PIK3CA-related overgrowth spectrum (PROS) zebrafish models reveal pan-lineage developmental dysregulation

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Abstract

Post-zygotic gain-of-function *PIK3CA* mutations arising during embryonic development cause disorders collectively known as the *PIK3CA*-related overgrowth spectrum (PROS). This ranges from minor, localized overgrowth to devastating multi-tissue overgrowth. Disease severity is widely attributed to a combination of PIK3CA genotype, affected cell type, and developmental timing of mutation acquisition. However, in PROS this explains neither the biased pattern of overgrowth - disproportionately affecting mesoderm and neuroectoderm-derived tissues - nor the typical low mutation burden within areas of extensive tissue overgrowth. Indeed, growing evidence suggests PROS mutations cause overgrowth non-cell-autonomously, although mechanisms of this are poorly understood. Here, we develop mosaic zebrafish models of PROS with overexpression of orthologous hotspot *pik3ca* mutations (*pik3ca*^{PROS}) to visualize and examine the effects of mutated cells on early development, in whole live animals. Reminiscent of PROS, we observe a spectrum of embryonic vasculature malformations (VMs), accompanied by larval muscle and bone overgrowth. Surprisingly, VMs only rarely expressed *pik3ca*^{PROS} in constituent endothelial cells, with *pik3ca*^{PROS}-expressing cells often closely abutting malformations instead. Single-cell transcriptomics of *pik3ca*^{PROS} mosaic zebrafish prior to VM onset revealed that most *pik3ca*^{PROS} cells are relatively immature and developmentally inert or constitute a small minority of mesodermal-derived cell types. Despite this constriction, global changes to cell fate were evident, alongside pervasive, pan-lineage abnormalities of gene expression, and rewiring of predicted ligand-receptor communication between lineages. We propose that targeting signals that indirectly propagate overgrowth through non-cell autonomous mechanisms - as well as PI3K activation itself - is worthy of therapeutic investigation.

Significance statement

Patchy, or mosaic, activating mutations in *PIK3CA* cause asymmetric human overgrowth due to aberrant hyperactivation of phosphoinositide 3-kinase (PI3K) signaling. Overgrowth prominently affects blood vessels and may be severely debilitating. While causation by *PIK3CA* mutations is clear, the explanation for the extent and pattern of associated overgrowth is not. Leveraging the power of zebrafish models for observation of early development, we now provide evidence for widely pervasive developmental effects of PIK3CA mutations extending beyond transcriptionally and phenotypically affected cells and lineages. This suggests potential therapeutic value of targeting secondary effects of PIK3CA activation as well as the activated PI3K itself.

Introduction

Coordination of lineage determination and growth rates within and among cell lineages is central to healthy human development. Class 1 phosphoinositide 3-kinases (PI3Ks) are one crucial group of signaling enzymes that mediate such coordination. PI3Ks transduce numerous upstream growth cues into widely ramifying downstream effects on cell growth, metabolism and other behaviors (1). They do this in a cell type, developmental stage and tissue specific manner. These growth cues include hormones and growth factors which activate receptor tyrosine kinases. The dominant class 1A PI3K subtypes regulating cell growth and metabolism in non-hematopoietic cell lineages include the p110α catalytic subunit encoded by *PIK3CA*, acting in concert with one of several regulatory subunits (2, 3). The heterodimeric enzyme is typically referred to as PI3Kα.

Hyperactivation of PI3Kα is common in solid human cancers, most often attributable to somatic activating *PIK3CA* mutations. The mutations with the greatest hyperactivity fall in 'hotspot' regions at the C terminal of the kinase domain (Histidine 1047) or in the helical domain (Glutamates 542 or 545) (4). These same mutations, if acquired during human development, also cause a wide range of asymmetric overgrowth disorders. Many different disease labels are used for these based on phenotypic descriptors, and the term *PIK3CA*-related overgrowth spectrum (PROS) is now used as an umbrella term for the whole range, reflecting its shared etiopathogenesis (5, 6). PROS ranges from very localized overgrowth, for example a circumscribed vascular malformation or single digit overgrowth, to severe and sometimes life-threatening multisystem disorders such as CLOVES syndrome (Congenital Lipomatous Overgrowth, Vascular Malformations, Epidermal Nevus, Spinal/Skeletal anomalies/Scoliosis) (5, 7). Vascular complications such as thromboembolism, hemorrhage, mass effects or high output heart failure are major causes of morbidity and mortality in PROS.

The wide phenotypic spectrum of PROS has most commonly been rationalized in terms of the timing and cell of origin of the founder *PIK3CA* mutation, which determines the anatomical site(s) of overgrowth, with the *PIK3CA* genotype (hotspot *vs* non hotspot) an important accessory factor determining the rate of overgrowth of affected tissues. This formulation fails to provide a complete account of the observed phenotypic heterogeneity, however. One unexplained observation is that not all tissues are equally affected in PROS. Mesoderm-derived veins, capillaries, lymphatics, adipose tissue, skeletal muscle, and bone are commonly and severely affected, for example, as are ectoderm-derived brain and peripheral nerves. In contrast, mesoderm-derived arteries and hematopoietic cells, and endoderm-derived organs are rarely affected (4, 7). Given the presumed stochastic nature of

35 spontaneous *PIK3CA* mutagenesis, this implies that mutations are excluded from, not
36 tolerated in, or not phenotypically penetrant in some lineages (4).

37 Another important observation is that the burden of *PIK3CA* mutation is variable, but often in
38 the range of <1-10% in tissue biopsies, even from macroscopically severely affected regions,
39 meaning that the bulk of sampled cells are genetically wild-type (WT) (4, 8-10). This will partly
40 reflect sampling of a genetically mosaic tissue, but the extent of this mismatch also suggests
41 that *PIK3CA* mutations may exert both cell-autonomous and non-cell-autonomous effects on
42 tissue growth. Work emerging using other model systems has shown that non-blood vessel
43 cells, such as PIK3CA-mutant lymphatic endothelial cells or Schwann cells, can increase PI3K
44 activation in neighboring endothelia to worsen vascular lesions and drive proliferation of WT
45 cells, through recruitment of macrophages or secretion of pro-inflammatory eicosanoids and
46 cytokines to drive proliferation of WT cells (11-14). Beyond its conceptual interest, identifying
47 mediators of such non-cell-autonomous mechanisms may be of translational value if these
48 secondary drivers of pathologic growth can be targeted. This is highly relevant in PROS as
49 available potent PI3K inhibitors have limited efficacy in only a subset of patients in studies so
50 far (15, 16), underscoring the need for better classification and understanding of the wider
51 signaling landscape within VMs to ensure optimal treatment strategies (17).

52 Dissecting how the presence of *PIK3CA*-mutated cells corrupts normal development is ideally
53 interrogated *in situ* in developing animals. Numerous animal models of PROS have indeed
54 been reported to date, using strategies based on endogenous (18-20) or transgenic
55 overexpression of *PIK3CA* hotspot variants (21-27), or a strongly activating but synthetic
56 *Pik3ca* allele (28, 29). However, although such strong genetic *Pik3ca* activation has been
57 shown to drive overgrowth in numerous lineages, the early events in overgrowth *in utero* are
58 not visible in mammals. We now present a zebrafish model of PROS circumventing this
59 limitation. The genetic tractability and transparency of zebrafish from their first cell divisions
60 into adulthood means that critical stages in disease onset and evolution hidden *in utero* can
61 be visualized and followed at cellular resolution from disease onset to adulthood in an intact
62 organism (30). The large clutch size and rapid development of zebrafish also offer high
63 experimental throughput to enable rapid testing of multiple PROS alleles in distinct cellular
64 lineages.

65 We use this new model to investigate the cellular and transcriptional landscape at the onset
66 of PROS vascular overgrowth. We show that overexpression of *pik3ca* hotspot alleles
67 (henceforth collectively "*pik3ca*^{PROS}") causes extensive vascular malformations, despite the
68 majority of *pik3ca*^{PROS} cells localizing adjacent to, rather than within lesions. Overexpression
69 limited to the mesoderm had widespread effects on subsequent development, manifesting as

aberrant partitioning of mutant cells among mesoderm-derived lineages. Single cell transcriptomics suggested more pervasive effects on gene expression and signaling in non-mesodermal lineages, with potential loosening of the normal pattern of developmental signal crosstalk among key embryonic structures. These findings consolidate evidence for developmental effects of *pik3ca*^{PROS} mosaicism that extend far beyond affected cells. This aligns with observations made in human PROS, presents a versatile animal model for further mechanistic interrogation, and opens new possibilities for accessory therapeutic targets beyond PI3K itself.

Results

Mosaic *pik3ca* hotspot transgenes cause vascular malformations in zebrafish embryos

Zebrafish Pik3ca protein has 91% homology with human PIK3CA, and AlphaFold predicts closely similar higher order structure to the experimentally determined structure of *PIK3CA*, evidenced by nearly identical positioning of PROS-related hotspot variants in both species **(Figure 1A)**. To develop a zebrafish *pik3ca-PROS* model, we generated Tol2-based vectors permitting *β-actin* promoter-driven (ubiquitous) expression of zebrafish *pik3ca*; either wild-type (WT) or harboring one of the hotspot mutations, namely *H1048R*, *H1048L* (orthologous to human *H1047R* and *H1047L* respectively), *E542K*, or *E545K* **(Figure 1B)**. A downstream IRES-*mScarlet* sequence enabled co-expression of the mScarlet reporter with mutant *pik3ca*. Additionally, the presence of an uncoupled secondary *cmcl2:GFP* cardiac reporter was included to provide a secondary readout of transgene integration. The *pik3ca*^{H1048R}*-mScarlet* construct was introduced into WT embryos to produce F0 mosaic transgenic embryos, which were raised and screened for transgene integration and overgrowth.

Many *pik3ca*^{H1048R}*-mScarlet*-injected embryos appeared morphologically normal, but 15% developed vascular lesions, observed as blood pooling and poor circulation most evident at 72 hours post-fertilization (hpf) **(Figure 1B)**. To investigate this phenotype further, we repeated *pik3ca*^{H1048R}*-mScarlet* transgene injections into the *Tg(fli1:eGFP)* endothelial reporter line, enabling co-visualization of blood vessels and transgene expression (31). *Fli1:eGFP* is first detectable during somitogenesis, during which angioblasts migrate from the lateral plate mesoderm (LPM) between the 10 and 18 somite stage (~16 hpf) towards the midline, with the main embryonic artery and vein being formed at 24 hpf, and trunk circulation being completed at 48 hpf (31, 32). In the trunk of zebrafish embryos at 72 hpf, arteries and veins are well established, with regularly-spaced intersegmental vessels (ISVs) connecting the ventral major aortic and venous vessels with the dorsal longitudinal anastomotic vessel (DLAV) (31, 33)

104 **(Figure 1C)**. Consistent with the variability observed in PROS, the severity of vascular
105 malformations resulting from transgene injection ranged from mild to severe, the latter obvious
106 from dramatic enlargement of the major blood vessels, causing blood pooling in the tail or
107 head, with some vascular overgrowths large enough to compromise swimming (**Figure 1D**).

108 In the same system, we next tested the propensity of other PROS hotspot variants to cause
109 vascular malformations, injecting WT, *H1048R, H1048L, E542K* or *E545K pik3ca*^{PROS}
110 constructs into *Tg*(*fli1:eGFP)* embryos. Transgenic expression of *pik3ca*^{WT}*-mScarlet* did not
111 induce overgrowth (**Supplementary Figure 1A**). However, all four hotspot variants, whether
112 affecting the kinase (*H1048R/L*) or helical domains (*E542K/E545K*) of Pik3ca, reproducibly
113 caused a spectrum of vascular anomalies, from mild mis-patterning of *fli1:eGFP*+ ISVs only
114 evident by fluorescent transgene expression pattern, to severe balloon-like overgrowth, of the
115 major vessels in the tail **(Figure 1D,E Supplementary Figure 1A)**. Severity scoring of
116 malformations for each of the four PROS alleles and of WT or uninjected controls showed that
117 transgenic expression of mosaic *pik3ca*^{PROS} variants in zebrafish caused vascular
118 malformations at similar levels of severity (**Figure 1F**).

119 To test whether our model could recapitulate other overgrowth elements seen in PROS, we
120 selected 72 hpf embryos displaying a high level of *pik3ca*^{PROS} transgene integration but
121 appearing otherwise normal, and grew them to larvae stages (to 14 days). Transgene
122 integration was assessed either by high numbers of *mScarlet*+ cells or by the surrogate marker
123 of expression of the co-injected heart-targeted *cmcl2:GFP* reporter in most cardiac cells. We
124 imaged these fish as they developed into larvae. Despite no visible vascular abnormality in
125 the embryos, these fish still developed vascular malformations by larval stages, seen as
126 thickening of vessels, or as vascular protrusions from the skin **(Figure 1G)**. Aside from
127 vascular malformations, some *pik3ca*^{PROS} zebrafish larvae also showed appreciable
128 *mScarlet*+ expression and overgrowth in the musculature, as well as premature mineralization
129 in the notochord, fusing and thickening of vertebrae, and abnormal patterning of hypural
130 cartilage in the tail **(Supplementary Figure 1B-D)**. These findings suggest that in the fish
131 model, as in human PROS, the trajectory of malformations is not exclusively determined in
132 embryos, and overgrowth can sometimes manifest or progress post-embryonically, notably in
133 tissues of mesodermal origin.

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137 **Evidence for cell-autonomous and non-cell-autonomous mechanisms of *pik3ca^{PROS}***
138 **vascular overgrowth**

139 Surprisingly, we observed that only a minority of endothelial cells within vascular
140 malformations expressed both mScarlet and eGFP fluorophores, proxies for *pik3ca^{PROS}* and
141 *fli1* expression, respectively. We used confocal microscopy to build a 3D image of the vascular
142 lesions in higher resolution to investigate this further **(Figure 2A; Movie 1)**. In one example of
143 a mild VM, we observed thickening of the DLAV without mScarlet cells in the lesion itself, but
144 with *pik3ca-PROS-mScarlet*+ cells abutting the overgrown region **(Figure 2A; Movie 2)**. In
145 other moderate and severe vascular malformations, *pik3ca^{PROS}-mScarlet*+ cells were again
146 most often found adjacent to malformations rather than comprising abnormal vessel walls
147 (**Figure 2A; Movie 3**). Indeed, GFP and mScarlet double positive cells accounted for <5% of
148 total lesion volume on average **(Figure 2B).** This provides circumstantial evidence for the
149 hypothesized non-cell autonomous mechanisms in PROS. Specifically, it argues that
150 unidentified *pik3ca^{PROS}* cells in or close to the vascular niche may exert non-cell-autonomous
151 effects upon WT endothelial cells.

152

153 **Early mesodermal *pik3ca^{PROS}* transgene expression causes endothelial mis-patterning**

154 Many tissues prominently affected in PROS are mesoderm-derived, indicating biases in cell
155 lineage contribution to overgrowth, and the possibility of spatial and temporal windows of
156 especial vulnerability to PROS mutations during development (4). We therefore sought to
157 investigate the consequences of disease onset in the early mesoderm, which has hitherto not
158 been possible to observe *in vivo* in mammalian systems, by swapping our ubiquitous *β-actin*
159 promoter for that of the pan-mesodermal marker *tbxta* (**Figure 3A, B**). The activation of *tbxta*
160 prior to endothelial specification would also address the possibility that *mScarlet*+ endothelial
161 cells had been present during vasculogenesis but had either silenced transgene expression
162 or died prior to imaging and scoring at 72 hpf. As all tested *pik3ca^{PROS}* alleles gave rise to VMs
163 with the same frequency and severity (**Figure 1F**), we focused on one hotspot variant for
164 subsequent work, *pik3ca^{E545K},* for studying mesodermal PI3K gain-of-function.

165 *pik3ca^{E545K}-mScarlet*+ expressed from the *tbxta* promoter was as effective as the *β-actin*
166 construct in inducing the *pik3ca^{PROS}* vascular phenotype in *Tg(fli1:eGFP)* embryos
167 (**Supplementary Figure 2**). *tbxta:pik3ca^{PROS}-mScarlet* expression was visible from early
168 gastrulation, before the earliest plausible stage of vascular malformation onset, as angioblasts
169 migrate from the lateral plate mesoderm (LPM) towards the midline during somitogenesis (10-
170 22 hpf) (31). At 18 hpf, *mScarlet* expression in injected controls was predominantly in the

171 notochord and myotome, but co-expressed with very few GFP+ endothelial cells, as expected
172 from known expression patterns of *tbxta* at this developmental stage (34) (**Figure 3C**). In
173 controls, very occasional ectopic *fli1:eGFP* cells were observed dorsal to the developing DA
174 and unattached to the nascent vasculature, thus seemingly independent of normal ISV
175 sprouting, which occurs from 20 hpf (**Figure 3C, Movie 4**). In embryos overexpressing
176 *pik3ca*^{PROS} however, the frequency of such ectopic *fli1:eGFP* cells was markedly increased
177 (**Figure 3D**). These ectopic cells persisted through ISV sprouting and subsequent branching
178 to form the DLAV at ~22 hpf. Occasionally, at this time point, we observed rare examples of
179 *eGFP* and mScarlet double positive cells dying (**Figure 3C,** orange arrow). By 28 hpf, we
180 observed DLAV cells forming premature dilated vessels (**Movie 5**).

181 We thus demonstrate that *pik3ca*^{PROS} expression in mesoderm alone disrupts the earliest
182 stages of vascular development, causing dysregulation of endothelial cell sprouting and
183 branching. Occasional *mScarlet*-expressing endothelial cells were present, some of which
184 were observed dying during aberrant development, which may indicate heightened sensitivity
185 of endothelial cells to high levels of *pik3ca*^{PROS} transgene overexpression. However, as in later
186 vascular development, vascular defects in this model appear to be largely a non-cell
187 autonomous effect of *pik3ca*^{PROS} expression on *fli1:GFP+, mScarlet+* endothelial cells.

188

189 **Mosaic mesodermal *pik3ca*^{PROS} expression induces pan-lineage dysregulation**

190 Given this evidence for non-cell autonomous effects of mesodermal *pik3ca*^{PROS} expression on
191 early vascular development, we next sought to interrogate more agnostically the extent of the
192 developmental disruption. We used FACS to sort live cells from *tbxta:pik3ca*^{PROS}*-mScarlet* and
193 *tbxta:mScarlet* injected controls at 19 hpf and subjected these to single-cell RNA sequencing
194 (scRNA-seq) (**Figure 4A**). Using Seurat, we identified 40 distinct cell populations common to
195 'control' and 'PROS mosaic' embryos. These were named using a combination of marker gene
196 expression patterns and projection of two previously published scRNA-seq datasets onto the
197 data (**Figure 4B, Supplementary Figure 3A, B**) (35, 36). This confirmed the presence of all
198 cell lineages expected at this developmental stage, including endothelial cells, CNS, epithelial,
199 lateral plate and presomitic mesoderm (LPM, PSM), and notochord (**Figure 4B, C**).

200 One clear difference between control and *pik3ca*^{PROS} embryos lay in so-called cluster '0', which
201 could not be mapped to a uniquely annotated lineage, instead featuring weak signatures of
202 many different cell types, hinting at a "confused" lineage identity (**Supplementary Figure 3C**).
203 We confirmed upregulation of the *pik3ca* transcript itself in this cluster, largely co-segregating
204 with *mScarlet* in PROS mosaic animals (**Figure 4D**). Velocity analysis showed a lack of clear

205 developmental directionality of transcription in PROS mosaic embryos compared to the small
206 corresponding cluster in control animals. In contrast, velocity in the PSM and neural lineages
207 was increased, suggesting non-cell autonomous perturbations of lineage transitions in both
208 mesodermal (*tbxta*-driven) and non-mesodermal (i.e. neural) lineages (**Supplementary**
209 **Figure 3D**).

210 The changes in relative sizes of different cell clusters between experimental conditions was
211 assessed by expressing the number of cells within each cluster as a percentage of the total
212 cell number for control and PROS datasets, respectively. After calculating the differences in
213 these percentages, a dramatic expansion of cluster 0 was observed in PROS mosaic embryos
214 (**Figure 4E; Supplementary Figure 3E**). Some clusters of mesodermal origin also showed
215 expansion in PROS mosaics, as may be expected from mesodermal activation of PI3K
216 signaling, but, surprisingly, this was not true of all mesoderm-derived clusters, and ectoderm-
217 derived neural clusters were also enriched in PROS mosaics (**Figure 4D, E; Supplementary**
218 **Figure 3E**). This suggests that *pik3ca*^{PROS} cells have developmental effects extending beyond
219 mesodermal lineages themselves, unbalancing cell fate decisions and thereby cell type
220 proportions in the embryo.

221 In PROS mosaic embryos, 319 genes were significantly upregulated in mScarlet+ PROS cells
222 relative to wildtype in PROS mosaics, including, as expected, *pik3ca, tbxta* and *mScarlet*, but
223 fewer were upregulated in PROS mosaics compared to control embryos (**Supplementary**
224 **Figure 4A**). Analysis of curated transcriptomic "footprints" of pathway activity indicated that
225 PI3K-coupled programs were significantly enriched in *mScarlet*+ cells in PROS mosaics
226 relative to their WT *mScarlet*- counterparts, indicating increased PI3K activity as well as
227 *pik3ca*^{PROS} overexpression in transgenic cells (**Supplementary Figure 4B**). Interestingly, the
228 same analysis revealed enrichment for signatures of apoptosis, DNA damage repair, G2/M
229 checkpoint activation and p53 activity, suggesting that *pik3ca*^{PROS} expression may ultimately
230 be toxic. Such negative selection of *pik3ca*^{PROS} has previously been invoked as a possible
231 explanation for the relative lack of PIK3CA mutations in some human cell lineages.
232 Surprisingly, few genes were significantly upregulated in PROS mosaic cluster 0 relative to
233 control, although footprint analysis did suggest significant enrichment of MYC-Targets and
234 Apical Junction components (**Supplementary Figure 4C**), although the uncertain nature of
235 cluster 0 in control embryos complicates interpretation of this comparison.

236 In other cluster groups, we found widespread enrichment of Kegg or Reactome pathway
237 components directly related to PI3K signaling in clusters from the PROS zebrafish, including
238 modules related to Pten, Insulin, PIP3, mTor and Foxo1 (**Figure 4F**). Other gene sets were
239 also frequently enriched, including Notch, Wnt and Mapk pathways, as well as shared

240 responses to cell metabolism and cell division processes. Strikingly, these transcriptional
241 changes extended to clusters in the PROS zebrafish of non-mesoderm origin that expressed
242 almost no mScarlet cells (**Figure 4F**). Moreover, these gene expression patterns persisted
243 when we removed the PROS *mScarlet*+ cells from the analysis entirely, indicating that
244 otherwise wild type mesodermal and non-mesodermal lineages are responding
245 transcriptionally to PROS cells even without expressing *pik3ca*^{PROS} themselves
246 (**Supplementary Figure 4D**).

247 ***pik3ca*^{PROS} elicits widespread transcriptional changes in ligand-receptor expression**
248 **patterns**

249 Finally, we sought evidence from single cell transcriptomic data implicating potential mediators
250 of the observed non-cell autonomous effects of genetic PI3K hyperactivation. We did this using
251 CellChat and NICHES as analytic tools to infer communication among cell lineages using the
252 proxy of expression patterns of ligand/receptor pairs (37, 38). Analyses of the top 10%
253 predicted cluster interactions by CellChat suggested major transcriptional changes in
254 signalling mediators in PROS embryos. In WT embryos the transcriptomic landscape
255 appeared dominated by reciprocal expression of ligand receptor pairs (loosely "signaling
256 traffic") among the notochord, and mixed epithelial/pharyngeal lineages, while in PROS
257 embryos a more distributed pattern was seen, with presomitic mesoderm and various neural
258 lineages much more prominent (**Figure 5A**).

259 To assess potential specific signaling pathways driving these predicted changes, we used the
260 NICHES algorithm, which analyses ligand-receptor mRNA expression in one-to-one pairs of
261 cells to infer communication (38). We subsetted our data to include the clusters found most
262 active by CellChat and ran NICHES to convert the single-cell gene expression dataset to a
263 single-cell signalling dataset based on LR signaling pattern genes, grouped by sending
264 clusters, receiving clusters, or individual pair-wise interactions (**Figure 5B)**. To identify
265 potential changes in cell signalling capacity between lineages in PROS mosaic animals
266 relative to control, we performed DE analysis on 'sending' and 'receiving' transcriptional
267 signatures for each cluster (**Supplementary Figure 4E**). To gain a comprehensive overview
268 of signaling changes, we simplified the outputs by grouping and averaging ligand and receptor
269 fold changes respectively (**Figure 5C**). We observed a marked increase in Notch, Wnt and
270 Ephrin ligand-receptor pairs in both mesodermal and non-mesodermal lineages, but again
271 cluster 0 exhibited general downregulation of sending and receiving signaling components.
272 This is in keeping with the non-cell autonomous responses to small numbers of *pik3ca*^{PROS}
273 cells in the PROS zebrafish, and the relatively inert state of C0 (**Figure 5C**; **Supplementary**
274 **Figure 3 C,D**).

275 We next calculated 'module scores' by averaging gene expression of all ligand-receptor pairs
276 within a pathway (e.g. all combinations of *jag/dll/dld-notch1a/1b/2/3* for notch signalling) and
277 compared the intensities of this score on cell-to-cell UMAPs, split by experimental condition
278 (**Figure 5D)**. Comparing the connectivity of PROS mosaics to control, we observed a reduction
279 in extent and intensity of mRNA expression of *egf* and *pdgf* ligand-receptor pairs in PROS,
280 further highlighting disruption of normal signaling through Pik3ca throughout the embryo
281 (**Figure 4F, 5D**). In contrast, we observed widespread upregulation of pairs of *notch*, *wnt* and
282 *ephrin* signaling genes (**Figure 5D**). Focusing on individual ligand-receptor pairs, we found
283 evidence for transcriptional "rewiring" of these pathways between lineages. For example, *dla-*
284 *notch3, wnt4-fzd5-lrp6* and *efnb3b-epha4a* ligand-receptor pair expression is increased for
285 neural, PSM, myotome and Epi/Ph nodes (**Figure 5D**).

286 We conceptualize these data to indicate that *pik3ca*^{PROS} has significant effects on cell fate,
287 behavior and likely signaling which ramify across many lineages, indicating pervasive and
288 disruptive non-cell-autonomous effects on early development. Overall, these analyses
289 highlight transcriptional changes in ligand and receptor gene expression that may contribute
290 to the non-cell-autonomous phenotypes observed and serve as potential drug targets worthy
291 of further investigation.

292

293 **Discussion**

294 Over ten years since mosaic, activating PIK3CA mutations were identified as the cause of a
295 group of asymmetric overgrowth disorders (39, 40) much has been learned. The wide human
296 clinical spectrum has been delineated, the natural history described, and nomenclature
297 harmonized (5, 6). Furthermore, case series and registry based open label studies have
298 established that the PI3Kα inhibitor alpelisib is of some clinical utility, leading to licensing in
299 the USA (16, 29) and establishment of an international randomized clinical trial still underway
300 (NCT04589650) (41). However, despite this major progress, important fundamental and
301 translational questions about PROS remain. Specifically, the explanation for the skewed
302 appearance of PROS-related pathology in some tissues but not others, and the involvement
303 and extent of non-cell-autonomous mechanisms of overgrowth in PROS pathogenesis have
304 eluded comprehensive analysis. The latter issue is of translational importance as it may yield
305 novel diagnostic and therapeutic targets; given current evidence that alpelisib has only partial
306 efficacy in only some patients with PROS (15), this is of great value.

307 Fundamental to translational progress is the fidelity of models to human pathology. A wealth
308 of murine models has advanced our understanding of PROS, by showing definitive causal

309 links between acquisition of PROS mutations and vascular malformations (22, 42), identifying
310 critical time windows during which endothelia are most sensitive to excessive PI3K signaling
311 (25), and demonstrating that PI3K and related pathway inhibition can mitigate or rescue
312 important disease manifestations (14, 21-23, 42, 43). More recently, mouse PROS or cancer
313 models have implicated signaling from non-endothelial cell types as drivers of vascular
314 overgrowth (11, 25, 27), but otherwise few animal studies have focused on non-cell-
315 autonomous contributors to PROS pathology to build a holistic view of multi-lineage
316 interactions during early development.

317 Overexpression of an activating PIK3CA allele has been shown to recapitulate several aspects
318 of PROS-related overgrowth in zebrafish (28), and the successful usage of zebrafish as a
319 preclinical model of lymphatic overgrowth or neurovascular malformations (44, 45)
320 encouraged us to develop patient-led zebrafish models to interrogate the complex interplay of
321 Pik3ca genotype and lineage context, and to examine overgrowth dynamics at early stages of
322 development. We now present a zebrafish model with mosaic overexpression of naturally
323 occurring hotspot *pik3ca*^{PROS} variants. This enables observation of the effects of PROS
324 mutations *in vivo*, from onset, at cellular resolution for the first time. Our model recapitulates
325 a spectrum of overgrowth severity affecting different cell lineages, as in PROS, with
326 vasculature most prominently affected, while scRNA-seq suggests that *pik3ca*^{PROS} expressing
327 cells appeared 'stuck' in a less differentiated cell state. This is reminiscent of iPSC models of
328 *PIK3CA*^{H1047R} activation, where homozygous mutant cells show transcriptional remodeling and
329 impaired differentiation (46). Our model also allows comparison of four different *pik3ca*
330 hotspot gain-of-function variants in parallel, which proved to cause vascular overgrowth with
331 similar frequency and severity. This argues against major differences in phenotypic expression
332 across different hotspot Pik3ca variants, whether affecting helical or kinase domains, although
333 the high level of expression in our model leaves open the possibility that differences may be
334 unmasked at endogenous expression levels.

335 The non-cell autonomous influences of *pik3ca*^{PROS} cells found in our *pik3ca*^{PROS} model is in
336 keeping with mounting evidence that paracrine signals from *PI3KCA*^{PROS} cells corrupt
337 neighboring wildtype cells. For example, eicosanoids secreted from *PIK3CA* mutant breast
338 cancer cells induce proliferation in WT cells (13), paracrine VEGF and IL-6 signaling from
339 mammary duct culture lead to dysfunction in neighboring innervating vasculature *in vitro* (12).
340 Moreover, *in vivo* activating Akt mutations in murine mammary tissue cause hyperplasia of
341 nearby WT cells in a paracrine manner (47), and lymphatic capillary endothelial cells
342 expressing *Pik3ca*^{H1047R} recruit VEGFC-producing macrophages which cause immune
343 activation and worsening of vascular malformations in a mouse lymphoma model (11). Lastly,
344 driving *Pik3ca*^{H1047R} in murine pericyte progenitors induced widespread capillary and venous

345 malformations as well as hypertrophy of Cre-targeted connective tissues (48). Paracrine
346 signals also have a role in normal endothelial and hematopoietic development, as a subtype
347 of endothelial cells derived from the paraxial mesoderm have been shown to support induction
348 of hematopoietic cells from neighboring hemogenic endothelial cells in zebrafish (49). It is
349 important to note that cell-autonomous and non-cell-autonomous contributions to VMs in our
350 model are not mutually exclusive, and we did consistently observe minorities of *fli1:eGFP*+
351 endothelia co-expressing *mScarlet* indicative of a mixture of signaling mechanisms occurring
352 in VMs. Likewise, gain-of-function *PIK3CA* mutations are found through genotyping
353 endothelial cells in PROS patients, and are thought to be a driver of overgrowth in their
354 wildtype counterparts. Therefore, both cell-autonomous and non-cell-autonomous signals
355 likely underpin PROS pathology.

356 A limitation of our model, as for many other published models, is that it relies on transgenic
357 *pik3ca* overexpression, although the variants we overexpressed were orthologues to human
358 disease-causing mutations. The extent of overactivation through transgenesis is likely higher
359 than seen in PROS where *PIK3CA* is regulated endogenously, meaning the lack of *mScarlet*
360 in cell types most sensitive to PI3K levels, such as the endothelia, may be due to an intolerably
361 high levels of PI3K signaling causing cell death, as we observed in some co-expressing cells,
362 and enrichment of apoptotic transcriptional signatures in *mScarlet*+ cells in PROS mosaic
363 embryos. Furthermore, the timing of p110α activation is important for phenotype severity, with
364 prenatal but not postnatal induction of $Pik3ca^{E545K}$ causing increased brain size, and timed
365 induction of $Pik3ca^{H1047R}$ in endothelial cells revealing critical windows of PI3K sensitivity
366 during development (25, 26). Further temporal resolution of $pik3ca^{PROS}$ during zebrafish
367 development, and to an extent the 'dosage' of PI3K activation would be possible using a drug-
368 inducible version of our transgene. Finally, further testing of our transgenic construct in older
369 fish, perhaps with more restricted spatial expression of PROS mutations, is required to see if
370 adipose tissue and other tissue defects observed in PROS occur, and to the same penetrance.

371 Our novel zebrafish $pik3ca^{PROS}$ model has enabled *in vivo* observation of early stages of
372 evolution of PROS-like vascular malformations at a cellular level. It has moreover offered
373 evidence for surprisingly pervasive developmental effects of mosaic *pik3ca* activation,
374 including extensive non-cell autonomous effects ramifying beyond the affected lineage. This
375 complements and extends existing models of PROS and provides a tractable platform for more
376 in-depth interrogation of specific candidate mediators of such remote effects of *pik3ca*
377 activation. Therapeutic targeting of these may in principle be a valuable adjunct or alternative
378 to PI3K inhibition in future.

379

380 **Materials and Methods**

381 **Structural comparison of zebrafish and human PIK3CA**

382 Protein structures for human PIK3CA (AF-P27986-F1-v4) and the predicted structure of
383 zebrafish Pik3ca (AF-F1QAD7-F1-v4) were visualized and annotated using PyMOL
384 (Schrodinger).

385

386 **Fish husbandry, fish lines**

387 Zebrafish were maintained in accordance with UK Home Office regulations, UK Animals
388 (Scientific Procedures) Act 1986, amended in 2013, and European Directive 2010/63/EU
389 under project licenses P8F7F7E52 and PP7317786. All experiments were approved by the
390 Home Office and AWERB (University of Edinburgh Ethics Committee). Fish stocks used were
391 wild-type AB, *Tg(fli1:eGFP)* (50), *casper* (51). Combined transgenic lines were generated by
392 crossing. Adult fish were maintained at ~28.5°C under 14:10 light-dark cycles. Embryos were
393 kept at 28.5°C and staged according to the reference table provided by (52) and (53).

394 ***Pik3ca^{PROS}* transgene plasmid construction**

395 First, the *eGFP* sequence in the p3E Gateway entry vector IRES-eGFP-pA (54) was swapped
396 for the *mScarlet* CDS (Thermo Fisher Scientific) with the HiFi DNA assembly kit (NEB) to make
397 the p3E entry vector p3E-IRES-mScarlet-pA. The wild type zebrafish *pik3ca* CDS was
398 introduced into pDonr221 (Invitrogen) using Gateway™ BP Clonase™ II (ThermoFisher
399 Scientific) to make pME-*pik3ca^{WT}*. *pik3ca* hotspot mutation sites were introduced into pME-
400 *pik3ca^{WT}* sequence by designing back-to-back mutagenesis primers incorporating hotspot
401 mutations *H1048R/L*, *E542K* or *E545K*, phosphorylating them using T4 PNK (NEB) according
402 to manufacturer's instructions, and amplifying using Q5 polymerase (NEB). T4 ligase (NEB)
403 was used to re-ligate the mutated amplicon, and the original template digested using Dpni
404 (NEB). LR Clonase II+ (ThermoFisher Scientific) was used to combine p5E entry vectors p5E-
405 *β-actin* (54), p5E-*fli1ep* (50), p5E- *tbxta* (this study, based off (55), with a *pik3ca-PROS* pME,
406 and p3E-IRES-mScarletpA (this study, modified from p3E-IRES-GFP) into pDestTol2CG2 (54)
407 which carries a *cmlc2:eGFP* reporter. Sanger sequencing was used to confirm successful
408 mutation of pME-*pik3ca^{WT}* to pME- *pik3ca$^{H1048/H1048L/E542K/E545K}$* and correct integration of all
409 components. Sequences of primers used for plasmid construction are found in
410 **Supplementary Table 1**.

411 **Generation of mosaic *pik3ca^{PROS}* fish**

412 Approx. 2 nl of 30 ng/µl transgenesis construct was co-injected with 35 ng/µl Tol2 mRNA into
413 zebrafish embryos at the 1 cell stage. Embryos were *Tg(fli1:eGFP)* to enable visualization of
414 blood vessels, in either a wildtype or *casper* (unpigmented) genetic background – the latter to
415 more easily image embryos beyond stages of melanocyte development. Embryos were
416 screened for successful PROS transgene integration from 30 hpf onwards by assessment of
417 *cmlc2:GFP* cardiac fluorescence and/or *mScarlet* fluorescence. Morphologically abnormal
418 embryos at this stage were discarded, as vascular development is disrupted due to defects in
419 somite and heart development, for example (56). Scoring of vascular malformations was
420 performed at 72 hpf. For studies on older fish, embryos were raised to juvenile stages.

421 **Imaging**

422 Embryos and juvenile fish were anaesthetized with MS:222 and if necessary, placed on 3%
423 methylcellulose. Fluorescent mesoscope images were acquired using a Leica M165FCA
424 fluorescence stereomicroscope fitted with a 0.63x and 1x Plan-Apochromat objective, and a
425 Leica LMT260 XY scanning stage (Leica Microsystems, Germany). Image acquisition was
426 performed using a Leica K8 monochrome cMOS camera in Leica Application Suite software
427 (LASX; version 3.7). Color images were acquired as above but using a Leica KC3 color cMOS
428 camera (Leica, Microsystems, Germany). Images were acquired in Leica Application Suite
429 software (LASX; version 3.7). Movies were generated using Imaris (v9.6.0). Images were
430 processed using FIJI (v2.9.0).

431 Confocal images were acquired using a 20X/0.75 lens on the multimodal Imaging Platform
432 Dragonfly (Andor technologies, Belfast UK) equipped with 488 and 561 lasers built on a Nikon
433 Eclipse TI-E inverted microscope body with Perfect focus system (Nikon Instruments, Japan.
434 Data were collected in Spinning Disk 25 µm pinhole mode on the iXon 888 EMCCD camera
435 using a Bin of 1 and no frame averaging, and using Andor Fusion acquisition software. Z
436 stacks were collected using the Nikon TiE focus drive. Movies were generated using Imaris
437 (v9.6.0). Images were processed using FIJI (v2.9.0).

438 **Bone and Cartilage staining**

439 To stain bone and cartilage in juvenile fish, the Juvenile No Acid Bone & Cartilage Stain was
440 followed as described (zfin.atlassian.net/wiki/spaces/prot/pages/352157857) using a 0.02%
441 Alcian Blue 8GX (Merck)/10 mM $MgCl_2$ solution to stain cartilage and then a 0.01% Alizarin
442 red S (Merck) solution to stain mineralized bone.

443 **FACS and library preparation**

444 *Tg(fli1:eGFP)* embryos were injected with either *tbxta:pik3ca*^{E545K}*-mScarlet* or *tbxta:mScarlet*
445 and >300 embryos per condition raised to 19 hpf. Each batch was dissociated to single cell
446 suspensions using the methods described in (57). Cells from stage matched wild-type
447 embryos were used as negative controls to gate fluorescence. Live cells were sorted using a
448 FACSAria SORP instrument (BD Biosciences UK), with GFP (525/50, BP/488 nm laser), RFP
449 (582/15 BP, 561 nm laser), and DAPI (450/20BP, 405 nm laser) filters into 2% BSA in PBS
450 and immediately processed using the Chromium platform (10x Genomics). Libraries were
451 prepared using v3.1 Standard Throughput kits according to 10x protocols (10x Genomics),
452 and quality control and quantification assays performed using High Sensitivity DNA kits on a
453 Bioanalyzer (Agilent). Libraries were initially sequenced on an Illumina NovaSeq SP Flow cell
454 with 100 cycles with two lanes per sample. The imbalances in cell number/sequencing depth
455 between the two samples necessitated re-sequencing of the PROS sample as before. This
456 resulted in an average of 120,316 reads/cell after normalization.

457 **Bioinformatics**

458 FastQ files were aligned using the CellRanger (v8.0.1, 10x Genomics) mkref, mkgtf and aggr
459 pipelines to custom zebrafish STAR genome index using annotations from Ensembl GRCz11
460 release 112 with manual addition of *eGFP* and *mScarlet*. The resultant h5 matrices were read
461 into R (v4.4.2) and subjected to quality control using Seurat (58) guidelines (v5.2.1, features
462 >200, <13000 and mitochondrial gene < 5%). This resulted in 10306 cells from PROS embryos
463 and 3505 from control. Data were scaled and regressed to cell cycle stage before performing
464 PCA, finding the dimensionality of the data by elbow plot (dims=50) to plot 40 clusters on
465 UMAP plots (SeuratExtend (v1.1.4 (59)). SingleR (60) (v2.8.0) was used with previously
466 published datasets GEO: GSE112294 (35) and NCBI SRA PRJNA940501 (36) to call clusters.
467 DE analyses were performed using zinbwave (v 1.28.0 (61)) and DESeq2 (v1.46.0(62)),
468 selecting genes with padj<0.05 before using clusterProfiler (v4.14.3 (63)) to perform gene
469 enrichment analyses searching Reactome, GO:BP and KEGG libraries, filtering by p.adj <0.05
470 (raw data are in **Supplementary Table 2**). Additional footprint enrichment analyses were
471 conducted using DecoupleR (v. 2.12.0 (64)) using the inbuilt Progeny databases and zebrafish
472 Hallmark datasets imported from MSigdDB according to package guidelines, with Seurat's
473 FindAllMarkers with wilcox testing to determine changes (p.adj < 0.1, p<0.05) to pathway
474 enrichment. Proportions of cells within cluster for PROS and control data were found by
475 dividing the number of cells per cluster/total cell number per experimental condition, with
476 Pearson's chi-squared test (two-tailed) used to determine significance (raw data are in
477 **Supplementary Table 3**). RNA velocity analysis was performed on loom files for control and
478 PROS data generated with velocyto (v. 0.17 (65)), and velocity computed using scVelo's
479 (v.0.2.5 (66)) dynamical modelling approach, and visualized using heatmaps and stream plots.

CellChat (v. 2.1.2 (37)) was used to infer potential signaling events between clusters according to relevant package guidelines. NICHES (v. 1.0.0 (38, 67)) was used to perform ligand-receptor expression analysis at single cell resolution, with differential expression analysis within Seurat, using the MAST test, performed to identify significant changes to LR gene expression (raw data are in **Supplementary Table 4**). Additionally, circuit diagrams were generated using code from https://github.com/RaredonLab/NICHESMethods. Plots were generated using Seurat, CellChat, NICHES, Complex Heatmap (v. 2.22.0 (68)) and ggplot2 (v. 4.0.0).

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Data and Code availability

scRNA-seq experiments generated in this study have been deposited at GEO and will become publicly available as of the date of publication. Computational code and plasmid constructs generated for this are available from the lead contact upon request.

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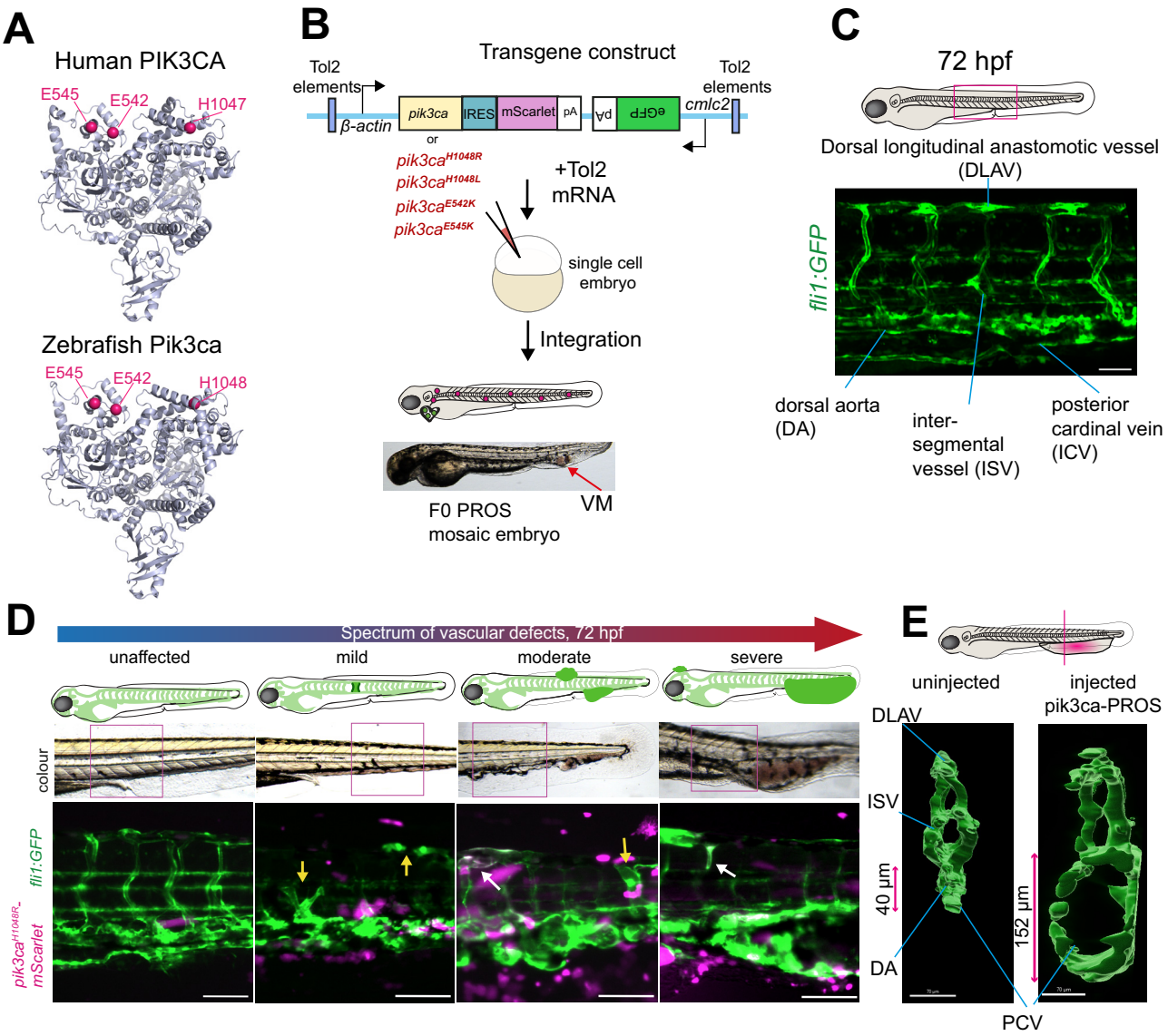
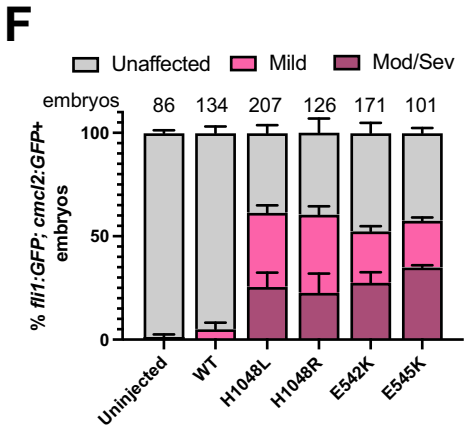

A
Human PIK3CA
E545
E542
H1047
Zebrafish Pik3ca
E545
E542
H1048
B
Transgene construct
Tol2 elements
β-actin
pik3ca
IRES
mScarlet
pA
pA
eGFP
cmlc2
Tol2 elements
or
pik3ca^H1048R
pik3ca^H1048L
pik3ca^E542K
pik3ca^E545K
+Tol2 mRNA
single cell embryo
Integration
F0 PROS mosaic embryo
VM
C
72 hpf
Dorsal longitudinal anastomotic vessel (DLAV)
fli1:GFP
dorsal aorta (DA)
inter-segmental vessel (ISV)
posterior cardinal vein (ICV)
D
Spectrum of vascular defects, 72 hpf
unaffected
mild
moderate
severe
colour
fli1:GFP
pik3ca^H1048R-mScarlet
E
uninjected
injected pik3ca-PROS
DLAV
ISV
40 µm
DA
152 µm
PCV


F
Unaffected
Mild
Mod/Sev
embryos
86
134
207
126
171
101
% fli1:GFP; cmcl2:GFP+ embryos
100
50
0
Uninjected
WT
H1048L
H1048R
E542K
E545K

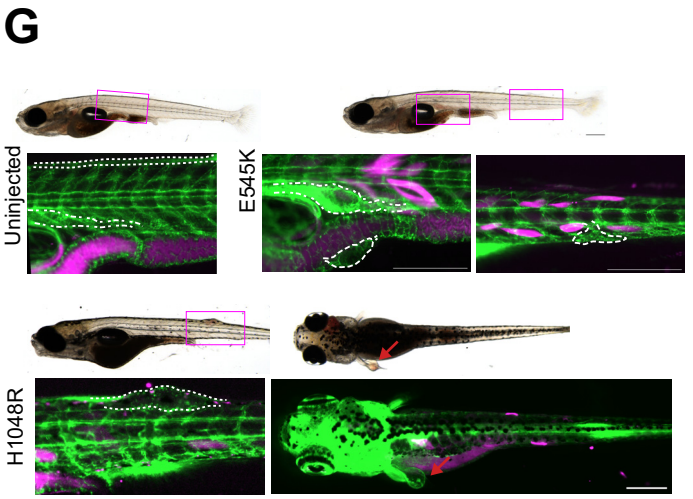

G
Uninjected
E545K
H1048R

Figure 1

Figure Legends

Figure 1: Mosaic *pik3ca* hotspot transgenes cause vascular malformations in zebrafish embryos

A) The structure of human PIK3CA and the predicted structure of the zebrafish Pik3ca orthologue is remarkably conserved, with one additional amino acid in zebrafish Pik3ca shifting the kinase domain amino acids by 1. The position of E542, E545 and H1047 residues found mutated in PROS (and the orthologous E542, E545 and H1048) are labelled in magenta and are predicted to occur in very similar structural regions of the protein in both human and fish.

B) Schematic representation of the transgenic *pik3ca*^{PROS} constructs generated for this study, containing *β-actin:pik3ca*^{PROS}*-mScarlet* and the cardiac reporter *cmlc2:GFP* flanked by Tol2 response elements, and is co-injected with *Tol2* mRNA into 1-cell zebrafish embryos. Embryos were screened for successful integration of the transgene from 48 hpf onwards, indicated by mosaic cardiac *cmlc2:GFP*, and *mScarlet* expression. At 72 hpf, some PROS mosaic embryos had tail vascular malformations.

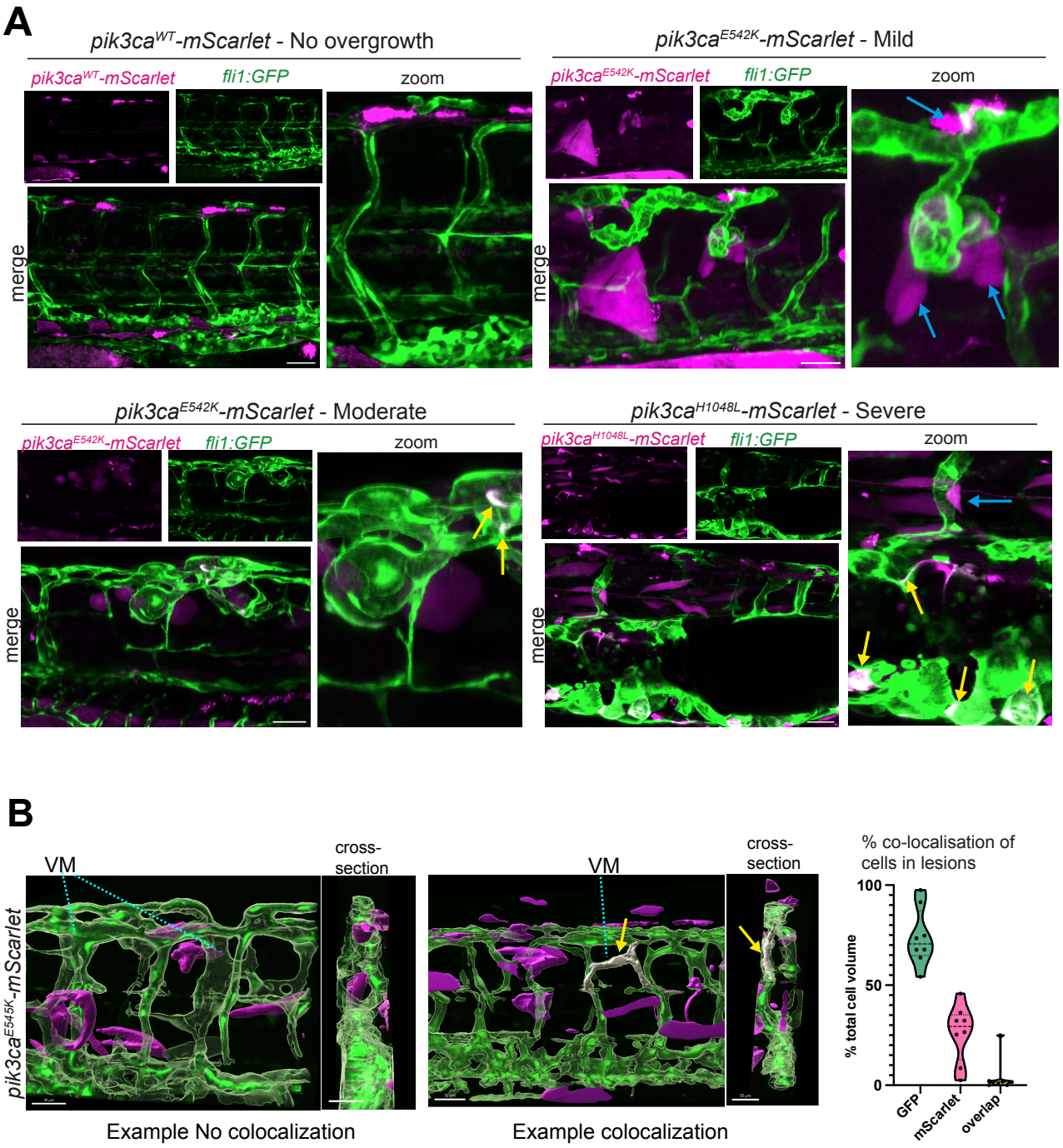
C) Maximum Z-projection of a 72 hpf *Tg(fli1:eGFP);casper* tail region, showing typical endothelial vessel structures marked by *fli1:eGFP* fluorescence (green). Scale bar = 50 µm

D) Representative diagrams and fluorescent microscopy images of the spectrum of vascular phenotypes seen in *Tg(fli1:eGFP)* embryos injected with *pik3ca*^{H1048R}*-mScarlet*. These ranged from unaffected and mild, to moderate and severe. *Fli1:eGFP* expression in unaffected embryos is as uninjected. Embryos scored 'mild' displayed expansion and mispatterning of isolated inter somitic vessels that was only visible when viewing eGFP fluorescence. Moderate and severe scores were applied to embryos where blood pooling was evident under brightfield, particularly obvious in the PCV. Yellow arrows indicate mis-patterned vessels in 'mild' embryos, and white arrows indicate overlap of *fli1:eGFP* (green) and *mScarlet* (magenta) expression in moderate and severe examples. Quantification of this and numbers screened can be seen in Figure 3. Scale bars = 100 µm.

E) Cross sections of trunk blood vessels at 72 hpf, showing the dramatic expansion of the PCV.

F) Quantification of these vascular malformation phenotypes for each construct, scored based on severity, as a percentage of the total numbers injected. Numbers indicate *fli1:eGFP; cmcl2:GFP*+ embryos screened for vessel malformations at 72 hpf, representative of three biological repeats per construct. Error bars represent S.E.M.

G) Representative images of juvenile *Tg(β-actin:pik3ca*^{E545K}*-mScarlet; fli1:eGFP); casper* (top) and *Tg(β-actin:pik3ca*^{H1048R}*-mScarlet; fli1:eGFP* fish that harboured no detectable vascular anomalies at 72 hpf, but were found to have vascular anomalies when imaged two weeks later (*E545K*-injected – 6/16, *H1048R*-injected – 3/9). Dotted lines encompass vessel malformations in PROS animals, and equivalent regions in control


A
pik3ca^WT-mScarlet - No overgrowth
pik3ca^WT-mScarlet
fli1:GFP
zoom
merge
pik3ca^E542K-mScarlet - Mild
pik3ca^E542K-mScarlet
fli1:GFP
zoom
merge
pik3ca^E542K-mScarlet - Moderate
pik3ca^E542K-mScarlet
fli1:GFP
zoom
merge
pik3ca^H1048L-mScarlet - Severe
pik3ca^H1048L-mScarlet
fli1:GFP
zoom
merge

B
VM
cross-section
pik3ca^E545K-mScarlet
Example No colocalization
VM
cross-section
Example colocalization
% co-localisation of cells in lesions
% total cell volume
100
50
0
GFP
mScarlet
overlap

Figure 2

Figure 2: Evidence for cell-autonomous and non-cell-autonomous mechanisms of *pik3ca^{PROS}* vascular overgrowth

A) Representative maximum projection confocal images of 72 hpf *Tg(fli1:eGFP);casper* embryos expressing either wildtype *pik3ca-mScarlet* (no overgrowth) or a *pik3ca^{PROS}* hotspot variant and exhibiting a mild, moderate or severe vascular phenotype. For each, separated *mScarlet* fluorescence (magenta), *fli1:eGFP* channels (green) and merged channels are shown, with a zoomed area to the right. Blue arrows indicate *mScarlet* cells in close proximity to deformed vessels. Yellow arrows indicate white cells co-expressing *fli1:eGFP* and *mScarlet*. Scale bars = 50 µm

B) Quantification of *mScarlet* cells by volume within GFP+ endothelial cells. Imaris was used to visualize confocal Z stacks of vessel overgrowth at 72 hpf. 3D structures of each channel, plus a co-localization channel were converted into surfaces, enabling volume calculations for vessels (green), mScarlet+ cells outside vessels (purple) or areas of colocalization (white). Volumes of each were expressed as percentage of total cell volume in 20x magnification view, dots indicate individual images (8 lesions were analysed in total).

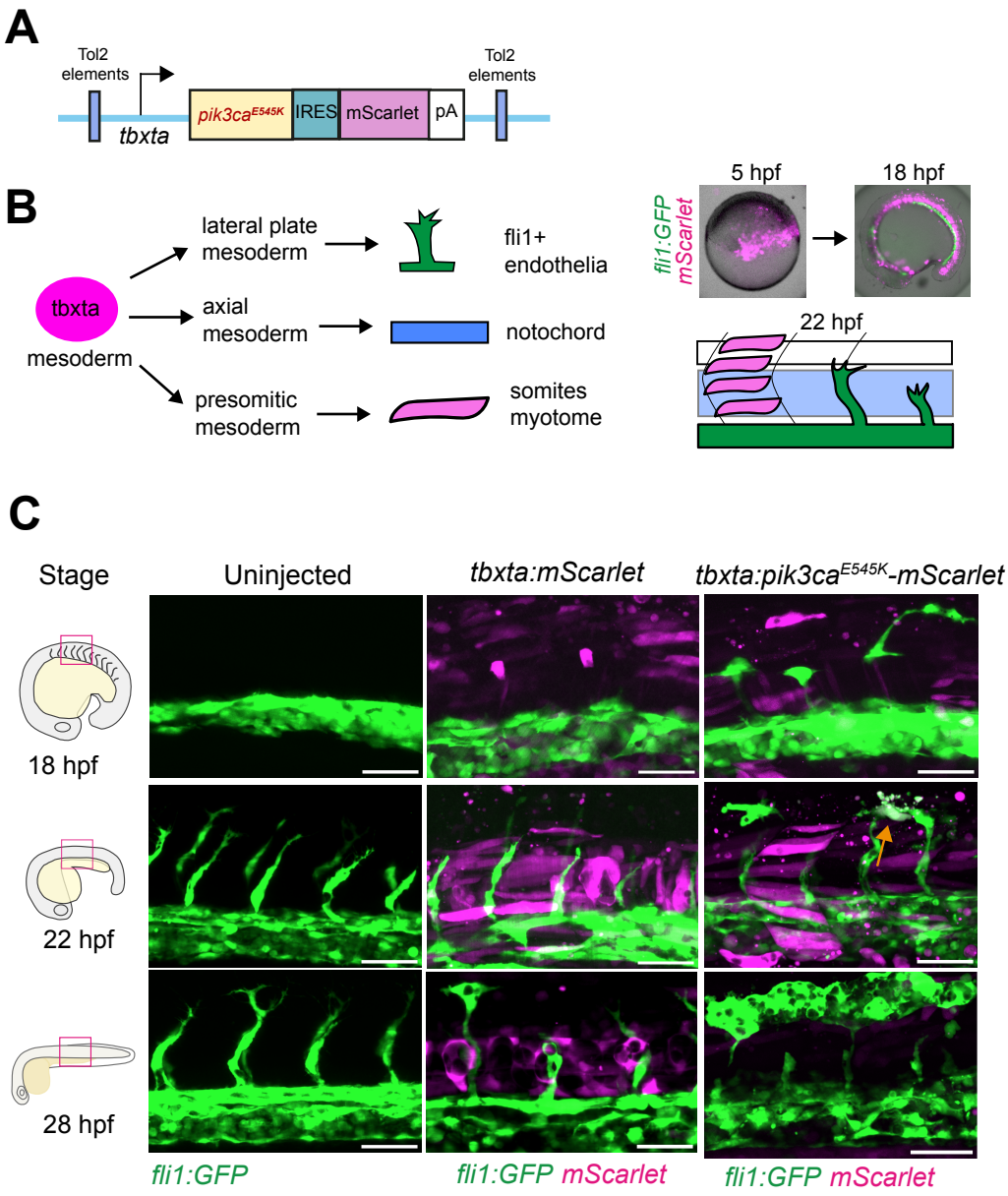

A
Tol2 elements
Tol2 elements
tbxta
pik3ca^{E545K}
IRES
mScarlet
pA
B
tbxta
mesoderm
lateral plate mesoderm
axial mesoderm
presomitic mesoderm
fli1+ endothelia
notochord
somites myotome
fli1:GFP mScarlet
5 hpf
18 hpf
22 hpf
C
Stage
Uninjected
tbxta:mScarlet
tbxta:pik3ca^{E545K}-mScarlet
18 hpf
22 hpf
28 hpf
fli1:GFP
fli1:GFP mScarlet
fli1:GFP mScarlet
D
18 hpf
****
****
ns
number ectopic eGFP+ cells
20
15
10
5
0
tbxta:mSc
tbxta:WT-mSc
tbxta:E545K-mSc

Figure 3

Figure 3: Early mesodermal *pik3ca*^{PROS} transgene expression causes endothelial mis-patterning

A) Plasmid design of the transgenic construct *tbxta:pik3ca*^{E545K}*-mScarlet*.

B) Simplified diagram of a *tbxta* progenitor cell which may differentiate into the lateral plate mesoderm, the notochord, or presomitic mesoderm which gives rise to endothelia, the notochord or muscle respectively. Mosaic *tbxta:mScarlet* is visible at 5 hpf (representative images) before onset of *fli1:eGFP* reporter expression. By 18 hpf, *tbxta* is most highly expressed in the notochord.

C) Representative maximum projection confocal images of embryos from a range of stages between somitogenesis (~18 hpf) and tailbud (~28 hpf) to visualise the start of *fli1:eGFP* expression and endothelial development. In uninjected embryos, lateral stripes of *fli1:eGFP* are present from 16hpf, with sprouting of ISVs occurring from anterior to posterior from around 19 hpf onwards. In *tbxta:pik3ca*^{E545K}*-mScarlet* injected embryos, ectopic *fli1:eGFP* cells can be seen dorsal to the initial lateral plate mesoderm stripe. Scale bars = 50 µm

D) Quantification of ectopic *fli1:eGFP*+ cells in injected *tbxta:pik3ca*^{E545K}*-mScarlet,* or injected with a similar construct lacking the *pik3ca-ires* element (unattached to the lateral mesoderm stripes of *fli1:eGFP*). 3 biological replicates shown with each dot representing 1 embryo. Significance was calculated with a two-tailed Mann Whitney test. ****=$p<0.001$.

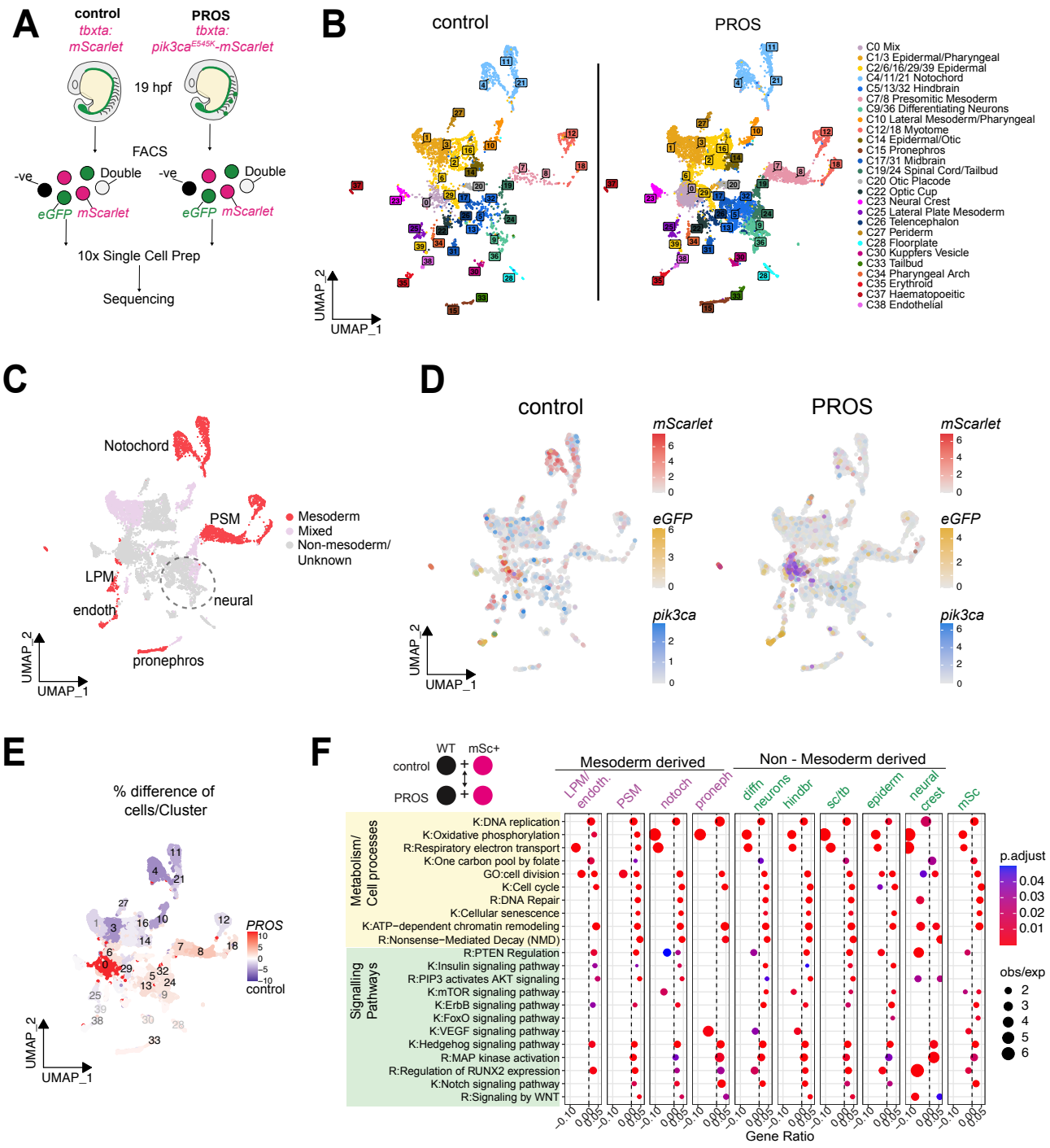

A
control
tbxta:
mScarlet
PROS
tbxta:
pik3ca^{E545K}-mScarlet
19 hpf
FACS
-ve
Double
eGFP
mScarlet
10x Single Cell Prep
Sequencing
B
control
PROS
UMAP_2
UMAP_1
C0 Mix
C1/3 Epidermal/Pharyngeal
C2/6/16/29/39 Epidermal
C4/11/21 Notochord
C5/13/32 Hindbrain
C7/8 Presomitic Mesoderm
C9/36 Differentiating Neurons
C10 Lateral Mesoderm/Pharyngeal
C12/18 Myotome
C14 Epidermal/Otic
C15 Pronephros
C17/31 Midbrain
C19/24 Spinal Cord/Tailbud
C20 Otic Placode
C22 Optic Cup
C23 Neural Crest
C25 Lateral Plate Mesoderm
C26 Telencephalon
C27 Periderm
C28 Floorplate
C30 Kuppfers Vesicle
C33 Tailbud
C34 Pharyngeal Arch
C35 Erythroid
C37 Haematopoeitic
C38 Endothelial
C
Notochord
PSM
LPM
endoth
neural
pronephros
Mesoderm
Mixed
Non-mesoderm/
Unknown
D
mScarlet
eGFP
pik3ca
E
% difference of
cells/Cluster
PROS
control
F
WT
mSc+
Mesoderm derived
Non - Mesoderm derived
LPM/ endoth.
PSM
notoch
proneph
diffn
neurons
hindbr
sc/tb
epiderm
neural crest
mSc
Metabolism/ Cell processes
K:DNA replication
K:Oxidative phosphorylation
R:Respiratory electron transport
K:One carbon pool by folate
GO:cell division
K:Cell cycle
R:DNA Repair
K:Cellular senescence
K:ATP−dependent chromatin remodeling
R:Nonsense−Mediated Decay (NMD)
Signalling Pathways
R:PTEN Regulation
K:Insulin signaling pathway
R:PIP3 activates AKT signaling
K:mTOR signaling pathway
K:ErbB signaling pathway
K:FoxO signaling pathway
K:VEGF signaling pathway
K:Hedgehog signaling pathway
R:MAP kinase activation
R:Regulation of RUNX2 expression
K:Notch signaling pathway
R:Signaling by WNT
Gene Ratio
p.adjust
obs/exp

Figure 4

Figure 4. Mosaic mesodermal *pik3ca*^{PROS} expression induces pan-lineage dysregulation

A) Schematic of experimental workflow for scRNA-seq. *Tg(fli1:eGFP)* embryos were injected with *tbxta:mScarlet* (control) or *tbxta:pik3ca*^{E545K}*-mScarlet* (PROS) and grown until 19 hpf, dissociated, and all live cells sorted by FACS before processing for 10x sequencing.
B) UMAP showing 40 cell clusters found the aggregated dataset, split by experimental condition.
C) UMAP of aggregated dataset coloured by mesodermal or other germ layer origin
D) FeaturePlots split by experimental condition, showing expression levels of *mScarlet* (red scale), *eGFP* (yellow scale) and *pik3ca* (blue scale)
E) The numbers of cells per cluster was expressed as a percentage of total cells for each experimental condition, and then the difference between percentages in PROS and control clusters visualised in a UMAP heat plot. Blue indicates a higher percentage of control cells relative to PROS, and red indicates a higher proportion of PROS relative to control cells.
F) Deseq2 DE analysis and gene enrichment analyses using GO (G:), KEGG (K:) and Reactome (R:) databases were performed to compare PROS mosaic vs control cells in various cluster groups, or in *mScarlet*+ cells across the dataset.

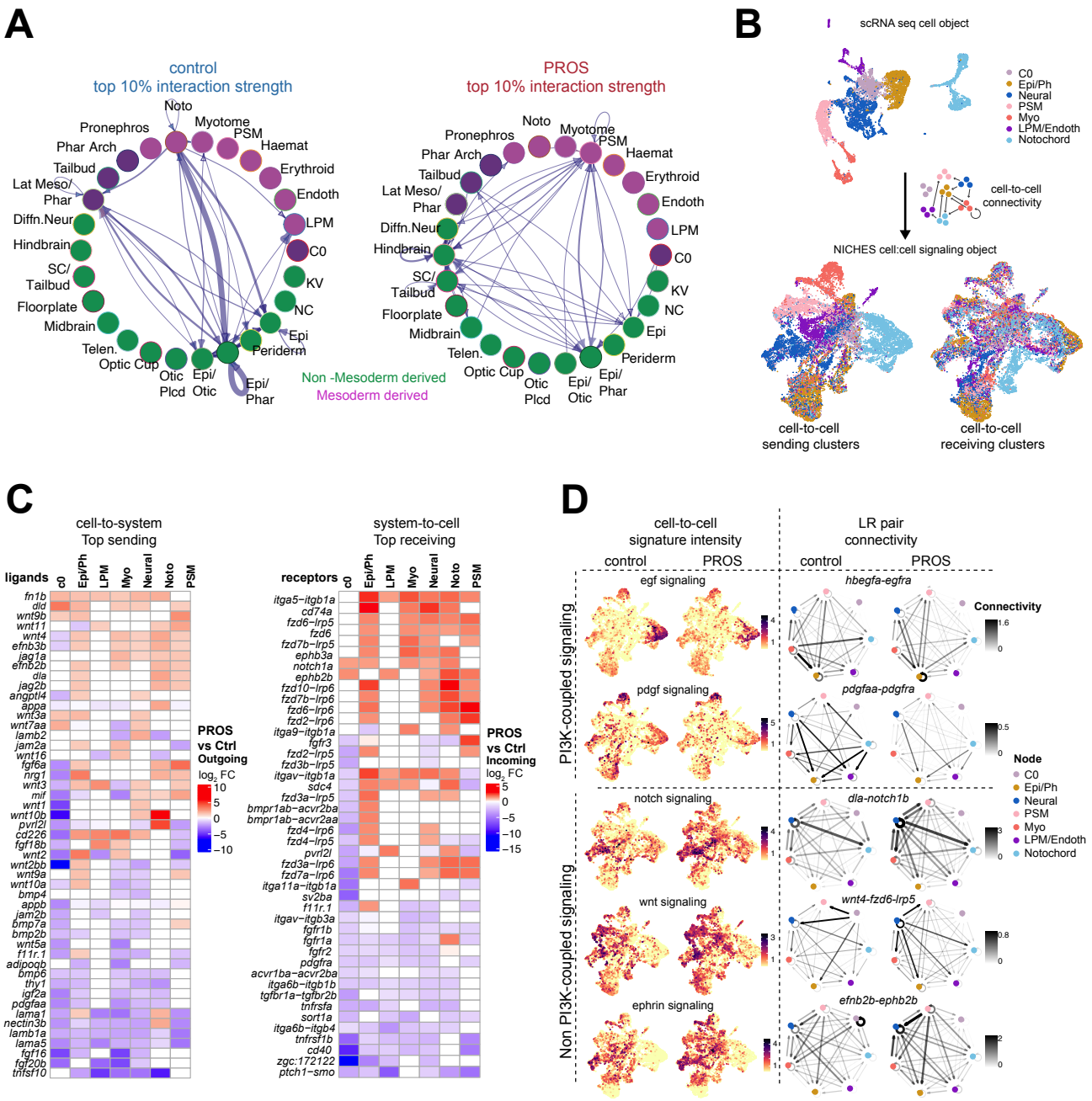

A
control
top 10% interaction strength
PROS
top 10% interaction strength
Noto
Myotome
PSM
Haemat
Erythroid
Endoth
LPM
C0
KV
NC
Epi
Periderm
Epi/ Phar
Epi/ Otic
Otic Plcd
Optic Cup
Telen.
Midbrain
Floorplate
SC/ Tailbud
Hindbrain
Diffn.Neur
Lat Meso/ Phar
Tailbud
Phar Arch
Pronephros
Non -Mesoderm derived
Mesoderm derived
B
scRNA seq cell object
C0
Epi/Ph
Neural
PSM
Myo
LPM/Endoth
Notochord
cell-to-cell connectivity
NICHES cell:cell signaling object
cell-to-cell sending clusters
cell-to-cell receiving clusters
C
cell-to-system
Top sending
system-to-cell
Top receiving
ligands
receptors
c0
Epi/Ph
LPM
Myo
Neural
Noto
PSM
PROS vs Ctrl Outgoing
PROS vs Ctrl Incoming
log2 FC
D
cell-to-cell signature intensity
LR pair connectivity
control
PROS
PI3K-coupled signaling
Non PI3K-coupled signaling
egf signaling
pdgf signaling
notch signaling
wnt signaling
ephrin signaling
hbegfa-egfra
pdgfaa-pdgfra
dla-notch1b
wnt4-fzd6-lrp5
efnb2b-ephb2b
Connectivity
Node
C0
Epi/Ph
Neural
PSM
Myo
LPM/Endoth
Notochord

Figure 5

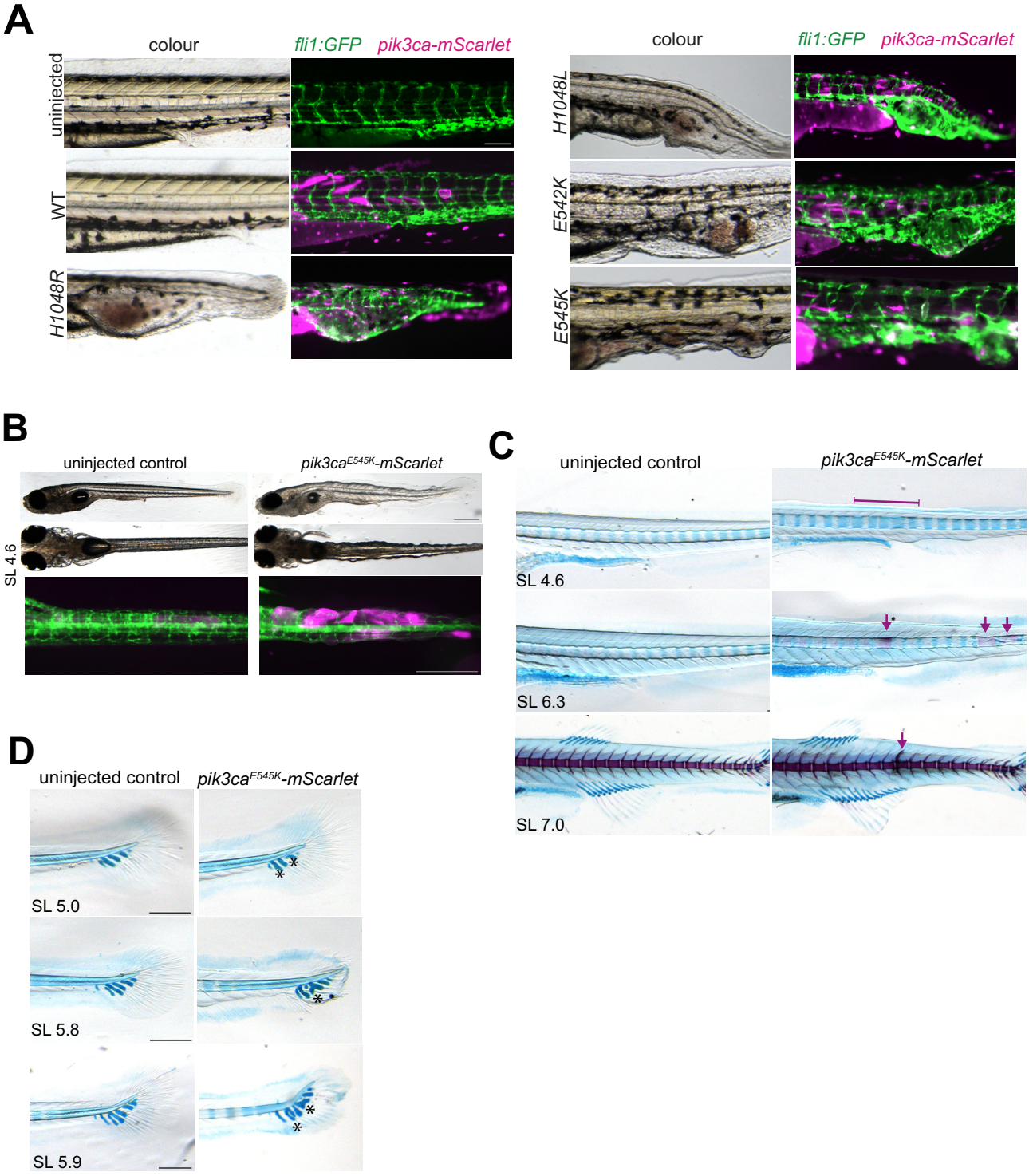
Figure 5. *pik3ca*^{PROS} promotes widespread transcriptional changes in ligand-receptor expression patterns

A) CellChat was used to identify potential interactions between ligand and receptor pairs in clusters. The top 10% strongest pathway interactions of 'control' cells relative to 'PROS' and vice versa shown, normalised to cell number. Blue arrows indicating whether a cluster is a sender or a receiver of the signal, with arrow thickness proportional to connection strength. Abbreviations: LPM=lateral plate mesoderm, PSM, presomitic mesoderm, Noto = notochord, Endoth=endothelial, KV = kuppfer's vesicle, NC=neural crest, Epi=epiderm, Epi/Phar epidermal/pharyngeal, Otic Plcd=otic placode, Telen=telencephalon, SC=spinal cord, Diffn. Neuron = differentiating neurons,

B) UMAP showing merged data to illustrate the methodology of NICHES, whereby a subset of clusters from Figure 4 is reclustered (Top UMAP). Ligand-receptor interactions between each group were quantified using NICHES, converting it into a signaling object of cell:cell interactions (Bottom UMAPs, grouped by either sending cluster type (left) or receiving cluster type (right). Colours in interaction maps were retained from the original clusters in the top UMAP. Epi/Ph = epiderm/pharyngeal mix, PSM = presomitic mesoderm, Myo=myotome, LPM/Endothelial = grouped lateral plate mesoderm and endothelial clusters.

C) Heatmap depicting $\log_2$ fold change in expression of the top differentially expressed "sending" ligand (left) and "receiving" receptor (right) genes in PROS relative to control embryos, clustered by row. Ligand-receptor mechanisms were aggregated by common ligand or common receptor (unaggregated data are shown in **Supplemental Figure 4C**). Only differences for which p.adj<0.05 by Wilcoxon test between PROS and WT are shown.

D) Examples of pathways showing dysregulated expression of ligand-receptor (LR) pairs. The expression values of LR pairs for each pathway were averaged as a module score. FeaturePlots (left) show intensity of this module for each cell:cell interaction, split by experimental condition. Examples of circus plots for each pathway are shown on the right. Signal direction between clusters (nodes) is shown by arrows, with darker and thicker arrows corresponding to greater connectivity strength. Connectivity scale intensity is matched between control and PROS plots for each LR pair.



Supplementary Figure 1

Supplementary Figure Legends

Supplementary Figure 1: Hotspot *pik3ca*^{PROS} transgenes cause vascular malformations and defects in muscle, cartilage and skeletal patterning in larval fish

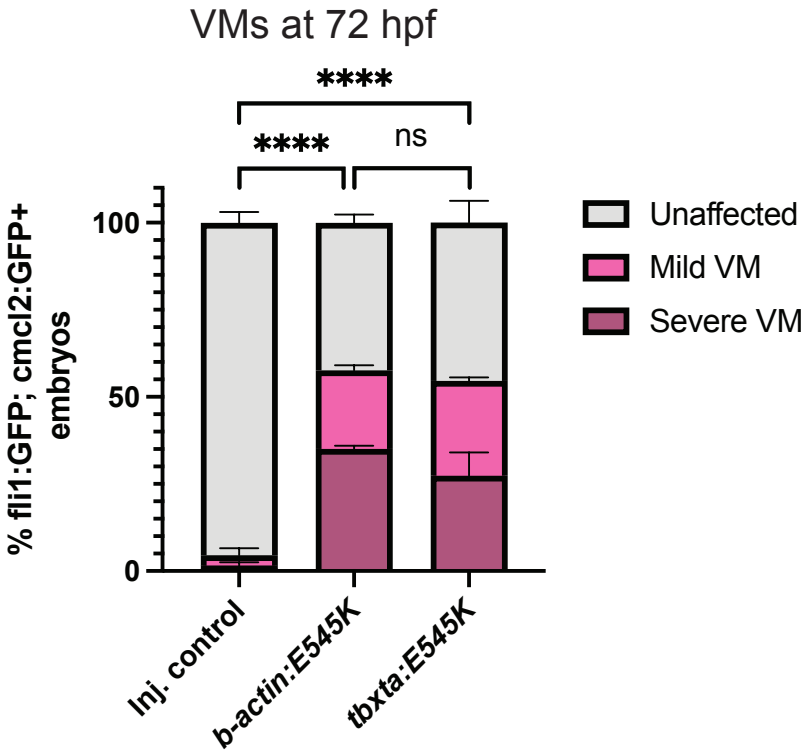
A) Representative images of severe vascular malformations found in 72 hpf *Tg(fli1:eGFP)* embryos previously injected with either *β-actin:pik3caH1048R*, *H1048L, E542K* or *E545K.* Uninjected embryos, and those injected with WT *pik3ca* showed no phenotype at this timepoint. In *pik3ca*^{PROS} embryos, colour panels on the left show blood pooling in malformed vessels, with matched merged fluorescence images of the same area on the right showing enlarged *fli1:eGFP+* vessels (green), and variable numbers of mosaic *pik3ca*^{PROS}*-mScarlet* cells (magenta). Scale bar = 100 µm.

B) *Tg(fli1:eGFP);casper* fish injected with *β-actin:pik3ca*^{E545K}*-mScarlet* but negative for vascular malformations at 72 hpf developed a 'rippled' appearance by two weeks of age (SL4.6) caused by protrusions of *pik3ca*^{PROS}*-mScarlet* cells in the embryo flank (10/25). Scale bar = 100 µm.

C) Patterning defects in the developing spine of staged matched *Tg(β-actin:pik3ca*^{E545K}*-mScarlet; fli1:eGFP); casper* injected embryos, showing irregular spacing of cartilage centra during early spine development (3/9 fish in which pattern had developed, prior to bone deposition), premature deposition of bone in patches posterior to stage-matched controls (1/3 fish at a stage where bone deposition is just starting in anterior and so therefore premature deposition is noticeable), and thickened vertebra structure (1/6 fish in which vertebrae were formed)

D) Comparison of stage-matched uninjected and *Tg(β-actin:pik3ca*^{E545K}*-mScarlet; fli1:eGFP); casper* injected fish during tail fin development with patterning defects, revealed by alcian blue staining for cartilage and alizarin red co-staining for bone. 3/9 cartilage hypural patterning defects, 5/7 tail shape defects.

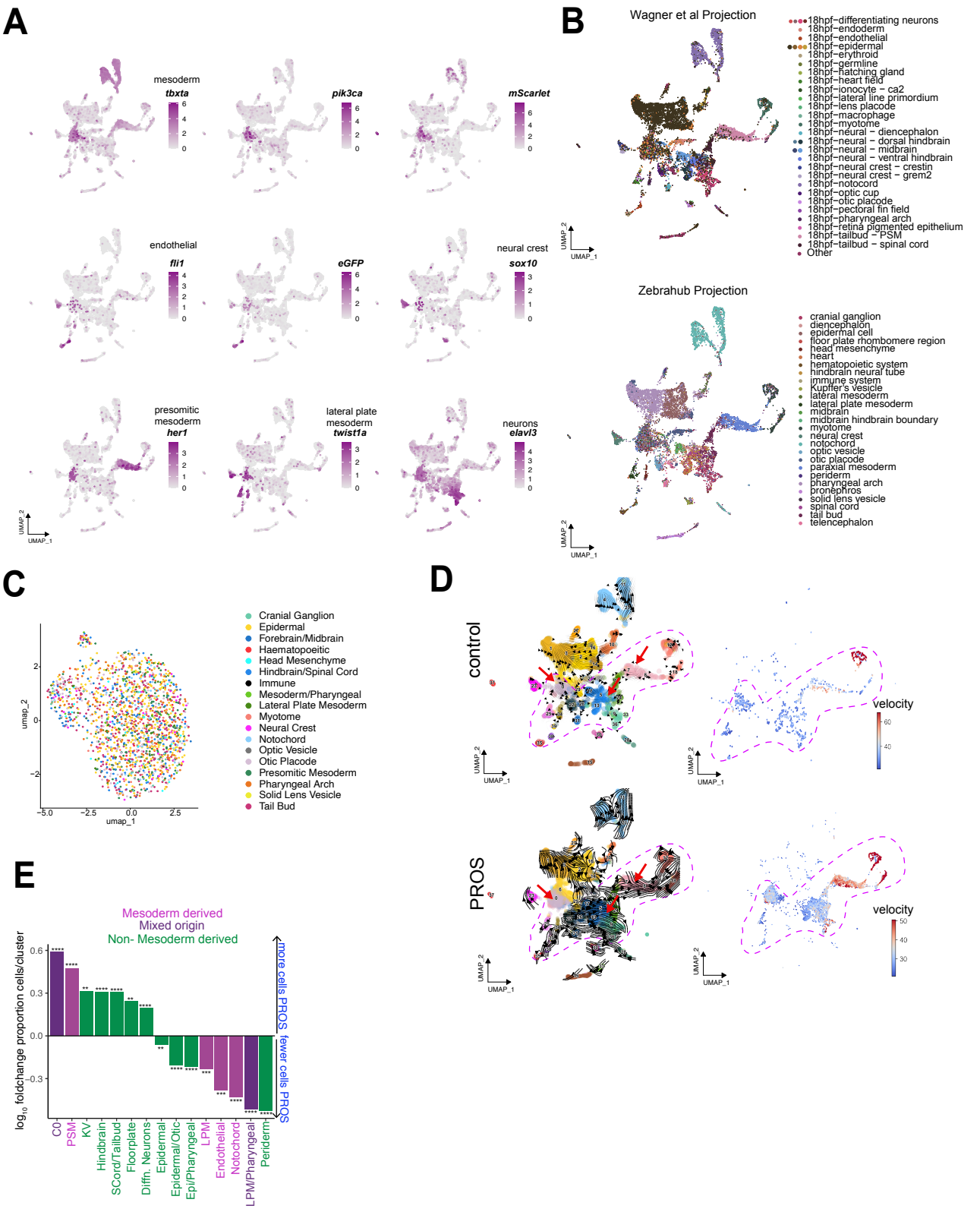
A



Supplementary Figure 2

Supplementary Figure 2: Ubiquitous and mesodermal expression of *pik3ca*^{PROS} causes vascular malformations

A) Quantification of vascular phenotypes in 72 hpf Tg(*fli1:eGFP*) embryos injected with *pik3ca*^{E545K}*-mScarlet* constructs driven by *β-actin* or *tbxta*, or an injected no-*pik3ca* control (*tbxta:mScarlet*). Represents 3 biological replicates. Error bars are S.E.M. p-values are from chi:squared tests on raw counts, * $p<0.05$, ***$p<0.001$, **** $p<0.0001$.



Supplementary Figure 3

Supplementary Figure 3: scRNA seq analysis of *pik3ca*^{PROS} mosaic embryos

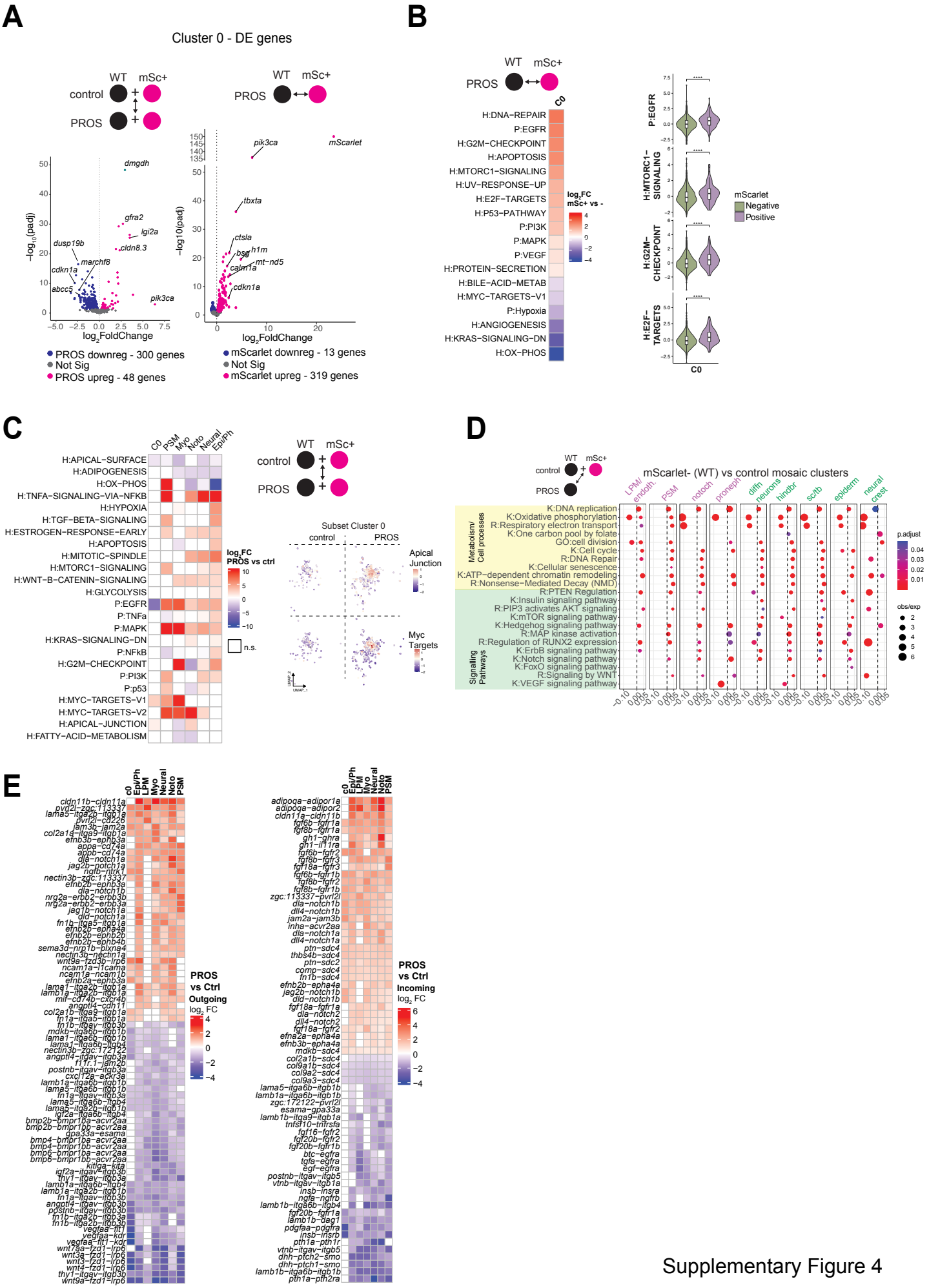
A) UMAP FeaturePlots showing expression intensity (purple) of transgenesis or cell lineage markers.

B) Mapping of 18hpf (top) and 19 hpf (bottom) published dataset identities onto these data to call clusters.

C) UMAP of re-clustered cluster 0, with identities from the zebrahub 19 hpf dataset mapped as in B.

D) Single cell velocity analysis was performed on PROS and control data, and the vector field overlaid on a UMAP projection, with arrow indicating velocity dynamics and strength across clusters. The greatest variance in arrow length and density between PROS and control is found in the cluster groups outlined (pink dashed). The velocity length of this subset is displayed on a heatmap, with cells showing the highest abundances of unspliced mRNA in red.

E) As Figure 4E, but to show the extent of the statistically significant changes in the proportion of cells within cluster groups. Two-sided Pearson's chi-squared proportion test. ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$



Supplementary Figure 4

Supplementary Figure 4: Mosaic *pik3ca*^{PROS} causes pan-lineage transcriptional changes, including to ligand-receptor signalling genes.

A) Volcano plots showing significantly differentially expressed genes after DE analysis of cells within cluster 0. Left – comparison of all cluster 0 PROS mosaics vs control, Right – mScarlet+ vs mScarlet- cells in PROS mosaics only.

B) Heatmap showing fold change enrichment (Seurat wilcox test $p<0.05$, $p.adj<0.1$) of Hallmark (H:) and Progeny (P:) pathways in PROS mosaic cluster 0 mSc+ vs mSc- cells. Violin plots confirm significant upregulation of selected Hallmark signatures in mScarlet+ cells (wilcox test, **** = $p<0.001$)

C) Heatmap showing fold change enrichment (Seurat wilcox test $p<0.05$, $p.adj<0.1$) of Hallmark (H:) and Progeny (P:) pathways in PROS mosaics relative to control N.S = non-significant, and Cluster 0 UMAP subset showing expression intensity of Hallmark terms, split by experimental condition.

D) As in Figure 4F), but comparing only *mScarlet-* (ie, WT) cells between experimental conditions.

E) Heatmaps depicting $\log_2$ fold change in expression of the top differentially expressed "sending" (left) and "receiving" (right) ligand-receptor gene pairs in PROS relative to control embryos, clustered by row. Only differences for which $p.adj<0.05$ by Wilcoxon test between PROS and WT are shown.

Movie Legends

Movie 1-3:

Movie 1 – Maximum projection movie of Figure 4A of uninjected *Tg(fli1:eGFP)* embryo showing the arrangement of vessels around the central notochord.

Movie 2 - Maximum projection movie of a Mild vascular lesion in a *Tg(fli1:eGFP)* embryo injected with *pik3ca*^{E545K}*-mScarlet* (see Figure 4B) showing first separated *pik3ca*^{PROS} *mScarlet* expression (magenta) and then merged with *fli1:eGFP*

Movie 3 – Maximum projection movie of a Moderate vascular lesion in a *Tg(fli1:eGFP)* embryo injected with *pik3ca*^{E545K}*-mScarlet* (see Figure 4B) showing first separated *pik3ca*^{PROS} *mScarlet* expression (magenta) and then merged with *fli1:eGFP.* Cells co-expressing *GFP* and *mScarlet* are white

Movie 4 – Timelapse of injected mosaic mosaic *tbxta:mScarlet Tg(fli1:eGFP)* control embryo between 16-28.5 hpf, showing normal development of blood vessels, with ISV sprouting and DLAV formation from anterior (left) to posterior (right).

Movie 5 – Timelapse of injected mosaic *pik3ca*^{PROS} *Tg(fli1:eGFP)* embryo between 16-28.5 hpf showing ectopic *fli1:eGFP*+ endothelial cells contributing to premature formation of the DLAV, and defects in ISV sprouting.

Supplementary Tables

Supplementary Table 1 – Primers used for plasmid construction

Supplementary Table 2 – Raw results from Deseq2 DE analyses used to generate Figure 4F

Supplementary Table 3 – Raw values used to generate proportion plots in Figures 4E and Supplementary Figure 3E.

Supplementary Table 4 – Raw results from DE analyses on NICHES signalling objects used to generate Figure 5C