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# Multiple congenital malformations arise from somatic mosaicism for constitutively active *Pik3ca* signalling

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13 **Keywords: neural crest, embryo, PI3K, cancer, birth defect, cleft palate, vascular anomaly**

## 14 1 Abstract

15 Recurrent missense mutations of the *PIK3CA* oncogene are among the most frequent drivers of  
16 human cancers. These often lead to constitutive activation of its product p110 $\alpha$ , a  
17 phosphatidylinositol 3-kinase (PI3K) catalytic subunit. In addition to causing a range of rare and  
18 common cancers, the H1047R mutation is also found in affected tissues of a distinct set of congenital  
19 tumours and malformations. Collectively termed *PIK3CA*-related disorders (PRDs), these lead to  
20 overgrowth of skin, brain, adipose, connective, musculoskeletal tissues and/or blood and lymphatic  
21 vessel components. Vascular malformations are frequently observed in PRD due to cell-autonomous  
22 activation of the PI3K signaling pathway within endothelial cells. These, like most muscle,  
23 connective tissue and bone, are derived from the embryonic mesoderm. However, important organ  
24 systems affected in PRDs are neuroectodermal derivatives. To further examine their development, we  
25 drove the most common post-zygotic activating mutation of *Pik3ca* in neural crest and related  
26 embryonic lineages. Effects in cells having once expressed Wnt1, including the brain roofplate and  
27 most neural crest, were most dramatic in the head. Outcomes included megalencephaly, cleft  
28 secondary palate and more subtle skull anomalies. Surprisingly, *Pik3ca*-mutant subpopulations of  
29 either mesodermal or neural crest origin was associated with widespread vascular anomalies, leading  
30 us to incidentally discover previously undescribed lineages that had expressed the transcription factor  
31 *Egr2* (*Krox20*) and that may be co-opted in pathogenesis. Schwann cell precursors having transcribed  
32 either *Krox20* or *Sox10* also gave rise to adult-onset vascular tumors and cancers, including  
33 melanoma, after *Pik3ca* activation. These murine phenotypes may aid discovery of new candidate  
34 human PRDs affecting craniofacial and vascular smooth muscle development as well as the  
35 reciprocal paracrine signaling mechanisms leading to tissue overgrowth.

## 36 2 Introduction

37 Inappropriate activation of signaling components between the cell membrane and its nucleus leads to  
38 pathologies with onset at any stage of life, ranging from before birth into old age. These include most  
39 cancers, but also numerous, individually rare diseases with a congenital basis.

40 Nearly two dozen distinct overgrowth disorders, characterized by somatic mosaicism for gene  
41 mutations that constitutively activate the phosphatidylinositol 3-kinase (PI3K) pathway, have been  
42 discovered over the last decade (Canaud et al., 2021). A striking majority of these diseases show the  
43 same hotspot, activating mutations in the *PIK3CA* gene as found in cancer, which has led to their  
44 qualification as PIK3CA-related disorders (PRDs). A large subset is known as “PIK3CA-related  
45 overgrowth syndromes” or PROS, where susceptible tissues such as the cortex (Alcantara et al.,  
46 2017), skeletal muscles (Frisk et al., 2019) or the face (Couto et al., 2017) develop segmental  
47 overgrowth. How imbalanced PI3K signaling affects normal homeostasis to cause these very  
48 different types of diseases is not understood because of a lack of information about its developmental  
49 effects on interdependent organ systems in the context of mosaicism.

50 Tumor progression, increased microvascular density and cancer invasiveness are widely associated  
51 with hyperactivity of PI3Ks and their downstream effectors. Normally, receptor tyrosine kinase-  
52 mediated recruitment and activation of PI3Ks lead to appropriate production of second messengers  
53 from lipid substrates. PI3Ks consist of a regulatory p85 subunit and one of three possible 110-kDa  
54 catalytic subunits (p110 $\alpha$ , p110 $\beta$ , p110 $\delta$ ). Mutations in *PIK3CA*, the gene encoding p110 $\alpha$ , are alone  
55 observed in 13% of all U.S. cancers (Mendiratta et al., 2021).

56 Once produced by an active PI3K, 3-phosphoinositide substrates can dock multiple possible  
57 intracellular signal transducers, including the three AKT protein isoforms. Distinct combinations of  
58 PI3Ks and AKTs seem to mediate a wide range of metabolic, trafficking, growth, survival,  
59 proliferation and motility processes to coordinate cellular responses with other signaling pathways.  
60 However, their specific effects have not been sufficiently characterized *in vivo* to understand how  
61 their mutations lead to congenital disease.

62 The normal potential of neural crest (NC) stem cells to both influence and differentiate as a function  
63 of surrounding tissues renders this population a prime target for growth factor receptor signaling  
64 anomalies (Le Lievre and Le Douarin, 1975; Bergwerff et al., 1998; Etchevers et al., 1999, 2001;  
65 Müller et al., 2008; Zachariah and Cyster, 2010). NC cells migrate away from the dorsal aspect of the  
66 future brain and spinal cord towards the end of the first month of human gestation and engender a  
67 wide variety of differentiated cell types (Le Douarin and Kalcheim, 1999). During their migration  
68 throughout the embryonic head and body, they encounter many distinct and changing  
69 microenvironments. Errors in the cross-talk between NC cells and their environment lead to a large  
70 class of diseases collectively known as neurocristopathies (Bolande, 1974; Etchevers et al., 2019).

71 Craniofacial neurocristopathies are collectively frequent birth defects, including isolated or  
72 syndromic cleft lip and/or palate (Juriloff and Harris, 2008) or the rarer craniosynostosis (Watt and  
73 Trainor, 2014). After birth, NC-derived Schwann cell precursors in peripheral nerves remain a latent,  
74 self-renewing multipotent stem cell pool that can be activated in response to multiple pathological  
75 stimuli (Petersen and Adameyko, 2017; Xie et al., 2019). Given the multiple steps in NC  
76 specification, migration and differentiation that are dependent on PI3K signaling (Ciarlo et al., 2017;  
77 Sittewelle and Monsoro-Burq, 2018), we sought to discover the *in vivo* consequences of  
78 constitutively active *Pik3ca* on the physiology of different populations of NC cells.

## 79 3 Materials and Methods

### 80 3.1 Mouse lines

81 All mice were obtained directly or through Charles River France from the Jackson Laboratories (Bar  
82 Harbor, ME, USA) and intentionally outbred over multiple generations to CD-1/Swiss mice  
83 purchased from Janvier Laboratories in order to phenocopy human genetic heterogeneity. Knock-in  
84 lines included conditional, floxed *Pik3ca*<sup>H1047R</sup> (RRID:IMSR\_JAX:016977) (Adams et al., 2011) or  
85 *RdTomato* reporter mice (RRID:IMSR\_JAX:007909); transgenics included the *Wnt1-Cre*  
86 (RRID:IMSR\_JAX:003829), *Krox20-Cre* (RRID:IMSR\_JAX:025744) and tamoxifen-inducible  
87 *Sox10-CreER<sup>T2</sup>* (RRID:IMSR\_JAX:027651) lines. 4-hydroxy-tamoxifen was solubilized in 10%  
88 ethanol, 90% corn oil and administered in a single intraperitoneal injection of 0.1 mL at 10 mg/mL.  
89 All mice were housed in individual, ventilated cages with 12-hr light/dark cycles with food and water  
90 *ad libitum*.

91 Mice were genotyped with 50 ng DNA purified from ear punch or tail clips using the primers  
92 described in the original reports and Phire Tissue Direct PCR Master Mix (Thermo Scientific).

### 93 3.2 Ethics approval

94 The animal study was reviewed and approved by the French national animal care and use committee  
95 (ACUC) C2EA-14 under the reference 9522-2017040517496865v5.

### 96 3.3 Histology, immunofluorescence and *in situ* hybridization

97 Embryos were staged taking embryonic day (E) 0.5 as the morning of the vaginal plug. Tissue  
98 biopsies were kept in ice-cold phosphate-buffered saline (PBS) until dissection, fixed in freshly  
99 thawed, neutral pH-buffered 4% paraformaldehyde for 20 minutes to overnight depending on tissue  
100 size, and rinsed again in PBS. Paraffin blocks were prepared according to standard embedding  
101 protocols, sections cut on a Leica microtome at 7  $\mu$ m, and deparaffinated and rehydrated to PBS  
102 through xylene and decreasing ethanol solutions. Alternatively, fluorescent tissues were equilibrated  
103 in 15% then 30% sucrose in PBS and positioned in liquid embedding compound (Leica) before snap-  
104 freezing in plastic molds over liquid nitrogen. Cryosections were cut at 12  $\mu$ m onto Superfrost Plus  
105 slides, dried, washed in PBS. All immunofluorescent sections were immersed for 20 minutes in 50  
106 mM glycine, 0.1 M ammonium chloride before pre-incubating in a blocking solution of 0.1% Tween-  
107 20, 2% fetal calf serum in PBS and diluting the primary antibodies at the indicated concentrations for  
108 overnight treatment under Parafilm coverslips at 4°C. Standard procedures were followed for DAPI  
109 counterstain, subsequent Alexa Fluor-coupled secondary antibody (ThermoFisher) incubation and  
110 mounting with Fluoromount G (SouthernBiotech) under coverslips.

111 The following primary antibodies were used in this study: rat anti-Pecam1/CD31 (ThermoFisher,  
112 RRID:AB\_467201), mouse anti-alpha-smooth muscle actin (Sigma-Aldrich, RRID:AB\_10979529),  
113 rabbit anti-phosphorylated-S6 ribosomal protein (Ser235/236) (Cell Signaling Technologies,  
114 RRID:AB\_2181035).

115 For the detection of *Pdgfra* transcripts on paraffin sections, we amplified a fragment as described by  
116 PCR (Orr-Urtreger et al., 1992) from cDNA prepared from a whole mouse embryo at E12.5. The  
117 reverse primer was prolonged by an additional T7 RNA polymerase recognition sequence  
118 (taatacgaactacataggaga) added at the 5' end. *In vitro* probe synthesis, purification and a standard  
119 chromogenic *in situ* hybridization protocol were carried out as described (Thomas et al., 2018).

120 Standard hematoxylin-eosin (HE; with or without Alcian blue to detect sulfated glycosaminoglycans)  
121 staining protocols were followed for designated sections.

### 122 **3.4 Microscopy**

123 Gross anatomy was photographed with a Leica MZ6 dissecting microscope and images captured with  
124 a DFC450 camera before analysis using the open-source ImageJ software (v1.53). Histology and *in*  
125 *situ* hybridization slides were photographed on a Zeiss AxioScan 7 and immunofluorescent sections  
126 on a Zeiss AxioZoom, Apotome or LSM800-Airyscan microscope equipped with Zen software (v2.3  
127 or 3.0). Centroid size of crania was quantified in ImageJ by delimiting the shape above a virtual line  
128 from the upper jaw to earlobe to occiput in the sagittal plane (Pilatti and Astúa, 2017).

### 129 **3.5 Micro-X-ray computed tomography ( $\mu$ CT) examination**

130 Late fetal (embryonic day [E]15.5-E20) specimens were genotyped and heads fixed overnight in 4%  
131 buffered paraformaldehyde at 4°C. They were stored in PBS + 0.1% w/v sodium azide before  
132 imaging on a X-ray micro-CT device (Quantum FX Caliper, Life Sciences, Perkin Elmer, Waltham,  
133 MA) hosted by the PIV Platform, EA2496, Montrouge, France. The X-ray source was set at 90 kV  
134 and 160  $\mu$ A. Tridimensional images were acquired with an isotropic voxel size of 20  $\mu$ m. Non-  
135 mineralized tissues were visualized after impregnating with Lugol's solution.

136 Tiff image stills were extracted from Dicom data frames using licensed 64-bit Irfanview imaging  
137 freeware (v4.59, <http://www.irfanview.com/>). Measurements were made in ImageJ after manual  
138 segmentation using contrast thresholding.

## 139 **4 Results**

### 140 **4.1 Constitutively active *Pik3ca* in most neural crest leads to perinatal death and craniofacial** 141 **malformations**

142 In order to understand the effects of PI3K signaling in neural crest (NC) derivatives, we mated  
143 conditional, floxed *Pik3ca*<sup>H1047R</sup> and/or Tomato (*RdT*) reporter (Madisen et al., 2010) knock-in lines  
144 with *Wnt1-Cre* transgenic mice, which express Cre recombinase in nearly all NC-derived cells from  
145 pre-migratory stages onwards (Danielian et al., 1998). This engendered somatic mosaicism for  
146 constitutive PI3K activation throughout the head and body of only those animals carrying both a  
147 floxed *Pik3ca* and a *Cre* allele in those tissues expressing the recombinase.

148 No *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup> mice were recovered at weaning; in fact, all *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup>  
149 mice died within the first day after birth. The dead neonates did not present a belly milk spot and had  
150 noticeably large heads and cleft palates (**Figure 1**). In order to better understand the causes and onset  
151 of death, we examined embryos at different stages of embryonic and fetal development up until birth.  
152 Mutants were compared to their unaffected control littermates expressing only one or neither of the  
153 *Wnt1-Cre* or *Pik3ca*<sup>H1047R/+</sup> alleles.

154 From E15.5 onwards, mutant crania were all visibly and significantly larger than controls (**Figure**  
155 **1G, 2(A, E)**). Body sizes were unaltered. Macrocephaly was present both at the level of the cranial  
156 vault and in the ossification of the mandible, which was significantly thicker in mutants (**Figure 2(B-**  
157 **D vs. F-H)**). The phenotypic variability present within litters at birth was exacerbated in mutant  
158 embryos, which all showed facial soft tissue asymmetry to degrees, and occasional premature  
159 ossification along with overgrowth (**Figure (2H)** is a littermate of **(B-D)** and **(F-G)**).

160 We sought additional causes of perinatal death because only approximately 50% of heads had  
161 developed cleft secondary palates (**Figure 2(A)** vs. **2(E)**). Two possibilities were adrenal dysfunction  
162 or cardiac malformation, both organs with vital neural crest-derived components for their  
163 morphogenesis. We compared their sizes on soft tissue contrast-enhanced micro-CT sections of  
164 E15.5-E16 embryos, as well as those of the pituitary, which controls adrenal function through the  
165 function of its corticotroph cells, and the thymus, another neural crest-dependent gland (Müller et al.,  
166 2008).

#### 167 **4.2 *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup> mice develop variable ocular and pituitary malformations**

168 In contrast to the megalencephaly observed in mutants by E16, the anterior pituitary gland was  
169 significantly smaller in micro-CT frontal sections of *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup> embryos than their  
170 control littermates (**Figure 2(I-O)**). No significant differences were measured in maximal cross-  
171 sectional areas of the adrenal glands or thymus (not shown). Variability in cardiac ventricular muscle  
172 wall thickness was observed but on average, was not significantly different between mutant and  
173 control embryos.

174 Initial cranial and trunk-level NC migration appeared to be unimpeded in *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup>;  
175 *RdT* mice versus their *Wnt1-Cre; RdT* littermates at E9.5 (**Figure 3(A-B)**). Likewise, at E13.5  
176 (**Figure 3(C-H)**), palatal shelves, head size and the position of the tongue had not yet developed  
177 obvious morphological differences. However, the eyes already appeared slightly malpositioned.  
178 Since signaling through the *Pdgfra* receptor for platelet-derived growth factor is known to be crucial  
179 for palatal development in both mice and humans (Tallquist and Soriano, 2003; Ding et al., 2004), we  
180 undertook *in situ* hybridization to its transcript. The expression pattern of *Pdgfra* was unaltered in the  
181 palatal NC-derived mesenchyme at E13.5, consistent with PI3K-mediated transduction being  
182 downstream of *Pdgfra* in these cells (He and Soriano, 2013). Lens coloboma and microspherophakia,  
183 an enlarged epithelial *Pdgfra* domain expression overlying a lack of primary fibers, and a thickened  
184 cornea were already evident in mutants (**Figure 3(I-M)**).

#### 185 **4.3 PI3K signaling in NC-derived vascular smooth muscle induces venous malformations**

186 One striking feature of *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup> mutants was the systematic presence of variable  
187 degrees of craniofacial vascular malformations from E13.5 onwards (**Figure 4**). These most  
188 resembled venous malformations in that they were low-flow, circumscribed congenital lesions within  
189 the NC-derived dermis over the frontal bones and, frequently, in the maxillary and retroorbital  
190 regions (**Figure 4 (A-D)**). The malformations often contained thromboses and were also observed in  
191 the heart, in both ventricles and atria.

192 In order to examine the composition of these abnormal vascular structures, we examined the  
193 distribution of the mural pericyte and smooth muscle marker,  $\alpha$ -smooth muscle actin (aSMA) and the  
194 endothelial marker Pecam-1 (CD31) in *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup> mutants using immunofluorescence  
195 (**Figure 4 (E-F)**). Vascular smooth muscle of the cardiac great arteries, derived from posterior  
196 rhombencephalic NC cells, was present but disorganized; the nuclei were not organized in their usual  
197 concentric layers.

198 Within the intracardiac lesions, *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup>; *RdT* mutants showed co-expression of the  
199 Tomato NC lineage marker with aSMA in a discontinuous manner around the vascular lacunae  
200 (**Figure 4 (G), arrow**). Where aSMA was present, the cells were somewhat but not entirely  
201 disorganized. They were somewhat cuboid rather than lamellar within the vascular media. A  
202 cutaneous vascular malformation in the skin over the parietal bone in a late fetus at E20.5 (**Figure 4**

203 (H) showed mutant aSMA-expressing, lineage-traced NC cells in dermal blood vessels surrounding  
204 the hair follicles. This observation was remarkable since the NC-derived dermis, vascular pericytes,  
205 meninges and craniofacial bones usually occupy the same territories of the ventral head and neck  
206 (Etchevers et al., 1999). Given that the mouse parietal bone is of mesodermal rather than NC origin,  
207 as shown by lineage tracing in a cross of the same *Wnt1-Cre* line with a conditional reporter allele  
208 (Jiang et al., 2002), this implies that PI3K signaling may enable some NC derivatives to spread to  
209 ectopic cranial regions before birth.

#### 210 4.4 PI3K signaling in muscle leads to widespread, progressive vascular anomalies

211 Despite the striking and lethal phenotype of *Pik3ca* constitutive activity in mesectodermal cephalic  
212 NC, we did not observe any changes in NC derivatives in the trunk in *Wnt1-Cre; Pik3ca<sup>H1047R</sup>*  
213 mutants at birth. This included the adrenal medulla and the enteric, autonomic or sensory nervous  
214 systems. In order to examine a subset of cardiac NC in the developing tricuspid valves (Odelin et al.,  
215 2018), we crossed the conditional, floxed *Pik3ca<sup>H1047R</sup>* and/or Tomato (*RdT*) reporter knock-in lines  
216 to the *Krox20-Cre* transgenic line, where Cre recombinase is expressed in the place of one allele of  
217 the conserved zinc finger transcription factor *Egr2* (Voiculescu et al., 2000).

218 Given the previously described expression of *Krox20* in tendons, chondrocytes and osteocytes in  
219 addition to a subpopulation of hindbrain NC cells and myelinating Schwann cells (Voiculescu et al.,  
220 2000; Maro et al., 2004), we examined *Krox20-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>* mice for signs of cardiac  
221 insufficiency from myxomatous valves, skeletal defects or peripheral neuropathy. *Krox20-Cre;*  
222 *Pik3ca<sup>H1047R/+</sup>* were initially healthy and viable, but over time post-weaning developed palpable  
223 lumps under the skin on the back, leg or tail and had soon reached a humane endpoint. Upon autopsy,  
224 we discovered widespread, lobular vascular structures filled with coagulated blood in the  
225 subcutaneous *panniculus carnosus* muscle, but also in and around skeletal, cardiac and smooth  
226 muscles, the lungs, the reproductive organs and many other densely vascularized tissues (**Figure 5**  
227 **(A-D, G-T)**). These vascular lesions had cavernoma-like fibrous septa, and the adjacent nerves were  
228 surrounded by loose fat. No phleboliths were observed and hearts appeared normal. Myelinated  
229 sciatic nerves showed no macroscopic or functional differences between mutant and control mice.  
230 However, mutant mice systematically developed splenomegaly (**Figure 5(E, F)**).

231 *Krox20-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>; RdT<sup>+/-</sup>* mice, like their *Krox20-Cre<sup>+/-</sup>; RdT<sup>+/-</sup>* counterparts, expressed  
232 the fluorescent Tomato marker in the smooth muscle of the gonad (**Figure 5(H)**), in finely ramified  
233 cells, probably reticular fibroblasts, in the spleen (**Figure 5(J)**), and in skeletal muscles throughout  
234 the body. As expected, cells in the aorta and pulmonary trunk were derived from *Krox20*-expressing  
235 progenitors (**Figure 5(L)**), but there were also scattered, filamentous cells throughout the walls of the  
236 ventricles and to a lesser extent, the atria, corresponding to the sites of developing cardiac vascular  
237 lesions in mutant animals (**Figure 5(D, I, M)**). A search for *Egr2* expression in a recent multi-organ  
238 database of single adult mouse fibroblasts and vascular mural cells  
239 (<https://betsholtzlab.org/Publications/FibroblastMural/database.html>) (Muhl et al., 2020)  
240 demonstrated that the cells that had expressed *Krox20-Cre* were likely to be endomysial and  
241 perimysial fibroblasts in cardiac and skeletal muscle, myelinating Schwann cells and endoneural  
242 fibroblasts in the peripheral nerves, and a subtype of vascular pericytes, fibroblasts and smooth  
243 muscle. Indeed, fascicles of the peripheral nerves, including autonomic, and a ring of probable  
244 pericytes (Topilko, 2019) at the base of hair follicles in the non-glabrous skin strongly expressed RdT  
245 in adulthood (**Figure 5(L, N, O)**), supporting the independent single-cell data and explaining the  
246 widespread and unexpected vascular phenotypes in *Krox20-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>* mutant mice.

247 **4.5 Egr2-driven *Pik3ca*<sup>H1047R/+</sup> expression induces postnatal pituitary and intramuscular**  
248 **artery remodeling**

249 Since *Krox20-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> mice survived to adulthood, we made a cursory examination of  
250 the pituitary gland for morphological or functional anomalies to compare to the *Wnt1-Cre*<sup>+/-</sup>;  
251 *Pik3ca*<sup>H1047R/+</sup> phenotype. Lineage tracing demonstrated that most if not all cells of the  
252 adenohypophysis, unlike the neurohypophysis, had once expressed the Krox20 transcription factor  
253 (**Figure 6(A, B)**). Examination of the capillary network in control mice with Pecam1 (CD31)  
254 immunofluorescence in confocal microscopy showed that even endothelial cell nuclei showed  
255 Tomato fluorescence (**Figure 6(C-F)**). Intriguingly, mutant pituitaries had many nuclei of cells that  
256 had replaced those descended from progenitors that had once expressed Krox20 but were not RdT+.  
257 There were fewer RdT+ cells overall and this was accompanied by a concomitant decrease in  
258 Pecam1+ / RdT+ cell density (**Figure 6(G-J)**) but not in organ size or, apparently, function.

259 A recent report has implicated increased PI3K signaling in the formation of cerebral cavernous  
260 malformations (CCMs) and phosphorylated S6 (p-S6) ribosomal protein expression as its endothelial  
261 intermediary (Ren et al., 2021). We therefore sought, but did not observe, similarly increased  
262 expression of p-S6 in the vascular lesions of the *Krox20-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> mice (**Figure 6(O, S)**).  
263 Slightly increased expression was sometimes observed in the abnormally shaped vascular smooth  
264 muscle cells of muscular cavernomas, co-expressing alpha-smooth muscle actin (**Figure 6(M, O,**  
265 **arrows)**), but not in the thickened, disorganized smooth muscle walls of the coronary artery (**Figure**  
266 **6(Q, S)**).

267 **4.6 Melanocytic and other tumors**

268 In many *Krox20-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> mutants, vascular anomalies were accompanied by  
269 widespread, extracutaneous pigmented melanocyte deposits (**Figure 7(A)**). In the meninges of the  
270 head, although some melanocytosis is physiological in mice (Gudjohnsen et al., 2015), the olfactory  
271 lobes (**Figure 7(B)**) and trigeminal nerves were covered in a melanocytic mesh. Pigmented  
272 melanocytes were also conspicuous in the capillary network of the lower incisor gingiva (**Figure**  
273 **7(C)**), which has not been described to our knowledge as a site for extracutaneous melanocytes.  
274 Some adults developed melanocytic tumors in addition to their vascular anomalies (**Figure 7(D)**).  
275 These regularly invested distant lymph nodes and were found in multiple sites, but without the  
276 typical tropism for brain, liver or lung, where tumors were never observed. Such mice rapidly  
277 reached humane endpoints. A rhabdomyomatous mesenchymal hamartoma was also observed in the  
278 inner thigh of one mutant mouse (**Figure 7(E)**).

279 We hypothesized that peripheral Schwann cells could be a source of such widely distributed  
280 extracutaneous melanocytes and that the expression of constitutively active *Pik3ca* therein would  
281 favor their phenotypic switch. To test this *in vivo*, *Sox10-CreER*<sup>T2</sup> mice were crossed with floxed  
282 *Pik3ca*<sup>fl(H1047R)/+</sup> mice to produce a tamoxifen-inducible Cre recombinase in *Sox10*-expressing cells.  
283 At adult stages, these include but are not restricted to nerve-resident peripheral glia and their  
284 precursors (Deal et al., 2021). Four female *Sox10-CreER*<sup>T2</sup>; *Pik3ca*<sup>H1047R/+</sup> from two litters were  
285 injected with 1 mg 40H-TAM at 15-19 weeks and compared to four similarly treated *Sox10-CreER*<sup>T2</sup>;  
286 *RdT* mice (three female, one male) and one female *Pik3ca*<sup>fl(H1047R)/+</sup> controls of the same age. Within  
287 five days, one mutant had died and the three others had attained a humane endpoint and were  
288 euthanized. Cause of death was not determined, but gross examination of the *Sox10-CreER*<sup>T2</sup>; *RdT*  
289 mice under a fluorescence binocular dissecting microscope demonstrated effective recombination had  
290 been induced in all. Although no obvious tumors had developed in the mutants, the superficially

291 pigmented axillary and Peyer's patch lymph nodes had enlarged germinal centers (not shown), and  
292 the ovaries were lumpy (**Figure 7(F)**).

293 *Sox10-CreER<sup>T2</sup>* males were then mated to three *Pik3ca*<sup>fl<sup>ox</sup>(1047R)/+</sup> females and the pregnant dams  
294 treated at E18.5 with 4OH-TAM to induce recombination in post-migratory NC and other Sox10-  
295 expressing cells just before birth. This led to recovery of a total of ten live births: five *Sox10-*  
296 *CreER<sup>T2</sup>*; *Pik3ca*<sup>H1047R/+</sup> (three female, two male), four *Sox10-CreER<sup>T2</sup>* and one male *Pik3ca*<sup>H1047R/+</sup>  
297 mouse. These animals were followed without incident for up to 1 year, when one male mutant rapidly  
298 developed an unpigmented, circumscribed tail tumor of 5 mm in diameter and showed signs of  
299 distress. After euthanasia, the tumor could be seen to contain varied cellular elements including  
300 smooth muscle and mucin-containing myxoid zones that stained with Alcian blue (**Figure 7(G)**). Our  
301 observations support other recent studies that highlight the importance of positional and lineage  
302 context for the carcinogenic potential of oncogenic mutations (Baggiolini et al., 2021) and imply that  
303 it may extend beyond MAP kinases to the PI3K signaling pathway.

## 304 **5 Discussion**

### 305 **5.1 Vascular tumor-like malformations arise from impaired endothelial-mural interactions**

306 *PIK3CA* gain-of-function mutations have been shown to lead to constitutive activation of Akt  
307 downstream of the TEK angiopoietin-1 receptor in human vascular endothelial cells (Limaye et al.,  
308 2015) as well as in targeted mouse models (Castillo et al., 2016), where TEK is known as Tie2.  
309 Nearly half of sporadic venous malformations (VMs) bear activating *TEK* mutations while others  
310 express activating *PIK3CA* H1047R, or E452K or C420R mutations, in a mutually exclusive manner  
311 (Castel et al., 2016). These, particularly H1047R, are the most frequent hotspot mutations for breast  
312 and colon cancer, but also malignancies in 45 other tissues and over 21,000 samples curated by  
313 COSMIC (v95) to date (Stone, 1926; Le Lièvre, 1978; Etchevers et al., 2019).

314 The work we present here is the first to demonstrate that activation of the same signaling pathway by  
315 the same mutation in adjacent perivascular pericytes and vascular smooth muscle also can induce  
316 congenital vascular malformations. It has long been understood that paracrine signaling between  
317 adluminal and abluminal cells is necessary for tissue-appropriate, functional blood and lymphatic  
318 vessel assembly. Pharmacological PI3K inhibition rescues inducible arteriovenous malformations in  
319 the context of an inducible animal model for a recurrent transforming growth factor- $\beta$  (TGF- $\beta$ )/bone  
320 morphogenetic protein (BMP) signalling pathway gene mutation known to cause hereditary  
321 hemorrhagic telangiectasia (Ola et al., 2016). Recent models for CCMs also feature upregulated  
322 PI3K activity and increased p-S6 in endothelial cells, unlike what we have initially observed in the  
323 vascular lesions induced before or after birth by increased PI3K signaling in mural cell progenitors  
324 with immunofluorescence. However, activation of pAkt, p-S6 and other pathway effectors in the  
325 connective tissues of patients with PIK3CA-mutated fibroadipose vascular anomalies (FAVA)  
326 indicate that the syndromic aspects are not dissociable from the vascular tumor-like malformations  
327 themselves (Hori et al., 2020). Further functional work will be needed to define the additional  
328 mediators and intracellular effectors of endothelial-mural paracrine exchanges in these new models

329 NC cells are a minority but crucial lineage in cardiac function and development. As contributors to  
330 melanocytic, glial, parasympathetic neuronal and a small fraction of pericytic and cardiomyocyte  
331 lineages, their role is essentially paracrine. The fact that both *Wnt1-Cre; Pik3ca*<sup>H1047R/+</sup> and *Krox20-*  
332 *Cre; Pik3ca*<sup>H1047R/+</sup> mice present vascular anomalies within cardiac tissue implies that PI3K signaling  
333 to a cell of NC origin in the heart has an impact on its subsequent secretory activity, with a similar  
334 effect on intracardiac vascular development as in the head and neck. In the future, single-nucleus

335 RNA sequencing of both cardiac ventricles and craniofacial mesenchyme could be an approach to  
336 identify such vasculogenic factors secreted by lineage-traced cardiac pericytes and glia in these  
337 mouse crosses.

## 338 **5.2 PI3K activity in NC cells also induces megalencephaly, jaw hyperplasia and cleft palate**

339 In contrast to blood and lymphatic vessels, where the endothelium is always of mesodermal origin,  
340 the constitutive activation of *Pik3ca* in cells having expressed *Wnt1* in the neuroepithelium before  
341 neural crest migration also led to apparently autonomous effects in the brain, while only some  
342 mesectodermal NC derivatives were affected (Le Lievre and Le Douarin, 1975). Lineage tracing with  
343 a floxed *Rosa-tomato* fluorescent reporter allele did not show any differences in NC distribution after  
344 migration into the face and head at E9.5, implicating PI3K signaling in the later differentiation of  
345 cephalic NC-derived mesenchyme into perivascular cells and other connective tissues, a potential  
346 outcome not available to truncal NC progeny in mammals (Etchevers et al., 2001; Deal et al., 2021).  
347 This may explain why there were no apparent effects at the level of the trunk in neonates, although  
348 we may have missed subtle effects on vagal innervation of the heart or gut.

349 Cranial NC mesenchyme and nerves normally secrete vascular endothelial growth factor (VEGF) to  
350 promote mandibular artery extension, stabilization through mural coverage and, thereby, support jaw  
351 elongation (Wiszniak et al., 2015). Interfering with signaling to the endothelial VEGF receptor, *Nrp1*,  
352 phenocopies mandibular artery loss and hemifacial microsomia in human patients.

353 We have seen in gross dissection and micro-CT that when cranial NC expresses constitutively active  
354 *Pik3ca*, there is the opposite phenotype: vascular and jaw hyperplasia. A clear association between  
355 vascular and craniofacial overgrowth has been reported clinically for decades, well before human  
356 genetics caught up (Krings et al., 2007). Facial capillary malformations found in Sturge-Weber  
357 syndrome are usually due to constitutively activating, somatic *GNAQ* mutations (Shirley et al., 2013)  
358 but are regularly associated with segmental overgrowth of the orbit or the jaw and in at least one  
359 report, additional somatic mutations, including one in *PIK3CA* (Lian et al., 2014). This missense  
360 mutation has been reported 134 times to date in COSMIC (<https://cancer.sanger.ac.uk/cosmic>) with a  
361 high pathogenicity score (0.97). G-protein and PI3K signaling cascades are both likely to mediate the  
362 exchanges of vascular cross-talk with NC-derived mesenchyme and the subsequent skull growth  
363 anomalies that can extend throughout life.

## 364 **5.3 Congenital overgrowth usually does not lead to malignancy but presents its own problems**

365 Somatic mutation of codon H1047 is frequently but not exclusively implicated in asymmetric, multi-  
366 systemic *PIK3CA*-related overgrowth disorders such as CLOVES [OMIM 612918; congenital  
367 lipomatous overgrowth, vascular malformations, epidermal nevi and skeletal abnormalities] and  
368 endophenotypic segmental overgrowth syndromes affecting muscle and fat, or fibroadipose  
369 hyperplasia. Another class of PRDs involve megalencephaly with various other features affecting  
370 musculoskeletal, vascular, connective and adipose tissues, of which megalencephaly-capillary  
371 malformation-polymicrogyria syndrome (MCAP) is emblematic (Lee et al., 2012; Kingsmore et al.,  
372 2013; Alcantara et al., 2017). PI3K inhibitors have been very promising in clinical trials for these  
373 conditions (eg. Dill et al., 2014; Roy et al., 2015; Venot et al., 2018; Hori et al., 2020)

374 Some patients develop supernumerary, hypertrophic muscles in the upper limbs; these are  
375 occasionally bilateral, indicating that the original somatic mutation may have developed in a cell  
376 whose progeny entered the paraxial mesoderm cell during gastrulation (Frisk et al., 2019). While  
377 nearly half of overgrowth PRD patients in one cohort presented vascular malformations, the

378 congenital tissue overgrowth in the vast majority continued to evolve postnatally (Keppler-Noreuil et  
379 al., 2014). Malignant tumors in PRD patients are nonetheless surprisingly rare, given that they  
380 express proven oncogenic mutations, and this finding was borne out in our mouse models. However,  
381 the resultant malformations themselves were not always compatible with viability.

382 We have observed that benign vascular and/or hamartoma-like tumors can arise postnatally in mice  
383 expressing constitutively active *Pik3ca* in a mosaic fashion, in cells having expressed the  
384 *Krox20/Egr2* or *Sox10* transcription factors. Both of these transcription factors are hallmarks of so-  
385 called “Schwann cell precursors” or SCP. SCP are NC-derived, non-myelinating cells that reside  
386 along or at the terminal ends of peripheral nerves, and that can respond to environmental changes  
387 such as injury or inflammation by differentiating into myelinating Schwann cells and endoneurial  
388 fibroblasts but also melanocytes in rodents (Adameyko et al., 2009). Interestingly, these resident,  
389 poised cells also normally contribute extracutaneous melanocytes to the heart, inner ear, ocular  
390 choroid plexus, to some normal skeletal muscles such as the quadriceps (evident in the gastrocnemius  
391 of pigmented mice, unpublished observations) and the CNS meninges (Kaucka et al., 2021).

392 Some melanocytosis was seen in the hypothalamic meninges and membranes surrounding the  
393 trigeminal nerves of adult pigmented *Krox20-Cre;RdT* mice without additional *Pik3ca* activity.  
394 However, in agouti or black tamoxifen-induced *Sox10-CreER<sup>T2</sup>* control mice, it did not appear to  
395 increase in extent from normal meningeal pigmentation (Gudjohnsen et al., 2015). This implies that  
396 dosage reduction of *Egr2* may be a prerequisite to SCP plasticity and would be a testable hypothesis  
397 for the future. Our findings show that these partially committed progenitors at any stage of life are  
398 particularly vulnerable to the effects of constitutively active PI3K signaling.

#### 399 **5.4 *Pik3ca*-related disorders may encompass previously unsuspected pathologies**

400 The wide variety and range in severity of PRD phenotypes is attributed in part to the location of the  
401 cells bearing the mutation and to the proportion of cells affected in each of any given patient’s  
402 tissues. We identified effects of constitutive PI3K signaling on pituitary and palatal development that  
403 are not features of the diverse PRDs already identified to date.

404 Adrenal insufficiency could contribute to perinatal mortality in our *Wnt1-Cre; Pik3ca<sup>H1047R/+</sup>* mice,  
405 since when constitutive Akt signaling is induced in the embryonic ectoderm, crucial proteins for  
406 differentiation of the corticotroph lineage, such as *Bmp4* and *Tbx19*, are significantly down-regulated  
407 in mice (Segrelles et al., 2008). If so, this would be another measure of paracrine NC effects on  
408 pituitary development. Interestingly, the fact that *Krox20-Cre; Pik3ca<sup>H1047R/+</sup>* survived to adulthood  
409 despite constant PI3K activity in all anterior pituitary cells would imply that it is the action of *Pik3ca*  
410 signaling in resident NC-derived cells in the pituitary or its meninges that led to the hypoplasia we  
411 regularly observed by birth. Such hypotheses and the nature of this paracrine activity exerted by NC-  
412 derived cells could be further investigated in these mouse models.

413 Lineage-traced *Krox20-Cre; RdT* mice showed a much broader distribution of cells in the body that  
414 had once or continued to express *Egr2* than previously described. Evidence exists that some of these  
415 are cutaneous vascular pericytes derived from Schwann cell precursors, themselves from a NC-  
416 derived “boundary cap cell” population residing adjacent to the spinal cord (Gresset et al., 2015;  
417 Topilko, 2019). However, many also appear to be specialized fibroblasts primed by this transcription  
418 factor (Muhl et al., 2020).

419 The sites of predilection for *Krox20-Cre; Pik3ca<sup>H1047R/+</sup>* vascular lesions were within the *panniculus*  
420 *carnosus* and epaxial skeletal muscle groups, compatible with intramuscular hemangioma, a tumor

421 rather than a vascular malformation (Tan et al., 2007; Kurek et al., 2012). Unlike soft-tissue  
422 angiomas, we did not observe a mature adipose or lipomatosis component to these lesions, or  
423 indeed in any of the other described mouse models. However, adipocytes do collect around  
424 peripheral nerves (**Figure 5(A-B)**) in our *Krox20-Cre; Pik3ca*<sup>H1047R/+</sup> mice. This observation may be  
425 relevant for the study of fibrolipomatous hamartoma, which like other PRDs is often associated with  
426 overgrowth of the innervated territory (Marek et al., 2021). Somatic or germline mutations in *PTEN*,  
427 leading indirectly to PI3K pathway activation, also predispose to cancer, localized tissue overgrowth  
428 and are frequently associated with intramuscular vascular anomalies (Tan et al., 2007; Ho et al.,  
429 2012).

430 Recently, gain-of-function *Pik3ca* mutations have been demonstrated to be sufficient to drive small,  
431 postnatal capillary hemangiomas in brain endothelial cells and are necessary, in combination with  
432 mutations of known CCM genes in mice or in humans, for the development of large postnatal  
433 cavernomas (Hong et al., 2021; Ren et al., 2021). Should *PIK3CA* mutations also be confirmed in  
434 human intramuscular hemangiomas or fibrolipomatous hamartomas, they would be the functional  
435 and tumoral counterpart of soft-tissue hamartomas due to *PTEN* mutations (Kurek et al., 2012; Luks  
436 et al., 2015; Tachibana et al., 2018). In these congenital and predisposing conditions, evolving  
437 anomalies are not always surgically accessible and can be aggravated in the case of incomplete  
438 resection. Targeted inhibitors of distinct pathway levels, potentially locally infused and in  
439 combination, show great promise and can now be tested in a wider range of tailored animal models.

## 440 **6 Figure legends**

441 **Figure 1.** Mutants compared to their unaffected control littermates expressing only one or neither of  
442 the *Wnt1-Cre* or *Pik3ca*<sup>H1047R/+</sup> alleles.

443 (A) At E20.5, fetal head from control littermate of (B) at same magnification, facing left.  
444 (B) At E20.5, mutant fetus like newborns had visible megalencephaly, displaced eyes above an  
445 enlarged maxillary primordium and skull vault, larger external ears, a longer nose, and facial vascular  
446 malformations (arrowheads). Bar, 1 mm.  
447 (C) Same fetus as in (A), skin removed and relative interocular distance indicated in square bracket.  
448 Bar, 2 mm.  
449 (D) Same fetus as in (B), skin removed to see hemorrhage from ruptured vascular malformations.  
450 Bar, 2 mm.  
451 (E) *Wnt1-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> mutant newborn at P0, view of cleft palate (bracket) after removal of  
452 skin and jaw. Bar, 2 mm.  
453 (F) *Wnt1-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> mutant newborn at P0, coronal view of cleft palate (bracket) after  
454 removal of skin and jaw, relative interocular distance indicated in square bracket at same scale as  
455 (C). Arrowhead, vascular malformation. Bar, 2 mm.  
456 (G) Maximum sagittal plane surface projection of lateral photographs from embryos at the indicated  
457 stages between embryonic day (E)15 and birth (P0), of either control (ctrl) littermate or *Wnt1-Cre*<sup>+/-</sup>;  
458 *Pik3ca*<sup>H1047R/+</sup> (mut) genotype. Through the last third of gestation, mutant heads were significantly  
459 larger than controls, as was the head of the sole neonate recovered.  
460 e, eye

462 **Figure 2.** Micro-computed tomography (micro-CT) of skull development in *Wnt1-Cre*<sup>+/-</sup>;  
463 *Pik3ca*<sup>H1047R/+</sup> mice at late fetal stages.

464 (A) Control littermate fetus at E15.5 in (E) – representative micro-CT frontal section through eyes,  
465 brain, palate, tongue and jaw.

466 (B, C, D) 3D projections in frontal view of the mineralization of three representative skulls of control  
467 littermate fetuses at E20.5 in (B-D). The arrow indicates mandibular bone thickness.  
468 (E) Control littermate embryo of of E15.5 embryo in (A) – representative micro-CT frontal section  
469 through eyes, brain, cleft palate, malpositioned tongue (arrow) and jaw.  
470 (F, G, H) 3D projections in frontal view of the mineralization of three representative skulls of control  
471 littermate fetuses at E20.5 in (F-H). The arrow indicates mandibular bone thickness.  
472 (I, J, K) Representative micro-CT frontal sections through the pituitary gland (boxed) in three control  
473 fetuses at E15.5.  
474 (L, M, N) Representative micro-CT frontal sections through the pituitary gland (boxed) in three  
475 *Wnt1-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> fetuses at E15.5. The morphology of the pituitary in N appears normal,  
476 unlike in L and M.  
477 (O) Segmentation analysis of the largest surface areas of the pituitary in the same sectional plane,  
478 shows a significant reduction in pituitary size at 15-16 embryonic days (p>0.01, n=6 controls versus  
479 6 mutants).  
480 a.u., arbitrary units; ctrl, control; mut, mutant.

481  
482 **Figure 3.** Phenotypes of *Wnt1-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> embryos from E9.5 to E13.5.

483 (A) When lineage-traced by co-expression of a *Rd1*<sup>+/-</sup> allele to transcribe Tomato red fluorescent  
484 protein in cells having expressed *Wnt1*, *Wnt1-Cre*<sup>+/-</sup>; *Rd1*<sup>+/-</sup> embryos at E9.5 show the normal  
485 distribution of neural crest (NC) mesenchyme in the face and pharyngeal arches. Bars = 0.5 mm for  
486 A, B.  
487 (B) *Wnt1-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup>; *Rd1*<sup>+/-</sup> embryos at E9.5 show unaltered distribution of NC-derived  
488 mesenchyme in the pharyngeal arches, frontonasal bud or body.  
489 (C) Left side of E13.5 control littermate to (D). Lack of pigment in retinal pigmented epithelium of  
490 eye is normal for a mouse that would have been born albino (*Tyr*<sup>c</sup>/*Tyr*<sup>c</sup>), a background allele  
491 (<http://www.informatics.jax.org/allele/MGI:1855976>). Bars = 2 mm for C, D.  
492 (D) Left side of E13.5 *Wnt1-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> littermate to C, showing vascular anomalies and  
493 cerebral hemorrhage.  
494 (E) Paraffin parasagittal section of control littermate head of (F) at E13.5, stained with HE. Bars =  
495 0.5 mm for E, F, G, H, L, M.  
496 (F) Paraffin sagittal section of mutant head at E13.5 showing enlarged frontonasal and mandibular  
497 tissues, cerebellar isthmus and choroid plexus, as well as a malpositioned Rathke's pouch relative to  
498 the infundibulum, stained with HE.  
499 (G) Paraffin frontal section of control head at E13.5 showing physiological position of tongue  
500 between palatal shelves and converging maxillary processes, stained with HE.  
501 (H) Paraffin frontal section of mutant littermate of (G) at E13.5, showing lens coloboma, thickened  
502 corneal epithelium and less convergent maxillary processes than in (G), stained with HE.  
503 (I) *In situ* hybridization with a *Pdgfra* probe in a control embryo at E13.5 shows transcript expression  
504 in blue in craniofacial mesenchyme around the ocular primordium, particularly in the lens epithelium  
505 and corneal stroma, but not in the lenticular primary fibers. Bars I, J, K = 0.2 mm.  
506 (J) Mutant embryos express *Pdgfra* normally within the ocular primordium at E13.5, but have  
507 microphakia.  
508 (K) An adjacent section to (J) shows an enlarged, *Pdgfra*<sup>+</sup> hyaloid vasculature relative to the control  
509 embryo section in (I).  
510 (L) A control frontal section at E13.5 after *Pdgfra in situ* hybridization.  
511 (M) A mutant frontal section of an embryo at E13.5 after *Pdgfra in situ* hybridization. Palatal, digital  
512 or mesenchymal expression are not qualitatively different.

513

514 **Figure 4.** Vascular lesions are present before birth with disorganized mural elements in *Wnt1-Cre*;  
515 *Pik3ca*<sup>H1047R/+</sup> mutants.

516 (A) Mutant embryo at E15.5 with periocular vascular anomalies.  
517 (B) Mutant littermate of (A) with segmental vascular anomalies in the maxillary region.  
518 (C, D) Mutant E20 fetuses. (C) was dead *in utero* and had a vascular lesion on the mandible. Both  
519 showed maxillary and posterior periocular vascular anomalies and megalencephaly. Scale bars A-D,  
520 2 mm.

521 (E) Control littermate and (F) mutant pulmonary trunk at E14.5; merged immunofluorescence (E',  
522 F'): yellow, endothelial marker Pecam-1 (CD31); (E'', F'') purple, smooth muscle marker,  $\alpha$ -smooth  
523 muscle actin (aSMA); (E''', F''') blue, nuclear marker DAPI. Bars E, F = 50  $\mu$ m.

524 (G) Cellular organization around an intracardiac vascular anomaly, showing a discontinuous (arrow)  
525 smooth muscle layer of mutant NC origin.

526 (H) Facial skin of E20.5 mutant fetus (not the ones in C, D) with numerous double-labeled, small  
527 capillary anomalies in upper dermis (arrow) around hair follicles.  
528 (G', H'): Tomato fluorescent protein; (G'', H''): smooth muscle marker,  $\alpha$ -smooth muscle actin  
529 (aSMA); (G''', H''') nuclear marker DAPI in blue. Bars G, H = 50  $\mu$ m.

530

531 **Figure 5.** Anatomy and histology of vascular anomalies in *Krox20-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> adult  
532 mutant mice.

533 (A) Subcutaneous vascular anomaly, epaxial (*longissimus dorsi*) muscle. Bars A-I: 2 mm.  
534 (B) Subcutaneous vascular anomaly, quadriceps.  
535 (C) Vascular anomalies around left gonad.  
536 (D) Mutant heart, small vascular anomaly at apex (arrow).  
537 (E) Control littermate spleens of those in (F).  
538 (F) Mutant spleens, same scale as (E).  
539 (G, H) Vascular tumor around gonad from different mouse than in (C), *Krox20-Cre*<sup>+/-</sup>;  
540 *Pik3ca*<sup>H1047R/+</sup>; *RdT*<sup>+/-</sup>. The fluorescent fibroblasts in gonad and lesional septa had expressed *Krox20*  
541 and thereby, constitutively active *Pik3ca*.  
542 (I) Intracardiac vascular anomalies were present in all mutant adults examined.  
543 (J) *Krox20-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup>; *RdT*<sup>+/-</sup> spleens as in (F) had numerous fluorescent ramifications  
544 consistent with reticular fibers, peripheral nervous or perivascular elements. Bar, 200  $\mu$ m.  
545 (K) Mutant femoral bone marrow in an adult mouse that had spontaneously died with multiple  
546 vascular anomalies was hypocellular with increased density of vascular sinuses rather than adiposity.  
547 Bar, 200  $\mu$ m.  
548 (L) Heart from a *Krox20-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup>; *RdT*<sup>+/-</sup> mouse showing recombined cells in a fine  
549 meshwork throughout the myocardium of all chambers with increased density in the ventral  
550 pulmonary trunk and strong expression in the outer wall of a sympathetic nerve (arrow). Bar, 200  
551  $\mu$ m.  
552 (M) Typical histology of vascular lacunae in the ventricular wall. Bar, 200  $\mu$ m.  
553 (N, O) Subcutaneous vascular tumor of tail. Bar, 2 mm.  
554 (P) Dorsal aspect of lineage-traced mutant hairy skin. Fluorescence is visible at the base of each hair  
555 follicle and in the vascular pericytes of capillaries underlying them. Bar, 200  $\mu$ m.  
556 (Q) Ventral aspect of lineage-traced mutant hairy skin. The *panniculus carnosus* muscle had  
557 expressed *Krox20* (striations) and the peripheral nerves express *Krox20Cre*-driven Tomato even  
558 more strongly in their myelinating Schwann cells. Bar, 0.5 mm.  
559 (R) Vascular lacunae separate disrupt the organization of muscle bundles and their external  
560 connective tissues in a mutant thigh. Bar, 200  $\mu$ m.  
561 (S) Disrupted vascular structures were also present in the mutant salivary gland.

562 (T) A profusion of small vascular sinuses were enlarged and necrosis was visible in the mutant liver.  
563 Bar = 100  $\mu$ m.  
564 ao, aorta; la, left atrium; lv, left ventricle; pt, pulmonary trunk; ra, right atrium; rca, right coronary  
565 artery; rv, right ventricle, sa, septal artery.  
566

567 **Figure 6.** Lineage tracing and immunofluorescence in *Krox20-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup> adult mutant  
568 mice.

569 (A) Dorsal view of control pituitary after removal of brain and meninges.

570 (B) Dorsal view of *Krox20-Cre*<sup>+/-</sup>; *RdT*<sup>+/-</sup> pituitary during dissection after removal of brain, showing  
571 highly fluorescent adenohypophysis under visible light. Bar, 1 mm.

572 (C-F) Section through adenohypophysis of *Krox20-Cre*<sup>+/-</sup>; *RdT*<sup>+/-</sup> mouse, showing normal cell  
573 density and that most or all cell types, including perivascular nuclei, had expressed *Krox20*, unlike  
574 the sparse recombination observed in the neurohypophysis (not shown). (C) Merged (D) Tomato (E)  
575 Pecam1 (CD31) (F) DAPI fluorescence. Bar, 20  $\mu$ m.

576 (G-J) Section through adenohypophysis of *Krox20-Cre*<sup>+/-</sup>; *Pik3ca*<sup>H1047R/+</sup>; *RdT*<sup>+/-</sup> mouse, showing  
577 slightly reduced cell density but a striking reduction in cells that had expressed *Krox20*, implying  
578 later compensation by endothelial and pituitary stem cells. Bar, 20  $\mu$ m.

579 (K, L) Expression of alpha-smooth muscle actin (aSMA, orange) around vascular tumors (K) and in  
580 a coronary artery and surrounding telangiectasias (L). Area magnified in (Q) indicated.

581 (M-P) Mural structure in representative vascular anomaly. Slight increase of phosphorylated S6  
582 kinase (O, purple, arrowheads) in a few among the disorganized and unusually shaped cells of the  
583 aSMA-expressing vascular wall (N, orange). (M) Merged. (P) DAPI. Bar = 10  $\mu$ m.

584 (Q-T) Mural structure of the coronary artery in (L). No apparent increase of phosphorylated S6  
585 kinase (S, purple) but presence of disorganized and unusually shaped cells in the aSMA-expressing  
586 vascular wall (R, orange), although some laminar structure is still present. (Q) Merged. (T) DAPI.  
587 Bar = 10  $\mu$ m.  
588

589 **Figure 7.** Widespread melanocytic anomalies in conjunction with *Krox20*- or *Sox10*-driven  
590 expression of constitutively active *Pik3ca*.

591 vascular anomalies were accompanied by widespread, extracutaneous pigmented melanocyte  
592 deposits (**Figure 7(A)**). In the meninges of the head, although some melanocytosis is physiological in  
593 mice (Gudjohnsen et al., 2015), the olfactory lobes (**Figure 7(B)**) and trigeminal nerves were covered  
594 in a melanocytic mesh. Pigmented melanocytes were also conspicuous in the capillary network of the  
595 lower incisor gingiva (**Figure 7(C)**), which has not been described to our knowledge as a site for  
596 extracutaneous melanocytes. Some adults developed melanocytic tumors in addition to their vascular  
597 anomalies (**Figure 7(D)**). These regularly invested distant lymph nodes and were found in multiple  
598 sites, but without the typical tropism for brain, liver or lung, where tumors were never observed.  
599 Such mice rapidly reached humane endpoints. A rhabdomyomatous mesenchymal hamartoma was  
600 also observed in the inner thigh of one mutant mouse (**Figure 7(E)**).

601 We hypothesized that peripheral Schwann cells could be a source of such widely distributed  
602 extracutaneous melanocytes and that the expression of constitutively active *Pik3ca* therein would  
603 favor their phenotypic switch. To test this *in vivo*, *Sox10-CreER*<sup>T2</sup> mice were crossed with floxed  
604 *Pik3ca*<sup>fl(H1047R)/+</sup> mice to produce a tamoxifen-inducible Cre recombinase in *Sox10*-expressing cells.  
605 At adult stages, these include but are not restricted to nerve-resident peripheral glia and their  
606 precursors (Deal et al., 2021). Four female *Sox10-CreER*<sup>T2</sup>; *Pik3ca*<sup>H1047R/+</sup> from two litters were  
607 injected with 1 mg 40H-TAM at 15-19 weeks and compared to four similarly treated *Sox10-CreER*<sup>T2</sup>;  
608 *RdT* mice (three female, one male) and one female *Pik3ca*<sup>fl(H1047R)/+</sup> controls of the same age. Within

609 five days, one mutant had died and the three others had attained a humane endpoint and were  
610 euthanized. Cause of death was not determined, but gross examination of the *Sox10-CreER<sup>T2</sup>; Rdt*  
611 mice under a fluorescence binocular dissecting microscope demonstrated effective recombination had  
612 been induced in all. Although no obvious tumors had developed in the mutants, the superficially  
613 pigmented axillary and Peyer's patch lymph nodes had enlarged germinal centers (not shown), and  
614 the ovaries were lumpy (**Figure 7(F)**).

615 *Sox10-CreER<sup>T2</sup>* males were then mated to three *Pik3ca<sup>flox(1047R)/+</sup>* females and the pregnant dams  
616 treated at E18.5 with 4OH-TAM to induce recombination in post-migratory NC and other *Sox10-*  
617 *expressing cells just before birth. This led to recovery of a total of ten live births: five *Sox10-**  
618 *CreER<sup>T2</sup>; Pik3ca<sup>H1047R/+</sup>* (three female, two male), four *Sox10-CreER<sup>T2</sup>* and one male *Pik3ca<sup>H1047R/+</sup>*  
619 mouse. These animals were followed without incident for up to 1 year, when one male mutant rapidly  
620 developed an unpigmented, circumscribed tail tumor of 5 mm in diameter and showed signs of  
621 distress. After euthanasia, the tumor could be seen to contain varied cellular elements including  
622 smooth muscle and mucin-containing myxoid zones that stained with Alcian blue (**Figure 7(G)**).

623 (A) Extracutaneous pigmented melanocyte deposits along nerves and muscle fascia in *Krox20-*  
624 *Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>* mice, close to vascular anomalies. Bar = 2 mm.

625 (B) Increased meningeal melanocytosis over ventromedial frontal lobes and olfactory bulbs in  
626 *Krox20-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>* mutants, not seen in 4OH-TAM-treated *Sox10-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>*  
627 mice. Bar = 2 mm.

628 (C) Pigmented, gingival melanocytosis over lower incisors of *Krox20-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>* mutants,  
629 not seen in 4OH-TAM-treated *Sox10-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>* mice. Bar = 1 mm.

630 (D) Melanoma in *Krox20-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>* mutant mouse near seminal gland. Bar = 2 mm.

631 (E) Rhabdomyomatous mesenchymal hamartoma in thigh of *Krox20-Cre<sup>+/-</sup>; Pik3ca<sup>H1047R/+</sup>* mutant  
632 mouse. Bar = 100  $\mu$ m.

633 (F) Ovary of *Sox10-CreER<sup>T2</sup>; Pik3ca<sup>H1047R/+</sup>* mouse induced at 15 weeks with 1 mg 4OH-TAM, after  
634 5 days.

635 (G) Unpigmented, myxoid melanoma in tail of induced *Sox10-CreER<sup>T2</sup>; Pik3ca<sup>H1047R/+</sup>* mouse, after  
636 nearly one year. Bar = 100  $\mu$ m.

## 637 **7 Conflict of Interest**

638 The authors declare that the research was conducted in the absence of any commercial or financial  
639 relationships that could be construed as a potential conflict of interest.

## 640 **8 Author Contributions**

641 EM, MM and ML planned and performed mouse crosses and dissections, and undertook the  
642 histology and immunofluorescence experiments as well as microscopy. AP contributed the data for  
643 Figure 2. GM and AB provided reagents and expertise on PI3K signaling. TF dissected pituitary  
644 glands and aided in the interpretation of the pituitary sections. NM reviewed the histology. HCE  
645 contributed the *in situ* hybridization and microscopy, conducted statistical analyses, obtained funding  
646 and wrote the manuscript. All authors reviewed the final manuscript.

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## 658 **11 Contribution to the field statement**

659 In this paper, my co-authors and I have developed multiple new mouse models to test the  
660 developmental function of a gene whose mutations are frequent and well known to cancer  
661 researchers, called PIK3CA. One particular mutation is present in 4 out of 10 common malignancies  
662 due to this gene, permanently activating the enzyme that it encodes and driving aggressive tumor  
663 growth. We have carefully observed and described the anatomical and molecular characteristics of  
664 the many malformations that can also be caused by the same oncogenic mutation. Mutations of  
665 PIK3CA have also been identified over the last decade in numerous rare disease syndromes with  
666 overlapping symptoms, among which musculoskeletal, brain and vascular malformations are  
667 regularly observed. By restricting PIK3CA activity to specific subsets of cells in the mouse, we have  
668 identified that their abilities to make or influence other cell types renders them more vulnerable to  
669 causing changes in tissue shape and size, or to developing cancer. These mouse models indicate  
670 additional candidate diseases in humans where PIK3CA may be locally active, opening new potential  
671 applications for existing treatments.

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