Cortical localization of APC2 plays a role in actin organization but not in Wnt signaling in *Drosophila*

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Accepted 17 January 2011
Journal of Cell Science 124, 1589-1600
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doi:10.1242/jcs.073916

Summary

The tumor suppressor Adenomatous polyposis coli (APC) has roles in both Wnt signaling and in actin and microtubule organization. Within the cell, APC proteins have been reported to localize in the cytoplasm, at the cell cortex and in the nucleus. How these localizations relate to the functions of the protein is an aspect of APC biology that is poorly understood. Using *Drosophila* S2 cells, we have dissected the structural and functional requirements for the cortical localization of *Drosophila* APC2. Here, we show that both the Armadillo repeats and a novel C-terminal domain are necessary for the cortical localization of APC2 in S2 cells and in the embryo, and that neither domain alone is sufficient for this localization. Furthermore, we show that the Armadillo repeats mediate self-association of APC2 molecules. To test the function of the cortical localization of APC2, we asked whether an APC2 protein deleted for the C-terminal localization domain could rescue APC mutant defects in Wnt signaling and actin organization in the *Drosophila* embryo. We show that although cortical localization is required for the APC2 function in organizing actin, cortical localization is dispensable for its role in regulating Wnt signaling.

Key words: Adenomatous polyposis coli, Wnt signalling, Actin, *Drosophila*, Cortex

Introduction

Adenomatous polyposis coli (APC) was first identified as a human colon cancer tumor suppressor associated with both sporadic and inherited forms of the disease (Groden et al., 1991; Joslyn et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). Extensive study revealed that APC proteins play essential roles in the negative regulation of Wnt signaling and in the organization and regulation of the actin and microtubule cytoskeletons. Despite this extensive study, there are still many unanswered questions about the mechanisms by which APC proteins affect these cellular functions, and why the disruption of these functions promotes tumorigenesis. One of these unanswered questions is how the subcellular localizations of APC proteins contribute to their functions.

In the absence of a Wnt signal, the 'destruction complex' prevents transcriptional activation of Wnt targets. This destruction complex, composed of a core of proteins including APC, GSK-3β and Axin, binds to and phosphorylates the key effector β-catenin, targeting it for destruction by the proteosome. Within this complex, APC binds directly to β-catenin, through its 15- and 20-amino-acid repeats (15Rs and 20Rs), and to Axin through its SAMP repeats (Fig. 1A). Although its exact subcellular location is not clear, the destruction complex may reside in cytoplasmic Axin puncta (Faux et al., 2008; Schwarz-Romond et al., 2005; Cliffe et al., 2003). APC proteins also localize to the cell cortex in a variety of cell types (Harris and Nelson, 2010; Grohmann et al., 2007; Maher et al., 2009; McCartney et al., 1999; Yu et al., 1999). Mutations in *Drosophila* APC2 that interfere with its cortical localization also affect its destruction complex function, suggesting that some destruction complex activity resides at the cortex (McCartney et al., 2006). Some studies of mammalian APC have supported this hypothesis (Maher et al., 2009). In addition to the cortex and the cytoplasm, APC is also present in the nucleus. There it might sequester nuclear β-catenin from the transcriptional machinery, preventing its interaction with TCF/LEF family of transcription factors necessary for activating Wnt target genes (reviewed in McCartney and Nathke, 2008). Upon receipt of a Wnt ligand, the receptors Frizzled and LRP6 are thought to promote the deactivation of the destruction complex, in part by sequestering Axin at the cortex through association with LRP6 (reviewed in Angers and Moon, 2009). This results in an accumulation of β-catenin that promotes the transcriptional activation of Wnt target genes.

In addition to its role as a negative regulator of Wnt signaling, APC proteins are implicated in cell migration and maintaining chromosome stability. In migrating cells, APC accumulates at the leading edge, interacting with a variety of cytoskeletal regulators to stabilize microtubules and/or promote actin polymerization. APC can bind microtubules directly (Munemitsu et al., 1994; Smith et al., 1994), or indirectly via EB1 (Su et al., 1995), to stabilize microtubules and promote cell migration (Nathke et al., 1996; Wen et al., 2004). Endogenous APC primarily associates with the plus-end tips of microtubules as 'clusters' in active membrane protrusions (Li et al., 2008; Matsui et al., 2008; Mimori-Kiyosue et al., 2000; Nathke et al., 1996). In addition, in polarized epithelia, APC localizes along the lengths of basal cortex microtubules and at the basal cortex itself, where it guides the formation of the basal microtubule network (Reilein and Nelson, 2005). APC also affects actin in migrating cells through interactions with effectors of Rho family GTPases, namely Asef and IQGAP (Kawasaki et al., 2000; Watanabe et al., 2004). Finally, APC promotes microtubule–kinetochore attachments in the nucleus and plays a role in maintaining chromosome stability during mitosis (Fodde et al., 2001; Kaplan et al., 2001; Zhang et al., 2007). Thus, APC proteins reside in distinct subcellular compartments: the cortex, the cytoplasm and the nucleus. How APC localizes to these compartments and how the localization affects APC function are not fully understood.

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Drosophila APC2 localizes to the cortex and to the cytoplasm (McCartney et al., 1999; Yu et al., 1999). It contains the conserved N-terminal Armadillo (Arm) repeats, and the 15Rs, 20Rs and SAMP repeats, but is missing the oligomerization, basic and EB1-binding domains, which are characteristic of vertebrate APC (Fig. 1A). We have shown previously that mutations in APC2 that disrupt cortical enrichment also disrupt destruction complex activity (McCartney et al., 2006). In addition, we have shown that APC2 together with the formin Diaphanous (Dia) promotes actin pseudocleavage furrow extension in the syncytial *Drosophila* embryo (Webb et al., 2009). Cortical enrichment of APC2 with actin plays a role in this process, as disruption of the cortical enrichment of APC2 by its mutation or by loss of Dia is correlated with furrow extension defects (Webb et al., 2009). These findings suggest that cortical enrichment plays an important role in both the Wnt signaling and the cytoskeletal functions of APC2. To test this hypothesis, we identified the domains of APC2 that are necessary and sufficient for cortical enrichment in *Drosophila* cells. Here, we show that the Armadillo repeats of APC2 and a novel domain consisting of its C-terminal 30 amino acids are necessary and sufficient for cortical enrichment. Furthermore, we show that although cortical enrichment of APC2 is required for the extension of actin pseudocleavage furrows in the *Drosophila* embryo, cortical enrichment of APC2 is dispensable for the negative regulation of Wnt signaling during embryonic development.

Results

Drosophila S2 cells have cellular machinery sufficient for the cortical enrichment of APC2

To dissect the proteins and domains necessary for cortical enrichment of APC2, we used Schneider's *Drosophila* cell line 2 (S2 cells) (Schneider, 1972). mCherry–APC2 (mCh-APC2) or EGFP–APC2 expressed in S2 cells under either the metallothionein promoter or the native APC2 promoter (McCartney et al., 2006), respectively (Fig. 1E,F), localized to the cell cortex in a manner similar to F-actin (Fig. 1C) and in a pattern comparable to that of the endogenous protein in the embryo (Fig. 1B). Expressed APC2 was sometimes observed in cytoplasmic puncta (Fig. 1F, arrow) that could be the result of endocytosis or phagocytosis. mCherry alone was found throughout the cell, with no apparent areas of enrichment (Fig. 1D). Unless indicated, all APC2 variants in S2 cells were tagged with mCherry and expressed under the metallothionein promoter. Because APC2 localized to cortical actin in S2 cells (Fig. 1G), we tested whether actin plays a role in this cortical enrichment of APC2. Cytochalasin D treatment disrupted the cortical actin network and the cortical enrichment of APC2 (Fig. 1H). Thus, actin is required to maintain the cortical enrichment of APC2 in S2 cells, consistent with reports in other systems (Rosin-Arbesfeld et al., 2001; Townsley and Bienz, 2000). These observations indicate that S2 cells have cellular machinery sufficient for the cortical enrichment of APC2 and can serve as a simple model system for studying cortical enrichment mechanisms.

Defining the domains of APC2 required for cortical enrichment

Vertebrate APC forms a complex with both α-catenin and β-catenin, suggesting that APC may associate with the adherens junction (Rubinfeld et al., 1993; Su et al., 1993b). Furthermore, α-catenin and Arm (the *Drosophila* β-catenin) might contribute to the association of APC2 with the cortical actin network in *Drosophila* syncytial embryos (McCartney et al., 2001). Axin, another component of the destruction complex, has also been implicated in the cortical enrichment of APC2 (Cliffe et al., 2003). However, knockdown of these proteins in S2 cells using RNA interference (RNAi) did not disrupt the cortical localization of APC2 (supplementary material Fig. S1).

To define the APC2 domains required for cortical enrichment, we examined the localization of mCh-APC2 fragments in S2 cells. Localization of APC2 mutant proteins in the embryo had previously suggested that both the N-terminal and C-terminal halves of APC2 are important for cortical enrichment (McCartney et al., 2006). Thus, we first divided APC2 into two halves. The N-terminal half

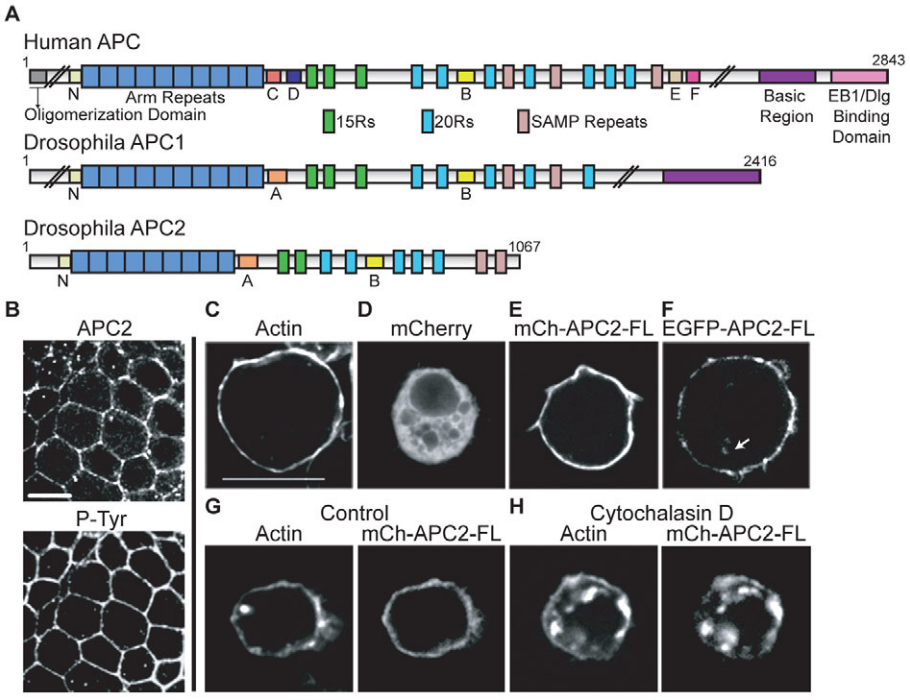

Fig. 1. S2 cells retain the machinery sufficient for cortical localization of APC2. (**A**) Schematic of human APC1 and *Drosophila* APC1 and APC2. (**B**) Wild-type APC2 is enriched at the apical cell cortex and is cytoplasmic in *Drosophila* embryonic epithelia. An antibody against phosphorylated tyrosine (P-Tyr) labels the cell cortex. (**C**) A fixed S2 cell stained with phalloidin reveals cortical actin. (**D**) mCherry alone localizes throughout the cytoplasm. mCh-APC2-FL expressed under either the *metallothionein* promoter (**E**) or the endogenous *APC2* promoter (**F**) is enriched at the cortex. Fixed control (**G**) and cytochalasin-D-treated (**H**) S2 cells expressing mCh-APC2-FL imaged for actin (phalloidin) and mCherry reveal that the cortical enrichment of APC2 depends upon actin. Scale bars: 10 μm.

(mCh-APC2-N) contains the N-terminal conserved region (N), the conserved region A (A) and the Arm repeats, a domain highly conserved among all APC proteins (Fig. 1A, Fig. 2A). The C-terminal half (mCh-APC2-C) contains the well-conserved 15Rs, 20Rs and the SAMP repeats (Fig. 1A, Fig. 2A). *Drosophila* APC2 does not contain the basic domain or the EB1-binding domains found in other APC proteins (Fig. 1A). When expressed in S2 cells, neither mCh-APC2-N nor mCh-APC2-C was strongly enriched at the cortex, unlike mCh-APC2-FL (full length) (Fig. 2C–E), indicating that both halves are required for the cortical enrichment of APC2.

To dissect further the C-terminal half, we deleted the 137 C-terminal amino acids, including the SAMP repeats (mCh-APC2-ΔSAMP; Fig. 2A). We refer to these sequences combined as the SAMP domain. mCh-APC2-ΔSAMP failed to enrich at the cortex (Fig. 2F), suggesting that the SAMP domain is necessary for the cortical enrichment of APC2. Consistent with the requirement for APC2-N, the SAMP domain alone is not sufficient for cortical enrichment (Fig. 2G). All of the fragments were expressed at their expected sizes, and no breakdown products were observed that might contribute to apparent mislocalization (Fig. 2B). Interestingly, linking the SAMP domain to mCh-APC2-N (mCh-APC2-N-SAMP; Fig. 2A) resulted in cortical enrichment, in the same manner as that of mCh-APC2-FL (Fig. 2H). This indicated that the Arm-binding 15Rs and 20Rs are dispensable for the cortical enrichment of APC2 in S2 cells. This is consistent with the fact that Arm and α-catenin are not required (supplementary material Fig. S1E,F).

As a more quantitative measure of cortical enrichment, we calculated a ratio of the cortical:cytoplasmic accumulation for each expressed protein on the basis of relative fluorescence intensity in fixed cells (Fig. 2I1,I2; see Materials and Methods). mCherry alone, mCh-APC2-N, mCh-APC2-ΔSAMP and mCh-APC2-SAMP had a cortical:cytoplasmic ratio of ~1, indicating no cortical enrichment. Interestingly, mCh-APC2-C exhibited a ratio of ~1.5, indicating a slight cortical enrichment. Consistent with our qualitative observations, there was no significant difference in cortical enrichment between mCh-APC2-FL and mCh-APC2-N-SAMP (Fig. 2I2).

A novel 30-amino-acid domain at the C-terminus is required for cortical enrichment

The above results indicate that the SAMP domain, containing both the SAMP repeats and additional C-terminal sequences, is necessary for the cortical enrichment of APC2. Although an RNAi experiment indicated that Axin is not required for cortical enrichment of APC2 (supplementary material Fig. S1G), other binding partners have been reported for SAMP repeats (Matsui et al., 2008). Consequently, we set out to determine whether the SAMP repeats are necessary for cortical enrichment of APC2. APC2 has two SAMP repeats (McCartney et al., 1999) (Fig. 3A); however, only the second repeat (SAMP2) has the key residues that are important for APC–Axin binding (Spink et al., 2000). Therefore, we first split the SAMP domain into two halves: one has the degenerate SAMP repeat (SAMP1) and the other has the conserved repeat (SAMP2)

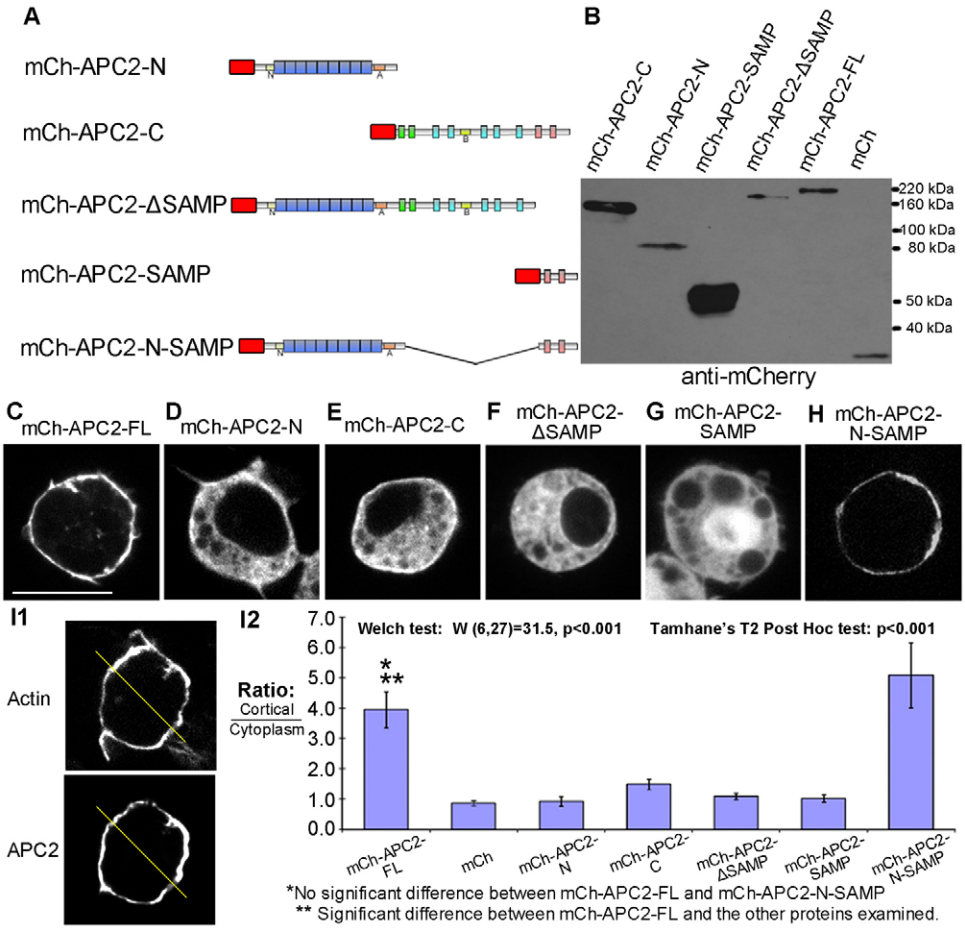

Fig. 2. Localization of APC2 N-terminal and C-terminal deletions. (**A**) Schematic of deletion constructs. The red boxes denote mCherry tags. (**B**) Western blot, using an anti-mCherry antibody, revealing that the APC2 mutants were expressed at the predicted molecular mass and that none showed substantial breakdown. (**C–H**) Live imaging revealed the localization of the indicated APC2 mutant proteins. Only the mCh-APC2-FL (C) and mCh-APC2-N-SAMP (H) showed substantial cortical enrichment. (**I**) I1 shows an example of the fixed cells used to quantify the cortical:cytoplasmic ratio as a measure of cortical enrichment. The ratio was calculated by comparing the average pixel intensity of the expressed protein at the cell cortex with that in the cytoplasm. Cortical pixels were defined as those overlapping with cortical actin. I2 shows a quantification of the experiment, confirming that only mCh-APC2-FL and mCh-APC2-N-SAMP have significant cortical enrichment. Error bars are the 95% confidence interval. Scale bar: 10 µm.

plus the C-terminal 30 amino acids (C30). When combined with APC2-N (Fig. 2A), only SAMP2-C30 retained the ability to restore cortical enrichment (Fig. 3B,C). We further split SAMP2-C30 into SAMP2 (mCh-APC2-N-SAMP2), and C30 (mCh-APC2-N-C30; Fig. 3A). Remarkably, C30 alone completely restored cortical enrichment (Fig. 3E), whereas SAMP2 alone did not (Fig. 3D). Consistent with this, APC2 lacking C30 (mCh-APC2-ΔC30; Fig. 3A) failed to exhibit cortical enrichment (Fig. 3F). Together with the RNAi experiment (supplementary material Fig. S1), these results indicate that Axin does not play a direct role in the cortical enrichment of APC2. Furthermore, we have identified C30 as a novel domain required for the cortical enrichment of APC2.

Examination of the APC2-C30 sequence revealed three serine residues that are potential phosphorylation sites (Fig. 3A). APC2 is a highly phosphorylated protein (McCartney et al., 1999), and the phosphorylation state of APC has strong functional implications, at least with respect to β-catenin binding (Ha et al., 2004; Rubinfeld et al., 1996; Xing et al., 2004). Therefore, we set out to determine whether these potential phosphorylation sites play a role in the cortical enrichment of APC2. We created mutant forms of mCh-APC2-N-C30 where the three serine residues were mutated to alanine (mCh-APC2-N-C30SA) or aspartate (mCh-APC2-N-C30SD) to abolish or mimic phosphorylation, respectively. Both of these mutants localized in a manner similar to mCh-APC2-N-C30 (Fig. 3G1,G2), indicating that phosphorylation, if any, at these sites does not influence the cortical enrichment of APC2.

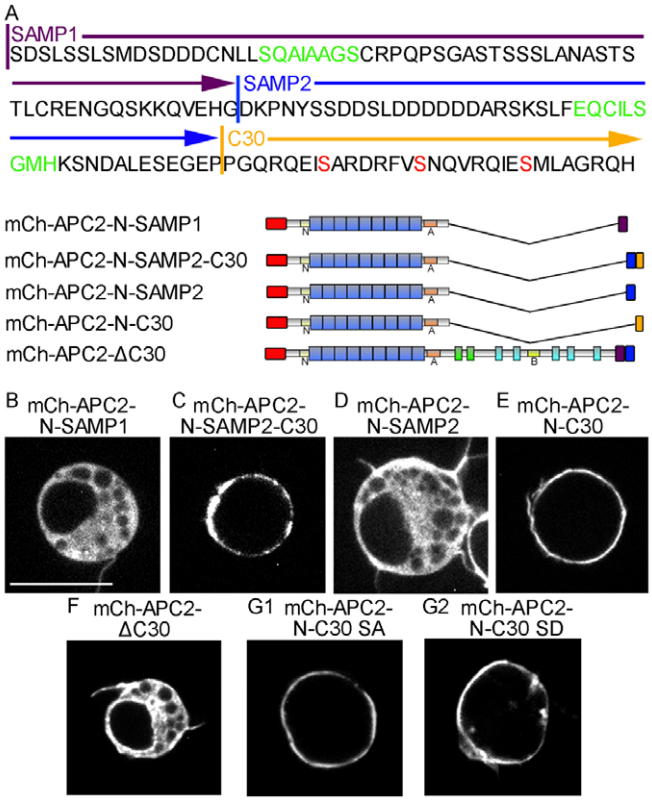

Fig. 3. Dissection of the APC2 C-terminal requirements for cortical enrichment. (**A**) Sequence of the C-terminal SAMP domain of APC2 and schematics of deletion constructs. The red boxes denote mCherry tags. Sequences in green indicate the SAMP repeats. The highlighted serine residues in C30 were replaced with either alanine (SA) or an aspartic acid (SD) residues. (**B**–**F**) Live imaging revealed the localization of the indicated APC2 mutant proteins. (**G**) Live imaging revealed the localization of mCh-APC2-N-C30 SA (G1) and mCh-APC2-N-C30 SD (G2), indicating that phosphorylation at these sites, if any, does not play a role in the cortical enrichment of APC2. Scale bar: 10 µm.

The Arm repeats are required for the cortical enrichment of APC2

The N-terminal half of APC2 contains the N-terminus (amino acids 1–17), conserved region N (amino acids 18–50), the Arm repeats (amino acids 51–424), which are conserved in all APC proteins, and conserved region A and surrounding sequences (amino acids 425–490), a domain conserved between *Drosophila* APC1 and APC2 (Fig. 1A). We first tested whether the N-terminus and conserved region N are important for the cortical enrichment of APC2 with two N-terminally deleted constructs: one lacking the N-terminus only (mCh-APC2-ΔN17; Fig. 4A) and the other lacking both N-terminal domains (mCh-APC2-ΔN50; Fig. 4A). Both mCh-APC2-ΔN17 and mCh-APC2-ΔN50 were strongly enriched at the cortex (Fig. 4C,D), in a manner similar to mCh-APC2-N-SAMP (Fig. 4B). Similarly, conserved region A was dispensable for cortical enrichment; an APC2 fragment containing only the Arm repeats and C30 (mCh-APC2-Arm-C30; Fig. 4A) was enriched at the cortex (Fig. 4E).

The Arm repeat domain is composed of nine Arm repeats (McCartney et al., 2006). Arm repeats are thought to form an integrated domain, and the proper folding of this domain is sensitive to deletions of individual repeats (Huber et al., 1997). However, on the basis of sequence alignment, the first two Arm repeats of APC2

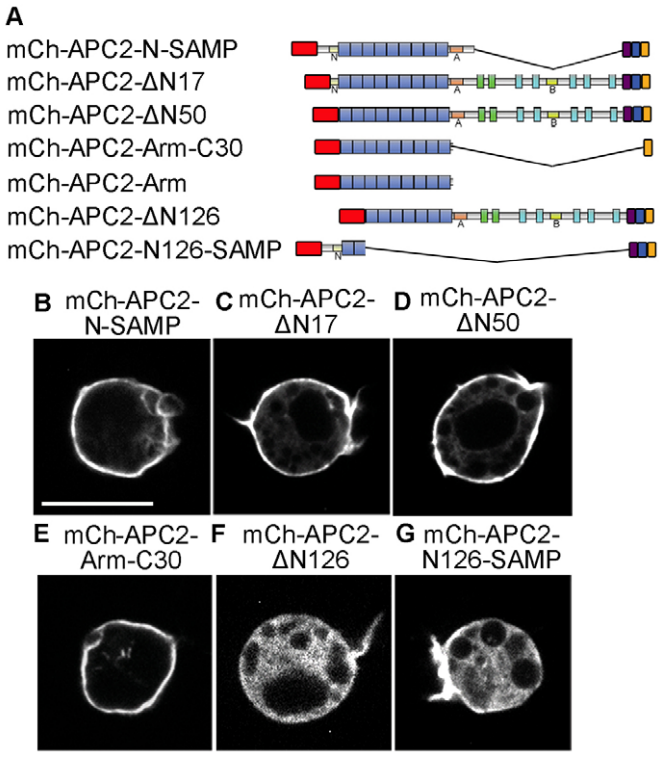

Fig. 4. Dissection of the N-terminal requirements for cortical enrichment. (**A**) Schematics of deletion constructs. The red boxes denote mCherry tags. (**B**–**G**) Live imaging revealed the localization of the indicated APC2 mutant proteins. From the N-terminal domain, the Arm repeats alone are required for the cortical enrichment of APC2. Scale bar: 10 µm.

(AR1,2) contain only two of the three helices characteristic of Arm repeats (Huber et al., 1997) and thus might not participate strongly in the folding of the entire domain. Deletion of the N-terminal domains and AR1,2 (mCh-APC2-ΔN126; Fig. 4A) resulted in the loss of cortical enrichment (Fig. 4F). However, a protein containing N126 and the SAMP domain (mCh-APC2-N126-SAMP; Fig. 4A) was also not sufficient for cortical enrichment (Fig. 4G). There are several alternative interpretations of the observation that AR1,2 are necessary, but not sufficient, in the context where the complete Arm repeat domain is sufficient. It is possible that AR1,2 are the portion of the repeats necessary for cortical localization but that, in isolation, disrupted folding of those repeats renders them insufficient. Alternatively, the deletion of AR1,2 might have disrupted the folding of the entire Arm repeat domain. Consequently, no further deletion analysis of the Arm repeats was attempted. Taken together, these experiments reveal that the Arm repeats and C30 are the minimal domains necessary for the cortical enrichment of APC2, and that neither domain alone is sufficient for the localization.

The Arm repeats mediate the formation of higher-order APC2 complexes

Vertebrate APC contains an oligomerization domain at its N-terminus (Fig. 1A) that is absent in both *Drosophila* APC1 and APC2 (McCartney et al., 2006). Therefore, we were surprised to find that APC2 appeared to form higher-order complexes in S2 cells: when mCh-APC2-ΔSAMP and mCh-APC2-N were coexpressed with EGFP-APC2-FL, the mCherry fragments were enriched at the cortex with EGFP-APC2-FL (Fig. 5A,B,F; data not shown). Interestingly, mCh-APC2-N, but not mCh-APC2-ΔSAMP, had a dominant negative effect on the cortical localization of EGFP-APC2-FL, resulting in a reduced cortical:cytoplasmic ratio (Fig. 5A,B,E; data not shown). mCh-APC2-Arm, containing just the Arm repeats (Fig. 4A), failed to substantially enrich together with EGFP-APC2-FL at the cortex (Fig. 5C,F), but had a similar dominant negative effect on EGFP-APC2-FL (Fig. 5C,E). mCh-APC2-C, which lacks the Arm repeats, did not redistribute to the cortex in the presence of EGFP-APC2-FL, nor did it cause EGFP-APC2-FL to redistribute to the cytoplasm (Fig. 5D–F). These observations suggest that the Arm repeats might mediate the formation of higher-order complexes between APC2 molecules. To test this hypothesis, we immunoprecipitated the mCh-APC2 fragments and examined their ability to co-precipitate expressed untagged APC2-FL. We observed that APC2-FL coprecipitated with mCh-APC2-Arm and mCh-APC2-N, but not with mCherry alone or mCh-APC2-C (Fig. 5G; data not shown). Furthermore, endogenous APC2 in S2 cells was also co-precipitated with mCh-APC2-N but not mCh-APC2-C (data not shown). Although this might seem inconsistent with the fact that the Arm-domain-containing fragments were unable to enrich at the cortex in the presence of endogenous APC2, we reasoned that endogenous APC2 is not sufficiently expressed to cause a visible enrichment of the exogenous fragments. Taken together, these findings suggest that APC2 molecules could form higher-order complexes through their Arm repeats. This type of association through the Arm repeats has not been reported for other APC proteins.

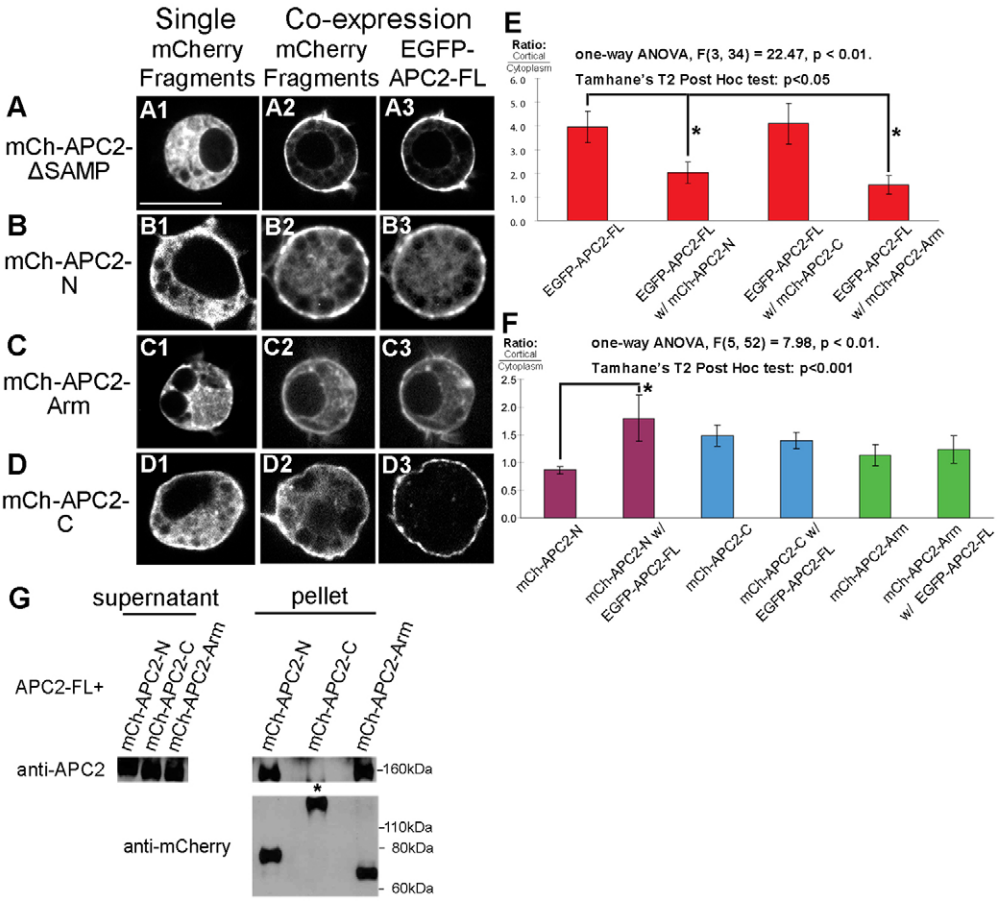

Fig. 5. APC2 self associates to form higher order complexes. (**A**–**D**) Live imaging reveals the localization of APC2 mutants, as indicated, with or without the coexpression of EGFP-APC2-FL. (**E**) The cortical:cytoplasmic ratio of EGFP-APC2-FL alone or in the presence of the indicated APC2 truncations. Asterisks indicate a significant decrease in the cortical:cytoplasmic ratio as compared with that of EGFP-APC2-FL alone. (**F**) The cortical:cytoplasmic ratio of mCh-APC2 truncations alone or in the presence of EGFP-APC2-FL as indicated. The asterisk indicates a significant increase in the cortical:cytoplasmic ratio as compared with that of EGFP-APC2-N alone. (**G**) Co-immunoprecipitation of untagged APC2-FL with mCh-tagged APC2 fragments from S2 cell lysates. The fragments were precipitated with anti-mCherry antibody and the blot (left and upper-right panels) was probed with anti-APC2 antibody. Immunoprecipitation of the mCh-APC2 fragments is shown in the lower-right panel. APC2-FL co-immunoprecipitates with APC2 fragments that contain the Arm repeats but not with APC2-C. Scale bar: 10 μm.

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APC2-C30 is required for cortical enrichment of APC2 in the embryo

To test whether C30 is also required for the cortical enrichment of APC2 in embryonic epithelia, we generated transgenic flies expressing different EGFP-tagged APC2 truncations under the native *APC2* promoter and examined their localization. We verified that all of the transgenes were expressed at levels comparable to endogenous APC2 (Fig. 6G). Endogenous APC2 in embryonic epithelia is localized to puncta at the cortex and in the cytoplasm (Fig. 1B, Fig. 6F) (McCartney et al., 1999). Consistent with our findings in S2 cells (Fig. 2), EGFP-APC2-FL and EGFP-APC2-N-SAMP were enriched at the apical cell cortex in stage 8 and 9 embryos null for endogenous APC2 ($APC2^{g10}$; Fig. 6A,B). Furthermore, we did not detect any substantial cortical enrichment of EGFP-APC2-ΔC30 in $APC2^{g10}$ embryos (Fig. 6C). Because APC2 can self-associate through the Arm repeats in S2 cells (Fig. 5), we predicted that EGFP-APC2-ΔC30 would localize to the cortex in the presence of endogenous APC2 in the embryo. Consistent with that prediction, the subcellular distribution of EGFP-APC2-ΔC30 was altered in the presence of endogenous APC2, and we observed some cortical enrichment (Fig. 6D). Analysis of cortical enrichment (see the Materials and Methods) revealed that in a wild-type background (Fig. 6D) EGFP-APC2-ΔC30 overlapped with 55% of the cortex, whereas in an *APC2*-null background (Fig. 6C) EGFP-APC2-ΔC30 overlapped with only 25% of the cortex ($P<0.05$; Student's *t*-test, Welch's variation). Although the EGFP-APC2-ΔC30 localization was altered by the presence of endogenous wild-type APC2, the localization of wild-type APC2 was not substantially altered by the presence of EGFP-APC2-ΔC30 (Fig. 6E,F). This is consistent with the finding that APC2-ΔSAMP did not influence cortical enrichment of APC2-FL in S2 cells (Fig. 5A; data not shown). Because the anti-APC2 antibody recognizes both wild-type APC2 and EGFP-APC2-ΔC30, we cannot rule out the possibility that wild-type APC2 in the presence of EGFP-APC2-ΔC30 has a more cytoplasmic localization.

Cortical enrichment of APC2 is not required for the negative regulation of Wnt signaling

The *Drosophila* embryo has been a long-standing model for Wnt signaling during development (reviewed by Logan and Nusse, 2004). A complete loss of function of *Drosophila* APC2 ($APC2^{g10}$) activates Wnt signaling in the embryo resulting in complete embryonic lethality (0% hatch rate to the larval stage; Table 1) (McCartney et al., 2006). In addition, these embryos exhibit phenotypes in the embryonic cuticle characteristic of Wnt activation (McCartney et al., 2006). These phenotypes include: a reduction in the cuticle size due to excess cell death, a hole in the anterior cuticle due to a failure in head involution, and the production of excess smooth cuticle at the expense of the denticles. Embryonic cuticles were scored for the severity of their defects on a scale of 0 (weakest) to 6 (strongest) (Fig. 7B) (McCartney et al., 2006), and a phenotypic average (PA) was calculated for each genotype. When the hatch rate was very high (>95%) cuticle phenotypes were not determined (nd; Table 1). $APC2^{g10}$ embryos have a PA of 3.6. To test the hypothesis that the cortical enrichment of APC2 is necessary for destruction complex function, we asked whether EGFP-APC2-ΔC30 could rescue the embryonic lethality and cuticle defects associated with $APC2^{g10}$. EGFP-APC2-FL completely rescues embryonic lethality (Table 1), resulting in a viable fertile stock of

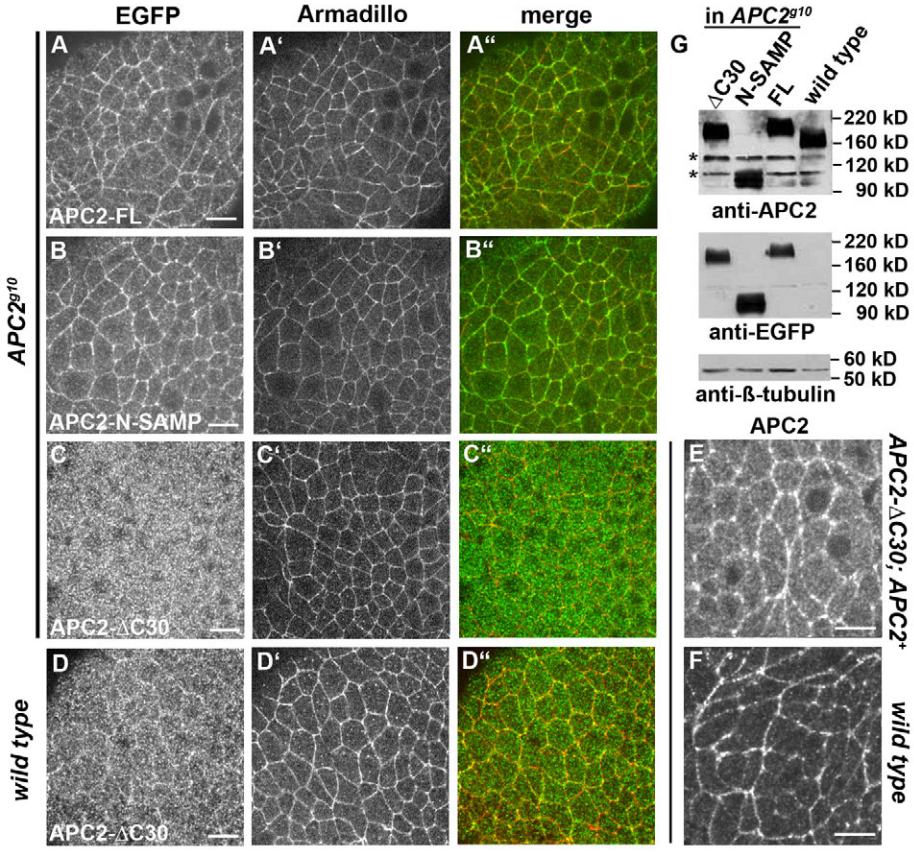

Fig. 6. C30 is required for the cortical enrichment of APC2 in embryonic epithelia. In an *APC2*-null background, EGFP-APC2-FL (**A**) and EGFP-APC2-N-SAMP (**B**) are enriched at the apical cortex with Arm. (**C**) EGFP-APC2-ΔC30 was not cortically enriched in an *APC2*-null background. (**D**) In a wild-type background, EGFP-APC2-ΔC30 exhibits some cortical enrichment. Immunolocalization of APC2 in wild-type embryos (**F**) or in wild-type embryos expressing EGFP-APC2-ΔC30 (**E**). Anti-APC2 recognizes both wild-type APC2 and APC2-ΔC30. (**G**) Western blots of embryonic lysate from either wild-type (w^{1118}) embryos, or $APC2^{g10}$ embryos expressing the indicated transgenes. Probing with the anti-APC2 antibody revealed the endogenous APC2 protein in wild-type embryos and the EGFP-tagged expressed proteins in the $APC2^{g10}$ embryos. The expression of the proteins from the transgenes was comparable to endogenous APC2. The anti-EGFP antibody recognized only the expressed proteins. β-tubulin was used as a loading control. Asterisks indicate background bands recognized by anti-APC2. Scale bar: 10 μm.

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Table 1. Hatch rate and phenotypic average for various *APC* mutants and rescue constructs

Genotype	Hatch rate (%) (n>250)	Phenotypic average (n>40)	Rescue of APC loss
APC2^{g10} (*APC2* null)	0	3.6	n.a.
P{EGFP-APC2-FL}; APC2^{g10}	96	n.d.	++
P{EGFP-APC2-ΔC30}; APC2^{g10}	96	n.d.	++
P{EGFP-APC2-N-SAMP}; APC2^{g10}	68	1.9	+/–
APC2^{g10} APC1^{Q8} (double null)	23[b]	3.3[c]	n.a.
P{EGFP-APC2-FL}; APC2^{g10} APC1^{Q8}	46[a]	0.8[d]	++
P{EGFP-APC2-ΔC30}; APC2^{g10} APC1^{Q8}	46[a]	0.3[e]	++
P{EGFP-APC2-N-SAMP}; APC2^{g10} APC1^{Q8}	17[b]	3.1[c]	–

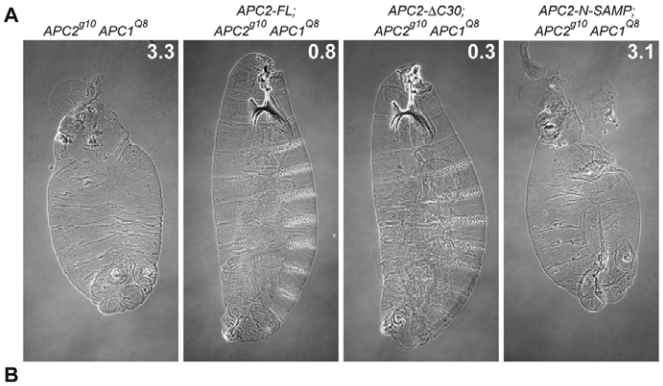
[a,b]No significant difference between each value marked with the same letter; significant difference between values marked with different letters.
[c,d,e]No significant difference between each value marked with the same letter; significant difference between values marked with different letters.
The level of rescue of *APC* loss is given qualitatively (+, a degree of rescue; –, a lack of rescue). For the statistical analysis of hatch rate in the double-null experiment a chi-square test gave χ^2=132.1 (d.f.=3, n=1655), P<0.001, followed with a Tukey-type post-hoc test, q(d.f.=∞, groups=4), P<0.05 per comparison. For statistical analysis of phenotypic average in the double-null experiment a Kruskal–Wallis test, χ^2=345.4 (d.f.=3, n=605), P<0.001, followed with a Dunn procedure using adjusted α levels of 0.008 (0.05/6) per comparison. n.a., not applicable; n.d., not determined.

the genotype *P{endoP-EGFP-APC2-FL}; APC2^{g10}*. EGFP-APC2-N-SAMP restores some APC2 activity resulting in a 68% hatch rate and a lower PA value (Table 1). Surprisingly, EGFP-APC2-ΔC30 rescued the *APC2^{g10}* mutant phenotypes to the same level as EGFP-APC2-FL, resulting in a viable fertile stock with no apparent defects in Wnt signaling (Table 1).

Complete loss of *APC1* in the embryo enhances the *APC2*-null phenotype (McCartney et al., 2006). We therefore reasoned that the ability of EGFP-APC2-ΔC30 to rescue the function of the destruction complex could depend upon the presence of APC1. Therefore, to further challenge EGFP-APC2-ΔC30 function in the destruction complex, we tested whether it could rescue the loss of both *APC2* and *APC1*. In the *APC2*-null experiment (above) all of the embryos are maternally and zygotically *APC2* null (supplementary material Fig. S2A). In the double-null experiment, 50% of the embryos are maternally and zygotically *APC2^{g10} APC1^{Q8}* (double null), and 50% of the embryos are maternally double null and zygotically heterozygous for the double-null chromosome (supplementary material Fig. S2B). With no rescuing transgene, 23% of the embryos in the double-null experiment hatched, owing to a partial paternal rescue (Table 1), whereas all of the maternally and zygotically mutant embryos died; of those 23% that hatched all were maternally mutant and zygotically heterozygous (data not shown). The EGFP-APC2-FL rescue construct suppressed the embryonic phenotypes, resulting in a 46% hatch rate and a significantly lower PA (Table 1; Fig. 7A). Remarkably, EGFP-APC2-ΔC30 provided the same degree of rescue as EGFP-APC2-FL (Table 1; Fig. 7A). Interestingly, the ability of EGFP-APC2-ΔC30 to rescue the cuticle defects in the double-null background was slightly, but significantly (P<0.05), stronger (Table 1), suggesting that the loss of cortical localization has a mild positive effect on the function of the destruction complex. By contrast, EGFP-APC2-N-SAMP provided no rescue in this background (Table 1 and Fig. 7A). Taken together, these data suggest that the cortical enrichment of APC2 is not required for destruction complex function.

Cortical enrichment of APC2 is required for actin pseudocleavage furrow extension

Early *Drosophila* embryos are syncytial, and, by nuclear cycle 10, most nuclei have migrated to the cortex creating the syncytial blastoderm. During the four cortical divisions before cellularization, actin undergoes dynamic and synchronized rearrangements (Sullivan and Theurkauf, 1995). In interphase, cortical actin is organized into ‘caps’ above each nucleus that expand during prophase to form rings surrounding each nucleus, as viewed from the surface (Fig. 8A). During late prophase and early metaphase these rings extend into the embryo to form pseudocleavage furrows (Fig. 8A). Furrows retract during anaphase forming new caps by the end of telophase. APC2 localizes with cortical actin throughout this actin cycle (McCartney et al., 1999), and *APC2*-null embryos exhibit defects in furrow extension (Fig. 8C,D) (Webb et al., 2009). To determine whether the cortical enrichment of APC2 is required for furrow extension, we first asked whether the C30 domain is required for the localization of APC2 to cortical actin in the



B

Score	Scoring Criteria
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, >3 patches of denticles remaining
4	Anterior hole, 50-60% wild type size, <2 denticle patches remaining
5	Anterior hole extends ventrally to ~30% cuticle length, 50-60% wild type size, no denticles
6	Anterior hole extends ventrally to ~50% cuticle length, cuticle round, 25-30% wild type size, no denticles

Fig. 7. Cortical localization of APC2 is not necessary for the regulation of Wnt signaling in the *Drosophila* embryo. (**A**) Representative embryonic cuticles for the genotypes as indicated. The number in the top right-hand corner indicates the phenotypic average. Higher numbers indicate stronger mutant phenotypes. All cuticles are shown to scale. (**B**) Scoring criteria used to generate the phenotypic average as described in McCartney et al. (McCartney et al., 2006).

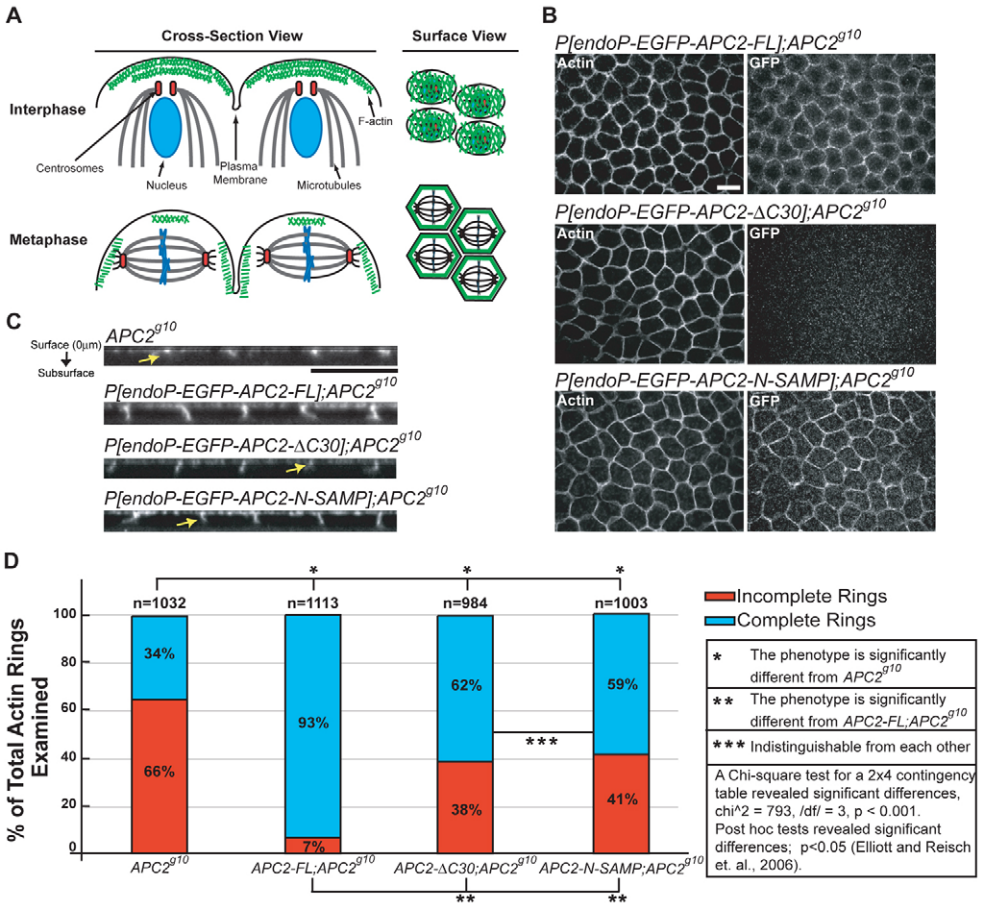


Fig. 8. Cortical localization of APC2 is required for actin furrow extension. (**A**) Schematic of cortical actin organization in the syncytial blastoderm stage embryo. (**B**) Surface views of cycle 12 metaphase embryos as indicated. (**C**) Cross-sectional view of *P{endo-EGFP-APC2-FL}*; $APC2^{g10}$, which appears wild type; however, furrow extension in *P{endo-EGFP-APC2-ΔC30}*; $APC2^{g10}$, *P{endo-EGFP-APC2-N-SAMP}*; $APC2^{g10}$ and $APC2^{g10}$ mutants is defective (yellow arrows). (**D**) The percentage of complete and incomplete rings was calculated for each genotype as a measure of furrow extension. APC2-ΔC30 and APC2-N-SAMP exhibit only a moderate rescue of furrow extension compared with that by APC2-FL. Scale bar: 10 μm.

syncytial embryo. As in the polarized epithelial cells of the embryonic epidermis (Fig. 6C), C30 was required for cortical localization, whereas the central 15Rs and 20Rs were dispensable (Fig. 8B).

To examine the role of the cortical enrichment of APC2 in actin furrow extension, we compared furrow extension in $APC2^{g10}$ embryos with that in $APC2^{g10}$ embryos expressing EGFP-APC2-FL, EGFP-APC2-ΔC30 or EGFP-APC2N-SAMP (Fig. 8C,D). To quantify furrow extension defects from tangential sections, we determined the percentage of incomplete rings at four different depths as described previously (Webb et al., 2009) (supplementary material Fig. S3A). $APC2^{g10}$ embryos expressing EGFP-APC2-FL had few defects in furrow extension compared with the number in $APC2^{g10}$ embryos (Fig. 8C,D); however, they exhibited some defects at the lowest depth (supplementary material Fig. S3B). $APC2^{g10}$ embryos expressing either EGFP-APC2-ΔC30 or EGFP-APC2-N-SAMP exhibited an intermediate phenotype compared with the phenotype of the EGFP-APC2-FL embryos (Fig. 8C,D; supplementary material Fig. S3B). These data suggest that the localization of APC2 with cortical actin is necessary for complete extension of actin pseudocleavage furrows.

Discussion

As the smallest APC protein, *Drosophila* APC2 has provided a relatively simple model for dissecting the complexity of APC function. It contains all of the core domains conserved among APC proteins (Arm repeats, 15Rs, 20Rs and SAMP repeats) but is missing the N-terminal oligomerization domain, and both the basic domain and the EB1-binding domain that, in part, mediate the association of some APC proteins with microtubules. *Drosophila* APC2 performs at least two of the conserved APC functions. We and others have shown that *Drosophila* APC2 is an essential negative regulator of Wnt signaling (McCartney et al., 1999; Yu et al., 1999); in addition, we have shown that APC2 influences the organization of the actin cytoskeleton in the early *Drosophila* embryo (Webb et al., 2009). Here, we have used *Drosophila* APC2 to address the role of cortical localization in APC function in Wnt signaling and cytoskeletal organization.

The role of cortical localization in the regulation of Wnt signaling

The subcellular localization of APC proteins has been controversial, owing, at least in part, to antibody cross-reactivity problems (Brocardo et al., 2005). It now seems clear that APC proteins are found in the nucleus, at the plus tips of microtubules during membrane extension, at the leading edge of migrating cells, in the cytoplasm and at the actin-rich cortex, where it sometimes associates with the adherens junctions (Harris and Nelson, 2010; Kawasaki et al., 2000; Nathke et al., 1996; Rosin-Arbesfeld et al., 2000; Watanabe et al., 2004; McCartney et al., 1999; Yu et al., 1999). Studies in both *Drosophila* and mammalian cells have suggested a functional link between the cortical localization of APC and its function in the destruction complex. We have shown that APC2 mutants that lose their cortical association also have diminished destruction complex activity (McCartney et al., 2006). Consistent with this hypothesis, Maher and colleagues showed that

Table 2. Summary of the localization and actions of the APC2 mutants in *Drosophila* S2 cells and embryos

APC2 construct	Localization in S2 cells	Cortical with APC2-FL in S2 cells	Disrupts cortical APC2-FL in S2 cells	IPs with APC2-FL in S2 cells	Localization in embryonic cells	Suppresses activation of Wnt signaling in *APC2* null	Suppresses activation of Wnt signaling in *APC2 APC1* double null	Suppresses furrow extension defects null in *APC2*
FL	**Cortical**	n.a.	n.a.	n.d.	**Cortical**	**Yes**	**Yes**	**Yes**
N	**Cytoplasmic**	**Yes**	**Yes**	**Yes**	n.d.	n.d.	n.d.	n.d.
C	**Cytoplasmic**	**No**	**No**	**No**	n.d.	n.d.	n.d.	n.d.
ΔSAMP	**Cytoplasmic**	**Yes**	**No**	n.d.	n.d.	n.d.	n.d.	n.d.
SAMP	**Cytoplasmic**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N-SAMP	**Cortical**	n.d.	n.d.	n.d.	**Cortical**	**Partial**	**No**	**Partial**
N-SAMP1	**Cytoplasmic**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N-SAMP2-C30	**Cortical**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N-SAMP2	**Cytoplasmic**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N-C30	**Cortical**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ΔC30	**Cytoplasmic**	n.d.	n.d.	n.d.	**Cytoplasmic**	**Yes**	**Yes**	**Partial**
Arm-C30	**Cortical**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arm	**Cytoplasmic**	**No**	**Yes**	**Yes**	n.d.	n.d.	n.d.	n.d.
ΔN17	**Cortical**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ΔN50	**Cortical**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ΔN126	**Cytoplasmic**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ΔN126-SAMP	**Cytoplasmic**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

IPs, immmunoprecipates; n.a., not applicable; n.d., not determined.

Axin, APC2 and GSK3β localized to cell–cell contacts, together with phosphorylated β-catenin, in mammalian cultured cells (Maher et al., 2009). Furthermore, two independent studies identified the association of APC with WTX (also known as AMER1 and FAM123B) (Grohmann et al., 2007; Major et al., 2007). Overexpression of WTX in cultured epithelial cells results in the redistribution of APC from microtubule tips to the cell cortex (Grohmann et al., 2007), whereas Major et al. (Major et al., 2007) demonstrated that WTX antagonizes Wnt signaling by promoting β-catenin degradation. Taken together, these reports again suggest that cortical APC plays a role in the negative regulation of Wnt signaling.

To test this hypothesis, we identified the domains of APC2 required for localization to the cell cortex (summarized in Table 2). Deletion of the C-terminal 30 amino acids resulted in the loss of cortical enrichment without disrupting any known functional domain, allowing us to test the function of cortical enrichment. To our surprise, loss of cortical enrichment appeared to have no effect on the activity of APC2 in the destruction complex. APC2-ΔC30 and APC2-FL performed equivalently well in our functional assays for the function of the destruction complex. One interpretation of this result is that cortical APC is playing an exclusive role in other cellular functions, such as actin organization, and does not function in the destruction complex. This does not rule out the possibility that the cortical pool can contribute protein to the cytoplasmic pool. Alternatively, the destruction complex with APC might usually reside at the cortex, but its location there is not essential to its function. In this case, the loss of cortical APC would necessitate a re-localization of the destruction complex to the cytoplasm with no adverse effect on function. Finally, cortical enrichment of the destruction complex might enhance the efficiency of its activity, rather than being essential for its activity. In that case, it is possible that our assays are not sensitive enough to detect very small losses in the function of the destruction complex. As we can detect a broad range of destruction complex activity using these assays (McCartney et al., 2006), we think that this is unlikely.

Does cortical APC2 play any role in Wnt signaling? The binding of Arm to Cadherin at the adherens junction can suppress Wnt signaling (Greaves et al., 1999; Cox et al., 2000), suggesting that cortical Arm negatively impacts Wnt signaling. By contrast, the cortical pool of APC2 could act as a sink to sequester APC2 away from the destruction complex and indirectly promote Wnt signaling. Our observation that APC2-ΔC30 provides a slightly enhanced rescue compared with that of APC2-FL (Table 1) suggests that blocking cortical localization might increase the amount of available APC2 for a cytoplasmic destruction complex function. Furthermore, Wnt-dependent relocalization of Axin to the cortex is one proposed mechanism for deactivation of the destruction complex (Cliffe et al., 2003; Metcalfe et al., 2010) and cortical localization of APC2 is not Wnt dependent (McCartney et al., 1999).

This implies that the APC2 cytoplasmic puncta are the sites of destruction complex activity. Consistent with this model, APC2-FL, APC2-N-SAMP and APC2-ΔC30 are all found in cytoplasmic puncta, and all provide at least some destruction complex function (Fig. 6; Table 1). Further support comes from the observation that APC2 and Arm colocalize in cytoplasmic puncta whose abundance and localization are influenced by the GSK3β homolog Zeste-white 3 (also known as Shaggy) (McCartney et al., 2001). Axin also resides in cytoplasmic puncta that have been implicated in the function of the destruction complex (Faux et al., 2008; Schwarz-Romond et al., 2005; Cliffe et al., 2003; Fagatto et al., 1999).

Given the predicted importance of APC2–Arm binding in the destruction complex, we were surprised to find that APC2-N-SAMP, a protein missing all known binding domains for Arm, displayed a moderate rescue of Wnt activation in the *APC2*-null background. This rescue was abolished in the absence of APC1 (Table 1). APC1 does provide some destruction complex function in the embryo (Akong et al., 2002a; Ahmed et al., 2002; McCartney et al., 2006). Overexpressed APC1 resides in centrosomes and on interphase microtubules (Akong et al., 2002a). Interestingly, overexpression of APC1 can drive APC2 to the centrosome and MTs (Akong et al., 2002a), and overexpression of APC2 can drive

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APC1 to the cortex (Akong et al., 2002b), suggesting that these proteins physically interact. Together with our identification of the Arm repeats of APC2 as an oligomerization domain (Fig. 5), we propose a model in which APC2 and APC1 form hetero-oligomers, through their Arm repeats, that function in the destruction complex. In wild-type embryos, APC2 probably predominates in these hetero-oligomers, as endogenous APC1 is weakly expressed (Akong et al., 2002a). Thus, hetero-oligomers of APC2-N-SAMP and APC1 provide partial destruction complex function, whereas APC2-N-SAMP homo-oligomers are non-functional in the destruction complex.

The role of cortical localization in actin furrow extension

Although cortical localization of APC2 is not essential for Wnt signaling, it is necessary for actin organization in the early *Drosophila* embryo. In this context, we have previously shown that the formin Dia is required to localize APC2 to actin furrows (Webb et al., 2009). Because Dia and APC2 directly bind, through the C-terminal halves of each protein (Webb et al., 2009), it is possible that APC2-C30 binds Dia. We previously proposed a model in which the binding of APC2 to Dia relieves the autoinhibition of Dia (Webb et al., 2009). This would activate Dia and promote its actin nucleation and elongation function, which is essential for actin furrow extension. Thus, APC2-ΔC30 would fail to fully rescue furrow extension because it cannot bind and activate Dia. If this simple model was correct, it would be predicted that APC2-N-SAMP, containing an intact C30 and localizing to the cortex (Fig. 8), would fully rescue furrow extension. However, APC2-N-SAMP also only results in a partial rescue indistinguishable from that of APC2-ΔC30 (Fig. 8), indicating that the region of APC2 containing the Arm-binding 15Rs and 20Rs plays a role in this process. One interpretation of these data is that Dia binding requires a complete C-terminus; thus, in both mutants binding is partially disrupted and only partial function is restored. Alternatively, the furrow extension function of APC2 might require other factors and not only binding of Dia. The requirement for the 15Rs and 20Rs suggests that an APC2–Arm interaction is necessary for normal furrow extension. Dissecting the role of the C-terminus of APC2 in furrow extension is the subject of future investigation.

The role of the Arm repeats and C30 in cortical localization and self association

Both the Arm repeats and C30 are necessary for the cortical localization of APC2, and neither is sufficient alone. These two domains might have distinct binding partners, and both are required for proper cortical association (Fig. 9A). Alternatively, the Arm repeats and C30 together may form a three dimensional structure that is recognized by a single binding partner (Fig. 9B). An mCh–APC2-N-C30 protein, where the Arm repeats are immediately adjacent to C30, localizes to the cortex in S2 cells (Fig. 3E). Although no complete APC protein has been crystallized, it is thought that the central repeat region is unstructured until association with binding partners such as β-catenin and Axin (Eklof Spink et al., 2001; Ha et al., 2004; Spink et al., 2000; Xing et al., 2004). This would provide the flexibility for C30 to interact with the Arm repeats in the intact molecule. This model predicts that APC2-C (with an intact C30) could associate with APC2-FL (with intact Arm repeats), and would thus localize to the cortex. This, however, is not what we observe (Fig. 5D). A third model suggests a relationship between the oligomerization of APC2 and its cortical localization (Fig. 9C).

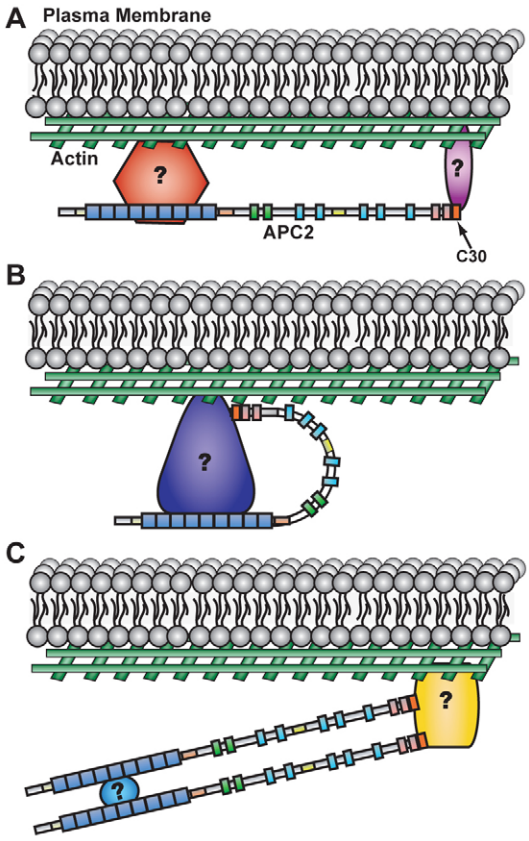

Fig. 9. Alternative mechanistic models for the cortical localization of APC2. (**A**) The Arm repeats (blue) and C30 (orange) of APC2 bind to different proteins that link APC2 to cortical actin. The binding of both is necessary and sufficient for cortical localization. (**B**) The Arm repeats and C30 form a three-dimensional docking site for a single protein that links APC2 to cortical actin. (**C**) The dimerization and/or oligomerization of the Arm repeats brings multiple C30 domains into close proximity. Together they form a docking site for a protein that links APC2 to cortical actin. In this model, dimerization and/or oligomerization is indirect and occurs through another unknown protein.

Vertebrate APC forms oligomers through the N-terminal oligomerization domain (Joslyn et al., 1993; Su et al., 1993a). Although it is clear that oligomerization can affect the function of truncated APC proteins (Green and Kaplan, 2003; Green et al., 2005; Tighe et al., 2004), the role of oligomerization in wild-type APC function is not well understood. Furthermore, the region of human APC between its Arm domain and the first 15R domain associates with itself and with the last C-terminal 300 amino acids (Li et al., 2008). Deletion of this region affected the clustering of APC at the plus-end tips of microtubules and was associated with defective migration (Li et al., 2008). *Drosophila* APC2 does not contain any of the self-association domains described for vertebrate APC. Instead, we have shown that APC2 forms higher-order complexes through the Arm repeats (Fig. 5). Given the requirement for the Arm repeats in self-association and cortical localization, it is possible that these aspects of APC2 behavior are causally linked. In this model, the Arm repeats facilitate the oligomerization of C30. Once oligomerized, C30 binds its target at the cortex (Fig. 9C). Additional analyses will reveal the oligomerization state of APC2 and its role in cortical localization.

A BLAST search revealed that the C30 domain is highly conserved among APC proteins in different *Drosophila* species but

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there is no substantial sequence conservation of this domain among other APC family members or other proteins. Nonetheless, identification of the putative cortical association factors might reveal a conserved mechanism for APC cortical localization. There is precedent for this among the APC proteins; the conservation of self-association without the conservation of the self-association domains is one example, and the SAMP repeats are another. In the *Drosophila* SAMP domain, the alanine residue is replaced by a glycine residue, and only the key methionine residue is conserved. APC binding to Axin is conserved through compensatory changes to the APC-binding site in Axin (Spink et al., 2000). Finally, although *Drosophila* APC2 does not contain the basic domain, which has been shown to mediate the association between vertebrate APC and Dia (Wen et al., 2004), it binds to *Drosophila* Dia through a different domain (Webb et al., 2009). Thus, the APC protein family provides an example of remarkable conservation of function without complete conservation of structure. Although it is clear that APC proteins affect a wide array of cellular functions, we are only just beginning to understand the mechanisms by which they carry out those roles.

Materials and Methods

Constructs

The specific amino acid positions of the *Drosophila* APC2 (Flybase annotation symbol: CG6193) fragments are as follows: APC2-N, 1–490; APC2-C, 491–1067; APC2ΔSAMP, 1–930; APC2-SAMP, 931–1067; APC2 N-SAMP, 1–490 plus 931–1067; APC2 N-SAMP1, 1–490 plus 931–992; APC2 N-SAMP2, 1–490 plus 993–1037; APC2 N-SAMP2-C30, 1–490 plus 993–1067; APC2 N-C30, 1–490 plus 1038–1067; APC2ΔC30, 1–1037; APC2ΔN17, 18–1067; APC2ΔN50, 51–1067; APC2 ARM-C30, 51–457 plus 481–490 plus 1038–1067; APC2ΔN126, 127–1067; APC2 N126-SAMP, 1–126 plus 931–1067; APC2-ARM, 51–457. The fragments were PCR amplified, sequenced and cloned into the *Eco*RI site in pRmHa-3 (containing the *metallothionein* promoter) and pCaSpeR-2 [containing the native *APC2* promoter (designated endoP)] (McCartney et al., 2006).

Antibodies and protein reagents

The antibodies against Arm (N27A1), β-tubulin (E7) and α-catenin (DCAT-1) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa and used as follows: anti-Armadillo antibody: 1:100 for western blotting, 1:500 for immunohistochemistry; anti-β-tubulin antibody (E7): 1:100 for western blotting, 1:500 for immunohistochemistry; and anti-α-catenin antibody: 1:1500. An anti-GFP antibody (Abcam) was used at 1:5000 and pre-absorbed with w^{1118} embryos before usage for immunohistochemistry; a different anti-GFP antibody (Roche) was used at 1:50 for western blotting. Anti-mCherry antibody (DsRed; Clontech) was used at 1:1000 for western blotting and 1:50 for immunoprecipitation. The antibody against phosphorylated tyrosine (Millipore) was used at 1:1000. Anti-APC2 antibody was used as previously described (McCartney et al., 2006). For actin staining, Alexa-Fluor-488–phalloidin (Invitrogen) was used at 1:500. DNA was stained with DAPI (1:1000, Sigma). Alexa-Fluor-conjugated secondary antibodies (Invitrogen) were used at 1:1000.

S2 cell culture, transfection and RNAi

Transfection was carried out with Effectene (Qiagen). For the *metallothionein* promoter experiments, expression was induced at 24 hours post-transfection by adding $CuSO_4$ to a final concentration of 40 μM for 14–16 hours. For the *endoP* experiments, cells were harvested ~40 hours after transfection. Cytochalasin D (Sigma) was used at final concentration of 10 μg/ml for 1 hour before cells were fixed. An equal amount of DMSO was used in control experiments.

For RNAi, the S2-DRSC cell line was used (*Drosophila* Genomics Resource Center). Genomic DNA from wild-type flies was extracted, according to standard protocol, to use as a template. Primer pairs used to amplify double-stranded RNA (dsRNA) were chosen from the *Drosophila* RNAi Screening Center at Harvard Medical School. The Amplicon IDs are as follows: α-catenin, DRSC11917; Arm, DRSC18738; and Axin, DRSC29423. The T7 promoter was added to the 5′ end of the primers. dsRNA was generated with the MEGAscript T7 kit (Ambion) and purified with the RNeasy kit (Qiagen). Experiments were performed as described previously (Rogers and Rogers, 2008). After RNAi treatment (5 days), cells were transfected with *endoP*-EGFP-APC2-FL. After imaging, cell lysate was prepared from the remaining cells for immunoblotting.

Fluorescence microscopy and image analysis

All S2 cells were imaged live unless otherwise noted. All fluorescent images were acquired with a spinning-disc confocal microscope with a Yokagawa scan head (Solamere Technology Group) and a QICAM-IR camera (Qimaging) on a Zeiss Axiovert 200M using QED InVivo software. For all images and image analysis of S2 cells, a single slice through the equator of the cell was acquired. For all images of embryonic tissue, a single optical slice at the level of the adherens junctions (Fig. 1B; Fig. 6A–D) or at the top of the furrows (Fig. 8B) is shown. Cross-sections were generated by reslicing the *z*-stacks in the *x*-*z* plane (ImageJ). To quantify cortical enrichment of mCh-APC2 fragments in S2 cells, cells were fixed with 4% paraformaldehyde and cortical actin was labeled with Alexa-Fluor-488–phalloidin. For each cell (n=10 for each condition), an image was acquired of both the actin and the mCherry. A straight line was drawn across the cell and the pixel intensity along the line in each channel was acquired (Image J). The cell cortex was defined using actin as a marker. The average pixel intensity of cortical and cytoplasmic mCh-APC2 was calculated for each cell, and the cortical:cytoplasm ratio was calculated. Cortical localization of APC2-ΔC30 in wild-type and $APC2^{g10}$ embryos was quantitatively assessed using Volocity software to find overlapping objects using the s.d. intensity. Arm was used to label the cortex. A total of 5–7 embryos per genotype was analyzed and the average percentage cortical overlap was calculated. Statistical analysis was performed using SPSS and Excel. Figures were prepared with Adobe Photoshop and Adobe Illustrator.

Immunoprecipitation and immunoblotting

After transfection, cells were lysed in buffer (50 mM HEPES pH 7.5, 115 mM potassium acetate, 2.5 mM magnesium acetate, 0.5% NP-40 substitute, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM sodium orthovanadate, 2 mM sodium metavanadate and 50 mM sodium fluoride) supplemented with 1× Complete protease inhibitor cocktail (Roche), and were preincubated with rec-G beads (Invitrogen) for 0.5 hours at 4°C. The pre-cleared lysate was then incubated with anti-mCherry antibody at 1:50 for 1 hour at 4°C. Rec-G beads were added to the mixture and incubated for another 0.5 hours at 4°C before washing. Gel electrophoresis and immunoblotting were performed using standard procedures. For dsRNA experiments, 10 μg of total protein was loaded per lane. For transgene expression in embryos, 50 μg of total protein was loaded per lane.

Genetics, hatch rate and cuticle preparations

Transgenic flies expressing *P{endoP-EGFP-APC2-FL}*, *P{endoP-EGFP-APC2-N-SAMP}* and *P{endoP-EGFP-APC2-ΔC30}* were generated using P-element mediated germline transformation (Model System Genomics of Duke University). Two independent second chromosome insertions for each transgene were crossed into the $APC2^{g10}$ and the *FRT* $APC2^{g10}$ $APC1^{Q8}$ backgrounds using standard methods. Double-null embryos maternally *FRT* $APC2^{g10}$ $APC1^{Q8}$ were generated using the FRT-FLP-DFS technique (Chou and Perrimon, 1996). Embryonic cuticles were prepared and the hatch rate analysis was performed as previously described (Wieschaus and Nusslein-Volhard, 1998). The cuticle phenotype scoring criteria (Fig. 7B) were as previously described (McCartney et al., 2006).

Analysis of pseudocleavage furrow extension

Embryos were collected for 2 hours at 27°C and fixed, stained and analyzed as described (Webb et al., 2009). Optical slices were acquired at every 0.2 μm. In brief, the first section where a clear actin ring was detected was designated as the 0 μm position. Three more subapical sections, at 0.8, 1.6 and 2.2 μm, were chosen for analysis (supplementary material Fig. S3A). Each section was scored for complete and incomplete actin rings. χ-squared analysis was used to assess significance.

We thank J. Minden, A. Linstedt, J. Hildebrand, G. Rogers and members of the Minden and McCartney laboratories for helpful suggestions, A. J. López for the S2 cells, T. Jarvela, R. Decal and J. Choe for help with constructs, D. Roberts and M. Peifer for sharing unpublished results and H. Teng for help with image analysis. We also thank FlyBase, the Bloomington Stock Center, the Developmental Studies Hybridoma Bank, the *Drosophila* Genomics Resource Center and the *Drosophila* RNAi Screening Center for stocks, reagents and information. This work was supported by NIH RO1 GM073891-01A2 to B.M.M. Deposited in PMC for release after 12 months.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/9/1589/DC1

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