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 (Gli3R) or activator (Gli3A) depending upon cellular context. Previously, we have shown that Gli3R 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 Gli3lox/lox mice. Importantly, mice genetically manipulated to misactivate Hh signaling displayed the same phenotypic spectrum demonstrating that the repressor function of Gli3R 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 Gli3R is critical for early placodal patterning and later mammary mesenchyme specification whereas positive Hh signaling occurs during nipple development.

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 buds 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 (Ptc1, Ptc2) on neighboring cells [26,27]. This event relieves the seven-pass transmembrane protein smoothened (Smo) from Ptc1-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, Pch 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 (GliA, GliR) forms of Gli proteins [31,32,33]. Gli2 is expressed independently of Hh signals in a functionally inactive form (Gli2R) but becomes processed in response to Hh signals into an activator (Gli2A) that initiates Hh gene transcription [34,35,36,37]. Gli1 is transcribed in a strictly Hh-dependent manner and once expressed, constitutively activates Hh signaling [38,39,40,41,42]. These features make it a pathway amplifier [38,39,40,41,42]. In the absence of an activator (Gli2A) that initiates Hh gene transcription [34,35,36,37]. Gli1 is transcribed in a strictly Hh-dependent manner and once expressed, constitutively activates Hh signaling [38,39,40,41,42]. These features make it a pathway amplifier [38,39,40,41,42]. In the absence of
of Hh signals, Gli3R is proteolytically processed into Gli3K. Hh signals prevent this proteolytic conversion and also transcriptionally downregulate Gli3 [27,35,43,44,45]. Most tissues maintain a specific Gli3R/Gli3K ratio by feedback mechanisms regulating downstream target gene expression. The processing of Gli proteins occurs within the primary vestigial organelle, primary cilium [46]. Intralellar 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 normal bud evagination, hair follicle encroachment and loss of sexual dimorphism. These data provide genetic evidence that repression of Hh signaling by Gli3K is required for MR#2 invagination, hair follicle suppression, and loss of male mammmary 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<sup>xt/xt</sup>) that lack Gli3 expression. To test whether the Gli3 phenotype results from loss of Gli3 repressor (Gli3K), or activator (Gli3K<sup>R</sup>) function we utilize two strains. Gli1 strictly dependent on Hh signals for its expression and thus is an excellent reporter of positive Hh signaling [31,57]. 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<sup>kit/kit</sup> or Gli2<sup>kit/kit</sup> mice) tests the effect of activating Hh signaling within the Gli2 field of expression. If misactivation (Gli2<sup>kit/kit</sup> mice) produces the same or exacerbates the phenotypes of Gli3<sup>xt/xt</sup> mice, this provides genetic evidence that the Gli3<sup>wt/xt</sup> phenotypes result from loss of Gli3K activity. In contrast, if the Gli2<sup>kit/kit</sup> and Gli3<sup>xt/xt</sup> 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<sup>wt/xt</sup> Embryos

Previously, we have shown that mice lacking Gli3 expression (Gli3<sup>kit/kit</sup>) or those genetically manipulated to misactivate Hh signaling (Gli2<sup>kit/kit</sup>;Gli3<sup>wt/xt</sup>) lack mammmary placodes #3 and #5 [51]. This demonstrated that for early embryonic mammary development Gli3K repression of Hh signaling is essential. As Gli3<sup>kit/kit</sup> mutants lack expression of positive placodal regulators within the MR#3 and #5 regions [51,54] we reasoned that Gli3K 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 <i>in situ</i> 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<sup>wt/xt</sup>: 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<sup>kit/kit</sup> 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<sup>wt/xt</sup> 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<sup>wt/xt</sup> embryos (n = 6) and wt (n = 6) siblings by <i>in situ</i> 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<sup>wt/xt</sup> 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<sup>kit/kit</sup> embryos (lacking Gli3), mammmary 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
During later development we examined these remaining MRs in skin whole-mounts from E17.5 and E18.5 female Gli3<sup>xt/xt</sup> embryos (n = 49). We also took advantage of the fact that hair follicles can be distinguished from MRs in control Gli1<sup>lzki/+</sup> and in mutant Gli3<sup>xt/xt</sup>; Gli1<sup>lzki/+</sup> 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<sup>lzki/+</sup> (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<sup>xt/xt</sup> 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<sup>lzki/+</sup> and mutant Gli3<sup>xt/xt</sup>; Gli1<sup>lzki/+</sup> embryos. Hair follicles were appropriately excluded from the presumptive nipple areolar zone of all MRs from control Gli1<sup>lzki/+</sup> embryos (Fig. 3D–F and Fig. 4C) as well as from MR#1 and MR#4 of mutant Gli3<sup>xt/xt</sup>; Gli1<sup>lzki/+</sup> embryos (Fig. 3G and I; Fig. 4A). In contrast, in mutant Gli3<sup>xt/xt</sup>; Gli1<sup>lzki/+</sup> 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).
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<sup>lox/lox</sup> to Conductin<sup>lacz/+</sup> 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<sup>lacz/+</sup> 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<sup>lox/lox</sup>;Conductin<sup>lacz/+</sup> embryos Conductin-LacZ expression was present only within the mesenchymal compartment (Fig. 5B). Next we examined serial sections of Gli3<sup>lox/lox</sup>;Gli1<sup>lox/+</sup> 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<sup>lox/+</sup> 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<sup>lox/lox</sup>;Gli1<sup>lox/+</sup> 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<sup>lacz/+</sup> embryos: being diminished within the mesenchyme and robustly upregulated within the mammary sprout (Fig. 6A). In contrast, Gli3<sup>lox/lox</sup>;Conductin<sup>lacz/+</sup> embryos maintained robust reporter expression in the MR#2 mammary mesenchyme but failed to upregulate expression within the epithelium (Fig. 6B). Control Gli3<sup>lox/lox</sup> embryos continued to show robust expression of ER, AR and Tenascin C within the mammary mesenchyme surrounding the MR#2 sprout (Fig. 6C, E, G). However, Gli3<sup>lox/lox</sup>Gli1<sup>lox/+</sup> 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<sup>lox/lox</sup> 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 [3,9,15,17,18,20,59]. 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<sup>lox/+</sup> embryos all MRs were appropriately lost by E16.5 (Table 2). Male Gli3<sup>lox/lox</sup> embryos, like their female counterparts failed to form MR#3 and MR#5. However, in Gli3<sup>lox/lox</sup> 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
(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 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 Gli3xt/xt;Conductinlz/+ 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+/- mice, which drives the expression of constitutively active Gli1 transactivator under the control of Gli2 promoter to Gli3xt/xt; Gli3x+/-; Gli1lzki/lzki mice. The genotypes arising from this cross alter the GliR:GliA ratio to progressively misactivate the pathway.

| MR# | Phenotype     | Gli3+/- (n=50) | Gli3+/- (n=90) | Gli3−/+ (n=58) |
|------|---------------|----------------|----------------|----------------|
| 1    | Loss          | 4              | 0              | 0              |
| 2    | Evagination   | 57             | 0              | 0              |
| 3    | Loss          | 27             | 0              | 0              |
| 4    | Evagination   | 6              | 0              | 0              |
| 5    | Imp. Invag.   | 4              | 0              | 0              |
| 6    | 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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Analysis of skin whole-mounts from E18.5 females showed that misactivation of the Hh pathway in \( Gli2\text{li}/\text{li}; Gli3\text{xt}/\text{xt}; Gli1\text{lz}/\text{li} \) embryos (\( n = 10 \)) produces the same spectrum of phenotypes seen in \( Gli3\text{xt}/\text{xt}; Gli1\text{lz}/\text{li} \) 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 \( Gli2\text{li}/\text{li}; Gli3\text{xt}/\text{xt}; Gli1\text{lz}/\text{li} \) embryos (Fig. 9A), in a manner similar to that shown previously for \( Gli3\text{xt}/\text{xt}; Gli1\text{lz}/\text{li} \) 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).

Figure 4. Evagination of MR\#2 and encroachment of hair follicles in Gli3\text{xt}/\text{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\text{xt}/\text{xt}; Gli1\text{lz}/\text{li} mutants and MR\#2 from control Gli1\text{lz}/\text{li} (C) embryos show normal invagination and appropriate exclusion of hair follicles. In contrast MR\#2 from Gli3\text{xt}/\text{xt}; Gli1\text{lz}/\text{li} mutants (B, D) showed prominent evagination and encroachment of hair follicles. doi:10.1371/journal.pone.0079845.g004

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\text{lz}/\text{li} 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\textsuperscript{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\textsuperscript{lz/+} embryos (A) show Wnt/\textbeta-catenin signaling pathway activity in the mammary mesenchyme and central epithelial cells whereas Gli3\textsuperscript{xt/xt}; Conductin\textsuperscript{lz/+} embryos (B) show activation only within the mesenchymal compartment. Immunohistochemical analysis of serial sections from control E13 Gli1\textsuperscript{lzki/+} (C, E, G) and mutant Gli3\textsuperscript{xt/xt};Gli1\textsuperscript{lzki/+} (D, F, H) embryos for expression of (C, D) p63, (E, F) ER and (G, H)AR. Note the epithelium of Gli3\textsuperscript{xt/xt};Gli1\textsuperscript{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).

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Figure 6. Gain of epithelial Wnt signaling and maintenance of mammary mesenchyme markers is compromised in MR#2 of Gli3<sup>xt/xt</sup> 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<sup>lz/+</sup> embryos whereas (B) mutant Gli3<sup>xt/xt</sup>; Conductin<sup>lz/+</sup> 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<sup>lz/+</sup> embryos. In contrast, ER was lost (D) and Tenascin C and AR expression were weakened (F, H) in mutant Gli3<sup>xt/xt</sup>;Gli1<sup>lz/+</sup> embryos. doi:10.1371/journal.pone.0079845.g006
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 Gli3R lies upstream of Bmp4/Tbx3 specification of placode #3. Second, that at later stages Gli3R 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.

Gli3R 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 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 of the site of placodal #3 points to the involvement of an intervening Hh suppressor, and previous studies suggest that Bmp4 is the most likely candidate for this role [12]. Tbx3 is a direct transcriptional target of Bmp4/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 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 hindgut, kidney and prostate [55,56,72,73,74]. Supporting the possibility that Bmp4 is a direct transcriptional target of positive Hh signaling and Gli3R repression, Gli binding sites are present in the murine Bmp4 promoter and transfection of cDNAs encoding Gli1 or Gli3Δ 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.

Gli3R Influences Mammary Mesenchyme Specification and Function

Our results show that although early Gli3R 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 is a unique set of regulatory requirements [1,77]. In Gli3/−/− 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/−/− 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 ß-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 Mst2 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

| Table 2. Percentages of MRs showing retention in male Gli3xt/xt embryos at E16.5 and E18.5. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| MR#            | Retention (E16.5) | Retention (E18.5) |
|                | Gli3ext/xt | Gli3ext/xt | Gli3+/− | Gli3ext/xt | Gli3+/− |
| (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

| Table 3. Percentages of evaginated MRs in male Gli3xt/xt embryos at E16.5 and E18.5. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| MR#            | Evagination (E16.5) | Evagination (E18.5) |
|                | Gli3ext/xt | Gli3ext/xt | Gli3+/− | Gli3ext/xt | Gli3+/− |
| (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
Our results show that Gli3<sup>xt/xt</sup> 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 compartments 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.

Figure 7. Sexual dimorphism is lost in Gli3<sup>xt/xt</sup> embryos. X-Gal stained skin whole-mounts from E18.5 male Gli3<sup>xt/xt</sup>;Gli1<sup>lzki/+</sup> 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<sup>xt/xt</sup>;Conductin<sup>lzki/+</sup> embryos at this stage (E).

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sexual dimorphism [15,17,18,19,20,59]. Our results show that Gli3<sup>xt/xt</sup> 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 compartments 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
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\(^{xt/xt}\)) 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 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\(^{40/40}\);Conductin\(^{lz/+}\) embryos retained mesenchymal Conductin-LacZ expression (A’) and showed weak Tenascin C (B’) and AR (C’) expression.

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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 in Gli3\(^{xt/xt}\) male embryos.
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/+};Gli1lzki/+ 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};Gli1lzki/+ embryos. Serial sections through a male MR#2 from Gli2^{1ki/1ki};Gli3^{xt/+};Gli1lzki/+ 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.

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

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

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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 epithelial 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 Gli2lkizki/lkizki 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 suggests 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 and 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. Gli2lkizki/lkizki, Gli2lk/zki, Gli3zikki mice were generously provided by Dr. Alexandra Joyner, Memorial Sloan Kettering Cancer Institute, and constructed as described [36,37] and Conductinlkizki/kizki (also called Axin2lkizki/zki) 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 Bnap4 or Tb3x3 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 Bnap4 expression

### Table 5. Percentages of MRs showing retention and evagination in male Gli2lkizki/lkizki, Gli3zikki embryos at E18.5.

| MR# | Phenotype | Gli2lkizki/lkizki | Gli2lk/zki | Gli3zikki | Gli2lkizki/lkizki; Gli3zikki | Gli2lkizki/lkizki; Gli2lk/zki | Gli2lkizki/lkizki; Gli3zikki | Gli2lkizki/lkizki; Gli2lk/zki; Gli3zikki |
|-----|-----------|-------------------|------------|----------|-----------------------------|-----------------------------|-----------------------------|---------------------------------|
| 1   | Retention | 69                | 25         | 0        | 0                            | 0                            | 0                           | 0                               |
|     | Evagination | 0                 | 0          | –        | –                            | –                            | –                           | –                               |
| 2   | Retention | 44                | 0          | 25       | 0                            | 0                            | 0                           | 0                               |
|     | Evagation | 44                | –          | 0        | –                            | –                            | –                           | –                               |
| 4   | Retention | 16                | 0          | 13       | 0                            | 0                            | 0                           | 0                               |
|     | Evagation | 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.

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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 Gli3xt/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+/- embryos (n = 6 each) using the student’s t test.

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+/- 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 expression is also found near and surrounding peripherin positive nerve tracts (M) and both Von Willebrand positive vessels (N). doi:10.1371/journal.pone.0079845.g010
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 alun 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) (1:1000), 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), Tenasin 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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