AD_________________

Award Number: W81XWH-04-1-0516

TITLE: Role of the Mouse Pygopus 2 Gene and Wnt Signaling in Normal and Malignant Development of Mammary Glands and Hair Follicles

PRINCIPAL INVESTIGATOR: Xing Dai, Ph.D.

CONTRACTING ORGANIZATION: The University of California
Irvine, CA 92697

REPORT DATE: May 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2006	Annual	26 APR 2005 - 25 APR 2006

4. TITLE AND SUBTITLE
Role of the Mouse Pygopus 2 Gene and Wnt Signaling in Normal and Malignant Development of Mammary Glands and Hair Follicles

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0516

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Xing Dai, Ph.D.

E-mail: xdai@uci.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of California
Irvine, CA 92697

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

14. ABSTRACT
The development of mammary gland is governed by multiple genetic pathways, although how these pathways are regulated and how they interact with each other is not yet fully understood. Previous studies have implicated the involvement of the canonical Wnt signaling pathway, which includes several key components that act together to activate other genes that are directly involved in controlling cell division or differentiation, in both normal and abnormal mammary gland development. The timing and overall activity of the pathway is key, as its disruption leads to aberrant mammary development and its overstimulation leads to breast cancer. We proposed genetic experiments to examine the role of a novel component of the Wnt pathway in mammary gland development and tumorigenesis. Specifically, we proposed to target genetic manipulations to a subset of the mammary epithelial cells that have stem cell characteristics. The mutant mice generated in this study should provide novel animal models to study the ontogeny and progression of mammary tumors. Furthermore, results from our studies should provide some insight into the role of epithelial stem cells in normal and diseased development. Finally, our studies might implicate possible drug target for treating breast cancer in the future.

15. SUBJECT TERMS
MPYGO2, PYGOPUS, WNT/BETA-CATENIN/LEF SIGNALING, GENE TARGETING, MICE
MAMARY GLAND DEVELOPMENT, MAMMARY TUMOR, HAIR FOLLICLE DEVELOPMENT, PILOMATRICOMA

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	44	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Cover……………………………………………………………………………………1

SF 298……………………………………………………………………………..……2

Table of Contents …………………………………………………….…………....3

Introduction…………………………………………………………….………….....4

Body…………………………………………………………………………………….4-5

Key Research Accomplishments………………………………………….………6

Reportable Outcomes……………………………………………………………….6

Conclusions…………………………………………………………………………..6

References…………………………………………………………………………….6-7

Appendices……………………………………………………………………………7

INTRODUCTION:

The canonical Wnt signaling pathway involves a complex series of intracellular events culminating in the stabilization of β-catenin, which enters the nucleus and binds to LEF/TCF transcription factors to stimulate gene expression.

The canonical Wnt signaling pathway, mediated by beta-catenin and members of the LEF/TCF family of proteins, is a conserved pathway required for multiple developmental processes in multiple tissues. This pathway regulates diverse cellular processes including proliferation and cell fate determination. Deregulated Wnt signaling leads to tumorigenesis in a variety of tissues including mammary gland and skin. Pygopus was identified as a genetic modifier of Wg signaling in Drosophila, and encodes a PHD-domain protein that associates with the β-catenin/LEF/TCF complex via an adaptor protein Legless [1-4]. Two murine pygopus paralogs, mpygo1 and mpygo2, have been identified, but their roles in developmental and tumorigenic processes that involve normal or aberrant Wnt/beta-catenin/LEF signaling remain elusive. Based on expression studies, we proposed that mpygo2 is the primary mediator of Wnt/beta-catenin/LEF signaling in mice and is therefore required for Wnt-involved developmental and tumorigenic processes. Our goal has been to focus on the development and morphogenesis of two epithelial tissues, mammary gland and skin, to test this hypothesis. We proposed two specific aims: 1- to characterize the function of mpygo2 in mammary gland and hair follicle development, and 2- to determinine the role of mpygo2 in Wnt/beta-catenin/LEF signaling-induced tumor formation.

BODY:

Task 1. To characterize the function of mpygo2 in mammary gland and hair follicle development. Months 1-36.

a. Generate an mpygo2 targeting vector and electroporate into ES cells to obtain homologous recombinants. This work has been completed.

b. Transfect the recombinant ES clones with a CMV-Cre expression vector and screen for mutant mpygo2 alleles where exon 3 (encoding the PHD domain) is deleted or flanked by loxP sites (a floxed allele). This work has been completed.

c. Inject ES clones containing the desired mutant (i.e., a classical deletion) or floxed mpygo2 alleles into blastocysts to obtain chimeric mice (to be performed by the UCI Transgenic Mouse Facility). This work has been completed.

d. Breed chimeric mice with wild-type C57BL/6 to obtain F1 heterozygotes. This work has been completed.

e. Intercross F1 mice heterozygous for mpygo2 deletion and genotype offspring. This work has been completed. Since homozygous null mutant pups are

born, we have analyzed them for mammary gland and hair follicle defects, as originally proposed. We observed an absence of mammary glands and a reduced number of hair follicles in these mice. We also crossed the mpygo2 mutant allele into a Wnt signaling reporter line, and show that mpygo2 deletion significantly reduced Wnt signaling output. These studies also led us to identify a previously recognized role for Wnt signaling in the proliferation of embryonic For detailed description of these findings, please see attached manuscript in preparation.

f. Cross F1 mice heterozygous for the floxed mpygo2 allele with K14-Cre transgenic mice to delete mpygo2 in an epithelial tissue-specific manner. Months 11-16. This effort is ongoing. Also see g.

g. Analyze defects in mammary glands and hair follicles from the tissue-specific mpygo2 knockout. Months 17-36. We have obtained several litters containing the desired skin-specific knockout mice, and are currently analyzing their mammary phenotypes. Specifically, we are performing functional tests to see if female mutants lactate. We are performing morphological analysis to see if branching and proliferation is defective in the mutant mammary glands. We are also studying the expression of K18 and K14, luminal and basal/myoepithelial markers, respectively, to follow a lead generated by the analysis of the null mutant that deletion of mpygo2, and by interference reduced Wnt signaling, might bias mammary epithelial cells towards a basal/myoepithelial fate.

Task 2. To determinine the role of mpygo2 in Wnt/beta-catenin/LEF signaling-induced tumorigenesis.

We have generated a K14-myc-mpygo2 transgenic construct. We made two attempts to inject this construct into mouse pronuclei for the production of transgenic mice, but unfortunately both resulted in no positives. We are currently modifying our construct and will try again. Due to this delay, we modified our plan accordingly. We purchased APCmin mice that are known to produce mammary tumors. We crossed our mpygo2 mutant allele into the APCmin background in order to determine whether a reduction in Wnt signaling can rescue or lessen the mammary tumor phenotype in APCmin mice, where Wnt signaling is aberrantly activated. We are in the process of acquiring N-beta-catenin transgenic mice. Once these mice arrive, we will perform similar experiments to see if mpygo2 mutation rescues or lessens delta N-beta-catenin–induced tumorigenesis.

KEY RESEARCH ACCOMPLISHMENTS:

- Successfully generated tissue-specific mpygo2 mutant mice.
- Obtained experimental evidence that mpygo2 is required for mammary gland development and Wnt signaling.
- Discovered that a reduction in Wnt signaling elicits mammary defects distinct from those of the loss of Wnt signaling.

REPORTABLE OUTCOMES:

- Finished a manuscript describing the initial analysis of mpygo2 function in mammary glands: Li B., Rhéaume, C., Veltmaat, J., Li, A., Bilanchone, V., Denmon, A., Teng, A., Munguia, J. E., Hu, M., Waterman, M. L., Lee, E.Y-H. P., and Dai, X. Mouse pygopus 2 is required for epithelial morphogenesis, controls the amplitude of canonical Wnt signaling, and regulates embryonic mammary epithelial cell proliferation.
- Gave a talk at Keystone Symposia on Wnt and beta-Catenin Signaling in Development and Disease, April, 2006, Snowbird Resort, Utah

CONCLUSIONS:

Our analyses of pygo2 knockout mice suggest that mpygo2 is required for maximum Wnt signaling activity in the mammary glands and for proliferation of the embryonic mammary progenitor cells. These findings nicely complement recent studies showing that elevated Wnt signaling leads to mammary tumors with stem/progenitor cell features (5, 6). Taken together, it is likely that Wnt signaling preferentially affects the expansion of mammary progenitor cells, which when goes uncontrolled, results in tumors with progenitor cell characteristics. Mpygo2 may present an additional drug target for treating breast cancer in the future.

REFERENCES:

1. Thompson, B., Townsley, F., Rosin-Arbesfeld, R., Musisi, H., and Bienz, M. (2002). A new nuclear component of the Wnt signalling pathway. Nat Cell Biol 4, 367-373.

2. Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S., and Basler, K. (2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. Cell 109, 47-60.

3. Belenkaya, T.Y., Han, C., Standley, H.J., Lin, X., Houston, D.W., and Heasman, J. (2002). pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. Development 129, 4089-4101.

4. Parker, D.S., Jemison, J., and Cadigan, K.M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. Development 129, 2565-2576.

5. Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., Tan, L.K., Rosen, J.M., and Varmus, H.E. (2003). Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci U S A 100, 15853-15858.

6. Liu, B.Y., McDermott, S.P., Khwaja, S.S., and Alexander, C.M. (2004). The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci U S A 101, 4158-4163.

APPENDICES: List of items attached

manuscript in preparation (Li et al.)

Mouse *pygopus 2* is required for epithelial morphogenesis and controls the amplitude of canonical Wnt signaling

Baoan Li[1#], Catherine Rhéaume[1], Jacqueline M. Veltmaat[2], Aihua Li[1], Andria Denmon[3], Andy Teng[1], Virginia Bilanchone[1], Ming Hu[1], Jesus E. Munguia[3], Stefano Piccolo, Eva Y-H. P. Lee[1], Marian L. Waterman[3], and Xing Dai[1,4,*]

[1]Department of Biological Chemistry
[3]Department of Microbiology and Molecular Genetics
[4]Developmental Biology Center
University of California, Irvine, CA 92697
[2] Developmental Biology Program
The Saban Research Institute of Childrens Hospital Los Angeles
Los Angeles, CA 90027

Running title: *mpygo2* in Wnt signaling and epithelial morphogenesis

Key words: *pygo2*, *pygopus*, Wnt/β-catenin signaling, mammary glands, hair follicles

[#] Present address:
School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, P. R. China.

[*]To whom correspondence should be addressed:
Department of Biological Chemistry
College of Medicine
D250 Med Sci I
University of California
Irvine, CA 92697-1700
Tel: 949-824-3101
Fax: 949-824-2688
E-mail: xdai@uci.edu

SUMMARY

The canonical Wnt signaling pathway involves a complex series of intracellular events culminating in the stabilization of β-catenin, which enters the nucleus and binds to LEF/TCF transcription factors to stimulate gene expression. *Pygopus* was identified as a genetic modifier of Wg signaling in *Drosophila*, and encodes a PHD-domain protein that associates with the β-catenin/LEF/TCF complex via an adaptor protein Legless (Lgs) [1-4]. Two murine *pygopus* paralogs, *mpygo1* and *mpygo2*, have been identified, but their roles in development and Wnt signaling remain elusive. Here we ablate the expression of *mpygo2* in mice and show that morphogenesis is defective for both ectodermally and endodermally derived tissues known to require Wnt signaling. In apparent contrast to a demonstrated role of Wnt signaling in the induction of epidermal appendages, *mpygo2* ablation allowed for normal specification of a mammary fate; instead the proliferation of embryonic mammary epithelial cells was compromised. Using transgenic Wnt reporter mice, we show that Wnt signaling is dramatically reduced but not absent in *mpygo2*-deficient tissues. In cultured mammary epithelial cells, the presence of mPygo2 conferred maximum effect on reporter expression to otherwise limiting amounts of β-catenin and Lef1. Taken together, our studies identify *mpygo2* as a key regulator of epithelial morphogenesis, and provide the first evidence that *mpygo2* is involved in Wnt signaling in mice. Importantly, we demonstrate that mPygo2 amplifies Wnt signaling, and that a reduction in Wnt signaling elicits defects distinct from those of the loss of Wnt signaling.

INTRODUCTION

The secreted Wnt proteins are known to trigger several distinct intracellular signaling pathways, the most extensively studied of which is the so-called canonical Wnt signaling pathway [5, 6]. This signaling pathway plays important roles in the normal development and adult homeostasis of myriad tissues and organs, and is also associated with diseases of the bone, eye, skin, and heart, as well as cancer [6, 7]. The exact cellular function of canonical Wnt signaling depends on tissue and developmental context. It has been shown to direct cell fate decisions during skin appendage formation [8-11], while during intestinal and lymphocyte development it regulates proliferation and survival of stem/progenitor cells [12-14].

The end point of an activated Wnt signaling cascade is the stabilization of β-catenin, a protein with dual functions at and away from the cell membrane. Consequently, β-catenin accumulates in the cytoplasm, enters the nucleus, and binds to a member of the LEF/TCF family of transcription factors to stimulate the expression of target genes involved in diverse cellular processes. Multiple factors have been found to interact with, and modulate the transcriptional regulatory activity of the nuclear β-catenin-LEF/TCF complex, thereby affecting the signaling output of the pathway [6]. Among these, Pygopus and Bcl9 (or Legless), identified by genetic means in *Drosophila*, are shown to be highly specific to and essential for Wg signaling [1-4]. Pygopus, a PHD-domain protein that associates with the β-catenin/LEF/TCF complex via adaptor protein Legless, is suggested to function as a devoted co-activator and/or by facilitating the nuclear retention of the complex [2, 15-18]. Two *pygopus* paralogs exist in mammals [1, 2]. Although suggested by cell culture experiments to share the function of their *Drosophila* ancestor, the in vivo roles of mammalian *pygopus* genes in Wnt signaling and development remain elusive.

Mammary glands and hair follicles are specialized epidermal appendages that arise from the multipotent surface ectoderm during mid-gestation. The morphogenesis of both organs requires reciprocal epithelial-mesenchymal communications, and appears to entail at least some common regulatory mechanisms. A number of studies have highlighted the involvement of canonical Wnt signaling in epidermal appendage morphogenesis, particularly in the initiation of an appendage fate [8-11, 19, 20]. For example, loss-of-function of β-catenin, which is believed to abolish canonical Wnt signaling, or transgenic expression of Dkk1, an inhibitor of Wnt signaling, in skin prior to appendage morphogenesis results in the lack of any mammary or hair follicle placodes [9-11]. While much has been learned in recent years about the molecular control of hair follicle morphogenesis [21-23], a comprehensive understanding of the genetic and differentiation pathways underlying mammogenesis is lacking [24]. The blockade of the very first step of mammogenesis in Wnt signaling mutants prevents the analysis of any possible function of the signaling pathway in subsequent events. It has been shown that aberrantly activated Wnt signaling leads to mammary tumors that are enriched in cells with features of stem and progenitor cells, raising the possibility that Wnt signaling targets mammary progenitor cells [25, 26]. In fact a common theme emerging from studies of different stem cell-containing tissues is that Wnt signaling preferentially affects the expansion of progenitor cells, which when goes uncontrolled, results in tumors with progenitor cell characteristics [27]. However, for reasons discussed above, no evidence is currently available to suggest a physiological role for Wnt signaling in the expansion or maintenance of mammary progenitor cells.

We have previously shown that murine *pygopus* 2 (*mpygo2*) is more broadly expressed than *pygopus* 1 (*mpygo1*) during embryonic development and in adult tissues [28]. This finding, plus the wide-spread involvement of Wnt signaling in development**,** led us to propose that

mpygo2 is likely an important candidate component of Wnt signaling in mice. In this study, we ablate the expression of *mpygo2* and demonstrate that *mpygo2* mutant mice show defects in a subset of Wnt-requiring tissues including the mammary glands. While mammary induction appears largely normal in these animals, the proliferation of epithelial cells within the developing mammary bud is compromised. We show that Wnt signaling is dramatically reduced but not absent in *mpygo2*-deficient tissues. Together, our studies provide the first genetic evidence that *mpygo2* is required for maximum Wnt signaling in mice. They further suggest that while affecting mammary gland morphogenesis, the loss of *mpygo2*, and by inference a reduction in the strength of Wnt signaling, elicit defects distinct from those of the complete loss of signaling.

MATERIALS AND METHODS

Generation of *mpygo2* mutant alleles and genotyping

mpygo2 genomic fragments were obtained by PCR using E14 ES cell DNA as a template, and were cloned into the pPGKneobpAlox2PGKDTA vector (a generous gift of Phil Soriano) to generate a targeting construct. Electroporation into E14 ES cells, screening for recombinants, and the generation of chimeric mice and germline mutants were performed as described [29]. PCR genotyping was performed using the following primers: a) for the detection of targeted, floxed, and “-”alleles, respectively, in ES DNA: 5’-CTCTAGCGTGTCTAAGGTCAGCCAGAGCG-3’ and 5’-AGGGCAAGCCATGTCAGTTCTCT-3’; b) for the detection of wild-type allele in ES and tail DNA: 5’-AGCGTGTCTAAGGTCAGCCAGAGGTTTG-3’ and 5’-GTAAAGCGTTGGGGGAGAGGAGGAGGAC-3’; c) for the detection of “-” allele in tail DNA: 5’-CTCTAGCGTGTCTAAGGTCAGCCAGAGCG-3’ and 5’-AGGGCAAGCCATGTCAGTTCTCT-3’. For Southern blot genotyping, E14 ES genomic DNA

was digested with Sph I, and the blot was probed with a 273-bp fragment downstream of the 3' arm (probe 1 in Fig. 1B), and tail DNA digested with Bam HI and blots probed with a 1-kb fragment upstream of exon 3 (probe 2 in Fig. 1B).

Northern, Western blot analysis and generation of α-mPygo2 antibody

Northern blot analysis was performed on RNA isolated from skin of E18.5 *mpygo2*$^{+/+}$ and $^{-/-}$ embryos as described [30] using a 270-bp cDNA fragment containing exon 1 of *mpygo2* as a probe. For Western blot analysis, skin lysates of E18.5 *mpygo2*$^{+/+}$ and $^{-/-}$ embryos were prepared by homogenization in lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 0.6M NaCl, 1% Triton X-100, 1 mM PMSF using a Tissue-Tearer (Biospec Products, Inc.), followed by centrifugation at 13,500 x g for 20 minutes at 4°C. Thirty μg was fractionated on 8% SDS-PAGE for Western blotting and immunodetection using a polyclonal rabbit α-mPygo2 antibody. This antibody was generated to a GST fusion protein containing amino acids 6-115 of mPygo2 (Harlan Bioproducts for Science) and affinity-purified.

Histology, morphology, immunostaining, and whole-mount in situ hybridization

For histological analysis, embryos or tissues were fixed in Bouin's fixative for 24 hours, processed and embedded in paraffin wax, sectioned at 6 μm and stained with hematoxylin/eosin. For whole-mount mammary gland staining, E18.5 skins were flattened on microscope slides, fixed overnight in Carnoy's solution (75% ethanol, 25% acetic acid) and stained with carmine alum as described by Sympson et al (1994). For quantification of the number of hair follicles, cross-sections of E18.5 skin were prepared and hair follicles counted in an area of $2.6x10^{5}$ μm^{2}. Statistical significance was determined using Student's t-test (n=20). Indirect immunofluorescence of embryonic tissues was performed as described [30] using rabbit α-

mPygo2 antiserum that has been preadsorbed with tissue powder prepared from E15.5 *mpygo2*$^{-/-}$ embryos. Whole-mount in situ hybridizations were performed as described [29] using a *Wnt10b* probe [31] or a 710-bp fragment corresponding to nucleotides 1034-1743 of *Lef1* mRNA (Accession number: NM_010703).

BrdU-labeling and immunodetection

Pregnant mice were intraperitoneally injected with 50 μg/g body weight of BrdU in PBS. Embryos were taken 1 hour after injection, cryosectioned at 10 μm, and fixed in 4% paraformaldehyde for 10 minutes, followed by immunohistochemistry as described [32]. A weak hematoxylin counterstaining was performed and cells within each mammary bud were counted to determine the percentage of BrdU-positive cells over the total number of cells. Statistical significance was determined using Student's t-test (n =20).

Analysis of BAT-gal expression

Embryos (E11.5 to E15.5) containing the BAT-gal transgene were fixed in 0.1 M phosphate buffer, pH 7.3, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA for 10~45 minutes at room temperature. After three washes in 0.1M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, they were transferred into freshly prepared X-gal staining solution containing 0.5 mg/ml X-gal, 10 mM potassium hexacyanoferrate (III), 10 mM potassium hexacyanoferrate (II), 5 mM EGTA, and 2 mM $MgCl_2$ in potassium phosphate buffer, and stained for 30 minutes at room temperature in the dark. After rinsing with PBS, embryos were post-fixed in 4% paraformaldehyde and examined under the microscope for photography. Transverse cryosections (10 μm) were counterstained with nuclear fast red and analyzed for the presence of mammary rudiments. For quantification of β-galactosidase activity, extracts were prepared by homogenizing the E18.5 embryo tissues in a lysis solution containing 100 mM

potassium phosphate (pH 7.8) and 0.2% Triton X-100 (10 ml/g tissue), followed by three freeze-thaw cycles. After centrifugation at 4°C, supernatant was taken and β-galactosidase activity measured using a chemiluminescence assay kit (Galacto-Light Kit from Tropix). The level of β-galactosidase activity was normalized against protein concentration of each sample, and expressed as fold changes over wild-type control. Statistical significance was determined using Student's t-test (n=3).

Transient transfection analysis

MCF-10A cells were seeded in 6-well plates 24 hours prior to transfection. Both TOP-FLASH (100 ng) and CMV-β-galactosidase (50 ng) reporter plasmids were co-transfected with expression plasmids for human LEF-1, *Xenopus* β-catenin, and mPygo2 using FuGENE6 transfection reagent (per manufacturer’s protocol with a 1:3 DNA:FuGENE ratio). Cells were harvested after 24 hours and processed for luciferase and β-galactosidase assays (Galacton Plus with Tropix Accelerator II). Data were normalized to β-galactosidase levels and fold activation by mPygo2 was calculated by reference to co-transfection with empty expression vector EVR2.

RESULTS

mpygo2 **is expressed in epithelial tissues that require canonical Wnt signaling for their morphogenesis**

In order to test whether mPygo2 protein is present in tissues that require Wnt signaling for their development, we generated a polyclonal antibody against the N-terminal domain (amino-acids 6-115) of mPygo2. Western blot analysis revealed a protein in skin extracts of wild-type but not *mpygo2*-deficient embryos (Fig. 1H; see below), confirming the specificity of this antibody. Using this antibody in indirect immunofluorescence, we detected mPygo2 protein in a number of embryonic tissues known to require Wnt signaling, e.g. lung, intestine, and

epidermal appendages. In E18.5 lung, nuclear staining was seen in epithelial cells lining the bronchioles and peripheral terminal alveolar tubules, with a particularly strong signal in some cells (arrow in Fig. S1A). In E18.5 intestine, nuclear staining was evident in cells of the intervillous epithelium, where future crypts form, and in a subset of intestinal mesenchymal cells (Fig. S1C).

Nuclear mPygo2 protein was detected in epithelial cells of the developing pelage and whisker hair follicles (Fig. 1A, left and middle, respectively), as well as in the developing mammary buds (Fig. 1A, right) and the basal and suprabasal cells of the interfollicular epidermis. Within the pelage hair follicle, the protein was particularly concentrated in the presumptive bulge region and the matrix/precortex cells, sites of active Wnt signaling [33, 34]. Nuclear staining was also observed in a subset of dermal cells, albeit at a reduced intensity, and particularly in the dermal papillae of pelage follicles. No nuclear staining was observed in *mpygo2*-deficient tissues (Figs. S1 and 1A; see below), indicating that the signals observed above are specific to the mPygo2 protein. In conclusion, and in accordance with the proposed nuclear function of mPygo2 in Wnt signaling, we found nuclear mPygo2 protein particularly abundant at sites where canonical Wnt signaling is active [33-36].

***mpygo2* is required for the morphogenesis of many epithelial tissues that need canonical Wnt signaling for their normal development**

To investigate *mpygo2*'s role in Wnt-mediated epithelial morphogenesis in vivo, we used the Cre/loxP technology to ablate *mpygo2* in mice. In the targeting vector, LoxP sites were introduced in positions flanking exon 3, which encodes amino acids 52-405 of the protein including a conserved PHD finger domain (Fig. 1B; [28]). Homologous recombination, followed by Cre-mediated excision in ES cells gave rise to clones containing either an *mpygo2* mutant

allele (where both exon 3 and neo are removed; from here on referred to as "mutant" or "-"), or a "floxed" allele (where neo is removed and exon 3 is flanked by LoxP sites), as confirmed by Southern blot and PCR analyses (Fig. 1C-D). ES cells containing the mutant allele were injected into C57BL/6 blastocysts, and the resulting chimeras were crossed with C57BL/6 mice to obtain germline transmission (Fig. 1E-F). By Northern blot analysis using a cDNA probe specific for exon 1, we found that small transcripts were generated from the mutated locus; however, the previously reported 3.2 kb *mpygo2* mRNA was completely absent in skin extracts from homozygous mutants (Fig. 1G). Western blot analysis did not reveal intact mPygo2 protein in mutant skin lysates (Fig. 1H). Moreover, under the experimental conditions, we did not detect any smaller, specific band that might be a truncated mPygo2 protein. These results suggest that the *mpygo2* locus was functionally inactivated as a consequence of the targeting event.

Consistent with the role of Wnt signaling in multiple developmental processes, we observed a pleiotropic phenotype in *mpygo2* mutant animals. Most mutants were born slightly runted (with an average reduction of 15-20% in body weight and ~10% in length), and nearly all mutants died shortly after birth. Approximately 14% of sixty-three mutant animals examined between E12.5 and term displayed an enlarged brain and exencephaly or a domed head (Fig. S2B, D). Furthermore, ~25% of the mutants, which were often distinct from those with brain defects, displayed small eyes that are externally visible (Fig. S2F,H). Histological analysis of mutant eyes at different embryonic stages revealed that lens was either smaller or completely absent. Additionally, the retina was abnormally organized, and often convoluted (Fig. S2J, L, N-O).

Consistent with a functional relevance for *mpygo2* expression in the embryonic lung, lungs from E18.5 and newborn *mpygo2*$^{-/-}$ animals were often pale, smaller than the wild type, and

showed no sign of air in their distal airways (data not shown). Examination of hematoxylin/eosin-stained sections revealed that the mutant alveoli were sometimes deflated, resembling atelectasis, a medical condition seen in human patients (Fig. S3B). In addition, more mesenchymal cells were observed between the alveoli. We next determined whether *mpygo2* mutants displayed defects in intestinal development, as previous studies of TCF-4 knockout mice suggest that Wnt signaling is required for the maintenance/proliferation of intestinal progenitor cells, which are located in the intervillous intestinal epithelium [14]. Surprisingly, the intervillous region of E18.5 and newborn *mpygo2*$^{-/-}$ animals appeared indistinguishable from the wild type (Fig. S3D, compare to S3C). By BrdU labeling, comparable levels of progenitor cell proliferation were observed between wild-type and mutant animals (Fig. S3E-F). Furthermore, staining with Alcian Blue and Grimelius to visualize goblet and enteroendocrine cells, respectively, did not reveal significant alterations in the mutant intestine (data not shown). Nearly all E18.5 mutant embryos (>90%) showed an average of ~10-15% reduction in the length of their small intestine compared to littermates. However, the correlation of this reduction to that in body weight /length makes it unlikely that this is a specific defect.

The well-established importance of Wnt signaling in epidermal appendage formation prompted us to examine the *mpygo2* mutants for possible defects in hair follicles and mammary glands. While a single mutant showed an arrest of hair follicle development at the bud stage, most mutants examined contained follicles that appeared grossly normal or slightly less elongated than the wild type (Fig. 2A-B and data not shown). However, when we quantified the number of hair follicles per skin sections of multiple wild-type and mutant E18.5 embryos, we observed a statistically significant decrease (30%) in the mutant (Fig. 2C-E). More striking defects were observed for the mammary glands of female E18.5 *mpygo2*$^{-/-}$ embryos, ranging

from impaired bud elongation and branching to lack of apparent mammary rudiment (Fig. 2F-O, Table 1). Most often, glands #2 and #3 were absent (Table 1, data not shown). Even in the least affected gland #4, branching morphogenesis was less extensive in the mutant compared to the wild type (Fig. 2M, compare to 2L, Table 1). Collectively, our analysis showed that *mpygo2* ablation results in defects in both ectodermally and endodermally derived organs, particularly those in which morphogenesis involves epithelial-mesenchymal interactions and requires canonical Wnt signaling. Compared to other tissues examined, the mammary gland defects appeared to be the most penetrant (100%), and were therefore the focus of subsequent studies.

mpygo2 ablation allows for mammary specification but causes reduced proliferation of embryonic mammary epithelial cells

Disruption of Wnt signaling by overexpressing Dkk1 abolishes the ability of the ectoderm to specify a mammary fate [10, 11]. To compare the *mpygo2* mutant phenotype with that of the existing Wnt mutant, we performed whole mount in situ hybridization using a *Wnt10b* probe, the earliest known ectodermal marker of mammary induction [31]. Similar levels of *Wnt10b* expression were observed in somite stage-matched wild-type and mutant embryos at E11.5, indicating that mammary-specific gene expression did indeed accompany an apparently timely morphological initiation of mammogenesis in the mutant (Fig. 3A, A', B, B'). We also examined the expression of K14 and K18, known to be expressed in the basal/myoepithelial and luminal mammary epithelial cells, respectively [37, 38], at later developmental stages (E18.5-P0). Compared to its wild-type counterpart, the mutant mammary epithelium expressed somewhat enhanced levels of K14, and weaker but still detectable levels of K18 (Fig. 3C-H and data not shown). These results suggest that in the absence of *mpygo2*, mutant mammary

epithelial cells maintain at least some aspects of their intrinsic differentiation potential despite their arrested elongation and branching.

To further explore the cellular basis of the mammary gland defect in *mpygo2* mutant, we next compared apoptosis and proliferation in the wild-type and mutant mammary fields. Overall, TUNEL-positive cells were barely detectable in wild-type buds at E15.5, and no increase was observed in mutant structures (data not shown). In contrast, we found less BrdU-positive cells in the mammary epithelium of mutant embryos (Fig. 3I-K), indicative of reduced proliferative activity when *mpygo2* was ablated. No such reduction was observed in the surrounding epidermis (data not shown), confirming that the mammary gland defect is specific. Therefore, the arrested growth of developing mammary buds in the mutant is likely due to decreased proliferation, but not increased apoptosis of the embryonic mammary epithelial cells.

Loss of *mpygo2* dramatically reduces but does not abolish Wnt signaling

Our finding that *mpygo2* ablation did not affect mammary specification is in apparent contrast to the previous observation that mammogenesis completely fails to initiate when Wnt signaling is disrupted by Dkk1 overexpression. Is *mpygo2* required for Wnt signaling after all? We directly addressed this question using double transgenic mice carrying wild-type or mutant *mpygo2* alleles and a transgenic BAT-gal Wnt reporter gene. In BAT-gal transgenics, LacZ is under the control of LEF/TCF-responsive elements, allowing β-galactosidase activity to be expressed at sites where canonical Wnt signaling is active [39]. In wild-type embryos, the five pairs of mammary placodes formed around E11.5 and exhibited canonical Wnt signaling as shown by BAT-gal expression (Fig. 4A, A' and data not shown). In *mpygo2* mutant embryos, all placodes appeared and had a similar size as in wild types as shown in transverse sections, but displayed greatly reduced BAT-gal expression (Fig. 4B, B' and data not shown). As

development proceeded, the wild-type placodes transformed into mammary buds consisting of epithelial cells that grew downward into the mesenchyme [24], and Wnt signaling persisted in these growing buds (Fig. 4C, C', E, E'). In the mutant however, mammary buds were now smaller than those in wild-type embryos, and continued to exhibit greatly reduced BAT-gal expression (Fig. 4D, D', F, F'). The extent of reduction was greatest in buds #2 and 3, correlating with the morphological finding that their subsequent development was most severely affected (Table 1). Importantly however, Wnt signaling was not completely abolished, as a close examination of transverse sections of the rudimentary mutant structures revealed the presence of faint BAT-gal expression (Fig. 4B', D', F'). The expression of *Lef1*, a marker of developing mammary epithelium and known target of canonical Wnt signaling [40, 41], was barely detectable in the mammary buds of E13 mutants (Fig. 4H, H', compare to G, G'). This result provided additional support that Wnt signaling is compromised in the mutant.

We next examined whether Wnt signaling is also reduced in other *mpygo2*-expressing tissues. Indeed, BAT-gal expression was greatly diminished, but still detectable in the hair follicles of mutants compared to control embryos (Fig. 4I-J). Furthermore, β-galactosidase activity in extracts of dorsal skin and lung from E18.5 homozygous mutant animals was considerably lower (>2-fold) than that of the wild type (Fig. 4K). Interestingly, despite the absence of any apparent phenotype in heterozygous mutants, a slight reduction in β-galactosidase activity was seen in their skin and lung extracts (Fig. 4K), suggesting that morphogenic consequences occur only when signaling activity falls below a certain critical threshold. Taken together, our results indicated that mPygo2 is required for maximum Wnt signaling in vivo; however a basal level of Wnt signaling is still achieved when *mpygo2* is ablated.

mPygo2 protein augments β-catenin/LEF-1-dependent transcriptional activation in cultured mammary epithelial cells

The requirement for *mpygo2* to achieve maximum levels of Wnt signaling in vivo suggests that mPygo2 protein serves as an amplifier of canonical Wnt signaling. To explore this possibility, we turned to reporter assays in cultured MCF10A mammary epithelial cells. Transfection of these cells with sub-optimal levels of expression plasmid for LEF-1 (200 ng) and β-catenin (400 ng) enabled only modest activation (2.2 fold) of the LEF/TCF target construct TOP-FLASH (Fig. 5). However, higher levels of activation, up to 160-fold, were observed when the amount of transfected LEF-1 and β-catenin plasmids was increased. Importantly, co-transfection of increasing amounts of *mpygo2* expression plasmid with the low levels of LEF-1 and β-catenin elicited dose-dependent increases in TOP-FLASH reporter activity to the highest level (193-fold). This activation was highly synergistic because expression of mPygo2 alone had no effect on reporter expression (data not shown). Activation was also specific because substitution of LEF-1 with a truncated mutant LEF-1 missing the β-catenin binding domain (dnLEF-1) nearly abolished activation (2-fold). These data show that canonical Wnt signaling in cultured mammary epithelial cells is sensitive and responsive to the presence of mPygo2, and that mPygo2 can dramatically amplify the activity of the otherwise limiting amounts of β-catenin and LEF-1.

DISCUSSION

Our study is the first to address the in vivo function of a mammalian *pygopus* gene. Consistent with the reported widespread involvement of Wnt signaling in organogenesis, we found mPygo2 protein expression in multiple epithelial tissues of organs that require Wnt

signaling for their development, and with a particular concentration at sites of active Wnt signaling. These findings indicate that mPygo2 has the correct spatio-temporal expression pattern to be a candidate component of Wnt signaling in mice. Interestingly and in contrast to the fact that *Drosophila pygo* mutants are phenotypically similar to the *armidilo* (fly β-catenin) or *Wg* mutants in multiple tissue and developmental sites [1-4], *mpygo2* mutant mice do not present a phenotype expected for a mutant with a complete loss of Wnt signaling. The early Wnt signaling-associated phenotypes, such as body axis and mesodermal defects displayed by the β-catenin knockout animals [42, 43], were not observed in *mpygo2*-deficient mice. Although the perinatal lethality of *mpygo2* mutant mice precludes us from being able to assess a possible role of *mpygo2* in adult intestine, our studies demonstrate that unlike TCF-4, *mpygo2* is not required for the formation and proliferation of intestinal stem cells in developing embryos. These said, a number of ectodermally or endodermally derived epithelial tissues that are known to require Wnt signaling for their normal development, including those of the lung, eye, and epidermal appendages, are clearly defective in *mpygo2* mutant animals. Together, our data suggest that *mpygo2* is required for a subset of the developmental processes that require Wnt signaling.

Using the BAT-gal Wnt reporter mice, we demonstrate that the Wnt signaling output is greatly reduced in *mpygo2*-deficient tissues compared to the wild type, providing the first in vivo evidence that *mpygo2* is involved in mammalian Wnt signaling. In contrast to studies in *Drosophila* that have emphasized an essential role of *pygopus* in Wg signaling, we observed residual Wnt signaling activity in the absence of *mpygo2*. This result indicates that a basal level of Wnt signaling in target tissues does not require *mpygo2*; instead *mpygo2* is required for a maximum signaling output. In cultured mammary epithelial cells, the strength of Wnt signaling, as measured by reporter gene expression, is indeed sensitive to the concentration of mPygo2

protein. In a dosage-dependent manner, mPygo2 is able to maximize the transcriptional activation potential of low levels of β-catenin and LEF-1. Collectively, our data argue that mPygo2 serves as an amplifier of the nuclear transcriptional regulatory activity of canonical Wnt signaling.

The view that *Drosophila pygo* and *mpygo2* have differential effect on Wg/Wnt signaling is consistent with the observed difference in the extent to which *Drosophila pygo* and *mpygo2* mutants resemble their corresponding β-catenin mutants. While flies have a single *pygopus* gene, mammals have two *pygopus* paralogs. It is possible that *mpygo1* plays a partially redundant role for *mpygo2*, especially during early developmental processes and in tissues that are least affected by the loss of *mpygo2*. However, the apparently restricted pattern of *mpygo1* expression is not in strong support of this notion. Moreover, we did not observe any significant increase in the level of *mpygo1* transcripts in *mpygo2*–deficient skin (data not shown). It is perhaps more likely that *pygopus* genes in mammals have evolved to play a non-essential but augmenting role in Wnt signaling, and this "amplifier" function is important to tailor to the needs of certain cellular processes that have a higher Wnt signaling threshold. However, definitive evidence awaits the generation and analysis of *mpygo1* and *mpygo2/mpygo1* double mutant. Furthermore, given the difference between *Drosophila* and mice with regard to *pygopus* function as well as complexity, it remains to be experimentally addressed whether or not mammalian Pygopus proteins are also involved in other developmental signaling pathways.

The extensive literature on Wnt signaling in epidermal appendage formation and the observation of a highly penetrant mammary gland phenotypes in *mpygo2* mutant mice allow us to compare the impact of loss of or reduction in Wnt signaling at a cellular level. In the absence of *mpygo2,* all five pairs of mammary placodes form, express a normal level of *Wnt10b*, and

develop to at least the early bud stage. This phenotype is distinctly different from that resulting from overexpressing *Dkk1* in the ectoderm, where mammagenesis fails to initiate [10, 11]. Furthermore, in contrast to other tissues where a role for Wnt signaling in stem/progenitor cell proliferation is clearly evident [12, 14], previous studies of embryonic epidermal appendages have not demonstrated a physiological involvement of this signaling pathway in proliferation. Here we report a significant decrease in the number of cycling cells in *mpygo2* mutant mammary buds, which we propose is at least partly responsible for their arrested development. Based on these observations, we surmise that a basal level of Wnt signaling in the absence of *mpygo2* is sufficient to direct cell fates for mammary induction. However, the proliferation of embryonic mammary epithelial cells requires a higher dose of Wnt signaling than that needed for their initial induction, and a function for Wnt signaling in proliferation can only be unmasked when an initial requirement is bypassed (Fig. 6). Is Wnt signaling also involved in differentiation within a specified mammary filed? The apparent imbalance between K14 and K18 expression in the mutant mammary rudiments raises the intriguing possibility that Wnt signaling may modulate the choice of a mammary epithelial cell to adopt a basal/myoepithelial or a luminal fate. This notion merits further investigation in adult mammary glands using our conditional "floxed" allele to bypass perinatal lethality.

The differential impact of loss of and reduction in Wnt signaling appears to take a different form during hair follicle morphogenesis. Whereas loss of Wnt signaling results in a complete failure in follicle initiation, we observed a reduction in the number of hair follicles per unit skin surface in *mpygo2* mutants. This is particularly interesting in light of the recent finding that homozygous transgenic mice overexpressing a stabilized form of β-catenin (ΔN-β-catenin) in skin produce increased number of hair follicles, whereas hemizygous transgenic mice do not

[8, 44]. Thus, the number of hair follicles increases when Wnt signaling increases above a critical threshold (as a result of doubling the levels of ΔN-β-catenin) and decreases when Wnt signaling falls below a critical threshold (as a result of *mpygo2* ablation). Together, these studies suggest that hair follicle initiation is a graded response that is sensitive to the dosage of Wnt signaling. They also predict that elevated levels of stabilized β-catenin might be able to compensate for the absence of mPygo2, a notion that can be experimentally tested in the future.

The skin defect in *mpygo2* mutant appears to be confined to the formation of mammary glands and hair follicles, as no consistent morphological abnormality was seen in the interfollicular epidermis, although mPygo2 protein was detected there. This situation is reminiscent of *Lef1*, which is expressed in developing interfollicular epidermis and budding epidermal appendages, yet its function is only manifested during appendage morphogenesis and differentiation [19]. In fact, existing literature has not yet revealed a role for canonical Wnt signaling in the interfollicular epidermis. It remains possible that mPygo2 functions in the epidermis in later processes in the adult that are not assayed here and/or in a Wnt-independent fashion. To a large extent, the mammary defects of *mpygo2*-deficient mice are similar to those observed in *Lef1* mutants, in that all mammary placodes are formed, but their development arrests in bud stage ([20], reviewed in [24]). Consistent with such morphological parallels is the finding that *Lef1* expression in mammary buds is severely reduced in *mpygo2*-deficient animals. Our studies together with previous reports suggest that *Lef1* is a physiological target of *mpygo2*-mediated Wnt signaling in mammary epithelium [40, 41], and may at least in part mediate the role of *mpygo2* in sustaining mammary gland development.

Collectively, our findings reinstate the widely accepted notion that Wnt signaling regulates multiple sequential cellular processes in organogenesis, ranging from lineage

specification to progenitor cell proliferation. More importantly, we have uncovered a molecule, namely mPygo2, that functions in vivo to affect the amplitude of canonical Wnt signaling. Our studies suggest that distinct cellular processes in a particular morphogenic event likely have different Wnt signaling thresholds. Finally, they implicate that a systematic comparison of *mpygo2* mutant phenotypes with those of mutants of other components of the signaling cascade will allow the examination of not only the dosage effect of Wnt signaling, but also unexplored roles of this important signaling pathway in morphogenesis and tumorigenesis of multiple tissues.

ACKNOWLEDGEMENTS

We thank Michele Massachio for technical assistance with ES cell electroporation and culture, Tom Fielder and Kai-Xuan Shi for blastocyst injection, all at the UCI Transgenic Mouse Facility. J. V. acknowledges support from CBCRP award 10-FB-0116 and a CHLA fellowship. A. D. was supported by the UCI-Minority Biomedical Research Support (MBRS) program, NIH grant # GM-55246. This work was supported by a DOD grant W81XWH-04-1-0516, and NIH Grants R01-AR47320 and K02-AR51482 awarded to X. D.

REFERENCES:

1. Thompson, B., Townsley, F., Rosin-Arbesfeld, R., Musisi, H., and Bienz, M. (2002). A new nuclear component of the Wnt signalling pathway. Nat Cell Biol *4*, 367-373.
2. Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S., and Basler, K. (2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. Cell *109*, 47-60.
3. Belenkaya, T.Y., Han, C., Standley, H.J., Lin, X., Houston, D.W., and Heasman, J. (2002). pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. Development *129*, 4089-4101.
4. Parker, D.S., Jemison, J., and Cadigan, K.M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. Development *129*, 2565-2576.
5. Veeman, M.T., Axelrod, J.D., and Moon, R.T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev Cell *5*, 367-377.

6. Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol *20*, 781-810.
7. Barker, N., and Clevers, H. (2000). Catenins, Wnt signaling and cancer. Bioessays *22*, 961-965.
8. Gat, U., DasGupta, R., Degenstein, L., and Fuchs, E. (1998). De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. Cell *95*, 605-614.
9. Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G., and Birchmeier, W. (2001). beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell *105*, 533-545.
10. Andl, T., Reddy, S.T., Gaddapara, T., and Millar, S.E. (2002). WNT signals are required for the initiation of hair follicle development. Dev Cell *2*, 643-653.
11. Chu, E.Y., Hens, J., Andl, T., Kairo, A., Yamaguchi, T.P., Brisken, C., Glick, A., Wysolmerski, J.J., and Millar, S.E. (2004). Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. Development *131*, 4819-4829.
12. Reya, T., O'Riordan, M., Okamura, R., Devaney, E., Willert, K., Nusse, R., and Grosschedl, R. (2000). Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity *13*, 15-24.
13. Timm, A., and Grosschedl, R. (2005). Wnt signaling in lymphopoiesis. Curr Top Microbiol Immunol *290*, 225-252.
14. Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P.J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet *19*, 379-383.
15. Townsley, F.M., Cliffe, A., and Bienz, M. (2004). Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. Nat Cell Biol *6*, 626-633.
16. Thompson, B.J. (2004). A complex of Armadillo, Legless, and Pygopus coactivates dTCF to activate wingless target genes. Curr Biol *14*, 458-466.
17. Krieghoff, E., Behrens, J., and Mayr, B. (2006). Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention. J Cell Sci *119*, 1453-1463.
18. Stadeli, R., and Basler, K. (2005). Dissecting nuclear Wingless signalling: Recruitment of the transcriptional co-activator Pygopus by a chain of adaptor proteins. Mech Dev *122*, 1171-1182.
19. Zhou, P., Byrne, C., Jacobs, J., and Fuchs, E. (1995). Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. Genes Dev. *9*, 700-713.
20. van Genderen, C., Okamura, R.M., Farinas, I., Quo, R.G., Parslow, T.G., Bruhn, L., and Grosschedl, R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. Genes Dev. *8*, 2691-2703.
21. Fuchs, E., and Raghavan, S. (2002). Getting under the skin of epidermal morphogenesis. Nat Rev Genet *3*, 199-209.
22. Fuchs, E., Merrill, B.J., Jamora, C., and DasGupta, R. (2001). At the roots of a never-ending cycle. Dev Cell *1*, 13-25.

23. Millar, S.E., Willert, K., Salinas, P.C., Roelink, H., Nusse, R., Sussman, D.J., and Barsh, G.S. (1999). WNT signaling in the control of hair growth and structure. Dev. Biol. *207*, 133-149.
24. Veltmaat, J.M., Mailleux, A.A., Thiery, J.P., and Bellusci, S. (2003). Mouse embryonic mammogenesis as a model for the molecular regulation of pattern formation. Differentiation *71*, 1-17.
25. Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., Tan, L.K., Rosen, J.M., and Varmus, H.E. (2003). Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci U S A *100*, 15853-15858.
26. Liu, B.Y., McDermott, S.P., Khwaja, S.S., and Alexander, C.M. (2004). The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci U S A *101*, 4158-4163.
27. Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843-850.
28. Li, B., Mackay, D.R., Ma, J., and Dai, X. (2004). Cloning and developmental expression of mouse pygopus 2, a putative Wnt signaling component. Genomics *84*, 398-405.
29. Mackay, D.R., Hu, M., Li, B., Rheaume, C., and Dai, X. (2006). The mouse Ovol2 gene is required for cranial neural tube development. Dev Biol *291*, 38-52.
30. Dai, X., Schonbaum, C., Degenstein, L., Bai, W., Mahowald, A., and Fuchs, E. (1998). The ovo gene required for cuticle formation and oogenesis in flies is involved in hair formation and spermatogenesis in mice. Genes Dev. *12*, 3452-3463.
31. Veltmaat, J.M., Van Veelen, W., Thiery, J.P., and Bellusci, S. (2004). Identification of the mammary line in mouse by Wnt10b expression. Dev Dyn *229*, 349-356.
32. Li, B., Nair, M., Mackay, D.R., Bilanchone, V., Hu, M., Fallahi, M., Song, H., Dai, Q., Cohen, P.E., and Dai, X. (2005). Ovol1 regulates meiotic pachytene progression during spermatogenesis by repressing Id2 expression. Development *132*, 1463-1473.
33. DasGupta, R., and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development *126*, 4557-4568.
34. Merrill, B.J., Gat, U., DasGupta, R., and Fuchs, E. (2001). Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. Genes Dev. *15*, 1688-1705.
35. Mucenski, M.L., Wert, S.E., Nation, J.M., Loudy, D.E., Huelsken, J., Birchmeier, W., Morrisey, E.E., and Whitsett, J.A. (2003). beta-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis. J Biol Chem *278*, 40231-40238.
36. van Noort, M., Meeldijk, J., van der Zee, R., Destree, O., and Clevers, H. (2002). Wnt signaling controls the phosphorylation status of beta-catenin. J Biol Chem *277*, 17901-17905.
37. Smith, G.H., Mehrel, T., and Roop, D.R. (1990). Differential keratin gene expression in developing, differentiating, preneoplastic, and neoplastic mouse mammary epithelium. Cell Growth Differ *1*, 161-170.
38. Mikaelian, I., Hovick, M., Silva, K.A., Burzenski, L.M., Shultz, L.D., Ackert-Bicknell, C.L., Cox, G.A., and Sundberg, J.P. (2006). Expression of terminal differentiation proteins defines stages of mouse mammary gland development. Vet Pathol *43*, 36-49.

39. Maretto, S., Cordenonsi, M., Dupont, S., Braghetta, P., Broccoli, V., Hassan, A.B., Volpin, D., Bressan, G.M., and Piccolo, S. (2003). Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci U S A *100*, 3299-3304.
40. Hovanes, K., Li, T.W., Munguia, J.E., Truong, T., Milovanovic, T., Lawrence Marsh, J., Holcombe, R.F., and Waterman, M.L. (2001). Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. Nat. Genet. *28*, 53-57.
41. Filali, M., Cheng, N., Abbott, D., Leontiev, V., and Engelhardt, J.F. (2002). Wnt-3A/beta-catenin signaling induces transcription from the LEF-1 promoter. J Biol Chem *277*, 33398-33410.
42. Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K., and Kemler, R. (1995). Lack of beta-catenin affects mouse development at gastrulation. Development *121*, 3529-3537.
43. Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C., and Birchmeier, W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. J Cell Biol *148*, 567-578.
44. Lowry, W.E., Blanpain, C., Nowak, J.A., Guasch, G., Lewis, L., and Fuchs, E. (2005). Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells. Genes Dev *19*, 1596-1611.

FIGURE LEGENDS

Figure 1. mPygo2 expression in skin and targeted deletion of *mpygo2*. (A) Immunofluorescence analysis of embryonic skin tissues (left and middle: E18.5; right: E15.5) using a rabbit α-mPygo2 antibody (green). DAPI staining was artificially colored red, so that nuclei of mPygo2-expressing cells were yellow. Insets are high magnification images showing nuclear presence of the protein. Arrow and arrowhead in top left panel indicates mPygo2 expression in the presumptive bulge area and in precortex, respectively. Arrow in top middle panel indicates mPygo2 expression in outer root sheath cells of a whisker follicle. White lines denote basement membrane. (B) Targeting vector design and the resulting *mpygo2* alleles. Boxes, exons; triangles, loxP sites. neo, neomycin; DTA, diphtheria toxin A; B, Bam HI; S, Sph I. Red bars above the wild-type (wt) locus indicate the positions of probes used for Southern blot analyses (see Experimental Procedures). (C) Southern and (D) PCR analysis of E14 ES cell clones containing wild-type or mutant alleles. (E) Southern and (F) PCR genotyping of embryos from intercrosses of heterozygous mutants containing the "-" allele. (G) Northern blot analysis of +/- and -/- E18.5 skin RNA using an exon 1-cDNA probe. Arrow points to low-molecular-weight RNA species that are present in the mutant. The same blot was stripped and re-probed with GAPDH to control for loading. (H) Western blot analysis of +/- and -/- E18.5 skin extracts using the α-mPygo2 antibody. "*"s indicate non-specific bands the presence of which varies from one experiment to another. Commassie staining (bottom) confirms equal loading of control and mutant extracts. Bar in A: left, 100 μm; middle, 30 μm; right, 50 μm.

Figure 2. Defective epidermal appendage morphogenesis in *mpygo2* mutant. Longitudinal (A, B) and cross-sections (C, D) of E18.5 skin were stained with

hematoxylin/eosin. The number of hair follicles (per $2.6x10^5$ μm^2) was determined in cross-sections and graphically represented in E (p=0.0017, n=20). (F-O) Whole-mount preparations of carmine-stained skins of E18.5 wild-type (F, H, J, L, N) and *mpygo2*$^{-/-}$ (G, I, K, M, O) females, showing the less severe phenotypes for individual mammary glands (MG) (see Table 1). Bar: 80 μm in A-B; 120 μm in C-D; 150 μm in F-O.

Figure 3. Mammary induction and differentiation is largely normal but epithelial cell proliferation is decreased in *mpygo2*-deficient embryos. (A, B) Whole-mount in situ hybridization for *Wnt10b* on developing mammary glands of wild-type (A) and mutant (B) embryos (E11). Corresponding sections of boxed areas in A-B are shown in A'-B'. (C-H) Immunofluorescence analysis of K14 (C-F) and K18 (G-H) expression in wild-type (C, E, G) and mutant (D, F, H) mammary glands at P0. Boxed areas in C and D are shown at higher magnifications in E and F, and their adjacent sections shown in G and H, respectively. (I,J) Immunohistochemical detection of BrdU incorporation in mammary buds of wild-type and mutant embryos at E15.5. (K) BrdU-labeling index, calculated as the percentage of BrdU-positive cells per total number of cells in mammary buds, of wild-type and mutant (n=20 for each genotype, $p=1.3x10^{-10}$). Bar: 300 μm in A-B; 110 μm in C, D; 55 μm in E-H; 15 μm in A'-B'; 18 μm in I-J.

Figure 4. Reduced Wnt signaling in *mpygo2*-deficient epithelial tissues. (A-F) Whole-mount LacZ staining of embryos at the indicated ages. Corresponding sections of boxed areas in A-F are shown in A'-F'. Filled and open arrows indicate externally visible and invisible mammary glands, respectively. (G-H) Whole-mount in situ hybridization for *Lef1* on developing

mammary glands of the wild-type (G, G') and mutant (H, H') embryos. Filled and open arrows indicate the presence and absence of hybridization signals, respectively. (I-J) Whole-mount LacZ staining showing stained hair follicles on back skin of wild-type (I) and mutant (J) E18.5 embryos. (K) Assays of β-galactosidase activity in extracts of skin (light purple) and lung (burgundy) of E18.5 embryos of the indicated genotypes. **Values for the wild type animals were set as 1, and mutant** "*" indicates statistically significant ($p<0.06$) differences from the wild type. Bar: 260 μm in A,B; 300 μm in C,D; 420 μm in E,F; 25 μm in A'-F'; 450 μm in G,H; 25 μm in G',H'; 120 μm in I,J.

Figure 5. mPygo2 potentiates β-catenin/LEF1-dependent transcription. MCF10-A mammary epithelial cells were transfected with the TOP-FLASH reporter plasmid, the CMV-β-galactosidase control plasmid, and expression vectors for LEF1, β-catenin, and mPygo2 at the indicated amounts (ng). Where indicated by "*", FOP-FLASH, which contains mutated LEF/TCF sites, was used instead of TOP-FLASH. Results are calculated as fold elevation of TOP-FLASH over that with co-transfected empty expression plasmid EVR2.

Figure 6. Model for the role of mPygo2 in modulating Wnt signaling strength and in mammary gland morphogenesis.

Figure S1. mPygo2 protein expression in wild-type (A, C) and mutant (B, D) lung (A, B) and intestine (C, D) of E18.5 embryos. Arrow in A points to strongly stained lung epithelial cells. Arrow and arrowhead in C indicate mPygo2-expressing intestinal epithelial and mesenchymal cells, respectively. Bar: 50 μm.

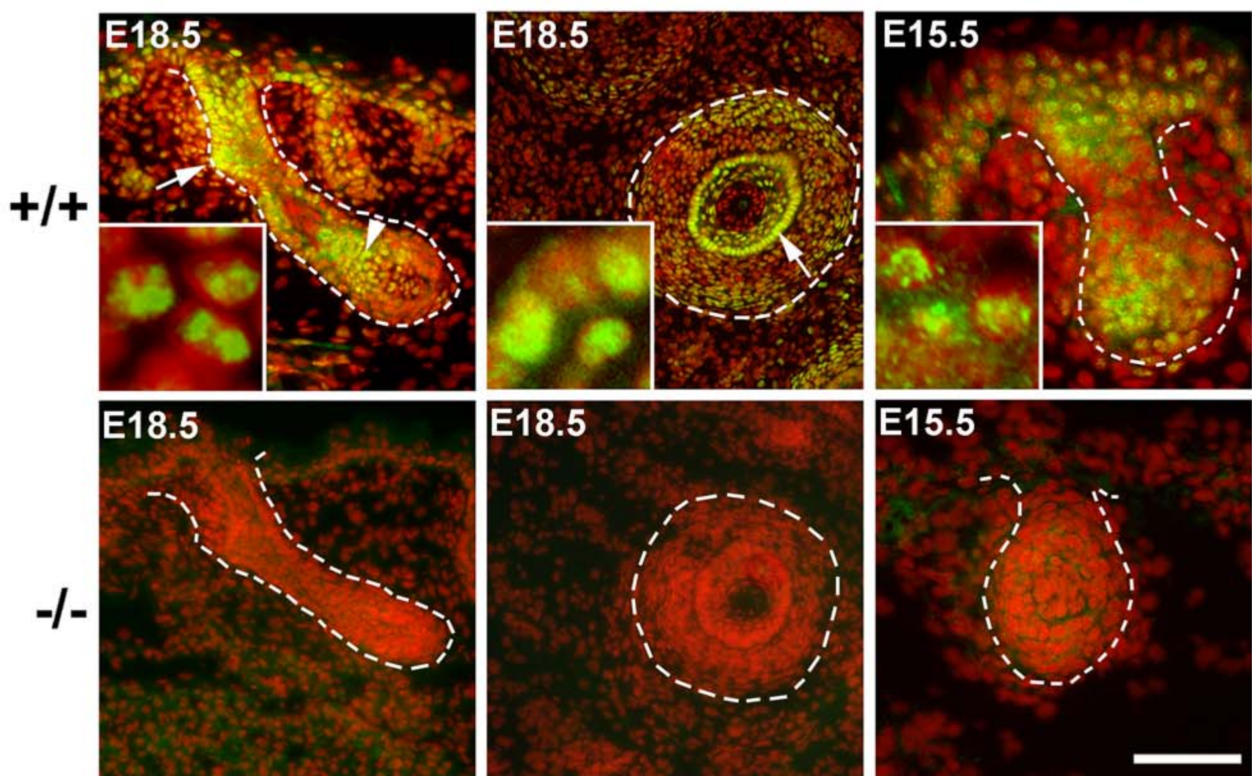
Figure S2. Brain (A-D) and eye (E-O) defects in *mpygo2*$^{-/-}$ mice. Shown are morphology (A-H) and histology (I-J) of wild-type (A, C, E, G, I, K, M) and mutant (B, D, F, H, J, L, N, O) animals. “*” and arrow indicate lens and convoluted retina, respectively. Note that lens is absent in one mutant (N) but smaller in another (O). Bar: 100 µm in I-J; 300 µm in K-O.

Figure S3. *mpygo2*$^{-/-}$ embryos show abnormal lung morphology but no apparent anomaly of presumptive intestinal crypts. Shown are results of histological analysis of lung (A,B) and intestine (C,D), and of BrdU-labeling experiments of intestine (E, F), respectively. Bar: 63 µm in A, B; 33 µm in C, D; 50 µm in E, F.

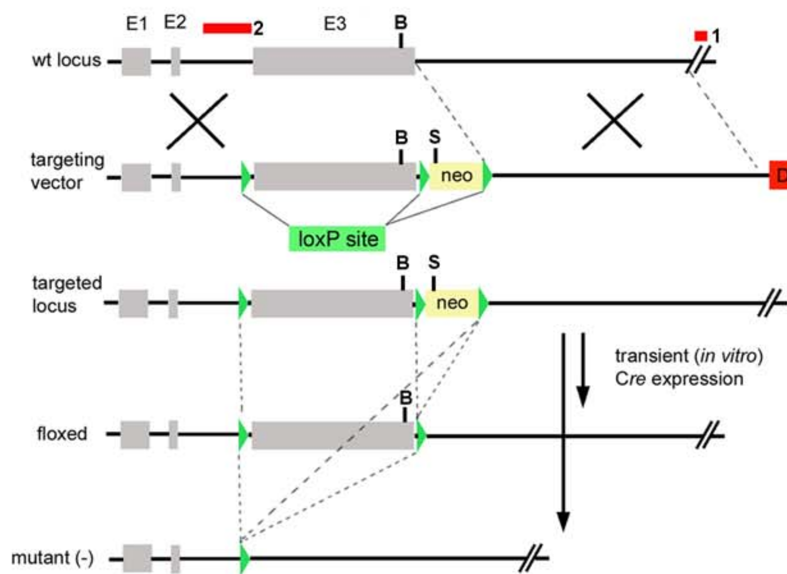
Table 1. Summary of morphological mammary gland defects in *mpygo2* mutant females.

Fig. 1

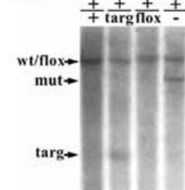
A



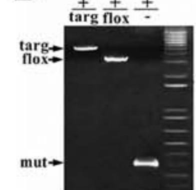
B



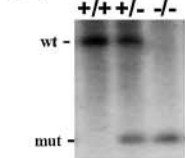
C



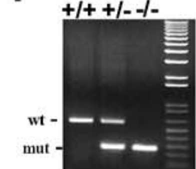
D



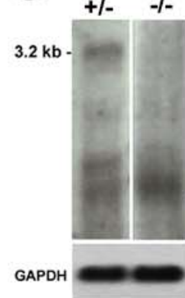
E



F



G



H

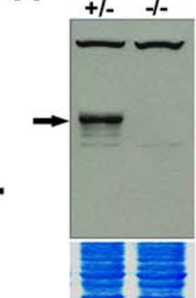


Fig.2

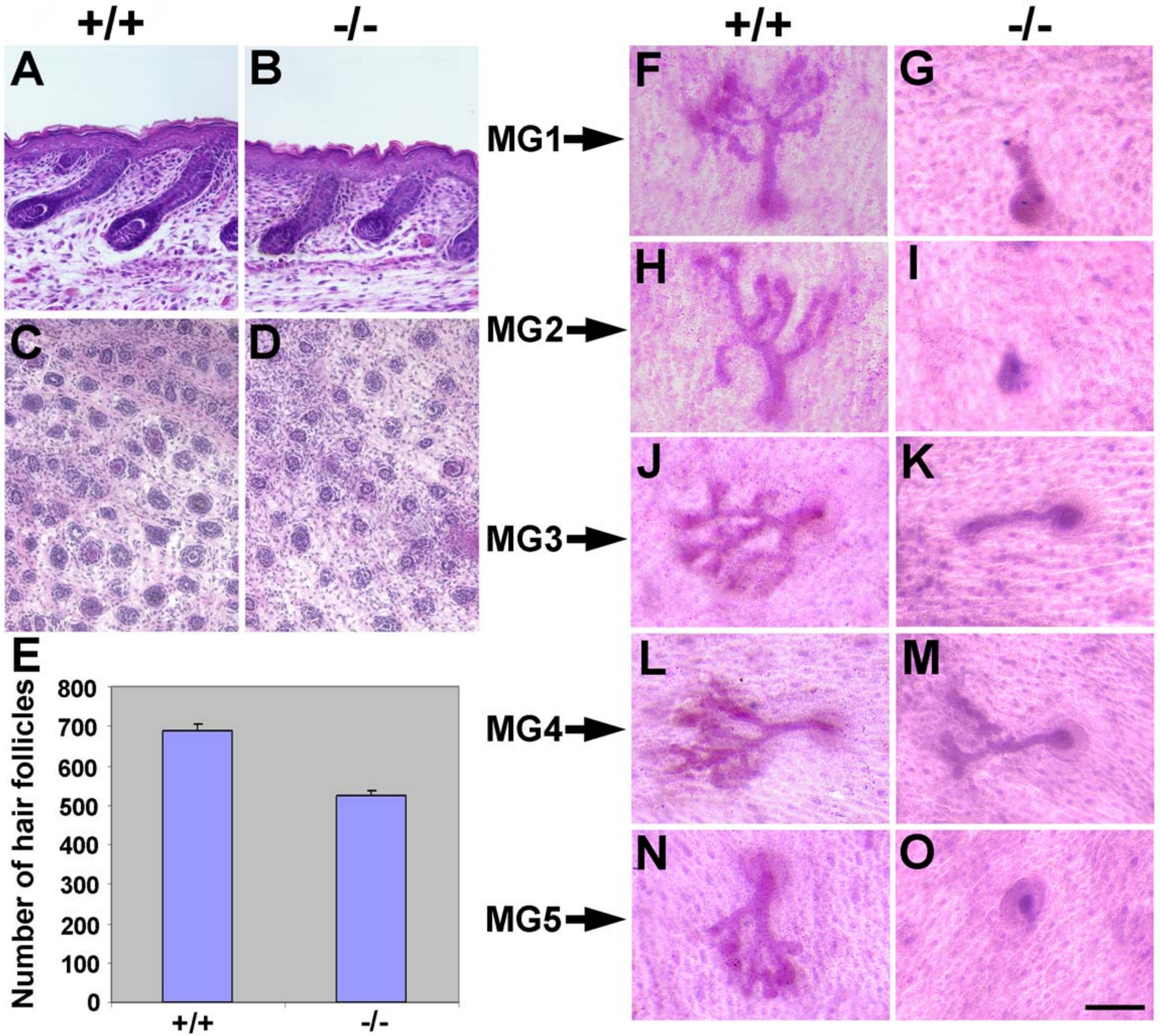


Fig. 3

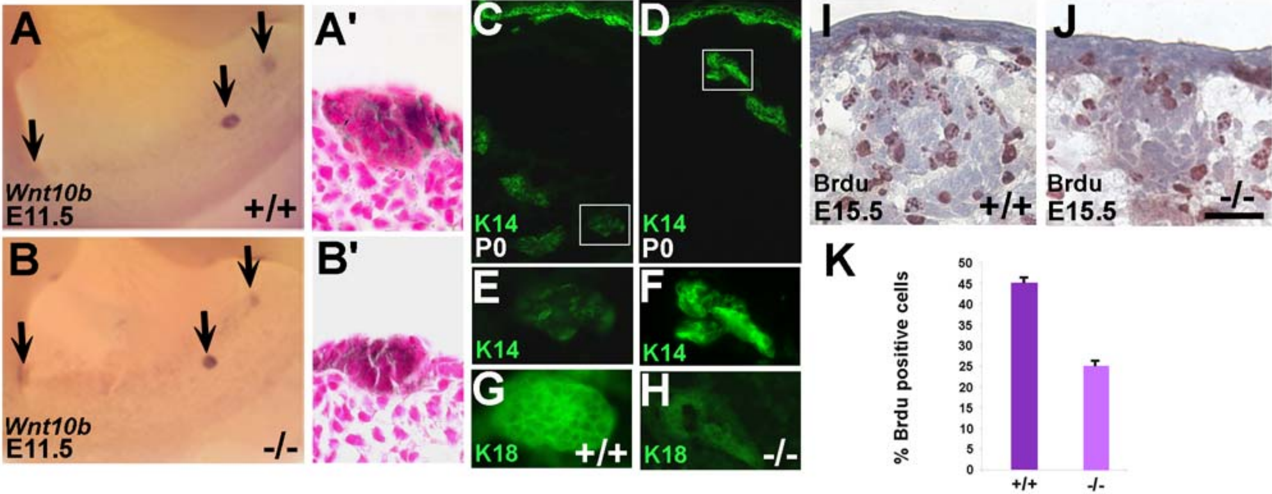


Fig.4

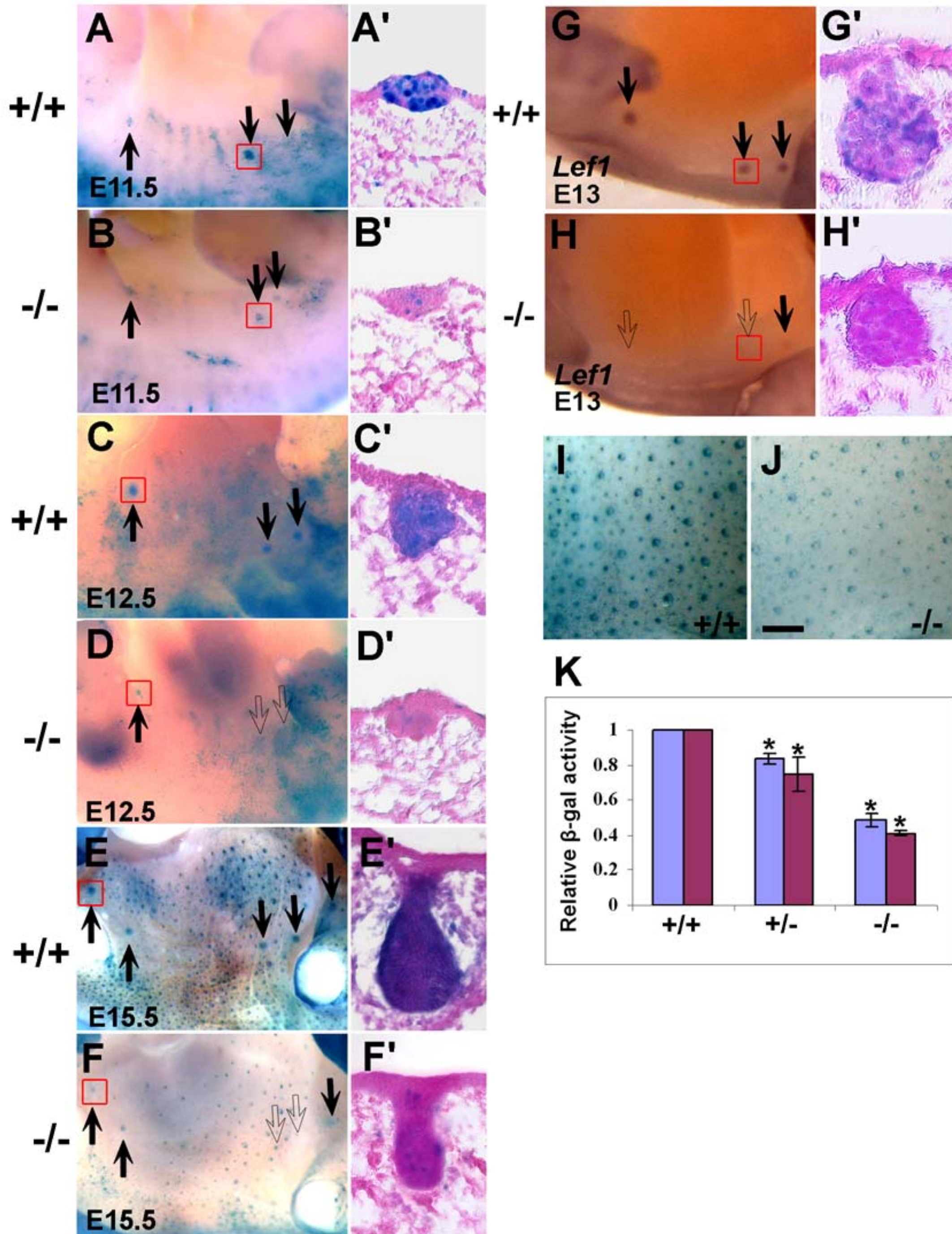


Fig. 5

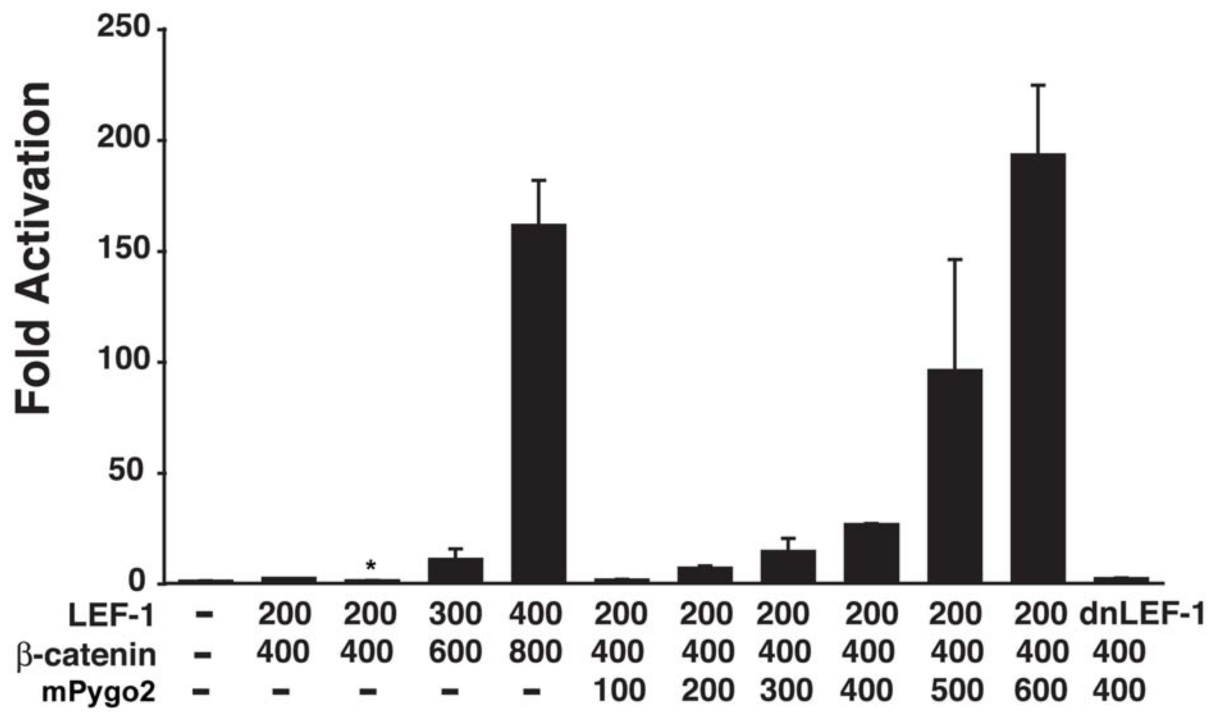


Fig. 6

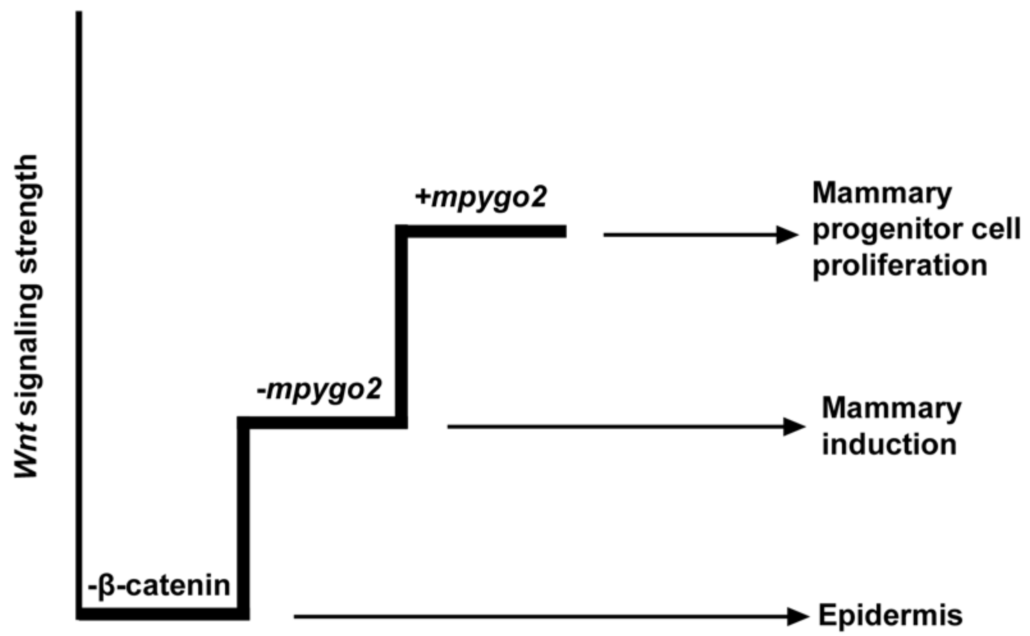


Fig. S1

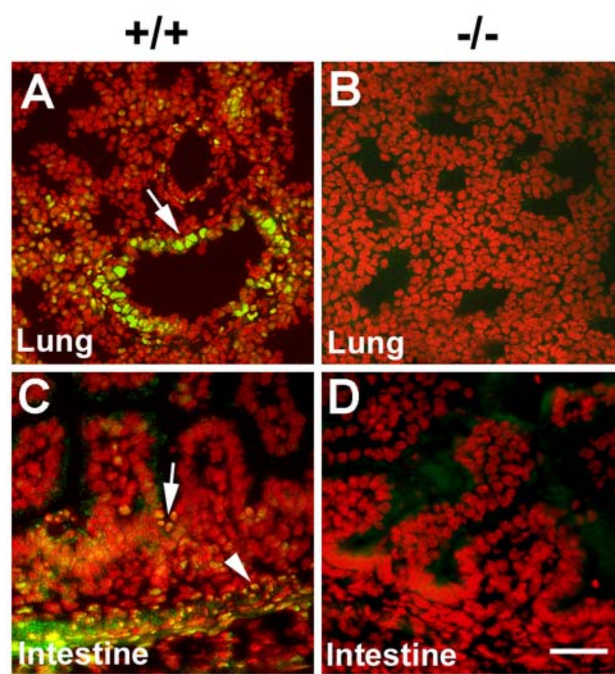


Fig. S2

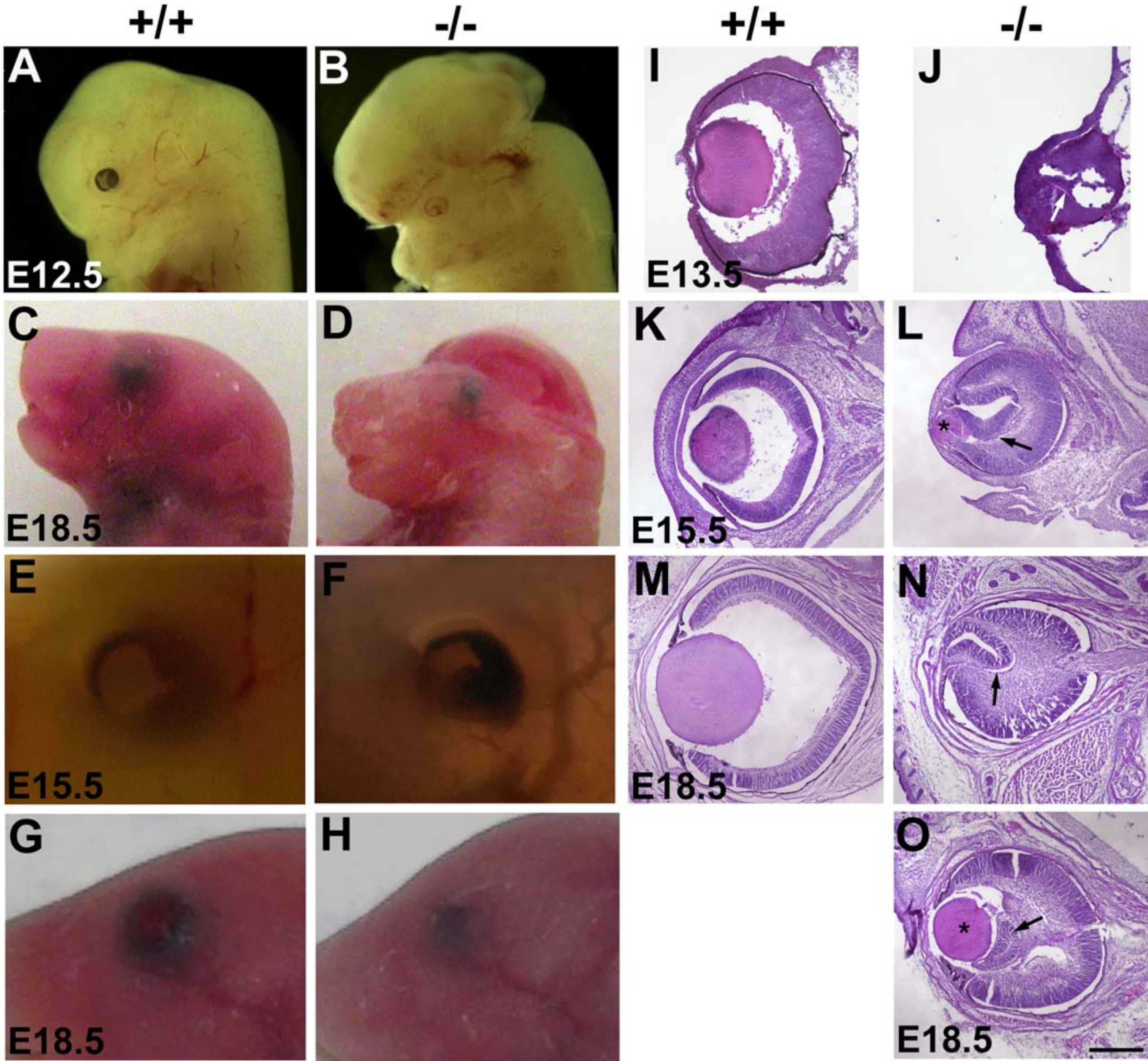


Fig. S3

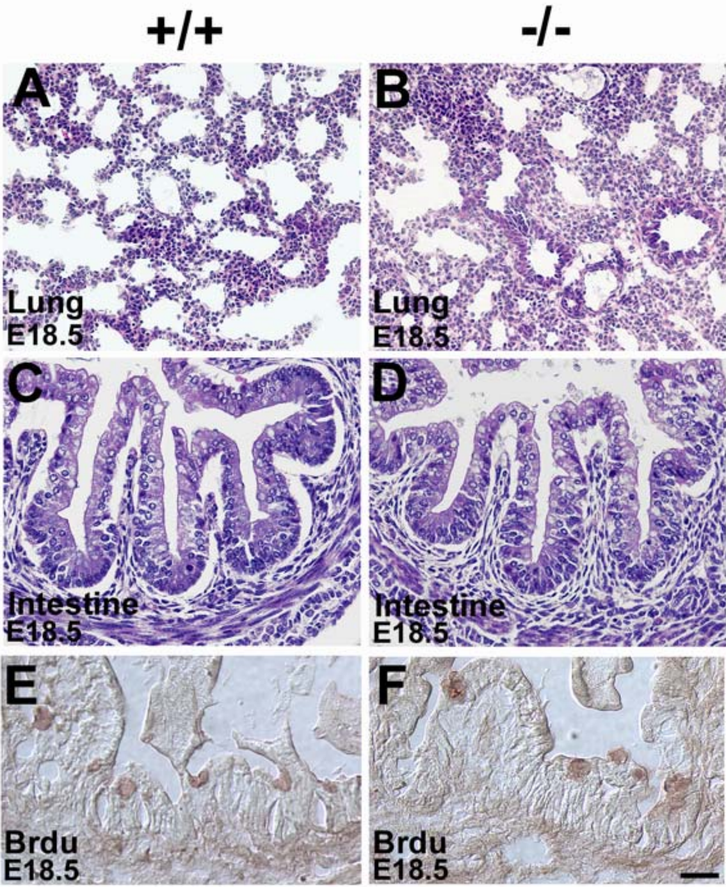


Table 1

Mammary gland pair	Not visible	Arrested at bud stage	Arrested at sprout stage	Branched
#1	4/8	1/8	3/8	0/8
#2	6/8	1/8	1/8	0/8
#3	5/8	0/8	3/8	0/8
#4	2/8	2/8	0/8	4[a]/8
#5	3/8	4/8	1/8	0/8

[a] The average branching point is calculated to be 3.25±0.63 in the mutant (n=4), while that of wild-type control is 6.5±1.04 (n=4) (p=0.04).