

LETTER

Developmental Phenotypes and Reduced Wnt Signaling in Mice Deficient for *Pygopus 2*Boan Li,^{1†} Catherine Rhéaume,^{1†} Andy Teng,¹ Virginia Bilanchone,¹ Jesus E. Munguia,² Ming Hu,¹ Shannon Jessen,¹ Stefano Piccolo,³ Marian L. Waterman,² and Xing Dai^{1,4*}¹Department of Biological Chemistry, University of California, Irvine, California²Department of Microbiology and Molecular Genetics, University of California, Irvine, California³Department of Histology, Microbiology and Medical Biotechnologies, Section of Histology and Embryology, University of Padua, Padua, Italy⁴Developmental Biology Center, University of California, Irvine, California

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Summary: Canonical Wnt signaling involves complex 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 (Wnt homolog) signaling in *Drosophila*, and encodes a PHD domain protein that associates with the β -catenin/LEF/TCF complex. Two murine *pygopus* paralogs, *mpygo1* and *mpygo2*, have been identified, but their roles in development and Wnt signaling remain elusive. In this study, we report that ablation of *mpygo2* expression in mice causes defects in morphogenesis of both ectodermally and endodermally derived tissues, including brain, eyes, hair follicles, and lung. However, no gross abnormality was observed in embryonic intestine. Using a BAT-gal reporter, we found Wnt signaling at most body sites to be reduced in the absence of *mpygo2*. Taken together, our studies show for the first time that *mpygo2* deletion affects embryonic development of some but not all Wnt-requiring tissues. *genesis* 45:318–325, 2007. © 2007 Wiley-Liss, Inc.

Key words: *pygo2*; *pygopus*; Wnt/ β -catenin signaling

INTRODUCTION

The secreted Wnt proteins trigger several intracellular signaling pathways, of which the most extensively studied is canonical Wnt signaling (Logan and Nusse, 2004; Veeman *et al.*, 2003). The canonical Wnt pathway functions in normal development and adult homeostasis of myriad tissues and organs, and is associated with diseases of the bone, brain, eye, skin, and heart, as well as cancer (Clevers, 2006; Logan and Nusse, 2004). Wnt signaling has been shown to direct patterning and cell fate decisions during skin appendage formation and lung morphogenesis (Andl *et al.*, 2002; Chu *et al.*, 2004; Gat *et al.*, 1998; Huelsken *et al.*, 2001; Shu *et al.*, 2005), while during intestinal and lymphocyte development, it

regulates proliferation and survival of stem/progenitor cells (Korinek *et al.*, 1998; Reya *et al.*, 2000; Timm and Grosschedl, 2005).

The critical event of activated Wnt signaling is stabilization of β -catenin, which then enters the nucleus and binds a member of the LEF/TCF family of transcription factors to stimulate expression of target genes involved in diverse cellular processes. *Pygopus*, a PHD domain protein identified by genetic screens in *Drosophila*, is suggested to function as a devoted coactivator for the β -catenin/LEF/TCF complex, and/or to facilitate nuclear retention of the complex (Kramps *et al.*, 2002; Kriehoff *et al.*, 2006; Stadel and Basler, 2005; Thompson, 2004; Townsley *et al.*, 2004). The *in vivo* role of mammalian *pygopus* genes in development and in Wnt signaling has not been explored. Of the two mammalian *pygopus* paralogs, murine *pygopus 2* (*mpygo2*) is more broadly expressed than *pygopus 1* (*mpygo1*) during embryonic development and in adult tissues (Li *et al.*, 2004).

To investigate the *in vivo* function of *mpygo2*, we used Cre/loxP technology to generate mice with deficient or conditional alleles of the gene. 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. 1a) (Li *et al.*, 2004). Homologous recombination followed by Cre-mediated excision in ES cells gave rise to clones containing either an *mpygo2* deletion allele (where both exon 3 and neo are removed; referred to herein as “mutant” or “–”), or a “floxed” allele (where neo is removed and exon 3 is

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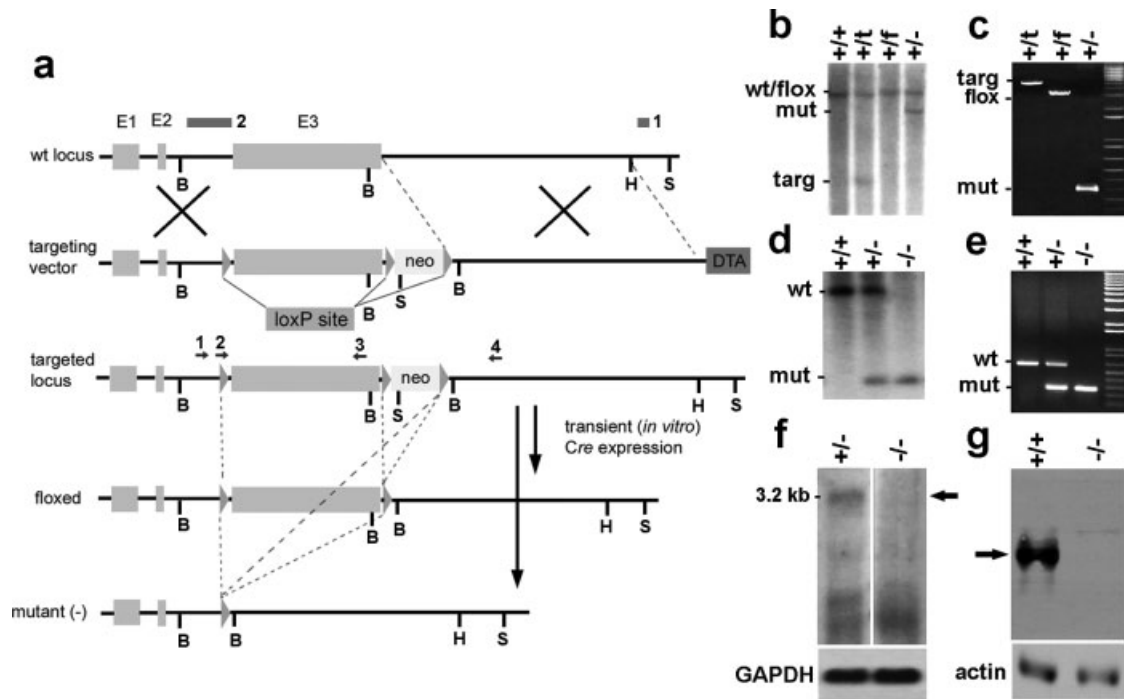


FIG. 1. Targeted deletion of *mpygo2*. (a) Targeting vector design and the resulting *mpygo2* alleles. Boxes, exons; triangles, loxP sites. neo, neomycin; DTA, diphtheria toxin A; B, Bam HI; S, Sph I; H, Hind III. Bars above the wild-type (wt) locus indicate the positions of probes used for Southern blot analyses, and small arrows above the targeted locus indicate positions of primers used in PCR genotyping (see Methods). (b) Southern and (c) PCR analysis of E14 ES cell clones containing wild-type or mutant alleles. (d) Southern and (e) PCR genotyping of embryos from intercrosses of heterozygous mutants containing the “-” allele. (f) Northern blot analysis of RNA from +/+ and -/- E18.5 skin using a cDNA probe specific for exon 1. Note absence of the 3.2-kb *mpygo2* transcript in the mutant (arrow). The same blot was stripped and reprobed with GAPDH to control for loading. (g) Western blot analysis of +/+ and -/- E18.5 skin extracts using the α -mPygo2 antibody. An anti-actin antibody was used as loading control (bottom).

flanked by LoxP sites), as confirmed by Southern blot (Fig. 1b) and PCR (Fig. 1c) analyses. ES cells containing the mutant or “floxed” allele were injected into C57BL/6 blastocysts, and the resulting chimeras were crossed with C57BL/6 mice to obtain germline transmission (Fig. 1d,e). Analyses here focused on *mpygo2*^{-/-} animals, in which *mpygo2* was ubiquitously deleted. Northern blot analysis of skin RNAs from homozygous mutants revealed a complete absence of the previously reported 3.2 kb *mpygo2* mRNA (Fig. 1f). Western blot analysis confirmed the absence of mPygo2 protein in mutant skin lysates (Fig. 1g). These results demonstrate that the *mpygo2* locus was functionally inactivated in *mpygo2*^{-/-} animals as a consequence of the targeting event.

We next performed immunofluorescence on select Wnt-requiring embryonic tissues (DasGupta and Fuchs, 1999; Merrill *et al.*, 2001; Mucenski *et al.*, 2003) to examine whether *mpygo2* is expressed there. Nuclear mPygo2 protein was detected in epithelial cells of the developing pelage and whisker hair follicles (Fig. 2a,c), respectively. Within the pelage hair follicle, the protein was primarily localized in the presumptive bulge, in matrix/precortex cells, and in dermal cells adjacent to these epithelial compartments. In E18.5 lung, most prominent nuclear staining was seen in a subset of epithelial cells lining the larger terminal airways (Fig. 2e); however,

scattered mPygo2-positive cells were also observed in epithelium of the smaller, peripheral terminal airways, as well as in the mesenchyme (Fig. 2e). In E18.5 intestine, weak, but detectable nuclear staining was seen in cells of the intervillous epithelium, where future crypts form, and in a subset of mesenchymal cells (Fig. 2g). No specific staining was observed in *mpygo2*-deficient tissues (Fig. 2b,d,f,h).

Consistent with the role of Wnt signaling in multiple developmental processes, we observed a pleiotropic phenotype in *mpygo2*^{-/-} animals. Most mutants were born slightly runted (with an average reduction of 15–20% in body weight and ~10% in length) and all exhibited perinatal lethality. Approximately 14% of 63 mutant animals examined between E12.5 and term displayed an enlarged brain and exencephaly or a domed head (Fig. 3b,d). Furthermore, macroscopic analysis of mutant pups revealed ~25% of mutants had smaller eyes; often these were distinct from the pups with brain defects (Fig. 3f,h). Histological analysis of eyes at different embryonic stages revealed several abnormalities. First, at all stages examined, mutant embryos contained smaller lens than the wild type or no lens at all (Fig. 3i,k,n,o). At E15.5, the wild-type lens was well-differentiated with cuboidal epithelial cells restricted to the anterior part of the lens (or capsule) (Fig. 3l, left). In contrast, the mu-

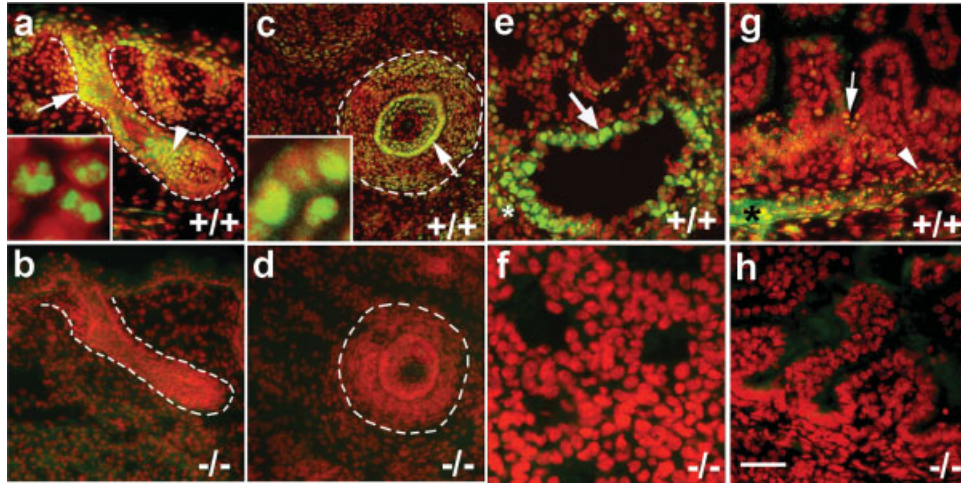


FIG. 2. mPygo2 protein expression in embryonic tissues. Shown are results of immunofluorescence analysis of E18.5 pelage hair (a, b) and whisker (c, d) follicles, lung (e, f), and intestine (g, h) using a rabbit α -mPygo2 antibody (green). DAPI staining was artificially colored red, so that nuclei of mPygo2-expressing cells were yellow. Arrow and arrowhead in (a) indicate mPygo2 expression in the presumptive bulge and precortex of a pelage follicle, respectively. Arrow in (c) indicates mPygo2 expression in outer root sheath cells of a whisker follicle. Arrow and “*” in (e) point to strongly stained lung epithelial cells and mesenchymal cells, respectively. Arrow and arrowhead in (g) indicate mPygo2-expressing intestinal epithelial and mesenchymal cells, respectively. “*” in (g) indicates nonspecific signals. Insets in (a) and (c) are high magnification images showing nuclear presence of the protein. White lines denote basement membrane. Bar: 65 μ m in a, b; 20 μ m in c, d; 25 μ m in e, f; 50 μ m in g, h.

tant lens was poorly differentiated, contained few lens fibers, and was surrounded by cuboidal epithelial cells (Fig. 3l, right). These defects persisted until later, as cuboidal cells with nuclei were still seen at both the anterior and posterior parts of the mutant lens at E18.5 (Fig. 3q). Second, eyelid development was delayed in the mutant, as at E15.5 mutant eyelids failed to form and close properly (Fig. 3k, arrowhead). Third, the cornea did not form properly at E15.5 (Fig. 3k,l; a single-layered surface ectoderm was seen where cornea was expected), and appeared deformed at E18.5 (Fig. 3n,q) in mutant embryos. Finally, the hyaloid cavity was not present and the retina was abnormally organized and often convoluted (Fig. 3i–o). Hair follicle defects were also seen but were relatively mild. A single mutant newborn showed an arrest of hair follicle development at the bud stage; however, most mutants examined contained grossly normal or slightly less elongated follicles than the wild type (Fig. 4a, b and data not shown). An \sim 30% decrease in hair follicle density was observed in the mutant (Fig. 4c–e). These results demonstrate that *mpygo2* is required for the proper morphogenesis of several ectodermally-derived tissues.

Endodermally derived tissues were also examined. Lungs from E18.5 and newborn *mpygo2*^{-/-} animals were often pale and smaller than the wild type (data not shown). Examination of hematoxylin/eosin (H/E)-stained sections revealed that at this stage wild-type lung contained well-developed terminal airways, characterized by the presence of small, irregularly shaped sacs lined with a wavy epithelium (Fig. 5a,c). In contrast, mutant lung contained airways that terminate in large, smooth-walled cylindrical structures (Fig. 5b,d). These defects are reminiscent of the reduced number of airway

saccules observed in mice overexpressing *Dkk1*, a Wnt inhibitor (Shu *et al.*, 2005), and suggest that loss of *mpygo2* also inhibited distal airway branching and formation in the embryonic lung. Furthermore, the expected thinning of the interstitial tissue, which normally occurs upon septation, appeared defective in the mutant (Fig. 5d). In the intestine, previous studies of TCF-4 knockout mice implicate Wnt signaling in the maintenance/proliferation of progenitor cells located in the intervillous intestinal epithelium (Korinek *et al.*, 1998). Surprisingly, the intervillous region of E18.5 and newborn *mpygo2*^{-/-} animals appeared indistinguishable from the wild type (Fig. 5f, compare with 5e). By BrdU labeling, comparable levels of progenitor cell proliferation were observed between wild-type and mutant animals (Fig. 5g,h). Furthermore, histological staining for goblet and enteroendocrine cells did not reveal significant alterations in the mutant intestine (data not shown). Nearly all E18.5 mutant embryos (>90%) showed an average of \sim 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.

To address whether *mpygo2* ablation affects Wnt signaling, we generated double transgenic mice carrying a transgenic BAT-gal Wnt reporter gene and wild-type or mutant *mpygo2* alleles. In BAT-gal transgenics, LacZ is under the control of LEF/TCF-responsive elements, allowing β -galactosidase activity to be detected at sites where canonical Wnt signaling is active (Maretto *et al.*, 2003). Whole-mount staining for β -galactosidase activity revealed a general reduction of BAT-gal expression in mutant embryos (Fig. 6a,b and data not shown). Section-

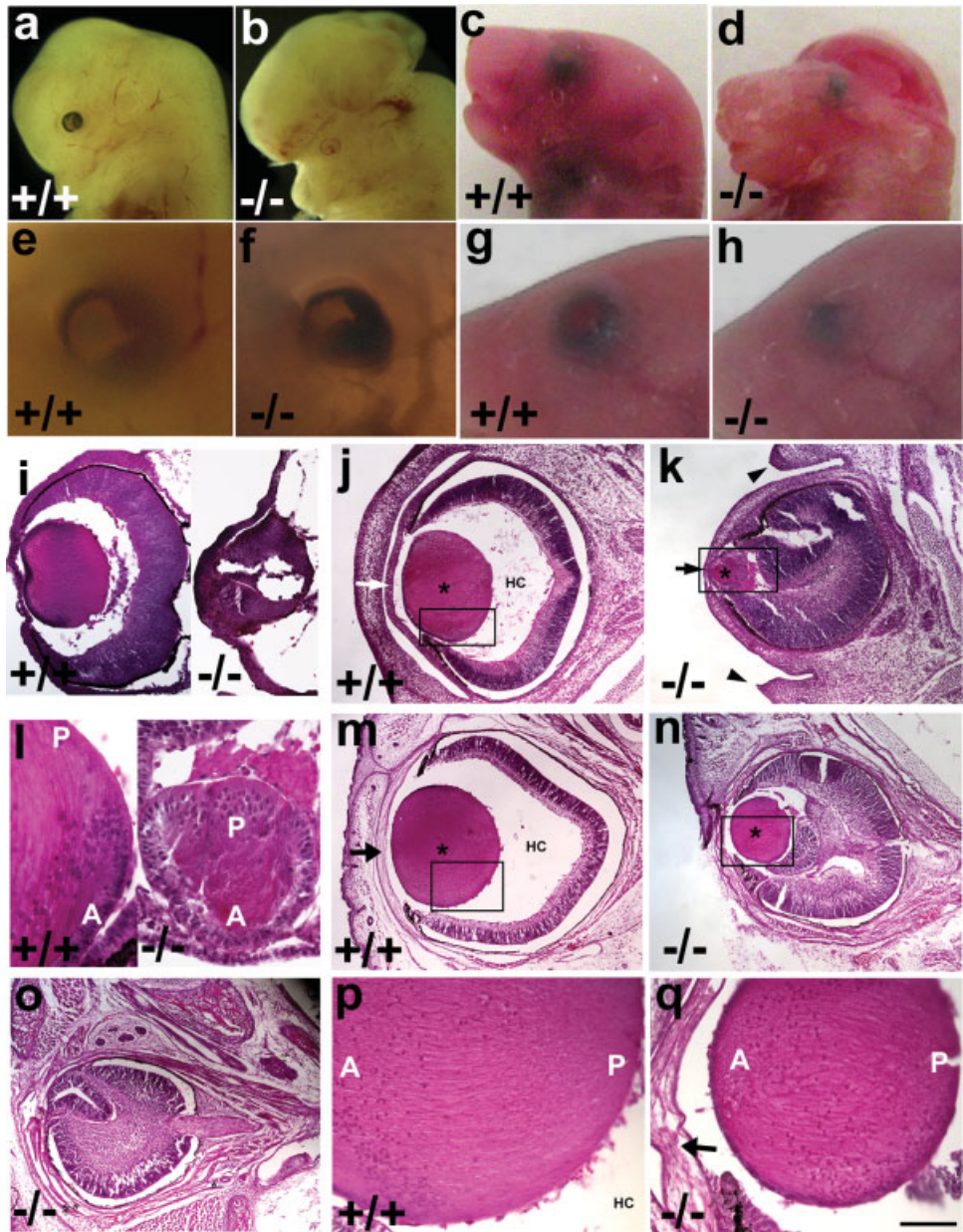


FIG. 3. Brain (a–d) and eye (e–q) defects in *mpygo2*^{-/-} mice. Shown are morphology (a–h) and histology (i–q) of wild-type (+/+) and mutant (-/-) animals at E12.5 (a–b), E13.5 (i), E15.5 (e–f, j–l), E18.5 (c–d, g–h, m–q). (l) contains high magnification images of boxes shown in j (left) and k (right). (p) and (q) are high magnification images of boxes shown in (m) and (n), respectively. Note that lens (indicated by “**”) is smaller in one mutant (n) but absent in another (o). Also note the presence of cuboidal epithelial cells at the posterior (P) part of the mutant lens (l, q). A, anterior part of the lens; HC, hyaloid cavity. Arrows indicate cornea, which is defective in the mutant. Bar: 130 μm in i; 225 μm in j, k; 55 μm in l; 285 μm in m–o; 75 μm in p, q.

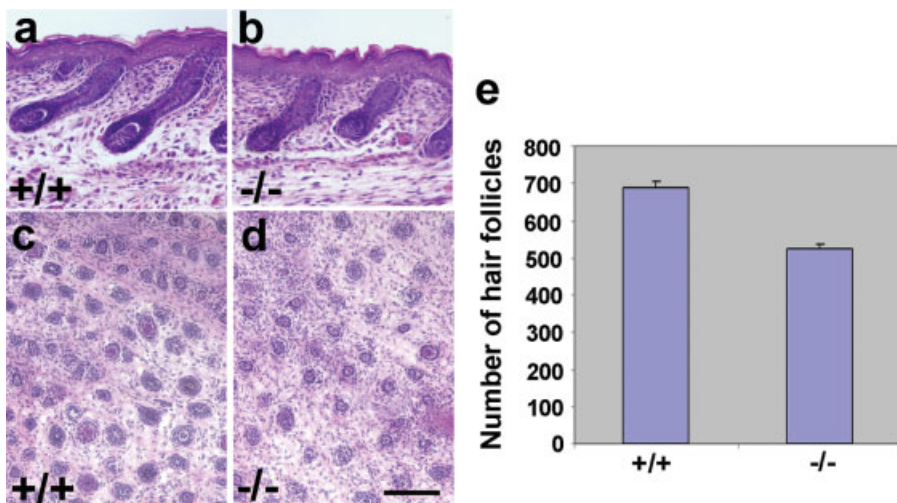


FIG. 4. Reduced hair follicle density in *mpygo2* mutant. Longitudinal (a, b) and cross-sections (c, d) of E18.5 skin were stained with hematoxylin/eosin. Cross-sections immediately underneath the skin surface were taken to quantify the total number of hair follicles (including those that may show delayed development), and values per $2.6 \times 10^5 \mu\text{m}^2$ were graphically represented in (e) ($P = 0.0017$, $n = 20$). Bar: 80 μm in a, b; 120 μm in c, d.

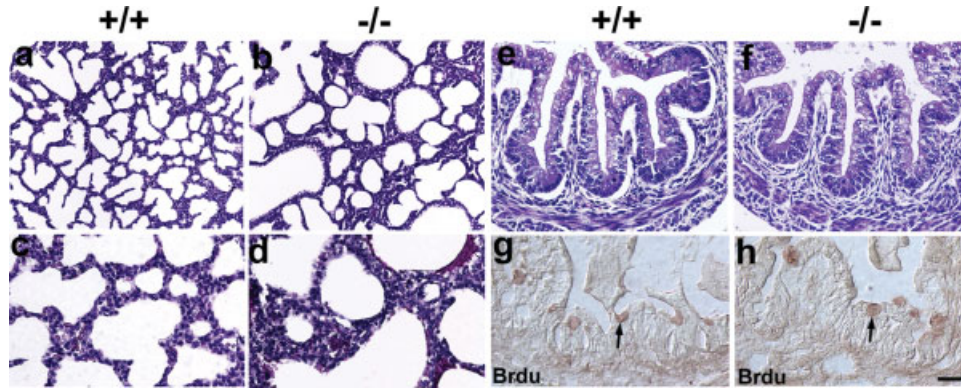


FIG. 5. *mpygo2*^{-/-} embryos show abnormal lung morphology but no apparent anomaly of presumptive intestinal crypts. Shown are results of histological analysis of lung (a–d) and intestine (e, f), and of BrdU-labeling experiments of intestine (g, h). Arrows in (g, h) point to BrdU-labeled cells in the intervillous region. Bar: 85 μ m in a, b; 30 μ m in c, d; 33 μ m in e, f; 50 μ m in g, h.

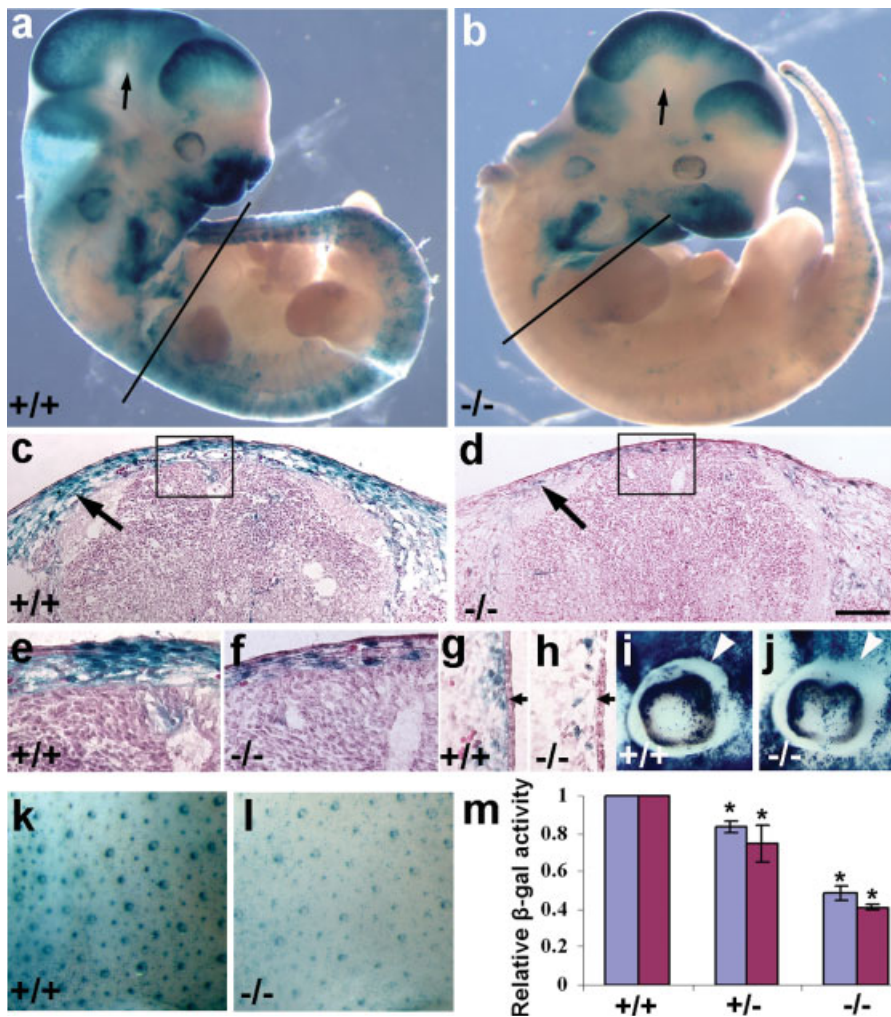


FIG. 6. Reduced Wnt signaling in some *mpygo2*-deficient embryonic tissues. (a, b) Whole-mount LacZ staining of E11.5 wild-type (+/+) and mutant (-/-) embryos. Note that reduction is particularly striking in posterior structures of the mutant, but is also visible in the head region (arrows). (c–h) H/E counter-stained sections of E11.5 embryos at positions indicated by lines in (a–b). (e) and (f) show high magnification images of boxed areas in (c) and (d), respectively. Arrows in (c–d) and (g, h) point to LacZ⁺-mesenchymal and LacZ⁻-surface ectodermal cells, respectively. (i, j) Prolonged LacZ staining to show expression in eyelids of E11.5 embryos. Note reduced staining in some (arrowhead) but not all areas of the mutant. (k, l) Whole-mount LacZ expression analysis showing stained hair follicles on back skin of E15.5 embryos. (m) Assays of β -galactosidase activity in extracts of skin (light purple) and lung (burgundy) from E18.5 embryos of the indicated genotypes. Values for the wild-type animals were set as 1, and “*” indicates statistically significant ($P < 0.06$) differences from the wild type. Bar: 125 μ m in c, d; 55 μ m in e, f; 55 μ m in g, h.

ing through stained E11.5 embryos revealed BAT-gal expression primarily in the mesenchymal cells between surface ectoderm and the neural tube and a decreased number of LacZ⁺ cells in the mutant (arrow in Fig. 6d;

compare with Fig. 6c). A subset of surface ectodermal cells above the neural tube were also positive, and again the number of positive cells appeared to be reduced in the mutant (Fig. 6e,f). The lateral surface ectoderm in

wild type or mutant did not show any BAT-gal expression, but its underlying mesenchyme stained positive, and there were more expressing cells in wild type than mutant (Fig. 6g,h). Curiously, BAT-gal expression was predominantly observed in pigmented cells of retina, a layer that was apparently unaffected by *mpygo2* deletion (Fig. 3k), and no reduction of lacZ staining was seen in the mutant (data not shown). Prolonged incubation with β -galactosidase substrates helped to visualize BAT-gal expression in surrounding eyelids, and it appeared that fewer cells were LacZ-positive in the mutant (arrowhead in Fig. 6j). Decreased BAT-gal expression in mutant hair follicles was evident in E15.5 embryos (Fig. 6k,l). Quantitative assay of β -galactosidase activity was performed on extracts of dorsal skin and lung from E18.5 embryos, and mutant tissues displayed considerably lower (>two-fold) activity than the wild type (Fig. 6m). Interestingly, despite the absence of any apparent phenotype in heterozygous mutants, a slight reduction in β -galactosidase activity was also seen in their skin and lung extracts (Fig. 6m), suggesting that morphogenic consequences occur only when signaling activity falls below a certain critical threshold. A very high level of β -galactosidase activity was detected in wild-type intestinal extracts, and no decrease was observed in the *mpygo2* mutants (data not shown). Taken together, our results indicate that mPygo2 is required in vivo for maximum Wnt signaling in some but not all tissues.

In summary, our data demonstrate that *mpygo2* is required for a subset of mammalian developmental processes that require Wnt signaling, particularly the proper morphogenesis of brain, eyes, hair follicles, and lung. This work is the first to address the in vivo function of a mammalian *pygopus* gene. Because *mpygo2*^{-/-} animals die perinatally, these experiments focused on embryonic defects caused by the deficient allele. Availability of the "floxed" *mpygo2* allele as reported here will facilitate future analysis of *mpygo2* function in specific adult tissues.

Our observation of reduced BAT-gal expression in most *mpygo2*-deficient tissues suggests that *mpygo2* functions in vivo to modulate Wnt signaling. However, phenotype appearance does not correlate 100% with reduced BAT-gal expression, particularly for eyes and brain. It is possible that Wnt signaling is affected in these tissues at an earlier developmental stage not examined here, or alternatively, that mPygo2 acts in these tissues in a Wnt-independent manner. Whether mPygo2 functions cell-autonomously and via protein-protein interactions to potentiate transcriptional activation by β -catenin/LEF/TCF complexes, as previously proposed (Kramps *et al.*, 2002; Kriehoff *et al.*, 2006; Stadel and Basler, 2005; Thompson, 2004; Townsley *et al.*, 2004), or that it regulates intermediate pathways or factors that in turn activate Wnt signaling remains to be addressed using more direct methods. With multiple attempts, we have not been able to detect nuclear β -catenin in wild-type tissues under study; thus we could not address a previously raised notion that Pygopus proteins facilitate

the nuclear accumulation of β -catenin (Thompson *et al.*, 2002; Townsley *et al.*, 2004). A considerable level of Wnt signaling remained in the absence of mPygo2; consistently, *mpygo2*-deficient animals 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 (Haegel *et al.*, 1995; Huelsken *et al.*, 2000), were not observed in *mpygo2*-deficient embryos. While β -catenin ablation or Dkk1 overexpression abolishes hair follicle initiation (Andl *et al.*, 2002; Huelsken *et al.*, 2001), *mpygo2* mutant newborns are able to produce hair follicles, albeit at a reduced density. Furthermore, unlike TCF-4 (Korinek *et al.*, 1998), *mpygo2* does not appear to be required for the formation and proliferation of intestinal stem cells in developing embryos. However, inactivation of β -catenin or overexpression of Dkk1 in lung epithelium results in expanded proximal airway formation at the expense of distal airways (Shu *et al.*, 2005), a phenotype shared by the *mpygo2* mutant embryos. While flies have a single *pygopus* gene, mammals have two *pygopus* paralogs. It is possible that *mpygo1* plays a partially redundant role for *mpygo2* during early developmental processes and in tissues that are least affected by the loss of *mpygo2*, although we did not observe any significant increase in the level of *mpygo1* transcripts in *mpygo2*-deficient embryonic skin (data not shown). Definitive evidence awaits the generation and analysis of *mpygo1* and *mpygo2/mpygo1* double mutant mice. Alternatively, *pygopus* genes in mammals may have evolved to play a nonessential but augmenting role in Wnt signaling, and requirements for such an augmentation role vary by tissue type. For example, mPygo2 may only be needed when a particular LEF or TCF factor is in use at a particular tissue site. In light of our findings and a recent study that describes Wg-independent association of *Drosophila* Pygo with target genes of dTCF (de la Roche and Bienz, 2007), it becomes clear that further studies are needed to fully understand the cellular and molecular function of mammalian *pygopus* genes and their relationship with Wg/Wnt signaling.

METHODS

Generation of *mpygo2* Mutant Alleles

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 (Mackay *et al.*, 2006). PCR genotyping was performed using the following primers: (a) for the detection of targeted, floxed, and "-" alleles, respectively, in ES DNA: 5'-CTCTAGCGTGTC-TAAGGTCAGCCAGAGCG-3' (primer no. 2 in Fig. 1a) and 5'-AGGGCAAGCTGCCATGTCAGTTCTCT-3' (primer no.

4 in Fig. 1a); (b) for the detection of wild-type allele in ES and tail DNA: 5'-AGCGTGTCTAAGGTCAGCCAGAG-GTTTG-3' (primer no. 1 in Fig. 1a) and 5'-GTAAAG-CGTTGGGGGAGAGGAGGAGGAC-3' (primer no. 3 in Fig. 1a); (c) for the detection of "-" allele in tail DNA: 5'-CTCTAGCGTGTCTAAGGTCAGCCAGAGCG-3' (primer no. 3) and 5'-AGGGCAAGCTGCCATGTCAGTTCTCT-3' (primer no. 4). 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); tail DNA was digested with Bam HI and blots probed with a 1-kb fragment upstream of exon 3 (probe 2 in Fig. 1b).

Histology and Immunostaining

Embryos or tissues were fixed in Bouin's fixative for 24 h, processed and embedded in paraffin wax, sectioned at 6 μm , and stained with hematoxylin/eosin. For quantification of the number of hair follicles, cross-sections of E18.5 skin were prepared and hair follicles were counted in an area of $2.6 \times 10^5 \mu\text{m}^2$. Statistical significance was determined using Student's *t*-test. Indirect immunofluorescence of embryonic tissues was performed as described (Dai *et al.*, 1998) using rabbit α -mPygo2 antiserum that was preadsorbed with tissue powder prepared from E15.5 *mpygo2*^{-/-} embryos. This antiserum was generated to a GST fusion protein containing amino acids 6–115 of mPygo2 (Harlan Bioproducts for Science) and affinity-purified.

BrdU-Labeling and Immunodetection

Pregnant mice were injected intraperitoneally with 50 $\mu\text{g/g}$ body weight of BrdU in PBS. Embryos were taken 1 h after injection, cryosectioned at 10 μm , and fixed in 4% paraformaldehyde for 10 min, followed by immunohistochemistry as described (Li *et al.*, 2005).

Analysis of BAT-gal Expression in Wild-Type and *mpygo2*-Deficient Embryos

mpygo2^{+/-} mice were bred with BAT-gal transgenics (Maretto *et al.*, 2003) to obtain *mpygo2*^{+/-} BAT-gal offspring, which were subsequently bred with *mygo2*^{+/-} mice to generate *mpygo2*^{+/+} BAT-gal and *mpygo2*^{-/-} BAT-gal littermates for analysis. E11.5 to E15.5 embryos were subjected to whole-mount staining for β -galactosidase activity as follows: embryos were fixed in 0.1 M phosphate buffer, pH 7.3, 0.2% glutaraldehyde, 2 mM MgCl₂, 5 mM EGTA for 10–45 min at room temperature. After three washes in 0.1 M phosphate buffer, pH 7.3, 2 mM MgCl₂, 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 MgCl₂ in potassium phosphate buffer, and stained for 30 min at room temperature in the dark. After rinsing with PBS, embryos were postfixed in 4% paraformaldehyde and examined under the microscope for photography. For quantifica-

tion of β -galactosidase activity, extracts were prepared by homogenizing E18.5 embryonic 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, β -galactosidase activity was measured from the supernatant using a chemiluminescence assay kit (Galacto-Light Kit from Tropix). β -galactosidase activity was normalized against protein concentration for each sample, and expressed as fold change over wild-type control. Statistical significance was determined using Student's *t*-test.

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