Deconstructing the ßcatenin destruction complex: mechanistic roles for the tumor suppressor APC in regulating Wnt signaling

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ABSTRACT **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 ßcatenin (ßcat) 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 ßcat-binding sites on APC play key roles. Instead, they suggest that multiple ßcat-binding sites act additively to fine-tune signaling via cytoplasmic 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 ßcat 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.**

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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-ß, Hedgehog, nuclear factor κB, and Wnt pathways. In fact, the last three pathways use the same SCF class E3 ligase, with the F-Box protein Slimb/ßTrCP as the recognition subunit (Maniatis, 1999). In each, a distinct multiprotein complex targets the effector for phosphorylation, triggering recognition by Slimb/ßTrCP. 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), 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 ßcatenin (ßcat) for phosphorylation, and ultimately ubiquitination and proteasomal destruction (Cadigan and Peifer, 2009). In the presence of Wnt signal, or in tumors lacking APC, ßcat levels rise, it enters the nucleus, binds T-cell

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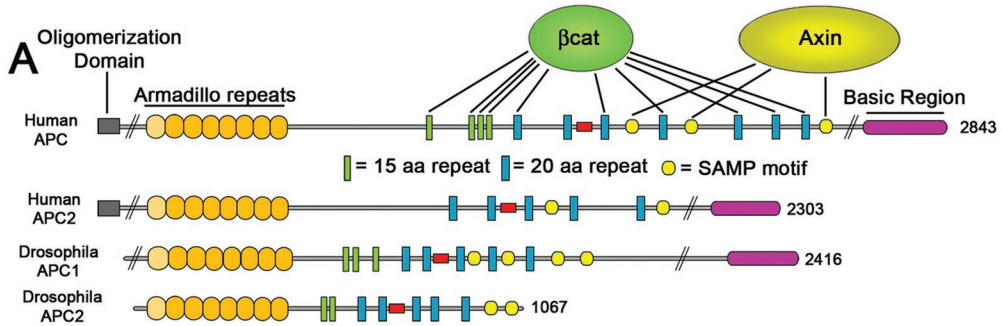
Abbreviations used: APC, adenomatous polyposis coli; Arm, Armadillo; ßcat, ßcatenin; CID, Catenin Inhibitory Domain; CK1, casein kinase 1; co-IP, coimmunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; GSK3, glycogen synthase kinase 3; MCR, mutation cluster region; NES, nuclear export sequence; NGS, normal goat serum; PBS, phosphate-buffered saline; RGS, regulator of G protein signaling; TCF, T-cell factor; Wg, Wingless.



B	SW480 cells			APC2 single mutant			APC2 APC1 double mutant
	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 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.

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 ßcat stability remain unclear.

Several hypotheses for APC function have been suggested. Some focus on destruction complex–independent roles of APC in nuclear trafficking of ßcat or action at promoters of Wnt target genes (Rubinfeld *et al.*, 1996; Bienz, 2002; Sierra *et al.*, 2006). Most studies agree, however, that APC's dominant role is to target ßcat for destruction as a part of the destruction complex. Because APC binds ßcat and coimmunoprecipitates (co-IPs) with glycogen synthase kinase 3 (GSK3) (Rubinfeld *et al.*, 1993), it was initially hypothesized that APC is a scaffold, templating ßcat phosphorylation. APC's structure is consistent with this hypothesis, as APC family members are large, complex proteins with multiple protein interaction motifs (Figure 1A; McCartney and Nathke, 2008). 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 ßcat, and SAMP repeats that bind Axin. It also contains the short conserved sequence B (McCartney *et al.*, 1999; called sequence B later in the text; also known as the Catenin Inhibitory

Domain [CID]; Kohler *et al.*, 2009), which we suspect binds an additional unknown partner. The C-terminal third of APC includes binding sites for cytoskeletal proteins (Nathke, 2004), 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).

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 ßcat, 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; Polakis, 2007). Recent biochemical experiments confirmed this, demonstrating that Axin can accelerate ßcat phosphorylation in vitro, in the absence of APC (Ha *et al.*, 2004). 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 ßcat for destruction. All must account for APC's complex structure, and in particular the fact that both APC and Axin have ßcat-binding sites. Unlike Axin, which has a single ßcat-binding site, APC has many of two different types, 15Rs and 20Rs. Important clues came from examining affinities of ßcat for both APC and Axin (Xing *et al.*, 2003; Ha *et al.*, 2004; Choi *et al.*, 2006; Liu *et al.*, 2006). Under basal conditions, ßcat'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). Phosphorylated APC has a higher affinity for ßcat than Axin (Ha *et al.*, 2004; Xing *et al.*, 2004; Choi *et al.*, 2006; Liu *et al.*, 2006).

These findings led Kimelman and Xu to propose that the destruction complex goes through a catalytic cycle of structural rearrangements to ensure ßcat phosphorylation and turnover (Kimelman and Xu, 2006). They suggest that the complex assembles with ßcat bound to Axin. ßcat is then phosphorylated by CK1 and GSK3, and APC is also phosphorylated in the process. In the catalytic cycle model, this triggers transfer of ßcat to APC, which is then suggested to facilitate ßcat transfer to the E3 ubiquitin ligase for destruction, with presumed dephosphorylation of APC resetting the system, allowing a new ßcat to be bound. Consistent with this catalytic cycle model, mammalian APC coimmunoprecipitates with ßcat and ßTrCP revealing that APC can indeed interact with the E3 ubiquitin ligase (Hart *et al.*, 1999). APC can also protect ßcat from dephosphorylation during this proposed transfer to ßTrCP (Su *et al.*, 2008), ensuring ubiquitination once ßcat is phosphorylated.

Weis et al. presented a different model for the role of multiple ßcat-binding sites in APC's activity (Ha *et al.*, 2004). Their in vitro data suggest that ßcat 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 ßcat with different affinities using distinct binding sites is key to modulating destruction of ßcat, allowing the destruction complex to accommodate the different intracellular ßcat levels found in the presence or absence of Wnt signaling (Ha *et al.*, 2004). In this model, high-affinity sites are key to maintain low levels of ßcat in the absence of Wnt signal, helping to target any free ßcat for destruction. In contrast, low-affinity binding sites capture ßcat when ßcat levels are elevated by Wnt signaling, facilitating rapid ßcat 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 ßcat-binding sites. Specifically, in the catalytic cycle model (Kimelman and Xu, 2006), 20R3 has a key role in ßcat destruction, because, when phosphorylated, its affinity for ßcat is 20-fold higher than the other 15Rs or 20Rs, thus exceeding that of Axin (Choi *et al.*, 2006; Liu *et al.*, 2006). Thus 20R3 may play a key role in transferring ßcat to the E3 ligase (ßcat 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 ßcat 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). Thus, in summary, mechanisms by which APC acts in the destruction complex to ensure ßcat 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 ßcat (Phelps *et al.*, 2009), whereas other experiments suggest that APC2 plays an unexpected positive role in Wnt signaling (Takacs *et al.*, 2008).

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 ßcat-binding sites, but lacking Axin binding sites (Polakis, 1997; Kohler *et al.*, 2008). Data in cultured colon cancer cells (Albuquerque *et al.*, 2002), mouse mutants (Gaspar *et al.*, 2009), and *Drosophila* embryos (McCartney *et al.*, 2006) suggest that these truncated proteins retain residual ability to target ßcat for destruction, but whether this suggests that APC can play an Axin-independent role in ßcat destruction or that the complex still targets ßcat for destruction without direct APC–Axin interactions (Peterson-Nedry *et al.*, 2008) remains unknown. Interestingly, in cultured colon cancer cells expressing truncated APC, overexpressing a central fragment of APC carrying 20Rs and SAMP repeats restores ßcat destruction (Munemitsu *et al.*, 1995; Rubinfeld *et al.*, 1997a). 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, 2010), 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 ßcat) 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; McCartney *et al.*, 1999; Yu *et al.*, 1999; Takacs *et al.*, 2008); 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; Akong *et al.*, 2002a). 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.

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 ßcat 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). The large size of these cells allowed us to assess effects of APC mutants on ßcat levels, localization, and subsequent downstream signaling. SW480 cells accumulate high levels of ßcat in the cytoplasm and nuclei (Supplemental Figure 1, A and B, arrowhead) and have high activity of the Wnt transcriptional reporter TOPFLASH (Supplemental Figure 1E; Korinek *et al*., 1997). ßcat destruction is rescued by full-length human APC, fragments of its middle region (Supplemental Figure 1, C–E; e.g., Munemitsu *et al*., 1995; Rubinfeld *et al*., 1997a), or by fly APC1 (Hayashi *et al*., 1997). We found that green fluorescent protein (GFP)-tagged APC2 restored ßcat destruction as effectively as human APC (Supplemental Figure 1, B vs. C and D). We also developed a method to quantify this, measuring ßcat fluorescence intensity in hundreds of transfected versus nontransfected cells with the Cellomics ArrayScan VTI, providing unbiased assessment of ßcat down-regulation (Supplemental Figure 1F). Fly APC2 also reduced TCF-regulated transcription (Supplemental Figure 1E).

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), in flies we can test mutant transgenes in the complete absence of APC2 (*APC2*^{g10} maternal and zygotic null single mutants) or in the complete absence of all APC family proteins (*APC2*^{g10} *APC1*^{Q8} maternal and zygotic double null mutants). Cell fates in the embryonic epidermis are an extremely sensitive readout of Wnt signaling (McCartney *et al*., 2006); anterior cells in each segment secrete hairlike denticles (Supplemental Figure 1G, arrowhead), whereas posterior cells receiving Wg signal secrete naked cuticle (Supplemental Figure 1G, arrow). APC2 is the primary player in the embryo (McCartney *et al*., 1999), but APC1 also contributes to Wnt regulation there (Ahmed *et al*., 2002; Akong *et al*., 2002a), and thus single and double mutants allow us to assess different parts of the phenotypic spectrum. In *APC2 APC1* double mutants, Arm (fly ßcat) levels rise dramatically, and all cells take on naked cuticle fates (Supplemental Figure 1I; Ahmed *et al*., 2002; Akong *et al*., 2002a). 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), due to residual APC1 activity. This modest rise in Arm levels changes most but not all cell fates to naked cuticle (Supplemental Figure 1H; 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). 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; Supplemental Figure 1L) rescues *APC2* single mutants to adult viability and fertility (Supplemental Figure 1K), and rescues *APC2 APC1* double mutants to embryonic viability and normal cell fates (Supplemental Figure 1J). We made site-directed mutants of GFP-tagged APC2 (Figure 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 (Supplemental Figure 2, 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 (Supplemental Figure 2, D–M). All transgenic constructs in the fly were expressed near endogenous levels.

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

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

To test these hypotheses, we deleted 20R3 from full-length *Drosophila* APC2 (APC2ΔR3; Figure 1B). In contrast to the predictions of these models, this had no effect on APC2's ability to down-regulate ßcat levels (Figure 2, A, B, and G) or TCF-regulated transcription in SW480 cells (Figure 2H). Furthermore, when introduced into flies, APC2ΔR3 fully rescued embryonic Wnt signaling defects of *APC2* single mutants (Figure 3, A and D) and restored adult viability (Supplemental Figure 3A). Finally, APC2ΔR3 also rescued embryonic viability, effects on cell fate choices (Figure 3, B and M), and destruction of Arm (Compare Figure 4, 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 4, E, I, and N). Thus, surprisingly, the highest affinity ßcat-binding site, 20R3, is fully dispensable for ßcat destruction.

To further explore the hypothesis that high- and medium-affinity 20Rs play key roles in ßcat destruction, we generated several other mutants. Deleting the medium-affinity ßcat-binding site, 20R1 (APC2ΔR1; Figure 1B) left APC2 fully functional in down-regulating ßcat levels (Figure 2, C and G) and TCF-regulated transcription (Figure 2H) 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 1B), or 20R1, 20R4, and 20R5 (APC2ΔR1,R4-R5; Figure 1B), were also fully functional in down-regulating ßcat levels in SW480 cells and in substantially reducing TCF-regulated transcription (Figure 2, 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 3, A, E, and F); and rescued embryos could survive to adulthood (Supplemental Figure 3, B and C). Embryonic viability, however, was reduced relative to wild-type APC2 or APC2ΔR3 (Figure 3, D–F), suggesting that they do not retain full function. These data refute models in which the highest affinity ßcat-binding sites play essential individual roles in ßcat destruction, and instead suggest 20Rs contribute additively to function in vivo.

If high-affinity ßcat binding either mediates transfer of ßcat from Axin to APC for handoff to the E3 ligase or helps deliver ßcat for destruction when ßcat levels are low, one might predict that the highest affinity ßcat-binding site, 20R3, would be sufficient for

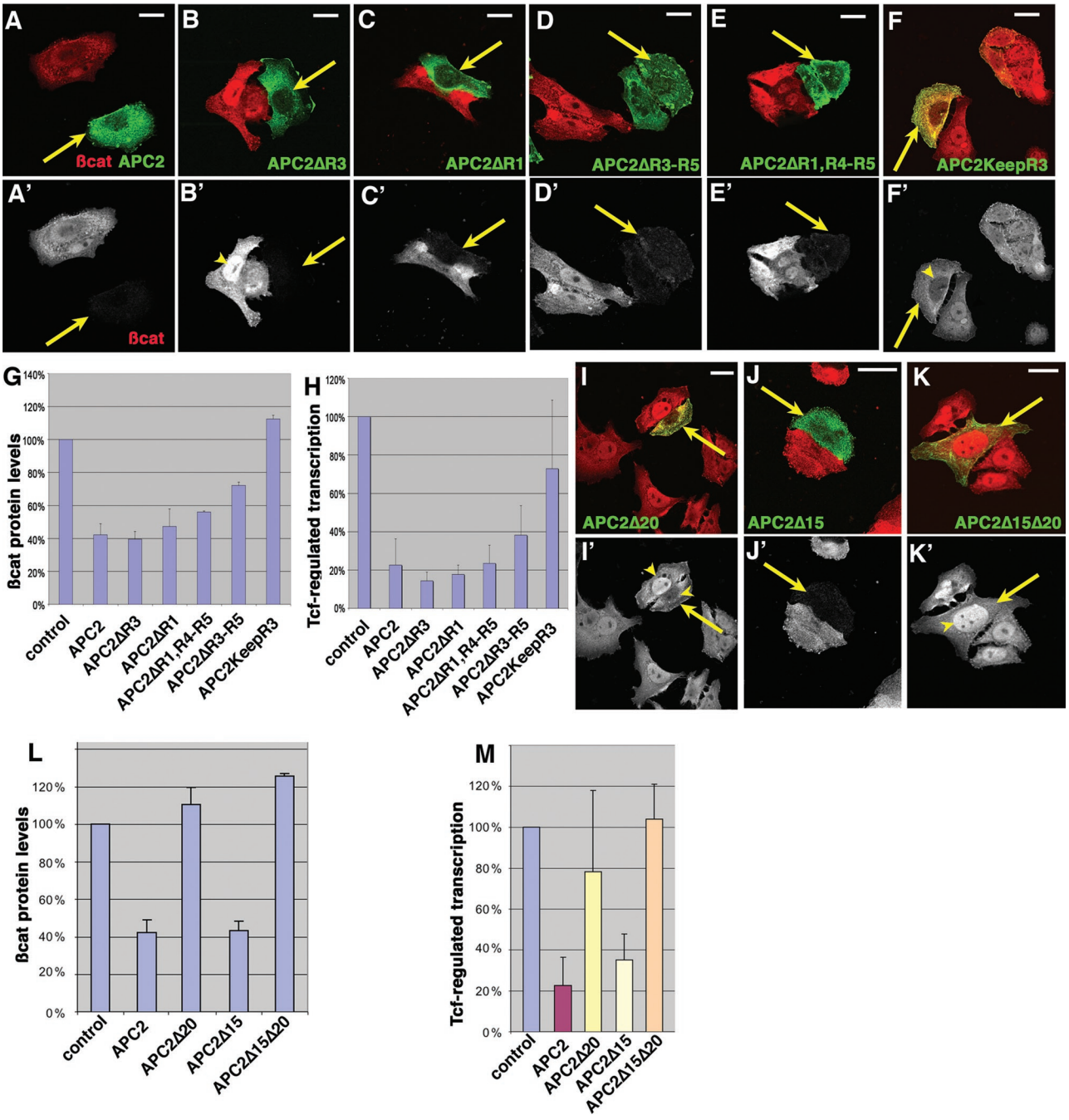


FIGURE 2: Individual high-affinity 20Rs are not essential for destruction but contribute with 15Rs to cytoplasmic retention. (A–F and I–K) SW480 cells transfected with GFP-tagged wild-type APC2 (A) or the indicated mutants. Arrows indicate representative cells transfected with wild type or mutant APC. Arrowheads = nuclear ßcat accumulation in control untransfected cells, and selected mutants that can (APC2KeepR3, APC2Δ20) or cannot (APC2Δ15Δ20) retain ßcat in the cytoplasm. (G and L) ßcat levels as quantified by Cellomics. (H and M) TOPFLASH assays revealing TCF-dependent transcription.

targeting ßcat for destruction. We tested this using an *APC2* mutant with all other 20Rs deleted (APC2KeepR3; Figure 1B). This provided two interesting surprises. In SW480 cells, APC2KeepR3 was completely inactive in targeting ßcat for destruction (Figure 2, F and G), and Wnt-mediated transcription was not reduced by a statistically significant level (Figure 2H). Thus ßcat binding provided by 20R3 is not sufficient for ßcat destruction.

We next examined APC2KeepR3 function in *Drosophila*. Consistent with the idea that APC2KeepR3 cannot rescue ßcat destruction, it had no ability to rescue the cell fate phenotype or the destruction of Arm in *APC2 APC1* double mutants (Figure 3, B and N; compare Figure 4, 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 3, A and G). To explore this further, we examined ßcat localization in SW480 cells expressing APC2KeepR3. Strikingly, although ßcat in SW480 cells is usually significantly enriched in nuclei (e.g., Figure 2B′ arrowhead), APC2KeepR3 was able to reduce nuclear ßcat enrichment (Figure 2F′, arrowhead).

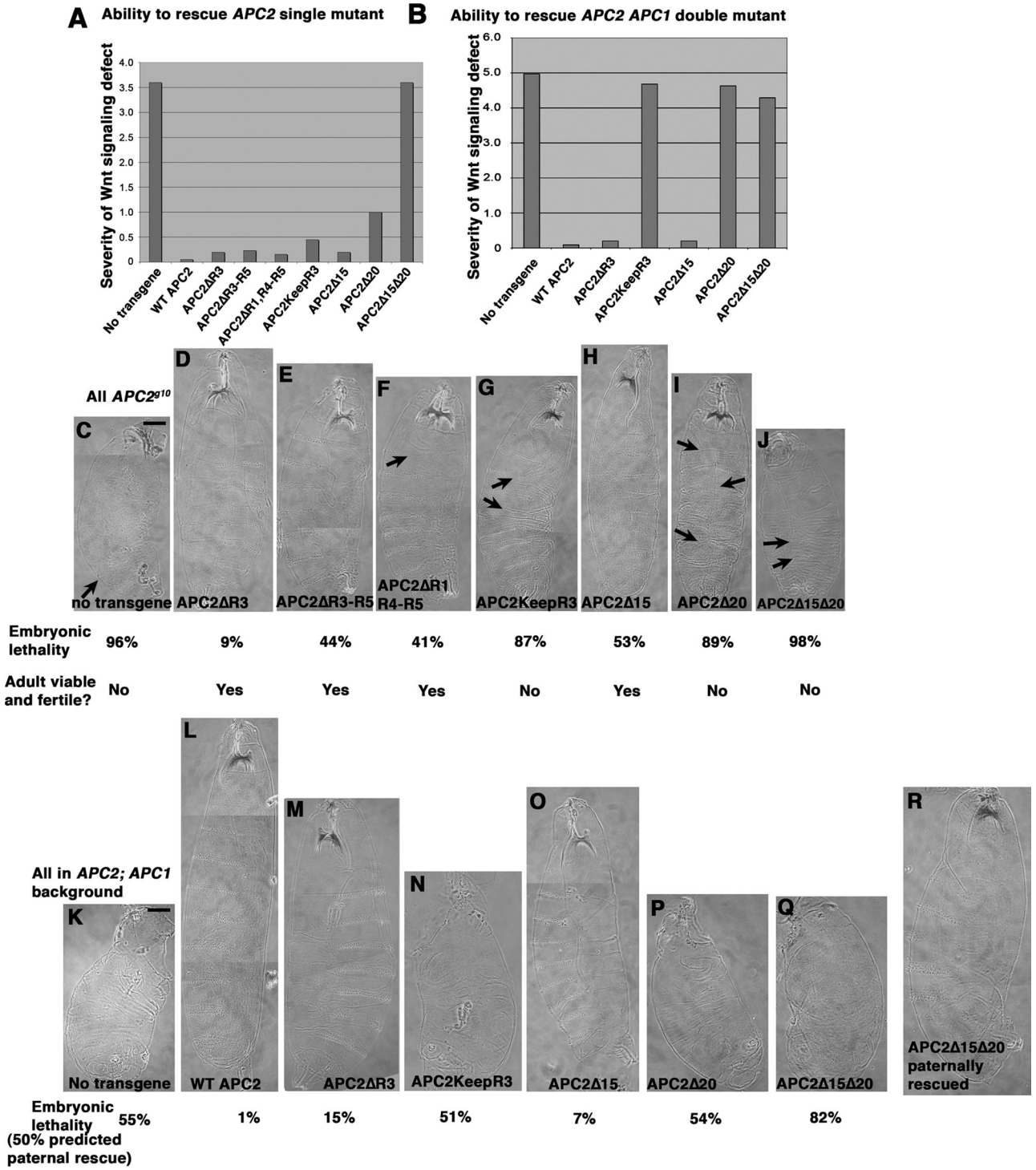


FIGURE 3: Many ßcat-binding sites are dispensable for Arm destruction but contribute additively to rescue *APC2* single mutants. (A and B) Rescue of Wnt-mediated cell-fate decisions in $APC2^{g10}$ maternal/zygotic single mutants (A) or $APC2^{g10}$ $APC1^{Q8}$ maternal/zygotic double mutants (B). Cuticles scored as in Supplemental Figure 5. 0 = wild-type and 6 = strongest loss-of-function phenotype observed. (C–J) Representative cuticles showing ability of transgenes to rescue $APC2^{g10}$ 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 $APC2^{g10}$ $APC1^{Q8}$ 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.

In *Drosophila APC2* single mutants, the remaining APC1 protein provides residual activity in regulating Arm levels (Ahmed *et al.*, 2002; Akong *et al.*, 2002a). 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

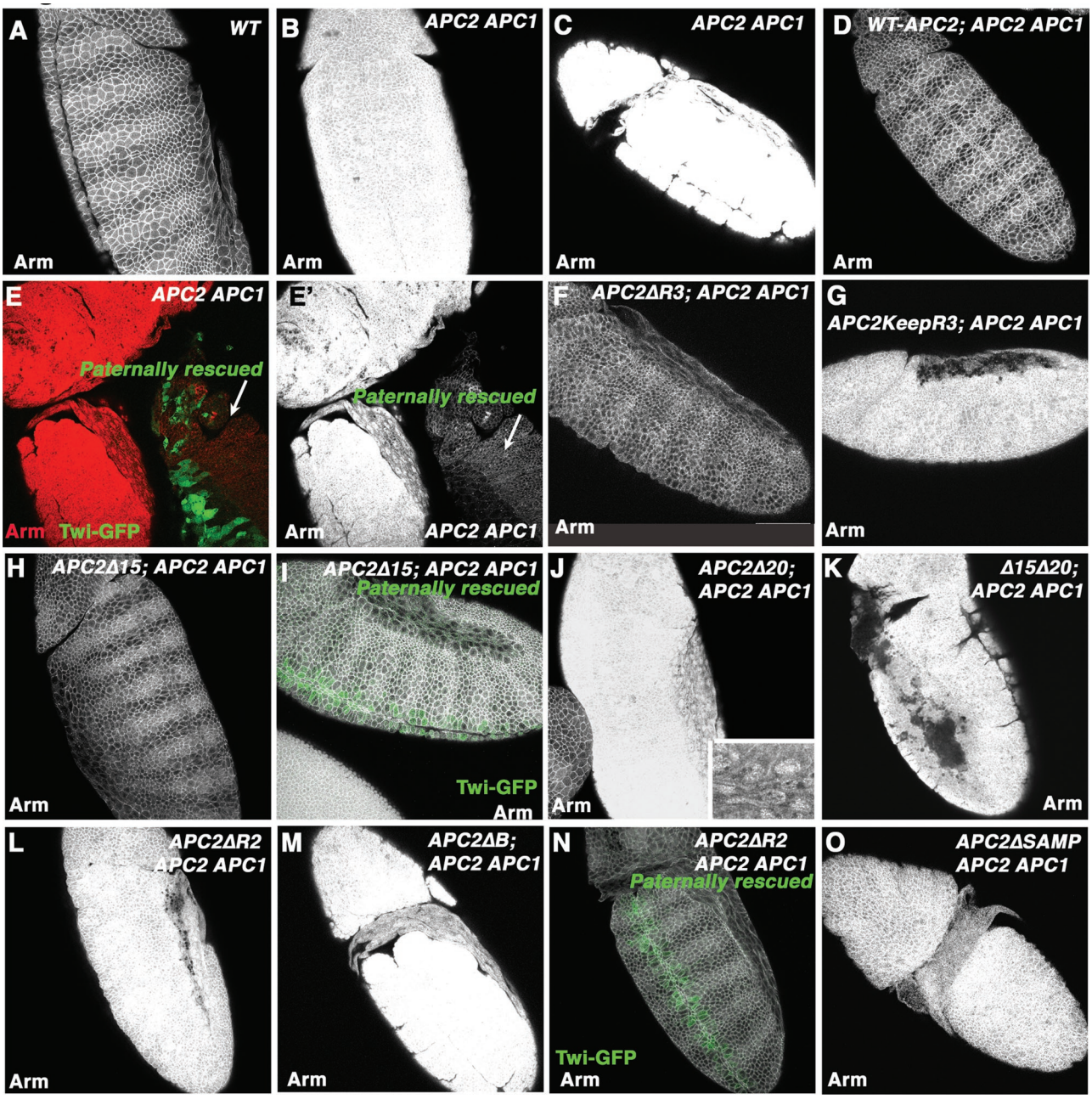


FIGURE 4: Roles of 20Rs and sequence B in targeting Arm for destruction in vivo. Stage 9–10 embryos *APC2*^{g10} *APC1*^{Q8} maternal/zygotic double mutants expressing indicated transgenes stained for Arm; Twist-GFP (Twi-GFP) on the Balancer was used to distinguish maternal/zygotic mutants and paternally 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.

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 ßcat-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 1B). In SW480 cells, APC2Δ20 behaved like APC2KeepR3—it could not rescue ßcat destruction (Figure 2, I and L) or reduce TCF-regulated transcription by a statistically significant amount (Figure 2M). We were surprised to find, however, that APC2Δ20 could still retain ßcat in the cytoplasm (Figure 2I′, compare

arrowheads). In *Drosophila* APC2Δ20 provided no rescue of Arm levels (Figure 4J) or cell fates in *APC2 APC1* double mutants (Figure 3, 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 3, 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 3A). Thus an APC2 protein lacking all 20Rs retains a small degree of function in vivo, correlating with its residual ability for cytoplasmic retention of ßcat, but importantly at least one 20R is essential for the destruction complex to mediate ßcat destruction.

We next hypothesized that the 15Rs might provide the ability to bind and retain ßcat in the cytoplasm, allowing APC2Δ20 to reduce Wnt signaling even though it was nonfunctional in ßcat destruction. Previous work explored the function of the 15Rs in ßcat regulation in the context of APC fragments (Kohler *et al.*, 2010), but not in the context of full-length APC. We thus first deleted them alone (APC2Δ15; Figure 1B). APC2Δ15 was fully functional in regulating ßcat destruction (Figure 2, J and L) and reducing TCF-regulated transcription in SW480 cells (Figure 2M). It was also highly functional in the animal, fully rescuing the cell fate defects of *APC2* single mutants (Figure 3, A and H) and restoring adult viability (Supplemental Figure 3D). It also fully rescued embryonic viability, cell fate choices, and Arm destruction of *APC2 APC1* double mutants (Figure 3, B and O; Figure 4H). 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 ßcat, might participate in cytoplasmic retention. To test this hypothesis, we generated a mutant lacking all 15Rs and 20Rs (APC2Δ15Δ20; Figure 1B); this mutant should be completely unable to bind ßcat. Strikingly, APC2Δ15Δ20 was fully inactivated in all contexts, in contrast to APC2Δ20. It provided no rescue of ßcat destruction (Figure 2, K and L) or TCF-mediated transcription (Figure 2M) in SW480 cells, and could not retain ßcat in the cytoplasm (Figure 2K, arrowhead). APC2Δ15Δ20 was also completely inactive in the animal, failing to provide any rescue activity in either *APC2* single mutants (Figure 3, A and J) or in *APC2 APC1* double mutants (Figure 3, B and Q; Figure 4K). 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 3, Q and R). Together, these data demonstrate that the 20Rs play an essential role in the ability of the destruction complex to target ßcat for destruction. They also suggest that cytoplasmic retention of ßcat by APC2 can play an important secondary role in negatively regulating ßcat transcriptional activity, and that both 15Rs and 20Rs contribute in an additive way to this ability.

20R2 and sequence B are essential for ßcat destruction

When examining the affinities of 15Rs and different 20Rs for ßcat (Choi *et al.*, 2006; Liu *et al.*, 2006), one surprise was that 20R2 lacks key residues for ßcat binding (Supplemental Figure 4, yellow arrows, red arrowheads), and does not detectably bind ßcat in vitro (Choi *et al.*, 2006; Liu *et al.*, 2006) or in cells (Kohler *et al.*, 2008), with or without phosphorylation. Despite the inability of 20R2 to bind ßcat, the divergent sequence of 20R2 is as or better conserved between flies and mammals than are high-affinity binding sites like 20R3 (Supplemental Figure 4). This prompted us to explore whether 20R2 is important for ßcat regulation. Given its inability to bind ßcat, we hypothesized it would be dispensable. To our surprise, however, removing 20R2 (APC2ΔR2; Figure 1B) completely blocked APC2's ability to rescue ßcat destruction in SW480 cells (Figure 5, A and D). APC2ΔR2 could retain ßcat in the cytoplasm (Figure 5A, compare arrowheads), however, consistent with the presence of numerous ßcat-binding sites, and thus it partially reduced TCF-regulated transcription (Figure 5E). In this it contrasted with APC2Δ15Δ20, which could not retain ßcat in the cytoplasm (Figure 5C, arrowhead) or reduce TCF-regulated transcription (Figure 5E).

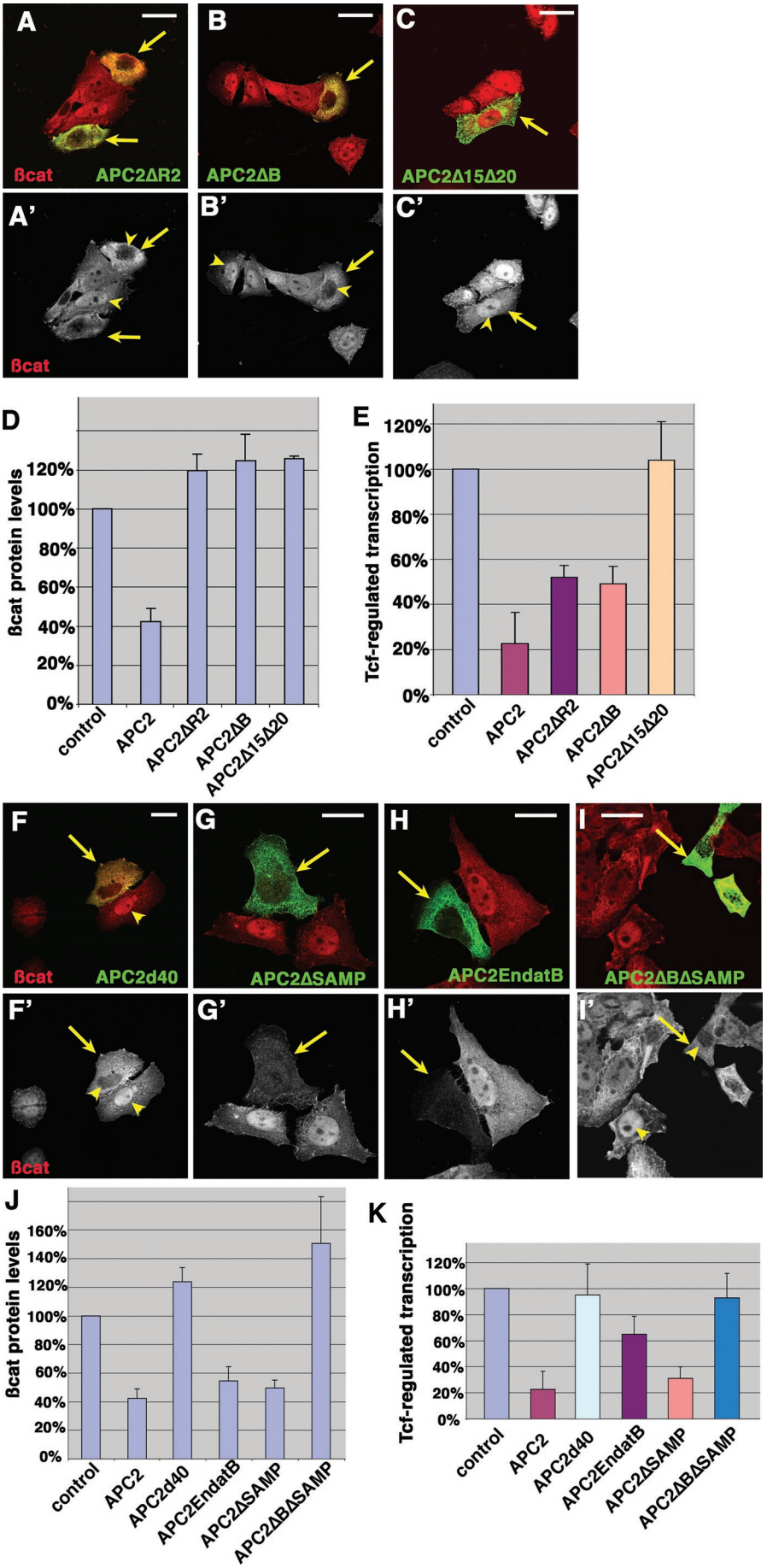
We next assessed APC2ΔR2 in the animal. Consistent with our observations in SW480 cells, APC2ΔR2 could not rescue Arm destruction in *APC2 APC1* double mutants (Figure 4L), and it also provided no cell fate rescue there (Figure 6, B and K). APC2ΔR2 retained substantial ability, however, to rescue cell fates, embryonic lethality, and adult viability of *APC2* single mutants (Figure 6, A and D), correlating with it retaining function in cytoplasmic retention. Thus 20R2 is essential for the ability of the destruction complex to target ßcat for destruction, but is dispensable for ßcat retention.

Immediately adjacent to 20R2 is another highly conserved sequence shared by all APC family members, sequence B (McCartney *et al.*, 1999; Supplemental Figure 4; also known as the CID; Kohler *et al.*, 2009). 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 1B) also abolished its ability to target ßcat for destruction in SW480 cells (Figure 5, B and D). Deleting sequence B, however, did not disrupt cytoplasmic retention (Figure 5B, compare arrowheads), and thus APC2ΔB retained the ability to partially reduce ßcat-dependent transcription (Figure 5E). When tested in the animal, APC2ΔB behaved similarly to APC2ΔR2. It could not rescue Arm destruction (Figure 4M), cell fates, or embryonic lethality in *APC2 APC1* double mutants (Figure 6, B and L). APC2ΔB retained, however, substantial ability to rescue cell fates, embryonic lethality, and adult viability of *APC2* single mutants (Figure 6, A and E; Supplemental Figure 3E), consistent with it retaining function in cytoplasmic retention. Thus these two adjacent conserved APC motifs, 20R2 and sequence B, play essential roles in ßcat 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 ßcat regulation in SW480 cells (hAPC2.8kb; Rubinfeld *et al.*, 1997a). This fragment spans from 20R1 through the end of the SAMPs (Figure 7A), and rescues ßcat destruction in SW480 cells (Figure 7B). We generated variants lacking 20R2, 20R3, or sequence B (Figure 7A). Deleting either 20R2 (hAPC2.8kbΔR2; Figure 7C) or sequence B (hAPC2.8kbΔB; Figure 7D; see also Kohler *et al.*, 2009) substantially reduced the ability to target ßcat for destruction, as assessed by immunofluorescence, whereas deleting 20R3 (hAPC2.8kbΔR3; Figure 7E) 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 7F). Thus both 20R2 and sequence B play conserved and essential roles in regulating ßcat destruction in flies and mammals.

Direct binding to Axin via the SAMPs is essential for Wnt regulation in *Drosophila* but dispensable in SW480 cells

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; Nakamura *et al.*, 1998; Spink *et al.*, 2000). 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), 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 1B) can no longer mediate ßcat destruction or down-regulate TCF-regulated transcription in SW480 cells (Figure 5, 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; Figure 6, 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; Figure 6, A and F)—this retention correlates with its ability to retain ßcat in the cytoplasm in SW480 cells (Figure 5F′, compare arrowheads).

These data are consistent with the idea that the SAMPs are essential for the ability of the destruction complex to target ßcat 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 1B). To our surprise, unlike APC2d40, APC2ΔSAMP largely restored ßcat destruction and down-regulated TCF-regulated transcription in SW480 cells (Figure 5, 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 6, B and N), or destruction of Arm (Figure 4O). In fact, like APC2Δ15Δ20, there was suggestion of dominant-negative activity, as many embryos that should have been paternally rescued died (Figure 6N). In *APC2* single mutants, APC2ΔSAMP retained detectable activity (Figure 6, 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

FIGURE 5: Sequence B and 20R2 are essential for ßcat 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 ßcat accumulation in control untransfected cells, compared to mutants that can retain ßcat in the cytoplasm (APC2ΔR2, APC2ΔB, APC2d40, APC2ΔBΔSAMP). (D and J) ßcat levels as quantified by Cellomics. (E and K) TOPFLASH assays revealing TCF-dependent transcription.

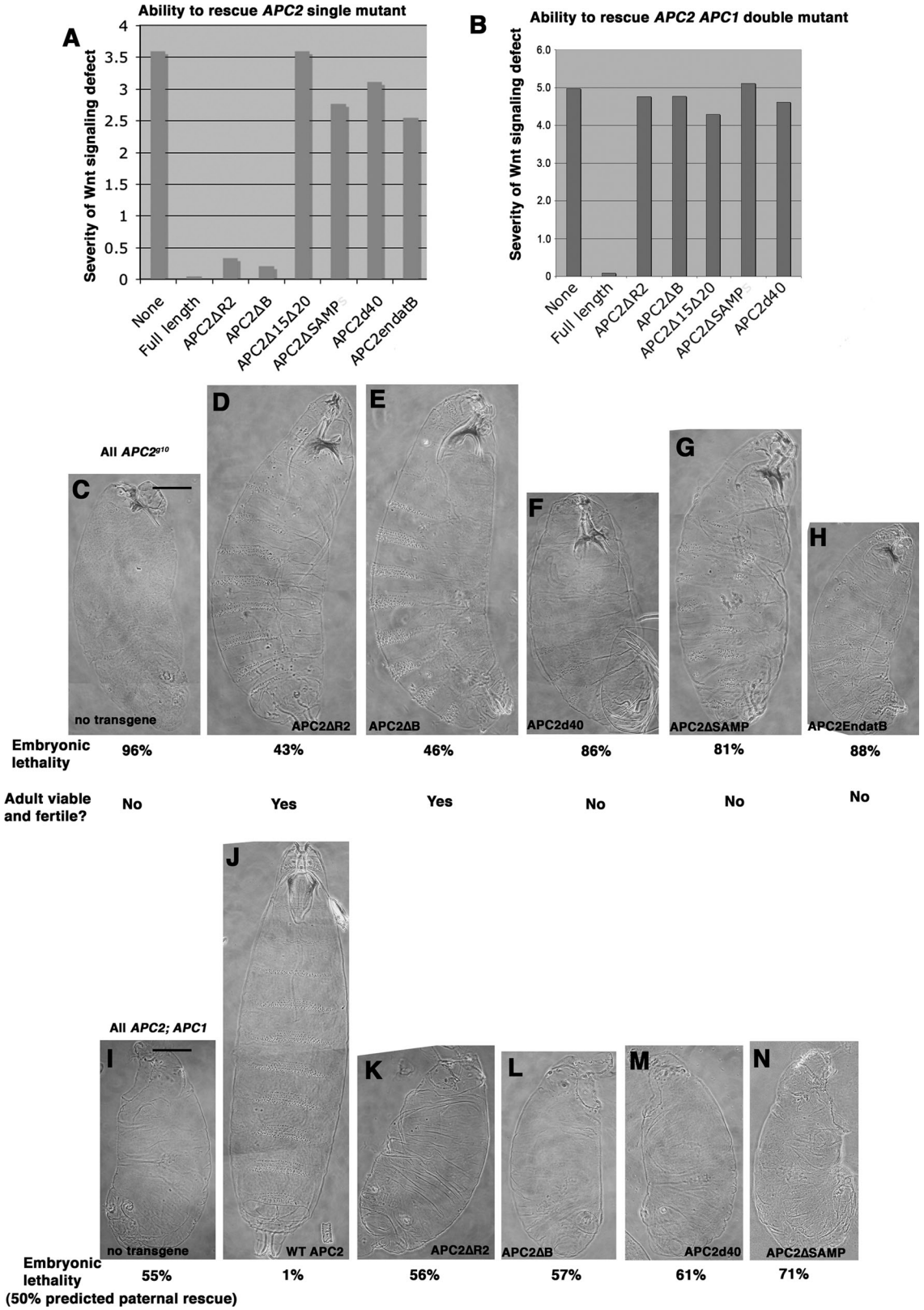


FIGURE 6: Sequence B, 20R2, and the SAMPs are essential for Arm destruction in vivo. (A and B) Rescue of Wnt-mediated cell-fate decisions in *APC2*^{g10} maternal/zygotic single mutants (A) or *APC2*^{g10} *APC1*^{Q8} maternal/zygotic double mutants (B). 0 is wild type and 6 the strongest loss-of-function phenotype observed. (C–H) Representative cuticles showing ability of transgenes to rescue *APC2*^{g10} 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 *APC2*^{g10} *APC1*^{Q8} maternal/zygotic double mutants. Below cuticles are rescue of embryonic lethality. Because fathers are heterozygous, 50% of embryos are paternally rescued.

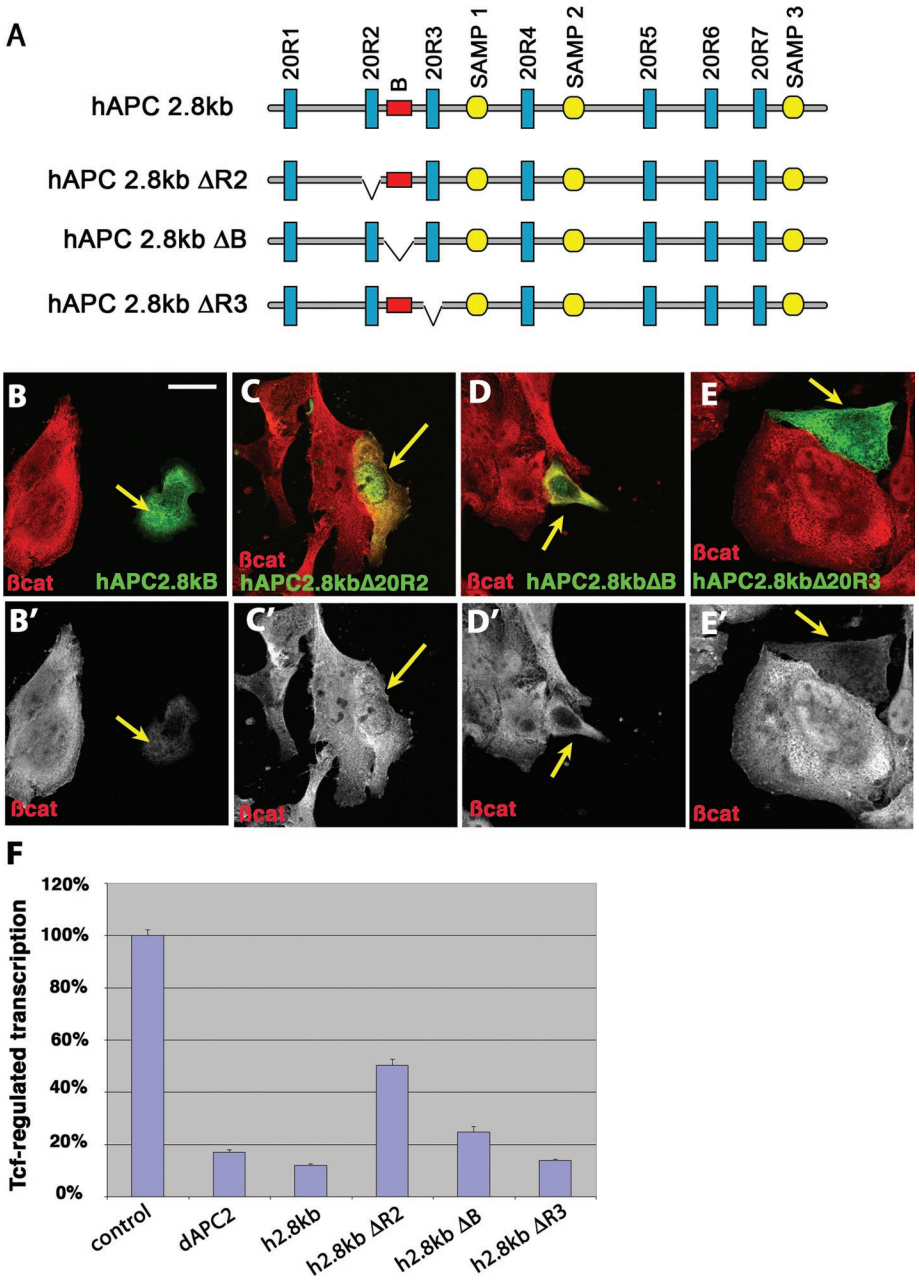


FIGURE 7: Sequence B and 20R2 are also essential for ßcat destruction by human APC. (A) Constructs used. (B–E) SW480 cells transfected with GFP-tagged fragments of human APC. Arrows indicate representative cells transfected with wild type or mutant APC fragments. (F) TOPFLASH assays revealing TCF-dependent transcription.

essential for the ability of the destruction complex to target ßcat 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 ßcat 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 8A; GFP-tagged APC2 transfected without Flag-Axin was a negative control). Co-IP was eliminated by deletion of the SAMPs (Figure 8B). 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 9A, inset), as are most APC2 mutants we analyzed earlier (unpublished data). In contrast, Flag-tagged *Drosophila* Axin localizes to cytoplasmic puncta (Figure 9B, inset), similarly to those previously observed on expression of mammalian Axin (Fagotto *et al.*, 1999; Smalley *et al.*, 1999). Interestingly, overexpressing fly Axin is sufficient to trigger ßcat destruction (Figure 9B, arrow), as was previously observed after overexpression of human Axin (Hart *et al.*, 1998; Nakamura *et al.*, 1998). Coexpressing *Drosophila* Axin and APC2 led to almost complete recruitment of APC2 into Axin puncta (Figure 9C, inset), consistent with a physical interaction: Similar results were previously observed with mammalian Axin and APC (Faux *et al.*, 2008). ßcat binding does not play a role in this interaction, as APC2Δ15Δ20 is also strongly recruited into Axin puncta (Figure 9D, inset). In contrast, recruitment of APC2ΔSAMP into Axin puncta was substantially reduced, although not completely eliminated (Figure 9E, 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, *APC2*^{d40}, mimics the tumor truncations, with a stop codon just after 20R2 (McCartney *et al.*, 2006). Although APC2d40 protein could not reduce ßcat levels (Figure 5, F and J) or Wnt regulated transcription (Figure 5K) in SW480 cells, to our surprise this mutant protein, which lacks all SAMPs, is readily recruited into Axin puncta (Figure 9F, inset). This interaction appears less robust than that mediated by the SAMPs, as it does not support co-IP of APC2d40 and Axin (Figure 8C). We next explored whether this SAMP-independent ability to interact with Axin was confined to *Drosophila* APC2. We cotransfected a truncated

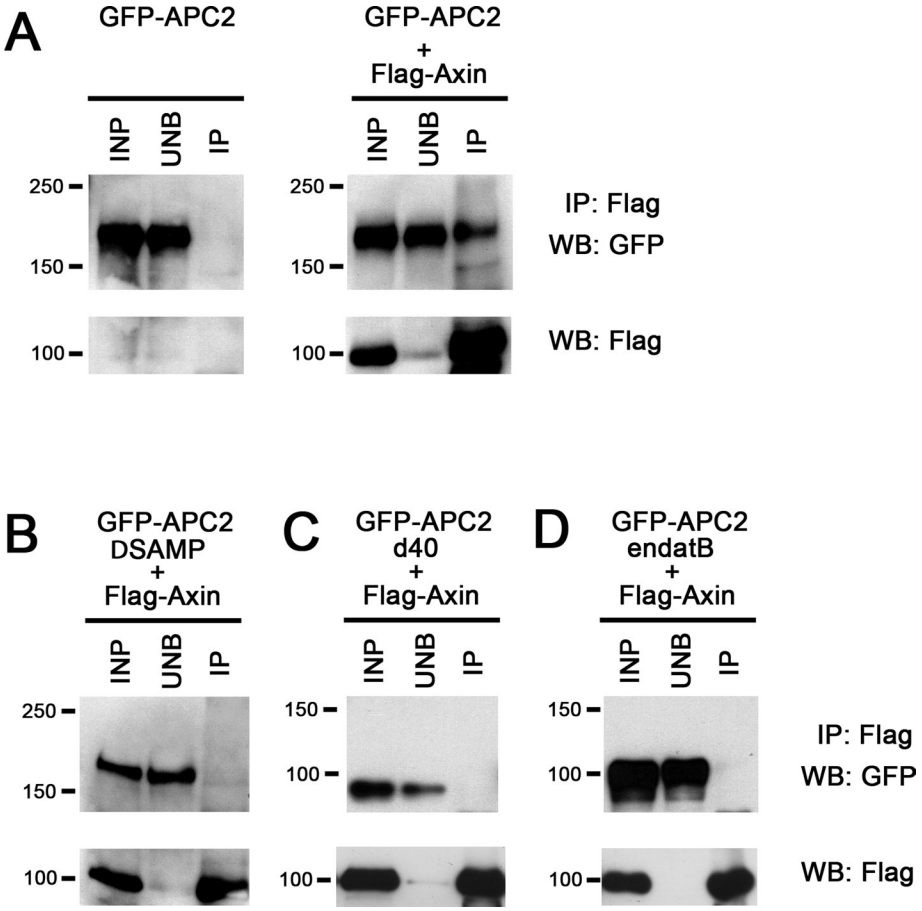


FIGURE 8: The SAMPs are necessary for APC2 to coimmunoprecipitate with Axin. Protein extracts from SW480 cells cotransfected with GFP-dAPC2 constructs and Flag-dAxin immunoprecipitated with a Flag antibody. (A) Full-length dAPC2 coimmunoprecipitates with dAxin. Cells transfected with GFP-dAPC2 alone 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.

human APC like that found in tumors (aa 1–1338) with human Axin. Strikingly, it also colocalized to Axin puncta (Figure 9I, 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 1B; only 56 amino acids longer than the truncation in APC2d40) drastically reduces localization in Axin puncta in SW480 cells (Figure 9G, inset); APC2EndatB also does not coimmunoprecipitate with Axin (Figure 8D). 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 ßcat for destruction in SW480 cells (Figure 5, H and J; although it was not fully effective at reducing TCF-regulated transcription; Figure 5K), but it could not restore substantial APC2 function in *APC2* single mutant flies (Figure 6, 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 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 9H, inset); however, like APC2ΔB, APC2ΔBΔSAMP cannot rescue ßcat regulation in SW480 cells (Figure 5, I, J, and K). Together, these data demonstrate that sequence B plays a key role in ßcat destruction, and suggest a speculative model in which it facilitates completion of a catalytic cycle that involves release of APC and perhaps ßcat 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 ßcat destruction complex operates and what role APC plays. Our data address these issues. They demonstrate that individual ßcat-binding sites are not essential for ßcat destruction, but instead suggest that these sites modulate Wnt signaling, acting collectively to retain ßcat in the cytoplasm. Furthermore, they demonstrate that 20R2 and sequence B are essential for targeting ßcat 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 ßcat for phosphorylation and ultimate destruction, and help guide thinking about analogous machines targeting other signaling effectors.

Individual ßcat-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 ßcat-binding sites of different affinities (Choi *et al.*, 2006; Liu *et al.*, 2006). 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; Kohler *et al.*, 2008, 2010). Several hypotheses were proposed to explain roles of these different sites, with special roles of high-affinity sites in regulating ßcat destruction a common feature. The catalytic cycle model suggested that the highest affinity binding sites are required to remove ßcat from Axin after ßcat and APC phosphorylation, facilitating ßcat transfer to the E3-ligase and subsequent destruction (Kimelman and Xu, 2006). In another model, different affinity binding sites play roles at the different ßcat concentrations in cells exposed to or not exposed to Wnt signals (Ha *et al.*, 2004). Thus both predict special roles for the highest affinity binding sites.

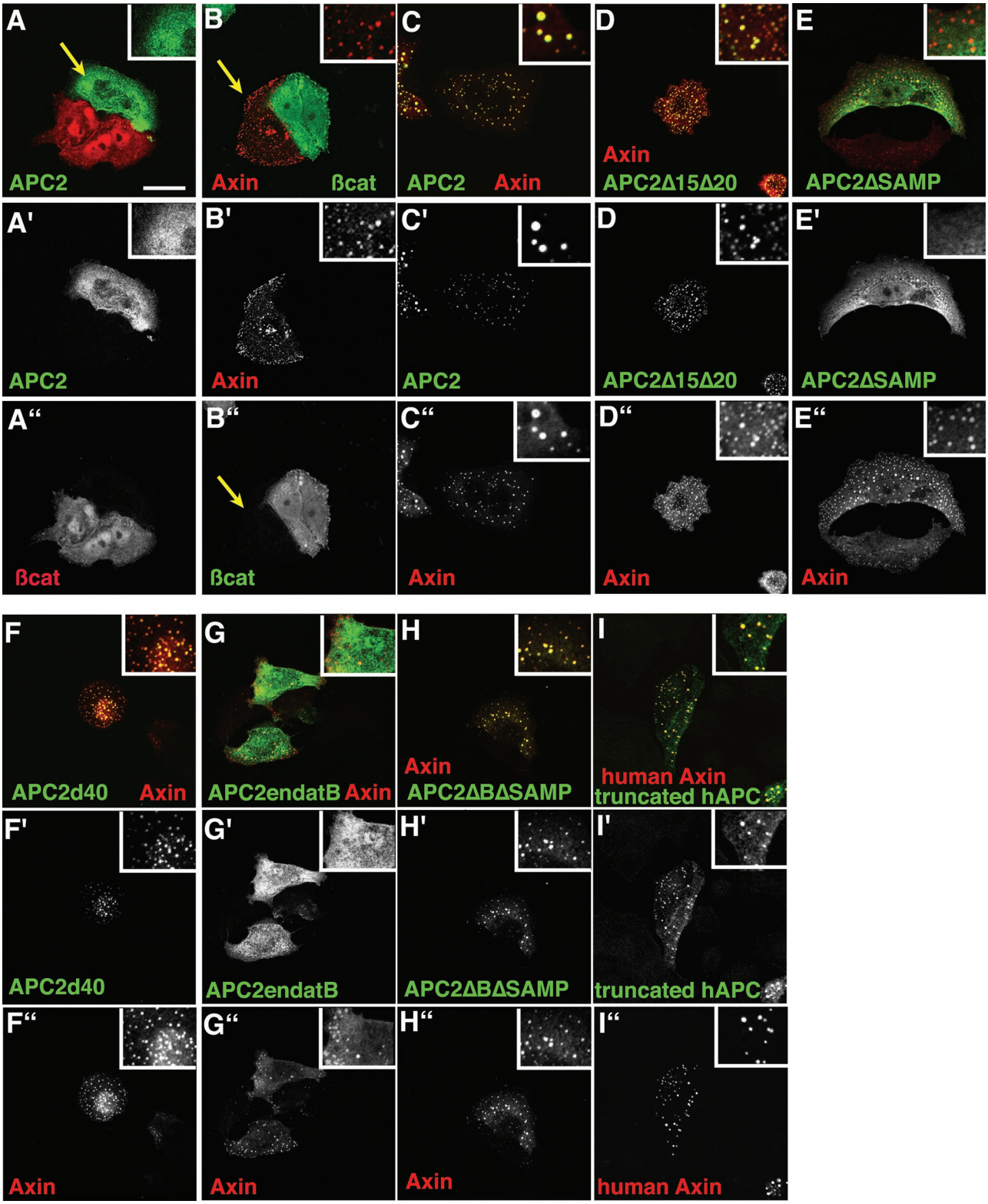


FIGURE 9: APC2 colocalization with Axin is SAMP-independent and is antagonized by sequence B. (A–H) SW480 cells transfected with GFP-tagged wild type or mutant APC2 and Flag-tagged fly Axin. Arrows in A and B = transfected cells. Insets = enlargements of puncta. (A) APC2 is diffusely cytoplasmic in SW480 cells. (B) Overexpressed Axin forms cytoplasmic puncta and can trigger ßcat destruction. (C) When coexpressed, Axin recruits APC2 into puncta. (D) APC2's ßcat-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.

We tested how the diverse ßcat-binding sites contribute to ßcat destruction. We were surprised to find that individual high-affinity binding sites are not essential for APC's mechanism of action. The highest affinity ßcat-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 ßcat binding 20R, could target ßcat 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 ßcat 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). Our data are consistent with the apparent lack of 15Rs in human APC2, which can down-regulate ßcat in SW480 cells (van Es *et al.*, 1999). Thus, no individual ßcat binding site appears essential for the destruction complex to target ßcat for destruction, although it remains possible that these ßcat binding sites play more subtle modulatory roles at the different ßcat concentrations in cells exposed to or not exposed to Wnt signals (e.g., Ha *et al.*, 2004). Eliminating all 20Rs, however, eliminates ßcat 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 ßcat-binding residues and does not detectably bind ßcat (Choi *et al.*, 2006; Liu *et al.*, 2006; Kohler *et al.*, 2008). 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 ßcat binding (Supplemental Figure 4), 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 ßcat 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 ßcat 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). 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 (Supplemental Figure 4) 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). 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) 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 ßcat 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 ßcat requires cycles of assembly and disassembly of the destruction complex, with transfer to the E3 ubiquitin ligase perhaps coincident with complex disassembly (Figure 10A). The idea of the necessity for a catalytic cycle builds on earlier suggestions by Kimelman and Xu (2006) 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 ßcat phosphorylation, separating APC and Axin and allowing the destruction complex to reset to phosphorylate additional ßcat. In mutants defective in disassembly (Figure 10B), perhaps ßcat 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 ßcat destruction—in contrast to the large APC pool, Axin is thought to be limiting (Salic *et al.*, 2000). 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 (Supplemental Figure 2D). Overexpressing Axin alone rescues ßcat 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), 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

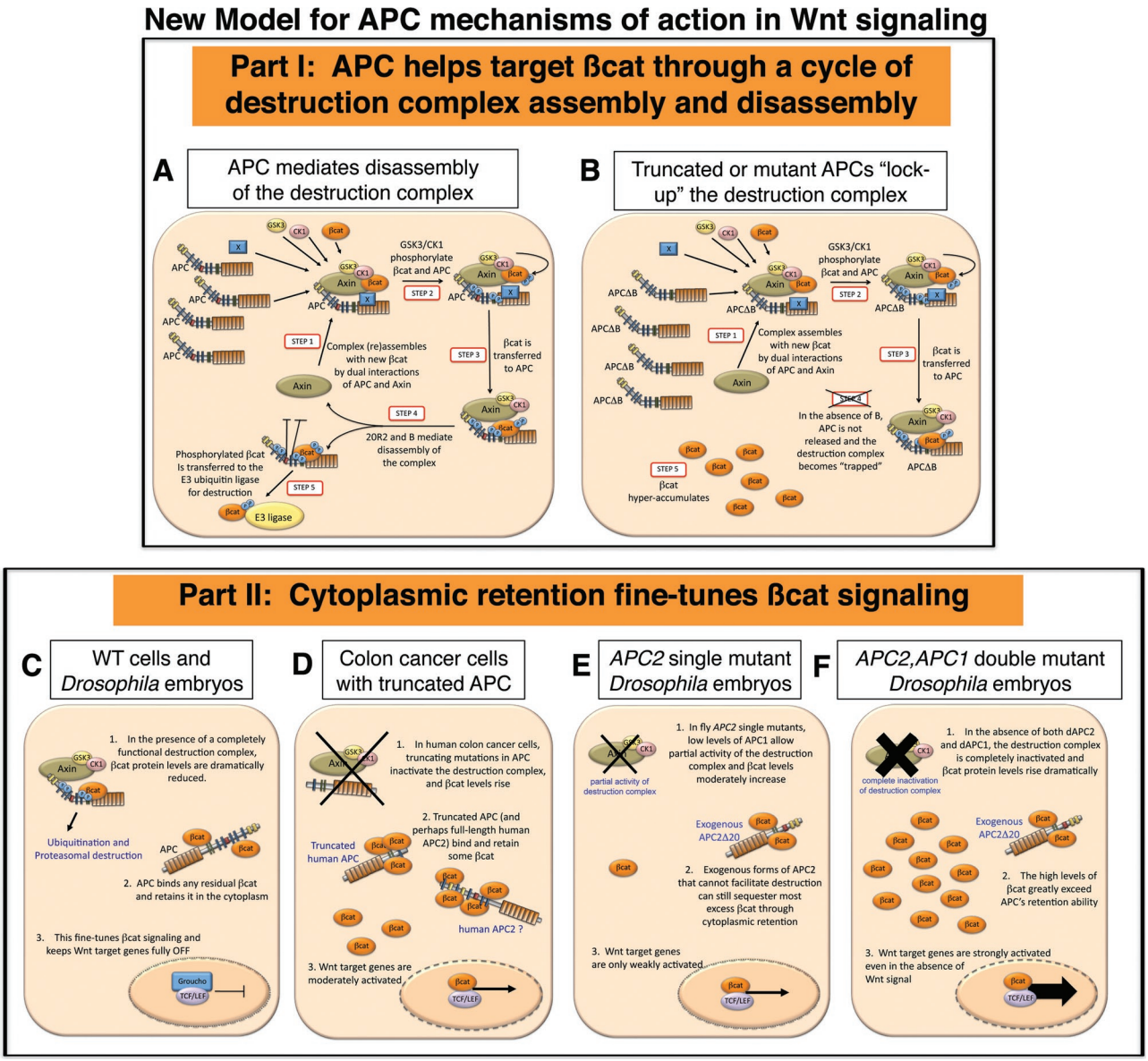


FIGURE 10: Model: APC regulates βcat signaling through a cycle of destruction complex assembly and disassembly, with cytoplasmic retention by APC playing a secondary role in fine-tuning signaling. This model builds on earlier suggestions of a catalytic cycle (Kimelman and Xu, 2006), of the role of multiple ßcat-binding sites as docking sites to accommodate different ßcat levels (Ha *et al.*, 2004), and of suggested roles for cytoplasmic retention in modulating signaling (Tolwinski and Wieschaus, 2001; Krieghoff *et al.*, 2006; McCartney *et al.*, 2006). Major new features are the essential natures of 20R2 and Sequence B in ßcat destruction, with the suggestion that they play roles in complex disassembly, and the idea that truncated human APC proteins cannot target ßcat for destruction but instead modulate signaling by cytoplasmic retention.

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

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

Comparing ßcat localization in wild type and APC mutant cells and tissues suggested that APC and Axin can retain ßcat in the cytoplasm (Tolwinski and Wieschaus, 2001; Krieghoff *et al.*, 2006; McCartney *et al.*, 2006). 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 ßcat destruction is APC's key role, with cytoplasmic retention playing a modulatory role in some circumstances. We found that high-affinity ßcat-binding sites are dispensable for ßcat destruction. Instead, the array of ßcat-binding sites in APC proteins appears to provide a sink for cytoplasmic retention of residual ßcat, preventing it from entering nuclei to activate gene expression, and thus modulating Wnt signaling (Figure 10C). Distinctions in rescue of *APC2* single mutants (Figure 3), combined with cell biological assessment of cytoplasmic retention in SW480 cells (Figure 2), 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

ßcat destruction were ineffective. We suspect that in the absence of all destruction complex activity, Arm levels exceed the buffering capacity of APC2's ßcat-binding sites (Figure 10F). In contrast, mutants retaining ßcat 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 10E). Because these same mutants also reduce TCF-regulated transcription in SW480 cells, ßcat 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 ßcat localization that we observed in SW480 cells were due to differential ability of APC2 mutants to export ßcat from the nucleus. APC can shuttle in and out of the nucleus, and some data support a role in assisting ßcat export (reviewed in Bienz 2002; Henderson and Fagatto, 2002). APC-mediated nuclear export of ßcat 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 ßcat nuclear export (Krieghoff *et al*., 2006). Second, only two of the identified nuclear export sequences (NESs) in human APC (Henderson and Fagatto, 2002) 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 ßcat 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 ßcat 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). 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), suggesting that truncated proteins have lost some but not all ability to target ßcat 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). 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 ßcat for destruction, in either SW480 cells or *Drosophila*. Instead, their ability to retain ßcat in the cytoplasm provides the most plausible explanation for their residual ability to regulate Wnt signaling in colon cancer cells (Figure 10D). 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). Cytoplasmic retention may help buffer ßcat, blunting the transcriptional response until the destruction complex is saturated with ßcat. 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). Retention may help turn graded signaling into a more binary response, with sharper thresholds. This possibility can now be explored.

MATERIALS 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) 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.

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: Function of APC2 mutants in vivo in *Drosophila*.

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 $APC2^{g10}$ single mutant and the $APC2^{g10}APC1^{Q8}$ double mutant backgrounds (McCartney *et al*., 2006). For analysis in the $APC2^{g10}$ single mutant background, embryos expressing the transgene but maternally/zygotically mutant for *APC2* were the progeny of *dAPC2 transgene;* $APC2^{g10}$ 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). Heat-shocked *dAPC2* transgene/+; *FRT82B* $APC2^{g10}$ APC^{Q8}/*FRT82B* ovo^{D} females were crossed to *dAPC2 transgene; FRT82B* $APC2^{g10}$ APC^{Q8}/*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). The level of embryonic cuticle rescue was assessed using previously established scoring criteria (McCartney *et al*., 2006).

IPs and Western blotting

IPs and Western blots were conducted as in Peifer et al. (1992). 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 $NaVO_3$, 0.4 mM $NaVO_5$). 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). 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). Briefly, embryos were dechorionated and fixed in 4% formaldehyde before devitelizination. 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.

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