Accumulation of differentiating intestinal stem cell progenies drives tumorigenesis

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Stem cell self-renewal and differentiation are coordinated to maintain tissue homeostasis and prevent cancer. Mutations causing stem cell proliferation are traditionally the focus of cancer studies. However, the contribution of the differentiating stem cell progenies in tumorigenesis is poorly characterized. Here we report that loss of the SOX transcription factor, Sox21a, blocks the differentiation programme of enteroblast (EB), the intestinal stem cell progeny in the adult Drosophila midgut. This results in EB accumulation and formation of tumours. Sox21a tumour initiation and growth involve stem cell proliferation induced by the unpaired 2 mitogen released from accumulating EBs generating a feed-forward loop. EBs found in the tumours are heterogeneous and grow towards the intestinal lumen. Sox21a tumours modulate their environment by secreting matrix metalloproteinase and reactive oxygen species. Enterocytes surrounding the tumours are eliminated through delamination allowing tumour progression, a process requiring JNK activation. Our data highlight the tumorigenic properties of transit differentiating cells.

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Maintenance of tissue homeostasis in the adulthood requires precise coordination of stem cell renewal and differentiation. Deregelation of these processes can lead to cancer. Stem cells live in a microenvironment and continuously receive signals from neighbouring heterologous cells composing the niche. Stem cell niches are complex, heterotypic and dynamic structures. Over the past few years, considerable progress has been made in elucidating how different niche factors promote stem cell maintenance during homeostasis and contributes to tissue regeneration upon damage. Stem cells usually divide asymmetrically to generate a self-renewing stem cell and a differentiating progenitor (or transit amplifying cell), which will eventually generate differentiated cells. Recent studies in flies and mammals have begun to establish that these differentiating progenitors are not simply a passive intermediate between stem cell and differentiated cells, but play active roles in regulating stem cell activity and regeneration.

Loss of proper differentiation is an important feature and likely a driver of cancer development. Historically, mechanistic studies of human cancers and regenerative medicine have focused almost exclusively on stem cells. The roles of the differentiating stem cell progeny in tumorigenesis remain largely unexplored. In this study, we analyse how a defect in the differentiating stem cell progeny in tumorigenesis remain largely unexplored. In this study, we show that the differentiating stem cell progeny in tumorigenesis remain largely unexplored.

Results

Sox21a is necessary for EB differentiation. In an RNA interference (RNAi) screen for factors regulating stem cell differentiation, we identified Sox21a, a gene encoding a transcription factor of the SOX family with homologues implicated in stem cell regulation in mammals. Silencing Sox21a with two independent RNAi constructs specifically in EBs using the conditional, temperature-sensitive Su(H)GAL4 UAS-GFP tub-Gal80TS system (hereafter referred to as GBE17) led to the accumulation of EBs in the adult (Fig. 1a–c). Consistent with this, Sox21a is specifically enriched in the midgut of adult Drosophila (Supplementary Fig. 1a–c). Moreover, examination of the expression pattern driven by this enhancer is homogenous from the anterior to the posterior midgut, and is identical to the expression of Escargot (Esg), a transcription factor with well-defined expression in progenitors.

To further study the function of Sox21a, we have generated two Sox21a mutations using CRISPR/Cas9-mediated genome editing. Both mutants carry a small deletion in the DNA-binding domain of Sox21a, the HMG domain, resulting in reading frameshift and premature stop (Fig. 1d). Thus, these alleles should be considered as null alleles. Strikingly, Sox21a mutant flies are viable and fertile with no apparent defects. To confirm the role of Sox21a in EB differentiation, we performed lineage tracing using mosaic analysis with a repressible cell marker technique (MARCM)22. While the wild-type clones (positively marked by green fluorescent protein (GFP)) contain both enterocytes and enteroendocrine cells, cells in Sox21a mutant clones along the whole midgut remained undifferentiated, as revealed by the absence of GFP-positive cells expressing the entocyte marker Pdm1 or the enteroendocrine cell marker Prospero (Fig. 1e,f). This differentiation defect is rescued by overexpressing Sox21a in the mutant clones (Fig. 1g). Quantification of clone size indicated that the Sox21a mutation reduces ISC division with a stronger effect in the posterior compared with the anterior midgut (Fig. 1h). Sox21a mutant clones generated in the posterior midgut barely grew, indicating a mandatory function of Sox21a for ISC division in the posterior midgut. In contrast, the existence of large Sox21a mutant clones in the anterior midgut indicates that Sox21a promotes to some extent stem cell division in this region but is less essential. The presence of a wild-type copy of Sox21a in the mutant clones also restored normal ISC division (Fig. 1h).

To further confirm the differential impact of Sox21a on ISC division in the anterior and posterior regions, we compared ISC proliferation rate in wild-type and Sox21a mutant flies overexpressing the JAK/STAT ligand unpaired 2 (Upd2) in the enterocytes with Myo1A-Gal4 UAS-GFP tub-Gal80TS system (hereafter referred to as Myo1A15). Unpaired are secreted proteins that have been shown to be potent inducers of ISC proliferation by activating JAK/STAT signalling in ISC23,24. Overexpression Upd2 strongly increased the number of mitotic ISCs in both the anterior and the posterior midgut in wild-type flies as revealed by the phospho-Histone H3 (PH3, a mitotic marker) count. In contrast, overexpressing Upd2 only increased the mitotic index in the anterior midgut of Sox21a mutant (Supplementary Fig. 4a–d). Collectively, our data show that Sox21a is essential for the differentiation of EBs into mature intestinal cells along the entire midgut. Its effect on ISC proliferation is more pronounced in the posterior midgut compared with the anterior midgut.

Overexpression of Sox21a drives EB differentiation. Since Sox21a is required to generate differentiated cells, we hypothesized that overexpressing this factor might force the progenitor cells to differentiate into mature intestinal cells. To test this, we created transgenic lines that enable its overexpression via the GAL4/UAS system (Supplementary Fig. 1g). Strikingly, overexpressing Sox21a in the progenitor cells with esg-Gal4 UAS-GFP tub-Gal80TS system (hereafter referred to as esg15) was sufficient to induce their differentiation into enterocytes and cause the loss of progenitors (Fig. 2a–c). Although Sox21a is required for the differentiation of both enterocytes and enteroendocrine cells, Sox21a overexpression induced progenitors to differentiate into enterocytes (Pdm1 positive and polyploid), but not enteroendocrine cells (Pros positive; Supplementary Fig. 2a,b). To further delineate the role of Sox21a in progenitor differentiation, we overexpressed Sox21a either specifically in EBs using GBE17 or in ISCs using Dl-Gal4 UAS-GFP tub-Gal80TS (Dl15). EBs were normally detected as small-nucleated cells with a polarized cell shape (Fig. 2d).
Overexpressing Sox21a in EBs for 4 days transformed most of them into large polyploid and round-shaped cells, indicative of a transformation into enterocyte (Fig. 2e,f). In contrast, overexpressing Sox21a with DI^TS in ISCs for 6 days did not induce ISC differentiation (Supplementary Fig. 2c,d). Using other insertions of the UAS-Sox21a transgene, we sometimes observed clusters of two to four ISCs (positive for DI-lacZ) when overexpressing Sox21a using esg^TS (Supplementary Fig. 2e,f). Thus, besides inducing progenitor cells to differentiate, Sox21a may also have a role in stem cell division. We conclude that Sox21a is a critical factor required for the transition from EBs to mature intestinal cells in the adult midgut.

Sox21a functions downstream of the JAK/STAT pathway. The JAK/STAT pathway plays a major role in ISC proliferation and differentiation in Drosophila. We therefore explored the relationship between Sox21a and JAK/STAT in stem cell differentiation. Quantitative reverse transcription–PCR (qRT–PCR) experiments showed that Sox21a expression is regulated by the JAK/STAT signalling pathway. Sox21a expression in the midgut was lower when Stat92E was silenced by RNAi using the esg^TS driver and was higher when expressing a gain-of-function allele of JAK (hop^trans); Supplementary Fig. 2g). Previous study has shown that MARCM clone cells mutant for Stat92E were also negative for enterocyte marker Pdm1 (Supplementary Fig. 2h), consistent with a mandatory role of JAK/STAT in progenitor differentiation. Moreover, overexpression of Sox21a restored the expression of the enterocyte marker Pdm1 in Stat92E null mutant clones, confirming the role of Sox21a as a major downstream effector of this pathway in mediating differentiation (Supplementary Fig. 2i). The position of Sox21a downstream of the JAK/STAT pathway and its role in EB differentiation were reinforced by two other observations. First, the expression of esg and its regulator mir-8, two genes encoding factors regulating the progenitor identity, was not affected in Sox21a flies (Fig. 3a,b; Supplementary Fig. 3a,b). Second, the expression of Pdm1, a transcription factor specifically expressed in enterocyte, was downregulated in Sox21a mutant EBs (see RNA sequencing (RNA-seq) experiment, described below).

Accumulation of EBs and formation of tumour in Sox21a flies. A striking feature of Sox21a flies is the presence of large clusters of progenitors in the anterior but not the posterior midgut (Supplementary Fig. 4a–n). This regional difference is not surprising, since the Sox21a mutation has a differential effect on ISC division rate in the anterior and posterior midgut. We have focused our subsequent studies on the anterior midgut to analyse the formation of these clusters. They contain both ISCs and EBs, but progressively become dominated by EBs, consistent with the function of Sox21a in EB differentiation (Fig. 3a–d; Supplementary Fig. 2h,i).
Figure 2 | Sox21a is sufficient to drive differentiation. (a,b) AMG of flies overexpressing GFP (control, a) or Sox21a (b) in progenitor cells using esg\textsuperscript{T5} driver for 2 days at 29°C. esg > GFP channel is also shown. Insets show a high-magnification view. Pdm1 staining is shown in red. (c) Quantification of the number of progenitor cells in a 10,000-\textmu m\textsuperscript{2} area in AMG of control and overexpressing Sox21a for 3 and 6 days (n = 12, 10 and 10, respectively). (d,e) AMG of flies overexpressing GFP (control, d) or Sox21a (e) in EBs using GBE\textsuperscript{T5} driver for 4 days at 29°C. GBE > GFP channel is also shown. (f) Quantification of the ratio of newly formed EC-like cells to all the GFP-positive cells in AMG of control and overexpressing Sox21a for 4 days (n = 5). Error bars indicate s.e.m. (c,f). P values from Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001) are shown in c and f, and the results represent three independent experiments. One representative image from 12 midguts tested in one experiment, which was repeated three times, is shown in a,b,d and e.

Sox21a tumour growth relies on ISC division. We next explored the mechanisms by which a simple defect in the differentiation program of EBs leads to tumour formation. ISCs are the only proliferating cells in the midgut in normal conditions\textsuperscript{17}. Although a study has reported that a small portion of EBs (< 5%) displays mitotic activity upon enteric \textit{Pseudomonas entomophila} infection, EBs do not divide under basal conditions\textsuperscript{17}. Using PH3 staining, we analysed the identity of mitotic cells in Sox21a mutant expressing a GBE > GFP to mark EBs. While we detected many ISCs undergoing mitosis, no mitotic EB was observed (n > 100; Supplementary Fig. 6a). We next explored whether tumour growth in Sox21a flies is sustained by stem cell division. \textit{Drosophila} ISC division relies on the epidermal growth factor receptor (EGFR) signalling\textsuperscript{28}. Blocking this pathway by expressing a dominant-negative form of EGFR in progenitors of Sox21a flies suppressed the formation of tumour (Supplementary Fig. 6b–d). The ingestion of enteric bacteria was previously shown to stimulate ISC proliferation in \textit{Drosophila} and promotes tumorigenesis in other models\textsuperscript{29}. Similarly, stimulating ISC proliferation by infecting Sox21a flies with bacteria increased the size and the numbers of tumours (Supplementary Fig. 6e–g). These observations indicate that ISC proliferation is essential for Sox21a tumour formation. This dependence on stem cell division again explains why tumours are only found in the anterior midgut but not the posterior midgut where Sox21a is required for both EB differentiation and ISC division.

Interestingly, ISC proliferation was markedly increased in the neighbourhood of Sox21a tumours (Supplementary Fig. 7a). Consistent with this, use of 10xStat-GFP\textsuperscript{D} reporter gene reveals higher JAK/STAT activity in the tumour (Fig. 4a). In addition, Sox21a mutant cells generated via MARCM triggered Ras/MAPK signalling in neighbouring wild-type...
cells, as revealed by a staining of phosphorylated ERK (dpERK) (Fig. 4b). Thus, Sox21a mutant cells induce a local hyperplasia by stimulating division in adjacent ISCs (Supplementary Fig. 7b). This suggests that EBs composing Sox21a tumours send a signal to neighbouring ISCs to drive their proliferation.

**EB-derived Upd2 is essential for Sox21a tumour growth.** ISC proliferation can be induced upon expression of secreted ligands of the EGFR pathway (Spitz, Vein and Keren), the JAK-STAT pathway (unpaired 1, 2 and 3) and the Wingless pathway (Wg)6,23,24,28,30,31. To identify the factor stimulating stem cell division in Sox21a tumour, we applied a candidate gene approach by knocking down genes encoding these ligands in either EBs with GBETS or in enterocytes with Myo1ATS. Depletion of the JAK/STAT ligand upd2 by RNAi in EBs but not in enterocytes strongly reduced tumour formation in Sox21a flies (Fig. 4c–e).

Similarly, upd2; Sox21a double mutant flies displayed a reduction in tumour burden (Fig. 4c). In contrast, inhibiting the other factors in EBs or enterocytes (Upd1, Keren and Wg) did not...
impair Sox21a tumour formation (Supplementary Fig. 8a,b). It should be noted that upd3 mutation and to a lesser extent depletion of Spitz in EBs had a modest effect on Sox21a tumours (Fig. 4c; Supplementary Fig. 8a). This indicates that ISC proliferation is mostly induced by Upd2 released from Sox21a EBs composing the tumour. The stimulation of ISC proliferation by EB-derived Upd2 produces more differentiation-defective EBs, providing a feed-forward loop underlying the expansion of Sox21a tumours. We hypothesize that tumours are initiated in Sox21a flies upon stochastic clustering of EBs, leading to local increase of the mitogen Upd2. This mechanism would explain the random localization of tumours observed in the anterior midgut of Sox21a flies.

In addition to Sox21a mutation, loss of Notch signalling in progenitors has been shown to induce tumours in the Drosophila midgut14,15,32. In contrast to the Sox21a EB tumours, Notch tumours are composed of ISCs that fail to differentiate into EBs. We have investigated the role of Upd2 in Notch tumour formation. Simultaneous depletion of upd2 and Notch by RNAi in the progenitors also suppressed Notch tumours (Fig. 4f,g), further emphasizing the role of Upd2 in tumour growth. Importantly, silencing upd2 in progenitor cells in an otherwise wild-type background led to a decrease in ISC numbers, pointing to a role of Upd2 in basal level stem cell maintenance (Fig. 4h–i).

Mmp2 is required for Sox21a tumour progression. We then investigated how a defect in the differentiation program transforms EBs into an aggressive tumour. For this, we compared gene expression of fluorescence-activated cell sorting (FACS)-sorted EBs of wild-type and Sox21a flies by RNA-seq33 (Fig. 5a). Of 1,080 differentially expressed genes (P<0.05, Robinson and Smyth Exact Test), 668 genes were reproducibly upregulated and 412 genes downregulated in Sox21a EBs (Fig. 5c). Many genes that were previously shown to be associated with tumorigenesis in other models were also identified in Sox21a tumour. For instance, the genes ImpL2, an insulin/insulin-like growth factor antagonist recently reported to mediate tumour-induced organ wasting34,35, and p53, which reprograms tumour metabolism36, were both upregulated in Sox21a EBs (Fig. 5d). Increased expression of breathless/fibroblast growth factor receptor (FGFR; btl) in Sox21a EBs was confirmed using btl-Gal4 UAS-actGFP (referred to as btl>actGFP; Supplementary Fig. 9a–f). While btl>actGFP never labels intestinal cells in wild-type midgut (Supplementary Fig. 9a), expression of the btl>actGFP reporter was observed in the anterior but not posterior midgut of Sox21a flies, in regions where tumours form (Supplementary Fig. 9b–f). The observation that some but not all the Sox21a EBs express btl>actGFP

Figure 4 | EB-derived upd2 is essential for tumour growth and ISC maintenance. (a) AMG of 10-day-old Sox21a flies stained for 10xSTAT-GFP (green) shows increased JAK/STAT activity in the tumour (indicated by a star). (b) AMG containing GFP-labelled Sox21a mutant clone stained for dPERK (red) at 16 days ACl. dPERK staining is observed around the Sox21a clone. (c) Tumour burden of flies with the indicated genotypes kept at 25 °C (mutant analysis) or 29 °C (overexpression analysis) for 21 days. Enh:Sox21a is a rescue construct with Sox21a under the control of its own enhancer sequence. ‘EB>’ refers to the EB driver GBE17, and ‘EC>’ refers to the enterocyte driver Myo1ATS15. Numbers of flies scored for each genotype are indicated. (d,e) Expressing GFP (d, control) or upd2-RNAi (e) in EBs of Sox21a flies placed for 21 days at 29 °C. Gut was stained with Armadillo (Arm; red, plasma membrane for progenitors) and Prospero (Pros, red, nuclear, for enteroendocrine cells). EBs are marked by GBE>GFP (green). (f–i) AMG of flies depleted for Notch (N) alone (f) or in combination with upd2 (g) for 4 days at 29 °C. The expression of upd2-IR in progenitors reduced tumour formation. (h,i) AMG of flies expressing GFP (control, h) or upd2-RNAi (i) in the progenitor cells using the esg-driver for 14 days at 29 °C shows that upd2 is required for basal stem cell maintenance. Progenitors are shown by esg>GFP in green (f–i) and by Arm immunostaining (h,i). P values in c (repeated three times) from χ²-test (*P<0.05; **P<0.01; ***P<0.001). Each other individual image shown in a, b and d–i represents 20 flies tested in one experiment repeated three times.
highlights the cellular heterogeneity of Sox21a tumours (Supplementary Fig. 9c).

Interestingly, genes encoding two matrix metalloproteinases, matrix metalloproteinase 2 (Mmp2) and to a lesser extent matrix metalloproteinase 1 (Mmp1) were upregulated in Sox21a EBs (Fig. 5d). Use of an endogenous Mmp2-GFP fusion confirmed an increased expression of Mmp2 specifically at the tumour site (Fig. 6a,b). Mmp1 and Mmp2 are downstream effectors of the JNK pathway that mediate tumour invasiveness in an imaginal disc tumour model38–40. Inactivating the JNK pathway by expressing a dominant-negative form of JNK (basket, bsk), depleting Mmp2 (but not Mmp1) or expressing the tissue inhibitor of metalloprotease (timp) in EBs of Sox21a flies reduced tumour burden and growth towards the lumen (Fig. 6c–f). Of note, Mmp2 but not Mmp1 was previously shown to be required for the invasive growth of larva air sac/trachea into tissues41.

**Tumour progression requires JNK activation in enterocytes.** Tumour progression in Sox21a flies was associated with the elimination of neighbouring enterocytes, as shown by the progressive disappearance of 4,6-diamidino-2-phenylindole (DAPI)-stained polyploid cells (Fig. 3g–j; Supplementary Fig. 5a–d). Tumour-induced elimination of normal cells has

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**Figure 5 | EB-specific transcriptomics.** (a), Transcriptional profiling of Sox21a/+/− (control) and Sox21a EBs by RNA-seq was performed with messenger RNA isolated from FACS-sorted EBs based on the presence of GBE-GFP fluorescent signal (see Method part for details). (b) Clustering of 1,080 differentially expressed genes (P<0.05, Robinson and Smyth exact test) between Sox21a/+/− and Sox21a EBs revealed that biological repeats (triplicates) cluster together. Columns correspond to replicate and rows to different genes. Relative expression level is indicated by the colour key shown at the top. (c) The 1,080 genes with differential expression were classified based on their gene ontology (GO) terms for biological process (BP). Enrichment of each GO term is shown with the P value. Red terms are the most significantly enriched ones. Redox and epithelial tube morphogenesis, described afterwards in the paper, are in red and blue, respectively. (d) A selection of genes upregulated in Sox21a EBs. Fold change (FC), the range of P values (*<0.05; **<0.01; ***<0.001) and the gene function groups are shown.
been observed in other cases and probably reflects a common feature of aberrantly proliferating cells\textsuperscript{52,43}. It is reminiscent of cell competition in Drosophila, a type of short-range cell–cell interaction, where the fitter cells eliminate the unfit cells by activating JNK signalling\textsuperscript{44}. Using a \textit{puffered} enhancer trap (\textit{puc-lacZ} and \textit{puc-Gal4}) as readout for JNK activity, we found that JNK signalling was induced in enterocytes surrounding Sox21a tumours from Sox21a flies or flies with EB-specific depletion of Sox21a by RNAi (Fig. 7a,b). Interestingly, JNK activation in enterocytes was coupled with the loss of the cell-polarity marker Discs large (Dlg) (Fig. 7a). In Notch ISC tumour\textsuperscript{43}, flanking enterocytes are eliminated by delamination into the lumen. Similarly, confocal microscopy revealed the presence of delaminating enterocytes in the lumen of Sox21a flies at the vicinity of tumour (Fig. 7c). However, in Sox21a tumour, EBs were also found intercalated with enterocytes (Fig. 7d). Several observations indicate that JNK activation in enterocytes flanking Sox21a mutant tumours is essential for tumour progression. First, Sox21a flies lacking one copy of \textit{hemipterous} (\textit{Hep}), a gene encoding the JNK kinase, have decreased tumour burden. In contrast, \textit{puc}\textsuperscript{6/+} heterozygote flies with enhanced JNK activity display an increase of tumour burden of Sox21a flies (Fig. 7e). Second, inactivation of JNK signalling specifically in enterocytes by expressing a dominant-negative form of JNK greatly suppressed tumour formation and the presence of delaminating enterocytes (Fig. 7e). Moreover, the elimination of enterocytes nearby the tumours does not involve caspase-dependent apoptosis, as tumour progression was not affected by expressing the caspase inhibitor P35 (Fig. 7e). Collectively, our data show that Sox21a tumour progression involves the elimination of enterocytes by JNK activation independent of caspase activation.

\textbf{Increase of ROS activity at the vicinity of Sox21a tumour.}

Reactive oxygen species (ROS) have been implicated in JNK activation and induction of cell death in Drosophila. For example, feeding flies with the ROS-generating compound Paraquat causes cellular damage and JNK activation in the midgut\textsuperscript{45,46}. A recent study reported that ROS from apoptotic cells propagate to and activate JNK in the nearby cells\textsuperscript{47}. This raises the possibility that tumour-derived ROS contribute to JNK activation in surrounding enterocytes, facilitating their elimination. We therefore investigated the role of ROS in tumorigenesis in Sox21a flies. Gene ontology analysis of the RNA-seq data set revealed enrichment in genes involved in redox homeostasis in Sox21a EBs (Fig. 5c). For instance, Sox21a EBs display increased expression of several \textit{Cytochrome P450} genes, the \textit{NADPH oxidase Dual oxidase (Duox)} and its regulator, the MAPK \textit{p38c} (refs 48, 49), as well as several genes encoding mitochondria and peroxisome components (Fig. 5d). Mitochondria and peroxisomes are two main sources of intracellular ROS\textsuperscript{50}. Using reporter constructs (\textit{mitoGFP} and \textit{peroxisome-GFP}), we observed an increase in mitochondrial and peroxisome signals at the tumour site, evocating a shift of metabolism (Fig. 8a–e; Supplementary Fig. 10a). Increase of peroxisomes in Sox21a EBs was further supported by the expression of Catalase (Cat), a protein localized to peroxisome (Fig. 8f,h). Consistent with these observations, \textit{in vivo} ROS detection using dihydroethidium (DHE) revealed a gradient of ROS peaking at the periphery of Sox21a tumours (Fig. 8i). Surprisingly, the level of ROS at the tumour site was lower, suggesting that EB tumour cells were less exposed to ROS compared with neighbouring enterocytes. Reporter genes and immunostaining analysis showed that many enzymes involved in ROS detoxification, including Catalase, Glutathione S-transferase D1 (GstD) and Superoxide dismutase 2 (SOD2) are enriched in progenitors of Sox21a flies (Fig. 8g,h);
We then investigated the role of ROS in tumour formation by feeding Sox21a flies with an antioxidant, N-acetylcysteine amide (AD4). We observed that N-acetylcysteine amide-fed Sox21a flies have reduced tumour burden, although the difference with untreated control did not reach statistical significance (Supplementary Fig. 8c). Interestingly, overactivation of the ROS-producing enzyme Duox specifically in EBs of wild-type flies led to increased JNK activity in the flanking cells, and often resulted in local hyperplasia (Fig. 8j–l). In these experiments, foci of JNK activation were not observed around individual EBs but only around clustered EBs (Fig. 8k). Clustering of EBs expressing Duox might thus lead to a local increase of ROS above a threshold sufficient to trigger JNK activity. While the relevance of ROS in Sox21a tumour progression requires further investigation, our data raise the possibility that tumour-derived ROS non-cell autonomously contribute to JNK activation and elimination of flanking enterocytes.

Discussion

In this work, we report the functional characterization of a new regulator of ISC differentiation, introduce a novel tumour model and provide mechanistic insights on how tumour may arise from a simple defect in the differentiation program of stem cell progeny (Fig. 9a,b).

Our data show that Sox21a, a previously uncharacterized transcription factor of the SOX family, plays a major role in the terminal differentiation of ISC progeny. Although the Drosophila genome encodes eight SOX genes, Sox21a is the only one enriched in the midgut. Sox21a is specifically expressed in progenitor cells along the entire midgut, and acts downstream of the JAK/STAT signalling to permit the transformation of EBs into enteroendocrine cells. Although Sox21a is required for both differentiated cell types, overexpression of Sox21a drives differentiation into enterocytes. It cannot be excluded that high level of Sox21a due to overexpression approach favours enterocyte rather than the enteroendocrine cell fate. Overexpression of Sox21a rescues the differentiation marker Pdm1 that is lost in JAK/STAT-deficient clones, demonstrating that Sox21a contributes significantly to EB differentiation downstream of this pathway. Consistent with this notion, our RNA-seq analysis demonstrates that Sox21a regulates a large set of genes including Pdm1, which encodes a transcription factor involved in the terminal differentiation of enterocytes. Our study also shows that Sox21a contributes to stem cell division notably in the posterior midgut. This is similar to the JAK/STAT pathway that impacts both stem cell division and differentiation. The observation that Sox21a flies are viable indicates that the role of Sox21a is likely restricted to the adult intestinal homeostasis.

Moving on, functional characterization of Sox21a target genes and identification of Sox21a-binding sites are now required to better understand the role of this transcription factor in ISC proliferation and progenitor differentiation.

An unexpected observation of our work was that Sox21a flies developed tumours that increase in size and grow towards the intestinal lumen over time. Sox21a tumours are mainly composed of post-mitotic progenitors, the EBs that are blocked in their differentiation. Our study shows that the growth of Sox21a tumours relies on ISC division. The requirement for ISC proliferation to drive Sox21a tumours explains why tumours are not observed in the posterior midgut and are more frequent when flies are infected with bacteria, a condition that stimulates stem cell proliferation. Our results indicate that the release of a mitogen, Upd2, by accumulating EBs provides a feed-forward loop stimulating ISCs to divide and differentiate, leading to a further increase in the number of EBs. It is likely that Sox21a

Figure 7 | Tumour-induced elimination of surrounding enterocytes requires JNK activation. (a) Two-week-old Sox21a intestine stained with the JNK activity reporter pucE69-Gal4 UAS-GFP (in green) and the basolateral cell-polarity marker Discs large (Dlg, in red). (b–d) Confocal section of intestine with EB-specific depletion of Sox21a for 2 weeks, stained with the JNK activity reporter pucE69-Gal4 UAS-GFP (in green) and Dlg (red, plasma membrane). EBs (GBETS>Sox21a-IR) are in green. (c, d) High-magnification view of two tumours defined in b. (e) Tumour burden in AMG of flies with the indicated genotypes monitored at 21 days. Flies were kept at 25 °C (for mutants) or 29 °C (for misexpression). Numbers of flies scored for each genotype (in biological triplicates) are indicated in e. P values from χ²-test (\(* < 0.05; ** < 0.01; *** < 0.001\)). Each other individual image shown in a–d represents 20 flies tested in one experiment, which was repeated three times.

Supplementary Fig. 10c–e). These observations suggest that these progenitor cells have an increased capacity to deal with ROS, and explain the lower level of ROS in Sox21a tumour.

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tumours are initiated at random sites where EB clustering leads to a local increase of Upd2. Like other cancer model, Sox21a tumours also express matrix metalloproteinase, which probably shapes the tumour environment to promote tumour progression. Accumulating EBs display a shift in metabolism with an increased expression of ROS-producing factors, such as Duox and a higher number of mitochondria and peroxisomes. This metabolic shift is likely to underlie the increase of ROS at the vicinity of the tumour. We observed that the progenitors express Sox21a6/+/ and Sox21a6/– tumours. This model highlights the active role of differentiating stem cell daughters in tumour formation. This model highlights the new concept highlighting the active role of differentiating stem cell daughters in tumour formation.

Mechanistic studies of several intestinal tumour models have been reported previously in *Drosophila*. The Sox21a tumour model is unique in its simplicity compared with other
Strains

Drosophila strains.(Driver strains) eg-Gal4, tub-Gal80; UAS-GFP (referred to as eg64); Myo1A-Gal4, tub-Gal80; UAS-GFP (referred to as Myo1A64); Sox21a/Gal4, tub-Gal80 (referred to as GBE73); Su(H)GAL4, UAS-nls-GFP, tub-Gal80; UAS-GFP (this study); DI-Gal4, UAS-GFP, tub-Gal80 (referred to as DI63); Act5C-Gal4 (BDSC53374); MARCM tester FRT2A; y,w,hsFlp, tub-Gal4, UAS-CDE-GFP; FRT2A, tub-Gal80 (gift from Yanruui Jiang) and y,w,hsFlp, tub-Gal4, UAS-nls-GFP; FRT2A, tub-Gal80 (this study); MARCM tester FRT82B; y,w,hsFlp, tub-Gal4, UAS-nls-GFP; FRT82B, tub-Gal80 (gift from Ernesto Sanchez-Herrero); GMR43E09-Gal4 (BDSC42471); puc69-Gal4 (ref. 59), mir-8-Gal4NP5247 (DGCR109497) and btl-Gal4, act-GFP (BDSC8807), Reporters: Su(H)-lacZ (ref. 60); puc-lacZ689 (DGCR109929); Dl-lacZ (gift from Michael Bautore); got-Dl-lacZ (ref. 61); 10xstat-GFP69 (ref. 62); Mmp2-GFP (BDSC56512); DI-GFP (BDSC59819); Cat-GFP (BDSC51646); Ap-GFP (BDSC7194); Mutants: upd2+, upd3+, and upd2-3 (ref. 63); FRT82B, Stat69 and hsp69, UAS lines: UAS-hop69; (gift from James Castelli-Gairola; UAS-Stat-RNAi (BDSC35600); UAS-Sox21a-RNAi (HMJ21395 and HMJ21325); UAS-N-RNAi (VDR100002); UAS-bak65 (BDSC6409); UAS-kay65 (BDSC7214); UAS-timp, UAS-Mmp1-RNAi, and UAS-Mmp2-RNAi (ref. 38); UAS-P35 (BDSC5072); UAS-EGFR69 (BDSC3646); UAS-GFP-SKL (BDSC8881); UAS-mito-HA-GFP (BDSC8442); UAS-Sox21a (this study); UAS-psi-RNAi (VDR103817); UAS-arr-RNAi (VDR142099); UAS-wg-RNAi (VDR13351 and 104579); UAS-upd1-RNAi (VDR13282); UAS-upd2-RNAi (BDSC3949 and NG5988R3-3) and UAS-upd3-RNAi (gift from Herve Agassie)

Methods

Tumour-burden quantification and statistical analysis. Tumour burden of flies with different genotypes was quantified as follows: Flies were grown either at 25°C (for mutant analysis) or at 18°C (for overexpression analysis) until adulthood. Sorted genotypes were kept at 25°C (for mutant analysis) or at 29°C (for overexpression analysis) for 20–22 days. The midguts of flies were dissected and scored for tumour burden based on the criteria described below. Grade 0 midguts carried no tumour (Grade 0) and had progenitor cells that were evenly interspersed by large multicellular enterocytes (Fig. 4g,k). Midguts with clusters of progenitors for cells spanning 3–4 enterocytes were designated grade 1 (Fig. 3h). Large tumour mass and multilayering cells growing towards the lumen were features of grade 2 and 3 tumours. While enterocytes were still present in grade 1 and 2 tumours, grade 3 tumours were only composed of progenitors. Tumour burden was defined as the place where the density of small-nucleated cells reduces to normal. The enterocytes surrounded by the grade 1 and 2 tumour cells were in the process of being eliminated through JNK activation, and we did not count them as part of the tumour. When a fly gut harboured several tumours, we assigned the final grade to the tumour with the highest grade. Overall, 20–30 flies were scored for each experiment and each experiment was repeated for at least three times. The results were pooled to generate graphs of tumour grade distribution in Excel. P values were calculated using two-tailed Student’s t-test, and indicated with *P<0.05; **P<0.01; or ***P<0.001.

To test the effect of bacterial infection on tumour burden, Gram-negative bacteria Drwminia carotovertina 15 (Ecc15) was orally ingested by 7-day-old Sox21a flies at OD600 = 100. The flies were returned to normal food after 2 days on Ecc15-containing medium, and scored for tumour burden after recovery for another 7 days. Other significance tests in the paper were done with Student’s t-test using the Prism 5 software.

Conditional expression of UAS-linked transgenes. The TARGET system was used in combination with the indicated Gal4 drivers to conditionally express UAS-linked transgenes. Flies were grown at 18°C to limit Gal4 activity. After 3–5 days, flies were transferred to 25°C to activate the UAS-linked transgenes.
at days 18 °C, adult flies with the appropriate genotypes were shifted to 29 °C, a temperature inactivating the temperature-sensitive Gal80s ability to suppress Gal4.

**MARCM clone induction.** The Sox21α9 allele was recombined to FRT2A site for MARCM analysis22. For clone induction, 3–5-day-old flies with the appropriate genotypes were heat-shocked for 30 min at 37.5 °C in a water bath. The flies were immediately transferred into a new tube and kept at 25 °C until dissection. Rescue experiments were performed by combining the UAS-Sox21α transgene with the FRT2A-sox21α in FRT2A-C176 flies. Note that UAS-Sox21α was only expressed in the clones indicated by the presence of GFP.

**Generation of Sox21α mutant and transgenes.** Sox21α mutant flies were generated with the method described before22 with the guide RNA sequence: 5′-GCCCTATCAGCTGTCCTCGGCG3′. The alleles originally named Sox21α9 and Sox21α10 were referred to as Sox21α1 and Sox21α2 in the paper. To generate the enh:Sox21α construct, the following primers (5′-acagcgacGAGCAGATCCTGCCCCTG-3′ and 5′-TCAATAGTTGGTGGCAGGCT-3′) were used to amplify the 2.8-kb Sox21α-RA-coding regions together with the intronic Sox21α enhancer from the genomic DNA of a wild-type fly. The PCR product was first cloned into pENTR-D-TOPO (Life Technologies) vector, and then swapped into either pFW destination vector to make UAS-Sox21αEnh::Sox21α or pTRW destination vector to make UAS-RFP-Sox21αEnh::Sox21α. Transgenic flies were established by standard P element-mediated germline transformation. At least three independent lines of each construct were tested for expression level. Note that without the presence of the Gal activator, the UAS-RFP-Sox21αEnh::Sox21α transgene drives the RFP reporter under the control of Sox21α cis-regulatory sequence. Despite the presence of RFP, this construct can rescue the Sox21α mutation (Fig. 4c).

**Histology and immunostaining.** Flies were transferred overnight into a classical fly food vial containing a filter paper soaked with a solution consisting of 5% sucrose to clear the digestive tract. Then, guts of adult females were dissected in PBS, and fixed for at least 1 h at room temperature in 4% paraformaldehyde in PBS. They were subsequently rinsed in PBS + 0.1% Triton X-100 (PBT), permeabilized and blocked in 2% bovine serum albumin PBT for 4 h. First, 1 h washing, secondary antibodies and DAPI were applied at room temperature for 2 h. Phalloidin was added to the secondary antibody incubation step in some experiments. Histology and staining on cross-sectioned guts were done as described previously65.

For FACS and RNA-seq analysis. Pair-end reads were mapped to the Drosophila melanogaster genome (release version 6.02) using Bowtie2 (ref. 66) with forward and reverse composition. Each sequencing experiment generated in total >40 million raw reads, and >80% was successfully mapped for each experiment. Gene expression was quantified by the number of paired reads that fall into the exons. Normalization was carried out using the size factor formula28. Differentially expressed genes were identified using the method described28. Over-represented GO terms were obtained by PANTHER28. GO terms were further filtered if the observed number of genes was <5% of the total input number of genes, and 25% of GO terms were discarded according to the ranking of fold enrichment.

**References**

1. Morrison, S. J. & Spradling, A. C. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 132, 598–611 (2008).

2. Lane, S. W., Williams, D. A. & Watt, F. M. Modulating the stem cell niche for tissue regeneration. Nat. Biotechnol. 32, 795–803 (2014).

3. Hsu, Y. C. & Fuchs, E. A family business: stem cell progeny join the niche to regulate homeostasis. Nat. Rev. Mol. Cell Biol. 13, 103–114 (2012).

4. Jiang, H. & Edgar, B. A. Intestinal stem cell function in Drosophila and mice. Curr. Opin. Genet. Dev. 22, 354–360 (2012).

5. Simons, B. D. & Clevers, H. Fetal homeostatic stem cell self-renewal in adult tissues. Cell 145, 851–862 (2011).

6. Cordero, J. B., Stefanatos, R. K., Scopelliti, A., Vidal, M. & Sansom, O. J. Inducible progenitor-derived Wingless regulates adult midgut regeneration in Drosophila. EMBO J. 31, 3901–3917 (2012).

7. Tian, A. et al. Injury-stimulated Hedgehog signaling promotes regenerative proliferation of Drosophila intestinal stem cells. J. Cell Biol. 208, 807–819 (2015).

8. Zhou, F., Rasmussen, A., Lee, S. & Agaisse, H. The UPD3 cytokine couples environmental challenge and intestinal stem cell division through modulation of JAK/STAT signaling in the stem cell microenvironment. Dev. Biol. 373, 383–393 (2013).

9. Li, Q. et al. The conserved misshapen-warts-Voricle pathway in enteroblasts to regulate intestinal stem cell in Drosophila. Dev. Cell 31, 291–304 (2014).

10. Hsu, Y. C., Li, L. & Fuchs, E. Transit-amplifying cells orchestrate stem cell activity and tissue regeneration. Cell 157, 935–949 (2014).

11. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. Cell 144, 646–674 (2011).

12. Barker, N. Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. Nat. Rev. Mol. Cell Biol. 15, 19–33 (2014).

13. Biteau, B., Hochmuth, C. E. & Jasper, H. Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. Cell Stem Cell 9, 402–411 (2011).

14. Ohsitne, B. & Spradling, A. The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature 439, 200–211 (2006).

15. Miccichè, C. A. & Perrimon, N. Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature 439, 475–479 (2006).

16. Antonello, Z. A., Reiff, T., Ballesta-Illean, E. & Domínguez, M. Robust intestinal homeostasis relies on cellular plasticity in enteroblasts mediated by miR-8-Escargot switch. EMBO J. 34, 2025–2041 (2015).

17. Kohlhaijer, A. et al. Src kinase function controls progenitor cell pools during regeneration and tumor onset in the Drosophila intestine. Oncogene 34, 2371–2384 (2015).

18. Sarkar, A. & Hochedlinger, K. The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. Cell Stem Cell 12, 15–30 (2013).

19. Zeng, X., Chauhan, C. & Hou, S. X. Characterization of midgut stem cell- and enteroblast-specific Gal4 lines in Drosophila. Genesis 48, 807–811 (2010).

20. Korzelius, J. et al. Escargot maintains stemness and suppresses differentiation in Drosophila intestinal stem cells. EMBO J. 33, 2967–2982 (2014).

21. Kondo, S. & Ueda, R. Highly improved gene targeting by germline-specific Cas9 expression in Drosophila. Genetics 195, 715–721 (2013).

22. Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci. 24, 251–254 (2001).

23. Jiang, H. et al. Cytokeine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell 137, 1343–1355 (2009).

24. Buchon, N., Broderick, N. A., Poidevin, M., Pradervand, S. & Lemaitre, B. Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Microbe 5, 37–40 (2009).

25. Beebe, K., Lee, W. C. & Mitchell, C. A. JAK/STAT signaling coordinates stem cell proliferation and multilineage differentiation in the Drosophila intestinal stem cell lineage. Dev. Biol. 338, 28–37 (2010).
37. Nagarkar-Jaiswal, S., Igaki, T., Pagliarini, R. A. & Xu, T. Loss of cell polarity drives tumor growth and invasion in Drosophila. Cell Stem Cell 8, 84–95 (2011).

39. Apidianakis, Y., Pitsouli, C., Perrison, N. & Rahme, L. Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. Proc. Natl Acad. Sci. USA 106, 20883–20889 (2009).

44. Moreno, E. Is cell competition relevant to cancer? Dev. Cell 18, 999–1011 (2010).

46. Buchon, N., Broderick, N. A., Kuraishi, T. & Lemaître, B. Drosophila EGF-R pathway coordinates stem cell proliferation and gut remodeling following infection. BMC Biol. 8, 152 (2010).

52. Bangi, E., Pitsouli, C., Rahme, L. G., Cagan, R. & Apidianakis, Y. Immune response to bacteria induces dissemination of Ras-activated Drosophila hindgut cells. EMBO. Rep. 13, 569–576 (2012).

54. Martorell, O. et al. Conserved mechanisms of tumorigenesis in the Drosophila adult midgut. PLoS ONE 9, e88413 (2014).

57. Brumby, A. M. & Richardson, H. E. scribble mutants cooperate with oncogenic Ras or notch to cause neoplastic overgrowth in Drosophila. EMBO J. 22, 5769–5779 (2003).

60. Furriols, M. & Bray, S. Dissecting the mechanisms of suppressor of hairless function. Dev. Biol. 227, 520–532 (2000).

64. Buchon, N. et al. Morphological and molecular characterization of adult midgut compartmentalization in Drosophila. Cell Rep. 3, 1725–1738 (2013).

65. Thomas, P. D. et al. PANTHER: a library of protein families and subfamilies indexed by function. Genome Res. 13, 2129–2141 (2003).

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Author contributions

Z.Z and B.L designed the research; S.K.R and U.K provided key reagents. Z.Z and J.P-B performed experiments; Z.Z, N.H and M.B analyzed the sequencing data. Z.Z and B.L. analyzed the data and wrote the manuscript.

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