1   **Dynamic adult tracheal plasticity drives stem cell adaptation to changes in intestinal**

2   **homeostasis.**

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13   **Abstract**

14   Coordination of stem cell function by local and niche-derived signals is essential to

15   preserve adult tissue homeostasis and organismal health. The vasculature is a prominent

16   component of multiple stem cell niches. However, its role in adult intestinal homeostasis

17   remains largely understudied. Here, we uncover a previously unrecognised crosstalk between

18   adult intestinal stem cells (ISCs) in *Drosophila* and the vasculature-like tracheal system, which

19   is essential for intestinal regeneration. Following damage to the intestinal epithelium, gut-

20   derived reactive oxygen species (ROS) activate tracheal HIF-1α and bidirectional FGF/FGFR

21   signaling, leading to reversible remodelling of gut-associated terminal tracheal cells and ISC

22   proliferation following damage. Unexpectedly, ROS-induced adult tracheal plasticity involves

23   downregulation of the tracheal specification factor *trachealess* (*trh*) and upregulation of IGF2

24   mRNA-binding protein (IGF2BP2/Imp). Our results reveal a novel intestine/vasculature inter-

25   organ communication program, which is essential to adapt stem cells response to the

26   proliferative demands of the intestinal epithelium.

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28   **Main**

29   Adult intestinal plasticity is largely owed to the action of stem cells, which must respond

30   to constant signals from the intestinal epithelium and its microenvironment, to fulfil global

31   tissue demands[1-3]. Surprisingly, little is known about the role of the vascular microenvironment

32   in adult intestinal homeostasis.

33   The *Drosophila* tracheal system is an oxygen-delivering interconnected tubular

34   network, functionally analogous to the mammalian vascular and respiratory systems[4].

35   Following specification from epidermal cells and the formation of a tracheal sac in the embryo,

36   tracheal cells undergo extensive cell rearrangements and cell shape changes, leading to the

37   formation of multicellular tubes that ramify into progressively thinner branches, culminating

38   with a terminal tracheal cell[5]. *Drosophila* terminal tracheal cells (TTCs), analogous to

39   mammalian vascular tip cells[6], extend prominent cytoplasmic projections, which supply

40   oxygen to their target tissues[4, 5, 7, 8]. While tracheal development and post-embryonic plasticity

41   have been significantly studied in *Drosophila* [5, 9, 10], there is scarce knowledge on the role and

42   regulation of the adult tracheal system.

43   The adult *Drosophila* midgut shares remarkable homology with the mammalian

44   intestine[11]. Critically, the midgut epithelium is maintained by intestinal stem cells (ISCs), which

45   self-renew and replenish the differentiated intestinal lineage —secretory enteroendocrine cells

46   and absorptive enterocytes— through the production of undifferentiated enteroblasts[12, 13]. As

47   its mammalian counterpart, the *Drosophila* gastrointestinal tract is densely tracheated[14].

48   Beyond the requirement for tracheal derived Dpp/BMP to restrain ISC proliferation[15], there is

49   no knowledge on the role of the tracheal system in adult midgut biology.

50   Here, we combine genetics and image analysis with *in vivo* functional and molecular

51   studies to characterise a novel inter-organ communication program between the adult

52   *Drosophila* midgut and its closely associated tracheal tissue, which is essential to shape stem

53   cell and tracheal plasticity during intestinal regeneration.

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Results

Intestinal damage leads to dynamic and reversible remodelling of gut associated adult TTCs in *Drosophila*.

The adult *Drosophila melanogaster* gut is extensively covered by TTCs, labelled with a *GAL4* reporter driven under the control of the *Drosophila* homologue of Serum Response Factor (dSRF)[16-18] (*dSRF>GFP*) (Fig.1a and Extended data Fig.1a). Transmission electron microscopy of adult posterior midguts denoted intimate contact between TTCs, enterocytes (ECs) (Fig.1b) and ISCs (Fig.1c). Oxygen and nutrient availability are two recognised determinants of TTC plasticity[10, 19]. We noticed that, damage to the adult *Drosophila* midgut epithelium caused by feeding animals with the pathogenic bacteria *Pseudomonas entomophila* (*Pe*)[20-23], the DNA-damaging agent Bleomycin[24-26], or the epithelial basement membrane disruptor Dextran Sulfate Sodium (DSS)[25, 27], led to a significant increase in TTC coverage within the posterior midgut (Fig.1d, e and Extended data Fig.1b). Consideration of gut resizing upon *Pe* damage did not impact the overall increase in tracheal coverage (Extended data Fig.1c-e). GFP labelled single terminal tracheal cell clones, unambiguously confirmed the increase in total number of cellular branches derived from individual TTCs in damaged (*Pe*) versus control (Sucrose) midguts (Fig.1f, g). Interestingly, observation of single TTC clones within *Pe* treated midguts revealed direct correlation between the number of individual TTC branches and nearby PH3^{+} ISCs (Fig.1h). Further quantification of tracheal phenotypes showed increase in primary, secondary and tertiary tracheal branches and total length of individual TTC extensions in damaged (*Pe*) versus control (Sucrose) midguts (Fig.1i-k). Quantification of TTC numbers or assessment of potential co-localization between TTC bodies and the cell proliferation marker PH3 revealed no evidence of TTC proliferation following midgut damage (Extended data Fig.1f, g). Collectively, these data suggest extensive cellular remodelling of TTCs in response to epithelial intestinal injury. A time course assessment of posterior midguts over a 16 hrs period of *Pe* infection (Damage phase) followed by 32 hrs on normal diet (Recovery phase) revealed direct correlation between tracheal

84   coverage and ISC proliferation (Fig.1l, m and Extended data Fig.1h, i). These results strongly

85   suggest that adult gut-associated-tracheal remodelling is a highly dynamic and reversible

86   process, which accompanies changes in intestinal homeostasis.

87   Low doses of whole body ɣ-irradiation in mice induce intestinal epithelial cell death,

88   followed by a strong peak of crypt cell proliferation between 72- and 96 hrs after irradiation[28].

89   Staining with anti-CD31, showed an increase in vascular endothelial cells in regenerating

90   (irradiated) versus control (non-irradiated) intestinal crypts (Fig.1n). These results indicate a

91   conserved phenomenology of vasculature/tracheal response to damage in the adult intestinal

92   epithelium.

93

94   **TTC remodelling is necessary for ISC proliferation following damage of the adult**

95   ***Drosophila* midgut**

96   An essential step in the intestinal regenerative response to damage, involves a robust

97   increase in ISC proliferation[1, 20 22, 25, 29] (Fig.1d, h). To address the functional role of the tracheal

98   system in adult intestinal regeneration, we severely reduced trachea by overexpressing the

99   pro-apoptotic gene *bax* (*UAS-bax*) in adult TTCs using temperature sensitive *dSRF-Gal4*

100 (*dSRF^{ts}>bax*) (Fig.2a, b). This caused a significant impairment of the regenerative response

101 of the intestine to multiple damages, as evidenced by an approximate 50% decrease in

102 damage induced ISC proliferation (Fig.2c). TTCs are best known for their role facilitating gas

103 exchange with their target tissues[4]. Thus, poor intestinal regeneration following TTC reduction

104 might reflect the need of oxygen in this process. Exposing animals to high and prolonged

105 hypoxic environmental conditions, induced activity of a reporter of *Drosophila* Hypoxia-

106 inducible factor-1α (HIF-1α/Sima)[30] and TTC remodelling in the adult midgut (Extended data

107 Fig.2a-c). However, while hypoxia clearly impaired damage induced ISC proliferation in the

108 adult midgut (Fig.2d), it did so to a lower extent than that observed upon TTC loss

109 (*dSRF^{ts}>bax*) (Fig.2c). This difference could be due to a compensatory effect of increased

110 trachea upon hypoxia (Extended data Fig.2a, c) or compromised ISC survival in *dSRF^{ts}>bax*

111 midguts. We assessed apoptosis and ISC numbers in hypoxic and *dSRF^{ts}>bax* midguts

112 through anti-caspase (Dcp-1) staining and the use of an ISC reporter (*Delta-LacZ*). Midguts

113 overexpressing *bax* in adult ECs (*NP1*^{ts}*>bax*), served as a 'cell-death' positive control

114 (Extended data Fig.2d, f). While we saw no evidence of cell death in hypoxic midguts,

115 *dSRF*^{ts}*>bax* midguts showed significant apoptosis (Extended data Fig.2e, g), which was

116 restricted to ECs, distinguished by their large nuclei (Extended data Fig.2e, lower panel,

117 magnified view). Consistently, this cell death phenotype did not translate into defective ISC

118 numbers (Extended data Fig.2h, i). Therefore, impaired midgut regeneration following the

119 hypoxia or TTC ablation regime used in our study is unlikely to be secondary to ISC loss.

120 Alternatively, differences in the regenerative response of hypoxic versus *dSRF*^{ts}*>bax* midguts

121 could be explained by the contribution of angiocrine factors to ISC proliferation, in addition to

122 oxygen availability. Dpp/BMP ligand has been identified as an angiocrine factor in the adult

123 midgut[15]. However, its action inhibits rather than induces ISC proliferation[15]. Therefore, a

124 potential role of Dpp cannot directly explain our results (see also Discussion).

125 Given that tracheal remodelling and ISC proliferation show almost the exact same

126 dynamics (Fig.1l, m and Extended data Fig. 1 h, i), it is unclear whether these events are part

127 of a feedforward mechanism or if one precedes the other. To address this, we assessed

128 tracheal remodelling following damage while blocking ISC proliferation by overexpressing

129 *UAS-myc RNAi* (*myc*$^{-IR}$)[31] using the stem/progenitor driver *escargot-GAL4* (*esg*^{ts}*>myc*$^{-IR}$)

130 (Fig.2e-g). As we needed to use the *GAL4/UAS* system to genetically manipulate gut cells,

131 we established a scoring method for assessing tracheal coverage through the use of light

132 microscopy, which was validated against our confocal microscopy tracheal quantification

133 approach (Extended Data Fig.3a-c). Gut-associated trachea remodelled normally in

134 *esg*^{ts}*>myc*$^{-IR}$ midguts following *Pe* damage, in spite of the almost complete absence of ISC

135 proliferation (Fig.2e-g). Therefore, TTC remodelling precedes midgut ISCs proliferation

136 following damage. We hypothesised that signals activated by damage upstream of ISC

137 proliferation might induce gut-tracheal remodelling.

138

Gut-derived ROS initiates tracheal remodelling through activation of HIF-1α/FGFR signaling in TTCs

Pathogen-induced intestinal damage triggers a strong oxidative burst and the production of reactive oxygen species (ROS) from the intestinal epithelium[32, 33]. We therefore tested whether ROS could trigger tracheal remodelling in the regenerating intestine. Feeding animals with the antioxidant N-acetyl cysteine (NAC) or genetically blocking ROS production in ECs by overexpressing the enzyme catalase[32] (*NP1ts>catalase*) significantly impaired damage-induced tracheal remodelling (Fig.2h, i and Extended data Fig.3d, e) in addition to the expected reduction in regenerative ISC proliferation (Fig. 2j and Extended data Fig.3f)[32]. Conversely, driving adult intestinal epithelial cell death through *bax* overexpression in ECs (*NP1ts>bax*) was sufficient to induce TTC remodelling and ISC proliferation (Extended data Fig.3g-i). Therefore, intestinal epithelial damage and ROS induce remodelling of gut associated trachea, which is in turn necessary to drive ISC proliferation during intestinal regeneration.

Exogenous H_2O_2 can stabilize HIF-1α —a key conserved driver of hypoxia-induced tracheal/vascular remodelling[9, 34, 35]— in normoxia[36]. The Sima/HIF-1α activity reporter *ldh-lacZ* was upregulated in gut-associated TTCs following midgut damage and in an ROS dependent manner (Fig.2k-m). Furthermore, midguts from *sima-/-* whole mutant animals or upon adult specific *sima* knockdown within TTCs (*dSRFts>sima-IR*) showed significantly impaired tracheal remodelling and ISC proliferation following damage (Fig.3a-f).

The *Drosophila* fibroblast growth factor receptor (FGFR), Breathless (Btl), is a well-known transcriptional target of HIF-1α during tracheal development and oxygen-driven tracheal remodelling[19, 37]. Consistently, a reporter of *breathless (btl)* expression (*btl-lacZ*) showed gene upregulation in TTCs following intestinal damage, which was abrogated by NAC (Fig.3g-i). TTC knockdown of *btl* (*dSRFts>btl-IR*) inhibited tracheal remodelling and ISC proliferation following damage (Fig.3j-l), without evidence of cell death or ISC loss (Extended data Fig.2e, g-i). Therefore, ROS-dependent activation of HIF-1α/FGFR signaling within TTCs following gut damage induces tracheal remodelling and regenerative ISC proliferation in the

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167 adult midgut. Consistently, expression of the HIF-1α/FGFR target gene *blistered* (*bs*)/*dSRF*[19,]

168 [38] was upregulated in damaged and hypoxic midguts (Extended data Fig.4a-e) and knocking

169 down *bs* in adult TTCs (*dSRF^ts^>bs^-IR^*) impaired tracheal remodelling and ISC proliferation

170 following damage (Extended data Fig.4f-h).

171

172 **ROS-dependent bidirectional FGF/FGFR signaling drives stem cell proliferation and**

173 **TTC remodelling during intestinal regeneration**

174   During development or hypoxia, the *Drosophila FGF*-like ligand Branchless (Bnl) is

175 upregulated in target tissues and signals paracrinally to its receptor FGFR/Breathless (Btl) in

176 the trachea to induce their remodelling[16, 19, 39]. Consistently, we observed upregulation of a *bnl*

177 reporter (*bnl-lacZ*) in ISCs/EBs and ECs following intestinal damage, which was impaired by

178 NAC (Fig.4a-c). These results suggest that ROS induces Bnl activation within the intestinal

179 epithelium following damage. Unexpectedly, using the same reporter, we observed that *bnl*

180 was also upregulated in TTCs following intestinal damage, in an ROS dependent manner

181 (Fig.4d-e). Expression of *bnl* in TTCs was confirmed by the use of an independent reporter

182 (Extended data Fig.5a, b). Overexpressing *bnl* in adult TTCs (*dSRF^ts^>bnl*) was sufficient to

183 induce ISC proliferation without TTC remodelling (Extended data Fig.5c, d).

184   We next assess the functional role of individual sources of FGF/Bnl in our system.

185 Consistent with our reporter expression data (Fig.4a-e), knocking down *bnl* from either TTCs

186 (*dSRF^ts^>bnl^-IR^*), ISCs/EBs (*esg^ts^>bnl^-IR^*) or ECs (*NP1^ts^>bnl^-IR^*) restrained ISC proliferation

187 following midgut damage but did not impair TTC remodelling (Fig.4f-h and Extended data

188 Fig.5e-j). This is in line with the high sensitivity of the regenerative intestine to discrete

189 fluctuations in individual signaling activity[31, 40]. Hence, small variations in Bnl levels, which are

190 insufficient to affect tracheal remodelling are enough to impact ISC proliferation following

191 damage. Instead, concomitant *bnl* knockdown from ECs and ISCs/EBs (*NP1>, esgts>bnl^-IR^*)

192 significantly impaired TTC remodelling and subsequent ISC proliferation (Fig.4i-k). Therefore,

193 the combined action of gut-derived sources of Bnl, is necessary to induce tracheal remodelling

194 following intestinal damage.

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195   Given that multiple sources of Bnl—from the midgut and TTCs—can individually

196   contribute to regenerative ISC proliferation independently of tracheal remodelling, we

197   hypothesised this may be through a non-tracheal receptor. Consistently, knocking down *btl*

198   from ISCs/EBs (*esg^{ts}>btl-IR*) significantly impaired ISC proliferation upon damage without

199   affecting TTC remodelling (Extended data Fig.6a-c). In the context of tracheal development,

200   Bnl/Btl signals through the MAPK/ERK pathway[5, 41], which is a key driver of ISC proliferation

201   in the adult *Drosophila* midgut[42-44]. Knocking down *btl* from ISCs/EBs (*esg^{ts}>btl^{-IR}*) impaired

202   damage-induced MAPK/ERK activation in the midgut (Extended data Fig.6d, e), suggesting

203   that activation of Btl in the midgut regulates regenerative ISC proliferation through MAPK/ERK

204   signaling.

205

206   **Identification of novel TTC intrinsic mechanisms triggered during intestinal**

207   **regeneration**

208   We next used Targeted DamID (TaDa) for TTC *in vivo* profiling of RNA Pol II chromatin

209   binding[45] in control (Sucrose) and *Pe* treated midguts (Fig.5a and Extended data Fig.7a).

210   TaDa is particularly advantageous in our system due to inherent difficulties to efficiently

211   separate tracheal tissue from the midgut. We identified 1747 and 1712 genes significantly

212   bound by RNA Pol II in TTCs from control (Sucrose) and *Pe* infected midguts, respectively

213   (Supplementary Table 1). Gene ontology (GO) analysis of areas with significant RNA Pol II

214   binding in control midguts revealed enrichment in components of the tracheal system, and

215   genes previously involved in epithelial tube morphogenesis and respiratory/tracheal system

216   development (Fig.5b) (Supplementary Table 2 and 3). This validated the sensitivity of TaDa

217   to reliably detect tracheal specific genes from combined gut and tracheal tissue samples.

218   Consistent with our reporter expression and functional data (Fig.4d-h), TaDa analysis

219   identified *bnl/FGF* as a gene with significant RNA Pol II binding in *Pe* treated midguts only

220   (Supplementary Table 1) (Extended Data Fig.7b). Unexpectedly, we were unable to detect

221   significant RNA Pol II binding to *btl* in adult TTCs of *Pe* treated midguts (Supplementary Table

222   1). This is counterintuitive given our gene expression and functional data on *btl* (Fig.3g-l).

223   Discrepancies between RNA pol II occupancy and mRNA transcript status are possible and

224   could be due to pausing of the polymerase[46], post-transcriptional mRNA regulation[47] or

225   temporally dynamic RNA pol II binding (*e.g* during intestinal damage), which may not be

226   captured by a single time point assessment.

227

228   **Imp/IGF2BP is a novel regulator of adult tracheal remodelling and intestinal**

229   **regeneration**

230   Interestingly, we noticed that, within the genes showing significant RNA pol II binding in TTCs

231   of *Pe* treated midguts only (Supplementary Table 1), there were several genes associated

232   with neuronal function (Fig.5c). Amongst them, was the highly conserved mRNA-binding

233   protein Imp/IGF2BP (Fig.5d) (Supplementary Table 1), which regulates axonal remodelling in

234   *Drosophila*[48, 49]. We confirmed upregulation of *Imp* transcription by qRT-PCR (Fig.5e) and Imp

235   increase in TTCs through the use of a protein trap (Imp::GFP) (Fig.5f, h). NAC treatment

236   showed that Imp upregulation in TTCs following intestinal damage depends on ROS

237   production (Fig.5g, h). Importantly, adult specific knock down of *Imp* from TTCs ($dSRF^{ts}>Imp^{-}$

238   ^{IR}) significantly impaired tracheal remodelling and ISC proliferation following midgut epithelial

239   damage (Fig.5i-k). Next, we investigated two well-known post-transcriptional targets of Imp in

240   our system: *chickadee/profilin*[48] and *myc*[49]. Functional experiments on the role of *chickadee*

241   led to inconclusive results (data not shown). However, Myc was upregulated in TTCs of *Pe*

242   treated midguts and this was abrogated by *Imp* knockdown ($dSRF^{ts}>imp^{-IR}$) (Fig.6a, b).

243   Moreover, adult specific knockdown of *myc* within TTCs using RNAi[31] ($dSRF^{ts}>imp^{-IR}$),

244   impaired tracheal remodelling and ISC proliferation following gut damage (Fig.6c-e).

245   Altogether, these results establish Imp as a novel regulator of TTC remodelling and ISC

246   proliferation during adult *Drosophila* midgut regeneration. This function of Imp is at least in

247   part through tracheal intrinsic control of Myc.

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249   *Tracheacless* **downregulation in TTCs is necessary for adult tracheal remodelling and**

250   **damage induced ISC proliferation**

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251 While our TaDa analysis revealed significant binding of RNA Pol II to *tracheless* (*trh*) in TTCs

252 of Sucrose treated midguts (Supplementary Table 1 and 3), this was not the case in the

253 damaged tissues (Fig.7a and Supplementary Table 1). This was surprising given that *trh* is

254 known a master regulator of tracheal gene expression and it is present in all tracheal cells

255 from the onset of embryonic development through adulthood[50-52]. Loss of *trh* during

256 development impairs tracheal cell specification and tube morphogenesis[51, 52]. However, qRT-

257 PCR confirmed downregulation of *trh* upon intestinal damage (Fig.7b). Antibody staining

258 (Fig.7c, d) and a transgenic reporter (*trh-lacZ*) (Fig.7e, f) further demonstrated protein and

259 gene downregulation in TTCs upon gut damage, respectively. Remarkably, *trh-lacZ* signal was

260 significantly restored upon *Pe* and NAC co-treatment or 32 hrs after removal of the damaging

261 agent (Fig.7e, f). This suggests that *trh* expression in adult TTCs is highly dynamic and its

262 downregulation upon intestinal damage is dependent on ROS. Importantly, consistent with our

263 gene and protein expression data, *trh* overexpression in adult TTCs (*dSRF^{ts}>trh*) significantly

264 impaired tracheal remodelling and ISC proliferation (Fig.7g-i), while *trh* knockdown

265 (*dSRF^{ts}>trh^{-IR}*) potentiates TTC remodelling and ISC proliferation following midgut damage

266 (Fig.7g-i). Altogether, these results suggest that ROS-induced *trh* downregulation in adult

267 TTCs is necessary to allow gut associated TTC plasticity and robust regeneration of the

268 intestine following damage.

269   Here, we reveal a novel inter-organ communication program in *Drosophila,* involving

270 the adult tracheal system and the midgut, which drives reciprocal adaptation of both tissues

271 to sustain a robust regenerative response of the intestine to injury (Fig.7j). Our results may

272 reflect vasculature/stem cell interactions in the mammalian intestine and other self-renewing

273 tissues.

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Discussion

The vasculature represents a prominent component of the gut microenvironment. However, its functional role in adult intestinal homeostasis remains largely unknown. Here, we report the cellular and molecular underpinnings of a novel inter-organ communication program between the adult *Drosophila* midgut and its closely associated vasculature-like tracheal tissue, which is fundamental to drive the regenerative response of ISCs following epithelial tissue damage.

ROS are key initiators of TTC remodelling and ISC proliferation, following intestinal damage by pathogenic infection (Fig. 7j). While our observations show a conserved phenomenology of TTCs/vasculature changes upon diverse intestinal insults and across species, it is highly conceivable that damage and species-specific molecular responses exist and that signals other than or in addition to ROS influence tracheal/vascular adaptations to intestinal damage.

HIF-1α/FGFR signaling drives tracheogenesis during development and in response to hypoxia following activation by FGF from target tissues [9, 34, 35]. As such, our findings suggest a repurposing of this developmental pathway during adult gut/tracheal crosstalk. However, the discovery of ROS-inducible angiocrine Bnl/FGF activating stem/progenitor cell FGFR signaling during adult intestinal regeneration is a novel finding from our study (Fig. 7j). FGF induces vascular endothelial cell differentiation in human intestinal organoids[53] and acts as an angiocrine factor in various tumour settings[54]. This highlights the great degree of conservation between the *Drosophila* and mammalian system and raises the possibility of a conserved angiocrine role of FGF ligands in mammalian intestinal regeneration.

The DPP/BMP ligand has been reported as an angiocrine factor required to restrain stem cell proliferation in the adult *Drosophila* midgut[15]. However, knocking down *dpp* in adult TTCs did not affect basal stem cell proliferation as reported upon global ligand downregulation in the adult trachea[15] (data not shown). Ligand compensation from main tracheal branches or

301   the intestinal epithelium[55] may explain discrepancies between our results and the published

302   study.

303   In addition to the well-established role of oxygen, nutrition regulates TTC remodelling

304   in the larval and adult *Drosophila* midgut[10]. In this context, a defined subset of enteric neurons

305   influence TTC remodelling through the delivery of Insulin- and Vasoactive Intestinal Peptide-

306   like neuropeptides[10]. Nutrient induced-tracheal remodelling involves activation of Insulin

307   Receptor (InR) signaling within TTCs. We observed no requirement for InR or enteric neuronal

308   activity in the plastic response of adult TTCs to intestinal damage (data not shown).

309   Reciprocally, FGF/FGFR signaling does not appear to mediate nutrient dependent TTC

310   remodelling[10]. These results suggest that molecular events driving tracheal tissue plasticity

311   are diverse and highly dependent on the biological context and/or stimuli.

312   We identified two novel tracheal-intrinsic molecular mechanisms triggered in response

313   to intestinal epithelial damage and necessary to induce TTC remodelling and ISC proliferation

314   (Fig. 7j). One, involving upregulation of *Drosophila* IGF2 mRNA-binding protein (Imp) and its

315   downstream target Myc (Fig. 7j). The other, requiring downregulation of the tracheal cell

316   specification factor *trh* (Fig. 7j). Known functions of Imp had been restricted to the induction of

317   neuronal remodelling and growth[48, 49]. Its mammalian orthologue, IGF2BP2, has been studied

318   for its involvement in metabolic disease[56] and its potential role in the vasculature remains to

319   be addressed. Trh, homologous to mammalian NPAS3[57], has been exclusively known for its

320   requirement in the specification of tracheal cells from undifferentiated progenitors in the

321   developing embryo[50-52]. Here, we present the first report for a role of Trh in terminally

322   differentiated adult tracheal cells, which involves its unexpected downregulation. Emerging

323   evidence suggests that adult tissues and cells, such as the intestine and neurons, lose

324   differentiation markers and acquire 'naïve' or 'foetal-like properties' during the process of

325   tissue regeneration[58-60]. Our work suggests the exciting possibility that this may also be the

326   case for the adult vasculature.

327   The vasculature is a largely uncharacterized component of the adult intestinal niche.

328   Vascularization of *in vitro* organ culture systems has been notoriously difficult, representing a

329   major roadblock in the field of tissue engineering. As such, our *in vivo* findings may be of broad

330   interest and impact to the vascular and intestinal research fields.

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349 **Methods**

350 **Fly stocks and rearing**. A complete list of fly lines and full genotypes used in this study can

351 be found in Supplementary Tables 5 and 6. In experiments using the *GAL4*/*GAL80^{ts}* system,

352 flies were crossed, F1 progenies reared, and adults aged for 5 days after eclosion at 18°C.

353 Animals were then transferred to 29°C for 5-7 days to allow activation of most transgenes prior

354 to phenotypic analysis. The exception was *bax*, which was overexpressed for only 3 days. If

355 not carrying temperature sensitive transgenes, crosses and offspring were kept at 25°C.

356 Overall, experimental animals were used 10-12 days following adult eclosion. Animals for

357 experiments were maintained in food vials at low densities (10-15 flies per vial) and were

358 transferred to fresh food every 2 days. Only adult posterior midguts from mated females were

359 analysed in this study.

360

361 **Damage induced intestinal regeneration.** Oral administration of *Pe*, Bleomycin or DSS was

362 performed as previously described[21, 25, 61] with minor modifications. Briefly, 10 day-old females

363 of the desired genotypes were starved in empty vials for 2hrs followed by feeding with a 5%

364 sucrose solution only (Sucrose), or Sucrose containing either *Pe* at OD 100, 25μg/ml

365 Bleomycin (Sigma-Aldrich, Cat#B2434), or 3% DSS (Sigma-Aldrich, Cat#42867) applied on

366 filter paper discs (Whatman). *Pe* infection was carried out for 16hrs, Bleomycin feeding was

367 done for 1-day and DSS feeding lasted for 2-days, with fresh media applied each day.

368 **NAC treatment.** Flies were placed in empty vials with a filter paper soaked with a 5% sucrose

369 solution containing 20mM NAC (Sigma-Aldrich, Cat#A7250) for 24 hrs. Animals were then fed

370 with either 5% sucrose + NAC or 5% sucrose + NAC + *Pe* (OD 100) for an additional period

371 of 24 hrs.

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373 **Mouse intestinal regeneration and IHC.** *C57BL/6* mice were subject to whole body 10 Gy

374 gamma-irradiation and the intestines were analysed 72 hrs post-irradiation, which represents

375 the proliferative phase of the regenerative response to damage in the mouse small intestine[28].

376   Small intestines were isolated and flushed with tap water. 10× 1cm portions of small intestine

377   were bound together with surgical tape and fixed in 4% neutral buffered formalin. Intestines

378   from 3 mice per condition were used. 4 μm sections of formalin–fixed paraffin–embedded

379   (FFPE) tissues were cut, mounted onto adhesive slides and incubated at 60°C overnight. Prior

380   to staining, sections were dewaxed for 5 minutes in xylene followed by rehydration through

381   decreasing concentrations of alcohol and final washing with H_2O for 5 minutes. FFPE sections

382   underwent heat–induced epitope retrieval in a Dako pre-treatment module. Sections were

383   heated in Target Retrieval Solution High pH (Dako, K8004) for 20 minutes at 97°C before

384   cooling to 65°C. Slides were removed and washed in Tris Buffered Saline with Tween (TbT)

385   (Dako, K8000) and loaded onto a Dako autostainer link48 platform where they were stained

386   with anti-CD31 antibody 1:75 (Abcam, ab28364) following standard IHC procedures. All

387   animal work has been approved by a University of Glasgow internal ethics committee and

388   performed in accordance with institutional guidelines under personal and project licenses

389   granted by the UK Home Office to J.B.C (PPL PCD3046BA).

390

391   **Single terminal tracheal cell clones**.

392   To generate single cell clones of terminal tracheal cells, parental lines were allowed to mate

393   for 2-3 days, after which adults were moved into new vials and F1 progenies where heat

394   shocked in a water bath for 1 hr at 37°C. Adults of the correct genotype, emerging from the

395   heat shocked animals, were selected and aged at 25°C for 10 days followed by feeding with

396   Sucrose (control) or *Pe* for 16 hrs to cause intestinal damage and induce regeneration.

397   Tissues were then dissected and processed for immunofluorescence staining and confocal

398   imaging.

399

400   **Hypoxia treatment.** Adult flies were aged at 25°C or 29°C in 21% O_2 (Normoxia) at a density

401   of 15-20 flies per vial. Then, animals were transferred overnight to 3% O_2 (Hypoxia) in a

402   Whitley Scientific H35 hypoxystation incubator.

403 ***Drosophila* immunohistochemistry.** Immunohistochemistry was carried out as described

404 previously[31]. The following antibodies were used: chicken anti-GFP 1:200 (Abcam, ab13970),

405 mouse anti-PH3 1:100 (Cell Signaling, 9706), rabbit anti-Dcp1 1:100 (Cell Signaling, #9578S),

406 rabbit anti-βgal 1:1000 (MP Biochemicals #559761), rabbit anti-DsRed 1:1000 (Clontech,

407 #632496), mouse anti-Arm 1:3 (Hybridoma Bank, N2 7A1), mouse anti-Dlg 1:100 (Hybridoma

408 Bank, 4F3), rabbit anti-p-Erk 1:100 (Cell Signaling, #9101), guinea pig anti-Myc 1:100 (gift

409 from Gines Morata) and rabbit anti-Trh 1:100 (gift from M. Llimargas). Chitin Binding Protein

410 (CBP 1:100; gift from M. Llimargas) was used to visualise all tracheal tissue. Alexa Fluor 488,

411 546 and 647 (Invitrogen) were used as secondary antibodies labels at 1:200 and 1:100

412 respectively. Guts were mounted in Vectashield anti-fade mounting medium for fluorescence

413 with DAPI (Vector Laboratories, Inc) to visualize all nuclei.

414

415 **Image acquisition.**

416 *Transmission Electron Microscopy*. Guts were dissected under Schneider's insect medium

417 and were subsequently fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for

418 1hr at room temperature. Samples were rinsed repeatedly in 0.1M sodium cacodylate buffer,

419 before fixation treatment in 1% Osmium Tetroxide/buffer for 1hr, followed by washing with

420 dH20 for 30mins. Samples were then stained in 0.5% Uranyl Acetate/dH20 for 1hr (in the dark)

421 and dehydrated by incubation in a graded series of Ethanol. Samples were subject to three

422 subsequent incubations in Propylene Oxide followed by Epon 812 resin/Propylene Oxide

423 (50:50) mix and left on a rotator overnight, followed by several incubations in pure Epon resin.

424 Samples were then embedded into blocks and oven incubated at 60°C for 48hrs. Ultrathin

425 sections (50-70nm thickness) were cut using a Leica Ultracut UCT. The sections produced

426 were collected on Formvar coated 100 mesh copper grids and subsequently contrast stained

427 in 2% Methanolic Uranyl Acetate for 5mins followed by Reynolds Lead Citrate for 5mins. Gut

428 samples were viewed using a JEOL 1200 EX TEM and Images captured using a Cantega 2K

429 x 2K camera and Olympus ITEM Software.

430   *Confocal microscopy (Zeiss LSM 780)*. Each image represents half-volume of the full posterior

431   midgut (area comprised between hindgut and Copper Cell Region) and were acquired with

432   20x, 40x or 63x lenses using identical acquisition conditions for all samples from a given

433   experiment. Images represent maximal intensity projection of a stable number of Z-Stacks and

434   were processed with ImageJ and Carl Zeiss Zen 3.0 to adjust brightness and contrast.

435   *Light microscopy (Axio observer Zeiss)*. Adult guts were dissected in PBS, mounted in 100%

436   glycerol and imaged immediately. Up to three pictures per posterior midgut were taken to

437   cover most of the area. Images were taken at their most apical plane to best detect TTCs with

438   a 20x lens. Images from mouse intestinal samples (Fig. 1n) were acquired under a 20x lens

439   using this microscope and were processed with ImageJ.

440

441   **Quantifications in the adult posterior midgut.**

442   *Quantification of ISC proliferation*. Antibodies against Phosphorylated Histone H3 (PH3) were

443   used to detect ISC proliferation in the adult midgut. The total number of PH3^{+ve} cells per

444   posterior midgut was quantified manually upon visual inspection using an Olympus BX51

445   microscope. The number of midguts analysed (n) for each experiment are indicated in the

446   figures.

447   *Quantification of tracheal coverage from immunofluorescence images*. Unless otherwise

448   noted, confocal images of *dSRF>GFP* expressing midguts were used and tracheal values

449   provided were obtained from quantification of tile scan images of the entire R4-R5 posterior

450   midgut regions, acquired with a 20x lens. Values of tracheal coverage represent pixel per area

451   and were obtained from maximum intensity Z-projections. Pictures were individually

452   processed on ImageJ as follows: 1) maximum intensity projection from Z stacks were

453   produced; 2) area of interest was cropped to eliminate Malpighian tubules, hindgut and copper

454   cell region; 3) "threshold" was adjusted to ensure the detection of most of terminal tracheal

455   branches; 4) function "skeletonize" was applied to generate a skeleton of the tracheal network;

456   5) maximum intensity of this skeleton was measured (Extended data Fig.1a). The number of

457   posterior midguts analysed (n) for each experiment are indicated in the figures.

458   *Quantification of tracheal branching form light-microscopy images*. Acquired images were

459   blindly scored using a 1 to 5 scoring system (Extended Data Fig.3a, c). The custom ImageJ

460   macro used for blind tracheal scoring is available at "Blind_scoring.ijm". Number of midguts

461   analysed (n) for each experiment are indicated in the figures.

462   *Quantification of total branches per TTC and TTC ramifications*. Maximum intensity projections

463   from confocal Z stacks were used. The number of primary, secondary and tertiary branches

464   derived from individual TTCs was assessed (Fig.1i). Due to the intrinsic complexity of the

465   tracheal, it is difficult to unambiguously assign cellular extensions/branches to a single TTC.

466   To circumvent this issue, we counted tracheal branches starting from a TTC body and defined

467   the end of a TTC extension when it touched the body of another TTC. Additionally, the

468   generation of single TTC clones allowed us to unambiguously quantify the total number of

469   branches from individual TTC, which we did manually (Fig.1f, h). These two approaches led

470   to same outcome. Number of TTC (n) and midguts analysed for each condition are indicated

471   in the corresponding figures and figure legends.

472   *Quantification of total tracheal length.* We used the plugin "NeuronJ" from ImageJ to quantify

473   the total length of all branches emerging from a TTC (Fig.1k). Number of TTC (n) and midguts

474   analysed for each condition are indicated in the corresponding figures and figure legends.

475   *Quantification of TTC nuclei*. Confocal images of posterior midguts from animals expressing

476   *dSRF^{ts}>RedStinger* to label TTC nuclei were used. The custom ImageJ macro used for

477   quantifying the number of TTC nuclei is available at "dSrf_pH3_overlap_for Jessica.ijm". This

478   macro was also used to quantify the number of TTC nuclei positive for PH3$^+$ staining

479   (Extended data Fig.1g). Number of midguts analysed (n) for each condition is indicated in the

480   corresponding figure and figure legend.

19

481   *Quantification of posterior midgut area*. Midgut tissue was visualized by DAPI staining and the

482   posterior midgut area (length x width) was measured with ImageJ (Extended Data Fig.1c, d).

483   Number of midguts analysed (n) for each condition is indicated in the corresponding figure

484   and figure legend.

485   *Quantification of lacZ reporters*. Antibodies against β-galactosidase were used to detect

486   *lactate dehydrogenase*-, *Delta*-, *breathless*-, *bs/dSRF*-, *branchless*- and *trachealess-lacZ*

487   reporters. Pictures were taken with confocal microscopy and staining was quantified using

488   ImageJ. For each gut quantified, the background staining signal was subtracted from the total

489   signal of β-galactosidase detected in TTCs, ISCs/EBs or ECs. This value was then divided by

490   the background signal to normalize the data. Number of cells (n) and midguts analysed for

491   each condition are indicated in the corresponding figures and figure legends.

492   *Quantification of cell death*. Antibodies against Dcp1 were used to assess cell death in

493   posterior midguts. Pictures were taken with confocal microscopy and Dcp1 staining intensity

494   was measured relative to the surface of the gut area analysed. Number of midguts analysed

495   (n) for each experiment are indicated corresponding in the figure and figure legend.

496   *Quantification of pERK, Imp::GFP, Myc and Trachealess staining*. Midguts stained with

497   antibodies to detect these proteins included a methanol fixation step between the PFA fixation

498   and PBST washing steps of the standard protocol, as described previously[40]. Images were

499   acquired with confocal microscopy and staining was quantified using ImageJ. For each gut

500   quantified, the background staining signal was subtracted from the total antibody signal within

501   DAPI positive cells. This value was then divided by the background signal in order to normalize

502   the data. The number of cells (n) and midguts analysed for each condition are indicated in the

503   corresponding figures and figure legends.

504   **Statistical analysis.** Unless otherwise noted, in all figures: NS, not significant ($p>0.05$); *$p <$

505   0.05, **$p < 0.01$, ***$p < 0.001$ ****$p< 0.0001$. Prism 6 software (GraphPad) was used for

506   statistical analyses. A two-tailed Student's t-test was conducted to compare two sample

507   groups. To test for significance in larger groups, two-way ANOVAs, corrected for multiple

508   comparisons using Sidak's statistical hypothesis testing, respectively, were used. P values

509   less than 0.05 were considered significant. Information on sample size and statistical tests

510   used for each experiment are indicated in figure legends. Unless otherwise noted, experiments

511   represent a minimum of 2 independent biological replicates. Please see reporting summary

512   for further details.

513

514   **qRT-PCR.**

515   Trizol (Invitrogen) was used to extract total RNA from 30 midguts per biological replicate.

516   cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit

517   (Applied Biosystems). QuantiNova SYBR Green (Qiagen) was used for qPCR. Data were

518   extracted and analysed using QuantStudio and Prism 6.0. Data from 5 biological replicates is

519   presented as the mean fold change with standard error. Expression of target genes was

520   measured and normalized to *gapdh1* or *act5c* using standard curves. Primer sequences can

521   be found in Supplementary Table 4.

522

523   **Targeted DamID (TaDa), library preparation, sequencing and data analysis.** *dSRF-GAL4;*

524   *tub-gal80^{ts} (dSRF^{ts}>)* animals were crossed to *UAS-LT3-Dam* or *UAS-LT3-Dam-Pol II* animals

525   at 18°C. F1 progeny were collected every 48 hrs and aged for a further 7 days at 18°C before

526   transferring to 29°C to induce adult restricted Dam protein expression for 24 hrs. During the

527   last 16 hrs at 29°C, flies were fed a Sucrose or Sucrose + *Pe* solution. 60 midguts per condition

528   per biological replicate were dissected in cold PBS and stored at -80°C. Methylated DNA

529   fragments were isolated and next generation sequencing libraries were prepared as described

530   previously[45]. Sequencing data from TaDa experiments were processed using a previously

531   described pipeline[62] and mapped to release 6.03 of the *Drosophila* genome. Transcribed

532   genes were annotated for Pol II binding data using a custom Perl script[46] (available at

533   https://github.com/tonysouthall/Dam-RNA_POLII_analysis) and release 6.11 of the annotated

534   *Drosophila* genome. Genes with significant RNA pol II binding were identified based on

535   meeting a threshold of 1% FDR and > 0.2 log2 ratios. Briefly, a log2 ratio of the Dam-RNA Pol

536   II read counts over control Dam-only read counts is calculated after quantile normalisation[62]

537   and if this ratio is higher than 0.2, then we would determine that this gene has significant RNA

538   Pol II binding (Supplementary Table 1). Significance was assigned based on the signal from

539   multiple GATC fragments and using a very stringent pipeline as a transcript had to have a

540   false discovery ratio (FDR) of less than 1% in both replicates to be called significant.

541   Gene Ontology (GO) term analysis was performed using the R package ClusterProfiler[63] to

542   search for enriched GO terms (Supplementary Table 2). Three independent biological

543   replicates were originally processed for each condition (Sucrose and *Pe*). However, after

544   visual inspection of the sequencing tracks, one replicate from each condition was excluded

545   from the analysis, due to poor DNA sample quality and unreliable sequencing data. Scattered

546   plots showing the correlation between samples for each condition are provided (Extended data

547   Fig.7a).

548

549   **Data availability**

550   TaDa sequencing data is available through the University of Glasgow institutional repositories

551   *DOI: 10.5525/gla.researchdata.994 (2020)*. The entire set of raw data supporting this study

552   will be made accessible through the same repository prior to publication.

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Author contributions.

J.P designed and carried out most experiments and analysed and interpreted the data. Y.Y. provided technical support throughout the study and perform RT-qPCRs. J.P. G.A, T.S and J.B.C. analysed the TaDa data. J.B.C conceived the project, designed experiments, analysed the data and supervised the study. J.P and J.B.C wrote the paper with contributions from the rest of the authors.

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733 **Fig. 1 – Adult tracheal plasticity following intestinal injury**. **a**, Confocal image of adult gut-

734 associated terminal tracheal cells (TTCs) (green) in *Drosophila*. Scale bar: 500μm. **b**, **c**,

735 Transmission electron microscopy of adult posterior midguts highlighting TTCs (green

736 pseudo-coloured), enterocyte (EC) and intestinal stem cell (ISC) (red pseudo- coloured); VM:

737 visceral muscle, Scale bars: 5μm. **d**, Confocal images of TTCs (green) and proliferating ISCs

738 (PH3; red) in control (Sucrose) or damaged (*Pe*) midguts. Scale bars: 100μm. **e,** Quantification

739 of tracheal coverage in midguts as in (d) or upon Bleomycin or DSS treatment. **f**,

740 Representative confocal images of single TTC clones (green) from control (Suc) or *Pe*

741 damaged posterior midguts and proliferating ISCs (PH3; red). Scale bar: 25μm. **g**,

742 Quantification of the total number of branches per TTC clone in midguts as in (f). **h, (top),**

743 Quantification of total number of branches per TTC clone and nearby PH3$^+$ ISCs upon *Pe*

744 infection. **(bottom),** Representative confocal images of TTC clones (green) and proliferating

745 ISCs (PH3; red) used for quantifications in top panel. Scale bars: 25μm. **f-h** <u>Statistics</u>:

746 Student's t test; n= number of TTC clones from 7-9 posterior midguts. **i,** Confocal image of a

747 TTC with pseudo-coloured primary (I^R), secondary (II^R) and tertiary (III^R) branches. Scale bar:

748 50μm. **j**, Quantification of number of branches per TTC from control or *Pe* infected posterior

749 midguts. <u>Statistics</u>: Two-way ANOVA followed by Sidak's multiple comparisons test; n=

750 number of TTCs from 6 midguts per condition. **k**, Quantification of total tracheal length per

751 TTC. <u>Statistics</u>: Student's t test; n= number of TTCs from 6 midguts per condition. **l**, **m**,

752 Quantification of tracheal coverage and PH3$^+$ cells in posterior midguts during 16 hrs of oral

753 *Pe* infection followed by 32 hrs standard food incubation. <u>Statistics</u>: Student's t test to compare

754 each timepoint of the damage and recovery stage against the 0 hrs and 16 hrs time points,

755 respectively. n= number posterior midguts. **n**, Immunohistochemical analysis of control (non-

756 irradiated) and regenerating (irradiated) mouse small intestines stained with anti-CD31 to

757 visualize endothelial cells. Scale bars: 100μm (main figure); 50μm (close up view).

758 Error bars: ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

759 **Fig. 2 – ROS induced tracheal remodelling is required for intestinal regeneration. a**,

760 Confocal images of adult posterior midguts from control animals or upon adult-specific *bax*-

761 driven TTC loss (green). Scale bar: 100μm. **b**, Quantification of tracheal coverage in posterior

762 midguts as in (a). Statistics: Student's t test; n= number of posterior midguts. **c-d**,

763 Quantification of PH3$^+$ ISCs in posterior midguts of the genotypes and conditions indicated. **e**,

764 Representative brightfield images of adult posterior midguts from Sucrose fed and *Pe* infected

765 animals without (control) or with adult-specific overexpression of *myc* RNAi in ISCs/EBs

766 (*esg^{ts}*>). Scale bar: 50μm. **f, g**, Quantification of PH3$^+$ ISCs (f) and scored tracheal branching

767 (g) in posterior midguts as in (e). **h**, Confocal images of control and *Pe* treated midguts in the

768 presence or absence of the antioxidant NAC. Scale bar: 100μm. **i, j**, Quantification of tracheal

769 coverage (i) and PH3$^+$ ISCs (j) in midguts as in (h). **c-j**, Statistics: Two-way ANOVA followed

770 by Sidak's multiple comparisons test; n= number of posterior midguts. **k,l,** Confocal images of

771 the HIF-1α/Sima activity reporter *ldh-lacZ* (grey) in control or *Pe* infected adult posterior

772 midguts without or with NAC (k, l, respectively). Scale bar: 50μm. **m**, Quantification of the

773 average *ldh-lacZ* staining intensity in TTC within a defined region of posterior midguts as in

774 (k, l). Statistics: Two-way ANOVA followed by Sidak's multiple comparisons test; n= number

775 of TTC quantified from 9 to 10 posterior midguts per condition.

776 Error bars: ±SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

777 **Fig. 3 – Activation of HIF-1α/FGFR signaling in TTCs is required for tracheal remodelling**

778 **and intestinal regeneration following damage. a,** Confocal images of TTCs (green) in

779 control (Sucrose) or *Pe* infected adult posterior midguts from wild type or *Sima$^{-/-}$* whole mutant

780 animals. Scale bar: 100μm. **b**, **c**, Quantification of tracheal coverage (b) and PH3$^+$ ISCs (c) in

781 posterior midguts as in (a). **d**, Confocal images of control (Sucrose) or *Pe* infected adult

782 posterior midguts from wild type animals or animals subject to adult-specific *sima* RNAi

783 overexpression (*sima^{-IR}*) within TTC (*dSRF^{ts}*>GFP). Scale bar: 100μm. **e**, **f**, Quantification of

784 tracheal coverage (e) and PH3$^+$ ISCs in posterior midguts as in (d). **a-f,** Statistics: Two-way

785 ANOVA followed by Sidak's multiple comparisons test; n= number of posterior midguts. **g**, **h**,

786   Representative confocal images of *btl* reporter expression (*btl-lacZ*; magenta) in control or *Pe*

787   infected adult posterior midguts without or with NAC (g, h, respectively). Dotted boxes

788   represent a higher magnification of the area pointed with an arrow. Scale bars: 50µm (main

789   figure); 20µm (close up view). **i,** Quantification of *btl-lacZ* staining intensity in TTC relative to

790   background within a defined region of posterior midguts as in (g, h). Statistics: Two-way

791   ANOVA followed by Sidak's multiple comparisons test; n= number of TTC from 9 to 10

792   posterior midguts/condition. **j,** Confocal images of control and damaged adult posterior

793   midguts from wild type animals or following *RNAi*-driven adult-specific *btl* knockdown (*btl^IR^*)

794   within TTCs. Scale bar: 100µm. **k, l,** Quantification of tracheal coverage (k) and PH3$^+$ ISCs in

795   posterior midguts as in (j). Statistics: Two-way ANOVA followed by Sidak's multiple

796   comparisons test; n= number of posterior midguts.

797   Error bars: ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

798   **Fig. 4 – Bidirectional FGF/FGFR signaling between the midgut and TTCs drives tracheal**

799   **remodelling and ISC proliferation during intestinal regeneration. a,** Representative

800   confocal images of *FGF/bnl* reporter expression (*bnl-lacZ*; magenta) in control and *Pe* treated

801   midguts in the presence or absence of NAC. Arrows point to reporter signal within ISCs/EBs

802   (small nuclei and stained with anti-Armadillo (Arm); pseudo-coloured in green). Asterisks point

803   to reporter signal within ECs (large nuclei and stained with anti-Discs large (Dlg); pseudo-

804   coloured in green). Dotted areas outline ISCs/EBs or ECs pointed by arrows or asterisks,

805   respectively. We noted consistent, unexplained downregulation of Dlg relative to Arm in

806   Sucrose and NAC treated midguts. Scale bars: 40µm (main figure); 9µm (close up view). **b,**

807   **c,** Quantification of *bnl-lacZ* staining within ISCs/EBs (b) and ECs (c) in posterior midguts as

808   in (a). **d,** Representative confocal images of *FGF/bnl* reporter expression (*bnl-lacZ*; magenta)

809   and TTCs (*dSRF>GFP*; green) in control (Sucrose) and regenerating (*Pe*) adult posterior

810   midguts in the presence or absence of NAC. Arrows indicate reporter signal within TTCs.

811   Dotted boxes show a magnified view of TTCs pointed by arrows. Scale bars: 40µm (main

812   figure); 20µm (close up view). **e,** Quantification of *bnl-lacZ* staining within TTCs in posterior

813   midguts as in (d). **a-e,** Statistics: Two-way ANOVA followed by Sidak's multiple comparisons

814   test; n= number of TTCs, ISC or ECs from 10-14 posterior midguts per condition. **f,** Confocal

815   images of control and *Pe* treated adult posterior midguts from wild type animals or upon RNAi-

816   driven adult-specific *bnl* knockdown (*bnl^IR^*) within TTCs. Scale bar: 50μm. **g, h,** Quantification

817   of tracheal coverage (g) and PH3$^+$ ISCs (h) in midguts as in (f). **i,** Representative brightfield

818   images of control (Sucrose) or regenerating (*Pe*) adult posterior midguts from wild type

819   animals or upon RNAi-driven adult *bnl* knockdown (*bnl^IR^*) within ISCs/EBs and ECs (*NP1>,*

820   *esg^ts^>GFP*). Scale bar: 50μm. **j, k,** Quantification of scored tracheal branching (j) and PH3$^+$

821   ISCs (k) in posterior midguts as in (i). **f-k,** Statistics: Two-way ANOVA followed by Sidak's

822   multiple comparisons test; n= number of posterior midguts.

823   Error bars: ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

824   **Fig.5 – Imp is a novel regulator of adult tracheal remodelling and intestinal**

825   **regeneration. a,** Schematic representation of targeted DamID approach used to profile RNA

826   pol II binding in adult TTCs. **b,** Gene Ontology term analysis corresponding to genes with

827   significant RNA pol II binding in adult TTCs from control midguts (Sucrose). **c,** List of genes

828   associated to neuronal processes showing significant RNA pol II binding in TTCs following *Pe*

829   treatment. Data is presented as the Log2 ratio of Dam-RNA Pol II/Dam (normalization control)

830   read counts. **d,** Profile of RNA Pol II binding to *Imp* in TTCs from control (Suc) and

831   regenerating (*Pe*) midguts. Boxes highlight areas with most prominent differences in RNA Pol

832   II binding. **e,** qRT-PCR for *Imp* mRNA expression from whole adult midguts and associated

833   tracheal tissue, in control (Sucrose) or *Pe* fed wild type animals. Statistics: Data are

834   represented as mean ± SEM of five biological replicates. Student's t test. **f, g,** Representative

835   confocal images of Imp protein reporter Imp::GFP (green) and TTCs (*dSRF>Cherry*; magenta)

836   in control (Sucrose) and regenerating (*Pe*) posterior midguts in the absence (f) or presence

837   (g) of NAC. Arrows indicate reporter signal within TTCs. Dotted boxes show magnified views

838   of TTCs pointed by arrows. Scale bars: 50μm (main figure); 12μm (close up view). **h,**

839   Quantification of Imp::GFP staining intensity relative to background within TTCs in posterior

840   midguts as in (f) and (g). Statistics: Two-way ANOVA followed by Sidak's multiple comparisons

841   test; n= number of TTC from 11-14 posterior midguts per condition. **i**, Representative confocal

842   images of control (Sucrose) or *Pe* treated adult posterior midguts from wild type animals or

843   upon RNAi-driven adult-specific *Imp* knockdown (*Imp^{-IR}*) within TTCs (green). Scale bar:

844   100μm. **j**, **k**, Quantification of tracheal coverage (j) and PH3$^+$ ISCs (k) in posterior midguts as

845   in (i). Statistics: Two-way ANOVA followed by Sidak's multiple comparisons test; n= number

846   of posterior midguts.

847   Error bars: ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

848   **Fig.6 – Imp dependent activation of Myc in TTCs is required for tracheal remodelling**

849   **and intestinal regeneration. a,** Representative confocal images of Myc staining (magenta)

850   and TTCs (*dSRF>GFP*; green) in control (Sucrose) or regenerating (*Pe*) midguts from wild

851   type animals or upon RNAi-driven adult-specific *Imp* knockdown (*Imp^{-IR}*) within TTCs. Arrows

852   indicate Myc signal in TTCs. Dotted boxes show magnified views of TTCs pointed by arrows.

853   Scale bars: 20μm (main figure); 10μm (close up view). **b**, Quantification of Myc staining

854   intensity relative to background in TTCs from posterior midguts as in (a). Statistics: Two-way

855   ANOVA followed by Sidak's multiple comparisons test; n= number of TTCs from 15-18

856   posterior midguts per condition. **c**, Representative confocal images of control (Suc) or *Pe*

857   treated adult posterior midguts from wild type animals or upon RNAi-driven adult-specific *myc*

858   knockdown (*myc^{-IR}*) within TTCs (green). Scale bar: 100μm. **d**, **e**, Quantification of tracheal

859   coverage (d) and PH3$^+$ ISCs (e) in posterior midguts as in (c). Statistics: Two-way ANOVA

860   followed by Sidak's multiple comparisons test; n= number of posterior midguts quantified.

861   Error bars: ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

862   **Fig.7 – *Trachealess* downregulation in TTCs is necessary for adult tracheal remodelling**

863   **and damage induced ISC proliferation. a,** Profile of RNA Pol II binding to *trachealess* (*trh*)

864   within TTCs of control (Suc) and regenerating (*Pe*) midguts. **b**, qRT-PCR for *trh* mRNA

865   expression from whole adult midguts and associated tracheal tissue of control (Sucrose) or

866   *Pe* fed animals. <u>Statistics</u>: Data are represented as mean ± SEM of five biological experimental

867   replicates. Student's t test. **c**, Representative confocal images of Trh staining (red) and TTCs

868   (*dSRF>GFP*; green) in control (Sucrose) or regenerating (*Pe*) midguts. Arrows indicate Trh

869   signal within TTCs. Dotted boxes show magnified views of TTCs pointed by arrows. Scale

870   bars: 20µm (main figure); 10µm (close up view). **d**, Quantification of Trh staining intensity

871   relative to background within TTCs in posterior midguts as in (c). <u>Statistics</u>: Student's t test;

872   n= number of TTC from 14 posterior midguts per condition. **e**, Representative confocal images

873   of *trh* reporter expression (*trh-lacZ*; magenta) and TTCs (*dSRF>GFP*; green) in control

874   (Sucrose) or *Pe* infected midguts without (Damage) or with NAC (Damage + NAC), and

875   following incubation in normal food during 32 hrs, post-infection (Recovery). Arrows indicate

876   *trh-lacZ* signal within TTCs. Dotted boxes show magnified views of TTCs pointed by arrows.

877   Scale bars: 50µm (main figure); 20µm (close up view). **f**, Quantification of *trh-lacZ* staining

878   intensity relative to background within TTCs of midguts as in (e). <u>Statistics</u>: Student's t test;

879   n= number of TTCs from 12-21 posterior midguts per condition. **g**, Representative confocal

880   images from control (Suc) or regenerating (*Pe*) adult posterior midguts from wild type animals

881   or upon adult-specific *trh* overexpression (*trh*) or RNAi-driven knockdown (*trh^{-IR}*) within TTCs

882   (green). Scale bar: 100µm. **h**, **i**, Quantification of tracheal coverage (h) and PH3$^+$ ISCs (i) in

883   posterior midguts as in (g). <u>Statistics</u>: Two-way ANOVA, followed by Sidak's multiple

884   comparisons test; n= number of posterior midguts. **j**, Schematic representation of working

885   model.

886   Error bars: ±SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

887

888   **Extended data Fig. 1 – Characterization and quantification of adult tracheal remodelling**

889   **following intestinal damage. a**, **(top)**, Representative confocal image of adult posterior

890   midgut and associated TTCs (green). **(bottom)**, illustration of the different steps followed for

891   the quantification of gut tracheal coverage as explained in Methods. Scale bar: 100µm. Box in

892   top panel highlights the area shown in the bottom panels **b,** Representative confocal images

893   of TTCs (green) from posterior midguts of control animals (Suc) or animals fed with Bleomycin

894   or DSS. Scale bar: 50μm. **c**, Representative confocal images of control (Suc) or damaged

895   (*Pe*) adult posterior midguts (shaded in brown). Scale bar: 100μm. **d**, **e**, Quantification of

896   posterior midgut area (d) and ratio of tracheal coverage over adult posterior midgut area (e).

897   **f**, Representative confocal images of TTCs (green) from control (Suc) or *Pe* damaged adult

898   posterior midguts stained with anti-PH3 to label proliferating ISCs (red). Bottom panels

899   represent orthogonal views of the midguts shown in top panels. Scale bar: 50μm. **g,**

900   Quantification of individual or combined TTC nuclei and PH3$^+$ cells in control or *Pe* infected

901   midguts. **c-g**, <u>Statistics</u>: Student's t test; n= number of posterior midguts; error bars ±SEM.

902   ****p< 0.0001. **h**, Confocal images of adult TTCs assessed at the indicated time points during

903   and after intestinal damage. White lines trace individual TTCs. Scale bar: 50μm. **i**, Correlation

904   graph between TTC coverage and ISC proliferation for each of the time points and conditions

905   presented in (h). <u>Statistics</u>: Pearson's correlation coefficient (r)=0.9660 and 0.9962;

906   **p=0.0075 and 0.0038 in damage and recovery phases, respectively.

907   **Extended data Fig. 2 – Hypoxia or impaired TTC remodelling does not affect ISC**

908   **survival. a**, Confocal images of TTCs (green) and the HIF-1α/Sima activity reporter *ldh-lacZ*

909   (grey) in adult posterior midguts from animals in normoxia (21% O_2) or subjected to 24 hrs of

910   hypoxia (3% O_2). Dotted boxes show a magnified view of TTCs pointed by arrows. Scale bars:

911   50μm (main figure); 20μm (close up view). **b**, Quantification of *ldh-lacZ* staining intensity within

912   TTCs, relative to background, in midguts as in (a). <u>Statistics</u>: Student's t test; n= number of

913   TTC from 9 posterior midguts per condition. **c**, Quantification of tracheal coverage in adult

914   posterior midguts as in (a). <u>Statistics</u>: Student's t test; n= number of posterior midguts. **d, e**,

915   Confocal images of adult posterior midguts stained with anti-Dcp1 (magenta) to visualize cell

916   death in control animals; upon adult-specific *bax* overexpression in ECs (d); animals subjected

917   to hypoxia or to the indicate genetic TTC disruptions (e). **d, e**, Upper panels (apical tissue

918   views); lower panels (longitudinal sections showing intestinal tube lumen). Scale bar: 50μm.

919   The high level of Dcp1 staining in the lumen of midguts subject to *bax* overexpression in ECs

920   (d) corresponds to many delaminating/dying cells. Dotted box in (e) shows a magnified view

921   of an apoptotic EC pointed by arrow in main figure and identified by its large nuclei and Dcp1

922   staining. Scale bars: 50μm (main figure); 20μm (close up view). **f, g,** Quantification of Dcp1

923   staining in midguts as in (d, e). Statistics: Student's t test; n= number of posterior midguts; **h,**

924   Confocal images of TTCs (green) and ISCs detected with a *Delta-lacZ* reporter (magenta) and

925   anti-Armadillo (Arm) staining (grey) in midguts as in (e). Dotted boxes in middle panels indicate

926   the magnified areas in the lower panels. Scale bars: 50μm (main figure); 20μm (close up view).

927   **i**, Quantification of the number of *Delta-lacZ* positive cells in midguts as in (h). Statistics:

928   Student's t test; n= number of posterior midguts.

929   Error bars: ±SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

930   **Extended data Fig. 3 – Gut associated TTCs are responsive to local signals from the**

931   **damaged intestinal epithelium. a**, Examples of tracheal branching levels assigned to each

932   of the scores used for the quantification of tracheal coverage from light microscopy images.

933   Scale bar: 50μm. **b**, **c (top panels)**, Quantification of tracheal branching from confocal (b) and

934   brightfield images (c) of control (Suc) or *Pe* damaged posterior midguts. **b**, **c (bottom panels)**

935   Representative confocal (b) or brightfield images (c) from midguts as in top panels. Scale bars:

936   50μm. **d**, Representative brightfield images from control (Suc) or *Pe* damaged posterior

937   midguts from wild type animals or upon adult-specific *catalase* overexpression within ECs.

938   Scale bar: 50μm. **e**, **f**, Scoring of tracheal branching (e) and quantification of PH3$^+$ ISCs (f) in

939   posterior midguts as in (d). Statistics: Two-way ANOVA followed by Sidak's multiple

940   comparisons test; n= number of posterior midguts quantified. **g**, Representative brightfield

941   images of posterior midgut from control animals or upon adult-specific overexpression of *bax*

942   in ECs. Scale bar: 50μm. **h**, **i**, Quantification of tracheal branching (h) and PH3$^+$ ISCs (i) in

943   posterior midguts as in (g). **b, c and g-i,** Statistics: Student's t test; n= number of posterior

944   midguts.

945   Error bars: ±SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

946   **Extended data Fig. 4 – *blistered/dSRF* expression is upregulated in adult TTCs following**

947   **intestinal damage or hypoxia and it regulates damage induced TTC remodelling and**

948   **ISC proliferation. a**, qRT-PCR for *blistered (bs)/dSRF* mRNA expression from adult midguts

949   and associated tracheal tissue, in control (Sucrose) or *Pe* treated midguts. Statistics: Data are

950   represented as mean ± SEM of five biological replicates; Student's t test. **b**, Representative

951   confocal images of *bs-lacZ* reporter expression (red) in control (Sucrose) and regenerating

952   (*Pe*) adult posterior midguts stained with Chitin Binding Protein (CBP, green) to visualise all

953   tracheal tissue. Dotted boxes show a magnified view of TTCs pointed by arrows. Scale bars:

954   50μm (main figure); 20μm (close up view). **c**, Quantification of *bs-lacZ* staining intensity within

955   TTCs in posterior midguts as in (b). **d**, Representative confocal images of *bs-lacZ* reporter

956   expression (red) in adult posterior midguts from animals housed in normoxia (21% O_2) or

957   subjected to 16 hrs of hypoxia (3% O_2) and stained with CBP (green). Dotted boxes show a

958   magnified view of TTCs pointed by arrows. Scale bars: 50μm (main figure); 20μm (close up

959   view). **e**, Quantification of *bs-lacZ* staining intensity within TTCs in posterior midguts as in (d).

960   **b-e,** Statistics: Student's t test; n= number of TTC from 12-15 posterior midguts per condition.

961   **f**, Confocal images of control (Sucrose) and damaged (*Pe*) adult posterior midguts from wild

962   type animals or following *RNAi*-driven adult-specific *bs* knockdown (*bs^{-IR}*) within TTCs. Scale

963   bar: 100μm. **g**, **h**, Quantification of tracheal coverage (g) and PH3^{+} ISCs in posterior midguts

964   as in (f). Statistics: Two-way ANOVA followed by Sidak's multiple comparisons test; n=

965   number of posterior midguts quantified.

966   Error bars: ±SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

967   **Extended data Fig. 5 – Multiple sources of Bnl individually contribute to regenerative**

968   **ISC proliferation in the adult *Drosophila* midgut, independently of tracheal remodelling.**

969   **a**, Representative confocal images of *FGF/bnl* reporter expression *bnl>GFPnlacZ* (green) in

970   control (Sucrose) and regenerating (*Pe*) adult posterior midguts stained with CBP (magenta)

971   to visualise all tracheal tissue and PH3 (magenta). Dotted boxes in left panels indicate the

972   area magnified in the right panels. Scale bars: 100μm (main figure); 40μm (close up view). **b**,

973   Quantification of *bnl>GFPnlacZ* staining intensity relative to background within TTCs in

974   posterior midguts as in (a). Statistics: Student's t test; n= number of TTC from 12 posterior

975   midguts per condition. **c, d** Quantification of tracheal coverage (c) and PH3$^+$ ISCs (d) from wild

976   type animals or upon adult-specific *bnl* overexpression within TTCs. Statistics: Student's t test;

977   n= number of posterior midguts. **e**, Representative brightfield images from control (Suc) or *Pe*

978   damaged posterior midguts from wild type animals or following RNAi-driven adult-specific *bnl*

979   knockdown (*bnl^{-IR}*) within ISCs/EBs. Scale bar: 50μm. **f, g**, Quantification of tracheal branching

980   (f) and PH3$^+$ ISCs (g) in posterior midguts as in (e). **h**, Representative brightfield images from

981   control (Suc) or *Pe* damaged posterior midguts from wild type animals or upon adult-specific

982   *bnl* knockdown (*bnl^{-IR}*) within ECs. Scale bar: 50μm. **i, j**, Quantification of tracheal branching

983   (i) and PH3$^+$ ISCs (j) in posterior midguts as in (h). **e-j,** Statistics: Two-way ANOVA followed

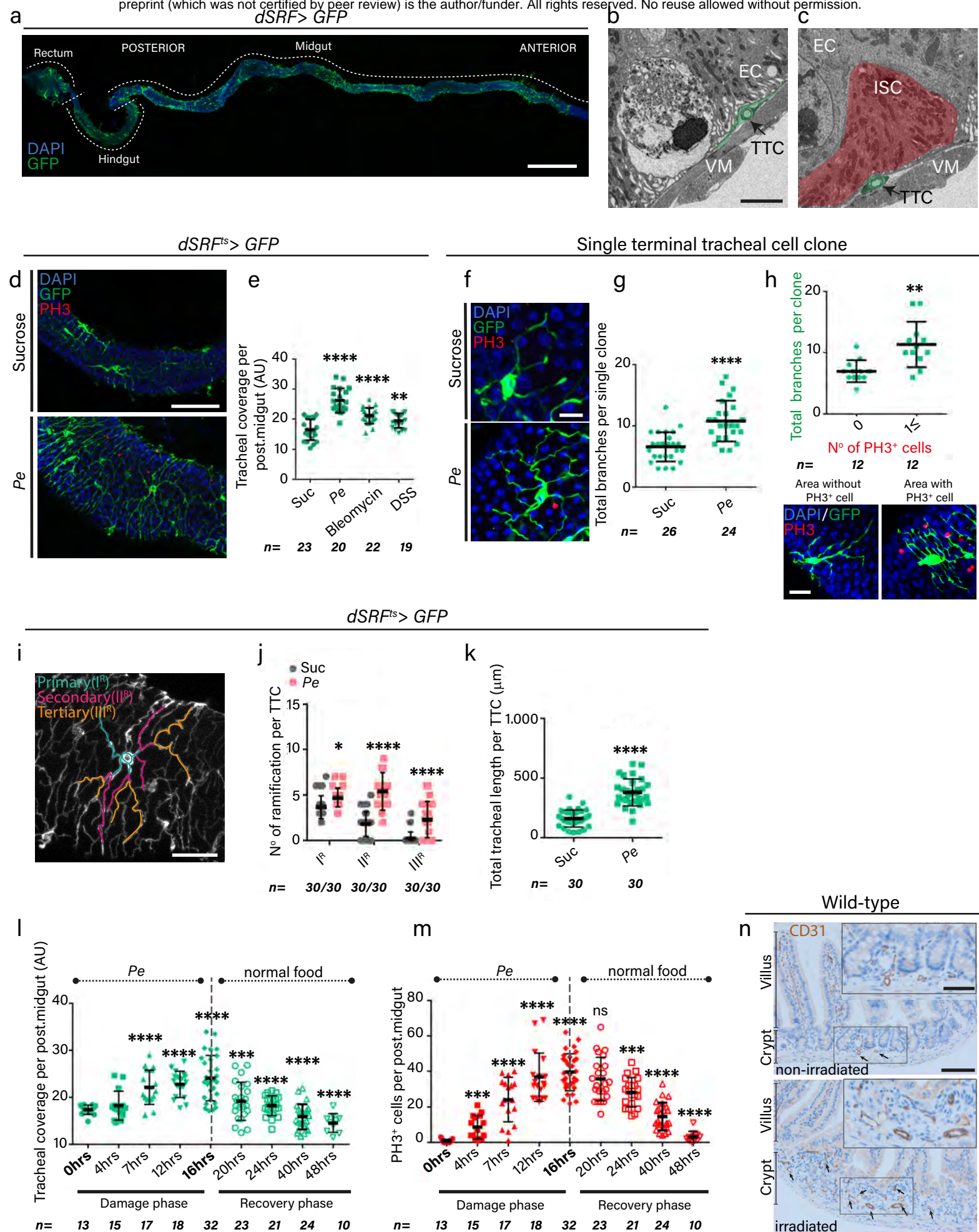984   by Sidak's multiple comparisons test; n= number of posterior midguts quantified.

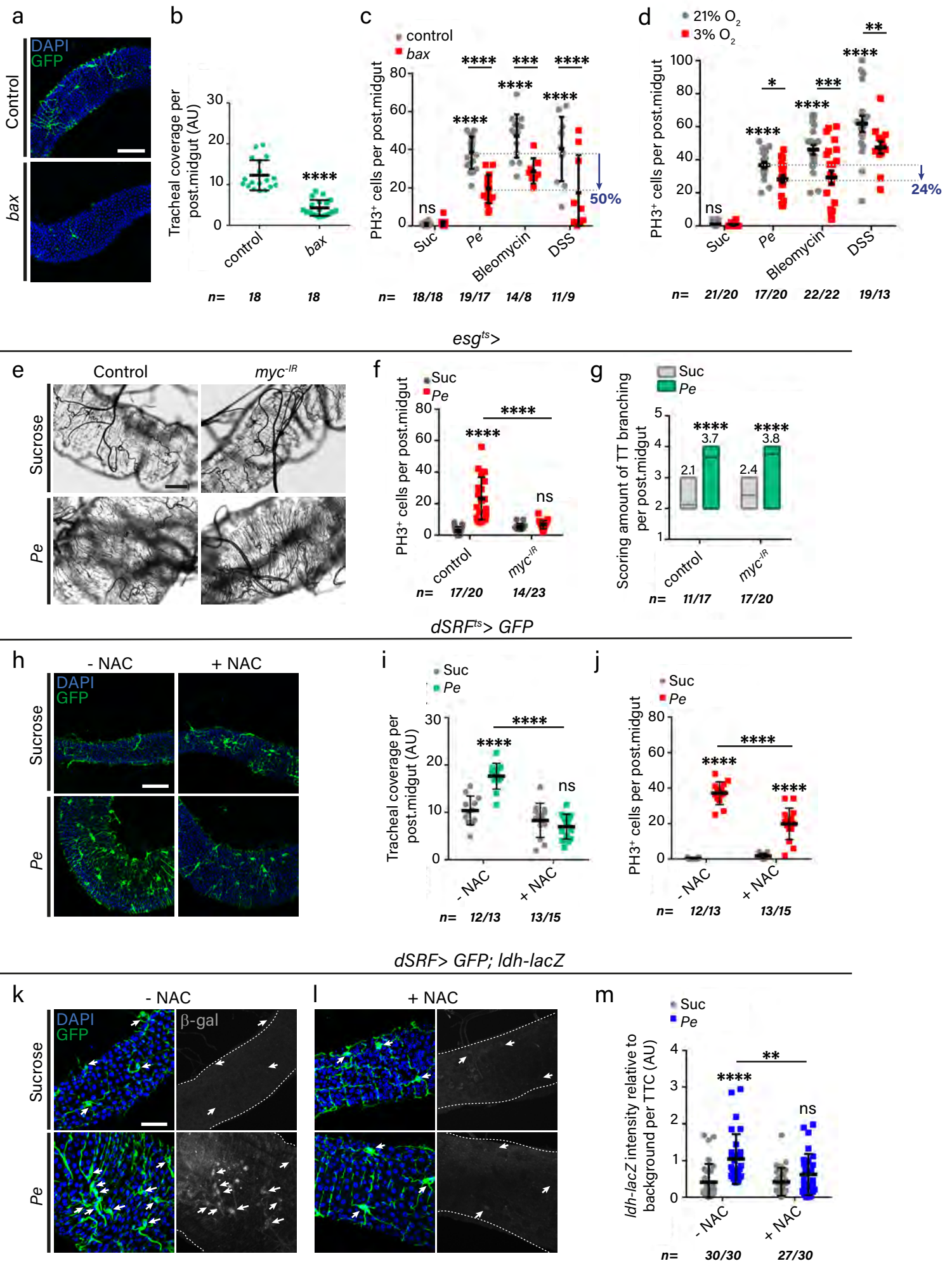985   Error bars: ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$.

986   **Extended data Fig. 6 – Individual sources of gut-derived Bnl are redundant for TTC**

987   **remodelling but required for damage induced ISC proliferation. a**, Representative

988   brightfield images of control (Sucrose) or regenerating (*Pe*) adult posterior midguts from wild

989   type animals or upon RNAi-driven adult-specific *btl* knockdown (*btl^{-IR}*) within ISCs/EBs. Scale

990   bar: 50μm. **b, c**, Quantification of tracheal branching (b) and PH3$^+$ ISCs (c) in posterior midguts

991   as in (a). Statistics: Two-way ANOVA followed by Sidak's multiple comparisons test; n=

992   number of posterior midguts. **d**, Representative confocal images of activated MAPK (pERK)

993   staining (red), Arm (grey) and ISCs/EBs (*esg>GFP*, green) in control (Sucrose) or

994   regenerating (*Pe*) adult posterior midguts from wild type animals or upon RNAi-driven adult-

995   specific *btl* knockdown (*btl^{-IR}*) within ISCs/EBs. Dotted boxes show a magnified view of the

996   ISCs/EBs pointed by arrows. Scale bars: 50μm (main figure); 40μm (close up view). **e**,

997   Quantification of pERK staining intensity relative to background within ISCs/EBs in posterior
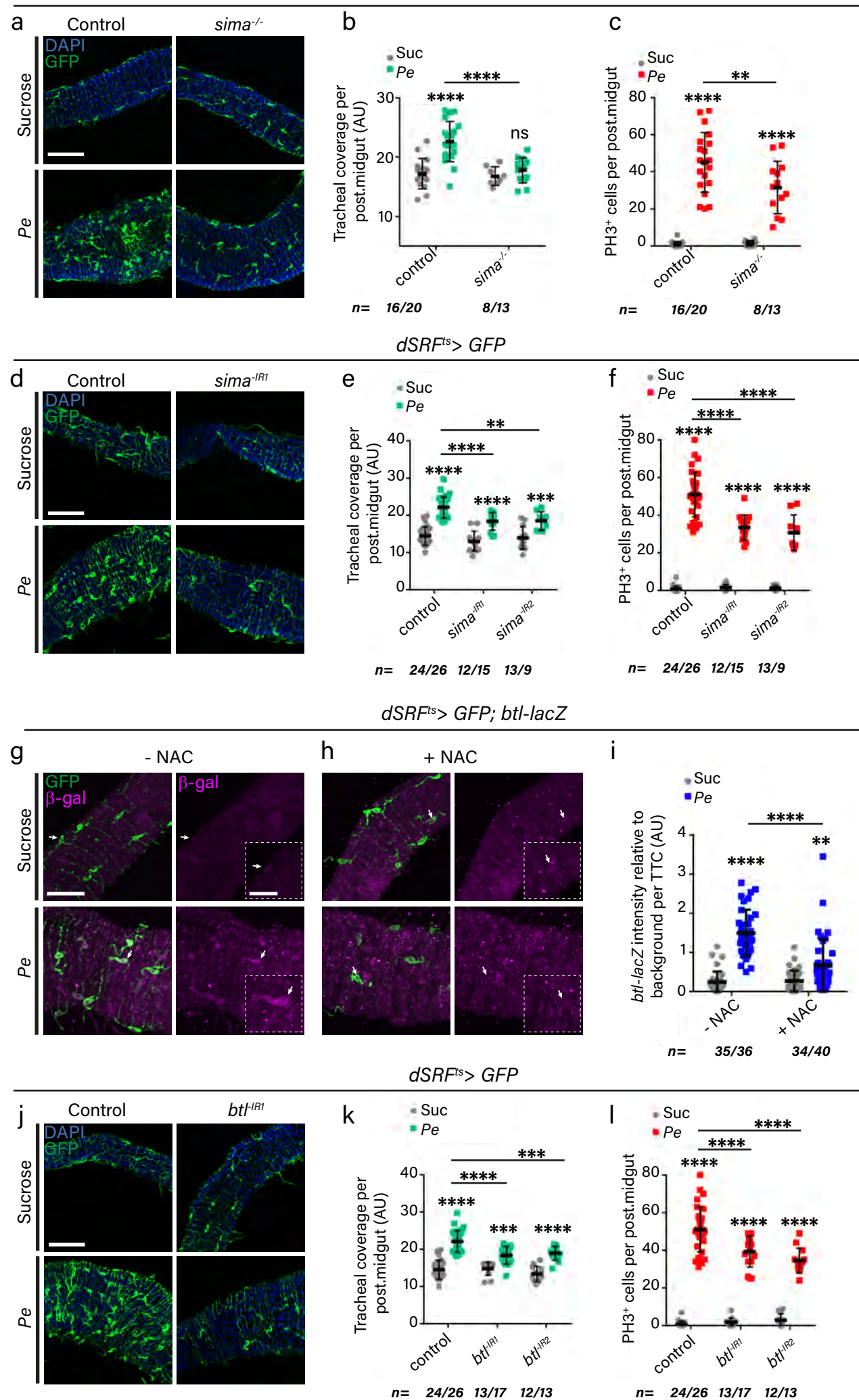
998   midguts as in (d). <u>Statistics</u>: Two-way ANOVA followed by Sidak's multiple comparisons test;

999   n= number of ISCs/EBs from 11 posterior midguts per condition.

1000   Error bars: ±SEM; *p < 0.05, **p < 0.01, ***p < 0.001 ****p< 0.0001.

1001   **Extended data Fig. 7 – Genome wide RNA pol II binding profile within adult TTC by**

1002   **targeted DamID (TaDa). a**, Scatterplots indicating correlation between TaDa replicates for

1003   each of the conditions used in this study. Significant correlation is observed between replicates

1004   of each condition. Each data point represents the average score for each gene (log2 Dam-pol

1005   II/ Dam-only). **b**, RNA Pol II binding profile to *bnl* in TTCs from control (Suc) and damaged

1006   (*Pe*) adult midguts. Boxes highlight areas with most prominent differences in RNA Pol II

1007   binding.

1008

1009

1010

1011

Perochon, Fig1

a *dSRF> GFP*

Rectum — POSTERIOR — Midgut — ANTERIOR — Hindgut — DAPI GFP

b — EC — VM — TTC

c — EC — ISC — VM — TTC

dSRF^{ts}> GFP

d — DAPI GFP PH3 — Sucrose — Pe

e — Tracheal coverage per post.midgut (AU) — Suc Pe Bleomycin DSS — n= 23 20 22 19

Single terminal tracheal cell clone

f — DAPI GFP PH3 — Sucrose — Pe

g — Total branches per single clone — Suc Pe — n= 26 24
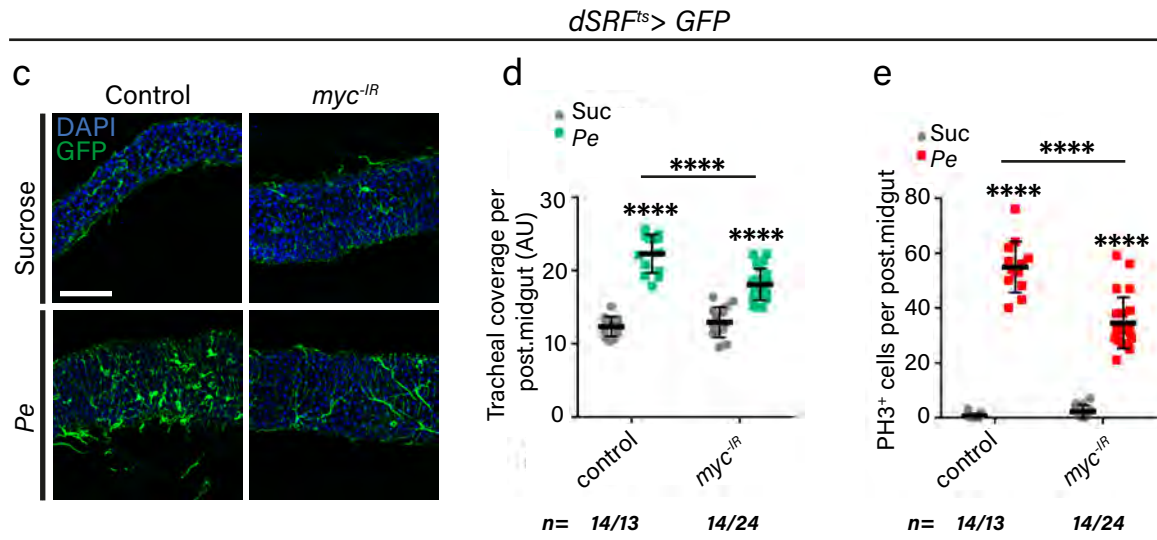
h — Total branches per clone — 0 1≥ — N° of PH3+ cells — n= 12 12 — Area without PH3+ cell — Area with PH3+ cell — DAPI/GFP PH3

dSRF^{ts}> GFP

i — Primary(I^R) Secondary(II^R) Tertiary(III^R)

j — N° of ramification per TTC — Suc Pe — I^R II^R III^R — n= 30/30 30/30 30/30

k — Total tracheal length per TTC (μm) — Suc Pe — n= 30 30

l — Tracheal coverage per post.midgut (AU) — Pe — normal food — 0hrs 4hrs 7hrs 12hrs 16hrs 20hrs 24hrs 40hrs 48hrs — Damage phase — Recovery phase — n= 13 15 17 18 32 23 21 24 10

m — PH3+ cells per post.midgut — Pe — normal food — 0hrs 4hrs 7hrs 12hrs 16hrs 20hrs 24hrs 40hrs 48hrs — Damage phase — Recovery phase — n= 13 15 17 18 32 23 21 24 10

n — Wild-type — CD31 — Villus — Crypt — non-irradiated — Villus — Crypt — irradiated

Figure 5

dSRF> GFP

a DAPI GFP — hindgut, posterior midgut, malpighian tubules

"treshold" → "skeletonize"

dSRF^{ts}> GFP

b Sucrose / Bleomycin / DSS — DAPI GFP

dSRF> GFP

c Sucrose / Pe — posterior midgut area

d Post. midgut area (mm²) — ns — Suc / Pe — n= 32 32

e tracheal coverage/ post.midgut area (AU) — **** — Suc / Pe — n= 32 32

f *dSRF> GFP* — Sucrose / Pe — DAPI GFP PH3 — Orthogonal view

g *dSRF^{ts}> GFP, RedStinger* — Suc / Pe — N° of cells/post.midgut — TTC (ns) / PH3+ (****) / TTC & PH3+ (ns) — n= 13/13 13/13 8/8

h *dSRF^{ts}> GFP* — DAPI GFP — 0hrs 4hrs 7hrs 12hrs 16hrs | 20hrs 24hrs 40hrs 48hrs — **Damage phase** (*Pe*) → Remove damage source → **Recovery phase** (normal food)

i Tracheal coverage per post.midgut (AU) — Damage phase / Recovery phase — 0hrs 4hrs 7hrs 12hrs 16hrs 20hrs 24hrs 40hrs 48hrs — Pearson r (**) — PH3+ cells per post.midgut

a

Score 1   Score 2   Score 3   Score 4

Sucrose phenotype   *Pe* phenotype

dSRF^{ts} > GFP

b

Tracheal coverage per post.midgut (AU)

****

Suc   Pe

DAPI
GFP

n=   9   10

Confocal microscopy

c

Scoring amount of TT branching

****
3.9

2.1

Suc   Pe

n=   10   8

vs   **Light microscopy**

NP1^{ts} >

d

Control   *catalase*

Sucrose

Pe

e

Scoring amount of TT branching per post.midgut

Suc
Pe

****
3.7

2.4

****

****
3.0

2.5

control   catalase

n=   14/17   17/18

f

PH3+ cells per post.midgut

Suc
Pe

****

****

****

****

control   catalase

n=   24/25   14/25

g

Control   *bax*

Sucrose

h

Scoring amount of TT branching per post.midgut

****
3.9

2.2

control   bax

n=   15   15

i

PH3+ cells per post.midgut

****

control   bax

n=   20   17

bs-lacZ

a — *bs* — Relative mRNA expression /*gapdh1*; Suc, Pe; *

b — Sucrose, Pe; CBP, β-gal

c — *bs-lacZ* intensity relative to background per TTC (AU); Suc, Pe; ****; n= 48, 56

d — 21% O$_2$, 3% O$_2$; CBP, β-gal

e — *bs-lacZ* intensity relative to background per TTC (AU); 21% O$_2$, 3% O$_2$; ****; n= 60, 56

dSRF^{ts}> GFP

f — Control, *bs^{-IR}*; Sucrose, Pe; DAPI, GFP

g — Tracheal coverage per post.midgut (AU); Suc, Pe; control, *bs^{-IR}*; ****, *; n= 14/17, 16/16

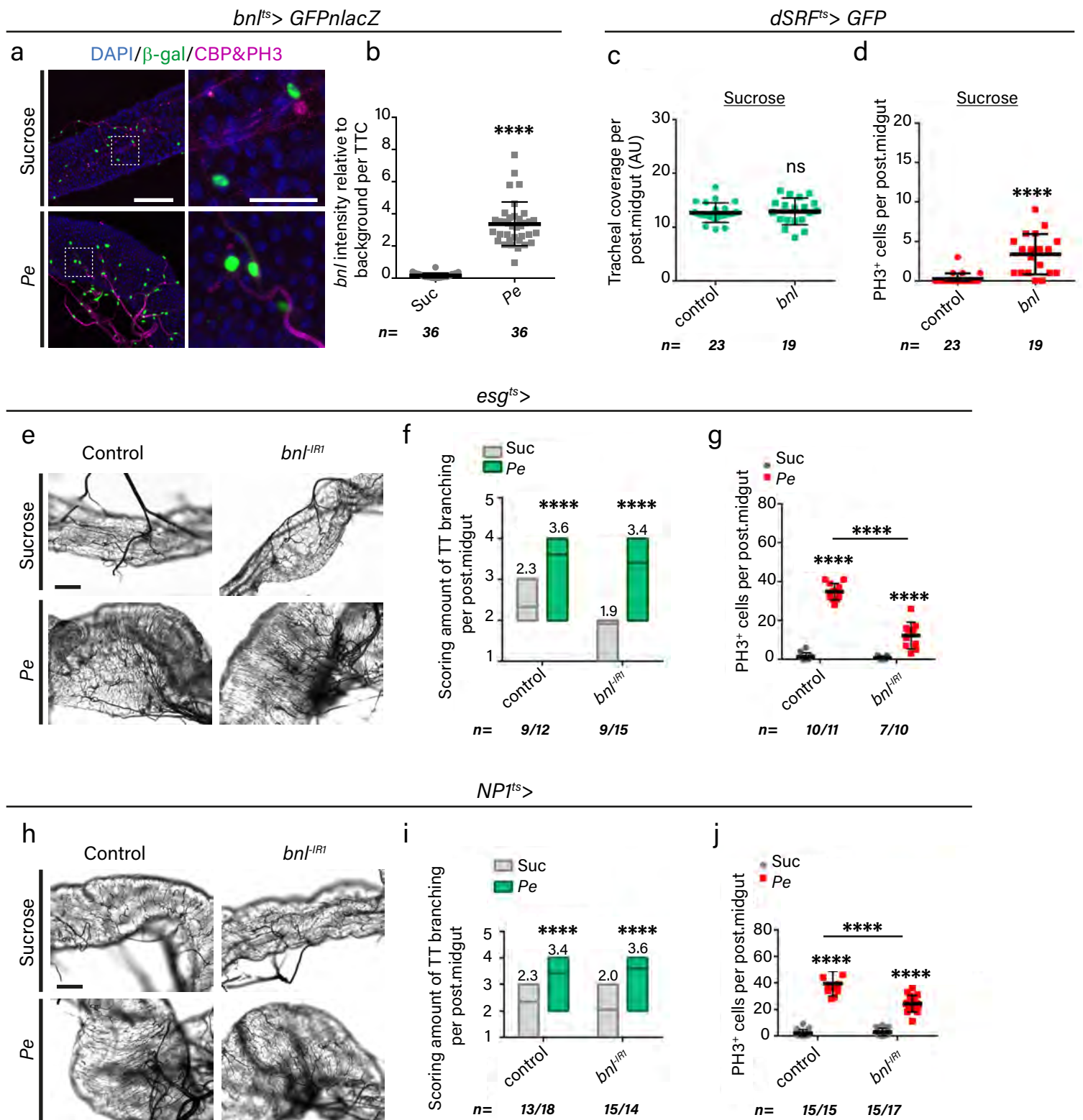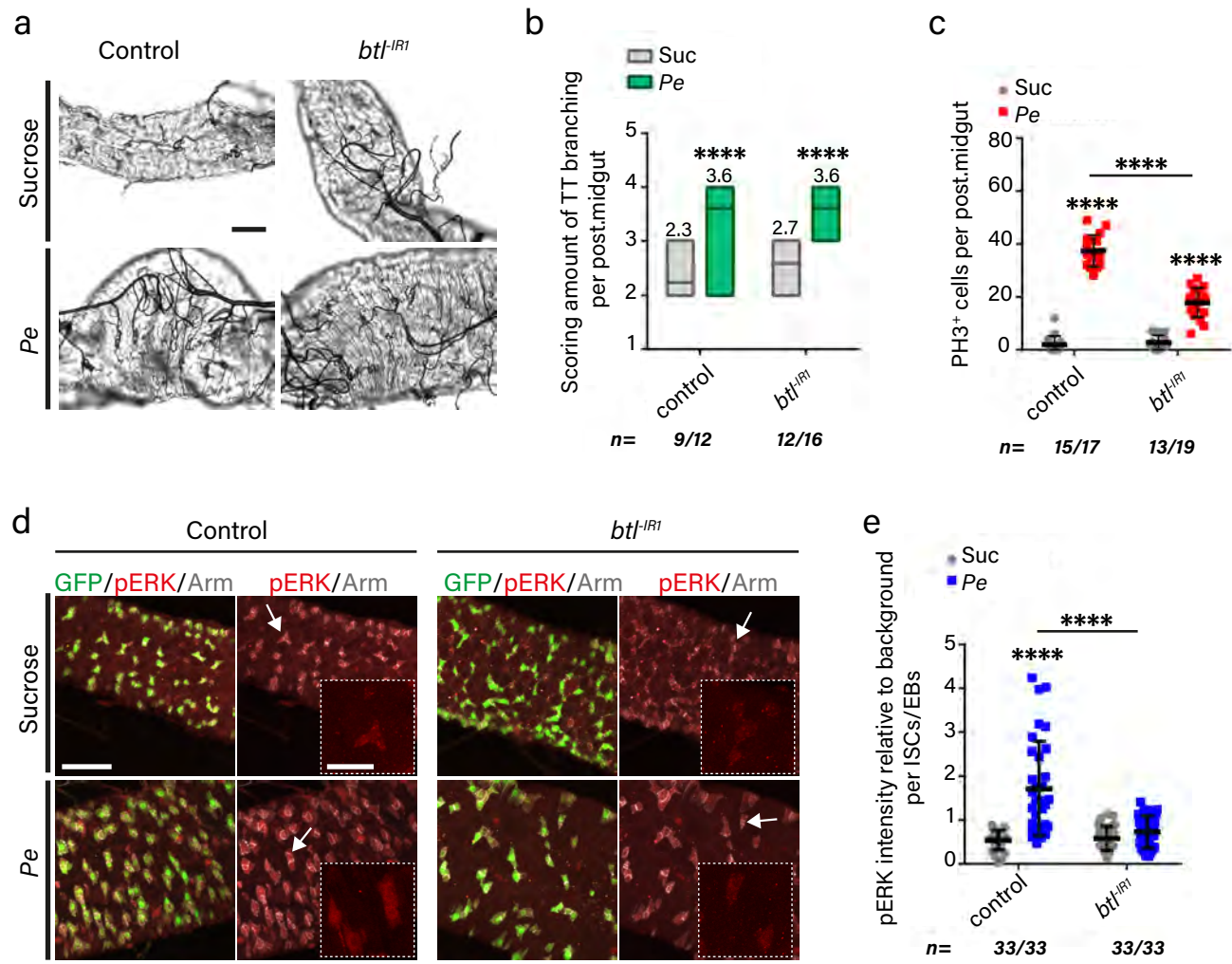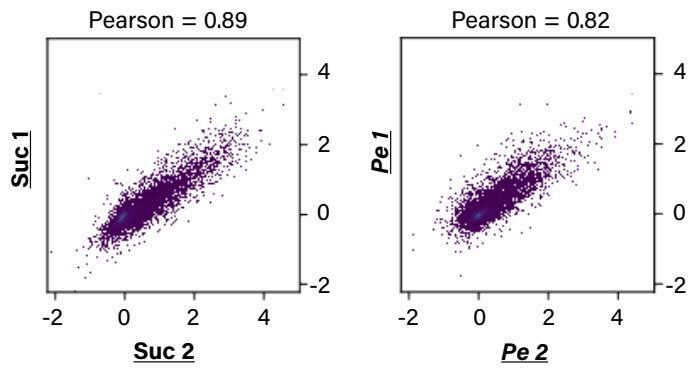**h** — PH3$^+$ cells per post.midgut; Suc, Pe; control, *bs^{-IR}*; ****, ****; n= 14/17, 16/16

esg^{ts}> GFP

a

Pearson correlation of average scores per genes

b