

Regulation of Wnt signaling by the tumor suppressor adenomatous polyposis coli does not require the ability to enter the nucleus or a particular cytoplasmic localization

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ABSTRACT Wnt signaling plays key roles in development and disease. The tumor suppressor adenomatous polyposis coli (APC) is an essential negative regulator of Wnt signaling. Its best-characterized role is as part of the destruction complex, targeting the Wnt effector β -catenin (β cat) for phosphorylation and ultimate destruction, but several studies suggested APC also may act in the nucleus at promoters of Wnt-responsive genes or to shuttle β cat out for destruction. Even in its role in the destruction complex, APC's mechanism of action remains mysterious. We have suggested APC positions the destruction complex at the appropriate subcellular location, facilitating β cat destruction. In this study, we directly tested APC's proposed roles in the nucleus or in precisely localizing the destruction complex by generating a series of APC2 variants to which we added tags relocalizing otherwise wild-type APC to different cytoplasmic locations. We tested these for function in human colon cancer cells and *Drosophila* embryos. Strikingly, all rescue Wnt regulation and down-regulate Wnt target genes in colon cancer cells, and most restore Wnt regulation in *Drosophila* embryos null for both fly APCs. These data suggest that APC2 does not have to shuttle into the nucleus or localize to a particular subcellular location to regulate Wnt signaling.

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INTRODUCTION

A few key signal transduction pathways shape cell fate decisions during normal development and maintain adult tissue homeostasis. These powerful pathways must be kept under tight control, as each also plays critical roles in oncogenesis, with virtually every tumor

exhibiting inappropriate activation of one or more pathways. One of the most interesting surprises in signal transduction has been the discovery of elaborate machinery that evolved to keep these pathways tightly off in the absence of ligands. This negative regulatory machinery is the target of inactivating mutations in human tumors.

The Wnt pathway provides a striking example (Cadigan and Peifer, 2009; Chien *et al.*, 2009). It is negatively regulated at many levels, from secreted Wnt antagonists to repressors binding T-cell factor (TCF) transcription factors at the end of the pathway. However, the primary mechanism keeping signaling off in cells not receiving Wnt ligands is regulated destruction of the key effector β -catenin (β cat; fly homologue Armadillo [Arm]). β cat is constitutively phosphorylated by a set of proteins known as the destruction complex, which triggers ubiquitination and proteasomal destruction. Wnt signals inactivate the destruction complex, stabilizing β cat and allowing it to enter nuclei to act as a transcriptional coactivator. In colon and other cancers, constitutive activation of Wnt signaling, by gain-of-function β cat mutations preventing its destruction or loss-of-function

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Abbreviations used: APC, adenomatous polyposis coli; Arm, Armadillo; β cat, β -catenin; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; IP, immunoprecipitation; MT, microtubule; NES, nuclear export signal; NGS, normal goat serum; NLS, nuclear localization signal; PBS, phosphate-buffered saline; SAMP, Ser-Ala-Met-Pro; TCF, T-cell factor.

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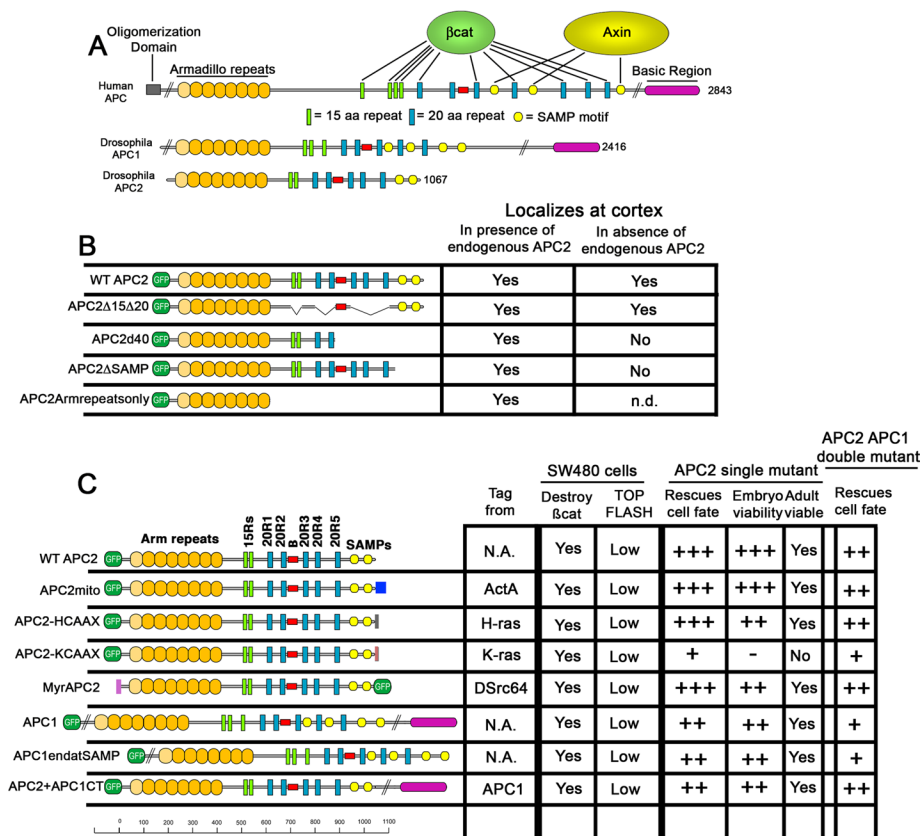


FIGURE 1: Diagrams of wild-type APC2 and the mutants used in both the localization studies and the functional analyses, and a summary of the functions of each mutant. Scale is in amino acids. (A) Both flies and mammals have two APC family members that share a core, including the highly conserved Arm repeats, as well as 15- and 20-amino acid repeats 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. Diagrams of mutants (B) or localization variants (C) used, and summaries of results of our cortical localization studies in *Drosophila* (B) and functional tests in SW480 cells and in *Drosophila* (C), as detailed in subsequent figures and Table 1. N.A., not applicable; n.d., not done.

mutations in the destruction complex proteins adenomatous polyposis coli (APC) or Axin, plays an important role (Polakis, 2007). APC mutations occur in >80% of all colon cancers, and thus APC's mechanistic roles in Wnt signaling are of significant interest.

APC is a multidomain protein regulating both Wnt signaling and the cytoskeleton (McCartney and Nathke, 2008; Bahmanyar et al., 2009; Figure 1A). N-terminal in APC family proteins is an Arm-repeat domain (so-called because it was first found in β cat/Arm), a protein interaction domain known to bind several partners, most with cytoskeletal functions, and also critical for regulating Wnt signaling, perhaps through an as yet unidentified protein partner. APC's middle region carries multiple binding sites for its destruction complex partner Axin (the Ser-Ala-Met-Pro [SAMP] repeats) and for β cat (the 15- and 20-aa repeats). C-terminal in mammalian APC is a region interacting directly and indirectly with microtubules (MTs). However, this latter region, while likely important for cytoskeletal regulation, is not essential for Wnt regulation, as mouse mutants lacking this region are adult-viable and not tumor-prone (Smits et al., 1999). Further, fly APC2, which can regulate Wnt signaling, ends after the SAMP motifs, thus lacking the direct MT-interacting domains (Figure 1A).

Despite 15 yr of work, key questions remain about APC's mechanistic role in Wnt regulation. APC is essential for effectively targeting

β cat for phosphorylation and destruction, but its role in the destruction complex is not clear. It was initially assumed to be the scaffold for presenting β cat to the kinases CK1 and GSK3 (Rubinfeld et al., 1996), but now it is clear that Axin plays this role (Ha et al., 2004). Another model, which we have favored (McCartney et al., 2006), suggests that APC binds the destruction complex and, via other protein interactions, localizes it to the correct place in the cell. However, it remains unclear where within the cell the destruction complex normally operates.

Mammalian APC has a complex subcellular localization mediated, at least in part, by cytoskeletal interactions. In isolated cultured cells it localizes to the cortex, particularly in cell protrusions, at which it clusters at ends of MTs (Näthke et al., 1996). It also can be transported along MTs and associate with MT plus ends (Mimori-Kiyosue et al., 2000). In epithelial cells and tissues, APC localization is more controversial, but most studies suggest at least a pool localizes to cell-cell junctions or the basolateral cell cortex (e.g., Langford et al., 2006a,b; Grohmann et al., 2007; Hendriksen et al., 2008; Maher et al., 2009). Fly APC2 localizes to the cell cortex (McCartney et al., 1999; Yu et al., 1999). The Arm repeats and C-terminal end of APC2 (McCartney et al., 2006; Zhou et al., 2011) are important for this cortical localization, but the mechanisms of cortical localization of APC family proteins remain mysterious.

This led to the hypothesis that APC localizes the destruction complex to the cell cortex, thus facilitating its function. A cortical location would put it in proximity to the Wnt receptors (Hendriksen et al., 2008), allowing rapid down-modulation after Wnt signaling. Both fly APC2 (McCartney et al., 1999) and human APC (Näthke et al., 1996) accumulate, at least in part, at the cell cortex. Consistent with the idea that cortical localization is essential for Wnt regulation, missense APC2 alleles exhibit a strong correlation between loss of cortical localization and loss of function in Wnt regulation (McCartney et al., 2006), and one, APC2^{Δ5}, is temperature-sensitive in phenotype and localization to the cell cortex (McCartney et al., 1999). However, other data mitigate against this model. While the two fly APC family members, APC1 and APC2, are redundant for Wnt regulation in many tissues (Ahmed et al., 2002; Akong et al., 2002a), their predominant intracellular localizations are distinct. APC2 is cortical, but APC1 primarily localizes to axons in neurons and to centrosomes and associated MTs in male germ line stem cells (Yamashita et al., 2003) or when overexpressed in the ectoderm or neuroblasts (Akong et al., 2002a,b). This calls the localization model into question. However, each APC can recruit the other to its "favorite location" when overexpressed (Akong et al., 2002a,b), and APC1 and APC2 interact in a two-hybrid assay (Mattie et al., 2010), raising the possibility that APC1 is recruited to the cortex at a low, but still functional, level. In this work, we test the localization model directly by altering APC2 localization and evaluating effects on its function.

In addition to suggesting a role for APC in the destruction complex, most reviews of Wnt signaling propose that APC also acts in nuclei in β cat regulation (Brocardo and Henderson, 2008; Neufeld, 2009). One model suggests that APC shuttles in and out of nuclei, exporting β cat from the nucleus to inactivate it (Bienz, 2002). Consistent with this, APC proteins have nuclear localization (NLS) and nuclear export signals (NES) and accumulate in nuclei after nuclear export is blocked by leptomycin B treatment (Henderson, 2000; Neufeld *et al.*, 2000; Rosin-Arbesfeld *et al.*, 2003). APC also can physically associate with Wnt target genes (Sierra *et al.*, 2006), raising the possibility that it plays a direct role in repressing Wnt target genes. Finally, APC can bind the transcriptional repressor C-terminal binding protein (CtBP; Hamada and Bienz, 2004; Schneikert *et al.*, 2011). While most functional tests of these nuclear roles have been indirect, involving misexpression of truncated proteins rather than genetic tests *in vivo*, Tolwinski (2009) found that membrane-tethered, myristylated APC2, which should be prevented from entering nuclei, cannot rescue Wnt regulation, consistent with an essential nuclear role. However, that study only used a single membrane-tethered version of APC2, and it is possible that the N-terminal tag inactivated protein function, leaving open the possibility that nuclear localization is not essential.

We thus set out to resolve whether APC plays a key role in targeting the destruction complex to a critical subcellular position, and whether APC needs to enter the nucleus to regulate Wnt signaling. We tested these hypotheses in both cultured colon cancer cells and the fruit fly *Drosophila*, allowing us to determine whether any such roles are conserved or might be divergent between flies and mammals.

RESULTS

Defining requirements for APC2 cortical localization

As outlined above, several models for APC function suggest its localization plays an important role in Wnt regulation, either because it localizes the destruction complex to the “right place” or because it shuttles in and out of nuclei in performing its functions. We thus began by defining domains of fly APC2 required for cortical localization, using a series of mutants in which particular protein–protein interaction sites had been deleted (Figure 1B; Roberts *et al.*, 2011). Wild-type APC2 localizes to the cell cortex of embryonic cells in a punctate manner (McCartney *et al.*, 1999; Yu *et al.*, 1999), and this localization is reduced or abolished in mutants that have missense changes in the Arm repeats or that are truncated after 20–amino acid repeat 2 (McCartney *et al.*, 2006). APC2–green fluorescent protein (GFP) localizes in a manner similar to wild-type APC2, both in the presence (unpublished data) and absence of endogenous APC2 (Figure 2A; anti-phosphotyrosine marks cell–cell junctions). We used a series of mutant forms of APC2 lacking particular protein interaction domains to determine which are essential for cortical localization. We assessed localization in the presence or absence of wild-type APC2, as endogenous APC2 coimmunoprecipitates with GFP-tagged APC2 (Figure 2I, lane 2; Zhou *et al.*, 2011), suggesting that APC2 can oligomerize.

We first tested an APC2 mutant lacking all Arm/ β cat binding sites (the 15- and 20-aa repeats; Figure 1B). This mutant, APC2 Δ 15 Δ 20, continued to localize to the cortex both in the presence (Figure 2B) and absence of endogenous APC2 (Figure 2C). Thus binding to β cat is not required for cortical localization. We next examined a truncated APC2 mutant, APC2^{d40}, in which a premature stop codon leads to a protein similar to the truncated APC proteins expressed in human colon tumors, which end in the “mutation cluster region” in the middle of APC’s central domain. APC2^{d40} ends after 20–amino

acid repeat 2 (Figure 1B). This mutant protein failed to localize to the cortex (McCartney *et al.*, 2006). We generated a GFP-tagged transgenic form of APC2^{d40}, which allowed us to assess its localization both in the presence and absence of wild-type endogenous APC2. As expected, it failed to localize to the cortex in the absence of endogenous APC2 (Figure 2E), but somewhat surprisingly, it localized to the cortex in a wild-type background (Figure 2D). We also examined APC2 Δ SAMP, which lacks both the Axin-binding sites (the SAMP motifs; Figure 1B) and the C-terminal 30 amino acids of APC2, the recently named C30 region (Zhou *et al.*, 2011). Like APC2^{d40}, APC2 Δ SAMP failed to localize to the cortex in the absence of endogenous APC2 (Figure 2G) but localized there in the wild-type background (Figure 2F). These data, together with previous studies (McCartney *et al.*, 2006; Zhou *et al.*, 2011), suggest the Arm repeats and C-terminal region, including the SAMPs and the C-terminal-most 30 amino acids, are each important for APC2 to localize to the cortex, as assessed in the absence of endogenous APC2, while the β cat-binding sites are dispensable. Further, since APC2 Δ 15 Δ 20 is not functional in Wnt regulation (Roberts *et al.*, 2011), cortical localization alone is not sufficient for Wnt regulation.

Strikingly, however, all the constructs retained at least some cortical localization in the presence of endogenous APC2. To further define the region sufficient for localization in the presence of wild-type APC2, we created a truncated form of APC2 encoding only the N-terminal Arm repeats (Figure 1B). Strikingly, this construct retained detectable cortical localization in the wild-type background (Figure 2H). The simplest explanation for this is that APC2^{Armrepeats} only associates with wild-type APC2. To test this hypothesis, we attempted to coimmunoprecipitate the two proteins. Endogenous APC2 coimmunoprecipitated with GFP-APC2^{Armrepeats} only (Figure 2I, lane 4), but not with GFP-APC2 Δ Armrepeats (Figure 2I, lane 3). The 15– and 20–amino acid repeats were also not essential for coimmunoprecipitation (coIP; Figure 2I, lane 5). Similar self-association mediated by the Arm repeats was recently reported by Zhou *et al.* (2011). These data suggest that the Arm repeats plus the short sequence N-terminal to them are necessary and sufficient for APC2 self-association, and can be recruited to the cortex by associating with wild-type APC2.

Approach and model systems to test importance of APC2 localization for function

To evaluate the role of APC localization in destruction complex function, and to test the hypotheses that APC proteins have nuclear roles or that they localize the destruction complex to the “correct” location, we generated modified versions of *Drosophila* APC2, designed to target the protein to distinct subcellular locations (Figure 1C). We then tested these in two parallel model systems: *Drosophila* embryogenesis and human colon cancer cells.

In *Drosophila* embryos we can completely eliminate function of both APC family members. Each localization mutant was GFP-tagged and expressed under the control of the endogenous APC2 promoter. Wild-type GFP-tagged APC2 rescued APC2 single mutants to viability and fertility and rescued Wnt signaling defects and embryonic lethality of APC2 APC1 maternal and zygotic double mutants (Roberts *et al.*, 2011), providing a baseline for our analysis. APC2 single and APC2 APC1 double mutant backgrounds provided sensitive tests for mutants with different degrees of residual function—the low levels of wild-type APC1 in APC2 single mutants (Ahmed *et al.*, 2002; Akong *et al.*, 2002a) allowed even partially functional APC2 proteins to provide detectable rescue, whereas double mutants required a fully or almost fully functional protein to provide rescue (McCartney *et al.*, 2006).

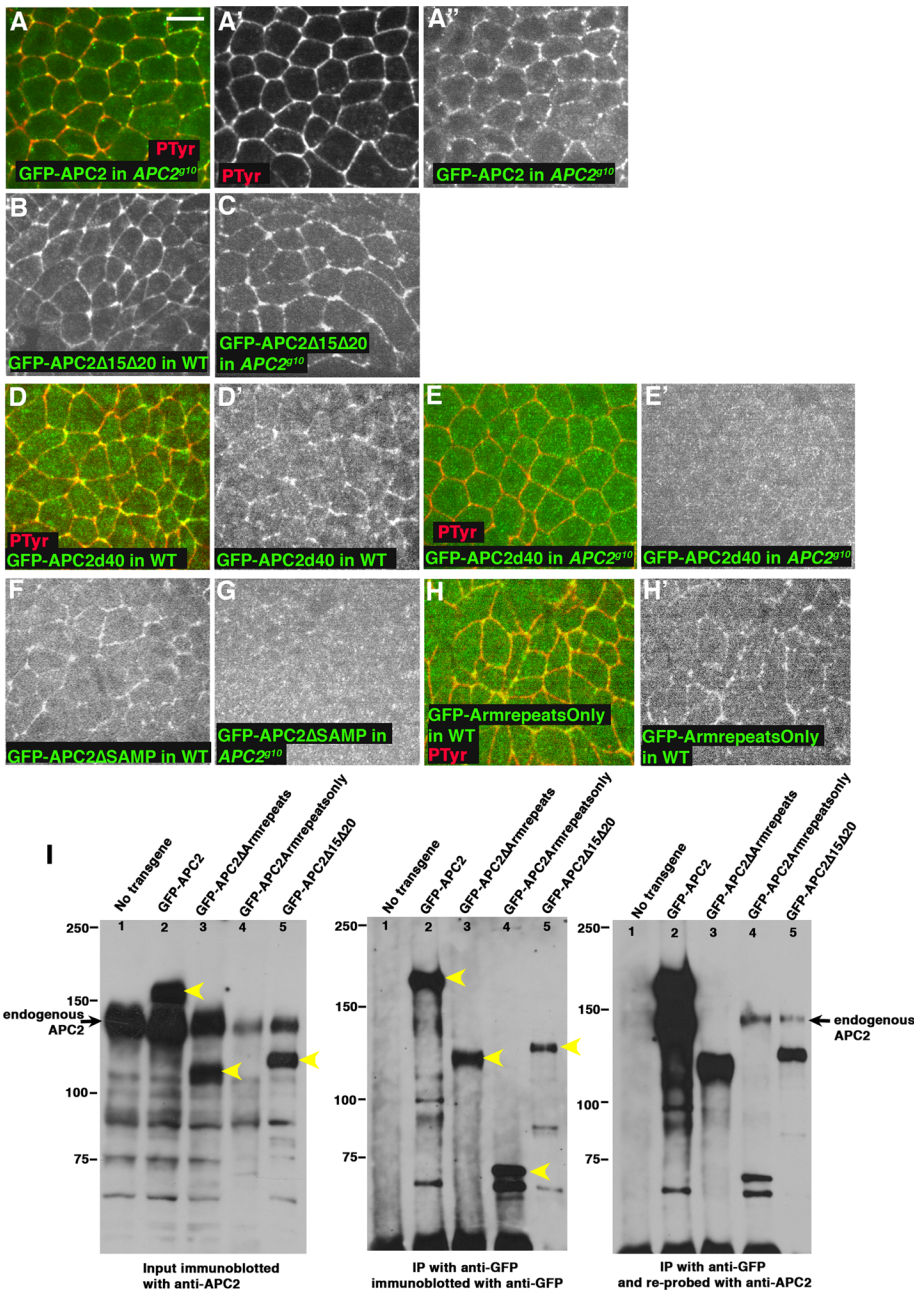


FIGURE 2: Defining determinants for APC2 cortical localization. (A–H) Images from the ectoderm of stage 9/10 wild-type or *APC2²⁰¹⁰* maternal/zygotic mutant embryos expressing the transgene indicated. Scale bar: 5 μ m. Anti-phosphotyrosine was used in some cases to mark cell outlines, and APC2 mutant proteins were visualized using the GFP tag. (A) GFP-APC2 localizes in a punctate pattern to the cell cortex in the absence of endogenous APC2; this localization resembles that of endogenous APC2 (McCartney *et al.*, 1999). Deleting all of the Arm/ β cat-binding sites (*APC2 Δ 15 Δ 20*) does not disrupt cortical localization in either the presence (B) or absence (C) of endogenous APC2. Truncation of APC2, producing a protein similar to that found in human colon tumors (*APC2d40*), does not eliminate cortical localization in the presence of endogenous APC2 (D) but does reduce or eliminate cortical localization in its absence (E). Deletion of the Axin-binding SAMP motifs and the C-terminal 30 amino acids of APC2 does not block cortical localization in the presence of endogenous APC2 (F) but does reduce or eliminate cortical localization in its absence (G). (H) A protein including only the APC2 Arm repeats can localize to the cortex in the presence of endogenous APC2. (I) The Arm repeats are necessary and sufficient for APC2 self-association. Embryonic extracts were prepared from transgenic flies expressing each of the constructs indicated, and immunoprecipitated with anti-GFP antibody. In the left blot, total cell extracts were analyzed by SDS–PAGE and immunoblotted with anti-APC2. Endogenous APC2 is found in all extracts (arrow). Arrowheads indicate the GFP-tagged wild-type or mutant versions of APC2. *APC2Armrepeatsonly* lacks the epitope used to raise the

In parallel, we tested each localization mutant in the human colon cancer cell line SW480. These cells carry a truncated allele of human APC, and as a result accumulate very high levels of β cat, which becomes enriched in nuclei and drives expression of Wnt-responsive target genes (Munemitsu *et al.*, 1995). Activation of Wnt-responsive genes in these cells is easily assessed via the well-characterized TOPflash assay (Korinek *et al.*, 1997). These human cells provide two additional advantages—they allow us to test phylogenetic conservation of mechanisms we are assessing, and they allow rapid and quantitative assessment of effects on β cat levels and TCF-regulated transcription.

The localization tags each effectively alter APC2 localization in *Drosophila*

One set of models for APC function suggests that it either shuttles β cat out of nuclei or acts directly at promoters of Wnt target genes in repression. Another model hypothesizes that APC localizes the destruction complex to a particular intracellular localization, such as the plasma membrane. We tested these hypotheses by creating a set of GFP-tagged full-length APC2 variants carrying different localization signals, designed to relocate APC2 to distinct intracellular locations (Figure 1C and Table 1), all of which should also prevent nuclear entry. To circumvent possible limitations in the ability of each tag to quantitatively relocate APC2, we used four different localization signals: 1) The C-terminal mitochondrial transmembrane anchor sequence of *Listeria ActA* (Pistor *et al.*, 1994), which we previously used to effectively target Enabled protein to *Drosophila* mitochondria (Gates *et al.*, 2007). 2) The C-terminal CAAX motif of H-ras, which in *Drosophila* appears to target largely to internal membranes (Gates *et al.*, 2007). 3) The C-terminal CAAX sequence of K-ras, which in *Drosophila* targets to both the

antibody and thus are not visualized. In the center and right blots, anti-GFP IPs were analyzed by SDS–PAGE and sequential immunoblotting with anti-GFP (center) and with anti-APC2 (right). Endogenous APC2 robustly coimmunoprecipitates with wild-type GFP-APC2 (lane 2), clearly coimmunoprecipitates with *APC2Armrepeatsonly* (lane 4) and *APC2 Δ 15 Δ 20* (lane 5), but does not detectably coimmunoprecipitate with *APC2 Δ Armrepeats* (lane 3) and is not immunoprecipitated from the negative control extract lacking a GFP-tagged transgene (lane 1).

Construct	Localization tag sequence	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 construct	N/A	96% (n = 398)	3.60 (n = 200)	55% (n = 284)	4.97 (n = 200)
Full-length APC2	N/A	8% (n = 414)	0.05 (n = 67)	1% (n = 357)	0.09 (n = 34)
APC2-HCAAX	H G C M S C K C V L S*	34% (n = 305)	0.10 (n = 33)	41% (n = 555)	0.91 (n = 308)
APC2-KCAAX	S K D G K K K K K K S K T K C V I M*	100% (n = 319)	1.92 (n = 209)	54% (n = 373)	2.62 (n = 127)
APC2mito	H T T L I L A M L A I G V F S L G A F I K I I Q L R K N N*	14% (n = 369)	0.13 (n = 91)	19% (n = 328)	0.24 (n = 30)
MyrAPC2	N-terminal-M G N K C C S K R Q G T M A G N I G L V I G A S	23% (n = 520)	0.08 (n = 178)	33% (n = 560)	0.66 (n = 166)
APC1	N/A	31% (n = 491)	0.69 (n = 161)	48% (n = 590)	3.04 (n = 110)
APC1endatSAMPs	Amino acids 1–1457 of APC1	28% (n = 316)	0.46 (n = 69)	33% (n = 315)	1.41 (n = 107)
APC2+APC1CT	Amino acids 1458– 2417 of APC1	21% (n = 242)	0.37 (n = 54)	24% (n = 329)	0.02 (n = 200)
APC2+3xNLS	3 × D P K K K R KV	ND	ND	ND	ND

*, stop codon.

TABLE 1: Localization constructs and rescue of Wnt signaling defects.

plasma membrane and internal membranes. We previously used this to effectively target Arm to the plasma membrane in *Drosophila* and prevent it from acting in nuclei (Cox *et al.*, 1999). 4) the N-terminal myristoylation sequence of fly Src64, which was previously used to effectively target Arm to the plasma membrane and thus prevent it from carrying out its nuclear functions (Zecca *et al.*, 1996; Tolwinski and Wieschaus, 2004). All localization-tagged APC2 constructs produced stable proteins of the appropriate size, as assessed by immunofluorescence and immunoblotting (see below).

We first assessed whether these targeting sequences altered APC localization in the expected way in *Drosophila* and in mammalian cells. We began by expressing our localization variants under GAL4:UAS control in the large cells of the *Drosophila* female germ line—their size facilitates assessing subcellular localization in detail, and these cells do not require Wnt signaling, thus simplifying things. Wild-type GFP-APC2 localizes to the cortex of both nurse cells and the oocyte (Figure 3, A and B, arrows), as previously observed for endogenous APC2 (McCartney *et al.*, 1999). In addition, GFP-APC2 also localized to the robust bundled actin filaments that are assembled in nurse cells during late oogenesis (Figure 3B, arrowhead), consistent with a role for actin in mediating APC2 cortical localization in other cell types (Townsend and Bienz, 2000).

Each localization tag effectively relocalized GFP-APC2 in *Drosophila* germ line cells, dramatically changing its localization, or in the case of tags targeting APC2 to the cortex, eliminating enrichment on nurse cell actin filaments. APC2mito no longer localized to the cortex (Figure 3, C and D, arrows) or to nurse cell actin filaments (Figure 3D)—instead it was relocalized to punctate cytoplasmic structures (Figure 3, C and D, arrowheads) that may be mitochondria. APC2-HCAAX also localized to large internal structures we suspect are vesicular in nature (Figure 3, E and F, arrowheads), as well as to smaller putative vesicles aligned along the cortex (Figure 3, E and F, arrows and insets). It did not localize to nurse cell actin fila-

ments (Figure 3F). APC2-KCAAX localized to the nurse cell cortex (Figure 3, G and H, arrows), thus overlapping wild-type APC2, but did not accumulate on nurse cell actin filaments (Figure 3H, arrowhead), suggesting it was effectively retained at the cortex by the CAAX tag. It also localized at low levels in punctate structures accumulating in oocytes (Figure 3G, arrowhead), which may be small vesicles. Finally, MyrAPC2 localized both to the cell cortex (Figure 3, I and J, arrows) and to large internal structures (Figure 3I, arrowhead) we suspect are vesicles. While these punctate structures sometimes aligned along actin filaments (Figure 3J, arrowhead and inset), MyrAPC2 did not otherwise colocalize with nurse cell actin filaments. Together, these data support the idea that the localization tags we added effectively relocalized most or all APC2.

We also tested whether these localization tags altered APC2 localization at two stages of embryogenesis. Once again, each had distinct effects on APC2 localization. In syncytial stage embryos, GFP-APC2 localized to the apical membrane (Supplemental Figure S1A, arrow) and to transient membrane furrows that invaginate to surround each mitotic spindle (Figure S1A, arrowhead), similar to endogenous APC2 (McCartney *et al.*, 2001). There also was a detectable cytoplasmic pool of GFP-APC2. APC2-KCAAX localized to invaginating membranes, especially at the invagination front (Figure S1B, arrow), and also surrounded large, internal, vesicle-like structures. APC2-HCAAX primarily localized to internal presumptive vesicles (Figure S1C, arrow), with some enrichment at tips of invaginating membranes (Figure S1C, arrowhead). MyrAPC2, like wild-type APC2, localized to the apical cortex and invaginating membranes (Figure S1D, arrows) and also localized to punctate putative vesicles in the internal cytoplasm. Finally, APC2mito localized exclusively to presumptive membrane-bound structures in the internal cytoplasm (Figure S1E, arrow). During extended germ band stages, when Wnt signaling regulates cell fates in the epidermis, the tags continued to alter APC2 localization. GFP-APC2, like

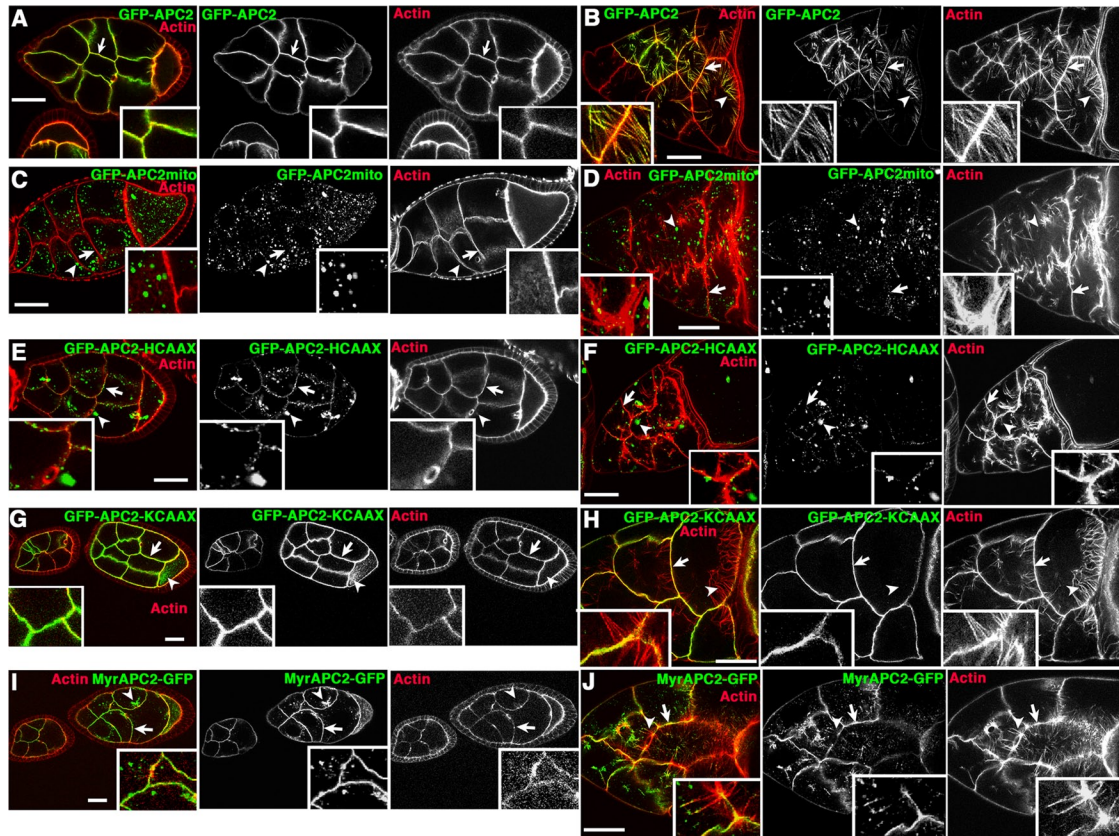


FIGURE 3: Our localization tags all effectively alter APC2 localization in *Drosophila*. Ovarian egg chambers, anterior left, with genotypes and antigens indicated. (A, C, E, G, I) Stages 6–8; (B, D, F, H, and J) stages 10b–11. All APC2 constructs were expressed in the germ line using an ovarian-specific GAL4 driver and visualized using their GFP tags. (A) Wild-type GFP-APC2 localizes to the cortex of nurse cells and the oocyte (arrow). (B) During late stages of oogenesis, robust actin filaments assemble in nurse cells and GFP-APC2 localizes to them (arrowhead and inset) as well as to the cell cortex (arrow). (C and D) In contrast, GFP-APC2mito is relocalized to punctate presumptive vesicular structures in the nurse cell (arrowheads) and oocyte cytoplasm and does not localize to the nurse cell cortex (arrows) or colocalize with actin filaments (inset). (E and F) GFP-APC2-HCAAX is relocalized to large and small presumptive vesicles in the nurse cell (arrowheads) and oocyte cytoplasm—some of these align along the cortex (E, inset) or nurse cell actin filaments (F, inset) while others do not. (G and H) GFP-APC2-KCAAX localizes to the plasma membrane of the nurse cells and oocyte (arrows). Unlike wild-type APC2, GFP-APC2-KCAAX also localizes to a cloud of small, presumptive vesicles in the oocyte (G, arrowhead) and does not localize to nurse cell actin filaments (H, arrowhead and inset). (I and J) MyrAPC2-GFP localizes to both the nurse cell (arrows) and oocyte plasma membrane and also to small cytoplasmic presumptive vesicles (I, arrowhead and inset), some of which coalign with nurse cell actin filaments (J, inset). Scale bars: 25 μ m.

endogenous APC2 (McCartney *et al.*, 1999; Yu *et al.*, 1999), is a peripheral membrane protein. It is not uniform at the cortex, but instead is punctate along the cortex and particularly enriched at tricellular junctions (Figure S1, F and F, close-up). Both APC2-HCAAX (Figure S1J) and APC2mito (Figure S1I) were dramatically different from endogenous APC2, as they localized to internal presumptive vesicles. While APC2-KCAAX (Figure S1G) and MyrAPC2 (Figure S1H) targeted to the plasma membrane, their localization was distinct from wild-type APC2 in that they were much more continuous along the membrane (Figure S1, G and H, close-ups), lacking pronounced enrichment at tricellular junctions, which is consistent with their being directly associated with the membrane via the lipid tags. Thus all of our tags effectively relocalize APC2 in *Drosophila*.

The localization tags also alter APC2 localization in human SW480 colorectal cancer cells

We next examined how the localization tags affected APC2 localization in the cultured human colon cancer cell line SW480 (all

were GFP-tagged to facilitate localization; Figure 1C). Immunoblotting revealed that each produced a GFP-tagged protein of proper size (Figure S2A; differences in transfection efficiency mean not all accumulated to identical levels). In SW480 cells, GFP-APC2 localized uniformly to the cytoplasm and was largely excluded from the nucleus (Figure 4B'; Roberts *et al.*, 2011). APC2mito was effectively relocalized to large perinuclear vesicular structures (Figure 4C'). We confirmed these are mitochondria by costaining with an antibody to a known mitochondrial protein, ATP synthase (Figure 4J), and confirmed effectiveness of mitochondrial tethering by fractionating cells. APC2mito cofractionated into mitochondria with ATP synthase, and was effectively removed from the cytoplasm, for which tubulin was a control (Figure 4K). We did not see detectable amounts of a cleaved product that might have lost the mitochondrial localization signal. Both APC2-KCAAX and APC2-HCAAX relocalized to small vesicular structures inside cells (Figure 4, D' and E'). APC2-HCAAX overlapped in localization with the Golgi protein GM130 (Figure S2B), while APC2-KCAAX localized to distinct vesicles (Figure S2C). MyrAPC2, in contrast,

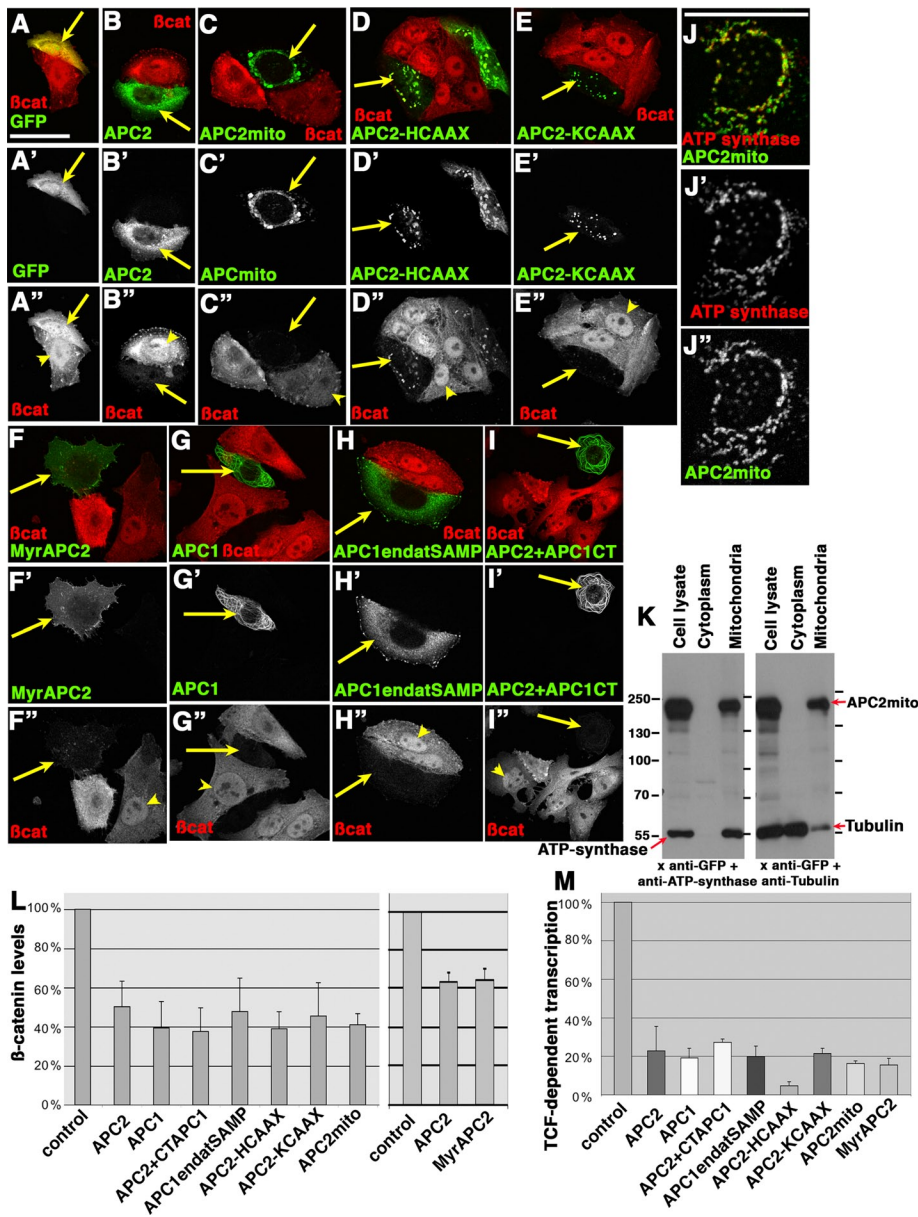


FIGURE 4: APC2 can restore β cat destruction and reduce Wnt-responsive gene expression from a variety of intracellular locations. (A–J) SW480 cells transfected with the indicated GFP-tagged constructs. GFP (green) and β cat (red). Transfected cells are indicated with arrows, and nuclei of transfected or nontransfected cells are indicated with arrowheads. (A) β cat levels are high, and it is enriched in nuclei of both untransfected SW480 cells and those transfected with GFP alone. (B) Wild-type APC2 is diffuse in the cytoplasm, and strongly reduces β cat levels. (C and J) APC2mito localizes to perinuclear structures (C) that also are labeled with the mitochondrial marker ATP-synthase (J). It strongly reduces β cat levels. Both APC2-HCCAX (D) and APC2-KCAAX (E) localize to punctate vesicular structures, and both strongly reduce β cat levels (some residual β cat colocalizes with APC2-HCCAX). (F) MyrAPC2 appears to localize diffusely to the plasma membrane and effectively reduces β cat levels. (G) APC1 localizes to MTs, and effectively reduces β cat levels. (H) Deletion of the C-terminal domain of APC1 results in a more diffuse cytoplasmic localization, but it still effectively reduces β cat levels. (I) Addition of APC1's C-terminal domain to APC2 relocates it to MTs, but it can still reduce β cat levels. (K) APC2mito is effectively relocated to mitochondria. Immunoblot of cell fractionation. Lanes are initial cell lysate, cytoplasmic fraction, and mitochondrial fraction. Anti-GFP recognizes GFP-APC2mito, ATP-synthase is a mitochondrial protein, and tubulin is a control cytoplasmic protein. (L) All constructs reduce β cat levels, regardless of their localization. β cat levels as quantified by Cellomics. (M) All constructs reduce expression of the Wnt-regulated reporter gene, TOPflash. (A–I) Scale bar (in panel A): 50 μ m; (J) scale bar: 50 μ m.

appeared to localize all over the plasma membrane (Figure 4F'). Thus all localization tags also effectively relocated APC2 in human cells.

In SW480 cells, we also explored localization of the other *Drosophila* APC family member, APC1. Like fly APC2, it can effectively down-regulate Wnt signaling (Hayashi *et al.*, 1997; Ahmed *et al.*, 1998, 2002; Akong *et al.*, 2002a). In vivo, APC1 localized to axons of the CNS (Hayashi *et al.*, 1997), while when overexpressed in either the embryonic epidermis or in larval neural stem cells, it localized to centrosomes and associated MTs (Akong *et al.*, 2002b). In SW480 cells, APC1-GFP decorated MTs (Figure 4G'). APC1 is significantly longer than APC2, and this C-terminal extension is both necessary for MT localization (Figure 4H'; APC1endat-SAMP), and sufficient, when added to full-length APC2 (Figure 4I'; APC2+APC1CT), to confer predominant localization to MTs.

APC tethered at a variety of cytoplasmic locations can negatively regulate β cat levels and TCF-dependent transcription in SW480 cells

These localization constructs allowed us to test two leading models for APC2 function. Our data given above suggest that each of our membrane tags effectively relocates APC2 to particular membrane compartments and thus should prevent APC2 from translocating to nuclei, thereby testing whether this is essential to regulation of Wnt signaling. The use of four different membrane tags, including one incorporating a transmembrane sequence (APC2mito), further strengthens this test. Second, if APC2 is essential for localizing the destruction complex to a particular subcellular location, then at most a subset should rescue Wnt regulation.

We first tested whether these localization mutants restored Wnt regulation in human SW480 cells. We transfected each localization mutant into these cells and assessed ability to reduce elevated levels of β cat protein, both by immunofluorescence of individual transfected cells (Figure 4, A–I) and by using an automated microscope to directly quantitate β cat levels in transfected cells versus untransfected neighbors on the same slide (Figure 4L). Strikingly, despite being relocated to distinct cytoplasmic locations and prevented from entering nuclei, all four localization mutants were fully functional at reducing β cat levels (Figure 4, C'–F' and L); in this they were as effective as human APC (Munemitsu *et al.*, 1995; Roberts *et al.*, 2011) or as *Drosophila* APC2 without a localization tag (Figure 4, B' and L; Roberts

et al., 2011). Thus APC2's ability to modulate β cat destruction in SW480 cells can occur at a variety of cellular locations.

Given previous reports suggesting that APC can act at promoters to assist in repressing Wnt-responsive genes (Sierra et al., 2006), we also tested the ability of each localization mutant to down-regulate Wnt-regulated transcription, using the TOPflash assay. All four localization mutants down-regulated TOPflash to the same degree as human APC or wild-type *Drosophila* APC2 (Roberts et al., 2011; Figure 4M). Together, these data suggest that in APC mutant SW480 cells, APC proteins that are tethered in the cytoplasm can rescue defects in Wnt regulation.

We also assessed the Wnt-regulatory abilities of full-length APC1, an APC1 mutant lacking its C-terminal MT-binding domain (APC1endat SAMP), and a fusion of the C-terminal MT-binding region of APC1 to full-length APC2 (APC2+APC1CT). The C-terminal region of APC1 was both necessary and sufficient for targeting to MTs (Figure 4, G–I). However, neither recruitment to nor lack of recruitment to MTs affected the ability to down-regulate β cat levels (Figure 4, G'–I' and L) or TCF-regulated transcription, as assessed by TOPflash (Figure 4M).

Taken together, this ability of APC proteins localized to diverse intracellular locations to each fully rescue Wnt regulation argues against our previous hypothesis that APC localizes the destruction complex to a special site that is essential for its function and suggests the ability to translocate into the nucleus may not be essential. However, since SW480 cells retain both truncated endogenous APC and a wild-type copy of human APC2, it remains possible that these could complement a function of APC not provided by our localization mutants. Further, the TOPflash reporter, because it is introduced by transfection, may not have a chromatin conformation that matches that of endogenous Wnt target genes. We thus extended these studies to transgenic *Drosophila*, where we could assess function in animals completely lacking APC family proteins, and where we could examine biological effects on cell fate choices that integrate effects on multiple endogenous target genes.

Mito-tethered APC2 fully rescues defects in regulation of Wnt signaling and embryonic lethality of *Drosophila* APC2 mutants

In *Drosophila* we can assess function in animals null mutant for APC2, or in double mutant animals completely lacking both fly APC proteins. Assessing constructs in both single and double mutant backgrounds also provided the ability to assess fine-scale differences in rescuing ability, as rescue of the double mutant requires a nearly completely functional APC2 protein (McCartney et al., 2006; Roberts et al., 2011). We focused on the embryonic epidermis, as the embryonic cuticle pattern provides an exceptionally sensitive assay of cell fate choices. Wild-type anterior cells in each segment secrete cuticle with hair-like protrusions called denticles (Figure 5A, arrows), while posterior cells secrete "naked cuticle" without hairs (Figure 5A, arrowheads). Loss of maternal and zygotic APC2 leads to fully penetrant embryonic lethality. Wnt signaling is strongly activated, with most cells in the embryonic epidermis taking on posterior fates and secreting only naked cuticle, although occasional denticles are present due to residual function of fly APC1 (McCartney et al., 1999; Figure 5, B, arrows, and M, and Table 1 show quantitation of Wnt signaling defects in different mutants). These cell fate defects are effectively rescued by wild-type GFP-APC2 (Figure 5, C and M; Roberts et al., 2011).

We tested each of our tethered constructs for their ability to rescue APC2 null mutants. Strikingly, APC2mito, with a C-terminal

membrane-spanning sequence from ActA, which localized APC2mito to internal vesicles in ovaries, embryos, and SW480 cells (Figures 2, S1, and S2) and conferred very reliable membrane tethering in cultured cells (Figure 4K), fully rescued Wnt-regulated cell fates in the epidermis (Figure 5, D and M). It also rescued APC2-null mutants to virtually full embryonic viability (86% viable vs. 92% for wild-type untethered GFP-APC2). In fact, APC2-null mutants expressing only APC2mito lived to adulthood (Figure 5K). Both APC2-HCAAX and MyrAPC2 also retained substantial rescue ability, with strong rescue of embryonic viability (66 and 77% viable, respectively); some rescued mutants survived to adulthood (Figure 5L), and both localization variants essentially completely rescued epidermal cell fates (Figure 5, E, G, and M). Together, these data strongly suggest that variants of APC2 that are tethered in the cytoplasm can retain substantial ability to regulate Wnt signaling. They also suggest localization to the plasma membrane is not essential for APC2 function.

Surprisingly, one variant that conferred largely plasma membrane localization in embryos (Figure S1), APC2-KCAAX, rescued significantly less well, though it did retain detectable ability to restore Wnt regulation. Embryonic viability was not restored (Figure 5C vs. 5F). However, APC2-KCAAX did possess some ability to rescue cell fates, though it was reduced (Figure 5, F and M); denticle bands reappeared (Figure 5F, arrows) but were often interrupted by naked cuticle (Figure 5F, arrowheads). Given the lower ability of APC2-KCAAX to rescue APC2 function, we examined whether this was due to substantially lower accumulation levels of the APC2-KCAAX protein. However, immunoblotting revealed that this protein accumulated at levels roughly comparable to that of wild-type APC2 (Figure 5N; both are GFP-tagged, making them run more slowly than endogenous APC2). APC2-KCAAX could also fail to rescue for two other reasons: 1) the localization it confers may be incompatible with full APC function, or 2) the localization tag itself might interfere with protein function for reasons not due to its effects on localization. We think the latter is less likely to be the sole reason in this case, as the C-terminal HCAAX tag did not impair APC2 function. Thus these data are consistent with the hypothesis that the KCAAX tag may localize APC2 to a place at which it is not fully functional.

Mito-tethered APC2 also rescues defects in regulation of Arm levels, cell fates, and embryonic lethality in the complete absence of APC family proteins

Drosophila APC2 and APC1 are partially redundant. In the embryonic epidermis, APC2 plays the primary role, but low levels of APC1 provide some residual function (Ahmed et al., 2002; Akong et al., 2002a). Because we have null mutations in both fly family members, we could test function of our tethered variants in the absence of both APC family proteins, using maternal and zygotic APC2 APC1 double mutants. APC2 APC1 maternal and zygotic double-null mutants are embryonic lethal (the 45% of embryonic progeny that live received wild-type APC2 and APC1 from their heterozygous fathers), with complete transformation of all surviving epidermal cells to posterior fates and thus no remaining denticles (Figure 6A) and highly elevated levels of Arm (fly β cat; Figure 7A vs. 7B) (Ahmed et al., 2002; Akong et al., 2002a). Wild-type GFP-APC2 effectively rescued embryonic viability (99% viable; rescue data from Roberts et al., 2011); restored embryonic cell fates, with alternating bands of denticles and naked cuticle (Figure 6B); and restored targeting of Arm for destruction in cells not receiving Wnt signals (Figure 7C, arrowheads).

Even in this background devoid of all APC function, APC2mito provided strong ability to rescue Wnt regulation and Arm

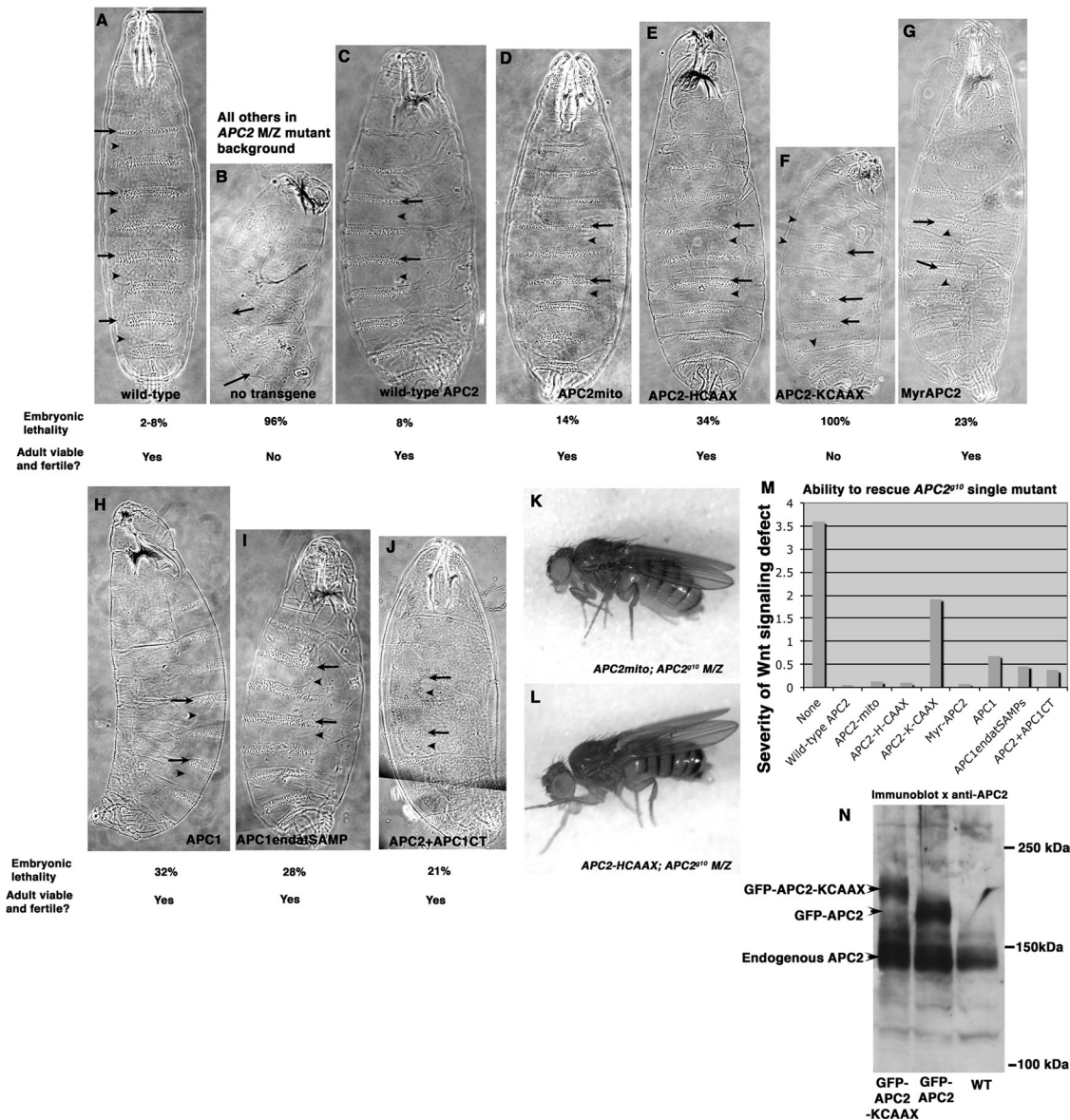


FIGURE 5: APC2 tethered at diverse cytoplasmic locations can rescue the Wnt signaling defects of APC2 single mutants. (A–J) Degree of rescue of Wnt-mediated cell fates by different tethered forms of APC2, as assessed by examining the embryonic cuticle. All except (A) are in the *APC2^{g10}* maternal/zygotic mutant background, and the transgene present is indicated. Cuticles are anterior up and dorsal or lateral surface toward viewer. Below each cuticle are the degrees of rescue of embryonic lethality, and whether the construct can rescue maternal/zygotic mutants to adult viability. (K and L) Rescued adults of indicated genotypes. (M) Quantitation of rescue of the Wnt signaling defects. Cuticles were scored according to the criteria in Roberts *et al.* (2011), with 0 being wild-type and 6 the strongest activation of Wnt signaling. Full data are in Table 1. (A) In wild-type embryos, anterior cells secrete hair-like denticles (arrows), while posterior cells secrete naked cuticle (arrowheads). (B) In *APC2^{g10}* maternal/zygotic mutants, Wnt signaling is activated, and almost all cells take on posterior fates and secrete naked cuticle. Only occasional cells secrete denticles (arrows). APC2mito (D) rescues cell fates (restoring alternating denticles and naked cuticle), embryonic lethality, and adult viability almost as well as wild-type APC2 (C). APC2-HCAAX (E) and MyrAPC2 (G) strongly rescue cell fates, substantially rescue embryonic viability, and in the case of APC2-HCAAX, rescue adult viability. (F) APC2-KCAAX retains some ability to rescue Wnt-regulated cell fates, restoring incomplete denticle bands (arrows; gaps are indicated by arrowheads), but this is substantially less than the rescue by the other constructs. It also fails to rescue embryonic or adult viability. (H–J) APC1, APC1endatSAMP, and APC2+APC1CT all rescue cell fates and embryonic viability reasonably well, and rescue adult viability. (N) Immunoblot of embryo extracts probed with anti-APC2. Embryos expressing GFP-APC2-KCAAX, GFP-APC2, or wild-type embryos for comparison. Scale bar for cuticles (in panel A): 75 μ m.

destruction. It substantially rescued embryonic viability (81% viable vs. 50% viable in the absence of the transgene due to paternal rescue). It also provided essentially complete rescue of epidermal cell fates (Figure 6A vs. 6, C and J; Table 1). In the double mutant back-

ground, we could also easily assess ability of the constructs to restore normal Arm regulation. APC2mito effectively restored normal down-regulation of Arm levels, thus restoring the normal striped pattern of Arm accumulation that results from the periodic expression of

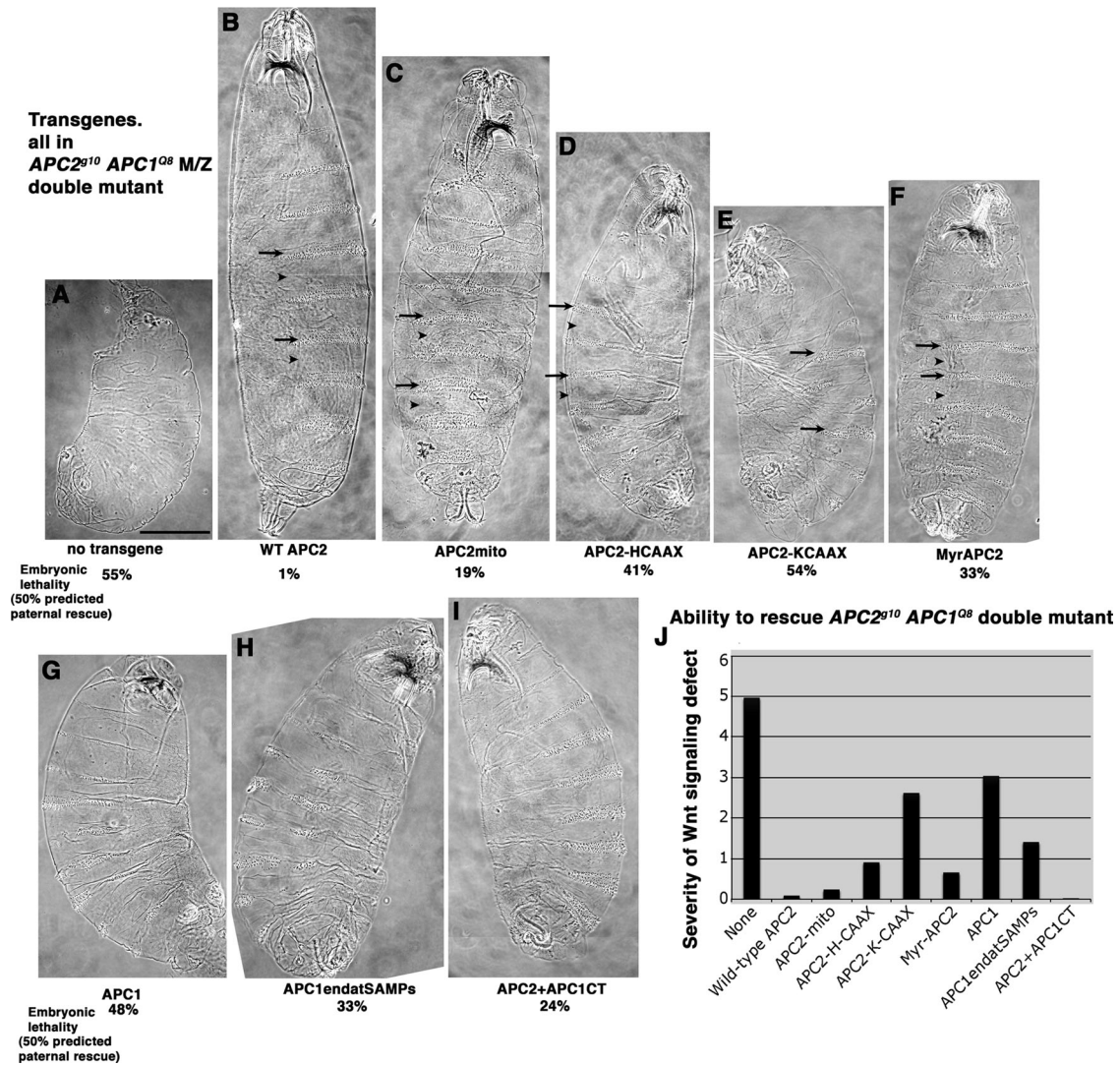


FIGURE 6: APC2mito, APC2-HCAAX, and MyrAPC2 substantially rescue Wnt signaling defects in embryos completely lacking APC family proteins. (A–I) Degree of rescue of Wnt-mediated cell fates by different tethered forms of APC2, as assessed by examining the embryonic cuticle. All are in the *APC2^{g10} APC1^{Q8}* maternal/zygotic mutant background, and the transgene present is indicated. Cuticles are anterior up and dorsal or lateral surface toward viewer. Anterior cell fates = denticles (arrows). Posterior cell fates = naked cuticle (arrowheads). Below each cuticle is the degree of rescue of embryonic lethality; because fathers are heterozygous, 50% of progeny get a wild-type copy of both APC1 and APC2 and thus are paternally rescued. (J) Quantitation of rescue of Wnt signaling defects in cell fate choice, assessed as in Figure 5. Full data are in Table 1. (A) *APC2^{g10} APC1^{Q8}* maternal/zygotic mutants have all cell fates converted to posterior fates and thus secrete only naked cuticle. (B) Wnt regulation is essentially completely rescued by a wild-type APC2 transgene. (C) APC2mito exhibits essentially complete rescue of Wnt-regulated cell fates and significant rescue of embryonic viability. APC2-HCAAX (D) and MyrAPC2 (F) also exhibit substantial rescue of Wnt-regulated cell fates, although they are not as effective in rescuing embryonic viability, and denticle belts are sometimes interrupted by small regions of naked cuticle (arrowheads). (E) APC2-KCAAX retains some ability to rescue anterior cell fates, as indicated by partial denticle belts (arrows) but is less effective than wild-type APC2 or APC2mito and also cannot rescue embryonic viability. Both APC1endatSAMP (H) and APC2+APC1CT (I) have significant rescuing ability, as demonstrated by restoration of cell fates and partial rescue of embryonic viability, while full-length APC1 is less effective (G). Scale bar: 75 μ m.

the Wnt ligand Wingless (Figure 7D; maternal zygotic mutants were distinguished from paternally rescued embryos using a GFP-marked Balancer chromosome expressed in the mesoderm [Figure 7F]). These data suggest that cytoplasmically tethered APC can strongly rescue Wnt regulation, even in the absence of any remaining endogenous APC activity.

All the other tethered constructs also retained function, although in many cases it was reduced from that of untethered wild-type APC2. Both APC2-HCAAX (Figure 6A vs. 6D) and MyrAPC2 (Figure 6A vs. 6F) strongly rescued embryonic cell fates (Figure 6J) and provided

some rescue of embryonic viability. Both also restored Arm degradation, reducing Arm levels and restoring the normal striped pattern of accumulation (Figure 7, E and I). One tethered APC2 construct was less functional than the others. APC2-KCAAX effectively restored regulation of Arm levels, with restoration of the striped pattern of Arm accumulation (Figure 7, G and H). Despite this, however, the rescue of epidermal cell fates was significantly weaker than that conferred by the other variants (Figure 6A vs. 6, E and J), with substantial gaps seen in the denticle belts. However, previous work with hypomorphic APC2 alleles demonstrated that small, nearly undetectable

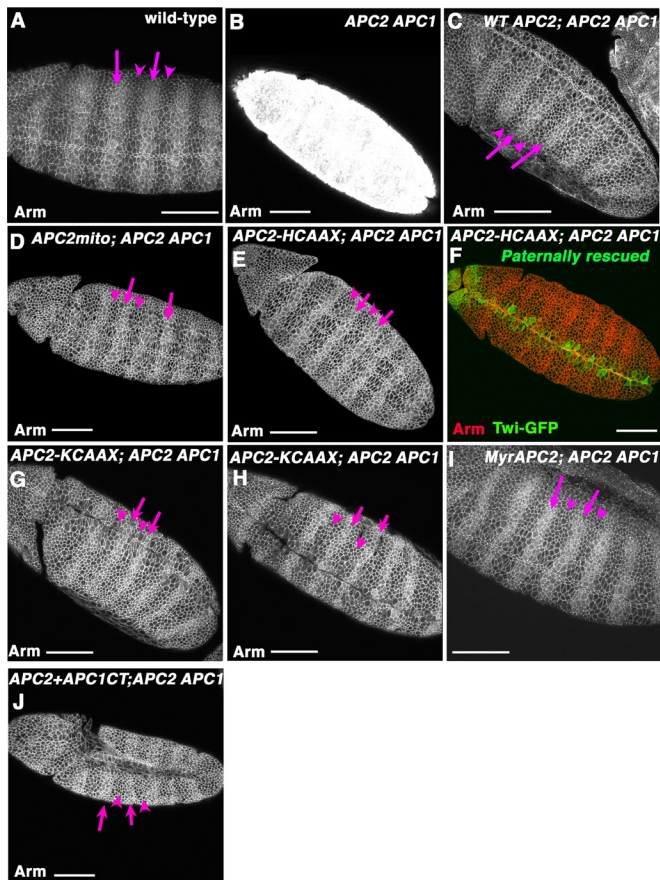


FIGURE 7: Cytoplasmically tethered APC2 restores down-regulation of Arm levels. stage 9–10 embryos, anterior left, stained to visualize Arm. Paternally rescued embryos were identified using a Balancer chromosome expressing GFP in the mesoderm (F). (A) Wild-type embryo showing accumulation of Arm in segmental repeated stripes of cells that receive Wingless signal (arrows), and the lower cytoplasmic/nuclear levels in cells that do not receive Wingless (arrowheads). (B) *APC2⁹¹⁰ APC1^{Q9}* double null maternal zygotic mutant embryo, thus lacking all endogenous APC proteins. Arm levels are highly elevated in all cells. (C) Wild-type APC2 expressed in the double mutant background rescues down-regulation of Arm levels. (D–I) All cytoplasmically tethered forms of APC2 restore normal down-regulation of Arm levels, with stripes of elevated Arm (arrows) and interstripes with lower Arm levels (arrowheads). (D) APC2mito, (E) APC2-HCAAX, (G and H) APC2-KCAAX, and (I) MyrAPC2. (J) APC2+APC1CT, relocalized to MTs by the addition of the C-terminus of APC1, also fully rescues Arm destruction. Scale bars: 75 μm .

changes in Arm levels can cause significant shifts in cell fate (McCartney *et al.*, 2006), so perhaps this is not that surprising. Together, these localization mutants reveal that cytoplasmically tethered forms of APC2 targeted to diverse cellular locations can restore full or nearly full Wnt regulation, even in the absence of all APC family members.

We also explored the function in *Drosophila* of variants and chimeras of the two APC family members, which localize quite differently: APC1, which in cultured cells localizes to MTs; APC1endat-SAMP, which deletes APC1's C-terminal MT-binding domain; and APC2+APC1CT, in which APC1's C-terminal MT-binding domain was added to APC2. All provided significant rescue of both embryonic viability and Wnt-regulated epidermal cell fates in the APC2 single mutant background (Figure 5, H–J and M). APC2+APC1CT also provided strong rescue of embryonic viability, cell fates, and

Arm levels (Figure 6A vs. 6, I and J; Figure 7J). Of course, this protein, while enriched on MTs, is not tethered there, so the reason for rescue by this construct could be residual function of protein that is not on MTs. APC1 itself retained some rescuing ability, although this was reduced; however, APC1 appeared to accumulate at lower levels *in vivo* (unpublished data), perhaps explaining this. Removal of the C-terminal MT-binding domain of APC1 largely restored its ability to rescue Wnt regulation (Figure 6, H and J).

Blocking nuclear export does not cause nuclear accumulation of our tethered APC2 variants

These data suggest that nuclear localization of APC is not essential for Wnt regulation, as variants that are tethered in the cytoplasm rescued Wnt regulation both in SW480 cells and in flies. However, it remained possible that low levels of our tethered variants were transiently entering and then leaving nuclei, as was demonstrated for wild-type human APC (Henderson, 2000; Neufeld *et al.*, 2000; Rosin-Arbesfeld *et al.*, 2003). To examine this issue, we assessed whether blocking nuclear export altered the localization of *Drosophila* APC2 or of our tethered APC2 variants, as it affects localization of human APC. We used leptomycin B, an inhibitor of Crm1-dependent nuclear export, which leads to accumulation of nuclear APC in human cells (Henderson, 2000; Neufeld *et al.*, 2000). We then visualized GFP-APC2, along with Hoechst as a DNA marker, and quantitated the number of cells with nuclear APC2, using three-dimensional stacks of sections through the cells. GFP alone served as a control, as it accumulated in the cytoplasm and nuclei of 94–95% of cells, with or without leptomycin treatment (Figure 8A vs. 8, B and M). In human SW480 cells, *Drosophila* APC2 is largely excluded from the nucleus, accumulating diffusely in the cytoplasm (Figure 8C; Roberts *et al.*, 2011); only 20% of cells scored had any nuclear APC2 (Figure 8M). In fact, even this may be an overestimate, as it was sometimes difficult to distinguish between truly nuclear APC2 and that accumulating in the cytoplasm above the nucleus (this was also a challenge for MyrAPC2, which localizes to the plasma membrane, and thus required distinguishing the apical plasma membrane from the underlying nucleus). Treatment of SW480 cells with leptomycin significantly altered APC2 localization—now 77% of cells showed both cytoplasmic and nuclear APC2 (Figure 8, D and M). We then tested each of our tethered variants. None of them exhibited significant nuclear enrichment *with or without* leptomycin treatment (Figure 8, E–L and M, and Table 2). These data suggest that while fly APC2 can move into and out of nuclei, our tethered proteins do not do so at any detectable level, and thus this does not explain their retention of function in Wnt regulation.

As a final test, we explored whether adding nuclear localization signals to APC2 could move it into nuclei and alter its function in Wnt regulation. To do so, we added three tandem copies of a strong nuclear localization signal from SV40 to the C-terminus of APC2 (Kalderon *et al.*, 1984; Table 1) and examined whether this altered APC2 localization in human SW480 cells. As noted above, fly APC2 is largely cytoplasmic and excluded from the nucleus when expressed in SW480 cells (Figure 9A; Roberts *et al.*, 2011). Strikingly, adding three tandem NLSs did not noticeably alter APC2 localization (Figure 9B). Further, it did not increase or decrease its ability to reduce βcat levels (Figure 9A vs. 9B) or its ability to turn down Wnt-regulated transcription, as assessed with the TOPflash reporter (Figure 9F). This failure to alter APC2 localization may reflect the fact that APC2 has many cytoplasmic partners, including Axin, which tether it in the cytoplasm. In earlier work, we saw similar results for fly Arm, as addition of a strong NLS was not sufficient to redirect it to nuclei in flies (Cox *et al.*, 1999).

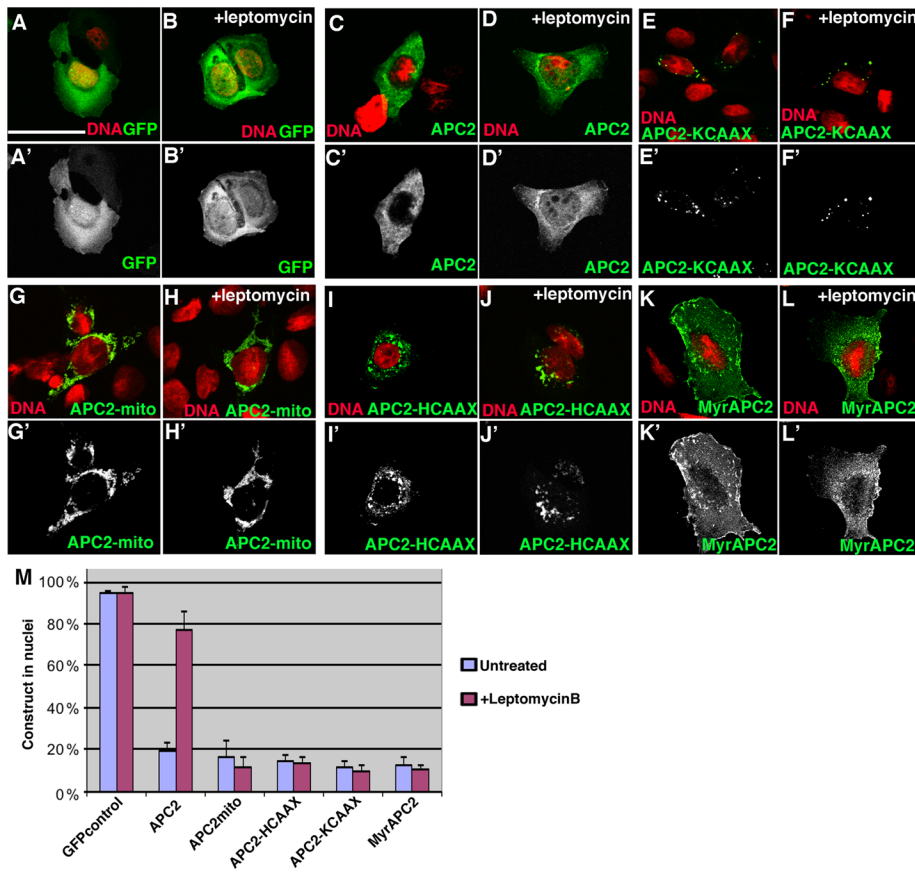


FIGURE 8: Blocking nuclear export does not lead to nuclear localization of our membrane-tethered APC2 variants. (A–L) SW480 cells transfected with the indicated constructs (all APC2 constructs were GFP-tagged) and then either treated with leptomycin B (B, D, F, H, J, and L) or with the methanol carrier as a control (A, C, E, G, I, and K). Proteins encoded by the transfected constructs were visualized by GFP fluorescence, and DNA was visualized with Hoechst. Scale bar: 50 μ m. (A and B) The GFP-alone control is both cytoplasmic and nuclear with or without leptomycin treatment. (C) GFP-APC2 is largely excluded from the nucleus. (D) Leptomycin treatment leads to nuclear accumulation of GFP-APC2. (E and F) APC2-KCAAX localizes to cytoplasmic vesicles and does not accumulate in nuclei with or without leptomycin treatment. (G and H) APC2mito localizes to presumptive mitochondria and does not accumulate in nuclei, even after leptomycin B treatment. (I and J) APC2-HCAAX localizes to perinuclear vesicles and does not accumulate in nuclei, even after leptomycin B treatment. (K and L) MyrAPC2 is localized diffusely to the plasma membrane, and leptomycin treatment does not induce nuclear accumulation. (M) Quantitation of mean fraction of cells with nuclear accumulation, \pm SD, as assessed by examination of confocal stacks of cells stained to visualize the GFP-tagged construct and Hoechst-labeled DNA. Means represent 100 cells counted in each of two to four separate experiments (see Table 2 for details).

One domain in APC family proteins that binds many cytoplasmic cytoskeletal partners is the Arm-repeat region. We previously found that deleting the Arm repeats from fly APC2

Drosophila APC2 tethered at different cytoplasmic locations and assessing their function in regulating Wnt signaling in both cultured mammalian cells and in vivo in *Drosophila*.

(APC2 Δ Armrepeats) eliminated its ability to down-regulate β cat levels and Wnt-regulated transcription in SW480 cells (Roberts et al., 2012). However, deleting the Arm repeats does subtly alter localization of APC2 in SW480 cells. APC2 Δ ArmRpt is evenly distributed between the cytoplasm and nucleus in some cells (Figure 9C, bottom), while remaining excluded from the nucleus in others (Figure 9C, top). This may reflect reduced cytoplasmic tethering. We then tested whether adding strong NLSs to this less well-tethered form of APC2 might drive it into nuclei. APC2 Δ ArmRpt+3xNLS localized to nuclei in most cells, and in many cells, it was strongly enriched in the nucleus (Figure 9, D' and E'). However, APC2 Δ ArmRpt cannot rescue β cat destruction or down-regulate a Wnt-dependent reporter with (Figure 9, D–F) or without (Roberts et al., 2012) an added tandem NLS sequence, reflecting the important role of the Arm repeats in APC2 function.

DISCUSSION

Despite 15 yr of study, the mechanistic role of the human tumor suppressor APC in Wnt signaling remains unclear. Most studies support the idea that its major role is as part of the β cat destruction complex, but its mechanistic role in that complex remains largely mysterious. One class of models suggests APC recruits the destruction complex to the correct place in the cell for its action (McCartney et al., 2006), with most studies supporting localization to the cell cortex. Other studies suggest APC plays critical roles in the nucleus (Neufeld, 2009), mediating β cat nuclear export or helping repress Wnt target genes. However, the localization hypothesis has not been directly tested, and the proposed nuclear roles are largely based on studies with overexpressed proteins, often in cells with deregulated Wnt signaling due to mutations in endogenous APC. In this study, we tested both models directly, generating forms of

Construct	Nuclear after leptomycin B treatment		Nuclear in control (methanol)		Number of experiments (100 cells scored/experiment)
	SD	SD	SD	SD	
GFP alone	94.49%	3.35%	94.67%	1.41%	n = 4
APC2	76.91%	9.35%	19.85%	3.95%	n = 4
APC2mito	11.32%	5.14%	17.00%	6.93%	n = 4
APC2-HCAAX	13.96%	2.58%	14.95%	2.48%	n = 3
APC2-KCAAX	9.87%	2.82%	11.45%	2.72%	n = 3
MyrAPC2	10.90%	1.84%	12.75%	3.40%	n = 2

TABLE 2: Effect of leptomycin B treatment on localization of APC variants in SW480 cells.

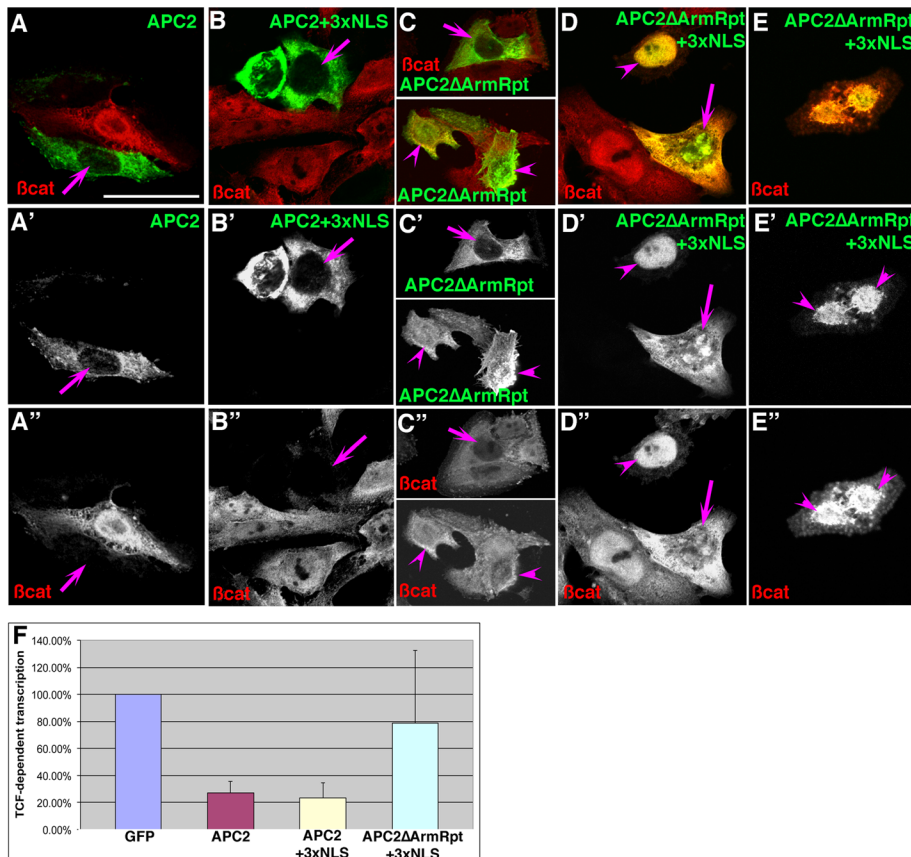


FIGURE 9: Added nuclear localization signals do not substantially relocalize APC2 but do relocalize APC2ΔArmRpts. (A–E) SW480 cells transfected with the indicated GFP-tagged constructs. GFP (green) and βcat (red). Transfected cells are indicated with arrows (cells in which the construct is largely excluded from the nucleus) or arrowheads (cells with noticeable nuclear accumulation). Scale bar: 50 μm. (A) GFP-APC2 is largely nuclear excluded (A', arrow) and restores βcat destruction (A'', arrow). (B) Adding three copies of a strong NLS (APC2+3xNLS) does not alter APC2 localization (it remains largely nuclear-excluded-B' arrow) or its ability to restore βcat destruction (B'', arrow). (C) Deleting APC2's Arm repeats reduces cytoplasmic anchoring—in some cells this protein remains largely excluded from the nucleus (C', arrow), but in many cells accumulation is even between cytoplasm and nuclei (C', arrowheads). APC2ΔArmRpt cannot restore βcat destruction (C''). (D and E) Adding three copies of a strong NLS results in increased nuclear accumulation of APC2ΔArmRpt+3xNLS, with many cells now showing preferential nuclear accumulation (D' and E', arrowheads), but this does not restore βcat destruction (D'' and E''). (F) TCF-dependent transcription of the TOPflash reporter is down-regulated by either APC or APC+3xNLS, but not by APC2ΔArmRpt+3xNLS. Scale bar: 50 μm.

APC2 that is tethered in the cytoplasm can properly regulate Wnt signaling

If APC proteins have essential nuclear roles, then tethering them in the cytoplasm should prevent nuclear entry with deleterious consequences for Wnt regulation. In fact, an earlier report suggested that a membrane-tethered form of APC2 was not functional in *Drosophila* (Tolwinski, 2009). We tethered APC2 to several different cytoplasmic locations, using a transmembrane sequence that is mitochondrially targeted, two different CAAX sequences from K-ras and H-ras, and a myristoylation signal from Src. We verified that these dramatically altered APC2 localization in the expected ways in both human SW480 cells and in *Drosophila* ovaries and embryos. In the case of APC2mito, we also confirmed effective targeting by cell fractionation. Further, we and others have previously shown that both the KCAAX tag and the myristoylation tag we used effectively tether other proteins with nuclear functions; when added to Arm each tag

eliminated the ability to rescue Arm's nuclear role in Wnt signaling, while allowing it to retain its role at the plasma membrane in adherens junctions (Cox et al. 1999; Tolwinski and Wieschaus, 2004).

Strikingly, all four of these cytoplasmically tethered APC2 proteins, including the one localized to mitochondria by a transmembrane domain, fully rescued Wnt regulation when transfected into SW480 cells. This included rescue of βcat destruction and reduction of a Wnt-responsive reporter gene. These data suggest that APC proteins do not have an essential nuclear function in Wnt regulation in these cells. However, this experiment has one significant caveat: these cells retain a truncated endogenous copy of human APC1, leaving open the possibility that this truncated protein can cover for any essential nuclear role. To address this, we also explored the ability of the tethered proteins to rescue Wnt regulation in *Drosophila*. Strikingly, APC2mito, APC2-HCAAX, and MyrAPC2 remained essentially fully functional, rescuing Wnt regulation in both APC2 single mutants and APC2 APC1 double mutants almost as effectively as wild-type APC2. These data further support the hypothesis that APC2 does not have an essential nuclear role.

Of course this does not rule out accessory, nonessential roles in Wnt regulation via nuclear export of βcat or transcriptional regulation, but these do not appear to be substantial. In particular, APC2mito rescued Wnt-dependent patterning as well as wild-type APC2 did, and the cuticle pattern is quite sensitive to subtle changes in Wnt regulation. However, APC2mito was not quite as effective at rescuing embryonic viability as wild-type APC2 (e.g., 86% vs. 92% viability when rescuing the APC2 single mutant). It is possible that this reflects a subtle defect in Wnt signaling, perhaps in a non-epidermal tissue, or it may reflect an effect of mito-tethering on APC2's known cytoskel-

etal roles. Our data remain quite consistent with data suggesting APC may regulate nuclear events indirectly by sequestering transcriptional regulators in the cytoplasm. There is strong support for the idea that APC sequesters βcat (Krieghoff et al., 2006; Seo and Jho, 2007; Roberts et al., 2011), and APC also may sequester the transcriptional repressor CtBP (Hamada and Bienz, 2004).

These data are also largely consistent with a recent test in knock-in mice. Zeineldin et al. (2012) generated a knock-in mutant of mouse APC mutating two NLS sequences in the protein, mutations that were previously shown to attenuate nuclear accumulation of human APC (Zhang et al., 2000). Strikingly, mice homozygous for this mutation are viable, without obvious effects on the many Wnt signaling processes required for normal development, thus ruling out an essential role for these sequences in Wnt regulation. The authors did observe subtle increases in proliferation in certain regions of the intestinal tract, consistent with possible subtle effects on Wnt signaling, and also

observed an increase in initiation of benign polyps in an APC-min heterozygous background, though once again this was confined to certain regions of the intestinal tract. Together, these effects are consistent with the possibility of subtle effects of mutating the APC NLS sequences on Wnt signaling, but it is also possible that subtle effects on the function of the SAMP motif in which one of these NLS sequences is embedded could also explain these differences.

APC2 does not appear to recruit the destruction complex to the “right location”

The mechanistic role of APC in the destruction complex remains mysterious. Originally, APC was thought to be a scaffold presenting β cat to the kinases CK1 and GSK3. However, it is now clear that Axin carries out this function (Ha *et al.*, 2004). Another model, which was proposed, suggested that APC acted to recruit the destruction complex to the “right” cellular location—perhaps a position at the cell cortex near the Wnt receptor complex, facilitating down-regulation. This was based on two sets of data. First, both *Drosophila* (McCartney *et al.*, 1999; Yu *et al.*, 1999) and human (Näthke *et al.*, 1996) APC proteins localize, at least in part, to the cell cortex. Second, we isolated a series of APC2 mutants with missense mutations in the Arm repeats—these exhibited a strong correlation between the degree of disruption of cortical localization and the degree of disruption of Wnt regulation (McCartney *et al.*, 2006). We thus hypothesized that APC2’s Arm repeats interacted with a cortical protein, and that APC2, via the SAMP repeats, then recruited Axin and the destruction complex to that location.

One prediction of this hypothesis would be that targeting APC2 to other cellular locations would disrupt, or at least attenuate, its function. Our membrane-tethered mutants allowed us to test this hypothesis. Strikingly, APC2mito, APC2-HCAAX, and MyrAPC2, which in mammalian cells are targeted to distinct intracellular compartments (mitochondria, Golgi bodies, and the plasma membrane respectively), effectively rescue Wnt regulation both in mammalian cells and in *Drosophila*. These data strongly suggest that there is not any unique intracellular location where APC2 must be to function in the destruction complex, and they also suggest that cortical localization per se is not essential. Consistent with this, Zhou *et al.* (2011) recently reported that deleting the 30 amino acids of APC2 C-terminal to the last SAMP repeat dramatically reduced cortical localization without disrupting Wnt regulation. These data also suggest that APC2 may not need to move from one cellular compartment to another to fulfill its essential functions.

One form of APC2 that was tethered to the plasma membrane, APC2-KCAAX, was less functional than APC2mito, APC2-HCAAX, and MyrAPC2, which localized to mitochondria, internal membrane vesicles, and the plasma membrane, respectively. This was surprising, given that the cell cortex is the predominant location at which we detect wild-type APC2. This may mean that only very restricted subcellular locations are incompatible with APC function—this is particularly striking when comparing APC2-KCAAX and MyrAPC2, which have quite similar localizations in stage 9 embryos, when Wnt signaling is occurring. One possibility is that certain cortical locations promote inactivation of the destruction complex via the Dishevelled-based mechanism that normally acts upon receipt of Wnt signaling. Recent work from the Bienz lab suggests that Axin localizes to different sites in cells receiving or not receiving Wnt signals (Fiedler *et al.*, 2011; Mendoza-Topaz *et al.*, 2011). In cells receiving Wnt signals, Axin localizes to plasma membrane puncta that colocalize with Dishevelled. This is presumably a “signalosome” complex in which Axin is inactive. In contrast, in cells not receiving Wnt signals Axin colocalizes with APC2 in the cytoplasm in complexes

that are presumptive active destruction complexes. Perhaps the KCAAX tag directs APC, and thus Axin, to a location at which it is too easily inactivated. Of course, it is also possible that the KCAAX tag inactivates APC2 in some other way, even though the similarly placed HCAAX tag does not do so.

How then do we reconcile these results with our previous work (McCartney *et al.*, 2006), which suggested that loss of cortical localization correlated with the severity of Wnt signaling defects in a series of APC2 mutants with missense changes in APC2’s Arm repeats? Structure-based sequence comparisons of the Arm repeats of APC and β cat suggest that most of the missense mutations we characterized affect the hydrophobic core, rather than surface exposed residues (McCartney *et al.*, 2006). We thus suspect that they destabilize the Arm-repeat domain as a whole, rather than affecting particular contact residues mediating binding with specific partners. If this hypothesis is correct, they would likely affect binding to all partners, rather than only particular ones. We further hypothesize that one partner affected by the mutations mediates cortical localization, perhaps modulating one of APC’s cytoskeletal roles, while a second, distinct partner is essential for APC’s role in the destruction complex and β cat regulation. In this model, rather than localizing the destruction complex to a particular subcellular site, the role of APC proteins in the destruction complex would be to bind Axin, β cat, and via APC’s Arm repeats, this hypothetical protein X. Identifying this unidentified, yet essential, member of the destruction complex, and determining its mechanistic role in Wnt regulation is therefore a high priority.

MATERIALS AND METHODS

APC constructs

Deletion constructs in Figure 1B are described in Roberts *et al.* (2011). For the localization constructs, full-length *Drosophila* APC2 was PCR-cloned into the pCR8/GW/TOPO Gateway Entry Vector (Invitrogen, Carlsbad, CA) by TOPO TA cloning. This entry vector served as the basis for further modification using PCR stitching to add the appropriate localization tags to full-length APC2. The added localization sequences are included in Table 1. Full-length *Drosophila* APC1 was built into a pCR8/GW/TOPO entry vector by a combination of PCR cloning and restriction digest cloning, using available cDNA clones and amplifying the rest from genomic DNA. APC1endatSAMPs was generated by PCR cloning off the full-length APC1 template, using primers that amplify amino acids 1–1457 of APC1. For APC2+APC1CT, amino acids 1458–2417 of APC1 were fused to the C-terminus of APC2 by PCR and engineered restriction cloning into the pCR8/GW/TOPO entry vector. All APC constructs were then recombined into expression vectors modified for Gateway cloning, using Gateway vectors provided by Terence Murphy. For expression in mammalian cells, 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 (CMV) promoter. To generate transgenic flies, APC2 constructs were Gateway-cloned into a modified pUASattB vector (GenBank accession number EF362409, Basler lab, University of Zurich) that added the endogenous APC2 promoter (McCartney *et al.*, 2006) and an EGFP-Gateway-3X STOP cassette and transgenics generated by BestGene (Chino Hills, CA). For APC2-KCAAX and APC2-HCAAX, the landing site was 58A (Bloomington stock #24484), while for APC2mito and MyrAPC2 it was 22A3 (Bloomington stock #9752). Additional details of cloning steps are available upon request.

Cell culture, transfections, and immunofluorescence

We cultured SW480 cells at 37°C under normal atmospheric conditions in L15 medium (Cellgro; Mediatech, Manassas, VA) + 10%

heat-inactivated fetal bovine serum and 1X penicillin–streptomycin. APC constructs were transfected into SW480s overnight using Lipofectamine 2000 (Invitrogen) per the manufacturer's protocol. After 24 h, 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 mouse anti- β cat (cat# 610153; 1:800; BD Transduction Laboratories, San Jose, CA), H-102 β catenin antibody (cat# sc-7199; 1:800; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-GM130 antibody (ab1299; 1:250; Abcam, Cambridge, MA; provided by Priscilla Siesser, University of North Carolina–Chapel Hill). Secondary antibodies were Alexa Fluor 568 or 647 (1:1000; Invitrogen).

Quantifying β cat protein levels

SW480 cells were transfected with GFP-APC2 constructs as described in the preceding section. Twenty-four hours later, cells were fixed and stained for β cat and 4',6-diamidino-2-phenylindole (DAPI) to detect DNA. Individual cells were defined by DAPI, and images of 1000–5000 cells per construct from three or more independent experiments were acquired and analyzed. The average total cellular fluorescence of β cat was determined for GFP-positive cells using an Array Scan V (Cellomics, Pittsburgh, PA) and vHCS View software (Cellomics).

TOP/FOP reporter assays

The TOP/FOPflash luciferase reporter and the pRL *Renilla* transfection control were kind gifts of Hans Clevers (Hubrecht Institute, The Netherlands). TOP/FOP reporter assays were carried out with the Dual-Glo Luciferase System (Promega, Madison, WI), following the manufacturer's instructions. Briefly, TOPflash or FOPflash (1 μ g) constructs were transiently cotransfected into SW480 cells together with the pRL transfection control (1 μ g) and the appropriate APC construct (2 μ g). Transcriptional activity was measured 24 h later, and TOPflash was normalized to *Renilla*. None of the APC constructs significantly affected FOPflash values.

Embryonic lethality assay and cuticle rescue

APC2 transgenes on the second chromosome were crossed into *APC2^{g10}* single mutant and *APC2^{g10}APC1^{Q8}* double mutant backgrounds (McCartney *et al.*, 2006). *APC2^{g10}* single mutant background: embryos expressing the transgene but maternally/zygotically mutant for *APC2* were progeny of *APC2 transgene; APC2^{g10}* females and males. *APC2^{g10}APC1^{Q8}* 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). *APC2 transgene / +; FRT82B APC2^{g10} APC^{Q8} / FRT82B ovo^D* females who had been heat-shocked on day 3 after egg-laying for 3 h at 37°C, were crossed to *APC2 transgene; FRT82B APC2^{g10} APC^{Q8}/TM3* males. All crosses were performed at 25°C. Embryonic lethality assays and cuticle preparations were as previously described (Wieschaus and Nüsslein-Volhard, 1986). Rescue of Wnt regulation was assessed by analyzing embryonic cuticles with previously established scoring criteria (McCartney *et al.*, 2006).

Immunoprecipitations (IPs) and immunoblotting

IPs and immunoblots were as in Peifer *et al.* (1992). Briefly, protein samples from tissue culture cells or dechorionated *Drosophila* embryos were ground on ice in 2X Laemmli buffer and boiled 5 min. For IPs, cells or dechorionated embryos were lysed in NET buffer (50 mM Tris, pH 7.5, 400 mM NaCl, 5 mM EDTA, 1% NP40) plus protease (Complete EDTA-free Protease Inhibitor tablets; Roche,

Indianapolis, IN) and phosphatase (1 mM NaF, 0.4 mM NaVO₃, 0.4 mM NaVO₅) inhibitors. Samples were run on 6% SDS–PAGE gels and blotted to nitrocellulose. Primary antibodies were anti-GFP (clone JL-8; 1:800 for SW480 experiments and 1:100 for embryo experiments; Clontech), anti-GFP (ab290 for IPs; 1:250; Abcam), anti-Flag (clone M2; Sigma-Aldrich, St. Louis, MO), anti-dAPC2 1:1000 (McCartney *et al.*, 1999), anti- α -tubulin (DM1A; 1:5000; Sigma-Aldrich), anti-aPKC γ (sc-216; 1:1000; Santa Cruz). Signal was detected with ECL-Plus (GE Health Sciences, Pittsburgh, PA).

Immunofluorescence/imaging of *Drosophila* embryos

Embryos were prepared and imaged as in Fox and Peifer (2007). Briefly, dechorionated embryos were fixed in 4% formaldehyde, methanol-devitelized, blocked in NGS, and sequentially incubated with anti-Arm (N27A1, DSHB; 1:50) and Alexa Fluor 568 secondary antibody (1:500; Invitrogen). Images were collected on either a Zeiss LSM 510 or Zeiss Pascal scanning confocal microscope. GFP-tagged endogenous promoter-driven deletion constructs in *Drosophila* embryos (Figure 2) were visualized live on a spinning disk confocal microscope. Adobe Photoshop 7.0 (Redmond, WA) was used to adjust input levels to span entire output gray scale, and to adjust brightness and contrast. When protein levels were compared, images were equally adjusted.

Leptomycin B treatment

SW480 cells were plated at 5×10^5 in six-well plates. On the second day, cells were transfected with the relevant constructs. On the third day, cells were treated with 24 ng/ml leptomycin B (dissolved in methanol; Sigma-Aldrich) for 6 h. The same amount of methanol alone was used for control cells. Cells were fixed after treatment and stained with Hoechst. Three independent experiments were conducted for each construct, and 100 cells were counted for each construct per experiment. To calculate the fraction of cells with nuclear localization of a given construct, Z-stacks of ~100 cells per construct per experiment were acquired using the 40x objective, and nuclear APC was quantitated by overlap of Hoechst and GFP.

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