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NOTCH activation interferes with cell fate specification in the gastrulating mouse embryo

Céline Souilhol^{1,2,*,*§}, Aitana Perea-Gomez^{3,§,¶}, Anne Camus^{3,‡}, Sarah Beck-Cormier^{1,2,‡}, Sandrine Vandormael-Pournin^{1,2}, Marie Escande^{1,2}, Jérôme Collignon³ and Michel Cohen-Tannoudji^{1,2,¶}

ABSTRACT

NOTCH signalling is an evolutionarily conserved pathway involved in intercellular communication essential for cell fate choices during development. Although dispensable for early aspects of mouse development, canonical RBPJ-dependent NOTCH signalling has been shown to influence lineage commitment during embryonic stem cell (ESC) differentiation. NOTCH activation in ESCs promotes the acquisition of a neural fate, whereas its suppression favours their differentiation into cardiomyocytes. This suggests that NOTCH signalling is implicated in the acquisition of distinct embryonic fates at early stages of mammalian development. In order to investigate *in vivo* such a role for NOTCH signalling in shaping cell fate specification, we use genetic approaches to constitutively activate the NOTCH pathway in the mouse embryo. Early embryonic development, including the establishment of anterior-posterior polarity, is not perturbed by forced NOTCH activation. By contrast, widespread NOTCH activity in the epiblast triggers dramatic gastrulation defects. These are fully rescued in a RBPJ-deficient background. Epiblast-specific NOTCH activation induces acquisition of neuroectoderm identity and disrupts the formation of specific mesodermal precursors including the derivatives of the anterior primitive streak, the mouse organiser. In addition, we show that forced NOTCH activation results in misregulation of NODAL signalling, a major determinant of early embryonic patterning. Our study reveals a previously unidentified role for canonical NOTCH signalling during mammalian gastrulation. It also exemplifies how *in vivo* studies can shed light on the mechanisms underlying cell fate specification during *in vitro* directed differentiation.

KEY WORDS: NOTCH, NODAL signalling, Mouse embryo, Mesoderm, Anterior-posterior patterning

INTRODUCTION

The evolutionarily conserved NOTCH signalling pathway is a key regulator of cell specification during embryonic development and adult tissue homeostasis (reviewed by Andersson et al., 2011; Artavanis-Tsakonas and Muskavitch, 2010; Guruharsha et al., 2012; Koch et al., 2013). In mammalian cells, there are four NOTCH receptors (NOTCH1–4) and several transmembrane ligands such as

Delta-like (DLL1, DLL3, DLL4) and Jagged (JAG1 and JAG2). The canonical NOTCH signalling pathway involves ligand binding to a NOTCH receptor resulting in its cleavage by a membrane-associated protease complex (γ -SECRETASE), which contains PRESENILIN. The released intracellular domain of NOTCH (NICD) is then translocated into the nucleus where it interacts with the DNA-binding protein CSL [also known as CBF1 in humans; Su(H) in *Drosophila*; LAG1 in *Caenorhabditis elegans*], also called RBPJ in vertebrates. Both proteins form a complex with other modulators, such as the transactivator Mastermind-like (MAML), and induce the expression of downstream target genes, which include members of the HES/HEY family. NOTCH signalling is modulated at different levels by numerous proteins and through integration with other signalling pathways, thus allowing delicate fine-tuning and context-dependent cellular responses to signals transmitted by NOTCH receptors.

The NOTCH pathway has been shown to influence cell fate choices leading to the formation of germ layers and the specification of distinct embryonic lineages in various organisms such as *C. elegans*, sea urchin, zebrafish and *Xenopus* (Acosta et al., 2011; Revinski et al., 2010; Sethi et al., 2012; reviewed by Shi and Stanley, 2006). In the mouse embryo, the earliest developmental event regulated by NOTCH signalling, in collaboration with the HIPPO pathway, is the formation, before implantation, of an extra-embryonic lineage, the trophoctoderm (Rayon et al., 2014). However, it is still unclear whether NOTCH signalling is involved in the specification of the embryonic lineages that arise from the pluripotent epiblast in mice to form the definitive germ layers. Mutant embryos defective in NOTCH signalling (*Rbpj*, *Notch1*, *Notch2*, *Jag1* and *Dll1* mutants) survive to approximately embryonic day (E) 9.5 and show severe defects in somitogenesis, neurogenesis, vasculogenesis and cardiogenesis. Nevertheless, they are indistinguishable from wild-type embryos at E8.0, by which stage all three germ layers have formed (reviewed by Yoon and Gaiano, 2005). Moreover, mutant embryos lacking maternal and zygotic RBPJ or O-fucosyltransferase 1 (POFUT1), both essential components of the canonical NOTCH signalling pathway, develop normally until E8.0 (Shi and Stanley, 2006; Souilhol et al., 2006).

Although loss-of-function analyses indicate that NOTCH signalling is dispensable for early embryogenesis in the mouse, these studies do not exclude a role for the pathway, alone or in collaboration with other signalling cascades, in the specification of mammalian embryonic lineages during gastrulation. Indeed, NOTCH signalling is likely to be active in early post-implantation embryos as suggested by the expression of the *CBF:H2B-Venus* NOTCH reporter and by the immunodetection of cleaved NICD in post-implantation embryos (Del Monte et al., 2007; Nowotschin et al., 2013). Moreover, *in vitro* studies on mouse (mESC) and human (hESC) embryonic stem cells support a role for NOTCH signalling in shaping early cell fate decisions. NOTCH is

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dispensable for mESC and hESC self-renewal. *Notch1* and *Rbpj* null mESCs can be established and maintained normally (Nemir et al., 2006; Schroeder et al., 2003) and blockade of the NOTCH pathway in hESCs by using a γ -SECRETASE inhibitor or a dominant negative form of MAML seems to enhance the growth of undifferentiated cells and to prevent their spontaneous differentiation (Noggle et al., 2006; Yu et al., 2008). However, canonical NOTCH signalling has been shown to regulate lineage commitment when ESCs are induced to differentiate. Constitutive expression of NICD in mESCs or treatment of hESCs with DLL1-expressing feeders favours the acquisition of a neuroectodermal fate (Das et al., 2010; Kurpinski et al., 2010; Lowell et al., 2006). By contrast, inactivation of the NOTCH pathway with a γ -SECRETASE inhibitor or via genetic deletion of *Notch1* and *Rbpj* accelerates ESC differentiation towards mesoderm, and enhances their cardiogenic potential (Jang et al., 2008; Nemir et al., 2006; Schroeder et al., 2003). Further revealing the complexity of NOTCH signalling, ligand-dependent cell type specification was observed when mESCs were exposed to JAG1 or DLL4 (Ramasamy and Lenka, 2010). Thus, NOTCH signalling stands as an important regulator of early cell fate choices *in vitro*, during the first steps of ESC commitment.

In order to investigate a potential role for NOTCH signalling during early embryonic cell fate specification *in vivo*, we constitutively activated the NOTCH pathway in the pre- and peri-implantation mouse embryo by using genetic approaches. We found that forced NOTCH activity has no dramatic effect before gastrulation. Anterior-posterior polarity is established normally despite widespread activation of the NOTCH pathway in the epiblast. By contrast, during gastrulation, NOTCH gain-of-function affects the differentiation of cell types from all three germ layers. Widespread NOTCH activity triggers acquisition of neuroectoderm identity and disrupts the formation of specific mesodermal precursors. In addition, we show that forced NOTCH activation results in misregulation of NODAL signalling, an essential pathway controlling cell fate decisions during gastrulation. Our approach reveals a new role for the NOTCH pathway at the onset of the gastrulation, which previous analyses of mutant embryos had not identified.

RESULTS

NOTCH activation does not trigger major developmental abnormalities before gastrulation

In order to study the effect of NOTCH gain-of-function during early mouse development, we analysed embryos derived from crosses between *Zp3-Cre^{tg/0}; Rosa26^{NIICD/+}* females and wild-type males. CRE expression in the oocytes of these females at the onset of follicular growth mediates the removal of a STOP cassette, thereby allowing expression from the *Rosa26* locus of a bi-cistronic mRNA coding for NOTCH1 intracellular domain (NIICD) and nuclear GFP. Normal ovulation rates were observed for these females, indicating that oogenesis was not perturbed by oocyte-specific NOTCH activation during follicular development and maturation (data not shown). At E3.5, blastocysts were recovered and cultured *in vitro* for 24 h. Embryos that expressed GFP and had therefore inherited the recombined *Rosa26* allele (designated NIICD^{ovo} hereafter) were obtained at the expected 50% ratio (106/191). NIICD^{ovo} embryos expressed GFP widely both in inner cell mass and trophectoderm cells and were morphologically indistinguishable from their control littermates (Fig. 1A–B').

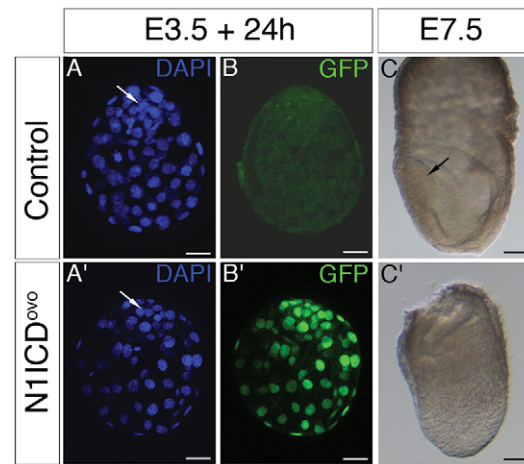


Fig. 1. Phenotype of embryos obtained from crosses between *Rosa26^{NIICD/+}; Zp3-Cre^{tg/0}* females and wild-type males. (A,A') DAPI staining, white arrows indicate the inner cell mass in control and NIICD^{ovo} E3.5 embryos cultured for 24 h. (B,B') GFP expression is absent in control embryos and present in all cells of NIICD^{ovo} embryos. (C,C') At E7.5, NIICD^{ovo} embryos do not show signs of headfold formation. Black arrow shows the left headfold in the control embryo. Scale bars: 25 μ m in A–B'; 100 μ m in C,C'.

NIICD^{ovo} embryos were recovered at expected frequencies after implantation (10 control versus 7 NIICD^{ovo} at E6.5; 7 versus 9 at E7.5; 6 versus 4 at E8.5 and 7 versus 5 at E10.5, respectively). However, morphological abnormalities could be detected as early as E7.5, with NIICD^{ovo} embryos reduced in size and showing no signs of neural plate and allantois bud formation (Fig. 1C,C'). These morphological abnormalities persisted at later stages, leading to developmental arrest by mid-gestation (data not shown).

Together, these observations indicate that although NOTCH activation is compatible with progression through the pre-implantation and peri-implantation periods, it becomes detrimental to post-implantation development at the time of gastrulation.

Correct regulation of canonical NOTCH signalling in the epiblast is essential for post-implantation development

To specifically analyse the impact of forced NOTCH activation on the process of germ layer formation during early post-implantation development, we generated embryos expressing NIICD exclusively in the epiblast and its derivatives. *Rosa26^{NIICD/NIICD}* females were crossed with *Sox2-Cre^{tg/0}* males to give rise to *Rosa26^{NIICD/+}; Sox2-Cre^{tg/0}* embryos (designated NIICD^{epi} hereafter). Using GFP expression as an indication of NIICD expression, we found that expression of NIICD began in a mosaic fashion in the E4.5 epiblast ($n=10$; Table 1, Fig. 2A–B'). Expression then became detectable in

Table 1. Morphological defects and GFP expression in embryos obtained from crosses between *Rosa26^{NIICD/NIICD}* females and *Sox2-Cre^{tg/0}* males

Developmental stage	Normal morphology	Abnormal morphology
E4.5	33 (10*)	0
E5.5	112 (58)	0
E6.5	194 (86)	0
E7.5	131 (0)	128 (128)
E8.5	66 (0)	59 (59)
E9.5	24 (0)	30 (30)

Number of GFP-positive embryos given in parentheses.

*Mosaic GFP expression in the epiblast.

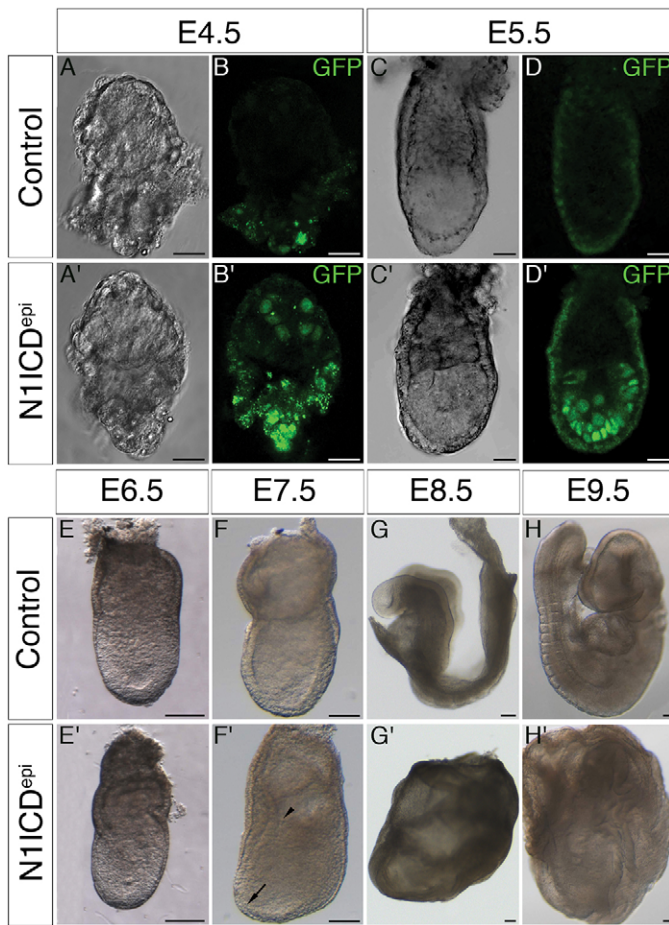


Fig. 2. Correct regulation of canonical NOTCH signalling pathway is essential for early post-implantation development. (A-D') Morphology and GFP expression of E4.5 and E5.5 control (A-D) and N1ICD^{epi} (A'-D') embryos obtained by crossing *Rosa26^{N1ICD/N1ICD}* females with *Sox2-Cre^{tg/0}* males. B, B', D, D' are single confocal sections. GFP is expressed first in a mosaic fashion (B) and then homogeneously (D') in the epiblast of the morphologically normal N1ICD^{epi} embryos (see also Table 1). (E-H') Morphology of E6.5 to E9.5 control (E-H) and N1ICD^{epi} (E'-H') embryos. At E7.5, N1ICD^{epi} embryos exhibited an elongated and thin antero-distal epiblast (arrow in F') and a delay or a lack of closure of the amnion (arrowhead in F' points to the proamniotic canal). At E8.5-E9.5, N1ICD^{epi} embryos do not show any sign of segmentation and are highly disorganised (G', H'). Scale bars: 25 μ m in A-D'; 100 μ m in E-H'.

all epiblast cells from E5.5 ($n=58$; Table 1, Fig. 2C-D'). Until E6.5, N1ICD^{epi} embryos were morphologically undistinguishable from control littermates (Table 1, Fig. 2E, E'). The first obvious anomalies were observed at E7.5, when N1ICD^{epi} embryos exhibited an elongated and thin anterodistal epiblast and a persistent proamniotic canal (Table 1, Fig. 2F, F'). At E8.5-E9.5, they did not show any sign of somitic segmentation and were highly disorganised (Table 1, Fig. 2G-H'). These results demonstrate that perturbation of NOTCH activity in the epiblast results in a dramatic gastrulation defect.

In order to determine if the phenotype observed in N1ICD^{epi} embryos resulted from a misregulation of the canonical NOTCH pathway, we analysed the expression of canonical NOTCH target genes, *Hes1* and *Hes5*. Both of them were significantly upregulated in the epiblast from E6.5 (Fig. 3), suggesting that the phenotype we observe in N1ICD^{epi} embryos is a direct consequence of elevated canonical NOTCH pathway activity. We then asked whether the phenotype of N1ICD^{epi} embryos could be rescued in an *Rbpj*-deficient background. RBPJ is an essential downstream component

of the canonical NOTCH pathway and its deletion should theoretically compensate for the dramatic effects of elevated NOTCH activity. We used mice that carry an *Rbpj* floxed allele, *Rbpj^{lox}*, which can be deleted upon Cre activity to give rise to a null allele, *Rbpj ^{Δ}* (Tanigaki et al., 2002). *Rosa26^{N1ICD/N1ICD}*; *Rbpj^{lox/\Delta}* females were crossed with *Sox2-Cre^{tg/0}*; *Rbpj^{+/\Delta}* males in order to collect N1ICD^{epi} embryos deficient for RBPJ (Fig. 4, Fig. S1, Table 2). Constitutive (*Rbpj ^{Δ/Δ}*) and epiblast-specific (*Sox2-Cre^{tg/0}*; *Rbpj^{lox/\Delta}*) *Rbpj* mutant embryos showed severe growth retardation, poor somite formation and an irregularly shaped neural tube (Fig. 4B, B', Fig. S1) (Oka et al., 1995). Remarkably, the post-implantation-specific activation of the NOTCH pathway in *Rbpj*-deficient mutants did not result in the severe defects observed in N1ICD^{epi} embryos (Fig. 4C, C', Fig. S1); instead their phenotype was similar to the one exhibited by *Rbpj* mutant embryos (Fig. 4D, D', Table 2, Fig. S1). This demonstrates that blocking the canonical NOTCH pathway is sufficient to rescue all the developmental anomalies induced by epiblast-specific NOTCH activation. Together, these results demonstrate that misregulation of the canonical NOTCH pathway in the epiblast impairs early post-implantation mouse development.

NOTCH activation does not affect anterior-posterior polarity establishment and gastrulation initiation

In order to determine the primary defect induced by the misregulation of the canonical NOTCH pathway in N1ICD^{epi} embryos, we analysed the expression of molecular markers characteristic of the different cell lineages that emerge during early post-implantation development. Activation of the NOTCH pathway did not perturb early epiblast identity as indicated by the normal expression of *Oct4* (also known as *Pou5f1*), *Fgf5*, *Nodal* and *Cripto* (also known as *Tdgf1*) (Ding et al., 1998; Mesnard et al., 2006; Pelton et al., 2002) at E5.5 in the epiblast of N1ICD^{epi} embryos ($n=5$, $n=3$, $n=3$ and $n=4$, respectively; Fig. 5A-D'). Extra-embryonic tissues were also correctly patterned at this stage. *Bmp4* expression was restricted to the distalmost part of the extra-embryonic ectoderm ($n=5$; Fig. 5E, E') (Lawson et al., 1999). In the visceral endoderm (VE), *Nodal* transcripts were found exclusively in the region covering the epiblast ($n=3$; Fig. 5C, C') (Mesnard et al., 2006), and the distal visceral endoderm (DVE) was induced as shown by the presence of *Lefty1* transcripts ($n=3$; Fig. 5F, F') (Yamamoto et al., 2004).

The proximodistal polarity of the E5.5 embryo is transformed into anterior-posterior polarity at E6.5 through the migration of DVE cells from distal to anterior positions, where they take the name of anterior visceral endoderm cells (AVE), and the formation of the primitive streak in the posterior epiblast (reviewed by Rossant and Tam, 2009; Takaoka and Hamada, 2012). The transcription factor brachyury (*T*) marked the posterior epiblast and the nascent primitive streak both in control and N1ICD^{epi} littermates ($n=3$; Fig. 5G, G') (Wilkinson et al., 1990). *Cer1*, encoding a NODAL antagonist expressed in AVE cells at the anterior pole of the embryo (Biben et al., 1998), was expressed normally in N1ICD^{epi} embryos ($n=4$; Fig. 5H, H'). Together these results demonstrate that misregulation of the canonical NOTCH pathway in N1ICD^{epi} embryos does not impair the establishment of anterior-posterior polarity and gastrulation initiation.

NOTCH activation impairs axial mesoderm formation and perturbs NODAL signalling

Despite the establishment of anterior-posterior polarity, specific molecular defects were observed in N1ICD^{epi} embryos. The

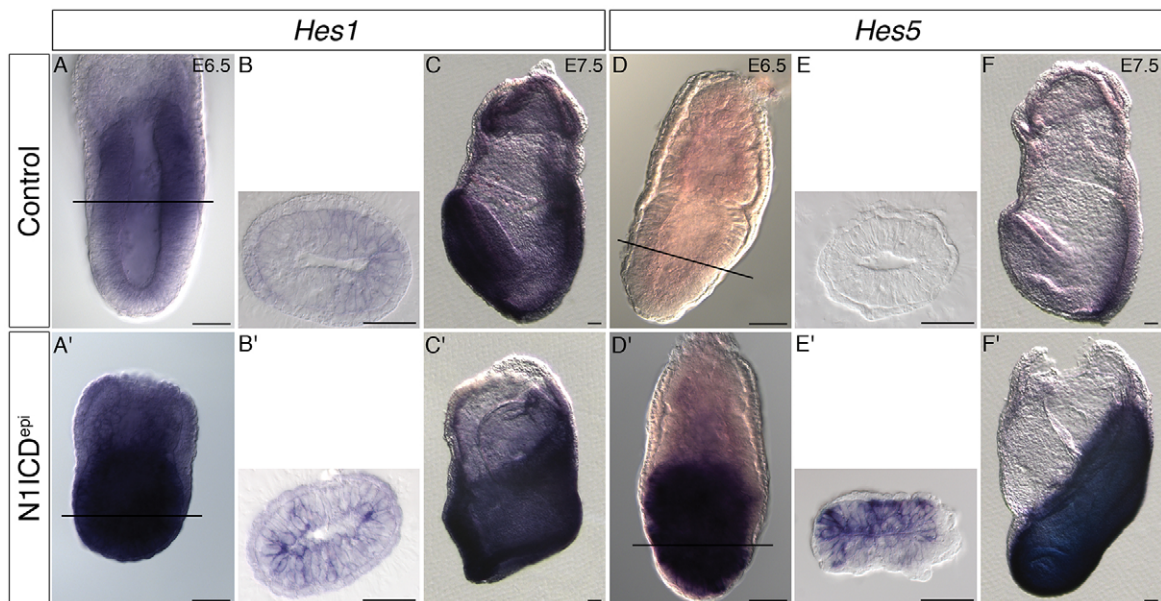


Fig. 3. Misregulation of NOTCH target genes in N1ICD^{epi} embryos. Expression of *Hes1* (A-C') and *Hes5* (D-F') in control (A-F) and N1ICD^{epi} (A'-F') embryos at E6.5 and E7.5. B, B' and E, E' are cryosections at the levels indicated by black lines in A, A' and D, D'. Expression of *Hes1* and *Hes5* is upregulated in the epiblast of N1ICD^{epi} embryos from E6.5. Scale bars: 50 μm.

transcription factor goosecoid (*Gsc*) is expressed in the AVE and in the anterior primitive streak (APS) at E6.5 (Faust et al., 1995). In N1ICD^{epi} embryos, *Gsc* transcripts were observed in the AVE but its expression in the APS was reduced ($n=2/7$) or absent ($n=5/7$; Fig. 6A, A'). In addition, *Lefty1* expression was not maintained in the AVE of E6.5 N1ICD^{epi} embryos ($n=11/12$; Fig. 6B, B'). *Gsc* and *Lefty1* expression in the APS and AVE respectively require high levels of NODAL signalling in the epiblast (Hoodless et al., 2001; Takaoka et al., 2006; Vincent et al., 2003; Yamamoto et al., 2001). We found that *Nodal* expression was reduced in the epiblast, the primitive streak and the VE of N1ICD^{epi} embryos ($n=4/5$; Fig. 6C, C'). These observations indicate that forced activation of the

canonical NOTCH pathway in the epiblast results in downregulation of *Nodal* and of its targets in the AVE and the APS at E6.5.

The downregulation of *Nodal* expression in N1ICD^{epi} embryos was not a result of a failure to sustain epiblast development as the markers *Otx2* and *Oct4* (Ang et al., 1994; Pelton et al., 2002) were expressed at normal levels in these embryos at E6.5 ($n=6$ and $n=3$, respectively; Fig. 6D, D', Fig. S2). The maintenance of *Nodal* expression in the epiblast, the primitive streak and the VE depends both on an autoregulatory loop involving NODAL signalling through its co-receptor CRIPTO (TDGF1), on the transcription factor OCT4, and on the WNT3/β-catenin pathway (Adachi et al., 1999; Ben-Haim et al., 2006; Granier et al., 2011; Norris et al.,

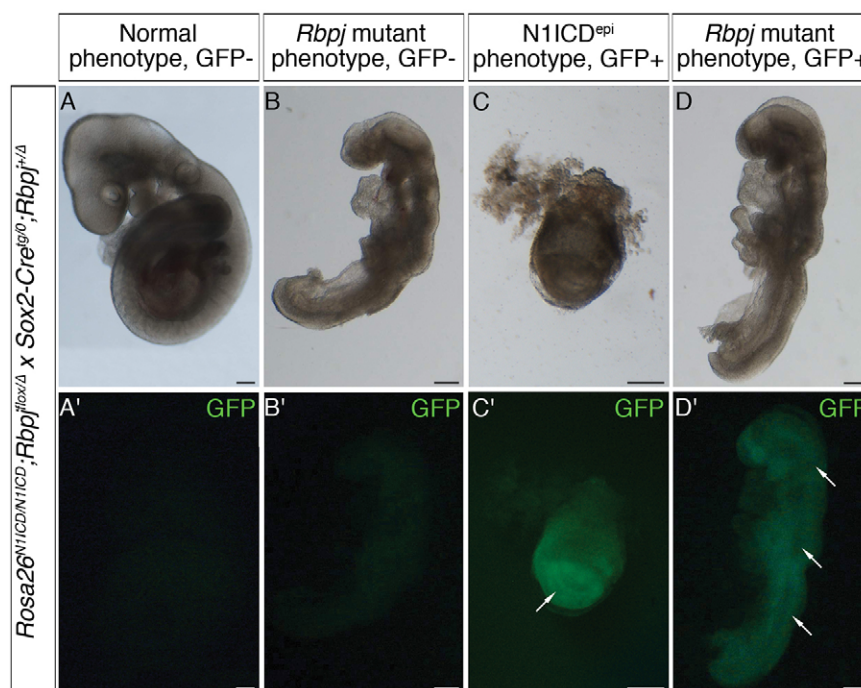


Fig. 4. The phenotype of N1ICD^{epi} embryos is dependent on RBPJ. (A-D') Morphology (A-D) and GFP expression (A'-D') in E9.5 embryos obtained from crosses between *Rosa26*^{N1ICD/N1ICD}; *Rbpj*^{flax/Δ} females and *Sox2-Cre*^{tg/tg}; *Rbpj*^{+1Δ} males. (A, A') Embryo showing a normal phenotype. (B, B') Embryo showing severe growth retardation, poor somite formation and tortuous neural tube, characteristic of the *Rbpj* mutant phenotype. (C, C') Embryo showing severe disorganisation, similar to N1ICD^{epi} embryos and expressing N1ICD-GFP in the epiblast (arrow). (D, D') Embryo with *Rbpj* mutant phenotype and expressing N1ICD-GFP in epiblast-derived tissues (arrows). Scale bars: 500 μm.

Table 2. Morphological defects and GFP expression in embryos obtained from crosses between *Rbpj*^{flx/Δ}, *Rosa26*^{N1ICD/N1ICD} females and *Rbpj*^{+/Δ}, *Sox2-Cre*^{tg/0} males

Genotype	Phenotype			
	Normal GFP negative	RBPJ mutant GFP negative	N1ICD ^{epi} GFP positive	RBPJ mutant GFP positive
<i>Rbpj</i> ^{flx/+} <i>Rosa26</i> ^{N1ICD/+}	4			
<i>Rbpj</i> ^{Δ/+} <i>Rosa26</i> ^{N1ICD/+}	5			
<i>Rbpj</i> ^{flx/Δ} <i>Rosa26</i> ^{N1ICD/+}	3			
<i>Rbpj</i> ^{Δ/Δ} <i>Rosa26</i> ^{N1ICD/+}		2		
<i>Rbpj</i> ^{flx/+} <i>Rosa26</i> ^{N1ICD/+} <i>Sox2-Cre</i> ^{tg/0}			5	
<i>Rbpj</i> ^{Δ/+} <i>Rosa26</i> ^{N1ICD/+} <i>Sox2-Cre</i> ^{tg/0}			4	
<i>Rbpj</i> ^{flx/Δ} <i>Rosa26</i> ^{N1ICD/+} <i>Sox2-Cre</i> ^{tg/0}				3
<i>Rbpj</i> ^{Δ/Δ} <i>Rosa26</i> ^{N1ICD/+} <i>Sox2-Cre</i> ^{tg/0}				5

2002; Norris and Robertson, 1999; Papanayotou et al., 2014). *Oct4*, *Cripto* and *Wnt3* were all normally expressed in E6.5 N1ICD^{epi} embryos ($n=3$, $n=5$ and $n=3$, respectively; Fig. S2, Fig. 6E-F'). The extra-embryonic ectoderm-derived BMP4, involved in *Cripto* and *Wnt3* induction (Beck et al., 2002; Ben-Haim et al., 2006), was also expressed in E6.5 N1ICD^{epi} embryos (data not shown). Moreover, the WNT3/β-catenin pathway was active in N1ICD^{epi} embryos, as shown by the expression of its targets *Axin2* and *Sp5* ($n=5$ and $n=6$, respectively; Fig. S2) (Tortelote et al., 2013). These observations indicate that signals upstream of *Nodal* expression are present in N1ICD^{epi} embryos. Together, our results suggest that NOTCH activation might either directly downregulate *Nodal* expression or interfere with the *Nodal* auto-regulatory loop.

Misexpression of N1ICD in epiblast cells and the accompanying downregulation of *Nodal* expression at E6.5 did not prevent the general process of gastrulation. The primitive streak (marked by the expression of brachyury) formed, and mesoderm cells were apparent both in the extra-embryonic and embryonic regions of E7.5 N1ICD^{epi} embryos ($n=6$; Fig. 6G-H'). Mesoderm formation in N1ICD^{epi} embryos was further confirmed by the presence of paraxial mesoderm cells marked by the expression of *Tbx6* at E8.0 ($n=7$; Fig. 7A,A') (Chapman et al., 1996), and of the somitic marker *Uncx* at E8.5-E9.5 ($n=6$; Fig. S3) (Mansouri et al., 1997).

Strikingly, no axial brachyury-expressing cells could be observed in E7.5 and E8.5 N1ICD^{epi} embryos ($n=6$; Fig. 6G' and data not shown). Chordin (*Chrd*), encoding a BMP-antagonist specifically expressed in the APS and in one of its derivatives, the notochord (Bachiller et al., 2000), was absent in N1ICD^{epi} embryos ($n=4$; Fig. 6I,I'). To further investigate the fate of the APS in N1ICD^{epi} embryos, we examined the formation of two additional derivatives of this tissue, the prechordal plate, marked by *Foxa2* (Ang et al., 1993), and the anterior definitive endoderm that will contribute to the foregut, marked by *Foxa2*, *Cer1* and *Sox17* (Biben et al., 1998; Kanai-Azuma et al., 2002). The expression of *Foxa2*, *Cer1* and *Sox17* was absent or dramatically reduced in N1ICD^{epi} embryos at E7.5 ($n=8$, $n=5$ and $n=2$, respectively; Fig. 6J-K', Fig. S2). At E8.5 and E9.5, *Shh*, a marker of axial mesoderm (notochord and prechordal plate), dorsal gut and floor plate (Echelard et al., 1993), was only expressed in rare scattered endoderm cells of N1ICD^{epi} embryos co-expressing SOX17, most likely corresponding to midgut or hindgut cells ($n=10$; Fig. S3).

It is well-established that the APS-derived tissues depleted in N1ICD^{epi} embryos are dependent on high levels of NODAL signalling for their specification (reviewed by Robertson, 2014; Vincent et al., 2003). Likewise, *Lefty2*, the expression of which we found missing in the primitive streak and nascent mesoderm cells of E7.0-E7.5 N1ICD^{epi} embryos ($n=11$; Fig. 6L,L'), is a direct target of NODAL

signalling (Saijoh et al., 2000). Our analysis therefore indicates that over-activating the NOTCH pathway in the epiblast results in defects normally associated with a dampening of NODAL signalling.

NOTCH signalling regulates the acquisition of distinct mesoderm fates

In vitro and *in vivo* studies have demonstrated a direct role for the NOTCH pathway in regulating the formation of distinct mesoderm types. Inducible activation of NICD in mESCs cultured under mesoderm differentiation conditions leads to a reduction of endothelial, haematopoietic and cardiac differentiation and favours the generation of smooth muscle cells (SMC) (Nemir et al., 2006; Schroeder et al., 2003, 2006). Activation of the NOTCH pathway in human mesenchymal or ESCs similarly induces SMC differentiation (Kurpinski et al., 2010). In the chick embryo, NOTCH activity in the ventral mesoderm mediates the balance between blood or endothelial and smooth muscle progenitors, and promotes the formation of the latter (Shin et al., 2009). In order to investigate whether NOTCH similarly regulates mesoderm cell fate acquisition in the gastrulating mouse embryo, we examined the formation of specific mesoderm types in N1ICD^{epi} embryos.

NKX2.5 is a transcription factor expressed in cardiac mesoderm precursors from E7.5 (Lints et al., 1993). In N1ICD^{epi} embryos, *Nkx2.5* transcripts were absent ($n=3/6$) or downregulated ($n=3/6$; Fig. 7B,B') at E8.0, indicating that cardiac mesoderm specification is abnormal in these embryos.

At E7.5, the VEGF receptor FLK1 (KDR) marks hemangioblasts located in the blood islands of the yolk sac, as well as endothelial precursors in the heart and allantois regions (Ema et al., 2006; Yamaguchi et al., 1993). The most prominent *Flk1* expression observed in N1ICD^{epi} embryos was found in extra-embryonic mesoderm cells that accumulated proximal to the primitive streak, suggesting that it corresponds to allantois precursors that failed to organise into an elongated allantois bud (Fig. 7C,C') (Inman and Downs, 2007). By contrast, *Flk1* expression was severely reduced in the cardiac and the yolk sac regions of N1ICD^{epi} embryos at E7.5 and E8.5, indicating that endothelial cell specification and blood island formation are impaired ($n=6$; Fig. 7C,C', Fig. S4).

The transcription factor HAND2 is expressed in cardiac, lateral and allantois precursors, as well as in extra-embryonic mesoderm cells lining the amnion and the chorion. HAND2 function is required for vascular development and in particular, for the differentiation of vascular mesenchyme into vascular smooth muscle cells, a cell type where *Hand2* is expressed from E9.0/E9.5 (Yamagishi et al., 2000). *Hand2* transcripts were normally detected in the proximal region of N1ICD^{epi} embryos from E7.5 and in lateral mesoderm cells surrounding the presumptive neural tube, and the mesoderm lining

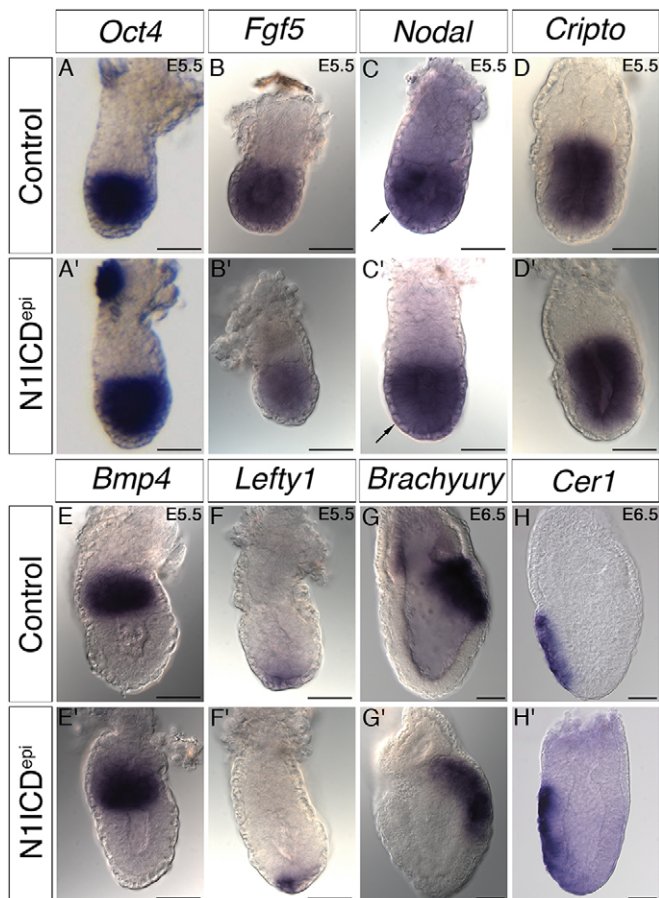


Fig. 5. NOTCH activation does not affect anterior-posterior polarity establishment. The expression patterns of the epiblast markers *Oct4* (A,A'), *Fgf5* (B,B'), *Nodal* (C,C') and *Cripto* (D,D') is normal in E5.5 N1ICD^{epi} embryos. Note that *Nodal* transcripts are also expressed in the VE covering the embryonic region (arrows in C,C'). *Bmp4* (E,E') and *Lefty1* (F,F') transcripts are expressed normally in the extra-embryonic ectoderm and DVE, respectively, at E5.5. At E6.5 the expression of *brachyury* (G,G') and *Cer1* (H,H') mark the nascent primitive streak and the AVE respectively, both in control and N1ICD^{epi} embryos. Scale bars: 50 μ m.

the chorion of N1ICD^{epi} embryos at E8.5 and E9.5 ($n=8$; Fig. 7D-E', Fig. S4). In addition, smooth muscle actin was also detected in the extra-embryonic region of E9.5 N1ICD^{epi} embryos ($n=3$; Fig. S4). Together, these observations suggest that upon NOTCH activation, cardiac and endothelial cell lineage formation is disrupted, whereas smooth muscle cells form normally.

NOTCH activation induces neural specification

The NOTCH pathway plays major sequential roles during mammalian neural development. NOTCH activity is notably required to maintain a pool of neural progenitors and to inhibit further neural differentiation, but can also regulate the acquisition of distinct cell fates by promoting gliogenesis or influencing binary fate choices during neurogenesis (reviewed by Pierfelice et al., 2011; Yoon and Gaiano, 2005). A role for the NOTCH pathway during early neural specification has been pinpointed by gain- and loss-of-function *in vitro* analyses showing that NOTCH activity promotes neural lineage commitment in pluripotent ESCs (Das et al., 2010; Kurpinski et al., 2010; Lowell et al., 2006). In order to investigate whether the NOTCH pathway can also play such an early

role during *in vivo* neural development, we analysed the formation of neuroectodermal tissue in N1ICD^{epi} embryos.

The transcription factor SOX1 is first expressed in the anterior region fated to become neuroectoderm when the neural plate forms at E7.5 (Cajal et al., 2012; Pevny et al., 1998). Strikingly, we found that in N1ICD^{epi} embryos *Sox1* was expressed in an anterior distal domain at a time when *Sox1* transcripts could not yet be detected in control littermates ($n=10/11$; Fig. 8A,A'). Quantitative PCR analysis on E7.5 single embryos demonstrated that *Sox1* expression levels were consistently higher in N1ICD^{epi} embryos ($n=6$) compared with their control littermates ($n=4$), whereas the expression of other ectoderm markers such as *Sox2* and *Dlx5* were not modified (Fig. 8D, Fig. S5) (Cajal et al., 2012). *Dlx5* expression was confined to the proximal ectoderm in E7.5 control and N1ICD^{epi} embryos ($n=5/6$; Fig. 8C). *Sox1* transcripts were maintained in a reduced distal domain of N1ICD^{epi} embryos at E8.5 ($n=3$; Fig. 8B,B'), and were found in a longitudinal tubular structure at E9.5 ($n=2$; Fig. S6), suggesting that a neural tube-like structure develops in N1ICD^{epi} embryos despite the overall disorganisation. The acquisition of neural identity did not result in neuronal differentiation, as demonstrated by the absence of expression of the proneural gene *Neurog2* (Gradwohl et al., 1996) and of the neuronal β 3 class III tubulin, TUJ1 (also known as TUBB3) (Easter et al., 1993) in N1ICD^{epi} embryos at E8.5 and E9.5 ($n=5$ and $n=7$, respectively; Fig. S6). This observation is in agreement with the known role of NOTCH signalling in maintaining neural precursors and inhibiting differentiation. Together, these results indicate that activation of NOTCH signalling promotes the acquisition of a neuroectodermal character by distal anterior epiblast cells.

NOTCH activation perturbs anterior ectoderm formation

We next investigated the regional identity of ectoderm and neuroectoderm cells present in N1ICD^{epi} embryos. At E7.5, the transcription factors coding genes *Otx2*, *Hesx1* and *Six3* are expressed in overlapping domains of the anterior surface ectoderm and anterior neural plate (Cajal et al., 2012). After gastrulation, their expression is found in the forebrain, with *Otx2* being also expressed in the midbrain (Oliver et al., 1995; Simeone et al., 1993; Thomas and Beddington, 1996). We found that at E7.5, *Hesx1* and *Six3* were not expressed in N1ICD^{epi} embryos, and *Otx2* transcripts were only detected in a much reduced anterior distal domain ($n=8$, $n=2$, $n=2$, respectively; Fig. 8D-G'). *Hesx1* and *Six3* transcripts were also absent at E8.5-E9.5 ($n=5$ and $n=2$, respectively; Fig. S6). Together, these observations indicate that the specification of the anterior-most neuroectoderm that will give rise to the forebrain is impaired in N1ICD^{epi} embryos, despite precocious neural specification.

Hoxb1 and *Gbx2*, two markers of posterior ectoderm and mesoderm with an anterior limit in the rostral hindbrain (Bouillet et al., 1995; Forlani et al., 2003), were normally expressed in the posterior ectoderm and primitive streak region of N1ICD^{epi} embryos ($n=4$ and $n=2$, respectively; Fig. 8H,H', Fig. S6). Therefore, the absence of anterior gene expression in the ectoderm of N1ICD^{epi} embryos is not associated with an expansion of the posterior neuroectoderm.

The abnormal anterior development of N1ICD^{epi} embryos was accompanied by aberrant gene expression in this region. Surprisingly, we found that *Nodal* and its target *Lefty2* were ectopically expressed in the anterior ectoderm and anterior mesoderm wings, opposite to the primitive streak and the amniochorionic fold in E7.5 N1ICD^{epi} embryos ($n=6/6$ and $4/7$, respectively; Fig. 8I-J', Fig. S7). This observation indicates that the anterior region of N1ICD^{epi} embryos has adopted some features of the gene expression program normally at play

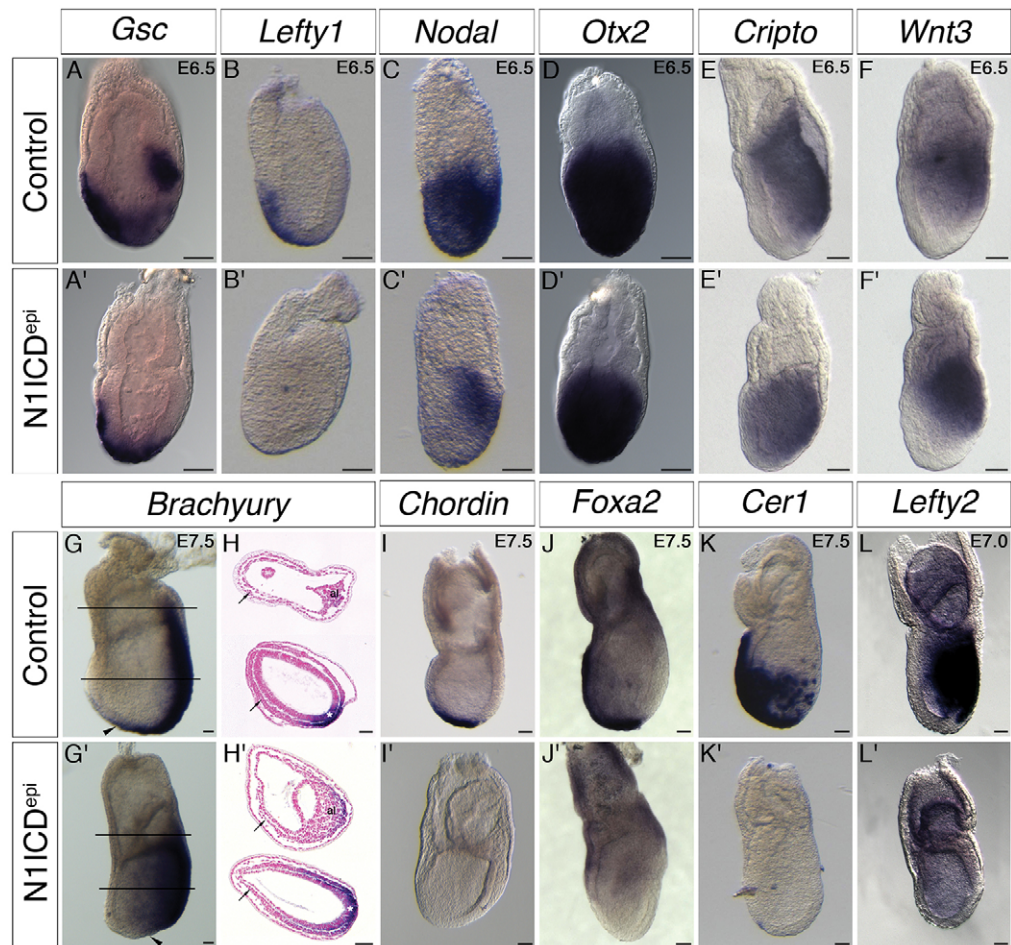


Fig. 6. NOTCH activation impairs axial mesoderm formation and perturbs NODAL signalling. (A,A') *Gsc* transcripts are absent in the anterior primitive streak of E6.5 N1ICD^{epi} embryos. (B,B') *Lefty1* transcripts are not maintained in the AVE of E6.5 N1ICD^{epi} embryos. (C,C') *Nodal* expression appears downregulated in the epiblast and VE of E6.5 N1ICD^{epi} embryos. (D-F') *Otx2*, *Cripto* and *Wnt3* transcripts are normally expressed in the epiblast and primitive streak region of E6.5 N1ICD^{epi} embryos. (G,G') *Brachyury* transcripts are expressed in the primitive streak and nascent mesoderm cells of E7.5 N1ICD^{epi} embryos. In contrast to control embryos, no axial expression is observed in N1ICD^{epi} embryos (arrowheads). Black lines indicate the level of sections shown in H and H'. (H,H') Histological sections showing *brachyury* expression in the primitive streak (white asterisks). Black arrows indicate mesoderm cells in extra-embryonic and embryonic regions; al, allantois. (I,I') *Chrd* transcripts mark the anterior primitive streak and the notochord in control embryos, but are absent in E7.5 N1ICD^{epi} embryos. (J,J') *Foxa2* is expressed in the anterior primitive streak, the entire axial mesoderm including the prechordal plate and the definitive endoderm. *Foxa2* expression is severely reduced in E7.5 N1ICD^{epi} embryos. (K,K') *Cer1* transcripts are severely reduced or absent in the definitive endoderm of E7.5 N1ICD^{epi} embryos. (L,L') The NODAL target *Lefty2* is not expressed in the forming lateral mesoderm of E7.5 N1ICD^{epi} embryos. Scale bars: 50 μ m.

in the posterior region of the E7.5 embryo. However, we could not detect any delamination of anterior ectoderm cells that would signal the formation of an ectopic primitive streak (Fig. 8I',J', Fig. S7). By contrast, the endogenous expressions of *Nodal* and *Lefty2* at this stage, in the primitive streak and nascent lateral mesoderm respectively, were absent in N1ICD^{epi} embryos (Fig. 6L,L', Fig. 8I-J', Fig. S7).

Together, our observations show that forced activation of NOTCH signalling in the epiblast interferes with the fine and dynamic regulation of *Nodal* expression in the early mouse embryo: NOTCH activation leads first to an abnormal downregulation of *Nodal* (and its target, *Lefty2*) in the primitive streak and nascent mesoderm from E6.5–E7.0, followed by an ectopic upregulation of both genes in the anterior ectoderm and mesoderm wings.

DISCUSSION

In this work we present evidence for a role of NOTCH signalling in regulating cell fate decisions during the formation of embryonic lineages in the gastrulating mammalian embryo.

Strikingly, forced expression of N1ICD in the oocyte during the growing phase and then in the zygote caused severe defects at post-implantation stages but did not lead to major perturbations during the pre- and peri-implantation periods, indicating that the earliest stages of mouse development are robust when facing NOTCH gain-of-function. These observations are consistent with a recent report demonstrating that whereas forced activation of NOTCH signalling in pre-implantation embryos can influence the segregation of the trophectoderm lineage, it does not prevent the formation of morphologically normal blastocysts at E3.5 (Rayon et al., 2014). Together with previous reports showing normal development up to E8.0 of embryos lacking maternal and zygotic expression of key components of the NOTCH pathway (Shi and Stanley, 2006; Souilhol et al., 2006), our results demonstrate that the NOTCH signalling pathway is not a crucial regulator of mouse embryonic development before gastrulation.

Our data indicates that pluripotent epiblast cell maintenance is not affected by NOTCH activation in the early post-implantation mouse

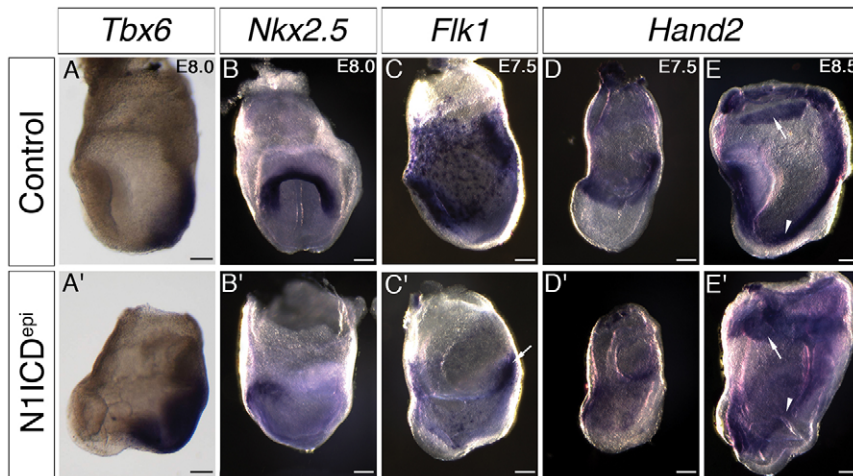


Fig. 7. NOTCH signalling regulates cell type specification of mesoderm precursors. (A,A') *Tbx6* transcripts are expressed in paraxial mesoderm cells of N1ICD^{epi} embryos. (B,B') *Nkx2.5* transcripts are downregulated in E8.0 N1ICD^{epi} embryos. B shows an anterior view, and B' a lateral view. (C,C') *Flk1* expression is severely reduced in E7.5 N1ICD^{epi} embryos except in the abnormal allantois bud (arrow). (D,D') *Hand2* is expressed in the proximal embryonic region of E7.5 N1ICD^{epi} embryos. (E,E') At E8.5 abundant *Hand2* transcripts are detected in lateral plate mesoderm (white arrowheads) and in mesoderm cells lining the chorion (white arrows). Scale bars: 100 μ m.

embryo. This is in agreement with *in vitro* studies showing that NOTCH activation in mESCs does not have a major impact on their maintenance and self-renewal (Lowell et al., 2006). A possibility would be that, similarly to the situation in the trophectoderm with the HIPPO pathway (Rayon et al., 2014), NOTCH cooperates with other signals to control subtle aspects of pluripotency in the post-implantation epiblast.

Immunostaining in wild-type embryos has revealed that N1ICD is first detected at the onset of gastrulation in mesoderm cells, and is only expressed in the neurectoderm after E8.0 (Del Monte et al., 2007). By contrast, N1ICD expression occurs in the entire epiblast of N1ICD^{epi} embryos from E6.5. Therefore, the phenotypes observed in N1ICD^{epi} embryos might result from ectopic and increased N1ICD activity in the ectoderm and mesoderm germ layers, respectively.

We have shown that NOTCH activation impairs the formation of the anterior primitive streak (APS) and its derivatives, the node and midline structures. The APS acts as an organising centre that patterns adjacent tissues and is thus considered the murine equivalent of the Spemann–Mangold organiser (Kinder et al., 2001). NOTCH signalling regulates the formation of organiser-derived midline structures in other vertebrates. In *Xenopus* and chick embryos, overexpression of NICD leads to an expansion of the floor plate at the expense of the notochord, suggesting that NOTCH signalling regulates a binary cell fate choice between these two derivatives of the organiser (Gray and Dale, 2010; López et al., 2003). In N1ICD^{epi} embryos, the notochord is similarly absent. However, we did not detect any sign of floor plate formation, indicating that in this case NOTCH activation led to a more general depletion of the organiser precursors. This is exemplified by the absence of axial mesoderm, definitive endoderm and floor plate markers, and the lack of a morphologically visible node. High levels of NODAL signalling are essential for the formation of the murine APS and its derivatives (reviewed by Robertson, 2014; Vincent et al., 2003). The expression of *Nodal* transcripts is reduced, and known NODAL targets such as *Gsc* and *Lefty2* are absent in the primitive streak of N1ICD^{epi} embryos. These observations indicate that NODAL signalling is dampened by NOTCH activation in the epiblast, providing a likely explanation for the complete lack of APS derivatives in N1ICD^{epi} embryos.

NOTCH activation in the epiblast triggers a premature expression of the neural plate marker *Sox1* in distal anterior epiblast cells when compared with the control situation. This observation is consistent with the results from *in vitro* studies showing that NOTCH

activation enhances and accelerates neural specification in mESCs and hESCs upon withdrawal of self-renewing stimuli (Lowell et al., 2006). In ESCs, NOTCH coordinates the response to neural inductive cues such as FGF, protects the cells against non-neural fates and stimulates proliferation of neural precursors in a cyclin D-dependent manner (Das et al., 2010; Lowell et al., 2006). In addition, *in vivo* studies have shown that *Sox1* expression in the spinal cord depends on NOTCH signalling (Genethliou et al., 2009). Direct induction of *Sox1* could similarly be involved in the premature *Sox1* expression induced by NOTCH activity in N1ICD^{epi} embryos. The neurectoderm tissue induced in N1ICD^{epi} embryos undergoes morphogenesis to form a neural tube-like structure at E9.5. However we could not detect any sign of neurogenesis, in agreement with the known role of NOTCH signalling in maintaining neural precursors and inhibiting further neuronal differentiation (reviewed by Pierfelice et al., 2011; Yoon and Gaiano, 2005).

Genetic and embryological studies have revealed that neural induction in the embryo is a multi-step process involving epiblast maturation, ectoderm specification and acquisition of neurectoderm fate (Li et al., 2013; reviewed by Stern, 2005). The identification of the regulatory networks controlling these sequential events has recently benefited from studies highlighting the similarities between the precursors present in the embryo and the *in vitro* cell types obtained in neural differentiation protocols of stem cells (Iwafuchi-Doi et al., 2012; Lupo et al., 2014). *In vivo* and *in vitro* evidence has demonstrated that inhibition of NODAL/ACTIVIN signalling promotes neural fate acquisition in the mouse embryo, as well as during differentiation of mESCs, mouse epiblast stem cells (mEpiSC), and hESCs (Camus et al., 2006; Chng et al., 2010; Li et al., 2013; Patani et al., 2009; Smith et al., 2008; Turner et al., 2014; Vallier et al., 2009; Watanabe et al., 2005). It is tempting to extrapolate that the attenuation of NODAL signalling observed in N1ICD^{epi} embryos from E6.5 contributes to the premature expression of *Sox1* in the distal anterior epiblast of these embryos. Therefore, it will be important to determine whether a similar effect of NOTCH on NODAL signalling is at play during the *in vitro* production of neural precursors from stem cells.

N1ICD^{epi} embryos exhibit abnormal anterior-posterior patterning of the ectoderm layer. In particular, *Hesx1* and *Six3*, which first mark the anterior ectoderm at E7.5 and subsequently the forebrain, are not expressed upon NOTCH activation in the epiblast. Genetic and embryological evidence has demonstrated that the formation of anterior structures in the mouse embryo requires sequential signals

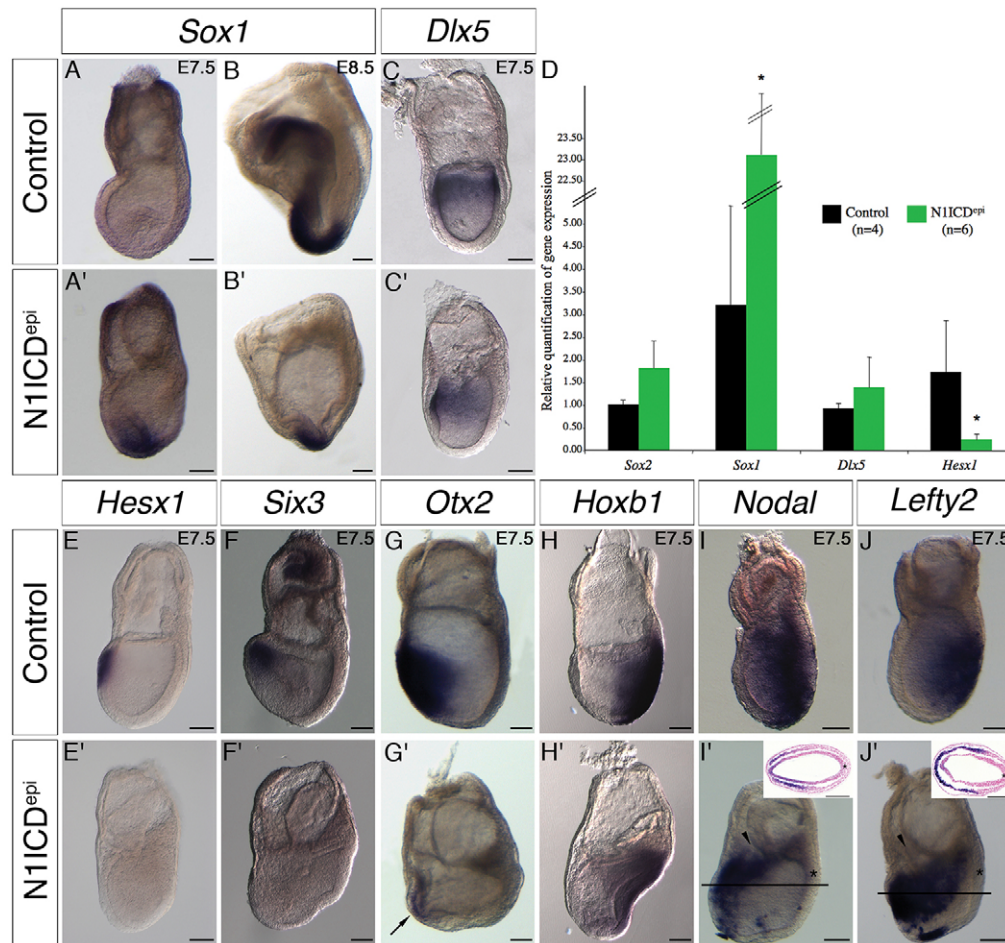


Fig. 8. NOTCH activation induces neural specification and perturbs anterior-posterior patterning. (A,A') In N1ICD^{epi} embryos *Sox1* was expressed in an anterior distal domain from the late streak/zero bud stage, at a time when *Sox1* transcripts could not be detected in control littermates. (B,B') *Sox1* transcripts were maintained in a reduced distal domain of N1ICD^{epi} embryos at E8.5. (C,C') The non-neural ectoderm marker *Dlx5* is normally expressed. (D) Quantitative RT-PCR analysis of *Sox2*, *Sox1*, *Dlx5* and *Hesx1* expression on single embryos. Mean values of gene expression relative to *Taf7* are represented, error bars indicate s.d. between individual samples (* $P < 0.05$, Student's 2-tailed *t*-test; $n = 4$ for controls, $n = 6$ for N1ICD^{epi} embryos). (E-G') *Hesx1*, *Six3* and *Otx2* transcripts are absent or very reduced in the anterior region of E7.5 N1ICD^{epi} embryos. Black arrow in G' indicates a reduced domain of *Otx2* expression. (H,H') *Hoxb1* is expressed normally in the posterior ectoderm and primitive streak region of N1ICD^{epi} embryos. (I-J') *Nodal* and *Lefty2* transcripts were absent in the primitive streak and nascent lateral mesoderm of E7.5 N1ICD^{epi} embryos, but were found ectopically in the anterior ectoderm and anterior mesoderm wings. Black arrowheads in I' and J' point to the proamniotic canal. Insets show sections at the level indicated by black lines in I', J'. Asterisks in I', J' and the corresponding insets indicate the primitive streak. Scale bars: 100 μ m.

from the AVE and the anterior mesendoderm to antagonise WNT, NODAL and BMP pathways (reviewed by Andoniadou and Martinez-Barbera, 2013; Arkell and Tam, 2012). The defective anterior specification observed in N1ICD^{epi} embryos could therefore be explained by the absence of the axial mesendoderm tissues required to maintain anterior identity in the ectoderm. However, the ectopic *Nodal* expression detected in the anterior ectoderm and mesoderm wings of N1ICD^{epi} embryos from E7.5 may also contribute to the absence of anterior structures in these embryos.

Perturbation of NODAL expression and signalling is a central aspect of the phenotype induced by NOTCH activation in the epiblast. However, the output of this interaction seems to depend on the cellular context as *Nodal* transcripts are abnormally downregulated in the epiblast and in the primitive streak of E6.5 N1ICD^{epi} embryos, and ectopically expressed in proximal anterior ectoderm and mesoderm from E7.5. In the wild-type embryo, fine-tuning of NODAL signalling is essential to orchestrate proliferation, migration and specification of embryonic and extra-

embryonic cell types during anterior-posterior polarity establishment, primitive streak formation, gastrulation, and left-right patterning (reviewed by Robertson, 2014; Shiratori and Hamada, 2014; Takaoka and Hamada, 2012). In agreement with its major role during early embryonic patterning, *Nodal* expression is extremely dynamic and tightly regulated by positive and negative inputs integrated on enhancer sequences. In the post-implantation epiblast at E5.5, *Nodal* expression is controlled by pluripotency factors, and notably by OCT4 (Papanayotou et al., 2014) and by an auto-regulatory loop involving NODAL signalling (Adachi et al., 1999; Norris et al., 2002; Norris and Robertson, 1999). In addition, WNT3/ β -catenin signalling triggers *Nodal* upregulation in the E6.5 posterior epiblast and primitive streak (Ben-Haim et al., 2006; Vincent et al., 2003), and NOTCH/RBPJ signalling controls its expression in the node from E7.5 (Krebs et al., 2003; Raya et al., 2003). The ectopic anterior expression of *Nodal* observed in E7.5 N1ICD^{epi} embryos might result from a direct action of N1ICD/RBPJ on the *Nodal* node-specific enhancer (NDE) (Adachi et al., 1999; Krebs et al., 2003; Norris and Robertson, 1999; Raya et al.,

2003). This direct regulation of *Nodal* by NOTCH signalling is also at play in pathological situations, as the NDE enhancer is involved in *Nodal* upregulation in response to elevated NOTCH signalling in melanoma and breast cancer cell lines (Hardy et al., 2010; Quail et al., 2011). Earlier downregulation of *Nodal* in the epiblast and primitive streak might result from the interference of NOTCH signalling with the positive regulators of *Nodal* expression at this stage. An antagonistic effect of NOTCH and WNT3/ β -catenin signalling has been demonstrated in various developmental systems (Acosta et al., 2011; Kwon et al., 2009, reviewed by Muñoz Descalzo and Martínez Arias, 2012). However, we could not detect such an effect in *NIICD*^{epi} embryos. Alternatively, NOTCH activation could interfere with the auto-regulatory loop that controls *Nodal* expression in the early epiblast (Adachi et al., 1999; Norris et al., 2002; Norris and Robertson, 1999). NICD has been shown to antagonise TGF β signalling in various cell types via direct binding to SMAD3 or its co-activators (Carlson et al., 2008; Masuda et al., 2005; Sun et al., 2005). A similar antagonistic activity of NICD on SMAD effectors could dampen the NODAL pathway leading in turn to reduced *Nodal* expression in *NIICD*^{epi} embryos.

In conclusion, the gain-of-function approach presented in this study provides the first demonstration that correct spatiotemporal regulation of the canonical NOTCH signalling pathway is essential for germ layer formation and patterning in the mouse embryo. Mutations resulting in abnormal activation of NOTCH signalling during gastrulation might similarly result in miscarriage in humans. Our results highlight the crucial role of NOTCH signalling in the modulation of NODAL/ACTIVIN signalling, a major determinant of early embryonic patterning. This interaction is bound to be important in other developmental situations as well as in tissue homeostasis and cancer. Our observations on how NOTCH modulates cell fate decisions *in vivo*, in coordination with other signalling pathways, will be relevant for the improvement of *in vitro* differentiation protocols aimed at obtaining cell types with therapeutic use.

MATERIALS AND METHODS

Generation of mice and embryos

Mice carrying the following alleles were used: Tg(*Zp3-cre*)82Kw, hereafter named *Zp3-Cre*^{tg} (de Vries et al., 2000), Tg(*Sox2-cre*)1Amc, hereafter named *Sox2-Cre*^{tg} (Hayashi et al., 2002), *Gt(ROSA)26Sor^{tm1(Notch1)Dam}*, hereafter named *Rosa26^{NIICD}* (Murtaugh et al., 2003), *Rbpj^{tm1.Hon}* and *Rbpj^{tm1.1.Hon}*, hereafter named *Rbpj^{fllox}* and *Rbpj^Δ* (Tanigaki et al., 2002). Genotyping of mice was performed using conditions described in Souilhoul et al. (2006) for the *Cre* lines and *Rbpj^{fllox}* and *Rbpj^Δ* alleles, and in Soriano (1999) for *Rosa26^{NIICD}* allele. Mice were maintained under a 12-h light cycle and embryos of different genotypes were obtained from natural matings. E0.5 to E3.5 embryos were dissected in M2 medium (Sigma). E4.5 to E9.5 embryos were dissected in DMEM, 10% fetal calf serum, 25 mM Hepes buffer pH 7.0 (Gibco). To monitor GFP expression upon recombination of the *Rosa26^{NIICD}* allele, E4.5 and E5.5 embryos were fixed in PBS/paraformaldehyde 4% at 4°C for 1 h, rinsed in PBS/Tween-20 0.01% and imaged directly using a LSM 710 confocal microscope (Zeiss) equipped with 40 \times /1.3NA lenses, or following anti-GFP staining. GFP expression in E6.5 to E9.5 embryos was analysed using a M165FC stereomicroscope (Leica). Experiments were performed in accordance with French Agricultural Ministry and European guidelines on the care and protection of laboratory animals.

In situ hybridisation and immunohistochemistry

Whole mount *in situ* hybridisation and immunohistochemistry were performed according to standard procedures (Burtscher and Lickert, 2009; Chazaud et al., 2006; Perea-Gomez et al., 2004). For histological analysis, embryos were embedded in paraffin and sectioned at 4 μ m. Antibodies are

listed in Table S1. E3.5-E5.5 embryos were imaged with an Axio Observer Z.1 microscope (Zeiss) equipped with 40 \times /1.3NA and 25 \times /0.8NA lenses. E6.5-E9.5 embryos were imaged with a Zeiss Lumar-V12 stereomicroscope.

Quantitative analysis of gene expression

Quantitative PCR assays were performed on cDNA obtained from single embryos as described (Cajal et al., 2014; Camus et al., 2006), except that mRNA quality was assessed using the RNA 6000 Nano-Assay on a BioAnalyser 2100 (Agilent), and reverse transcription was performed using the SuperScript Vilo cDNA Synthesis Kit (Invitrogen). The normalisation procedure is described in supplementary materials and methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.S., A.P.-G., A.C. and M.C.-T. conceived and coordinated the project. C.S., A.P.-G., A.C., M.C.-T., S.B.-C., S.V.-P. and M.E. performed experiments. A.P.-G. and M.C.-T. wrote the manuscript with contributions from C.S., A.C. and J.C.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.121145/-/DC1>

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