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Notch destabilises maternal β-catenin and restricts dorsal-anterior development in *Xenopus*

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SUMMARY

The blastula *chordin*- and *noggin*-expressing centre (BCNE) is the predecessor of the Spemann-Mangold's organiser and also contains the precursors of the brain. This signalling centre comprises animal-dorsal and marginal-dorsal cells and appears as a consequence of the nuclear accumulation of β-catenin on the dorsal side. Here, we propose a role for Notch that was not previously explored during early development in vertebrates. Notch initially destabilises β-catenin in a process that does not depend on its phosphorylation by GSK3. This is important to restrict the BCNE to its normal extent and to control the size of the brain.

KEY WORDS: Notch, β-catenin, BCNE, Dorsal-anterior development, Brain, *Xenopus*

INTRODUCTION

In vertebrates, the Wnt/β-catenin pathway is essential for formation of the main body axis, as demonstrated in different model organisms, such as *Xenopus*, zebrafish and mouse (Heasman et al., 1994; Schneider et al., 1996; Moon and Kimelman, 1998; Huelsken and Birchmeier, 2001; Marikawa, 2006). The frog embryo provided great knowledge about the events leading to the initial dorsal-ventral (D-V) asymmetry. The key step is the stabilisation of β-catenin, the downstream effector of the canonical Wnt pathway, at the dorsal side during early stages of development; at the ventral side β-catenin is actively destroyed (Sokol, 1999; Weaver and Kimelman, 2004).

Studies in *Drosophila*, *Xenopus* and mammalian cells helped to understand Wnt transduction. In the absence of canonical Wnt signalling, as is the case in the unfertilised frog egg, β-catenin is incorporated into a destruction complex coordinated by the scaffold protein Axin, whereupon phosphorylation of serine residues 33 and 37 by glycogen synthase kinase 3 (GSK3) targets β-catenin for degradation by the ubiquitin-proteasome system (Hayward et al., 2008; Verheyen and Gottardi, 2010).

Maternal dorsalising components of the Wnt pathway, including *wnt11* mRNA, Dishevelled (Dsh) and GSK3-binding protein (GBP), are originally deposited in the vegetal pole of the frog egg. Fertilisation triggers a process called cortical rotation during which maternal dorsalising determinants are translocated to the equatorial region, opposite to the sperm entry point, through a parallel array of oriented microtubules. During the first cleavages, Wnt11 protein secreted by the dorsal vegetal cells binds its receptor Frizzled (Xfz7), launching canonical Wnt signalling in the dorsal side of the embryo. Consequently, Dsh interacts with Axin and recruits GBP, which in turn displaces GSK3 from the destruction complex, and GSK3 is degraded (Weaver and Kimelman, 2004; Tao et al., 2005). The main consequence of this local decrease of GSK3 activity is the stabilisation of hypophosphorylated β-catenin, which increases in the cytoplasm of the dorsal cells and afterwards in their nuclei, where it begins to accumulate at the 16-32 cells stage. At mid to late blastula stages, animal-dorsal and vegetal-dorsal cells are enriched with nuclear β-catenin (Larabell et al., 1997; Schohl and Fagotto, 2002).

Nuclear β-catenin recruits the methyl transferase Prmt2, which methylates histone H3 in the promoters of target genes. This is important to establish a poised chromatin architecture, which prompts the genes of the dorsal programme to be immediately ready for transcription at high levels when zygotic transcription begins at midblastula transition (MBT) (Blythe et al., 2010). By interacting with the transcription factor TCF-LEF, nuclear β-catenin activates target genes in the dorsal cells, ultimately leading to the specification of the Spemann-Mangold's organiser at the beginning of gastrulation (Molenaar et al., 1996).

An important signalling centre comprising animal-dorsal and marginal-dorsal cells precedes the Spemann-Mangold's organiser in *Xenopus*. The blastula chordin- and noggin-expressing centre (BCNE) appears when zygotic transcription begins and its specification depends on the dorsal accumulation of nuclear β-catenin (Wessely et al., 2001; Wessely et al., 2004; Kuroda et al., 2004; De Robertis and Kuroda, 2004).

The BCNE contains the precursors of the brain (including the whole forebrain and most of the midbrain and hindbrain) and also gives rise to the Spemann-Mangold's organiser, derivatives of which will form the embryonic dorsal midline structures (Spemann and Mangold, 2001; Le Douarin and Halpern, 2000; López et al., 2003). The BCNE expresses the neural inducers Noggin, Chordin (Chd) and Nodal-3 (Xnr3) and the transcription factors Siamois (Sia), Twin (Twi) and FoxA4a. Nuclear β-catenin activates the dorsal transcription of *sia* and *twi*. The homeodomain proteins Sia and Twi repress *bmp4* transcription and transiently activate the expression of *Xnr3* and also the expression of the BMP antagonists *chd* and *nog* in the prospective anterior neuroectoderm. This double inhibition of BMP signalling at blastula stages is crucial for brain formation (Kuroda et al., 2004; Ishibashi et al., 2008).

The transmembrane receptor Notch often acts by lateral inhibition, preventing equipotent cells from all adopting the same fate. In the absence of Notch signalling, a repressor complex containing the factor of the CBF1/Su(H)/LAG1 (CSL) family inhibits the transcription of Notch-target genes. During neurogenesis, for example, all cells within a proneural cluster are competent to adopt

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the neuronal fate, but only one finally differentiates as a neuron. This precursor is the source of the ligand Delta, which binds Notch on the surface of the neighbouring cells, triggering two successive proteolytic cleavages of the receptor, the second one in charge of the γ-secretase enzymatic complex, which cleaves Notch within the transmembrane domain. As a result, the intracellular domain of Notch (NICD) enters the nucleus, the co-repressors previously bound to CSL are displaced and co-activators are recruited in a new complex, now containing NICD and CSL. This complex activates the transcription of Notch-target genes that lead to the suppression of the neuronal fate in the cells surrounding the neuronal precursor (Lai, 2004; Fortini, 2009; Jorissen and De Strooper, 2010; Kovall and Blacklow, 2010; Tanigaki and Honjo, 2010).

There is growing evidence that Notch sometimes functions through non-canonical mechanisms that do not rely on CSL transcription factors. Other ligands, distinct from the Delta-Serrate family, might participate, and even non-nuclear mechanisms, perhaps involving cytoskeleton interactions, were proposed for Notch-dependent axon guidance in *Drosophila* (Brennan et al., 1999; Hu et al., 2003; Hori et al., 2004; Langdon et al., 2006; Mizutani et al., 2007; D'Souza et al., 2008; Le Gall et al., 2008; D'Souza et al., 2010).

Notch and Wnt (Wingless in *Drosophila*) can interact in synergic or antagonistic ways, depending on the context. The synergistic interactions generally involve the canonical, CSL-dependent transcriptional activation of Notch-target genes (for a review, see Hayward et al., 2008). Remarkably, the CSL-independent pathway is preferentially associated with antagonism of Wingless in *Drosophila* (Lawrence et al., 2001; Martínez Arias et al., 2002; Langdon et al., 2006; Hayward et al., 2008). In recent years, evidence has been found that in the wing imaginal disc, Notch initially acts through a non-transcriptional pathway by promoting the degradation of Armadillo (Arm, the fly homologue of β-catenin) without the intervention of GSK3 (Hayward et al., 2005; Hayward et al., 2008; Sanders et al., 2009).

Notch transcripts are present both maternally and zygotically in *Xenopus* (Coffman et al., 1990) and there are some hints of Notch activity in the animal hemisphere before the beginning of gastrulation (Mir et al., 2008). We have previously described a role for Notch in development of the dorsal midline derivatives of the Spemann-Mangold's organiser, which might be related to germ layer segregation during gastrulation (López et al., 2003; López et al., 2005; Revinski et al., 2010). Although the importance of Wnt signalling during the first steps of development is well established in vertebrates, Notch function has not been thoroughly addressed so far at these stages.

We wondered whether Wnt and Notch interact during establishment of the main body axis in *Xenopus*. Here, we show that Notch antagonises Wnt signalling by destabilising β-catenin in a process that does not require phosphorylation of β-catenin by GSK3. This interaction is important to restrict the extension of the BCNE in the animal hemisphere and to control the size and anterior-posterior (A-P) pattern of the brain.

MATERIALS AND METHODS

Embryological manipulations, RNA synthesis, morpholinos and injections

Albino and wild-type *Xenopus laevis* embryos were obtained using standard methods, staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) and fixed with MEMFA (Harland, 1991). Embryos were injected into one dorsal or ventral cell at the 4-cell stage or at the animal hemisphere before the first mitotic division.

Synthetic capped mRNAs were obtained as described (Franco et al., 1999). The *nicd-mt*, *su(H)1*^{DBM}, β-*catenin-GFP* and *Δβ-catenin-GFP* constructs were described previously (Chitnis et al., 1995; Yost et al., 1996; Wettstein et al., 1997). The Notch morpholino antisense oligonucleotide modified with 3′-fluorescein (Notch Mo) was used as previously described (López et al., 2003; Revinski et al., 2010). As control morpholino (control Mo) we used the standard control oligonucleotide modified with 3′-fluorescein or the random control oligonucleotide 25-N (Gene Tools, LLC, OR, USA). Some injections included as tracer 10 ng of Dextran Oregon Green 488, MW 10,000, anionic lysine fixable (DOG, Molecular Probes, Invitrogen).

In situ hybridisation and immunodetections

The preparation of digoxigenin-labelled antisense RNA probes and the whole-mount in situ hybridisation procedure (WMISH) were performed as described previously (Pizard et al., 2004), except that the proteinase K step was omitted and 10% of polyvinyl alcohol was included during colour development for stage 9 embryos.

The c-myc epitope of the *nicd-mt* construct, the GFP epitope of the β-*catenin-GFP* and Δβ-*catenin-GFP* constructs, the 3′-fluorescein tag of the morpholinos and the Dextran Oregon Green (DOG) tracer were revealed by horseradish peroxidase (HRP) or alkaline phosphatase immunostaining, or by immunofluorescence as described previously (López et al., 2005; Revinski et al., 2010).

Endogenous β-catenin was detected as described previously (Cuykendall and Houston, 2009) with some modifications. Embryos were fixed for 90 minutes with MEMFA, permeabilised for 72 hours at –20°C with 80% methanol, 20% DMSO, and then with pre-hybridisation solution in the same conditions as for WMISH. After washing twice for 15 minutes each with TBSE (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 6.5) and once for 15 minutes with TBSET (TBSE + 0.1% Triton X-100), embryos were incubated for 3 hours in blocking buffer (TBSET + 0.6 % bovine serum albumin), then overnight at 4°C with an anti-β-catenin antibody (rabbit polyclonal IgG, Sigma, C2206) diluted 1/100 in blocking buffer. After four washes in TBSET for 1 hour each, embryos were incubated for 1 hour in blocking buffer and then overnight at 4°C with anti-rabbit IgG-HRP (Santa Cruz Biotech) diluted 1/1000 in blocking buffer. After washing twice for 5 minutes each, with TBS (10 mM Tris-HCl, 150 mM NaCl, pH 6.5), embryos were equilibrated for 30 minutes at room temperature with DAB solution (0.5 mg/ml of 3,3′-diaminobenzidine in 10 mM Tris-HCl, pH 6.5) and staining was revealed with 0.009% of H_2O_2 in DAB solution. The reaction was stopped in methanol. Embryos were cleared and photographed in Murray's solution (1 volume benzyl alcohol, 2 volumes benzyl benzoate) diluted 75% in methanol or were rehydrated and photographed in PBS.

RESULTS

Notch restricts the expression of BCNE markers to the dorsal region

We activated Notch signalling by unilaterally injecting 4-cell-stage embryos with an mRNA encoding the *Xenopus* intracellular domain of Notch fused to the C-myc epitope (*nicd-mt*) and analysed the expression of the BCNE markers *chd* and *Xnr3* at late blastula. NICD notably decreased the expression of both markers on the injected side (*chd*: 90%, *n*=59, Fig. 1B; *Xnr3*: 78%, *n*=36, Fig. 1H), suggesting that Notch is able to inhibit the development of the BCNE.

To corroborate the suggestion that Notch normally modulates the expression of these BCNE markers, we knocked-down the entire Notch pathway. Unilateral dorsal injections of Notch Mo in 4-cell-stage embryos expanded the domains of both BCNE markers (*chd*: 79%, *n*=19, Fig. 1D; *Xnr3*: 61%, *n*=23, Fig. 1J; compare the area depicted by the dotted yellow line on the injected side with the area depicted by the dotted red line on the non-injected side). In some ventrally injected embryos we observed a few ectopic cells expressing *chd* or *Xnr3*, and they were dispersed or formed small

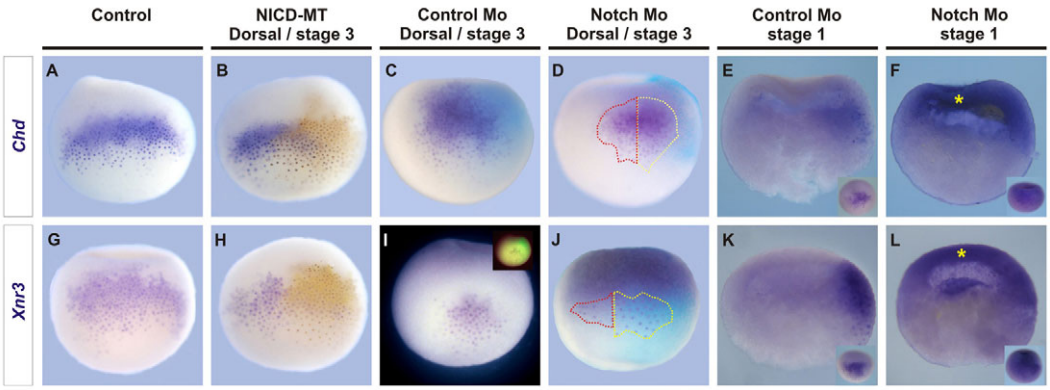

Fig. 1. Notch restricts the expression of blastula *chordin*- and *noggin*-expressing centre (BCNE) markers to the dorsal region. Expression of *chd* (A-F) and *Xnr3* (G-L) in stage 9 *Xenopus* embryos. (**A**,**B**,**G**,**H**) Embryos injected with 1 ng of *nicd-mt* mRNA into a dorsal cell at the 4-cell stage (stage 3; B,H) and control siblings (A,G). Brown dots correspond to horseradish peroxidase immunolocalisation of the c-myc epitope fused to the intracellular domain of Notch (NICD). (**C**,**D**,**I**,**J**) Embryos injected with 20 ng of control morpholino (Mo; C,I) or Notch Mo (D,J) into a dorsal cell at stage 3. The injected side was revealed by alkaline-phosphatase immunostaining of the fluorescein tag (BCIP, turquoise staining in C,D,J) or by immunofluorescence (inset in I). Red and yellow dotted lines depict the area of expression of the BCNE markers on the non-injected and on the injected side, respectively. (**E,F,K,L**) Hemisectioned embryos injected with 20 ng of control Mo (E,K) or Notch Mo (F,L) before the first cleavage. The dorsal side is oriented to the right, animal up. Insets in E, F, K and L show the corresponding whole embryos. Whole embryos are shown in dorsal views, animal up.

patches (see Fig. S1 in the supplementary material). We reasoned that if maternal Notch activity restricted the expression of *chd* and *Xnr3* to the dorsal side, perhaps earlier injections of Notch Mo could result in more robust ectopic expression of these markers. Indeed, injections of Notch Mo before the first cleavage led to more notable expansions of the area of expression of both BCNE markers (*chd*: 97%, *n*=41, compare Fig. 1F with 1E; *Xnr3*: 85%, *n*=26, compare Fig. 1L with 1K). These expansions occurred throughout the animal hemisphere (yellow asterisks, Fig. 1F,L). Injections of control Mo in sibling embryos did not affect the expression of either marker (Fig. 1E,K; *n*=31). Strikingly, when we injected $su(H)1^{DBM}$ mRNA at the 1-cell stage to impair CSL-dependent, canonical Notch activity, we did not observe an expansion of the *chd* or *Xnr3* domains on the animal hemisphere at stage 9 (*chd:* 100%, *n*=32; *Xnr3:* 100%, *n*=15; see Fig. S2 in the supplementary material), although we observed an increase in the number of primary neurons at neural plate stages and an impairment in somitogenesis at the tadpole stage (data not shown), as previously described for this construction in *Xenopus* (Wettstein et al., 1997; Jen et al., 1997). These results confirm that Notch normally restricts the expression of BCNE markers to the dorsal side in the animal hemisphere, probably by a mechanism that does not rely on CSL-mediated transcription.

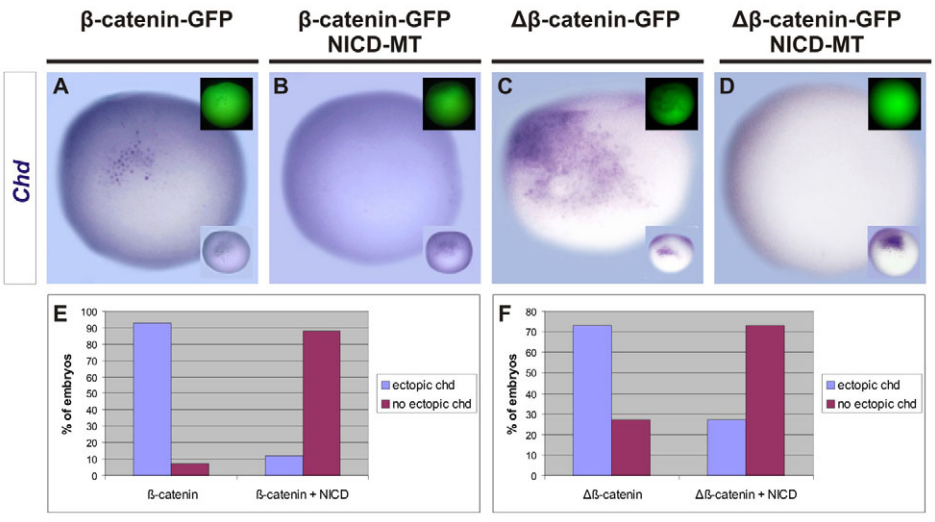

Fig. 2. Notch impairs the ability of β- and Δβ-catenin to induce ectopic expression of the blastula *chordin*- and *noggin*-expressing centre (BCNE) marker *chd* at the ventral side. (**A-D**) Expression of the BCNE marker *chd* in whole *Xenopus* embryos at stage 9. Embryos were injected into one ventral blastomere at the 4-cell stage with the following mRNAs: 1 ng of β-*catenin-GFP* (A), 1 ng of β-*catenin-GFP* + 1 ng of *nicd-mt* (B), 1 ng of Δβ-*catenin-GFP* (C) or 1 ng of Δβ-*catenin-GFP* + 1 ng of *nicd-mt* (D). All embryos are shown in ventral views. The upper insets show the immunofluorescence revealing the DOG tracer at the ventral side. The lower insets show the corresponding dorsal views with the normal expression of *chd* in the BCNE. (**E**,**F**) Percentage of embryos expressing (blue bars) or not expressing (red bars) ectopic *chd* at the ventral side. (E) Comparison of β-*catenin-GFP* (left) and β-*catenin-GFP* + *nicd-mt* injections (right). (F) Comparison between Δβ-*catenin-GFP* (left) and Δβ-*catenin-GFP* + *nicd-mt* injections (right).

Notch impairs the induction of an ectopic BCNE by interfering with the β-catenin pathway

Because the formation of the BCNE depends on a cascade triggered by maternal β-catenin (Kuroda et al., 2004; Ishibashi et al., 2008), one possibility is that Notch is interfering with this pathway. Alternatively, Notch might be repressing the expression of BCNE markers independently of the β-catenin pathway.

To begin to understand this problem, we wondered whether NICD could interfere with the induction of an ectopic BCNE by ventral injections of β-*catenin* mRNA. Overexpression of β-*catenin* mRNA is sufficient to induce the expression of BCNE markers, including *chd*, at the blastula stage (Wessely et al., 2001). Thus, we compared the expression of *chd* in embryos ventrally injected with β-*catenin-GFP* mRNA with or without *nicd-mt* mRNA. Whereas β-*catenin* alone induced ectopic *chd* expression at the ventral side in 93% of the cases (*n*=27; Fig. 2A,E), 88% of the embryos co-injected with *nicd-mt* mRNA did not show ventral expression of *chd* (*n*=16; Fig. 2B,E). The remainder 12% only exhibited scant *chd*+ cells at ectopic sites. We conclude that Notch impairs the ability of β-catenin to induce an ectopic BCNE.

Notch has ventralising properties and abolishes the dorsalising activity of β-catenin

To corroborate further our previous results, we injected *nicd-mt* RNA prior to the first mitotic division and allowed embryos to develop until the tailbud stage in order to evaluate effects on D-V patterning. Embryos were scored according to the dorsoanterior index (DAI) (Kao and Elinson, 1988). Notch gain of function ventralised 100% of the injected embryos. Ventralisation occurred at different grades, with a higher frequency of microcephalic and reduced eyes and forehead phenotypes (Fig. 3; *n*=47).

Next, we wondered whether Notch could rescue the dorsalising effect of β-catenin. With this purpose, we compared the external phenotypes of embryos ventrally injected with β-*catenin-GFP* mRNA with or without *nicd-mt* mRNA, which were allowed to develop until tadpole stages. As expected, injection of β-*catenin* mRNA alone produced different grades of dorsalisation in 82% of the embryos (*n*=74; Fig. 4B,C). It is noteworthy that NICD rescued embryos from dorsalisation by β-catenin in the majority of cases (82%, *n*=92; Fig. 4B,D).

In conclusion, Notch can ventralise embryos by interfering with the dorsalising activity of β-catenin.

Notch decreases the steady state levels of β-catenin before MBT

To investigate whether Notch could be affecting the steady state levels of β-catenin, we injected embryos with β-*catenin-GFP* mRNA with or without *nicd-mt* mRNA. Embryos were fixed at early, mid and late blastula, and were processed for immunostaining with antibodies against GFP or c-myc.

β-Catenin-GFP protein was detected at the three stages analysed when its encoding mRNA was injected alone (75%, *n*=320; Fig. 5A,E,I,M). By contrast, when we added *nicd-mt* mRNAs, the β-catenin-GFP protein completely disappeared from the cytoplasm as well as from the nuclei in the majority of the embryos (71%, *n*=385; Fig. 5B,F,J,M). Thus, Notch can decrease the steady state levels of β-catenin, probably by impairing its stability.

In embryos injected with *nicd-mt* mRNA alone, the NICD-MT protein was found both in the cytoplasm and the nuclei (100%, *n*=20; Fig. 5D,H,L,N), as expected. However, when co-injected with β-*catenin-GFP* mRNA, the NICD-MT protein was not detected in the nuclei anymore but was instead relocated to cell-cell junctions in the majority of the embryos (76%, *n*=55; Fig. 5C,G,K,N). These observations together suggest that, as a result of their interaction, β-catenin is destabilised and the nuclear function of Notch might be impeded.

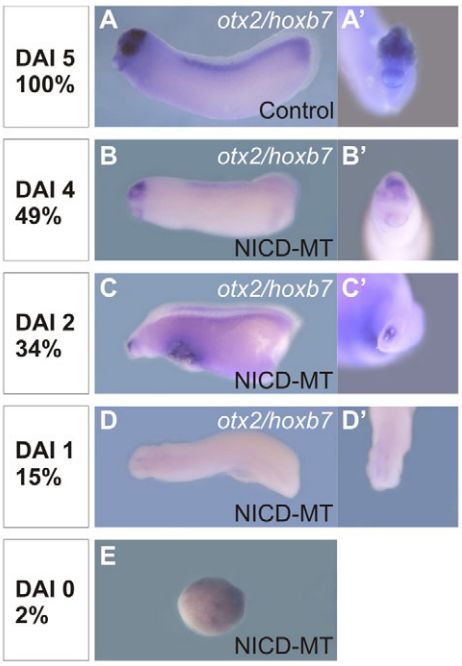

Fig. 3. Notch gain of function produces ventralised phenotypes. *Xenopus* embryos were injected with 1 ng of *nicd-mt* RNA before the first mitotic division and were allowed to develop until stage 28, when they were scored according to the dorsoanterior index (DAI) (Kao and Elinson, 1988). DAI 5, normal; DAI 4, reduced eyes and forehead; DAI 2, microcephalic; DAI 1, acephalic; DAI 0, bauchstück. The percentage corresponding to each phenotype is shown at the left (*n*=47). (**A**,**A'**) Lateral (A) and frontal (A′) views of a sibling control embryo hybridised with the cephalic marker *otx2* (Pannese et al., 1995; Blitz and Cho, 1995) and the posterior marker *hoxb7* (López and Carrasco, 1992; Godsave et al., 1994). (**B-D'**) Lateral (B-D) and frontal (B′-D′) views of embryos injected with *nicd-mt* RNA and hybridised with *otx2* and *hoxb7*. Note the loss of cephalic structures at different grades, as revealed by *otx2* expression. (**E**) An extremely ventralised embryo injected with *nicd-mt* mRNA.

To determine whether endogenous Notch can modulate the steady state levels of β-catenin, embryos were injected before the first cleavage with β-*catenin-GFP* mRNA with or without Notch Mo or control Mo. Immunodetection with an antibody against GFP revealed higher levels of the β-catenin-GFP protein at early, mid and late blastula in embryos co-injected with Notch Mo (61%, *n*=202; compare Fig. 6B with 6A). These results indicate that an endogenous Notch activity already present before MBT can decrease the steady state levels of β-catenin, further suggesting that Notch impairs β-catenin stability by a mechanism that does not rely on transcriptional activation of Notch target genes.

To confirm whether Notch actually modulates the steady state levels of endogenous β-catenin, we employed an antibody raised against amino acids 768-781 of human and mouse β-catenin, which are 100% conserved in the *Xenopus* cognate (Cukyendall and Houston, 2009). When development starts, maternal β-catenin protein increases in the cytoplasm of the dorsal cells and

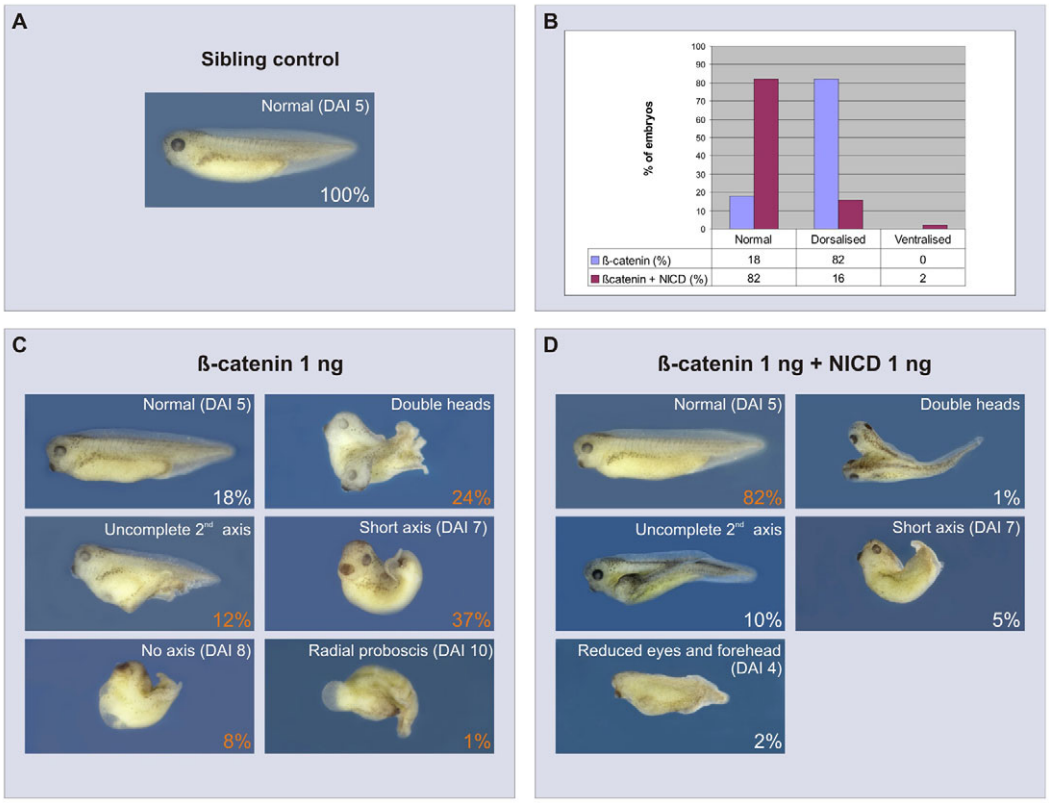

Fig. 4. Notch abolishes the dorsalising activity of β-catenin. (**A**,**C**,**D**) External phenotypes of *Xenopus* tadpoles at stage 35. A shows a control tadpole. Sibling embryos were injected into one ventral blastomere at the 4-cell stage with 1 ng of β-*catenin-GFP* mRNA (C) or with 1 ng of β-*catenin-GFP* mRNA + 1 ng of *nicd-mt* mRNA (D) and fixed at the tadpole stage (stage 35). Embryos were scored according to the dorsoanterior index (DAI) (Kao and Elinson, 1988). Dorsalised phenotypes also include double heads and incomplete secondary axis. Most embryos injected with β-*catenin-GFP* mRNA were dorsalised (82%, *n*=74; the percentage of each dorsalised phenotype is shown in orange; C). Most embryos injected with 1 ng of β-*catenin-GFP* mRNA + 1 ng of *nicd-mt* mRNA were indistinguishable from sibling controls (82%, *n*=92; percentage in orange in D). (**B**) Frequency distribution of normal, dorsalised or ventralised phenotypes, expressed as percentage of injected embryos for β-*catenin-GFP* mRNA alone (blue bars, *n*=74) or β-*catenin-GFP* mRNA + 1 ng of *nicd-mt* mRNA (red bars, *n*=92), as described for C and D.

begins to accumulate in their nuclei at the 16-32 cells stage, so that by mid-to-late blastula, animal-dorsal and vegetal-dorsal cells become highly enriched with nuclear β-catenin (Larabell et al., 1997; Schohl and Fagotto, 2002). At both early and late blastula, uninjected controls or embryos injected with control Mo showed a D-V gradient of β-catenin immunoreactivity (Fig. 7A,C,E,F,I,J) with fainter levels ventrally (yellow asterisks) and maximum levels dorsally (red asterisks). By stage 9, this gradient is clearly nuclear (Fig. 7J). NICD dramatically decreased the levels of endogenous β-catenin throughout the embryo at early and late blastula (90%, *n*=20; compare Fig. 7B,D with 7A,C). On the other hand, the D-V gradient of endogenous β-catenin was impaired after injection of Notch Mo, which increased the levels of the protein on the ventral side relative to control Mo-injected siblings at both stages (66%, *n*=50; compare yellow asterisks in Fig. 7E,F and 7G,H for stage 6.5 and in 7I,J and 7K,L for stage 9). Notably, whereas in the animal cells of Control Mo- injected embryos, β-catenin was predominantly distributed in the peripheral cytoplasm at early blastula (Fig. 7E,F, black arrows), Notch Mo increased the nuclear β-catenin levels (white arrow in Fig. 7H) and appeared to clear β-catenin from the zones of cell-cell contact (compare Fig. 7G,H with 7E,F). Therefore, if Notch is attenuated, nuclear β-catenin accumulation expands towards the ventral side of the embryo and this can already be seen before the burst of zygotic transcription. In conclusion, these results confirm that by destabilising maternal β-catenin protein, maternal Notch restricts nuclear accumulation of β-catenin to the dorsal side of the embryo.

Notch modulates the levels and activity of β-catenin independently of its phosphorylation by GSK3

Next, we wondered whether Notch favours the typical degradation pathway of β-catenin that involves phosphorylation by GSK3 at its N-terminus and whether this impairs its dorsal induction activity. To answer these questions, we made use of a Δβ-catenin-GFP construct, in which the fragment encoding the first 47 amino acids of β-catenin were removed. Δβ-catenin-GFP lacks the three GSK3 target residues on its N-terminus and can induce dorsal development even in the presence of GSK3 in excess (Yost et al., 1996; Verheyen and Gottardi, 2010).

At late blastula, whereas Δβ-*catenin-GFP* mRNA alone induced ectopic *chd* expression at the ventral side in 73% of the cases (*n*=59; Fig. 2C,F), 73% of the embryos co-injected with *nicd-mt* mRNA did not show ventral expression of *chd* (*n*=36; Fig. 2D,F). Thus, NICD inhibits the ability of β-catenin to induce an ectopic BCNE even when its phosphorylation by GSK3 is disabled.

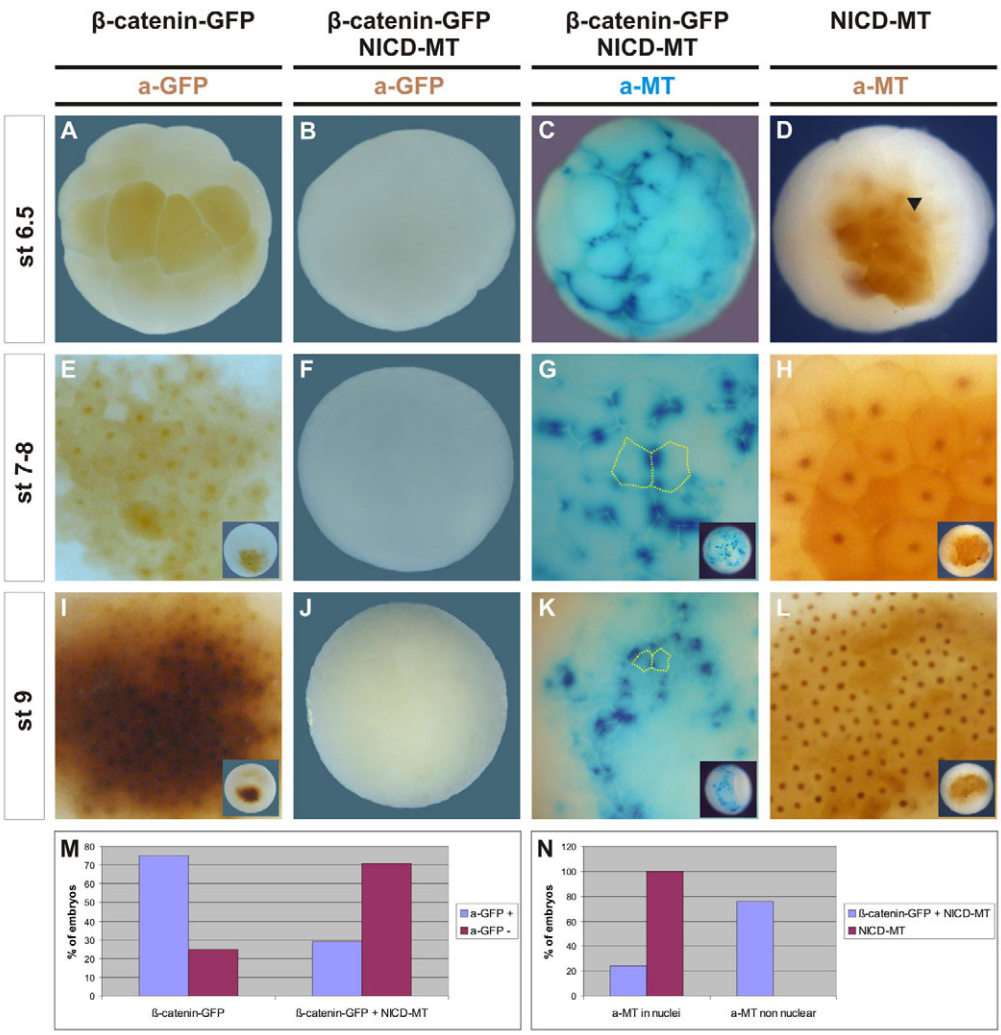

Fig. 5. Notch decreases the steady state levels of exogenous β-catenin. (**A-L**) *Xenopus* embryos were injected with 1 ng of β-*catenin-GFP* mRNA (A,E,I), 1 ng of β-*catenin-GFP* mRNA + 1 ng of *nicd-mt* mRNA (B,C,F,G,J,K) or 1 ng of *nicd-mt* mRNA alone (D,H,L). Embryos were fixed at stage 6.5 (A-D), 7-8 (E-H) or 9 (I-L). They were processed for immunohistochemistry with antibodies against GFP (A,B,E,F,I,J) or c-myc (C,D,G,H,K,L) and revealed with HRP/DAB (A,B,D-F,H-J,L; brown) or alkaline phospatase/BCIP (C,G,K; turquoise). All embryos are shown in animal views. E, G, H, I, K and L are magnifications of the embryos shown in the insets. The arrowhead in D points to the nuclear localisation of NICD-MT. The dotted yellow line in G and K depicts the intercellular boundary around two neighbouring animal cells. Notice the turquoise foci revealing the localisation of NICD-MT at both sides of the boundary, in cell-cell junctions. (**M**) Percentage of embryos expressing the recombinant β-catenin-GFP protein (a-GFP+, blue bars) or not expressing it (a-GFP–, red bars). Comparison of β-*catenin-GFP* (left) and β-*catenin-GFP* + *nicd-mt* injections (right). (**N**) Percentage of embryos with nuclear (left) or without nuclear expression of the recombinant NICD-MT protein, which is instead relocated to cell-cell junctions (right). Comparison of β-*catenin-GFP* + *nicd-mt* injections (blue bars) and *nicd-mt* injections (red bars).

Moreover, when we compared the expression of Δβ-catenin-GFP by immunodetection with the antibody against GFP, we found that the chimerical protein disappeared after co-injections with *nicd-mt* mRNA. This was already observed at early blastula (81%, n=22; compare Fig. 6D with 6C) and also at mid blastula (84%, n=38; data not shown). This strongly suggests that Notch impairs the dorsal inducer activity of β-catenin by decreasing its steady state levels through a mechanism that does not require phosphorylation of its N-terminus by GSK3.

To ascertain whether endogenous Notch actually decreases the steady state levels of the β-catenin form resistant to GSK3-mediated degradation, embryos were injected before the first cleavage with Δβ-*catenin-GFP* mRNA with or without Notch Mo or control Mo. After immunodetection with the antibody against GFP, we found that levels of Δβ-catenin-GFP protein were increased in embryos co-injected with Notch Mo relative to embryos co-injected with control Mo. This was already evident at early blastula (100%, n=27; compare Fig. 6F with 6E) and was also observed at mid blastula (75%, n=24; data not shown).

In conclusion, the endogenous Notch activity already present before MBT is able to destabilise β-catenin through a mechanism that does not rely on phosphorylation by GSK3.

Notch activity controls the size of the brain, a main derivative of the BCNE

The changes that we observed in the expression of BCNE markers at stage 9 as a consequence of manipulating Notch signalling suggest that in the animal hemisphere, Notch normally restricts the formation of the BCNE to the dorsal side of the embryo. As the whole forebrain and most of the midbrain and hindbrain derive

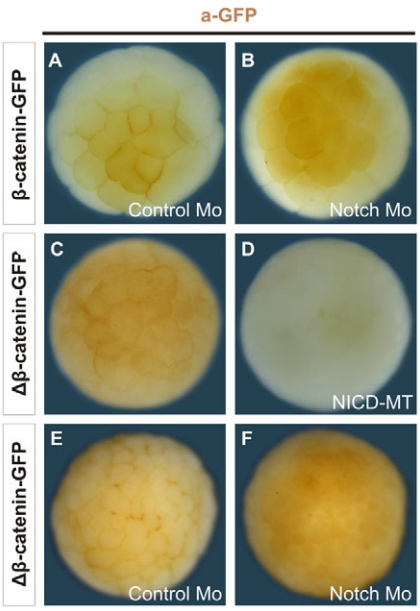

Fig. 6. Endogenous Notch can decrease the steady state levels of β-catenin before midblastula transition (MBT) through a mechanism that does not require its phosphorylation by GSK3. (**A-F**) Immunodetection of the GFP tag of β-catenin-GFP (A,B) or the GFP tag of Δβ-catenin-GFP (C-F). *Xenopus* embryos were injected before the first cleavage with 1 ng of β-*catenin-GFP* mRNA + 20 ng of control morpholino (Mo; A), 1 ng of β-*catenin-GFP* mRNA + 20 ng of Notch Mo (B), 1 ng of Δβ-catenin-GFP mRNA (C), 1 ng of Δβ-catenin-GFP mRNA + 1 ng of *nicd-mt* mRNA (D), 1 ng of Δβ-catenin-GFP mRNA + 20 ng of control Mo (E) or 1 ng of Δβ-catenin-GFP mRNA + 20 ng of Notch Mo (F). All embryos were co-injected with DOG as tracer and classified by fluorescence. Animal views of embryos fixed at early blastula (stage 6.5-7) were photographed in PBS.

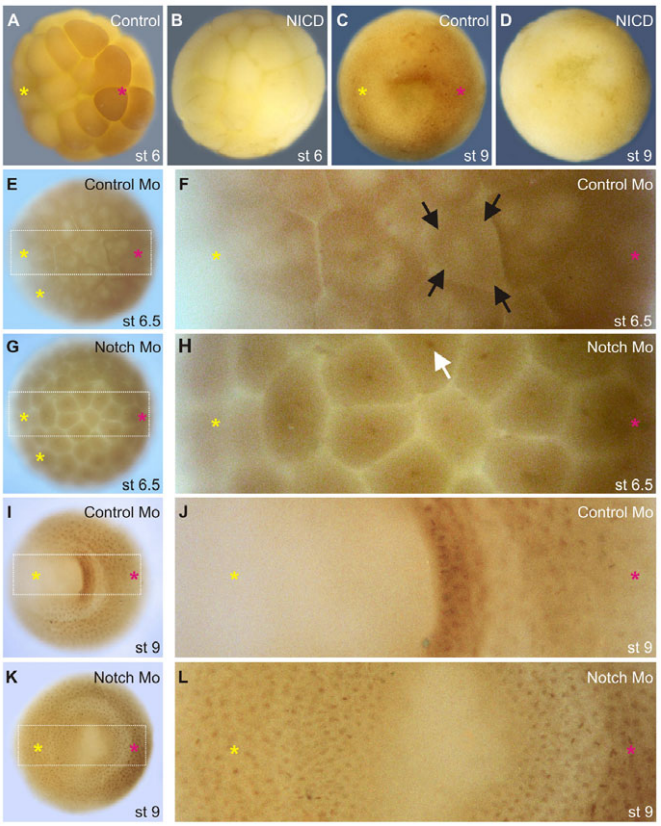

Fig. 7. Notch modulates the levels and the intracellular distribution of endogenous β-catenin. (**A-L**) Immunolocalisation of endogenous β-catenin in whole *Xenopus* embryos. Embryos were injected before the first mitotic division with 1 ng of *nicd-mt* mRNA (B,D), 20 ng of control morpholino (Mo; E,F,I,J) or 20 ng of Notch Mo (G,H,K,L). Injected embryos and their corresponding uninjected siblings (A,C) were fixed at early blastula (A,B,E-H) and late blastula (C,D,I-L) and were processed for whole-mount immunohistochemistry with an antibody raised against the last C-terminal 14 amino acids of β-catenin (brown). All embryos are shown in animal views, with the dorsal side oriented to the right, except in B, where it was not possible to assign the dorsal-ventral (D-V) orientation. F, H, J and L are magnifications of the area enclosed by the rectangular frame in E, G, I and K, respectively. Embryos were photographed in PBS (A-D) or in 75% Murray's solution in methanol (E-L). Uninjected controls (A,C) or embryos injected with control Mo (E,I) show a D-V gradient of β-catenin immunoreactivity, with fainter levels ventrally (yellow asterisks) and maximum levels dorsally (red asterisks). Notch Mo increased the levels of β-catenin protein on the ventral side relative to control Mo-injected siblings (compare yellow asterisks in E,F and G,H for stage 6.5 and in I,J and K,L for stage 9). Black and white arrows indicate the distribution of β-catenin in the peripheral cytoplasm and in the nuclei, respectively.

from the BCNE (Kuroda et al., 2004), we tested whether the size of these derivatives is also changed after attenuation of Notch from very early stages. For this purpose, we injected Notch Mo or control Mo before the first cleavage and cultured the embryos until the neurula stage to study the expression of different A-P markers.

At the neural plate stage, the homeobox gene *Xanf1* is expressed in the future ventral and dorso-rostral part of the diencephalon and in the entire prospective telencephalon (Zaraisky et al., 1995; Kazanskaya et al., 1997; Ermakova et al., 2007) (Fig. 8E). The homeobox transcription factor *otx2* is expressed in a manner complementary to *Xanf1*, depicting a ring-shaped domain that encircles an expression hole (asterisk, Fig. 8A), which coincides with the area in which *Xanf1* transcripts are located (asterisk, Fig. 8E). The rostral portion of the *otx2* domain corresponds to the superficial layer in the rostral margin of the anterior neural plate and to both layers of the ectodermal region, including the stomodeal-hypophyseal and cement gland anlage, adjacent to the anterior margin of the neural plate. The posterior and lateral components of the *otx2* domain are localised within the anterior neural plate, but immediately posterior and lateral to the *Xanf1* domain (Pannese et al., 1995; Blitz and Cho, 1995; Ermakova et al., 1999; Ermakova et al., 2007). At this stage, *otx2* marks the presumptive mesencephalon and diencephalon and the presumptive midline of the dorsal telencephalon (Blitz and Cho, 1995; Ermakova et al., 2007). Immediately juxtaposed to the posterior border of *otx2*, *engrailed-2* (*en2*) is expressed in two bilateral transverse stripes demarcating the future midbrain-hindbrain boundary (Hemmati-Brivanlou et al., 1991) (Fig. 8E). In the central nervous system, *hoxb7* is expressed in the prospective spinal cord (Fig. 8I, left side) with an anterior limit located at the posterior-most part of the prospective hindbrain (green arrowhead in Fig. 8I) (López and Carrasco, 1992; Godsave et al., 1994).

Whereas in Notch Mo-injected embryos, the expression of *otx2* was more diffuse than in control Mo-injected siblings, the ring-shaped domain and its enclosed expression hole were notably expanded in the morphants (91%, n=22; Fig. 8B). A complementary change was observed in the pattern of *Xanf1*,

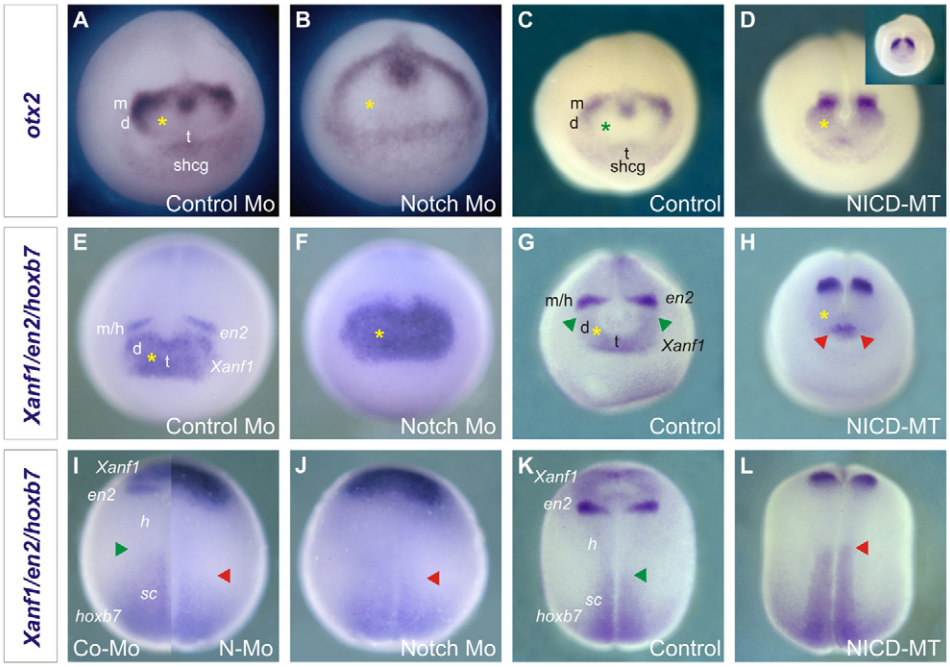

Fig. 8. Attenuation of Notch from the beginning of development produces an expansion of the brain, whereas gain of function of Notch produces the opposite results. (**A-L**) Expression of anterior-posterior (A-P) neural markers at stage 14 (A,B,E,F,I,J) or stage 15 (C,D,G,H,K,L). *Xenopus* embryos were injected prior to the first mitotic cleavage in the animal hemisphere with 20 ng of control morpholino (Mo; A,E, left side of I), 20 ng of Notch Mo (B,F,J, right side of I) or 1 ng of *nicd-mt* RNA (D,H,L). Embryos injected with control Mo and Notch Mo are siblings. C, G and K are the corresponding uninjected sibling controls of D, H and L, respectively. Specimens were processed for whole-mount in situ hybridisation for the following markers: *otx2* (A-D); *Xanf1*, *en2* and *hoxb7* (E-L). A-H show anterior views, dorsal up. I-L show dorsal views, anterior up. The inset in D shows the expression of *otx2* in a more severely affected *nicd-mt* RNA-injected embryo. I is a photocomposition showing a control Mo (Co-Mo)-injected embryo (left) and a Notch Mo (N-Mo)-injected sibling (right). F, right side of I and J show Notch Mo-injected embryos displaying different grades of expansion of the *Xanf1* territory, with gradual extinction of the *en2* stripes and caudal shift of the *hoxb7* domain. Asterisks in A-C highlight the expression hole encircled by *otx2*, coincident with the area of expression of *Xanf1* (asterisks in E-G). Red arrowheads in H indicate the reduction of the *Xanf1* domain in *nicd-mt* RNA-injected embryos relative to control siblings (G, green arrowheads). Red arrowheads in I and J indicate the posterior shift of the *hoxb7* domain in Notch Mo-injected embryos relative to control Mo-injected siblings (I, green arrowhead). The red arrowhead in L indicates the anterior shift of the *hoxb7* domain in *nicd-mt* RNA-injected embryos relative to control siblings (K, green arrowheads). shcg, stomodeal-hypophyseal and cement gland anlage; t, presumptive telencephalon; d, presumptive diencephalon; m, presumptive mesencephalon; m/h, presumptive boundary between midbrain and hindbrain; h, presumptive hindbrain; sc, presumptive spinal cord.

expression of which was increased and clearly expanded by Notch Mo, coinciding with the larger expression hole left by *otx2* (92%, *n*=24; Fig. 8B,F). *en2* expression was more diffuse or nearly missing, and the gap between the *Xanf1* and *en2* domains was reduced or hardly noticeable in Notch Mo-injected embryos in comparison with control Mo-injected siblings (100%, *n*=24; Fig. 8F,J and right side of 8I). Notch Mo produced a posterior shift of *hoxb7* (83%, *n*=24; compare red arrowheads in Fig. 8I, right side, and 8J with green arrowhead in 8I, left side).

Embryos injected with Notch Mo prior to the first cleavage were allowed to develop until more advanced stages and their external morphology was examined to evaluate effects on the D-V axis. We observed an increase in dorso-anterior development in 58% of Notch Mo-injected tadpoles when compared with siblings injected with control Mo (Fig. 9A, *n*=26). Embryos appeared to have bigger heads (35%) or developed a bent axis (DAI 6, 19%). Secondary axes were rarely observed (4%).

When assessed with the help of regional markers, embryos injected with Notch Mo showed an even more obvious increase in dorso-anterior development. Normally, *otx2* expression decreases in the presumptive diencephalon prior to tailbud stage 28, when *otx2* marks the presumptive telencephalon and mesencephalon and, also, the eyes primordia (Blitz and Cho, 1995) (Fig. 9B). When compared in frontal views, the more anterior brain structures of Notch Mo-injected tailbuds were notably expanded relative to control Mo-injected siblings (77%, *n*=22). The prospective anterior brain was expanded in the medio-lateral axis, resulting in more separated eyes and also caudally, as revealed by the larger presumptive telencephalic domain of *otx2* and the more caudal position of the presumptive mesencephalic domain (compare Fig. 9C with 9B). Moreover, the anterior limit of *hoxb7* was caudally displaced relative to the cement gland and the head/trunk ratio was significantly increased in tailbuds injected with Notch Mo in comparison with control Mo-injected siblings (100%, *n*=11; Fig. 9D,E).

These results together indicate that attenuation of Notch from the beginning of development enhances dorso-anterior development, increasing the size of the neural derivatives of the BCNE. This is consistent with the boost of maternal β-catenin signalling and the expansion of BCNE markers at late blastula observed in this condition. Notch seems to modulate the A-P character of these derivatives, because Notch-Mo expands the *Xanf1* domain at the expense of prospective mesencephalic territories, suggesting that the forebrain, which derives entirely from the BCNE, is the main structure expanded. The caudal shift of *hoxb7* suggests that the hindbrain is caudally enlarged, probably at the expense of the spinal cord, which does not derive from the BCNE (Fig. 10C,F).

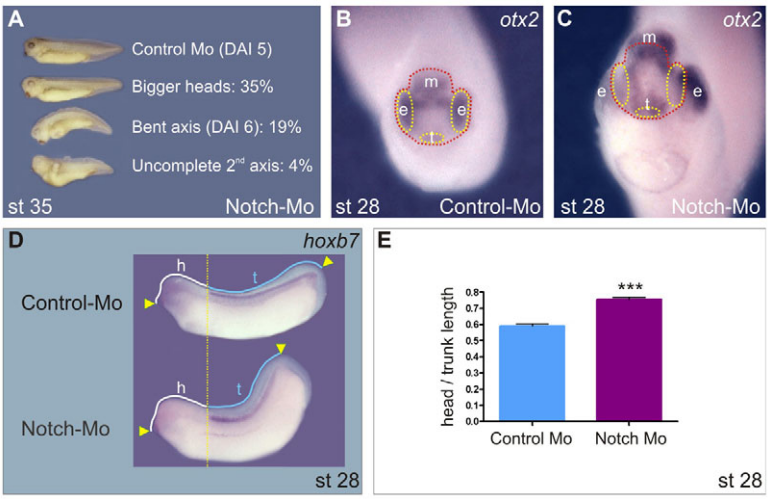

Fig. 9. Attenuation of Notch from the 1-cell stage onwards enhances dorso-anterior development. Sibling *Xenopus* embryos were injected prior to the first cleavage with 20 ng of control morpholino (Mo) or 20 ng of Notch Mo. (**A**) External morphology of stage 35 tadpoles injected with control Mo (upper embryo) or Notch Mo. Three examples of enhanced dorso-anterior development produced by Notch Mo are shown below the control Mo-injected tadpole. (**B**,**C**) Expression pattern of *otx2* at stage 28 in a control Mo-injected tailbud (B) and a Notch Mo-injected sibling (C), shown in frontal views. The dotted red line in B depicts the contour of cephalic structures marked by *otx2* in a control Mo-injected embryo, including the prospective telencephalic domain (t, encircled by a yellow dotted line), the prospective eye domains (e, encircled by yellow dotted lines) and the prospective mesencephalic domain (m). These domains were projected on the Notch Mo-injected sibling shown in C, taking the telencephalic *otx2* domain as reference, to emphasise the expansion of the most cephalic structures. (**D**) Lateral view of a control Mo-injected tailbud (upper embryo) and a Notch Mo-injected sibling (lower embryo) at stage 28, showing the expression of *hoxb7*, aligned with respect to the anterior limit of *hoxb7* (vertical yellow line). The left arrowheads point to the cement gland, and the right arrowheads to the tip of the tail. Head length (h, white line) was measured from the cement gland to the *hoxb7* anterior limit. Trunk length (t, blue line) was measured from the *hoxb7* anterior limit to the tip of the tail. (**E**) Notch Mo-injected tailbuds (red bar) display a significantly higher head/trunk length ratio than control Mo-injected siblings (blue bar). $P<0.0001$, two-tailed *t*-test.

To confirm this hypothesis, we performed gain-of-function experiments by injecting *nicd-mt* mRNA into 1-cell-stage embryos and analysed the expression of the same set of A-P markers at neurula stages. In contrast to Notch Mo, NICD clearly reduced the area encircled by *otx2*. Moreover, the normal expression hole observed in sibling controls disappeared and was filled by *otx2* transcripts (100%, *n*=15), notably replacing *Xanf1*, whose domain was complementarily decreased (compare asterisks in Fig. 8C,D,G,H). Instead of the horseshoe-shaped area depicted in sibling controls at this stage (green arrowheads, Fig. 8G), the *Xanf1* domain became reduced to a small anterior spot, far from the *en2* domain (91%, *n*=11; red arrowheads, Fig. 8H). In addition, the prominent *en2* stripes and the anterior limit of *hoxb7* were displaced anteriorly in *nicd-mt* RNA-injected embryos when compared with sibling controls (compare arrowheads in Fig. 8K,L; *en2:* 100%, *n*=11; *hoxb7:* 73%, *n*=11). In conclusion, Notch gain of function produced a loss of the most anterior landmarks of the neural plate with a concomitant displacement of more posterior markers to more anterior positions. These changes are opposite to those obtained with Notch Mo, confirming that Notch normally controls the size of the brain and modulates the A-P pattern of the central nervous system.

Remarkably, when we injected *su(H)1*^{DBM} mRNA at the 1-cell stage to impair CSL-dependent, canonical Notch activity, the ring shaped domain of *otx2* and its enclosed expression hole were not expanded in comparison with sibling controls at neurula stages (90%, *n*=20; see Fig. S3A,B in the supplementary material). Moreover, in *su(H)1*^{DBM}-injected embryos, the *Xanf1* domain was not expanded, the gap between the *Xanf1* and *en2* domains was conserved and there was not an apparent shift in the anterior limit of *hoxb7* in comparison with sibling controls at stage 15 (82%, *n*=11; see Fig. S3C-F in the supplementary material). These results suggest that CSL-mediated transcription is unlikely to be involved in modulating the size of the brain and the A-P patterning of the BCNE derivatives.

DISCUSSION

The Wnt/β-catenin pathway is the core of an evolutionarily conserved mechanism that establishes the main body axis of vertebrates. Despite the increasing evidence about the interactions between Notch and Wnt, the question of whether Notch could affect dorsal-anterior development had not been addressed before.

The formation of the BCNE depends on maternal β-catenin activity (Wessely et al., 2001; Kuroda et al., 2004) and here we show that Notch initially behaves as an inhibitor of dorsal-anterior development in the animal hemisphere by restricting the BCNE to its normal extent. This is explained by the degradation of β-catenin in the ventral region through a mechanism that does not involve its phosphorylation by GSK3. This role for Notch during early development was not previously recognised in vertebrates.

Notch as an antagonist of β-catenin

Endocytosis is indispensable for canonical Notch signalling. Both the ligand-activated receptor and ligand-unbound Notch are continually internalised into endocytic vesicles, which are routed for trafficking in a complex pathway. It is believed that the γ-secretase-dependent cleavage that renders the NICD fragment, which is responsible for activating transcription through CSL, can occur at different points during endosomal trafficking (Chitnis, 2006; Fortini and Bilder, 2009; Yamamoto et al., 2010).

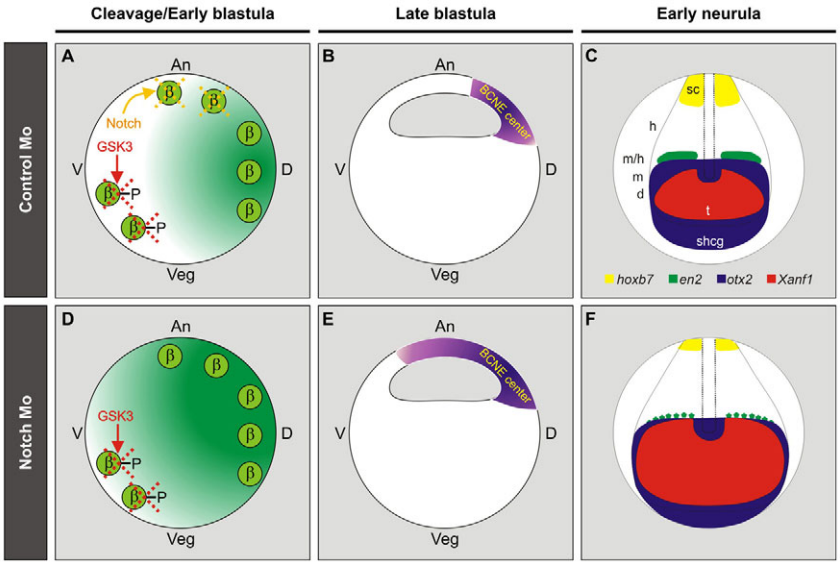

Fig. 10. Model for Notch function during early development of *Xenopus*. (**A-C**) Normal development of a *Xenopus* embryo injected with control Mo, showing β-catenin dynamics at cleavage or early blastula stages (A), the blastula *chordin*- and *noggin*-expressing centre (BCNE) at late blastula (B) and the anterior-posterior (A-P) pattern of the neural plate at early neurula (C). During cleavage stages or early blastula, β-catenin is normally degraded at the ventral side but accumulates in the nuclei of the dorsal side, forming a dorsal-to-ventral gradient, as illustrated in green (A). For simplicity, only the GSK3 and Notch pathways are shown (in red and orange, respectively). GSK3 phosphorylates serine residues 33 and 37 of β-catenin. Phosphorylated β-catenin (β-P) is targeted for fast degradation by the ubiquitin-proteasome system (red dotted lines). Notch targets hypophosphorylated β-catenin (β) for degradation, probably by the endocytic-lysosomal pathway (orange dotted lines). Nuclear β-catenin activity in the dorsal animal/marginal cells of the blastula promotes the formation of the BCNE (purple area in B). The whole forebrain and most of the midbrain and the hindbrain derive from the BCNE (C). (**D-F**) Changes observed in β-catenin dynamics at cleavage or early blastula stages (D), in the extent of the BCNE at late blastula (E) and in the A-P pattern of the neural plate at early neurula (F) when Notch is attenuated by injection of Notch Mo prior to the first cleavage. When Notch is attenuated, hypophosphorylated β-catenin is further stabilised in more ventral locations (D). This results in a ventral expansion of the BCNE at late blastula (E) and BCNE derivatives are enlarged in the early neurula (F). C and F are diagrams of embryos in anterior-dorsal views, summarising the results shown in Fig. 8A-F. An, animal; Veg, vegetal; D, dorsal; V, ventral; shcg, stomodeal-hypophyseal and cement gland anlage; t, presumptive telencephalon; d, presumptive diencephalon; m, presumptive mesencephalon; m/h, presumptive boundary between midbrain and hindbrain; h, presumptive hindbrain; sc, presumptive spinal cord.

Studies in the epithelium of the *Drosophila* wing disc have identified another endocytic pathway for Notch which requires neither the classical ligands nor cleavage by γ-secretase. Although it depends on structural motifs present in the intracellular domain of Notch, this pathway does not involve CSL-mediated transcription. Immunoprecipitation assays demonstrated that Notch and Arm/β-catenin are associated in the same protein complex. Notch associates near the adherens junctions with hypophosphorylated Arm/β-catenin, which has escaped tagging by GSK3 for ubiquitin-proteasome degradation. Because this complex enters endosomal trafficking and becomes degraded, this non-canonical, non-transcriptional function of Notch would be important to buffer activated Arm/ß-catenin in order to keep low levels of spontaneous Wnt activity in the system (Hayward et al., 2005; Sanders et al., 2009). This antagonistic interaction of Notch and Wnt was proposed as an example of how biological systems decrease their own noise (Hayward et al., 2008). This is in agreement with the results we show here during early *Xenopus* development.

Some evidence that Notch similarly antagonises β-catenin in vertebrates has been emerging recently. In mouse embryos, targeted deletion of Notch1 in cardiac progenitor cells increases the levels of nuclear β-catenin and the resulting consequences in the developing heart are similar to the effects of stabilised β-catenin. Consistent with in vivo observations, knockdown of Notch1 in cardiac progenitor cells in vitro increases the levels of hypophosphorylated β-catenin and stimulates transcription from a Wnt/β-catenin promoter (Kwon et al., 2009). In mouse cell lines in culture, the intracellular fragment of Notch1 (NICD1) inhibits osteoblastogenesis, decreases the cytoplasmic levels of endogenous β-catenin and impairs canonical Wnt signalling, even when provided by a β-catenin mutant resistant to GSK3-mediated degradation (Deregowski et al., 2006). NICD1 also functions as tumour suppressor in mouse skin by preventing the nuclear accumulation of hypophosphorylated β-catenin, which is associated with the development of basal-cell carcinoma (Nicolas et al., 2003).

Other authors employed two Notch constructs to address this issue in mammalian cells in culture. One was tethered to the membrane and was unable to activate transcription through CSL. The other was spontaneously cleaved by γ-secretase and activated CSL-dependent transcription. Both were able to interfere with the transcriptional activity of an oncogenic form of β-catenin, which cannot be phosphorylated by GSK3, suggesting that this function of Notch does not require the release of NICD nor transcription mediated by CSL (Hayward et al., 2005).

Therefore, although in *Drosophila* and in the few examples studied in vertebrate cells the intracellular domain of Notch promotes β-catenin instability, it is not clear whether this function is carried out by the γ-secretase-cleaved fragment or by uncleaved Notch. In our experiments, NICD is able to decrease the steady state levels of endogenous β-catenin and also destabilises the truncated β-catenin form resistant to GSK3 phosphorylation. From

our results, we cannot discern whether the full length Notch or the cleaved NICD fragment are normally responsible for this function in early *Xenopus* development.

When NICD was overexpressed alone, we found it in the cytoplasm and nuclei of the *Xenopus* blastula cells, as expected. However, when co-expressed with β-catenin, NICD was not detected in the nuclei anymore but was instead located around cell-cell junctions, whereas the β-catenin protein disappeared. This strongly resembles the situation in the epithelium of the *Drosophila* imaginal discs, when hypophosphorylated Arm/β-catenin associates with Notch near the adherens junctions to enter endosomal trafficking for degradation (Sanders et al., 2009). Our results suggest that when Notch interacts with β-catenin, it is not involved in a nuclear function. Instead, β-catenin is degraded, perhaps through endosomal trafficking, in a GSK3-independent way.

In addition to its transcriptional function, β-catenin also participates in the adhesive role of adherens junctions. Together with α- and γ-catenin/plakoglobin, β-catenin operates as a cytoplasmic adaptor, linking transmembrane cadherin cell adhesion molecules to the actin cytoskeleton (Miller and Moon, 1996). Interestingly, we observed that in the animal cells of the early blastula, endogenous β-catenin disappears from the peripheral cytoplasm and becomes preferentially located to the nuclei when Notch is attenuated. This suggests that Notch might be controlling the β-catenin pools available for cell adhesion or transcription in such way that low levels of this non-canonical Notch activity in the dorsal cells would assure higher nuclear localisation and transcriptional function of β-catenin.

In conclusion, we propose that a maternal, non-transcriptional Notch activity, higher at the ventral side of the early embryo, restricts the BCNE to the dorsal region by promoting degradation of β-catenin that escapes phosphorylation by GSK3, and that this occurs even before MBT (Fig. 10). This is supported by the observation that interfering with CSL-mediated transcription did not result in an expansion of the BCNE markers on the animal hemisphere at stage 9 or an increase in the size of the BCNE derivatives at later stages.

The forkhead transcription factor FoxI1e is initially expressed on the dorsal side of the animal hemisphere (Mir et al., 2008). Unlike *chd* and *Xnr3* (Kuroda et al., 2004; Ishibashi et al., 2008), the spatial regulation of this gene is independent of canonical Wnt signalling at the late blastula stage. However, maternal depletion of *notch* mRNA from oocytes with a phosphorothioate-modified antisense oligonucleotide, followed by the host transfer method, results in an expansion of *foxI1e* expression towards the ventral side at this stage, and this is mimicked by the dominant-negative construct $Su(H)1^{DBM}$, which interferes with canonical Notch signalling (Mir et al., 2008). Notwithstanding that this is most likely to represent a transcriptional regulation of *foxI1e* by Notch, these results support the hypothesis that Notch activity is spatially regulated in the animal hemisphere, with lower levels at the dorsal side and higher levels at the ventral side. Therefore, both canonical and transcriptional mechanisms triggered at MBT, and degradation of β-catenin that begins to operate earlier contribute to the landscape of Notch activity before gastrulation.

Apart from the very well known destruction of GSK3-phosphorylated β-catenin through the ubiquitin-proteasome system and the Notch-dependent pathway that we describe here, there is evidence that at least another two mechanisms contribute to the confinement of dorsal development in *Xenopus* by modulating β-catenin dynamics at different points. First, maternal Jun NH2-terminal kinase (JNK) restricts nuclear accumulation of β-catenin by regulating nuclear-cytoplasmic transport (Liao et al., 2006). Second, nuclear factor of activated T cells (XNFAT) mediates destabilisation of cytoplasmic β-catenin through the non-canonical Wnt/Ca^{2+} pathway, and this occurs downstream of Dsh but upstream of GSK3 and TCF3 (Saneyoshi et al., 2002).

Notch restricts the size of the brain and participates in the A-P patterning of the neural plate

The whole forebrain derives from the BCNE, but the latter also contributes to part of the midbrain and hindbrain (Kuroda et al., 2004). The observation that Notch Mo expands the *Xanf1* domain at the expense of prospective mesencephalic territories indicates that, among the BCNE derivatives, the forebrain is the structure that is preferentially enlarged (Fig. 10C,F). This is correlated with a caudal shift of *hoxb7* expression, suggesting also an enlargement of the hindbrain at the expense of the spinal cord, which does not derive from the BCNE (Fig. 10C,F). Therefore, when Notch is attenuated from the beginning of development, a general anteriorisation seems to affect the entire neural plate. The fates of the dorsal animal blastomeres are significantly mixed in terms of brain or spinal cord fate (Dale and Slack, 1987). Therefore, it seems plausible that concomitantly to the restriction in the formation of the BCNE, Notch modulates the A-P character of the central nervous system, favouring more posterior values among BCNE derivatives themselves and between BCNE derivatives and the spinal cord.

Finally, the emergence of the *anf* gene was coincident with the appearance of vertebrates. It was proposed that through the inhibition of diencephalic and mesencephalic programmes in the anterior neural plate, the acquisition of this new gene allowed the development of the rostral forebrain (Ermakova et al., 2007). It will be interesting to elucidate whether attenuation of Notch signalling in the dorsal side of the embryo was relevant to the emergence of the rostral forebrain during evolution.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
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