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

Samara L. Lewis[1], Poh-Lynn Khoo[1], R. Andrea De Young[1], Kirsten Steiner[1], Chris Wilcock[2], Mahua Mukhopadhyay[3], Heiner Westphal[3], Robyn V. Jamieson[1,2], Lorraine Robb[4] and Patrick P. L. Tam[1,2,*]

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. The 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) (Enkhmandakh et al., 2006). This complex interacts synergistically with OTX2 to activate target genes (Nakano et al., 2000). Interestingly, loss of the functions of *Ldb1*, *Ssdp1* 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 *Hesx1* 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 *Wnt8a* and acts in concert with *Dkk1* to attenuate WNT signalling in the anterior tissues (Lewis et al., 2007).

[1]Embryology Unit, Children's Medical Research Institute, University of Sydney, Locked Bag 23, Wentworthville, New South Wales, NSW 2145, Australia. [2]Faculty of Medicine, University of Sydney, Locked Bag 23, Wentworthville, New South Wales, NSW 2145, Australia. [3]Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institute of Health, Bethesda, MD 20892, USA. [4]The Walter and Eliza Hall Institute of Medical Research, 1G, Royal Parade, Parkville, Victoria 3050, Australia.

*Author for correspondence (e-mail: ptam@cmri.usyd.edu.au)

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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 Magnuson, 2005). *Wnt2b* 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 *Dkk1* and *Wnt3* activity at early gastrulation is essential for head development.

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×C57BL6 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 mutant phenotype. Embryos were also collected from pregnant mice of the $Dkk1^{+/-}$ intercross for the analysis of phenotype and marker expression. For detecting the response of cells to WNT signalling in embryos of different *Dkk1* genotypes, two lines of transgenic mice, TOPGal (DasGupta and Fuchs, 1999) and BATGal (Maretto et al., 2003), were crossed with $Dkk1^{+/-}$ mice to introduce the *lacZ* transgene onto a $Dkk1^{+/-}$ background. Mating of the resultant mice generated wild-type, $Dkk1^{+/-}$ and $Dkk1^{-/-}$ embryos that express one of the transgenic reporters, which can be detected by X-Gal staining for β-galactosidase activity. For transplantation experiments to examine the differentiation of $Dkk1^{-/-}$ cells, a stock of mice was produced by crossing $Dkk1^{+/-}$ mice with transgenic mice that co-express two transgenes (pCAGG-EGFP and *Hmgcr-nls-lacZ*) widely in embryonic tissues (Kinder et al., 2001). Offspring from this mating were selected for the $Dkk1^{+/-}$*;EGFP;lacZ* genotype. Embryos containing $Dkk1^{-/-}$ cells that harboured the *EGFP* and *lacZ* transgenes were obtained from pregnant female mice generated by intercrossing $Dkk1^{+/-}$*;EGFP;lacZ* mice.

Genotyping by PCR of *Dkk1* and *Wnt3* was performed on tail tissues of newborn mice and the yolk sac of embryos (primer sequences and PCR conditions available upon request). Mice harbouring the *lacZ* and *EGFP* transgenes were identified by X-Gal staining and visualizing the fluorescence of ear tissues.

Phenotypic analysis of *Dkk1* mutant embryos

Whole-mount in situ hybridization

Embryos were processed for in situ hybridization (ISH) according to the protocol of Wilkinson and Nieto (Wilkinson and Nieto, 1993) with the following modifications. Riboprobes were labelled with digoxigenin-11-UTP (Roche) using the AmpliScribe kit (Epicentre Technologies) or the MAXIscript T7/T3 Kit (Ambion). SDS was used in place of CHAPS in both prehybridization and hybridization, no RNA digestion was performed after hybridization, and formamide was omitted from post-hybridization washes. The following riboprobes (and the source) were used: *Dkk1*, *Sfrp1* and *Sfrp5* (H. Westphal); *Lhx1*, *Wnt3* and *Chrd* (R. Behringer); *Six3* (O. Guillermo); *Fgf8* (G. Martin); *Hesx1* (S. Dunwoodie); *T* (B. Hermann); *Tbx6* (D. Chapman); *En1* (A. Joyner); *Dlx5* (J. Rubenstein); *Sox17* (J. Gad); *Cer1*, *Foxa2* and *Sfrp5* (W. Shawlot); *Wnt2b* and *Wnt8a* (S. Aizawa); *Mixl1* (L. Robb); *Nkx2.1* (S. L. Ang); *Pax6* (K. Backs); *Axin2* (J. Martinez-Barbera); and *Shh* (B. St-Jacques).

Immunofluorescence

Mid-streak stage (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. Phallotoxin (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-streak stage $Dkk1^{-/-}$*;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-streak stage ARC/s 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.

Recipient embryos were cultured (Sturm and Tam, 1993) for 24 hours until they reached the six- to eight-somite stage. The host embryos were examined by fluorescence microscopy to visualise the distribution of the graft-derived EGFP-expressing cells. 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.

Electroporation of the endoderm

Mid-streak stage 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). The embryos were then cultured in vitro (Sturm and Tam, 1993). The sites of labelling were ascertained by the localization of the EGFP-expressing cells 3 hours after electroporation. After 24 hours of in vitro culture, the distribution of the EGFP-expressing cells along the anterior-posterior body axis was recorded.

RT-PCR analysis of *Dkk1* induction

NIH3T3 cell lines containing pLNCX retroviral vector expressing full-length cDNA, encoding *Wnts* or *lacZ* (Kispert et al., 1998) were used to test the induction of *Dkk1*. The expression of β-galactosidase, *Wnt1*, *Wnt3a*, *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).

Quantitation of *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 $Dkk1^{+/+};Wnt3^{+/+}$, $Dkk1^{+/+};Wnt3^{+/-}$, $Dkk1^{+/-};Wnt3^{+/+}$ and $Dkk1^{+/-};Wnt3^{+/-}$ genotypes (from the $Dkk1^{+/-};Wnt3^{+/-}$ intercross). Total RNA was isolated using the RNeasy Micro Kit (50) (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 posterior germ layer tissues of the gastrulating embryo and is normally absent from the progenitor of the rostral forebrain and frontonasal tissues.

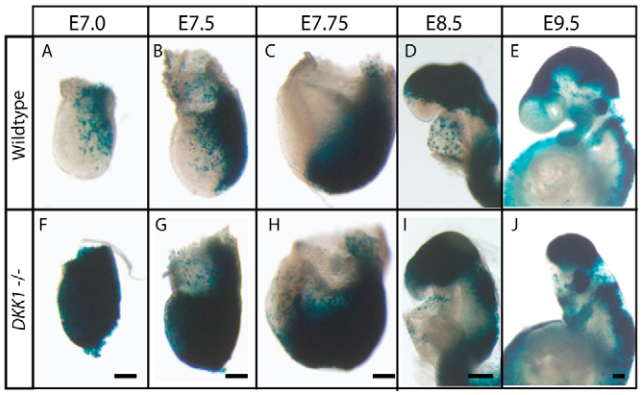

Fig. 1. Enhanced expression of the WNT reporter in the Dkk1$^{-/-}$ embryo. (**A-J**) Expression pattern of the BATGal reporter in (A-E) wild-type and (F-J) $Dkk1^{-/-}$ embryos (A,F, E7.0; B,G, E7.5; C,H, E7.75; D,I, E8.5; E,J, E9.5). In the mutant embryos, the *lacZ* expression domain expands to the anterior germ layer tissues at E7.0-7.75, encompasses the whole head folds at E8.5 and reaches the rostral-most region of the truncated head. Scale bar: 50 μm.

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. S1I,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 (*Mixl1*, *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

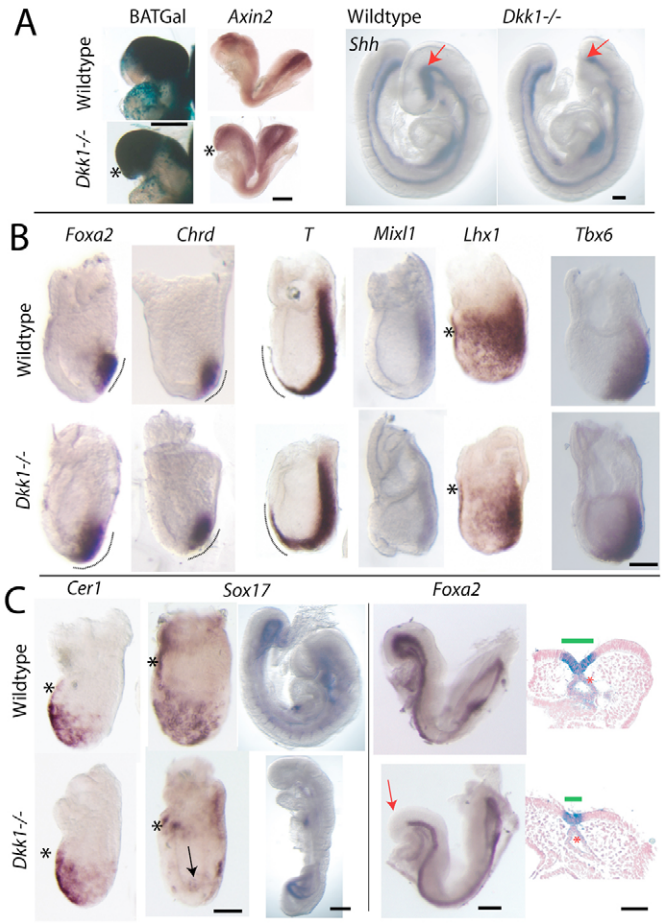

Fig. 2. Impact of loss of Dkk1 on mesoderm and endoderm differentiation and anterior morphogenesis. (**A**) *Dkk1*$^{-/-}$ embryo shows an expanded domain (black asterisk) of BATGal (2/2 embryos) and *Axin2* expression (3/3 embryos) in the head folds and anterior tissues, and loses head structures anterior to the upper hindbrain level (red arrow) with an abrupt truncation of *Shh*-expressing axial mesoderm and floor plate. Scale bar: 100 μm. (**B**,**C**) Marker analysis of the wild-type and *Dkk1*$^{-/-}$ embryos at E7.0 (B: *Foxa2*, *Chrd and Lhx1*; C: *Cer1* and *Sox17*), E7.5 (B: *Mixl1*, *T* and *Tbx6*), E8.5 (C: *Foxa2*) and E9.0 (C: *Sox17*). Scale bar: 50 μm. Markers of anterior visceral endoderm are expressed in the mutant embryos (black asterisk). The domain (indicated by lines) of *Foxa2* and *Chrd* expression in the node area is broader, and *T* expression extends more anteriorly in the head process mesoderm. The endoderm is formed (*Cer1*), but fewer *Sox17*-expressing cells are present in the mutant embryo (black arrow). *Sox17* expression is absent in the foregut of the E9.0 mutant embryo. At E8.5, the head truncation is clearly visible (*Foxa2*: whole-mount embryo, red arrow), and the histology (transverse section of the head) shows reduced *Foxa2* expression in the foregut (red asterisk) and the neural plate (green bar).

extensively to anterior structures, including the foregut and anterior axial mesoderm. Similarly, *Dkk1*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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) (Kimura-Yoshida et al., 2005). 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*$^{-/-}$ embryo, the expression of *Sfrp1* is reduced but *Sfrp5* does not change (Fig. 3F,F′,G,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*$^{-/-}$ embryo. *Dkk1* is not expressed in four out of five null-mutant embryo, and the remaining

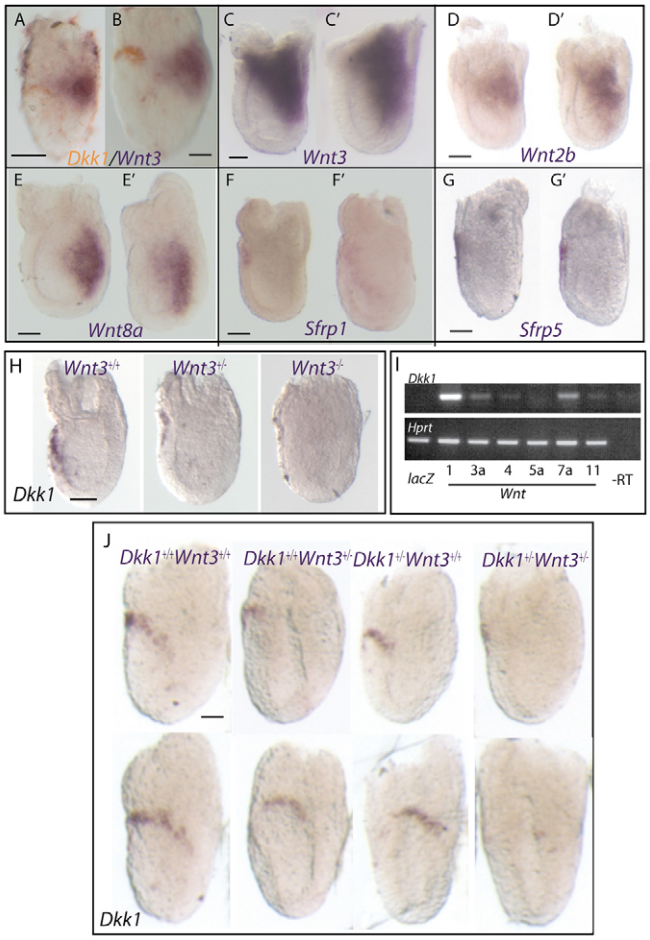

Fig. 3. Expression of WNT signalling pathway components in the mutant embryo. (**A**,**B**) Double in situ hybridization showing *Dkk1* (orange) and *Wnt3* (purple) expression domains. (A) At E6.5, the *Dkk1* expression domain is juxtaposed to the *Wnt3* domain. (B) At E7.25, the two domains become wider apart. (**C-G′**) Wild-type (C-G) and *Dkk1*$^{-/-}$ (C′-G′) E7.0 embryos: the domain of *Wnt3* (C,C′) remain unchanged, but that of *Wnt2b* (D,D′; six out of six mutant embryos) and *Wnt8a* (E,E′; one out of three mutant embryos) are slightly expanded in *Dkk1*-null embryos. The expression domain of *Sfrp1* (F,F′) is reduced while that of *Srfp5* (G,G′) is unchanged. (**H**) The domain of *Dkk1* expression becomes more restricted with the decrease in *Wnt3* gene dose. (**I**) RT-PCR analysis of *Dkk1* expression in NIH3T3 cells expressing *lacZ, Wnt1,Wnt3a, Wnt4, Wnt5a, Wnt7a* and *Wnt11*. –RT, no reverse transcription control; *Hprt*, loading control. (**J**) Reduced domain of *Dkk1* expression in the *Dkk1*$^{+/-}$;*Wnt3*$^{+/-}$ embryo, compared with *Dkk1*$^{+/+}$;*Wnt3*$^{+/+}$, *Dkk1*$^{+/+}$;*Wnt3*$^{+/-}$ and *Dkk1*$^{+/-}$;*Wnt3*$^{+/+}$ embryos (top row, lateral view; bottom row, frontal view). In situ hybridization for H and J was performed under the same conditions so that the staining result can be compared among embryos of each set. Scale bar: 50 μm.

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. 3J, *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 *Wnt5a* 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. 3I). 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).

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

Genotype of embryo		Expression level†	
		Dkk1 (*n*)	*Wnt3* (*n*)
A. *Wnt3*$^{+/-}$ × *Wnt3*$^{+/-}$			
Dkk1$^{+/+}$	*Wnt3*$^{+/+}$	99032±10365 (7) [100%]	6039±1294 (7) [100%]
Dkk1$^{+/+}$	*Wnt3*$^{+/-}$	101383±12083 (6) [102%]	5111±1995 (6) [84%]
Dkk1$^{+/+}$	*Wnt3*$^{-/-}$	5552±1871** (8) [5.6%]	118±60** (8) [1.9%]
B. *Dkk1*$^{+/-}$;*Wnt3*$^{+/-}$ × *Dkk1*$^{+/-}$;*Wnt3*$^{+/-}$			
Dkk1$^{+/+}$	*Wnt3*$^{+/+}$	2265±275 (8) [100%]	306±86 (8) [100%]
Dkk1$^{+/+}$	*Wnt3*$^{+/-}$	2038±449 (9) [90%]	175±49 (9) [57%]
Dkk1$^{+/-}$	*Wnt3*$^{+/+}$	1617±317 (5) [71%]	201±110 (5) [66%]
Dkk1$^{+/-}$	*Wnt3*$^{+/-}$	1027±167* (7) [45%]	149±34 (7) [49%]

*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 [%].

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

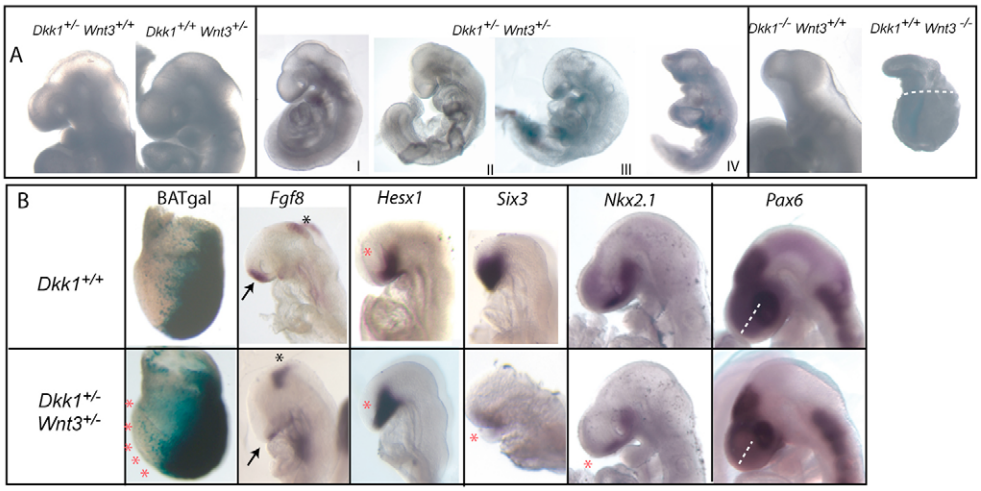

Fig. 4. Anterior defects of the compound *Dkk1*$^{+/-}$;*Wnt3*$^{+/-}$ mutant embryo. (**A**) The E9.5 *Dkk1*$^{+/-}$;*Wnt3*$^{+/-}$ embryos display the four categories of phenotypes (Class I-IV) with different degrees of head and trunk defects, compared with single heterozygous (*Dkk1*$^{+/-}$;*Wnt3*$^{+/+}$ and *Dkk1*$^{+/+}$;*Wnt3*$^{+/-}$) embryo with normal head morphology and single homozygous mutants showing head truncation (*Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$) or arrested development at gastrulation (*Dkk1*$^{+/+}$;*Wnt3*$^{-/-}$, broken white line marks the embryonic/extra-embryonic border of the embryo). (**B**) BATgal reporter is expressed ectopically in cells in the anterior region (red asterisks) of the E7.75 compound *Dkk1*$^{+/-}$;*Wnt3*$^{+/-}$ mutant embryo. Analysis of marker expression reveals the loss of forebrain tissue at E8.5 (*Fgf8*, *Hesx1* and *Six3*). In the *Dkk1*$^{+/-}$;*Wnt3*$^{+/-}$ embryos, *Fgf8* expression is reduced in the commissural plate (arrow) but not altered in the isthmus (black asterisk). Tissues anterior to the *Hesx1*-expressing domain are reduced (red asterisk). *Six3* expression is reduced in the ventral forebrain (red asterisk). At E9.5, the expression domain of *Nkx2.1*, which marks the ventral forebrain is reduced but that of *Pax6* for dorsal forebrain tissues is maintained. Broken line indicates the length of the forebrain, which is slightly shorter in the mutant embryo).

Table 2. The number of E7.0-E10.5 embryos of different genotypes derived from *Dkk1*$^{+/-}$;*Wnt3*$^{+/-}$ intercross

	Number of embryos of each genotype* (number displaying abnormalities)									
Age (E)	*Dkk1*$^{+/+}$ *Wnt3*$^{+/+}$	*Dkk1*$^{+/+}$ *Wnt3*$^{+/-}$	*Dkk1*$^{+/-}$ *Wnt3*$^{+/+}$	*Dkk1*$^{+/-}$ *Wnt3*$^{+/-}$	*Dkk1*$^{+/+}$ *Wnt3*$^{-/-}$	*Dkk1*$^{+/-}$ *Wnt3*$^{-/-}$	*Dkk1*$^{-/-}$ *Wnt3*$^{+/+}$	*Dkk1*$^{-/-}$ *Wnt3*$^{+/-}$	*Dkk1*$^{-/-}$ *Wnt3*$^{-/-}$	Total
7.0-8.0	1 (0)	3 (0)	1 (1)	8 (8)	5 (5)	4 (4)	1 (1)	4 (4)	3 (3)	30
8.0-9.0	4 (0)	12 (1)	4 (1)	8 (6)	7 (7)	6 (6)	2 (2)	3 (3)	2 (2)	48
9.5	12 (0)	24 (1)	17 (1)	45 (31)	12 (12)	12 (11)	4 (4)	10 (10)	3 (3)	139
Total number	17 (0)	39 (2)	22 (3)	61 (45)	24 (24)	22 (21)	7 (7)	17 (17)	8 (8)	217
% Abnormal	0	5	14	74	100	95	100	100	100	
10.5	11 (0)	19 (1)	21 (2)	26 (3)	0	0	7 (7)	22 (16)	0	106
% Abnormal	0	5	10	12	N/A	N/A	100	73	N/A	

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 (in parentheses) that displayed abnormal phenotype; the percentage of abnormal embryos in each genotype class is shown in the last row of each intercross.
*χ^2 analysis was performed on the frequency of each genotype across all age groups, but genotypes were not present at the expected Mendelian frequency (χ^2=30.5, significant difference at P<0.05) due to the loss of *Wnt3*-null embryos, which were arrested at gastrulation.
N/A, not applicable as no embryos of this genotype were found.

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 forebrain and branchial arch defects. Consistent with the rescue of the mutant phenotype, expression of several markers is restored in the *Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$ embryo (Fig. 5B). *Fgf8* expression, which is absent from the commissural plate of the *Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$ mutant, re-appears in the ventral forebrain of the *Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$ embryo. The other domain of *Fgf8* expression in the head of *Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$ embryo is localized to the equivalent of isthmus (asterisks in Fig. 5B, *Fgf8*). *Six3*, which is not expressed in the *Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$ embryo, is re-expressed in the presumptive forebrain of the rescued embryo (Fig. 5B, *Six3*). In the E9.5 *Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$ embryo, *En1* expression is reduced. The presence of a broader domain of *En1* expression in the *Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$ embryo (Fig. 5B: *En1*) (Rowitch et al., 1995) suggests that midbrain development may be restored. Whereas *Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$ embryos (7/7 embryos) had malformed branchial arches, morphologically normal *Dlx5*-expressing arches were formed in *Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$ embryos (three out of six embryos; Fig. 5B, *Dlx5*) (Liu et al., 1997). In the E8.5 *Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$ embryo, TOPGal-free tissue, which is greatly reduced in the *Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$ embryo, is restored in the rostral part of the head folds (Fig. 5B, TOPGal), suggesting that a WNT-signal free zone is re-established. In summary, lowering the level of *Wnt3* activity has partially compensated for the loss of *Dkk1* function, suggesting that *Dkk1* modulation of *Wnt3* activity at gastrulation is crucial for normal head development.

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

Genotype	Age	I	II	III	IV	Total
Dkk1$^{+/-}$;*Wnt3*$^{+/-}$	E8.5-9.5	16 (30)	27 (51)	4 (8)	6 (11)	53
	E10.5	23 (88)	3 (12)	0	0	26
Dkk1$^{-/-}$;*Wnt3*$^{+/-}$	E8.5-9.5	0	0	12 (92)	1 (8)	13
	E10.5	6 (27)	6 (27)	10 (46)	0	22

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.

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.

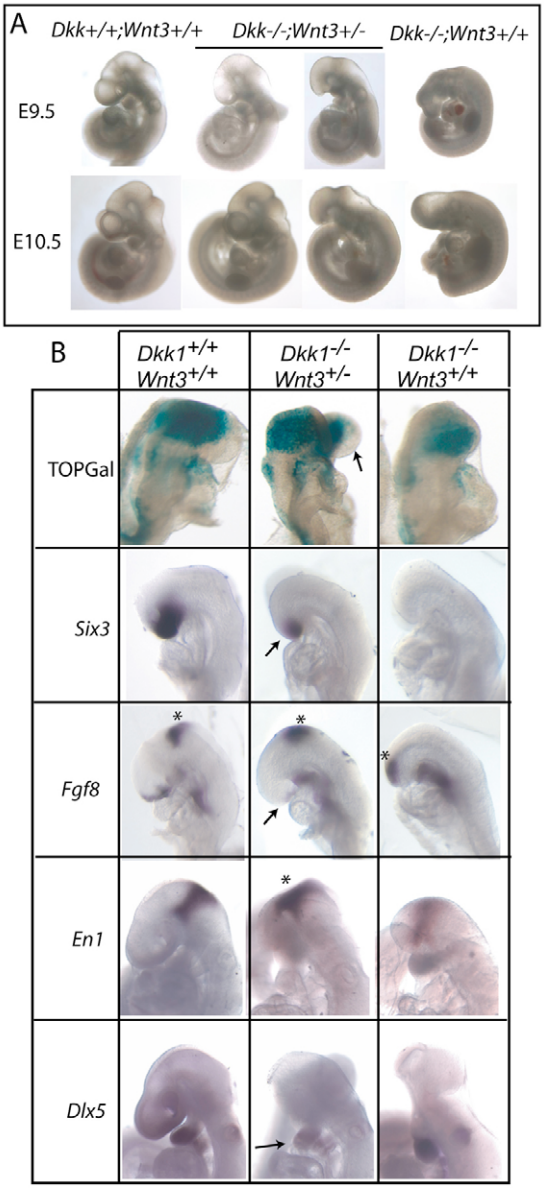

Fig. 5. Partial rescue of the head and forebrain defects in the *Dkk1*$^{-/-}$ embryo with reduced *Wnt3* gene dose. (**A**) Comparison of the morphology of the *Dkk1*$^{+/+}$;*Wnt3*$^{+/+}$, *Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$ and *Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$ embryos showing the almost complete to partial restoration of head structures in the *Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$ embryo at E9.5 and E10.5. (**B**) Marker analysis of wild-type (*Dkk1*$^{+/+}$;*Wnt3*$^{+/+}$), 'rescued' (*Dkk1*$^{-/-}$;*Wnt3*$^{+/-}$) and *Dkk*-null (*Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$) embryos (E8.5: TopGal, *Six3* and *Fgf8*; E9.5: *En1* and *Dlx5*). The presence of TOPGal-free and *Six3*-expressing domains (arrows) show the restoration of forebrain tissue in the 'rescued' embryos. Expression of *Fgf8* is present in the commissural plate (arrow) of the rescued embryo but is weaker than the wild type, whereas the isthmus is restored to a normal position (asterisk) that is shifted rostrally in the *Dkk1*$^{-/-}$;*Wnt3*$^{+/+}$ embryo. *En1* is restored to nearly wild-type pattern in the midbrain of the rescued embryo (asterisk). *Dlx5* expression (arrows) reveals the improved development of the branchial arch in the rescued embryo.

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*$^{+/-}$;*Wnt3*$^{+/-}$ 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*$^{+/-}$ 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*$^{+/-}$;*Wnt3*$^{+/-}$ 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*$^{+/-}$;*Wnt3*$^{+/-}$ embryos also display posterior defects, indicating that the WNT signal may not have been maintained at the required level in the posterior tissue 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*$^{-/-}$ 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, descendants of $Dkk1^{-/-}$ APS cells were absent from the posterior mesoderm of the wild-type host, which may reflect a preferential anterior localization of *Dkk1*-deficient mesoderm cells. These findings of two different embryo models raise the possibility that DKK1 may have cell-autonomous and/or a germ-layer (endoderm versus mesoderm)-specific role in navigating cell movement.

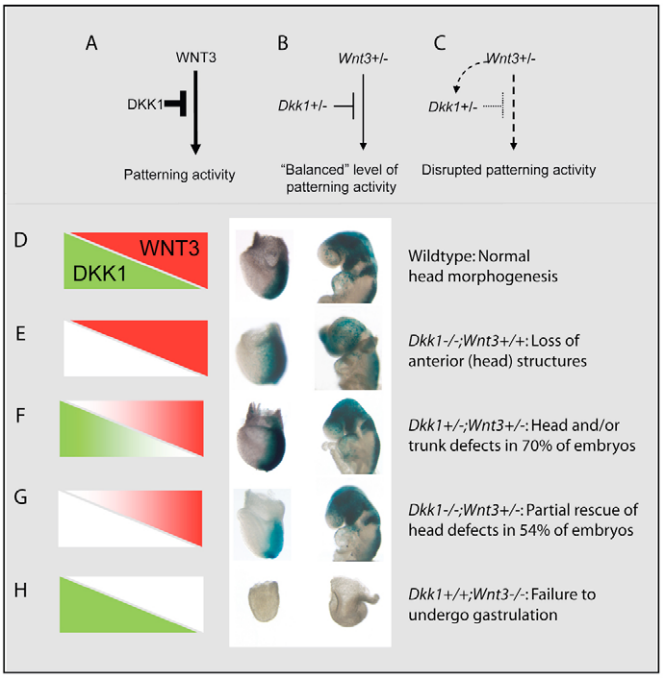

Fig. 6. The scheme of genetic interaction and the requirement of *Dkk1* and *Wnt3* activity in anterior morphogenesis. (**A**) The modulation of WNT3 signalling by the antagonistic action of DKK1 maintains a correct level of patterning activity. (**B**) Halving the dose of both genes in the $Dkk1^{+/-};Wnt3^{+/-}$ embryo would theoretically produce a 'balanced' level of patterning activity but at a reduced level (thinner arrow and inhibitory connector). (**C**) Because of the requirement of WNT3 signalling to regulate the expression of *Dkk1*, the reduced level of signalling associated with the $Wnt3^{+/-}$ genotype and the loss of one copy of transcription target *Dkk1* gene in the $Dkk1^{+/-};Wnt3^{+/-}$ embryo have resulted in a reduction of DKK1 activity to below the heterozygous ($Dkk1^{+/-}$) level, which may be inadequate for antagonizing WNT3 signalling (dotted inhibitory connector). This may lead to an unbalanced (and reduced) WNT3 signalling activity (broken arrow) that disrupts head morphogenesis in the $Dkk1^{+/-};Wnt3^{+/-}$ embryos. (**D-H**) The schematic profile of agonist (WNT3) and antagonist (DKK1) activity (red, WNT3 signal; green, DKK1 activity; white, no activity; shaded, reduced activity) in embryos of different *Dkk1* and *Wnt3* genotypes, correlated with the pattern of WNT reporter (TOPGal) expression in the E7.75 and E9.5 embryos, and the phenotypic consequences. (D) A balanced *Dkk1* and *Wnt3* activity in the wild-type embryo enables normal pattering and morphogenesis of anterior (head) structures. (E,F) The unbalanced agonist and antagonist activity leads to an elevated WNT signalling (revealed by the expanded TOPGal expression domain) and abnormal phenotypic outcome in embryos of (E) $Dkk1^{-/-};Wnt3^{+/+}$ (*Dkk1* null) and (F) $Dkk1^{+/-};Wnt3^{+/-}$ (compound heterozygous) embryos. (G) In $Dkk1^{-/-};Wnt3^{+/-}$ (rescued) embryos, the TOPGal expression domain is very similar to that of (D) the wild-type embryo. (H) *Wnt3*-null embryos do not develop beyond gastrulation and show no TOPGal expression when examined at E7.75 or E9.0, which may indicate a complete shutdown of WNT signalling function owing to arrested development.

It is likely that the canonical and non-canonical WNT pathways, and the processes that they control, namely cell differentiation and morphogenesis, are more likely to be interdependent than separate (Dabdoub and Kelley, 2005). A switch between the WNT/β-catenin and the WNT/PCP pathway may be achieved by regulating the level of *Dkk1* activity. It may be envisaged that as DKK1 sequesters LRP6 to block the β-catenin-LEF/TCF pathway, the frizzled receptors may become available for engagement with the PCP pathway. Presently, it is not known whether the PCP pathway is activated whenever *Dkk1* activity is absent and whether *Dkk1/Wnt3*/β-catenin activity has a direct role in endoderm movement. In the zebrafish, a switch may be mediated by the binding of Dkk1 to Knypek, thereby enhancing PCP activity that influences gastrulation cell movement (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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Supplementary material

Supplementary material for this article is available at
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