Hypomorphic expression of *Dkk1* in the *doubleridge* mouse: dose dependence and compensatory interactions with *Lrp6*

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Summary

doubleridge **is a transgene-induced mouse mutation displaying forelimb postaxial polysyndactyly. We have cloned the** ***doubleridge*** **transgene insertion site and demonstrate that** ***doubleridge*** **acts in** ***cis*** **from a distance of 150 kb to reduce the expression of dickkopf 1 (*Dkk1*), the secreted Wnt antagonist. Expression of *Dkk1* from the *doubleridge* allele ranges from 35% of wild-type level in E7.0 head to <1% of wild type in E13.5 tail. *doubleridge* homozygotes and *doubleridge/*null compound heterozygotes are viable. An allelic series combining the wild-type, *doubleridge* and null alleles of *Dkk1* demonstrates the effect of varying *Dkk1* concentration on development of limb, head and vertebrae. Decreasing expression of *Dkk1* results in hemivertebral fusions in progressively more anterior positions, with severity increasing from tail kinks to spinal curvature. We demonstrated interaction between *Dkk1* and the Wnt co-receptors *Lrp5* and *Lrp6* by analysis of several types of double mutants. The polydactyly of *Dkk1*$^{d/d}$ mice was corrected by reduced expression of *Lrp5* or *Lrp6*. The posterior digit loss and axial truncation characteristic of *Lrp6* null mice was partially corrected by reduction of *Dkk1*. Similarly, the anterior head truncation characteristic of *Dkk1* null mice was rescued by reduction of *Lrp6*. These compensatory interactions between *Dkk1* and *Lrp6* demonstrate the importance of correctly balancing positive and negative regulation of Wnt signaling during mammalian development.**

Key words: Dkk1, Lrp6, Wnt, Limb, Polydactyly

Introduction

The Wnt signal transduction pathways direct cell proliferation, cell identity and cell polarity from embryonic patterning to adult homeostasis. The mechanism of Wnt signaling involves formation of a complex between an extracellular Wnt ligand and two membrane proteins, a seven-transmembrane Frizzled (Fz) receptor, and a lipoprotein-receptor related protein, Lipoprotein receptor-related protein 5 (Lrp5) or Lrp6. Formation of this complex leads to cytoplasmic accumulation of the active form of β-catenin and its translocation to the nucleus (Huelsken and Behrens, 2002). Nuclear β-catenin binds to the transcription factors Tcf and Lef1 and activates the transcription of target genes (Moon et al., 2002). The extracellular protein Dkk1 inhibits the canonical Wnt pathway by binding the Lrp5/6 receptor (Mao et al., 2001; Bafico et al., 2001; Semenov et al., 2001). Recent experiments indicate that the Dkk1-Lrp complex is removed from the membrane via interaction with the membrane proteins Kremen 1 or Kremen 2 (Mao et al., 2002; Davidson et al., 2002).

Dkk1 was identified in a screen for head inducers in *Xenopus* (Glinka et al., 1998). Overexpression of *Dkk1* in early *Xenopus* blastomeres led to embryos with enlarged anterior head structures, whereas injection of antibody to *Dkk1* induced microcephaly. The role in head development is conserved in mammals. Mice lacking *Dkk1* exhibit incomplete development of structures anterior to the midbrain, resulting in perinatal death (Mukhopadhyay et al., 2001). The null mice also display polydactyly and syndactyly, demonstrating the role of *Dkk1* in limb development.

Mammalian Dkk1 is a 266-residue protein with two cysteine-rich domains (Fedi et al., 1999). The second cysteine-rich domain is required for binding to Lrp6 and Kremen 2 (Li et al., 2002; Mao and Niehrs, 2003). Three vertebrate paralogs, *Dkk2*, *Dkk3* and *Dkk4*, were identified by sequence homology (Glinka et al., 1998; Krupnik et al., 1999). Dkk2 and Dkk4 bind Lrp5/6 and inhibit Wnt signaling with lower affinity than Dkk1. Dkk2 can activate Wnt signaling in cells lacking Krm2 (Mao and Niehrs, 2003). Dkk3 does not bind to Lrp5/6 or Kremen, but may play a role in Wnt signaling (Krupnik et al., 1999; Mao et al., 2001; Mao et al., 2002).

Lrp5 and *Lrp6* are widely expressed during embryonic development and in many adult tissues (Brown et al., 1998; Hey et al., 1998; Pinson et al., 2000). In in vitro assays, the affinity of Dkk1 for Lrp6 is greater than for Lrp5 (Bafico et al., 2001). The phenotypic consequences of mutations in Wnt proteins and Lrp receptors are closely related. *Lrp6* null mice exhibit defects in neural tube closure and midbrain/hindbrain like *Wnt1* null mice, axial truncation and loss of hindlimbs like *Wnt3a* hypomorphs, and urogenital defects, loss of posterior digits and double ventral forelimbs like *Wnt7a* null mice (Pinson et al., 2000).

Deficiency of *Lrp5* results in low bone mass, failure of postnatal regression of eye vasculature, and abnormal metabolism of cholesterol and glucose (Kato et al., 2002;

Fujino et al., 2003). Loss-of-function mutations of human *LRP5* result in osteoporosis-pseudoglioma syndrome, a recessive disorder with low bone mass and disruptions in ocular vasculature (Gong et al., 2001), whereas gain-of-function mutations in *LRP5* result in excess bone density (Little et al., 2002; Boyden et al., 2002; Van Wesenbeck et al., 2003). Dkk inhibitors have been suggested as potential therapeutic agents for osteoporosis (Patel and Karsenty, 2002).

The *doubleridge* mutant mouse was identified in a screen for recessive, transgene-induced insertional mutants (Adamska et al., 2003). The *doubleridge* transgene randomly inserted into an SJL-derived segment of chromosome 19 in a microinjected (C57BL/6×SJL)F_2 fertilized egg. Homozygous *doubleridge* mice exhibit defective limb development. In this report we describe cloning of the *doubleridge* insertion site and demonstration that *doubleridge* is a hypomorphic allele of *Dkk1*. We used this viable mutant to investigate the interaction of *Dkk1* with other components of Wnt signaling during development.

Materials and methods

Cosmid library

Genomic DNA from spleen of homozygous *doubleridge* adults was prepared as described by Lu et al. (Lu et al., 1999) and partially digested with MboI (New England Biolabs). Digested genomic DNA was separated by size on a 5-40% sucrose gradient centrifuged at 89,000 ***g*** for 16 hours. Fragments between 35-45 kb were selected as inserts for a cosmid library using the SuperCos I vector (Stratagene) and packaged into phage heads using Gigapak XL III packaging extracts (Stratagene). Cosmid clones (200,000, equivalent to 2×genomic coverage) were screened by filter hybridization (Lu et al., 1999) using two transgene probes, a 290 bp fragment of the rat Cacnb4 cDNA and a 320 bp fragment of rat NSE intron 1 (Kearney et al., 2001). Cosmid clones positive for both probes were end-sequenced and subcloned to isolate the transgene junction fragments.

Genotyping

Dkk1 genotype of *doubleridge* mice was determined by amplification of the proximal transgene junction with three primers, F1 (5′ GTT TCA GCC CCA AAG ACT GCA TAG), R1-genomic (5′ TTC ATT GAC GCT TTC CTT TCC AAG) and R-2 transgenic (5′ GAA TGT TGA GAG TCA GCA GTA GCC) using the following PCR conditions: incubation at 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes, followed by a final extension of 7 minutes at 72°C. The Dkk1 targeted null allele was detected with primers for the Neomycin-resistance cassette: Dkk1-Neo F: 5′CTT GGG TGG AGA GGC TAT TC and Dkk1-Neo R: 5′AGG TGA GAT GAC AGG AGA TC. Genotypes for Lrp5 and Lrp6 null mutations were determined with the following primers: Lrp5-NeoF, 5′GCA GCG CAT CGC CTT CTA TC; Lrp5-genomicF, 5′GAG CTC TCA AGC TCA GCC AG; Lrp5-genomicR, 5′CTT CTC TCC AGA CTC CCA AAG C; Lrp6-genomic3F, 5′CAG GCA TGT AGC CCT TGG AG; Lrp6-genomic4R, 5′ACT ACA AGC CCT GCA CTG CC and Lrp6-insert R, 5′GTA GAG TTC CCA GGA GGA GCC.

Primer extension/chain termination assay

Embryos from timed matings were dissected as follows: E7.0 whole embryo; E8.5 head and tail; E9.5 head, forelimb and tail; E10.5 head, forelimb, hindlimb and tail; and E13.5 head, forelimb, hindlimb and tail. Genomic DNA was prepared from extraembryonic membranes for genotyping. Tissues from littermates with identical genotypes were pooled and RNA was prepared with the TRIzol reagent (Invitrogen). RNA (≤1 μg) was treated with Dnase I (Invitrogen) and first-strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen) using random hexamer primers. The 3′ untranslated region (UTR) of Dkk1 was amplified with primers 1 (5′AGG GGA AAT TGA GGA AAG CAT C) and 2 (5′TTG GAA GGT ATT GTC GGA ATG C) using PfuTurbo DNA polymerase (Stratagene). The 499 bp RT-PCR product was separated from the 570 bp product of genomic DNA by gel purification. Thermosequenase (Stratagene) was used for the extension reactions from primer 3 (5′TGC CAG AGA CAC TAA ACC GAC AGT C) in the presence of dATP, dCTP, dCTP and α^{33}P-ddGTP. The relative amount of 31 bp product from the C3H allele and 33 bp product from the SJL allele was determined by densitometry using the BioRad Molecular Imager FX with Quantity One software, as described previously (Kearney et al., 2002; Buchner et al., 2003).

Wholemount in situ hybridization

Embryos were genotyped by PCR of genomic DNA from embryonic membranes. E0.5 was considered to be noon of the day when the vaginal plug was found. Hybridization was performed with a single digoxygenin-labelled probe (Bober et al., 1994) using BM Purple (Roche) as the alkaline phosphatase substrate. The Dkk1 probe was described by Glinka et al. (Glinka et al., 1998).

Skeletal preparations

Samples were prepared and stained as described (Kimmel and Trammell, 1981), using alcian blue to stain cartilage and alizarin red to stain bone.

Northern blots

RNA was prepared from adult tissues and analyzed as described (Kearney et al., 2001). cDNA probes were amplified by RT-PCR. The 972 bp mannose binding lectin 2 (*Mbl2*) fragment was amplified with primers F (5′CTT GCC TCC TGA GTC TTT GCT G), R (5′TTT TCA GAA CAA ACT GCG GAC G); Prkg1 (818 bp) F (5′CAT TTA CAG GGA CCT CAA GCC G), R (5′GCT TTG CTT CAG GAC CAC CAT G); AK006729 (359 bp) F (5′TCT GGC AAC ATA AAC GGA AGT G) R (5′TGG ATT GAG AAG CGT GTA GGA G).

Histology

After fixation in Bouin's solution, E17.5 embryos were embedded in paraffin and sectioned. Six micron cross-sections of forelimbs were stained with hematoxylin and eosin.

Results

Cloning and characterization of the *doubleridge* insertion site

A library of genomic DNA from homozygous *doubleridge* mice was constructed in a cosmid vector and screened by hybridization with transgene-specific probes. Subclones of positive cosmids were sequenced to identify the junctions between transgene and mouse genomic DNA (Fig. 1A). Mouse genomic sequence obtained from the ends of the overlapping cosmids 4-1 and 5-2 matched the sequence of mouse chromosome 19 (http://www.ensembl.org/Mus_musculus/). There is a gap of 60 kb between the location of the two junction sequences in the assembled genome (Fig. 1B). To determine whether the intervening 60 kb was deleted from the *doubleridge* insertion site, we designed primer pairs at 10-kb intervals across the region. The predicted PCR fragments were amplified from wild-type SJL genomic DNA, but not from *doubleridge* genomic DNA (Fig. 1C). The data demonstrate a 60-kb deletion at the *doubleridge* insertion site on chromosome 19.

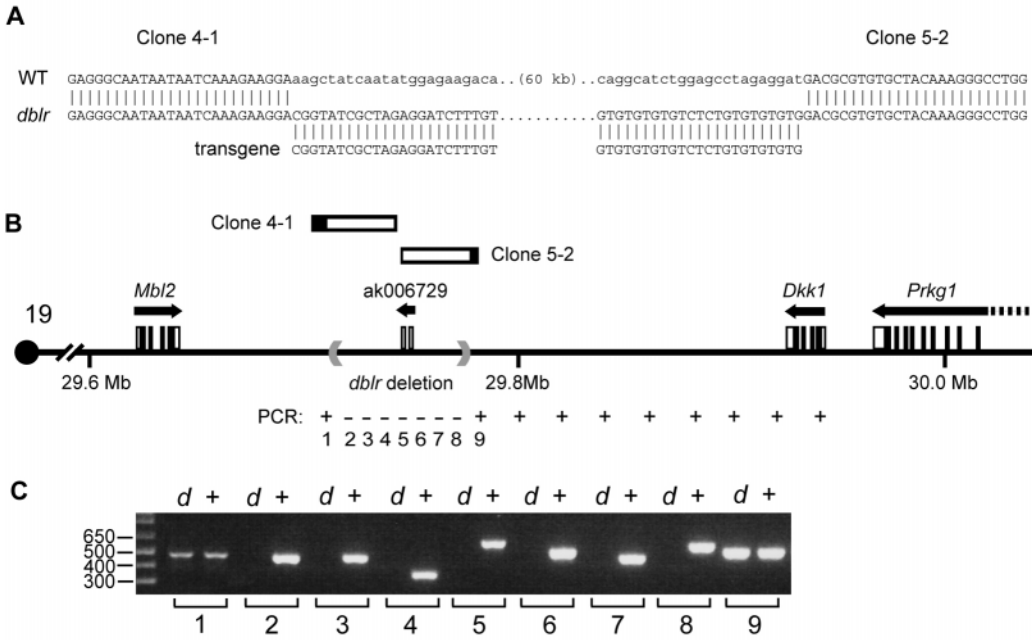

Fig. 1. Structure of the transgene insertion site in the *doubleridge* mutant. (A) Sequence of the two junction fragments from overlapping cosmid clones. (B) Position of the mouse genomic sequence in cosmid clones (black) aligned with the assembled genomic sequence of a 400-kb region of mouse chromosome 19. The position of the 60-kb *doubleridge* deletion and nearby genes is shown. Open box, noncoding exon; filled box, coding exon; arrow, direction of transcription. The promoter of *Prkg1* is located at 31.3 Mb. (C) Confirmation of the 60-kb deletion in *doubleridge* genomic DNA. Primer pairs located between the cosmid junction sequences do not amplify *doubleridge* genomic DNA.

Analysis of the *doubleridge* chromosome region

The *doubleridge* transgene inserted into a gene-poor region of chromosome 19 that corresponds to human chromosome band 10q21.1. The mouse genomic DNA was queried to multiple DNA analysis programs using the NIX site at the UK Human Genome Mapping Project Resource Centre (http://www.hgmp.mrc.ac.uk/NIX/). The closest known genes to the insertion site are *Dkk1* and *Mbl2*, located at a distance of 150 kb and 90 kb, respectively (Fig. 1).

The only transcript deleted by the *doubleridge* mutation is a testis EST represented by a singleton clone, AK006729, in the NCBI database. This EST contains two exons but lacks an open reading frame. There is no corresponding human EST, and the lack of sequence conservation in the human genome (<50% sequence identity) suggests that this is not a functional transcript. An LTR element of the RMER15 class in exon 1 accounts for 74/148 bp of exon 1 and a SINE repeat accounts for 56/259 bp in exon 2. RT-PCR from 20 wild-type mouse tissues indicated that transcription is restricted to testis. The fertility of *doubleridge* males demonstrates that the transcript is not required for reproduction. It is unlikely that deletion of this EST contributes to the *doubleridge* phenotype.

We compared the human and mouse sequences spanning the 60-kb deletion using the programs PipMaker and Vista, in order to detect functional noncoding elements (Schwartz et al., 2000; Mayor et al., 2000). Seven conserved non-coding elements ≥100 bp in length with ≥75% sequence identity were identified (data not shown), but none of these were conserved in the corresponding regions of the *Fugu* or zebrafish genomes (Ensembl sequence assembly of 3/3/03 for *Fugu* and 7/2/03 for zebrafish).

Non-complementation of *doubleridge* and *Dkk1*

The limb abnormality in *doubleridge* mice is very similar to that of *Dkk1* null mice (Mukhopadhyay et al., 2001). To determine whether *doubleridge* is an allele of *Dkk1*, we performed a complementation test with the null allele. Homozygous *doubleridg*e females were crossed with a heterozygous $Dkk1^{+/-}$ male and forelimbs were examined at E18.5. Two phenotypic classes of offspring were obtained in equal numbers: 9/18 with normal limbs (Fig. 2A) and 9/18 with postaxial polysyndactyly of the forelimb (Fig. 2B). Genotyping demonstrated that all of the offspring with normal forelimbs inherited the *Dkk1* wild-type allele, whereas all of the offspring with polysyndactyly inherited the *Dkk1* null allele from the heterozygous parent. The forelimb abnormalities in the affected offspring are similar to those in *doubleridge* and *Dkk1* null homozygotes (Fig. 2C,D). The failure of the *doubleridge* chromosome to complement the limb abnormality, together with its chromosomal location, demonstrate that the *doubleridge* mutation generated a new allele of *Dkk1*, designated $Dkk1^{d}$. The $Dkk1^{d/-}$ compound heterozygotes are fully viable on the mixed genetic background of this cross.

Lack of rearrangement of *Dkk1* exons in *doubleridge* mice

To determine whether the Dkk1 coding sequence is rearranged or mutated in *doubleridge* mice, we amplified a 2.7-kb fragment of genomic DNA extending from 71 bp upstream of the first exon to 93 bp downstream of the last exon (Fig. 1). No differences in size or sequence were detected between the PCR products from *doubleridge* homozygotes and wild-type SJL mice. Southern blots of genomic DNA digested with

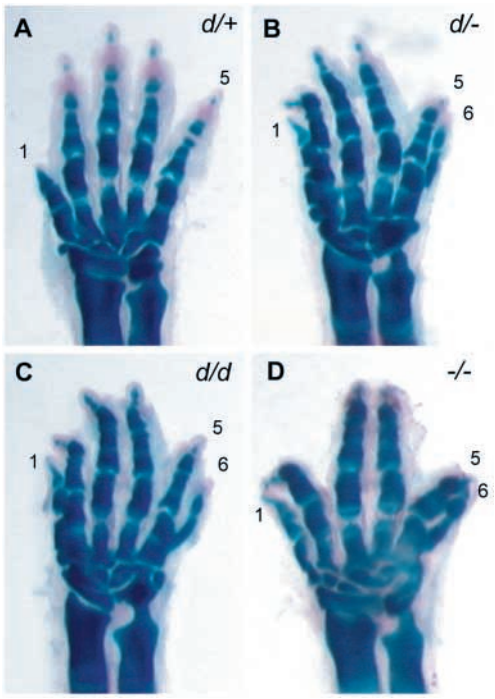

Fig. 2. Non-complementation between the *doubleridge* mutation and a null allele of *Dkk1*. (A) Normal limb development in $Dkk1^{d/+}$ heterozygote; (B) Postaxial polysyndactyly in $Dkk1^{d/-}$ compound heterozygote; (C) Postaxial polysyndactyly in $Dkk1^{d/d}$ homozygote, (D) Postaxial polysyndactyly in $Dkk1^{-/-}$. Forelimbs from E18.5 embryos were stained with alcian blue and alizarin red.

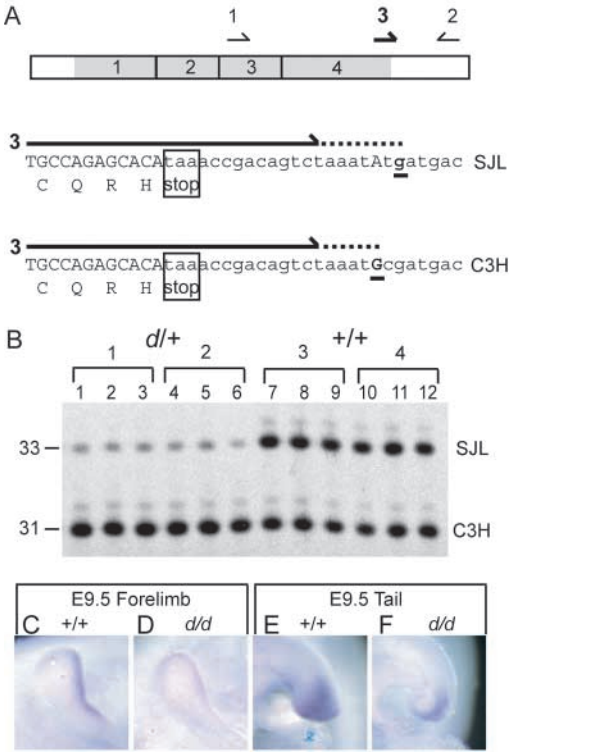

Fig. 3. Hypomorphic expression of the $Dkk1^{d}$ (*doubleridge*) allele detected by primer extension and wholemount in situ hybridization. (A) Quantitative assay of allelic *Dkk1* transcripts in heterozygous mice. Primers 1 and 2 are used for RT-PCR amplification of transcripts, and the 25 bp primer 3 for primer extension of the amplified RT-PCR product. The single nucleotide polymorphism in the 3′ UTR that distinguishes the C3H and SJL transcripts is shown in bold. The extension products obtained in the presence of α^{33}P-ddGTP are 31 bp in length for the C3H allele and 33 bp in length from the SJL allele, the parental allele for the *doubleridge* insertion. (B) Primer extension products are separated on a 10% acrylamide gel and visualized using BIOMAX film. Triplicate assays of four RNA samples from pooled hindlimbs at E13.5. Samples 1 and 2, (C3H X SJL)F1-$Dkk1^{d/+}$ *doubleridge* heterozygotes; 3 and 4, (C3H X SJL)F1 wild-type heterozygotes. (C-F) Wholemount in situ hybridization of $Dkk1^{d/d}$ embryos and +/+ littermates with a Dkk1 cDNA probe demonstrates reduced expression in multiple domains. (C,D) E9.5 forelimb; (E,F) E9.5 tail bud.

BamH1, EcoRI, HindIII and ScaI were hybridized with a full-length Dkk1 cDNA probe. No differences were observed in the lengths or hybridization intensity of restriction fragments (data not shown). These results demonstrate that there are no mutations or rearrangements of the Dkk1 exons and introns as a result of the *doubleridge* mutation.

Quantitation of *Dkk1* expression from the *doubleridge* allele

The *doubleridge* mutation arose on an SJL chromosome (Adamska et al., 2003). To develop an assay for quantitation of *Dkk1* transcripts, we first identified a single nucleotide polymorphism in the 3′ UTR that differed between the founder strain SJL and a control strain, C3H. The relative abundance of the two allelic transcripts in (SJL X C3H) F1 heterozygous mice was determined with a primer extension/chain termination assay based on the polymorphic nucleotide (Fig. 3A). RNA from heterozygous embryos was amplified by RT-PCR using primers 1 and 2, followed by a primer extension step using primer 3 in the presence of α^{33}P-ddGTP. The radiolabelled product obtained from the C3H allele is 31 bp in length, whereas the product of the SJL allele is 33 bp in length. The two products were separated by gel electrophoresis and visualized on X-ray film or with a phosphorimager.

C3H mice were crossed with wild-type SJL and with *doubleridge* homozygotes. In RNA from wild-type (SJL X C3H) F1 embryos, the amount of product from the SJL and C3H alleles is equal (Fig. 3B, lanes 7-12). When the SJL chromosome was inherited from a *doubleridge* homozygote, the abundance of the SJL allele product in the (SJL X C3H) F1-$Dkk1^{d/+}$ heterozygote is significantly lower than the C3H allele product (Fig. 3B, lanes 1-6). The reduction in relative expression of the $Dkk1^{d}$ allele demonstrates that the *doubleridge* mutation acts in *cis* to reduce the expression of *Dkk1*. To quantitate the reduction in expression of the $Dkk1^{d}$ allele, RNA from dissected embryonic tissues representing the major *Dkk1* expression domains was assayed and primer extension products were quantitated with a phosphoimager. Expression of the *doubleridge* allele ranged from 35% to <1% of wild-type in embryonic tissues between E7.0 and E13.5 (Table 1).

Table 1. The *doubleridge* mutation reduces *Dkk1* expression

	% of wild-type expression				
	E7.0	E8.5	E9.5	E10.5	E13.5
Head	34±5	25±2	11±2	16±3	13±4
Tail		23±4	22±4	23±3	<1
Forelimb			4±0.2	7±2	12±1
Hindlimb				8±2	8±1

Dkk1$^{d/d}$ mice, which are homozygous for the 3′ UTR from the SJL allele of *Dkk1*, were crossed with wild-type C3H mice. RNA samples were prepared from pooled tissues of the *Dkk1*$^{d/+}$ F1 littermates. Primer extension assays were carried out as described in Fig. 3. Values represent the radioactivity in the 33 bp SJL allele product as percent of radioactivity in the 31 bp C3H allele product, mean±s.d. (n=3). In control tissues from F1 offspring of wild-type SJL mice, examined at E11.5 and E13.5, the amount of SJL transcript was equal to 100% of the C3H transcript (see text for discussion).

Distribution of *Dkk1* transcripts in *doubleridge* embryos

The effect of the *doubleridge* mutation on specific *Dkk1* domains was examined by wholemount in situ hybridization. *Dkk1*$^{d/+}$ heterozygotes were intercrossed, and littermate *Dkk1*$^{d/d}$ and *Dkk1*$^{+/+}$ offspring were processed and stained in parallel. Expression of *Dkk1* was reduced in the ventral ectoderm and mesoderm of the forelimb bud (Fig. 3C,D). Reduced expression was also evident in the presomitic mesoderm of the E9.5 tail bud (Fig. 3E,F). Widespread reduction of *Dkk1* expression was observed at E11.5 and E13.5 in the mutant embryos, in agreement with the quantitative data in Table 1.

Other genes are not affected by the *doubleridge* transgene insertion

To determine whether the *doubleridge* insertion affects the expression of other genes in the *Dkk1* region, we examined the expression of the two closest genes, *Mbl2* and *Prkg1 (*cGMP-dependent protein kinase 1) (Fig. 1B). Northern blot analysis of RNA from adult tissues demonstrated no difference between mutant and wild-type expression of *Mbl2* in adult liver, and no difference in expression of *Prkg1* in brain and heart (data not shown). The size and abundance of the transcripts was consistent with previous reports (Pfeifer et al., 1998; Hansen et al., 2000). *Prkg1* expression in E13.5 limb bud was examined by semiquantitative RT-PCR and did not differ between *doubleridge* and wild-type embryos. The adjacent genes thus appear to be unaffected by the transgene insertion.

Effect of reduced *Dkk1* expression on head development

In the allelism tests described above, *Dkk1*$^{d/–}$ compound heterozygotes on a mixed genetic background were viable and fertile, with complete head development like the *Dkk1*$^{d/d}$ homozygote (Fig. 4A). On a predominantly C57BL/6J genetic background, however, a variety of severe cranial defects were observed in compound heterozygotes (Fig. 4B), including hydrocephaly with micrognathia (Fig. 4C). The most severely affected *Dkk1*$^{d/–}$ compound heterozygotes exhibit neonatal lethality with anophthalmia and hypoplastic anterior head structures (Fig. 4E). Nonetheless, these defects are less severe than those of *Dkk1* null mice, which exhibit anterior truncation of the head (Fig. 4F,G). The level of *Dkk1* expression in compound heterozygotes appears to be close to the threshold required for development of anterior head structures.

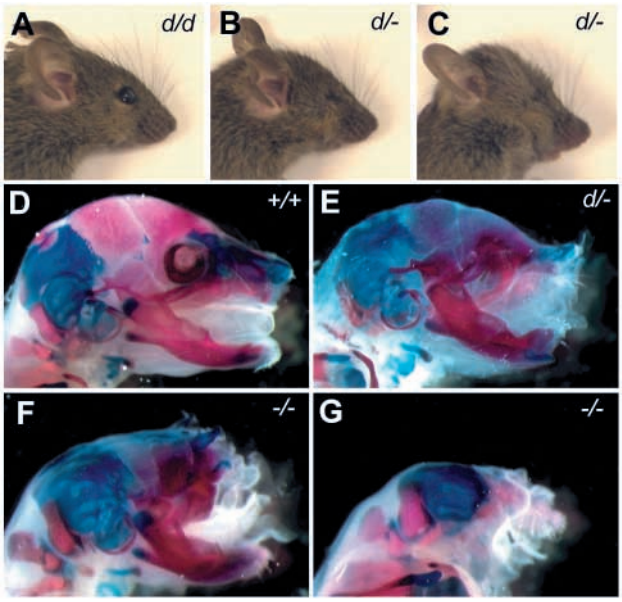

Fig. 4. Effect of varying *Dkk1* expression on development of anterior head structures. (A) Normal head development in *Dkk1*$^{d/d}$ homozygote; (B,C) two viable *Dkk1*$^{d/–}$ compound heterozygotes with closed eyes and hydrocephaly. (D-G) Skeletal preparations stained with Alcian Blue and Alizarin Red: (D) wild type at P0; (E) severely affected *Dkk1*$^{d/–}$ compound heterozygote with anophthalmia at P0; (F,G) head development in two *Dkk1*$^{–/–}$ null homozygotes at E18.5.

Effect of reduced *Dkk1* expression on vertebral development

Both tail kinks and fused vertebrae were observed in *Dkk1*-deficient mice. In *Dkk1*$^{d/d}$ homozygotes, tail kinks are rare and restricted to the tip of the tail (Fig. 5A). Most *Dkk1*$^{d/–}$ compound heterozygotes display kinked tails, with some defects extending to the most anterior portion (Fig. 5B,C). Cartilaginous fusions are visible in the newborn tail (Fig. 5D). In some cases, there are hemivertebrae in the lumbar region with the appearance of trapezoidal vertebral bodies (Fig. 5F, arrow). The vertebral abnormalities in *Dkk1*$^{–/–}$ null homozygotes extend from the lumbar region to the tip of the tail. Hemivertebral fusions in the lumbar/sacral region were present in all of the *Dkk1*$^{–/–}$ null homozygotes (8/8) (Fig. 5G). Decreasing expression of *Dkk1* is thus correlated with higher incidence, increased severity, and more anterior location of vertebral defects.

Amelioration of the *doubleridge* limb phenotype by reduced expression of *Lrp6*

If postaxial polysyndactyly in *doubleridge* mice is the result of excess Wnt signaling through the Lrp5/6 coreceptors, then compensatory reduction in Wnt signaling by decreasing the amount of receptor is predicted to restore the correct digit number. To test this hypothesis, we generated F2 mice from a cross between *Dkk1*$^{d/d}$ homozygotes and *Lrp6*$^{+/–}$ mice (Pinson et al., 2000). Forelimb structure was examined at E16.5 and classified as no rescue (polysyndactyly, Fig. 6A), partial rescue

Fig. 5. Vertebral defects in *Dkk1* mutants. (A-C) Kinked tail in adult mice. (D) Vertebral fusions in the tail of $Dkk1^{d/-}$ compound heterozygote, E18.5. (E-G) Vertebral fusions in sacral and lumbar regions of E18.5 embryos; arrow, hemivertebra. *Dkk1* genotypes are indicated.

(small postaxial digit, Fig. 6B) or complete rescue (normal digit number) (Fig. 6C). Normal digit number was restored in one-third of the $Dkk1^{d/d}Lrp6^{+/-}$ mice and an additional one-third exhibited partial rescue of the *doubleridge* phenotype (Table 2). Thus, the phenotype resulting from hypomorphic expression of *Dkk1* can be corrected by genetically reducing the amount of *Lrp6*.

Partial rescue of *doubleridge* limb abnormalities in *Lrp5* double mutants

To evaluate genetic interaction between *Dkk1* and *Lrp5*, *doubleridge* homozygotes were crossed with mice carrying the *Lrp5* targeted null mutation. $Lrp5^{+/-}$ and $Lrp5^{-/-}$ mice are viable with normal limbs. Genetic reduction of one *Lrp5* allele had little effect on the *doubleridge* phenotype (Table 2). Complete elimination of *Lrp5* partially rescued the *doubleridge* forelimb phenotype in $Dkk1^{d/d}Lrp5^{-/-}$ mice. Reduction of *Lrp5* was less effective than reduction of *Lrp6* in rescuing the *doubleridge* phenotype (Table 2).

Improved viability of *Lrp6* null embryos with reduced expression of *Dkk1*

Homozygous $Lrp6^{-/-}$ embryos are recovered in reduced numbers late in gestation (Stump et al., 2003) (B.T.M., unpublished). To determine whether reduction of the Wnt antagonist *Dkk1* would compensate for the loss of the *Lrp6* receptor, we generated *Lrp6* null embryos that were $Dkk1^{d/+}$ or $Dkk1^{+/-}$. A total of 82 embryos were collected at E16.5, five

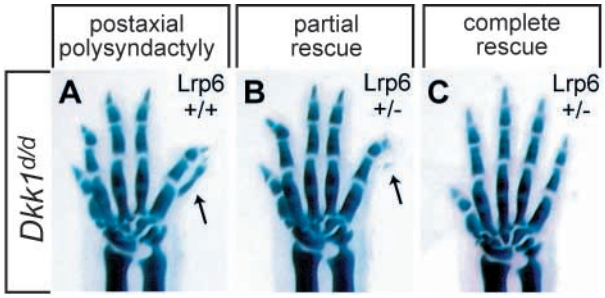

Fig. 6. (A-C) Genetic interaction between *Dkk1* and *Lrp6* indicated by rescue of postaxial polysyndactyly in double mutants. All mice are homozygous for the *doubleridge* allele, $Dkk1^{d}$. The extra digit in the *doubleridge* single mutant is marked by the arrow. The frequency of partial and complete rescue is dependent on Lrp genotype (Table 2). E16.5 forelimbs were stained with alcian blue to reveal skeletal elements.

litters from the cross $Lrp6^{+/-}$ X $Lrp6^{+/-}Dkk1^{d/+}$ and five litters from the cross $Lrp6^{+/-}$ X $Lrp6^{+/-}Dkk1^{+/-}$. Nine of the $Lrp6^{-/-}$ embryos were heterozygous for one of the *Dkk1* mutations, consistent with the prediction of 10/82 ($P>0.8$). Only three of the $Lrp6^{-/-}$ embryos were wild-type for *Dkk1*, significantly fewer than the predicted 10/82 ($P<0.05$). Thus, reduction of *Dkk1* increased the viability of *Lrp6* null embryos at E16.5.

Reduction of *Dkk1* levels improves axial development in the *Lrp6* null embryo

Homozygous *Lrp6* null embryos exhibit pleiotropic defects including axial truncation distal to the lumbar/sacral vertebrae similar to that seen in hypomorphic *Wnt3a* mutants (Pinson et al., 2000). Diminished signaling through Wnt3a in the presomitic mesoderm is the probable cause of the axial truncations. *Dkk1* is also expressed in the presomitic mesoderm during somitogenesis. Reduction of the Wnt inhibitor Dkk1 is predicted to increase Wnt3a signaling and correct axial development in the *Lrp6* null embryos. To test this hypothesis, we generated an allelic series of *Lrp6* null embryos with decreasing levels of *Dkk1* expression.

$Lrp6^{-/-}$ homozygotes display axial truncation, fusion of thoracic ribs, and severely defective hind limbs (Fig. 7A). As predicted, axial development was improved in $Dkk1^{d/+}Lrp6^{-/-}$ and $Dkk1^{d/d}Lrp6^{-/-}$ double mutants (Fig. 7B,C). The rescue of hindlimb development is striking, and is more extensive in the $Dkk1^{d/d}$ homozygotes, which have lower expression of *Dkk1* than the $Dkk1^{d/+}$ heterozygotes. Formation of caudal vertebrae is also more complete in the $Dkk1^{d/d}$ mice (Fig. 7C). Nonetheless, the $Dkk1^{d/d}Lrp6^{-/-}$ double mutants do not survive beyond P1 and are significantly smaller than their $Dkk1^{d/d}$ littermates (Fig. 7D). The lumbar vertebrae of $Dkk1^{d/d}Lrp6^{-/-}$ embryos exhibit spondylosis (anterior-posterior fusion) (Fig. 7E, bracket), and the distinct organization of individual vertebrae is lost in the sacral region. The major improvement in the axial structure of $Dkk1^{d/d}Lrp6^{-/-}$ mice compared with the *Lrp6* null provides additional evidence of in vivo interaction between *Dkk1* and *Lrp6*.

Rescue of anterior-posterior defects in $Dkk1^{d/d}Lrp6^{-/-}$ limbs

Forelimbs of *Lrp6* null mice are defective in the anterior-

Table 2. Reduction of *Lrp5* or *Lrp6* rescues polydactyly in developing forelimbs of *Dkk1*$^{d/d}$ homozygotes

Genotype	Total number of limbs	Postaxial polysynda ctyly (extra digit)	Small postaxial digit (partial rescue)	Normal digit number (complete rescue)	% with partial or complete rescue
Dkk1$^{d/d}$	48	48	0	0	0
Dkk1$^{d/d}$ *Lrp6*$^{-/-}$	14	0	0	14	100%
Dkk1$^{d/d}$ *Lrp6*$^{+/-}$	28	9	10	9	68%
Dkk1$^{d/d}$ *Lrp5*$^{-/-}$	20	11	9	0	45%
Dkk1$^{d/d}$ *Lrp5*$^{+/-}$	32	28	4	0	12%

Embryos were harvested at E16.5 and stained with Alcian Blue. Numbers represent individual forelimbs, two from each embryo. Limbs with partial or complete rescue are demonstrated in Fig. 6. *Dkk1*$^{d/d}$ *Lrp6*$^{-/-}$ forelimbs are shown in Fig. 7.

posterior and dorsal-ventral patterning. We analyzed the forelimbs of double mutants to determine the effect of reducing *Dkk1* levels in the *Lrp6* null embryo. Homozygous *Lrp6* null embryos fail to maintain the apical ectodermal ridge (AER) and usually lack posterior digits 4 and 5 (Fig. 7F). Reducing the dosage of *Dkk1* on the *Lrp6* null background compensated for the receptor deficiency, with a single missing digit in *Dkk1*$^{d/+}$*Lrp6*$^{-/-}$ forelimbs and no missing digits in *Lrp6*$^{-/-}$ *Dkk1*$^{d/d}$ mice (Fig. 7F). The limbs of *Dkk1*$^{d/d}$ homozygotes display normal dorsal-ventral patterning (Fig. 7H) (Adamska et al., 2003). Examination of dorsal-ventral patterning in the *Dkk1*$^{d/d}$ *Lrp6*$^{-/-}$ forelimb reveals the presence of ectopic ventral tendons (Fig. 7G) as also observed in *Lrp6*$^{-/-}$ null mice (Pinson et al., 2000). Hindlimbs are present in *Dkk1*$^{d/d}$ *Lrp6*$^{-/-}$ embryos with normal digit number and ventral characteristics in the dorsal compartment. Reduction of *Dkk1* is thus sufficient to rescue the digit number and anterior-posterior defects in the limbs of *Lrp6* null mice, but not dorsal-ventral patterning. Both allele series support the hypothesis that Dkk1 and Lrp6 serve opposing functions in Wnt signaling (Table 3).

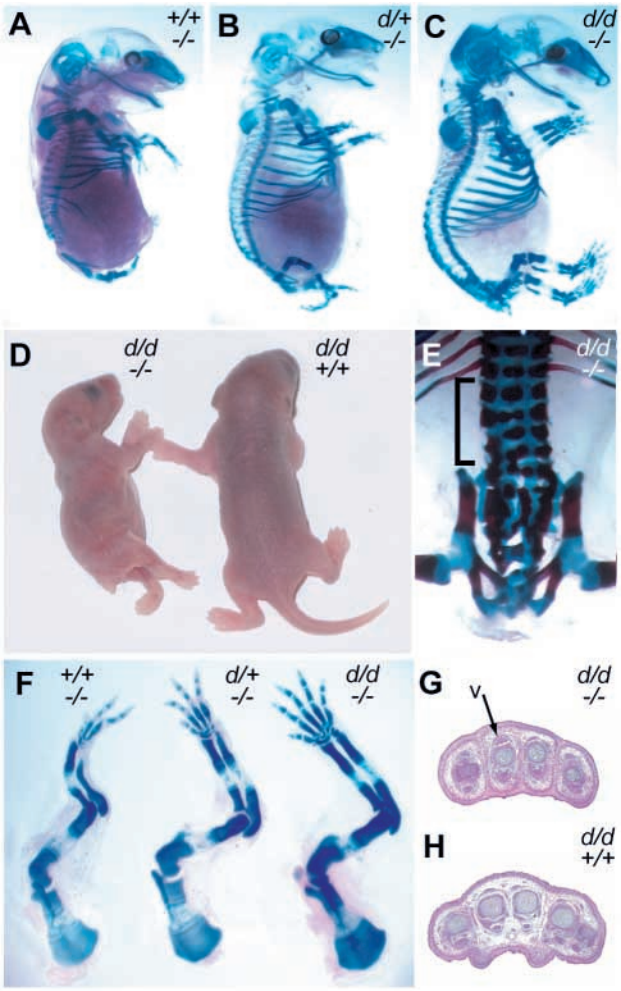

Fig. 7. Rescue of axial truncation in *Lrp6* null mice by reduced expression of *Dkk1*. Genotypes are indicated with *Dkk1* above and *Lrp6* below. (A-C) E16.5 embryos demonstrating axial truncation; (D) small size of *Dkk1*$^{d/d}$*Lrp6*$^{-/-}$ double mutant at P1; (E) fusion of lumbar and sacral vertebrae, compare with wild-type control in Fig. 5E; (F) Correction of posterior digits in E16.5 forelimbs. (G) Cross-section of forelimb at E17.5 stained with hematoxylin and eosin. Arrow, ectopic ventral tendon (v). (H) Normal dorsal tendons in *Dkk1*$^{d/d}$ homozygote.

Rescue of head structures in *Dkk1* null mice by reduced expression of *Lrp6*

Wnt inhibition mediated through *Dkk1* is required for early head induction. Lack of *Dkk1* in the mouse results in anterior head truncations and neonatal death (Fig. 8A). Genetic reduction of *Lrp6* in the *Dkk1* homozygous null mice improves anterior head development. *Dkk1*$^{-/-}$*Lrp6*$^{+/-}$ double mutants examined at E16.5 displayed rostral cranial structures not found in *Dkk1* null embryos (Fig. 8B). The better developed anterior head structures enabled three of the *Dkk*$^{-/-}$*Lrp6*$^{+/-}$ double mutants to suckle and survive beyond 14 days. One double mutant had extensive development of the anterior head, with open right eye and complete nasal structures (Fig. 8C). The skeleton revealed lateral deviation of the lumbar vertebrae, or scoliosis (Fig. 8D, arrow). Another double mutant has survived to three months of age with no visible abnormalities other than small size. The rescue of anterior head truncation and neonatal lethality in the *Dkk1*$^{-/-}$*Lrp6*$^{+/-}$ double mutant

Table 3. Opposing roles of *Dkk1* and *Lrp6* in determination of digit number

		Forelimb digits	
Dkk1	*Lrp6*	Number	Description
+/+	–/–	3	Missing digits 4 and 5
d/+	–/–	4	Missing digit 5
d/d	–/–	5	Normal digit number
d/d	+/–	5+	Occasional 6th digit
d/d	+/+	6	Consistent 6th digit

Deficient Wnt signaling in *Lrp6*-null mice results in missing digits. Excessive Wnt signaling in *Dkk1*$^{d/d}$ mice produces an extra posterior digit. Intermediate phenotypes in three types of double mutants are dependent on the relative levels of *Dkk1* and *Lrp6*.

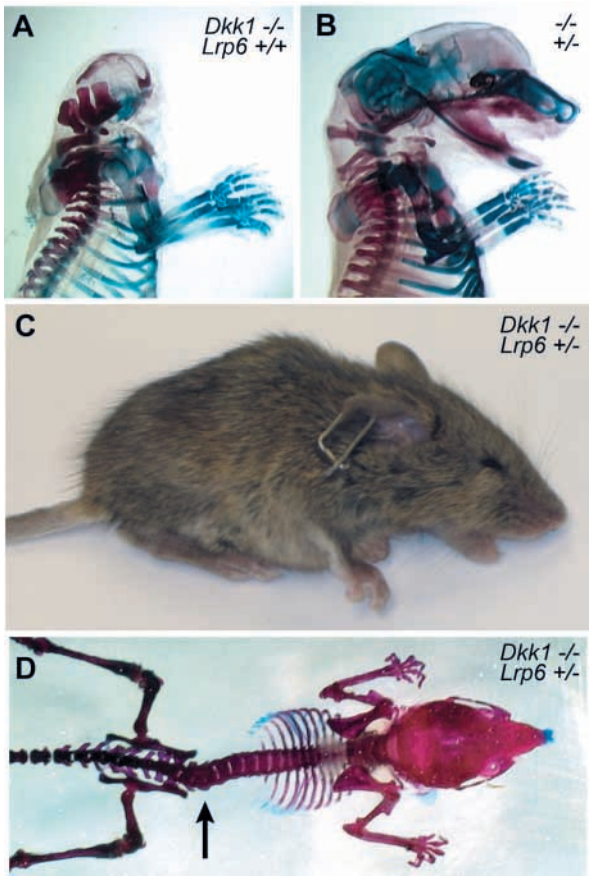

Fig. 8. Rescue of anterior head truncation in *Dkk1* null mice by reduced expression of *Lrp6*. (A) Single mutants that are null for *Dkk1* lack anterior structures of the head, which results in neonatal lethality. The null phenotype is rescued in double mutants which are heterozygous for the null allele of *Lrp6*, shown at E16.5 (B) and P30 (C). Lateral deviation of the lumbar vertebrae in the P30 double mutant is evident in the skeletal preparation (D).

provides further evidence of in vivo interaction between Dkk1 and Lrp6.

Discussion

doubleridge is a hypomorphic allele of *Dkk1*

In the *doubleridge* mutation, insertion of a 50-kb transgene composed of tandem copies of a 6.5-kb construct was accompanied by deletion of 60 kb of genomic DNA from the insertion site. The insertion site is located 150-kb downstream of the *Dkk1* gene. Allelism between *doubleridge* and the *Dkk1* null allele is demonstrated by the limb abnormalities in compound heterozygotes. The sequence and intron/exon structure of *Dkk1* was not affected by the mutation, but reduced gene expression was detected by wholemount in situ hybridization and quantitative analysis of *Dkk1* transcripts. The hypomorphic effect is evident throughout *Dkk1* expression domains, with transcript levels below 35% of normal (Table 1). By combining wild-type, $Dkk1^{d/d}$ and null alleles of *Dkk1* we generated an allelic series of six genotypes with *Dkk1* expression decreasing from 100% of normal to zero. No abnormalities are observed in $Dkk1^{d/+}$ heterozygotes, or in null heterozygotes with 50% of normal expression. Head and vertebral defects are mild in $Dkk1^{d/d}$ homozygotes and more severe in $Dkk1^{d/-}$ compound heterozygotes. Nonetheless, the survival of compound heterozygotes demonstrates that a low level of *Dkk1* is sufficient for many developmental processes. The threshold for survival is below the level in compound heterozygotes, and can be influenced by strain background (B.T.M., unpublished).

Two mechanisms may be considered to explain the negative effect of the *doubleridge* insertion on *Dkk1* expression. The replacement of 60 kb of endogenous DNA with 50 kb of transgene may have affected chromatin structure. In some cases, insertion of multiple transgene copies has been shown to induce DNA methylation and silencing of nearby genes (Garrick et al., 1998; McBurney et al., 2002). However, the normal expression of the adjacent genes in *doubleridge* mice suggests that the effect of this insertion is restricted to the *Dkk1* gene.

A second reasonable hypothesis is that the 60-kb deletion removed a transcriptional enhancer of *Dkk1* located 150 to 200 kb downstream of the 3′ UTR. Several examples of transcriptional regulatory elements located more than 100 kb from the gene promoter are now known (Bedell et al., 1995; Calhoun and Levine, 2003). In another recently described transgene-induced limb mutant, the *Sasquatch* mouse, the insertion site is located 1 Mb from the promoter and disrupted a *cis*-acting regulatory element (Lettice et al., 2002; Lettice et al., 2003). The sequence of the *Sasquatch* enhancer of Sonic hedgehog is well-conserved in fish, and mutations in the enhancer were identified in human patients with limb defects. We attempted to identify an evolutionarily conserved regulatory element in the 60-kb *doubleridge* deletion by sequence comparison with the corresponding region of human chromosome 10q21. We found seven segments of 100 bp with sequence identity greater than 75%, but none were conserved in fish genomic sequences. When more sequence is available from chicken and other vertebrate genomes, we may be able to find a *doubleridge* enhancer by this method. There is no match for the *Sasquatch* enhancer in the *doubleridge* deletion.

Head development in *Dkk1* mutants

Head induction in the vertebrate embryo is dependent on inhibition of Wnt and Bmp signaling (Niehrs, 2001). The Six3 transcription factor represses *Wnt1* expression, and loss of *Six3* results in anterior head defects because of excess Wnt signalling (Lagutin et al., 2003). The null mutation of the Wnt antagonist *Dkk1* also results in failure to develop anterior structures of the head (Mukhopadhyay et al., 2001). In contrast, $Dkk1^{d/d}$ homozygotes express one-third of normal *Dkk1* levels during head induction, and this provides sufficient reduction in Wnt signalling to permit normal head development in most individuals. $Dkk1^{d/-}$ compound heterozygotes exhibit variable development of rostral head structures, suggesting that the threshold requirement for *Dkk1* during head induction is in the range of 15 to 20%.

Dkk1 and vertebral development

Segmentation in the vertebrate embryo is achieved through the oscillating expression of Notch signaling genes and the intracellular Wnt inhibitor *Axin2* (Pourquie et al., 2003). Expression of *Wnt3a* in the presomitic mesoderm is required

for caudal somitogenesis, as demonstrated by the missing somites in the *vestigial tail* mouse mutant, a hypomorph of *Wnt3a* (Greco et al., 1996; Ikeya and Takada, 2001). Overexpression of *Wnt3a* in the posterior presomitic mesoderm reduced the size of the somites in chick embryos (Aulehla et al., 2003). The expression of *Dkk1* in the presomitic mesoderm overlaps the *Wnt3a* expression domain. We observed small, irregularly shaped vertebrae in mice deficient for *Dkk1*, which progressed to more anterior positions with lower levels of Dkk1 in the allele series. Reduced *Dkk1* expression thus results in a phenotype similar to overexpression of *Wnt3a* in the chick, indicating that *Dkk1* modulates Wnt signaling during development of vertebral structures.

Interaction between *Dkk1* and *Lrp6*

Functional interaction of *Dkk1* and *Lrp6* has been observed in *Xenopus* embryos and in cultured mammalian cells, and direct biochemical interaction between the two proteins was demonstrated (Mao et al., 2001; Bafico et al., 2001; Semenov et al., 2001). We provide genetic evidence for interaction of *Dkk1* and *Lrp6* during mammalian development, based on analysis of double mutants. The ratio of ligand (Dkk1) to receptor (Lrp6) appears to determine the extent of abnormalities in developing forelimb (Table 3). Reduction of *Dkk1* results in extra digits, whereas reduction of *Lrp6* reduces the number of digits. In $Dkk1^{d/d}$ $Lrp6^{-/-}$ double mutants, normal digit number is restored (Table 3). This in vivo 'titration' provides genetic evidence that the genes are active in the same pathway and is consistent with a model of direct interaction. Other aspects of the *Lrp6* null phenotype such as the presence of ectopic ventral tendons are not rescued, perhaps because *Dkk1* is not normally expressed in the dorsal compartment.

When Lrp6 is absent, Lrp5 is thought to mediate canonical Wnt signaling. Reduction of Lrp5 was less effective in rescuing digit abnormalities, suggesting that Lrp6 is more important in the developing limb. In *Xenopus* embryos, injection of *Lrp6* but not *Lrp5* induces a secondary axis (Tamai et al., 2000). Humans and mice with null mutations in *Lrp5* do not exhibit morphological abnormalities, and phenotypes are limited to postnatal defects of low bone density and impaired regression of the hyaloid vessels in the eye (Gong et al., 2001; Kato et al., 2002). Dkk inhibitors have been considered as possible treatments for these conditions. The $Dkk1^{d/d}$ $Lrp5^{-/-}$ mice provide a viable animal model for evaluating the effect of reduced *Dkk1* on these phenotypes.

The axial truncations in $Lrp6^{-/-}$ embryos are located caudal to the lumbar region and resemble those of hypomorphic *Wnt3a* mutants (Pinson et al., 2000). Caudal development is more complete in $Dkk1^{d/d}$ $Lrp6^{-/-}$ double mutants. No additional improvement was observed with further reduction of *Dkk1* expression in $Dkk1^{d/-}$ and $Dkk1^{-/-}$ double mutants (B.T.M., unpublished). The $Dkk1^{d/d}$ $Lrp6^{-/-}$ double mutants were able to suckle but exhibited perinatal lethality. The accumulation of urine in the kidneys and ureter of affected animals suggests that the lethality may be the result of neurogenic bladder or obstruction of the urethra. In mice with low levels of *Wnt3a*, reduced urinary and excretory function were associated with defects in the S2-S4 autonomic ganglia (Greco et al., 1996).

Dkk1 null mice exhibit anterior truncation of the head that is incompatible with postnatal survival. This defect can be ameliorated in $Dkk1^{-/-}$ $Lrp6^{+/-}$ double mutants (Fig. 8). Thus, simultaneous reduction of an antagonistic ligand and a receptor can restore Wnt signaling to a balance compatible with life. The dramatic correction of the severe developmental defects of *Lrp6* null and *Dkk1* null mice observed in double mutants provides support for a direct role of Dkk1 in reducing Wnt signaling through the Lrp6 receptor.

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