1   ***Drosophila scribble* mutant tumors undergo a transition from a growth arrest state**

2   **to a proliferative state over time**

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25

26   **Summary:**

27

28   The *Drosophila* neoplastic tumor suppressor gene (nTSG) mutant tumors have

29   successfully modeled many aspects of human tumor progression. However, the fly nTSG

30   mutant tumors progress rapidly over days. This is in contrast with most human tumors

31   which develop slowly, harbor heterogeneous cell populations for selection and undergo

32   an evolution-like process. Whether the fast-growing fly nTSG mutant tumors have

33   capacity for evolution remains unclear. Through quantitative analysis of the *scrib* mutant

34   tumor growth, we found that the *scrib* mutant tumors evolve to display different growth

35   rates and cell cycle profiles over time. Multiple growth-regulatory signaling pathways

36   show quantitative differences in early versus late *scrib* mutant tumors. These data suggest

37   that the *scrib* mutant tumors undergo a transition from a growth arrest state to a

38   proliferative state. Through longitudinal single cell RNA (scRNA) data analysis we found

39   that the *scrib* mutant tumors harbor heterogeneous cell populations likely of distinct

40   proliferative states, which are available for potential selection. This study raises the

41   possibility of studying tumor evolution in a genetically accessible and fast-growing

42   invertebrate tumor model.

43

44   **Introduction**

45

46   Proteins essential for maintaining epithelial structures, such as cell polarity complexes,

47   are involved in growth control (Bilder, 2004; Boggiano and Fehon, 2012; Sun and Irvine,

48   2016). For example, the basolateral Scribble complex, composed of Scribble (Scrib),

49   Discs large (Dlg) and Lethal giant larvae (L(2)gl), were discovered as a group of

50   "neoplastic tumor suppressor genes" (nTSGs) in *Drosophila* (Bilder et al., 2000; Bilder

51   and Perrimon, 2000; Gateff, 1978; Woods and Bryant, 1991). *Drosophila* larvae

52   homozygous mutant for any of the nTSGs grow into giant larvae with tumorous imaginal

53   discs and optic lobes. These mutant tumors fail to differentiate and grow into masses that

54   survive serial transplantations, induce cachexia and eventually kill the hosts (Figueroa-

55   Clarevega and Bilder, 2015; Gateff, 1978). Studies of *Drosophila* nTSGs over decades

56   have provided valuable insights into the mechanisms of growth control and tumorigenesis

57   (Bilder, 2004; Gonzalez, 2013; Pastor-Pareja and Xu, 2013; Richardson and Portela,

58   2018; Sonoshita and Cagan, 2017). For example, analyses of the nTSG mutant clonal

59   growth have revealed cell competition-mediated tumor suppression mechanisms, the

60   cooperative actions of multiple conserved signaling pathways during tumor development,

61   and tumor microenvironment influences (Brumby and Richardson, 2003; Chen et al.,

62   2012; Cordero et al., 2010; Igaki et al., 2006; Katheder et al., 2017; Pagliarini and Xu,

63   2003; Vaughen and Igaki, 2016; Yamamoto et al., 2017).

64

65   Interestingly, while the fly nTSG mutant tumors have successfully modeled many aspects

66   of human epithelial cancers, it was noted that for the fly nTSG tumors that progress

67   rapidly over days, a single gene mutation is sufficient to cause tumorigenesis. This is in

68   contrary to human tumors which typically develop slowly over months and years and

69   supported a multiple-hit model (Bilder, 2004; Hanahan and Weinberg, 2000; Nordling,

70   1953). Human tumors have been shown to display a variable degree of genetic and

71   epigenetic intratumor heterogeneity (ITH) that provides a foundation for selection and

72   tumor evolution (McGranahan and Swanton, 2017). Whether the fast-growing fly nTSG

73   mutant tumors have capacity for evolution has remained unclear.

74

75   Through quantitative analysis of the *scrib* mutant tumor growth, we found that over time

76   the *scrib* mutant tumors display different growth rates and cell cycle profiles. Moreover,

77   multiple signaling pathways display quantitative differences in early versus late *scrib*

78   mutant tumors. We demonstrated that high JNK signaling activity is a primary cause of

79   growth arrest in early *scrib* mutant tumors. These data suggest that the *scrib* mutant cells

80   undergo a transition from a growth arrest state to a proliferative state during tumor

81   progression. Longitudinal scRNA data analysis further reveals heterogeneity in the *scrib*

82   mutant tumors that potentially provides opportunities for selection and drives the

83   transition from a growth arrest state to a proliferative state as a population.

84

85   **Results**

86

87   **The *scrib* mutant tumors display different growth rates over time.**

88

89 To explore potential evolving traits during the *scrib* mutant tumor progression, we first

90 monitored the growth of wing imaginal discs derived from 3-hour egg collection of a

91 *scrib[1]*/TM6B stock (Bilder and Perrimon, 2000) (Figure 1A-B). On average, at 4-day and

92 5-day after egg laying (AEL), the growth rate of the *scrib* mutant tumors is around 25%-

93 30% of that of control imaginal discs raised at identical conditions (Figure 1B). By 7-day

94 AEL the growth rate of the *scrib* mutant tumors is comparable with that of the 5-day AEL

95 control group (Figure 1B). Note that the *scrib* mutant tumors would continue growth to

96 sizes consistent with previous reports (Bilder et al., 2000; Gateff, 1978). Using phospho-

97 Histone H3 (PH3) as a marker for mitotic cells, we detected the 4-day AEL *scrib* mutant

98 tumors harbor much less PH3+ cells per unit volume than the control group (Figure 1C-D

99 and 1G). By 5-day AEL the *scrib* mutant tumors contain comparable number of PH3+

100 cells per unit volume with the control larvae as the wild type control group approached

101 the end of growth period (Figure 1E-F and 1G).

102

103 To test whether the initial slow-growth phenotype we observed in the early *scrib* mutant

104 tumors is specific to the *scrib[1]* allele, we generated tumors depleted of Scrib or Dlg at the

105 posterior region of wing discs through *engrailed*-Gal4 mediated RNAi. Although the

106 mosaic clones depleted of Scrib or Dlg are eliminated through cell competition (Brumby

107 and Richardson, 2003; Igaki et al., 2006; Vaughen and Igaki, 2016; Yamamoto et al.,

108 2017), the cell competition process does not cross segmentation boundary (Johnston,

109 2009; Morata and Ripoll, 1975; Simpson, 1979; Simpson and Morata, 1981). Therefore,

110 we can analyze the growth of the posterior *scrib RNAi* and *dlg RNAi* tumors independent

111 of the influence of cell competition. At 4-day and 5-day AEL, the volume of the *scrib*

112 RNAi and *dlg* RNAi tumors is much smaller than the size of the posterior region in

113 control wing discs (Figure 1H-Q). Meanwhile, the anterior regions of the *scrib* RNAi, *dlg*

114 RNAi and control imaginal discs have comparable average volumes (Figure 1H-Q),

115 indicating that the slow-growth phenotype observed in early *scrib* tumors is likely to be

116 independent of specific alleles used and an overall larval developmental delay.

117 Interestingly, from 4-day to 6-day AEL we could detect an increase of PH3+ cell number

118 in the posterior *scrib* RNAi and *dlg* RNAi tumors (Figure 1H-U), indicative of changes in

119   growth rates during tumor progression. Note that larvae harboring *scrib* RNAi or *dlg*

120   RNAi imaginal discs turned into pupae by 7-day AEL, preventing further measurement

121   of growth rates.

122

123   **The *scrib* mutant tumors show cell cycle defects that resolve over time.**

124

125   The small volume of early *scrib* mutant tumors can be caused through increased

126   apoptosis, defects in cellular growth or defective cell proliferation. We found that

127   prevention of apoptosis by overexpressing p35 could not rescue the growth arrest of the

128   posterior *scrib* RNAi cells (Figure S1A-H). Moreover, while we were able to detect

129   apoptotic cells in the posterior *scrib* RNAi region, a similar number of apoptotic cells can

130   also be detected in the anterior control region (Figure S1I). Therefore, the small volume

131   of early *scrib* mutant tumors is unlikely to be caused by increased apoptosis. The cell

132   volume of individual *scrib* mutant cells is larger than that of the control wing disc cells

133   (Figure S2A-B), consistent with a loss of epithelial packing and an elevation of mTOR

134   signaling activity in the *scrib* mutant cells (Figure S2C-F). Therefore, the growth arrest of

135   early *scrib* mutant tumors is also unlikely to be caused by defects in cellular growth.

136

137   Next, we examined additional proliferation markers in the *scrib* mutant tumors. Using 30-

138   min EdU incorporation as an indicator for S-phase cells, we noticed a significant decrease

139   in EdU incorporation in the 4-day AEL posterior *scrib* RNAi cells (Figure 2A, 2B and

140   2E). However, in 5-day AEL wing discs, we noticed that the posterior *scrib* RNAi cells

141   show similar EdU incorporation rate as the control group (Figure 2C, 2D and 2E). Flow

142   cytometry analysis showed a significant decrease of G1/S population in the posterior

143   *scrib* RNAi cells in comparison with the anterior control cells from 4-day AEL wing

144   discs (Figure 2F). By 5-day and 6-day AEL, the G1/S population in the posterior *scrib*

145   RNAi cells showed progressive recovery to a level comparable with that of the anterior

146   control cells (Figure 2G and 2H). We also observed a similar pattern of G1/S population

147   decrease from 4-day AEL *scrib* mutant tumors and recovery from 5-day *scrib* mutant

148   tumors in comparison with the control group (Figure 2I and 2J). Notably, flow cytometry

149   analysis detected a population of cells with higher DNA content than normal cells in the

150   4-day AEL Scrib-depleted cells (Figure 2F and 2I). Using the FUCCI system (Zielke et

151   al., 2014), we detected a population of G2/M cells (GFP+RFP+) with enlarged cell nuclei

152   in 4-day AEL Scrib-depleted cells (Supplemental Movie 1). Taken together, the above

153   data suggested that a population of 4-day AEL *scrib* mutant cells is arrested during G2/M

154   transition and a subset of these cells might re-initiate the DNA replication process before

155   entering mitosis.

156

157   *Drosophila* larval brain neuroblasts are an excellent model for analyzing mitosis due to

158   its accessibility for live imaging (Cabernard and Doe, 2013). We observed that the *scrib*

159   mutant neuroblasts displayed a significant prolonged entry into mitosis (Figure S3),

160   consistent with the cell cycle defects we observed in 4-day AEL *scrib* mutant wing disc

161   cells.

162

163   We conclude that the growth arrest of early *scrib* mutant tumors is most likely caused by

164   defects in cell cycle progression, yet the cell cycle defects observed in the early *scrib*

165   mutant tumors can resolve over time.

166

167   **The *scrib* mutant tumors display quantitative differences in multiple signaling**

168   **pathway activities over time.**

169

170   To investigate the reason why early and late *scrib* mutant tumors display different growth

171   rates and cell cycle profiles, we examined transcriptomes of *scrib* mutant tumors

172   collected at different time points. Principal component analysis (PCA) showed the

173   biological repeats of the *scrib* mutant tumors from the same stage are well-clustered and

174   the *scrib* mutant tumors form a transition trajectory along time (Figure 3A). Hierarchical

175   clustering further showed that the early and late *scrib* mutant tumors show distinctive

176   gene expression pattern (Figure 3B).

177

178   Interestingly, the time-course analysis showed that several signaling pathways previously

179   implicated in modulating the *scrib* mutant cell growth show quantitative changes in the

180   *scrib* mutant tumors over time. For example, the *unpaired* (*upd*) genes, the JAK/STAT

181 pathway ligands, are significantly regulated in the 8-day *scrib* mutant tumors in

182 comparison with the wild type control, with fold changes consistent with those reported

183 in a previous study (Figure S4A)(Bunker et al., 2015). Time-course analysis of the *upd*

184 family gene transcription reveals a peak expression in the 5-day AEL *scrib* mutant

185 tumors that decreases over time (Figure S4A). Meanwhile, the expression of *mirror*,

186 which is repressed by JAK/STAT transcriptional activity (Zeidler et al., 1999), shows

187 lowest expression level in 5-day AEL *scrib* mutant tumors that recovers over time

188 (Figure S4A). Notch, EGFR, JNK and Hippo signaling activities have been previously

189 shown to modulate the growth outcomes of the *scrib* mutant mosaic clones (Brumby and

190 Richardson, 2003; Chen et al., 2012; Igaki et al., 2006; Pagliarini and Xu, 2003). Similar

191 to the JAK/STAT pathway, a time-course analysis of well-established transcriptional

192 targets of Notch, EGFR and JNK signaling pathways reveals quantitative changes during

193 the *scrib* mutant tumor progression (Figure 3C-D, Figure S4). Therefore, it is likely that

194 quantitative changes in these signaling activities over time determine the differential

195 growth rates and cell cycle profiles we observed in early and late *scrib* mutant tumors.

196

197 In particular, the abnormally high JNK signaling activity or low Notch or low EGFR

198 signaling activity could be the underlying reason for the slow growth phenotype we

199 observed in early *scrib* mutant tumors. We found that the growth arrest phenotype in 4-

200 day and 5-day AEL posterior *scrib* tumors were rescued through overexpression of a

201 dominant-negative form of Basket (Bsk^{DN}) (Igaki et al., 2006)(Figure 3E-L) or a

202 dominant-negative form of Tak1 (Figure S5), which block JNK signaling activity.

203 Overexpression of Ras^{V12} and Notch intracellular domain (NICD) in combination with

204 *scrib* RNAi through *engrailed*-Gal4 caused lethality during embryogenesis or severe

205 overall developmental delay even with a temperature-sensitive form of tubulin-Gal80.

206 We therefore performed the growth analysis of *scrib* RNAi tumors in combination with

207 overexpression of Ras^{V12} using a C885a-Gal4 which expresses early to induce

208 tumorigenesis and does not cause overall developmental delay (Hrdlicka et al., 2002). We

209 found that overexpression of Ras^{V12} or NICD or Yki^{S168A} (an active form of Yki) cannot

210 rescue the slow growth phenotype in early *scrib* RNAi tumors (Figure 3M-S), even

211 though overexpression of Ras^{V12} and Yki^{S168A} showed growth-promoting effects in later

212   stages (Figure 3T). Note that overexpression of NICD with C885a-Gal4 still lead to

213   larvae of overall small body size and therefore small tumor size (Figure 3T). Taken

214   together, these data suggest that the slow growth phase in early *scrib* tumors is primarily

215   caused by high JNK signaling activity.

216

217   **The *scrib* mutant tumors harbor heterogeneous cell populations of different**

218   **proliferative states.**

219

220   The *scrib* mutant tumors display changes in growth rate and cell cycle profiles, as well as

221   quantitative differences in activities of multiple signaling pathways over time. These data

222   suggest that the *scrib* mutant tumors undergo a transition from a growth arrest state to a

223   proliferative state. To gain further insights into this transition, we built a

224   spatiotemporally-resolved transcriptomic landscape from staged *scrib* mutant tumors.

225

226   We profiled a minimum of 3000 cells per stage from 4-day, 5-day and 8-day AEL *scrib*

227   mutant tumors. We then pooled the *scrib* mutant cells from different stages together for

228   clustering and examined the distribution of four cell types defined by the expression

229   levels of the JNK signaling activity reporter *Mmp1* and the EGFR signaling reporter *kek1*.

230   *Mmp1*^{high} cell number decreases and *kek1*^{high} cell number increases over time, consistent

231   with the bulk RNA-seq data and validating the scRNA data quality (Figure 4A-B).

232   Moreover, *Mmp1* and *kek1* show significant biased distribution in single cells from

233   different stages (Figure S6), suggesting that the *scrib* mutant tumors might harbor

234   heterogeneous populations of cells at different proliferative states.

235

236   To further explore the distribution of potential cell states, we first used *Mmp1* and *kek1* as

237   marker genes to perform semi-supervised cell ordering in pseudotime with Monocle

238   (Trapnell et al., 2014) (Figure 4C-E). The expression of JNK and EGFR signaling

239   reporter genes show progressive changes along the pseudotime trajectory (Figure S7).

240   Interestingly, the Hippo signaling activity reporters *Diap1*, *expanded(ex)* and *Cyclin*

241   *E(CycE)* also show increase along the pseudotime trajectory (Figure S7), while these

242   genes do not show obvious changes over time (Figure S6). Similarly, genes that promote

243   cell cycle progression, such as *Cyclin B*, *Cdc25/string,* and *Cdc20/fizzy,* also show

244   increase along the pseudotime trajectory (Figure S7). Taken together, the pseudotime

245   trajectory likely reflects an arrest-to-proliferation state transition trajectory for the *scrib*

246   mutant cells. We found that single cells from tumors of different ages are scattered along

247   the pseudotime trajectory. Notably, the 4-day and 5-day AEL *scrib* mutant cells are more

248   enriched towards the arrested state of the pseudotime trajectory and the 8-day AEL *scrib*

249   mutant cells are enriched towards the opposite end along the trajectory (Figure 4E).

250

251   The above analysis is based on semi-supervised learning and the method assumes a tree-

252   like structure to distinguish cell states (Trapnell et al., 2014). To gain an unbiased view of

253   the *scrib* mutant cell states, we further adopted the scTDA, a nonlinear, model-

254   independent, and unsupervised topological data analysis (TDA) method for analyzing and

255   visualizing single-cell data (Rizvi et al., 2017). We randomly selected the same number

256   of cells from each stage and pooled these cells from all stages for analysis. Interestingly,

257   different random experiments robustly capture well-clustered cells that likely represent

258   the arrested state based on inspection of marker genes such as *Mmp1*, *kek1*, *CycE* and

259   *string* (Figure 4F and Figure S8). Moreover, cells from different ages are scattered on the

260   topological representation, again indicating that the *scrib* mutant tumors from all profiled

261   stages harbor heterogeneous cell populations. Notably, the cells at the arrest state are

262   more likely to be from the early stage tumors (Figure 4F and Figure S8). Interestingly,

263   while the arrest state is well-clustered and easily detectable, other cell states form

264   complex structures in the topological representations, indicating heterogeneous cell states

265   presented in the *scrib* mutant tumors are unlikely to be along a linear transition from the

266   least proliferative to the most proliferative state.

267

268   **Discussion**

269

270   Here we demonstrated that the *scrib* mutant tumors undergo a transition from a growth-

271   arrest state to a proliferation state over time and constructed a spatiotemporally resolved

272   evolution landscape for the *scrib* mutant tumors. Our data suggest that the *scrib* mutant

273   tumors harbor heterogeneous cell populations, providing a foundation for potential

274   selection and transition into a proliferation state as a population over time.

275

276   We do not yet know how cells of different proliferative states arise in undifferentiated

277   *scrib* mutant tumors. Notably, in flies a class of spindle assembly checkpoint mutations in

278   combination with apoptosis blockage can induce neoplastic tumor growth (Morais da

279   Silva et al., 2013). It is therefore interesting to speculate if the two types of neoplastic

280   tumor growth might partially share a common basis of generating heterogeneous cells

281   through genome instability. It is also possible that cells of different states arise from

282   stochasticity and the differences are amplified through complex feedback loops within

283   the cell-signaling network. It will be interesting to explore whether and how cell states

284   defined by combinatorial signaling activities are passed to daughter cells through

285   epigenetic markers. It will be also interesting to explore whether and how cells can transit

286   among different proliferative states.

287

288   The clonal *scrib* mutant cells are eliminated through cell competition when they are

289   surrounded by wild-type neighbors (Brumby and Richardson, 2003; Vaughen and Igaki,

290   2016; Yamamoto et al., 2017). It is noteworthy that the clonal *scrib* mutant cells are

291   likely to be in a different state from *scrib* mutant cells in early homozygous tumors.

292   Studies have shown overexpression of Ras^{V12}, NICD and p35 can effectively block the

293   clonal *scrib* mutant cells from apoptosis induced by cell competition (Brumby and

294   Richardson, 2003; Pagliarini and Xu, 2003). In our study, we found that overexpression

295   of Ras^{V12}, NICD and p35 have little effects in relieving the early *scrib* mutant tumors

296   from growth arrest. It will be interesting to profile the clonal *scrib* mutant cells in the

297   future and compare how the clonal *scrib* mutant cells change cell state in response to cell

298   competition signals.

299

300   **Experimental Procedures**

301

302   **Fly stocks**

303

304 The fly strains used in this study were: $scrib^1$ FRT82B/TM6B (Bilder et al., 2000), UAS-

305 *scrib* RNAi on the 2nd chromosome (Bloomington/BL38199), UAS-*scrib* RNAi on 3rd

306 chromosome (BL35748), UAS-*dlg* RNAi on the 3rd chromosome (BL35772), y[1] v[1];

307 P{y[+t7.7]=CaryP}attP2 (BL36303, the 3rd chromosome TRiP line background strain),

308 y[1] v[1]; P{y[+t7.7]=CaryP}attP40 (BL36304, the 2nd chromosome TRiP line

309 background strain), engrailed-Gal4 UAS-GFP (BL25752), engrailed-Gal4 (BL30564),

310 UAS-p35 (Hay et al., 1994)(BL6298), Fly-FUCCI (BL55098), worGal4, UAS-

311 cherry::Jupiter, Sqh::GFP (Cabernard and Doe, 2013), c885a-Gal4 (Hrdlicka et al.,

312 2002)(BL6990), UAS-Ras^{V12} (Karim and Rubin, 1998), UAS-Yki^{S168A} (Oh and Irvine,

313 2009)(BL28818), UAS-NICD (Rebay et al., 1993), UAS-Bsk^{DN}(Igaki et al., 2006)(a kind

314 gift from Jose C Pastor-Pareja) , UAS-Tak1^{DN} (BL58811).

315

316 **Immunohistochemistry**

317

318 Around 50 embryos collected within 3 hours were put in an individual vial of fly food to

319 avoid crowding and the larvae were raised at 25-degree incubator for appropriate lengths

320 of time before dissection. Imaginal discs were fixed and stained according to standard

321 protocols. The primary antibodies used were mouse anti-phospho-Histone3 (1:1000, Cell

322 Signaling), rabbit anti-Dcp-1(1:50, Cell Signaling), rabbit anti-phospho-*drosophila* S6

323 (Romero-Pozuelo et al., 2017), goat anti-GFP (1:1000, Abcam) and rabbit anti-DsRed

324 (1:500, Takara). The secondary antibodies conjugated with various Alexa Fluor dyes

325 (ThermoFisher) were used at 1:500. Phalloidin conjugated with Alexa Fluor dyes

326 (1:1000, ThermoFisher) and Hoechst (1:10000, ThermoFisher) were used to stain F-actin

327 and DNA, respectively. For EdU incorporation assay, we labeled the dissected imaginal

328 discs for 30 min before fixation using the Click-iT^{TM} Plus EdU Alexa Fluor^{TM}594 Imaing

329 Kit (ThermoFisher). All images were acquired on a Leica TCS SP8 confocal microscope.

330

331 **Western blotting**

332

333   About 30 larvae were dissected in PBS. Cell lysates were homogenized in 1X RIPA

334   (Millipore) with protease inhibitors (Roche). The primary antibodies used were mouse

335   anti-MMP1 (1:100, DSHB) and mouse anti-alpha-tubulin (1:5000, DSHB).

336

337   **Image processing and data analysis**

338

339   Images were taking as z-stacks with a step size of 1 μm. Tissue volume was measured

340   with Measure Stack plugin in Fiji. PH3+ cell number was calculated with Cell Counter

341   plugin in Fiji. EdU intensity were measured in unit areas from the posterior and anterior

342   region respectively.

343

344   **Fluorescence-activated cell-sorting (FACS) analysis**

345

346   Wing discs were dissected from staged larvae and dissociated for FACS analysis

347   according to standard protocol (Neufeld et al., 1998). Cells were sorted with

348   BDFACSAria IIIu and data were analyzed with FlowJo.

349

350   ***Drosophila* neuroblast live imaging**

351

352   Female virgins of hsFLP; worGal4,Sqh::GFP,UASCherry::jupiter; FRT82B/TM6B were

353   crossed with males of *scrib*FRT82B/TM6B. Progeny were heat shocked at 38 degree (in

354   the water bath) for 1 hour and subsequently raised at 25 degree until imaging. Five-day

355   AEL larvae were then dissected and imaged according to standard protocol (Cabernard

356   and Doe, 2013). For wild type control, larvae expressing wt;

357   worGal4,Sqh::GFP,UASCherry::jupiter; Dr/TM6B were dissected and imaged with the

358   same laser setting as that of the *scrib* mutant neuroblasts.

359

360   **Bulk RNA-seq and data analysis**

361

362   Total RNA was extracted from control and *scrib^{1}* wing imaginal discs with RNeasy Mini

363   Kit (Qiagen). Construction of cDNA libraries and 150bp paired-end sequencing on

364   Illumina HiSeq platform were performed by Novogene. Cleaned raw reads were mapped

365   to the reference genome using STAR and counts are generated by featuresCounts

366   available in Subread package. PCA analysis was performed in DESeq2. Count

367   normalization was performed using edgeR before hierarchical clustering (hclust function

368   in R).

369

370   **10x Genomics single cell RNA-seq and data analysis**

371

372   Staged *scrib^1* wing imaginal discs were dissected and transferred to DPBS

373   (ThermoFisher). The wing imaginal discs were dissociated in 0.25% Trypsin-EDTA

374   solution at 37 ℃ for 10 min. Cells were then washed in DPBS and passed through 35 μm

375   filter before library preparation. Construction of 10x single cell libraries and sequencing

376   on Illumina Hiseq platform were performed by Novogene. Raw data mapping and

377   primary analysis was performed in the Cell Ranger pipeline. Secondary analysis for

378   marker gene expression pattern was performed with Cell Ranger R kit and Seurat. Cell

379   type clustering and construction of single-cell pseudotime trajectory using semi-

380   supervised DDRTree method was performed with Monocle (Trapnell et al., 2014).

381   Unstructured and unsupervised topological data analysis (TDA) method was performed

382   for ordering cells (Rizvi et al., 2017). To balance the cell number among stages, we

383   randomly selected 500 cells per stage for each experiment and repeated the experiment

384   for three times. Figures from different repeats were shown in Supplementary Figure 8.

385

386   **Author contributions:**

387

388   Y.Y., T.T.J. and L.Z. designed the experiments. T.T.J., L.Z. and Y.W. performed all the

389   experiments except Figure S3. Y.Y., M.X.D. and T.T.J. analyzed all the data and

390   prepared all the figures except Figure S3. S.S.H. and J.G.W provided RNA sequencing

391   data analysis tools, performed TDA, prepared Figure 4F, Figure S6 and Figure S8. P.T.,

392   A.A.S, V.S and C.C. performed experiments for Figure S3 and prepared Figure S3. Y.Y.

393   wrote the manuscript with input from J.G.W. and C.C.

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406 **References:**

407 Bilder, D. (2004). Epithelial polarity and proliferation control: links from the
408 Drosophila neoplastic tumor suppressors. Genes Dev *18*, 1909-1925.
409 Bilder, D., Li, M., and Perrimon, N. (2000). Cooperative regulation of cell polarity and
410 growth by Drosophila tumor suppressors. Science *289*, 113-116.
411 Bilder, D., and Perrimon, N. (2000). Localization of apical epithelial determinants by
412 the basolateral PDZ protein Scribble. Nature *403*, 676-680.
413 Boggiano, J.C., and Fehon, R.G. (2012). Growth control by committee: intercellular
414 junctions, cell polarity, and the cytoskeleton regulate Hippo signaling. Dev Cell *22*,
415 695-702.
416 Brumby, A.M., and Richardson, H.E. (2003). scribble mutants cooperate with
417 oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. EMBO J *22*,
418 5769-5779.
419 Bunker, B.D., Nellimoottil, T.T., Boileau, R.M., Classen, A.K., and Bilder, D. (2015). The
420 transcriptional response to tumorigenic polarity loss in Drosophila. Elife *4*.
421 Cabernard, C., and Doe, C.Q. (2013). Live imaging of neuroblast lineages within
422 intact larval brains in Drosophila. Cold Spring Harb Protoc *2013*, 970-977.
423 Chen, C.L., Schroeder, M.C., Kango-Singh, M., Tao, C., and Halder, G. (2012). Tumor
424 suppression by cell competition through regulation of the Hippo pathway. Proc Natl
425 Acad Sci U S A *109*, 484-489.
426 Cordero, J.B., Macagno, J.P., Stefanatos, R.K., Strathdee, K.E., Cagan, R.L., and Vidal, M.
427 (2010). Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor
428 promoter. Dev Cell *18*, 999-1011.
429 Figueroa-Clarevega, A., and Bilder, D. (2015). Malignant Drosophila tumors
430 interrupt insulin signaling to induce cachexia-like wasting. Dev Cell *33*, 47-55.
431 Gateff, E. (1978). Malignant neoplasms of genetic origin in Drosophila melanogaster.
432 Science *200*, 1448-1459.
433 Gonzalez, C. (2013). Drosophila melanogaster: a model and a tool to investigate
434 malignancy and identify new therapeutics. Nat Rev Cancer *13*, 172-183.

435   Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.
436   Hay, B.A., Wolff, T., and Rubin, G.M. (1994). Expression of baculovirus P35 prevents
437   cell death in Drosophila. Development *120*, 2121-2129.
438   Hrdlicka, L., Gibson, M., Kiger, A., Micchelli, C., Schober, M., Schock, F., and Perrimon,
439   N. (2002). Analysis of twenty-four Gal4 lines in Drosophila melanogaster. Genesis
440   *34*, 51-57.
441   Igaki, T., Pagliarini, R.A., and Xu, T. (2006). Loss of cell polarity drives tumor growth
442   and invasion through JNK activation in Drosophila. Curr Biol *16*, 1139-1146.
443   Johnston, L.A. (2009). Competitive interactions between cells: death, growth, and
444   geography. Science *324*, 1679-1682.
445   Karim, F.D., and Rubin, G.M. (1998). Ectopic expression of activated Ras1 induces
446   hyperplastic growth and increased cell death in Drosophila imaginal tissues.
447   Development *125*, 1-9.
448   Katheder, N.S., Khezri, R., O'Farrell, F., Schultz, S.W., Jain, A., Rahman, M.M., Schink,
449   K.O., Theodossiou, T.A., Johansen, T., Juhasz, G., *et al.* (2017). Microenvironmental
450   autophagy promotes tumour growth. Nature *541*, 417-420.
451   McGranahan, N., and Swanton, C. (2017). Clonal Heterogeneity and Tumor
452   Evolution: Past, Present, and the Future. Cell *168*, 613-628.
453   Morais da Silva, S., Moutinho-Santos, T., and Sunkel, C.E. (2013). A tumor suppressor
454   role of the Bub3 spindle checkpoint protein after apoptosis inhibition. J Cell Biol *201*,
455   385-393.
456   Morata, G., and Ripoll, P. (1975). Minutes: mutants of drosophila autonomously
457   affecting cell division rate. Dev Biol *42*, 211-221.
458   Neufeld, T.P., de la Cruz, A.F., Johnston, L.A., and Edgar, B.A. (1998). Coordination of
459   growth and cell division in the Drosophila wing. Cell *93*, 1183-1193.
460   Nordling, C.O. (1953). A new theory on cancer-inducing mechanism. Br J Cancer *7*,
461   68-72.
462   Oh, H., and Irvine, K.D. (2009). In vivo analysis of Yorkie phosphorylation sites.
463   Oncogene *28*, 1916-1927.
464   Pagliarini, R.A., and Xu, T. (2003). A genetic screen in Drosophila for metastatic
465   behavior. Science *302*, 1227-1231.
466   Pastor-Pareja, J.C., and Xu, T. (2013). Dissecting social cell biology and tumors using
467   Drosophila genetics. Annu Rev Genet *47*, 51-74.
468   Rebay, I., Fehon, R.G., and Artavanis-Tsakonas, S. (1993). Specific truncations of
469   Drosophila Notch define dominant activated and dominant negative forms of the
470   receptor. Cell *74*, 319-329.
471   Richardson, H.E., and Portela, M. (2018). Modelling Cooperative Tumorigenesis in
472   Drosophila. Biomed Res Int *2018*, 4258387.
473   Rizvi, A.H., Camara, P.G., Kandror, E.K., Roberts, T.J., Schieren, I., Maniatis, T., and
474   Rabadan, R. (2017). Single-cell topological RNA-seq analysis reveals insights into
475   cellular differentiation and development. Nat Biotechnol *35*, 551-560.
476   Romero-Pozuelo, J., Demetriades, C., Schroeder, P., and Teleman, A.A. (2017).
477   CycD/Cdk4 and Discontinuities in Dpp Signaling Activate TORC1 in the Drosophila
478   Wing Disc. Dev Cell *42*, 376-387 e375.
479   Simpson, P. (1979). Parameters of cell competition in the compartments of the wing
480   disc of Drosophila. Dev Biol *69*, 182-193.

481 Simpson, P., and Morata, G. (1981). Differential mitotic rates and patterns of growth
482 in compartments in the Drosophila wing. Dev Biol *85*, 299-308.
483 Sonoshita, M., and Cagan, R.L. (2017). Modeling Human Cancers in Drosophila. Curr
484 Top Dev Biol *121*, 287-309.
485 Sun, S., and Irvine, K.D. (2016). Cellular Organization and Cytoskeletal Regulation of
486 the Hippo Signaling Network. Trends Cell Biol *26*, 694-704.
487 Trapnell, C., Cacchiarelli, D., Grimsby, J., Pokharel, P., Li, S., Morse, M., Lennon, N.J.,
488 Livak, K.J., Mikkelsen, T.S., and Rinn, J.L. (2014). The dynamics and regulators of cell
489 fate decisions are revealed by pseudotemporal ordering of single cells. Nat
490 Biotechnol *32*, 381-386.
491 Vaughen, J., and Igaki, T. (2016). Slit-Robo Repulsive Signaling Extrudes
492 Tumorigenic Cells from Epithelia. Dev Cell *39*, 683-695.
493 Woods, D.F., and Bryant, P.J. (1991). The discs-large tumor suppressor gene of
494 Drosophila encodes a guanylate kinase homolog localized at septate junctions. Cell
495 *66*, 451-464.
496 Yamamoto, M., Ohsawa, S., Kunimasa, K., and Igaki, T. (2017). The ligand Sas and its
497 receptor PTP10D drive tumour-suppressive cell competition. Nature *542*, 246-250.
498 Zeidler, M.P., Perrimon, N., and Strutt, D.I. (1999). Polarity determination in the
499 Drosophila eye: a novel role for unpaired and JAK/STAT signaling. Genes Dev *13*,
500 1342-1353.
501 Zielke, N., Korzelius, J., van Straaten, M., Bender, K., Schuhknecht, G.F., Dutta, D.,
502 Xiang, J., and Edgar, B.A. (2014). Fly-FUCCI: A versatile tool for studying cell
503 proliferation in complex tissues. Cell Rep *7*, 588-598.
504

505   **Figure legends**

506

507   **Figure 1 The Scrib-depleted cells display different growth rates over time.**

508

509   (A) Examples of a control 5-day AEL imaginal disc and $scrib^1$ mutant wing imaginal

510   discs from 4-day AEL to 10-day AEL stained for actin (red) and DNA (blue). Control

511   genotype: *FRT82B*. Scale bar: 10μm.

512

513   (B) Quantification of volumes for control and $scrib^1$ mutant wing imaginal discs over

514   time. Control genotype: *FRT82B* raised at identical conditions. Control, **4d** n = 10,

515    $5\pm1\times10^5\mu m^3$, **5d** n =19, $1.9\pm0.4\times10^6\mu m^3$; $scrib^1$ mutant, **4d** n = 16, $1.4\pm0.3\times10^5\mu m^3$, **5d** n

516   = 18, $5\pm2\times10^5\mu m^3$, **6d** n = 19, $7\pm2\times10^5\mu m^3$, **7d** n = 17, $2.0\pm0.7\times10^6\mu m^3$, **8d** n = 14,

517    $3\pm1\times10^6 \mu m^3$, **9d** n = 17, $4\pm1\times10^6 \mu m^3$, **10d** n = 11, $6\pm2\times10^6 \mu m^3$. Note that larvae from

518   the control group become pupae at 5-day AEL.

519

520   (C-F) 4-day AEL (C-D) and 5-day AEL (E-F) control (C and E) and $scrib^1$ mutant (D and

521   F) wing imaginal discs stained for PH3(green), actin (red) and DNA (blue). Control

522   genotype: *FRT82B*. Scale bar: 10μm.

523

524   (G) Quantification of PH3+ cell number per unit volume ($10^5\mu m^3$) in 4-day AEL and 5-

525   day AEL control and $scrib^1$ mutant wing imaginal discs. Statistical analysis was

526   performed by unpaired t-test. Control, *FRT82B*, **4d** n = 10, 37 ± 5, **5d** n =19, 26 ± 3; $scrib^1$

527   mutant, **4d** n = 16, 22 ± 4, **5d** n = 18, 25 ± 3.

528

529   (H-U) 4-day (H-J), 5-day (M-O) and 6-day (R-S) AEL *scribRNAi* (H, M, R), *dlgRNAi* (I,

530   N, S) and control (J, O) imaginal discs stained for PH3 (gray), actin(red) and GFP

531   (green). Quantification of volumes (K, P, T) and PH3+ cell number per unit volume

532   ($10^5\mu m^3$) (L, Q, U) for 4-day (K, L), 5-day (P, Q) and 6-day (T, U) AEL *scribRNAi*,

533   *dlgRNAi* and control imaginal discs. Genotype for the *scribRNAi* group: *engrailed-Gal4*

534   *UAS-GFP/+; UAS-scribRNAi/+*. Genotype for the *dlgRNAi* group: *engrailed-Gal4 UAS-*

535   *GFP/+; UAS-dlgRNAi/+*. Genotype for the control group: *engrailed-Gal4 UAS-GFP/+;*

536   *P{y[+t7.7]=CaryP}attP2 /+* (the 3rd chromosome TRiP line background strain). The

537   *scribRNAi* group, **4d** n = 15, anterior, $2.6\pm0.5\mathrm{x}10^5\mu m^3$, PH+ cell number 37±5, posterior,

538    $4\pm1\mathrm{x}10^4\mu m^3$, PH+ cell number 14±8, **5d** n = 14, anterior, $4.3\pm0.9\mathrm{x}10^5\mu m^3$, PH+ cell

539   number 29±5, posterior, $8\pm4\mathrm{x}10^4\mu m^3$, PH+ cell number 21±6, **6d** n = 15, anterior,

540    $8\pm2\mathrm{x}10^5\mu m^3$, PH+ cell number 13±3, posterior, $3\pm2\mathrm{x}10^5\mu m^3$, PH+ cell number 25±4; The

541   *dlgRNAi* group, **4d** n = 16, anterior, $3.0\pm0.5\mathrm{x}10^5\mu m^3$, PH+ cell number 34±9, posterior,

542    $5\pm2\mathrm{x}10^4\mu m^3$, PH+ cell number 11±8, **5d** n = 17, anterior, $8\pm2\mathrm{x}10^5\mu m^3$, PH+ cell number

543   16±5, posterior, $3\pm1\mathrm{x}10^5\mu m^3$, PH+ cell number 25±9, **6d** n = 16, anterior, $9\pm2\mathrm{x}10^5\mu m^3$,

544   PH+ cell number 16±5, posterior, $4\pm2\mathrm{x}10^5\mu m^3$, PH+ cell number 33±7; The control

545   group, **4d** n = 16, anterior, $2.5\pm0.6\mathrm{x}10^5\mu m^3$, PH+ cell number 44±8, posterior,

546    $1.3\pm0.3\mathrm{x}10^5\mu m^3$, PH+ cell number 43±6, **5d** n = 13, anterior, $6\pm1\mathrm{x}10^5\mu m^3$, PH+ cell

547   number 25±4, posterior, $4\pm1 \mathrm{x}10^5\mu m^3$, PH+ cell number 28±4. Scale bar: 10μm.

548   Statistical analysis was performed by unpaired t-test. Note that larvae from the control

549   group become pupae at 5-day AEL.

550

551   **Figure 2 The Scrib-depleted cells display different cell cycle profiles over time.**

552

553   (A-E) 4-day AEL (A-B) and 5-day AEL (C-D) control (A and C) and *scribRNAi* (B and

554   D) wing imaginal discs stained for EdU (red in A, B, C, D and gray in A', B', C', D'),

555   GFP (green) and DNA (blue). (E) Quantification of posterior/anterior EdU intensity ratio

556   for 4-day and 5-day AEL control and *scribRNAi* wing imaginal discs. Genotype for the

557   *scribRNAi* group: *engrailed-Gal4 UAS-GFP/+; UAS-scribRNAi/+*. Genotype for the

558   control group: *engrailed-Gal4 UAS-GFP/+; P{y[+t7.7]=CaryP}attP2 /+*. The *scribRNAi*

559   group, 4d n = 8, 5d n = 10; The control group, 4d n = 9, 5d n = 10. Statistical analysis

560   was performed by unpaired t-test. Scale bar: 10μm.

561

562   (F-H) FACS analysis of DNA contents of control and *scribRNAi* cells from 4-day (F), 5-

563   day (G) and 6-day(H) AEL wing imaginal discs. Genotype for FACS analysis: *engrailed-*

564   *Gal4 UAS-GFP/+; UAS-scribRNAi/+*. The anterior GFP- cells serve as control and the

565   posterior GFP+ cells are the *scribRNAi* cells. Because the number of GFP- cells is much

566   larger than GFP+ cells, the histogram overlay is normalized using the unit distribution

567 mode in FlowJo. At least five thousand cells were recorded for each cell group. Each

568 experiment is replicated for at least three times.

569

570 (I-J) FACS analysis of DNA contents of control and *scrib[1]* mutant cells from 4-day (I)

571 and 5-day (J) AEL wing imaginal discs. Genotype for the control group: *FRT82B* raised

572 at identical conditions. At least five thousand cells were recorded for each cell group.

573 Each experiment is replicated for at least three times.

574

575 **Figure 3 High JNK signaling activity causes growth arrest in early *scrib* mutant**

576 **tumors.**

577

578 (A) Principle component analysis (PCA) of transcriptomes from 5-day AEL control

579 imaginal discs and 5-day, 6-day, 8-day and 10-day AEL *scrib* mutant wing imaginal

580 discs. Control genotype: *FRT82B*. Four biological replicates are plotted for each time

581 point except for 6-day AEL groups which only three biological replicates are recovered.

582

583 (B) Hierarchical clustering of transcriptomes from 5-day, 6-day, 8-day and 10-day AEL

584 *scrib* mutant wing imaginal discs.

585

586 (C) Plot of JNK signaling reporter genes in staged *scrib* mutant wing discs normalized by

587 those in 5-day AEL control imaginal discs using $\log_2$(FPKM fold change) values. Control

588 genotype: *FRT82B*.

589

590 (D) Western blot analysis of Mmp-1 protein level in 5-day AEL control imaginal discs,

591 5-day and 8-day AEL *scrib* mutant wing imaginal discs. Control genotype: *FRT82B*.

592

593 (E-J) 4-day (E-G) and 5-day (H-J) AEL *bsk^DN* (E, H), *scribRNAi* (F, I) and *scribRNAi*

594 *bsk^DN* (G, J) imaginal discs stained for actin(red) and GFP (green). Genotype for (E) and

595 (H): *engrailed-Gal4 UAS-GFP/+; UAS- bsk^DN/+*. Genotype for (F) and (I): *engrailed-*

596 *Gal4 UAS-GFP/UAS-scribRNAi.* Genotype of (G) and (J): *engrailed-Gal4 UAS-*

597 *GFP/UAS-scribRNAi; UAS- bsk^DN/+*. Scale bar: 10μm.

598

599   (K-L) Quantification of volumes for 4-day (K) and 5-day (L) AEL bsk^{DN}, $scribRNAi$ and

600   $scribRNAi$ bsk^{DN} imaginal discs. bsk^{DN}, **4d** n = 17, anterior, $1.7\pm0.7\text{x}10^5\mu\text{m}^3$, posterior,

601   $8\pm4\text{x}10^4\mu\text{m}^3$, **5d** n = 17, anterior, $5\pm2\text{x}10^5\mu\text{m}^3$, posterior, $4\pm1\text{x}10^5\mu\text{m}^3$. $scribRNAi$, **4d** n =

602   20, anterior, $2.0\pm0.5\text{x}10^5\mu\text{m}^3$, posterior, $4\pm1\text{x}10^4\mu\text{m}^3$, **5d** n = 15, anterior, $4\pm1\text{x}10^5\mu\text{m}^3$,

603   posterior, $8\pm4\text{x}10^4\mu\text{m}^3$. $scribRNAi$ bsk^{DN}, **4d** n = 14, anterior, $1.4\pm0.4\text{x}10^5\mu\text{m}^3$, posterior,

604   $6\pm2\text{x}10^4\mu\text{m}^3$, **5d** n = 8, anterior, $4.2\pm0.5\text{x}10^5\mu\text{m}^3$, posterior, $2.4\pm0.8\text{x}10^5\mu\text{m}^3$. Statistical

605   analysis was performed by unpaired t-test.

606

607   (M-R) 4-day AEL control (M), $scribRNAi$ (N), $scribRNAi$ $Tak1^{DN}$(O), $scribRNAi$

608   Yki^{S168A}(P), $scribRNAi$ Ras^{V12}(Q), and $scribRNAi$ $NICD$ (R) imaginal discs stained for

609   actin (red). Genotypes are as follows: (M) $C885a\text{-}Gal4/+; UAS\text{-}GFP/+;$ (N) $C885a\text{-}$

610   $Gal4/+; UAS\text{-}scribRNAi /+;$ (O) $C885a\text{-}Gal4/UAS\text{-}Tak1^{DN}; UAS\text{-}scribRNAi/+;$ (P)

611   $C885a\text{-}Gal4/UAS\text{-}Yki^{S168A}; UAS\text{-}scribRNAi/+;$ (Q) $C885a\text{-}Gal4/UAS\text{-}Ras^{V12}; UAS\text{-}$

612   $scribRNAi/+;$ (R) $C885a\text{-}Gal4/UAS\text{-}NICD; UAS\text{-}scribRNAi/+$. Scale bar: 10μm.

613

614   (S) Barplot of volumes for 4-day AEL control (n = 33, $1.0\pm0.3\text{x}10^6\mu\text{m}^3$), $scribRNAi$ (n =

615   30, $5\pm1\text{x}10^5\mu\text{m}^3$), $scribRNAi$ $Tak1^{DN}$ (n = 35, $1.1\pm0.2\text{x}10^6\mu\text{m}^3$), $scribRNAi$ Yki^{S168A} (n =

616   26, $7\pm2 \text{x}10^5\mu\text{m}^3$), $scribRNAi$ Ras^{V12} (n = 30, $4\pm2\text{x}10^5\mu\text{m}^3$), and $scribRNAi$ $NICD$ (n =

617   18, $2.1\pm0.4\text{x}10^5\mu\text{m}^3$) imaginal discs.

618

619   (T) Quantification of volumes for control, $scribRNAi, scribRNAi$ $Tak1^{DN}, scribRNAi$

620   $Yki^{S168A}, scribRNAi$ $Ras^{V12},$ and $scribRNAi$ $NICD$ imaginal discs over time. Control, **4d** n

621   = 33, $1.0\pm0.3\text{x}10^6\mu\text{m}^3$; **5d** n = 26, $2.1\pm0.6\text{x}10^6\mu\text{m}^3$. $scribRNAi$, **4d** n = 30, $5\pm1\text{x}10^5\mu\text{m}^3$;

622   **5d** n = 27, $1.4\pm0.5\text{x}10^6\mu\text{m}^3$; **6d** n = 34, $3\pm1\text{x}10^6\mu\text{m}^3$; **7d** n = 34, $6\pm2\text{x}10^6\mu\text{m}^3$; **8d** n = 32,

623   $8\pm3\text{x}10^6\mu\text{m}^3$. $scribRNAi$ $Tak1^{DN}$, **4d** n = 35, $1.1\pm0.2\text{x}10^6\mu\text{m}^3$; **5d** n = 39, $3\pm1\text{x}10^6\mu\text{m}^3$; **6d**

624   n = 32, $6\pm3\text{x}10^6\mu\text{m}^3$. $scribRNAi$ Yki^{S168A}, **4d** n = 26, $7\pm2\text{x}10^5\mu\text{m}^3$; **5d** n = 30,

625   $1.9\pm0.8\text{x}10^6\mu\text{m}^3$; **6d** n = 32, $5\pm1\text{x}10^6\mu\text{m}^3$; **7d** n = 28, $9\pm3\text{x}10^6\mu\text{m}^3$; **8d** n = 34,

626   $1.1\pm0.4\text{x}10^7\mu\text{m}^3$. $scribRNAi$ Ras^{V12}, **4d** n = 30, $4\pm2\text{x}10^5\mu\text{m}^3$; **5d** n = 35, $1.9\pm0.4\text{x}10^6\mu\text{m}^3$;

627   **6d** n = 30, $7\pm2\text{x}10^6\mu\text{m}^3$; **7d** n = 28, $1.1\pm0.4\text{x}10^7\mu\text{m}^3$; **8d** n = 28, $1.6\pm0.6\text{x}10^7\mu\text{m}^3$.

628   *scribRNAi NICD*, **4d** n = 18, 2.1±0.4x10^{5}µm^3; **5d** n = 20, 3±2x10^{5}µm^3; **6d** n = 21,

629   5±3x10^{5}µm^3; **7d** n = 18, 2.5±0.7x10^{5}µm^3; **8d** n = 25, 2.2±0.4x10^{5}µm^3.

630

631   **Figure 4 Visualization of single cells from the *scrib* mutant tumors in the reduced**

632   **dimensional space.**

633

634   (A-B) Visualization of single cells ordered in the t-SNE reduced dimensional space

635   colored by cell types defined by *Mmp1* and *kek1* expression level (A) (normalized

636   expression level=1 as threshold), and tumor age (B). Single cells pooled from 4-day, 5-

637   day and 8-day AEL *scrib* mutant tumors. 3000 single cells per time point are randomly
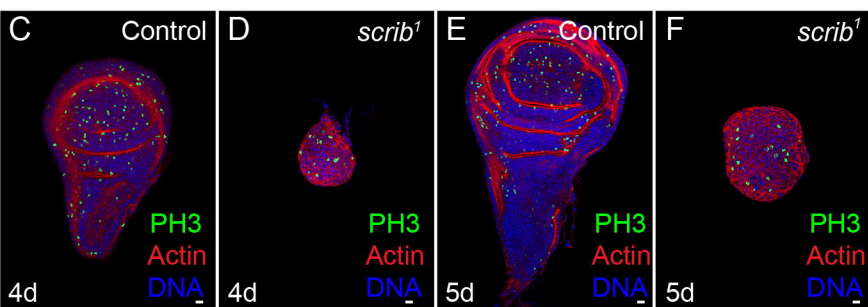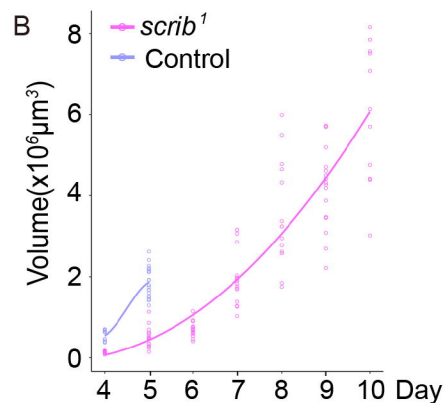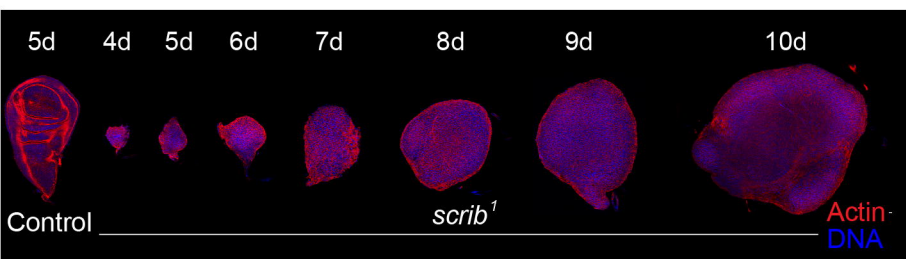
638   sampled.

639

640   (C-E) Visualization of single cells ordered along a pseudotime trajectory in the reduced

641   dimensional space colored by pseudotime (C), cell types defined by *Mmp1* and *kek1*

642   expression level (D) (normalized expression level=1 as threshold), and tumor age (E).

643   3000 single cells per time point are randomly sampled.

644

645   (F) Topological representation of single cells ordered using scTDA. 500 cells per time

646   point are randomly sampled. The nodes (circles) are clusters of single cells with similar

647   global expression profiles, and the node size corresponds to the number of cells in that

648   cluster. Edges (lines) connect clusters that have at least one cell in common. The node

649   color in the left panel indicates the time line of the *scrib* mutant tumor progression. A

650   node composed of a mixture of cells from early and late stages has an intermediate color.

651   The node color in the right panel indicates the expression level of marker genes labeled in

652   each panel.

Figure 1
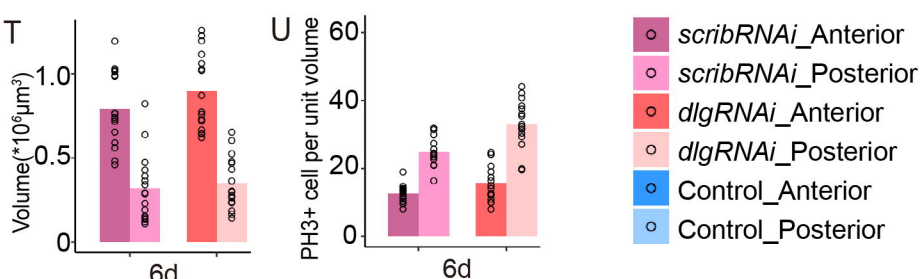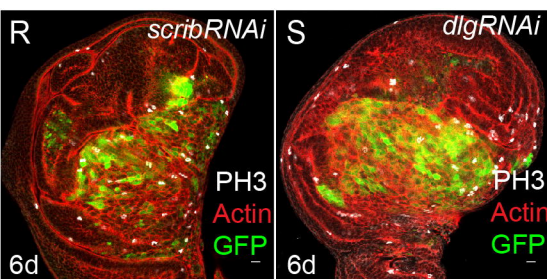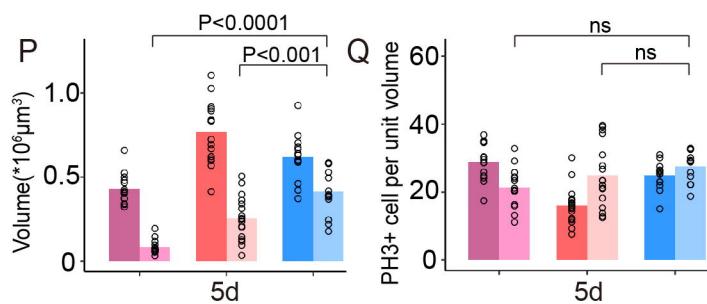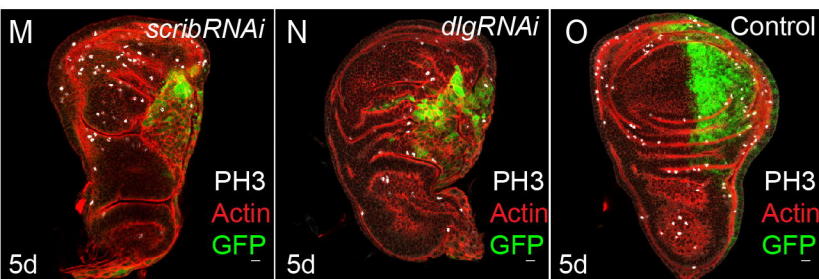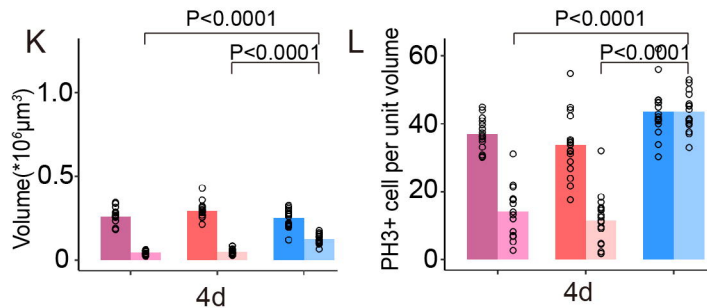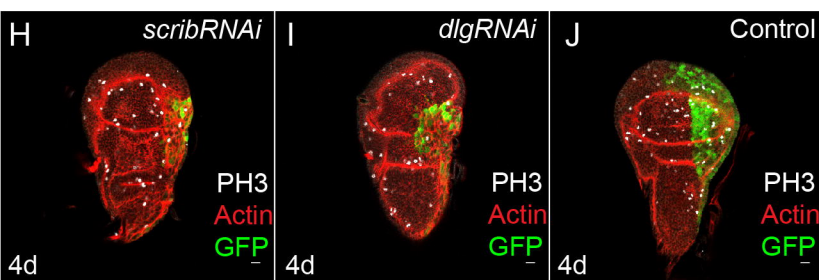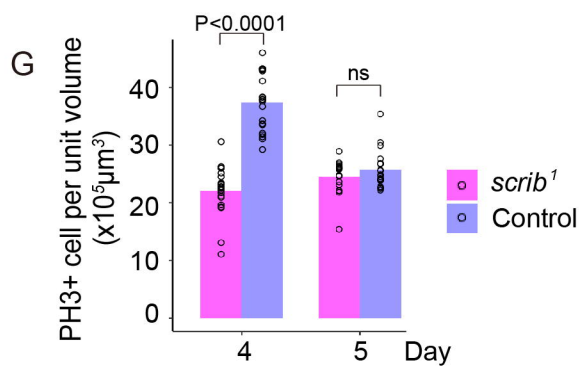
Figure 2

Figure 3

Figure 4

A

Cell type
- *Mmp1*^high *kek1*^high
- *Mmp1*^low *kek1*^low
- *Mmp1*^low *kek1*^high
- *Mmp1*^high *kek1*^low

B

Day
- 4
- 5
- 8

C

Pseudo-time
30
0

D

Cell type
- *Mmp1*^high *kek1*^high
- *Mmp1*^low *kek1*^low
- *Mmp1*^low *kek1*^high
- *Mmp1*^high *kek1*^low

E

Day
- 4
- 5
- 8

F

Time
Early   Late

Mmp1

kek1

CycE

string

Expression
High

Low