Development 133, 3661-3670 (2006) doi:10.1242/dev.02542

Gli3-mediated repression of Hedgehog targets is required for normal mammary development

Sarah J. Hatsell[1,2] and Pamela Cowin[1,2,*]

The Hedgehog pathway is vital for the development of many epidermal appendages, but its role in mammary development has been unclear. Here, we show that although *Gli2* and *Gli3* are expressed during embryonic mammary development, transcriptional reporters of positive Hedgehog signaling are absent. Nevertheless, $Gli3^{xt/xt}$ embryos show aberrant early mammary marker expression and lack two pairs of mammary buds, demonstrating that Gli3 is essential for mammary bud formation and preceding patterning events. Misactivation of the Hedgehog pathway by targeted expression of the constitutive activator *Gli1,* from the *Gli2* promoter in $Gli3^{xt/+}$ mice, also induces mammary bud loss. Moreover, loss of *Gli3* expression induces *Gli1* misexpression in mammary mesenchyme. These results establish that the essential function of Gli3 during embryonic mammary development is to repress Hedgehog/Gli1-inducible targets. During postnatal mammary development, *Gli2* and *Gli3* are expressed in stromal and myoepithelial cells, and Gli3 is also found within the lumenal epithelium. Again, transcriptional reporters of positive Hedgehog signaling are absent from these cell types, yet are expressed robustly within mammary lymphatics. Thus, positive Hedgehog signaling is absent throughout mammary development, distinguishing the mammary gland from other epidermal appendages, such as hair follicles, which require Hedgehog pathway activity.

KEY WORDS: Mammary, Breast, Gli, Hedgehog, Wnt

INTRODUCTION

The Hedgehog (Hh) family of secreted morphogens controls the patterning, growth, morphogenesis and homeostasis of many tissues, including vertebrate and invertebrate epidermal appendages (Ingham and McMahon, 2001). The Hh pathway was elucidated and is best understood in the development of cuticular denticle belts of the fruitfly, *Drosophila melanogaster* (Hammerschmidt et al., 1997). Flies express a single Hh protein that binds to Patched (Ptc), a twelve-pass transmembrane receptor, on neighboring cells. Hh-Ptc association relieves the seven-pass transmembrane protein, Smoothened (Smo), from Ptc-mediated repression. Smo then promotes phosphorylation of Cubitus interruptus (Ci), a microtubule-bound transcription factor, inhibiting its proteolysis into a transcriptional repressor, Ci^R, and converting it into a full-length transcriptional activator, Ci^A (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998). Thus, the Hh morphogen gradient is translated into position-specific gene expression by modulating the Ci^A:Ci^R ratio (Aza-Blanc and Kornberg, 1999).

In mammals, the Hh pathway is far more complicated. Mammals express three Hh genes [sonic (*Shh*), Indian (*Ihh*) and desert (*Dhh*) hedgehog] and two patched genes (*Ptc1* and *Ptc2*) (Echelard et al., 1993; Goodrich et al., 1997; Lewis et al., 1999a; Motoyama et al., 1998; Pathi et al., 2001; Pearse et al., 2001). Moreover, the transcriptional activator and repressor roles of Ci have been subdivided in complex ways among three homologues: Gli1, Gli2 and Gli3 (Hui et al., 1994). Gli2 is expressed in the absence of Hh signals and, in this situation, is either inactive or functions as a weak transcriptional repressor (Aza-Blanc et al., 2000; Bai and Joyner, 2001; Sasaki et al., 1999; Sheng et al., 2002). Hh signals activate Gli2 initiating transcription of Hh target genes, including *Ptc1* and *Gli1* (Bai et al., 2002). *Gli1* is strictly dependent on Hh signals for its expression and thus is an excellent reporter of positive Hh signaling (Bai et al., 2002; Bai et al., 2004). It lacks a transcriptional repressor domain and is a strong activator of Hh target genes, including itself. It can effectively substitute for Gli2 and antagonize Gli3, yet it appears to be dispensable for Hh signaling as demonstrated by the normal phenotype of $Gli1^{-/-}$ mice (Bai et al., 2002; Bai and Joyner, 2001; Dai et al., 1999; Hynes et al., 1997; Lee et al., 1997; Park et al., 2000). Gli3 can be expressed in the absence of Hh signals. However, Shh signaling suppresses both *Gli3* transcription and the N-terminal proteolytic processing that produces the Gli3 repressor ($Gli3^R$) (Aza-Blanc et al., 2000; Li et al., 2004; Marigo et al., 1996; Wang et al., 2000). Gli3 can function as a transcriptional activator or repressor of Gli1 and other target genes depending upon the cell context (Bai et al., 2004; Wang et al., 2000). In the simplest model, Gli2 acts at the top of the pathway to induce expression of the amplifier Gli1, which antagonizes the repressor activity of Gli3. However, cell context-specific roles of Gli2 and Gli3 mean that activator and repressor functions cannot be assumed for these proteins but must be determined empirically.

The Shh pathway plays a central role in the formation of many vertebrate epidermal appendages (sweat, sebaceous, lachrymal and salivary glands, hair, whiskers, feathers, scales, teeth and nails) that arise as a result of epithelial-mesenchymal interactions (Chuong et al., 2000; Cobourne and Sharpe, 2005; Dassule et al., 2000; Gallego et al., 2002; Michno et al., 2003; Pispa and Thesleff, 2003; Ting-Berreth and Chuong, 1996). The requirement for Shh during embryonic and adult hair follicle development and downward growth has been particularly well documented (Chiang et al., 1999; Mill et al., 2003; St-Jacques et al., 1998). Hair follicles and mammary glands co-evolved and share many local inductive pathways (Wnt, Fgf, Bmp and Pthlh) (Andl et al., 2002; Chu et al., 2004; Hens and Wysolmerski, 2005; Mailleux et al., 2002; Oftedal, 2002; Wysolmerski, 2002; Wysolmerski et al., 1998). Many similarities exist between the cyclical development of the mammary

[1]Department of Cell Biology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA. [2]Department of Dermatology, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA.

*Author for correspondence (e-mail: cowinp01@med.nyu.edu)

Accepted 19 July 2006

gland during pregnancy, lactation and involution, and the rounds of hair follicle growth (anagen), regression (catagen) and resting (telogen). Mammary glands first form around embryonic day 10 (E10.5) as bilateral ectodermal thickenings between the fore and hindlimbs, known as milk lines (Veltmaat et al., 2003). At about E11, the lines fragment and cells coalesce into five pairs of mammary placodes that, within 1 day, form elevated mammary buds. Between E13-E14 the mammary buds invaginate, forming a bulb below the surface of the epithelium. This structure induces the underlying stroma to condense and differentiate into mammary mesenchyme. In males, fetal androgens stimulate mammary mesenchymal fibroblasts to constrict around the epidermal buds, choking further development (Dunbar et al., 1999). In females, epithelial buds elongate at E16, forming the mammary sprout, which penetrates the underlying fat pad precursor and branches to form 5-6 primary ductules by E18. Further development does not occur until puberty, when estrogen and growth hormone induce stromal IGF secretion, stimulating proliferation of cells at the tips of each duct within terminal end buds, leading to ductal elongation (Marshman and Streuli, 2002). During each cycle of pregnancy, progesterone and prolactin stimulate several local paracrine pathways that promote extensive ductal side-branching and alveologenesis (Brisken et al., 2000; Brisken et al., 1998; Henninghausen and Robinson, 1998; Robinson et al., 2000). At the end of lactation, the mammary gland involutes by a sequential process involving epithelial apoptosis, extensive matrix remodeling and a wave of adipogenesis, which replenishes the mammary fatpad (Lund et al., 1996).

Both *Shh* and *Ihh* mRNAs have been detected within embryonic mammary bud epithelium by in situ hybridization, but elimination of either gene has no effect on bud development (Gallego et al., 2002; Michno et al., 2003). Thus, the function of Hh signaling within the mammary gland is obscure, and questions remain as to whether signaling by Shh and Ihh is redundant or dispensable for embryonic mammary gland development. To explore the role of the Hh pathway further, we determined the expression of the three downstream transcription factors, Gli1, Gli2 and Gli3, and examined the effects of altering the Gli activator/repressor ratio during mammary development. Our results demonstrate that, contrary to previous suggestion, and, in contrast to other epidermal appendages, positive Hh signaling is absent throughout mammary development. Furthermore, we show that Gli3-mediated transcriptional repression is essential for the formation of two pairs of mammary buds, and misactivation of the Hh pathway, by targeted expression of Gli1, induces bud loss.

MATERIALS AND METHODS

Mice

The following mice, maintained on an outbred background, were a kind gift from Dr Alexandra Joyner, Skirball Institute, NYU School of Medicine. $Gli1^{lzki/+}$ mice were constructed as described (Bai et al., 2002). Mice carrying *Gli1* or *lacZ* knocked into the *Gli2* locus ($Gli2^{1nki/+}$ and $Gli2^{lzki/+}$) were constructed as described (Bai and Joyner, 2001). $Gli3^{xt/+}$, $Ptc1^{lzki/+}$and TOP-Gal mice were obtained from Jackson Laboratories (Bar Harbor, ME). $Ptc1^{lzki/+}$ mice were as described (Goodrich et al., 1997).

Whole-mount analysis

For detection of *lacZ* expression, mammary glands or embryos were fixed in 4% paraformaldehyde (PFA) diluted in phosphate-buffered saline (PBS) for 1 hour, followed by three 1-hour washes in rinse buffer (2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% NP-40 in PBS). X-gal staining was carried out overnight in staining buffer (50 μg/ml X-gal in rinse buffer containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide). Mammary glands were washed in PBS, post-fixed for 1 hour in 4% PFA, dehydrated through a graded series of ethanol, cleared for ~30 minutes in Citrisolve (Fisher Scientific, Pittsburg, PA) and mounted in Securemount (Fisher Scientific) and viewed under a Leica dissecting microscope (Bannockburn, IL).

Histology

For histological analysis, mammary glands and embryos were stained as above with X-gal then post-fixed with 4% PFA overnight at 4°C. They were embedded in paraffin and sectioned and in some cases processed further for immunohistochemistry as described below.

Immunohistochemistry

Sections (4 μm) were deparaffinized with xylene and rehydrated through a graded series of ethanol. Citric acid antigen retrieval was performed for all antibodies by placing slides in 1.92 g/l of sodium citrate (pH 6.0) and microwaving for 20 minutes. Rabbit anti-Keratin 14 (K14) (Covance, Berkeley, CA) (1:400) primary antibody was detected by using the DAKO EnVision$^+$ Kit comprising horse-radish peroxidase (HRP) coupled anti-rabbit IgG followed by diaminobenzidine (DAB) following the manufacturer's protocol (DAKO, Carpinteria, CA). Mouse anti-p63 (Neomarkers, Freemont, CA) (1:500) was detected using biotin labeled goat anti-mouse IgG (Vector Labs, Burlingame, CA) and rabbit anti-Gli3 (Santa Cruz Biotechnology, Santa Cruz, CA) (1:100) was detected using biotin labeled goat anti-rabbit IgG (Vector Labs) (1:1000) followed by streptavidin-HRP, which was detected using DAB.

Whole-mount in situ hybridization

Embryos were fixed overnight in 4% PFA diluted in PBS, dehydrated in methanol and stored at –20°C. Before hybridization embryos were rehydrated, bleached by incubating for 30 minutes in 6% H_2O_2, treated with 6 μg/ml proteinase K for 10 minutes, washed in 2 mg/ml glycine, then fixed in 4% PFA for 20 minutes. All solutions were made up in PBS-T (PBS, 1% Tween-20) and three 5 minute PBS-T washes followed each step. Embryos were prehybridized for 2-3 hours in 50% formamide 5× SSC, 50 μg/ml tRNA, 1% SDS, 50 μg/ml heparin then hybridized overnight at 70°C in the same buffer containing 2 μg/ml of digoxigenin (DIG)-labeled Gli3 probe. Following several washes, DIG was detected by overnight incubation at 4°C in alkaline phosphatase (AP) labeled anti-DIG Fab' fragments (Roche Indianapolis IN). Color was developed with BM-purple AP substrate (Roche). Further protocol details are available at http://saturn.med.nyu.edu/research/dg/joynerlab/protocols.html

Section in situ hybridization

Sections were dewaxed in xylene and rehydrated, fixed with 4% PFA, treated with 1 μg/ml proteinase K for 15 minutes at 37°C, post-fixed in 4% PFA and dehydrated. Sections were hybridized overnight at 55°C in 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 300 mM NaCl, 0.02 M Tris-HCl pH 8.0, 5 mM EDTA, 0.01% sarkosyl, 250 μg/ml yeast tRNA containing 1 μg/ml DIG labeled Gli3 probe. DIG was detected as above.

Northern analysis

Total RNA was isolated from mammary gland using the ToTALLY RNA kit (Ambion, Austin, Texas) (Imbert et al., 2001). mRNA was purified from 30 μg of total RNA using the Poly(A)Pure kit (Ambion). Northern analysis was carried out on these mRNA samples using the NorthernMax-Gly kit (Ambion). The Gli3 cDNA probe was obtained from Dr Alexandra Joyner and the K18 cDNA probe was obtained from Caroline Alexander (University of Wisconsin, Madison, WI).

RESULTS

Gli2-lacZ is expressed in the dermal mesenchyme and the basal epithelium and surrounding condensed stroma of embryonic mammary buds

Gli2 is converted into a transcriptional activator by Hh signals and functions as the primary Hh transducer in many tissues (Aza-Blanc et al., 2000; Bai et al., 2002; Sasaki et al., 1999; Sheng et al., 2002). To determine the activity of the *Gli2* promoter during embryonic mammary development, we examined expression of a *lacZ* reporter knocked into the *Gli2* locus ($Gli2^{lzki/+}$) (Bai and Joyner, 2001). In the

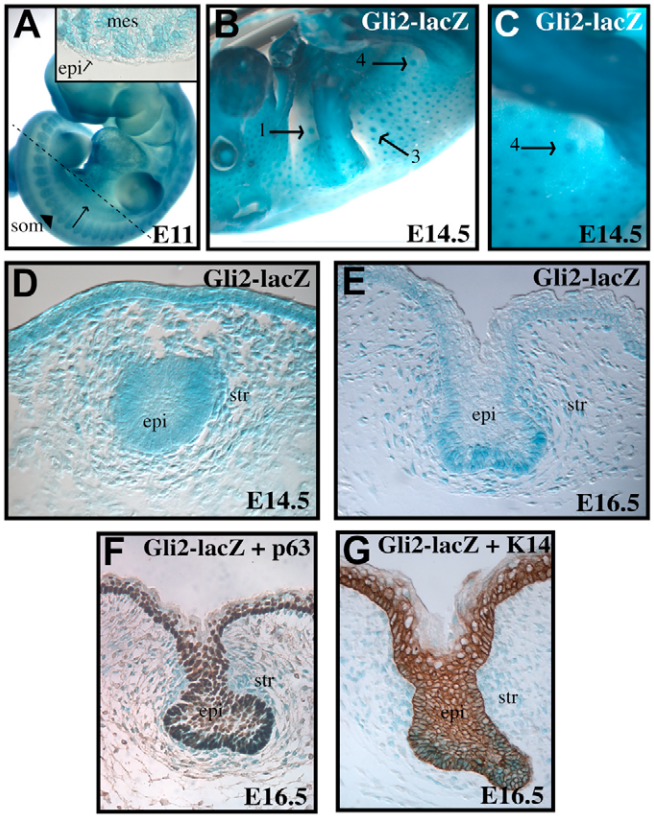

Fig. 1. *Gli2-lacZ* expression in embryonic mammary buds. (**A-C**) Whole-mount X-gal staining of *Gli2-lacZ* embryos. (A) At E11, expression is seen in an arc between the fore- and hindlimbs (arrow) and in the somites (som, arrowhead). (A, inset) A cross-section through this embryo shows staining is found within the dermal mesenchyme (mes) but not in the epithelial layer (epi, arrow). (B,C) At E14.5 strong staining is seen in both hair placodes and mammary buds (1,3,4, arrows). (**D-G**) Cross-sections through *Gli2-lacZ* embryos stained with X-gal. Staining is seen within the basal epithelial layer (epi) and surrounding stroma (str) of E14.5 mammary buds (D) and E16.5 mammary sprout (E). Epithelial *Gli2-lacZ* expression is restricted to a basal subset of p63- (F) and K14- (G) positive cells.

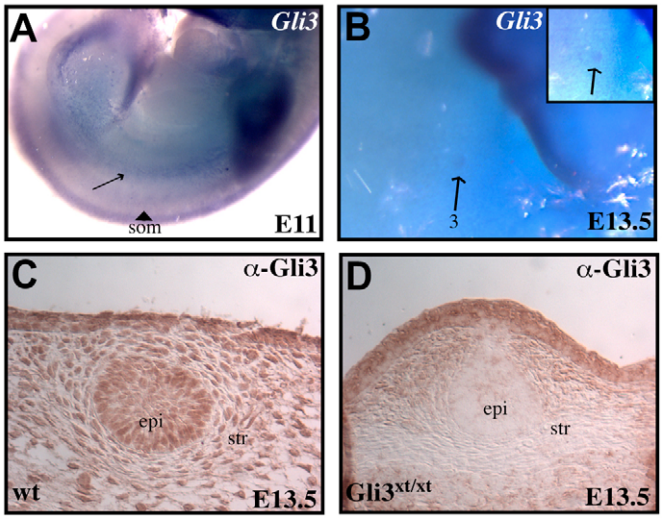

Fig. 2. Detection of *Gli3* mRNA in embryos by whole-mount in situ hybridization. (**A**) At E11 a broad band of staining is seen between the fore- and hindlimbs in the region of the developing mammary line (arrow) and in the somites (som, arrowhead). (**B**) At E13.5 weak staining is seen in and around all five pairs of mammary buds but expression is strongest in bud pair number 3, inset shows bud number 3 at higher magnification. (**C**) Immunohistochemistry for Gli3 showed nuclear staining in the mammary bud epithelium (epi) and surrounding stroma (str) in wild type. (**D**) Staining was not seen in the mammary bud epithelium from a *Gli3*$^{xt/xt}$ control embryo, although some background staining was present in the stroma.

region of the developing mammary line, E11 *Gli2*$^{lzki/+}$ embryo whole mounts and sections showed mesenchymal *lacZ* expression in a diffuse arc between the fore and hind limb and in the underlying somites (Fig. 1A). By E14, strong expression was observed within all five pairs of mammary buds as well as in hair follicles and whisker pads (Fig. 1B,C). Histological sections of E14 embryos revealed intense *Gli2-lacZ* expression within the epidermis, the mammary bud epithelium and the surrounding condensed mammary mesenchyme (Fig. 1D). By E16.5, epithelial *Gli2-lacZ* expression became restricted to the basal layer of the epidermis and mammary sprout (Fig. 1E). At this stage, p63 and keratin 14 (K14) expression within the mammary sprout epithelium is uniform (Fig. 1F,G), but becomes restricted, within the adult mammary gland, to basal myoepithelial cell types.

Gli3 is expressed in embryonic mammary bud epithelium and mesenchyme

Gli3 is a strong transcriptional repressor of Hh target genes during lung and limb development but is able to weakly activate Hh target genes during ventral spinal cord patterning (Bai et al., 2004). A reporter allele of *Gli3* has not been described. Therefore, we examined expression of *Gli3* mRNA by in situ hybridization. *Gli3* mRNA was detected in E11 embryo whole mounts in a diffuse arc between the fore and hind limb and the underlying somites, a pattern similar to that observed for *Gli2-lacZ* (Fig. 2A). This pattern is consistent with recent reports of somitic and weaker dermal mesenchymal *Gli3* mRNA expression (Veltmaat et al., 2006). E13.5 embryo wholemounts showed weak *Gli3* expression in the vicinity of all mammary buds with stronger expression in pair number 3 (Fig. 2B). Gli3 protein was localized by immunohistochemistry within the nuclei of mammary bud epithelial cells and surrounding stromal cells (Fig. 2C,D), in a similar expression pattern to that of *Gli2-lacZ*.

Lack of Hh target gene expression distinguishes mammary buds from other embryonic epidermal appendages

Gli1 is a direct transcriptional target of positive Hh signaling, and its expression is strictly dependent on Hh signals transduced by either Gli2 or Gli3 activators. Thus, reporters of *Gli1* promoter activity provide reliable indicators of positive Hh pathway activation (Bai et al., 2002; Bai et al., 2004). *Gli1-lacZ* expression in *Gli1*$^{lzki/+}$ mice was absent throughout embryonic mammary development yet was prominent in embryonic hair follicles and whisker pads (Fig. 3A,B). The absence of *Gli1-lacZ* reporter expression in mammary buds and robust activity in hair follicles strongly suggests that Hh pathway activation and Gli-dependent gene expression diverges among distinct types of epidermal appendages. To test this hypothesis further, we examined the activity of a second Hh target gene, *Ptc1* (Goodrich et al., 1997). Hh signals upregulate *Ptc1* expression as part of a negative feedback mechanism. Although Ptc1 provides a receptor for Hh proteins, at high levels it suppresses Hh activity by sequestering Smo (Casali and Struhl, 2004). *Ptc1-lacZ* expression

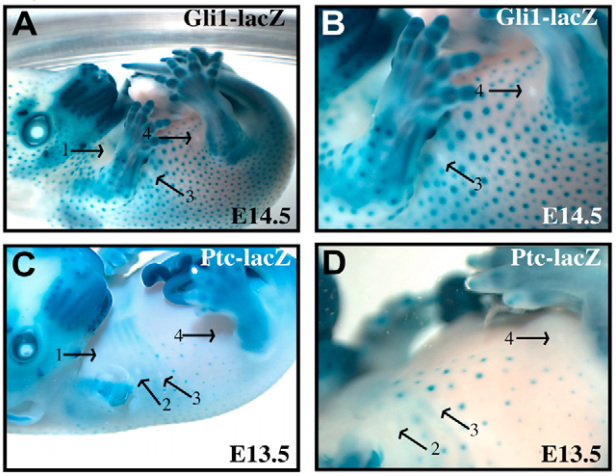

Fig. 3. Hh transcriptional targets are not expressed in embryonic mammary buds. Whole-mount X-gal staining of *Gli1-lacZ* in E14.5 (**A**,**B**) and *Ptch-lacZ* in E13.5 (**C**,**D**) embryos. Hair follicles show staining in these embryos but mammary buds (1-4, arrows) are unstained. B,D are higher magnification images of A,C.

was observed in hair follicles but was absent throughout embryonic mammary development (Fig. 3C,D). These results confirm that the Hh pathway is active in epidermal appendages, such as hair follicles, but is either inactive or repressed throughout embryonic mammary development.

Postnatal mammary *Gli2-lacZ* expression is observed continuously in stromal cells and cyclically in myoepithelial cells

At birth *Gli2-lacZ* expression was found in both basal epithelial and stromal layers of the mammary gland (Fig. 4A,B). At the onset of puberty, it was lost from the epithelial cells but was expressed in spikes along the entire ductal system (Fig. 4C,E), and was concentrated around the neck of the terminal end buds, giving a thistle-like appearance to these structures (Fig. 4E, arrow). *Gli2-lacZ* was also prominently expressed in mammary lymphatics (Fig. 4E, asterisk). Histological sections showed that *Gli2-lacZ* periductal spikes comprise groups of tightly adherent stromal cells that triangulate between adipocytes and the myoepithelium (Fig. 4D,F). Immunohistochemical analysis confirmed that in virgin (Fig. 4G,H) and early pregnant (P8) mice all *Gli2-lacZ*-positive cells lay beneath the K14/p63-positive myoepithelial layer and thus were stromal. P14 glands, however, showed additional myoepithelial expression surrounding alveoli as revealed by *Gli2-lacZ* colocalization with K14 and p63 (Fig. 5A-D). This *Gli2-lacZ*-positive myoepithelial population persisted during lactation but was lost during involution (data not shown).

Postnatal Gli3 is expressed in mammary ducts and alveoli and surrounding stroma

Northern analysis detected *Gli3* mRNA expression in mammary glands from virgin and pregnant mice (Fig. 6A). In situ hybridization showed diffuse *Gli3* mRNA expression throughout the mammary gland, with stronger expression in mammary ducts and alveolar clusters (Fig. 6B,D). Immunohistochemistry confirmed prominent nuclear expression of Gli3 within lumenal and myoepithelial cell types (Fig. 6F,G) and also detected Gli3 expression in stromal cells and adipocytes (Fig. 6F,G, arrows). Thus, Gli2 and Gli3 expression overlap in stromal and

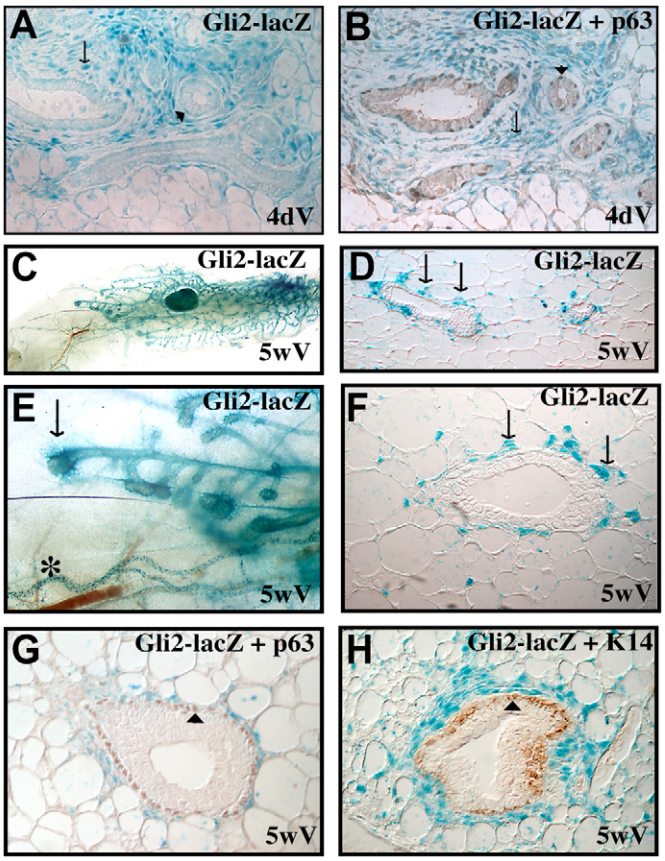

Fig. 4. Postnatal mammary expression of *Gli2-lacZ* in 4 day and 5-week-old virgins. Strong X-gal staining is seen in the stromal cells (arrows) surrounding the mammary ducts in glands of 4-day-old virgin mice (4dV) (**A**,**B**). Weaker expression is seen in the myoepithelial cells identified by p63 immunohistochemistry (arrowheads, B). In 5-week-old virgins (5wV), *Gli2-lacZ* is found in the stromal cells encasing the ducts and is especially strong in the condensing stroma around the terminal end bud (**C**,**E**, arrow). Expression is also found in lymphatic ducts at all stages of mammary development (E, asterisk). Cross-section through ducts (**D**) and terminal end buds (**F**) of 5-week-old mice show spikes of stromal cells (arrows) between the adipocytes and myoepithelial cells (D,F). Immunohistochemistry for p63 (**G**) and K14 (**H**) demonstrates that, at this developmental stage, all *Gli2-lacZ* staining lies beneath the myoepithelial layer (arrowheads) and is, thus, stromal.

myoepithelial compartments, but only Gli3 is expressed in lumenal epithelial cells, suggesting that regulation of the Hh pathway differs in these distinct cell types.

Postnatal mammary expression of Hh target genes, *Gli1-lacZ* and *Ptc1-lacZ*, is restricted to lymphatics

In contrast to the pattern of *Gli2-lacZ* and *Gli3* expression, mammary glands from $Gli1^{tzki/+}$ mice showed no *Gli1-lacZ* reporter expression within epithelial, stromal, myoepithelial or adipocyte cell-types at any stage of development. Yet, in all cases a subset of mammary vessels stained intensely (Fig. 7A,B). *Ptc1-lacZ* was also expressed exclusively within the same subset of mammary vessels (data not shown). Many of these vessels had a large diameter and extended from the periphery of the fatpad towards the lymph node. Inspection at higher magnification showed that *Gli1-lacZ* and *Ptc1-lacZ* were expressed only in vessels lacking red blood cells (Fig. 7B). Similar

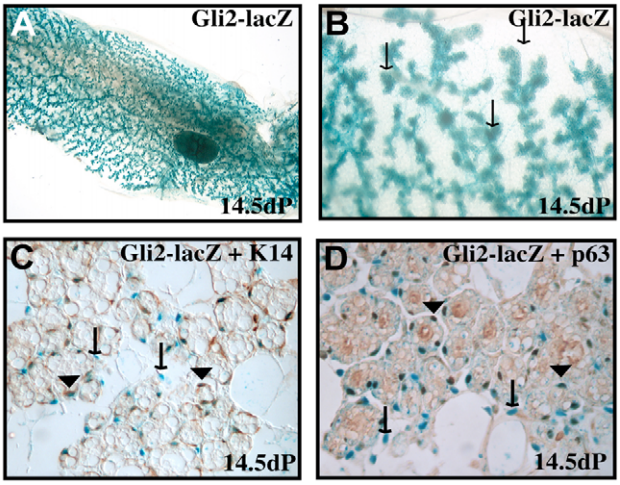

Fig. 5. Mammary *Gli2-lacZ* expression during pregnancy. At 14.5 days mid-pregnancy (14.5dP) *Gli2-lacZ* expression is especially strong around alveoli (arrows) (**A**,**B**). Cross-sections of glands show that at this stage of pregnancy *Gli2-lacZ* remains in stromal cells (arrows) and is also found in myoepithelial cells (arrowheads) that stain positive for K14 (**C**) and p63 (**D**).

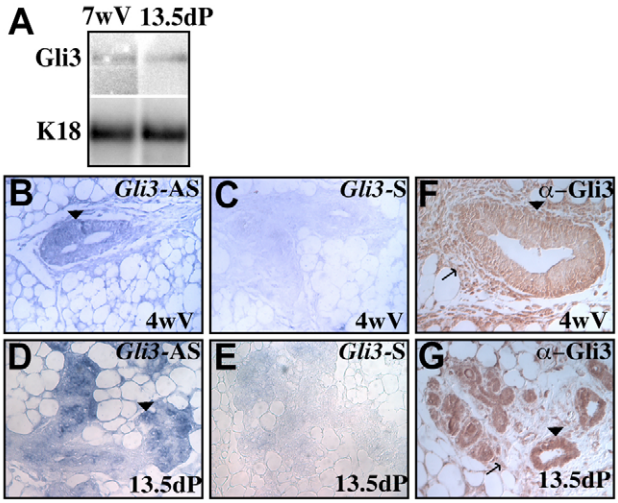

Fig. 6. *Gli3* expression in postnatal mammary gland. (**A**) *Gli3* mRNA was detected by northern blot analysis of mRNA isolated from mammary glands of both 7-week-old virgin (7wV) and mid-pregnant (13.5dP) mice. Keratin 18 (K18) was used as a control for mRNA integrity and loading. (**B**) *Gli3* mRNA was detected by in situ hybridization in mammary epithelium (arrowhead) of 4-week-old virgin (4wV) mice. (**D**) Expression continues during pregnancy within both the luminal and myoepithelial cells (arrowhead). Sense control hybridization showed no signal in glands from 4-week-old virgins (**C**) or 13-day pregnant mice (**E**). Immunohistochemistry for Gli3 showed nuclear staining in both luminal and myoepithelial cells (arrowheads) of 4-week-old virgins (**F**) and 13-day pregnant mice (**G**). Staining was also seen in stromal cells at both stages (arrows).

vessels were observed in many other tissues, including the surface of the heart and omentum (Fig. 7C,D). *Gli1-lacZ* and *Ptc1-lacZ*-positive vessels stained with anti-LYVE, a specific marker of lymphatics (data not shown). The absence of Hh target gene expression within the adult mammary tree again shows that positive Hh signaling is absent or repressed, challenging previous reports that suggest positive Hh signaling is active in postnatal mammary gland development (Lewis et al., 2001; Lewis et al., 1999b).

Gli3$^{xt/xt}$ mice show aberrant placode development and lack two pairs of mammary buds

After establishing the expression patterns of the three Gli genes, we tested the consequences of removing their function on embryonic mammary development. In keeping with its lack of expression within the mammary tree, loss of *Gli1* (*Gli1*$^{lzki/lzki}$) had no effect on mammary development: all ten mammary glands were present in newborn *Gli1*$^{lzki/lzki}$ mice and showed normal function, exemplified by the ability of adults to raise normal size litters. Mice lacking Gli2 (*Gli2*$^{lzki/lzki}$) die perinatally but form all ten buds at E13, and no obvious mammary phenotype is observed in histological sections (Table 1). The spontaneously occurring *extra-toes*^{J} mouse mutant (*Gli3*$^{xt/xt}$) carries an intragenic deletion in *Gli3* resulting in loss of Gli3 expression (Hui and Joyner, 1993). *Gli3*$^{xt/+}$ mice showed normal embryonic development of all ten mammary glands and, as adults, could successfully feed their litters (Table 1). By contrast, all *Gli3*$^{xt/xt}$ embryo whole-mounts lacked bud pair number 5 and the majority lacked bud number 3. A small percentage showed reduction and/or misplacement of bud number 3 (Fig. 8B,D; Table 1). To analyze which stage of mammary bud formation is affected by loss of Gli3 function, we examined the embryos of *Gli3*$^{xt/+}$ mice crossed to *Gli3*$^{xt/+}$;TOP-Gal mice harboring the TOP-Gal transgenic reporter of Lef/Tcf transcriptional activity (DasGupta and Fuchs, 1999). TOP-Gal is expressed during the earliest stages of mammary line formation and later in the mammary placodes and buds (Chu et al., 2004). At E11 TOP-Gal expression was seen in the developing placode pairs number 3 and number 4 in all wild-type and *Gli3*$^{xt/+}$ embryos (Fig. 8A). By contrast, 80% of *Gli3*$^{xt/xt}$ embryos lacked TOP-Gal expression from the placode 3 region of the mammary line (Fig. 8B; Table 1). Fourteen percent of embryos showed normal marker expression on one side and absence of marker expression on the contralateral side. Similar results were observed by in situ

Table 1. Embryonic mammary phenotype of Gli2 and Gli3 mutant mice

Genotype	Number buds analyzed	Number of buds with phenotype
Wild type	90	All normal
Gli2$^{lzki/lzki}$	14	All normal
Gli3$^{xt/+}$	192	All normal
Gli3$^{xt/xt}$	80	Fifty-eight missing bud 3, 13 hypoplastic/misplaced bud 3, 80 missing bud 5
Gli2$^{lzki/lzki}$; *Gli3*$^{xt/+}$	6	All normal
Gli2$^{lzki/+}$; *Gli3*$^{xt/xt}$	8	Seven missing bud 3, one hypoplastic bud 3, eight missing bud 5
Gli2$^{1nki/1nki}$	18	All normal
Gli2$^{1nki/+}$; *Gli3*$^{xt/+}$	16	All normal
Gli2$^{1nki/1nki}$; *Gli3*$^{xt/+}$	14	Thirteen missing bud 3, one hypoplastic bud 3, 12 missing bud 5, two hypoplastic bud 5

hybridization for *Wnt10b*, another early marker of the mammary line and placodes (Veltmaat et al., 2004) (data not shown). These data demonstrate that early patterning of the mammary line and placode 3 formation is compromised in *Gli3*$^{xt/xt}$ embryos. By E12.5, TOP-Gal was expressed in all five pairs of mammary buds in wild-type and *Gli3*$^{xt/+}$ embryos (Table 1; Fig. 8C). TOP-Gal staining in *Gli3*$^{xt/xt}$ embryos revealed that bud pair number 5 was always missing and bud number 3 was absent 83% of the time or was small and/or misplaced in the remaining 17% (Fig. 8D; Table 1). Thus, Gli3 function is essential for the normal complement and positioning of mammary buds. A similar percentage of absence of bud pairs number 3 and 5 was observed in E14.5 *Gli3*$^{xt/xt}$ embryos. Histological sections of *Gli3*$^{xt/xt}$ embryos showed that the remaining bud pairs are normal at E14.5 (Fig. 2D).

Misactivation of Hh signaling results in selective mammary bud loss

In several developmental processes, Gli3 is a strong transcriptional repressor of Hh target genes in the absence of Hh but has recently been shown to activate Hh target genes weakly in ventral spinal cord (Bai et al., 2004). To investigate whether the repressor or activator function of Gli3 is essential for formation of mammary bud pairs number 3 and number 5, we examined a series of double mutant mice (Table 1). Gli2 requires positive hedgehog signaling for activation and, in the absence of signaling, Gli2 is present in an inactive or weakly repressive state. Gli1, however, lacks a repressor domain and is a strong amplifier of the pathway (Dai et al., 1999; Park et al., 2000). Thus, driving expression of the constitutive *Gli1* activator under the control of the *Gli2* promoter (*Gli2*$^{1nki/+}$ and *Gli2*$^{1nki/1nki}$) tests the effect of progressively increasing the activator to repressor ratio within the Gli2 field of expression (embryonic mammary bud and mesenchyme). In similar experiments, substitution of *Gli2* by *Gli1* has been shown to exacerbate the *Gli3*$^{xt/+}$ limb phenotype (Bai and Joyner, 2001). In the E13.5 mammary gland, substituting one or even two copies of *Gli2* with *Gli1* (*Gli2*$^{1nki/+}$ and *Gli2*$^{1nki/1nki}$) had no effect on mammary development (Fig. 8E-G; Table 1). Likewise, replacing a single copy of *Gli2* by *Gli1* and at the same time lowering *Gli3* expression (*Gli2*$^{1nki/+}$;*Gli3*$^{xt/+}$) failed to produce a mammary phenotype. However, when both *Gli2* copies were replaced by *Gli1,* and *Gli3* was simultaneously reduced (*Gli2*$^{1nki/1nki}$;*Gli3*$^{xt/+}$), mammary bud pairs number 3 and number 5 were lost in the majority of embryos (Fig. 8H-J; Table 1). Thus, in the mammary gland, two copies of the *Gli1* activator expressed from the *Gli2* promoter are sufficient to antagonize Gli3 repressor expressed from a single copy of *Gli3*. This establishes that the function of Gli3, within the embryonic mammary gland, is to repress Hh target genes. Our results further demonstrate that the Gli activator/repressor ratio of hedgehog signaling is crucial for correct mammary gland patterning and that buds 3 and 5 are particularly susceptible to changes in this ratio.

Analysis of Gli2 function in double mutants

The lack of phenotype in *Gli2*$^{lzki/lzki}$ mice suggests that Gli2 is either inactive, functions redundantly with other mammary Gli proteins (Gli3) or is completely antagonized by Gli3. We used double

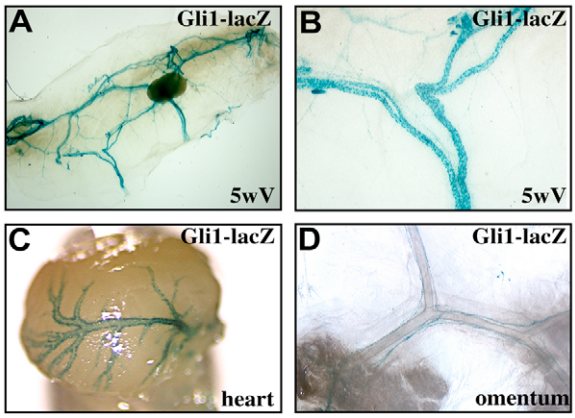

Fig. 7. Lymphatic expression of *Gli1-lacZ* in adult tissue. (**A**,**B**) Expression in mammary glands from 5-week-old virgins (5wV) is restricted to lymphatic vessels. Similar X-gal staining is seen in vessels in various organs including surrounding the heart (**C**) and the omentum (**D**).

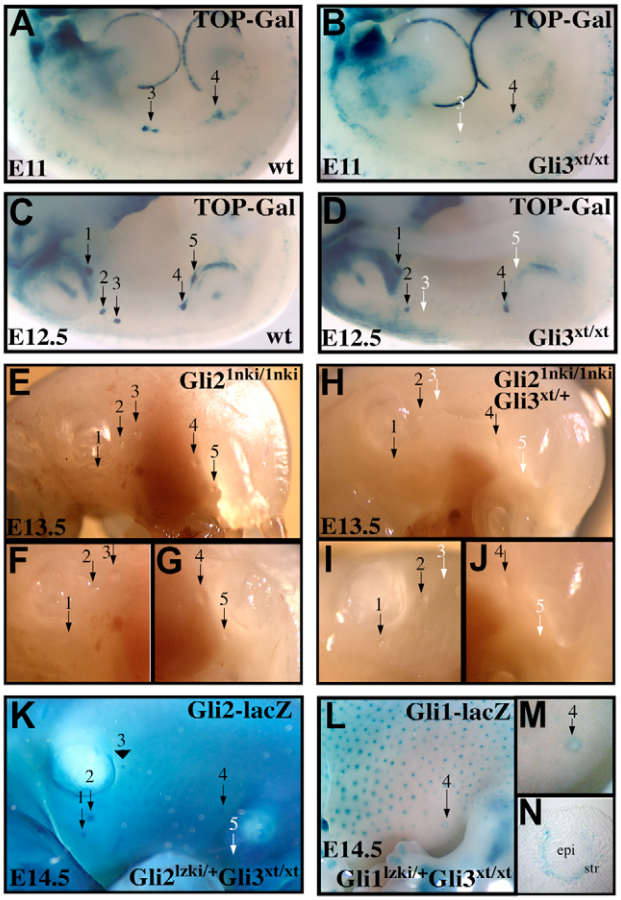

Fig. 8. Mammary bud formation is impaired in *Gli3*$^{xt/xt}$ mice and after mis-activation of the hedgehog pathway. TOP-Gal expression is seen in the developing mammary placodes number 3 and number 4 in wild-type E11 embryos (arrows) (**A**). TOP-Gal expression is absent from the region of bud 3 in E11 *Gli3*$^{xt/xt}$ embryos (white arrow) but is present in the developing bud 4 (black arrow) (**B**). In E12.5 wild-type embryos, TOP-Gal expression reveals the development of five pairs of mammary buds (**C**, arrows), whereas *Gli3*$^{xt/xt}$ embryos lack bud pairs 3 and 5 (**D**, predicted position of missing buds is marked by white arrows). E13.5 *Gli2*$^{1nki/1nki}$ embryos (**E**) show normal development of five pairs of mammary bud, but *Gli2*$^{1nki/1nki}$; *Gli3*$^{xt/+}$ embryos (**H**) show loss of buds 3 and 5 (white arrows). Higher magnification of the thoracic buds (**F**,**I**) and inguinal buds (**G**,**J**). A hypoplastic bud 3 (arrowhead) is seen in some *Gli2*$^{lzki/+}$; *Gli3*$^{xt/xt}$ embryos (**K**). Misactivation of *Gli1-lacZ* is seen in the stroma (str) surrounding buds 1 and 4 (arrow) in *Gli3*$^{xt/xt}$ embryo wholemounts (**L**,**M**) and sections (**N**).

mutants to examine these possibilities (Table 1). If Gli2 and Gli3 function redundantly, then Gli2 loss could exacerbate the Gli3 phenotype by causing loss of mammary buds in a $Gli3^{xt/+}$ background. No mammary phenotype was observed in $Gli2^{lzki/lzki};Gli3^{xt/+}$ embryos (Table 1). If Gli2 and Gli3 antagonize one another, then Gli2 reduction or loss could rescue the loss of mammary buds seen in $Gli3^{xt/xt}$ phenotype. Although a hypoplastic bud number 3 was seen in one out of eight $Gli2^{lzki/+};Gli3^{xt/xt}$ embryos, the frequency of this phenotype did not exceed that observed in $Gli3^{xt/xt}$ embryos. (Fig. 8K; Table 1). We were unable to test whether total removal of *Gli2* would be restorative because $Gli2^{lzki/lzki};Gli3^{xt/xt}$ mice die ~E10.5 prior to mammary anlagen formation. However, to test further whether Gli3 directly represses Gli2-mediated activation of Hh target genes we crossed $Gli1^{lzki/+}$ reporter mice to $Gli3^{xt/+}$ mice and examined the effect of loss of Gli3 expression on the *Gli1-lacZ* reporter. At E14.5 *Gli1-lacZ* was detected in the stroma surrounding bud pairs 1 and 4 of $Gli3^{xt/xt}$ embryos, n=4 (Fig. 8L-N) but not wild-type embryos (Fig. 3A,B). As Gli1 expression is dependent on Gli2-mediated transcriptional activation, we conclude that Gli2 is present in an activator form but is fully antagonized by Gli3R in normal embryos. These experiments further demonstrate that, in addition to affecting early patterning events, which govern placode formation, Gli3R continues to repress Hh target genes within the stroma surrounding mammary buds after their formation.

DISCUSSION

Our key findings are that Gli3-mediated repression is essential for the normal complement of mammary buds and that contrary to previous suggestion Hh target gene expression is absent throughout embryonic and postnatal mammary development. Repression of Hh signaling distinguishes mammary glands from other epidermal appendages, which require Hh pathway activation. These conclusions are based on the following results: *lacZ* reporters of positive Hh activity remain silent throughout mammary development; mice lacking expression of Hh activators, Gli1 or Gli2, show normal mammary development; $Gli3^{xt/xt}$ mutants lack two pairs of mammary placodes and buds; and misactivation of the pathway by targeted expression of *Gli1* to the Gli2 locus in $Gli3^{xt/+}$ mice phenocopies complete loss of Gli3 function.

Positive Hh signaling is absent during embryonic mammary development

The Hh pathway is crucial for the patterning and growth of many epidermal appendages (Chuong et al., 2000). An absolute requirement for its activity has been documented in the development of hair, teeth and feathers (Hardcastle et al., 1998; St-Jacques et al., 1998; Ting-Berreth and Chuong, 1996). The fact that epidermal appendages have a common origin and share many developmental pathways has prompted several recent investigations into the role of Hh signaling in mammary development. These studies detected *Shh* and *Ihh* mRNA within mammary bud epithelium, but, nevertheless, showed that $Shh^{-/-}$ and $Ihh^{-/-}$ embryos develop ten mammary buds, which undergo normal postnatal development if transplanted into adult mice (Gallego et al., 2002; Michno et al., 2003). These data suggest that Hh signaling is either redundant or dispensable for mammary development. The presence of *Ptc1* mRNA within $Shh^{-/-}$ mammary glands was interpreted as evidence for Hh redundancy (Michno et al., 2003). However, in comparison with the hair follicle, where the Hh pathway is unquestionably active, mammary *Ptc1* mRNA levels are barely detectable. Such basal levels are consistent with absence of Hh signaling resulting in repression of *Ptc1* transcription. Consistent with the viewpoint that Shh and Ihh are dispensable for mammary bud development, our results show that two well-characterized and sensitive reporters of positive Hh signaling, *Gli1-lacZ* and *Ptc1-lacZ*, are absent during embryonic mammary development. Further challenging the concept that positive Hh signaling is required for mammary development, our analyses show that *Gli1* and *Gli2* mutants have no obvious defects in mammary bud formation.

Gli3 acts as a repressor during embryonic mammary development

In stark contrast to the lack of mammary phenotypes in $Gli1^{lzki/lzki}$ and $Gli2^{lzki/lzki}$ embryos, the majority of $Gli3^{xt/xt}$ mutants lack mammary placodes number 3 and 5, and the remainder show hypoplastic, asymmetric or lateral displacement of placode number 3. Marker analysis indicates that *Gli3* is essential for the earliest stages of embryonic mammary development, affecting the positioning and formation of mammary placodes. Gli3 is present in the absence of Hh signaling and thus can have Hh-independent functions. However, Hh signals downregulate *Gli3* transcription and inhibit Gli3 proteolysis into $Gli3^{R}$, a process referred to as negative Hh signaling. Although Gli3 often acts as a potent repressor of the Hh pathway, several studies have shown that in certain contexts it functions as a weak activator ($Gli3^{A}$). For example, Gli3 is essential for the correct patterning of the ventral spinal cord where its activation of *Gli1* is involved in the development of motoneurons (Bai et al., 2004). Additional examples of $Gli3^{A}$ function are found in the development of the glandular epithelium of the embryonic stomach and skeletal muscle (Kim et al., 2005; McDermott et al., 2005). To determine whether Gli3 functions as an activator or repressor of Hh regulated genes within the embryonic mammary gland, we conducted double *Gli* mutant experiments. Targeted replacement of *Gli2* by *Gli1* within $Gli3^{xt/+}$ mice resulted in loss of mammary bud pairs number 3 and number 5. The ability of the constitutive Gli1 activator to antagonize Gli3 reveals that Gli3 functions as a repressor in this developmental context. Whether $Gli3^{R}$ functions independently of Hh signals or is modulated by them during embryonic mammary development remains to be determined. However, the induction of the Hh target gene *Gli1* in the stroma of the remaining $Gli3^{xt/xt}$ E14.5 mammary buds suggests that, in normal embryos, Gli3 is actively repressing Hh signaling at this stage.

Mammary placodes arise in a distinct order: number 3, number 4, number 1 + number 5, number 2. Pairs 3 and 4 form at the anterior and posterior ends of the mammary line. Pairs 1 and 5 form from independent streaks of cells that encircle the fore- and hindlimbs (Veltmaat et al., 2004). Pair 2 develops last from streaks of Wnt10b-positive cells extending from placodes 1 and 3. Wnt/catenin signaling is the earliest known marker of embryonic mammary development, and mice misexpressing the Wnt inhibitor Dkk within the epidermis fail to form any mammary placodes (Andl et al., 2002; Chu et al., 2004). Lack of expression of the Wnt signaling reporter TOP-Gal in the central region of the mammary line in E11 $Gli3^{xt/xt}$ embryos demonstrates that Gli3 repression is required prior to these early patterning events that precede mammary placode formation. It has been proposed that cells migrate along the mammary line and perilimbal streaks and coalesce to form placodes (Veltmaat et al., 2004). Intriguingly, genetic interaction has been reported between Gli3 and Twist, a regulator of epithelial-mesenchymal transitions that are critical for cell migratory processes (O'Rourke et al., 2002). The loss of only two pairs of mammary buds upon misactivation of the Hh pathway in $Gli3^{xt/xt}$ mice reinforces previous data showing that specific combinations of molecular cues govern the formation

of different pairs of placodes (Veltmaat et al., 2003). Analysis of inbred mouse strains and, more recently, of scaramanga (Ska) mice implicates variable susceptibility of specific bud pairs to both loss and supernumerary formation (Howard et al., 2005; Little and McDonald, 1945; Veltmaat et al., 2003). In these studies, correct regulation of morphogenic interactions involved in mammary line and placode formation appears to be most crucial for the formation of bud pair 3 and least crucial for that of bud pair 4. Further examples are found in studies on loss of Tbx3, which in humans produces mammary hypoplasia and nipple loss, as well as in studies on Lef1 and Fgf pathways. Mice lacking *Tbx3* show loss of mammary buds but occasional retention of bud number 2 (Bamshad et al., 1997; Davenport et al., 2003; Eblaghie et al., 2004). *Lef1*$^{-/-}$ mice form small mammary placodes that degenerate and occasionally retain bud 4 (van Genderen et al., 1994). Mice that lack Fgf10, which is expressed within the ventrolateral portion of somites, or its receptor Fgfr2b, which is expressed in the mammary placodes, fail to develop mammary buds 1, 2, 3 and 5, yet retain bud number 4 (Mailleux et al., 2002). Recently, a genetic requirement for Gli3 in the ventral somitic expression of Fgf10 has been described (Veltmaat et al., 2006). Our demonstration that Gli3 acts as a transcriptional repressor now shows that this Gli3-mediated induction of Fgf10 expression must be indirect.

The studies described above suggest that different placodes vary in their susceptibility to a crucial developmental threshold during the earliest stages of mammary development (Veltmaat et al., 2003). Our experiments reveal that the Gli^A/Gli^R ratio provides such a crucial developmental threshold for buds number 3 and number 5. Yet there are no reports of mammary bud loss in mouse models or human syndromes (basal cell nevus syndrome) where the Hh pathway is aberrantly activated (Johnson et al., 1996; Nilsson et al., 2000; Sheng et al., 2002). However, this is not surprising because, in mice, mammary bud loss occurs only under conditions that result in embryonic lethality (complete loss of *Gli3* or partial loss of *Gli3* in conjunction with pathway misactivation) and are likely to have the same outcome in humans. Nevertheless, there are occasional reports of rare human syndromes with features suggestive of pathway misactivation, such as polydactyly associated with hypoplastic nipples (Teebi and Druker, 2001).

Epidermal appendages show distinct requirements and proliferative responses to Hh signaling: repression of hedgehog signaling is essential for mammary development

The results of this study show that, despite their common origin, mammary glands differ from other epidermal appendages in their requirement for Hh pathway repression rather than activation. Hh-positive activity is essential for tooth and hair follicle development (Cobourne et al., 2001; Dassule et al., 2000; Gritli-Linde et al., 2002; Hardcastle et al., 1998; Mill et al., 2003; St-Jacques et al., 1998). For example, hair follicles express high levels of *Shh*, show pathway activation, as evidenced by *Gli1-lacZ* expression, and proliferate in response. In the absence of positive Hh signaling, hair placodes form but fail to undergo downward growth and arrest at the hair plug stage (Chiang et al., 1999; Mill et al., 2003; St-Jacques et al., 1998). By contrast, mammary buds express low levels of *Shh* and *Ihh*, fail to activate the pathway, as evidenced by lack of *Gli1-lacZ* expression, yet proliferate when signals are repressed. Repression of the Hh pathway is essential for the correct morphogenesis of several other organs. For example, in the limb, loss of Gli3 function in *Gli3*$^{xt/xt}$ mutants results in polydactyly, resembling a Shh gain-of-function phenotype (Hui and Joyner, 1993; Litingtung et al., 2002).

Hh target gene expression is absent during development of the adult mammary tree but is robust within lymphatics

In addition to defining the roles of Hh signaling in embryonic mammary development, our results provide new insight into the role of the Hh pathway during adolescent and adult mammary gland development. *Gli2-lacZ* expression within the stroma encasing virgin mammary ducts is entirely consistent with a previous report of *Gli2* mRNA expression (Lewis et al., 2001). However, the greater sensitivity and clarity afforded by the *lacZ* reporter, combined with immunohistochemical marker analysis, allows us to define that *Gli2* expression occurs cyclically in mid-pregnant mice within myoepithelial cells and not within all epithelial cells as reported previously (Lewis et al., 2001). Furthermore, we show that although Gli3 is co-expressed with Gli2 in stroma and myoepthelia, it is the only Gli found within the lumenal epithelium of the adult gland. Lack of expression of *Gli1-lacZ* and *Ptc1-lacZ* within mammary epithelia, myoepithelial, stroma or adipocytes leads us to conclude that Hh signaling is absent or stifled within the parenchyma of the adult gland yet is robust within lymphatics. This finding is at odds with a previous report of *Ptc1* mRNA expression in the epithelial layers of virgin ducts and a haploinsufficient phenotype in *Ptc1*$^{+/-}$ mice involving minor changes in terminal end bud clefting and transient ductal hyperplasia that rectifies during pregnancy (Lewis et al., 1999b). Of note, our examination of *Ptc1-lacZ* expression employs the same mouse, albeit on an outbred background. We have looked extensively and have seen no evidence of these phenotypic aberrations. Moreover, our observations are entirely consistent with other studies reporting lack of Gli1 expression in normal human breast tissue (Kubo et al., 2004). Intriguingly, these studies reported Gli1 protein upregulation in 52/52 epithelial breast cancers and 4/6 epithelial breast tumor cell lines probably resulting from epigenetic regulatory events, as mutations in Hh pathway components are infrequent in breast tumors (Chang-Claude et al., 2003; Kubo et al., 2004; Vorechovsky et al., 1999; Wicking et al., 1998; Xie et al., 1997). Our results show that Gli3 is the only Gli expressed in normal lumenal epithelial cells. Whether *Gli1* misexpression in breast tumors results from loss of $Gli3^R$ activity is an important issue for future investigation.

We thank Alexandra Joyner and Brian Bai for their generous gift of the mice. We thank Brigitte Teissedre and Minoti Hiremath for critical reading of the manuscript. Research described in this article was supported by the Susan G. Komen Foundation (P.C.), by a pilot and feasibility grant from the NYU Cancer Center (P.C.), and by Philip Morris USA and by Philip Morris International.

References

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.

Aza-Blanc, P. and Kornberg, T. B. (1999). Ci: a complex transducer of the hedgehog signal. *Trends Genet.* **15**, 458-462.

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.

Aza-Blanc, P., Lin, H. Y., Ruiz i Altaba, A. and Kornberg, T. B. (2000). Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities. *Development* **127**, 4293-4301.

Bai, C. B. and Joyner, A. L. (2001). Gli1 can rescue the in vivo function of Gli2. *Development* **128**, 5161-5172.

Bai, C. B., Auerbach, W., Lee, J. S., Stephen, D. and Joyner, A. L. (2002). Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. *Development* **129**, 4753-4761.

Bai, C. B., Stephen, D. and Joyner, A. L. (2004). All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. *Dev. Cell* **6**, 103-115.

Bamshad, M., Lin, R. C., Law, D. J., Watkins, W. C., Krakowiak, P. A., Moore, M. E., Franceschini, P., Lala, R., Holmes, L. B., Gebuhr, T. C. et al. (1997).

Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. *Nat. Genet.* **16**, 311-315.
Brisken, C., Park, S., Vass, T., Lydon, J. P., O'Malley, B. W. and Weinberg, R. A. (1998). A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc. Natl. Acad. Sci. USA* **95**, 5076-5081.
Brisken, C., Heineman, A., Chavarra, T., Elenbaas, B., Tan, J., Dey, S. K., McMahon, A. P. and Weinberg, R. (2000). Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev.* **14**, 650-654.
Casali, A. and Struhl, G. (2004). Reading the Hedgehog morphogen gradient by measuring the ratio of bound to unbound Patched protein. *Nature* **431**, 76-80.
Chang-Claude, J., Dunning, A., Schnitzbauer, U., Galmbacher, P., Tee, L., Wjst, M., Chalmers, J., Zemzoum, I., Harbeck, N., Pharoah, P. D. et al. (2003). The patched polymorphism Pro1315Leu (C3944T) may modulate the association between use of oral contraceptives and breast cancer risk. *Int. J. Cancer* **103**, 779-783.
Chiang, C., Swan, R. Z., Grachtchouk, M., Bolinger, M., Litingtung, Y., Robertson, E. K., Cooper, M. K., Gaffield, W., Westphal, H., Beachy, P. A. et al. (1999). Essential role for Sonic hedgehog during hair follicle morphogenesis. *Dev. Biol.* **205**, 1-9.
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.
Chuong, C. M., Patel, N., Lin, J., Jung, H. S. and Widelitz, R. B. (2000). Sonic hedgehog signaling pathway in vertebrate epithelial appendage morphogenesis: perspectives in development and evolution. *Cell Mol. Life Sci.* **57**, 1672-1681.
Cobourne, M. T. and Sharpe, P. T. (2005). Sonic hedgehog signaling and the developing tooth. *Curr. Top. Dev. Biol.* **65**, 255-287.
Cobourne, M. T., Hardcastle, Z. and Sharpe, P. T. (2001). Sonic hedgehog regulates epithelial proliferation and cell survival in the developing tooth germ. *J. Dent. Res.* **80**, 1974-1979.
Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M. and Ishii, S. (1999). Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J. Biol. Chem.* **274**, 8143-8152.
DasGupta, R. and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**, 4557-4568.
Dassule, H. R., Lewis, P., Bei, M., Maas, R. and McMahon, A. P. (2000). Sonic hedgehog regulates growth and morphogenesis of the tooth. *Development* **127**, 4775-4785.
Davenport, T. G., Jerome-Majewska, L. A. and Papaioannou, V. E. (2003). Mammary gland, limb and yolk sac defects in mice lacking Tbx3, the gene mutated in human ulnar mammary syndrome. *Development* **130**, 2263-2273.
Dunbar, M. E., Dann, P. R., Robinson, G. W., Hennighausen, L., Zhang, J. P. and Wysolmerski, J. J. (1999). Parathyroid hormone-related protein signaling is necessary for sexual dimorphism during embryonic mammary development. *Development* **126**, 3485-3493.
Eblaghie, M. C., Song, S. J., Kim, J. Y., Akita, K., Tickle, C. and Jung, H. S. (2004). Interactions between FGF and Wnt signals and Tbx3 gene expression in mammary gland initiation in mouse embryos. *J. Anat.* **205**, 1-13.
Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.
Gallego, M. I., Beachy, P. A., Hennighausen, L. and Robinson, G. W. (2002). Differential requirements for shh in mammary tissue and hair follicle morphogenesis. *Dev. Biol.* **249**, 131-139.
Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.
Gritli-Linde, A., Bei, M., Maas, R., Zhang, X. M., Linde, A. and McMahon, A. P. (2002). Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. *Development* **129**, 5323-5337.
Hammerschmidt, M., Brook, A. and McMahon, A. P. (1997). The world according to hedgehog. *Trends Genet.* **13**, 14-21.
Hardcastle, Z., Mo, R., Hui, C. C. and Sharpe, P. T. (1998). The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants. *Development* **125**, 2803-2811.
Henninghausen, L. and Robinson, G. W. (1998). Think globally, act locally: the making of a mouse mammary gland. *Genes Dev.* **12**, 449-455.
Hens, J. R. and Wysolmerski, J. J. (2005). Key stages of mammary gland development: molecular mechanisms involved in the formation of the embryonic mammary gland. *Breast Cancer Res.* **7**, 220-224.
Howard, B., Panchal, H., McCarthy, A. and Ashworth, A. (2005). Identification of the scaramanga gene implicates Neuregulin3 in mammary gland specification. *Genes Dev.* **19**, 2078-2090.
Hui, C. C. and Joyner, A. L. (1993). A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nat. Genet.* **3**, 241-246.
Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162**, 402-413.
Hynes, M., Stone, D. M., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A. and Rosenthal, A. (1997). Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. *Neuron* **19**, 15-26.
Imbert, A., Eelkema, R., Jordan, S., Feiner, H. and Cowin, P. (2001). ΔN89ß-catenin induces precocious development, differentiation, and neoplasia in mammary gland. *J. Cell Biol.* **153**, 555-568.
Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epsitein, E. H., Jr et al. (1996). Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* **272**, 1668-1671.
Kim, J. H., Huang, Z. and Mo, R. (2005). Gli3 null mice display glandular overgrowth of the developing stomach. *Dev. Dyn.* **234**, 984-991.
Kubo, M., Nakamura, M., Tasaki, A., Yamanaka, N., Nakashima, H., Nomura, M., Kuroki, S. and Katano, M. (2004). Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. *Cancer Res.* **64**, 6071-6074.
Lee, J., Platt, K. A., Censullo, P. and Ruiz i Altaba, A. (1997). Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. *Development* **124**, 2537-2552.
Lewis, K. E., Concordet, J. P. and Ingham, P. W. (1999a). Characterisation of a second patched gene in the zebrafish Danio rerio and the differential response of patched genes to Hedgehog signalling. *Dev. Biol.* **208**, 14-29.
Lewis, M. T., Ross, S., Strickland, P. A., Sugnet, C. W., Jimenez, E., Scott, M. P. and Daniel, C. W. (1999b). Defects in mouse mammary gland development caused by conditional haploinsufficiency of Patched-1. *Development* **126**, 5181-5193.
Lewis, M. T., Ross, S., Strickland, P. A., Sugnet, C. W., Jimenez, E., Hui, C. and Daniel, C. W. (2001). The Gli2 transcription factor is required for normal mouse mammary gland development. *Dev. Biol.* **238**, 133-144.
Li, Y., Zhang, H., Choi, S. C., Litingtung, Y. and Chiang, C. (2004). Sonic hedgehog signaling regulates Gli3 processing, mesenchymal proliferation, and differentiation during mouse lung organogenesis. *Dev. Biol.* **270**, 214-231.
Litingtung, Y., Dahn, R. D., Li, Y., Fallon, J. F. and Chiang, C. (2002). Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* **418**, 979-983.
Little, C. and McDonald, H. (1945). Abnormalities of the mammae in the house mouse. *J. Hered.* **36**, 285-288.
Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K. and Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* **122**, 181-193.
Mailleux, A. A., Spencer-Dene, B., Dillon, C., Ndiaye, D., Savona-Baron, C., Itoh, N., Kato, S., Dickson, C., Thiery, J. P. and Bellusci, S. (2002). Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo. *Development* **129**, 53-60.
Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996). Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. *Dev. Biol.* **180**, 273-283.
Marshman, E. and Streuli, C. H. (2002). Insulin-like growth factors and insulin-like growth factor binding proteins in mammary gland function. *Breast Cancer Res.* **4**, 231-239.
McDermott, A., Gustafsson, M., Elsam, T., Hui, C. C., Emerson, C. P., Jr and Borycki, A. G. (2005). Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation. *Development* **132**, 345-357.
Michno, K., Boras-Granic, K., Mill, P., Hui, C. C. and Hamel, P. A. (2003). Shh expression is required for embryonic hair follicle but not mammary gland development. *Dev. Biol.* **264**, 153-165.
Mill, P., Mo, R., Fu, H., Grachtchouk, M., Kim, P. C., Dlugosz, A. A. and Hui, C. C. (2003). Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. *Genes Dev.* **17**, 282-294.
Motoyama, J., Takabatake, T., Takeshima, K. and Hui, C. (1998). Ptch2, a second mouse Patched gene is co-expressed with Sonic hedgehog. *Nat. Genet.* **18**, 104-106.
Nilsson, M., Unden, A. B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P. G. and Toftgard, R. (2000). Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. *Proc. Natl. Acad. Sci. USA* **97**, 3438-3443.
Oftedal, O. T. (2002). The mammary gland and its origin during synapsid evolution. *J. Mammary Gland Biol. Neoplasia* **7**, 225-252.
Ohlmeyer, J. T. and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* **396**, 749-753.
O'Rourke, M. P., Soo, K., Behringer, R. R., Hui, C. C. and Tam, P. P. (2002). Twist plays an essential role in FGF and SHH signal transduction during mouse limb development. *Dev. Biol.* **248**, 143-156.

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M. and Joyner, A. L. (2000). Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* **127**, 1593-1605.

Pathi, S., Pagan-Westphal, S., Baker, D. P., Garber, E. A., Rayhorn, P., Bumcrot, D., Tabin, C. J., Blake Pepinsky, R. and Williams, K. P. (2001). Comparative biological responses to human Sonic, Indian, and Desert hedgehog. *Mech. Dev.* **106**, 107-117.

Pearse, R. V., 2nd, Vogan, K. J. and Tabin, C. J. (2001). Ptc1 and Ptc2 transcripts provide distinct readouts of Hedgehog signaling activity during chick embryogenesis. *Dev. Biol.* **239**, 15-29.

Pispa, J. and Thesleff, I. (2003). Mechanisms of ectodermal organogenesis. *Dev. Biol.* **262**, 195-205.

Robinson, G. W., Henninghausen, L. and Johnson, P. E. (2000). Side-branching in the mammary gland: the progesterone-Wnt connection. *Genes Dev.* **14**, 889-894.

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M. and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* **126**, 3915-3924.

Sheng, H., Goich, S., Wang, A., Grachtchouk, M., Lowe, L., Mo, R., Lin, K., de Sauvage, F. J., Sasaki, H., Hui, C. C. et al. (2002). Dissecting the oncogenic potential of Gli2: deletion of an NH(2)-terminal fragment alters skin tumor phenotype. *Cancer Res.* **62**, 5308-5316.

St-Jacques, B., Dassule, H. R., Karavanova, I., Botchkarev, V. A., Li, J., Danielian, P. S., McMahon, J. A., Lewis, P. M., Paus, R. and McMahon, A. P. (1998). Sonic hedgehog signaling is essential for hair development. *Curr. Biol.* **8**, 1058-1068.

Teebi, A. S. and Druker, H. A. (2001). Brachycephaly, cutis aplasia congenita, blue sclerae, hypertelorism, polydactyly, hypoplastic nipples, failure to thrive, and developmental delay: a distinct autosomal recessive syndrome? *Clin. Dysmorphol.* **10**, 69-70.

Ting-Berreth, S. A. and Chuong, C. M. (1996). Sonic Hedgehog in feather morphogenesis: induction of mesenchymal condensation and association with cell death. *Dev. Dyn.* **207**, 157-170.

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**, 2691-2704.

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.

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.

Veltmaat, J. M., Relaix, F., Le, L. T., Kratochwil, K., Sala, F. G., van Veelen, W., Rice, R., Spencer-Dene, B., Mailleux, A. A., Rice, D. P. et al. (2006). Gli3-mediated somitic Fgf10 expression gradients are required for the induction and patterning of mammary epithelium along the embryonic axes. *Development* **133**, 2325-2335.

Vorechovsky, I., Benediktsson, K. P. and Toftgard, R. (1999). The patched/hedgehog/smoothened signalling pathway in human breast cancer: no evidence for H133Y SHH, PTCH and SMO mutations. *Eur. J. Cancer* **35**, 711-713.

Wang, B., Fallon, J. F. and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423-434.

Wicking, C., Evans, T., Henk, B., Hayward, N., Simms, L. A., Chenevix-Trench, G., Pietsch, T. and Wainwright, B. (1998). No evidence for the H133Y mutation in SONIC HEDGEHOG in a collection of common tumour types. *Oncogene* **16**, 1091-1093.

Wysolmerski, J. J. (2002). The evolutionary origins of maternal calcium and bone metabolism during lactation. *J. Mammary Gland Biol. Neoplasia* **7**, 267-276.

Wysolmerski, J. J., Philbrick, W. M., Dunbar, M. E., Lanske, B., Kronenberg, H. and Broadus, A. E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development* **125**, 1285-1294.

Xie, J., Johnson, R. L., Zhang, X., Bare, J. W., Waldman, F. M., Cogen, P. H., Menon, A. G., Warren, R. S., Chen, L. C., Scott, M. P. et al. (1997). Mutations of the PATCHED gene in several types of sporadic extracutaneous tumors. *Cancer Res.* **57**, 2369-2372.