Destruction Complex Function in the Wnt Signaling Pathway of *Drosophila* Requires Multiple Interactions Between Adenomatous Polyposis Coli 2 and Armadillo

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

WNT signal transduction plays essential roles in both normal embryonic development and the maintenance of adult tissues by directing cell fates, promoting proliferation, and influencing morphogenesis (reviewed in Logan and Nusse 2004). Consistent with its essential nature, the Wnt signaling pathway is well studied. However, many of the molecular mechanisms that regulate Wnt signaling are poorly understood. In the absence of a Wnt ligand, the pathway is negatively regulated by the activity of the degradation or destruction complex. Loss of negative regulation and

constitutive activation of Wnt target genes is associated with a variety of diseases, the most well understood of which is colorectal cancer (reviewed in Logan and Nusse 2004 and Polakis 2007). The destruction complex, which includes the kinases GSK3 β and CK1, the scaffolding protein Axin, and the colon cancer tumor suppressor Adenomatous polyposis coli (APC), acts to phosphorylate β -catenin (β cat), the key effector of the Wnt pathway, and target it for ubiquitination and degradation by the proteasome (reviewed in Kennell and Cadigan 2009). Loss of function of any one of these core components of the destruction complex results in the ligand-independent activation of Wnt target genes. Activation of the Frizzled receptor by a Wnt ligand results in the deactivation of the destruction complex through LRP6/Arrow and the cytoplasmic protein Dishevelled. This allows for the accumulation of β cat that enters the nucleus to promote the activation of Wnt target genes in complex with members

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of the TCF/LEF family of transcription factors. Thus, the destruction complex is a nexus of Wnt pathway regulation.

Within the destruction complex itself there are myriad protein–protein interactions. Axin, thought to act as a scaffolding protein in the complex, can bind directly to β cat, APC, GSK-3 β , and Dishevelled and is thought to promote interactions within the complex (reviewed in Cadigan and Peifer 2009). The precise role of APC in the complex is less well understood (reviewed in McCartney and Nathke 2008). APC directly binds β cat through its 15-amino-acid repeats (15Rs) and 20-amino-acid repeats (20Rs) (Rubinfeld *et al.* 1993, 1995; Su *et al.* 1993b). The vertebrate APC protein contains four 15Rs and seven 20Rs (Figure 1). The role of the 15Rs in β -catenin degradation is controversial. Studies of mutant APC fragments in cultured cells have yielded contradictory results, where one study concluded that the 15Rs are necessary and sufficient for β cat degradation (Kohler *et al.* 2010) and another suggested that they do not contribute to degradation (Munemitsu *et al.* 1995). Recent *in vivo* studies of 15R mutants in the context of full-length APC2 in *Drosophila* concluded that the 15Rs are largely dispensable for β cat degradation, but may contribute to its cytoplasmic retention (Roberts *et al.* 2011). APC also binds Axin directly through the SAMP repeats, of which there are three in vertebrate APC (Figure 1) (Behrens *et al.* 1998; Hart *et al.* 1998). Conserved sequence B or CID (sequence B), that lies between 20R1 and 20R2 in both vertebrate and *Drosophila* APCs has recently been shown to play a significant role in β cat degradation, although the mechanism is unknown (Kohler *et al.* 2009; Roberts *et al.* 2011). In cancer, most APC mutations are found in what is termed the mutational cluster region (MCR) that results in the production of truncated APC proteins that are missing five or more of the 20Rs and the SAMP repeats, retaining the N-terminal domains, the 15Rs, approximately two of the 20Rs, and centers over conserved sequence B (Figure 1). Such mutations disrupt destruction complex activity, resulting in the constitutive activation of the Wnt pathway (reviewed in Logan and Nusse 2004). The kinases in the complex phosphorylate β cat to target it for degradation, but also phosphorylate Axin and APC (Rubinfeld *et al.* 1996; Hart *et al.* 1998; Ikeda *et al.* 1998). In the case of APC, phosphorylation regulates the APC– β cat interaction (Rubinfeld *et al.* 1996; Liu *et al.* 2006).

Cocrystallization of β cat with the third 20R (20R3) of vertebrate APC revealed the specific sites of phosphorylation in the 20Rs that play a role in direct contact with β cat *in vitro* (Ha *et al.* 2004; Xing *et al.* 2004). While both phosphorylated and nonphosphorylated APC (P-APC and non-P-APC, respectively) bind β cat, phosphorylation of APC by CK1 and GSK3 β increases the affinity of APC for β cat by 300- to 500-fold (Xing *et al.* 2004). The binding of Axin to APC enhances GSK3 β and CK1 phosphorylation of APC, suggesting that the phosphorylation of APC may take place within the destruction complex (Ikeda *et al.* 2000). Mutation of three conserved serine residues in 20R2 significantly decreased its activity in SW480 cells, arguing that phosphorylation of

APC plays a role in β cat degradation (Rubinfeld *et al.* 1997). Interestingly, vertebrate 20R2 does not bind β cat (Choi *et al.* 2006; Liu *et al.* 2006; Kohler *et al.* 2008), suggesting that it may have a distinct role in Wnt signaling. Most APC 20Rs are composed of two regions referred to as the N-terminal extended region and a phospho-region. The phospho-region of the 20R binds to Armadillo repeats 1–5 of β cat, and the extended region of APC binds to Armadillo repeats 5–9 of β cat (Ha *et al.* 2004; Xing *et al.* 2004). The interface between the 20R extended region and β cat is primarily composed of hydrophilic interactions including two key salt bridges between APC and two lysines in β cat referred to as the “charged buttons.” These charged buttons play a critical role in the interaction between β cat and a number of its binding partners (Omer *et al.* 1999; Graham *et al.* 2000; Von Kries *et al.* 2000; Fasolini *et al.* 2003). These structural findings have led to a number of speculative models for the assembly and mechanism of the destruction complex. While we now understand at the structural level how APC and β cat interact *in vitro*, the significance of these interactions to destruction complex activity *in vivo* has not been established.

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

Materials and Methods

Constructs

The specific amino acid positions of the *Drosophila* APC2 (FlyBase annotation symbol: CG6193) fragments are as follows:

APC2- Δ 15R Δ 20R Δ B: 1–490 plus 931–1067.

APC2- Δ 20R Δ B: 1–592 plus 931–1067.

APC2-R1–R5SA: S610A, D613A, N616A, T619A, S660A, S663A, D666A, S752A, T755A, S758A, S761A, S799A, P802A, S805A, S808A, S877A, S880A, S883A, T886A.

APC2-R1–R5SD: S610D, D613D, N616D, T619D, S660D, S663D, V669D, S752D, T755D, S758D, S761D, S799D, P802D, S805D, S808D, S877D, S880D, S883D, T886D.

APC2-R1–R5ExR: E595Q, Y600A, E603Q, T605A, P606A, T645A, D650A, L653A, T655A, P656A, D737N, F742A, E745Q, T747A, P748A, D784N, Y789A, E792Q, T794A, T795A, D862N, Y867A, E870Q, S872A, T873A.

APC2-R3-R5SA: S752A, T755A, S758A, S761A, S799A, P802A, S805A, S808A, S877A, S880A, S883A, T886A.
APC2-R3-R5SD: S752D, T755D, S758D, S761D, S799D, P802D, S805D, S808D, S877D, S880D, S883D, T886D.
APC2-R3-R5ExR: D737N, F742A, E745Q, T747A, P748A, D784N, Y789A, E792Q, T794A, T795A, D862N, Y867A, E870Q, S872A, T873A.

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

Genetics, hatch rate, and cuticle preparations

Transgenic flies expressing *P[endoP-EGFP-APC2-FL]*, *P[endoP-EGFP-APC2-Δ15Δ20RΔB]*, *P[endoP-EGFP-APC2-Δ20RΔB]*, *P[endoP-EGFP-APC2-R1-R5SA/SD/ExR]*, and *P[endoP-EGFP-APC2-R3-R5SA/SD/ExR]* were generated using *P*-element-mediated germline transformation (Model System Genomics; Duke University, Durham, NC). Two independent second chromosome insertions for each transgene were crossed into the *APC2^{g10}* (APC2 null) and the *FRT APC2^{g10} APC1^{Q8}* (APC double null) backgrounds, using standard methods. Embryos maternally and zygotically *FRT APC2^{g10} APC1^{Q8}* were generated using the FRT/FLP/DFS technique (Chou and Perrimon 1996). Relevant crosses and genotypes are shown in Figure 3.

Embryonic cuticles were prepared and hatch rate analysis was performed as previously described (Wieschaus and Nusslein-Volhard 1998). The cuticle phenotype scoring criteria were previously described (McCartney *et al.* 2006). In brief, each cuticle for a given genotype was given a score between 0 (wild type) and 6 (most severe). Details for each scoring category are as follows: 0, wild-type cuticle, but did not hatch; 1, head defects, but no head hole, wild-type length, 15% of denticles missing; 2, head defects or small head hole, >70% wild-type length, most denticle bands still represented by at least one patch of denticles; 3, anterior hole, 50–60% wild-type size, more than three patches of denticles remaining; 4, anterior hole, 50–60% wild-type size, fewer than two denticle patches remaining; 5, anterior hole extends ventrally to ~30% cuticle length, 50–60% wild-type size, no denticles; and 6, anterior hole extends ventrally to ~50% cuticle length, 25–30% wild-type size, no denticles. Cuticles with features of more than one category were designated by halves. For example, a cuticle with features of both a 2 and a 3 was designated as a 2.5. A phenotypic average (PA) was calculated from these data.

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

Embryos were collected for 6 hr at 27° and fixed and stained as described in McCartney *et al.* (1999). Antibodies that were used for this analysis are as follows: Anti-Armadillo (N27A1, 1:250) and anti-Engrailed (4D9, 1:50) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Anti-GFP (Abcam, 1:5000) was preabsorbed against *w¹¹¹⁸* embryos before using for immunohistochemistry. Anti-APC2 was used as previously described (McCartney *et al.* 2006). Secondary antibodies were conjugated with various Alexa dyes [Invitrogen (Carlsbad, CA), 1:1000].

Subcellular localization was determined for *P[endoP-EGFP-APC2-FL]* and *P[endoP-EGFP-APC2-Δ15Δ20RΔB]*, *P[endoP-EGFP-APC2-Δ20RΔB]*, *P[endoP-EGFP-APC2-R1-R5SA/SD/ExR]*, and *P[endoP-EGFP-APC2-R3-R5SA/SD/ExR]* in the APC2 null background. Armadillo (Arm) accumulation and Engrailed (En) expression were also assessed in these genotypes. For these analyses, two independent staining experiments were performed and five embryos from each experiment were imaged. Representative embryos are shown.

Image analysis

Images were acquired with a spinning disc confocal microscope with a Yokagawa scan head (Solamere Technology Group) with a QICAM-IR camera (Qimaging) on a Zeiss (Thornwood, NY) Axiovert 200M, using QED InVivo software. For Figure 5, multiple images at 25× were merged by using Adobe Photoshop to generate whole embryo images. Figures were prepared with Adobe Photoshop and Adobe Illustrator.

S2 cell culture, transfection, and immunoprecipitation

S2 cells were cultured and transfected with Effectene, using standard protocols (QIAGEN, Valencia, CA). Expression was induced at 24 hr post-transfection by adding CuSO₄ to a final concentration of 40 μM for 14–16 hr. Co-immunoprecipitation (co-IP) was performed as in Zhou *et al.* (2011). In brief, after induction cells were lysed and the lysate was preincubated with rec-G beads (Invitrogen) for 0.5 hr at 4°. The precleared lysate was incubated with anti-Armadillo antibody at 1:50 for 1 hr at 4°. Rec-G beads were added and incubated for another 0.5 hr at 4° prior to washing. SDS-PAGE and immunoblotting were performed using standard procedures.

Results

Mutation of the extended region and phosphorylated residues in *Drosophila* APC2

We generated targeted mutations in *Drosophila* APC2 that disrupt or mimic phosphorylation or disrupt the conserved residues in the extended region (ExR), in all five of the 20Rs (Figure 1, B and C). The selection of those residues was based on the alignment between human 20R3 that was

Figure 1 (A) Schematic representations of vertebrate APC, βcat, and Axin with domains and binding partners indicated. (B) Schematic representations of *Drosophila* APC proteins with the sites of relevant null mutations indicated (*APC1^{Q8}* and *APC2^{g10}*). Deletion mutants and targeted point mutants are shown. (C) Sequence alignment between human APC 20R3 and the 20Rs of *Drosophila* APC2. Designation as a phosphorylation residue, a salt bridging residue, or other conserved residue is based on Ha *et al.* (2004) and Xing *et al.* (2004).

cocrystallized with βcat (Ha *et al.* 2004; Xing *et al.* 2004) and the 20Rs of APC2 (Figure 1C). We have designated these mutants as APC2-R1–R5SA, APC2-R1–R5SD, and APC2-R1–R5ExR.

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

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

To begin the characterization of these mutant proteins, we expressed them in *Drosophila* S2 cells. We have previously shown that APC2-FL localizes to the cortex in S2 cells, similar to its endogenous localization in embryonic cells (Zhou *et al.* 2011). This localization requires both the Arm repeats and the C-terminal C30 domain (Zhou *et al.* 2011), all of which are intact in our mutants. As a test of the stability and functionality of our mutants, we asked whether they localized to the S2 cell cortex. All the mutants localize to the cell cortex like APC2-FL (Figure 2A). We further tested the ability of the deletions and the 20R1–R5 mutants to associate with Armadillo in S2 cell lysate. While we have perturbed direct APC2–Arm interactions in all of the mutants, the mutant forms of APC2 retain the ability to bind to Axin through their SAMP domain (Behrens *et al.* 1998; Hart *et al.* 1998) and to endogenous wild-type APC2 in the S2 cells through their Arm domain (Zhou *et al.* 2011). Thus, the targeted mutants that are unable to bind Armadillo directly may still

Figure 2 Characterization of APC2 mutants in S2 cells and in the *Drosophila* embryo. (A) Expression of mCherry (mCh)-tagged versions of the targeted APC2 mutants revealed that they localize to the cortex like APC2-FL in S2 cells. (B) All of the mCherry-tagged APC2 mutants co-immunoprecipitate with EGFP-Armadillo from S2 cells, suggesting that they retain the ability to interact directly or indirectly with Armadillo. mCherry alone does not co-immunoprecipitate with EGFP-Armadillo. (C and D) In the embryo, expression of the APC2 mutants was driven by the native APC2 promoter. (C) All of the APC2 mutants localize to the cell cortex and to the cytoplasm in embryonic epithelia. (D) Immunoblot of embryonic lysates probed with anti-GFP. Equal amounts of embryonic lysate were loaded in each lane. This demonstrated that while most of the mutant transgenes are expressed at levels comparable to APC2-FL, there were some exceptions. APC2-R3-R5SA (line 27) was expressed at very low levels. In the case of deletions, we obtained lines that express at levels higher than APC2-FL and lines that express at levels lower than APC2-FL. We have previously shown that APC2-FL expresses at levels comparable to endogenous APC2 (Zhou *et al.* 2011). The functional experiments shown in Figures 4–7 were conducted with all of the lines shown with the exception of those marked with an *. Bars, 10 μ m.

associate indirectly with Armadillo in a complex through Axin or wild-type APC2. When coexpressed with EGFP-Arm, the deletion mutants and the R1–R5 mutants could be co-immunoprecipitated with Armadillo (Figure 2B). This suggests that while we have disrupted specific residues and domains within APC2, the mutated forms of APC2 retain the ability to interact with other binding partners in the cell.

To test the functional significance of these domains in the destruction complex function of APC2, we generated transgenic flies expressing the EGFP-tagged APC2 mutants under the native APC2 promoter (McCartney *et al.* 2006). This has been previously shown to be sufficient to drive wild-type expression of APC2 transgenes (McCartney *et al.* 2006).

We introduced all of these transgenes into an APC2 null background (APC2^{g10}; Figure 3A) or into the APC2 APC1 double-null background (APC2^{g10} APC1^{Q8}; Figure 3B). Thus in contrast to some other structure-function types of studies with APC, in this study the mutant forms of APC2 are expressed at physiological levels in a background lacking all other APC2 or APC2 and APC1. Consistent with our observations in S2 cells, all of the APC2 mutant proteins expressed in APC2 null embryos are enriched at the cell cortex of embryonic epithelia similar to APC2-FL or the endogenous APC2 protein (Figure 2C and Zhou *et al.* 2011). Western blot demonstrates that expression of most of the proteins from the transgenes is comparable to endogenous

A APC2 Null Experiment

P= Transgene carrying wild type or mutant EGFP tagged APC2 under the control of its native promoter

APC2^{g10}= null allele of APC2 (McCartney *et al.*, 2006)

APC1^{Q8}= null allele of APC1 (Ahmed *et al.*, 1998)

B APC2 APC1 Double Null Experiment

Figure 3 Schematics of the genetic crosses employed in this study. In the APC2 null rescue experiment (A), all of the progeny are homozygous for the APC2 transgene (P) and maternally and zygotically null (MZ) for endogenous APC2. In the APC double-null rescue experiment (B), all of the progeny are homozygous for the APC2 transgene. Half of the progeny are maternally and zygotically null for the double-null chromosome (*FRT APC2^{g10} APC1^{Q8}*), and the other half are maternally double null and zygotically heterozygous for the double-null chromosome (MZ+). This figure is modified from Zhou *et al.* (2011).

APC2 (Figure 2D). One striking difference is APC2-R3–R5 SA that expresses at very low levels (Figure 2D and data not shown). While APC2-R3–R5 SA expresses at low levels in the embryo overall, we do find some variation in expression within the epithelium. We have shown these more highly expressing cells to demonstrate the cortical localization of the protein (Figure 2C). For the APC2-Δ15RΔ20RΔB and APC2-Δ20RΔB mutants, we tested two independent lines that express below the level of APC2-FL (low) or above the level of APC2-FL (high).

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

In *Drosophila*, APC2 and APC1 have redundant functions in Wnt signaling throughout development (Ahmed *et al.* 2002; Akong *et al.* 2002a,b). In the embryo, APC2 plays a primary role in the destruction complex, presumably due to its significantly higher expression level compared to APC1 (Ahmed *et al.* 1998, 2002; McCartney *et al.* 1999). However, complete loss of APC1 enhances the APC2 null Wnt-dependent embryonic phenotypes (Ahmed *et al.* 2002; Akong *et al.* 2002a). In the first set of experiments, we asked whether the APC2 deletion mutants could restore destruction complex activity in an APC2 null background where APC1 is wild type. We have previously shown that differences in Wnt pathway regulation can be observed for different APC2 mutant alleles in a background wild type for APC1 (McCartney *et al.* 2006).

Complete loss of *Drosophila* APC2 (*APC2^{g10}*) constitutively activates Wnt signaling in the embryo, resulting in embryonic lethality [0% hatch rate to the larval stage (McCartney *et al.* 2006)]. These embryos exhibit embryonic cuticle defects characteristic of excessive Wnt activation, including reduction in size due to excess apoptosis, an anterior hole due to a failure of head involution, and the production of excess smooth cuticle at the expense of denticles due to a Wnt-dependent

change in cell fate (McCartney *et al.* 2006). Due to phenotypic variability, we classify these phenotypes on the basis of a 0–6 scale and calculate an average cuticle phenotype (PA) for each genotype examined (see *Materials and Methods* and McCartney *et al.* 2006). The PA for *APC2^{g10}* embryos is 3.4 (Figure 4 and Supporting information, Figure S1). In addition, complete loss of APC2 is characterized by an accumulation of Arm in the embryonic epidermis (McCartney *et al.* 2006). Due to a segmentally repeated pattern of Wingless (Wg, a *Drosophila* Wnt) expression in the embryo, Arm normally accumulates in stripes that correspond to cells that are activating the pathway (Figure 5 and Peifer *et al.* 1994). All cells localize Arm to the adherens junction. In APC2 null embryos, all cells accumulate Arm equally and no stripes are observed (McCartney *et al.* 2006) (Figure 5). One molecular outcome of the uniform accumulation of Arm and activation of the Wnt pathway is the expansion of the expression domain of the Wg target gene Engrailed (Figure 5). Expression of APC2-FL in the *APC2^{g10}* embryos completely restores destruction complex activity, resulting in a 98% hatch rate, very weak cuticle defects in the few embryos that fail to hatch to larvae (PA = 0.2), Arm accumulation in stripes, and a wild-type pattern of En expression (Figures 4, A and B, and 5). The phenotypic profile for a given genotype is consistent between the different assays, although some variation does exist.

To test the hypothesis that the central repeats of APC2 are necessary for destruction complex function, we asked whether APC2-Δ15RΔ20RΔB and APC2-Δ20RΔB could rescue the hatch rate and cuticle defects associated with *APC2^{g10}*. We predicted that complete loss of these domains would result in a nonfunctional APC2 protein. Surprisingly, both deletion mutants provided enough activity to moderately rescue the hatch rate and cuticle phenotype of *APC2^{g10}* (Figure 4, A and B, and Table 1). The level of rescue was dependent on the expression levels of the transgenes (Figure 4, A and B, and

Figure 4 *APC2* deletion mutants suppress *APC2* null defects, but fail to rescue *APC2 APC1* double-null defects. (A–D) Hatch rate and embryonic cuticle analysis of *APC2* null embryos and *APC2 APC1* double-null embryos with transgenes expressing *APC2*-FL, *APC2*- $\Delta 15R\Delta 20R$, and *APC2*- $\Delta 20R\Delta B$. High/Low indicates whether the transgene was high or low expressing (refer to Figure 2D). Statistical comparisons of these data are shown in Tables 1 and 2. (A) Hatch rate of *APC2* null embryos alone or expressing indicated transgenes. (B) The cuticle phenotype of embryos that failed to hatch was assessed and a phenotypic average (PA) was calculated for *APC2* null embryos and *APC2* null embryos expressing the indicated transgenes. (C) Hatch rate of *APC2 APC1* double-null embryos alone or expressing indicated transgenes. (D) The cuticle phenotype of embryos that failed to hatch was assessed and a PA was calculated for *APC2 APC1* double-null embryos and *APC2 APC1* double-null embryos expressing the indicated transgenes. Cuticles were classified as either MZ+ (maternally null and zygotically heterozygous) or MZ (maternally and zygotically null). (E) Representative cuticles for indicated genotypes. Orientation: anterior is toward the top and ventral is either up or to the right. The PA and the class (MZ or MZ+) are shown for each genotype. All cuticles are shown at the same scale.

Table 1). Higher expression of the deletion mutants significantly reduced the degree of rescue (Figure 4, A and B, and Table 1). In addition, *APC2*- $\Delta 20R\Delta B$ provided more significant rescue than *APC2*- $\Delta 15R\Delta 20R\Delta B$, suggesting that 15R-dependent Arm binding plays a role in the Wnt signaling function of *APC2*. Analysis of Arm accumulation and En expression revealed a consistent trend relative to the hatch rate and cuticle phenotypes; stripes of Arm accumulation were weakly restored and the En expression domain was weakly reduced compared to *APC2^{g10}* (Figure 5). Taken together, these data suggest that deletion of

either the 20Rs alone or both the 15Rs and 20Rs results in an *APC2* protein that retains some function in the destruction complex in the presence of *APC1*.

We predicted that the surprising ability of *APC2*- $\Delta 15R\Delta 20R\Delta B$ and *APC2*- $\Delta 20R\Delta B$ to provide some rescue in the *APC2* null might be due to the presence of *APC1*. Thus, to further challenge the function of *APC2*- $\Delta 20R\Delta B$ and *APC2*- $\Delta 15R\Delta 20R\Delta B$, we tested their ability to rescue the Wnt-dependent defects in the *APC* double-null background (*APC2^{g10} APC1^{Q8}*). In the *APC* double-null background,

Figure 5 Armadillo and Engrailed expression in wild-type and *APC2* null embryos expressing *APC2* wild-type and mutant transgenes. Representative embryos of indicated genotypes stained for Armadillo or Engrailed are shown. In the case of embryos expressing transgenes, the genotype is *P[endoP-EGFP-X]; APC2^{g10}*, where endoP is the native *APC2* promoter and X indicates a particular *APC2* transgene. “High” refers to the expression level of the transgene (refer to Figure 2D). Orientation: anterior is to the left and dorsal is up. Representative high-magnification images of two stripes were selected at the same dorso-ventral position for comparison. Bar, 25 μ m.

the *APC2* mutants are responsible for all destruction complex activity. The *APC* double-null embryos were generated using the FRT/FLP/DFS technique (Chou and Perrimon 1996). On the basis of this method, 50% of the embryos were maternally

and zygotically double null for *APC* (MZ), and 50% were maternally double null but zygotically heterozygous (MZ⁺; Figure 3B). In the absence of any rescuing transgene, 23% of the progeny hatched into larvae, and 25% displayed weak cuticle

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

Genotype	Hatch rate (%)	0–1 (%)	1.5–2.5 (%)	3–4 (%)	4.5–6 (%)	PA	Category ^a
<i>APC2-FL; APC2^{g10}</i>	98	2				0.2	1
<i>APC2 R3-R5 SD; APC2^{g10}</i>	97	3	<1			0.6	1
<i>APC2 R3-R5 SA; APC2^{g10}</i>	93	7				0.2	2
<i>APC2 R3-R5 ExR; APC2^{g10}</i>	91	8	1			0.3	2
<i>APC2 R1-R5 SD; APC2^{g10}</i>	89	11	<1			0.5	3
<i>APC2 Δ20RΔB(low); APC2^{g10}</i>	85	12	2	1		0.9	3
<i>APC2 R1-R5 SA; APC2^{g10}</i>	72	18	<1			0.5	4
<i>APC2 Δ15RΔ20RΔB(low); APC2^{g10}</i>	68	15	8	7		1.8	5
<i>APC2 Δ20RΔB(high); APC2^{g10}</i>	52	31	17	<1		1.1	6
<i>APC2 R1-R5 ExR; APC2^{g10}</i>	34	50	16			1.0	7
<i>APC2 Δ15RΔ20RΔB(high); APC2^{g10}</i>	36	18	15	31		2.4	8
<i>APC2^{g10}</i>	0	14	6	74	6	3.4	9

The *n* for the hatch rate experiments was >200. For cuticle analysis, *n* > 19.

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

defects (ranging from 0 to 2.5; Figure 4, C–E, and Table 2). These progeny represent the MZ⁺ class and display relatively weak phenotypes due to the paternal contribution of an APC2⁺ APC1⁺ chromosome (Figure 4, D and E, and data not shown). The remaining 52% of the progeny are maternally and zygotically null (the MZ class). These are embryonic lethal and display strong cuticle defects (ranging from 3 to 6; Figure 4, D and E, and Table 2). Expression of APC2-FL in the double-null background resulted in a partial rescue with an overall shift in the severity of the phenotype toward wild type: 46% of the progeny hatch and the remaining progeny exhibit a very weak cuticle phenotype (Figure 4, D and E, PA = 0.7). This suggests that all of the MZ⁺ embryos hatch, and the MZ embryos die with a significantly suppressed phenotype. Interestingly, neither APC2-Δ20RΔB nor APC2-Δ15RΔ20RΔB provided any activity in the APC double-null background. The hatch rate and cuticle phenotype of the progeny were indistinguishable from the double null alone (Figure 4, C–E, and Table 2). These results suggest that direct APC2–Arm interactions are essential for APC2's destruction complex function, consistent with previous work. Furthermore, the simplest interpretation of the ability of the APC2 deletion mutants to function only in the presence of APC1 is that APC2 and APC1 act cooperatively, rather than in independently functioning destruction complexes (see Discussion).

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

To test the hypothesis that phosphorylation of the 20Rs of APC2 is essential for its destruction complex function, we tested the ability of the phospho-dead (SA) and phospho-mimetic (SD) forms of APC2 to rescue the Wnt-dependent defects in APC2 null and in APC double-null embryos. Mutants that alter phosphorylation in 20R3–20R5, but leave 20R1 and 20R2 unchanged (APC2-R3–R5SA and APC2-R3–

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

Examination of the activity of APC2-R1–R5SA and APC2-R1–R5SD in the APC double-null background revealed an intriguing pattern of rescue. In both cases, the lethality was suppressed, resulting in a hatch rate of ~50%—very close to that provided by APC2-FL (Figure 6C; Table 2). However, when we examined the cuticle defects of the remaining embryos, we found that the vast majority exhibited a very severe phenotype characteristic of the MZ

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

Genotype	Hatch rate (%)	Weak cuticle class			Strong cuticle class			Category ^a
		0–1 (%)	1.5–2.5 (%)	PA	3–4 (%)	4.5–6 (%)	PA	
APC2-FL; APC2 ^{g10} APC1 ^{Q8}	46	45.5	6	0.7	2	0.5	NA	1
APC2 R3–R5 SA; APC2 ^{g10} APC1 ^{Q8}	68	3	23	1.8	6	0	NA	1
APC2 R3–R5 SD; APC2 ^{g10} APC1 ^{Q8}	40	57	0	0.5	3	0	NA	1
APC2 R3–R5 ExR; APC2 ^{g10} APC1 ^{Q8}	50	50	0	0.5	0	0	NA	1
APC2 R1–R5 SA; APC2 ^{g10} APC1 ^{Q8}	51	2	1	NA	40	6	4.2	2
APC2 R1–R5 SD; APC2 ^{g10} APC1 ^{Q8}	49	2	0	NA	38	11	4.4	2
APC2 R1–R5 ExR; APC2 ^{g10} APC1 ^{Q8}	21	15	16	1.4	26	22	4.5	3
APC2 Δ20RΔB; APC2 ^{g10} APC1 ^{Q8}	24	12	4	0.7	58	2	4.0	3
APC2 Δ15RΔ20RΔB; APC2 ^{g10} APC1 ^{Q8}	18	15	13	1.3	50	4	4.0	3
APC2 ^{g10} APC1 ^{Q8}	23	16	8	1.2	42	11	4.3	3

The *n* for the hatch rate experiments was >100. For cuticle analysis, *n* > 40. NA, not applicable.

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

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

class (PA = 4.2–4.4; Figure 6, D and E, and Table 2). This is in contrast to APC2-FL where the remaining embryos exhibit very weak cuticle defects (PA = 0.7; Figure 6, D and E, and Table 2). These data suggest that APC2-R1–R5SA and APC2-R1–R5SD have no function in the absence of all APC2 and APC1, as ~50% of the progeny in the APC double-null experiment exhibit no rescue. However, in the presence of the paternally provided APC2 and APC1 (MZ+), APC2-R1–R5SA and APC2-R1–R5SD appear to rescue as well as APC2-FL (Table 2).

Taken together with the analysis of the deletions, we find that R3–R5 SA and SD are largely indistinguishable from APC2-FL (Figures 4–6; Tables 1 and 2). APC2 R1–R5 SA and SD are similar to the deletions in that they fail to rescue in the absence of all APC. However, the phospho-mutants

appear to provide more function than the deletions in the APC2 null embryos. This difference may reflect the dominant negative activity of the deletions. Furthermore, APC2 R1–R5 SA and SD are able to rescue the MZ+ class in the double-null experiment to hatching, while the deletion mutants have no effect on this class (Figures 4–6; Tables 1 and 2).

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

In the absence of phosphorylation, APC interacts with β cat through the N-terminal extended region of a 20R (Ha *et al.* 2004; Xing *et al.* 2004). To test the role of this interaction in the destruction complex function of APC2, we disrupted the conserved residues either in the extended region (Figure 1C)

Table 3 Summary of characterization of APC2 proteins and their ability to rescue destruction complex function in APC2 null and APC2 APC1 double-null embryos

Genotype	Cortical localization in S2 cells and embryos	Co-IP with Arm from S2 cells	Rescue of APC2 null defects in Wnt signaling	Rescue of APC double-null defects in Wnt signaling in the MZ ⁺ progeny	Rescue of APC double-null defects in Wnt signaling in the MZ progeny
<i>Wild type</i>	+	+	NA	NA	NA
<i>APC2-FL</i>	+	+	++	+	+
<i>APC2R3-R5SA</i>	+	+	++	+	+
<i>APC2R3-R5SD</i>	+	+	++	+	+
<i>APC2R3-R5ExR</i>	+	+	++	+	+
<i>APC2R1-R5SA</i>	+	+	+	+	—
<i>APC2R1-R5SD</i>	+	+	+	+	—
<i>APC2R1-R5ExR</i>	+	+	+/-	—	—
<i>APC2-Δ20RΔB</i>	+	+	+/-	—	—
<i>APC2-Δ15RΔ20RΔB</i>	+	+	+/-	—	—

NA, not applicable.

of R3–R5, leaving R1 and R2 intact (APC2-R3–R5ExR), or in all five repeats (APC2-R1–R5ExR). Consistent with what we observed with the phospho-mutants, mutation of R3–R5 alone resulted in a protein that was largely indistinguishable from APC2-FL (Figures 5 and 7, A and B; Table 1). Again, this shows that R1–R2 interactions are sufficient for APC2's destruction complex function. In contrast, disruption of the extended region in all of the repeats significantly reduced activity in the destruction complex. In the APC2 null background, the hatch rate was only modestly rescued (34%) and the cuticle phenotype was suppressed (Figure 7, A and B, Figure S1, and Table 1). Consistent with this weak rescue, the En expression domain remained expanded and Arm accumulation was apparent (Figure 5). This rescue profile is more similar to that of the deletions than to that of the phospho-mutants (Table 1). Consistent with that observation, in the APC double-null background APC2-R1–R5ExR provided no rescue (Figure 7, C–E; Table 2), similar to Δ20RΔB or Δ15RΔ20RΔB (Figure 4, C–E; Table 2). Taken together, these results suggest that the extended region interactions may play a more significant role in APC2's destruction function than phospho-based interactions *in vivo*.

Discussion

Despite significant interest in understanding the mechanisms that govern the regulation of Wnt signaling, there are numerous outstanding questions regarding the fundamental molecular mechanisms that promote the degradation of βcat and the role that APC proteins play. We have tested the model that (1) the 20Rs with the highest affinity for Arm are essential for APC2's destruction complex activity, (2) phosphorylation of APC2 is essential for destruction complex activity, and (3) the 20R extended region plays a role in destruction complex activity. Our findings have revealed not only that the high-affinity binding sites are not essential, contrary to previous predictions (Liu *et al.* 2006), but also that multiple interactions between APC2 and Arm are nec-

essary for destruction complex function. Finally, our data strongly suggest that APC proteins self-associate in the destruction complex with implications for complex assembly and function.

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

20R3 of vertebrate APC has the highest affinity for βcat *in vitro*, suggesting that it may play the most significant role in the APC–βcat interaction (Liu *et al.* 2006). Comparison of vertebrate 20R3 to the 20Rs of *Drosophila* APC2 revealed that 20R3, 20R4, and 20R5 were indistinguishable relative to their conservation with vertebrate 20R3 (Figure 1C). Thus, we predicted that disruption of 20R3–R5 would significantly impair destruction complex function. We were surprised to find that all of our 20R3–R5 mutants (SA, SD, and ExR) have nearly wild-type destruction complex activity in both the APC2 single mutant and the APC2 APC1 double mutant (Table 3). This indicates that 20R1 and 20R2 are sufficient for APC2's destruction complex function. 20R1 is similar to 20R3–R5 in that the key residues in the extended region are well conserved, as are the key phosphorylation residues (Figure 1C). 20R2 has some interesting differences; while it is similar to 20R1 within the phospho-region, 20R2 is missing the key salt bridging residues found in the other repeats (Figure 1C). Although vertebrate 20R2 does not appear to bind to βcat (Choi *et al.* 2006; Liu *et al.* 2006; Kohler *et al.* 2008), Rubinfeld *et al.* (1997) suggested that vertebrate 20R2 does play a role in βcat destruction in SW480 colon cancer cells. Consistent with this idea, Roberts *et al.* (2011) recently demonstrated that deletion of 20R2 alone produces a protein that is unable to promote βcat degradation in either SW480 cells or the *Drosophila* embryo. Together with their other observations, the authors suggest that 20R2 and the adjacent region B [also known as CID (Kohler *et al.* 2009)] together form the binding site for an unknown destruction complex protein.

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

Researchers have often pondered the question of why APC has so many 20Rs. In cancer, typically five or more of the 20Rs are deleted, leaving approximately two 20Rs and all of the 15Rs (Polakis 1997). Rubinfeld *et al.* (1997) demonstrated that while only one 20R of vertebrate APC was required for binding to β cat, three 20Rs were necessary for the downregulation of β cat, and four or more were required for complete activity. The picture that has emerged from more recent studies is that not all 20Rs are created equal and that in some cases they may have distinct roles to play in the regulation of β cat. Our work and that of Roberts *et al.* (2011) suggest that the differences in 20R function are unlikely due exclusively to differences in binding affinity to

β cat. Some may promote β cat degradation through direct β cat binding, or through other binding partners, and some may participate in other functions such as the cytoplasmic retention of β cat (Roberts *et al.* 2011) that may negatively regulate its transcriptional function.

The role of *APC2* in the cytoplasmic retention of Armadillo

Roberts *et al.* (2011) proposed that APC negatively regulates Wnt signaling not only by promoting β cat destruction, but also by retaining β cat in the cytoplasm. In this way, APC acts as a brake on Wnt signaling by preventing the transcriptional activity of nuclear β cat. In light of this model, Roberts

et al. (2011) proposed an explanation for why some APC2 mutants suppress Wnt signaling in APC2 null embryos, but not in double-null embryos as we have also observed (Figure 5). In the APC2 null background, the level of cytoplasmic Arm is modestly increased. If an APC2 mutant has retention ability it will retain that excess Arm in the cytoplasm, suppress Wg signaling, and rescue the defects. In APC double-null embryos, the level of cytoplasmic Arm is too high for cytoplasmic retention alone to prevent transcriptional activation of Wnt targets. Roberts *et al.* (2011) suggested that the 15Rs and 20Rs are additively responsible for the cytoplasmic retention of β cat.

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

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

An alternative explanation for the single-null vs. double-null rescue differences is the ability of APC2 and APC1 to act cooperatively in the destruction complex. Expression and genetic analysis in the embryo revealed that APC2 is the primary APC protein in the embryonic epidermis, but that nearly undetectable amounts of APC1 also contribute to destruction complex function in that tissue (Ahmed *et al.* 2002; Akong *et al.* 2002b). The fact that APC2 is enriched at the cortex and is found in cytoplasmic puncta (McCartney *et al.* 1999), while APC1 appears to localize to centrosomes and microtubules (Akong *et al.* 2002a,b), suggested that APC2 and APC1 reside in independent destruction complexes localized in distinct subcellular compartments. One caveat to

this interpretation is that APC1 could be detected only when overexpressed and that overexpression could influence its subcellular distribution. Our observations of rescue in APC2 null vs. APC2 APC1 double-null embryos (this work and Zhou *et al.* 2011) suggest that we reevaluate the relationship between APC2 and APC1. The strongest APC2 mutants, including the deletions and all of the R1–R5 mutants, provide some rescue in APC2 null embryos, but no rescue in the complete absence of APC1 (Figure 6). If the activity in APC2 null embryos was due to the combined action of APC1-based destruction complexes and independent APC2 mutant-based destruction complexes, taking APC1 away would not affect the activity of the APC2 mutant destruction complexes. In the APC2 APC1 double-null background, however, the strongest APC2 mutants provide no APC-dependent destruction complex activity (Figure 4). This suggests that APC1 and APC2 may be present in the same destruction complexes.

We propose that the basis for this cooperativity is APC self-association within the destruction complex and that self-association is necessary for destruction complex assembly and/or activity. We have recently shown that *Drosophila* APC2 self-associates via the Armadillo repeats (Zhou *et al.* 2011). Because APC1 also contains the conserved Armadillo repeats (Figure 1B), we predict that APC2 and APC1 may have the ability to form hetero-oligomers (Figure 8A). There is no precedent in the literature for direct association of Arm repeats, suggesting the activity of a linker protein. Consistent with the idea of self-association, overexpression of APC1 in the *Drosophila* embryo or larval brain redistributes APC2 from the cell cortex to the centrosome and microtubules where APC1 is localized, and overexpression of APC2 increases cortical localization of overexpressed APC1 (Akong *et al.* 2002a,b). Self-association may also explain why high levels of the APC2 deletion mutants have a dominant negative effect on rescue (Figure 4, A and B): because APC1 expression is low in the embryo, overexpressed APC2 deletions form homo-oligomers that are nonfunctional and compete for Axin binding in the destruction complex. If APC2 and APC1 associate in the destruction complex, they should colocalize, but we observe this colocalization only when APC1 is overexpressed. Furthermore, our studies of APC2 (Zhou *et al.* 2011), combined with other work on Axin (Cliffe *et al.* 2003), strongly suggest that the destruction complex exists as cytoplasmic puncta. Endogenous APC2 localizes to cytoplasmic puncta (McCartney *et al.* 1999), and given the findings presented here, we predict that endogenous APC1 is found there as well, below the level of detectability.

Vertebrate APC self-associates through at least three distinct domains not conserved in *Drosophila* APCs, including the N-terminal dimerization domain (Figure 1A) (Joslyn *et al.* 1993; Su *et al.* 1993a; Day and Alber 2000). Furthermore, Li *et al.* (2008) have shown that a novel domain C terminal to the Arm repeats (N3) and the last 300 amino acids of the protein (C3) also promote self-association.

Figure 8 Models of interactions within the destruction complex (A) and the catalytic cycle of the destruction complex (B). (A) On the basis of the ability of Axin and APC to self-associate *in vivo*, we predict that these interactions contribute to the assembly of the functional “destructosome”. We have depicted Axin and APC here as dimers, but the formation of higher-order assemblages has not been ruled out. (B) Step 1: the APC-Axin-kinase complex assembles onto the Axin scaffold. APC is in its non-P state. Step 2: βcat enters the complex via interactions with Axin and Arm repeats 1–5 and with the extended region of a 20R of APC through Arm repeats 5–9. Step 3: GSK3β and CKI phosphorylate both βcat and APC. This results in the loss of the Axin-βcat interaction as P-APC outcompetes Axin. Step 4: The P-APC-P-βcat complex dissociates from Axin. Step 5: Proteasome-associated chaperones separate APC and βcat. βcat proceeds to degradation by the proteasome while P-APC is released. Step 6: P-APC is dephosphorylated, perhaps via PP2A. It is now able to reenter the Axin-kinase complex.

These N3–C3 and N3–N3 interactions of APC play a role in the regulation of peripheral clusters of APC in vertebrate cultured cells (Li *et al.* 2008). Within the basic domain, ANS2 promotes dimerization and is implicated in the actin nucleation function of APC *in vitro* (Okada *et al.* 2010). Despite the abundant evidence that APC proteins can form dimers, a role for dimerization in APC’s destruction complex function has not been demonstrated.

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

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

Speculative models have suggested that APC with phosphorylated 20Rs (P-APC) functions in multiprotein complex assembly (Ha *et al.* 2004) or as an essential step in a catalytic cycle (Kimelman and Xu 2006; Roberts *et al.* 2011). Our *in vivo* data indicate that phosphorylation of the 20Rs is essential for the normal destruction of Arm. In the absence of all

other APC, the 20R1–R5SA mutant provides no destruction activity consistent with an essential role in complex assembly or promotion of a catalytic cycle. To test whether P-APC is sufficient, we generated the phospho-mimetic form of APC2 where the conserved Ser and Thr in the 20Rs were altered to aspartic acid (Figure 1C). We found that the APC2 20R1–R5SD mutant acts as a loss of function: when APC2 20R1–R5SD is the only APC protein present, embryos express phenotypes consistent with ligand-independent activation of Wnt targets (Figure 6). Taken together, these data indicate that both P-APC2 and non-P-APC2 are required for destruction complex function.

When APC is not phosphorylated, it binds βcat through the 20R extended region (Ha *et al.* 2004; Xing *et al.* 2004). When phosphorylated, APC binds through both the extended region and through the phospho-region, increasing the binding affinity between APC and βcat (Ha *et al.* 2004; Xing *et al.* 2004). The fact that APC2 20R1–R5SA and APC2 20R1–R5SD mutants are nonfunctional despite their ability to interact through the extended region suggests that the extended region interactions are not sufficient for APC2’s destruction complex function. Our data indicate that the extended region interactions are necessary for normal destruction function. In the complete absence of all extended region interactions (APC2 20R1–R5ExR), the mutant APC2 has no activity in the absence of endogenous APC2 and APC1 (Figures 5 and 7). In fact, the APC2 20R1–R5ExR mutant that still retains the ability to be phosphorylated has less activity than either of the APC2 20R1–R5 phospho-mutants; it provides significantly less rescue in APC2 null embryos (Table 1). In double-null embryos the APC2 20R1–R5 phospho-mutants rescue the zygotically heterozygous class (MZ⁺) to hatching,

whereas the APC2 20R1–R5ExR mutant has no effect on either the MZ⁺ or the MZ class (Table 2). Taken together, our data are consistent with a model for destruction complex function that requires both P-APC and non-P-APC and requires binding of the extended region to Arm.

The model of destruction complex assembly and action

Using other models as a foundation (Ha *et al.* 2004; Kimelman and Xu 2006; Roberts *et al.* 2011), we propose the following model for the assembly and activity of the destruction complex (Figure 8B). We predict that the APC–Axin–kinase complex preassembles in part through interactions between the SAMP repeats of APC2 and the RGS domain of Axin (Figure 8B, step 1). This is consistent with an essential role for the SAMP repeats in APC2's destruction complex function (Roberts *et al.* 2011) and an essential role for the APC-binding RGS domain of Axin in the mouse (Chia *et al.* 2009). Preassembly of an APC–Axin–kinase complex is also consistent with the fact that when cytoplasmic levels of β cat are low (in the absence of signal), the affinity between non-P-APC and β cat is too low to promote their direct binding (Ha *et al.* 2004). Here, β cat assembles into the complex (Figure 8B, step 2) through the interaction with Axin via Arm repeats 3–5 and the extended region of the 20Rs through Arm repeats 5–9. While others have proposed that the 15Rs of APC serve in this function (Kimelman and Xu 2006), Roberts *et al.* (2011) have shown that the 15Rs are dispensable for APC2 function in the embryo in the presence of intact 20Rs. Further, as we have shown that the extended region is necessary, we favor a model in which the extended region of the 20Rs plays this role. Phosphorylation of the N-terminal sites in Arm, and the 20Rs of APC by CK1 and GSK3 β , displaces P-Arm from Axin and transfers it to P-APC (Figure 8B, step 3). The notion that APC is phosphorylated within the complex is consistent with the findings that the direct binding of Axin to APC stimulates GSK phosphorylation of APC and that the presence of β cat further enhances APC phosphorylation (Ikeda *et al.* 2000). The displacement from Axin and disassembly of the complex (Figure 8B, step 4) also involve the action of 20R2 specifically and region B (Roberts *et al.* 2011). Because PP2A associates with the complex via Axin, it has been proposed that it may dephosphorylate APC within the complex, facilitating the turnover of β cat to the proteasome (Xing *et al.* 2003). The inability of PP1 to dephosphorylate APC in the presence of β cat *in vivo* suggests that phospho-APC may not be accessible to phosphatase within the complex (Ha *et al.* 2004). Instead, the interaction with the proteasome machinery may act to separate P-APC from P-Arm (Ha *et al.* 2004) (Figure 8B, step 5), sending P-Arm farther down the path to proteasomal degradation and releasing P-APC to be dephosphorylated (Figure 8B, step 6). Non-P-APC is then available to reassemble with Axin and reinitiate the cycle.

In conclusion, the destruction complex of the Wnt signaling pathway plays a vital role in the negative regulation of Wnt signaling. Loss of this regulatory mechanism con-

tributes to diseases such as colon cancer. As a complex molecular machine, it has been difficult to dissect its many moving parts to determine how the destruction complex assembles and how precisely these different parts contribute to β cat phosphorylation and subsequent degradation by the proteasome. The combination of biochemical and structural studies with rigorous *in vivo* testing is now beginning to reveal important factors that govern the activity of this essential molecular machine.

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Note added in proof: Consistent with our model that APC1 and APC2 can homo-dimerize via their Arm repeats, Mattie *et al.* (2010) has shown by yeast two-hybrid analysis that these domains can interact.

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GENETICS

Supporting Information

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Destruction Complex Function in the Wnt Signaling Pathway of *Drosophila* Requires Multiple Interactions Between Adenomatous Polyposis Coli 2 and Armadillo

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