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1 **Notch3 is a genetic modifier of NODAL signalling for patterning asymmetry during mouse**
2 **heart looping**

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23 Short title: Notch3 role in left-right asymmetry

24

25 **Abstract**

26 The TGF β secreted factor NODAL is a major left determinant required for the asymmetric
27 morphogenesis of visceral organs, including the heart. Yet, when this signalling is absent,
28 shape asymmetry, for example of the embryonic heart loop, is not fully abrogated, indicating
29 that there are other factors regulating left-right patterning. Here, we used a tailored
30 transcriptomic approach to screen for genes asymmetrically expressed in the field of heart
31 progenitors. We thus identify *Notch3* as a novel left-enriched gene and validate, by
32 quantitative in situ hybridization, its transient asymmetry in the lateral plate mesoderm and
33 node crown, overlapping with *Nodal*. In mutant embryos, we analysed the regulatory hierarchy
34 and demonstrate that *Nodal* in the lateral plate mesoderm amplifies *Notch3* asymmetric
35 expression. The function of *Notch3* was uncovered in an allelic series of mutants. In single
36 neonate mutants, we observe that *Notch3* is required with partial penetrance for the
37 development of ventricles, in addition to its known role in coronary arteries. In compound
38 mutants, we reveal that *Notch3* acts as a genetic modifier of *Nodal*, able to modulate heart
39 looping direction and the curvature of the outflow tract. Whereas *Notch3* was previously
40 associated with the CADASIL syndrome, its contribution to asymmetric organogenesis is now
41 relevant to severe laterality defects such as the heterotaxy syndrome.

42 **Abbreviations**

43 Ao = Aorta

44 AVC = Atrioventricular canal

45 BH = Benjamini-Hochberg

46 BMP = bone morphogenetic protein

47 E = Embryonic day

- 48 HREM = High Resolution Episcopic Microscopy
- 49 HT = Heart tube
- 50 ISH = In situ hybridisation
- 51 IPCCC ICD = International Paediatric and Congenital Cardiac Code and International
- 52 Classification of Diseases
- 53 L = Left
- 54 LA = Left atrium
- 55 LPM = Lateral plate mesoderm
- 56 LV = Left ventricle
- 57 Myo = Myocardium
- 58 No = Node
- 59 n.s. = non-significant
- 60 NT = Neural tube
- 61 P = Postnatal day
- 62 R = Right
- 63 RA = Right atrium
- 64 RT-qPCR = Reverse transcription quantitative polymerase chain reaction
- 65 RV = Right ventricle
- 66 SHF = Second Heart Field
- 67 TGF β = Transforming growth factor- β
- 68 VSD = Ventricular Septal Defect
- 69

70 Introduction

71 Left-right patterning of the embryo is essential for the formation of visceral organs (1,2).
72 Anomalies in this process lead to the heterotaxy syndrome, a severe condition including
73 complex congenital heart defects which determine the prognosis of patients (3). The
74 mechanisms of symmetry-breaking are now well established in the mouse (4). Chirality of
75 tubulin underlies the rotational movement of motile cilia in the pit of the left-right organiser,
76 also referred to as the node. This generates a leftward fluid flow sensed by crown cells,
77 resulting in the asymmetric expression of the left determinant NODAL in the lateral plate
78 mesoderm. Genetic alterations to the formation of the node, ciliogenesis or NODAL signalling
79 are associated with heterotaxy in both mice (2,5–8) and humans (9,10). NODAL signalling is
80 then sensed by organ precursors to modulate morphogenesis, as shown in the intestine (11)
81 and heart (12). In the absence of *Nodal*, some features are symmetrical (spleen, bronchi, lung
82 lobes, atrial appendages), but many others (e.g heart looping, stomach, gut) retain some level
83 of asymmetry, although abnormal (6,12–15). This indicates that *Nodal* is not always required
84 to initiate asymmetry and that there are other factors of asymmetry in addition to NODAL
85 signalling.

86 The heart is the first organ to undergo asymmetric morphogenesis in the embryo. This
87 manifests as a rightward looping of the heart tube primordium, at E8.5 in the mouse (16). We
88 have previously reconstructed the dynamics of this process and defined morphological and
89 quantitative staging criteria from E8.5c to E8.5j, including the repositioning of ventricles from
90 cranio-caudal to left-right, and the left displacement of the venous pole (17). We have shown
91 that heart looping is primarily a buckling mechanism, when the heart tube elongates between
92 fixed poles (17). We found that *Nodal* is not required for buckling and thus to initiate looping.
93 In contrast, *Nodal* biases buckling, by amplifying and coordinating asymmetries independently

94 at the two poles of the heart tube (12). Thus, in the absence of *Nodal* in the lateral plate
95 mesoderm, asymmetries are reduced and the helical shape of the looped heart tube is
96 abnormal. Because two asymmetries, at the arterial and venous poles, have a randomised
97 orientation, *Nodal* mutants display 4 classes of abnormal heart loop. We observed that NODAL
98 signalling is transient, before the formation of the heart tube (E8.5c-d), in precursor cells of
99 the heart tube poles. Cardiac precursor cells are progressively incorporated into the heart
100 tube, as a main driver of its elongation. Patterning of the field of cardiac precursors has been
101 studied in terms of molecular profiling (18) and fate (19). However, the left-right patterning of
102 cardiac precursors has remained centered on the *Nodal* pathway.

103 Other players of asymmetry have been identified, including BMP (20–22), HH (23), NOTCH
104 (24–26) and WNT (27–29) signalling. Few of these are reported to be asymmetric: *Wnt3* in the
105 left side of the node (28), and BMP signalling in the right lateral plate mesoderm (21). They
106 were all shown to be required for *Nodal* asymmetry. Thus, the factors which can provide
107 asymmetry besides *Nodal* have remained enigmatic.

108 NOTCH signalling is an example of a pathway required both for left-right asymmetry and for
109 heart morphogenesis (30), but playing multiple roles. Among the four paralogues, *Notch2* is
110 expressed in the node, and *Notch1* around the node in the caudal epiblast and presomitic
111 mesoderm (24,31). *Notch1;Notch2* double mutants disrupt heart looping asymmetry, in
112 keeping with a role in the left-right organiser (25). NOTCH1 and the transcriptional NOTCH
113 effector RBPJ are required in the heart field for cardiomyocyte differentiation (32–34). *Notch1*
114 is later expressed in the endocardium, where it is important to regulate myocardial
115 trabeculation (35) and valve formation (36,37). *Notch3* mutant mice are viable and fertile (38),
116 but display defects in the maturation of the smooth muscle wall of coronary arteries (39).
117 *Notch3* is a broad marker of pericytes and controls the formation of smooth muscles in non-

118 cardiac arteries and intestinal lacteals (40–42). Gain-of-function of NOTCH3 in CADASIL
119 syndrome is responsible for smooth muscle degeneration in the brain (40,43,44). *Notch3* is
120 also involved in the regulation of quiescence and stemness of neural, skeletal muscle and
121 mammary stem cells (45–48). Finally, *Notch4* is expressed together with *Notch1* in endothelial
122 cells to regulate vascular remodelling (49). Thus *Notch1/2* but not *Notch3/4* have been
123 previously associated with left-right patterning, at the level of the left-right organiser.

124 To uncover asymmetric factors in organ specific precursors, rather than the left-right
125 organiser, recent transcriptomic screens were performed on micro-dissected tissues excluding
126 the node (50,51). Whereas NODAL signalling is transient (12,52), these screens were based on
127 pooled embryos, which limits their resolution. The asymmetric candidate genes that have
128 been identified have not yet been analysed for functional significance.

129 Here, we focused on asymmetric expression in the field of heart progenitors, at the time of
130 heart looping. We compared paired left/right samples in single embryos to avoid confounding
131 individual or stage variations. Our screen and quantitative in situ validations reveal *Notch3* as
132 a novel gene enriched in the left lateral plate mesoderm. *Notch3* is co-expressed with *Nodal*,
133 although with a slight time-delay and partial spatial overlap. In mutants, we show that *Notch3*
134 asymmetry is amplified by NODAL signalling. Single *Notch3* mutants do not develop
135 heterotaxy, but show ventricle and coronary artery defects after birth. Analysis of compound
136 mutants indicate that *Notch3* is a genetic modifier of NODAL signalling during heart looping.
137 Overall, we identify not only a novel asymmetric gene, but also a novel player in the left-right
138 patterning of the lateral plate mesoderm, relevant to congenital heart defects.

139

140 Results

141 Identification of *Notch3* as a novel asymmetric marker

142 To screen for left-right asymmetric gene expression in cardiac cells, aside the *Nodal*
143 pathway, we used a transcriptomic approach in the mouse embryo. To reduce confounding
144 factors, the approach was targeted in space and time and left/right comparisons were
145 conducted on single rather than pooled embryos. We focused on the heart field, given that it
146 is a tissue which can be bisected at the midline and is patterned by NODAL signalling (12). In
147 contrast, the heart tube changes its position during looping (17) and has a left-right origin
148 difficult to dissect (19). We have previously shown that NODAL signalling is transient in heart
149 precursors (12), highlighting the importance of specifically staging embryos. We focused on
150 the E8.5f stage, when we have detected morphological asymmetry at the arterial pole of the
151 heart tube (17). Thus, we micro-dissected the heart field of E8.5f wild-type embryos (Fig. 1A
152 and S1A-D), in the dorsal pericardial wall and down to the second somite, in agreement with
153 landmarks of fate mapping experiments (19) and profiling of single cardiac cells (18). Bulk RNA
154 sequencing of paired left and right samples were compared to identify differential asymmetric
155 expression in the heart field. As expected, targets of NODAL signalling, *Pitx2* and *Lefty2*, were
156 significantly enriched on the left side (Fig. 1B). A total set of 597 genes were predicted to be
157 differentially expressed (Table S1). Ingenuity Pathway analysis showed NOTCH signalling as the
158 strongest asymmetric pathway and left-sided (Fig. 1C). We controlled the transcriptomic levels
159 of individual genes associated with NOTCH in this differential analysis and their cardiac
160 expression in a published single cell transcriptomic dataset at the same stage (18): whereas
161 *Dtx4* was found to be expressed only in ectodermal cells (Fig. S1E), *Notch3*, *Hes1*, *Jag1*, *Ncstn*
162 were identified as asymmetric candidate genes in the heart field (Fig. 1D).

163 We selected *Notch3* for further validation, because it is the gene with highest expression, it
164 encodes a receptor and transcription co-factor central to NOTCH signalling. This *Notch*
165 paralogue has not previously been associated with left-right patterning. We mapped *Notch3*
166 expression in whole mount E8.5 wild-type embryos using sensitive RNAscope in situ
167 hybridization. *Notch3* was detected throughout the lateral plate mesoderm, as well as in the
168 crown of the node, with higher expression on the left side (Fig. 1E-F). *Notch3* was not
169 expressed in the myocardium of the forming cardiac tube. We then assessed the dynamics of
170 *Notch3* expression within the heart field (Fig. 1G). Our quantification shows that *Notch3* is
171 transiently asymmetric, reaching 4.5-fold left-sided enrichment in the heart field at E8.5d, a
172 stage when the myocardium bulges out to form a tube (Fig. 1H). Although *Nodal* asymmetry
173 starts one stage earlier (E8.5c, (12)), *Notch3* and *Nodal* overlapped in the left lateral plate
174 mesoderm at E8.5d (Fig. 1I, Video S1), and were found co-expressed in single cells (Fig. S1F).
175 We have thus identified *Notch3* as a novel left-sided gene in the heart field.

176 **Fig 1. RNA-sequencing of paired samples identifies a left-sided enrichment of *Notch3* at**
177 **E8.5.**

178 (A) Outline of the left (light blue) and right (light red) heart field micro-dissected for RNA-
179 sequencing at the indicated stage of mouse heart looping. (B) MA-plot representing relative
180 gene expression between paired left and right heart field at E8.5f. Non-significant differential
181 expression is represented in red, differential expression in green (p -value <0.05), and blue
182 (Benjamini-Hochberg (BH) corrected p -value <0.05 , LimmaVoom, $n=4$ embryos). (C) Ingenuity
183 Pathway Analysis on the gene list shown in Table S1 ordered according to significance and
184 colour-coded for the activation state (z -score: blue, active pathway on the left; red, on the
185 right). (D) Normalised read counts of genes involved in the *Notch* pathway in the left (blue)
186 and right (red) heart field. The dotted line indicates the threshold of background expression.
187 Whisker plots show the median, 25th- and 75th quartiles (boxes), and the extreme data points
188 (whiskers). * p -value < 0.05 , ** Benjamini-Hochberg corrected p -value < 0.05 (LimmaVoom,
189 $n=4$). (E-F) Expression of *Notch3* (white) detected by whole mount RNAscope ISH in E8.5d wild-
190 type embryos, shown in frontal views (e1, cranial part of the embryo, f1, caudal part of the
191 embryo) and transverse sections (e2-e3, f2, at the levels indicated in e1-f1) ($n=6$). Filled and
192 empty arrowheads point to high and low expression, respectively. (G) Segmentation of the
193 cardiac region in 3D images, to quantify gene expression in the left (white) or right (yellow)
194 heart field. The heart tube is coloured in red. Somites are outlined by white dotted lines.
195 Expression of *Notch3* within the segmented second heart field is extracted in the right panel.
196 (H) Quantification of normalised *Notch3* asymmetric expression in the heart field at sequential
197 stages. The means are shown on a red dotted line and standard deviations are in blue ($n=3$ at

198 E8.5c, 6 at E8.5d, 6 at E8.5e, 3 at E8.5f). (I) *Notch3* (white) and *Nodal* (magenta) co-expression
199 detected by double whole mount RNAscope ISH in E8.5d wild-type embryos, shown in a frontal
200 view (i1) and transverse section (i2) (n=5). The midline is indicated by a yellow dotted line. HT,
201 heart tube; L, left; LPM, lateral plate mesoderm (green dotted outline); Myo, myocardium (red
202 dotted outline); No, node; R, right; SHF, second heart field. See also Video S1.
203

204 ***Nodal* is required in the lateral plate mesoderm to amplify *Notch3* asymmetric expression**

205 Given that *Notch3* asymmetry overlaps with that of *Nodal*, we investigated whether *Notch3*
206 and *Nodal* depend on each other. NOTCH signalling has been shown to directly activate *Nodal*
207 in the node, in *Dll1* and *Rbpj* mutants (26). In contrast, we found that *Nodal* was correctly
208 patterned in the node and left lateral plate mesoderm of *Notch3*^{-/-} mutants (Fig. S2A-B). In
209 reverse, in mutants in which *Nodal* is inactivated in the lateral plate mesoderm but not in the
210 node (12), *Notch3* expression was impacted (Fig. 2A-B). Quantification of *Notch3* expression
211 at sequential stages in the heart field shows that *Notch3* asymmetry is severely decreased in
212 *Nodal* mutants compared to controls (Fig. 2C). Yet, some significant asymmetry is still
213 detectable. In keeping with a partial dependency of *Notch3* on *Nodal*, we observed that the
214 expression domain of *Notch3* partially overlaps with that of *Nodal*, extending more medially
215 and less laterally (Fig. S2C-D). Thus, *Nodal* is required in the left lateral plate mesoderm to
216 amplify, rather than initiate, *Notch3* asymmetric expression.

217 **Fig 2. Decreased *Notch3* asymmetry in *Nodal* mutants.**

218 (A-B) Whole mount RNAscope ISH of *Notch3* in control (A) and *Nodal* mutants (B) at E8.5d in
219 frontal views. Expression of *Notch3* within the segmented heart field is extracted in right
220 panels. The midline of the embryo is indicated by a yellow dotted line. (C) Corresponding
221 quantification of normalised *Notch3* asymmetric expression in the heart field at sequential
222 stages in littermate controls (n= 3 at E8.5c, 5 at E8.5d, 3 at E8.5e) and *Nodal* mutants (n= 5 at
223 E8.5c, 3 at E8.5d, 6 at E8.5e). Means and standard deviations are shown. *p-value<0.05
224 between controls and mutants, ♦p-value<0.05 to compare mutant levels with a symmetry
225 hypothesis (Log2 ratio =0) (Pairwise Mann-Whitney Wilcoxon tests). L, left; R, right; SHF,
226 second heart field.
227

228

229 **Single *Notch3* mutants have normal heart looping but later congenital heart defects**

230 We next investigated the role of *Notch3* in heart development, in mutants inactivated for it
231 (Fig. S3). Given its asymmetric expression when the cardiac tube forms, we looked for a
232 potential effect on the rightward looping of the heart tube. E9.5 mutant embryos were
233 collected at Mendelian ratio (Fig. 3A) and no heart looping defect was detected, both
234 qualitatively (Fig. 3B-D) and quantitatively (Fig. 3E-F). *Notch3*^{-/-} mutant embryos were
235 indistinguishable from controls, with a normal orientation of the right ventricle-left ventricle
236 axis relative to the midline, and a normal left displacement of the venous pole.

237 **Fig 3. Normal heart looping in single *Notch3* mutants.**

238 (A) Histogram showing the percentage of genotypes recovered in E9.5 litters of *Notch3*^{+/-} x
239 *Notch3*^{+/-} crosses. The observed frequency is not significantly different from the expected
240 Mendelian ratio (dotted lines) (p-value = 0.30, chi-square test, n as indicated). (B-D) Brightfield
241 ventral views (b1-d1), and 3D segmented heart tubes shown in ventral (b2-d2) and cranial (b3-
242 d3) views, of littermate *Notch3*^{+/+} (B), *Notch3*^{+/-} (C) and *Notch3*^{-/-} (D) E9.5 embryos. Cardiac
243 regions are colour coded: outflow tract in green, right ventricle in red, left ventricle in blue,
244 atrioventricular canal and left atrium in yellow, right atrium in magenta. The notochord (cyan)
245 is used as a reference axis to align samples. (E) Quantification of the orientation of the RV/LV
246 axis relative to the notochord, as schematised in b2. (F) Quantification of the displacement of
247 the venous pole relative to the notochord, normalised by the tube length, as schematised in
248 b3. Means and standard deviations are shown (Pairwise Mann-Whitney Wilcoxon tests with
249 Benjamini-Hochberg correction, n=11 *Notch3*^{+/+}, 10 *Notch3*^{+/-} and 16 *Notch3*^{-/-}). LV, left
250 ventricle; n.s., non-significant; RV, right ventricle.

251

252 We then collected *Notch3* mutants after birth. In agreement with previous reports (38),
253 they were collected at Mendelian ratio (Fig. 4A). *Notch3*^{-/-} mutants did not show heterotaxy,
254 unlike *Nodal* mutants, but had congenital heart defects with partial penetrance (67%),
255 including peri-membranous and muscular ventricular septal defects, thinning of the right
256 ventricular wall and coronary artery dilatation (Fig. 4B-C). Defects of coronary arteries are in
257 keeping with fetal expression of *Notch3* in pericytes and smooth muscle cells (Fig. S4). Overall,
258 our results show that *Notch3* is required for ventricle and coronary artery development.

259 However, removal of *Notch3* alone does not uncover a role in left-right asymmetric
260 organogenesis.

261 **Fig 4. Congenital heart defects in *Notch3* mutants.**

262 (A) Histogram showing the percentage of genotypes recovered in P14 litters of *Notch3*^{+/-} x
263 *Notch3*^{+/-} crosses. The observed frequency is not significantly different from the expected
264 Mendelian ratio (dotted lines) (p-value = 0.64, chi-square tests, n as indicated). (B-C) Sections
265 of *Notch3*^{-/-} mutant hearts at P0, imaged by HREM. An example of a mutant heart with no
266 phenotype is shown (B, n=4/12). Defects (C) include peri-membranous ventricular septal
267 defect (VSD) (yellow arrowhead, n=1/12), muscular VSD (red arrowheads, n=4/12), dilatation
268 of the septal coronary artery (pink arrowhead, n=1/12) and thin RV wall (green arrow, n=3/12).
269 AVC, atrioventricular canal; Ao, aorta; LA, left atrium; LV, left ventricle; RA, right atrium; RV,
270 right ventricle.

271

272 ***Notch3* is a genetic modifier of *Nodal* in heart looping**

273 Since *Notch3* overlaps with and is amplified by *Nodal*, we investigated their interaction in
274 the context of left-right asymmetry. We generated double heterozygote mutants and
275 quantified heart looping. Compared to single heterozygotes (Fig. 5A-B), *Notch3*^{+/-}; *Hoxb1*^{Cre/+};
276 *Nodal*^{flox/+} double heterozygotes (Fig. 5C) did not show any heart looping defects, including
277 normal orientation of the right ventricle-left ventricle axis relative to the midline, and normal
278 left displacement of the venous pole (Fig. 5D-E). Thus, we did not detect a genetic interaction
279 between *Notch3* and *Nodal*.

280 We then analysed the role of *Notch3* in a sensitized background. We generated a large
281 cohort of mutants, with a knock-down of *Notch3* within the context of *Nodal* inactivation in
282 the left lateral plate mesoderm. *Notch3*^{+/-}; *Hoxb1*^{Cre/+}; *Nodal*^{flox/null} mutants lost the randomized
283 direction of heart looping, typical of *Nodal* mutants (Fig. 5F-G). Rightward looping was more
284 frequent when *Notch3* was reduced in *Nodal* mutants. However, this did not correspond to a
285 rescue, since all rightward mutant heart loops were abnormal, compared to controls.
286 Compared to *Nodal* mutants, additional defects in the outflow tract were observed with partial

287 penetrance. The outflow tract was straighter in 40% (7/18) of *Notch3*^{+/-}; *Hoxb1*^{Cre/+}; *Nodal*^{flox/null}
288 mutants within looping Class 4 (Fig. 5H-J), which is the less severe class. These genetic analyses
289 thus uncover a role for *Notch3* as a genetic modifier of *Nodal* during left-right asymmetric
290 organogenesis.

291 **Fig 5. Heart looping variations in *Notch3* and *Nodal* compound mutants.**
292 **(A-C)** 3D segmented heart tubes shown in ventral (a1-c1) and cranial views (a2-c2) of single
293 *Nodal* heterozygote (A), single *Notch3* heterozygote (B) and double heterozygote (C) embryos
294 at E9.5. Cardiac regions are colour coded as in Fig. 3. The notochord (cyan) is used as a
295 reference axis to align samples. **(D)** Quantification of the orientation of the RV/LV axis relative
296 to the notochord. **(E)** Quantification of the displacement of the venous pole relative to the
297 notochord, normalised by the tube length. Means and standard deviations are shown.
298 (Pairwise Mann-Whitney Wilcoxon tests with Benjamini-Hochberg correction, n=8 *Hoxb1*^{Cre/+};
299 *Nodal*^{flox/+}, 14 *Notch3*^{+/-}; *Nodal*^{flox/+}, 12 *Notch3*^{+/-}; *Hoxb1*^{Cre/+}; *Nodal*^{flox/+}). **(F)** Brightfield images
300 of homozygote *Nodal* mutants with decreased *Notch3* (*Notch3*^{+/-}; *Hoxb1*^{Cre/+}; *Nodal*^{flox/null}) at
301 E9.5, in a ventral view. **(G)** Corresponding quantification of their looping direction frequency
302 (right bar), which, in contrast to *Nodal* mutants with normal *Notch3* levels (left bar), differs
303 from a randomised looping direction (dotted line). *p-value<0.05 (Chi-square test with Yates'
304 continuity correction, n=76 *Hoxb1*^{Cre/+}; *Nodal*^{flox/null} and 47 *Notch3*^{+/-}; *Hoxb1*^{Cre/+}; *Nodal*^{flox/null}).
305 **(H-J)** Comparison of Class 4 abnormal heart loops in homozygote *Nodal* (I), *Nodal*; *Notch3*
306 compound (J) mutants compared to control *Notch3*^{+/-} embryos (H), seen in brightfield right-
307 sided views. Filled and empty arrowheads point to curved and straight outflow tract,
308 respectively. L, Left; LV, left ventricle; R, Right; RV, right ventricle.
309

310 Discussion

311 We have identified *Notch3* as a novel left asymmetric factor. We show that it is amplified by
312 NODAL signalling. We do not detect a genetic interaction between *Nodal* and *Notch3*, but
313 rather show that *Notch3* acts as a genetic modifier of *Nodal*. Knock-down of *Notch3*
314 exacerbates looping defects observed in *Nodal* mutants. Single *Notch3* mutant neonates have
315 other cardiac defects in coronary arteries, transmural growth and septation of ventricles.

316 Left-right asymmetry is mainly analysed in terms of patterning of the major left
317 determinant, *Nodal*, and its downstream pathway including *Lefty2* and *Pitx2* (4,53,54).
318 However, in the absence of *Nodal*, organ asymmetry is not fully abrogated (6,13–15). Heart
319 looping, which is the first asymmetric morphogenesis, still occurs to some extent in *Nodal*
320 mutants, producing helical heart tubes, even if abnormal (12). This indicates that there are
321 other factors than NODAL signalling required for heart asymmetry. BMP (20–22), HH (23),
322 NOTCH (24–26) and WNT (27–29) signalling have been shown to be required upstream of
323 *Nodal* for asymmetry. Transcriptomic screens have been performed to identify asymmetric
324 genes in embryos outside the node or in cardiac precursors (50,51). Yet, asymmetric candidate
325 genes were either not validated or not analysed functionally, leaving open the question of
326 which are genuine asymmetry factors. Here, we have used a transcriptomic screen tailored
327 spatially in the heart field, temporally at the stage of the first morphological asymmetry, and
328 specifically on left-right asymmetry based on paired comparison in single embryos. This has
329 successfully identified a novel left factor, *Notch3*. We have characterised by quantitative and
330 whole mount sensitive in situ hybridisation its dynamic spatio-temporal expression pattern
331 relative to *Nodal*. And we have demonstrated its role in left-right asymmetry based on an allelic
332 series of mutants. Similarly to *Nodal*, *Notch3* is transiently asymmetric, highlighting the
333 importance of careful staging to monitor left-right patterning. In contrast to *Nodal*, *Lefty2* and

334 *Pitx2*, which are exclusively expressed on the left of the lateral plate mesoderm, *Notch3* is also
335 expressed on the right, with a 4.5-fold enrichment on the left side. Thus, quantitative and
336 sensitive approaches are critical to uncover factors which have lower asymmetry levels
337 compared to the *Nodal* pathway.

338 *Nodal* plays multiple roles in asymmetry. *Nodal* is well known to act as a bias, able to set
339 the laterality of asymmetry, so that its absence leads to a randomized orientation. This is the
340 case for the direction of heart looping (12) or the stomach position (14). In addition, *Nodal*,
341 upstream of *Pitx2*, acts as a regulator of identity, able to confer a left anatomical structure to
342 the atria and lungs or to induce the formation of the spleen (14,55,56). Finally, we had shown
343 that *Nodal* can also act as an amplifier of asymmetry (12). This had been detected at a
344 morphological level, with a reduced rotation of the arterial pole during heart looping in *Nodal*
345 mutants. We now show this at a molecular level, with the amplification of *Notch3* asymmetry
346 by *Nodal*.

347 NOTCH signalling is required at different levels for left-right asymmetry. *Notch1/2* were
348 shown previously to play a role in the formation of the left-right organiser and the induction
349 of *Nodal* (24–26). We now identify the role of another NOTCH paralogue, NOTCH3. Whereas
350 *Notch1* is expressed around the node in the caudal epiblast and presomitic mesoderm, and
351 *Notch2* in the node pit (24,31), we have detected *Notch3* in the node crown and lateral plate
352 mesoderm. Our conditional mutants permit to dissect a role of *Notch3* in the lateral plate
353 mesoderm for left-right asymmetry. How *Notch3* regulates heart looping direction and the
354 curvature of the outflow tract remains an open question. *Notch3* regulates muscle
355 differentiation and maturation in other tissues, such as the smooth muscles of arteries,
356 coronary arteries and intestinal lacteals, or skeletal muscles, and is expressed in precursors
357 cells such as pericytes or satellite stem cells (39–42,45). It will be interesting to investigate

358 whether *Notch3* in the heart field similarly regulates the differentiation and maturation of the
359 cardiac muscle to impact heart looping. Elongation of the heart tube is important for heart
360 looping, as a driver of a buckling mechanism (17). Thus, asymmetries in myocardial cell
361 differentiation, when precursor cells incorporate into the heart tube, can provide a left-right
362 bias to orient looping. We have shown previously that *Nodal* modulates a large set of genes
363 involved in myocardial cell differentiation, some of which like *Tnnt1* are asymmetrically
364 expressed within the wild-type heart tube (12). Whether *Notch3* acts similarly remains to be
365 investigated. For later transmural growth and septation of ventricles, it is possible that a
366 potential role of *Notch3* on muscle differentiation and maturation intervenes. However, since
367 *Notch3* is expressed in cardiac precursors and not cardiomyocytes, that would imply a long
368 delay from expression to phenotype. Another possibility is that ventricle growth in *Notch3*
369 mutants is a secondary consequence of defective coronary vascularisation (57).

370 Although *Notch3* expression is sensitive to *Nodal*, our observations support the idea that
371 *Notch3* is not a component of the *Nodal* pathway. The expression pattern of *Notch3* differs
372 from *Nodal*, with a right sided and more medial left sided localization. In contrast to *Pitx2* or
373 *Lefty2* (12), *Notch3* expression is not erased in *Nodal* mutants. Functionally, we do not detect
374 a genetic interaction between *Notch3* and *Nodal*, and show that knock-down of *Notch3* can
375 exacerbate the phenotype of *Nodal* mutants. Thus, *Notch3* comes out as a novel genetic
376 modifier of *Nodal*. The underlying mechanism is still unknown. A link between the two
377 pathways has been detected based on the direct binding of NOTCH3 to the NODAL co-receptor
378 TDGF1, which enhances NOTCH proteolytic maturation and sensitization to ligand-induced
379 NOTCH signalling (58). Given that the disease associated with *Nodal* dysfunction, heterotaxy,
380 has a heterogenous phenotypic spectrum, the identification of genetic modifiers of known

381 genes involved in heterotaxy opens novel perspectives to fill gaps in the genetic diagnosis of
382 60% of patients (59).

383 **Methods**

384 EXPERIMENTAL MODEL AND SUBJECT DETAILS

385 C56Bl6J mice were used as wild-type embryos. *Notch3*^{tm1Grid/tm1Grid} mutants (abbreviated
386 *Notch3*^{-/-}, (38)) were kept in a C56Bl6J background; they are viable and fertile as homozygotes.
387 *Nodal*^{null/+}; *Hoxb1*^{Cre/+} males (12) were maintained in a mixed genetic background and crossed
388 with *Nodal*^{flox/flox} females (60) to generate *Nodal* conditional mutants. Both male and female
389 embryos were collected and used randomly for experiments, except for RNA sequencing, in
390 which only male embryos were used to reduce variability. Embryonic day (E) 0.5 was defined
391 as noon on the day of vaginal plug detection. Embryonic stages were determined based on the
392 morphology of the heart according to (17) and (12). Somite numbers were evaluated from
393 brightfield images; samples with less than 18 somites were excluded from heart looping
394 quantifications. All embryos were genotyped by PCR, using primers listed in Table S2. Animals
395 were housed in individually ventilated cages containing tube shelters and nesting material,
396 maintained at 21°C and 50% humidity, under a 12h light/dark cycle, with food and water ad
397 libitum, in the Laboratory of Animal Experimentation and Transgenesis of the SFR Necker,
398 Imagine Campus, Paris. Animal procedures were approved by the ethical committees of the
399 Institut Pasteur, Université Paris Cité and the French Ministry of Research.

400 METHOD DETAILS

401 **RNA isolation and sequencing**

402 Paired left and right heart fields of E8.5f embryos were micro-dissected, from below the
403 headfolds to the end of the second somite (19), after removal of the heart and the back. The
404 tissue was flash frozen in liquid nitrogen. All samples were collected within 1h30 of sacrificing

405 the mother. Total RNA was extracted in TRIzol-Chloroform and purified using the RNeasy micro
406 kit (QIAGEN) including DNase treatment. RNA quality and quantity were measured on
407 Fragment Analyzer (Agilent Technologies). All RQN were >9.7. The libraries were established
408 using the Nugen Universal Plus mRNA-Seq kit, using 15ng of total RNA per sample. The
409 oriented cDNAs produced from the poly-A+ fraction were PCR amplified (15-18 cycles). An
410 equimolar pool of the final indexed RNA-Seq libraries was sequenced on an Illumina
411 NovaSeq6000, with paired-end reads of 130 bases and a mean sequencing depth of 37.15
412 million reads per sample.

413 **Embryo dissection**

414 Embryos were dissected in 1xDPBS and fixed in 4% paraformaldehyde either for 6h at room
415 temperature or 24h at 4°C. Yolk sac or tail pieces were collected for genotyping. For embryos
416 dissected at E9.5, hearts were arrested in diastole by treatment with cold 250mM KCl for 5
417 minutes. Fixed embryos were gradually dehydrated into methanol and stored at -20°C. For
418 pups collected at P0, they were euthanised, immersed in a cardioplegia solution (110mM NaCl,
419 16mM KCl, 16mM MgCl₂, 1.5mM CaCl₂, 10mM NaHCO₃) for 5 minutes and fixed in 4% PFA 24h
420 at 4°C.

421 **RNA in situ hybridization (ISH)**

422 ISH was performed whole mount as in (12). *Wnt11* and *Bmp2* antisense riboprobes were
423 transcribed from plasmids. Signals were detected by alkaline phosphatase (AP)-conjugated
424 anti-DIG antibodies (1/2000), which were revealed with the BM purple (magenta) substrate.
425 The samples were washed in 1x DPBS, post-fixed and imaged by HREM.

426 Wholmount RNAscope ISH were performed using the Mutliplex Fluorescent v2 Assay
427 (Biotechne) and the protocol of (61). *Notch3* (425171-C1, 425171-C2) and *Nodal* (436321-C1)
428 probes were used. Amplification steps were performed using the TSA cyanine5 and cyanine3
429 amplification kit. Hoechst (1/1000) was used as a nuclear counterstain. Samples were then
430 transferred in R2 CUBIC clearing reagents and embedded in R2 reagent containing agarose
431 (62). Multi-channel 16-bit images were acquired with a Z.1 lightsheet microscope and a
432 20X/1.0 objective.

433 **RT-qPCR**

434 Reverse transcription was performed on RNA isolated from entire (left and right) micro-
435 dissected heart fields using a Reverse Transcription kit (QuantiTect, Qiagen). Quantitative PCR
436 was carried out using the ViiA7 real-time PCR system. mRNA expression levels were measured
437 relatively to *Polr2b* and normalised with a reference cDNA sample, taken as a pool of 7 whole
438 embryos at stage E8.5c-g, using the standard $\Delta\Delta C_t$ method. Primers are listed in Table S2.

439 **High Resolution Episcopic Microscopy (HREM)**

440 E9.5 embryos or P0 hearts were imaged in 3D by HREM after embedding in methacrylate
441 resin (JB4) containing eosin and acridine orange as contrast agents (12). Two channel images
442 of the surface of the resin block were acquired using the optical high-resolution episcopic
443 microscope and a 1X Apo objective repeatedly after removal of on average 1.75 μm or 2.63
444 μm thick sections. The datasets comprise 742-1869 images of 0.90-4.48 μm resolution in x and
445 y depending on the stage. Icy (63) and Fiji (ImageJ) softwares were used to crop and scale the
446 datasets. 3D reconstructions and analysis were performed using Imaris.

447

448 QUANTIFICATION AND STATISTICAL ANALYSIS

449 **Bioinformatics Analyses of bulk RNA sequences**

450 FASTQ files were mapped to the ENSEMBL Mouse GRCm38/mm10 reference using Hisat2
451 and counted by featureCounts from the Subread R package. Due to a high number of
452 duplicates (on average 89%), duplicates were excluded. Flags were computed from counts
453 normalized to the mean coverage. All normalized counts < 20 were considered as background
454 (flag 0) and ≥ 20 as signal (flag = 1). P50 lists used for the statistical analysis gather genes
455 showing flag=1 for at least half of the samples. For the analysis of gene expression, normalized
456 read counts below 250 were considered as background, based on known markers included or
457 excluded from the micro-dissected tissue (Fig. S1B-D). Differential gene expression analysis
458 was performed using three independent methods (DESeq2, edgeR and LimmaVoom) followed
459 by thresholds on absolute fold change ≥ 1.2 and p-value ≤ 0.05 , leading to 597 genes (Table S1).
460 Differential gene expression with LimmaVoom is plotted, and grouped in three categories
461 depending on p-value and corrected p-value (Benjamini-Hochberg procedure). For each
462 differential analysis method, functional analyses were carried out using the Ingenuity Pathway
463 analysis on the list of differentially expressed genes, using delta between conditions and p-
464 value of differential analysis. Transcriptomic data have been deposited in NCBI Gene
465 Expression Omnibus (GEO) with the accession number GSE237126.

466 **Bioinformatics Analyses of published single cell RNA sequences**

467 Data from single cardiac wild-type cells at E8.5 (18) were downloaded from
468 <https://crukci.shinyapps.io/heartAtlas/> analysed using scran (64) and visualized using Seurat
469 v3.1.2 (65). Data from single cardiac wild-type cells at E11.5-P9 (66) were downloaded from
470 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193346>, and visualized similarly.

471 **Quantification of RNAscope ISH signal**

472 3D images were analyzed using Imaris. For quantification of left-right asymmetry in the
473 second heart field, the second heart field was manually segmented in the lateral plate
474 mesoderm layer, using headfolds and the second somite pair as cranial and caudal boundaries,
475 respectively, and separated into left and right following the midline (using neural tube closure
476 as a landmark). Both Hoechst signal and the signal of the gene of interest were extracted. To
477 account for potential biases between the left and right during imaging, the spot detector
478 function was used on the Hoechst channel to generate a normalisation score between the two
479 sides, using as a threshold a coverage of 50% of spots on each side. To calculate the left/right
480 ratio of the gene of interest, its number of spots were measured on the left and right sides,
481 normalised to Hoechst signal and to the volume of the segmented region.

482 **Quantification of the geometry of the heart tube at E9.5**

483 HREM images were used to segment the different compartments of the heart tube in
484 Imaris. Eight landmarks along the tube were extracted and used for quantifications as
485 described previously (12) : one at the exit of the outflow tract, one at the sulcus between the
486 two ventricles, and one at the bifurcation of the two atria (taken as the venous pole). The five
487 other landmarks are centroids of cardiac regions: outflow tract, right ventricle, left ventricle,
488 atrioventricular canal and left atrium, and right atrium, segmented according to the expression
489 patterns of *Wnt11* and *Bmp2* and anatomical landmarks such as cushion boundaries or the
490 interventricular or interatrial sulcus. Heart shapes were aligned in 3D using an in-house
491 MATLAB code so that the Z-axis corresponds to the notochord axis and the x-axis to a
492 perpendicular dorso-ventral axis. The orientation of the axis between the left and right

493 ventricles, the distance of the venous pole to the notochord and the tube length were
494 measured as in (17).

495 **Phenotyping of congenital heart defects**

496 Hearts of *Notch3* mouse mutants were phenotyped at P0 in 3D images acquired by HREM,
497 based on the segmental approach (67) and IPCCC ICD-11 clinical code.

498 **Statistical Analyses**

499 Statistical tests and p-values are described in figure legends and Table S3. Group allocation
500 was based on PCR genotyping. All sample numbers (n) indicated in the text refer to biological
501 replicates, i.e. different embryos or different cells. Investigators were blinded to allocation
502 during imaging and phenotypic analysis, but not during quantifications. Pairwise Mann-
503 Whitney-Wilcoxon tests were used to compare experimental groups, with a Benjamini-
504 Hochberg correction applied on p-values when more than 2 populations are compared. A chi-
505 square test was used to compare percentage distributions, or a chi-square test with Yates'
506 continuity correction for comparison with an expected value. Tests were performed with R and
507 Excel. Embryos which had been damaged during experiments were excluded for
508 quantification.

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519 Conceptualization, S.M.M., A.D., T.H.B.; Formal Analysis, T.H.B., A.D., M-A.C., E.P.; Funding
520 Acquisition, S.M.M.; Investigation, T.H.B., A.D., M-A.C.; Methodology, T.H.B., A.D., E.P., S.M.M.;
521 Project Administration, S.M.M.; Software, T.H.B., E.P. ; Supervision, S.M.M., A.D., T.H.B.;
522 Visualization T.H.B., A.D., M-A.C., E.P.; Writing – Original Draft, S.M.M. ; Writing – Review &
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722 **Supporting Information**

723 **Figure S1 related to Fig. 1. Transcriptomic approach. (A)** Brightfield images of wild-type
724 embryos used for RNA sequencing at E8.5f. In the left panel, an outline of the dissected areas
725 is shown. The identification number of embryos is given. **(B)** Normalised read counts of genes
726 used to validate the threshold of expression in the transcriptomic analysis. The osteocyte gene
727 *Dmp1*, inner ear marker *Oc90*, neuronal marker *Neurod1* are used as negative controls and
728 *Mmp9* as a positive control, lowly expressed in left heart progenitors. Whisker plots show the
729 median, 25th- and 75th quartiles (boxes), and the extreme data points (whiskers). **(C)**
730 Normalised read counts of genes used as markers to control sample micro-dissection. *En2*,
731 *Wnt8b* are anterior markers, *Hoxa5*, *Hoxb6* posterior markers, *Rfx4*, *Tfap2b* back markers, *Isl1*,
732 *Six2*, *Fgf8* second heart field markers, *Tbx5*, *Mab21l2* juxta-cardiac field (JCF) markers and
733 *Myh7b*, *Myoz1*, *Tnnt1* cardiomyocyte markers. The dotted line indicates the threshold of
734 background expression. **(D)** Normalised read counts of genes used as markers to validate the
735 left-right dissection of samples. NODAL targets *Lefty2* and *Pitx2*, as well as *Six2* label the left
736 side. *Nodal* is turned off at E8.5f. *p-value between the left and right sides < 0.05, ***
737 Benjamini-Hochberg corrected p-value < 0.00001 (LimmaVoom, n=4). **(E)** Violin plot of *Dtx4*
738 expression in single cells at E8.5 from (18), clustered as annotated (n= 89 Ec2, 59 Me2, 713
739 Me3, 221 Me4, 355 Me5, 65 Me6, 514 Me7). Dots are normalised reads per cell. **(F)** Violin plot
740 of *Notch3* expression in single *Nodal*-negative (n=1559) and *Nodal*-positive (n=309) cells of
741 cardiac clusters (Me3-7) of (18) at E8.5 (stage 1 to Late Heart Tube, wild-type embryos). 75%
742 (234/309) of *Nodal*-positive cells also express *Notch3*.

743 **Figure S2 related to Fig. 2. Normal *Nodal* expression in *Notch3* mutants. (A-B)** Whole
744 mount RNAscope ISH of *Nodal* in *Notch3*^{-/-} mutant embryos at E8.5c in anterior (A) and
745 posterior (B) frontal views. The midline of the embryo is indicated by a yellow dotted line. A

746 transverse section at the level of the node (see b1) is shown in b2. Filled and empty
747 arrowheads point to high and absent expression, respectively. **(C-D)** Transverse section of the
748 left lateral plate mesoderm, labelled by double wholemount RNAscope ISH of *Nodal* (red) and
749 *Notch3* (white). The region of *Notch3* and *Nodal* co-expression is outlined in yellow (n=3). L,
750 left; LPM, lateral plate mesoderm; No, node; NT, neural tube; R, right.

751 **Figure S3 related to Fig. 3. *Notch3* inactivation in mutants. (A)** Schema of *Notch3* alleles in
752 wild-types and *Notch3*^{-/-} mutants, indicating the localisation of exons and primers used for RT-
753 qPCR. **(B)** Relative expression of *Notch3* in micro-dissected heart fields of littermate wild-types
754 (n=5 E8.5e, 3 E8.5g), *Notch3*^{+/-} (n=6 E8.5e, 6 E8.5g) and *Notch3*^{-/-} (n=6 E8.5e, 4 E8.5g) embryos,
755 quantified by RT-qPCR using the indicated primer pairs and normalised to wild-types. **p-
756 value<0.01, ***p-value<0.001 (Pairwise Mann-Whitney Wilcoxon tests with Benjamini-
757 Hochberg correction).

758 **Figure S4 related to Fig. 4. *Notch3* expression after heart looping.** Violin plot of *Notch3*
759 expression in single cardiac cell transcriptomic between E11.5 and P9 (from (66)), clustered as
760 annotated (n= 5422 atrial cardiomyocytes, 10493 ventricular cardiomyocytes, 2361
761 endocardium, 1176 vascular endocardium, 1012 epicardium, 3309 fibroblast like cells, 182
762 smooth muscle cells, 349 pericytes, 1032 macrophages, 33 B cells, 42 T cells, 17 dendritic cells,
763 23 natural killer cells, 22 neutrophils, 244 blood cells). Dots are normalised reads per cell.

764 **Video S1 related to Fig. 1. Co-expression of *Notch3* and *Nodal*.** *Notch3* (white) and *Nodal*
765 (magenta) double whole-mount RNAscope ISH in a wild-type E8.5d embryo imaged in 3D by
766 lightsheet microscopy.

767 **Table S1 related to Fig. 1.** List of 597 differentially expressed genes used for Ingenuity
768 Pathway analysis, based on a fold change ≥ 1.2 and p-val ≤ 0.05 with either the LimmaVoom,

769 DESeq2 or edgeR methods. Flags indicate the number of samples in which normalised read
770 counts are ≥ 20 (n=4 embryos). Lf, left heart field at E8.5f; Rf, right heart field at E8.5f.

771 **Table S2. List of primers** used for genotyping and RT-qPCR.

772 **Table S3. Data.** Numerical data used to generate figure graphs along with statistical tests.

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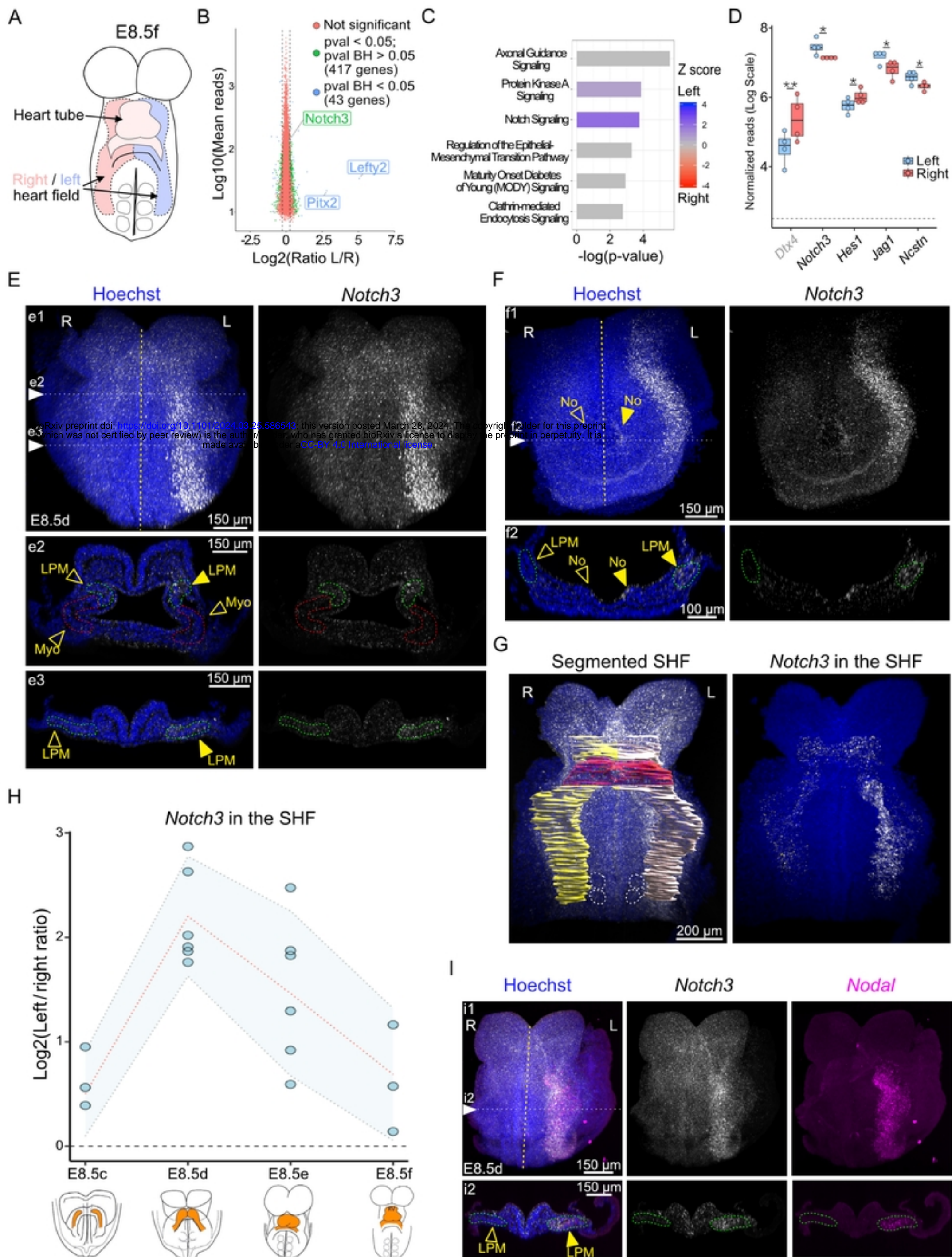


Figure 1

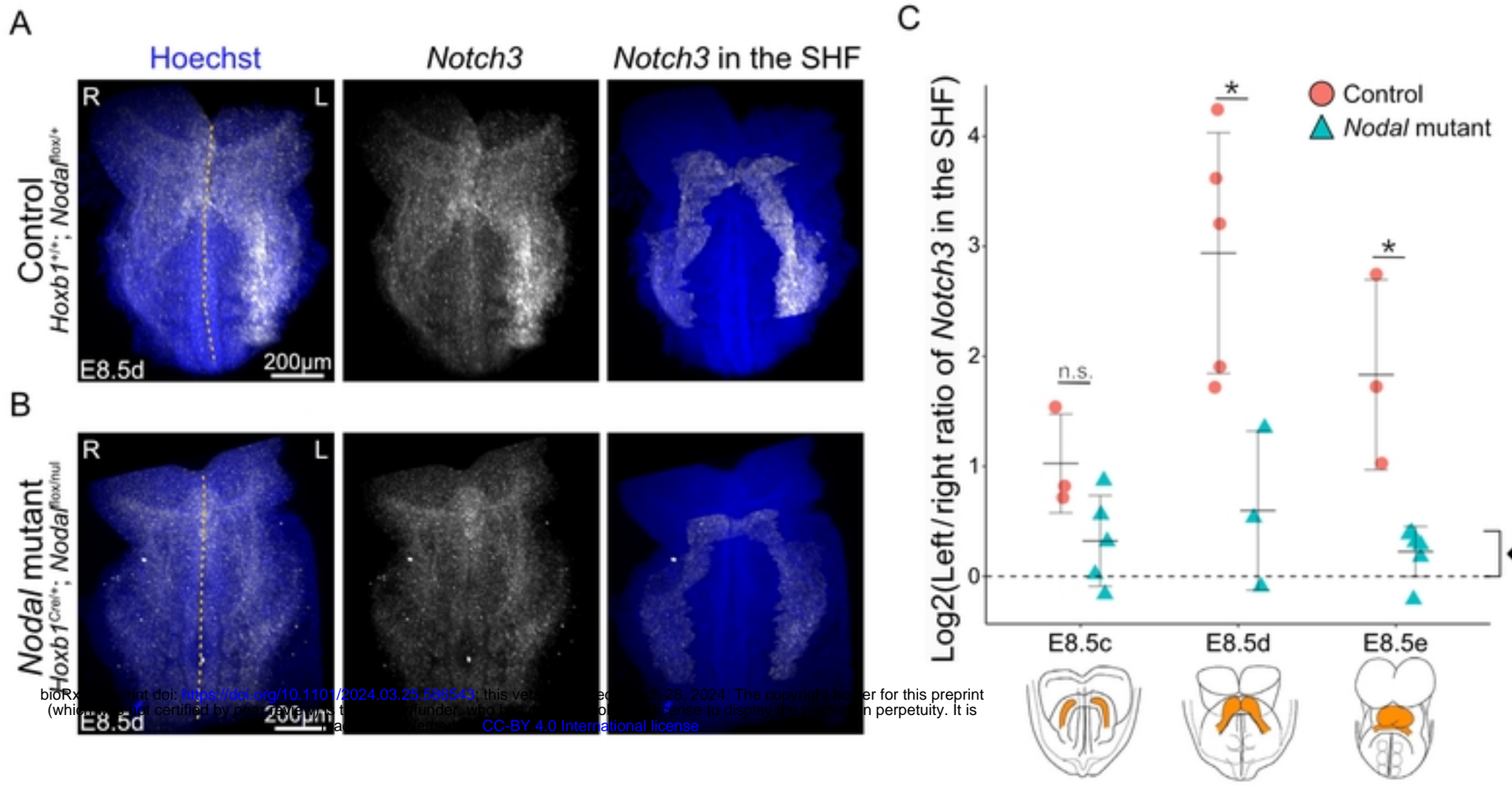


Figure 2

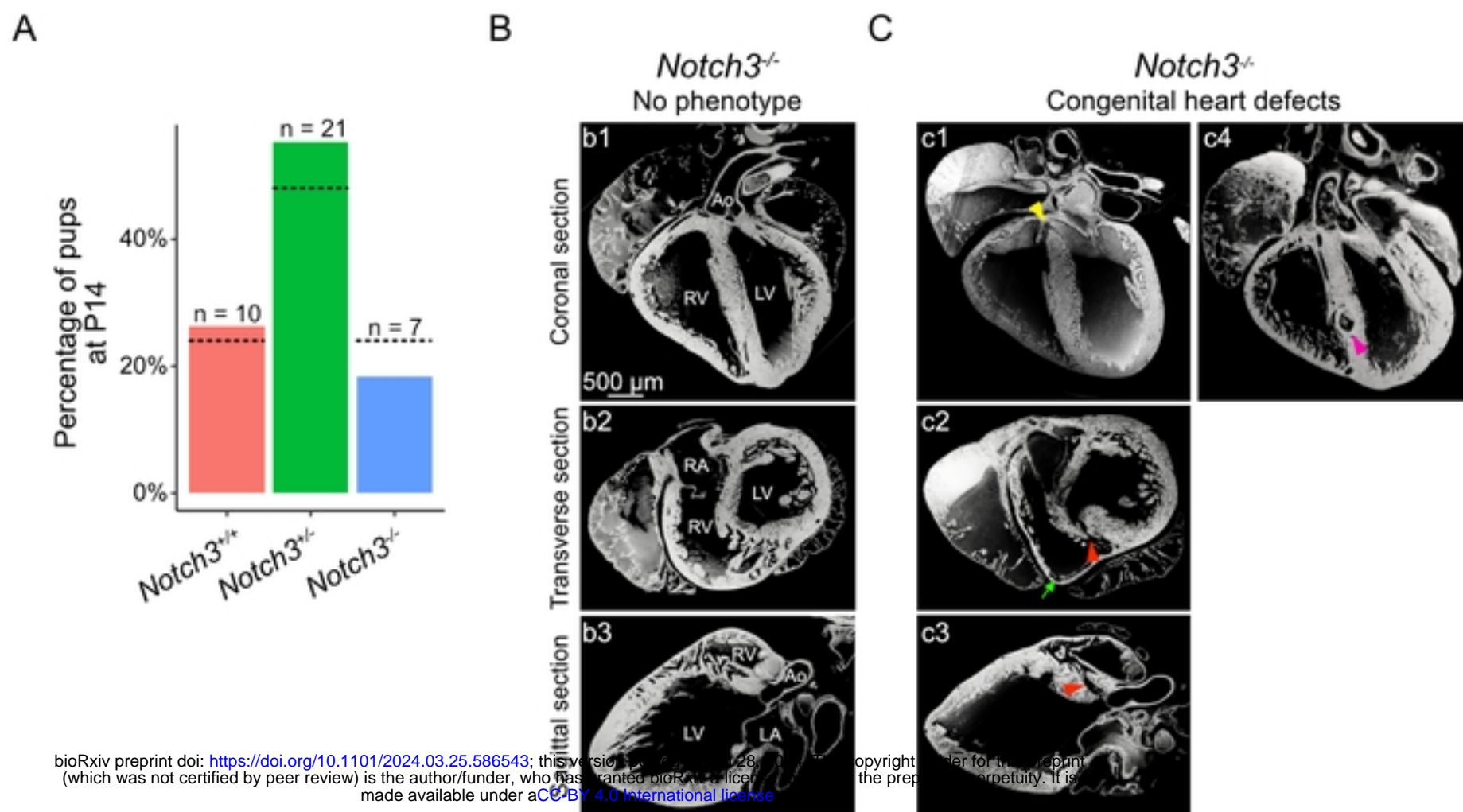


Figure 4

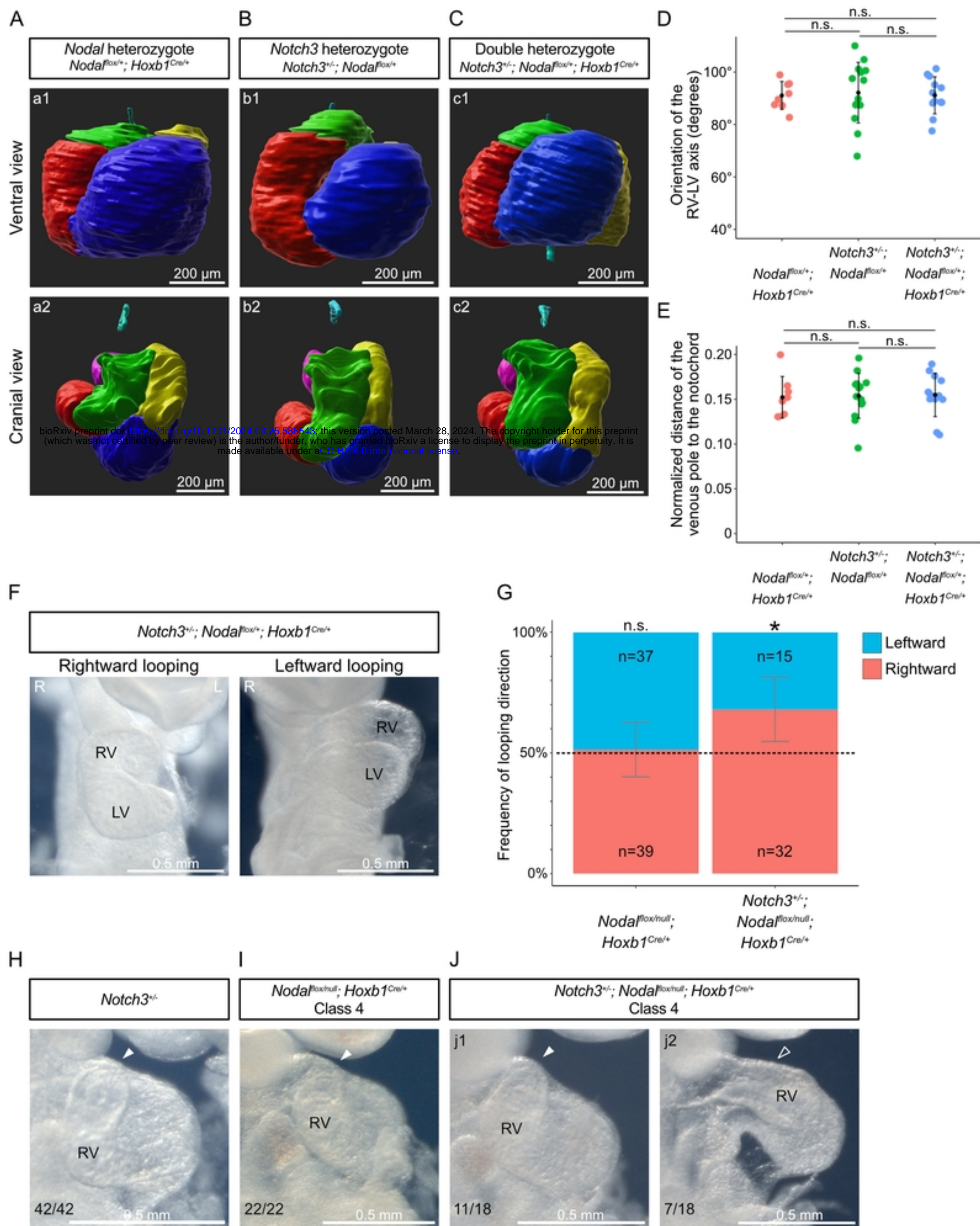


Figure 5