Context-specific function of the LIM homeobox 1 transcription factor in head formation of the mouse embryo

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ABSTRACT

Lhx1 encodes a LIM homeobox transcription factor that is expressed in the primitive streak, mesoderm and anterior mesendoderm of the mouse embryo. Using a conditional Lhx1 flox and three different Cre deleters, we demonstrated that LHX1 is required in the anterior mesendoderm, but not in the mesoderm, for formation of the head. LHX1 enables the morphogenetic movement of cells that accompanies the formation of the anterior mesendoderm, in part through regulation of Pcdh7 expression. LHX1 also regulates, in the anterior mesendoderm, the transcription of genes encoding negative regulators of WNT signalling, such as Dkk1, Hesx1, Cer1 and Gsc. Embryos carrying mutations in Pcdh7, generated using CRISPR-Cas9 technology, and embryos without Lhx1 function specifically in the anterior mesendoderm displayed head defects that partially phenocopied the truncation defects of Lhx1-null mutants. Therefore, disruption of Lhx1-dependent movement of the anterior mesendoderm cells and failure to modulate WNT signalling both resulted in the truncation of head structures. Compound mutants of Lhx1, Dkk1 and Cntnb1 show an enhanced head truncation phenotype, pointing to a functional link between LHX1 transcriptional activity and the regulation of WNT signalling. Collectively, these results provide comprehensive insight into the context-specific function of LHX1 in head formation: LHX1 enables the formation of the anterior mesendoderm that is instrumental for mediating the inductive interaction with the anterior neuroectoderm and LHX1 also regulates the expression of factors in the signalling cascade that modulate the level of WNT activity.

KEY WORDS: Head formation, Anterior mesendoderm, LHX1, Transcription factor, Protocadherin, WNT signalling, CRISPR

INTRODUCTION

WNT activity is important for regulating cell proliferation, differentiation and polarity (van Amerongen and Nusse, 2009), and for the anterior-posterior patterning of vertebrate embryos (Kiecker and Niehrs, 2001; Petersen and Reddien, 2009; Fukase and Sokol, 2013). In the mouse, formation of the embryonic head requires stringent control of the level of WNT signalling during early postimplantation development (Fossat et al., 2011b, 2012; Arkell et al., 2013). Elevated WNT signalling is incompatible with anterior development. In Six3-deficient mutants, ectopic expression of Wnt1 is associated with the truncation of forebrain structures (Lagutin et al., 2003). In embryos lacking Dkk1, which encodes a secreted WNT antagonist, the region of the head rostral to the midbrain is truncated (Mukhopadhyay et al., 2001). DKK1 forms a complex with LRP6-KREMEN1, which sequesters the WNT co-receptor LRP6, preventing the formation of a functional frizzled-LRP receptor complex for signal transduction via β-catenin, thereby blocking WNT signalling (Zorn, 2001). Point mutations of Lrp6 and Ctnnb1 (which encodes β-catenin), which result in gain of function of the co-receptor and the transducer, respectively, are associated with head truncation (Fossat et al., 2011b). The interaction between Dkk1, Lrp6 and Cntnb1 mutations (Fossat et al., 2011b) points to a crucial role of WNT/β-catenin signalling in head development and that elevated WNT activity underpins the failure to form the embryonic head.

Consistent with the concept that suppression of WNT signalling activity is required for head morphogenesis, the expression of several WNT inhibitors (Dkk1, Cer1, Sfrp1 and Sfrp5) is regionalised to anterior germ layer tissues, whereas the expression of ligands (Wnt3, Wnt3a and Wnt5) is principally in posterior tissues (Mukhopadhyay et al., 2003; Kemp et al., 2005; Petersen and Reddien, 2009). Before gastrulation, cells of the extra-embryonic anterior visceral endoderm (AVE) express WNT inhibitors (Pfister et al., 2007). During gastrulation, the AVE is replaced by a mixed population of cells called the anterior mesendoderm (AME) that comprises the axial mesendoderm and the definitive endoderm. The AME, like the AVE, produces WNT inhibitors and is instrumental in inducing and maintaining the anterior characteristics of the neuroectoderm during the morphogenesis of head structures (Camus et al., 2000).

Lhx1 (also known as Lim1) encodes a LIM homeobox transcription factor. Loss of Lhx1 function results in head truncation (Shawlot and Behringer, 1995) that phenocopies loss of Dkk1 function. Chimera studies show that normal head development requires Lhx1 function in both the AVE and the epiblast (Shawlot et al., 1999). Cre-mediated ablation of Lhx1 specifically in epiblast-derived tissues also produces a head truncation phenotype (Kwan and Behringer, 2002; Tanaka et al., 2010), but the developmental process underpinning the mutant phenotype has not been investigated and it is not known whether Lhx1 is required specifically in either the mesoderm or the AME, which are both derived from the epiblast. LHX1 forms a complex with LDB1 and SSBP3 (also known as SSDP1) that regulates the expression of target genes (Hobert and Westphal, 2000; Enkhmandakh et al., 2006). Like Lhx1−/− embryos, Ldb1−/− and Ssbp3−/− embryos also display truncation of head structures.
In Ldb1 mutants, the expression of genes that encode WNT inhibitors (Frzb, Sfrp1, Sfrp2, Cer1 and Dkk1) is reduced; and in Ssbp3 mutants, Dkk1 expression is lost from the prechordal plate (Mukhopadhyay et al., 2003; Nishioka et al., 2005). These findings suggest that elevated WNT signalling might be contributing to the truncation of anterior structures in embryos that have lost Lhx1-related function.

In the present study, we show that loss of Lhx1 activity in the epiblast causes head truncation. Before gastrulation, Lhx1 is expressed in the extra-embryonic AVE (supplementary material Fig. S1). During gastrulation, Lhx1 expression is initially detected in the proximal-posterior epiblast where the primitive streak will form, and subsequently in the nascent mesoderm and the AME (supplementary material Fig. S1). Lhx1 expression is not detected in the ectoderm (supplementary material Fig. S1).

In mid-streak stage mutant embryos, Lhx1 expression was maintained in the AVE (Fig. 1A) but was drastically reduced in the mesoderm. In early-bud stage Lhx1-epiCKO embryos, Lhx1 expression was completely lost from the mesoderm and the AME (supplementary material Fig. S1). Lhx1 expression is not detected in the ectoderm (supplementary material Fig. S1).

The head truncation phenotype of E9.5 Lhx1-epiCKO embryos. All panels show a lateral view with anterior to the left. ant, anterior; pos, posterior; prx, proximal; dis, distal; AVE, anterior visceral endoderm; M, mesoderm; PS, primitive streak; AME, anterior mesendoderm. Scale bars: 100 µm.

RESULTS

Loss of Lhx1 activity in the epiblast causes head truncation

Before gastrulation, Lhx1 is expressed in the extra-embryonic AVE (supplementary material Fig. S1). During gastrulation, Lhx1 expression is initially detected in the proximal-posterior epiblast where the primitive streak will form, and subsequently in the nascent mesoderm and the AME (supplementary material Fig. S1). Lhx1 expression is not detected in the ectoderm (supplementary material Fig. S1).

To investigate the tissue-specific requirement of Lhx1 in the epiblast and its derivatives, we studied the phenotype of embryos in which Lhx1 activity was ablated in the epiblast by Meox2-Cre activity (Lhx1flox/−;Meox2+/Cre, hereafter Lhx1-epiCKO embryo). In mid-streak stage mutant embryos, Lhx1 expression was maintained in the AVE (Fig. 1A) but was drastically reduced in the mesoderm. In early-bud stage Lhx1-epiCKO embryos, Lhx1 expression was completely lost from the mesoderm and the AME (Fig. 1A). At gastrulation, Lhx1-epiCKO embryos were similar in size and morphology to controls (Fig. 1A) and formed a primitive streak (that expressed T; Fig. 1B). However, little or no expression of Otx2 was detected in the anterior ectoderm (Fig. 1B), indicating a loss of anterior neural tissue potency (Iwafuchi-Doi et al., 2012). Marker analysis of E8.5 Lhx1-epiCKO embryos revealed the loss
of the precursor of the dorsal and ventral forebrain (indicated by Six3, Hesx1, Fgf8 expression), the midbrain (En1) and the midhindbrain junction (En1, Fgf8), whereas that of the hindbrain [Gbx2, Krox20 (also known as Egr2)] was relatively intact (Fig. 1C). At E9.5, the Lhx1-epiCKO embryos displayed a fully penetrant head truncation phenotype (Fig. 1D; supplementary material Table S1).

**Loss of Lhx1 disrupts the formation of anterior midline tissues**

In the anterior region of Lhx1-epiCKO embryos at E7.5-E7.75, before the manifestation of the abnormal head phenotype, the expression of Dkk1, Hesx1, Cer1 and Gsc was lost or reduced (Fig. 2A). Although cells expressing Nog, Chrd and Foxa2 were present in the node and in the midline tissue immediately anterior to
the node of the gastrula stage embryo, *Foxa2* and *Shh* expression was missing from the rostral tissues of the early-somite stage embryo (Fig. 2A). No *Shh*-expressing cells were present in the midline beneath the floor plate of the neural folds (Fig. 2Ai-iv). These findings suggest that *Lhx1*-deficient cells might be unable to form the AME.

To further investigate this possibility, we labelled the endoderm overlying the anterior segment of the primitive streak of the mid-streak stage embryo, where the precursors of the AME initially reside following recruitment from the epiblast (Tam et al., 2007) (Fig. 2B). After 24 h of *in vitro* culture, the labelled cells in control embryos (*n* = 8) were distributed along the full extent of the anterior-posterior axis, whereas their counterparts in *Lhx1*-epiCKO embryos (*n* = 7) failed to extend anteriorly but remained in the posterior region of the embryo (Fig. 2B). During gastrulation, the axial mesendoderm and the definitive endoderm are formed by recruiting *Foxa2*-expressing cells from the epiblast, which then transit through the primitive streak and expand after integration into the endoderm layer to populate the AME (Fig. 2C; *n* = 5). The absence of *Shh* and *Foxa2* expressing cells that were transfected with constructs encoding *LHX1* alone or *LHX1*Δ on both alleles of *Lhx1*−/− embryos (Fig. S2A) suggested that this defect might be a consequence of the impaired morphogenetic movement of the *Lhx1*-deficient AME progenitor cells.

In *Xenopus* embryos, knockdown of *Xlim1* (the *Lhx1* orthologue) leads to a headless phenotype that is accompanied by a lack of anterior extension of the chordamesoderm (the equivalent of the axial mesendoderm of the mouse). This morphogenetic defect can be rescued by expressing *XPapc*, a protocadherin in the planar cell polarity pathway, which promotes anterior extension of the *Xlim1*-deficient chordamesoderm tissue (Hukriede et al., 2003). In the mouse embryo, *Pcdh8* (the orthologue of *XPapc*) is expressed only in the mesoderm (supplementary material Fig. S2C) (Hukriede et al., 2003) and, although it is downregulated in the *Lhx1*-null mutant (Hukriede et al., 2003), loss of *Pcdh8* function has no impact on head development (Yamamoto et al., 2000). We therefore examined the possibility that, in mouse, *LHX1* controls the expression of other protocadherins that may be required for head formation.

*Pcdh8* belongs to the *Pcdh* family, which has nine members (Morishita and Yagi, 2007). It also has two putative paralogues, *Pcdh12* and *Pcdh20* (Fliche et al., 2013). Expression of all of these *Pcdh* genes was detectable by RT-PCR in E7.75 mouse embryos (supplementary material Fig. S2A). In P19 embryonal carcinoma cells that were transfected with constructs encoding *LHX1* alone or in combination with the two co-factors, LDB1 and SSBP3, only *Pcdh7*, *Pcdh8* and *Pcdh19* were activated by *LHX1* or *LHX1*Δ as compared with control transfections (Fig. 3A; supplementary material Fig. S2B).

We next investigated the expression of these *Pcdh* genes in embryos. During gastrulation, *Pcdh7* is expressed, like *Lhx1*, in the mesoderm and the AME (Fig. 3B), but *Pcdh19* and *Pcdh8* are expressed only in the mesoderm (supplementary material Fig. S2C). Focusing on *Pcdh7*, we found that its expression was reduced in *Lhx1*-epiCKO embryos (Fig. 3C). We identified two regions in the *Pcdh7* locus that are conserved between mouse and human and contain *LHX1* recognition motifs (YTAATNN; where Y is C or T and NN is TA, TG, CA, GG or GA) (Mochizuki et al., 2000; Sudou et al., 2012; Yasuoka et al., 2014); they were localised −0.2 kb upstream (−0.2R) and −8.5 kb downstream (+8.5R) of the *Pcdh7* START codon (Fig. 3D; supplementary material Fig. S3).

Chromatin immunoprecipitation (ChIP)-qPCR analysis was performed with an anti-HA antibody on P19 cells that were transfected with a plasmid expressing HA-tagged wild-type (*Lhx1*-HA) or HA-tagged mutant (*LHX1A-HA, lacking DNA- and protein-binding domains) *LHX1* protein. Both −0.2R and +8.5R regions were bound by *LHX1* but not *LHX1A* (Fig. 3E). Furthermore, both regions could mediate the activation of a luciferase reporter by *LHX1*, but not *LHX1A* (Fig. 3F).

We then tested the functional requirement for *Pcdh7* in embryonic development. Using CRISPR-Cas9 editing (San et al., 2013), two independent embryonic stem cell (ESC) clones, each harbouring frameshift mutations immediately downstream of the START codon on both alleles of *Pcdh7* (supplementary material Fig. S4), were generated. Chimeric embryos were produced by introducing these ESC clones into 8-cell host embryos expressing a *Rosa26-lacZ* transgene, which allowed an unequivocal assessment of the contribution of the *lacZ*-negative ESCs to the embryo proper (see Materials and Methods; Fig. 3G). E9.5 chimeras that were composed almost entirely of mutant ESCs displayed a reduced forebrain and an open neural tube (Fig. 3G; *Pcdh7*−/−/− *n* = 3, *Pcdh7*−/−/− *n* = 4). This phenotype was not observed in control chimeras generated with the parental ESC line (Fig. 3G; wild type *n* = 3). In 7/>9 E7.75 embryos generated with the mutant ESCs, a shorter *Shh*-positive midline structure was formed than in stage-matched control embryos (*n* = 5) (Fig. 3H). Altogether, these results suggest that the reduction of *Pcdh7* expression in *Lhx1*-epiCKO embryos might contribute to the defect in AME formation and the head truncation phenotype.

### Lhx1 function is required in the AME for head formation

In gastrula stage embryos, *Lhx1* is expressed in the mesoderm and the AME (supplementary material Fig. S1). To study the requirement for *Lhx1* in these two tissues, we generated mutant embryos in which *Lhx1* was ablated either in the mesoderm or the AME. To inactivate *Lhx1* flox in the mesoderm, we used a Cre recombinase that is expressed from the *Mesp1* locus (Saga et al., 1999). Mesoderm cells that express *Mesp1-Cre* have been shown to contribute extensively to the cranial mesenchyme (Saga et al., 1999; Bildsoe et al., 2013). In the mesodermal conditional mutant (*Lhx1*floxC−/−; *Mesp1*cre, hereafter *Lhx1*-mesCKO) embryos, *Lhx1* expression was detected initially in the nascent mesoderm adjacent to the primitive streak (supplementary material Fig. S5A, LS) but was lost in the fully formed mesoderm, whereas expression was retained in the AME (supplementary material Fig. S5A, OB, EB-LB). Despite the loss of *Lhx1* expression in the mesoderm, *Lhx1*-mesCKO embryos were morphologically indistinguishable from controls (supplementary material Fig. S5B), suggesting that *Lhx1* function in the mesoderm is unlikely to be crucial for head development.

To assess the function of *Lhx1* in the AME, the *Lhx1* flox allele was ablated by tamoxifen-activated MerCreMer (mcm) recombinase expressed from the *Foxa2* locus (Park et al., 2008). *Foxa2* is expressed in the AME and the endoderm, but not the mesoderm (Bertscher and Lickert, 2009). Expression of the *lacZ* reporter in tamoxifen-treated *Foxa2flox/mcm;Rosa26lcre/2mcm* embryos showed that MerCreMer was activated widely in the endoderm layer of the embryo, which encompassed the *Lhx1*-expressing AME tissues (Fig. 4A) (Park et al., 2008; Ip et al., 2014). When *Lhx1*floxflox mice were crossed with *Lhx1*1/−; *Foxa2*/mcm mice or *Lhx1*1/−; *Foxa2mcm/mcm* mice, embryos of four genotypes (*Lhx1*1/−; *Foxa2*/mcm, *Foxa2*/−; *Lhx1*1/−; *Foxa2*/mcm, *Lhx1*1/−; *Foxa2*/−, *Lhx1*1/−; *Foxa2*/−, *Lhx1*1/−; *Foxa2*/mcm, *Lhx1*1/−; *Foxa2*/mcm) were obtained. Twenty-eight hours after injection at E6.5, *Lhx1* expression was markedly reduced or absent in the AME.
of tamoxifen-treated \textit{Lhx1}\textsuperscript{lox/−};\textit{Ftxa2}\textsuperscript{+/mcm} (\textit{Lhx1}-ameCKO) embryos, as compared with mock-treated (vehicle only) \textit{Lhx1}\textsuperscript{lox/−};\textit{Ftxa2}\textsuperscript{+/mcm} or tamoxifen-treated \textit{Lhx1}\textsuperscript{lox/−};\textit{Ftxa2}\textsuperscript{+/+} controls (Fig. 4B). At E9.5, 90% of the \textit{Lhx1}-ameCKO embryos displayed some degree of head truncation (Fig. 4C,D; supplementary material Table S2). The expression of forebrain markers was lost or reduced in the \textit{Lhx1}-ameCKO embryos (Fig. 4E). These results highlight an essential role for \textit{Lhx1} in the AME for head formation.

We next examined the AME of the \textit{Lhx1}-ameCKO embryos. In contrast to \textit{Lhx1}-epiCKO embryos, the \textit{Lhx1}-ameCKO embryos formed an \textit{Shh}-expressing midline structure similar to the anterior midline of control embryos (Fig. 4F). However, \textit{in situ} hybridisation showed that the expression of Dkk1, Hexx1, Cer1 and Gsc was
reduced in the mutant embryos (Fig. 5A), which was confirmed by RT-qPCR analysis (Fig. 5B). Furthermore, we showed that Lhx1 expression, in combination with Ldb1 and Ssbp3, could activate these four genes in P19 cells (Fig. 5C).

Hesx1 is a known LHX1 target in mouse. LHX1 binds the dTAAT and pTAAT elements (referred to hereafter as the dp region) of the Hesx1 locus (Fig. 5D) (Chou et al., 2006). As expected, ChIP-PCR in P19 cells using the anti-HA antibody and the LHX1-HA expression constructs confirmed the binding of LHX1 to the dp region of the Hesx1 locus (Fig. 5E).

Cer1 and Gsc have also been reported to be directly regulated by XLIM1 in Xenopus, which binds to a region referred to as U1 (Sudou et al., 2012). The U1 region of Gsc is conserved in mouse and is also recognised by LHX1 (Fig. 5D,E; supplementary material Fig. S6A). However, no equivalent of the U1 region of Xenopus Cer1 (Sudou et al., 2012) was found in mouse. Instead, we showed that LHX1 could bind to a conserved region of the Cer1 locus containing LHX1 recognition motifs located ∼4 kb (+4R) after the START codon (Fig. 5D,E; supplementary material Fig. S6B).

Whether Dkk1 is a direct downstream target of LHX1 has not previously been investigated. A conserved region of the mouse Dkk1 locus, referred to as H1, has been shown to be necessary for the control of Dkk1 expression (Kimura-Yoshida et al., 2005). This region is bound by another homeobox transcription factor, OTX2 (Kimura-Yoshida et al., 2005; Ip et al., 2014), which can directly interact with LHX1 to activate target genes involved in head formation (Nakano et al., 2000; Yasuoka et al., 2014). We analysed the H1 region of Dkk1 and found two putative LHX1 recognition motifs, one localised at the distal (dH1) end and one at the proximal end (Fig. 5D; supplementary material Fig. S6C). Our results showed that LHX1 binds to dH1 but not to the proximal region in H1 (Fig. 5E; data not shown). LHX1Δ did not bind to any of the target sequences in the four genes tested (Fig. 5E). We further analysed the interaction of LHX1 with Dkk1 and showed that the H1 region can mediate the activation of luciferase reporter by LHX1 or LHX1+LDB1+SSBP3 but not LHX1Δ (Fig. 5F). Furthermore, a point mutation in the LHX1 recognition motif in dH1 abolished its trans-activating function (Fig. 5G).
Fig. 5. The expression of genes associated with the AME is modulated by LHX1. (A) In situ hybridisation of Dkk1, Hesx1, Cer1 and Gsc in neural-groove (NG) to head-fold (HF) stage control (Lhx1\textsuperscript{flox+}, Foxa2\textsuperscript{Δexon1} mock treated or Lhx1\textsuperscript{flox+}, Foxa2\textsuperscript{Δexon1} tamoxifen treated) embryos collected 32±2 h after mock or tamoxifen treatment at E6.5. Lateral views with anterior to the left. Scale bars: 100 µm. (B) RT-qPCR analysis of the expression of Dkk1, Hesx1, Cer1 and Gsc (relative to β-actin) in the anterior germ layer tissues of neural-groove to head-fold stage control (Lhx1\textsuperscript{flox+}, Foxa2\textsuperscript{Δexon1} or Lhx1\textsuperscript{flox+}, Foxa2\textsuperscript{Δexon1}) and Lhx1-ameCKO (Lhx1\textsuperscript{flox−}, Foxa2\textsuperscript{Δexon1}) embryos collected 32±2 h after tamoxifen treatment at E6.5. (C) RT-qPCR analysis of the expression of Dkk1, Hesx1, Cer1 and Gsc (relative to β-actin) in P19 cells transfected with different combinations of vectors expressing a mock protein, Lhx1, Ldb1+Ssbp3 and Lhx1+Ldb1+Ssbp3. (D) Genomic structure of the mouse Dkk1, Hesx1, Cer1 and Gsc loci. Coordinates are indicated relative to the START codon. Grey boxes, exons; orange lines, the conserved regions analysed in E-G; green circles, LHX1 recognition motifs; dH1 and H1, distal H1 region and H1 region; dp, Hesx1 dTAAT and pTAAT containing region; +4R, conserved region of Hesx1, Cer1 and Gsc. (E) ChiP-PCR analysis of the conserved regions shown in D following transfection of P19 cells with plasmids expressing Ldb1+Ssbp3+Lhx1-ΔHA or Ldb1+Ssbp3+Lhx1-ΔHA, and PCR detection of the target sequence in input and anti-HA (α-HA) immunoprecipitated chromatin. (F,G) Firefly luciferase activity (relative to Renilla luciferase) in P19 cells transfected with pGL3-promoter plasmid containing the Dkk1 dH1 region (F) or the Dkk1 dH1 region with mutated LHX1 recognition motif (G), a Renilla luciferase expression vector and different combinations of vectors expressing a mock protein, Lhx1+ΔHA, Lhx1, Ldb1 and/or Ssbp3. Data represent the means±s.e. of n=5 independent samples for each condition of transfection (C,F,G). *P<0.05, **P<0.01, ***P<0.001, no significant difference (ns) by t-test.

Altogether, these results suggest that LHX1 is required in the AME for head formation, where it may directly regulate the transcription of Dkk1, Hesx1, Cer1 and Gsc.

Elevated WNT signalling activity contributes to the head phenotype in Lhx1 mutants

Lhx1-epiCKO and Lhx1-ameCKO embryos displayed head defects similar to those of embryos affected by an excess of WNT/β-catenin signalling activity (Figs 1 and 4) (Mukhopadhyay et al., 2001; Lewis et al., 2008; Fossat et al., 2011b). In both conditional mutants, loss of Lhx1 results in the reduced expression of genes encoding WNT antagonists (Figs 2 and 5). This finding points to a potential gain of WNT activity in the Lhx1 mutants. Using the BATGal lacZ transgene, which is a reporter of WNT activity (Maretto et al., 2003), and by measuring the expression of two direct WNT targets (Axin2 and Lef1) by RT-qPCR, we showed that both the reporter and the target genes are significantly upregulated in the anterior tissues of the Lhx1-epiCKO embryo (Fig. 6A,B). A similar, albeit not statistically significant, trend was observed for the target genes in Lhx1-ameCKO embryos (Fig. 6C). This is likely to be related to the incomplete penetrance of the abnormal head phenotype in these mutants (Fig. 4D; supplementary material Table S2), a conjecture that is compatible with our previous finding that the degree of head truncation is correlated with the extent of elevation of WNT activity (Fossat et al., 2011b).

To test whether enhanced WNT signalling underlies the Lhx1 mutant phenotype, we examined the phenotypic effect of an increase of WNT signalling in conjunction with reduced Lhx1 activity in compound mutant embryos. Ctnnb1 encodes β-catenin, which is the key transcriptional mediator for the activity of the WNT/β-catenin signalling cascade (Petersen and Reddien, 2009). The Ctnnb1 bflace (Bfc) gain-of-function allele is associated with an excess of WNT/β-catenin signalling activity and the homozygous Bfc mutant embryo displays a headless phenotype (Fossat et al., 2011b). To determine whether the effect of Lhx1 loss is enhanced by an increase of WNT/β-catenin signalling activity, we examined the phenotype of embryos heterozygous for the Lhx1-null allele and the Ctnnb1 Bfc allele. We found that 71% of Lhx1\textsuperscript{+/−}; Ctnnb1\textsuperscript{Bfc+} compound mutant embryos displayed various degrees of head defect, whereas only 19% of Lhx1\textsuperscript{+/−} embryos and 24% of Ctnnb1\textsuperscript{Bfc−} embryos were affected (Fig. 6D; supplementary material Fig. S7A and Table S3).

We also tested whether the increase in WNT signalling due to the inactivation of one allele of Dkk1 (Mukhopadhyay et al., 2001) could also enhance the head truncation phenotype of Lhx1 mutant embryos. We found that 74% of Dkk1\textsuperscript{+/−}; Lhx1\textsuperscript{+/−} compound
heterozygous embryos displayed abnormal head morphology, compared with 9% of Lhx1−/− embryos and 0% of Dkk1+/− embryos (Fig. 6E; supplementary material Fig. S7C and Table S4). At E7.75, Axin2 expression was significantly elevated in the anterior region of the Dkk1+/−;Lhx1−/− embryos as compared with wild type (supplementary material Fig. S7D). We also generated compound Lhx1+−/−;Dkk1+/−;Meox2+/Cre embryos by crossing Lhx1+−/−;Dkk1+/−;Meox2+/Cre mice with Lhx1−/−;Meox2+/Cre mice. In these mutants, the flox allele of Lhx1 is ablated only in the epiblast-derived tissues that are also heterozygous for the Dkk1-null allele. They displayed a higher frequency of head truncation compared with the single-heterozygous embryos (supplementary material Fig. S7E,F and Table S5). The enhancement of the head phenotype might therefore be due to the additive effects of elevated WNT signalling caused by reduced Lhx1 activity and exacerbated by a further increase in signalling activity in Dkk1+/− epiblast derivatives. Taken together, these results support the proposition that an increase in WNT signalling activity underpins the head truncation phenotype of Lhx1 loss-of-function embryos.

**DISCUSSION**

Our study has provided several novel insights into the role of LHX1 in head morphogenesis. We have demonstrated that LHX1 acts upstream in the WNT pathway by regulating genes encoding factors that negatively modulate the level of signalling activity. We have also shown that LHX1 is required for the formation of the anterior midline tissues in which these WNT-modulating factors are expressed. Therefore, the loss of Lhx1 function leads to a reduction in WNT antagonistic activity, possibly via a direct effect on the transcriptional regulation of the target genes and an indirect effect resulting from the loss of tissues expressing the downstream genes (Fig. 7).

The tissue requirement of Lhx1 for head formation during gastrulation has been studied in mouse chimeras (Shawlot et al., 1999). In that study, chimeras with an Lhx1+/− visceral endoderm and a wild-type epiblast displayed head defects similar to Lhx1−/− embryos, which is consistent with an essential requirement of Lhx1 in the visceral endoderm for head formation (Shimono and Behringer, 2003). Complementary chimeras with wild-type visceral endoderm and Lhx1−/− epiblast (Lhx1−/− ↔ +/+ chimeras) have head defects resembling those of Lhx1−/−epiCKO embryos (Shawlot et al., 1999; Kwan and Behringer, 2002; the present study). The formation of the AME was not specifically addressed in the Lhx1−/− ↔ +/+ chimeras (Shawlot et al., 1999). Our study on the formation of the AME was prompted by the observations that XLim1 is necessary for the formation of the equivalent structure in Xenopus and that Lhx1−/− anterior primitive streak tissue displays impaired tissue extension activity when transplanted into a wild-type host (Hukriede et al., 2003). We have shown that no midline structure resembling the AME is present in Lhx1−/−epiCKO embryos. Although Foxa2−, Nog- and Chrd-expressing progenitors of the AME are specified despite the loss of Lhx1 from their precursors in the epiblast, these progenitor cells do not participate effectively in the morphogenetic movements that accompany the formation of the AME. The loss of Lhx1 function might have affected the morphogenetic capacity of the Foxa2-expressing AME progenitors. This phenotype could be partly explained by the downregulation of Pcdh7, a potential LHX1 transcriptional target. Pcdh7 is co-expressed with Lhx1, its expression is reduced in Lhx1−/−epiCKO mutants and Pcdh7−/− deficient embryos display head and AME defects. This demonstrates a novel requirement for Lhx1 in the formation of the AME that might be mediated by PCDH7 (Fig. 7). In Xenopus, the orthologue of Pcdh7 has been shown to play a role in cell adhesion and ectodermal cell sorting (Bradley et al., 1998; Rashid et al., 2006). Human PCDH7 is involved in the regulation of cell shape and cell adhesion (Yoshida, 2003) and inactivation of PCDH7 inhibits breast cancer cell migration and invasion (Li et al., 2013). Furthermore, Xenopus embryos in which Pcdh7 expression is disrupted display defective neural tube closure (Rashid et al., 2006), a phenotype also observed in Pcdh7−/− mutant mouse embryos.

Previous attempts to study the role of Lhx1 in the AME have analysed the anterior tissue of Lhx1−/− embryos (Shawlot et al., 1999; Shimono and Behringer, 1999). However, the findings of our present study show that the AME is absent in the anterior tissue of the embryo when Lhx1 is inactivated in the epiblast (which contains the progenitors of the AME), suggesting that the AME was not part of...
the level of WNT signalling activity for head formation. The WNT signalling (Martinez-Barbera et al., 2000; Andoniadou et al., 2011) demonstrates, for the first time, a requirement for genes in the AME, which encode secreted molecules (e.g. Dkk1, Cer1) and transcription factors (Gsc, Hesx1) that negatively modulate (antagonise) the level of WNT signalling activity for head formation. Gsc has been shown to directly repress the expression of Wnt8. OTX2 has been shown to directly regulate the transcription of Lhx1. LHX1 also regulates the expression of Pcdh7, a protocadherin that may be involved in the morphogenetic activity of FOXA2-expressing cells during the formation of the AME. The functional relationship of Pcdh7 with WNT signalling is presently unknown.

The tissue fragment analysed previously. In the present work, the AME does form in Lhx1-ameKO embryos and the loss of Lhx1 in this tissue results in the development of an abnormal head. This demonstrates, for the first time, a requirement for Lhx1 in the AME. Loss of Lhx1 in the AME results in the downregulation of genes that encode WNT signalling antagonists, such as Dkk1, Hesx1, and Gsc. Hesx1 is a direct target of LHX1 (Chou et al., 2006) and Hesx1−/− embryos display a truncated head that is associated with an increase in WNT signalling (Martinez-Barbera et al., 2000; Andoniadou et al., 2007, 2011). The Hesx1 mutant phenotype is rescued by β-catenin inactivation and exacerbated by the deletion of Tcf7l1 (also known as Tcf3), a negative regulator of WNT activity (Andoniadou et al., 2011). In Xenopus, Cer1 and Gsc are bound and activated by XLM1 (Sudou et al., 2012). Xenopus Cer1 encodes a secreted factor that antagonises WNT signalling (Piccolo et al., 1999). In mouse, Cer1 activity maintains the neural characteristics of the anterior ectoderm (Shawlot et al., 2000). We have previously demonstrated that Gsc activity is required for patterning the forebrain (Canus et al., 2000). A reduction of both Dkk1 and Gsc in the mouse embryo results in head truncation (Lewis et al., 2007), presumably owing to increased WNT signalling activity in the compound mutant with reduced activity of the antagonist and repressor of WNT ligand expression (Yao and Kessler, 2001). Results of RT-qPCR and ChIP-qPCR show that LHX1 can bind to a newly identified regulatory region in Cer1, and Dkk1 is likely to be a novel transcriptional target of LHX1 that acts in concert with LDB1 and SSBP3 (Mukhopadhyay et al., 2003; Nishioka et al., 2005; Enkhamdakh et al., 2006). Dkk1−/− embryos fail to develop a head due to an excess of WNT3 signalling (Lewis et al., 2008) and Dkk1 and Lhx1 mutations interact synergistically to enhance head truncation. Loss of WNT antagonists, and Dkk1 in particular, is therefore a causative factor of head truncation in Lhx1 mutants (Fig. 7).

Our study has shown that LHX1 enables the morphogenetic cell movements that are instrumental in the formation of the AME, which is the source of factors that fine-tune the level of WNT signalling activity (Arkell and Tam, 2012; Fossat et al., 2012; Arkell et al., 2013). In the AME, LHX1 function intersects with the WNT pathway via transcriptional control of modulators of WNT signalling activity. LHX1 binds to a regulatory region of Dkk1 that also interacts with another transcription factor, OTX2 (Kimin-Yoshida et al., 2005; Ip et al., 2014), and OTX2 and LHX1 are part of the same activator complex (Nakano et al., 2000; Yaukawa et al., 2014). OTX2 can directly regulate Cer1 and Gsc in Xenopus (Sudou et al., 2012; Yaukawa et al., 2014) and Hesx1 in chicken (Spieler et al., 2004). Lhx1 is also a target of OTX2 and its expression in the AME is regulated by OTX2 (Ip et al., 2014). Together, our findings reveal an upstream function of LHX1, which may act in conjunction with OTX2, in regulating the expression of genes that encode secreted molecules (e.g. Dkk1, Cer1) and transcription factors (e.g. Gsc, Hesx1) that are involved in the modulation of WNT/β-catenin signalling (Fig. 7).

**MATERIALS AND METHODS**

**Mouse strains, genotyping and crosses**

Lhx1fl/fl and Lhx1−/− (Kwan and Behringer, 2002), Meox2−/Cre (Tallquist and Soriano, 2000), Mesp1−/− (Sage et al., 1999), Foxa2−/− (Park et al., 2008), Rosa26−/−R26R (Soriano, 1999), CMV-Cre (Schwenk et al., 1995), BATGal (Maretto et al., 2003), Cnnb1flox/flox (Fossat et al., 2011b) and Dkk1fl/fl (Mukhopadhyay et al., 2001) mice were used. Genotyping by PCR followed established protocols and was performed on DNA extracted from tail tissues of newborn or the yolk sac of embryos. Breeding strategies for the production of mutants are outlined in supplementary material Tables S1-S5. Animal experimentation was approved by the Animal Ethics Committee of the Children’s Medical Research Institute and the Children’s Hospital at Westmead.

**Generation of Pcdh7 mutant chimeric embryos using the CRISPR-Cas9 technology**

Oligonucleotides 5′-CACCGCCAACAGCATGCTCCGGTAC and 5′-AAA-CTGACGGGACGCTGTGTTGGC were cloned into the pSpCas9(BB)-2A-GFP (PX458) plasmid [Addgene plasmid #48138 (Ran et al., 2013)], a gift from Feng Zhang, to express the sgRNA targeted to R1-129 ESCs. GFP-positive clones were isolated and analysed for mutation of the Pcdh7 locus. Two clones with biallelic frameshift mutations (Pcdh7−1081−2; supplementary material Fig. S3A) and the parental R1-129 ESC line were used to generate chimeras. Eleven to thirteen ESCs were injected into pseudopregnant mice and stained with X-Gal (for E9.5 embryos).

**Embryo collection, staging, head morphology analysis and tamoxifen injection**

Embryos were collected at the required gestational age or at specific time points after tamoxifen or mock treatment. Embryos were staged by morphology (Downs and Davies, 1993) or somite number. The morphology of E8.5-E10.5 mutant embryos was compared with stage-matched control

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**Fig. 7. The input of LHX1 function to WNT signalling in head formation.** LHX1, in conjunction with co-factors LDB1 and SSBP3, and potentially OTX2, activates genes in the AME, which encode secreted molecules (Dkk1, Cer1) and transcription factors (Gsc, Hesx1) that negatively modulate (antagonise) the level of WNT signalling activity for head formation. Gsc has been demonstrated to directly repress the expression of Wnt8. OTX2 has been shown to directly regulate the transcription of Lhx1. LHX1 also regulates the expression of Pcdh7, a protocadherin that may be involved in the morphogenetic activity of FOXA2-expressing cells during the formation of the AME. The functional relationship of Pcdh7 with WNT signalling is presently unknown.
embryos with specific attention to embryo and head size, number of somites and morphology of the head. Embryos were assigned to one of five categories based on the size of the forebrain and midbrain (see Fig. 4D). Tamoxifen (Sigma-Aldrich) was administered to pregnant mice by intrauterine injection of 1 mg (100 µl of 10 mg/ml in canola oil) per 20 g body weight. For the mock control, an equivalent volume of canola oil was administered.

In situ hybridisation, immunostaining, X-Gal staining and histology
These followed standard protocols; details are provided in the supplementary Materials and Methods.

Cell labelling and embryo culture experiments
Embryos were collected at mid-streak stage. Cells in the endoderm layer associated with the anterior and the posterior segment of the primitive streak were labelled with CM-DiI and DiO (Molecular Probes, respectively) (Fossat et al., 2011a). Embryos were cultured in vitro for 24 h (Fossat et al., 2011a). Embryos were imaged under bright-field and fluorescent light (merged image) using a Leica SP5 confocal microscope before and after culture.

Cell transfection assays for RT-qPCR and ChIP-PCR
P19 cells were transfected with different combinations of GFP, a mock plasmid, pLhx1-HA, pLhx1Δ-HA, pLab1 and/or pSshp3 in equimolar quantity (see supplementary Materials and Methods for details) and sorted by flow cytometry using a BD FACS Aria III cell sorter.

Molecular cloning and luciferase assay
The generation of expression vectors and plasmids for the luciferase assay is described in the supplementary Materials and Methods. P19 cells were transfected with pGL3-promoter (empty or containing a genomic region), a pRL vector and different combinations of mock plasmid, pLhx1-ΔHA, pLhx1Δ-ΔHA, pLab1 and/or pSshp3 in equimolar quantity (see supplementary Materials and Methods for details). The luciferase assay was performed as described previously (Ip et al., 2014).

Sampling for RT-qPCR analysis
The following were sampled for RT-qPCR analysis: 15 Lhx1-epiCKO (Lhx1flox-/-;Meox2+/+Cre) and 15 control (Lhx1flox-/-;Meox2+/+Cre) stage-matched (early-bud to head-fold) E7.75 embryos; five Lhx1-ameCKO (Lhx1flox-/-;Foxa2-/-;Meox2+/+Cre) and five control (Lhx1flox-/-;Foxa2-/-;Meox2+/+Cre) stage-matched (neural groove to head-fold) E7.75 embryos. 32±2 h after tamoxifen injection at E6.5; five Lhx1+/+;Dkk1+/+; three Lhx1+/+;five Dkk1+/+; and five wild-type stage-matched (early-bud to head-fold) E7.75 embryos. Each embryo was bisected longitudinally to isolate anterior germ layer tissues for analysis.

For cell experiments, flow-sorted GFP-expressing cells were collected in triplicate for each condition of transfection.

RNA isolation and RT-qPCR conditions are described in the supplementary Materials and Methods.

ChIP-PCR analysis
The EZ-Magna ChIP Kit (Millipore) was used (see supplementary Materials and Methods for details). For Pcdh7, a previously established ChIP-qPCR protocol was used (Ip et al., 2014). Primer sequences were as published (Ip et al., 2014) or are listed in supplementary material Table S6.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary material
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