**Dkk1 and Wnt3 interact to control head morphogenesis in the mouse**

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Loss of Dkk1 results in ectopic WNT/β-catenin signalling activity in the anterior germ layer tissues and impairs cell movement in the endoderm of the mouse gastrula. The juxtaposition of the expression domains of Dkk1 and Wnt3 is suggestive of an antagonist-agonist interaction. Downregulation of Dkk1 when Wnt3 activity is reduced reveals a feedback mechanism for regulating WNT signalling. Compound Dkk1;Wnt3 heterozygous mutant embryos display head truncation and trunk malformation, which are not found in either Dkk1+/− or Wnt3+/− embryos. Reducing the dose of Wnt3 gene in Dkk1+/+ embryos partially rescues the truncated head phenotype. These findings highlight that head development is sensitive to the level of WNT3 signalling and that DKK1 is the key antagonist that modulates WNT3 activity during anterior morphogenesis.

**KEY WORDS:** Head development, WNT signalling, DKK1, WNT3, Mouse

**INTRODUCTION**

Truncation of the head or loss of anterior structures is found in a plethora of mouse mutants. One of these, which is associated with the loss of Lhx1 (Lim1), displays complete loss of craniofacial structures rostral to the upper hindbrain (Shawlot and Behringer, 1995). Lhx1 encodes a LIM class homeodomain protein (Li et al., 1999) that forms complexes with LIM-domain binding protein (LDB1) and single-strand DNA binding protein 1 (SSDP1) (Enkhmndakh et al., 2006). This complex interacts synergistically with OTX2 to activate target genes (Nakano et al., 2000). Interestingly, loss of the functions of Ldb1, Sdp1 and Otx2 individually, like that of Lhx1, also leads to head truncation in the mouse embryo, albeit with different degrees of severity (Mukhopadhyay et al., 2003; Nishioka et al., 2005; Acampora et al., 1995; Acampora et al., 1997; Ang et al., 1996; Matsuo et al., 1995). The activity of the genes that influence head morphogenesis is potentially linked to WNT signalling, principally via effects on the expression of antagonistic factors. Mutant embryos that lack Otx2 or Foxa2 (which directly trans-activates Otx2) also lose the expression of a WNT antagonist, Dkk1, in the visceral endoderm (Kimura et al., 2001; Zakin et al., 2000; Kimura-Yoshida et al., 2007). Similarly, loss of Ssdp1 or Ldb1 function affects the expression of Dkk1, as well as of other WNT antagonists such as Sfrp1, Sfrp2 and Frzb/Sfrp3 (Hoang et al., 1998; Nishioka et al., 2005; Mukhopadhyay et al., 2003).

To date, the most compelling evidence for a role of WNT antagonist activity in head formation is that the loss of Dkk1 results in anterior truncation (Mukhopadhyay et al., 2001). Dkk1 encodes a secreted protein that inhibits canonical WNT signalling by sequestering the LRP6 co-receptor so that it can no longer function alongside the frizzled receptors to activate the WNT signalling cascade (Glinka et al., 1998; Mao et al., 2001; Zorn, 2001). Dkk1 is expressed first in a girth of visceral endoderm in the mid-region of the embryonic day (E) 5.5 embryo. It is then expressed in a crescent-shaped domain of visceral endoderm in the anterior region of the E6.0-6.5 embryo (Kimura-Yoshida et al., 2005) and similarly in the anterior endoderm of E7.0-7.5 embryos (Lewis et al., 2007; Pfister et al., 2007). Dkk1 is later restricted to the prechordal plate and the ventral foregut endoderm (Lewis et al., 2007). At every stage of development from E5.5 to E7.5, Dkk1-expressing cells appear to demarcate the anterior and lateral border of the expression domain of Fzd8 (which encodes a frizzled WNT receptor) in the anterior endoderm (Lu et al., 2004). Taken together, these findings imply a link between the modulation of WNT signalling by the antagonists and the development of anterior structures. Consistent with this concept, blocking WNT signalling in embryonic stem cells by DKK1 promotes the formation of precursors of forebrain neurones (Watanabe et al., 2005). By contrast, ectopic expression of Wnt1 in the anterior tissues, in conjunction with the loss of Six3 or Hexx1 function, is associated with truncation of anterior structures or posteriorization of the forebrain tissues (Lagutin et al., 2003; Andoniadou et al., 2007).

Despite the demonstration of a crucial requirement for Dkk1 activity for head formation, it is not clearly known when its function is required during embryogenesis. Some findings suggest that an earlier function of Dkk1 in the visceral endoderm may be dispensable at least for the induction of forebrain tissues. These include: (1) that in the Dkk1-null mutant embryo, the molecular markers for anterior visceral endoderm are expressed correctly (Mukhopadhyay et al., 2001); (2) that chimeric embryos comprising Dkk1-deficient extra-embryonic tissues, including the visceral endoderm, develop normally (Mukhopadhyay et al., 2001); and (3) that, although the enforced expression of Dkk1 in the Otx2-null mutant can restore the patterning of the visceral endoderm, it is not sufficient to rescue the truncated head phenotype (Kimura-Yoshida et al., 2005). There is, however, indirect evidence for a role of Dkk1 later in the anterior mesendoderm for head formation, which is revealed by a synergistic interaction of Dkk1 and Gsc via their negative action on WNT signalling. Both genes are expressed in the prechordal plate and the foregut endoderm. Gsc may suppress the transcription of Wnt1a and acts in concert with Dkk1 to attenuate WNT signalling in the anterior tissues (Lewis et al., 2007).
As the function of Dkk1 is to antagonize WNT signalling, the phenotypic effect of the loss of Dkk1 function may be caused by an inadequate control of the level of WNT signals. Several genes encoding WNT ligands are expressed in the mouse embryo during the immediate postimplantation period (Yamaguchi, 2001; Kemp et al., 2005). Of these, Wnt3 is one of the first to be expressed: transiently in the posterior visceral endoderm, then restricted to the posterior proximal epiblast and later to the nascent primitive streak (Rivera-Perez and Magnunson, 2005). Wnt7b and Wnt8a are expressed primarily in the posterior epiblast, Wnt3a in the primitive streak and Wnt5a in the mesoderm (Yamaguchi, 2001; Kemp et al., 2005). In contrast to the posterior localization of the ligand transcripts, genes coding for WNT antagonists, such as Dkk1, Sfrp1 and Sfrp5 (Kemp et al., 2005; Finley et al., 2003; Kimura-Yoshida et al., 2005; Lewis et al., 2007), are expressed in the anterior visceral endoderm and later the anterior definitive endoderm. The regionalization of antagonist and ligand expression in the embryo is consistent with the maintenance of a graded signalling activity from low in the progenitor tissues of the head to high in the posterior germ layer and the primitive streak (Pfister et al., 2007). However, it is not known which of these WNT activities is modulated by DKK1 to control head morphogenesis.

In the present study, we have shown by marker and reporter analysis of mutant embryos that loss of Dkk1 leads to ectopic activation of WNT/β-catenin signalling during gastrulation. Our results demonstrate that Dkk1 and Wnt3 activities are regulated in a negative feedback manner in vivo. Analysing the development of compound Dkk1;Wnt3 mutants reveals that Dkk1 and Wnt3 interact genetically, and that balancing the expression in the embryo is consistent with the maintenance of a graded signaling activity from low in the progenitor tissues of the head to high in the posterior germ layer and the primitive streak (Pfister et al., 2007).

MATERIALS AND METHODS

Mouse strains

To test for the genetic interaction between Dkk1 and Wnt3, Dkk1+–/– (Mukhopadhyay et al., 2001) and Wnt3+–/– (Liu et al., 1999) mice, maintained on a 129×C57BL/6 background, were intercrossed to generate compound Dkk1×Wnt3 mutant mice. These mice were then interbred to produce mutant embryos of different combinations of Dkk1 and Wnt3 genotype. A total of 323 embryos were collected at E7.75 to E10.5 for the analysis of compound Dkk1;Wnt3 mutant phenotype. Embryos were also collected from pregnant mice of the mutant genotype. 323 embryos were collected at E7.75 to E10.5 for the analysis of compound Dkk1;Wnt3 mutant phenotype. Embryos were also collected from pregnant mice of the mutant genotype.

Immunofluorescence

Mid-stage streak (E7.0) wild-type and Dkk1−/− embryos were processed for whole-mount immunofluorescence according to the protocol of Ciruna and Rossant (Ciruna and Rossant, 2001) using anti-β-catenin (rabbit) (Abcam) and Alexa 488-conjugated secondary antibody (Invitrogen). Nuclei were counterstained by propidium iodide. Phalloidin (Invitrogen) was used to stain the F-actin to reveal the cell outline. Stained embryos were dissected into anterior and posterior halves, and mounted separately for confocal fluorescence microscopy.

Cell transplantation experiment

Pregnant Dkk1–/–EGFP;lacZ mice were euthanized at E7.0 to harvest embryos for isolating cells for transplantation. Embryos were genotyped by PCR on yolk sac tissues (primer sequences available upon request). Tissue fragments were dissected from the anterior region of the primitive streak [APS, containing progenitors of mesoderm and endoderm (Kinder et al., 2001)] of the mid-stage streak Dkk1−/−;EGFP;lacZ embryos. Similar APS fragments were obtained from Dkk1+–/–;EGFP;lacZ for the control experiment. These fragments were dissected into clumps of about 10-15 cells, which were transplanted into the APS of wild-type mid-stage stage ARCs host embryos for assessing the differentiation of Dkk1−/− APS cells. The recipient embryos were examined by fluorescence microscopy 2 hours after transplantation to ascertain the location of the EGFP-expressing graft. Embryos showing incorrect positioning of the graft were excluded from further analysis.

Electroporation of the endoderm

Mid-stage streak wild-type and Dkk1−/− embryos were harvested from E7.0 pregnant mice. Cells in the endoderm layer of the embryo were marked by electroporating a pCMV-EGFP expression vector (Davidson et al., 2003). Embryos were then brieﬂy fixed in 4% paraformaldehyde, stained in X-Gal solution overnight to detect the lacZ-positive graft-derived cells and then processed for paraffin wax histology. The number and the distribution of X-Gal stained cells in the host tissues were scored in serial sections of the specimen.

RT-PCR analysis of Dkk1 induction

NIH3T3 cell lines containing pNCX retroviral vector expressing full-length EDNA, encoding Wnts or lacZ (Kispert et al., 1998) were used to test the induction of Dkk1. The expression of β-galactosidase, Wnt1, Wnt5a, Wnt4, Wnt5a, Wnt7a and Wnt11 in respective cell lines was confirmed by RT-PCR using primers described by Lako et al. (Lako et al., 2001). Total RNA was prepared from the cultured cells using the RNeasy Kit (Qiagen) following the manufacturer’s instructions. RNA was reverse-transcribed using SuperScript III Reverse Transcriptase using oligo(dT) as a primer. RT-PCR for Dkk1 and Hprt was performed on the products of the reverse transcription (primer sequences available upon request).
Quantitative Dkk1 and Wnt3 expression level

Quantitative RT-PCR analysis was performed to determine the level of Dkk1 and Wnt3 expression of E7.0-7.5 embryos of mice of Wnt3+/−, Wnt3+/− and Wnt3+/− genotypes (from Wnt3−/- inter-cross) and of Dkk1+/−;Wnt3+/−, Dkk1−/-;Wnt3+/− and Dkk1−/-;Wnt3+/− genotypes (from the Dkk1−/-;Wnt3+/− intercross). Total RNA was isolated using the RNeasy Micro Kit (Qiagen). cDNA was generated using the SuperScript III First Strand Synthesis System (Invitrogen) with oligo-dT primers. Quantitative RT-PCR of Dkk1 transcript was performed using the Rotorgene 6000 thermal cycler (Corbett Research) with SYBR green I (Molecular Probes) using Platinum Taq DNA Polymerase (Invitrogen). Primer sequences and reaction conditions are available upon request. The levels of Gapdh were used for the normalisation of sample results. Initially, PCR products were run in a 2% gel to confirm correct band size in order to validate the results, and routine melting curve analysis was performed later to verify the presence of a single amplified product.

RESULTS

Ectopic WNT signalling activity in the Dkk1 mutant embryo

To assess the effect of the loss of Dkk1 activity on WNT signalling in the gastrulating embryos at E7.0-7.75, the patterns of expression of two lacZ reporters containing TCF/LEF-response elements (TOPGal and BATGal) were studied in the Dkk1−/- embryo. In the wild-type embryos, expression of the BATGal reporter was detected first at E7.0 in the posterior germ layer and the extra-embryonic tissues adjacent to the primitive streak (Fig. 1A). During gastrulation, BATGal expression extends distally in step with the elongation of the primitive streak (Fig. 1B), eventually to the full length of the primitive streak and in the adjacent posterior germ layer tissues (Fig. 1C). At the early-somite and early-organogenesis stages, BATGal expression is detected widely in the brain and anterior tissues, except those in the most rostral part of the forebrain and the prospective frontonasal region (Fig. 1D,E). The domain of TOPGal expression is more restricted to the posterior germ layer tissues and the primitive streak during gastrulation, and to more caudal parts of the brain, and the intensity of X-Gal staining reaction is weaker than that of the BATGal reporter (see Fig. S1A-E in the supplementary material). Taken together, the expression pattern of the two reporters reveals that WNT signalling activity is strong in the anterior tissues, except those in the most rostral part of the forebrain and frontonasal tissues.

In the E7.0-7.75 Dkk1−/- embryos, BATGal reporter is expressed in a wider domain encompassing more anterior germ layer tissues (11/14 embryos) or even the whole embryo (3/14 embryos) (Fig. 1F-H). Stronger and broader TOPGal expression is found in the posterior proximal region and the primitive streak of Dkk1−/- embryos (see Fig. S1F-H in the supplementary material). At both the early-somite and early-organogenesis stages, BATGal- or TOPGal-free tissues are reduced or absent from the rostral region of the mutant embryo (Fig. 1I,J; see Fig. S1L,J in the supplementary material). To further assess the cellular response to WNT signalling in the Dkk1-null embryo, we examined the overall level of expression and the subcellular localization of β-catenin in the anterior endoderm of the mid-streak (E7.0) embryos. In five Dkk1-null embryos, endoderm cells in the anterior-proximal, the anterior-distal and the posterior regions (see Fig. S1L,N,P in the supplementary material) are stained more strongly for β-catenin, which was also localized more frequently in the nucleus of the endoderm cells than in the equivalent population in the wild-type embryo (see Fig. S1K,M,O in the supplementary material). The abundance of stabilised form of β-catenin in the anterior endoderm of the Dkk1−/- embryo and the elevated expression of WNT activity reporters in the anterior germ layer tissues are consistent with an elevated response to WNT signalling in the progenitor tissues of the embryonic head. Concurrent with the expanded expression domain of BATGal (Fig. 2A: BATGal) and TOPGal (see Fig. S1I,J in the supplementary material), a WNT-downstream gene, Axin2, is expressed more widely in the head folds of the early-somite-stage Dkk1−/- embryo (Fig. 2A, Axin2) (Jho et al., 2002). By E9.5, Dkk1−/- mutant embryo lost all craniofacial structures rostral to the upper hindbrain (Fig. 2A, Shh) (Echelard et al., 1993).

Loss of Dkk1 activity does not affect the allocation of germ layer precursors but may affect cell spreading in the endoderm

Marker expression was analysed to test whether the truncation of the head is associated with an inappropriate allocation and/or patterning of progenitor tissues (Fig. 2B,C). No significant difference in the expression pattern of markers of the primitive streak (Mx1, T) (Robb et al., 2000; Wilkinson et al., 1990), the organizer (Foxa2, Chrd) (Ang and Rossant, 1994; Klingensmith et al., 1999), the mesoderm (Lhx1, Tbx6) (Shawlot and Behringer, 1995; Chapman et al., 1996) and the endoderm (Cer1) (Belo et al., 1997) was found between the wild-type and mutant embryos. There is, however, a more extended domain of T expression in the head process mesoderm (Fig. 2B, T). Sox17 expression is reduced in the definitive endoderm of the early-bud stage mutant embryo, and the expression of both Foxa2 and Sox17 is reduced in the anterior foregut endoderm of the early-somite stage mutant embryo (Fig. 2C, Sox17, Foxa2) (Kanai-Azuma et al., 2002; Ang and Rossant, 1994). The differentiation of the endoderm may therefore be affected by the loss of Dkk1. It is not known, however, whether the loss of Dkk1 may have affected the allocation of the germ layer progenitors to the endoderm and mesoderm or cell fate specification.

To further test the impact of loss of Dkk1 on cell differentiation, cells from the APS of the mid-streak (E7.0) Dkk1−/- embryos were transplanted orthotopically to the APS of the E7.0 wild-type host embryo and the distribution of the descendants of the transplanted cells in the host was examined (see Fig. S2A in the supplementary material). Cells from the wild-type donor embryos contributed
extensively to anterior structures, including the foregut and anterior axial mesoderm. Similarly, Dkk1<sup>−/−</sup> cells were able to colonize the cranial paraxial mesoderm, the anterior axial mesoderm and the foregut endoderm of the host embryo (see Fig. S2A-D in the supplementary material). Loss of Dkk1 therefore does not affect the ability of APS cells to colonize the anterior tissues that are missing in the Dkk1-null mutant. Intriguingly, most graft-derived Dkk1-null cells were found in the cranial mesenchyme and few in the trunk and posterior region of the host embryo (see Fig. S2A in the supplementary material, boxed section). The lineage analysis results show that Dkk1-null cells can contribute to both mesoderm and endoderm of the host embryo. It is likely that the altered expression of cell markers is related to defective differentiation of mutant cells after allocation to the respective lineages.

Previously, it has been shown that cells in the visceral endoderm of the pre-gastrula embryo can be mobilized from sites of strong WNT signal towards sites of high DKK1 activity (Kimura-Yoshida et al., 2005). To test whether loss of Dkk1 affects cell movement in the endoderm, groups of cells in five different regions of endoderm of the E7.0 Dkk1<sup>−/−</sup> embryo were electroporated with a pcMV-EGFP expression vector so that the descendants of these cells can be tracked as the embryo develops in vitro (e.g. Fig. S3A,B in the supplementary material). In contrast to the extensive displacement of endoderm cells in the wild-type embryos, endoderm cells of the Dkk1<sup>−/−</sup> were more restricted in their distribution in the anterior-posterior axis of the embryo. Cells in the anterior endoderm were not wholly displaced to the yolk sac (see Fig. S3C in the supplementary material) or tended to localize more frequently to the mid-embryonic sites than those in the wild-type embryo (see Fig. S3D in the supplementary material). F-actin immunostaining revealed that endoderm cells in the anterior region of the E7.0 Dkk1<sup>−/−</sup> embryo have a smaller apical cell surface area, suggesting that these cells may be more tightly packed together (see Fig. S3H,I in the supplementary material). Cells in the distal endoderm failed to extend along the anterior-posterior axis (see Fig. S3E in the supplementary material) and those in the posterior-distal site were restricted to the posterior region and did not move to anterior sites (see Fig. S3F in the supplementary material). Only cells in the posterior-proximal region of Dkk1-null mutant embryo behaved like the wild type (see Fig. S3G in the supplementary material). Loss of Dkk1 function may therefore impede the anterior-posterior spreading of endoderm cells in the anterior and middle parts of the embryonic gut.

Wnt3 activity influences Dkk1 expression

In the early to mid-streak (E6.5-7.25) embryo, Dkk1-expressing cells are distributed in a crescent-shaped domain that delimits the anterior and lateral border of the anterior endoderm (Fig. 3A,B) (Kimura-Yoshida et al., 2005; Pfister et al., 2007). At similar stages, Wnt3 is expressed in the visceral endoderm, with the domain extending anteriorly along the mid-girth of the pre-streak embryo (Fig. 3A) (Rivera-Perez and Magnuson, 2005). During gastrulation, Wnt3 is expressed in the posterior epiblast and the primitive streak (Fig. 3B,C), where two other WNT genes (Wnt2b and Wnt8a) are also expressed (Fig. 3D,E). Among these genes, Wnt3 displays the broadest expression domain, which extends anteriorly to the vicinity of the Dkk1-expressing endoderm cells (Fig. 3A,B). In embryos that lack Dkk1 activity, the expression domain of Wnt3 remains unchanged (Fig. 3C), whereas those of Wnt2b (6/6 embryos) and Wnt8a (1/3 embryo) expand slightly (Fig. 3D,E). In addition to Dkk1, two WNT antagonists, Sfrp1 (Kemp et al., 2005) and Sfrp5 (Finley et al., 2003), are also expressed in the anterior region of the embryo. In the Dkk1<sup>−/−</sup> embryo, the expression of Sfrp1 is reduced but Sfrp5 does not change (Fig. 3F,G).

Dkk1 is directly targeted by WNT/β-catenin signalling (Gonzalez-Sancho et al., 2005; Niida et al., 2004; Chamorro et al., 2005) through the binding of TCF/β-catenin complex to the Lef/TCf sites in the Dkk1 promoter (Chamorro et al., 2005). To test whether Dkk1 expression is influenced by Wnt3 activity in vivo, we examined Dkk1 expression in the E7.0 Wnt3<sup>−/−</sup> embryo. Dkk1 is not expressed in four out of five null-mutant embryo, and the remaining...
embryo shows very weak and restricted expression in the anterior endoderm (Fig. 3H, Wnt3+/–). Wnt3-null embryos fail to gastrulate but they do form the anterior visceral endoderm (Liu et al., 1999), suggesting that the lack of Dkk1 expression is not due to the loss of this tissue. Dkk1 expression domain is more restricted in the Wnt3+/– embryo (Fig. 3H, Wnt3+/–). Q-RT-PCR analysis of embryos of different Wnt3 genotypes showed that Dkk1 expression is not changed when Wnt3 dose is halved but is markedly reduced in the absence of Wnt3 (Table 1A). In view of the fact that Dkk1 expression depends on Wnt3 activity, we predict that halving the gene dose of Dkk1 in Wnt3+/– embryo might reduce the expression of Dkk1. This was found to be the case: Dkk1 expression in the Dkk1+/–;Wnt3+/– is significantly downregulated (Table 1B). In situ hybridization was also performed on embryos of different Dkk1;Wnt3 genotypes. The results show that in the Dkk1+/–;Wnt3+/– embryo, the domain of Dkk1 expression in the anterior endoderm is reduced (seven out of eight embryos; Fig. 3I, Dkk1+/–;Wnt3+/–) to a similar extent as in the Dkk1+/–;Wnt3+/– embryo (five out of five embryos; Fig. 3J, Dkk1+/–;Wnt3+/–). The Dkk1+/–;Wnt3+/– embryos display weaker and more restricted Dkk1 expression than both Dkk1+/–;Wnt3+/– and Dkk1–/–;Wnt3+/– embryos (five out of five embryos; Fig. 3J, Dkk1–/–;Wnt3+/–).

To test whether Dkk1 could respond to the activity of specific WNTs reputed to be of the canonical versus non-canonical pathway, we test its expression in NIH3T3 cells that express six Wnt genes (Wnt1, Wnt3a, Wnt4, Wnt5a, Wnt7a and Wnt11). We found that Dkk1 was induced most robustly by Wnt1 and Wnt7a, less effectively by Wnt3a, and not by the other three WNT genes (Fig. 3I). Wnt1 is expressed in the neural tissue after head-fold formation and Wnt7a is expressed during late organogenesis. As neither gene is expressed at gastrulation, they are not likely to compensate for loss of Wnt3. Wnt3a and Wnt3a are expressed in the gastrula, and could have an effect on Dkk1 expression. However, the cell culture data show that they are ineffective in activating Dkk1 (Fig. 3J). Although the in vitro cell culture experiments may not mimic the induction of Dkk1 in the endoderm, and factors such as Wnt3, Wnt2b and Wnt8a had not been tested, our results on the six WNT genes show that there is a certain degree of upstream specificity in activating Dkk1 expression. These findings suggest that Wnt3 activity may be the key for regulating Dkk1 expression in the pre-gastrulation and gastrula stage embryos, and that it could not be substituted for by other WNT signals. In E7.75-9.0 Wnt3-null embryos, the TOPGal reporter is not expressed (n=7, Fig. 6H), indicating that WNT signalling activity is generally curtailed in the mutant embryo after gastrulation.

**Dkk1 and Wnt3 interact genetically in head morphogenesis**

To test whether a reduced level of Dkk1 activity may affect head morphogenesis, we undertook a phenotypic study of the compound Dkk1+/–;Wnt3+/– mutant embryo. Like Dkk1+/– embryos, some Dkk1+/–;Wnt3+/– embryos have smaller head folds at E8.5 and forebrain at E9.5 (Fig. 4A). Altogether, about 70% of E8.0-9.5 Dkk1+/–;Wnt3+/– embryos display abnormal head morphology, compared with 5.5% of Dkk1+/–;Wnt3+/– and 9.5% of Dkk1+/–;Wnt3+/– embryos from the same cross (Table 2), a finding consistent with the degree of Dkk1 downregulation for these genotypes (Table 1B). Analysis of BATGal reporter expression (Fig. 4B: BATGal) revealed that ectopic signalling activity is perceived by cells in the anterior region of the E7.75 compound heterozygous embryo, suggesting that the elevated canonical WNT signalling may precede or be coincidental with the emergence of anterior defects. The phenotype of Dkk1+/–;Wnt3+/– embryos is variable but can be categorized in four ways:

- **Class I**, normal (30%);
- **Class II**, reduced forebrain size (51%);
- **Class III** forebrain truncation with additional patterning defect such as malformed eye and branchial arches (8%); and
- **Class IV**, severe head truncation and/or trunk defects (11%) (Fig. 4A, Table 3).
In the Class II mutant embryos, the expression domains of Fgf8, Hesx1, and Six3, which mark the rostral forebrain tissues, are reduced (Fig. 4B; Fgf8, Hesx1, Six3) (Crossley and Martin, 1995; Thomas and Beddington, 1996; Oliver et al., 1995). In the mutant embryo that developed a relatively intact forebrain, Nkx2.1 expression was reduced, suggesting that the brain tissues lack the molecular characteristics of the ventral forebrain (Fig. 4B; Nkx2.1) (Camus et al., 2000). By contrast, expression of Pax6 is similar to that in the wild-type embryo (Fig. 4B; Pax6) (Inoue et al., 2000), suggesting the dorsal forebrain tissues are probably not affected. The most severely malformed embryos (Class IV) display an open neural tube, reduced head size and the formation of a solitary tissue mass in place of the paired branchial arches (see Fig. S4 in the supplementary material). The increase in the proportion of the Class I embryos from 30% at E9.5 to 88% at E10.5 might be due to the loss of the severely malformed (Class III-IV) embryos (Table 3). Alternatively, the morphological defects of forebrain might have been corrected during the development of some Class II embryos. The results of the compound heterozygote study suggest that Dkk1 and Wnt3 interact genetically, and that the reduction of Dkk1 activity in Dkk1+/−;Wnt3+/− embryos leads to abnormal head morphogenesis.

**Rescue of the Dkk1-null mutant phenotype by lowering the Wnt3 activity**

To test whether WNT3 signalling is targeted by DKK1 antagonistic activity, we examined the head phenotype of Dkk1−/−;Wnt3+/− embryos to see whether reducing the level of

![Fig. 4. Anterior defects of the compound Dkk1+/−;Wnt3+/− mutant embryo.](image)

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![Fig. 4. Anterior defects of the compound Dkk1+/−;Wnt3+/− mutant embryo.](image)

### Table 1. Levels of Dkk1 and Wnt3 expression in embryos of (A) Wnt3 and (B) Dkk1;Wnt3 crosses as determined by quantitative RT-PCR

<table>
<thead>
<tr>
<th>Genotype of embryo</th>
<th>Expression level†</th>
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<tr>
<td></td>
<td>Dkk1 (n)</td>
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<tr>
<td><strong>A. Wnt3+/− × Wnt3+/−</strong></td>
<td></td>
</tr>
<tr>
<td>Dkk1+/− Wnt3+/−</td>
<td>9903±10365 (7) [100%]</td>
</tr>
<tr>
<td>Dkk1−/− Wnt3−/−</td>
<td>10138±12083 (6) [102%]</td>
</tr>
<tr>
<td>Dkk1−/− Wnt3−/−</td>
<td>5552±1871** (8) [5.6%]</td>
</tr>
<tr>
<td><strong>B. Dkk1+/−;Wnt3+/− × Dkk1+/−;Wnt3+/−</strong></td>
<td></td>
</tr>
<tr>
<td>Dkk1+/−;Wnt3+/−</td>
<td>2265±275 (8) [100%]</td>
</tr>
<tr>
<td>Dkk1+/−;Wnt3−/−</td>
<td>2038±449 (9) [90%]</td>
</tr>
<tr>
<td>Dkk1−/−;Wnt3−/−</td>
<td>1617±317 (5) [71%]</td>
</tr>
<tr>
<td>Dkk1−/−;Wnt3−/−</td>
<td>1027±167* (7) [45%]</td>
</tr>
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*P<0.01, **P<0.01 by Student’s t-test compared with Dkk1+/−;Wnt3+/− level. The difference in the absolute values of the expression level between datasets A and B is due to the use of RT-PCR machines with different settings. However, each set is internally consistent as the samples were analyzed under similar conditions using the same instrument.

†Expression level: mean±s.e. arbitrary units normalized relative to GAPDH [%].
Table 2. The number of E7.0-E10.5 embryos of different genotypes derived from Dkk1+/−;Wnt3+/− intercross

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>7.0-8.0</td>
<td>1 (0)</td>
<td>3 (0)</td>
<td>1 (1)</td>
<td>8 (8)</td>
<td>5 (5)</td>
<td>4 (4)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>8.0-9.0</td>
<td>4 (0)</td>
<td>12 (1)</td>
<td>4 (1)</td>
<td>8 (6)</td>
<td>7 (7)</td>
<td>6 (6)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>9.5</td>
<td>12 (0)</td>
<td>24 (1)</td>
<td>17 (1)</td>
<td>45 (31)</td>
<td>12 (12)</td>
<td>11 (11)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Total</td>
<td>17 (0)</td>
<td>39 (2)</td>
<td>22 (3)</td>
<td>61 (45)</td>
<td>24 (24)</td>
<td>22 (21)</td>
<td>7 (7)</td>
</tr>
<tr>
<td>% Abnormal</td>
<td>0</td>
<td>5</td>
<td>14</td>
<td>74</td>
<td>100</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>10.5</td>
<td>11 (0)</td>
<td>19 (1)</td>
<td>21 (2)</td>
<td>26 (3)</td>
<td>0</td>
<td>7 (7)</td>
<td>22 (16)</td>
</tr>
<tr>
<td>% Abnormal</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>12</td>
<td>N/A</td>
<td>N/A</td>
<td>100</td>
</tr>
</tbody>
</table>

Embryos of three age groups (E7.0-8.0, E8.0-9.0 and E9.0-10.0) were collected. The number of embryos of each genotype is shown, followed by the number (% of embryos in each genotype) shown in the last row of each intercross.

Table 3. Distribution of embryos in the four categories of mutant phenotype in two types of compound mutant embryos at E8.5-9.5 and E10.5

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dkk1+/−;Wnt3+/−</td>
<td>E8.5-9.5</td>
<td>16 (30)</td>
<td>27 (51)</td>
<td>4 (8)</td>
<td>6 (11)</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>E10.5</td>
<td>23 (88)</td>
<td>3 (12)</td>
<td>0</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Dkk1+/−;Wnt3−/−</td>
<td>E8.5-9.5</td>
<td>0</td>
<td>12 (92)</td>
<td>1 (8)</td>
<td>13</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>E10.5</td>
<td>6 (27)</td>
<td>26 (27)</td>
<td>10 (46)</td>
<td>0</td>
<td>22</td>
</tr>
</tbody>
</table>

Data are presented as the number (%) of embryos in each category. Embryos are classified based on the following phenotypes: I, normal (indistinguishable from the wild type); II, reduced forebrain size or discernible decrease in the amount of brain tissue; III, similar to II, plus additional defect of the branchial arches; IV, severe head truncation and/or trunk defects.

WNT3 signalling may compensate for the complete loss of antagonist activity. All Dkk1+/−;Wnt3−/− embryos display anterior truncation at E8.5-9.5 (Table 3; Fig. 5A,B), but not as severe as that of Dkk1+/−;Wnt3+/− embryos. Surprisingly, by E10.5, about 27% of embryos develop morphologically normal head (Table 3, Fig. 5A), suggesting that there has been amelioration of the mutant phenotype in two types of compound mutant embryos, is restored in the rostral part of the head folds (Fig. 5B, asterisks in Fig. 5B, asterisks in Fig. 5B). In the E8.5-9.5 (Liu et al., 1997). In the E8.5... The activity of Dkk1 is therefore to delineate the boundary of the prospective head and/or forebrain field. In the skin, WNT and DKK proteins are shown to act as molecules in a reaction-diffusion mechanism for the spatial patterning of hair follicles (Sick et al., 2006). The activity of Dkk1 and Wnt3 in the anterior endoderm may be involved in a similar mechanism for delimiting the tissue domain of the head from the body.

DISCUSSION

Dkk1 activity may mark the site of WNT ligand-receptor interaction in the germ layers

In the early-streak stage embryo, Wnt3 expression in the visceral endoderm extends from the proximal-posterior region across the lateral region to reach the anterior proximal region of the embryo where Dkk1 is expressed. During gastrulation, Wnt3 is also expressed in the cells of the primitive streak. Dkk1-expressing cells are distributed in a crescent-shaped domain in the anterior endoderm (Kimura-Yoshida et al., 2005; Lewis et al., 2007), which demarcates the proximal and lateral border of the Fzd8 expression domain (Lu et al., 2004). The alignment of the Dkk1-expressing cells with the border between the Wnt3 and the Fzd8 domains suggests that Dkk1 activity may be induced by WNT3 signalling in cells that also express the Fzd8 receptor. A potential role for Dkk1 is therefore to delineate the boundary of the prospective head and/or forebrain field. In the skin, WNT and DKK proteins are shown to act as molecules in a reaction-diffusion mechanism for the spatial patterning of hair follicles (Sick et al., 2006). The activity of Dkk1 and Wnt3 in the anterior endoderm may be involved in a similar mechanism for delimiting the tissue domain of the head from the body.

Dkk1 expression in vivo is responsive to Wnt3 gene dosage

In the Dkk1-null mutant, the expression domain of Wnt3 and Sfrp5 remains unchanged, whereas that of Wnt2b and Wnt8a is slightly expanded and that of Sfrp1 is reduced. Overall, these changes are associated with an elevated level of WNT signalling perceived by cells in the anterior germ layer tissues. On the contrary, Dkk1 expression changes with decreasing Wnt3 gene dose: the expression domain is slightly altered with a halved dose of Wnt3 (Wnt3+/−/− embryo), significantly reduced to a ‘hypomorphic’ level in Dkk1−/−;Wnt3+/−/− embryo and almost completely lost in the Wnt3−/−/− embryo. The induction of Dkk1 by Wnt signal, which in turn antagonizes the signalling activity, constitutes a negative-feedback mechanism for regulating WNT signalling. That Dkk1 can be induced specifically by Wnt1 in the NIH3T3 cells (this study) and that this regulatory loop can be blocked by RNAi against Dkk1 in 293T cells (Niida et al., 2004) suggests that Dkk1 is the key component in modulating Wnt1 signals in these cell models. A similar negative-feedback mechanism is found for Axin2, which is directly activated by β-catenin/Tcf and inhibits WNT signalling by downregulating β-catenin (Jho et al., 2002; Aulehla et al., 2003).
It is possible that the activity of other canonical WNT genes [Wnt2, Wnt2b, Wnt6 and Wnt8a (Kemp et al., 2005)] that are expressed at similar developmental stages to Wnt3 may contribute to outcome of loss of Dkk1. However, the finding that reducing Wnt3 gene dose alone may substantially rescue the head truncation phenotype suggests that other WNT genes may have a relatively minor role in the initial phase of head induction.

**Dkk1 interacts specifically with Wnt3 signalling to regulate head formation**

Dkk1 and Wnt3 are expected to have opposing actions in establishing a regionalized and balanced WNT signalling activity for embryonic patterning (Fig. 6A,D). The loss of function of one of these two genes leads to totally different phenotypes: embryos that lack Dkk1 develop a truncated head (Mukhopadhyay et al., 2001) (Fig. 6E), whereas those lacking Wnt3 fail to gastrulate (Liu et al., 1999) (Fig. 6H). By studying the phenotype of the compound mutant embryo, we showed that the genetic interaction of Dkk1 and Wnt3 during gastrulation is essential for head morphogenesis. However, the interaction is more complex than a simple stoichiometric balance of gene dose. Halving the dose of each gene in the Dkk1<sup>+/−</sup>;Wnt3<sup>+/−</sup> embryo is expected to produce a reduced but otherwise balanced agonist versus antagonist activity (Fig. 6B). On the contrary, the level and the domain of Dkk1 expression in the compound heterozygous mutant embryo are lower and more restricted, respectively, than those in the Dkk1<sup>−/−</sup> embryo. In view of that, Dkk1 and Wnt act in a negative-feedback pathway, the loss of one allele of Wnt3 may result in a reduced level of agonist activity that may then induce less Dkk1 activity when only one wild-type Dkk1 allele present (Fig. 6C). The head phenotype of Dkk1<sup>−/−</sup>;Wnt3<sup>+/−</sup> embryo may be caused by the unbalanced WNT3 signalling resulting from the ‘hypomorphic’ Dkk1 activity, which cannot be substituted for by other antagonists. The phenotype of the compound heterozygous embryo is variable, which may suggest that the reduced gene dose has resulted in an unstable but generally excessive signalling activity (Fig. 6F). As well as anterior defects, some Dkk1<sup>+/−</sup>;Wnt3<sup>+/−</sup> embryos also display posterior defects, indicating that the WNT signal may not have been maintained at the required level in the posterior region of these embryos for the formation of trunk structures. This suggests that a proper level of interaction between Dkk1 and Wnt3 is essential for maintaining a balanced signalling activity for both anterior and posterior morphogenesis. Our results further show that reducing the Wnt3 dose in Dkk1<sup>+/−</sup> embryo can partially rescue the brain and pharyngeal defect, despite the absence of Dkk1 (Fig. 6G). This finding suggests that Wnt3 signal is specifically targeted by Dkk1 in the anterior germ layers to maintain at a proper level of activity for head morphogenesis.

**Dkk1 and cell movement in the endoderm**

In the Otx2-null mutant embryo, absence of Dkk1 activity is accompanied by the accumulation of nuclear and cytoplasmic β-catenin, and by the lack of cell movement in the visceral endoderm. Defects of cell movement can, however, be rescued by expressing Dkk1 from the Otx2 locus or lowering the level of WNT/β-catenin signalling by reducing the gene dose of Ctnnb1 [which encodes β-catenin (Kimura-Yoshida et al., 2005)].

The definitive endoderm is reputed to be an essential source of inductive signals for patterning the anterior region of the embryo and for the specification of forebrain tissues. A delay or failure to move the definitive endoderm to the anterior region of the embryo may underpin the loss of anterior structures in mutant embryos, owing to the lack of provision of patterning cues to the overlying tissues (Lewis and Tam, 2007). In the Lhx1-null mutant, definitive endoderm cannot move anteriorly to populate the foregut because of the immobility of the pre-existing anterior visceral endoderm (Shimono and Behringer, 2003; Tam et al., 2004). In the Mixl1-null
mutant, abnormal anterior development is associated with impaired endoderm cell movement owing to the lack of propulsive action caused by inefficient tissue accretion in the posterior endoderm (Tam et al., 2007). By tracking the movement of the endoderm cells in the Dkk1-null embryo, we have shown that loss of Dkk1 impedes the movement of the definitive endoderm. It is interesting that in the Dkk1-deficient zebrafish embryo, there is an accelerated anterior movement of mesoderm cells (Caneparo et al., 2007). In the mouse, Dkk1-deficient zebrafish embryo, there is an accelerated anterior movement of mesoderm cells (Caneparo et al., 2007). In the mouse, the expression of glypican 4 (the Knypek homologue) overlaps with that of Dkk1 in the anterior region of the embryo (Ybot-Gonzalez et al., 2005). This raises the possibility that Dkk1 acting in conjunction with glypican 4 may regulate cell movement in the endoderm in addition to its other role in modulating WNT3/β-catenin activity.

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Wnt3 signalling and head morphogenesis


