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ACTIVATION OF ERK BY SONIC HEDGEHOG INDEPENDENT OF CANONICAL HEDGEHOG SIGNALLING

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Abstract

Hedgehog (Hh) signalling is mediated through the Patched-1 (Ptch1) receptor. Hh-binding to Ptch1 blocks the inhibitory effects of Ptch1 on the activity of the transmembrane protein, Smoothened (Smo), resulting induction of target genes by the Gli-family of transcription factors. We demonstrate here that Hh-binding to Ptch1 stimulates activation of Erk1/2. This activation is insensitive to the small molecule Smo antagonists and occurs in a cell line that does not express *Smo*. Specifically, the C-terminus of Ptch1 harbors motifs encoding Class I and II SH3-binding sites. SH3-domain binding activity was verified using GST-c-src^{SH3}, -Grb2^{SH3} and -p85β^{SH3} fusion-proteins. Ectopically-expressed Grb2 or p85β could also be co-immunoprecipitated with the Ptch1 C-terminus. Addition of Shh to serum-starved human mammary epithelial cells and Shh Light II fibroblasts stimulated phosphorylation of Erk1/2. Erk1/2 activation was observed in cells where Smo activity had been inhibited using cyclopamine and in the breast epithelial cell line, MCF10A, that does not express *Smo*. These data reveal novel binding activities for the C-terminal region of Ptch1 and define a signalling pathway stimulated by the Hh-ligands operating independently of pathways requiring Smo.

Keywords

Hedgehog; smoothened; patched-1; Erk; SH3-domain; signal transduction

INTRODUCTION

The Hedgehog (Hh)-signalling pathway is an essential developmental pathway controlling cell fate and morphogenesis. In vertebrates, three Hh-ligands, Sonic (Shh), Indian (Ihh) and Desert Hedgehog (Dhh) (Echelard et al., 1993, Krauss et al., 1993), all closely related to Hedgehog (Hh) in Drosophila, regulate a well defined molecular-genetic signal transduction pathway. In its basic form, the 12-pass transmembrane receptor, Patched-1 (Ptch1), regulates the activity of the transcriptional effectors of Hh-signalling, Gli2 and Gli3 (Ruppert et al.,

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1988, Mo et al., 1997, Borycki et al., 1998, Ding et al., 1998, Sasaki et al., 1999), these latter transcription factors are closely related to Drosophila Cubitus interuptis (Ci) (Orenic et al., 1987, Orenic et al., 1990, Ruppert et al., 1990, Tiniakow and Terentieva, 1933). In the absence of Hh-ligands, the principal activity associated with Ptch1 is repression of the activity of a 7-pass transmembrane protein, Smoothened (Smo) (Alcedo et al., 1996). Inhibition of Smo by Ptch1 results in the maintenance of a cytoplasmic proteolytic complex containing Suppressor of Fused (SuFu). The proteolytic activity of this complex cleaves the C-terminal domains of the Gli proteins (Gli2 or Gli3), yielding transcriptional repressor forms (Aza-Blanc et al., 1997, Sasaki et al., 1999, Ruiz i Altaba, 1999). Upon binding of the Hh-ligands to Ptch1 (Chen and Struhl, 1996, Alcedo et al., 1996) inhibition of Smo is relieved. Smo activation, in turn, blocks the proteolytic activity of the SuFu-containing complex, thereby maintaining the Gli proteins in their transcriptional activator state and ultimately driving expression of Hh-target genes (Ohlmeyer and Kalderon, 1998, Lum et al., 2003, Pham et al., 1995, Therond et al., 1996, Hepker et al., 1997, Sisson et al., 1997, Monnier et al., 1998, Robbins et al., 1997, Forbes et al., 1993). Despite Ptch1-dependent regulation of Smo activity playing a central role in mediating Hh-signalling, the mechanisms by which this regulation occurs are not well understood.

Patched-1 is known to contain domains that suggest it may participate in the regulation of additional molecular pathways. Previous studies, for example, have defined a region in the sterol-sensing domain of Ptch1 (Loftus et al., 1997, Martin et al., 2001) that complexes cyclin B1 in a Hh-dependent manner (Barnes et al., 2001). This association suggests that the Hh-pathway may be involved in control of the cell division cycle specifically at mitosis. While genetic evidence for the significance of this Ptch1-mediated activity has not been shown, it may represent a Hh-dependent check-point control mechanism consistent with a mitogenic role for the Hh-signalling pathway (Forbes et al., 1996, Dahmane and Ruiz i Altaba, 1999, St-Jacques et al., 1999, Kenney and Rowitch, 2000, Fu et al., 2004, Mill et al., 2005). Distinct from the domain responsible for binding cyclin B1, the C-terminal cytoplasmic domain of Ptch1 binds to the molecular chaperone, Tid1 (Wakabayashi et al., 2007). Tid1 activity has been implicated in signalling pathways that involve ras (Tarunina et al., 2004, Trentin et al., 2001), Smads (Torregroza and Evans, 2006) and ErbB2 (Kim et al., 2004) as well as in the control of apoptosis (Syken et al., 1999, Edwards and Munger, 2004) and cell senescence (Tarunina et al., 2004). The significance of the interaction between Tid1 and Ptch1 has been shown using the *FVB* mouse strain. *FVB* harbors a polymorphism in the C-terminus of Ptch1 effecting its ability to complex Tid1 (Wakabayashi et al., 2007). Here, susceptibility of Kras-induced skin squamous cell carcinomas in *FVB* mice segregates with the mutant *Ptch1* allele. How binding of the Hh-ligands to Ptch1 effects the association of Tid1 with Ptch1 and alters signalling through downstream pathways has not been characterized however.

As we published previously (Moraes et al., 2009), the role of the Hh-signalling pathway in control of the growth and morphogenesis of the mammary gland was studied using mice carrying the *Mesenchymal dysplasia* (*mes*) allele of *Ptch1* (Ptch1^{mes}) (Sweet et al., 1996). Ptch1^{mes} lacks most of the cytoplasmic C-terminal region of Ptch1 due to a 32 bp deletion in the last exon, resulting in a frameshift and truncation of the last 220 amino acids (Makino et al., 2001). Despite this mutation, mice homozygous for the *mes* allele of *Ptch1* are viable but sterile and exhibit polydactyly, a white belly spot, precocious hair follicle development (Nieuwenhuis et al., 2007) and, as we have recently shown, severe defects in mammary gland development during puberty (Moraes et al., 2009).

Previous studies of Ptch1^{mes} mice showed that, in the dermis of these animals, no significant alterations were evident in the levels of *Ptch1* or *Gli1* (Nieuwenhuis et al., 2007), both transcriptional targets of the canonical Hh-signalling cascade (Dahmane et al., 1997, Lee et

al., 1997, Alexandre et al., 1996, Marigo et al., 1996, Forbes et al., 1993). We speculated, therefore, that Hh-signalling may recruit or activate other signalling cascades through the C-terminal region of Ptch1 independent of its Smo-dependent functions. Our results show that the C-terminal region of Ptch1 binds to SH3-encoding domains of a number of proteins, including Grb2, c-src and p85β. We demonstrate further that Shh can stimulate a U0126-sensitive signalling cascade that activates Erk1/2. Furthermore, activation of Erk1/2 occurs in cell lines lacking *Smo* or in the presence of the small chemical inhibitors of Smo.

MATERIALS AND METHODS

Mice

Mesenchymal dysplasia (*mes*) mice are an inbred strain of mice harboring a 32 bp deletion of the coding region of the C-terminal cytoplasmic domain of the *Ptch1* gene, resulting in truncation of the Ptch1 protein at the beginning of the cytoplastic domain (Makino et al., 2001). These mice were obtained from Jackson Labs and backcrossed onto the C57BL/6 background (Charles River) for >10 generations.

Expression Constructs

RNA from Ptch1$^{wt/mes}$ heterozygote animals was isolated using Trizol according to the manufacturer's instructions. The cytoplastic domains of Ptch1^{wt} and Ptch1^{mes} were ampified by RT-PCR and the cDNA cloned into in frame with the pEGFP-C1 vector in order to express N-terminally GFP-tagged C-terminal domains of Ptch1^{wt} and Ptch1^{mes} fusion proteins. The previously described (Nieuwenhuis et al., 2007) expression constructs encoding the full length wild type or Ptch1^{mes} Ptch1 proteins tagged at their N-terminus with GFP were a kind gift of Dr. C. C. Hui (Hospital For Sick Children Research Institute, Toronto, Canada)

Cell Culture

Human mammary gland epithelial cells (HMEC) and MCF10A cell lines were cultured in DMEM/F12 medium with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocordisone, 5 μg/ml transferin, 1 ng/ml cholora toxin, 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere containing 5% CO2. Shh Light II fibroblasts (Sasaki et al., 1997), which harbor a Gli-responsive luciferase transgene, were obtained from ATCC. Cells were grown in DMEM/F12 medium with 10% fetal bovine serum plus 100 μg/ml streptomycin and 100 U/ml penicillin. For serum starvation, HMEC or Shh Light II cells were switched to media containing 0.1% horse serum, for 48 hours. For MCF10A cells, cells were trypisinized and replated in growth conditions for 4 hours in order for the cells to re-attach. Media was then changed to DMEM/F12 without fetal bovine serum for 24 hours.

Shh Peptide and Chemical Inhibitors

N-Shh peptide was purchased from R&D System. Before stimulation, cultured cells were serum starved using DMEM/F12 medium with 0.1% horse serum for 48 hours and thereafter stimulated with 1μg/ml of N-Shh for indicated time points. For chemical inhibitors, 5 μM of the Smo inhibitor, cyclopamine (LC Laboratories),was added to cells 24 hours before stimulation with the N-Shh peptide for HMEC and Shh Light II cells. 5 nM of the farnesyl transferase inhibitor, H-Cys-4-Abz-Met-OH (FTase II; Sigma) was added to cells 1 hour before exposure to N-Shh peptide. The MEK-specific inhibitor, U0126 (Sigma), was added to a final concentration of 10nM to cells one hour prior to addition of Shh peptide. For inhibition of N-Shh activity, 20 μl of conditioned media containing the α-Shh monoclonal antibody expressed from the hybridoma cell line, 5E1, (Developmental Studies Hybridoma

Bank, University of Iowa), was mixed with the N-Shh peptide and added to serum-starved cells.

Phospho-Erk and Luciferase Assays

For phospho-Erk assays, cells were trypsinized and replated in complete medium for 4 hours. Attached cells were then switched to media lacking serum for 48 hours. Serum starved cells were then stimulated with N-Shh. For measurement of the activation of Erk1/2, cells were harvested at various times points and lysed in buffer containing 0.5% Nonidet P-40, 120 mM NaCl, 50 mM Tris, pH 8.0. Following centrifugation, 40 μg of whole cell lysate was run on 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose. Blots were then probed for using an α-phospho-Erk antibody (Cell Signalling). Blots were then stripped and reprobed with an antibody directed to total Erk (Cell Signalling).

For luciferase activity in Shh Light II cells, luciferase activity was determined as previously described (Sasaki et al., 1997). Briefly, cells were lysed in lysis buffer at room temperature for 15 minutes followed by centrifugation at 13,000rpm for 1 minute. Cell lysates were transferred to a new tube and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase was used as the reporter gene and Renilla luciferase was used as control for normalization. Luciferase activity in lysates was read in plates using a Berthold Technologies microplate luminometer. Luciferase assays were performed three times with each sample assayed in triplicate. For MCF10A mammary epithelial cells, cells grown in complete medium were transfected using Lipofectamine 2000 (Invitrogen) preincubated at room temperature for 20 minutes either with 1μg of the 8xGli-Luc reporter plasmid, 1 μg of CMV-βgalactosidase (control for transfection efficiency) and either 1μg of activated Gli2 (Gli2ΔN) expression plasmid or 1μg of pcDNA3. Twenty-four hours after transfection cells were switched to serum free medium and starved for 24 hours. N-Shh peptide was added at the final concentration of 1μg /ml, grown for another 24 hours and the measured luciferase activity normalized to βgal activity.

GST Binding Assay

The GST fusion constructs encoding the SH3 domains of Grb2, p85 or c-src were kindly provided by Dr. M. Moran (University of Toronto). GST-fusion proteins were expressed in bacteria and purified on glutathione sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. An equal amounts (2 μg) of purified GST or GST-Grb2^{SH3}, GST-P85^{SH3}, GST-P85^{SH3} were incubated with glutathione beads for 2 hours at 4°C. After washing, the beads were incubated with whole cell lysates containing GFP-Ptch1^{wt} or GFP-Ptch1^{mes} protein and were rocked for 2 hours at 4°C. The beads were washed five times with Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 120 mM NaCl, 50 mM Tris, pH 8.0). Bound proteins were eluted by boiling in SDS buffer for 5 minutes before loading onto 10% SDS-polyacrylamide gels.

Immunoprecitations and Co-immunofluorescence

COS-1 or 293 cells were transfected with plasmids expressing triple-HA-tagged Ptch$^{C\text{-}term}$ and either GFP-tagged full length Grb2 (Yamazaki et al., 2002)(a kind gift of Dr. L. Samelson (National Institutes of Health, Bethesda Maryland, U.S.A.), flag-tagged PIK3R2 (p85β), wild type, flag-tagged Smurf2 or the catalytically-defective Smurf2 mutant, Smurf CA ((Ogunjimi et al., 2005); the latter three expression constructs obtained from Dr. J. Wrana, Samuel Lunenfeld Res. Inst., Toronto, ON, Canada). Forty-eight hours following transfection, cell lysates were prepared as described above for the phospho-Erk analysis. Anti-GFP, anti-flag or anti-HA antibodies were added to 200 μg of lysate from transfected cells. Immune complexes were then isolated on protein G-sepharose beads and isolated proteins identified by western analysis.

HMEC cells grown on coverslips were transfected with expression vectors expressing GFP-tagged, full length Ptch1, full length Ptch1^{mes} or GFP-fusion proteins with the C-terminal region of Ptch1 or Ptch1^{mes}. Forty-eight hours post transfection, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After washing with PBS 3 times, cells were permeabilized with 0.1% Triton X-100 for 2 minutes then treated with 3% BSA. Cells were probed with α-EEA-1 (Applied Biological Materials Inc.) for 1 hour at room temperature. After three washes with PBS, cells were incubated with a TRITC-conjugated goat anti-mouse secondary antibody for 1 hour at room temperature, then washed in PBS and embedded in Vectashield mounting medium. Digital photographs were taken on a Nikon Eclipse 80i Fluorescence microscope using Q Imaging monochrome digital camera and Q Capture Pro software.

RESULTS

Canonical Hh-signalling and localization of Ptch1 appears normal in mammary cells of mice

The cytoplasmic C-terminal region of Ptch1 is truncated in *mes* mice. While development of some tissues is altered, the overall phenotype of these animals is relatively normal suggesting that Smo-mediated Hh-signalling is generally uncompromised. We tested, therefore, if distinct activities might be attributable to the C-terminus of the *mes* variant of Ptch1 relative to the wild type protein. The subcellular localization of full length Ptch1 from wild type and *mes* mice were compared to the distribution of the isolated C-termini from these same proteins (Figure 1). Full length mutant Ptch1^{mes}, tagged at its N-terminus with GFP, localized to vesicles similar to the localization of the full length wild type protein (Figure 1A–B). As Figure 1C showed further, the GFP-tagged C-terminal cytoplasmic domain of wild type Ptch1 also localized to vesicles in human mammary epithelial cells (HMECs). Co-immunofluorescence revealed that these vesicles harbored the endosomal marker, EEA-1 (Figure 1E–G), consistent with the previously reported cellular localization of endogenous Ptch1 (Martin et al., 2001,Incardona et al., 2002). However, in contrast to the isolated wild type C-terminus and the full length Ptch1^{mes} protein, GFP-tagged C-terminus of Ptch1^{mes} did not localize to any structures within the cell despite retaining the first 50 amino acids (Figure 1D). Thus, while the full length Ptch1^{mes} protein appeared to localize normally, the C-terminus of Ptch1 from *mes* mice was defective for tethering to EEA1-containing vesicles.

C-terminus of Patched-1 interacts with SH3-containing factors

The requirement for an intact C-terminus for localization of this isolated domain to vesicles suggested that sequences in this region might facilitate tethering of this domain to vesicles. As Figure 2 illustrates, the C-terminus of Ptch1 harbours proline-rich sequences encoding consensus binding sites for Class I and Class II SH3-domains (Feng et al., 1995,Rickles et al., 1995,Yu et al., 1994) or WW-domains (Sudol et al., 1995,Macias et al., 1996,Jager et al., 2006). These sites in Ptch1 are highly conserved among mammals and, for one of these (^{1288}RPPPYRPRR), among vertebrates.

To more formally test whether Ptch1 might form complexes with SH3-containing proteins, a GFP-Ptch$^{C\text{-}term}$ (amino acids 1163–1435) fusion protein was expressed in COS1 cells. Whole cell lysates were then added to glutathione-sepharose beads binding bacterially-expressed GST-fusion proteins encoding the SH3-domains of p85β, Grb2 and c-src. As Figure 3B illustrates, the cytoplasmic C-terminal domain of Ptch1 bound specifically to SH3-domains of Grb2, c-src and p85β but not to GST alone.

Further delineation of the regions in the C-terminus of Ptch1 required for binding to these SH3-domains is illustrated in Figure 3C. Using a series of deletion mutants of GFP-Ptch1$^{C\text{-}term}$, as well as the C-terminus from Ptch1^{mes} (see Figure 3A), binding to GST-Grb2 was determined. All of the GFP-Ptch1 $^{C\text{-}term}$ deletion mutants formed complexes with this SH3-domain while binding of the GFP-Ptch1 ^{mes} fusion protein could not be detected. Thus, Grb2 appeared to bind to the C-terminus of Ptch1 through at least two independent domains. However, binding required sequences downstream of the position of the altered/truncated mutant *mes* Ptch1 protein.

Binding of the C-terminus of Ptch1 to Grb2 was also determined in co-immunoprecipitation assays (Figure 4). GFP-tagged full length Grb2 was co-transfected with HA_3-tagged Ptch1$^{C\text{-}term}$. Figure 4A illustrates co-immunoprecipitation of GFP-Grb2, detected with an α-GFP antibody, following immunoprecipitation of HA_3-Ptch1$^{C\text{-}term}$ with α-HA. Figure 4B illustrates further that flag-tagged p85β (PIK3R2) could be co-immunoprecipitated with GFP-tagged wild type Ptch1$^{C\text{-}term}$ but not the *mes* Ptch1$^{C\text{-}term}$, consistent with the binding data in Figure 3B. The PPXY motif in the fourth polyproline region of the C-terminus (see Figure 2) has also been shown to mediate binding to WW-domains present in the HECT-family of E3 ubiquitin ligases (Ogunjimi et al., 2005,Wiesner et al., 2007,Qiu et al., 2000). We tested whether a candidate E3 ubiquitin ligase, Smurf2, or its catalytically-defective variant, Smurf2 CA (Ogunjimi et al., 2005), could associate with the C-terminus of Ptch1. As Figure 4B shows, Smurf2 was co-immunoprecipitated with the C-terminus of wild type but not *mes* Ptch1.

Thus, a number of candidate proteins harbouring SH3-domains or WW-domains were able to bind to the C-terminal region of the Ptch1. However, binding of these same candidate factors to the C-terminal region of Ptch1 derived from the Ptch1^{mes} was not apparent.

Shh stimulates activation of Erk1/2 independent Smo-mediated canonical Hh-signalling

Shh Light II cells harbour a luciferase reporter transgene sensitive to stimulation by the Hh-ligands (Taipale et al., 2000). To determine if the Hh-ligands utilized signalling cascades employing SH3-containing components, serum starved Shh Light II cells were stimulated with N-Shh and assayed for phosphorylation of Erk1/2. As Figure 5A illustrates, addition of the N-Shh ligand at levels of 0.5 and 1.0 μg/ml caused a significant induction of phospho-Erk1/2 after two hours. The same concentrations of N-Shh also induced expression of luciferase from the Gli-luc reporter stably integrated in this cell line (data not shown). In order to determine that activation was independent of Smo activity, increasing concentrations of N-Shh were added to Shh Light II cells in the absence or presence of the Smo inhibitor, cyclopamine (Incardona et al., 1998, Incardona et al., 2000) (Figure 5B). Erk1/2 activation was still apparent in cyclopamine-treated cells despite the inhibition of the canonical Hh-signalling pathway, the latter verified by the inhibition of luciferase activity in these cells (Figure 5C).

Activation of Erk1/2 by N-Shh was also tested in human mammary epithelial cells (HMEC). As Supplementary Figure 1 illustrates, activation or Erk1/2 was detected within 2 minutes following activation by N-Shh and peaks at about 15 minutes. A second wave of stimulation occurs after 2 hours. Figure 5D also demonstrates the stimulation of phospho-Erk1/2 within 15 minutes following addition of N-Shh to serum-starved HMEC. To begin to determine the pathways leading to activation of Erk1/2, stimulation of Erk1/2 was performed in the presence of the farnesyl transferase inhibitor, FTase II. N-Shh-dependent activation of Erk1/2 was blocked in the presence of FTase II.

As Figure 6 illustrates, N-Shh-dependent activation of Erk1/2 was not due to contamination of the N-Shh preparation with factors that might signal through alternative pathways.

Serum-starved Shh Light II cells or NIH3T3 fibroblasts were treated with N-Shh in the presence of the α-Shh antibody, 5E1. While Erk1/2 activation was seen for both cell types when N-Shh was added, mixing of N-Shh with the 5E1 antibody prior to addition to the cells blocked activation of Erk1/2 (Figure 6B). For Shh Light II, inhibition of Smo-mediated canonical signalling was also observed, as determined by the failure to stimulate expression of the Gli-luciferase reporter gene (Figure 6A). Thus, activation of Erk1/2 was dependent specifically on N-Shh and was not the consequence of a contaminant in the N-Shh preparation.

While inhibition of N-Shh-dependent activation of Erk1/2 in the presence of cyclopamine supports the notion that activation occurs through a Ptch1-mediated pathway that is independent of Smo activity, a more definitive test for this pathway was demonstrated using *Smo*-deficient cells. Like several other cell lines derived from mammary epithelial cells or breast cancers, the human mammary epithelial cell line, MCF10A, has been shown previously to express *Ptch1*, *Gli2* and *Gli3* but lacks *Smo* expression (Zhang et al., 2008). We verified by RT-PCR that MCF10A do not express detectable levels of *Smo* while *Smo* expression was detected in T47D cells, as also shown previously, as well as in HMEC (Figure 7A). The lack of *Smo* expression predicts that N-Shh signalling through the canonical Hh-signalling pathway would be absent. This prediction was tested in MCF10A cells transiently transfected with the Gli-luciferase reporter construct. As Figure 7B illustrates, addition of N-Shh to these cells did not cause any stimulation of the Hh-pathway reporter gene. The Gli-Luc reporter gene could be activated, however, by co-transfection with a vector expressing a constitutively active version of Gli2, Gli2ΔN. To ensure that the N-Shh used to stimulate MCF10A cells was active was determined by adding the same preparation to Shh Light II cells (Figure 7C); activation of the endogenous reporter gene was observed.

Given that MCF10A cells do not express *Smo* and that N-Shh does not stimulate the canonical Hh-signalling pathway, we tested next whether N-Shh in these cells could activate Erk1/2. N-Shh was added to serum starved MCF10A cells and changes in the levels of phospho-Erk1/2 determined. As Figure 7D demonstrates, stimulation of MCF10A cells with N-Shh resulted in a 3.03 +/− 0.66 fold increase in phospho-Erk1/2 levels. This level of activation was similar to that seen in HMEC and Shh Light II cells (Figure 7E). To more definitively assign the pathway induced by N-Shh leading to activation of Erk1/2, MCF10A cells were first treated with 10nM of the MEK1 inhibitor, U0126 (Duncia et al., 1998, Favata et al., 1998). As the last lane in the Figure 7D illustrates, no activation of Erk1/2 by N-Shh was evident in the presence of this MEK1 inhibitor.

Taken together, these observations reveal an apparent bifurcation of the Hh-signalling pathway at the level of Ptch1. Specifically, these data define a novel signalling cascade operating through SH3-domain-encoding factors directly stimulated by the Hh-ligands. This cascade can be stimulated by the Hh-ligands in the absence of *Smo* or in the presence of the small chemical inhibitors of the Smo-mediated Hh-signalling.

DISCUSSION

The requirement of the Hedgehog signalling pathway directing cell fate and morphogenesis in a large number of tissues across phyla has been well characterized. Both genetic and molecular studies have defined a relatively well conserved molecular pathway that transmits signalling upon stimulation by the Hh-ligands. Central to this signal transduction pathway is the receptor for the Hh-ligands, Ptch1, whose activity is directed toward the regulation of the activity of a 7-pass, integral membrane protein, Smo. The mechanism for this regulation has not been clearly delineated nor has a physical interaction between Ptch1 and Smo been

demonstrated. Regardless, in the absence of Hh-signalling, Ptch1 acts to inhibit Smo activity, facilitating the conversion of the transcriptional effectors of the Hh pathway, Gli2/Gli3, to their repressor forms.

Additional signal transduction pathways also interact with the Hh-signalling pathway, although to date, these have typically been described in relation to changes in Smo activity . For example, evidence exists suggesting that stimulation of both heterotrimeric and small G-proteins specifically through activation of Smo activity may occur in at least some cellular contexts (Kasai et al., 2004, Riobo et al., 2006d, Oro, 2007). Likewise, potentiation of the activation of the Gli proteins may include activation of AKT via PI3k, although this may arise through pathways independent of the canonical Hh-signalling pathway (Riobo et al., 2006b). Another pathway more specifically relevant to the data presented in this paper involves MEK-1. Activation of MEK-1 was shown to synergise with the canonical Hh pathway resulting in significant enhancement of Gli-dependent transcriptional activation, the latter determined using a fibroblast cell line harboring a Gli-responsive luciferase-reporter construct (Riobo et al., 2006a). This activation was suggested to be dependent on the PKCδ activity, as determined by rottlerin-dependent repression of Hh-mediated activation of the Gli-luciferase reporter; repression of this activation was as effective as the Smo-specific inhibitor, cyclopamine. It is further interesting to note that, as was recently summarised (Riobo and Manning, 2007), these pathways may have been added to the Hh-signalling cascade evolutionarily more recently since Hh-signalling in *Drosophila* does not appear to invoke or depend on signalling through these mechanisms (Riobo et al., 2006c).

The observation that Ptch1 can stimulate Erk1/2 phosphorylation upon stimulation by Shh in the absence of Smo or in the presence of the Smo inhibitor, cyclopamine, suggests that the Hh-signalling pathway may have acquired additional characteristics in vertebrates where direct regulation of signalling through pathways leading to Erk1/2 activation arises. Inspection of the sequence of the C-terminus of Ptch1 from *xenopus*, chickens, rodents and humans reveals a highly conserved, Pro-rich sequence predicted to bind to factors harbouring SH3- and WW-domains. Additional Pro-rich motifs in this region are also conserved among mammals. While significant Pro-rich sequences exist in the Cterminal domain of *Drosophila* Ptch, they do not encode for the motifs observed in vertebrates, in general, and mammals in particular. The ability of these motifs in *Drosophila* to facilitate binding to SH3-containing factors remains to be determined.

It is clear, however, that sequences in C-terminus of murine Ptch1 facilitate association of this region to factors inhabiting EEA-1-containing vesicles. Truncations encoded by the Ptch1^{mes} allele prevented the GFP-Ptch$^{C\text{-}term}$-fusion protein from localizing to these structures. Importantly, when full length Ptch1 protein, with either the wild type or Ptch1*mes* C-termini were expressed, both proteins localised to vesicles in HMEC's. This localization for full length Ptch1^{mes} is consistent with the observation that in mice homozygous for the Ptch1^{mes} allele, Hh-signalling through the canonical pathway appears to be intact in skin (Nieuwenhuis et al., 2007) and the mammary gland (H. Chang; unpublished observation). We propose that the Ptch1^{mes} allele results in segregation at the level of Ptch1 of at least two pathways regulated by the Hh-ligands; A) Ptch1 regulates the activity of Smo in a Hh-dependent manner, although the mechanisms here remains undetermined, B) Ptch1 regulates a signalling cascade independent of Smo and that is sensitive to farnesyl transferase inhibitors and the MEK-specific inhibitor, U0126. Evidence for another Ptch1-dependent pathway regulating cell cycle progression has also been reported (Barnes et al., 2001). Based on our binding data, we suggest further that Shh-induced activation of Erk1/2 may employ a cascade of signalling factors, including MEK, that depend on SH3-containing factors binding to the C-terminus of Ptch1. It will be important to determine if differences in activation of Erk1/2 arise in cells derived from Ptch1$^{mes/mes}$ mice. Attempts to assay these

changes were unsuccessful since serum starvation of primary mammary epithelial cells or mammary fibroblasts resulted in cell death precluding determination of changes in Erk1/2 activation upon stimulation with N-Shh (data not shown).

Our previous molecular-genetic analysis of the *mes* mouse revealed a mammary gland-specific role for Hh-signalling (Moraes et al., 2009). Similarly, alteration in morphogenesis of another skin appendage, the hair follicle, arises in *mes* mice (Nieuwenhuis et al., 2007). However, despite changes in the morphogenesis of hair follicles in the postnatal animal, expression of transcriptional targets of the canonical Hh-signalling pathway, *Ptch1* and *Gli1*, appeared unchanged relative to wild type animals (Nieuwenhuis et al., 2007). Likewise, we also failed to detect changes in the levels of *Ptch1* in epithelial cells or stromal fibroblasts isolated from the Ptch1^{mes} mammary gland (H. Chang; unpublished observation), suggesting that canonical Hh-signalling is not significantly altered in this tissue. In contrast to these skin appendages, a reduction in the mass of white fat as well as in the size of adipose cells (non-mammary gland) was shown to be associated with a very strong increase in *Ptch1* and *Gli1* levels in adipocytes in Ptch1^{mes} mice (Li et al., 2008). Furthermore, the polydactyly exhibited by mice homozygous for the *mes* allele of Ptch1 is consistent with altered activity of the canonical Hh-signalling pathway. Taken together, these observations suggest that altered Hh-signalling due to the truncation of the C-terminus of Ptch1 may have different effects on the growth and/or development of distinct tissues. We propose, however, that these differential effects for the Hh-pathway may arise through utilization of distinct signalling cascades working through the Ptch1 receptor upon stimulation by the Hh-ligands and that these include signalling through a ras/MEK-mediated signal transduction cascades that activates Erk1/2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

Shh	sonic hedgehog
Smo	smoothened
Ptch1	Patched-1
Hh	Hedgehog
HMEC	human mammary epithelial cells
FTase II	H-Cys-4-Abz-Met-OH
GST	glutathione S transferase
GFP	green fluorescent protein
MEK	mitogen-activated protein kinase kinase
Erk	extracellular signal-regulated kinase

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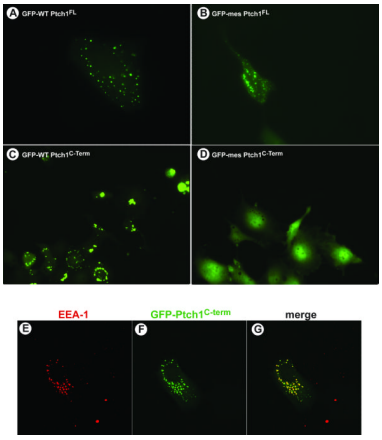


Figure 1. The C-terminus of wild type by not *mes* Ptch1 localizes to vesicles

Human mammary epithelial cells were transfected with plasmids expressing GFP-tagged (**A**) full length, wild type Ptch1, (**B**) full length *mes* Ptch1, (**C**) wild type, Ptch1$^{C\text{-term}}$ or (**D**) *mes* Ptch1$^{C\text{-term}}$. Localization of these GFP-tagged fusion proteins was then visualized by direct immunofluorescence in live cells in culture. Both wild type and *mes* full length Ptch1 localize to the vesicles. Similarly, the isolated C-terminus of Ptch1 also localizes to vesicles. In contrast the C-terminus from the *mes* variant of Ptch1 is distributed randomly and does not localize to specific cellular structures. Human mammary epithelial cells grown on cover slips were transfected with a plasmid expressing GFP-Ptch$^{C\text{-term}}$. Cells were fixed and endosomal localization of EEA-1 (**E**) determined by indirect immunofluorescence. Localization of GFP-Ptch$^{C\text{-term}}$ (**F**) was determined by direct fluorescence. The merged images (**G**) reveal the overlapping localization of EEA-1 and GFP-Ptch$^{C\text{-term}}$.

Figure 2. Conserved SH3- and WW-domain binding motifs in the C-terminus of Ptch1

Comparison of the C-terminal region of Ptch1 from mouse, rat, human, chicken and frogs (Xenopus) reveal the presence of conserved Class I and Class II SH3-binding motifs.

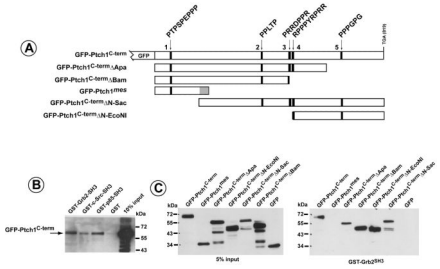


Figure 3. Association of the C-terminus of Ptch1 to the SH3-domains of Grb2, c-src and p85

A) Schematic of GFP-fusion proteins containing the Ptch1 C-terminal region. Polyproline motifs are designated and numbered sequentially for reference. **B**) GFP-Ptch1$^{C\text{-}term}$ was expressed in HMECs and cell lysates added to GST-fusion proteins containing the SH3 domains of Grb2, c-src or p85β. GFP-Ptch1$^{C\text{-}term}$ could bind specifically to the SH3-domains of each of these signal transduction factors but not to GST alone. **C**) GFP-fusion proteins with full length Ptch1$^{C\text{-}Term}$, Ptch1^{mes} or deletions of the C-terminus were expressed in HMECs, added GST-Grb2^{SH3} and binding determined by western blot directed against GFP. The ^{mes} mutant of Ptch1 does not bind to the SH3-domain of Grb2. Binding of all other Ptch1 deletion mutants to Grb2^{SH3} reveals that Grb2^{SH3} binds to Ptch1 through more than one site but requires sequences deleted in the Ptch1^{mes} mutant.

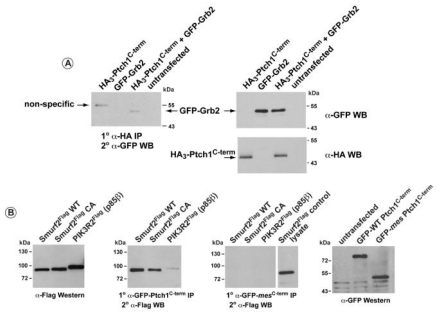


Figure 4. Co-immunoprecipitation of full length Grb2, p85β and Smurf2 with Ptch1$^{C\text{-term}}$

A) 293 cells were transfected with HA-tagged Ptch1$^{C\text{-term}}$, GFP-tagged, full length Grb2 or both together. HA_3-Ptch1$^{C\text{-term}}$ was then immunoprecipitated using and α-HA antibody and co-immunoprecipitated GFP-Grb2 detected by western analysis using an α-GFP antibody. Left panel shows that Grb2 is co-immunoprecipitated with the Ptch1$^{C\text{-term}}$. Western blots in the right panels show the relative level of expression GFP-Grb2 (top) or HA_3-Ptch1$^{C\text{-term}}$ (bottom).

B) 293 cells were transfected with flag-tagged Smurf2, Smurf2 CA or PIK3R2 (p85β) and GFP-Ptch1 $^{C\text{-term}}$ (second panel) or GFP-mes Ptch1$^{C\text{-term}}$ (third panel). Wild type and *mes* GFP-Ptch1$^{C\text{-term}}$ was immunoprecipitated with an α-GFP antibody. Co-immunprecipitated Smurf2, Smurf2 CA and p85β, detected by western analysis using an α-flag antibody. The second panel reveals that all three factors were co-immunprecipitated with wild type GFP-Ptch1$^{C\text{-term}}$. No binding was evident for these same factors using the GFP-tagged Ptch1$^{C\text{-term}}$ from the *mes* mice (third panel). First and last panels, respectively, show similar levels of expression for flag-tagged Smurf2, Smurf2 CA and p85β or GFP-WT-Ptch1$^{C\text{-term}}$ and GFP-*mes*-Ptch1$^{C\text{-term}}$.

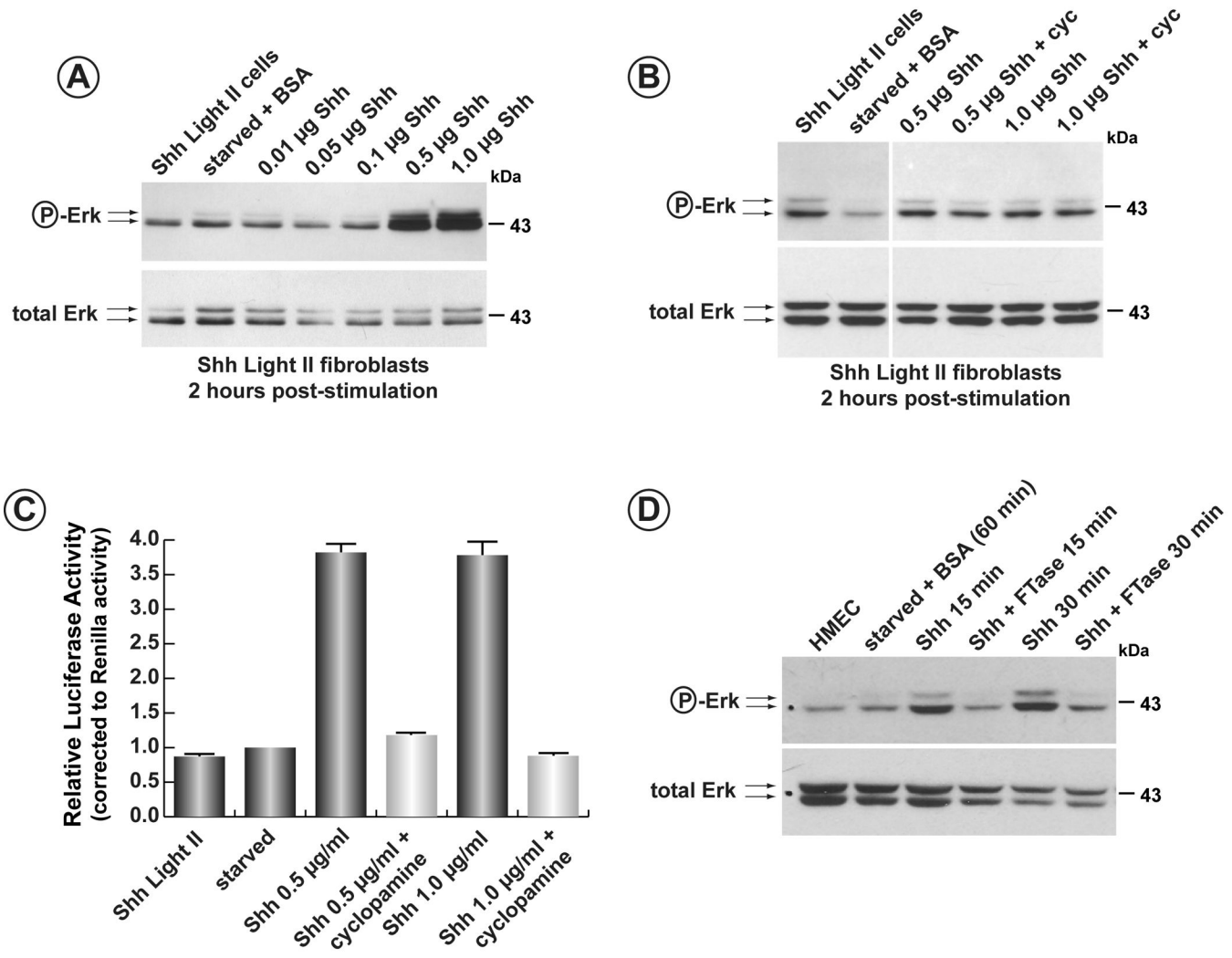


Figure 5. Activation of Erk1/2 by Shh in HMECs

A) Shh Light II cells were serum starved for 48 hours and increasing concentrations of N-Shh were added. Cells were then harvested after 2 hours and phospho-Erk1/2 detected by western blot (upper panel). The blot was then stripped and reprobed for total Erk1/2 (lower panel). **B)** Induction of phospho-Erk1/2 by N-Shh is repressed in the presence of FTase II. Serum-starved HMECs were induced with N-Shh in the presence or absence of the farnesyl transferase inhibitor, FTase II. Induction of phospho-Erk1/2 was blocked in the presence of this inhibitor. **C)** Induction of phospho-Erk1/2 is insensitive to the presence of cyclopamine. Serum-starved Shh Light II cells were stimulated in the presence or absence of the Smo-inhibitor, cyclopamine. Induction of phospho-Erk1/2 by N-Shh was unaffected by the presence of the inhibitor. **D)** Cyclopamine inhibits canonical Shh-signalling. The same cells used in panel C) were also assessed for induction of the Smo-mediated Hh-signalling pathway. Induction of luciferase from the Gli-luciferase reporter stably integrated in this line shows a 3.5-fold activation following addition of N-Shh. In the presence of cyclopamine, induction of luciferase is blocked. The block in the canonical Hh-pathway occurs despite induction of phospho-Erk1/2 under the same conditions.

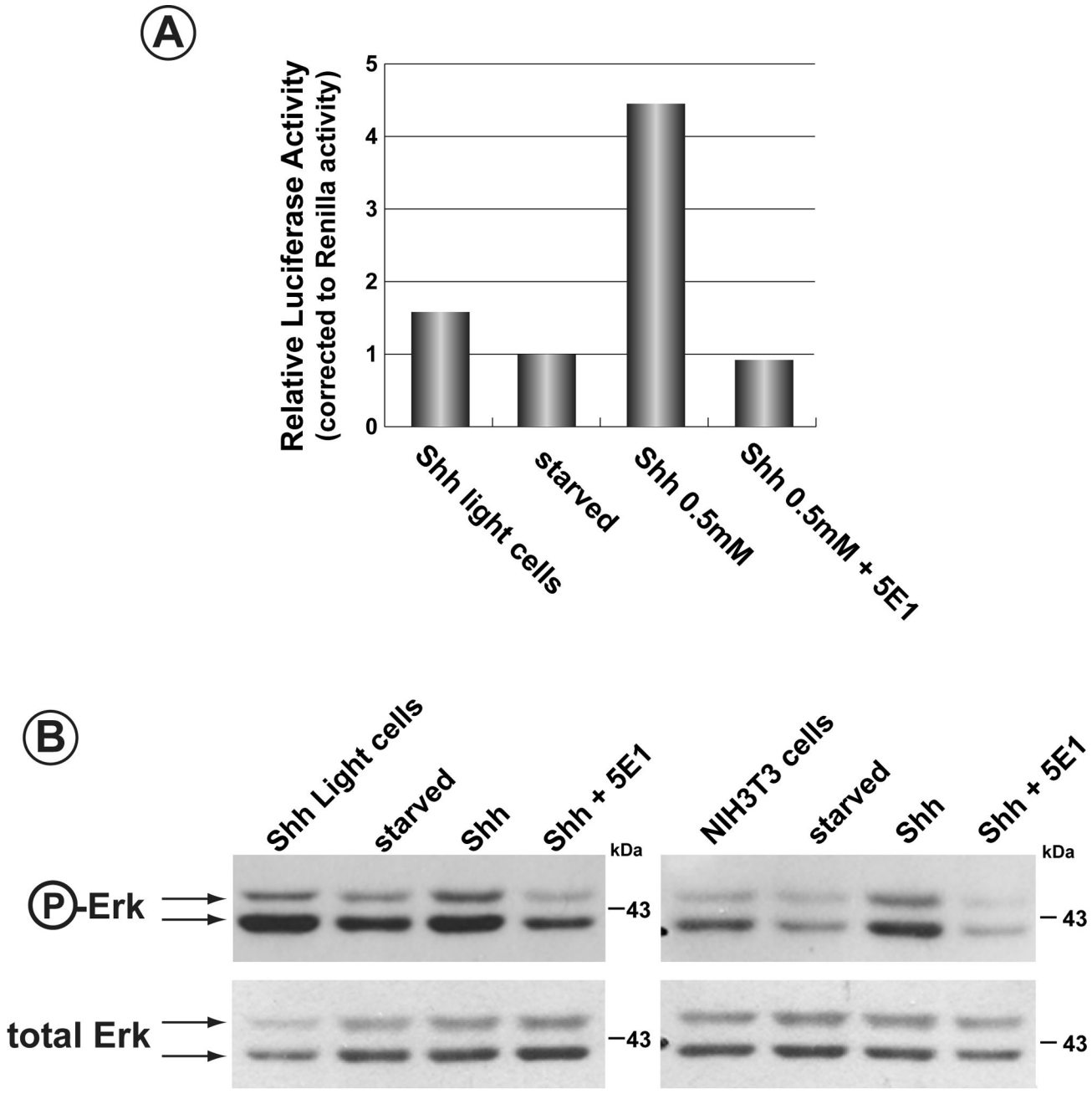
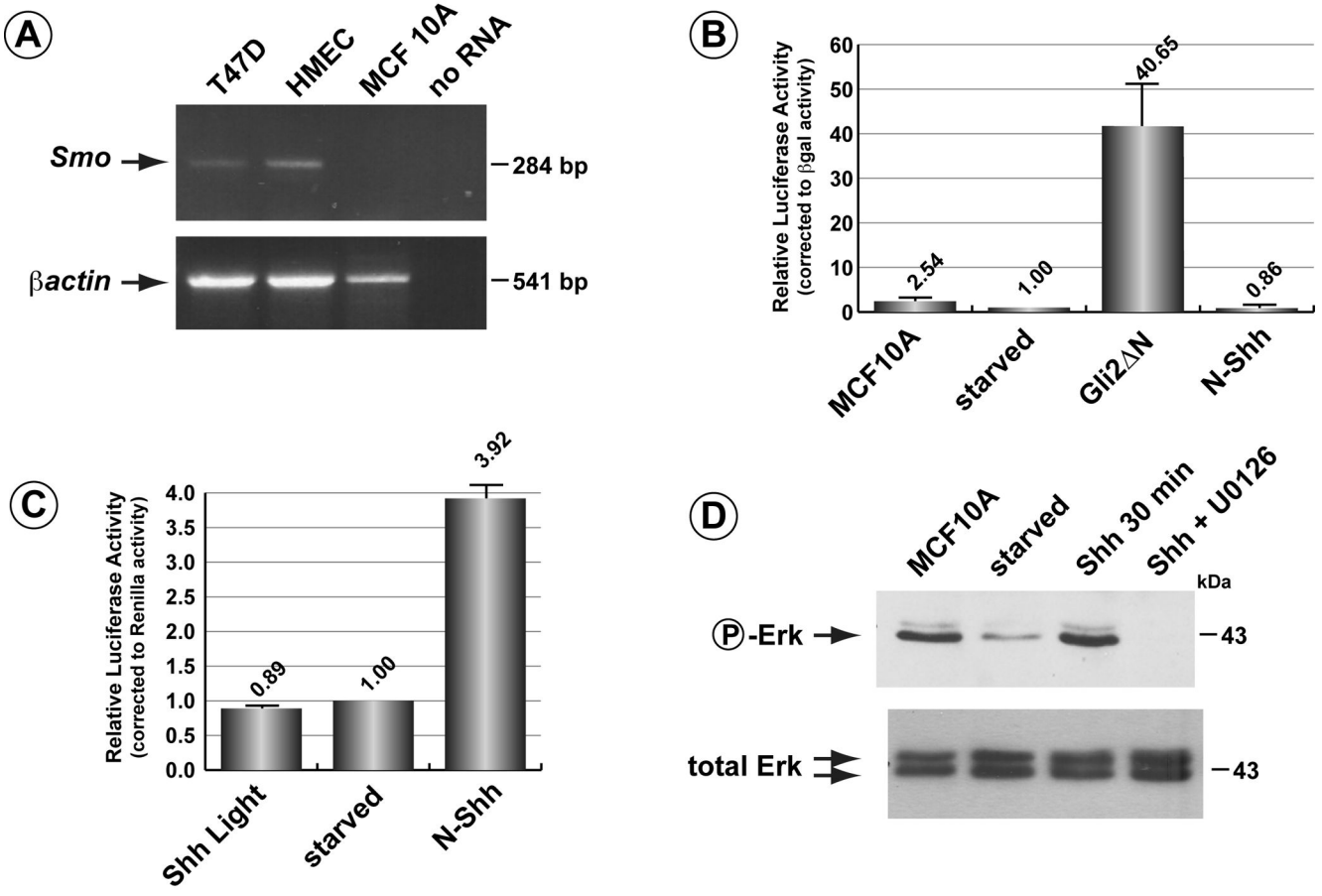


Figure 6. The α-Shh antibody, 5E1, blocks Shh-dependent activation of Erk1/2

A) Specificity of N-Shh-activation of the canonical Hh-pathway was determined using an antibody, 5E1, against N-Shh. N-Shh stimulated lucferase expression from the Gli-luciferase reported gene in Shh Light II cells. This Shh-dependent induction was blocked in the presence of the 5E1 antibody. **B)** Induction of Erk1/2 is specific to N-Shh. In order to verify that induction of Erk1/2 by N-Shh was not due to contaminants in the N-Shh preparation, the α-Shh antibody, 5E1, was first added to N-Shh and then used to stimulate serun-starved Shh Light II cells or NIH3T3 fibroblasts. Induction of phospho-Erk1/2 by N-Shh was blocked in the presence of 5E1.



E

Cell Line	Fold Erk1/2 Activation
All Cell Lines (15)	2.71 +/- 0.76
HMEC (7)	2.38 +/- 0.43
Shh Light II (4)	2.99 +/- 1.38
MCF10A (4)	3.03 +/- 0.66

Figure 7. Shh activates Erk1/2 in the Smo-deficient cell line, MCF10A

A) Lack of *Smo* expression in MCF10A cells. RT-PCR for *Smo* or β*actin* was performed on RNA isolated from the breast epithelial lines, T47D, HMEC and MCF10A. As shown previously, Smo is not expressed in MCF10A cells. **B)** Induction of phospho-Erk1/2 by N-Shh in MCF10A cells. Despite the lack of *Smo*, Erk1/2 is activated by N-Shh. The last lane shows further that induction of Erk1/2 is blocked in the presence of the MEK-inhibitor, U0126. **C**) Smo-deficient MCF10A cells transiently transfected with the Gli-luc reporter were stimulated with N-Shh. As expected, no activation through the canonical Hh-pathway was observed (Shh lane). Co-transfection of Gli-Luc with constitutively active Gli2ΔN did activate the reporter construct. **D**) That the N-Shh preparation that failed to activate the Hh-

signalling pathway in MCF10A cells was active was determined by stimulating Shh Light II cells. **E**) Summary of levels of activation of Erk1/2 by N-Shh in all cells lines tested. Number of experiments for each cell line is denoted in brackets.