Stringent requirement of a proper level of canonical WNT signalling activity for head formation in mouse embryo

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SUMMARY
In mouse embryos, loss of Dickkopf-1 (DKK1) activity is associated with an ectopic activation of WNT signalling responses in the precursors of the craniofacial structures and leads to a complete truncation of the head at early organogenesis. Here, we show that ENU-induced mutations of genes coding for two WNT canonical pathway factors, the co-receptor LRP6 and the transcriptional co-activator β-catenin, also elicit an ectopic signalling response and result in loss of the rostral tissues of the forebrain. Compound mutant embryos harbouring combinations of mutant alleles of Lrp6, Ctnnb1 and Dkk1 recapitulate the partial to complete head truncation phenotype of individual homozygous mutants. The demonstration of a synergistic interaction of Dkk1, Lrp6 and Ctnnb1 provides compelling evidence supporting the concepts that (1) stringent regulation of the level of canonical WNT signalling is necessary for head formation, (2) activity of the canonical pathway is sufficient to account for the phenotypic effects of mutations in three different components of the signal cascade and (3) rostral parts of the brain and the head are differentially more sensitive to canonical WNT signalling and their development is contingent on negative modulation of WNT signalling activity.

KEY WORDS: Dkk1, Lrp6, β-Catenin, ENU mutant, WNT signalling, Head formation, Mouse, Gene dosage

INTRODUCTION
Formation of the head of the mouse embryo is accomplished by the coordinated regulation of specification, differentiation and movement of the progenitor cells, culminating in an orchestration of orderly growth and patterning of the brain and the facial primordia. These processes are driven by the morphogenetic activity elicited by inductive interactions between the primordial tissues of the head mediated by intercellular signalling. Analysis of gene expression patterns reveals that a multitude of genes encoding transcription factors and signalling molecules, among them inhibitors and components of the WNT signalling pathway, are activated in these tissues during head formation (Robb and Tam, 2004).

WNT (wingless and int) factors signal to the target cells by one of three transduction mechanisms: canonical WNT-β-catenin (for tissue patterning), WNT-planar cell polarity (WNT-PCP; for controlling cell shape and tissue remodelling) and WNT-calcium pathways (for calcium signalling and cell adhesion (Logan and Nusse, 2004)). Signalling via the WNT-β-catenin pathway involves a cascade of molecular activity from binding of the WNT ligand to the cell surface receptor complex of frizzled (FZD) and lipoprotein receptor-related proteins (LRPS and LRP6) and transduction of the signalling activity to the subsequent activation of responsive genes mediated by the transcriptional co-activator β-catenin (Fig. 1A). WNT signalling activity is negatively regulated by secreted proteins such as secreted frizzled-related proteins (SFRP) and DKK1. DKK1 dampens signalling activity by disengaging LRP6 from the receptor complex thereby blocking the WNT ligand-receptor interaction (Brott and Sokol, 2002; MacDonald et al., 2009).

Fate-mapping studies of mouse embryos at gastrulation and early organogenesis reveal that progenitors of the prospective telencephalon and diencephalon are always found in the region of the embryo with no or weaker WNT-reporter activity (Fig. 1B), suggesting that negative modulation of signalling activity is a prerequisite for normal development of the head. In the mouse, embryos lacking Dkk1 activity display ectopic and elevated WNT signalling activity in precursor tissues of the embryonic head and fail to form brain and head structures (Lewis et al., 2008; Mukhopadhyay et al., 2001), whereas reduced Dkk1 activity in the hypomorphic Dkk1-doubleridge mutant is associated with head malformation (MacDonald et al., 2004). Analysis of the phenotype of compound mutant embryos has revealed that the severity of head truncation defects caused by loss of Dkk1 function might be enhanced by reducing the gene dosage of Gsc (Lewis et al., 2007), which acts to repress the transcription of WNT ligand genes (Yao and Kessler, 2001). By contrast, reducing the level of Wnt3 activity is sufficient to allow normal head morphogenesis in some embryos that completely lack Dkk1 activity (Lewis et al., 2008). A precedent study has also shown that the loss of Dkk1 antagonist function might be compensated for by reduction of the function of the LRP6 co-receptor (MacDonald et al., 2004).

To assess further the impact of canonical WNT signalling activity in head development, we have conducted a genetic study of the interaction of DKK1 activity with two components of the signalling cascade: the LRP6 co-receptor (encoded by Lrp6) that...
forms a complex with the frizzled receptor and the WNT ligand, and β-catenin (encoded by Ctnnb1), which is the co-activator acting with LEF and TCF transcription factors to regulate the expression of WNT target genes (Fig. 1A). To study the genetic interaction between Dkk1, Lrp6 and Ctnnb1, we utilised two mutants that were identified in screens of n-ethyl-n-nitrosourea (ENU)-mutagenised mice: gwazi (Bogani et al., 2004) and batface (Nolan et al., 2000). These two mutants harbour a point mutation in Lrp6 and Ctnnb1, respectively, and they are associated with a gain of canonical WNT signalling activity and head truncation defects. Our analysis of compound mutants for Dkk1, Lrp6 and Ctnnb1 showed that repression of canonical WNT signalling by DKK1, mediated by interaction with the co-receptor LRP6 and the downstream transcriptional co-activator β-catenin, is essential for embryonic head formation. We also found that development of the rostral parts of the brain is particularly sensitive to canonical WNT signalling, underpinning the necessity of a stringent regulation of the domain and level of the WNT signalling response for craniofacial morphogenesis.

**MATERIALS AND METHODS**

**Characterisation and genotyping strategy of the gwazi mutant mice**

The gwazi (Gw) mutant was identified in an ENU mutagenesis screen for semi-dominant mutant mice (Bogani et al., 2004) by the observation that heterozygous carriers of the mutation have a crooked tail (see Fig. S1A in the supplementary material). Genetic mapping localised the mutation to the mid portion of Mmu6. The genomic DNA of the non-recombinant region was scanned for nucleotide changes by sequence analysis of PCR amplified products of the coding region of the Lrp6 gene. Of the 23 exons analysed, one showed a single base difference between the affected individuals and the pre-ENU treated parental (BALB/c) strains (Fig. 2A). A further 30 affected individuals were sequenced and all were found to harbour this nucleotide change. The variant product spanned exon 7 and was caused by a mis-sense mutation at nucleotide 1862 (accession #BC060704; see Fig. S1B in the supplementary material) resulting in D549G change (Fig. 2B) of a conserved region of the LRP6 protein (see Fig. S1B in the supplementary material). Five other strains of mice (Mus castaneus, Mus spreitus, C57BL/6J, 129Sv and 101/H) were analysed and none was found to contain the variant allele, suggesting that it is not a naturally occurring polymorphism. In the colony, which was maintained as heterozygotes on a C57/HeH background, co-segregation of the mutation and phenotype was observed in over 200 meioses. These mice are heretofore referred to as Lrp6-Gw mice.

For genotyping, two independent PCRs were performed from the same DNA sample extracted from tail or yolk sac. Primers NF19.10 (5'-ACTATGGTATCTGGGACACTA-3') and NF21.1 (5'-GATCTAACTCATACAGCACAC-3') amplified a fragment of 293 bp from the Lrp6* allele only. Conditions used were: 94°C for 30 seconds, 55°C for 60 seconds, 72°C for 45 seconds for 35 cycles. Primers NF19.1 (5'-ACTATGTCTT-ACCTGACCTG-3') and NF21.1 (5'-GATCTAACTCATACAGCACA-3') amplified a fragment of the same size from the Lrp6* allele only (see Fig. S1C in the supplementary material). Conditions used were: 94°C for 30 seconds, 58°C for 60 seconds, 72°C for 45 seconds for 35 cycles.

**Characterisation and genotyping strategy of the batface mutant mice**

The batface (Bfc) mutant was isolated in a genetic screen for dominant mutations and the heterozygous mutants were identified by craniofacial dysmorphology (see Fig. S2A in the supplementary material) and aberrant neurological behaviour (Nolan et al., 2000). Genetic mapping localised the mutation to the distal region of Mmu9 and sequencing of genes in the non-recombinant interval identified a C to A transversion in the Ctnnb1 gene resulting in a T653K change in the β-catenin protein (Fig. 3A,B) (P.M.N., unpublished). These mice are heretofore referred to as Ctnnb1-Bfc mice, which were maintained as heterozygotes separately on a C57/BL6 and BALB/c background.

For genotyping, two independent PCR amplifications were performed from the same DNA sample extracted from tail or yolk sac. Primers NF19.3 (5'-AAAGAGTGACTGCAGGCGG-3') and NF18.1 (5'-GGACAGCTGCTGCTGTATG-3') amplified a fragment of 235 bp from the Ctnnb1+ allele only. Primers NF19.3 (5'-AAAGAGTGACTGCAGGCGG-3') and NF19.2 (5'-GGACAGCTGCTGCTGTATG-3') amplified a fragment of 236 bp from the Ctnnb1+ allele only (see Fig. S2C in the supplementary material). Conditions used were: 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds for 35 cycles.

**RNA isolation and RT-qPCR**

To assess the expression of Axin2, embryonic day (E)8.5 stage-matched (two to six somites) embryos harvested from pregnant mice of Ctnnb1Bfc/+ × Ctnnb1Bfc/+ crosses were dissected in PBS, staged for developmental status and frozen whole. The embryos were genotyped using the yolk sac material. To determine the level of expression of Axin2, Dkk1 and Sfrp1, E9.5 stage-matched (20-25 somites) embryos harvested from pregnant mice of Ctnnb1Bfc/+ × Dkk1Bfc/+ × Lrp6Gw/Gw crosses were dissected in PBS, staged and categorised. Head and body parts were separated by cutting transversely at the level immediately posterior to the otic vesicle (Fig. 4A) and were frozen separately. The embryos were genotyped using the yolk sac material. Extraction of total RNA was carried out independently for...
Fig. 3. Ctnnb1-Bfc mutation. (A) A C to A change in the Ctnnb1Bfc allele leads to the T653K mutation of the ß-catenin protein. Wt, wild type. (B) Localisation of the mutation in the last armadillo repeat of the ß-catenin protein. Functional domains of the protein are colour-coded.

each sample (whole E8.5 embryo; head or body part of E9.5 embryo) using the RNeasy Micro Kit (Qiagen). First strand cDNA was synthesised using 100 ng (whole E8.5 embryo, head part of E9.5 embryo) or 300 ng (body part of E9.5 embryo) of mRNA and the SuperScript III First Strand System (Invitrogen). Quantitative PCR was performed in triplicate from 1:3 dilution of cDNA of each sample using the Rotorgene 6000 thermal cycler (Corbett Research), SYBR Green I (Molecular Probes) and Platinum Taq DNA Polymerase (Invitrogen). Primers 5’-ACCTCAAGTGCCAAAATCTCTCA-3’ and 5’-GTGATAAGGATTGACTGGGT-3’ amplify a fragment of 140 bp from Actin1 cDNA. Primers 5’-TTGTAATGACCATGCTGGGA-3’ and 5’-GTGATAAGGATTGACTGGGT-3’ amplify a fragment of 237 bp from Dkk1 cDNA. Primers 5’-CCATGCAGAGTGGAGA-3’ and 5’-GTGATAAGGATTGACTGGGT-3’ amplify a fragment of 195 bp from Sfrp1 cDNA. Primers 5’-AGCCCTGTGCTCTACA-3’ and 5’-GTGATAAGGATTGACTGGGT-3’ amplify a fragment of 145 bp from ß-actin cDNA. Conditions were similar for all the PCRs: 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 20 seconds for 40 cycles. Fluorescence was read at the end of each elongation step. A melting curve was plotted at the end of the run to check that only the fragment of interest had been amplified.

Fig. 4. Phenotype analysis of mutant embryos. (A) Morphometric analysis of head size of E9.5 embryos. The measurement is expressed as the ratio of the linear distance along the silhouette of the head from the border of the mandibular arch and maxillary prominence to the rostral margin of the otic vesicle (pink dashed line), and the diameter (blue line) of the otic vesicle (brown circle); the latter is relatively constant among embryos of the different genotypes (see also Lewis et al., 2007). The computed values of wild type and the most truncated (Dkk1-null-like) heads were normalized as 0% and 100% reduction of head size, respectively. (B) Phenotypic classification of the mutant embryo based on the morphology and degree of reduction of the head: Category (Cat.) I, normal; II-IV, partial truncation; V, most severe truncation. The range of the percentage of head size reduction (Red.) and the scores of the specimen (I, Lrp6Gw/+; II and III, Lrp6Gw/+, Ctnnb1Bfc/+; IV and V, Dkk1+/–;Lrp6Gw/+, Ctnnb1Bfc/+; for each catagory are shown.

RESEARCH ARTICLE 669

RESULTS

Lrp6-Gw mutation causes a gain of WNT signalling function

Gwazi (Gw) mice were identified in an ENU-mutagenesis screen by their kinked tail, which is caused by the intercalation of a sesamoid bone between the caudal vertebrae (see Fig. S1A in the supplementary material) (Bogani et al., 2004). We identified the causative mutation in the seventh coding exon of the Lrp6 gene, resulting in the substitution of a highly conserved aspartic acid with glycine (D549G) in the last YWTD repeat of the second propeller domain of the Lrp6 protein (Fig. 2A,B). A bent tail was found in 67.5% of Lrp6Gw/+ and 85.5% of Lrp6Gw/Gw mice (see Table S1A in the supplementary material). The frequency of tail kinks increased to 100% in Dkk1+/–;Lrp6Gw/Gw mice, showing that the Lrp6-Gw mutant phenotype is enhanced with the elevation of WNT signalling activity caused by reduced DKK1. By contrast, the frequency of tail kinks dropped to 21.5% owing to the reduction of WNT signalling activity in Wnt3+/–;Lrp6Gw/Gw mice (see Table S1B in the supplementary material). These results strongly suggest that the tail kink is associated with a gain of WNT function due to the Lrp6-Gw mutation, which is enhanced by the Dkk1-null mutation and suppressed by reduced Wnt3 activity. Further support of the gain-of-function effect of the Lrp6-Gw mutation is the expanded expression domain of Lef-Tcf-promoter-lacZ reporter (BATGal) (Maretto et al., 2003). BATGal expression is normally regionalised to the posterior germ layers of the E7.5 embryo and is absent from the genotype. X-Gal staining was performed as described by Lewis et al. (Lewis et al., 2008). Specimens of different genotypes were processed in the same run to ensure that the staining results could be compared.

Phenotypic analysis

The mutant embryos were examined for head morphology in comparison with wild-type embryos of the same age. The degree of head truncation was determined by morphometric measurement of the head size of E9.5 embryos (see legend for Fig. 4A). Based on the degree of head truncation, embryos of various genotypes were assigned to one of the five phenotype categories: [I] normal head size (0%) and morphology comparable to the wild-type embryo, [II] ≤25 % reduction of the forebrain, [III] 26-75% reduction of the forebrain, [IV] >75% reduction but with recognisable remnant of the forebrain and [V] phenoctomy of the complete head truncation (100%) of Dkk1-null embryo (Fig. 4B).

In situ hybridisations with Six3, Fgf8, Emx2, Hex1, Tcf4, En2, Cer1, Foxa2, Lhx1 and Shh riboprobes were performed on between two and eight specimens of each specific genotype as described by Simeone et al. (Simeone et al., 1992), Fossati et al. (Fossati et al., 2007), Lewis et al. (Lewis et al., 2008) and Lavado et al. (Lavado et al., 2008).
the progenitors of the prospective forebrain (Fig. 1B). In Lrp6\textsuperscript{Gw/Gw} embryos at E7.5, BATGal expression expanded to the anterior germ layer tissues (8/14=57% embryos; Fig. 5A), reminiscent of previous observations in Dkk1\textsuperscript{−/−} embryos (see Fig. S3A in the supplementary material) (Lewis et al., 2008). By E8.5-9.5, the BATGal expression domain extended further anteriorly (Fig. 1B; see Fig. S1D in the supplementary material). The Lrp6\textsuperscript{Gw} allele is therefore associated with a gain of WNT signalling function.

Lrp6\textsuperscript{Gw/Gw} mutant embryos display head defects

Embryos lacking Dkk1 activity displayed fully penetrant and complete truncations of the head (see Fig. S3B in the supplementary material) (Lewis et al., 2007; Lewis et al., 2008; Mukhopadhyay et al., 2001). To test if the gain of WNT function caused by the Lrp6\textsuperscript{Gw} mutation affects embryonic head formation similarly to that of loss of DKK1 function, we examined early organogenesis-stage embryos produced by crossing heterozygous Lrp6\textsuperscript{Gw/+} mice.

In the E10.0-11.0 litters of Lrp6-Gw mice, wild type, Lrp6\textsuperscript{Gw/+} and Lrp6\textsuperscript{Gw/Gw} embryos were present in the expected Mendelian ratio (Table S2 in the supplementary material). A reduced forebrain size (Class II and III) was found in 38.5% of E9.5 (Fig. 5B) and 9.5% of the E10.5 embryos (Fig. 5C,D) suggesting that, like the Dkk1\textsuperscript{−/−} embryos, there is a reduction of the size of the telencephalon. An increase in the penetrance of the head phenotype (to 78.5%) was found in the Dkk1\textsuperscript{−/−};Lrp6\textsuperscript{Gw/+} embryos, which was comparable to the frequency of 33% observed for Lrp6\textsuperscript{Gw} embryos (Fig. 5C; see Table S3A in the supplementary material). Marker analysis revealed very similar changes in the Six3, Fgf8, Emx2, Hexx1 and Tcf4 expression domain in the Dkk1\textsuperscript{−/−};Lrp6\textsuperscript{Gw/+} and Lrp6\textsuperscript{Gw} embryos (Fig. 5C,D) suggesting that, like the Lrp6\textsuperscript{Gw} abnormal embryos, there is a reduction of the size of the telencephalon. An increase in the penetrance of the head phenotype (to 78.5%) was found in the Dkk1\textsuperscript{−/−};Lrp6\textsuperscript{Gw/+} embryos (see Table S3A in the supplementary material). These findings are consistent with the hypothesis that the Lrp6\textsuperscript{Gw} and Dkk1\textsuperscript{−/−} alleles interact synergistically in a gene-dosage-dependent manner to influence the head size, reinforcing the idea that DKK1 interacts functionally with LRP6 in regulating WNT signalling activity for head morphogenesis.

Ctnnb1-8fc mutation causes a gain of WNT signalling function and head defects

The demonstration of a positive genetic interaction of Lrp6 and Dkk1 implies the involvement of the canonical signalling pathway in regulating head morphogenesis and that a gain of WNT function underpins the abnormal phenotype. However, it is necessary to test more specifically whether the phenotypic effect is mediated by the intracellular transcriptional co-activator, \(\beta\)-catenin, which is downstream of LRP6 and DKK1 in the canonical signalling cascade.

Batface (Bfc) mice were identified in an ENU-mutagenesis screen by their bat-like squashed face (see Fig. S2A in the supplementary material) (Nolan et al., 2000). We mapped the causative mutation to the thirteenth coding exon of the Ctnnb1

<table>
<thead>
<tr>
<th>Genotype of E9.0-10.0 embryos</th>
<th>Number of embryos with head defects/number analysed</th>
<th>Percentage of embryos with head defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1/73</td>
<td>1.5</td>
</tr>
<tr>
<td>Lrp6\textsuperscript{Gw/+}</td>
<td>8/124</td>
<td>6.5</td>
</tr>
<tr>
<td>Dkk1\textsuperscript{−/−}</td>
<td>2/23</td>
<td>8.5</td>
</tr>
<tr>
<td>Ctnnb1\textsuperscript{8fc/+}</td>
<td>9/34</td>
<td>26.5*</td>
</tr>
<tr>
<td>Lrp6\textsuperscript{Gw/Gw}</td>
<td>8/27</td>
<td>29.5*</td>
</tr>
<tr>
<td>Dkk1\textsuperscript{−/−};Lrp6\textsuperscript{Gw/+}</td>
<td>19/44</td>
<td>43*</td>
</tr>
<tr>
<td>Dkk1\textsuperscript{−/−};Lrp6\textsuperscript{Gw/Gw}</td>
<td>11/14</td>
<td>78.5*</td>
</tr>
<tr>
<td>Lrp6\textsuperscript{Gw/+};Ctnnb1\textsuperscript{8fc/+}</td>
<td>37/43</td>
<td>86*</td>
</tr>
<tr>
<td>Dkk1\textsuperscript{−/−};Lrp6\textsuperscript{Gw/Gw}</td>
<td>23/25</td>
<td>92*</td>
</tr>
<tr>
<td>Ctnnb1\textsuperscript{8fc/+}</td>
<td>10/10</td>
<td>100*</td>
</tr>
<tr>
<td>Dkk1\textsuperscript{−/−};Lrp6\textsuperscript{Gw/Gw}</td>
<td>3/3</td>
<td>100*†</td>
</tr>
<tr>
<td>Dkk1\textsuperscript{−/−};Lrp6\textsuperscript{Gw/Gw};Ctnnb1\textsuperscript{8fc/+}</td>
<td>22/22</td>
<td>100*†</td>
</tr>
</tbody>
</table>

\*Significantly different from wild type at P<0.001 by \(\chi^2\)-test.
†Sample size too small for testing.
homozygous offspring were found. In litters produced by crossing Ctnnb1<sup>Bfc/c</sup> and Ctnnb1<sup>+/-</sup> mice, heterozygous offspring were under-represented (27% on C57BL/6 background and 38.5% on BALB/c background, versus the expected 50%; see Table S4A in the supplementary material), suggesting that the viability of heterozygotes is compromised. On C57BL/6 and BALB/c background, all heterozygous Ctnnb1<sup>Bfc/c</sup> mice displayed a bat-like face with squashed snout. Furthermore, 23% Ctnnb1<sup>Bfc/c</sup> (C57BL/6) and 10.5% Ctnnb1<sup>Bfc/c</sup> (BALB/c) heterozygous mice also displayed ocular defects (Fig. S2A; see Table S4A in the supplementary material), including microphthalmia, anophthalmia and cataract-like clouding of the lens. There was a distortion of the genotype ratio of the offspring produced by crosses of Dkk1<sup>+/-</sup> and Ctnnb1<sup>Bfc/c</sup> mice, with a greatly reduced number of compound heterozygous Dkk1<sup>+/-</sup>;Ctnnb1<sup>Bfc/c</sup> mice (5.5% versus the expected 25%; see Table S4B in the supplementary material). Dkk1<sup>+/-</sup>;Ctnnb1<sup>Bfc/c</sup> mice also showed a higher incidence of ocular defects (71.5%); see Table S4B in the supplementary material) than Ctnnb1<sup>Bfc/c</sup> mice of either the C57BL/6 or the BALB/c background. The enhanced impact on viability and ocular development of the Ctnnb1<sup>Bfc/c</sup> mice caused by genetically ablating one Dkk1 allele thus suggests that the Ctnnb1<sup>Bfc</sup> allele is associated with an increase of WNT signalling activity. Analysis of BATGal expression in the Ctnnb1<sup>Bfc/c</sup> mutant embryos revealed an expansion of the expression domain to anterior tissues at E7.5 (7/8 embryos), E8.5 and E9.5 (all embryos) (Fig. 6A; see Fig. S2D in the supplementary material), similar to that found in the Lrp6<sup>Gw/Gw</sup> and Dkk1<sup>+/-</sup> mutants. In addition, RT-qPCR analysis showed upregulation of a WNT downstream target, Axin2, in E8.5 Ctnnb1<sup>Bfc/c</sup> and Ctnnb1<sup>Bfc/c</sup> embryos (see Fig. S2E in the supplementary material). Ctnnb1<sup>Bfc</sup> allele is therefore likely to be a gain of WNT function mutation.

As the Ctnnb1<sup>Bfc</sup> mutation might cause a gain of WNT function, we examined whether it affects embryonic head formation similarly to the Lrp6<sup>Gw/Gw</sup> and Dkk1<sup>+/-</sup> mutants. Of the heterozygous Ctnnb1<sup>Bfc/c</sup> embryos, 37.5% at E8.0-9.0 and 89.5% at E9.0-11.0 displayed head defects characterised by a reduced forebrain (Class II; Fig. 6B; see Fig. S2F in the supplementary material), which is reminiscent of the Lrp6<sup>Gw/Gw</sup> phenotype. Ctnnb1<sup>Bfc/c</sup> embryos recovered in E7.0-11.0 litters (19.5% of all embryos) on the C57BL/6 background (see Table S5 in the supplementary material) all displayed the strong phenotype (Class IV) of forebrain truncation (E9.5, Fig. 6B; see Fig. S1A in the supplementary material). Analysis of Six3 and Fgf8 expression revealed a marked reduction of these telencephalon markers in the Ctnnb1<sup>Bfc/c</sup> embryos and very weak to no expression in the Ctnnb1<sup>Bfc/c</sup> embryos (Fig. 6C). These findings suggest that the telencephalon is reduced in Ctnnb1<sup>Bfc/c</sup> embryos and is absent in Ctnnb1<sup>Bfc/c-Bfc</sup> embryos, which also have a smaller diencephalon.

To study the interaction between Ctnnb1<sup>Bfc</sup> and Dkk1<sup>+</sup> alleles, two intercrosses were performed to produce embryos of six genotypes (see Table S3B in the supplementary material). On the BALB/c background, 23% Ctnnb1<sup>Bfc/c</sup> embryos displayed deficiency of head tissues (Class II). This frequency was increased to 95% in Dkk1<sup>+/-</sup>;Ctnnb1<sup>Bfc/c</sup> embryos (Fig. 6B; see Table S3B in the supplementary material), whereas 9% of Dkk1<sup>+/-</sup> embryos and
expression in E9.5 Ctnnb1Bfc/Bfc 100% of Dkk1+/– and schematic diagrams to highlight the changes in the expression domain matched by somite number (s). The gene expression data are collated in embryos compared with wild-type embryos. Embryos were stage-Dkk1+/– and

Fig. 6. Phenotype of Ctnnb1-Bfc mutant embryos. (A) Expression of the Tcf-Lef-lacZ reporter (BATGal) in E7.5 (early bud stage) and E8.5 (early somites stage) wild-type and Ctnnb1Bfc/Bfc mutant embryos. (B) Morphological appearance of wild-type, Ctnnb1Bfc/+; Ctnnb1Bfc/Bfc and Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/+ E9.5 embryos. (C) Six3, Fgf8, Hexx1 and Tcf4 expression in E9.5 Ctnnb1Bfc/+; Ctnnb1Bfc/Bfc and Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/+ embryos compared with wild-type embryos. Embryos were stage-matched by somite number (s). The gene expression data are collated in schematic diagrams to highlight the changes in the expression domain of the markers (colour-coded) in the head of Ctnnb1Bfc/+; Ctnnb1Bfc/Bfc and Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/+ embryos compared with wild-type embryos.

100% of Ctnnb1Bfc/bc and Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/bc embryos showed head reduction defects (see Table S3B in the supplementary material). E9.5 Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/+ embryos showed a reduced expression domain of Six3, Fgf8 and Hexx1 in the forebrain, which was intermediate in degrees between that of Ctnnb1Bfc/+ and Ctnnb1Bfc/bc embryos (Fig. 6C). The enhanced manifestation of an abnormal head phenotype in Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/bc embryos provides compelling evidence for a positive genetic interaction of the Ctnnb1Bfc and Dkk1 alleles in head formation.

**Gene dosage-dependent effects on the manifestation of mutant phenotypes**

The functional connection between Lrp6, Dkk1 and Ctnnb1 in head morphogenesis was investigated by examining different combinations of the three alleles in embryos of eight genotypes obtained from four intercrosses (see Table S6 in the supplementary material). As expected from previous results showing an interaction between Dkk1 and Lrp6<sup>6Gw</sup> alleles and between Dkk1 and Ctnnb1Bfc alleles, a genetic interaction between Ctnnb1Bfc and Lrp6<sup>6Gw</sup> alleles was revealed by a severe head deficiency phenotype in 86% of the Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> double heterozygous mutants, compared with 5-15% of the single heterozygous (Lrp6<sup>6Gw/+</sup> and Ctnnb1Bfc/+ mutants). The double heterozygous Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> embryos phenocopied the deficiency of the compound Dkk1<sup>1</sup>/–; Lrp6<sup>6Gw/+</sup> and Dkk1<sup>1</sup>/–; Ctnnb1Bfc/+ embryos, and displayed similar changes in gene expression in the telencephalon (Fig. 7A, B and Fig. 6C). Furthermore, we observed that all of the triple compound Dkk1<sup>1</sup>/–; Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> embryos developed head defects (see Table S6 in the supplementary material), with 23% of them showing a phenocopy of the complete truncation (Class V) of the Dkk1<sup>1</sup>– embryos (Fig. 7A; see Fig. S3B in the supplementary material). Analysis of marker expression showed that these severely affected embryos completely lacked Fgf8- and Tcf4-expressing forebrain structures, and the En2-expressing tissues (the presumptive mesencephalon) were localised at the rostral-most position in the head (Fig. 7B). In the triple-mutant brain, Fgf8 expression at the presumptive mesencephalon/metencephalon boundary appeared to be unaffected (Fig. 7B). These results point to a positive genetic interaction of the Dkk1<sup>1</sup>, Lrp6<sup>6Gw</sup> and Ctnnb1Bfc alleles in head formation.

**Head truncation is associated with elevated canonical WNT signalling activity**

Collectively, the outcomes of the genetic interaction study infer that these three components of the canonical WNT signalling pathway are functionally integrated into the molecular mechanism of head morphogenesis in the mouse embryo. These data also suggest that proper regulation of the canonical WNT signalling activity is essential for the formation of the rostral parts of the brain and the head. The collated data (Table 1) showed that the impact of mutation, as revealed by enhancement of the mutant phenotype, is influenced by the gene dosage, with the severity of defects increasing in the order of Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> double heterozygous, Lrp6<sup>6Gw/+</sup> single mutant, Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> (triple heterozygous, 100%). Among the triple-mutant embryos, with 23% of them showing a phenocopy of the complete truncation (Class V) of the Dkk1<sup>1</sup>– embryos (Fig. 7A; see Fig. S3B in the supplementary material). Analysis of marker expression showed that these severely affected embryos completely lacked Fgf8- and Tcf4-expressing forebrain structures, and the En2-expressing tissues (the presumptive mesencephalon) were localised at the rostral-most position in the head (Fig. 7B). In the triple-mutant brain, Fgf8 expression at the presumptive mesencephalon/metencephalon boundary appeared to be unaffected (Fig. 7B). These results point to a positive genetic interaction of the Dkk1<sup>1</sup>, Lrp6<sup>6Gw</sup> and Ctnnb1Bfc alleles in head formation.

100% of Ctnnb1Bfc/bc and Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/bc embryos showed head reduction defects (see Table S3B in the supplementary material). E9.5 Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/+ embryos showed a reduced expression domain of Six3, Fgf8 and Hexx1 in the forebrain, which was intermediate in degrees between that of Ctnnb1Bfc/+ and

Dkk1<sup>1</sup>/–; Ctnnb1Bfc/+ and Dkk1<sup>1</sup>/–; Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> embryos. The enhanced manifestation of an abnormal head phenotype in Dkk1<sup>1</sup>/–; Ctnnb1Bfc/bc embryos provides compelling evidence for a positive genetic interaction of the Ctnnb1Bfc and Dkk1 alleles in head formation.

**Gene dosage-dependent effects on the manifestation of mutant phenotypes**

The functional connection between Lrp6, Dkk1 and Ctnnb1 in head morphogenesis was investigated by examining different combinations of the three alleles in embryos of eight genotypes obtained from four intercrosses (see Table S6 in the supplementary material). As expected from previous results showing an interaction between Dkk1 and Lrp6<sup>6Gw</sup> alleles and between Dkk1 and Ctnnb1Bfc alleles, a genetic interaction between Ctnnb1Bfc and Lrp6<sup>6Gw</sup> alleles was revealed by a severe head deficiency phenotype in 86% of the Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> double heterozygous mutants, compared with 5-15% of the single heterozygous (Lrp6<sup>6Gw/+</sup> and Ctnnb1Bfc/+ mutants). The double heterozygous Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> embryos phenocopied the deficiency of the compound Dkk1<sup>1</sup>/–; Lrp6<sup>6Gw/+</sup> and Dkk1<sup>1</sup>/–; Ctnnb1Bfc/+ embryos, and displayed similar changes in gene expression in the telencephalon (Fig. 7A, B and Fig. 6C). Furthermore, we observed that all of the triple compound Dkk1<sup>1</sup>/–; Lrp6<sup>6Gw/+; Ctnnb1Bfc/+</sup> embryos developed head defects (see Table S6 in the supplementary material), with 23% of them showing a phenocopy of the complete truncation (Class V) of the Dkk1<sup>1</sup>– embryos (Fig. 7A; see Fig. S3B in the supplementary material). Analysis of marker expression showed that these severely affected embryos completely lacked Fgf8- and Tcf4-expressing forebrain structures, and the En2-expressing tissues (the presumptive mesencephalon) were localised at the rostral-most position in the head (Fig. 7B). In the triple-mutant brain, Fgf8 expression at the presumptive mesencephalon/metencephalon boundary appeared to be unaffected (Fig. 7B). These results point to a positive genetic interaction of the Dkk1<sup>1</sup>, Lrp6<sup>6Gw</sup> and Ctnnb1Bfc alleles in head formation.

**Head truncation is associated with elevated canonical WNT signalling activity**

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100% of Ctnnb1Bfc/bc and Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/bc embryos showed head reduction defects (see Table S3B in the supplementary material). E9.5 Dkk1<sup>1</sup>+/–; Ctnnb1Bfc/+ embryos showed a reduced expression domain of Six3, Fgf8 and Hexx1 in the forebrain, which was intermediate in degrees between that of Ctnnb1Bfc/+ and Ctnnb1Bfc/bc embryos (Fig. 6C). The enhanced manifestation of an abnormal head phenotype in Dkk1<sup>1</sup>/–; Ctnnb1Bfc/bc embryos provides compelling evidence for a positive genetic interaction of the Ctnnb1Bfc and Dkk1 alleles in head formation.
observed (see Fig. S4 in the supplementary material). These findings suggest that mutations of these three components of the canonical WNT signalling pathway do not affect the formation or the function of the tissues that are known to influence head formation and that the head truncation observed is primarily caused by changes in the response of the head tissues to elevated WNT signalling activity.

To determine whether the manifestation of the head deficiency/truncation is influenced by allele-specific mutant effects, we analysed the complete set of E9.0-10.0 embryo data by categorising the mutant phenotypes by the degree of severity (Fig. 4B). Analysis of the relative frequency of the different phenotype categories revealed a correlation of the severity of the head phenotype and the genotype: there is increasing disruption of the morphogenesis of the prosencephalon when there are mutations of more than one component of the signalling cascade, and with increasing dosage of mutant alleles individually and in combination (Fig. 8A). It is likely that this phenotypic outcome reflects the degree of gain of canonical WNT function in the different genotype combinations.

To test whether the degree of head truncation is correlated with the gain of WNT signalling function, we measured the level of expression of two known canonical WNT target genes (Axin2 and Dkk1; Fig. 1A) in individual embryos of the five phenotypic categories. To ensure that the embryos were on a comparable strain background, they were generated by crossing Dkk1+/–;Lrp6Gw/Gw male and Ctnnb1Bfc/+ female mice. The overall distribution of phenotypic categories of embryos derived from this cross was similar to that of the embryos pooled from previous crosses (see Table S7 in the supplementary material). Thirty-four embryos at the 20- to 25-somite stage were selected, categorised and samples of head and body parts were analysed separately by RT-qPCR. Quantification of the level of Axin2 mRNA in the head samples revealed that expression of this WNT target gene was significantly enhanced with increased degree of head truncation (Fig. 8B). Based on the observation that the response of Dkk1 to WNT activity is related to the dosage of wild-type allele in the genome (higher in Dkk1+/+ than Dkk1+/– embryos) (Lewis et al., 2008), Dkk1 expression was analysed separately for mutant embryos of the Dkk1+/+ and Dkk1+/– genotype (Fig. 8B). The findings revealed a significant increase in Dkk1 expression with the severity of the truncation phenotype for compound mutant embryos with the Dkk1+/– genotype. Although not statistically significant, Dkk1 expression had a tendency to increase in a similar manner in embryos with the Dkk1+/– genotype (categories I to III only; Fig. 8B). Despite the elevated activity of Dkk1 in the compound mutant, the manifestation of the head phenotype indicates that this is not sufficient to antagonise the excessive WNT signalling activity. There was no change in the expression of Sfrp1, another WNT antagonist, in embryos of the five phenotype categories (see Fig. S5 in the supplementary material), suggesting that there is not any compensatory activity of other antagonists. To rule out a secondary effect on gene expression level due to loss of tissues in the truncated head, we also determined Axin2 and Dkk1 expression in the body of the embryo (which showed no evident loss of tissues) to verify the gain of WNT function effect. Similar to the head, the expression of both WNT targets increased in the body samples with the severity of the truncation phenotype (Fig. 8B). These results strongly suggest that the manifestation of the head truncation phenotype is associated with a gain of WNT function due to Dkk1-null, Lrp6-Gw and Ctnnb1-Bfc mutations individually and in combination. Furthermore, the degree of loss of the head or forebrain structures is correlated with the extent of elevation of WNT signalling activity during the formative phase of head morphogenesis (Fig. 8C).
The findings of this study highlight a functional impact of the level of canonical WNT signalling activity perceived by the progenitor tissues on their differentiation to specific parts of the forebrain and head. The loss of DKK1 has been shown to result in increased WNT signalling activity and causes head truncation (Lewis et al., 2008). In Xenopus and zebrafish, DKK1 inhibition of the canonical WNT signalling by sequestration of LRPs (Mao et al., 2001; Semenov et al., 2001) is required for proper head formation (Caneparo et al., 2007; Kazanskaya et al., 2000; Shinya et al., 2000). In the present study, ENU-mutant mice, harbouring mutations in two components of the canonical WNT signalling cascade – the co-receptor LRPs and the transcriptional co-activator β-catenin – were used to test for genetic interaction with the Dkk1 loss-of-function mutant allele. We have shown that in both ENU mutants the expression domain of the LeF-Tcf-lacZ reporter expands ectopically to the anterior embryonic tissues that contain the prospective prosencephalon during organogenesis. This ectopic reporter activity can be discerned before the manifestation of the head phenotype, indicating that cells in these tissues are responding inappropriately to LEF-TCF-mediated WNT signalling activity prior to head morphogenesis. Further tests by genetic crosses showed that the tail phenotype of Lrp6Gw/+ mice can be decreased or enhanced by reducing the dosage of Wnt3a or Dkk1 genes, respectively. For the Ctnnb1-Bfc mutant mice, the viability of the Ctnnb1Bfc/+ mice was reduced and the incidence of ocular defects was increased when these heterozygotes also carried a Dkk1-null allele. Furthermore, expression of a WNT target, Axin2, was increased in Ctnnb1Bfc/+ and Ctnnb1Bfc/Bfc mutants. Taken together, these findings strongly suggest that the ENU-mutant allelic combinations of Lrp6 and Ctnnb1 are causing an increased level of WNT signalling activity, similar to the effect of loss of function of an antagonist such as DKK1.

The impact of the ENU-induced mutation on the function of LRPs and β-catenin can be deduced from the known function of the specific domains of protein (He et al., 2004; Schneider et al., 2003). Among the Lrp6 mutations, some are natural point mutations such as the Lrp6<sup>cd</sup> allele resulting in gain of WNT signalling activity (Carter et al., 2005; Kokubu et al., 2004; Pinson et al., 2000). Like the Lrp6<sup>cd</sup> mutation, the Lrp6-Gw mutation is localised in the second propeller domain (PD) coding region of the Lrp6 gene. As LRPs and DKK1 inhibition of the canonical WNT signalling activity, similar to the effect of loss of function of an antagonist such as DKK1.

The Ctnnb1<sup>Bfc</sup> allele is unique and contrasts with other Ctnnb1 mutations (Haegel et al., 1995; Huelsken et al., 2000) in its enhancing effect on WNT signalling activity and in that the mutant phenotype encompasses head deficiency and truncation. The Ctnnb1<sup>Bfc</sup> allele has any effect on WNT or DKK1 binding either. However, the Lrp6-Gw mutation might also alter the interaction of LRP6 with MESD and other transcriptional co-regulators (Stadeli et al., 2006; Willert and Jones, 2006), and also affects the interaction with cadherins to mediate cell adhesion (Yap et al., 1997). A previous study has shown that a conditional ablation of β-catenin in the forebrain results in head truncation (Junghans et al., 2005) similar to the
phenotype described in the present study. However, head truncations in this case might be caused by disruption of the interaction of β-catenin with N-cadherin (which affects cell adhesion and the maintenance of epithelial architecture of the neuroepithelium, and triggers cell death leading to the loss of brain structures) as no changes in β-catenin-WNT signalling activity were detected in the prospective telencephalon (Junghans et al., 2005). Our preliminary study indicates that the β-catenin protein with T635K mutation is functional regarding its interaction with N-Cadherin (data not shown). This finding and the upregulation of an endogenous WNT-target (Axin2) suggest that in the Ctnnb1-Bfc mutant the forebrain truncation phenotype is more likely to be caused by the excessive WNT activity in the telencephalon, a region which would normally not perceive any WNT signal (Fig. 1B).

Although the precise effect of these specific ENU-induced mutations on the function of LRP6 and β-catenin is not fully known, the fact that they both lead to a gain of WNT signalling activity provided a unique opportunity to test the interaction of these signalling pathway components with DKK1. We demonstrated a positive genetic interaction of Dkk1, Lrp6 and Ctnnb1 highlighting that the antagonist, the co-receptor and the transcriptional co-activator are integrated functionally in the signalling pathway for head formation. In 23% of the triple compound Dkk1+/−;Lrp6GW/−;Ctnnb1Bfc/− mutants, the combined effect of the mutant alleles of these three factors is sufficient to account for the phenotypic consequences of the elevated signalling response caused by the complete loss of antagonistic function in the Dkk1−− mutant. This finding suggests that DKK1 function for head development in mouse might essentially be to repress the canonical WNT pathway, in contrast to zebrafish and Xenopus in which DKK1 activity might also influence the WNT-PCP activity (Caneparo et al., 2007).

Our study has also provided insights into the dosage-dependent requirement of canonical WNT signalling activity in head morphogenesis. The different degrees of loss of the forebrain and head structures in the compound mutants of different genotype combinations (ranging from the presence of one mutant allele each of two genes [compound Dkk1;Lrp6, Dkk1;Ctnnb1, Lrp6;Ctnnb1 heterozygotes], two mutant alleles of the Lrp6 or the Ctnnb1 genes and one mutant allele for Dkk1 [compound Lrp6 homozygote with Dkk1 heterozygote or compound Ctnnb1 homozygote with Dkk1 heterozygote] to triple heterozygotes) provides an unique experimental readout of the impact of disrupted WNT signalling activity on forebrain development. Quantitative analysis of the expression of two endogenous WNT targets in these mutants has shown that the outcome of head development is tightly linked to canonical WNT signalling activity, the manifestation of the gradual head truncation phenotype being directly correlated to the progressive increase in the level of canonical WNT signalling activity. Therefore, our results reveal the importance of precise control of the level of canonical WNT signalling activity during normal head formation, which also underlies a differential sensitivity of the rostral tissues of the head and forebrain to the level of WNT signalling activity. The molecular basis of this sensitivity is not known. However, it might be related to the expression of inherently active signal receptors (e.g. those encoded by Fzd5 and Fzd8) and the signal transducers (e.g. LRP5, LRP6 and β-catenin) in the tissues of the anterior region of the embryo (Kemp et al., 2007; Lu et al., 2004; Kelly et al., 2004; Kimura-Yoshida et al., 2005) in which response to WNT proteins (e.g. WNT3) would normally be blocked by the antagonist (e.g. DKK1) (Lewis et al., 2008). Reduction of the antagonist activity or changes in the function of co-receptor and transcription co-activator could raise the signalling activity (Fig. 8) to the threshold level required to elicit WNT downstream activity (Fig. 9), which consequently disrupts morphogenesis during a crucial phase of head formation.

The exceptional sensitivity of the forebrain and associated craniofacial structures to WNT activity highlights the fact that the formation of the embryonic head is contingent on fine-tuned regulation of the domain and level of canonical WNT signalling activity in the progenitor tissues (Lagutin et al., 2003; Lewis et al., 2007; Lewis et al., 2008). In contrast to the impact of loss of WNT antagonist on anterior development, loss of WNT ligand (Yamaguchi et al., 1999) or β-catenin activity, particularly in the posterior embryonic tissues (Yap et al., 1997), leads to failure of the development of posterior structures. It transpires that the anterior-posterior patterning of body parts (in this case, the embryonic head) in the mouse can be mapped onto a WNT landscape where ligands, receptors/co-receptors, trans-activators and antagonists are regionalised and act in concert to establish an ascending gradient of signalling activity in the head-tail dimension (Fig. 9). The organisation of the body plan of the mammalian embryo might, therefore, be accomplished by deploying a WNT-mediated morphogenetic mechanism that is conserved among vertebrate species (Niehrs, 2004; Petersen and Reddien, 2009).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Fig. 9. The WNT landscape and signalling gradient in the head region of early mouse embryo. The left-hand panel depicts the regionalisation of the activity of the ligand (WNT3) (Lewis et al., 2008), the receptor (frizzled 5, frizzled 8; Fz5/8) (Kemp et al., 2007; Lu et al., 2004), the antagonist (DKK1) (Lewis et al., 2008), the co-receptor (LRP6) (Kelly et al., 2004) and the transcriptional co-activator (β-catenin) (Kimura-Yoshida et al., 2005), which translates into a resultant anterior-posterior gradient of signalling activity in the head region of the embryo (depicted in the right-hand panel). Cells in the anterior region of the embryo might be inherently poised to respond to the WNT signal but are suppressed from responding by the activity of the antagonist(s). A low threshold level of signalling activity for eliciting cellular response in the telencephalon and diencephalon might account for the findings that an elevation of the WNT activity leads to preferential loss/reduction of these brain parts. Ant, anterior; Post, posterior; d, diencephalon; m, mesencephalon; r, rhombencephalon; t, telencephalon.
References