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Published in:
EMBO Journal

DOI:
10.15252/embj.201696247

Publication date:
2017

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Ret receptor tyrosine kinase sustains proliferation and tissue maturation in intestinal epithelia

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Abstract

Expression of the Ret receptor tyrosine kinase is a defining feature of enteric neurons. Its importance is underscored by the effects of its mutation in Hirschsprung disease, leading to absence of gut innervation and severe gastrointestinal symptoms. We report a new and physiologically significant site of Ret expression in the intestine: the intestinal epithelium. Experiments in Drosophila indicate that Ret is expressed both by enteric neurons and adult intestinal epithelial progenitors, which require Ret to sustain their proliferation. Mechanistically, Ret is engaged in a positive feedback loop with Wnt/Wingless signalling, modulated by Src and Fak kinases. We find that Ret is also expressed by the developing intestinal epithelium of mice, where its expression is maintained into the adult stage in a subset of endocrine/enterochromaffin cells. Mouse organoid experiments point to an intrinsic role for Ret in promoting epithelial maturation and regulating Wnt signalling. Our findings reveal evolutionary conservation of the positive Ret/Wnt signalling feedback in both developmental and homeostatic contexts. They also suggest an epithelial contribution to Ret loss-of-function disorders such as Hirschsprung disease.

Keywords Drosophila; enteroendocrine; intestine; Ret; stem cell

Subject Categories Development & Differentiation; Signal Transduction; Stem Cells

DOI 10.15252/embj.201696247 | Received 5 December 2016 | Revised 26 July 2017 | Accepted 28 July 2017 | Published online 12 September 2017
The EMBO Journal (2017) 36: 3029–3045

Introduction

The identification of the Ret receptor over 20 years ago, at the time enabled by its oncogenic potential when ectopically activated, spurred a flurry of research into this highly conserved transmembrane receptor with tyrosine kinase activity (Takahashi et al, 1985; Takahashi & Cooper, 1987; Ibanez, 2013). It is now well established that the endogenous activity of Ret is required for the formation and/or maintenance of a variety of cell types including neuronal, kidney, lymphoid, hematopoietic and testis germ cells (Schuchardt et al, 1994; Naughton et al, 2006; Veiga-Fernandes et al, 2007; Ibanez, 2013; Fonseca-Pereira et al, 2014). In these cells, Ret signalling is involved in a diverse range of developmental processes including proliferation, migration survival and/or differentiation (Sasselli et al, 2012; Ibanez, 2013). The importance of maintaining appropriate levels of Ret signalling—and doing so in the right place and at the right time—is highlighted by the severity of disorders resulting from its dysregulation. First found in a human lymphoma (Takahashi et al, 1985), ectopic activation of Ret signalling is now a recognised and significant feature of several other cancers (Ibanez, 2013; Santoro & Carlomagno, 2013; Romei et al, 2016). The digestive system is the site of perhaps one of the most striking clinical manifestations resulting from Ret dysregulation: Hirschprung disease (or HSCR). Frequently resulting from Ret loss-of-function mutation and affecting ca. 1 in 5,000 humans, HSCR leads to a variety of severe gastrointestinal symptoms such as abnormal peristalsis and bowel obstruction, which have been attributed to a striking absence of enteric innervation in the distal part of the gut (Martucciochi et al, 2000; Sasselli et al, 2012). Here, we report an unexpected and important role for Ret in a different gastrointestinal cell population: the intestinal epithelium.

Results

Ret is expressed by the adult somatic progenitors of the Drosophila intestine

Expression of Ret has been reported in developing neurons of many animals including Drosophila (Pachnis et al, 1993; Sugaya et al,
Having established that Ret is required to sustain stem cell proliferation, we sought to explore its sufficiency. pH3 quantifications indicated that misexpression of Ret, achieved by expression of a UAS-Ret transgene, either clonally or in all adult intestinal progenitors, resulted in increased mitoses (Fig 2E and H). Clone size quantifications revealed that the increased proliferation triggered by Ret misexpression gave rise to a larger number of progeny (Fig 2H).

Together, loss- and gain-of-function experiments indicate that adult intestinal stem cell proliferation is modulated by Ret; normal proliferation requires endogenous Ret levels, and can be further enhanced by increasing Ret expression cell-intrinsically above wild-type levels.

**A Ret/wingless positive feedback loop promotes stem cell proliferation in the adult Drosophila intestine**

Several mitogenic signals have been shown to promote stem cell proliferation when mis- or over-expressed from adult intestinal progenitors (Jiang & Edgar, 2012; Guo et al, 2016; Jiang et al, 2016). To begin to explore the signalling context in which Ret may function, we tested the ability of such mitogens to stimulate proliferation of adult intestinal progenitors with reduced Ret expression. To this end, we expressed ligands for the Jak-Stat, Egfr and Wingless (Wg)/Wnt signalling pathways [unpaired 1 (Upd1), secreted Spitz (sSpitz) and Wg, respectively] together with a Ret-RNAi transgene from adult intestinal progenitors. Ret downregulation did not render stem cells unable to respond to all mitogenic signals, but selectively reduced wg-induced proliferation (as revealed by pH3 quantifications, Fig 3A). Co-expression of Ret-RNAi and a dominant-negative form of Shaggy (Sgg), the Drosophila orthologue of glycosyn thase kinase 3β (Gsk3b), indicated that Ret is also required for the increased proliferation resulting from activating Wg signalling in adult intestinal progenitors downstream of the Wg receptor (Fig 3A).

We then explored the interaction between these two pathways in the converse scenario: Ret-induced proliferation. We found that Ret over-expression in adult intestinal progenitors leads to striking upregulation of Wg ligand in these cells (Fig 3B). Experiments in which we simultaneously over-expressed Ret and downregulated Wg signalling in adult intestinal progenitors [achieved by co-expression of RNAi transgenes the downstream Wg pathway component dishevelled (dsh), or by expression of a dominant-negative version of the transcription factor target pangolin/TCF (Pan)] indicated that Ret-driven proliferation requires active Wg signalling in adult intestinal progenitors (Fig 3C).

Although several studies have reported contributions of Wg signalling to intestinal stem cell homeostasis (Lin et al, 2008; Cordero et al, 2012; Tian et al, 2016), they have reached different conclusions regarding the source of Wg protein. To test whether the Ret-induced Wg upregulation results from cell-intrinsic Wg production by adult intestinal progenitors, we first quantified wg transcript by RT-qPCR in dissected midguts following over-expression of Ret in adult intestinal progenitors. In contrast to the wg transcript upregulation detected following wg expression in these cells (positive control), Ret over-expression did not result in detectable wg transcript induction (Fig 3D). This suggested that the increased Wg protein observed following Ret expression may result from a non-transcriptional mechanism. To confirm this, we analysed wg
transcription with cell specificity by making use of \( \text{wg}^{\text{KO}} \)-Gal4: a \( \text{wg} \) transcription reporter in which Gal4 is expressed under the control of the endogenous \( \text{wg} \) promoter (Alexandre et al., 2014). As described in a previous study (Tian et al., 2016), we found no expression of this reporter in adult intestinal stem cells (Fig 3E), but were able to detect it at intestinal compartment boundaries (data not shown). Consistently, Ret expression from this Gal4 driver failed to promote intestinal stem cell proliferation (Fig 3E).

Together, these results indicate that a positive, reciprocal interaction between Ret and Wg pathways promotes intestinal stem cell proliferation. Ret expression in adult intestinal progenitors promotes \( \text{wg} \) signalling in these cells via a non-transcriptional mechanism that increases Wg protein on their surface.

**Ret-dependent phosphorylation of Src42A and Fak kinases affects proliferation of adult intestinal progenitors**

What cellular events downstream of Ret promote Wg signalling and intestinal stem cell proliferation? Previous studies have revealed a complex and sometimes contradictory interplay between phosphorylation events resulting from Ret receptor activation involving binding to phosphorylation of Src and focal adhesion kinases (Melillo et al., 1999; Encinas et al., 2001, 2004; Sandilands et al., 2012). To explore the significance of these interactions in the context of intestinal homeostasis in vivo, we first tested whether phosphorylation of Ret receptor was required to promote intestinal proliferation. To this end, we generated transgenic flies expressing a
kinase-dead Ret protein harbouring a substitution of Lys805 into Met (RetK805M): a mutation previously shown to abrogate the high levels of Tyr autophosphorylation observed following expression of wild-type Drosophila Ret in COS cells (Abrescia et al., 2005; see Materials and Methods for details). In contrast to the mitogenic effects of wild-type Ret, RetK805M failed to promote proliferation of...
adult intestinal progenitors, despite efficient expression of this mutated Ret protein in these cells (Fig 4A). This indicates that phosphorylation of Ret is required for its mitogenic effects in the intestine.

Several ex vivo studies have shown that, following its phosphorylation and activation, Ret can bind the non-receptor tyrosine kinase Src (Melillo et al., 1999; Encinas et al., 2004). Consistent with this biochemical interaction, we observed that, in addition to upregulating Wg, Ret over-expression in adult intestinal progenitors also resulted in enhanced phospho-Src immunoreactivity (Fig 4B), suggestive of Src kinase activation. Activation of Src cytoplasmic tyrosine kinases is frequently implicated in signal transduction...
downstream of transmembrane receptors, and modulation of Src levels in intestinal progenitors results in phenotypes similar to those described above for Ret depletion/over-expression (Cordero et al., 2014; Kohlmaier et al., 2015), suggesting that Ret-induced hyperproliferation may require Src kinases. Experiments in which we simultaneously over-expressed Ret and downregulated Src42A or Src64B (the two Src kinases in Drosophila) from adult intestinal progenitors confirmed that Src42A, but not Src64B, is required for both Ret-driven hyperplasia and activation of Wg ligand in intestinal progenitors (Fig 4C). The existence of positive feedback between Ret and Wg pathways via Src42A was further suggested by the fact that activation of Src42A upregulated Wg ligand in intestinal progenitors (Fig 4D), and by our finding that the previously reported proliferation increase resulting from Src42A activation

Figure 4.
Is the epithelial expression of Ret confined to the fly intestine? RT–qPCR expression analysis of Ret transcript in mice revealed strong Ret expression in all three portions of the mouse small intestine (duodenum, jejunum and ileum, Fig 5A). To determine whether some of the Ret transcript detected in these whole-tissue samples originated from the intestinal epithelium and to further investigate its developmental expression, we derived epithelial cultures from both developing and mature small intestinal tissue. Epithelial cells from the adult small intestine of mice can be cultured ex vivo as budding organoids with distinct proliferative and differentiated domains (Sato et al, 2009). If epithelial cells are indeed derived from the foetal epithelium or the proximal region of the neonatal intestine, they grow like spheroids (foetal enterospheres—FEnS) when subject to the same treatment. These FEnS lack differentiated lineages and consist of a seemingly homogenous proliferative cell population, seen as precursors to the adult stem cell population (Fordham et al, 2013). RT–qPCR analysis of epithelial cultures derived from embryonic or mature small intestine indicated that Ret transcript is present in both kinds of epithelial cultures (FEnS and organoids, respectively, Fig 3B). This suggested that the source of Ret transcript in the intestinal tissue preparations is not only enteric neurons/mesenchymal cells, but also epithelial cells.

To characterise the epithelial cell types contributing to Ret expression, we conducted in situ hybridisation and immunohistochemical analyses of small intestinal tissue. Scattered Ret-positive epithelial cells were consistently detected using both methods, both in immature small intestine (postnatal day 1 (P1) intestine) by in situ hybridisation (Fig 5C), and in mature epithelium by immunofluorescence (co-staining with epithelial marker E-cadherin in Fig 5D, specificity of epithelial signal validated in Fig EV2A and B). We were not able to detect Ret protein in the epithelium of immature intestines (data not shown), possibly because of relatively lower expression as suggested by the ex vivo cultures (Fig 5B).

Scattered Ret-positive cells were detected in the jejunal but were most prominent in the ileum (Fig 5D and E and data not shown). Their position within the epithelium is variable; we observed them at positions ranging from the top of the villi to the upper portion of crypts, but were typically located in the lower half of the villi (Figs 5D and E, and EV2A, C and D). Immunohistochemical analyses with cell type-specific markers failed to reveal an overlap with Paneth or goblet cell markers (lysozyme and mucin-2, respectively, Fig EV2C and D), but consistently indicated that most,
if not all, the Ret-positive epithelial cells correspond to a subset of chromogranin-A (CgA)-positive cells (Fig 5E), suggesting that Ret-positive cells are enteroendocrine in nature (Rindi et al., 2004; Bellono et al., 2017). Similarly scattered, Ret-, E-cadherin- and CgA-positive cells were also observed at equivalent positions in organoid cultures (Fig 5F and G).
Together, these data indicate that Ret is expressed in the developing intestinal epithelium. Ret expression is maintained in the mature intestinal epithelium in a subset of secretory, chromogranin-A-positive cells.

Ret promotes maturation and Wnt signalling in the developing mouse epithelium

Having established that the mouse intestinal epithelium expresses Ret, we sought to assess the proliferation and behaviour of mouse epithelial cells with reduced Ret function. To this end, we used intestinal epithelial cultures derived from Ret51 mice (de Graaff et al., 2001). We chose this mouse model because it lacks Ret9: one of two isoforms of the Ret gene, and the most abundant isoform in both whole intestinal tissue and epithelial organoids (Fig EV3A and B). Importantly, unlike Ret−/− mice that die perinatally, Ret51 mice are viable for up to 7 days postnatally, allowing recovery of neonatal intestinal tissue. As previously reported, we observed that epithelial cultures derived from the proximal portion of neonatal (P1) small intestinal tissue of control mice comprise a mixed population of FEnS and organoids (Fordham et al., 2013; Fig 6B and C). We first confirmed that total Ret mRNA expression was reduced to 20% of its wild-type levels in the epithelium of FEnS/organoids derived from Ret51 mice (Fig 6A). We then quantified the proportion of Ret51 organoids relative to FEnS and found that it was reduced when compared to that of cultures derived from control littermates (Fig 6B and C). The increased relative abundance of primitive enterospheres in primary cell cultures derived from Ret51 mutant intestine was further reflected in their reduced branching relative to cultures derived from control tissue (Fig 6D).

Analyses of cells isolated from the primary cell cultures indicated that both the fraction of viable Ret51 cells and their cell cycle state were comparable to controls (Fig EV4A–C). This pointed to defective maturation, rather than proliferation or viability, as an underlying mechanism accounting for the Ret51 epithelial phenotype. Consistent with this idea, transcriptional analysis of FEnS/organoid cultures derived from control and Ret51 mice using a panel of epithelial differentiation markers revealed that, at a time point where most differentiation markers were only marginally reduced in cultures derived from Ret51 mice (Fig EV4D), there was a strong reduction in Axin2 transcript levels in these Ret51 cultures (Fig 6E). Axin2 is a commonly used readout of Wnt pathway activity: a signalling pathway that sustains intestinal stem cell proliferation and promotes both differentiation of Paneth cells in vivo and the maturation of FEnS to organoids ex vivo (Jho et al., 2002; van Es et al., 2005; Fordham et al., 2013; Clevers et al., 2014). To explore a possible positive feedback between Ret and Wnt signalling further, we treated immature cultures derived from wild-type E16.5 small intestines, consisting exclusively of FEnS, with GDNF: a Ret ligand. Exposure of FEnS to this Ret ligand robustly increased Axin2 levels and promoted branching: a hallmark of maturation (Fig 6F–H). We further established that the effects of Ret on epithelial maturation were not confined to the ex vivo FEnS/organoid system. Instead, immunohistochemical analysis of the neonatal small intestinal tissue of Ret51 mice revealed reduced expression of interstitial lysozyme: a Paneth cell marker and one of the first hallmarks of tissue maturation, first detected in interstitial regions within the first week postnatally (Fordham et al., 2013; Fig 6I).

Together, these findings indicate that Ret impacts the transition between the ever expanding foetal epithelium and adult tissue homeostasis. Like in flies, Ret signalling can promote Wnt signalling to sustain epithelial maturation.

Discussion

A new role for Ret in intestinal epithelia: positive feedback between Ret and Wg/Wnt signalling

Our findings in Drosophila indicate that Ret is expressed not only by enteric neurons, but also by the adult somatic stem cells of the intestinal epithelium. In contrast to known Ret functions in other progenitor cell types—for example, in spermatogonia or the hematopoietic system (Naughton et al., 2006; Hofmann, 2008; Fonseca-Pereira et al., 2014)—Ret is not required for the survival of adult somatic stem cells in the intestine, but sustains both their homeostatic and regenerative proliferative capacity. Our gain- and loss-of-function experiments point to the existence of positive feedback between Ret and Wg signalling. Despite abundant genetic evidence that Wg signalling promotes stem cell proliferation in flies (Lin et al., 2008; Cordero et al., 2012; Tian et al., 2016), the source of Wg has remained unclear. Using new, improved tools to visualise Wg expression (Alexandre et al., 2014), our findings lend further support to recent data (Tian et al., 2016) indicating that the source of Wg ligand is not the stem cells themselves, despite the striking Ret-driven upregulation of Wg on their surface. How might Ret signalling in adult intestinal progenitors lead to Wg protein upregulation in these cells without affecting its transcript? Two possible ways in which it might do so is by upregulating the expression of Wg receptor(s) on their surface, and/or by promoting signalling from stem cells to the Wg-producing cells at the intestinal boundaries and/or the visceral muscles (Buchon et al., 2013; Tian et al., 2016) to increase Wg release/trafficking.

Epithelial Ret is not a peculiarity of the fly intestine; we find that Ret is also expressed in the developing intestinal epithelium of mice, prior to the maturation of enteroendocrine or Lgr5-positive stem cells. Although our immunohistochemical analyses have not revealed a specific Ret-positive cell population at this stage, our ex vivo experiments using epithelial cultures devoid of enteric neuron or mesenchymal point to an intrinsic role for Ret at this stage in promoting epithelial maturation. The mechanism underlying the maturation-promoting effects of Ret may involve positive feedback between Ret and Wnt signalling similar to those we have found in flies. Indeed, the Wnt pathway target Axin2 is reduced in epithelial cultures derived from Ret51 mice and upregulated when wild-type FEnS are treated with the Ret ligand GDNF: a treatment that also promotes their branching. These data are consistent with the previous finding that elevated Wnt signalling promotes FEnS to organoid maturation (Fordham et al., 2013) and is reminiscent of the Wnt11/Ret autoregulatory loop promoting ureteric branching during kidney development (Majumdar et al., 2003). The relevant source of Wnt driving tissue maturation is currently unknown and is most likely not epithelial (Farin et al., 2012; Kabiri et al., 2014; San Roman et al., 2014; Aoki et al., 2016; Valenta et al., 2016). Our Drosophila finding that Wg ligand upregulation is not transcriptional underscores the importance of considering tissue crosstalk...
and non-autonomous signalling in any future studies addressing Wnt contributions to epithelial maturation in mice.

At first sight, the Ret effects on developmental maturation in mice appear to be different from its homeostatic role in flies. However, we find that Ret continues to be expressed in the adult small intestine, where Ret expression is prominent in a subset of enteroendocrine cells positive for the secretory marker chromogranin-A. Based on their position, these cells may correspond to enterochromaffin cells: intriguing cells that contribute 90% of the serotonin in circulation, control gastrointestinal motility and secretions and have recently been shown to be chemosensory (Roth & Gordon, 1990; Gershon, 2003; Rindi et al, 2004; Gershon & Tack, 2007; Bellono et al, 2017). A very recent study has blurred the distinction between enteroendocrine cells and their precursors by revealing expression...
overlaps between markers of enteroendocrine precursor identity and differentiated fate (including chromogranin-A), and by suggesting that differentiated enteroendocrine cells can have stem cell-like properties (Yan et al., 2017). This is exciting because it suggests that, whilst the cellular classification of Ret-positive cells based on known markers may differ between flies (ISCs) and mice (enteroendocrine), Ret-enabled stem cell functionality may contribute to regeneration in both epithelia. Intriguingly, endocrine tumours derived from the small intestine, ileal carcinoids, secrete serotonin and have been reported to express high levels of Ret (Cunningham & Janson, 2011). Conditional deletion of Ret in adult intestinal epithelium will, in future, determine its contribution to enteroendocrine fate and will help establish possible enteroendocrine contributions to intestinal homeostasis and tumour formation.

**Signalling events downstream of Ret**

Consistent with *ex vivo* transfection studies in mammalian cells, pointing to physical association between Ret and c-Src (Melillo et al., 1999; Encinas et al., 2004), we find that Src kinase Src42A is required downstream of Ret to activate Wg signalling. These findings therefore strengthen the link between two pathways previously known to control ISC proliferation in flies—Src and Wg (Lin et al., 2008; Cordero et al., 2012, 2014; Kohlmaier et al., 2015)—and may provide a physiological context for the previously reported, Src-dependent mitogenic effects of mutated, oncogenic Ret in the *Drosophila* developing retina (Read et al., 2005). The finding that, in the *Drosophila* intestine, Src kinases control expression of a mitogenic module consisting of String/Cdc25 and cyclin E (Kohlmaier et al., 2015) provides a simple link between Ret activation and its pro-proliferative effects. Overactive Src kinases do, however, lead tointestinal tumours (Kohlmaier et al., 2015) so mechanisms must be in place to limit the positive Ret/Wg feedback loop so that it sustains homeostatic proliferation, but does not result in tumour formation. Availability of Wg ligand may be an extrinsic mechanism, but the focal adhesion kinase Fak may provide a cell-intrinsic break downstream of Ret/Src activation. Consistent with this idea, Ret expression leads to both Src42A and Fak phosphorylation, but we find that the two kinases have opposing effects on proliferation: Src42A promotes proliferation downstream of Ret, whereas Fak blocks it. Hence, despite the fact that blocking Fak function may represent a therapeutic opportunity in some cancers (Ashton et al., 2010; Lee et al., 2015), our findings are more aligned with a previous study (Macagno et al., 2014) that suggested that, at least in the context of Ret-driven tumorigenesis, Fak can act as a tumour suppressor. In future, it will also be of interest to explore how the Ras/Raf/Erk pathway, activated by Ret in other contexts and previously shown to affect ISC proliferation in flies (Buchon et al., 2010; Biteau & Jasper, 2011; Jiang et al., 2011), intersects with Src/Fak/Wg signalling in response to Ret activation.

Both Wnt and Src pathways can have strong effects on proliferation, differentiation and/or tumorigenesis in the murine intestine (Reya & Clevers, 2005; Jiang & Edgar, 2012; Cordero et al., 2014). Src is required for mouse intestinal tumourigenesis following upregulation of Wnt signalling (Cordero et al., 2014). Based on our functional findings in *Drosophila* and our expression data in mice, a possible contribution of the Ret- and Cga-positive cells to this process deserves further investigation. It will also be of interest to investigate how Src/Fak kinase signalling contributes to the maturation of foetal intestinal epithelial cells and whether this is important in the development of intestinal disorders.

**Epithelial Ret signalling in intestinal homeostasis and pathology**

Ret expression is one of the defining features of enteric neurons (Sasselli et al., 2012). We have found another evolutionarily conserved and physiologically significant site of Ret expression: the intestinal epithelium. The presence of Ret in these two gastrointestinal cell types of very different developmental origin raises the possibility that the development and/or physiology of enteric neurons, intestinal epithelial progenitors and, in mammals, Ret-expressing intestinal lymphoid cells (Ibiza et al., 2016) is coordinated. Such coordination may, for example, help ensure a match between the size of the intestinal epithelium, the number of innervating neurons during development and the transition from an immature foetal epithelium into a functional epithelium involved in nutrient uptake and interorgan signalling. In mammals, Ret ligands of the glial cell line derived neurotrophic factor (Gdnf) family may orchestrate Ret signalling in these three tissues (Sasselli et al., 2012; Ibiza et al., 2016). In flies (which lack these Ret ligands), integrins have been shown to interact with Ret in sensory neurons (Soba et al., 2015). Interfering with integrin expression in the *Drosophila* intestine can have different effects on intestinal progenitor proliferation, survival and/or orientation depending on whether the integrins are removed from the progenitors or their niche—the visceral muscles (Goulas et al., 2012; Lin et al., 2013). Intriguingly, integrin downregulation in adult intestinal progenitors reduces their normal proliferation and can suppress their overproliferation in response to overactive Wingless signalling (Lin et al., 2013; and our own observations): phenotypes strikingly similar to those resulting from Ret downregulation. In the light of the known links between integrins and Fak/Src signalling in both normal and cancer cells (Mitra & Schlaepfer, 2006) and the effects that we have found for Src and Fak downstream of Ret activation, Ret could provide a new route for the integrin activation of the Src/Fak complex. Alternatively, the recent finding that GDF15, a divergent member of the TGF-β superfamily, signals through a GDNF family receptor z-like in a Ret-dependent way (Yang et al., 2017; Mullican et al., 2017; Emmerson et al., 2017) also raises the intriguing possibility that TGF-β-like ligands modulate Ret signalling in the intestinal epithelium, potentially linking intestinal regeneration with the known GDF15 roles in food intake/body weight.

The crucial requirement for Ret in enteric nervous system development is underscored by disorders such as HSCR, in which Ret loss of function leads to almost complete absence of enteric innervation in varying lengths of the distal gut (Schuchardt et al., 1994; Sasselli et al., 2012). Whilst the contribution of enteric aganglionosis to HSCR is unquestionable, our findings raise the possibility that, if the epithelial expression of Ret is conserved in humans, dysregulation of epithelial signalling may contribute to disorders that, like HSCR, result from Ret mutation. Epithelial Ret signalling might also contribute to other aspects of gastrointestinal physiology previously shown to be affected by reduced Ret function, such as intestinal motility, gut–microbiota interactions and the compensatory response to massive small bowel resection (Gianino et al., 2003; Hitch et al., 2012; Dey et al., 2015). Interestingly, HSCR is typically
diagnosed around birth due to defects in gastrointestinal functions. This coincides with the first demands on intestinal function, which could reflect not only neuronal defects related to peristalsis, but also defects associated with the transition from a foetal into a functional adult epithelium. Given that many of the pathways that drive tissue expansion and the maintenance of non-differentiated progenitor populations during foetal development are deregulated in cancer (Hu & Shivdasani, 2005), a possible contribution of Ret signalling to colorectal tumours also deserves further investigation.

**Materials and Methods**

**Fly husbandry**

Fly stocks were reared on a standard cornmeal/agar diet (6.65% cornmeal, 7.15% dextrose, 5% yeast, 0.66% agar supplemented with 2.2% nipagin and 3.4 ml/l propionic acid). All experimental flies were kept in incubators at 25°C, 65% humidity and on a 12-h light/dark cycle, except for those containing tub-Gal80TS transgenes, which were set up at 18°C (restrictive temperature) and transferred to 29°C (permissive temperature) at the time when Gal4 induction was required. This depended on the specific experimental requirements but typically, for loss-of-function (RNAi) or gain-of-function (UAS) experiments, flies were raised and aged as adults for 4 days at 18°C, were then shifted to 29°C to induce transgene expression, and adult midguts were dissected after 10–20 days (as indicated in each figure panel). Flies were transferred to fresh vials every 3 days, and fly density was kept to a maximum of 15 flies per vial. Only virgin females were used for all experiments.

For mutant ISC clonal analyses (MARCM clones), 4-day-old adults (raised and aged at 25°C) were heat-shocked for 1 h at 37°C to induce clones and were then kept at 29°C until dissection (8 days or 10 days thereafter as indicated in each figure panel). Flies were transferred to fresh vials every 3 days.

For damage-induced regeneration assays using RNAi transgenes, 4-day-old adult virgin flies were collected and raised at 18°C and were then shifted to 29°C for 10 days on standard media. Flies were transferred to fresh vials every 3 days. Flies were then transferred in an empty vial containing a piece of 3.75 cm × 2.5 cm paper. 500 ml of 5% sucrose solution (control) or 5% sucrose + 3 dextran sulphate sodium (DSS) solution was used to wet the paper, used as feeding substrate. Flies were transferred to fresh vial with fresh feeding paper every day for 3 days prior to dissection.

Damage-induced regeneration assays using mutant animals were conducted in an almost identical manner, but 4- to 10-day-old adult virgin flies reared at 25°C were used instead.

**Fly stocks**

**UAS transgenes**

UAS-Flybow 1.1 [Bloomington Drosophila Stock Centre (BDSC): 35537, (Hadjicekononou et al, 2011)], UAS-Stg::GFP (UAS-Stinger, (Barolo et al, 2000), gift from M. Landgraf), UAS-Dcr2 (UAS-Dicer2, (Dietzl et al, 2007), gift from I. Salecker), UAS-Ret [UAS-Ret-3xFLAG-6xHis (Kallijarvi et al, 2012)]; UAS-upd1 (Jiang et al, 2009; gift from J. Cordero); UAS-sspitz [Schweitzer et al, 1995; gift from J. Treisman]; UAS-wg (UAS-wg::HA, BDSC:5918); UAS-sggΔ (UAS-sgg-A81T, BDSC: 5560, (Bourouis, 2002)]; UAS-dshΔN (UAS-dshAB21-2, (Axelrod et al, 1998), gift from J. Axelrod); UAS-panΔN (van de Wetering et al, 1997, gift from F.J. Díaz-Benjumea); UAS-Src42CA1 (BDSC: 6410); UAS-Dcr2 (VDRC Stock Centre: 60007); UAS-Ret-RNAi (VDRC: GD 843); UAS-dsh-RNAi (VDRC: KK 101525); UAS-Src42A-RNAi (VDRC:KK 100708); UAS-Src64B-RNAi (VDRC: GD 35252), UAS-Fak (Palmer et al, 1999).

**Reporters and Gal4 drivers**


**MARCM stocks**

FRT40A: w, hs-flp, tub-Gal4, UAS-GFP; tub-Gal80, FRT40A (gift from J. de Navascués).

**Generation of a Ret knock-out allele and a kinase-dead Ret**

A targeted knock-out allele of Ret (RetKO) was generated using ends-out genomic engineering (Gong & Golic, 2003; Huang et al, 2009). Briefly, the Ret gene was replaced by a targeting cassette containing 5 kb and 3 kb of genomic sequences flanking the 5′ and 3′ region of Ret exons 3–8, respectively. RetKO candidate alleles were identified by correct chromosomal insertion of the targeting cassette containing a GMR-white marker cassette (pRK2; Huang et al, 2009). Candidate flies were analysed by PCR amplification and sequencing of genomic 5′ and 3′ regions flanking the targeting cassette to validate correct gene replacement. RetKO animals were viable and absence of Ret protein was further validated by immunohistochemistry of larval body wall preparations. A single fully validated RetKO allele was used for all subsequent experiments.

To generate flies expressing a kinase-dead version of Ret, we generated a Ret coding sequence harbouring a mutation resulting in a Lys to Met substitution at position 805 of the protein sequence. We fused this mutant Ret coding sequence with a sequence coding for a V5 tag and cloned the fusion into a pUAST vector.

Detailed description and analysis of both reagents will be provided elsewhere (N. Hoyer, P. Ziehlke, M. Petersen, K. Sauter, C. Hu, R. Scharrenberg, Y. Peng, C. Han, J.Z. Parrish, P. Soba, manuscript in preparation).

**Cell, clone and proliferation quantifications in adult Drosophila midguts**

For quantifications of the number of intestinal progenitors (e.g. Fig 2A), confocal images were obtained with a Leica TCS SP5 upright confocal microscope using a 40× oil immersion objective. The images (1,024 × 512) were loaded into ImageJ, and they were reconstructed using maximum projection from the first three Z stack planes. These maximum projections were cropped into a new file of defined dimensions (width: 600 pixels, height: 150 pixels) to control for size differences, and the number of GFP-positive cells was then analysed (ImageJ function: Plug-ins> Analyze>Cell counter).

The number of cells in MARCM clones was quantified using a nuclear reporter. Images were acquired using a 63× oil immersion
objective. Z-stacks of four adjacent, but non-overlapping segments of the midgut were imaged, starting at the midgut–hindgut boundary and moving anteriorly, and the number of cells per clone was counted in all clones found in these stacks (ImageJ function: Plugin=Analyze>Cell counter).

*Mitotic indices* were quantified by counting pH3-positive cells using a Nikon Eclipse 90i Fluorescence microscope through a 40× objective.

**Analysis of GFP and Wg levels**

Confocal images were obtained with a Leica SP5 upright confocal microscope using a 40× oil immersion objective. A single stack (1,024 × 512) immediately posterior to the midgut–hindgut boundary was acquired with a Z resolution of 0.8 μm. ImageJ was used to generate a maximum projection using half of the Z stack planes. For GFP quantifications, the threshold was adjusted (ImageJ function: Image>Adjust>Threshold) to subtract background for the GFP channel and selected areas were then analysed (ImageJ function: analyse particles) to quantify the % of area above the threshold. To quantify Wg levels, the same procedure was applied for the red channel.

**Mouse strains and husbandry**

Ret51 mice, their control littersmates or wild-type mice (C57BL/6j obtained from Charles River or C57BL/6Nrij obtained from Janvier) were used as indicated in each experiment. Both male and female mice were used. All animals were placed under a 12-h light–dark cycle in an SPF unit in individually ventilated cages. Food and water were provided *ad libitum*. All mouse experiments performed in Copenhagen were approved by “Dyreforsøgstilsynet” (the national board approving animal experiments in Denmark). The experiments performed in the UK were conducted following guidelines from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R) under project licenses granted by the UK Home Office Animals (Scientific Procedures) Act 1986.

**Immunohistochemistry**

**Drosophila**

Dissected *Drosophila* guts were fixed at room temperature for 20 min in PBS, 4% paraformaldehyde. All subsequent incubations were done in PBS, 4% horse serum, 0.2% Triton X-100 at 4°C following standard protocols.

The following primary antibodies were used at the following dilutions: guinea pig anti-Ret 1:1,000 (Soba *et al*., 2015), mouse anti-Pdm1 1/20 [kind gift from Steve Cohen, generated by Yeо *et al* (1995)], mouse anti-Wg 1/20 (4D4 Developmental Studies Hybriddoma Bank), mouse anti-Arm 1/100 (N2 7A1 Developmental Studies Hybriddoma Bank), mouse anti-ProS 1/50 (MR1A, Developmental Studies Hybriddoma Bank), chicken anti-beta galactosidase 1/200 (ab39631, Abcam), goat anti-HRP 1/600 (123-095-021 Jackson Immunoresearch), rabbit anti-p-Src 1/100 (44660C; Invitrogen), rabbit anti-p-hGF (Ser10) 1/500 (9701L, Cell Signalling Technology), mouse anti-GFP 1/1,000 (1181446001, Roche), rabbit anti-V5 1/200 (V9137, Sigma) and rabbit anti-phospho-FAK (Tyr397) 1/250 (3283, Cell Signalling Technology).

Fluorescent secondary antibodies (FITC-, Cy3- and Cy5-conjugated) were obtained from Jackson Immunoresearch. Vectashield with DAPI (Vector Labs) was used to stain DNA.

**Mouse**

P1 intestines were dissected from male and female *Ret51* mice, and littermate controls. Tissue was fixed overnight at 4°C in 4% paraformaldehyde and was subsequently embedded in paraffin. Fixed and embedded tissue was sectioned (5 μm) and was subsequently dewaxed. Then, the slides were processed for antigen retrieval by incubating them in Diva Decloaker (Biocare Medical) using 2100 Antigen Retriever. Following permeabilisation and blocking with 0.3% Triton X-100, 10% adult bovine serum and 5% skim milk powder, samples were incubated overnight at 4°C with the primary antibody. The next day, the slides were washed in PBS and incubated with secondary antibodies PBS 0.1% BSA for 1.5 h at room temperature. This was followed by washes with PBS and mounting with Vectashield-DAPI mounting medium.

Adult small intestines were dissected from male and female wild-type mice (C57BL/6j) obtained from Charles River. Small intestines were fixed overnight at 4°C in 4% paraformaldehyde. They were then incubated in 30% sucrose solution and embedded in a mix of 7.5% gelatin (Sigma, G2500) and 15% sucrose in PBS until set. They were then snap-frozen in isopentane (Sigma, 154911) cooled at −60°C. Frozen sections were sectioned (14 μm thick) transversally using a Microm HM 560 Cryostar cryostat (Thermo Fisher Scientific). Sections were mounted onto SuperFrost Plus (Thermo Fisher Scientific) glass microscope slides. The sections were air-dried for 1–2 h at room temperature and were subsequently processed for antigen retrieval treatment by incubating them in sodium citrate buffer (10 mM sodium citrate, pH 6, Sigma-W302600) at 80°C for 15 min. Organoid cultures were fixed for 15 min with 4% paraformaldehyde a room temperature and processed whole mount. Following permeabilisation with 0.3% PBT and blocking in 10% horse serum, samples were incubated overnight at 4°C with the primary antibodies. The next day, they were incubated after a few PBT washed with appropriate fluorophore-conjugated secondary antibodies for 2 h at room temperature. This was followed by washes with PBT and mounting with Vectashield-DAPI mounting medium.

Antibodies used were as follows: Ret 1/300 (Immune Systems GT-15002), chromogranin-A 1/1,000 (Immunostar 20085), lysozyme 1/500 (Thermo Fisher Scientific PA1-29680), mucin 2 1/500 (Santa Cruz sc-15334) and/or E-cadherin ECD2 1/300 (Thermo Fisher Scientific 13-1900 (Invitrogen)). Alexa-488 and Alexa-555 (Thermo Fisher Scientific) were used as secondary antibodies.

**Image acquisition**

Confocal images were obtained with a Leica TCS SP5 upright confocal microscope. Z-stacks planes were typically collected every 0.8 μm, and the images (1,024 × 1,024) were reconstructed using maximum projection. Confocal maximum projections were loaded into ImageJ, and level and channel adjustments were applied to the confocal images shown in the figures (the same correction was applied to all images belonging to the same experiment), but all quantitative analyses were carried out on unadjusted raw images or maximum projections.
Statistics and data presentation

Sample size was not limiting in Drosophila experiments. Typically, 10–20 midguts were used for quantifications of midgut-level features such as the number of intestinal progenitors, expression of markers (e.g. Fig 1) or mitotic indices. An average of 30–40 clones was counted per midgut in experiments involving MARCM clones. In mouse experiments, power calculations to determine sample size were performed using expected changes in mean and standard deviation, available from the literature or from preliminary experiments conducted in our laboratories. A power of 0.8 was used in all power calculations. All experiments were performed using at least three independent biological replicates to allow for post hoc statistical analyses, and all animals were included in the analyses. For experiments involving organoids, lines were derived from independent animals, and treatment responses were normalised to the same line. Samples were not randomised, and the analyses were not blinded.

All statistical analyses were carried out in the R environment (R Core Team, 2014). Comparisons between two genotypes/conditions were analysed with the Mann–Whitney–Wilcoxon rank sum test (R function wilcox.test). All graphs were generated using Adobe Illustrator using the scatter graph tool. All confocal and bright field images belonging to the same experiment and displayed together in our figure were acquired using the exact same settings. For visualisation purposes, level and channel adjustments were applied using ImageJ to the confocal images shown in the figure panels (the same correction was applied to all images belonging to the same experiment), but all quantitative analyses were carried out on unadjusted raw images or maximum projections. In all figures, values are presented as average ± standard error of the mean (SEM), P-values from Mann–Whitney–Wilcoxon test (non-significant (ns): \( P > 0.05 \); *: \( 0.05 > P > 0.01 \); **: \( 0.01 > P > 0.001 \); ***\( P < 0.001 \)).

In situ hybridisation of mouse intestinal tissue

P1 intestines were fixed overnight at 4°C in 4% paraformaldehyde and were subsequently embedded in paraffin. Intestines were sectioned at 16 μm thickness. Digoxigenin-labelled antisense probes were generated from pBS SK plasmid with 2.5 kb of cDNA representing the entire intracellular and part of the extracellular domain of mouse Ret mRNA.

Quantification of transcript composition and levels in mouse intestine/ex vivo cultures

Ret gene expression levels were analysed by reverse transcription–quantitative polymerase chain reaction (RT–qPCR). Total RNA was isolated from dissected intestines or intestinal cultures using Qiagen® MicroKit RNaseasy. cDNA was synthesised using Invitrogen SuperScript III Reverse Transcriptase kit using random primers. For Ret expression in Fig 5A and B, TaqMan probes against a region common to both Ret isoforms were purchased from Thermo Fisher Scientific (Ret probe Mm00436304_m1 and Actb control Mm02619580_g1 for normalisation) and used together with a TaqMan Gene Expression Mastermix (Thermo Fisher Scientific). For αg expression in Fig 3D, the same procedure was used with TaqMan probes against αg (Dm02361072_s1) for normalisation. For all other RT–qPCR experiments, specific gene expression levels were measured using Invitrogen EXPRESS SYBR® GreenER™ qPCR Supermix with premixed Rox with optimised primer pairs in a Thermo Fisher Scientific QuantiStudio™ 6 Flex Real-Time PCR System. Gene expression was normalised to Gapdh transcript levels. The primers used were as follows:

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-TGTTCTACATCCCCACGTG-3'</td>
<td>5'-TGAGGAGAATGCTGCTA-3'</td>
</tr>
<tr>
<td>Lgr5</td>
<td>5'-CAGGTCCTTCACCTTACC-3'</td>
<td>5'-AGTAGGGACAGCCTAGT-3'</td>
</tr>
<tr>
<td>Fabp1</td>
<td>5'-TGGAAAGAGGACCTTCTTGG-3'</td>
<td>5'-GCAGGAGTCTGCGTAC-3'</td>
</tr>
<tr>
<td>Muc2</td>
<td>5'-CACACCAGCCATGGTAG-3'</td>
<td>5'-AGACCTGGTTGATCC-3'</td>
</tr>
<tr>
<td>Chga</td>
<td>5'-GAACACGGCCATGACAGAAG-3'</td>
<td>5'-TCGGAGATACCTGAGAC-3'</td>
</tr>
<tr>
<td>Ngn3</td>
<td>5'-GTTTCAAATTTCCACCCACAC-3'</td>
<td>5'-GTTTGGCTGACGCAACATG-3'</td>
</tr>
<tr>
<td>Lyz1</td>
<td>5'-GCAAAGGCTCTGCAATGCTG-3'</td>
<td>5'-CAGTTGACGACTGCCTTGA-3'</td>
</tr>
<tr>
<td>Olfn4</td>
<td>5'-GCACGAGCTGAGCTG-3'</td>
<td>5'-GCAGCGAGAAGGAAAC-3'</td>
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<tr>
<td>Ascl2</td>
<td>5'-CTACTCTGTCGGAGAAG-3'</td>
<td>5'-ACTCAACAGATGCTGTAAC-3'</td>
</tr>
<tr>
<td>Axin2</td>
<td>5'-TGAAACTGAGCTCTGAAA-3'</td>
<td>5'-AGAGCTGGTGCTCGAAATG-3'</td>
</tr>
</tbody>
</table>

To determine the relative abundance of Ret isoforms, RNAseq reads from libraries originating from either adult small intestinal tissue (ileum of female mice) or adult organoids (derived from female whole small intestine) were aligned to mouse UCSC mm9 genome and gene models from Illumina’s IGenomes using Tophat version 2.12. Relative transcript abundance Of Ret isoforms was calculated using Cufflinks version 2.11. For visualisation of isoform abundance, reads were allocated to transcripts using the GenomicAlignments Bioconductor package following the Cufflinks models and the assigned isoform signal visualised with the Gviz Bioconductor package.

FEnS/organoid cultures

Proximal parts of small intestines (age as indicated for each experiment) of male and female Ret57 mice, their littermate controls or wild-type animals (C57BL/6JR) obtained from Janvier) were isolated and then rinsed in PBS. The tissue was cut into 2–4 mm sections, transferred to clean PBS with 2 mM ethylene-diaminetetraacetic acid (EDTA) and incubated by rocking it on ice for 45 min. The tissue was then re-suspended in clean PBS and dissociated by pipetting. Supernatant was collected and filtered through a 100- to 70-μm-pore-diameter nylon mesh. Crypts were seeded on 48-well plates in 25 μl of Corning® Matrigel® Growth Factor Reduced (GFR) basement membrane mix, phenol red-free and ENR-Advanced DMEM/F1 (Gibco) (hEGF 50 ng/ml; Noggin 100 ng/ml, R-spondin1 500 ng/ml). The ENR medium was supplemented with murine GDNF 100 ng/ml (Peprotech) in the GDNF treatment experiments.

FEnS/organoid branching quantifications

Branching coefficient was assessed from epithelial culture pictures using this formula in FIJI software:

\[
\text{Branching coefficient} = 1 - \frac{\text{perimeter}^2}{\text{area}}.
\]
Cell cycle and cell death quantifications using dissociated cells

Single-cell suspensions from FEnS/organoids were obtained by incubation for 5–15 min at 37°C in 5 ml of Gibco® TrypLE®. For cell cycle analyses, cells were fixed in cold ethanol, washed in PBS and subsequently stained with DAPI. To detect apoptosis, single cell suspensions were stained using Annexin V Apoptosis detection Kit from BD Bioscience. Flow cytometry was performed using a BD® LSR2, and data were analysed using FlowJo® software.

Expanded View for this article is available online.

Acknowledgements
We thank Yasuko Antoku (BRIC core Facility) and the Center for Advanced Bioimaging (CAB) Denmark, University of Copenhagen for technical assistance, and Julia Cordero and Vassiliis Pachnis for helpful discussions. Susumu Hirabayashi provided comments on the manuscript. We are grateful to Jeff Axelrod, Luis Alberto Baena-Lopez, Sarah Bray, Steve Cohen, Joaquín de Navascués, Fernando Díaz-Benjumea, Matthias Landgraf, Bruno Lemaire, Ruth Palmer, Iris Salecker and Jessica Treisman for providing reagents. This work was funded by an ERC Starting Grant and a Wellcome Trust Research Career Development fellowship to I.M.-A. (ERCStG 310411 and WT083559, respectively), a Lundbeck foundation fellowship to K.B.J. (R105-A9755), a Spanish Ministry of Education Fellowship to D.P., a Marie Curie Fellowship to J.C., and an Academy of Finland Postdoctoral Fellowship to J.K. and MRC intramural funding. B.H. holds an EMBO advanced fellowship, and both K.B.J. and I.M.-A. were supported by the EMBO Young Investigator Programme.

Author contributions
DP and IM-A designed research. DP, JG, BH, CK and AM performed the experiments, with contributions from DH and DN. TC analysed the RNAseq isoform data. NH, JK, JAW and PS generated fly reagents (transgenics/mutants). NT and AJB provided the RetS2 mice. DP, BH, KBJ and IMA analysed and interpreted the data, with contributions from JG, CK and AM. IM-A wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References

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Retin in intestinal epithelia  

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