Non-autonomous crosstalk between the Jak/Stat and Egfr pathways mediates Apc1-driven intestinal stem cell hyperplasia in the Drosophila adult midgut

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SUMMARY
Inactivating mutations within adenomatous polyposis coli (APC), a negative regulator of Wnt signaling, are responsible for most sporadic and hereditary forms of colorectal cancer (CRC). Here, we use the adult Drosophila midgut as a model system to investigate the molecular events that mediate intestinal hyperplasia following loss of APC in the intestine. Our results indicate that the conserved Wnt target Myc and its binding partner Max are required for the initiation and maintenance of intestinal stem cell (ISC) hyperproliferation following Apc1 loss. Importantly, we find that loss of Apc1 leads to the production of the interleukin-like ligands Upd2/3 and the EGF-like Spitz in a Myc-dependent manner. Loss of Apc1 or high Wg in ISCs results in non-cell-autonomous upregulation of upd3 in enterocytes and subsequent activation of Jak/Stat signaling in ISCs. Crucially, knocking down Jak/Stat or Spitz/Egfr signaling suppresses Apc1-dependent ISC hyperproliferation. In summary, our results uncover a novel non-cell-autonomous interplay between Wnt/Myc, Egfr and Jak/Stat signaling in the regulation of intestinal hyperproliferation. Furthermore, we present evidence suggesting potential conservation in mouse models and human CRC. Therefore, the Drosophila adult midgut proves to be a powerful genetic system to identify novel mediators of APC phenotypes in the intestine.

KEY WORDS: Apc, Drosophila midgut, Egfr, Myc, Stat, Intestinal stem cells

INTRODUCTION
Canonical, or β-catenin-dependent, Wnt signaling, which we will refer to as Wnt signaling, is an essential regulator of cell proliferation and differentiation in the vertebrate intestine (Ireland et al., 2004; Korinek et al., 1998; van de Wetering et al., 2002). Inactivating mutations in APC, a negative regulator of Wnt signaling, are detected in 80% of hereditary and sporadic forms of colorectal cancer (CRC) (Kinzler et al., 1991; Korinek et al., 1997). Mouse models have shown that inactivation of ApC is sufficient to drive intestinal hyperplasia (Andreu et al., 2005; Sansom et al., 2004). Moreover, Apc deletion within murine intestinal stem cells (ISCs) results in rapid adenoma formation, suggesting that they can act as cells of origin in CRC (Barker et al., 2009).

Multiple Wnt target genes have been identified in CRC cell lines, mouse models and human tumor samples carrying loss-of-function mutations in APC (Holstege and Clevers, 2006; Sansom et al., 2007; Van der Flier et al., 2007). Indeed, 4 days of Apc loss in the murine intestine is sufficient to lead to significant dysregulation of hundreds of genes (Sansom et al., 2004). Thus far, very few of these genes have been functionally tested in vivo to directly address their contribution to CRC. The identification of Wnt target genes required for intestinal proliferation in vivo holds great potential for the development of targeted CRC therapies, as well as for regenerative medicine.

Owing to its remarkable resemblance to the vertebrate intestine (Casali and Batlle, 2009), the Drosophila adult midgut is emerging as a useful model with which to study intestinal homeostasis, regeneration and abnormal proliferation. Importantly, the fly intestinal epithelium is replenished by its own ISCs (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Drosophila ISCs are randomly scattered along the basolateral membrane of the intestinal tube and, following division, they give rise to an undifferentiated progenitor, the enteroblast (EB), which differentiates into either the secretory cell lineage represented by the enteroendocrine (ee) cells or the absorptive epithelial cell lineage represented by the enterocytes (ECs). The Wnt signaling pathway shows a significant degree of conservation in the fly intestine. Loss-of-function analyses indicate that Wnt signaling is required for ISC proliferation during homeostasis and regeneration of the Drosophila midgut (Lin et al., 2008; Cordero et al., 2012), whereas overexpression of Wg or inactivating mutations in the two Drosophila Apc homologs, Apc1 (Apc – FlyBase) and Apc2, drive ISC hyperproliferation (Cordero et al., 2009; Lee et al., 2009; Lin et al., 2008). In contrast to the mouse phenotype (Sansom et al., 2004), hyperactivation of Wnt signaling in the Drosophila midgut does not affect cell lineage differentiation (Cordero et al., 2009; Lee et al., 2009).

Here we use the posterior adult Drosophila midgut to address the molecular mechanisms that mediate ISC hyperproliferation following loss of Apc in the intestine. We demonstrate that the Egfr and Jak/Stat signaling pathways are activated in a Myc-dependent manner and are required to drive ISC hyperproliferation in response to Apc1 loss. Importantly, we provide direct in vivo evidence for paracrine crosstalk between the Egfr and Jak/Stat pathways in Apc1-driven intestinal hyperplasia. Finally, our results suggest that the pathways that are ectopically activated in the fly midgut might also be activated in human CRC.
MATERIALS AND METHODS

Fly stocks
The following fly stocks were kindly provided by colleagues: the null alleles Apcl\(^{108}\), Apcl\(^{177}\), Apcl\(^{131}\) and Apcl\(^{233}\) (Yash Ahmed, Dartmouth Medical School), escargot-gal4, UAS-gfp (Shigeo Hayashi, RIKEN Center for Developmental Biology), UAS-max-IR (Peter Gallant, Universität Würzburg), MyoIA\(^{+}\)gfp, UAS-Upd and 10\(^{\times}\)Stat-gfp (Bruce Edgar, ZMBH, Heidelberg; the latter previously made by Erika Bach, New York University School of Medicine), upd3.1-lacZ (Huaiqin Jiang, UT Southwestern Medical Center), MARCM 82B line (David Bilder, Berkeley), UAS-DEER\(^{DN}\) and UAS-Spitz (Matthew Freeman, MRC LMB, Cambridge). The remainder of the lines used were obtained from the Drosophila Genomics Resource Center (DGRC), Vienna Drosophila RNAi Center (VDRC) and the Bloomington Stock Center. For overexpression of UAS-wg, we used Bloomington Stock number 5918. VDRC ID numbers: UAS-stat-IR (43866), UAS-dome-IR (2947), UAS-dome-IR (2612), UAS-apcl-1 (1333), UAS-spitz-IR (3920).

Fly maintenance
Croses were maintained at either 18°C or 22°C in standard medium. Animals of the desired genotypes were collected within 48 hours of eclosion, allowed to age for an additional 3-5 days and then switched to the desired temperature. All experiments involving the activation of a transgene under the control of gal4/gal80\(^{108}\) were switched from 18°C to 29°C; the rest of the experiments were carried out at 25°C. Animals were kept in incubators with controlled 12-hour light:dark cycles. Flies were changed into new food every 2-3 days. Only posterior midguts from females were analyzed in this study.

Fly genotypes
yw; escargot-gal4, UAS-gfp+/++; Apcl\(^{108}\)FRT82B/++; yw; escargot-gal4, UAS-gfp+/++; Apcl\(^{108}\)FRT82B/+/Apcl\(^{108}\)FRT82B/yw; escargot-gal4, UAS-gfp+/++; tub-gal80ts/+; FRT82B, tub-gal80/Apc1Q8, tub-gal80/lacZ/females were analyzed in this study.

The following fly stocks were kindly provided by colleagues: the null stocks of Apc\(^{108}\)FRT82B (Peter Gallant, University of Cambridge). The remainder of the lines used were obtained from the Drosophila Genomics Resource Center (DGRC), Vienna Drosophila RNAi Center (VDRC) and the Bloomington Stock Center. For overexpression of UAS-wg, we used Bloomington Stock number 5918. VDRC ID numbers: UAS-stat-IR (43866), UAS-dome-IR (2947), UAS-dome-IR (2612), UAS-apcl-1 (1333), UAS-spitz-IR (3920).

Histology and tissue analysis
For immunofluorescence, tissues were dissected and stained as described (Cordero et al., 2012). Primary antibodies were: chicken anti-GFP, 1:4000 (Abcam); mouse anti-ΔF508, 1:2000 and Alexa 594, 1:100 (Invitrogen); Cy5, 1:50 (Jackson Laboratories). Secondary antibodies were: Alexa 488, 1:100 (Invitrogen); Alexa 594, 1:150 (Invitrogen); Alexa 647, 1:200 (Invitrogen); Cy3, 1:50 (Jackson Laboratories). Confocal images were captured using a Zeiss 710 confocal microscope and processed with Adobe Photoshop CS to adjust brightness and contrast. Images represent a maximum intensity projection of z-stacks.

For immunohistochemistry, fly intestines were dissected and fixed in 10% formalin and then paraffin embedded to allow sectioning and Hematoxylin and Eosin (H+E) staining.

Clonal analysis
Recombinant clones were generated using the MARCM technique as previously described (Lee and Luo, 2001). Crosses were maintained at 22°C. Then, 3- to 5-day-old adults of the desired genotypes were selected and subject to three 30-minute heat shocks at 37°C in 1 day. Flies were then incubated at 25°C and their guts dissected for analysis 2 weeks after clonal induction. ISC proliferation was scored by the number of cells per clone and percentage of clones with pH\(^{3+}\) cells.

Regression of Apcl\(^{108}\) phenotypes
Whole gut
We analyzed the midguts of animals carrying the following alleles and transgenes: (1) esg-gfp; Apcl\(^{108}\); (2) esg-gfp; Apcl\(^{108}\); (3) Apcl\(^{108}\) carrying the esg\(^{+}\) gfp driver in combination with...
UAS-myc-IR2 or UAS-max-IR6 transgenes (Apc1\textsuperscript{108} + myc-IR2 and Apc1\textsuperscript{108} + max-IR6, respectively). Adults were kept at room temperature for 5 days, after which their midguts were analyzed or transferred to 29°C for 7 days to allow RNAi expression (Apc1\textsuperscript{108} + myc-IR2 and Apc1\textsuperscript{108} + max-IR6). Midguts were dissected and the total number of pH3\textsuperscript{+} cells per posterior midgut was quantified. See Fig. 3.

**MARCM clones**

Clones carrying the following alleles and transgenes were induced and allowed to develop at 22°C for 14 days: (1) control (iacZ); (2) Apc1\textsuperscript{Q8}; (3) UAS-myc-IR2; Apc1\textsuperscript{Q8} (myc-IR); (4) UAS-max-IR6; Apc1\textsuperscript{Q8} (max-IR). After the first 14 days, all flies were switched to 29°C and midguts were dissected after 1 day (15-day-old clones; 15d), 7 days (21-day-old clones; 21d) and 14 days (28-day-old clones; 28d) of incubation at 29°C. The latter incubation was only performed for UAS-myc-IR2; Apc1\textsuperscript{Q8} and UAS-max-IR6; Apc1\textsuperscript{Q8} MARCM clones. ISC proliferation was scored by the number of cells per clone and percentage of clones with pH3\textsuperscript{+} cells. See supplementary material Fig. S3.

**Lifespan studies**

For each study, 30-50 flies were used. Flies were collected within 48 hours after eclosion and kept at a density of 20 flies per vial at 22°C or 29°C in a controlled 12-hour light:dark cycle. Every 2-3 days, vials were changed and the number of dead flies was counted, from which mean and maximum lifespan (MLS, the last 10% of surviving flies) were calculated. Prism (GraphPad) software was utilized to build survival curves analyzed using the Kaplan Meier log-rank test.

**RNA quantification**

TRIZOL (Invitrogen) was used to extract total RNA from 6-10 midguts per biological replicate. cDNA synthesis from three biological replicates was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). MAXIM SYBR Green Master Mix (Fermentas) was used for quantitative (q) PCR. Data were extracted and analyzed using Applied Biosystems 7500 software version 2.0 and Prism. Data are presented as the mean fold change with standard error. Primers are listed in supplementary material Table S1.

**Quantifications and statistics**

In each experiment, 5-15 midguts were analyzed. Clonal sizes were quantified as the total number of cells per clone (scored by DAPI staining). ISC proliferation was quantified as the total number of pH3\textsuperscript{+} cells per posterior midgut. Different cell lineages in control and Apc1\textsuperscript{Q8} MARCM clones were quantified as percentage of Ecs, ee or ISCs (scored by nuclear size, Prospero or Delta staining, respectively) among the total number of cells per clone (scored by DAPI). Quantification of ISCs was also represented by the number of Delta\textsuperscript{-} clones per cell. For the quantification of Delta\textsuperscript{-} cells in whole Apc1\textsuperscript{Q8} and Apc1\textsuperscript{Q8} midguts, we counted the total number of Delta\textsuperscript{-} cells within a consistent region of the posterior midgut, which was imaged with a 40× lens and comprised a field of 0.04 mm\textsuperscript{2}. The percentage of Myc\textsuperscript{-}esg-gfp\textsuperscript{+} cells in posterior midguts was calculated as the number of esg-gfp\textsuperscript{+} cells that showed Myc staining over the total number of esg-gfp\textsuperscript{+} cells within a consistent region of the posterior midgut imaged as above. Results are presented in graphs created by Prism 5. We used Student’s t-test or one-way ANOVA with Bonferroni’s multiple comparison test for statistical analysis.

**Mouse and human tissue staining**

All experiments were performed following UK Home Office guidelines. Alleles and induction protocols used were as previously described (Sansom et al., 2007; Ireland et al., 2004; Barker et al., 2007).

We performed standard immunohistochemistry of formalin-fixed intestinal sections. Primary antibodies were c-Myc (1:100; Santa Cruz sc-40) and p-Stat3 (1:1000; Santa Cruz sc-7993-B).

Colon carcinoma tissue arrays (CO804a, Biomax.us) were stained with the indicated primary antibodies and individual cores scored based on the percentage of each core with a staining intensity of 0, 1, 2 or 3. Staining intensities were analyzed using the Spearman’s rank correlation coefficient.

**RESULTS**

**Apc1 but not Apc2 is required for ISC hyperproliferation in the Drosophila adult midgut**

Drosophila Apc1 and Apc2 are redundant during development (Ahmed et al., 2002). Nevertheless, both genes have been shown to independently regulate stem cell divisions in the male Drosophila germ line (Yamashita et al., 2003).

Concomitant knockdown of the two Drosophila Apc homologs in progenitor cells results in ISC hyperproliferation (Cordero et al., 2009; Lee et al., 2009) within the Drosophila adult midgut. We therefore examined whether independent roles of Apc1 and Apc2 can be distinguished in the regulation of ISC proliferation. We
examined the posterior midguts from animals carrying the null allele Apc1Q8 (Ahmed et al., 1998) in combination with the stem/progenitor cell driver escargot-gal4, UAS-gfp (esg-gfp) (Michell and Perrimon, 2006). We will refer to the Apc1Q8 allele as Apc1. We observed that the midguts of Apc1 animals displayed a hyperplastic phenotype, which was characterized by ISC hyperproliferation as demonstrated by a significant increase in phosphorylated Histone H3-positive (pH3+) and esg-gfp+ cells (Fig. 1A,B,E). This phenotype was comparable to that resulting from overexpression of Wg or the activated form of β-catenin/Armadillo (Arms10) in stem/progenitor cells under the inducible escargot-gal4, UAS-gfp; tub-gal80 driver (esg>wg and esg>arm10, respectively) (Fig. 1C-E; supplementary material Fig. S2G,J,L). Furthermore, whole animals homozygous for loss-of-function alleles of Apc1 displayed a significantly decreased lifespan when compared with heterozygote controls (Fig. 1F). No intestinal hyperproliferation or decrease in lifespan was observed in animals lacking Apc2 only (data not shown).

To confirm the role of Apc1 in ISC proliferation and to test whether there was any contribution from Apc2, we created MARCM clones (Lee and Luo, 2001) of cells deficient for Apc1 or Apc2 only and compared them with Apc1, Apc2 double-mutant clones (Fig. 1G-J) (Cordero et al., 2009; Lee et al., 2009). Consistent with the whole mutant phenotype, intestinal Apc1 clones had more cells than control (lacZ) clones (Fig. 1G,H,K). Importantly, no difference was observed between Apc2 and control clones (Fig. 1G,I,K). Furthermore, Apc1 clones showed no significant difference in size when compared with Apc1, Apc2 double-mutant clones (Fig. 1H,J,K). As previously reported in Apc1, Apc2 double mutants (Cordero et al., 2009; Lee et al., 2009), loss of Apc1 in the Drosophila midgut resulted in increased ISC proliferation with no major differentiation defects (supplementary material Fig. S1). We did, however, note that MARCM Apc1 clones had a slight increase in the percentage of ECs and increased numbers of Delta+ ISCs (Ohlstein and Spradling, 2007), which was proportional to the increase in the total number of cells (supplementary material Fig. S1). These phenotypes mimic the accumulation of ECs due to impaired sloughing off and upregulation of Lgr5+ ISCs observed in the Apc mouse intestine (Barker et al., 2009; Sansom et al., 2004).

Taken together, these results indicate that loss of Apc1 is responsible for ISC hyperproliferation in Apc1, Apc2 MARCM clones in the Drosophila midgut (Cordero et al., 2009; Lee et al., 2009). This phenotype strongly resembles that of the mammalian intestine (Sansom et al., 2004), indicating a significant degree of evolutionary conservation in the biology of this tissue. Therefore, the Drosophila midgut is a useful system for the identification of key downstream signaling events following Apc1 loss.

**Myc is upregulated and required for ISC hyperproliferation downstream of Wg signaling**

Myc proteins are well-known proto-oncogenes and general, although not universal, regulators of normal growth and proliferation (Johnston et al., 1999; Trumpp et al., 2001). Myc dysregulation is observed in a wide range of human tumors (Vita and Henriksson, 2006). In the mouse, c-Myc is an essential mediator of the Apc phenotypes in the intestine (Sansom et al., 2007) but not in the liver (Reed et al., 2008). Drosophila Myc [also known as dMyc or Diminutive (Dm) – FlyBase] acts downstream of Wg during epithelial regeneration in the larval wing disc (Smith-Bolton et al., 2009), although it is not required for cells with high Wnt signaling to act as supercompetitors (Vincent et al., 2011).

Therefore, the interactions between Wnt signaling and Myc seem to be highly context dependent.

We tested whether there was a role for Myc in Wnt-driven hyperplasia in the Drosophila midgut. First, we examined the levels of Myc expression in esg>wg, esg>arm10 and Apc1 intestines and observed that Myc protein was significantly upregulated in all cases compared with wild-type tissues (Fig. 2A-D; supplementary material Fig. S2A,B’). Importantly, the ISC-EB cell population, labeled by esg-gfp, displayed the highest levels of Myc (Fig. 2E,E’; supplementary material Fig. S2A-B’). Previous work shows that Apc-driven ISC proliferation in the Drosophila midgut involves the Arm (β-catenin) co-factor Pangolin (Tcf) (Lee et al., 2009). Consistently, Myc upregulation was dependent on Pangolin (supplementary material Fig. S2C-D’). Myc staining of Apc1...
MARCM clones revealed autonomous as well as non-autonomous upregulation of Myc outside the clones (Fig. 2F,F', arrows).

In order to functionally test the importance of Myc upregulation in response to Apc1 loss, we used the MARCM system to target RNA interference (RNAi) for myc (myc-IR) or its transcriptional partner Max (max-IR) (Steiger et al., 2008) within Apc1 mutant clones. myc-IR and max-IR overexpression completely suppressed hyperproliferation of Apc1 MARCM clones (Fig. 2G-L). Even though clones of myc-IR had reduced ISC proliferation when compared with lacZ control clones (Fig. 2J,L), overexpression of either myc-IR or max-IR in Apc1 clones resulted in clonal sizes and proliferation levels comparable to those of the RNAi transgenes alone (Fig. 2L). Furthermore, knockdown of myc also suppressed hyperproliferation induced by Wg overexpression (supplementary material Fig. S2E-L). Together, these results demonstrate that Myc has a conserved role in mediating Apc1-driven hyperproliferation in the intestine.

**myc proto-oncogene addiction in Apc1-driven hyperproliferation**

Oncogene addiction is a process in cancer biology through which tumors may become dependent on certain pathways not just for proliferation and growth but also for maintenance and survival (Felsher, 2008). Studies of oncogene addiction have generally been performed by overexpression of exogenous transgenes (e.g. Kras and Myc), which are then switched off within the tumors that they initiated, leading to tumor regression (Felsher and Bishop, 1999). A more relevant question for human tumorigenesis is whether switching off endogenous levels of proteins driven by an oncogenic or tumor suppressor mutation will also cause tumor regression. Recent studies have shown that transient overexpression of the dominant-negative Myc protein ‘Omo-Myc’, which is thought to prevent Myc/Max dimerization, can cause tumor regression in established lung adenomas driven by KRAS mutation (Soucek et al., 2008). We have previously shown that Apc deficiency in the mouse intestine requires Myc at the onset of hyperplasia (Athineos and Sansom, 2010; Sansom et al., 2007). Nevertheless, no studies have shown whether there is a sustained requirement for Myc in this context.

We tested whether the Apc1-driven hyperproliferation in the adult Drosophila midgut is dependent on Myc once established. We first used Apc1 whole mutant animals to assess whether downregulation of myc and Max within the esg− cell population could affect hyperproliferation across the entire intestinal epithelium. We created temperature-sensitive myc and Max RNAi (tub-gal80ts, UAS-myc-IR and tub-gal80ts, UAS-max-IR, respectively), which we placed in the background of whole Apc1 animals carrying the esg−>gfp driver (Fig. 3). Animals of the desired genotype were allowed to age at the non-permissive temperature for 5 days, following which we ‘turned on’ the RNAi
for an additional 7 days (Fig. 3A). Before turning on the RNAi, Apc1 midguts displayed the expected hyperproliferative phenotype (Fig. 3C,D,G). After 7 days of RNAi for either myc or Max, hyperproliferation was almost completely reverted (Fig. 3E-G). A similar outcome was observed in the context of MARCM Apc1 clones (supplementary material Fig. S3). Taken together, these experiments indicate that the Apc1 hyperproliferative phenotype not only requires Myc for its establishment but also for its maintenance. Strikingly, myc or Max knockdown in esg$^{+}$ cells enhanced total survival from whole Apc1 mutant animals (Fig. 3H,I).

**Loss of Apc1 leads to Jak/Stat pathway activation**

The non-cell-autonomous upregulation of Myc in MARCM clones of Apc1 (Fig. 2F,F’) suggested the production of secreted factors within Apc1 mutant tissues, which might contribute to hyperproliferation of the adult Drosophila midgut. We took a candidate approach and performed RT-qPCR from Apc1 whole midguts to look at levels of known regulators of ISC proliferation, which may be secreted (Biteau and Jasper, 2011; Jiang et al., 2011; Karpowicz et al., 2010; Ren et al., 2010; Shaw et al., 2010; Buchon et al., 2009; Jiang et al., 2011).

Jak/Stat signaling is required for Drosophila intestinal homeostasis and regeneration. Overexpression of the ligand Upd is sufficient to drive ISC hyperproliferation (Beebe et al., 2010; Buchon et al., 2009; Jiang et al., 2009; Lin et al., 2010). Additionally, Jak/Stat mediates hyperproliferation induced by loss of the tumor suppressor Hippo or hyperactivation of Egrf signaling (Biteau and Jasper, 2011; Jiang et al., 2011; Jiang et al., 2009; Karpowicz et al., 2010; Ren et al., 2010; Shaw et al., 2010).

RT-qPCR of whole Apc1 and esg$^{P>B} >$wg midguts indicated significant upregulation of the interleukin-like ligands upd2 and upd3, as well as the downstream target of the pathway Socs36E (Fig. 4A). As upd3 showed the highest upregulation, we investigated its tissue expression domain using a lacZ reporter (Jiang et al., 2009). This showed that, either whole tissue loss of Apc1 or overexpression of Wg in ISCs resulted in activation of upd3-lacZ almost exclusively in ECs. These are characterized by their large nuclei, lack of Delta and Prospero staining and absence of esg$^{gfp}$ (Fig. 4B-E’; supplementary material Fig. S4). In a similar scenario to Myc upregulation (Fig. 2F,F’), MARCM clones of Apc1 revealed non-autonomous activation of upd3 expression (Fig. 4F,F’, arrows). A transgenic reporter of Jak/Stat pathway activation, Stat-GFP (Bach et al., 2007), showed that loss of Apc1 in the midgut led to upregulation in Jak/Stat pathway activity primarily within stem/progenitor cells (Fig. 4G-J). Together, these data suggest that, in the context of Apc1 loss, Upd3 is secreted from ECs and activates Jak/Stat signaling in ISCs.

**Jak/Stat mediates Apc1-dependent hyperproliferation**

To test the functional relevance of Jak/Stat signaling activation upon Apc1 loss we used the MARCM system to drive RNAi for Stat (stat-IR; Apc1) or the receptor domeless (dome-IR; Apc1) within clones of Apc1 (Fig. 5A-C). Stat or dome knockdown on its own does not prevent ISC proliferation but affects differentiation (Beebe et al., 2010; Buchon et al., 2009; Jiang et al., 2009). Remarkably, loss of either component of the Jak/Stat signaling pathway significantly suppressed the hyperproliferative phenotype of Apc1 clones (Fig. 5A-D), as well as that of Wg-overexpressing midguts (Fig. 5E-J). Therefore, Jak/Stat signaling upregulation is essential to drive ISC hyperproliferation by high Wg signaling in the Drosophila midgut.

We next investigated the epistatic relationship between Apc1 and Jak/Stat signaling in the adult Drosophila midgut. First, we looked at Jak/Stat signaling pathway activation in Apc1 intestines with myc knockdown. RT-PCR of whole midguts indicated that activation of Jak/Stat signaling in Apc1 midguts is almost entirely dependent on Myc activation in ISCs (Fig. 6A). We next examined Myc levels in stat-IR; Apc1 and dome-IR; Apc1 MARCM clones (Fig. 6B-D’).
Myc upregulation upon Apc1 loss (Fig. 6B,B’) was diminished in dome-IR; Apc1 clones (Fig. 6C,C’) but still significantly upregulated in stat-IR; Apc1 clones (Fig. 6D-D’). These results were confirmed upon dome and Stat knockdown from wg-overexpressing midguts (Fig. 6E-H). Moreover, upd1 overexpression induced upregulation of Myc (Fig. 6I-J’), which might at least partly explain the non-autonomous activation of Myc in Apc1 clones (Fig. 2). Upregulation of Myc was required for Upd-dependent ISC hyperproliferation (Fig. 6K). Together, these results suggest the presence of a positive-feedback crosstalk between Myc and Jak/Stat activation in response to Apc1 loss in the Drosophila midgut.

**Egfr signaling mediates Wnt/Myc and Jak/Stat signaling crosstalk**

The non-autonomous activation of upd3 in ECs in response to high levels of Wnt/Myc signaling in ISCs (Figs 4, 5) suggested the existence of paracrine signaling between ISCs and ECs. Crosstalk between Jak/Stat and Egfr/Ras/MAPK signaling mediates ISC proliferation during homeostasis and regeneration of the adult Drosophila midgut. Overexpression of Egfr-like ligands leads to upregulation of upd1-3 and subsequent Jak/Stat pathway activation. Conversely, ectopic upd1 overexpression results in upregulation of Egfr-like ligands and activation of Egfr signaling (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang et al., 2011). Therefore, we hypothesized that the Egfr pathway could provide a link between Wnt/Myc and Jak/Stat signaling.

RT-qPCR of whole Apc1 and esg>wg midguts showed selective upregulation of the EGFR-like ligands spitz and vein (Fig. 7A; supplementary material Fig. S5A). Of these, only the upregulation of spitz was dependent on Myc expression in ISCs (Fig. 7A). Like Myc, spitz is normally expressed in ISCs/EBs (supplementary material Fig. S5B-C), whereas vein is predominantly expressed in the visceral muscle (supplementary material Fig. S5D,D’). (Buchon et al., 2010; Biteau and Jasper, 2011; Jiang et al., 2011). This suggested that Spitz might be functionally required downstream of Myc, causing the paracrine activation of Upd3. To test this, we overexpressed a secreted form of Spitz in progenitor cells (esg>spitz), which led to upd3 upregulation in ECs (Fig. 7B; supplementary material Fig. S5E-E’), in a similar fashion to esg>wg midguts (Fig. 4). Given that Jak/Stat signaling is absolutely required for Apc1-dependent ISC proliferation, if the activation of Spitz is required for upd3 expression then it should also be necessary for ISC hyperproliferation in this context. We made use of RNAi for spitz (spitz-IR) (Buchon et al., 2010) and Apc1 (Apc1-IR) (Cordero et al., 2009) to test whether Spitz from ISCs/EBs is required for Apc1-dependent ISC hyperproliferation (Fig. 7D,G). Owing to the chromosomal location of the available transgenes, we could not test this hypothesis in a MARCM setting. Consistent with the Apc1 loss-of-function phenotype, overexpression of Apc1-IR in progenitor cells (esg>Apc1-IR) resulted in ISC hyperproliferation (Fig. 7D,G), which was dependent on Myc upregulation (supplementary material Fig. S8). Importantly, concomitant knockdown of spitz and Apc1 (esg>spitz-IR; Apc1-IR) completely suppressed ISC hyperproliferation from esg>Apc1-IR midguts (Fig. 7F,G). These results suggest that Egfr activation by Spitz mediates ISC hyperproliferation in response to Apc1 deficiency in the midgut. Consistently, overexpression of a dominant-negative form of Egfr in the background of Apc1 MARCM clones (DERN; Apc1G8) completely suppressed the phenotype of Apc1 MARCM clones (supplementary material Fig. S5L).

We next designed a strategy to directly address the role of Egfr signaling in the upregulation of upd3 in ECs. Egfr activation in ECs has been shown to regulate gut remodeling in response to bacterial infection (Buchon et al., 2010). We tested whether preventing Egfr function in ECs affects upd3 upregulation in the same cells and ISC proliferation in the context of high Wnt signaling. As in the case of other secreted ligands (Jiang et al., 2011), ectopic overexpression of Wg in different cell types led to ISC hyperproliferation (Fig. 1E). Therefore, we used the temperature-inducible EC-specific driver Myo1A-gal4, UAS-gfp; tub-gal80ts (Myo1A>gfp) (Jiang et al., 2009) to overexpress either wg (Myo1A>wg) or combined wg and DERDN (Myo1A>DERDN; wg) in ECs (Fig. 7H-K). Myo1A>wg midguts displayed ISC hyperproliferation and upregulation of upd3 (Fig. 7L,M). Myo1A>DERDN; wg displayed suppression of both Wg-dependent ISC proliferation and upd3 upregulation (Fig. 7K-M).
Together, these data suggest that Egfr signaling is a key mediator in the paracrine crosstalk between Wnt/Myc and Jak/Stat signaling in the Drosophila midgut.

Conserved Stat3 activation in mouse models and human CRC
Apc loss from mouse ISCs results in rapid adenoma formation (Barker et al., 2009) (Fig. 8A), and widespread deletion from the intestinal epithelium leads to a ‘crypt-progenitor cell-like’ hyperplastic phenotype (Sansom et al., 2004) (Fig. 8E,F). We used the inducible Cre/Lox system (Sauer, 1998) to knockout Apc from the mouse intestine and stained to detect levels of phosphorylated Stat3 (p-Stat3) [Stat3 is the closest homolog of Drosophila Stat92E (marelle)]. p-Stat3 was normally restricted to the proliferative ‘crypt’ (Fig. 8A, boxed area C; Fig. 8E; supplementary material Fig. S6A, dashed line), while differentiated villi showed low levels of activated protein (Fig. 8A, boxed area B). We observed ectopic p-Stat3 in intestinal adenomas of Lgr5-CreER; Apc1Q8 mice (Barker et al., 2009) (Fig. 8A, arrows and boxed area D; supplementary material Fig. S6B), as well as in the expanded hyperproliferative crypts of Ah-Cre; Apc1Q8 (Sansom et al., 2007) intestines (Fig. 8F, dashed line). Suppression of Apc-dependent hyperproliferation by c-Myc depletion restored the normal p-Stat3 staining pattern (Fig. 8G, dashed line). Furthermore, immunohistochemical staining of a human CRC tissue microarray for p-Stat3 and c-Myc showed significant positive correlation between protein levels (P<0.0001, Spearman’s rank correlation coefficient, n=72; supplementary material Fig. S6C,D). Together, these results point to potential conservation in the role of Jak/Stat signaling in Apc-dependent intestinal hyperplasia.

DISCUSSION
Using the Drosophila adult midgut as a model system we have uncovered a key set of molecular events that mediate Apc-dependent intestinal hyperproliferation. Our results suggest that paracrine crosstalk between Egfr and Jak/Stat signaling is essential for Apc1-dependent ISC hyperproliferation in the Drosophila midgut (Fig. 9).
Dependency on Myc and Max

We have previously demonstrated that Myc depletion prevents Apc-driven intestinal hyperplasia in the mammalian intestine (Sansom et al., 2007; Athineos and Sansom, 2010). In this study, we provide evidence that such a dependency on Myc is conserved between mammals and Drosophila. We further demonstrate that endogenous Myc or Max depletion causes regression of an established Apc1 phenotype in the intestine. Taken together, these data highlight the importance of developing Myc-targeted therapies to inhibit Apc1-deficient cells. Since not all roles of Myc are Max dependent (Steiger et al., 2008), present efforts are focused on developing inhibitors that interfere with Myc binding to Max and would therefore be less toxic. Our data provide the first in vivo evidence in support of the Myc/Max interface as a valid therapeutic target for CRC.

Myc in ISC proliferation and growth

Recent work showed that loss of the tuberous sclerosis complex (TSC) in the Drosophila midgut leads to an increase in cell size and inhibition of ISC proliferation (Amcheslavsky et al., 2011). Reduction of endogenous Myc in TSC-deficient midguts restored normal ISC growth and division. These results might appear contradictory to ours, where Myc is a positive regulator of ISC proliferation. However, in both scenarios, modulation of Myc levels restores the normal proliferative rate of ISCs.

Previous work in mouse showed that Myc upregulation is essential for Wnt-driven ISC hyperproliferation in the intestine (Sansom et al., 2007). However, Myc overexpression alone only recapitulates some of the phenotypes of hyperactivated Wnt signaling (Murphy et al., 2008; Finch et al., 2009). Here, we show that overexpression of Myc is capable of mimicking some aspects of high Wnt signaling in the Drosophila midgut, such as the activation of Jak/Stat (supplementary material Fig. S7), but is not sufficient to drive ISC hyperproliferation (supplementary material Fig. S7). Multiple lines of evidence have shown that forced overexpression of Myc in Drosophila and vertebrate models results in apoptosis partly through activation of p53 (Montero et al., 2008; Murphy et al., 2008; Finch et al., 2009). Therefore, driving ectopic myc alone is unlikely to parallel Apc deletion in the intestine, where the activation of multiple pathways downstream of Wnt signaling is likely to contribute cooperatively to hyperproliferation.
upregulation in a dome-dependent manner. Apc1/Myc-dependent activation of Egfr and Jak/Stat signaling leads to intestinal hyperproliferation. Alternative possibilities for Upd3 upregulation by Spitz/Egfr signaling are shown in gray. EB, enteroblast; EC, enterocyte; ISC, intestinal stem cell; VM, visceral muscle.

Fig. 9. Paracrine Egfr and Jak/Stat signaling crosstalk mediates Apc1-dependent intestinal hyperproliferation. Loss of Apc1 in the midgut leads to the production of Spitz and Upd3 in ISCs and ECs, respectively. Upregulation of both ligands requires previous activation of Myc in ISCs. Activated Jak/Stat signaling in turn contributes to Myc upregulation in a dome-dependent manner. Apc1/Myc-dependent activation of Egfr and Jak/Stat signaling leads to intestinal hyperproliferation. Alternative possibilities for Upd3 upregulation by Spitz/Egfr signaling are shown in gray. EB, enteroblast; EC, enterocyte; ISC, intestinal stem cell; VM, visceral muscle.

results show that activation of Wnt/Myc signaling in ISCs leads to non-autonomous upregulation of upd3 within ECs. Furthermore, Spitz/Egfr signaling appears to mediate the paracrine crosstalk between Wnt/Myc and Jak/Stat in the midgut. Overexpression of a dominant-negative Egfr in ECs blocks Upd3 upregulation and ISC hyperproliferation in response to high Wnt signaling (Fig. 7). A previous EC-specific role for Egfr has been demonstrated during midgut remodeling upon bacterial damage (Buchon et al., 2010). Nevertheless, the downstream signaling that mediates such a role of Egfr remains unclear given that the activation of downstream MAPK/ERK occurs exclusively within ISCs (Buchon et al., 2010; Biteau and Jasper, 2011; Jiang et al., 2011) (data not shown). Therefore, the current evidence would suggest that Egfr activity in ECs does not involve cell-autonomous ERK activation. Consistent with these observations, we did not detect p-ERK (Rolled – FlyBase) localization outside ISCs in response to either Apc loss or overexpression of wg in the Drosophila midgut (data not shown).

Reports on the Apc murine intestine have also failed to detect robust ERK activation (Sansom et al., 2006; Haigis et al., 2008). Since MAPK/ERK is only one of the pathways activated downstream of Egfr, it is possible that ERK-independent mechanisms are involved. It is important to explore this further because ERK-independent roles of Egfr signaling have not yet been reported in Drosophila. Thus, what mediates Upd3 upregulation in ECs in response to Egfr signaling activation and whether Spitz-dependent upregulation of Upd3 involves a direct role of Egfr in ECs remain unclear. A potential alternative explanation is that intermediate factors induced in response to Spitz/Egfr activation in ISCs might drive Upd3 expression (Fig. 9).

In summary, we have elucidated a novel molecular signaling network leading to Wnt-dependent intestinal hyperproliferation. Given the preponderance of APC mutations in CRC, the integration of Egfr and Jak/Stat activation might be a conserved initiating event in the disease.

**Jak/Stat signaling activation in Apc-driven intestinal hyperplasia**

Understanding the contribution of Jak/Stat signaling to the Apc phenotype in the mammalian intestine has been complicated by genetic redundancy between Stat transcription factors. Constitutive deletion of Stat3 within the intestinal epithelium slowed tumor formation in the ApcMin mouse, but the tumors that arose were more aggressive and ectopically expressed Stat1 (Mustea et al., 2010). Using the Drosophila midgut we provide direct in vivo evidence that activation of Jak/Stat signaling downstream of Apc1/Myc mediates Apc1-dependent hyperproliferation.

Our data on the Drosophila midgut and in mouse and human tissue samples suggest that blocking Jak/Stat activation could represent an efficacious therapeutic strategy to treat CRC. Currently, there are a number of Jak2 inhibitors under development (Che et al., 2009; Quintás-Cardama et al., 2010) and it would be of great interest to examine whether any of these could modify the phenotypes associated with Apc loss.

**Non-autonomous production of Upd and the role of Egfr signaling**

Previous studies have demonstrated that ECs are the main source of Upds/interleukins in the midgut epithelium (Jiang et al., 2009). Our
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Competing interests statement

The authors declare no competing financial interests.

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References


Mechanisms of Apc hyperplasia


