

Single cell, whole embryo phenotyping of pleiotropic disorders of mammalian development

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Abstract

Mouse models are a critical tool for studying human diseases, particularly developmental disorders, as well as for advancing our general understanding of mammalian biology. However, it has long been suspected that conventional approaches for phenotyping are insufficiently sensitive to detect subtle defects throughout the developing mouse. Here we set out to establish single cell RNA sequencing (sc-RNA-seq) of the whole embryo as a scalable platform for the systematic molecular and cellular phenotyping of mouse genetic models. We applied combinatorial indexing-based sc-RNA-seq to profile 101 embryos of 26 genotypes at embryonic stage E13.5, altogether profiling gene expression in over 1.6M nuclei. The 26 genotypes include 22 mouse mutants representing a range of anticipated severities, from established multisystem disorders to deletions of individual enhancers, as well as the 4 wildtype backgrounds on which these mutants reside. We developed and applied several analytical frameworks for detecting

48 differences in composition and/or gene expression across 52 cell types or trajectories. Some
49 mutants exhibited changes in dozens of trajectories (*e.g.*, the pleiotropic consequences of altering
50 the *Sox9* regulatory landscape) whereas others showed phenotypes affecting specific subsets of
51 cells. We also identify differences between widely used wildtype strains, compare phenotyping
52 of gain vs. loss of function mutants, and characterise deletions of topological associating domain
53 (TAD) boundaries. Intriguingly, even among these 22 mutants, some changes are shared by
54 heretofore unrelated models, suggesting that developmental pleiotropy might be “decomposable”
55 through further scaling of this approach. Overall, our findings show how single cell profiling of
56 whole embryos can enable the systematic molecular and cellular phenotypic characterization of
57 mouse mutants with unprecedented breadth and resolution.

58

59 Introduction

60

61 For over 100 years, the laboratory mouse (*Mus musculus*) has served as the quintessential animal
62 model for studying both common and rare human diseases¹⁻⁴. For developmental disorders in
63 particular, mice have been transformative, as a mammalian system that is nearly ideal for genetic
64 analysis and in which the embryo is readily accessible⁵.

65

66 In the first decades of the field, mouse genetics relied on spontaneous or induced mutations
67 resulting in visible physical defects that could then be mapped. However, gene-targeting
68 techniques subsequently paved the way for “reverse genetics”, *i.e.* analysing the phenotypic
69 effects of intentionally engineered mutations. Through systematic efforts such as the International
70 Knockout Mouse Consortium, knockout models are now available for thousands of genes⁶.
71 Furthermore, with the emergence of CRISPR/Cas genome editing^{7,8}, it is increasingly practical to
72 delete individual regulatory elements or otherwise modify the *cis*-regulatory landscape, and to
73 then study the *in vivo* consequences of these alterations^{9,10}.

74

75 Phenotyping has also grown more sophisticated. Conventional investigations of developmental
76 syndromes typically focus on one organ system at a specific stage of development, *e.g.* combining
77 expression analyses, histology, and imaging to investigate a visible malformation^{1,11,12}. However,
78 pleiotropy is a pervasive phenomenon in mammalian development, and focusing on one aspect
79 of a phenotype may come at the expense of detecting or characterising others, particularly if they
80 are subtle or masked by lethality. The concept of the Mouse Clinic, in which a given model is
81 subjected to a battery of standardised tests, reflects a more systematic approach¹³. However,
82 such clinics are expensive and time-consuming to conduct in practice. Furthermore, many kinds
83 of phenotypes detected through such tests (*e.g.*, behavioural, electrophysiological) may require
84 years of additional work to link to their molecular and cellular correlates. It is also the case that
85 knockouts of even highly conserved coding or regulatory sequences frequently result in no
86 detectable abnormality or only minor transcriptional changes¹⁴⁻¹⁶. In such instances, it remains
87 unknown whether there is truly no phenotype, or whether the methods used are simply
88 insufficiently sensitive. In sum, phenotyping has become “rate limiting” in mouse genetics.

89

90 The recent emergence of single cell molecular profiling technologies (*e.g.*, sc-RNA-seq) offer a
91 potential path to overcome this barrier. As a first step, we and others have extensively applied sc-
92 RNA-seq to profile wildtype mouse development at the scale of the whole embryo¹⁷⁻²². Applying
93 sc-RNA-seq to mouse mutants, several groups have successfully unravelled how specific
94 mutations affect transcriptional networks and lead to altered cell fate decisions in individual
95 organs²³⁻²⁶. However, there is still no clear framework for analysing such data at the scale of the
96 whole embryo, nor for how such data from multiple mutants might be combined to better
97 understand the molecular and cellular basis of classic phenomena like pleiotropy.

98

99 Here we set out to establish sc-RNA-seq of whole embryos as a scalable framework for the
100 systematic molecular and cellular phenotyping of mouse genetic models. We profiled 101
101 embryos of 22 different mouse mutants and 4 wildtype backgrounds at E13.5. The resulting
102 mouse mutant cell atlas (MMCA) includes over 1.6M sc-RNA-seq profiles. To analyze these data,

103 we develop and apply new strategies for detecting differences in composition and/or gene
104 expression across 52 cell types or trajectories spanning the whole mid-gestational embryo.

105
106 Single-cell RNA-seq of 101 mouse embryos

107
108 We collected a total of 103 mouse embryos, including 22 different mutants and four wildtype (WT)
109 strains (C57BL/6J, G4, FVB, and BALB/C) at embryonic stage E13.5, and generally four
110 replicates per strain (**Fig. 1a**). The mouse mutants were chosen to represent a spectrum of
111 phenotypes ranging from very severe pleiotropic developmental disorders (e.g., *Sox9*, which we
112 expected to affect many organ systems) to knockouts of individual, noncoding regulatory
113 elements (many of which we expected to result in, at best, subtle defects).

114
115 We grouped the 22 mutants, all homozygous, into four rough categories (**Supplementary Table**
116 **1**): 1) pleiotropic mutants, representing knockouts of developmental genes expressed in multiple
117 organs (*Ttc21b* KO, *Carm1* KO, *Gli2* KO), as well as two mutations of the *Sox9* regulatory
118 landscape suspected to have pleiotropic effects, both of which effectively result in the introduction
119 of a boundary element between endogenous *Sox9* enhancers and the *Sox9* promoter (*Sox9* TAD
120 boundary KI; *Sox9* regulatory INV)^{27–30}. 2) developmental disorder mutants, intended to model
121 specific human diseases (*Scn11a* GOF, *Ror2* KI, *Gorab* KO, *Cdkl5* -Y)^{31–33}, 3) mutations of loci
122 associated with human disease (*Scn10a/Scn11a* DKO, *Atp6v0a2* KO, *Atp6v0a2* R755Q,
123 *Fat1TAD* KO)^{34,35}. 4) prospective deletions of cis-regulatory elements, including of TAD
124 boundaries in the vicinity of developmental transcription factors including *Smad3*, *Twist1*, *Tbx5*,
125 *Neurog2*, *Sim1*, *Smad7*, *Dmrt1*, *Tbx3*, and *Twist1*³⁶, and, as a positive control, the ZRS distal
126 enhancer (Zone of polarizing activity Regulatory Sequences) which regulates sonic hedgehog
127 (SHH) expression and results in absent distal limb structures³⁷.

128
129 The 103 flash-frozen embryos (26 genotypes x 4 replicates; one embryo was lost in transport), all
130 staged at E13.5, were sent by five groups to a single site, where they were subjected to sci-RNA-
131 seq3 as previously described¹⁷. After removing potential doublets, we profiled 1,671,245 nuclei
132 altogether (16,226 +/- 9,289 per embryo; 64,279 +/- 18,530 per strain; median UMI count of 843
133 per cell and median genes detected of 534 at 75% duplication rate).

134
135 Applying principal components analysis (PCA) to “pseudobulk” profiles of the 103 embryos
136 resulted in two roughly clustered groups corresponding to genetic background (**Fig. 1b**). In
137 particular, wildtype and mutant FVB embryos clustered separately from C57BL/6J, G4, and
138 BALB/C embryos. However, embryos corresponding to individual mutants did not cluster
139 separately, suggesting that none were affected with severe, global aberrations and highlighting
140 the inadequacy of bulk RNA-seq for detecting mutant-specific effects. A single outlier embryo
141 (#104) was aberrant with respect to cell recovery (n = 1,047) as well as appearance
142 (**Supplementary Fig. 1**).

143
144 We next sought to validate the staging of these embryos, leveraging our previous mouse
145 organogenesis cell atlas (MOCA), which spans E9.5 to E13.5¹⁷. PCA of pseudobulk profiles of 61
146 wildtype embryos from MOCA resulted in a first component (PC1) that was strongly correlated

147 with developmental age (**Fig. 1c**). Projecting pseudobulk profiles of the 103 MMCA embryos to
148 this embedding resulted in the vast majority of MMCA embryos clustering with E13.5 embryos
149 from MOCA along PC1, consistent with accurate staging. However, five embryos from MMCA
150 appeared closer to E11.5 or E12.5 embryos from MOCA. Four of these were retained as their
151 delay might be explained by their mutant genotype, while one from a wildtype background
152 (C57BL/6; #41) was designated as a second outlier. We removed cells from the two outlier
153 embryos (#104; #41) as well as cells with high proportions of reads mapping to the mitochondrial
154 genome (>10%) or ribosomal genes (>5%). This left 1,627,857 cells, derived from 101 embryos
155 (**Fig. 1d**).

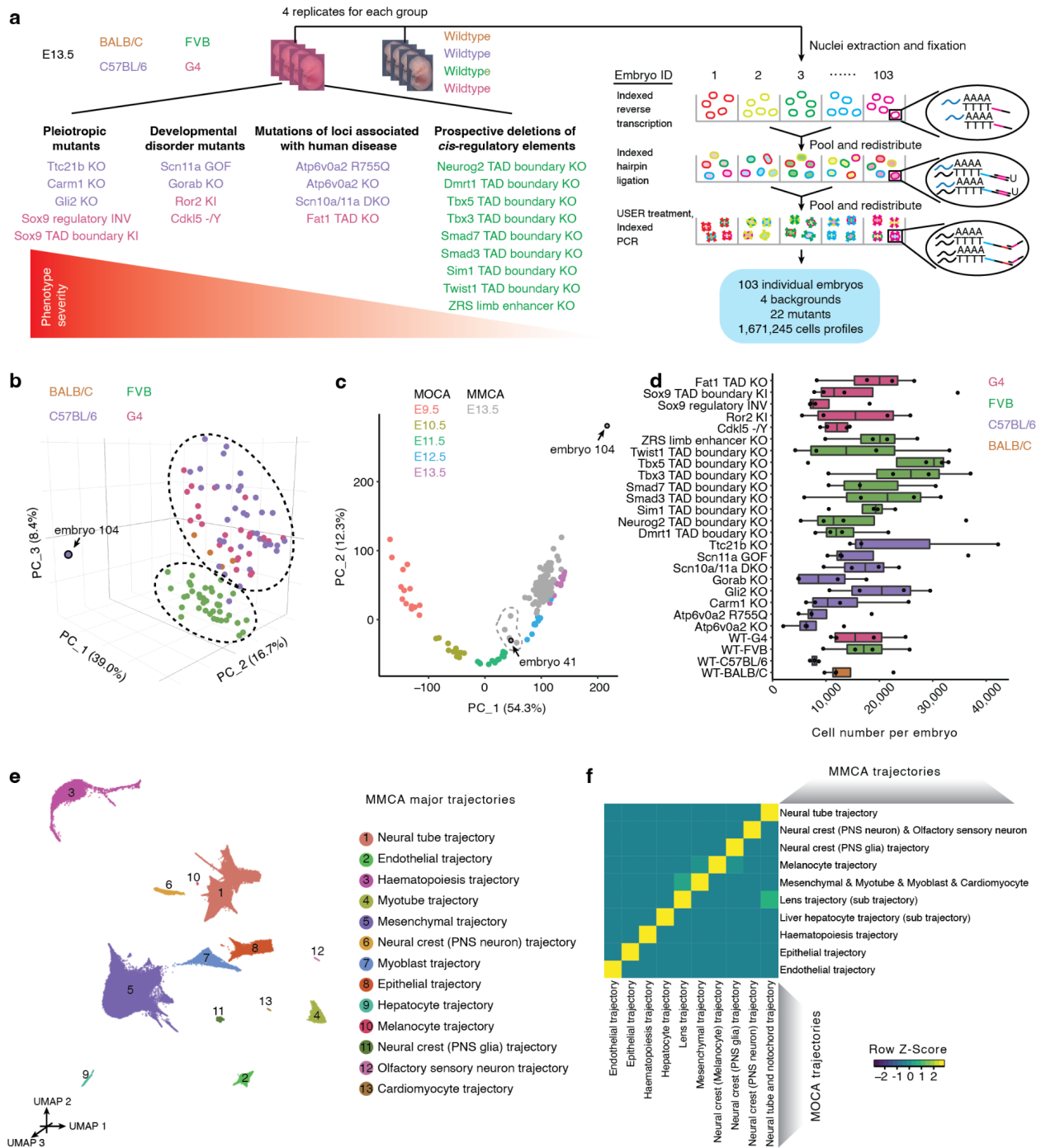
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157 To facilitate an integrated analysis, we sought to project cells from all genotypes to a wildtype
158 derived “reference embedding” (**Supplementary Fig. 2; Methods**). We first applied principal
159 components (PC) dimensionality reduction to cells from wildtype genotypes only ($n = 215,575$;
160 13.2% of dataset). We then projected cells from mutant genotypes to this embedding, followed
161 by alignment on the combined data to mitigate the effects of technical factors. Next, we applied
162 the UMAP algorithm to the aligned principal components of wildtype cells, followed by Louvain
163 clustering and manual annotation of the resulting major trajectories and sub-trajectories based on
164 marker gene expression. Finally, we projected mutant cells into this UMAP space and assigned
165 them major trajectory and sub-trajectory labels via a k -nearest neighbour (k -NN) heuristic.

166

167 Altogether, we identified 13 major trajectories, 8 of which could be further stratified into 59 sub-
168 trajectories (**Fig. 1e; Supplementary Fig. 3; Supplementary Table 2**). These were generally
169 consistent with our annotations of MOCA, albeit with some corrections as we have described
170 elsewhere^{38,39}, as well as greater granularity for some cell types that is likely a consequence of
171 the deeper sampling of E13.5 cells in these new data (**Fig. 1f; Supplementary Fig. 4**). For
172 example, what we had previously annotated as the excitatory neuron trajectory could be further
173 stratified into a di/mesencephalon (*Slc17a6+*, *Barhl1+*, *Shox2+*), thalamus (*Ntng1+*, *Gbx2+*)
174 and spinal cord (*Ebf1+*, *Ebf3+*) sub-trajectories, while skeletal muscle could be further
175 stratified into myoblast (*Pax7+*) and myotube (*Myh3+*, *Myog+*) sub-trajectories.

176



177
178

179 **Figure 1. Single-cell RNA-seq of 103 whole mouse embryos staged at E13.5.** **a**, We applied sci-RNA-
180 seq3 to profile 1.6M single cell transcriptomes from 103 individual E13.5 embryos, derived from 22 mutants
181 and four wildtype strains, in one experiment. **b**, Embeddings of pseudobulk RNA-seq profiles of MMCA
182 mouse embryos in PCA space with visualisation of top three PCs. Briefly, single cell data from individual
183 embryos of MMCA were aggregated to create 103 pseudobulk samples. Embryos are colored by
184 background strain. The black dotted circles highlight two major groups corresponding to FVB vs. other
185 backgrounds. Embryo #104 was a clear outlier. **c**, Embeddings of pseudobulk RNA-seq profiles of MOCA¹⁷
186 and MMCA mouse embryos in PCA space defined solely by MOCA, with MMCA embryos (gray) projected

187 onto it. The top two PCs are visualised. Colored points correspond to MOCA embryos of different stages
188 (E9.5-E13.5), and grey points to MMCA embryos (E13.5). Embryos #104 and #41 were labelled as outliers
189 and removed from the dataset, as discussed in the text. The dashed line (manually added) highlights five
190 MMCA embryos which are colocalized with E11.5 or E12.5 embryos from MOCA. Three are *Scn11a* GOF
191 (#33, #34, #36), one is *Carm1* KO (#101), and one is C57BL/6 wildtype (#41). **d**, The number of cells
192 profiled per embryo for each strain. The centre lines show the medians; the box limits indicate the 25th and
193 75th percentiles; the replicates are represented by the dots. **e**, 3D UMAP visualisation of wildtype subset
194 of MMCA dataset (215,575 cells from 15 wildtype E13.5 embryos). Cells are colored by major trajectory
195 annotation. **f**, Correlated developmental trajectories between MOCA¹⁷ and MMCA based on non-negative
196 least-squares (NNLS) regression (**Methods**). Shown here is a heat map of the combined regression
197 coefficients (row-scaled) between 10 developmental trajectories from MMCA (rows) and 10 corresponding
198 developmental trajectories from the MOCA (columns). PNS: peripheral nervous system.
199

200 Mutant-specific differences in cell type composition

201
202 Analogous to how there are many assays for phenotyping a mouse, there are many computational
203 strategies that one might adopt in order to investigate mutant-specific differences in these
204 embryo-scale sc-RNA-seq data. Here we pursued three main approaches: 1) quantification of
205 gross differences in cell type composition (this section); 2) investigation of more subtle differences
206 in the distribution of cell states within annotated trajectories and sub-trajectories; and 3) analysis
207 of the extent to which phenotypic features are shared between mutants.

208
209 To systematically assess cell type compositional differences, we first examined the proportions
210 of cells assigned to each of the 13 major trajectories across the 4 wildtype and 22 mutant strains.
211 For the most part, these proportions were consistent across genotypes (**Supplementary Fig. 5a**).
212 However, some mutants exhibited substantial differences. For example, compared to the
213 C57BL/6 wildtype, the proportion of cells falling in the neural tube trajectory decreased from 37.3%
214 to 33.7% and 32.6% in the *Gli2* KO and *Ttc21b* KO mice, respectively, while the proportion of
215 cells falling in the mesenchymal trajectory decreased from 44.1% to 37.1% in the *Gorab* KO mice.
216 These changes are broadly consistent with the gross phenotypes associated with these
217 mutations^{28,33,40,41}, but are caveated by substantial interindividual heterogeneity within each
218 genotype (**Supplementary Fig. 5b**). Also of note, we observe differences in major trajectory
219 composition between the four wildtype strains. For example, relative to BALB/C and C57BL/6, the
220 FVB and G4 wildtype mice consistently had substantially lower proportions of cells in the
221 mesenchymal trajectory and higher proportions of cells in the neural tube trajectory
222 (**Supplementary Fig. 5c**).

223
224 To increase resolution, we sought to investigate compositional differences at the level of sub-
225 trajectories. For each combination of background (C57BL/6, FVB, G4) and sub-trajectory (n = 54),
226 we performed a regression analysis to identify instances where a particular mutation was
227 nominally predictive of the proportion of cells falling in that sub-trajectory (uncorrected p -value <
228 0.05; beta-binomial regression; **Methods**). Across the 22 mutants, this analysis highlighted 300
229 nominally significant changes (**Fig. 2a**; **Supplementary Table 3**). Due to the limited number of
230 replicate embryos per wildtype and mutant strain, our power to detect changes is limited,
231 particularly in the smaller trajectories. Nevertheless, several patterns were clear:

232
233 First, it is evident that *Atp6v0a2* KO and *Atp6v0a2* R755Q, two distinct mutants of the same gene³⁴,
234 are assigned very similar patterns by this analysis, both with respect to which sub-trajectories are
235 nominally significant as well as the direction and magnitude of changes (first two rows of **Fig. 2a**).
236 Although perhaps expected, the consistency supports the validity of this analytical approach.

237
238 Second, the mutants varied considerably with respect to the number of sub-trajectories that were
239 nominally significant for compositional differences. At the higher extreme, the proportions of cells
240 falling in 30 of 54 sub-trajectories were nominally altered by the *Sox9* regulatory INV mutation,
241 consistent with the wide-ranging roles of *Sox9* in development^{42,43}. On the other hand, other
242 mutants, such as the TAD boundary knockouts, exhibited comparatively few changes, consistent
243 with the paucity of gross phenotypes in such mutants¹⁶. Nonetheless, all TAD boundary knockouts

244 did show some changes, including specific ones, e.g. the lung epithelial and liver hepatocyte
245 trajectories were decreased in the *Dmrt1* and *Tbx3* TAD boundary KOs, respectively, but not in
246 other TAD boundary knockouts. At the lower extreme, the *Sim1* TAD boundary KO exhibited just
247 two altered sub-trajectories.

248

249 Third, some sub-trajectories exhibited altered proportions in many mutants (e.g. the
250 mesencephalon/MHB trajectory in 12 mutants) while others were changed only in a few (e.g. the
251 definitive erythroid trajectory in *Ror2* KI only). In some cases, such patterns were “block-like” by
252 background strain (e.g. all B6 mutants exhibited gains in endothelial cells and losses in
253 endocardium). Although particular sub-trajectories might be vulnerable to disruption in a strain-
254 specific way, it is also possible that this is a technical artefact (e.g. if the four wildtype replicates
255 that we profiled for a given strain were atypical).

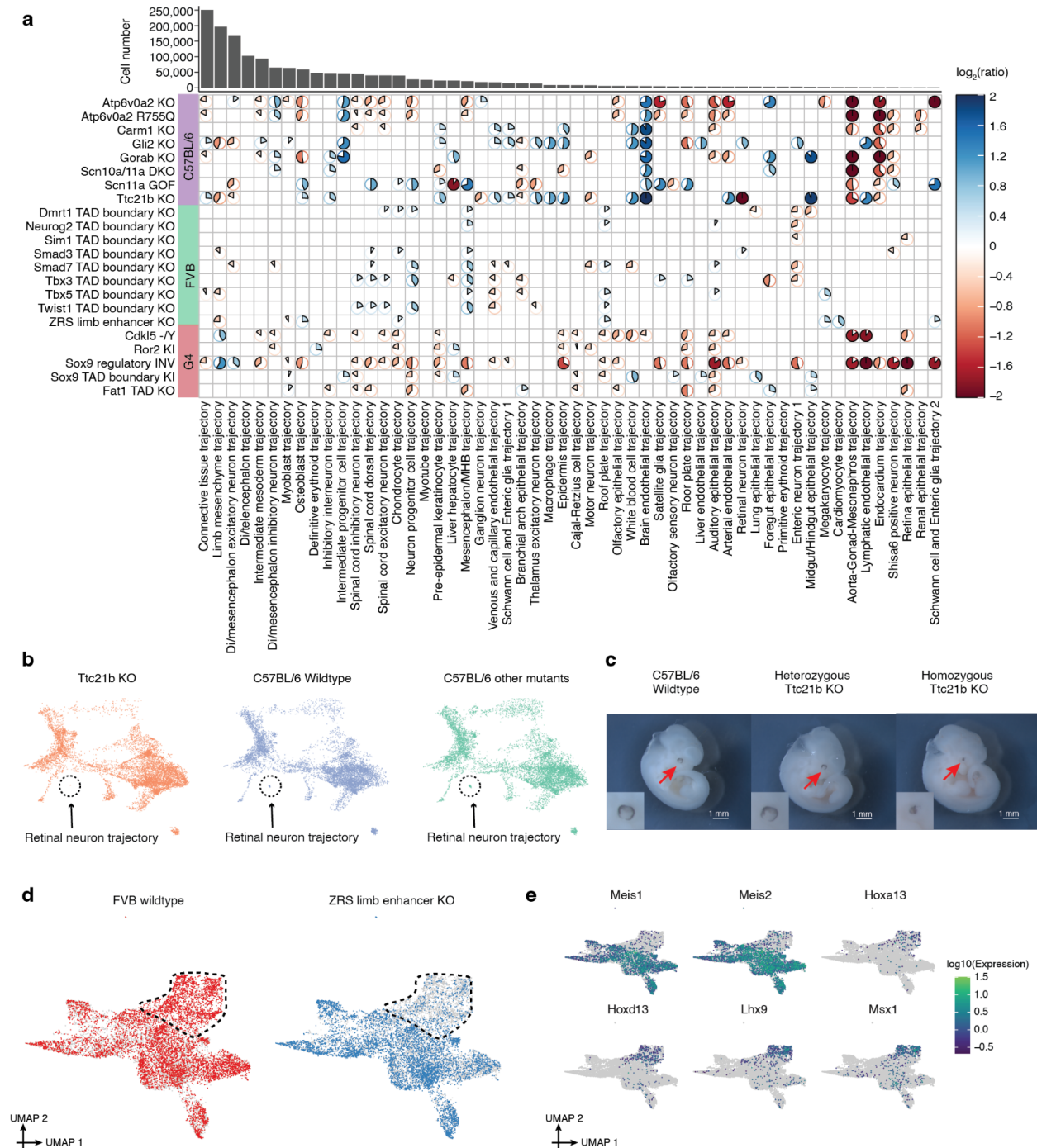
256

257 There were a few extreme examples, e.g. where a sub-trajectory appeared to be fully lost in a
258 specific mutant. For example, *Ttc21b*, which encodes a ciliary protein and whose knockout is
259 associated with brain, bone and eye phenotypes^{28,44,45}, exhibited a dramatic reduction in the
260 proportion of cells in the retinal neuron trajectory ($\log_2(\text{ratio}) = -6.69$; unadjusted p -value = 0.028;
261 beta-binomial regression) (**Fig. 2b**), as well as the lens ($\log_2(\text{ratio}) = -2.64$) and retina epithelium
262 ($\log_2(\text{ratio}) = -2.32$) trajectories (**Supplementary Fig. 6**). Validating this finding, the developing
263 eye appears diminished in the homozygous *Ttc21b* mutant at E11.5 embryos compared to the
264 wildtype or heterozygous mutant (**Fig. 2c**).

265

266 However, most changes were relatively subtle. For example, the ZRS limb enhancer KO is a well-
267 studied mutant which shows a loss of the distal limb structure at birth⁴⁶. This analytical framework
268 highlighted eight sub-trajectories whose proportions were nominally altered in the ZRS limb
269 enhancer KO, most of which were mesenchymal. However, although the most extreme, the
270 reduction in limb mesenchymal cells was only about 30% ($\log_2(\text{ratio}) = -0.49$; unadjusted p -value
271 = $6.32e-3$; beta-binomial regression). To assess whether further subpopulations of the limb
272 mesenchyme were more substantially changed, we performed co-embedding of limb
273 mesenchyme cells from the ZRS limb enhancer KO and the FVB wildtype. Indeed, a
274 subpopulation of the limb mesenchyme was much more markedly affected (**Fig. 2d**;
275 **Supplementary Fig. 7a**), and this subpopulation specifically expressed markers of the distal
276 mesenchyme of the early embryonic limb bud, such as *Hoxa13* and *Hoxd13* (**Fig. 2e**)⁴⁷. Of note,
277 we did not observe such heterogeneity when we examined the seven other sub-trajectories whose
278 proportions were nominally altered in the ZRS limb enhancer KO (**Supplementary Fig. 7b**),
279 consistent with the specificity of this phenotype.

280



281
282

283 **Figure 2. Cell composition changes for individual mutants across developmental trajectories.** **a**,
284 Heatmap shows log₂ transformed ratios of the cell proportions between each mutant type (y-axis) and its
285 corresponding wildtype background, across individual sub-trajectories (x-axis). Sub-trajectories with a
286 mean number of cells across individual embryos of less than ten were excluded from this analysis, leaving
287 54 (columns). Only those combinations of mutant and sub-trajectory which were nominally significant in the
288 regression analysis are shown (see text and **Methods**; uncorrected *p*-value < 0.05; beta-binomial
289 regression). For calculating the displayed ratios, cell counts from replicates were merged. The pie color and
290 direction correspond to whether the log₂ transformed ratio is above 0 (blue, clockwise) or below 0 (red,

291 anticlockwise), while the pie size and colour intensity correspond to the scale of log2 transformed ratio. A
292 handful of log2 transformed ratios with > 2 (or < -2) were manually set to 2 (or -2) for a better visualisation.
293 The number of cells assigned to each developmental trajectory in the overall dataset is shown above the
294 heatmap. **b**, 3D UMAP visualisation of the neural tube trajectory, highlighting cells from either the *Ttc21b*
295 KO (left), C57BL/6 wildtype (middle), or other mutants on the C57BL/6 background (right). The three plots
296 were randomly downsampled to the same number of cells ($n = 8,749$ cells). **c**, Homozygous *Ttc21b* KO
297 mice embryo (E11.5) showed abnormal eye development. **d**, UMAP visualisation of co-embedded cells of
298 limb mesenchyme trajectory from the ZRS limb enhancer KO and FVB wildtype. The same UMAP is shown
299 twice for both, highlighting cells from either FVB wildtype (left) or ZRS limb enhancer KO (right). The subset
300 of cells in this co-embedding exhibiting more extreme loss in the ZRS limb enhancer KO is highlighted. **e**,
301 The same UMAP as in panel **d**, colored by gene expression of marker genes which appear specific to
302 proximal limb development (*Meis1*, *Meis2*)^{48,49} and distal limb development (*Hoxa13*, *Hoxd13*, *Lhx9*,
303 *Msx1*)^{47,50,51}. Gene expression was calculated from original UMI counts normalised to size factor per cell,
304 followed by 10-log transformation. PNS: peripheral nervous system. MHB: midbrain-hindbrain boundary.
305 Di: Diencephalon.
306

307 LochNESS analysis reveals differences in transcriptional state within cell type trajectories

308

309 Given that most of the mutants that we studied did not exhibit macroscopic anatomical defects or
310 otherwise severe phenotypes at E13.5, we next sought to develop a more sensitive approach for
311 detecting deviations in transcriptional programs within cell type trajectories. Specifically, we
312 developed “lochNESS” (local cellular heuristic Neighbourhood Enrichment Specificity Score),
313 score that is calculated based on the “neighbourhood” of each cell in a sub-trajectory co-
314 embedding of a given mutant (all replicates) vs. a pooled wildtype (all replicates of all backgrounds)
315 (**Fig. 3a; Methods**; although developed independently, this approach is similar to recent work by
316 Dann and colleagues⁵²). Briefly, we took the aligned PC features of each sub-trajectory, as
317 described above, and found k-NNs for each cell, excluding cells from the same mutant replicate
318 from consideration. For each mutant cell, we then computed the ratio of the observed vs. expected
319 number of mutant cells in its neighbourhood, with expectation simply based on the overall ratio of
320 mutant vs. wildtype cells in co-embedding. In the scenario where mutant and wildtype cells are
321 fully mixed, the resulting ratio should be close to 1. The final lochNESS was defined as the ratio
322 minus 1, equivalent to the fold change of mutant cell composition.

323

324 Visualisation of lochNESS in the embedded space highlights areas with enrichment or depletion
325 of mutant cells. For example, returning to the previously discussed ZRS limb enhancer KO mice,
326 we observed markedly low lochNESS in a portion of the limb mesenchymal trajectory
327 corresponding to the distal limb (**Fig. 3b; Fig. 2d**). This highlights the value of the lochNESS
328 framework, as within the sub-trajectory (limb mesenchyme), an effect could be detected and also
329 assigned to a subset of cells in a label-agnostic fashion.

330

331 Plotting the global distributions of lochNESS for each mutant across all sub-trajectories, we further
332 observed that some mutants (e.g. most TAD boundary knockouts; *Scn11a* GOF) exhibit
333 unremarkable distributions (**Fig. 3c**). However, others (e.g. *Sox9* regulatory INV; *Scn10a/11a*
334 DKO) are associated with a marked excess of high lochNESS, consistent with mutant-specific
335 effects on transcriptional state across many developmental systems. Of note, we confirmed that
336 repeating the calculation of lochNESS after random permutation of mutant and wildtype labels
337 resulted in bell-shaped distributions centred around zero (**Supplementary Fig. 8a**). As such, the
338 deviance of lochNESS can be summarised as the average euclidean distance between lochNESS
339 vs lochNESS under permutation (**Supplementary Fig. 8b**).

340

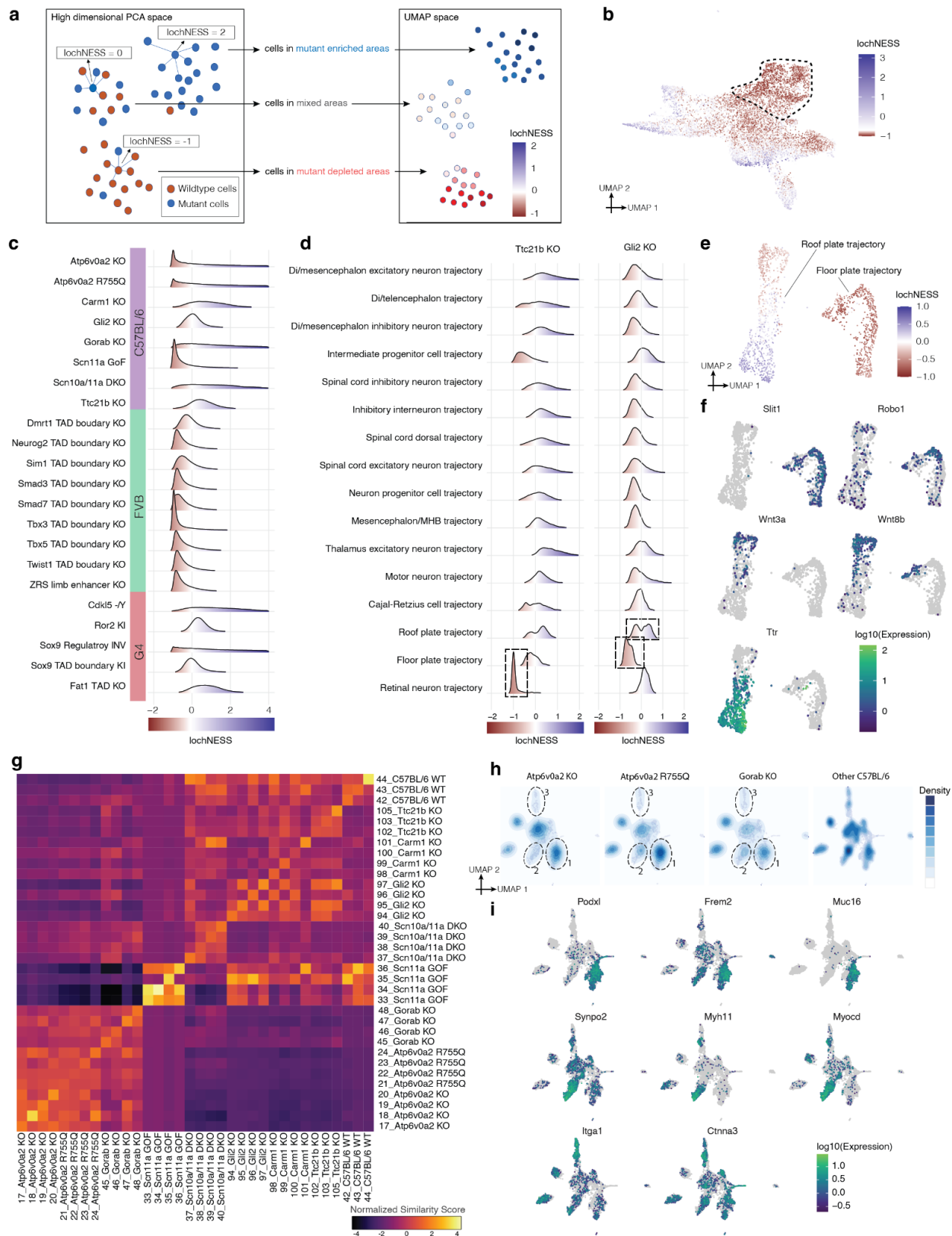
341 We next examined lochNESS within each mutant of each sub-trajectory to identify system-specific
342 phenotypes. For example, consistent with results shown above, we observed low lochNESS
343 within the retinal neuron sub-trajectory in the *Ttc21b* KO (**Fig. 3d; Supplementary Fig. 8c**). We
344 also observed a strong shift towards low scores for the floor plate sub-trajectory in the *Gli2* KO,
345 and interestingly, a more subtle change in lochNESS distribution for the roof plate trajectory,
346 which is forming opposite to the floor plate along the D-V axis of the developing neural tube (**Fig.**
347 **3d; Supplementary Fig. 8c**). To explore this further, we extracted and reanalyzed cells
348 corresponding to the floor plate and roof plate. Within the floor plate, *Gli2* KO cells consistently
349 exhibited low lochNESS (**Fig. 3e**). However, there were only a handful of differentially expressed
350 genes between wildtype and mutant cells, and no significantly enriched pathways within that set.

351 For example, genes like *Robo1* and *Slit1*, both involved in neuronal axon guidance, are
352 specifically expressed in the floor plate relative to the roof plate (**Fig. 3f; Supplementary Fig. 8g**),
353 but are not differentially expressed between wildtype and *Gli2* KO cells of the floor plate.
354 Alternatively, our failure to detect substantial differential expression may be due to power, as there
355 were fewer floor plate cells in the *Gli2* KO (~60% reduction). Overall, these observations are
356 consistent with the established role of *Gli2* in floor plate induction and the previous demonstration
357 that *Gli2* knockouts fail to induce a floor plate (Matise et al. 1998; Ding et al. 1998).

358
359 Less expectedly, this focused analysis also revealed two subpopulations of roof plate cells, one
360 depleted and the other enriched for *Gli2* KO cells (**Fig. 3e; Supplementary Fig. 8d-f**). To annotate
361 these subpopulations, we examined genes whose expression was predictive of lochNESS via
362 regression (**Methods**). The mutant-enriched group of roof plate cells was marked by *Ttr*, a marker
363 for choroid plexus and dorsal roof plate development⁵³, as well as genes associated with the
364 development of cilia (e.g. *Cdc20b*, *Gmnc*, *Dnah6* and *Cfap43*), while the mutant-depleted group
365 was marked by Wnt signaling-related genes including *Rspo1/2/3* and *Wnt3a/8b/9a* (**Fig. 3f;**
366 **Supplementary Fig. 8g; Supplementary Table 3**)⁵⁴⁻⁵⁷. It has been shown that ventrally-
367 expressed *Gli2* plays a central role in dorsal-ventral patterning of the neural tube by antagonising
368 Wnt/Bmp signalling from the dorsally-located roof plate⁵⁸. Our results are consistent with this, and
369 also define two subpopulations of roof plate cells on which *Gli2* KO appears to have differential
370 effects. Of note, the relatively subtle and opposing effects on these roof plate subpopulations
371 were missed by our original analysis of cell type proportions, and only uncovered by the
372 granularity of the lochNESS strategy.

373
374 LochNESS distributions can be systematically screened to identify sub-trajectories exhibiting
375 substantial mutant-specific shifts. For example, while all TAD boundary KO mutants have similarly
376 unremarkable global lochNESS distributions, when we plot these distributions by sub-trajectory,
377 a handful of shifted distributions are evident (**Supplementary Fig. 9a**). Such deviations,
378 summarised as the average euclidean distances between lochNESS and lochNESS under
379 permutation, are visualised in **Supplementary Fig. 9b**. For example, multiple epithelial sub-
380 trajectories, including pre-epidermal keratinocyte, epidermis, branchial arch, and lung epithelial
381 trajectories, are most shifted in *Tbx3* TAD boundary KO cells. Co-embeddings of mutant and
382 wildtype cells of these sub-trajectories, together with regression analysis, identify multiple keratin
383 genes as positively correlated with lochNESS, consistent with a role for *Tbx3* in epidermal
384 development (**Supplementary Fig. 9c-d; Supplementary Table 4**)^{59,60}. The lung epithelial cells
385 were separated into two clusters, with the cluster more depleted in *Tbx3* TAD boundary KO cells
386 marked by *Etv5*, a transcription factor associated with alveolar type II cell development, as well
387 as *Bmp* signalling genes that regulate *Tbx3* during lung development (*Bmp1/4*), and distal airway
388 markers *Sox9* and *Id2*⁶⁰⁻⁶².

389



390
391

392 **Figure 3. LochNESS analysis identifies mutant related changes.** **a**, Schematic of lochNESS calculation
393 and visualisation. **b**, UMAP visualisation of co-embedded cells of limb mesenchyme trajectory from the ZRS
394 limb enhancer KO and FVB wildtype, colored by lochNESS, with colour scale centred at the median of
395 lochNESS. The subset of cells in this co-embedding that corresponds to the area exhibiting more extreme
396 loss in the ZRS limb enhancer KO cells in Fig. 2d is highlighted (dashed circle). **c**, Distribution of lochNESS
397 across all 64 sub-trajectories in each mutant. **d**, Distribution of lochNESS in the neural tube sub-trajectories
398 of the *Ttc21b* KO and *Gli2* KO mutants. Dashed boxes highlight the shifted distributions of the retinal neuron
399 sub-trajectory of the *Ttc21b* KO mutant and the floor plate and roof plate sub-trajectories of the *Gli2* KO
400 mutant. **e**, UMAP visualisation of co-embedded cells of the floor plate and roof plate sub-trajectories from
401 the *Gli2* KO mutant and pooled wildtype, colored by lochNESS. **f**, same as in panel **e**, but colored by
402 expression of selected marker genes. **g**, Heatmap showing similarity scores between individual C57BL/6
403 embryos in the mesenchymal trajectory. Rows and columns are grouped by genotype and labelled by
404 embryo id and genotype. **h**, UMAPs showing the co-embedding of the intermediate mesoderm sub-
405 trajectory for mutants from the C57BL/6 background strain, with cell density and distributions overlaid.
406 Dashed circles highlight three clusters of cells where *Atp6v0a2* KO, *Atp6v0a2* R755Q and *Gorab* KO mice
407 exhibit enrichment (cluster 1) or depletion (clusters 2 & 3), compared to other mutants in the C57BL/6
408 background strain. **i**, same as in panel **h**, but colored by expression of marker genes of the clusters
409 highlighted in panel **h**.
410

411 Identification of mutant-specific and mutant-shared effects

412

413 Pleiotropy, wherein a single gene influences multiple, unrelated traits, is a pervasive phenomenon
414 in developmental genetics, and yet remains poorly understood⁶³. A corollary of pleiotropy is that
415 there are also specific traits that appear to be influenced by multiple, unrelated genes. For the
416 most part, the characterization of the sharing of phenotypic features between multiple Mendelian
417 disorders has remained coarse. For example, many disorders share macrocephaly as a feature,
418 but it remains largely unexplored whether the molecular and cellular basis for macrocephaly is
419 shared between them, unique to each, or somewhere in between.

420

421 Although here we have “whole embryo” molecular profiling of just 22 mutants, we sought to
422 investigate whether we could distinguish between mutant-specific and mutant-shared effects
423 within each major trajectory. In brief, within a co-embedding of cells from all embryos from a given
424 background strain, we computed k-NNs as in **Fig. 3a**, and then calculated the observed vs.
425 expected ratio of each genotype among a cell’s k-NNs. The “similarity score” between one
426 genotype vs. all others is defined as the mean of these ratios across cells of the genotype. To
427 assess whether any observed similarities or dissimilarities are robust, we can also calculate
428 similarity scores between individual embryos. For example, for the mesenchymal trajectory of
429 C57BL/6 mutants, similarity scores are generally higher for pairwise comparisons of individuals
430 with the same genotype (**Fig. 3g; Supplementary Fig. 10a-b**).

431

432 The *Scn11a* GOF mutant exhibited the most extreme similarity scores, in terms of both similarity
433 between replicates and dissimilarity with other genotypes (**Fig. 3g; Supplementary Fig. 10a**).
434 The *Scn11a* GOF mutant carries a missense mutation in the *Scn11a* locus which is reported to
435 result in reduced pain sensitivity both in mice and men without obvious signs of
436 neurodegeneration, suggesting altered electrical activity of peripheral pain-sensing neurons and
437 impaired synaptic transmission to postsynaptic neurons (Leipold et al. 2013). However, at least
438 grossly, the mutant does not seem to be associated with mesenchymal phenotypes. Noting that
439 the *Scn11a* GOF mutant embryos clustered with E12.5 embryos instead of E13.5 embryos in our
440 pseudobulk analysis (**Fig. 1c**), we speculated that its extreme similarity scores might be
441 attributable to developmental delay of the *Scn11a* GOF mutant at the scale of the whole embryo.
442 To investigate this further, we co-embedded *Scn11a* GOF mutant cells with pooled wildtype cells
443 and MOCA cells from the neural tube trajectory. While wildtype cells were distributed near E13.5 cells
444 from MOCA, the *Scn11a* GOF cells were embedded closer to cells from earlier developmental
445 timepoints (**Supplementary Fig. 10d**). As a more systematic approach, we calculated a “time score”
446 for each cell from the MMCA dataset by taking the k-NNs of each MMCA cell in the MOCA dataset
447 and calculating the average of the developmental time of the MOCA cells. The relative time score
448 distributions of *Scn11a* GOF cells and wildtype cells suggest that *Scn11a* GOF cells are significantly
449 delayed in all major trajectories examined (single sided student’s t-test, raw p-value < 0.01;
450 **Supplementary Fig. 10e**). As such, the apparently unique signature of *Scn11a* GOF cells might be
451 attributable to these embryos simply being earlier in development, suggesting a more global role for
452 sodium ion channels not only for neuronal function but also early development and cell fate
453 determination⁶⁴. Incorrect staging is formally possible, but unlikely because the embryos derived from
454 three independent litters.

455
456 In sharp contrast with the relative uniqueness of the *Scn11a* GOF mutant, we also observed that
457 the similarity scores between three mutants -- *Atp6v0a2* KO, *Atp6v0a2* R755Q and *Gorab* KO --
458 was consistent with shared effects, in the mesenchymal, epithelial, endothelial, hepatocyte and
459 neural crest (PNS glia) trajectories in particular; in other main trajectories, such as neural tube
460 and hematopoiesis, *Atp6v0a2* KO and *Atp6v0a2* R755Q exhibited high similarity scores with one
461 another, but not with *Gorab* KO (**Fig. 3g; Supplementary Fig. 10a,c,f**). Such sharing is perhaps
462 expected between the *Atp6v0a2* KO and *Atp6v0a2* R755Q mutants, as they involve the same
463 gene. In human patients, mutations in *ATP6V0A2* and *GORAB* cause overlapping connective
464 tissue disorders, which is reflected in the misregulation of the mesenchymal trajectory of *Atp6v0a2*
465 and *Gorab* mutants³⁴⁻³³. However, only the *ATP6V0A2*-related disorder displays a prominent CNS
466 phenotype, consistent with the changes in the neural tube trajectory seen only in both *Atp6v0a2*
467 models (**Supplementary Fig. 10a,c,f**).

468
469 In order to explore phenotypic sharing between these genotypically distinct mutants at greater
470 granularity, we co-embedded cells of the intermediate mesoderm sub-trajectory from C57BL/6
471 strains. We identified three subclusters of intermediate mesoderm where *Atp6v0a2* KO, *Atp6v0a2*
472 R755Q and *Gorab* KO mice are similarly distributed compared to other C57BL/6 genotypes (**Fig.**
473 **3h,i**). In particular, cluster 1 is enriched for cells from *Atp6v0a2* KO, *Atp6v0a2* R755Q and *Gorab*
474 KO mice and is marked by genes related to epithelial-to-mesenchymal transition, cell-cell
475 adhesion and migration, such as *Podxl*, *Frem2* and *Muc16*⁶⁵⁻⁶⁷. Clusters 2 and 3 are depleted in
476 cells from *Atp6v0a2* KO, *Atp6v0a2* R755Q and *Gorab* KO mice and are marked by muscular
477 development related genes like *Synpo2*, *Myh11* and *Myocd* (cluster 2), and cell-cell adhesion
478 related genes like *Itga1* and *Ctnna3* (cluster 3)⁶⁸⁻⁷².

479
480 Altogether, these analyses illustrate how the joint analysis of mutants subjected to whole embryo
481 sc-RNA-seq has the potential to reveal sharing of molecular and cellular phenotypes. This
482 includes global similarity (e.g. *Atp6v0a2* KO vs. *Atp6v0a2* R755Q) as well as instances in which
483 specific aspects of phenotypes are shared between previously unrelated mutants (e.g. *Atp6v0a2*
484 mutants vs. *Gorab* KO).

485 486 Global developmental defects in *Sox9* regulatory mutant

487
488 About half of the mutants profiled in this study model disruptions of regulatory, rather than coding,
489 sequences. Among these, the *Sox9* regulatory INV mutant stands out in having a dramatically
490 shifted lochNESS distribution, particularly in the mesenchymal trajectory (**Fig. 3c; Fig. 4a**). The
491 *Sox9* locus encodes a pleiotropic transcription factor that plays a central role during the
492 development of the skeleton, the brain, in sex determination as well as several other tissues
493 during embryogenesis, orchestrated by a complex regulatory landscape⁷³⁻⁸¹. This particular
494 mutant features an inversion of a 1Mb region upstream of *Sox9* that includes several distal
495 enhancers and a TAD boundary, essentially relocating these elements into a TAD with *Kcnj2*,
496 which encodes a potassium channel (**Fig. 4b**)^{27 82,83}. Consistent with the heterozygous and
497 homozygous *Sox9* knockout, the homozygous *Sox9* regulatory INV is perinatally lethal, with
498 extensive skeletal phenotypes including digit malformation, a cleft palate, bowing of bones and

499 delayed ossification. In addition to the loss of 50% of Sox9 expression, the inversion was
500 previously shown to lead to pronounced misexpression of *Kcnj2* in the digit anlagen in a wildtype
501 Sox9 pattern²⁷. However, the extent to which *Kcnj2* and Sox9 are mis-expressed elsewhere, as
502 well as the molecular and cellular correlates of the widespread skeletal phenotype, have yet to be
503 deeply investigated.

504
505 At the level of mesenchymal sub-trajectories, shifts in lochNESS distribution for Sox9 regulatory
506 INV were consistently observed, but the limb mesenchyme and connective tissue were
507 particularly enriched for cells with extremely high lochNESS (**Fig. 4a**, right). Of relevance, 2 of the
508 3 major enhancers (E250 and E195) known to drive Sox9-mediated chondrogenesis in
509 mesenchymal stem cells are located within the inverted region (**Fig. 4b**)⁷⁵. Cell type composition
510 analysis (**Fig. 2a**) showed that Sox9 regulatory INV mutants harbor considerably larger numbers
511 of cells classified as limb mesenchyme, at the expense of osteoblasts, intermediate mesoderm,
512 chondrocytes and connective tissue trajectory. This shift can also be seen in a UMAP embedding
513 (**Fig. 4c**), a topic that we revisit further below.

514
515 These changes in cell type composition were accompanied by reduced expression of Sox9 and
516 increased expression of *Kcnj2* in bone (aggregate of chondrocyte, osteoblast, limb mesenchyme;
517 **Supplementary Fig. 11a**), although the number of cells expressing *Kcnj2* was generally low. This
518 suggests that the Sox9 regulatory inversion is resulting in increased *Kcnj2* expression (via Sox9
519 enhancer adoption) and Sox9 reduction (via boundary repositioning) not only in the digit anlagen,
520 but in skeletal mesenchyme more generally. To validate this, we performed RNA *in situ*
521 hybridization (RNAscope) on sections of developing bones of the rib cage at E13.5, comparing a
522 heterozygous Sox9 regulatory INV mouse with a wildtype littermate. Consistent with our sc-RNA-
523 seq data derived from homozygous mutants, we observe a Sox9-patterned increase in *Kcnj2*
524 levels, together with losses in Sox9 expression, in the developing bone (**Fig. 4d**; **Supplementary**
525 **Fig. 11b**).

526
527 Since the inverted Sox9 regulatory region also hosts multiple enhancers active in other tissues
528 (e.g. E161-lung; E239-cerebral cortex)⁷⁵, we wondered whether these patterns were also seen in
529 other tissues. Indeed, both sc-RNA-seq expression analysis and RNAscope quantification show
530 increased *Kcnj2* levels in all other tissues examined. While reductions in Sox9 expression, clear
531 in bone, were not observed in most other tissues in our single cell data, RNAscope quantification
532 showed reductions in Sox9 expression in the telencephalon and lung as well (**Supplementary**
533 **Fig. 11**). Taken together, these data suggest marked changes in mesenchyme due to reductions
534 of Sox9 expression (presumably due to separation from key enhancers), together with broader
535 increases in *Kcnj2* expression (presumably due to the appropriation of Sox9 enhancers).

536
537 To explore the apparent effects of the Sox9 regulatory inversion on mesenchyme in more detail,
538 in particular the apparent accumulation of limb mesenchyme (**Fig. 4c**), we reanalyzed mutant and
539 wildtype cells from the limb mesenchyme sub-trajectory on their own, which revealed subsets
540 corresponding to condensing mesenchyme, perichondrium, and undifferentiated mesenchyme
541 (**Supplementary Fig. 12a,b**). This analysis further revealed that the vast majority of limb
542 mesenchyme “accumulation” in mutant embryos was due to a large proportion of cells that appear

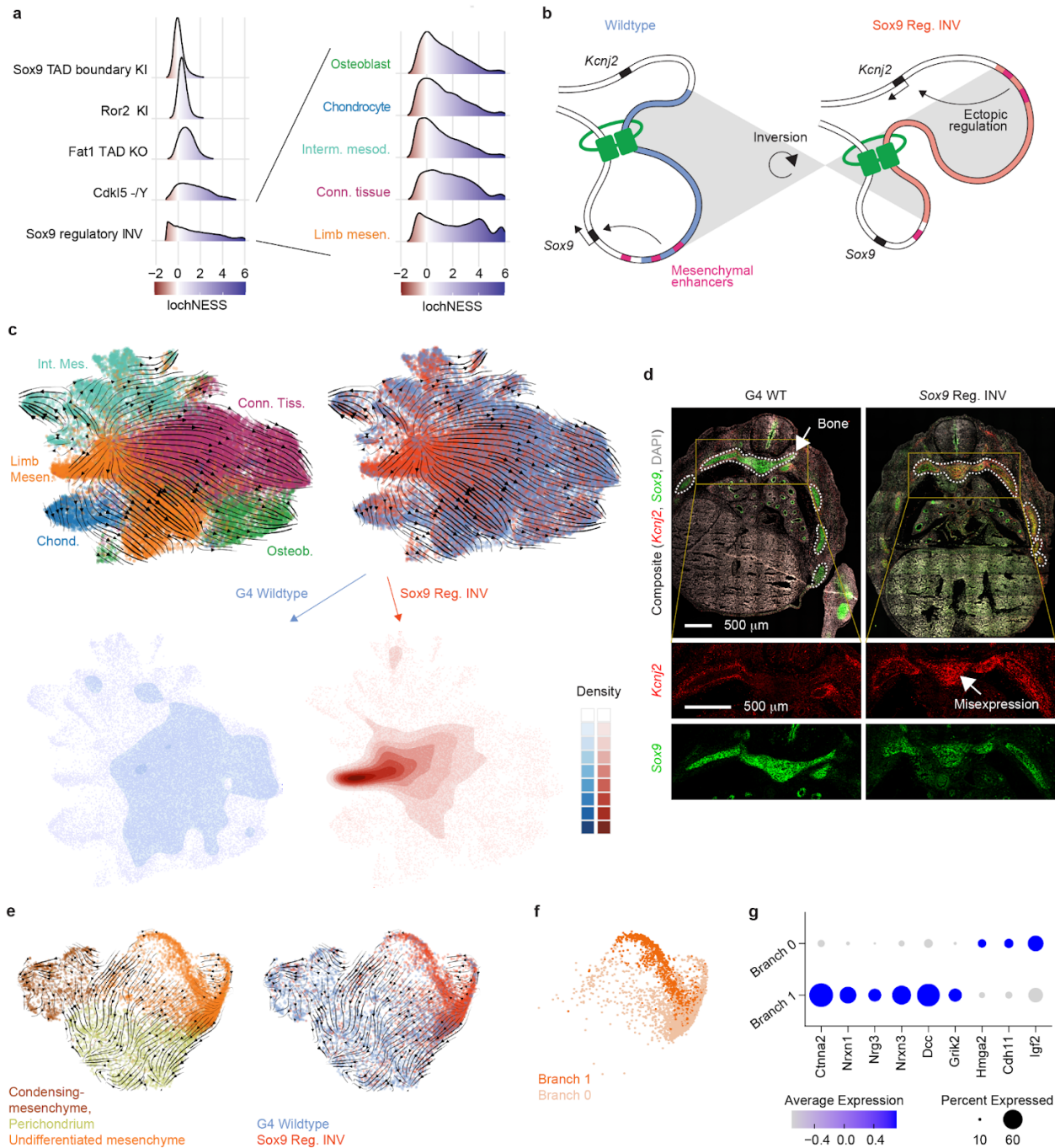
543 delayed or stalled in an undifferentiated or stem-like state, rather than an accumulation of more
544 advanced limb mesenchyme (**Fig. 4c**, bottom panels; **Supplementary Fig. 12a**). Of note,
545 because the annotation of “limb mesenchyme” for this sub-trajectory was propagated forward
546 from earlier stages of development during the creation of MOCA, we cannot rule out that other,
547 non-limb mesenchymal populations contribute to this expanded, undifferentiated pool in the Sox9
548 regulatory INV embryos as well.

549
550 Inspection of density plots and RNA velocity suggested that wildtype undifferentiated
551 mesenchymal cells (a subset of cells annotated as limb mesenchyme in **Fig. 4c**) are poised to
552 undergo differentiation into diverse subtypes (**Fig. 4c**; **Supplementary Fig. 12a**). In sharp
553 contrast, undifferentiated mesenchymal cells from Sox9 regulatory INV embryos accumulate at
554 the “source” of differentiation, and also appear to acquire a distinct state (high density region in
555 bottom right sub-panel of **Fig. 4c**). This accumulation is even more apparent in integrated views
556 of the limb mesenchyme sub-trajectory, where we observe two distinct branches, each heavily
557 enriched for Sox9 regulatory INV mutant cells, within undifferentiated mesenchyme (**Fig. 4e**;
558 **Supplementary Fig. 13a**).

559
560 To investigate these two branches further, we performed sub-clustering of Sox9 regulatory INV
561 undifferentiated mesenchyme cells, followed by differential expression analysis (**Fig. 4f,g**).
562 Interestingly, the most differentially expressed genes in “branch 1” were neuronal, *e.g.* several
563 neurexins and neuregulin 3, an observation that was supported by single-sample gene set
564 enrichment analysis (ssGSEA)⁸⁴, which further highlighted KRAS and other signalling pathways
565 (**Fig. 4g**; **Supplementary Fig. 13b,c**). Of note, mesenchymal stem cells can be differentiated to
566 neuronal states *in vitro*⁸⁵. Although further investigation is necessary, we note that cells
567 contributing to “branch 0” as well as the neuronal-trending “branch 1” are present in wildtype
568 embryos, albeit at much reduced frequencies compared to the Sox9 regulatory INV mutant
569 (**Supplementary Fig. 13a**, left).

570
571 In sum, consistent with what is known about the role of Sox9 as a driver gene in cartilage and
572 skeletal development, our data reveals a redirection in the differentiation of osteoblast,
573 chondrocytes and other derivatives of the undifferentiated mesenchyme in the Sox9 regulatory
574 INV mutant. Among mutants on the G4 background, the observed pattern is specific to the Sox9
575 regulatory INV mutant (**Supplementary Fig. 14**). Remarkably however, when we examine the
576 entire dataset (**Supplementary Fig. 14-16**), we observe a similar accumulation of undifferentiated
577 mesenchymal cells in the *Atp6v0a2* KO, *Atp6v0a2* R755Q, and *Gorab* KO mutants, indicating
578 sharing of this sub-phenotype amongst 4 of 22 mutants examined (**Supplementary Fig. 16-17**).
579 This observation further illustrates the potential for systematic, whole embryo analysis to reveal
580 sharing of molecular and cellular sub-phenotypes across pleiotropic developmental mutants in
581 unexpected ways.

582



583
584

585 **Figure 4. Apparent stalling and redirection of mesenchyme differentiation in the Sox9 regulatory**
586 **INV mutant. a**, LochNESS distributions for all G4 mutants in the mesenchymal trajectory (left) and the Sox9
587 regulatory INV mutant in mesenchymal sub-trajectories (right). **b**, Model of Sox9 regulatory INV mutation
588 depicting ectopic *Kcnj2* expression due to adoption of chondrogenesis and osteogenesis specific
589 enhancers. **c**, top: RNA velocity on UMAP embedding of mesenchymal G4 wildtype and Sox9 regulatory
590 INV cells labelled by annotation (left) or sample (right). bottom: 2D density plots of the same UMAP
591 embedding for G4 wildtype (left) and Sox9 regulatory INV cells (right). **d**, Sox9 regulatory INV heterozygous
592 mutant and littermate wildtype RNA scope images (red: *Kcnj2*; green: Sox9), with insets below highlighting
593 a region corresponding to developing bone (white circled area) **e**, RNA velocity on UMAP embedding of G4

594 wildtype and Sox9 regulatory INV cells in the limb mesenchymal trajectory labelled by annotation (left) or
595 sample (right). **f.** UMAP embedding of Sox9 regulatory INV cells in the undifferentiated mesenchyme,
596 visualised in the same embedding as in panel **e.** **g.** Dot plot of the top six (where available) significantly
597 differentially expressed genes between the two branches.
598

599 Discussion

600

601 In this study, we set out to establish whole embryo sc-RNA-seq as a new paradigm for the
602 systematic, scalable phenotyping of mouse developmental mutants. In one experiment, we
603 generated ~1.6M single cell transcriptomes from just over 100 E13.5 embryos corresponding to
604 22 mutant genotypes and 4 wildtype strains. To investigate the resulting dataset, we developed
605 analytical approaches to identify deviations in cell type composition, subtle differences in gene
606 expression within cell types (“lochNESS”), and sharing of sub-phenotypes between mutants
607 (“similarity scores”). We also evaluated how a range of gross phenotypic severities manifest at
608 the molecular and cellular levels, and show how global analysis can in some cases reveal
609 molecular and cellular phenotypes that may be missed by conventional phenotyping. Such “*in*
610 *silico* developmental biology”, wherein global profiles of developmental mutants are subjected to
611 systematic, outcome-agnostic computational analyses, may complement and guide conventional
612 phenotyping, which can be impractical to scale to all physiological systems even for a single
613 mutant.

614

615 We emphasise that the concurrent analysis of many mutants proved essential to the
616 contextualization of particular observations, *i.e.* to understand how specific or non-specific any
617 apparent deviation really was, against a background of dozens of genotypes and over 100
618 embryos. This aspect of the study also enabled us to discover shared aspects of phenotypes
619 between previously unrelated genotypes, *e.g.* between *Gorab* and *Atp6v0a2* mutants. Looking
620 forward, profiling of additional mouse mutants might enable the further “decomposition” of
621 developmental pleiotropy, a poorly understood phenomenon, into “basis vectors” (*e.g.* the stalling
622 of undifferentiated mesenchyme in 4 of 22 mutants examined).

623

624 Our mouse mutant cell atlas (MMCA) has limitations. First, we only profiled 4 replicates per mutant
625 at a single developmental time point. We can't exclude that some subtle effects were missed that
626 might have been captured through profiling of a larger number of replicate embryos. Second, we
627 profiled only ~15,000 cells per embryo, which is only a small fraction of the millions of cells that
628 are present in E13.5 embryos, which may also have limited sensitivity. A counterweight to these
629 limitations is that for any given mutant, we had over 1.5M cells from other genotypes (wildtype or
630 other mutants), which facilitated the detection of mutant-specific phenotypes for even rare cell
631 types, *e.g.* in the retina (*Ttc21b* KO) and roof plate (*Gli2* KO).

632

633 Third, although we performed more detailed *in silico* analyses of selected mutants and
634 phenotypes, we were not able to explore all mutants in detail, nor to thoroughly investigate other
635 aspects of the data (*e.g.* the differences between wildtype strains). Even for these 22 mutants,
636 but also looking to the future, we anticipate the community input and domain expertise will be
637 essential to extract full value from these data, including the development of additional analytical
638 strategies. To facilitate this, we created an interactive browser that allows exploration of mutant-
639 specific effects on gene expression in trajectories and sub-trajectories, together with the
640 underlying data (https://atlas.gs.washington.edu/mmca_v2/).

641

642 In 2011, the International Mouse Phenotyping Consortium (IMPC) set out to drive towards the
643 “functionalization” of every protein-coding gene in the mouse, by generating thousands of
644 knockout mouse lines⁹². Although over 7,000 lines have already been analysed, thousands more
645 still await phenotyping, and even what phenotyping has been done is not necessarily
646 comprehensive⁹³. In principle, the whole embryo sc-RNA-seq phenotyping approach presented
647 here could be extended to all Mendelian genes or even to all 20,000 mouse gene KOs, to advance
648 our understanding of the molecular and cellular basis of human developmental disorders, to
649 decompose pleiotropy, and to shed light on the function(s) of mammalian genes.
650

651 **Methods**

652

653 **Data reporting**

654

655 No statistical methods were used to predetermine sample size. Embryos used in experiments
656 were randomised before sample preparation. Investigators were blinded to group allocation during
657 data collection and analysis. Embryo collection and sci-RNA-seq3 analysis were performed by
658 different researchers in different locations.

659

660 **Embryo collection**

661

662 Mutants were generated through conventional gene editing tools and breeding or tetraploid
663 aggregation and collected at the embryonic stage E13.5, calculated from the day of vaginal plug
664 (noon = E0.5). Collection and whole embryo dissection was performed as previously described⁹⁴.
665 The embryos were immediately snap-frozen in liquid nitrogen and shipped to the Shendure Lab
666 (University of Washington) in dry ice. Sets of animals with the same genotype were either all male
667 or half male-half female. All animal procedures were in accordance with institutional, state, and
668 government regulations.

669

670 **Nuclei isolation and fixation**

671

672 Snap frozen embryos were processed as previously described¹⁷. Briefly, the frozen embryos were
673 cut into small pieces with a blade and further dissected by resuspension in 1 ml ice cold cell lysis
674 buffer (CLB, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% IGEPAL CA-630, 1%
675 SUPERase In and 1% BSA) in a 6 cm dish. adding another 3ml CLB, the sample was strained
676 (40 µm) into a 15 ml Falcon tube and centrifuged to a pellet (500g, 5 min). Resuspending the
677 sample with another 1 ml CLB, the isolation of nuclei was ensured. Pelleting the isolated nuclei
678 again (500g, 5 min) was followed by a washing step by fixation in 10 ml 4% Paraformaldehyde
679 (PFA) for 15 minutes on ice. The fixed nuclei were pelleted (500g, 3 min) and washed twice in the
680 nuclei suspension buffer (NSB) (500g, 5 min). The nuclei finally were resuspended in 500µl NSB
681 and split into 2 tubes, each containing 250 µl sample. The tubes were flash frozen in liquid nitrogen
682 and stored in a -80°C freezer, until further use for library preparation. The embryo preparation
683 was preceded randomly for nuclei isolation in order to avoid batch effects.

684

685 **sci-RNA-seq3 library preparation and sequencing**

686

687 The library preparation was performed previously described^{17,95}. In short, the fixed nuclei were
688 permeabilized, sonicated and washed. Nuclei from each mouse embryo were then distributed into
689 several individual wells into 4 96-well plates. We split samples into four batches (~25 samples
690 randomly selected in each batch) for sci-RNA-seq3 processing. The ID of the reverse transcription
691 well was linked to the respective embryo for downstream analysis. In a first step the nuclei were
692 then mixed with oligo-dT primers and dNTP mix, denatured and placed on ice, afterwards they
693 were proceeded for reverse transcription including a gradient incubation step. After reverse
694 transcription, the nuclei from all wells were pooled with the nuclei dilution buffer (10 mM Tris-HCl,

695 pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1% SUPERase In and 1% BSA), spun down and redistributed
696 into 96-well plates containing the reaction mix for ligation. The ligation proceeded for 10 min at
697 25°C. Afterwards, nuclei again were pooled with nuclei suspension buffer, spun down and washed
698 and filtered. Next, the nuclei were counted and redistributed for second strand synthesis, which
699 was carried out at 16°C for 3h. Afterwards tagmentation mix was added to each well and
700 tagmentation was carried out for 5 minutes at 55°C. To stop the reaction, DNA binding buffer was
701 added and the sample was incubated for another 5 minutes. Following an elution step using
702 AMPure XP beads and elution mix, the samples were subjected to PCR amplification to generate
703 sequencing libraries.

704

705 Finally after PCR amplification, the resulting amplicons were pooled and purified using AMPure
706 XP beads. The library was analysed by electrophoresis and the concentration was calculated
707 using Qubit (Invitrogen). The library was sequenced on the NovaSeq platform (Illumina) (read 1:
708 34 cycles, read 2: 100 cycles, index 1: 10 cycles, index 2: 10 cycles).

709

710 Processing of sequencing reads

711

712 Read alignment and cell-x-gene expression count matrix generation was performed based on the
713 pipeline that we developed for sci-RNA-seq³¹⁷ with the following minor modifications: base calls
714 were converted to fastq format using Illumina's *bcl2fastq/v2.20* and demultiplexed based on PCR
715 i5 and i7 barcodes using maximum likelihood demultiplexing package *deML*⁹⁶ with default settings.
716 Downstream sequence processing and cell-x-gene expression count matrix generation were
717 similar to sci-RNA-seq⁹⁷ except that the RT index was combined with hairpin adaptor index, and
718 thus the mapped reads were split into constituent cellular indices by demultiplexing reads using
719 both the the RT index and ligation index (Levenshtein edit distance (ED) < 2, including insertions
720 and deletions). Briefly, demultiplexed reads were filtered based on the RT index and ligation index
721 (ED < 2, including insertions and deletions) and adaptor-clipped using *trim_galore/v0.6.5* with
722 default settings. Trimmed reads were mapped to the mouse reference genome (mm10), using
723 *STAR/v2.6.1d*⁹⁸ with default settings and gene annotations (GENCODE VM12 for mouse).
724 Uniquely mapping reads were extracted, and duplicates were removed using the unique
725 molecular identifier (UMI) sequence (ED < 2, including insertions and deletions), reverse
726 transcription (RT) index, hairpin ligation adaptor index and read 2 end-coordinate (*i.e.* reads with
727 UMI sequence less than 2 edit distance, RT index, ligation adaptor index and tagmentation site
728 were considered duplicates). Finally, mapped reads were split into constituent cellular indices by
729 further demultiplexing reads using the RT index and ligation hairpin (ED < 2, including insertions
730 and deletions). To generate the cell-x-gene expression count matrix, we calculated the number
731 of strand-specific UMIs for each cell mapping to the exonic and intronic regions of each gene with
732 *python/v2.7.13 HTseq* package⁹⁹. For multi-mapped reads, reads were assigned to the closest
733 gene, except in cases where another intersected gene fell within 100 bp to the end of the closest
734 gene, in which case the read was discarded. For most analyses, we included both expected-
735 strand intronic and exonic UMIs in the cell-x-gene expression count matrix.

736

737 The single cell gene count matrix included 1,941,605 cells after cells with low quality (UMI <= 250
738 or detected gene <= 100) were filtered out. Each cell was assigned to its original mouse embryo

739 on the basis of the reverse transcription barcode. We applied three strategies to detect potential
740 doublet cells. As the first strategy, we split the dataset into subsets for each individual, and then
741 applied the *scrublet/v0.1* pipeline¹⁰⁰ to each subset with parameters (min_count = 3, min_cells =
742 3, vscore_percentile = 85, n_pc = 30, expected_doublet_rate = 0.06, sim_doublet_ratio = 2,
743 n_neighbors = 30, scaling_method = 'log') for doublet score calculation. Cells with doublet scores
744 over 0.2 were annotated as detected doublets (5.5% in the whole data set).

745
746 As the second strategy, we used an iterative clustering strategy based on *Seurat/v3*¹⁰¹ to detect
747 the doublet-derived subclusters for cells. Briefly, gene count mapping to sex chromosomes was
748 removed before clustering and dimensionality reduction, and then genes with no count were
749 filtered out and each cell was normalized by the total UMI count per cell. The top 1,000 genes
750 with the highest variance were selected. The data was log transformed after adding a pseudo
751 count, and scaled to unit variance and zero mean. The dimensionality of the data was reduced
752 by PCA (30 components) first and then with UMAP, followed by Louvain clustering performed on
753 the 10 principal components (resolution = 1.2). For Louvain clustering, we first fitted the top 10
754 PCs to compute a neighbourhood graph of observations (k.param = 50) followed by clustering the
755 cells into sub-groups using the Louvain algorithm. For UMAP visualisation, we directly fit the PCA
756 matrix with min_distance = 0.1. For subcluster identification, we selected cells in each major cell
757 type and applied PCA, UMAP, Louvain clustering similarly to the major cluster analysis.
758 Subclusters with a detected doublet ratio (by *Scrublet*) over 15% were annotated as doublet-
759 derived subclusters.

760
761 We found the above *Scrublet* and iterative clustering-based approach is limited in marking cell
762 doublets between abundant cell clusters and rare cell clusters (e.g. less than 1% of the total cell
763 population), thus, we applied a third strategy to further detect such doublet cells. Briefly, cells
764 labeled as doublets (by *Scrublet*) or from doublet-derived subclusters were filtered out. For each
765 cell, we only retain protein-coding genes, lincRNA genes, and pseudogenes. Genes expressed
766 in less than 10 cells and cells expressing less than 100 genes were further filtered out. The
767 downstream dimension reduction and clustering analysis were done with *Monocle/v3*¹⁷. The
768 dimensionality of the data was reduced by PCA (50 components) first on the top 5,000 most highly
769 variable genes and then with UMAP (max_components = 2, n_neighbors = 50, min_dist = 0.1,
770 metric = 'cosine'). Cell clusters were identified using the Leiden algorithm implemented in
771 *Monocle/v3* (resolution = 1e-06). Next, we took the cell clusters identified by *Monocle/v3* and first
772 computed differentially expressed genes across cell clusters with the *top_markers* function of
773 *Monocle/v3* (reference_cells=1000). We then selected a gene set combining the top ten gene
774 markers for each cell cluster (filtering out genes with fraction_expressing < 0.1 and then ordering
775 by pseudo_R2). Cells from each main cell cluster were selected for dimension reduction by PCA
776 (10 components) first on the selected gene set of top cluster-specific gene markers, and then by
777 UMAP (max_components = 2, n_neighbors = 50, min_dist = 0.1, metric = 'cosine'), followed by
778 clustering identification using the Leiden algorithm implemented in *Monocle/v3* (resolution = 1e-
779 04). Subclusters showing low expression of target cell cluster-specific markers and enriched
780 expression of non-target cell cluster-specific markers were annotated as doublets derived
781 subclusters and filtered out in visualisation and downstream analysis. Finally, after removing the

782 potential doublet cells detected by either of the above three strategies, 1,671,270 cells were
783 retained for further analyses.

784

785 Whole mouse embryo analysis

786

787 As described previously¹⁷, each cell could be assigned to the mouse embryo from which it derived
788 on the basis of its reverse transcription barcode. After removing doublet cells and another 25 cells
789 which were poorly assigned to any mouse embryo, 1,671,245 cells from 103 individual mouse
790 embryos were retained (a median of 13,468 cells per embryo). UMI counts mapping to each
791 sample were aggregated to generate a pseudobulk RNA-seq profile for each sample. Each cell's
792 counts were normalised by dividing its estimated size factor, and then the data were log2-
793 transformed after adding a pseudocount followed by performing the PCA. The normalisation and
794 dimension reduction were done in *Monocle/v3*.

795

796 We previously used sci-RNA-seq3 to generate the MOCA dataset, which profiled ~2 million cells
797 derived from 61 wild-type B6 mouse embryos staged between stages E9.5 and E13.5. The
798 cleaned dataset, including 1,331,984 high quality cells, was generated by removing cells with
799 <400 detected UMIs as well as doublets (<http://atlas.gs.washington.edu/mouse-rna>). UMI counts
800 mapping to each sample were aggregated to generate a pseudobulk RNA-seq profile for each
801 embryo. Each cell's counts were normalised by dividing its estimated size factor, and then the
802 data were log2-transformed after adding a pseudocount, followed by PCA. The PCA space was
803 retained and then the embryos from the MMCA dataset were projected onto it.

804

805 Cell clustering and annotation

806

807 After removing doublet cells, genes expressed in less than 10 cells and cells expressing less than
808 100 genes were further filtered out. We also filtered out low-quality cells based on the proportion
809 of reads mapping to the mitochondrial genome (MT%) or ribosomal genome (Ribo%) (specifically,
810 filtering cells with MT% > 10 or Ribo% > 5). We then removed cells from two embryos that were
811 identified as outliers based on the whole-mouse embryo analysis (embryo 41 and embryo 104).
812 This left 1,627,857 cells (median UMI count 845; median genes detected 539) from 101 individual
813 embryos that were retained for all subsequent analyses.

814

815 To eliminate the potential heterogeneity between samples due to different mutant types and
816 genotype backgrounds, we sought to perform the dimensionality reduction on a subset of cells
817 from the wildtype mice (including 15 embryos with 215,575 cells, 13.2% of all cells) followed by
818 projecting all remaining cells, derived from the various mutant embryos, onto this same
819 embedding. These procedures were done using *Monocle/v3*. In brief, the dimensionality of the
820 subset of data from the wildtype mice was reduced by PCA, retaining 50 components, and all
821 remaining cells were projected onto that PCA embedding space. Next, to mitigate potential
822 technical biases, we combined all cells from wildtype and mutant mice and applied the *align_cds*
823 function implemented in *Monocle/v3*, with MT%, Ribo%, and log-transformed total UMI of each
824 cell as covariates. We took the subset of cells from wildtype mice, using their "aligned" PC features
825 to perform UMAP (max_components = 3, n_neighbors = 50, min_dist = 0.01, metric = 'cosine') by

826 *uwot/v0.1.8*, followed by saving the UMAP space. Cell clusters were identified using the Louvain
827 algorithm implemented in *Monocle/v3* on three dimensions of UMAP features, resulting in 13
828 isolated major trajectories (**Fig. 1e**). We then projected all of the remaining cells from mutant
829 mouse embryos onto the previously saved UMAP space and predicted their major-trajectory
830 labels using a *k*-nearest neighbour (*k*-NN) heuristic. Specifically, for each mutant-derived cell, we
831 identified its 15 nearest neighbour wildtype-derived cells in UMAP space and then assigned the
832 major trajectory with the maximum frequency within that set of 15 neighbours as the annotation
833 of the mutant cell. We calculated the ratio of the maximum frequency to the total as the assigned
834 score. Of note, over 99.9% of the cells from the mutant mice had an assigned score greater than
835 0.8. The cell-type annotation for each major trajectory was based on expression of the known
836 marker genes (**Supplementary Table 2**).

837
838 Within each major trajectory, we repeated a similar strategy, but with slightly adjusted PCA and
839 UMAP parameters. For the major trajectories with more than 50,000 cells, we reduced the
840 dimensionality by PCA to 50 principal components; for the other major trajectories of more than
841 1,000 cells, we reduced the dimensionality by PCA to 30 principal components; for the remaining
842 major trajectories, we reduced the dimensionality by PCA to 10 principal components. UMAP was
843 performing with `max_components = 3`, `n_neighbors = 15`, `min_dist = 0.1`, `metric = 'cosine'`. For
844 the mesenchymal trajectory, we observed a significant separation of cells by their cell-cycle phase
845 in the UMAP embedding. We calculated a *g2m* index and a *s* index for individual cells by
846 aggregating the log-transformed normalised expression for marker genes of the G2M phase and
847 the S phase and then included them in *align_cds* function along with the other factors. Applying
848 these procedures to all of the main trajectories, we identified 64 sub trajectories in total. Similarly,
849 after assigning each cell from the mutant mice with a sub-trajectory label, we calculated the ratio
850 of the maximum frequency to the total as the assigned score. Of note, over 96.7% of the cells
851 from the mutant mice had an assigned score greater than 0.8. The cell-type annotation for each
852 sub-trajectory was also based on the expression of known marker genes (**Supplementary Table
853 2**).

854
855 Identification of inter-datasets correlated major and sub trajectories using non-negative least-
856 squares (NNLS) regression

857
858 To identify correlated cell trajectories between MOCA and MMCA datasets, we first calculated an
859 aggregate expression value for each gene in each cell trajectory by summing the log-transformed
860 normalised UMI counts of all cells of that trajectory. For consistency during the comparison to
861 MOCA, we manually regrouped the cells from the MMCA dataset into 10 cell trajectories, by
862 merging the olfactory sensory neuron trajectory into the neural crest (PNS neuron) trajectory,
863 merging the myotube trajectory, the myoblast trajectory, and the cardiomyocyte trajectory into the
864 mesenchymal trajectory, splitting the hepatocyte trajectory into the lens epithelial trajectory and
865 the liver hepatocyte trajectory. Next, for the two datasets, we applied non-negative least squares
866 (NNLS) regression to predict gene expression in a target trajectory (T_a) in dataset A based on the
867 gene expression of all trajectories (M_b) in dataset B: $T_a = \beta_{0a} + \beta_{1a}M_b$, based on the union of the
868 3,000 most highly expressed genes and 3,000 most highly specific genes in the target trajectory.
869 We then switched the roles of datasets A and B, *i.e.* predicting the gene expression of target

870 trajectory (T_b) in dataset B from the gene expression of all trajectories (M_a) in dataset A: $T_b = \beta_{0b}$
871 $+ \beta_{1b}M_a$. Finally, for each trajectory a in dataset A and each trajectory b in dataset B, we combined
872 the two correlation coefficients: $\beta = 2(\beta_{ab} + 0.001)(\beta_{ba} + 0.001)$ to obtain a statistic, where high
873 values reflect reciprocal, specific predictivity. We repeated this analysis on sub-trajectories within
874 each major trajectories.

875

876 Identification of significant cell composition changes in mutant mice using beta-binomial
877 regression

878

879 A cell number matrix of all 64 developmental sub-trajectories (*rows*) and 101 embryos (*columns*)
880 was created and the cell number were then normalised by the size factor of each column which
881 was estimated by *estimate_size_factors* function in *Monocle/v3*. 10 sub-trajectories with a mean
882 of cell number across individual embryo < 10 were filtered out. The beta-binomial regression was
883 performed using the *VGAM* package of *R*, based on the model “(trajectory specific cell number,
884 total cell number of that embryo - trajectory specific cell number) \sim genotype”. Of note, embryos
885 from the four different mouse strain backgrounds were analysed independently.

886

887 Defining and calculating lochNESS

888

889 To identify local enrichments or depletions of mutant cells, we aim to define a metric for each
890 single cell to quantify the enrichments or depletions of mutant cells in its surrounding
891 neighbourhood. For these analyses, we consider a mutant and a pooled wildtype combining all 4
892 background strains in a main trajectory as a dataset. For each dataset, we define “lochNESS” as:

$$893 \text{lochNESS} = \frac{\# \text{ of mutant cells in } k\text{NNs}}{k} / \frac{\# \text{ of mutant cells in dataset}}{N} - 1$$

894 Where N is the total number of cells in the dataset, $k = \frac{\sqrt{N}}{2}$ scales with N and the cells from the
895 same embryo as the cell are excluded from the k -NNs. Note that this value is equivalent to the
896 fold change of mutant cell percentage in the neighbourhood of a cell relative to in the whole main
897 trajectory. For implementation, we took the aligned PCs in each sub-trajectory as calculated
898 above and for each cell in an embryo we find the k -NNs in the remaining mutant embryo cells and
899 wildtype cells. We plot the lochNESS in a red-white-blue scale, where white corresponds to 0 or
900 the median lochNESS, blue corresponds to high lochNESS or enrichments, and red corresponds
901 to low lochNESS or depletions. For reference, we simultaneously create a null distribution of
902 lochNESS using random permutation of the mutant and wildtype cell labels, simulating datasets
903 in which the cells are randomly mixed.

904

905 Identifying lochNESS associated gene expression changes

906

907 To identify gene expression changes associated with mutant enriched or depleted areas, we find
908 differentially expressed genes through fitting a regression model for each gene accounting for
909 lochNESS. We use the *fit_models()* function implemented in *monocle/v3* with lochNESS as the
910 *model_formula_str*. This essentially fits a generalized linear model for each gene: $\log(y_i) = \beta_0 +$
911 $\beta_n * x_n$, where y_i is the gene expression of $gene_i$, β_n captures the effect of the lochNESS x_n on
912 expression of $gene_i$ and β_0 is the intercept. For each $gene_i$, we test if β_i is significantly different

913 from zero using a Wald test and after testing all genes, we adjust the p-values using the Benjamini
914 and Hochberg procedure to account for multiple hypotheses testing. We identify the genes that
915 have adjusted p-value<0.05 and large positive β_i values as associated with mutant enriched
916 areas, and those with large negative β_i values as associated with mutant depleted areas.

917

918 Calculating mutant and embryo similarity scores

919

920 We can extend the lochNESS analysis, which is computed on each mutant and its corresponding
921 wildtype mice, to compute “similarity scores” between all pairs of individual embryos from the
922 same background strain. We consider all embryos in the same background in a main trajectory
923 as a dataset. For each dataset, we take define a “similarity score” between $cell_n$ and $embryo_j$ as:

$$924 \text{ similarity score}_{cell_n, embryo_j} = \frac{\# \text{ of cells from embryo}_i \text{ in kNNs of } cell_n}{k} / \frac{\# \text{ of cells from embryo}_j \text{ in dataset}}{N}$$

925 Where N is the total number of cells in the dataset and $k = \frac{\sqrt{N}}{2}$. We take the mean of the similarity
926 scores across all cells in the same embryo, resulting in an embryo similarity score matrix where
927 entries are:

$$928 \text{ similarity score}_{embryo_i, embryo_j} = \frac{1}{n_i} \sum_{n=1}^{n_i} \text{ similarity score}_{cell_n, embryo_j}$$

929 Where n_i is the number of cells in $embryo_i$. The embryo similarity score matrix can be visualised
930 in a square heatmap where rows and/or columns are hierarchically clustered.

931

932 Identifying and quantifying developmental delay

933

934 To identify potential mutant related developmental delay, we integrate MMCA with MOCA. We
935 consider a mutant and its corresponding wildtype in a sub trajectory as a dataset. We take the
936 cells from E11.5-E13.5 with similar annotations from MOCA and co-embed with the MMCA cells.
937 We take the raw counts from both datasets, normalise, and process the data together without
938 explicit batch correction as both datasets were generated with sci-RNA-seq3 and were similar in
939 dataset quality. We visualise the co-embedded data in 3D UMAP space and check for
940 developmental delay in the mutant cells (*i.e.* mutant cells embedded closer to early MOCA cells
941 compared to wildtype cells). To quantify the amount of developmental delay, we find k-NNs in

942 MOCA for each cell in MMCA and calculate $time\ score = \frac{\sum_{n=1}^k T_n}{k}$, where T_n is the developmental
943 time of MOCA $cell_n$ in the k-NNs of the MMCA cell. Afterwards, we test if the average time scores
944 of mutant cells are significantly different from that of wildtype cells using a student’s t-test.

945

946 RNAscope *in situ* Hybridization

947

948 For RNAscope, embryos were collected at stage E13.5 and fixed for 4 hours in 4% PFA/PBS at
949 room temperature. The embryos were washed twice in PBS before incubation in a sucrose series
950 (5%, 10% and finally 15% sucrose (Roth) /PBS) each for an hour or until the embryos sank to the
951 bottom of the tube. Finally, the embryos were incubated in 15% sucrose/PBS and O.C.T. (Sakura)
952 in a 1:1 solution before embedding the embryos in O.C.T in a chilled ethanol bath and put into -
953 80°C for sectioning. The embryos were cut into 5 μ m thick sections on slides for RNAscope.

954

955 Simultaneous RNA *in situ* hybridization was performed using the RNAscope® technology
956 (Advanced Cell Diagnostics [ACD]) and the following probes specific for Mm-Kcnj2 (Cat. No.
957 476261, ACD) and Mm-Sox9 (Cat. No. 401051-C2, ACD) on five µm sections of the mouse
958 embryos. RNAscope probes were purchased by ACD and designed as described by Wang *et*
959 *al.*¹⁰². The RNAscope® assay was run on a HybEZ™II Hybridization System (Cat. No. 321720,
960 ACD) using the RNAscope® Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323100, ACD) and
961 the manufacturer's protocol for fixed-frozen tissue samples with target retrieval on a hotplate for
962 5 minutes. Fluorescent labelling of the RNAscope® probes was achieved by using OPAL 520 and
963 OPAL 570 dyes (Cat. No. FP1487001KT + Cat. No. FP1488001KT, Akoya Biosciences,
964 Marlborough, MA, USA) and stained sections were scanned at 25x magnification using a LSM
965 980 with Airyscan 2 (Carl Zeiss AG, Oberkochen, DE).

966

967 Image analysis

968

969 For quantitative analysis of the RNAscope images, representative fields of view for each stained
970 section were analysed using the image processing software Fiji¹⁰³. Each organ of interest mRNA
971 signal was counted in a defined area (1 x 1 mm²) with an n=6 per condition. Statistics were
972 calculated using student t-Test and evaluated (- p > 0,05 = non-significant, p < 0,05 - ≥ 0,01 = * ,
973 p < 0,01 - ≥ 0,001= ** - p < 0,001= ***).

974

975 Clustering and annotation limb mesenchyme trajectory

976

977 *Seurat/v4.0.6* was used for the analysis. Wildtype cells in the limb mesenchyme trajectory from
978 all wild-type mice (n = 15 mice, n = 25,211 cells) were used to first annotate the cells. The raw
979 counts were log-normalised after which PCA was performed with default parameters on top 2000
980 highly variable genes selected using the “vst” method. Nearest neighbours were computed on the
981 PCA space, with default parameters, except that all the principal components computed earlier
982 were used. Clustering was performed using the Louvain community detection algorithm with a
983 resolution of 0.1, resulting in three clusters. Positive marker genes for these clusters were
984 identified using the Wilcoxon Rank Sum test, where only the genes expressed in at least 20% of
985 the cells in either cell groups were considered. The clusters were annotated based on biologically
986 relevant markers (**Supplementary Fig. 12b**). The newly assigned cell annotations for the Limb
987 mesenchyme trajectory cells in the wildtype dataset were transferred to the corresponding cells
988 in the *Sox9* regulatory INV mutant using the *FindTransferAnchors* and *TransferData* functions
989 using default parameters, except that all the computed principal components were used. 92.3%
990 of the transferred annotations had a score (prediction.score.max) greater than or equal to 0.8.

991

992 Density visualization and RNA velocity analysis

993

994 Using *Seurat/v4.0.6*, the raw counts were log-normalised, and PCA was performed with default
995 parameters on top highly variable genes 2000 genes, selected using the “vst” method.
996 Dimensionality reduction was performed using PCA using default parameters, after which the
997 UMAP embedding was carried out on all computed PC components. Density plots were created
998 using the *stat_2d_density_filled* function in *ggplot2/v3.3.5*. For RNA velocity analysis using

999 *scVelo/v0.2.4*, the total, spliced, and unspliced count matrices, along with the UMAP embeddings
1000 were exported as an h5ad file using *anndata/v0.7.5.2* for R. The count matrices were filtered and
1001 normalised using *scv.pp.filter_and_normalize*, with *min_shared_counts=20* and
1002 *n_top_genes=2000*. Means and variances between 30 nearest neighbours were calculated in the
1003 PCA space (*n_pcs=50*, to be consistent with default value in *Seurat*). The velocities were
1004 calculated using default parameters and projected onto the UMAP embedding exported from
1005 *Seurat*.

1007 Single sample Gene Set Enrichment Analysis

1008
1009 Single-sample Gene Set Enrichment Analysis (ssGSEA) was applied to sc-RNA-seq data using
1010 the *escape* R-package⁸⁴. The *msigdb* and *getGeneSets* functions were used to fetch and filter
1011 the entire Hallmark (H, 50 sets) or the Signature Cell Type (C8, 700 sets) *Mus musculus* gene
1012 sets from the MSigDB^{104,105}. *enrichIt* with default parameters, except for using 10000 groups and
1013 variable number of cores, was performed on the *seurat*-object containing data corresponding to
1014 the undifferentiated mesenchyme cells from the *Sox9* regulatory INV mutant, after converting the
1015 feature names to gene symbols as necessitated by the *escape* package. The obtained enrichment
1016 scores for each gene set were compared between the two branches (**Fig. 4f**) using the two sample
1017 Wilcoxon test (*wilcox_test*) with default parameters and adjusted for multiple comparisons using
1018 Bonferroni correction.

1020 Data availability

1021
1022 The data generated in this study can be downloaded in raw and processed forms from the NCBI
1023 Gene Expression Omnibus under accession number GSE199308. Other intermediate data files,
1024 code and an interactive app to explore our dataset will be made freely available via
1025 https://atlas.gs.washington.edu/mmca_v2/.

1027 Code availability

1028
1029 All code will be made freely available through a public GitHub repository.

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1051

1052 **Author contributions**

1053

1054 J.C., M.S. and J.S. conceptualized, supervised and funded the project. D.R.B., W.C., A.D., D.E.D.,
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1056 embryos. J.C. and J.H. extracted and fixed the nuclei from embryos and performed the sci-RNA-
1057 seq experiment. S.U., R.B., R.H., N.Hans. and J.H. performed RNAscope experiment and image
1058 analysis. X.H., C.Q., J.H., V.S. and S.B. performed all computational analyses. C.M. created the
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1060 with data analysis and results interpretation. X.H., C.Q., J.H. and V.S. wrote the first draft of the
1061 manuscript, which was finalized together with J.C., M.S. and J.S. and input from all authors.

1062

1063 **Competing interests**

1064

1065 J.S. is a SAB member, consultant and/or co-founder of Cajal Neuroscience, Guardant Health,
1066 Maze Therapeutics, Camp4 Therapeutics, Phase Genomics, Adaptive Biotechnologies and Scale
1067 Biosciences.

1068

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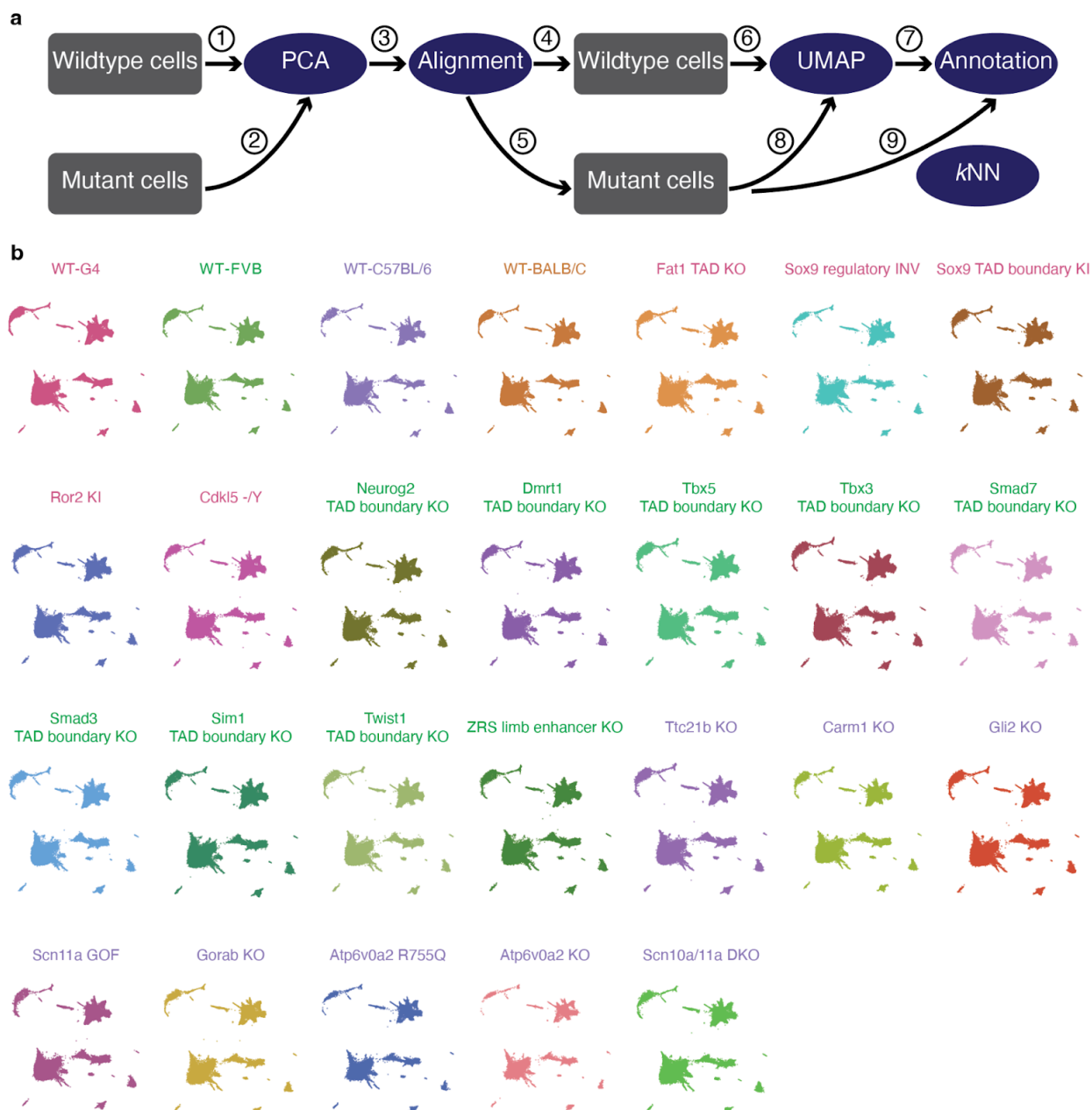
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|-------|----------------------|-------|-------------------------|---------|-----------------------|
| 1-4 | G4 WT strain | 37-40 | Scn10a/11a DKO | 74-77 | Dmrt1 TAD boundary KO |
| 5-8 | Ror2 KI | 41-44 | C57BL/6 WT strain | 78-81 | Tbx3 TAD boundary KO |
| 9-12 | Cdk15 -Y | 45-48 | Gorab KO | 82-85 | ZRS limb enhancer KO |
| 13-16 | Fat1 TAD KO | 50-53 | Smad3 TAD boundary KO | 86-89 | FVB WT strain |
| 17-20 | Atp6v0a2 KO | 54-57 | Twist1 TAD boundary KO | 90-93 | BALB/C WT strain |
| 21-24 | Atp6v0a2 R755Q | 58-61 | Tbx5 TAD boundary KO | 94-97 | Gli2 KO |
| 25-28 | Sox9 TAD boundary KI | 62-65 | Neurog2 TAD boundary KO | 98-101 | Carm1 KO |
| 29-32 | Sox9 regulatory INV | 66-69 | Sim1 TAD boundary KO | 102-105 | Ttc21b KO |
| 33-36 | Scn11a GOF | 70-73 | Smad7 TAD boundary KO | | |

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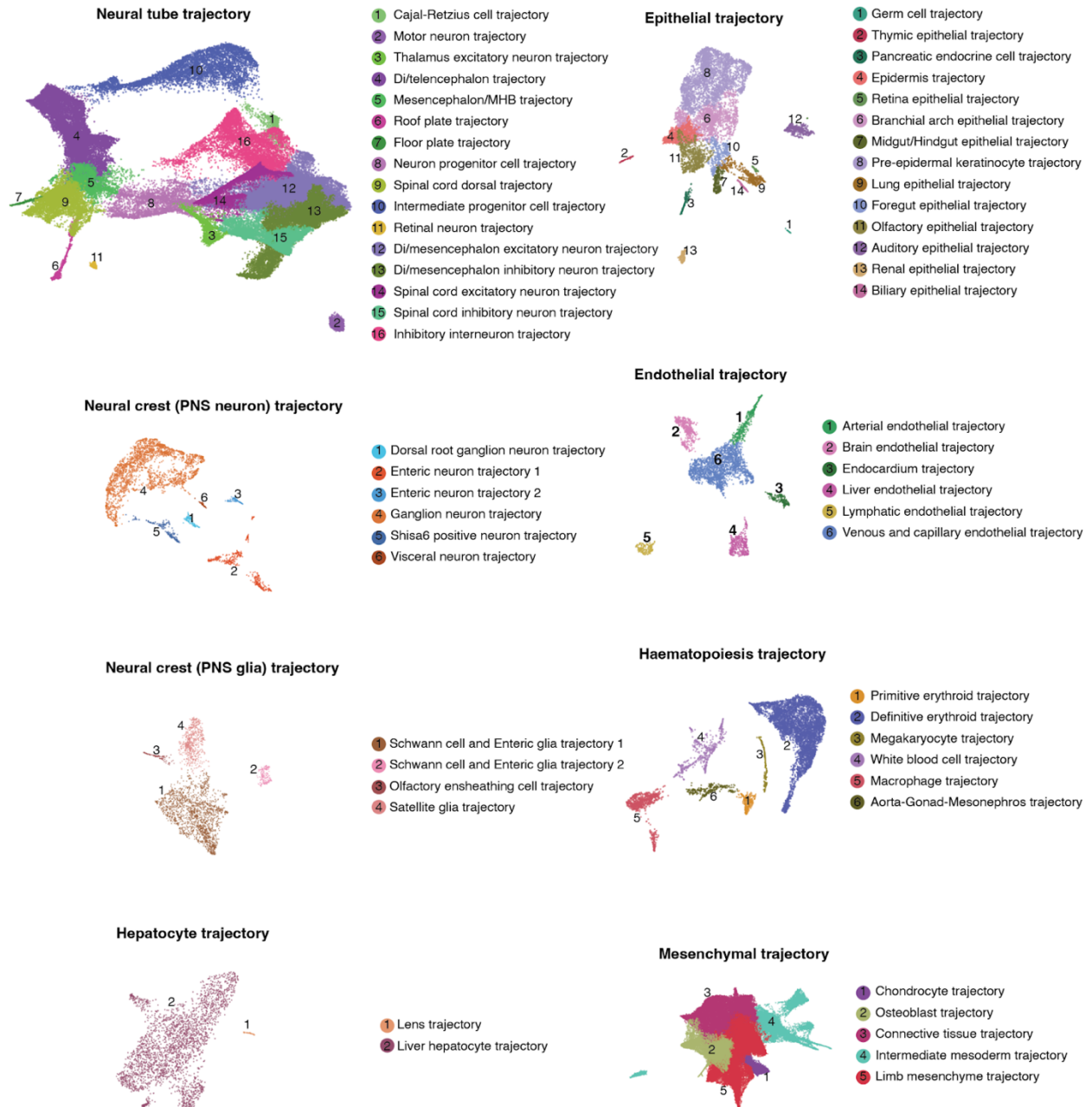
1281 **Supplementary Figure 1. Images of mouse embryos.** 104 embryos (26 genotypes x 4 replicates) were
 1282 staged at E13.5 and sent by five groups to a single site. #49 was accidentally skipped in our numbering
 1283 systems. Embryo #70 was lost in transport. Pictures of embryos #1, #5, #9, #13 and #91 were not taken,
 1284 but the embryos were included in the sci-RNA-seq3 experiment. As discussed in the text, embryos #41 and
 1285 #104 were labelled as outliers based on computational analyses and their data discarded, while data from
 1286 the remaining 101 embryos were retained and analysed further. Of note, in addition to the computational
 1287 analyses suggesting that embryo #104 was an outlier, it was also relatively small in size upon visualisation.
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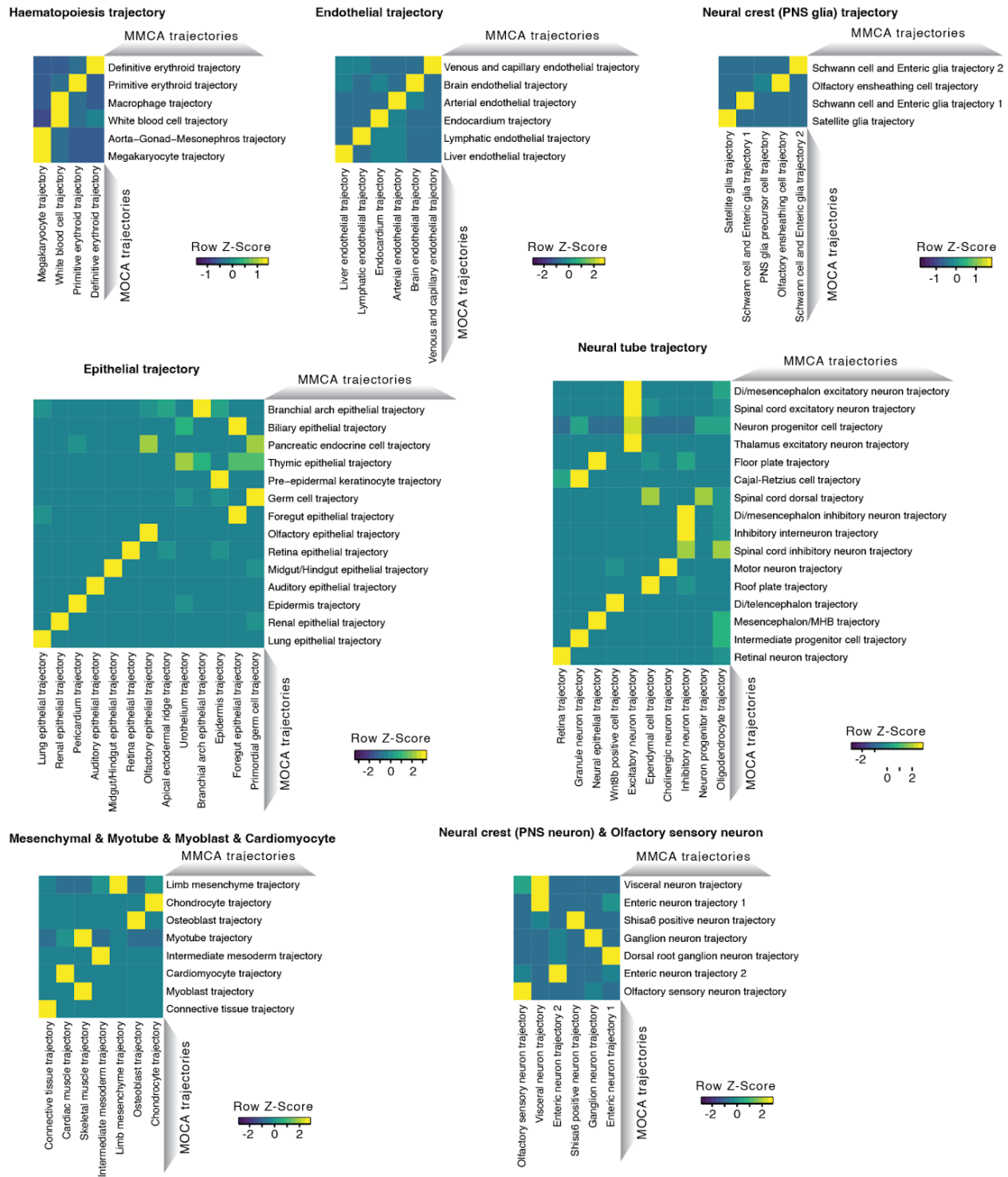
1291 **Supplementary Figure 2. Integrating cells derived from embryos of multiple genetic backgrounds**
 1292 **to a single, wildtype-based “reference embedding”.** **a**, Schematic of approach. We first applied principal
 1293 components-based dimensionality reduction to cells from wildtype genotypes only (①). We then projected
 1294 cells from the mutant embryos to this PCA embedding (②). Next, to mitigate potential biases from technical
 1295 factors, we applied the *align_cds* function in *Monocle/v3*, with the MT%, Ribo%, and log-transformed total
 1296 UMIs of each cell as covariates (③). We then split wildtype and mutant cells again (④ & ⑤), and applied
 1297 the UMAP algorithm to wildtype cells only using their “aligned” PC features (⑥), followed by Louvain
 1298 clustering and manual annotation of individual clusters based on marker gene expression to identify major
 1299 trajectories, and then iterative clustering and annotation to identity and annotate sub-trajectories (⑦).
 1300 Finally, cells from mutant embryos were projected to this wildtype-based UMAP embedding, again using
 1301 their aligned PC features (⑧). Major trajectory labels were assigned to mutant cells via a *k*-nearest

1302 neighbour (k -NN) heuristic, and these last steps were repeated to further assign sub-trajectory labels to
1303 mutant cells (9). **b**, 3D UMAP visualisations of cells from each wildtype or mutant background within the
1304 shared “reference embedding” resulting from the aforescribed procedures.
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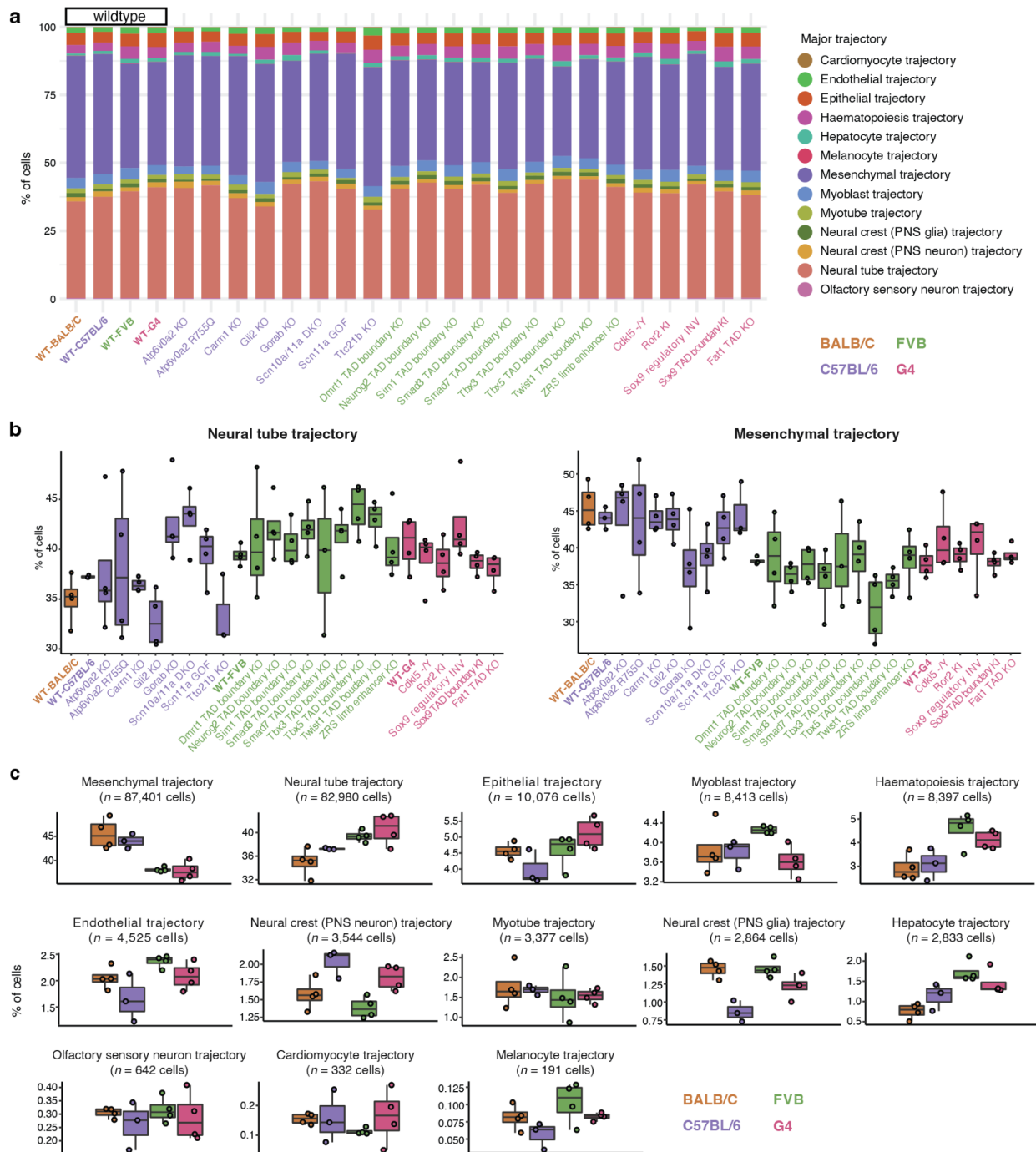
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1308 **Supplementary Figure 3. Annotation of sub-trajectories in data from wildtype E13.5 embryos.** From
1309 215,517 single cell profiles of wildtype E13.5 embryos of four strains in MMCA, we annotated 13 major
1310 trajectories. For 8 of these 13 major trajectories, iterative analysis identified the additional sub-trajectories
1311 shown here as 3D UMAP visualisations. Cells are colored by sub-trajectory annotations. PNS: peripheral
1312 nervous system. MHB: midbrain-hindbrain boundary. Di: Diencephalon.
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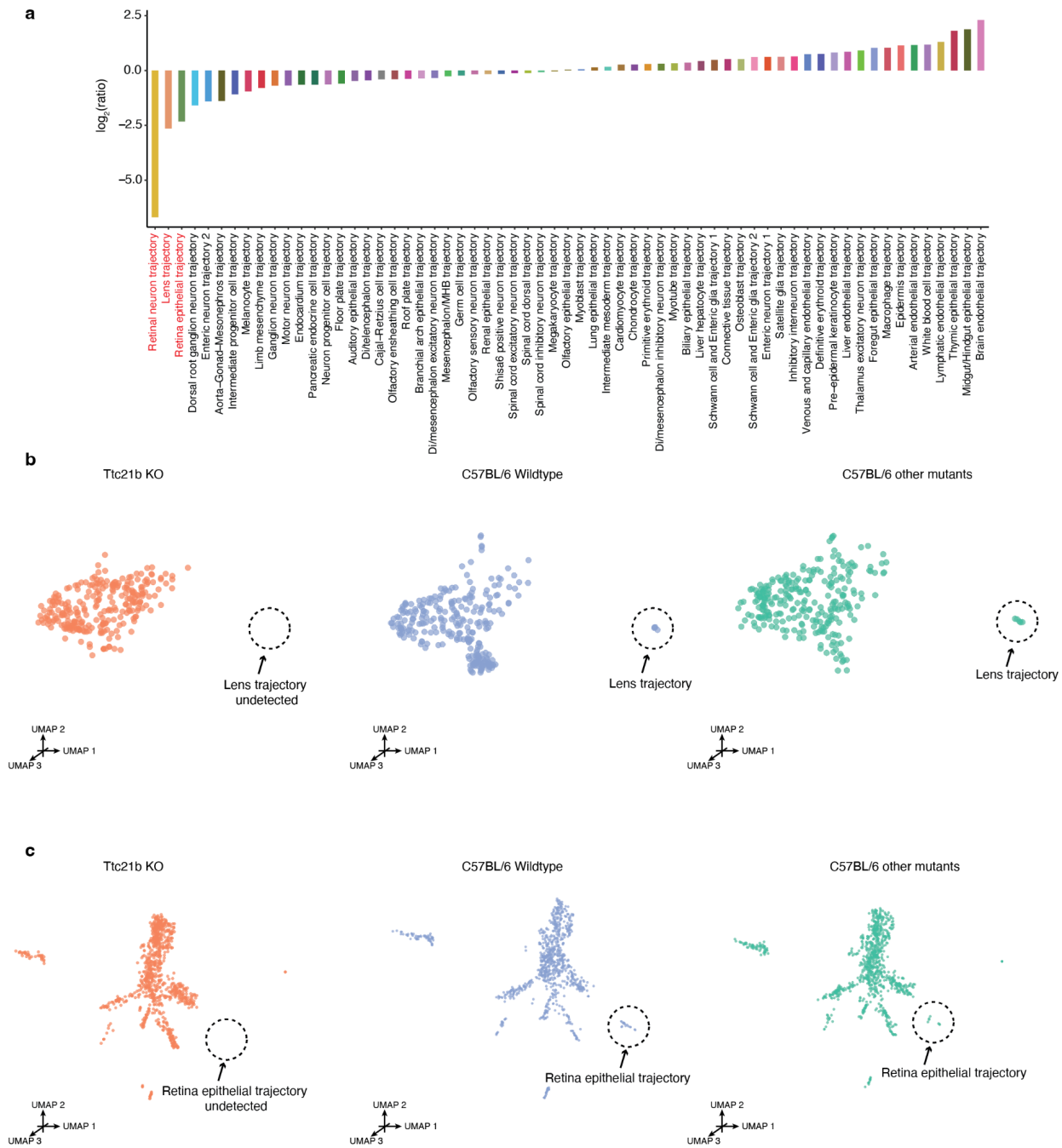
Supplementary Figure 4. Correlated developmental sub-trajectories between MOCA (E9.5 - E13.5) and MMCA (E13.5 only) based on non-negative least-squares (NMLS) regression. Similar to Fig. 1f, shown here are heat maps of the combined β values (row-scaled) between developmental sub-trajectories from MMCA (rows) and developmental sub-trajectories from the MOCA (columns), within each major trajectory. PNS: peripheral nervous system. MHB: midbrain-hindbrain boundary. Di: Diencephalon.



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1324 **Supplementary Figure 5. Cell composition for individual wildtype and mutant embryos across**
 1325 **developmental trajectories.** **a**, Cell composition across 13 major trajectories of embryos from different
 1326 wildtype or mutant strains. Cells from all replicates for each strain were pooled for this visualisation. **b**,
 1327 Boxplots of cell proportions falling into neural tube (left) or mesenchymal (right) trajectories for different
 1328 wildtype or mutant strains. Each point corresponds to an individual embryo. **c**, Boxplots of cell proportions
 1329 falling into each of the 13 major trajectories for the four wildtype strains. Each point corresponds to an
 1330 individual embryo. The total number of cells from each major trajectory profiled from wildtype embryos is

1331 also listed. In the boxplots (panels b & c), the centre lines show the medians; the box limits indicate the
1332 25th and 75th percentiles; the replicates are represented by the dots. PNS: peripheral nervous system.
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1336 **Supplementary Figure 6. Multiple retinal trajectories are diminished in *Ttc21b* KO mice. a**, The log₂
1337 transformed ratio of the cell proportions of each sub-trajectory, comparing *Ttc21b* KO and C57BL/6 wildtype
1338 embryos, are shown. Although reductions in the retina epithelial and lens trajectories were excluded from
1339 the regression analysis due to their low numbers, they were, together with the retinal neuron trajectory, the
1340 most extreme in magnitude. **b**, 3D UMAP visualisation of the hepatocyte major trajectory, highlighting cells
1341 from either the *Ttc21b* KO (left), C57BL/6 wildtype (middle), or other mutants on the C57BL/6 background
1342 (right). The three plots were randomly downsampled to the same number of cells ($n = 264$ cells) **c**, 3D
1343 UMAP visualisation of the epithelial major trajectory, highlighting cells from either the *Ttc21b* KO (left),

1344 C57BL/6 wildtype (middle), or other mutants on the C57BL/6 background (right). The three plots were
1345 randomly downsampled to the same number of cells ($n = 937$ cells).
1346

a

ZRS limb enhancer KO

UMAP 2
↑
→ UMAP 1

FVB wildtype

UMAP 2
↑
→ UMAP 1

b

Myoblast trajectory

Osteoblast trajectory

UMAP 2
↑
→ UMAP 1

UMAP 2
↑
→ UMAP 1

Chondrocyte trajectory

Roof plate trajectory

UMAP 2
↑
→ UMAP 1

UMAP 2
↑
→ UMAP 1

Megakaryocyte trajectory

Cardiomyocyte trajectory

UMAP 2
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→ UMAP 1

UMAP 2
↑
→ UMAP 1

Schwann cell and Enteric glia trajectory 2 trajectory

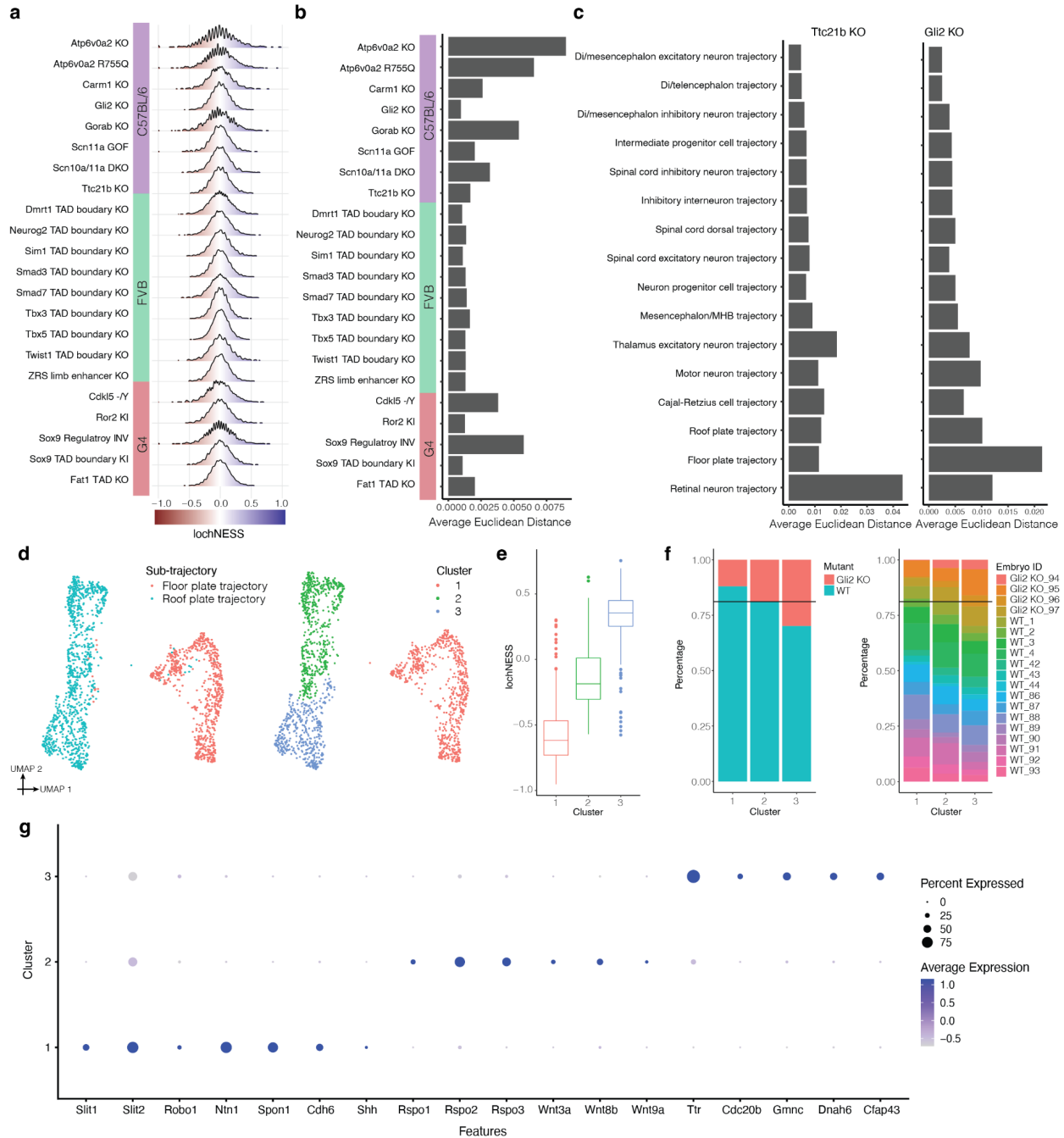
● FVB wildtype

● ZRS limb enhancer KO

UMAP 2
↑
→ UMAP 1

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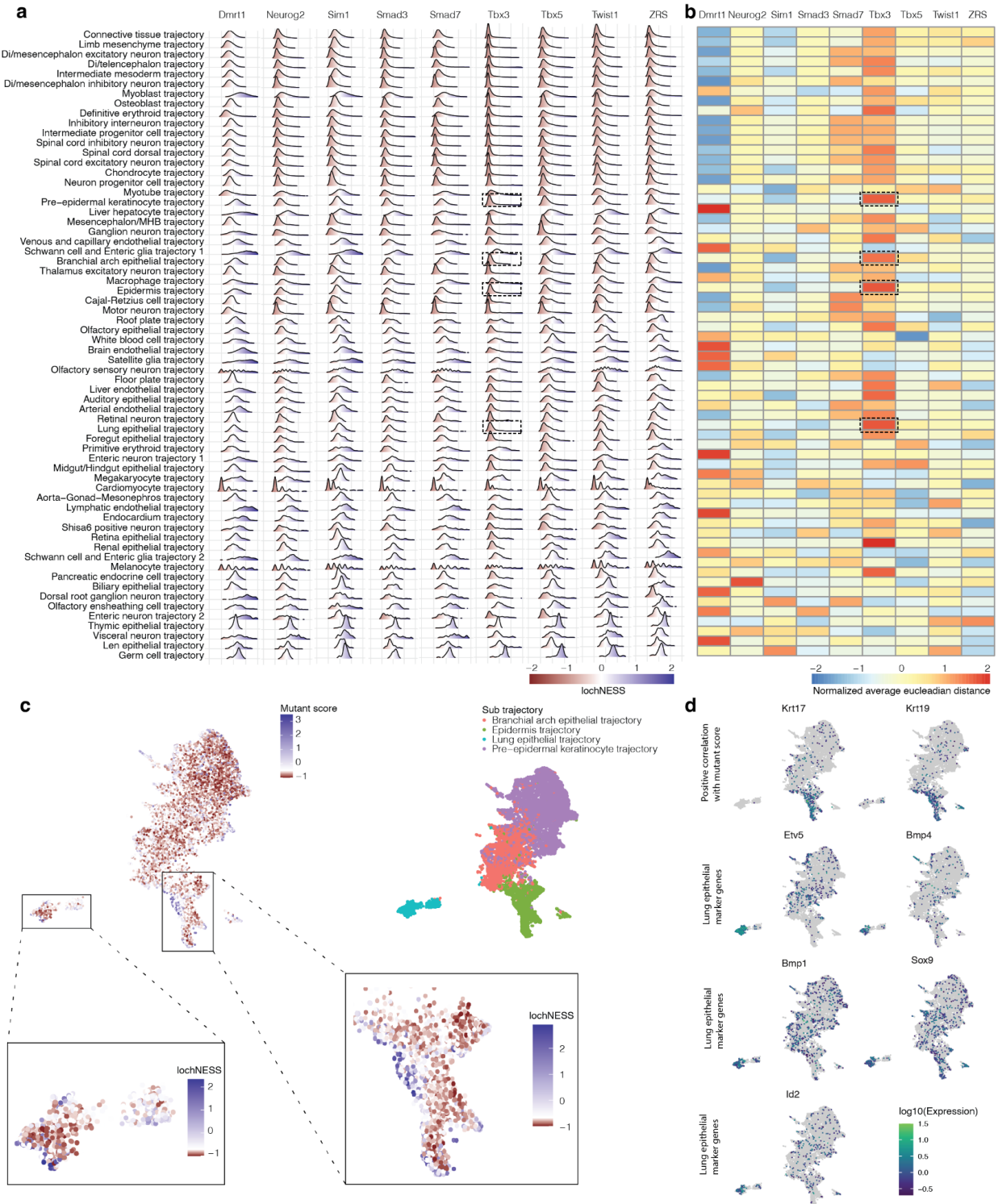
1349 **Supplementary Figure 7. Co-embedding cells from nominally altered trajectories from ZRS limb**
1350 **enhancer KO and FVB wildtype. a**, UMAP visualisation of co-embedded cells of limb mesenchyme
1351 trajectory from the ZRS limb enhancer KO and FVB wildtype. The same UMAP is shown eight times,
1352 highlighting cells from either ZRS limb enhancer KO (top row) or FVB wildtype (bottom row), and breaking
1353 out the four individual replicates for each strain. **b**, UMAP visualisation of co-embedded cells of various
1354 sub-trajectories from the ZRS limb enhancer KO and FVB wildtype. The same UMAP is shown twice for
1355 each, highlighting cells from either FVB wildtype (left) or ZRS limb enhancer KO (right). These are the seven
1356 sub-trajectories in which, in addition to limb mesenchyme, we detected nominally significant differences in
1357 cell type proportions for the ZRS limb enhancer KO.
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1361 **Supplementary Figure 8. Quantitative analysis of lochNESS distributions and analysis of *Gli2* KO in**
 1362 **the roof plate and floor plate trajectories. a,** Distribution of lochNESS in all cells of each mutant under
 1363 random permutation of mutant labels. **b,** Barplot showing the average euclidean distance between
 1364 lochNESS vs. lochNESS under permutation across all cells within a mutant. **c,** Barplots showing the
 1365 average euclidean distance between lochNESS and lochNESS under permutation, across all cells in neural
 1366 tube sub-trajectories of the *Ttc21b* KO and *Gli2* KO mutants. **d,** UMAP visualisation of co-embedded cells
 1367 of the floor plate and roof plate sub-trajectories from the *Gli2* KO mutant and pooled wildtype, colored by
 1368 sub-trajectory (left) or cluster number (right). **e,** Boxplot showing the lochNESS distribution in each cluster
 1369 shown on the right of panel d. **f,** Barplots showing the cell composition of each cluster shown on the right

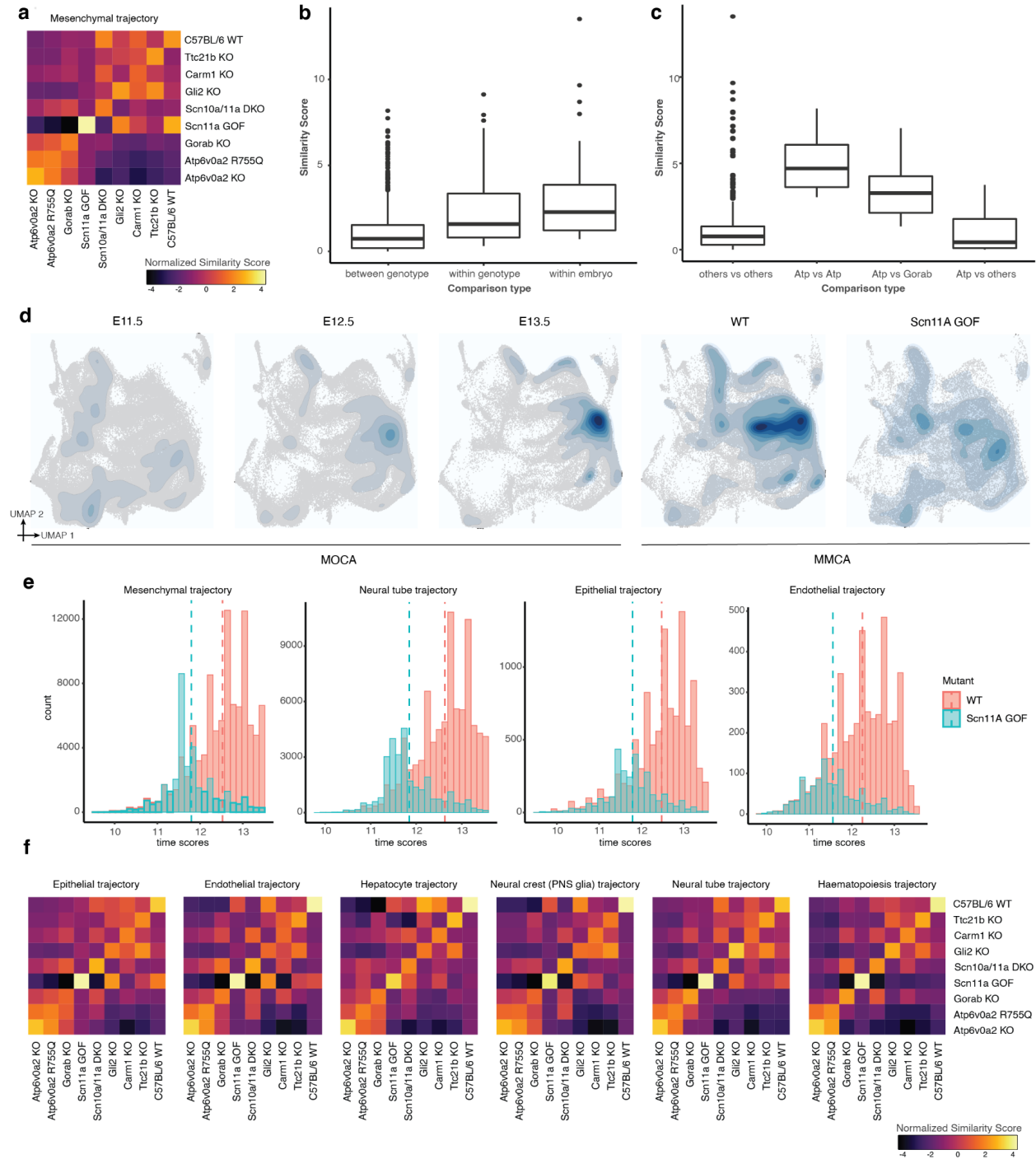
1370 of panel **d**, split by mutant vs. wildtype (left) or individual embryo (right), with a reference line at the overall
1371 wildtype cell proportion. **g**, Dotplot summarising the expression of and percent of cells expressing selected
1372 marker genes in each cluster shown on the right of panel **d**.
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1376 **Supplementary Figure 9. Systematic screening of lochNESS distributions identifies altered**
 1377 **epithelial sub-trajectories in the *Tbx3* TAD Boundary KO mutant.** **a**, Distribution of lochNESS in each
 1378 sub-trajectory of the mutants in the FVB background strain, all of which are TAD boundary KOs. Dashed
 1379 boxes in the sixth column highlight the most deviated epithelial sub-trajectories in the *Tbx3* TAD Boundary
 1380 KO mutant. **b**, Row-normalised heatmap showing the average euclidean distance between lochNESS and

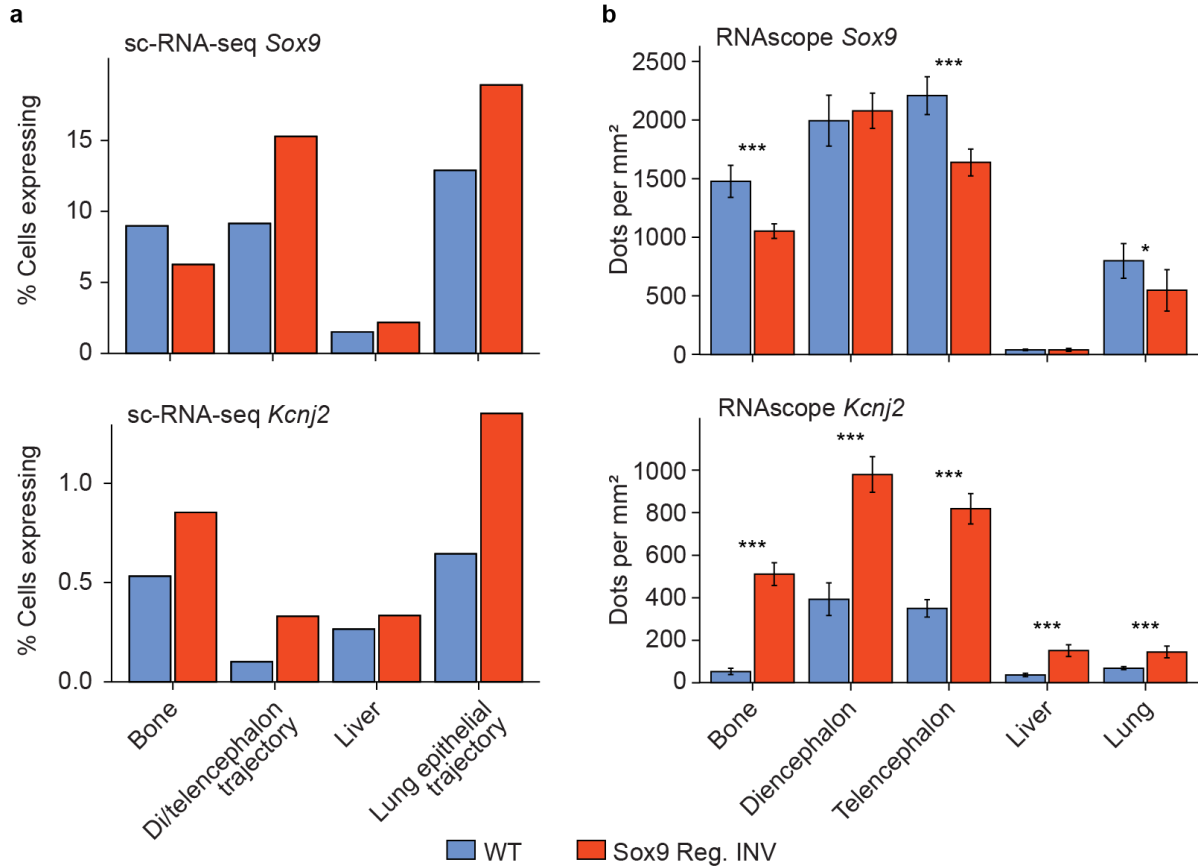
1381 lochNESS under permutation in each sub-trajectory for the same mutants shown in panel **a**, centred and
1382 scaled by row. Dashed boxes in the sixth column again highlight the most deviated epithelial sub-
1383 trajectories in the *Tbx3* TAD Boundary KO mutant. **c**, UMAP showing co-embedding of *Tbx3* TAD Boundary
1384 KO and pooled wildtype cells in the pre-epidermal keratinocyte, epidermis, branchial arch, and lung
1385 epithelial sub trajectories, colored by lochNESS (top left) [with blown up insets showing lochNESS in lung
1386 epithelial (bottom left) and epidermis (bottom right) sub-trajectories], or by sub-trajectory identity (right).
1387 LochNESS colour scale is centred at the median of lochNESS. **d**, same as in panel **c**, but colored by
1388 expression of selected mutant related genes and marker genes.
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1392 **Supplementary Figure 10. Similarity scores reveal mutant-shared and mutant-specific effects.**
 1393 **a**, Heatmap showing similarity scores between C57BL/6 genotypes in the mesenchymal trajectory. **b**,
 1394 Boxplot showing the similarity scores of comparisons between embryos of different genotypes (left),
 1395 between embryos of the same genotype (middle), and within the same embryos (right) for C57BL/6
 1396 genotypes in the mesenchymal trajectory. **c**, Boxplot showing the similarity scores of comparisons
 1397 between *Atp6v0a2* KO vs. *Atp6v0a2* R755Q (left), *Atp6v0a2* KO or *Atp6v0a2* R755Q vs. *Gorab* KO
 1398 (middle), *Atp6v0a2* KO or *Atp6v0a2* R755Q vs. other C57BL/6 genotypes, in the mesenchymal

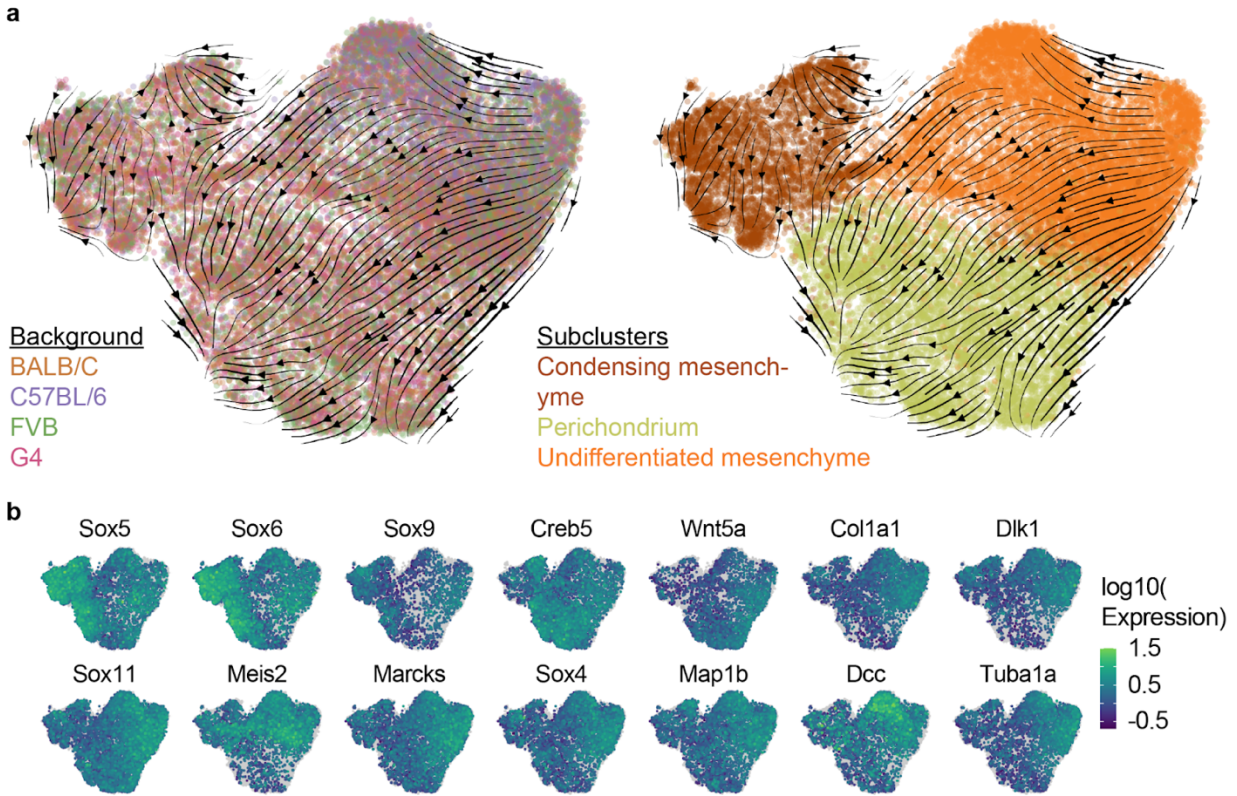
1399 trajectory. Genotype names are simplified in the x-axis legend (“Atp” = *Atp6v0a2* KO or *Atp6v0a2*, “Gorab”
1400 = *Gorab* KO, “others” = *Carm1* KO, *Gli2* KO, *Scn10a/11a* DKO, *Scn11a* GOF, *Ttc21b* KO or C57BL/6
1401 wildtype). **d**, UMAPs showing co-embedding of *Scn11a* GOF cells with pooled wildtype cells and
1402 E11.5-E13.5 MOCA cells, in the neural tube trajectory, split by mutant (MMCA) and time point (MOCA),
1403 with cell density and distributions overlaid. **e**, Barplots showing the distribution of “time scores” for
1404 *Scn11a* GOF cells and pooled wildtype cells in the mesenchyme, neural tube, endothelial and
1405 epithelial main trajectories, with reference lines at the mean value of time scores. **f**, Heatmaps showing
1406 similarity scores between C57BL/6 genotypes in selected main trajectories. *Gorab* KO exhibits high
1407 similarity to the two *Atp6v0a2* genotypes in the epithelial, endothelial, hepatocyte and neural crest
1408 (PNS glia) trajectories, but not the neural tube and hematopoiesis trajectories.
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1412 **Supplementary Figure 11. Misregulation of *Sox9* and *Kcnj2* in the *Sox9* regulatory INV mutant. a,**
1413 Quantification of *Sox9* (top row) and *Kcnj2* (bottom row) expression in sc-RNA-seq data in the wildtype
1414 (blue) and *Sox9* regulatory INV (red) genotypes in selected trajectories. For “bone” and “liver”, multiple sub-
1415 trajectories were pooled to match the tissue labels in the RNAscope data in panel b. Specifically, “bone”
1416 refers to cells from chondrocyte, osteoblast, and limb mesenchyme trajectories, whereas “liver” refers to
1417 cells from the liver endothelial and liver hepatocyte trajectories. b, Quantification of *Sox9* and *Kcnj2*
1418 expression based on RNAscope images of heterozygous E13.5 wildtype and *Sox9* regulatory INV mutant
1419 embryos (images not shown; available upon request). The mRNA signal was counted in a defined area (1
1420 x 1 mm²), n=6 each condition. Statistics were calculated using student t-test and evaluated the following:
1421 p > 0.05 = non-significant; p < 0,05 - ≥ 0.01 = *; p < 0,01 - ≥ 0.001= **; p < 0.001= ***.

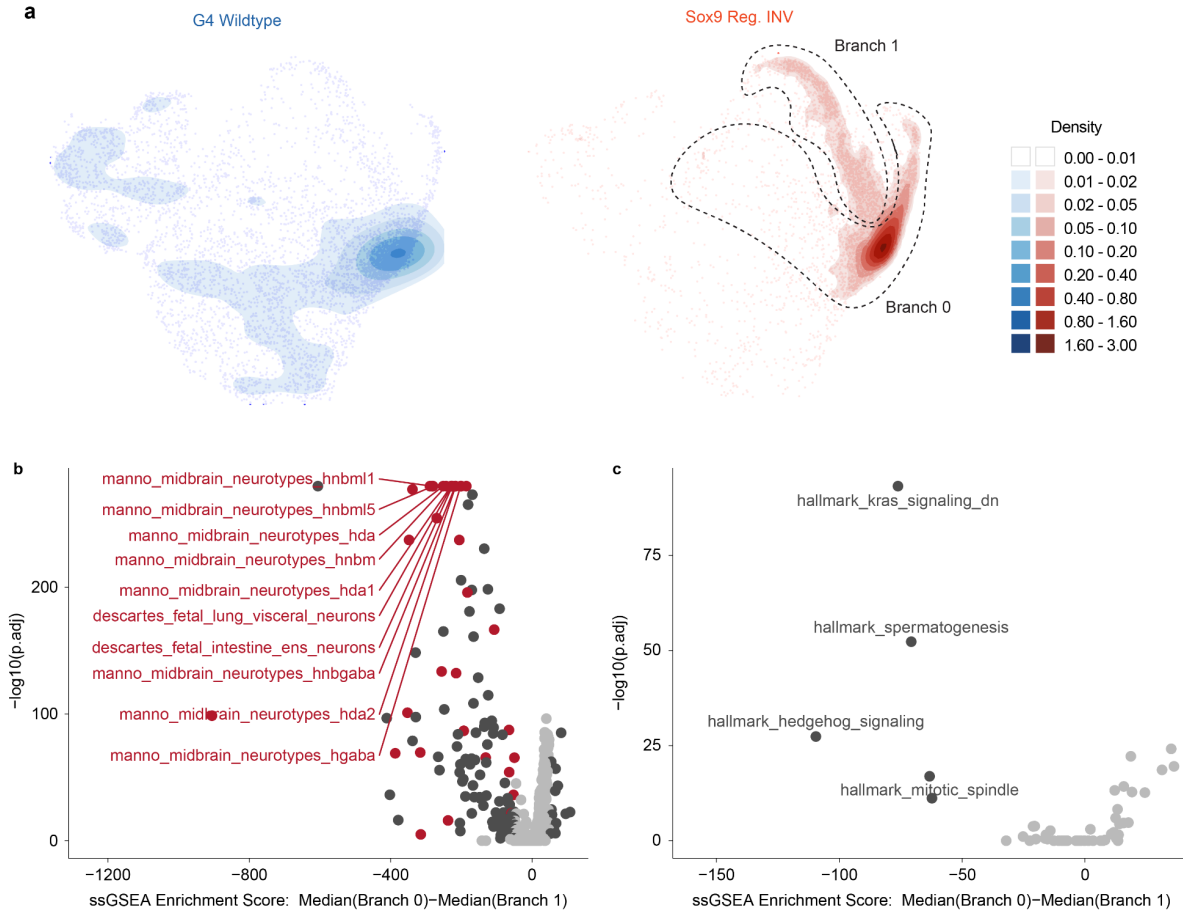
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1425 **Supplementary Figure 12. Sub-clustering and annotation of the wildtype limb mesenchyme.** **a**, Sub-
1426 clustering of the limb mesenchyme trajectory based on cells from pooled wildtype. RNA velocity arrows
1427 generated using scVelo (Methods) indicate the transition of undifferentiated mesenchyme (marked by
1428 *Meis2*, *Marcks*, *Map1b*) into perichondrium (*Wnt5a*, *Creb5*) and condensing mesenchyme (*Sox5*, *Sox6*,
1429 *Sox9*) in all wildtype samples^{86–91}. **b**, Marker gene expression used to annotate limb mesenchyme sub-
1430 clusters. All except *Dcc* and *Tuba1a* are literature-based markers of the three cell types.

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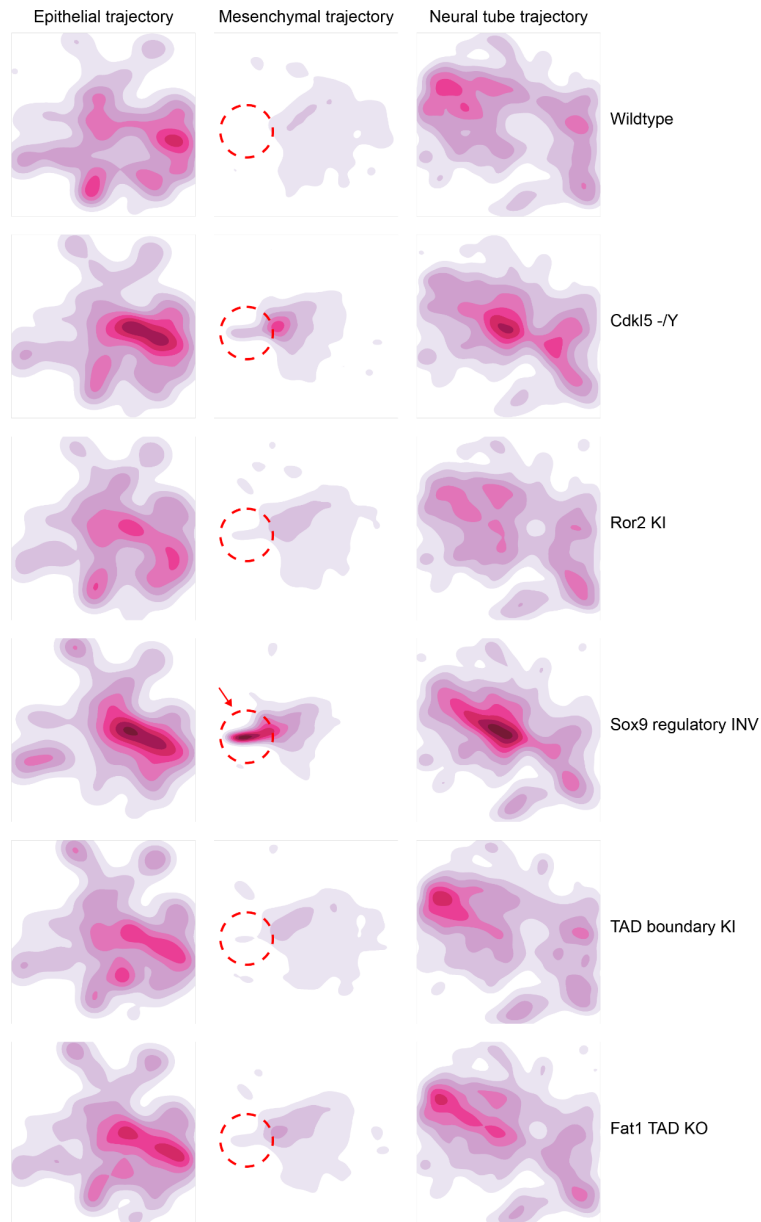
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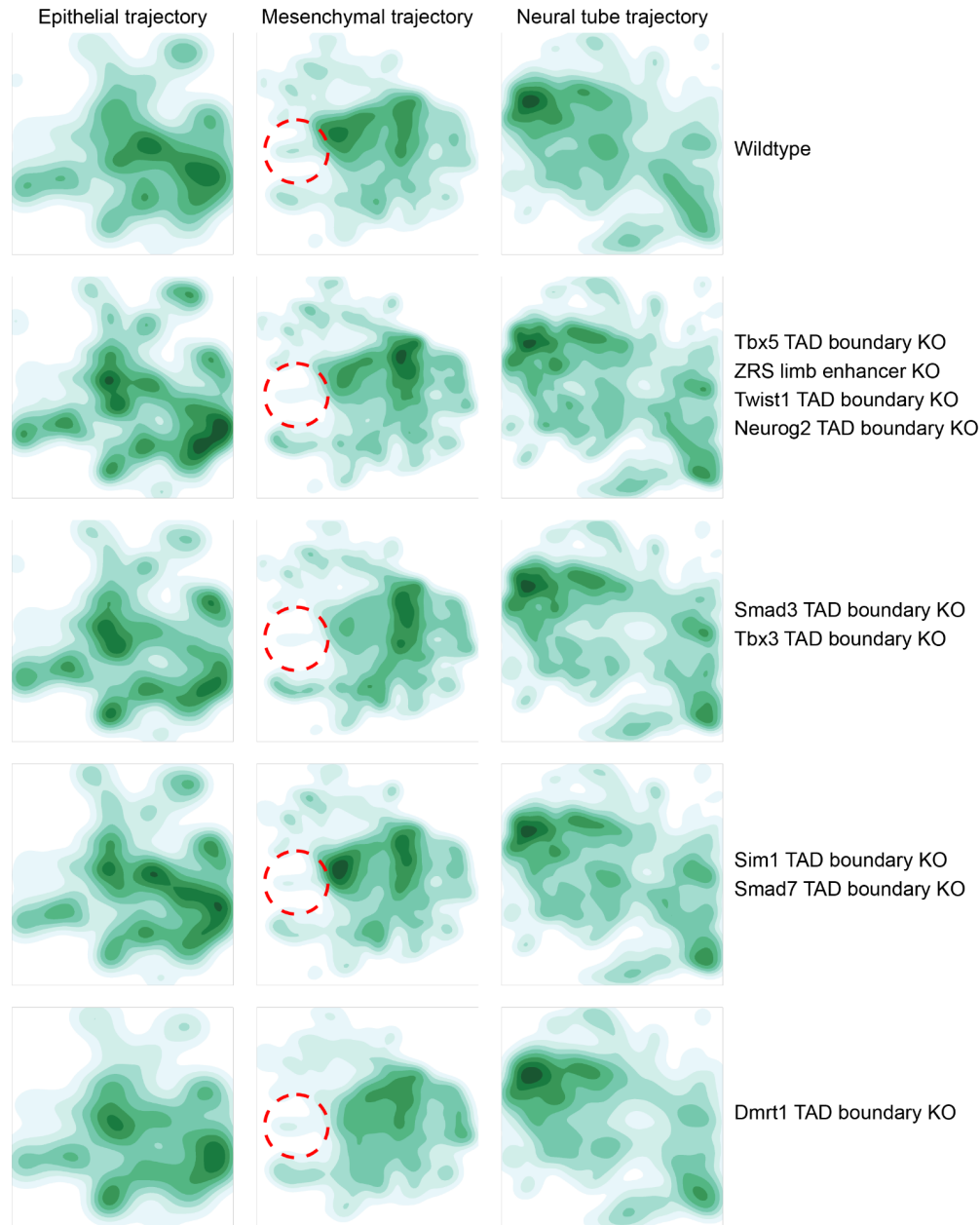
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Supplementary Figure 13. Stalling of Sox9 regulatory INV cells in the undifferentiated mesenchyme and gene set enrichment analysis on these cells. **a**, Density plots for UMAP embedding of G4 wildtype and Sox9 regulatory INV cells in the limb mesenchymal trajectory (same embedding as **Fig. 4e**). Dotted black lines demarcate the two branches of the undifferentiated mesenchyme, based on the sub-clustering shown in **Fig. 4f**. **b,c**, Comparison of the ssGSEA⁸⁴ scores between the two branches of undifferentiated mesenchyme for Sox9 regulatory INV cells for (a) cell type signature (C8) and (b) Hallmark gene sets. Gene sets that are both significantly different between the two branches and that have a difference in median ssGSEA scores greater than 50 are highlighted in dark grey, and the ten most significantly different gene sets are also labelled. In panel **b**, all significantly different gene sets with names containing “neuro” are highlighted in red.



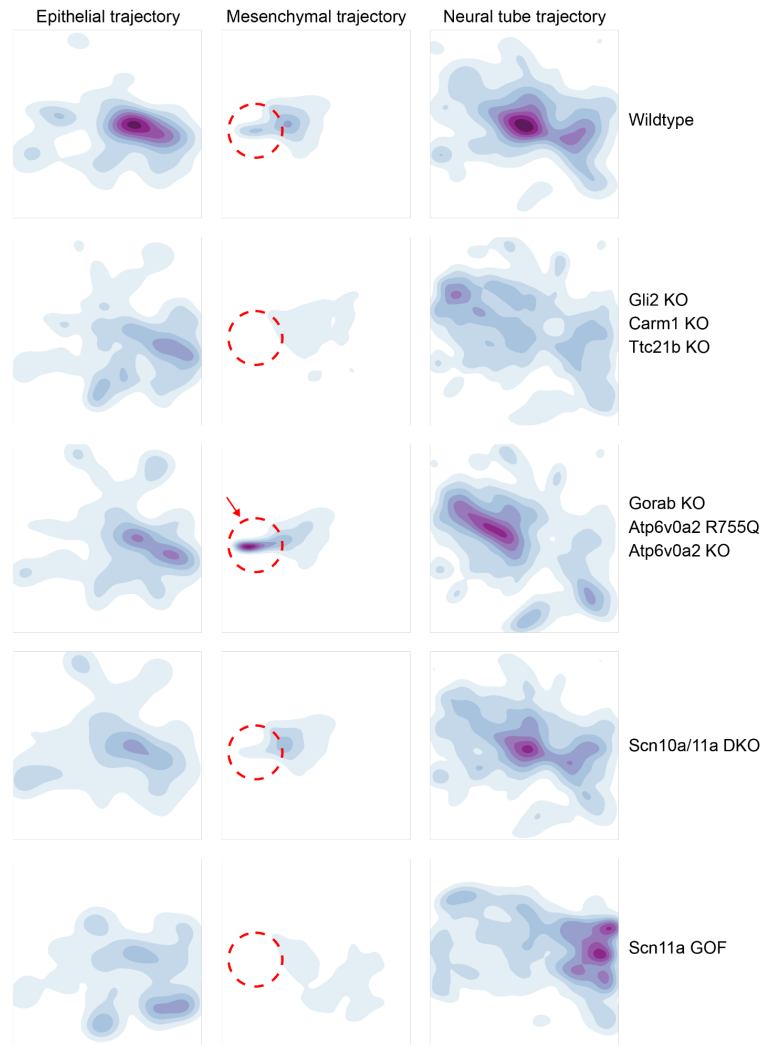
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Supplementary Figure 14. Density plots of the UMAP co-embedding of wildtype and mutant samples from G4 mouse background. We focus on the epithelial, mesenchymal and neural tube main trajectories, which are the three largest. The densities are corrected for the total number of cells. The colour scale is kept consistent across mutants (rows), but varied across the trajectories (columns). Arrow points to the accumulation of cells in the Sox9 regulatory INV mutant. Dotted circles demarcate the location of cellular accumulation in Sox9 regulatory INV mutant in the same embedding across all the other mutants.



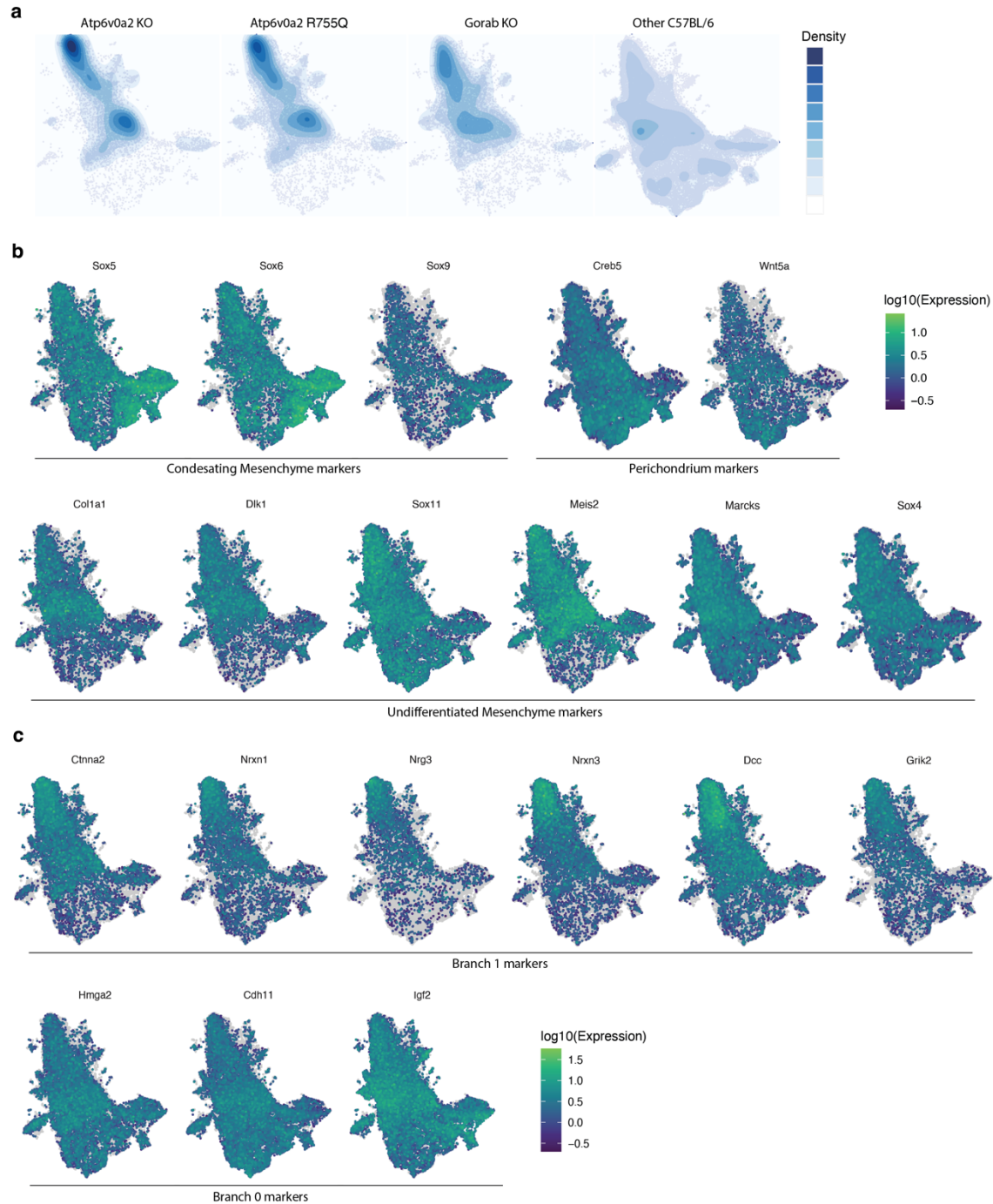
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Supplementary Figure 15. Density plots of the UMAP co-embedding of wildtype and mutant samples from FVB mouse background. We focus on the epithelial, mesenchymal and neural tube main trajectories, which are the three largest. The same embedding as in Supplementary Fig. 14 was used. Mutants with visually similar UMAP embeddings were combined for presentation. The densities are corrected for the total number of cells. The colour scale is kept consistent across mutants (rows), but varied across the trajectories (columns). Dotted circles demarcate the location of cellular accumulation in *Sox9* regulatory INV mutant in the same embedding across all the other mutants.



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Supplementary Figure 16. Density plots of the UMAP co-embedding of wildtype and mutant samples from C57BL/6 mouse background. We focus on the epithelial, mesenchymal and neural tube main trajectories, which are the three largest. The same embedding as in Supplementary Fig. 14 was used. Mutants with visually similar UMAP embeddings were combined for presentation. The densities are corrected for the total number of cells. The colour scale is kept consistent across mutants (rows), but varied across the trajectories (columns). Dotted circles demarcate the location of cellular accumulation in *Sox9* regulatory INV mutant in the same embedding across all the other mutants. Arrow highlights a similar accumulation of cells in the *Gorab* KO, *Atp6v0a2* R755Q, and *Atp6v0a2*KO mutants.



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1477 **Supplementary Figure 17. Density and marker gene expression plots of UMAP co-embeddings of**
1478 **wildtype and mutant samples from C57BL/6 mouse background in the limb mesenchyme trajectory.**
1479 **a**, UMAPs showing the co-embeddings of the limb mesenchyme trajectory for wildtype and mutant
1480 genotypes from the C57BL/6 background strain, with cell density and distributions overlaid. **b**, same as in
1481 panel **a**, but colored by expression of limb mesenchyme sub-cluster marker genes. The accumulation of
1482 cells in the *Gorab* KO, *Atp6v0a2* R755Q, and *Atp6v0a2*KO mutants express markers of undifferentiated
1483 mesenchyme. **c**, same as in panel **a**, but colored by expression of significantly differentially expressed

1484 genes between the two branches of Sox9 regulatory INV undifferentiated mesenchyme cells as shown in
1485 Fig. 4g.
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