APC2 self-association is necessary for proper ß-cat regulation in cultured cells, we asked whether APC2 self-association is also necessary for destructosome activity and the negative regulation of Wnt signaling in the more physiologically relevant context of the Drosophila embryo. We expressed GFP-tagged APC2-FL or APC2-ΔASAD in the embryo under the native APC2 promoter (McCartney *et al.*, 2006), and found that the two tagged proteins are expressed at levels comparable to the endogenous wild type protein (Fig. 6A). As previously shown, APC2-FL protein expressed in *APC2* null (*APC2*^{g10}) embryos is enriched at the cell cortex of embryonic epithelia similar to endogenous APC2 (McCartney *et al.*, 1999; Zhou *et al.*, 2011). However, APC2-ΔASAD exhibited limited enrichment at the cortex (Fig. 6B), consistent with our observations in S2 cells (Fig. S4). We have previously demonstrated that the localization of APC2 to the cell cortex requires both the N-terminal region (aa 1-490) and the most C-terminal 30 amino acids (C30; Fig. 1A; (Zhou *et al.*, 2011). Because we have now demonstrated that the function of C30 requires APC2 dimerization

(McCartney and Molinar, unpublished data), it is not surprising that the ASAD is necessary for cortical localization. We have previously shown that cortical localization of APC2 is not required to regulate Wnt signaling (Zhou *et al.*, 2011), therefore, the lack of APC2-ΔASAD cortical localization will not affect its destructosome activity.

Between 4-6 hrs after egg laying, Wnt signaling is activated in a subset of ectodermal cells within each developing segment. Cells receiving Wnt produce smooth cuticle, while cells not receiving Wnt produce microtubule and actin based apical projections that result in the formation of cuticular outgrowths called denticles. Thus, Wnt signaling results in a segmentally repeated pattern of denticles and smooth cuticle on the ventral surface of the embryo (like Fig. 6D *APC2-FL; APC2^{g10}*). This segmentally repeated pattern is reflected in the accumulation of Arm in "stripes" of cells receiving Wnt (Fig. 6E), and in the patterned expression of the Wnt target gene *engrailed* (*en*; Fig. 6E). Embryos activating Wnt signaling uniformly throughout the ectoderm, as in the null *APC2^{g10}*, were embryonic lethal (0% hatch rate to the larval stage; Fig, 6C), produced excess smooth cuticle at the expense of denticles (Fig. 6D), accumulated Arm uniformly across the ectoderm (Fig. 6E2), and exhibited an expanded expression domain of *en* (Fig. 6E2). Addition of APC2-FL into the null background rescued all of these defects to a virtually wild type phenotype (Fig. 6 C-E). In contrast, expression of APC2-ΔASAD in the *APC2* null suppressed, but failed to fully rescue, these defects. While 98% of *APC2-FL; APC2^{g10}* embryos hatched (Fig. 6C), only 39% of *APC2-ΔASAD; APC2^{g10}* embryos hatched to the larval stage (Fig. 6C). The 61% of *APC2-ΔASAD; APC2^{g10}* embryos that failed to hatch exhibited a suppressed phenotype compared to the null alone (Fig. 6D); denticle bands were restored, but were frequently incomplete, and the overall size of the embryo increased, but not to the level of APC2-FL rescue (Fig. 6D). Consistent with the incomplete rescue, cells not receiving the Wnt signal in the *APC2-ΔASAD; APC2^{g10}* embryos still exhibited elevated Arm (Fig. 6E4), and the *en* expression domain remained expanded (Fig. 6E4). In conclusion, the APC2-ΔASAD protein supports only weak destructosome activity in the Drosophila embryo, consistent with our results in SW480 cells (Fig. 4).

Drosophila express a splice form of APC2 lacking the ASAD

While we have shown that self-association is necessary for APC2's destructosome function, self-association may interfere with other aspects of APC function and it may be regulated. Although little is known about the regulation of self-association via OD-1 in human APC, splice variants of APC that alter that domain have been reported (Carson et al., 2004; Santoro and Groden, 1997). In addition, exon 9, containing the human ASAD, can be alternatively spliced (Groden *et al.*, 1991; Joslyn *et al.*, 1991) Remarkably, we identified a splice variant of *APC2* in the modENCODE database that selectively removes a region within the ASAD. We confirmed at the mRNA level that both isoforms are found in 4-8 hr Drosophila embryos, albeit at dramatically different levels (Fig. S5). These data suggest that a monomeric form of APC2 may have a functional role during development.

Discussion

As core members of the β-cat destruction complex, APC proteins are indispensible negative regulators of Wnt signaling. APC loss leads to unregulated accumulation of β-cat and Wnt pathway activation. Previous studies focusing on APC's binding to ß-cat and Axin demonstrated the importance of these interactions for APC's destructosome activity (Kunttas-Tatli et al., 2012; Roberts et al., 2011). Here, we reveal a novel role for APC proteins in destructosome function by promoting destructosome assembly and stability through self-association. While we have known for almost 20 years that APC proteins are essential negative regulators of Wnt signaling (Munemitsu *et al.*, 1995), we are still uncovering novel roles for APC in this process.

Role of APC in the formation of the destructosome

Axin is thought to drive destructosome assembly through polymerization via its C-terminal DAX domain (Fiedler *et al.*, 2011). Furthermore, Axin's direct binding to all other core members of the complex suggests that its primary function is scaffolding (Luo & Lin, 2003). Surprisingly, Mendoza-Topaz *et al.* (2011) demonstrated that APC is essential for Axin complex assembly *in vivo*; without APC, Axin failed to form functional destructosomes in Drosophila embryos. The authors suggested two scenarios to

explain this. First, Axin is unstable without APC, resulting in the observed reduction in Axin protein that may be below the minimum concentration needed for polymerization. Second, APC is a cofactor clustering Axin via its multiple Axin interaction domains (SAMPs), APC self-association, or both. Our data support the model that APC promotes destructosome assembly by stimulating Axin polymerization via APC self-association (Fig. 7). With APC2, Axin formed larger and more stable structures in cultured cells (Fig. 3 and 4). Disrupting APC2 self-association by removing the ASAD had no effect on APC2-Axin interactions, but resulted in fragmented and significantly more dynamic Axin puncta (Fig. 3-5). One might expect that loss of APC2 self-association would result in Axin-GFP puncta similar to those in cells lacking additional APC2. We predict that APC2-ΔASAD fragments the Axin puncta because monomeric APC2 (APC2m) retains its interaction with Axin and interferes with Axin polymerization (Fig. 7). This might be due to the stronger APC-Axin interaction (50 nM K_D *in vitro*) versus the weaker Axin-Axin interaction (5-20 μM K_D *in vitro*) (Lee *et al.*, 2003; Schwarz-Romond *et al.*, 2007). This dramatic affinity difference further supports the idea that a cofactor like APC is required to efficiently polymerize the less abundant Axin (Fig. 7) (Lee *et al.*, 2003).

Our data also suggest that once APC drives Axin polymerization, it stabilizes Axin in the complex (Fig. 5). Because of Axin's ability to bind the other core components of the complex, stabilized Axin may in turn stabilize the presence of GSK3ß and CK1, leading to more efficient ß-cat phosphorylation and degradation. This is consistent with our functional data in cultured cells and during Drosophila embryogenesis; the expression of monomeric APC2 resulted in significant reduction, but not complete loss, of destructosome activity (Fig. 4 and 6). The low level of APC1 in the embryo does support destructosome function (Ahmed *et al.*, 2002; Akong *et al.*, 2002; Kunttas-Tatli *et al.*, 2012), and we predict that this activity is due in part to APC1's association with the more abundant APC2 through the ASAD, and through APC1's self-association. Taken together, we conclude that while APC self-association is not strictly essential for destructosome activity, it is necessary for normal function. Because even slight elevations in Wnt signaling due to the reduction of negative regulation can lead to dramatic defects (Komori et al., 2014; McCartney et al., 2006), we predict that

maximally efficient destructosome activity is essential for both normal development and to prevent Wnt signaling mediated cancers.

Our findings appear to contrast with some previous work about the role of APC's N-terminal domains in destructosome function. Over-expression of hAPC internal fragments containing at least three 20Rs but lacking OD1, OD2, and the Arm repeats, rescues ß-cat destruction in SW480 cells (Rubinfeld et al., 1997; Roberts et al., 2011; Li et al., 2012). This appears to suggest that APC's N-terminal domains are dispensable. In contrast, over-expression of analogous dAPC2 fragments or dAPC2 N-terminal deletions failed to rescue destructosome function in SW480 cells (Roberts et al., 2011 and unpublished results). Consistent with these results and our findings here, we previously demonstrated that the N-terminus of APC2 is essential *in vivo* (Roberts et al., 2011; Roberts et al., 2012). Moreover, hypomorphic point mutations in the Arm repeats of dAPC2 (McCartney et al., 1999 and 2006), and mouse studies of colon cancer (Crist et al., 2010), suggest that the N-terminus is functionally important. It is unclear how internal fragments of hAPC rescue destruction while the analogous Drosophila fragments do not; however, this collection of *in vivo* data provides a compelling argument that the N-terminus of APC is essential for ß-cat destruction in Drosophila and mammals

The self-association of APC proteins significantly affects their functions

Previously, the only functions ascribed to APC dimerization were in cytoskeletal regulation. ANS2 in the basic domain is necessary for dimerization and APC's actin nucleation activity (Okada *et al.*, 2010). Phosphorylation enhances OD-2 dimer formation, which in turn enhances the assembly of microtubule-associated APC clusters at the cell periphery (Li *et al.*, 2008). Dispersing these APC clusters by disrupting OD-2 reduced cell migration. The potential parallels in APC function in these clusters and in destructosomes are intriguing; in both cases, blocking self-association disrupts the assembly of these prominent macromolecular assemblages.

It is unclear what role OD1 plays in normal APC function. Clinically relevant APC C-terminal truncations forming dimers with wild type APC through OD1 in heterozygous

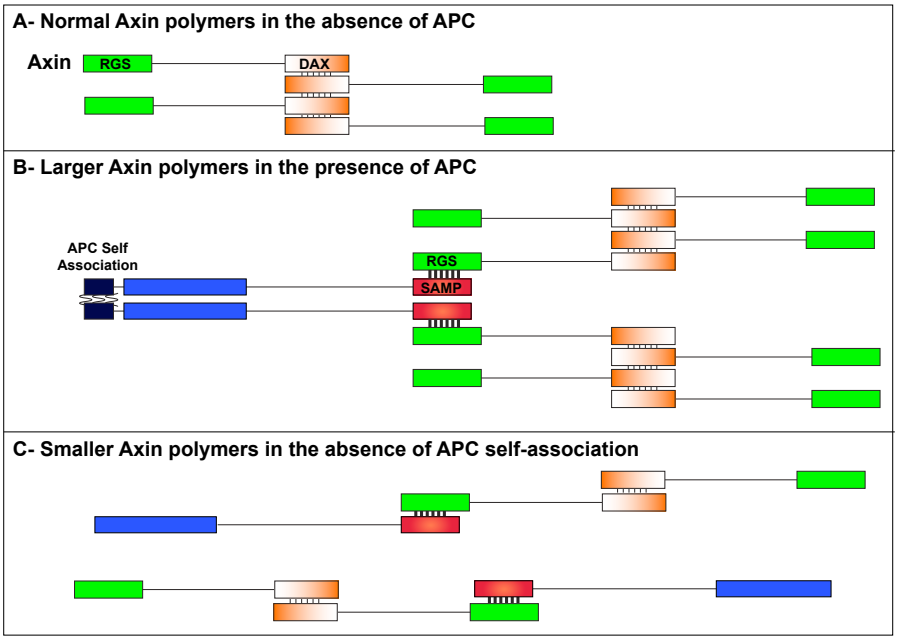

Figure 7: Model for the role of APC self-association in promoting Axin puncta formation. A) Normal Axin polymers form via the weak DAX interactions (thin lines) in the absence of APC. B) Larger Axin polymers form via the stronger interaction between APC's SAMP repeats and Axin's RGS domains (thick lines) due in part to APC's ability to self-associate (unknown binding affinity; wavy lines). C) Smaller Axin polymers form in the absence of APC self-association.

cells may promote chromosomal instability and aneuploidy by interfering with microtubule functions (Green & Kaplan, 2003; Green *et al.*, 2005). Interestingly, a splice isoform of APC that skips exon-1 encoding OD1 is enriched in mouse and human brain and heart (Thliveris *et al.*, 1994). A splice variant of hAPC that deletes a portion of the ASAD has also been observed (Groden *et al.*, 1991; Joslyn *et al.*, 1991), and there are some reports of colorectal cancer associated mutations in this region that may result in increased production of the ASAD lacking isoform (van der Luijt *et al.*, 1995). However, the data are limited and the functional consequences unclear. Our intriguing observation that Drosophila express an alternate splice form of *APC2* lacking the ASAD (Fig. S5) suggests that Drosophila may be a relevant and simple system in which to examine the functional consequences of these alternative APC isoforms.

While the function of APCm is currently unknown, our observations suggest that it may complex more efficiently with Arm repeat binding proteins such as Kap3 (Fig. 2E). Similarly, the binding of OD2 and the C-terminal domains of vAPC (aa 2545-2843) decreased Kap3 association with the Arm repeats (Li & Näthke, 2005). This suggests that APCm may exhibit enhanced binding to a broad array of Arm repeat partners including Kap3, Asef, IQGAP and the PP2A regulatory subunit (Jimbo et al., 2002; Kawasaki, 2000; Seeling et al., 1999; Watanabe et al., 2004). The cytoskeletal functions of the first three suggest that APCm may have enhanced cytoskeletal roles. APC's association with PP2A is thought to be in the context of the destructosome (Seeling *et al.*, 1999), suggesting that APCm may also have destructosome function.

Methods:

Constructs and Molecular Biology:

Site-directed mutagenesis primers were designed and a standard PCR-based mutagenesis protocol was followed. The resulting *APC2* mutants were cloned into the pGEM-T Easy (Promega) shuttle vector and then into the EcoRI site in pRmHa-3 (metallothionein promoter vector for S2 cells), pCS2(+) (CMV promoter vector for SW480 cells), and pCaSpeR-2 modified to contain the native APC2 promoter and GFP for expression in whole Drosophila (McCartney *et al.*, 2006). The mutant constructs were confirmed by sequencing. The specific amino acid positions of the Drosophila

APC2 (FlyBase annotation symbol: CG6193) fragments are as follows: APC2-ΔASAD, 1-69 plus 100-1067; APC2-ASADPro, Pro81Leu, Pro84Leu, Pro94Leu, Pro98Leu; APC2-N175K, Asn175Lys APC2-N, 1–490; APC2-C, 491–1067; APC2-ΔSAMP, 1–930 plus 1037-1067; APC1-N, 1-904. Full length Kap3 (aa 1-945) was PCR amplified from DGRC cDNA clone LD13052 and shuttled through pGEM-T Easy to EcoR1 of pRmHa-3. For the Axin-GFP construct, GFP-Gateway-3X STOP cassette was inserted downstream of the pMT promoter in pMT V5/His (Invitrogen). Full length Axin was then cloned into the pCR8 Gateway entry vector, and gateway cloned into the pMT GFP-W destination vector. For the Axin-HA construct, 3XHA-Gateway-3X STOP cassette was inserted downstream of the pMT promoter in pMT V5/His (Invitrogen) and full length Axin was then cloned into the EcoRI and Xho sites of the pMT HA destination vector.

For validation of the newly identified APC2 isoform forward (5'GCACAACATCGTCCACAATAATCC-3') and reverse (5'GCTCCCAGTTCGCACATAGTCTG-3') primers were used to amplify the region of APC2 containing the putative intron encompassing the ASAD. 4-8 hr embryonic cDNA was used to PCR amplify the region, and the unspliced isoform (231 bp) and the spliced form (168 bp) were identified on a GelStar™ (Lonza) agarose gel. The less abundant spliced isoform was then isolated from the agarose gel (with some contamination from the unspliced form), and re-amplified using the same primers. Both isoforms were directly sequenced for validation.

Yeast Two-Hybrid Analysis

Yeast Two-Hybrid (Y2H) analysis was performed using the Matchmaker System (Clontech). Briefly, the pGBKT7 and pGADT7 yeast vectors were engineered to be Gateway compatible by inserting a Gateway-3X STOP cassette downstream of the Gal4 DNA Binding Domain or Gal4 Transcriptional Activation Domain respectively. APC2 constructs containing the Arm repeats were TOPO-TA cloned into the pCR8/GW/TOPO vector (Life Technologies) and gateway cloned into pGBKT7-W and pGADT7-W. Resulting constructs were transformed into the Y2HGold and Y187 yeast strains respectively using the SC Easy Transformation kit (Life Technologies). Following

transformation and selection, appropriate yeast colonies were mated in 2X YPAD media for 24 hrs and plated on double selection –Leu –Trp plates. Resulting yeast colonies were inoculated in liquid –Leu –Trp media, and β-galactosidase assays performed using the Yeast β-galactosidase Assay Kit (Thermo Scientific, Pierce). Several different colonies were tested per experiment, and each experiment was conducted independently three times. β-galactosidase activity was calculated using the equation: activity = (1000 x OD420)/(T x V x OD660) where T= duration of the reaction in minutes and V=volume of the reaction in mls.

S2 cell culture, transfections, and co-immunoprecipitation experiments:

S2 cells were cultured at 25°C in Schneider's Media (Lonza) with 10% heat-inactivated fetal bovine serum (FBS) and 1X penicillin-streptomycin. DNA constructs were transfected into S2 cells using Effectene (Qiagen) and standard protocols at a cell density of 2.5 x 10^5 cells in 6-well plates. Expression of constructs was induced 24 hrs post-transfection with $CuSO_4$ (40mM final concentration) for 14-16 hours. Co-immunoprecipitation experiments were performed as described in Zhou *et al.*, 2011. Briefly, cells were lysed and pre-incubated with Rec-G beads (Invitrogen) for 30 minutes at 4°C. mCherry antibody (Clontech, cat# 632496.) was used to pull-down tagged proteins from the pre-cleared lysate. Rec-G beads were then added and incubated for 1 hour at 4°C. After washing the beads several times, SDS-PAGE and immunoblotting were performed using standard procedures. Anti-APC2 antibody (McCartney *et al.*, 1999) was used to visualize the various APC2 constructs. To visualize Axin-HA localization, the same transfection procedure was applied and S2 cells were fixed with 4% paraformaldehyde 14-16 hrs after induction and labeled with phalloidin (to label cortical actin) and anti-HA (mouse 1:200, gift from Adam Linstedt's lab, Carnegie Mellon University).

Cell-sorting:

BD FacsVantage Diva option (laser 488) was used to sort the high (33%), medium (33%) and low (33%) expressing S2 cells 24 hrs after induction for the indicated constructs. Cells were sorted into PBS and images of live cells were taken immediately under identical imaging conditions for puncta size measurements. Imaris

(Bitplane) was then used to measure the area of the Axin puncta in μm^2.

SW480 cell culture, transfections, and immunofluorescence

SW480 cells were cultured in Dulbecco's Modified Eagle Media with High Glucose (DMEM-H) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1X Pen/Strep/Glutamine. Cells were maintained at 37°C and 5% CO_2. For transfections, SW480 cells were plated at a density of 2.5 x 10^5 cells in 6-well plates and grown overnight. pCS2(+)-APC2 DNA constructs were transfected using TurboFect (Thermo Fisher) according to the manufacturer's instructions. For immunoflourescence, cells were plated on glass coverslips, transfected with 4 μg of the relevant mCherry-tagged APC2 DNA construct, and fixed 24 hrs post-transfection with 4% formaldehyde in 1X Phosphate Buffered Saline (1X PBS) for 10 mins. Cells were washed three times with 1X PBS, blocked for 15 mins in 1X PBTN (1X PBS containing 1% Normal Goat Serum and 0.1% Triton-100), and then antibody stained as previously described (Roberts *et al.*, 2011). The primary antibody was mouse anti-β-cat (BD Transduction Laboratories, cat# 610153, 1:1000) and the secondary was goat anti-mouse Alexa 488 (Life Technologies, 1:1000). For quantification of β-cat levels, 30 cell images were taken for each condition using identical settings on the spinning disc confocal microscope (see below). The fluorescent intensities for three circular regions of interest (1264 sq. pixels) were measured for each cell (using ImageJ) and were averaged for each condition.

TOP/FOP Luciferase Reporter Assay

The TOP/FOP Flash Luciferase constructs and the pRL Renilla transfection control were provided by Hans Clevers (Hubrecht Institute, The Netherlands). Luciferase assays were performed using the Dual Glo Luciferase System (Promega) according to the manufacturer's protocol. Briefly, SW480 cells were transiently co-transfected with either 1 μg of the TOP or FOP Flash Luciferase reporter, 1 μg of pRL, and 2 μg of the appropriate APC2 construct. 24 hrs post-transfection, cells were lysed in a hypotonic 0.1X PBS solution and subjected to a 5 min freeze-thaw at -80°C. Cells were scraped and cellular debris pelleted at 3000 x g in a microcentrifuge. Lysates were mixed with the provided luciferase substrate, and luciferase activity was measured using a Perkin Elmer EnSpire platereader. Luciferase signal was normalized to Renilla

activity, and overall values normalized to the mCherry only control. All samples were measured in triplicate per experiment, and three independent experiments were performed. None of the constructs displayed significant FOP Flash activity.

Fly genetics, hatch rate and cuticle analysis:

Transgenic flies expressing *P[endoP-EGFP-APC2-FL]* (Zhou *et al.*, 2011), and *P[endoPEGFP-APC2-ΔASAD]* were generated using P-element–mediated germline transformation (Model System Genomics; Duke University, Durham, NC). Two independent second chromosome insertions for each transgene were crossed into the $APC2^{g10}$ *(APC2 null)* background using standard methods. Embryonic cuticles were prepared and hatch rate analysis was performed as previously described (Wieschaus and Nusslein-Volhard, 1998). Scoring criteria for the cuticle phenotype was previously described (McCartney *et al.*, 2006). Cuticle images were taken with darkfield illumination at 20X zoom with a Spot RT Color Model 2.2.0 camera from Diagnostic Instruments.

Immunohistochemistry in the Drosophila embryo:

Embryos were collected 4-6 hrs at 27ºC and fixed and stained as previously described (McCartney *et al.*, 1999). Anti-Armadillo (Arm; ms, N27A1, 1:250) and anti-Engrailed (En; ms, 4D9, 1:50), were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Anti-GFP (Abcam, 1:5000) was pre-absorbed against w^{1118} embryos before using for immunohistochemistry. Anti-APC2 (rt, 1:1000) was used as previously described (McCartney *et al.*, 1999). Secondary antibodies were conjugated with various Alexa dyes [Invitrogen (Carlsbad, CA), 1:1000].

Imaging and image 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. For images of whole embryos stained for Arm and En, multiple images acquired at 25X were merged using Adobe Photoshop to generate whole embryo images.

Sequence Alignments of oligomerization domain 1 and ASAD

Sequences for various species used in the study were acquired from the NCBI pblast database. *Drosophila melanogaster (AAF56249.1)*, *Nasonia vitripennis* (XP001602839.2), *Capitella teleta (*ELU12449.1), *Lottia gigantea* (ESO95067.1), *Strongylocentrotus purpuratus* (XP783363.3), *Saccoglossus kowalevskii* (XP002738523.1), *Ciona intestinalis* (XP002124987.2) and *Homo sapiens* (AAA03586.1). The Nasonia, Capitella, Lottia genomes have not been annotated, therefore we relied on manual confirmation of the APC sequences based on conservation of the protein. ClustalW was used for generating sequence alignments. We considered a minimum of 50% conservation to decide the presence of OD-1 or ASAD.

Live imaging and FRAP analysis:

FRAP experiments were carried out using a Zeiss LSM510 confocal microscope with ZEN software. After 2 pre-bleach scans at 3X zoom, 10 bleaching scans with 100% intensity of 488nm over the region of interest (15px-15px circle) were performed. This area was kept constant and similar sized puncta were bleached for the analysis. After photobleaching, the fluorescence recovery was monitored every 3.96 sec for 100 frames. The recoveries of the fluorescence intensities of each image series were quantified with Image J and processed using Excel. $\Delta F/F$ was calculated by taking the difference between the time zero fluorescence measurement (arbitrary units) and the fluorescence measurement at each time point divided by the time zero fluorescence measurement. This showed the degree of recovery post bleaching normalized by the time zero fluorescence. The three groups were compared to each other in a regression analysis model. The analysis was protected overall by ANOVA and individual pairwise comparisons were assessed by the Tukey-Kramer HSD post-hoc test. In order to assess that rate of fluorescence recovery, we calculated the sum of least squares best fit regression line for each site and compared them with ANOVA and the Tukey-Kramer HSD post-hoc test. Time zero normalized degree of recovery at our last time point (384.12 sec) for each condition was compared using ANOVA and the Tukey-Kramer HSD post-hoc test. All analyses were conducted using JMP 11.

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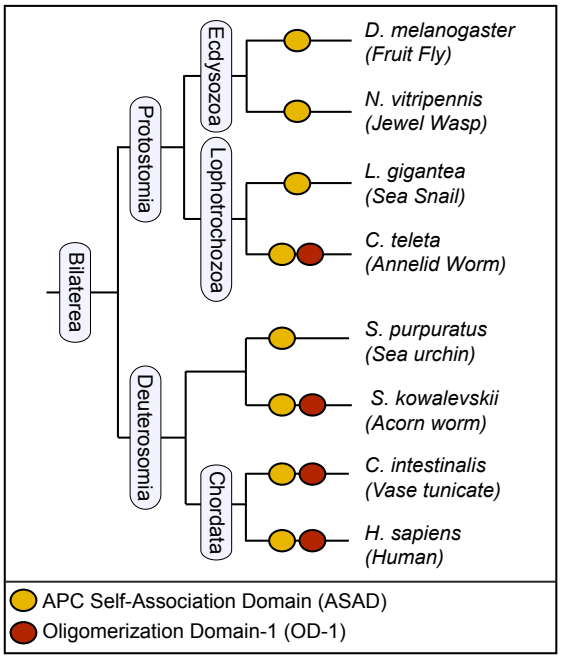
Supplemental Figures:

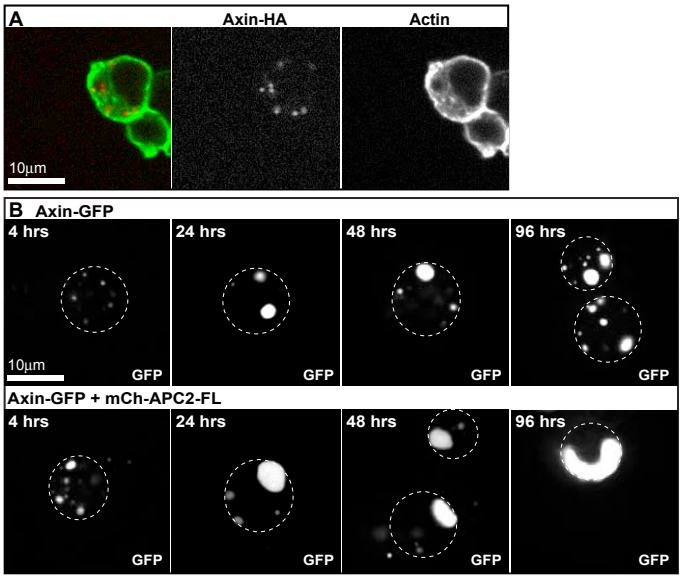


Percent Sequence Similarity to hAPC(AAA03586.1)

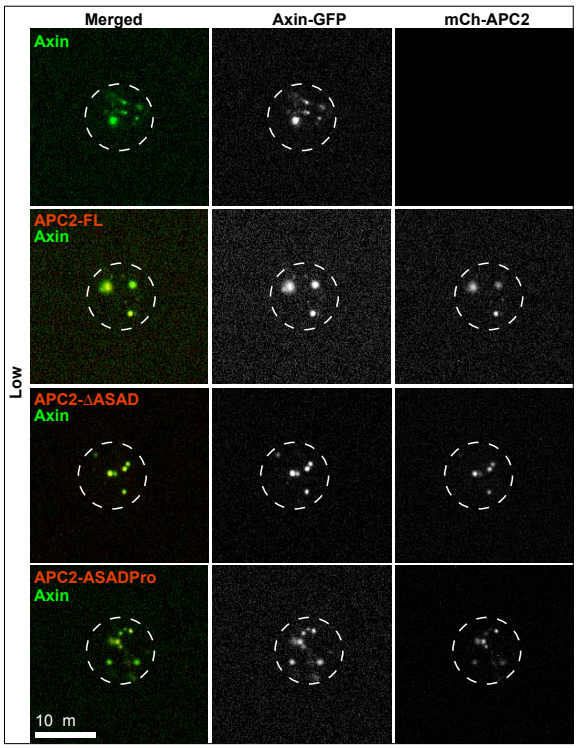
Species Name & Accession #	hAPC-OD-1	hAPC-ASAD
C. intestinalis (XP002124987.2)	83%	55%
S. kowalevskii (XP002738523.1)	96%	78%
S. purpuratus (XP783363.3)	NA	78%
L. gigantea (ESO95067.1)	NA	81%
C. teleta (ELU12449.1)	93%	74%
N. vitripennis (XP001602839.2)	NA	81%
D. melanogaster (AAF56249.1)	NA	68%

NA: Not Applicable

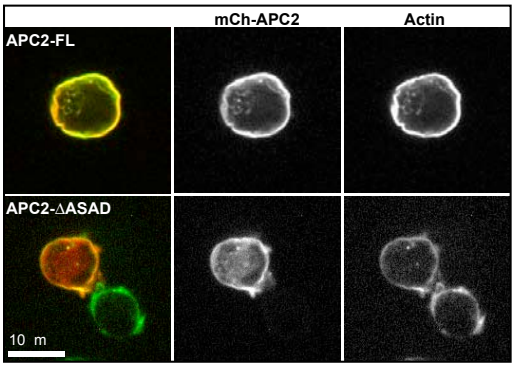
Supplemental Figure 1: The APC Self-Association Domain (ASAD) is conserved across bilaterian phyla. A) Phylogenetic tree depicting the conservation of the ASAD (orange circles) and human oligomerization domain 1 (OD-1) (burgundy circles) across bilaterian phyla. OD-1 is primarily present in deutorostomes. The exception is the sea urchin APC, which does not have an OD-1 sequence, but instead contains an N-terminal insertion of a poly-glutamine repeat. All phyla examined contained the ASAD.



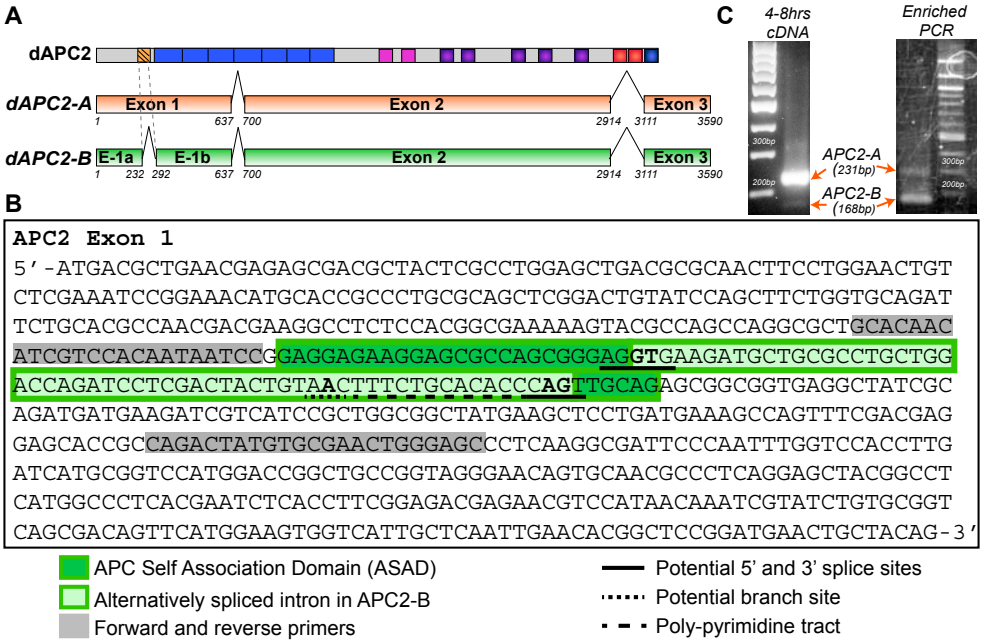
Supplemental Figure 2: A) Similar to Axin-GFP, HA-tagged Axin forms cytoplasmic puncta in S2 cells. B) APC2-FL promotes the formation of larger Axin puncta. Puncta size increases over time in cells expressing Axin-GFP alone, but appears to reach a plateau after 48hrs. Conversely, Axin-GFP/ APC2-FL puncta are larger than Axin-GFP puncta at all time points and their size increased even after 48 hrs. Surprisingly, we frequently observed that Axin-GFP/APC2-FL puncta are abnormally shaped by 96 hrs. This was never observed for Axin-GFP puncta. Scale bar: 10µm



Supplemental Figure 3: FACS sorted cells were divided into three different groups based on the expression of Axin-GFP (high, medium and low) and puncta size and number was assessed. The striking alteration in destructosome morphology of the self-association mutants allowed quantification of their size only at low expression level. Scale bar: 10μm



Supplemental Figure 4: Disruption of APC2 self-association leads to the disruption of cortical localization in Drosophila S2 cells. APC2-FL is cortical while APC2-ΔASAD is mostly cytoplasmic; actin is used to label the cortex. Scale bar: 10μm



Supplemental Figure 5: Drosophila embryos express a splice form of APC2 that disrupts the ASAD. A) Schematic representationof the APC2 protein and both APC2 splice forms APC2-A and APC2-B. B), Exon 1 of APC2 with the ASAD (dark green), the alternatively spliced intron (light green), potential 5' and 3' acceptor sites (line), and the branch site and polypyrimidine tract (dashed lines). C) PCR was used to specifically amplify the two splice variants from cDNA prepared from 4-8 hr wild type embryos. While the full-length version of APC2 (APC2-A) containing the ASAD appears to be expressed at significantly higher levels, we did identify a lower molecular weight product that corresponds in size to the expected product from APC2-B amplification. We extracted the putative APC2-B band, subjected it to 2 additional rounds of PCR (enriched PCR), and sequenced both the putative APC2-B and APC2-A bands to confirm their identity.The low expression of APC2-B relative to APC2-A might be due to various factors including tissue specificity. Splice site consensus sequences are YAG|G at the 3' splice site, preceded by a polypryrimidine tract, and MAG|GTRAGT (M= A or C, R= A or G) at the 5' splice site (Mount et al., 1992). The 5' and 3' splice sites in the APC2-B splice isoform generally follow the consensus albeit not perfectly. Although the 5' splice site contains the most important GT dinucleotide at the first two positions of the intron, it only matches the overall consensus at 6 of 9 positions. Interestingly, while Drosophila APC1 does contain the ASAD (Fig. 1B), it does not appear to express a self-association incompetent isoform based on the modENCODE database.

Chapter 5

Dissecting the mechanism of APC2-Diaphanous dependent actin assembly in the Drosophila syncytial embryo

* Manuscript in preparation

Dissecting the mechanism of APC2-Diaphanous dependent actin assembly in the Drosophila syncytial embryo

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Abstract

Cell division, cell shape change and cell migration are a few of the many cellular processes that require precise cytoskeletal rearrangements mediated by an orchestra of actin and microtubule-associated proteins. The colon cancer tumor suppressor Adenomatous polyposis coli (APC) is a well-known negative regulator of the Wnt signaling pathway, but a poorly understood regulator of the actin cytoskeleton. Work from our lab and others has recently revealed that APC proteins can collaborate with formins to promote actin assembly *in vitro* and *in vivo*. We previously demonstrated that a complex between APC2 and the formin Diaphanous is necessary for the formation of actin-based pseudocleavage furrows in the early Drosophila embryo. To understand the molecular mechanisms driving this collaboration, we have taken a combination of structure-function and live imaging approaches. Here we demonstrate that APC2's ß-catenin-binding 20 amino acid repeats (20Rs) are sufficient for direct binding to Dia, and this domain is necessary for actin-furrow extension in the embryo. Because the 20Rs are highly phosphorylated, and phosphorylation regulates APC-ß-catenin binding, we predicted that 20R phosphorylation may regulate the activity of the APC2-Dia complex. Consistent with that hypothesis, targeted mutants that disrupt or mimic phosphorylation both interfere with APC2 dependent actin furrow extension. Furthermore, manipulation of the kinase GSK3ß that targets the 20Rs also interferes with furrow extension. Taken together, these data suggest a model by which direct binding between APC2 and Dia through the 20Rs is required for the activity of the complex that is regulated by GSK3ß through phosphorylation of the 20Rs. As a complement to these studies in the actin furrow, we examined the effects of APC2 and Dia on actin dynamics in a different population of early embryonic actin called the actin cap. Our studies revealed a novel role for the APC2-Dia complex in regulating the actin cap dynamics that hadn't been previously described and suggested a potential crosstalk between branched and linear actin filaments.

Introduction

The actin cytoskeleton is essential for many developmental processes including cytokinesis, cell motility, and morphogenesis. Actin rearrangements are orchestrated by vast number of proteins. Functions of many of these factors have been examined only *in vitro*, leaving a significant gap in our understanding as to how they function in an intact animal. The Drosophila syncytial embryo is a powerful *in vivo* system to study dynamics of the actin cytoskeletal (Fig. 1B). Early Drosophila divisions occur without cytokinesis that leads to a formation of a syncytium (reviewed in Sullivan and Theurkauf, 1995). Following nuclear migration to the cortex in cycle 10, four more rounds of synchronous divisions occur prior to cellularization. Cortical actin is highly dynamic at this time in concert with nuclear divisions. In interphase, “actin caps” form above each nucleus that promote centrosome separation and proper spacing of the nuclei (Cao et al., 2010; Stevenson et al., 2002) (Fig 1B). During metaphase, actin cytoskeleton is reorganized into the actin-based extensions called “pseudocleavage furrows”. Furrows function to separate the dividing nuclei to ensure mitotic fidelity (Webb et al., 2009) (Fig 1B). Furrows regress rapidly during anaphase and reorganized into caps during telophase. Each nuclear /actin cycle happens very quickly and takes ~10-20 minutes.

It has been proposed that two pools of cortical actin exist: branched in the caps and linear in the furrows. The size of the overall G-actin pool is stable as monomers are recycled between the cap and the furrow (Cytrynbaum et al., 2005; Stevenson et al., 2002). However, the molecular mechanisms driving this actin reorganization from the caps to the furrows are poorly understood. The branched actin nucleator Arp2/3, and its activator Scar are required for actin cap formation and expansion, and loss of function mutants display defects in both actin cap expansion and centrosome separation (Cao et al., 2010; Stevenson et al., 2002). These mutants also display furrow defects, which might be an indirect consequence of the expansion defects and the lack of coordination between the two pools of actin (Stevenson et al., 2002). Pseudocleavage furrows form and extend into the embryo between neighboring nuclei where caps meet. They reach their deepest length during metaphase, to ensure the separation of the neighboring

nuclei. Previous studies suggested a role for the recycling endosome pathway Nuclear

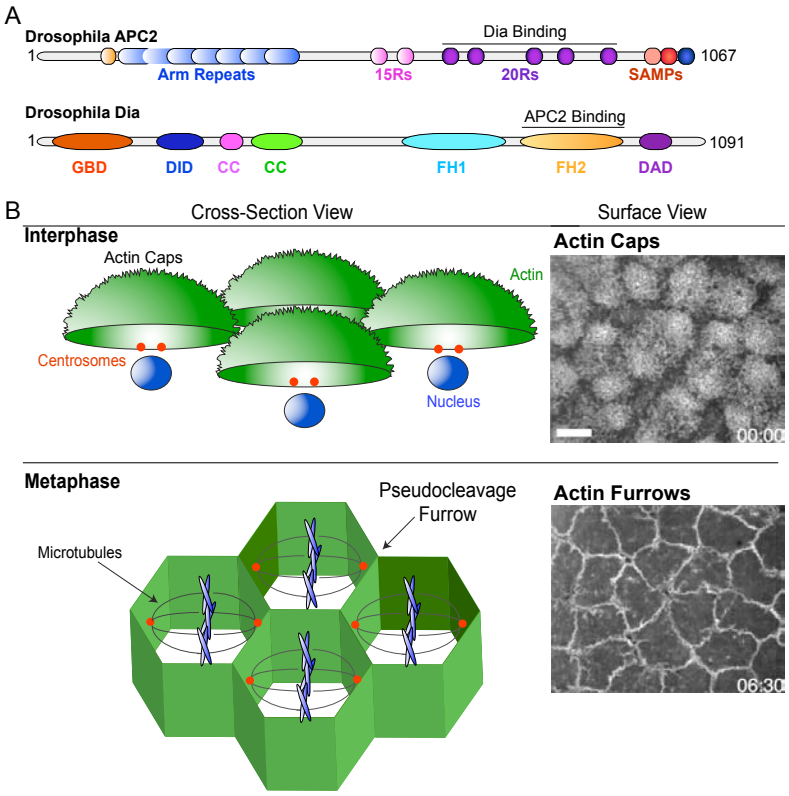


Figure 1: A) Schematic representation of Drosophila APC2 (dAPC2) (ASAD: APC self-association domain (orange), Arm repeats: Armadillo repeats (blue) 15Rs: 15 amino acid repeats (pink), 20Rs: 20 amino acid repeats (purple), SAMP Repeats (red), C30 (dark blue)) Diaphanous (Dia) (GTPase Binding Domain (GBD) red, Dia inhibitory domain (DID) blue, Formin homology domain 1 (FH1) (light blue), FH1 (orange), Dia activation domain (DAD) purple.) B) Actin dynamics during Drosophila syncytial development. During interphase actin is organized in caps, then into furrows during metaphase.

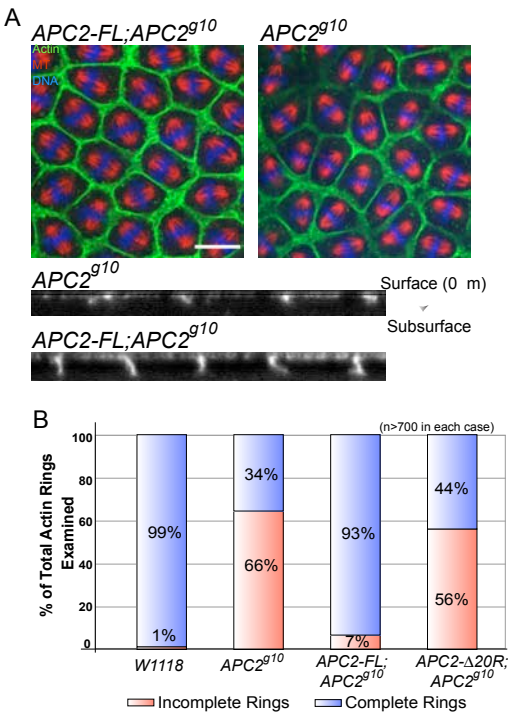


Figure 2: APC2 null (APC2g10) displays furrow extension defects, which can be rescued by introducing the full-length transgene into the background. B) Removal of Dia interaction domains (APC2-20Rs) display similar degree of furrow extension defects.

fall out (Nuf), Rab11 and Discontinuous actin hexagons (Dah) in the formation of furrows (Albertson et al., 2005; Cao et al., 2008).

Previous studies from our laboratory demonstrated a novel collaboration between Drosophila Adenomatous polyposis coli 2 (APC2) and the formin Diaphanous (Dia) to extend actin furrows (Webb et al., 2009). Loss of either Dia or APC2 leads to defects in the extension of these furrows. To understand the molecular mechanisms driving this collaboration, first we have taken a combination of structure-function analysis. Here we show that the APC2 20Rs, that are sufficient for direct binding to Dia, are necessary for actin-furrow extension in the embryo. Furthermore, we have shown that phosphorylation of the 20Rs plays a role in their activity in actin furrow extension, and that this is likely regulated by GSK3ß activity. Finally, we have initiated live imaging experiments of the actin cap in APC2 and dia mutants to dissect their role in actin dynamics. Through these experiments we have made intriguing observations regarding the role of the APC2-Dia complex, and how linear and branched actin networks may functionally intersect. 3

Results:

Removal of the Dia binding domain of APC2 exhibits furrow defects

Previously, we have shown that complete loss of APC2 (*APC2^{g10}*) leads to severe defects in actin furrow extension in the syncytial embryo (Webb et al., 2009) (Fig 1C). We recently discovered that the 20 amino acid repeat (20R) region of APC2 is sufficient to mediate the interactions with the FH2 domain of Dia (Molinar and Stepanik, unpublished data) (Fig 1A). In the light of this finding, we tested the ability of the APC2 mutant that lacked the Dia interaction domain (APC2-Δ20R) to rescue the actin furrow defects of the null *APC2^{g10}*. We have previously shown that the native promoter that drives endogenous levels of APC2 *in vivo* is sufficient for complete rescue of APC2 lethality and rescue of furrow extension defects (Zhou et al., 2011) (Fig 2). We predicted that the removal of 20Rs would fail to rescue the null defects of *APC2^{g10}* and consistent with our hypothesis, the APC2-Δ20R mutant displayed furrow defects similar to the complete loss of APC2 (Fig. 2). This suggests that the 20Rs, through mediating the

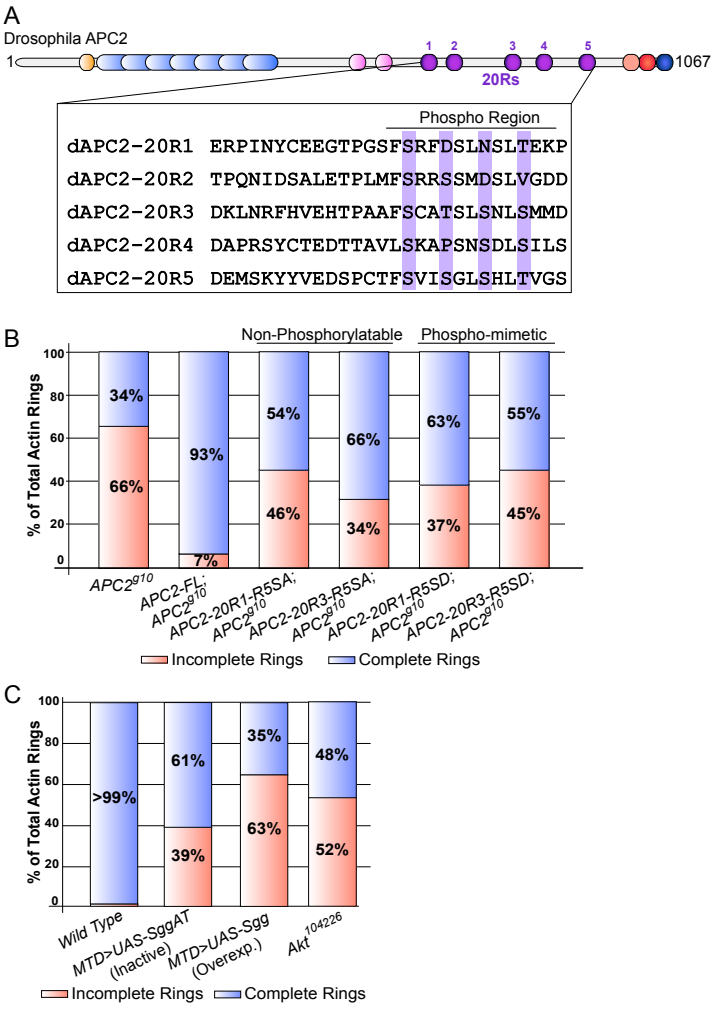


Figure 3: A) Sequence alignments of all five dAPC2 20Rs. Phospho-residues that manipulated in this study are highlighted in purple. B) APC2 20R phospho-mutants display furrow extension defects. C) Manipulation of GSK3 (Sgg) and Akt also displays furrow defects. Maternal driver was used to drive the expression of UAS-SggAT (Inactive Sgg) and UAS-Sgg. Akt^{104226} hypomorph also displayed defects.

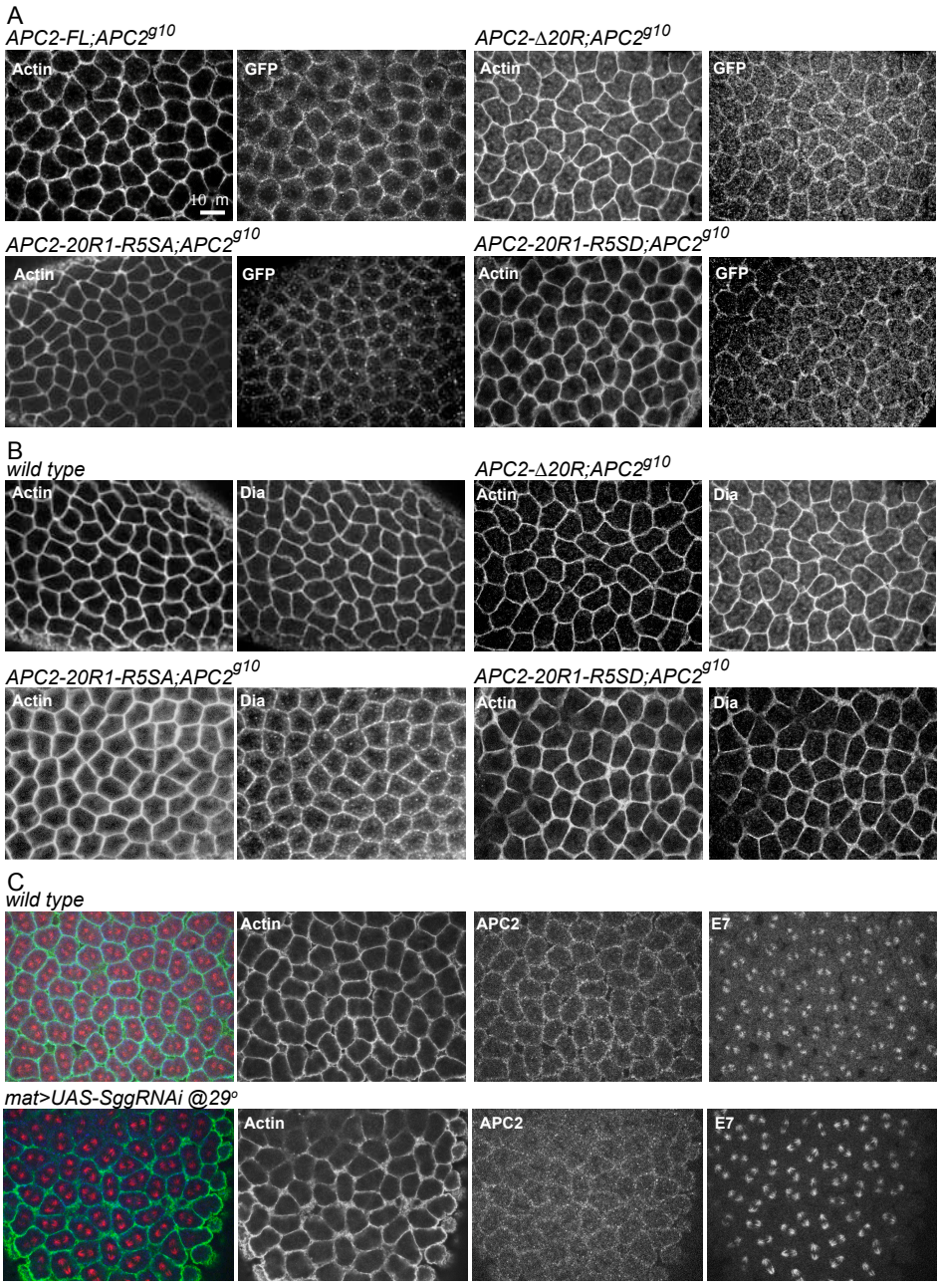


Figure 4: A, B) Localization of GFP-tagged mutant transgenes in the early embryo. Phalloidin labels the actin, GFP labels the various mutants, and Dia. C) Disrupting Sgg in the early embryo disrupts APC2s cortical localization.

interaction with Dia, are essential for APC2's activity in the formation of the actin furrows.

Phosphorylation of the Dia binding domain regulates the activity of the APC2-Dia complex.

Drosophila APC2 is a phosphoprotein (McCartney et al., 1999; Zhai et al., 2008) and phosphorylation of the 20Rs is an important regulatory mechanism in the context of the Wnt signaling destruction complex *in vivo* (Kunttas-Tatli et al., 2012). GSK3ß and CK1 phosphorylate multiple sites within each repeat that modulates binding affinity to ß-cat (Liu et al., 2006). Given the fact that the 20Rs also mediate Dia interaction, and the activity and colocalization of the APC2-Dia complex is cell cycle dependent and occurs only during metaphase (Webb et al., 2009), we wanted to test if the phosphorylation status of these domains plays a role in regulating the activity of the APC2/Dia complex during actin furrow extension. To determine whether phosphorylation at these sites also impacts APC2's actin furrow extension function, we used mutant flies that disrupted (S to A) or mimicked (S to D) phosphorylation of the 20Rs in all five repeats (20R1-R5) or only in a subset (20R3-R5), and assessed the ability of these mutants to rescue actin furrow defects in *APC2* null embryos (Fig. 3A). We have previously shown that both phospho-20Rs and non-phospho-20Rs are required for APC2's destructosome function (chapter 2) as both the phospho-mimetic and the non-phosphorylatable mutants of 20R1-R5 do not support destructosome function in the absence of all other APC (Kunttas-Tatli et al., 2012). On the other hand, the phosphorylation status of 20R3-R5 did not affect APCs Wnt signaling function. In contrast to these results in Wnt signaling, both the APC2-20R1-R5 and the 20R3-R5 mutants displayed partial rescue of furrow extension defects, albeit weaker than that of the APC2-Δ20R mutant. This suggests that the phosphorylation of all five 20Rs may affect APCs actin furrow extension function and both forms of APC2, phospho-20Rs and non-phospho-20Rs, are required (Fig. 3B). This might suggest the importance of the cyclic phosphorylation and dephosphorylation as a regulatory mechanism.

We have previously shown that APC2 and Dia both localize to actin throughout

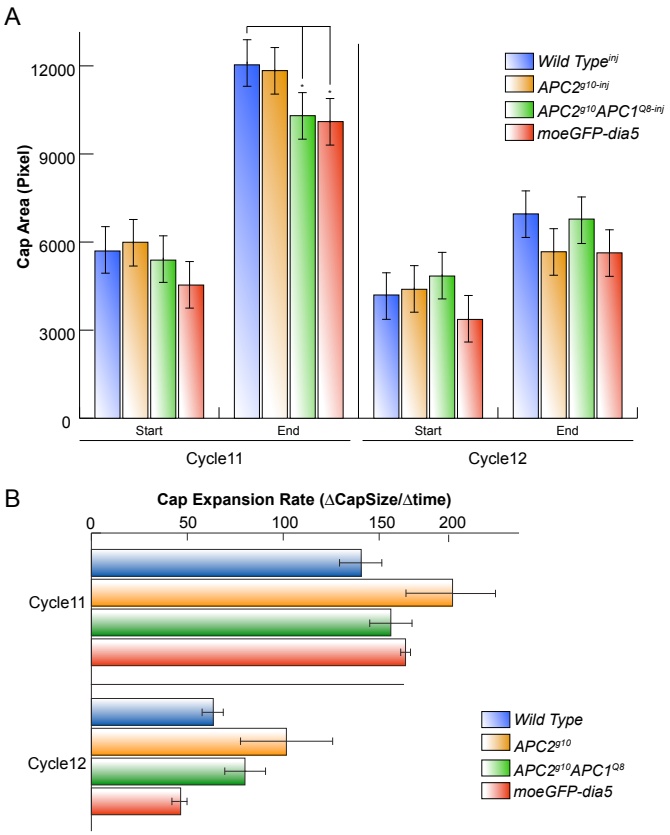


Figure 5: A) Actin cap areas were measured during cycle 11 and 12 for wild type and mutant embryos. Wild type (blue), APC2g10 (orange), APC2g10APC1Q8 (green) and dia5 (red) B) Cap expansion rates were calculated for the same set of embryos.

the actin cycle, but they only colocalize strongly at metaphase (Webb et al., 2009). Therefore, we tested if APC2 mutants that exhibit defective furrow extension failed to colocalize with Dia in actin furrows, or exhibited global defects in localization to cortical actin throughout the actin cycle. Our colocalization analysis revealed that neither APC2-Δ20R nor the phospho-mutants displayed defects in cortical localization; suggesting removal or phosphorylation status of the 20Rs have no effect on cortical localization as well as Dia colocalization (Fig. 4A, B).

Drosophila GSK3ß regulates APC phosphorylation and APC2's activity in furrow extension

Many of the 20R phosphorylation sites in hAPC are phosphorylated by GSK3ß (Drosophila Shaggy, Sgg) *in vitro* (Liu et al., 2006). Therefore, we predicted that manipulating this kinase in the early embryo would modulate the activity of the APC2-Dia complex during furrow extension and result in furrow extension defects. To manipulate the kinase, we used germline specific GAL4 drivers (MTDGal4 or matVP67; matVP15) to drive the expression of UAS-lines during oogenesis and consequently perturbing early embryogenesis. Consistent with our hypothesis, both inactivating (UAS-SggAT) and hyperactivating (UAS-Sgg) Sgg disrupts furrow extension. Expression of the inactive form of Sgg may be less effective as it must act as a dominant negative. Sgg activity is negatively regulated by phosphorylation by Akt. Consistent with the UAS-Sgg overexpression, reduction of Akt produced similar furrow extension defects.

We have previously shown that APC2 loses its cortical association in syncytial embryos mutant for Sgg (McCartney et al., 2001). Thus, we wanted to test if Sgg mutants also exhibited furrow defects. After testing various maternal drivers at various temperatures, we were able to generate mutant embryos by using the UAS-SggRNAi flies that lacked any maternal Sgg contribution. We were able to recapitulate the loss of cortical APC2 localization in cycle 12 metaphase embryos compared to wild type (Fig. 4C).

APC and Dia are required for the production of dynamic actin bundles in the interphase actin cap

We have clearly established that a complex of APC2 and Dia promotes the

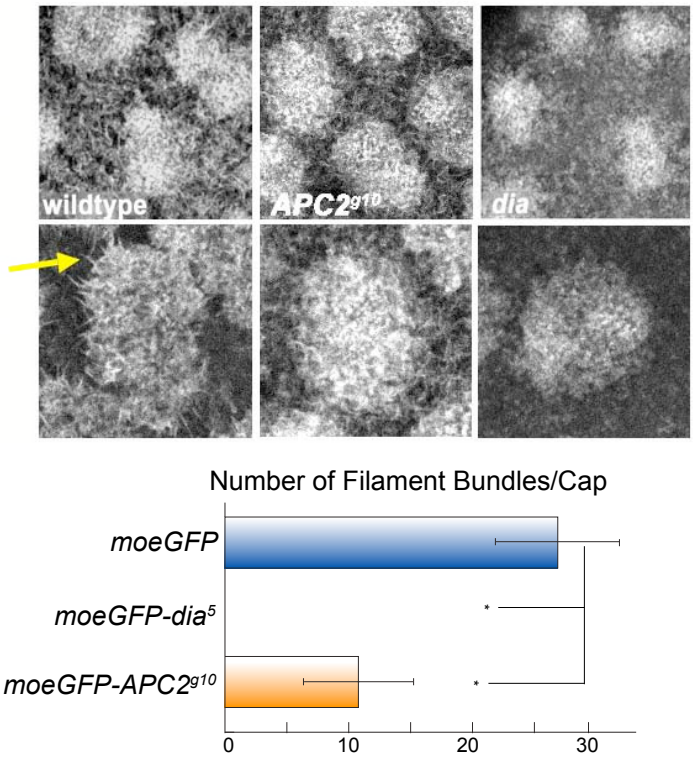
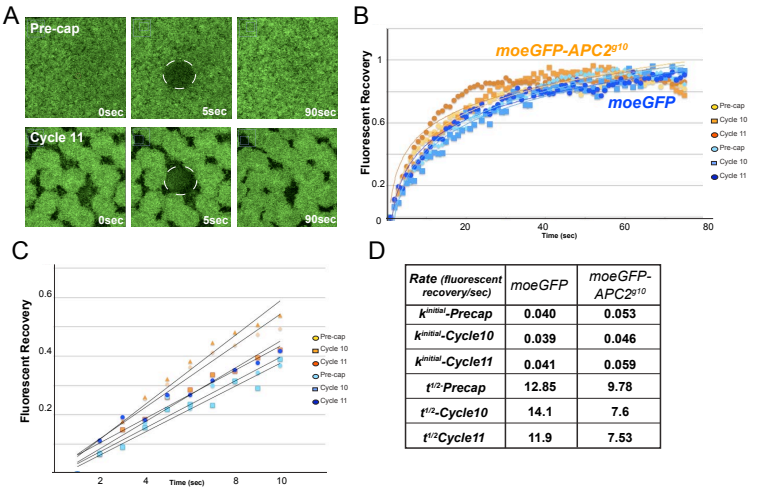


Figure 6: Actin bundle at the periphery of the actin caps were counted. While dia mutants don't have any, APC2 mutants exhibit reduced numbers.



Rate (fluorescent recovery/sec)	moeGFP	moeGFP-APC2^{g10}
$k^{initial}$-Precap	0.040	0.053
$k^{initial}$-Cycle10	0.039	0.046
$k^{initial}$-Cycle11	0.041	0.059
$t^{1/2}$-Precap	12.85	9.78
$t^{1/2}$-Cycle10	14.1	7.6
$t^{1/2}$Cycle11	11.9	7.53

Figure 7: A,B) The rate of fluorescent recovery after photobleaching in moeGFP (wild type) (blue) and moeGFP-APC2 mutants (orange). C,D) The initial rate of recovery within the first 10 seconds (kinitial) is higher in APC2 mutants compared to wild type embryos, resulting in a smaller t1/2.

formation of actin furrows in the syncytial embryo, and we have begun to dissect the mechanisms behind that activity. As a complement to our established actin furrow extension assays, we examined the role that APC2 and Dia play in regulating actin dynamics in the interphase actin cap. Live imaging of actin caps is a tractable approach that may reveal important details that are otherwise undetectable in fixed embryos. In order to image APC2 and Dia mutants live, we either recombined moe-GFP (labels actin) into the null APC2 (*APC2*^{g10}), APC double null (*APC2*^{g10}*APC1*^{q8}) or dia (*dia*^{5}) backgrounds, or we injected mutant embryos with fluorescently (rhodamine) labeled actin.

We were surprised to see that the surface of the actin cap is comprised in part of a population of highly dynamic linear actin bundles that we have not seen described in the literature. These bundles both extend and retract prior to and during cap expansion, and strongly resemble filopodia. It is not clear what role these projections play in actin cap function. Given the role of Dia in nucleating and elongating linear actin filaments, we predicted that APC2 and Dia would be necessary for the assembly of these structures. Consistent with our predictions, *APC2* mutants have significantly reduced numbers of these actin projections and in *dia* mutants they are completely absent (Fig. 6). This suggests that Dia and APC2 collaborate in the actin caps to promote the formation of these linear actin filaments, consistent with their collaboration to assemble actin furrows. In order to understand this cooperation, we examined APC2 and Dia localization in the cap. Interestingly, two proteins do not significantly colocalize in the cap (Fig). APC2 forms various sized puncta structures within the cap. On the other hand, Dia localizes mostly to the inter-cap area in the apical sections, but colocalizes with actin in the periphery of the cap (Fig 9). This pattern of localization is reminiscent to the rocket launcher mechanism, which was proposed for vAPC-Dia relationship (Breitsprecher et al., 2012). The use of triple-color single molecule fluorescence microscopy experiments revealed that upon filament nucleation, APC-Dia complex separate, with Dia moving on the growing barbed ends while APC remained at the site of nucleation. We think, the inter-cap Dia labels the molecules at the tip of the actin bundles (growing end), while APC2 puncta labels the site of nucleation. However, due to low image resolution and the fact that these bundles don't fix well, we were unable to

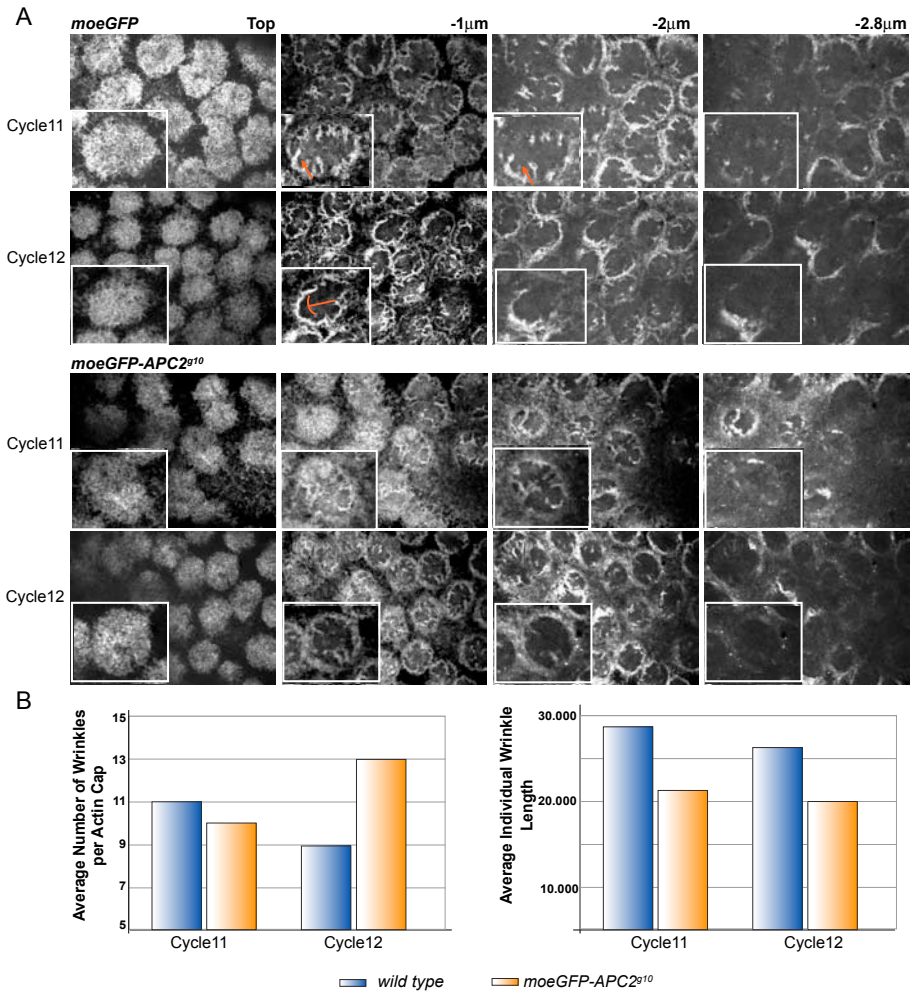


Figure 8: A) The actin in wild type caps forms protrusions into the center of the cap that we termed "wrinkles". B) Both number and the length of the wrinkles displayed differences in wild type (blue), and APC2g10 (orange) embryos in a cycle dependent manner.

confirm this similarity.

Overall cap structure is altered in *APC2* mutants

If the linear actin bundles are necessary for overall cap structure, we predicted that APC2 and dia mutant caps would display other structural defects. To test this, we first examined wild type (moe-GFP) actin caps along the z-axis over time and found that below the surface at cycle 11, the actin in wild type caps forms protrusions into the center of the cap that we termed “wrinkles” (Fig. 8). These can extend for at least 1 μm into the embryo along the z-axis. We predicted that these wrinkles may provide structural integrity to the cap. Therefore, stronger actin caps would have fewer wrinkles than weaker actin caps. Cycle 11 caps are larger in area compared to cycle 12 caps, and thus we expected that cycle 11 caps would have a greater number of structural wrinkles to support their larger area. Consistent with this hypothesis, cycle 11 caps have a greater number of wrinkles Because APC2 mutant caps have a reduced number of linear filament bundles, we predicted that this would lead to a weaker cortex that would exhibit increased number of wrinkles to compensate. At cycle 12, APC2 mutants do exhibit significantly more wrinkles per cap despite the fact that cap area appears to be unchanged. In addition, APC2 mutant wrinkles are significantly shorter at both cycles 11 and 12, which may also suggest a weaker actin cortex. We are continuing to analyze these phenotypes to understand both the origin and function of the wrinkles, and how APC2 and Dia influence the structure of the cap.

Disruption of APC and Dia does not significantly affect cap area, but does result in variable rates of cap expansion

We predicted that the reduction or loss of the dynamic linear actin bundles from the cap could negatively affect cap behavior. Consequently, we asked if loss of *APC* or *dia* altered cap assembly and dynamics. First, we measured the area of actin caps at the beginning and end of nuclear cycles 11 and 12. We found no significant difference between the mutants and wild type with the exception of the end of cycle 11 where *APC* double mutant and *dia* mutant embryos exhibited a slight, but statistically significant, reduction in cap area (Fig. 5A). This suggests that the caps do not fully expand in those mutants. Interestingly, *APC2* and *dia* mutants exhibited an increase in the cap

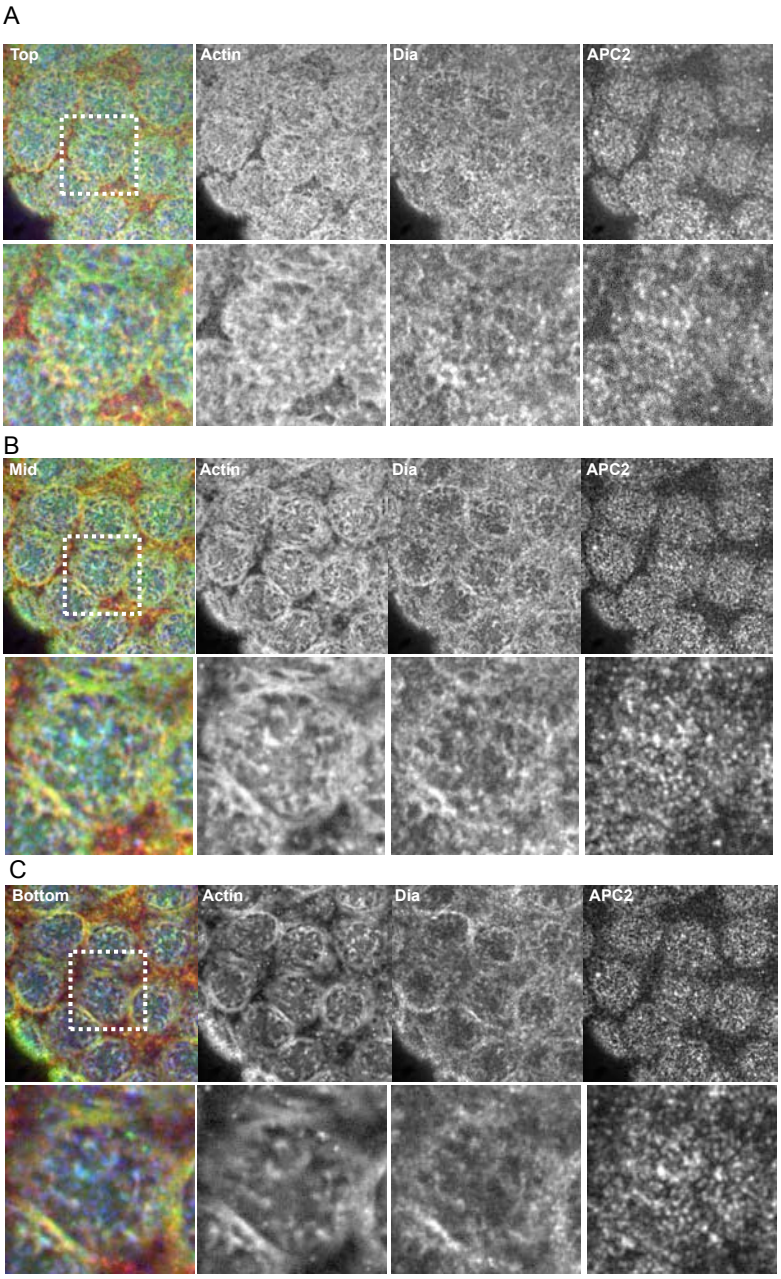


Figure 9: Colocalization of actin (green), APC2 (blue) and Dia (red) in the actin cap. Top, mid and bottom slices were selected as representative to show the localization of these proteins both in the peripheral actin bundles (A) and also deeper actin wrinkles (B, C)x.

expansion rate at cycles 11 compared to wild type, while the *APC* double mutants did not (Fig. 5B). In cycle 12, both *APC2* mutants and the *APC* double mutants showed an increase in cap expansion rate, but in contrast, *dia* mutants exhibited a reduced rate of cap expansion. Taken together, these data suggest that one role of the APC2-Dia dependent linear actin bundles may be to provide a framework to regulate Scar-Arp2/3 dependent cap expansion. In the absence of that framework, the rate of cap expansion appears to become more variable. This suggests that the rate of branched actin assembly and disassembly may be regulated by the presence of the linear filament bundles.

This observation suggested that the rate of actin filament turnover in *APC2* and *dia* mutant caps may also be more variable. To begin to test this, we measured the rate of fluorescent recovery after photobleaching in moeGFP (wild type) and moeGFP-APC2 mutants (Fig. 7). We found that the rate of recovery within the first 10 seconds ($k^{initial}$) is higher in *APC2* mutants compared to wild type embryos, resulting in a smaller t1/2. By 80 seconds post-bleach, maximum fluorescence recovery was achieved in all cases, and the overall t1/2 was not significantly different between the mutants or the times analyzed. This suggests that reduction of linear actin bundles in the cap leads to an increased rate of filament turnover in the remaining branched actin population. This could be due to the presence of a larger pool of actin monomers, or could reflect a more direct regulation of branched actin assembly by linear actin filaments.

Future Directions

Crosstalk between linear and branched actin filaments

Our actin cap data suggested potential cross talk between linear and branched actin filaments. We would like to study this further but manipulating these two pools of actin in the early embryo and utilize some of the assays we mentioned above. In order to manipulate the linear actin pool, in addition to APC2 and Dia mutants, we would like to express constitutively active form of Dia in the early embryo. My initial attempts to achieve this have been unsuccessful as expression of this form of Dia using different GAL4 driver and temperatures resulted in either no phenotype, or oogenesis failure. To manipulate the branched actin pool, we tried RNAi to knockdown various components of

the Arp2/3 complex or its regulator Scar. However, the reagents did not appear to knockdown these components effectively as we could not recapitulate the published loss of function phenotypes. To overcome this obstacle, we decided to use an Arp2/3 inhibitor. Future studies will be required to use this inhibitor effectively in the early embryo. In addition to these experiments, I wanted to look at the localization of Scar in conjunction with Dia and APC2 in the cap and also in the furrow (Fig. 10). Consistent with previous studies, Scar localizes to the cap, but there is little colocalization between Scar and APC2 or Dia. Surprisingly, we also found Scar in the furrow, suggesting that there may be an unappreciated role for branched actin there as well.

The role of APC2-Arm complex in the early embryo

Given the fact that the Dia binding 20Rs also mediate interactions with ß-catenin (Armadillo or Arm in Drosophila), we wanted to test if Arm could be also in the complex with APC2 and Dia. Given the well-established role of APC proteins in the destruction complex during Wnt signaling, we wanted to test if loss of APC2 or removing the Arm interaction domains would have an affect on the levels of Arm in the early embryo (Fig 11). Surprisingly, levels of Arm were significantly elevated in APC mutant backgrounds, indicative of destruction complex activity in the early embryo. Because there is no embryonic transcription at this time, it's interesting to see an active destruction complex. Currently we don't know if the elevated levels of Arm could activate transcription, however, we wanted to test if it had an affect on furrow extension. In order to mimic elevated levels of Arm in the early embryo, we again used the UAS/Gal4 system to express a constitutively active form of Arm (Arm^{s10}). Future studies will be performed to determine if these embryos will display actin defects in the cap or the furrow.

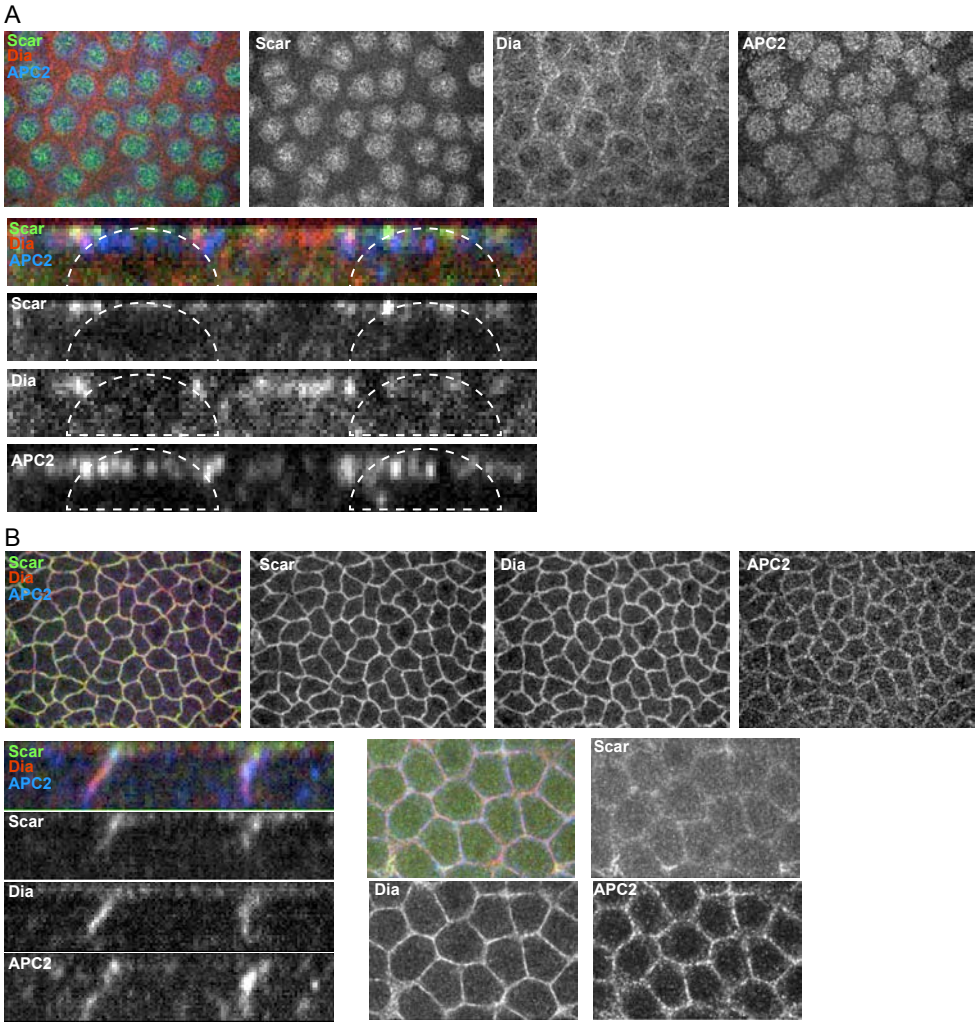


Figure 10: Colocalization between Scar, Dia, and APC2. A) While during interphase, Scar and APC2 localizes to the cap, while Dia is mostly in the intercap area. B) During metaphase all three proteins colocalize apically, while only APC2 and Dia colocalize basally.

Mechanistic properties of APC2 and Dia mutant embryos

In order to gain more insight into how loss or reduction of linear actin filaments might affect the overall mechanics of the embryos, we will conduct microrheology experiments in collaboration with Lance Davidson. This technique can be used to measure the rheological properties of the syncytial embryos, like viscosity or strain. In order to obtain these measurements, I will inject fluorescent beads and track the movement of multiple beads relative to each other, and compare wild type embryos to *dia* and *APC2* mutants. The relative movement of beads at the cortex will in turn can be used to extract information about viscosity. My initial experiments were successful in injecting the beads and visualizing them at the cortex.

Methods

Fly genetics and Immunohistochemistry in the Drosophila embryo:

Transgenic flies expressing *P[endoP-EGFP-APC2-FL], P[endoPEGFP-APC2-Δ20R], P[endoPEGFP-APC2-R1R5SA]*, *P[endoPEGFP-APC2-R1R5SD], P[endoPEGFP-APC2-R3R5SA]*, and *P[endoPEGFP-APC2-R3R5SD]* ((Kunttas-Tatli et al., 2012)) were generated using P-element–mediated germline transformation (Model System Genomics; Duke University, Durham, NC). UAS/Gal4 system was used to express TRIP RNAi lines (Bloomington) as well as other UAS constructs. Maternal drivers used are: MTDGal4 (Dr. Lynn Cooley), nosGal4 (Bloomington), matVP67; matVP15 (Dr. Adam Martin).

Embryos were collected 4-6 hrs at room temperature (RT) and fixed and stained as previously described (McCartney *et al.*, 1999). Anti-APC2 (rt, 1:1000) (McCartney *et al.*, 1999), Anti-Dia (rb, 1:5000), Anti-Scar (p1c1 DSHB, 1:50). Anti-GFP (Abcam, 1:5000) was pre-absorbed against w^{1118} embryos before using for immunohistochemistry. Phalloidin Secondary antibodies were conjugated with various Alexa dyes [Invitrogen (Carlsbad, CA), 1:1000].

Furrow Depth Analysis:

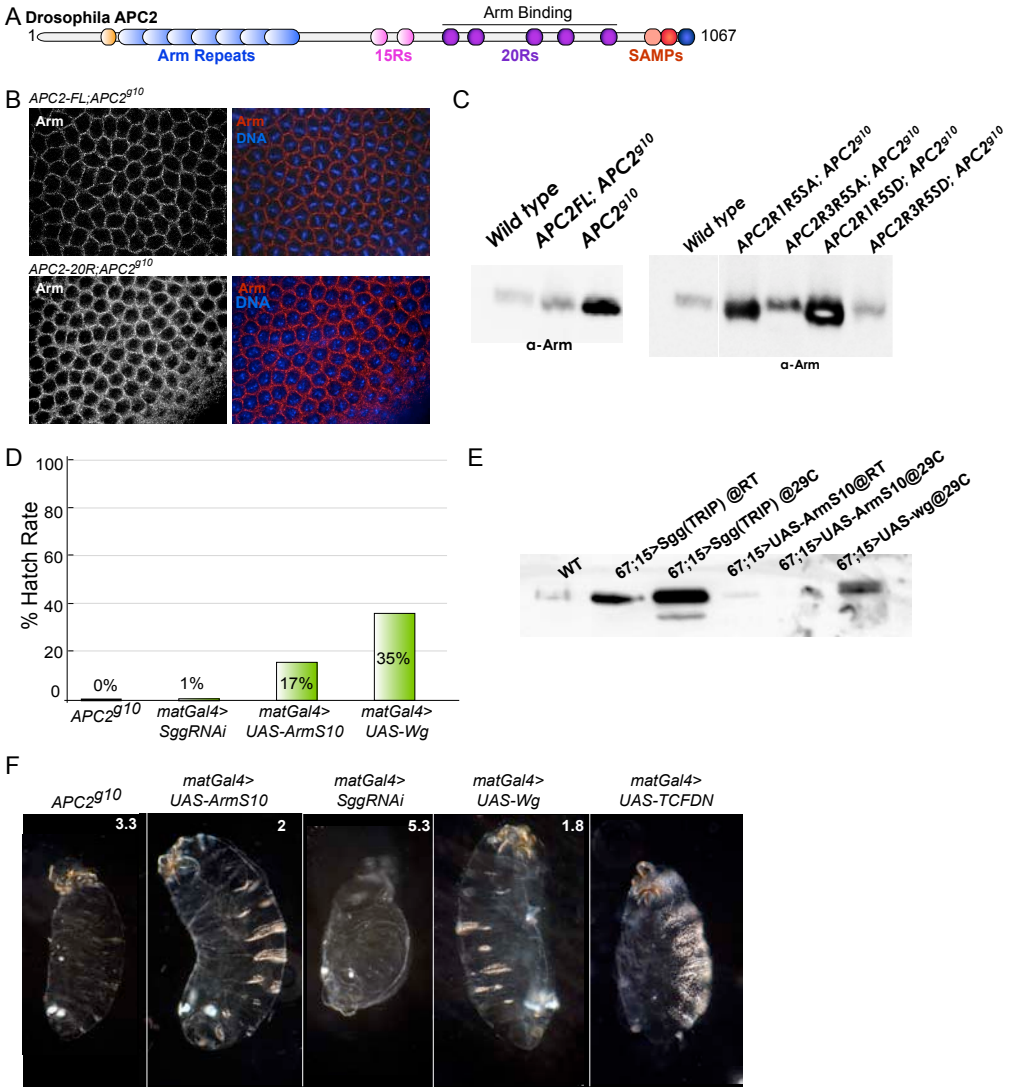


Figure 11: A) Schematic representation of dAPC2, highlighting the Arm binding domain (20Rs). B) Arm localization is not disrupted in APC2-Δ20R mutants. C) Arm protein levels are upregulated in APC2 mutants in the earl embryo. D,F) Manipulating Wnt signaling components in the early embryo by using a maternal driver (mat67;mat15) elicit Wnt signaling defects (hatch rate and cuticle) as well as cause upregulation of Arm protein levels (E).

We collected 0-2hr embryos and fixed and hand-peeled as previously described ((Webb et al., 2009)). We then stained with Alexa-phalloidin to reveal actin structures. DAPI and an anti-tubulin-E7 antibody was used to stage the embryos. We then acquired stacks of nuclear cycle 12, metaphase embryos to assess the depth of furrow extension as previously described (Webb et al., 2009). Based on these x-y images we calculate the percentage of complete and incomplete rings as a measure of the success of furrow extension.

Live imaging, FRAP and image analysis:

We collected 0-1 hr embryos, dechorionated and prepared as previously described. In order to study live actin cap dynamics, we used genetically encoded moe-GFP embryos or injected wild type or mutant embryos with rhodamine actin (Cytoskeleton) as previously described (Cao et al., 2010). Movies 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. Cap area was measured for 5 different caps during cycle 11 and cycle12 for 2 movies for each genotype.

FRAP experiments were carried out using a Zeiss LSM510 confocal microscope with ZEN software. After 5 pre-bleach scans, 10 bleaching scans with 100% intensity of 488nm over the region of interest were performed. This area was kept constant across wild type and APC2 mutant embryos. After photobleaching, the fluorescence recovery was monitored every 2 sec for 40 frames. The recoveries of the fluorescence intensities of each image series were quantified with Image J and halftimes and initial rates were calculated using Excel.

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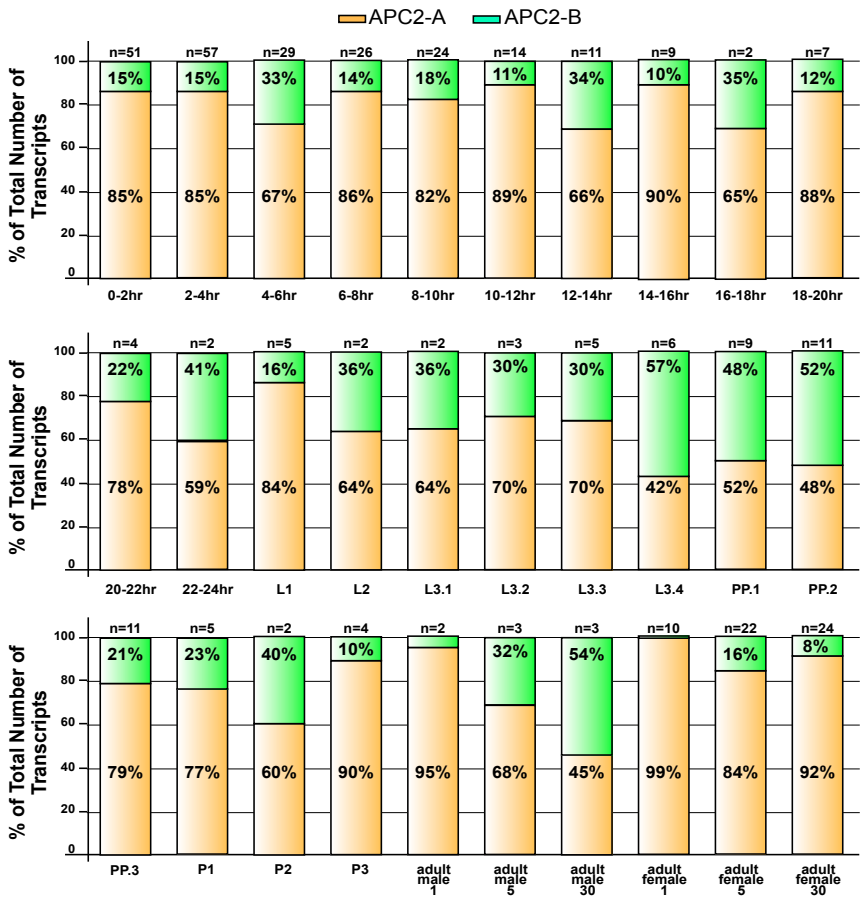
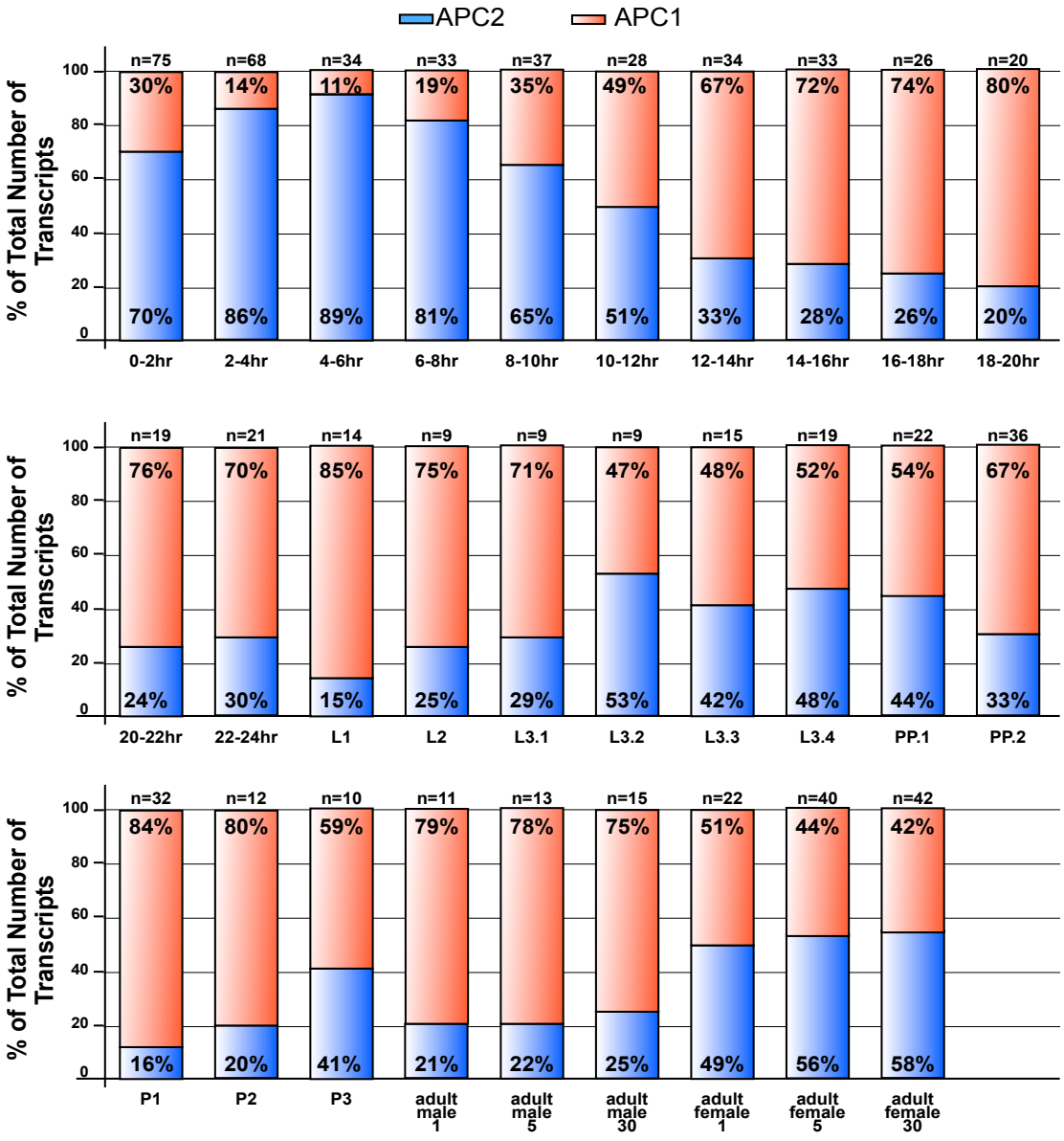
Appendix

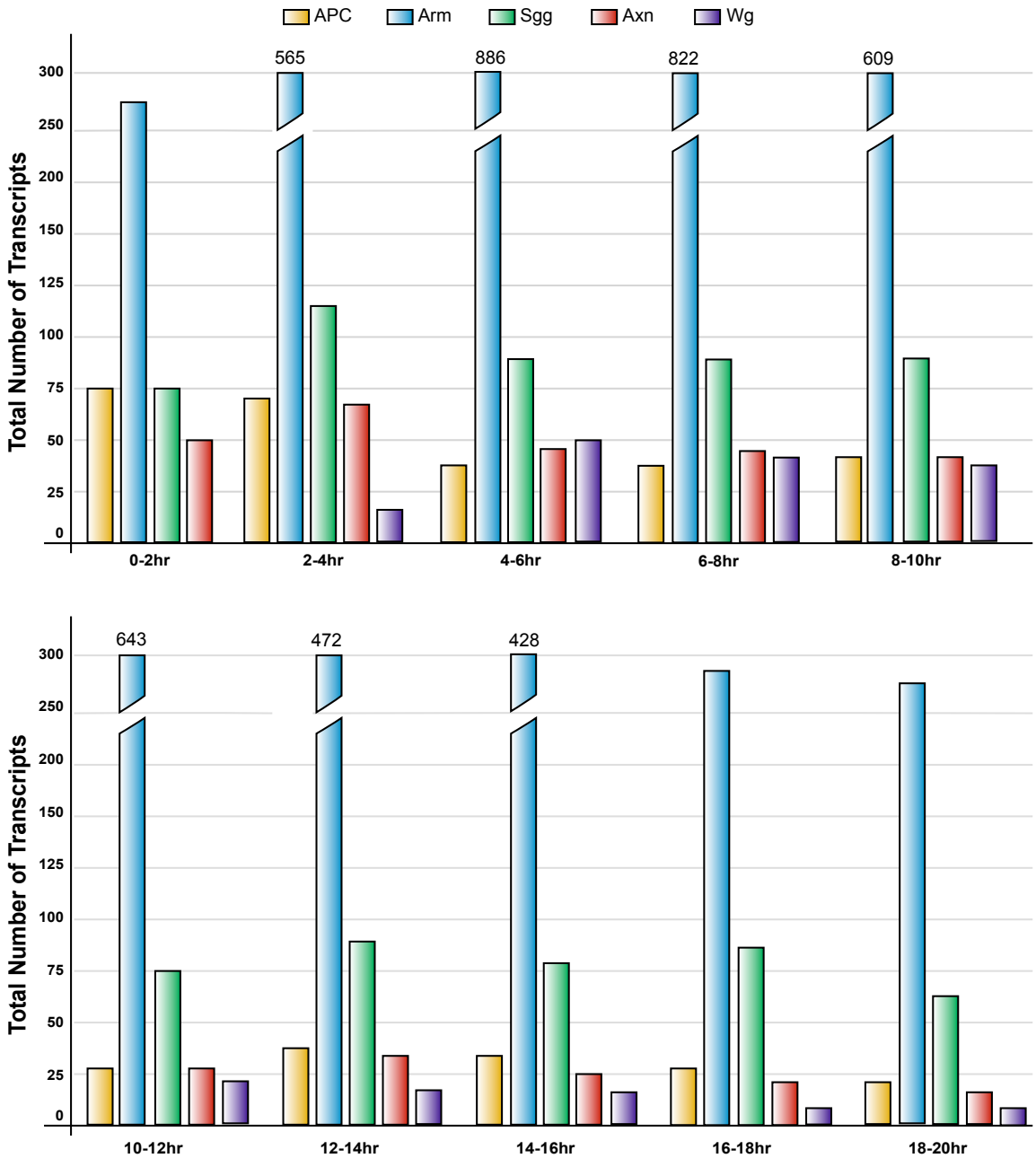
Appendix 1: Modencode- APC2A vs APC2-B Isoforms



Appendix 2: Modencode- APC2 vs APC1



Appendix 3: Modencode- Wnt components



Appendix 4: Early Embryo- Actin and MT

Actin Related					
Protein	**Function**	**TRIP**	**Mutant**	**Ab**	**Expected Phenotype in the Syncytial Embryo**
APC2	Microtubule and actin regulation	Yes	Yes	Yes	Defects in furrow formation, nuclear loss, spindle retention defect
Dia	Unbranched actin nucleator	Yes	Yes	Yes	Defects in furrow formation
Formin 3	Unbranched actin nucleator	Yes	No	No	Unknown
Arp3 (Arp66B)	Branched actin nucleator	Yes	Yes	Yes	No actin rings/furrows but actin caps
Arp5	Branched actin nucleator	Yes	No	No	No actin rings/furrows but actin caps
Arpc1 (Sop2)	Branched actin nucleator	Yes	Yes	Yes	No actin rings/furrows but actin caps
Scar	Arp2/3 activator/mediator	Yes	Yes		Defects in actin rings and furrow extension
Wsp	Arp2/3 activator/mediator	Yes	Yes		Defects in actin rings and furrow extension
Nuf	Endosome recycling pathway	Yes	No	Yes	Defects in actin rings and furrow formation
Rab11	Functions with Nuf and Dah	Yes	Yes	Yes	Defects in actin rings and furrow formation
Dah	Functions with Nuf and Dah	No	Yes	Yes	Defects in actin rings and furrow formation
Tsr (cofilin)	Actin filament diassembly	Yes	Yes		Defects in furrow formation
Scra (Annilin)	Actin binding protein/scaffolding protein	Yes	Yes	Yes	Cellularization defects
Pnut	Septin/actin bundling	Yes	Yes	Yes	Cellularization defects
Chic	Profilin/ actin polymerization	Yes	Yes	Yes	
RhoGEFII	GTP exchange factor	Yes	Yes	Yes	Defects in actin rings and cellularization
Rho1	Small GTPase	Yes	Yes	Yes	Cellularization defects
Swa	mRNA binding/dynein binding	Yes	Yes		Defects in actin rings and furrow formation
Spg	Unconventional RacGEF	Yes	Yes		Defects in actin caps, rings and furrow formation
Dal	Unknown	Yes	Yes	No	Defects in actin rings and furrow extension, spindle collisions
Sced	Actin org., involved in cell cycle	Yes	Yes	Yes	No actin rings/furrows but actin caps
Actin 79B	Actin related protein	Yes	No	No	Unknown
Moe	Actin binding protein	Yes	No	Yes	Unknown
Ena	Actin modulator/effector of Abl	Yes	Yes	Yes	Unknown
Abl	Non-receptor Tyr kinase/regulates Ena	Yes	Yes	Yes	Excess apical actin in rings, furrow defects
		Yes			
Asp	Microtubule binding/kinase activity	Yes	Yes		Spindle formation defects
Mars	Spindle stab./org.	Yes	?		Defects in spindle to centrosome attachment

Cnn	Spindle org./centrosome org.	Yes	Yes	Yes	Actin cap defects
EB1	+Tip MT binding protein	Yes	Yes	Yes	Furrow defects,
Jar	Unconventional myosin VI (-end directed motor)	Yes	Yes	Yes	Defects in furrow formation, spindle collisions
Centrosomal protein 190kD	Nuclear axial expansion/microtubule binding	Yes	No	No	Unknown
Cdc2	Cell cycle; G2/M transition of mitotic cell cycle	Yes	Yes	Yes	Cell cycle defects
Grp (Chk1)	Cell cycle arrest; mitotic cell cycle regulation of syncytial embryo; spindle assembly	Yes	Yes	Yes	Defects in furrows and nuclear, fail to cellularize, cell cycle defects
Cyclin T	Regulation of cell shape; cell cycle; actin filament org.	Yes	No	No	Unknown
Cdk5	Cell cycle, axonogenesis	Yes	No	Yes	Unknown
Cdc2c	Cell cycle; JAK-STAT cascade	Yes	No	No	Unknown
Arm	Cell adhesion; cell proliferation; cell fate specification	No	No	Yes	Nuclear Loss
APC1	Wnt pathway regulation, cell adhesion	Yes	No	No	Unknown
Axn	Wnt pathway regulation; stem cell maintenance	Yes	No	No	Nuclear Loss
Zw3 (sgg)	Regulation of Wnt pathway	Yes	No	Yes	Nuclear Loss
α-cat	Actin binding, cytoskeletal anchoring at plasma membrane	Yes	No	Yes	Unknown