The WT1-like transcription factor Klumpfuss maintains lineage commitment of enterocyte progenitors in the *Drosophila* intestine

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In adult epithelial stem cell lineages, the precise differentiation of daughter cells is critical to maintain tissue homeostasis. Notch signaling controls the choice between absorptive and entero-endocrine cell differentiation in both the mammalian small intestine and the *Drosophila* midgut, yet how Notch promotes lineage restriction remains unclear. Here, we describe a role for the transcription factor Klumpfuss (Klu) in restricting the fate of enteroblasts (EBs) in the *Drosophila* intestine. Klu is induced in Notch-positive EBs and its activity restricts cell fate towards the enterocyte (EC) lineage. Transcriptomics and DamID profiling show that Klu suppresses enteroendocrine (EE) fate by repressing the action of the proneural gene Scute, which is essential for EE differentiation. Loss of Klu results in differentiation of EBs into EE cells. Our findings provide mechanistic insight into how lineage commitment in progenitor cell differentiation can be ensured downstream of initial specification cues.
n many tissues, somatic stem cells respond to tissue injury by increasing their proliferative potential and producing new differentiating cell progeny. To maintain homeostasis during such periods of regeneration, cell specification and differentiation need to be precisely coordinated within a dynamic environment. Studies in the mammalian intestine have demonstrated a surprising plasticity in such specification events, showing that even differentiated cells can revert into a stem cell state during times in which tissue homeostasis is perturbed1−2. These findings highlight the critical role of gene regulatory networks in establishing and maintaining differentiated and committed cell states in homeostatic conditions.

The *Drosophila* midgut is an excellent model to study lineage differentiation of adult stem cells both in homeostasis as well as during regeneration and aging. The *Drosophila* midgut is maintained by intestinal stem cells (ISCs), which can generate differentiated enteroctyes (EC) or enteroendocrine (EE) cells3−4. Upon injury or infection, ISC proliferation is dramatically increased in response to mitogenic signals from damaged enterocytes5, leading to substantial dysfunction, as evidenced in aging intestine. These differences have been proposed to underlie the determination of lineage fate and lineage differentiation. ISCs produce the Notch-ligand Delta and Delta-positive ISCs can give rise to clones with both ECs and EEs5, whereas Delta-negative ISCs can only give rise to enterocytes, whereas Delta-positive ISC descendants give rise to clones with both ECs and EEs14,15. To trace the fate of Klu-expressing cells, we crossed the *klu-Gal4, UAS-GFP, tub-Gal80ts* FlipOut (F/O) lineage-tracing cassette16, with *Su(H)GBE-Gal4ts* to express the enhancer-trap line by performing a knock-in replacement of the Klu Coding Sequence (CDS) with the Gal4 CDS (Supplementary Fig. 1, see Methods). In contrast, *Dl-lacZ* staining was mostly found in small, diploid cells neighboring the GFP-positive cells (Fig. 1d, i, arrowheads). To confirm their identity, we combined the *klu-Gal4, UAS-lacZ* line with the Notch activity reporter Su(H)GBE-Gal4ts, which is exclusively activated in EEs10. In addition, we used *Delta-lacZ* (Dl-lacZ) as a marker for ISCs. The expression of *klu-Gal4, UAS-GFP* overlapped almost exclusively with *Su(H)GBE-Gal4ts* (Fig. 1d, i, arrowheads). To further confirm the expression of Klu in EEs, we used a FISH-probe for *klu* mRNA: this labeled *klu* mRNA in Su(H)GBE-Gal4ts-UAS-GFP marked EEs (Supplementary Fig. 1h, i, arrowheads).

Lineage-tracing experiments have shown that Notch-positive EB precursor cells exclusively give rise to enterocytes, whereas Delta-positive ISCs can give rise to clones with both ECs and EEs14,15. To trace the fate of Klu-expressing cells, we crossed the *klu-Gal4* enhancer-trap line to an Actin promoter-driven FlipOut (F/O) lineage-tracing cassette (UAS-GFP, tub-Gal80ts; UAS-Flip, Act >STOP> Gal4). As expected, *Dl-Gal4*-expressing ISCs gave rise to both ECs as well as EEs, marked by expression of the transcription factor Pros promoter (Pros) (Fig. 1, m, arrows). In contrast, Notch-positive EEs (Su(H)GBE-Gal4ts) only gave rise to ECs, but not EEs (Fig. 1n, o, arrowheads). Similar to Notch-positive EEs, *klu-Gal4*-labeled cells gave rise exclusively to EEs, not to EEs (Fig. 1p, q). We conclude that Klu is expressed in the EC-generating EEs in the *Drosophila* midgut.

**Klu loss of function leads to excess EE differentiation.** To determine the role of Klu in the specification and/or differentiation of cells in the ISC lineage, we first inhibited Klu function using the temperature-inducible TARGET-system to express RNAi constructs in specific lineages25. We used eg-Gal80ts to express *kluRNAi* in ISCs and EEs, and Su(H)GBE-Gal4ts to express *kluRNAi* in EEs only. In both conditions, knockdown of Klu increased EE numbers in the posterior midgut (Fig. 2a−d, quantification in Fig. 2i), suggesting that knockdown of Klu promoted the adoption of EE over EC fates in these cells. To confirm this, we used EB-specific FlipOut lineage tracing in combination with *kluRNAi* to trace the fate of *kluRNAi*-expressing EEs. We induced cobalt for 10 days at 29 °C, followed by a short 16-hour infection with the pathogen *Erwinia carotovora* (*Ec*) to induce gut turnover. Pros-positive EEs are seldom found in such EB-derived Su(H)GBE-F/O clones in control backgrounds, yet we found a significant increase of such
cells in clones expressing kluRNAi (Fig. 2e, f, quantification in Fig. 2j). To further confirm these results, we generated GFP-marked clones homozygous for a null allele of Klu, kluR51 using the MARCM technique and quantified EE numbers. Quantiﬁcation showed that kluR51 MARCM clones had more EE cells/clone (Fig. 2g, h, quantiﬁcation in Fig. 2k). Interestingly, the GFP-negative tissue also contained more EEs in kluR51 MARCM animals than in control animals (FRT2A, Fig. 2g, compare with Fig. 2h). This is likely due to the fact that in this genotype, the GFP-negative tissue is heterozygous for kluR51. Accordingly, MARCM RNAi (FRT40A; kluRNAi) clones (in which the surrounding tissue is wild type for Klu) had an increase in the number of EE cells/clone, but no diﬀerence in EE cells in the non-clonal surrounding tissue (Supplementary Fig. 2g, h, quantiﬁcation in Supplementary Fig. 2i, j). These results strongly suggest that Klu acts cell-autonomously in preventing EE differentiation of EB.

Interestingly, EB-to-EC diﬀerentiation could still occur in klu-deficient lineages: esg-F/O clones expressing kluRNAi (esg-F/O; kluRNAi) still contained cells with large nuclear size and positive for the EC marker Pdm1 (refs. 21,27) (Supplementary Fig. 2a–f). In summary, our results indicate that loss of Klu alters the EE-to-EC ratio in ISC lineages, but does not fully impair EC differentiation.

Ectopic Klu blocks proliferation and EB diﬀerentiation. Based on these observations, we hypothesized that constitutive Klu overexpression could reduce EE diﬀerentiation in the ISC lineage and might trigger ectopic diﬀerentiation of ISCs into ECs. To test this, we used the esg-F/O system to express full-length Klu in ISC-derived clones. Wild-type esg-F/O clones take up most of the posterior midgut 2 weeks after induction, containing a mixture of ECs and EEs (Fig. 3a). In contrast, clones expressing full-length Klu remained very small, containing only a few cells that did not exhibit any hallmarks of differentiation into either EEs or ECs (Fig. 3b). Klu is thought to act mainly as a repressor of transcription based on studies in other organs. To ask whether this repressor function of Klu would elicit the phenotypes observed, we expressed the zinc-finger DNA-binding domain of Klu fused to either a VP16 activation domain (Klu-VP16) or the repressor domain from Engrailed (Klu-ERD) 28. Whereas clones grew normally and diﬀerentiation still occurred in clones expressing the activating Klu-VP16, clone size was smaller and diﬀerentiated cells were not observed in clones expressing the repressing Klu-ERD, conﬁrming that transcriptional repression of genes regulated by Klu is suﬃcient to limit growth of ISC-derived clones (Fig. 3c, d, quantiﬁcation in Fig. 3e). Similarly, UAS-klu expression in esg-F/O clones inhibited proliferation of ISCs (measured by quantifying mitotic ﬁgures in the gut) both in homeostatic and infected conditions (infection with Ecc15; Supplementary Fig. 3a). Restriction of Klu expression solely to ISC (using esgO combined with Su(H)-Gal80) (ref. 29) showed that the repression of mitosis upon Ecc15

Fig. 1 Klu is speciﬁcally expressed in enteroblast cells. a–c The klu-Gal4, UAS-GFP reporter line shows expression in the midgut epithelium. ISCs (arrows) and EBs (arrowheads) are visualized by egsp-lacZ (beta-galactosidase, red). Cells are outlined with Armadillo/beta-catenin (Arm, grayscale). Representative area of posterior midgut is shown. n = 3 animals. d–i The klu-Gal4, UAS-GFP was combined with Su(H)-GBE-lacZ (enteroblast (EB) marker) or Dl-lacZ (intestinal stem cell (ISC) marker). Expression of Klu largely overlaps with the EB marker Su(H)-GBE-lacZ (d–f), and Klu-positive cells are found adjacent to the Delta-positive ISCs (g–i). j Quantification of EB-marker gene overlap of the genotypes displayed in d–f. n = 10 guts/animals (Su(H)-GBE-lacZ, n = 572 cells counted). k Quantification of ISC-marker gene overlap of the genotypes displayed in g–i. n = 7 guts/animals (Dl-lacZ, n = 1370 cells counted). l–q Lineage-tracing of cells in the intestine using diﬀerent cell-speciﬁc drivers. EEs are marked by antibody staining for the transcription factor Prospero (Pros, red). Arrows indicate GFP-Pros double-positive EEs in the clonal area, whereas arrowheads indicate EEs outside the clonal area. l–m The Dl-Gal4-positive ISCs give rise to both differentiated cell types of the intestinal lineage (enterocytes (EC) and enteroendocrine (EE) cells). n, o Su(H)-GBE-positive EBs, klu-Gal4-positive cells give rise exclusively to ECs. Representative areas of posterior midgut are shown. n = 7 guts examined for l, m, n = 7 guts examined for n, o and n = 10 guts examined for p, q. Scale bar = 25 μm, except in a–c, scale bar is 25 μm.
infection is mainly due to the ectopic expression of Klu in ISCs, although we do observe a small but significant decrease if we express Klu using the EB-driver Su(H)\(^{D}\) (Supplementary Fig. 3b).

We also combined expression of Klu (UAS-klu) with expression of the oncogenic Ras\(^{V12}\) variant (UAS-Ras\(^{V12}\)) in esg-F/O clones. Whereas esg-F/O\(\times\)Ras\(^{V12}\) clones occupy the entire posterior midgut 2 days after induction and contribute to a rapid loss of viability of the animal, co-expression of UAS-klu markedly reduced clonal size and rescued viability (Supplementary Fig. 3c–g). This is consistent with an anti-mitotic effect of ectopic Klu expression in ISCs.

To ask whether sustained expression of Klu in EBs would influence their differentiation, we performed lineage-tracing experiments in control and klu\(^{RNAi}\)-expressing Su(H)-F/O clones in Fig. 3c and d, n = 10 for control and n = 10 for klu\(^{RNAi}\) guts. Quantification of the number of Pros-positive EE cells/clone and the total number of Pros-positive EE cells/ROI for the genotypes in g and h, n = 15 guts (FRT2A control) and n = 17 guts (klu\(^{RNAi}\)). For quantifications in i–k, error bars represent mean \(\pm\) SD. Significance was calculated using Student’s t-test with Welch’s correction. Scale bars = 50 \(\mu\)m.

To further characterize the gain-of-function phenotype, we combined UAS-klu with the ISC-marker Dl-lacZ and the EB-marker Su(H)\(^{D}\)GE-lacZ. Interestingly, esg-F/O clones expressing UAS-klu did not stain positive for Dl-lacZ (Fig. 4a–d) or Su(H)\(^{D}\)GE-lacZ (Fig. 4e–h), suggesting that ectopic Klu expression in ISCs interferes with normal Dl-Notch signaling in ISC-EB pairs. To investigate this interaction between Notch signaling and Klu activity further, we performed epistasis experiments: Klu overexpression prevented the formation of large tumors in Notch loss of function esg-F/O clones (Supplementary Fig. 4a–l) and UAS-klu can repress the excess mitosis seen in the esg\(^{RNAi}\)/Su(H)\(^{D}\), consistent with the inhibition of ISC proliferation upon Klu expression.

To test whether Notch is required for Klu expression in EBs, we performed qRT-PCR for klu on progenitor cells expressing

Fig. 2 Loss of Klu leads to excess EE differentiation. a–d RNAi-mediated knockdown of Klu results in an excess of Pros-positive EE cells. Expression of klu\(^{RNAi}\) using the ISC + EB driver esg-Gal4\(^{S}\) (Pros in green, compare a with b) or the EB-specific Su(H)\(^{D}\)GE-Gal4\(^{S}\) driver (Pros in red, compare c with d). e, f Su(H)-GBE-driven FlipOut (Su(H)-F/O) clones expressing klu\(^{RNAi}\) show an increased number of Pros-positive EE cells in the clonal area upon Ecc15 infection compared to controls (compare f with e). g, h Clonal analysis of control FRT2A (g) or FRT2A, klu\(^{R51}\) (h) null mutant MARCM clones. Representative areas of posterior midguts are shown. i EE cell quantification of the posterior midgut for the genotypes in a–d. Number of midguts n = 12 (control w\(^{1118}\)) and n = 16 (klu\(^{RNAi}\)) for a and b and n = 15 (control w\(^{1118}\)) and n = 18 (klu\(^{RNAi}\)) in c and d. j Quantification of GFP-Pros double-positive cells/ROI in control and klu\(^{RNAi}\)-expressing Su(H)-F/O clones in e and f. n = 10 for control and n = 10 for klu\(^{RNAi}\) guts. k Quantification of the number of Pros-positive EE cells/clone and the total number of Pros-positive EE cells/ROI for the genotypes in g and h. n = 15 guts (FRT2A control) and n = 17 guts (klu\(^{RNAi}\)). For quantifications in i–k, error bars represent mean \(\pm\) SD. Significance was calculated using Student’s t-test with Welch’s correction. Scale bar = 50 \(\mu\)m.
**Fig. 3** Klu overactivation results in a loss of Delta-Notch signaling and ISC differentiation. a–d Clonal expression of different Klu isoforms using the esg-Gal4-driven FlipOut (esg-F/O) system to generate ISC clones. a Control esg-FO clones grow to occupy most of the posterior midgut 2 weeks after clonal induction. b–d Clones expressing either full-length Klu (UAS-kluFL, b) or the Klu zinc-finger DNA-binding domain fused to the Engrailed Repressor Domain (UAS-ERD-kluZF, d) resulted in a block of differentiation. This was not observed when expressing the Klu zinc-finger DNA-binding domain fused to the VP16 transcriptional activator domain (UAS-VP16-kluZF, c). Representative areas of posterior midguts are shown. e Quantification of genotypes in a–d. n = 5 midguts for each genotype. f–i Su(H)-F/O control clones contain GFP-Pdm1 double-positive cells, representative of EB > EC differentiation (f, g closeup in h, i). j–m Su(H)-F/O > UAS-klu clones contained much less GFP-Pdm1 double-positive cells, indicative of impaired EB > EC differentiation upon Klu expression. n Quantification of the percentage of GFP-Pdm1 double-positive cells in images of posterior midguts from control (f–i) and UAS-klu Su(H)-F/O (j–m) clones. n = 7 midguts for each genotype. For quantifications in e and n: Error bars represent mean ± SD. Significance was calculated using Student’s t-test with Welch’s correction. Scale bar = 50 µm.

\( N^{\text{RNAi}} \) for 1 week (\( \text{esg}^{\text{ds}} > N^{\text{RNAi}} \)). Consistent with the formation of Pros\(^+\) cell tumors, loss of Notch leads to a 5.5-fold upregulation of \( \text{pros} \) mRNA in Esg\(^+\) cells. However, klu expression is almost absent from \( N^{\text{RNAi}} \) cells (Fig. 4j), strongly suggesting that Klu expression depends on Notch activity.

Ectopic activation of Notch in stem-progenitor cells using the Intracellular domain of Notch (\( \text{esg}^{\text{ds}} > \text{UAS-N}^{\text{ICD}} \)) results in a loss of the stem-progenitor compartment due to premature differentiation into EC cells. \( \text{UAS-N}^{\text{ICD}} \) expression resulted in klu mRNA expression in large Esg\(^+\) cells that seem to be differentiating into ECs based on their nuclear size (Fig. 4m, n, compare with Fig. 4k, l), suggesting that Notch activation is sufficient to induce Klu expression. However, combining \( \text{UAS-N}^{\text{ICD}} \) with \( klu^{\text{RNAi}} \) did not alter the premature differentiation phenotype of \( \text{UAS-N}^{\text{ICD}} \) (Supplementary Fig. 4m–t).

Since Notch activation is thus sufficient to induce differentiation of Esg\(^+\) progenitors into ECs even in the absence of Klu, we conclude that induction of Klu by Notch in EBs is important to prevent specification of EBs into EE progenitors, but is not essential for other steps in EC differentiation.

Altogether, our results indicate that the Notch-mediated induction of Klu in EBs is required to restrict lineage commitment of EBs to the EC fate. Reciprocally, ectopic Klu expression interferes with normal Delta-Notch signaling between ISC and EB and inhibits proliferation. We propose that ISC-derived EB daughter cells that express Klu enter a cell cycle arrested, undifferentiated state, and that Klu needs to be downregulated for EC differentiation to proceed. To test this hypothesis, and to understand how Klu expression controls the EB state, we decided to explore the transcriptional program downstream of Klu.

**RNA-Seq supports role of Klu in Notch and EE differentiation.** To gain a comprehensive overview of the genes controlled by Klu in the intestine, we performed RNA-Sequencing (RNA-Seq) on FACS-sorted Esg\(^+\) progenitor cells expressing either \( klu^{\text{RNAi}} \) or \( \text{UAS-klu}^{\text{30}} \) (see Fig. 5a, and Methods for details). Principal component analysis on the transcriptome of these populations showed that all sample groups form distinct clusters and that group replicates cluster closely together (Supplementary Fig. 5a). We also...
Fig. 4 Klu is regulated by Notch and represses Notch-induced tumor formation. a, b Control esg-F/O clones always contain 1 or more ISCs expressing Dl-lacZ. c, d esg-F/O+UAS-klu clones have no detectable Dl-lacZ expression. e, f Similarly, control esg-F/O clones contain EBs expressing Su(H)-GBE-lacZ, but not esg-F/O clones expressing UAS-klu (g, h). Representative areas of posterior midgut are shown. n = 3 midguts examined/genotype in a-h. i Quantification of the number of mitoses per midgut in esgFi flies expressing NRNAi alone or in combination with UAS-klu. Klu expression also reduced mitosis compared to control midguts (esgFi × w1118, control average = 4.2 mitoses/midgut, compared to UAS-klu: 0.48 mitoses/midgut, P < 0.0001). n = 42 for control (crossed with w1118) esgFi flies and NRNAi animals, n = 33 for UAS-klu and n = 24 for UAS-klu NRNAi. j Quantitative real-time PCR of sorted esgFi GFP+ cells expressing NRNAi for the EE-marker Pros and Klu. cDNA was derived from two replicates/genotype, each replicate containing mRNA isolated from esgFi GFP+ cells from 100 midguts/genotype. k–n Fluorescent in situ hybridization for a klu probe showed induced expression in esgFi GFP+ cells that overexpress constitutively active Notch intracellular domain (NICD, m, n) compared to control esgFi cells (k, l). Representative areas of posterior midgut are shown. n = 4 midguts examined/genotype in k–n. Scale bar = 50 μm

noticed that the distance between control and kluRNAi sample groups and the UAS-klu group in the largest principal component PC1 is much larger (Supplementary Fig. 5a). This indicates more profound transcriptional changes in the UAS-klu samples compared to controls than between kluRNAi and controls. This is also reflected in the FACS-profile of EsgFi cells expressing kluRNAi and UAS-klu: whereas ISC and EB population sizes appeared similar between control and kluRNAi, the UAS-klu-expressing EsgFi cells showed a loss of clearly distinguishable ISC and EB compartments (Supplementary Fig. 5b–d). We first confirmed that the transcriptome of esgFi>kluRNAi sorted cells indeed reflects the excess EE differentiation phenotype seen in kluRNAi animals by performing qRT-PCR for prospero (pros) and scute (scu). The EE marker pros was upregulated 5-fold upon kluRNAi (Fig. 5b). The proneural transcription factor Scute (scu) is necessary and sufficient for EE generation in the Drosophila midgut14,31,32 and many upstream factors impinge on the expression of scu to regulate EE differentiation33. mRNA levels of scu increased ~2.5-fold upon kluRNAi expression, and UAS-klu expression completely abolished sc mRNA expression in stem-progenitor cells (Fig. 5b). In addition, we checked klu mRNA levels to verify knockdown and overexpression efficiency. As expected, we saw a 70% reduction in klu mRNA upon kluRNAi. Surprisingly, however, expression of klu mRNA in UAS-klu-expressing progenitor cells
**a**
esg-Gal4<sup>T</sup>, UAS-GFP>; w<sup>1118</sup>-<br/>- klu<sup>RNAi</sup>-<br/>- UAS-klu<br/>
Elastase dissociation<br/>
FACS Sorting of GFP<sup>+</sup> cells<br/>
RNA- isolation and amplification<br/>
qRT-PCR

**b**
qRT-PCR

**c**
qRT-PCR

**d**
klu<sup>RNAi</sup> UP UAS-klu DOWN

329 28.9%
81 7.1%
728 64%

**e**
RNAi vs Con UAS vs Con

**f**
Control

**g**
Control closeup

**h**
klu<sup>RNAi</sup>

**i**
UAS-klu

**j**
FACS

miraP-GFP control
miraP-GFP klu<sup>RNAi</sup>
miraP-GFP UAS-klu

**miraP-GFP intensity**
was almost completely abolished (Fig. 5c). This was contrary to the expected klu overexpression, but was explained by the fact that the UAS-klu construct does not carry the endogenous klu 3′ UTR, which our primers targeted. Primers that solely target the coding region of klu (klu CDS), in turn, readily detected a ~12-fold upregulation of klu transcript. Hence, while transgenic Klu was induced as expected, endogenous Klu expression was repressed, indicating that Klu may repress its own expression. This notion of a negative autoregulatory loop was confirmed in our RNA-seq data, as we detected a high number of reads in the coding region of the gene in UAS-klu samples, and no reads in the 3′UTR (Supplementary Fig. 6).

Comparing the transcriptomes of wild-type progenitors with the experimental samples, we found 410 genes upregulated in kluRNAi and 809 genes downregulated in UAS-klu-expressing Esq+ cells (Padj < 0.05, log2 FC > 0.5 or < −0.5). We also found 283 genes downregulated in kluRNAi and 1025 genes upregulated in UAS-klu with the same criteria (Padj < 0.05 and log2 FC < −0.5 or > 0.5), Wald significance test with Benjamini and Hochberg correction, see Methods and Supplementary Data 1). Given that only the repressor form (kluZF-ERD) of Klu could recapitulate the phenotype of the expression of full-length Klu in esq-F/O clones (Fig. 3), we focused our analysis on genes that would be upregulated in the absence of Klu, but downregulated upon klu RNAi expression. In this category of 81 genes, many genes involved in the regulation of Notch signaling (the Hairy/ Enhancer of Split (E(Spl)) complex genes m6, m7, m8, and the HES-like transcription factor Deadpan), as well as several previously described regulators of EE differentiation (encoding the proneural proteins Aesense (ase), Scute (sc), and the adaptor protein Phyllopod, phyl)) could be identified (Fig. 5e). Additional E(Spl) genes (E(Spl)-m6 and E(Spl)-my) were significantly upregulated in kluRNAi samples, but did not change significantly in UAS-klu samples (Fig. 5e). E(Spl)-genes are a group of genes activated by Notch that mediate its downstream transcriptional response44. Phyl, in turn, acts to destabilize Trastrack (ttk), a strong repressor of the achaete-scute complex genes scute and asense, loss of which leads to a dramatic increase in EE numbers33,35. Reciprocally, loss of phyl stabilizes Ttk and results in a complete loss of EE36. The induction of phyl in klu loss of function conditions thus explains the increase in EEs. We also found that expression of Charlatan (chnt) was downregulated by UAS-klu. Chn is a transcription factor that positively regulates Achaete and Scute, and loss of Chn causes proliferation and differentiation defects in the midgut stem-progenitor compartment37-39. Hence, Klu represses the expression of several genes that have reported roles in EE differentiation.

Our transcriptome data also revealed changes downstream of Klu that may explain the Klu-induced exit from the stem cell state: ISC maintenance depends on the Class I bHLH-family member daughterless (Da)/E47-like, since loss of Da results in loss of ISC fate and EC differentiation32. The gene miranda (mira) is a Da/proneural target gene that is also highly expressed in ISCs and to a lesser extent in EEs (Fig. 5f, g). Proneural factors such as Ase and Sc require Da to dimerize and regulate transcription.40 kluRNAi resulted in a slight but significant upregulation of mira in ISC/EB clusters, whereas Klu overexpression resulted in a 2.3-fold downregulation (Fig. 5e). To confirm this, we used a mira- Promoter-GFP (mira-GFP) line32 and combined this with kluRNAi and UAS-klu. Confocal microscopy and FACS sorting of cells expressing either kluRNAi and UAS-klu confirmed that UAS-klu expression could reduce mira-GFP levels in Esq+ cells, whereas a slight induction is seen in kluRNAi cells (Fig. 5i, j). FACS sorting indicated an increase in GFP intensity of the EB cells (Fig. 5j), rightmost peak) in kluRNAi Esq+ cells. This suggests that physiologically, Klu acts to inhibit mira expression in EEs and that ectopic expression of Klu in ISC is sufficient to repress the expression of stem cell markers like miranda.

Klu acts upstream of Scute in EE differentiation. Scute plays a critical role in a transcriptional loop that regulates both ISC proliferation and the initiation of EE differentiation.16 Our genetic and transcriptional profiling experiments suggest that Klu likely acts downstream of Notch, but upstream of the proneural genes Ase and Sc in repressing EE differentiation (Figs. 3–5, Supplementary Fig. 4). We performed epistasis experiments with Klu and Sc to test this hypothesis. We generated esq-F/O clones that express kluRNAi in the presence or absence of scRNAi. Clones expressing kluRNAi contained more EE cells compared to control clones (Fig. 6c, d compared with Fig. 6a, b), whereas clones expressing scRNAi are almost completely devoid of EE cells (Fig. 6e, f). The combination of kluRNAi and scRNAi also resulted in clones with little or no EE differentiation (Fig. 6f, h, quantification in Fig. 6i). This suggests that excess EE differentiation in kluRNAi-expressing clones depends on Scute. To confirm that Scute would act downstream of Klu in determining EE fate, we combined overexpression of Scute and Klu. Clonal expression of Scute using the esq-F/O system resulted in clones consisting almost entirely of Pros-positive EE cells whereas clones expressing UAS-klu are completely devoid of EE cells (quantification in Fig. 6f), images in Supplementary Fig. 7a–l). Co-expression of Klu and Scute leads to a marked reduction in clone size (Supplementary Fig. 7m) but EE differentiation was observed in a large fraction of the clones, although the percentage of differentiated cells is reduced compared to UAS-Sc alone (Fig. 6j). We conclude that Scute can still induce EE differentiation, even in Klu gain-of-function conditions.

We observed an increase in the number of Pros-pH3 double-positive cells in UAS-Sc compared to control, likely representing the EE-progenitor cells (EEp) undergoing a final round of division16. Strikingly, this percentage increased in esq-F/O > UAS-klu + UAS-sc clones (Supplementary Fig. 7n).

Note: The article text is a summary of research findings and does not include detailed figure references or data tables. The figures and data mentioned are part of the original research paper and are not included in this text summary.
over-expressing clones (Supplementary Fig. 7n), indicating that these cells might be arrested in mitosis. This suggests that although Klu expression cannot completely repress UAS-Scute-induced EE differentiation, the effect of Klu on cell cycle progression interferes with the proliferation-inducing capacity of Scute.

**Klu binds to genes regulating EE fate, cell cycle and Notch.** The differentially expressed genes (DEGs) from our RNA-Seq analysis might reflect genes that are direct target genes of Klu. Alternatively, the transcriptional changes might be the consequence of a change in cell populations due to the loss or overexpression of Klu. To distinguish between these possibilities and to identify genes directly regulated by Klu, we performed targeted DamID of Klu. To distinguish between these possibilities and to identify genes directly regulated by Klu, we performed targeted DamID of Klu.

We considered these peaks as high-confidence Klu-binding sites. Our transcriptomics data on Klu indicated that Klu controls many genes involved in Notch signaling, EE differentiation, and cell cycle regulation. We identified a cluster of binding sites at the centrosomal end of the E(Spl)-locus around the E(Spl)-mδ and E(Spl)-my genes (Fig. 7a). Since our RNA-Seq data showed that many of the E(Spl)-genes change expression in both kluRNAi and UAS-klu conditions (Fig. 5e), this suggests that Klu could possibly regulate the expression of multiple members of the E(Spl)-complex through this binding peak at the centrosomal end of the E(Spl)-locus. Furthermore, we identified a Klu-Dam binding peak at the klu locus, supporting our hypothesis that Klu acts in an autoregulatory loop by negatively regulating its own expression (Fig. 7b).

Previous work has shown that Scute and the E(Spl)-complex member E(Spl)m8-ΔHLH act in a regulatory loop to generate an EE precursor directly from the ISCl16. Since our results indicate that Scute is upregulated upon loss of Klu and acts epistatically to Klu in EE formation, we first looked for Klu binding in and around the scute locus. We did not observe binding of Klu-Dam around any of the genes in the Achaete/Scute complex. However, we did identify a DamID peak around the *sina* and *sinah* loci (Fig. 7c). Together with the adaptor protein Phylllopop, the Sina and Sinah E3-ubiquitin ligases are able to degrade the transcriptional repressor Tramtrack (*ttk*), which represses EE fate13,36. *sina* transcript levels are upregulated 2.2-fold upon klu RNAi and *phyl* levels are upregulated 8-fold as well as downregulated 15-fold upon UAS-klu expression (Fig. 5e, Supplementary Data File 1). Hence, we propose that Klu represses EE fate determination in EBs upstream of Scute by stabilizing Tramtrack, since Klu directly represses the members of the

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**Fig. 6** Scute acts downstream of Klu in enteroendocrine differentiation. **a, b** Control esg-F/O clones 14 days after clonal induction. **c, d** Expression of kluRNAi lead to increased EE differentiation in clones, marked by increased numbers of Pros+ cells (red). **e, f** scRNAi clones showed almost no EE differentiation. **g, h** Similarly, the combination of kluRNAi with scRNAi resulted in clones lacking EE differentiation. Representative areas of posterior midgut are shown. **i, j** Quantification of GFP+/Pros+ double-positive cells/clone of the genotypes in **a–h**. n = 10 for control, n = 14 for kluRNAi, n = 10 for scRNAi, and n = 12 for scRNAi; kluRNAi. **j** Quantification of GFP+/Pros+ double-positive cells/clone of esg-F/O clones expressing either UAS-sc, UAS-klu, or the combination. See Supplementary Fig. 7 for images. n = 5 for control, n = 6 for UAS-klu, UAS-sc, and UAS-klu/UAS-sc. Error bars represent mean ± SD. Significance was calculated using Student’s t-test with Welch’s correction. Scale bar = 50 μm.
E3-ligase complex Sina, Sinah, and (indirectly) Phyl that can normally target Ttk for destruction.

In addition to genes involved in Notch signaling and EE specification, we find evidence for direct repression of critical cell cycle regulators by Klu. We find Klu-binding peaks at both the Cyclin B (CycB) and Cyclin E (CycE) loci (Fig. 7d, e), two Cyclins that are essential for G1–S and G2–M progression, respectively. CycE is also upregulated upon klu RNAi expression. Notch activation is essential for the mitotic-to-endocycle switch in follicle cells of the Drosophila ovary, and polyploidization is a critical step in the normal process of EB-to-EC differentiation. We propose that Klu plays a role in remodeling the cell cycle in response to Notch activation by directly repressing two critical cell cycle regulators. Furthermore, this explains how Klu acts as a potent suppressor of cell proliferation (Fig. 3, Supplementary Fig. 3).

Altogether, our data suggest a model (Fig. 8) in which Klu acts as a Notch effector in the EB that acts to restrict the duration of the Notch transcriptional response (through negative regulation of the E(Spl)-complex members and Klu itself). Second, Klu prevents activation of the Scute-E(Spl)m8 transcriptional circuit that triggers EE differentiation. Finally, we find evidence that Klu can bind and repress critical cell cycle regulators such as Cyclin B and Cyclin E, likely promoting the switch from a mitotic to an endoreplicating cell cycle in differentiating ECs.

Discussion

Our work identified a mechanism by which lineage decisions are cemented through the coordinated repression of alternative fates and of cell proliferation in somatic stem cell daughter cells. Notch-induced expression of Klu in EBs is necessary to repress EE fates in EBs, but also to restrict Notch target gene expression. Hence, its own expression has to be self-regulated to allow differentiation to ECs to proceed. We find that Klu represses several genes that are critical for EE differentiation; most notably genes that influence the level of Scute. Transient expression of Scute is necessary and sufficient for EE differentiation and this is accomplished by a double-negative feedback loop between Scute and E(Spl)m8 (ref. 16). Klu expression results in the repression of both transcription factors in EBs, inactivating the transcriptional circuit that governs EE differentiation (Fig. 8). Previous work has shown that Klu is directly regulated by Su(H) and acts as a Notch effector in hemocyte differentiation. We find that overexpression of Klu results in the loss of Notch signaling activity in stem-progenitor cells, and that Klu is able to repress several Notch effector genes (such as the HES/E(Spl) family and HES/E(Spl)-like genes such as Deadpan). We thus propose that Klu acts in a negative feedback loop downstream of Notch signaling to ensure that Notch effector gene activity is transient in EBs, mirroring the transient nature of EE specification by Scute and E(Spl)m8.

Klu is a zinc-finger transcription factor with some similarity to WT1 (refs. 24,47). While the sequence similarity between these factors is limited, our data suggest that functional parallels can be drawn: Loss of WT1 in the mouse kidney results in glomerulosclerosis and is accompanied by ectopic expression of HES/E(Spl) family genes and in zebrafish kidney podocytes Notch expression induces WT1 transcription, while the Notch intracellular domain (NICD) and WT1 synergistically promote transcription at the promoter of the HES/E(Spl) family gene Hey1 (ref. 19). This suggests that the negative feedback between Notch and its effectors Klu or WT1 might be conserved between species, even though conservation at the sequence level between these transcription factors is low.

Our data also support a role for Klu for regulating cell cycle progression. Overexpression of Klu results in a strong block in cell proliferation in RasV12-induced tumors and our DamID data suggest that Klu can directly regulate Cyclins B and E. The phenotype of Klu in EBs is in stark contrast to its role in the neuroblast stem cell lineage, where overexpression of Klu leads to a strong overproliferation of immature...
neural progenitor cells and the formation of brain tumors. However, this likely reflects the different role for Notch in the NB lineage, where continuous activation of Notch similarly leads to INP overproliferation and tumor formation. Thus, the role of Klu in promoting either lineage differentiation or stem-progenitor cell proliferation seems to be context-dependent. Similarly, Wt1 was initially identified as a tumor-suppressor gene mutated in the rare pediatric kidney cancer Wilms’ Tumor. However, expression of Wt1 was found to be elevated in many solid tumors and in acute myeloid leukemias. During development, Wt1 plays a role in the formation of many different tissues of mesodermal and neuroectodermal origin. Although Wt1 expression seems restricted in adult animals at first glance, whole-body knock-out of Wt1 in adult mice results in rapid demise of the animals with kidney, spleen, bone, and fat tissue defects as well as defective erythropoiesis. Furthermore, recent results in zebrafish have shown that Wt1b can be re-activated in specific mesenchymal cells upon damage, suggesting that Wt1b re-expression is involved in regeneration upon damage. In addition, Wt1 is often transiently expressed in both nephric and hematopoietic lineages in committed progenitor cell types, similar to the expression of Klu in the EB, raising the possibility that to fully understand the role of Wt1-like proteins in tumorigenesis, cell lineage relationships, as well as cell proliferation and differentiation events in tumors need to be taken into account.

Critically, our work highlights the role for transient transcriptional rewiring events during cell specification in stem cell lineages. This rewiring seems to be required to ensure lineage commitment downstream of initial symmetry breaking signals like Notch, and ensure commitment to cell differentiation into a defined lineage. As such, it can be expected that similar transcriptional rewiring needs to happen for cells to undergo de-differentiation into stem cells in regenerating tissues. Understanding this transcriptional rewiring process will substantially advance efforts to control tissue repair and regeneration in mammals, including humans.

Methods

Fly strains and husbandry. The following strains were obtained from the Bloomington Stock Center: BL28731 (klu RNAi on 3rd), BL60669 (klu RNAi on 2nd), BL56355 (UAS-klu[Neo]), BL11651 (UAS-klu[Neo]), BL51672 (USAC-sc), BL1997 (w*; F[w/+; mC = UAS-FLP]D5), BL54433 (y1 w*; M[w/+; mC = hs.min(FRT.STOP1) dam]ZH51C), BL1672 (w1118); snail/Sco[CyO], P[ry + > t7.2] = en1[wg(en1)])

VDCR: v27228 (N RInA). Other stocks: klu-Gal4 UAS-GFP, FRT2A kluRSi/ Tm6B, hs-Fly, UAS-GFP/Pm7,FRT2A, UabGal4 (C. Klein, Düsseldorf) UAS-Klu2F, UAS-ERD-Klu2F, UAS-VP16-Klu2F (all constructs inserted into ZH51C on 2nd, C.Y. Lee, U. Michigan) eg-F0 (w; eg-Gal4, tub-Gal80ts, UAS-GFP; Act > CD2 > Gal4(UAS-GFP/TM6B), esg (w;wcl-Gal4, UAS-GFP;CyO/tub-Gal80ts/Tm3), Su(H) (w;Su(H)Gal4, UAS-C8D- GEF/Cre, UAS-DIP86B-Gal4-p650/Gal4); Su(H) > F/O (w; Su(H)-F/O (Wig); UAS-FLP/D5])[J22]; Tubstocks generated in this study for the posterior midgut on a Zeiss Apotome microscope or Zeiss LSM710 confocal at either x20 or x40 magnification. Images were captured as Z-stacks with 8–10 slices of 0.22–1.0 µm thickness. Images were converted to maximum-intensity projections in Fiji (https://fiji.sc) and quantifications were performed using the CellCounter Fiji plugin. ROIs in quantifications are defined as images taken from the posterior midgut R4-R5 region at x20 magnification in which all cells/clones were quantified. Scale bar = 50 µm in all images, except in Fig. 1a: scale bar = 25 µm. Graphing, statistical analysis, and survival curves were produced in GraphPad Prism. Significance was calculated using Student’s t-test. In case of unequal variances, Student’s t-test with Welch’s correction was used.

Cloning and transgene generation. We used the Inducible DamID system from the Van Steensel lab to generate klu-Dam41. To this end, we amplified the klu Full-length cDNA (derived from BDGP Cold clone FI1015) using Act and Nofl-containing primers and cloned the fragment into the vector p-attB-min isp70-PFR-STOP91-FRT-DamMyo[open] (Addgene plasmid #71809). Transgenic lines were generated by GenetiVision Inc. using the ph313 integrate-mediated site-specific transgenesis system. The finished construct was injected into Bloomington stock BL24882 (ZH51C at position on 2nd) and resulting transgenic line were tested by genotyping PCR. Both control Dam-only (BL56433) and klu-Dam transgenic lines were crossed to BL1672 (w1118); snail/Sco[CyO], P[ry + > t7.2] = en1[wg(en1)]) before use. The klu-CDS CRISPR line was generated by Rainbow Transgenics (Camarillo, CA, USA). A targeting construct was designed to replace the klu CDS with the Gal4 CDS at the klu ATG. Two independent transformants were obtained that both showed identical EB-specific expression.

DamID. Control Dam-only (BL56433) and klu-Dam male flies were crossed to w; ex-gal4, tub-Gal80ts, UAS-GFP/Cyo, w[ + ; mC = UAS-FLP]D5]J22/TM6B virgins. Crosses were maintained at 18°C and progeny was shifted to 29°C for 24 h to induce the FLP-mediated recombination of the STOP-Cassette. Thirty to 50 midguts of Dam-only and klu-Dam were dissected in 1× PBS in three different batches and used for genomic DNA extractions. The amplified GATC-sequences and subsequent amplification was done according to the protocol published by Marshall et al.57 until Step 34, from which we continued NGS library
preparation using the Illumina TruSeq nano DNA kit LT. After library quality control, samples were sequenced as 50 bp single-end on an Illumina HiSeq2500.

Midgut FACS RNA isolation and sequencing. For RNA-Seq, UAS-expression of UAS-klu or klu

RNA-RNA isolation was performed using a protocol adapted from the FISH Tag RNA Multicolor kit (Invitrogen Cat. No. MP 32956) using the Sp6 aDNA fragment was synthesized and cloned into a pUCIDT plasmid (IDT).

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The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding authors upon reasonable request. DamID data have been deposited in the GEO database under the accession code: GSE131878. RNA-Seq data have been deposited in the GEO database under the accession code: GSE132243.

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Quantiﬁcative real-time PCR. Quantitative real-time PCR (qRT-PCR) was performed using ampliﬁed RNA from FACS-sorted Esg+ cell populations (see above) as template. cDNA was generated using the QuantTect Reverse Transcription Kit.

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For DamID, we used the damid_seq pipeline42 to generate binding proﬁles for Klu-Dam. Triplicate samples for Klu-Dam (34.9, 33.5, and 34.1 million reads) and Dam-only control (34.7, 34.5, and 35.6 million reads) were aligned to the Drosophila genome (UCSC dnmt). Overall aligning rate was between 86% and 91% across all samples. First, gat.track-maker.pl pipeline used to build a GATC fragment ﬁle. Then the main utility damidseq_pipeline was used to align the reads to the genome using bowtie2, bin and count reads, normalize counts, and compute log2 ratio between corresponding DamID and control Dam-only samples42. The pipeline identiﬁed 1707, 1663, 1681 peaks with FDR < 0.01 per replicate. To test for reproducibility we ﬁrst used the damid_pipeline42 to identify peaks with weaker conﬁdence (FDR < 0.1) and the idr python package (https://github.com/abdely/idr) to identify 1169 peaks with IDR < 0.05 between replicate1 and replicate2. We used an in-house developed script to annotate peaks in proximity to genes. In total, 1667 genes found around reproducible peaks using the FIMO tool61. Reads were visualized using IGV as overlayed triplicate Klu-Dam (log2FC over Dam-only) tracks.

The in-house developed script to annotate peaks in proximity to genes from damid_pipeline data is available in the file Supplementary Data 2.

For RNA-Seq, UAS-expression of UAS-klu or klu

RNA-Seq and DamID data analysis. The 15–21 million quality-passed reads per sample were mapped to the D. melanogaster reference genome (BGDp6) with TopHat2 (version 2.1.0)58. Of each sample, approximately 80% of the reads was mapped to the genome. From this, 90% could be assigned to genes using Fea-

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Author contributions
J.K. and H.J. conceived the project and designed experiments. J.K., S.A., L.A.R.-F., M.B., and E.M. performed experiments and collected data. M.G. helped with optimizing the RNA-Seq library amplification protocol and DamID protocol. T.R.-O. and P.K. performed data analysis on the RNA-Seq and DamID samples. P.S.-V. provided preliminary data for the study. J.K. and H.J. wrote the manuscript.

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