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

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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 neurectoderm identity and disrupts the formation of specific mesodermal precursors including the derivatives of the anterior primitive streak, the mouse organism. 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; Revisinski 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 trophectoderm (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
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 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 during early embryonic cell fate specification in vitro, 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<sup>0/0</sup>; Rosa26<sup>NICD<sup>0/0</sup></sup> 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 (NICD) 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 NICD<sup>ovo</sup> hereafter) were obtained at the expected 50% ratio (106/191). NICD<sup>ovo</sup> 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<sup>NICD<sup>0/0</sup></sup> females and wild-type males. (A,A') DAPI staining, white arrows indicate the inner cell mass in control and NICD<sup>ovo</sup> E3.5 embryos cultured for 24 h. (B,B') GFP expression is absent in control embryos and present in all cells of NICD<sup>ovo</sup> embryos. (C,C') At E7.5, NICD<sup>ovo</sup> embryos do not show signs of headfold formation. Black arrow shows the left headfold in the control embryo. Scale bars: 25 µm in A-B'; 100 µm in C-C'.](Image)

NICD<sup>ovo</sup> embryos were recovered at expected frequencies after implantation (10 control versus 7 NICD<sup>ovo</sup> 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 NICD<sup>ovo</sup> 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 NICD exclusively in the epiblast and its derivatives. Rosa26<sup>NICD<sup>0/0</sup></sup> females were crossed with Sox2-Cre<sup>0/0</sup> males to give rise to Rosa26<sup>NICD<sup>0/0</sup></sup>; Sox2-Cre<sup>0/0</sup> embryos (designated NICD<sup>epi</sup>) hereafter). Using GFP expression as an indication of NICD expression, we found that expression of NICD began in a mosaic fashion in the E4.5 epiblast (n=10; Table 1, Fig. 2A-B'). Expression then became detectable in the epiblast.

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<tr>
<th>Developmental stage</th>
<th>Normal morphology</th>
<th>Abnormal morphology</th>
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<tr>
<td>E4.5</td>
<td>33 (10*)</td>
<td>0</td>
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<tr>
<td>E5.5</td>
<td>112 (58)</td>
<td>0</td>
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<tr>
<td>E6.5</td>
<td>194 (86)</td>
<td>0</td>
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<tr>
<td>E7.5</td>
<td>131 (0)</td>
<td>128 (128)</td>
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<tr>
<td>E8.5</td>
<td>66 (0)</td>
<td>59 (59)</td>
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<tr>
<td>E9.5</td>
<td>24 (0)</td>
<td>30 (30)</td>
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Number of GFP-positive embryos given in parentheses. *Mosaic GFP expression in the epiblast.
all epiblast cells from E5.5 (n=58; Table 1, Fig. 2C-D'). Until E6.5, N1ICD<sup>Δ</sup> embryos were morphologically undistinguishable from control littermates (Table 1, Fig. 2E,E'). The first obvious anomalies were observed at E7.5, when N1ICD<sup>Δ</sup> embryos exhibited an elongated and thin anterior-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<sup>Δ</sup> embryos do not show any sign of segmentation and are highly disorganised (G'-H'). Scale bars: 25 µm in A-D'; 100 µm in E-H'.

### 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<sup>Δ</sup> 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 Tgfα1) (Ding et al., 1998; Mesnard et al., 2006; Pelton et al., 2002) at E5.5 in the epiblast of N1ICD<sup>Δ</sup> 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<sup>Δ</sup> 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<sup>Δ</sup> embryos (n=4; Fig. 5H,H'). Together these results demonstrate that misregulation of the canonical NOTCH pathway in N1ICD<sup>Δ</sup> 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<sup>Δ</sup> embryos. The...
transcription factor goosecoid (Gsc) is expressed in the AVE and in the anterior primitive streak (APS) at E6.5 (Faust et al., 1995). In N1ICDepi embryos, Gsc transcripts were observed in the AVE but its expression in the APS was reduced \( (n=2/7) \) or absent \( (n=5/7) \; (\text{Fig. } 6A, A') \). In addition, Lefty1 expression was not maintained in the AVE of E6.5 N1ICDepi embryos \( (n=11/12; \text{Fig. } 6B, B') \). 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 N1ICDepi embryos \( (n=4/5; \text{Fig. } 6C, C') \). 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 N1ICDepi 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 \( (n=6 \text{ and } n=3, \text{ respectively; } \text{Fig. } 6D, D') \). 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., 2007).
2002; Norris and Robertson, 1999; Papanayotou et al., 2014). Oct4, 
Crypt and Wnt3 were all normally expressed in E6.5 N1ICD<sup>Δ</sup>
embryos (n=3; n=5 and n=3, respectively; Fig. S2, Fig. 6E–F). The 
extra-embryonic ectoderm-derived BMP4, involved in 
derivation (Beck et al., 2002; Ben-Haim et al., 2006), was also 
expressed in E6.5 N1ICD<sup>Δ</sup> embryos (data not shown). Moreover, 
the WNT3/β-catenin pathway was active in N1ICD<sup>Δ</sup> 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<sup>Δ</sup> 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 brachury) formed, and mesoderm cells were 
expressed in the APS and in one of its derivatives, the notochord 
(Nkx2.5 is a transcription factor expressed in cardiac mesoderm 
precursors in the heart and allantois regions (Ema et al., 2006; 
Yamaguchi et al., 1993). The most prominent 
expression 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<sup>Δ</sup> 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<sup>Δ</sup> embryos at E7.5 
and E8.5, indicating that endothelial cell specification and blood 
island formation are impaired (n=6; Fig. 7C, C). (Inman and 
Downs, 2007).

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<sup>Δ</sup> embryos from E7.5 and in lateral mesoderm cells 
surrounding the presumptive neural tube, and the mesoderm lining 

Table 2. Morphological defects and GFP expression in embryos obtained from crosses between Rbpj<sup>loxΔ</sup>, Rosa26<sup>N1ICD/Δ</sup> females and Rbpj<sup>loxΔ</sup>, 
Sox2-Cre<sup>Δ</sup> males

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<tr>
<th>Genotype</th>
<th>Phenoype</th>
<th>Normal</th>
<th>RBPJ mutant</th>
<th>N1ICD&lt;sup&gt;Δ&lt;/sup&gt;</th>
<th>RBPJ mutant</th>
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<td>Rbpj&lt;sup&gt;loxΔ&lt;/sup&gt; Rosa26&lt;sup&gt;N1ICD/+&lt;/sup&gt;</td>
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<td>Rbpj&lt;sup&gt;loxΔ&lt;/sup&gt; Rosa26&lt;sup&gt;N1ICD/+ Sox2-Cre&lt;sup&gt;Δ&lt;/sup&gt;&lt;/sup&gt;</td>
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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 NIDC 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<sup>Δ</sup> embryos.

NKKX2.5 is a transcription factor expressed in cardiac mesoderm 
precursors from E7.5 (Lints et al., 1993). In N1ICD<sup>Δ</sup> embryos, 
Nkkx2.5 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.
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 N1ICDepi 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 N1ICDepi embryos. Scale bars: 50 µm.

the chorion of N1ICDepi 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 N1ICDepi 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 notoriously 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 N1ICDepi embryos.

The transcription factor SOX1 is first expressed in the anterior region fate to become neuroectoderm when the neural plate forms at E7.5 (Cajal et al., 2012; Pevny et al., 1998). Strikingly, we found that in N1ICDepi 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 N1ICDepi 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 N1ICDepi embryos (n=5/6; Fig. 8C). Sox1 transcripts were maintained in a reduced distal domain of N1ICDepi 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 N1ICDepi 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 class III tubulin, Tuj1 (also known as Tubb3) (Easter et al., 1993) in N1ICDepi 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 N1ICDepi 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 N1ICDepi 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 N1ICDepi 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 N1ICDepi embryos (n=4 and n=2, respectively; Fig. 8H,H', Fig. S6). Therefore, the absence of anterior gene expression in the ectoderm of N1ICDepi embryos is not associated with an expansion of the posterior neuroectoderm.

The abnormal anterior development of N1ICDepi 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 N1ICDepi embryos (n=6/6 and 4/7, respectively; Fig. 8I-J', Fig. S7). This observation indicates that the anterior region of N1ICDepi embryos has adopted some features of the gene expression program normally at play...
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 N1ICDepi embryos (Fig 6L, L′). 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 N1ICDepi 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 N1ICDepi embryos. (K, K′) Cer1 transcripts are severely reduced or absent in the definitive endoderm of E7.5 N1ICDepi embryos. (L, L′) The NODAL target Lefty2 is not expressed in the forming lateral mesoderm of E7.5 N1ICDepi embryos. Scale bars: 50 µm.

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; Soulhhol 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...
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 N1ICDepi embryos from E6.5. Therefore, the phenotypes observed in N1ICDepi 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 N1ICDepi 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 N1ICDepi 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 N1ICDepi 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 N1ICDepi embryos. The neurectoderm tissue induced in N1ICDepi 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 (Iwasuchi-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 N1ICDepi 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.

N1ICDepi 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...
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 N1ICDepi 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 N1ICDepi 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 N1ICDepi 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 N1ICDepi 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.,

Fig. 8. NOTCH activation induces neural specification and perturbs anterior-posterior patterning. (A,A’) In N1ICDepi 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 N1ICDepi 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 Tal7 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 N1ICDepi embryos). (E-G) Hesx1, Six3 and Otx2 transcripts are absent or very reduced in the anterior region of E7.5 N1ICDepi 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 N1ICDepi embryos. (I-J’) Nodal and Lefty2 transcripts were absent in the primitive streak and nascent lateral mesoderm of E7.5 N1ICDepi 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’. Asterisks in I’,J’ and the corresponding insets indicate the primitive streak. Scale bars: 100 µm.
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 N1ICD™ 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 TGFβ 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 N1ICD™ 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)82Krn, hereafter named Zp3-Cre® (de Vries et al., 2000), Tg(Sox2-cre)1Amc, hereafter named Sox2-Cre® (Hayashi et al., 2002), Gt(Rosa)26Sor1<tm1(Notch1-DN)Kum> hereafter named Rosa26N1ICD (Murtaha et al., 2003), Rbpj<tm1.Hon> and Rbpj<tm1.1Hon>, hereafter named RbpjHon and Rbpj™ (Tanigaki et al., 2002). Genotyping of mice was performed using conditions described in Souilhol et al. (2006) for the Cre lines and RbpjHon and Rbpj™ alleles, and in Soriano (1999) for Rosa26N1ICD 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 Rosa26N1ICD allele, E4.5 and E5.5 embryos were fixed in PBS/parafomaldehyde 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×/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 μm. Antibodies are listed in Table S1. E3.5-E5.5 embryos were imaged with an Axio Observer Z.1 microscope (Zeiss) equipped with 40×/1.3NA and 25×/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 BioAnalyzer 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. and M.C.-T. wrote the manuscript with contributions from C.S., A.C. and J.C.

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Supplementary information
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**RESEARCH ARTICLE**

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