Testing hypotheses for the functions of APC family proteins using null and truncation alleles in Drosophila

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Adenomatous polyposis coli (APC) is mutated in colon cancers. During normal development, APC proteins are essential negative regulators of Wnt signaling and have cytoskeletal functions. Many functions have been proposed for APC proteins, but these have often rested on dominant-negative or partial loss-of-function approaches. Thus, despite intense interest in APC, significant questions remain about its full range of cellular functions and about how mutations in the gene affect these. We isolated six new alleles of Drosophila APC2. Two resemble the truncation alleles found in human tumors and one is a protein null. We generated ovaries and embryos null for both APC2 and APC1, and assessed the consequences of total loss of APC function, allowing us to test several previous hypotheses. Surprisingly, although complete loss of APC1 and APC2 resulted in strong activation of Wingless signaling, it did not substantially alter cell viability, cadherin-based adhesion, spindle morphology, orientation or selection of division plane, as predicted from previous studies. We also tested the hypothesis that truncated APC proteins found in tumors are dominant negative. Two mutant proteins have dominant effects on cytoskeletal regulation, affecting Wnt-independent nuclear retention in syncytial embryos. However, they do not have dominant-negative effects on Wnt signaling.

KEY WORDS: Mutation cluster region, Familial adenomatous polyposis, Adherens junctions, β-Catenin

INTRODUCTION

Many genes mutated in human cancers are crucial components of signal transduction pathways regulating normal development. For example, Wnt signaling controls cell fate, asymmetric cell division and stem cell behavior (reviewed by Logan and Nusse, 2004), while the Wnt regulator APC is the tumor suppressor mutated in the hereditary colon cancer syndrome familial adenomatous polyposis (FAP) (reviewed by Gaspar and Fodde, 2004). APC mutations also underlie >70% of sporadic colon cancers.

APC encodes a multidomain protein with several functions (Fig. 1A). It regulates Wnt signaling as part of the ‘destruction-complex’, which maintains low levels of cytoplasmic β-catenin, the key Wnt effector, in the absence of Wnt signals (reviewed by Polakis, 2000). Within this complex, APC binds both β-catenin, via its 15- and 20-amino acid repeats, and the scaffolding protein Axin, via its SAMP repeats (Fig. 1A). Axin and APC present β-catenin to Casein kinase I and GSK3β (fly Zw3), which sequentially phosphorylate β-catenin, targeting it for ubiquitination and destruction. Interestingly, most colon tumors retain an APC protein truncated in the ‘mutation-cluster region’ (MCR, Fig. 1A), lacking the SAMP repeats and all sequences located between that region and the C-terminal end (Polakis, 2000). Wnt ligands like Drosophila Wingless (Wg) inactivate the destruction-complex and stabilize β-catenin by engaging a Frizzled/LRP5/LRP6 receptor complex. The mechanism of destruction-complex inactivation is not well understood, but involves Dishevelled and interactions between LRP5/6 and Axin. Wnt signals may alter Axin localization or stability (Cliffe et al., 2003; Tolwinski et al., 2003).

Both mammals and Drosophila have two APC proteins with shared and divergent structures (Fig. 1). One key question about APC function concerns the relative roles of family members. Mammalian APC is broadly expressed and homozygous mutants die during gastrulation (Moser et al., 1995). Mammalian APC2 is strongly expressed in the CNS (Nakagawa et al., 1998; van Es et al., 1999; Yamanaka et al., 2002), but its mutant phenotype has not been reported. Drosophila APC1 is strongly expressed in the CNS and germline, and homozygous mutants are viable and fertile with defects confined to eye development (Ahmed et al., 1998; Hayashi et al., 1999). Drosophila APC2 is broadly expressed. Zygotic mutants are viable and normal, but maternal/zygotic (MZ) mutants die with defects in Wg signaling during embryogenesis (McCartney et al., 1999). Fly APC1 and APC2 are partially redundant in post-embryonic Wg signaling and Wg-independent brain development (Ahmed et al., 2002; Akong et al., 2002a; Akong et al., 2002b). This occurs despite the fact that their domain structures and subcellular localizations are distinct. APC1 carries the basic domain, and localizes to centrosomes and microtubules, whereas APC2 lacks that domain and localizes to the cortex (Akong et al., 2002b).

In addition to regulating Wnt signaling, APC family proteins have proposed functions in cytoskeletal regulation (reviewed by Nathke, 2004). These are reflected in its binding partners (Fig. 1A). The N-terminal third of APC encodes a series of Armadillo (Arm) repeats, binding sites for cytoskeletal regulators, including the Rac-GEF ASEF, kinesin-associated KAP3, and IQGAP. The C-terminal third encodes a basic region that binds microtubules and the formin Diaphanous (Wen et al., 2004), and a binding site for the microtubule plus-end-binding protein EB1.

Many different cytoskeletal functions have been proposed for APCs. Loss-of-function studies using putative hypomorphic alleles suggest that APC2 helps mediate interactions between mitotic...
spindles and cortical actin in early embryos (McCarty et al., 2001), and works with APC1 to regulate mitotic spindle orientation in the male germline (Yamashita et al., 2003). Certain APC2 alleles also affect cadherin-based adhesion (Hamada and Bienz, 2002; Townsley and Bienz, 2000). RNAi of APC2 alters the symmetric divisions of ectodermal epithelial cells (Lu et al., 2001). Studies in cultured cells using either truncations or dominant-negative approaches suggest roles for mammalian APC in kinetochore function (Fodde et al., 2001; Kaplan et al., 2001) and microtubule organization in polarized cells (Etienne-Manneville and Hall, 2003; Kawasaki et al., 2003; Shi et al., 2004; Wen et al., 2004; Zhou et al., 2004). Finally, in vitro studies suggest APC functions in spindle assembly (Dikovskaya et al., 2004). However, in many cases these effects were subtle. Because these studies did not use null alleles, and some used ‘dominant-negative’ approaches, two distinct possibilities remain: (1) APC family proteins may play essential roles in some of these cytoskeletal processes, which were not fully disrupted using the partial loss-of-function approaches employed; or (2) APC family proteins may be non-essential for some of these processes, but the truncated APC proteins expressed by the mutant alleles or the ‘dominant-negative’ constructs may disrupt them because of their ability to bind and inactivate other essential players. Consistent with the latter possibility, siRNA inactivation of mammalian APC led to less severe spindle defects than those observed in a truncation mutant (Green et al., 2005).

To fully assess APC function, one must assess the consequences of completely eliminating one or both APC proteins. Few studies have used null alleles, and none have removed or reduced function of both APC family members. Although studies of Drosophila APC1 (Ahmed et al., 2002) included a protein-null allele, the only allele encodes a truncated APC protein ending in the MCR (Fig. 1A). This contrasts with most tumor suppressors. The truncated proteins cannot correctly regulate β-catenin, although whether they are null for this function is unclear. Data from tumors and engineered mouse mutations (Smits et al., 1999) suggest that truncated proteins are selected for the deletion of all Axin-binding SAMP repeats, reducing their ability to regulate Wnt signaling. While this explains the function lost by truncation, it does not explain why truncations are inevitable. Bi-allelic null mutations would eliminate function, but are not found in human tumors. There is substantial controversy over whether the truncated proteins have dominant effects or simply reduce APC function. We used genetic and cell biological tools in Drosophila to address these crucial questions.

MATERIALS AND METHODS
Genetic screen for new APC2 alleles
"ru h th st cu sr e ca/TM6 Tb" (referred to as ru cu ca/TM6 Tb) was isogenized prior to the screen. The cross scheme for generating new APC2 alleles is diagrammed (see Fig. 2). ru cu ca/TM6 Tb males were mutagenized with 25 mM EMS (Grigliatti, 1998) and crossed to females carrying TM3 Sb. Individual F1 males carrying the mutagenized chromosome (++) over TM3 Sb were crossed to virgin APC22S females at 27°C (its non-permissive temperature). Embryos maternally and zygotically APC2 mutant die as embryos, whereas paternally rescued embryos survive to adulthood. In crosses where the mutagenized chromosome carried a new allele of APC2, half of the progeny should die as embryos. After three days, each vial was scored for the presence of unhatched embryos whose cuticles had turned brown; these arise when eggs are fertilized but embryos die after having produced cuticle. Approximately 9400 fertile crosses were scored in six rounds of mutagenesis. For lines that exhibited significant embryonic lethality, males were recovered from the crosses and individually crossed to virgin females carrying TM6 Tb to recover the mutagenized chromosome. 110 lines were generated and re-tested for the failure to complement APC22S. Nineteen lines failed to complement APC22S a second time and the mutant embryos exhibited cuticle phenotypes consistent with activation of Wg signaling. Of these mutant chromosomes, we detected molecular lesions in seven (APC22S, APC22S, APC22S, APC22S, APC22S, APC22S and APC22S) upon sequencing the APC2 gene from PCR-amplified genomic DNA from APC22S/Df(3R)crb 84-7 adults.

Hatch rates and cuticle analysis
Embryos were the progeny of APC2allele/Df(3R)crb 84-7 females × APC2allele/+ males. All alleles were scored at 27°C and 18°C. Double-mutant embryos maternally FRT APC2allele APC1Q8 were generated using the FRT/FLP/DfS technique (Chou and Perrimon, 1996). Cuticle preparations and hitch rate analysis were as described by Wieschaus and Nusslein-Volhard (Wieschaus and Nusslein-Volhard, 1998).
**RESULTS**

**Genetic screen for new APC2 alleles**

We generated new alleles using the mutagen EMS (see Fig. 2), selecting them by their failure to complement the M/Z embryonic lethality of APC2<sup>Δ5</sup> (McCartney et al., 1999). This screen would allow us to identify null alleles, as deficiencies removing APC2 fail to complement APC2<sup>Δ5</sup>.

We obtained seven mutations affecting the coding region of APC2 (Fig. 1C). Three are missense mutations in the Arm repeats (APC2<sup>Δ90</sup> repeat 3, APC2<sup>Δ9</sup> repeat 6, and APC2<sup>Δ5</sup> repeat 7; altered amino acids are shown in Fig. 1C). Four alleles result from premature stop codons that should truncate the protein. Two truncate APC2 within Arm repeats 8 or 9: APC2<sup>Δ50</sup> and APC2<sup>Δ70</sup>. Two others truncate APC2 within the MCR: APC2<sup>Δ40</sup> [amino acid 677]; its phenotype has been reported previously (Ahmed et al., 2002; McCartney et al., 2001) and APC2<sup>Δ41</sup> (amino acid 728). Together with APC2<sup>Δ5</sup> (McCartney et al., 1999), which deletes a single serine in Arm repeat 5, and APC2<sup>Δ1758</sup>, identified by Hamada and Bientz (Hamada and Bientz, 2002), a missense mutation in Arm repeat 3, there are nine APC2 alleles. Several mimic those in human tumors or those engineered in mice. APC2<sup>Δ50</sup> resembles mouse APC<sup>Δ710</sup> and APC<sup>ΔMIN</sup> that truncate just C terminal to the Arm repeats (Oshima et al., 1995a; Su et al., 1995). The APC2<sup>Δ40</sup> and APC2<sup>Δ41</sup> truncations are similar to those in the human APC MCR.

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**Immunolocalization, immunoblots and imaging**

Embryos and ovaries were from adults maintained at 27°C. Tissues were fixed as described previously (McCartney et al., 1999). Antibodies/probes used were as follows: for actin, Alexa-488-phallolidin (1:500, Molecular Probes); rat anti-APC2-CT (1:1000) (McCartney et al., 1999); mouse anti-tubulin-E7 (1:1000); anti-Arm-N27A1 (1:500); anti-DE-cadherin-DCAD2 (1:50); anti-En (1:50, all Developmental Studies Hybridoma Bank); for DNA, propidium iodide (25 μg/ml, Molecular Probes) or DAPI (10 μg/ml, Sigma-Aldrich). DAB reactions were carried out as described by McEwen et al. (McEwen et al., 2000). Anti-APC2-NT antisera were raised in guinea pigs by Pocono Rabbit Farms & Laboratory (Canadensis, PA) against GST-APC2 (amino acids 1-126). Immunoblotting used anti-APC2 at 1:1000, detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). For nuclear loss, DAPI-stained nuclei near the cortex but below the cortical monolayer of nuclei were scored as lost – one surface of each embryo was analyzed. The percentage of nuclei lost was determined as the number of lost nuclei/total number of nuclei (estimated from nuclear number in 1 μm<sup>2</sup> of the cortex).

Imaging was performed using various microscope systems. The images in Figs 4, 5 were produced using a Zeiss LSM510 with gain, offset, and pinhole size kept constant for each wavelength; those in Fig. 6 and Fig. 9A-H used a Zeiss LSM510 or LSM410, respectively, with internal wild-type controls expressing histone-GFP co-stained with mutants to control for staining variations. The images in Fig. 7A-C,H-J were produced using a Spinning-disc confocal microscope (Solamere Technology Group) with a Yokogawa scanhead and a Hamamatsu OrcaAG CCD camera on a Zeiss Axiovert200M; those in Fig. 7D,E and Fig. 8 used a Zeiss Axioskop2 Plus and a Canon Powershot G5 camera. Analysis of Fig. 6E,F and Fig. 7G used Adobe Image-J. Figures were prepared using Adobe Shop.

**APC2 rescuing transgene**

Genomic DNA (688 bp) from the gene upstream of APC2 (CGI3608) to the AUG (the ‘endogenous promoter’) of APC2 was PCR amplified and inserted into the EcoRI site of pCaSpeR-2 (modified to include the ADH-polyadenylation signal of pRmHa-3). The APC2 coding region (cDNA LD18122) was inserted 3′ to the endogenous promoter. P-element-mediated transformation generated several lines. Two independent second-generation lines rescued the maternal-effect lethality of APC2<sup>Δ5</sup> at 27°C (data not shown).

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**Both the Arm repeats and the C terminus are required for cortical association**

APC2 localizes to the cortex, co-localizing with cortical actin and overlapping adherens junctions (Fig. 4A) (McCartney et al., 1999; Yu and Bienz, 1999; Yu et al., 1999). We used our mutants to determine whether particular domains are essential or dispensable for cortical localization (we did not examine APC2<sup>Δ90</sup> and APC2<sup>Δ70</sup>, as anti-APC2-NT does not work in tissue). APC2<sup>Δ5</sup> protein fails to localize to the cortex (McCartney et al., 1999), implicating Arm repeat 5 in correct localization. APC2<sup>Δ9</sup>, affecting Arm repeat 6, and...
APC<sup>b5</sup>, affecting repeat 7, encode exclusively cytoplasmic proteins (data not shown), supporting a role for Arm repeats 5-7 in localization. The two proteins truncated in the MCR, APC<sup>2b50</sup> and APC<sup>2b51</sup>, are also exclusively cytoplasmic (Fig. 4; data not shown), supporting a role for the C terminus of APC2 (including the Axin-binding sites) in cortical localization. APC2<sup>g41</sup> and APC2<sup>N175K</sup> proteins weakly associate with the cortex (Fig. 4B; data not shown).

**Complete loss of APC proteins does not disrupt adhesion**

Hemizygous APC<sup>N175K</sup> or APC<sup>2b5</sup> mutants exhibit reduced Arm at adherens junctions, resulting in reduced cadherin-based adhesion, although this effect was not as dramatic as eliminating essential junctional proteins (Hamada and Bienz, 2002; Townsley and Bienz, 2000). One hypothesis is that eliminating APC2 and APC1 function might disrupt adhesion completely.

We first tested this in ovaries. Each egg chamber has 15 nurse cells and an oocyte surrounded by the somatic follicular epithelium (Fig. 5A,C). Arm normally accumulates strongly in follicle cell adherens junctions, and weakly at nurse cell junctions (Peifer et al., 1993). We examined females null for APC2 and reduced for APC1 function (APC2<sup>g10</sup> APC1<sup>Q8</sup>/APC2<sup>g10</sup>), thus reducing function equally in both germ cells and somatic follicle cells, and also egg chambers in which the germline was homozygous APC2<sup>g10</sup> APC1<sup>Q8</sup> (Fig. 5B,D,F; data not shown). In some mutant egg chambers, cytoplasmic Arm levels were elevated (Fig. 5B, F; data not shown), consistent with a loss of destruction-complex.

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**Table 1. Mutant phenotypes of progeny of APC2 allele/deficiency females crossed with APC2 allele/+ males**

<table>
<thead>
<tr>
<th>APC2 allele</th>
<th>27°C</th>
<th>18°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotypic average (n)</td>
<td>Hatch rate (n)</td>
<td>Size of protein</td>
</tr>
<tr>
<td>APC2&lt;sup&gt;b5&lt;/sup&gt;</td>
<td>2.0 (215)</td>
<td>52% (549)</td>
</tr>
<tr>
<td>APC2&lt;sup&gt;g41&lt;/sup&gt;</td>
<td>3.1 (189)</td>
<td>52% (399)</td>
</tr>
<tr>
<td>APC2&lt;sup&gt;g50&lt;/sup&gt;</td>
<td>3.3 (227)</td>
<td>49% (889)</td>
</tr>
<tr>
<td>APC2&lt;sup&gt;g51&lt;/sup&gt;</td>
<td>3.5 (247)</td>
<td>53% (539)</td>
</tr>
<tr>
<td>APC2&lt;sup&gt;g55&lt;/sup&gt;</td>
<td>3.6 (197)</td>
<td>38% (1269)</td>
</tr>
<tr>
<td>APC2&lt;sup&gt;g56&lt;/sup&gt;</td>
<td>3.8 (214)</td>
<td>46% (920)</td>
</tr>
<tr>
<td>APC2&lt;sup&gt;g57&lt;/sup&gt;</td>
<td>4.2 (317)</td>
<td>48% (585)</td>
</tr>
<tr>
<td>APC2&lt;sup&gt;g58&lt;/sup&gt;</td>
<td>4.4 (263)</td>
<td>51% (449)</td>
</tr>
</tbody>
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Phenotypic scoring criteria were: 0, wild-type cuticle, but did not hatch; 1, head defects but no head hole/wild-type size/15% of denticles missing; 2, head defects or small 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 denticle bands remaining; 4, 3 as 3, but =2 denticle patches remaining; 5, size as 3, but anterior hole extends ventrally to ~30% cuticle length/no denticles; 6, anterior hole extends ventrally to ~50% cuticle length/cuticle round, 25-30% wild-type size/no denticles.

nd, not determined; pa, phenotypic average.
function. However, cortical Arm localization was largely unaltered in mutant germ cells and follicle cells, regardless of whether (Fig. 5B′,D′) or not (Fig. 5F′) they had elevated Arm levels.

Cadherin-based adhesion positions the oocyte at the posterior end of each egg chamber (Godt and Tepass, 1998). In APC2 Δ5 or APC2 Δ175K mutants, oocytes are occasionally mispositioned (Hamada and Bienz, 2002; Townsley and Bienz, 2000). To determine the effect of loss of APC function on oocyte position, we compared wild type, APC2 Δ10, APC2 Δ10 APC1 Δ5/APC2 Δ10, and APC2 Δ10 APC1 Δ8 mutant germ lines. We distinguished the oocyte from nurse cells by the presence of four ring canals (Fig. 5B, arrow) and its elevated accumulation of cortical actin. There was no difference in the frequency of mispositioned oocytes between wild type and mutants (Fig. 5E). Thus a complete loss of APC function does not substantially reduce cadherin-based adhesion in ovaries.

Embryonic adherens junctions are established during cellularization, and total loss of cadherin-based adhesion results in catastrophic defects in epithelial architecture (Cox et al., 1996; Tepass et al., 1996). We did not observe defects in epithelial structure in M/Z APC2 APC1 double null embryos (Fig. 6B,D,F). Furthermore, we did not observe disruption of cortical localization of DE-cadherin (Fig. 6A,B) or α-catenin (Fig. 6C-F) in embryonic epithelia. In fact, if anything, cortical localization was slightly elevated. Arm levels were highly elevated (Fig. 6B,D), but cortical Arm was still present. Finally, the cuticles of these embryos (see below) did not display the total disruption seen when adhesion is strongly compromised. The discrepancy with the earlier results of the Bienz Laboratory suggests that the proteins they tested may have dominant-negative effects on cadherin-catenin function; consistent with this, APC2 Δ5 has dominant-negative effects on cortical nuclear retention (see below).

Assessing roles of APC proteins in nuclear retention, spindle morphology and orientation

After fertilization, Drosophila embryos undergo a series of nuclear divisions without cytokinesis. As microtubules form spindles, actin lines transient furrows separating adjacent nuclei, preventing spindle collisions (Fig. 7A). Thus, defects in actin or microtubule function can result in abnormal nuclear divisions with resulting abnormal
nuclei transported into the embryo interior. In APC2\textsuperscript{4S} mutants, a subset of peripheral nuclei are lost without significant actin or microtubule defects (McCartney et al., 2001), suggesting that APC2 helps tether actin to microtubules, thereby tethering nuclei to the cortex.

Our new alleles allowed us to examine how complete loss of APC2 or complete loss of both APC proteins affects this process, revealing whether the partial nuclear loss phenotype of APC2\textsuperscript{4S} and APC2\textsuperscript{d40} (McCartney et al., 2001) was enhanced. We quantified nuclear loss in APC2 mutant embryos or in embryos doubly null for APC2 and APC1 (Fig. 7F). We defined an abnormal embryo as one in which \( \geq 2\% \) of the cortical nuclei have moved into the embryo interior (Sullivan et al., 1993). In our two wild-type strains (\( y\ w \) and Oregon-R), 0-3\% of embryos are abnormal, consistent with previous reports (2-3\%) (Sullivan et al., 1993).

We first examined the effects of a complete lack of APC2. Thirty-five percent of APC2\textsuperscript{g10} maternally mutant embryos are abnormal (Fig. 7E,F). This is partially rescued by \( P[\text{APC2}^+], \) an APC2 transgene driven by the endogenous promotor (14\%; Fig. 7F); this incomplete rescue may be due to reduced levels of APC2 expression from the transgene. Seven percent of the progeny of mothers heterozygous for APC2\textsuperscript{g10} are abnormal – this may reflect slight haploinsufficiency or may be within the wild-type range. To determine whether APC1 also has syncytial functions, we examined nuclear loss in APC2\textsuperscript{g10} APC1\textsuperscript{G8} maternally double-mutant embryos (Fig. 7B,C,F). Thirty-nine percent of the embryos were abnormal, similar to APC2\textsuperscript{g10} alone (35\%), suggesting that if APC1 functions in this process, its contribution is relatively minor. In APC2\textsuperscript{4S}, nuclear loss appears to be largely due to disrupted tethering (Mc CARTney et al., 2001). In APC2\textsuperscript{g10} and in double null mutants, this may be compounded by spindle collisions resulting from compromised actin furrows (Fig. 7B,B', arrow). However, even in the most severe cases, many nuclei remain at the cortex.

Our mutant alleles also allowed us to assess which domains of APC2 are important for function in nuclear retention, and to examine whether truncated proteins have dominant effects on this process. We previously documented nuclear retention defects in APC2\textsuperscript{d40} (McCartney et al., 2001), which is truncated in the MCR (Fig. 1C, Fig. 7F). APC2\textsuperscript{N175K} and APC2\textsuperscript{c9} maternal mutants have relatively weak phenotypes (12\% and 8\% abnormal embryos). By contrast, 58\% of APC2\textsuperscript{d40} embryos are abnormal. This is reduced to 15\% by \( P[\text{APC2}^+], \) which rescues the null allele to the same degree. This indicates that the dramatic effect of APC2\textsuperscript{d40} is due to its effect...
on APC2. In progeny of mothers heterozygous for APC2<sup>AS</sup>, the frequency of abnormal embryos (21%) was substantially higher than in wild type (2-3%), and was much higher than that caused by heterozygosity for the null allele (7%). This suggests that APC2<sup>S</sup> protein, like APC2<sup>AS</sup>, is dominant negative in this process.

In Xenopus extracts, APC is required to form robust spindles (Dikovskaya et al., 2004). In syncytial Drosophila embryos, thousands of nuclei divide synchronously, providing an excellent system in which to examine whether fly APCs are crucial for spindle structure. In APC2<sup>AS</sup>/H9004<sup>S</sup> mutants, nuclei and spindles are lost from the cortex without significant defects in spindle morphology (McCartney et al., 2001). To determine whether complete loss of APC2 and APC1 affects syncytial spindles, we compared spindle morphology in embryos maternally APC2<sup>g10</sup> APC1<sup>Q8</sup> double mutant with nuclear- and cell-cycle stage-matched wild types. APC2APC1 null embryos did not have significant defects in overall spindle morphology (Fig. 7B’). Astral microtubules are not easily observed in syncytial embryos (Foe et al., 1993), and thus we did not examine them. The one change in spindles we noted was a slight but significant lengthening of the pole-to-pole distance (Fig. 7G).

After cellularization, ectodermal cells divide in synchronous regions called mitotic domains (Foe et al., 1993). We next compared spindle orientation and division plane in wild-type and APC2<sup>g10</sup> APC1<sup>Q8</sup> M/Z mutant ectoderm. Wild-type spindles are oriented perpendicular to the apical-basal axis (92%, Fig. 7H,K) and divisions are symmetric, resulting in two equal daughter cells (100%, Fig. 7H,K). A previous RNAi study (Lu et al., 2001) suggested that disrupting APC2 function results in mis-oriented...
spindles and asymmetric cytokinesis in the ectoderm. However, spindles in APC2<sup>+/+</sup> APC1<sup>Q8</sup> MIZ mutant embryos are oriented normally (90-92%, Fig. 7J,K), and all divisions were symmetric (100%, Fig. 7J,K). We also examined whether truncated APC2 proteins have dominant effects, examining divisions in APC2<sup>d40/+</sup> embryos derived from APC2<sup>d40/g10</sup> homozygous mothers. We observed no dominant-negative effects (Fig. 7I,K). One possible explanation of the discrepancy with the results of Lu et al. (Lu et al., 2001) is off-target RNAi effects of their 1 kb dsRNA. Thus, APC family proteins do not play a key role in spindle structure, orientation or division plane selection in the Drosophila ectodermal epithelium.

Relating APC2 structure to function in Wnt signaling
APC proteins play an essential role in regulating Wnt signaling, but key questions remain about the relationship between structure and function. We used our APC2 mutations to assess the requirement for different domains in Wg regulation, and to determine the level of function retained by truncated proteins and their potential for dominant-negative effects on Wg signaling.

The embryonic cuticle provides a sensitive readout of cell fate choices. We examined the cuticle phenotype of MIZ mutants, placing each allele in trans to a deletion of APC2 to reduce concerns about other background mutations. We initially assessed phenotypes at 27°C, in case any mutations were temperature sensitive. Global activation of Wg signaling has several consequences for embryogenesis. Three phenotypes vary roughly in parallel. (1) Epidermal cell fates – increased Wg signaling leads to fewer denticle-producing cells and more smooth cuticle (Fig. 8D, black arrow). (2) Cuticle size – elevated Wg signaling results in fewer epidermal cells, due to apoptosis (Pazdera et al., 1998). (3) Head morphology – elevated Wg signaling disrupts head involution (Fig. 8D, open arrow). We scored >180 embryos per genotype, assigned each to a phenotypic category from weak (0) to strong (6; representative cuticles are in Fig. 8), and calculated a phenotypic average (pa) for each allele. The nine APC2 alleles form a phenotypic series with phenotypic averages ranging from 2.0 for APC2<sup>e90</sup> to 4.5 for APC2<sup>g10</sup> (Table 1). This divided the alleles into three categories based on Wg activation: (1) weak – APC2<sup>e90</sup>, APC2<sup>d40</sup> and APC2<sup>g10</sup>; (2) moderate – APC2<sup>c9</sup>, APC2<sup>d40</sup> and APC2<sup>g41</sup>; and (3) strong – APC2<sup>f90</sup>, APC2<sup>g90</sup> and APC2<sup>g10</sup> [our APC2<sup>N175K</sup> stock has a somewhat weaker phenotype than had been previously reported (Hamada and Bienz, 2002)].

We also assessed effects on Wg signaling directly, examining Arm stability. In wild type, Arm accumulates at cell-cell junctions in all cells, whereas in cells receiving Wg Arm also accumulates in the cytoplasm and nucleus, due to inactivation of the destruction-complex (Fig. 9A). APC2<sup>d40</sup> mutants have elevated Arm levels (McCartney et al., 1999), but not as elevated as those seen when the destruction-complex is completely inactivated by MIZ loss of Zw3 kinase (Peifer et al., 1994; Siegfried et al., 1994). The less severe phenotype of APC2<sup>d40</sup> is due, in part, to slight APC1 activity (Ahmed et al., 2002; Akong et al., 2002a), but might also suggest that APC2<sup>d40</sup> does not fully inactivate APC2.

The cuticle phenotypes of our APC2 alleles correlate well with Arm levels. The weakest allele, APC2<sup>e90</sup>, has only a slight elevation in Arm levels; stripes are still readily apparent (Fig. 9B). Other weak to moderate alleles with missense mutations in the Arm repeats (APC2<sup>c9</sup> and APC2<sup>b5</sup>) have a slightly greater elevation of interstripe Arm levels, but stripes remain detectable (Fig. 9C,D) – they resemble APC2<sup>d40</sup> (McCartney et al., 1999). The alleles truncating APC2 in the MCR, APC2<sup>d40</sup> (Fig. 9E) (Akong et al., 2002a) and APC2<sup>g41</sup> (Fig. 9F), are stronger still, showing uniformly high levels equivalent to those in wild-type Arm stripes. Finally, APC2<sup>g90</sup> and
the null allele APC2g10 accumulate Arm levels in all cells higher than those in wild-type stripes (Fig. 9G,H). The highest Arm levels, however, are only seen when APC1 is also removed, in APC2g10 APC1Q8 double null mutants (Fig. 9I) – these resemble the extremely high levels previously seen in APC2d40 APC1Q8 double mutants (Ahmed et al., 2002; Akong et al., 2002a) and in zw3 M/Z mutants (Peifer et al., 1994; Siegfried et al., 1994) (Fig. 9J). We also examined the expression of a Wg target gene, engrailed (en), in our strongest alleles, APC2g41, APC2f90 and APC2g10. en is activated in additional cells posterior to its normal domain, but it is not activated in all cells (see Fig. S1 in the supplementary material), results that are identical to what was previously observed in APC2/H9004S (McCartney et al., 1999) or M/Z zw3 mutants (Siegfried et al., 1992).

APC2g41 is temperature sensitive, exhibiting M/Z embryonic lethality at 25°C and viability at 18°C (McCartney et al., 1999). To determine whether other APC2 alleles are temperature sensitive, we assessed their cuticle phenotypes and hatch rates at 18°C (Table 1). Strikingly, all mutants with missense mutations in the Arm repeats, except APC2N175K, are temperature sensitive; many M/Z mutants hatch as larvae. By contrast, the truncation alleles APC2d40, APC2g41 and APC2g90, and the null allele APC2g10 are not temperature sensitive.

We also assessed whether mutants had dominant-negative effects on Wg signaling. None affect adult patterning in zygotic heterozygotes or homozygotes, ruling out strong dominant-negative effects. As a more sensitive test, we assessed paternal rescue of M/Z embryonic lethality, which requires that paternal wild-type APC2 can counter the effects of M/Z mutant protein. If paternal rescue is fully effective, 50% of the progeny of mutant females crossed to heterozygous males should hatch. For eight alleles, ~50% (46-52%, Table 1) of the offspring hatch as larvae. The exception is APC2g9, where only 38% hatch. Thus, most alleles, including those producing proteins truncated in the MCR, do not have apparent dominant-negative effects on Wg signaling; however, APC2g9 may have some dominant effect on viability, such that paternal rescue is incomplete.

APC1 acts redundantly with APC2 in Wg regulation in many tissues (Ahmed et al., 2002; Akong et al., 2002a; Akong et al., 2002b). In embryos, low levels of APC1 in the epidermis provide a small amount of residual function when APC2 is reduced. Interestingly, the cuticle phenotypes of the strongest APC2 single mutants (Fig. 8) are roughly as severe as those of APC2g41 APC1Q8 double mutants (Ahmed et al., 2002; Akong et al., 2002a), although their effects on Arm levels (Fig. 9) suggest residual APC1 function in APC2 null single mutants. To further explore this, we examined the cuticle phenotypes of embryos M/Z double mutant for a null allele of APC1 (APC1Q8) and several APC2 alleles (Table 1). All show approximately the same severity of cuticle phenotype [phenotypic average (pa)=3.7-4.0; Fig. 8H,I], suggesting that, in the absence of APC1, all are so disabled that Wg regulation drops below the threshold of function measurable in our cuticle assay. Alternatively, the partial activity of some mutant proteins may depend on APC1 function in some way. APC2g10 APC1Q8 double null mutants (pa=3.8, n=238; Fig. 8I) are quite similar to axin (axn) null embryos (pa=4.1, n=239; Fig. 8J), which fully inactivate the destruction complex. In fact, this analysis slightly underestimates the double null phenotype, because some paternally rescued embryos die (data not shown). APC2g10 APC1Q8 M/Z mutants selected using a GFP marker have a more severe phenotype (pa=4.7, n=96).

**DISCUSSION**

Despite substantial interest in Wnt signaling and its regulation in development and disease, important questions remain about the nature of the null phenotype and thus the full range of processes in which APC family proteins play a crucial role.
Assessing roles for APC proteins in cell adhesion and spindle function

Experiments in vitro, in cultured cells and in Drosophila suggested novel roles for APCs in cadherin-based adhesion (Hamada and Bienz, 2002; Townsley and Bienz, 2000), spindle structure and chromosome segregation (e.g. Green and Kaplan, 2003). Although some of these effects were subtle, APC family function was not completely eliminated, suggesting that APCs may play essential roles in one or more of these processes. Alternatively, because these phenotypes were assessed in cells expressing truncated or otherwise mutant proteins, or expressing transfected APC fragments, it is possible that these effects result from dominant interference with binding partners of APC that work in a process in which APC proteins themselves are not essential.

To distinguish between these possibilities, null mutations removing the function of both APCs must be characterized. In mammals, all work has been done in single mutants and most was done with cells or animals expressing one truncated APC allele. Recently, Cre-lox technology was used to generate mouse APC alleles that may be null; these delete exon 14, and are predicted to truncate APC before the Arm repeats (Colnot et al., 2004; Shibata et al., 1997). Although the phenotype of homozygous animals has not been reported, Cre induction was used to create homozygous mutant clones of colon cells. This triggers polypl hypomorphism (Shibata et al., 1997), with mutant cells assuming stem cell properties consistent with Wnt activation (Andreu et al., 2005; Sansom et al., 2004). Other phenotypes were not assessed, however, and tests to confirm that this allele is protein null were not reported, so splicing variations might produce residual mutant protein.

We examined ovaries and embryos null for APC2, or double null for both APC2 and APC1, for essential roles in cadherin-based adhesion. We did not observe phenotypes consistent with substantial disruption of cadherin-catenin function, which disrupts both oogenesis and embryonic epithelial integrity (Cox et al., 1996; Tepass et al., 1996). In ovaries, loss of APC2 and APC1 had no apparent effect on adhesion, and in embryos we did not observe significant alterations in DE-cadherin or α-catenin localization at adhesions junctions. Thus APC family proteins do not play an essential role in cell adhesion. However, we cannot rule out subtle modulatory effects.

We also tested proposed roles for APC proteins in spindle assembly and orientation. Embryos null for both APC proteins had no defects in spindle structure in syncytial embryos, apart from those in regions of spindle detachment or defective metaphase furrows, and no defects in spindle orientation or cell division symmetry in the ectoderm during gastrulation. Thus APC family proteins are not essential for spindle function in these tissues. We did see a subtle but significant lengthening of syncytial spindles during cycle 13. We did not assess subtle defects in chromosome segregation, which might lead to a slow accumulation of aneuploid cells in tumors – this will require other assays. How can we reconcile the earlier data suggesting that APC family proteins have roles in adhesion and cytoskeletal regulation when our full loss-of-function experiments indicate that they do not? One possibility is that truncated fragments of APC may have dominant effects on processes in which APC does not play an essential role – our data on the phenotype of APC2-G5 in spindle tethering, which is discussed in more detail below, provide an example of this.

Are truncated APC proteins dominant negative?

Unlike most other tumor suppressors, APC homozygous null colon tumors are either rare or non-existent. Instead, one allele encodes a protein truncated in the MCR, suggesting strong selection for this event during tumor development. Several models propose that the truncated APC proteins found in tumors are dominant negative. One suggests that this affects Wnt signaling, with truncated APC proteins promoting stem cell proliferation (Kim et al., 2004). Most models suggest that truncated proteins affect cytoskeletal functions. Different studies come to different conclusions, however. For example, some suggest that truncated APC interferes with microtubule-kinetochore attachments, leading to genomic instability (Green and Kaplan, 2003; Green et al., 2005; Tighe et al., 2001), but others suggest these effects are subtle (Sieber et al., 2002a). A dominant-negative role of truncated APC is not essential for disease, as some FAP patients inherit germline-null APC mutations (Laken et al., 1999; Sieber et al., 2002b). Their adenomas carry truncating mutations in the other allele; in this case, there was no wild-type APC to be affected by a dominant-negative truncation. Furthermore, the putative dominant-negative effect is not sufficient for oncogenesis – mice engineered to express truncated APC in a wild-type background do not develop polyps or tumors (Oshima et al., 1995b).

Our genetic data provide new insight into this question. We saw little evidence for dominant-negative effects on Wg signaling. Heterozygotes are viable and adults are wild type in phenotype, and wild-type paternal APC2 effectively rescues eight of the nine mutants, suggesting that mutant proteins cannot be strongly dominant negative. The exception is APC2-G5, where there appears to be some interference with paternal rescue. Likewise, our data suggest that truncated APC2 does not substantially affect spindle structure (we did not address whether APC1 truncations behave dominantly).

We did, however, find compelling evidence for dominant-negative effects on nuclear retention in syncytial embryos. APC2-G5 and APC2-G46 heterozygotes exhibit elevated levels of nuclear loss, and the frequency of abnormal embryos is higher in APC2-G5 homozygotes than in APC2 null mutants. Thus, the cytoskeletal functions of APCs may be more sensitive to dominant-negative effects of truncated proteins, and this may affect chromosome segregation and contribute to tumor progression.

Our data also illuminate the mechanisms of dominant-negative activity. Loss of APC1 did not enhance the nuclear-loss phenotype of APC2 null embryos, suggesting that APC1 does not play a significant role in this process. This suggests that the dominant-negative effect is not on maternally contributed APC1 (Hayashi et al., 1997). As nuclear retention is not completely disrupted in double null mutants, alternative mechanisms of nuclear retention partially compensate for the lack of APC1 and APC2. Because APC2-G5 has a nuclear retention defect more severe than that of embryos MZ null for both APCs, this mutant protein may not only block residual APC2 function, but may also interfere with a parallel, APC2-independent means of nuclear retention.

Our data also provide insights into the domains of APC2 required for nuclear retention, suggesting roles for the Arm repeats and the C terminus (Fig. 10). Because APC2-G5 mutants exhibit much more nuclear loss than do APC2-G5/W and APC2-G5, Arm repeat 5 may have a special importance, perhaps by interfering with binding of a particular partner; APC2-G5 may also more profoundly affect the overall structure of the Arm repeats.

**APC structure and function in Wnt signaling**

Our experiments provide an in vivo test of the function of proteins truncated in the MCR (Fig. 10). APC2-G46 and APC2-G47 strongly reduce the ability to regulate Wg signaling, but are not as strong as the null APC2-G5, or APC2-G46, truncating APC2 at the end of the Arm
Testing hypotheses of APC function

Fig. 10. Structure/function of APC2. Wild-type APC2 is shown in the middle. (Above) Domains defined by mutations (black bars) important for cortical localization or nuclear retention. (Below) Truncated proteins that are null or have reduced function, domains important for Wnt signaling, and a region conferring a more severe phenotype on APC2<sup>291</sup>.

repeats. A similar severely truncated allele of mammalian APC led to higher levels of Wnt reporter activity in cultured cells than did a truncation in the MCR (Kielman et al., 2002). Our data support the ‘just right’ hypothesis (Albuquerque et al., 2002), which posits selection in tumors for mutations in which Wnt signaling is elevated, but not too much. In this model, proteins truncated in the MCR retain some ability to regulate β-catenin, resulting in levels of Wnt signaling that are above the threshold for polyp formation but not ‘too high’, which might be cell lethal. Our study is the first direct test of this hypothesis using null alleles.

Our nine alleles also allow us to begin to assess the roles of different domains in Wg regulation. The role of the Arm repeats of APC has been unclear. In APC mutant tumor cells, transfection of constructs encoding the 15- and 20-amino acid repeats and the SAMP repeats alone restores β-catenin turnover (Munemitsu et al., 1995). However, because these cells retain the Arm repeats-containing truncated protein found in the tumor, these two APC fragments might exhibit intra-allelic complementation. Our data suggest that the Arm repeats are crucial for full function of APC2 in the destruction complex (Fig. 10). As our missense mutations are not null for Wg regulation, APC proteins may retain residual function in Wg signaling without the Arm repeats. Alternatively, the mutations we isolated may not fully disrupt the repeats. Each Arm repeat comprises three alpha-helices (e.g. Huber et al., 1997). Together, the Arm repeats form a superhelical structure with a large repeat comprises three alpha-helices (e.g. Huber et al., 1997).

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Supplementary material
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