**Adenomatous polyposis coli regulates Drosophila intestinal stem cell proliferation**

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Adult stem cells define a cellular reserve with the unique capacity to replenish differentiated cells of a tissue throughout an organism’s lifetime. Previous analysis has demonstrated that the adult *Drosophila* midgut is maintained by a population of multipotent intestinal stem cells (ISCs) that resides in epithelial niches. *Adenomatous polyposis coli (Apc)*, a tumor suppressor gene conserved in both invertebrates and vertebrates, is known to play a role in multiple developmental processes in *Drosophila*. Here, we examine the consequences of eliminating *Apc* function on adult midgut homeostasis. Our analysis shows that loss of *Apc* results in the disruption of midgut homeostasis and is associated with hyperplasia and multilayering of the midgut epithelium. A mosaic analysis of marked ISC cell lineages demonstrates that *Apc* is required specifically in ISCs to regulate proliferation, but is not required for ISC self-renewal or the specification of cell fate within the lineage. Cell autonomous activation of Wnt signaling in the ISC lineage phenocopied *Apc* loss and *Apc* mutants were suppressed in an allele-specific manner by abrogating Wnt signaling, suggesting that the effects of *Apc* are mediated in part by the Wnt pathway. Together, these data underscore the essential requirement of *Apc* in exerting regulatory control over stem cell activity, as well as the consequences that disrupting this regulation can have on tissue homeostasis.

**KEY WORDS:** *Drosophila*, Intestinal stem cells, *Apc*

**INTRODUCTION**

Many adult tissues require the activity of tissue-specific stem cell populations to maintain homeostasis throughout the course of an organism’s lifetime. The dual characteristics of self-renewal and multipotency make stem cells ideally suited for this central role. Adult stem cells reside in specialized microenvironments called niches, which regulate stem cell behavior at baseline homeostasis and dynamically respond to changing environmental stimuli by modulating lineage output (reviewed by Jones and Wagers, 2008). Tight control of stem cell proliferation is essential to ensure that homeostatic balance is maintained; disruption of stem cell proliferation can lead to homeostatic imbalance, compromised wound healing, and disease. However, little is currently known about the factors that constrain stem cell proliferation.

The precise location and cellular architecture of the adult stem cell niche has been defined with high resolution in only a small number of tissues (reviewed by Morrison and Spradling, 2008). The ability to identify, manipulate and mark individual stem cell lineages has made *Drosophila* an excellent model system with which to dissect stem cell regulation. For example, the adult *Drosophila* midgut contains approximately 2000 intestinal stem cells (ISCs) distributed along the anteroposterior (AP) axis of the organ (Fig. 1A,B) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs have a pyramidal morphology and are located in an epithelial niche distant from the midgut lumen, adjacent to the basement membrane and the visceral musculature surrounding the midgut. ISCs are multipotent and give rise to a lineage that consists of two types of differentiated daughters, the enteroendocrine (ee) cells and the enterocytes (ECs), which together form a cellular monolayer lining the length of the adult midgut.

*Adenomatous polyposis coli (Apc)* encodes an evolutionarily conserved protein, which was first identified by positional cloning as one of the genes commonly deleted in the hereditary colon cancer syndrome familial adenomatous polyposis (FAP) (Groden et al., 1991; Kinzler et al., 1991). Mutations in both copies of *Apc* are also detected in many spontaneous colorectal adenomas (Miyoishi et al., 1992; Powell et al., 1992; Ichii et al., 1992). As the majority of *Apc* mutations are loss of function, *Apc* is thought to function as a tumor suppressor gene. Early insight into the molecular function of *Apc* came from the identification of β-catenin, a Wnt pathway effector, as a binding partner for *Apc* (Su et al., 1993; Rubinfeld et al., 1997). The requirement for *Apc* in the Wnt signaling pathway is now well characterized and current models suggest that *Apc* together with Axin, Glycogen Synthase Kinase 3 and Casein Kinase 1 comprise a multiprotein β-catenin destruction complex. In the absence of Wnt ligands, the destruction complex phosphorylates β-catenin, targeting it for proteasomal degradation.

The *Drosophila* genome, like that of both mouse and human, contains two *Apc* family members, *Apc1* (also known as *Apc* – FlyBase) and *Apc2* (Hayashi et al., 1997; Ahmed et al., 1998; McCartney et al., 1999; Hamada et al., 1999a). Previous analysis in *Drosophila* has implicated *Apc* in a number of developmental processes, including cell survival, cell fate specification and proliferation. Yet, the role of *Apc* in maintaining adult *Drosophila* midgut homeostasis has not been examined. In this study, we investigate the consequences of eliminating *Apc* function on adult midgut homeostasis and ISC behavior.

**MATERIALS AND METHODS**

*Drosophila* strains and culture

*Apc2C9/TM6 Tb, Hu (Apc2C9)* is a temperature-sensitive allele with a permissive temperature of 18°C and non-permissive temperature of 27°C; gift from B. McCartney, Carnegie Mellon University, Pittsburgh, PA, USA. *w; FRT18A Apc2C9, Apc1Q8/TM6C (Apc2C9)* is null for Armadillo degradation but retains the ability to promote Wnt signaling in the eye; *Apc1Q8* is a null allele; gift from B. McCartney. *w; FRT18A Apc2C9, Apc1Q8/TM6C (Apc2C9)* is a strong hypomorph resulting from a deletion; gift from Y. Ahmed, Dartmouth...
College, Hanover, NH, USA), w; FRT82B AsxI042420/TM3 Sh (AsxI042420 is a
tissue-specific allele; gift from Y. Ahmed), y; w; UAS-GFP, hsflp; tubGal4,
FRT82B tubGal80/TM6B (gift from N. Perrimon, Harvard Medical School,
Boston, MA, USA), y; esgGal4/Cyo (gift from S. Hayashi, RIKEN, Kobe,
Japan), y; UAS-NK4Δ, UAS-GFP, y, y; esgK606/Cyo (esg-lacZ), y; FRT82B
hsFLP (this stock was used as a wild-type control in the mosaic analysis
performed in this study), y; UAS-arm510 (activated arm), y; UAS-
pan.dTCF4N4 (dominant-negative allele). Unless indicated, all strains were
obtained from the Bloomington Stock Center. Crosses were cultured on
standard dextrose media and were transferred to fresh food augmented with
yeast paste every 1-2 days during the experimental period. Crosses were reared
at 18, 25, or 29°C, in passively illuminated and humidified incubators. In this
study, only adult female flies of the following genotypes were analyzed.

Figure 2:
/+ /w; esgK606/+ ; FRT82B Apc2Δ10, Apc1ΔQ8 /+ . Apc1ΔQ8 / Apc2Δ10.
/+ /w; FRT82B Apc2Δ10, Apc1ΔQ8 / tubGal4, FRT82B

Figures 3, 4 and S2:
y; hsFLP, UAS-GFP / w; + ; FRT82B hsπM / tubGal4, FRT82B
y; hsFLP, UAS-GFP / w; + ; FRT82B Apc2Δ10, Apc1ΔQ8 /+ ;
tubGal80.

Figure 5:
y; hsFLP, UAS-GFP / w; esgK606/+ ; FRT82B hsπM / tubGal4, FRT82B
tubGal80.

Figures 6, S3 and S4:
y; hsFLP, UAS-GFP / w; UAS-NK4Δ; + ; FRT82B hsπM / tubGal4, FRT82B
y; hsFLP, UAS-GFP / w; + ; FRT82B Apc2Δ10, Apc1ΔQ8 /+ ;
tubGal4, FRT82B

Figures 7 and S5:
y; hsFLP, UAS-GFP / w; + ; FRT82B hsπM / tubGal4, FRT82B
y; hsFLP, UAS-GFP / w; UAS-pan.dTCF4N4 /+ ; FRT82B
y; hsFLP, UAS-GFP / w; + ; FRT82B Apc2Δ10, Apc1ΔQ8 /+ ;
tubGal4, FRT82B

Mosaic analysis
The MARCM system was used to generate marked ISC lineages or
‘clones’. MARCM was used to produce both marked wild-type and mutant
lineages; in addition, all manipulations involving the misexpression of UAS
transgenes were also performed using the MARCM system to ensure that
only ISC lineages were analyzed. To induce clones, experimental animals
were subjected to a 37°C heat pulse for 35-45 minutes within the first week
of adulthood. Induction protocols varied from one to three heat pulses
within a 24-hour period, depending on the desired rate of mitotic
recombination.

Temperature shift experiments
Croses were established and cultured at 18°C until adulthood. F1 progeny
were divided into two equal pools; controls were cultured at 18°C and the
experimental group was shifted to 29°C for 10 days. BrdU was administered
ad libitum in Drosophila media (200 μl of 6 mg/ml BrdU in 20% sucrose
per vial) for the 24-hour period immediately preceding the 10-day time point.

Histology
Adult flies were dissected in 1× PBS (Sigma, USA). The gastrointestinal
trac tract was removed and fixed in a final solution of 0.5× PBS (Sigma, USA)
and 4% electron microscopy grade formaldehyde (Polysciences, USA) for
a minimum of 30 minutes. Samples were washed in 1× PBS with 0.1%
Trition X-100 (PBST) for 2 hours and then incubated with primary antibodies
overnight. Samples were washed in PBST for 2 hours and then incubated with
secondary antibodies for 3 hours. Finally, samples were washed in
PBST overnight. Mounting media containing DAPI (Vectorshield, USA) was
added and samples were allowed to clear for 1 hour prior to mounting. All
steps were completed at 4°C, with no mechanical agitation.

Antisera
Primary antibodies
Chicken anti-GFP (Abcam, USA) used at a dilution of 1:10,000; rabbit anti-
β-Gal (Cappel, USA), 1:2000; mouse anti-Pros (Developmental Studies
Hybridoma Bank; DSHB), 1:100; mouse anti-DI (DSHB), 1:10; mouse anti-
BrdU (Becton Dickinson, USA), 1:100; rabbit anti-Pdm1 (gift of W. Chia
(Yeo et al., 1995)), 1:1000; rabbit anti-Tachykinin (gift of D. Niesel (Siviter
et al., 2000)). 1:1000.

Secondary antibodies
Goat anti-chicken Alexa 488 (Molecular Probes, USA) used at a dilution of
1:2000; goat anti-mouse Alexa 568 (Molecular Probes, USA), 1:2000; goat
anti-mouse Alexa 633 (Molecular Probes, USA), 1:2000; goat anti-rabbit
Alexa 568 (Molecular Probes, USA), 1:2000.

Dyes and mounting media
Alexa 594-conjugated Phalloidin (Molecular Probes, USA) diluted 1:500;
Vectashield+ DAPI mounting media (Vector, USA).

Microscopy and imaging
Samples were examined on a Leica DMS5000 upright fluorescent
microscope. Confocal images were collected using a Leica TCS SP5
confocal microscope system. Images were processed for brightness and
contrast, and assembled in Photoshop CS (Adobe, USA).

Cell counts, measurements and statistical analysis
In the temperature-shift analyses, entire midguts were scored. BrdU+ esg-
lacZ+ positive pairs of cells were scored as a single event; clusters of three
cells were scored as two events. In those TS experiments lacking esg-lacZ
only small BrdU clusters were scored (Fig. 2D). Midgut area was
determined by first acquiring digital images of the posterior midgut on a
compound microscope; Leica application suite (LAS) software was then
used to determine the area of the posterior region. Next, the maximal number
of nuclear layers and maximal epithelial height was determined based on
confocal micrographs taken from the same posterior midguts. Only those
regions of the epithelium from the outer face of posterior midgut, which has
a larger circumference, were analyzed to minimize secondary distortion of
the epithelium due to the coiled morphology of the midgut (Fig. 3E,F).

In our mosaic analysis, the number of cells per clone was scored in either
anterior and/or posterior midgut frames 5 days following induction (Fig. 4A;
see also Fig. S1 in the supplementary material; Fig. 4DF, Fig. 6G). Data
from the anterior and posterior midgut are combined in Fig. 4D, but
separated by region in Fig. 4F. Unless indicated, counts were collected from
posterior frames. Clones within selected frames were defined as clusters of
contiguous cells, as assessed at 40× magnification on compound or confocal
microscopes. For a clone to be scored it had to lie completely in the field of
view; clones that partially wrapped around the ‘edge’ of the midgut sample
were excluded from the analysis to minimize counting inaccuracy. To
determine the number of dividing cells per clone, GFP+ pH3+ cells were
counted from the middle frame of the posterior midgut 5 days after heat-
shock induction (Fig. 4E). To assay ISC self-renewal, the number of clones
RESULTS

Apc regulates adult midgut homeostasis

Previous studies of Apc have demonstrated that functional redundancy exists between Apc1 and Apc2 in a number of Drosophila tissues, including the embryonic epidermis, the wing and eye imaginal discs, and the larval brain (Ahmed et al., 2002; Akong et al., 2002a; Akong et al., 2002b; McCartney et al., 2006). We therefore reasoned that simultaneous reduction of both Apc1 and Apc2 would be a direct means by which to initially assess the requirement for Apc in the adult midgut. To globally reduce Apc function in a conditional manner, we used a temperature-sensitive allele encoded by escargot (esg) and eye imaginal discs, and the larval brain (Ahmed et al., 2002; Akong et al., 2002a; Akong et al., 2002b; McCartney et al., 2006). Unshifted control animals grown at the permissive temperature were compared with experimental animals shifted to the non-permissive temperature for 10 days during adulthood. In these experiments, we observed an increase in the number of BrdU+ cells in experimental samples that often appeared as clusters of two to three small cells (Fig. 2A-C). Previous analysis has demonstrated that the transcriptional repressor encoded by esg can be used to identify ISCs and their nascent daughters, called enteroblasts (EBs) (Fig. 2A,B) (Michellini and Perrimon, 2006). To quantify the Apc(+/+), we counted the number of small BrdU+ clusters in the midgut following a 24-hour BrdU pulse that immediately preceded the 10-day time point (BrdU administered ad libitum in food media). Global reduction in the levels of Apc1 and Apc2 resulted in a significant increase in the number of BrdU+ small cell clusters (Fig. 2D; n=11). This analysis suggested that Apc is necessary for the maintenance of midgut homeostasis in the adult.

Apc is required in ISC lineages to maintain homeostasis

Although the TS analysis suggested a role for Apc in regulating midgut homeostasis, it did not directly establish a requirement for Apc in the midgut. To test the requirement for Apc specifically in the midgut, we next conducted a mosaic analysis of Apc double mutants (Apc(2)10, Apc(1)08; subsequently referred to as Apc clones, except where indicated). Positively marked ISC lineages lacking Apc function were generated in the adult using the MARCM system (Lee and Luo, 1999) and identified on the basis of GFP expression. At 20 days after induction, midguts containing Apc clones were associated with gross anatomical changes, including midgut hyperplasia and multilayered cellular masses that distorted the luminal surface of the midgut (Fig. 3A-D). Plotting the maximal number of nuclear layers as a function of midgut area revealed an inverse correlation in Apc mosaics and an increase in each parameter compared with wild type (Fig. 3E; wild type, n=11; Apc, n=12). At 20 days after induction, it was often difficult to unambiguously identify individual marked Apc mutant lineages because of the changes in overall midgut morphology. However,
by plotting the maximal number of nuclear layers as a function of maximal epithelial height, it was evident that up to five layers could be detected in mosaic Apc midguts (Fig. 3F; wild type, n=11; Apc, n=12). Finally, in those cases in which individual clones could be definitively identified, Apc mutant lineages were found to produce multilayered masses more frequently than were wild type (Apc, 31.5%, n=181; wild type, 1.3%, n=227).

The analysis of Apc clones was extended to determine the number of labeled cells in individual ISC lineages. As we observed little or no distortion of the midgut due to multilayering at early time points, a 5-day post-induction time point was selected to quantify the number of cells per ISC clone. Cell counts were performed by scoring clones at two defined regions within each midgut analyzed (Fig. 4A; see Materials and methods and Fig. S1 in the supplementary material for experimental criteria). Mosaic analysis revealed a significant increase in the number of cells per clone in Apc lineages compared with wild-type controls (Fig. 4B-D; n=76). This increase in clone size suggested that Apc loss leads to an increase in proliferation. Consistently, we observed that Apc mutant lineages were associated with a significant increase in the number of phospho-histone H3 positive (pHH3+) cells when compared with wild-type cell lineages (Fig. 4E; wild type, n=18; Apc, n=17).

An analysis of Apc clone size along the AP axis of the gastrointestinal tract was performed to determine whether the requirement for Apc was dependent on midgut region. This analysis revealed a significant increase in Apc clone size in both the anterior and posterior midgut compared with wild-type controls (Fig. 4F). Although the trend towards increased clone sizes in ISCs lacking Apc was observed throughout the midgut, the average size of Apc clones was found to be greater in the posterior. Taken together, our mosaic analysis demonstrates that Apc is required in the ISC cell lineage throughout the midgut to maintain homeostasis.

**Apc loss does not affect ISC self-renewal**

One possible explanation for the hyperplasia associated with Apc loss is that Apc affects ISC self-renewal. Early studies of Drosophila germ line stem cells employed a lineage-tracing assay to measure stem cell self-renewal (Margois and Spradling, 1995). A pulse/chase experiment was used to determine the number of marked stem cell lineages retained in the tissue at defined intervals following induction. To determine whether Apc loss affected ISC self-renewal in the midgut, we generated labeled ISCs and counted the number of marked ISC lineages per midgut at 5 and 10 days after induction. When the number of ISC lineages per gut lacking Apc was compared with wild type, no significant differences were detected (Fig. 4G). These data show that Apc loss does not detectably affect the fidelity of ISC self-renewal.

**ISCs lacking Apc generate the differentiated cells of the lineage**

A second possible explanation for the hyperplasia associated with Apc loss is that Apc is required for differentiation in the ISC lineage. Inspection of Apc clone morphology initially suggested that cell fate in the lineage was correctly specified. To more rigorously analyze cell fate in Apc lineages, we examined a panel of molecular markers to label distinct cell types in the ISC lineage. The transcriptional
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The presence of esg− cell populations in Apc lineages suggested that ee cell and EC fates had been specified in the absence of Apc. To test this directly, we examined Apc lineages for the presence of ee cells and ECs using molecular markers. To determine whether ee cell fate is specified in lineages lacking Apc, we examined the expression of Prospero (Pros), a marker of the ee cell population (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006). The presence of Pros+ cells was detected in Apc clones (Fig. 5D), as observed in wild-type cell lineages (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Similarly, examination of Tachykinin (Tk) expression, which marks a specific subset of ee cells (Ohlstein and Spradling, 2006), demonstrated that Apc is not necessary for this ee cell subtype (Fig. 5E). Finally, differentiated ECs are distinguished by their large, polyploid nuclei (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006), demonstrated that Apc clones revealed the presence of large Pdm1+ nuclei within mutant lineages (Fig. 5F). Taken together, this analysis demonstrates that ISCs lacking Apc are capable of producing both ee cell and EC fates.

Apc is required in ISCs to regulate proliferation

Our experiments show that a reduction or loss of Apc leads to ISC lineages of increased size, as well as an increase in the number of both S-phase and M-phase markers. Over time, this leads to hyperplasia and multilayering of the midgut. Yet, no alteration in ISC self-renewal or cell fate specification was detected using lineage-tracing analysis. Collectively, these findings raised the possibility that Apc is required specifically in ISCs to regulate proliferation. Previous analyses demonstrated that targeted knockdown of Notch (N) in ISCs leads to an expansion of esg+ cell number in the midgut (Michelli and Perrimon, 2006). Similarly, the generation of ISC lineages completely lacking N function leads to an expanded clonal population of undifferentiated cells (Ohlstein and Spradling, 2006). Thus, reduction of N function generates a population of ectopic cells that exhibit the characteristics of midgut ISCs.

If Apc functions specifically in ISCs to limit proliferation, then we predict that the loss of Apc should enhance the severity of the N loss-of-function phenotype. To directly test this possibility, we compared the number of cells per clone generated by ISCs lacking N with the number generated by ISCs lacking both N and Apc. Using the MARCM system, we created Apc mosaics that simultaneously expressed a N RNAi transgene (Presente et al., 2002) (N RNAi, Apc2g10; subsequently referred to as N RNAi, Apc clones). At 10 days after induction, the cell proliferation and multilayering observed in N RNAi, Apc clones was often extensive (Fig. 6A–D; see also Fig. S3 in the supplementary material), suggesting that Apc enhances the N RNAi phenotype.

A quantitative analysis was performed to compare the number of ISCs generated in N RNAi, Apc clones with the number of ISCs in marked N RNAi lineages 5 days after induction (Fig. 6E–G; see also
Apc lacking. Anti-Tachykinin (red) marks a subset of ee cells in the clone. Anti-pros (red) marks ee cells in the clone. (E) ISC lineages bar: 50 μm. This analysis revealed a significant increase in the mitotic index of ISCs lacking both N and Apc compared with a lack of N alone (Fig. 6H; N<sup>RNAi</sup>, Apc, n=16; N<sup>RNAi</sup>, Apc, n=18). This finding, together with the increased size of N<sup>RNAi</sup>, Apc mutant clones, demonstrates that the loss of Apc can affect ISC proliferation in the midgut independently of N-mediated cell fate specification.

Wnt signaling regulates homeostasis in ISC lineages

The requirement for Apc in the β-catenin destruction complex suggested that the Wnt signaling pathway might function to regulate the ISC lineage. To investigate this possibility, we first examined the effects of activating Wnt signaling on the size of marked ISC lineages. Wnt signaling was activated by generating mosaic animals expressing a constitutively active form of β-catenin, arm<sup>510</sup> (Pai et al., 1997). As in the case of Apc loss, arm<sup>510</sup> clones appeared abnormally large (see Fig. S5A-C in the supplementary material).

To quantify the arm<sup>510</sup> phenotype, we compared the number of cells labeled in ISC lineages expressing arm<sup>510</sup> to the number in marked wild-type lineages 5 days after induction. This analysis revealed a significant effect of arm<sup>510</sup> on ISC clone size compared with wild-type controls (see Fig. SSD in the supplementary material). A comparison of the Apc and arm<sup>510</sup> phenotypes revealed that in both cases there was a significant increase in clone size relative to wild type. Nevertheless, the magnitude of the increase was greater following Apc loss than in the presence of arm<sup>510</sup>, as has been observed in other contexts (e.g. Pai et al., 1997; Hayden et al., 2007) (see Fig. SSD in the supplementary material). Thus, Wnt pathway activation leads to an increase in the size of marked ISC lineages.

Apc hyperplasia is suppressed by reductions in Wnt signaling

The finding that constitutive Wnt activation resembled Apc loss raised the possibility that hyperplasia observed in ISC lineages lacking Apc resulted from Wnt activation. To test this directly, we examined the effect of blocking Wnt signaling in Apc mutants. In these studies, Wnt signaling was reduced by generating mosaic animals expressing a dominant-negative form of pangolin (pan), pan<sup>ΔN</sup>, a transcription factor necessary for Wnt signaling (Brunner et al., 1997; van de Wetering et al., 1997). Control experiments showed first that, in contrast to wild-type lineages, mosaic expression of pan<sup>ΔN</sup> resulted in reduced clone size 5 days after induction (Fig. 7A,B), as has previously been reported for the loss of other Wnt pathway components upstream of pan (Lin et al., 2008). Second, loss of a negative regulator in the Wnt pathway, Axin (Hamada et al., 1999b; Willert et al., 1999), phenocopied both Apc loss and arm<sup>510</sup> expression (Fig. 7C). Third, ISC lineages simultaneously expressing pan<sup>ΔN</sup> and lacking Axin led to complete suppression of the Axin loss-of-function phenotype (Fig. 7D). Together, these control experiments established that pan<sup>ΔN</sup> can suppress robust activation of the Wnt signaling pathway resulting from Axin loss. We next investigated whether pan<sup>ΔN</sup> was sufficient to suppress the Apc double mutant phenotype. Simultaneous expression of pan<sup>ΔN</sup> in ISC lineages lacking Apc<sup>ΔN</sup> and Apc<sup>ΔN</sup> led to a complete suppression of Apc hyperplasia (Fig. 7E,F). However, simultaneous expression of pan<sup>ΔN</sup> in ISC lineages lacking Apc<sup>ΔN</sup> and Apc<sup>ΔN</sup> resulted only in a partial suppression of the Apc phenotype (Fig. 7G,H). One potential explanation for these differences is that Apc<sup>ΔN</sup>, in contrast to Apc<sup>ΔN</sup>, encodes a protein that retains an amino-terminal fragment, which has been shown to have an activating role in the Wnt signaling pathway (Takacs et al., 2008). Similarly truncated alleles of Apc are known  to be associated with human adenomas (Lamllum et al., 1999; Rowan et al., 2000; Albuquerque et al., 2002; Cheadle et al., 2002). Taken
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DISCUSSION

In the current study, we report that loss of Apc results in a disruption of midgut homeostasis and is associated with hyperplasia and multilayering of the midgut epithelium. Our mosaic analyses show that Apc is required specifically in ISCs to regulate stem cell proliferation. By contrast, loss of Apc did not detectably affect self-renewal or cell fate specification in the ISC lineage. Activation of Wnt signaling in the ISC lineage phenocopied Apc loss and Apc mutants were suppressed in an allele-specific manner by abrogating Wnt signaling, suggesting that the effects of Apc are mediated in part by the Wnt pathway. The finding that Apc differentially affects ISC proliferation without obviously altering self-renewal or multipotency highlights the ability of the stem cell to fine-tune lineage output to meet homeostatic need; loss of Apc appears to short-circuit this regulation, providing increased cellular output in the absence of true physiological demand for new cells.

Previous analysis of Wnt signaling in the midgut has led to the assertion that Wnt functions as the primary maintenance signal for ISCs; cell-autonomous loss of Wnt transduction components results in a failure of ISC maintenance, while ectopic expression of Wnt ligand leads to an increase in the number of DI-expressing cells (Lin et al., 2008). These observations led to the following model: transduction of the Wnt signaling pathway in ISCs adjacent to a Wnt source maintains the stem cell population by preventing lineage differentiation. A central prediction of the model is that cell-autonomous activation of the Wnt signaling pathway in ISCs should lead to the production of daughter cells, which constitutively transduce the Wnt signal and, as such, remain undifferentiated. The predicted consequence of this manipulation is an expansion in the number of ISCs at the expense of differentiated cells within the lineage, as has been observed in the case of N loss (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). In this study, we directly tested this prediction by analyzing marked ISC lineages. Our experiments clearly demonstrate that in contrast to N loss, ISCs lacking Apc generate both differentiated cell types of the adult midgut: ee cells and ECs. Importantly, these findings suggest that ISCs and their daughters are not distinguished solely on the basis of Wnt signal transduction.

Several explanations could account for these apparent disparities. First, it is worth noting that although DI might be a reliable marker for certain stem cells in wild-type midguts, this might not be the case in every mutant background examined. For example, a previous analysis in Drosophila has demonstrated that Wnt activation is sufficient to stimulate high levels of DI expression in a cell-autonomous manner (Michelli et al., 1997). Thus, it is possible that Wnt pathway activation can uncouple DI expression from stem cell identity, thereby diminishing the utility of DI as a reliable stem cell marker. A second possible explanation is methodological; the use of genetic mosaic analyses to analyze individual ISC lineages, as in this study, might provide a different view of the Wnt pathway activation phenotype to that seen following the use of Gal4 driver lines. Third, it is possible that Wnt can affect stem cell maintenance upstream of Apc via a non-canonical pathway. For example, studies have demonstrated that Wnt signaling can act directly via dishevelled (dsh) to inhibit the N signaling pathway (Axelrod et al., 1996; Rulifson et al., 1996). Such effects might not be detected in the Apc, Axin or activated armS10 ISC lineages analyzed here.

Together, our studies show that the abrogation of Wnt signaling in ISC lineages is sufficient to suppress the effect of Apc loss in an allele-specific manner.

Fig. 6. Apc is required in ISCs to regulate proliferation. (A-F) The MARCM system was used to positively identify ISC lineages with GFP. (A,B) Clone outlines indicated in red, nuclei in blue (DAPI), and dividing cells in white (phospho-histone H3). (A) ISC clones expressing N-RNAi 10 days after induction. (B) ISC clones expressing N-RNAi and lacking Apc, 10 days after induction. (C,D) ISC lineages marked with GFP (anti-GFP, green; DAPI, blue); dashed red line indicates midgut outline. (C) ISC clones expressing N-RNAi 10 days after induction. (D) ISC clones expressing N-RNAi and lacking Apc, 10 days after induction. Extensive multilayering is evident in cross section. Asterisk indicates hyperplastic cells in lower focal planes extending up into the luminal space. (E-H) Quantitation of ISC proliferation phenotype. (E) ISC clones expressing N-RNAi 5 days after induction. (F) ISC clones expressing N-RNAi and lacking Apc, 5 days after induction. (G) Comparison of N-RNAi and N-RNAi, Apc clone size 5 days after induction. N-RNAi, Apc loss leads to an increase in the number of cells per clone (N-RNAi, n=16; N-RNAi, Apc, n=110). (H) Comparison of N-RNAi and N-RNAi, Apc mitotic index 5 days after induction. Apc loss leads to an increase in the ISC mitotic index (N-RNAi, n=18; N-RNAi, Apc, n=16). Error bars denote s.e.m. Scale bars: 50 μm.
In this study, we have demonstrated an increase in the number of dividing cells following 
Apc loss, which is consistent with what has previously been reported for mutations that activate the Wnt
pathway (Lin et al., 2008). And yet, as discussed above, the presence of differentiated cells within marked
Apc mutant lineages strongly suggested that the increase in dividing cells could not be explained
solely by a N-dependent change in cell fate within the ISC lineage

as was proposed by Lin et al. Based on our analysis of Apc, we
hypothesized that the increased proliferation following Apc loss
reflects a cell-autonomous requirement for Apc specifically in ISCs.
This view is further supported by the observation that Apc loss can
lead to an increase in mitotic index when N and Apc are
simultaneously removed from the ISC lineage. Thus, there is a
requirement for Apc specifically in ISCs to regulate proliferation
that is separable from N-dependent cell fate specification. Taken
together, we conclude that a primary requirement for Apc in the ISC
lineage is to autonomously restrict the proliferation of ISCs, and not
to regulate the choice of cell fate.

On the basis of our findings we propose that ISC activity is
regulated by the level of Wnt signal transduction (Fig. 8). In this
model, Wnt functions as a permissive signal for ISC self-renewal,
as we show that constitutive Wnt activation is not a sufficient
criterion to convert all ISC progeny to stem cells, nor is activation
sufficient to alter the fidelity of ISC self-renewal. Intermediate
levels of Wnt define an adaptive homeostatic range, which permits
the midgut to respond to environmental changes that the organism
encounters. ISCs transducing Wnt at levels outside this range
appear refractory to homeostatic input, as low levels of Wnt are
associated with ISC loss, whereas Wnt activation leads to
hyperplasia.

Nevertheless, our data do not rule out additional roles for Apc
in the ISC lineage. For example, it is possible that the hyperplasia
observed in Apc mutants reflects the combined requirement of Apc
to regulate proliferation both in the ISCs and in nascent EB
daughters. Similarly, Apc might also play a role in regulating cell
turnover in the midgut. Indeed, several of our observations support
the view that Apc might, in fact, be required for EC differentiation.
First, mosaic analysis of Apc shows that cells of the lineage
contribute to the multilayering phenotype, which suggests that
mutant cells might have failed to properly establish appropriate
adhesive contacts with the monolayer and/or the surrounding
extracellular matrix (Fig. 3D,F). Second, although ECs lacking Apc
appear to have large, polyplody nuclei and express molecular
markers such as Pdm1, their nuclei are often detectably smaller than
those of wild-type ECs (Fig. 4B,C). Furthermore, ECs lacking Apc	enably exhibit a restricted basal profile, failing to develop the tiled
morphology that is characteristic of wild-type ECs (Fig. 4B,C).
Finally, in the absence of Apc, ECs often display reduced
cytoplasm, suggesting a requirement for cellular growth, a
requirement that is not observed in either Axin mutant or armSc10
expressing ICS lineages (Fig. 7; see also Fig. S5 in the
supplementary material).

The discovery that Apc is somatically mutated in cells of the
smallest human adenomas (Miyoshi et al., 1992; Powell et al.,
1992) and that the frequency of Apc mutations detected among
early adenomas is roughly the same as the frequency of Apc
mutations in more advanced carcinomas (Powell et al., 1992) were
among the seminal observations that established Apc as the rate-
limiting step for gastrointestinal tumor initiation (reviewed by
Kinzler and Vogelstein, 1996; Clevers, 2006). Subsequently, a
number of Apc models have been established in both mouse and
zebrafish to study gastrointestinal tumorigenesis (Su et al., 1992;
Fodde et al., 1993; Oshima et al., 1995; Haramis et al., 2006). Yet,
the precise cell(s) in which Apc is required remained unknown.
Recently, through the use of refined genetic cell lineage tracing
methodologies in the mouse, specific subpopulations of cells in the
intestinal mucosa have been identified (e.g. Lgr5+ and Bmi1+),
which display the ability to self-renew and undergo multilineage
differentiation (Barker et al., 2007; Sangiorgi and Capecchi, 2008).
Subsequent studies have demonstrated that deletion of Apc specifically within the Lgr5+ cell population leads to the formation of rapidly proliferating cells in both the large and small intestine (Barker et al., 2009). Thus, in the case of both Drosophila ISCs and mouse Lgr5+ cells, loss of Apc leads to a disruption of homeostasis in the intestinal stem cell lineage. The remarkable parallels that exist between the dipteran and mammalian gastrointestinal tract suggest that the Drosophila midgut will continue to be a powerful genetic model system with which to dissect the molecular mechanisms underlying tumor initiation.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/3/2263/DC1

References


