

1 **Human axial progenitors generate trunk neural crest cells**

2 Thomas J. R. Frith¹, Ilaria Granata², Erin Stout¹, Matthew Wind¹, Oliver Thompson¹, Katrin
3 Neumann³, Dylan Stavish¹, Paul R. Heath⁴, James O.S. Hackland¹, Konstantinos
4 Anastassiadis³, Mina Gouti⁵, James Briscoe⁶, Val Wilson⁷, Mario R. Guarracino², Peter W.
5 Andrews¹, Anestis Tsakiridis^{1*}

6 ¹Centre for Stem Cell Biology, Department of Biomedical Science, The University of Sheffield, Alfred
7 Denny Building, Western Bank, Sheffield S10 2TN United Kingdom

8 ²Computational and Data Science Laboratory (CDS-LAB), High Performance Computing and
9 Networking Institute (ICAR), National Research Council of Italy (CNR), Via Pietro Castellino, 111,
10 Napoli, Italy

11 ³Stem Cell Engineering, Biotechnology Center, TU Dresden, Tatzberg 47, 01307 Dresden, Germany

12 ⁴Sheffield Institute for Translational Neuroscience, University of Sheffield, 385a Glossop Road,
13 Sheffield S10 2HQ United Kingdom

14 ⁵Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin, Germany

15 ⁶The Francis Crick Institute, 1 Midland Road, London, NW1 1AT United Kingdom

16 ⁷MRC Centre for Regenerative Medicine, Institute for Stem Cell Research, School of Biological
17 Sciences, University of Edinburgh, 5 Little France Drive, Edinburgh EH16 4UU United Kingdom

18 *Correspondence: a.tsakiridis@sheffield.ac.uk

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29 **Abstract**

30 The neural crest (NC) is a multipotent embryonic cell population generating distinct cell types
31 in an axial position-dependent manner. The production of NC cells from human pluripotent
32 stem cells (hPSCs) is a valuable approach to study human NC biology. However, the origin
33 of human trunk NC remains undefined and therefore current in vitro differentiation strategies
34 induce only a modest yield of trunk NC cells. Here we show that hPSC-derived axial
35 progenitors, the posteriorly-located drivers of embryonic axis elongation, give rise to trunk
36 NC cells and their derivatives. Moreover, we define the molecular signatures associated with
37 the emergence of human NC cells of distinct axial identities in vitro. Collectively, our findings
38 indicate that there are two routes toward a human post-cranial NC state: the birth of cardiac
39 and vagal NC is facilitated by retinoic acid-induced posteriorisation of an anterior precursor
40 whereas trunk NC arises within a pool of posterior axial progenitors.

41

42 **Introduction**

43 The neural crest (NC) is a multipotent cell population which arises in the dorsal neural
44 plate/non-neural ectoderm border region during vertebrate embryogenesis. Studies utilising
45 chick and amphibian embryos have indicated that different levels of BMP, WNT and FGF
46 signals, emanating from the mesoderm/non-neural ectoderm, orchestrate NC induction and
47 specification (1). This occurs via the action, first of neural plate border-specific transcription
48 factors such as PAX3/7, MSX and ZIC family members, and then via definitive NC-specifiers
49 (e.g. SOX9/10) (2). Once specified, NC cells undergo epithelial-to-mesenchymal transition
50 (EMT), exit the neural tube, and migrate to generate various cell types. The identity of NC
51 products correlates to their position along the anteroposterior (A-P) axis, which is in turn
52 reflected by the expression of paralogous HOX gene groups. Cranial NC cells give rise to
53 mesoectodermal derivatives (e.g. dermis, cartilage, bone), melanocytes, neurons and glia
54 colonizing the head (3) and are divided into an anterior HOX-negative and a posterior HOX
55 group(1-3)-positive domain. The latter also includes cells contributing to heart structures
56 (termed cardiac NC) (3, 4). Vagal NC cells, which are located between somites 1-7, are

57 marked by the expression of HOX group(3-5) members (5-7) and generate the enteric
58 nervous system (ENS) (8). HOX group(5-9)-positive NC cells at the trunk level (5, 9-11)
59 produce sympathoadrenal cells, which in turn give rise to sympathetic neurons,
60 neuroendocrine cells, and melanocytes (12).

61 An attractive approach for studying human NC biology and modelling NC-associated
62 developmental disorders (neurocristopathies) involves the in vitro differentiation of human
63 pluripotent stem cells (hPSCs) toward NC cells. Conventional protocols to obtain NC from
64 hPSCs are conceptually based on the production of a neurectodermal intermediate, via
65 TGF β signalling inhibition, which is subsequently steered toward a NC fate, usually through
66 stimulation of WNT activity combined with the appropriate BMP level (13-16). These
67 strategies yield NC cells of an anterior cranial character lacking HOX gene expression and
68 the generation of more posterior HOX+ NC subtypes typically relies on the addition of
69 retinoic acid (RA) and/or further WNT signalling stimulation (17-20). However, these signals
70 fail to efficiently induce a high number of NC cells of a HOX(5-9)+ trunk identity from an
71 anterior cranial progenitor. Therefore, the generation of trunk NC derivatives such as
72 sympathoadrenal cells often requires the flow cytometry-based purification of small cell
73 populations positive for lineage-specific fluorescent reporter (18) or cell surface markers
74 (21), a time-consuming and laborious approach.

75 A number of studies in chicken and mouse embryos employing both fate mapping
76 and lineage tracing have shown the existence of a posterior NC progenitor entity, which is
77 distinct from its more anterior counterparts and potentially co-localises with a pool of
78 caudally-located axial progenitors (22-27) . These progenitors include a bipotent stem cell-
79 like population that fuels embryonic axis elongation through the coordinated production of
80 spinal cord neurectoderm and paraxial mesoderm (PXM) (28) (reviewed in (29) and (30)). In
81 both mouse and chick embryos these neuromesodermal progenitors (NMPs) are located in
82 the node/streak border and the caudal lateral epiblast during early somitogenesis, and later
83 in the chordoneural hinge within the tailbud (TB) (27, 31-34). No unique NMP markers have
84 been determined to date and thus phenotypically NMPs are defined by the co-expression of

85 the pro-mesodermal transcription factor Brachyury (T) and neural regulator SOX2 (35-37).
86 Furthermore, they express transcripts which are also present in the primitive streak (PS) and
87 TB marking committed PXM and posterior neurectodermal progenitors such as *Cdx* and *Hox*
88 gene family members, *Tbx6* and *Nkx1-2* (23, 24, 31, 38, 39). T and SOX2 have a critical
89 role, in conjunction with CDX and HOX proteins, in regulating the balance between NMP
90 maintenance and differentiation by integrating inputs predominantly from the WNT and FGF
91 signalling pathways (27, 38-41). The pivotal role of these pathways has been further
92 demonstrated by recent studies showing that their combined stimulation results in the robust
93 induction of T+SOX2+ NMP-like cells from mouse and human PSCs (42-44).

94 NMPs/axial progenitors appear to be closely related to trunk NC precursors in vivo.
95 Specifically, trunk NC production has been shown to be controlled by transcription factors
96 which also regulate cell fate decisions in axial progenitors such as CDX proteins (45-47) and
97 NKX1-2 (48). The close relationship between bipotent axial and posterior NC progenitors is
98 further supported by fate mapping experiments involving the grafting of a portion of E8.5
99 mouse caudal lateral epiblast T+SOX2+ cells (27) and avian embryonic TB regions (22, 34)
100 as well as lineage tracing experiments (23, 24) which have revealed the presence of cell
101 populations exhibiting simultaneously mesodermal, neural and NC differentiation potential.
102 Together these findings suggest that the trunk/lumbar NC is likely to originate from a subset
103 of axial progenitors arising near the PS/TB.

104 Here we sought to determine whether trunk NC is also closely related to axial
105 progenitors in the human and thus define a robust improved protocol for the production of
106 trunk NC cells and their products from hPSCs. We show that hPSC-derived, “pre-neural”
107 axial progenitors contain a subpopulation that displays a BMP-dependent, mixed early
108 NC/NMP transcriptional signature and thus is likely to represent the earliest trunk NC
109 precursors. We demonstrate that neuromesodermal-potent axial progenitor cultures are
110 competent to generate efficiently trunk NC cells, marked by thoracic HOX gene expression.
111 This transition to trunk NC appears to take place via the maintenance of a CDX2/posterior
112 HOX-positive state and the progressive amplification of an NC gene regulatory network. We

113 also show that “caudalisation” via RA treatment of anterior NC precursors leads to the
114 acquisition of a mixed cardiac and vagal NC identity rather than a trunk NC character and
115 define novel markers of distinct posterior NC subtypes. Finally, we utilise our findings to
116 establish a protocol for the in vitro generation of PHOX2B+ sympathoadrenal cells and
117 sympathetic neurons at high efficiency from cultures of posterior axial progenitor-derived
118 trunk NC cells without the need for FACS-sorting to select for minor precursor
119 subpopulations. Taken together these findings provide an insight into the mechanisms
120 underpinning the “birth” of human NC cells at different axial levels and pave the way for the
121 modelling of trunk NC-linked diseases such as neuroblastoma.

122

123 **Results**

124 **Transcriptome analysis of human axial progenitors**

125 We and others have previously shown that combined stimulation of the WNT and FGF
126 signalling pathways in PSCs leads to the production of a high (>80%) percentage of
127 T+SOX2+ cells. The resulting cultures resemble embryonic posterior axial progenitors,
128 including NMPs, both in terms of marker expression and developmental potential (38, 42-44,
129 49). To interrogate the transcriptome changes associated with the induction of such
130 progenitors in a human system and identify the presence of trunk NC precursors, we carried
131 out RNA sequencing (RNAseq) following 3-day treatment of hPSCs with recombinant FGF2
132 and the WNT agonist/GSK-3 inhibitor CHIR99021 (CHIR). As reported previously, most cells
133 emerging under these conditions co-expressed T and SOX2 as well as CDX2 (**Figure 1A**).
134 We found that the transcriptomes of hNMPs and hPSCs were markedly distinct from each
135 other (**Figures S1A, S1B**) and dramatic global gene expression changes accompanied the
136 acquisition of an axial progenitor character: 1911 and 1895 genes were significantly
137 ($p_{adj} < 0.05$; Fold Change ≥ 2) up- and down-regulated compared to hPSCs respectively
138 (**Table S1**). Predictably, the top-downregulated genes were associated with undifferentiated
139 hPSCs (e.g. NANOG, GDF3, POU5F1), anterior neurectoderm (OTX2) and lateral/ventral
140 mesoderm (KDR). The vast majority of the top-upregulated genes were well-established

141 drivers of axis elongation (e.g. *TBRA*, *CDX1/2*, *EVX1*, *MSGN1*, *TBX6*) and
142 WNT/FGF/NOTCH/RA signalling pathway components, known to be expressed at high
143 levels in the late PS/TB regions in vivo (e.g. *WNT3A/5B*, *RSPO3*, *FGF4/8*, *FGF17*, *HES7*)
144 (**Figures 1B, S1C, S1D, Table S1**). A large fraction of upregulated genes were
145 transcriptional regulators (**Figure S1D, Table S1**) and we found that members of HOX gene
146 clusters belonging to paralogous groups 1-9 were strongly differentially expressed between
147 the two groups (**Figure 1C, S1E, Table S1**). The upregulation of posterior thoracic (group 5-
148 9) HOX transcripts as well as the presence of many transcripts (23/32) marking “late” E9.5
149 mouse embryonic NMPs such as *CYP26A1*, *FGF17* and *WNT5A* (38) suggest that day 3
150 WNT-FGF-treated hPSC cultures may correspond to a more developmentally advanced
151 axial progenitor state. Overall, these data confirm our previous observations that treatment
152 of hPSCs with WNT and FGF agonists gives rise to cultures resembling embryonic posterior
153 axial progenitors.

154

155 **A BMP-dependent neural crest signature in human axial progenitor cultures**

156 We next sought evidence pointing to the potential links between trunk NC and human axial
157 progenitors. The RNAseq analysis revealed that a considerable number of genes known to
158 collectively mark the neural plate border and early NC in vivo (“NC/border” e.g. *SOX9*,
159 *PAX3*, *MSX1/2*, *SNAI1/2*, *ZIC1/3*) were also significantly upregulated in axial progenitors
160 (**Figure 2A**), a finding which was further verified using quantitative real time PCR (qPCR)
161 (**Figure S2A**). To exclude the possibility that the presence of such markers was the result of
162 spontaneous differentiation of NM bipotent axial progenitors and their neural derivatives we
163 examined their co-expression with *T*, a marker of both NMPs and prospective PXM.
164 Immunostaining of d3 WNT-FGF-treated hPSC cultures showed that a considerable fraction
165 of *T*+ cells also expressed the early NC markers/specifiers *SOX9*, *SNAI2* and *PAX3* (**Figure**
166 **2B**). Moreover, *SOX9*-positive cells were found to express low or no *MSGN1* or *TBX6*
167 (**Figure S2B**) suggesting that the upregulation of border/NC markers following WNT-FGF
168 treatment of hPSCs was unlikely to reflect the presence of committed PXM cells given that

169 some of these genes are also expressed in the mesoderm during axis elongation.
170 Collectively, these findings indicate that a NC/border state probably arises within multipotent
171 posterior axial progenitors which have not committed to a neural or mesodermal fate.

172 A number of both in vivo and in vitro studies have pointed to an optimal level of
173 low/intermediate BMP signalling acting as an inducer of a NC/border character in
174 conjunction with WNT and FGF (14, 16, 48, 50-53). We therefore examined whether hPSC-
175 derived axial progenitor cultures exhibit endogenous BMP activity. RNAseq revealed that
176 many BMP pathway -associated transcripts (BMP2/4/6/7 and ID1/4) were significantly
177 upregulated compared to hPSCs (**Figure 2A**). Moreover, antibody staining showed
178 expression of phosphorylated SMAD1/5, a readout of BMP activity (**Figure 2C**). This is
179 extinguished upon treatment with the BMP antagonist LDN193189 (LDN) (54) during the in
180 vitro differentiation of hPSCs to day 3 axial progenitors (**Figure 2C**). Interestingly, BMP
181 inhibition also caused a decrease in the transcript levels of most border/NC-specific
182 transcripts with PAX3 and MSX1 being the most severely affected (**Figure 2D**). We also
183 observed a reduction in the NMP/late PS/TB markers CDX2 and WNT3A whereas the PXM
184 specifier TBX6 remained relatively unaffected by LDN treatment suggesting that emergence
185 of prospective PXM is not influenced by BMP inhibition (**Figure 2D**). By contrast, SOX2
186 expression was markedly increased upon LDN treatment (**Figure 2D**). Taken together these
187 data indicate a correlation between endogenous BMP activity and the acquisition of a
188 SOX2+low border/NC identity by posterior axial progenitors while transition toward a SOX2+
189 high neural fate relies on BMP antagonism in vitro.

190

191 **In vitro-derived axial progenitors are a source of trunk neural crest cells**

192 We reasoned that if posterior axial progenitors with border/NC features correspond to
193 pioneer trunk NC precursors then they should be competent to generate definitive trunk
194 neural crest when placed in the right culture environment. We have recently reported a
195 protocol for the efficient generation of anterior cranial NC cells from hPSCs involving the
196 combined stimulation of WNT signalling, TGF β signalling inhibition and moderate BMP

197 activity via the parallel addition of BMP4 and the BMP type 1 receptor inhibitor DMH1 (16).
198 Culture of day 3 WNT-FGF-treated hPSCs under these NC-inducing conditions for 5-6 days
199 gave rise to a high number (average percentage=50% of total cells) of cells co-expressing
200 the definitive NC marker SOX10 together with HOXC9, a readout of trunk axial identity
201 (**Figure 3A, 3B, 3C**). A large proportion of the cultures were also SOX9+HOXC9+ further
202 confirming an trunk NC character whereas the percentage of neural cells marked by SOX1
203 expression remained very low throughout the course of the differentiation (**Figure 3B, S3A,**
204 **S3B**). This may indicate that posterior NC progenitors do not progress through neural
205 commitment but rather diverge from an earlier pre-neural, border-like stage reflecting
206 previous reports which show that NC specification takes place prior to definitive neurulation
207 (48, 55, 56). Furthermore, during the transition toward trunk neural crest, the NMP/pre-
208 neural marker NKX1-2 was rapidly extinguished followed shortly after by T, while CDX1
209 transcript levels declined more slowly (**Figure S3A, S3B**). By contrast, the expression of
210 CDX2 and SOX9 was maintained at high levels (>70% of total cells) throughout the course
211 of differentiation of axial progenitors to trunk NC while SOX10 expression appeared only
212 after day 7 of differentiation (Day 0 defined as the start of axial progenitor induction from
213 hPSCs) (**Figure S3C, data not shown**). We also confirmed the NM potency of the starting
214 axial progenitor cultures as treatment with high levels of FGF2/CHIR and RA led to the
215 production of TBX6+ PXM and SOX1+ spinal cord, posterior neurectoderm (PNE) cells
216 respectively (**Figure 3A, 3D**). Taken together these data suggest that hPSC-derived NM-
217 potent axial progenitor cultures are competent to produce trunk NC at high efficiency.

218 Similar to popular in vitro neural induction strategies, most current NC differentiation
219 protocols aiming to generate posterior (e.g. trunk) cell populations from hPSCs rely on the
220 caudalisation of an anterior ectodermal precursor via treatment with RA and/or WNT
221 agonists (15, 17-20). Therefore, we compared our axial progenitor-based approach for
222 generating trunk NC to a conventional strategy involving the generation of anterior cranial
223 NC (ANC) precursor cells (16) followed by RA addition in the presence of WNT and BMP
224 signalling (**Figure 4A**). The axial identity of the resulting cells was assessed by qPCR assay

225 of *HOX* transcripts corresponding to different levels along the A-P axis. In line with previous
226 findings (17, 19) RA-treated cells expressed high levels of *HOX* group 1-5 members
227 compared to untreated NC suggesting a posterior cranial and vagal/cardiac NC character
228 (**Figure 3E**). However, efficient induction trunk of *HOXC8* and 9 transcripts was only
229 achieved when posterior axial progenitors were employed as the starting population for NC
230 generation (**Figure 3E**). Furthermore, axial progenitor-derived NC cells were marked by
231 increased expression of the trunk NC marker *HES6*, but did not express the cranial markers
232 *OTX2*, *DMBX1* and *LHX5* although they were positive for the “late” cranial NC transcripts
233 (*TFA2B*, *ETS1*, *SOX8*) (57) (**Figure 3F**). We thus conclude that posterior axial progenitors
234 are the ideal starting population for efficiently generating trunk NC in vitro whereas RA
235 treatment of anterior NC precursors predominantly produces posterior cranial and
236 cardiac/vagal NC cells. These data also serve as evidence supporting the notion that trunk
237 NC precursors are likely to arise within cells with axial progenitor/NMP features rather than a
238 caudalised anterior progenitor.

239

240 **Efficient A-P patterning of human neural crest cells reveals molecular signatures of** 241 **distinct axial identities**

242 To further discern the identity of posterior NC subtypes induced either via RA treatment or
243 an axial progenitor intermediate as well as identify unique associated molecular signatures
244 we carried out analysis of the transcriptomes of NC cells arising under these conditions as
245 well as those of their precursors using microarrays (**Figure 4A**). We found that axial
246 progenitor-derived NC cells (NMP-NC d9) and their precursors (NMP-NC d6) grouped
247 together and were distinct from a cluster containing d6 anterior cranial NC (ANC) and +RA
248 NC cells and their common d3 progenitor (ANC d3) (**Figure 4B, S4A**). Although the three
249 final populations exhibited distinct transcriptional profiles they all expressed pan-NC genes
250 including “early” NC/border (*MSX1/2*, *PAX3/7*) and “late” NC (*SOX10*, *SNAI1/2*) transcripts
251 (**Figure 4C, S4B, S4C, Table S2**). In line with our previous observations (**Figure 3C**), ANC
252 cells failed to express any *HOX* transcripts, RA treatment induced anterior *HOX* genes and

253 only WNT-FGF-treated hPSCs gave rise to NC cells positive for thoracic HOX group (5-9)
254 members (**Figure 4D**) reflecting an anterior cranial, posterior cranial/cardiac/vagal and trunk
255 NC fate respectively. The axial identity of the resulting posterior NC subtypes was further
256 confirmed by the observation that some of the most-upregulated transcripts in +RA cells
257 were established posterior cranial (e.g. *ALX1/3* (58)), cardiac (e.g. *FOXC1* and 2 (59);
258 *PDGFRa* (60); *TBX2/3* (61)) and vagal/enteric NC markers (*PHOX2A* (62); *KITLG* (63);
259 *ARPC1B* (64) (**Figure 4E**). In contrast, known trunk NC, sympathoadrenal and
260 sympathetic/sensory neuron regulators such as *CDX2* (47), *INSM1* (65), *ISL1* (66) and
261 *NEUROG1* (67) were induced only in NC cells derived from axial progenitors (**Figure 4F**,
262 **Table S2**). We also identified *ASLX3*, the human homologue of the *Drosophila* polycomb
263 protein *asx* (68) which has been recently linked to the developmental syndrome Bainbridge-
264 Ropers (69) as a novel trunk NC marker (**Figure 4F**, **Table S2**). Transcription factors
265 specifically induced in anterior cranial NC cells included the forkhead gene *FOXS1* which
266 has been shown to be expressed in mouse NC derivatives (70) and *TCF7L2*, a WNT
267 signalling effector which has been reported to harbour a NC-associated enhancer (71)
268 (**Tables S2, S3**). Collectively these data support the idea that a mixed posterior
269 cranial/vagal/cardiac NC character arises upon treatment of anterior NC precursors with RA
270 whereas a *bona fide* trunk NC identity can be achieved only via an axial progenitor
271 intermediate.

272 One of the most over-represented gene categories in all three axial NC subtypes
273 were transcription factors and a common NC-specific transcription factor module was found
274 to be expressed regardless of axial character (**Figure S4B**, **Table S3**, **Table S4**). This
275 included well-established border/NC regulators such as *PAX3/7*, *MSX2*, *SOX9/10*, *TFAP2A*-
276 *C* and *SNAI1/2* (**Figure S4C**, **Tables S3, S4**). However, the expression levels of many of
277 these transcription factors varied between the three groups (**Figure S4C**). The highest levels
278 of *HES6*, *FOXD3* and *MSX1/2* were found in axial progenitor-derived trunk NC cells whereas
279 high *PAX7* and *SNAI1/ SOX9* expression was more prevalent in the anterior cranial and RA-
280 treated samples respectively (**Figure S4C**). Comparison of the day 6 trunk and d3 ANC

281 precursor transcriptomes also revealed that expression of LHX5 and DMBX1 marks an
282 anterior NC state whereas HES6 is associated exclusively with a trunk fate (**Figure S4D**)
283 indicating that diversification of axial identity in NC cells starts at an early time point via the
284 action of distinct molecular players.

285

286 **Distinct routes to posterior neural crest fates**

287 To identify candidate genes mediating the gradual lineage restriction of trunk NC precursors
288 present in axial progenitor cultures we compared the transcriptomes of d6 trunk NC
289 precursors and day 3 WNT-FGF-treated hPSCs (=“NMPs”). We found that dramatic global
290 gene expression changes take place during the axial progenitor-trunk NC transition (**Figure**
291 **4G, S4E**). Some of the most upregulated transcripts were the NC-specific TFAP2A/B, ETS1,
292 SOX5 and SOX10 together with the established trunk NC specifier CDX2, the novel trunk
293 NC marker ASLX3, the nuclear receptors NR2F1/2 and thoracic HOX genes (HOXB7, B9)
294 (**Figure 4G, Table S4**). In contrast, signature axial progenitor transcription factors such as
295 *T*, *MIXL1*, *NKX1-2*, anterior HOX genes (*HOXA1/B1*) and some WNT signalling components
296 (*WNT8A/5B*) were significantly downregulated (**Figure 4G, Table S4**). Thus differentiation of
297 trunk NC precursors appears to involve the transition from an axial progenitor-associated
298 gene regulatory network to a NC-specifying one that incorporates factors which potentially
299 act as general determinants of posterior cell fate (*CDX2*, *HOXB9*).

300 We also examined transcriptome changes during the transition from an anterior NC
301 precursor state (ANC d3) to RA-posteriorised vagal/cardiac NC cells (+RA d6). The most-
302 highly induced transcripts in posterior cranial/cardiac/vagal NC cells included the RA
303 receptors beta and gamma (*RARB/g*) which have been involved in hindbrain and neural crest
304 patterning (72) and the T-box transcription factor *TBX2*, a marker of cardiac NC and in vitro
305 derived vagal/enteric NC progenitors (19) (**Table S4**). Other upregulated transcripts included
306 the planar cell polarity (PCP) component *PRICKLE1*, a regulator of cardiac NC cell function
307 (73) and the TGF β signalling-associated gene *TGFBI* (**Table S4**). Anterior NC d3 precursor
308 specific –transcripts included the border markers *PAX7* and *ZIC3* as well as the early cranial

309 NC transcription factors *OTX2* and *LHX5* (57) (**Table S4**). These results indicate that, in
310 contrast to trunk NC cells, posterior crania/ cardiac/vagal NC cells arise from an anterior
311 neural plate border precursor through posteriorisation under the influence of RA and possibly
312 the non-canonical WNT and TGF β pathways.

313

314 **Efficient in vitro generation of sympathoadrenal cells from axial progenitors**

315 We next sought to determine whether trunk NC cells derived from axial progenitors are the
316 optimal source of sympathoadrenal (SA) progenitors and their derivatives. The BMP and
317 sonic hedgehog (SHH) signalling pathways have been shown to be critical for the
318 specification of these lineages from NC cells (74, 75). Therefore we cultured d8 trunk NC
319 cells generated from axial progenitors carrying a GFP reporter within the SA/sympathetic
320 neuron regulator *PHOX2B* locus (18, 76), in the presence of BMP and sonic hedgehog
321 (SHH) signalling agonists (**Figure 5A**). GFP expression was assayed after 4 days of culture
322 of trunk NC cells in BMP4 and SHH agonists (i.e. day 12 of differentiation) (**Figure 5B**).
323 FACS analysis revealed that the majority of cells were *PHOX2B* expressing (average
324 percentage from four independent experiments=73.5%, s.d.= 6.344) (**Figure 5B**) and a
325 large proportion of them were also positive for the early SA progenitor marker *ASCL1* (77)
326 indicating that they had acquired a sympathoadrenal identity (**Figure S5A**). Further
327 maturation of the resulting SA progenitors in the presence of neurotrophic factors (BDNF,
328 GDNF and NGF) for a further 6-8 days resulted in the induction of a high yield of
329 sympathetic neurons/progenitors co-expressing *PHOX2B* together with the sympathetic
330 neuron regulator *GATA3* (78) (average of 40% of total cells), *ASCL1* (63%) and the SA
331 differentiation regulator *ISL1* (64%) (**Figure 5C, 5D**). A high proportion of the resulting cells
332 also expressed the dopamine production-associated enzyme/sympathetic neuron marker
333 tyrosine hydroxylase (TH) (79) (**Figure 5D**). Furthermore, the cultures widely expressed the
334 peripheral nervous system marker *PERIPHERIN* (PRPH) (80) together with the trunk axial
335 marker *HOXC9* (**Figure S5B**). We also detected dramatic induction of *GATA3*, *ASCL1*, *TH*
336 and *PHOX2B* transcripts (between 1000 and 1,000,000-fold) as well as other SA lineage

337 markers such as *GATA2*, *DBH* (dopamine- β -hydroxylase) and to a lesser extent *PHOX2A*
338 using qPCR (**Figure 5E**). Together, these results suggest that the optimal route toward the
339 efficient production of sympathoadrenal cells and sympathetic neurons from hPSCs relies on
340 the induction of posterior axial progenitors.

341

342 **Discussion**

343 Despite progress in the optimisation of current NC differentiation protocols the in vitro
344 generation of trunk NC cells from hPSCs remains challenging and requires FACS-sorting of
345 selected progenitor subpopulations, a time-consuming and laborious process also
346 associated with increased cell death. This bottleneck prevents the dissection of the
347 mechanisms directing human NC emergence at different axial levels as well as the efficient
348 isolation of cell types for modelling trunk NC-specific neurocristopathies such as
349 neuroblastoma. Previous work in amniote embryos suggested that posterior
350 (trunk/lumbosacral) NC cells arise independently from their anterior counterparts, within a
351 pool of axial progenitors localised near the primitive streak and the tailbud during axis
352 elongation (22, 25-28). Here we utilised these findings and exploited our ability to induce T+
353 NM-potent axial progenitors from hPSCs in order to use them as the optimal starting point
354 for the efficient in vitro derivation of trunk NC (~50% HOXC9+SOX10+), SA progenitors
355 (~70% PHOX2B-GFP+) and sympathetic neurons without the use of FACS sorting. This
356 strategy represents a considerable improvement over current approaches, which typically
357 yield 5-10% PHOX2B-GFP+ cells (18) and is in line with a recent study reporting the
358 successful production of chromaffin-like cells through the use of an NC-induction protocol
359 which transiently produces T+SOX2+ cells (20, 21).

360 We show that, similar to neural cells a HOX-positive posterior identity is acquired by
361 human NC cells via two distinct routes: posterior cranial/vagal/cardiac HOX(1-5)+ NC cells
362 emerge through the RA/WNT-induced posteriorisation of a default anterior precursor,
363 reflecting Nieuwkoop's "activation-transformation" model, whereas HOX(5-9)+ trunk NC cells
364 arise from a separate WNT/FGF-induced posterior axial progenitor exhibiting caudal lateral

365 epiblast/NMP features mixed with a BMP-dependent neural plate border/neural crest
366 identity (**Fig. 6**). This finding offers an explanation for the failure of current RA
367 posteriorisation-based in vitro differentiation protocols (17, 19) to yield high numbers of
368 HOX9+ trunk NC cells and should serve as the conceptual basis for the design of
369 experiments aiming to generate NC cells of a defined A-P character from hPSCs.

370 Our data indicate that a subpopulation of in vitro derived human axial progenitors
371 acquires border/early NC characteristics in response to the WNT and FGF signals present in
372 the differentiation culture media, and under the influence of autocrine BMP signalling. This is
373 in line with bulk and single cell transcriptome data showing that mouse embryonic axial
374 progenitors/NMPs express border and early NC markers (38, 41). Furthermore, our data
375 reflect findings in the chick embryo showing that an “unstable”, pre-neural plate border
376 domain, potentially defined by the co-localisation of pre-neural (*Nkx1-2*) (81) and border
377 markers such as *Pax3* (82) and *Msx1*, arises in the avian embryonic caudal lateral epiblast
378 in response to autocrine BMP, FGF (51) and possibly WNT signalling (83). We also found
379 that CDX2 expression is maintained at high levels during the generation of trunk NC
380 indicating that this transcription factor might be critical in inducing an NC character in axial
381 progenitors. CDX2 has been shown, together with β -catenin, to bind and activate neural
382 plate border/early NC specifiers such as MSX1 and ZIC1 (47, 84) and, intriguingly, ChiP-Seq
383 data from in vitro-derived mouse NMPs have revealed that many border/NC genes are direct
384 targets of CDX2 often jointly with T (e.g. PAX3, SOX9, ZIC3) (39). Collectively these findings
385 raise the possibility that β -catenin and CDX2, in conjunction with FGF/BMP signalling, may
386 be critical for the establishment of a border/NC identity in T+ axial progenitors and further
387 work is required to test this hypothesis.

388 We show that BMP/WNT treatment of human axial progenitors promotes the
389 induction of a definitive trunk NC state. This transition appears to coincide with the
390 progressive extinction of key axial progenitor genes and their replacement by a battery of
391 NC-specific transcription factors such as TFAP2B, SOX10, NR2F2 and NR2F1 while the
392 levels of some “common” axial progenitor-NC markers (e.g. SOX9, PAX3, TFAP2A and

393 SNAI2) remain high (**Figure 4, S3, Table S4**). TFAP2A has been previously reported to act
394 as a master NC transcription factor whose binding on key enhancers, together with
395 NR2F1/2, appears to initiate transcription of NC-specific genes (71). This finding raises the
396 possibility that these transcription factors are the molecular drivers of the transition from an
397 early posterior axial progenitor state to a lineage-restricted trunk NC fate in response to
398 BMP/WNT. How is a trunk axial character specified? Our transcriptome analysis data
399 suggest that a generic trunk identity is first installed during the emergence of multipotent
400 CDX2+trunk HOX+ axial progenitors under the influence of WNT-FGF activity and then
401 “converted” into posterior NC through the progressive accumulation of neural plate
402 border/definitive NC markers within BMP-responsive cells. The posterior character of these
403 incipient trunk NC precursors and their progeny is likely to be maintained via continuous
404 CDX2 expression and further potentiation of trunk HOX activities as indicated by the
405 expression profiles of these genes in both d6 precursor and d9 trunk NC cells (**Figure 4, S4,**
406 **Tables S2-4**) and their reported roles in trunk NC specification. However, other trunk NC-
407 specific regulators may also be involved in this process and loss-/ gain-of-function
408 approaches are required to dissect their exact involvement in programming trunk identity.

409

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423

424

425 **Author Contributions**

426 Conceptualization: TJRF, AT

427 Data Curation: IG

428 Formal Analysis: TJRF, IG, AT

429 Resources: JB, MG, TJRF, KN, KA, AT

430 Funding Acquisition: AT, PWA, VW, JB, MG, KA, MRG

431 Investigation: TJRF, IG, MW, ES, OT, JOSH, DS, PH, MG, AT

432 Project Administration: AT

433 Supervision: AT, PWA

434 Writing – Original Draft Preparation: AT with input from TJRF, IG

435 Writing – Review & Editing: All co-authors

436

437 **Competing interests**

438 The authors have no competing interests to declare

439

440 **Figure legends**

441 **Figure 1. Transcriptome analysis of in vitro-derived human axial progenitors**

442 (A) Immunofluorescence analysis of expression of indicated markers in day 3 hPSC-derived
443 axial progenitors. Magnified regions corresponding to the insets are also shown. (B)

444 Heatmap showing the expression values of selected markers in three independent axial
445 progenitor and hPSC sample replicates. The expression values (FPKM) were scaled to the
446 row mean. The color key relates the heat map colors to the standard score (z-score). (C)

447 Induction of all significantly upregulated HOX transcripts in axial progenitors relative to
448 hPSCs. Paralogous HOX groups corresponding to different axial levels such as cervical

449 (groups 1-4), brachial/thoracic (5-9) and lumbosacral (10-13) are indicated. See also Figure
450 S1 and Table S1.

451 **Figure 2. hNMP cultures exhibit a BMP-dependent neural crest/border signature**

452 (A) Log-fold induction of representative neural crest/neural plate border and BMP-associated
453 transcripts in axial progenitors compared to hESCs. (B) Immunofluorescence analysis of
454 expression of indicated markers in axial progenitos. Magnified regions corresponding to the
455 insets are also shown. (C) Immunofluorescence analysis of expression of phosphorylated
456 SMAD1/5 (p-SMAD) in the presence and absence of the BMP inhibitor LDN193189 (LDN).
457 (D) qPCR expression analysis of indicated markers in axial progenitors in the presence (+)
458 or absence (-) of LDN. Error bars=S.D. (n=2). In all cases nuclei were counterstained with
459 DAPI. PXM, paraxial mesoderm; NC, neural crest. See also Figure S2.

460 **Figure 3. In vitro derived axial progenitors generate trunk neural crest efficiently**

461 (A) Diagram depicting the culture conditions employed to direct trunk NC, posterior
462 neurectoderm (PNE) and paraxial mesoderm (PXM) differentiation from hPSC-derived axial
463 progenitors. (B) Immunofluorescence analysis of the expression of indicated NC (SOX10,
464 SOX9) and neural (SOX1) and the thoracic/trunk marker HOXC9 in trunk NC (TNC) cells
465 derived from axial progenitors after 8 days of differentiation. Magnified regions
466 corresponding to the insets are also shown. Note that the HOXC9/SOX10 and SOX10/SOX1
467 image sets correspond to the same cells. (C) Quantitation of cells marked by different
468 combinations of HOXC9 and SOX10 expression in day 8 trunk NC cultures derived from
469 axial progenitors following image analysis. Six randomly selected fields (two independent
470 experiments) were used for analysis (total number of cells scored= 5366, average number of
471 cells/field=894, error bars=s.d.). (D) Immunofluorescence analysis of TBX6 (left) or SOX1
472 (right) expression in axial progenitors treated with CHIR-FGF2 (pro-PXM conditions) and RA
473 (pro-PNE conditions) respectively. (E) qPCR expression analysis of indicated HOX genes in
474 hPSC-derived anterior cranial (ANC), retinoic acid (RA)-treated NC (+RA), and axial
475 progenitor-derived NC cells (NMP-NC) relative to hPSCs. Error bars=S.E.M. (n=3). (F)
476 qPCR expression analysis of indicated NC markers in +RA and axial progenitor-derived NC

477 cells relative to untreated anterior cranial NC cells. Error bars=S.E.M. (n=3). See also Figure
478 S3.

479 **Figure 4. Transcriptome analysis of in vitro derived neural crest cells corresponding**
480 **to distinct axial levels**

481 (A) Diagrams showing the culture conditions employed for generating NC cells of distinct
482 axial identities using hPSCs. Asterisks indicate the timepoints used for sample harvesting
483 and transcriptome analysis. D, day of differentiation. ANC, Anterior neural crest. (B) Principal
484 component analysis depicting variance between different samples used for microarray
485 analysis (timepoints shown in A). (C) Venn diagram showing the overlap between all
486 significantly upregulated (≥ 2 -fold relative to undifferentiated hESCs, $FDR \leq 0.05$) in each
487 indicated NC group. (D) Log fold induction of HOX genes in indicated NC populations
488 relative to hPSCs. (E) Log fold induction of representative significantly upregulated (≥ 2 -fold
489 relative to undifferentiated hPSCs, $FDR \leq 0.05$) transcripts marking day 6 RA-treated NC
490 cells. (F) Log fold induction of representative significantly upregulated (≥ 2 -fold relative to
491 undifferentiated hPSCs, $FDR \leq 0.05$) transcripts marking day 9 axial progenitor-derived NC
492 cells. (G) Log fold changes in the expression of the most-upregulated and most-
493 downregulated transcripts in day 6 axial progenitor-derived NC precursors compared to d3
494 hPSC-derived axial progenitors. See also Figure S4, Tables S2, S3, and S4.

495 **Figure 5. Axial progenitor-derived trunk neural crest is an optimal source of**
496 **sympathoadrenal cells**

497 (A) Diagram depicting the culture conditions employed to direct axial progenitors (“NMPs”)
498 toward trunk NC and subsequently sympathoadrenal progenitors (SAP) and sympathetic
499 neurons. (B) FACS analysis of PHOX2B-GFP expression in SAP cells derived from axial
500 progenitors as shown in A. Below: Immunofluorescence analysis of PHOX2B-GFP and
501 PHOX2B protein expression following antibody staining. (C) Quantitation of d18
502 differentiated cells positive for the indicated markers in relation to PHOX2B-GFP expression
503 following antibody staining. In each case four randomly selected representative fields were
504 used to obtain the average number of cells/marker. Total numbers of cells scored: GATA3

505 (N=3003), ASCL1 (N=2575), ISL1 (N=2963). (D) Immunofluorescence analysis of PHOX2B-
506 GFP together with the indicated markers in day 18 differentiated SAP/sympathetic neurons
507 derived from axial progenitors as shown in A. (E) qPCR expression analysis of indicated
508 SAP/sympathetic neuron markers in d12 and d18 cultures. Error bars=S.E.M. (n=3). See
509 also Figure S5.

510 **Figure 6. A-P patterning of in vitro derived human NC cells**

511 Diagrammatic model summarising our findings on the in vitro generation of NC subtypes of
512 distinct A-P identity from hPSCs. Examples of unique genes that were found to mark each
513 NC population exclusively are shown in red.

514

515 **Supplemental Figure Legends**

516 **Figure S1**

517 (A) Dendrogram showing the clustering of the individual axial progenitor (NMP) and hESC
518 sample replicates based on RNAseq expression data. (B) Principal Component Analysis
519 (PCA) plot indicates a good separation, in terms of gene expression, between the two
520 conditions and a good replication among samples. ((C) Volcano plot reporting pvalue (y axis)
521 as a function of log₂ fold change (x axis) between the axial progenitor and hPSC groups.
522 The red dots depict significant genes (pvalue<=0.05). Top differentially expressed
523 transcriptional regulators of axis elongation and WNT/FGF/NOTCH/RA signalling pathway
524 components are shown. (D) Top gene ontology groups significantly enriched in axial
525 progenitors vs hPSCs. (E) Hierarchical clustering heatmap showing the expression values of
526 HOX genes in axial progenitor and hPSC sample replicates. The expression values (FPKM)
527 were scaled to the row mean. The color key relates the heat map colors to the standard
528 score (z-score).

529 **Figure S2.**

530 (A) qPCR expression analysis of indicated NC/border and pluripotency markers in axial
531 progenitors vs hPSCs. Error bars=S.E.M. (n=3). (B) Immunofluorescence analysis of SOX9

532 expression together with MSGN1-VENUS (top) and TBX6 (bottom) in axial progenitors
533 derived from a MSGN1-VENUS or wild type hPSCs respectively.

534 **Figure S3**

535 (A) Immunofluorescence analysis of indicated markers one (day 4 of differentiation) or two
536 (day 5 of differentiation) days after re-plating axial progenitors into NC inducing conditions.
537 (B) qPCR expression analysis of indicated axial progenitor (NMP) and neural markers during
538 differentiation toward TNC. Error bars=s.e.m. (n=3) (C) Immunofluorescence analysis of
539 indicated NC markers one (day 4 of differentiation) or two (day 5 of differentiation) days after
540 re-plating axial progenitors into NC inducing conditions.

541 **Figure S4**

542 (A) Heatmap showing Pearson's signal correlation between indicated samples. (B) Venn
543 diagram showing the overlap between transcription factors (TFs) enriched (≥ 2 -fold relative to
544 undifferentiated hPSCs, $FDR \leq 0.05$) in each indicated NC group of distinct axial identity.
545 The detailed list of all TFs can be found in Table S3. (C) Heatmap showing relative
546 expression of representative NC markers in NC populations of distinct axial identity and
547 hPSCs. (D) Heatmap showing relative expression of representative NC markers in trunk and
548 anterior cranial NC precursors and hPSCs. (E) Volcano plot depicting upregulated (red) and
549 downregulated (green) genes in d6 trunk NC precursors (NMP-NC) vs axial progenitors
550 (=NMPs).

551 **Figure S5**

552 (A) Immunofluorescence analysis of PHOX2B-GFP and ASCL1 expression in d12 SAP cells
553 derived from axial progenitors as shown in Fig. 5A. (B) Immunofluorescence analysis of
554 PRPH and HOXC9 expression in sympathetic neurons derived from axial progenitors as
555 shown in Fig. 5A.

556

557 **Table S1.** Significantly up- and downregulated transcripts, GO enrichment and TF
558 signatures from RNAseq analysis

559 **Table S2.** List of genes upregulated in different NC populations and GO enrichment analysis

560 **Table S3.** List of transcription factors shared between different NC populations

561 **Table S4.** List of all genes up- and down-regulated in indicated NC populations and their
562 progenitors.

563 **Table S5.** List of primers

564

565 **METHODS**

566 **Cell culture and differentiation**

567 We employed the following hPSC lines: a Shef4-derived Sox2-GFP reporter hESC line (44),
568 the H9-derived SOX10-GFP (15) and PHOX2B-GFP (18) reporter hESC lines, the MSGN1-
569 VENUS reporter hiPSC line, and the unmodified Mastershef7 hESC line (44) and an iPSC
570 line (MIFF-1) derived from a healthy individual (85). The MSGN1-Venus reporter line was
571 generated by Transposon mediated BAC transgenesis using protocols described by (86). In
572 brief, a human BAC (RP11-12L16) with piggyBac transposon repeats flanking the bacterial
573 backbone and with Venus inserted directly after the initiating methionine of MSGN1 was
574 transfected together with a piggyBac Transposase into NCRM1 iPSCs. Cells were cultured
575 in feeder-free conditions in either Essential 8 (Thermo Fisher) or mTeSR1 (Stem Cell
576 Technologies) medium on laminin 521 (Biolamina) or vitronectin (Thermo Fisher). All
577 differentiation experiments were carried out in at least three different hPSC line. For
578 NMP/axial progenitor differentiation hPSCs were dissociated using PBS/EDTA and plated at
579 a density of 55,000 cells/cm² (density optimised for 12-well plates) on fibronectin (Sigma) or
580 vitronectin (Thermo Fisher)-coated wells, directly into NMP-inducing medium containing
581 CHIR99021 (Tocris), FGF2 (20 ng/ml, Peprotech) and ROCK inhibitor Y-27632 for the first
582 only day (10 μM, Tocris). We observed some variation in terms of induction of T+SOX2+
583 NMPs both between hPSC lines and also batches of CHIR99021 and thus the concentration
584 of the latter was varied between 3-4 μM. BMP inhibition was carried out using LDN193189
585 (Tocris) at 100 nM. For trunk NC differentiation day 3 hPSC-derived axial progenitors were
586 dissociated using accutase and re-plated at a density 30,000 cells /cm² on Geltrex (Thermo
587 Fisher)-coated plates directly into NC-inducing medium containing DMEM/F12 (Sigma), 1x

588 N2 supplement (Thermo Fisher), 1x Non-essential amino acids (Thermo Fisher) and 1x
589 Glutamax (Thermo Fisher), the TGFb/Nodal inhibitor SB431542 (2 μ M, Tocris), CHIR99021
590 (1 μ M, Tocris), DMH1 (1 μ M, Tocris), BMP4 (15 ng/ml, Thermo Fisher) and Y-27632 (10
591 μ M, Tocris). The medium was replaced at days 5 and 7 of differentiation but without the
592 ROCK inhibitor and trunk NC cells were analysed either at day 8 or 9. For cranial neural
593 crest differentiation hPSCs were dissociated using accutase and plated under the same NC-
594 inducing conditions as described above for 5-6 days. For posterior cranial/vagal/cardiac NC
595 generation d4 differentiated anterior NC progenitors induced as described above were
596 treated with retinoic acid (1 μ M, Tocris) in the presence of the NC-inducing medium till day 6
597 of differentiation. For sympathoadrenal progenitor (SAP) differentiation d8 trunk NC cells
598 were re-suspended at a density of 200-300,000 cells /cm² on Geltrex (Thermo Fisher)-
599 coated plates directly into medium containing BrainPhys neuronal medium (Stem Cell
600 Technologies), 1x B27 supplement (Thermo Fisher), 1x N2 supplement (Thermo Fisher), 1x
601 Non-essential amino acids (Thermo Fisher) and 1x Glutamax (Thermo Fisher), BMP4 (50
602 ng/ml, Thermo Fisher), recombinant SHH (C25II) (50 ng/ml, R&D) and purmorphamine
603 (1.25-1.5 μ M, Millipore) and cultured for 4 days (d12 of differentiation). For further
604 sympathetic neuron differentiation d12 SAP cells were switched into a medium containing
605 BrainPhys neuronal medium (Stem Cell Technologies), 1x B27 supplement (Thermo Fisher),
606 1x N2 supplement (Thermo Fisher), 1x Non-essential amino acids (Thermo Fisher) and 1x
607 Glutamax (Thermo Fisher), ascorbic acid (200 μ M, Sigma) , NGF (10 ng/ml, Peprtech),
608 BDNF (10 ng/ml, Peprtech) and GDNF (10 ng/ml, Peprtech) and cultured for a further 6-8
609 days. For paraxial mesoderm differentiation d3 axial progenitor cultures were treated with
610 accutase and replated at a density of 45,000/cm² on 12-well Geltrex-coated plates in N2B27
611 containing FGF2 (40 ng/ml, Peprtech) and CHIR99021 (8 μ M, Tocris) for two days. For
612 neural differentiation d3 axial progenitor cultures were treated with accutase and replated at
613 a density of 45,000/cm² on 12-well Geltrex-coated plates in N2B27 containing 100 nM
614 retinoic acid (Tocris) for 2-3 days.

615 **RNA sequencing**

616 *Sample preparation*

617 For RNA sequencing we employed hESCs or axial progenitors following culture on
618 fibronectin in FGF2 (20 ng/ml) and CHIR99021 (3 μ M). Total RNA from NMPs and hESCs
619 was harvested using the RNeasy kit (Qiagen) according to the manufacturer's instructions.

620 *Library preparation/sequencing*

621 Total RNA was processed according to the TruSeq protocol (Illumina). Three separate RNA
622 libraries (biological replicates) were barcoded and prepared for hPSCs and D3 axial
623 progenitors. Library size, purity and concentration were determined using the Agilent
624 Technologies 2100 Bioanalyzer. For sequencing, four samples were loaded per lane on an
625 Illumina Genome Analyzer Hiseq2500.

626 *RNAseq quality control and mapping*

627 The quality of raw reads in fastq format was analyzed by FastQC
628 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Adapter contamination and poor
629 quality ends were removed using Trim Galore v. 0.4.0 (Babraham Bioinformatics - Trim
630 Galore! Available at: http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/).
631 Single-end clean reads were aligned to the human reference genome (hg38 assembly) using
632 Tophat2 v2.0.13 (87).

633 *RNA seq data analysis*

634 Read alignments were sorted with SAMtools v1.1 before being counted to genomic features
635 by HTSeq version 0.6.0 (88). The average overall read alignment rate across all samples
636 was 94.3%. Differential gene expression was performed using DESeq2 version 1.16.1 (89).
637 in R version 3.3.3. Genes were considered significantly differentially expressed (DE) with a
638 Benjamini-Hochberg adjusted pvalue ≤ 0.05 and a \log_2 FoldChange $> |1|$. Gene Ontology
639 (GO) biological processes (BP) enrichment analysis was carried out for DE genes using the
640 DAVID gene ontology functional annotation tool (<https://david.ncifcrf.gov/>)
641 (90, 91) with default parameters. We considered as significant terms having a FDR adjusted
642 pvalue ≤ 0.05 , which is derived from a modified Fisher's exact test.

643

644 **Microarrays**

645 *Sample preparation and processing*

646 Samples were prepared according to the Affymetrix WT Plus protocol for Gene Chip ®
647 Whole Transcript Expression Arrays. Briefly 200ng of high quality total RNA, (RNA integrity
648 number (RIN) greater than 9), was converted to double stranded cDNA with the introduction
649 of a T7 polymerase binding site. This allowed the synthesis of an antisense RNA molecule
650 against which a sense DNA strand was prepared. The RNA strand was digested and the
651 resulting single stranded DNA fragmented and biotin labelled. Along with appropriate
652 controls the labelled fragmented DNA was hybridised to Affymetrix Clariom D arrays
653 overnight using the Affymetrix 640 hybridisation oven; 16 hours with rotation at 60 rpm at
654 45°C. The arrays were washed and stained according to standard protocols which allowed
655 the introduction of streptavidin-phycoerythrin in order to generate a fluorescent signal from
656 the hybridised biotinylated fragments. The washed and stained arrays were scanned using
657 the Affymetrix 3000 7G scanner with autoloader. The generated CEL files were taken
658 forward for analysis.

659 *Data analysis*

660 Data were analysed using the Affymetrix Transcriptome Analysis Console 4.0 software.
661 Analysis of Expression (Gene+Exon) was used to generate lists of all differentially
662 expressed genes showing >2; <-2 fold Log Change and P<0.05. For the distance matrix
663 (**Figure S4A**), Exploratory Grouping analysis was used. Log₂ normalised intensity data
664 values were mapped in R using the package 'pheatmap' with correlation clustering by gene.
665 Gene ontology analysis was carried out using the ToppGene suite
666 (<https://toppgene.cchmc.org/enrichment.jsp>) (92). Area proportional 3-Venn diagrams were
667 drawn using the eulerApe software (93).

668 **Quantitative real time PCR**

669 Total RNA from different samples was harvested using the RNeasy kit (Qiagen) according to
670 the manufacturer's instructions and digested with DNase I (Qiagen) to remove genomic
671 DNA. First strand cDNA synthesis was performed using the Superscript III system (Thermo

672 Fisher) using random primers. Quantitative real time PCR was carried out using the Applied
673 Biosystems QuantStudio™ 12K Flex thermocycler together with the Roche UPL system.
674 Primer sequences are shown in Table S5.

675 **Flow cytometry**

676 Cells were lifted into a single cell suspension using Accutase (as previously described) and
677 resuspended in FACS buffer (DMEM with 10% v/v FCS) to neutralise Accutase before
678 centrifugation at 1100rpm/4min. Cells were then resuspended in FACS buffer at 1x10⁶
679 cells/ml and 100,000 cells were placed in FACS tubes (Falcon 352053). A GFP baseline was
680 set using unmodified wild type control cells.

681 **Immunofluorescence**

682 Cells were fixed for 10 minutes at 4°C in 4% paraformaldehyde (PFA) in phosphate buffer
683 saline (PBS), then washed in PBST (PBS with 0.1% Triton X-100) and treated with 0.5 M
684 glycine/PBST to quench the PFA. Blocking was then carried for 1-3 h in PBST supplemented
685 with 3% donkey serum/1% BSA at room temperature or overnight at 4°C. Primary and
686 secondary antibodies were diluted in PBST containing in PBST supplemented with 3%
687 donkey serum/1% BSA. Cells were incubated with primary antibodies overnight at 4°C and
688 with secondary antibodies at room temperature for 2 h in the dark. Cell nuclei were
689 counterstained using Hoechst 33342 (1:1000, Thermo Fisher) and fluorescent images were
690 taken using the InCell Analyser 2500 system (GE Healthcare). We used the following
691 antibodies: anti-T (1:200; AF2085, R&D), anti-SOX2 (1:200; ab92494, Abcam), anti-SOX9
692 (1:200; 82630, CST), anti-SNAI2 (1:400; C19G7, CST), anti-PAX3 (1:50; DSHB), anti-
693 phosphoSMAD1/5/9 (1:100; D5B10, CST), anti-SOX10 (1:200; D5V9L, CST), anti-SOX1
694 (1:100; AF3369, R&D), anti-TBX6 (1:50, AF4744, R7D) anti-TH (1:1000; T1299, SIGMA),
695 HOXC9 (1:50; ab50839, Abcam) anti-PRPH (1:100; AB1530, Millipore), anti-CDX2 (1:200;
696 ab76541, Abcam), anti-ASCL1 (1:100; 556604, BD Pharmingen), anti-GATA3 (1:100; sc-269,
697 Santa Cruz), anti-GFP (1:1000; ab13970, Abcam), anti-ISL1 (1:100, DSHB), anti-PHOX2B
698 (1:100; sc-376997, Santa Cruz). Images were processed using Photoshop and Fiji. Nuclear

699 segmentation followed by single cell fluorescence quantification was performed as described
700 previously using Fiji and the MultiCell3D application as described previously (35, 94).

701 **Data availability**

702 The microarray and RNAseq data have been deposited to GEO (GSE109267 and
703 GSE110608).

704

705

706

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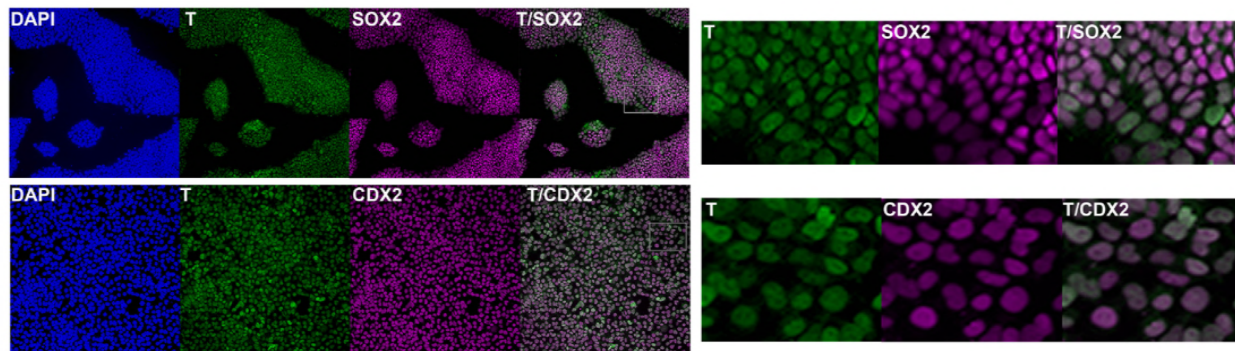
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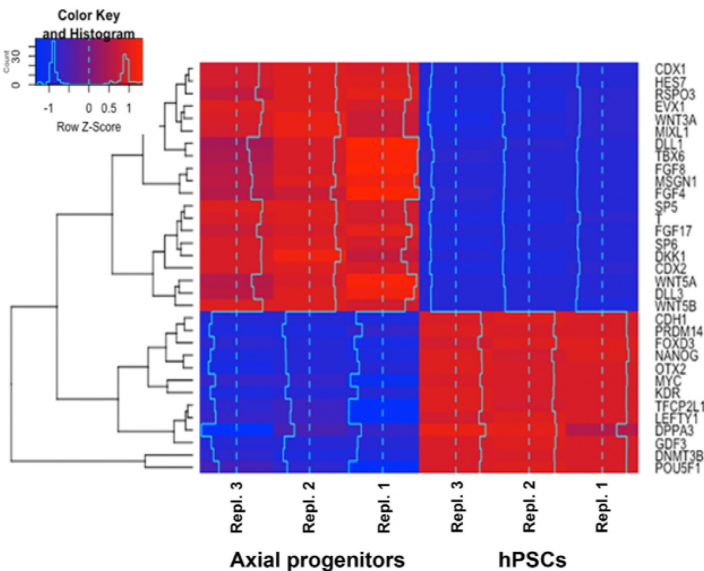
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Fig.1

A



B



C

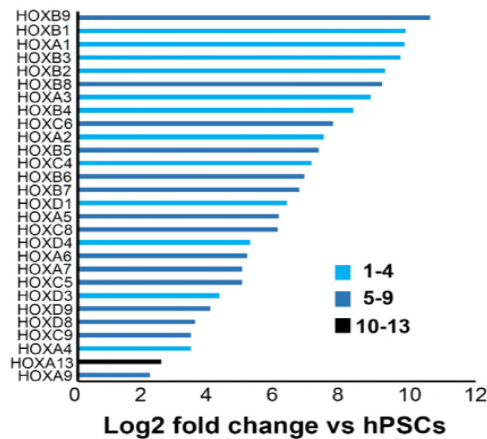


Fig. 2

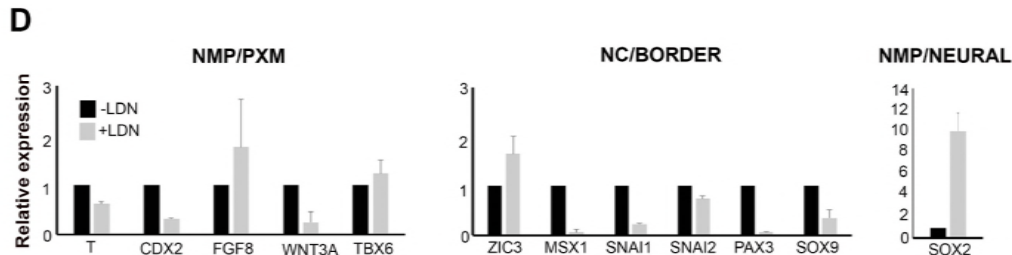
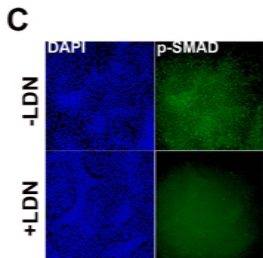
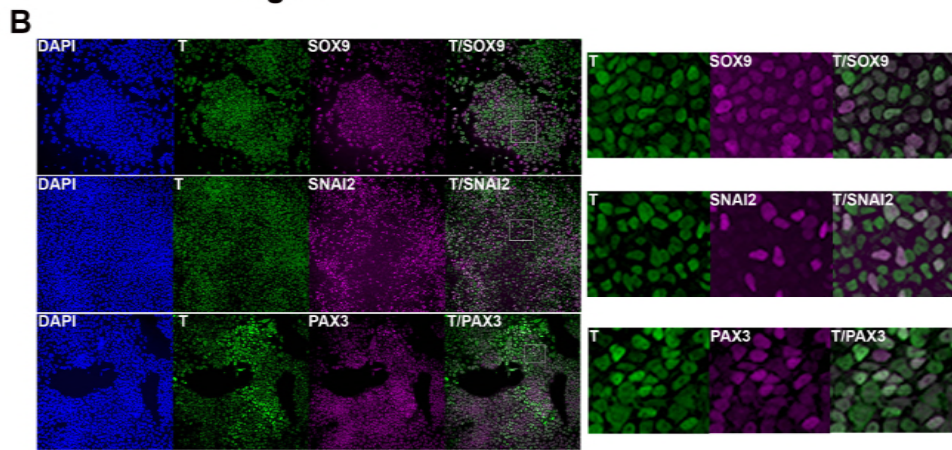
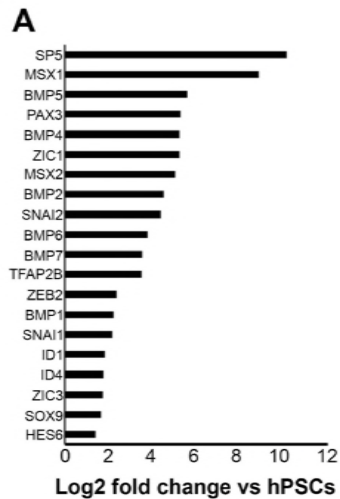


Fig. 3

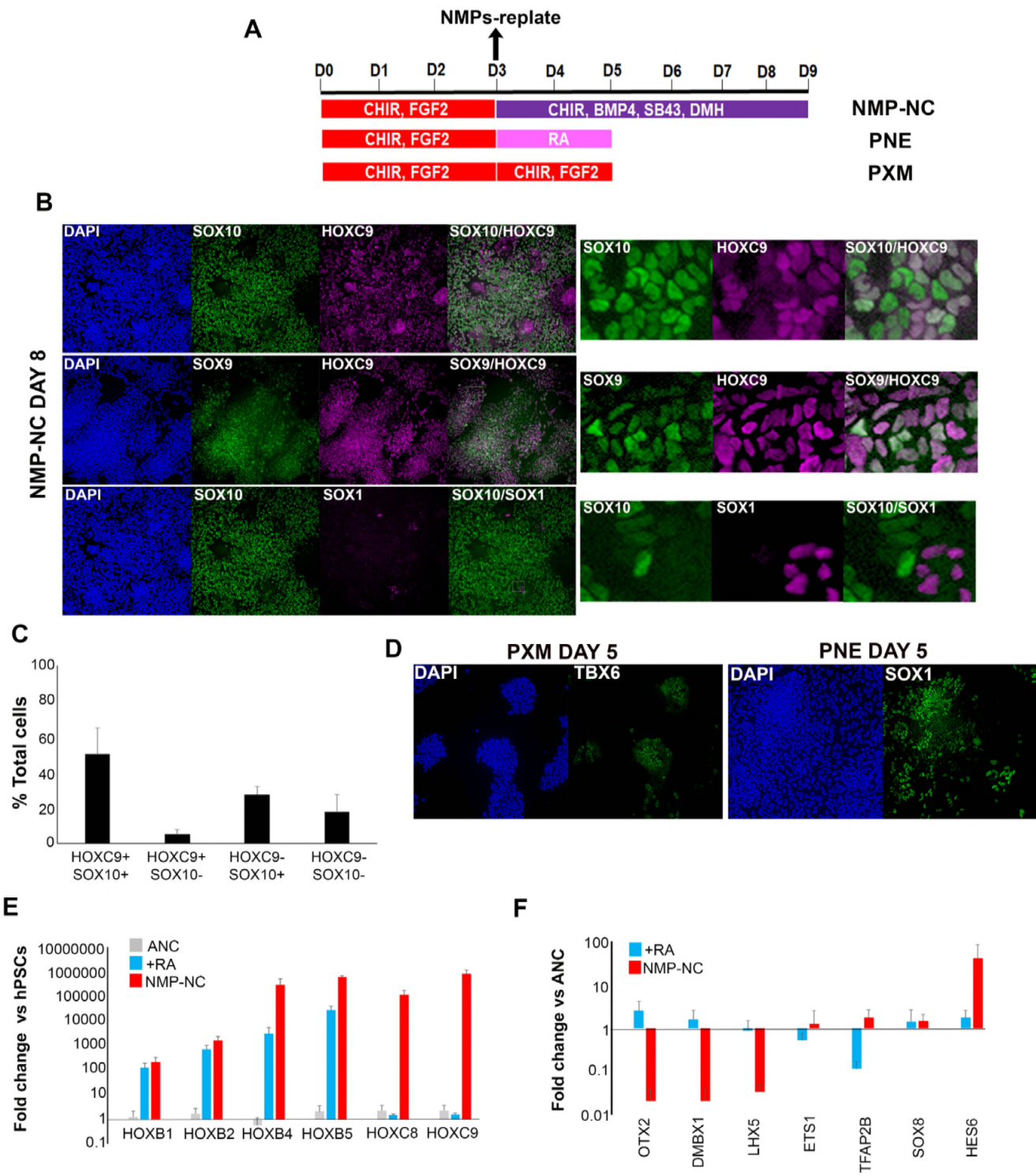


Fig. 4

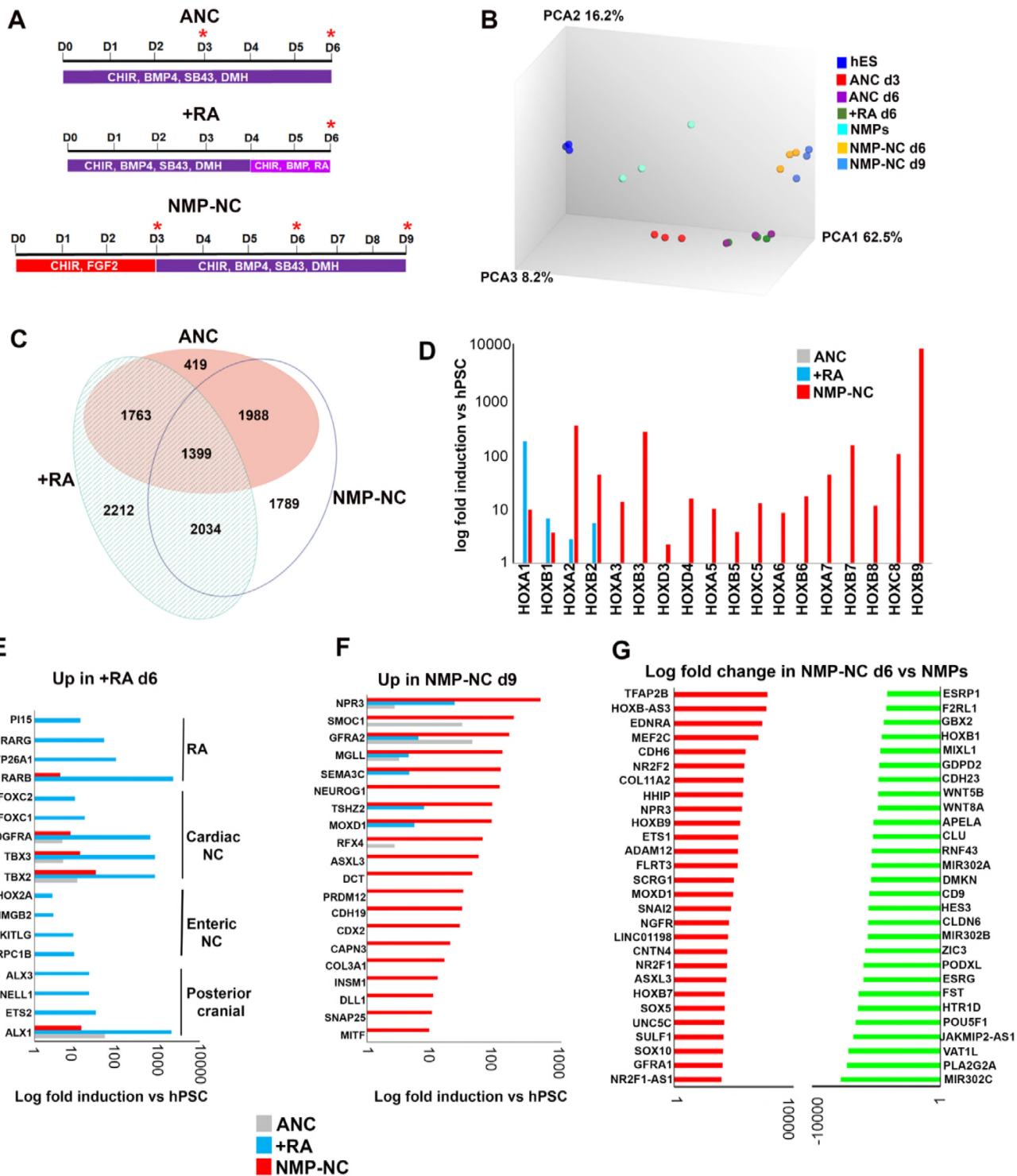


Fig. 5

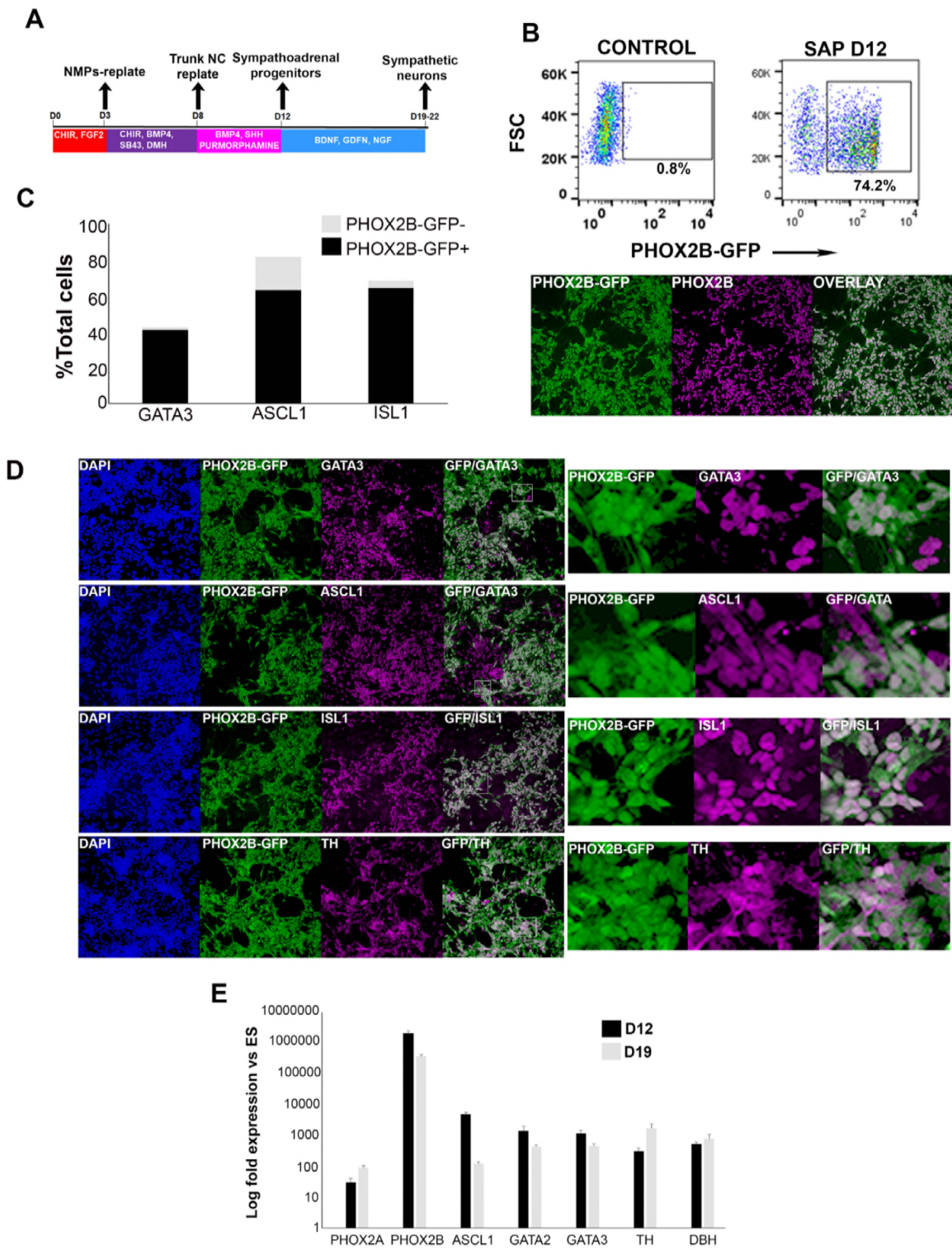


Fig. 6

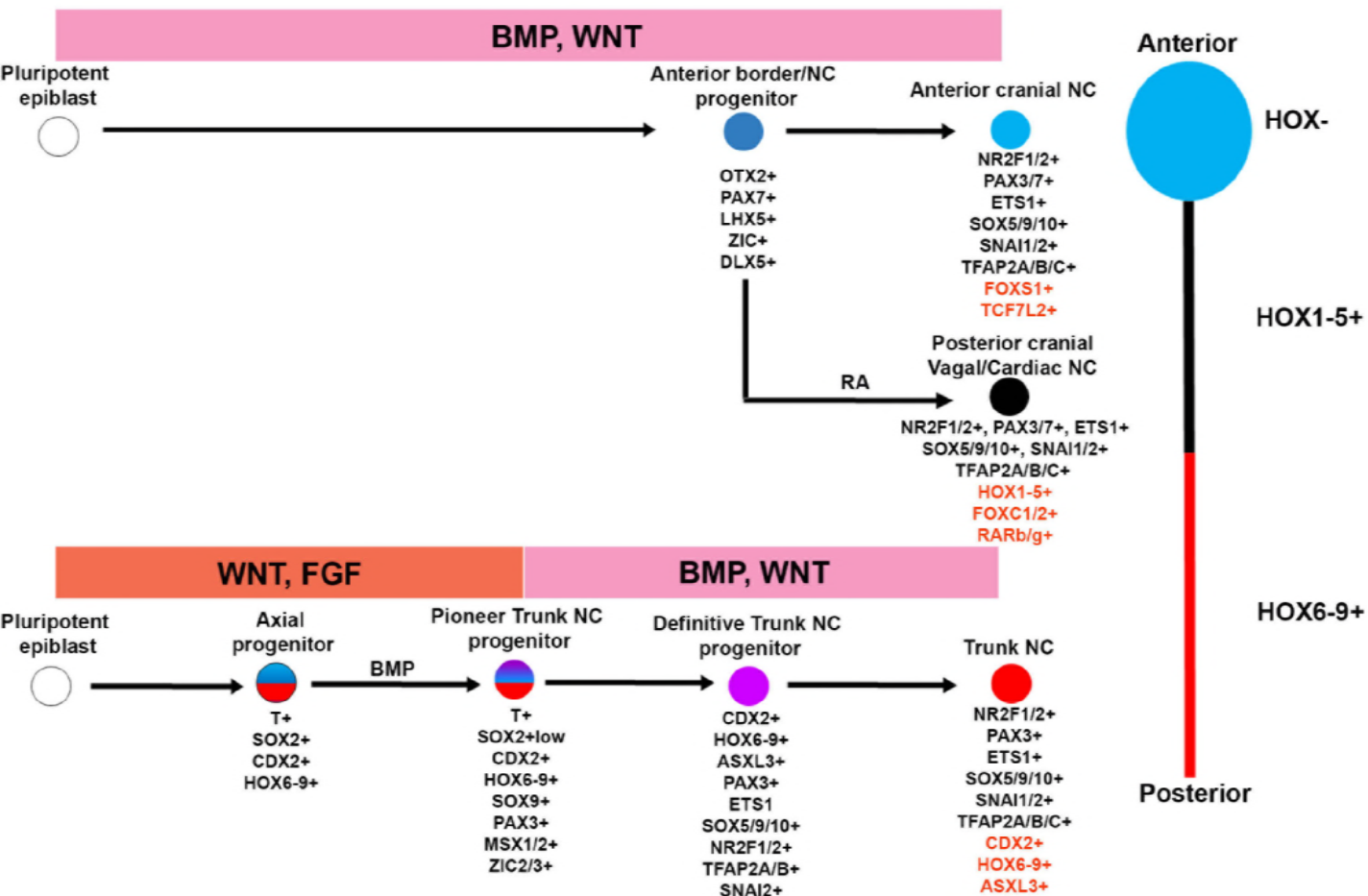


Fig. S1

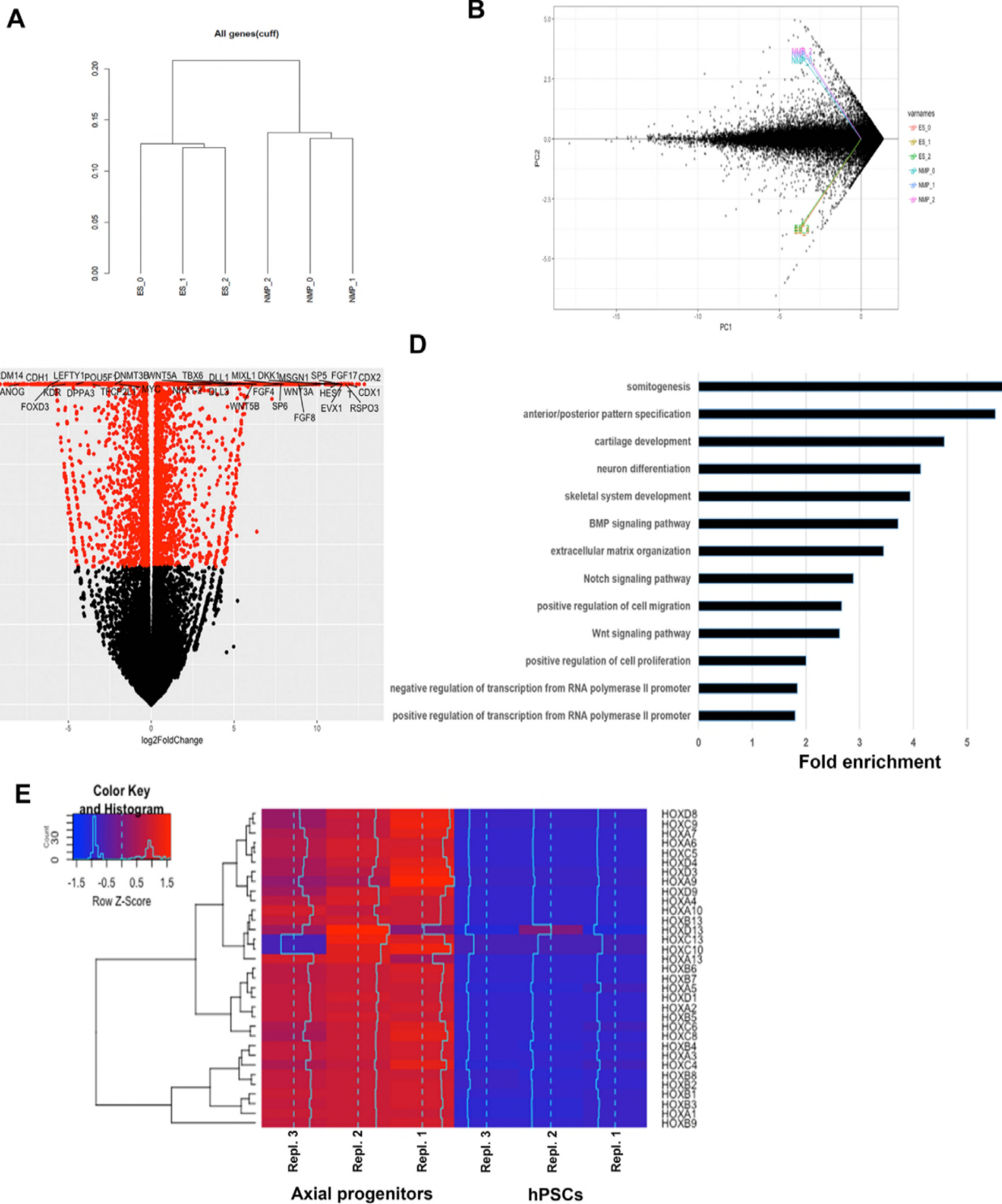
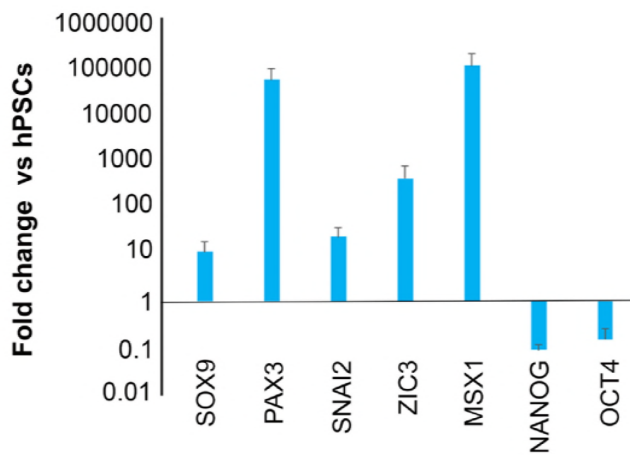


Fig. S2

A



B

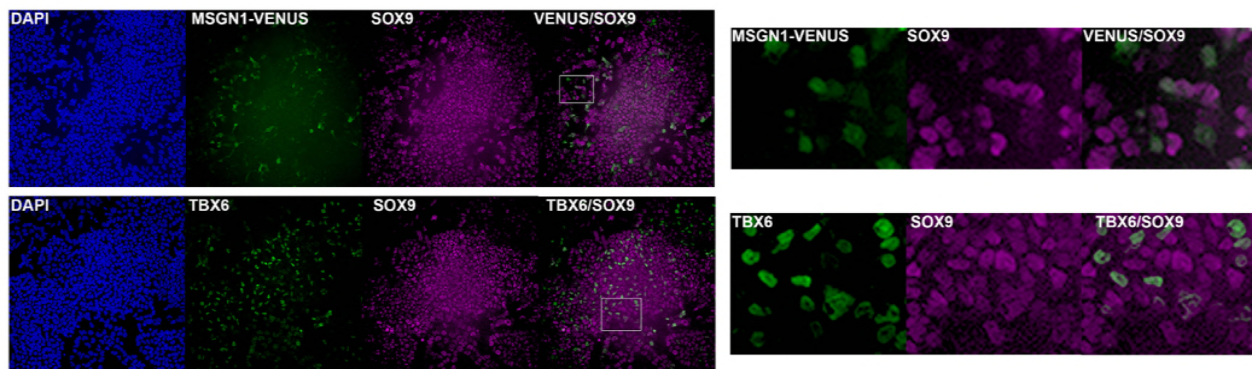
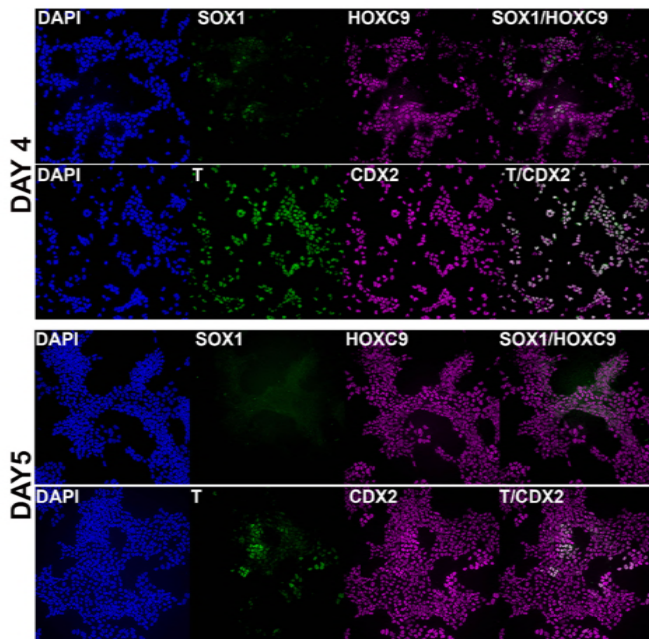
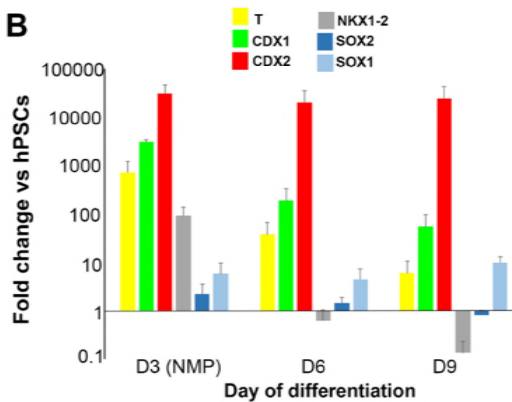


Fig. S3

A



B



C

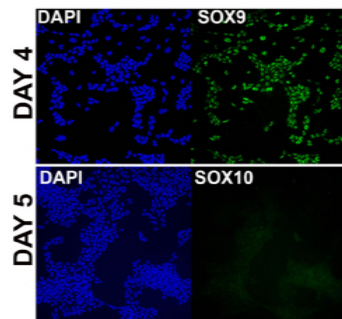


Fig. S4

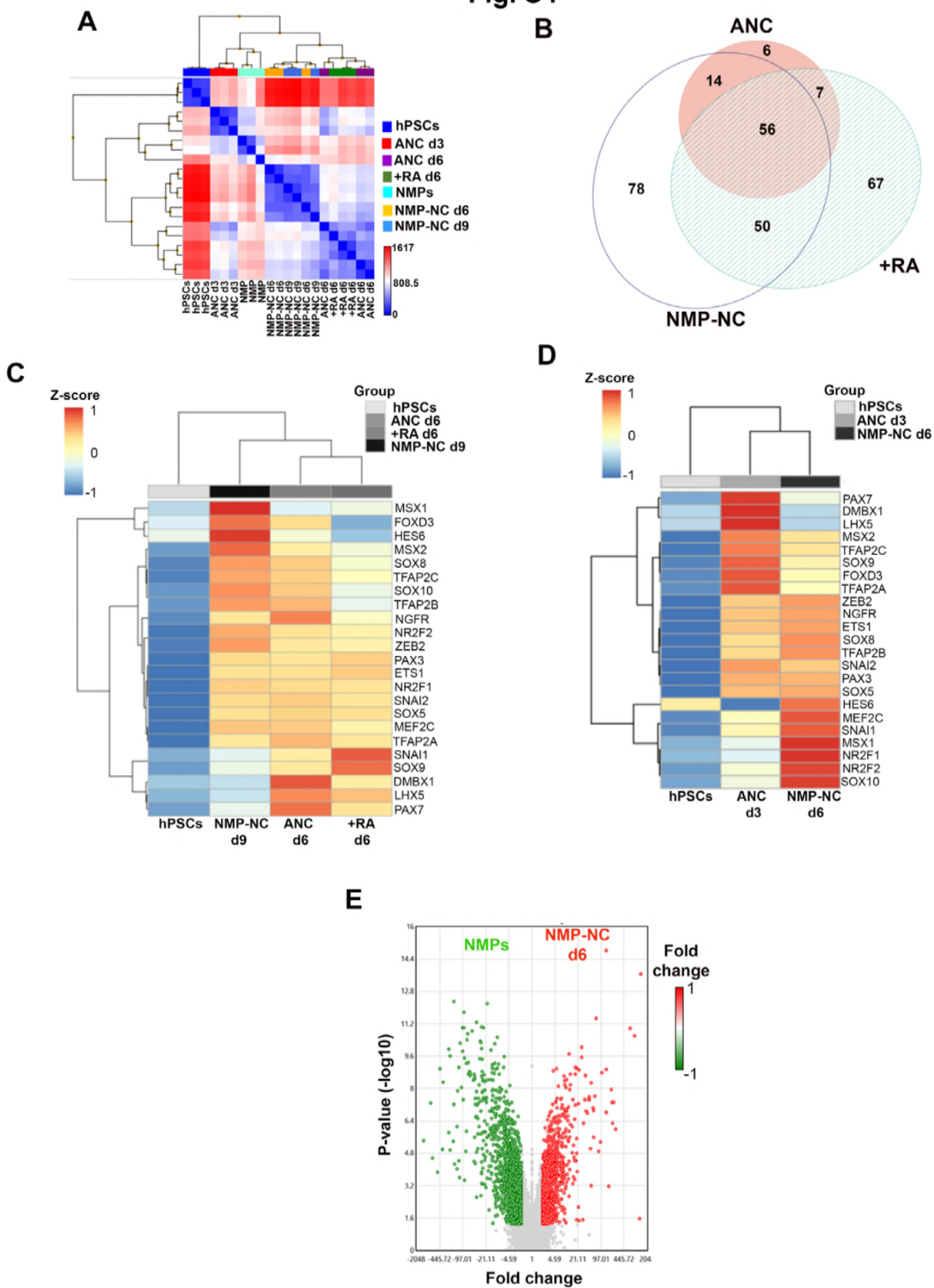
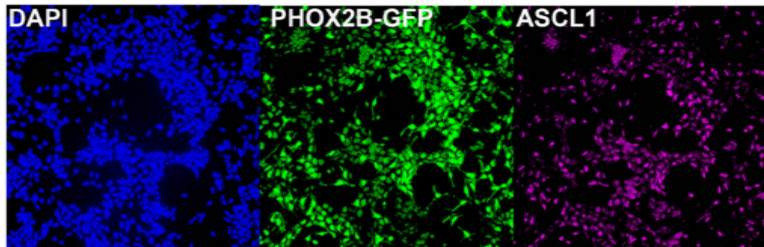


Fig. S5

A



B

