Novel Roles for Adenomatous Polyposis Coli Family Members and Wingless Signaling in Cell Adhesion and Axon Outgrowth During Drosophila Brain Development.

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ABSTRACT

MELISSA A. HAYDEN: Novel roles for Adenomatous Polyposis Coli family members and Wingless signaling in cell adhesion and axon outgrowth during *Drosophila* brain development.
(Under the direction of Mark Peifer)

Mutation in the tumor suppressor gene, *adenomatous polyposis coli (APC*), leads to the initiation of colon cancer. Both *Drosophila* and mammals have two APC family members, *APC1* and *APC2*. Our lab and others have characterized both *Drosophila* family members in the developing embryonic epidermis. In addition, we characterized the expression of both in the developing larval brain. Construction of the brain is one of the most complex developmental challenges. Wnt signals shape all tissues, including the brain, and the APC is a key negative regulator of Wnt/Wingless (Wg) signaling. We carried out the first assessment of the role of APC proteins in brain development, simultaneously inactivating both *APC1* and *APC2* in clones of cells in the *Drosophila* larval optic lobe. Loss of both *APC*s triggers dramatic defects in optic lobe development. Double mutant cells segregate from wild-type neighbors to form epithelial "loops", while double mutant neurons form tangled axonal "knots", suggesting changes in cell adhesion. Activation of Wg signaling downstream of APC mimics these phenotypes, a dominant-negative TCF construct blocks them, and a known Wg target, *Decapentaplegic*, is activated in double mutant clones. These results strongly suggest that the phenotypes result from activated Wg signaling. We also explored the roles of classic cadherins in differential adhesion. Finally, we propose a

model suggesting that Wg signaling regulates fine scale cell fates along the anterior-posterior axis in the developing larval brain, in part by creating an adhesion gradient.

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INTRODUCTION

Development and Disease

Development is a special time in all of our lives. As soon as the egg is fertilized a complex yet coordinated set of events ensues in an exquisitely precise, temporally controlled manner. It is here, during development, where small alterations in a single protein may cause larger, often detrimental, changes to occur. For example, a single mistake in one gene can be disruptive enough to lead to the death of the developing embryo. Despite this fragility, Nature has ensured that this process is overall a robust one. After all, we made it through, even if there were minor mistakes along the way. But once we develop into an adult, many of those developmental processes are not obsolete. In fact, many of the same signal transduction pathways that are crucial during development remain key in the maintenance of our adult tissues.

It is often these same developmental processes that tend to go awry in disease states such as cancer. By learning about the normal processes required for proper development, and how those processes are regulated, we gain insight into what goes wrong in disease. This information can then facilitate our harnessing these biological mechanisms, perhaps via development of specific pharmacological therapies or other innovative techniques such as gene therapy, to intervene and one day ameliorate the condition. Just like a long journey starts with one single step, so does learning about a gene's function. A single gene product could be important for one specialized function or for many separate events throughout our lives. But where do we start the analyses? A

common practice in developmental biology is to start with a mutant gene and examine how it affects a known normal process.

To do this in a simple model system like *Drosophila* is much easier than to do so in a more complex animal (reviewed in Adams and Sekelsky, 2002). *Drosophila* is used for a multitude of reasons. It is small, inexpensive and simple to propagate, with a short life span. More importantly, flies have homologs to numerous human genes, and often have fewer redundant genes within a gene family than higher organisms. A wealth of information has been gained over the last 100 years on the Drosophila genome. The discovery of genes with visible phenotypes, such as white eyes, forked bristles or tubby shaped bodies, allowed those characteristics to be used as markers, providing an easy way for the researcher to track their gene(s) of interest. This idea of genetic markers facilitated the development of a number of new technologies allowing the integration of genes from other species into the fly. For example, yeast genes such as *Gal4* and *heat shock flipase* have been introduced into fly lines. In fruit flies, it is now simple to use such tools to turn a particular gene "on" or "off" in the whole animal, or even in specific tissues, during different stages in development.

Genetics has proved to be powerful tool not only for learning how basic biological processes work, but in the diagnosis of human disease. This was true in the case of Familial Adenomatous Polyposis (FAP). This heritable disease is characterized by adenomatous polyps carpeting the colon and rectum (reviewed in Radtke and Clevers, 2005). These benign epithelial polyps, if left untreated, will undergo additional genetic changes resulting in a deadly form of colorectal cancer. Patients typically present with carcinoma with an average age of 40 years old, but cases as early as late childhood have

also been identified. Drastic surgical intervention, such as resection of the colon, is often times the only method of treatment.

Familial Adenomatous Polyposis and the *Adenomatous Polyposis Coli* gene

In 1991, several groups demonstrated that mutations in the human *adenomatous polyposis coli* gene (*APC*) are the causative event in FAP *(*Kinzler *et al.,* 1991; Groden *et al.*, 1991; Nishisho *et al.*, 1991) . FAP shows an autosomal dominant pattern of inheritance with almost complete penetrance but does have variations in expression. This variability may be due to where the mutations in the *APC* gene occur (Figure 1.1). APC mutations occurring in the first or last third of the gene have been associated with a late onset of disease and a small number of polyps (attenuated polyposis) (Spirio *et al*., 1993; van der Luijt *et al*., 1996; Friedl *et al*., 1996). However, most mutations occur in the central region of the APC gene called the mutation cluster repeat region (MCR).

Figure 1.1: Mutation Cluster Region (MCR) in Human *APC*

These mutations correlate with a more severe phenotype leading to thousands of polyps at a young age and even some additional extra-colonic manifestations (such as polyps in the upper GI tract and malignancies in other sites, *e.g.* the brain). Mutations in the MCR are invariably premature stop codons, generating a truncated form of APC missing almost half of the normal protein (the COOH terminal half). This suggests that this region of the

gene is somehow more susceptible to mutation or that tumor initiation is perhaps more likely to occur when a mutation arises in the MCR of APC (Polakis, 1995). In addition to familial cases of colon cancer, it was subsequently found that many sporadic cases of colon cancer also harbor mutations in the *APC* gene, indicating APC's important role as a tumor suppressor in colon epithelial cells (Nishisho *et al.*, 1991).

Interestingly, FAP patients are not homozygous mutant for the *APC* tumor suppressor gene. Kinzler and Vogelstein (1996) proposed a 2-hit model, suggesting that patients inherit one mutant allele and the wild type *APC* allele is lost or mutated in colorectal tumors. The heterozygous patients, who have a predisposition for colon carcinoma, acquire an additional "second hit" mutation in the cells of the gut epithelium leading to the generation of tumors that are homozygous mutant for *APC*. Patients can inherit a null allele of *APC* and have a second hit resulting in a truncation of *APC*, or more commonly, can inherit a truncated allele of *APC* and receive a second hit that is null for *APC*.

The idea that tumor initiation is perhaps more likely to occur when a mutation arises in the MCR of *APC* led to the hypothesis that the expression of COOH-terminally truncated proteins, like the ones in seen in FAP, act in a dominant-negative manner, resulting in a greater propensity for tumor growth. Experimental evidence for this dominant-negative effect was provided by Dihlmann *et al.* (1999). Here normal APC regulation of β-cat/TCF-mediated transcription was shown to be strongly inhibited by an ectopically expressed MCR mutant *APC* gene product (truncated at codon 1309). A weaker effect on transcription was seen when using mutant *APC* gene products associated with attenuated polyposis, such as those at codon 386 or 1465. This provides a potential molecular explanation for the genotype-phenotype correlation seen with different mutations in APC associated with FAP.

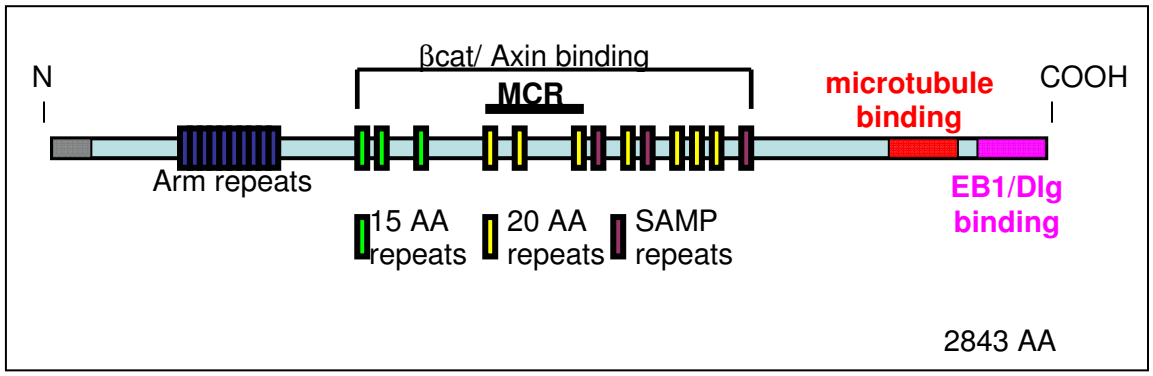
However, it should be noted that in a mouse model of FAP, mice inheriting a null allele of *APC* develop colorectal adenomas and FAP, so it is not essential to have a dominant negative effect for tumor initiation to occur (Shibata *et al.*, 1997; Colnot *et al.*, 2004).

A role for APC in Wnt/Wg signaling

This leads to the question of what is the function of the *APC* gene and how is that disrupted in colorectal tumorigenesis? Characterization of APC revealed many clues to the potential mechanisms for its tumor suppressor function. The best characterized function for APC is as an integral part of a signaling pathway that is essential during normal development and maintenance of adult tissues. That signaling pathway is the Wnt/Wg (Wingless) pathway (reviewed in Fogerty *et al.*, 2005). The inappropriate activation of this pathway through loss of APC function contributes to cancer progression.

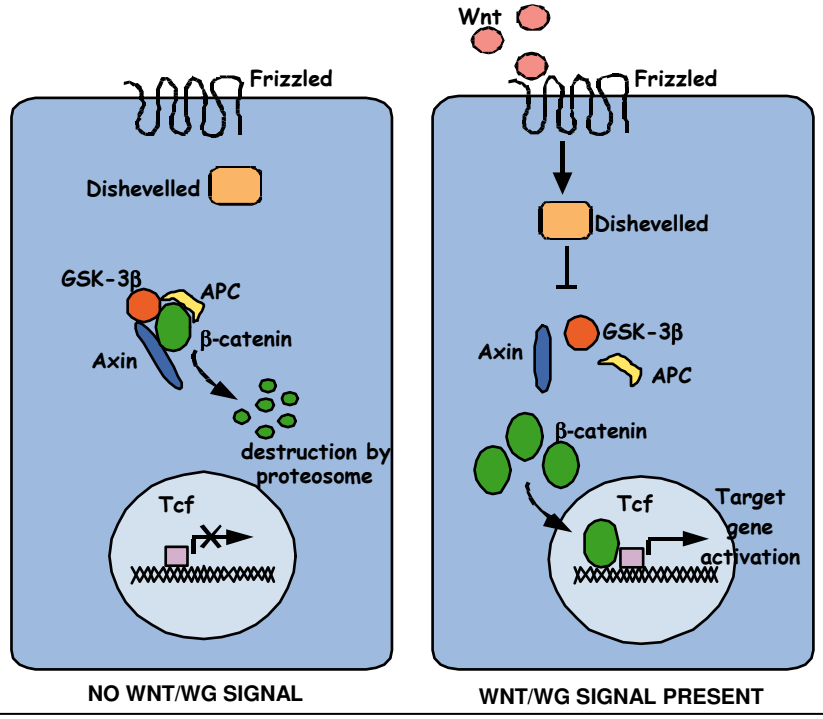
APC is a large protein with many different protein interaction motifs. A number of these, such as the 15 and 20 amino acid repeats, and SAMP repeats, are important for the direct binding and degradation of the key Wnt signaling effector, β-catenin (βcat; in *Drosophila* known as Armadillo (Arm) (reviewed in Peifer and Polakis 2000; Figure 1.2).

Figure 1.2: Functional Domains of Human *APC*



When no secreted Wnt/Wg ligands are available to bind Wnt/Wg receptors (Frizzled), cytoplasmic βcat is bound in a destruction complex with APC and several other proteins, including the scaffolding protein Axin and the kinase GSK-3β (Figure 1.3). This allows βcat to be phosphorylated by Casein kinase I and GSK-3β, targeting it for ubiquitination and destruction by the proteosome. However, in the presence of a Wnt/Wg signal, engagement of the Wnt receptors activates the Dishevelled protein to inactivate the destruction complex,

Figure 1.3: APC functions as a negative regulator of Wnt/Wg signaling



allowing the hypo-phosphorylated βcat to accumulate in the cytoplasm and be imported into the nucleus. Here βcat interacts with DNA binding proteins of the TCF/LEF family to form bipartite transcription factors and activate Wnt/Wg target genes (Nelson and Nusse, 2004).

This canonical Wnt/Wg signaling pathway has been extensively characterized as

essential for many early embryonic developmental processes (*e.g*. axis specification, primitive streak formation, and neural patterning; (Logan and Nusse, 2004) and as well as in the regulation of adult processes (*e.g*. normal stem cell differentiation in the gut-described below). In the case of FAP, the inactivating mutations of *APC* cause aberrant accumulation of both cytoplasmic and nuclear βcat, leading to the improper activation of Wnt target genes (Korinek *et al.*, 1997; Rubinfeld *et al.*, 1997; Morin *et al*., 1997). This affects downstream target gene activation leading to profound effects on the regulation of the cell cycle and proliferation, processes known to contribute to tumor initiation. Inappropriate activation of the Wnt pathway in the colon blocks differentiation of the cells that normally migrate from the crypt to the villi, and effectively locks the cells into a self-renewing stem cell-like fate (reviewed in Reya and Clevers 2005).

Stem Cells and Wnt Signaling

Cancer is thought by many to be a disease of stem cells. Many precursor/stem cell populations utilize Wnt signaling. Depending upon intrinsic characteristics of the particular stem cell population, stem cells interpret Wnt signaling in different ways. Wnt signaling can drive proliferation and promote self renewal, while in other tissues it promotes differentiation along a particular lineage pathway. In the colon, Wnt signaling maintains epithelial stem cells in a stem cell fate (Reya and Clevers, 2005).

The small and the large intestine (colon) are lined by an epithelial sheet of cells. In pocketing of this epithelial layer gives rise to the familiar crypt-villus structures. The slowly proliferating, undifferentiated stem cells reside near the U-shaped bottom of this epithelium, constituting the crypt. Activated Wnt signaling from neighboring mesenchymal cells is required to maintain these self-renewing epithelial stem cells throughout our lives. These

stem cells divide to produce rapidly proliferating "transit-amplifying proliferative progenitors," capable of differentiating into all gut epithelial lineages. These progenitor cells are also exposed to different levels of Wnt signaling along the crypt-villus axis. As they migrate up to the top of the U-shaped structure, towards the villus, they are progressively exposed to less and less Wnt signal. Once these committed progenitors reach the top one-third of the crypt-villus, they undergo cell cycle arrest and differentiate into mature Goblet cells (secreting protective mucins), enteroendocrine cells (secreting hydrolases and absorbing nutrients), and absorptive epithelial cells. The life cycle of one of these epithelial cells spans less than a week. These differentiated cells at the villus are eliminated by apoptosis, and thus a constant repopulation must occur. It must be noted that while all cells in the crypt are derived from one stem cell (monoclonal), each villus receives cells from multiple crypts and is thus polyclonal (Reya and Clevers,2005 ; Radtke and Clevers, 2005).

Current evidence shows a clear link between Wnt signaling, proliferation in the normal colon, and colorectal cancer. Nuclear βcat, an indicator of active Wnt signaling, is normally present in crypts (Battle *et al.*, 2002), *TCF-/-* mice are missing the crypt progenitor compartment entirely (Korinek *et al.*, 1998), and inhibiton of Wnt receptors by transgenic Dickkopf-1 induces the loss of the crypt compartment entirely (Pinto *et al.*, 2003). These data suggest a model where cells that have lost APC function maintain their crypt progenitor fate, due to inappropriate activation of the Wnt pathway. This allows for the formation of the adenomatous polyps, which can persist there for many years, allowing ample opportunity for additional mutations to occur that lead to transformation and carcinoma.

There is evidence that Wnt signaling is equally as important in other stem cells. Embryonic stem cells also rely on Wnt signaling to maintain their undifferentiated, self-

renewing state. Activation of Wnt signaling is sufficient to maintain pluripotency and suppress differentiation in both human and mouse ES cells in vitro (Sato *et al.*, 2004). Wnt signaling also plays an important role in stem cells of the hematopoietic system. Activation of signaling in normal hematopoietic stem cells promotes multi-lineage differentiation potential in both lymphoid and myeloid progenitor populations (Baba *et al.*, 2005). In the skin, the role of Wnt signaling is more complex (reviewed in Alonso and Fuchs, 2003). Here it helps specify the hair follicle. Removal of ßcat blocks the formation of hair follicles altogether. Transgenic mice expressing stabilized ßcat exhibit *de novo* follicle morphogenesis, allowing committed interfollicular epidermal cells to revert back to a follicular stem cell-like state. It has been proposed that different levels of Wnt activity direct different outcomes, *i.e*. stem cell activation versus hair follicle differentiation (Blanpain *et al.*, 2004).

Wnt signaling also plays critical roles in neural stem cells and brain development (Ciani and Salinas, 2005). Making an adult brain is vital for the developing animal, and a number of precisely timed events must happen in a coordinated manner (reviewed in Tissir and Goffinet, 2003). For example, in mammals, stem/progenitor cells, like those in the ventricular zone in the developing cortex, must first proliferate extensively to give rise to large numbers of neurons. Next, the neuronal daughters must exit the cell cycle and migrate to appropriate locations; *e.g*., neurons move out of the ventricular and subventricular zones with the help of radial glial cell fibers. These cells must also know where to stop their migration, *i.e*. which layer of the cerebral cortex they will populate. Finally, neurons differentiate into specific subtypes and extend axons and neurites. This will establish the network of connectivity required for integrated control of the brain and the entire animal.

Numerous signal transduction pathways in addition to Wnt signaling are utilized for organization and patterning necessary to generate this complex organ.

The developing mammalian brain has a complex pattern of expression of Wnt ligands, and Wnts pattern cell fates there during early development (Ciani *et al.*, 2005). For example, loss of mouse *Wnt1* results in defects in the midbrain, cerebellum, and spinal cord (McMahon *et al.*, 1990) while mutation of *Wnt3a* leads to a complete loss of the hippo-campus (Lee *et al*., 2000). When Wnt1 and Wnt3a are ectopically expressed, overgrowth results, without altering cell fates along the dorsoventral axis of the neural tube. This is consistent with a role in precursor cell proliferation (Megason *et al*., 2002). Further evidence of this came from expression of constitutively-active ßcat in neuronal progenitor cells. This led to an enlarged cerebral cortex with more neuroepithelial precursors. Despite this, precursors followed a normal differentiation program, giving rise to neurons in relatively normal spatial patterns (Chenn and Walsh, 2002).

Since Wnt signaling is so important for the formation of the brain, it is not unexpected that mammalian APC family members are also expressed there. Both flies and humans have two APC family members, and both family members are expressed in the brain. APC is broadly expressed during early embryogenesis and in the adult (Groden *et al*., 1991; Bhat *et al*., 1994; Chazaud *et al*., 2006). Mice homozygous mutant for *APC* die with patterning defects during gastrulation (Moser *et al*., 1995). A second mammalian APC family member, APC2, is also highly expressed in the CNS (Yamanaka *et al*., 2002), but *APC2* mutant mice have yet to be reported. Our lab and others (Ahmed *et al*., 1998, McCartney *et al*., 1999, Akong *et al*., 2002a) have seen expression of both APC family members in developing *Drosophila* larval brains, which will be discussed in detail later.

In addition to their normal roles in brain development, mutations in the Wnt pathway also are associated with predisposition for primitive neuroectodermal tumors such as medulloblastoma, which are thought to be derived from multipotent, cerebellar precursor cells (Fogarty *et al*., 2005). Another familial disorder characterized by germline mutations of APC called Turcot's syndrome shows an increased frequency of medulloblastoma. Mutations in APC, βcat and Axin1 have also been shown to occur in sporadic medullo-blastomas, with a cumulative frequency of about 15% (Fogarty *et al*., 2005).

Other Functional Roles for APC

APC also has roles in cell migration, cell-cell adhesion, chromosome segregation, spindle assembly (Hanson and Miller, 2005). These roles are thought to be Wnt/Wg signaling independent. In some of these roles, APC continues to use βcat as a binding partner. In addition to its role in signal transduction, βcat is well known to be involved in organizing epithelial tissue architecture and cellular polarity. It is important for the function of the transmembrane protein E-cadherin (E-cad), a Ca^{++}-dependent adhesion molecule necessary in the formation and maintenance of adherens junctions (AJ). During development, AJ are crucial in maintaining cell-cell adhesion. They play a similar role in the adult animal. These multi-protein complexes help to link the actin cytoskeleton of adjacent epithelial cells into a coordinated network via the homophillic interactions of E- cad. βcat/Arm binds the cytoplasmic tail of E-cad and can also bind to α-catenin. α-catenin can interact with the actin cytoskeleton both indirectly, via α-actinin and vinculin, and directly, by binding F-actin filaments, to link AJ to the cytoskeleton (Perez-Moreno *et al*., 2003). AJ are highly dynamic structures. Their modulation is necessary for normal morphogenesis and cell migration to occur during development and later in the adult epithelium. APC colocalizes with β-catenin,

E-cadherin, and α-catenin in AJ in *Drosophila* (Figure 1.4; McCartney *et al.,* 2001; Yu *et al*., 1999). It is not known whether APC regulates AJ function, however evidence from Hamada *et al*., (2002) suggests it may play a role in modulating cell-cell adhesion by regulating the association of βcat/Armadillo with the AJ.

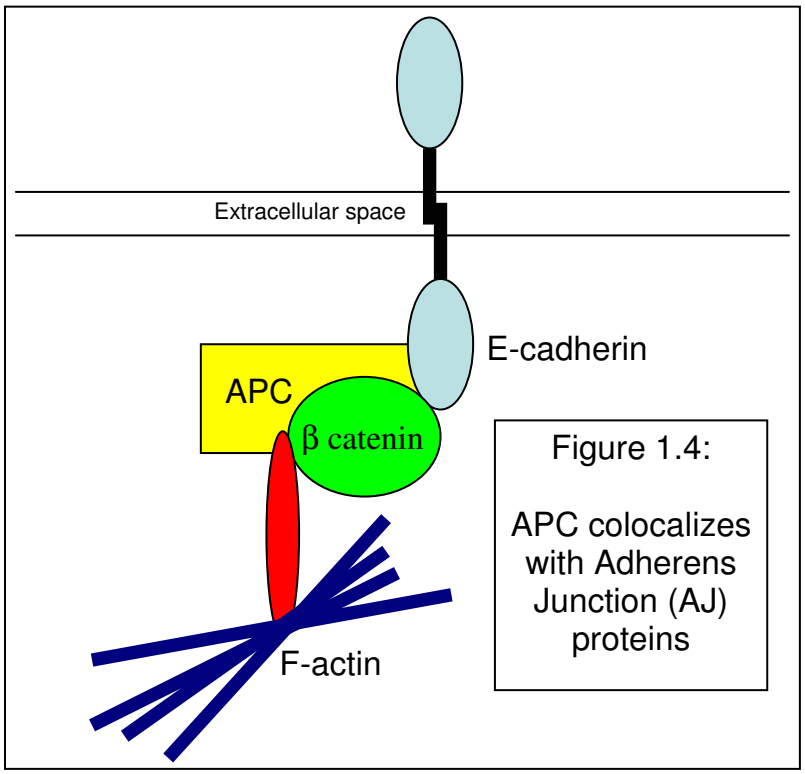


Figure 1.4:

APC colocalizes with Adherens Junction (AJ) proteins

Other functional domains present in APC allow its direct interaction with microtubules and other microtubule associated proteins (Figure 1.2; reviewed in Hanson and Miller, 2005; Akiyama and Kawasaki, 2006). It has been shown that mammalian APC can bind to and bundle microtubules *in vitro*, and localizes at their plus ends *in vivo* (Munemitsu *et al.,* 1994; Nathke *et al*., 1996; Mogensen *et al*., 2002). APC has also been shown to be able to stabilize microtubule plus ends, *in vitro* and *in vivo* and this stabilization was regulated by phosphorylation of APC by GSK3-β (Zumbrunn *et al.*, 2001). In *Xenopus*, APC-GFP can dynamically track along a subset of microtubules and again concentrate at

their plus ends (Mimori-Kiyosue *et al.*, 2000). The C-terminal region of APC contains a basic motif thought to mediate interactions with the microtubule cytoskeleton (reviewed in Mimori-Kiyosue and Tsukita 2001), as well as binding sites for the tumor suppressor Discs Large (Dlg) (Matsumine *et al.*, 1996) and EB-1, a microtubule plus end-binding protein (Su *et al.,* 1995). The biological relevance of the interactions between APC and microtubules is still not completely understood.

However, these interactions may affect microtubule attachment to other cellular structures. APC mutant ES cells display chromosomal and spindle aberrations (Fodde *et al.,* 2001, Dikovskaya *et al.,* 2004). During metaphase, APC accumulates at the kinetochore where it is believed to help link the plus ends of spindle microtubules. APC mutant cell lines have been shown to form mitotic spindles with an excess of microtubules that inefficiently connected to the kinetochore. This data is consistent with a role for APC in kinetochore-microtubule attachment and suggests that truncation mutations in APC which eliminate microtubule binding may be a contributing factor to the chromosomal instability seen in cancer cells. APC may also have a role in linking dynein and dynactin to the cell cortex (Allen and Nathke, 2001). Interestingly, overexpression of Lis1 disrupts dynein localization, causing spindle positioning defects similar to those seen in APC mutant cells (Faulkner *et al*., 2000; Smith *et al*., 2000).

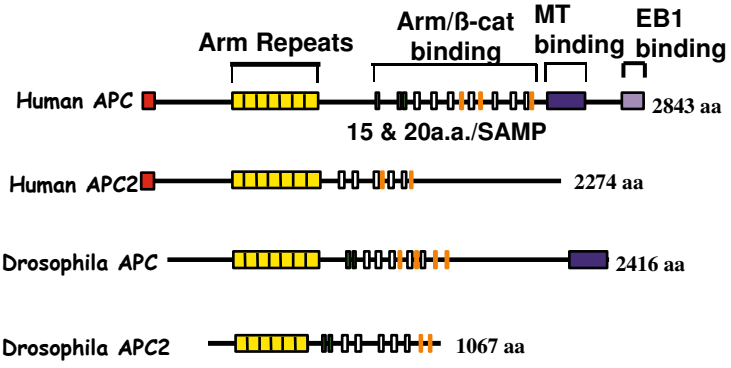
APC is also proposed to serve as a link between the microtubule and actin cytoskeleton via its interaction with Armadillo. APC localizes to the cell cortex of polarized epithelial cells. Disruption of the actin cytoskeleton in this context leads to delocalization of APC (Reinacher-Schick and Gumbiner, 2001; Rosin-Arbesfeld *et al*., 2001). Another APC family member, *Drosophila* APC2, co-localizes with AJ components in the furrows that form

in the early embryo between mitotic syncytial nuclei. Previous work in our lab showed removal of APC2 function at this stage in development leads to weakened attachment of the mitotic spindle microtubules to the ingressed membrane furrows, and subsequent detachment of some nuclei from the cortex of the embryo (a "nuclear fallout" phenotype) (McCartney *et al*., 2001). Thus, APC2 can mediate the attachment of spindle microtubules to the cell cortex, perhaps via the AJ complex and cortical actin. In *Drosophila* testis, cortically localized APC in germline stem cells helps orient mitotic spindles to ensure asymmetric division occurs properly (Yamashita, *et al*. 2003).

APC family members expression

The conservation of APC family members in humans, mice, and *Drosophila* illustrates its important cellular functions. As previously mentioned, both mammals and flies have two APC family members (Figure 1.5). The first identified *Drosophila* APC (APC1) is 27% identical and 46% similar to human APC. Both human and *Drosophila* APC share multiple functional domains, such as the Arm repeats, 15- and 20-amino acid repeats, SAMP repeats,

Figure 1.5: Domain structure of vertebrate and *Drosophila* APC proteins



and a microtubule binding domain. Aside from these conserved functional domains, APC family members share little sequence similarity.

Initial studies of Drosophila *APC1* suggested its expression in embryos is restricted to the axons of the CNS and the developing germ cells (Hayashi *et al*., 1997). Genetic analysis of *APC1* mutants proved surprising. Homozygous mutants are viable and fertile, with the only apparent defect in eye development. Activation of Wg signaling in the photoreceptor cells of the eye triggered inappropriate apoptosis (Ahmed *et al*., 1998). This limited phenotype was not expected, given the essential role Wnt/Wg signaling was known to play in patterning the epidermis of the early Drosophila embryo and other tissues. Several groups consequently identified a second Drosophila APC gene, *APC2* (Hamada *et al*., 1999; McCartney *et al*., 1999; Yu *et al*., 1999). APC2 retains many of the domains present in APC1, *e.g.* Armadillo repeats, the 15- and 20-amino acid repeats and the SAMP repeats. However, it does not contain the basic microtubule-binding motif found in APC1.

APC2 has a more widespread expression pattern throughout development. Maternal and zygotic *APC2* mutant embryos die with a patterning defect consistent with APC2 negatively regulating Wg signaling in the embryonic epidermis (McCartney *et al*., 1999). *Drosophila* APC2 was found to only be essential during embryogenesis, since zygotic mutants are viable (the maternal contribution is sufficient to complete embryogenesis). *Drosophila* APC1 seems to be important only in specific cell populations in the CNS. Since APC family members are thought to play an essential role in Wnt/Wg signaling we suspected the relatively restricted phenotypes observed in zygotic *APC1* and *APC2* mutants was due to redundancy. Our lab and the Wieschaus lab independently identified overlapping redundant roles for APC1 and APC2 in regulating Wg signaling in the embryonic and adult epidermis

(Akong *et al*., 2002b; Ahmed *et al*., 2002), as well as potential Wg-independent roles for both APC1 and APC2 in the developing CNS (Akong *et al*.,2002a). As no one had previously defined the effects completely eliminating APC2 function, our lab generated a number of new alleles to do so in the living animal. The experiments here investigate the effects of different mutations in *APC2* on Wg signaling in the epidermis as well as the requirements for both APC1 and APC2 in the developing CNS. The role of both APC1 and APC2 in cell-cell adhesion during the development of the CNS is also addressed.

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PREFACE TO CHAPTER II

Chapter II describes how *Drosophila* APC1 and APC2 can function redundantly in some tissues during development. Both APC1 and APC2 had been shown to act independently as regulators of Wg signaling in a subset of tissues during development. APC1 does so in the photoreceptors of the developing *Drosophila* eye (Ahmed *et al*., 1998), but this was the only tissue altered by the null mutation. *APC2* zygotic mutants are also viable to adulthood. It is only when *APC2* is also removed maternally that one sees that APC2 functions in the embryonic epidermis to regulate Wg signaling (McCartney *et al*., 1999). However, mutations in *APC2* do not result in as severe an effect on either the cuticle phenotype or the levels of Armadillo accumulation seen in the embryonic epidermis when another Wg signaling regulator, *zeste white 3 kinase (zw3),* is completely eliminated. Further, in some tissues, such as the imaginal discs (a precursor of the adult epidermis) or the larval brain, mutation of either *APC* family members has no phenotypic effect. This led to the hypothesis that both *APC1* and *APC2* may have overlapping function in some tissues. We test this hypothesis here.

For this work, published in Developmental Biology in 2002, I generated cuticle preparations for maternal/zygotic mutants in the APC^{d40} and $APC^{\Delta S}$ alleles. I also helped with the examination of levels of Armadillo accumulation in the embryonic epidermis of APC^{d40} and *zw3* mutants.

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Drosophila APC2 and APC1 Play Overlapping Roles in Wingless Signaling in the Embryo and Imaginal Discs

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The regulation of signal transduction plays a key role in cell fate choices, and its disregulation contributes to oncogenesis. This duality is exemplified by the tumor suppressor APC. Originally identified for its role in colon tumors, APC family members were subsequently shown to negatively regulate Wnt signaling in both development and disease. The analysis of the normal roles of APC proteins is complicated by the presence of two APC family members in flies and mice. Previous work demonstrated that, in some tissues, single mutations in each gene have no effect, raising the question of whether there is functional overlap between the two APCs or whether APC-independent mechanisms of Wnt regulation exist. We addressed this by eliminating the function of both *Drosophila* APC genes simultaneously. We find that APC1 and APC2 play overlapping roles in regulating Wingless signaling in the embryonic epidermis and the imaginal discs. Surprisingly, APC1 function in embryos occurs at levels of expression nearly too low to detect. Further, the overlapping functions exist despite striking differences in the intracellular localization of the two APC family members.

Key Words: **APC; β-catenin; Armadillo; Wnt; Wingless; *Drosophila*; tumor suppressor.**

INTRODUCTION

Signal transduction plays a key role in setting cell fates in embryogenesis. When inappropriately activated by mutation, however, signal transduction pathways often help trigger oncogenesis. The Wnt pathway provides an excellent example of this. Wnt signaling regulates diverse developmental decisions in all animals studied, and inappropriate activation of Wnt signaling leads to colon and other cancers. The most common mechanism by which Wnt signaling is activated in tumors is by loss-of-function mutations in the tumor suppressor APC (reviewed in Polakis, 2000).

Transduction of Wnt signals occurs via regulation of the levels of cytoplasmic Arm/βcat (the pool not assembled into cell–cell adhesive junctions; reviewed in Polakis, 2000). In the absence of signal, cytoplasmic Arm/βcat is rapidly targeted for destruction in a two-step process. Arm/βcat is first captured by a multiprotein complex that includes APC and a second scaffolding protein Axin. This complex targets Arm/βcat for phosphorylation by Zeste-white3 (Zw3)/glycogen synthase kinase-3 (GSK3). Phosphorylated Arm/βcat is a substrate for a ubiquitin ligase, targeting it for proteasomal destruction. Wnt signaling turns off the APC/Axin/GSK3 complex by an unknown mechanism. This triggers the accumulation of Arm/βcat, which enters the nucleus and works with DNA-binding proteins of the TCF/LEF family to activate Wnt target genes. These data helped explain APC's tumor suppressor role. In the colon, the absence of functional APC allows βcat levels to rise, activating Wnt target genes such as *cyclinD1* and *c-myc*, promoting cell proliferation. This model fits the data well, though questions remain. For example, since APC is expressed in most tissues, why is the colon the primary tissue in which tumors arise in *APC* heterozygous people or mice?

APC's role in tumors led to the hypothesis that it is a key

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negative regulator of Wnt signaling in other contexts (APC family proteins also play Wnt-independent cytoskeletal roles; see accompanying paper for details). Supporting a key role in Wnt regulation, mammalian APC is broadly expressed, and mice homozygous for loss-of-function mutations in *APC* die as early embryos (Fodde *et al.*, 1994; Moser *et al.*, 1995). This hypothesis was further tested in *Drosophila*, where a firm connection to Wnt signaling was established. Loss-of-function mutations in fly *APC1* activate Wnt signaling in the photoreceptors of the eye, triggering their apoptosis (Ahmed *et al.*, 1998). Unlike mammalian APC, however, mutations in fly *APC1* are not lethal, and its protein product is not ubiquitous. In embryos, high level expression is limited to CNS axons and developing germ cells (Hayashi *et al.*, 1997), suggesting that APC1 is absent from many tissues where Wnt signaling is required.

One possible explanation for the limited effect of mutations in fly *APC1* as well as the limited tissue-spectrum of tumors resulting from mutations in mammalian *APC* was functional redundancy with other, as yet undiscovered APC family members. This led several labs to identify second *APC* relatives in both mammals (APC2 or APC-L; (Nakagawa *et al.*, 1998; van Es *et al.*, 1999) and flies (APC2 or E-APC; Hamada *et al.*, 1999; McCartney *et al.*, 1999; Yu *et al.*, 1999). Fly APC2 is broadly expressed in embryonic and postembryonic development, consistent with the redundancy hypothesis (McCartney *et al.*, 1999; Yu *et al.*, 1999). To test this further, mutations in *APC2* were characterized. Animals maternally and zygotically mutant for *APC2* die as embryos with inappropriate stabilization of Arm and activation of Wg signaling in the embryonic epidermis (McCartney *et al.*, 1999), supporting the idea that different APC family members function in different tissues.

However, there are many tissues where neither single mutant has an effect (Ahmed *et al.*, 1998; McCartney *et al.*, 1999). Most striking are the larval imaginal discs, precursors of the adult epidermis, which are affected by neither single mutant. Two hypotheses might explain this. First, APC proteins may not be essential for all Wnt regulation—in cultured cells, Axin overexpression partially compensates for loss of APC (von Kries *et al.*, 2000). Alternatively, in some *Drosophila* tissues, APC1 and APC2 may play redundant roles, and thus mutation in one would not disrupt function. We have tested these hypotheses by creating situations where animals or tissues are double mutant for both *APC* genes. These experiments reveal that APC1 and APC2 play redundant roles in both the embryonic epidermis and the larval imaginal discs, where they cooperate to regulate Wg signaling.

MATERIALS AND METHODS

Genetic and Phenotypic Analysis

Alleles used were: $APC2^{\Delta S}$ (McCartney *et al.*, 1999), $APC2^{\Delta 40}$ (McCartney *et al.*, 2001), and $APC1^{Q8}$ (Ahmed *et al.*, 1998). $APC2^{810}$ was generated in an EMS mutagenesis screen, identified by failure to complement $APC2^{\Delta S}$ (unpublished data). Double mutant *APC2 APC1* chromosomes were generated by meiotic recombination. All stocks were kept at 25°C. Embryo collections were done at 27°C. Transgenic lines used for misexpression and overexpression studies were UAS-APC2-GFP (R. Rosin-Arbesfeld and M. Bienz), UAS-APC1 (E. Wieschaus). Transgenes were expressed by crossing to Engrailed-GAL4 at 27°C. Canton S was the wild type. To generate germline clones, larvae of genotype $FRT82B\ ovo^{D}/APC2^{\Delta 40}APC^{Q8}$ were γ−irradiated with 1000 rads at 32–48 h after egg-laying (AEL) at 25°C. Females were crossed to $APC2^{\Delta 40}\ APC^{Q8}/TM3actinGFPSer$ males at 27°C. Embryos produced from this cross were either maternally and zygotically mutant, or paternally rescued; these were distinguished by the presence of GFP. To generate imaginal disc clones, larvae of the genotype $FRT82B\ myc\ Sb^{63}/APC2^{\Delta 40}\ APC^{Q8}$ were γ−irradiated with 1100 rads at 24–36 h AEL at 25°C. Clones in the discs of wandering third instar larvae were identified by the absence of APC2 and myc. Adult wings with clones were mounted on slides in Faure's solution.

Immunolocalization

Imaginal discs were fixed in 4% paraformaldehyde for 20 min. Embryos were fixed in 1:1 3.7% formaldehyde in PBS:heptane for 20 min. All were blocked in 1% normal goat serum/0.1% Triton X-100 in PBS for at least 2 h. Primary antibodies were as follows: rat polyclonal anti-APC2 (1:1000), mouse monoclonal anti-Arm N27A1 (DSHB, 1:200), and rabbit polyclonal anti-Armadillo N2 (1:200) and anti-APC1 (1:1000).

RESULTS

APC1 and APC2 Play Overlapping Roles in Regulating Wg Signaling during Embryogenesis

Both APC1 and APC2 (McCartney *et al.*, 1999) have phenotypes that suggest that they act as regulators of Wg signaling, but each only affects a subset of the tissues where Wg signaling regulates development. Further, there are certain tissues, such as the imaginal discs, where neither gene has a phenotype. One possible explanation is partial redundancy between the two genes. We first addressed this issue in the embryo. Embryos maternally and zygotically mutant for *APC2* exhibit Arm stabilization and thus activation of Wg signaling in the epidermis (McCartney *et al.*, 1999; Hamada and Bienz, 2002). However, neither their cuticle phenotype nor the level of Arm accumulation are as drastic as those of *zw3* maternal and zygotic mutants (Peifer *et al.*, 1994; Siegfried *et al.*, 1992). Three hypotheses seemed possible: (1) The *APC2* allele tested retained some function, (2) APC1 might provide some function in the absence of APC2, or (3) APC proteins might not be absolutely essential for Arm destruction.

To test the redundancy hypothesis, we generated embryos that were maternally and zygotically $APC2^{\Delta 40}$ $APC1^{Q8}$, and thus double mutant for both APCs (Figs. 1E and 1F). Wild-type embryos have a segmentally reiterated pattern of anterior denticles (Fig. 1A, arrow) and posterior naked cuticle (Fig. 1A, arrowhead) on their ventral surfaces. Dorsally, cells secrete different hair types at different posi-

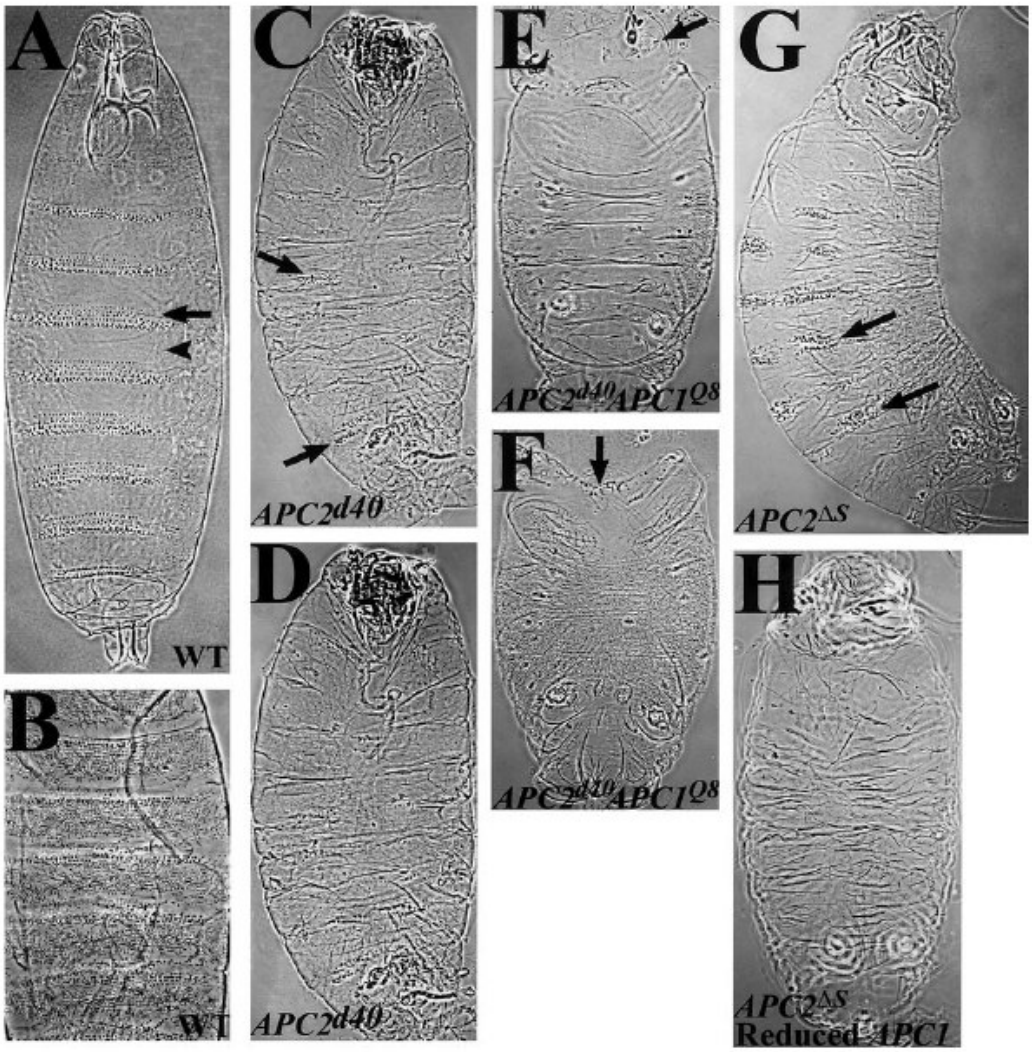

FIG. 1. *APC1* mutations enhance the embryonic phenotype of *APC2*. Cuticle preparations, anterior up. (A, B) Wild-type ventral (A) and dorsal (B) cuticles. Ventrally, note the segmentally reiterated anterior denticle belts (arrow) and posterior naked cuticle (arrowhead). Dorsally, different hair types are secreted at different positions along the anterior–posterior axis. (C, D) $APC2^{d40}$ maternal and zygotic mutant. Ventral (C) and dorsal (D) cuticles. Ventrally, most cells are converted to posterior fates but some cells still secrete denticles (e.g., arrows). (E, F) $APC2^{d40}$ $APC1^{Q8}$ maternal and zygotic double mutant. Ventral (E) and dorsal (F) cuticles. Ventrally, all cells secrete naked cuticle, and dorsally, all cells secrete fine hairs characteristic of posterior cells. Head involution is disrupted (E, arrow) and dorsal closure is partially disrupted (F, arrow). (G) $APC2^{\Delta S}$ maternal and zygotic mutant. Ventrally, most cells choose posterior fates but some cells still secrete denticles (arrows). (H) Progeny of $APC2^{\Delta S}$ $APC1^{Q8}/APC2^{\Delta S}$ females and males. All ventral cells secrete naked cuticle.

tions (Fig. 1B). In $APC2^{d40}$ maternal and zygotic single mutants, most cells take on posterior fates and secrete naked cuticle, indicative of excess Wg signaling (Fig. 1C). However, unlike *zw3* maternal and zygotic mutants, some cells still adopt anterior fates and secrete denticles. (e.g., Fig. 1C, arrows), In contrast, $APC2^{d40}$ $APC1^{Q8}$ maternal and zygotic double mutants exhibit a much stronger embryonic cuticle phenotype (Fig. 1E). Maternal and zygotic double mutant cuticles were shorter, and, unlike $APC2^{d40}$ maternal and zygotic single mutants, most embryos completely lack

denticles. Further, maternal and zygotic double mutants exhibit a complete failure of head involution (Fig. 1E, arrow). Dorsally, all cells are transformed to the fate normally adopted by posterior cells, and thus all secrete fine dorsal hairs (Fig. 1F), and maternal and zygotic double mutants exhibit slight abnormalities in dorsal closure not seen in the single mutant (Fig. 1F, arrow). Thus, the cuticle phenotype of the maternal and zygotic double mutant is much more similar to that of embryos maternally and zygotically mutant for *zw3* (Siegfried *et al.*, 1992), suggesting that APC1 partially compensates for loss of APC2 in the embryonic epidermis. Interestingly, embryos receiving paternal wild-type copies of *APC2* and *APC1* are rescued to viability.

We also looked at zygotic *APC2 APC1* double mutants, combining $APC1^{Q8}$ with $APC2^{\Delta 40}$, $APC2^{\Delta S}$, and $APC2^{E10}$. All of these zygotic double mutant combinations were embryonic viable and exhibited a wild-type cuticle pattern (data not shown). This suggests that maternally contributed APC1 and APC2 are sufficient for embryonic Wg signaling; this is similar to what we previously observed for *APC2* zygotic single mutants (McCartney *et al.*, 1999). Zygotic *APC2 APC1* double mutants die later as larvae, with defects in brain development (see accompanying paper).

APC1 and APC2 Both Regulate Arm Levels in Embryos

To further test the functional overlap between APC1 and APC2, we examined Arm accumulation in maternal and zygotic double mutants. In wild-type embryos, Arm accumulates at adherens junctions of all cells, but in cells that do not receive Wg signal, Arm levels in the cytoplasm and nucleus are low (Fig. 2I, arrowheads). Wg signal stabilizes cytoplasmic and nuclear Arm (Fig. 2I, arrows). In $APC2^{\Delta 40}$ maternal and zygotic single mutants, Arm levels are elevated (Fig. 2D), but do not become uniformly high as is observed in *zw3* maternal and zygotic mutants (Fig. 2G; Peifer *et al.*, 1994); in this, $APC2^{\Delta 40}$ resembles $APC2^{\Delta S}$ (McCartney *et al.*, 1999). In contrast, in $APC2^{\Delta 40}$ $APC1^{Q8}$ maternal and zygotic double mutants, Arm levels become extremely elevated (Figs. 2B and 2E), thus more precisely matching *zw3* maternal and zygotic mutants.

We next looked more closely at subcellular localization. In $APC2^{\Delta 40}$ maternal and zygotic single mutants, the levels of Arm are elevated but one can see further Arm stabilization by Wg signaling (Fig. 2L, arrows). In maternal and zygotic double mutants (when we turned down the brightness to compensate for increased Arm levels), we observed that Arm is highly elevated in both the cytoplasm and nuclei (Fig. 2K), with no differences seen between cells that receive Wg signal and those that do not. Further, in $APC2^{\Delta 40}$ $APC1^{Q8}$ maternal and zygotic double mutants, Arm becomes somewhat enriched in nuclei relative to the cytoplasm; this is most evident in cells of the amnioserosa (Figs. 2H and 2K, arrowheads). In this regard, $APC2^{\Delta 40}$ $APC1^{Q8}$ maternal and zygotic double mutants are more similar to *Axin* maternal and zygotic mutants (Tolwinski and Wieschaus, 2001) than to *zw3* maternal and zygotic mutants, in which levels of Arm in the cytoplasm and nuclei are equal (Peifer *et al.*, 1994). Tolwinski and Wieschaus (2001) previously suggested that Axin acts as a cytoplasmic anchor for Arm, and that this plays an important role in regulating Wg signaling. Our data suggest that APC proteins may act with Axin to form this cytoplasmic anchor. We also noted that in $APC2^{\Delta 40}$ $APC1^{Q8}$ maternally double mutant but zygotically rescued siblings, cells that do not receive Wg signal (Fig. 2J, arrowheads) have slightly elevated Arm levels relative to wild type. This may result from Arm stabilization before the onset of zygotic gene expression, as we previously observed in *zw3* (Peifer *et al.*, 1994).

APC1 Is Expressed at Very Low Levels in the Epidermis during Embryogenesis

These data, along with the lack of an embryonic phenotype of the *APC1* single mutant (Ahmed *et al.*, 1998), suggest that, while APC2 plays an essential role in the embryonic epidermis, APC1 plays an accessory role, ameliorating the *APC2* mutant phenotype. APC2 is uniformly expressed by all cells in the embryonic epidermis, accumulating at the apicolateral cell cortex (McCartney *et al.*, 1999; Figs. 3A and 3B). Previous analysis of APC1 suggested that in the embryo it accumulates to high levels only in the axons and the developing germline (Hayashi *et al.*, 1997), although uniformly expressed maternal mRNA is present. We thus reexamined APC1 expression in the epidermis, using the zygotic double mutant $APC2^{\Delta 40}$ $APC1^{Q8}$ as a negative control. While we could easily detect APC1 accumulation in CNS axons (see accompanying paper) and the germline (data not shown), the level of accumulation in the epidermis was relatively low, and it appeared diffusely cytoplasmic. At early stages of embryogenesis, the epidermal staining we observed in wild type and $APC2^{\Delta 40}$ $APC1^{Q8}$ was very similar, perhaps reflecting maternally contributed APC1, while in late embryos we could detect expression above background in the wild-type versus the mutant epidermis (Figs. 3C and 3D). Thus, a very low level of APC1 seems to be able to provide some residual function in Arm destruction.

This activity of APC1, despite its low level of expression, prompted us to examine whether there might be dose-sensitive interactions between the two *APC* genes. We thus reduced the dose of *APC1* in embryos mutant for *APC2*, by crossing males and females of the genotype $APC2^{\Delta S}$ $APC1^{Q8}/APC2^{\Delta S}$ $APC1^{+}$. The progeny of this cross (Fig. 1H) had a more severe cuticle phenotype than the progeny mutant only for $APC2^{\Delta S}$ (Fig. 1G). We saw similar dose-sensitive interactions using the $APC2^{\Delta 40}$ $APC1^{Q8}/APC2^{\Delta 40}$ genotype (data not shown).

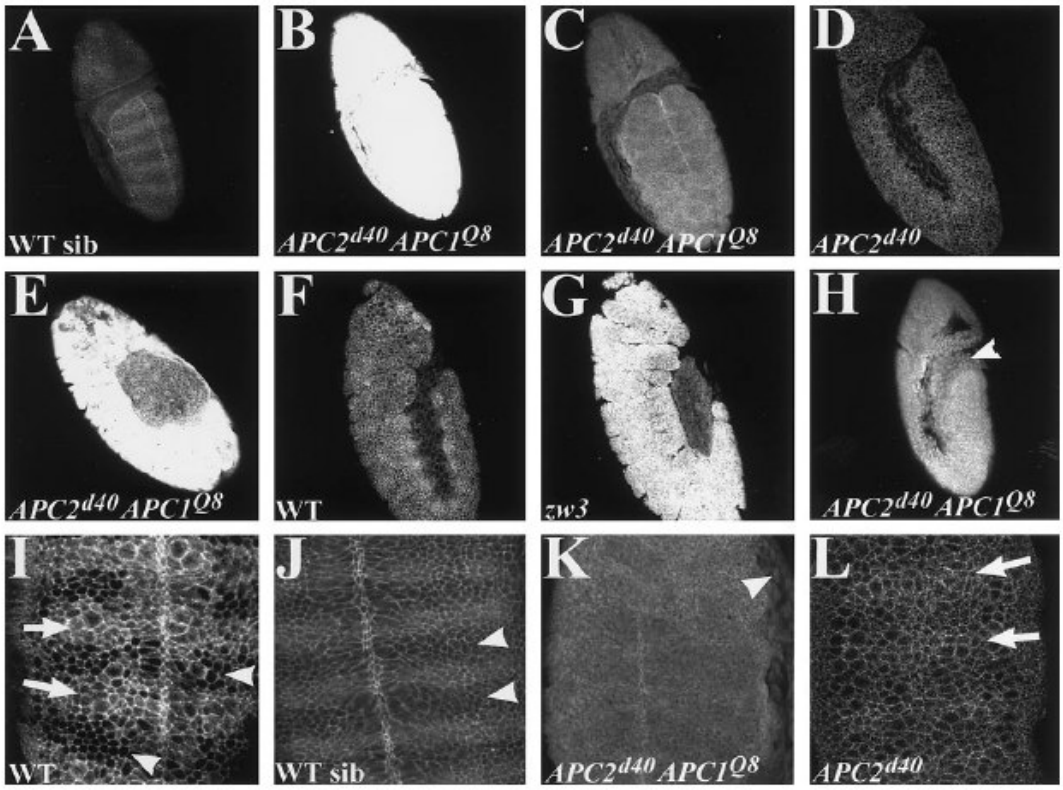

FIG. 2. In *APC2 APC*1 maternal and zygotic double mutant embryos, Arm levels are highly elevated. Embryos stained to reveal Arm. Anterior is up. (A–D) Stage 9. (A, B) Both are progeny of a mother whose germline was $APC2^{d40}$ $APC1^{Q8}$ double mutant. They were prepared for immunofluorescence and imaged together. (A) Zygotically rescued sibling. Stripes of cells in which Wg has stabilized Arm are clearly visible. (B) $APC2^{d40}$ $APC1^{Q8}$ maternal and zygotic double mutant. Arm levels are highly elevated. (C) Same as (B) with brightness reduced to allow visualization of subcellular localization. (D) $APC2^{d40}$ maternal and zygotic single mutant. Levels of Arm are somewhat elevated in all cells. (E) Stage 14 $APC2^{d40}$ $APC1^{Q8}$maternal and zygotic double mutant. (F) Stage 11 wild-type. (G) Stage 11 *zw3* maternal and zygotic mutant. (H) Lateral view of stage 9 $APC2^{d40}$ $APC1^{Q8}$ maternal and zygotic double mutant. Arrowhead, nuclear enrichment in amnioserosa. (I–L) Close-ups, stage 9. Brightness in (K) was reduced to allow visualization of subcellular localization. (I) Wild-type. Arrowheads, cells not receiving Wg– Arm is only in adherens junctions. Arrows, cells that received Wg, have stabilized cytoplasmic and nuclear Arm. (J) $APC2^{d40}$ $APC1^{Q8}$ maternal mutant that was zygotically rescued. Cells that have not received Wg signal accumulate slightly elevated levels of Arm (arrowheads). (K) $APC2^{d40}$ $APC1^{Q8}$maternal and zygotic double mutant. All cells accumulate high levels of Arm; some nuclear enrichment is observed (e.g., arrowhead). (L) $APC2^{d40}$ maternal and zygotic single mutant. While Arm levels are somewhat elevated in all cells, Wg signal still can further stabilize Arm (arrows).

APC1 and APC2 Accumulate at Very Different Intracellular Locations When Mis-Expressed in the Embryonic Epidermis

Endogenous APC2 localizes to the cell cortex in most cells throughout development, including those of the embryonic epidermis during the establishment of segment polarity (McCartney *et al.*, 1999). We thus have suggested that the cell cortex is the likely location of the destruction complex, and that the function of APC2 might be to recruit the destruction complex to this site. In support of this, the mutant proteins encoded by $APC2^{d40}$and $APC2^{\Delta S}$, that lack function in Wg regulation, no longer localize to the cortex (McCartney *et al.*, 1999, 2001).

As the data above suggest that APC1 can contribute to destruction complex function, we suspected that it should localize similarly to APC2. As the endogenous level of expression of APC1 was too low to reliably assess intracellular localization, we mis-expressed both APC1 and APC2-GFP in stripes in the embryonic neurectoderm using en-

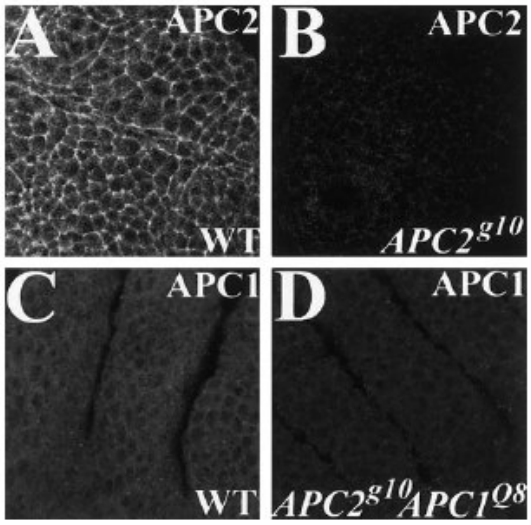

FIG. 3. APC1 and APC2 localization in embryos. (A, B) APC2 expression in the embryonic epidermis of stage 11 wild-type (A) and $APC2^{g10}$ maternal and zygotic mutant (B; negative control) embryos. APC2 is enriched at the apicolateral cell cortex. (C, D) APC1 expression in the epidermis of stage 15 wild-type (C) and $APC2^{g10}APC1^{Q8}$ zygotic double mutant (D; negative control) embryos. APC1 accumulates at low levels diffusely in the cytoplasm.

grailed (en)-GAL4. Expression levels exceeded those of endogenous APC2, as revealed by staining embryos expressing APC2-GFP with anti-APC2, which recognizes both endogenous and exogenous protein. Levels of staining were much higher in the segmental stripes where en-GAL4 is expressed (Fig. 4A). When overexpressed, APC2 localized to the cell cortex (Figs. 4A–4C), resembling endogenous APC2. In contrast, overexpressed APC1 was diffusely cytoplasmic, with strong enrichment at what appear to be centrosomes and associated microtubules of both epidermal cells (Figs. 4D–4G and 4I) and neuroblasts (Figs. 4J and 4K). We saw similar differences in localization of the two proteins in the larval brain (see accompanying paper). These data raise questions about the normal localization of the destruction complex, as the primary location of APC1 upon overexpression is different from that of APC2. We also made one additional observation that may be relevant to this discussion. When we overexpressed APC1 in the embryonic epidermis, we found that endogenous APC2 now became less cortical and more diffusely cytoplasmic (Figs. 4G and 4H, white bracket vs red bracket). We also observed recruitment of endogenous APC2 to centrosomes (Figs. 4G–4L, white arrowheads) in epidermal cells and neuroblasts overexpressing APC1. This suggests that the two APCs may each be able to recruit the other to new locations; this is further supported by similar observations after mis-expression in the larval brain (see accompanying paper).

APC1 and APC2 Play Redundant Roles during Imaginal Disc Development

The overlapping functions of APC1 and APC2 in the embryo raised the possibility that redundancy might help explain why neither *APC1* (Ahmed *et al.*, 1998) nor *APC2* (McCartney *et al.*, 1999) single mutants have defects in patterning of the imaginal discs, precursors of the adult epidermis. To test this, we induced mitotic recombination to produce clones of double mutant cells in animals heterozygous for a wild-type and a double mutant chromosome. In animals in which we induced clones, we found alterations of imaginal disc patterning consistent with activation of Wg signaling (Fig. 5). In the wing, one function of Wg is to specify the wing margin. Activation of Wg signaling by removing *zw3* function was previously observed to transform cells from a wing blade to a wing margin fate, leading to patches of margin bristles in the blade (Blair, 1992). We observed similar patches of bristles in the wing blades of animals in which $APC2^{d40}$ $APC1^{Q8}$ double mutant clones were induced (Figs. 5B, 5C, 5E, and 5F); they varied in size from tens to hundreds of cells. We also saw regions of the notum where too many cells adopted bristle fates (Fig. 5H, arrow); this was also previously observed in clones mutant for *zw3* (Simpson and Cateret, 1989).

These data suggest that loss of both APC family members activates Wg signaling in imaginal discs, presumably via Arm stabilization. To test this hypothesis, we examined the effect of clonal loss of both *APC* genes on Arm levels. We generated clones of cells that were homozygous $APC2^{d40}$ $APC1^{Q8}$ double mutant in an $APC2^{d40}$ $APC1^{Q8}$/+ heterozygous background. In doing so, one generates adjacent "twin spots"—clones of homozygous wild-type cells. We detected mutant clones by the absence of APC2 staining; both mutant clones (Fig. 5J, arrows) and their homozygous wild-type twin spots (with elevated APC2; Fig. 5J, arrowheads) could be easily distinguished from their heterozygous neighbors. In $APC2^{d40}$ $APC1^{Q8}$ double mutant clones, we saw elevated Arm accumulation. Thus, loss-of-function of both APC family members is sufficient to deregulate Arm accumulation in the imaginal discs.

DISCUSSION

APC is a tumor suppressor that negatively regulates Wnt signaling, functioning in a multiprotein complex that targets the Wnt effector Arm/β-cat for proteolytic destruction (reviewed in Polakis, 2000). This model of APC function was strongly supported by genetic analysis of the roles of APC proteins during *Drosophila* development (Ahmed *et al.*, 1998; McCartney *et al.*, 1999). However, this relatively simple picture recently became more complex. There are two APC family members in both flies and mammals, with both conserved and divergent structural elements, raising questions about their overlapping or divergent functions in both cell biological and biological events.

A Complex Functional Relationship between APC1 and APC2

Our data reveal a complex pattern of overlapping functions between APC1 and APC2. When we began, we knew that, in certain tissues, individual APCs play critical roles: APC1 is essential in photoreceptors (Ahmed *et al.,* 1998), while APC2 plays critical roles in syncytial embryos and the embryonic epidermis (McCartney *et al.,* 1999, 2001). This functional partition is paralleled by distinct embryonic expression patterns, with APC1 on at high levels in axons (Hayashi *et al.,* 1997) and APC2 on at high levels in the ectoderm (McCartney *et al.,* 1999). In other tissues, such as imaginal discs, neither APC family member is essential, even though both Wg signaling and regulation of Arm stability are critical there. This raised the question of whether the two proteins are redundant in these tissues or whether APC-independent means of regulating Arm stability exist.

Our data suggest a complex picture in which some tissues depend exclusively on one APC family member (photoreceptors), others depend primarily on one family member (the embryonic ectoderm), while in some, either family member provides sufficient function (the larval brain or imaginal discs). The embryonic ectoderm provides a striking example of this complexity. APC2 plays an important role there, with the Wg pathway activated in *APC2* mutants (McCartney *et al.,* 1999). However, certain aspects of the *APC2* phenotype were puzzling; embryos null for another destruction complex component, *zw3*, have a more severe phenotype and more highly elevated Arm levels (Peifer *et al.,* 1994), suggesting that residual destruction complex activity remains in APC2's absence.

We considered several explanations for this. First, the *APC2* allele we used is not a protein null allele. Second, residual destruction complex function might remain in the absence of all APC family members. However, our data support a third possibility: in the absence of APC2, APC1 provides function in the embryonic epidermis that is not sufficient for wild-type pattern, but does allow residual activity of the destruction complex. This is somewhat surprising in view of the expression pattern of APC1 in embryos, which was initially thought to be restricted to axons and primordial germ cells. However, the uniform maternal mRNA (Hayashi *et al.,* 1997) and low levels of APC1 protein (Fig. 3) appear to provide a low level of APC1 function in epidermal cells. While this paper was under review, a similar study of the redundancy of the two fly APC proteins was published (Ahmed *et al.,* 2002). These authors also document redundancy between the *APC1* and *APC2* in both the embryo and the imaginal discs. They further demonstrate that raising the level of expression of *APC1* can rescue the embryonic defects of *APC2*, and that elevated expression of *APC2* can rescue the eye phenotype of *APC1* mutants. These data further underscore the functional overlap between the two proteins.

Together, these two studies raise questions about the possible overlap in function of the mammalian APC proteins in development and oncogenesis and also raise interesting questions about the evolution of small multigene families. The two APC proteins in flies and in mammals appear to have been derived from independent gene duplication events in each lineage. After duplication, the two genes diverged in their patterns of expression and their domain structures (APC2 appears to have lost certain domains, such as the putative microtubule-binding domain). It thus remains to be determined whether there is selective pressure to retain low levels of APC1 in the embryonic ectoderm, or whether residual expression is simply a relict of a time when flies may have had only a single APC. APC family proteins also have Wnt-independent cytoskeletal roles, and may have overlapping roles in these as well; the accompanying paper describes an apparent example of this during brain development.

Overlapping Functions and Divergent Localization

The functional overlap of the two APC family members is even more striking given their distinct structures and intracellular localizations. All APC family proteins share a core structure: a block of highly conserved Arm repeats, which in APC2 is essential for cortical localization and function in the destruction complex, and a set of short repeated sequences that bind Arm/β-cat or Axin, the number and arrangement of which are variable. APC2 contains only this core, which is thus sufficient for both regulating Arm levels as well as for APC2's Wnt-independent role in spindle anchoring. APC1 is longer at its N and C termini, and shares with mammalian APC (at least by sequence similarity) a domain known to bind microtubules. These structural differences confer strikingly different cell biological properties upon the two fly APCs. When overexpressed, APC2 localized almost exclusively to the cell cortex, resembling its endogenous localization. In contrast, overexpressed APC1 localized to the region of the centrosome and to cytoplasmic microtubules, a localization potentially mediated by its putative microtubule-binding domain.

This striking difference in localization raises several interesting issues. We suspect that the localization of APC proteins depends on the availability of and the affinity for different binding partners, as we previously observed for Arm. We hypothesize that the core APC domains are sufficient for cortical localization, but that the additional domain(s) in APC1 redirect it to microtubules. We further hypothesize that if APC1 were detached from microtubules, it would exhibit a default localization to the cortex. This hypothesis is supported by Bienz and colleagues, who showed that one could shift mammalian APC from association with microtubules to actin by disrupting microtubules (Rosin-Arbesfeld *et al.,* 2001).

These data also have implications for the destruction complex. Two mutations that disrupt the localization of APC2 to the cell cortex also disrupt its function in Arm

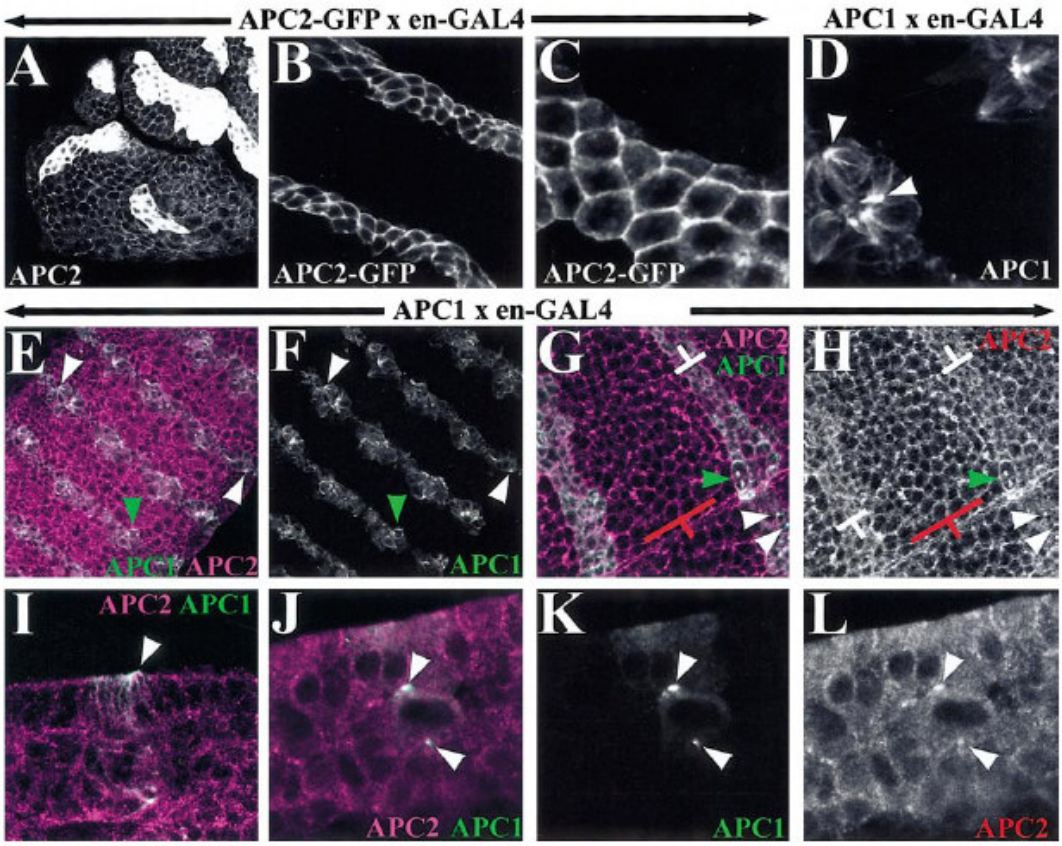

FIG. 4. APC1 and APC2 localize differently when overexpressed in embryos. (A–H) Surface views of extended germ-band embryos. (I–L) Optical cross-sections. (A–C) Embryos expressing APC2-GFP under the control of *en*-GAL4. (A) Anti-APC2 antibody. (B, C) GFP visualized directly. (A) *en*-GAL4-driven APC2-GFP accumulates in segmental stripes, at levels much higher than that of endogenous APC2. (B, C) APC2-GFP localizes to the cell cortex, similar to endogenous APC2. (D–L) Embryos expressing APC1 under the control of *en*-GAL4. (D) Close-up. APC1 localizes to centrosomes and associated microtubules (arrowheads). (E–L) Double-labeled embryos: APC1 (E, G, I, J green, F, K), endogenous APC2 (E, G, I, J purple, H, L). Overexpressed APC1 does not accumulate at the cortex—instead, it accumulates diffusely in the cytoplasm with stronger localization to structures that appear to be centrosomes and microtubules (white arrowheads). (E, F) Green arrowhead, cell with separated centrosomes. (G, H) Green arrowhead, cell in the late stages of mitosis. Endogenous APC2 is normally cortical (G, H, red bracket). In cells expressing APC1, endogenous APC2 becomes diffusely cytoplasmic (G, H, white bracket) and is recruited to presumptive centrosomes with APC1 (white arrowheads). (I) Ectodermal cell, cross-section. APC1 and APC2 colocalize to the apical centrosome and the microtubules radiating from it (white arrowhead). (J–L) Mitotic neuroblast, cross-section. APC1 and APC2 accumulate at the separated centrosomes (white arrowheads).

regulation (McCartney *et al.*, 1999, 2001), suggesting that cortical localization of the destruction complex is important for function. However, the distinctive localization of APC1 raises questions about this conclusion. The simplest interpretation of our data are that APC1 and APC2 localize the destruction complex to distinct places, but that the complex functions at either location. There are several caveats to this interpretation, not least of which is that the predominant localization of APC1 upon overexpression may mask a small amount localized to the cortex; our genetic experiments suggest that nearly undetectable levels of APC1 can confer Arm regulatory activity. It is also possible that both APC2 and APC1 bind to similar partners but with different affinities. Thus, in the absence of APC2 (in *APC2* mutants or in tissues where it is not normally expressed), APC1 may localize to places where APC2 is normally found.

Finally, our data suggest that APC1 and APC2 may

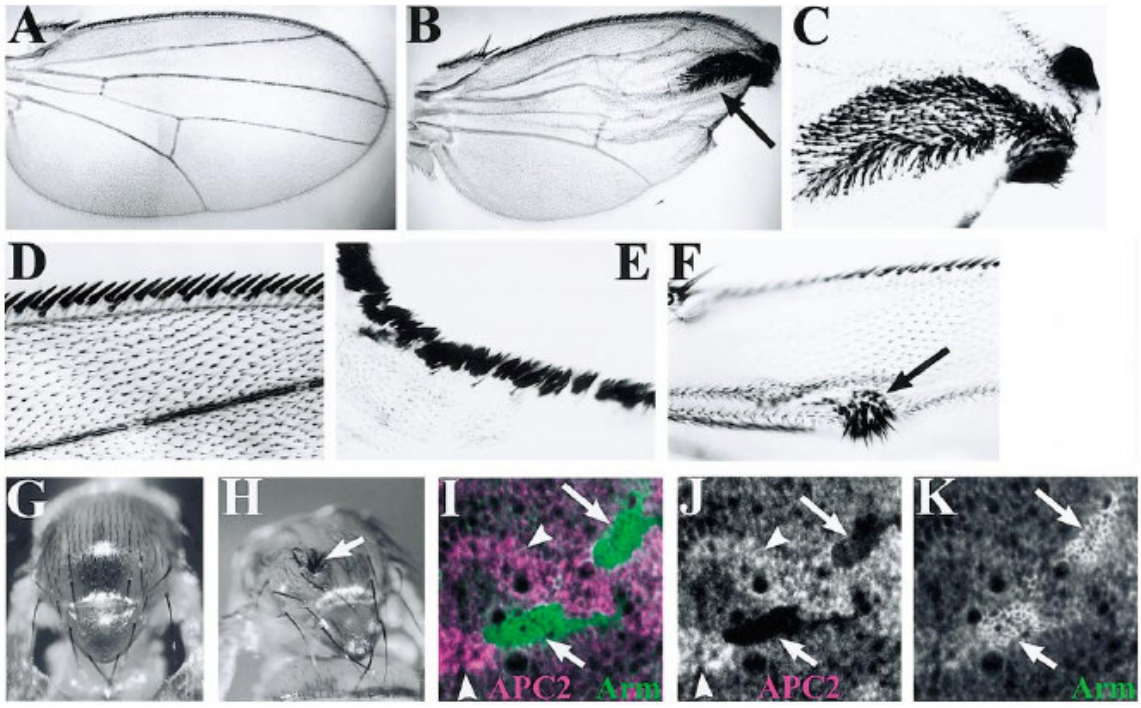

FIG. 5. *APC1* and *APC2* play redundant roles in imaginal disc development. (A–F) Wings from wild-type adults (A, D), or adults in which we induced clones of cells double mutant for $APC2^{q40}$ $APC1^{Q8}$ (B, E, F; C, close up of region in B indicated by arrow). Variable size patches of ectopic wing margin bristles occur in wing blades of animals in which clones were induced (arrows). (D) Wild-type wing margin. (E) Excess cells adopting the wing margin bristle fate. (G, H) Notums. Wild-type (G) and adult in which we induced clones of cells double mutant for $APC2^{q40}$ $APC1^{Q8}$ (H). Note ectopic notal bristles (arrow). (I–K) Wing imaginal disc with clones of cells double mutant for $APC2^{q40}$ $APC1^{Q8}$. Double labeled: APC2 (I purple, J), Arm (I green, K). Double-mutant cells can be identified by reduced APC2 levels (J, arrow); homozygous wild-type twin spots have increased APC2 (J, arrowheads). Double-mutant cells have elevated Arm levels (K, arrows) relative to heterozygous mutant or wild-type cells; this was most evident when one focused below the apical-most plane.

interact. Overexpression of APC1 triggered relocalization of endogenous APC2 from the cortex to centrosomes and microtubules. When both APCs were overexpressed, APC2 recruited APC1 to the cortex (accompanying paper). The mechanism by which this occurs is not clear. Mammalian APC homodimerizes via an N-terminal coiled-coil (Day and Alber, 2000). A clear match to this region is found in mammalian APC2, raising the possibility that mammalian APC and APC2 heterodimerize. However, there is only a weak, partial similarity to this region in fly APC1, and fly APC2 lacks this region entirely. Other mechanisms of oligimerization may exist, or a linker protein or proteins could mediate this putative interaction.

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PREFACE TO CHAPTER III

Chapter III describes an analysis of the biological roles of APC family members, using the *Drosophila* embryonic epidermis and ovary as a model system. APC family members are was known to act as negative regulators of Wnt/Wg signaling. A number of other functions had been attributed to APC family members. Many of these were based on studies using dominant negative constructs in cultured cells or using partially functional alleles. The phenotype of complete loss of both family members had not been characterized in either mammals or *Drosophila*.

The Peifer lab, in collaboration with the Besjovec lab, generated and characterized multiple alleles of *APC2*, both missense and truncation mutations. Here we report the identification of the first molecular null allele of *APC2*, the $APC2^{g10}$ allele. This discovery was essential for my work illustrating the effects of complete elimination of *APC* family members in the developing larval brain. We examined how this and other *APC2* alleles disrupt Wg signaling. We also use the *APC2 APC1* double null animals to examine proposed roles in cytoskeletal regulation. This work was published in Development in 2006.

I contributed to this work in several ways. I generated maternal/zygotic (M/Z) mutant animals for each of the different *APC2* alleles, and made cuticle preparations (to examine effects on Wg signaling in the embryonic epidermis) and analyzed the percent embryonic lethality at both permissive (18^0C) and non-permissive temperatures (27^0C). Meredith Price did the phenotypic scoring of the cuticle preparations. I also examined the localization and levels of Armadillo in all the different alleles, again using M/Z mutants. These effects were

compared to what was observed in *zeste White 3 kinase* (the fly homolog of *GSK-3β*) and *APC* double null mutant embryos. In addition, I looked at how expression of a known downstream Wg target gene, *engrailed*, was affected in several of the *APC2* maternal/zygotic mutants.

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

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SUMMARY

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 often rest 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 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, while 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 were 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.

INTRODUCTION

Many genes mutated in human cancers are critical components of signal transduction pathways regulating normal development. For example, Wnt signaling controls cell fate, asymmetric cell division, and stem cell behavior (reviewed in 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 in 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 ß-catenin, the key Wnt effector, in the absence of Wnt signals (reviewed in (Polakis, 2000). Within this complex, APC binds both ß-catenin, via its 15- and 20-amino acid repeats, and the scaffolding protein Axin, via its SAMP repeats (Fig. 1A). Axin and APC present ß-catenin to Casein kinase-I and GSK-3ß (fly Zw3), which sequentially phosphorylate ß-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 further C-terminal (Polakis, 2000). Wnt ligands like *Drosophila* Wingless (Wg) inactivate the destruction-complex and stabilize ß-catenin by engaging a Frizzled/LRP5/6 receptor complex. The mechanism of destruction-complex inactivation is not well understood, but it 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

different 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*., 1997). *Drosophila* APC2 is broadly expressed. Zygotic mutants are viable and normal, but maternal/zygotic (M/Z) 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, while 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 in Nathke, 2004). These are reflected in its binding partners (Fig. 1A). The N-terminal third of *APC* encodes a series of Arm repeats, binding sites for cytoskeletal regulators including the Rac-GEF ASEF, the 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 APC proteins. Loss-of-function studies using putative hypomorphic alleles suggest that APC2 helps mediate interactions between mitotic spindles and cortical actin in early embryos (McCartney *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 truncation alleles 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 plays a role 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 by 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 were seen 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 removed or reduced function of both APC family members. While studies of *Drosophila APC1* (Ahmed *et al*., 2002) included a protein-null allele, the only tissue defective in single mutants

is the eye, where loss of APC1 triggers apoptosis, precluding assessment of other cell biological roles.

A second striking but unanswered question is why, while both copies of *APC* are invariably mutated in colon tumors, one allele encodes a truncated APC protein ending in the MCR (Fig. 1A). This contrasts with most tumor suppressors. The truncated proteins cannot correctly regulate ß-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 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 critical questions.

Materials and methods

Genetic screen for new *APC2* alleles

See Suppl. Fig. 1.

Hatch rates and cuticle analysis

Embryos were progeny of *APC2allele/Df(3R)crb 87-4* females X *APC2allele/+* males. All alleles were scored at 27ºC and 18ºC. Double-mutant embryos maternally *FRT APC2allele APC1Q8* were generated using the FRT/FLP/DFS technique (Chou and Perrimon, 1996). Cuticle preparations and hatch rate analysis were as in Wieschaus and Nusslein-Volhard, (1998).

Immunolocalization, Immunoblots, and imaging

Embryos and ovaries were from adults maintained at 27ºC. Tissues were fixed as in (McCartney *et al*., 1999). Antibodies/probes: actin=Alexa-488-phalloidin (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), DNA=propidium iodide (25ug/ml, Molecular Probes) or DAPI (10ug/ml; Sigma-Aldrich). DAB reactions were as in (McEwen *et al*., 2000). Anti-APC2-NT antisera were raised in guinea pigs by Pocono Rabbit Farms and Laboratory (Canadenisis, 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. Percent nuclei lost=number of lost nuclei/total number of nuclei (estimated from nuclear number in 1µm2 of the cortex). Imaging: Fig. 3-4—Zeiss LSM510 with gain, offset, and pinhole size kept constant for each wavelength; Fig. 5, 8A-H—Zeiss LSM510 or LSM410 respectively, with internal wild-type controls expressing histone-GFP co-stained with mutants to control for staining variations; Fig. 6A-C, H-J—Spinning-disc confocal microscope (Solamere Technology Group) with Yokogawa scanhead and Hamamatsu OrcaAG CCD camera on a Zeiss Axiovert200M; Fig 6D,E, Fig. 7—Zeiss Axioskop2plus and Canon PowershotG5 camera. Analysis of Fig. 5E",F",6F,G,K used Image-J. Figures were prepared using Adobe Photoshop.

APC2 rescuing transgene

688bp of genomic DNA extending from the gene upstream of *APC2* (CG13608) to *APC2's* AUG (the "endogenous promoter") 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 2nd-chromosome lines rescued the maternal-effect lethality of $APC2^{\Delta S}$ at 27ºC (data not shown).

RESULTS

Genetic Screen for new *APC2* alleles

We generated new alleles using the mutagen EMS (Suppl. Fig. 1), selecting them by their failure to complement the M/Z embryonic lethality of $APC2^{\Delta S}$ (McCartney et al., 1999). This screen would allow us to identify null alleles, as Deficiencies removing *APC2* fail to complement $APC2^{\Delta S}$.

We obtained seven mutations affecting *APC2's* coding region (Fig. 1C). Three are missense mutations in the Arm repeats ($APC2^{e90}$ repeat 3, $APC2^{c9}$ repeat 6, and $APC2^{b5}$ repeat 7; altered amino acids are in Fig. 1C). Four alleles result from premature stop codons that should truncate the protein. Two truncate APC2 within Arm repeats 8 or 9: $APC2^{g10}$ and $APC2^{f90}$. Two others truncate APC2 within the MCR: $APC2^{d40}$ (amino acid 677; its phenotype was first reported in (Ahmed *et al*., 2002; Akong *et al*., 2002a; McCartney *et al*., 2001) and $APC2^{g41}$ (amino acid 728). Together with $APC2^{\Delta S}$ (McCartney et al., 1999), which deletes a single serine in Arm repeat 5, and $APC2^{N175K}$, identified by Hamada and Bienz (2002), a missense mutation in Arm repeat 3, there are nine *APC2* alleles. Several

mimic those in human tumors or engineered in mice. *APC2*^{f90} resembles mouse *APC*$^{\Delta 716}$ and *APC*^{MIN} that truncate just C-terminal to the Arm repeats (Oshima *et al*., 1995a; Su *et al*., 1995). The *APC2*^{d40} and *APC2*^{g41} truncations are similar to those in the human *APC* MCR.

We next examined how the mutations affect APC2 protein, using homozygous or hemizygous tissues to remove wild-type APC2 (Fig. 2, Table 1). We first examined the two alleles with the earliest stop codons, *APC2*^{f90} and *APC2*^{g10}. Early stop codons often lead to nonsense-mediated mRNA decay and thus we anticipated that these might produce little or no protein. No protein was detected by immunoblotting with APC2 antibodies recognizing a C-terminal epitope downstream of the predicted stop codons (amino acids 491-1067; (McCartney *et al*., 1999). To determine whether *APC2*^{f90} and *APC2*^{g10} are protein null, we utilized antibodies directed against an N-terminal APC2 epitope (amino acids 1-126, Fig. 1C) that would be present in the predicted truncated proteins. This antibody recognized background bands at 59 and 44kDa, and recognized strongly reduced levels of a 41kDa truncated protein in *APC2*^{f90} (Fig. 2C), close to its predicted MW (Table 1). No APC2g10 protein at or near the expected size is detected (Fig. 2C), suggesting that *APC2*^{g10} is protein null. Consistent with this, it is among our strongest alleles (see below), and its cuticle phenotype over a Deficiency resembles its homozygous phenotype (data not shown), the genetic test for a null allele.

Truncated human APC proteins in tumors, with stop codons in the MCR, accumulate at near normal levels. Our alleles mimicking these, *APC2*^{d40} and *APC2*^{g41}, produce 79kDa and 92kDa proteins accumulating at reduced levels (Fig. 2B), consistent with the stop codons observed (Fig. 1C). All alleles carrying point mutations in the Arm repeats produce slightly-reduced levels of wild-type size protein (Fig. 2A).

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 (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 $APC2^{f90}$ and $APC2^{g10}$, as anti-APC2-NT does not work in tissue). $APC2^{\Delta S}$ protein fails to localize to the cortex (McCartney *et al*., 1999), implicating Arm repeat 5 in correct localization. $APC2^{c9}$, affecting Arm repeat 6, and $APC2^{b5}$, 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, $APC2^{d40}$ and $APC2^{g41}$, are also exclusively cytoplasmic (Fig. 3C and data not shown), supporting a role for APC2's C-terminus (including the Axin-binding sites) in cortical localization. $APC2^{e90}$ and $APC2^{N175K}$ proteins can weakly associate with the cortex (Fig. 3B', arrow, and data not shown).

Complete loss of APC proteins does not disrupt adhesion

Hemizygous $APC2^{N175K}$ or $APC2^{\Delta S}$ mutants exhibit reduced Arm at adherens junctions, resulting in reduced cadherin-based adhesion, though 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. 4A,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 ($APC2^{g10}$

APC1^{Q8}/*APC2*^{g10}), thus reducing function equally in both germ cells and somatic follicle cells, and also egg chambers in which the germline was homozygous *APC2*^{g10} *APC1*^{Q8} (Fig. 4B,D,F; data not shown) In some mutant egg chambers, cytoplasmic Arm levels were elevated (Figure 4B',D'), consistent with loss of destruction-complex function. However, cortical Arm localization was largely unaltered in mutant germ cells and follicle cells, regardless of whether (Fig. 4B',D') or not (Fig. 4F') 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*$^{\Delta S}$ or *APC2*^{N175K} 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*^{g10}, *APC2*^{g10} *APC1*^{Q8}/*APC2*^{g10}, and *APC2*^{g10} *APC1*^{Q8} mutant germlines. We distinguished the oocyte from nurse cells by the presence of 4 ring canals (Fig. 4B, arrow) and its elevated accumulation of cortical actin. There was no difference in the frequency of mispositioned oocytes between wild-type and mutants (Fig. 4E). Thus 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. 5B,D,F). Furthermore, we did not observe disruption of cortical localization of DE-cadherin (Fig. 5A,B) or α-catenin (Fig. 5C,D,E,F) in embryonic epithelia. In fact, if anything, cortical localization was slightly elevated. Arm levels were highly elevated (Fig. 5B,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 lab suggests that the proteins they tested may have dominant-negative effects on cadherin-catenin function; consistent with this, *APC2*$^{\Delta S}$ 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. 6A). Thus, defects in actin or microtubule function can result in abnormal nuclear divisions with resulting abnormal nuclei transported into the embryo interior. In *APC2*$^{\Delta S}$ 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*$^{\Delta S}$ and *APC2*^{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. 6F). We defined an abnormal embryo as one in which >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 complete lack of *APC2*. 35% of *APC2*^{g10} maternally-mutant embryos are abnormal (Fig. 6E,F). This is partially rescued by *P[APC2+],* an *APC2* transgene driven by the endogenous promotor (14%; Fig. 6F); this

incomplete rescue may be due to reduced levels of APC2 expression from the transgene. 7% of the progeny of mothers heterozygous for *APC2*^{g10} are abnormal—this may reflect slight haploinsufficiency or may be within the wild-type range. To ask whether APC1 also has syncytial functions, we examined nuclear loss in *APC2*^{g10} *APC1*^{Q8} maternally double-mutant embryos (Fig. 6B,C,F). 39% of the embryos were abnormal, similar to *APC2*^{g10} alone (35%), suggesting that if APC1 functions in this process, its contribution is relatively minor. In *APC2*$^{\Delta S}$, nuclear loss appears to be largely due to disrupted tethering (McCartney *et al*., 2001). In *APC2*^{g10} and in double null mutants, this may be compounded by spindle collisions resulting from compromised actin furrows (Fig 6B,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*^{d40} (McCartney *et al*, 2001), which is truncated in the MCR (Fig. 1). 18% of *APC2*^{d40} maternally-mutant embryos are abnormal (Fig. 6F). Interestingly, progeny of *APC2*^{d40} heterozygous mothers had a similar phenotype (19% abnormal), suggesting that *APC2*^{d40} has dominant-negative effects on this process.

We also assessed nuclear loss for three missense mutations affecting the Arm repeats (*APC2*^{N175K}, *APC2*^{c9}, and *APC2*$^{\Delta S}$; Fig. 1C, 6F). *APC2*^{N175K} and *APC2*^{c9} maternal-mutants have relatively weak phenotypes (12% and 8% abnormal embryos). In contrast, 58% of *APC2*$^{\Delta S}$ embryos are abnormal. This is reduced to 15% by *P[APC2+],* which rescues the null allele to the same degree. This indicates that the dramatic effect of *APC2*$^{\Delta S}$ is due to its affect on APC2. In progeny of mothers heterozygous for *APC2*$^{\Delta S}$ 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*$^{\Delta S}$ protein, like *APC2*^{d40}, 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 place to examine whether fly APCs are critical for spindle structure. In *APC2*$^{\Delta S}$ 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*^{g10} *APC1*^{Q8} double mutant with nuclear- and cell-cycle stage-matched wild-types. *APC2 APC1* null embryos did not have significant defects in overall spindle morphology (Fig. 6B',C'). 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. 6G).

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*^{g10} *APC1*^{Q8} M/Z mutant ectoderm. Wild-type spindles are oriented perpendicular to the apical-basal axis (92%, Fig. 6H,K) and divisions are symmetric, resulting in two equal daughter cells (100%, Fig. 6H,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*^{g10} *APC1*^{Q8} M/Z mutant embryos are oriented normally (90-92%, Fig. 6J,K), and all divisions were symmetric (100%; Fig. 6J,K). We also examined whether truncated APC2 proteins have dominant effects,

examining divisions in $APC2^{d40/+}$ embryos derived from $APC2^{d40}$ homozygous mothers. We observed no dominant-negative effects (Fig. 6I,K). One possible explanation of the discrepancy with the results of Lu *et al*. (2001) are off-target RNAi effects of their 1kb 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 M/Z mutants, placing each allele in trans to a deletion of *APC2* to reduce concerns about other background mutations. We initially assessed phenotypes at 27°, 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. 7D, black arrow). 2) Cuticle size—elevated Wg signaling results in fewer epidermal cells, due to apoptosis (Pazdera *et al*., 1998), and 3) Head morphology—elevated Wg signaling disrupts head involution (Fig. 7D, 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. 7), and calculated a phenotypic average for each allele. The nine *APC2* alleles form a phenotypic series with phenotypic averages ranging from 2.0

for *APC2*^{e90} to 4.5 for *APC2*^{g10} (Table 1). This divided the alleles into three categories based on Wg activation: 1) weak: *APC2*^{e90}, *APC2*^{b5}, and *APC2*^{N175K} 2) moderate: *APC2*^{c9}, *APC2*$^{\Delta S}$, and *APC2*^{d40} 3) strong: *APC2*^{g41}, *APC2*^{f90}, and *APC2*^{g10} (our *APC2*^{N175K} stock has a somewhat weaker phenotype than 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, while in cells receiving Wg Arm also accumulates in the cytoplasm and nucleus, due to inactivation of the destruction-complex (Fig. 8A). *APC2*$^{\Delta S}$ mutants have elevated Arm levels (McCartney *et al*., 1999), but not as elevated as those seen when the destruction-complex is completely inactivated by M/Z loss of *Zw3 kinase* (Peifer *et al*., 1994; Siegfried *et al*., 1994). The less severe phenotype of *APC2*$^{\Delta S}$ is due, in part, to slight APC1 activity (Ahmed *et al*., 2002; Akong *et al*., 2002a), but might also suggest that *APC2*$^{\Delta S}$ does not fully inactivate APC2.

The cuticle phenotypes of our *APC2* alleles correlate well with Arm levels. The weakest allele, *APC2*^{e90}, has only a slight elevation in Arm levels; stripes are still readily apparent (Fig. 8B). Other weak to moderate alleles with missense mutations in the Arm repeats (*APC2*^{c9} and *APC2*^{b5}) have slightly greater elevation of interstripe Arm levels, but stripes remain detectable (Fig 8C,D)—they resemble *APC2*$^{\Delta S}$ (McCartney *et al*., 1999). The alleles truncating APC2 in the MCR, *APC2*^{d40} (Fig. 8E, Akong *et al*., 2002a) and *APC2*^{g41} (Fig. 8F), are stronger still, showing uniformly high levels equivalent to those in wild-type Arm stripes. Finally, *APC2*^{f90} and the null allele *APC2*^{g10} accumulate Arm levels in all cells higher than those in wild-type stripes (Fig. 8G,H). The highest Arm levels, however, are only seen when APC1 is also removed, in *APC2*^{g10} *APC1*^{Q8} double null mutants (Fig. 8I)—these resemble the extremely high levels previously seen in *APC2*^{d40} *APC1*^{Q8} double mutants

(Ahmed *et al*., 2002; Akong *et al*., 2002a; Peifer *et al*., 1994; Siegfried *et al*., 1994) and in *zw3* M/Z mutants (Fig. 8J). We also examined the expression of a Wg target gene, *engrailed* (*en*) in our strongest alleles, $APC2^{g41}$, $APC2^{f90}$, and $APC2^{g10}$. *en* is activated in additional cells posterior to its normal domain but it is not activated in all cells (Supplemental Fig. 2), results identical to what was previously observed in $APC2^{\Delta S}$ (McCartney *et al*., 1999) or M/Z *zw3* mutants (Siegfried *et al*., 1992).

$APC2^{\Delta S}$ is temperature-sensitive, exhibiting M/Z embryonic lethality at ≥25ºC and viability at 18ºC (McCartney *et al*., 1999). To determine whether other *APC2* alleles are temperature-sensitive, we assessed their cuticle phenotypes and hatch rates at 18ºC (Table 1). Strikingly, all mutants with missense mutations in the Arm repeats, except $APC2^{N175K}$, are temperature-sensitive; many M/Z mutants hatch as larvae. In contrast, the truncation alleles $APC2^{d40}$, $APC2^{g41}$, and $APC2^{f90}$, and the null allele $APC2^{g10}$ 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 $APC2^{c9}$, 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, $APC2^{c9}$ 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. 7) are roughly as severe as those of $APC2^{d40}$ $APC1^{Q8}$ double mutants (Ahmed *et al*., 2002; Akong *et al*., 2002a), although their effects on Arm levels (Fig. 8) 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* ($APC1^{Q8}$) and several *APC2* alleles (Table 1). All show approximately the same severity of cuticle phenotype (pa = 3.7-4.0; Fig. 7H,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. $APC2^{g10}$ $APC1^{Q8}$ double null mutants (pa = 3.8, n = 238; Fig. 7I) are quite similar to axin null embryos (pa = 4.1, n= 239; Fig. 7J), 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). $APC2^{g10}$ $APC1^{Q8}$ 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 critical 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 APC proteins in cadherin-based cell adhesion (Hamada and Bienz, 2002; Townsley and Bienz, 2000), spindle structure, and chromosome segregation (Green and Kaplan, 2003). While some of these effects were subtle, APC family function was not completely eliminated, suggesting the possibility that APCs 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 with 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). While the phenotype of homozygous animals has not been reported, Cre induction was used to create homozygous mutant clones of colon cells. This triggers polyp formation (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 cell 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 cell adhesion and in embryos we did not observe significant alterations in DE-cadherin or alpha-catenin localization at adherens 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 slow accumulation of aneuploid cells in tumors—this will require other assays. How can we reconcile the earlier data that suggested that APC family proteins have these roles in cell adhesion and cytoskeletal regulation, while our full loss-of-function experiments indicate that they do not? One possibility is that 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 $APC2^{\Delta S}$ in spindle tethering, 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 that could be affected by a dominant-negative truncation. Further, the putative dominant-negative effect is not sufficient for oncogenesis—mice engineered to express truncated APC proteins 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 8 of the 9 mutants, suggesting that mutant proteins cannot be strongly dominant-negative. The exception is $APC2^{c9}$, 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. $APC2^{\Delta S}$ and $APC2^{d40}$ heterozygotes exhibit elevated levels of nuclear loss, and the frequency of abnormal embryos is higher in $APC2^{\Delta S}$ 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, alternate mechanisms of nuclear retention partially compensate for the lack of APC1 and APC2. Since $APC2^{\Delta S}$ has a nuclear retention defect more severe than embryos M/Z null for both APCs, this mutant protein may not only block residual APC function, but also may interfere with a parallel, APC-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. 9). Since $APC2^{\Delta S}$ mutants exhibit much more nuclear loss than $APC2^{N175K}$ and $APC2^{c9}$, Arm repeat 5 may have special importance, perhaps by interfering with binding of a particular partner; $APC2^{\Delta S}$ 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. 9). $APC2^{d40}$ and $APC2^{g41}$ strongly reduce ability to regulate Wg signaling, but are not as strong as the null, $APC2^{g10}$, or as $APC2^{f90}$, truncating APC2 at the end of the Arm repeats. A similar severely truncated allele of mammalian APC led to higher levels of Wnt reporter activity in cultured cells than 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 ß-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 Wnt regulation. The role of APC's Arm repeats has been unclear. In *APC* mutant tumor cells, transfection of constructs encoding the 15- and 20-amino acid and SAMP repeats but lacking the Arm repeats restores ß-catenin turnover (Munemitsu *et al*., 1995). However, because these cells retain the Arm repeat-containing truncated protein found in the tumor, these two APC fragments might exhibit intra-allelic complementation. Our data suggest that the Arm repeats are critical for full function of APC2 in the destruction-complex (Fig. 9). Since our missense mutations are not null for Wnt regulation, APC proteins may retain residual function in Wnt signaling without the Arm repeats. Alternatively, the mutations we isolated may not fully disrupt the repeats. Each Arm repeat is comprised of three alpha-helices (*e.g*., (Huber *et al*., 1997). Together, the Arm repeats form a superhelical structure with a large groove where partner proteins bind. Structure-based sequence alignments (Jennifer Stamos and Bill Weis, personal communication) indicate that four of five missense mutations ($APC2^{e90}$, $APC2^{N175K}$, $APC2^{c9}$, and $APC2^{b5}$) are in helix 3 of Arm repeats 3,6, and 7. These mutations affect residues predicted to be in the protein core rather than those predicted to form the binding surface. They may thus destabilize individual Arm repeats or the Arm repeat domain, without totally eliminating its function. This is consistent with the temperature-sensitivity of four of the five Arm repeat mutations.

APC2 and APC1 function redundantly in Wg signaling throughout *Drosophila* development, despite differences in domain structure and subcellular localization. This redundancy suggests that the shared domains—the Arm repeats, 15- and 20-amino acid repeats, SAMP repeats and conserved sequences A and B—are sufficient for Wg regulation. We hypothesize that the Arm repeats are the docking site for a binding partner important for destruction-complex function. Using the temperature-sensitive allele $APC2^{\Delta S}$ we previously found that the phenotype and the membrane-association of the mutant protein varied in parallel. Two of our weakest new alleles also exhibit residual membrane-association—thus the Arm repeats may bind a partner mediating cortical localization of the destruction-complex. However, this does not explain how APC1 and APC2 can have different predominant localizations (Akong *et al*., 2002a) and yet be redundant. Perhaps low-level cortical accumulation of APC1, especially in APC2's absence, is sufficient for function. Future tests of this model and identification of the relevant binding-partner are needed. We will further explore the function of the Arm repeats and other conserved regions as we continue our analysis of this complex, multi-functional protein family.

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Figure Legends

Fig. 1. Human and fly APC1 and APC2. There are nine mutant alleles of fly *APC2*: 5 point-mutants in the Arm repeat region (black arrows) and 4 truncations (blue arrows). We used a null allele of *APC1* ($APC1^{Q8}$) resulting from a nonsense mutation in Arm repeat 4 (Ahmed *et al*., 1998).

Fig. 2. APC2 mutant proteins. Protein samples derived from ovaries of *APC2* allele/Deficiency females, or from embryos whose mothers and fathers were *APC2* allele/Deficiency, so only mutant protein was present (see Methods), were immunoblotted with C-terminal antibody (A,B) or N-terminal antibody (C). Arrows, mutant proteins. Arrowheads, background bands.

Fig. 3. APC2 mutant proteins lose cortical association. Germ-band extended embryos stained for phosphotyrosine labeling the cortex (green) and APC2 (red). (A, A') Wild-type APC2 localizes to the cortex (arrow) and in the cytoplasm of the ectoderm. Mutant proteins fall into two categories: (C) those that have no detectable cortical localization ($APC2^{g41}$ is shown) and (B) those with some residual cortical localization (arrow; $APC2^{N175K}$ is shown). Scale bar=10µm.

Fig. 4. APC proteins are not essential for cell adhesion in ovaries. (A-D',F,F') Wild-type (A,C) and $APC2^{g10}$ $APC1^{Q8}/APC2^{g10}$ (B,D,F) stage 7-8 egg chambers double-labeled for actin and Arm as indicated. Follicle cells (fc), ring canals (rc), and oocytes (o). E. Frequency of mispositioned oocytes. Scale bars=10µm (A,B), 5µm (C,D,F)

Fig. 5. APC proteins are not essential for embryonic cell adhesion. A-F. Stage-matched stage 9-10 wild-type and mutant embryos labeled for Arm, DE-cadherin, alpha-catenin and Dlg (a basolateral marker), as indicated. To select M/Z *APC2*^{g10} *APC1*^{Q8} embryos, females carrying germline clones were crossed to *moeGFP APC2*^{g10} *APC1*$^{Q8/+}$ males. Wild-types=his-GFP-marked embryos stained in the same tubes. E",F". Z-axis cross-sections of E,F. Arrows, adherens junctions. Scale bar=10µm (A-F'), 2.5µm (E",F").

Fig. 6. Nuclear loss, spindle morphology and spindle orientation. (A-C,H-J) Syncytial embryos in nuclear cycle 13 (A-C) and dividing ectoderm in germband-extended embryos (H-J); actin (red), microtubules (green), and DNA (blue); lower rows=microtubules alone. (A-C) *APC2*^{g10} *APC1*^{Q8} syncytial embryos (B,C) do not have significant defects in spindle morphology. Note weak actin rings (arrow, B) and nuclear loss evidenced by small, empty actin rings (arrow, C). (D,E). Syncytial wild-type (D) and *APC2*^{g10}. DNA (DAPI). Arrowheads=out-of-focus yolk nuclei; arrows=nuclei lost from surface. (F) Quantitation of nuclear loss in *APC2* and *APC2 APC1* mutant syncytial embryos. Bars=% embryos with ≥2% of cortical nuclei lost--see Methods. Purple=*APC2*^{c9}, yellow=*APC2*^{N175K}, red=*APC2*$^{\Delta S}$, green= *APC2*^{d40}, blue= *APC2*^{g10} (G). Quantification of syncytial spindle length (pole-to-pole). (H-J) Wild-type spindles are parallel to the epithelium (arrowheads, H) and divisions are symmetric (arrow, H). Spindle orientation (arrowheads, I,J) and division plane (arrows, I.J) are normal in *APC2*^{g10} *APC1*^{Q8} M/Z mutants (J) and embryos maternally *APC2*^{d40} and zygotically *APC2*$^{d40/+}$ (I). (K) Quantification of these phenotypes. Scale bars=10µm

Fig. 7. *APC2* mutant alleles vary widely in their effect on Wg signaling. Embryonic cuticles scored using phenotypic criteria in Table 1. Representative pictures of each class are presented.

Fig. 8. Effects on Arm stability match effects on cuticle phenotype. Arm levels, stage 9. A-H. Ventral views, anterior to the left. Wild-type (A) and *APC2* M/Z mutants (B-H). Confocal settings were normalized using his-GFP wild-type controls. I-J. Wild-type, $APC2^{g10}$ $APC1^{Q8}$ M/Z mutants, paternally-rescued embryos, and *zw3* M/Z mutants. Scale bars=20µm.

Fig. 9. Structure/function of APC2. Wild-type APC2 is 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 $APC2^{g41}$.

Supplemental Figure Legends

Suppl. Fig. 1. Generation of new alleles of *APC2*. 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. *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 chomosome (*) over *TM3 Sb* were crossed to virgin $APC2^{\Delta S}$ females at 27ºC (its non-permissive temperature). Embryos maternally and zygotically

APC2 mutant die as embryos, while 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 3 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 retested for the failure to complement $APC2^{\Delta S}$. 19 lines failed to complement $APC2^{\Delta S}$ 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 7 ($APC2^{b5}$, $APC2^{c9}$, $APC2^{d40}$, $APC2^{e90}$, $APC2^{f90}$, $APC2^{g10}$, and $APC2^{g41}$) upon sequencing the *APC2* gene from PCR-amplified genomic DNA from *APC2-/Df(3R) crb* 87-4 adults.

Suppl. Fig. 2. En expression in wild-type (A) and the indicated *APC2* maternal and zygotic mutants (B-D). Scale bar=20µm.

Table 1

	27° C					18°C	
APC2 allele	phenotypic average (n)	hatch rate (n)	Size of protein	Protein localization	*APC2- APC1*^{Q8} pa (n)	phenotypic average (n)	hatch rate (n)
APC2^{e90}	2.0 (215)	52% (549)	Wild type	Reduced membrane, cytoplasmic	nd	0.9 (220)	85% (673)
APC2^{b5}	3.1 (189)	52% (399)	Wild type	cytoplasmic	nd	0.6 (102)	80% (311)
APC2^{N175K}	3.3 (227)	49% (889)	Wild type	Reduced membrane, cytoplasmic	3.7 (323)	3.4 (214)	48% (872)
APC2$^{\Delta 5}$	3.5 (247)	53% (539)	Wild type	cytoplasmic	4.0 (243)	0.6 (161)	90% (877)
APC2^{c9}	3.6 (197)	38% (1269)	Wild type	cytoplasmic	nd	0.9 (161)	77% (338)
APC2^{d40}	3.8 (214)	46% (920)	79 kDa truncation predicted 75 kD	cytoplasmic	3.7 (176)	3.8 (365)	48% (329)
APC2^{g41}	4.2 (317)	48% (585)	92 kDa truncation predicted 80 kD	cytoplasmic	3.8 (205)	4.4 (204)	nd
APC2^{f90}	4.4 (263)	51% (449)	41 kDa truncation predicted 45kD	cytoplasmic	3.7 (204)	4.6 (178)	42% (432)
APC2^{g10}	4.5 (409)	49% (418)	No protein predicted 43 kD	cytoplasmic	3.8 (238) M/Z 4.7 (96)	4.0 (285)	48% (409)

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 denticles remaining. 4 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.

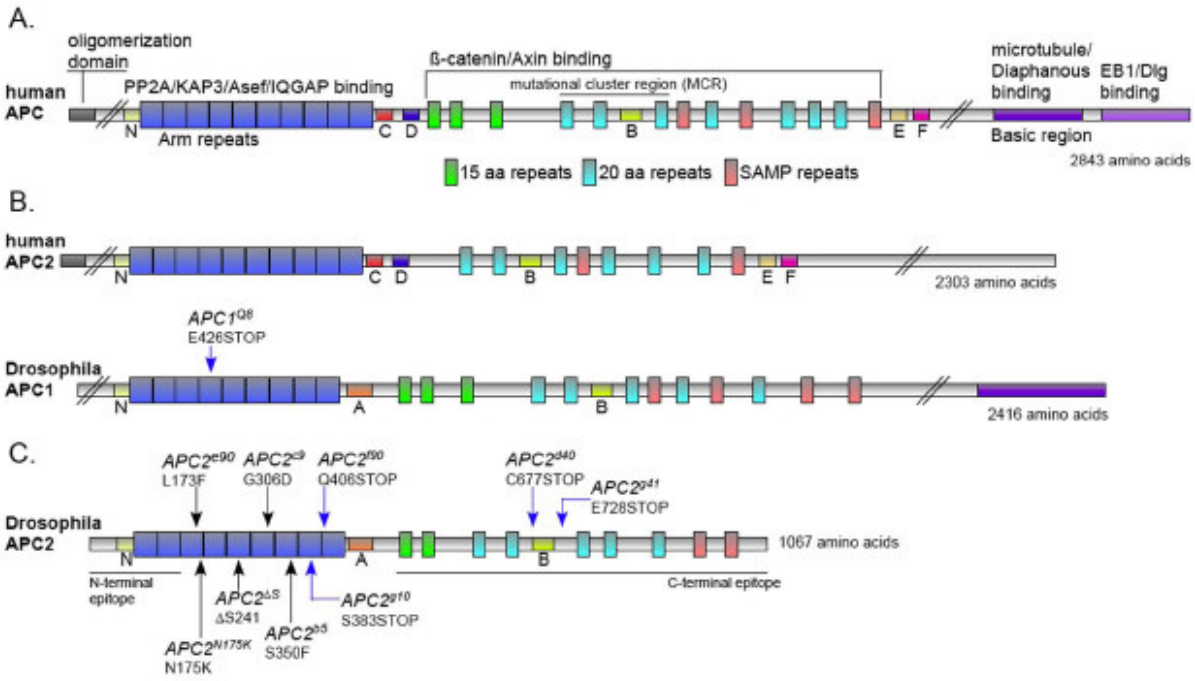


Fig. 3.1 Human and fly APC1 and APC2.

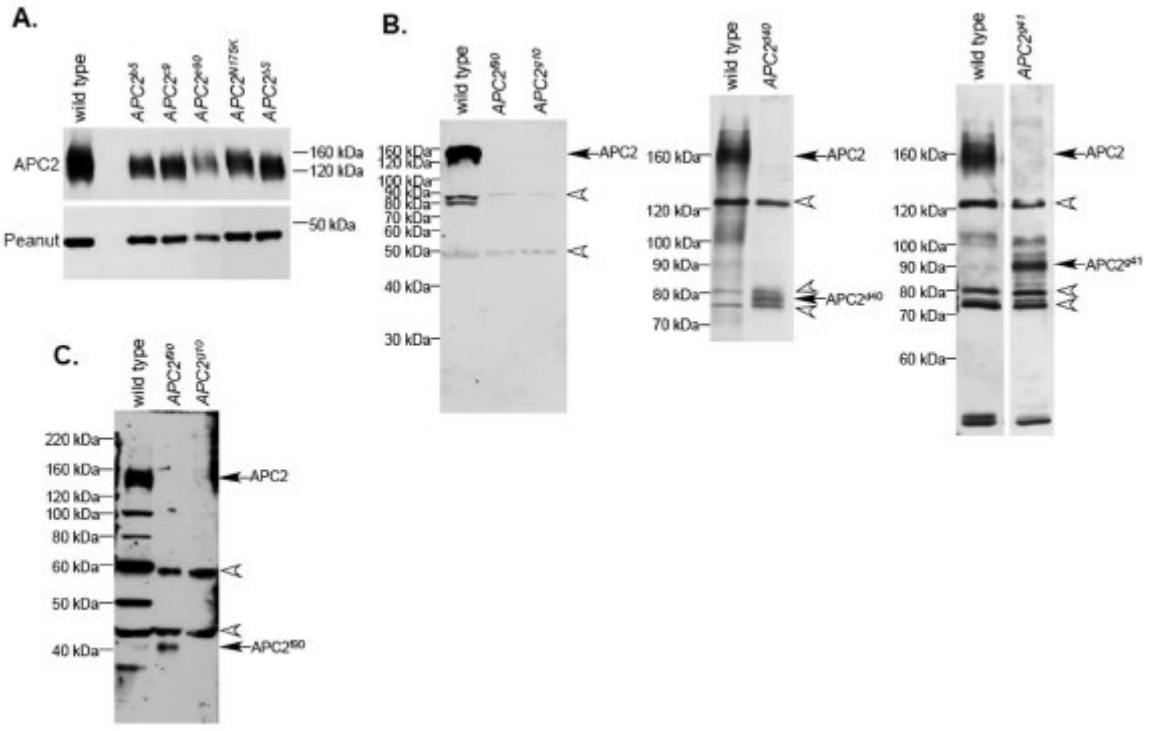


Fig. 3.2 APC2 mutant proteins.

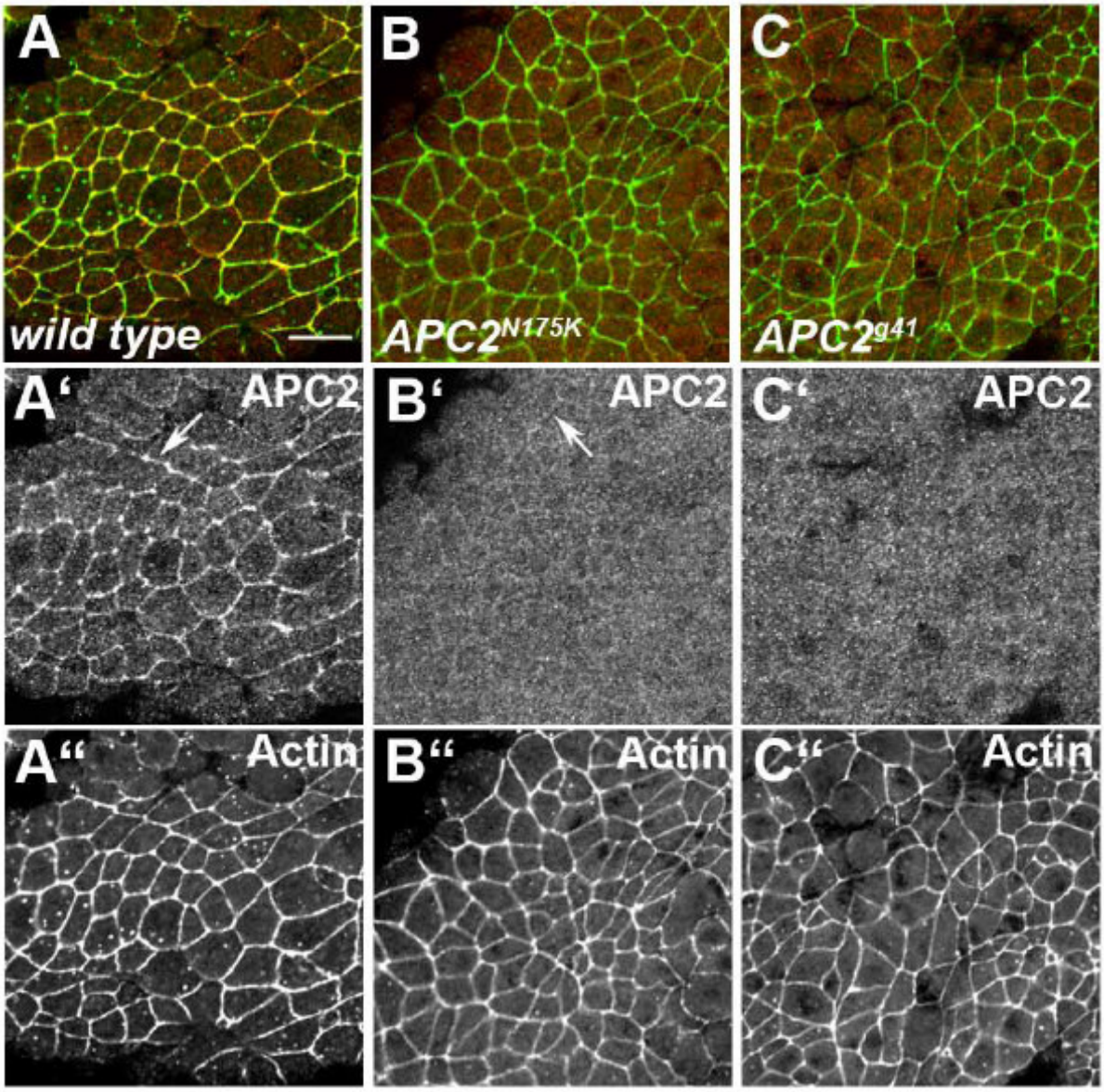
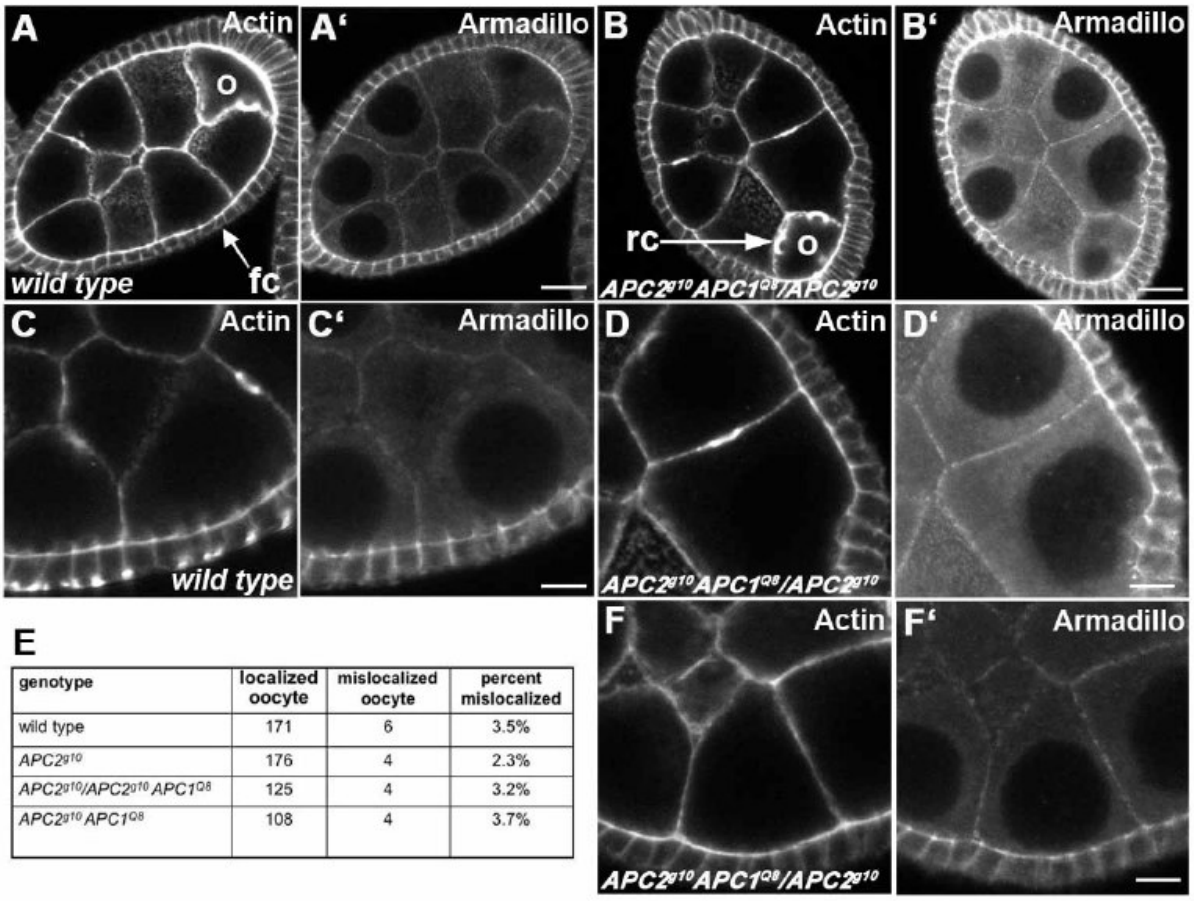


Fig. 3.3 APC2 mutant proteins lose cortical association.



genotype	localized oocyte	mislocalized oocyte	percent mislocalized
wild type	171	6	3.5%
APC2g10	176	4	2.3%
APC2g10/APC2g10 APC1Q8	125	4	3.2%
APC2g10 APC1Q8	108	4	3.7%

Fig. 3.4 APC proteins are not essential for cell adhesion in ovaries.

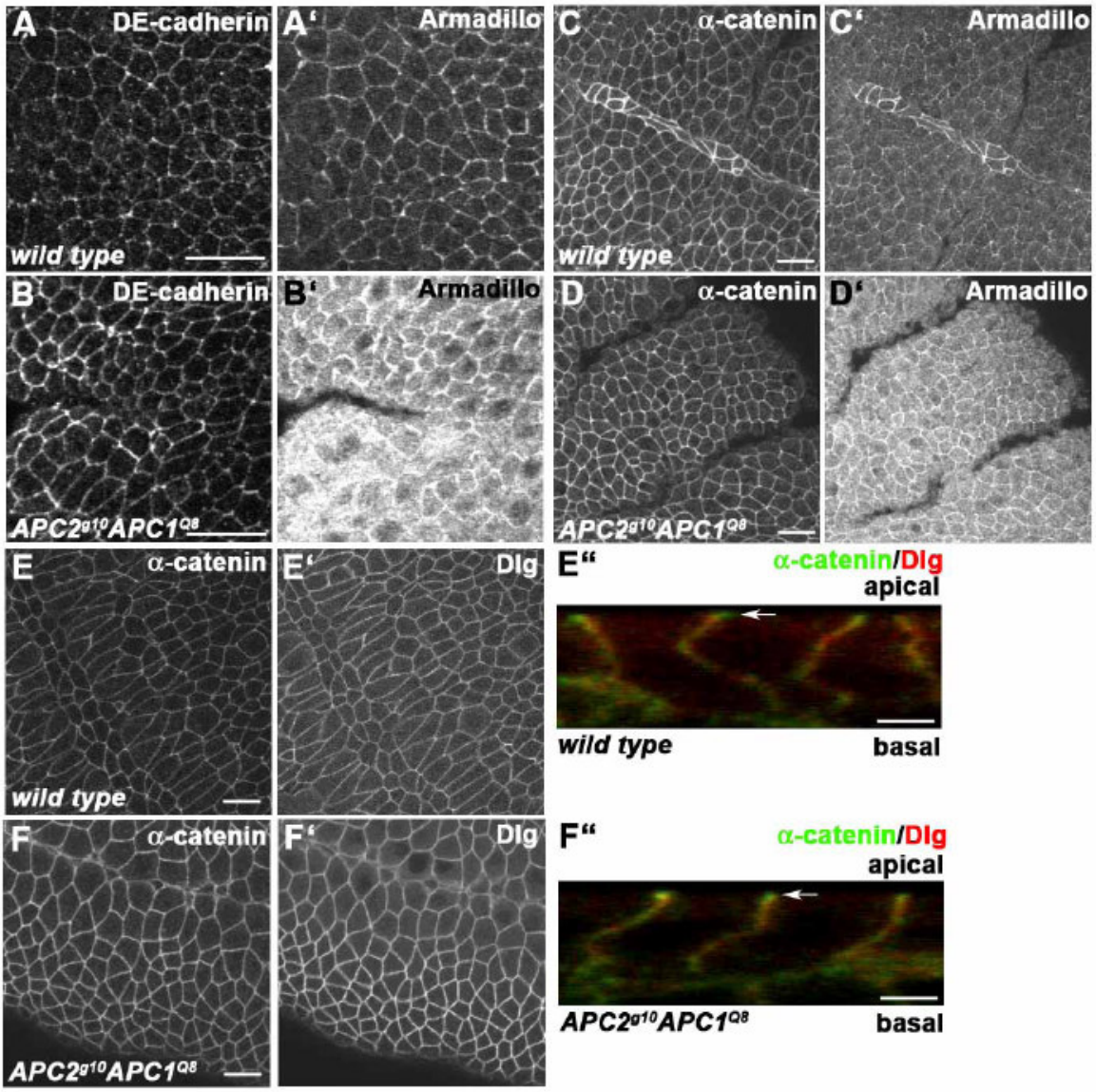
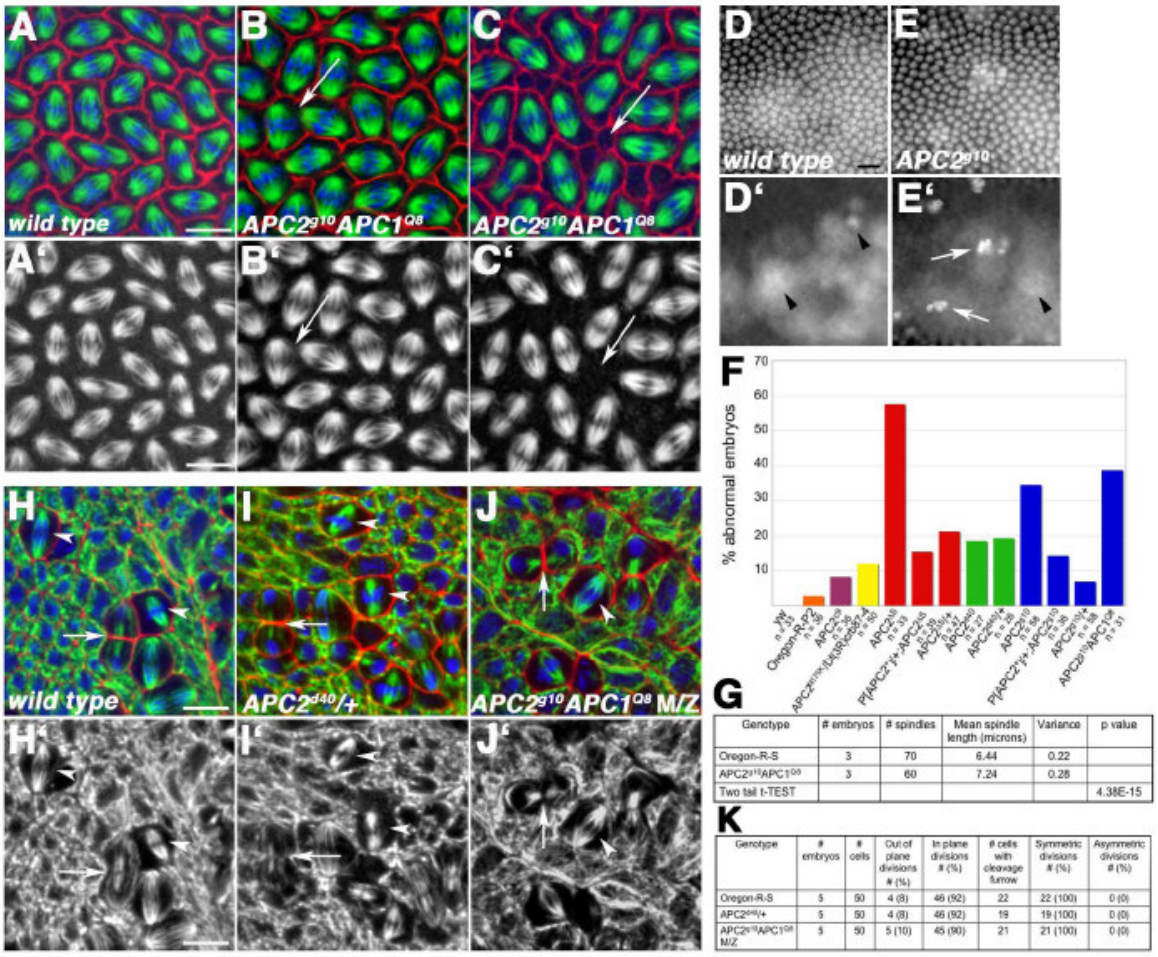


Fig. 3.5 APC proteins are not essential for embryonic cell adhesion.



Genotype	# embryos	# spindles	Mean spindle length (microns)	Variance	p value
Oregon-R-S	3	70	6.44	0.22	
APC2g10APC1Q8	3	60	7.24	0.28	
Two tail t-TEST					4.38E-15

Genotype	# embryos	# cells	Out of plane divisions # (%)	In plane divisions # (%)	# cells with cleavage furrow	Symmetric divisions # (%)	Asymmetric divisions # (%)
Oregon-R-S	5	50	4 (8)	46 (92)	22	22 (100)	0 (0)
APC2d40/+	5	50	4 (8)	46 (92)	19	19 (100)	0 (0)
APC2g10APC1Q8 M/Z	5	50	5 (10)	45 (90)	21	21 (100)	0 (0)

Fig. 3.6 Nuclear loss, spindle morphology and spindle orientation.

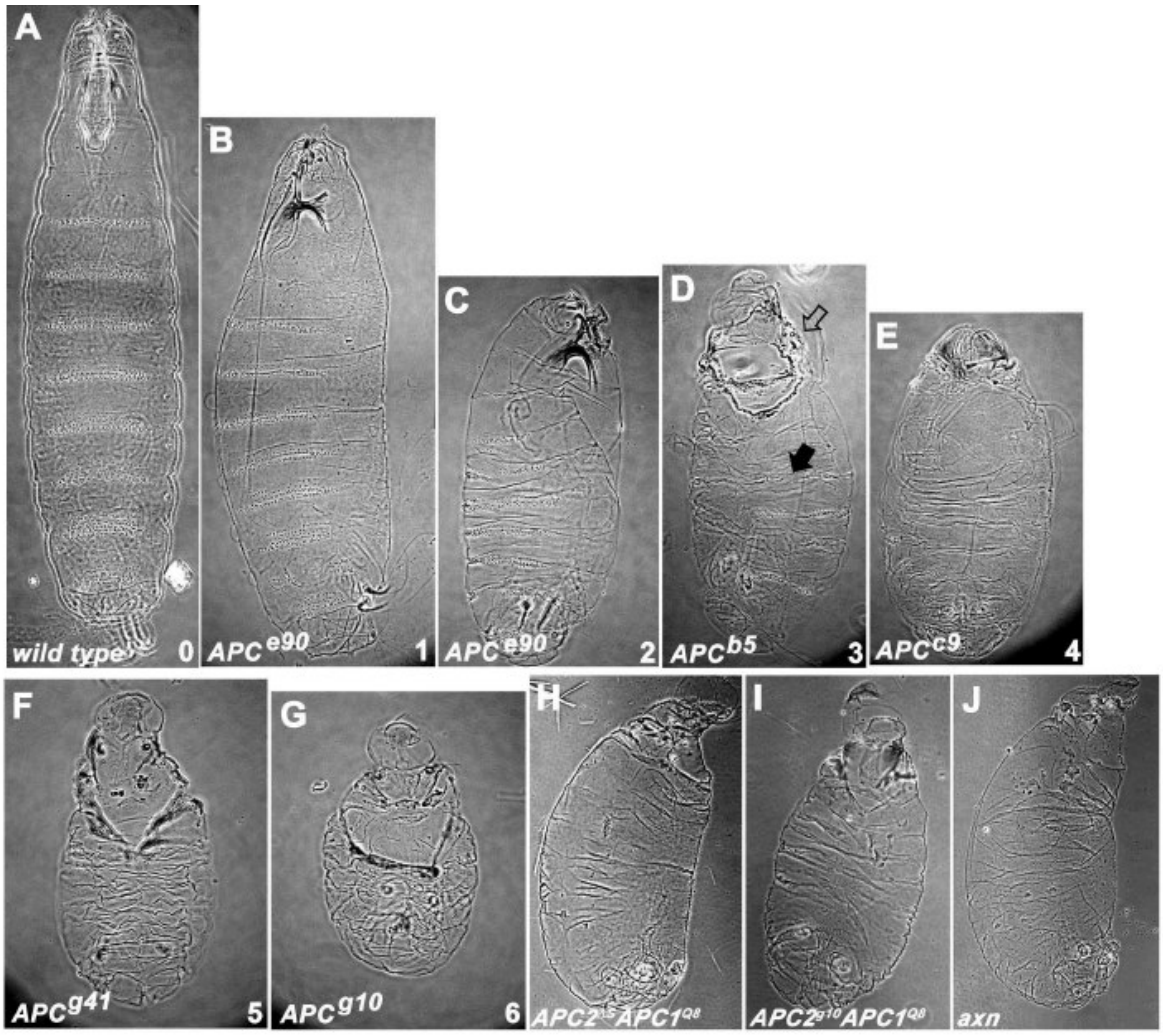


Fig. 3.7 *APC2* mutant alleles vary widely in their effect on Wg signaling.

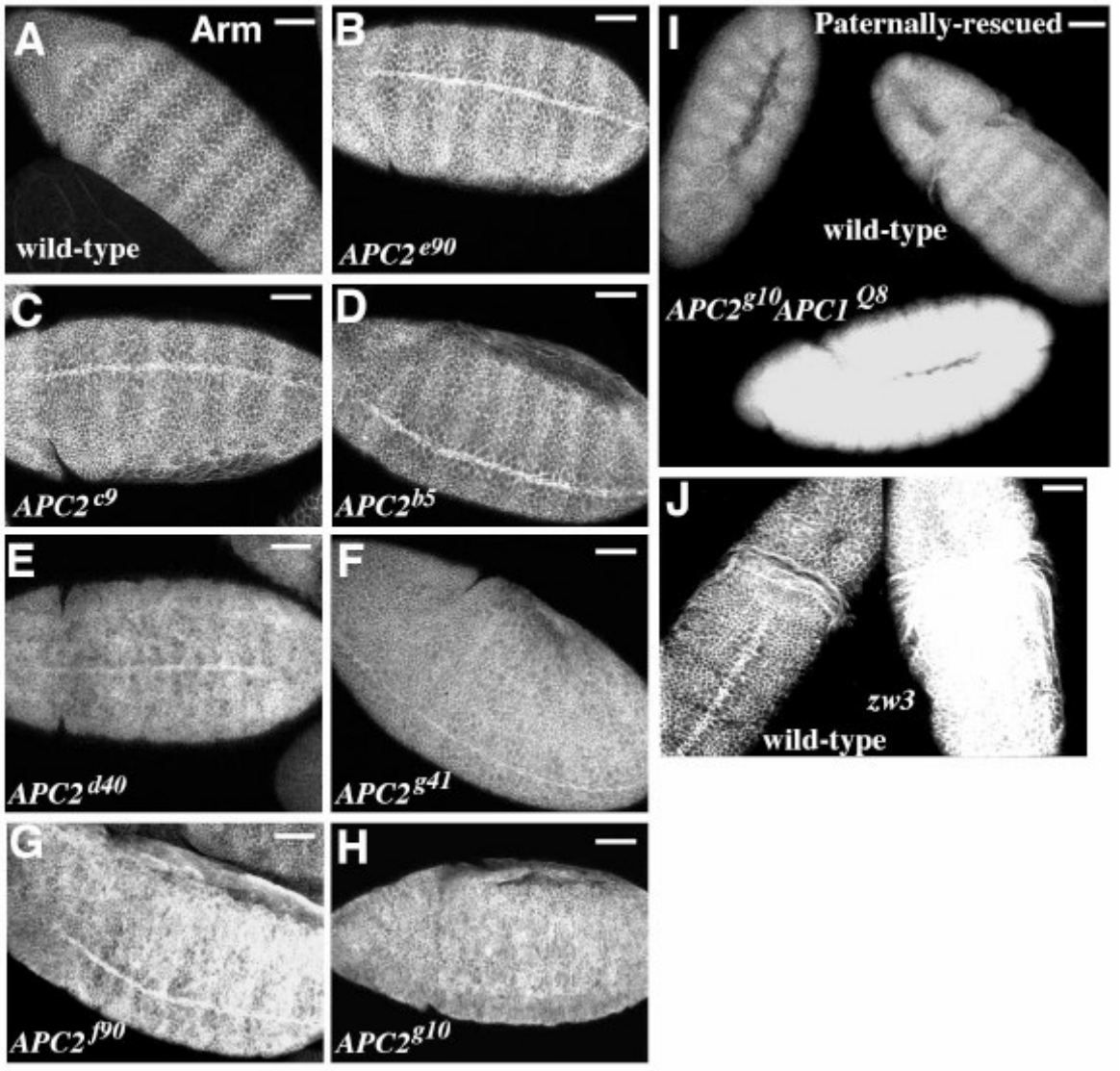


Fig. 3.8 Effects on Arm stability match effects on cuticle phenotype.

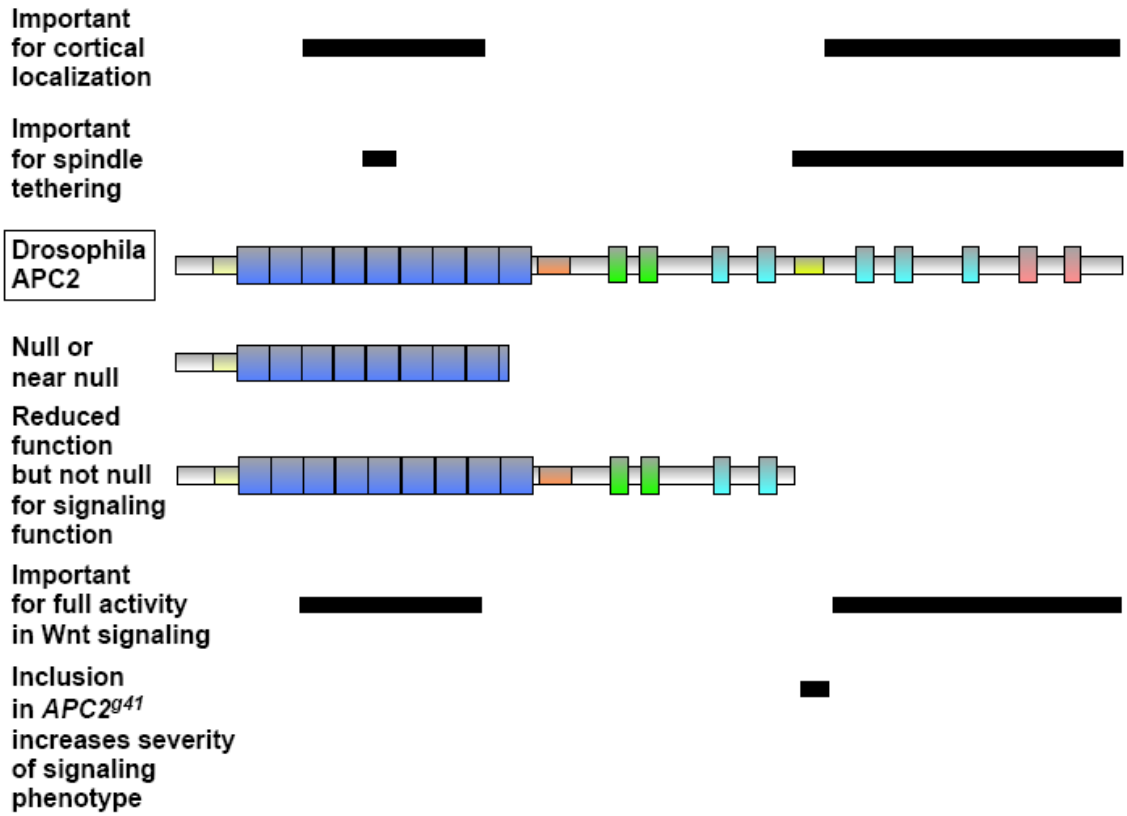
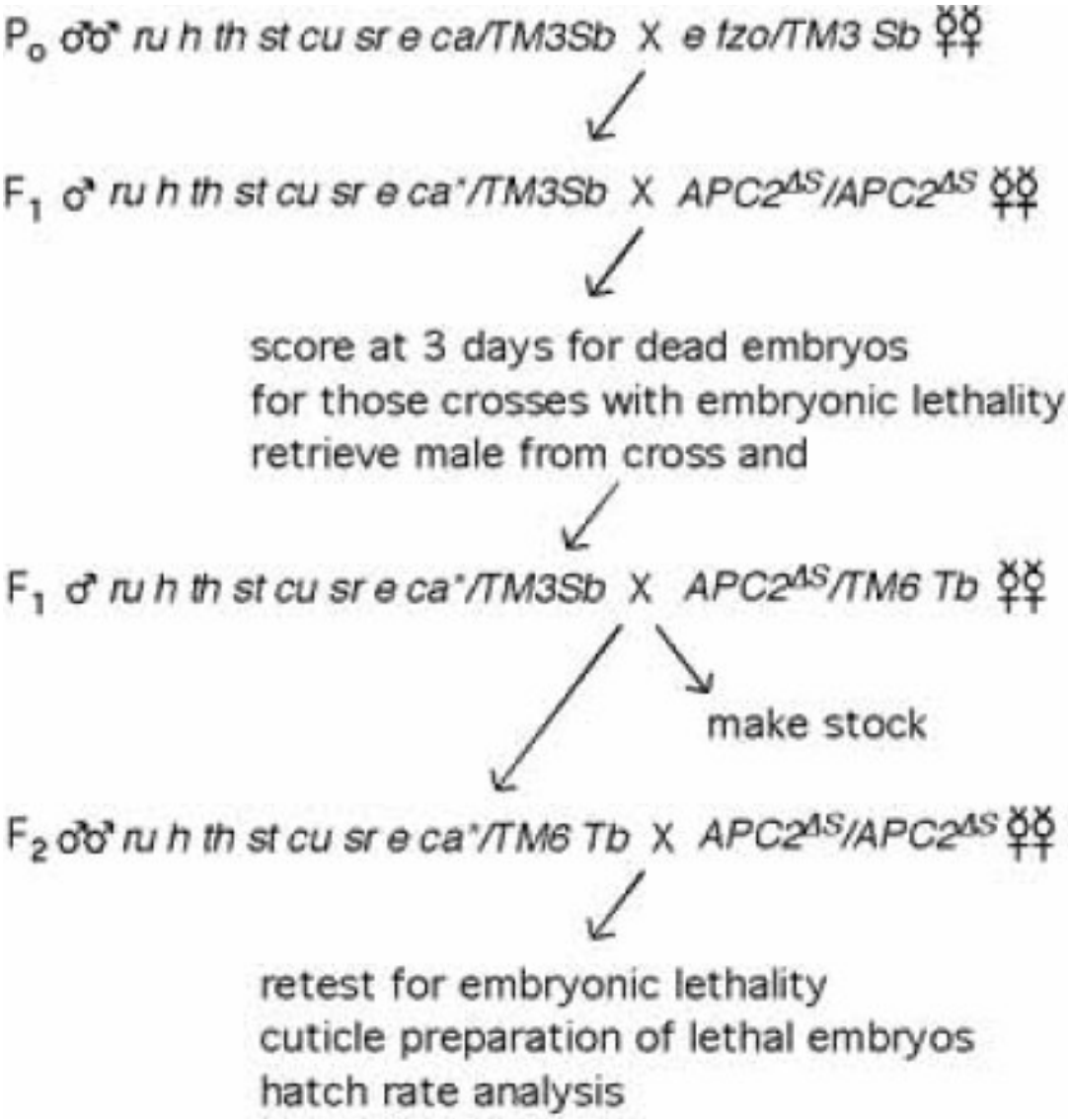
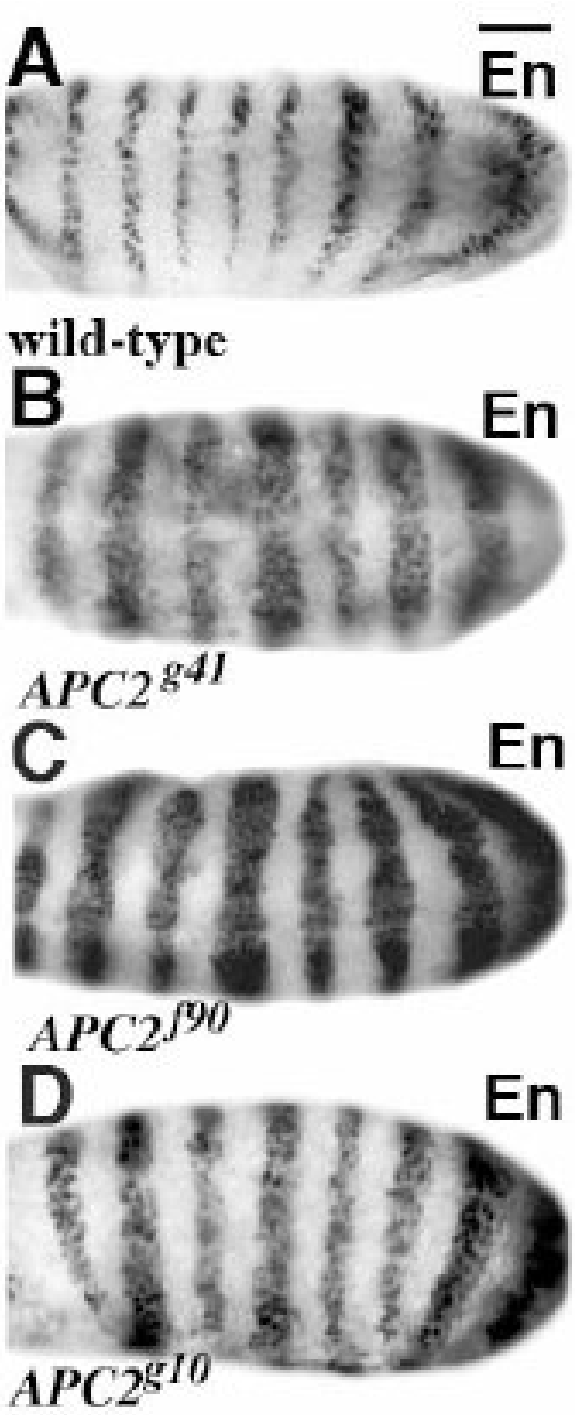


Fig. 3.9 Structure/function of APC2.



Suppl. Fig. 3.1 Generation of new alleles of *APC2*.



Suppl. Fig. 3.2 En expression in wild-type (A) and the indicated *APC2* maternal and zygotic mutants (B-D).

PREFACE TO CHAPTER IV

In the following chapter I provide the first analysis of APC function in the developing brain and describe a novel role *APC* family proteins play there. The presence of two partially redundant family members in humans and mice has thus far prevented such an analysis. We utilized tools in *Drosophila* allowing us to simultaneously eliminate the function of both *APCs* to address questions that have been impossible to address in other systems. The elimination of both APCs in flies leads to the death of the animal during the second instar, presumably due to a defect in neuroblast proliferation. Using the MARCM technique allowed us to circumvent this problem by generating patches (clones) of cells that were mutant for both APC proteins in an otherwise normal (heterozygous) animal. This also more closely approximates what is happening in the gut epithelium during the initial stages of colon cancer, when a few previously heterozygous cells lose APC function due to mutational inactivation.

The developing *Drosophila* larval brain is a complex organ with many cell types, including epithelial like stem cells. The developing optic lobe is particularly interesting because unlike the embryonic brain or the larval central brain, which develop from isolated neural stem/progenitor cells called neuroblasts, the optic lobe develops from an epithelial sheet of neuroblasts, and therefore more closely resembles the development of the mammalian cortex. To define the roles of APC family members here we first needed to

describe the development of the optic lobe using modern molecular and cell biological tools, complementing classic histological work done in flies 15-20 years ago.

Next, we examined the function of *APC* family members during larval brain development. Akong et. al. (2002) showed that both *APC* family members could function redundantly during the development of the larval brain. Animals that were zygotically mutant for both *APC* family members also had defects in neuroblast proliferation that presumably lead to their death during the second larval instar. Using the MARCM technique to generate clones of cells that were double mutant for both *APCs* (in an otherwise normal brain) allowed the first *in vivo* examination of *APCs* roles there.

Kathryn Akong generated FRT fly lines for MARCM analysis using different alleles of *APC2* combined with a null allele of *APC1*, $APC1^{Q8}$. She carried out the initial characterization of the mutant phenotypes of several *APC* double mutant lines, focusing in particular on the truncation allele $APC2^{d40}$. I focused my work on the *APC* double null mutants, $APC2^{g10}$ $APC1^{Q8}$. Loss of APC function has dramatic consequences, affecting the adhesion of epithelial neuroblasts and axon outgrowth. One potential molecular mechanism that could explain the observed phenotype was altered cell-cell adhesion. To address this question, both Kathryn and I attempted to mimic the mutant phenotypes seen in *APC* double mutants using E-cadherin null mutants (shg^{R69}) and E-cadherin overexpression MARCM clones. I also examined DE-cadherin and DN-cadherin accumulation in APC double mutant cells. Another molecular mechanism that could give rise to the observed phenotypes was activation of Wg signaling. I addressed that possibility by mimicking activated Wg signaling in clones, and by blocking Wg signaling in the *APC* double null mutant clones using a *UAS*-

inducible dominant-negative construct for the Wg responsive transcription factor *TCF*. I further examined whether there was activation of known downstream Wnt/Wg target genes, such as Dpp, in the *APC* double null mutant clones. Since Wnt signaling target gene activation has been shown to influence proliferation, I also addressed this in *APC* double null mutant clones. This work has been submitted and accepted for publication in Developmental Biology.

Novel roles for APC family members and Wingless/Wnt signaling during *Drosophila* brain development

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ABSTRACT

Construction of the brain is one of the most complex developmental challenges. Wnt signals shape all tissues, including the brain, and the tumor suppressor Adenomatous Polyposis Coli (APC) is a key negative regulator of Wnt/Wingless (Wg) signaling. We carried out the first assessment of the role of APC proteins in brain development, simultaneously inactivating both *APC1* and *APC2* in clones of cells in the Drosophila larval optic lobe. We focused on the medulla, where epithelial neural progenitors shift from symmetric to asymmetric divisions across the lateral-medial axis. Loss of both APCs triggers dramatic defects in optic lobe development. Double mutant cells segregate from wild-type neighbors, while double mutant neurons form tangled axonal knots, suggesting changes in cell adhesion. Strikingly, phenotypes are graded along the anterior-posterior axis. Activation of Wg signaling downstream of APC mimics these phenotypes, a dominant-negative TCF blocks them, and a known Wg target, *decapentaplegic,* is activated in double mutant clones, strongly suggesting that the phenotypes result from activated Wg signaling. We also explored the roles of classic cadherins in differential adhesion. Finally, we propose a model suggesting that Wg signaling regulates fine scale cell fates along the anterior-posterior axis, in part by creating an adhesion gradient, and consider possible alternate explanations for our observations.

Key Words:
Mutation cluster region, familial adenomatous polyposis, adherens junctions, beta-catenin

INTRODUCTION

The developmental processes required to make an animal are often the same processes that go awry in cancer. The Wnt signaling pathway provides an excellent example. Wnt signaling is required for many essential events of embryonic development, including axis specification, neural patterning, and brain development (reviewed in Logan and Nusse, 2004). However, Wnt signaling is also inappropriately activated in several tumor types, most notably colorectal cancer (reviewed in Nathke, 2004).

Powerful developmental regulatory pathways like the Wnt pathway are kept off by stringent negative regulation. The tumor suppressor Adenomatous polyposis coli (APC), mutated in both familial adenomatous polyposis (FAP), a heritable form of colon cancer, and in many sporadic colorectal tumors, is a key part of the negative regulatory machinery for Wnt signaling. This machinery destabilizes ß-catenin (ßcat; *Drosophila* Armadillo; Arm; reviewed in Nelson and Nusse, 2004), the key Wnt effector. In the absence of Wnt signals, ßcat is held in a complex with APC, a scaffolding protein Axin, and the kinases GSK-3ß and casein kinase I. While in this destruction complex, ßcat is phosphorylated by Casein kinase I and GSK-3ß and thus targeted for ubiquitination by an E3-ubiquitin ligase and proteasomal destruction. However, Wnt signals inactivate the destruction complex, allowing ßcat to accumulate in the cytoplasm and enter the nucleus. There it interacts with TCF/LEF family proteins to form bipartite transcription factors and activate downstream targets. In colon tumors, inactivating mutations of APC cause aberrant accumulation of cytoplasmic and nuclear ßcat, leading to the improper activation of downstream target genes, some of which regulate the cell cycle and promote proliferation. This blocks differentiation of colon cells that normally migrate from the crypts to the villi, and effectively locks them into a self-

renewing stem cell-like fate (reviewed in Reya and Clevers, 2005).

We are interested in the normal roles of Wnt signaling during development, and how it is regulated. Wnt signaling plays key roles in virtually every tissue (reviewed in Logan and Nusse, 2004), including brain development (reviewed in Ciani and Salinas, 2005). Making an adult brain requires exquisitely precise temporal and spatial controls (reviewed in Tissir and Goffinet, 2003). Stem/progenitor cells, like those in the ventricular zone in the mammalian cortex, must proliferate extensively to give rise to large numbers of neurons. Next, neuronal daughters exit the cell cycle and migrate to appropriate locations; e.g., neurons must move out of the ventricular and subventricular zones along radial glial cell fibers and halt migration in the proper layer of the cortex. Finally, neurons differentiate into specific subtypes and extend axons and neurites, establishing the network of connectivity required for integrated control of the brain and the entire animal. Numerous signal transduction pathways act combinatorially to specify the diverse set of cell types found in the brain.

The developing mammalian brain has a complex pattern of expression of Wnt ligands that help specify cell fate and proliferation (Ciani and Salinas, 2005). For example, mouse *Wnt1* regulates fates in the midbrain, cerebellum, and spinal cord (McMahon and Bradley, 1990) while *Wnt3a* helps specify the hippocampus (Lee et al., 2000). When Wnt1 and Wnt3a are ectopically expressed, overgrowth results, without altering cell fates along the dorsoventral axis of the neural tube, consistent with a role in progenitor cell proliferation (Megason and McMahon, 2002). Expression of constitutively-active ßcat in neuronal progenitor cells leads to an enlarged cerebral cortex with more neuroepithelial progenitors. Despite this, progenitors differentiated, giving rise to neurons in relatively normal spatial

patterns (Chenn and Walsh, 2002).

As key regulators of Wnt signaling, it is not surprising that both mammalian APC family members are expressed in the brain. APC is broadly expressed with high levels in the brain during embryogenesis and adulthood (Bhat et al., 1994; Groden et al., 1991). Mice homozygous mutant for APC die with patterning defects during early gastrulation (Chazaud and Rossant, 2006; Moser et al., 1995). Mammalian APC2 is also highly expressed in the CNS (Yamanaka et al., 2002), but *APC2* mutant mice have not been reported. Further, no one has explored the potential normal roles of mammalian APCs in brain development.

In addition to regulating normal brain development, activated Wnt signaling is also associated with a subset of primitive neuroectodermal tumors such as medulloblastoma, which are thought to be derived from multipotent, cerebellar progenitor cells (Fogarty et al., 2005). There is an increased frequency of medulloblastoma in FAP patients, and mutations in APC, ßcat, and Axin1 also occur in sporadic medulloblastoma, with a cumulative frequency of ~15% (Fogarty et al., 2005). Understanding the normal role of Wnt signaling in the brain may help us better understand these tumors.

Drosophila is excellent model system to examine the roles of Wnt signaling in brain development. During embryogenesis, the fly Wnt Wingless (Wg) patterns neuroblasts along the anterior-posterior (A/P) axis (reviewed in Logan and Nusse, 2004). During larval development it helps direct the dramatic remodeling of the brain necessary to meet the new sensory and motor needs of the adult (Kaphingst and Kunes, 1994). For example, the larval optic lobe produces the adult optic processing center. It invaginates from the procephalic ectoderm during embryogenesis (Green et al., 1993). The 30-40 proliferative progenitor cells of the 1st instar optic lobe form an epithelial sphere (reviewed in (Meinertzhagen and

Hanson, 1993). By the end of the 2nd instar they develop into two distinct populations: the outer proliferative center (OPC; ~700 neuroblasts) and the inner proliferative center (IPC; ~400 neuroblasts), and production of postmitotic neurons begins. A medial proliferation zone at the edge of the OPC gives rise to ~40,000 cells that make up the medulla, while a second proliferative zone, separated from the medulla by the laminar furrow, gives rise to the lamina (Hofbauer and Campos-Ortega, 1990). The OPC provides an excellent place to identify molecular mechanisms governing the behavior of progenitor cells and their progeny, coordinating proliferation, migration, and cell-cell interactions.

The secreted signals Hedgehog, Wg, and Decapentaplegic (Dpp) help confer identity to cells in the larval brain, as they do in other tissues. Hedgehog made by photoreceptor neurons regulates proliferation and development of laminar cells (Huang and Kunes, 1998). Wg is expressed by cells at the posterior dorsal and ventral tips of the horseshoe-shaped OPC, and helps organize the A/P axis, promoting proliferation and mediating nested zones of gene expression (Kaphingst and Kunes, 1994; Song et al., 2000; The upward bending of the anterior CNS makes the definition of anterior-posterior versus dorsal-ventral complex, and Kunes and colleagues refer to this axis as the dorsal/ventral axis. Both descriptions are reasonable--below we use the conventions of Meinertzhagen and Hanson (1993), referring to this as the anterior-posterior axis). Wg promotes the expression of *dpp* in cells adjacent to those expressing Wg. *dpp* mutants also disrupt the nested zones of gene expression, decrease proliferation, and reduce the medullar neuropil from its posterior ends. Thus Wg and Dpp help set up fates and regulate proliferation along the A/P axis (Kaphingst and Kunes, 1994).

Drosophila has two APC family members that play largely redundant roles in Wg signaling and also have Wnt-independent roles (Ahmed et al., 1998; Ahmed et al., 2002;

Akong et al., 2002a; McCartney et al., 1999; McCartney et al., 2001; McCartney et al., 2006). Both APC1 and APC2 are broadly expressed in the developing larval brain (Akong et al., 2002b), with APC1 accumulation highest in axons, and APC2 accumulating in axons and neural progenitor cells. The two proteins have an early, redundant role in regulating re-entry of neuroblasts into the cell cycle at the beginning of larval development.

Many progenitor/stem cell populations utilize Wnt signaling. Depending upon intrinsic characteristics of the stem cell population, stem cells interpret Wnt signals in different ways. They can drive proliferation and promote self-renewal, or can promote differentiation along a particular lineage. In the colon, Wnt signaling maintains cells in a stem cell fate (Reya and Clevers, 2005). Embryonic stem cells also rely on Wnt signaling to maintain their pluripotent, self-renewing state in vitro (Sato et al., 2004). During hematopoiesis, Wnt signaling promotes multi-lineage differentiation potential in lymphoid and myeloid progenitors (Baba et al., 2005). In the skin, Wnt signaling's role is more complex (reviewed in Alonso and Fuchs, 2003). It has been proposed that different levels of Wnt activity direct different outcomes, i.e. maintaining stem cell populations in the hair follicle versus regulating hair follicle differentiation (Blanpain et al., 2004).

Here we explored the role of APC family members and Wnt signaling in the larval brain, addressing APC function in the neural progenitor cells (neuroblasts) within the optic lobe.

MATERIALS AND METHODS

Fly strains/genetics

Alleles and stocks are described at Flybase [www.flybase.org]. All experiments were performed at 25°C. Clones were generated by MARCM (Lee and Luo, 2001), using hsFlp

and a 3 hour heat shock at 37°C at ~48 AEL. Briefly, clones double mutant for *APC2* and *APC* were generated by crossing *Elav-Gal4 hsFlp mCD8:GFP/Y; ArmGal4/+; FRT 82B Gal80/+* males to *FRT82B APC2 APC^{Q8}/TM6 Tb* females. Non-Tb, female larvae with Gal80 (assessed by lack of global GFP expression) were dissected. Two possible genotypes were present: they could inherit both Elav-Gal4 and ArmGal-4, or only Elav-Gal4. For MARCM clones over-expressing Arm^{S10} *y w; UAS-Arm^{S10}/+; FRT 82B $^{w+}$ /+* females were crossed to *Elav-Gal4 hsFlp mCD8:GFP/Y; ArmGal4/+; FRT 82B Gal80/+* males, and female larvae with GAL80 were dissected. To generate MARCM clones over-expressing TCF-DN and mutant for *APC2^{g10} APC^{Q8}*, *Elav-Gal4 hsFlp mCD8:GFP/Y; UAS-TCF-DN/+; FRT 82B Gal80/+* males were crossed to *FRT82B APC2^{g10} APC^{Q8}/TM6 Tb* females. Non-Tb, female larvae with Gal80 were dissected. 50% of these females should carry *APC2^{g10} APC^{Q8}* mutant clones expressing UAS-TCF-DN, while the other 50% should have *APC2^{g10} APC^{Q8}* mutant clones that did not have UAS-TCF-DN. To generate MARCM clones with *dpp-LacZ* that were mutant for *APC2^{g10} APC^{Q8}*, *Elav-Gal4 hsFlp mCD8:GFP/Y; dpp-LacZ/+; FRT 82B Gal80/+* males were crossed to *FRT82B APC2^{g10} APC^{Q8}/TM6 Tb* females. To generate MARCM clones with *shg-LacZ* that were mutant for *APC2^{g10} APC^{Q8} Elav-Gal4 hsFlp mCD8:GFP/Y; shg-LacZ/+; FRT 82B Gal80/+* males were crossed to *FRT82B APC2^{g10} APC^{Q8}/ TM6 Tb* females. To generate MARCM clones mutant for *shg^{R69}*, *FRT42D shgR69/Cyo* females were crossed to *Elav-Gal4 hsFlp mCD8:GFP/Y; FRT 42D Gal80/ FRT 42D Gal80* males. To over-express DE-cadherin in MARCM clones *UAS-DE-cad/+; FRT82B w^{+} /+* females were crossed to *Elav-Gal4 hsFlp mCD8:GFP/Y; ArmGal4/+; FRT 82B Gal80/+* males.

Immunolocalization and Microscopy

Larval tissues were dissected in Schneider's Drosophila Medium (GIBCO)+10% fetal bovine serum. Brains were fixed 20 minutes in 3.7% formaldehyde in PBS and blocked ≥ 2h in PBS/1% normal goat serum/0.3% TritonX-100. Primary antibodies were: rat polyclonal anti-APC2 (1:1000; McCartney et al., 1999), rat monoclonals anti-DE cadherin DCAD2 (1:200) and anti-N-cadherin (1:500), mouse monoclonals anti-Arm N271A (1:200), anti-myc 9E10 (1:100), BP102 (1:5), anti-fasciclin III (1:500; all from DSHB), anti-Miranda (Matsuzaki, 1:2000), anti-phosphohistone III (Upstate Biotechnology, 1:500), anti-cyclin E (H. Richardson 1:1000), and rabbit anti-ßgalactosidase (Promega, 1:1000). Alexa-phalloidin (Molecular Probes) was used to image F-actin. Secondary antibodies were from Molecular Probes. Following PBS washes, brains were mounted on slides in AquaPolyMount (Polysciences, Inc.), and imaged using a 510 confocal microscope (Carl Zeiss MicroImaging, Inc.), a 40X objective (Plan-NeoFluor; NA 1.3) and LSM 510 AIM software. Adobe Photoshop7.0 was used to adjust input levels so the main range of signals spanned the entire output grayscale, and was used to adjust brightness and contrast.

RESULTS

Wild-type medullar development—epithelial neuroblasts, symmetric and asymmetric divisions and neuronal differentiation

We focused on the OPC, which forms the lamina and medulla. Before analyzing the role of APC proteins in the OPC, we needed to understand the anatomy and development of this structure. In the 3rd instar optic lobe, OPC neuroblasts form an epithelial sheet in the shape of a horseshoe, which wraps around the lateral part of each brain lobe, encircling the entering optic nerve (Fig. 1A; Meinertzhagen and Hanson, 1993; Nassif et al., 2003). This

sheet is continuous on the anterior side (Fig. 1A,B,D) but has a cleft on the posterior side (Fig. 1A,E). The epithelial sheet is divided into two regions by the laminar furrow, which runs circumferentially (Fig. 1D). The region lateral to the furrow forms the lamina, while the region medial to the furrow forms the medulla (Fig. 1D'). The IPC is also epithelial at this stage, but has a more complex shape. Its posterior horn reaches the brain surface on the posterior side of the brain near the more ventral arm of the OPC (Fig. 1A,E).

Previous work used histological approaches combined with BrdU and ^{3}H-thymidine labeling to establish proliferation patterns (Hofbauer and Campos-Ortega, 1990; Ito and Hotta, 1992; Truman and Bate, 1988; White and Kankel, 1978). We used genetic and cell biological approaches now available to extend this classic work. Together these data reveal that epithelial medullar neuroblasts are divided into two classes with striking differences in both cell biology and developmental potential; lateral neuroblasts are more epithelial in character and divide symmetrically to increase the neuroblast pool, and more medial neuroblasts that primarily divide asymmetrically to generate neurons. All cells accumulate cortical actin (Fig. 1D,F) and APC2 (Fig. 1G). However, lateral cells (adjacent to the laminar furrow) are highly epithelial in architecture, and express high levels of the adherens junction proteins DE-cadherin (DE-cad; Fig. 1F, blue bracket) and Arm (Fig. 1D,G, blue brackets). Previous ^{3}H-thymidine labeling suggested that these lateral neuroblasts near the laminar furrow undergo symmetric divisions in the plane of the epithelium, producing two neuroblast daughters. More medial neuroblasts have substantially reduced DE-cad and Arm levels (Fig. 1D,F,G, white brackets). Instead, they express proteins indicating neuronal character, like Elav-GAL4 driving mCD8-GFP (Fig. 1J.K, white brackets; Elav-GAL4 is expressed by medial neuroblasts while Elav protein itself appears to be restricted to the

neuronal daughters (data not shown)) or express proteins indicating that they are dividing asymmetrically, like the asymmetrically localized anchor Miranda (Mira; Fig. 1L,M, white brackets). This is also consistent with earlier ^{3}H-thymidine labeling, which suggested that a band of medial neuroblasts produce ganglion cell daughters by asymmetric divisions. Mira allows us to visualize these; division plane is not rigidly perpendicular to the epithelium, as Mira crescents are seen in many orientations (Fig. 1L" inset). This pattern of lateral epithelial character coupled with symmetric divisions, and medial "neuronal" character coupled with asymmetric divisions is seen in both the anterior (Fig. 1D.F.G,J,L) and posterior (Fig. 1E,K,M) medulla.

Deeper in the optic lobe, one finds the cell bodies of medullar neurons, progeny of the asymmetric divisions (Fig.1I1-I4 are successively deeper sections anterior to posterior). In the anterior medulla, these neurons send out axons in small fasciculated bundles (seen in longitudinal section in Fig. 1H, arrowhead) that project parallel to the overlying epithelium and target the large medullar neuropil (Fig.1H,arrow) that is just medial to the laminar furrow. Like the overlying OPC, this neuropil is horseshoe-shaped when viewed from the vantage point of the entering optic nerve (Fig. 1I1); the horns of the neuropil lie beneath the horns of the OPC on the posterior side of the brain. Due to this architecture, posterior medullar neurons send axons in fasciculated bundles that are roughly perpendicular to the overlying epithelium (seen in cross-section in Fig. 1I2,I3, red arrowheads), but also converge on the medullar neuropil (Fig. 1I2, green and yellow arrowheads). We also noted that in the dorsal anterior medulla, neuronal cell bodies accumulated elevated levels of Arm (Fig. 1N).

We also examined OPC development during earlier larval instars, using cell biological markers to supplement earlier work (Meinertzhagen and Hanson, 1993; Nassif et

al., 2003). 1st instar optic lobe precursors form epithelial spheres within each brain lobe (Fig. 2A), and begin proliferating in the late 1st instar (Hofbauer and Campos-Ortega, 1990). The optic lobe then subdivides in two, forming the IPC and OPC progenitors. These can be distinguished by expression of the adhesion molecule Fasciclin III (FasIII) specifically in the IPC (Fig. 2B1-B3 are sections from posterior to anterior). In 2nd instars, they begin to assume their distinctive morphologies: the OPC is horseshoe-shaped with a posterior cleft (Fig. 2B2), and the IPC has a posterior arm reaching the brain surface (Fig. 2B1). The morphology seen in the late 3rd instar becomes recognizable by the early 3rd instar (Fig. 2C). Two differences from the late 3rd instar are notable. First, the entire epithelial OPC expresses high levels of adherens junction proteins (Fig. 2C, blue bracket), similar to the most lateral cells of late 3rd instars (Fig. 1F). ^{3}H-thymidine labeling previously revealed that the first asymmetric divisions occur along the medial edge of the OPC at the end of the 2nd instar, and this zone then expands (Hofbauer and Campos-Ortega, 1990), ultimately producing the broad region expressing Mira that we observed in late 3rd instars (Fig. 1L). Second, the laminar furrow only forms during the mid 3rd instar, at which point a second zone of asymmetric divisions appears that will produce the lamina (Hofbauer and Campos-Ortega, 1990).

To complement earlier analyses of proliferation patterns in the brain by BrdU and ^{3}H-thymidine labeling (Hofbauer and Campos-Ortega, 1990; Ito and Hotta, 1992; Truman and Bate, 1988; White and Kankel, 1978), and to provide a base-line for our studies of *APC* mutants, we analyzed proliferation of wild-type medullar neuroblasts using MARCM (Lee and Luo, 2001). This allows us to mark single cells and their descendents with GFP (using the ubiquitously expressed Arm-GAL4 driver combined with Elav-GAL4). When clones of

marked wild-type cells arose on the anterior side of the medulla, most were roughly wedge-shaped, extending along the lateral to medial axis (Fig. 3A1-A2; numbered panels are successive sections through the same brain lobe, from the anterior surface moving deeper into the brain). We suspect that these arise from a marked neuroblast in the lateral, epithelial region, which divides symmetrically, with daughters displaced medially. Based on the earlier analysis described above, it is likely that some descendents of the initial marked cell initiate asymmetric divisions in the late 2nd instar; their daughters internal to the brain surface differentiate as medullar neurons. These are visualized by focusing deeper into the brain; GFP-marked progeny form a wedge-shaped clone of medullar neurons that send small bundles of fasciculated axons (Fig. 3A3, arrow) into the medullar neuropil (Fig. 3A3, arrowhead). At the brain surface, clones had irregular shapes, suggesting that clonal cells are relatively free to disperse among non-clonal cells. Occasional anterior clones arose in medial medullar neuroblasts (Fig. 3B1, arrow); their GFP-marked progeny also formed wedge-shaped clones of medullar neurons (Fig. 3B2, arrow). Lateral clones have similar morphologies (data not shown).

In the posterior horns of the horseshoe-shaped medulla, two types of clones with distinct morphologies arose. One resembled clones on the anterior side, and thus likely arose in lateral epithelial neuroblasts. These clones had very complex shapes (Fig. 3C1,D1,arrows), suggesting that marked and unmarked cells mix freely in this region. The other clone type extended in a tight line along the edge of one of the horns of the medulla (Fig. 3E2, arrowhead). Both clone types have medullar neuronal descendents, extending axons to the medullar neuropil (Fig. 3C2,arrowhead,E3,arrow). Taken together, our cell biological and clonal analyses confirm and extends classic observations: lateral medullar

neuroblasts in the laminar furrow are epithelial in character, express high levels of adherens junctions proteins, and primarily divide symmetrically, with their neuroblast progeny pushed medially. Neuroblasts in the more medial medulla downregulate epithelial markers, upregulate neuronal markers and divide asymmetrically to give rise to the medullar neurons.

APC proteins play important roles in medullar development

Animals zygotically singly mutant for either *APC2* or *APC1* are adult viable (Ahmed et al., 1998; McCartney et al., 1999) and have normal brains (Akong et al., 2002b). In contrast, zygotic double mutants die at the beginning of the 2nd instar, with defects in neuroblast proliferation (Akong et al., 2002b), precluding examination of APC roles in later optic lobe development. We thus used MARCM to create GFP-marked clones of cells double mutant for both *APC2* and *APC1* throughout larvae, including in the larval brain. We induced clones in early 2nd instars, and initially analyzed them in late 3rd instars. We carefully analyzed animals double mutant for null alleles of *APC2* and *APC1* ($APC2^{g10}$ and $APC1^{Q8}$; Suppl. Fig. 1A, Ahmed et al., 1998; McCartney et al., 2006), or double mutant for the $APC1^{Q8}$ and $APC2^{d40}$, encoding a truncated protein similar to those in human colon tumors (Suppl. Fig. 1A; McCartney et al., 2001).

Loss of both APC2 and APC1 resulted in several striking defects in medullar development. The phenotypes of different double mutant combinations were similar in nature but differed significantly in their strength. Only certain regions of the medulla along the A/P axis are prone to these phenotypic effects—here we discuss phenotypes in those regions, and we examine the regional differences in sensitivity in more detail below. In regions prone to phenotypic effects, phenotypes are highly penetrant (e.g., 27/27 $APC2^{d40}$ $APC1^{Q8}$ double mutant clones in the region most susceptible to effects had phenotypes;

Suppl. Table 1).

In *APC2*^{d40} *APC1*^{Q8} double mutants two predominant phenotypes were seen. When *APC2*^{d40} *APC1*^{Q8} double mutant clones arose in lateral epithelial neuroblasts, double mutant cells exhibited a striking segregation from wild-type neighbors, consistent with differential adhesion between mutant and wild-type cells. Mutant cells formed epithelial loops emerging from the laminar furrow (Fig. 4A,B,E; contrast with wild-type clone in Fig. 3A) or in some cases epithelial balls entirely separated from the wild-type epithelium (Fig. 4G). We confirmed that clones had lost wild-type APC2 by their reduced staining with APC2 antibodies (Fig. 4B, inset).

When *APC2*^{d40} *APC1*^{Q8} double mutant clones arose in more medial neuroblasts, double mutant cells also behaved in a way consistent with differential adhesion: clones were usually round rather than irregular in shape (Fig. 4D1,F1), consistent with inhibition of mixing of wild-type and mutant cells. These neuroblasts could still give rise to neuronal progeny, producing clones of marked neurons that extended axons. However, rather than sending out axons in small fasciculated bundles to the medullar neuropil, mutant axons associate with one another, forming large axon knots in the clone center or at its periphery (Fig. 4D2,F2; compare to wild-type clone in Fig. 3B2). We confirmed that knots were made up of axons by using the axon marker BP102, which normally accumulates in medullar axons (Fig. 4H,I arrowheads) and the medullar neuropil (Fig. 4H,I arrows). BP102 specifically labels axon knots (Fig. 4J,J'). Like wild-type medullar axons, axon knots also accumulate actin (Fig. 4D2'), DE-cad (Fig. 4D2"), and tubulin (data not shown). Interestingly, double mutant cells may not have totally lost axon guidance information. In some clones, rather than forming axon knots, mutant cells sent hyperfasciculated axons to the medullar neuropil

(Fig. 4K, arrow). In addition, occasional axons escaped from knots and targeted the medullar neuropil appropriately (Fig. 4G,L arrowheads). Both knots and loops were highly penetrant, and roughly equal in frequency—occasional clones formed both a large loop and a knot (Suppl. Table 1).

APC2^{g10} *APC1*^{Q8}double null mutant clones had similar but more severe phenotypes. Many double null mutant clones extended from the laminar furrow across the medulla to the medial border (Fig. 5B1,C1), and produced both large epithelial loops (Fig. 5B1,C1) and axon knots (Fig. 5B2,C2 arrows). Loss of APC function in many tissues triggers the failure to destroy Arm. We observed elevated levels of cytoplasmic Arm in both *APC2*^{d40} *APC1*^{Q8} (Fig. 4A", arrow vs. arrowhead), and *APC2*^{g10} *APC1*^{Q8}double null mutant clones (Fig. 5C1', arrow vs. arrowhead) with levels of Arm similar in both genotypes. It is worth noting that Arm levels were only modestly increased over wild-type, unlike the dramatic increase in Arm levels in the epidermis of *APC2*^{d40} *APC1*^{Q8} double mutant embryos (Ahmed et al., 2002; Akong et al., 2002a).

We also analyzed several other *APC2* mutants in less detail—these have effects on Wg signaling ranging from strong to weak (McCartney et al, 2006). Their brain phenotypes were qualitatively similar to those of *APC2*^{d40} *APC1*^{Q8} and *APC2*^{g10} *APC1*^{Q8}, but varied in severity. We examined two additional truncated alleles: *APC2*^{g41} resembles truncations found in colon tumors while *APC2*^{f90} is a more severe truncation at the end of the Arm repeat domain (Suppl. Fig. 1A). Both *APC2*^{g41} *APC1*^{Q8} double mutants (Suppl. Fig. 1B-D) and *APC2*^{f90} *APC1*^{Q8} double mutants (Suppl. Fig. 1E) were roughly similar to *APC2*^{d40} *APC1*^{Q8}, with epithelial loops or axon knots. Both were less severe than the protein null *APC2*^{g10}. We analyzed two weaker alleles with point mutations in APC2's Arm repeats (Suppl. Fig. 1A);

APC2^{N175K} *APC1*^{Q8} and *APC2*$^{\Delta S}$ *APC1*^{Q8} were less severe than *APC2*^{d40} *APC1*^{Q8} double mutants. In both we occasionally saw clones in the center of the anterior medulla with no phenotype (e.g., Suppl. Fig. 1G)— this was never seen in *APC2*^{d40} *APC1*^{Q8} double mutants. *APC2*$^{\Delta S}$ *APC1*^{Q8} double mutant clones produced epithelial balls, loops, and axon knots (Suppl. Fig. 1F), while *APC2*^{N175K} *APC1*^{Q8} had only milder phenotypes like hyperfasciculated axons (Suppl. Fig. 1H). The phenotypic severity of these alleles largely parallel the strength of their effect on Wg signaling, as assessed in the embryonic epidermis (McCartney et al., 2006). This is consistent with the idea that the APC brain phenotype is due to effects on Wg signaling. We address this further below.

There is differential sensitivity to APC loss along the anterior-posterior axis

The striking phenotypes described above do not occur in all double mutant clones. Instead, the phenotypic consequences of loss of both APC family members exhibited a strikingly graded difference in severity along the A/P axis of the medulla. We mapped clones onto the medulla (Fig. 5A), defining the tip of the dorsal horn of the posterior medulla as 0°, the middle of the anterior side of the medulla as 180°, and the tip of the ventral horn of the medulla as 360°. Double null mutant clones on the anterior side of the medulla exhibited the phenotypes described above (e.g., Fig. 5B,C; 30/30 clones in which at least part of the clone was between 90-216° had loops or knots; compare to wild-type clones in Fig. 3A,B). Within the anterior medulla there was a gradient of phenotypic severity; clones located on the ventral (next to the ventral nerve cord; Fig. 5E) or dorsal sides (Fig. 5D) of the medulla often exhibited less severe phenotypes, including hyperfasciculated axons (Fig. 5D,E arrows) rather than axon knots. In contrast, clones of double mutant cells in the posterior horns of the medulla were much more normal (Fig. 5F-H; 18/18 clones entirely within the region of 0-72°

on the dorsal horn of the medulla were normal (compare to top wild-type clone in Fig. 5K), and 61/62 clones entirely within the region of 225-360° were normal (compare to wild-type clones in Fig. 5J,K)). Posterior double mutant cells did not form epithelial loops, and even very large clones (Fig. 5F) sent out normal axons that projected to the medullar neuropil in a fashion indistinguishable from wild-type. However, we did note one phenotype consistent with a more modest degree of differential adhesion between wild-type and posterior double mutant cells. Posterior clones of wild-type cells are usually quite irregular in shape (Fig. 5I-K), presumably due to cell mixing. In contrast, posterior double mutant clones were often rounded rather than irregular in shape (Fig. 5F-H,L). Interestingly, posterior double mutant clones did accumulate elevated Arm levels (Fig. 5L, arrow), though the difference may not be as striking as in the anterior medulla. $APC2^{d40}\,APC1^{Q8}$ double mutant clones showed a similar graded severity (Suppl. Table 1); while most posterior $APC2^{d40}\,APC1^{Q8}$ clones were normal in phenotype, occasional clones in this region (<5%) segregated, forming balls. $APC2^{d40}$ may have "dominant-negative" effects, as previously observed in $APC2^{d40}$ mutant embryos (McCartney et al., 2006)). Overall, this analysis reveals a strikingly graded response to loss of APC function, with anterior cells much more sensitive.

Wg is normally expressed in the posterior tips of the medulla (Kaphingst and Kunes, 1994), the regions we find least sensitive to APC loss. In contrast, the cells most sensitive to APC loss are in the center of the anterior side of the medulla, farthest from cells that express Wg. Wg helps pattern the A/P axis of the OPC (Kaphingst and Kunes, 1994). The differential sensitivity is consistent with the idea that inappropriate activation of Wg signaling underlies the effects of APC loss-of-function. Double mutant cells should activate Wg signaling regardless of their position. If double mutant cells are adjacent to the cells

expressing Wg, they should assume fates roughly in concert with their position and be relatively normal in phenotype. However, double mutant cells on the anterior side of the medulla, far from Wg-expressing cells, may assume fates inconsistent with their position and the fates of the wild-type neighbors, causing defects. We test this further below.

Loss of APC function does not lock cells in a stem cell fate

Wnt signaling and APC play diverse roles in stem/progenitor cells. Loss of APC in the human colon locks cells into a stem cell fate (van de Wetering et al., 2002). To assess whether APCs play similar roles in neural progenitor cells in the medulla, we examined whether loss of APC2 and APC1 affects the ability of progenitor cells to self-renew or produce differentiated daughters. In the wild-type medulla, there is a graded program of cell fates from lateral to medial. Lateral epithelial neuroblasts in the laminar furrow undergo symmetric divisions, increasing the neuroblast pool. In contrast, more medial neuroblasts express proteins like Mira and undergo asymmetric divisions, producing neuronal daughters as well as additional neuroblasts.

APC2 APC1 double mutant neuroblasts appear to undergo an essentially normal program of self-renewal, and produce seemingly normal numbers of differentiated daughters that send out axons. When $APC2^{d40}$ $APC1^{Q8}$ double mutant clones arise in the laminar furrow, the cells predominantly divide in the plane of the epithelium, like their wild-type neighbors; these cells form the epithelial loops (e.g., Fig. 4A). They retain epithelial architecture, like wild-type lateral neuroblasts, as indicated by polarized DE-cad (Fig. 4E, arrow vs. arrowhead) and actin (Fig. 4A', arrow vs. arrowhead) accumulation. They also can undergo asymmetric divisions, yielding neuronal daughters. We observed this in two ways. While most of our MARCM experiments included the ubiquitously-expressed Arm-GAL4

driver, allowing us to visualize all cells in the clone, we also generated clones expressing only Elav-GAL4, which in the medulla is only expressed in cells as they enter the region of asymmetric divisions (Fig. 1J', white bracket)—thus only cells with neuronal character activate GFP. When clones only expressed Elav-GAL4, $APC2^{d40}$ $APC1^{Q8}$ double mutant epithelial loops were surrounded by halos of cells adopting the neuronal fate (Fig. 4A, arrowhead). $APC2^{d40}$ $APC1^{Q8}$ double mutant clones in more medial positions sometimes had a central epithelial ball (Fig. 4F1, arrowhead; marked by elevated actin and DE-cad) surrounded by a similar halo of Elav-GAL4 expressing cells (Fig. 4F1 arrow). The larger loops in double null clones had a similar gradient of differentiation, with a central epithelial region (e.g., Fig. 5B1, M arrows), a surrounding region expressing moderate levels of DE-cad (thus resembling the region just medial to the laminar furrow; Fig. 5B1, arrowheads), and a surrounding halo of cells adopting neural fates, expressing both Elav-GAL4 and Mira (Fig 5M, arrowhead). Thus APC double mutant cells retain the normal lateral to medial gradient from epithelial to neuronal, but it is now is observed from the center of the loop to its periphery.

Loss of APC function increases clone growth rate

Wnt signaling and APC help regulate proliferation in many tissues of flies and mammals. Wnt signaling often promotes proliferation, but it can also inhibit it. We thus examined whether $APC2^{g10}$ $APC1^{Q8}$ double null mutant clones have differences in growth rate relative to wild-type. We measured clone volume of wild-type and double mutant clones induced in the same experiments, measuring clone area in each confocal section, and taking into account section thickness. We examined clones in the anterior third of the medulla, where phenotypes were always observed, and clones on the posterior side of the medulla,

where phenotypes were confined to subtle effects on cell sorting. Anterior *APC2*^{g10} *APC1*^{Q8}double mutant clones were significantly larger in volume than controls—mean volume was about twice that of wild-type (Fig. 6A; $p<0.002$). In contrast, posterior double mutant clones were not statistically different from wild-type (Fig. 6A; $p>0.8$).

Clone volume measures growth rate over most of the 2nd and 3rd instars, in cells with a cell cycle time of ~8-9 hours. Relatively small differences in growth rate might significantly increase clone volume. To look directly at proliferation, we used phospho-histoneH3 (P-His), a mitotic marker. In *APC2*^{g10} *APC1*^{Q8}double null mutant clones (Fig. 6B-E2), there were no striking differences in P-His staining relative to wild-type. Cyclin E (CycE) is a key regulator of proliferation, including in the developing brain (Bello et al., 2006; Betschinger et al., 2006). We thus examined CycE expression in wild-type and *APC2*^{g10} *APC1*^{Q8}double null mutant clones. In wild-type CycE accumulates at high levels in nuclei of both epithelial and medial medullar neuroblasts (Fig. 6F,G arrows), but is not expressed at high levels in medullar neurons (Fig. 6G, arrowhead). *APC2*^{g10} *APC1*^{Q8}double null mutant clones did not have increased CycE relative to wild-type (Fig. 6H-J, compare arrowheads). This is in contrast to what is seen in *brat* mutant clones, which have increased proliferation and consistent CycE accumulation in all cells of the clone (Bello et al., 2006; Betschinger et al., 2006). Once again, the morphological changes caused by loss of APC function displaced CycE-expressing cells to unusual places—for example, in clones making axon knots, peripheral, more epithelial cells expressed CycE even when displaced within the brain (Fig. 6I, right arrowhead), but neuronal cells in the clone center did not (Fig. 6J, right arrowhead). Thus loss of APC function leads to a statistically significant increase in growth rate in anterior clones, without a dramatic increase in mitotic index or CycE expression.

However, we cannot rule out small differences. Small changes in growth and proliferation could affect clone volume. For example, a 10% increase in growth rate in mutant clones per cell cycle would roughly double clone volume.

Activating Wg signaling is necessary and sufficient for the phenotypes seen

APC family proteins are multifunctional—they negatively regulate Wnt signaling but also have Wnt-independent cytoskeletal effects (Nathke, 2004). Each is a plausible cause of changes in cell adhesion and axon outgrowth. Based on the correlation between sensitivity to loss of APC and endogenous expression of Wg described above, we first tested the hypothesis that the phenotypes we observe result from changes in Wg signaling in double mutant cells. We ectopically activated Wg signaling downstream of APC, using Arm^{S10}, a form of Arm lacking GSK-3 phosphorylation sites, preventing its destruction (Pai et al., 1997). We expressed this in clonal patches using MARCM and examined the resulting clones (Arm^{S10} is myc-tagged, allowing us to directly examine Arm^{S10} expression levels). Arm^{S10} could mimic the effects of loss of APC; clones in anterior or lateral regions exhibited both epithelial segregation (Fig. 7A1) and axon knots (Fig.7A2, B2). Further, there was an A/P gradation of phenotypes, with loops and knots seen in anterior and lateral clones (Fig. 7A,B), and posterior clones having only the "rounded clone" phenotype (Fig. 7D), without defects in axons or more severe cell sorting. Levels of Arm^{S10} varied from clone to clone (as assessed by levels of myc-staining). Clones with moderate to high-level myc-expression (Fig. 7A,B,D) had phenotypes (including the posterior rounded-clone phenotype), while clones with low-level myc-expression did not (Fig. 7C, E). The frequency and average severity of Arm^{S10} phenotypes were less than those of *APC2 APC1* double null clones, but the phenotypes were qualitatively similar. Thus activation of Wg signaling is sufficient for at

least the basic phenotypic response.

To test whether transcriptional output of the canonical Wnt pathway is necessary for the effects of loss of APC function, we generated *APC2 APC1* double null mutant clones that simultaneously expressed a dominant-negative form of TCF (TCF-DN=TCFΔN). This form of TCF cannot bind Arm and thus acts as a constitutive repressor (van de Wetering et al., 1997). In these crosses only half of the larvae express TCF-DN, and thus at best we could expect suppression in half of the clones. We focused on clones in the anterior third of the medulla, as in the absence of TCF-DN, 100% of double mutant clones in this region (21/21 entirely within 108-252°) had a strong phenotype (an epithelial loop or axon knot, and sometimes both), and thus any suppression in this region should be apparent. We analyzed 18 clones in this region of the medulla, in larvae of which half should express TCF-DN. Four clones had an essentially normal morphology (Fig. 7G), not forming an epithelial loop or axon knot—this was never observed in double mutant clones without TCF-DN expression. Three additional clones had weaker phenotypes than the vast majority of the double mutant clones in this region that did not express TCF-DN, forming only a very small knot (Fig. 7F2) or hyperfasciculated axons. Eleven resulted in either a loop or knot. Suppression was not complete (i.e., it did not reach 50%), perhaps because of delay in build-up of the TCF-DN protein after clone induction. However, TCF-DN could block or ameliorate effects of APC loss, consistent with activation of Wg signaling playing an important role in the phenotype.

Loss of APC leads to activation of the Wg target gene *dpp*

To further test the hypothesis that phenotypes induced by loss of APC function result from activated Wg signaling, we examined whether a known Wg target gene is up-regulated in *APC2 APC1* double mutant clones. The best-characterized Wg target in 3rd instar brains

is the fly BMP homolog *dpp* (Kaphingst and Kunes, 1994; Song et al., 2000). We utilized a *dpp*-LacZ reporter to address whether *dpp* expression is altered in $APC2^{g10}\,APC1^{Q8}$double mutant clones. *dpp*-lacZ is normally expressed in the posterior ventral and dorsal horns of the OPC, in cells adjacent to the Wg-expressing cells (Kaphingst and Kunes, 1994; Song et al., 2000; Fig. 8A,B arrows) but is absent from medullar cells on the anterior side of the brain.

We observed strong activation of *dpp*-LacZ in $APC2^{g10}\,APC1^{Q8}$double mutant clones, with an excellent correlation with phenotype. Double mutant cells in the anterior medulla (which have a mutant phenotype) show ectopic expression of *dpp*-LacZ (Fig. 8C1,C2). Levels of *dpp*-LacZ expression in the more epithelial cells (Fig. 8C1) were as high or higher than those in the normal *dpp*-LacZ expression domain (Fig. 8B), but *dpp*-LacZ expression was lower in cells taking on neuronal character (as indicated by expression of Elav-GAL4; Fig. C2,D, arrow vs. arrowhead). Lateral clones in regions where clones had morphological phenotypes also had ectopic *dpp*-LacZ expression (Fig. 8D). Small clones deep in the medulla, which did not have morphological phenotypes, often lacked *dpp*-lacZ expression (data not shown). Double mutant clones in the posterior medulla sometimes overlapped the normal *dpp*-LacZ expression domain. In clones with a rounded phenotype, we sometimes saw slightly augmented expression levels in the normal expression domain (Fig. 8B vs. E, arrows), and expression expanded to regions of the clone that might not normally express *dpp*-LacZ (Fig. 8B vs. E, arrowheads). We observed one additional effect. Clones at the very ends of the posterior horns of the medulla (overlapping the region that expresses Wg and does not express *dpp*-LacZ) did not express *dpp*-LacZ, and, more surprising, exhibited a very sharp boundary with wild-type *dpp*-LacZ -expressing cells (Fig. 8F, arrows). *dpp*-LacZ

was activated in 2nd and early 3rd instar larvae, as early as we saw morphological phenotypes in anterior (Fig. 8G, arrow) and lateral (Fig. 8H, arrow) clones. Since *dpp*-LacZ is also expressed in the IPC, we examined expression of the IPC marker fasciclin III in $APC2^{g10}$ $APC1^{Q8}$double mutant clones. It is not expressed in these clones (data not shown), suggesting that $APC2^{g10}$ $APC1^{Q8}$double mutant cells are not transformed to IPC identity. These data are consistent with transformation of medullar cells from anterior to posterior fates, and support the idea that loss of APC function activates Wg signaling.

Loss of APC does not dramatically alter levels of classic cadherins

Many phenotypes observed in $APC2^{g10}$ $APC1^{Q8}$double mutant clones—rounded clone shapes, epithelial loops and axon knots—are consistent with changes in adhesion between mutant cells and wild-type neighbors. One obvious set of candidates that could explain the apparent differential adhesion of mutant and wild-type cells are the classic cadherins, DE-cad and DN-cadherin (DN-cad). In the medulla, DN-cad is normally only expressed by medullar neurons (Suppl. Fig. 2A1,A2), and its expression levels are not consistently altered within $APC2^{g10}$ $APC1^{Q8}$double mutant clones (Suppl. Fig 2B,C; see Figure legend for details). Thus DN-cad is unlikely it is a major cause of the cell sorting seen.

We next examined DE-cad as a candidate cause of cell segregation. In the wild-type medulla, DE-cad is expressed in high levels in lateral epithelial neuroblasts, at low levels in medial neuroblasts, and also accumulates in medullar axons (Fig. 1). If DE-cad mediated cell sorting during wild-type development, one might expect to see graded expression along the A/P axis, as is seen along the dorsal-ventral axis of wing discs (Jaiswal et al., 2006). We saw no obvious difference in DE-cad expression along the A/P axis of the medulla (Fig. 1F,J,K), but subtle, continuous changes might not be apparent. We thus closely examined

DE-cad accumulation in $APC2^{g10}$ $APC1^{Q8}$double mutant clones from the late 2nd instar (Suppl. Fig. 3A,B), when morphological phenotypes just become apparent, through the early 3rd instar (Suppl. Fig. 3C,D), to the late 3rd instar, where phenotypes are most obvious (Suppl. Fig. 3E-H; see Fig. legend for details). In late 3rd instars, DE-cad levels differ more dramatically across the wild-type medulla. Like wild-type cells, $APC2^{g10}$ $APC1^{Q8}$double mutant cells downregulate DE-cad accumulation as they differentiate (compare Suppl. Fig. 3E,F, arrowheads to Suppl. Fig. 3G1,G2, arrowheads). When we compared wild-type and mutant cells of similar fate, however, there was no strong increase or decrease in DE-cad levels. Occasionally levels appeared slightly reduced (Suppl. Fig. 3C, arrowheads, F, arrowheads), but it was difficult to rule out that this resulted from altered morphology of double mutant cells. Thus, DE-cad protein levels are either unchanged, or at most slightly reduced. We also examined a transcriptional reporter for DE-cad, *cad-lacZ (=shg-lacZ)*, which can report subtle differences in transcription of the gene encoding DE-cad (e.g., Jaiswal et al., 2006). Double mutant cells had similar levels of cadherin-reporter expression on both the anterior (Suppl. Fig. 3I-L) and posterior (Suppl. Fig. 3M) sides of the medulla, and, like wild-type medullar cells, down-regulated the cadherin-reporter in the transition from epithelial (Suppl. Fig. 3I'-K', arrows) to neuronal (Suppl. Fig. 3K', arrowhead, L2; in these experiments, the Arm-GAL4 driver could not be used, so not all double mutant cells express GFP). Thus there are not dramatic changes in DE-cad transcription in response to loss of APC function.

Reduction in DE-cad levels can partially mimic the phenotype

These data rule out large-scale changes in classic cadherin expression, but do not rule out the idea that subtle changes more subtle changes in adhesion. We thus tested the

hypothesis that changes in adhesion could produce phenotypes like those of *APC2 APC1* double mutant clones. We first reduced cadherin-based adhesion by generating MARCM clones of cells mutant for a null allele of the gene encoding DE-cad (*shotgun; shg*^{R69}; Uemura et al., 1996). We recovered *shg* null clones in both the medulla and the central brain. However, within the medulla, we did not recover any clones in more epithelial regions near the laminar furrow (0/23 clones were in this region), suggesting that loss of DE-cad where it is expressed at high levels may compromise clone viability. However, we did recover clones in more medial regions of the medulla. Clones were small, and were restricted to very medial positions at the brain surface (Fig. 9A-C). Deeper in the brain, however, mutant cells could differentiate into neurons and send axons into the neuropil (Fig. 9B1, arrowhead, C2, arrow). However, *shg*^{R69} mutant clones shared some phenotypes with *APC2 APC1* double mutant clones—most striking, these phenotypes also were graded in severity along the A/P axis of the medulla. Clones in anterior and lateral positions in the medulla sometimes differentiated normally (3/8 clones; data not shown), sometimes produced mildly hyperfasciculated axons (2/8 clones; data not shown), and sometimes gave rise to axon knots (3/8 clones; Fig. 9A2,B2). In contrast, *shg* mutant clones on the posterior side of the medulla were normal in morphology, but clones were rounded rather than irregular in shape (Fig. 9C vs. D), consistent with some degree of differential adhesion. Thus reducing cadherin-based adhesion can partially mimic loss of APC function, suggesting that it may be one contributing cause of the phenotypes observed.

In parallel, we explored whether increasing DE-cad expression might mimic loss of APC function. We over-expressed DE-cad in MARCM clones, using UAS-DE-cad (Oda and Tsukita, 2001). Staining with DE-cad antibodies confirmed over-expression (Fig. 9E,F

arrows). In contrast to loss of DE-cad, increased DE-cad expression had no phenotypic effects. Clones over-expressing DE-cad were seen in anterior, lateral and posterior positions (Fig. 9E,F, data not shown), sent out correctly targeted axons and did not form knots (Fig. 9E2,F2 arrows).

Discussion

The role of Wnt signaling in medullar development

Earlier work by the Kunes lab revealed important roles for Wg signaling in medullar development (Kaphingst and Kunes, 1994; Song et al., 2000). Wg has a restricted expression pattern from the 1st instar onward. Cells expressing Wg reside at the tips of the medullar horseshoe, on the posterior side of the brain. These cells serve as a signaling center, creating nested zones of gene expression that expand anteriorly. By loss-of-function analysis Kunes and colleagues found that the nested zones of gene expression depend on Wg signaling. Wg is important for proliferation of OPC progenitors—when it is disrupted early, the medullar neuropil is much smaller. In many ways this resembles the roles Wg plays in imaginal discs, where it acts early to promote proliferation, and has a later role in setting up nested domains of gene expression. Our data extend this earlier analysis in two ways. First, by eliminating APC function we activated rather than inactivated Wg signaling. Second, by carrying out clonal analysis, we could analyze later roles of Wg signaling in medullar development, and also could analyze the effects of Wg signaling in different regions of the medulla. This revealed that Wg signaling regulates fine scale patterning, perhaps by regulating differential adhesion and axon outgrowth.

Our ability to conduct clonal analysis in different medullar regions revealed striking regional differences in the effects of loss of APC function (Fig. 4; Suppl. Table 1). *APC2*

APC1 double mutant clones in the anterior medulla have dramatic defects in epithelial segregation and axon outgrowth. Posterior double mutant clones, in contrast, have only subtle differences in cell mixing, while clones in intermediate regions have intermediate phenotypes. These data are consistent with a role for Wg signaling in directing fine scale cell fate choices in a graded fashion all along the A/P axis, reminiscent of the role Wg plays in the developing wing imaginal disc (Cadigan, 2002). Double mutant clones should experience high levels of Wg signaling even when distant from the normal Wg source. If these cells are found in the posterior horns, where cells normally experience high levels of Wg (Kaphingst and Kunes, 1994), their fates match those of their neighbors and there are not strong defects in epithelial organization or axon outgrowth. Double mutant cells further and further from the normal source of Wg will be more and more "out of place" and will differ more in fate from their wild-type neighbors. Apparently, the greater the mismatch in fate, the more association with neighbors is perturbed. The subtle cell segregation of double mutant clones in the vicinity where normal Wg signal is maximal suggests that double mutant cells may have higher levels of Wg activation than are ever experienced by wild-type cells, even those exposed to the highest levels of Wg. This is consistent with what we and others observed in embryos, where *APC2 APC1* double mutants accumulate higher levels of Arm than are accumulated by wild-type cells seeing maximal levels of normal Wg signaling (Ahmed et al., 2002; Akong et al., 2002a).

Our data also suggest that Wg signaling affects not only gene expression patterns and proliferation of OPC progenitors (Kaphingst and Kunes, 1994), but also affects the final differentiated fate of their neuronal progeny, once again acting in a graded fashion along the A/P axis. Double mutant clones (and Arm^{S10}-expressing clones in the same region) exhibit

defects in axon outgrowth, forming axon knots. These phenotypes are strongly graded along the A/P axis, with anterior axon knots, hyperfasciculated axons in intermediate regions, and wild-type axon outgrowth in posterior clones. This suggests that neurons may have fairly finely graded fates along the A/P axis, with proper axon outgrowth ensured by these graded fates.

Some aspects of medullar development are reminiscent of roles of Wg and Dpp in imaginal discs, where they act to regulate both pattern and growth/proliferation (Cadigan, 2002). Clones with activated Wg or Dpp signaling can exhibit cell segregation and overgrowth (Haerry et al., 1998; Heslip et al., 1997; Martin-Castellanos and Edgar, 2002; Nellen et al., 1996). Further, cells expressing an activated Dpp receptor proliferate faster than wild-type cells, but levels of P-His and CycE are not dramatically altered in expression (Martin-Castellanos and Edgar, 2002). Thus the roles of Wnt signaling in growth and patterning may be parallel in the medulla and imaginal discs.

Is differential adhesion a cause of the phenotypes seen and a feature of the wild-type brain?

Double mutant epithelial neuroblasts segregate from wild-type neighbors, consistent with differential adhesion between wild-type and mutant cells. These phenotypic effects are graded along the A/P axis, with loops on the anterior side and subtle but distinguishable effects on cell mixing on the posterior. One possible model to explain this is that a gradient of differential cell adhesion along the wild-type A/P axis helps shape proper medullar architecture. This could be particularly important as cells move medially from the laminar furrow. Marked clones of wild-type cells on the anterior side are wedge-shaped, suggesting that progeny of epithelial neuroblasts may move orthogonally to the laminar furrow. This could be facilitated by graded adhesion along the A/P axis, inhibiting cells from moving up

or down this axis to positions inappropriate for their fate. Of course this putative differential adhesion could be directly regulated by Wg signaling, or it could be well downstream of this signaling pathway—future research is needed to address this.

The medulla is the target field for a subset of photoreceptor axons, and correct axon targeting is critical for proper visual processing (Mast et al., 2006). The broad zones of gene expression created by local Wg expression (Kaphingst and Kunes, 1994; Song et al., 2000) likely help shape this field. However, it was unclear whether this phenotypic specification is fine-grained. Double mutant neurons send out axons that form axon knots. Once again, this phenotype is graded along the A/P axis, with axon knots on the anterior side, hyperfasciculated axons correctly targeting the medullar neuropil in intermediate positions, and normal axon outgrowth in posterior clones. One speculative possibility is that axons also have graded differences in adhesive properties along the wild-type A/P axis. Wild-type medullar neurons send axons in small fasciculated bundles directly to the medullar neuropil, consistent with graded adhesive behavior helping prevent axons from targeting regions inappropriate for their fates. In this model, the guidance information allowing axons to find the medullar neuropil would be largely unaffected by loss of APC, explaining the occasional "escaper axons" that target the medullar neuropil. An alternate possibility is that Wg hyperactivation interferes with the ability of cells to appropriately respond to existing guidance information.

If such differential adhesion exists, what molecules might mediate it? In the simplest model, a single molecule might do so, with its expression/activity graded along the A/P axis. Classic cadherins were obvious candidates. In the medulla, DN-cad is accumulating only on medullar axons and incoming photoreceptor axons (Lee et al., 2001). This restricted

expression pattern, and the lack of consistent changes in DN-cad expression in double mutant clones suggest that it is not likely to be a critical mediator of the effects we observed.

DE-cad is expressed by all cells affected by loss of APC function, and thus it was a more plausible target. However, there are not dramatic differences in DE-cad expression along the A/P axis, though it is difficult to rule out subtle, graded quantitative changes. Changes in cadherin levels are sufficient to mediate cell sorting both in vitro (Steinberg and Takeichi, 1994) and in vivo (Godt and Tepass, 1998; González-Reyes and St Johnston, 1998). Reducing DE-cad levels, by making clones of cells mutant for a null *shg* allele, was incompatible with cell survival or maintenance in epithelial neuroblasts, and thus we could not test this hypothesis there. However, loss of DE-cad in more medial regions and in neurons partially mimicked loss of APC function, leading to rounded clones and small axon knots. This is consistent with the possibility that differential adhesion underlies both axon knots and loops, but testing this hypothesis will require further experimentation. Loss of APC function did not result in large changes in DE-cad expression or accumulation. Subtle differences are more difficult to rule out, due to the changes in expression of DE-cad across the medulla and the alterations in brain architecture caused by loss of APC function—in fact subtle reduction in DE-cad expression levels sometimes was seen, though not in all clones. This leaves open the possibility that subtle reduction in DE-cad levels is a contributing cause. DE-cad may play similar roles in cell segregation along the dorsal-ventral axis of the developing Drosophila wing (Jaiswal et al., 2006; Wodarz et al., 2006).

APC proteins could also affect cadherin function by two other mechanisms. APC loss elevates Arm levels; if Arm levels are limiting this might increase cadherin-based adhesion. Second, being homozygous for certain *APC2* alleles during oogenesis reduces

cadherin-based adhesion (Hamada and Bienz, 2002). However, complete loss of APC function does not have detectable effects on cadherin-based adhesion during oogenesis or embryogenesis (McCartney et al., 2006), making it less likely that this mechanism is critical.

Of course changes in DE-cad function are only one possible explanation for the cell segregation and axon abnormalities we observe. Many other cell adhesion molecules exist, and one or more of those may be regulated directly or indirectly by Wg signaling. For example, ephrins/Eph signaling, which is modulated by Wnt signaling in the colon (Batlle et al., 2002), and which can alter axon pathfinding in the larval brain (Dearborn et al., 2002; Boyle et al. 2006), is a possible candidate. It will be important to explore these and other possible players in the future.

APC function, Wnt signaling and stem cell behavior

Wnt signals play key roles in many tissues arising from tissue stem cells, and also can regulate ES cell pluripotency. This led to widespread interest in the possibility that Wnt signals might act as a master regulator of stem cell self renewal. In certain tissues, like the colon and hematopoietic system Wnt signaling plays a key role in stem cell self-renewal in vivo or can do so in vitro (Reya and Clevers, 2005). In other tissues, however, Wnt signaling plays more complex roles. In the skin, Wnt signaling plays several roles at different stages in the process, regulating stem cell maintenance in the hair follicle, but also regulating terminal differentiation of hair and maintaining the balance between hair follicle and sebaceous gland fates (Blanpain and Fuchs, 2006). There are also tissues where Wnt signaling plays no apparent role in stem cell maintenance—e.g., *Drosophila* female germline stem cells (Song et al., 2002). APC family proteins also play Wnt-independent roles in stem cell maintenance, through effects on spindle orientation in asymmetric cell divisions

(Yamashita et al., 2003).

What role does Wnt signaling play in neural stem cells during brain development? In the mammalian cortex, Wnt signaling regulates proliferation, but activated Wnt signaling is not incompatible with neural differentiation and patterning (Chenn and Walsh, 2002). Our data further illuminate the role Wnt signaling and APC's have in neural stem cells in the *Drosophila* brain. As in the mammalian brain, Wnt signaling promotes growth and/or proliferation (Kaphingst and Kunes, 1994; our data), but Wnt signaling does not lock cells into a stem cell fate. Our data clearly show that cells with activated Wnt signaling can undergo essentially normal pathways of self-renewing divisions balanced with asymmetric divisions, and can differentiate as neurons and send out axons. Our data suggest that during medullar development, Wnt signaling helps establish finely graded cell fates and translate them into the intricate architecture of the medulla. Interestingly, Wnt regulation of differential cell adhesion and thus fine-scale tissue architecture is seen in the colon, where regulation of ephrin signaling sets up graded cell fates along the crypt-villus axis (Batlle et al., 2002). Further work will allow us to define in molecular terms the mechanisms governing differential fates and differential cell adhesion, providing a molecular map of this aspect of brain architecture.

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Figure legends

Fig. 1. Development of the medulla. A-C. Diagrams , 3rd instar larval brain. A. Entire brain. B. Anterior surface view of single optic lobe (as in D). C. Cross section of optic lobe in B (as in H). D-N, Wild-type late 3rd instar larval brain lobes, dorsal to top, antigens indicated. D-G, J-M. Anterior (D,F,G,J,L) or posterior (E,K,M) surface views. Blue brackets, epithelial neuroblasts; white brackets, medial neuroblasts. H. Cross-section through medulla (as in B). Arrowhead, fasciculated bundles of medullar axons projecting to medullar neuropil (arrow). I1-I4. Section series, from below anterior surface to just below posterior surface. D',I2, L=lamina, m=Medulla. Green and yellow arrowheads, dorsal and ventral horns of neuropil. I2,I3. Red arrowheads=fasciculated medullar neurons. M, arrow=Mira-positive cells in IPC. N. Anterior, just below surface. Arm levels are higher in dorsal neuronal cell bodies. Scale bar=30µm.

Fig. 2. Larval development of the optic lobe. A. Late 1st/early 2nd instar. Arrows=epithelial optic lobe progenitors. B1-B3. 2nd instar. Section series, posterior surface (B1) to anterior surface (B3). One horn of the IPC reaches the posterior surface (B1), while the OPC is prominent on the anterior surface (B3). C. Early 3rd instar. Blue bracket, all neuroblasts are epithelial and express high levels of DE-cad. Scale bar=30µm.

Fig. 3. Proliferation pattern and cell sorting of wild-type medullar cells. GFP-marked clones of wild-type cells. 3rd instar brain lobes, dorsal to top. A1-A4. Serial sections, clone on the anterior side of the medulla. A2, arrow=roughly wedge-shaped clone. A3, Fasciculated medullar neurons (arrow) projecting to the medullar neuropil (arrowhead). B1-B2. Clone

arising on the medial edge of the medullar neuroblasts (B1, arrow), which forms a wedge-shaped clone of neurons (B2, arrow). C1-C2,D1-D2. Surface and deeper sections of clones on the ventral horn of the posterior medulla. Note complex shapes (arrows) due to free sorting with wild-type cells, and normal neuronal projections. E1-E3. Serial sections, lateral clone (bottom right, E2 arrow) and an "edge clone" on the dorsal horn of the posterior medulla (E2,arrowhead). E3,arrow=normal neurons from lateral clone. Scale bar=30µm.

Fig. 4. Reduction in APC function leads to epithelial segregation and defects in axon outgrowth. GFP-marked $APC2^{d40}$ $APC1^{Q8}$ double mutant clones in 3rd instar brain lobes, dorsal up (H,I=wild-type). Antigens indicated. A,B,D-F. Anterior views of medulla. Numbered panels are confocal sections from anterior surface successively deeper. A,B,E. Epithelial loops. C, left. Diagram of epithelial loops. D2,F2. Axon knots. C, right. Diagram of axon knot. G. Epithelial ball (arrow) beneath surface and escaper axons (arrowhead). H-J. BP102 accumulates in wild-type (H,I) axons (arrowheads) and medullar neuropil (arrows), and in mutant axon knots (J, arrow). K. Hyperfasciculated axons (arrow). L. Escaper axons (arrowhead). Scale bar=30µm.

Fig. 5. Loss of APC function has differential effects along the anterior-posterior axis. A. Diagram of medulla showing regions with strong phenotypes (red), milder phenotypes (yellow) or only mild effects on cell sorting (green). B-M. GFP-marked $APC2^{g10}$ $APC1^{Q8}$ double mutant clones in 3rd instar brain lobes, dorsal up (J-L are wild-type). Antigens and clone position in degrees indicated. Numbered panels are confocal sections from anterior surface successively deeper. B,C. Epithelial loops and knots. D,E. Clones with

hyperfasciculated axons (arrows). F-H,L. Posterior clones exhibiting round shape but normal in morphology. Note increased Arm accumulation in L (arrow). I-K. Comparable wild-type clones. M. Epithelial loop. Mira is absent in lateral epithelial region (arrow) and present in medial cells of clone (arrowhead). Scale bar=30µm.

Fig. 6. Clones lacking APC function are larger than wild-type clones. A. Volumes of position-matched wild-type and *APC2*^{g10} *APC1*^{Q8} double mutant clones, with means and SD. B-J. 3rd instar brain lobes, dorsal up. Antigens indicated. B-E,H-J. GFP-marked *APC2*^{g10} *APC1*^{Q8} double mutant clones, outlined in yellow. F,G are wild-type. B-E. Phospho-histoneH3, showing mitosis. B-D. Anterior clones. E. Posterior clone. In wild-type, at any given time a fraction of the neuroblasts in the superficial layers of the medulla are mitotic, as assessed by P-His (B,C arrows), with slightly lower levels in epithelial neuroblasts adjacent to the laminar furrow. In deeper sections through medullar neurons, few cells are mitotic (E2, arrow), as expected. Since double null mutant cells form loops and knots, cells are often displaced from their normal positions; at times this leads to apparent reductions or increases in P-His, but double mutant cells generally matched comparable wild-type cells (compare arrowheads in B-E). F-J, CycE expression in wild-type brain (F,G) and anterior double mutant clones (H-J). Scale bar=30µm.

Fig. 7. Activation of Wg signaling is necessary and sufficient for formation of epithelial knots and loops. 3rd instar brain lobes, dorsal up. Antigens indicated. A-E. GFP-marked MARCM clones expressing myc-tagged Arm^{S10}. A. Anterior clone expressing high levels of Arm^{S10}. B. Intermediate-region clone expressing high levels of Arm^{S10}. C. Anterior clone

expressing low levels of Arm^{S10}. D. Posterior clone expressing high levels of Arm^{S10}. E. Posterior clone expressing moderate levels of Arm^{S10}. F, G. GFP-marked $APC2^{g10}$ $APC1^{Q8}$ double mutant clones—50% should also express TCF-DN. F. Anterior clone with very mild phenotype. G. Anterior clone with a wild-type morphology. Scale bar=30µm.

Fig. 8. The Wg target gene *dpp* is activated in clones lacking APC function. Antigens indicated. A. Diagram showing wild-type Wg and Dpp expression domains. B-F. 3rd instar brain lobes, dorsal up. B. Wild-type, *dpp*-lacZ expression pattern. C-H. GFP-marked $APC2^{g10}$ $APC1^{Q8}$ double mutant clones. Arrows indicate mutant clones with ectopic *dpp*-lacZ expression. C. Anterior clones. D. Intermediate-region clone. E. Posterior clone (arrows and arrowheads are for comparison with wild-type pattern in B). F. Posterior clone in the tip of the medullar horns (the putative Wg-expression domain). G,H. Early 3rd instars. Scale bar=30µm.

Fig. 9. Loss of DE-cad function can cause axon knots, but DE-cad overexpression does not. 3rd instar brain lobes, dorsal up. Antigens indicated. A-C. GFP-marked shg^{R69} mutant clones (arrows). A. Anterior clone forming axon knot. B. Intermediate region clone forming axon knot (inset, actin alone). C. Posterior clone with rounded margin but no axon defects. D. Wild-type control clone. E-F. GFP-marked MARCM clones overexpressing DE-cad (arrows). In each case several sections from surface inward are shown. E inset=close-up or region marked with arrow). Scale bar=30µm.

Suppl. Fig. 1. Different alleles of *APC2* have phenotypic consequences consistent with their effects on Wg signaling. 3rd instar brain lobes, dorsal up. Antigens indicated. A. Diagram of APC proteins and alleles used. B-H. GFP-marked clones double mutant for $APC1^{Q8}$ and the indicated *APC2* allele. Scale bars=30µm.

Suppl. Fig. 2. DN-cad accumulation is not substantially altered by loss of APC function. 3rd instar brain lobes, dorsal up. Antigens indicated. A1,A2. Surface and deeper sections. DN-cad is normally absent from lateral epithelial neuroblasts near the laminar furrow, and is also absent from most medial medullar neuroblasts (A1,A2 white arrows) but accumulates in medullar neurons (A2, arrowhead) and the medullar neuropil (red arrow), as well as in progeny of the large central brain neuroblasts (A1, arrowhead) and in axons entering from the photoreceptors (data not shown), as described by others (Lee et al., 2001). B,C. GFP-marked $APC2^{g10}$ $APC1^{Q8}$ double mutant clones. DN-cad does not accumulate in most double mutant epithelial cells (B,C arrows), and thus they resembled wild-type epithelial neuroblasts. Double mutant cells resembling more medial neuroblasts also did not accumulate DN-cad (C, arrow), while double mutant cells adopting neuronal identities expressed DN-cad, and it accumulated in axon knots (C, arrowhead), as it did in the wild-type medullar neuropil. Occasional cells adjacent to epithelial portions of clones near or in the laminar furrow did express DN-cad (B,arrowhead), but similar isolated cells also expressed other neuronal markers (e.g., Elav-GAL4), suggesting these cells were differentiating as medullar neurons. Scale bar=30µm.

Suppl. Fig. 3. There are not dramatic changes in DE-cad expression in cells lacking APC function. GFP-marked *APC2*^{g10} *APC1*^{Q8} double mutant clones. Antigens indicated. Arrows and arrowheads discussed in text. A-H. Accumulation of DE-cad protein. A,B. 2nd instars. Insets, DE-cad with clones circled. C,D. Early 3rd instars, clone circled (C) or bracketed (D). At these stages all medullar cells are epithelial. DE-cad levels in *APC2*^{g10} *APC1*^{Q8}double mutant clones are similar to wild-type (A,B,D; compare regions indicated by arrowheads). Occasionally levels appeared slightly reduced (C, arrowheads), but it was difficult to rule out that this resulted from altered morphology of double mutant cells. E-H. Late 3rd instar brain lobes, dorsal up. E-G, anterior clones (circled in red). E,G insets, DE-cad. H. Posterior clone, outlined in red. In late 3rd instars, DE-cad levels differ more dramatically across the medulla. Like wild-type cells, *APC2*^{g10} *APC1*^{Q8}double mutant cells downregulate DE-cad accumulation as they differentiate—levels were highest in cells equivalent to lateral epithelial neuroblasts (E,F, arrowheads), remained high in cells equivalent to more medial neuroblasts (F', arrows), and dropped substantially in the most medial neuroblasts (G1, arrowheads) and neurons (G2, arrowheads). DE-cad levels in double mutant cells did not differ dramatically from those in wild-type, either on the anterior (E-G) or posterior (H) sides of the medulla, although in occasional clones levels appeared slightly reduced relative to wild-type (F, arrowheads). I-M. Expression of *cad*-lacZ. Late 3rd instar brain lobes, dorsal up. Clones outlined in red. When clones are induced, they activate Elav-GAL4, which is only expressed in neuronal cells, triggering GFP expression there. I-L. Anterior clones. L1, L2. Surface and deeper sections, insets= *cad*-lacZ. M. Posterior clone. Levels of *cad-lacZ* were high in epithelial neuroblasts in the laminar furrow (I', bottom arrow), and dropped in medial neuroblasts (I', bottom arrowhead) and medullar neurons (K', bottom arrowhead).

Double mutant cells had similar levels of cadherin-reporter expression on both the anterior (I-L) and posterior (M) sides of the medulla, and, like wild-type medullar cells, down-regulated the cadherin-reporter in the transition from epithelial (I'-K', arrows) to neuronal (K', arrowhead, L2; in these experiments, the Arm-GAL4 driver could not be used, so not all double mutant cells express GFP). Occasionally reporter gene expression seemed slightly diminished in double mutant clones (I',K', compare arrows), but we also observed (though less frequently) examples where reporter gene expression appeared higher in double mutant cells (J', arrows). Scale bar=30µm.

Suppl. Table 4.1. Phenotypes of *APC2*^{d40} *APC1*^{Q8} double mutant clones.

Clone position	No Hyperfasciculated Phenotype	Ball + knot or knot	Epithelial loop or lateral ball	Loop + knot	Axons
Anterior (n=27)	0	11	12	3	1
Lateral (n=15)	1	10	0	0	4
Posterior dorsal (n=16)	16	0	0	0	0
Posterior ventral (n=27)	26	1	0	0	0

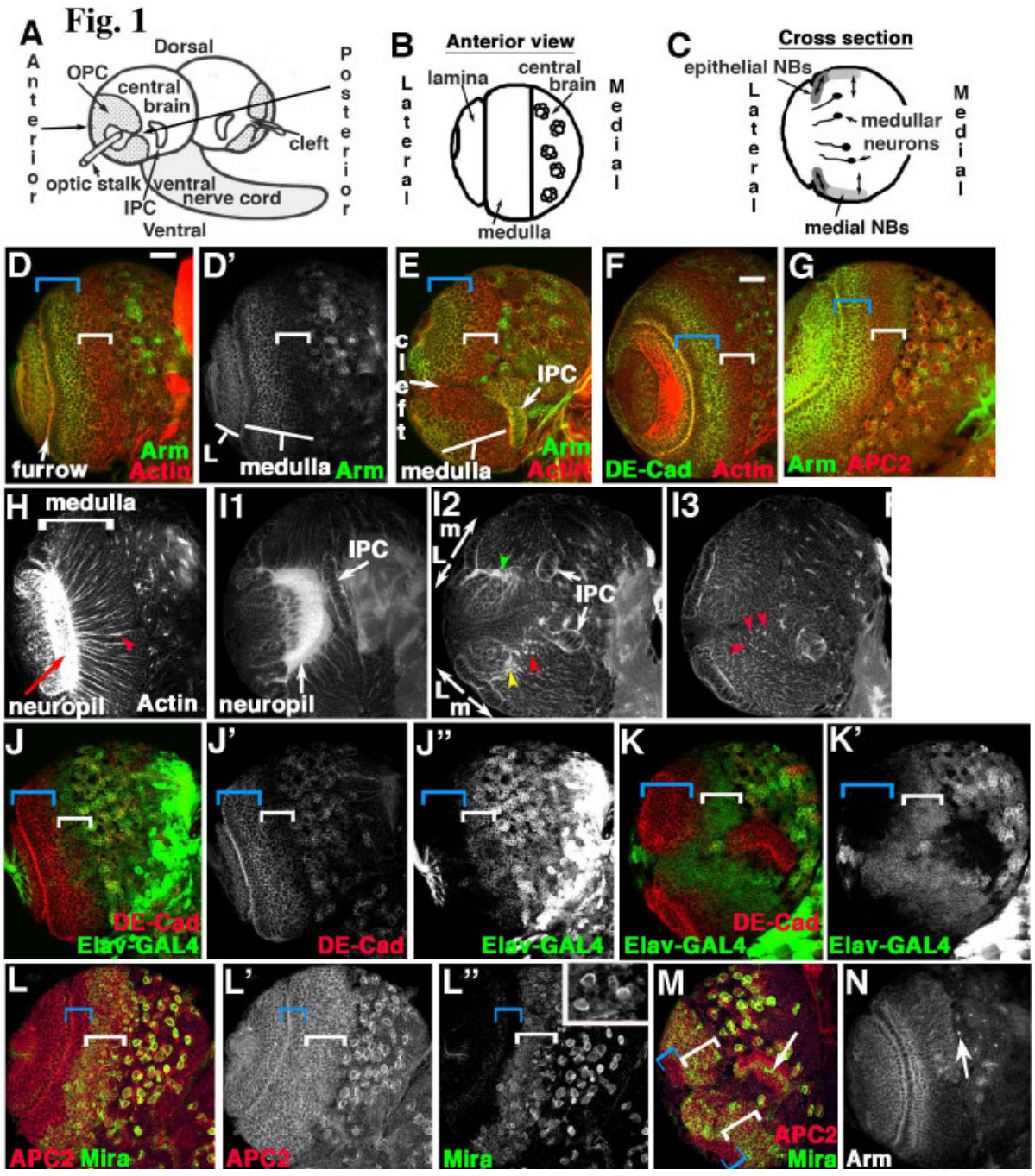


Fig. 4.1. Development of the medulla.

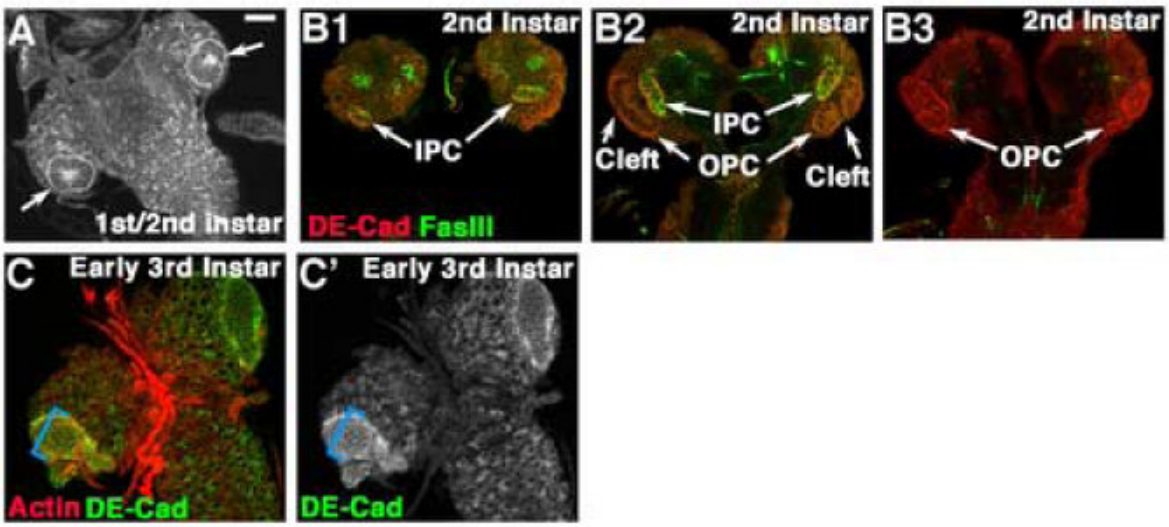


Fig. 4.2. Larval development of the optic lobe.

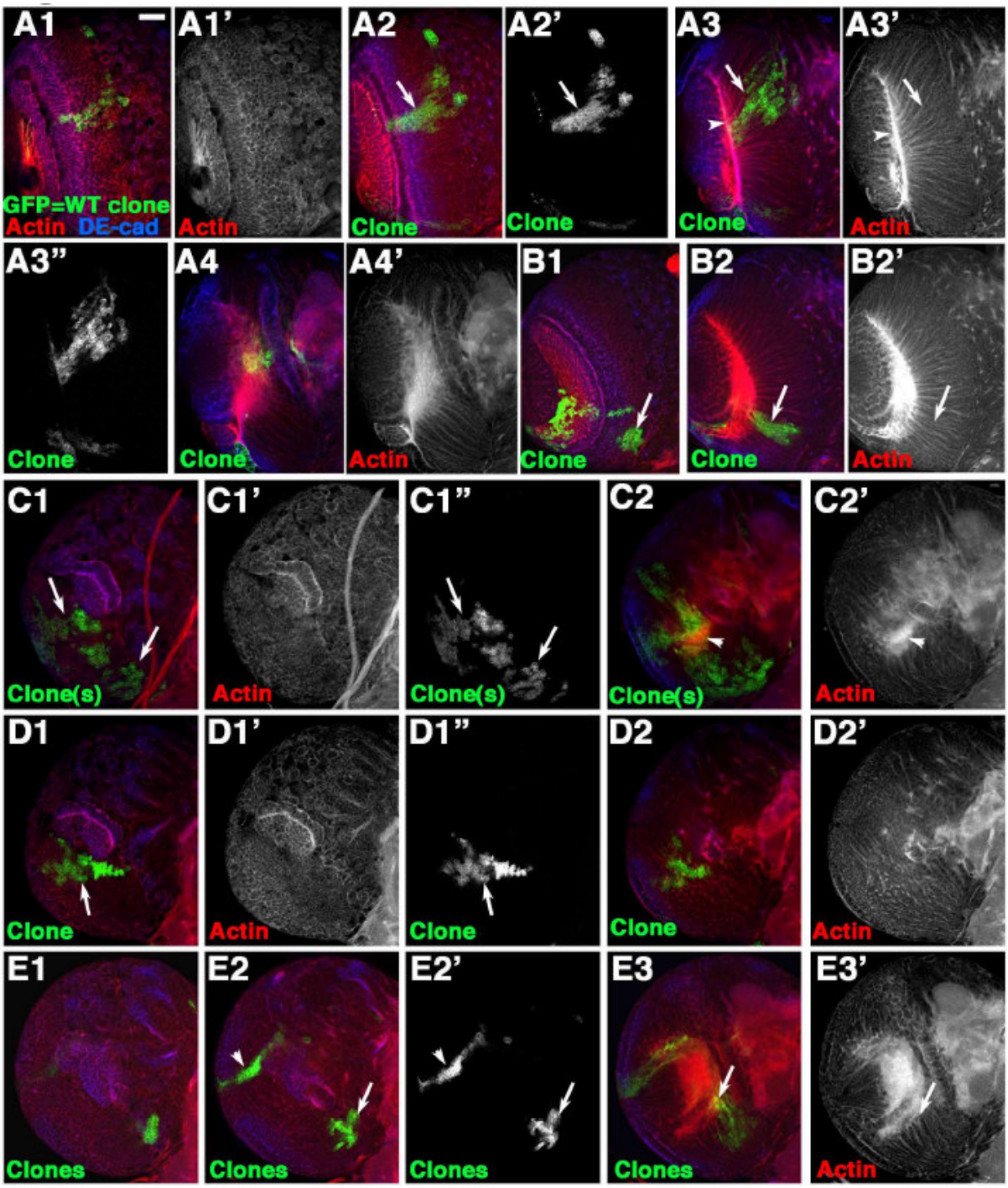


Fig. 4.3. Proliferation pattern and cell sorting of wild-type medullar cells.

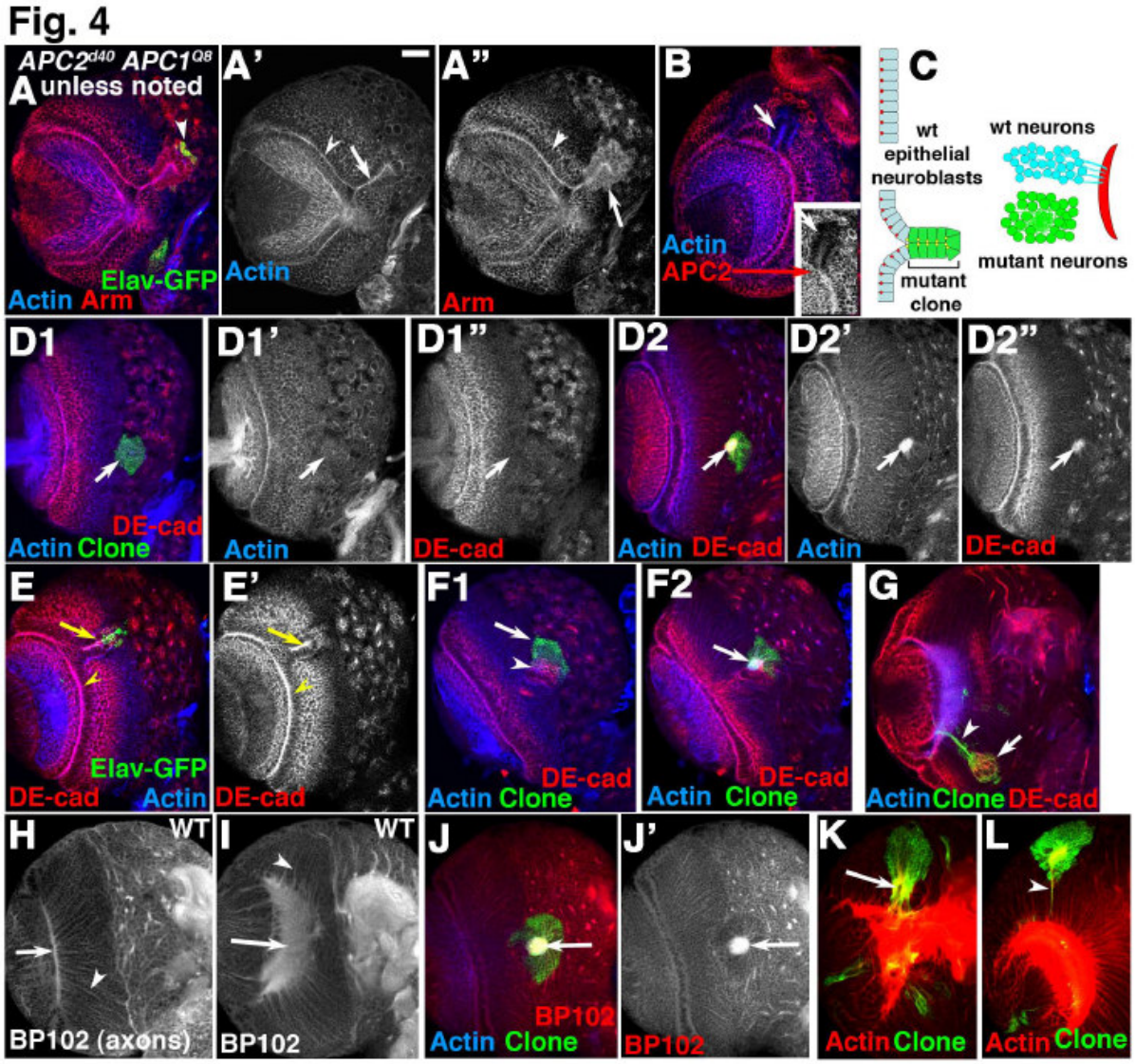


Fig. 4.4. Reduction in APC function leads to epithelial segregation and defects in axon outgrowth.

Fig. 5

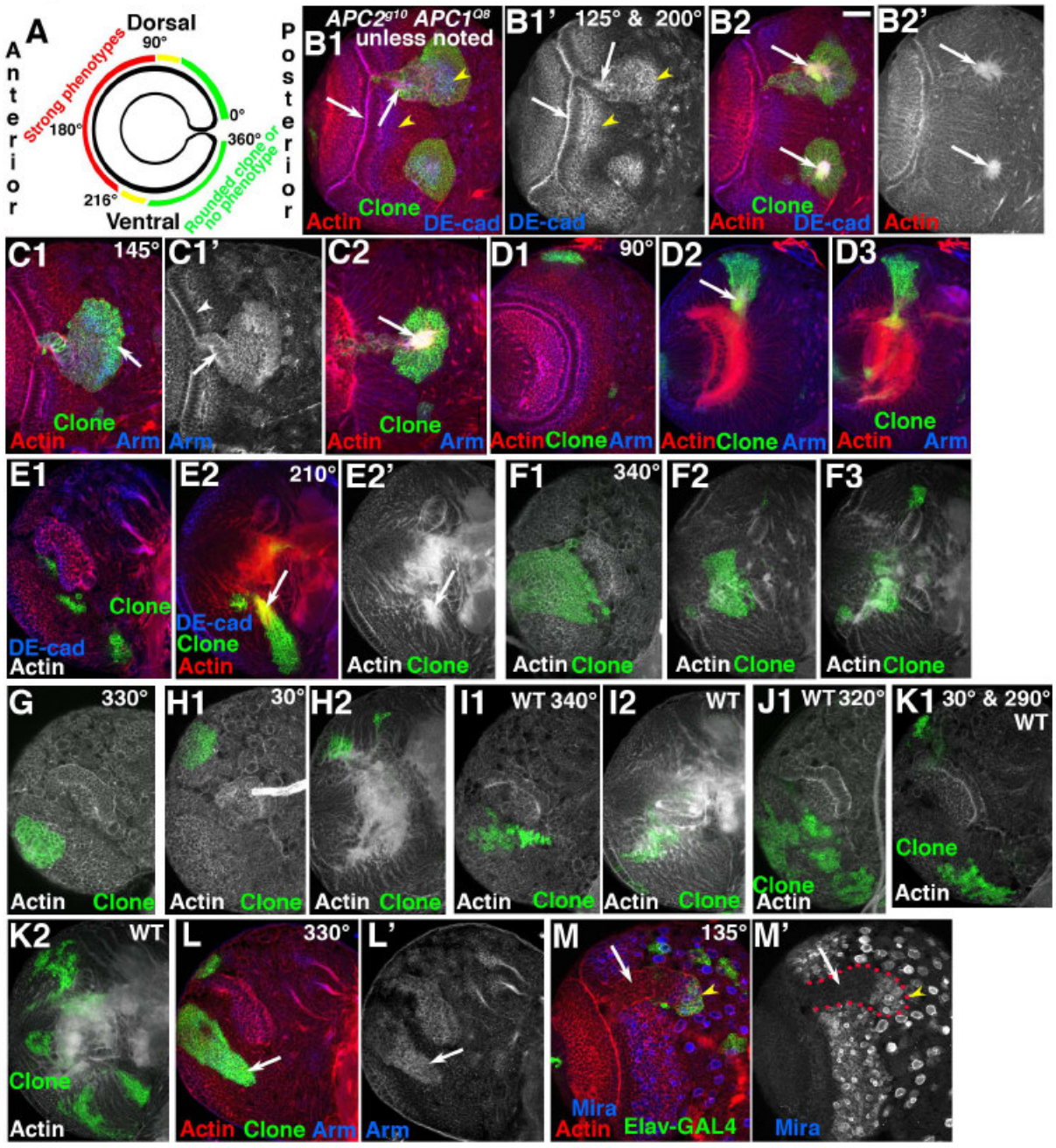


Fig. 4.5. Loss of APC function has differential effects along the anterior-posterior axis.

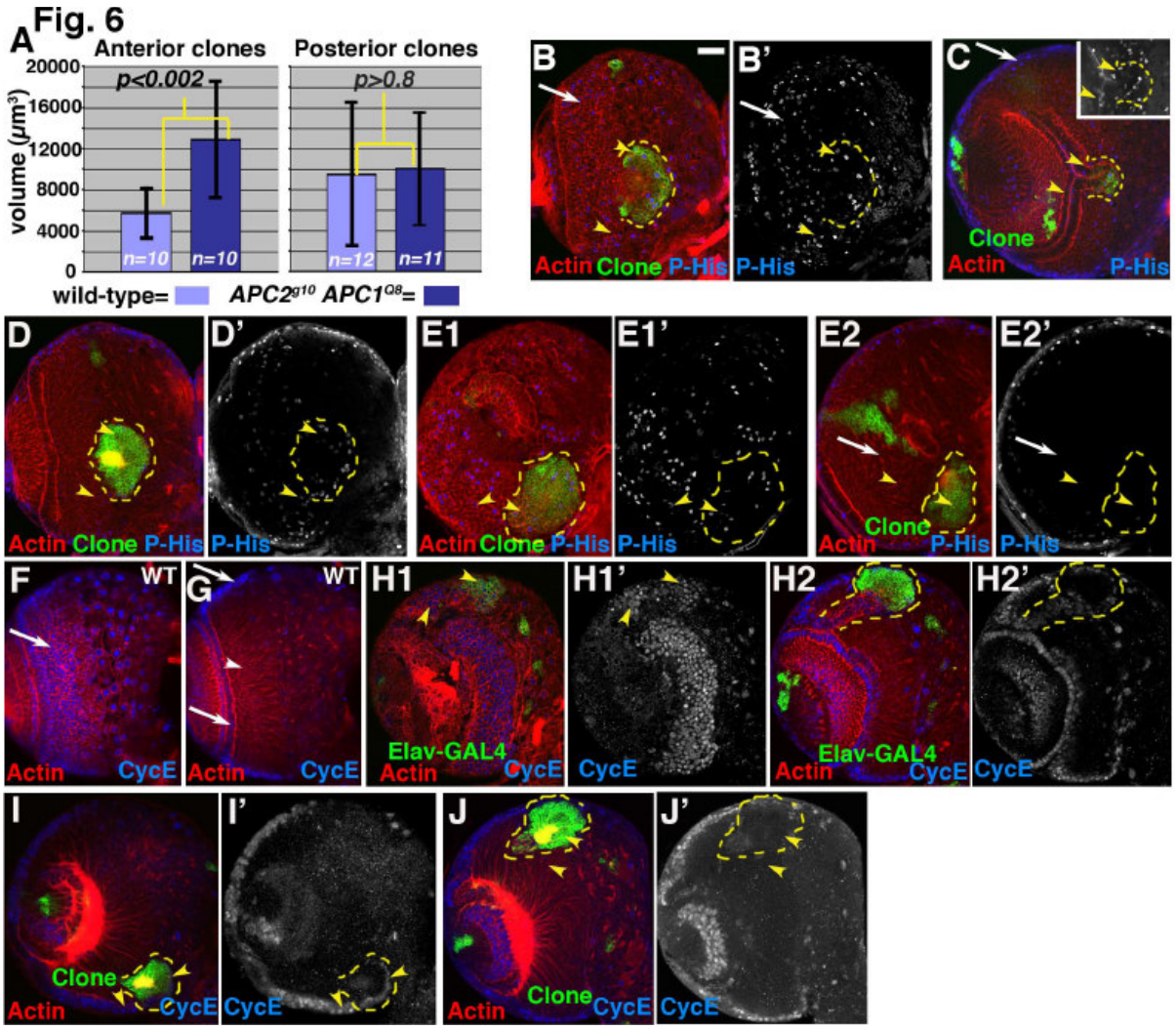


Fig. 4.6. Clones lacking APC function are larger than wild-type clones.

Fig. 7

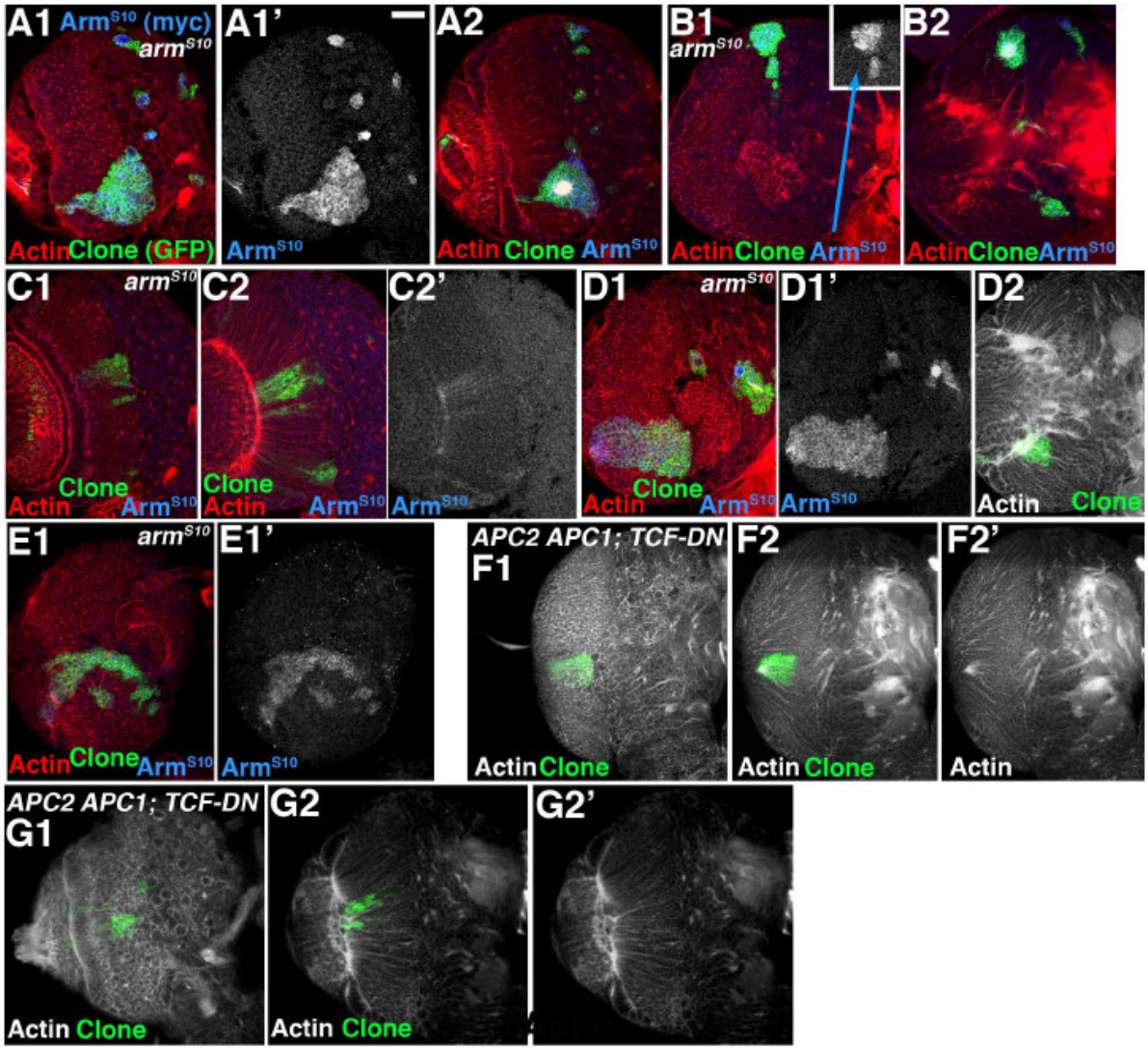


Fig. 4.7. Activation of Wg signaling is necessary and sufficient for formation of epithelial knots and loops.

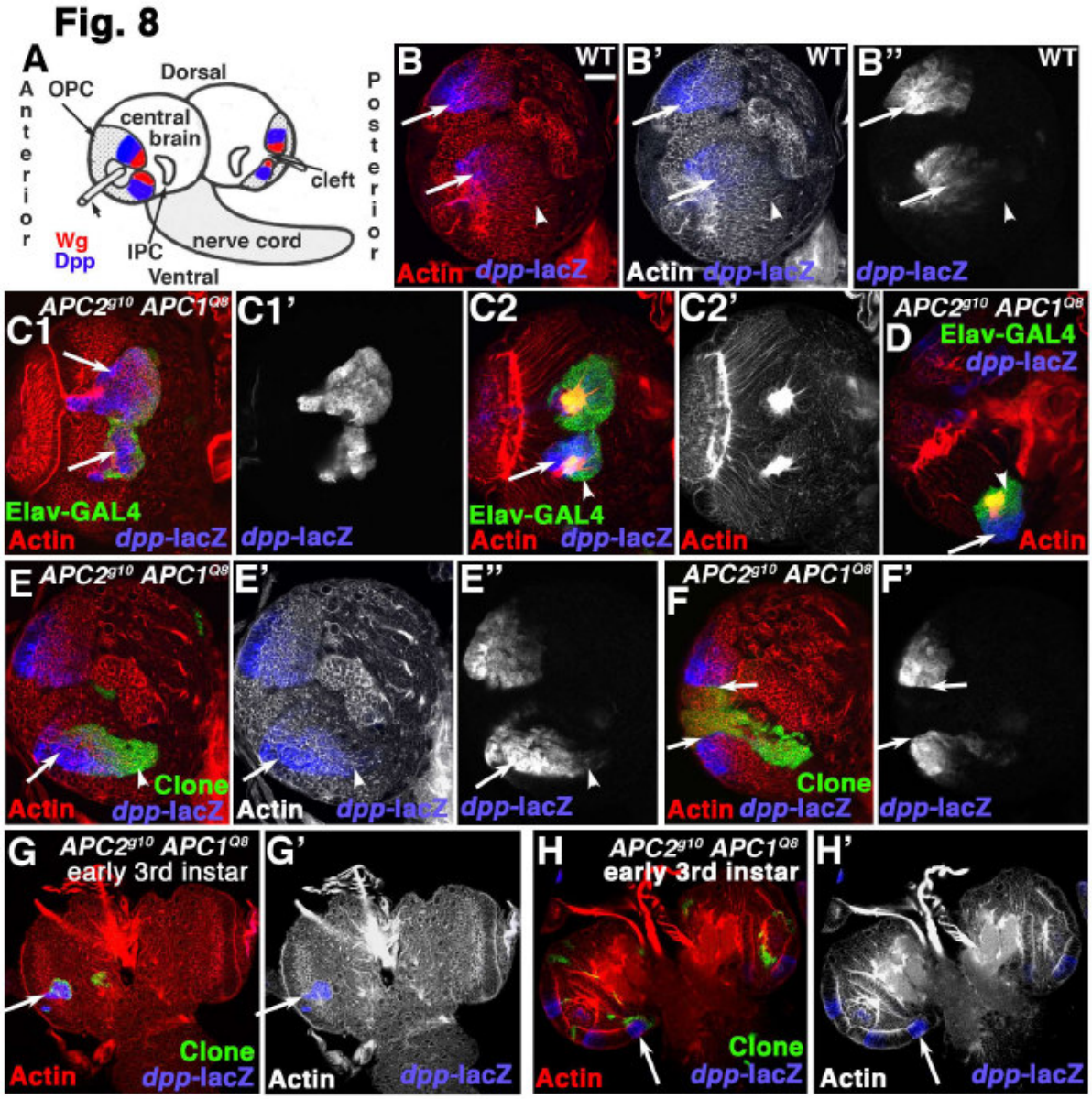


Fig. 4.8. The Wg target gene *dpp* is activated in clones lacking APC function.

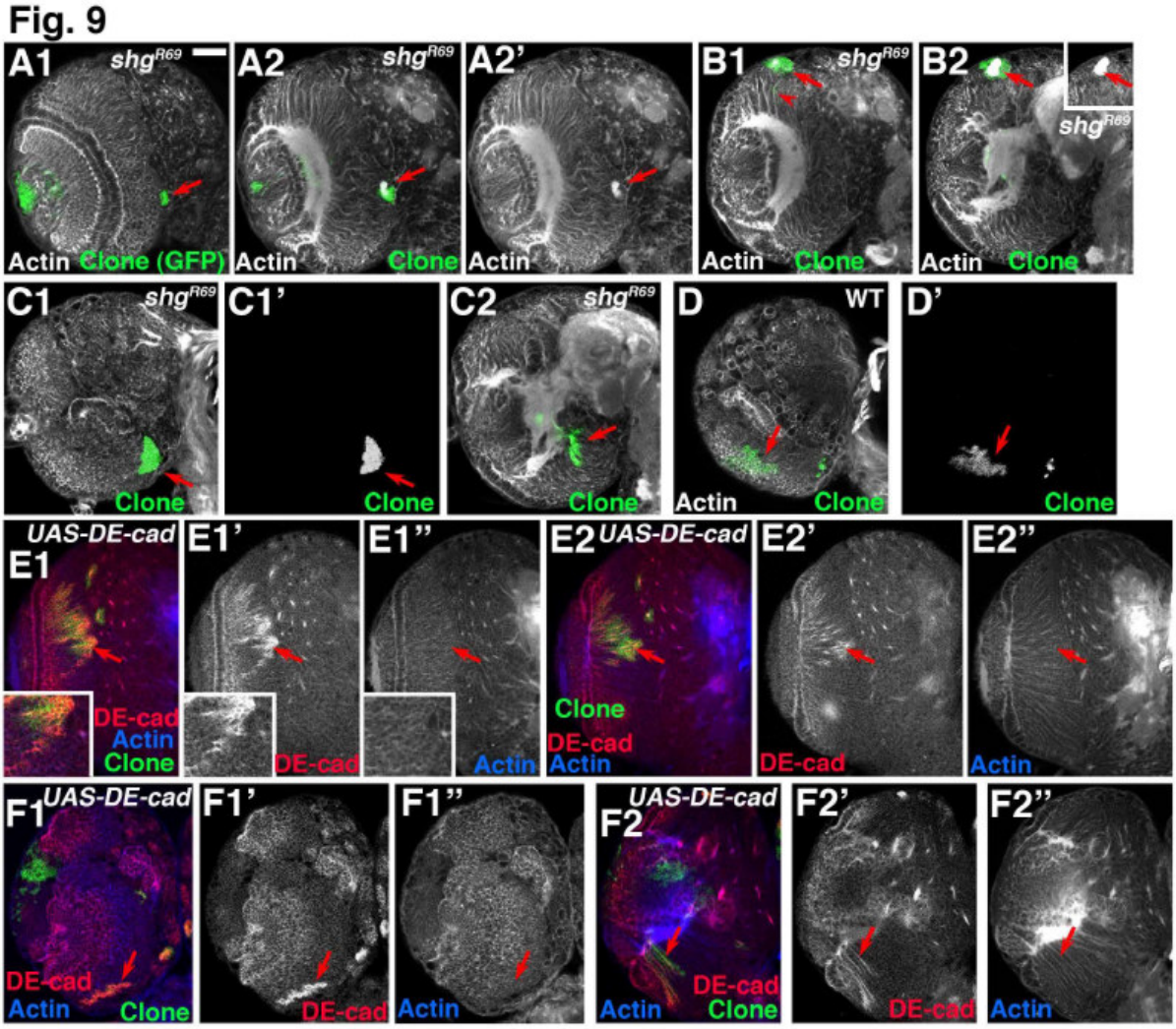
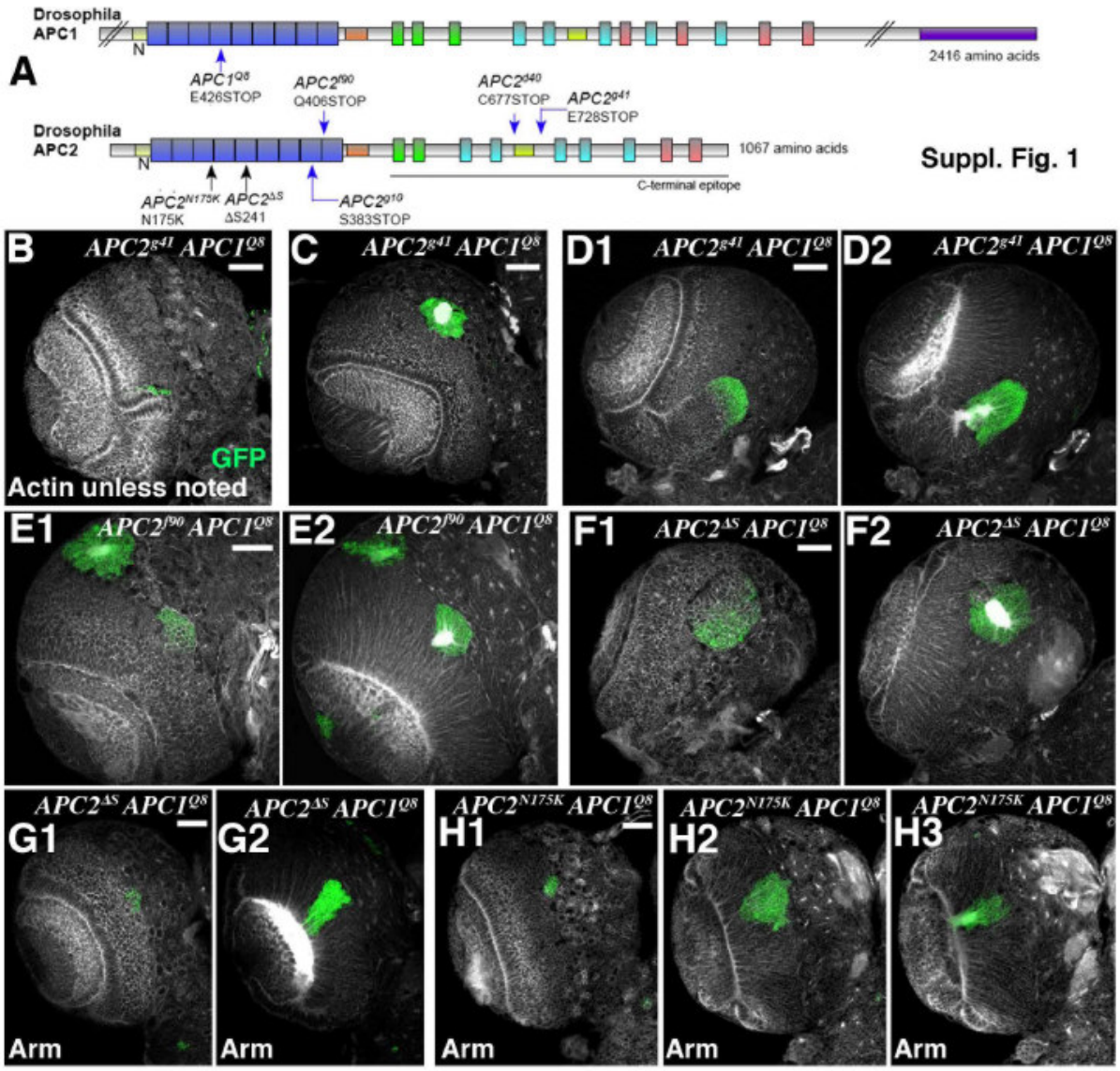
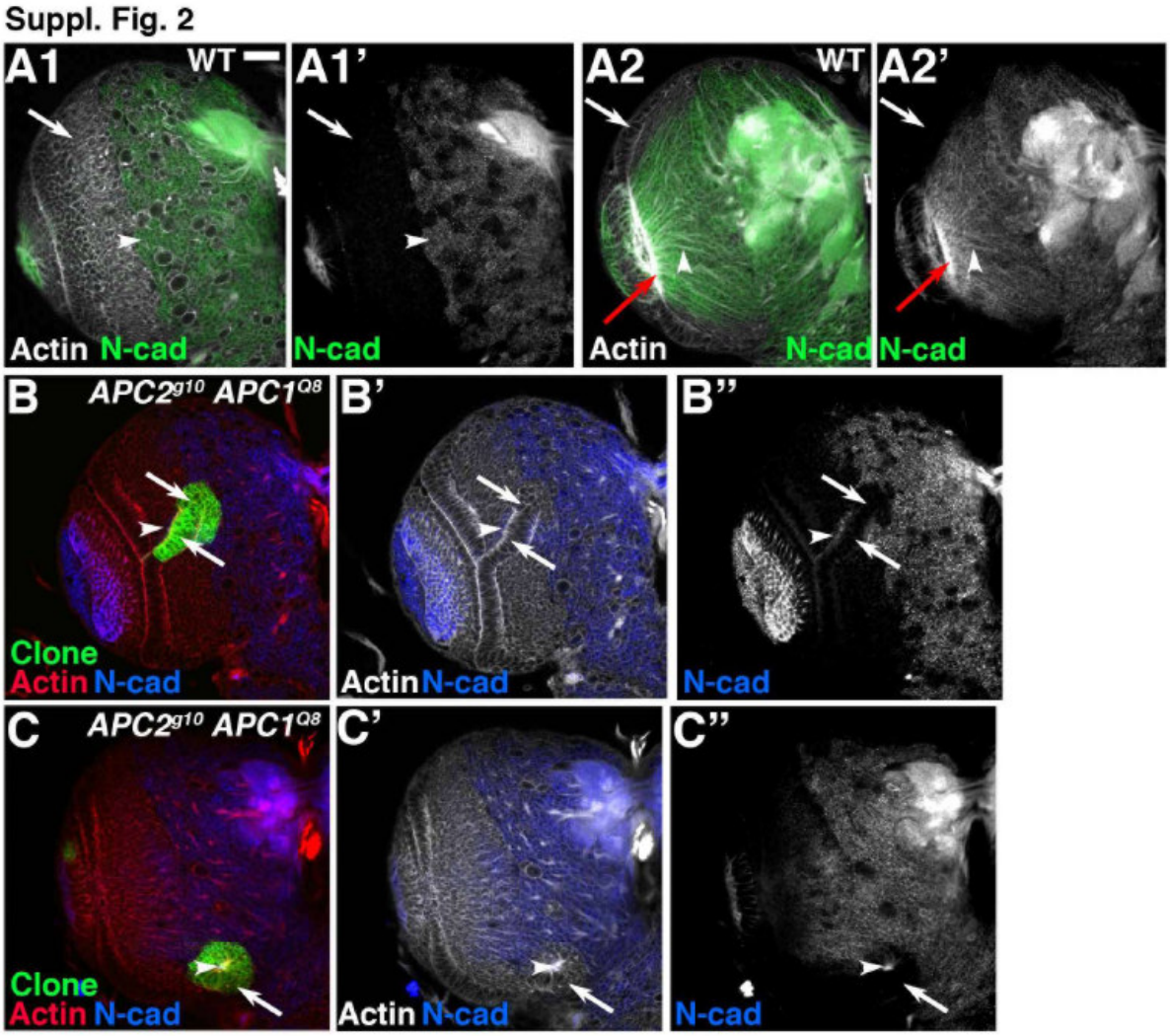


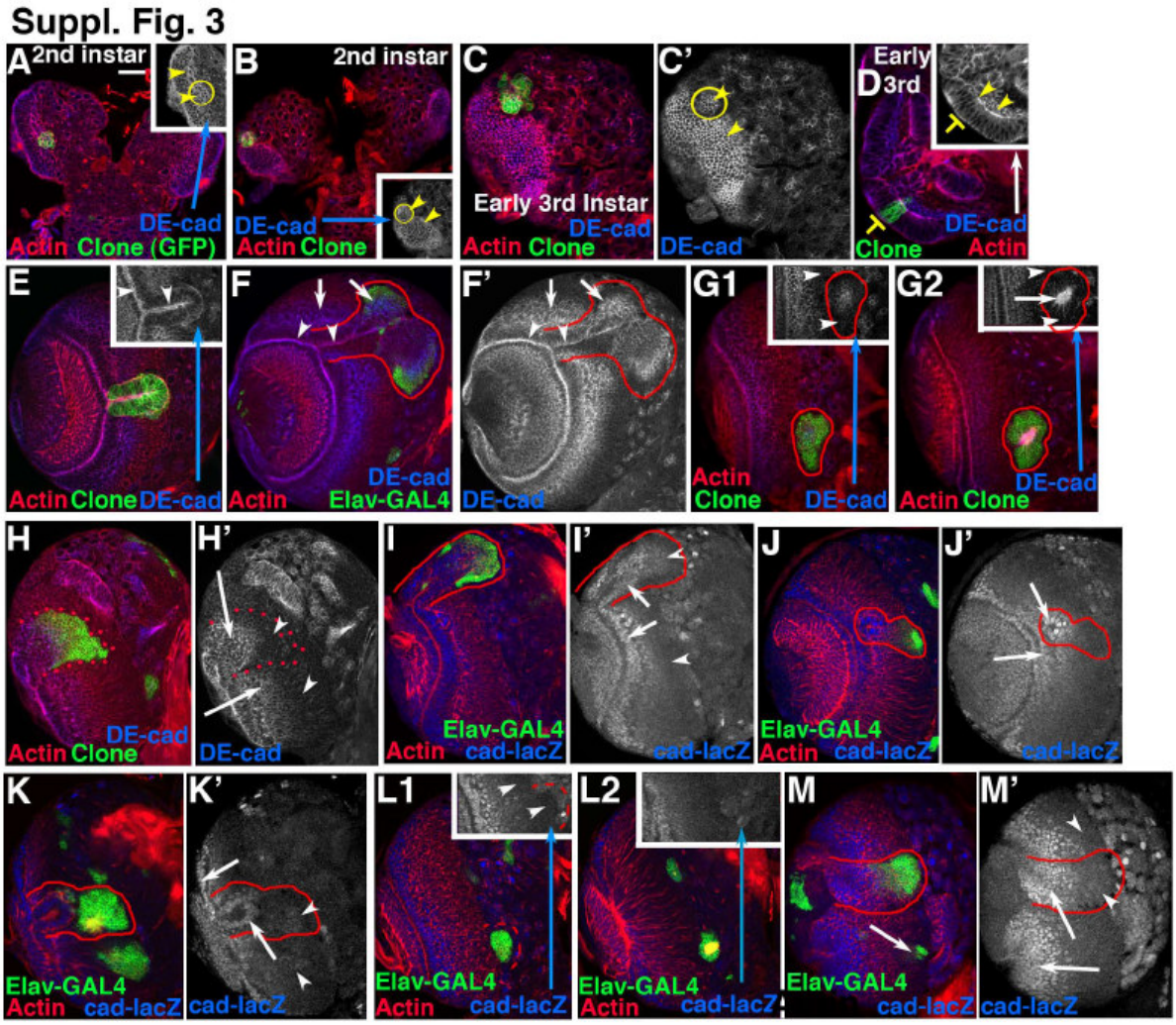
Fig. 4.9. Loss of DE-cad function can cause axon knots, but DE-cad overexpression does not.



Suppl. Fig. 4.1. Different alleles of *APC2* have phenotypic consequences consistent with their effects on Wg signaling.



Suppl. Fig. 4.2. DN-cad accumulation is not substantially altered by loss of APC function.



Suppl. Fig. 4.3. There are not dramatic changes in DE-cad expression in cells lacking APC function.

DISCUSSION

Many exciting discoveries have helped to illuminate the role of the tumor suppressor gene *adenomatous polyposis coli*. In an effort to understand a deadly disease, colon cancer, which results from mutation in the *APC* gene, a lot of interesting cell biology has been observed in the process. APC family members have roles in cytoskeletal regulation of actin and the microtubules. It can also have roles at the cell cortex that are important for cell signaling (Zhou *et al.,* 2004). But the best characterized role thus far has been its role in regulating a signaling pathway known to be important for development and disease, the Wnt /Wg signaling pathway.

The cellular communication networks must integrate a number of signals into defined lines of action to achieve the desired outcome, always checking to see "can you hear me now?" Even the absence of communication often initiates a set of events to make sure the cell is never disconnected from its environment. A multifunctional protein such as APC has different roles that can have different implications for both proper development and disease.

Both flies and humans have two *APC* genes. In each animal one APC family member clearly has a broader expression pattern than the other, however, in many tissues one family member can compensate functionally for the other. For example, both our lab and the Wieschaus lab demonstrated that in places such as the developing embryonic epidermis and the larval brain, that despite distinct localizations, the two APC family members can cooperate towards one role and function redundantly there (Akong *et al*., 2002; Ahmed *et al.,*

2002). At first glance it seems like a great plan to have a "backup", but the structural differences between the APC family members suggest things are more complicated. What is it about those differences within the two proteins that explains their distinct pattern of expression and perhaps their "specialized" functions in these tissues?

One way to answer this question is to undertake a structure function study, which is part of the cell biological work on APC2 in which I participated in. This was the first attempt to map those different functional domains in a living animal, using a variety of mutations, including truncation mutations which completely eliminate certain regions or functional domains of the protein. Mapping the domains necessary for certain functions will help define the portions of the proteins required for each specialized cell biological task. These studies may also help us identify other protein partners that are required for those specialized functions that reside within a particular domain of the APC protein. Finally, this may also give us an insight into why some APC mutations in humans are more detrimental than others, and perhaps provide the molecular mechanism behind those effects.

While it is important to define functions for particular regions of the proteins, it is equally important to determine the consequences of complete loss of both APC proteins. The elimination of both *Drosophila* APCs leads to the death of the animal during the second instar, presumably due to a defect in neuroblast proliferation (Akong 2002a). While this revealed an important shared function of APC proteins in regulating this event, it precluded analysis of later events in development. This was circumvented by generating clones of cells that were mutant for both APC proteins using the MARCM technique in an otherwise normal (heterozygous) animal. Using MARCM in the developing larval brain allowed us to address the potential roles of *APC* family members in multiple cell biological events such as Wg

signaling and cell-cell adhesion. We can also address the extent to which each of those mechanisms contributes to the phenotypes we observed in our *APC* double mutants.

This approach also allowed us to create a situation similar to what happens in the gut of FAP patients, where a few previously heterozygous cells in the crypt lose APC function due to mutational inactivation. When this happens in a patient, a colon polyp forms. When we make *APC* mutant clones in the brain we see a phenotype that shares some phenotypic similarities to a colon polyp: the epithelial loop phenotype. The progenitor/stem cells (neuroblasts) in the laminar furrow region proliferate to give rise to daughter cells faster than their wild type counterparts, ultimately resulting in an expansion of their normal "niche," to areas of the brain they would not normally reside in. In doing so, these mutant cells segregate away from their wild type neighbors but maintain their contacts with their *APC* double mutant neighbors. As is the case in *APC* mutant polyps, inappropriate Wnt/Wg target gene activation also occurs in the *APC* double mutant epithelial loops (in this case, *Dpp-lacZ*).

This and the other phenotypes we observed could be explained by two possible scenarios. First, the changes seen in APC double mutant cells could be due to altered Wg signaling. Alternatively, the phenotypes observed could be the result of change in cell-cell adhesion, possibly due to APC function in the regulation the AJ. A third possibility was one that initially worried me: what if the phenotypes involved both functions? This could pose a significant challenge! How could I distinguish between the defects in adhesion and defects due to aberrant signaling? And which process is affected by which function? In the end we learned that things are even more complex. We found that we need to understand how one signaling pathway, Wg, affects another signaling pathway, Dpp (and perhaps other

unidentified pathways), while at the same time regulating adhesion and cell growth. Activated Wg signaling could downregulate E-cadherin, directly affecting adhesion as it does in some mammalian cell lines (Birchmeier et al, 1993; Takeichi 1993; Birchmeier and Behrens, 1994), all the while affecting Dpp and/or other signaling pathways concurrently to regulate the number and viability of the cells affected.

The use of the advanced genetic and molecular genetic tools available in *Drosophila* helped to tease apart this whirlwind of events. By changing one condition, for example increasing or eliminating one protein like E-cadherin, we got one piece of the puzzle. The overexpression of E-cad in MARCM clones in the larval brain seemed to have no effect on normal morphology or adhesion in the developing brain. However, removal of E-cad did have an effect, resulting in the formation of axon knots. This was not unexpected, as many studies in tissue culture revealed segregation of cells when cadherin levels were altered (Friedlander *et al*., 1989; Steinberg *et al*., 1994). What was unexpected was the fact that loss of E-cadherin did not result in segregation of epithelial cells or the presence of the epithelial loop phenotype. In fact we saw a complete lack of *E-cad* null clones in the area of the laminar furrow of the developing larval brain. One possibility is these cells are eliminated by apoptosis because they are somehow recognized as defective. Another possibility is that due to their lack of adhesion, they are no longer properly tethered or positioned in the laminar furrow region, an area that normally has high level of E-cadherin expression. Perhaps these *E-cad* mutant cells migrate (or are pushed by the normal, proliferating neuroblasts) more medially and/or deeper into the brain to positions where there is normally less E-cad. (We did observe *E-cad* null clones in the medial OPC). The latter possibility could be addressed by examining *E-cad* null clones at an earlier developmental stage, *i.e.* second instar, to see if

any mutant cells are present in/near the laminar furrow region. Tunnel labeling at this time point may also allow us to address whether the *E-cad* mutant clones are also undergoing cell death.

As previously mentioned, we did recover *E-cad* null clones in the more medial areas of the OPC. Cells within these clones differentiated as neurons, extended axons, and their mutant axons formed axon knots similar to those seen in *APC* double mutant cells. Because we were able to mimic the *APC* axon knot phenotype by eliminating E-cad, we concluded that cell adhesion is likely to play a role in a least one of the observed *APC* mutant phenotypes. The *E-cad* null clones, whose only presumed defect is in cell adhesion, can still either receive signals from their environment that tell them to differentiate, or follow a preprogrammed plan to differentiate into neurons. Axon pathfinding in mammalian cells is known to depend on cadherins (reviewed in Ranscht, 2000). As these E-cad deficient neurons attempt to send out there axons and target appropriately, perhaps they are unable to make stable contacts with their neighbors, to correctly interact with their surroundings and target appropriately. Instead, the elongating *E-cad* null axons coil upon themselves, generating the abnormal structures we call axon knots. Another way we could examine this hypothesis is to address axon guidance in the *Drosophila* larval brain mushroom body. Previous studies have defined exactly where the mushroom body axons innervate (Ito *et al*., 1997; Lee *et al*., 1999). Using mushroom body specific *Gal4* lines to generate MARCM clones, we could directly address the importance of E-cad in targeting *Drosophila* axons in the developing larval brain.

Our second exploration of mechanisms focused on Wg signaling. We activated Wg signaling by overexpressing Arm^{S10}, an inducible form of Armadillo that can not be

destroyed by the normal proteosomal pathway. This activates downstream Wg target gene expression, which can then initiate a multitude of effects. By doing so, we were able to mimic both the epithelial loop and axon knot phenotypes seen in our *APC* double mutants. However this did not generate loops that were as large or expansive as those seen in *APC* double null mutants, although the effect was qualitatively similar. These studies indicated that inappropriate activation of the Wg signaling pathway is sufficient to generate both in the phenotypes seen in *APC* mutant clones. In these studies we observed differences in the levels of Arm^{S10} expression, as assessed by the myc tag staining. Perhaps the use of a "stronger" Gal4 driver, such as *tubulin-Gal4* instead of *elavGal4*, would generate higher levels of Arm^{S10} expression in the MARCM clones and thus give a more robust epithelial loop phenotype. The difference in phenotypic strengths may not be surprising as loss of both APCs has much stronger effects in embryonic Wg signaling than expression of Arm^{S10} (Pai *et al*.,1997, Akong *et al*., 2002b)

To expand upon the idea that Wg signaling plays a role in generating the *APC* mutant phenotypes, I blocked Wg signaling, using a dominant negative version of the Wg regulated transcription factor *TCF*, expressed in the *APC* double null mutant clones. The expression of this construct ameliorated the *APC* double null phenotypes. In some cases, no mutant morphology was observed in the *APC* double null clones, something that was <u>never</u> seen in the absence of expression of the dominant negative *TCF* construct. The incomplete penetrance seen with the dominant negative *TCF* construct is again due to the difference in levels of expression generated by the *elavGal4* driver in different clones. The use of a stronger Gal4 driver, such as *tubulin-Gal4*, might give a more consistent level of expression of the dominant negative construct, and thus produce a more uniform elimination of the

mutant phenotypes. However, it must be noted that even in the *APC* double null mutant clones there are a range of phenotypic severities. Perhaps the *APC* mutant clones with no mutant phenotype in these experiments would have had the less severe version of the phenotype without the expression of the dominant negative construct. Conversely, the *APC* mutant clones in these experiments that showed mild phenotypes may have had the most severe version of the phenotype had they not had expression of the dominant negative *TCF* construct.

One known direct Wg target gene in certain mammalian cells is *E-cad* (Jamora *et al.,* 2003*)*. Most studies link activation of signaling with the increase of E-cad. Our studies suggested that overexpressing E-cad alone had no effect. We did not find any apparent increase in E-cad proteins, nor did we see any increase in gene expression using a transcriptional reporter construct, *E-cad-lacZ* in our *APC* double mutant clones. We did sometimes see what could be interpreted as a decrease in *E-cad-lacZ* expression, which would be consistent with our studies where we specifically removed E-cad. Perhaps *E-cad* is not a Wg target gene in the developing larval brain. It is possible that a Wg independent function of *APC* is to somehow stabilize E-cad at AJ. In this case, removal of APC could disrupt normal E-cad localization perhaps leading to axon knots and changes in migration.

We also examined a number of other well characterized Wg target genes in the larval brain. We found that several proteins such as Engrailed and Vestigal, encoded by genes that are Wg targets in other tissues such as the embryonic epidermis and the imaginal disc, are not expressed in the larval brain.

The best characterized direct Wg target gene expressed in the developing brain is the TGFβ homolog, *Dpp*. Like Wg, Dpp is a secreted ligand that binds to a transmembrane

receptor to stimulate its signal transduction pathway. Within the developing wing disc epithelium, Dpp signaling is crucial in the establishment and maintenance of the cell shape and epithelial integrity. The transmembrane TGF-β type I receptor Thickveins (Tkv) is primarily responsible for transduction of the DPP signal. Using a transcriptional reporter, *Dpp-lacZ*, we find that *APC* double null clones inappropriately activate and express high levels of Dpp.

In the *Drosophila* wing disc, clonal overexpression of either Dpp or a constitutively activated Dpp receptor, Tkv^{Q235D}, give rise to phenotypes similar to our *APC* double mutant epithelial loop phenotype. Large bulging clones with smooth borders that appear to minimize contact with their wild type neighbors result. An overgrowth phenotype results as well, as is described in more detail below (Martin-Castellanos and Edgar, 2002). Tkv^{Q235D} mutant phenotypes are affected by their position along the anterior-posterior axis of the disc, with the most severe phenotypes resulting when clones are induced in areas of the disc furthest from the endogenous Dpp signal. We see a similar effect in *APC* double null clones. The most severe *APC* mutant phenotypes are present on the anterior side of the brain, the region furthest from the endogenous Wg and Dpp signals (posterior side of the brain). *APC* double mutant clones close to the endogenous Wg and Dpp signals, along the posterior side of the brain, also express Dpp, however no epithelial loops or axon knots were observed here. In the imaginal discs, it is known that cells at different distances from the Wg or Dpp source are exposed to different concentrations of the morphogen, and in some cases can activate different sets of target genes (Zecca *et al.,*1996; Nellen *et al.,* 1996, Lecuit *et al.,* 1996). For example, in the wing disc *spalt* is only expressed in cells along the Anterior/Posterior boundary (A/P) where high levels of Dpp are observed. Conversely, the cells farther from

the A/P boundary express a different target gene, *optomotor-blind (omb)* which requires less Dpp for activation. This sets up different regions within the disc, each expressing a different Dpp target gene. The phenotype differences we see in *APC* double mutant clones along the Anterior/Posterior (A/P) axis of the brain may be due to the differences in Wg or Dpp target gene activation resulting from changes in the levels of Wg and/or Dpp. Since omb is expressed in larval brains, it would be useful to address its activation using *omb-lacZ* in the *APC* double mutant clones.

Effects on cell growth after activation of the *Dpp* pathway have also been documented. In the case of an activated Dpp receptor (Tkv^{Q235D}), cell growth was increased by approximately 20% without affecting Cyclin E, String, or phospho-histone H3 levels. This is again strikingly similar to our findings. *APC* double null mutants show consistent and strong activation of Dpp, as measured by *Dpp-lacZ*, in areas of the brain that generate mutant phenotypes (with respect to the anterior-posterior axis). When cell growth was assessed in *APC* double null clones, measurement of *APC* double mutant clones in the anterior portion of the brain showed a greater than 2 fold increase in volume with respect to wild type clones but no changes in Cyclin E or phospho-histone H3 were seen. While there is no measurable increase in proliferation, a shortening of the cell cycle by 20% (as was seen in the activated Tkv^{Q235D} clones) over 10 rounds of division will result in a 2 fold increase in the volume of the clone. Perhaps the "overgrowth" observed in *APC* double mutant clones in the anterior region of the brain is due to activated Dpp signaling that shortens the cell cycle.

This has led us to examine the effects of overexpression of the Dpp ligand and overexpression of the constitutively activated version of Tkv (Tkv^{Q235D}) in MARCM clones in the larval brain. Preliminary results show minor, if any, defects associated with

overexpression of the Dpp ligand (data not shown). There are no apparent changes in the clone volume and we did not observe the formation of axon knots. The only phenotype observed was seen in a single clone, which had a slight disruption in the laminar furrow region of the brain that is consistent with disruptions seen in some of the *APC* double mutant epithelial loops. One caveat to this experiment is the assumption that Tkv receptors are present and can respond to excess Dpp ligand overexpressed in this region of the larval brain. This makes the overexpression of the activated Tkv receptor, Tkv^{Q235D}, a more relevant comparison to what we believe is happening in the APC double mutant clones. We are currently addressing this question by making Tkv^{Q235D} MARCM clones in the larval brain.

While there is ample evidence in the wing disc that activation of Dpp signaling can result in overproliferation and outgrowths, it must be noted that there is contradictory evidence that mutational inactivation of Dpp signaling can also generate a similar segregation phenotype. Clonal analysis of cells lacking *Tkv* revealed changes in cell shape and segregation of the *tkv* mutant cells away from their wild type neighbors, producing cystlike epithelial extrusions in the wing disc. These extruded cells form structures reminiscent of the rosettes seen in our *APC* double mutant clones in the larval brain. This extrusion was shown to be independent of cell death, and blocking apoptosis did not rescue the phenotype (Gibson & Perrimon 2005). Ectopic F-actin was observed at the apical adherens junctions of *tkv* mutant cells and at the interface between tkv^{-} and wild type neighbors, a result similar to what we saw at the boundary between *APC* double mutant and wild type cells. Martin-Castellanos and Edgar (2002) also examined $tkv^{-/-}$ clones and compared their results to clones with activated Dpp signaling. They found inhibition of Dpp signaling (via mutational inactivation) had the strongest effect in medial areas of the wing disc, where Dpp levels are

normally high, as opposed to what was found in clones of cells with activated Dpp signaling, where the strongest phenotypes were seen in the lateral regions of the disc, far from the endogenous Dpp source. It is unclear how similar effects result from opposing Dpp signaling events. Identifying the Dpp targets responsible for growth effects and determining how those targets are integrated with inputs from other signaling pathways is necessary to explain this apparent paradox.

Unlike mammals, *Drosophila* Dpp is the only BMP ortholog (being most similar to mammalian BMP4; reviewed in van den Brink, 2004). Interestingly, BMP signals do regulate proliferation in the intestinal epithelium, but here it works to oppose Wnt signaling. BMP signaling has been shown to normally regulate cell death via apoptosis in intestinal epithelial cells. In fact, loss of function mutations in the BMP receptor, *BMPR1A*, and its signaling intermediate *SMAD4*, result in another familial form of colorectal cancer called juvenile polyposis syndrome (JPS). Intestinal polyps are classical characterized based on their histology. Adenomatous polyps are defined as neoplasms with clonal expansion of dedifferentiated epithelial cells. In contrast, hamartomatous polyps, which are typical in JPS, are not necessarily considered neoplastic in nature and seem to differentiate normally, with expansions of both epithelial and other mucosal cell types. Some reports speculate that in intestinal crypts both BMP and Wnt signaling are acting in opposition to regulate proliferation of the intestinal stem cells. One report, using a conditional knockout of BMP signaling in the intestinal epithelium of mice, links the two pathways via PI3 kinase signaling (He *et al*., 2004). This hypothesis that a loss in BMP signaling leads to activation of PI3 kinase-Akt signaling, which in turn activates βcat is quite controversial. However it is very interesting that the "polyps" (epithelial loops) we observed in our *APC* double null mutant

clones do not seem to dedifferentiate, as is the case with adenomatous polyps, nor do they have dramatic overproliferation. Instead, the neuroblasts comprising the epithelial loops give rise to progeny that can differentiate into neurons that can fasciculate axons. Thus it is possible that our *APC* mutant phenotype does not recapitulate what is happening in adenomatous polyps, but in fact is more phenotypically similar to hamartomatous polyps. Our results with Dpp-lacZ staining however show that Dpp signaling is dramatically activated, as opposed to BMP signaling being inactivated as is the case in JPS. Perhaps this again reflects a difference in the milieu, resulting in different downstream target genes that are specifically activated by the two or more pathways within a specific tissue.

This work has attempted to dissect APCs functions in the living animal, *Drosophila melanogaster*. The developing larval brain is a complex tissue with a number of different cell types, including dividing stem cells that may have analogous regulatory mechanisms to those in the human gut. Combined with the powerful genetic tools available in flies, this is an excellent place to study APC function. We have described roles for both cell-cell adhesion and altered Wg (and perhaps Dpp) signaling in the formation of abnormal structures in the brain such as epithelial loops and axon knots. This data provoke new questions. Are the changes in cell growth due to Dpp signaling or does Wg have additional target genes yet to be defined in the larval brain that can regulate growth? Perhaps one of the biggest questions is how relevant are our finding with respect to the molecular mechanisms that are involved in the initiation and formation of colon polyps and medulloblastomas. If these pathways are conserved from fly to human, ą investigation into the Dpp signaling pathway in medulloblastoma may be a fruitful avenue of research.

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