LOOKING INSIDE THE WNT/BETA-CATENIN DESTRUCTION COMPLEX: MECHANISMS AND THE MINIMAL MACHINE

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Genetics and Molecular Biology.

Chapel Hill
2016

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ABSTRACT

Mira I. Pronobis: Looking inside the Wnt/beta-catenin destruction complex: mechanisms and the minimal machine
(Under the direction of Mark A. Peifer)

The Wnt/beta-catenin pathway is one of the most studied signaling pathways. It is essential throughout development, and its dysregulation is linked to various diseases including cancer. We have learned much about on how Wnt signaling is activated, but less is known about its downregulation. Wnt signals are transduced via effects on levels of the transcriptional co-activator β-catenin (βcat). This is achieved by the destruction complex which consists of Adenomatous polyposis coli (APC), the scaffold Axin, and the kinases GSK3 and CK1 which target βcat for ubiquitination and subsequent proteasomal degradation. APC and Axin are the key negative regulators of Wnt signaling. While Axin acts as the scaffold of the destruction complex, APC's role remained unknown. In our work we first explored APC's βcat binding sites and their role in regulation of Wnt signaling. We found that the βcat binding sites act additively in the sequestration of βcat. Next we explored APC's mechanistic function in the destruction complex. We found that the destruction complex is not a static entity but a dynamic structure in which assembly and conformational change drive βcat degradation. My work revealed the internal structure of the APC:Axin complex, in which Axin forms strands and sheets, while APC stimulates Axin multimerization. Based on my data, we concluded that APC plays two roles inside the destruction complex: (1) APC promotes efficient Axin multimerization through one

known and one novel APC:Axin interaction site, and (2) APC promotes turnover of βcat to the E3-ligase by a GSK3-regulated mechanism. In my third project we investigated the interplay of APC and Axin and their functional relationship. Both APC and Axin are essential at endogenous levels. However, in APC deficient cell lines overexpression of Axin compensates for mutation of APC and reduces βcat, suggesting APC and Axin act redundantly to facilitate βcat destruction. Based on my data I found that (1) several combinations of non-functional APC and Axin mutants can complement one another in stimulating βcat degradation, suggesting that the APC:Axin complex is a robust machine, (2) that a total of 5 regions in APC and Axin are essential for a functional destruction complex, and (3) that these 5 essential regions can reconstitute the wildtype APC:Axin complex in features and functions, thus representing the minimal βcat destruction machine.

ACKNOWLEDGEMENTS

First I want to thank Mark Peifer for being a brilliant mentor.
He made me a better scientist.

A big thanks go to the people in the Peifer lab
and all the other labs at UNC that I worked with!

Then I would like to thank my committee:
Bob, Vicki, Ben and Steve,
who always put me on the right path when I drifted away

To my non-scientific community:

First I like to acknowledge my two dogs
Mike and Nala,
who reminded me to relax, and taught me how to live every moment

My friends
who helped to balance my private life with my work life:
Josi, Sophie and Josh

My parents and my brother, to
whom I am forever grateful for believing in me
and for all the support you gave me.

Thanks to all of you for making this possible!!

PREFACE

Chapter 2 describes my early work on APC. This second author paper was published in Molecular Biology of the Cell in 2011 (Molecular biology of the cell 22.11 (2011): 1845-1863). Here we investigated the role of APC's βcat binding sites using mammalian tissue culture cells and the fruit fly. Dave Roberts conducted the fly experiments and did the cloning of various constructs. John Poulton, Jon Waldmann, Elise Stephenson and Shahnaz Hanna contributed to the fly experiments. This work was completed under the direction of Mark Peifer.

My contribution to the work was the mammalian tissue culture experiments. I measured the βcat levels and Wnt signaling activity, and I generated different APC mutants. Additionally, I found that colocalization of APC to Axin is negatively regulated by 20R2 and region B.

Chapter 3 describes the core of my research during my PhD. This first author paper was published in eLIFE in 2015 (eLife 4 (2015): e08022). Here we investigated the mechanistic role of APC in the destruction complex. We used mammalian tissue culture cells and the fruit fly. Nasser Rusan acquired a subset of the high resolution SIM images, and Mark Peifer analyzed the SIM images and measured the volume. This work was completed under the direction of Mark Peifer.

I conducted all the experiments in the tissue culture system and in the fly. I acquired the FRAP data and did the biochemical experiments. I also acquired some high resolution SIM images and I assisted with analyzing the SIM image volume measurements.

Chapter 4 describes another project that I started during my PhD. This first author paper is not published yet, but it will be submitted this summer. Here we investigated the cooperative function of APC and Axin in βcat destruction. For this we used mammalian tissue culture cells. Natalie Deuitch conducted the reporter gene assays with help from Vinya Posham. Natalie Deuitch also helped with βcat immunostaining and analysis, and with the cloning of different constructs. This work was completed under the direction of Mark Peifer.

I conducted all biochemical experiments and acquired the SIM high resolution images and the FRAP data. I also analyzed the FRAP data and the SIM images. Further I generated different APC and Axin mutants, and did the βcat Immunostaining and analysis. I also helped with the reporter gene assays.

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APC	Adenomatous polyposis coli
βcat	beta-catenin, βcatenin
CK1	Casein kinase 1
Frzd	Frizzled
GSK3	Glycogen synthase kinase 3
Lef	Lymphoid enhancer factor
LRP 5/6	Lipoprotein related receptor
Tcf	T-cell factor

CHAPTER 1: INTRODUCTION

Cell signaling pathways in development and disease

Every cell in our body has to communicate with the outside environment. The fundamental process in single and multicellular organisms involves receiving, processing and responding to outside cues. For this cells developed signaling pathways. The text book core principles of cell signaling involves 3 components: (1) A membrane or cytoplasmic receptor, which receives outside cues and transmits information into the cytoplasm, (2) a set of transducing components, which interacts with the intracellular part of the membrane receptor and transduces the signal into to the cell nucleus, and (3) a transcriptional regulator, which triggers the cell's response by activating or repressing transcription of target genes.

To this day the number of signaling pathways that are discovered is still increasing; but a subset of these pathways are fundamental in development and disease (Cooper et. al., 2000, Pires-daSilva and Sommer, 2003). The canonical Wnt/β-catenin pathway, Hedgehog pathway, retinoic acid signaling, the FGF pathway, Notch-Delta signaling, and the TGF-β pathway regulate cell proliferation, stem cell pluripotency and cell fate choices during development. They play major roles in axis determination, gastrulation, neural tube formation and other fundamental processes. Interestingly, the same pathways that are essential in development are also the ones that have severe consequences in adulthood when dysregulated (Vogelstein et al., 2013).

Human cancer is the leading cause of death worldwide (WHO, 2012). Genomic analyses of different types of cancer revealed that approximately 140 genes are considered to promote

tumorigenesis, and that mutations in only two to eight of these promoting genes are sufficient to drive tumorigenesis in a given cell type (Vogelstein et al., 2013). Interestingly, these 140 genes affect only 12 signaling pathways suggesting that dysregulation of these core cellular pathways is the driving force leading to cancer development. These signaling pathways can be divided into those driving cell survival, genome maintenance, and cell fate, such that when altered they allow cells a selective growth advantage. Interestingly, in some signaling pathways one core component is the major target of mutation.

The Wnt signals and cancer

The canonical Wnt signaling pathway is one of these 12 powerful oncogenic pathways. In the early 1980s two independent studies found the first member of the Wnt pathway: The gene Wnt1, the first known ligand of the canonical Wnt receptors, was discovered by studying the Mouse Mammary Tumor Virus, which randomly integrates in the mouse genome (Nusse and Varmus, 1982). The first identified integration site that led to the development of mammary tumors was named Integration site-1 (Int-1). After sequencing Int1, its *Drosophila* homolog gene *wingless* was identified. Regulatory mutations in the fly gene generated flies without wings (wingless), while null alleles led to embryonic lethality with defects in embryonic cell fates (Nusslein-Volhard and Wieschaus, 1980). Thus, the pathway was named Wingless-int pathway or Wnt pathway. In 1989, axis duplication studies in Xenopus embryos confirmed the conservation of the canonical Wnt pathway among animals (McMahon and Moon, 1989), and through work in several model organisms its major components were very quickly identified.

Today Wnt signaling remains one of the most studied pathways. This is not only because of its essential function throughout development, but more its important role in tumorigenesis.

Negative regulators of Wnt signaling are frequently mutated in various forms of cancer like colorectal cancer, hepatocellular carcinomas, ovarian cancer, and adenocarcinoma (Vogelstein et al., 2013). In some cancers dysregulation of Wnt signals is the actual primary cause of tumorigenesis, and in others dysregulation occurs later and contributes greatly to tumor development and transition into a metastatic state.

One negative regulator of Wnt signaling, Adenomatous polyposis coli (APC), has been found to be the major cause of intestinal adenomas when mutated. In fact, 85% of sporadic colorectal tumors are caused by APC mutation (Hisamuddin and Yang, 2004). Others are caused by mutations in the DNA Mismatch Repair System, but interestingly they acquire subsequent mutations in genes that are negative regulators of the Wnt pathway; thus dysregulation of Wnt seems to be key in the occurrence of intestinal cancers (Hisamuddin and Yang, 2004). The role of Wnt in the maintenance of the intestinal stem cell niche is very likely an explanation for its leading role in colorectal cancer, and also one reason for its high frequency of dysregulation in other forms of cancer (Nusse, 2008).

The Wnt signaling pathway

The Wnt signaling pathway follows the core principal of signaling pathways. Reception of ligands in the outside environment is achieved by the Wnt receptor complex, which consists of Lipoprotein receptor-related protein 5/6 (LRP 5/6) and Frizzled (Frzd) (Figure 1.1, Roberts et al.,2007, Tamai et al., 2000; Wehrli et al., 2000). The key transducer of Wnt signaling is the adhesion protein βcatenin (βcat). Although βcat has a role in adherens junctions, it acts as a transcriptional co-activator in the Wnt pathway (Pronobis and Peifer, 2012). When present at high levels, it shuttles from the cytoplasm into the nucleus and acts as a co-activator of Wnt

target genes, thus βcat plays the role of transducer and activator in the Wnt pathway. When Wnt signal is inactive βcat levels are tightly regulated by a protein complex called the "destruction complex". This complex has multiple components which vary among animals. However, there are 4 core players that are found among all animals (Figure 1.1): APC, the scaffold protein Axin and two kinases, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) (Clevers and Nusse, 2012; Nakamura et al., 1998). APC and Axin recruit βcat into the destruction complex and the kinases phosphorylate specific residues in βcat, creating a binding site for the F-box protein of the $SCF^{betaTrCP}$ E3-ligase complex (Kitagawa et al., 1999). Once bound to the E3-ligase βcat gets ubiquitinated and is subsequently transferred to the proteasome for degradation. Thus the destruction complex regulates the intracellular levels of βcat by priming it for destruction.

Wnt signaling is activated when a Wnt ligand binds to the seven transmembrane receptor Frzd inducing clustering of Frzd and LRP 5/6 (Figure 1.1, Clevers and Nusse, 2012; Fiedler et al., 2011; Hsu et al., 1999). This in turn activates Wnt positive regulators such as Dishevelled (Dsh), and Protein Phosphatase 1 or 2A (PP1/PP2A) and others, and leads to inhibition of the destruction complex (Fiedler et al., 2011; Hsu et al., 1999). Thus βcat is no longer primed for proteasomal degradation and its levels rise. Then it transfers into the nucleus where it binds to transcription factors of the T-cell factor/ Lymphoid enhancer factor (Tcf/Lef) family, hereby replacing the repressor Groucho (Cavallo et al., 1998). Although substantial research has been done on the canonical Wnt pathway there are still many mechanisms and functions that remain mysterious.

Turning the destruction complex off

There are 6 major mechanisms that have been proposed to allow Wnt signals to inhibit the function of the destruction complex allowing βcat to accumulate: (1) Axin sequestration via LRP 5/6, (2) Axin-Dvl hetero-polymerization, (3) alterations in the intramolecular conformation of Axin, (4) changes in Axin levels, (5) GSK3 inhibition, and (6) inhibition of βcat transfer to the E3-ligase (Cselenyi et al., 2008; Fiedler et al., 2011; Huang et al., 2009; Li et al., 2012; Luo et al., 2005). I will now discuss these one by one.

(1) Upon Wnt ligand binding, the LRP 5/6 cytoplasmic C-terminus gets phosphorylated, which creates a binding site for Axin (Cselenyi et al., 2008). Axin is a key player and has been proposed to be the limiting factor of the destruction complex. Thus binding to LRP sequesters Axin and inhibits the degradation of βcat (Itoh et al., 1998).

(2) Axin forms the scaffold of the destruction complex by self-polymerization via its DIX domain (Figure 1.2, Fiedler et al., 2011). The Wnt positive regulator Dvl contains also a DIX domain and has been shown to form homo-polymers (Kishida et al., 1999). Axin and Dvl can also hetero-polymerize via their DIX domains and the formation of hetero-polymers interferes with the dynamic properties of Axin molecules in the destruction complex. While Axin dynamics are usually slow in the complex co-expression of Dvl accelerated the dynamics of the scaffold Axin weakening the βcat platform in its stability. Dvl is “activated” upon Wnt ligand binding and could therefore inhibit βcat degradation by interfering with Axin self-polymerization (Schwarz-Romond et al., 2007).

(3) Another mechanism proposed to interfere with Axin’s ability to self-polymerize was recently discovered. Axin has the ability to form intramolecular loops, restricting the availability

of the DIX domain and thus inhibiting formation of polymers (Luo et al., 2005). This closed conformation is regulated by GSK3. In the presence of GSK3 Axin is in an “open” conformation allowing it to serve as the scaffold of the destruction complex. However with Wnt signaling activation, Axin is dephosphorylated by a phosphatase and this transitions Axin into its closed conformation in which its DIX domain is unavailable to self-polymerize. Thus the destruction complex would lose its scaffolding function and βcat would no longer be targeted for degradation.

(4) Axin is proposed to be the limiting factor in the destruction complex due to its low protein levels (Salic et al. 2000). Thus degradation of Axin has been suggested as another mechanism for regulation of destruction complex function (Huang et al., 2009). In this study long term treatment with a Wnt ligand reduced Axin levels via Tankyrase-mediated degradation. However, it remains unclear whether Axin degradation occurs only in tissues treated over many hours with Wnt or whether it occurs also in tissues where Wnt signaling is only activated for a short time.

(5) Phosphorylation of βcat by GSK3 is a key step in the degradation of βcat. Although GSK3 has multiple targets besides βcat, and therefore is highly expressed in cells, a local decrease of GSK3 activity has been shown in experiments and mathematical modeling to be sufficient to interfere with the constitutive degradation of βcat (Cselenyi et al., 2008; Hernandez et al., 2012). GSK3 activity or availability has been suggested to be downregulated by Dvl, by sequestration in multi-vesicular bodies, or by the cytoplasmic tail of LRP 5/6, or by other kinases (Cliffe et al., 2003; Cselenyi et al., 2008; Taelman et al., 2010). Thus targeting the key kinase GSK3 in its ability to phosphorylate βcat is another mechanism to interfere with

destruction complex function.

(6) The last mechanism proposed to shut down the destruction of βcat targets the hand-over of βcat from the destruction complex to the E3-ligase (Li et al., 2012). This study showed that the destruction complex does not get disassembled (via Dvl or intramolecular "closed" conformation), and also revealed no difference in the phosphorylation activity of GSK3, instead it suggested that βcat transfer to the E3-ligase is reduced and this drives the accumulation of βcat in the cytoplasm.

All these proposed mechanisms affect key steps of βcat destruction, however it remains unclear which step is the most important one, in which order they occur, or whether they are spatially and temporally separate from one another. Nevertheless all these steps could interfere with the function of the destruction complex to allow βcat to transduce Wnt signals into the nucleus and activate Wnt target genes.

Facilitating βcat destruction

Degradation of βcat involves 4 steps: (1) Binding and recruitment into the destruction complex, (2) phosphorylation of βcat by CK1 and GSK3, (3) ubiquitination of βcat by the $SCF^{betaTrCP}$ E3-ligase, and (4) βcat destruction by the proteasome. The recruitment of βcat into the complex is facilitated by APC and Axin, which both have βcat binding sites (Behrens et al., 1998). However, it remains unknown whether there is a specific order or role for APC's or Axin's βcat sites or whether they act redundantly. Once in the complex, βcat gets first phosphorylated by CK1 (Liu et al., 2002). Hereby, the Serine at positon 45 (S45) on βcat is the first residue that undergoes phosphorylation. Once βcat is primed by CK1, GSK3 phosphorylates S41, S37 and subsequently S33. This dual-kinase mechanism is key in preparing βcat for ubiquitination.

Phosphorylation at residues S33 and S37 create a binding site for the F-box protein beta-transducin repeat containing protein (bTrCP), which recognizes phosphorylated βcat (Sadot et al., 2002). It still remains unclear how βcat is transferred to the E3-ligase and whether any core component of the destruction complex, APC or Axin assists or even facilitates the transfer. Further the transfer of ubiquitinated βcat to the proteasome remains largely mysterious.

While the major role of the kinases inside the destruction complex is the phosphorylation of βcat, studies suggest that they also regulate and impact the interaction of APC and Axin with each other and with βcat (Seo and Jho, 2007; Yamamoto et al., 1999). Thus phosphorylation could be used to regulate the activity of the destruction complex and thus of βcat turnover.

Axin and its role in the βcat destruction complex and disease

Since Axin has the ability to self-polymerize via it's DIX domain it has been suggested that it is the scaffold of the destruction complex (Figure 1.2, Kishida et al., 1999). However, Axin does much more than that. It is not only a scaffold, but also acts as a hub by recruiting all the components necessary to facilitate βcat destruction. It has binding sites for APC, GSK3, βcat, CK1 and other Axin proteins (Behrens et al., 1998). Thus studies have shown that overexpressing Axin in an APC-mutant cell line can reduce βcat levels (Nakamura et al., 1998), by stimulating phosphorylation of βcat by both kinases (Ikeda et al., 2000). Its ability to bind GSK3, CK1 and βcat, and bringing them into close proximity to each other might likely be the reason why Axin stimulates βcat phosphorylation.

From an evolutionary point of view Axin is found in the simplest multicellular animals which have all Wnt signaling (Srivastava et al., 2008). Thus the Axin protein itself and its role in

the destruction complex to serve as a hub is highly conserved throughout animals. The evolutionary conservation of Axin and the role of Wnt signaling in disease is also the reason why Axin is a common target in a wide variety of cancers. Mutations in Axin occur throughout the gene with no apparent hot spot for mutations (Salahshor and Woodgett, 2005). Studies have found mutations in the APC binding site, the regulator of G-protein signaling (RGS) domain, in the βcat or GSK3 binding site of Axin, all in the same type of cancers, suggesting that no cancer-type specificity exists. Instead all Axin mutations appear to dysregulate βcat destruction and activate Wnt signaling (Salahshor and Woodgett, 2005). Additionally, since no types of tumors are known that are caused and initiated by Axin mutations, mutations in Axin are very likely late occuring and are acquired during tumorigenesis (Salahshor and Woodgett, 2005). Thus initial loss of Axin function may be too strong of an alteration in βcat levels since high levels of βcat have been associated with apoptosis (Albuquerque et al., 2002). Therefore only if the cell is already in a tumor-like state can Axin mutations drive tumorigenesis.

APC and its role in the destruction complex

APC was first defined as the scaffold of the destruction complex, but since the discovery of Axin its role in the destruction complex remained largely speculative. There are three major models that have been proposed for the function of APC in the destruction complex. (1) Since APC has multiple βcat binding sites that display different affinities, all of which are increased upon phosphorylation, one model suggested a competition between APC and Axin in βcat binding (Liu et al., 2006). In this model βcat is recruited by Axin, but phosphorylation of APC's strongest βcat binding site outcompetes Axin for βcat. APC bound βcat is then released to the E3-ligase. However, this model appears questionable for the following reasons: since Axin has

only one βcat binding site while APC proteins have up to 10 βcat binding sites it appears more likely that APC is sweeping the cytoplasm and recruiting βcat into the destruction complex rather than Axin. Additionally Axin's role as a scaffold in which Axin's dynamic in the destruction complex are slow, would also mitigate against the model.

(2) The second model suggests that APC's role in βcat destruction is indeed the recruitment of βcat into the destruction complex, and that the different affinities of its βcat binding sites reflect the need for recruitment when different levels of βcat are present in the cytoplasm (Ha et al., 2004). When βcat levels are low APC needs phosphorylation of its highest affinity binding sites to sequester the few βcat molecules in the cytoplasm, while after Wnt activation when βcat levels are high APC could use all its βcat binding sites to capture and sequester βcat. Thus this model places APC's major role in the recruitment of βcat into the destruction complex.

(3) The most recent model suggests that APC protects βcat from dephosphorylation by PP1/PP2A (Su et al., 2008). In this model βcat binding to APC, but interestingly not Axin, interferes with dephosphorylation. Thus βcat could then be safely handed-over to the E3-ligase for ubiquitination. However, this model contradicts the finding that some APC mutant cell lines exhibit high levels of phosphorylated βcat (Sadot et al., 2002). Thus, although it may be one of APC's functions to act as a protector for phosphorylated βcat it is very unlikely that this is its major function. Taken together, although a lot of research has been done no study has addressed the mechanistic function of APC in the Axin complex.

APC and colorectal cancer

The *APC* gene was initially discovered in Familial Adenomatous Polyposis (FAP) patients,

who are prone to develop colorectal cancer (Kinzler et al., 1991; Soravia et al., 1998). These patients carry a mutation in one of the APC alleles. Although the mechanism remains unclear, patients develop overtime a second deletetion in the other APC allele in a subset of colorectal cells. Since APC is a tumor suppressor losing both copies is necessary to initiate tumor progression. Loss of APC function leads to formation of colon polyps, which are the initial stage of colorectal cancer progression (Dekker and van Gulik, 2005). Thus different than Axin, APC mutations are not only late occuring mutations, but instead can start tumorigenesis.

Nonsense or frameshift mutations in the APC gene, which truncate the protein, are most frequently detected in colon tumors, while full gene deletions are rarely seen (Kohler et al., 2008). Interestingly, the most frequent truncating mutations that are found in APC are located in a hot spot region in the APC gene where they preserve the N-terminus with a few βcat binding sites, but delete the Axin binding sites (Figure 1.2, Kohler et al., 2008). The partial preservation of the N-terminus of APC could be explained by the 'just right signaling' hypothesis in which the preserved βcat binding sites sequester βcat and keep its levels high to activate Wnt signaling but not too high to induce apoptosis (Albuquerque et al., 2002). Thus rapid proliferation is one consequence of APC mutation and this in turn enables cells to acquire additional mutations which benefit tumorigenesis further.

The hot spot region of mutations in APC is called the Mutation Cluster Region (MCR). It includes two highly conserved regions of unknown function, 20 amino acid R2 (20aaR2) and region B, along with 20aa R3 the strongest βcat binding site (Kohler et al., 2008). Since the Axin binding sites are at the C-terminus, truncations in APC in the MCR would suggest that binding to Axin is critical for APC's function and βcat degradation. Another reason for the hot spot could

be the disruption of the highly conserved regions 20R2 and region B, which might be involved in APC's key function. In this regard it remains very curious that mutations in the two APC alleles do not occur randomly, but are interdependent. For instance if the first mutation truncates 20R2 or B, the second allele is very likely to preserve 20R2 and B, and vice versa (Kohler et al., 2009). Thus there seems to be some functional reason for this occurrence which can only be explained by defining the function of both regions. Interestingly, 20R2 and B are critical for APC's ability to target βcat for degradation, since APC mutants lacking 20R2 and region B cannot destroy βcat, while mutants that preserve both regions can still reduce βcat levels (Roberts et al., 2011).

Remaining questions in the regulation of Wnt signaling

In this work we addressed several questions about how cells maintain low levels of βcat in the absence of Wnt signaling. APC and Axin are the core components of the destruction complex. Without either one βcat levels remain high and Wnt signals are constitutively active. Thus we need to understand APC's and Axin's explicit functions in the destruction complex as well as how they function cooperatively together.

First we focused on APC's role in the destruction complex: In Chapter 2 we tested the competition model as well as the βcat recruitment model by addressing the function of different APC mutants that lack βcat binding sites in both mammalian cell culture and in the fruit fly. Based on our data we proposed a new model on the function of APC's βcat binding sites in down regulation of Wnt signaling and which helped to explain the occurrence of specific mutations found among colorectal cancer patients. These data also focused our attention on 20R2/B.

In our second study described in Chapter 3, we deepen our understanding of APC's function further. One former study proposed that APC protects βcat in the destruction complex from dephosphorylation. However, many colon cancer cells with mutated APC have high levels of phosphorylated βcat suggesting that shielding βcat from dephosphorylation cannot be APC's major function. In Chapter 3 we focused on APC's mechanic role inside the destruction complex, its impact on the scaffold Axin, and the role of its essential regions, 20R2/B and their function.

Lastly, we investigated the cooperative function of APC and Axin in the destruction complex in Chapter 4. Since overexpression of Axin can rescue βcat levels in APC mutant cell lines, we investigated the redundancy of regions in APC and Axin. Further, we wanted to identify what regions are truly required for a functional destruction complex to design the minimal βcat destruction machine.

Figure 1.1

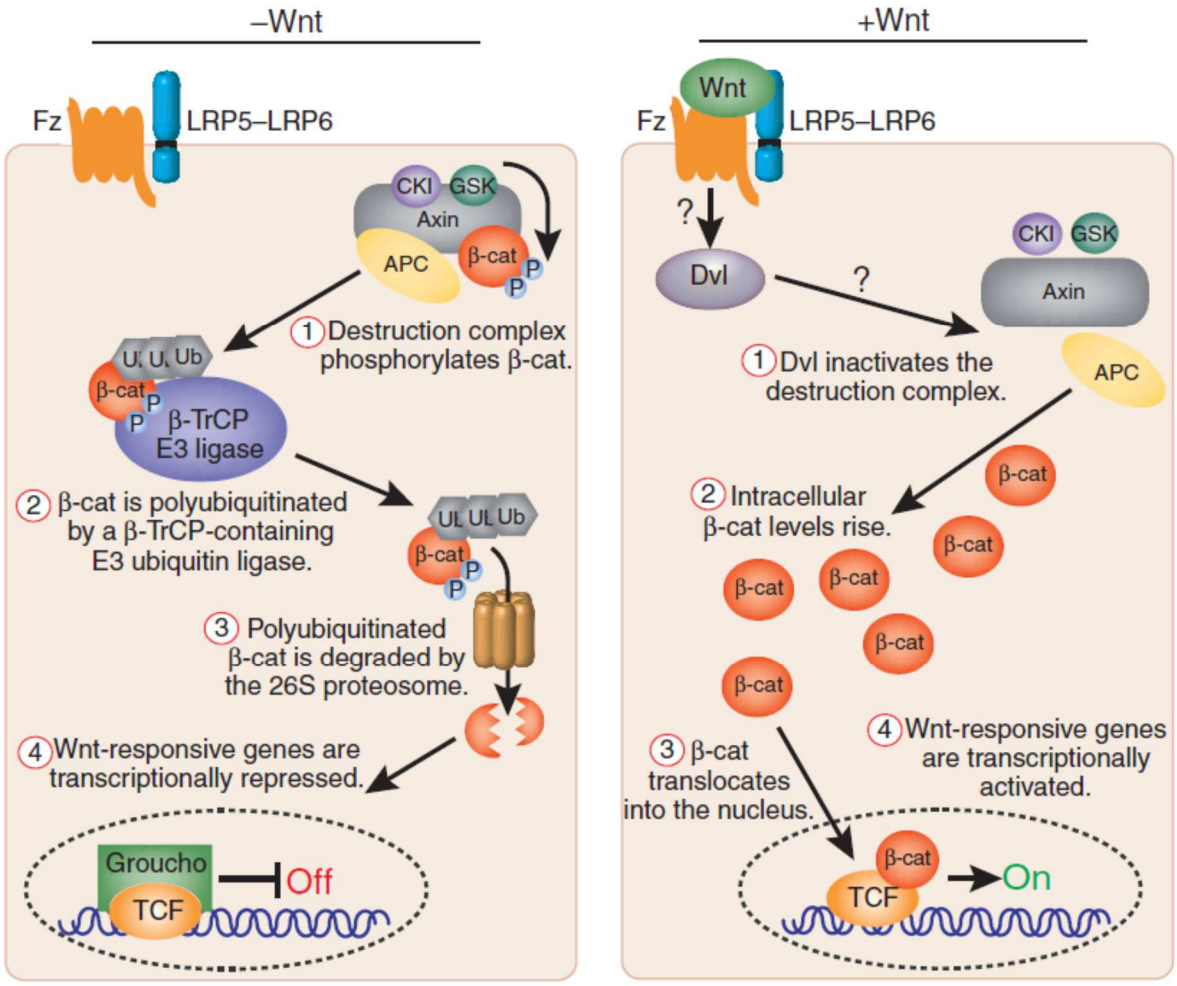

Figure 1.1: The Wnt signaling pathway. Left: Wnt off state: βcat is constantly targeted for degradation. (1)Phosphorylation of βcat by the destruction complex initiates transfer to the E3-Ligase complex, (2)which polyubiquitinates βcat and turns it over to the proteasome for destruction. (4) Groucho, a transcriptional repressor blocks activation of Wnt target genes by binding to Tcf in the absence of βcat.
Right: Wnt on state: (1) The function of the destruction complex is inhibited by a many different proposed mechanisms. (2) βcat levels rise and (3) it translocates into the nucleus. (4) βcat binds to Tcf and activates Wnt target genes.(Roberts et al. 2007)

Figure 1.2

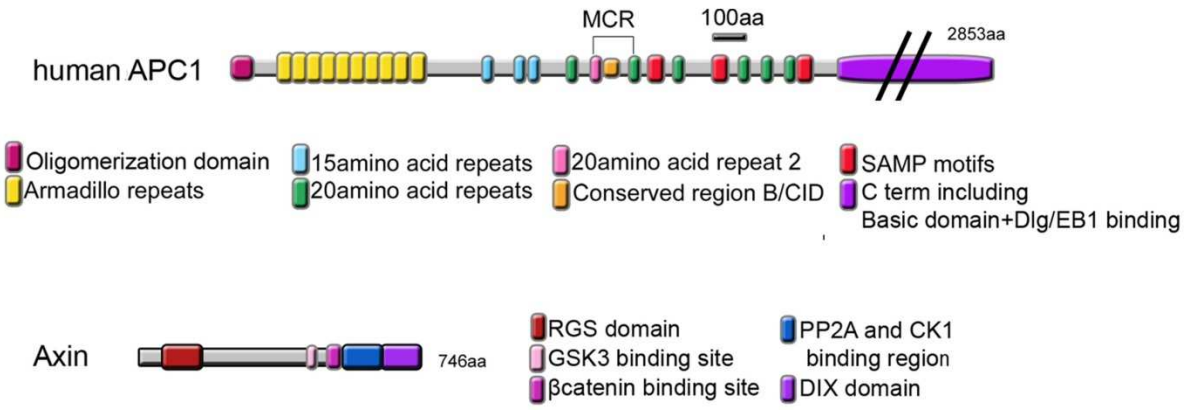

Figure 1.2. Schematic representation of human APC and Axin proteins. APC1 protein. Regions are indicated. MCR=Mutational cluster region, the hot spot of APC truncations. Bottom: Axin protein. Regions are indicated

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CHAPTER 2: DECONSTRUCTING THE BETA-CATENIN DESTRUCTION COMPLEX: MECHANISTIC ROLES FOR THE TUMOR SUPPRESSOR APC IN REGULATING WNT SIGNALING[1]

Overview

Negatively regulating signaling by targeting key effectors for ubiquitination/destruction is essential for development and oncogenesis. The tumor suppressor adenomatous polyposis coli (APC), an essential negative regulator of Wnt signaling, provides a paradigm. *APC* mutations occur in most colon cancers. Acting in the "destruction complex" with Axin, glycogen synthase kinase 3, and casein kinase, APC targets βcatenin (βcat) for phosphorylation and recognition by an E3 ubiquitin-ligase. Despite 20 years of work, the internal workings of the destruction complex and APC's role remain largely mysterious. We use both *Drosophila* and colon cancer cells to test hypotheses for APC's mechanism of action. Our data are inconsistent with current models suggesting that high-affinity βcat-binding sites on APC play key roles. Instead, they suggest that multiple βcat-binding sites act additively to fine-tune signaling via cytoplasmic

[1] This chapter previously appeared as an article in the Journal of Molecular biology of the cell. The original citation is as follows: David M. Roberts, Mira I. Pronobis , John S. Poulton, Jon D. Waldmann, Elise M. Stephenson, Shahnaz Hanna, and Mark Peifer. "Deconstructing the beta-catenin destruction complex: meachnistic roles for the tumor suppressor APC in regulating Wnt signaling, Molecular biology of the cell 22.11 (2011): 1845-1863

retention. We identify essential roles for two putative binding sites for new partners—20-amino-acid repeat 2 and conserved sequence B—in destruction complex action. Finally, we demonstrate that APC interacts with Axin by two different modes and provide evidence that conserved sequence B helps ensure release of APC from Axin, with disassembly critical in regulating βcat levels. Using these data, we suggest a new model for destruction complex action in development, which also provides new insights into functions of truncated APC proteins in cancer.

Introduction

Tumor suppressors that negatively regulate proliferation and other properties of cancer cells play key roles in oncogenesis, emphasizing the built-in negative regulation that keeps powerful signaling pathways in check. One common mechanism of negatively regulating cell signaling is via ubiquitin-mediated destruction. E3 ligases target key components of many critical signaling pathways, including the receptor tyrosine kinase, transforming growth factor-β, Hedgehog, nuclear factor NFκB, and Wnt pathways. In fact, the last three pathways use the same SCF class E3 ligase, with the F-Box protein Slimb/βTrCP as the recognition subunit (Maniatis, 1999 blue right-pointing triangle). In each, a distinct multiprotein complex targets the effector for phosphorylation, triggering recognition by Slimb/βTrCP. This outline provided significant insight into these developmentally critical pathways that also contribute to oncogenesis. The complexity of the multiprotein complexes targeting signaling effectors for destruction, however, poses a challenge for scientists trying to understand mechanisms of signal transduction.

Wnt signaling provides a paradigm illustrating these issues. The tumor suppressor adenomatous polyposis coli (APC; Kinzler et al., 1991 blue right-pointing triangle), mutated in most cases of colon cancer, is a key negative regulator of Wnt signaling. It is part of a protein complex that, in the absence of Wnt signals, targets βcatenin (βcat) for phosphorylation, and ultimately ubiquitination and proteasomal destruction (Cadigan and Peifer, 2009 blue right-pointing triangle). In the presence of Wnt signal, or in tumors lacking APC, βcat levels rise, it enters the nucleus, binds T-cell factor (TCF)-family DNA binding proteins, and thus activates Wnt-responsive target genes. This outline of signaling was a major advance, but despite intense interest for more than 20 years in Wnt signaling and APC function in normal development and cancer, the mechanisms by which the destruction complex acts and the mechanistic role that APC plays in regulating βcat stability remain unclear.

Several hypotheses for APC function have been suggested. Some focus on destruction complex–independent roles of APC in nuclear trafficking of βcat or action at promoters of Wnt target genes (Rubinfeld et al., 1996 blue right-pointing triangle; Bienz, 2002 blue right-pointing triangle; Sierra et al., 2006 blue right-pointing triangle). Most studies agree, however, that APC's dominant role is to target βcat for destruction as a part of the destruction complex. Because APC binds βcat and coimmunoprecipitates (co-IPs) with glycogen synthase kinase 3 (GSK3) (Rubinfeld et al., 1993 blue right-pointing triangle), it was initially hypothesized that APC is a scaffold, templating βcat phosphorylation. APC's structure is consistent with this hypothesis, as APC family members are large, complex proteins with multiple protein interaction motifs (Figure 2.1A; McCartney and Nathke, 2008 blue right-pointing triangle). APC's N-terminal third includes a block of Armadillo (Arm) repeats that bind multiple partners, with

the full repertoire still to be defined. The middle third of APC carries a series of short binding sites for proteins involved in Wnt regulation, including 15- and 20-amino-acid repeats (15Rs and 20Rs) that bind βcat, and SAMP repeats that bind Axin. It also contains the short conserved sequence B (McCartney et al., 1999 blue right-pointing triangle; called sequence B later in the text; also known as the Catenin Inhibitory Domain [CID]; Kohler et al., 2009 blue right-pointing triangle), which we suspect binds an additional unknown partner. The C-terminal third of APC includes binding sites for cytoskeletal proteins (Nathke, 2004 blue right-pointing triangle), but this region is not essential for Wnt regulation, as a truncated mutant lacking it is viable and not tumor prone (Smits et al., 1999 blue right-pointing triangle).

Although initial biochemical data suggested that APC served a scaffolding role in the destruction complex, the subsequent discovery of Axin and the realization that it carries binding sites for βcat, APC, GSK3, CK1, for proteins involved in reception of Wnt signals (LRP5/6, Dishevelled), and for other putative components of the Wnt pathway, suggested that Axin is the scaffold (Kimelman and Xu, 2006 blue right-pointing triangle; Polakis, 2007 blue right-pointing triangle). Recent biochemical experiments confirmed this, demonstrating that Axin can accelerate βcat phosphorylation in vitro, in the absence of APC (Ha et al., 2004 blue right-pointing triangle). These findings left the mechanistic role of APC in the destruction complex mysterious.

Several alternative models of the inner functioning of the destruction complex emerged, each suggesting different mechanistic roles for APC in targeting βcat for destruction. All must account for APC's complex structure, and in particular the fact that both APC and Axin have βcat-binding sites. Unlike Axin, which has a single βcat-binding site, APC has many of two

different types, 15Rs and 20Rs. Important clues came from examining affinities of βcat for both APC and Axin (Xing et al., 2003 blue right-pointing triangle; Ha et al., 2004 blue right-pointing triangle; Choi et al., 2006 blue right-pointing triangle; Liu et al., 2006 blue right-pointing triangle). Under basal conditions, βcat's affinity for Axin is higher than that for APC. APC, however, is a casein kinase 1 (CK1) and GSK3 substrate, with phosphorylation sites within the 20Rs (Rubinfeld et al., 1997b blue right-pointing triangle). Phosphorylated APC has a higher affinity for βcat than Axin (Ha et al., 2004 blue right-pointing triangle; Xing et al., 2004 blue right-pointing triangle; Choi et al., 2006 blue right-pointing triangle; Liu et al., 2006 blue right-pointing triangle). These findings led Kimelman and Xu to propose that the destruction complex goes through a catalytic cycle of structural rearrangements to ensure βcat phosphorylation and turnover (Kimelman and Xu, 2006 blue right-pointing triangle). They suggest that the complex assembles with βcat bound to Axin. βcat is then phosphorylated by CK1 and GSK3, and APC is also phosphorylated in the process. In the catalytic cycle model, this triggers transfer of βcat to APC, which is then suggested to facilitate βcat transfer to the E3 ubiquitin ligase for destruction, with presumed dephosphorylation of APC resetting the system, allowing a new βcat to be bound. Consistent with this catalytic cycle model, mammalian APC coimmunoprecipitates with βcat and βTrCP revealing that APC can indeed interact with the E3 ubiquitin ligase (Hart et al., 1999 blue right-pointing triangle). APC can also protect βcat from dephosphorylation during this proposed transfer to βTrCP (Su et al., 2008 blue right-pointing triangle), ensuring ubiquitination once βcat is phosphorylated.

Weis et al. presented a different model for the role of multiple βcat-binding sites in APC's activity (Ha et al., 2004 blue right-pointing triangle). Their in vitro data suggest that βcat

binding to APC protects APC from dephosphorylation, thus precluding the catalytic cycle outlined in the preceding paragraph. Instead, they suggest that APC's ability to bind βcat with different affinities using distinct binding sites is key to modulating destruction of βcat, allowing the destruction complex to accommodate the different intracellular βcat levels found in the presence or absence of Wnt signaling (Ha et al., 2004 blue right-pointing triangle). In this model, high-affinity sites are key to maintain low levels of βcat in the absence of Wnt signal, helping to target any free βcat for destruction. In contrast, low-affinity binding sites capture βcat when βcat levels are elevated by Wnt signaling, facilitating rapid βcat destruction. This results in the pathway being quickly turned off after Wnt ligands are no longer present.

Both the Kimelman and Xu and Weis models suggest an essential role for high-affinity βcat-binding sites. Specifically, in the catalytic cycle model (Kimelman and Xu, 2006 blue right-pointing triangle), 20R3 has a key role in βcat destruction, because, when phosphorylated, its affinity for βcat is 20-fold higher than the other 15Rs or 20Rs, thus exceeding that of Axin (Choi et al., 2006 blue right-pointing triangle; Liu et al., 2006 blue right-pointing triangle). Thus 20R3 may play a key role in transferring βcat to the E3 ligase (βcat affinity of all 20Rs decreases >100× without phosphorylation, but 20R3 still has a 50-fold higher affinity than the next best 20R). Lower affinity binding sites might act as docking sites for transfer of βcat to Axin. In Weis's model, in contrast, high- and low-affinity sites play different roles, with high-affinity sites modulating baseline destruction in the absence of Wnt signal and low-affinity sites playing a role in winding down Wnt signaling after ligand is withdrawn (Ha et al., 2004 blue right-pointing triangle). Thus, in summary, mechanisms by which APC acts in the destruction complex to ensure βcat destruction and thus regulate Wnt signaling remain an open question. In fact,

recent work suggested that loss of APC alone may not even lead to accumulation of nuclear βcat (Phelps et al., 2009 blue right-pointing triangle), whereas other experiments suggest that APC2 plays an unexpected positive role in Wnt signaling (Takacs et al., 2008 blue right-pointing triangle).

Another puzzling yet critical aspect of APC function is intimately tied to its tumor suppressor role. Colon tumors carry one APC allele encoding a truncated protein retaining the Arm repeats and some of the βcat-binding sites, but lacking Axin binding sites (Polakis, 1997 blue right-pointing triangle; Kohler et al., 2008 blue right-pointing triangle). Data in cultured colon cancer cells (Albuquerque et al., 2002 blue right-pointing triangle), mouse mutants (Gaspar et al., 2009 blue right-pointing triangle), and Drosophila embryos (McCartney et al., 2006 blue right-pointing triangle) suggest that these truncated proteins retain residual ability to target βcat for destruction, but whether this suggests that APC can play an Axin-independent role in βcat destruction or that the complex still targets βcat for destruction without direct APC–Axin interactions (Peterson-Nedry et al., 2008 blue right-pointing triangle) remains unknown. Interestingly, in cultured colon cancer cells expressing truncated APC, overexpressing a central fragment of APC carrying 20Rs and SAMP repeats restores βcat destruction (Munemitsu et al., 1995 blue right-pointing triangle; Rubinfeld et al., 1997a blue right-pointing triangle). Many studies extended this work, further evaluating the ability of different APC fragments to complement the truncated proteins in cancer cells (e.g., Kohler et al., 2008 blue right-pointing triangle, 2010 blue right-pointing triangle), with interesting but complex results, but the mechanism of this apparent complementation remains mysterious.

Drosophila provides a powerful system to test different hypotheses for APC's function in the destruction complex during development and to explore function of truncated APC proteins. Flies have two APC proteins sharing human APC's conserved core domains. Both fly APCs regulate Arm (Arm = fly βcat) stability and thus Wnt signaling (the primary Wnt in most fly tissues is Wingless [Wg]). Each APC plays individual roles in tissues where it is primarily expressed (Ahmed et al., 1998 blue right-pointing triangle; McCartney et al., 1999 blue right-pointing triangle; Yu et al., 1999 blue right-pointing triangle; Takacs et al., 2008 blue right-pointing triangle); for example, APC2 plays an essential role in regulating Wnt signaling in the embryonic epidermis. Many tissues requiring Wg signaling, however, are not affected in either single mutant, due to functional redundancy (Ahmed et al., 2002 blue right-pointing triangle; Akong et al., 2002a blue right-pointing triangle). In Drosophila we have null mutations in both APC family members and thus can produce animals completely lacking APC function. We combined the power of this system with parallel assays in the simpler colon cancer cell line system, giving us the ability to test which features of APC function are conserved between flies and mammals. Together, these studies allowed us to test different hypotheses for APC function, using mutants altering its structure in specific ways.

Material and Methods

APC constructs

Full-length Drosophila APC2 and a large internal piece of human APC (amino acids 1230–2130) were PCR cloned into the pCR8/GW/TOPO Gateway Entry Vector (Invitrogen, Carlsbad, CA) by TOPO TA cloning. These entry vectors served as the basis for further deletion

mutagenesis, which was accomplished using a combination of standard restriction cloning, PCR stitching, and site-directed mutagenesis. The sites/domains deleted in the various APC constructs are included in Table 1. APC constructs were then recombined into expression vectors modified for Gateway cloning, using Gateway vectors provided by Terence Murphy (Carnegie Institution for Science, Baltimore MD). For expression in mammalian cells, dAPC2 and hAPC constructs were recombined into a modified ECFP-N1 vector (Clontech, Mountain View, CA) with an EGFP-Gateway-3X STOP cassette restriction cloned downstream of the cytomegalovirus promoter. A similar ECFP-N1 vector with a 3X Flag-Gateway-3X STOP cassette was generated for Axin expression in mammalian cells. To generate transgenic flies, dAPC2 constructs were Gateway cloned into a modified pUAStattB vector (Basler lab, GenBank accession number EF362409) that added the endogenous dAPC2 promoter (McCartney et al., 2006 blue right-pointing triangle) and an EGFP-Gateway-3X STOP cassette. Details of the cloning are available upon request.

Cell culture, transfections, and immunofluorescence

SW480 cells were cultured in L15 medium (Cellgro, Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bozerum and 1X Pen/Strep. SW480s were grown at 37°C under normal atmospheric conditions. APC constructs were transfected into SW480s overnight using Lipofectamine 2000 (Invitrogen) per manufacturer's protocol. After 24 h, cells were processed for analysis. For immunofluorescence, cells were fixed in 4% formaldehyde/1X phosphate-buffered saline (PBS) for 5 min, blocked with 1% normal goat serum (NGS)/0.1% Triton-100/1X PBS, and then antibody stained. Primary antibodies were H-102 βcat antibody (cat# sc-7199; Santa Cruz Biotechnology, Santa Cruz, CA) used at 1:8000 vol/vol and anti–FLAG

M2 antibody (cat# F1804; Sigma, St. Louis, MO) at 1:1000 vol/vol. Secondary antibodies were Alexa 568 or 647 (Invitrogen) used at 1:1000 vol/vol.

Quantifying βcat protein levels

To quantify βcat protein levels, SW480 cells were transfected with GFP–APC constructs overnight. Twenty-four hours later, cells were fixed and antibody stained for βcat and 4',6-diamidino-2-phenylindole (DAPI). Individual cells were defined by DAPI, and the average total cellular intensity of βcat was determined for GFP-positive cells using an Array Scan V (Thermo Scientific Cellomics, Pittsburgh, PA) and the vHCS View software (Thermo Scientific Cellomics). Images of 1000–5000 cells per construct from ≥3 independent experiments were acquired and analyzed.

TOP/FOP reporter assays

The TOP/FOP Flash Luciferase reporter constructs and the pRL Renilla transfection control were gifts from Hans Clevers (Hubrecht Institute, Utrecht, The Netherlands). TOP/FOP reporter assays were conducted using the Dual Glow Luciferase System (Promega, Madison, WI) per the manufacturer's instructions. Briefly, TOP Flash or alternately FOP Flash (1 μg) constructs were transiently cotransfected into SW480 cells together with the pRL transfection control (1 μg) and the appropriate APC construct (2 μg). Transcriptional activity was measured 24 h posttransfection and was defined as the ratio of TOP Flash normalized to Renilla. None of the APC constructs significantly affected FOP Flash values.

Transgenic fly lines, embryonic lethality assay, and cuticle rescue

Transgenic fly lines were generated by Best Gene (Chino Hills, CA). dAPC2 transgenes on the second chromosome were crossed into the APC2g10 single mutant and the

APC2g10APC1Q8 double mutant backgrounds (McCartney et al., 2006 blue right-pointing triangle). For analysis in the APC2g10 single mutant background, embryos expressing the transgene but maternally/zygotically mutant for APC2 were the progeny of dAPC2 transgene; APC2g10 females and males. In the double mutant background, embryos expressing the transgene but maternally/zygotically mutant for both APCs were generated using the FRT/FLP/DFS technique (Chou and Perrimon, 1996 blue right-pointing triangle). Heat-shocked dAPC2 transgene/+; FRT82B APC2g10 APCQ8/FRT82B ovoD females were crossed to dAPC2 transgene; FRT82B APC2g10 APCQ8/TM3 males. Heat shocks were performed on day 3 after egg laying for 3 h at 37°C. All crosses were performed at 25°C. Embryonic lethality assays and cuticle preparations were performed as previously described (Wieschaus and Nüsslein-Volhard, 1986 blue right-pointing triangle). The level of embryonic cuticle rescue was assessed using previously established scoring criteria (McCartney et al., 2006 blue right-pointing triangle).

IPs and Western blotting

IPs and Western blots were conducted as in Peifer et al. (1992 blue right-pointing triangle). Briefly, protein samples from either tissue culture cells or dechorionated Drosophila embryos were prepared by directly adding 2X Laemmli buffer, grinding on ice with a plastic pestle, and boiling for 5 min. For IPs, cells or dechorionated embryos were first lysed in NET buffer (50 mM Tris, pH 7.5, 400 mM NaCl, 5 mM EDTA, 1% NP40) containing protease inhibitors (Complete EDTA-free Protease Inhibitor tablets; Roche, Basel, Switzerland) and phosphatase inhibitors (1 mM NaF, 0.4 mM NaVO3, 0.4 mM NaVO5). Protein samples were resolved on a 6% SDS–PAGE gel and blotted to nitrocellulose. Primary antibodies were anti-GFP (clone JL-8; Clontech), anti-GFP (ab290 for IPs; Abcam, Cambridge, MA), anti-Flag (clone M2; Sigma), and

anti-dAPC2 (McCartney et al., 1999 blue right-pointing triangle). Signal was detected with ECL-Plus (Amersham, Piscataway, NJ).

Immunofluorescence/imaging of Drosophila embryos

Embryos were prepared and imaged as in Fox and Peifer (2007 blue right-pointing triangle). Briefly, embryos were dechorionated and fixed in 4% formaldehyde before devitelization. Embryos were then blocked in NGS and sequentially incubated with anti-Arm (N27A1, DSHB used at 1:50) and Alexa 568 secondary antibody (Invitrogen) used at 1:500. Images were collected on either a Zeiss LSM 510 or Zeiss Pascal scanning confocal microscope (Carl Zeiss, Thornwood, NY). Adobe Photoshop 7.0 was used to adjust input levels to span entire output grayscale, and to adjust brightness and contrast. When protein levels were compared, images were equally adjusted.

Results

Model systems to assess APC function in human cells and flies

To test hypotheses of APC's mechanism of action in Wnt regulation, we used two model systems, each with unique advantages. To rapidly assess the ability of APC mutants to target βcat for destruction, and test whether Wnt regulatory mechanisms are similar in human and fly cells, we used human SW480 colon cancer cells, the endogenous APC of which is truncated (ending at aa1338, after 20R1; Nishisho et al., 1991 blue right-pointing triangle). The large size of these cells allowed us to assess effects of APC mutants on βcat levels, localization, and subsequent downstream signaling. SW480 cells accumulate high levels of βcat in the cytoplasm and nuclei (Figure 2.2, A and B, arrowhead) and have high activity of the Wnt transcriptional

reporter TOPFLASH (Figure 2.2E; Korinek et al., 1997 blue right-pointing triangle). βcat destruction is rescued by full-length human APC, fragments of its middle region (Figure 2.2, C–E; e.g., Munemitsu et al., 1995 blue right-pointing triangle; Rubinfeld et al., 1997a blue right-pointing triangle), or by fly APC1 (Hayashi et al., 1997 blue right-pointing triangle). We found that green fluorescent protein (GFP)-tagged APC2 restored βcat destruction as effectively as human APC (Figure 2.2, B vs. C and D). We also developed a method to quantify this, measuring βcat fluorescence intensity in hundreds of transfected versus nontransfected cells with the Cellomics ArrayScan VTI, providing unbiased assessment of βcat down-regulation (Figure 2.2F). Fly APC2 also reduced TCF-regulated transcription (Figure 2.2E).

In parallel, we tested APC function in vivo during Drosophila embryonic development. In contrast to SW480 cells, in which the truncated APC may retain function, and which also express human APC2 (Maher et al., 2009 blue right-pointing triangle), in flies we can test mutant transgenes in the complete absence of APC2 (APC2g10 maternal and zygotic null single mutants) or in the complete absence of all APC family proteins (APC2g10 APC1Q8 maternal and zygotic double null mutants). Cell fates in the embryonic epidermis are an extremely sensitive readout of Wnt signaling (McCartney et al., 2006 blue right-pointing triangle); anterior cells in each segment secrete hairlike denticles (Figure 2.2G, arrowhead), whereas posterior cells receiving Wg signal secrete naked cuticle (Figure 2.2G, arrow). APC2 is the primary player in the embryo (McCartney et al., 1999 blue right-pointing triangle), but APC1 also contributes to Wnt regulation there (Ahmed et al., 2002 blue right-pointing triangle; Akong et al., 2002a blue right-pointing triangle), and thus single and double mutants allow us to assess different parts of the phenotypic spectrum. In APC2 APC1 double mutants, Arm (fly βcat) levels rise dramatically, and

all cells take on naked cuticle fates (Figure 2.2I; Ahmed et al., 2002 blue right-pointing triangle; Akong et al., 2002a blue right-pointing triangle). In this stringent background, transgenes must retain substantial function to rescue Arm destruction and thus cell fates. In contrast, in APC2 single mutants, Arm levels rise only slightly (McCartney et al., 1999 blue right-pointing triangle), due to residual APC1 activity. This modest rise in Arm levels changes most but not all cell fates to naked cuticle (Figure 2.2H; arrows = remaining denticles). This background allows us to assess subtle differences in APC function, as even quite impaired mutants provide some rescue of cell fates in the presence of APC1 (McCartney et al., 2006 blue right-pointing triangle). As we show later in this article, this allowed us to identify other functions of APC2, beyond its role in targeting Arm for destruction. GFP-tagged APC2 under control of its endogenous promoter (expressed at the same level as endogenous APC2 (Figure 2.2L) rescues APC2 single mutants to adult viability and fertility (Figure 2.2K), and rescues APC2 APC1 double mutants to embryonic viability and normal cell fates (Figure 2.2J). We made site-directed mutants of GFP-tagged APC2 (Figure 2.1B) and examined their function in mammalian cells and transgenic flies, directly testing different hypotheses for APC's mechanism of action. We confirmed expression and stability of mutant constructs transfected into SW480 cells by fluorescence of the GFP tag (see figures) and by immunoblotting (Figure 2.3, A–C). To confirm expression and stability of mutant constructs in Drosophila, we used immunoblotting of embryo extracts with either anti-APC2, using endogenous APC2 as a level comparison, or anti-GFP antibody, with a different GFP-tagged and expression-confirmed construct for level comparisons (Figure 2.3, D–M). All transgenic constructs in the fly were expressed near endogenous levels.

Individual high-affinity βcat-binding sites are not essential for βcat destruction

To understand how βcat is targeted for destruction, we must understand how the destruction complex works. In the catalytic cycle model (Kimelman and Xu, 2006 blue right-pointing triangle), the destruction complex assembles with βcat bound to Axin. GSK3 phosphorylation of both βcat and APC creates higher affinity βcat-binding sites on APC, and thus βcat is transferred from Axin to APC, facilitating final transfer to the E3 ligase. In the most explicit versions of this hypothesis, the highest affinity βcat-binding site, 20R3, should be essential—its affinity for βcat is 20-fold higher than the other 20Rs when phosphorylated, and it is the only 20R with significant βcat affinity when not phosphorylated. In the Weis model (Ha et al., 2004 blue right-pointing triangle), high-affinity binding sites like 20R3 should be essential for baseline regulation of the low βcat levels present in the absence of Wnt signal. Both hypotheses suggest that 20R3 will be critical for proper βcat destruction.

To test these hypotheses, we deleted 20R3 from full-length Drosophila APC2 (APC2ΔR3; Figure 2.1B). In contrast to the predictions of these models, this had no effect on APC2's ability to down-regulate βcat levels (Figure 2.4, A, B, and G) or TCF-regulated transcription in SW480 cells (Figure 2.4H). Furthermore, when introduced into flies, APC2ΔR3 fully rescued embryonic Wnt signaling defects of APC2 single mutants (Figure 2.5, A and D) and restored adult viability (Figure 2.6A). Finally, APC2ΔR3 also rescued embryonic viability, effects on cell fate choices (Figure 2.5, B and M), and destruction of Arm (Compare Figure 2.7, A, B, C, and F) in APC2 APC1 double mutants (in assessing Arm levels, paternally rescued embryos were eliminated from analysis using a GFP-marked Balancer chromosome; e.g., Figure 2.7, E, I, and N). Thus, surprisingly, the highest affinity βcat-binding site, 20R3, is fully dispensable for βcat destruction.

To further explore the hypothesis that high- and medium-affinity 20Rs play key roles in βcat destruction, we generated several other mutants. Deleting the medium-affinity βcat-binding site, 20R1 (APC2ΔR1; Figure 2.1B) left APC2 fully functional in down-regulating βcat levels (Figure 2.4, C and G) and TCF-regulated transcription (Figure 2.4H) in SW480 cells. Even more striking, two mutants lacking different subsets of three of the highest affinity binding sites, 20R3, 20R4, and 20R5 (APC2ΔR3-R5; Figure 2.1B), or 20R1, 20R4, and 20R5 (APC2ΔR1,R4-R5; Figure 2.1B), were also fully functional in down-regulating βcat levels in SW480 cells and in substantially reducing TCF-regulated transcription (Figure 2.1, D, E, G, and H). We next assessed APC2ΔR3-R5 and APC2ΔR1,R4-R5 in vivo in Drosophila. Both retained substantial function in vivo; both rescued cell fate choices in APC2 single mutant embryos (Figure 2.5, A, E, and F); and rescued embryos could survive to adulthood (Figure 2.6, B and C). Embryonic viability, however, was reduced relative to wild-type APC2 or APC2ΔR3 (Figure 2.5, D–F), suggesting that they do not retain full function. These data refute models in which the highest affinity βcat-binding sites play essential individual roles in βcat destruction, and instead suggest 20Rs contribute additively to function in vivo.

If high-affinity βcat binding either mediates transfer of βcat from Axin to APC for handoff to the E3 ligase or helps deliver βcat for destruction when βcat levels are low, one might predict that the highest affinity βcat-binding site, 20R3, would be sufficient for targeting βcat for destruction. We tested this using an APC2 mutant with all other 20Rs deleted (APC2KeepR3; Figure 2.1B). This provided two interesting surprises. In SW480 cells, APC2KeepR3 was completely inactive in targeting βcat for destruction (Figure 2.4, F and G), and Wnt-mediated transcription was not reduced by a statistically significant level (Figure 2.4H).

Thus βcat binding provided by 20R3 is not sufficient for βcat destruction.

We next examined APC2KeepR3 function in Drosophila. Consistent with the idea that APC2KeepR3 cannot rescue βcat destruction, it had no ability to rescue the cell fate phenotype or the destruction of Arm in APC2 APC1 double mutants (Figure 2.5, B and N; compare Figure 2.7, A, B, and G). When we tested this mutant in APC2 single mutants, however, to our surprise, it retained significant ability to rescue cell fates, although it did not substantially rescue embryonic or adult viability (Figure 2.5, A and G). To explore this further, we examined βcat localization in SW480 cells expressing APC2KeepR3. Strikingly, although βcat in SW480 cells is usually significantly enriched in nuclei (e.g., Figure 2.4B' arrowhead), APC2KeepR3 was able to reduce nuclear βcat enrichment (Figure 2.4F', arrowhead).

In Drosophila APC2 single mutants, the remaining APC1 protein provides residual activity in regulating Arm levels (Ahmed et al., 2002 blue right-pointing triangle; Akong et al., 2002a blue right-pointing triangle). Based on these data, we hypothesized that cytoplasmic retention by APC2KeepR3 attenuates signaling by sequestering the only slightly elevated Arm levels in APC2 single mutants, thus partially rescuing cell fates, but that it cannot sequester the highly elevated Arm levels seen in APC2 APC1 double mutants. We next discuss our reasons for favoring cytoplasmic retention over alternative models.

Multiple βcat-binding sites modulate Wnt signaling regulation via cytoplasmic retention

To test the hypothesis that the residual function of APC2KeepR3 in Drosophila APC2 single mutants results from the ability of 20R3 to bind and retain some Arm in the cytoplasm, we further explored the correlation between cytoplasmic retention and ability to rescue APC2 single mutants. We first deleted all 20Rs from APC2 (APC2Δ20; Figure 2.1B). In SW480 cells,

APC2Δ20 behaved like APC2KeepR3—it could not rescue βcat destruction (Figure 2.4, I and L) or reduce TCF-regulated transcription by a statistically significant amount (Figure 2.4M). We were surprised to find, however, that APC2Δ20 could still retain βcat in the cytoplasm (Figure 2.4I', compare arrowheads). In Drosophila APC2Δ20 provided no rescue of Arm levels (Figure 2.7J) or cell fates in APC2 APC1 double mutants (Figure 2.5, B and P), demonstrating that at least one of the 20Rs plays an essential role in destruction complex function. APC2Δ20, however, still largely rescued cell fates in APC2 mutants (Figure 2.5, A and I), although it did not rescue embryonic or adult viability, and in the cell fate assay it was not as effective as APC2KeepR3 (Figure 2.5A). Thus an APC2 protein lacking all 20Rs retains a small degree of function in vivo, correlating with its residual ability for cytoplasmic retention of βcat, but importantly at least one 20R is essential for the destruction complex to mediate βcat destruction.

We next hypothesized that the 15Rs might provide the ability to bind and retain βcat in the cytoplasm, allowing APC2Δ20 to reduce Wnt signaling even though it was nonfunctional in βcat destruction. Previous work explored the function of the 15Rs in βcat regulation in the context of APC fragments (Kohler et al., 2010 blue right-pointing triangle), but not in the context of full-length APC. We thus first deleted them alone (APC2Δ15; Figure 2.1B). APC2Δ15 was fully functional in regulating βcat destruction (Figure 2.4, J and L) and reducing TCF-regulated transcription in SW480 cells (Figure 2.4M). It was also highly functional in the animal, fully rescuing the cell fate defects of APC2 single mutants (Figure 2.5, A and H) and restoring adult viability (Figure 2.5D). It also fully rescued embryonic viability, cell fate choices, and Arm destruction of APC2 APC1 double mutants (Figure 2.5, B and O; Figure 2.7H). Thus the 15Rs are not essential for APC's mechanism of action in the destruction complex.

These results, however, did not rule out the hypothesis that 15Rs, by binding βcat, might participate in cytoplasmic retention. To test this hypothesis, we generated a mutant lacking all 15Rs and 20Rs (APC2Δ15Δ20; Figure 2.1B); this mutant should be completely unable to bind βcat. Strikingly, APC2Δ15Δ20 was fully inactivated in all contexts, in contrast to APC2Δ20. It provided no rescue of βcat destruction (Figure 2.4, K and L) or TCF-mediated transcription (Figure 2.4M) in SW480 cells, and could not retain βcat in the cytoplasm (Figure 2.4K, arrowhead). APC2Δ15Δ20 was also completely inactive in the animal, failing to provide any rescue activity in either APC2 single mutants (Figure 2.5, A and J) or in APC2 APC1 double mutants (Figure 2.5, B and Q; Figure 2.7K). In fact, in double mutants there was indication of dominant-negative activity, as APC2Δ15Δ20 substantially reduced the ability of paternal APC2 and APC1 to rescue embryonic viability of APC2 APC1 maternal mutants (Figure 2.5, Q and R). Together, these data demonstrate that the 20Rs play an essential role in the ability of the destruction complex to target βcat for destruction. They also suggest that cytoplasmic retention of βcat by APC2 can play an important secondary role in negatively regulating βcat transcriptional activity, and that both 15Rs and 20Rs contribute in an additive way to this ability.

20R2 and sequence B are essential for βcat destruction

When examining the affinities of 15Rs and different 20Rs for βcat (Choi et al., 2006 blue right-pointing triangle; Liu et al., 2006 blue right-pointing triangle), one surprise was that 20R2 lacks key residues for βcat binding (Figure 2.7, yellow arrows, red arrowheads), and does not detectably bind βcat in vitro (Choi et al., 2006 blue right-pointing triangle; Liu et al., 2006 blue right-pointing triangle) or in cells (Kohler et al., 2008 blue right-pointing triangle), with or

without phosphorylation. Despite the inability of 20R2 to bind βcat, the divergent sequence of 20R2 is as or better conserved between flies and mammals than are high-affinity binding sites like 20R3 (Figure 2.7). This prompted us to explore whether 20R2 is important for βcat regulation. Given its inability to bind βcat, we hypothesized it would be dispensable. To our surprise, however, removing 20R2 (APC2ΔR2; Figure 2.1B) completely blocked APC2's ability to rescue βcat destruction in SW480 cells (Figure 2.8, A and D). APC2ΔR2 could retain βcat in the cytoplasm (Figure 2.8A, compare arrowheads), however, consistent with the presence of numerous βcat-binding sites, and thus it partially reduced TCF-regulated transcription (Figure 2.8E). In this it contrasted with APC2Δ15Δ20, which could not retain βcat in the cytoplasm (Figure 2.8C, arrowhead) or reduce TCF-regulated transcription (Figure 2.8E).

We next assessed APC2ΔR2 in the animal. Consistent with our observations in SW480 cells, APC2ΔR2 could not rescue Arm destruction in APC2 APC1 double mutants (Figure 2.7L), and it also provided no cell fate rescue there (Figure 2.9, B and K). APC2ΔR2 retained substantial ability, however, to rescue cell fates, embryonic lethality, and adult viability of APC2 single mutants (Figure 2.9, A and D), correlating with it retaining function in cytoplasmic retention. Thus 20R2 is essential for the ability of the destruction complex to target βcat for destruction, but is dispensable for βcat retention.

Immediately adjacent to 20R2 is another highly conserved sequence shared by all APC family members, sequence B (McCartney et al., 1999 blue right-pointing triangle (Figure 2.7; also known as the CID; Kohler et al., 2009 blue right-pointing triangle). Given its proximity to 20R2 and the strongly conserved spacing between them, we hypothesized that it would also be required for APC2 activity in the destruction complex. Strikingly, deleting sequence B from full-

length APC2 (APC2ΔB; Figure 2.1B) also abolished its ability to target βcat for destruction in SW480 cells (Figure 2.8, B and D). Deleting sequence B, however, did not disrupt cytoplasmic retention (Figure 2.8B, compare arrowheads), and thus APC2ΔB retained the ability to partially reduce βcat-dependent transcription (Figure 2.8E). When tested in the animal, APC2ΔB behaved similarly to APC2ΔR2. It could not rescue Arm destruction (Figure 2.7M), cell fates, or embryonic lethality in APC2 APC1 double mutants (Figure 2.9, B and L). APC2ΔB retained, however, substantial ability to rescue cell fates, embryonic lethality, and adult viability of APC2 single mutants (Figure 2.9, A and E; Figure 2.6E), consistent with it retaining function in cytoplasmic retention. Thus these two adjacent conserved APC motifs, 20R2 and sequence B, play essential roles in βcat destruction.

Sequence B and 20R2 are among the most highly conserved sequences in APC proteins. We thus tested whether they play important roles in human APC, deleting them from a minimal fragment that rescues βcat regulation in SW480 cells (hAPC2.8kb; Rubinfeld et al., 1997a blue right-pointing triangle). This fragment spans from 20R1 through the end of the SAMPs (Figure 2.10A), and rescues βcat destruction in SW480 cells (Figure 2.10B). We generated variants lacking 20R2, 20R3, or sequence B (Figure 2.10A). Deleting either 20R2 (hAPC2.8kbΔR2; Figure 2.10C) or sequence B (hAPC2.8kbΔB; Figure 2.10D; see also Kohler et al., 2009 blue right-pointing triangle) substantially reduced the ability to target βcat for destruction, as assessed by immunofluorescence, whereas deleting 20R3 (hAPC2.8kbΔR3; Figure 2.10E) did not impair function of the 2.8 kb fragment. Likewise, removing 20R3 did not impair the ability to reduce TCF-regulated transcription, whereas deletion of either 20R2 or sequence B attenuated this activity (Figure 2.10F). Thus both 20R2 and sequence B play conserved and essential roles in

regulating βcat destruction in flies and mammals.

Direct binding to Axin via the SAMPs is essential for Wnt regulation in Drosophila

In the current model, the destruction complex assembles by direct interactions between APC's SAMPs and Axin's regulator of G protein signaling (RGS) domain (Kishida et al., 1998 blue right-pointing triangle; Nakamura et al., 1998 blue right-pointing triangle; Spink et al., 2000 blue right-pointing triangle). In mice, a truncated APC retaining a single SAMP can regulate Wnt signaling during both normal development and oncogenesis, as assessed by viability to adulthood and lack of a tumor-prone phenotype (Smits et al., 1999 blue right-pointing triangle), supporting the hypothesis that tumor truncations are selected for loss of the SAMPs to inactivate the destruction complex. Consistent with these results, a mutant fly APC2 protein truncated after 20R2 and thus lacking the SAMPs (APC2d40; Figure 2.1B) can no longer mediate βcat destruction or down-regulate TCF-regulated transcription in SW480 cells (Figure 2.8, F, J, and K). These data are also consistent with the complete inability of APCd40 to rescue cell fates in APC2 APC1 double mutants (McCartney et al., 2006 blue right-pointing triangle; Figure 2.9, B and M), suggesting that it is defective in destruction. APC2d40 retains, however, some residual ability to rescue cell fates in APC2 single mutants (McCartney et al., 2006 blue right-pointing triangle; Figure 2.9, A and F)—this retention correlates with its ability to retain βcat in the cytoplasm in SW480 cells (Figure 2.8F', compare arrowheads).

These data are consistent with the idea that the SAMPs are essential for the ability of the destruction complex to target βcat for destruction. APC2d40 and the previously characterized human truncation mutants eliminating the SAMPs also delete some 20Rs, leaving open the question of whether the SAMPs are essential. To test the hypothesis that the SAMPs

are essential for destruction complex activity, we generated a mutant APC2 cleanly deleting them without removing any 20Rs (APC2ΔSAMP; Figure 2.1B). To our surprise, unlike APC2d40, APC2ΔSAMP largely restored βcat destruction and down-regulated TCF-regulated transcription in SW480 cells (Figure 2.8, G, J, and K).

We next investigated APC2ΔSAMP function in the animal. In contrast to our SW480 results, APC2ΔSAMP was totally nonfunctional in APC2 APC1 double mutants, providing no rescue of cell fate choices, embryonic viability (Figure 2.9, B and N), or destruction of Arm (Figure 2.7O). In fact, like APC2Δ15Δ20, there was suggestion of dominant-negative activity, as many embryos that should have been paternally rescued died (Figure 2.9N). In APC2 single mutants, APC2ΔSAMP retained detectable activity (Figure 2.9, A and G), but this was substantially reduced from wild type or many of the mutants lacking different combinations of 20Rs. Together these data suggest that interaction with Axin via the SAMP repeats is essential for the ability of the destruction complex to target βcat for destruction in Drosophila, but not in SW480 cells. We discuss possible reasons for this in the Discussion section.

APC colocalizes with Axin by a second mechanism independent of its SAMP repeats

The ability of APC2ΔSAMP to down-regulate βcat levels in SW480 cells and retain at least some function in APC2 single mutants was surprising, because APC–Axin interaction is key to all proposed models for destruction complex action. We thus examined whether deleting the SAMPs fully eliminates APC2–Axin interactions. We explored this in two ways—by co-IP of tagged proteins from extracts of SW480 cells and by examining colocalization in these cells. By co-IP, we could readily detect interaction between GFP-APC2 and fly Flag-Axin (Figure 2.11A; GFP-tagged APC2 transfected without Flag-Axin was a negative control). Co-IP was eliminated

by deletion of the SAMPs (Figure 2.11B). We also analyzed colocalization of APC2 and Axin. When expressed in SW480 cells, GFP-tagged wild-type APC2 is largely diffuse in the cytoplasm (Figure 2.12A, inset), as are most APC2 mutants we analyzed earlier (unpublished data). In contrast, Flag-tagged Drosophila Axin localizes to cytoplasmic puncta (Figure 2.12B, inset), similarly to those previously observed on expression of mammalian Axin (Fagotto et al., 1999 blue right-pointing triangle; Smalley et al., 1999 blue right-pointing triangle). Interestingly, overexpressing fly Axin is sufficient to trigger βcat destruction (Figure 2.12B, arrow), as was previously observed after overexpression of human Axin (Hart et al., 1998 blue right-pointing triangle; Nakamura et al., 1998 blue right-pointing triangle). Coexpressing Drosophila Axin and APC2 led to almost complete recruitment of APC2 into Axin puncta (Figure 2.12C, inset), consistent with a physical interaction: Similar results were previously observed with mammalian Axin and APC (Faux et al., 2008 blue right-pointing triangle). βcat binding does not play a role in this interaction, as APC2Δ15Δ20 is also strongly recruited into Axin puncta (Figure 2.12D, inset). In contrast, recruitment of APC2ΔSAMP into Axin puncta was substantially reduced, although not completely eliminated (Figure 2.12E, inset). Together, these data support the importance of the SAMPs in destruction complex assembly.

Given these results, we were puzzled by the partial activity of APC2ΔSAMP in both SW480 cells and in the animal. We thus considered two hypotheses to explain this residual function: 1) that APC2 has some Axin-independent ability to regulate Wnt signaling, or 2) that APC2 and Axin retain some ability to interact even in the absence of the SAMPs. These hypotheses would be consistent with the small amount of residual colocalization when overexpressed in SW480 cells.

In examining the other mutants we generated, we found to our surprise that APC2 and Axin can colocalize independently of the SAMPs. As discussed earlier in the text, most colon tumors retain a truncated protein that has lost the SAMPs but retains the 15Rs and one to three 20Rs. The Drosophila mutant, APC2d40, mimics the tumor truncations, with a stop codon just after 20R2 (McCartney et al., 2006 blue right-pointing triangle). Although APC2d40 protein could not reduce βcat levels (Figure 2.8, F and J) or Wnt regulated transcription (Figure 2.8K) in SW480 cells, to our surprise this mutant protein, which lacks all SAMPs, is readily recruited into Axin puncta (Figure 2.12F, inset). This interaction appears less robust than that mediated by the SAMPs, as it does not support co-IP of APC2d40 and Axin (Figure 2.11C). We next explored whether this SAMP-independent ability to interact with Axin was confined to Drosophila APC2. We cotransfected a truncated human APC like that found in tumors (aa 1–1338) with human Axin. Strikingly, it also colocalized to Axin puncta (Figure 2.12I, inset).

Sequence B may regulate APC–Axin interactions

These data suggest that two mechanisms exist by which APC2 and Axin can interact, one SAMP-dependent and one SAMP-independent. Interestingly, the truncations in APC2d40 and most tumor proteins remove not only the SAMPs but also sequence B. Given the important role we found for sequence B earlier in the text, we explored whether it might regulate APC2–Axin interactions. Strikingly, truncating APC2 after sequence B (APC2EndatB; Figure 2.1B; only 56 amino acids longer than the truncation in APC2d40) drastically reduces localization in Axin puncta in SW480 cells (Figure 2.12G, inset); APC2EndatB also does not coimmunoprecipitate with Axin (Figure 2.11D). These data suggested that sequence B may actually inhibit interaction with Axin in SW480 cells, perhaps because it is involved in releasing APC2 from the Axin

complex during the catalytic cycle. Like APC2ΔSAMP, APC2EndatB restored the ability to target βcat for destruction in SW480 cells (Figure 2.8, H and J; although it was not fully effective at reducing TCF-regulated transcription; Figure 2.8K), but it could not restore substantial APC2 function in APC2 single mutant flies (Figure 2.9, A and H).

To test the hypothesis that sequence B antagonized interaction with Axin, we generated an additional mutant in which both the SAMPs and sequence B were deleted (APC2ΔBΔSAMP; Figure 2.1B). If sequence B is important for releasing APC2 from the Axin complex, we predicted that APC2ΔBΔSAMP would once again colocalize with Axin in SW480 cells. This was indeed the case (Figure 2.12H, inset); however, like APC2ΔB, APC2ΔBΔSAMP cannot rescue βcat regulation in SW480 cells (Figure 2.8, I, J, and K). Together, these data demonstrate that sequence B plays a key role in βcat destruction, and suggest a speculative model in which it facilitates completion of a catalytic cycle that involves release of APC and perhaps βcat from Axin.

Discussion

Wnt signaling is a paradigm for negatively regulating key developmental and oncogenic signaling pathways by targeting effectors for destruction. Despite models of Wnt signaling in every cell biology text, major questions remain about how the βcat destruction complex operates and what role APC plays. Our data address these issues. They demonstrate that individual βcat-binding sites are not essential for βcat destruction, but instead suggest that these sites modulate Wnt signaling, acting collectively to retain βcat in the cytoplasm. Furthermore, they demonstrate that 20R2 and sequence B are essential for targeting βcat for destruction, and support a model in which they help regulate APC–Axin interactions to

complete a cycle of destruction complex activity. Together, they provide novel insights into inner workings of the machine targeting βcat for phosphorylation and ultimate destruction, and help guide thinking about analogous machines targeting other signaling effectors.

Individual βcat-binding sites are dispensable for destruction complex function

APC's complex structure provides clues to its mechanism of action. One mysterious feature of APC proteins is that they share multiple βcat-binding sites of different affinities (Choi et al., 2006 blue right-pointing triangle; Liu et al., 2006 blue right-pointing triangle). Their function in Wnt regulation remained unclear; most studies transfected small APC fragments into colon cancer cells already expressing truncated mutant APCs retaining a subset of these sites (e.g., Munemitsu et al., 1995 blue right-pointing triangle; Kohler et al., 2008 blue right-pointing triangle, 2010 blue right-pointing triangle). Several hypotheses were proposed to explain roles of these different sites, with special roles of high-affinity sites in regulating βcat destruction a common feature. The catalytic cycle model suggested that the highest affinity binding sites are required to remove βcat from Axin after βcat and APC phosphorylation, facilitating βcat transfer to the E3-ligase and subsequent destruction (Kimelman and Xu, 2006 blue right-pointing triangle). In another model, different affinity binding sites play roles at the different βcat concentrations in cells exposed to or not exposed to Wnt signals (Ha et al., 2004 blue right-pointing triangle). Thus both predict special roles for the highest affinity binding sites.

We tested how the diverse βcat-binding sites contribute to βcat destruction. We were surprised to find that individual high-affinity binding sites are not essential for APC's mechanism of action. The highest affinity βcat-binding sites, 20R3 and 20R1, are each dispensable for full-length APC2. Even proteins lacking 20R3-R5 or 20R1+R4-R5, and thus retaining only a single

βcat binding 20R, could target βcat for destruction. Furthermore, 15Rs are fully dispensable for full-length APC2. This finding contrasts with those of previous studies using small human APC fragments, in which 15Rs were necessary and sufficient to target βcat for destruction. The necessity of the 15Rs was revealed, however, only in fragments with no SAMPs—in the presence of SAMPs, 15Rs were not necessary (Kohler et al., 2010 blue right-pointing triangle). Our data are consistent with the apparent lack of 15Rs in human APC2, which can down-regulate βcat in SW480 cells (van Es et al., 1999 blue right-pointing triangle). Thus, no individual βcat binding site appears essential for the destruction complex to target βcat for destruction, although it remains possible that these βcat binding sites play more subtle modulatory roles at the different βcat concentrations in cells exposed to or not exposed to Wnt signals (e.g., Ha et al., 2004 blue right-pointing triangle). Eliminating all 20Rs, however, eliminates βcat destruction, suggesting that at least one 20R is essential for destruction complex activity.

20R2 and sequence B are essential for APC's role in the destruction complex

One surprise emerging from biochemical analysis of APC was that 20R2, unlike other 20Rs, lacks key βcat-binding residues and does not detectably bind βcat (Choi et al., 2006 blue right-pointing triangle; Liu et al., 2006 blue right-pointing triangle; Kohler et al., 2008 blue right-pointing triangle). This finding suggested that 20R2 might not be important for APC function. Instead, we found that 20R2 and sequence B are essential for destruction complex activity, likely as binding site(s) for novel partners.

The sequence of 20R2 is highly conserved through evolutionary time, with strong conservation of the very residues explaining its lack of βcat binding (Figure 2.13), suggesting that it is a binding site for a novel partner. Our data help explain this strong sequence

conservation—20R2 is essential for APC2 to down-regulate βcat levels in both colon cancer cells and Drosophila. Furthermore, this feature is not unique to fly APC2, as deleting 20R2 from a human APC fragment also strongly diminished its activity in βcat destruction.

Adjacent to 20R2 is sequence B, also known as the CID. Earlier work revealed an important role for it in the function of APC fragments transfected into SW480 cells (Kohler et al., 2009 blue right-pointing triangle). Our data confirm this and further demonstrate that it is essential for full-length APC2 and critical for destruction complex activity in vivo. Sequence B's length and sequence conservation (Figure 2.13) are consistent with it also being a binding site for an unknown partner essential for destruction complex activity. Because 20R2 and sequence B are adjacent, they may act together as a single protein-binding site. The partner(s) identity will be revealing.

Cyclic assembly/disassembly of Axin–APC complexes may regulate destruction complex function

In the current model, APC and Axin are key to destruction complex action, and they bind solely by interactions between APC's SAMPs and Axin's RGS domain. This model was recently called into question by the observation that Drosophila Axin lacking the RGS domain retained some function (Peterson-Nedry et al., 2008 blue right-pointing triangle). Our data also suggest that this model is oversimplified and offer an alternate view.

We confirmed that removing the SAMPs alters APC–Axin interactions, substantially reducing both APC2–Axin co-IP and APC recruitment into large puncta forming upon Axin overexpression. Our data further suggest, however, that residual APC–Axin interaction remains in the absence of the SAMPs, as some enrichment of APC2ΔSAMP in larger Axin puncta remains. Confirming this finding, removing additional C-terminal sequences including 20R3-R5

and sequence B restored robust recruitment of APCd40 into Axin puncta. The nature of this interaction must be different, however, as it does not sustain co-IP—perhaps this complex disassembles upon cell lysis. Thus our data and that on AxinΔRGS (Peterson-Nedry et al., 2008 blue right-pointing triangle) support the existence of two distinct modes of APC–Axin interaction.

Sequence B and 20R2 clearly play essential, conserved roles in APC action in the destruction complex—APC2 cannot target βcat for destruction in their absence. Our data suggest a speculative model for their action. Removing sequence B alone restores APC2ΔSAMP recruitment into Axin puncta, suggesting that sequence B normally modulates APC–Axin interactions. We speculate that productive destruction of βcat requires cycles of assembly and disassembly of the destruction complex, with transfer to the E3 ubiquitin ligase perhaps coincident with complex disassembly (Figure 2.14A). The idea of the necessity for a catalytic cycle builds on earlier suggestions by Kimelman and Xu (2006) blue right-pointing triangle but suggests a new mechanism by which this occurs. In our speculative model, sequence B and perhaps 20R2 would be essential for complex disassembly after βcat phosphorylation, separating APC and Axin and allowing the destruction complex to reset to phosphorylate additional βcat. In mutants defective in disassembly (Figure 2.14B), perhaps βcat could be captured, phosphorylated, and even transferred to APC, but not released from the complex. This might ultimately trap all destruction complexes in this state, slowing or halting further βcat destruction—in contrast to the large APC pool, Axin is thought to be limiting (Salic et al., 2000 blue right-pointing triangle). Of course, this is speculative, and other possibilities exist. For example, 20R2 and sequence B may simply facilitate a conformational change driving the

reaction forward.

It is also important to note that our data suggest that interaction of Axin and APC via the SAMPs, although dispensable in SW480 cells, is essential for APC2's action in the destruction complex in Drosophila. One possible reason for the difference is that our and most other experiments in SW480 cells involve substantially overexpressing exogenous proteins, with APC at levels more than 50 times that seen in the fly (Figure 2.3D). Overexpressing Axin alone rescues βcat destruction in these cells. Perhaps even weak interaction of APC2ΔSAMP and Axin is sufficient when APC2ΔSAMP concentrations are artificially elevated. Similarly, elevating Axin levels may allow partially productive interactions with truncated APC present in tumor cells. In Drosophila at normal expression levels, in contrast, a more stable interaction mediated by the SAMPs may be critical for initially assembling destruction complexes, with an unknown mechanism then abrogating this interaction to release APC from Axin and reset the destruction complex. This idea is consistent with the fact that truncated mouse APC mutants lacking SAMPs are strongly reduced in their ability to regulate Wnt signaling (Smits et al., 1999 blue right-pointing triangle), suggesting that at normal expression levels SAMPs are also essential for mammalian APC. It will be exciting to test predictions of this model and further uncover the mechanisms of action of the destruction complex and possible novel partners involved.

Our data also raise questions about the oligomeric state of APC in the destruction complex. Drosophila APCs lack the N-terminal coiled-coil found in mammalian APCs, but the dominant-negative activity of APC2Δ15Δ20 in Drosophila and the ability of APC2ΔSAMPs to retain function in SW480 cells may suggest that APC2 oligomerizes in vivo with itself, with fly APC1, or even with mammalian APC—Drosophila APC1 and APC2 can recruit one another to

alternate locations if overexpressed (Akong et al., 2002b blue right-pointing triangle). It will be important to explore the mechanisms and role of oligomerization in assembly and function of the destruction complex.

Cytoplasmic retention: a regulatory mechanism in normal development and oncogenesis?

Comparing βcat localization in wild type and APC mutant cells and tissues suggested that APC and Axin can retain βcat in the cytoplasm (Tolwinski and Wieschaus, 2001 blue right-pointing triangle; Krieghoff et al., 2006 blue right-pointing triangle; McCartney et al., 2006 blue right-pointing triangle). The two biological systems we used allowed us to explore the mechanistic basis and biological roles of cytoplasmic retention during both normal development and in cancer cells.

Our data suggest that regulating βcat destruction is APC's key role, with cytoplasmic retention playing a modulatory role in some circumstances. We found that high-affinity βcat-binding sites are dispensable for βcat destruction. Instead, the array of βcat-binding sites in APC proteins appears to provide a sink for cytoplasmic retention of residual βcat, preventing it from entering nuclei to activate gene expression, and thus modulating Wnt signaling (Figure 2.14C). Distinctions in rescue of APC2 single mutants (Figure 2.5), combined with cell biological assessment of cytoplasmic retention in SW480 cells (Figure 2.4), suggest that multiple 15R and 20Rs mediate cytoplasmic retention, acting in an additive fashion.

In APC2 APC1 double mutants, in which no endogenous APC function remains, mutants restoring cytoplasmic retention but not βcat destruction were ineffective. We suspect that in the absence of all destruction complex activity, Arm levels exceed the buffering capacity of APC2's βcat-binding sites (Figure 2.14F). In contrast, mutants retaining βcat in the cytoplasm

significantly rescued cell fate choices in APC2 single mutants presumably because low-level APC1 in these embryos reduces Arm levels into the range where cytoplasmic retention can effectively limit signaling (Figure 2.14E). Because these same mutants also reduce TCF-regulated transcription in SW480 cells, βcat levels in SW480 cells must also still be within the range able to be restrained by cytoplasmic retention, at least when APC proteins are overexpressed.

Another interpretation of our data is that the differences in βcat localization that we observed in SW480 cells were due to differential ability of APC2 mutants to export βcat from the nucleus. APC can shuttle in and out of the nucleus, and some data support a role in assisting βcat export (reviewed in Bienz 2002 blue right-pointing triangle; Henderson and Fagatto, 2002 blue right-pointing triangle). APC-mediated nuclear export of βcat remains a possibility. We think, however, that it is less likely for several reasons. First, data from fluorescence recovery after photobleaching suggest that human APC may not affect the rate of βcat nuclear export (Krieghoff et al., 2006 blue right-pointing triangle). Second, only two of the identified nuclear export sequences (NESs) in human APC (Henderson and Fagatto, 2002 blue right-pointing triangle) are conserved in fly APC2—these reside in 20R3 and 20R4 (neither N-terminal NES sequence is conserved in fly APC2). APC2Δ20, which removes both of these sequences, can still trigger βcat accumulation in the cytoplasm instead of the nucleus of SW480 cells. These data are more consistent with a role in retention rather than nuclear export, but, given the degenerate nature of NES sequences, we cannot rule out a role for APC in βcat nuclear export.

Cytoplasmic retention: helping explain selection of truncated APC proteins in cancer

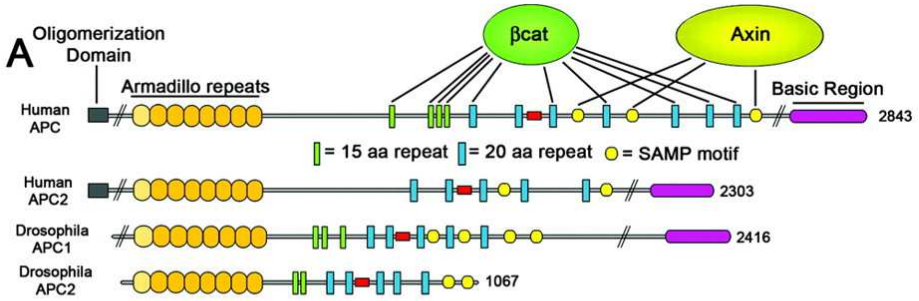
Unlike most tumor suppressors, APC mutant colon tumors are not homozygous mutant for null mutations. Instead, essentially all carry at least one allele encoding a truncated protein (Polakis, 1997 blue right-pointing triangle). Most are truncated in the mutation cluster region (MCR) between the end of 20R1 and the beginning of 20R3. There has been much discussion of reasons for this truncation. Hypotheses range from suggesting that truncated proteins play dominant-negative roles in Wnt regulation, to those suggesting they have a "gain-of-function." One prominent hypothesis with considerable experimental support is the "just right" hypothesis (Albuquerque et al., 2002 blue right-pointing triangle), suggesting that truncated proteins have lost some but not all ability to target βcat for destruction. Consistent with this hypothesis, tumor cell lines truncated earlier than the MCR have higher levels of TCF-regulated transcription than do those truncated in the MCR. Our earlier data on the function of similar truncation mutants in Drosophila APC2 were consistent with this (McCartney et al., 2006 blue right-pointing triangle). Somewhat surprisingly, however, truncated APC2 did not rescue function in APC2 APC1 double mutants. Thus, despite support for the "just-right" model, the mechanism by which truncated proteins provide residual Wnt regulation remained unknown.

Our new data provide a strong hypothesis for the mechanism by which truncated APC proteins retain partial function. Our data demonstrate that truncated proteins are completely unable to function in the destruction complex to target βcat for destruction, in either SW480 cells or Drosophila. Instead, their ability to retain βcat in the cytoplasm provides the most plausible explanation for their residual ability to regulate Wnt signaling in colon cancer cells (Figure 2.14D). Intriguingly, our data also suggest that loss of SAMPs and some 20Rs reduces

cytoplasmic retention more than does loss of those 20Rs alone. This finding further emphasizes how selection for particular truncations fine-tunes Wnt signaling in cancer cells.

Our data also open the possibility that cytoplasmic retention plays an important role in normal Wnt regulation. In at least some circumstances, Wnt signals act in a graded fashion over a field of cells (e.g., Zecca et al., 1996 blue right-pointing triangle). Cytoplasmic retention may help buffer βcat, blunting the transcriptional response until the destruction complex is saturated with βcat. In fact, in the fly embryonic epidermis, a substantial amount of Arm is in the cytoplasm of cells receiving Wg signal, as well as in nuclei (Peifer et al., 1994 blue right pointing triangle). Retention may help turn graded signaling into a more binary response, with sharper thresholds. This possibility can now be explored.

Figure 2.1



	SW480 cells			APC2 single mutant			APC2 APC1 double mutant
B	Destroy ßcat	Retain ßcat	TOP FLASH	Rescues cell fate	Embryo viability	Adult viable	Rescues cell fate and embryo viability
WT APC2	Yes	N.A.	Low	+++	+++	Yes	Yes
APC2ΔR3	Yes	N.A.	Low	+++	+++	Yes	Yes
APC2ΔR1	Yes	N.A.	Low	N.D.	N.D.	N.D.	N.D.
ΔR1,R4-R5	Yes	N.A.	Low	+++	++	Yes	N.D.
ΔR3-R5	Yes	N.A.	Low	+++	++	Yes	N.D.
Keep R3	No	Yes	High	++	-	No	No
APC2Δ20	No	Yes	High	++	-	No	No
APC2Δ15	Yes	N.A.	Low	+++	++	Yes	Yes
Δ15Δ20	No	No	High	-	-	No	No
APC2ΔR2	No	Yes	Medium	+++	++	Yes	No
APC2ΔB	No	Yes	Medium	+++	++	Yes	No
APC2d40	No	No	High	+/-	+/-	No	No
EndatB	Yes	N.A.	Medium	+	+/-	No	N.D.
ΔSAMP	Yes	N.A.	Low	+	+/-	No	No
ΔBΔSAMP	No	Yes	High	N.D.	N.D.	N.D.	N.D.

Figure 2.1. Diagrams of wild-type APC2 and the mutants used, and summary of the functions of each mutant. Scale bar is in amino acids. Both flies and mammals have two APC family members that share a core including the highly conserved Arm repeats, as well as 15Rs, 20Rs, and SAMPs. The C-terminal regions of APC family proteins are much more divergent, both within and between animal phyla, and Drosophila APCs lack the N-terminal coiled-coil oligomerization domain found in mammalian APCs. Summaries of the results of functional tests in SW480 cells and in Drosophila, as detailed in the subsequent figures and Table 1. N.A., not applicable; N.D., not done.

Figure 2.2

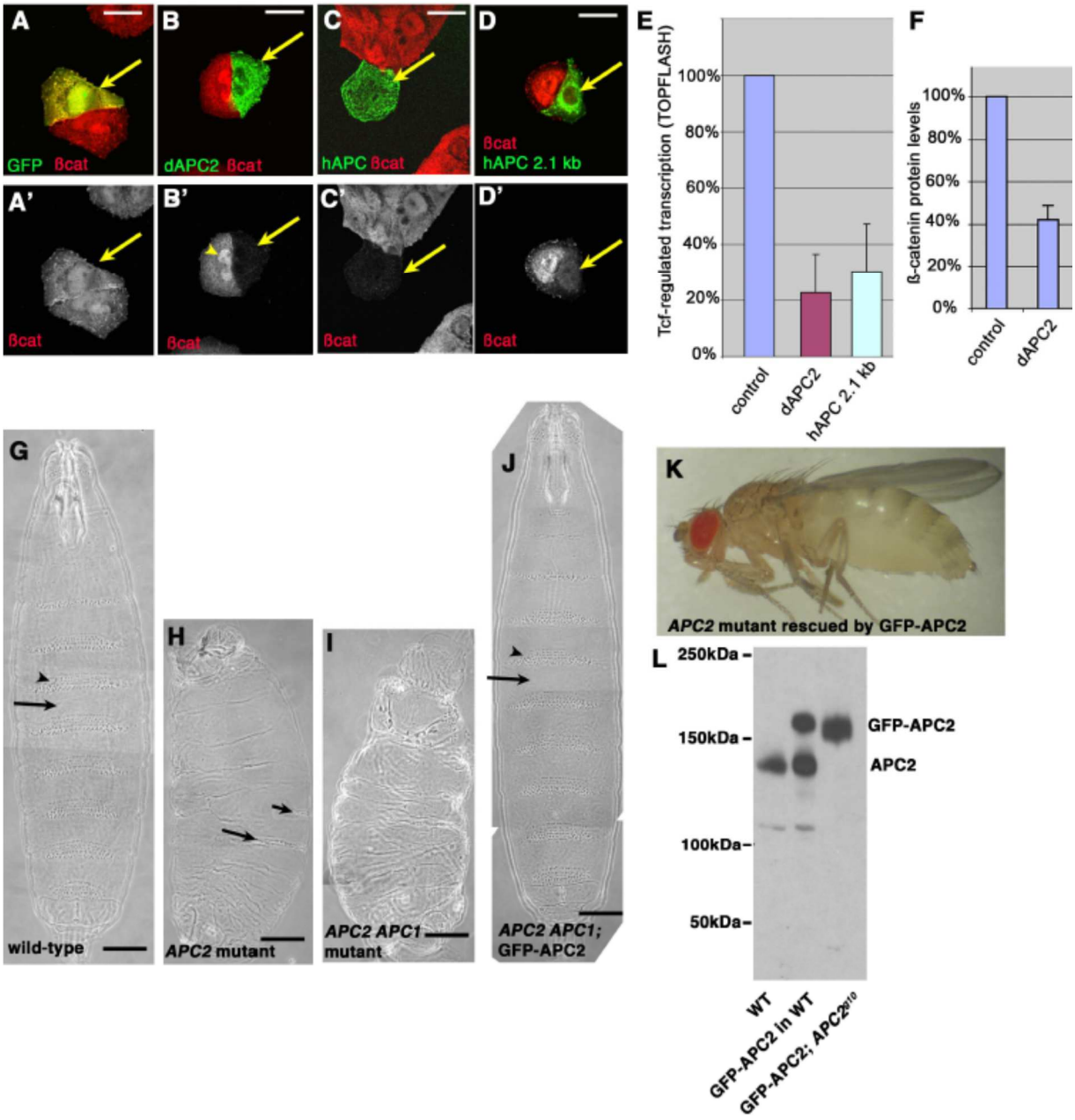


Figure 2.2. Fly and human APC proteins reduce βcat levels in SW480 cells, and fly APC restores cuticle patterning in APC single and double mutant flies. (A-D) SW480 cells transfected with GFP-tagged wild-type APC2 (A) or the indicated mutants. Arrows indicate representative cells transfected with wild type or mutant APC. (E) TOPFLASH assays revealing TCF-dependent transcription.(F) βcat levels as quantified by Cellomics. (G-J) Rescue of Wnt-mediated cell-fate decisions in APC2g10 maternal/zygotic single and APC2g10APC1Q8 double mutants.(K) APC2 expression rescues to adulthood. (L) Expression of APC2 transgene is similar to endogenous APC2 levels.

Figure 2.3

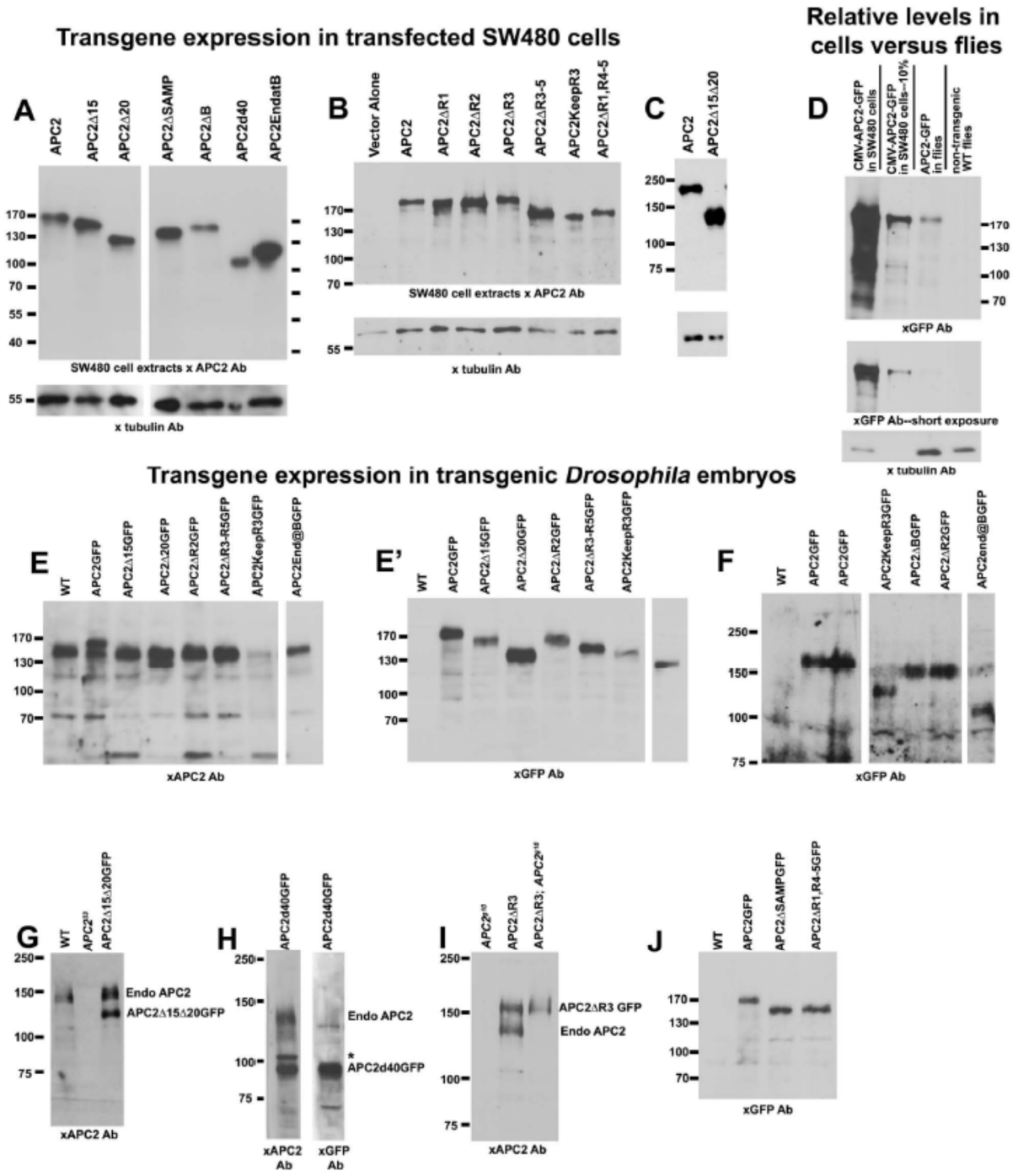


Figure 2.3. Expression levels of transgenes in SW480 cells and in Drosophila embryos

Figure 2.4

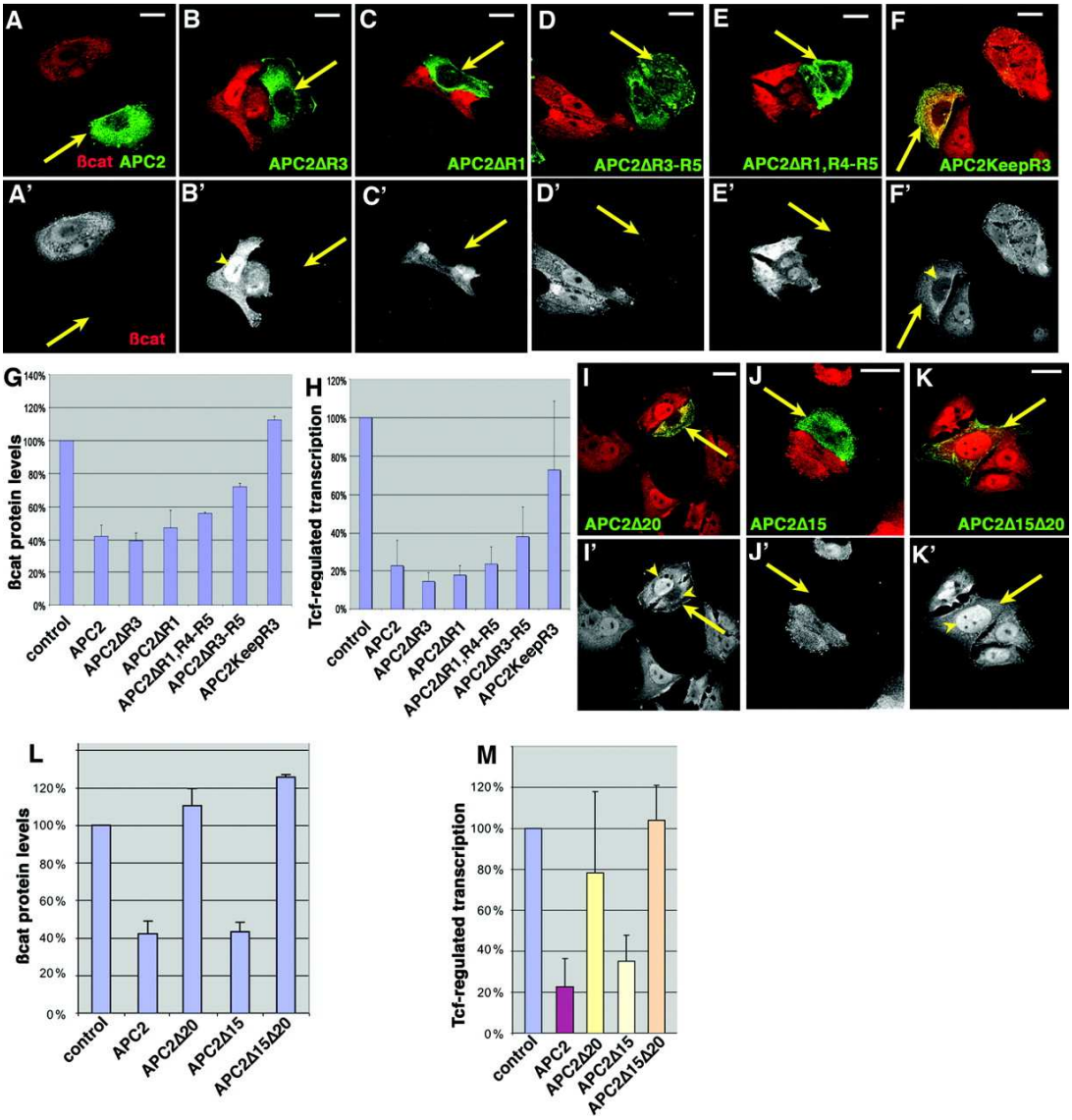


Figure 2.4. Individual high-affinity 20Rs are not essential for destruction but contribute with 15Rs to cytoplasmic retention. (A–F and I–K) SW480 cells transfected with GFP-tagged wild-type APC2 (A) or the indicated mutants. Arrows indicate representative cells transfected with wild type or mutant APC. Arrowheads = nuclear βcat accumulation in control untransfected cells, and selected mutants that can (APC2KeepR3, APC2Δ20) or cannot (APC2Δ15Δ20) retain βcat in the cytoplasm. (G and L) βcat levels as quantified by Cellomics. (H and M) TOPFLASH assays revealing TCF-dependent transcription.

Figure 2.5

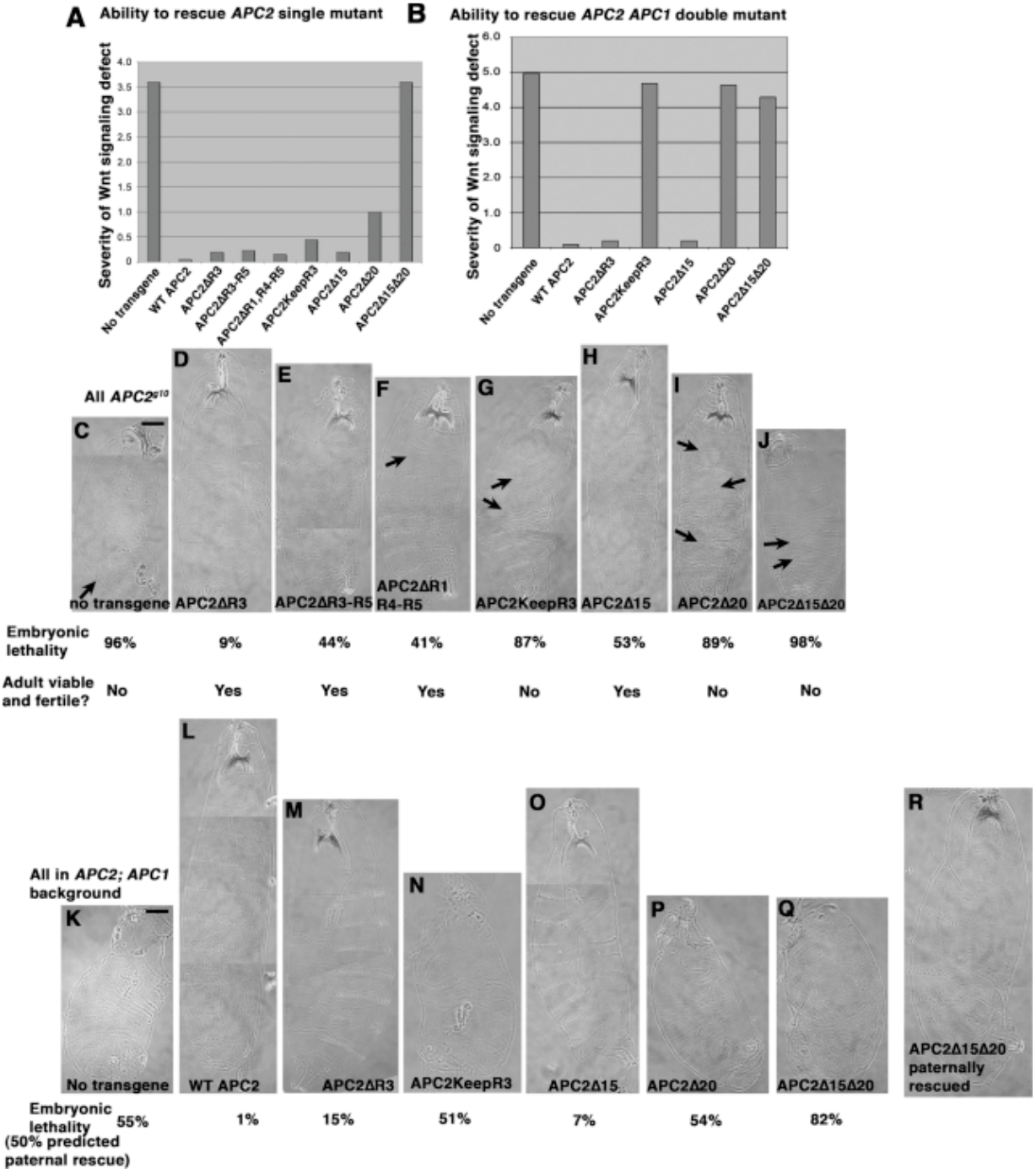

A
Ability to rescue APC2 single mutant
Severity of Wnt signaling defect
No transgene
WT APC2
APC2ΔR3
APC2ΔR3-R5
APC2ΔR1,R4-R5
APC2KeepR3
APC2Δ15
APC2Δ20
APC2Δ15Δ20
B
Ability to rescue APC2 APC1 double mutant
Severity of Wnt signaling defect
All APC2s10
C
D
E
F
G
H
I
J
no transgene
APC2ΔR3
APC2ΔR3-R5
APC2ΔR1 R4-R5
APC2KeepR3
APC2Δ15
APC2Δ20
APC2Δ15Δ20
Embryonic lethality
96%
9%
44%
41%
87%
53%
89%
98%
Adult viable and fertile?
No
Yes
Yes
Yes
No
Yes
No
No
All in APC2; APC1 background
K
L
M
N
O
P
Q
R
No transgene
WT APC2
APC2ΔR3
APC2KeepR3
APC2Δ15
APC2Δ20
APC2Δ15Δ20
APC2Δ15Δ20 paternally rescued
Embryonic lethality (50% predicted paternal rescue)
55%
1%
15%
51%
7%
54%
82%

Figure 2.5. Many βcat-binding sites are dispensable for Arm destruction but contribute additively to rescue APC2 single mutants. (A and B) Rescue of Wnt-mediated cell-fate decisions in APC2g10 maternal/zygotic single mutants (A) or APC2g10 APC1Q8 maternal/zygotic double mutants (B). Cuticles scored as Figure 2.15. 0 = wild-type and 6 = strongest loss-of-function phenotype observed. (C–J) Representative cuticles showing ability of transgenes to rescue APC2g10 maternal/zygotic single mutants. Below cuticles are rescue of embryonic lethality and of adult viability and fertility (ability of rescued flies to go at least two generations). Arrow in C and J = residual denticles. Arrows in F, G, and I = missing denticles. (K–R) Representative cuticles showing ability of transgenes to rescue APC2g10 APC1Q8 maternal/zygotic double mutants. Below cuticles are rescue of embryonic lethality. Because fathers are heterozygous, 50% of embryos are paternally rescued. APC2Δ15Δ20 had >50% lethality, suggesting that some paternally rescued embryos die. A putative embryo like this is shown in R.

Figure 2.6

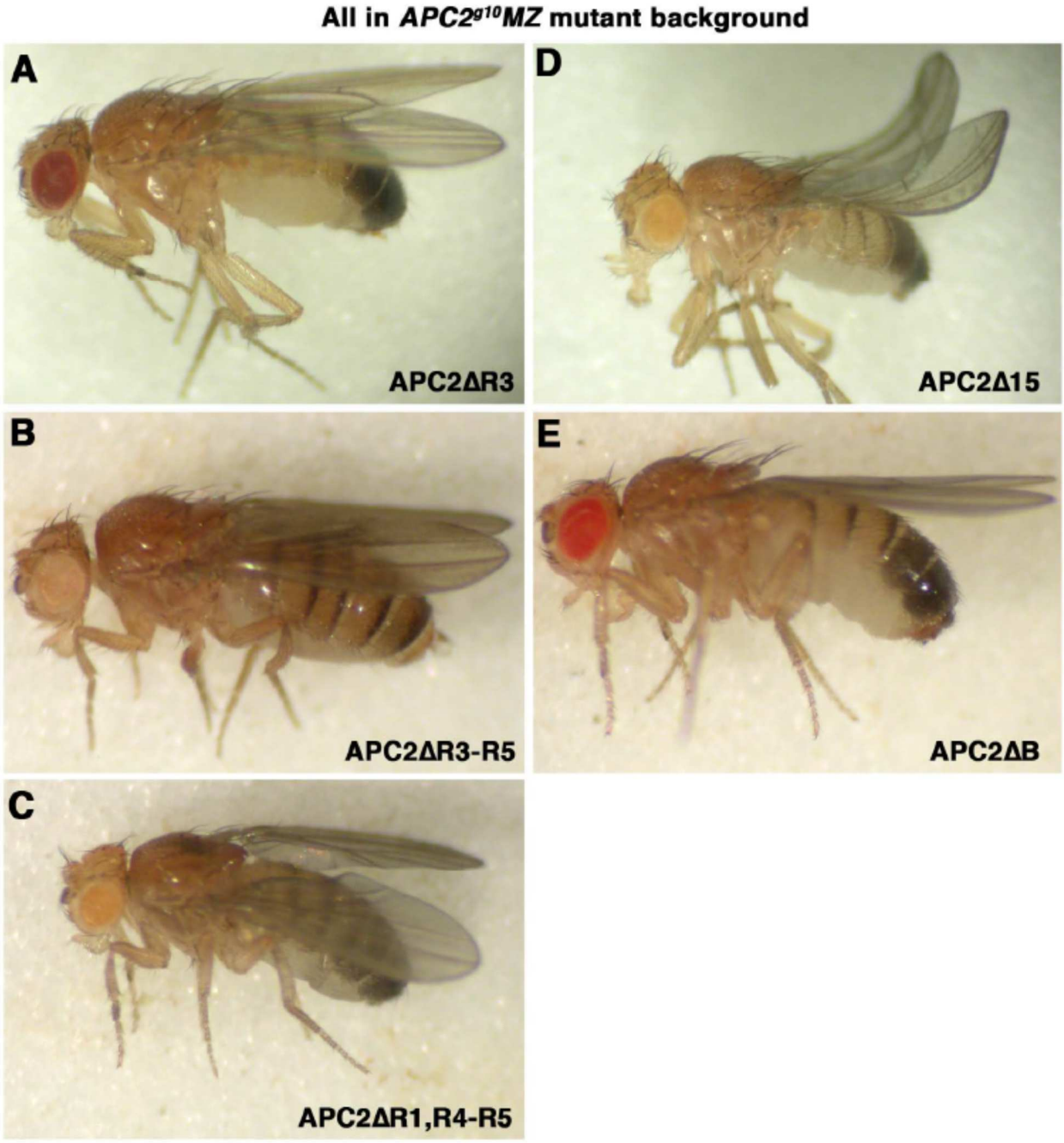


Figure 2.6. APC2 mutant transgenes rescue APC2g10 single mutants to adulthood

Figure 2.7

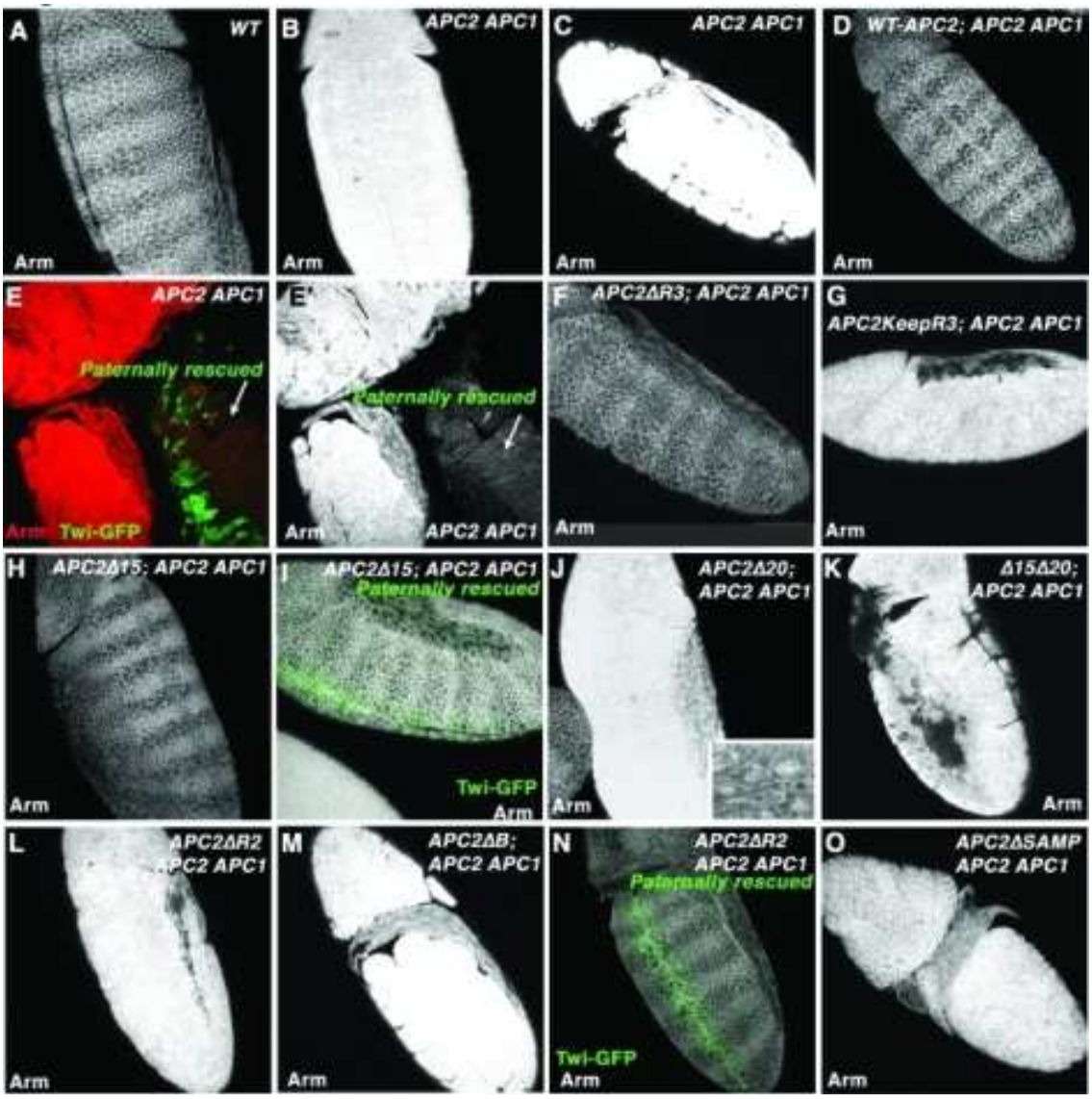


Figure 2.7. Roles of 20Rs and sequence B in targeting Arm for destruction in vivo. Stage 9–10 embryos *APC2*^{g10} *APC1*^{Q8} maternal/zygotic double mutants expressing indicated transgenes stained for Arm; Twist-GFP (Twi-GFP) on the Balancer was used to distinguish maternal/zygotic mutants and paternally rescued embryos. (A) Wild type. Normal Arm stripes, stabilized by segmentally repeated stripes of Wg signal. (B) Highly elevated Arm levels in stage 9 (B) or stage 10 (C) *APC2*^{g10} *APC1*^{Q8} maternal/zygotic double mutants. (D) Wild-type APC2 transgene restores normal Arm levels. (E) Adjacent *APC2*^{g10} *APC1*^{Q8} maternal/zygotic double mutants and paternally rescued embryos. (F and H). APC2ΔR3 (F) and APC2Δ15 (H) restore normal Arm levels. (G, I–L, and N) APC2KeepR3 (G), APC2Δ20 (J), APC2Δ15Δ20 (K), APC2ΔR2 (L), APC2ΔB (M), and APC2ΔSAMP (O) all cannot restore Arm destruction. (I, inset) Arm accumulates in nuclei of amnioserosal cells when destruction is inactivated. (I and N) Paternally rescued embryos illustrating diagnostic Twi-GFP pattern and normal Arm levels.

Figure 2.8

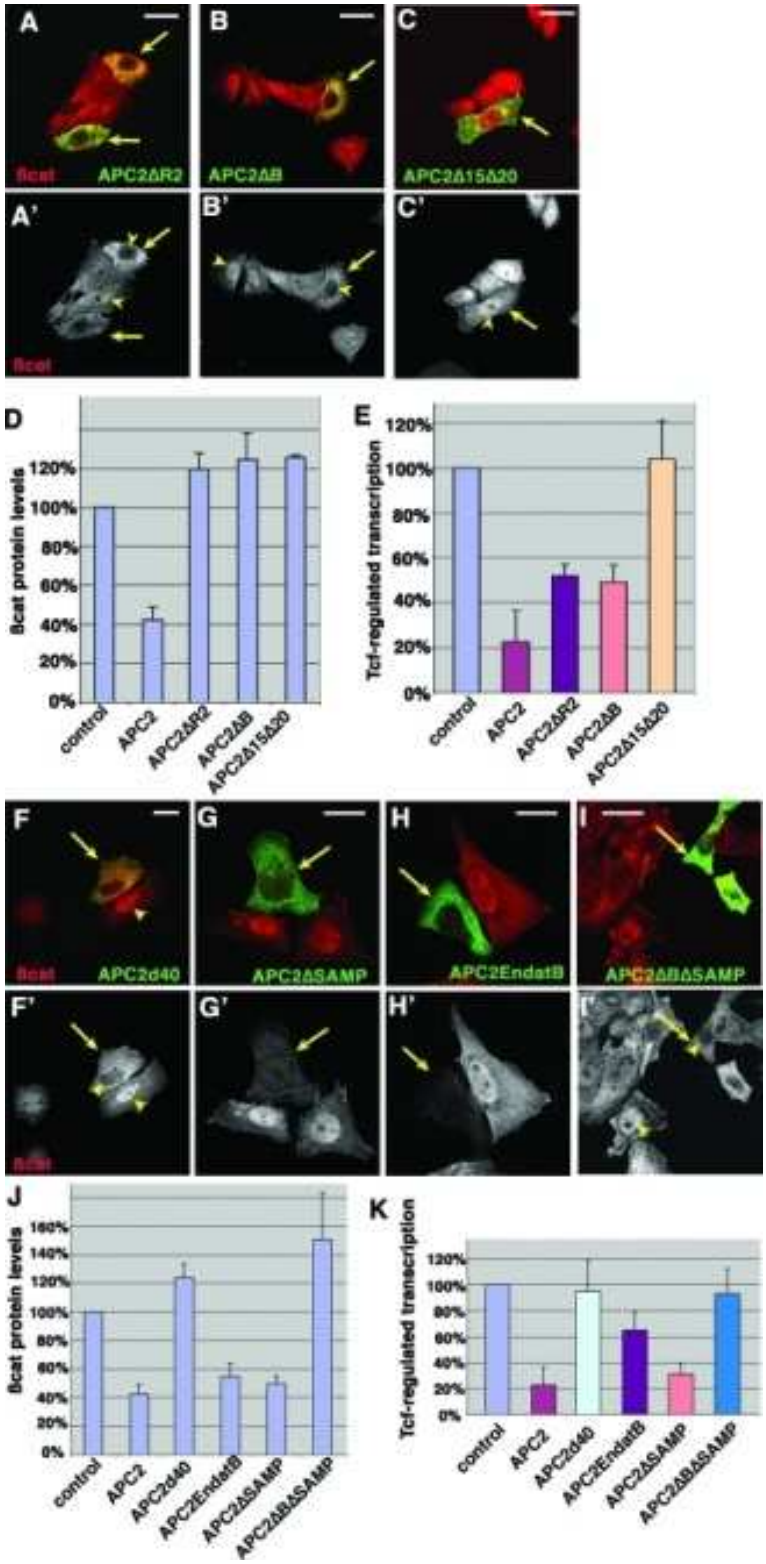

A
B
C
βcat
APC2ΔR2
APC2ΔB
APC2Δ15Δ20
A'
B'
C'
βcat
D
βcat protein levels
120%
100%
80%
60%
40%
20%
0%
control
APC2
APC2ΔR2
APC2ΔB
APC2Δ15Δ20
E
Tcf-regulated transcription
120%
100%
80%
60%
40%
20%
0%
control
APC2
APC2ΔR2
APC2ΔB
APC2Δ15Δ20
F
G
H
I
βcat
APC2d40
APC2ΔSAMP
APC2EndatB
APC2ΔBΔSAMP
F'
G'
H'
I'
βcat
J
βcat protein levels
160%
140%
120%
100%
80%
60%
40%
20%
0%
control
APC2
APC2d40
APC2EndatB
APC2ΔSAMP
APC2ΔBΔSAMP
K
Tcf-regulated transcription
120%
100%
80%
60%
40%
20%
0%
control
APC2
APC2d40
APC2EndatB
APC2ΔSAMP
APC2ΔBΔSAMP

Figure 2.8. Sequence B and 20R2 are essential for βcat destruction by APC2 in SW480 cells. (A–C and F–I) SW480 cells transfected with GFP-tagged APC2 mutants. Arrows indicate representative cells transfected with mutant APC proteins. Arrowheads = nuclear βcat accumulation in control untransfected cells, compared to mutants that can retain βcat in the cytoplasm (APC2ΔR2, APC2ΔB, APC2d40, APC2ΔBΔSAMP). (D and J) βcat levels as quantified by Cellomics. (E and K) TOPFLASH assays revealing TCF-dependent transcription.

Figure 2.9

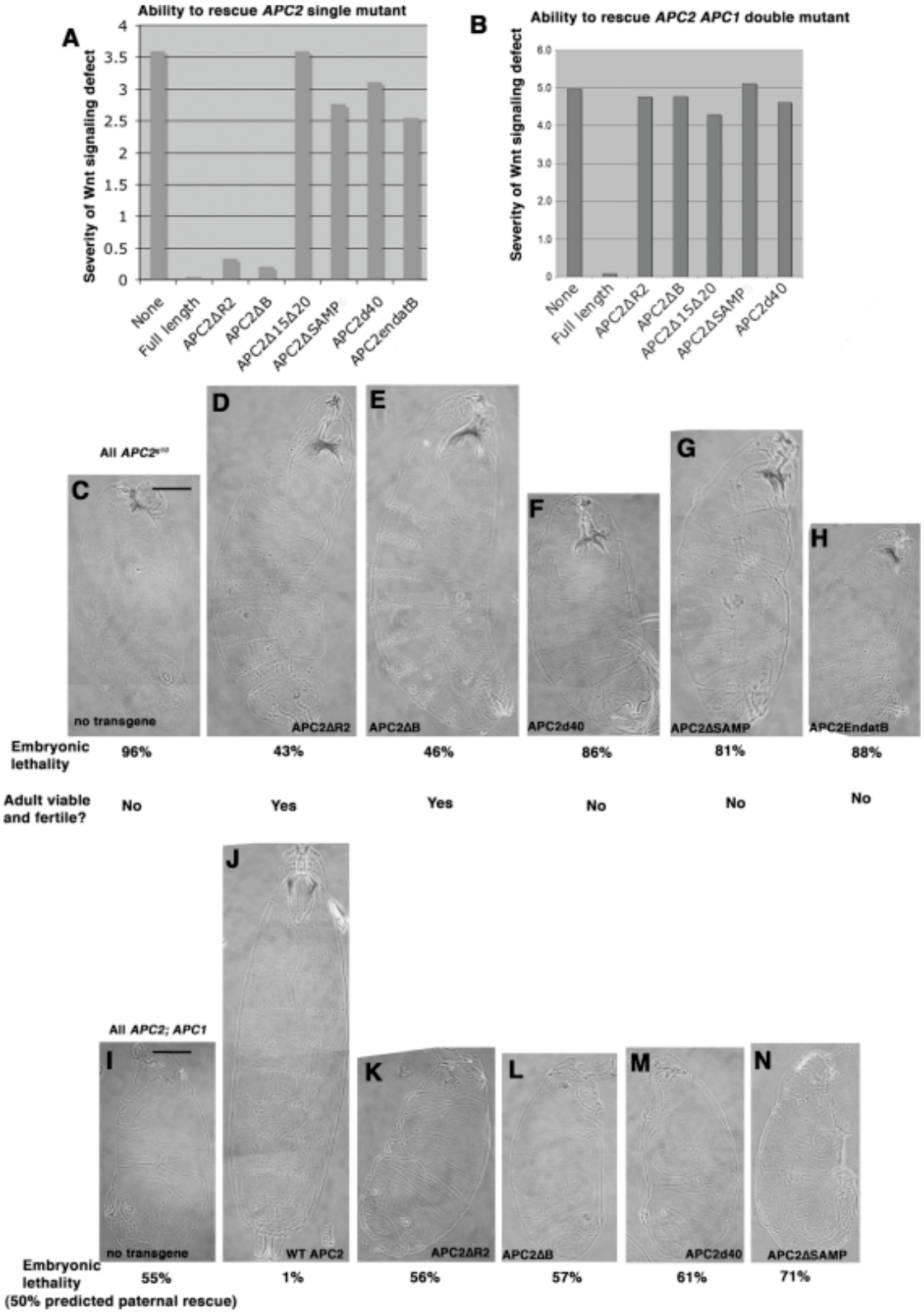

A
Ability to rescue APC2 single mutant
Severity of Wnt signaling defect
4
3.5
3
2.5
2
1.5
1
0.5
0
None
Full length
APC2ΔR2
APC2ΔB
APC2Δ15Δ20
APC2ΔSAMP
APC2d40
APC2endatB
B
Ability to rescue APC2 APC1 double mutant
Severity of Wnt signaling defect
6.0
5.0
4.0
3.0
2.0
1.0
0
None
Full length
APC2ΔR2
APC2ΔB
APC2Δ15Δ20
APC2ΔSAMP
APC2d40
All APC2
C
no transgene
D
APC2ΔR2
E
APC2ΔB
F
APC2d40
G
APC2ΔSAMP
H
APC2EndatB
Embryonic lethality
96%
43%
46%
86%
81%
88%
Adult viable and fertile?
No
Yes
Yes
No
No
No
All APC2; APC1
I
no transgene
J
WT APC2
K
APC2ΔR2
L
APC2ΔB
M
APC2d40
N
APC2ΔSAMP
Embryonic lethality
(50% predicted paternal rescue)
55%
1%
56%
57%
61%
71%

Figure 2.9. Sequence B, 20R2, and the SAMPs are essential for Arm destruction in vivo. (A and B) Rescue of Wnt-mediated cell-fate decisions in APC2g10 maternal/zygotic single mutants (A) or APC2g10 APC1Q8 maternal/zygotic double mutants (B). 0 is wild type and 6 the strongest loss-of-function phenotype observed. (C–H) Representative cuticles showing ability of transgenes to rescue APC2g10 maternal/zygotic single mutants. Below cuticles are rescue of embryonic lethality and of adult viability and fertility (ability of rescued flies to go at least two generations). (I–N) Representative cuticles illustrating ability of transgenes to rescue APC2g10 APC1Q8 maternal/zygotic double mutants. Below cuticles are rescue of embryonic lethality. Because fathers are heterozygous, 50% of embryos are paternally rescued.

Figure 2.10

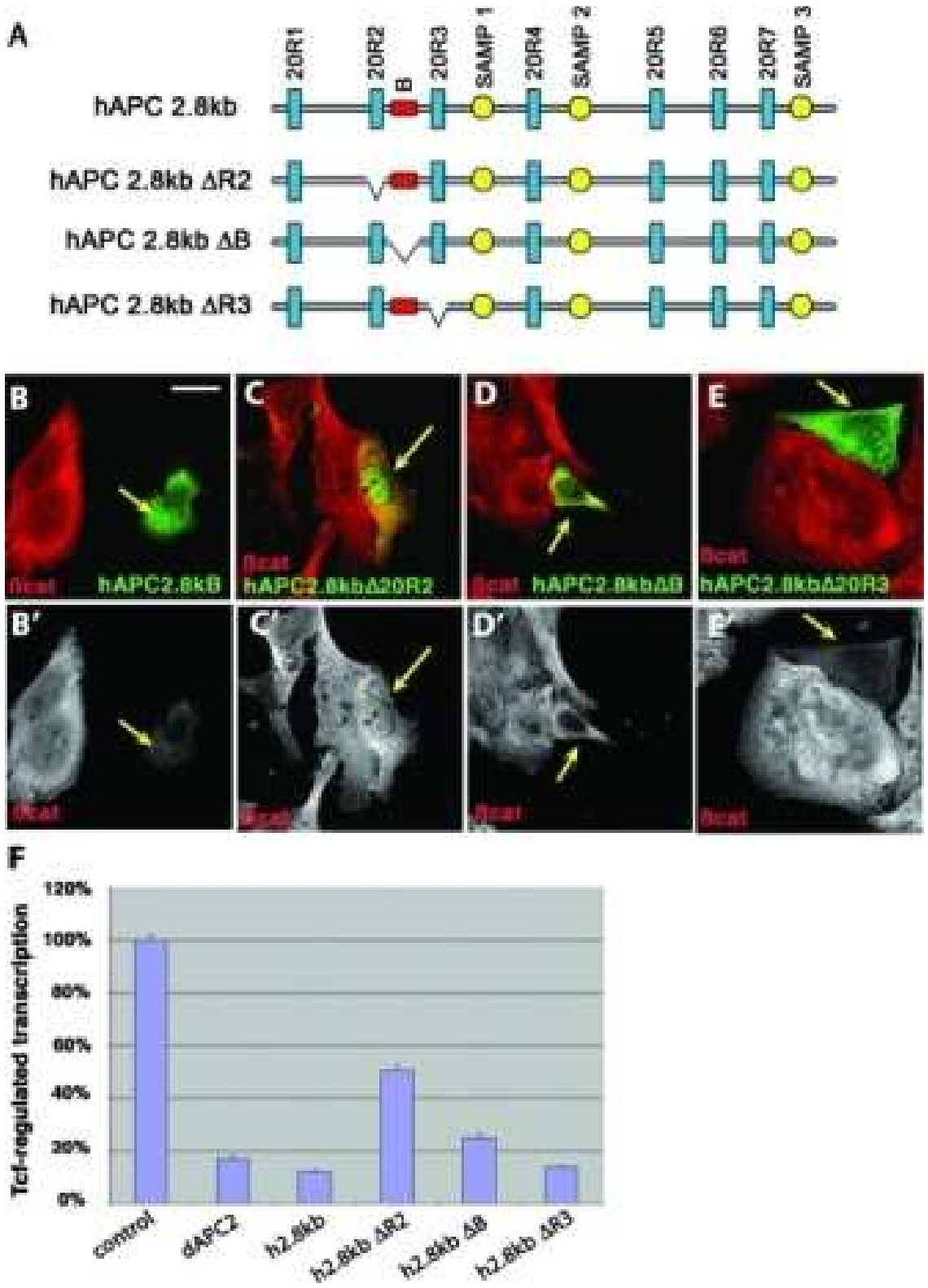

A
20R1
20R2
B
20R3
SAMP 1
20R4
SAMP 2
20R5
20R6
20R7
SAMP 3
hAPC 2.8kb
hAPC 2.8kb ΔR2
hAPC 2.8kb ΔB
hAPC 2.8kb ΔR3
B
C
D
E
hAPC2.8kB
hAPC2.8kbΔ20R2
hAPC2.8kbΔB
hAPC2.8kbΔ20R3
B'
C'
D'
E'
F
Tcf-regulated transcription
120%
100%
80%
60%
40%
20%
0%
control
dAPC2
h2.8kb
h2.8kb ΔR2
h2.8kb ΔB
h2.8kb ΔR3

Figure 2.10. Sequence B and 20R2 are also essential for βcat destruction by human APC. (A) Constructs used. (B–E) SW480 cells transfected with GFP-tagged fragments of human APC. Arrows indicate representative cells transfected with wild type or mutant APC fragments. (F) TOPFLASH assays revealing TCF-dependent transcription.

Figure 2.11

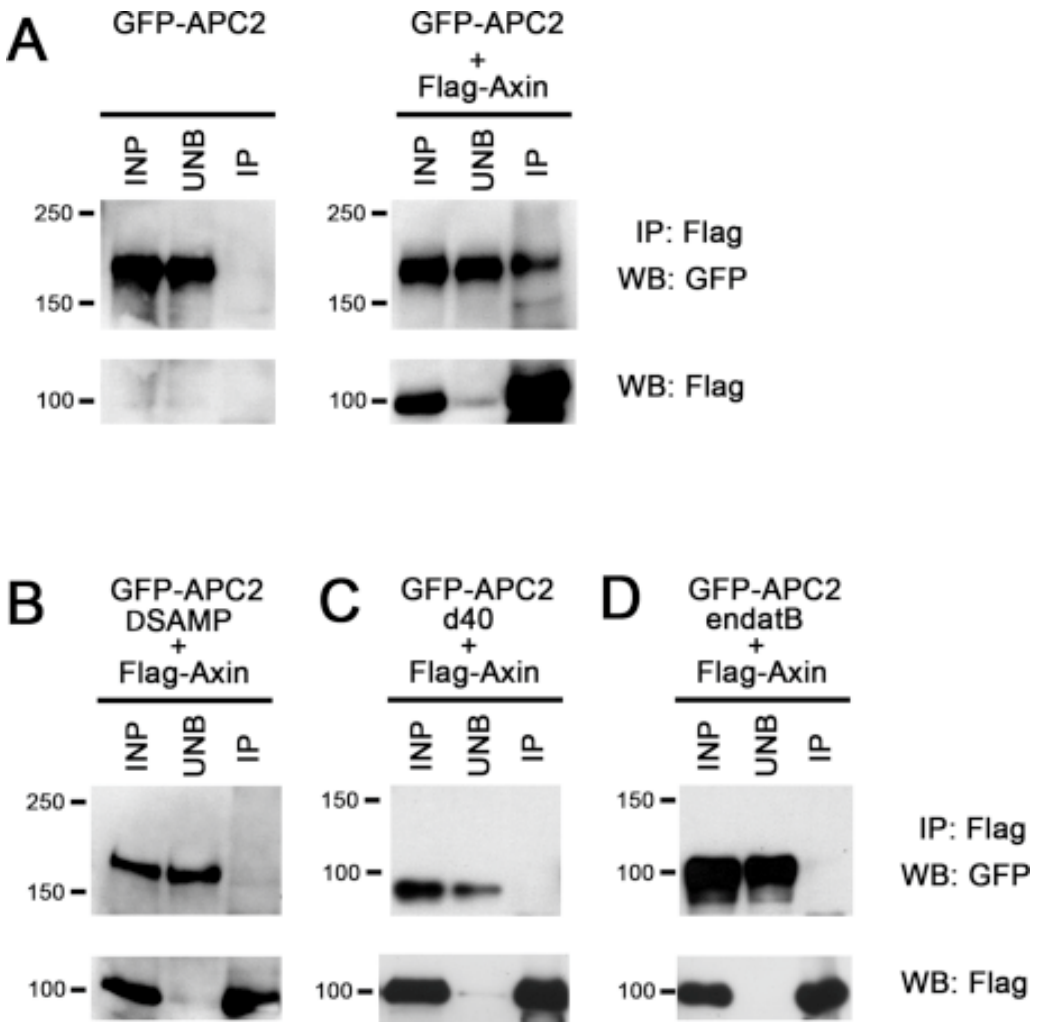


Figure 2.11. The SAMPs are necessary for APC2 to coimmunoprecipitate with Axin. Protein extracts from SW480 cells cotransfected with GFP-dAPC2 constructs and Flag-dAxin immunoprecipitated with a Flag antibody. (A) Full-length dAPC2 coimmunoprecipitates with dAxin. Cells transfected with GFP-dAPC2 alone were used as a negative control. (B–D) GFP-APC2ΔSAMPs (B), GFP-APC2d40 (C), and GFP-APC2End at B (D) all fail to coimmunoprecipitate with Flag-dAxin despite the observation that APC2-d40 colocalizes with dAxin in SW480 cells.

Figure 2.12

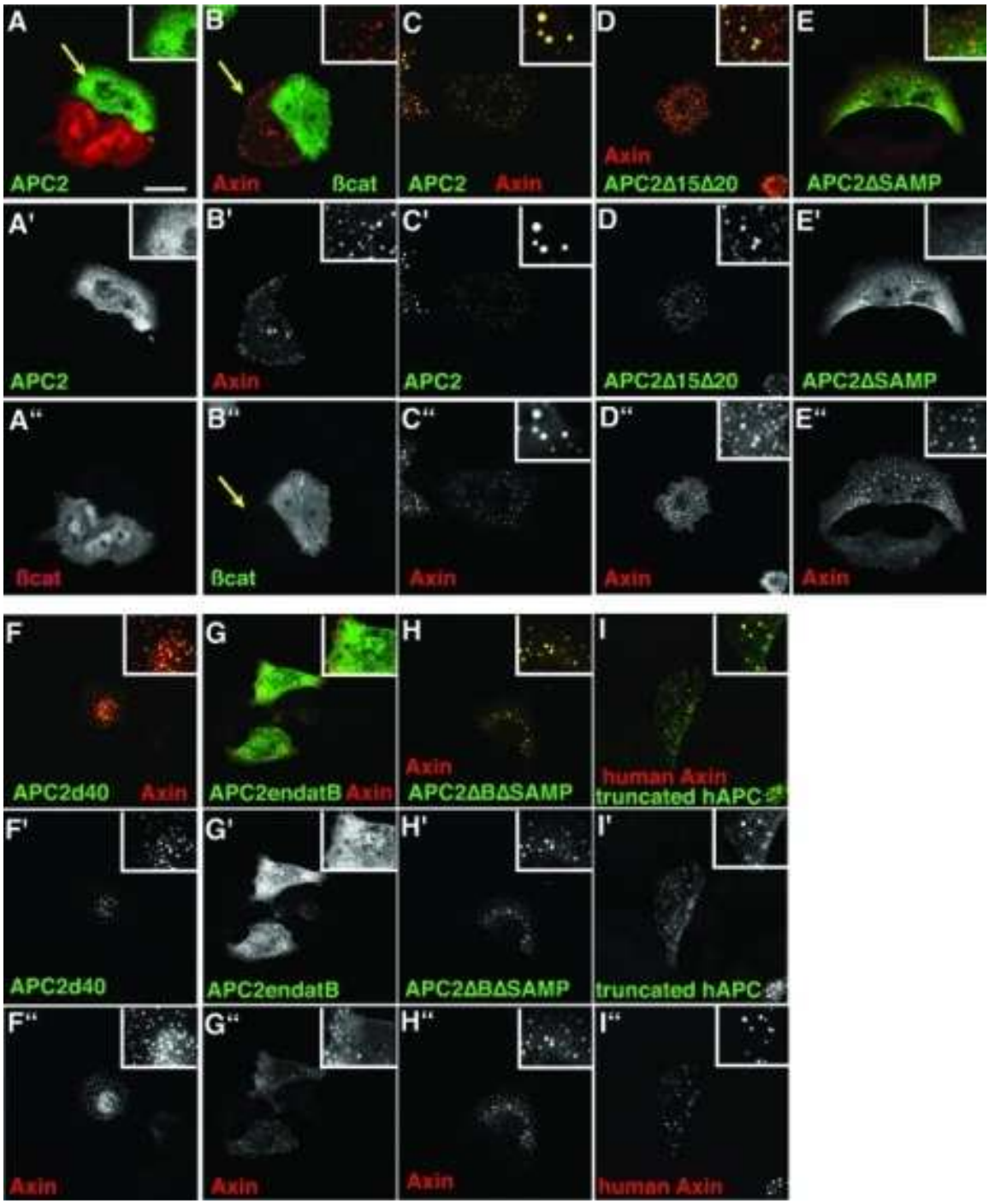

A
APC2
B
Axin
ßcat
C
APC2
Axin
D
Axin
APC2Δ15Δ20
E
APC2ΔSAMP
A'
APC2
B'
Axin
C'
APC2
D
APC2Δ15Δ20
E'
APC2ΔSAMP
A''
ßcat
B''
ßcat
C''
Axin
D''
Axin
E''
Axin
F
APC2d40
Axin
G
APC2endatB
Axin
H
Axin
APC2ΔBΔSAMP
I
human Axin
truncated hAPC
F'
APC2d40
G'
APC2endatB
H'
APC2ΔBΔSAMP
I'
truncated hAPC
F''
Axin
G''
Axin
H''
Axin
I''
human Axin

Figure 2.12. APC2 colocalization with Axin is SAMP-independent and is antagonized by sequence B. (A–H) SW480 cells transfected with GFP-tagged wild type or mutant APC2 and Flag-tagged fly Axin. Arrows in A and B = transfected cells. Insets = enlargements of puncta. (A) APC2 is diffusely cytoplasmic in SW480 cells. (B) Overexpressed Axin forms cytoplasmic puncta and can trigger βcat destruction. (C) When coexpressed, Axin recruits APC2 into puncta. (D) APC2's βcat-binding sites are not essential for recruitment into puncta. (E) Deletion of the SAMPs greatly reduces recruitment into puncta. (F) A protein truncated after 20aaR2 is once again recruited into Axin puncta. (G) Recruitment of a protein truncated after sequence B is much less robust. (H) Deletion of sequence B restores recruitment into Axin puncta of an APC2 mutant lacking the SAMPs. (I) Truncated human APC (aa 1–1338) also colocalizes to human Axin puncta.

Figure 2.13

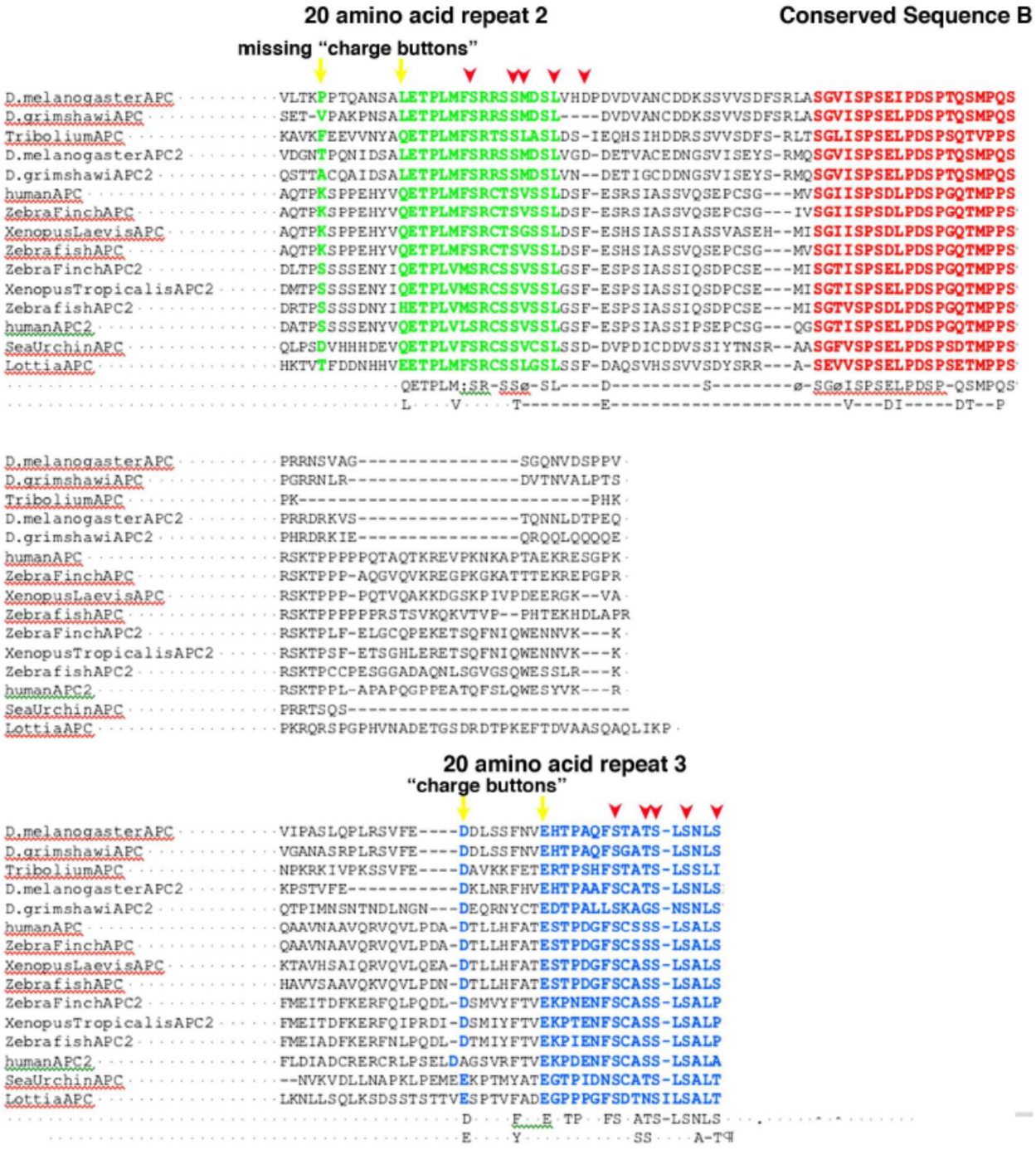


Figure 2.13. Alignment of the MCR region of APC of different species.

Figure 2.14

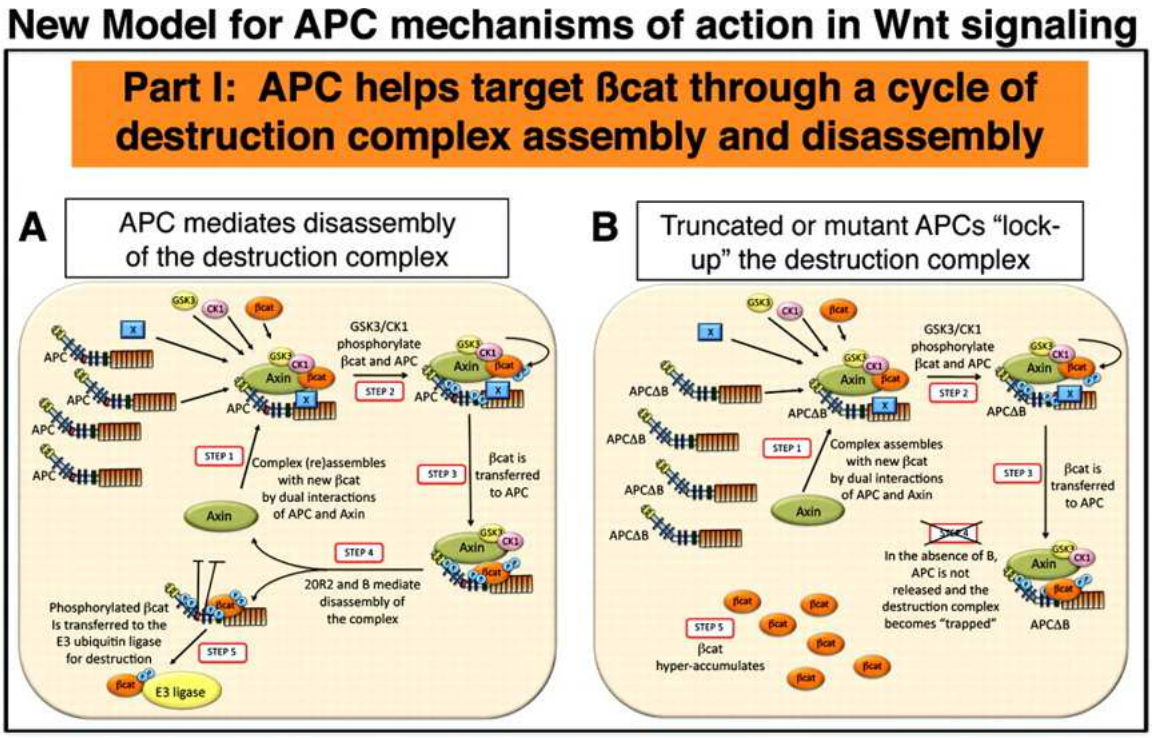


Figure 2.14. Model: APC regulates βcat signaling through a cycle of destruction complex assembly and disassembly. This model builds on earlier suggestions of a catalytic cycle (Kimelman and Xu, 2006 blue right-pointing triangle. Major new features are the essential natures of 20R2 and Sequence B in βcat destruction, with the suggestion that they play roles in complex disassembly, and the idea that truncated human APC proteins cannot target βcat for destruction but instead modulate signaling by cytoplasmic retention.

Figure 2.15

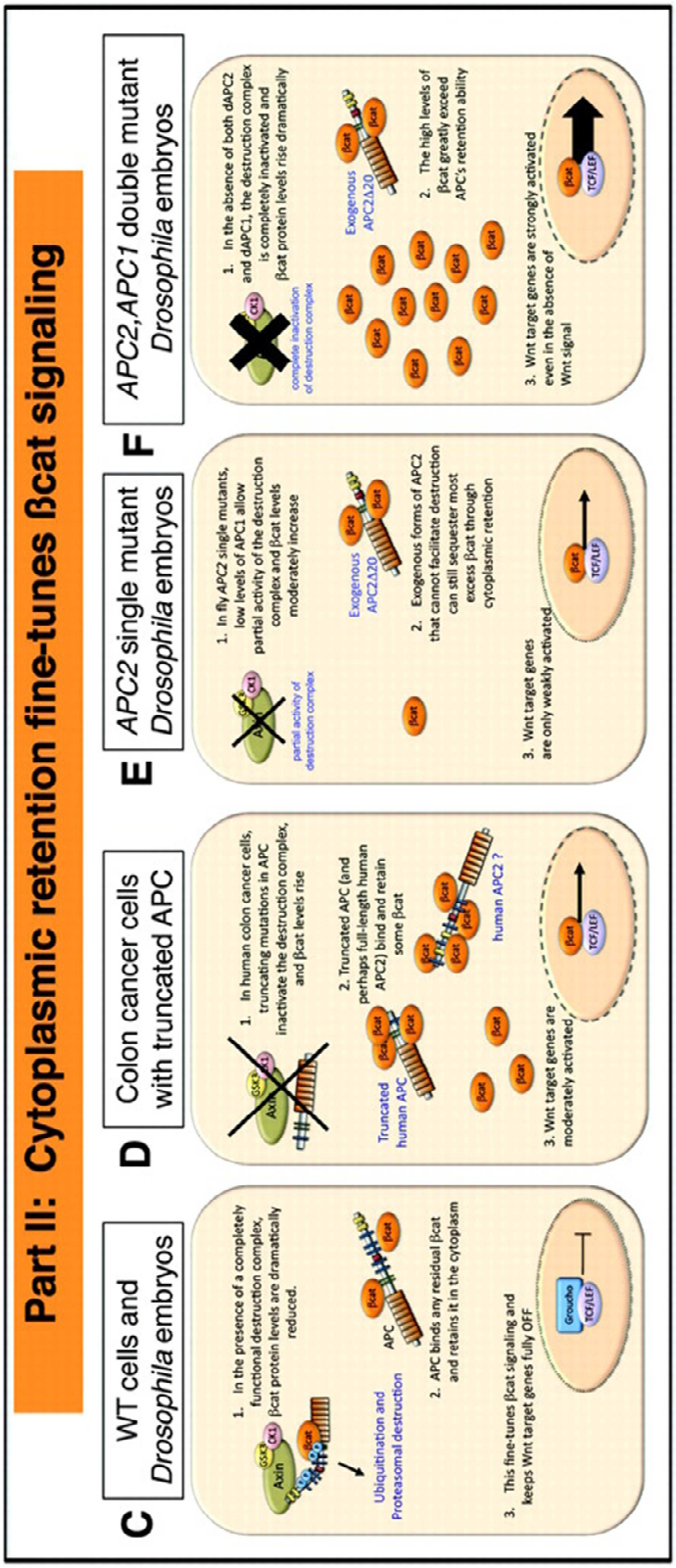
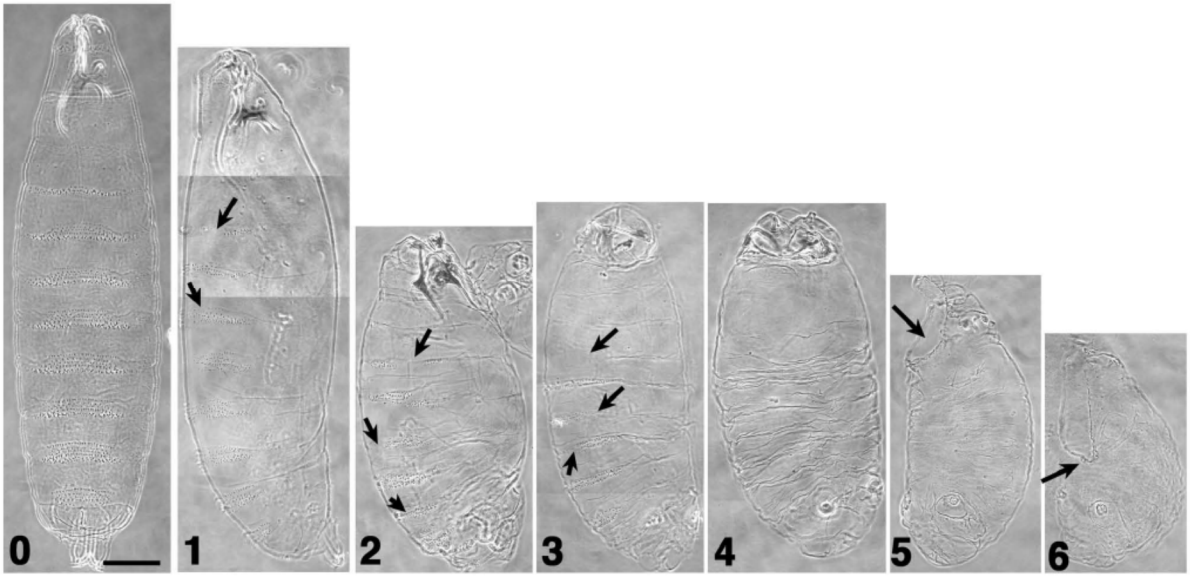

Part II: Cytoplasmic retention fine-tunes βcat signaling
C
WT cells and Drosophila embryos
1. In the presence of a completely functional destruction complex, βcat protein levels are dramatically reduced.
Ubiquitination and Proteasomal destruction
2. APC binds any residual βcat and retains it in the cytoplasm
3. This fine-tunes βcat signaling and keeps Wnt target genes fully OFF
Groucho
TCF/LEF
D
Colon cancer cells with truncated APC
1. In human colon cancer cells, truncating mutations in APC inactivate the destruction complex, and βcat levels rise
2. Truncated APC (and perhaps full-length human APC2) bind and retain some βcat
Truncated human APC
human APC2 ?
3. Wnt target genes are moderately activated.
E
APC2 single mutant Drosophila embryos
1. In fly APC2 single mutants, low levels of APC1 allow partial activity of the destruction complex and βcat levels moderately increase
partial activity of destruction complex
Exogenous APC2Δ20
2. Exogenous forms of APC2 that cannot facilitate destruction can still sequester most excess βcat through cytoplasmic retention
3. Wnt target genes are only weakly activated.
F
APC2,APC1 double mutant Drosophila embryos
1. In the absence of both dAPC2 and dAPC1, the destruction complex is completely inactivated and βcat protein levels rise dramatically
complete inactivation of destruction complex
Exogenous APC2Δ20
2. The high levels of βcat greatly exceed APC's retention ability
3. Wnt target genes are strongly activated even in the absence of Wnt signal

Figure 2.14. Model: Cytoplasmic retention by APC plays a secondary role in fine-tuning signaling. This model builds on the role of multiple βcat-binding sites as docking sites to accommodate different βcat levels (Ha et al., 2004 blue right-pointing triangle), and of suggested roles for cytoplasmic retention in modulating signaling (Tolwinski and Wieschaus, 2001 blue right-pointing triangle; Krieghoff et al., 2006 blue right-pointing triangle; McCartney et al., 2006 blue right-pointing triangle).

Figure 2.16



0 Wild-type
1 At most 1 denticle band or part of two bands absent. Head OK.
2 More than half of total denticles present. Head skeleton still present
3 Less than half of total denticles left, but more than 3 substantial portions of a denticle belt remaining. Strong head defects or head hole
4 A small patch of denticle to none. Head hole
5 No denticles. "V-neck" head hole.
6 No denticles. Cuticle very reduced in size, head/dorsal hole almost to spiracles.

Figure 2.16. Cuticle scoring chart

Table 1.1

Construct	Deleted residues	Embryonic lethality in APC2 single mutant	Cuticle score in APC2 single mutant	Embryonic lethality in APC2 APC1 double mutant	Cuticle score in APC2 APC1 double mutant
No transgene	N/A	96% (n = 398)	3.60 (n = 200)	55% (n = 284)	4.97 (n = 200)
APC2	N/A	8% (n = 414)	0.05 (n = 67)	1% (n = 357)	0.09 (n = 34)
ΔR3	737–764	9% (n = 489)	0.20 (n = 41)	15% (n = 333)	0.20 (n = 44)
ΔR1,R4-R5	595–622, 784–889	41% (n = 295)	0.16 (n = 263)	ND	ND
ΔR3-R5	737–889	44% (n = 346)	0.23 (n = 119)	ND	ND
KeepR3	595–672, 784–889	87% (n = 349)	0.45 (n = 200)	51% (n = 74)	4.68 (n = 75)
Δ20	595–672, 737–889	89% (n = 494)	1.00 (n = 273)	54% (n = 312)	4.63 (n = 130)
Δ15	497–535	53% (n = 291)	0.20 (n = 200)	7% (n = 371)	0.20 (n = 41)
Δ15Δ20	497–535, 595–672, 737–889	98% (n = 325)	3.60 (n = 171)	82% (n = 186)	4.29 (n = 129)
ΔR2	645–672	43% (n = 279)	0.33 (n = 223)	56% (n = 329)	4.76 (n = 234)
ΔB	677–714	46% (n = 382)	0.21 (n = 65)	57% (n = 496)	4.77 (n = 204)
d40	676–1067	81% (n = 287)	3.11 (n = 209)	61% (n = 135)	4.61 (n = 54)
ΔSAMPs	930–1067	86% (n = 338)	2.77 (n = 184)	71% (n = 168)	5.11 (n = 109)
End at B	715–1067	88% (n = 391)	2.55 (n = 177)	ND	ND

Cuticle scoring as in Supplemental Figure 5. n = number of embryos scored. N/A, Not applicable; ND, not done.

Table 1.1. Function of APC2 mutants in vivo in Drosophila.

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CHAPTER 3: A NOVEL GSK3-REGULATED APC:AXIN INTERACTION REGULATES WNT SIGNALING BY DRIVING A CATALYTIC CYCLE OF EFFICIENT BETA-CATENIN DESTRUCTION[1]

Overview

APC, a key negative regulator of Wnt signaling in development and oncogenesis, acts in the destruction complex with the scaffold Axin and the kinases GSK3 and CK1 to target βcatenin for destruction. Despite 20 years of research, APC's mechanistic function remains mysterious. We used FRAP, super-resolution microscopy, functional tests in mammalian cells and flies, and other approaches to define APC's mechanistic role in the active destruction complex when Wnt signaling is off. Our data suggest APC plays two roles: (1) APC promotes efficient Axin multimerization through one known and one novel APC:Axin interaction site, and (2) GSK3 acts through APC motifs R2 and B to regulate APC:Axin interactions, promoting high-throughput of βcatenin to destruction. We propose a new dynamic model of how the destruction complex regulates Wnt signaling and how this goes wrong in cancer, providing insights into how this multiprotein signaling complex is assembled and functions via multivalent interactions.

[1] This chapter previously appeared as an article in the Journal of eLife. The original citation is as follows: Mira I. Pronobis , Nasser R. Rusan, and Mark Peifer. "A novel GSK3-regulated APC:Axin interaction regulates Wnt signaling by driving a catalytic cycle of efficient beta-catenin destruction". eLife (2015): e08022.

Introduction

The Wnt signaling pathway is one of the most critical in animals, controlling both cell proliferation and fate (Clevers and Nusse, 2012). Deregulated Wnt signaling plays roles in several cancers (Polakis, 2007; Kandoth et al., 2013); most strikingly, mutations in Adenomatous polyposis coli (APC), a key negative regulator of Wnt signaling, initiate ~80% of colon cancers. Although APC was identified in 1991, its mechanistic role in Wnt regulation remains mysterious.

Wnt signaling regulates levels of the transcriptional co-activator βcatenin (βcat; Clevers and Nusse, 2012). Like other powerful signaling pathways driving development and oncogenesis, animals evolved dedicated machinery to keep the Wnt pathway off in the absence of signal. In the OFF-state, βcat levels are kept low by the action of the multiprotein destruction complex, which includes APC (Figure 3.1A), the scaffold Axin (Figure 3.1A) and the kinases GSK3 and CK1. In the current model, βcat is recruited into the destruction complex by binding APC or Axin and sequentially phosphorylated by CK1 and GSK3. This creates a binding site for the E3-Ligase SCFβTrCP and βcat is ubiquitinated and destroyed. Wnt ligand binding to the Wnt receptor inhibits the destruction complex, through a series of events whose order and relative importance remains unclear (MacDonald and He, 2012). βcat levels rise and it activates Wnt target genes.

Despite this attractive model, several key issues remain. APC and Axin are destruction complex core components. Both are protein interaction hubs, combining folded domains with intrinsically disordered regions carrying peptide motifs that bind protein partners. APC was

initially thought to be the scaffold templating βcat phosphorylation but subsequent work revealed that Axin plays this role (Hart et al., 1998; Ha et al., 2004). Axin has binding sites for βcat, APC, GSK3, CK1 and PP2A, and self-polymerizes via its DIX domain (Figure 3.1A), which allows it to locally increase the concentration of proteins mediating βcat phosphorylation (Ikeda et al., 1998; Fagotto et al., 1999; Liu et al., 2002; Fiedler et al., 2011). Thus while APC is essential for destruction complex function, its role in the complex remained a mystery.

APC combines an N-terminal Armadillo repeat domain (Arm rpts) with a more C-terminal intrinsically disordered region; each contains binding sites for multiple partners (Figure 3.1A). The Arm rpts bind cytoskeletal regulators (Nelson and Nathke, 2013), and also help regulate Wnt signaling but the relevant binding partner(s) for this is unclear (Roberts et al., 2012). APC's SAMP repeats bind Axin's RGS region, helping mediate destruction complex assembly. Both the Arm rpts and SAMPs are essential for APC function. APC also has multiple βcat binding sites, the 15 and 20 amino acid repeats (15Rs and 20Rs), which have different affinities for βcat, and act redundantly to sequester βcat and fine tune Wnt signaling (Liu et al., 2006; Roberts et al., 2011). However, surprisingly, these binding sites are not essential for APC's mechanistic role in βcat destruction (Yamulla et al., 2014).

Recent work led to significant insights into how Wnt signaling turns off the destruction complex. Axin turnover and Dvl-Axin hetero-polymerization may inhibit destruction complex function by disassembling the destruction complex (Schwarz-Romond et al., 2007; Fiedler et al., 2011). However, recent studies revealed that the destruction complex remains intact for some time after Wnt signals are received, and can continue to phosphorylate βcat (Hernandez et al., 2012; Li et al., 2012b; Kim et al., 2013). Thus these studies suggest that Wnt signaling leads to

either reduction in the rate of βcat phosphorylation by the destruction complex, or that it inhibits a key regulated step after phosphorylation, perhaps transfer to the E3 ligase, thus turning down an intact destruction complex, leading to high levels of βcat.

In contrast, less attention has been devoted to the active destruction complex when Wnt signaling is off, the state most relevant to what happens in colon tumors. Strikingly, these tumors lack wild-type APC but instead invariably express a truncated APC protein retaining some but not all functional domains—the reason for this remains controversial (Albuquerque et al., 2002). Further, while tumor cells do not effectively target βcat for destruction, it is less clear at what step destruction is blocked by APC truncation. In the simplest model, mutant destruction complexes wouldn't template βcat phosphorylation, but Axin's ability to do this in vitro in APC's absence cast doubt on this (Ha et al., 2004). Further, cancer cells with truncated APC have high levels of phosphorylated βcat, suggesting that the step blocked is after βcat phosphorylation (Yang et al., 2006; Kohler et al., 2008). One intriguing study proposed that APC acts in the destruction complex to protect βcat from dephosphorylation and then targets it to the E3 ligase (Su et al., 2008). However phospho-βcat is elevated in APC-mutant cell lines, despite loss of APC function suggesting that protecting βcat from dephosphorylation is not APC's only role in the destruction complex (Yang et al., 2006). Thus APC's key mechanistic function in the destruction complex remains largely unknown.

In exploring APC's mechanism of action when Wnt signaling is inactive, we recently focused on two conserved binding sites in APC, 20R2 (R2) and motif B (B; this is also known as the catenin interaction domain = CID; Kohler et al., 2009; Roberts et al., 2011; Schneikert et al., 2014; Choi et al., 2013; Figure 3.1A). R2 is related to the 20R βcat binding sites, but lacks a key

interacting residue and cannot bind βcat (Liu et al., 2006). B is immediately adjacent to R2—its function was initially unknown, though it was recently shown to bind α-catenin, and thus play a role in Wnt regulation (Choi et al., 2013). Strikingly, although other 20Rs are individually dispensable, both R2 and B are essential for the destruction complex to target βcat for destruction (Kohler et al., 2009; Roberts et al., 2011; Schneikert et al., 2014). Our data further suggested that R2/B negatively regulates APC/Axin interaction, a somewhat surprising role for an essential part of the destruction complex.

This prompted us to broaden our analysis. Most destruction complex models, even those that consider the kinetics of initial destruction complex assembly (Lee et al., 2003), portray it as a static entity that binds, phosphorylates and hands off βcat. Recent work prompted us to consider an alternative hypothesis, viewing the destruction complex as a complex multiprotein entity whose assembly, structure and dynamics are key to its function in maintaining low βcat levels when Wnt signaling is off. To test this hypothesis, we used FRAP, super-resolution microscopy and other approaches to explore the structure and dynamics of the destruction complex. This provided new insights into APC's mechanism of action, providing evidence that it plays two roles inside the active destruction complex: (1) APC promotes efficiency of the destruction complex by enhancing complex assembly through two separate APC:Axin interaction sites, one of which is novel, (2) the novel APC:Axin interaction is dynamic, and regulation of this interaction by GSK3 acting via motifs R2 and B is essential to send phosphorylated βcat to destruction. More broadly, our data also provide insights into how intrinsically disordered regions assist in the assembly and dynamics of multiprotein signaling complexes.

Material and Methods

Constructs

Drosophila APC2, human APC's Arm rpts (1–1012aa), and Axin were cloned as in Roberts et al. (2011). In short, constructs were cloned into pECFP-N1 (Clontech, Mountain View CA) via Gateway (Invitrogen, Waltham MA) with either an N-terminal 3XFlag-tag or GFP-tag (Roberts et al., 2011). The C-terminal-RFP vector was generated by cloning the Gateway cassette and TagRFP from pTag-RFP (Evrogen, Russia) into pECFP-N1. For Phospho-APC2 constructs Serine 688 and 692 were changed using PCR stitching. Drosophila Axin fragments were N-term (1–54aa), RGS (55–171aa), GSK3 binding region (171–494aa), βcat binding region (494–532aa), PP2A binding region (532–666aa), and DIX (666–747aa).

Antibodies

1° antibodies: βcat (BD Transduction, San Jose CA, 1:1000), FlagM2 (Sigma, St. Louis MO, 1:1000, 1µg/ml IP), GFP (Novus Biologicals, Littleton CO, 1:2000), βcat-S33/37 (Abcam, UK, 1:1000), γ-tubulin (Sigma, 1:5000), tagRFP (Evrogen, 1:5000), hAPC1 (Calbiochem, Billerica MA, 1:1000), hAxin1 (Cell Signaling, Danvers MA, 1:2000), aPKCγ (Santa Cruz Biotechnology, Dallas TX, 1:2000). 2° antibodies: Alexa 568 and 647 (Invitrogen, 1:1000), HRP anti-mouse and anti-rabbit (Pierce, Rockford IL, 1:50,000).

Cell culture

SW480 cells were cultured in L15 medium (Corning, Tewksbury MA) + 10% heat-inactivated FBS+1X Pen/Strep (Gibco, Waltham MA) at 37 °C without CO2. Lipofectamine 2000 (Invitrogen, Waltham MA) was used for transfections following manufacturer's protocol. For immunostaining and IP cells were processed after 24 hr. Immunostaining was as described in

Roberts et al. (2011). In short cells were fixed 5 min in 4% formaldehyde/1XPBS, rinsed 5 min in 0.1% Triton-100/1XPBS, blocked with 1% normal goat serum/1XPBS, and incubated in antibody. Samples were mounted in Aquapolymount (Polysciences, Warrington PA). For drug treatment 30 µM LiCl (Sigma; dissolved in L15), 2 µM BIO (Tocris, UK) or 25 µM MG132 (Calbiochem; both dissolved in 99% EtOH) were added 24 hr after transfection, and incubated 6h.

Microscopy

Immunostained samples were imaged on a LSM Pascal microscope (Zeiss) and processed with the LSM image browser (Zeiss, Germany). SIM microscopy was carried out on the Deltavision OMX (GE Healthcare Life Sciences, Pittsburgh PA) using 4% formaldehyde fixed samples mounted in Vectashield (Vector, Burlingame CA) following manufacturer's protocol. Images were processed using Imaris 5.5, ImageJ and the LSM Image Browser. PhotoshopCS4 (Adobe, San Jose, CA) was used to adjust levels so that the range of signals spanned the entire output grayscale and to adjust brightness and contrast.

Estimating relative over-expression levels of Axin and APC

We roughly calculated levels of expression by comparative immunoblotting, suggesting that expression levels were in the order: hAxin1-GFP > fly Axin-GFP > endogenous hAxin1. Since fly Axin is not recognized by hAxin1 antibodies we did this in two steps, first comparing levels of tagged human Axin1 vs Drosophila Axin using antibodies against the GFP epitope tag, and then comparing the levels of the transfected human Axin1 vs the endogenous Axin1 protein in SW480 cells, using antibodies against human Axin1 (Figure 3.2C). Because differences in expression levels took us out of the linear range of film, we diluted the more concentrated sample by a known amount. Protein bands in immunoblots of diluted samples (Figure 3.2C3–4)

were quantitated in ImageJ. Levels were normalized by (1) determining γ-tubulin levels in Figure 3.2C1–2 where samples were equally loaded, (2) by measuring γ-tubulin of diluted samples, and by calculating in the dilution factor. Once all samples were normalized to γ-tubulin the ratio between GFP-tagged fly and human Axin was calculated. Next, the ratio between hAxin1-GFP and endogenous hAxin1 was determined. Lastly the overexpression levels of fly GFP-Axin to endogenous hAxin1 were determined using the formula: ((ratio GFP-FlyAxin to hAxin1-GFP) × (ratio hAxin1-GFP to endogenous hAxin1)) / Transfection efficiency.

We then carried out a similar procedure for APC2, comparing levels of tagged human APC1 cloned so as to mimic the truncated APC1 seen in SW480 cells vs tagged Drosophila APC2 using antibodies to the Flag epitope, and then levels of tagged truncated human APC1 vs that of the endogenous truncated APC1 protein (Figure 3.2D). Lastly the overexpression levels of fly GFP-Axin to endogenous hAxin1 were determined using the formula ((ratio Flag-fly APC2 to Flag-hAPC1-1338) × (ratio Flag-hAPC1-1338 to endogenous hAPC1-1338))/transfection efficiency.

Quantification

Z-projections of cell image stacks were generated using ImageJ. βcat fluorescent intensity: Cells were outlined, mean intensity measured, background subtracted, and βcat average intensity of a transfected cell normalized to mean of the βcat intensity of 2–3 adjacent untransfected cells. 10 cells were each measured in 3 independent experiments. Puncta colocalization of APC2ΔSAMPs with Axin in BIO/LiCl treated cells were determined by scoring for puncta formation in the APC2ΔSAMPs channel. 100 cells were scored in 3 independent experiments. APC:Axin complex size: Particle Analyzer of ImageJ was used. Background was

subtracted and threshold for particles set to 200. Cytoplasmic puncta of 10 cells were averaged. Cell images were taken with LSM Pascal (Zeiss) with a resolution of 5.7 pixel/µM. Mean number of particles per cells was calculated from size measurements. Puncta volumes were measured using Imaris Software (Bitplane, Concord MA) from image z-stacks acquired on the Deltavision OMX (GE Healthcare Life Sciences). For comparing sequences, ClustalW2 (EMBL, Germany) was used for alignment. Statistical tests used the Student's t-test.

FRAP

FRAP was conducted using Eclipse TE2000-E microscope (Nikon, Japan) 24–72 hr after transfection. Drug treated samples were measured 6–48 hr after drug treatment (30–72 hr after transfections). Movies were taken at 1 frame/3 s or 1 frame/6 s for 20 min and bleaching was conducted for 8 s with 100% laser. Movies were processed using FRAP analyzer in ImageJ. Bleached area and cell were outlined, background was subtracted and the movie was processed with FRAP profiler. Values were normalized and recovery plateau and standard error were calculated by averaging 10 movies. For t1/2 values were processed in GraphPad (La Jolla CA) using non-linear regression (curve fit)-one phase decay. t1/2 of 10 movies was averaged and standard error calculated.

Protein work

IPs were as described in Li et al. (2012b). In short cells were lysed on ice 15 min in 150 mM NaCl, 30 mM Tris pH 7.5, 1 mM EDTA, 1% Triton-X-100, 10% glycerol, 0.5 mM DTT, 0.1 mM PMSF + proteinase/phosphatase inhibitors (EDTA-free, Pierce), lysates cleared by centrifugation at 13,200 rpm 30 min at 4 °C, and preincubated with Sepharose beads 2h at 4 °C. 1 µg/ml antibody was added and incubated overnight at 4 °C. Beads were blocked in 5% BSA/1xPBS

overnight at 4 °C, added to antibody-lysis mix, incubated 1–2 h at 4 °C, washed 5× with lysis buffer 4 °C, mixed with 2xSDS and incubated 10 min at 96 °C . Cell lysis for immunoblotting was similar. For βcat protein levels centrifugation was at 13,200 rpm for 30 min or 1000 rpm for 10 min at 4 °C. Drug treated cells were harvested after 6h. Proteins were run on 8% or 6% (hAPC1) SDS gels and blotted to nitrocellulose. IP quantification was in ImageJ using Gel plot analyzer. IP baits were normalized to amount of IPed protein, and βcat protein levels were normalized to loading control γ-tubulin.

In vitro kinase assay

R2/B fragments of hAPC1 (1355aa–1465aa) and fly APC2 (632aa–733aa) were cloned via Gateway into pdest15-GST tagged vector. Protein expression was induced via IPTG (Apex, San Diego CA) and protein purification was conducted using Glutathione beads (Sigma). Kinase assays using human GSK3β (Sigma#G4296) were conducted following the manufacturer's protocol. The GSK3 substrate peptide YRRAAVPPSPSLSRHSSPHQ(pS)EDEE (based on human muscle glycogen, Signalchem, Canada) was used as a positive control. Samples were run using Tricine SDS-PAGE (Schagger, 2006), and the gel was Coomassie stained and measured for radioactivity by exposing to film.

Fly work

Transgenic fly lines were generated by Best Gene (Chino Hills, CA). APC2 transgenes were crossed into the APC2g10 APC1Q8 double mutant backgrounds as described previously (McCartney et al., 2006). Maternal/zygotic double mutants for both APCs were generated using the FRT/FLP/DFS technique (Chou and Perrimon, 1996). Fly crosses and heat-shock conditions were as described in Roberts et al. (2011). Embryonic lethality and cuticle preparations were

conducted as described in Wieschaus and Nüsslein-Volhard (1998).

Results

Goal and system used

Our goal is to define APC's mechanistic role in βcat regulation when Wnt signaling is off. In recent work, we discovered that two motifs in APC's intrinsically unstructured region, R2 and B, are essential for promoting βcat destruction in human cells and in Drosophila (Roberts et al., 2011). Here we sought to define the mechanism by which these motifs and APC itself act, using as a model SW480 colon cancer cells. These cells have high βcat levels, as they lack wildtype human APC1 (hAPC1) and instead express a truncated APC1 protein ending before the mutation cluster region (MCR; Figure 3.1B). SW480 cells also express human APC2 (Maher et al., 2009), but this is not sufficient to help mediate βcat destruction. We express in these cells fly APC2, the homolog of hAPC1, a full length APC that shares all conserved regions important for Wnt regulation with hAPC1 but is significantly smaller in size (Figure 3.1B). Fly APC2 effectively reduces βcat levels in SW480 cells (Roberts et al., 2011), and thus can interact with all human destruction complex proteins needed to target βcat for degradation.

There is abundant evidence that the functional destruction complex is a multimer of the individual destruction complex proteins. One important underpinning of this idea is that Axin oligomerizes via self-polymerization of the DIX domain, and this multimerization is critical for its Wnt regulatory function (Kishida et al., 1999; Schwarz-Romond et al., 2007). Endogenous Axin forms small puncta in cultured cells and when overexpressed these puncta become more prominent, in a DIX-domain dependent fashion (Fagotto et al., 1999; Faux et al., 2008; Figure

3.2A). The level of Axin over-expression needed to trigger more prominent Axin puncta is not dramatic—for example, treatment of SW480 cells with tankyrase inhibitors increased levels of AXIN1 3-5x and AXIN2 5-20x and this was sufficient to trigger formation of Axin puncta (de la Roche et al., 2014). Axin puncta are dynamic multiprotein complexes that can recruit APC and other destruction complex proteins, and previous data from many labs are consistent with the idea that the puncta can serve as useful models of the smaller endogenous destruction complexes, based on correlations between puncta formation, dynamics, and function in βcat destruction (e.g. Faux et al., 2008; Fiedler et al., 2011).

We and others previously identified the key structural domains of APC and Axin that are essential for destruction complex function and βcat destruction (e.g. Roberts et al., 2011). Our current goal was to define how these proteins' domains function together to facilitate APC and the destruction complex's mechanisms of action. To do so, we used the APC:Axin puncta formed in SW480 cells as a visible and thus measurable read-out to study mechanisms underlying destruction complex structure, assembly, dynamics and function.

Our experiments and those of many earlier investigators used transfected human or in our case fly proteins to study Wnt signaling in cultured mammalian cells (e.g., Bilic et al., 2007; Fiedler et al., 2011; Kim et al., 2013). This strategy likely leads to both variable expression levels between cells and elevated expression relative to endogenous protein. We first investigated variation from cell to cell within a transfection, by quantitating whole cell fluorescence of GFP- or RFP-tagged APC2 or Axin and investigating whether different levels of Axin or APC2 expression altered the ability to down-regulate βcat levels (Figure 3.2B). There was a substantial range of APC2 or Axin expression levels among cells (5- to 10-fold). Importantly,

βcat levels were substantially reduced at all levels of APC2 or Axin expression, even the lowest levels assessed—this was true for Axin alone, APC2 alone, or Axin plus APC2 (Figure 3.2B). In all cases, ability to reduce βcat levels was somewhat diminished at the highest levels of expression (Figure 3.2B)—this may be because at very high expression levels, the transfected protein forms non-functional complexes with only a subset of the destruction complex proteins, as was previously suggested (Lee et al., 2003).

We next used immunoblotting to get order of magnitude estimates for the level of expression of our transfected constructs relative to the endogenous proteins. We describe the procedure used in detail in the Materials and methods. We began with Axin, to determine the level of over-expression of fly Axin vs endogenous human Axin. Since fly Axin is not recognized by hAxin1 antibodies we did this in two steps, first comparing levels of tagged human Axin1 vs Drosophila Axin using antibodies against the epitope tag, and then comparing the levels of the transfected human Axin1 vs the endogenous Axin1 protein in SW480, using antibodies against human Axin1 (Figure 3.2C). We then used these ratios and the transfection efficiencies to calculate the average ratio of Drosophila Axin:endogenous hAxin1. Transfected Axin accumulated at roughly 80- to 120-fold that of endogenous hAxin1 (Table 1). For APC, estimating 'overexpression' was more problematic, as SW480 cells do not accumulate wild-type APC1—instead they accumulate a truncated APC1 ending at amino acid 1338 (Figure 3.1B). We thus used a similar two-step process, comparing levels of tagged human APC1 cloned so as to mimic the truncated APC1 seen in SW480 cells vs tagged Drosophila APC2 using antibodies to the epitope, and then levels of tagged truncated human APC1 vs that of the endogenous truncated APC1 protein (Figure 3.2D). This ratio was roughly 1400–2400 (Table 1). We suspect

this is an over-estimate of the relative ratio of Drosophila APC2 to normal levels of hAPC1 in a wild-type colon cell, as the truncated APC1 protein present in SW480 cells would accumulate at lower levels than wild-type APC1 if it is subjected to nonsense-mediated mRNA decay, like many other proteins with early stop codons and like other truncated APC1 alleles (Castellsagué et al., 2010; Popp and Maquat, 2013). Further, we also may need to reduce the ratio by a further factor of two as it is probable SW480 cells carry only one copy of the truncated APC allele, as most colorectal tumors either have the second allele mutated early enough to not produce a truncated protein or have lost the second allele by deletion (Christie et al., 2013). Regardless, it is important to remember that while the puncta provide a useful and visualizable model of the destruction complex, we are examining over-expressed proteins.

A novel Axin:APC association site regulated by APC motifs R2 and B

To probe destruction complex structure and dynamics, we first need to define mechanisms of complex assembly. In the current model, destruction complex assembly is mediated by interaction of APC2's SAMPs and Axin's RGS domain (Fagotto et al., 1999; Figure 3.1A). Consistent with this, deleting the SAMPs disrupted APC2 recruitment into Axin puncta. However a shorter APC2 mutant, lacking both the SAMPs and R2 and B and thus resembling the truncated hAPC1 in tumors (Figure 3.1B), did co-localize with Axin (Roberts et al., 2011). We further found that deleting R2 and/or B, both of which are essential for APC function in flies and mammals, restored colocalization of Axin and APC2ΔSAMPs (Roberts et al., 2011). Thus a second means of mediating APC2:Axin interactions must exist, that is separate from the known SAMP:RGS interaction.

To identify the protein sequences mediating this putative R2/B regulated mechanism by

which APC2 and Axin can interact, we truncated APC2 from its C-terminus, and assessed APC2 mutant colocalization with Axin. APC2's Arm rpts (Figure 3.1B) formed cytoplasmic puncta when expressed alone (Figure 3.3A,B), consistent with APC2's ability to self-oligimerize (Kunttas-Tatli et al., 2014). When co-expressed with Axin, the Arm rpts and Axin puncta associate, suggesting the Arm rpts are the Axin association site (Figure 3.1C,D arrows; intriguingly they often did not precisely colocalize, in contrast with the full length proteins (Roberts et al., 2011)). Next we defined where on Axin the Arm rpts associate, by expressing Axin fragments and conducting co-immunoprecipitation (co-IP; Figure 3.1E,F). Both full length Axin and a region including the GSK3 binding site co-IPed with APC's Arm rpts (Figure 3.1E,F). Axin's DIX domain was weakly detected in co-IPs, but this may occur by polymerization with full-length endogenous human Axin1 (hAxin1). Thus fly APC2's Arm rpts can mediate association with Axin's middle region. This was exciting, as it may explain the Arm rpts essential role in Wnt regulation (McCartney et al., 2006; Roberts et al., 2012).

To determine if this second APC:Axin interaction mechanism is conserved in humans and whether it occurs between proteins expressed at endogenous levels, we used SW480 cells, in which endogenous hAPC1 is truncated after 20R1 at 1338aa, thus lacking the SAMPs, R2 and B (Figure 3.1B). hAPC1-1338aa co-IPed with endogenous hAxin1 (Figure 3.1G). To verify that association was through the Arm rpts, we tested whether hAPC1's Arm rpts alone (Figure 3.1B) colocalized with hAxin1 in SW480 cells. hAPC1Arm rpts and hAxin1 each formed cytoplasmic puncta (Figure 3.3C–E). When coexpressed, they colocalized in cytoplasmic puncta (Figure 3.1H,I arrows), suggesting both hAPC1 and fly APC2 can associate with the Axin complex in two ways: the known interaction via the SAMPs and this novel interaction via the Arm rpts.

Axin and APC2 form structured macromolecular complexes in vivo

Given this new data on destruction complex assembly, we next explored the structures assembled by Axin vs Axin plus APC, using the puncta formed in SW480 cells as a visualizable destruction complex model. While current data suggest Axin polymerization and APC recruitment are essential for destruction complex function, previous microscopy provided only limited information about the internal structure of Axin–APC complexes in vivo. In these images, Axin and APC colocalized in puncta without resolvable internal structure (e.g., Mendoza-Topaz et al., 2011; Roberts et al., 2011; Figure 3.4A,B). Recent advances increased the resolution possible with light microscopy. We thus used the superresolution approach Structured Illumination Microscopy (SIM) to visualize APC:Axin complexes, revealing a striking and previously undescribed internal structure. Cytoplasmic complexes formed by Axin alone appear to consist of Axin cables/sheets, assembling into hollow structures (Figure 3.4C–F). Interestingly, coexpressing APC2 substantially altered the structure of many complexes, increasing the length/complexity of Axin cables (Figure 3.4G, H–J = three representative puncta), with APC2 and Axin cables intertwined and in some puncta APC bridging Axin cables (Figure 3.4H–J, arrows).

These data suggest APC2 may enhance assembly of Axin oligomers, stimulating formation of larger complexes. To test this hypothesis, we quantified puncta cross-sectional area in Axin vs APC + Axin expressing cells, making z-projections of transfected cells and using the ImageJ particle analyzer. This revealed APC2:Axin puncta average almost twice the cross-sectional area of puncta formed by Axin alone (Figure 3.2K, left). We confirmed this by comparing the volume of Axin and APC:Axin complexes, as measured from our SIM data—once

again APC + Axin puncta were on average significantly larger than those assembled from Axin alone, and the largest APC + Axin puncta were larger than any of the Axin puncta (Figure 3.2L). When we compared the average number of complexes in Axin alone vs APC2 + Axin expressing cells, we found twice as many complexes in cells expressing Axin alone (Figure 3.4K, right), suggesting that in the absence of functional APC, Axin forms more numerous but smaller puncta. These data support the hypothesis that APC2 induces changes in the structure of Axin complexes formed in vivo, enhancing Axin assembly into higher order complexes.

APC2 stabilizes the Axin complex and promotes destruction complex throughput of βcat

The effect of APC on destruction complex structure might suggest APC helps regulate Axin turnover within the destruction complex. Previous analyses suggested that Axin dynamics within the destruction complex puncta correlate with function. Axin puncta are dynamic protein assemblies, and the Wnt effector Dishevelled significantly increases Axin dynamics in puncta, suggesting it negatively regulates Axin self-association (Schwarz-Romond et al., 2007), consistent with its known negative regulatory role in destruction complex function. To directly assess APC2 and Axin dynamics in the destruction complex and their influence on one another, we used Fluorescent Recovery After Photobleaching (FRAP) to measure dynamics of both APC2-GFP and Axin-RFP (Figure 3.5A–D). We assessed both recovery (mobile) fraction and t1/2, using unbleached puncta as controls. Recovery fraction assesses what percentage of molecules in a complex turnover in the experimental time frame, and t1/2 reflects the rate at which the dynamic fraction is exchanged—they are not necessarily dependent on one another. We found APC2 is also a dynamic component of destruction complex puncta, reaching a recovery plateau of 40% and a t1/2 of 150 s (Figure 3.5B); however, APC2 is not as dynamic as Axin (Figure 3.5C).

To test the hypothesis that APC stabilizes assembly of Axin monomers into the multimeric destruction complex, we compared Axin dynamics in puncta containing Axin alone with those containing Axin plus APC2. Interestingly, Axin expressed alone was quite dynamic, with a recovery plateau of almost 90% and a t1/2 = 150 s (Figure 3.5C,E). However, when Axin was coexpressed with APC2, Axin dynamics were significantly reduced (recovery plateau = 40% and t1/2 = 300 s; Figure 3.5D,E), suggesting APC stabilizes Axin assembly within puncta.

Axin cannot target βcat for destruction in APC's complete absence, even when Axin is overexpressed (Mendoza-Topaz et al., 2011). However, in SW480 cells, which express both truncated hAPC1 and endogenous hAPC2 (Maher et al., 2009), Axin overexpression can increase βcat destruction (Nakamura et al., 1998). If APC's role in the active destruction complex is to stabilize Axin assembly and thus the destruction complex scaffold, then APC should enhance Axin's ability to target βcat for destruction. We thus examined whether Axin alone fully restores βcat destruction, or whether adding APC2, either to endogenous wild-type Axin or co-expressed with fly Axin, further facilitates this. We began by measuring βcat fluorescence intensity in z-projections of cells transfected with either APC2 + Axin or Axin alone, using untransfected cells as internal controls (Figure 3.5F–H). Interestingly, while Axin reduced total βcat levels (Figure 3.5F,G), βcat was further reduced in cells expressing both APC2 and Axin (Figure 3.5F,I) suggesting that APC2 promotes more effective destruction complex activity.

Cells expressing APC2 ‘alone’ also had strong βcat reduction (Figure 3.5F,H), presumably due to interaction with endogenous human Axin. We thus tested whether fly APC2 can and does interact with human Axin. Fly GFP-APC2 colocalizes in puncta with exogenous hAxin1-RFP (Figure 3.5J), and more importantly, endogenous hAxin1 coIPs with Flag-APC2 expressed in

SW480 cells (Figure 3.5K). One further caveat is that we were assessing the ability of APC2 and Axin to promote βcat destruction after over-expression. To determine if differing levels of over-expression might explain the differences between Axin and APC2 + Axin (or 'APC2 alone'), we quantitated level of Axin or APC2 expression in a given cell by measuring levels of GFP/RFP fluorescence, and in parallel assessed levels of βcat in that cell (via fluorescence intensity). Strikingly, βcat levels were more effectively reduced by APC2 + Axin or by 'APC2 alone' than by Axin at all levels of expression assessed. As noted above, in all cases, ability to reduce βcat levels was somewhat diminished at the highest levels of expression (Figure 3.2B)—this may be because at very high expression levels, the transfected protein forms non-functional complexes with only a subset of the destruction complex proteins, as was previously suggested (Lee et al., 2003). Together these data support the hypothesis that APC2 enhances Axin's ability to promote βcat destruction.

Interestingly, in Axin-alone transfected cells, much of the excess βcat that accumulated (Figure 3.5F) was in Axin puncta (Figure 3.5G', inset, arrow). In contrast, puncta in cells co-expressing APC2 and Axin had almost undetectable βcat levels (Figure 3.5I', inset). We thus hypothesized that APC enhances Axin's ability to promote βcat exit from the destruction complex, and thus the destruction complex's βcat throughput. To further explore this, we examined phospho-βcat levels. SW480 cells, like other colon cancer cell lines with hAPC1 truncated before the Mutation Cluster Region (MCR; Figure 3.1B), have high phospho-βcat levels (Yang et al., 2006). This suggests that while Axin can facilitate βcat phosphorylation in these cells, Axin is less efficient at targeting βcat for destruction in the absence of wild-type APC1, and thus phosphorylated βcat accumulates. Strikingly, expressing APC2 alone or APC2 +

Axin dramatically decreased phospho-serine 33/37 βcat (to ~20% that in untransfected cells), while Axin alone reduced phospho-βcat to only 60% (Figure 3.5L,M). These data further support the hypothesis that APC2 enhances Axin's ability to promote βcat destruction.

One potential caveat to the increased accumulation of βcat in puncta of cells expressing Axin alone vs those expressing APC2 plus Axin is that the former cells may simply have higher overall levels of βcat, thus resulting in higher accumulation in puncta. To address this, we inhibited βcat destruction using the proteasome inhibitor MG132. As others have previously observed (Sadot et al., 2002), proteasome inhibition elevates βcat levels. Proteasome inhibition elevates βcat levels both in cells expressing Axin alone and in those expressing APC2 plus Axin (Figure 3.6C). Strikingly, this allows it to accumulate in puncta even in cells expressing Axin + APC2 (Figure 3.6A vs B). However, cells expressing Axin still accumulate significantly higher levels of βcat than those expressing Axin plus APC2 (Figure 3.6C). Together, these data are consistent with a model in which APC increases βcat throughput of the destruction complex by stabilizing Axin assembly.

Both APC2's Arm rpts and SAMPs are required to stabilize APC2:Axin complexes

Since APC associates with Axin via two regions, the Arm rpts and SAMP motifs (Figure 3.1), we hypothesized each interaction helps stabilize destruction complex assembly. To test this, we first measured APC2 dynamics when either the Arm rpts or SAMPs were individually deleted (Figure 3.7A). Deleting either region increased APC2 dynamics in Axin puncta; APC2ΔArm and APC2ΔSAMPs turnover reached higher plateaus (Figure 3.7B; 80–90% vs 40% for wild-type) in shorter times (Figure 3.7B; t1/2 wildtype APC2 150 s; APC2ΔArm 75 s; APC2ΔSAMPs <25 s). Thus, APC2 needs both the Arm rpts and the SAMPs to stably associate

with Axin complexes. Next we examined Axin dynamics in the presence of each APC2 mutant. Both the Arm rpts and SAMPs were required to stabilize Axin in destruction complexes, since Axin coexpressed with either APC2ΔArm or APC2ΔSAMPs exhibited the fast dynamics characteristic of Axin expressed alone (Figure 3.7C). Thus APC2 stabilizes APC:Axin complexes through multivalent interactions mediated by the Arm rpts and SAMPs.

Our SIM imaging suggested APC stabilization of Axin complexes altered their substructure. We thus tested whether both Axin interaction sites were essential for the effects on the structure of Axin puncta. APC2ΔArm, which retains the SAMPs, closely colocalized with Axin, even at SIM resolution, rather than forming filaments of its own within puncta, like wild-type APC2 (Figure 3.7D–F vs Figure 3.7H). Further, Axin within these puncta remained simple in structure, similar to puncta formed by Axin alone (Figure 3.7G). APC2ΔSAMPs, which is much less tightly associated with Axin by either confocal localization or coIP (Hart et al., 1998; Roberts et al., 2011), did not strongly colocalize with Axin, instead forming a diffuse network surrounding Axin puncta. Importantly, APC2ΔSAMPs did not alter Axin structure within puncta as visualized by SIM. In the presence of APC2ΔSAMPs, Axin puncta retained the simpler structure of those formed by Axin (Figure 3.7I–K). Further, neither APC2ΔArm nor APC2ΔSAMPs increased Axin puncta size or reduced Axin puncta number (Figure 3.7L–N). Thus APC's ability to stabilize destruction complexes and stimulate growth of Axin cables requires both sites mediating Axin complex interaction, the Arm rpts and SAMPs. Both sites are also required to allow APC2 to efficiently downregulate βcat levels; APC2ΔSAMPs did not stimulate βcat destruction below Axin-alone mediated levels, while APC2ΔArmrpts could not downregulate βcat levels (Figure 3.7O). Since both APC2's Arm rpts and the SAMPs are essential for Wnt

regulation in Drosophila (Roberts et al., 2011, 2012), this suggests that APC2's ability to stabilize destruction complex assembly through its multivalent interactions is critical for destruction complex throughput of βcat.

R2 and B regulate APC2 dynamics and APC2's ability to enhance βcat throughput

Our earlier steady state analysis revealed that APC2 motifs R2 and B antagonized APC:Axin interaction when the SAMPs were deleted. Since both R2 and B are essential for APC function in targeting βcat for destruction in vivo, we extended our work to determine whether and how R2 and B affect the dynamics of APC:Axin interactions. Our data above reveal the new Axin complex-association site is in APC's Arm rpts (Figure 3.1). Since removing R2/B restored APC/Axin interaction even in the absence of the SAMPs (Figure 3.8A–E), we hypothesized R2/B negatively regulates APC2 Armrpt:Axin interaction and that release of this interaction is essential to allow phosphorylated βcat to be moved on to destruction.

Our hypothesis predicted deleting either R2 or B should stabilize APC2:Axin interaction, by enabling APC2's Arm rpts to also productively mediate association with Axin. To test this we assessed how deleting R2 or B (Figure 3.9A) affected APC2 dynamics by FRAP. Deleting either R2 or B had the predicted effect, decreasing APC2 turnover rate dramatically (Figure 3.9B), suggesting R2 and B regulate APC2 dynamics in the active destruction complex. While deleting R2 and B strongly stabilized APC2 in the complex, it did not significantly diminish APC2's ability to stabilize Axin, destruction complex size and structure (Figure 3.8F–K).

We next asked which step in the cycle of destruction complex function is blocked by removing R2 or B, and thus altering APC dynamics. To do so, we examined if βcat was retained in puncta containing these mutants. βcat was nearly undetectable when APC2 and Axin are

coexpressed (Figure 3.9C, arrows). Interestingly, deleting either R2 or B led to βcat accumulation in APC2:Axin complexes (Figure 3.9D,E, arrows). Deleting R2 also abolished APC2's ability to enhance the reduction of βcat levels given by Axin alone (Figure 3.9F), suggesting that APC2 without R2 is not functional and therefore inhibited in its ability to promote βcat destruction via Axin complexes. βcat levels increased even further when motif B was deleted (Figure 3.9F) suggesting that APC2ΔB may interfere with βcat degradation via Axin. These data suggest that R2 and B regulate APC2 dynamics in the destruction complex and that APC2 lacking them cannot effectively support Axin in targeting βcat for destruction.

R2 and B regulate APC2:Axin association via the Arm rpts

R2 or B thus regulate APC2:Axin interactions. We next determined which Axin association site, APC2's Arm rpts or SAMPs, was regulated. Deleting the SAMPs substantially reduced APC2 recruitment into Axin complexes while further deleting either R2 or B restored strong Axin:APC2ΔSAMPs colocalization (Figure 3.8); in APC2ΔSAMPs the only remaining means of interacting with the Axin complex was via the Arm rpts, suggesting Arm rpts:Axin association is regulated by R2/B. In contrast, deleting APC2's Arm rpts (Figure 3.10A) did not reduce Axin colocalization (Figure 3.10B arrows); thus not surprisingly deleting either R2 or B in APC2ΔArm did not further alter this (Figure 3.10A,C,D arrows).

To directly test the hypothesis that R2 and B regulate dynamics of Arm rpts:Axin association, we returned to our FRAP assay. Consistent with effects on colocalization, deleting R2 or B (Figure 3.11A) decreased APC2ΔSAMPs dynamics (Figure 3.11B; t1/2 increased significantly for both mutants; intriguingly recovery fraction was only significantly affected by deleting R2, perhaps reflecting the unaltered destruction complex structure in these mutants).

Co-IPs extended the FRAP results, revealing more stable APC2ΔSAMPs:Axin interaction when R2 was deleted (Figure 3.11C; quantified in Figure 3.11D; the change after deleting motif B and the SAMPs did not reach statistical significance). Thus without R2, APC2's Arm rpts associate more robustly with Axin complexes, slowing APC2 dynamics. In contrast, SAMPs:Axin interaction was not regulated by R2 or B: APC2ΔArm (in which the only remaining interaction with Axin was via the SAMPs), APC2ΔArmΔR2, and APC2ΔArmΔB all had similar recovery plateaus and t1/2 (Figure 3.10E), and co-IPs showed no difference in APC2:Axin association among these constructs (Figure 3.10F,G). In fact, APC2ΔR2 and APC2ΔB, which retain both the Arm rpts and SAMPs, coIPed with Axin as or more robustly than wild-type APC2 (Figure 3.10H,I) consistent with enhanced APC:Axin interaction through increased Arm rpts:Axin association when R2 and B were deleted. Thus APC2 and Axin have two distinct interaction interfaces with different properties: strong association via the SAMPs is independent of R2/B and a second interaction via APC2's Arm rpts is controlled by R2/B.

APC2:Axin association via the Arm repeats is regulated by GSK3 kinase

The known essential role of R2 and B in the active destruction complex in targeting βcat for destruction suggested their ability to regulate APC:Axin interaction is critical. We thus further explored the mechanism by which they regulate destruction complex dynamics and function, by comparing sequences of these adjacent motifs in mammalian and fly APCs (Figure 3.12A). Strikingly, threonines and serines were among the most highly conserved residues. In the destruction complex, GSK3 and CK1 phosphorylate not only βcat but also Axin and other sites on APC (Ikeda et al., 1998; Yamamoto et al., 1999; Ha et al., 2004). Motif B has multiple serines matching GSK3's phosphorylation consensus and one match to the CK1 consensus,

while R2 has several matches to both GSK3 and CK1 consensuses (Figure 3.12A). GSK3 kinase plays multiple roles in promoting destruction complex activity, phosphorylating βcat to target it to the E3 ligase and also regulating the destruction complex by phosphorylating the βcat binding sites on APC, increasing their affinity, and phosphorylating Axin. We hypothesized GSK3 also phosphorylates R2 and/or B, to trigger release of APC2's Arm rpts from Axin.

To test this we first determined whether blocking GSK3 activity using LiCl, a well-known GSK3 inhibitor (Klein and Melton, 1996), or BIO, a very specific GSK3 inhibitor (Meijer et al., 2003) affected APC2ΔSAMPs:Axin interaction (we verified GSK3 inhibition by assessing βcat accumulation in APC2:Axin puncta; Figure 3.13A–F; Stambolic et al., 1996). APC2ΔSAMPs (Figure 3.12B) is only weakly recruited into Axin puncta (Figure 3.12C). If GSK3 phosphorylation of R2/B antagonizes Arm rpts:Axin association, inhibiting GSK3 should increase colocalization of APC2ΔSAMPs with Axin, as did deleting R2 or B (Figure 3.10). Strikingly, while APC2ΔSAMPs is largely diffusely cytoplasmic (Figure 3.12C), BIO treatment strongly increased Axin:APC2ΔSAMPs colocalization (Figure 3.12D arrows), in a concentration dependent manner (Figure 3.12E). Consistent with this, only 16% of untreated control cells had APC2ΔSAMPs:Axin colocalization (Figure 3.12F), while inhibiting GSK3 with BIO increased colocalization to 75% of cells (Figure 3.12F). LiCl also led to robust Axin:APC2ΔSAMPs colocalization (Figure 3.13G vs Figure 3.13H; quantified in Figure 3.13I). We used coIP to verify that APC2ΔSAMPs associates more robustly with Axin upon GSK3 inhibition. Deleting the SAMPs drastically reduced APC2:Axin coIP (Figure 3.12G; quantified in Figure 3.12H). GSK3 inhibition by either LiCl or BIO increased APC2ΔSAMPs coIP with Axin (Figure 3.12G,H). Thus GSK3 inhibition stabilizes steady state Axin:APC2ΔSAMPs association, as did deleting R2 or B, consistent with our model that

phosphorylating these motifs normally antagonizes Axin:Arm rpts association.

The hypothesis that association of APC2's Arm rpts with Axin is regulated by GSK3 also predicts inhibiting GSK3 should affect APC2 dynamics in the destruction complex, as did deleting R2 or B. Consistent with this, inhibiting GSK3 dramatically decreased APC2's dynamics (Figure 3.12I; plateau reduced from 40% to 10%; t1/2 increased from 150 to >1000 s). This suggests APC2 turnover in Axin complexes is regulated by GSK3. Our model further predicts that GSK3 regulates APC2 dynamics by regulating the APC2Arm rpts:Axin association. Thus GSK3 inhibition should stabilize APC2ΔSAMPs:Axin interactions and reduce APC2ΔSAMPs dynamics. As predicted, APC2ΔSAMPs rapid turnover was dramatically decreased by GSK3 inhibition (Figure 3.12J). In contrast, GSK3 inhibition had no effect on APC2ΔArm turnover (Figure 3.13J). These data suggest that GSK3 activity promotes release of APC2's Arm rpts from the Axin complex.

Our data are consistent with GSK3 acting via phosphorylating R2 and/or B, but could also be mediated via other effects of GSK3. To assess this, we examined whether inhibiting GSK3 affected turnover of an APC2ΔSAMPs mutant lacking both R2 and B (Figure 3.12B), reasoning this would assess the effect of GSK3 on regulation of the Axin:APC2Arm rpts interaction. Strikingly, the recovery plateau and t1/2 of APC2ΔR2/BΔSAMPs were insensitive to GSK3 inhibition (Figure 3.12K), in contrast to APC2ΔSAMPs. These data are consistent with GSK3 affecting APC2 residence time in the destruction complex through R2/B. However, deleting R2/B and blocking GSK3 activity had different effects on APC2 recovery fraction, suggesting that not all effects of GSK3 are mediated through R2/B. GSK3 phosphorylates other targets in the destruction complex, and thus it is very likely GSK3 inhibition has additional means of altering

destruction complex dynamics. Further, an unphosphorylated R2/B motif may affect APC's dynamics differently than deleting R2/B. Taken together, however, our data suggest that GSK3 acts in part through R2 and B to promote release of APC2's Arm rpts from the Axin complex.

If GSK3 inhibition stabilizes the interaction of APC2 and Axin, and as noted above, APC2 also can stabilize Axin in puncta, increasing their size (and thus decreasing puncta number), then GSK3 inhibition might synergize with APC, further increasing the size of Axin puncta. We thus inhibited GSK3 with either BIO or LiCl in cells co-expressing Axin and APC2 and examined both puncta size and number. GSK3 inhibition did not further increase puncta size or further decrease puncta number (Figure 3.12L). These data are consistent with our analysis above of APC2ΔR2 and APCΔB—these mutations also stabilize APC2 in the destruction complex (Figure 3.9B) but do not further increase puncta size or decrease puncta number (Figure 3.10F–J). We also tested whether proteasome inhibition might trigger enlargement of the APC + Axin complexes—this also did not further increase puncta size or further decrease puncta number (Figure 3.12L). Thus, while APC2 can stabilize Axin complexes, inhibition of GSK3 or the proteasome does not synergize with this.

Mutating putative phosphorylation sites in B disrupts APC2's function in regulating βcat

We hypothesized that R2/B phosphorylation by GSK3 is a key step in APC2's mechanism to target βcat for destruction. Thus we tested whether R2/B can be phosphorylated by GSK3. A GST-fusion containing just R2 and B from either fly APC2 or human APC1 can be phosphorylated in vitro by human GSK3 (Figure 3.14A). Human R2/B was strongly phosphorylated, whereas fly R2/B was more weakly phosphorylated. Thus the potential phosphorylation sites in R2/B can be phosphorylated by GSK3, possibly at the GSK3 consensus sites (Figure 3.14B).

R2 and B are essential for APC2's function in βcat degradation. Our hypothesis suggests phosphorylation of R2/B promotes release of APC2's Arm rpts from the Axin complex, and that this would be essential for the catalytic cycle of the destruction complex—thus mutating these putative GSK3 phosphorylation sites in motif B would reduce APC2 function in helping mediate βcat destruction. To begin to test this, we replaced 2 (APC2AA; Figure 3.14B, red arrows), 4 or 6 (Figure 3.14B, magenta arrowheads) conserved serines in B that match the GSK3 consensus with alanine, to prevent phosphorylation. All reduced function in downregulating βcat levels (see below and data not shown). We thus focused on the least altered of these, the mutant that replaced the more N-terminal two serine residues with alanine (APC2AA; Figure 3.14B, red arrows), thus preventing phosphorylation. We also created a mutant that replaced these same two residues with aspartic acid, creating a phosphomimetic APC2 (APC2DD). Strikingly, while APC2DD effectively reduced βcat levels (Figure 3.14C,F), APC2AA was unable to do so. APC2AA cells accumulated βcat at levels as high or higher than adjacent untransfected cells (Figure 3.14D,F), suggesting these two amino acid missense mutations substantially reduced APC2 function. APC2DD substantially reduced βcat levels (to ∼30%), but was not quite as effective as wildtype APC2 (Figure 3.14F). This statistically significant difference may suggest dephosphorylation of these residues is also required for full APC2 function. We also found mutating a single conserved serine residue in R2 to alanine (APC2 R2S->A (=APC2S660A); Figure 3.14B) strongly diminished APC2 function in reducing βcat levels (Figure 3.14E,F). Together, these data are consistent with a model in which phosphorylation of conserved serines in APC2 motifs R2 and B are important for APC's function in the destruction complex to target βcat for degradation. Finally, we tested whether the first of these putative phosphomutants affected

APC2 dynamics in the FRAP assay. Since GSK3 inhibition slowed APC's dynamics, we predicted APC2AA should have a lower turnover rate than wildtype APC or APC2DD. We saw a subtle but statistically significant reduction in APC2AA recovery fraction (Figure 3.14G). However this was not nearly as dramatic as that of deleting R2 or B (Figure 3.9B); perhaps this due to the fact that we only altered two of several potential phosphorylation sites. Together these data are consistent with the idea that phosphorylation of conserved serine residues in R2/B regulates APC's function in the destruction complex, but since the effect on dynamics was substantially less dramatic than that of deleting R2 or B, it suggests other residues in R2 and B may also contribute to regulating APC2 dynamics. Further, it is clear that GSK3 has other effects on the complex, complicating interpretation of its inhibition. It will be important to examine this in the future.

Mutating putative phosphorylation sites in B severely reduces APC2's function in regulating βcat

To test the role of these two putative phosphorylation sites in APC function in an in vivo context where we can examine both cells receiving and not receiving Wnt signals, we turned to Drosophila, where we can express mutant APC2 under control of the endogenous APC2 promoter in the complete absence of all endogenous APC function, using embryos maternally and zygotically APC2 APC1 double mutant. We generated transgenics expressing APC2DD or APC2AA using the endogenous APC2 promotor (as in Roberts et al., 2011), and crossed them into the APC2 APC1 mutant background. All progeny were maternally APC2 APC1 mutant and 50% of progeny were also zygotically mutant, while the other 50% were paternally rescued (we cannot generate homozygous double mutant males). In the absence of the transgene, 43% of progeny hatch (Figure 3.15A), consistent with ~50% zygotic rescue. A wild-type APC2 transgene

expressed in APC2 APC1 mutants led to 95% survival, comparable to wild-type flies (93–99%; Figure 3.15A). APC2DD was as effective as wild-type APC2 at restoring embryonic viability (96% survival; Figure 3.15A). In contrast, APC2AA expressed in APC2 APC1 mutants only weakly rescued embryonic lethality (64% viability; Figure 3.15A).

We then examined rescue of cell fates. In wildtype embryos, a row of cells in each body segment expresses the Wnt homolog Wingless (Wg), thus regulating cell fate. In cells not receiving Wg signal, the destruction complex effectively destroys βcat that is not sequestered in cadherin-catenin complexes at the cell membrane, and cells choose anterior fates and secrete cuticle covered with denticles (Figure 3.15B1). In contrast, cells receiving Wg signal accumulate cytoplasmic and nuclear βcat, choose posterior fates, and secrete naked cuticle (Figure 3.15B1). Maternal/zygotic APC2 APC1 mutants cannot destroy βcat and thus all cells accumulate extremely high levels of βcat, resulting in a smaller embryo in which all surviving cells choose posterior fates and secrete naked cuticle (Figure 3.15B2; Ahmed et al., 2002; Akong et al., 2002). Like our wildtype APC transgene (Figure 3.15B3), APC2DD fully restored alternating anterior (denticle) and posterior (naked cuticle) fates, resembling wild-type (Figure 3.15B4). In contrast, APC2AA largely failed to restore cell fates. Most embryos lost nearly all denticles (Figure 3.15B5–7), thus resembling embryos expressing an indestructible form of βcat (Pai et al., 1997). This suggested APC2AA has severely reduced regulatory function, though it is not completely dead.

The final test was to examine how well these APC2 mutants restored βcat destruction. In wildtype (Figure 3.15C,J), Wg signal expressed in segmental stripes turns down destruction complex activity, leading to successive stripes of cells with only cortical βcat (destruction

complex on) or with elevated cytoplasmic and nuclear βcat (destruction complex turned down). In contrast APC2 APC1 maternal/zygotic mutants have exceptionally high βcat levels in all cells (Figure 3.15D). We scored mutant embryos blinded to genotype. Consistent with its rescue of viability and cell fate, APC2DD fully rescued βcat destruction (Figure 3.15E vs Figure 3.15F,K,O; 30/30 APC2DD embryos were scored as wild-type). In contrast, APC2AA had substantially reduced destruction complex function (Figure 3.15O; 14/29 embryos scored as mutant, consistent with 50% zygotic rescue). Maternal/zygotic APC2 APC1 double mutants expressing APC2AA did not totally lose destruction complex function or the ability to respond to Wg signal. Stripes of cells with relatively reduced βcat levels were still present, but overall levels of βcat were substantially elevated and interestingly, levels were especially elevated in a subset of cells receiving Wg signal (Figure 3.15G–I,L–N). Together, these data suggest that these two putative phosphorylation sites in B are critical for APC2 function in vivo.

Discussion

Mutations in APC disrupt embryonic development from its onset, and are the first step in most colon cancers. Although we know APC functions in the destruction complex to help target βcat for degradation, its mechanism of action remains unknown. Our study offers novel insights into APC's function in the destruction complex, providing the first glimpses of the internal structure of the APC:Axin complex, and leading to a novel dynamic and testable model of a regulated catalytic cycle of destruction complex function. Based on our data we propose an explicit mechanistic model for APC2 function inside the destruction complex (Figure 3.16). In step 1, APC2 stabilizes destruction complex assembly via its Arm rpts and SAMPs motifs. The

assembled destruction complex then facilitates βcat phosphorylation (step 2) and protects it from dephosphorylation. GSK3, which phosphorylates βcat, Axin and other sites on APC, also phosphorylates APC2's R2 and B (step 3; CK1 may also be involved, as it is in the other phosphorylation events in the complex). We hypothesize this induces a conformational change in APC2 that releases the Arm rpts from association with Axin (step 4); since only a subset of APC2 molecules in the destruction complex would release the Arm rpts at any given time, the overall destruction complex would remain stable. We propose this conformational change allows transfer of phospho-βcat to the E3-ligase (step 5). Alternately, GSK3 may regulate the release of a complex of APC and phospho-βcat, allowing APC to shield phospho-βcat from dephosphorylation (Su et al., 2008) and guide it to the E3-ligase. The catalytic cycle of APC would then be reset by dephosphorylation (step 6). This provides a testable model for the mechanisms regulating a key signaling pathway. It also may provide insights into the assembly and dynamics of other large multiprotein complexes that assemble via dynamic multivalent interactions involving motifs within intrinsically disordered regions, leading to phase transitions (Li et al., 2012a; Toretsky and Wright, 2014).

APC stabilizes the Axin complex and alters complex assembly, thus increasing destruction

While textbook diagrams often depict the destruction complex as a four protein 1:1:1:1 Axin: APC:GSK3:CK1 complex, many lines of data suggest the functional destruction complex is a large multimeric complex (e.g., Fiedler et al., 2011). One mechanism involved was already known: the DIX domain of Axin polymerizes in a head-to-tail fashion, in which beta-sheet 2 of one DIX domain interacts with beta-sheet 4 of the next monomer, thus forming filaments, and this polymerization is essential for destruction complex function (Schwarz-Romond et al., 2007;

Fiedler et al., 2011). These Axin polymers are responsible for assembly of the cytoplasmic puncta we use as a model. Previous and current data from a number of labs are consistent with the idea that the puncta serve as useful models of the smaller endogenous destruction complexes (Faux et al., 2008), based on correlations between puncta formation, dynamics, and function in βcat destruction. We used the puncta to visualize assembly and dynamics of Axin:APC complexes in parallel with functional studies in colon cancer cells and Drosophila to define APC's role in destruction complex assembly and function. It is important to note that our data in SW480 cells involve significant over-expression—it will be useful in the future to examine destruction complex structure and function at endogenous levels, perhaps by tagging endogenous loci using CRISPR.

APC is absolutely essential for the destruction complex to reduce βcat levels when Wnt signaling is off (Mendoza-Topaz et al., 2011), but the mechanism by which it acts remained unclear. Our FRAP and super-resolution microscopy data support a model in which one role of APC is to promote/stabilize Axin self-assembly and slow Axin turnover in the destruction complex, thus increasing destruction complex multimerization and its ability to process βcat. We found APC does so by interacting with Axin via two different kinds of interaction sites: the known direct interaction with the SAMPs, and a novel interaction via APC's Arm rpts, which may be direct or indirect. Both interactions are critical for targeting βcat for destruction since APC without either SAMPs or Arm rpts cannot reduce βcat levels effectively. Almost all truncations in colon cancers remove the SAMPs (Kohler et al., 2008). Earlier data from our lab also implicated APC's Arm repeats in Wnt regulation—our new results provide a mechanistic basis for this effect.

APC2:Axin complexes were previously only resolved as co-localized spots. We provide the first glimpses inside the destruction complex. Super-resolution microscopy revealed that Axin puncta consist of Axin cables/sheets, which we hypothesize are bundled Axin polymers, assembled by the previously observed DIX domain polymerization (Schwarz-Romond et al., 2007). Consistent with our observation that APC2 promotes growth and reduces dynamics of Axin complexes, APC2 cables intertwine with and bridge Axin cables. Both Axin interaction sites are required for these effects. Stimulating growth of Axin complexes via destruction complex stabilization by APC2 may be essential when proteins are expressed at endogenous levels, increasing local concentrations of all destruction complex components, accounting for the highly efficient βcat destruction observed in the presence of APC. It will be interesting to explore the nature of the protein network involved at even higher resolution. As noted above, thus far we have visualized complexes of APC2 and Axin expressed at significantly elevated levels—it will be important to verify and extend these studies to the more modest size complexes found in vivo during normal development, using CRISPR to tag endogenous loci.

Axin and APC cooperate to ensure efficient βcat destruction

APC mutations in tumors do not eliminate APC; instead the N-terminus, Arm rpts and some βcat binding sites remain (Kohler et al., 2008). Truncations cluster in the mutation cluster region (MCR), suggesting this region is critical. R2 and B, which are removed by truncations in the MCR, are essential for βcat downregulation (Kohler et al., 2009; Roberts et al., 2011). In the textbook model, the destruction complex phosphorylates βcat and thus targets it to an E3 ligase. It was thus surprising that colon cancer cells with truncations disrupting R2/B have high levels of phosphorylated βcat, in contrast to tumors retaining R2/B (Yang et al., 2006). Why do

cells with non-functional APC have high levels of phosphorylated βcat?

Our data, together with earlier work, suggest Axin, the scaffold of the destruction complex, can mediate βcat phosphorylation even in cells with truncated APC, like the SW480 cells we use as a model, in which R2 and B are lost. Overexpressing Axin reduced total βcat levels, suggesting Axin can partially compensate for APC truncation, but phospho-βcat levels remained elevated inside the destruction complex. Interestingly, introducing APC2 reduced βcat accumulation in puncta and reduced phospho-βcat levels. These data suggest that without functional APC, Axin can mediate βcat phosphorylation, but transfer of βcat out of the destruction complex toward destruction is less efficient. It remains to be determined whether the accumulated phospho-βcat is actively transferred by Axin to the E3-ligase in the absence of functional APC, or if it is passively transferred due to a substantial increase in phospho-βcat. Thus while Axin can template βcat phosphorylation and can, at least in the presence of the truncated APC1 present in tumor cells, send it on to destruction, our data suggest APC promotes the rate at which βcat is transferred out of the destruction complex and sent to the proteasome. Future use of photoactivatible βcat constructs will further clarify this.

Based on our model APC mediated βcat transfer is only possible when R2 and B are maintained in the truncated APC (Figure 3.16). R2 and B are essential for function in the absence of endogenous APC function in vivo in Drosophila (Roberts et al., 2011). In SW480 cells our data suggest they are essential for APC2 to further simulate the rate of βcat destruction mediated by Axin transfection. Consistent with this, tumor cells that retain R2/B in the truncated APC have low levels of phospho-βcat (Yang et al., 2006), suggesting APC is still able to facilitate βcat transfer out of the destruction complex due to the presence of the Arm rpts, R2

and B (although the transfer would be less efficient due to loss of the SAMPs, the Axin binding sites). In contrast, truncated APC mutants that disrupt R2 and B function can associate with the Axin complex via the Arm rpts, but would not be able to assist in βcat transfer out of the destruction complex. The development of CRISPR knockout technology will allow future examination of the importance of truncated APC1, as well as the endogenous APC2 and Axin expressed in colon cancer cells in destruction complex assembly and function.

Destruction complex function requires dynamic APC2:Axin interactions regulated by R2/B

APC's Arm rpts bind cytoskeletal regulators (Nelson and Nathke, 2013), but their mechanism of action in Wnt signaling remained unclear. The fact that overexpressing hAPC1 fragments lacking the Arm rpts in SW480 cells rescued Wnt regulation initially suggested the Arm rpts were dispensable (Rubinfeld et al., 1997). However these fragments were only tested in the presence of the truncated endogenous hAPC1 in these cells, which retains the Arm rpts. Thus hAPC1 fragments without Arm rpts may work with endogenous truncated APC, restoring partial function. In contrast, in flies, in the complete absence of endogenous APC, APC2 requires its Arm rpts for Wnt regulation (McCartney et al., 2006; Roberts et al., 2012). Our data provide the first mechanistic role for the Arm rpts in Wnt function, demonstrating they act as a regulated Axin interaction site, and revealing that this interaction is conserved in humans. Whether this interaction is direct or indirect remains to be determined.

What then is the mechanism by which APC facilitates βcat destruction? Two conserved APC motifs, R2 and B, are essential to target βcat for degradation (Kohler et al., 2009; Roberts et al., 2011). Deleting either leads to βcat accumulation in the destruction complex, suggesting R2 and B are critical for destruction complex throughput of βcat. Our colocalization, FRAP, and

co-IP assays further suggest R2/B controls APC2:Axin association via APC2's Arm rpts. These data are consistent with a model in which APC2's R2 and B trigger an intramolecular conformational change in APC2, releasing the Arm rpts from association with the Axin complex (Figure 3.16). In contrast, the SAMPs bind Axin independently of R2/B. In our model binding via the SAMPs would keep APC associated with Axin complexes when the Arm rpts are released, maintaining a functional destruction complex and facilitating βcat transfer to the E3-ligase. However, as noted above, it is also possible GSK3 may regulate the release of a complex of APC and phospho-βcat, allowing APC to shield phospho-βcat from dephosphorylation (Su et al., 2008) on the way to the E3-ligase.

Phosphorylation of R2/B may be the trigger for release of the APC Arm repeat:Axin interaction

Motifs R2 and B include highly conserved serine/threonines matching the GSK3 and CK1 consensuses, and this region is phosphorylated by GSK3 in vitro. GSK3 increases APC2 dynamics, destabilizing the Arm rpts:Axin association via a mechanism that requires R2 and B. Strikingly, mutating two conserved serines in B to alanine blocked APC2's ability to reduce βcat levels, while a parallel phosphomimetic mutant did not disrupt APC2 function. We saw a similar reduction in APC2 function after mutating a conserved serine residue in R2. Consistent with our data, CK1epsilon phosphorylation of hAPC1 R2 occurs in an Axin-dependent fashion, and site directed mutagenesis blocking phosphorylation of two conserved serines in R2 (hAPC1 S1389 and S1392; distinct from and just C-terminal to the residue we mutated in APC2 R2) reduced the ability of a human APC1 fragment to down regulate Wnt signaling (Rubinfeld et al., 2001), further suggesting R2 phosphorylation also is important for APC function. Our data are consistent with a model in which GSK3 phosphorylation of R2 and B could be one major

regulatory step in APC2's dynamic cycle in the destruction complex, triggering release of the Arm rpts from the Axin complex, and thus allowing APC2 to promote βcat release for destruction (Figure 3.16). However, our data also show that mutating two residues in motif B to prevent their phosphorylation had only a subtle effect on APC2 dynamics. This may suggest additive roles for multiple phosphorylated residues, or may suggest the connection between phosphorylation, dynamics and function is more complex. Further, GSK3 can phosphorylate most of the other proteins in the destruction complex, and thus it clearly plays multiple roles in its function.

Phosphorylation of R2/B itself could trigger release of APC's Arm rpts from the Axin complex, or alternately, phosphorylation may create a binding site for a binding partner that carries out this function. Intriguingly, a recent study found that α-catenin co-IPs with motif B of hAPC1, and suggests α-catenin is an essential player in destruction complex function (Choi et al., 2013). Perhaps R2/B phosphorylation by GSK3 regulates association of α-catenin with APC, and its binding induces alterations in APC:Axin interactions. It will be exciting to test this hypothesis.

The multiple potential phosphorylation sites in R2/B, the hypomorphic nature of our double point mutant in Drosophila vs the complete loss of function after deleting either R2 or B (Roberts et al., 2011), and the residual function of our two-residue phosphomimetic mutant may suggest different combinations of phosphorylated residues in R2 and B help tune and regulate APC function in the destruction complex. Our results also suggest GSK3 is likely to affect destruction complex structure and dynamics via several mechanisms, consistent with its known role in phosphorylating Axin and other sites in APC. Mathematical modeling suggests

that a reduction in GSK3 ability to phosphorylate βcat is one key step in Wnt signaling activation, placing GSK3 activity in the center of 'destruction complex inactivation' (Lee et al., 2003). It is intriguing to speculate about GSK3-mediated APC regulation in the context of Wnt signaling. Since GSK3 activity is inhibited by Wnt signaling, key residues in R2 and B that drive APC:Axin interaction dynamics would no longer be phosphorylated. Our data suggest this would decrease APC dynamics in the Axin complex (since APC's Arm rpts would more strongly associate with Axin), perhaps reducing βcat transfer out of the destruction complex. Thus the drop in GSK3 activity upon Wnt signaling would not only downregulate the destruction complex via its main target, βcat, but through a core destruction complex component, APC, by acting via R2 and B. It will be of interest to explore further these complex interactions in both Wnt off and Wnt on states.

One intriguing property of the APC2AA mutant in Drosophila is that Wnt regulation of βcat was maintained, but βcat levels were elevated in both Wnt off and Wnt on regions, presumably reflecting a less efficient destruction complex. This re-focused our attention on the much higher levels of βcat seen in APC2 APC1 mutants than occur in wildtype cells where Wg signal turns the destruction complex 'OFF'. These results suggest the destruction complex remains active when Wnt signal is on, but operates less effectively—this is very consistent with recent in vitro work (Hernandez et al., 2012). A destruction complex operating at reduced levels could lead to high enough βcat levels to activate Wnt target genes but could maintain low enough levels to be quickly shut down when needed; it would also prevent the apoptosis seen in some cell types at extremely high βcat levels. The drop in GSK3 activity upon Wnt signaling would both decrease phosphorylated βcat and, based on our model, inhibit APC facilitating βcat

transfer to the E3-Ligase. This speculative hypothesis would merge two recent studies proposing either reduced βcat phosphorylation (Hernandez et al., 2012) or a key regulated step involving transfer of βcat to the E3-ligase (Li et al., 2012b) as key steps in Wnt regulation of the destruction complex. It will be intriguing to further probe the role of phosphorylation of R2/B in the mechanism of destruction complex action.

Figure 3.1

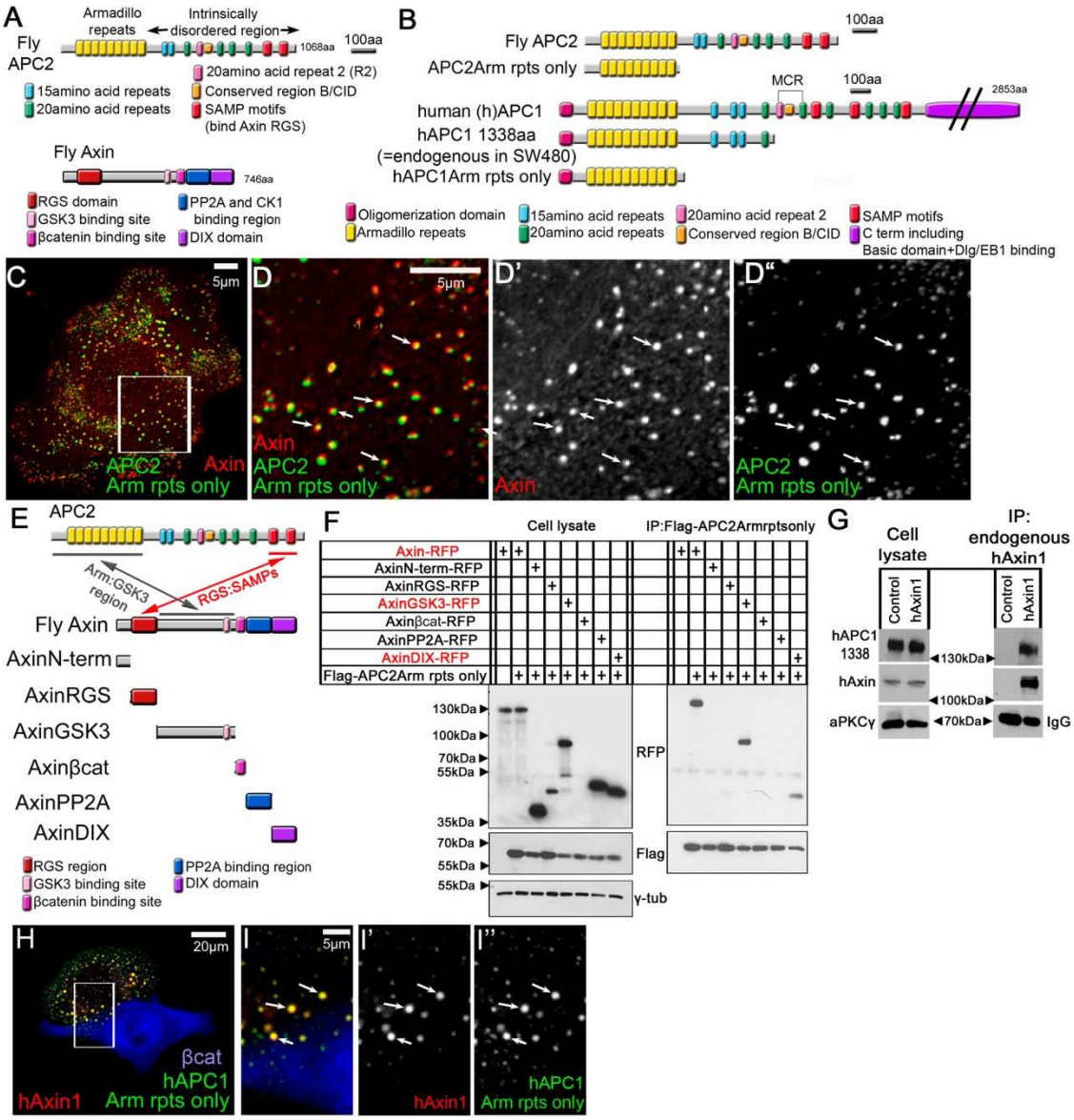

Figure 3.1. APC2's Arm rpts provide a second means of interacting with the Axin complex. (A) Fly APC2 and Axin. (B) Constructs used. hAPC1-1338 = the endogenous truncated hAPC1 in SW480 cells. (C and D) SW480 cells coexpressing GFP-APC2Arm rpts only and Axin-RFP, which localize adjacent to one another (arrows). (D) Insets = box in (C). (E) Known and novel APC:Axin interaction sites (top) and Axin constructs (bottom). (F and G) IPs from SW480 cells. (F) APC's Arm rpts coIP with Axin's middle region that contains the GSK3 binding site. Axin's DIX domain was weakly detected. The βcat binding site fragment was not detected in either immunoblots or immunofluorescence (not shown), suggesting rapid degradation. (G) IP of endogenous hAxin1 or control Ig. Truncated endogenous hAPC1-1338 coIPs with hAxin1 at endogenous levels. (H) GFP-hAPC1Arm rpts only and hAxin1-RFP colocalize in puncta (arrows). (I) Insets = box in (H).

Figure 3.2

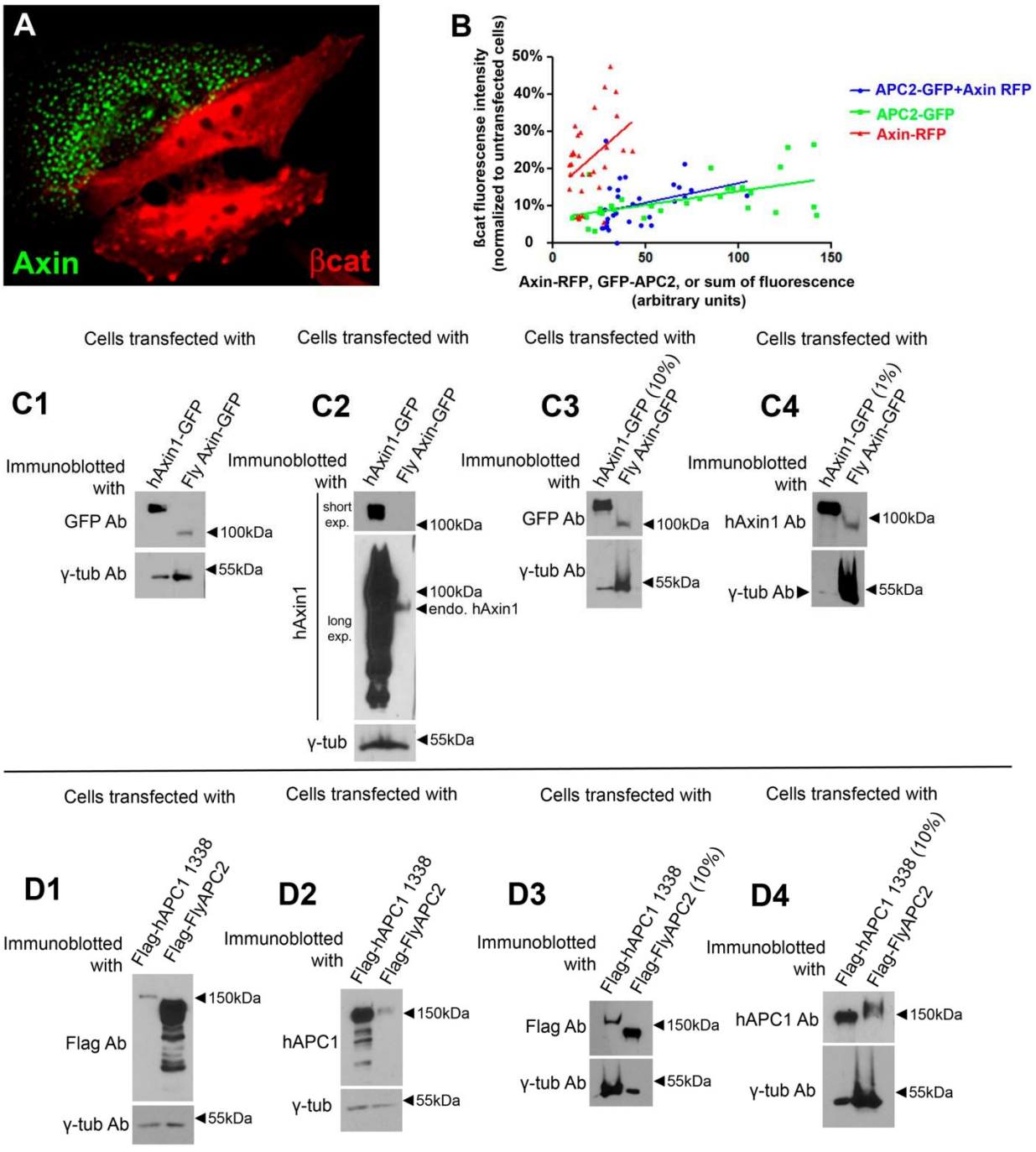
A
Axin
βcat
B
βcat fluorescense intensity (normalized to untransfected cells)
50%
40%
30%
20%
10%
0
0
50
100
150
Axin-RFP, GFP-APC2, or sum of fluorescence (arbitrary units)
APC2-GFP+Axin RFP
APC2-GFP
Axin-RFP
Cells transfected with
C1
hAxin1-GFP
Fly Axin-GFP
Immunoblotted with
GFP Ab
γ-tub Ab
100kDa
55kDa
C2
short exp.
long exp.
hAxin1
endo. hAxin1
γ-tub
C3
hAxin1-GFP (10%)
C4
hAxin1-GFP (1%)
hAxin1 Ab
D1
Flag-hAPC1 1338
Flag-FlyAPC2
150kDa
Flag Ab
D2
hAPC1
D3
Flag-hAPC1 1338 (10%)
D4

Figure 3.2. Assessing levels of over-expression of Axin and APC. (A) When overexpressed in SW480 cells, fly Axin forms puncta. (B) Plot of immunofluorescence intensities in SW480 cells transfected with GFP-APC2, or Axin-RFP, or GFP-APC2 + Axin–RFP, and stained for βcat via antibody. βcat intensities of transfected cells were normalized to adjacent untransfected cells and plotted against the GFP, or RFP, or sum of GFP and RFP intensities. 10 cells were measured each time in 3 independent experiments. (C) Measuring the levels of Axin overexpression in SW480 cells. Immunoblot analysis of cells transfected with human Axin1-GFP (hAxin1-GFP) or fly Axin-GFP with the indicated antibodies. γ-tubulin was used as a loading control. (C1,C2) Equal volumes of cell lysate were loaded. (C3) 10% the amount of hAxin1-GFP lysate was loaded. (C4) 1% of the amount of hAxin1-GFP lysate was loaded. One representative immunoblot of 3 independent experiments. Details of calculations used are in the Results and Materials and methods—full data is in Table 1. (D) Measuring the levels of APC2 overexpression in SW480 cells. Immunoblot analysis of cells transfected with Flag-tagged truncated human APC1-1338 (see Figure 1B) or fly APC2 with the indicated antibodies. γ-Tubulin was used as a loading control. (D1,D2) Equal volumes of cell lysate were loaded. (D3) 10% the amount of Flag-flyAPC2 lysate loaded. (D4) 10% the amount of Flag hAPC1-1338 lysate was loaded. One representative immunoblot of 3 independent experiments.

Figure 3.3

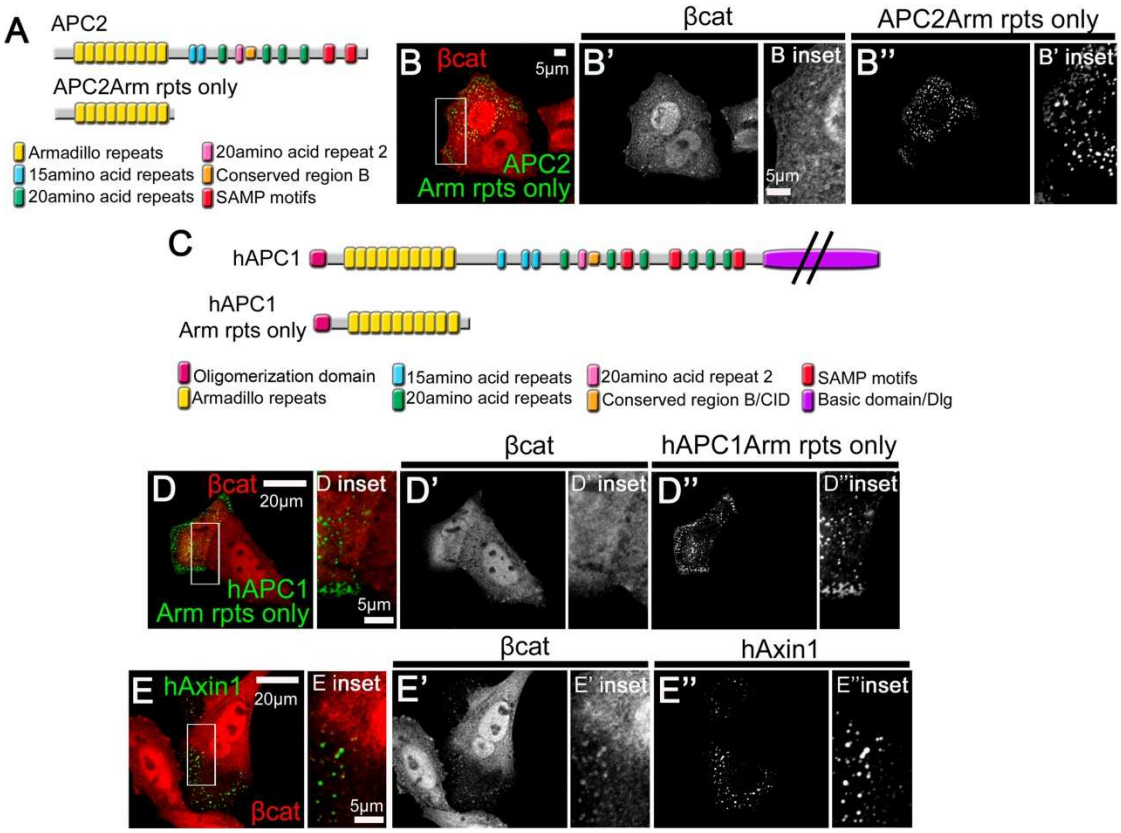

Figure 3.3. Human APC's Arm repeat domain colocalizes with Axin. (A) Diagrams of fly APC2 and APC2Arm rpts only. (B) SW480 cells expressing GFP-APC2Arm rpts only. APC2Arm rpts only forms cytoplasmic puncta and is unable to reduce βcat levels (insets = box in B). (C) Diagrams of hAPC1 and hAPC1Arm rpts only mutant. (D) GFP-hAPC1Arm rpts only expressed in SW480 cells. hAPC1Arm rpts also forms cytoplasmic puncta and is unable to reduce βcat levels (insets = box in D). (E) GFP-hAxin1 expressed in SW480 cells forms cytoplasmic puncta, and does reduce βcat levels, but βcat accumulates in the puncta (insets = box in D).

Figure 3.4

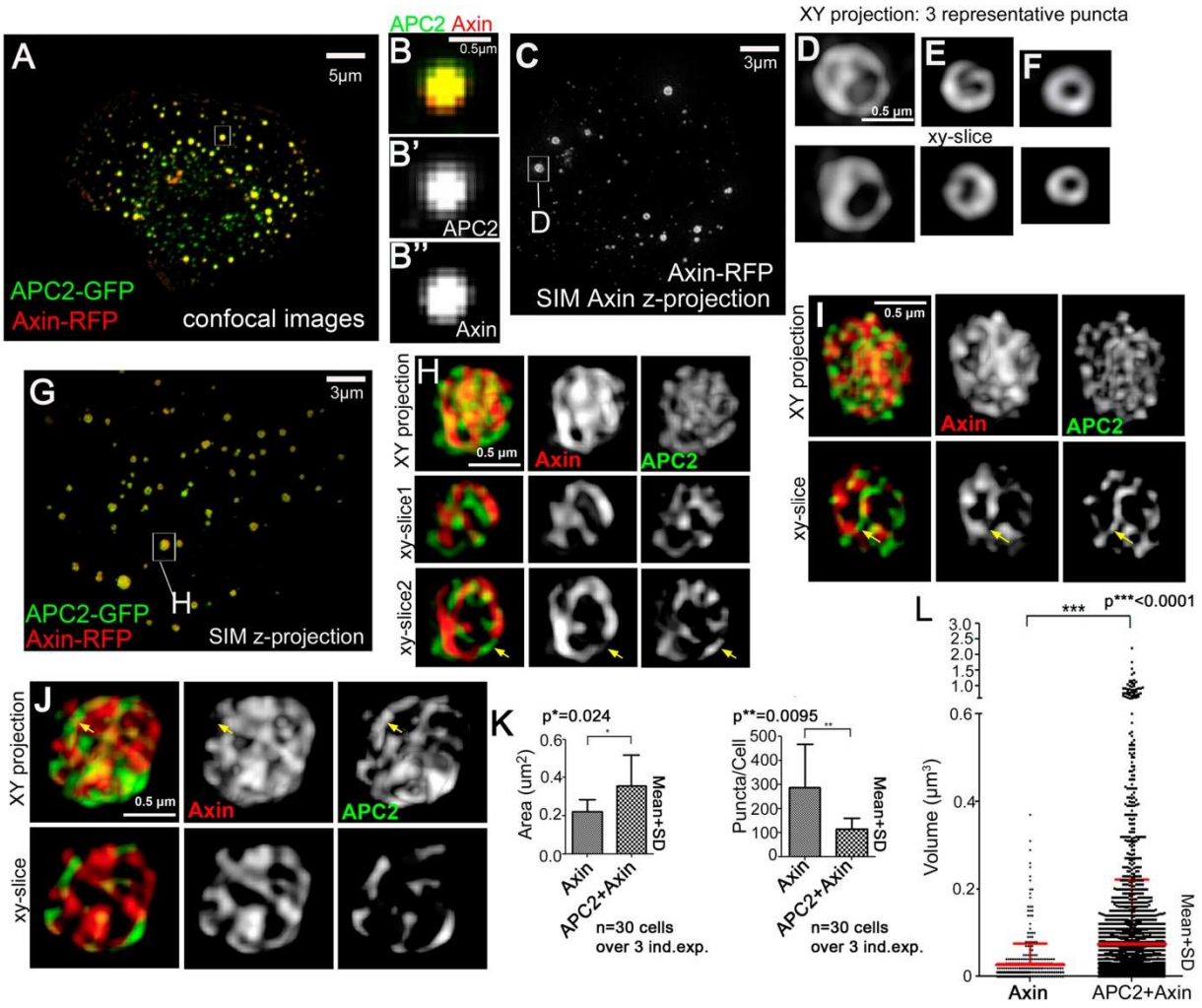

Figure 3.4. Axin and APC2 form structured macromolecular complexes in vivo.SW480 cells. (A) Confocal image, GFP-APC2 and Axin-RFP. APC2 is recruited into Axin puncta. (B) Closeups, showing failure to resolve internal structure. (C–J) SIM super-resolution. (C) Axin-RFP alone. (D–F) Closeups, Axin complexes from different cells. D = punctum boxed in C. Axin cables assemble into spheres/sheets. (G) Cell coexpressing GFP-APC2 and Axin-RFP. (H–J) Closeups of APC2:Axin complexes from different cells. H = punctum boxed in G. Axin cables increase in complexity and APC2 forms cables intertwined with Axin (arrows). (K) Analysis of confocal images. Complexes formed by APC2 and Axin average nearly twice the cross-sectional area of complexes formed by Axin alone (left). Axin-expressing cells have twice as many complexes as cells coexpressing APC2 + Axin (right). Student's t-test. (L) Puncta volume in Axin expressing cells (n = 3) vs APC2 + Axin expressing cells (n = 11) showing volumes across puncta population. Volume differences expressing cells are consistent with area quantification in (K). ANOVA-Bonferroni was used.

Figure 3.5

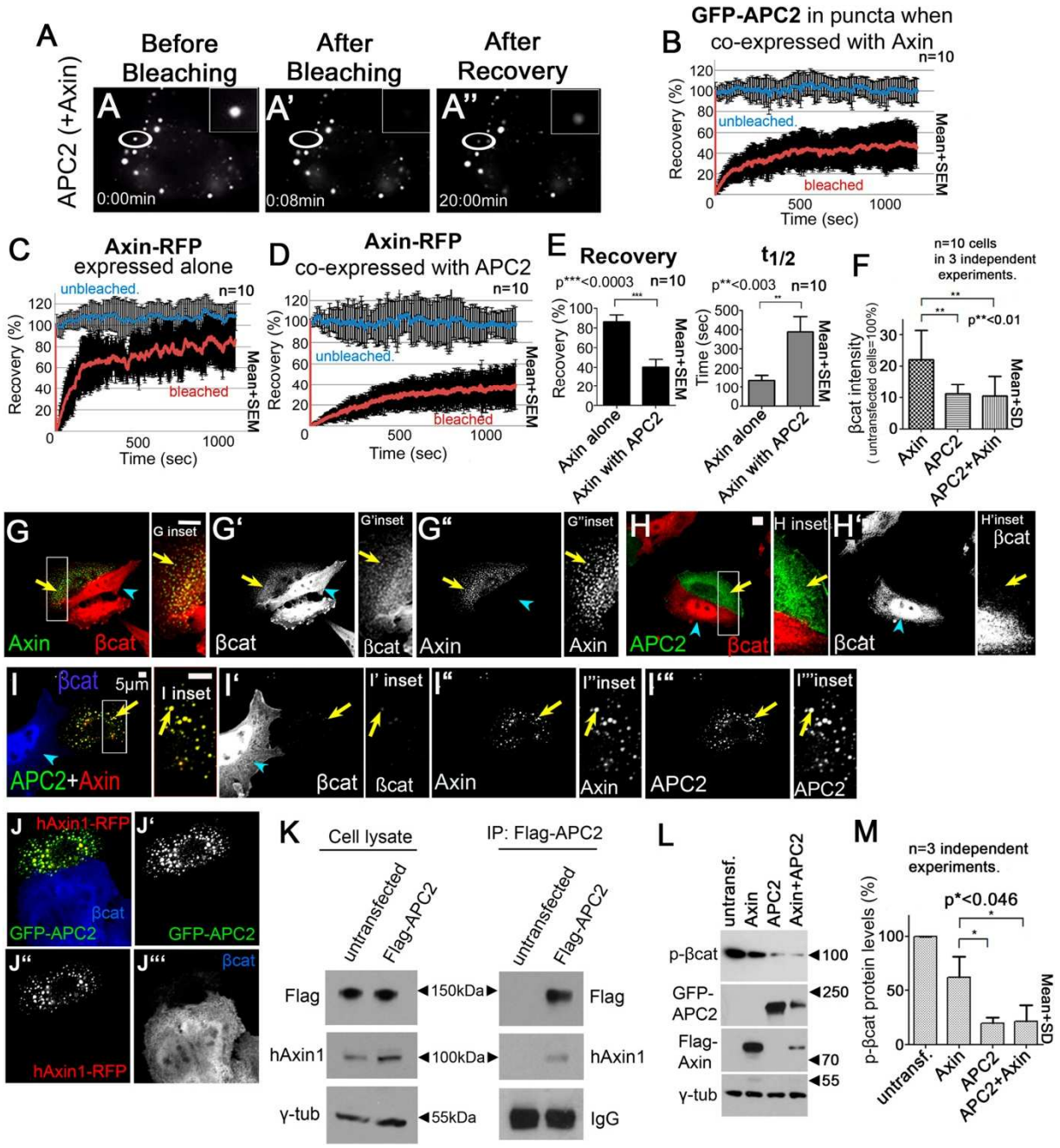
A
Before Bleaching
After Bleaching
After Recovery
APC2 (+Axin)
A
A'
A''
0:00min
0:08min
20:00min
B
GFP-APC2 in puncta when co-expressed with Axin
n=10
Recovery (%)
unbleached.
bleached
Mean+SEM
Time (sec)
C
Axin-RFP expressed alone
D
Axin-RFP co-expressed with APC2
E
Recovery
p***<0.0003 n=10
t1/2
p**<0.003 n=10
Axin alone
Axin with APC2
Time (sec)
F
n=10 cells in 3 independent experiments.
p**<0.01
βcat intensity (untransfected cells=100%)
Axin
APC2
APC2+Axin
Mean+SD
G
G inset
G'
G'inset
G"
G"inset
Axin
βcat
H
H inset
H'
H'inset
APC2
I
5μm
I inset
I'
I' inset
I"
I"inset
I'"
I'"inset
APC2+Axin
J
hAxin1-RFP
J'
GFP-APC2
J"
J'"
K
Cell lysate
IP: Flag-APC2
untransfected
Flag-APC2
Flag
hAxin1
γ-tub
150kDa
100kDa
55kDa
IgG
L
untransf.
Axin
APC2
Axin+APC2
p-βcat
GFP-APC2
Flag-Axin
γ-tub
100
250
70
55
M
n=3 independent experiments.
p*<0.046
p-βcat protein levels (%)
untransf.
Axin
APC2
APC2+Axin
Mean+SD

Figure 3.5. APC2 stabilizes Axin complexes and promotes efficient βcat destruction. (A) Stills, FRAP movie, SW480 cells transfected with GFP-APC2 (shown) and Axin-RFP. Inset = magnified APC2 signal in punctum. (B) APC2 recovers to ∼40% when in Axin puncta. Recovery curve (red); unbleached control (blue). (C) Axin expressed alone plateaus at ∼80%. (D and E) Axin is stabilized when coexpressed with APC2. (F) Total cell βcat fluorescent intensity normalized to untransfected cells (= 100%). APC2 or APC2 + Axin expression lead to stronger βcat reduction than Axin alone. (G–I) Indicated constructs expressed in SW480 cells. Insets = regions boxed. (G) GFP-Axin forms puncta and reduces βcat levels in this hAPC1 mutant cell line. βcat is detectable in puncta (arrows). (H) GFP-APC2 expressed alone is dispersed throughout the cell and βcat levels are low overall and in puncta. (I) Axin-RFP + GFP-APC2 coexpressed. βcat is reduced in APC2:Axin puncta (arrow) relative to puncta with Axin alone (G). (J) GFP-APC2 is recruited into puncta formed by human hAxin1-RFP. (K) Endogenous human hAxin1 co-IPs from SW480 cells with transfected Flag-APC2. Untransfected cells serve as a negative control. (L and M) Phospho-S33/37-βcat levels are more reduced when either APC2 or APC2 + Axin are expressed relative to Axin alone. (L) Immunoblot , transiently transfected SW480 cell extracts, centrifuged at 1000 rpm. (M) Quantification, phospho-S33/37-βcat protein levels from (L) and 2 replicates. Student's t-test.

Figure 3.6

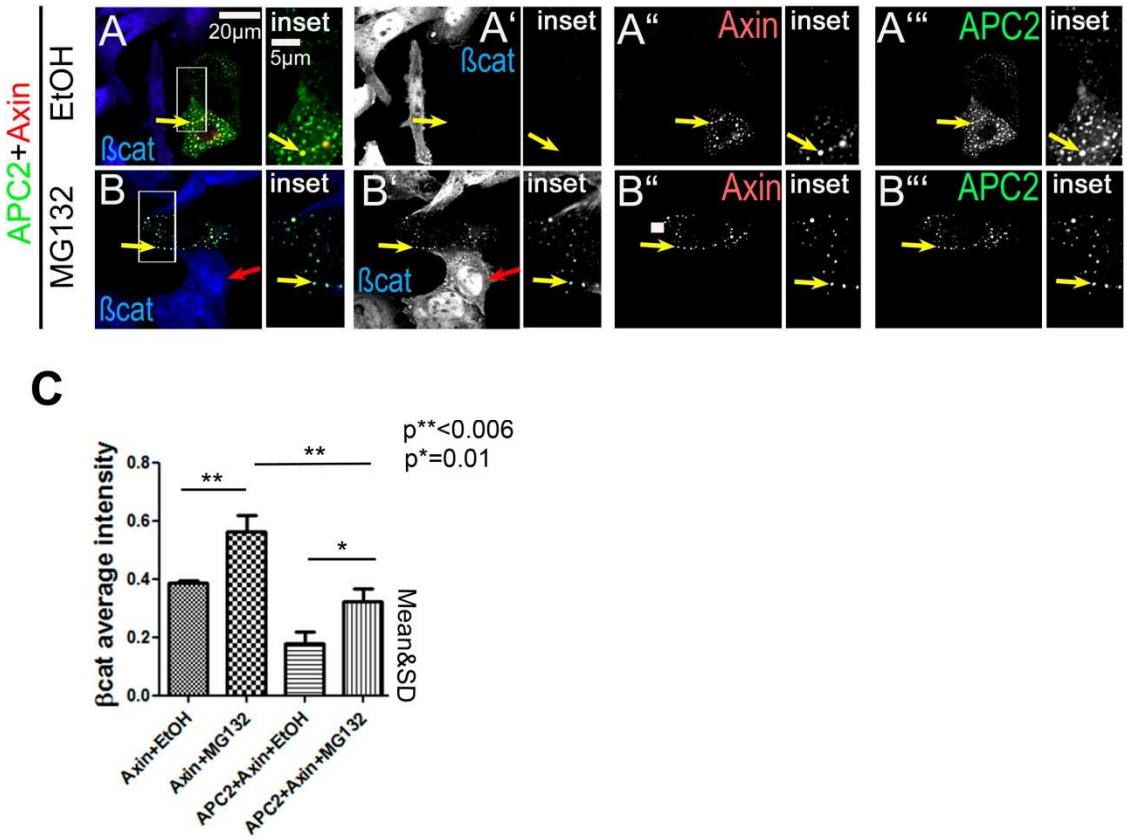

Figure 3.6. While proteasome inhibition reduces βcat destruction and causes βcat to detectably accumulate in APC2 + Axin puncta, it does not abolish the ability of APC2 to enhance Axin function in this regard.(A and B) SW480 cells transfected with APC2 and Axin and treated with either ethanol as a control or with the proteasome inhibitor MG132. MG132 treatment elevates overall βcat levels and leads detectable accumulation in APC2 + Axin puncta (yellow arrows). Red arrows indicate untransfected cells. (C) Quantitation of total cell βcat fluorescent intensity normalized to untransfected cells (= 100%). MG132 treatment increases βcat levels in both Axin and APC2 + Axin treated cells. However, APC2 + Axin expression still leads to stronger βcat reduction than Axin alone**.**

Figure 3.7

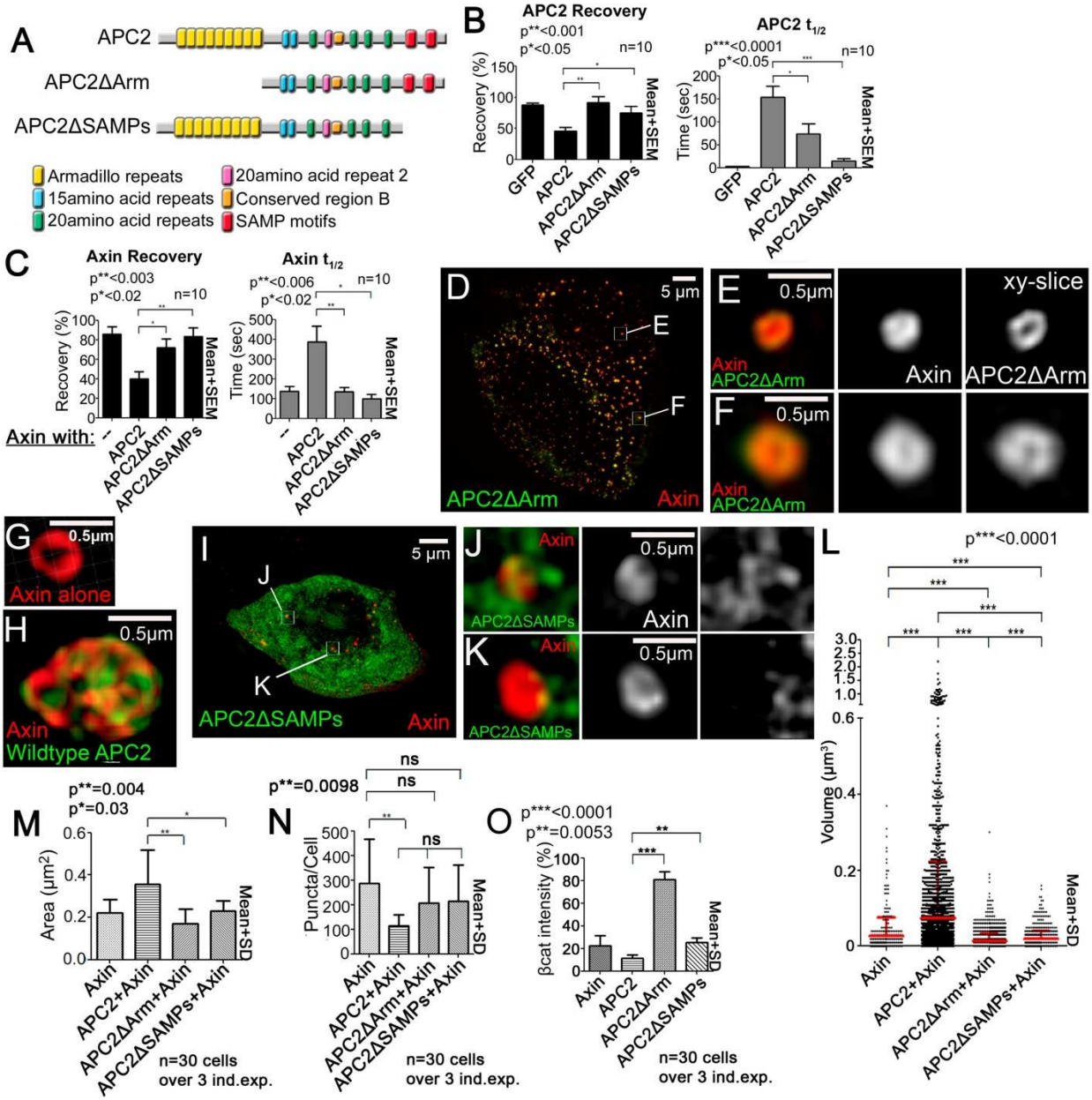
A
APC2
APC2ΔArm
APC2ΔSAMPs
Armadillo repeats
15amino acid repeats
20amino acid repeats
20amino acid repeat 2
Conserved region B
SAMP motifs
B
APC2 Recovery
p**<0.001
p*<0.05
n=10
Recovery (%)
Mean+SEM
GFP
APC2
APC2ΔArm
APC2ΔSAMPs
APC2 t1/2
p***<0.0001
p*<0.05
n=10
Time (sec)
C
Axin Recovery
p**<0.003
p*<0.02
n=10
Axin t1/2
p**<0.006
p*<0.02
n=10
Axin with:
D
5 µm
E
F
APC2ΔArm
Axin
E
0.5µm
xy-slice
Axin
APC2ΔArm
F
G
0.5µm
Axin alone
H
0.5µm
Axin
Wildtype APC2
I
5 µm
J
K
APC2ΔSAMPs
Axin
J
Axin
0.5µm
APC2ΔSAMPs
K
L
p***<0.0001
Volume (µm3)
Axin
APC2+Axin
APC2ΔArm+Axin
APC2ΔSAMPs+Axin
M
p**=0.004
p*=0.03
Area (µm2)
n=30 cells
over 3 ind.exp.
N
p**=0.0098
ns
Puncta/Cell
n=30 cells
over 3 ind.exp.
O
p***<0.0001
p**=0.0053
βcat intensity (%)
n=30 cells
over 3 ind.exp.

Figure 3.7. APC2's Arm rpts and SAMPs each are required to stabilize APC2:Axin complexes. (A) APC2 mutants. (B and C) FRAP analyses, SW480 cells. (B) APC2 needs both the Arm rpts and SAMPs to robustly associate with Axin puncta. Student's t-test. (C) Axin stabilization by APC2 is abolished when either APC2's Arm rpts or SAMPs are deleted. (D,K) SIM super-resolution images, SW480 cells expressing indicated constructs. (D–F) GFP-APC2ΔArm and Axin-RFP. (E–F) Close-ups, X–Y slice. Axin structure resembles complexes formed by Axin alone. (G and H) Axin-RFP puncta and APC2:Axin puncta for comparison. (I–K) GFP-APC2ΔSAMPs and Axin-RFP. (J,K) Close-ups. Axin does not form a complex internal structure when APC2 ΔSAMPs is expressed. (L) Puncta volume in SIM images of Axin (n = 3 cells), APC2 + Axin (n = 11), APC2ΔArm + Axin(n = 9), and APC2ΔSAMPs + Axin (n = 5) expressing cells. Deleting either the Arm rpts or the SAMPs inhibits APC2's ability to enhance puncta volume. ANOVA-Bonferroni. (M) Puncta area, confocal images. Area differences are consistent with volumes in (L). Student's t-test. (N) Puncta number, confocal images. Deleting Arm rpts or SAMPs in APC2 fails to decrease number of APC2:Axin puncta as does wildtype APC2. (O) APC mutants lacking the Arm rpts or SAMP motif show decreased ability to reduce βcat levels in SW480 cells. Quantification, total cell βcat fluorescent intensity normalized to untransfected cells.

Figure 3.8

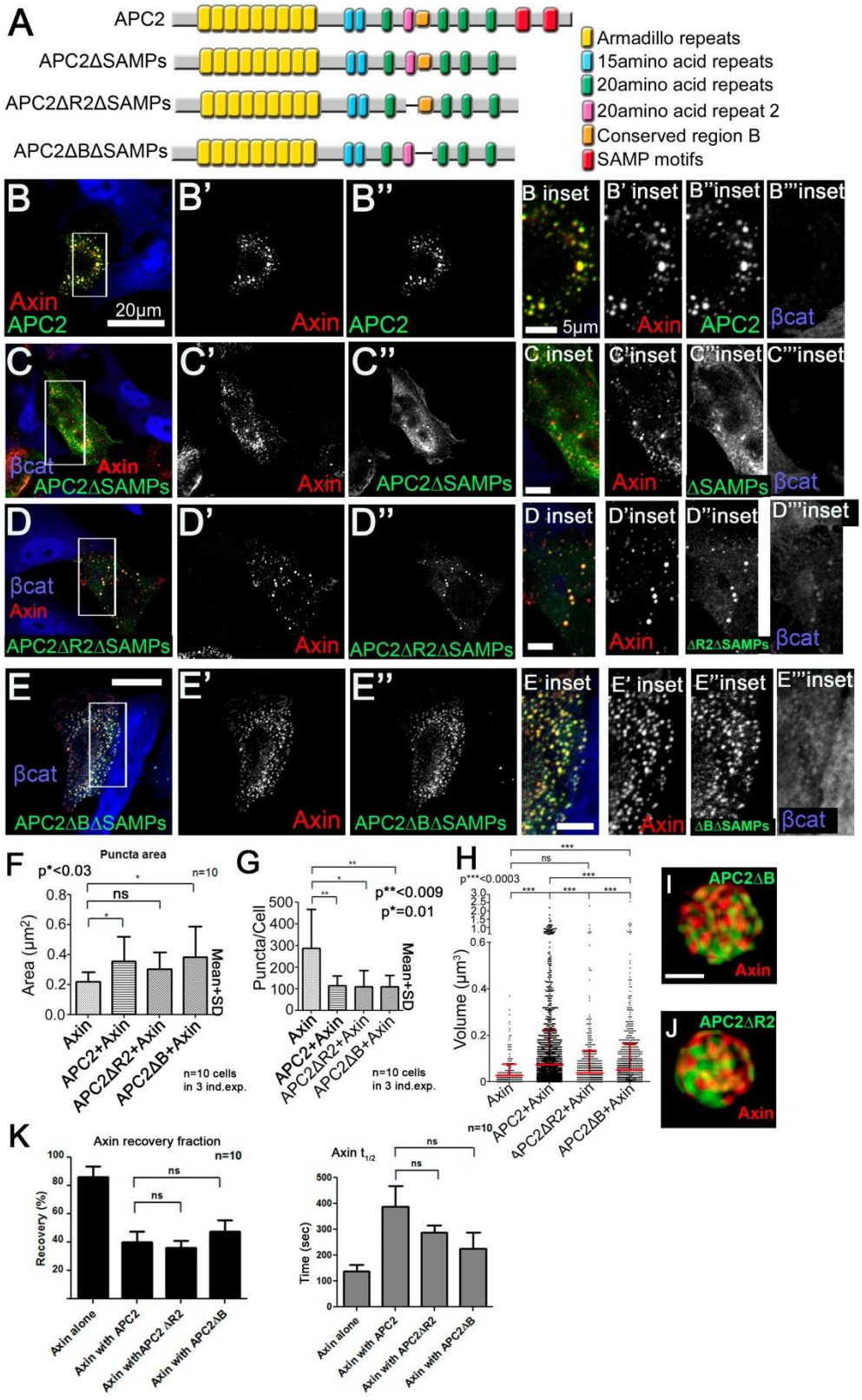
A
APC2
APC2ΔSAMPs
APC2ΔR2ΔSAMPs
APC2ΔBΔSAMPs
Armadillo repeats
15amino acid repeats
20amino acid repeats
20amino acid repeat 2
Conserved region B
SAMP motifs
B
B'
B''
B inset
B' inset
B''inset
B'''inset
Axin
APC2
20μm
5μm
βcat
C
C'
C''
C inset
C'inset
C''inset
C'''inset
APC2ΔSAMPs
ΔSAMPs
D
D'
D''
D inset
D'inset
D''inset
D'''inset
APC2ΔR2ΔSAMPs
ΔR2ΔSAMPs
E
E'
E''
E inset
E' inset
E''inset
E'''inset
APC2ΔBΔSAMPs
ΔBΔSAMPs
F
Puncta area
p*<0.03
n=10
ns
Area (μm²)
Mean+SD
Axin
APC2+Axin
APC2ΔR2+Axin
APC2ΔB+Axin
n=10 cells
in 3 ind.exp.
G
p**<0.009
p*=0.01
Puncta/Cell
H
p***<0.0003
Volume (μm³)
I
APC2ΔB
J
APC2ΔR2
K
Axin recovery fraction
Recovery (%)
Axin alone
Axin with APC2
Axin withAPC2 ΔR2
Axin with APC2ΔB
Axin t1/2
Time (sec)
Axin with APC2ΔR2

Figure 3.8. Colocalization of APC2's Arm repeat domain with Axin is controlled by R2 and B and APC2 without R2 or region B still stabilizes Axin complexes. (A) Diagram of APC2 constructs. (B) GFP-APC2 and Axin-RFP colocalize with one another in SW480 cells and reduce βcat levels (inset = box in B). (C) Deleting APC2's SAMPs reduces colocalization with Axin (inset = box in C). (D) Deleting R2 from APC2ΔSAMPs enhances colocalization with Axin (inset = box in D). (E) APC2ΔSAMPs lacking B colocalizes strongly with the Axin (inset = box in E). (F) Puncta area of APC2ΔR2 + Axin and APC2ΔB + Axin is similar to wildtype APC2 + Axin and differs from puncta formed by Axin alone. Analysis of confocal images, total of n = 30 cells. Student's t-test was used. (G) Wildtype APC2, APC2ΔR2 and APC2ΔB all reduce Axin puncta number. Quantitation of puncta number of confocal images used in (F). (H) Comparison of puncta volumes using SIM images reveals that APC2ΔR2 + Axin and APC2ΔB + Axin form puncta that are in between those formed by Axin alone and those formed by APC2 + Axin puncta. Volumes across puncta population. Axin (n = 3), APC2 + Axin (n = 11), APC2ΔR2 + Axin (n = 6), and APC2ΔB + Axin (n = 9). ANOVA-Bonferroni was used. (I,J) APC2ΔR2 + Axin and APC2ΔB + Axin puncta maintain complex internal structure. Closeups of puncta of SIM high resolution images of APC2ΔB + Axin (I) and APC2ΔR2 + Axin expressed in SW480 cells. (K) Deleting R2 or B does not significantly diminish the ability of APC2 to stabilize Axin in the destruction complex.

Figure 3.9

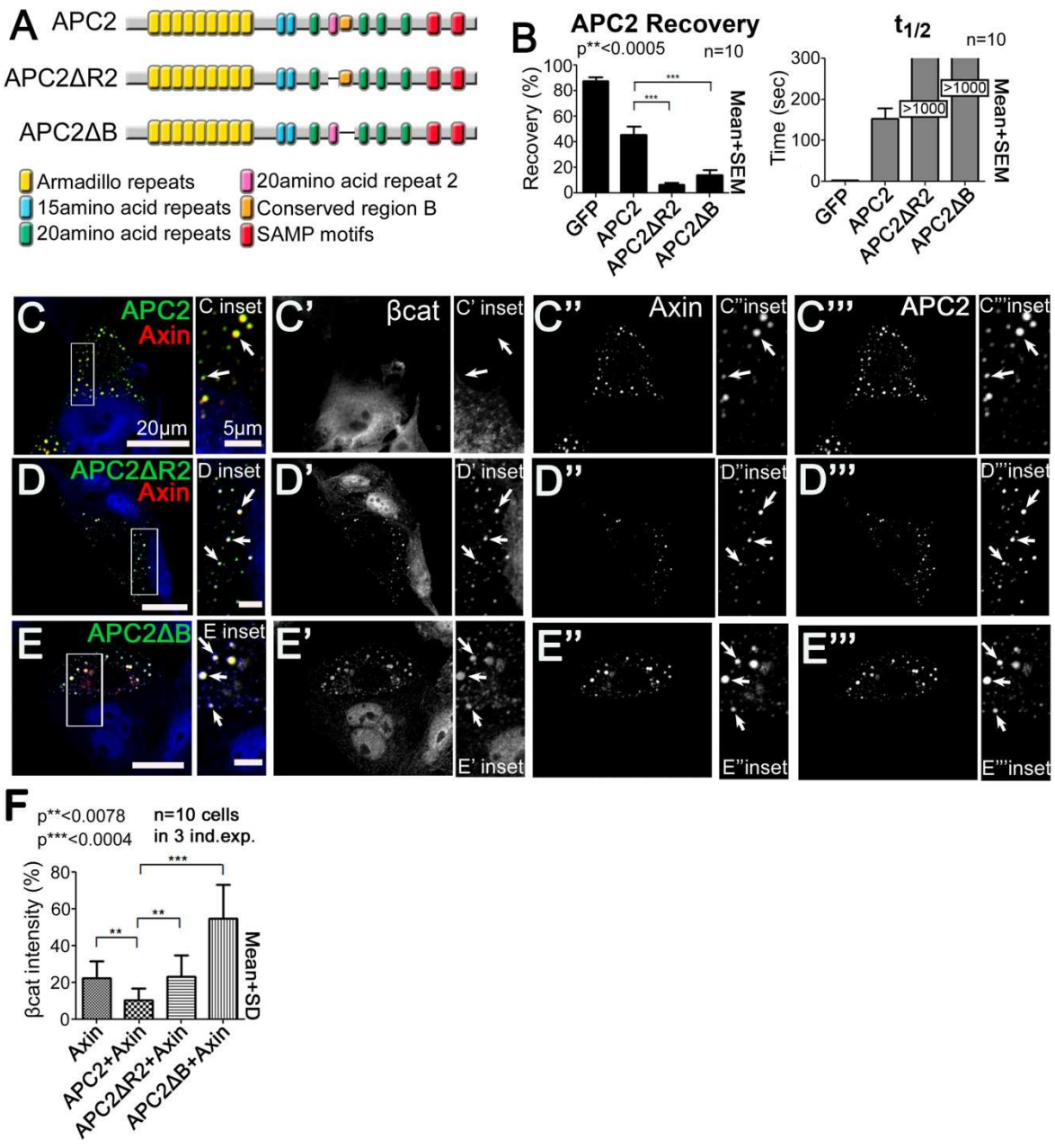

Figure 3.9. R2 and B regulate APC2 dynamics in the destruction complex, and regulate βcat removal from the destruction complex. (A) APC2 mutants. (B–F) SW480 cells transfected with Axin-RFP and indicated GFP-APC2 constructs (B) FRAP assay. Deleting either R2 or B slows APC2's turnover in Axin puncta. (C–E) Axin-RFP, GFP-APC2 constructs, βcat (inset = boxes). (C) βcat is essentially undetectable in APC2:Axin puncta (arrows). (D) βcat accumulates in APC2ΔR2:Axin puncta (arrows). (E) βcat strongly accumulates in APC2ΔB:Axin puncta (arrows). (F) Deletion of R2 or B impair the ability of APC2 to aid Axin in reducing βcat fluorescent intensity.

Figure 3.10

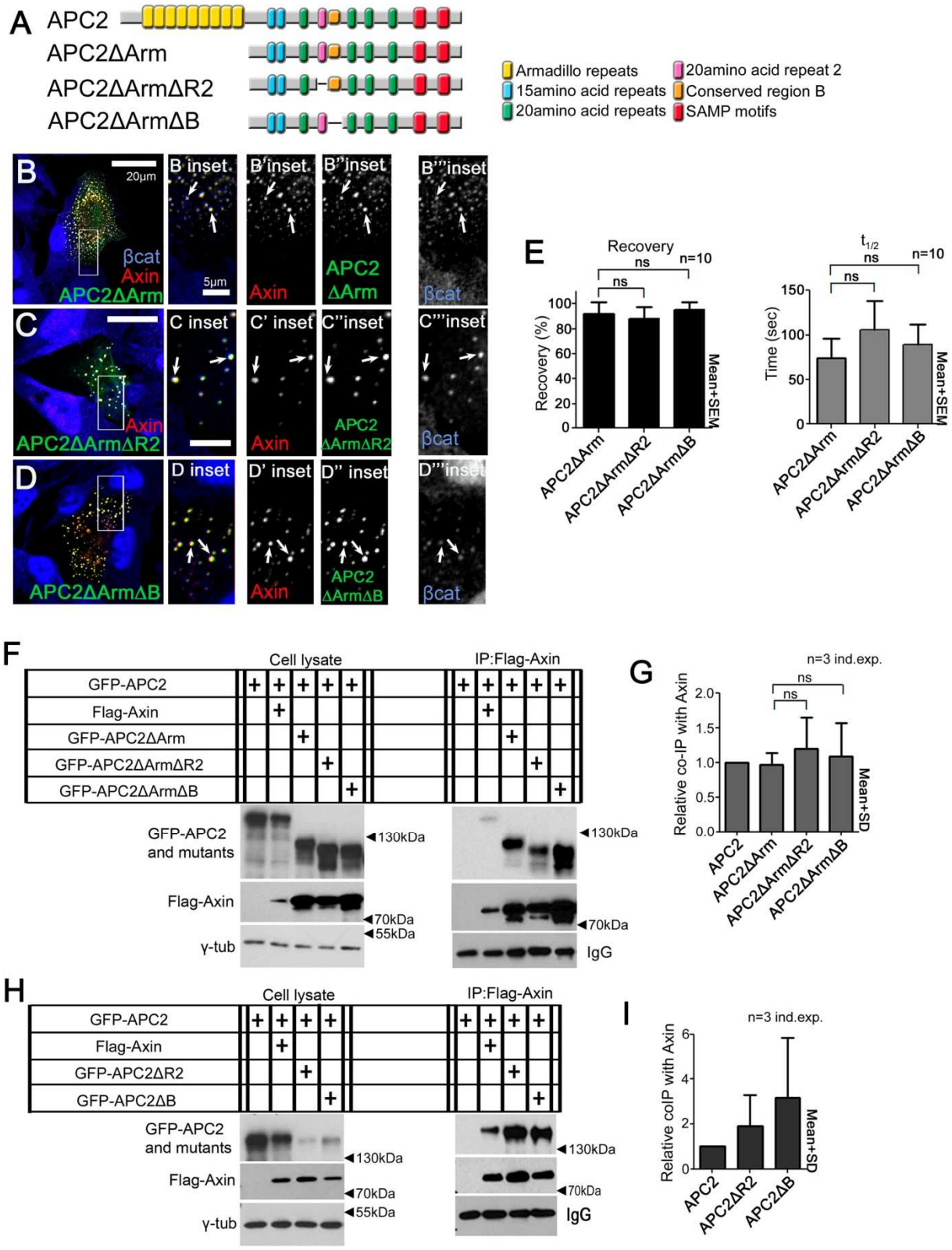
A
APC2
APC2ΔArm
APC2ΔArmΔR2
APC2ΔArmΔB
Armadillo repeats
15amino acid repeats
20amino acid repeats
20amino acid repeat 2
Conserved region B
SAMP motifs
B
20μm
B inset
B' inset
B'' inset
B''' inset
βcat
Axin
APC2ΔArm
5μm
C
C inset
C' inset
C'' inset
C''' inset
APC2ΔArmΔR2
D
D inset
D' inset
D'' inset
D''' inset
APC2ΔArmΔB
E
Recovery
ns
n=10
Recovery (%)
Mean+SEM
t1/2
Time (sec)
F
Cell lysate
IP:Flag-Axin
GFP-APC2
Flag-Axin
GFP-APC2ΔArm
GFP-APC2ΔArmΔR2
GFP-APC2ΔArmΔB
GFP-APC2 and mutants
130kDa
Flag-Axin
70kDa
55kDa
γ-tub
IgG
G
n=3 ind.exp.
Relative co-IP with Axin
Mean+SD
H
GFP-APC2ΔR2
GFP-APC2ΔB
I
Relative coIP with Axin

Figure 3.10. Binding of APC2's SAMP motif to Axin is not regulated by R2 and B. (A) Diagram of APC2 constructs. (B) GFP-APC2ΔArm colocalizes with Axin-RFP in SW480 cells (inset = box in B). βcat is detectable in APC2ΔArm:Axin complexes. (C) Deleting R2 does not alter colocalization of APC2ΔArm with Axin (inset = box in C). βcat remains detectable in the APC2ΔArmΔR2:Axin puncta. (D) APC2ΔArm lacking B colocalizes with Axin (inset = box in D). βcat is detectable in the APC2ΔArmΔB:Axin complexes. (E) FRAP assay of GFP-APC2ΔArm mutants with Axin-RFP in SW480 cells. Deletion of either R2 or B does not alter turnover of APC2ΔArm. Student's t-test was used. (F) Co-Immunoprecipitations of GFP-APC2ΔArm mutants with Flag-Axin. IP of Axin via anti-Flag antibody. APC2 binding to Axin via the SAMP motifs is not altered when R2 or B are deleted. Representative blot of 3 independent experiments. (G) Quantification Co-IP of indicated APC2 mutants with Flag-Axin ((F) and 2 replicates), normalized to coIP with wild-type APC2. Binding via the SAMPs is not altered when R2 or B are deleted. Student's t-test was used. (H) Co-IP of indicated APC2 mutants with Flag-Axin. Deletion of either R2 or B in full length APC2 increases its association with Axin. Representative blot of 3 independent experiments. (I) Quantification, Co-IP of indicated APC2 mutants with Flag-Axin ((H) and 2 replicates), normalized to coIP with wild-type APC2. Student's t-test was used.

Figure 3.11

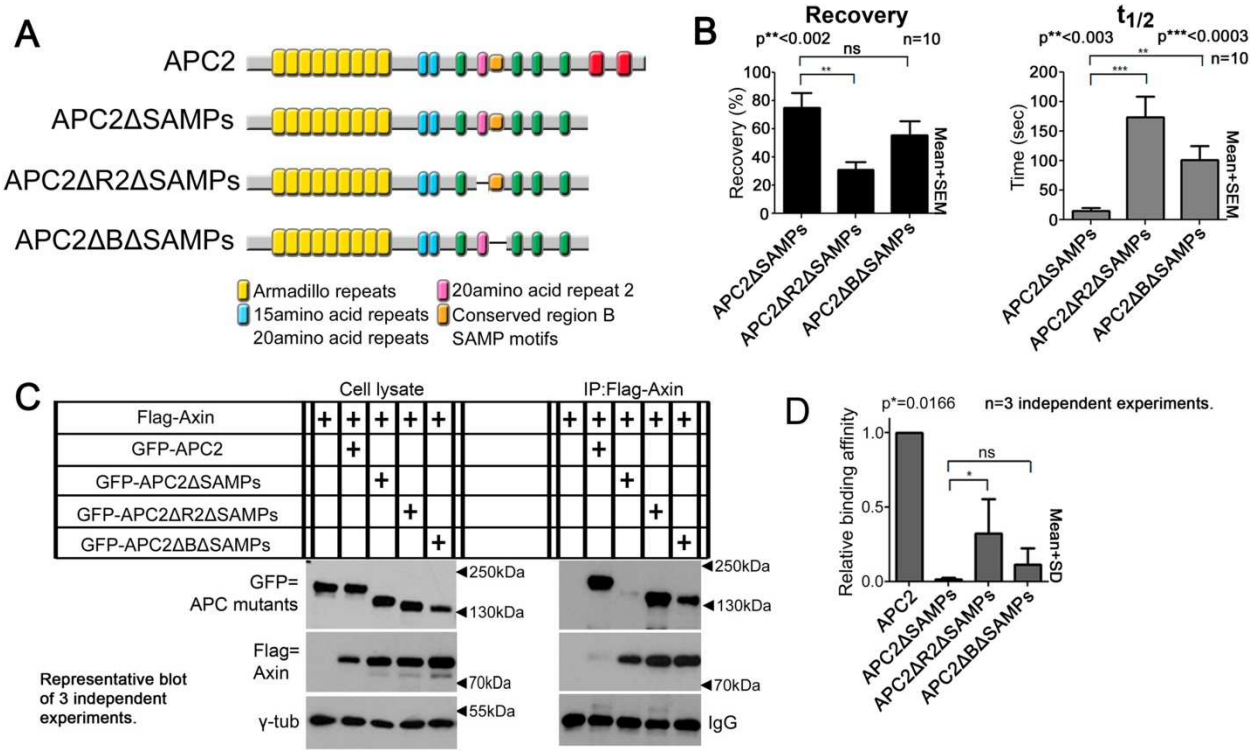

Figure 3.11. Association of Axin with APC2's Arm rpts is controlled by R2 and B. (A) APC2 constructs. (B) Deleting either R2 or B in APC2ΔSAMPs slows APC2 recovery time and reduces recovery fraction. FRAP assay, SW480 cells transfected with GFP-tagged APC2 constructs and Axin-RFP. (C) IPs of indicated constructs. Deleting the SAMPs substantially decreases APC2:Axin coIP, but this is partially restored by deleting R2. (D) Quantification, >2 replicates, normalized to Axin pull down. Student's t-test.

Figure 3.12

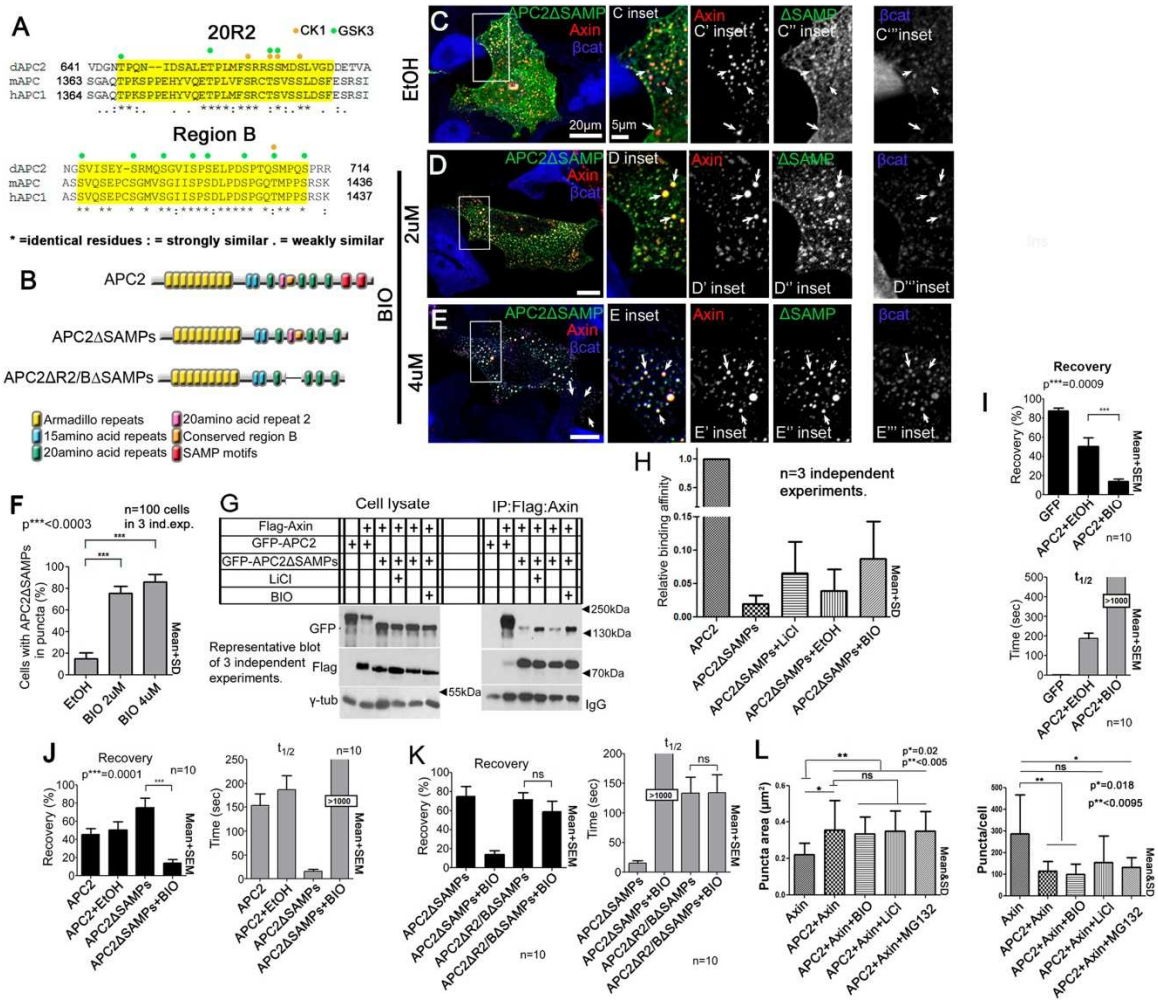

Figure 3.12. Axin:APC2 Arm rpts association is regulated by GSK3.(A) R2 and B of Drosophila dAPC2, mouse mAPC1 and human hAPC1. Potential CK1(orange) and GSK3(green) phosphorylation sites. (B) APC2 mutants. (C–E) SW480 cells expressing GFP-APC2ΔSAMPs and Axin-RFP. Insets = boxes in C–E (C) Control (Ethanol treated (EtOH)). Deleting the SAMPs reduces APC2:Axin colocalization (arrows). (D) 2 μM BIO enhances APC2ΔSAMPs recruitment into Axin puncta. (E) Increasing BIO to 4 μm further boosts APC2ΔSAMPs recruitment into Axin puncta. (F) Quantification of (C–E). (G) CoIP of APC2ΔSAMPs with Axin in SW480 cells ± LiCl or BIO. Full length APC2 is a control. Deleting the SAMPs drastically reduces coIP but GSK inhibition partially restores this. (H) Quantification of coIP in G, >2 replicates, normalized to Axin. (I) FRAP assay, SW480 cells transfected with Axin-RFP + GFP-APC2. GSK3 inhibition decreases APC2 dynamics. (J) GSK inhibition also slows APC2ΔSAMPs dynamics. (K) Deleting R2/B in APC2ΔSAMPs abolishes effect of GSK3 inhibition on dynamics. Student's t-test. (L) GSK3 inhibition with either BIO or LiCl does not further increase the size or decrease the number of APC plus Axin puncta, nor does treatment with the proteasome inhibitor MG132. Puncta area and puncta number, from confocal images.

Figure 3.13

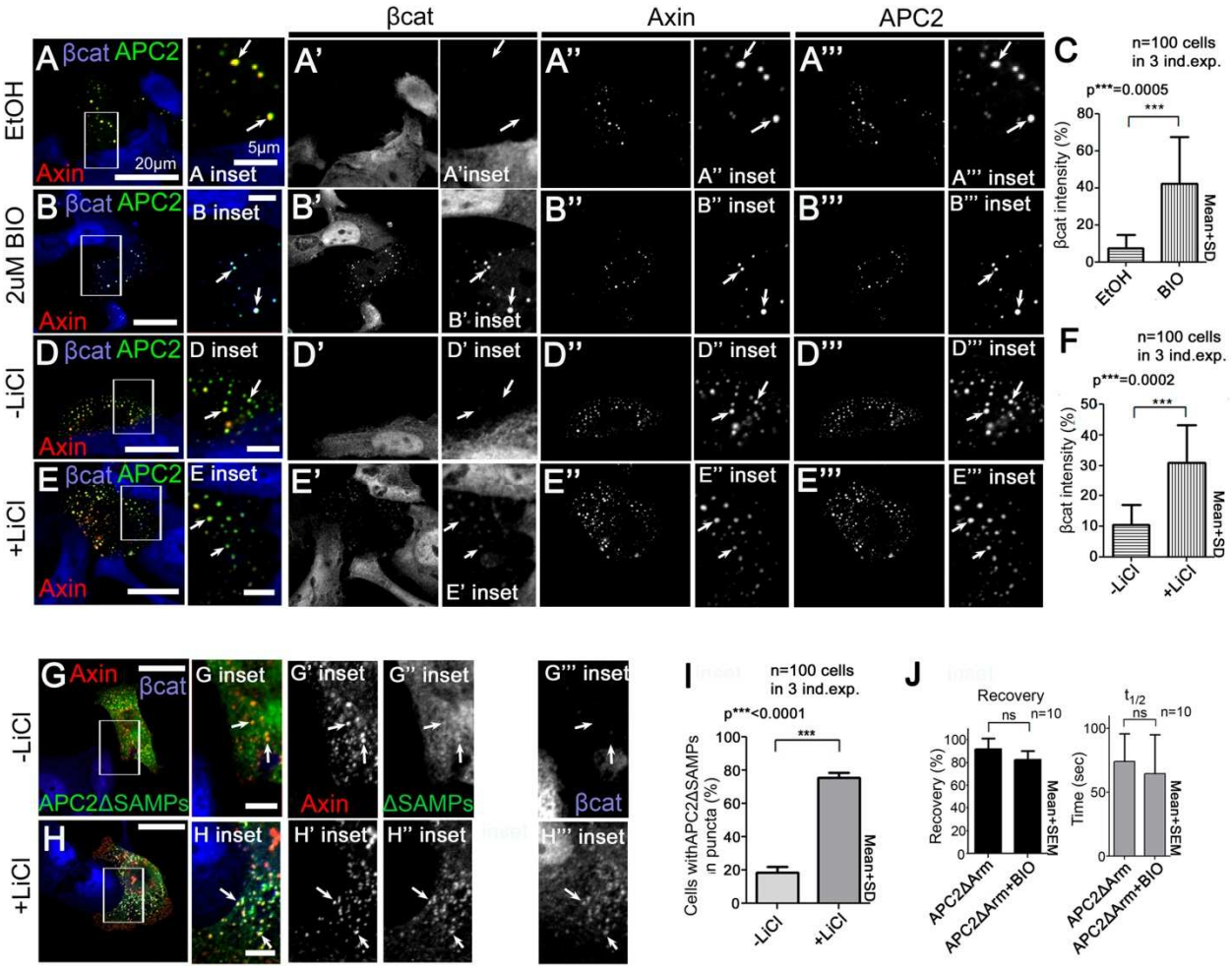

Figure 3.13. GSK3 regulates association of APC2's Arm repeats with the Axin complex. (A, B, D and E) GFP-APC2 and Axin-RFP expressed in SW480 cells. βcat stained via antibody. (A) APC2 and Axin strongly colocalize in cytoplasmic puncta in cells treated with EtOH as control for BIO. (Inset = box in A) βcat levels in APC2:Axin complexes are very low. (B) GSK3 inhibition via BIO does not alter colocalization of APC2 with Axin, but leads to βcat accumulation in APC2:Axin puncta (inset = box in B). (C) Quantification of total βcat fluorescent intensity in (A and B). (D) In control cells for LiCl treatment, βcat levels in APC2:Axin complexes are very low (inset = box in D). (E) βcat accumulates in APC2:Axin complexes when GSK3 is inhibited via LiCl (inset = box in E). (F) Quantification of total βcat fluorescent intensity in (D and E). (G) GFP-APC2ΔSAMPs is diffuse and only associates weakly with Axin (inset = box in G) in control cells for LiCl treatment. (H) GSK3 inhibition with LiCl enhances association of APC2ΔSAMPs with Axin (inset = box in H). (I) Quantification of APC2ΔSAMPs colocalization with Axin (G vs H). Student's t-test was used. (J) FRAP assay in SW480 cells transfected with Axin-RFP and GFP-APC2ΔArm. GSK3 inhibition via BIO does not alter APC2's turnover rate when association is mediated by the SAMPs. Student's t-test was used.

Figure 3.14

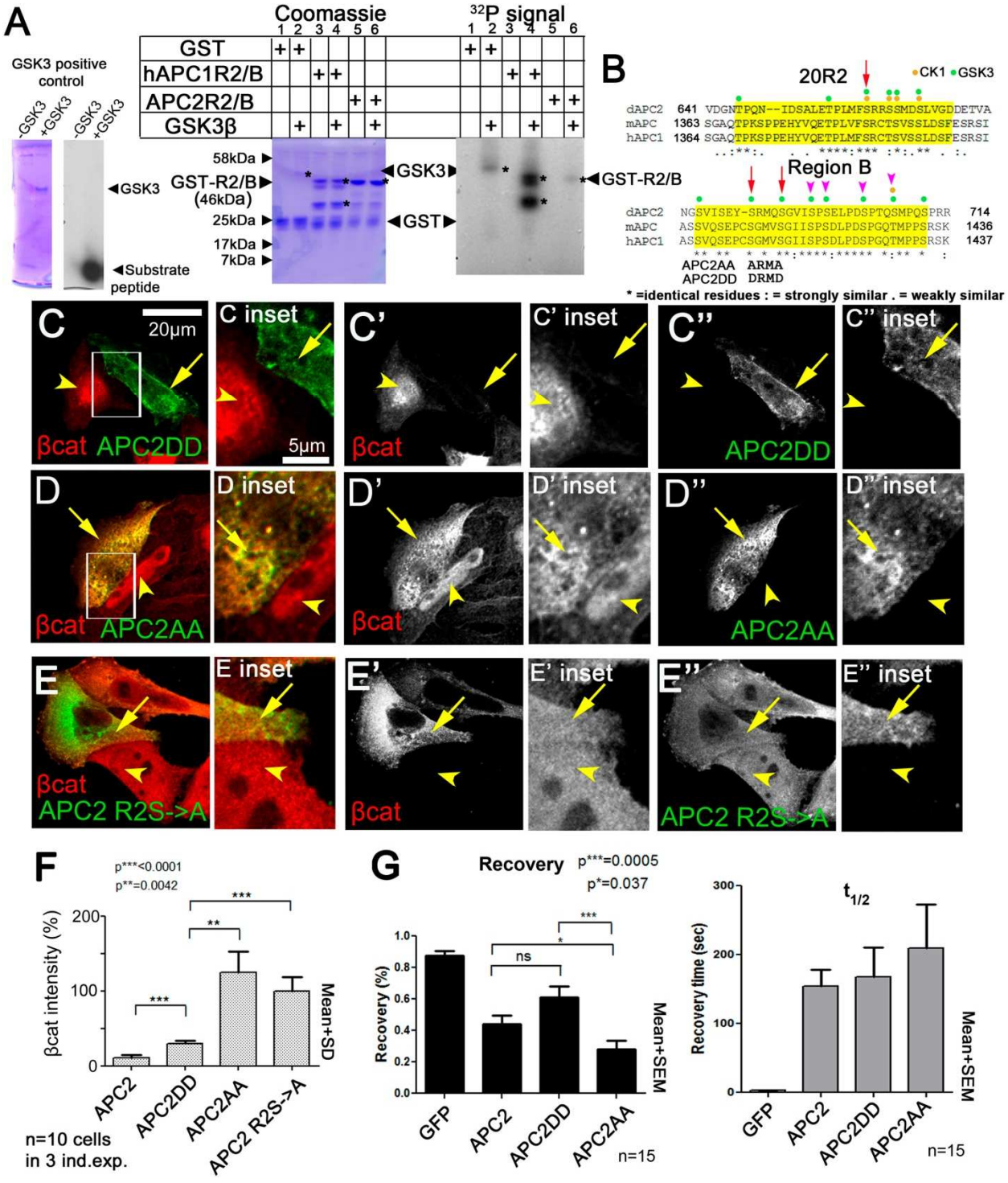
A
Coomassie
32P signal
GST
hAPC1R2/B
APC2R2/B
GSK3β
GSK3 positive control
-GSK3
+GSK3
58kDa
GST-R2/B (46kDa)
25kDa
17kDa
7kDa
GSK3
GST
GST-R2/B
Substrate peptide
B
20R2
CK1
GSK3
Region B
dAPC2 641 VDGNTPQN--IDSALETPLMFSRRSSMDSLVGDDETVA
mAPC 1363 SGAQTPKSPPEHYVQETPLVFSRCTSVSSLDSFESRSI
hAPC1 1364 SGAQTPKSPPEHYVQETPLMFSRCTSVSSLDSFESRSI
dAPC2 NGSVISEY-SRMQSGVISPSELPDSPTQSMPQSPRR 714
mAPC ASSVQSEPCSGMVSGIISPSDLPDSPGQTMPPSRSK 1436
hAPC1 ASSVQSEPCSGMVSGIISPSDLPDSPGQTMPPSRSK 1437
APC2AA ARMA
APC2DD DRMD
* =identical residues : = strongly similar . = weakly similar
C
20μm
C inset
5μm
C'
C' inset
C''
C'' inset
βcat APC2DD
βcat
APC2DD
D
D inset
D'
D' inset
D''
D'' inset
βcat APC2AA
βcat
APC2AA
E
E inset
E'
E' inset
E''
E'' inset
βcat APC2 R2S->A
βcat
APC2 R2S->A
F
p***<0.0001
p**=0.0042
βcat intensity (%)
Mean+SD
APC2
APC2DD
APC2AA
APC2 R2S->A
n=10 cells in 3 ind.exp.
G
Recovery
p***=0.0005
p*=0.037
Recovery (%)
Mean+SEM
ns
GFP
APC2
APC2DD
APC2AA
n=15
t1/2
Recovery time (sec)
Mean+SEM
n=15

Figure 3.14. Mutating putative phosphorylation sites in B disrupts APC2 function.(A) R2/B of human or fly APCs can be phosphorylated by human GSK3. In vitro kinase assay, GSK3 substrate peptide (positive control, left panels), GST-tagged humanAPC1R2/B or fly APC2R2/B fragments. GST was a negative control. Left of each pair: Coomassie stained gel, right: Phosphorylation detected using P32. Asterisks (*) indicate Coomassie-stained bands that align with P32-labeled proteins. In the presence of GST alone, GSK3 autophosphorylates (lane 2). HumanAPC1R2/B is strongly phosphorylated (lane 4) while fly APC2R2/B was more weakly phosphorylated (lane 6). Representative of two experiments. (B) R2 and B of Drosophila dAPC2, mouse mAPC1 and human hAPC1. Potential CK1 (orange) and GSK3 (green) phosphorylation sites. Red Arrow in R2 = serine mutated to alanine in mutant assessed in panel E. Red arrows in B = serines mutated to aspartic acid or alanine in APC2AA or APC2DD. Magenta arrowheads = additional serines mutated in 4 serine and six serine mutations (data not shown). (C) APC2DD (2 serines in B changed to aspartic acid; arrows in B) effectively reduces βcat levels in SW480 cells (arrow vs arrowhead). Inset = box in A. (D) APC2AA (2 serines in B changed to alanine, arrows in B) is unable to target βcat for destruction (arrow vs arrowhead). (E) APC2 R2S->A (single serine in R2 changed to alanine, arrow in B) is unable to target βcat for destruction (arrow vs arrowhead). (F) Quantification, total βcat fluorescent intensity. Student's t-test. (G) FRAP, Axin-RFP + GFP-APC2 constructs. APC2AA reaches a lower recovery plateau than either wild-type APC or APC2DD.

Figure 3.15

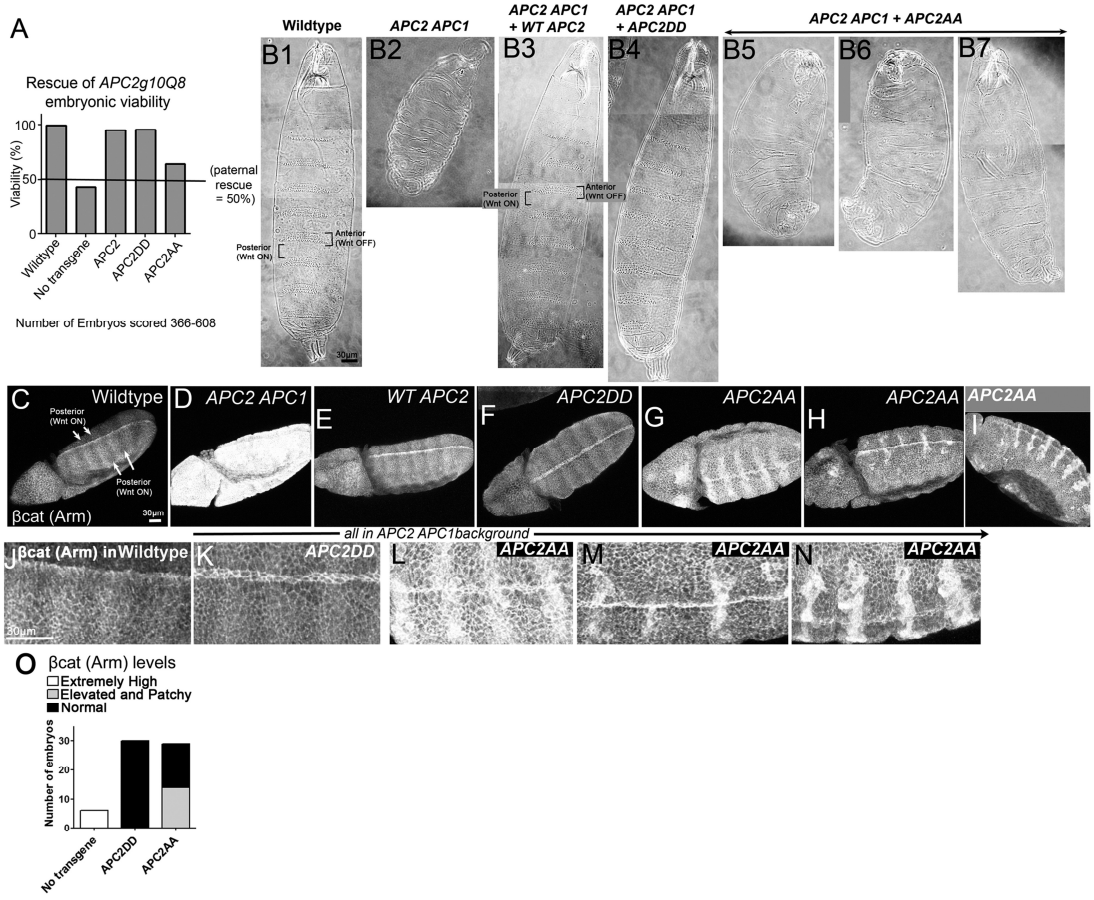

Figure 3.15. Blocking potential phosphorylation at 2 conserved serines in B disrupts APC2 function in the fly. APC2DD and APC2AA (Figure 8B) were expressed with the endogenous APC2 promoter in APC2 APC1 maternal/zygotic double mutants. (A) APC2 APC1 maternal/zygotic double mutants die as embryos (50% of embryos are zygotically rescued). APC2DD rescues embryonic viability as well as wildtype APC2. In contrast, APC2AA has only weak rescue ability. (B) Cuticles. (B1) Wildtype. Note pattern of anterior denticles (Wnt inactive) and posterior naked cuticle (Wnt active). (B2) Loss of APC2 and APC1 leads to denticle loss and expanded naked cuticle. (B3) Wildtype APC2 fully restores Wnt regulated cell fates of alternating denticles and naked cuticle. (B4) APC2DD similarly restores cell fates. (B5–B7) APC2AA largely fails to restore Wnt-regulated cell fates, and thus most cells secrete naked cuticle. Images = range of rescue ability. (C–I) βcat (fly Armadillo (Arm)) levels. Stage 9–10 embryos. (J–N) Close-ups of C–I. (C,J) Wildtype. Striped pattern of βcat indicative of Wg (fly Wnt) active and Wg inactive regions. (D) Loss of APC1 and APC2 leads to uniform very high levels of βcat. (E) WT APC2 restores normal βcat regulation, with higher levels in cells receiving Wg signal, and lower levels in other cells. However, Wg signal does not elevate βcat levels to those seen in embryos lacking functional APC. (F and K) APC2DD also rescues normal βcat regulation. (G–I and L–N) APC2AA restores some Wnt responsiveness, but βcat levels are elevated in all cells and especially elevated in a subset of cells receiving Wnt signal. (O) Quantification, embryos blind-scored.

Figure 3.16

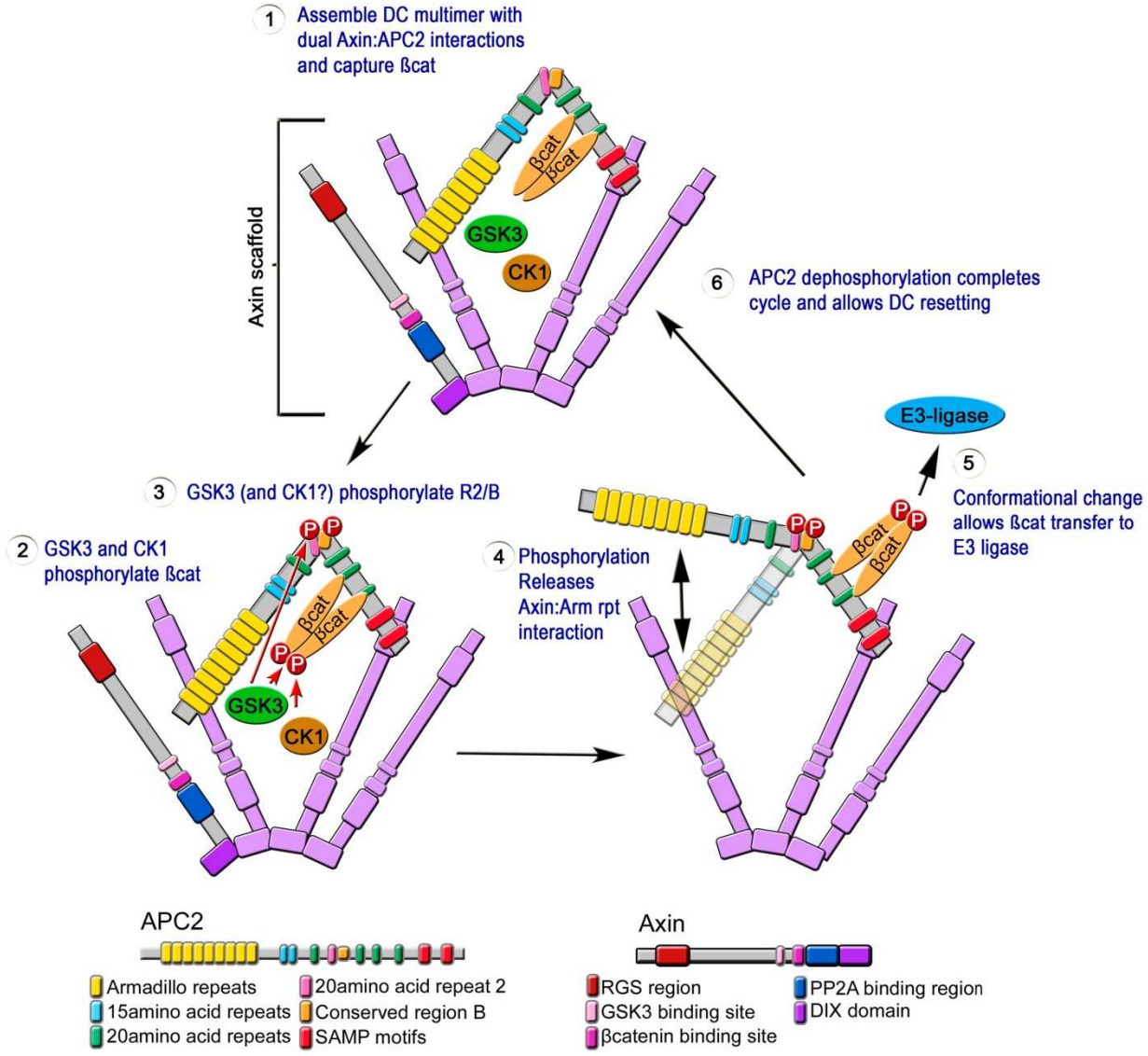

Figure 3.16. Speculative Model of APC2's catalytic cycle inside the destruction complex.(1) APC2 assembles with Axin via its Arm rpts and SAMPs. (2) APC2-bound βcat is phosphorylated by CK1 and GSK3. (3) GSK3 and CK1 phosphorylate R2/B region in APC2. (4) This induces a conformational change that releases APC2's Arm rpts from Axin. (5) APC2-bound βcat is released to the E3-ligase—alternately an APC–βcat complex is released. (6) Dephosphorylation resets the cycle.

Table 3.1

Summary Axin overexpression

Experiment	I	II	III
Ratio GFP-FlyAxin to GFP-hAxin1	1.04	0.11	0.48
Ratio GFP-hAxin1 to endo hAxin1	33.87	431.67	62.82
Transfection efficiency	30%	42%	36%
Fold overexpression level of GFP-FlyAxin to endo hAxin1	117.41	113.06	83.76

Summary APC2 overexpresssion

Experiment	I	II	III
Ratio Flag-FlyAPC2 to Flag-hAPC1-1338	17.31	138.45	28.02
Ratio Flag-hAPC1-1338 to endo hAPC1-1338	25.17	5.86	14.69
Transfection efficiency	30%	33%	25%
Fold overexpression level of Flag-FlyAPC2 to endo hAPC1-1338	1452.30	2458.54	1646.46

DOI: 10.7554/eLife.08022.006

Table 3.1. Quantitation of relative expression levels of transfected versus endogenous APC and Axin

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CHAPTER 4: ENGINEERING THE MINIMAL BETA-CATENIN DESTRCUTION MACHINE OF THE CANONICAL WNT PATHWAY

Overview

The βcatenin destruction complex consists of APC and Axin, which are the key negative regulators of the canonical Wnt signaling pathway. While Axin is the scaffold of the destruction complex, APC has been suggested to function in the efficient transfer of βcatenin from the destruction complex to the E3-ligase. At endogenous levels both are needed to downregulate the levels of the transcriptional co-activator βcatenin. However, in APC deficient cell lines overexpression of Axin compensates for loss of APC and reduces βcatenin levels suggesting APC and Axin act redundantly to facilitate βcatenin destruction. Here we investigated the redundant functions of APC and Axin in the βcatenin destruction complex. We found that (1) non-functional APC and Axin mutants can complement each other in reducing βcatenin levels, suggesting that the APC:Axin complex is a robust machine, (2) a total of 5 regions shared by APC and Axin are essential for a functional destruction complex, and (3) these 5 essential regions can reconstitute the wildtype APC:Axin complex in its internal complex structure, size, complex dynamics and in βcatenin destruction efficiency. Thus by engineering the minimal βcatenin destruction machine we identified the features required to build the complex scaffold, the binding and recruitment of βcatenin into the complex, and the efficient transfer of βcatenin to the E3-ligase as the key functions of the APC:Axin destruction complex.

Introduction

The canonical Wnt pathway is one of the most studied signaling pathways in animal cells. Its key roles during development and in diseases such as cancer underline the importance of fully understanding Wnt signaling regulation (Kandoth et al., 2013; Nusse, 2012). Cells have evolved mechanisms to keep powerful signaling pathways like this one in an off state in the absence of ligand. Two key negative regulators of Wnt signaling, Adenomatous polyposis coli (APC) and Axin, are critical for embryonic development and also act as important tumor suppressors in the colon and other tissues. (The Wnt homepage http://web.stanford.edu/group/nusselab/cgi-bin/wnt/ ; (Holstein, 2012)). 80% of sporadic colorectal cancers begin with mutations in APC (Kandoth et al., 2013), while mutations in Axin are found in hepatocellular carcinomas, ovarian cancer, and adenocarcinoma (Salahshor and Woodgett, 2005; Satoh et al., 2000).

Canonical Wnt signaling culminates by regulating the intracellular levels of the transcriptional co-activator βcatenin (βcat)(Nusse, 2012). APC and Axin, together with two kinases, GSK3 and CK1, are core components of the βcat destruction complex, which constitutively phosphorylates βcat. Phosphorylation creates a binding site for the SCFβTrCP E3-ligase complex which ubiquitinates βcat and hands it over to the proteasome for degradation. When Wnt signaling is activated through binding of a Wnt ligand to the Wnt receptor complex, the function of the destruction complex is inhibited, and βcat levels rise. βcat transfers then into the nucleus where it binds to Tcf/Lef transcription factors and drives expression of Wnt target genes (MacDonald and He, 2012).

Extensive research on the activation of Wnt signaling has led to a series of different models and mechanisms on how the destruction complex is inhibited (MacDonald and He, 2012). However, the mechanisms by which Wnt signaling is kept off is less well understood. The destruction complex is key in maintaining low levels of βcat, but we are just starting to understand the mechanistic function of each core component (Stamos and Weis, 2013). We know that Axin serves as the scaffold of the destruction complex. It self-polymerizes with other Axin molecules, forming a hub into which it then recruits APC, βcat, and the two kinases, the proteins needed for βcat phosphorylation and hand off to the E3-ligase (Kishida et al., 1999). APC's role inside the Axin complex has been less clear. Several hypotheses, including a role in localizing the destruction complex to a particular subcellular location, or an essential role in shuttling in and out of the nucleus, have been ruled out. APC can protect βcat from dephosphorylation, and our recent work suggests that APC functions as a stabilizer of the Axin scaffold and is also involved in efficient transfer of βcat to the E3-ligase by a GSK3 regulated mechanism (Pronobis et al., 2015; Su et al., 2008).

Elucidating the individual functions of Axin or APC contributes to our understanding, but to fully understand the downregulation of Wnt signaling by the destruction complex, we need to view the APC:Axin complex as an entity in which APC and Axin work cooperatively together to reduce levels of βcat. Our recent super resolution microscopy provided new insights into the cooperative assembly of these two proteins into a large multiprotein machine (Pronobis et al., 2015)--we now want to carry this further, defining the minimal component for its function. Both Axin and APC are large multidomain scaffolding proteins. Each has one or more large globular domains that mediate multiple protein interactions, and each also contains long

intrinsically unstructured regions that contain peptide binding sites for other protein partners. Surprisingly, some features of the two proteins seem overlapping--e.g., both contain βcat binding sites and each has a domain that facilitates self-oligimerization.

We hypothesized that if we could identify the essential regions required for APC:Axin cooperative function, and then use these insights to try and design the minimal βcat destruction machine this would provide new insights into the core mechanisms of the destruction complex function, and a paradigm for understanding large multiprotein complexes that assemble by multivalent interactions. More broadly this might provide valuable information about what goes wrong when APC or Axin are mutated in cancer cells.

Here we investigated the destruction complex as an entity by studying both APC and Axin, and their cooperative mechanistic function in the degradation of βcat. We found that (1) APC and Axin can complement each other and thus share redundant function inside the βcat destruction complex. (2) The core mechanism of the destruction machine depends on a total of 5 regions shared by APC and Axin that are sufficient to degrade βcat as effectively as wildtype APC:Axin complexes, and that (3) these 5 regions can reconstitute the wildtype APC:Axin complex in its internal structure, complex size and complex dynamics. Based on our data we propose a new model on the mechanistic function of the destruction complex as an entity.

Material and Methods

Constructs

Drosophila APC2 and Axin constructs were cloned using pECFP-N1 (Clontech) as a backbone vector via Gateway (Invitrogen) as described in (Pronobis et al., 2015; Roberts et al.,

2011). N-terminal GFP-tag (Roberts et al., 2011)and C-terminal-RFP were used (Pronobis et al., 2015). APC mutants were APC2ΔARB (494-536 aa, 595-621 aa, 733-1068 aa), APC2ARB (1-494 aa, 536-595 aa,621-733 aa), APC2ΔR2/B (Δ645-715 aa), Axin mutants were AxinΔβcat (Δ449-531aa), AxinΔDIX, Axinβcat-DIX (Δ666-746 aa), AxinN-term-GSK3 (1-494aa). The Chimera consisted of APC2's Arm rpts (1-495 aa), R2 and region B (621-733 aa) linked with a 6aa Gly-Ser-Gly linker to Axin's C-terminus (493-746 aa).

Antibodies

Primary antibodies: βcat (BD Transduction, 1:1000), GFP (Abcam, 1:10,000), γ-tubulin (Sigma, 1:5000), tagRFP (Evrogen, 1:5000), aPKCγ (Santa Cruz, 1:2000). Secondary antibodies: Alexa 568 and 647 (Invitrogen, 1:1000), HRP anti-mouse and anti-rabbit (Pierce, 1:50,000).

Cell culture

SW480 cells were cultured in L15 medium (Corning) with 10% heat-inactivated FBS+1X Pen/Strep(Gibco) at 37 °C without CO2. Lipofectamine 2000 (Invitrogen) was used for transfections following manufacturer's protocol. For immunostaining cells were processed after 24 hr. Immunostaining was conducted as described in Roberts et al. (2011)

Microscopy

Immunostained samples were imaged on a LSM Pascal microscope (Zeiss) and processed with the LSM image browser (Zeiss). SIM microscopy was carried out on the SIM high resolution microscope (Nikon) using 4% formaldehyde fixed samples mounted in Aquapolymount. Images were processed using Imaris 5.5, ImageJ and the LSM Image Browser.

Quantifications

Z-projections of cell image stacks were generated using ImageJ. βcat fluorescent

intensity was measured as described in (Pronobis et al., 2015). Shortly, cells were outlined, mean intensity measured, background subtracted, and βcat average intensity of a transfected cell normalized to mean of the βcat intensity of 2–3 adjacent untransfected cells.

APC:Axin complex area and volume quantification was measured as described (Pronobis et al., 2015) using the ImageJ Particle Analyzer and Imaris Software (Bitplane).

FRAP

FRAP was conducted using Eclipse TE2000-E microscope (Nikon) 24–72 hr after transfection. Movies were taken at 1 frame/6 s for 20 min and bleaching was conducted for 8 s with 100% laser power. Movies were processed using FRAP analyzer in ImageJ and GraphPad as described previously (Pronobis et al., 2015).

Reporter Gene Assay for Wnt activity

The TOP/FOP Flash Luciferase and the pRL Renilla constructs (transfection control) were gifts from Hans Clevers (Hubrecht Institute, Utrecht, The Netherlands). Dual Glow Luciferase System (Promega, Madison, WI) was used for reporter assays following manufacturer's instructions and has been described previously (Roberts et al., 2011). Transcriptional activity was defined as the ratio of TOP Flash normalized to Renilla. None of the constructs affected FOP Flash values.

Protein work

Cells were lysed in 2xSDS buffer and incubated at 96°C for 10min. Proteins were run on 8% or 7% SDS gels and blotted to nitrocellulose membrane.

Results

APC and Axin share regions with redundant functions

APC and Axin are both needed for efficient βcat degradation in SW480 cells but over-expressing Axin can partially compensate for a truncated APC protein. In our effort to design the minimal destruction machine and thus probe the mechanism of the APC:Axin complex, we used the human colorectal cancer cell line SW480. These cells have high levels of βcat due to a truncation of the endogenous human APC1 at 1338aa (Fig. 1D, (Rubinfeld et al., 1997)). In this APC mutant cell line, transfection of exogenous APC can restore βcat destruction. In our previous work we established a system that makes use of the Drosophila homologs APC2 and Axin -- this allowed us to assess full length proteins, as human APC1 (2853aa) is too large be effectively transfected and expressed in cells (Figure 4.1. D). Fly APC2 has all the functional regions needed to downregulate Wnt signaling and can reduce βcat effectively in the human SW480 cells, emphasizing the conservation of the Wnt pathway in all animals (Figure 4.1D, (Roberts et al., 2011)). Fly Axin also shares all regions found in human Axin (Figure 4.1C, (Willert et al., 1999)). Overexpression allowed us to study the βcat destruction machine in its core features and independent of limiting protein levels, and also to directly visualize the destruction complex, which assembles into large protein puncta that can be readily visualized.

Although Axin is unable to function in the degradation of βcat in the complete absence of APC (Mendoza-Topaz et al., 2011), elevating expression of wildtype Axin can also reduce βcat levels in this cell line carrying a truncated APC (Figure 4.1A - I,II (Cliffe et al., 2003)). These data suggested that Axin can compensate for reduced APC function when its levels are elevated, reflecting the suggestion that Axin is the rate limiting component (Salic et al., 2000). However

our previous data revealed that Axin overexpression alone is not as effective in downregulating βcat levels as is co-expression of APC plus Axin (Nakamura et al., 1998), consistent with the idea that APC and Axin need to work cooperatively to create the most effective βcat destruction machine.

APC and Axin have 5 regions that are essential for their function in βcat degradation

To define the core mechanisms underlying destruction complex function, we first identified the regions in APC and Axin that are essential for their individual function in reducing βcat levels (Figure 4.2). Our previous work revealed that 3 regions in APC2 are critical: the Armadillo repeats (Arm rpts), 20 amino acid repeat 2 (R2), and conserved region B (or catenin-inhibitory domain (CID) in human APC, Figure 4.3A, Figure 4.2A, (Pronobis et al., 2015; Roberts et al., 2012; Roberts et al., 2011)). We confirmed that all APC2 mutants were effectively expressed in SW480 cells, as previously reported (Pronobis et al., 2015; Roberts et al., 2012; Roberts et al., 2011). APC2ΔArm, APC2ΔR2, or APC2ΔB were unable to reduce βcat levels, while wildtype APC2 decreased βcat effectively (Figure 4.2B-E). Quantification of βcat immunofluorescence confirmed that levels were still >80% of those in control SW480 cells, and Wnt signaling activity measured by the TOP/FOP Flash transcriptional reporter was only weakly reduced (to about 70%; Figure 4.2F,G--the remaining βcat binding sites can sequester some βcat (Roberts et al., 2011)). We next identified the regions in Axin that are essential for its ability to reduce βcat levels in this APC mutant cell line (Figure 4.3A, S1 H). Overexpressing wildtype Axin reduced βcat in the SW480 cells (Figure 4.2I,(Nakamura et al., 1998)). However, consistent with studies in Axin mutant flies(Peterson-Nedry et al., 2008) deleting either Axin's βcat binding site or its DIX domain inhibits Axin's function (Figure 4.2H-K); both Axin mutants

are expressed at levels similar to that of wildtype Axin (Figure 4.2N). AxinΔβcat and AxinΔDIX failed to reduce βcat levels or Wnt-regulated transcription, suggesting both regions are also critical for Axin function in APC mutant human cells (Figure 4.2L,M,). Thus APC has 3 regions and Axin has 2 regions that are essential for their individual function in the destruction complex (Figure 4.3A).

Complementation suggests APC and Axin share regions with redundant functions

APC's and Axin share several potentially redundant functions (Figure 4.1B, C, upper part). These include 1) the ability to multimerize/self-polymerize, mediated by APC's Arm rpt regions or Axin's DIX domain, respectively, 2) the ability to bind and recruit βcat into the complex, APC via its 15 amino acid repeats (15aaRs) and 20 amino acid repeats (20aaRs) and Axin via its βcat binding site, and 3) two mechanisms by which APC and Axin interact, direct interaction of APC's SAMP motifs with the RGS region of Axin, and association of APC's Arm rpts with the GSK3 binding region of Axin (Kishida et al., 1999; Liu et al., 2006; Nakamura et al., 1998; Pronobis et al., 2015; Roberts et al., 2012; Spink et al., 2000). However, there are also certain functions that appear unique to APC or to Axin, such as Axin's ability to bind to GSK3, CK1 and the phosphatases PP2A/PP1 (the precise binding sites for CK1 and the phosphatases have not been defined (Amit et al., 2002; Hsu et al., 1999)) or APC's ability to increase transfer of βcat to the E3-ligase, and (Figure 4.1B,C, bottom part, (Nakamura et al., 1998; Pronobis et al., 2015)).

To examine whether some of these potentially redundant regions are in fact dispensible when APC and Axin work together, and to define other functional redundancies, we examined whether APC and Axin mutants that lack their essential regions can complement one another in

βcat destruction and Wnt signaling inhibition. In earlier work we were surprised to find that APC's multiple βcat binding sites are dispensible for targeting βcat for destruction, though they do modulate signaling by retaining βcat in the cytoplasm (Roberts et al., 2011). We hypothesized that Axin's βcat binding site might compensate, and the strongly reduced activity of AxinΔβcat supported this. To see if the βcat binding sites in APC could compensate for loss of the site in Axin, we co-expressed AxinΔβcat with each APC mutant (APC2ΔArm, APC2ΔR2, APC2ΔB). To test for other less obvious functional redundancies, we also expressed each APC mutant with AxinΔDIX (Figure 4.3B). Strikingly, APC and Axin mutants that lack essential regions and thus couldn't reduce βcat when expressed separately were often able to facilitate βcat destruction when co-expressed (Figure 4.3B-O). However, some combinations reduced βcat and Wnt transcriptional activity better than others. Based on their rescue ability we classified them into 3 groups (Figure 4.3B). Group 1 (APC2ΔArm+ AxinΔβcat, APC2ΔR2+ AxinΔDIX) showed full complementation ability in βcat levels and Wnt transcriptional activity (Figure 4.3B-G). The levels of βcat intensity and inhibition of Wnt signaling were indistinguishable from wildtype APC+Axin suggesting that these APC and Axin mutants can fully complement each other (Figure 4.3F-G). Group 2 (APC2ΔR2+ AxinΔβcat, APC2ΔArm+ AxinΔDIX) was classified as strong complementation ability (Figure 4.3H-K). This group had reduced βcat levels and Wnt signaling activity, however the reduction was weaker when compared to wildtype APC2+Axin transfected cells (Figure 4.3J,K). Weak complementation ability was seen in group 3, which consists of APC2ΔB coexpressed with either AxinΔβcat or AxinΔDIX (Figure 4.3L,O). Although, a reduction in βcat was detected, Wnt transcriptional activity was as high as when APC2ΔB, AxinΔβcat or AxinΔDIX were expressed individually suggesting that loss of certain regions like

region B interferes with complementation (Figure 4.3N,O, vs. 4.2A,E-M). The deletion of Axin's Δβcat binding site or the self-polymerization domain in Axin was compensated for by APC2ΔArm and APC2ΔR2. Interestingly, since APC2ΔArm and AxinΔDIX lack the predicted self-polymerization domains and so do not form any cytoplasmic puncta (Figure 4.3J,K), it is surprising that both could strongly complement each other. However, APC2ΔB was less effective in complementing both Axin mutants. Although APC2ΔB still maintains all βcat binding sites it reduced βcat levels and co-transcriptional activity only weakly. This suggests that region B might have a unique status in the cooperative function of APC and Axin. Thus, when working together APC and Axin can complement functional deficits in the other to efficiently target βcat for destruction.

APC's and Axin's essential regions each make semi-independent contributions to destruction

The data above suggest that APC2 and Axin create a robust destruction machine in which some regions are partially redundant, and that co-expression can lead to complementation. To further probe this, we assessed the function of the destruction complex as we systematically decreased the number of essential regions, by co-expressing different APC and Axin mutants (Figure 4.5A,B; we confirmed via immnuoblot expression of APC and Axin mutants that had not been previously tested (Pronobis et al., 2015; Roberts et al., 2012; Roberts et al., 2011, Figure 4.5). When we sorted these APC2+ Axin combinations based on the number of essential regions present, we found a generally gradual decrease in destruction complex function and thus increase in βcat levels as we progressed from 5 essential regions present to 0 regions present (Figure 4.5B). However, within a group defined by number of essential regions present we saw variations in βcat reduction-in particular mutant combinations

seemed strongly affected by deleting APC2's region B. When we averaged the βcat levels within a group, we found that deletion of 3 regions increased βcat levels significantly while missing 2 regions the APC:Axin complex was still able to sufficiently lower βcat levels (Figure 4.5C). Thus the destruction complex is a robust machine that can withstand on average the loss of 2 essential regions. Taken together, our data suggests that the 5 essential regions play an important role in the destruction complex.

The essential regions of APC and Axin are sufficient to downregulate Wnt signaling

Having defined regions of APC2 and Axin that are necessary for their function, we next tested whether APC's and Axin's essential regions are sufficient to facilitate downregulation of βcat and Wnt signaling. To do so, we generated APC2ARB, which has only the Arm rpts, R2 and region B, and Axinβcat-DIX, which consists of the C-terminal half of Axin that contains its two essential regions (Figure 4.4A). We tested their function in the reduction of βcat and in the inhibition of Wnt signaling. Both retained significant function, reducing βcat levels to about 40% of the control, though they were not as effective as wild-type APC2 or wildtype Axin, respectively (Figure 4.4A,G,H,K vs S1). Strikingly, both minimized constructs were indistinguishable from wildtype APC2 or Axin in inhibiting Wnt-regulated transcription. Thus the essential regions of APC and Axin have a moderate ability to reduce βcat levels, but can fully downregulate Wnt signaling (Figure 4.4L).

Covalently linking the essential regions of APC and Axin increases their negative-activity

Next we tested whether we could decrease βcat levels further by co-expressing APC2ARB and Axinβcat-DIX. Co-expression of both did not significantly reduce βcat levels further (Figure 4.3I,K,L). However, in their natural state, APC binds to Axin's RGS region via its

SAMP motif (Spink et al., 2000), both of which are deleted in our minimized constructs. We thus hypothesized that linking APC2ARB to Axinβcat-DIX physically might improve rescue ability (Figure 4.3A, bottom), we therefore created a chimera linking the C-terminus of APC2ARB to the N-terminus of Axinβcat-DIX. This mimics the interaction of APC's C-terminal SAMP motifs and Axin's N-terminal RGS domain. Strikingly, the APC:Axin chimera strongly decreased βcat Levels (to ~10%), and Wnt activity was also effectively inhibited (Figure 4.3J, M,N). Thus the Chimera downregulates βcat levels and Wnt signaling better than APC2ARB and Axinβcat-DIX expressed individually or co-expressed. In fact, the Chimera downregulated βcat levels and Wnt-regulated transcription as effectively as wildtype APC+Axin (Figure 4.3M,N). Strikingly, when we compared the Chimera to Axin, we found that the Chimera rescued better than expression of Axin alone, suggesting that substituting the N-terminal part of Axin with the essential regions of APC is more efficient than Axin in downregulation of βcat and Wnt signaling (Figure 4.3M,N).

Next we assessed whether the reduction of βcat is dose-sensitive, thus depending on protein levels of the Chimera, APC or Axin. In our previous work, we found that there was actually an inverse relationship for expression of wild-type APC2 or Axin, suggesting that at very high levels they may assemble into partial, non-functional complexes. We observed that the Chimera exhibited the same inverse relationship between levels of GFP-tagged protein and of βcat, thus suggesting it was effective at the lowest levels of expression we assessed (Figure 4.3O). Consistent with our overall assessment, this experiment also revealed that the Chimera appears more effective at promoting βcat destruction than is Axin, across the entire concentration range. Thus none of the 3 proteins showed dosage dependency. Thus, covalently

linking the 5 essential regions of APC and Axin together is sufficient to reduce βcat and inhibit Wnt signaling in SW480 cells as efficiently as co-expressing wild-type APC and Axin.

APC's and Axin's essential regions remain indispensible in the Chimera

Since we physically linked APC's and Axin's regions together into an artificial chimeric protein, we next assessed whether all 5 essential regions remain indispensible when linked in the Chimera. We sequentially deleted each of the 5 regions and tested the ability of the Chimera mutants to facilitate βcat destruction and to repress Wnt-dependent transcription (Figure 4.6A). Deleting APC2' s Arm rpts, R2, or region B, or Axin's βcat binding site or DIX domain substantially reduced the ability of the Chimera to downregulate βcat levels and Wnt-dependent transcription (Figure 4.6B-I). Each of the chimera mutants had βcat levels over 50% and Wnt signaling activity over 40% (Figure 4.6H,I). We observed one additional intriguing thing--most of the Chimera mutants accumulated βcat in the complexes , but ChimΔβcat did not (Figure 4.6F, arrow heads vs S3C-E,G, arrow heads) suggesting that the βcat binding site of Axin is able to recruit βcat into the Chimera complex. Thus the Chimera needs all 5 essential regions to fully function in βcat destruction.

The internal structure and the complex size of the Chimera are similar to APC:Axin complexes

Since the Chimera reduced the destruction complex to 5 regions, we wanted to investigate whether those 5 regions are able to reconstitute the APC:Axin complex. If we really understand the destruction complex machinery the features of the Chimera complex should be similar to those of APC+Axin complexes. First we used high resolution SIM microscopy to investigate the internal structure. As previously reported, Axin alone forms simple structures that consists of circular sheets or cables (Figure 4.7A-D, Pronobis et al., 2015). Co-expression of

APC altered the Axin complex in which more cable associate with one another as previously documented (Figure 4.7E-H). Interestingly, larger complexes displayed a more complex structure than smaller ones (Figure 4.7F vs. H). Thus APC helps build the βcat destruction complex by enabling Axin to form larger complexes. When we looked at the internal structure of the Chimera complex we found similarities to APC:Axin complexes (Figure 4.7I-L). The internal structure compromised several cables that were intertwined with one another. Thus the Chimera is able to reconstitute the internal structure of the destruction complex.

Next we compared the volumes of the APC+Axin and Axin cytoplasmic puncta using the SIM images. We found that as previously described APC+Axin formed larger complexes than Axin alone (Figure 4.7M, Pronobis et al., 2015). Consistent with the internal structure the Chimera complexes also resembled APC+Axin complexes more in their volume than Axin complexes. Comparison of the area using LSM images confirmed the volume measurement in which also the Chimera complexes had an area consistent with the native APC:Axin complex (Figure 4.7N). Interestingly, the number of puncta where different from cells that have been transfected with Axin alone, and were similar to APC:Axin transfected cells (Figure 4.7O). These data suggest that the larger complexes are formed by reducing the number of complexes in cells. Thus the Chimera that consists of the 5 essential regions of APC and Axin is able to reconstitute the internal structure, size and number of complexes of APC:Axin complexes.

The Chimera mimics dynamics of the APC:Axin complex

As a final comparison of the Chimera to co-expression of Axin and APC2, we examined the dynamic behavior of the chimeric complex using Fluorescence Recovery After Photobleaching (FRAP). We previously found that large puncta formed by Axin alone are highly

dynamic, with a recovery plateau of 90% and a t1/2 of 220sec (Fig4.8A,B, (Pronobis et al., 2015)). However, in the presence of APC Axin's dynamics slow down - the recovery plateau decreases to 30% and the t1/2 extends to almost 600 sec, suggesting that APC stabilizes Axin multimerization (Figure 4.8B, Pronobis et al., 2015). Interestingly, although APC's recovery plateau when in complex with Axin is similar to that of Axin, APC shows a faster turnover (t1/2=100sec; Figure 4.8C). This is consistent with the model that Axin is the scaffold and therefore its turnover is slower, while APC constantly re-assembles into the complex, perhaps bringing in new βcat. When we tested the Chimera for its dynamics, we found that its recovery plateau is very similar to that of both APC and Axin, suggesting a stable core of Chimera polymers. Strikingly, however, the t1/2 of the Chimera is 100sec and therefore more similar to APC (100sec) than Axin (600sec, Figure 4.8C). Thus, the artificial Chimera reconstitutes different aspects of the dynamics of the wildtype APC:Axin complex.

Since the Chimera reconstituted the native APC and Axin complex in dynamics, structure, size and βcat regulation efficiency, but does not have a GSK3 binding site, we wanted to investigate whether the Chimera is able to interact with the endogenous human Axin, and whether this explains why it does not need a GSK3 binding site. When we IP-end for GFP-tagged Chimera we found that endogenous Axin can be pulled down with the Chimera (Figure 4.8D). Thus although the Chimera recues as well as APC+Axin, it might still depend on endogenous Axin.

Discussion

The βcat destruction complex is key in maintaining Wnt signaling activity. By studying the cooperative mechanism of APC and Axin in the destruction complex, our study revealed new insights into how the destruction complex functions.

Our data suggests that although Axin overexpression has been shown to reduce βcat in the APC mutant SW480 cell line βcat downregulation is not as efficient as in APC2+Axin, or APC2(+endogenous hAxin) transfected cells (Figure 4.8E). Since APC and Axin mutants can complement each other, we suspect that the truncated hAPC1 in SW480 cells does not have all the functional regions such as R2 and B that are required to assist Axin (Figure 4.8E). This data is consistent with experiments in the fly where overexpression of Axin or in APC2 mutant flies (point mutation N175K in Arm rpts region) is able to restore wildtype phenotype, but is unable to do so in APC2APC1 null fly mutants (Mendoza-Topaz et al., 2011). Thus Axin relies partially on APC for its function in which a functional APC protein is not required, but instead the presence of certain regions in APC.

In our complementation assay some combination of APC and Axin mutants reach wildtype APC+Axin levels in βcat destruction or inhibition of Wnt signaling. However, although some mutants are able to fully complement, many showed higher levels of βcat and Wnt activity (Figure 4.8E). Further when we systematically deleted the 5 essential regions in APC and Axin, and tested for βcat reduction we found that on average 2 essential regions were dispensable in the destruction complex. These data suggest that the APC:Axin complex does not use all of its regions at the same time to facilitate βcat destruction, but instead has multiple ways in which a combination of different regions leads to downregulation of Wnt signaling

(Figure 4.8F). Such mechanism could be important to secure low levels of βcat when mutations occur or when some regions are inhibited in their function by modifications or inhibitors. Since this would only slow down the machinery instead of turning it off completely, it would allow in case of mutations to maintain low Wnt signaling, and in case of inhibition to fine tune Wnt activity. Thus we propose that the combination of different regions is reflected in the redundant function of APC and Axin to create a robust βcat destruction complex that can be easily fine-tuned in its destruction rate.

The 3 essential regions in APC or the 2 essential regions of Axin repressed Wnt signaling as well as wildtype APC or Axin, respectively. These data suggest that the essential regions may have a yet unknown mechanism in which they interfere with βcat activated transcription. Since βcat is a co-activator of transcription recruiting several components of the transcription machinery, and APC and Axin have been shown to shuttle into the nucleus, it would be interesting to investigate how the essential regions repress Wnt activity ((Cong and Varmus, 2004; Neufeld et al., 2000; Pronobis and Peifer, 2012).

Based on our data linking the essential regions of APC and Axin together facilitates βcat destruction and inhibition of Wnt signaling as efficient as wildtype APC+Axin, and better than Axin alone. We show that all regions in the Chimera are important for keeping the Wnt pathway downregulated and that the APC:Axin Chimera can reconstitute the destruction complex in its internal complex structure, its size, and its dynamics. Thus the regions in the Chimera combine the key functions of the destruction complex (Figure 4.8G): (1) Multimerize/polymerize via APC's Arm rpts and Axin's DIX domain allows the generation of a compartment in which all other destruction complex components are recruited to allow efficient βcat destruction. This so

called phase transition model in which multivalent proteins create a compartment-like structure inside the cytoplasm has been recently proposed to play a major role in signaling transduction (Brangwynne et al., 2009; Li et al., 2012). It would be interesting to test whether the polymerization domains in the Chimera could be replaced by non-Wnt related polymerization regions. (2) The recruitment of βcat into the APC:Axin complex is another key feature. Since it has been suggested that APC's βcat binding sites act redundantly, our data suggest that Axin's binding site is sufficient to pull βcat into the complex. Thus APC's βcat sites can be replaced by Axin's βcat binding site (Figure 4.8F). It would be interesting to test whether APC's 20R3 which has been proposed to have the highest affinity to βcat could replace Axin's βcat binding site, or whether there is something unique about Axin's binding site (Liu et al., 2006). (3) The Arm rpts, R2 and region B have been recently proposed to function in the efficient transfer of βcat to the E3-ligase- another key step in βcat degradation. The Chimera dependents on these three regions since deletion of any regions resulted in loss of βcat downregulation and accumulation of βcat in the destruction complex. However, in case of the Arm rpts it would be interesting to test whether they have dual function in the complex-multimerization and βcat transfer- or only one of both are required for the minimal βcat machine.

Mutations in APC or in Axin have been associated in the occurrence of cancers (Kandoth et al., 2013; Salahshor and Woodgett, 2005; Satoh et al., 2000). Our study broadens the understanding of the destruction complex as an entity and provides new insights into the mechanism on how APC and Axin facilitate βcat destruction together. By engineering the destruction complex we simplified and replaced regions in the APC:Axin Chimera (Figure 4.8H):

APC's SAMPs and Axin's RGS motif was replaced by a linker. This suggests that APC and Axin could be permanently connected to each other without any impact on their βcat destruction efficiency. Thus in case of colorectal cancer where APC gets truncated, linkage of the mutant APC (if all 3 essential regions are present) to Axin might increase the efficiency in βcat destruction and restore Wnt regulation.

Recruitment of GSK3 has been shown to be a key feature of the destruction complex, however although the Chimera strongly reduces Wnt signaling it lacks a GSK3 binding site. We propose that binding to endogenous hAxin1 is sufficient to recruit GSK3 into the complex, since fly APC2 and fly Axin has been shown to bind to endogenous hAxin1, and our co-IP experiments confirmed that the Chimera also associated with human Axin (Pronobis et al., 2015). However, there is still the possibility that since we are overexpressing the Chimera GSK3 recruitment may not be necessary due to its high abundance.

Thus we show that the APC:Axin complex is a robust machinery that uses the redundancy of regions to facilitate βcat destruction in multiple ways, and that its key function can be reduced to 5 essential regions that can reconstitute the destruction complex when Wnt signaling is turned off. It would be interesting to test whether and how the Chimera complex responds to Wnt activation.

Figure 4.1

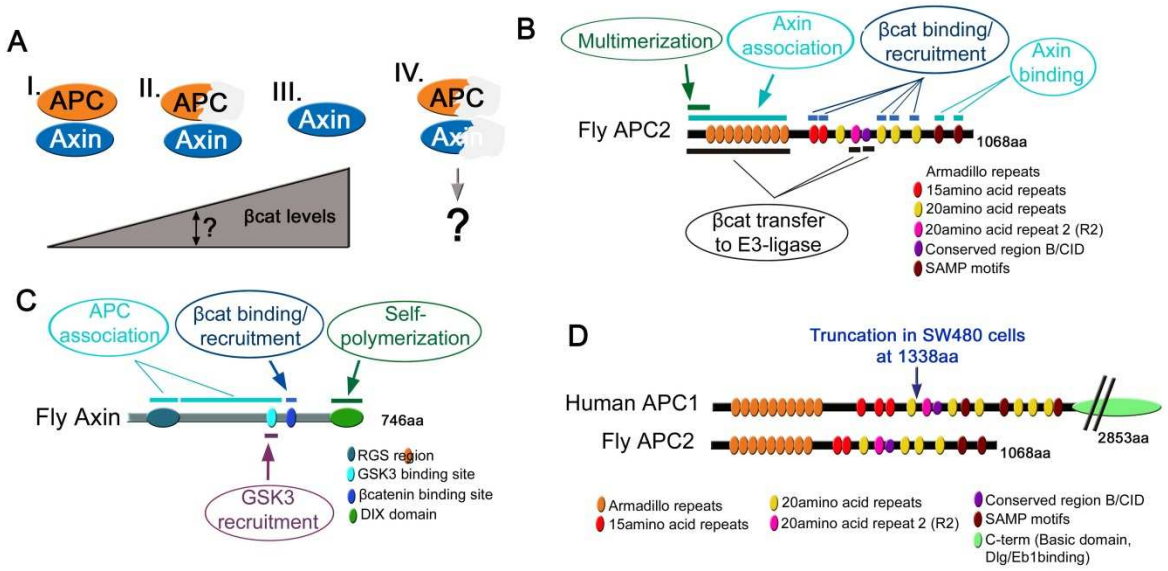

Figure 4.1. APC and Axin have regions with redundant function. (A) Representation of βcat levels with different APC and Axin combinations. (I) Wildtype APC and Axin are most efficient in βcat degradation. (II) It remains unknown how efficient βcat reduction is when Axin and a truncated APC are present. (III) Axin alone is unable to destroy βcat (Mendoza-Topaz et al., 2011). (IV) APC and Axin mutants have not been tested for their ability to reduce βcat levels. (B) Schematic representation of fly APCs. Top: Regions that have redundant function with Axin. Bottom: Unique functions in APC. (C) Schematic representation of fly Axin. Top: Regions that may have redundant function with APC. Bottom: Regions with unique function. (D) Schematic representation and comparison of human APC1 and fly APC2.

Figure 4.2

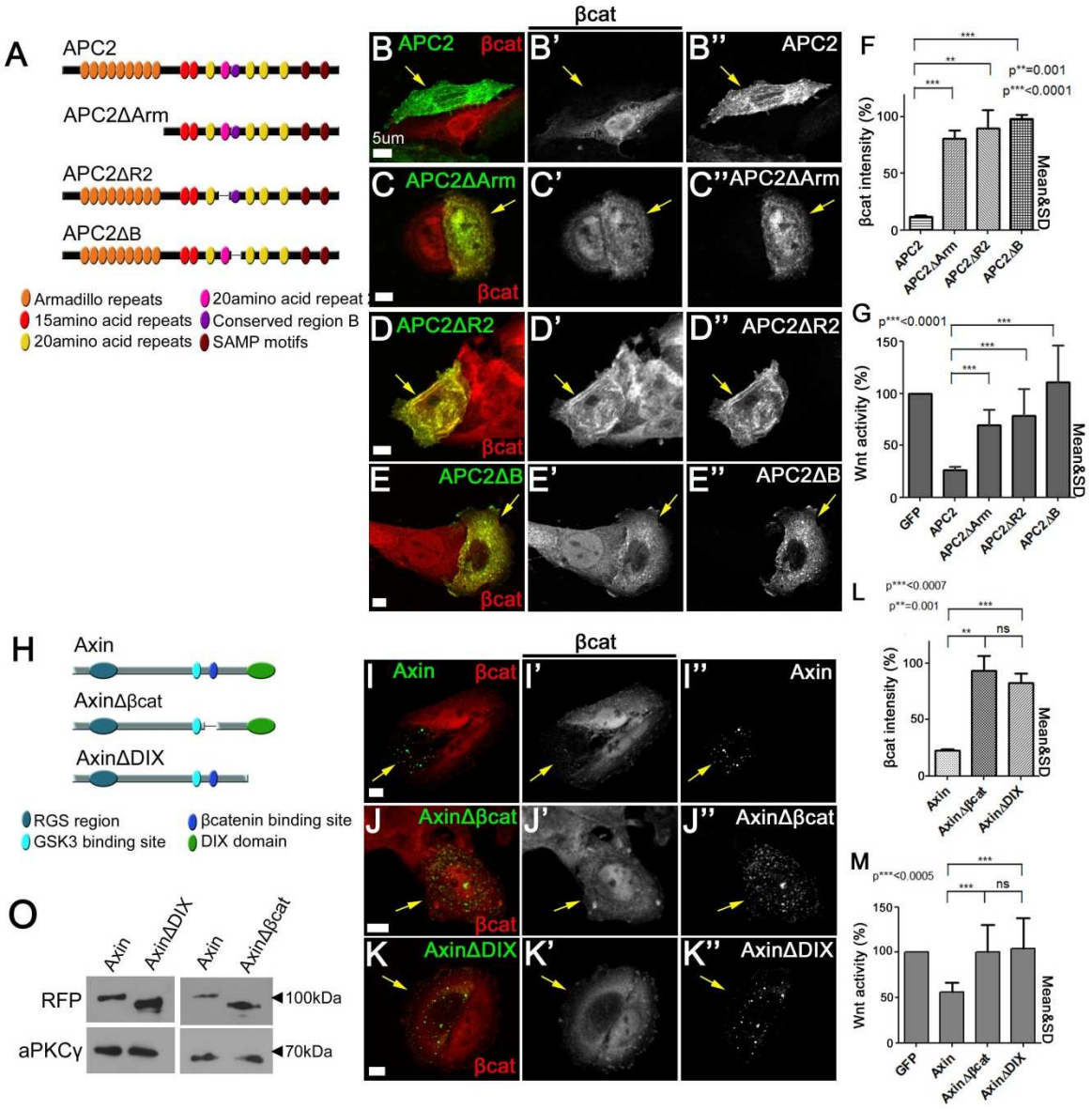
A
APC2
APC2ΔArm
APC2ΔR2
APC2ΔB
Armadillo repeats
15amino acid repeats
20amino acid repeats
20amino acid repeat
Conserved region B
SAMP motifs
βcat
B APC2 βcat
B' B''
C APC2ΔArm
C' C''
D APC2ΔR2
D' D''
E APC2ΔB
E' E''
5um
F
p**=0.001
p***<0.0001
βcat intensity (%)
Mean&SD
APC2
APC2ΔArm
APC2ΔR2
APC2ΔB
G
p***<0.0001
Wnt activity (%)
GFP
APC2
APC2ΔArm
APC2ΔR2
APC2ΔB
H
Axin
AxinΔβcat
AxinΔDIX
RGS region
GSK3 binding site
βcatenin binding site
DIX domain
I Axin βcat
I' I''
J AxinΔβcat
J' J''
K AxinΔDIX
K' K''
L
p***<0.0007
p**=0.001
ns
M
p***<0.0005
O
Axin
AxinΔDIX
Axin
AxinΔβcat
RFP
aPKCγ
100kDa
70kDa

Figure 4.2. APC and Axin have regions that are essential to facilitate βcat destruction. (A) Schematic representation of APC mutants. (B) APC2 reduces βcat levels. Immunofluorescence images of GFP-APC2 overexpressed in SW480 cells and stained via antibody for βcat (red). Arrows point to transfected cells. (B) GFP-APC2ΔArm is unable to facilitate βcat destruction. (D) GFP-APC2ΔR2 is unable to destroy βcat. (E) GFP-APC2ΔB is unable to facilitate βcat reduction. (F) Deleting Arm rpts, R2 or region B interferes with APC's ability to reduce βcat levels. Quantification of βcat levels of indicated constructs in SW480 cells. 10 cells each in 3 independent experiments were measured. (G) Wnt regulated transcription is not inhibited when Arm rpts, R2, or region B is deleted in APC. Quantification of TOP/FOP Flash reporter gene assay of indicated constructs in SW480 cells. Triplicates were measured in 3 independent experiments. (H) Schematic representation of Axin mutants. (I) Wildtype Axin can reduce βcat levels. Axin-RFP expressed in SW480 cells. (J) AxinΔβcat-RFP is unable to reduce βcat levels. (K) AxinΔDIX-RFP is unable to facilitate βcat destruction. (L) Deletion of either the βcat binding site or DIX domain in Axin impairs its ability to reduce βcat levels. Quantification of βcat intensity. (M) Wnt regulated transcription remains high in cells expressing Axin mutants that delete βcat or DIX domain. Student t-test was used. (O) Western Blot of indicated Axin constructs.

Figure 4.3

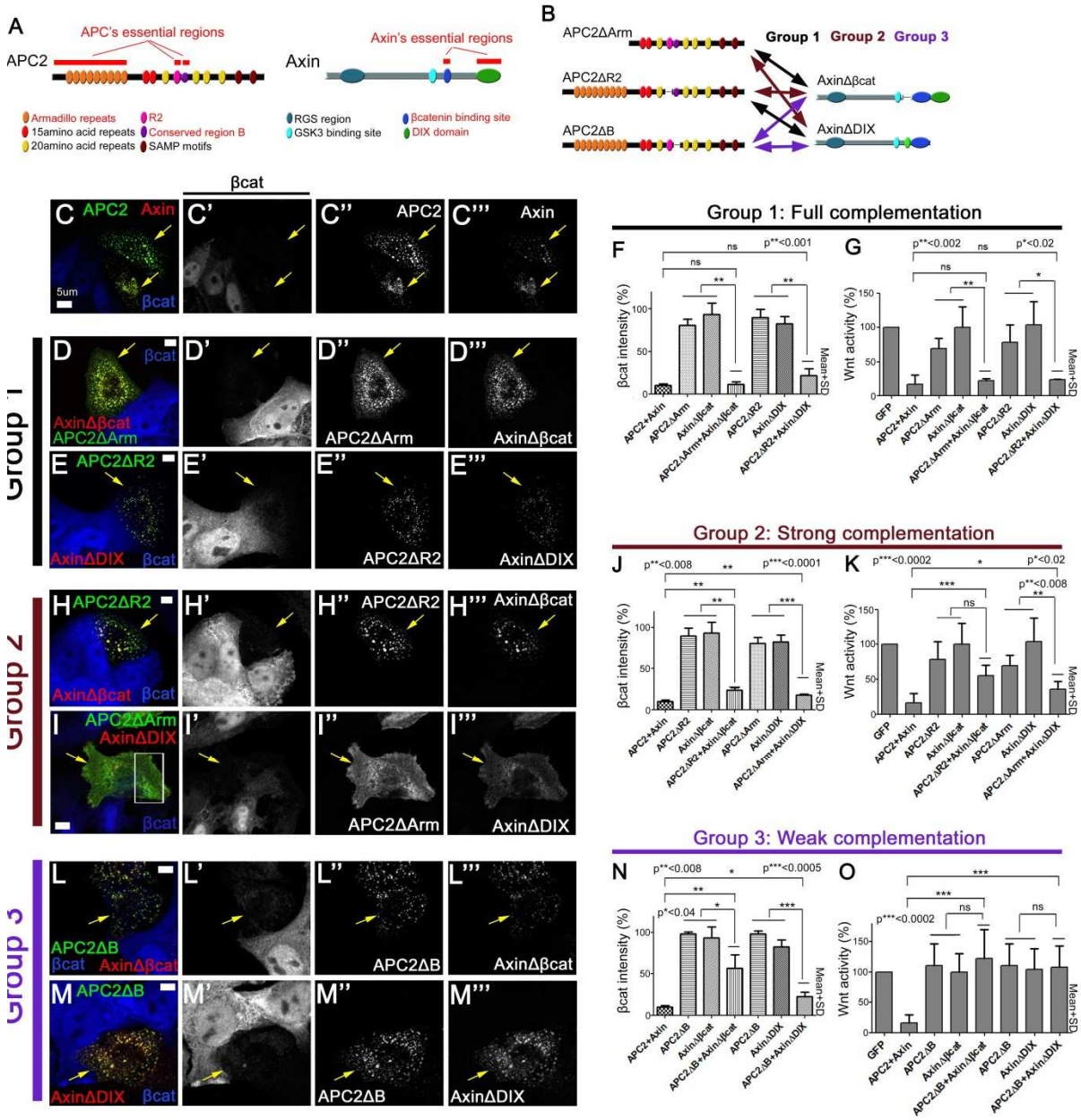
A
APC's essential regions
APC2
Armadillo repeats
15amino acid repeats
20amino acid repeats
R2
Conserved region B
SAMP motifs
Axin's essential regions
Axin
RGS region
GSK3 binding site
βcatenin binding site
DIX domain
B
APC2ΔArm
APC2ΔR2
APC2ΔB
Group 1
Group 2
Group 3
AxinΔβcat
AxinΔDIX
βcat
C APC2 Axin
5um
βcat
C'
C'' APC2
C''' Axin
Group 1
D βcat
AxinΔβcat
APC2ΔArm
D'
D''
D'''
AxinΔβcat
E APC2ΔR2
AxinΔDIX
βcat
E'
E''
APC2ΔR2
E'''
AxinΔDIX
Group 2
H APC2ΔR2
AxinΔβcat
βcat
H'
H''
APC2ΔR2
H''' AxinΔβcat
I APC2ΔArm
AxinΔDIX
βcat
I'
I''
APC2ΔArm
I'''
AxinΔDIX
Group 3
L
APC2ΔB
βcat
AxinΔβcat
L'
L''
APC2ΔB
L'''
AxinΔβcat
M APC2ΔB
AxinΔDIX
βcat
M'
M''
APC2ΔB
M'''
AxinΔDIX
Group 1: Full complementation
F
βcat intensity (%)
ns
p**<0.001
**
Mean+SD
APC2+Axin
APC2ΔArm
AxinΔβcat
APC2ΔArm+AxinΔβcat
APC2ΔR2
AxinΔDIX
APC2ΔR2+AxinΔDIX
G
Wnt activity (%)
p**<0.002
ns
p*<0.02
*
GFP
APC2+Axin
APC2ΔArm
AxinΔβcat
APC2ΔArm+AxinΔβcat
APC2ΔR2
AxinΔDIX
APC2ΔR2+AxinΔDIX
Group 2: Strong complementation
J
βcat intensity (%)
p**<0.008
p***<0.0001
***
APC2+Axin
APC2ΔR2
AxinΔβcat
APC2ΔR2+AxinΔβcat
APC2ΔArm
AxinΔDIX
APC2ΔArm+AxinΔDIX
K
Wnt activity (%)
p***<0.0002
p*<0.02
p**<0.008
GFP
APC2+Axin
APC2ΔR2
AxinΔβcat
APC2ΔR2+AxinΔβcat
APC2ΔArm
AxinΔDIX
APC2ΔArm+AxinΔDIX
Group 3: Weak complementation
N
βcat intensity (%)
p**<0.008
p***<0.0005
p*<0.04
APC2+Axin
APC2ΔB
AxinΔβcat
APC2ΔB+AxinΔβcat
APC2ΔB
AxinΔDIX
APC2ΔB+AxinΔDIX
O
Wnt activity (%)
p***<0.0002
ns
GFP
APC2+Axin
APC2ΔB
AxinΔβcat
APC2ΔB+AxinΔβcat
APC2ΔB
AxinΔDIX
APC2ΔB+AxinΔDIX

Figure 4.3. Certain APC and Axin mutants can complement each other in βcat degradation. (A) Schematic representation of APC2 and Axin. Essential regions are highlighted in red. (B) Combinations of APC and Axin mutants tested. Colors of arrows correspond to group1-3. (C) Wildtype APC2 and Axin form cytoplasmic complexes and reduce βcat. Immunofluorescence of GFP-APC2 and Axin-RFP overexpressed in SW480 cells and stained for βcat via antibody (blue). Arrows point to transfected cells. (D) GFP-APC2ΔArm and AxinΔβcat-RFP show full complementation in βcat reduction, and are thus categorized into group1. (E) GFP-APC2ΔR2 and AxinΔDIX-RFP show full complementation and are thus categorized into group1. (F) Quantification of βcat fluorescence intensity in SW480 cells of group1. Constructs are indicated. 10 cells each in 3 independent experiments were measured. (G) Quantification of Wnt regulated transcription in SW480 cells of group1. Indicated APC and Axin mutants are indistinguishable from wildtype APC2 and Axin in their ability to inhibit Wnt. 3 triplicates were measured in 3 independent experiments. (H) GFP-APC2ΔR2 and AxinΔβcat-RFP show strong complementation in βcat reduction and are categorized into group2. (I) GFP-APC2ΔArm and AxinΔDIX-RFP show strong complementation and are thus categorized into group2. Interestingly, GFP-APC2ΔArm and AxinΔDIX-RFP do not form large puncta. (J) Quantification of βcat levels of indicated constructs of group2. See (F) for details. (K) Quantification of Wnt regulated transcription activity in group2. Constructs are indicated. See (G) for details. (L) GFP-APC2ΔB and AxinΔβcat-RFP show weak complementation in βcat reduction and are thus categorized into group3. (M) GFP-APC2ΔB and AxinΔDIX-RFP show weak complementation and are categorized into group3. (J) Quantification of βcat levels of indicated constructs of group3. See (F). (K) Quantification of Wnt signaling activity in group3. Constructs are indicated. Student t-test (two-tailed) was used.

Figure 4.4

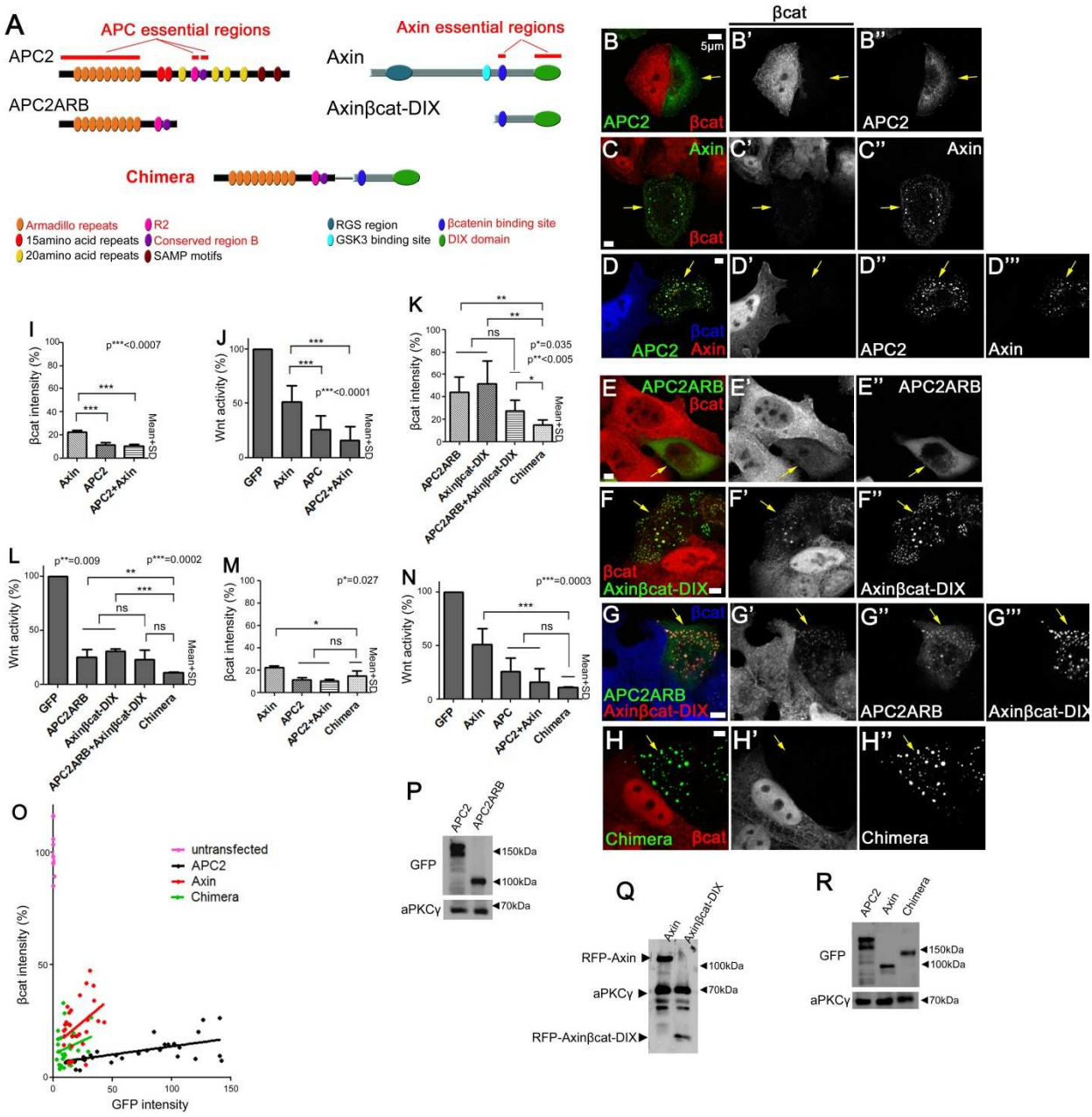
A
APC essential regions
APC2
APC2ARB
Axin essential regions
Axin
Axinβcat-DIX
Chimera
Armadillo repeats
15amino acid repeats
20amino acid repeats
R2
Conserved region B
SAMP motifs
RGS region
GSK3 binding site
βcatenin binding site
DIX domain
βcat
5μm
APC2
Axin
APC2ARB
Axinβcat-DIX
Chimera
I
J
K
L
M
N
O
P
Q
R
βcat intensity (%)
Wnt activity (%)
Mean+SD
GFP intensity
untransfected
GFP
aPKCγ
RFP-Axin
RFP-Axinβcat-DIX
150kDa
100kDa
70kDa

Figure 4.4. The APC:Axin Chimera reduces βcat as efficient as wildtype APC and Axin. (A) Schematic representation of APC and Axin constructs. Top: Wildtype APC and Axin with essential regions indicated. Bottom: Essential regions were combined into a single polypeptide to create the Chimera. (B) GFP-APC2 reduces βcat levels. Immunofluorescence of APC2 overexpressed in SW480 cells and stained with βcat via antibody (red). Arrows point to transfected cells. (C) GFP-Axin also reduces βcat levels. (D) Co-expressing GFP-APC and Axin-RFP reduces βcat levels (blue). (E) GFP-APC2ARB which consists of APC's 3 essential regions is able to moderately reduce βcat levels. (F) Axinβcat-DIX-RFP also shows moderate reduction in βcat levels. (G) Coexpression of APC2ARB and Axinβcat-DIX. (H) Expression of GFP-Chimera in SW480 cells. (I) Axin is unable to reduce βcat levels as effectively as APC2, or APC2+Axin. Quantification of βcat fluorescence intensity of indicated constructs in SW480 cells. 10 cells each in 3 independent experiments were measured. (J) Axin is unable to inhibit Wnt regulated transcription as well as APC2, or APC2+Axin. Quantification of Wnt regulated transcription in SW480 cells. Constructs are indicated. 3 triplicates were measured in 3 independent experiments. (K) Linking the essential regions of APC and Axin together increases the reduction of βcat. Quantification of βcat intensity, see (I) for details. (L) The Chimera strongly inhibits Wnt regulated transcription. Quantification of Wnt regulated transcription of indicated constructs, see (J) for details. (M) The Chimera reduces βcat levels better than wildtype Axin. Constructs are indicated, quantification details in (I). (N) Wnt regulated transcription is as effectively inhibited in Chimera expressing cells as it is in wildtype APC2+Axin, or APC2 expressing cells. See quantification details in (J). (O) APC2, Axin, or the Chimera are not dosage dependent in βcat degradation. Plot of GFP and βcat signal of indicated constructs and untransfected cells. 30 cells total for each condition. (P-R) Western blot for expression levels of indicated constructs. Student t-test (two-tailed) was used.

Figure 4.5

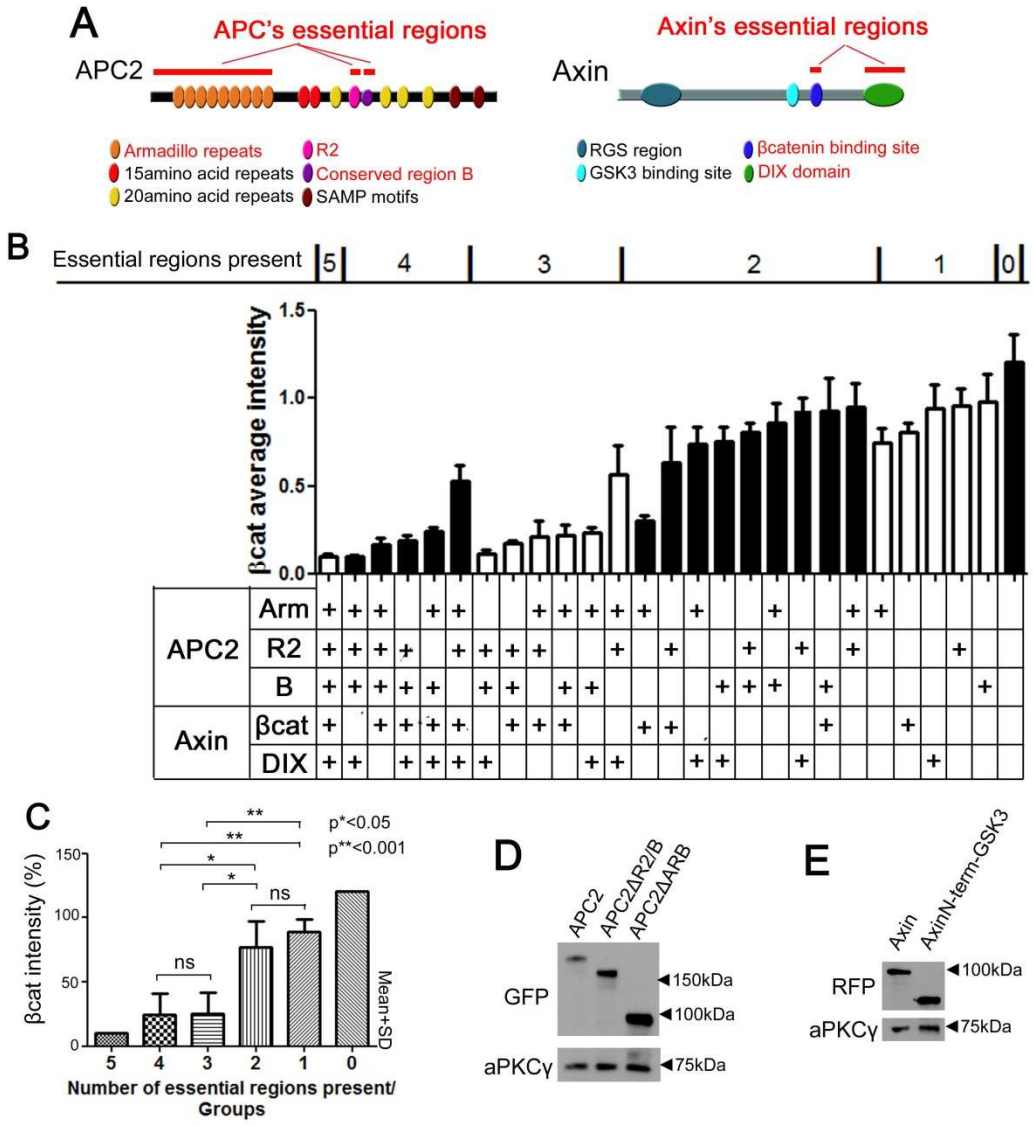

Figure 4.5. The essential regions in APC and Axin are important for a function destruction complex. The essential regions in APC and Axin are important for a functional destruction complex. (A) Schematic representation of APC and Axin. Essential regions are indicated in red. (B) Loss of the essential regions in APC and Axin results in high βcat levels. Co-expression of APC and Axin mutants in SW480 cells, followed by quantification of βcat fluorescence intensity. Top: Mutants are sorted into groups which are defined by how many essential region are present. Middle: βcat average intensity. Bottom: Table describing which essential regions are present. (C) For βcat reduction up to 2 essential regions are dispensable. Average of the groups that were defined in (B). (D) Western blot analysis of expression levels of indicated APC2 mutants used. (E) Expression levels of indicated Axin mutants that delete either the βcat binding site or the DIX domain. Student t-test (two-tailed) was used.

Figure 4.6

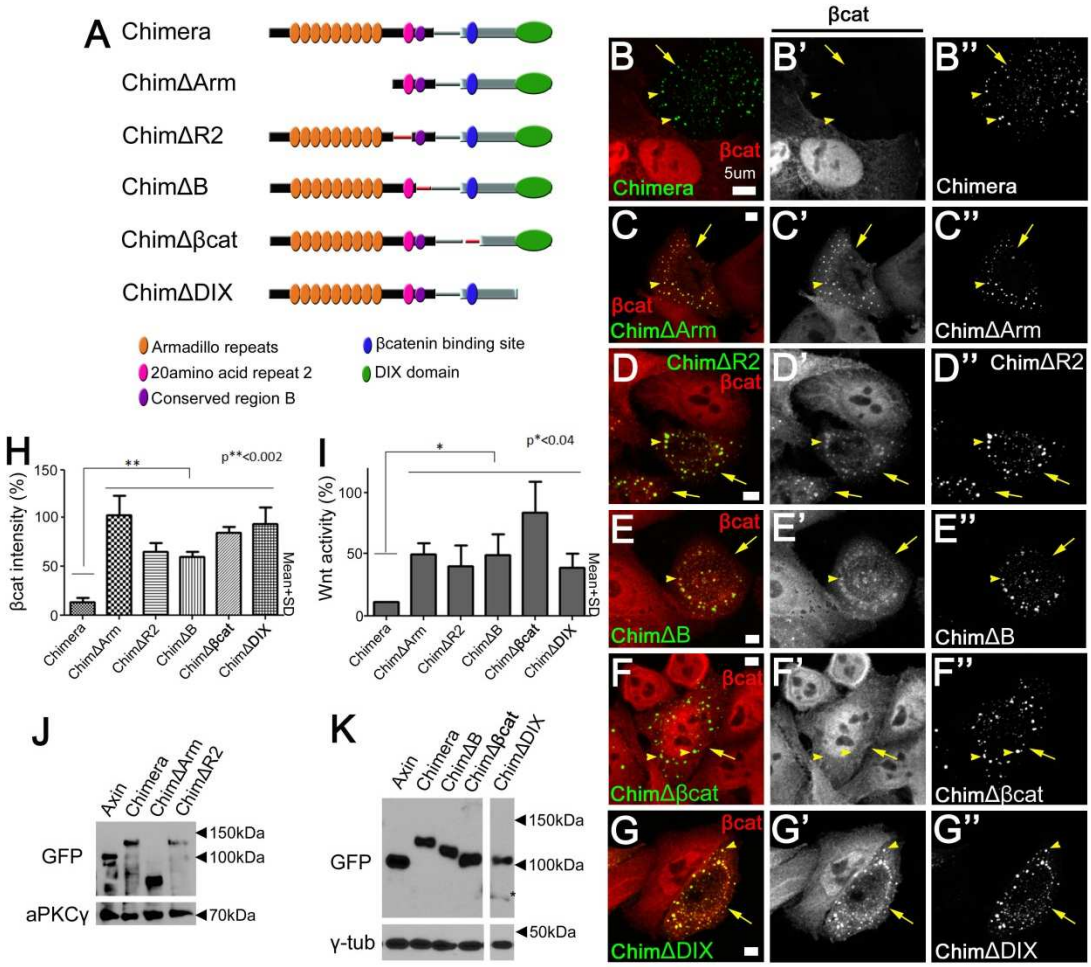

Figure 4.6. All regions in the Chimera are important for its efficient function in βcat degradation. (A) Schematic representation of Chimera mutants. (B) The Chimera reduces βcat levels effectively. Immunofluorescence images of GFP-Chimera expressed in SW480 cells and stained for βcat via antibody (red). Arrows point to transfected cells. (C) Deletion of the Arm rpts impairs Chimera function in βcat reduction. GFP-ChimΔArm expressed inSW480 cells. (D) GFP-ChimΔR2 is unable to reduce βcat. (E) GFP-ChimΔB has high levels of βcat. (F) Deltion of the βcat binding site in the Chimera inhibits its ability to reduce βcat levels. Interestingly, in contrast to the other Chimera mutants, no accumulation of βcat is seen in GFP-ChimΔβcat complexes. (G) GFP-ChimΔDIX is inhibited in its ability to destroy βcat. (H) βcat levels are elevated in Chimera mutants. Quantification of βcat levels of indicated constructs in SW480 cells. 10 cells each in 3 independent experiments were measured. (I) Wnt regulated transcription is high in Chimera mutants. Quantification of Wnt regulated transcription in SW480 cells of indicated constructs. Triplicates were measured in 3 independent experiments**.** (J) Expression levels of indicated Chimera mutants by Western Blot. (K) Western Blot analysis of indicated Chimera mutants. Student t-test was used.

Figure 4.7

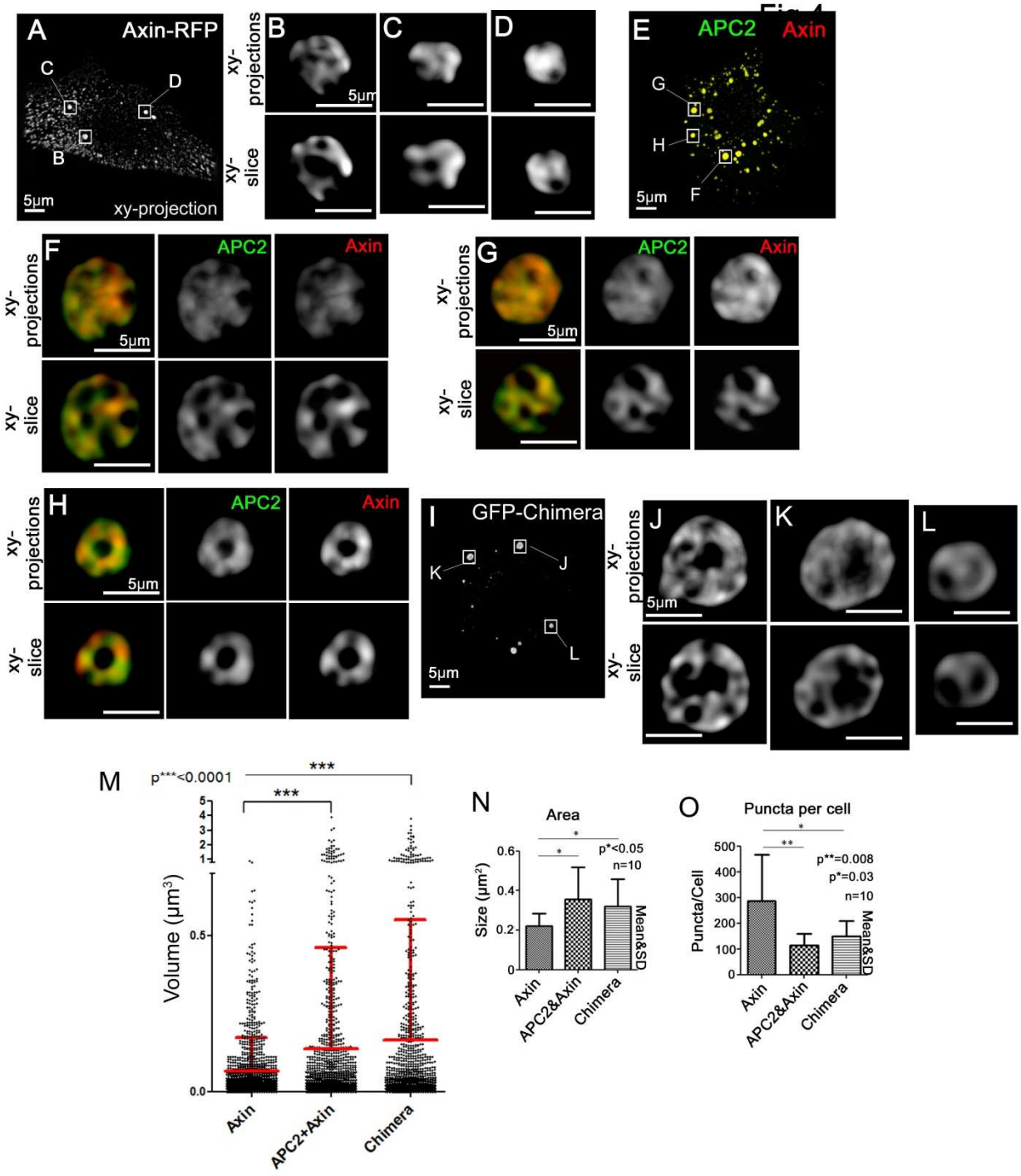
A
Axin-RFP
C
D
B
5μm
xy-projection
xy-projections
xy-slice
B
C
D
5μm
E
APC2
Axin
G
H
F
5μm
F
APC2
Axin
5μm
G
APC2
Axin
5μm
H
APC2
Axin
5μm
I
GFP-Chimera
K
J
L
5μm
J
K
L
5μm
M
p***<0.0001
***
***
Volume (μm³)
Axin
APC2+Axin
Chimera
N
Area
*
*
p*<0.05
n=10
Size (μm²)
Mean&SD
Axin
APC2&Axin
Chimera
O
Puncta per cell
*
**
p**=0.008
p*=0.03
n=10
Puncta/Cell
Mean&SD
Axin
APC2&Axin
Chimera

Figure 4.7. The Chimera complex reconstitutes the APC:Axin complex in the internal structure and size. (A) SIM images of SW480 cells transfected with Axin-RFP. Axin complexes consist of circular sheets/cable. (B-D) Enlarged SIM images of puncta indicated in (A). (E) APC co-expression leads to a more internal complex structure of Axin puncta. SIM images of GFP-APC2 and Axin-RFP transfected cells. (F-H) Close ups of complexes indicated in (E). (I) The Chimera has a similar internal structure as APC:Axin complexes. GFP-Chimera expressed inSW480 cells. (J-L) Close ups of Chimera puncta indicated in (I). (M) Chimera puncta are similar in volume to APC:Axin complexes. Puncta volume comparison of SIM images of indicated constructs. Axin (n=4 cells), APC2+Axin, Axin (n=10 cells) were quantified. (N) The cross-section area of the chimeric complexes is similar to APC+Axin. Area comparison of indicated constructs using LSM images.10 cells were quantified for each construct. (O) The number of puncta found in Chimera transfected cells is similar to APC+Axin transfected cells, and reduced relative to Axin puncta number. Quantification of number of puncta in 10 cells for each condition. Student t-test was used.

Figure 4.8

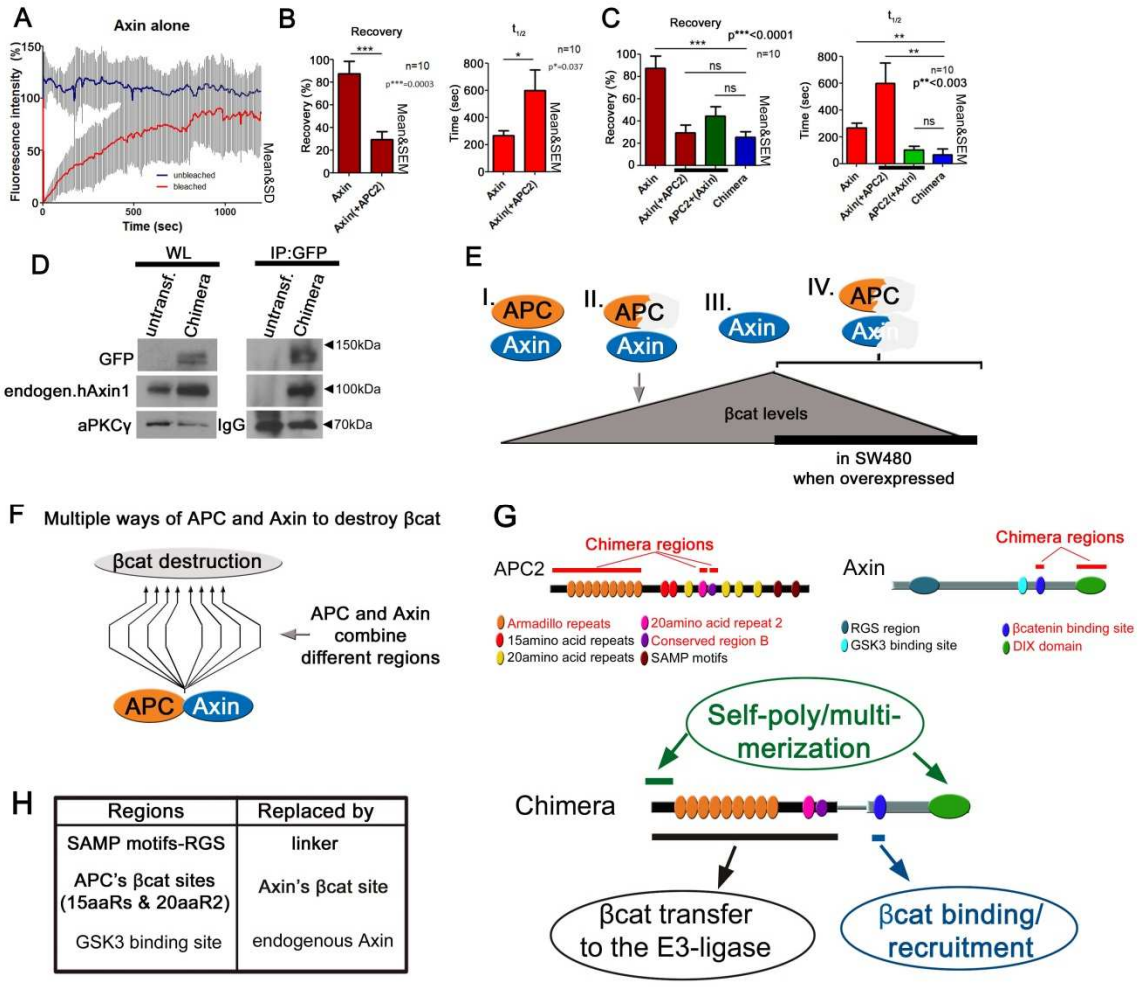

Figure 4.8. The dynamics of the chimera complex are similar to APC2+Axin complexes, and new models of the destruction complex and its key function. (A) Axin complexes are dynamic. FRAP analysis of complexes formed by Axin-RFP in SW580 cells. Unbleached (blue) and bleached (red). 10 complexes in 10 cells of 3 independent experiments were measured. (B) Axin's dynamics slow down when APC is co-expressed. GFP-APC2 and Axin-RFP were expressed in SW480 cells. Left: Recovery plateau, Right: t1/2. (C) APC2 and the Chimera have similar dynamics. The recovery plateau of the Chimera is similar to that of Axin, or APC. However, the time needed to recover is similar to APC. (D) Reused model of fig1A. (II) Axin working with a truncated APC2 does not downregulate βcat as efficiently as wildtype APC+Axin. (IV) APC and Axin mutants can complement each other. Different mutants show different efficiencies in βcat destruction. (E) APC and Axin use a combination of different regions to secure low levels of βcat. The nature of combination defines efficiency. (F) The Chimera combines all the essential function of the APC:Axin complex. (G) In engineering the Chimera we replaced several regions in APC and Axin.

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CHAPTER 5: DISCUSSION

Throughout development and in tumorigenesis Wnt signaling plays essential roles. Highly conserved from the first multicellular animals to humans, it is one of the building blocks that organize cells into tissue. The regulation of βcat is key in maintaining proper Wnt signaling activity. βcat levels are kept low when Wnt signaling is off, and this is accomplished by the destruction complex which primes βcat for destruction by phosphorylation. Although much research has been done on the Wnt pathway there are still many key things that remain unclear. During my PhD we addressed some of the key questions about how the destruction complex functions. This provided a better understanding of the APC:Axin complex, but it also opened the door to new areas where more research is needed to fully understand the complicated dynamics and mechanisms that occur inside the βcat destruction complex.

The recruitment of βcat into the APC:Axin complex and its release to the E3-ligase

In chapter 2 we investigated the function of APC's βcat-binding sites and found that they act redundantly and additively in facilitating βcat degradation. Thus although APC's βcat binding sites have different affinities, there is no apparent difference in APC's ability to target βcat for destruction between constructs that maintain lower affinity or higher affinity binding sites; instead the number of sites is critical.

In chapter 4 we attempted to reduce the redundancy of APC and Axin to the minimal machine. This revealed that all of the βcat binding sites in APC are dispensable. These results suggest that there is no functional difference in the origin of the βcat binding site in the

destruction complex as long as they bind βcat. However, since we used Axin's βcat binding site in the APC:Axin chimera, we don't know whether the function of Axin's βcat site has particular importance or whether it could be replaced by one of APC's βcat binding sites. In a fully functional complex they may have different functions. APC's βcat sites could be used to capture βcat in the cytoplasm to bring it into the complex, while Axin's binding site could serve as a gate-keeper for regulating βcat destruction levels - monitoring βcat levels by sensing it via its βcat binding site. Crystal structures revealed that although both APC and Axin bind to the Armadillo repeats 3-7 in βcat, they can bind βcat simultaneously when APC's βcat binding sites are unphosphorylated (Ha et al., 2004; Xing et al., 2003). Thus it would be interesting to test whether all βcat binding sites in the destruction complex serve exclusively in sequestration of βcat, or whether some exhibit additional functions. Replacing the βcat binding site in Axin by one of APC's sites would address this question. Moreover creating a destruction complex with multiple but identical binding sites is an intriguing idea.

Once βcat is recruited into the complex and phosphorylated, βcat is released from APC and Axin to the E3-ligase. Some studies suggested that βcat cannot be released at all once it is bound by phosphorylated APC, due to the high affinity of phosphorylated binding sites for βcat (Ha et al., 2004). However, there must be a mechanism that triggers the release of βcat from APC and Axin. This suggests that the βcat binding sites in APC and Axin must have some unique features. Thus, could we replace all βcat binding site in APC and Axin with βcat binding sites from other proteins like E-cadherin, Tcf, or α-catenin? Since these proteins each cover different regions of βcat when bound - E-cadherin covering all of the Armadillo repeats, TCF binding 7 Arm repeats, and α-catenin occupying just one-- these studies would address whether βcat

sites are indeed redundant or whether a special release mechanism is built into them.

The Armadillo repeats in APC and their role in Wnt signaling

In chapter 3 we identified a role for APC's Arm rpts in the transfer of βcat to the E3-ligase by identifying the Arm rpts domain as a second Axin association site. The Arm rpts have been shown to interact with multiple proteins without roles in Wnt signaling - most of them are cytoskeletal regulators (Jimbo et al., 2002; Kawasaki et al., 2000; Tirnauer, 2004). SAM68, a TCF splicing factor has also been shown to directly bind to the Arm rpts, but the explicit function of this interaction needs to be determined (Morishita et al., 2011). However, APC's Arm rpts have not been assigned a specific mechanistic role in the destruction complex. Identifying the Arm rpts as a Axin association site raises new questions. Is the association direct or indirect? And where do the Arm rpts bind within Axin's GSK3 binding region? Answering these questions could reveal a new player with yet unknown function that bridges the interaction of Axin with the Arm rpts, if the association is accomplished by a different protein. If the association is direct it would be interesting to test what regions in the Arm rpt domain are participating in binding to Axin. For example, is the N-terminal polymerization helix is needed or is the entire Arm rpt domain is used. Further the fact that the Arm rpts bind Axin in a region close to the GSK3 binding site could be an explanation for why APC gets effectively phosphorylated by GSK3 although it lacks a GSK3 binding site. APC phosphorylation is Axin dependent (Ha et al., 2004). However, if the Arm rpts bind to the GSK3 binding site itsel, it would suggest that the Arm rpts might play an additional role in competing with GSK3 in the association with Axin. Thus it would be interesting to investigate the Arm rpts - Axin association further.

APC proteins have been shown to multimerize using either a coiled-coiled

oligomerization domain found in human APC proteins or an N-terminal helix in the fly homologs (Joslyn et al., 1993; Polakis, 1997). Thus the ability to multimerize contributes to complex formation and potentially to the complex dynamics. Thus it would be intriguing to investigate how deletion of the N-terminal helix impacts the internal structure of the APC:Axin complex and its dynamics. Since overexpression of just the Arm rpt domain leads to formation of cytoplasmic puncta while full length proteins are distributed throughout the cytoplasm it would be interesting to investigate what mechanism keeps APC from hyper-polymerization and forming cytoplasmic puncta (Roberts et al., 2012). Is it the amino acids just C-terminal to the Arm rpts or does a different region in APC regulate this behavior? R2 and B are potential candidates that could regulate multimerization, since they control the binding of APC's Arm rpts to Axin and the dynamics of APC in the Axin-complex.

APC and the exclusion from the nucleus

Another field that needs additional research is the exclusion of APC from the nucleus. Studies in the past revealed that APC has the ability to shuttle in and out of the nucleus (Brocardo and Henderson, 2008). Nuclear localizing sequences (NLS) and nuclear export signals have been identified in human APC, but are not conserved throughout the APC proteins in different animals. Moreover, APC's ability to shuttle into the nucleus seems to be less critical since our experiments showed that altering APC's intracellular localization, thus excluding it from the nucleus, still promoted proper downregulation of βcat (Roberts et al., 2012). Thus rather than being essential in the nucleus, APC can act independently of its nuclear function. Interestingly, when APC is expressed in mammalian cells it tends to stay out of the nucleus and accumulates in the cytoplasm. However, when the Arm rpts are deleted APC proteins are

uniformly distributed through the entire cell (unpublished data). Thus it seems that the Arm rpts serve as an anchor keeping APC in the cytoplasm; this might be essential for its function. Therefore it would be interesting to investigate how the Arm rpts tether APC in the cytoplasm and what happens to βcat levels and Wnt activity when APC is exclusively in the nucleus. These experiments might identify the exclusion of APC from the nucleus as an important function of the Arm rpts in Wnt regulation.

Wnt regulated transcription and the role of the essential regions in APC and Axin

In chapter 4 we describe the strong down regulation of Wnt regulated transcription by a construct retaining only APC's essential regions. Although the βcat levels were higher than that of wildtype APC+Axin, Wnt regulated transcription was the same. Similarly, a construct retaining Axin's essential region reduced βcat levels only moderately, but decreased Wnt regulated transcription as well as wildtype Axin. Thus the essential regions might have an important role in Wnt regulated gene expression, that is in addition to their function in the destruction complex. Identifying which nuclear proteins interact with the essential regions would allow speculation about the mechanism of action.

The importance of polymerization in the destruction complex

Deleting the DIX domain in Axin or the Arm rpts in APC inhibits their function in facilitating βcat degradation. In chapter 4 we build the minimal βcat destruction machine, creating the Chimera, and we showed that both Arm rpts and the DIX domain are essential for its function. However, questions still remain about whether they exclusively function in polymerization, or whether these two regions each polymerize in a unique way to build the platform for βcat phosphorylation and transfer to the E3-ligase. Thus it would be interesting to

test whether the DIX domain or the Arm rpts can be replaced by different polymerization domains of proteins unrelated to Wnt signaling. Wildtype APC, and Axin, and the Chimera could be then tested for their ability to destroy βcat. These experiments would provide insights into whether polymerization has to build an ordered structure to allow the destruction complex to function, or whether polymerization and the binding to other molecules in an unordered fashion is sufficient to build the βcat destruction complex. Another intriguing experiment would be to swap the DIX domain with the Arm rpts region, or even create APC and Axin proteins that have just the Arm rpts (replacing Axin's DIX domain with the Arm rpts) or just the DIX domain (replacing the Arm rpts with the DIX domain) present.

The ratio of APC and Axin in the destruction complex

The Chimera targets βcat efficiently for destruction. Interestingly in the Chimera APC and Axin are permanently linked to each other, thus setting the ratio of APC and Axin to 1:1. This suggests that APC and Axin wildtype could be permanently physically linked and this would not interrupt the destruction machinery. However, intriguingly when measuring the dynamics of APC and Axin we measured faster dynamics and a faster turnover rate of APC than Axin in the destruction complex. This fits very well into the model of Axin as the scaffold of the destruction complex and thus having a slower turnover rate, while APC is constantly shuttling in and out the complex to recruit new βcat in. Therefore it would be interesting to test what happens to dynamics when full length APC and Axin are permanently linked and to define how this influences βcat degradation and Wnt transcriptional activity.

Phosphorylation and dephosphorylation of APC and Axin

In Chapter 3 we looked at the dynamics of the destruction complex. We found that

blocking GSK3 activity resulted in slow dynamics of APC. Interestingly, we also found that Axin's dynamics are influenced by GSK3 activity (data not shown). When high concentrations of BIO, the GSK3 inhibitor, were used, Axin's dynamics also slowed down, suggesting that this scaffolding protein also relies on GSK3 activity. The effects on Axin's dynamics were seen with a high dosage of the inhibitor, while APC's dynamics were affected at low concentrations. These data would suggest that when GSK3 activity is inhibited due to Wnt activation the lack of GSK3 would first impact APC's function. Thus the high levels of βcat when Wnt is turned on might be achieved by inhibiting APC's ability to facilitate βcat degradation, since APC appears to be more sensitive to small changes in GSK3 activity than Axin. This would support the model in which Axin is the main machinery that targets constitutively βcat for destruction, while APC is a regulatory unit which determines the destruction rate of βcat. This model is supported by our fly data in chapter 3 where Armadillo levels in Wnt-on regions of wildtype embryos are much lower than Armadillo levels in Wnt-on regions of APC2AA mutants, suggesting that in wildtype βcat continues to be destroyed although Wnt signaling is turned on. This suggests that kinase activity may regulate βcat destruction rates via APC.

APC and Axin and the combinatorial code of regions that leads to βcat degradation

Our study in Chapter 4 revealed that APC and Axin not only have regions of redundant functions, but also have regions with unique functions that can facilitate βcat destruction in various ways when present in different combinations. Thus when we look at the destruction complex as an entity in which different regions function together in the reduction of βcat, different mutant combinations would lead to different efficiencies in securing low levels of βcat. By using mathematical modeling of our data, we might be able to identify how different

combinations of regions that facilitate βcat degradation. This would provide insights into how the machinery functions and what happens to the efficiency of the destruction complex when regions are missing or non-functional due to mutations. We have initiated a collaboration with Walter Fontana's lab at Harvard to investigate the combinatorial nature of the destruction complex.

Axin and its microtubule binding site

We were surprised to find that the C-terminal fragment of Axin that we used to generate the Chimera has a very curious localization in the cell. This Axin fragment localizes along microtubules (unpublished data). Alignment of Axin proteins from different species revealed that there are conserved residues in the PP2A/PP1/CK1 region that could facilitate the microtubule interaction. Interestingly, when we used nocadozole to depolymerize the microtubules, Axin changed its localization from along microtubules to formation of cytoplasmic puncta (data not shown). Thus Axin maybe able to associate with the microtubule network. Wildtype Axin forms cytoplasmic puncta and has not yet been described to associate along microtubules. However, it is possible that the N-terminal part of Axin could inhibit the microtubule interaction, and that the interaction only occurs when Axin is modified in a specific way, making the binding site accessible to the microtubule network. It would be interesting to test what role the microtubule association plays in Wnt-off and Wnt-on state. Since recruitment of Axin to the cell membrane has been identified as one of the steps to shut down βcat destruction, association of Axin with the microtubule network might be the mechanism by which Axin is recruited to the membrane (Bilic et al., 2007). Thus point mutations and deletion could test how the loss of microtubule association influences Wnt activation.

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