

# 1 **The embryonic node functions as an instructive stem cell niche**

2

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9

10 **In warm-blooded vertebrate embryos (mammals and birds), the body forms from a**  
11 **growth zone at the tail end. Hensen's node, a region which induces and patterns the**  
12 **neural axis is located within this growth zone. The node also contains the precursors of**  
13 **neural, mesodermal and endodermal structures along the midline and has been suggested**  
14 **to contain a small population of resident stem cells. However, it is unknown whether the**  
15 **rest of the node constitutes an instructive stem cell niche, specifying stem cell behaviour.**  
16 **Here we combine transplantation of a single cell in vivo with single-cell mRNA sequencing**  
17 **in the chick and show that when made to enter the node, non-node-progenitor cells**  
18 **become resident and gain stem cell behaviour. These cells preferentially express G2/M**  
19 **phase cell-cycle related genes and are concentrated in posterior sub-regions of the node.**  
20 **The posterior part of the node therefore behaves as an instructive stem cell niche. These**  
21 **results demonstrate a new function for the vertebrate node during development.**

22

23 In higher vertebrate embryos, the body axis forms in head-to-tail direction from a growth zone  
24 at the tail end which is present from gastrula stages through to the end of axis elongation.  
25 During gastrulation (in chick: stage HH3+ to HH4)<sup>1</sup>, epiblast cells lateral to the anterior tip of  
26 the streak/node ingress into it. After this, the node begins to regress caudally<sup>2</sup> as cells exit the  
27 node to lay down the midline of the developing head-tail axis (Fig. 1 a-c)<sup>3-6</sup>. However,  
28 transplantation of cell groups and fate mapping experiments in chick<sup>4,7-9</sup> and mouse<sup>10-14</sup> during  
29 early development have suggested that the node contains some resident, self-renewing cells  
30 that persist during axial elongation in the node, while other cells leave (Fig. 1c, ‘RC’). Could  
31 the former be stem cells<sup>15</sup>, specified by neighbouring node cells?

32

33 There are two possibilities: either there is a special population of stem cells set aside during  
34 early development that is maintained by the node environment (“permissive”), or the node  
35 constitutes a special niche that can instruct any cell to acquire self-renewing stem cell  
36 characteristics<sup>16-18</sup>. To demonstrate self-renewal and to test whether the node is an instructive  
37 stem cell niche, it is critical to challenge the responses of an individual cell to the node  
38 environment. Here we use transplantation of single cells *in vivo* and single-cell RNA  
39 sequencing (scRNA-seq) to describe a cell’s response to the node.

40

#### 41 **Non-node cells can become resident**

42 To test whether the node environment can impart resident behaviour onto other cells, we  
43 grafted anterior epiblast (which never normally enters the node<sup>5,19-21</sup>) to a position adjacent to  
44 the HH3+/4 node, so that transplanted cells would be carried into the node by gastrulation  
45 movements (Fig. 1f). Graft-derived cells (from the transgenic GFP-donor) give rise to axial  
46 tissues and express appropriate molecular markers of node, notochord and somite (Fig. 1g,

47 Extended Data Fig. 1a-j). Importantly, the contribution of this anterior epiblast to cells with  
48 resident behaviour (88%, n=30/34) is similar to that of lateral epiblast (Fig1. d-e, Extended  
49 Data Fig. 1k) (89%, n=8/9), which does normally enter the node. These results show that the  
50 node can confer resident behaviour and axial identity.

51

### 52 **Prospective node cells are plastic**

53 To test whether cells are intrinsically committed to node and axial identities, we prevented  
54 lateral epiblast cells from entering the node by grafting them into a remote anterior position  
55 (Fig. 1j). After culture to HH8-10, graft-derived cells localize to and resemble head structures  
56 rather than node derived tissues and fail to express the node marker, chordin (Fig. 1k). Lateral  
57 cells therefore develop according to their new anterior position<sup>5,19-21</sup> (Fig. 1h-i), demonstrating  
58 that cells normally destined to give rise to node and axial identities are not committed to these  
59 before they enter the node.

60

### 61 **Self-renewal specified by a node niche**

62 We then asked whether resident cells specified by the node are stem cells by testing for self-  
63 renewal, a key characteristic of a stem cell<sup>22-24</sup>. First, anterior epiblast was made to enter the  
64 node by grafting adjacent to it at HH3+/4 (Extended Data Fig. 2a-b). Following culture to HH8-  
65 10, two to ten GFP-positive cells remaining in the node were re-grafted into a second, younger  
66 (HH3+/4) host node (Extended Data Fig. 2c-f) to determine whether the GFP-positive cells can  
67 self-renew and contribute daughters to the developing axis for a second time. GFP-positive  
68 cells contributed to both node and axis in 17% of embryos (n=4/23) (Extended Data Fig. 2g-i)  
69 suggesting self-renewal.

70

71 To demonstrate this at the single-cell level we repeated the re-grafts (again using two  
72 successive hosts) with just a single GFP-positive resident cell (Fig. 2). After culture of the  
73 second host to HH8-10 (Fig. 2e-f), GFP-positive cells were detected in 21% of grafted embryos  
74 (n=11/53) (Extended Data Fig. 2j), of which 36% had multiple GFP-positive cells, showing  
75 that cell division had occurred; 18% had GFP-positive cells in both node and axis revealing  
76 that the resident cells both self-renewed and contributed to the axial midline (Fig. 2f-g). The  
77 node's ability to specify self-renewing resident behaviour from cells not normally destined to  
78 enter it clearly demonstrates the properties of an instructive niche<sup>16-18</sup>.

79

80 To test whether this niche relies on neighbouring supporting cells, re-grafts of an individual  
81 GFP-positive cell were performed alone (n=26), or attached to a few neighbouring GFP-  
82 negative cells from the first host (n=27). While survival and division of GFP-positive cells was  
83 comparable between these two conditions, GFP-positive cells attached to GFP-negative  
84 neighbours showed an increased contribution to the node when compared to GFP-positive cells  
85 grafted alone (40% versus 17%) (Extended Data Fig. 2j). This finding is also consistent with  
86 the idea that the node behaves like an instructive niche, inducing self-renewal behaviour on  
87 other cells.

88

### 89 **Properties of node sub-regions**

90 Does the entire node act as a niche, or is this property located in a particular sub-region? To  
91 identify the regions containing long-term resident cells, we constructed a fate map of the node  
92 by labelling each of six sub-regions using a lipophilic dye (DiI) at HH8 (Extended Data Fig.  
93 3a). After culture to HH11-12, all cells from anterior sub-regions had come out from the node  
94 and the middle sub-regions contributed to anterior parts of the later node (chordoneural hinge),  
95 while only the posterior sub-regions continued to contribute to the entire older node and its

96 derivatives (Fig. 3a-b, Extended Data Fig. 3b-g). This suggests that resident cells remaining in  
97 the node the longest are confined to posterior sub-regions. Consistent with this, in the re-  
98 grafting experiments described earlier, the single cell remaining in the node was always found  
99 in the posterior sub-region (Fig. 2f-g). Furthermore, live imaging of embryos in which a mosaic  
100 of cells was fluorescently-labelled revealed endogenous resident cells remaining in the  
101 posterior node as it regresses (HH5-9), whereas most cells from other regions of the node were  
102 left behind to contribute to the axis (Supplementary Movie 1). This is consistent with fate  
103 mapping results of the mouse node, showing that some labelled cells remain in the node-  
104 primitive-streak border as the axis forms<sup>14,25</sup>. The posterior node is therefore the strongest  
105 candidate for an axial stem cell niche in amniotes. This is further supported by findings that  
106 when the posterior part of the node is removed, axial elongation is impaired<sup>9,25</sup>.

107

### 108 **Molecular properties of resident cells**

109 What are the molecular characteristics of cells residing in this posterior niche? We grafted  
110 GFP-epiblast from next to the node to the same position in a wild-type host and cultured the  
111 embryos to HH8. Single graft-derived cells were collected from the posterior, middle and  
112 anterior regions of the HH8 node, and processed for scRNA-seq using SmartSeq (Fig. 3c). The  
113 data were examined by Principal Component (PC) analysis (Extended Data Fig. 5a); the first  
114 component (PC1) groups cells into two clusters, one composed largely of posterior cells (Fig.  
115 3d). To identify the genes causing this clustering, we calculated the correlation coefficient of  
116 genes for PC1. Thirty-seven genes have significant expression in the posterior cluster  
117 (correlation coefficient  $<-0.8$ ); the majority of these (31/37) encode proteins of the G2/M  
118 phases of the cell cycle (Fig. 3e-f, Extended Data Fig. 4). This suggests that these cells are  
119 preparing to divide. Graft-derived cells isolated from other parts of the node appear to be  
120 randomly distributed in other phases of the cell cycle (Extended Data Fig. 5b-f).

121

122 To determine whether the posterior region of the node is a unique site for cells at the G2/M  
123 phases of the cycle, we compared the transcriptomes of entire node sub-regions at HH8  
124 (Extended Data Fig. 7a). Half of the cell-cycle related genes identified in the scRNA-seq were  
125 also enriched in the posterior node as a whole, while the other half, including CDK1, were  
126 instead enriched in anterior regions (Fig. 3f versus 3g, Extended Data Fig. 6). Expression of  
127 these G2/M cell-cycle related genes is therefore specific to individual resident cells, but not  
128 necessarily to their micro-environment. This suggests that dividing resident cells may make up  
129 only a small proportion of the node niche. Interestingly, the transcriptomes of the sub-regions  
130 reveal an enrichment of genes involved in Wnt, Notch and FGF signalling in the posterior part  
131 of the node (Extended Data Fig. 7). These three pathways have been implicated in other stem  
132 cell niches<sup>18</sup>.

133

#### 134 **Competence to respond to the node niche**

135 If anterior epiblast cells can acquire resident, self-renewing behaviour in response to the node  
136 environment, can any epiblast cell be similarly induced? To answer this, epiblast from older  
137 stage embryos (HH4+/5, corresponding to prospective neural plate) was used as donor tissue  
138 (Fig. 4a-b)<sup>3,26</sup>. This later HH4+/5 epiblast was forced to enter a younger (HH3+/4) node by  
139 grafting just adjacent to it (Fig. 4c-e). After culture to HH8-11, graft-derived cells from these  
140 late-to-early transplants contributed to the same axial and paraxial structures as the grafts from  
141 younger donors described earlier (Extended Data Fig. 8a). However, late epiblast gave rise to  
142 caudal node and/or chordoneural hinge in fewer embryos (55%) than lateral or anterior epiblast  
143 (89% and 88% respectively) suggesting that late epiblast cells are less able to respond to the  
144 node environment. Late epiblast cells also give rise to mesodermal structures (notochord and  
145 PSM/medial somite) less frequently than either lateral or anterior epiblast, and some that do,

146 fail to express appropriate mesodermal genes (Fig. 4e). In contrast, late grafts contribute more  
147 frequently to neural structures (floorplate, 45% and lateral neural plate, 27%) than younger  
148 lateral epiblast (33% and 11% respectively) (Extended Data Fig. 8a). At the time of grafting,  
149 donor late epiblast cells already express neural plate markers (including SOX2 and ZEB2)  
150 (Extended Data Fig. 8b-g), but following grafting they lose expression of these genes except  
151 for descendants contributing to neural structures (Extended Data Fig. 8h-k). This suggests that  
152 the node environment causes late epiblast cells to lose their neural plate identity but is not  
153 sufficient to convert them fully into axial mesoderm. This transition away from mesodermal  
154 and towards neural fates appears to take place around stages HH5- to HH5 (Extended Data Fig.  
155 8l) and suggests that older, neural plate epiblast has lost its competence to respond to signals  
156 from the node niche that induce axial identity and resident behaviour.

157

158 What molecular changes underlie this loss of competence? To address this we performed  
159 scRNA-seq on cells with resident behaviour at HH8, originating from non-competent cells  
160 (from late-to-early grafts) and compared these to cells that are competent (from grafts of lateral  
161 or anterior epiblast) (Extended Data Fig. 9a-c). Irrespective of origin, the most significant  
162 variation among the cells could still be accounted for by their expression of G2/M-phase related  
163 cell-cycle genes (Extended Data Fig. 9d-g, compare with Fig. 3d-e; clustering by PC1-2).  
164 However, PC3-4 clusters cells into overlapping groups according to their donor origin (lateral,  
165 anterior or late epiblast) (Fig. 4f). A subset of late-epiblast-derived cells that is associated with  
166 limited competence is characterised by specific expression of 15 genes (correlation coefficient  
167  $>0.55$  with PC3) (Extended Data Fig. 10a). Of these genes, seven have known roles in cell  
168 adhesion and/or in neural development including NCAM1 and CLDN1, normally expressed  
169 mainly in the neural plate (Fig. 4g-i and Extended Data Fig. 10b-i). This suggests that these

170 later cells may have lost their competence to respond to the node environment because they  
171 have already initiated their differentiation into neural plate.

172

### 173 **Conclusion**

174 Until now, apart from a few studies using prospective single cell fate mapping<sup>4,10,11</sup>, evidence  
175 for resident cells in the node was based on studies of cell populations<sup>8,9,12-14</sup>. Without  
176 challenging behaviour at the single cell level it has not been possible to test whether the node  
177 represents an instructive stem cell niche. Here, using single cell grafts we discovered that the  
178 node can specify resident and self-renewing behaviour. Thus, in addition to its well-known  
179 roles as an ‘organizer’ of the amniote embryo<sup>27-29</sup> and its ability to dorsalize mesoderm<sup>30,31</sup>,  
180 this key embryonic structure also functions as a stem cell niche that can specify resident stem  
181 cells for the developing head-tail axis.

182

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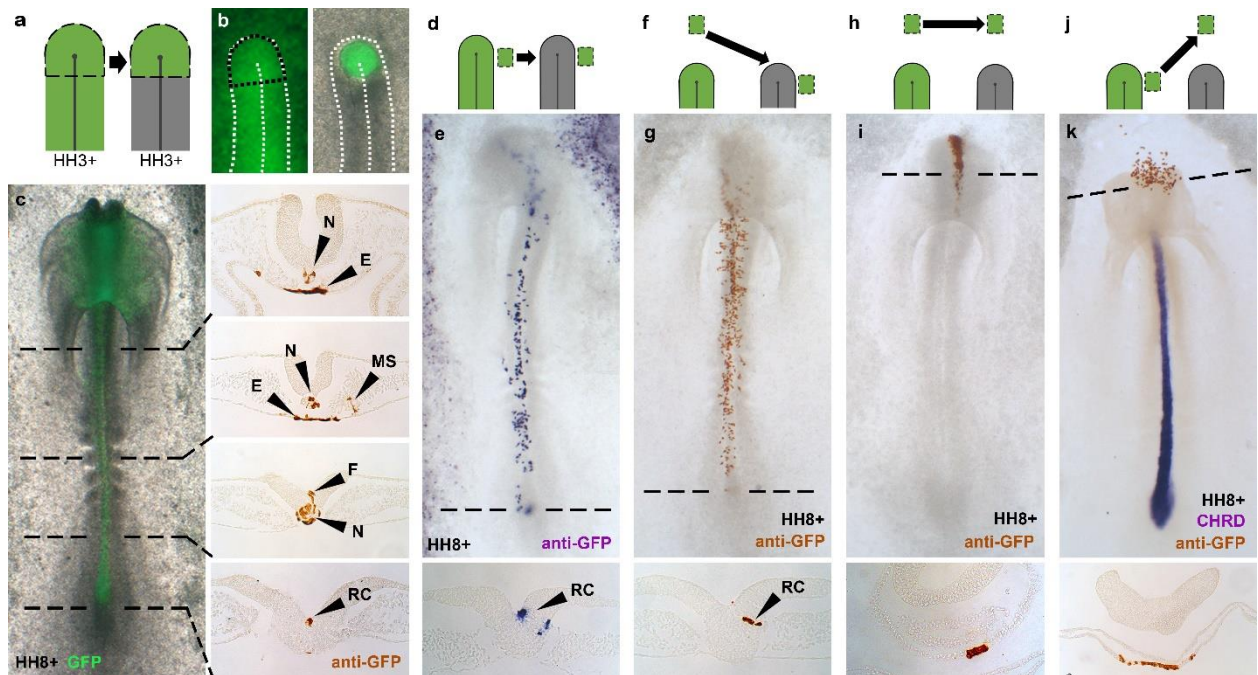


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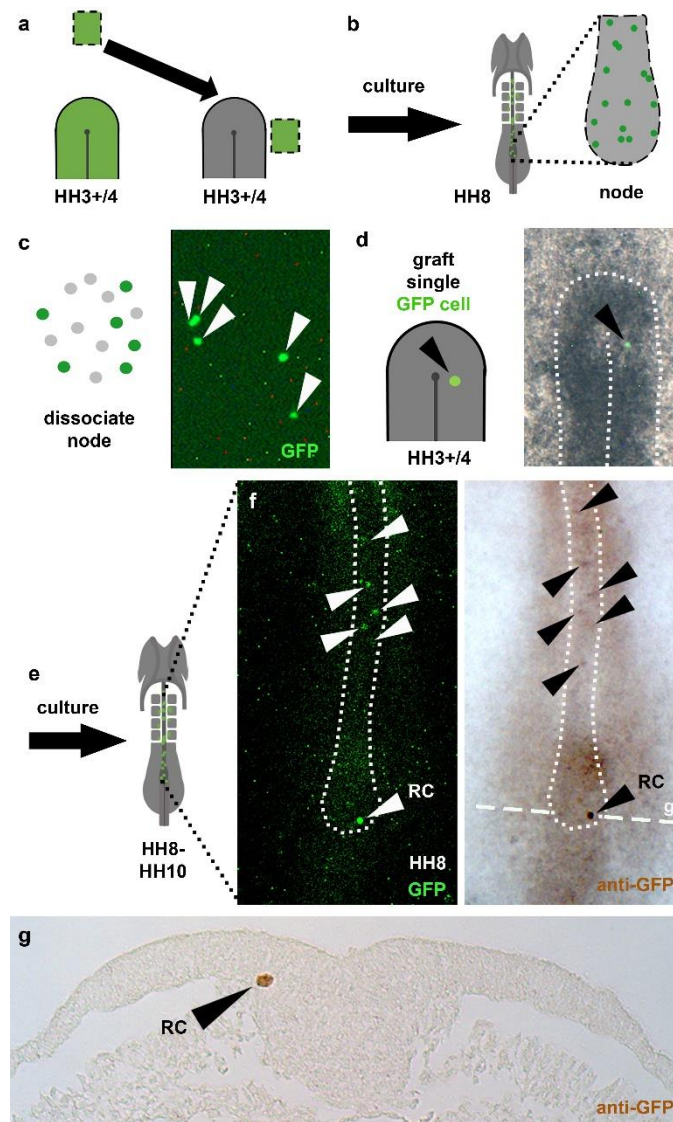
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260

261 **Figure 1 | The node confers resident behaviour and axial fates.** a-c, Node replacement using  
 262 a GFP-donor showing normal node axial fates. d-e, Epiblast lateral to the HH3+/4 node  
 263 ingresses into it and gives rise to the axis and to regressing node as ‘resident cells’ (RC). f-g,  
 264 Anterior epiblast not normally fated to enter the node behaves as lateral epiblast when forced  
 265 to do so. h-i, Anterior epiblast normally gives rise to head structures. j-k, Lateral epiblast no  
 266 longer gives rise to node-derived axial structures when prevented from entering the node.  
 267 N=notochord; E=endoderm; MS=medial-somite; F=floorplate; RC=resident cell. Transverse  
 268 dashed lines show levels of accompanying sections.



269

270 **Figure 2 | Single cell re-grafts reveal that the node can specify self-renewing resident cells.**

271 **a**, Anterior epiblast not normally fated to enter the node was made to do so. **a-c**, After culture,

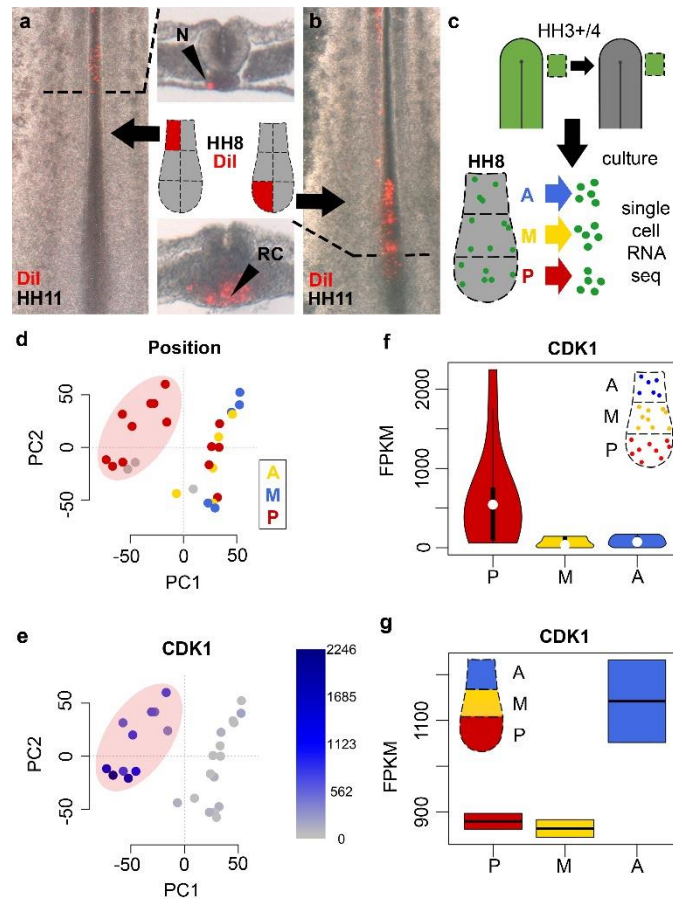
272 cells still residing in the HH8 node (b) were dissociated (c). **d**, A single GFP-positive cell was

273 isolated and grafted into a second younger, HH3+/4 host node. **e-g**, After culture (e), the single

274 GFP-positive cell had proliferated and descendants were found resident in the node and along

275 the axis (f-g). Transverse dashed line in (f) shows level of section in (g). RC=resident cell.

276



277

278 **Figure 3 | Long-term resident cells reside in the posterior node and are enriched in G2/M-**

279 **phase cell-cycle genes. a-b,** Fate mapping of six domains reveals that only posterior sub-

280 regions continue to contribute to resident cells. **c,** Individual resident cells originating from

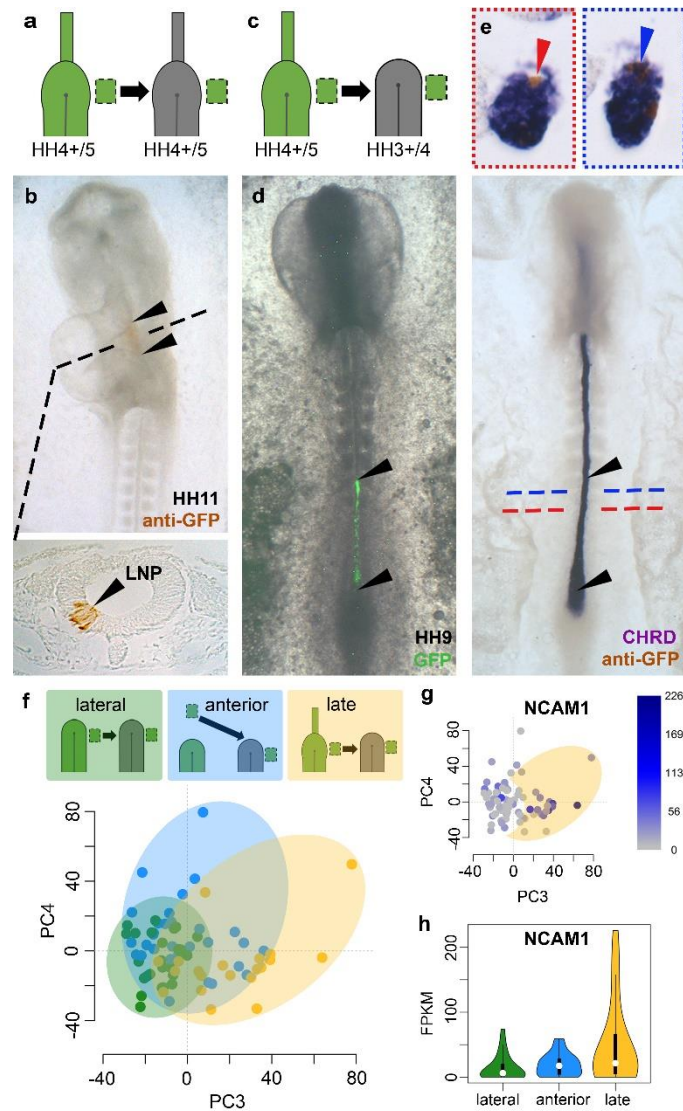
281 lateral epiblast were isolated from anterior (blue), middle (yellow) and posterior (red) HH8

282 node sub-regions and processed for scRNA-seq. **d-e,** PC analysis reveals a cluster of posterior

283 cells (pink oval) (d) enriched in G2/M-phase-related genes, including CDK1 (e) (intensity of

284 blue reflects FPKM levels). **f-g,** While single posterior resident cells are enriched in CDK1 (f),

285 bulk-RNA-seq shows that this is not the case in all cells of that region.



286

287 **Figure 4: Older epiblast is not competent to respond to the node.** a-b, ('Late') epiblast  
288 lateral to the HH4+/5 node normally contributes to lateral neural plate (LNP). c-e, When made  
289 to enter the younger node (c), 'late' epiblast contributes to the axial midline (d-e). Black  
290 arrowheads show the extent of head-to-tail contribution. While some graft-derived cells  
291 (visualised by anti-GFP) in the notochord express the notochord marker CHRD (blue arrow),  
292 others do not (red arrow). f-h, Single cells from the HH8 node plotted using PC3/4 cluster  
293 according to their epiblast origin (lateral, anterior or late) (f). Expression of neural plate-like  
294 genes, including NCAM1, correlates with 'late' cells outside of the control (green) cluster  
295 (yellow oval) (g-h). Intensity of blue in (g) reflects FPKM levels.

296 **Methods**

297 **Embryos:** Wild type chicken embryos were obtained from Brown Bovan Gold hens (Henry  
298 Stewart Farm). Transgenic cytoplasmic GFP chicken embryos were supplied by the avian  
299 transgenic facility at The Roslin Institute, Edinburgh<sup>8</sup>. All eggs were incubated at 38°C in  
300 humidified incubators and staged according to Hamburger and Hamilton<sup>1</sup>. For grafting  
301 experiments, *ex-ovo* embryo cultures were prepared using the New technique<sup>32</sup> with  
302 modifications as described by Stern and Ireland<sup>33</sup>.

303

304 **Epiblast grafts:** Donor embryos were isolated in Tyrode's solution<sup>34</sup>. The donor embryo was  
305 turned ventral side up, underlying endodermal and mesodermal layers were peeled away and a  
306 piece of epiblast (~20-50 cells) was cut out using 30G syringe needles. The epiblast piece was  
307 checked to ensure no mesodermal/endodermal cells remained. An equal sized piece of epiblast  
308 was removed from the host in the desired location and replaced with the donor epiblast. For  
309 'lateral-to-lateral' grafts (see Fig. 1d), epiblast was grafted into the equivalent position in the  
310 host as its donor origin (i.e. 'left-to-left' or 'right-to-right'). The 'lateral epiblast' was taken  
311 from immediately adjacent to the tip of the streak/node. 'Anterior' epiblast was taken from a  
312 midline position, about half-way between the tip of the streak/node and the anterior area opaca.

313

314 **Re-grafts of groups of cells:** The first graft was an epiblast graft from a GFP-donor to a non-  
315 GFP host (see Extended Data Fig. 2a-b). The second graft (re-graft) included a group of cells  
316 from the first host's node, containing 2-10 GFP-positive cells alongside some neighbouring  
317 GFP-negative cells. A small 'nick' was made in the node (ventral side) of the second host into  
318 which this group of cells was inserted using 30G syringe needles to carefully manoeuvre the  
319 small pieces of tissue (see Extended Data Fig. 2c-f). The grafted embryos were left at room



320 temperature for ~15 min to aid attachment of the graft to the host before further incubation.

321 Embryos were cultured to HH8-10.

322

323 **Single cell re-grafts:** The first host had an ‘anterior-to-lateral’ graft (see Fig. 2a). After culture  
324 to HH8-10, a single GFP-positive cell was collected from the host node (see ‘single cell  
325 manipulation’ below) and then transferred using a micropipette made from a pulled 50  $\mu$ l  
326 calibrated micropipette (Drummond Scientific, Cat 2-000-050) attached to an aspirator tube,  
327 into the second host (HH3+/4) (see Fig. 2b-d). For some re-grafts, a single GFP-positive cell  
328 was transferred attached to one or more neighbouring GFP-negative cells from the first host  
329 (but there was never more than one GFP-positive cell). A small ‘nick’ was made in the node  
330 of the second host. The GFP-positive cell was maneuvered into this nick by gently ‘blowing’  
331 saline on the cell with a micropipette. Ideally, once placed into its pocket, a flap of tissue would  
332 be used to cover the transplant site. The grafted embryo was then left at room temperature for  
333 ~15 min to aid attachment of the cell to the host. Each New culture was checked by  
334 fluorescence microscopy again just prior to incubation to ensure that the grafted GFP-positive  
335 cell was still in place. Embryos were cultured to HH8-10.

336

337 **DiI labelling:** The lipophilic dye, DiI (DiI-CellTracker CM, Molecular Probes Life  
338 Technologies, # C7001) was used for fate mapping of the HH8 node. For 10  $\mu$ l of working  
339 solution, 8.5  $\mu$ l of 0.3M sucrose and 1  $\mu$ l of 1:20,000 Tween-20 were used with 0.5  $\mu$ l of 2mM  
340 DiI (in dimethylformamide). All components were first pre-heated at 65°C, thoroughly mixed  
341 and dissolved. The protocol for preparation and application of DiI was adapted from<sup>4,35,36</sup>. The  
342 embryo was first prepared for New culture<sup>32</sup> and kept submerged in Tyrode’s. The node sub-  
343 region to be labelled was cut out using 30G syringe needles and transferred to a drop of

344 Tyrode's containing DiI (~9:1 Tyrode's : DiI working solution) and kept in the dark for 1-2  
345 min. The tissue piece was then removed and washed in successive drops of Tyrode's to remove  
346 any excess DiI before verifying that sufficient labelling had taken place, by fluorescence  
347 microscopy. The tissue piece was replaced into its original position, preserving the original  
348 dorsoventral orientation. Labelled embryos were cultured to ~HH11-12. After culture, embryos  
349 were fixed in 4% paraformaldehyde in PBS for at least 4 days at 4°C. To assess the location of  
350 the descendants of the DiI-labelled cells, several thick transverse sections were cut from each  
351 embryo by hand, using a scalpel, with the embryo pinned securely using insect pins in a silicon  
352 rubber-bottomed dish<sup>34</sup>.

353

354 ***In situ* hybridization:** *in situ* hybridization with digoxigenin (DIG-)-labelled riboprobes was  
355 carried out following established protocols<sup>37-39</sup>. Antisense DIG-riboprobes were synthesized  
356 by restriction digest and *in vitro* transcription. Plasmids used: CHR<sup>40</sup>, FOXA2<sup>41,42</sup>;  
357 PARAXIS/TCF-15<sup>43</sup>; ZEB2<sup>26</sup>; SOX2<sup>44</sup>; TBX6<sup>45</sup>; DLL1<sup>46</sup>; RSPO3 (ChEST784h18); NKAIN4  
358 (ChEST110n2); DRAXIN (ChEST54511); CLDN1 (ChEST168n2); NCAM1 (ChEST845i20);  
359 MSGN1 (ChEST90p23); CHST15 (ChEST391h17); AKAP12 (ChEST376j15); FOXM1  
360 (ChEST313o15); TOP2A (ChEST849a2); NUF2 (ChEST450j22); CENPL (ChEST97i12);  
361 MAD2L1BP (ChEST365n5).

362

363 **Immunohistochemistry:** anti-GFP antibody staining largely followed the methods described  
364 by Stern<sup>38</sup> and Streit and Stern<sup>39</sup>. Embryos were processed for anti-GFP antibody staining either  
365 immediately after collection (and fixing in 4% PFA overnight at 4°C) or following *in situ*  
366 hybridization.

367

368 **Histology:** Some embryos processed for *in situ* hybridization and/or anti-GFP antibody  
369 staining were embedded in paraffin wax and sectioned using a microtome. Methods largely  
370 followed those of Izpisúa-Belmonte et al.<sup>37</sup>. All sections were transverse and 10  $\mu$ M thick.  
371 Slides were mounted using a 3:1 solution of Canada balsam (Merck, # 1016910100) and  
372 HistoClear (HS-202 HISTO-CLEAR II, National Diagnostics).

373

374 **Microphotography:** Images of all whole-mount embryos and thick sections were recorded  
375 using transmitted light with an Olympus SZH10 stereomicroscope with epifluorescence optics.  
376 Paraffin sections were examined on an Olympus Vanox-T optical microscope. A QImaging  
377 Retiga 2000R Fast 1394 camera and QCapture Pro software was used for all image capture.

378

379 **Live imaging and cell tracking:** Electroporation mixture containing 1 mg.ml<sup>-1</sup> pDsRed-  
380 Express plasmid, 6% (wt/vol) sucrose and 0.04% (wt/vol) Fast Green FCF was applied  
381 dorsally, just lateral to the node of HH4- embryos to transfect ingressing cells. Electroporation  
382 was performed in a custom-made chamber with four pulses of 5 V, 50 msec width, 500 ms  
383 interval. Embryos were then cultured using a modification of New's method<sup>32,33</sup> in 35 mm  
384 plastic dishes with a glass coverslip base, and imaged with a Zeiss LSM 880 inverted  
385 microscope using a Plan-Apochromat 20x, 0.8 NA objective. Images were acquired at 10 min  
386 intervals using 3x5 tiling (10% overlap) to achieve coverage of the whole embryo. Image  
387 analysis and cell tracking were performed using Imaris (Bitplane) software. The embryo was  
388 imaged from the epiblast (dorsal) side but the output from Imaris is displayed as a mirror image  
389 (pseudo-ventral view).

390

391 **Single cell manipulation:** For collection of single cells for single cell re-grafts and scRNA-  
392 seq, the cultured embryo was first submerged in Tyrode's solution (for re-grafts) or sterile  
393 molecular grade PBS with 0.1% glucose (for scRNA-seq). The node was then divided into  
394 anterior, middle and posterior sub-regions of equal rostro-caudal length. Each of these regions  
395 containing GFP-positive cells was cut out, in turn, and placed in a drop of non-enzymatic  
396 dissociation medium (Sigma, # C5914-100ML), kept over ice. Each piece was washed twice  
397 in drops (~30  $\mu$ l) of this dissociation medium while over ice. To help with dissociation, after  
398 ~5 min, the tissue was gently aspirated up and down using a micropipette (made from a pulled  
399 50  $\mu$ l calibrated micropipette (Drummond Scientific, Cat 2-000-050) attached to an aspirator  
400 tube). The micropipette was broken at the tip to have a diameter just narrower than the width  
401 of the tissue piece. Once the piece of tissue was fragmented, a capillary with a narrower tip  
402 was used for further dissociation to single cells in suspension. GFP-positive cells were  
403 identified by fluorescence under a dissection microscope (x70 magnification) and were  
404 individually aspirated using a micropipette. The cell was transferred into a drop of Tyrode's  
405 (for re-grafts) or of sterile molecular grade PBS (for scRNA-seq) to replace the dissociation  
406 medium and to verify that there was only a single GFP-positive cell. Once verified, the cell  
407 was transferred (using a fresh pulled micropipette) to the second host (for re-grafts) or into a  
408 200  $\mu$ l tube containing 5  $\mu$ l of lysis buffer and 5% RNase inhibitor (for scRNA-seq) (lysis  
409 buffer and RNase inhibitor from SMART-Seq v4 Ultra Low Input RNA Kit, Takara, # 634892).  
410 Once the dissociation process began, cells were collected for ~20 min, after which time any  
411 remaining dissociated tissue was discarded, and a new tissue piece taken from the embryo.

412

413 **scRNA-seq:** The SMART-Seq v4 Ultra Low Input RNA Kit (Takara, # 634892) targeting  
414 mRNA, was used for preparing the single cells for sequencing. Amplified cDNA was purified  
415 using AMPure magnetic purification beads (Agencourt AMPure XP, Beckman Coulter #

416 A63880). The DNA concentration of purified cDNA was checked by Qubit (dsDNA HS Assay  
417 Kit, Thermofisher # Q33230). All samples yielding at least 5 ng of cDNA were sheared by  
418 sonication (Covaris, S220/E220 focused-ultrasonicator, settings set to: 10% duty factor, 200  
419 cycles per burst, 120 second treatment time, 175W peak incident power) to obtain ~500bp  
420 fragments for library preparation. The ThruPLEX DNA-seq, Dual Index Kit (Takara, #  
421 R400406) was used to construct dual indexed libraries for each sample. Libraries were  
422 individually purified, using AMPure magnetic purification beads (Agencourt AMPure XP,  
423 Beckman Coulter # A63880). DNA concentration of purified libraries was checked by Qubit  
424 (dsDNA HS Assay Kit, Thermofisher # Q33230) and size distribution of cDNA measured  
425 using TapeStation (Agilent High sensitivity D1000 screen tape, # 5067-5584). All libraries  
426 were individually diluted to 10 nM in elution buffer before pooling together. Pooled libraries  
427 were sequenced by UCL Genomics using an Illumina NextSeq sequencer with a 75bp single  
428 end read cycle kit. The average number of reads per cell was ~10 Million (range: 6,838,400-  
429 15,404,851). The single cell RNA-seq raw data have been deposited in EBI Array Express  
430 (accession number E-MTAB-9116).

431

432 **RNA-seq of tissues:** Tissues (HH8 node sub-regions) were isolated from transgenic-GFP  
433 embryos using 30G syringe needles in sterile molecular grade PBS. Tissues were collected into  
434 RNAlater (Invitrogen, # AM7020). For each sample, tissues were collected from thirteen to  
435 seventeen embryos. RNA was extracted using the Micro Total RNA Isolation Kit (Invitrogen,  
436 # AM1931) and concentration and quality measured using TapeStation (Agilent High  
437 sensitivity RNA screen tape, # 5067-5579). The NEBNext Single Cell/Low Input RNA Library  
438 Prep Kit for Illumina (# E6420) was used for cDNA and library synthesis (performed by UCL  
439 Genomics). Libraries were sequenced by Illumina NextSeq using a 75bp single end read cycle  
440 kit. The average number of reads per sample (node sub-region) was ~22 Million (range:

441 19,572,310 to 24,523,360). The bulk RNA-seq raw data have been deposited in EBI Array  
442 Express (accession number E-MTAB-9115).

443

444 **RNA-seq data processing:** Raw data were checked using FastQC<sup>47</sup> to assess overall quality.  
445 Cutadapt<sup>48</sup> was used to remove low-quality bases (Phred quality score <20) at the 3' and 5'  
446 ends, adapter sequences, primer sequences, and poly-A tails of each read. Reads were aligned  
447 to the galGal6 chicken genome using TopHat2<sup>49</sup>, alignment rates were 91.9%±0.3% (for  
448 scRNA-seq) and 86.3%±0.65% (for RNA-seq of tissues). Transcripts were counted and  
449 normalized using Cufflinks<sup>50</sup> programs *cuffquant* and *cuffnorm* respectively. Data analysis was  
450 performed in the R environment (R-3.5.1).

451

452 For scRNA-seq, all sequenced cells passed quality control. The matrix of transcript FPKMs  
453 (Fragments Per Kilobase of transcript per Million mapped reads) contains expression of 24,353  
454 genes in 77 samples (cells). Of these, 13,817 are expressed (with an FPKM >0.5) in at least  
455 two cells in our data. The top 5000 most variable of these genes were used for principal  
456 component (PC) analysis. PC analysis was carried out on two datasets: data from single cells  
457 from the HH8 node originating only from lateral epiblast (n=27) and data from single cells  
458 from the HH8 node originating from anterior, lateral and late epiblast (n=77). Correlation  
459 coefficients were calculated for the relationship between gene expression and a given principal  
460 component.

461

462 For RNA-seq of tissues, all node sub-regions passed quality control. Mitochondrial RNAs,  
463 ribosomal RNAs and microRNAs were excluded. Fold changes for each gene in posterior

464 sub-regions against its expression in all other regions were calculated to find genes with the  
465 most marked differential expression between sub-regions (see Extended Data Fig. 7b).

466

#### 467 **Data availability**

468 The scRNA-seq and bulk-RNA-seq raw datasets generated during the current study are  
469 available in the EBI Array Express (<https://www.ebi.ac.uk/arrayexpress/>). Accession numbers:  
470 E-MTAB-9116 and E-MTAB-9115.

471

472

#### 473 **References for Methods and Extended Data**

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686

687 **Author contributions**

688 H-CL processed raw RNA-seq data, AM and NP made and analysed the movie. TS performed  
689 all other experiments and analysis. CDS coordinated the research and obtained the funding. TS  
690 and CDS designed the project and wrote the manuscript.

691

692 **Competing interests:** Authors declare no competing interests.

693

694 **Supplementary information** is available for this paper.

695

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699 **Supplementary Information**

700

701 **Supplementary Movie**

702 **Supplementary movie 1 | Live tracking of cells in the node from HH5 to HH9.** Time-lapse

703 movie showing a mosaic of cells labelled with DsRed (pseudo-colour encoded as green).

704 Selected cells originating from the anterior part of the node at HH5 are highlighted in blue and

705 some originating from the posterior part of the node are highlighted in red. The outline of the

706 node is highlighted by a white dashed line.

707

708 **Supplementary Data**

709 **Supplementary data 1 | FPKM table for scRNA-seq data.** Transcriptional profiling of single

710 cells individually harvested from the HH8 node, originating from the three experimental

711 conditions described in Fig. 4f. Details on the epiblast origin (developmental stages and

712 position in the donor) of the cells and their positions within the host node at the time of

713 collection are included in the raw data submitted to EBI Array Express (accession number E-

714 MTAB-9116).

715

716 **Supplementary data 2 | FPKM table for bulk-RNA-seq data.** Transcriptional profiling of 6

717 sub-regions of the HH8 node (see Extended Data Fig. 7a). The raw data were submitted to EBI

718 Array Express (accession number E-MTAB-9115). Abbreviations for the node regions at stage

719 8: S15\_AL8: anterior-left; S16\_AR8: anterior-right; S17\_ML8: middle-left; S18\_MR8:

720 middle-right; S19\_PL8: posterior-left; S20\_PR8: posterior-right.

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