HESX1- and TCF3-mediated repression of Wnt/β-catenin targets is required for normal development of the anterior forebrain

Cynthia L. Andoniadou*,1, Massimo Signore*,1, Rodrigo M. Young2, Carles Gaston-Massuet1, Stephen W. Wilson2, Elaine Fuchs3 and Juan Pedro Martinez-Barbera1,‡

SUMMARY
The Wnt/β-catenin pathway plays an essential role during regionalisation of the vertebrate neural plate and its inhibition in the most anterior neural ectoderm is required for normal forebrain development. Hesx1 is a conserved vertebrate-specific transcription factor that is required for forebrain development in Xenopus, mice and humans. Mouse embryos deficient for Hesx1 exhibit a variable degree of forebrain defects, but the molecular mechanisms underlying these defects are not fully understood. Here, we show that injection of a hesx1 morpholino into a ‘sensitised’ zygotic headless (tcf3) mutant background leads to severe forebrain and eye defects, suggesting an interaction between Hesx1 and the Wnt pathway during zebrafish forebrain development. Consistent with a requirement for Wnt signalling repression, we highlight a synergistic gene dosage-dependent interaction between Hesx1 and Tcf3, a transcriptional repressor of Wnt target genes, to maintain anterior forebrain identity during mouse embryogenesis. In addition, we reveal that Tcf3 is essential within the neural ectoderm to maintain anterior character and that its interaction with Hesx1 ensures the repression of Wnt targets in the developing forebrain. By employing a conditional loss-of-function approach in mouse, we demonstrate that deletion of β-catenin, and concomitant reduction of Wnt signalling in the developing anterior forebrain of Hesx1-deficient embryos, leads to a significant rescue of the forebrain defects. Finally, transcriptional profiling of anterior forebrain precursors from mouse embryos expressing eGFP from the Hesx1 locus provides molecular evidence supporting a novel function of Hesx1 in mediating repression of Wnt/β-catenin target activation in the developing forebrain.

KEY WORDS: Hesx1, Tcf3 (Tcf7l1), Wnt/β-catenin, Forebrain, Mouse, Zebrafish

INTRODUCTION
Suppression of posteriorising signals, and in particular of Wnt signalling, is necessary for correct forebrain specification (Buchert et al., 2010; Felix and Aboobaker, 2010; Fredieu et al., 1997; Heisenberg et al., 2001; Houart et al., 2002; Kimura et al., 2000; Kudoh et al., 2002; Perea-Gomez et al., 2001; van de Water et al., 2001; Wilson and Houart, 2004). Inhibitors that are able to sequester Wnt molecules or to bind their receptors are secreted either locally within the neuroectoderm (Houart et al., 2002) or from underlying tissues (Glimka et al., 1997; Kazanskaya et al., 2000; Piccolo et al., 1999) to abolish Wnt signalling in the forebrain. In addition, other molecules act within the receiving cell to either bind to β-catenin (Garaventa et al., 1999; Satoh et al., 2004) or to interfere with receptor maturation (Yamamoto et al., 2005) to ensure that the pathway is inactive within the anterior neural plate. The combination of these cell- and non-cell-autonomous mechanisms is thought to lead to the establishment of a high-posterior to low-anterior gradient of Wnt signalling that provides the positional information required for the regionalisation of the incipient neural plate into the fore, mid and hindbrain. However, less is known about how Wnt/β-catenin target genes are repressed in forebrain precursors.

β-catenin, the product of Ctnnb1, is at the centre of the canonical Wnt pathway. In the absence of Wnt molecules, β-catenin is phosphorylated by a ‘destruction complex’ formed by several proteins, including GSK3β, CK1α, AXIN1 and APC, and targeted for ubiquitylation and subsequent proteasome-mediated degradation. Binding of secreted Wnt ligands to Frizzled and LRP membrane receptors causes the disassembly of the destruction complex and inhibition of β-catenin phosphorylation. This results in the cytoplasmic accumulation of β-catenin, which can enter the nucleus to interact with DNA-binding TCF/LEF transcription factors and activate transcription of Wnt target genes (reviewed by van Amerongen and Nusse, 2009).

Among the four TCF/LEF factor family members, only Tcf3 (Tcf7l1) is expressed in the developing forebrain primordium of the mouse at presomitic and early somite stages (Galceran et al., 1999; Merrill et al., 2004). Experiments in Xenopus laevis indicate that tcf3 acts as a repressor of Wnt targets and interacts with Groucho corepressors (Brannon et al., 1999; Brantjes et al., 2001; Houston et al., 2002). In zebrafish, knockdown of both paralogues of Tcf3, tcf3a (tcf7l1a) and tcf3b (tcf7l1b), results in loss of repressor activity and in anterior truncations (Kim et al., 2000), indicating that there is a requirement for the β-catenin-independent Tcf repressor activity in head morphogenesis (Dorsky et al., 2003). In mouse, Tcf3-deficient embryos undergo gastrulation but exhibit variable degrees of defects, including primitive streak and axis duplications, supernumerary neural folds, and neural patterning defects involving expansion of

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midbrain at the expense of forebrain and hindbrain tissues (Merrill et al., 2004). However, the mechanisms underlying these defects are not fully understood because Tcfβ is expressed prior to the onset of gastrulation in the epiblast, anterior mesendoderm and anterior neuroectoderm, and defects in any of these could lead to aberrant neural patterning. Specifically, whether Tcfβ plays a role within forebrain tissue remains unknown (Merrill et al., 2004).

HESX1 is a paired-like homeobox transcription factor that is expressed in the anterior regions of the vertebrate embryo but is absent from invertebrates, including close relatives such as ascidians, amphioxus and Ciona intestinalis (Kazanskaya et al., 1997; Martinez-Barbera et al., 2000). Using genetic fate mapping, we have previously shown that derivatives of Hesx1-expressing precursors that are normally destined to populate the anterior forebrain (cerebral cortex, basal ganglia, ventral diencephalon and eyes) change their fate in Hesx1-deficient embryos and colonise posterior forebrain regions as well as the neural crest lineage (Andoniadou et al., 2007). A similar posterior transformation of anterior forebrain is observed in knockdown experiments of the Hesx1 orthologue Xanf in Xenopus (Ermakova et al., 1999). The molecular mechanisms responsible for the lack of anterior identity and cell fate transformation are not known. Understanding the pathogenesis of these early defects is also of clinical relevance, as mutations in HESX1 result in forebrain, eye and pituitary defects (Dattani et al., 1995). In this study we sought to specifically address the molecular function of Hesx1 in anterior forebrain precursors. Combining genetic and molecular approaches we reveal a novel role for Hesx1 as an antagonist of the Wnt/β-catenin pathway in the mouse and zebrafish forebrain. In addition, we demonstrate a requirement for Tcfβ in forebrain progenitors, where it genetically interacts with Hesx1 to promote anterior character by repressing the transcriptional activation of Wnt/β-catenin target genes.

MATERIALS AND METHODS

Animals

Wild-type and zhdph0381 zebrafish embryos were raised at 28°C and staged according to Kimmel et al. (Kimmel et al., 1995). Single-cell embryos were injected with 5 nl of 0.5 pmol/nl hex1 morpholino (5'-TGCAAGAGAAGCCATTGCTAAACTC-3') and/or 1 pg/ml mouse Hesx1 mRNA. hdph0381 mutant embryos were genotyped as described (Kim et al., 2000).

Hesx1-Cre, Ctnb1lox(ex2-6), Tcfβ-flox, R26-YFP, BAT-gal, Six3-lacZ, Hesx1lox/+ and Ctnb1-lox(ex2-6) mice have been described previously (Andoniadou et al., 2007; Brault et al., 2001; Lagutin et al., 2003; Maretto et al., 2003; Nguyen et al., 2009; Srinivas et al., 2001; Dattani et al., 1998; Haegel et al., 1995). Tcfβ-flox animals were crossed with the Actb-Cre strain to generate Tcfβlox/– animals, which were bred further on C57BL/6 in order to remove the Cre transgene from the background. Breeding of genetically modified animals and all animal procedures were carried out under the UK Home Office Animals (Scientific Procedures) Act 1986. A mixed background, backcrossed onto C57BL/6, was used for all strains. Embryos and pups were genotyped by PCR on DNA from yolk sacs, tail buds or ear biopsies as described previously (Andoniadou et al., 2007). Briefly, the thermal profile comprised a single step for 2 minutes at 94°C, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds. The wild-type and mutant alleles yield bands of ~500 bp and 250 bp, respectively. For primers, see supplementary material Table S9.

The Hesx1-eGFP targeting vector was generated using homologous regions obtained from plasmids carrying the mouse Hesx1 gene (Dattani et al., 1998). A cassette containing eGFP followed by (1) four SV40 and one PGK polyadenylation sites flanked by frt sequences, (2) the diphtheria toxin A (DTA) coding sequence (Ivanova et al., 2005) and (3) a PGK-Neo cassette flanked by frt sequences (Andoniadou et al., 2007) was cloned into a vector containing ~6.5 kb and 1.3 kb of 5′ and 3′ homologous regions, respectively (Fig. 5). The linearised targeting vector was electroporated into CCE ES cells (129/SvEv; kindly provided by E. Robertson, Sir William Dunn School of Pathology, Oxford, UK) and ~400 colonies were picked, expanded and screened by PCR and Southern blot, as described previously (Andoniadou et al., 2007). Two correctly targeted clones were isolated and injected into blastocysts from C57BL/6J(Harlan) mice. Male chimeras were backcrossed to C57BL/6J females to establish the F1 generation of heterozygous mice. F1 animals were crossed to the Actb:FLPe strain (Rodriguez et al., 2000), kept on a C57BL/6J background, to excise the PGK-Neo cassette. After backcrossing with C57BL/6J animals to remove the FLPe transgene, Hesx1eGFP+ heterozygotes were kept on a C57BL/6J background.

Flow sorting

For microarray gene profiling, 3- to 5-somite embryos from Hesx1eGFP- intercrosses were selected for eGFP expression, phenotyped and grouped into normal (Hesx1eGFP+/+) and mutant (Hesx1eGFP+/–). Pieces of each embryo were retained for genotyping to ensure the integrity of each pool. Embryos were manually dissociated, and single cells were flow sorted using a MoFlo XDP (Beckman Coulter, Fullerton, CA, USA) directly into Buffer RLT (Qiagen) for RNA extraction. Fluorescence was detected using a 530/540 filter. Cell sorting data were analysed using Summit software (Dako).

Microarray analysis

Total RNA was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer’s recommendations with the addition to the lysis buffer of 500 ng bacterial ribosomal RNA (Roche) per reaction as carrier. cDNA synthesis, linear amplification and labelling of cRNA were carried out according to the manufacturer’s protocols using the GeneChip 3′ IVT Express Kit (Affymetrix). Gene expression profiling was performed to compare eGFP-positive purified anterior forebrain precursors from Hesx1eGFP+ (normal) and Hesx1eGFP+/– embryos on the Affymetrix Mouse430_2 platform using the GeneChip Hybridisation, Wash and Stain Kit (Affymetrix). This analysis was carried out in triplicate for each genotype, over a ~1 year period, using independently isolated pools of cells from several embryos (n=20-30 for each replicate) and was validated on independent biological samples by qRT-PCR (supplementary material Fig. S3). Gene expression data are deposited at ArrayExpress with ID: E-MEXP-2586. Files were processed in MATLAB (Mathworks) and GeneSpring GX (Agilent Technologies). GC-RNA normalisation was carried out and significantly differentially expressed genes were identified after the Benjamini and Hochberg false discovery rate was applied as a multiple testing correction method. GeneSpring Gene Ontology and DAVID databases were used to generate pathway lists. Where multiple probe sets were available for a single gene, the value of the unique probe set was used, or, in the case of multiple unique probe sets, that with the highest raw intensity levels.

In situ hybridisation, immunofluorescence and X-Gal staining

Whol mount in situ hybridisation, X-Gal staining and immunofluorescence on paraffin sections were performed as previously described (Andoniadou et al., 2007). Antibodies against GFP (Invitrogen, 1:350), cleaved caspase 3 (Cell Signalling Technology, 1:200) and phospho-histone H3 (Upstate, 1:300) were detected with Alexa Fluor-conjugated secondary antibodies (Invitrogen, 1:350).

Quantitative real-time (qRT) PCR

RNA was extracted using the RNeasy Micro Kit (Qiagen) following the manufacturer’s protocols and including on-column DNAse digestion. Up to 2 μg RNA was used for cDNA synthesis using Omniscript reverse transcriptase (Qiagen) with random hexamers (Promega).

qRT-PCR was carried out to validate microarray results, on independent biological replicates in triplicate. Briefly, 10-50 ng template cDNA was used per reaction on an ABI 7500 Fast Cycler employing SYBR-based technology (Invitrogen, 1:350). 2-ΔΔCt was calculated for each gene relative to the internal control β-actin. qRT-PCR was carried out to validate microarray results, on independent biological replicates in triplicate. Briefly, 10-50 ng template cDNA was used per reaction on an ABI 7500 Fast Cycler employing SYBR-based technology (Invitrogen, 1:350). 2-ΔΔCt was calculated for each gene relative to the internal control β-actin.
to denature UNG and activate Taq polymerase, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Results were normalised to endogenous levels of Gapdh and were analysed using the ΔΔCt method.

We assessed excision in the forebrain by quantitative PCR on DNA using primers specifically designed against exon 3 of Ctnnb1, which should be excised in Ctnnb1-lox(ex2-6) embryos in the presence of Cre recombinase. DNA was extracted from dissected forebrains (anterior to the telencephalic/diencephalic boundary) of 9.5 dpc embryos using the DNA Micro Kit (Qiagen) using standard protocols. Primers against endogenous control and results were analysed using the ΔΔCt method.

Microscopy
Images of live embryos or after fixation or wholemount in situ hybridisation were captured using a Leica TCS camera and IM50 software (Leica). Images were processed using Photoshop (Adobe).

RESULTS
HESX1 can antagonise Wnt signalling, a role conserved between zebrafish and mouse
To test whether HESX1 acts to maintain anterior forebrain identity by antagonising Wnt signalling, we investigated the effect of reducing hesx1 (previously anf) levels in zygotic headless (hdl; tcf3a; tcf7l1a) zebrafish mutants. The hdl phenotype, which results from a mutation in one of the two zebrafish tcf3 paralogues, was initially characterised as a maternal zygotic (mz) mutant that displayed a severe anterior truncation phenotype, including the absence of eyes (Kim et al., 2000). However, this phenotype is not observed in zygotic (z) hdl mutants owing to compensation by tcf3b, which is also expressed in the anterior neural plate of the zebrafish embryo (Dorsky et al., 2003) (Fig. 1B). Indeed, the severe mzhdl forebrain defects can be induced in zhdll mutants upon injection of tcf3b-specific morpholinos (MOs) (Dorsky et al., 2003). We took advantage of this ‘sensitised’ zhdll background to investigate the function of hesx1 in zebrafish.

Injection of hesx1 MO into wild-type embryos had no effect (Fig. 1C; 2.5 pmol/embryo, n=100). However, when we injected embryos from hdl+/− intercrosses, we found anterior forebrain defects in ~25% of cases, which were confirmed to be homozygous mutants by hdl genotyping (Table 1; Fig. 1D). No defects were induced in other genotypes. The absence of eyes was apparent in hdl+/− hesx1 morphant embryos, as was the absence of the telencephalon and part of the diencephalon, indicating that the phenotype is similar to the mzhdl phenotype (Kim et al., 2000). This phenotype was specific to the knockdown of Hesx1, as coinjection of hesx1 MO in embryos from hdl+/− embryos (arrowhead in D), which are sensitised to increased levels of Wnt signalling. Injection of hesx1 MO into control hdl+/− embryos has no effect (E).

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Table 1. Injection of zebrafish embryos from hdl+/− intercrosses with zebrafish hesx1 MO, with or without mouse hesx1 mRNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Without eyes</th>
<th>Normal development</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>hdl+/−</td>
<td>18</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Other genotypes</td>
<td>0</td>
<td>63</td>
<td>80</td>
</tr>
<tr>
<td>hdl+/−+hesx1 MO</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hdl+/−+hesx1 mRNA</td>
<td>25</td>
<td>55</td>
<td>80</td>
</tr>
</tbody>
</table>

Treatment was with 2.5 pmol/embryo hesx1 MO with or without 5 pg mouse Hesx1 mRNA.

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showed only unilateral microphthalmia and the telencephalon developed normally; and class II defects, in embryos with bilateral microphthalmia and/or unilateral anophthalmia in conjunction with a reduction in size of the telencephalic vesicles (n=33; Table 2; supplementary material Fig. S1C-E).

In situ hybridisation for specific regional markers revealed a reduction of their expression domains concomitant with the progressive loss of anterior tissue. Expression of the forkhead box-containing transcription factor Foxg1, which is essential for normal development of the telencephalon, was markedly reduced in class II embryos (n=5; Fig. 2E). Likewise, expression of Fgf8, a crucial signalling molecule during forebrain development (Meyers et al., 1998; Shimamura and Rubenstein, 1997), at the anterior tip of the telencephalon (ANR, anterior neural ridge) was reduced only in class II Hesx1Cre+/–;Tcf3+/– embryos (n=6; Fig. 2D,D’). The expression domain of Pax6, a paired box transcription factor involved in forebrain patterning and boundary formation, was also reduced in the telencephalon and eye of class I and II embryos but extended normally to the posterior forebrain-midbrain boundary, suggesting that more caudal regions were unaffected (n=7; Fig. 2F).

To reduce gene dosage further, Hesx1Cre+/–;Tcf3+/– embryos were generated. Hesx1Cre+/–;Tcf3+/– embryos usually displayed a fully penetrant phenotype with variable expressivity, extending from class II to a group with more severe defects than those described above, termed class III, which include bilateral anophthalmia as well as a reduction in telencephalic tissue (Andoniadou et al., 2007) (Table 2; supplementary material Fig. S1F,G). When a copy of Tcf3 was removed in Hesx1Cre+/–;Tcf3+/– embryos, the severity was dramatically increased, resulting in loss of most of the anterior forebrain, which was designated a class IV defect (n=13; Table 2; Fig. 2G-I; supplementary material Fig. S1H,I). Wholemount in situ hybridisation against Foxg1 and Pax6 demonstrated the specific loss of anterior forebrain (n=3 per marker; Fig. 2H,I). Likewise, the expression domain of Fgf8 at the ANR was absent, but normal Fgf8 staining was observed at the mid-hindbrain boundary in all embryos analysed, confirming the loss of forebrain but not midbrain tissue (n=3; Fig. 2G,G’).

Together, these experiments suggest that a minimum gene dosage of Hesx1 and Tcf3 is required for normal development of the forebrain.

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**Table 2. Genotypes of embryos from Hesx1Cre+/–;Tcf3+/– × Hesx1+/– crosses, classified according to severity of anterior defects**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of embryos</th>
<th>Class of forebrain defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesx1+/–;Tcf3+/–</td>
<td>24</td>
<td>None I II III IV</td>
</tr>
<tr>
<td>Hesx1+/–;Tcf3+/–</td>
<td>27</td>
<td>– – – – –</td>
</tr>
<tr>
<td>Hesx1+/–;Tcf3+/–</td>
<td>20</td>
<td>19 1 – – –</td>
</tr>
<tr>
<td>Hesx1+/–;Tcf3+/–</td>
<td>22</td>
<td>4 9 9 – –</td>
</tr>
<tr>
<td>Hesx1+/–;Tcf3+/–</td>
<td>11</td>
<td>4 2 5 – –</td>
</tr>
<tr>
<td>Hesx1+/–;Tcf3+/–</td>
<td>24</td>
<td>– – 11 13 – –</td>
</tr>
<tr>
<td>Hesx1+/–;Tcf3+/–</td>
<td>13</td>
<td>– – – – 13</td>
</tr>
<tr>
<td>Hesx1+/–;Tcf3+/–</td>
<td>20</td>
<td>20 – – – –</td>
</tr>
<tr>
<td>Total</td>
<td>161</td>
<td></td>
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</tbody>
</table>

Defects were classified as: Class I, unilateral microphthalmia with normal telencephalic vesicles; class II, bilateral microphthalmia or unilateral anophthalmia with reduction in size of the telencephalic vesicles; class III, bilateral anophthalmia with reduction in size of the telencephalic vesicles; and class IV, complete absence of anterior forebrain development.

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**Fig. 2. Gene dosage-dependent forebrain defects in mouse embryos genetically deficient for Hesx1 and Tcf3.** In situ hybridisation with Fgf8, Foxg1 and Pax6 antisense riboprobes on 9.5 dpc embryos. (A,A’) Fgf8 expression in the wild-type brain is restricted to the anterior neural ridge (ANR, arrows) and at the mid-hindbrain boundary (MHB, arrowhead). Lateral view in A, frontal in A’. (B) Foxg1 is expressed in the normal developing telencephalic vesicles (arrows). (C) In the wild-type brain, Pax6 is expressed in the dorsal telencephalon (arrow), posterior forebrain (arrowheads) and eye. (D,D’) In Hesx1Cre+/–;Tcf3+/– double heterozygotes, Fgf8 expression in the MHB (arrowheads) is normal but is reduced in the ANR (arrows). (E) Foxg1 expression in the telencephalon is severely reduced in Hesx1Cre+/–;Tcf3+/– embryos (arrows). (F) Pax6 expression in the telencephalon and eye is decreased but expression in the posterior forebrain is normal (arrowheads). (G-I) Forebrain defects are very severe in Hesx1Cre+/–;Tcf3+/– embryos and most of the forebrain is missing, as evidenced by the lack of Fgf8 (G,G’) and Foxg1 (H) expression. Note the normal expression of Fgf8 in the MHB (arrowhead in G) and the minimal expression of Pax6 (arrowhead in I).
**Tcf3 is required in anterior neural progenitors for normal forebrain development**

In mouse embryos, *Hesx1* is initially expressed in the anterior visceral endoderm at the onset of gastrulation at 6.5 dpc, in the anterior mesendoderm by 7.5 dpc and subsequently in the anterior neural ectoderm from 7.5-8.0 dpc (Thomas and Beddington, 1996). Tcf3 is expressed in the epiblast at pre-gastrulation stages and in the anterior mesendoderm and anterior neural plate at 7.5 dpc in a broader domain than *Hesx1* (Merrill et al., 2004). Therefore, the genetic interaction between *Hesx1* and Tcf3 might occur at the anterior mesendoderm and/or the anterior neural plate with weak expression in the anterior mesendoderm (Dorsky et al., 2003; Kim et al., 2000; Spieler et al., 2004). Since, in zebrafish, *tcf3*-mediated repression of Wnt/β-catenin targets is required for normal brain development (Dorsky et al., 2003; Kim et al., 2000), we reasoned that if a derepression of Wnt targets is required for normal brain development, this should be evidenced by anteriorisation of targets activated by Wnt expression. The direct Wnt/β-catenin/TCF/LEF target *Sp5* (Weidinger et al., 2005) is normally expressed in the midbrain but is absent from the anterior forebrain (Fig. 3C). By contrast, the *Sp5* expression domain was rostrally expanded in the anterior forebrain of *Hesx1*; *Tcf3*–/– embryos (n=4; Fig. 3D). Expression of *Six3*, an essential repressor during normal forebrain development, is negatively regulated by the Wnt signalling pathway (Braun et al., 2003; Lagutin et al., 2003). In agreement with this notion and the ectopic expression of *Sp5* in the anterior forebrain, the *Six3* expression domain in the anterior neural plate was markedly reduced in *Hesx1*; *Tcf3*–/– embryos at the 5- to 8-somite stage (Fig. 3B, B'). Often, *Six3* expression was asymmetric in *Hesx1*; *Tcf3*–/– embryos (Fig. 3B). It is important to note that in the anterior forebrain of *Hesx1*–/– mutants, *Sp5* is ectopically expressed, whereas the *Six3* expression domain is reduced and asymmetric defects are also observed (Andoniadou et al., 2007; Dattani et al., 1998).

In summary, three main conclusions can be drawn from these results: (1) in mouse, Tcf3 is required in anterior forebrain precursors, where it prevents the ectopic expression of Wnt/β-catenin targets; (2) there is a genetic interaction between *Hesx1* and Tcf3 in the anterior neuroectoderm; and (3) the similar molecular and morphological defects observed in *Hesx1*; *Hesx1*; *Tcf3*–/– and *Hesx1*; *Tcf3*–/– embryos suggest that both factors synergise in a gene dosage-dependent manner to maintain the anterior character of anterior forebrain progenitors.

**Conditional removal of β-catenin partially rescues the anterior forebrain defects of *Hesx1* null mutants**

The genetic analyses described above on *Hesx1*-deficient and *Hesx1*Tcf3 compound embryos suggest that the posteriorisation of anterior forebrain precursors leading to anterior truncations is driven by the derepression of Wnt/β-catenin targets and the ectopic activation of the pathway in the anterior neural plate. To test this
Fig. 3. Tcf3 is required within the anterior neuroectoderm for normal mouse forebrain development. (A-D) In situ hybridisation with Six3 and Sp5 antisense riboprobes on 8.5 dpc Hesx1\textsuperscript{Cre+};Tcf3\textsuperscript{loxP} (B, B') and control Hesx1\textsuperscript{Cre+};Tcf3\textsuperscript{loxP} (A, A') embryos. Expression of Six3, a marker of the anterior forebrain primordium in 8.5 dpc embryos, is reduced in the Hesx1\textsuperscript{Cre+};Tcf3\textsuperscript{loxP} mutant compared with the control. By contrast, the expression domain of Sp5, a direct Wnt/β-catenin target gene, is rostrally expanded into the prospecitive forebrain region of the Hesx1\textsuperscript{Cre+};Tcf3\textsuperscript{loxP} mutant (arrow in D) as compared with a control embryo, which does not express Sp5 in the prospective forebrain (arrow in C). The arrowheads in C and D denote Sp5 expression in the midbrain (black) and tailbud (white). (E-J) In situ hybridisation with Fgf8, Foxg1 and Pax6 antisense riboprobes on 9.5 dpc Hesx1\textsuperscript{Cre+};Tcf3\textsuperscript{loxP} embryos with severe (class IV) or mild (class I-III) forebrain defects. Stage-matched wild-type controls are shown in Fig. 2A-C. (E, E') Fgf8 expression at the ANR is severely reduced (arrow in E), with only a minimal domain of expression remaining (arrow in E'); however, expression at the MHB remains normal (arrowhead in E). In an embryo with severe anterior truncation (F), Foxg1 expression, which is normally at the telencephalic vesicles, is lost. Similarly, the anterior Pax6 expression domain is almost absent due to the loss of forebrain tissue and only a small patch of Pax6-positive cells, probably corresponding to posterior forebrain, is detectable (arrow in G). In mildly affected Hesx1\textsuperscript{Cre+};Tcf3\textsuperscript{loxP} mutants, the Fgf8 expression domain at the ANR is reduced and restricted to the midline (arrows in H, H'), but MHB expression is unaffected (arrowhead in H). Foxg1 expression in the telencephalon is severely reduced in a mildly affected Hesx1\textsuperscript{Cre+};Tcf3\textsuperscript{loxP} embryo (arrow in I). In these embryos, Pax6 expression in the telencephalon and eye is decreased but expression in the posterior forebrain is normal (arrowheads in J).

hypothesis further, we used the BATgal transgenic mouse line, which provides a lacZ reporter for active β-catenin/TCF/LEF signalling (Maretto et al., 2003). X-Gal staining of Hesx1\textsuperscript{Cre+};BATgal and BATgal embryos revealed no significant differences in the β-galactosidase staining pattern and the anterior forebrain remained unstained, demonstrating the low or absent signalling mediated by β-catenin/TCF/LEF in the anterior region of the neural plate (Fig. 4A, A'; data not shown). By contrast, Hesx1\textsuperscript{Cre-};BATgal mutant embryos displayed a clear aniorisation of X-Gal staining into the rostral neural plate, relative to controls (Fig. 4B, B'). This ectopic β-galactosidase expression in the absence of Hesx1 strongly suggests that there is an activation of Wnt/β-catenin signalling in the anterior forebrain of these mutants.

We reasoned that downregulation of this signalling pathway would result in the improvement of the defective patterning and development of the anterior neural plate in Hesx1-deficient mutants. We used a genetic approach to reduce the levels of β-catenin (Ctnnb1) expression, and therefore ameliorate the levels of Wnt/β-catenin signalling in Hesx1-deficient mutants. Previously described Hesx1\textsuperscript{+/–} and Ctnnb1\textsuperscript{+/–} strains, each carrying a null allele, are both viable and fertile (Dattani et al., 1998; Haegel et al., 1995). We generated Hesx1\textsuperscript{+/–};Ctnnb1\textsuperscript{+/–} compound mice, which were also normal and fertile. By crossing these animals to Hesx1\textsuperscript{Cre+};Ctnnb1\textsuperscript{LOF/–}, we generated Hesx1\textsuperscript{Cre+};Ctnnb1\textsuperscript{LOF/–} and control Hesx1\textsuperscript{Cre+};Ctnnb1\textsuperscript{+/–} embryos. Morphological comparisons did not reveal significant differences in the severity of anterior forebrain defects between these two genotypes (supplementary material Table S1; n=181 embryos), and haploinsufficiency of Ctnnb1 did not restore forebrain development in the Hesx1\textsuperscript{Cre+};Ctnnb1\textsuperscript{LOF/–} mutants relative to Hesx1\textsuperscript{Cre+};Ctnnb1\textsuperscript{+/–} controls.

To remove both copies of β-catenin, we used a conditional approach resulting in the specific deletion of Ctnnb1 only in Hesx1-expressing cells. By crossing the Hesx1-Cre driver with a Ctnnb1 loss-of-function strain in which exons 2-6 are flanked by loxP sites [Ctnnb1-lox(ex2-6)], hereafter Ctnnb1-LOF (Brault et al., 2001), we overcame the gastrulation defect of Ctnnb1–/– embryos (Haegel et al., 1995). We generated embryos completely lacking Hesx1 and conditionally null for Ctnnb1 in the forebrain (Hesx1\textsuperscript{Cre+};Ctnnb1\textsuperscript{LOF/–}) through crossing Hesx1\textsuperscript{Cre+};Ctnnb1\textsuperscript{LOF/–} with Hesx1\textsuperscript{Cre+};Ctnnb1\textsuperscript{LOF/–} animals (supplementary material Fig.
S5). Analysis of Hesx1Cre;Ctnnb1LOF/− embryos between 9.5 dpc and 15.5 dpc demonstrated a shift in the range of forebrain defects to a milder spectrum (ranging from normal to class II, Table 4; and 15.5 dpc demonstrated a shift in the range of forebrain defects.

Our results demonstrate that conditional removal of Ctnnb1 in the anterior neural plate leads to the ectopic activation of multiple Wnt/β-catenin target genes in anterior forebrain progenitors. Our results demonstrate that conditional removal of Ctnnb1 in the anterior forebrain leading to a reduction of Wnt/β-catenin signalling is sufficient to improve forebrain patterning in Hesx1-deficient mutants, strongly suggesting that the mechanisms underlying the forebrain defects in Hesx1-deficient embryos are mediated by the ectopic activation of Wnt/β-catenin signalling in the anterior neural plate. We investigated this hypothesis further by performing gene profiling analyses, comparing anterior forebrain precursors expressing and not

Role of Hesx1 and Tcf3 in forebrain development

**Fig. 4.** Loss of function of β-catenin is sufficient to improve forebrain patterning in mouse Hesx1Cre;Ctnnb1LOF/− embryos. (A,A’) X-Gal staining reveals BATgal activity in the neural plate of Hesx1Cre;BATgal control embryo at 8.5 dpc, but the anteriormost forebrain is not stained (arrows). (B,B’) By contrast, in the Hesx1Cre mutant, the anterior forebrain is BATgal positive, suggesting ectopic activation of the Wnt/β-catenin signalling pathway (arrows). (C-E) The conditional inactivation of β-catenin in a Hesx1Cre;Ctnnb1LOF/− embryo (D) leads to a significant improvement of telencephalic (arrowheads) and eye (arrow indicating the presence of an optic vesicle) development compared with a Hesx1Cre;Ctnnb1LOF/− embryo (E). However, compared with a wild-type control embryo (C), this is not a full restoration to normal development. (F-H) Frontal views of embryos after in situ hybridisation with antisense riboprobes against Fgf8. Normal expression at the ANR of a wild-type embryo at 3-5 somites (arrows in f). In Hesx1Cre mutants, the expression of Fgf8 at the ANR is reduced and restricted to the midline (arrowheads in H). In the Hesx1Cre;Ctnnb1LOF/− embryo (G) there is an asymmetric expansion in Fgf8 expression compared with the homozygous Hesx1 mutant (H).

This phenotypic rescue of forebrain development was supported by an overall improvement of anterior neural plate patterning in Hesx1Cre;Ctnnb1LOF/− embryos. The Fgf8 expression domain in the ANR of Hesx1Cre/− mutants is smaller than in the control embryo (I). Expression of the Wnt/β-catenin direct target gene Sp5 is normally excluded from the anterior forebrain (arrow in M), and it remains so in the Hesx1Cre;Ctnnb1LOF/− embryo (arrow in N). However, ectopic Sp5 expression is detected in the anterior forebrain of Hesx1Cre;Ctnnb1+/− and Hesx1Cre/− mutants (arrowheads in O,P).
expressing Hesx1 at the 3- to 5-somite stage, just when the first morphological and molecular abnormalities are detectable in Hesx1-deficient mutants. Because the Hesx1 expression domain is very restricted at this stage, we generated a Hesx1-eGFP knock-in mouse line by replacing the Hesx1 coding region with eGFP as a tool to flow sort this small population of forebrain progenitors (Fig. 5A). This enabled us to avoid the contamination from Hesx1 non-expressing cells that would be present if performing this analysis using whole embryos or micro-dissected regions.

Hesx1<sup>eGFP/+</sup> mice were normal and fertile and embryos showed fluorescence in the anterior neural plate (Fig. 5C) in a pattern identical to endogenous Hesx1 expression. By contrast, Hesx1<sup>eGFP/eGFP</sup> embryos (Fig. 5D) showed the anterior forebrain defects observed in Hesx1-deficient embryos and the pattern of fluorescence was faithful to the residual anterior forebrain domain in these mutants, which is restricted to the most anteromedial region of the neural plate and is marked by Six3 expression (Martinez-Barbera et al., 2000).

### Table 4. Genotypes of embryos from Hesx1Cre<sup>+/+</sup>;Ctnnb1<sup>LOF/+</sup> × Hesx1<sup>+/+</sup>;Ctnnb1<sup>LOF/+</sup> crosses, classified according to severity of anterior defects

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<th>Class of forebrain defects</th>
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*Only relevant genotypes are shown. For a full list of genotypes, see supplementary material Table S2.

‡See Table 2 for class definitions.

**Fig. 5.** Purification of anterior forebrain precursors by flow sorting from Hesx1<sup>eGFP/+</sup> and Hesx1<sup>eGFP/eGFP</sup> mouse embryos. (A) Targeting strategy for the generation of the Hesx1-eGFP allele. Top to bottom: structure of the murine Hesx1 locus; Hesx1-eGFP targeting vector; targeted allele prior to and after flipase-mediated excision of the Neo cassette; expected bands for the targeted and wild-type alleles after Southern blot analysis of DNA samples digested with EcoRI and hybridised with an external probe (red line in top schematic). The DTA cassette is irrelevant to this study as it has not been activated in the presence of Cre in the experiments presented here. (B) Southern blot analysis of wild-type (Hesx1<sup>+/+</sup>) and Hesx1<sup>eGFP/+</sup> ES cell clones digested with EcoRI and hybridised with an external probe (red line in A). Only the 4.3 kb wild-type band is detected in the Hesx1<sup>+/+</sup> sample, whereas both wild-type and mutant (3.9 kb) bands are detected in three correctly targeted Hesx1<sup>eGFP/+</sup> clones. (C) Dorsal view of the neural plate of a 3-somite stage Hesx1<sup>eGFP/+</sup> embryo showing eGFP fluorescence in the anterior forebrain primordium during normal development (arrowheads). (D) In the Hesx1<sup>eGFP/eGFP</sup> embryo, in which there is no Hesx1 expression, the anterior forebrain domain marked by eGFP fluorescence becomes medially restricted (arrowheads). (E) Flow sorting of dissociated whole embryos between 3 and 5 somites allows the specific isolation of cells from the prospective anterior forebrain through eGFP fluorescence from the Hesx1 locus. Scatter plots from a representative experiment are shown. Purified cells from heterozygous Hesx1<sup>eGFP/+</sup> (normal) or homozygous Hesx1<sup>eGFP/eGFP</sup> mutant embryos were used for RNA isolation and subsequent microarray analysis.
Microarray analysis of anterior forebrain precursors isolated by flow sorting from Hesx1eGFP/+ and Hesx1eGFP/eGFP 3- to 5-somite embryos (Fig. 5) confirmed changes in gene expression that we had previously characterised by wholemount in situ hybridisation on Hesx1 null mutants (Andoniadou et al., 2007). For example, by wholemount in situ hybridisation, Foxg1 and Pax6 expression domains were reduced in the Hesx1+/- mutant anterior forebrain compared with Hesx1+/+ or Hesx1 +/- controls. In the microarray (full data can be found at ArrayExpress with ID: E-MEXP-2586), levels of Foxg1 and Pax6 expression were found to be 1.86-fold and 1.81-fold higher, respectively, in the Hesx1+/-/+ heterozygous relative to the Hesx1+/-/+ homozygous anterior forebrain precursors. Conversely, the expression domains of genes that were expressed at significantly lower levels (Table 5). This is line with previous research suggesting that HESX1 normally functions as a transcriptional repressor (Carvalho et al., 2010; Dasen et al., 2001; Ermakova et al., 1999; Ermakova et al., 2007). Validation by qRT-PCR on independent samples was carried out for a selection of genes from this shortlist and all 19 genes queried displayed the same trend observed in the microarray (supplementary material Fig. S6). Among the upregulated genes, a significant proportion (20%) were associated with the Wnt pathway, including Tnfrsf19, Dixo1, Sp5, Apcdd1 and Tnik. A further 46% were associated with processes positively regulated by enhancement of Wnt signalling, namely neural crest specification (16%; e.g. Sox10, Twist1, Ednra, Ednrb) and neural differentiation (30%; Neurog1, Neuro, Mef2c, Nr2f1, Nrp2).

Certain genes that are known to be upregulated in the forebrain of the Hesx1-deficient embryos, as judged by in situ hybridisation, e.g. the Wnt target Axin2 (Andoniadou et al., 2007), did not pass the strict criteria that we set for statistically significant differences so we also analysed all genes by their Gene Ontology annotations and grouped them by involvement in specific pathways, with differences of 1.6-fold as cut-off. Key components, main effectors and downstream target genes of the major morphogenetic pathways dependent on SHH, FGF or BMP/TGFβ were largely unaffected (supplementary material Tables S3-S5). By contrast, multiple components of the canonical Wnt signalling pathway were upregulated in Hesx1eGFP/+ cells. When subdivided into receptors, ligands, intracellular components, negative regulators and target genes, it became clear that a large number of Wnt targets were expressed at higher levels in the absence of the repressor HESX1 (15 out of 37 genes queried, over 1.6-fold upregulated; supplementary material Table S6). The levels of Wnt receptors and

### Table 5. Genes expressed at significantly different levels in pairwise microarray comparison of Hesx1eGFP/+ and Hesx1eGFP/eGFP anterior forebrain cells

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Positive value denotes higher expression in Hesx1eGFP/+ and negative value denotes higher expression in Hesx1eGFP/+. Known targets, components and regulators of the Wnt pathway are in bold.
Wnt ligands remained unchanged, except for an increase in the expression of Wnt1, Wnt3a and Wnt6 (Table 5; supplementary material Table S7). Notably, expression of Cmnb1 was unaffected in Hesx1eGFP/eGFP cells, as was the expression of genes encoding components of the destruction complex, such as Axin1, Gsk3b, Csk1a1 and Apc, demonstrating that the pathway was not affected at the level of regulation of Cmnb1 expression or of components affecting degradation of β-catenin protein (supplementary material Table S8).

Together, this gene expression analysis demonstrates that the absence of HESX1 leads to the ectopic activation of numerous Wnt target genes in anterior forebrain progenitors.

**DISCUSSION**

In this study, we provide novel genetic and molecular data demonstrating that the homeobox gene Hesx1 antagonises the activation of Wnt/β-catenin signalling in early forebrain progenitors in zebrafish and mouse embryos. In addition, using genetic approaches we reveal a previously unidentified requirement for Tcf3 in these progenitors, where it interacts with Hesx1 to promote anterior character.

**Novel function of hesx1 in zebrafish forebrain development**

Hesx1 has previously been shown to play an essential role during normal forebrain and pituitary development in Xenopus, mice and humans. Here, we show that hesx1 zebrafish morphants display forebrain defects in the sensitised hdl background that are reminiscent of those observed in Hesx1-deficient mouse embryos. These defects are rescued by injection of murine Hesx1 mRNA, suggesting a functional conservation in both species. Significant amino acid sequence homology between Hesx1 proteins of zebrafish and mouse is restricted to the homeobox (DNA-binding domain) at the C-terminus and the engrailed homology 1 (eh1) domain at the N-terminus, which interacts with Groucho/TLE members to mediate transcriptional repression (Carvalho et al., 2010; Dasen et al., 2001). This suggests that the main molecular function of Hesx1/Anf family members is to act as transcriptional repressors.

Members of the TCF/LEF family, including TCF3, activate transcription of Wnt target genes upon association with β-catenin. They are also able to repress genes through association with Groucho/TLE1 co-repressors, an interaction that displaces their association with β-catenin. In the zebrafish embryo, loss of Tcf3a repressor activity was shown to be solely responsible for the anterior truncations displayed in the hdl mutants. Injection of mRNA encoding a truncated form of Tcf3a that lacks the N-terminal β-catenin-interacting domain was capable of rescuing the mutant phenotype through its repressor function. Furthermore, overexpression of the DNA-binding domain fused to the engrailed repressor domain was also able to rescue the hdl anterior defects; however, when fused to the VP16 activator domain it not only failed to rescue this phenotype but also induced forebrain truncations in wild-type embryos (Kim et al., 2000).

Together, these experiments and our MO injections on a sensitised hdl background suggest that there is a functional interaction between Hesx1 and Tcf3a factors that prevents the expression of Wnt targets in the zebrafish embryo. Therefore, the lack of a phenotype in the hesx1 zebrafish morphant could be a consequence of genetic redundancy, whereby Tcf3 factors compensate for the lack of Hesx1.

**Tcf3 and Hesx1 genetically interact in the zebrafish and mouse embryo to antagonise Wnt/β-catenin signalling activation in anterior forebrain progenitors**

In the zebrafish embryo, Tcf3 acts independently of β-catenin for normal forebrain development by maintaining the repression of Wnt/β-catenin target genes. Our data extend this analysis and provide evidence that Tcf3 plays this role specifically within the forebrain neuroectoderm. Compound embryos carrying a distinct gene dosage of Hesx1 and Tcf3 show telencephalic and eye defects that are similar to those observed in single-mutant embryos deficient for either Hesx1 or Tcf3. This genetic interaction suggests a requirement not only for TCF3 but also for HESX1 in the inhibition of Wnt/β-catenin target expression in anterior forebrain precursors. We cannot rule out a weak interaction in the axial mesendoderm, but our Dkk1 and Shh analyses suggest that this tissue is unlikely to be affected.

Confirming a novel function of HESX1 in antagonising Wnt signalling, we show that the Hesx1Cre/+ forebrain defects are partially rescued and forebrain patterning improved in Hesx1Cre-/Cmnb1LOF/– embryos, in which aberrant Wnt signalling is prevented specifically in anterior forebrain precursors. This illustrates that the forebrain abnormalities in Hesx1-deficient mutants are indeed caused by an ectopic response to this pathway. Deletion of Cmnb1 in Foxg1-Cre;Cmnb1LOF/– embryos has previously been shown to cause forebrain defects (Junghans et al., 2005; Wang et al., 2010), which result from an increase in apoptosis following disruption of structural integrity due to the absence of β-catenin in the adherence junctions of neuroepithelial cells (Junghans et al., 2005) as well as the loss of Fgf8 expression at the ANR (Paek et al., 2011; Wang et al., 2010). By contrast, we did not observe any forebrain phenotype in Hesx1Cre+/Cmnb1LOF/– embryos. This is possibly due to the different expression patterns of Foxg1 and Hesx1, or is a potential additive effect due to Foxg1 haploinsufficiency in the Foxg1-Cre knock-in line used, as even heterozygous animals have telencephalic defects (Eagleson et al., 2007).

Analysis of double-heterozygous embryos for Hesx1 and another Wnt antagonist, Six3, have also revealed forebrain defects comparable to those of Hesx1;Tcf3 compound mutant embryos (supplementary material Fig. S7) (Gaston-Massuet et al., 2008). Similar to the demonstration that Hesx1 can antagonise Wnt signalling in the zebrafish forebrain, mice Six3 mRNA is able to rescue the hdl phenotype through antagonising Wnt signalling (Lagutin et al., 2003).

Finally, gene profiling analysis revealed a significant enhancement in the expression of genes relevant to Wnt signalling in the Hesx1-deficient anterior forebrain precursors relative to Hesx1LOF/+ heterozygous controls. The increase in expression of several target genes of the Wnt/β-catenin pathway in the Hesx1LOF/+ forebrain cells demonstrates the ectopic activation of this pathway in the absence of Hesx1 in a tissue that would normally be unresponsive to Wnt signals. Indeed, in the Hesx1LOF/+ population, we observe an increase in the Wnt effector genes Lef1 (1.7-fold) and Tcf1 (1.57, 1.6-fold), which are not normally expressed in the anterior forebrain. The ectopic expression of Sp5 in the forebrain of Hesx1Cre+/Tcf3LOF/– embryos leads to the notion of a similar underlying defect in these mutants. This supports the notion that Hesx1 may act, in concert with Tcf3 and Six3, as a negative regulator of the Wnt pathway in the anterior forebrain, furthering our understanding of the mechanisms required to establish forebrain identity. In addition, the microarray data
provide a valuable resource because they define the normal molecular signature of early anterior forebrain precursors. This information can be used for comparative studies with other mouse mutants or to assess the efficiency of protocols for in vitro differentiation of stem cell lines into neurons.

The variability in the forebrain defects observed in compound mutants also exists in human patients with mutations in *HESX1*. To date, more than 15 mutations have been identified in human *HESX1* in association with variable degrees of forebrain and pituitary defects (Kelberman et al., 2009). Our mouse rescue opens the possibility for mutations in genes with synergistic action, such as *TCF3*, to be candidates for modifying these phenotypes.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


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Role of Hesx1 and Tcf3 in forebrain development

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