

Gli Activity Is Critical at Multiple Stages of Embryonic Mammary and Nipple Development

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Abstract

Gli3 is a transcriptional regulator of Hedgehog (Hh) signaling that functions as a repressor (Gli3^R) or activator (Gli3^A) depending upon cellular context. Previously, we have shown that Gli3^R is required for the formation of mammary placodes #3 and #5. Here, we report that this early loss of Gli3 results in abnormal patterning of two critical regulators: *Bmp4* and *Tbx3*, within the presumptive mammary rudiment (MR) #3 zone. We also show that Gli3 loss leads to failure to maintain mammary mesenchyme specification and loss of epithelial Wnt signaling, which impairs the later development of remaining MRs: MR#2 showed profound evagination and ectopic hairs formed within the presumptive areola; MR#4 showed mild invagination defects and males showed inappropriate retention of mammary buds in Gli3^{xt/xt} mice. Importantly, mice genetically manipulated to misactivate Hh signaling displayed the same phenotypic spectrum demonstrating that the repressor function of Gli3^R is essential during multiple stages of mammary development. In contrast, positive Hh signaling occurs during nipple development in a mesenchymal cuff around the lactiferous duct and in muscle cells of the nipple sphincter. Collectively, these data show that repression of Hh signaling by Gli3^R is critical for early placodal patterning and later mammary mesenchyme specification whereas positive Hh signaling occurs during nipple development.

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Introduction

Mammary development becomes apparent in mice around E10.5 with expression of *Wnt10b* in mammary lines between the fore- and hind-limbs and in axillary and inguinal streaks [1,2,3]. Between E10.5 and E11.5, influx of epithelial cells towards and along these mammary lines and streaks leads to the formation of five pairs of placodes [4]. Analyses of knock-out mice and of human syndromes involving loss of mammary rudiments (MRs) or abnormal nipple number have identified more than a dozen factors essential for early mammary placodal development [3,5,6,7,8,9,10,11]. Among these factors, MR#3 formation depends upon reciprocal antagonism between ventrally expressed *Bmp4* and dorsal *Tbx3* [12]. At ~E12.5 the placodes form elevated buds. These buds sink below the periderm ~E13.5 to form bulb-like structures, which induce underlying fibroblasts to become mammary mesenchyme [13,14]. In females proliferation beginning at E15–E16, causes the mammary bulbs to sprout, penetrate the underlying developing fat-pad, and branch to form a small ductal tree (~E18) [11,13,14,15,16]. The mammary mesenchyme in turn signals to the overlying epidermis to suppress hair follicles and form the nipple sheath [11,13,14,15,16]. In male embryos, intrinsic androgen response within the mesenchyme leads to atrophy of the buds [3,9,17,18,19,20].

The Hedgehog (Hh) pathway plays a central role in the patterning and proliferation of many tissues, and its requirement

in epidermal appendages, such as hair follicles and teeth, has been particularly well documented [21,22,23,24,25]. The mammalian Hh ligands, Sonic (Shh), Indian (Ihh) and Desert (Dhh), bind to twelve-pass transmembrane receptors Patched (Ptch1, Ptch2) on neighboring cells [26,27]. This event relieves the seven-pass transmembrane protein smoothed (Smo) from Ptch-mediated repression and generates signals that are transduced by the Gli family of transcriptional activators and repressors (Gli1-3) [27,28,29]. Downstream Gli target genes, *Ptch* and *Hhip* (Hedgehog interacting protein), together with molecules acting at the level of ligand-binding such as cell surface bound Ig/fibronectin family members *Cdo* and *Boc* provide feedback mechanisms at various levels to keep the pathway in check [30]. Hh target gene expression is determined by the ratio of activator to repressor (Gli^A: Gli^R) forms of Gli proteins [31,32,33]. Gli2 is expressed independently of Hh signals in a functionally inactive form (Gli2^R) but becomes processed in response to Hh signals into an activator (Gli2^A) that initiates Hh target gene transcription [34,35,36,37]. Gli1 is transcribed in a strictly Hh-dependent manner and once expressed, constitutively activates Hh target genes, including itself, and is considered to be a non-essential pathway amplifier [38,39,40,41,42]. These features make it a useful and reliable indicator of Hh pathway activation [31,37]. Gli3 functions as a transcriptional activator (Gli3^A) or repressor (Gli3^R) depending on the cellular context [31,43]. In the absence

of Hh signals, Gli3^A is proteolytically processed into Gli3^R. Hh signals prevent this proteolytic conversion and also transcriptionally downregulate *Gli3* [27,35,43,44,45]. Most tissues maintain a specific Gli^R:Gli^A ratio by feedback mechanisms regulating downstream target gene expression. The processing of Gli proteins occurs within the primitive vestigial organelle, primary cilium [46]. Intraflagellar transport proteins (Ift) associate with kinesins or dyneins and are responsible for the formation and maintenance of primary cilia [47,48].

Although hair follicles and mammary glands share many local inductive pathways, these appendages undergo strikingly different responses to Hh signaling [21,22,24,49,50,51,52,53]. Hair and teeth require Hh signaling for downgrowth. We have shown that Gli3-mediated repression of Hh signaling is essential for the formation of MR#3 and #5 [51]. Loss of Gli3 exerts milder effects on the development of remaining MRs. However the molecular consequences of Gli3 action and whether it functions as an activator or repressor of Hh signaling or via Hh-independent functions at later stages have not been addressed.

Here we have investigated the effects of *Gli3* inactivation on factors involved early in the specification of MR#3 and found that Gli3 is required for the correct patterning of *Bmp4* and *Tbx3*. We further show that later in embryonic mammary development Gli3 loss or genetic misactivation of Hh signaling produce the same phenotypic spectrum of abnormal bud evagination, hair follicle encroachment and loss of sexual dimorphism. These data provide genetic evidence that repression of Hh signaling by Gli3^R is required for MR#2 invagination, hair follicle suppression, and loss of male mammary glands. Our results show that although mesenchymal Wnt signaling is activated in the absence of Gli3, later aspects of mammary mesenchymal specification are impaired and estrogen signaling and epithelial Wnt signaling fails. Finally, we show that positive Hh signaling is induced within specialized mesenchymal cell populations surrounding the lactiferous duct and is dynamically regulated within the smooth muscle cells of the nipple sphincter during the reproductive cycle.

Results

In our experiments below we utilized a number of genetic approaches to define the function of Gli3 during mammary development. First we examined Gli3 extra-toes mutant mice (*Gli3^{xt/xt}*) that lack Gli3 expression. To test whether the Gli3 phenotype results from loss of Gli3 repressor (Gli3^R) or activator (Gli3^A) function we utilize two strains. Gli1 strictly dependent on Hh signals for its expression and thus is an excellent reporter of positive Hh signaling [31,37]. Therefore Gli1-LacZ reporter expression indicates where positive Hh signaling is activated. Although Gli2 initiates Hh signaling it is present prior to this event in an inactive or weakly repressive state. In contrast, Gli1, lacks any repressor domain or function and once expressed is a strong amplifier of the pathway [40,41]. Thus, driving expression of the constitutive *Gli1* activator under the control of the *Gli2* promoter (*Gli2^{1ki/+}* or *Gli2^{1ki/1ki}*) tests the effect of activating Hh signaling within the Gli2 field of expression. If misactivation (*Gli2^{1ki/1ki}* mice) produces the same or exacerbates the phenotypes of *Gli3^{xt/xt}* mice, this provides genetic proof that the *Gli3^{xt/xt}* phenotypes result from loss of Gli3^R activity. In contrast, if the *Gli2^{1ki/1ki}* and *Gli3^{xt/xt}* phenotypes differ then Gli3 could be acting as a transcriptional activator of Hh signaling or in a manner independent of its role in Hh signaling.

Bmp4 and *Tbx3* Expression is Distorted within the Presumptive Mammary Placode #3 Region in *Gli3^{xt/xt}* Embryos

Previously, we have shown that mice lacking Gli3 expression (*Gli3^{xt/xt}*) or those genetically manipulated to misactivate Hh signaling (*Gli2^{1ki/1ki};Gli3^{xt/+}*) lack mammary placodes #3 and #5 [51]. This demonstrated that for early embryonic mammary development Gli3^R repression of Hh signaling is essential. As *Gli3^{xt/xt}* mutants lack expression of positive placodal regulators within the MR#3 and #5 regions [51,54] we reasoned that Gli3^R must repress an intervening negative regulator. We hypothesized that *Bmp4* could be a legitimate target of Gli3-mediated repression because it has been shown to antagonize the positive mammary placodal regulator *Tbx3* in the mammary gland and to be a target of Hh signaling in development of other organs [12,55,56]. To test this hypothesis, we examined the effect of Gli3 loss on *Bmp4* mRNA expression by *in situ* hybridization. As there were no significant differences in the distance between the fore- and hind-limb buds between the two genotypes (wt: 1.5±0.16 mm; *Gli3^{xt/xt}*: 1.5±0.18 mm; student's t test p=0.934) we assessed changes in the zone of *Bmp4* expression in terms of distance from the axilla to the most distal point of expression as indicated by white dotted lines in Fig. 1B, E. In wild-type (wt) embryos (n=6) (~E10.5–E11.5) we observed an arc of *Bmp4* expression on the ventral flanks closely abutting the axilla of the 40-somite (Fig. 1A) and 45-somite stages (0.5±0.15 mm) (Fig. 1B) in accord with previous studies documenting *Bmp4* expression ventral to the mammary line [12]. Sections through these embryos showed *Bmp4* mRNA expression within the ectoderm and the mesenchyme (Fig. 1C, C'). By comparison, *Gli3^{xt/xt}* embryos (n=6) showed significant displacement of *Bmp4* mRNA expression between the developing fore- and hind-limb buds at the 40-somite stage (Fig. 1D) that became more pronounced by the 45-somite stage (0.8±0.22 mm; student's t test p=0.037) (Fig. 1E, F, F') resulting in inappropriate *Bmp4* mRNA expression within the presumptive mammary placode #3 region (Fig. 1 arrow) that falls between somites 16 and 17 in wt. Cho et al have provided evidence that *Bmp4* establishes the site of development of mammary placode #3 on the flank through mutual antagonism with Tbx transcription factors [7,12]. Based on this model we proposed that if Gli3 functions upstream of the *Bmp4*/*Tbx3* mechanism then *Gli3^{xt/xt}* embryos would also show distortions in the *Tbx3* expression pattern. To determine if this was the case, we compared the *Tbx3* mRNA expression pattern in *Gli3^{xt/xt}* embryos (n=6) and wt (n=6) siblings by *in situ* hybridization. Consistent with previous studies of wt embryos, we observed a wide zone of *Tbx3* expression between the fore- and hind-limbs at the 40-somite stage (Fig. 2A), that by the 45-somite stage, was reduced in intensity but strongly concentrated within the developing mammary placodes (0.5±0.1 mm) (Fig. 2B). In contrast, the *Tbx3* expression zone was narrower at both the 40- and 45-somite stages in *Gli3^{xt/xt}* embryos (0.3±0.05 mm; student's t test p=0.004) (Fig. 2C, D) and failed to concentrate within the presumptive bud regions at the 45-somite stage (Fig. 2D). Taken together with observations of *Bmp4*, we conclude that Gli3 acts upstream of the *Bmp4*/*Tbx3* mechanism of mammary specification within the presumptive mammary placode #3 region.

Gli3 is Required for MR#2 Invagination and Suppression of Surrounding Hair Follicles

Although placodes #3 and #5 fail to develop in *Gli3^{xt/xt}* embryos (lacking Gli3), mammary placodes #1, #2 and #4 are clearly visible on the surface of E14.5 embryos, albeit with a consistent delay in #4 [51,54]. To determine if Gli3 is required

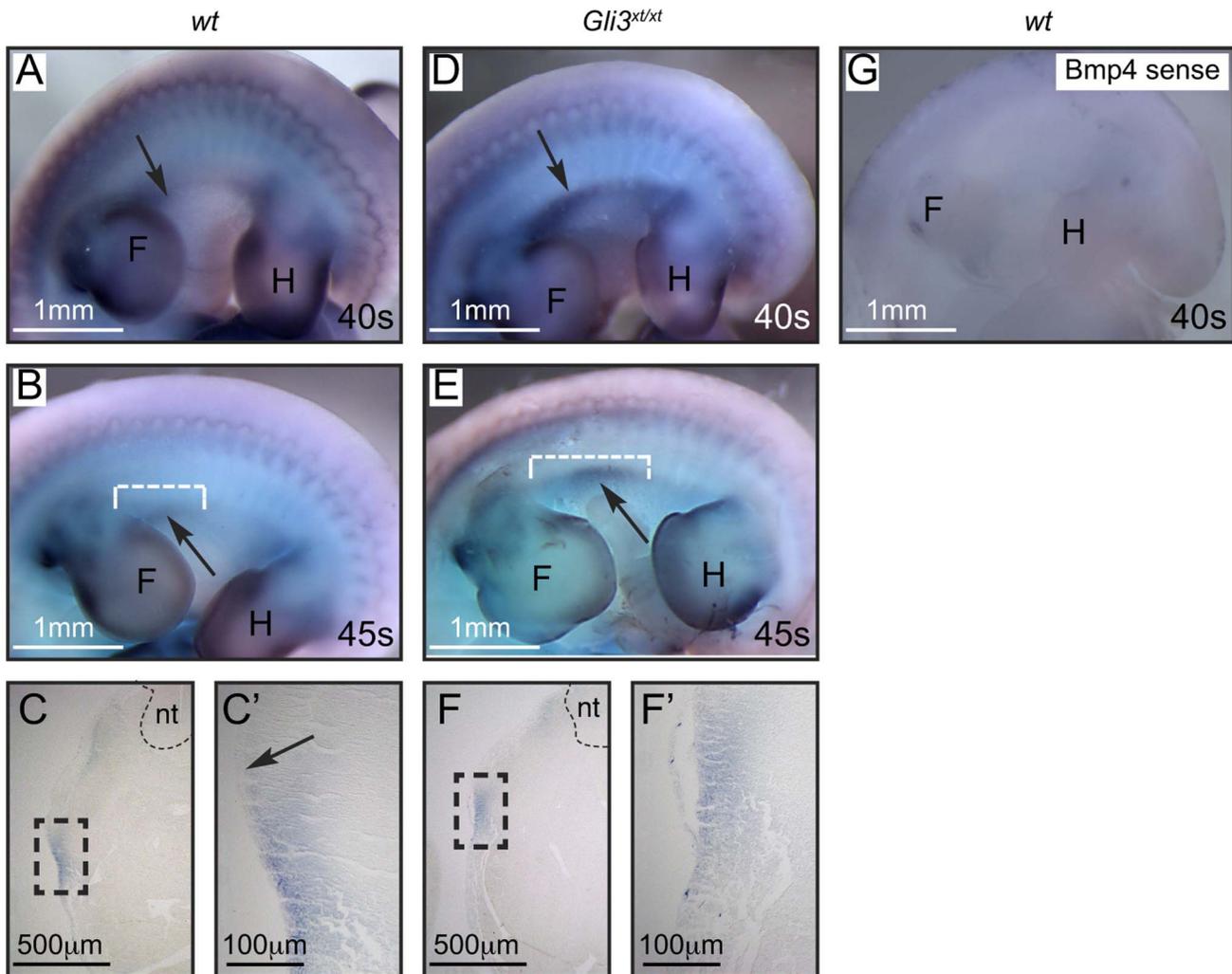


Figure 1. *Bmp4* mRNA expression is displaced in *Gli3^{xt/xt}* embryos. Whole-mount *in situ* hybridization for *Bmp4* in wt (A, B) and *Gli3^{xt/xt}* (D, E) embryos. In 40-somite stage wt embryos (A) *Bmp4* is expressed ventrally. This expression is more intense in *Gli3^{xt/xt}* embryos at this stage (D). At the 45-somite stage, expression is reduced in wt embryos (B arrow), but expression is displaced dorsally and centrally into the locale of the developing mammary placode #3 in *Gli3^{xt/xt}* embryos (E arrow). Distance from the axilla to the most distal point of *Bmp4* expression (B, E white dotted lines) was measured in mm. Sections through a 45-somite wt embryo (C) and higher power (C') show mesenchymal expression ventral to the developing placode #3 (arrow). Sections through a 45-somite *Gli3^{xt/xt}* embryo (F) and higher power (F') show the displaced *Bmp4* expression. Control *in situ* hybridization with *Bmp4* sense probe is shown on a wt embryo in (G). Abbreviations: F – fore-limb, H – hind-limb, nt – neural tube, s – somite. doi:10.1371/journal.pone.0079845.g001

during later development we examined these remaining MRs in skin whole-mounts from E17.5 and E18.5 female *Gli3^{xt/xt}* embryos (n = 49). We also took advantage of the fact that hair follicles can be distinguished from MRs in control *Gli1^{l2ki/+}* and in mutant *Gli3^{xt/xt}*; *Gli1^{l2ki/+}* embryos by their engagement in Hh signaling and consequent expression of the Gli1-LacZ reporter [51]. Inspection of the inner side of the skin of control wildtype (wt) (Fig. 3A–C) and *Gli1^{l2ki/+}* (Fig. 3D–F) mice showed that 100% of MR#1, #2 and #4 had sprouted, with #1 and #2 penetrating the underlying dense fat pad and branching to form small ductal trees. In *Gli3^{xt/xt}* mutant embryos, although MR#1 developed normally (Fig. 3G) MR#2 failed to sprout and arrested prior to invagination in 84% of cases (Fig. 3H) (Table 1). MR#4 was affected in a minority of cases (Fig. 3I) (Table 1). Examination of the outside of skin whole-mounts and histological sections confirmed that mutant MR#1 invaginated normally (Fig. 4A). However mutant MR#2 evaginated as a prominent bulge

projecting from the epidermal surface (57%) (Fig. 4B and D) or was lost altogether (27%) (Table 1).

Suppression of hair follicle formation within the designated nipple sheath is an important aspect of late embryonic mammary development [11]. To determine if this process was affected by loss of Gli3 activity, we analyzed hair follicle suppression in control *Gli1^{l2ki/+}* and mutant *Gli3^{xt/xt}*; *Gli1^{l2ki/+}* embryos. Hair follicles were appropriately excluded from the presumptive nipple areolar zone of all MRs from control *Gli1^{l2ki/+}* embryos (Fig. 3D–F and Fig. 4C) as well as from MR#1 and MR#4 of mutant *Gli3^{xt/xt}*; *Gli1^{l2ki/+}* embryos (Fig. 3G and I; Fig. 4A). In contrast, in mutant *Gli3^{xt/xt}*; *Gli1^{l2ki/+}* embryos, ectopic hair follicles expressing Gli1-LacZ were observed inappropriately close to the base (Fig. 4D) and even at the tip of the evaginated MR#2 (Fig. 4F).

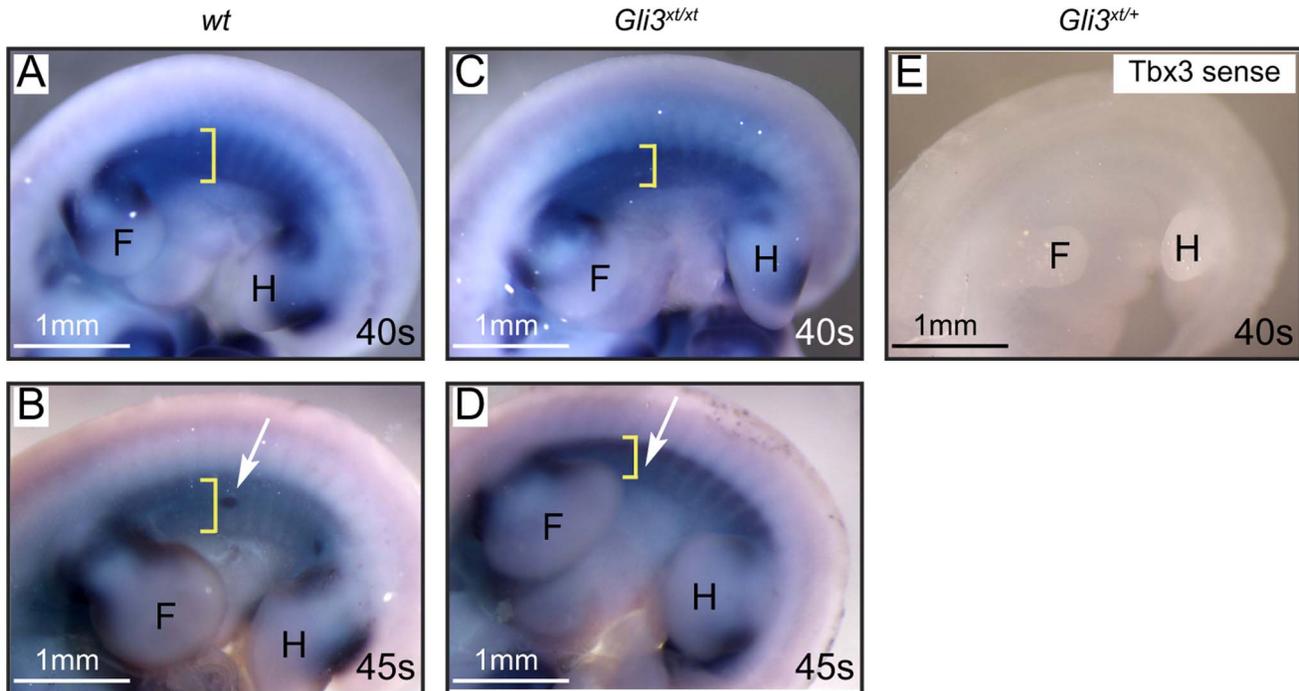


Figure 2. *Tbx3* mRNA expression in *Gli3^{xt/xt}* embryos. Whole-mount *in situ* hybridization for *Tbx3* in wt (A, B) and *Gli3^{xt/xt}* (C, D) embryos. At the 40-somite stage, wt embryos (A) express *Tbx3* mRNA in the mesenchyme between the fore- and hind-limb. This band of expression is narrower in *Gli3^{xt/xt}* embryos at this stage (yellow bracket). At the 45-somite stage *Tbx3* is reduced within the mesenchyme but is induced within the epithelium of developing mammary placodes #3 (white arrow) in wt embryos (B). *Gli3^{xt/xt}* embryos (D), show a narrower band of *Tbx3* expression between the fore- and hind-limb and fail to concentrate epithelial expression within placode #3 (white arrow). The control *in situ* hybridization for *Tbx3* sense probe is shown in (E). Abbreviations: F – fore-limb, H – hind-limb, s – somite. doi:10.1371/journal.pone.0079845.g002

Loss of Gli3 Impairs MR#2 Mammary Mesenchyme Specification

A series of elegant experiments has demonstrated that complex reciprocal epithelial-mesenchymal signaling regulates MR invagination and suppression of surrounding hair follicles [3]. To investigate the status of the mammary mesenchyme specification we first investigated Wnt/ β -catenin signaling pathway activity by crossing *Gli3^{xt/+}* to *Conductin^{l2/+}* heterozygous LacZ knock-in reporter lines. *Conductin* is expressed constitutively in response to canonical Wnt/ β -catenin signaling and its product negatively regulates the pathway [57,58]. In control *Conductin^{l2/+}* mice the *Conductin*-LacZ reporter was expressed in the mammary mesenchyme and within central epithelial cells of MR#2 (Fig. 5A) whereas in the evaginated MR#2 of *Gli3^{xt/xt};Conductin^{l2/+}* embryos *Conductin*-LacZ expression was present only within the mesenchymal compartment (Fig. 5B). Next we examined serial sections of *Gli3^{xt/xt};Gli1^{l2ki/+}* E13 embryos stained estrogen receptor (ER), and androgen receptor (AR) antibodies as markers of mammary mesenchyme specification and with p63 antibodies to detect the epithelial layer. In control *Gli1^{l2/+}* embryos MR#2 comprised a compact p63-positive epithelial bulb beneath the epidermis (Fig. 5C), surrounded by a condensed ring of ER-positive and AR-positive mammary mesenchymal cells (Fig. 5E and G respectively). In *Gli3^{xt/xt};Gli1^{l2ki/+}* embryos, MR#2 comprised a raised epithelial placode of p63-positive cells (Fig. 5D). However few underlying mesenchymal cells expressed ER and AR (Fig. 5F and H respectively), and these failed to condense. By E18.5, *Conductin*-LacZ reporter expression had switched in control *Conductin^{l2/+}* embryos: being diminished within the mesenchyme and robustly upregulated within the mammary

sprout (Fig. 6A). In contrast, *Gli3^{xt/xt};Conductin^{l2/+}* embryos maintained robust reporter expression in the MR#2 mammary mesenchyme but failed to upregulate expression within the epithelium (Fig. 6B). Control *Gli1^{l2/+}* embryos continued to show robust expression of ER, AR and Tenascin C within the mammary mesenchyme surrounding the MR#2 sprout (Fig. 6 C, E, G). However, *Gli3^{xt/xt};Gli1^{l2/+}* embryos had lost mesenchymal ER expression (Fig. 6D) and showed weak Tenascin C and AR expression (Fig. 6F and H) in evaginated MR#2 and lacked all histological signs of mammary mesenchyme condensation (Fig. 6C–H). Collectively these results show that, although mammary mesenchymal specification is initiated normally, maintenance of mammary mesenchymal markers and gain of epithelial Wnt signaling is compromised in MR#2 of *Gli3^{xt/xt}* embryos.

Gli3 is Required for Sexual Dimorphism during Mammary Development

Next we asked whether the observed impairment in the mammary mesenchymal specification program has functional consequences for MR formation in males. Around E13 a surge of secreted androgens in male embryos induces mammary mesenchymal cells to encapsulate and cause the mammary bulb to atrophy [3,9,15,17,18,19,20,59]. To determine whether Gli3 activity influences this process we looked for evidence of inappropriate retention of MRs in E14.5 male embryos. In control *Gli3^{+/+}* embryos all MRs were appropriately lost by E16.5 (Table 2). Male *Gli3^{xt/xt}* embryos, like their female counterparts failed to form MR#3 and MR#5. However, in *Gli3^{xt/xt}* males 66% of MR#1 were retained at E16.5 but only 15% by E18.5 suggesting that their normal atrophy occurred but was delayed

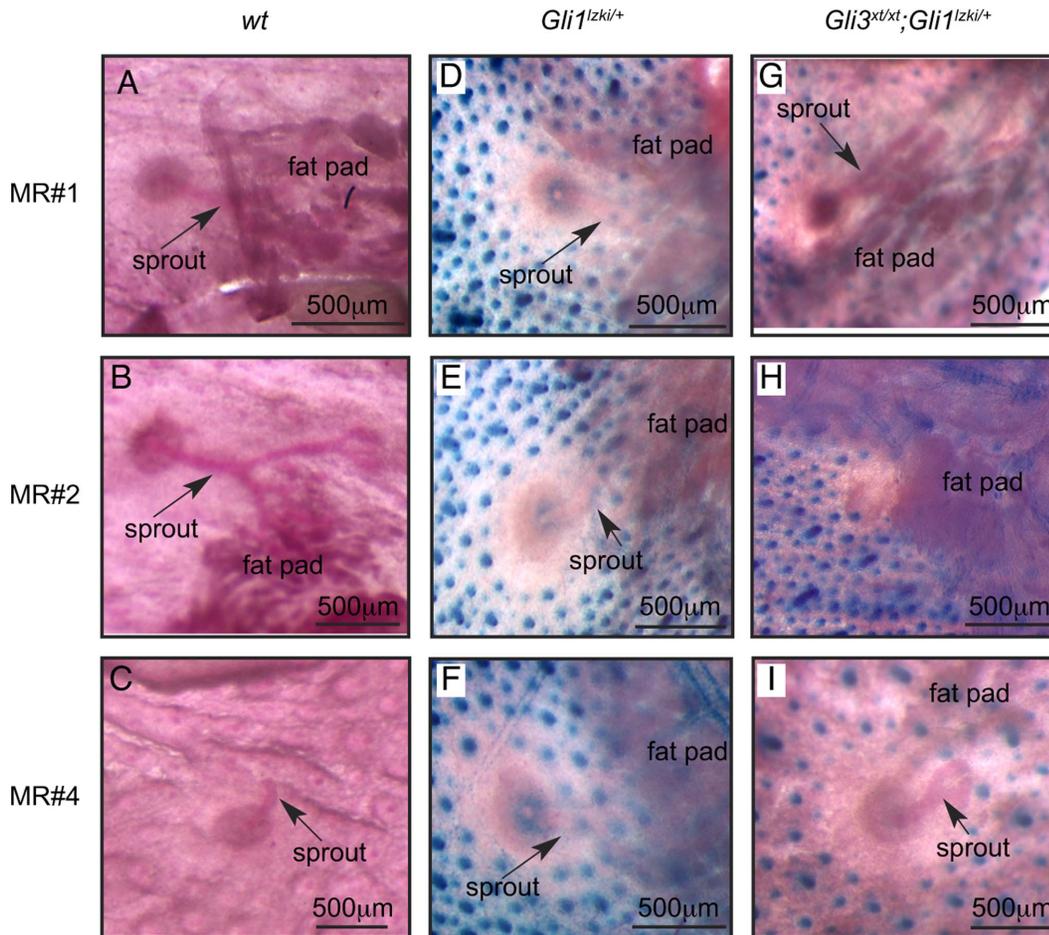


Figure 3. Failure of MR#2 invagination in *Gli3*^{xt/xt} embryos. Analysis of the inner surface of skin whole-mounts from E18.5 wt (A, B, C) and *Gli1*^{l2ki/+} (D, E, F) and *Gli3*^{xt/xt}; *Gli1*^{l2ki/+} (G, H, I) embryos stained with X-Gal to detect *Gli1-LacZ* reporter expression (blue) within hair follicles and counterstained with carmine alum (pink). MRs#1 (A, D, G) and #4 (C, F, I) show comparable development at E18.5 in all three genotypes: sprouts are clearly visible (arrow). In control wt (B) and *Gli1*^{l2ki/+} (E) embryos sprout #2 has elongated and branched several times but in *Gli3*^{xt/xt}; *Gli1*^{l2ki/+} embryos (H), MR#2 shows no evidence of sprouting towards the fat pad. doi:10.1371/journal.pone.0079845.g003

(Table 2). However, MR#2 and MR#4 showed very high rates of retention at both E16.5 and E18.5 (Table 2). The majority of these

Table 1. Percentages of MRs showing phenotypic abnormalities in female *Gli3*^{xt/xt} embryos at E18.5.

MR#	Phenotype	<i>Gli3</i> ^{xt/xt}	<i>Gli3</i> ^{xt/+}	<i>Gli3</i> ^{+/+}
		(n = 49)	(n = 90)	(n = 58)
1	Loss	4	0	0
2	Evagination	57	0	0
	Loss	27	0	0
4	Evagination	6	0	0
	Imp. Invag.	4	0	0
	Loss	4	0	0

Numbers represent percentages of MRs showing loss, evagination or impairment in invagination from a total 'n'. Abbreviations: Imp. Invag: Impaired Invagination; MR: mammary rudiment; xt: extra toe mutation; n: total number of MRs analyzed.

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retained MRs#2 and #4 were evaginated (Table 3; Fig. 7C, D). In control E14.5 *Gli3*^{+/+} male embryos MRs showed robust expression of *Conductin-LacZ*, *Tenascin C* and *AR* in the zone of mesenchymal constriction around the epithelial bulb (Fig. 8A–C). Mutant *Gli3*^{xt/xt}; *Conductin*^{l2/+} embryos showed mesenchymal *Conductin-LacZ* expression in the three remaining MRs but epithelial *Wnt* signaling was absent (Fig. 7E and Fig. 8), *Tenascin C* and *AR* were expressed in very few cells and the MRs were frequently evaginated (Fig. 8A'–C', Table 3). Thus *Gli3* activity exerts a significant influence on sexual dimorphism.

Gli3 Acts as a Repressor of Hh Signaling during Late Mammary Development

Our results show that lack of *Gli3* expression severely compromises MR#2 invagination in both sexes and leads to inappropriate retention of MRs#1, #2 and #4 in males. To test if *Gli3* functions as an activator or repressor of Hh signaling during these later stages of mammary development we crossed *Gli2*^{1ki/+} mice, which drives the expression of constitutively active *Gli1* transactivator under the control of *Gli2* promoter to *Gli2*^{1ki/+}; *Gli3*^{xt/+}; *Gli1*^{l2ki/l2ki} mice. The genotypes arising from this cross alter the *Gli*^R:*Gli*^A ratio to progressively misactivate the pathway

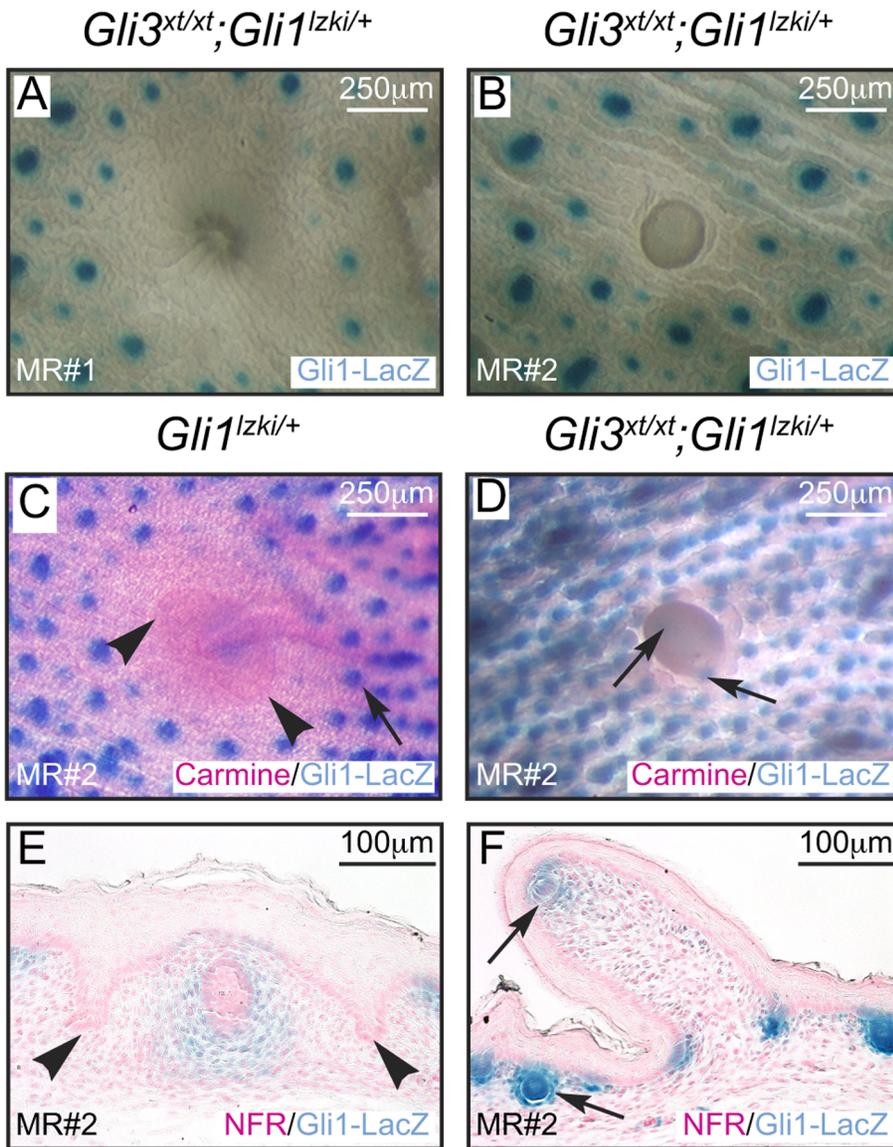


Figure 4. Evagination of MR#2 and encroachment of hair follicles in *Gli3^{xt/xt}* embryos. Analysis of outer surface of E18.5 skin whole-mounts (A–D) stained with carmine (C, D) and sections stained with nuclear fast red (E, F) and X-Gal (blue) to detect hair follicles expressing the Gli1-LacZ reporter (A–F). MR#1 (A) from *Gli3^{xt/xt}; Gli1^{lzk1/+}* mutants and MR#2 from control *Gli1^{lzk1/+}* (C) embryos show normal invagination and appropriate exclusion of hair follicles. In contrast MR#2 from *Gli3^{xt/xt}; Gli1^{lzk1/+}* mutants (B, D) showed prominent evagination and encroachment of hair follicles. doi:10.1371/journal.pone.0079845.g004

[36,51]. Analysis of skin whole-mounts from E18.5 females showed that misactivation of the Hh pathway in *Gli2^{lki/1ki}; Gli3^{xt/+}; Gli1^{lzk1/+}* embryos (n = 10) produces the same spectrum of phenotypes seen in *Gli3^{xt/xt}; Gli1^{lzk1/+}* embryos: MR#2 was prominently evaginated (60% (Fig. 9A) and MRs#1 and #4 showed mild impairment of invagination in 20% of cases (Table 4). Hair follicles, demarcated by expression of the Gli1-LacZ reporter, inappropriately encroached around the protruding MR#2 of *Gli2^{lki/1ki}; Gli3^{xt/+}; Gli1^{lzk1/+}* embryos (Fig. 9A), in a manner similar to that shown previously for *Gli3^{xt/xt}; Gli1^{lzk1/+}* embryos (Fig. 4). Similarly, in male embryos, Hh pathway misactivation leads to significant rates of retention of MR#1, MR#2 and MR#4 (Fig. 9B–E; Table 5), evagination of MR#2 and reduced expression of Tenascin C and AR (Fig. 9F–H).

The Hh Pathway is Activated in Developing and Adult Nipple

In contrast to the requirement for repression of Hh signaling in mammary rudiment development, we found that positive Hh signaling occurs within the developing nipple. A small ring of Gli1-LacZ expression was observed at E18.5 in *Gli1^{lzk1/+}* embryonic skin whole-mounts (Fig. 10A) and histological sections (Fig. 10B) within the mesenchyme around the neck of the lactiferous duct and under the nipple sheath. Postnatally, the mammary mesenchyme develops into highly specialized nipple mesenchyme (Fig. 10C–F). Immunohistochemical analysis of nipple sections defined a number of cell types undergoing Hh signaling in the postnatal nipple. Des+;SMA+;Vim- smooth muscle cells, showed Gli1-LacZ expression during puberty, pregnancy and involution but lost reporter expression during lactation (Fig. 6G, H, I). Gli1-LacZ-positive Des-;SMA-;Vim+ fibroblasts surrounded the lactiferous

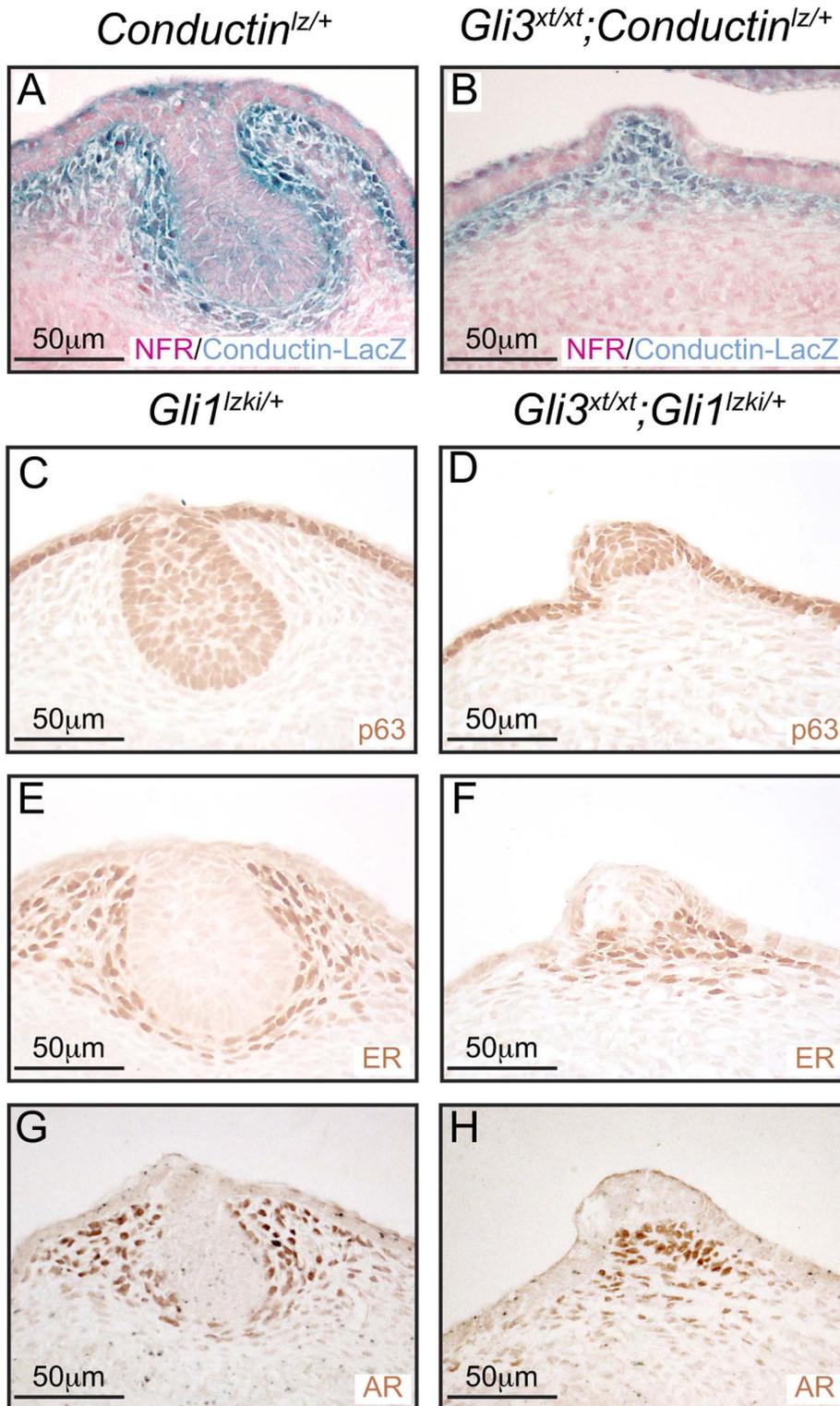


Figure 5. Mammary mesenchyme specification in E14.5 *Gli3*^{xt/xt} embryos. (A, B) Analysis of sections of MR#2 stained with X-Gal (blue) for expression of Conductin-LacZ reporter and counterstained with NFR. Control *Conductin*^{lz/+} embryos (A) show Wnt/ β -catenin signaling pathway activity in the mammary mesenchyme and central epithelial cells whereas *Gli3*^{xt/xt}; *Conductin*^{lz/+} embryos (B) show activation only within the mesenchymal compartment. Immunohistochemical analysis of serial sections from control E13 *Gli1*^{lzki/+} (C, E, G) and mutant *Gli3*^{xt/xt}; *Gli1*^{lzki/+} (D, F, H) embryos for expression of (C, D) p63, (E, F) ER and (G, H) AR. Note the epithelium of *Gli3*^{xt/xt}; *Gli1*^{lzki/+} mutant embryos fails to invaginate (D), the mammary mesenchyme shows no histological evidence of condensation and few cells express ER (F) and AR (H). doi:10.1371/journal.pone.0079845.g005

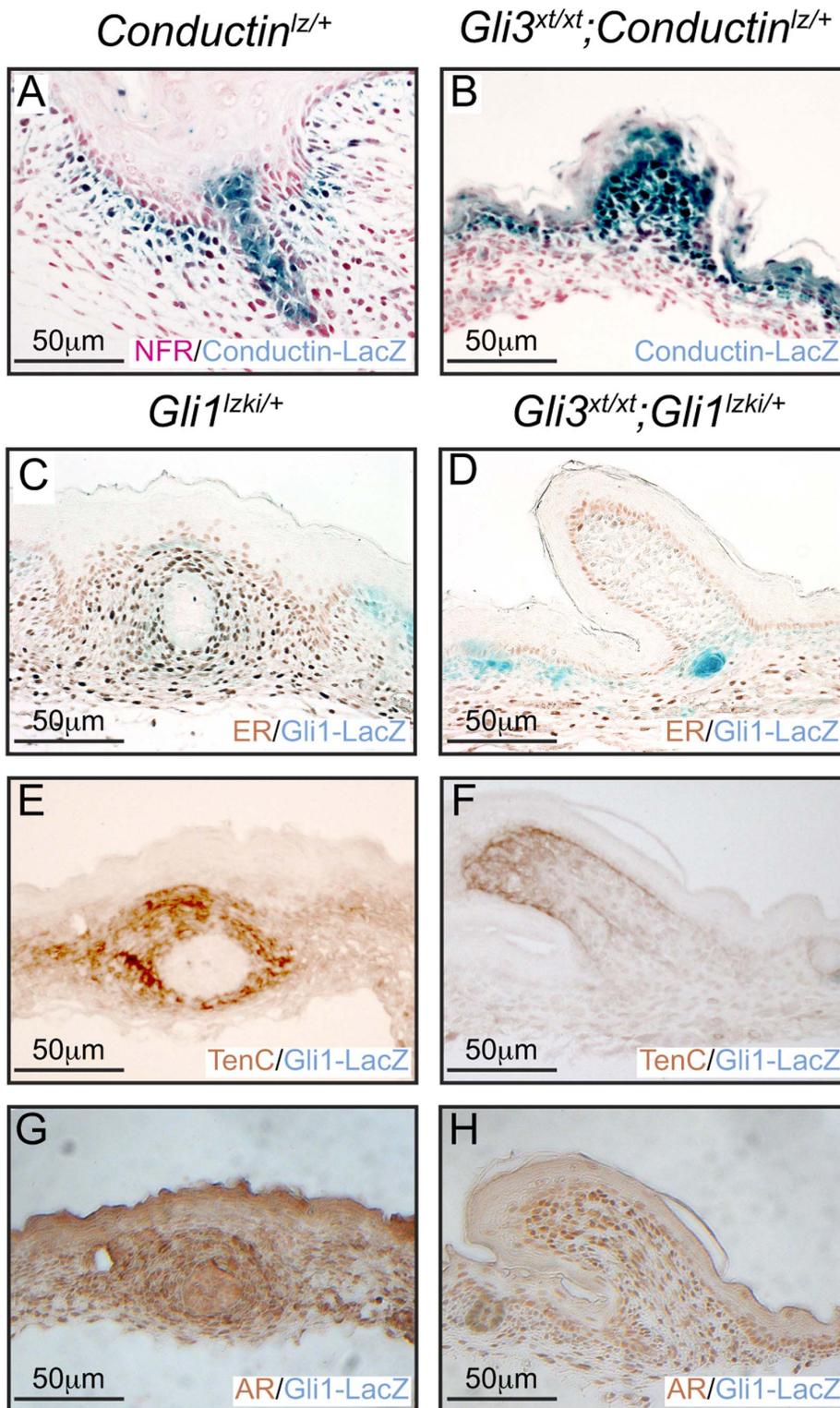


Figure 6. Gain of epithelial Wnt signaling and maintenance of mammary mesenchyme markers is compromised in MR#2 of *Gli3*^{xt/xt} embryos. Analysis of X-Gal and NFR stained sections from MR#2 at E18.5. (A) *Conductin-LacZ* is robustly expressed within the epithelial mammary sprout of control *Conductin*^{l^z/+} embryos whereas (B) mutant *Gli3*^{xt/xt}; *Conductin*^{l^z/+} embryos lack expression within the epithelium and maintain robust mesenchymal expression. (C–J) Analysis of serial sections for mammary mesenchyme markers by immunohistochemistry revealed that ER (C), Tenascin C (E) and AR (G) are maintained in control *Gli1*^{l^zki/+} embryos. In contrast, ER was lost (D) and Tenascin C and AR expression were weakened (F, H) in mutant *Gli3*^{xt/xt}; *Gli1*^{l^zki/+} embryos.
doi:10.1371/journal.pone.0079845.g006

Table 2. Percentages of MRs showing retention in male *Gli3^{xt/xt}* embryos at E16.5 and E18.5.

MR#	Retention (E16.5)			Retention (E18.5)		
	<i>Gli3^{xt/xt}</i>	<i>Gli3^{xt/+}</i>	<i>Gli3^{+/+}</i>	<i>Gli3^{xt/xt}</i>	<i>Gli3^{xt/+}</i>	<i>Gli3^{+/+}</i>
	(n = 30)	(n = 58)	(n = 18)	(n = 54)	(n = 88)	(n = 56)
1	66	0	0	15	0	0
2	77	0	0	69	0	0
4	78	0	0	61	0	0

Numbers represent percentages of MRs retained on male skin whole-mounts from a total 'n'. Abbreviations: E: embryonic day; n: total number of putative sites for MRs on male skin whole mounts.

doi:10.1371/journal.pone.0079845.t002

duct (Fig. 10 J, K, L). Minor subsets of Vim+ Gli1-LacZ-positive fibroblasts were found encasing peripherin-positive nerve tracts (Fig. 6M) and in close association with Von-Willebrand factor positive vessels (Fig. 6N).

Discussion

The main findings of our study are threefold. First, that *Gli3^R* lies upstream of *Bmp4/Tbx3* specification of placode #3. Second, that at later stages *Gli3^R* significantly influences the maintenance of mammary mesenchyme specification and function. Third, that *Gli3* impinges on these developmental events via repression of Hh signaling. In contrast we document that positive Hh signaling occurs during embryonic and postnatal nipple development.

Gli3^R Acts Upstream of *Bmp4/Tbx3* Patterning

Our data show that loss of *Gli3* results in inappropriate expansion of *Bmp4* and failure of *Tbx3* to concentrate within the presumptive MR#3 region. The positive role of *Tbx3* in MR development is well documented [7,60,61]. Firstly, *Tbx3* is expressed within mammary placodes at E11.75 and *Tbx3^{-/-}* mice lack most mammary buds [7,12]. During postnatal mammary development, haploinsufficient *Tbx3^{+/-}* mice display significantly underdeveloped ductal trees at puberty and conversely, inducible *Tbx3* overexpression accelerates mammary epithelial cell proliferation resulting in mammary hyperplasia [7,62]. In humans, heterozygous mutations in *TBX3* result in Ulnar-Mammary Syndrome, which is characterized by mammary hypoplasia [63]. Aberrations in human *Tbx3* gene have also been implicated in breast cancer [64,65,66,67,68]. The fact that loss of *Gli3* repression results in loss of upregulation at the site of placodal #3 points to the involvement of an intervening *Tbx3* suppressor, and previous studies suggest that *Bmp4* is the most likely candidate for this role [12]. *Tbx3* is a direct transcriptional target of *Bmp*/*Smad* activity during the development of limb buds, retina and adult brain [69,70,71]. Antagonism between *Bmp4* and *Tbx3* has been shown to be critical for formation of the mammary line within the presumptive MR#3 region [12]. Previous studies have placed *Gli3* upstream of the positive placodal regulator *Fgf10* [54]. However we have shown that gain of Hh signaling negatively regulates placodes #3 formation [51]. Hence *Gli3* must repress an intervening Hh-dependent placodal repressor. The finding that loss of *Gli3^R* expands the zone of *Bmp4*, a known antagonist of the positive placodal regulator *Tbx3*, suggests that *Bmp4* may be this intervening repressor. Consistent with this concept, connections between Hh signaling and mesenchymal *Bmp4* expression have been documented during development of other tissues such as the

Table 3. Percentages of evaginated MRs in male *Gli3^{xt/xt}* embryos at E16.5 and E18.5.

MR#	Evagination (E16.5)			Evagination (E18.5)		
	<i>Gli3^{xt/xt}</i>	<i>Gli3^{xt/+}</i>	<i>Gli3^{+/+}</i>	<i>Gli3^{xt/xt}</i>	<i>Gli3^{xt/+}</i>	<i>Gli3^{+/+}</i>
	(n = 30)	(n = 58)	(n = 18)	(n = 54)	(n = 88)	(n = 56)
1	7	0	0	2	0	0
2	60	0	0	63	0	0
4	56	0	0	61	0	0

Numbers represent percentages of MRs that protrude from the surface of male skin whole-mounts from a total 'n'. Abbreviations: E: embryonic day; n: total number of putative sites for MRs on male skin whole mounts.

doi:10.1371/journal.pone.0079845.t003

hindgut, kidney and prostate [55,56,72,73,74]. Supporting the possibility that *Bmp4* is a direct transcriptional target of positive Hh signaling and *Gli3^R* repression, Gli binding sites are present in the murine *Bmp4* promoter and transfection of cDNAs encoding Gli1 or *Gli3^A* has been shown to activate a human *BMP4* promoter-reporter construct in COS-7 cells [75,76]. Collectively these findings suggest a model in which *Gli3* acts upstream of *Fgf10* and also upstream of *Bmp4/Tbx3* in the latter case by acting to repress Hh-activation of *Bmp4* thereby relieving antagonism on the positive placodal regulator *Tbx3*.

Gli3^R Influences Mammary Mesenchyme Specification and Function

Our results show that although early *Gli3^R* function is essential for the formation of placodes #3 and #5, it is not required for the early development of placodes #1, #2 and #4 [51,54]. It has been well documented that mammary placodes form in a specific temporal order (#3, #4, #1, #5, #2) and that each pair has a unique set of regulatory requirements [1,77]. In *Gli3^{xt/xt}* mutants placodes #1 and the majority of #4 go on to sprout and branch despite loss of pathway repression [51]. However, loss of *Gli3* activity in *Gli3^{xt/xt}* mutants produces profound effects on the later development of MR#2, which forms a large evaginated protrusion. A previous report has suggested that higher proliferation of mesenchymal cells and inability of the adjacent ectoderm to undergo apoptosis contributes to this protrusion and that MR#2 nevertheless sprouts [4]. In contrast, our results show that 84% of MR#2 fail to invaginate or sprout and remain evaginated or are lost altogether. A series of elegant experiments has shown that invagination and sprout downgrowth are regulated by reciprocal epithelial-mesenchymal signaling that lead to specialization and subsequent condensation of mammary mesenchyme together with suppression of surrounding hair follicles. These studies showed: (1) PTHrP released from the bud specifies the surrounding condensed mammary mesenchyme inducing expression of reporters of β -catenin signaling and a suite of mesenchymal markers including Lef1, hormone receptors and Tenascin C [9,11,13,14,16,59,77,78,79]; (2) Lef-independent Wnt signaling is first required in the mesenchyme for mammary mesenchyme specification but Wnt signaling occurs in both compartments and Lef-dependent activity is required later for sprouting [5,59,80,81]; (3) In females PTHrP-dependent upregulation of mesenchymal *BmpR1a* expression also increases mesenchymal *Mx2* expression, which inhibits hair follicle cell fate within the overlying nipple epidermis [11,13,14,16,82]; (4) In males androgen stimulation of AR expression leads to their detachment and loss of this PTHrP-dependent AR expression in PTHrP^{-/-} mice manifests as loss of

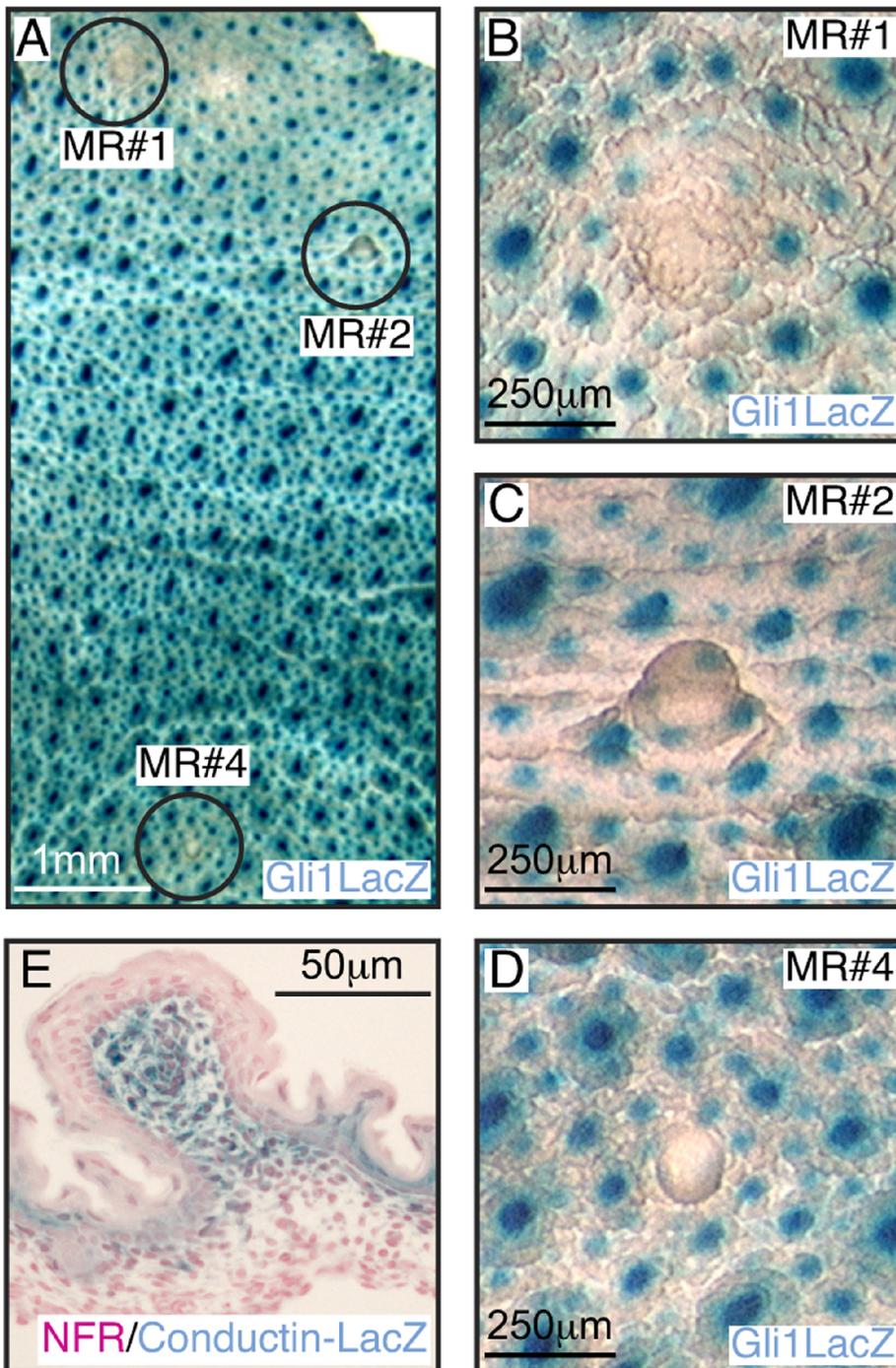


Figure 7. Sexual dimorphism is lost in *Gli3*^{xt/xt} embryos. X-Gal stained skin whole-mounts from E18.5 male *Gli3*^{xt/xt};*Gli1*^{Izki/+} embryos show retention of MR#1, #2 and #4 (A). Examination of skins at high power revealed that MR#1 does not protrude from the surface of the skin (B), whereas MR#2 and #4 clearly evaginate (C, D). Elevated Wnt signaling activity can be seen in mesenchymal cells of protruding MR#2 from *Gli3*^{xt/xt};*Conductin*^{Iz/+} embryos at this stage (E). doi:10.1371/journal.pone.0079845.g007

sexual dimorphism [15,17,18,19,20,59]. Our results show that *Gli3*^{xt/xt} mutants initiate mammary mesenchyme specification, evidenced by mesenchymal expression of Conductin-LacZ Wnt reporter, but fail to maintain mammary mesenchymal ER expression or to upregulate epithelial Wnt signaling. The loss of these markers suggests that the feedback signaling loop between the mammary mesenchyme and overlying epithelial compart-

ments is defective. This likely accounts for the failure to establish nipple identity during subsequent development and the reversion of the overlying epithelium to an epidermal fate evidenced by aberrant formation of ectopic hair follicles within the epidermis of the evaginated bud. The functional significance of this mammary mesenchyme impairment is also reflected by the loss of sexual dimorphism. Despite the low levels of expression of AR and

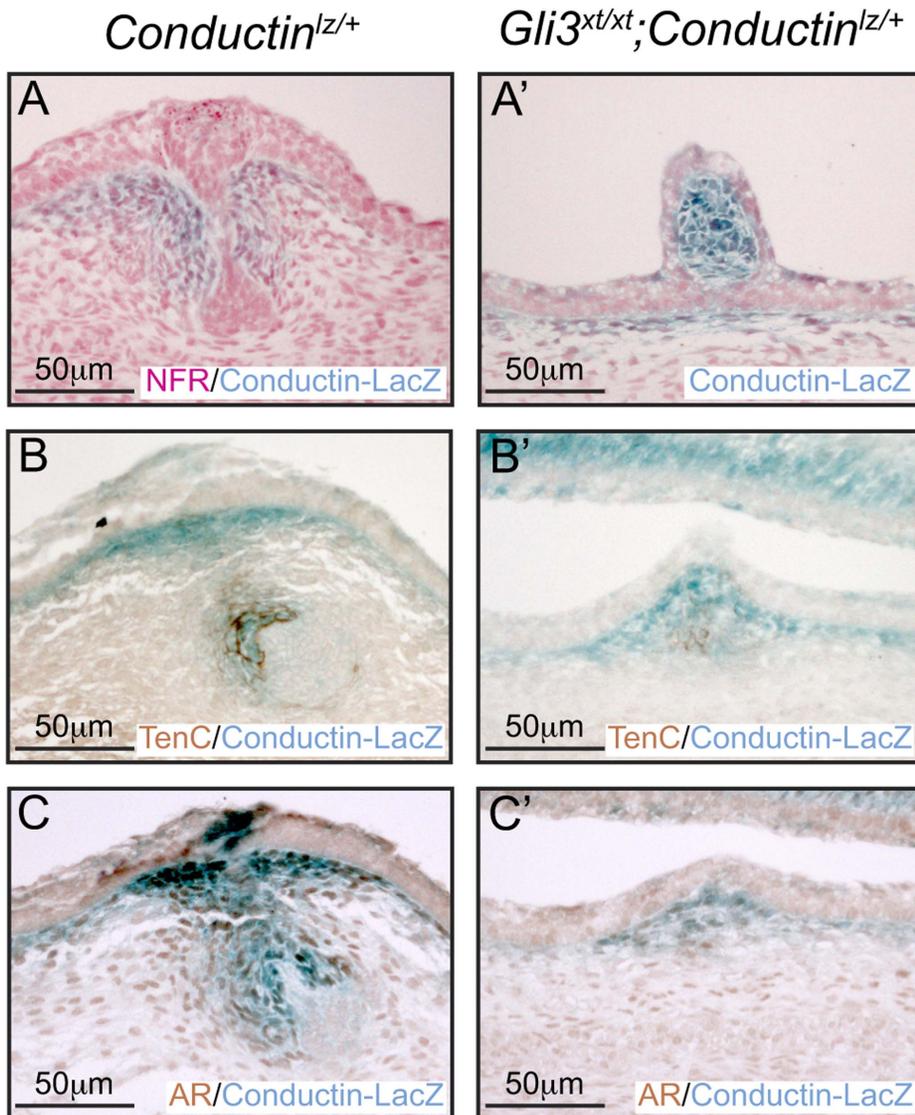


Figure 8. Mammary mesenchyme specification is impaired in male *Gli3*^{xt/xt} embryos. X-Gal stained sections from control *Conductin*^{lz/+} embryos showed appropriate constriction of mesenchymal cells coincident with the expression of Conductin-LacZ reporter (A), Tenascin C (B) and AR (C). However, mesenchymal cells surrounding the protruding MR#2 of *Gli3*^{xt/xt};*Conductin*^{lz/+} embryos retained mesenchymal Conductin-LacZ expression (A') and showed weak Tenascin C (B') and AR (C') expression. doi:10.1371/journal.pone.0079845.g008

Tenascin C clearly designating the sites for MR formation in both sexes, the failure to maintain robust expression of these markers likely impairs the androgenic response that would normally induce their demise in males leading to the aberrant retention of primitive and frequently evaginated MRs#1, #2 and #4 in *Gli3*^{xt/xt} male embryos.

Gli3 Acts via Repression of Hh Signaling during Late Embryonic Mammary Development

Gli3 proteins occur within mammalian cells either in a Hh-dependent full-length transcriptional-activator capacity (*Gli3*^A) or in the absence of Hh signals, are proteolytically cleaved into truncated repressor proteins (*Gli3*^R). Highly cell-contextual and opposing functions of Gli3 have been documented in different mammalian systems: For example, in spinal cord, skeletal muscle and stomach Gli3's primary function is that of Hh-activation whereas *Gli3*^R is the critical repressor of Hh signaling pathway in

hair, teeth, limb and lung development [35,43,44,45,83,84,85,86,87]. A critical balance of *Gli3*^R:*Gli3*^A ratio is maintained within mammalian cells for proper execution of Hh signaling pathway (reviewed in [88]). Our results show that deliberate Hh-pathway misactivation (*Gli2*^{1ki/1ki};*Gli3*^{xt/+}) produces the same spectrum of phenotypic aberrations as loss of Gli3 in both sexes: MR#2 fails to invaginate, to upregulate epithelial Wnt reporter expression, to appropriately condense mammary mesenchyme and to suppress surrounding hair follicles. In addition, expression of mesenchymal markers AR and Tenascin C is more prominently impaired in misactivated MRs. These results provide genetic evidence that Gli3 acts as a repressor of Hh signaling and not in some Hh-independent or Hh transactivator capacity. We conclude that Gli3 functions as a repressor of Hh signaling and significantly influences three events in mammary development: MR invagination, hair follicle suppression and eradication of MRs in males.

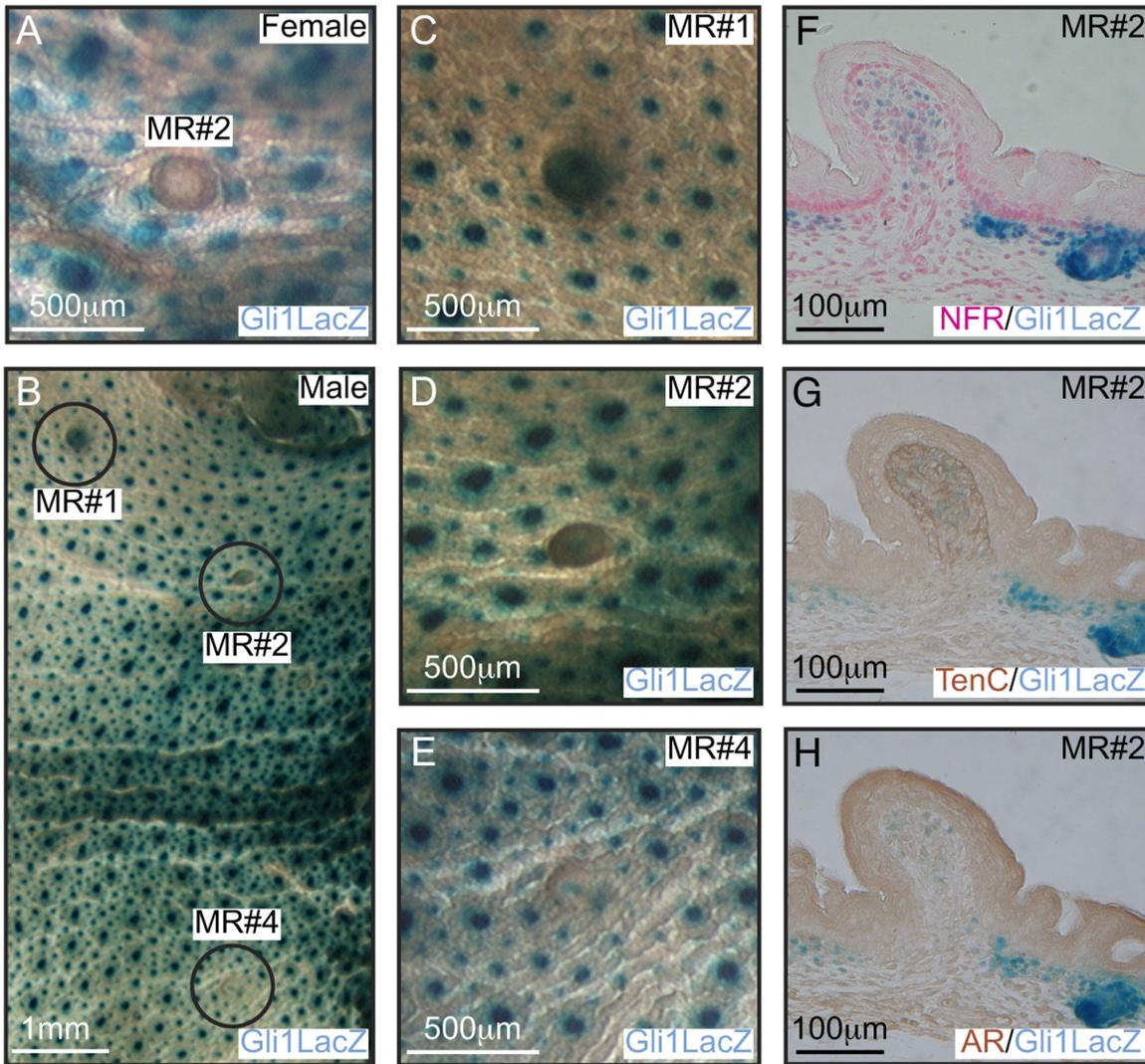


Figure 9. Misactivation of Hh signaling detrimentally affects MR invagination and hair follicle suppression in females and eradication of MRs in males. X-Gal stained whole-mounts (A–E) and sections (F–H) of *Gli2*^{1ki/1ki};*Gli3*^{xt/+};*Gli1*^{l2ki/+} embryos were examined at E18.5. In whole-mounts of female skins, MR#2 protruded prominently and showed encroachment of hair follicles inappropriately close to the evaginated MR (A). Examination of male skin whole-mounts revealed retention of MR#1, #2 and #4 at low (B) and high power (C, D, E respectively), similar to that seen in *Gli3*^{xt/xt};*Gli1*^{l2ki/+} embryos. Serial sections through a male MR#2 from *Gli2*^{1ki/1ki};*Gli3*^{xt/+};*Gli1*^{l2ki/+} embryos showed Gli1-LacZ-positive hair follicles close to the protruding bud (F; NFR counterstain) and weak expression of Tenascin C (G) and loss of AR (H) by immunohistochemistry. doi:10.1371/journal.pone.0079845.g009

Table 4. Percentages of MRs showing phenotypic abnormalities in female *Gli2*^{1ki/1ki};*Gli3*^{xt/+} embryos at E18.5.

MR#	Phenotype	<i>Gli2</i> ^{1ki/1ki} ; <i>Gli3</i> ^{xt/+}	<i>Gli2</i> ^{1ki/1ki}	<i>Gli2</i> ^{1ki/+} ; <i>Gli3</i> ^{xt/+}	<i>Gli2</i> ^{1ki/+}	<i>Gli3</i> ^{xt/+}	<i>Gli3</i> ^{+/+}
		(n = 10)	(n = 14)	(n = 8)	(n = 8)	(n = 8)	(n = 10)
1	Imp. Invag.	20	0	0	0	0	0
2	Evagination	60	0	0	0	0	0
4	Evagination	10	0	0	0	0	0
	Imp. Invag.	10	0	0	0	0	0
	Loss	0	0	13	0	0	0

Numbers represent percentages of MRs showing loss, evagination or impairment in invagination from a total 'n'. Abbreviations: Imp. Invag: Impaired Invagination; n: total number of MRs analyzed. doi:10.1371/journal.pone.0079845.t004

Table 5. Percentages of MRs showing retention and evagination in male *Gli2*^{1ki/1ki}; *Gli3*^{xt/+} embryos at E18.5.

MR#	Phenotype	<i>Gli2</i> ^{1ki/1ki} ; <i>Gli3</i> ^{xt/+}	<i>Gli2</i> ^{1ki/1ki}	<i>Gli2</i> ^{1ki/+} ; <i>Gli3</i> ^{xt/+}	<i>Gli2</i> ^{1ki/+}	<i>Gli3</i> ^{xt/+}	<i>Gli3</i> ^{xt/+}
		(n = 32)	(n = 10)	(n = 8)	(n = 14)	(n = 16)	(n = 16)
1	Retention	69	25	0	0	0	0
	Evagination	0	0	–	–	–	–
2	Retention	44	0	25	0	0	0
	Evagination	44	–	0	–	–	–
4	Retention	16	0	13	0	0	0
	Evagination	9	–	0	–	–	–

Numbers represent percentages of MRs that are retained or protrude [in brackets] from the surface of male skin whole-mounts from a total 'n'. Abbreviations: n: total number of putative sites for MRs on male skin whole mounts.

doi:10.1371/journal.pone.0079845.t005

Positive Hh Signaling in Embryonic Nipple Development

Although this study shows that repression of the Hh pathway is essential for both early and late embryonic mammary development, we found evidence that the Hh pathway becomes activated during embryonic nipple development. *Gli1-LacZ* expression occurs ~E17.5 within the mammary mesenchyme underlying the developing nipple sheath and surrounding the lactiferous ducts of all wild-type glands. Nipples are sites of regional epidermal specialization and their formation is dependent upon inductive signals from the underlying ventral dermis to the overlying ventral epidermis [11,13,14,78,89]. Due to the poor survival of *Gli2*^{lzki/lzki} embryos at this stage we have been unable to address whether positive Hh signaling is essential for embryonic nipple development [36,90]. However the timing of the *Gli1-LacZ* reporter expression suggest the possibility that positive Hh signaling may participate in this patterning process. Positive Hh signaling continues postnatally within the nipple connective tissue and is robust in cell-types that distinguish nipple stroma from adjacent ventral dermis. For example, *Gli1-LacZ* is expressed within cells running circumferential to the lactiferous duct that provide mechanical support to the nipple during suckling. Hh signaling is also active in smooth muscle of the nipple sphincter that play important functions during the milk let down response. *Gli1-LacZ* is also expressed in cells surrounding capillaries, which are abundant in nipple and serve to nourish the thickened epidermis, and surrounding nerve tracts that send stimuli leading to oxytocin release [14,82,89]. These histological specializations of the nipple connective tissue form during the first few weeks after birth but are predetermined during embryonic exposure of the mammary mesenchyme to PTHrP, as demonstrated by their *de novo* induction in the entire ventral dermis of female mice overexpressing PTHrP under the control of the keratin 14 promoter [82,91]. The relationship between Hh activity and the development of the nipple stroma remains an important question for future study.

Experimental Procedures

Mice

The following mice were maintained on an outbred background. *Gli1*^{lzki/+}, *Gli2*^{1ki/+}, *Gli3*^{xt/+} mice were generously provided by Dr. Alexandra Joyner, Memorial Sloan Kettering Cancer Institute, and constructed as described [36,37] and *Conductin*^{lz/+} (also called *Axin2*^{g2/+}) mice were a gift from Dr. Franke Costantini, Columbia University [57,58]. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of New York University School of

Medicine (NYUSOM). The animals were monitored and cared for daily at the NYUSOM Skirball Central Animal Facility (SCAF), which were maintained to be sterile and clean, requiring full gowning (head covers, masks, gowns, gloves and booties) procedures to ensure that the animals are not exposed to outside pathogens. Before sacrifice, mice were first anaesthetized using carbon dioxide and then euthanized by cervical dislocation. All animal care and euthanasia procedures adhered to the guidelines specified by the NYUSOM Division of Laboratory Animals Resources (DLAR: www.med.nyu.edu/dlar).

Whole-mount X-Gal Staining

For detection of *LacZ* expression, embryos or tissues were fixed in 4% paraformaldehyde (PFA, Sigma Aldrich, St. Louis, MO) diluted in phosphate buffered saline (PBS) for 30 minutes, followed by four 15 minutes washes in rinse buffer (2 mM MgCl₂, 0.1% sodium deoxycholate, 0.2% NP40 prepared in PBS). X-Gal staining was carried out at room temperature for 2–3 hours in staining buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal, Denville Scientific, South Plainfield, NJ) prepared in rinse buffer). After staining, embryos and tissues were washed in PBS, post-fixed for overnight in 4% PFA at 4°C and viewed under a Zeiss Axiovert (Oberkochen, FRG) brightfield dissecting microscope.

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% hydrogen peroxide, treated with 4 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 5X SSC, 50 µg/ml tRNA, 1% SDS and 50 µg/ml heparin followed by hybridization overnight at 70°C in the same buffer containing 2 µg/ml of digoxigenin (DIG) labeled *Bmp4* or *Tbx3* 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). Embryos were postfixed in 4% PFA, embedded in paraffin and sectioned. The distance between the fore- and hind- limb was relatively uniform in all genotypes therefore we determined the extent of *Bmp4* expression

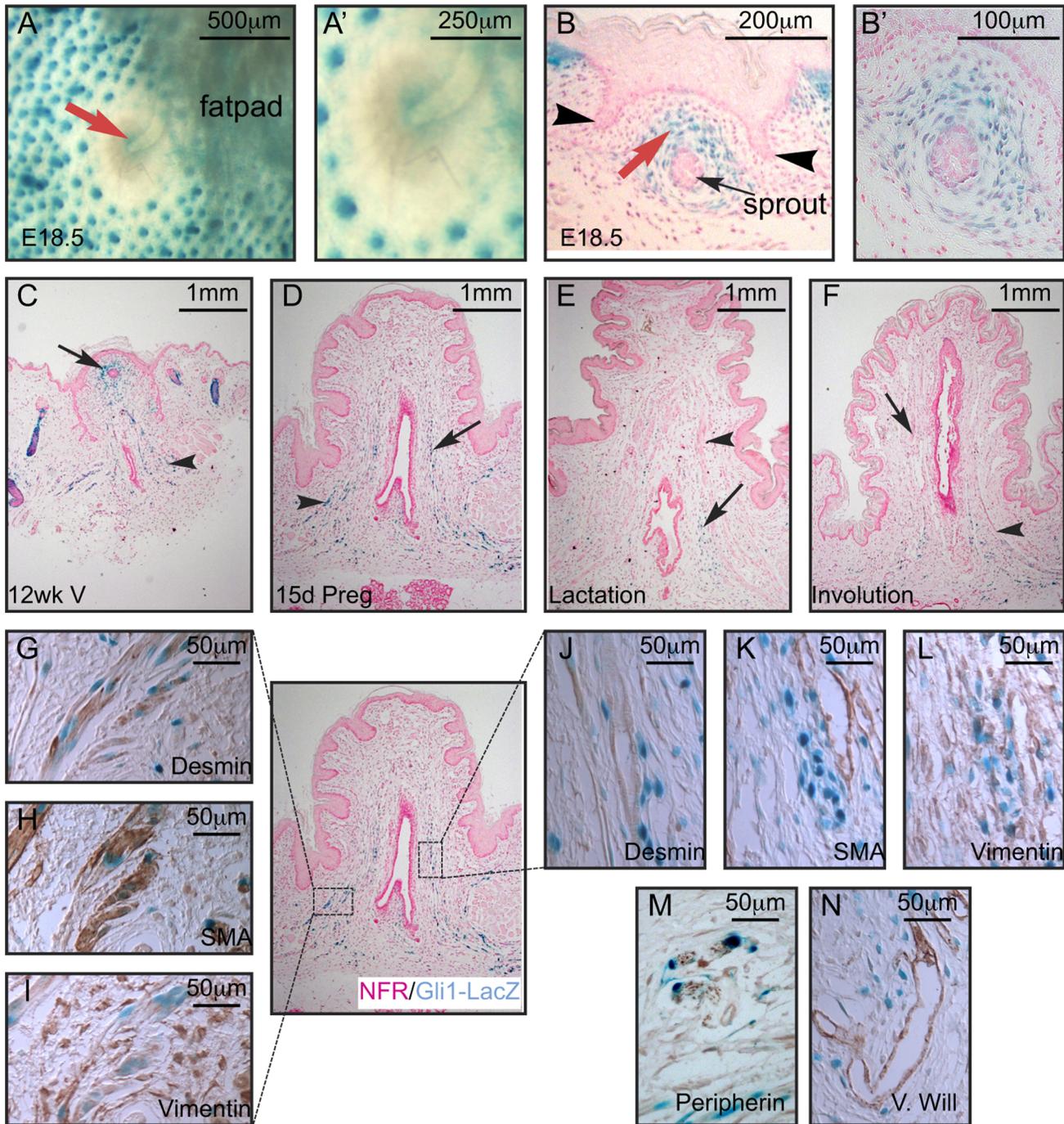


Figure 10. Expression and modulation of *Gli1-LacZ* in the adult nipple during the pregnancy cycle. *Gli1-LacZ* expression is visible at the neck of the mammary sprout (red arrow) in skin whole-mounts of E18.5 *Gli1^{l2kl/+}* embryos at low (A) and higher power (A'). Histological section through the sprout shows expression of *Gli1-LacZ* within the stroma (red arrow) surrounding the sprout (black arrow) underneath the nipple sheath (black arrowheads) at low (B) and higher power (B'). *Gli1-lacZ* is expressed within the dermal component but not the epithelium in virgin (C), mid pregnant (D), lactating (E) and involuting (F) nipples. Immunohistochemistry for desmin (G, J), SMA (H, K) and vimentin (I, L) on serial sections of a 15.5 day pregnant nipple demonstrated that *Gli1-lacZ* was expressed in both smooth muscle cells and fibroblasts but not myofibroblasts. *Gli1-lacZ* was also found near and surrounding peripherin positive nerve tracts (M) and both Von Willebrand positive vessels (N). doi:10.1371/journal.pone.0079845.g010

by measuring the distance between the base of the fore-limb bud (axilla) and the most posterior tip of expression (indicated by white dotted lines in Fig. 1B, E) and compared the distances in mm between wt and *Gli3^{xt/xt}* embryos (n = 6 each) using the student's t test. The breadth of the band of *Tbx3* expression was measured (in

mm) at the location of mammary placode 3 (that falls between somites 16 and 17 in wt embryos [54]) and compared between wt and *Gli3^{xt/xt}* embryos (n = 6 each) using the student's t test.

Carmine Staining

For detection of mammary sprouts in E18 embryos, skins were removed from the embryos and fixed in 4% PFA for 1 hour. The skins were washed in PBS then stained for 1 hour in carmine solution diluted 1:5 in water. Carmine was prepared by boiling 1 g carmine alum and 25 g aluminum potassium sulfate in 500 mL of water for 20 minutes followed by filtration.

Histology

For histological analysis, embryos and tissues were stained as above with X-Gal, post-fixed with 4% PFA overnight at 4°C then embedded in paraffin and sectioned. Isopropanol was substituted for xylene to prevent diffusion of the X-Gal stain during processing.

Immunohistochemistry

Four μm sections were deparaffinized by baking at 60°C and incubating slides in Citrisolv (Fisher Scientific, Pittsburgh, PA) and rehydrated through a graded series of ethanol. Citric acid antigen retrieval was performed for all antibodies by placing slides in 10 mM sodium citrate pH 6.0 and boiling in a microwave at 90 W power for 30 minutes. Primary rabbit antibodies against AR (Santa Cruz Biotechnologies, Santa Cruz, CA) (1:100), Desmin (Abcam, Cambridge, MA) (1:50), Peripherin (Chemicon, Temecula CA) (1:1000) and Von Willebrand Factor (Sigma Aldrich) (1:1000), mouse antibodies against p63 (Neomarkers, Fremont,

CA) (1:500), SMA (Sigma Aldrich) (1:5000), Tenascin C (Immuno Biological Laboratories, Gunma, Japan) (1:500) and ER (Novocastra, Newcastle, U.K.) (1:500) and guinea pig antibodies against Vimentin (Progen, Heidelberg, Germany) (1:1000) were added overnight at 4°C. Biotin-labeled secondary antibodies (Vector Laboratories, Burlingame, CA) (1:1000) and streptavidin-HRP (Vector Laboratories) (1:200) were added for 30 minutes each, and colorimetrically detected using diaminobenzidine (Vector Laboratories). Sections were counterstained for better visualization in 0.1% solution of Nuclear Fast Red (NFR, Polyscientific, Bayshore, NY) for 1 minute and washed in a stream of running water for 5 minutes. Sections were then dehydrated and dipped in Citrisolv (Fisher Scientific) before being mounted in Cytoseal (VWR, Radnor, PA).

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Author Contributions

Conceived and designed the experiments: SH PC. Performed the experiments: AC SH AP LK. Analyzed the data: AC SH AP LK PC. Wrote the paper: AC SH AP LK PC.

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