1   **The WT1-like transcription factor**

2   **Klumpfuss maintains lineage commitment**

3   **in the intestine**

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23   **Abstract**

24

25   Stem cell (SC) lineages in barrier epithelia exhibit a high degree of plasticity.
 26   Mechanisms that govern the precise specification of SC daughter cells during
 27   regenerative episodes are therefore critical to maintain homeostasis. One
 28   such common mechanism is the transient activation of the Notch (N) signaling
 29   pathway. N controls the choice between absorptive and entero-endocrine cell
 30   fates in both the mammalian small intestine and the *Drosophila* midgut, yet
 31   how precisely N signaling promotes lineage restriction in progenitor cells
 32   remains unclear. Here, we describe a role for the WT1-like transcription factor
 33   Klumpfuss (Klu) in restricting the fate of *Drosophila* enteroblasts (EBs)
 34   downstream of N activation. Klu is transiently induced in Notch-positive EBs
 35   and its transient activity restricts cell fate towards the enterocyte (EC) lineage.
 36   Transcriptomics and DamID profiling show that Klu suppresses
 37   enteroendocrine (EE) cell fates by repressing E(Spl)m8-HLH and Phyllopod,
 38   both negative regulators of the proneural gene Scute, which is essential for
 39   EE differentiation. At the same time, Klu suppresses cell cycle genes,
 40   committing EBs to differentiation. Klu-mediated repression of its own
 41   transcription further sets up a negative feedback loop that ensures temporal
 42   restriction of Klu-mediated gene regulation, and is essential for subsequent
 43   differentiation of ECs. Our findings define a transient cell state in which EC
 44   lineage restriction is cemented, and establish a hierarchy of transcriptional
 45   programs critical in executing a differentiation program downstream of initial
 46   induction events governed by N signaling.

47

48 Introduction

49 In many tissues, somatic SCs respond to tissue injury by increasing their
50 proliferative potential and producing new differentiating cell progeny. To
51 maintain homeostasis during such periods of regeneration, cell specification
52 and differentiation need to be precisely coordinated within a dynamic
53 environment. Studies in the mammalian intestine have demonstrated a
54 surprising plasticity in such specification events, showing that even
55 differentiated cells can revert into a stem cell state in conditions in which
56 tissue homeostasis is perturbed ^{1,2}. These findings highlight the critical role of
57 gene regulatory networks in establishing and maintaining differentiated and
58 committed cell states in homeostatic conditions.

59 The *Drosophila* midgut is an excellent model to study lineage differentiation of
60 adult stem cells both in homeostasis as well as during regeneration and
61 aging. The *Drosophila* midgut is maintained by intestinal stem cell (ISCs),
62 which can generate differentiated enterocytes (EC) or entero-endocrine (EE)
63 cells ^{3,4}. Upon injury or infection, ISC proliferation is dramatically increased in
64 response to mitogenic signals from damaged enterocytes ⁵⁻⁷. Mis-regulation
65 of cell specification and differentiation in this lineage can lead to significant
66 dysfunction, as evidenced in aging intestines, where disruption of normal N
67 signaling due to elevated Jun-N-terminal Kinase (JNK) signaling leads to an
68 accumulation of mis-differentiated cells that contribute to epithelial dysplasia
69 and barrier dysfunction ^{8,9}.

70

71 Notch signaling plays a central role in both ISC proliferation and lineage
72 differentiation. ISCs produce the Notch-ligand Delta and activate Notch in the

EB daughter cell. Levels of Delta vary markedly between ISCs in the homeostatic intestine. These differences have been proposed to underlie the decision between EC and EE differentiation in a specific ISC lineage¹⁰. It has been proposed that higher N activity is associated with differentiation into the EC fate, while lower N activity promotes EE differentiation^{10,11}. Loss of N in ISC lineages leads to the formation of tumors that consist of highly Delta-expressing ISCs and of Prospero (Pros)-expressing EEs^{10,12,13}. These tumors are likely a consequence of impaired EB differentiation, resulting in an increased frequency of symmetric divisions, as well as of differentiation of a subset of EBs into EE, suggesting that EE differentiation is a default fate in the lineage when N signaling activity is reduced.

Interestingly, recent work has shown that lineage specification in ISC daughter cells is likely more complex than a simple model in which EB fates are determined stochastically or by ‘lateral-inhibition’ – like N-mediated mechanisms. It has been shown that pre-determined ISCs exist that express the EE marker Prospero and generate daughter cells that differentiate into EEs^{14,15}.

The exact cell state in which the decision between EE and EC fates is cemented, however, remains unclear. A transient specification step has been identified in EE differentiation, in which cells transiently express Scute, a transcription factor that negatively regulates N responsive genes such as E(Spl)m8, as well as its own expression¹⁶. Furthermore, EBs have been shown to remain in a transient state for a prolonged period of time before differentiating into an EC fate¹⁷. Mechanisms that regulate and maintain this transient state remain unclear.

98

99 Here we describe a role for the Wilms' Tumour 1 (WT1) – like transcription
 100 factor Klumpfuss (Klu) in lineage commitment during EC differentiation in the
 101 adult fly intestine. In transcriptome studies of FACS sorted ISC and EBs, we
 102 found Klu to be expressed in an Esg-dependent manner specifically in EBs.
 103 Klu is critical for leg and bristle development, as well as for lineage
 104 differentiation of Type II neuroblast stem cells^{18,19}. Klu is related to the
 105 mammalian tumor suppressor gene Wilms' Tumour 1 (WT1), and its
 106 overexpression in neuroblast stem cells leads to tumorous overgrowths that
 107 can grow autonomously when transplanted in the abdomen of flies^{20,21}. In the
 108 intestine, we find that loss of Klu leads to aberrant entero-endocrine
 109 differentiation of EB cells, whereas ectopic activation of Klu results in a failure
 110 to differentiate. Transcriptomics and DNA-binding studies reveal that Klu
 111 controls EE differentiation by repressing genes involved in Notch signaling, as
 112 well as by indirectly controlling the levels of the Achaete-Scute complex
 113 members *asense* and *scute*. Klu further represses its own expression,
 114 defining a transient state of EBs in which specification into ECs is cemented
 115 by precise temporal regulation of N signaling. We propose that the transient
 116 expression of Klu 'locks in' the EC fate in EBs, preventing ectopic proneural
 117 gene activation and thus ensuring lineage commitment into the EC fate.

118

119 Results

120 The transcription factor Klu is expressed in the enteroblast precursor 121 cells

122 We identified Klumpfuss (Klu) transcripts to be specifically enriched in
123 transcriptomes isolated from sorted progenitor cells²² and to be significantly
124 downregulated upon loss of the stem and progenitor specific transcription
125 factor Escargot (Esg)²³. To confirm and better characterize *klu* expression in
126 the *Drosophila* posterior midgut, we used a *klu-Gal4*, *UAS-GFP* reporter line
127 that reflects Klu expression in the wing and eye discs of wandering 3rd instar
128 larvae^{18,24}. In the midgut, GFP expression was seen in the larger cells of the
129 stem-progenitor nests (ISC+EB). These cells resemble EBs based on both
130 nuclear and cellular size (Figure 1A arrowheads). To confirm their identity, we
131 combined the Klu reporter line with the Notch activity reporter *Su(H)GBE-lacZ*,
132 which is exclusively activated in EBs, but not in ISCs¹⁰. In addition, we used
133 *Delta-lacZ* (*DI-lacZ*) as a marker for ISCs. The expression of *klu-Gal4*, *UAS-*
134 *GFP* overlapped almost exclusively with the EB-specific *Su(H)GBE-lacZ*
135 reporter. In contrast, ISC-specific Delta-lacZ staining was mostly found in
136 small, diploid cells neighboring the GFP-positive cells (Figure 1 B, C,
137 quantification in D, E).

138 Lineage tracing experiments have previously shown that Notch-positive EB
139 precursor cells exclusively give rise to enterocytes, whereas Delta-positive
140 ISCs can give rise to clones with both ECs and EEs^{14,15}. To trace the fate of
141 Klu-expressing cells, we crossed the *klu-Gal4* enhancer-trap line to a Actin
142 promoter-driven FlipOut lineage tracing cassette (*UAS-GFP*, *tub-*
143 *Gal80ts*; *UAS-Flp*, *Act>STOP>Gal4*). Upon temperature shift, the UAS-Flp-

144 inducible *Act>STOP>Gal4* will be activated, marking all cells that express Klu
145 as well as all differentiated progeny from these cells. As expected, *DI-Gal4*
146 expressing ISCs give rise to both ECs (visible as large GFP-positive cells with
147 a large, polyploid nucleus) as well as EEs, marked by expression of the
148 transcription factor Prospero (Pros) (Figure 1F, arrows). In contrast, Notch-
149 positive EBs (*Su(H)GBE-Gal4*) only gave rise to ECs, but not EEs (Figure 1G,
150 arrowheads). Similar to Notch-positive EBs, *klu-Gal4*-traced cells gave rise
151 exclusively to ECs, but not EEs (Figure 1H). We conclude that Klu is
152 specifically expressed in the EC-generating EBs in the *Drosophila* midgut.

153

154 **Klu loss of function leads to excess EE differentiation**

155 To determine the role of Klu in the specification and/or differentiation of cells
156 in the ISC lineage, we first inhibited Klu function using RNAi. We used the
157 TARGET-system to express UAS-driven RNAi constructs in specific lineages
158 in a temperature-dependent manner²⁵. We used the *esg-Gal4^{ts}* driver to
159 express Klu RNAi in ISCs and EBs and the *Su(H)GBE-Gal4^{ts}* driver that drives
160 expression in EBs only. In both conditions, knockdown of Klu lead to an
161 increase of EEs in the posterior midgut (Figure 2A-D, quantification in E). To
162 confirm the EE differentiation phenotype, we used Mosaic analysis with a
163 repressible cell marker (MARCM)²⁶ to generate marked lineages
164 homozygous for a null allele of Klu, *klu^{R51}*¹⁸ and traced the fate of *klu^{R51}*
165 mutant cells. Quantification showed that loss of Klu leads to more EE
166 cells/clone (Figure 2F-I). Interestingly, the GFP-negative tissue also contained
167 more EEs in *klu^{R51}* MARCM animals than in the control animals (*FRT2A*,
168 Figure 2F, compare with 2G). This is likely due to the fact that in this

169 genotype, the GFP-negative tissue is heterozygous for the *klu*^{R51} null allele. In
 170 addition, we generated stem cell clones expressing *klu*^{RNAi} using the *escargot*
 171 promoter-driven FlipOut system (*esg-F/O*). Enterocyte differentiation still
 172 occurred in *esg-F/O* > *klu*^{RNAi} clones based on nuclear size of cells within the
 173 clones and staining for the enterocyte marker Pdm1^{23,27} (Figure S1).
 174 In summary, these results indicate that loss of Klu function alters the EE-to-
 175 EC ratio, but does not impair EC differentiation.

176

177 **Ectopic Klu expression inhibits stem cell differentiation and impairs**

178 **Delta-Notch signaling**

179 We hypothesized that constitutive Klu expression would impair EE
 180 differentiation. To test this, we used the *esg-F/O* system to express full-length
 181 Klu in ISC-derived clones. Wild-type *esg-F/O* clones take up most of the
 182 posterior midgut 2 weeks after induction, containing a mixture of ECs and EEs
 183 (Figure 3A). In contrast, clones expressing full-length Klu did not exhibit any
 184 hallmarks of differentiation into either EEs or ECs, but contained only a few
 185 small, round cells (Figure 3B). Klu is thought to act mainly as a repressor of
 186 transcription, based on studies in neuroblast stem cells and in sensory organ
 187 precursor (SOP) cells in the developing wing^{20,24,28}. To ask whether this
 188 repressor function of Klu would elicit the phenotypes observed, we expressed
 189 the zinc-finger DNA-binding domain of Klu fused to either a VP16 activation
 190 domain (Klu-VP16) or fused to the repressor domain from Engrailed (Klu-
 191 ERD)²⁸. Whereas differentiation still occurred in clones expressing the
 192 activating Klu-VP16, differentiation was significantly impaired in clones
 193 expressing the repressing Klu-ERD, confirming that transcriptional repression

194 of genes regulated by Klu is sufficient to impair differentiation (Figure 3C-D,
195 quantification in E).

196 Together with the temporally restricted endogenous expression pattern of Klu,
197 these data indicate that Klu is acting in early EBs to restrict EE differentiation,
198 but that it has to be downregulated and that its sustained expression keeps
199 cells in an undifferentiated state. To specify this state, we combined *UAS-Klu*
200 with the ISC-marker *DI-lacZ* and the EB-marker *Su(H)GBE-lacZ*. Interestingly,
201 *esg-F/O* clones expressing *UAS-klu* did not stain positive for either *DI-lacZ* or
202 *Su(H)GBE-lacZ*. This suggests that Klu expression promotes an exit from the
203 (DI^+) stem cell state, but also interferes with transcriptional programs induced
204 by Delta-Notch signaling in EBs. To further investigate the interaction between
205 Notch signaling and Klu activity in ISC differentiation, we performed epistasis
206 experiments: the formation of large tumors consisting of proliferating ISCs and
207 Pros-positive EEs in N loss of function conditions could be prevented by
208 expression of Klu (*UAS-klu* expression in N^{RNAi} *esg-F/O* clones), which
209 resulted in a complete block of both cell proliferation and EE differentiation
210 (Figure S2). This suggests that Klu acts downstream of Notch in EC
211 differentiation, but additionally acts as a potent inhibitor of cell proliferation.

212 We further combined expression of Klu (*UAS-Klu*) with expression of the
213 oncogenic *Ras*^{V12} variant (*UAS-Ras*^{V12}) in *esg-F/O* clones. Whereas *esg-F/O*>
214 *Ras*^{V12} clones occupy the entire posterior midgut 2 days after induction and
215 contribute to a significant loss of viability of the animal, co-expression of *UAS-*
216 *klu* markedly reduced clonal size at this timepoint and rescued viability (Figure
217 S3).

218 Altogether, our results indicate that the N-mediated transient expression of
219 Klu in EBs is critical to restrict lineage commitment to the EC fate and inhibit
220 proliferation, but that normal differentiation can only proceed once Klu is
221 downregulated. To test this hypothesis, and to better understand the
222 mechanism by which transient Klu expression controls EB cell fate, we
223 decided to explore the progenitor cell-specific transcriptional program
224 downstream of Klu.

225

226 **Transcriptome profiling supports a role for Klu in regulating Notch** 227 **signaling and EE fate repression**

228 To gain a comprehensive overview of the genes controlled by Klu in the
229 intestine, we performed RNA-Sequencing on biological triplicate populations
230 of FACS sorted Esg-positive progenitor cells in which we expressed either a
231 *klu^{RNAi}* construct or *UAS-klu* (see Figure 4A, Supplementary Figure S5 and
232 Methods for details). To perform these studies, we used an approach
233 previously described in ²⁹. We first confirmed that the transcriptome of sorted
234 cells indeed reflects the excess EE differentiation phenotype seen in *klu^{RNAi}*
235 animals by performing qRT-PCR for *prospero* (*pros*) and *scute* (*sc*). The EE
236 marker *pros* is upregulated 5-fold upon *klu^{RNAi}* (Figure 4B). The proneural
237 transcription factor Scute (*sc*) is necessary and sufficient for EE generation in
238 the *Drosophila* midgut ^{14,30,31} and many upstream factors impinge on the
239 expression of Sc to regulate EE differentiation ³². mRNA levels of *sc* increase
240 ~2.5-fold upon *klu^{RNAi}* animals and *UAS-klu* expression completely abolishes
241 *sc* mRNA expression in stem-progenitor cells (Figure 4B).

242 In addition, we checked the mRNA levels for Klu to verify knockdown and
 243 overexpression efficiency. As expected, we see a 70% reduction in mRNA
 244 levels upon *klu*^{RNAi}. Surprisingly, we found that the expression of *klu* mRNA in
 245 *UAS-klu*-expressing progenitor cells was almost completely abolished
 246 compared to control (Figure 4C). This was contrary to our expectation of
 247 detecting increased expression upon Gal4/UAS-mediated overexpression, but
 248 was explained by the fact that the *UAS-klu* construct does not carry the
 249 endogenous *klu* 3'UTR, which our primers targeted. Accordingly, primers that
 250 target the coding region of *klu* (*klu* CDS) readily detect a ~12-fold upregulation
 251 of *klu* transcript. Hence, while transgenic Klu was induced as expected, the
 252 endogenous Klu gene was repressed, indicating that Klu may repress its own
 253 expression. The notion of a negative autoregulatory loop was confirmed in our
 254 RNA-seq data, as we saw increased reads in the CDS of the gene, and no
 255 reads in the 3' UTR (Figure S5).

256 Comparing the transcriptomes of wild-type progenitors with the experimental
 257 samples, we found 410 genes significantly upregulated in *klu*^{RNAi} and 809
 258 genes significantly downregulated in *UAS-klu*-expressing *esg*⁺ cells (*P*_{adj} <
 259 0.05, log₂FC > 0.5 or < -0.5). Given the role of Klu as a repressor of
 260 transcription, we focused our RNA-Seq data analysis on genes that would be
 261 upregulated in the absence of Klu, but downregulated upon *UAS-klu*
 262 expression in *Esg*-positive stem-progenitor cells (Figure 4D). In this category
 263 of 81 genes, many genes involved in the regulation of Notch signaling (the
 264 HES/*E(Spl)*- complex genes *m6*, *m7*, *m8*, and the HES-like transcription factor
 265 Deadpan), as well as several previously described regulators of EE
 266 differentiation (encoding the proneural proteins Asense (*ase*), Scute (*sc*), and

the adaptor protein Phyllopod, *phyl*) could be identified (Figure 4E). Additional *E(Spl)* genes (*E(Spl)-mδ* and *E(Spl)-mγ*) were significantly upregulated in *klu*^{RNAi} samples, but did not significantly change in *UAS-klu* samples (Figure 4E). *E(Spl)*-genes are a group of genes activated by Notch that mediate its downstream transcriptional response³³. *Phyl*, in turn, acts to destabilize Tramtrack (*ttk*), a strong repressor of the *achaete-scute* complex genes *scute* and *asense*, and loss of which leads to a dramatic increase in EE numbers^{32,34}. Reciprocally, loss of *phyl* stabilizes Ttk and results in a complete loss of EE cells in the midgut³⁵. The induction of *phyl* in *klu* loss of function conditions thus explains the increase in EEs.

We also found that the expression of the transcription factor Charlatan (*chn*) is significantly downregulated by *UAS-klu* expression. Chn is a transcription factor that positively regulates the proneural genes Achaete and Scute and loss of Chn in the midgut leads to proliferation and differentiation defects in the stem-progenitor compartment^{36–38}. Hence, Klu represses the expression of several genes that have reported roles in EE differentiation.

Our transcriptome data also revealed transcriptome changes downstream of Klu that may explain the Klu-induced exit from the stem cell state: stem cell maintenance is dependent on the Class I bHLH-family member Daughterless (*Da*)/E47-like, since loss of *da* results in loss of ISC fate and differentiation into enterocytes³¹. The gene *miranda* (*mira*) is a *Da*/proneural target gene that is also highly expressed in ISCs^{31,38}. Proneural factors such as Ase and Sc require *Da* to dimerize and regulate transcription³⁹. We found that loss of Klu results in a slight but significant upregulation of *mira* mRNA, whereas Klu overexpression results in a 2.3-fold downregulation (Figure 4E). To confirm

292 this, we used the *mira-Promoter-GFP* (*mira-GFP*) line and combined this with
 293 *klu^{RNAi}* and *UAS-klu*. Confocal microscopy and FACS-sorting of cells
 294 expressing either *klu^{RNAi}* and *UAS-klu* confirmed that *UAS-klu* expression
 295 could reduce *mira-GFP* levels whereas a slight induction is seen in *klu^{RNAi}*
 296 cells (Figure 4F-I).

297 Taken together, we conclude that Klu has three main functions in EBs: 1) To
 298 turn off the Notch transcriptional response by repressing E(Spl)-gene
 299 expression, 2) to promote the exit from the stem cell state by repressing
 300 genes like *mira*, and 3) To repress aberrant EE differentiation in the EB by
 301 repressing proneural gene activation. By responding to N activity and
 302 repressing its own expression, Klu further serves as a ‘timer’ for N-mediated
 303 transcriptional responses in EBs.

304

305 **Klu acts upstream of the transcription factor Scute in EE differentiation**

306 Our genetic and transcriptional profiling experiments suggest that Klu likely
 307 acts downstream of Notch, but upstream of the proneural genes Ase and Sc
 308 in repressing EE differentiation (Figure 3, Figure 4, Figure S2). Scute plays a
 309 critical role in a transcriptional loop that regulates both ISC proliferation and
 310 the initiation of EE differentiation¹⁶. Our data indicate that Klu can act to
 311 inhibit both proliferation and EE differentiation by affecting factors that
 312 genetically act upstream of Scute (Figure 3 and Figure 4). Therefore, we
 313 performed epistasis experiments with Klu and Sc to confirm this hypothesis.
 314 We generated *esg-F/O* clones that express *klu^{RNAi}* in the presence or absence
 315 of *sc^{RNAi}*. Clones expressing *klu^{RNAi}* contained more EE cells compared to
 316 control clones (Figure 5A-B), whereas clones expressing *sc^{RNAi}* are almost

completely devoid of EE cells (Figure 5C). The combination of *klu*^{RNAi} and
sc^{RNAi} also resulted in clones with little or no EE differentiation, as quantified
by the number of Pros-positive cells (Figure 5D, quantification in 5E). This
suggests that the appearance of extra EE cells in *klu*^{RNAi}-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 *esg-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 (Figure 5F, Figure S6A-D). Co-expression
of Klu and Scute leads to a marked reduction in clone size (Figure S6F) 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
(Figure 5F). 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
esg-F/O > UAS-klu+UAS-sc clones (Figure S6E). Since the clonal size in this
genotype is no larger than in *esg-F/O>UAS-klu* single overexpression clones
(Figure S6F), indicating that these cells might be arrested in mitosis. This
suggests that although Klu expression cannot completely repress *UAS-Sc*-
induced EE differentiation, the effect of Klu on cell cycle progression interferes
with the proliferation-inducing capacity of Scute.

342 **Klu directly represses several genes that regulate EE fate, Notch** 343 **signaling and the cell cycle**

344 To identify genes that are directly regulated by Klu, we performed targeted
345 DamID of Klu in esg-positive stem-progenitor cells ⁴⁰. We used the DamID-
346 seq pipeline ⁴¹, see Methods) to identify 1667 genes that had 1 or more Klu
347 binding peak(s) within 2 KB of their gene body in all 3 replicates. Using 2
348 published position weight matrices for Klu-binding ⁴², we could establish that
349 692 of the 1667 genes (41.5%) had 1 or more Klu-binding motif(s) present in
350 their binding peaks. We considered these peaks as high-confidence Klu-
351 bound sites. Our transcriptomics data on Klu indicated that Klu controls many
352 genes involved in Notch signaling, EE differentiation and cell cycle regulation.
353 We identified a cluster of binding sites at the centrosomal end of the *E(Spl)*-
354 locus around the *E(Spl)-mδ* and *E(Spl)-mγ* genes (Figure 6E). Since our
355 RNA-Seq data showed that many of the *E(Spl)*-genes change expression in
356 both *klu*^{RNAi} and *UAS-klu* conditions (Figure 4E), this suggests that Klu could
357 possibly regulate the expression of multiple members of the *E(Spl)*-complex
358 through this binding peak at the centrosomal end of the *E(Spl)*-locus.
359 Furthermore, we identified a Klu-Dam binding peak at the *klu* locus,
360 supporting our hypothesis that Klu acts in an autoregulatory loop by
361 negatively regulating its own expression (Figure 6A). Previous work has
362 shown that Scute and the *E(Spl)*-complex member *E(Spl)m8-HLH* act in a
363 regulatory loop to generate an EE precursor directly from the ISC ¹⁶. Since
364 our results indicate that Scute is upregulated upon loss of Klu and acts
365 epistatically to Klu in EE formation, we first looked for Klu binding in and
366 around the *scute* locus. We did not find significant 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 (Figure 6B). Together with the adaptor protein Phyllopod, the Sina and Sinah E3-ubiquitin ligases are able to degrade the transcriptional repressor Tramtrack (*ttk*), which represses EE fate^{32,35}. *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 (Figure 4E). 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 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 (Figure 6C and D), 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^{43,44}. We propose that Klu plays a role in remodeling the cell cycle in response to Notch activation by directly repressing 2 critical cell cycle regulators. Furthermore, this explains how Klu acts as a potent suppressor of cell proliferation, even in combination of the strongly oncogenic RasV12 overexpression (Figure 3 and Figure S3).

391 Altogether, our data suggest a model (Figure 6G) in which Klu acts as a Notch
 392 effector in the EB that acts to restrict the duration of the Notch transcriptional
 393 response (through negative regulation of the E(Spl)-complex members and
 394 Klu itself). Second, Klu prevents activation of the Scute-E(Spl)m8
 395 transcriptional circuit that triggers EE differentiation. Finally, we find evidence
 396 that Klu can bind and repress critical cell cycle regulators such as Cyclin B
 397 and Cyclin E, likely promoting the switch from a mitotic to an endoreplicating
 398 cell cycle in differentiating ECs.

399

400

401   **Discussion**

402   Our work identified a mechanism by which lineage decisions are cemented
403   through the coordinated repression of alternative fates and of cell proliferation
404   in somatic stem cell daughter cells. N-induced expression of Klu in EBs is
405   necessary to repress EE fates in EBs, but also to temporally restrict N target
406   gene expression. Hence, its own expression has to be self-regulated to allow
407   differentiation to ECs to proceed. We find that Klu represses several genes
408   that are critical for EE differentiation; most notably genes that influence the
409   accumulation of the transcription factor Scute. Transient expression of Scute
410   is necessary and sufficient for EE differentiation and the transient nature of
411   Scute activation is accomplished by a double-negative feedback loop between
412   Scute and E(Spl)m8¹⁶. The expression of Klu results in the repression of both
413   transcription factors in EBs, inactivating the transcriptional circuit that governs
414   EE differentiation (Figure 6F). Previous work on Klu has shown that Klu is
415   directly regulated by Su(H) and acts as a Notch effector in hemocyte
416   differentiation⁴⁵. In the ISC lineage, we find that overexpression of Klu results
417   in the loss of Notch signaling activity in progenitor cell clones, and that Klu is
418   able to repress several Notch effector genes (such as the HES/E(Spl) family
419   and HES/E(Spl)-like genes such as Deadpan). We thus propose that Klu acts
420   in a negative feedback loop downstream of Notch signaling to ensure that
421   Notch effector gene activity is transient in EBs, mirroring the transient nature
422   of EE specification by Scute and E(Spl)m8.

423   Loss of WT1 in the mouse kidney results in glomerulosclerosis and is
424   accompanied by ectopic expression of HES/E(Spl) family genes⁴⁶ and in
425   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*⁴⁷. This suggests that the negative feedback between Notch and its effector Klu/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 *N^{RNAi}* or oncogenic *Ras^{V12}*-induced tumors and our DamID binding studies suggest that Klu can directly regulate Cyclins B and E. This 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 resulting formation of brain tumors that can be transplanted to distant regions of the body and continue to grow^{20,21}. However, this likely reflects the different role for Notch in the NB lineage, where continuous activation of Notch also 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. This is reminiscent of the context-specific rules for Notch as either a tumor suppressor or an oncogene in mammalian adult stem cell lineages. For instance, activating Notch1 mutations are the main driver of T-cell acute lymphoblastic leukemia⁴⁸, whereas Notch-inactivating mutations are found in many epithelial-derived solid tumor types such as head-and-neck or skin carcinomas (see⁴⁹ and references therein).

Similarly, *Wt1* was initially identified as a tumor-suppressor gene mutated in the rare pediatric kidney cancer Wilms' Tumor⁵⁰. Recently, WT1 was also identified as a strong inhibitor of proliferation in a genome-wide gain-of-

451 function screen for tumor suppressors and oncogenes⁵¹. However,
 452 expression of WT1 was found to be elevated in many solid tumors and in
 453 acute myeloid leukemia^{52,53}. Thus, similar to Notch, the role of WT1 in
 454 tumorigenesis seems to be highly context-dependent. Intriguingly, in both
 455 nephric and hematopoietic lineages WT1 is often transiently expressed in
 456 committed progenitor cells, similar to the expression of Klu in the EB
 457 progenitors, raising the possibility that to fully understand the role of WT1-like
 458 proteins in tumorigenesis, cell lineage relationships, as well as cell
 459 proliferation and differentiation events in tumors need to be taken into
 460 account.

461 Critically, our work highlights the role for transient transcriptional ‘rewiring’
 462 events during cell specification in somatic stem cell lineages. Such events
 463 seem to be required to ensure lineage commitment downstream of initial
 464 symmetry breaking signals like Notch, and ensure progression of cell
 465 differentiation into a defined lineage. As such, it can be expected that similar
 466 transcriptional feedback loops need to be reversed for cells to undergo de-
 467 differentiation into stem cells in mammalian regenerating tissues. We propose
 468 that understanding their makeup and regulation will significantly advance
 469 efforts to control tissue repair and regeneration in mammals, including
 470 humans.

471 **Methods**

472 **Fly strains and husbandry**

473 The following strains were obtained from the Bloomington Stock Center:

474 BL28731 (*klu* RNAi on 3rd) BL60469 (*klu* RNAi on 2nd), BL56535 (*UAS-*
475 *klu*^[Hto]), BL11651 (*Df*⁰⁵¹⁵¹-*lacZ*) BL26206 (*sc* RNAi), BL51672 (*UAS-sc*),
476 BL1997 (*w*^[*]; *P*{*w*[+*mW.hs*]=*FRT(w[hs])*}2A), BL4540 (*w*^[*]; *P*{*w*[+*mC*]=*UAS-*
477 *FLP.D*}JD2). BL65433 (*y*[1] *w*^[*]; *M*{*w*[+*mC*]=*hs.min(FRT.STOP1)dam*}ZH-
478 51C) BL1672 (*w*[1118]; *sna*[*Sco*]/*CyO*, *P*{*ry*[+*t7.2*]=*en1*}*wg*[*en11*]).
479 VDRC: v27228 (*N* RNAi). Other stocks: *klu-Gal4 UAS-GFP, FRT2A*
480 *kluR51/Tm6B, hs-Flp, Tub-Gal4, UAS-GFP/Fm7;FRT2A,*
481 *TubGal80ts/Tm2,Ubx* (T. Klein, Düsseldorf) *UAS-kluFL, UAS-ERD-kluZF,*
482 *UAS-VP16-kluZF* (C.Y. Lee, U. Michigan) *esg-F/O (w; esg-Gal4, tub-Gal80ts,*
483 *UAS-GFP; UAS-flp, Act>CD2>Gal4(UAS-GFP)/TM6B), esg^{ts} (y,w;esg-Gal4,*
484 *UAS-GFP/CyO;tub-Gal80ts/Tm3), Su(H)^{ts} (w;Su(H)GBE-Gal4,UAS-CD8-*
485 *GFP/CyO;tub-Gal80^{ts}/TM3), ISC-specific esg^{ts} (Wang et al., 2014) w;esg-*
486 *GAL4,UAS-2XEYFP/CyO;Su(H)GBE-GAL80,tub-Gal80ts/TM3,Sb, w;esg-*
487 *gal4, tub-Gal80ts, UAS-GFP/CyO,wg-lacZ;P{w[+mC]=UAS-FLP.D}JD2/Tm6B.*

488 Stocks generated in this study: *w;Klu-Dam(ZH-51C) M4M1/CyO,*

489 *P{ry[+t7.2]=en1}wg[en11]*

490

491 **Immunostaining and microscopy**

492 Midgut immunostaining was performed as described in ⁵⁴. Antibodies used
493 include: Chicken anti-GFP (1:1000, ThermoFisher A10262), mouse anti-
494 Prospero (MR1A, 1:50, DSHB), mouse anti-β-galactosidase (40-1a, 1:200,
495 DSHB), rabbit anti-β-galactosidase (1:200, ThermoFisher A11132) mouse

496 anti-Armadillo (N2 7A1, 1:20, DSHB), rabbit anti-phosphorylated Histone H3-
 497 Ser10 (pH3S10, 1:500, sc8656-R, Santa Cruz Biotechnology). Images were
 498 taken from the R5 and R4 regions of the posterior midgut on a Zeiss Apotome
 499 microscope or Zeiss LSM710 confocal at either 20X or 40X magnification.
 500 Images were captured as Z-stacks with 8-10 slices of 0.22-1.0 μm thickness.
 501 Images were converted to maximum-intensity projections in Fiji (<https://fiji.sc>)
 502 and quantifications were performed using the CellCounter Fiji plugin. Scale
 503 bar = 50 μm in all images, except in Figure 1A: scale bar = 25 μm . Graphing,
 504 statistical analysis and survival curves were produced in GraphPad Prism.

505

506 **Cloning and transgene generation**

507 We used the Inducible DamID system from the Van Steensel lab to generate
 508 *klu-Dam*⁴⁰. To this end, we amplified the Klu Full-length cDNA (derived from
 509 BDGP Gold clone F101015) using *AscI* and *NotI*-containing primers and
 510 cloned the fragment into the vector *p-attB-min.hsp70P-FRT-STOP#1-FRT-*
 511 *DamMyc[open]* (Addgene plasmid #71809). Transgenic lines were generated
 512 by Genetivision Inc. using the ϕC31 integrase-mediated site-specific
 513 transgenesis system⁵⁵. The finished construct was injected into Bloomington
 514 stock BL24482 (*ZH-51C* attP-site on 2nd) and the resulting transgenic lines
 515 were tested by genotyping PCR. Both control (Dam-only, BL65433) and *klu-*
 516 Dam transgenic lines were crossed to BL1672 (*w[1118]; sna[Sco]/CyO,*
 517 *P{ry[+t7.2]=en1}wg[en11]*) before use.

518

519 **DamID**

Control Dam-only (BL65433) and *klu*-Dam male flies were crossed to *w;esg-gal4, tub-Gal80ts, UAS-GFP/CyO, wg-lacZ; P{w[+mC]=UAS-FLP.D}JD2/Tm6B* virgins. Crosses were maintained at 18°C and progeny was shifted to 29°C for 24 hours to induce the Flp-mediated recombination of the STOP-Cassette. 30-50 midguts of Dam-only and *klu*-Dam were dissected in 1X PBS in 3 different batches and used for isolation of total genomic DNA. Isolation of methylated GATC-sequences and subsequent amplification was done according to the protocol published by Marshall et al.⁵⁶ 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.

531

532 **Midgut FACS, RNA-isolation and Sequencing**

Midgut dissociation and FACS was performed as described in²⁹. UAS-expression of *UAS-klu* or *klu*^{RNAi} was induced for 2 days, followed by 16 hours of *Ecc15* infection to stimulate midgut turnover. We dissected 100 midguts/genotype in triplicate and for each sample 20,000-40,000 cells were sorted into RNase-free 1X PBS with 5 mM EDTA. RNA was isolated using the Arcturus PicoPure™ RNA Isolation Kit. Subsequently, the entire amount of isolated RNA was used as input for RNA-amplification using the Arcturus™ RiboAmp™ HS PLUS Kit. 200 ng of amplified aRNA was used as input for RNA-Seq library preparation using the TruSeq Stranded mRNA Library Prep Kit (Illumina) and samples were subsequently sequenced as 50 bp single-end on an Illumina HiSeq2500.

544

545 **Quantitative real-time PCR**

546 Quantitative real-time PCR (qRT-PCR) was performed using aRNA from
 547 FACS-sorted *esg*⁺ cell populations (see above) as template. qRT-PCR was
 548 performed using the TaqMan FAM-MGB system in a 10 µl reaction on a
 549 BioRad CFX384 C1000 Touch Cyclor using the following probes: *klu*
 550 (*dm02361358 s1*), *pros* (*dm02135674 g1*), *sc* (*dm01841751 s1*) and *Act5C*
 551 (*dm02361909 s1*) was used for normalization. The *klu* CDS primer assay was
 552 ordered as a Custom TaqMan Assay. Reactions were performed in triplicate
 553 on 3 independent biological replicates. Relative expression was quantified
 554 using the $\Delta\Delta C_t$ method. Data were calculated using Microsoft Excel and
 555 plotted as relative fold-changes +/- SEM in Graphpad Prism.

556

557 **RNA-Seq and DamID data analysis**

558 The 15-21 million quality-passed reads per sample were mapped to the *D.*
 559 *melanogaster* reference genome (BDGP6) with TopHat2 (version 2.1.0)⁵⁷. Of
 560 each sample, approximately 80% of the reads was mapped to the genome.
 561 From this, 90% could be assigned to genes using FeatureCounts resulting in
 562 11-15 million analysis-ready reads per sample⁵⁸.
 563 The table of raw counts per gene/sample was analyzed with the R package
 564 DESeq2 (version 1.16.1) for differential expression⁵⁹. Both sample groups of
 565 interest (UAS & RNAi) were pair-wise contrasted with the control sample
 566 group (control). For each gene of each comparison, the p-value was
 567 calculated using the Wald significance test. Resulting p-values were adjusted
 568 for multiple testing with Benjamini & Hochberg correction. Genes with an
 569 adjusted p-value <0.05 are considered differentially expressed (DEGs).

570 For DamID, we used the damid_seq pipeline ⁴¹ to generate binding profiles for
571 Klu-Dam. Triplicate samples for Klu-Dam (34.9, 33.5 and 34.1 millions reads)
572 and Dam-only control (34.7, 34.5, and 35.6 million reads) were aligned to the
573 *Drosophila* genome (UCSC dm6). Overall aligning rate was between 86% and
574 91% across all samples. First, 'gat.track.maker.pl' script was used to build a
575 GATC fragment file. Then the main utility 'damidseq_pipeline' was used to
576 align the reads to the genome using bowtie2, bin and count reads, normalize
577 counts, and compute log2 ratio between corresponding DamID and control
578 Dam-only samples. The pipeline identified 1,707, 1,663, 1,681 peaks with
579 FDR<0.01 per each replicate. To test for reproducibility we first used Marshall
580 OJ's damid_pipeline to identify peaks with weaker confidence (FDR< 0.1) and
581 the 'idr' python package (<https://github.com/nboley/idr>) to identify 1,169
582 peaks with IDR<0.05 between replicate1 and replicate2. We used an in-house
583 developed script to annotate peaks in proximity to genes. 1,667 genes found
584 to be in proximity to at least one reproducible peak. To find Klu binding motifs
585 in our reproducible peak set, we scanned for 2 different Klu PWM (described
586 in ⁴²) around reproducible peaks using the FIMO tool ⁶⁰. Reads were
587 visualized using IGV as overlaid triplicate Klu-Dam (log₂FC over Dam-only)
588 tracks.
589

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599

600 **Author contributions**

601 J.K and H.J conceived the project and designed experiments. J.K., M.B. and
602 E.M. performed experiments and collected data. T.R-O. performed data
603 analysis on the RNA-Seq and DamID samples. P.S-V. provided preliminary
604 data for the study. J.K. and H.J. wrote the manuscript.

605

606 **Competing interests**

607 The authors declare no competing financial interests.

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842
843
844

845 **Figure legends**

846 **Figure 1. The transcription factor Klu is specifically expressed in**
 847 **enteroblast cells. A-A'** The *klu-Gal4*, *UAS-GFP* reporter line shows
 848 expression in the midgut epithelium. ISCs (arrows) and EBs (arrowheads) are
 849 visualized by *esg-lacZ*. Cells are outlined with Armadillo/ β -catenin. **B-C.** The
 850 *klu-Gal4*, *UAS-GFP* was combined with *Su(H)-GBE-lacZ* (enteroblast (EB)
 851 marker) or *DI-lacZ* (intestinal stem cell (ISC) marker). Expression of Klu
 852 largely overlaps with the EB marker *Su(H)-GBE-lacZ* (B), and Klu-positive
 853 cells are found adjacent to the Delta-positive ISC (C). **D-E.** Quantification of
 854 marker gene overlap of the genotypes displayed in (B-C) $n = 7$ guts (*DI-lacZ*,
 855 $n = 1370$ cells counted) and $n = 10$ guts (*Su(H)-GBE-lacZ*, $n = 572$ cells
 856 counted). **F-H.** Lineage-tracing of cells in the intestine using different cell-
 857 specific drivers. **F-F'.** The *DI-Gal4*-positive ISCs give rise to both differentiated
 858 cell types of the intestinal lineage (enterocytes (EC) and entero-endocrine
 859 (EE) cells). EEs are marked by antibody staining for the transcription factor
 860 Prospero (Pros, in red). Arrows indicate GFP-Pros double-positive EEs in the
 861 clonal area, whereas arrowheads indicate EEs outside the clonal area. **G-G'.**
 862 *Su(H)-GBE*-positive EB cells exclusively give rise to ECs, but not to EEs
 863 (arrowheads). **H-H'.** Similar to *Su(H)-GBE*-positive EBs, Klu-positive cells also
 864 give rise exclusively to ECs, but not EEs.

865

866 **Figure 2. Loss of Klu leads to excess EE differentiation. A-E.** RNAi-
 867 mediated knockdown of Klu results in an excess of Pros-positive EE-cells.
 868 Expression of *klu^{RNAi}* using either the EB-specific *Su(H)GBE-Gal4^{ts}* driver
 869 (Pros in red, compare A with B) or the ISC+EB driver *esg-Gal4^{ts}* (Pros in

870 green, compare C with D) results in more EE cells in the posterior midgut. **E.**
 871 EE cell quantification of the posterior midgut for the genotypes in **A-D**. Error
 872 bars represent mean $\pm$ S.D. Significance was calculated using Student's t-
 873 test with Welch's correction. Number of midguts $n = 15$ (control w^{1118}) and $n =$
 874 18 (klu^{RNAi}) for experiments with the $Su(H)GBE-Gal4^{ts}$ driver and $n = 12$
 875 (control w^{1118}) and $n = 16$ (klu^{RNAi}) for $esg-Gal4^{ts}$ **F-I**. Clonal analysis of the
 876 klu^{R51} null mutant allele using the MARCM technique. klu^{R51} mutant clones
 877 have more EE cells compared to FRT2A control clones (compare F and G).
 878 **H-I**. Quantification of the number of Pros-positive EEs/clone (H) and the total
 879 number of Pros-positive EEs/ROI for the genotypes in **F-G**. $n = 15$ guts
 880 (FRT2A control) and $n = 17$ guts (klu^{R51}). Error bars represent mean $\pm$ S.D.
 881 Significance was calculated using Student's t-test with Welch's correction.

882

883 **Figure 3. Klu overactivation results in a loss of intestinal stem cell**
 884 **differentiation and Delta-Notch signaling. A-D**. Clonal expression of
 885 different Klu isoforms using the esg -FlipOut ($esg-F/O$) system to generate ISC
 886 clones. **A**. Control $esg-FO$ clones grow to occupy most of the posterior midgut
 887 2 weeks after clonal induction. **B-D**. Clones expressing either full-length Klu
 888 ($UAS-kluFL$, **B**) or the Klu zinc-finger DNA-binding domain fused to the
 889 Engrailed repressor domain ($UAS-ERD-kluZF$, **D**) resulted in a block of
 890 differentiation. This was not observed when expressing the Klu zinc-finger
 891 DNA-binding domain fused to the VP16 transcriptional activator domain ($UAS-$
 892 $VP16-kluZF$, **C**). **E**. Quantification of genotypes in **A-D**. Error bars represent
 893 mean $\pm$ S.D. Significance was calculated using Student's t-test with Welch's
 894 correction. $n = 5$ for each genotype. **F-I**. Klu expression in ISC clones leads to

895 a loss of Notch signaling activity in ISC-EB pairs. **F.** Control clones always
 896 contain 1 or more ISCs that are positive for the Notch ligand Delta (*DI-lacZ*,
 897 red). **G.** *DI-lacZ* staining is absent from clones expressing *UAS-kluFL*. **H.**
 898 Control clones have EB cells that are marked by presence of the Notch
 899 activity reporter *Su(H)-GBE-lacZ* (red). **I.** Notch transcriptional activity is
 900 absent from *UAS-kluFL*-expressing *esg-F/O* clones.

901

902 **Figure 4. Transcriptome profiling indicates that Klu acts to repress**

903 **Notch signaling output and genes critical to EE differentiation. A.**

904 Overview of the experiment: *esg^{ts}* GFP⁺ cells either expressing *klu^{RNAi}* or
 905 *UAS-kluFL* were sorted in triplicate and their transcriptome was compared to
 906 control *esg^{ts}* GFP cells (see Methods for more details). **B.** qRT-PCR analysis
 907 of sorted cells for Klu and the critical EE fate regulators Scute (*sc*) and
 908 Prospero (*pros*). **C.** qRT-PCR analysis of *klu* mRNA expression with a primer
 909 pair that targets the endogenous 3' UTR coding sequence (*klu UTR*) and a
 910 primer pair that targets the coding region only (*klu CDS*). **D.** Overlap of
 911 significantly upregulated genes in *klu^{RNAi}* and significantly downregulated
 912 genes in *UAS-kluFL*. **E.** Volcano plots comparing expression of a selection of
 913 genes from the overlap of 81 genes shown in (D). Genes upregulated in the
 914 *klu^{RNAi}* VS control set (left) are downregulated in the *UAS-klu* VS control set
 915 (right). **F-I.** Klu represses Da-dependent *miraP-GFP* expression in ISC and
 916 EB. Control *miraP-GFP* expression (F) is high in ISCs and EBs and slightly
 917 increased in *klu^{RNAi}* midguts (G). *UAS-kluFL* expression in reduced levels of
 918 *miraP-GFP* (H). **I.** GFP-intensity of *miraP-GFP*-positive cells for the
 919 genotypes in (F-H) by FACS. *n* = 50 midguts per genotype.

920

921 **Figure 5. Scute acts downstream of Klu in the induction of entero-**
 922 **endocrine differentiation. A-D.** Expression of *klu*^{RNAi} lead to increased EE
 923 differentiation in clones 14 days after clonal induction, marked by increased
 924 numbers of Pros⁺-cells (red) (A-B, quantification in E-F). *sc*^{RNAi} clones
 925 showed almost no EE differentiation (C). Similarly, the combination of *klu*^{RNAi}
 926 with *sc*^{RNAi} resulted in clones lacking EE differentiation (D). **E** Quantification of
 927 GFP⁺/Pros⁺ double-positive cells/clone of the genotypes in (A-D). **F.**
 928 Quantification of GFP⁺/Pros⁺ double-positive cells/clone of *esg-F/O* clones
 929 expressing either *UAS-sc*, *UAS-klu* or the combination. See Figure S6 for
 930 images. Error bars represent mean +/- S.D. Significance was calculated using
 931 Student's t-test with Welch's correction. For E: *n* = 10 for control and *sc*^{RNAi}, *n*
 932 = 14 for *klu*^{RNAi}, and *n*=12 for *sc*^{RNAi}; *klu*^{RNAi}. For F: *n* = 5 for control, *n* = 6 for
 933 *UAS-klu*, *UAS-sc* and *UAS-klu*; *UAS-sc*.

934

935 **Figure 6. Klu directly represses genes involved in Notch signaling, cell**
 936 **cycle and EE differentiation. A-E.** Klu-Dam binding tracks (in triplicate) for
 937 the *klu* locus (A), *sina* locus (B), *Cyclin E* (C) and *Cyclin B* (D) as well as the
 938 E(Spl)-complex genes *E(Spl)-mδ* and *E(Spl)-mγ* (E). Tracks are displayed
 939 using Integrated Genome Viewer (IGV) as overlaid tracks of the triplicate
 940 Klu-Dam VS Dam-only control comparisons. Arrows indicate direction of
 941 transcription. Numbers indicate the average maximum height of the peak
 942 (log₂FC of Klu-Dam over Dam-only control) for each of the 3 replicates. **F.**
 943 Model of Klu function in lineage differentiation in the intestine. Klu expression
 944 is activated by Delta-Notch signaling in the EB cell, together with other

945 members of the Hairy/Enhancer of Split family of Notch target genes. Klu
 946 accumulation in EBs results in a subsequent repression of these target genes,
 947 including the repression of its own expression. Additionally, Klu acts as a
 948 safeguard to repress erroneous EE differentiation in the enteroblast-
 949 enterocyte lineage by indirectly repressing the accumulation of proneural
 950 genes such as Asense and Scute through inhibition of the E3-complex
 951 members Phyllopod (*phyl*) and Seven-in Absentia (*sina*) that repress the
 952 accumulation of Scute and thereby inhibit EE fate. Finally, Klu acts in the
 953 regulation of the cell cycle in EB cells, as the cell remodels its cell cycle from
 954 a mitotic to an endocycle.

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Figure 2 Korzelius et al.

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Figure 5 Korzelius et al.

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