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Drosophila Apc1 and Apc2 regulate Wingless transduction throughout development

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SUMMARY

Inactivation of the Adenomatous Polyposis Coli (APC) tumor suppressor triggers the development of most colorectal carcinomas. APC is required for targeted degradation of β-catenin, the central transcriptional activator in the Wnt/Wingless (Wg) signal transduction pathway; however, the precise biochemical functions of APC remain uncertain. The two *Drosophila* homologs of APC (Apc1 and Apc2) appear to have predominantly different tissue distributions, different subcellular localizations and mutually exclusive phenotypes upon inactivation. Unexpectedly, we have found that despite these differences, simultaneous reduction in both *Drosophila* Apc proteins results in the global nuclear accumulation of β-catenin and the constitutive activation of Wg transduction throughout development. This redundancy extends even to functions previously thought to be specific to the individual Apc homologs. Together, these results reveal that the combined activity of Apc1 and Apc2 allows a tight regulation of transcriptional activation by β-catenin and suggest that APC proteins are required for the regulation of Wnt transduction in all cells.

Key words: *Drosophila*, APC, Wingless, β-catenin, Transduction

INTRODUCTION

The *Adenomatous Polyposis Coli* (*APC*) tumor suppressor was identified as the gene mutated in specific families with a hereditary predisposition towards developing colorectal adenomatous polyps and carcinomas (Joslyn et al., 1991; Kinzler et al., 1991). Truncation mutations in *APC* were subsequently also found in greater than 80% of sporadic colonic adenomatous polyps and carcinomas (Miyoshi et al., 1992). Homozygous inactivation of *APC* appears to be the earliest molecular event in colonic epithelial cells that underlies their transformation through the stages of hyperproliferative epithelium, neoplastic adenoma and finally carcinoma (Powell et al., 1992; Kinzler and Vogelstein, 1998). The tumor suppressor function of APC relies in part on its ability to promote the degradation of β-catenin, a protein that functions both in epithelial cell adherens junctions, and as a transcriptional transactivator in the Wnt signal transduction pathway (reviewed by Polakis, 2000). The APC-mediated regulation of β-catenin links APC to Wnt signaling, and thus to a developmental pathway that plays central roles in cell fate determination and patterning in organisms ranging from fruit flies to mammals.

In the absence of Wnt signaling, β-catenin is relatively rapidly degraded by ubiquitin-mediated targeting to the proteasome (Polakis, 2000). The targeting of β-catenin for degradation depends not only on APC, but also on Glycogen Synthase Kinase 3β (GSK3β) and Axin. Together, these proteins form a complex that catalyzes the phosphorylation of β-catenin by GSK3β. Phosphorylated β-catenin is recognized by the SCF ubiquitin ligase complex, ubiquitinated and targeted for degradation (Aberle et al., 1997; Jiang and Struhl, 1998). Biochemical studies, including the in vitro reconstitution of β-catenin degradation in *Xenopus* egg extracts, demonstrate a requirement for Axin, GSK3β and APC in β-catenin degradation, which does not occur in extracts depleted for any of these three proteins (Ikeda et al., 1998; Salic et al., 2000). Efficient phosphorylation of β-catenin by GSK3β requires the binding of both β-catenin and GSK3β to Axin. Axin binds β-catenin with low affinity, however, and APC greatly enhances the Axin/β-catenin interaction. APC has also been implicated in a second ubiquitin-mediated β-catenin degradation pathway that is not dependent on either GSK3β or Axin (Liu et al., 2001).

Much of the Wnt pathway has been conserved from flies to mammals, and *Drosophila* has served as an excellent model organism in which to dissect genetically the roles of the many Wnt/Wingless (Wg) signaling components, as well as to place their activities in a hierarchical order (Cadigan and Nusse, 1997). Wg transduction events are required for specification of cell fate in most tissues, and require the induction of transcriptional transactivation by Armadillo (Arm), the fly homolog of β-catenin. In the embryo, Wg transduction is required for cell fate specification within the ectoderm, mesoderm and endoderm. Wg transduction is also required at postembryonic stages within most imaginal precursors of adult

tissues, specifying dorsoventral and/or anteroposterior identity. Homologs of the 'negative' effectors of the pathway, APC, Axin and GSK3β, also exist in *Drosophila*. Inactivation of *Axin* or of *zeste-white 3* (*zw3*), the *Drosophila* homolog of GSK3β, results in the constitutive activation of Wg transduction in both embryonic and imaginal disc tissues (Siegfried et al., 1992; Diaz-Benjumea and Cohen, 1994; Jiang and Struhl, 1996; Willert et al., 1999; Hamada et al., 1999). These studies reveal that Axin and Zw3 are active in the absence of Wg transduction, and must be inactivated upon Wg signaling.

In both humans and in *Drosophila*, there are two homologs of APC (Nakagawa et al., 1998; van Es et al., 1999). The *Drosophila* Apc homologs Apc1 and Apc2 share the greatest sequence similarity with each other and with the human APC homologs in regions required for protein-protein interactions: the Arm repeats, the β-catenin binding sites and the Axin-binding sites (Hayashi et al., 1997; Yu et al., 1999; McCartney et al., 1999) (Fig. 1A). In addition, both *Drosophila* Apc proteins have been shown to interact physically with Arm and Axin (Hayashi et al., 1997; Hamada et al., 1999; McCartney et al., 1999). However, the *Drosophila* Apc proteins differ from each other in several important respects, raising questions of whether these two proteins have evolved to assume distinct functions. First, Apc1 is expressed predominantly in the nervous system, while Apc2 is found ubiquitously (Hayashi et al., 1997; McCartney et al., 1999; Yu et al., 1999). Second, the subcellular distribution of Apc1 and Apc2 are largely distinct; Apc2 is associated with actin-based structures (Yu et al., 1999; McCartney et al., 1999), while Apc1 appears excluded from these structures (Hayashi et al., 1997; Ahmed et al., 1998) (Y. A., A. N. and E. W., unpublished). Third, the C-terminal half of Apc1, which contains a putative microtubule-binding domain, is completely missing in Apc2 (see Fig. 1A). Finally, loss-of-function mutations in both *Drosophila* Apc genes have revealed mutually exclusive phenotypes. Inactivation of *Apc1* results in retinal neuronal apoptosis that is dependent on Arm hyperactivation, yet loss of *Apc1* has no effect on Wg-dependent patterning at any developmental stage (Ahmed et al., 1998). By contrast, inactivation of *Apc2* results in the constitutive activation of Wg signaling; however, this phenotype is restricted to embryogenesis, with no effect on the many postembryonic Wg transduction events (McCartney et al., 1999; Yu et al., 1999). Thus, inactivation of either Apc gene results in a limited range of phenotypes with regard to Wg transduction.

Inactivation of human *APC* also appears to induce phenotypes in a limited number of tissues. Although human APC is widely expressed, germline mutations of *APC* result in disease that is restricted primarily to the gastrointestinal tract, retina, jaw and long bones, and abdominal connective tissue (Fearnhead et al., 2001). Together, these observations raise several questions about the *Drosophila* and human APC proteins. Are the APC proteins required in the regulation of Wnt/Wg signaling in all cells, or just a subset? Do the two APC proteins ever function within the same cell? Have the two APC proteins evolved to assume completely distinct functions, or instead can they act in a functionally redundant manner, such that the full range of APC activity would be revealed only upon simultaneous inactivation of both *APC* genes? We undertook this study to address these questions. Unexpectedly, we found that despite their different properties, simultaneous reduction in the activity of the two *Drosophila* Apc proteins results in the stabilization and nuclear accumulation of Arm in virtually all cells, and the constitutive activation of Wg transduction in most tissues throughout development. We also found that the limited Arm hyperactivation phenotypes revealed by inactivation of either *Drosophila* Apc gene singly are not the result of unique functions of one of the two *Drosophila* Apc proteins, but rather reflect the requirement for threshold levels of Apc1 and Apc2 in the regulation of Arm activity. Together, these results reveal that the combined activity of Apc1 and Apc2 within the same cell allows a tight regulation of transcriptional activation by Arm, and suggest that APC proteins are required for the regulation of Wnt/Wg transduction in all cells.

MATERIALS AND METHODS

Mutations and chromosomal deletions

The $Apc1^{Q8}$ (Ahmed et al., 1998), $Apc2^{d40}$ (McCartney et al., 2001), $Axin^{S044230}$ (Hamada et al., 1999), $zw3^{M11\text{-}1}$ (Siegfried et al., 1992), and osk^{166} (Lehmann and Nusslein-Volhard, 1991) mutant alleles were used. *w6* is a deficiency on the right arm of chromosome 3 at cytological position 95E that eliminates the *Apc2* gene (gift from M. Bienz).

Transgenes

The genomic *Apc2* transgene was made using a 7 kb *Swa*I/*Spe*I restriction fragment from P1 clone DS 00648 (Berkeley *Drosophila* Genome Project), which includes the *Apc2* gene. This fragment includes nucleotides 1.3 kb upstream and 1.3 kb downstream from sequences encoding the full-length *Apc2* transcript. This fragment was subcloned into the Casper vector for P-element-mediated transformation (Rubin and Spradling, 1982).

The *Apc1* transgene, which contains sequences encoding the full-length *Apc1* cDNA (Hayashi et al., 1997), was subcloned into the pUAST vector and used for generating *UAS-Apc1* transgenic flies (S. Hayashi, A. N., A. Levine and E. W., unpublished) by P-element-mediated transformation (Rubin and Spradling, 1982).

Overexpression of Apc1 in the embryo was driven by the maternal-GAL4 line 67, which contains a second chromosomal insert of a *GAL4-VP16* fusion transgene under control of the maternal *α-tubulin* promoter (gift from D. St. Johnston).

Generation of somatic and germline mutant clones

Clones of mutant somatic and germ cells were generated by the FLP-mediated recombination method (Xu and Rubin, 1993; Chou and Perrimon, 1996). Clones of $Apc1^{Q8}$ $Apc2^{d40}$ mutant cells were marked in the adult by loss of either a *yellow* or *white* transgene in the body cuticle and eye, respectively. Clones were induced by subjecting first or second instar larvae to a 37°C heat shock for 1-2 hours. In addition, eye-specific clones were induced using *eyeless*- (*ey*) *flp* (gift from M. Brodsky). Pharate and adult structures were placed in 70% ethanol, boiled in 10% sodium hydroxide, rinsed in water, dissected and mounted in Faure's medium.

Genotypes for generating $Apc1^{Q8}$ $Apc2^{d40}$ mutant clones are listed below.

Apc1 Apc2 somatic clones

y hsp70-flp/+; FRT 82B e $Apc2^{d40}$ $Apc1^{Q8}$/ FRT 82B hsp70-CD2, y+

Apc1 Apc2 germline clones

y hsp70-flp/+; FRT 82B e $Apc2^{d40}$ $Apc1^{Q8}$/ FRT 82B ovo^{D1}

Apc1 Apc2 eye clones

w; ey-flp/+; FRT 82B e $Apc2^{d40}$ $Apc1^{Q8}$/ FRT 82B P{w+} 90E

Histology

To generate anti-Apc2 sera, a 498 bp PCR fragment encoding amino acids 722 to 887 of the Apc2 protein was amplified from a *Drosophila* expressed sequence tag (clone LD18122, Berkeley *Drosophila* Genome Project) and cloned into the pET-29a vector (Novagen). S-TAG fusion protein was used as an immunogen in rabbits. As an internal control for variability in immunostaining, and as a means of differentiating signal from background, we fixed and stained homozygous $Apc2^{d40}$ maternal/zygotic mutant embryos in the same tube as embryos that have wild-type Apc2, but which are mutant for $oskar^{166}$ (Lehmann and Nusslein-Volhard, 1991). The $oskar^{166}$ mutant embryos were then identified by a lack of staining with an anti-Vasa antibody, and a comparison of Apc2 immunostaining was made between the wild-type Apc2 (*oskar* mutant) embryos and the $Apc2^{d40}$ mutant embryos.

The other primary antibodies used were Armadillo N2 (Riggleman et al., 1990) (Developmental Studies Hybridoma Bank) anti-Apc1 (Hayashi et al., 1997), Engrailed mAb 4D9 (Developmental Studies Hybridoma Bank) and anti-Vasa (gift from Girish Deshpande). The crude anti-Apc1 sera was presorbed against embryos and used at a 1:1000 dilution. Goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 546 (Molecular Probes) were used as secondary antibodies. Fluorescent images were obtained on a Zeiss LSM510 confocal microscope. Adult eyes were fixed, embedded in plastic resin, sectioned and stained with Toluidine Blue (Cagan and Ready, 1989).

RESULTS

Characterization of an *Apc2* mutation

The $Apc2^{d40}$ mutation was isolated in a chemical mutagenesis screen and results in a change in the Apc2 protein at amino acid 677 from a cysteine to a stop codon (McCartney et al., 2001). This stop codon is located within the β-catenin-binding site region, after the second 20 amino acid repeat. It is predicted to eliminate both of the Axin binding sites (Fig. 1A). To confirm that this mutation reduces translation of the full length Apc2 protein, we analyzed Apc2 protein in both wild-type and $Apc2^{d40}$ mutant embryos with an antisera directed against an epitope that is C-terminal to the site of the $Apc2^{d40}$ truncation (Fig. 1A). Whole-mount immunostaining of wild-type embryos with the anti-Apc2 sera revealed that Apc2 was found within the cytoplasm (Fig. 1B,E), actin caps (Fig. 1B,D) and at the adherens junctions (Fig. 1E) of all epithelial cells. Adults that were homozygous for the $Apc2^{d40}$ mutation survived to adulthood, allowing us to obtain embryos that were both maternally and zygotically deficient for the wild-type Apc2 protein. Immunostaining of these maternal/zygotic $Apc2^{d40}$ mutant embryos with the anti-Apc2 sera revealed that Apc2 staining was eliminated in both the cytoplasm and at the membrane (Fig. 1C). This analysis verifies both the specificity of the Apc2 antisera, and the loss of full-length Apc2 protein caused by the $Apc2^{d40}$ truncation.

Previous analyses have revealed roles for *Apc2* in the regulation of Wg-dependent epidermal patterning (Yu et al., 1999; McCartney et al., 1999; McCartney et al., 2001). To quantitate the degree to which the mutation in $Apc2^{d40}$ results in the functional inactivation of Apc2 activity, we examined $Apc2^{d40}$ embryos for epidermal patterning defects that are indicative of ectopic Wg transduction. The ventral epidermal cells of wild-type embryos secrete a segmented cuticle, comprised of stereotyped rows of patterned hairs that are separated by 'naked' cuticle, which lacks these hairs (Fig. 1F). Wg signaling is required for the specification of cells that generate the naked cuticle. Inactivation of the negative regulators of Wg signaling, Axin and Zw3, constitutively activates Wg transduction, and expands the naked cuticle to encompass the entire embryonic ventral surface (Siegfried et al., 1992; Hamada et al., 1999). Consistent with ectopic activation of Wg transduction, $Apc2^{d40}$ mutant embryos have a naked cuticle similar to that found in *Axin* and *zw3* mutant embryos, though to a lesser degree (Fig. 1G). Immunostaining of $Apc2^{d40}$ maternal/zygotic mutants revealed expansion of all Engrailed stripes, and a dramatic reduction in the striped accumulation of Arm protein in the epidermis, also consistent with the constitutive activation of Wg transduction (Fig. 1I-L). The naked cuticular phenotype was enhanced by replacing one zygotic and one maternal $Apc2^{d40}$ allele with a deficiency that completely eliminated the *Apc2* gene. In such embryos, nearly all denticle-forming cells were transformed into those that secrete naked cuticle; the majority of embryos are completely naked (Fig. 1H; see also Fig. 4J). This finding indicates that $Apc2^{d40}$ retains some wild-type activity, and behaves like a strong hypomorphic allele, rather than a genetic null of *Apc2*. Our subsequent analysis of Apc1 and Apc2 function uses this feature to create a sensitized genetic background in which to assay interaction between the two Apc genes.

Redundant roles for Apc1 and Apc2 in regulation of Wg transduction throughout post-embryonic development

As inactivation of either Apc gene results in a limited range of phenotypes with regard to Wg transduction, we wished to determine whether any situations existed in which they functioned in a redundant manner. To identify functional redundancy between the two APC proteins, we examined $Apc1^{Q8}$ (Ahmed et al., 1998) (see Fig. 1A) $Apc2^{d40}$ double mutant flies. In contrast to either the $Apc1^{Q8}$ or the $Apc2^{d40}$ mutants, which survive to adulthood, $Apc1^{Q8}$ $Apc2^{d40}$ double mutant homozygotes derived from heterozygous parents showed normal Wg responses during embryogenesis and differentiated a normal larval cuticle, but died during larval stages. This lethality raises the possibility that there are some tissues in which Apc activity is required, but can be supplied by either of the two Apc genes. As this early lethality precludes the analysis of later developmental stages, we generated marked mitotic clones of cells that were homozygous for both the $Apc1^{Q8}$ and $Apc2^{d40}$ mutations within imaginal discs. We found that in contrast to the absence of patterning defects detected when either wild-type Apc1 or Apc2 were eliminated singly, simultaneous reduction in both Apc1 and Apc2 function during larval stages resulted in phenotypes consistent with the constitutive activation of Wg transduction in many different tissues (Fig. 2).

Wg transduction is required for the patterning of the wing margin (Couso et al., 1994). Constitutive activation of Wg transduction results in the production of ectopic marginal bristles within the wing blade (Blair, 1992; Diaz-Benjumea and Cohen, 1995; Zecca et al., 1996). In the wing blade, $Apc1^{Q8}$ $Apc2^{d40}$ double mutant clones marked with a *yellow* mutation cell autonomously assume the fate of those marginal cells that are closest to the mutant clone (Fig. 2A-C). In the anterior compartment, such clones form thick bristles with sockets

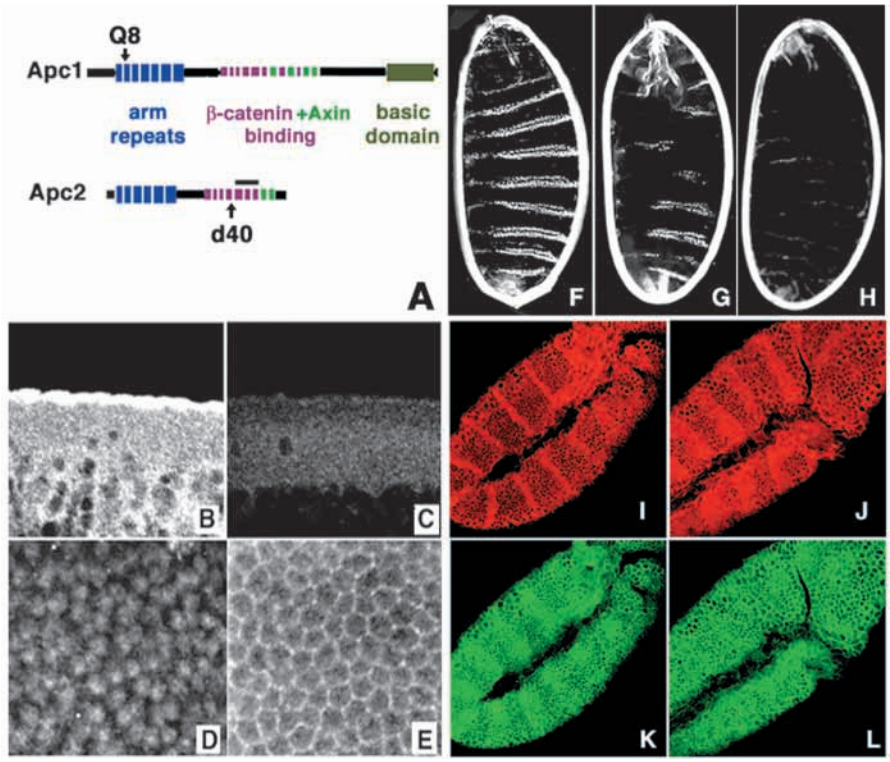

Fig. 1. Ectopic embryonic Wg transduction in a hypomorphic *Apc2* mutant. (A) The Apc1 and Apc2 proteins. The conserved Armadillo repeat region (blue), β-catenin-binding sites (purple) and Axin binding sites (green) are indicated. The position of the stop codon in $Apc1^{Q8}$ and $Apc2^{d40}$, as well as the epitope to which the anti-Apc2 sera was raised (black bar) are indicated. (B-E) Immunostaining of wild-type (B,D,E) and $Apc2^{d40}$ maternal/zygotic mutant embryos (C) with the anti-Apc2 sera. (B,C) Embryos at similar stages obtained using the same confocal microscope settings. A dramatic reduction in Apc2 staining is found in the $Apc2^{d40}$ mutant embryos. (F-H) Dark field images of cuticles of homozygous *Apc2* mutant embryos from $Apc2^{d40}/Apc2^{d40}$ mothers and wild-type fathers (F), $Apc2^{d40}/Apc2^{d40}$ mothers and $Apc2^{d40}$ /+ fathers (G), and $Apc2^{d40}$/Df (w6) mothers and Df (w6)/+ fathers (H). Introducing a deficiency for *Apc2* in the mothers and fathers results in an embryo that nearly completely lacks ventral epidermal denticles, and thus reveals the hypomorphic nature of the $Apc2^{d40}$ mutation. (I-L) Immunostaining of heterozygous *Apc2* embryos from $Apc2^{d40}/Apc2^{d40}$ mothers and wild-type fathers (I,K) or homozygous *Apc2* mutant embryos from $Apc2^{d40}/Apc2^{d40}$ mothers and $Apc2^{d40}$/+ fathers (J,L) with anti-Engrailed (red, I,J) or anti-Arm (green, K,L) antibodies. $Apc2^{d40}$ maternal/zygotic mutants have an expansion of naked cuticle, and an expansion of Engrailed stripes, as well as a reduction in the striped accumulation of Arm protein, all consistent with the ectopic activation of Wg transduction.

characteristic of the anterior margin, while in the posterior compartment, the bristles formed have a long tapered morphology characteristic of that region (Fig. 2B,C). The clones in either compartment can be quite large, occupying up to one third of the wing's surface. We found that $Apc1^{Q8}$ $Apc2^{d40}$ double mutant clones were similar in morphology to those we generated with a null allele of *Axin* (Fig. 2D,F) (Hamada et al., 1999). The sizes of mutant clones that we recovered upon inactivation of *Axin* or *Apc1 Apc2* were considerably larger than those found upon inactivation of *zw3* (Diaz-Benjumea and Cohen, 1995; Heslip et al., 1997) (Fig. 2E,H), probably owing to roles for Zw3 in Wg-independent cell proliferation pathways that are not shared by Axin/Apc (Cross et al., 1995).

In the leg, Wg transduction is required for the specification of ventral structures. Constitutive activation of Wg transduction in the leg produces ventralization of normally dorsal structures, which can result in the formation of a secondary axis in the distal leg, and supernumerary outgrowths, rather than complete appendages, in the proximal leg (Diaz-Benjumea and Cohen, 1994). We found both outgrowths in the proximal leg and duplications in the distal leg in marked $Apc1^{Q8}$ $Apc2^{d40}$ double mutant clones (Fig. 2H,I). These duplications arose from the dorsal side of the leg and were associated with the presence of marked mutant cells, but often included the neighboring, genetically heterozygous bristles as well. These findings are similar to those seen upon inactivation of *zw3* or *Axin*, and are consistent with the constitutive activation of Wg transduction in the dorsal and dorsolateral regions of the leg disc (Diaz-Benjumea and Cohen, 1994; Heslip et al., 1997; Hamada et al., 1999). In cells that had differentiated into dorsal structures (i.e. the edge bristle and preapical bristles of the second leg), $Apc1^{Q8}$ $Apc2^{d40}$ mutant clones were rarely detected, consistent with the observation that simultaneous inactivation of *Apc1* and *Apc2* results in the ventralization of normally dorsal structures. $Apc1^{Q8}$ $Apc2^{d40}$ mutant clones could, however, be found frequently in ventrally derived structures (i.e. the apical bristles). $Apc1^{Q8}$ $Apc2^{d40}$ mutant clones that marked ventral bristles often had a normal morphology. The distribution of $Apc1^{Q8}$ $Apc2^{d40}$ double mutant clones is therefore complementary to that previously described for cells containing mutations in *arm*, for which clones are recovered primarily in dorsally derived structures (Peifer et al., 1991). The complementary distribution of $Apc1^{Q8}$ $Apc2^{d40}$ clones and of *arm* clones suggests that Apc activity is required to regulate Arm activity negatively in dorsal regions of the leg disc, but may be dispensible in the ventral most regions, where Wg signal transduction is strongest and Armadillo levels are normally high.

Wg transduction at the anterolateral margins of the eye imaginal disc prevents ectopic neuronal differentiation from these positions (Ma and Moses, 1995; Treisman and Rubin, 1995; Lee and Treisman, 2001). Constitutive activation of Wg transduction induces cells that would normally differentiate as neurons to instead produce cuticle and sensory bristles secreted by cells at the dorsal margin of the eye (Heslip et al., 1997; Lee and Treisman, 2001). Ectopic head cuticle was found within the eye surface of $Apc1^{Q8}$ $Apc2^{d40}$ double mutant clones (Fig. 2J). The $Apc1^{Q8}$ $Apc2^{d40}$ mutant clones recovered can be

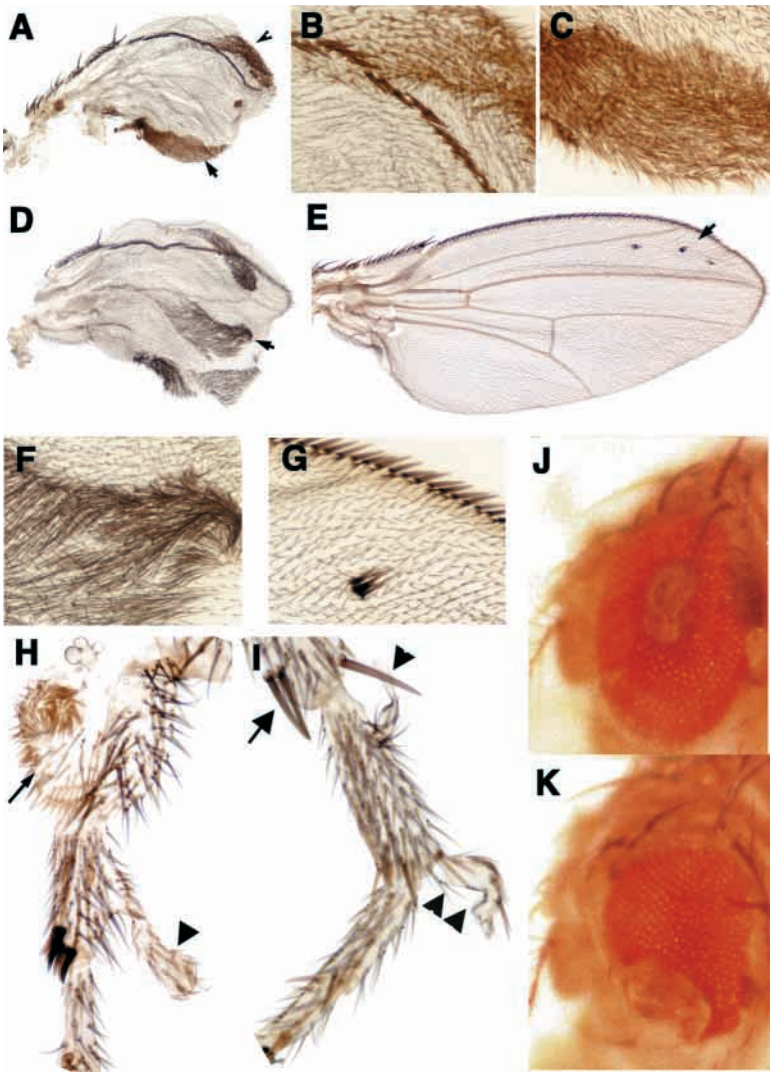

Fig. 2. Ectopic Wg transduction in the wing, leg and eye upon simultaneous reduction of *Apc1* and *Apc2*. (A-C) Constitutive activation of Wg transduction in the adult wing upon simultaneous reduction of Apc activity in $Apc1^{Q8}$ $Apc2^{d40}$ double mutant clones that are marked by a mutation in the *yellow* gene. $Apc1^{Q8}$ $Apc2^{d40}$ clones in anterior wing margin (arrowhead in A) form bristles (higher magnification shown in B), whereas in the posterior margin (arrow in A) form thin tapered hairs (higher magnification shown in C). Adult wing with mutant clones homozygous for $Axin^{S04423}$ (D,F) or $zw3^{M11\text{-}1}$ (E,G) show the same cell fate transformations, but only the *Axin* mutant clones are similar in size to those produced by $Apc1^{Q8}$ $Apc2^{d40}$. In the adult legs (H,I), marked $Apc1^{Q8}$ $Apc2^{d40}$ mutant clones are associated with outgrowths (arrow in H) and incomplete duplications (arrowhead in H) in regions containing dorsal and lateral pattern elements. In I, there is a duplication of a dorsal structure, the pre-apical bristle (arrow), and an incomplete distal duplication (double arrowhead), as well as a mutant clone of cells that includes the normally formed apical bristle, which is a ventral structure (single arrowhead). Constitutive activation of Wg transduction in the adult eye in an $Apc1^{Q8}$ $Apc2^{d40}$ mutant clone (J) or an *Axin* mutant clone (K) transforms ommatidial cells within the clone to cuticular fates.

large, comprising up to half of the eye. As noted for the wing clones, the sizes of mutant clones that we recovered in the eye upon inactivation of either *Apc1 Apc2* or *Axin* were considerably larger than those obtained upon inactivation of *zw3*, though the cell fate transformations were phenotypically identical (Fig. 2J,K and data not shown). The marking of $Apc1^{Q8}$ $Apc2^{d40}$ mutant clones in the eye by the absence of the *white* gene product revealed that there was a complete transformation of cell fate in the mutant tissue; none of the $Apc1^{Q8}$ $Apc2^{d40}$ double mutant cells within the eye differentiated as neurons.

In addition to the patterning defects in the wing, leg and eye imaginal discs, we also found mutant phenotypes associated with $Apc1^{Q8}$ $Apc2^{d40}$ clones located in the head cuticle, antennae, labial disc derivatives, notum, tergites and genitalia, all of which are consistent with the constitutive activation of Wg transduction in these tissues (data not shown). Thus simultaneous reduction in Apc1 and Apc2 ectopically activated Wg transduction in nearly all tissues. The only tissue in which we did not see activation of Wg transduction upon Apc reduction was in the abdominal sterna. We suspect that rather than indicating that Apc does not function in this tissue, this result probably reflects either the difficulty in isolating mutant clones that results from the late division of abdominal precursor cells, or the perdurance of wild-type Apc1 and/or Apc2 protein within these cells.

In summary, contrary to expectations based on differences in their immunostaining patterns, this clonal analysis reveals that simultaneous reduction in both Apc proteins results in ectopic Wg transduction in most tissues, and induces patterning defects similar to those that result from inactivation of the other negative components in the Wg signal transduction pathway, Axin and Zw3. Together, these clonal analyses reveal that the Apc1 and Apc2 proteins have a crucial role in preventing ectopic Wg transduction in many if not all cells, but that either Apc1 or Apc2 alone is sufficient to provide this regulation throughout most of post-embryonic development.

Apc1 protein is ubiquitously expressed

Previous immunostaining experiments have revealed that Apc1 is detected predominantly in the central and peripheral nervous system, with only low if any Apc1 in the epidermis (Hayashi et al., 1997; Ahmed et al., 1998). However, as the above phenotypic analysis clearly revealed the presence of Apc1 activity in non-neuronal tissues, we re-analyzed both heterozygous and homozygous $Apc1^{Q8}$ mutant embryos for Apc1 protein with an anti-Apc1 sera. We performed these experiments by adjusting the antibody concentration to detect Apc1 in tissues outside the nervous system. Under these conditions, we again found Apc1 to be most prominent in the central and peripheral nervous system. In addition, we consistently detected higher levels of Apc1 in nearly all tissues in the heterozygous $Apc1^{Q8}$ embryos when compared with their homozygous mutant sibs (Fig. 3). Even in the embryonic epidermis, where the intensity of Apc1 staining is relatively low, we detected a reproducible Apc1 staining pattern that was distinguishable from background (Fig. 3B,E). These data

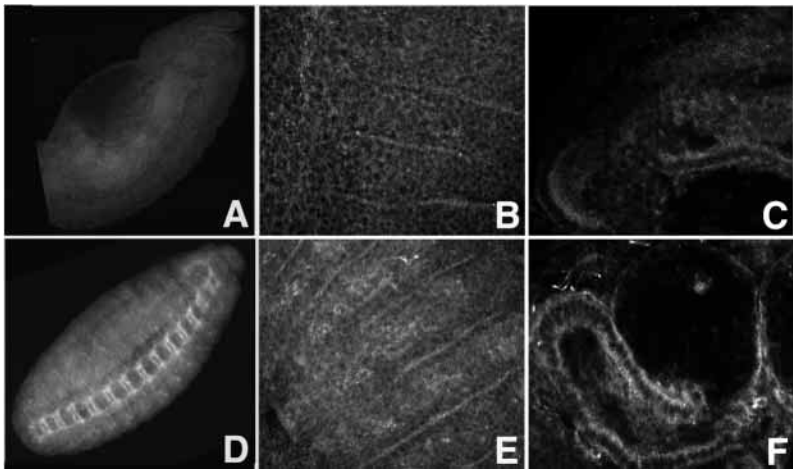

Fig. 3. Apc1 is ubiquitously expressed. (A-F) Whole-mount immunostaining of *Apc1^{Q8}/Apc1^{Q8}* (A-C) and *Apc1^{Q8}/+* (D-F) embryos with an anti-Apc1 sera. Whole embryos (A,D), epidermis (B,E) and gut (C,F) are shown. For each paired set of images, the same confocal microscope settings have been used. Although Apc1 staining is most prominent in the nervous system, there is a low, but consistent increase in the intensity of staining for Apc1 in all cells in *Apc1^{Q8}* heterozygous embryos when compared with their homozygous *Apc1^{Q8}* mutant siblings.

reveal that not only Apc2, but also Apc1, was ubiquitously expressed within embryonic tissues. Given this ubiquitous expression, it is remarkable that inactivation of either Apc protein ever leads to a specific phenotype. We thus performed the following experiments to determine whether functions that are unique to either Apc1 or Apc2 underlie these mutant phenotypes.

Apc1 and Apc2 have partially redundant roles in regulating Wg transduction even in the embryonic epidermis

Unlike the constitutive activation of Wg transduction that is induced by maternal and zygotic Apc2 loss, inactivation of Apc1 has no effect on Wg transduction during embryogenesis (Ahmed et al., 1998). We reasoned, however, that in the embryonic epidermis, as in the imaginal discs, Apc2 activity might compensate for defects induced by Apc1 loss, and thus mask a role for Apc1 in the regulation of Wg transduction. We thus examined embryos that are maternally and zygotically *Apc2^{d40}* mutant, and in addition have a reduction in either the maternal or zygotic dose of *Apc1*. We found that in this sensitized genetic background, simultaneous reduction in the activity of the *Apc1* and *Apc2* genes resulted in cell fate transformations in the epidermis that were more severe than reduction in the activity of either gene alone; the ventral cuticle lacked nearly all denticles (Fig. 4A-C). This enhanced ectopic Wg transduction was revealed when the *Apc1* gene dose was decreased by half, either maternally or zygotically in *Apc2^{d40}* mutant embryos; in either situation, the majority of embryos were nearly completely naked (Fig. 4J). Thus, Apc1 has an unexpected role in preventing the ectopic activation of Wg transduction in embryonic epidermal cells that is revealed only when Apc2 activity is compromised.

To determine whether there are circumstances in which Apc1 activity in the embryonic epidermis can be detected in the presence of some wild-type Apc2, we simultaneously reduced both maternally provided Apc1 and maternally provided Apc2, but provided some wild-type Apc2 zygotically. Embryos that lack wild-type maternally provided Apc2, but have wild-type zygotic Apc2, develop normally (McCartney et al., 1999) (Fig. 1F). Embryos that lacked both maternally provided Apc1 and Apc2 developed with some cuticular defects, but would often hatch and survive to adulthood (Fig. 4D). By contrast, if embryos mutant for maternally provided Apc2 also lacked maternally and paternally provided Apc1, they died either during embryonic or larval stages after differentiating partially naked cuticles and expanded Engrailed stripes, despite the presence of wild-type zygotic Apc2 (Fig. 4F,I). Thus zygotic Apc2 allows the normal development of embryos that lack maternal Apc2 only if wild-type levels of Apc1 are also present. Together, these data reveal that even during embryogenesis, which is the only stage of development in which mutations in *Apc2* induce a Wg hyperactivation phenotype, Apc1 can function in a subsidiary role to prevent transcriptional activation by Arm.

To determine whether reduction in the levels of one of the Apc proteins results in an increase in the levels of the other one, we examined embryos that are mutants of either *Apc1* or *Apc2*. In homozygous *Apc1^{Q8}* mutant embryos, we found no increase in Apc2 immunostaining in the embryonic epidermis. Similarly, in embryos that were maternally and zygotically *Apc2^{d40}* mutant, we found no increase in Apc1 immunostaining in the embryonic epidermis (data not shown). Thus, a substantial increase in the levels of either Apc protein upon loss of the other one does not appear to underlie their functional redundancy.

Apc1 overexpression can prevent ectopic Wg transduction induced by Apc2 loss in the embryonic epidermis

Given that Apc1 can function in a subsidiary role in the embryonic epidermis, we wished to investigate why Apc1 is normally unable to compensate for loss of Apc2 in this tissue. Perhaps it is the relatively low levels of Apc1 in the embryonic epidermis that prevent Apc1 from fully compensating for Apc2 loss. Alternatively, there may exist specific roles for Apc2 in regulating Wg transduction events that cannot be substituted for by Apc1. If specific roles for Apc2 did exist, then merely an increase in the absolute levels of Apc1 would not enable Apc1 to substitute functionally for Apc2. To address this question, we generated transgenic flies in which overexpression of *Apc1* specifically during embryogenesis was achieved under UAS/GAL4 control (Brand and Perrimon, 1993). We found that overexpression of *Apc1* during embryogenesis was sufficient to prevent the constitutive activation of Wg transduction induced by Apc2 loss.

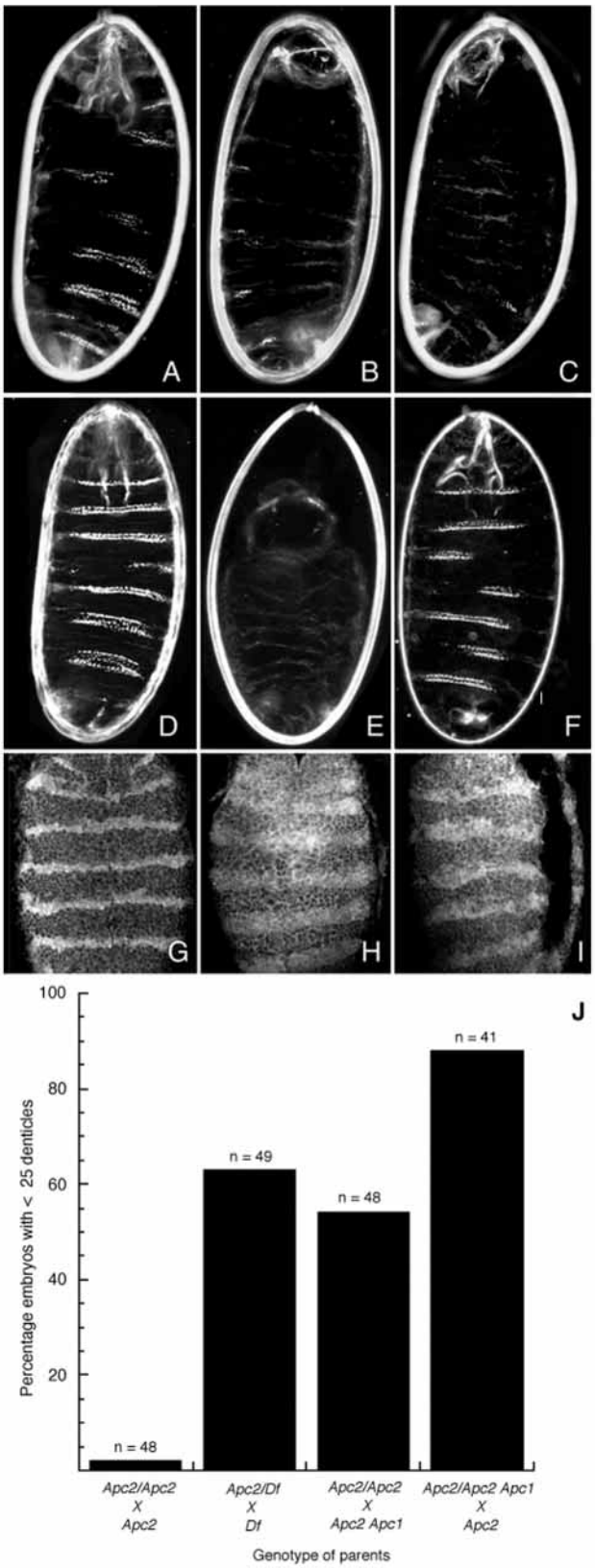

Fig. 4. Reduction in *Apc1* enhances ectopic Wg transduction in the *Apc2* mutant. (A-C) Cuticles of embryos from $Apc2^{d40}/Apc2^{d40}$ mothers and $Apc2^{d40}$ fathers (A); $Apc1^{Q8}Apc2^{d40}/Apc2^{d40}$ mothers and $Apc2^{d40}$ fathers (B); $Apc2^{d40}/Apc2^{d40}$ mothers and $Apc1^{Q8}$ $Apc2^{d40}$ fathers (C). Most of the denticles that remain in $Apc2^{d40}$ mutants are eliminated by reducing the maternal or zygotic dose of *Apc1* by half. (D-I) Embryos from $Apc1^{Q8}$ $Apc2^{d40}$ germline clones, which lack maternally provided wild-type Apc1 and Apc2. Cuticles (D-F) and Engrailed stripes (G-I) in embryos from $Apc1^{Q8}$ $Apc2^{d40}$ germline clones with a wild-type zygotic allele of *Apc1* and *Apc2* (D,G), $Apc1^{Q8}$ $Apc2^{d40}$ germline clone embryo homozygous for only $Apc2^{d40}$ (E,H) or homozygous for only $Apc1^{Q8}$ (F,I). The ectopic Wg activation caused by simultaneous homozygous reduction of *Apc1* and *Apc2* maternally is made more severe by elimination of either zygotic wild-type *Apc1* or zygotic wild-type *Apc2*. (J) A quantitative analysis of embryonic cuticular patterning defects that result from the $Apc1^{Q8}$ and $Apc2^{d40}$ mutations.

Maternal/zygotic $Apc2^{d40}$ mutant embryos that would otherwise die with a naked cuticle survive to adulthood upon *Apc1* overexpression. Thus, a burst of *Apc1* expression that is restricted to embryogenesis allowed $Apc2^{d40}$ maternal/zygotic mutants to survive to adulthood with no patterning defects. This result is consistent with previous data (McCartney et al., 1999) suggesting that embryogenesis is the only stage of development for which there is a mutant phenotype associated with *Apc2* inactivation. These data suggest that it is the absolute levels of Apc1 and Apc2, rather than an inherent specificity in function, that underlies differences in the relative contributions of the two Apc proteins in regulating Wg transduction in the embryonic epidermis.

Apc2 overexpression in the pupal retina can prevent neuronal apoptosis induced by Apc1 loss

Inactivation of *Apc1* results in the apoptotic death of all retinal neurons during pupation (Ahmed et al., 1998) (Fig. 5B). The apoptosis induced by Apc1 loss was dependent on transcriptional activation by Arm, as reducing the levels of Arm inhibited apoptosis in the *Apc1* mutant. Apc2 was also present in pupal retinal neurons yet was not sufficient to prevent the apoptosis induced by *Apc1* loss. In addition, $Apc2^{d40}$ homozygous mutant flies had a normal number of retinal neurons (data not shown). We sought to determine whether the negative regulation of Arm by Apc1 in retinal neurons was a unique property of Apc1 that was not shared by Apc2. Alternatively, this may reveal another situation that can be accounted for by differences in the relative levels of Apc1 and Apc2. We thus generated transgenic flies that expressed one additional copy of the *Apc2* gene, under control of its endogenous promoter. Two independent *Apc2* insertions rescued the lethality of the $Apc2^{d40}$ maternal/zygotic mutant. These *Apc2* insertions reveal that introduction of one extra copy of the *Apc2* gene was sufficient to partially prevent retinal neuronal apoptosis in the *Apc1* mutant pupal retina (Fig. 5C). The same *Apc2* transgenes had no effect on Wg transduction in wild-type flies, arguing against an artifactual result due to possible dominant negative effects of overexpression (Fig. 5A and data not shown). Thus, Apc2 is able to substitute for Apc1 in the pupal retina, revealing that even in neurons, the two Apc proteins are functionally equivalent. These results, coupled with those described in the previous section, suggest that functional redundancy between the two Apc genes in the regulation of Arm exists in many tissues. The limited Arm hyperactivation phenotypes revealed by inactivation of either Apc gene alone are not the result of functions unique to either

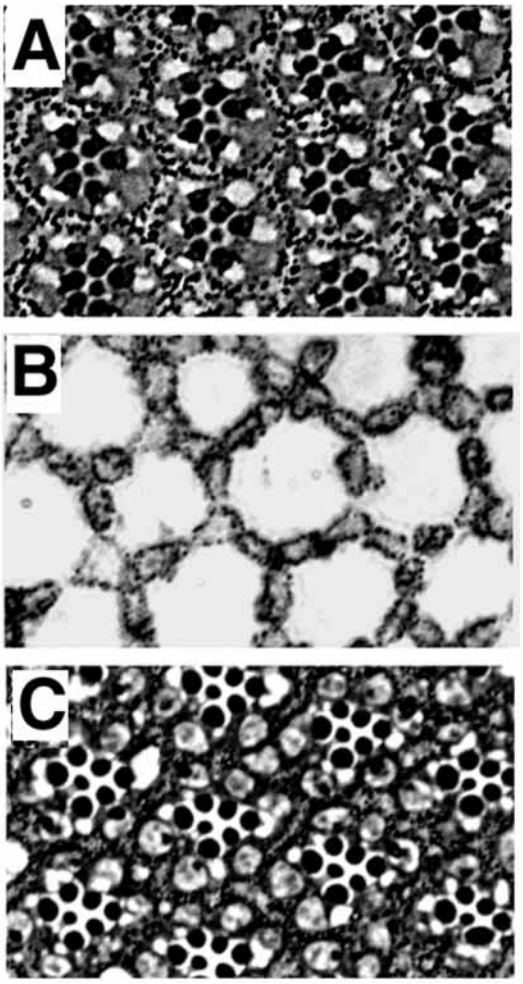

Fig. 5. Rescue of retinal neuronal apoptosis in the *Apc1*^{Q8} mutant by overexpression of *Apc2*. Genotypes shown are as follows: (A) P[*Apc2*]/+; +/+; (B) *Apc1*^{Q8}/*Apc1*^{Q8}; (C) P[*Apc2*]/Y; *Apc1*^{Q8}/*Apc1*^{Q8}. (A) Tangential section through the eye of a fly with one extra copy of an *Apc2* transgene. As in wild-type eyes, in each ommatidium there are eight photoreceptor neurons located in a highly ordered pattern. Seven neurons are seen in the plane of focus. Each group of photoreceptor cells is surrounded by a lattice of pigment cells, identified by the small, darkly stained pigment granules they contain. (B) In the homozygous *Apc1*^{Q8} mutant, there is apoptotic death of all retinal neurons in all ommatidia. The pigment cell lattice remains intact. (C) One extra copy of the *Apc2* gene is sufficient to partially prevent the neuronal cell death that is induced by *Apc1* loss. The degree to which the apoptosis is prevented is greater in *Apc1* mutant males than in females. As the *Apc2* transgene is inserted on the X chromosome, we infer that this difference is the result of dose compensation (Kelley and Kuroda, 1995).

of the Apc proteins. Rather, these phenotypes reflect the requirement for threshold levels or total intracellular 'dose' of Apc in the regulation of Arm activity.

Simultaneous reduction in Apc1 and Apc2 results in nuclear accumulation of Arm in all epithelial cells

The activities of both Zw3 and Axin are required for the targeting of Arm for degradation. Inactivation of either of these proteins results in a marked increase in total Arm levels. However, genetic experiments reveal that Zw3 and Axin differ in their effects on the intracellular distribution of Arm (Peifer et al., 1994; Hamada et al., 1999). In *zw3* mutant embryos, there is an increase in Arm that is equally dispersed between the cytoplasm and the nucleus of all epithelial cells (Peifer et al., 1994). By contrast, elimination of *Axin* results in a preferential accumulation of Arm in the nucleus (Tolwinski and Wieschaus, 2001). These results support a model in which Axin serves as a cytoplasmic anchor for Arm, in addition to its role in targeting Arm for degradation (Tolwinski and Wieschaus, 2001), thus exerting an additional level of control on transcriptional activation by Arm. APC has also been proposed to have a dual role in the regulation of Arm, not only in the targeting of Arm for degradation, but also in the nuclear export of Arm in a CRM1-dependent pathway (Henderson, 2000; Rosin-Arbesfeld et al., 2000; Neufeld et al., 2000) (see Discussion).

In this context, we sought to determine the intracellular localization of Arm upon simultaneous reduction of both *Drosophila* Apc proteins. As noted above, in wild-type embryos Arm is found primarily at the adherens junctions of all epithelial cells, and in addition is equally dispersed in the cytoplasm and nucleus of those cells responding to a Wg signal, forming segmental 'stripes' of Arm protein (Riggleman et al., 1990) (Fig. 1K). By contrast, in *Apc1*^{Q8} *Apc2*^{d40} maternal/zygotic double mutant embryos, there is both a marked increase in the overall intensity of Arm staining (Fig. 6A,B), and in addition an accumulation of Arm preferentially in the nucleus (Fig. 6C-H). The nuclear accumulation of Arm is observed in all epithelial cells that lack both maternal and zygotic Apc1 and Apc2.

In the *Apc1 Apc2* double mutant, Arm accumulates within the nucleus during gastrulation. In embryos that are both maternally and zygotically mutant for *Apc1* and *Apc2*, Arm maintains this nuclear accumulation throughout embryonic development. By contrast, if these double mutant embryos receive a zygotic wild-type allele of *Apc1*, the nuclear localization of Arm is drastically reduced, arguing that the continued absence of all Apc protein is required to maintain Arm in the nucleus (data not shown). These results reveal that the Apc1 and Apc2 proteins have a redundant role not only in regulating the intracellular levels of Arm, but also in preventing the nuclear accumulation of Arm, in a temporal and spatial manner that appears similar to that previously demonstrated for Axin (Tolwinski and Wieschaus, 2001).

DISCUSSION

Biochemical experiments have revealed APC to be a key member of a multiprotein complex that is required for the phosphorylation of β-catenin, and the subsequent targeting of β-catenin to the proteasome. Conclusive evidence for the biochemical functions of APC in this complex has been in part hampered by the inability to obtain pure, full-length APC protein to use in reconstitution experiments (Salic et al., 2000). A further impediment to dissecting APC function has been the difficulty in establishing a genetically tractable in vivo system to complement biochemical studies. Analyses of APC function in *Xenopus* embryos, a well characterized in vivo system for studying Wnt transduction, have been complicated by

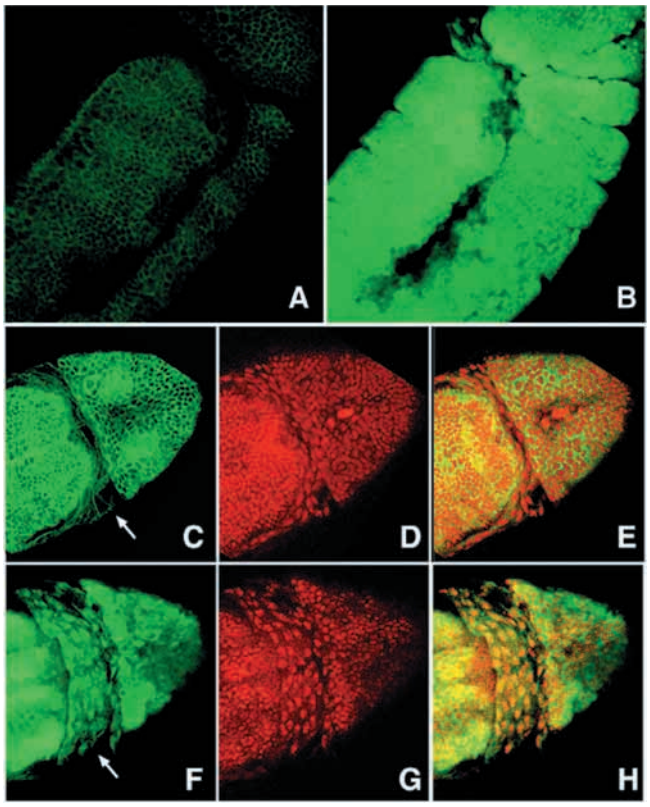

Fig. 6. Stabilization and nuclear accumulation of Arm in the *Apc1*^{Q8} *Apc2*^{d40} double mutant. Embryos from *Apc1*^{Q8} *Apc2*^{d40} germ cells that are wild type for both *Apc1* and *Apc2* zygotically (A), or mutant for *Apc1*^{Q8} *Apc2*^{d40} zygotically (B), stained with anti-Arm antibody. (A,B) Same confocal microscope settings. There is a dramatic increase in the intensity of Arm staining in the combined maternal and zygotic *Apc1 Apc2* double mutant embryos. (C-H) Embryos from homozygous *Apc1*^{Q8} *Apc2*^{d40} germ cells that are wild-type for both *Apc1* and *Apc2* zygotically (C-E), or mutant for *Apc1*^{Q8} *Apc2*^{d40} zygotically (F-H) stained with anti-Arm antibody in green (C,F), or with Hoechst dye to detect nuclei in red (D,G) or both (E,H). Staining of the amnioserosa (arrows in C,F) of a stage 9 mutant embryo reveals a nuclear accumulation of Arm that persists throughout embryogenesis only in the *Apc1*^{Q8} *Apc2*^{d40} maternal/zygotic double mutant.

dominant negative effects that result from the inadvertent generation of truncated APC fragments, rather than full-length APC, and the overexpression of these fragments (Farr et al., 2000).

In this regard, the in vivo analyses of loss-of-function mutations in the two *Drosophila* homologs of *Apc* have been crucial in providing conclusive evidence that transcriptional transactivation by β-catenin can in fact be negatively regulated by APC (Ahmed et al., 1998; McCartney et al., 1999). However, previous studies using loss-of-function mutations in either of the two *Drosophila* Apc genes have failed to establish an absolute requirement for Apc in regulating Wg signaling throughout development, as many Wg transduction events proceed normally, particularly during post-embryonic stages. These findings raised questions as to whether Apc is required to prevent the constitutive activation of Wg transduction in only a subset of cells, and whether Apc function could be compensated for by other mechanisms elsewhere. We report the consequences of simultaneously reducing the activities of both *Drosophila* Apc proteins. We find an absolute requirement for Apc proteins in preventing the constitutive activation of Wg signaling in many epithelial cells throughout development. We also find that in those limited situations for which the inactivation of one of the two *Drosophila* Apc proteins does lead to hyperactivation of transcriptional activation by Arm, the other Apc protein can functionally substitute if provided in sufficient quantity. This result argues against a specific function for either Apc protein in regulating Wg transduction.

Both *Drosophila* Apc proteins regulate Wg transduction within the same cells throughout development

Apc1 is highly (though not exclusively) expressed in neurons, while Apc2 is highly (though not exclusively) expressed in most epithelial cells, leading to the proposal that the two Apc proteins function in a tissue-specific manner (Yu et al., 1999). The data presented here argue against a tissue-specific division in Apc expression or function. The dramatic and global constitutive activation of Wg transduction that is revealed only by simultaneous reduction in both *Drosophila* Apc proteins demonstrates that both Apc proteins are found and function in many tissues that are not restricted by cell type or developmental stage.

These results reveal that the combined activity of Apc1 and Apc2 within the same cell enables these two proteins to tightly regulate Arm levels. Thus, specific phenotypes that are found upon inactivation of either *Apc1* or *Apc2* singly (leading to cell death in pupal retinal neurons and cell fate transformation in the embryonic epidermis, respectively) denote the relatively rare situations in which the activity of one of the two Apc proteins is not sufficient to compensate for reduction in the other. Our data reveal that even in the embryonic epidermis, Apc1 and Apc2 function to prevent the ectopic activation of Wg transduction. When Apc2 activity is reduced, ectopic Wg transduction is very sensitive to the dose of *Apc1*, as cutting the wild-type dose of *Apc1* in half either maternally or zygotically has dramatic effects. In this tissue, Apc1 has a subsidiary role though, and the normal levels of Apc1 are not sufficient to compensate for Apc2 loss. These data, coupled with the rescue of Apc2 reduction by *Apc1* overexpression, suggest that the absolute levels of Apc1 and Apc2 are important in enabling the two Apc proteins to compensate for each other.

We were not able to determine whether the converse situation is also true, i.e. whether reducing levels of endogenous Apc2 would exacerbate defects resulting from mutations in *Apc1*, because we do not have a hypomorphic allele of *Apc1* to use as a sensitized background for genetic interaction tests. We were, however, able to demonstrate that retinal neuronal apoptosis is exquisitely sensitive to total Apc2 activity, as increasing the dose of *Apc2* by only one copy was sufficient to prevent apoptosis in the *Apc1* mutant. Together, these data suggest that the absolute levels, or total ‘dose’ of intracellular Apc1 and Apc2 is important in preventing the hyperactivation of Arm. Whether the dose sensitivity that is revealed in these situations reflects differences not only in total levels, but also in the relative binding affinities of the two Apc proteins for Arm, Axin or Zw3 remains to be investigated.

The functional redundancy in the Apc proteins suggests that

the C-terminal half of Apc1 might not be required for targeted degradation of Arm, as this region of the protein is completely lacking in Apc2. However, this region of Apc1 might be important in previously proposed roles for APC that might be independent from β-catenin degradation. These include the alteration of cell migration through regulation of the actin cytoskeleton (Kawasaki et al., 2000), the planar positioning of mitotic spindles with respect to the polarized epithelial cell membrane (Lu et al., 2001), and in kinetochore-microtubule attachment (Kaplan et al., 2001; Fodde et al., 2001). While our data demonstrate that both *Drosophila* Apc proteins function in the regulation of Wg transduction, further analysis employing the *Apc1 Apc2* double mutant will be required to address their possible redundancy in functions that are independent of β-catenin degradation.

An absolute requirement for APC in the degradation of β-catenin

Previous studies have raised questions as to whether an absolute requirement for APC exists in the targeting of β-catenin to a degradation pathway (Behrens et al., 1998; Hart et al., 1998). In cell culture experiments, overexpressed Axin is able to downregulate β-catenin levels even in cells that lack wild-type APC. Furthermore, even after deletion of its RGS domain, which is required for the interaction of Axin with APC, overexpressed Axin is still able to induce the degradation of β-catenin. These data have led to the hypothesis that APC may facilitate, but not be absolutely necessary for, the Axin-mediated degradation of β-catenin. If APC were to merely facilitate Axin mediated degradation of β-catenin, we would expect that phenotypes found upon reduction in APC would not be as severe as those found upon inactivation of Axin, as residual Axin-mediated degradation of β-catenin would persist in the absence of APC. Instead, we find that inactivation of APC results in phenotypes that completely mimic inactivation of Axin, with respect to both their scope and their severity. Our data argue against a secondary role for APC in the degradation of β-catenin, and provide in vivo evidence for an absolute requirement for APC in preventing the constitutive activation of Wg transduction in virtually all epithelial cells.

APC and the nuclear export and cytoplasmic anchoring of β-catenin

Human APC has been found to shuttle between the nucleus and cytoplasm (Henderson, 2000; Rosin-Arbesfeld et al., 2000; Neufield et al., 2000). Nuclear export of human APC is dependent on both nuclear export sequences within APC and on the CRM1 export receptor (Fornerod et al., 1997; Stade et al., 1997). Treatment of cells in culture with the CRM1-specific export inhibitor leptomycin B results in the nuclear accumulation of APC, as well as the nuclear accumulation of β-catenin. These findings have led to the proposal that APC is required for the nuclear export of β-catenin. However this hypothesis must be reconciled with studies employing oocytes and semipermeabilized cultured cells to investigate β-catenin export, which reveal that β-catenin can be exported from the nucleus in a manner that is independent of the CRM1 pathway and independent of APC (Eleftheriou et al., 2001; Wiechens and Fagotto, 2001).

We find that in epithelial cells that lack both wild-type *Drosophila* Apc1 and Apc2, Arm accumulates within the nucleus. Nuclear accumulation of Arm is found only in the $Apc1^{Q8}$ $Apc2^{d40}$ maternal/zygotic double mutant, and occurs during gastrulation. The nuclear localization of Arm, and the temporal pattern of the nuclear accumulation of Arm in the absence of wild-type Apc1 and Apc2, is similar to that seen upon inactivation of Axin (Tolwinski and Wieschaus, 2001), and in contrast to that seen upon inactivation of Zw3 (Peifer et al., 1994), in which the increased levels of Arm appear uniformly dispersed between nucleus and cytoplasm. Our data are therefore completely consistent with the model that there is a second role for APC in the nuclear export of β-catenin, in addition to the role APC serves in the targeting of β-catenin to degradation.

However, an alternate model for APC function in Arm localization incorporates three observations: (1) a similar temporal pattern of nuclear Arm accumulation is seen in *Axin* mutants and in *Apc1 Apc2* double mutants; (2) the interaction of Axin with β-catenin is critically dependent on APC (Salic et al., 2000); and (3) β-catenin is freely diffusible from nucleus to cytosol (Wiechens and Fagotto, 2001). In this model, an Axin/APC complex would serve as a cytoplasmic anchor for β-catenin and would dictate, in part, the steady-state subcellular localization of β-catenin. Axin would serve as the primary cytoplasmic anchor for β-catenin, but its physical interaction with β-catenin would be greatly enhanced by APC. The elimination of either Axin or APC, or their functional inactivation in the presence of Wg transduction, would not only increase the total levels of β-catenin, but would also shift the steady state localization of β-catenin to the nucleus. While further experiments will be necessary to distinguish between roles for APC in the nuclear export and/or cytoplasmic anchoring of β-catenin, our data suggest that together, APC and Axin exercise two levels of control of β-catenin activity: APC and Axin not only initiate the destruction of β-catenin, but also modulate the ability of β-catenin to accumulate in the nucleus where it can serve as a transcriptional activator.

Implications for the two human APC proteins in disease

Our results reveal an absolute requirement for APC in the targeting of β-catenin for destruction and may have implications for the function of the human APC proteins in the regulation of Wnt transduction. In mouse and humans, as in *Drosophila*, there are two known APC homologs, APC and APC2/APCL. The mammalian APC homologs are expressed at high levels in the nervous system, with lower levels in many other tissues analyzed (Smith et al., 1993; Brakeman et al., 1999; Nakagawa et al., 1998; van Es et al., 1999). Although human APC is widely expressed (Smith et al., 1993; Midgley et al., 1997), germline mutations in APC result in a relatively narrow spectrum of disease. This includes the development of adenoma in the gastric and small and large bowel epithelia, as well as osteomas, desmoid fibromatosis, and lesions in retinal neurons and pigment epithelium (Fearnhead et al., 2001). While hyperactivating mutations in β-catenin are also associated with colonic carcinoma and desmoid fibromatosis, these hyperactivating mutations have been found in several carcinomas that are not detected in people with germline mutations in APC (Polakis, 2000). Several scenarios could account for this discrepancy in sites of disease induced by APC loss versus β-catenin hyperactivation. Perhaps human APC has a key role in controlling the degradation of β-catenin in only a

subset of epithelial tissues. Alternatively, in a manner directly analogous to that we find for the two *Drosophila* Apc proteins, inactivation of one human APC homolog might be compensated for by the activity of the other in most tissues. Homozygous inactivation of human *APC* would induce disease states in only those tissues in which *APC*, rather than *APC2*, is the predominantly expressed gene, and would be dependent on the absolute levels of the two APC proteins in any given cell.

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