Niche appropriation by *Drosophila* intestinal stem cell tumours

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Mutations that inhibit differentiation in stem cell lineages are a common early step in cancer development, but precisely how a loss of differentiation initiates tumorigenesis is unclear. We investigated *Drosophila* intestinal stem cell (ISC) tumours generated by suppressing Notch (*N*) signalling, which blocks differentiation. Notch-defective ISCs require stress-induced divisions for tumour initiation and an autocrine EGFR ligand, Spitz, during early tumour growth. On achieving a critical mass these tumours displace surrounding enterocytes, competing with them for basement membrane space and causing their detachment, extrusion and apoptosis. This loss of epithelial integrity induces JNK and Yki/YAP activity in enterocytes and, consequently, their expression of stress-dependent cytokines (Upd2, Upd3). These paracrine signals, normally used within the stem cell niche to trigger regeneration, propel tumour growth without the need for secondary mutations in growth signalling pathways. The appropriation of niche signalling by differentiation-defective stem cells may be a common mechanism of early tumorigenesis.

As in mammals, *Drosophila* ISCs maintain gut homeostasis by dividing to replace lost cells. ISCs generate transient progeny, enteroblasts (EBs), that can differentiate into absorptive enterocytes (ECs) or secretory enteroendocrine cells1,2 (EEs). These epithelial cells grow on a basement membrane (BM) overlaying the visceral muscle (VM), which together with ECs, EEs and EBs comprises the stem cell niche. Fly midgut homeostasis is regulated by Notch, cytokine/JAK–STAT, EGFR/Ras/MAPK, JNK, Hippo, insulin, Wnt, PDGF/VEGF, Hh and BMP/TGFβ signalling3. The various niche cells (EBs, ECs and VM) collectively provide these signals to regulate ISC growth, self-renewal and differentiation. Delta-Notch signalling is the primary trigger of EB to EC differentiation1–4. ISCs express a Notch (N) ligand, Delta (Dl), which activates the Notch receptor in EBs and promotes their differentiation into ECs (refs 2,4). Loss of N, Dl or other Notch pathway components in progenitor cells (ISCs and EBs) results in the rapid expansion of differentiation-defective, escargot-positive (*esg*)+, Delta-positive (Dl+) ISC-like cells that form multi-layered neoplastic tumours, typically admixed with excess Prospero-positive (Pros+) EE cells that are also produced1,2,4–6.

According to current models differentiation-defective stem cells should remain dependent on growth and survival factors in their niche1, and require secondary mutations to initiate the run-away growth characteristic of tumours7. Although the importance of immune cells, fibroblasts and vasculature recruited as a tumour builds its microenvironment is appreciated8, how tumour-initiating cells interact with the stem cell niche before microenvironment formation is poorly understood. In flies, differentiation-defective larval neural or adult germline stem cell tumours can overgrow in their respective organs, but whether the stem cell niche is required to propel the growth of these cells has not been tested10. In the fly midgut, the growth of ISC-derived tumours (for example, *Apc-* or *Ras*12,13,dlg−) has been proposed to require niche signals10–12; however, the mechanisms controlling the activation of these signals, and the functional importance of the niche cells that produce them, have not been directly tested. In the mouse intestine, stem-like tumour-initiating cells (*Lgr5*+, *Apc*−) in early adenomas remain associated with Paneth cells—an essential part of the normal ISC niche14—but whether these tumour-initiating cells are dependent on growth signals from Paneth cells is unknown15. Here we examine how differentiation-defective Notch− ISC tumours grow in the adult fly ISC niche.

**Notch− ISC tumour cells are proliferative endocrine progenitors** We first investigated the identity of the tumour cells that arise from depleting Notch with RNA interference (RNAi) in progenitors, using the *esg*10,14,15 GAL4–UAS system (*esg*14,15). We found N− tumours to be an admixture of Pros*high* EE-like cells and neoplastic ISC-like cells that express high levels of ISC markers (Dl*high* *esg*14,15) but also low levels of EE markers (Pros; Supplementary Fig. 1a–a′, c–c′). ISC-like tumour cells did not show markers of active Notch signalling (*SuH–lacZ*) or EC differentiation (Pdm1; Supplementary Fig. 1b–b′, d–d′).
mRNA-seq analysis showed that ISC-like $N^-$ tumour cells (D)$^{\text{high}}$ and esg$^{\text{high}}$ expressed many ISC-associated genes (such as $Dl$ and $spdo$; refs 2,4,6,16) and EE-associated genes (such as pros and Allatostatin, AstA; refs 1,2,17; Supplementary Fig. 1g–j), and functional tests showed that these cells differentiated into EEs, rather than ECs; refs 2,4,6,16) and EE-associated genes (such as AstA; refs 1,2,17; Supplementary Fig. 1g–j), and functional tests showed that these cells differentiated into EEs, rather than ECs, when $N^{\text{RKK}}$ expression was extinguished (Supplementary Fig. 1e–f). Together these data indicate that ISC-like tumour cells are actually committed EE precursors rather than multipotent progenitors. We did not find mitotic (phospho-Ser 10 histone 3-positive) Pros$^+$ EE-like cells within the tumours; indeed, only esg$^+$ ISC-like tumour cells were proliferative (Figs 3a and 5c). To determine the role of the excess EEs in tumour growth, we suppressed Notch signalling by expressing RNAi directed against Notch receptors (Supplementary Fig. 5d). In this case large esg$^+$ tumours formed similar to those seen with $N^-$ tumours containing EEs, indicating that the excess EEs present in most $N^-$ tumours have little if any role in tumour growth.

**Figure 1** Tissue stress promotes ISC tumour initiation. (a) System to independently express transgenes in ECs with Myo1A$^+$ and subsequently initiate ISC-derived tumours by heat-shock-induced FLP-FRT mediated recombination. (b) Mean number of tumours per midgut with s.e.m. 14 days (18°C) after tumour induction following GFP ($n=20$ midguts), JNKK (hep) ($n=12$ midguts, Mann–Whitney: $P=0.0203$) or activated JNKK (hep$^{+\text{AT}}$) ($n=10$ midguts, Mann–Whitney: $P=0.0002$) expression in ECs with Myo1A$^+$ for 5 days (29°C). Midguts pooled from 2 independent experiments. (c) Mean percentage of flies with tumours with s.e.m. from $n=3$ independent experiments 14 days (18°C) after tumour induction following GFP, JNKK (hep) or activated JNKK (hep$^{+\text{AT}}$) (paired $t$-test: $P=0.0076$) expression in ECs with Myo1A$^+$ for 5 days (29°C). (d) Cells per neur$^{+\text{F585}}$: tumour with mean (red line) and s.e.m. 14 days (18°C) after tumour induction following GFP ($n=13$ tumours pooled from 20 midguts), JNKK (hep) ($n=31$ tumours pooled from 12 midguts) or activated JNKK (hep$^{+\text{AT}}$) ($n=77$ tumours pooled from 10 midguts) expression in ECs with Myo1A$^+$ for 5 days (29°C). Midguts pooled from 2 independent experiments. (e) Mean percentage of flies with tumours with s.e.m. from $n=3$ independent experiments in flies expressing GFP and $N^{\text{RKK}}$ or $N^{\text{RKA}}$, Cycloheximide (CycE) and cdc25/string (stg) (paired $t$-test: $P=0.0185$) with esg$^+$ (weak) for 3 days. (f) Mean percentage of flies with tumours with s.e.m. from $n=3$ independent experiments after being fed food alone, $Pseudomonas entomophila$ (P.e.) containing food (paired $t$-test: $P=0.0034$), sucrose alone or P.e. containing sucrose (paired $t$-test: $P=0.0007$) for 1 day before tumour induction by expressing $N^{\text{RKA}}$ with esg$^+$ (weak) for 3 days (food alone). Significance notation is explained in 'Statistical analysis'.

**Gut epithelial stress promotes ISC tumour initiation**

To understand how the niche might contribute to ISC-derived tumorigenesis, we investigated how $N^-$ tumours initiate. Despite a presumably uniform block to differentiation by the expression of $N^{\text{RKK}}$ using esg$^+$, tumours were not detected in all experimental animals. This indicated that simply blocking differentiation was not sufficient for tumour initiation. Hence, we investigated whether tumour initiation might be dependent on environmental factors. Previous reports showed that stress or damage to the midgut (for example, by enteric infection) activates JNK and Yki/YAP signalling and that this stimulates the production of cytokines (Unpaired 2,3; Upd2,3) by enteric infection (Supplementary Fig. 1e–f). For instance, it was found that the frequency of tumour initiation from $N^-$ ISC could be enhanced by enteric infection15. We confirmed these results by performing enteric infection with $Pseudomonas entomophila$ before initiating ISC tumours by inducing
Figure 2 Spi/EGFR/MAPK signalling is induced in ISC tumours and the niche. (a–d) Di-phosphorylated ERK (a, c, b, d, red) in midguts expressing GFP (a, b, green) or GFP and N^Kras (c, d, green) with esg\(^{n}\) for 2 days. (e–f) β-galactosidase (e, f, red) in midguts of flies bearing vn-lacZ (vn\(^{+}\)) and expressing GFP (e, green) or GFP and N^Kras (f, green) with esg\(^{n}\) for 2 days. (g) Mean normalized expression (NE) value (RPKM, log\(_{2}\)) from \(n\) = 2 independent experiments of EGFR ligand (spi, Km and vn) and signalling target (aos) mRNA determined by mRNA sequencing of midguts expressing GFP (control, red) or GFP and N^Kras (blue) with esg\(^{n}\) for 3 days. The adjusted fold change in gene expression in tumorous midguts (blue), normalized to control midguts (red), is indicated. The Benjamini–Hochberg adjusted \(P\) value for esg is 7.71 \times 10^{-3}; vn, \(P\) = 3.25 \times 10^{-16}; aos, \(P\) = 2.84 \times 10^{-24}.

\(N^{Kras}\) (Fig. 1f and Supplementary Fig. 6f). As JNK signalling can be activated by enteric infection, we tested whether JNK signalling in ECs might influence the frequency of tumour initiation, as suggested previously\(^{13}\). We expressed a brief pulse of activated Hemipterous (Hep\(^{Akt}\), Jun Kinase Kinase), with the EC-specific Myo1A\(^{GAL4–UAS}\) system (Myo1A\(^{GAL4–UAS}\)), and then afterwards used FLP/FRT-mediated recombination to induce ISC clones mutant for the Notch signalling component, neuralized (neur), to inhibit stem cell differentiation (Fig. 1a and Supplementary Fig. 3a–c). Neur is an E3 ubiquitin ligase required for Notch signalling\(^{5}\) and its loss results in ISC tumours.
Figure 3 ISC tumour initiation and outgrowth requires autonomous Spi/EGFR signalling. (a–c) Phosphorylated histone H3 Ser 10 (PH3, red) in midguts expressing GFP (green) and N\textsuperscript{RNAi} (a), GFP, EGFR\textsuperscript{RNAi} and N\textsuperscript{RNAi} (b) or GFP, spi\textsuperscript{RNAi} and N\textsuperscript{RNAi} (c) with esg\textsuperscript{ts} for 3 days. (d) Phosphorylated histone H3 Ser 10 (red) in midguts expressing GFP (green), spi\textsuperscript{RNAi} and N\textsuperscript{RNAi} with esg\textsuperscript{ts} for 14 days. (e) Mean number of phosphorylated histone H3 Ser 10-positive cells per midgut with s.e.m. after expression with esg\textsuperscript{ts} of GFP and N\textsuperscript{RNAi} (n=30 midguts), GFP, N\textsuperscript{RNAi} and Egfr\textsuperscript{RNAi} (n=30 midguts, Mann–Whitney; P=0.0001), GFP, N\textsuperscript{RNAi} and spi\textsuperscript{RNAi} for 3 (n=33 midguts, Mann–Whitney; P<0.0001) or 30 days, or GFP, N\textsuperscript{RNAi} and Mkp3 (5 or 10 days). Midguts were pooled from 3 independent experiments. (f) Survival after expression of GFP alone (control), GFP and spi\textsuperscript{RNAi}, GFP and N\textsuperscript{RNAi}, or GFP, N\textsuperscript{RNAi} and spi\textsuperscript{RNAi} with esg\textsuperscript{ts} (n=100 flies per genotype pooled from 2 independent experiments). DNA is a–d (blue). Scale bars in a–c, 40\mu; d, 50\mu. Significance notation is explained in ‘Statistical analysis’.

consisting of DI+ ISC-like cells and Pros+ EE cells\textsuperscript{4}, similar to N\textsuperscript{RNAi}. Similarly to infection, delivering a pulse of JNK activity to ECs before Notch suppression resulted in more tumours per midgut (Fig. 1b) and more flies bearing tumours (Fig. 1c). However, the JNK pulse did not affect ISC outgrowth after initiation (Fig. 1d), indicating that this transient stress specifically promoted tumour initiation. As JNK is known to induce the Upd cytokines\textsuperscript{13} and thereby activate ISCs for division, we examined whether mutant ISC division might be a prerequisite for tumour initiation. To test this we co-expressed Cyclin E (CycE) and string (Cdc25, stg), a gene combination that promotes ISC division\textsuperscript{24}, together with N\textsuperscript{RNAi} in progenitor cells. This also greatly increased tumour incidence, confirming that ISC division is sufficient to promote tumour initiation (Fig. 1e and Supplementary Fig. 6g). These results suggest that, in addition to loss of differentiation capacity, the formation of small clusters of ISC-like cells by stress-induced stem cell divisions may be a prerequisite for tumour formation.

ISC tumour initiation and outgrowth requires autonomous Spi/EGFR signalling

We next sought to define the tumour autonomous factors that drive tumour growth after initiation. EGFR signalling is required for ISC proliferation\textsuperscript{25–28}, so we checked its role. N\textsuperscript{−} or neur\textsuperscript{−} tumour growth increased the expression of the EGFR ligands spitz (spi) and vein (vn) (Fig. 2g and Supplementary Fig. 2b) and mRNA-seq of esg\textsuperscript{+} tumour cells isolated by fluorescence-activated cell sorting revealed that the tumour cells themselves produced more spi but not vn (Fig. 2h). Notably, the highest level of spi expression in normal midguts was in DI\textsuperscript{+} ISCs (Supplementary Fig. 2a). The expression of the EGFR pathway target argos was also increased in the tumour cells (Fig. 2h), which had higher levels of activated MAPK (ppMAPK) than normal ISCs (Fig. 2a–d). To determine whether EGFR/MAPK signalling was necessary for N\textsuperscript{−} tumour growth, we expressed MAPK phosphatase 3 (Mkp3), or RNAi against Egfr or spi in the tumour cells themselves. These treatments suppressed tumour growth. Thus, N\textsuperscript{−} tumour cells, like normal ISCs, require EGFR/MAPK activity to grow (Fig. 3a–e). Depleting spi for 2 weeks in wild-type animals did not deplete progenitor cells, suggesting that Spi, unlike the EGFR (refs 25,26) is not an essential survival factor for normal ISCs. Remarkably, although flies bearing N\textsuperscript{−} ISC tumours died within 10 days, depleting spi in the tumour cells resulted in viable flies with tumours that remained small even after 30 days (Fig. 3e,f). These results indicate that autocrine Spi/EGFR/MAPK signalling is required for ISC tumour growth. As tumour initiation also requires ISC mitosis (Fig. 1), we speculate that Spi signalling may be effective only when multiple ISC-like Spi-expressing tumour cells are juxtaposed in a cluster, and that mitosis generates these clusters.

ISC tumours promote enterocyte detachment, extrusion and death

Tumour cells might compete with normal epithelial cells for adhesion to the BM, which in the midgut consists of extracellular matrix (ECM) components including collagen IV, laminin and perlecian\textsuperscript{29,30}. Cell extrusion due to cell overcrowding has been observed in fish and mouse epithelia, and proposed as a mechanism for maintaining tissue homeostasis\textsuperscript{15}. Accelerating epithelial replacement in the midgut results in the rapid loss of older ECs (ref. 25), possibly also by cell extrusion. However, there is little data available on the extrusion of normal cells at tumour boundaries, or how this might impact tumour growth. In our case, light and electron microscopy showed that ECs adjacent to and juxtaposed in a cluster, and that mitosis generates these clusters.

ARTICLES

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(a, b) Transmission electron micrograph of anterior midgut expressing GFP (a) or N\textsuperscript{TRAM} (b) with esg\textsuperscript{ts} for 3 days. (c) System to independently initiate ISC-derived tumours by heat-shock-induced FLP–FRT-mediated recombination and subsequently express transgenes in ECs with Myo1\textsuperscript{ATsGFP;} neur IF65/-. (d–g) \textbeta-galactosidase (d–e; red) in midguts of flies bearing puc–lacZ and expressing GFP (d, green) or GFP and D\textsubscript{IRNA} (e, green) with esg\textsuperscript{ts} for 8 days. High \textbeta-galactosidase (e; red; arrow) was observed in ECs adjacent to ISC tumours; lower \textbeta-galactosidase (arrowhead) in ECs further away. (f, g) \textbeta-galactosidase (f, green; g, red) in midguts of flies bearing ex–lacZ and expressing GFP (f, green) or GFP and N\textsuperscript{TRAM} (g, green) with esg\textsuperscript{ts} (weak) for 7 days.

(h) Cells per tumour amongst ECs expressing GFP (control, n=35 tumours from 12 midguts, skewness = 1.117, kurtosis = 0.4573) or GFP and p35 (n=42 tumours from 28 midguts, P=0.0019, skewness = 1.586, kurtosis = 1.707) at 29°C. (i) GFP (control, n=83 tumours from 32 midguts, skewness = 2.650, kurtosis = 8.662) and bsk\textsuperscript{E35R} (n=82 tumours from 45 midguts, P=0.0346, skewness = 3.805, kurtosis = 17.430) and puc (n=29 tumours from 19 midguts, P=0.0046), GFP and bsk\textsuperscript{E35R} (n=57 tumours from 44 midguts, P<0.0001, skewness = 3.857, kurtosis = 16.530) or GFP and hep (n=191 tumours from 68 midguts, P=0.0045, skewness = 1.508, kurtosis = 2.082) at 29°C or GFP (control, n=51 tumours from 23 midguts, skewness = 2.414 and kurtosis = 5.719) or GFP and hep\textsuperscript{ts} (n=42 tumours from 14 midguts, P<0.0001, skewness = 1.413 and kurtosis = 1.724) at 29°C (i); GFP (control, n=79 tumours from 41 midguts, skewness = 2.017, kurtosis = 4.577), GFP and wts (n=92 tumours from 33 midguts, P=0.0088) or GFP and yki\textsuperscript{IRNA} (n=55 tumours from 17 midguts, P<0.0001, skewness = 5.693, kurtosis = 36.85) at 29°C (j) with Myo1\textsuperscript{ATsGFP; } 7–8 days. In (h–j), midguts were pooled from 3 independent experiments; P values from Mann-Whitney test; mean (red line) and s.e.m. (blue) are shown. DNA in d, e, f (blue). Scale bars in a, b, 5 μm; in d–d, 25 μm; in e–e, 20 μm; in f–g, 60 μm. Significance notation is explained in ‘Statistical analysis’.

**Figure 4** Growing ISC tumours induce changes in the niche. Growing ISC tumours induce changes in the niche. Figure 4 A RT I C L E S

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effects on tumour initiation these JNK suppressors were activated (Bsk).

JNK and Yki activity spread several EC diameters away from tumours (refs 18,20,21,23). Using reporters for JNK (Epithelial stress activates both JNK and Yki/YAP signalling in midgut epithelia (Fig. 4h and Supplementary Fig. 3d,e), indicating that EC apoptosis was not due directly to increased JNK activity, because rapid EC detachment or pyknotic nuclei were not observed in regions without tumours. By determining the skewness and kurtosis for each tumour population we found that tumour populations were positively skewed, such that most tumours were smaller than the mean size (see Figs 4 and 6 and Supplementary Fig. 7 legends). Decreasing tumour growth by JNK suppression increased the positive skew in the tumour size distribution, such that even more tumours were smaller than the mean and even fewer were larger. In contrast, enhancing tumour growth by overexpressing Hep or HepRNAi in ECs consistently shifted tumour sizes closer to a normal distribution. Using the same approach, we tested the non-tumour autonomous function of Hippo signalling in ISC tumour growth by expressing ykiRNAi or Warts (Wts), an inhibitor of Yki, in ECs. This also inhibited neurons and Yki activity in ECs surrounding larger ISC tumours contributes indirectly to tumour growth.

Tumour growth induces mitogenic signals in the niche

In studying the tumorous neurons clones we noted increased mitoses both inside and outside of the tumours (Fig. 5a,b). In addition, most of the mitoses in tumour cells were at tumour boundaries rather than tumour interiors (Fig. 5c,d). Both observations suggested that the tumours might stimulate the production of diffusible mitogenic signals by surrounding niche cells. Previous studies showed that EC apoptosis or ectopic JNK or Yki activity in ECs can stimulate the expression of the EGFR ligands Vn and Keratin (Krn), and the Upd2 and Upd3 cytokines, all of which are stress-inducible ISC mitogens. Thus, we suspected that stress from the N– neurons might also activate these niche-derived mitogens. Indeed, mRNA-seq and quantitative PCR experiments on tumour-bearing midguts revealed increases in Vn, upd2 and upd3 (Figs 2g and 6e and Supplementary Figs 2b and 5a,d). mRNA-seq experiments on sorted tumour cells showed that both Vn and upd3 expression increased in the surrounding niche rather than in tumour cells (Figs 2h and 6e and Supplementary Fig. 5b) whereas upd2 increased in tumour cells and also the niche (EC, VM; Fig. 6e and Supplementary Fig. 5b). In contrast to a previous report, upd1 induction was detected neither inside nor outside N– tumours (Fig. 6e and Supplementary Fig. 5b–d). Using a reporter we observed high Vn expression in VM adjacent to the tumours (Fig. 2f–g), but not in regions devoid of tumours (Fig. 2e–c). In situ hybridization showed that many ECs adjacent to or located apically to ISC tumours expressed high levels of upd3 mRNA (Fig. 6b–c and Supplementary Fig. 6a–b′), which was undetectable in controls (Fig. 6a–a′). Cytokine induction was not due to EE cell expansion nor to altered enteric bacterial load (Supplementary Figs 5d and 6c–e), and so we infer that it was a direct result of tumour-induced EC stress. mRNA-seq experiments also revealed that the PDGF/VEGF (PVR) ligand Pvr2, the insulin-like peptide Dilp3 and Wg, were non-autonomously induced by N– tumours, presumably in ECs and/or VM (Supplementary Fig. 2c,d).
These signals have also been implicated as ISC mitogens or survival factors\textsuperscript{2,3,3-3\textsuperscript{7}} and might also promote tumour growth.

**Upd cytokines produced by tumour-adjacent enterocytes drive tumour growth**

The most potent known effectors of ISC proliferation are the Upd cytokines. We found that tumour cells had high STAT activity (Fig. 6d,d’ and Supplementary Fig. 5h-i’), expressed the STAT target Socs36E (Fig. 6e), and could be stimulated to grow faster by increasing Upd signalling (Fig. 6f). Expressing oncogenic Ras (Ras\textsuperscript{V12G}) in ECs—a treatment that induces JNK activity (Supplementary Fig. 7) and Upd3 (ref. 25) in ECs—also strongly accelerated \(N^\text{\textsuperscript{-}}\) tumour growth (Supplementary Figs 7c and 3l,m). Previous reports\textsuperscript{8,39} showed that STAT is required in \(N^\text{\textsuperscript{-}}\) ISC tumours for them to produce EEs, but the role of JAK–STAT signalling in tumour growth was not addressed. Expressing a dominant-negative form of the Upd receptor Domeless (Dome\textsuperscript{ACYT}) in \(N^\text{\textsuperscript{-}}\) tumours strongly suppressed their growth without altering cell identity (Fig. 6f and Supplementary Fig. 5g-g’,i-j’), indicating that Upd signalling was required for tumour growth.

A recent study of \(Apc^\text{\textsuperscript{-}}\) tumours in the fly midgut\textsuperscript{11} also reported the induction of an \(upd3\) reporter gene (\(upd3\)-lacZ) in...
tumour-adjacent ECs, and showed that *domeless* and *Stat92E* were required in the tumour cells for optimal tumour growth. However, the requirement for *upd3* was not functionally tested, and the *Apc* tumour cells themselves also expressed *upd3-lacZ* (ref. 11), leaving it unclear whether niche-derived Upd3 was important for tumour growth. To resolve this question we directly tested the requirement for niche-derived Upd3 and Upd2. First, we measured tumour growth. To determine whether EC detachment from the BM and VM was involved in the induction of JNK and Yki activity and Upd3 expression, we first scored how detachment correlated with these markers. However, we did not observe increased dpMAPK staining in ECs tumour-adjacent enterocytes that were fully detached, partially detached or fully detached in midguts bearing *upd3.1-lacZ* (yellow, *n* = 4 z-stacks), *puc-lacZ* (green, *n* = 3 z-stacks) or ex-*lacZ* (red, *n* = 3 z-stacks) and expressing GFP and *N^{RNAi}* with *esg* for 3 days. (b) Mean percentage with s.e.m. of *N* tumour-adjacent enterocytes in each category (fully attached, partially detached and fully detached) that had high β-galactosidase positivity, in midguts bearing *upd3.1-lacZ* (yellow, *n* = 4 z-stacks), *puc-lacZ* (green, *n* = 3 z-stacks) or ex-*lacZ* (red, *n* = 3 z-stacks) and expressing GFP and *N^{RNAi}* with *esg* for 3 days. (c) β-galactosidase (red) in midguts bearing *upd3.1-lacZ* expressing GFP (green) and *N^{RNAi}* with *esg* for 3 days. (d) βPS1 integrin (d, d′, magenta) in midguts bearing puc-*lacZ* and expressing *Mmp1* and GFP (d, green) with *Myo1Ab* for 2 days. β-galactosidase (d′, d″, red (nuclear)) in ECs and phalloidin (actin) (d′; d″, red) in VM of midguts bearing puc-*lacZ* and expressing *Mmp1* and GFP (d′′, green) with *Myo1Ab* for 2 days. β-galactosidase (e, e′, red) in ECs and phalloidin (e′, white) in VM of midguts bearing ex-*lacZ* and expressing *Mmp1* and GFP (e′, green) with *Myo1Ab* for 2 days. β-galactosidase (f, f′, red) in ECs of midguts bearing *upd3.1-lacZ* and expressing *Mmp1* and GFP (f, green) with *Myo1Ab* for 2 days. In (a, b, z-stacks acquired from 2 independent experiments. DNA in (c′, d′, e′ and f′ (blue). Scale bars in (c–c′, 40 μm; d–d′, 35 μm; e–e′, 25 μm; f–f′, 60 μm.

**Figure 7** Tumour-induced enterocyte detachment induces JNK and Yki activity and *upd3* expression. (a) Mean percentage with s.e.m. of β-galactosidase (β-gal)-positive *N* tumour-adjacent enterocytes that were fully attached, partially detached or fully detached in midguts bearing *upd3.1-lacZ* (yellow, *n* = 4 z-stacks), *puc-lacZ* (green, *n* = 3 z-stacks) or ex-*lacZ* (red, *n* = 3 z-stacks) and expressing GFP and *N^{RNAi}* with *esg* for 3 days. (b) Mean percentage with s.e.m. of *N* tumour-adjacent enterocytes in each category (fully attached, partially detached and fully detached) that had high β-galactosidase positivity, in midguts bearing *upd3.1-lacZ* (yellow, *n* = 4 z-stacks), *puc-lacZ* (green, *n* = 3 z-stacks) or ex-*lacZ* (red, *n* = 3 z-stacks) and expressing GFP and *N^{RNAi}* with *esg* for 3 days. (c) β-galactosidase (red) in midguts bearing *upd3.1-lacZ* expressing GFP (green) and *N^{RNAi}* with *esg* for 3 days. (d) βPS1 integrin (d, d′, magenta) in midguts bearing puc-*lacZ* and expressing *Mmp1* and GFP (d, green) with *Myo1Ab* for 2 days. β-galactosidase (d′, d″, red (nuclear)) in ECs and phalloidin (actin) (d′; d″, red) in VM of midguts bearing puc-*lacZ* and expressing *Mmp1* and GFP (d′′, green) with *Myo1Ab* for 2 days. β-galactosidase (e, e′, red) in ECs and phalloidin (e′, white) in VM of midguts bearing ex-*lacZ* and expressing *Mmp1* and GFP (e′, green) with *Myo1Ab* for 2 days. β-galactosidase (f, f′, red) in ECs of midguts bearing *upd3.1-lacZ* and expressing *Mmp1* and GFP (f, green) with *Myo1Ab* for 2 days. In (a, b, z-stacks acquired from 2 independent experiments. DNA in (c′, d′, e′ and f′ (blue). Scale bars in (c–c′, 40 μm; d–d′, 35 μm; e–e′, 25 μm; f–f′, 60 μm.

**JNK, Yki and Upd3 are induced by detachment of enterocytes from the VM**

It was proposed that hyperplastic *Apc* tumours induce *upd3* expression by stimulating EGFR signalling in surrounding ECs. However, we did not observe increased dpMAPK staining in ECs surrounding *N* ISCs tumours, even though these tumours produced high levels of Spi (Fig. 2a–d,h). Rather, we found high JNK and Yki activity and *upd3* expression in ECs surrounding ISCs tumours. To determine whether EC detachment from the BM and VM was involved in the induction of JNK and Yki activity and *upd3* expression, we first scored how detachment correlated with these markers. Tumour-adjacent ECs positive for puc-*lacZ*, ex-*lacZ* or *upd3.1-lacZ* were scored in three categories: fully attached, partially detached or fully detached. *upd3.1-lacZ* was observed nearly exclusively in fully or partially detached ECs, whereas puc-*lacZ* and ex-*lacZ* were...
Figure 8 | Integrin loss from enterocytes induces JNK activity and promotes ISC tumour growth. (a,b) β-galactosidase (nuclear) (a,b, a’, b, white) and βPS1 (a,b, red) in ECs of midguts bearing puc–lacZ and expressing GFP (a’, green) or GFP and αPS3+4tsRNAi (b’, green) with Myo1AtsGFP for 7 days. (c) Mean number with s.e.m. of phosphorylated histone H3 Ser 10-positive cells per midgut in flies expressing GFP (control, n = 18 midguts), GFP and αPS3+4tsRNAi (n = 19 midguts, Mann–Whitney test: P < 0.0006) or βPS1tsRNAi (n = 17 midguts, Mann–Whitney: P < 0.0004) with Myo1AtsGFP for 7 days. (d) βPS1 integrin (d,d, red) in midguts bearing neuRts65– tumours (yellow dashed line) and expressing GFP (d’, green) with Myo1AtsGFP for 10 days. Arrowheads indicate detached ECs apical to tumours that have lost basal βPS1 expression. (e) Cells per neuRts65– tumour with mean (red line) and s.e.m. amongst ECs expressing either GFP (control, n = 74 tumours from 35 midguts, skewness = 1.876, kurtosis = 4.283) or GFP and αPS3+4tsRNAi (n = 63 tumours from 10 midguts, Mann–Whitney: P = 0.00116, skewness = 1.126) or GFP and βPS1tsRNAi (n = 54 tumours from 23 midguts, Mann–Whitney: P = 0.0004, skewness = 1.262, kurtosis = 1.093) with Myo1AtsGFP for 7 days. (f) Model for Notch-dependent tumorigenesis in the adult Drosophila midgut. In c and e, midguts pooled from 3 independent experiments. DNA in a,b,d,d- (blue). The scale bars in a–b, d–d’, 20 μm. Significance notation is explained in ‘Statistical analysis’.

also observed in fully attached tumour-adjacent ECs (Fig. 7a,c–c’). When we scored only very strong signals for these markers, the trends were more obvious: high upd3.1–lacZ signal was predominantly confined to partially and fully detached ECs, whereas high JNK and Yki signals were less tightly correlated with detachment and spread further from the tumours (Figs 7b and 4e–e’). These data indicate that JNK and Yki activity and upd3 expression are induced in detached ECs, and also that the induction of JNK and Yki activity can spread to neighbouring fully attached ECs (Supplementary Fig. 8a).

To determine whether EC detachment is sufficient to induce JNK and Yki activity in ECs we expressed the matrix metalloproteinase Mmp1, which is known to cleave ECM components. We also depleted integrins, which mediate cell adhesion to the ECM, from ECs. Loss of integrins from ISCs has been shown to affect their asymmetric division, proliferation and maintenance, whereas loss of β integrin subunits from ECs can induce ISC proliferation. Mmp1 expression in ECs resulted in their detachment from the VM (Fig. 7d–d’). Detaching ECs in this way caused them to lose βPS1 (Myospheroid, Mys) and to induce JNK and Yki activity (Fig. 7d–d’,e–e’) and upd3
expression (Fig. 7f–f’). Interestingly, we also found that ECs detached by tumours had reduced βPS1 expression (Fig. 8d–d’). Our mRNA-seq analysis of ECs revealed that in addition to βPS1 (mys), αPS1 (multiple edematous wing, mew) and αPS3 (scab, scb) were highly expressed in ECs (Supplementary Fig. 8b–c”), consistent with previous reports.41 We found that depleting βPS1, αPS1 or both αPS3 and αPS4, but not αPS2 (inflated, if), in ECs increased the number of detaching ECs and induced ISC proliferation (Fig. 8a–c). These ECs showed decreased basal surfaces and increased JNK activity (Fig. 8b–b’). JNK activity was often observed in partially detached ECs (Figs 7a and 8b–b’) suggesting that decreased ECM adhesion, rather than decreased proximity to the VM, induces stress signalling. Notably, activating JNK directly by expressing Hep4 in ECs did not result in their rapid detachment, indicating that JNK activation is not likely to be causal for EC detachment. Together these data demonstrate that EC detachment from the BM is sufficient to stimulate JNK and Yki activity, and upd3 expression (Supplementary Fig. 8a).

ISC tumours and enterocytes compete for the substratum

A previous study in the fly midgut found that reducing integrins in N− or Apc− tumour cells inhibited their growth, and that these tumours were eliminated from the epithelium.41 We confirmed this interesting observation, which suggests that tumour cells may compete with normal cells for BM attachment. To test the importance of tumour/EC competition in another way, we generated tumours amongst ECs in which integrins were depleted by targeted RNAi. We expected that this might reduce the ability of these ECs to compete with the tumour cells for BM attachment. Indeed, we found that these treatments enhanced tumour growth (Fig. 8e). Together, these data support the idea that tumour cells compete with ECs for attachment to the BM, through integrin-mediated adhesion.

DISCUSSION

Here we describe a step-wise series of events during the earliest stage of tumour development in a stem cell niche (Fig. 8f). First, the combination of environmentally triggered mitogenic signalling and a mutation that compromises differentiation generates small clusters of differentiation-defective stem-like cells. Autocrine (Spi/EGFR) signalling between these cells then promotes their expansion into clusters, which quickly reach a size capable of physically disrupting the surrounding epithelium and driving the detachment and apical extrusion of surrounding epithelial cells (that is, ECs). This loss of normal cells seems to involve tumour cell/epithelial cell competition through integrin-mediated adhesion. Subsequently, the loss of epithelial integrity (specifically, EC detachment) triggers stress signalling (JNK, Yki/YAP) in the surrounding epithelium and underlying VM, and these stressed tissues respond by producing cytokines (Upd2,3) and growth factors (Vn, Pvf, Wg, dILP3). These signals are normally used within the niche to activate stem cells for epithelial repair, but in this context they further stimulate tumour growth in a positive feedback loop. It is noteworthy that in this example a single mutation that blocks differentiation is sufficient to drive early tumour development, even without secondary mutations in growth signalling pathways that might make the tumour-initiating cells growth-factor- and niche-independent (for example, Ras, PTEN).

Thus, tumour cell–niche interactions can be sufficient to allow tumour-initiating cells to rapidly expand, increasing their chance to acquire secondary mutations that might enhance their growth or allow them to survive outside their normal niche.42 Our study highlights the importance of investigating the factors that control paracrine stem cell mitogens and survival signals in the niche environment. Tumour-niche interactions may be important to acquire a sizable tumour mass before the recruitment of a tumour-specific microenvironment that supports further tumour progression. A careful analysis of similar interactions in other epithelia, such as in the lung, skin or intestine could yield insights relevant to the early detection, treatment and prevention of cancers in such tissues.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

The project was conceived and developed by P.H.P. and B.A.E. P.H.P. contributed Figs 1–8 and Supplementary Figs 1–8. D.D. contributed Figs 2a,c,d, 4b,c, 5b and 8b. mRNA-seq data were analysed by P.H.P. and NIH R01 GM051186, DKFZ A220, DFG SFB 673 and ERC Advanced Grant 268351 to B.A.E.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Fly stocks. All experiments were performed using 5–10-day-old, adult female Drosophila melanogaster. The following fly stocks were used: eg-GAL4; tubGAL80/ UAS–GFP (eg-GAL4, Myo1AL4; tubGAL80/UAS–GFP (Myo1AL4)), tubGAL80/ UAS–GFP; heat shocked at 37 °C until dissection. For data describing mitoses per midgut, cells per tumour, and tumour frequency, the Mann–Whitney test (two-sided) was applied to determine statistical significance. For data describing tumour incidence or mitoses at the tumour interior/boundary, the paired t-test (two-sided) was applied to determine statistical significance. The significance level is indicated as * for P < 0.05, ** for P < 0.01, and *** for P < 0.001. For P > 0.05, no significance was indicated.

In situ hybridization. Fluorescent in situ hybridization for upd3 mRNA was performed as described in ref. 25. Midguts were dissected, fixed in 8% paraformaldehyde/PBS overnight at 4 °C, permeabilized 3 times for 10 min in PBST (PBS, 0.1% Triton X-100) and then stored in 70% ethanol. Midguts were then hybridized with a fluorescently labelled (CAL Fluor Red 590) DNA 20-nucleotide probe (pool of 48; Stellaris) against the upd3 transcript.

Light microscopy. Samples were analysed using Nikon Eclipse Ti, Leica DM5000B, Zeiss LSM 510 and Leica SP5 microscopes. Images were processed with ImageJ (NIH) and Adobe Photoshop CS5. Confocal images are presented as maximal intensity projection of images obtained every 0.25–1.0 µm. When comparing protein or transcript levels, each z-stack was acquired with the same laser intensity and gain, maximal intensity projections of identical dimensions were created, and were further similarly processed with Adobe Photoshop CS5, except in the case of Supplementary Fig. 5, which was significantly brightened. Representative images presented were obtained from ≥2 independent experiments.

Transmission electron microscopy. Midguts were dissected, fixed in 0.1% Karnovsky's fixative and post-fixed in OsO4. Midguts were then dehydrated in ascending concentrations of ethanol, embedded in epoxy and sectioned to obtain 70–90 nm sections. Samples were analysed with a FEI Tecnai 1230 transmission electron microscope with an Orius SC1000 Gatan CCD.

mRNA sequencing of whole midguts and sorted midgut cell populations. Whole midgut RNA was isolated from 15 guts using the RNAeasy RNA isolation kit (Qiagen) following the manufacturer's protocol. Cell-type-specific profiling was performed as previously described41. Briefly, 100 guts were dissected in RNAfree PBS and treated with 7.5 mg ml−1 collagenase or 4 mg ml−1 elastase 1 h at 27 °C. Dissociated cells were pelleted at 300 g for 15 min, resuspended in 1X RNAase-free PBS, filtered using 25 µm filters (BD Falcon) and sorted using a FACS Aria II sorter (BD Biosciences) with 70 µm nozzle size. To exclude auto-fluorescence, gates were set using control midguts from w1118 flies. Cell-type-specific GAL4 drivers (ex: Myo1A–GAL4 (EEs)) were used to express green or yellow fluorescent protein (GFP or YFP) to sort each cell population. GFP- or YFP-positive cells were sorted on the basis of fluorescence intensity and cell size. A total of 2,000 cells were sorted for each sample. Total RNA from each sorted cell type was isolated using the PicoPure RNA isolation kit (Arcturus). Two nanograms of isolated total RNA was used for RNA amplification using the Arcturus RibonH Plus RNA Amplification Kit for whole midgut and cell-type-specific RNA profiling. Total RNA from each sample was reverse transcribed using a T7 promoter sequence containing oligo(dT) primer and SuperScript III Reverse Transcriptase (200U) enzyme. Random hexamers were used for the second strand synthesis. Amplified RNA (aRNA) was then produced by in vitro transcription using T7 RNA polymerase at 42 °C for 6 h. The aRNA integrity was determined with an Agilent 2100 bioanalyzer, enriched for 200–400bp mRNA and used directly for RNA sequencing. Amplified mRNA was then shunted by magnesium-catalysed hydrolysis and used for cDNA library preparation. Adapters were ligated and mRNA sequencing was performed using an Illumina HiSeq2500 sequencer with 50 bp read length.

mRNA sequencing data analysis. All reads were inspected using Fastqc version 0.11.0 with default settings. As no trimming was needed, the raw reads were mapped to the reference genome version 70 (ENSEMBL) using tophat2 (version 2.0.9) with default parameters (2 mismatches allowed), boost library 1.54.0, bowtie2 2.1.0 and samtools version 0.1.19. The resulting bam files were converted to sam files using samtools, and subsequently counted using HTSeqCount (0.5.4p3). Ambiguous reads, reads with low quality, unaligned reads, reads with non-unique alignment and reads aligning to non-features were discarded. Differential expression analysis was conducted using edgeR (3.2.4) with filtering for low expressed genes showing a c.p.m. value above 1 in two biological replicates. P values were adjusted using Benjamini–Hochberg correction. Genes with a c.p.m. > 1, a 1.5-fold change and an adjusted percentage of affected flies obtained from 3 independent experiments and s.e.m. are presented for each genotype.
P value < 0.05 were considered significantly deregulated. The mean normalized expression (RPKM, log2) value from 2 independent experiments, adjusted fold change and adjusted P value for each gene are presented. A principle component analysis (PCA) was conducted using the function prcomp in the stats package of R (3.1.0) with scaling the variables to have unit variance and zero centrering based on the mean RPKM values per cell type. Only genes showing RPKM values greater than zero were used for calculation.

Quantitative RT–PCR. RNA was isolated from 15 to 20 midguts using either TRIzol or RNAeasy kit (Qiagen); 250–500 ng of RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad) or the Quantitect cDNA synthesis kit (Qiagen); 250–500 ng of RNA was used for cDNA synthesis using the iScript One Step RT–PCR kit with using the iScript cDNA synthesis kit (Bio-Rad) or the Quantitect cDNA synthesis kit (Qiagen). qPCR was performed using the iScript One Step RT–PCR kit with the iScript One Step RT–PCR kit with SYBR green (Bio-Rad) or with LightCycler 480 SYBER Green I Master (Roche). SYBR green incorporation during PCR was detected using the iQ5 system (Bio-Rad) or Roche 480 II Lightcycler. The following primers were used: upd1 F: 5′-CCACGTAAGTTTGCATGTTG-3′, upd1 R: 5′-CTAAACAGTAGGACAGGC-3′, upd2 F: 5′-CACAAGTGCCGGAAGCTCAA-3′, upd2 R: 5′-GGGCTTCTTGCGGATGCTCTTG-3′, upd3 F: 5′-GCCCTCTTCAACAAAATCGA-3′, upd3 R: 5′-TCTCTCCTTGCAGCTCCTTG-3′, spi F: 5′-CTCTTCTTGTGCCGTTTGG-3′, spi R: 5′-CGCATGTGAAAGTGGTAGCTT-3′, Km F: 5′-CGTGTCTTTGCGAACAAACAGT-3′, Km R: 5′-TGTCGCAATGCAGTTTAAGG-3′, vn F: 5′-AA CGCAGAGGTCAAGAAAGAT-3′, vn R: 5′-GGGCACATTTAGCCTCGGAAC-3′, Dl F: 5′-TCGTCTTTGACAGGAGGTCCT-3′, Dl R: 5′-AAGCTGCAAGCATTAG TGTC-3′, Ast F: 5′-CCTCGGGCTCTAATACTCG-3′ and Ast R: 5′-GATCTCG TTGTCTCTGTCTG-3′. We found that cnr gene expression did not change after ISC tumour growth and thus the expression of each target gene was normalized to the threshold cycles for the control gene. The raw mRNA-seq data for control midguts (+, GFP) and +, GFP and control midguts (−, lacZ) were scored every 2 days and surviving adults were transferred to fresh food vials. Flies expressing with lacZ were scored until 50 flies were found dead. For all other genotypes, the number of dead flies was scored for 14 days.

Survival analysis. One hundred adult flies per genotype were transferred to food vials (10 flies per vial) and shifted to 29°C. The number of dead flies in each vial was scored every 2 days and surviving adults were transferred to fresh food vials. Flies expressing with lacZ were scored until 50 flies were found dead. For all other genotypes, the number of dead flies was scored for 14 days.

Antibiotic treatment and detection of enteric bacteria levels. Fifty adults were fed for 3–4 days normal fly food or food containing antibiotics (10,000 U ml−1 penicillin, 10 mg ml−1 streptomycin (1:150)). Midguts were dissected from 20 to 25 flies, homogenized and used for total genomic DNA isolation. gDNA was then used for PCR for bacterial 16S rDNA with the following primers: F: 5′-AAGAGTTGAT CCTGGCTTCAG-3′ and R: 5′-GTTATCCGTTCGACTT-3′. Twenty to twenty-five flies from the same cohort were shifted to 29°C for 2–3 days to express either GFP or GFP and N1105 with esg+ to assess tumour growth in midguts with normal or low luminal bacteria levels.

Accesion code. The raw mRNA-seq data for control midguts (esg+, GFP), midguts with tumours (esg+, N1105, GFP), esg+ cells sorted from control midguts (esg+, GFP) and esg+ cells sorted from tumorous midguts (esg+, N1105, GFP) have been deposited at the National Center for Biotechnology Information Sequence Read Archive (NCBI-SRA) under the primary accession number, SRP049937.
**Supplementary Figure 1** ISC tumor cells differentiate towards the secretory fate. (a) Prospero (a, red) in midguts expressing GFP (a, green) with \( esg^{ts} \) for 3 days. (b) Pdm-1 (b, red) in midguts expressing GFP (b, green) with \( esg^{ts} \) for 2 days. (c) Delta (c') and Prospero (Pros, c'') in midguts expressing GFP and \( N^{RNAi} \) (c) with \( esg^{ts} \) for 3 days. \( esg^{ts}Dl^{ts} \) cells expressed high levels of Prospero (c-c'', arrowheads); \( esg^{ts}Dl^{ts} \) ISC-like cells expressed lower levels of Prospero (c-c'', arrows). (d) Pdm-1 (d'; d'', red) in midguts expressing GFP (d; d'', green) and \( N^{RNAi} \) with \( esg^{ts} \) for 2 days. (e-f) Prospero (e', f', red) in midguts expressing GFP (e, green) and \( N^{RNAi} \) with \( esg^{ts} \) for 5 days or in midguts recovering for 7 days at 18°C after expression of GFP (f, green) and \( N^{RNAi} \) for 5 days with \( esg^{ts} \). (g-j) Mean normalized expression (NE) value (RPKM, log2) from \( n=2 \) independent experiments of ISC (g) or EE cell (j) associated genes determined by mRNA sequencing of midguts expressing GFP (control, red) or GFP and \( N^{RNAi} \) (tumorous, blue) with \( esg^{ts} \) for 3 days. The adjusted fold change in gene expression in tumorous midguts (blue), normalized to control midguts (red), is indicated. Benjamini-Hochberg adjusted \( p \)-value for \( Dl, spdo, esg, shg, sc, pros, Rab3 \) and AstA is 7.71e-322; arm, \( p=1.05e-50 \). The mean NE value (RPKM, log2) from \( n=2 \) independent experiments of ISC (h) and EE (i) associated genes determined by mRNA sequencing of \( esg^{ts} \) cells sorted from control midguts expressing GFP (red) or from tumorous midguts expressing GFP and \( N^{RNAi} \) (blue) with \( esg^{ts} \) for 3 days. The adjusted fold change in gene expression in sorted \( esg^{ts} \) tumor cells (blue), normalized to \( esg^{ts} \) cells from control midguts, is indicated. NS, not significant. Benjamini-Hochberg adjusted \( p \)-values for \( Dl \) is 2.53e-05; \( spdo \), 1.81e-05; \( sc \), 3.48e-14; \( pros \), 1.26e-14; \( Rab3 \), 2.52e-19 and AstA, 1.01e-09. (k) Principal component analysis of transcriptional profiles of ISC tumors, intestinal stem cells (ISCs), \( esg^{ts} \) progenitors, \( SuH^{+} \) enteroblasts (EB), enterocytes (EC), enteroendocrine cells (EE) and visceral muscle (VM). DNA is in a', b', c''', d'''; e-f' (blue). Scale bars in a-a', b-b', c-c'' and d-d'', 25 \( \mu \)m; e-f', 30 \( \mu \)m.
**Supplementary Figure 2** ISC tumors induce EGF, insulin, PDGF-VEGF and Wnt signaling in the niche. (a) Mean normalized expression values (RPKM, log2) of \( \text{spi} \) determined by mRNA sequencing of sorted midgut cell types from \( n=2 \) independent experiments. (b) Mean fold change (log2) with s.e.m. from \( n=4 \) independent experiments of EGFR ligand mRNA levels determined by qPCR on midguts bearing 7 day \( \text{neur}^{F65/} \) clones. Fold changes were normalized to control midguts bearing 7 day wild-type clones. (c-d) Mean normalized expression (NE) value (RPKM, log2) from \( n=2 \) independent experiments of other midgut signaling factors determined by mRNA sequencing of midguts expressing GFP (control, red) or GFP and \( \text{N}^{\text{RNAi}} \) (tumorous, blue) with \( \text{esg}^{ts} \) for 3 days (c). The adjusted fold change in gene expression in tumorous midguts (blue), normalized to control midguts (red), is indicated. Benjamini-Hochberg adjusted p-value for \( \text{Ilp}3 \) is \( 2.47 \times 10^{-35} \); \( \text{Pvf}2 \), \( 4.00 \times 10^{-46} \) and \( \text{wg} \), 0.0016. The mean NE value (RPKM, log2) from \( n=2 \) independent experiments of midgut signaling factors determined by mRNA sequencing of \( \text{esg}^{+} \) cells sorted from control midguts expressing GFP (red) or from tumorous midguts expressing GFP and \( \text{N}^{\text{RNAi}} \) (blue) with \( \text{esg}^{ts} \) for 3 days (d). NS, not significant.
Supplementary Figure 3 Tumor frequency and incidence after inhibiting apoptosis, JNK and Yki activity and cytokine expression in enterocytes. (a) 8 midgut regions used to determine the frequency of \textit{neur}\textsuperscript{F65\textsuperscript{L}}- tumor incidence (b) and tumor size (c) along the midgut anterior (A)–posterior (P) axis. (b) Mean percent of total \textit{neur}\textsuperscript{F65\textsuperscript{L}}- tumors (n=45) found in each of 8 midgut regions. (c) Mean number of cells per \textit{neur}\textsuperscript{F65\textsuperscript{L}}- tumor (n=45) with s.e.m. found in each of 8 midgut regions. (d, f, h, j, l) Mean tumors per midgut with s.e.m. 7 or 8 days after tumor induction in midguts expressing GFP (n=6 midguts) or GFP and \textit{p35} (n=12 midguts) (d), GFP (n=16 midguts) or GFP and \textit{bskK53R} (n=17 midguts), \textit{puc} (n=6 midguts), \textit{bskRNAi} or \textit{hep} (n=28 midguts) (f), GFP (control, n=16 midguts) or GFP and \textit{wts} (n=19 midguts) or \textit{ykiRNAi} (n=9 midguts) (h), GFP (control, n=17 midguts) or GFP and \textit{upd2RNAi} (1) (n=1 midguts), \textit{upd2RNAi} (2) (n=3 midguts), \textit{upd3RNAi} (1) (n=12 midguts) or \textit{upd3RNAi} (2) (n=10 midguts) (j), and GFP (control, n=25 midguts) or GFP and \textit{RasV12} (n=26 midguts) (l) in ECs with \textit{Myo1Ats}. (e, g, i, k, m) Mean percent flies with tumors with s.e.m. 7 or 8 days after tumor induction in midguts expressing GFP or GFP and \textit{p35} (e), GFP or GFP and \textit{bskK53R}, \textit{puc}, \textit{bskRNAi} or \textit{hep} (g), GFP or GFP and \textit{wts} or \textit{ykiRNAi} (i), GFP or GFP and \textit{upd2RNAi} (1), \textit{upd2RNAi} (2), \textit{upd3RNAi} (1) or \textit{upd3RNAi} (2) (k), and GFP or GFP and \textit{RasV12} (m) in ECs with \textit{Myo1A\textsuperscript{C}}. In b-c, tumors pooled from 2 independent experiments; in d, f, h, j and l, midguts were pooled from 3 independent experiments; in e, g, i, k, and m, mean determined from n=3 experiments.
**Supplementary Figure 4** ISC tumor growth induces enterocyte detachment and loss. (a) DNA (a, blue) in midguts containing neurIF65/− tumors (yellow dashed line) amongst ECs expressing GFP (a’, green) with Myo1A for 8 days. Arrowheads indicate ECs overlying tumors (a, a’). (b-c) Mean normalized expression (NE) value (RPKM, log2) from n=2 independent experiments of pro-apoptotic factors (hid, rpr, grim and skl) determined by mRNA sequencing of midguts expressing GFP (control, red) or GFP and esgts (blue) for 3 days (b). The adjusted fold change in gene expression in tumorous midguts (blue), normalized to control midguts (red), is indicated. The Benjamini-Hochberg adjusted p-value for grim is 5.86e-127. The mean NE value (RPKM, log2) from two independent experiments of apoptosis factors determined by mRNA sequencing of esg+ cells sorted from control midguts expressing GFP (red) or from tumorous midguts expressing GFP and NRNAi (blue) with esgts for 3 days (c). NS, not significant. (d) Transmission electron micrograph of an apoptotic EC (anterior) from midguts expressing GFP and NRNAi (blue) with esgts for 3 days. White arrowheads highlight abnormal EC microvilli. Scale bars in a-a’, 30 μm; in d, 2 μm.
Supplementary Figure 5  Cytokines are induced by ISC tumor growth and not by enteroendocrine cell expansion. (a) Mean fold change (log2) with s.e.m. from n=4 independent experiments in Delta and cytokine ( upd1, upd2, upd3) mRNA levels determined by quantitative PCR on midguts bearing 7 day old neu65F6S clones. Fold changes were normalized to control midguts bearing 7 day old wild-type clones. (b) The mean normalized expression (NE) value (RPKM, log2) from n=2 independent experiments of cytokines ( upd1-3) and JAK-STAT target socs36E determined by mRNA sequencing of esg+ cells sorted from control midguts expressing GFP (red) or from tumorous midguts expressing GFP and N RNAi (blue) for 3 days. The adjusted fold change in gene expression in sorted esg+ tumor cells (blue), normalized to esg+ cells from control midguts, is indicated. NS, not significant. The Benjamini-Hochberg adjusted p-value for upd2 is 1.36e-10. (c) β-galactosidase (c; c', red) in midguts of flies carrying upd-lacZ and expressing N RNAi with esgts for 2 days. (d) Mean fold change (log2) with s.e.m. from n=4 independent experiments in Delta and cytokine ( upd 1-3) and EGF ligand ( spi, km, vn) mRNA levels determined by quantitative PCR on midguts expressing N RNAi for 2 days (n=3 independent experiments), sc for 7 days (n=3 independent experiments), and Suh RNAi for 5 days (n=4 independent experiments) with esgts. Fold changes were normalized to control whole midguts overexpressing GFP with esgts for 2 (for N RNAi), 7 (for sc) and 5 days (for Suh RNAi), ND, not determined; NS, not significant. Overexpression of the pro-secretory factor Scute (Sc) expanded the number of Pros+ EEs (Ast+). (e-f) Phospho-histone H3 Ser 10 (red) in control (e) or upd2-3 mutant (Δ2-3) (f) midguts expressing GFP (green) and N RNAi for 14 days with esgts (weak). (g) Delta (g; g', red) and Prospero (g'') in midguts expressing GFP (g', green) and N RNAi with esgts for 4 days. (h) β-galactosidase (red) and D-GFP (green) in midguts of flies carrying Dl-lacZ and 10X STAT-DGFP. (i-j) D-GFP (i) or N RNAi and domeΔCYT (j) with esgts for 4 days. Rare, large ISC tumor shown in j. DNA in c', e, f, h, i, and j' (blue). Scale bars in c-c’, 35 μm; e, 25 μm; f, 30 μm; g-g’, 20μm; h, 4μm; i-j’, 30μm.
Supplementary Figure 6 Cytokine induction by ISC tumors is not due to changes in luminal bacteria load. (a-b) *in situ* hybridization to *upd3* mRNA in midguts from flies infected with *Pseudomonas entomophila* for 2 days (b; b', red) and control midguts (no infection) (a; a', red). Cells with high *upd3* mRNA are indicated with arrows; with lower *upd3* mRNA, arrowheads (b-b’). (c) PCR for bacterial 16S rDNA from total genomic DNA isolated from midguts of flies fed normal food or antibiotics for 3 days (undiluted genomic DNA, 1:1). Tumor growth did not increase luminal bacterial load. (d) PCR for the *crq* gene to detect fly genomic DNA input. (e) Mean number of phosphorylated histone H3 Ser10 positive cells per midgut with s.e.m. of flies fed food alone (n=31 midguts), *Pseudomonas entomophila (P.e.)* containing food (n=24 midguts; Mann-Whitney: p<0.0001), sucrose alone (n=33 midguts) or P.e. containing sucrose (n=15 midguts; Mann-Whitney: p=0.0001) for 1 day prior to tumor induction by expressing *NRNAi* with *esg*3 (weak) for 3 days (food alone). (f) Mean number of phosphorylated histone H3 Ser10 positive cells per midgut with s.e.m. in flies expressing GFP (control, -antibiotics: n=20 midguts ; +antibiotics: n=22 midguts) or *NRNAi* (-antibiotics: n=26 midguts; +antibiotics: n=28 midguts) with *esg*3 for 3 days fed normal food or food containing antibiotics. Reducing gut luminal bacteria to negligible levels had no significant effect on tumor growth. Thus Upd2,3 induction after tumor growth was not due to alterations in luminal bacteria but was a response to tumor growth. (f) Mean number of phosphorylated histone H3 Ser10 positive cells per midgut with s.e.m. of flies fed food alone (n=31 midguts), *Pseudomonas entomophila (P.e.)* containing food (n=24 midguts; Mann-Whitney: p<0.0001), sucrose alone (n=33 midguts) or P.e. containing sucrose (n=15 midguts; Mann-Whitney: p=0.0001) for 1 day prior to tumor induction by expressing *NRNAi* with *esg*3 (weak) for 3 days (food alone). (g) Mean number of phosphorylated histone H3 Ser10 positive cells per midgut with s.e.m. in flies expressing GFP and *NRNAi* (n=36 midguts) or GFP, *NRNAi*, cyclin E (*cycE*) and *cdc25*/*stringy* (*stg*) (n=29 midguts, Mann-Whitney: p<0.0001) with *esg* weak (weak) for 3 days. In e, midguts pooled from 2 independent experiments; in f-g, from 3 independent experiments. DNA in a’ and b’ (blue). Scale bars in a-a’, 25μm; b-b’, 35μm.
Supplementary Figure 7  Oncogenic RasV12 expression in ECs induces JNK activity and stimulates ISC tumor growth. (a-c) β-galactosidase (a-c; a', c', red) in midguts bearing puc-lacZ and expressing GFP (a', b', c'; green) alone for 5 days (a) or GFP and hepAct for 2 days (b) or GFP and RasV12 for 5 days (c) with Myo1Ats. (d) Mean number of phosphorylated histone H3 Ser10 positive cells per midgut with s.e.m. in flies expressing GFP alone (control, n=11 midguts), GFP and hep (n=9 midguts, Mann-Whitney: p=0.0064) or RasV12 (n=15 midguts, Mann-Whitney: p<0.0001) for 7 days or GFP and hepAct (n=11 midguts) for 2 days with Myo1Ats. (e) Cells per neuIf65- tumor with mean (red line) and s.e.m. amongst ECs expressing GFP (control, n=56 tumors from 25 midguts, skewness= 2.929, kurtosis= 12.39) or GFP and RasV12 (n=35 tumors from 26 midguts, Mann-Whitney: p=0.0004, skewness= 2.049, kurtosis= 4.029) with Myo1Ats for 7 days. In d, midguts pooled from 2 independent experiments; in e, from 3 independent experiments. DNA in a', b' and c' (blue). Scale bars in a-c' are 45 μm.
**Supplementary Figure 8** Integrin subunit expression and βPS1 basal localization in ECs. (a) Tumor-induced EC detachment induces graded JNK and Yki activity and upd3 expression. fa, fully attached; pd, partially detached; fd; fully detached. (b) Mean normalized expression (NE) values (RPKM) from two independent experiments of α and β integrin subunits determined by mRNA sequencing of sorted midgut ECs. (c) β-PS1 (c’; c” and c’’’, red) and Actin (phalloidin) (c’’’-c’’’’, white) in midguts expressing GFP (c; c’ and c’’’, green) with Myo1Ats. Scale bars in c-c’’’’, 15μm.
Supplementary Video Legends

**Supplementary Video 1. Induction of JNK activity in ECs surrounding ISC tumors (1-Channel).**
β-galactosidase (red) in midguts bearing puc-lacZ and expressing N0RNAi and GFP with esg23 for 3 days.

**Supplementary Video 2. Induction of JNK activity in ECs surrounding ISC tumors (2-Channel).**
β-galactosidase (red) in midguts bearing puc-lacZ and expressing N0RNAi and GFP (green) with esg23 for 3 days.

**Supplementary Video 3. Induction of JNK activity in ECs surrounding ISC tumors (3-Channel).**
β-galactosidase (red) in midguts bearing puc-lacZ and expressing N0RNAi and GFP (green) with esg23 for 3 days. DNA is in blue.