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Lrp4 and Wise interplay controls the formation and patterning of mammary and other skin appendage placodes by modulating Wnt signaling

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SUMMARY

The future site of skin appendage development is marked by a placode during embryogenesis. Although Wnt/β-catenin signaling is known to be essential for skin appendage development, it is unclear which cellular processes are controlled by the signaling and how the precise level of the signaling activity is achieved during placode formation. We have investigated roles for Lrp4 and its potential ligand Wise (Sostdc1) in mammary and other skin appendage placodes. *Lrp4* mutant mice displayed a delay in placode initiation and changes in distribution and number of mammary precursor cells leading to abnormal morphology, number and position of mammary placodes. These *Lrp4* mammary defects, as well as limb defects, were associated with elevated Wnt/β-catenin signaling and were rescued by reducing the dose of the Wnt co-receptor genes *Lrp5* and *Lrp6*, or by inactivating the gene encoding β-catenin. *Wise*-null mice phenocopied a subset of the *Lrp4* mammary defects and *Wise* overexpression reduced the number of mammary precursor cells. Genetic epistasis analyses suggest that Wise requires Lrp4 to exert its function and that, together, they have a role in limiting mammary fate, but Lrp4 has an early Wise-independent role in facilitating placode formation. *Lrp4* and *Wise* mutants also share defects in vibrissa and hair follicle development, suggesting that the roles played by Lrp4 and Wise are common to skin appendages. Our study presents genetic evidence for interplay between Lrp4 and Wise in inhibiting Wnt/β-catenin signaling and provides an insight into how modulation of Wnt/β-catenin signaling controls cellular processes important for skin placode formation.

KEY WORDS: Wnt/β-catenin signaling, Lrp4, Sostdc1, Wnt antagonists, Mammary placodes, Skin appendages, Vibrissae, Limb

INTRODUCTION

Skin appendages such as teeth, hair and mammary glands develop from the surface ectoderm and underlying mesenchyme during embryogenesis. Despite the differences in the final structures, these skin appendages arise through similar morphological processes and tissue interactions in the early stages of their development (Mikkola and Millar, 2006). The future site of appendage development is initially marked by a thickening of the epithelium, which gives rise to a more localized placode. Subsequently, invagination of the placodal epithelium and condensation of the underlying mesenchymal cells leads to bud formation. Interactions within and between epithelial and mesenchymal tissues are essential for the proper growth and patterning of placode development. Genetic disruptions of genes encoding components of signaling pathways (Wnt, FGF, BMP, Eda, etc.) often cause developmental defects in multiple skin appendages, suggesting that patterning processes are shared among these appendages at the molecular level (Pispa and Thesleff, 2003; Mikkola and Millar, 2006).

Although many aspects of early patterning are similar, the spatial and temporal dynamics of placode development appear to be unique among the appendages. For example, hair placode formation begins with broad, regularly spaced epithelial thickenings, which are gradually refined to smaller circular placodes (Schmidt-Ullrich and Paus, 2005). By contrast, mammary placodes develop along the mammary lines, two lines of transient epithelial thickening, which appear between the fore- and hindlimb buds. Within one day, five pairs of mammary placodes form in a defined order as the mammary lines resolve (Robinson, 2007; Cowin and Wysolmerski, 2010) (Fig. 1C). The molecular and cellular basis of this transition is still unclear. However, earlier morphological studies in rabbits and recent cell-tracing experiments in mice suggested that the formation and growth of mammary placodes involve migration and reassembly of the mammary epithelial cells (Propper, 1978; Lee et al., 2011). This dynamic mode of placode formation suggests that mammary glands may have adopted a distinct molecular mechanism for placode induction.

Genetic studies in mouse have provided insights on signaling pathways required for embryonic mammary development (Robinson, 2007). In particular, Wnt signaling plays an important role in formation of mammary placodes. In the Wnt/β-catenin signaling pathway, interaction of Wnt ligands with Frizzled (Fz) receptors and the Wnt co-receptors Lrp5 and Lrp6 initiates a series of intracellular events leading to stabilization and nuclear accumulation of β-catenin. Subsequently, β-catenin forms complexes with TCF/LEF transcription factors and activates expression of target genes (MacDonald et al., 2009). Ectopic expression of the Wnt inhibitor Dickkopf 1 (Dkk1) blocks placode formation (Chu et al., 2004) and lack of Lef1, Lrp5 or Lrp6 disrupts normal placode development (van Genderen et al., 1994; Boras-Granic et al., 2006; Lindvall et al., 2006; Lindvall et al., 2009). It has been shown that Wnt/β-catenin signaling is initially activated in a broad domain along the mammary line, coincident with the expression pattern of a number of Wnt genes, but rapidly

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becomes restricted to mammary placodes (Chu et al., 2004; Veltmaat et al., 2004). This suggests that spatiotemporal control of the signaling activity is tightly coupled to placode formation. However, little is known about how precise control of Wnt signaling is achieved during embryonic mammary development.

Modulation of Wnt/β-catenin signaling in the extracellular space is often mediated by secreted Wnt antagonists, which interact with Wnts, Fz receptors or Lrp5/6 co-receptors (MacDonald et al., 2009). For example, Dkk1, Sost and Wise (Sostdc1 – Mouse Genome Informatics) can bind to the extracellular domain of Lrp5/6 and inhibit Wnt signaling presumably by disrupting the formation or activity of Wnt-induced Fz-Lrp5/6 complexes (Semënov et al., 2001; Itasaki et al., 2003; Li et al., 2005; Semënov et al., 2005). Another layer of complexity was added by recent findings on a low-density lipoprotein (LDL) receptor-related protein, Lrp4. The extracellular domain of Lrp4 resembles that of Lrp5/6, but its intracellular domain is distinct from that of Lrp5/6, suggesting that it may have different inputs on Wnt signaling (Herz and Bock, 2002; Weatherbee et al., 2006). In humans, *LRP4* mutations cause limb, kidney and tooth malformations in Cenani-Lenz syndrome and are associated with bone overgrowth in two isolated cases of sclerosteosis (Li et al., 2010; Leupin et al., 2011). The role for Lrp4 appears to be conserved in mammals as mice deficient for *Lrp4* also display defects in limbs, kidney and teeth (Johnson et al., 2005; Weatherbee et al., 2006; Ohazama et al., 2008).

In *Lrp4* mutant mice, limb and tooth defects were associated with abnormal Wnt signaling activity. Furthermore, Lrp4 can antagonize activation of Wnt signaling when overexpressed in cultured cells, and this inhibitory activity is lost in mutant proteins (Johnson et al., 2005; Li et al., 2010). However, studies in bone and kidney development revealed no apparent elevation of Wnt signaling in *Lrp4* mutants (Choi et al., 2009; Karner et al., 2010). In addition, Lrp4 is implicated in regulation of Bmp signaling in some contexts and functions as a co-receptor for Agrin in the neuromuscular junction (Kim et al., 2008; Ohazama et al., 2008; Zhang et al., 2008). Therefore, whether Lrp4 directly inhibits the Wnt pathway or controls another pathway to indirectly affect Wnt signaling *in vivo* is unclear.

Similar to Lrp5/6, Lrp4 can bind *in vitro* to Dkk1, Sost and Wise, suggesting that roles for Lrp4 in Wnt signaling may be modulated by binding of these antagonists (Ohazama et al., 2008; Choi et al., 2009; Karner et al., 2010). This is consistent with the observation that Lrp4 facilitates the Wnt inhibitory function of Sost in *in vitro* bone mineralization (Leupin et al., 2011). In addition to this potential cell-autonomous role as a membrane receptor, Lrp4 is also postulated to modulate Wnt signaling by releasing its extracellular domain, and hence sequestering Wnt antagonists (Choi et al., 2009; Dietrich et al., 2010). It remains to be determined whether interaction between Lrp4 and the Wnt antagonists plays a significant role *in vivo*.

Wise is known as a context-dependent modulator of Wnt signaling and an inhibitor of Bmp signaling (Itasaki et al., 2003; Laurikkala et al., 2003; Lintern et al., 2009). The strong genetic interaction of *Wise* with *Lrp5* and *Lrp6* suggested that Wise controls tooth number and patterning by inhibiting Wnt signaling (Ahn et al., 2010). In this study, we present *in vivo* evidence for interaction between Lrp4 and Wise in mammary glands and other skin appendage development. Our genetic analyses revealed that Lrp4 has an early role in facilitating placode initiation and, together with Wise, has later roles in placode patterning. Our data demonstrate that tight control of Wnt/β-catenin signaling is crucial for timely initiation and patterning of mammary and vibrissae placodes, and provide insights into interplay between Lrp4 and Wnt antagonists in Wnt inhibition.

MATERIALS AND METHODS

Mouse strains

Lrp4^{mdig}, *Lrp4^{mitt}*, *Lrp4^{mte}*, *TopGal*, *TCF/LEF:H2B-GFP*, *Lrp5*, *Lrp6*, *Ctnnb1^{fx}*, *K14cre*, *R26-floxstop-lacZ* and *Wise* mice have been described previously (DasGupta and Fuchs, 1999; Soriano, 1999; Dassule et al., 2000; Pinson et al., 2000; Brault et al., 2001; Kato et al., 2002; Simon-Chazottes et al., 2006; Weatherbee et al., 2006; Ahn et al., 2010; Ferrer-Vaquer et al., 2010). All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Stowers Institute for Medical Research (Protocol 2010-0062).

Generation of *Lrp4-lacZ*, *K14-tTA*, *TCF-tTA* and *tetO-Wise* transgenic mice

For *Lrp4-lacZ* BAC reporter, a mouse BAC clone, RP23-276H15, was modified to contain an 134 kb genomic region that covers the whole *Lrp4*-coding region and neighboring upstream (36 kb) and downstream (44 kb) sequences using the bacterial recombination technology (Lee et al., 2001). *lacZ* was then inserted in frame into the first coding exon of *Lrp4*. The *K14-tTA* was generated by inserting the *K14* promoter (Ahn et al., 2010) and a synthetic intron (IVS) (Clontech) upstream of *VP22-tTA-SV40pA* (Gossen and Bujard, 1992). For *TCF-tTA*, the *K14* promoter of *K14-tTA* was removed except the basal promoter region (–120 to +13) and replaced with the multiple TCF-binding sites from TOPFLASH vector (Millipore). To make *tetO-Wise*, *G-CaMP2* of *tetO-G-CaMP2* (He et al., 2008) was replaced with a *Wise* ORF, and then *IRES-eGFP* was subcloned between *Wise* and *SV40pA*. Transgenic founders were generated by pro-nuclear injection of linearized constructs into C57Bl/10J×CBA-F1 embryos.

β-Gal staining, *in situ* hybridization and BrdU analysis

To detect β-galactosidase activity, embryos were fixed in either 0.1% paraformaldehyde/0.2% glutaraldehyde (E11.5-E13.5) or 4% paraformaldehyde (PFA) (E14.0 or older) for 30-60 minutes on ice. After several washes in phosphate-buffered saline, samples were stained in X-Gal for 4-20 hours at 4°C or at room temperature. Whole-mount *in situ* hybridization was performed with embryos fixed in 4% PFA overnight according to standard protocols using DIG-labeled antisense riboprobes. Histological samples were paraffin wax-embedded after post-fixation in 4% PFA, sectioned at 8 μm and counterstained with nuclear Fast Red. For analysis of cell proliferation and cell death, embryos were harvested 2 hours after intraperitoneal injection of BrdU (50 μg/g body weight) into pregnant females, sectioned and stained with a mouse anti-BrdU antibody (Amersham), a mouse E-cadherin antibody (BD Biosciences) or a rabbit caspase 3 antibody (Cell Signaling).

Confocal microscopy and cell counting

Fluorescent images were obtained by the LSM 710 confocal microscope (Carl Zeiss). Nuclei with fluorescence above basal level were counted using the Imaris software (Bitplane).

RESULTS

Abnormal development of the mammary glands in Lrp4 mutant mice

Lrp4 is known to be expressed in placodes of skin appendages such as mammary glands, hair follicles and vibrissae (Weatherbee et al., 2006; Fliniaux et al., 2008). This prompted us to look into potential roles for Lrp4 in development of these tissues. Mice homozygous for null alleles of *Lrp4* (*Lrp4^{mitt}* and *Lrp4^{mte}*) die after birth, but mice homozygous for a hypomorphic allele (*Lrp4^{mdig}*) survive to reach adulthood (Simon-Chazottes et al., 2006; Weatherbee et al., 2006). Our analyses of both *Lrp4$^{mitt/mdig}$* and *Lrp4$^{mdig/mdig}$* females revealed a variety of abnormalities in the number, position and morphology of nipples (Fig. 1A,B and data not shown). In *Lrp4* mutant females, nipples 2 and 3 were frequently fused and the

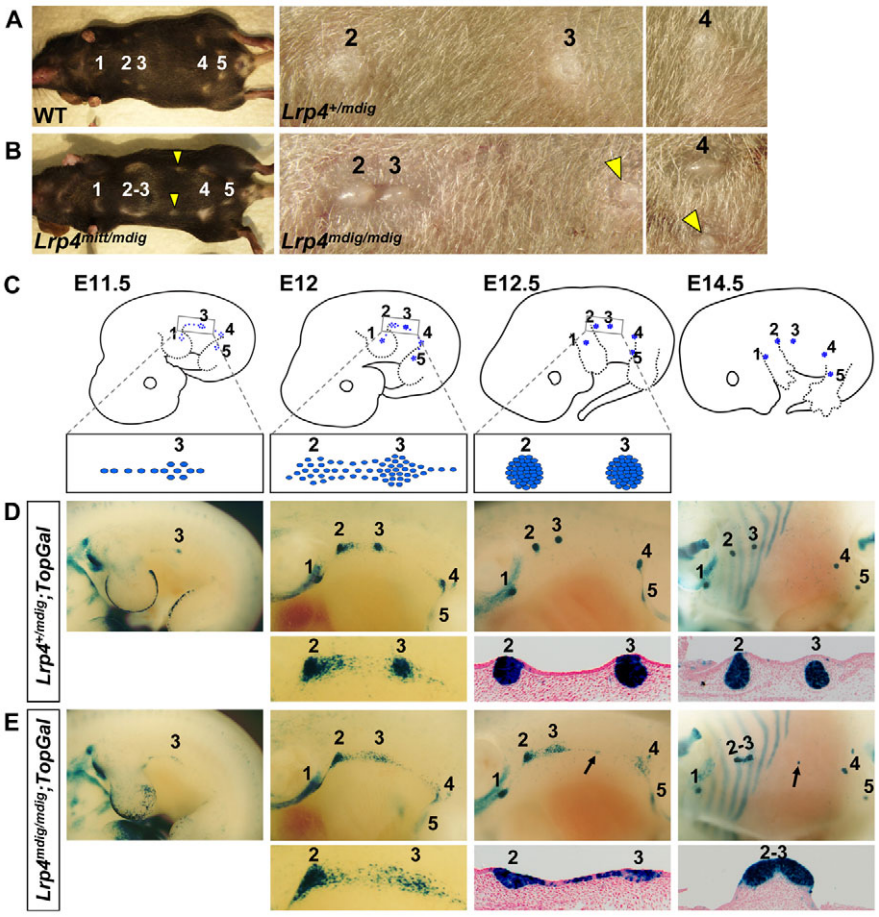

Fig. 1. Abnormal mammary development in *Lrp4* mutant mice. (**A**) Five pairs of nipples in a pregnant control female. (**B**) *Lrp4* mutant female displays ectopic nipples (arrowheads) and fusion of nipples 2 and 3. (**C**) The appearance of mammary placodes during embryogenesis (top) and distribution of mammary epithelial cells around placodes 2 and 3 (bottom). (**D**,**E**) *TopGal*-expressing epithelial cells are gradually restricted to placodes in controls. In *Lrp4* mutants, delayed placode formation (E12.0) is followed by ectopic placodes (arrows) and fusion of placodes 2 and 3. Higher magnification images (E12.0) and histological sections (E12.5 and E14.5) of the placode 2/3 area are shown below.

individual nipples were enlarged compared with those of control females. In addition, ectopic nipples were present in the region between nipples 3 and 4, and around nipple 4 (yellow arrowheads in Fig. 1B). The ectopic nipples were smaller than normal nipples and were associated with little or no fat pads, suggesting that they are non-functional (data not shown).

Lrp4 is essential for patterning of the mammary placodes

The mammary defects in *Lrp4* mutants suggest that Lrp4 plays a role in embryonic mammary development. The number and position of the nipples and associated mammary glands is primarily determined around embryonic day 12 (E12) when the mammary placodes develop (Cowin and Wysolmerski, 2010). We used the *TopGal* reporter mouse line (DasGupta and Fuchs, 1999) to follow the progress of mammary development and to monitor changes in the activity of Wnt/β-catenin signaling (Fig. 1C,D,E). Consistent with the previous report (Chu et al., 2004), in control embryos, *TopGal*-expressing epithelial cells were spread along the mammary lines at E11.5, and within a day they sequentially became restricted to placodes in a defined order (3, 1/4, 5 and, finally, 2). *TopGal* expression was gradually lost in the inter-placodal regions; after E12.5, *TopGal* expression was seen only in the epithelial cells of the mammary buds.

In $Lrp4^{mdig/mdig}$ embryos, *TopGal*-expressing cells were more loosely organized around the developing placodes at E12.0, suggesting that placode assembly was delayed (compare Fig. 1D with 1E). This is particularly apparent in placodes 3 and 4 at this stage. Consistent with a delay, the mutant placodes displayed broader but shallower epithelial invagination at E12.5, which is typical of an earlier stage placode. Furthermore, there were ectopic *TopGal*-expressing cells spread along the mammary line. A large proportion of these cells were found around the underdeveloped placodes, especially placodes 2, 3 and 4. It appeared that some of these cells later give rise to supernumerary placodes (arrows in Fig. 1E) in the interplacodal region, which correspond to the site of supernumerary nipples. Similar changes were observed in $Lrp4^{mitt/mdig}$ and $Lrp4^{mitt/mitt}$ mice, indicating that the observed defects are loss-of-function phenotypes (supplementary material Fig. S1).

We investigated whether the abnormal mammary patterning in *Lrp4* mutants is associated with changes in the number of mammary epithelial cells using the *TCF/LEF:H2B-GFP* reporter, which marks mammary placodes similar to *TopGal* (Ferrer-Vaquer et al., 2010). Confocal imaging of the placode 2/3 region revealed a 40% increase in the total number of GFP-expressing cells (Fig. 2A-C; supplementary material Movies 1, 2). Together, these data indicate that Lrp4 is required for facilitating the assembly of mammary placodes and for limiting the number of mammary epithelial cells.

Reduced proliferation of the misplaced mammary epithelial cells causes placode fusion

Although mammary placodes 2 and 3 were developmentally delayed and morphologically abnormal in *Lrp4* mutants, they were centered at fairly normal positions at E12.0 (Fig. 1E). However, afterwards the distance between the two placodes was reduced compared with controls, leading to fusion later in development (Fig. 1E). To

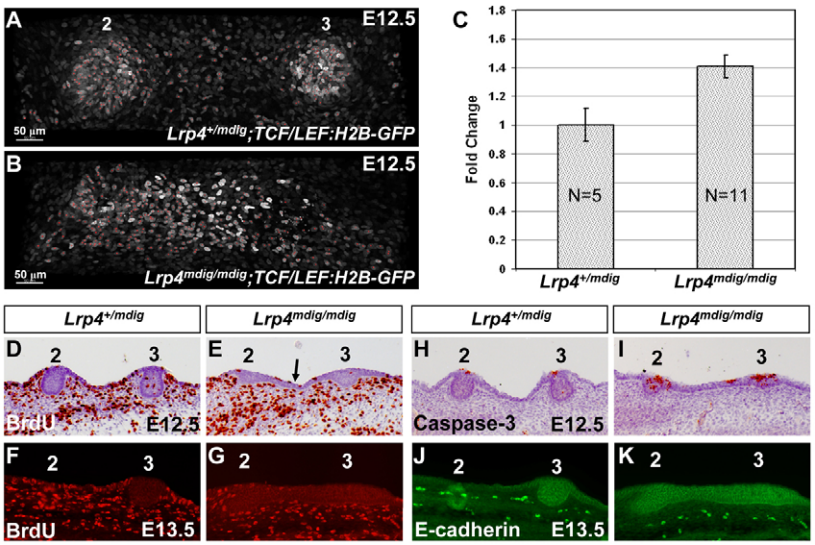

Fig. 2. Increased number of mammary epithelial cells in *Lrp4* mutants. (**A**,**B**) Confocal images of the placode 2/3 region from *TCF/LEF:H2B-GFP* embryos. (**C**) Relative number of GFP-positive cells as marked by a red dot in A,B. Data are mean±s.d. (**D-G**) BrdU staining is reduced in the interplacodal region (arrow) in *Lrp4* mutants. (**H**,**I**) Caspase 3 staining. (**J**,**K**) E-cadherin staining. Note that placode 2 is out of the focal plane in F and J.

investigate the underlying basis of this placode fusion, we examined the rate of cell proliferation and cell death. As previously reported (Balinsky, 1950), in control mice non-mammary epithelial cells surrounding the placodes were actively proliferating whereas the placodes themselves displayed a very low level of proliferation (Fig. 2D). By contrast, in *Lrp4*$^{mdig/mdig}$ mice, cell proliferation was greatly reduced in the interplacodal region (Fig. 2E; supplementary material Fig. S2). The interplacodal region continued to show reduced proliferation and was thickened at E13.5 in the mutants (Fig. 2F,G,J,K). Combined with the *TopGal* and *TCF/LEF:H2B-GFP* expression data, our results indicate that cells in the interplacodal region in *Lrp4* mutants possess mammary fate.

With respect to cell death, in control mice, a small number of apoptotic cells were observed mostly around the neck of the buds, but not in the interplacodal epithelium (Fig. 2H). In *Lrp4* mutants, more apoptotic cells were observed in the interplacodal region and also around the sites of invagination (Fig. 2I). Together, these results suggest that placode fusion in the mutants is largely due to relatively slow growth of ectopic mammary epithelial cells in the interplacodal region that forms part of the large extended placode, but removal of some epithelial cells by cell death also contributes to the fusion.

Reduction in the dose of the Wnt co-receptors ameliorates the *Lrp4* mutant defects in limb and mammary patterning

To examine which signaling pathways were misregulated in the mammary placodes of *Lrp4* mutants, placodes 2 and 3 were dissected from E12.5 embryos and expression analysis was performed using qPCR assays designed for components of Wnt, FGF, TGFβ/BMP and Eda pathways (supplementary material Fig. S3). Differential expression of genes in Wnt (*Dkk1*, *Dkk4* and *Lef1*) and TGFβ/BMP (*Bmp3*, *Msx1* and *Msx2*) pathways suggests that signaling activity of the two pathways is changed in *Lrp4* mutants.

The increased number and abnormal distribution of cells expressing the Wnt reporters (Figs 1, 2) and *Lef-1* (supplementary material Fig. S3) in *Lrp4* mutants raises the possibility that elevation of Wnt/β-catenin signaling is causally related to the mammary defects. To explore this idea, we examined genetic interactions between *Lrp4* and the Wnt co-receptor genes *Lrp5* and *Lrp6*. In crosses between *Lrp4* and *Lrp5/6* mutants, we first focused on examining limb defects as a means to score for genetic interactions (Fig. 3A). All the known *Lrp4* mutants have been characterized by abnormal patterning of the apical ectodermal ridge (AER) and polysyndactyly, whereas *Lrp5;Lrp6* compound mutants displayed limb defects in a dose-dependent manner (Holmen et al., 2004; Johnson et al., 2005; Simon-Chazottes et al., 2006; Weatherbee et al., 2006). At E13.5, *TopGal*-expressing cells were normally confined to AER as a thin line, but in *Lrp4* mutants these cells were scattered in the distal limb buds owing to broadening of AER. Interestingly, inactivating two copies of *Lrp5* or single copy of *Lrp6* ameliorated the AER defects of *Lrp4* mutants and fairly normal limb patterning was observed in *Lrp4*$^{mdig/mdig}$*;Lrp5*$^{+/–}$*;Lrp6*$^{+/–}$ mice. Loss of *Lrp6* resulted in severe limb defects with a stronger effect on hind limbs (Pinson et al., 2000; Zhou et al., 2010). Such limb defects of *Lrp6*-null mice were significantly rescued in *Lrp4*$^{mdig/mdig}$*;Lrp6*$^{–/–}$ mice (n=5) (Fig. 3C,C′), indicating that Lrp4 and Lrp6 act antagonistically.

It has been shown that embryonic mammary development is delayed or severely impaired in *Lrp5*$^{–/–}$ and *Lrp6*$^{–/–}$ mice, respectively, in association with reduced Wnt signaling activity. (Lindvall et al., 2006; Lindvall et al., 2009). We observed that reduced doses of *Lrp5* and *Lrp6* can rescue the mammary defects of *Lrp4* mutants (Fig. 3B,B'). When both copies of *Lrp5* or a single copy of *Lrp6* were inactivated in *Lrp4* mutants, *TopGal* expressing cells were more confined around the sites of bud formation, indicating amelioration of *Lrp4* mutant phenotypes. Furthermore, in *Lrp4*$^{mdig/mdig}$*;Lrp5*$^{+/–}$*;Lrp6*$^{+/–}$ mice, the buds appeared to be fairly normal, and buds 2 and 3 were fully separated in the majority of cases (Fig. 3D). These genetic interactions indicate that the abnormal limb and mammary development in *Lrp4* mutants is largely due to elevated Wnt signaling and support the idea that Lrp4 inhibits Wnt/β-catenin signaling *in vivo*.

Lrp4 facilitates placode formation and restricts mammary fate by inhibiting Wnt/β-catenin signaling

We investigated whether the normal timing of placode initiation is restored in *Lrp4*$^{mdig/mdig}$*;Lrp5*$^{+/–}$*;Lrp6*$^{+/–}$ mice. Indeed, the compound mutants displayed placodes of almost normal morphology and size with few *TopGal*-expressing cells in the interplacodal region at E12.5 (Fig. 4A-C′). This suggests that a reduction in Wnt/β-catenin signaling can compensate for loss of Lrp4 function and facilitate placode formation in *Lrp4* mutants.

DEVELOPMENT

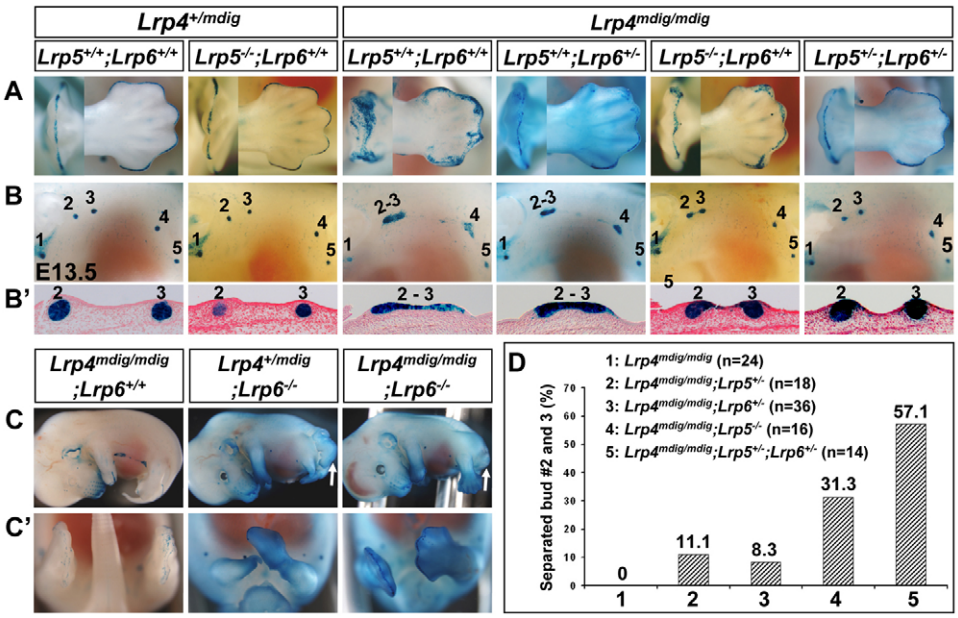

Fig. 3. Genetic interaction of *Lrp4* with *Lrp5* and *Lrp6*. *TopGal* expression at E13.5. A low level of broad β-galactosidase activity is detectable from the *Lrp6* mutant allele. (**A-B′**) Reduced dosages of *Lrp5* and *Lrp6* rescue the limb (A) and mammary (B,B′) defects of *Lrp4* mutants. A proximal (left, dorsal to the right) and a dorsal (right) view of a forelimb bud are shown with anterior to the top (A). (**C**,**C′**) *Lrp4* and *Lrp6* compensate for loss of each other in limbs. Note that hindlimb defects of *Lrp6*$^{-/-}$ mice were rescued by inactivation of *Lrp4*, but other defects such as loss of tail remain the same (arrows). (**D**) Separation of mammary bud #2 and 3 by reduced dosages of *Lrp5* and *Lrp6* in *Lrp4* mutants.

To further explore a role for Wnt/β-catenin signaling in controlling the number of mammary epithelial cells, we inactivated the β-catenin gene (*Ctnnb1*) in the epithelium after placode initiation using a conditional allele of β-catenin combined with a *Cre* line driven by a Keratin 14 promoter (*K14cre*). *K14cre* can induce recombination in a subset of epithelial cells along the mammary line at E11.5-E12.0 (Fig. 4D,E). By E12.5, Cre activity is detected in most epithelial cells in and around the mammary buds (Fig. 4F,F′). In *β-catenin*$^{fx/-}$*;K14cre* mice, all the buds formed at their normal position, consistent with the late onset of Cre activity, but they were smaller at E12.5 and remained growth-retarded afterwards (Fig. 4G,H,G′,H′; supplementary material Fig. S4). This suggests that Wnt/β-catenin signaling is required for producing a sufficient pool of the mammary precursor cells and for facilitating growth of the buds at later stages.

We then tested whether inactivation of *Ctnnb1* has effects on the *Lrp4* mutant phenotypes (Fig. 4I,J,I′,J′). Interestingly, a greater reduction in placode size was observed in *Lrp4* mutants than in control mice when *Ctnnb1* was inactivated. *Lrp4*$^{mitt/mdig}$*;β-catenin*$^{-/fx}$*;K14cre* mice often developed a variable number of small placodes in the placode 2/3 region. We interpret this to mean that due to the delay in placode formation in *Lrp4* mutants, *Ctnnb1* was inactivated at a relatively earlier stage of placode development, resulting in further reduction in mammary precursor cells. These genetic analyses further support the idea that Wnt/β-catenin is essential for inducing or maintaining the mammary fate in the epithelial cells before placode assembly. Taken together, these data suggest that Lrp4 normally facilitates placode formation and limits the number of mammary epithelial cells by inhibiting Wnt/β-catenin signaling.

Lrp4 is required for development of hair and vibrissal follicles

We next investigated whether our findings in mammary gland development reflect related roles for Lrp4 in other skin appendages.

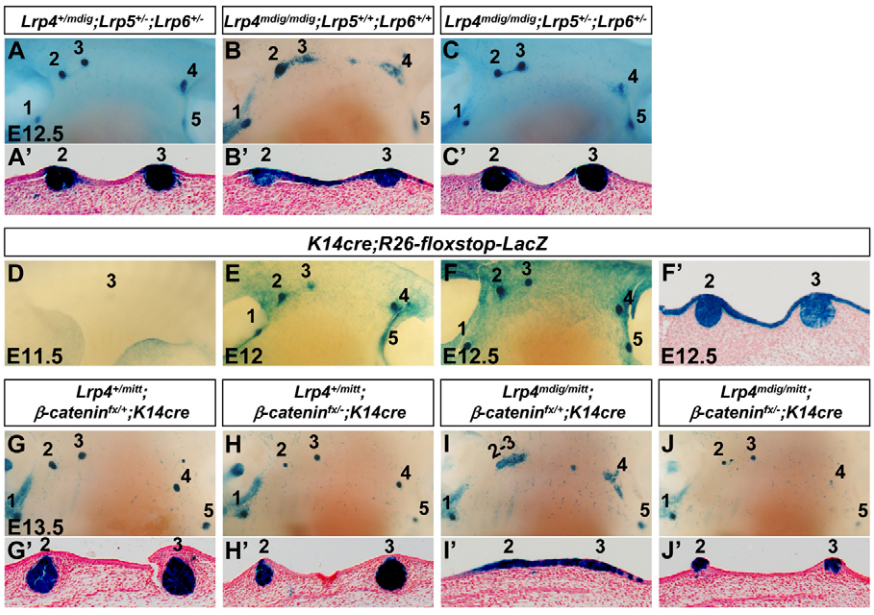

Fig. 4. Lrp4 facilitates placode initiation and controls the number of mammary epithelial cells via inhibition of Wnt/β-catenin signaling. (**A-C′**) Reduced dose of *Lrp5* and *Lrp6* restores normal timing of placode initiation and reduces ectopic *TopGal*-expressing cells in *Lrp4* mutants. (**D-F′**) Detection of Cre activity from *K14cre* transgene. (**G-J′**) Conditional inactivation of *Ctnnb1* in control mice results in smaller mammary buds. In *Lrp4* mutants, inactivation of *Ctnnb1* results in separated, but much smaller, buds.

Primary hair follicles were marked by *Wnt10b* transcripts at E14.5 in control mice, but in the *Lrp4* mutant skin, *Wnt10b* was undetectable (Fig. 5A). Our *Lrp4-lacZ* BAC reporter line marked newly forming hair placodes at E13.5 and continued to express in the primary hair follicles (Fig. 5B,C), mimicking endogenous *Lrp4* expression pattern (Fliniaux et al., 2008). In *Lrp4* mutants, *Lrp4-lacZ* expression was not observed at E13.5, and hair placodes were less developed at E14.5, indicating that hair follicle development is delayed (Fig. 5C,E).

Groups of vibrissae develop in different regions of the mouse head (Yamakado and Yohro, 1979), and supernumerary vibrissal follicles were observed for each group in *Lrp4* mutants (Fig. 5H-I; supplementary material Fig. S5). In particular, we detected extra interramal vibrissal follicles, which form along a transverse line under the chin at E14.5 (Fig. 5H-I). One day earlier, there was a delay in morphogenesis of the follicles in *Lrp4* mutants with less condensed domains of *TopGal* expression (Fig. 5F-G′). In general, these phenotypes were milder and less penetrant in $Lrp4^{mdig/mdig}$ mice compared with $Lrp4^{mitt/mitt}$ mice (data not shown). Our analyses revealed that Lrp4 is required for timely formation of hair and vibrissal follicles, and suggest that Lrp4 normally facilitates morphogenesis of these skin placodes similar to its role in mammary placodes.

Wise is required for development of mammary glands and vibrissae

As Wise is a potential ligand for Lrp4 and mice deficient for *Wise* or *Lrp4* displayed similar tooth defects, we investigated roles for Wise in the mammary glands and other skin appendages. Earlier studies have shown that, in developing skin appendages, *Wise* is excluded from the epithelial signaling centers where *Lrp4* is expressed (Laurikkala et al., 2003; Weatherbee et al., 2006). During mammary placode formation, *Lrp4* was expressed in the placodal epithelial cells similar to *Lef-1*, whereas *Wise* expression was strong in the surrounding epithelial and mesenchymal cells (Fig. 6A,A′). Comparison of *TopGal*, which marks the epithelial signaling centers, and our *Wise-lacZ* reporter further demonstrates that the complementary expression pattern of *Lrp4* and *Wise* is a common feature of skin appendage formation (supplementary material Fig. S5).

In *Wise*-null females, we observed changes in the position and number of nipples (Fig. 6B). In control females, there was only a modest level of variation in the distance between nipples 2 and 3 (data not shown). However, in the majority of *Wise*-null females the distance was greatly reduced, and with a low frequency (4/18) the two nipples were fused or juxtaposed next to each other. In addition, *Wise*-null females frequently displayed supernumerary nipples around normal ones. We next examined changes in *TopGal* expression in *Wise*-null mice (Fig. 6C,D). In the mutants at E12.0, the placodes appeared modestly enlarged, but formed at the normal positions with no clear sign of delay in placode assembly. However, by E12.5, mutant placodes were further enlarged with an increased number of *TopGal*-expressing cells. Some of these *TopGal*-expressing cells were observed outside the placodes, in particular in the region between placodes 2 and 3, and formed a bridge connecting the two placodes. Histological sections revealed that the expanded *TopGal* expression was associated with abnormal morphology of the mammary epithelium in the mutant. The distance between the two placodes/buds became gradually reduced in most mutants, often leading to fusion by E14.5 (4/12) consistent with the adult nipple phenotypes. Similar to the observation in $Lrp4^{mdig/mdig}$ mice, this abnormal spacing between the placodes was associated with reduced proliferation in the interplacodal region (supplementary material Fig. S5). These data indicate that Wise and Lrp4 have a similar role in controlling the distribution and number of the mammary epithelial cells, but Wise is largely dispensable for placode initiation.

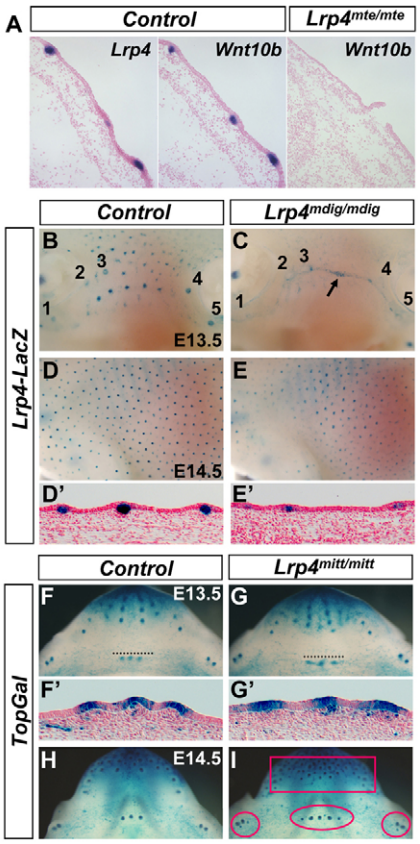

Fig. 5. *Lrp4* is required for development of other skin appendages. (**A**) Delayed formation of the primary hair follicles in *Lrp4* mutants, as evidenced by lack of *Wnt10b* expression. (**B-E′**) Expression of the *Lrp4-lacZ* BAC reporter line in the primary hair follicles and mammary buds (1-5) at E13.5-E14.5. Focalized reporter expression is normally observed in mature hair placodes of back skin at E14.5. In *Lrp4* mutants, *Lrp4-lacZ* expression is spread along the mammary line (arrow) with no sign of hair placodes at E13.5 (C) and hair follicle morphogenesis is delayed (E,E′). (**F-G′**) Abnormal patterning of interramal vibrissal follicles in *Lrp4* mutants. Frontal sections (F′-G′) were obtained along the broken line. (**H**,**I**) *Lrp4* mutants display supernumerary vibrissal follicles in the submental (rectangle), postoral (circle) and interramal (oval) regions.

In addition to the mammary defects, *Wise*-null mice displayed supernumerary vibrissal follicles with a frequency lower than that of $Lrp4^{mitt/mitt}$ mice (supplementary material Fig. S5; data not shown). Overall, our data suggest that Lrp4 and Wise are required for common processes in skin appendage development, but Lrp4 has additional roles. During the preparation of this manuscript, Närhi et al. independently reported similar defects in mammary glands and vibrissae of *Wise*-null mice (Närhi et al., 2012). Relatively milder mammary defects, such as lack of fusion described by Närhi et al., are probably due to difference in strain background.

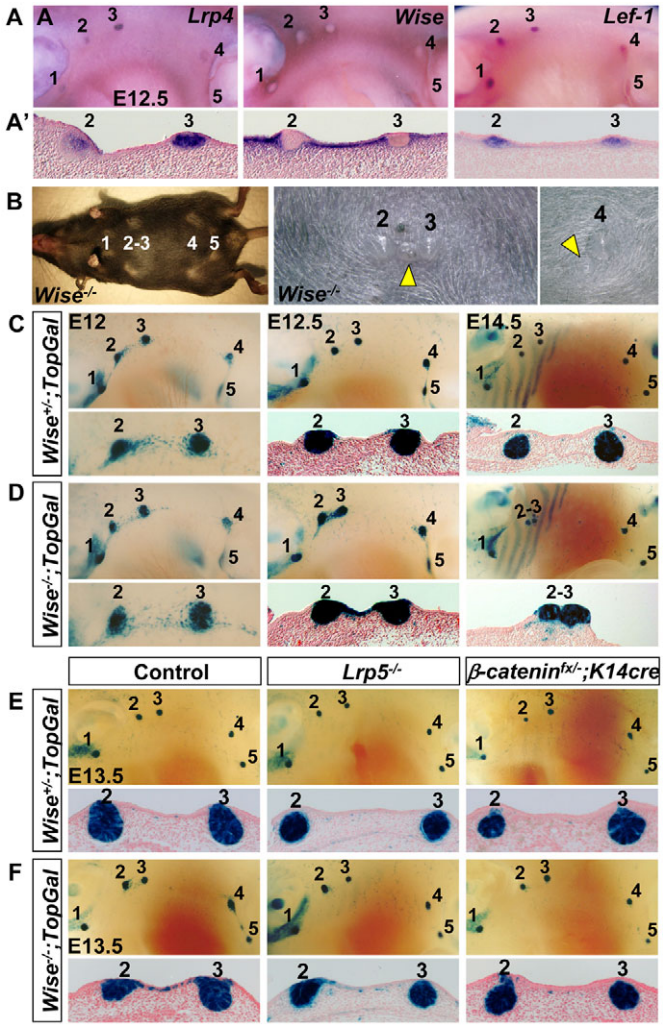

Fig. 6. Wise controls patterning of mammary placodes via the Wnt/β-catenin pathway. (**A**,**A′**) Whole-mount *in situ* hybridization (A) and cross-section across mammary placodes 2 and 3 (A′). (**B**) *Wise*-null females display abnormal spacing between nipples and supernumerary nipples (arrowheads). (**C**,**D**) In mutants (D), abnormal size and morphology of the placodes is apparent by E12.5. The distance between placodes 2 and 3 is reduced, often leading to fusion at later stages. *TopGal*-expressing cells are ectopically observed in the interplacodal regions. (**E**,**F**) Loss of *Lrp5* or epithelial inactivation of *Ctnnb1* restores normal spacing between mammary buds 2 and 3 in *Wise*-null mice.

Wise controls the number and distribution of the mammary epithelial cells via inhibition of Wnt/β-catenin signaling

We genetically tested whether changes in Wnt/β-catenin signaling account for the *Wise*-null mammary defects. We first found that removing both copies of *Lrp5* significantly rescues the abnormal spacing and ectopic *TopGal* expression of *Wise*-null mammary buds (Fig. 6E,F). In addition, epithelial inactivation of *Ctnnb1* eliminated the ectopic *TopGal* expression around the buds and restored the normal spacing between the buds 2 and 3 in *Wise*-null mice (Fig. 6E,F). These genetic interactions suggest that elevated Wnt/β-catenin signaling is the primary cause of mammary defects in *Wise*-null mice.

To complement and validate predicted roles for Wise based on loss-of-function analyses, we investigated whether overexpression of Wise using the keratin 14 promoter (*K14-Wise*) (Ahn et al., 2010) can reduce the number of placodal epithelial cells. *K14-Wise* embryos showed defects in development of hair/vibrissal follicles, mammary placodes and limbs with reduced *TopGal* expression (supplementary material Fig. S6). Owing to the challenge in maintaining viable *K14-Wise* mice, we established a bi-transgenic system in which expression of tetracycline-controlled transactivator (tTA) is driven by the keratin 14 promoter (*K14-tTA*) in a driver line and tTA activates expression of *Wise* together with *eGFP* in an expressor line (*tetO-Wise*) (Fig. 7A) (Gossen and Bujard, 1992). Using a strong (32) or a moderate (87) *K14-tTA* driver, the mammary defects of *K14-Wise* embryos were reproduced (Fig. 7B-E). *Wise* overexpression led to a significant reduction in the number of mammary epithelial cells present around the placodes (Fig. 7B′,C′). Importantly, *Wise* overexpression in the epithelium was sufficient to restore the normal morphology and spacing of the placodes in *Wise*-null mice (Fig. 7D-G′). Using the promoter with multiple TCF-binding sites (*TCF-tTA*), similar mammary defects were observed even when *Wise* was overexpressed specifically in the placodes (Fig. 7H-K′). As *Wise* is not normally expressed in the placodes, these gain-of-function phenotypes are consistent with the non-cell-autonomous function of Wise as a secreted protein. Together, our loss- and gain-of-function analyses suggest that Wise controls the number and distribution of the mammary epithelial cells during placode formation by inhibiting Wnt/β-catenin signaling.

Wise requires Lrp4 to exert its function *in vivo*

The overall similarities in skin defects and elevated Wnt signaling in *Lrp4* and *Wise* mutants raise the issue of whether Lrp4 and Wise act through a common or parallel pathway. To genetically test this idea, we generated combinatorial mutants of the two genes. No mammary defect was observed in transheterozygotes, and defects in double homozygous mutants were indistinguishable from those of $Lrp4^{mdig/mdig}$ mice during embryonic mammary development (Fig. 8A-C). A similar genetic interaction was observed with $Lrp4^{mitt}$ (data not shown). This epistasis analysis suggests that inactivating *Wise* does not exacerbate the defects in *Lrp4* mutants.

Considering the close genetic interaction of both genes with the components of Wnt/β-catenin pathway, this lack of synergy or additive effect between the two mutants suggests that Lrp4 and Wise may be acting on the same pathway to inhibit Wnt/β-catenin signaling and Lrp4 acts downstream of Wise. Alternatively, it is possible that Lrp4 and Wise function independent of each other, but Lrp4 has a larger role in modulating the level of signaling activity. To distinguish between these possibilities, we overexpressed *Wise* in *Lrp4* mutants. Elevated *Wise* expression would rescue the *Lrp4* mutant phenotypes if Wise and Lrp4 function primarily in an independent manner. However, overexpression of *Wise* resulted in no changes in the limb and mammary defects of *Lrp4* mutants (Fig. 8D-I; Fig. 7G′-I′). Although *Wise* overexpression reduced the number of vibrissal follicles in control mice, $Lrp4^{mdig/mdig}$*;K14tTA;tetO-Wise* mice still displayed supernumerary vibrissal follicles (Fig. 8J-M). *Wise* overexpression also disrupted *TopGal* expression in the tongue, consistent with the essential role of Wnt signaling in the taste papilla development (Iwatsuki et al., 2007) (Fig. 8J′,L′). However, in $Lrp4^{mdig/mdig}$*;K14tTA;tetO-Wise* mice, only minor changes in *TopGal* expression were observed in the tongue (Fig. 8K′,M′). These data suggest that, in the mammary placodes and other contexts, Wise depends on Lrp4 for its function; they also support the idea that Lrp4 acts downstream of Wise to inhibit Wnt signaling.

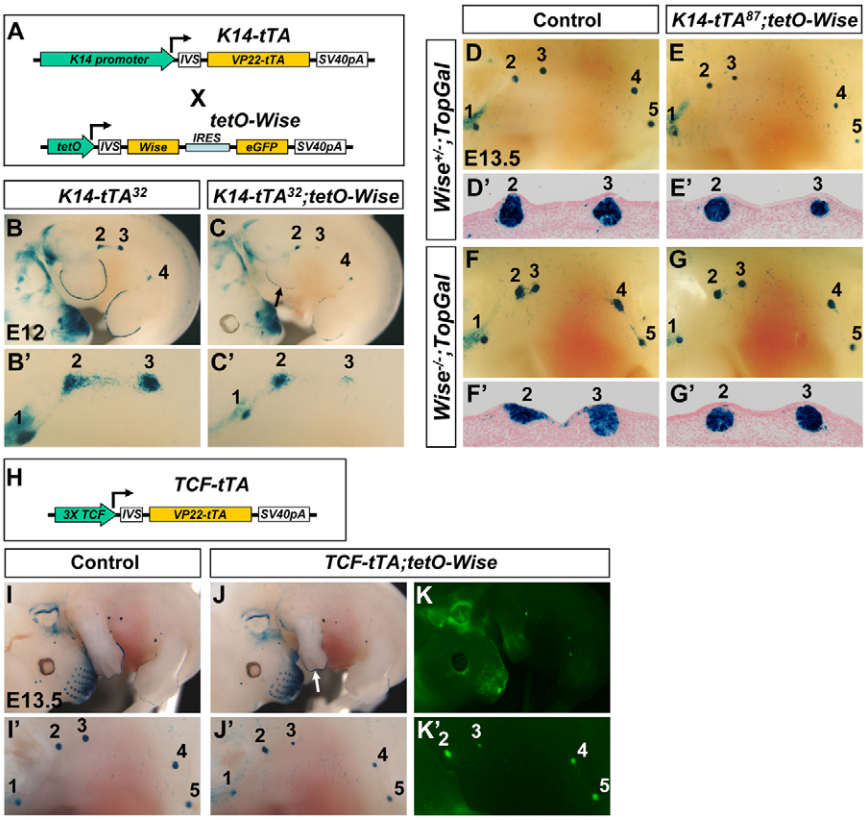

Fig. 7. *Wise* overexpression disrupts mammary development. (**A**) *K14-tTA* and *tetO-Wise* constructs. (**B-C′**) With a strong driver, *Wise* overexpression disrupts limb development (arrow) and results in smaller mammary placodes. (**D-G′**) *Wise*-null mammary defects are rescued by a moderate level of *Wise* expression in the ectoderm. (**H**) *TCF-tTA* construct. (**I-K′**) *TCF-tTA;tetO-Wise* mice display limb and mammary defects. *TopGal* (B-G′,I-J′) and *eGFP* (K,K′).

DISCUSSION

Our genetic analyses have revealed that Lrp4 and Wise play stage-specific roles for proper patterning and morphogenesis of the murine mammary glands and other skin appendages through their ability to modulate Wnt/β-catenin signaling. Lrp4 has an early role in facilitating placode initiation and together Lrp4 and Wise have later roles in induction and/or maintenance of precursor cells. Through loss-, gain-of-function and epistasis analyses, we found that Wise requires Lrp4 to exert its activity. Together, our data suggest a model whereby Wise and Lrp4 work in concert to modulate the activity of Wnt signaling though a common mechanism. These findings have important implications for a mechanistic understanding of how Wnt antagonists participate in the precise control of Wnt signaling to regulate cellular processes involved in ectodermal placode formation.

Lrp4 and Wise control patterning of the mammary placodes

Development of mammary glands provides an opportunity to study spatiotemporal patterning of ectodermal organs as multiple placodes form along the mammary lines in a fairly well-defined order. Our analyses of *Lrp4* and *Wise* mutant mice have provided insight on the cellular processes that control the transition from stretches of thickened epithelium into precisely spaced placodes.

First, initiation of the placodes requires assembly of the precursor cells. In *Lrp4* mutants, even when comparables number of cells were present around the site of placode formation, they were loosely assembled with a smaller degree of invagination compared with those of control mice. This delay in placode assembly suggests that Lrp4 normally facilitates aggregation of the precursor cells.

Second, the number of the precursor cells needs to be tightly controlled for proper morphogenesis of individual placodes and maintenance of spacing between them. The significant increase in the number of Wnt reporter-positive cells in *Lrp4* and *Wise* mutants suggests that both Lrp4 and Wise have a role in limiting the mammary fate to a defined number of epithelial cells. This may be achieved by suppressing maintenance of mammary fate in existing precursor cells or by blocking induction of new precursor cells as mammary epithelial cells tend to proliferate at a very low rate.

In addition, migration of the mammary precursor cells may play an important role in placode initiation and morphogenesis. The sustained presence of the precursor cells in the interplacodal regions of *Lrp4* and *Wise* mutants suggest that these cells fail to migrate to the normal sites of placode formation. These ectopic precursor cells then interfere with morphogenesis of normal placodes and give rise to supernumerary placodes. The extent of migration along the mammary line is not well characterized. It is possible that cell movement is limited to cells near the sites of placode formation and cells farther away from the placodes lose their potential to become mammary epithelial cells.

Disruption in any of the above processes would lead to defects in the number, morphogenesis and position of the mammary placodes. Mutant phenotypes suggest that initially Lrp4 is predominantly required for assembly of the placodes, and later both Lrp4 and Wise play a role in the number of the precursor cells.

Lrp4 and Wise are required for development of other skin appendages

Consistent with the idea that the molecular mechanisms for early morphogenesis are shared among the skin appendages, both *Lrp4* and *Wise* mutants display similar abnormalities in patterning of hair and vibrissal follicles, with stronger defects observed in *Lrp4* mutants. Interestingly, the formation of supernumerary vibrissal follicles is preceded by delayed placode morphogenesis with a

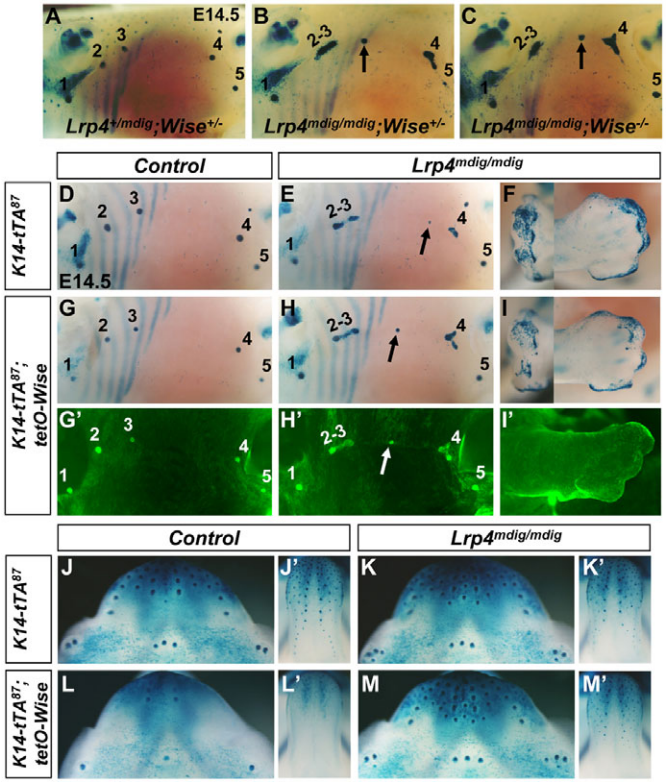

Fig. 8. Genetic interaction between *Lrp4* and *Wise*. (**A-C**) *TopGal* expression in *Lrp4;Wise* double mutant mice. Transheterozygotes display normal mammary patterning, and inactivation of *Wise* does not exacerbate *Lrp4* mutant defects such as fusion of buds 2 and 3, and ectopic buds (arrows). (**D-I'**) *Wise* overexpression, as evidenced by *eGFP* expression (G'-I'), fails to rescue the mammary (D,E,G,H) and forelimb (F,I) defects of *Lrp4* mutants, as shown by no significant change in *TopGal* expression. Arrows indicate ectopic mammary placodes. (J-M') *Wise* overexpression causes reduction in the number of vibrissal follicles (J,L) and taste papilla (J',L'), but not in *Lrp4* mutants (K,M,K',M'). All at E14.5 except F,I,I', which are at E13.5.

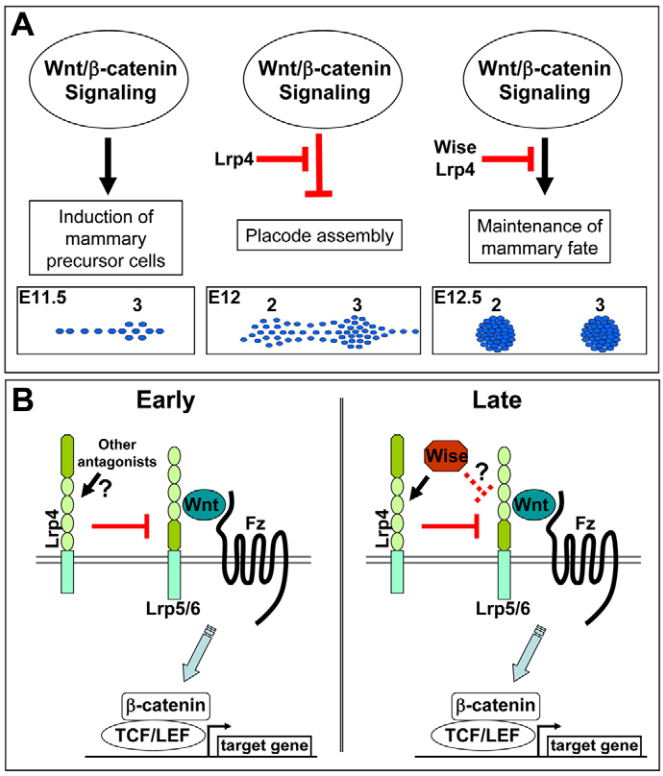

Fig. 9. Model for function of Lrp4 and Wise in mammary development. (**A**) Wnt/β-catenin signaling modulates multiple steps of placode development. Initially, Lrp4 facilitates placode initiation, and later Lrp4 and Wise together limit the number of mammary precursor cells by inhibition of Wnt/β-catenin signaling. (**B**) Early (left), Lrp4 functions in a Wise-independent manner, but later (right), Lrp4 and Wise act together to inhibit Wnt/β-catenin signaling.

broader distribution of the Wnt-active precursor cells in *Lrp4* mutants. A delay in placode formation was also observed in the primary hair follicles of *Lrp4* mutants. These delays are reminiscent of the defects observed during the mammary placode formation. Focalization of the epithelial precursor cells and associated Wnt activity is commonly seen during the formation of the skin placodes as well as AER (Mikkola and Millar, 2006; Fernandez-Teran and Ros, 2008). It is possible that Lrp4 and its ligands modulate Wnt signaling in those precursor cells to control cellular processes such as cell movement, cell shape change, cell-cell adhesion and cell proliferation, which are important for patterning and morphogenesis of the skin placodes (Jamora et al., 2003).

Lrp4 and Wise inhibit Wnt/β-catenin pathway during mammary development

We showed that the mammary defects of *Lrp4* and *Wise* mutants can be rescued by reducing the dose of *Lrp5/6* and *Ctnnb1*. This genetic interaction indicates that elevated Wnt/β-catenin signaling is responsible for the mammary defects and suggests that Lrp4 and Wise directly antagonize Wnt/β-catenin signaling instead of acting indirectly via another signaling pathway. This is consistent with the previous studies that provided genetic evidence that Wise functions as a Wnt inhibitor in tooth development (Munne et al., 2009; Ahn et al., 2010).

Our genetic analyses also demonstrate that Wnt/β-catenin signaling is essential for induction and/or maintenance of mammary precursor cells, but its activity needs to be tightly controlled to achieve a proper number of these cells (Fig. 9A). Early inhibition of Wnt/β-catenin signaling would lead to loss or reduction of the precursor cells disrupting placode formation as seen in *K14-Dkk1* (Chu et al., 2004) and *K14-Wise* mice. Conversely, elevated Wnt/β-catenin signaling in *Lrp4* and *Wise* mutants results in increase in the number of mammary epithelial cells. Another important implication of our study is that a temporal reduction of Wnt/β-catenin signaling is necessary to facilitate initiation of mammary placodes and this seems to be applicable to other skin appendages. Thus, our study provides additional insight into the diverse roles played by Wnt signaling throughout placode development and underscores the importance of Wnt inhibitory function of Lrp4 and Wise in these processes.

Wise requires Lrp4 to modulate Wnt/β-catenin signaling

Similar to other LDL receptor-related proteins, Lrp4 is implicated in regulating different signaling pathways (May et al., 2007; Willnow et al., 2007). With its multiple ligand binding motifs, Lrp4 has the ability to bind to secreted Wnt and Bmp antagonists (Ohazama et al., 2008; Choi et al., 2009). Interestingly, in both

humans and mice, *Lrp4* mutations phenocopy defects caused by deficiency of individual Wnt antagonists in a tissue-specific manner. For example, limb defects of *Lrp4* mutants are similar to those of *Dkk1* mutant mice (MacDonald et al., 2004), and bone overgrowth of humans with *LRP4* mutations is reminiscent of bone defects caused by *SOST* and *DKK1* mutations (Balemans et al., 2001; Morvan et al., 2006). Last, *Lrp4* and *Wise* mutant mice share defects in the skin appendages (Ohazama et al., 2008) (this study). These observations imply that interplay between Lrp4 and the Wnt antagonists may play an important role in modulating Wnt/β-catenin signaling in many developmental and physiological contexts.

Although genetic evidence for such interplay has been lacking, our observation that *Wise* gain-of-function phenotypes depend on Lrp4 in various tissue contexts provides an important insight on this issue. Based on our loss- and gain-of-function analyses, we propose that Lrp4 and Wise act through a common mechanism where Lrp4 lies downstream of Wise in a pathway leading to inhibition of Wnt/β-catenin signaling (Fig. 9B). In this model, Lrp4 is required to mediate or potentiate the Wnt inhibitory function of Wise and possibly other Wnt antagonists. This model is consistent with the lack of synergy or additive effects between *Lrp4* and *Wise* mutants in our epistasis analyses. The earlier Wise-independent role for Lrp4 and the relatively milder mammary defects of *Wise*-null mice might be attributed to function of Lrp4 alone or compensation by other antagonists. It remains to be explored how Lrp4, the Wnt antagonists and Lrp5/6 interact with each other to modulate Wnt/β-catenin signaling.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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