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Regulation of Gli proteins by the Hedgehog Signaling Pathway

presented by Lyle Vincent Villamater Lopez

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Regulation of Gli proteins by the Hedgehog Signaling Pathway

A dissertation presented

by

Lyle Vincent Villamater Lopez

to

The Division of Medical Sciences

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Regulation of Gli proteins by the Hedgehog Signaling Pathway

Abstract

Hedgehog signaling is essential during embryogenesis and in the maintenance of adult stem cells. Deregulation of this pathway leads to developmental abnormalities and cancer. In mammals, the Gli transcription factors transduce the Hedgehog signal to effect changes in gene expression, yet an understanding of signal transduction through Gli is still incomplete. In this dissertation, I describe my studies on the mechanisms of Gli protein regulation. Specifically, I investigated how the Hedgehog signal regulates Gli protein localization and activation through the primary cilium, and I identified a new role for the proteasome in regulating Gli transcriptional activity.

Primary cilia are antenna-like protrusions that act as organizing centers where Hedgehog pathway components meet to facilitate signal transduction. I showed that Gli proteins complex with the cytoplasmic protein Suppressor of Fused (SuFu) and rapidly accumulate in cilia tips after activation of the transmembrane protein Smoothened. I also demonstrated that Gli dissociates from SuFu in response to the Hedgehog signal, leading to the initiation of Gli transcriptional activity. I found that activation of Protein Kinase A (PKA) blocks Gli-SuFu ciliary recruitment and dissociation, providing a new mechanistic explanation for PKA's antagonistic action on the pathway.

Gli proteins are negatively regulated by the proteasome, the cell's proteolytic destruction machine, not only by modulating the levels of Gli proteins through complete degradation but also by partially processing Gli-activator forms into shorter Gli-repressors. I found that

pharmacological proteasome inhibitors are potent inhibitors of Gli transcriptional activity, blocking Hedgehog pathway activation. I showed that proteasome inhibition reduces Gli occupancy on DNA promoters, preventing transcription of target genes. I therefore identified an unexpected positive role for the proteasome in Gli-mediated pathway activation. Finally, I provide preliminary evidence for the potential therapeutic use of proteasome inhibitors in Hedgehog-activated cancers.

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Acknowledgements

I am sometimes asked, since I am a scientist, whether I believe in God. My answer is 'Yes!' I see proof of God's existence in the intricate and beautiful workings of cells and the molecules within it. I am blessed and privileged to see His creation everyday.

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I dedicate this dissertation to my family:

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- to my father Hernando for convincing me I can do anything

- to my stepkids Felicia, Adriana and Alexy for putting up with my nerdiness, and to my grandson Jeremiah – I can't wait for you to grow up so I can show you what grandpa did

- to my beautiful wife Maria, I am who I am because of you, I strive to be a better man each minute because of you, I love you very much

CHAPTER ONE:

GENERAL INTRODUCTION

Introduction

How does an animal form starting from a single-celled zygote? The answer is fundamentally complex and wondrous. Two biological concepts that help explain but a small portion of this answer is at the heart of this dissertation: cell communication and the regulation of gene expression.

Multicellular organisms have evolved distinct pathways that provide a means for communication between cells (elegantly reviewed in Gerhart, 1998). During embryogenesis, cell-to-cell communication is crucial for the precise spatial and temporal orchestration of signals that lead to differential expression of genes that determines each cell's fate. One such pathway in the early development of animals is the Hedgehog (Hh) signaling pathway. Hh plays critical roles in the cell-fate determination and patterning of many bodily structures. These roles are exemplified by studies in the fly imaginal wing disc and the mouse neural tube and the anterior-posterior axis of the limb bud (reviewed in McMahon et al., 2003; Hooper and Scott, 2005). Aberrant Hh signaling results in many congenital defects (reviewed in Nieuwenhuis and Hui, 2005). In the adult, Hh is important in the maintenance of stem and progenitor cells, and help mediate repair mechanisms in response to injury (Trowbridge et. al., 2006; reviewed in Beachy et al., 2004). It is not surprising that deregulation of this developmental signaling pathway can lead to cancer in humans (reviewed in Taipale and Beachy, 2001; Barakat et al., 2010).

Cells typically receive external stimuli from mitogens and morphogens via receptors on the cell membrane. These growth signals are transduced through the cytoplasm and ultimately effect a response in the nucleus in the form of changes in gene expression. In the Hh signaling pathway, the transcription factors Ci (Cubitus interruptus in flies) and three Gli homologs (Gli1, Gli2 and Gli3 in mammals) control the expression of target genes. Transcription factors bind

promoter regions in DNA and recruit the transcriptional machinery to induce gene expression. Equally as important as activation, transcriptional repression ensures that gene expression occur only at the right time and place. Activation and repression can be achieved by controlling the amount of transcriptional activators that reach the nucleus by regulating subcellular localization. In chapter 2, I investigate the changes in localization of Gli in an important cellular organelle – the primary cilia. I demonstrate that recruitment of Gli to cilia is a required step for its activation.

Another way to control activation and repression is by modulating the concentration of the transcription factor in the cell. The proteasome is one of the cell's degradation machines and completely proteolyzes proteins in a regulated manner. In an unusual and rare mechanism, the proteasome also incompletely proteolyzes Ci/Gli from a full-length transcriptional activator into a shorter repressor. In this way, Ci/Gli exist in both an activator and repressor form. In chapter 3, I study the effects of increasing cellular concentrations of Gli on target gene transcription when the proteasome is inhibited. I found that proteasome inhibition antagonizes the transcription of Gli target genes despite the concurrent accumulation of Gli. I discovered that intact proteasome function is required for Gli activation.

Gli transcription factors have both activator and repressor roles

The Ci/Gli protein topology is composed of an N-terminal DNA binding domain and a C-terminal transactivation domain (Figure 1.1).

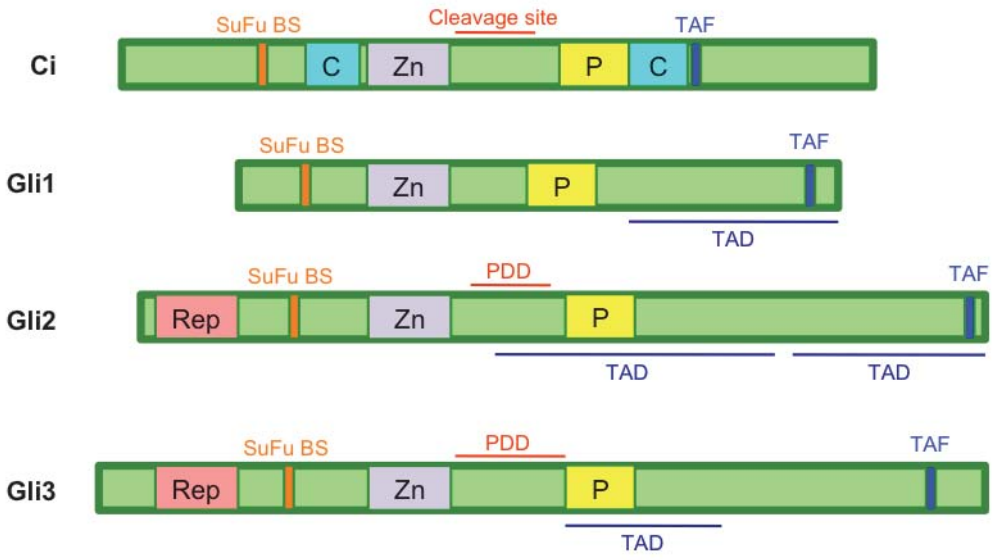

Figure 1.1 Topology of Ci and Gli proteins.
Schematic diagram of domains and motifs in the *Drosophila* Cubitus interruptus (Ci) and the mouse homologs Gli1, Gli2 and Gli3. Figure adapted from Hui and Angers, 2011.

Zn, Zinc-finger DNA binding domain	SuFu BS, SuFu-binding site
P, Phosphorylation cluster	TAF, TAF-binding site acidic activation motif
C, Cos2-binding sites	PDD, Processing determinant domain
Rep, Repressor domain	TAD, Transcription activation domain

The DNA binding domain contains five highly conserved zinc-fingers that recognizes and bind a consensus DNA sequence consisting of a nine-nucleotide core (GACCACCCA) (Kinzler and Vogelstein, 1990). While the DNA binding domains of Ci and Gli proteins are highly conserved, the rest of the protein shows more divergence in sequence. Full length Ci is robustly processed into a shorter repressor form in the absence of Hh signaling (Aza-Blanc et al., 1997). The Hh signal blocks this processing, allowing full-length Ci activators to accumulate. In vertebrates, this dual role of Ci is distributed among the Gli homologs. Differences in transcriptional function of each Gli protein may be explained by sequence differences with Ci outside the DNA binding domain. In these regions, Gli3 is most similar in sequence to Ci, and is robustly processed into the repressor form. Gli2 shows less similarity with Ci, and is poorly processed. Gli1 shows least

similarity with Ci, and is not processed. Therefore, based on their individual propensities to be proteolytically processed, Gli1 is exclusively an activator; Gli2 is mostly an activator; while Gli3 is mostly a repressor.

It is clear that the C-terminal transactivation domain is required for gene transcription. However studies on the mechanism for transcriptional activation and repression by Ci/Gli are scarce. A stretch of 18 acidic amino acids in the most C-terminal region of the transactivation domain resembles a binding motif for the transcriptional coactivator TAFII31 (TATA Binding Protein Associated Factor II 31) and was found to be required for transcriptional activation (Yoon et al., 1998). In the fly, Ci promotes the recruitment of the histone acetyltransferase CBP (cAMP response element binding protein) to induce target gene expression (Akimaru et al., 1997). Though Gli3 is only weakly activating, its transcriptional activation also involves direct interaction with CBP (Dai et al., 1999). Through sequence homology, it was proposed that Gli2, but not Gli1, might also interact with CBP (Dai et al., 1999, Kasper et al., 2006). Further, it was found that Gli3 transcriptional activation involved direct association with Mediator, a large multi-protein coactivator complex, and the alleviation of a repressive subunit MED12 within it (Zhou et al., 2006).

Repressor forms of Ci/Gli contain only the DNA-binding domain and lack the C-terminal transactivation domain. Thus, Ci/Gli repressors presumably bind DNA, compete with activator forms, and occupy target gene promoters to inhibit transcription. Gli3 was also found to bind the TGF-β pathway repressor Ski and recruit the histone deacetylase HDAC1, both proteins associated with transcriptional repression (Dai et al., 2002).

The role of Gli proteins in development and cancer

Loss of function studies in mice highlights the duality of Gli proteins. Gli2 loss mimics defects resulting from loss of Hh signaling, while Gli3 loss phenocopies constitutive Hh activation.

The mammalian homolog Sonic hedgehog (Shh) is required for the proper development of many organ systems such as central nervous system (CNS), lungs, and limbs (Chiang et al., 1996). Loss of Shh in mice leads to numerous morphological defects appearing at embryonic day 9.5. Embryos are smaller than their wildtype littermates. Cells in the neural tube exhibit an expansion of dorsal markers (such as Pax3) into ventral regions concurrent with loss of ventral markers (such as HNF3-β and ISL1/2). Gross dorsalization of the CNS leads to cyclopia in mice similar to humans who are heterozygous mutant for *SHH* (Chiang et al., 1996). These mice also have bone defects and loss of anterior-posterior patterning in the limb producing only one digit. The lungs, trachea and esophagus are fused in these mice (Litingtung et al., 1998; Pepicelli et al., 1998).

Constitutive pathway activation can result from the loss of Patched (Ptc), a major negative regulator of Hh signaling. In mice lacking functional Ptc, the CNS is ventralized, manifested by the reduction of dorsal markers and expansion of ventral markers (such as HNK3-β and Shh) (Goodrich et al., 1997). Ptc null homozygous embryos die at embryonic day 9.5. Ptc heterozygotes survive; however, they have a high incidence of tumors such as medulloblastoma and a small percentage show abnormal limb patterning such as extra digits and syndactyly (Goodrich et al., 1997; Hahn et al., 1998).

Gli2 loss of function

Deletion of the zinc finger domain results in a complete loss of function of Gli2. Mice homozygous mutant for Gli2 die at birth and exhibit loss of the floorplate in the spinal cord corresponding to loss of expression of HNF3-β and Shh (Ding et al., 1998; Matise et al., 1998). While the expression of Shh in the notochord remains normal, the notochord fails to regress from the ventral spinal cord (Ding et al., 1998; Matise et al., 1998). There is dramatic reduction in lung tissue, with the remaining lobes being fused. Lungs also show abnormal lobe outgrowth and complete loss of accessory lobe (Motoyama et al., 1998a). These mice show normal anterior-posterior patterning of the limbs resulting in the normal number of digits. However the long bones of limbs are shortened and fused (Mo et al., 1997). In humans, loss of function mutations in *GLI2* can lead to developmental anomalies resembling holoprosencephaly, a failure of the forebrain to develop two hemispheres (Roessler et al. 2003). Defects due to loss of Gli2 are reminiscent of reduced Hh signaling, indicating Gli2's primary role as an activator.

Mice with loss of Gli1 function are viable, suggesting that Gli1 is not required for normal development. However, Gli1 and Gli2 double mutants show defects indicative of reduced Hh signaling suggesting that Gli2 can compensate for Gli1 (Park et al., 2000).

Gli3 loss of function

A spontaneous mutation in mice called XTJ (extra toes J) consisting of a chromosomal deletion affecting *Gli3* was so-named due to the abnormal increase in digit number (Schimmang et al., 1992; Hui and Joyner, 1993; Buscher et al., 1998). Heterozygous mutant mice are viable but have an extra preaxial digit and mild craniofacial abnormalities. Homozygous mutants die at birth, have 7-8 digits as well as moderate to severe exencephaly (Hui and Joyner, 1993; Schimmang et al., 1992; Schimmang et al., 1993). Loss of Gli3 also led to ectopic Shh

expression in the anterior limb bud at embryonic day 11.5 that corresponds to the formation of additional digits (Buscher et al., 1997; Masuya et al., 1995). In addition, Hh target gene expression was observed in several tissues in Gli3 null mice indicating that Gli3 functions mostly as a repressor (Buttitta et al., 2003; Hu et al., 2006). In humans, the truncation of Gli3 to a shorter protein missing the zinc fingers led to loss of function. This was found to be responsible for Greig Cephalo Polysyndactyly Syndrome (GCPS), an autosomal dominant disorder characterized by craniofacial abnormalities, and postaxial and preaxial polydactyly and syndactyly (Vortkamp et al., 1991; Wild et al., 1997).

A common theme underlying the pathogenesis of cancer is the reactivation of silenced developmental pathways. Hh pathway activation is observed in certain cancers. Gli1, the founding member of the Gli family of genes, was first identified and named because it was amplified in a human glioblastoma cell line (Kinzler et al., 1987). Gli1 itself is a direct target of the Hh pathway and is overexpressed in a growing list of cancers such as gliomas, medulloblastomas, osteosarcomas and basal cell carcinomas (Fuller and Bigner, 1992; Roberts et al., 1989; Dahmane et al., 1997). Although Gli2 amplification is not readily observed in human tumors, studies in mice demonstrate that its ectopic expression instigates basal cell carcinoma in the skin (Grachtchouk et al., 2000).

Gli activation in cancer can be brought about by mutations in upstream Hh components such as loss of negative regulators Ptc or Suppressor of Fused (SuFu), or the oncogenic activation of positive regulators such as Shh or Smo (reviewed in Barakat et al., 2010; Teglund and Toftgard, 2010; Yang et al., 2010). Additionally, there is increasing evidence that Gli

activation can occur independently of upstream Hh pathway components (reviewed in Stecca and Ruiz i Altaba, 2010; Yang et al., 2010).

Overview of vertebrate Hedgehog signal transduction

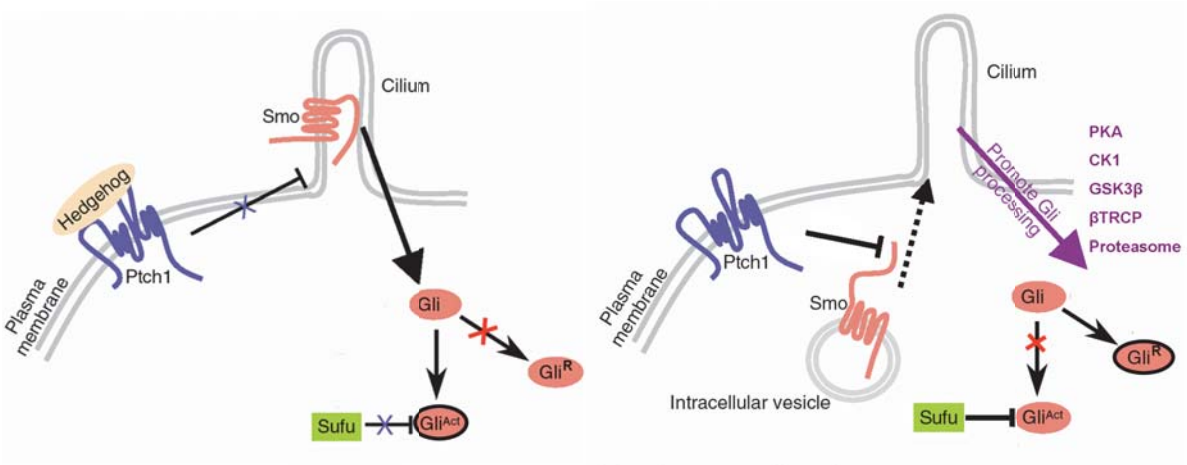

Figure 1.2 Overview of vertebrate Hedgehog signal transduction
Left: Pathway activation: the Hedgehog ligand binds its receptor Patched (Ptch1) leading to Smoothened (Smo) activation. Gli activator forms (Gli-Act) accumulate to upregulate target gene expression. Right: Inactive pathway: In the absence of Hh signals, Patched inhibits Smo. Suppressor of Fused (Sufu) inhibits Gli-A and Gli is targeted to partial proteolysis by the proteasome. Gli repressor forms (Gli-R) accumulate to repress target genes. Figure adapted from Huangfu and Anderson, 2006.

The vertebrate Hh pathway is briefly summarized in Figure 1.2. On the cell surface, the Hh ligand binds its receptor Patched (Ptc). This relieves the inhibition of another transmembrane protein Smoothened (Smo). In vertebrate cells, these membrane events occur at the primary cilium. Once activated, Smo translocates to cilia and initiates a series of events that leads to the upregulation of Hh target genes by the transcription factor Gli. In the absence of Hh, Ptc is active and inhibits Smo. Gli activity is repressed and transcription of Hh target genes is kept low.

Gli activity can be functionally defined as its ability to promote target gene transcription. This activity of Gli is antagonized by at least three mechanisms: (1) Gli is bound and kept

inactive by the small cytoplasmic protein Suppressor of Fused (SuFu); (2) Gli is completely degraded; and (3) full length activator forms of Gli are partially processed into shorter repressor forms. The Hh signal must alleviate these inhibitory mechanisms to produce active Gli.

Studies in the past decade have revealed the importance of the primary cilia and the ubiquitin proteasome system in regulating the switch between active and inactive Gli. In the remaining sections I will review our current mechanistic understanding of the core Hh pathway components and the roles of the primary cilia and the proteasome in regulating Gli.

Hedgehog signaling in the fly

Hh signal transduction was first discovered and best characterized in the fly *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus, 1980). The effector of the Hh pathway is the transcription factor Cubitus interruptus (Ci) (Alexandre et al., 1996). In the absence of Hh, Ci is tethered away from the nucleus in a microtubule-associated complex comprised of the kinesin protein Costal2 (Cos2) (Whittle, 1976; Sisson et al., 1997) and the kinase Fused (Fu) (Preat et al., 1990). Three additional kinases are recruited to this complex to regulate Ci: protein kinase A (PKA), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) (Zhang et al., 2005). To keep expression of Hh target genes low, Ci is partially proteolyzed into a truncated 75kDa transcriptional repressor (Ci75). Ci75 is missing its C-terminal transactivation domain and competes for binding to target gene promoters with its full-length 155kDa activator form (Ci155) (Aza-Blanc et al., 1997; Methot and Basler, 1999). Ci processing is initiated by sequential phosphorylation events, first by PKA (Chen et al., 1998) which then primes Ci for further phosphorylation by CK1 and GSK3 (Jia et al., 2002; Price and Kalderon, 2002; Jia et al., 2005). These phosphorylated residues serve as docking sites for the E3 ubiquitin ligase complex SCF-

Slimb (Jiang and Struhl, 1998; Noureddine et al., 2002; Smelkinson and Kalderon, 2006; Smelkinson et al., 2007), after which Ci is targeted to the proteasome to be incompletely degraded (Aza-Blanc et al., 1997).

Intracellular Hh signaling is activated when the Hh ligand binds and inhibits Ptc leading to activation of Smo (van den Heuvel and Ingham, 1996; Taipale et al., 2002). The mechanisms for signal transduction between Smo and Ci are unclear but possibly include phosphorylation events and conformational changes of Smo's cytoplasmic tail (Zhang et al., 2004; Zhao et al., 2007), which interacts with the inhibitory Ci/Cos2/Fu complex (Stegman et al., 2000; Jia et al., 2003). These events ultimately lead to the release and stabilization of Ci155 from its inhibitory complex where it is free to enter the nucleus and promote target gene transcription (Methot and Basler, 2000; Wang and Holmgren, 2000; Aikin et al., 2008).

Notable differences in Hh signal transduction between flies and mammals

The core components of Hh signaling in the fly are conserved in mammals with some notable differences. First, single genes in the fly have expanded into gene families in vertebrates. For instance, in mammals there are three *Hh* genes (*Sonic Hedgehog*, *Indian Hedgehog* and *Desert Hedgehog*) (Echelard et al., 1993), two *Patched* genes (*Patched1* and *Patched2*) (Motoyama et al., 1998b), and three *Ci* homologs (*Gli1*, *Gli2* and *Gli3*) (Ruppert et al., 1988). These gene duplications may be important for tissue-specific expression and developmental timing. In the case of the Hh homologues, Shh has the most widespread role during embryogenesis (Chiang et al., 1996), though Ihh shows some redundancy with Shh (Zhang et al., 2001). Ihh and Dhh have more tissue-specific roles in bone and sperm development, respectively (St-Jacques et al., 1999; Echelard et al., 1993). As for Ci, transcriptional activity has been

delegated to the three Gli proteins, with Gli3 being mostly a repressor and Gli2 and Gli1 being mostly activators.

Second, there are differences in the core pathway members between flies and mammals. Suppressor of Fused (SuFu) is not essential in the fly (Preat, 1992), while SuFu is indispensable for proper embryonic development and viability in mammals (Cooper et al., 2005; Svard et al., 2006). In contrast, the kinase Fu is essential in the fly (Preat et al., 1990) but its mammalian homolog is not required (Chen et al., 2005; Merchant et al., 2005).

Hh signaling is transduced through the primary cilium in mammals (Huangfu and Anderson, 2005), while cilia are not required in flies (Wong and Reiter, 2008). It is worth noting that Ci processing in flies requires the microtubule-associated kinesin Cos2, while in vertebrates, Gli processing requires cilia. We can perhaps think of the intraflagellar transport (IFT) system within cilia as the functional orthologues of Cos2 wherein both bring together the components of the Hh pathway to enable efficient signal transduction.

Hedgehog signaling at the vertebrate cell membrane

Hedgehog signaling begins with the production and secretion of the Hh morphogen. Hh is produced as a larger precursor protein that undergoes autocatalytic cleavage mediated by its C-terminal intein-like domain into a smaller N-terminal fragment (Porter et al., 1995). This smaller fragment is doubly modified with two lipid adducts. Unique to all known proteins, a cholesterol moiety is attached to the C-terminus concurrent with autocleavage (Porter et al., 1996a, Porter et al., 1996b). To the N-terminus, a palmitate is added by Hedgehog acetyltransferase (HHAT) (Chamoun et al., 2001; Micchelli et al., 2002). Finally, secretion of the processed Hh ligand is

mediated by the transmembrane protein Dispatched (Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002).

Patched (Ptc) is the Hh receptor. In the absence of the Hh signal, this 12-pass transmembrane protein inhibits the activity of another transmembrane protein, the 7-spanner Smoothened (Smo) through mechanisms that are not yet understood. Upon Hh ligand binding, Ptc is inhibited, leading to Smo activation (van den Heuvel and Ingham, 1996; Taipale et al., 2002). This mechanism of inhibition is perhaps connected to the changes in subcellular localization of Ptc and Smo. Ptc resides within the primary cilium in the absence of signal and moves out of cilia upon Hh binding (Rohatgi et al., 2007). Conversely, Smo is initially excluded from cilia and moves into cilia upon pathway activation (Corbit et al., 2005; Rohatgi et al., 2007). Interestingly, the synthetic Smo agonist SAG causes Smo to move into cilia while Ptc ciliary localization remains unchanged. In addition, certain Hh pathway antagonists, such as the naturally occurring Smo antagonist cyclopamine and the PKA activator forskolin, bring Smo into cilia without concurrent Hh pathway activation. The nature and identity of the endogenous signal between Ptc and Smo is yet to be discovered.

Intracellular Hedgehog signal transduction in vertebrates

It is still unknown how Smo signals to Gli proteins. But at least three proteins have been identified that help mediate signal transduction between Smo and Gli: these are the small cytoplasmic protein Suppressor of Fused (SuFu), the kinesin protein Kif7, and protein kinase A (PKA).

SuFu is a major negative regulator of the Hh pathway in mammals. In mice, the loss of SuFu phenocopies ectopic Hh pathway activation (Cooper et al., 2005; Svard et al., 2006). SuFu

binds Gli proteins in an inhibitory complex that sequestered away from the nucleus (Ding et al., 1999; Kogerman et al., 1999; Methot and Basler, 2000; Dunaeva et al., 2003; Merchant et al., 2004). SuFu also promotes the efficient processing of Gli repressor although the mechanism for this is unclear (Kise et al., 2009; Humke et al., 2010; Wang et al., 2010). SuFu localizes to cilia but its function is independent of cilia. SuFu still inhibits Gli in cells deficient in IFT proteins (Jia et al., 2009). Moreover, the loss of SuFu leads to Hh pathway activation even in cells lacking cilia (Chen et al., 2009).

Kif7 is the mammalian homologue of *Drosophila* Costal2 (Cos2). While Cos2 has a clear role in tethering Ci away from the nucleus, the role of Kif7 in mammalian Hh signal transduction is less clear. Kif7 has been shown to interact with Smo as well as Gli proteins (Tay et al., 2005; Cheung et al., 2009), however it can have both positive and negative effects on the Hh pathway (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). As a negative regulator, Kif7 is proposed to promote Gli2 and Gli3 degradation by providing a scaffold for recruitment of pro-degradation proteins. As a positive regulator, Kif7 is proposed to be required for trafficking and accumulation of Gli proteins to ciliary tips. Interestingly, Smo is required for the ciliary localization of Kif7 (Endoh-Yamagami et al., 2009). It is yet to be demonstrated whether Kif7 is a bona fide kinesin motor and it is not known how Smo regulates Kif7 movement into cilia (Wilson and Chuang, 2007).

The cAMP-dependent protein kinase A (PKA) has many important roles in the cell including the regulation of cellular metabolism. PKA plays a negative role in the Hh pathway (Li et al., 1995; Jiang and Struhl, 1995; Hammerschmidt et al., 1996). Loss of PKA in mice produces phenotypes reminiscent of Hh activation (Tuson et al., 2011). This negative role of PKA is ascribed to the regulation of Ci and Gli. PKA phosphorylates Gli creating docking sites for the

E3 ubiquitin ligase βTRCP and promoting the formation of Gli repressors (Wang et al., 2000; Pan et al., 2006; Tempe et al., 2006; Wang and Li, 2006). PKA has also been shown to phosphorylate Gli1 at zinc fingers preventing its nuclear accumulation (Sheng et al., 2006). Further, PKA inhibits the transcriptional activities of Ci and Gli1 (Wang et al., 1999; Kaesler et al., 2000). In chapter 2, I elucidate an additional mechanism for PKA in inhibiting Hh signaling – it prevents the trafficking of Gli/SuFu complexes into cilia, inhibiting dissociation and subsequent Gli activation (Tukachinsky et al., 2010). Concurrent with my findings, another group found that PKA blocked nuclear entry of full length Gli3 (Humke et al., 2010). Recently, PKA was found to localize to the ciliary base (Tuson et al., 2011). An alternative model has been proposed from studies in PKA null mice: in the absence of the Hh signal, Gli/SuFu complexes enter the cilia and are somehow modified or marked within cilia. Just following their exit from cilia, PKA acts on this marked Gli/SuFu complex to promote Gli3 processing and to keep Gli2 activators tightly associated with and inhibited by SuFu (Tuson et al., 2011).

The primary cilium as a signaling organelle

Primary cilia are found in nearly all vertebrate cells. Even before the connection between cilia and Hh was discovered, these antenna-like structures were already appreciated as a distinct and functional signaling organelle (reviewed in Pazour and Witman, 2003). Cilia are non-motile and are assembled during G_0 and are enriched in non-proliferating, quiescent cells. In cell culture, cilia assembly is promoted by contact inhibition due to confluency and by serum starvation. Cilia are formed as a microtubule-based outgrowth called the axoneme originating from the mother centriole, the "older" of the pair of centrioles that organizes the mitotic spindle during cell division (Sorokin, 1962). The axoneme contains a "9+0" configuration of nine outer

microtubule doublets but missing a central pair that is typically found in motile cilia. The membrane enveloping primary cilia is continuous with the plasma membrane, but the composition of proteins within it is distinct. Intraflagellar transport (IFT) proteins mediate the assembly of primary cilia and, in conjunction with kinesin and dynein motors, facilitate the transport of proteins in and out of cilia (Rosenbaum and Witman, 2002; Hsiao et al., 2012). Recently, evidence of a diffusion barrier at the base of cilia was found to restrict access to the ciliary membrane thereby defining cilia as a compartment physically separate from the rest of the cell (Hu et al., 2010; Chih et al., 2011). We can now think of cilia as a distinct organelle with regulated and selective transport of proteins into and out of it.

The notion of cilia as signaling organelles is not novel. Signaling that occurs within the cilia of specialized sensory neurons allow us to see and smell. Ciliated cells in the retina are photoreceptor cells that express G-protein coupled receptors (GPCRs) such as rhodopsin. Ciliated cells in olfactory sensory neurons also contain GPCRs that are required for olfaction (Jones et al., 1988; Pace et al., 1985). In non-sensory neurons, primary cilia are the sites of somatostatin and serotonin reception and signaling (Handel et al., 1999; Brailov et al., 2000). In the kidney, primary cilia are enriched in polycystin-2, which is important in calcium signaling and the detection fluid flow (Pazour et al., 2002).

Role of primary cilium in the Hedgehog pathway

A forward genetic screen in mice identified mutants with developmental defects that mimicked the loss of Hh signaling. These mutants were found to be in *Ift88* and *Ift172*, genes that encode intraflagellar transport (IFT) proteins, which are essential to build primary cilia (Huangfu et al., 2003). This and subsequent studies have established that primary cilia and the

IFT system are required for Hh signaling in mammals (Huangfu et al., 2003; Haycraft et al., 2005; Huangfu and Anderson, 2005). Gli proteins themselves are regulated by IFTs. A hypomorphic *Ift88* mutant displayed polydactyly and reduced target gene expression; a phenotype indicating reduced Hh signaling despite normal expression of the Shh. This suggested that Ift88 is required for transcriptional activation by Gli (Liu et al., 2005). In addition, efficient proteolytic processing of Gli3 into repressor forms appears to require fully functioning IFTs (Liu et al., 2005; May et al. 2005). Defects in Hh signaling are not merely a result of defects in cilia assembly, however. A recent study showed that Ift25, while not involved in cilia assembly, is required for the export of Smo and Ptch out of cilia (Keady et al., 2012). *Ift25* mutants show a diminished response to Hh due to the accumulation of both Smo and Ptch in cilia. Furthermore, Gli2 localization to cilia and Gli3 repressor processing is also deficient indicating that Ift25 directly couples to Hh signal transduction (Keady et al., 2012).

All core Hh pathway components traffic through cilia in a regulated and signal-dependent manner. Ptc localizes to cilia in the absence of signal and is forced out of the cilium after Hh ligand binding (Rohatgi et al., 2007). Smo accumulates along the ciliary membrane in response to the Hh signal (Corbit et al., 2005). All three mammalian Gli proteins localize to the distal tip of primary cilia although the mechanism for their ciliary recruitment remains unclear (Haycraft et al., 2005).

Primary cilia are therefore an organizing center where Gli/SuFu complexes are brought in close proximity of Smo. In the absence of SuFu, Gli is constitutively activated and the cilia are no longer required (Jia et al., 2009). In cells that lack cilia, loss of SuFu still leads to Gli activation (Chen et al., 2009). Thus cilia are required insomuch as SuFu is around.

The role of the proteasome in Gli regulation

The proteasome is an ATP-dependent protease that carries out the degradation of proteins. It clears damaged or misfolded proteins. It is part of the adaptive immune response, by digesting both self and foreign proteins into smaller peptides for eventual antigen presentation. It is crucial for the quick modulation of the concentrations of regulatory proteins in response to internal and extracellular signals. For example, the rapid accumulation or degradation of specific cyclins determines progression through the cell cycle. Also, the tumor suppressor p53, so-called guardian of the genome, is usually kept in low abundance through proteasomal degradation. In response to stress, such as DNA damage, p53 proteins are rapidly stabilized by the inhibition of its degradation.

The ubiquitin proteasome system is an important regulator of Gli proteins in two ways. First, the general stability of both Gli activator and repressor forms is modulated by complete degradation. Second, in a rare mechanism, Gli activator forms are incompletely proteolyzed into repressor forms. Thus the balance of Gli activator and repressors forms that dictate a cell's response to Hh signaling are maintained and regulated by the proteasome. It is interesting to note that proteasomes have been found to localize to the centrosomes that form the ciliary base (Wigley et al., 1999; Doxsey et al., 2005). It is therefore speculated that the base of cilia are possible sites of Gli proteolysis although no concrete evidence for this has been reported.

Complete proteolysis of Gli

Three E3 ubiquitin ligase complexes have been identified that mediate the complete proteolysis of Gli proteins. First, speckle-type POZ protein (SPOP), an adapter protein, together with a cullin 3-based E3 ubiquitin ligase, mediates the targeting of full-length Gli2 and Gli3 to

the proteasome (Zhang et al., 2006; Chen et al., 2009; Zhang et al., 2009; Wang et al., 2010). SuFu antagonizes SPOP by competing with binding sites within Gli2 and Gli3 (Chen et al., 2009; Wang et a., 2010). Loss of SuFu therefore destabilizes Gli2 and Gli3 proteins, presumably because it leaves Gli exposed to SPOP-mediated degradation (Chen et al., 2009; Jia et al., 2009; Wang et al., 2010). However, neither SPOP nor SuFu seem to regulate the degradation of Gli repressor forms (Wang et al., 2010).

Second, the adaptor protein Numb, with the E3 ligase Itch, mediates the degradation of Gli1 (Di Marcotullio et al., 2006; Di Marcotullio et al., 2011). Mutation of Numb/Itch binding sites within Gli1 caused its stabilization and enhanced oncogenicity in *in vitro* assays (Di Marcotullio et al., 2011).

Third, the E3 ligase complex SCF-βTrCP mediates the complete degradation of Gli1 and Gli2 (Bhatia et al., 2006; Pan et al., 2006). Gli2 is targeted to the proteasome by SCF-βTrCP in the absence of Hh signaling, while Hh pathway activation stabilizes Gli2 (Pan et al., 2006). The complete proteolysis of Gli1 is mediated by SCF-βTrCP, through a mechanism that is promoted by PKA (Huntzicker et al., 2006).

Partial proteolysis of Gli

The proteasome partially proteolyzes full-length Gli proteins into shorter repressor forms with varying efficiencies that reflects each Gli protein's main function. Gli3 is robustly processed in the absence of the Hh signal and is mainly a repressor (Wang et al., 2000). Gli2 is processed inefficiently (Pan et al., 2006), while Gli1 is not processed (Dai et al., 1999; Kaesler et al., 2000; Park et al., 2000), reflecting Gli1 and Gli2 function as mostly activators. The processing of Gli into repressor forms is crucial for proper embryonic development. Gli3 mutants

deficient in processing leads to polydactyly (Wang et al., 2007) while Gli2 mutants lead to embryonic lethality (Pan et al., 2009).

The determinants for the processing of Gli2 and Gli3 are similar to that of Ci. They require the same kinases and E3 ligases. In the absence of Hh signaling, PKA phosphorylates multiple sites within Gli, which primes the region for additional phosphorylation by CK1 and GSK3 (Wang et al., 2000; Pan et al., 2006; Tempe et al., 2006; Wang and Li, 2006; Schrader et al., 2011). In turn, these phosphorylated sites serve as recognition sites for the E3 ligase complex SCF-βTrCP (Pan et al., 2006; Tempe et al., 2006; Wang and Li, 2006).

Unlike Ci, Gli processing is enhanced by additional vertebrate components. The efficient processing of Gli3 requires localization to primary cilia (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005). The activity of SuFu also promotes Gli3 processing (Kise et al., 2009; Humke et al., 2010; Wang et al., 2010). Although Kif7 both inhibits and enhances developmental markers of Hh signaling in knockout mice, it was found to promote Gli3 processing (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009).

A strong determinant of partial processing also resides within the primary amino acid sequence and the resulting secondary structure. For Ci, the domain requirements for partial proteolysis are represented by the zinc fingers and the sequences following it (Methot and Basler, 1999; Tian et al., 2005; Wang and Price, 2008). Gli proteins arose as duplications of Ci and the function of each Gli protein then diverged. Gli3's sequence is most closely related to Ci, and, like Ci, it is processed robustly (Wang et al., 2000). Gli2's sequence is more diverged and is not processed as efficiently as Gli3 (Pan et al., 2006). Gli1 is least similar to Ci and is not processed at all (Dai et al. 1999; Kaesler et al. 2000; Park et al. 2000). Gli1 likely lost the sequence features required for processing.

Domain deletion and swapping studies identified a region of about 200 amino acids immediately following the zinc fingers in Gli3 as the processing determinant domain (PDD) (Pan and Wang, 2007). Replacing a corresponding region within Gli2 with the Gli3 PDD gave Gli2 the ability to be processed efficiently. Further, a fusion protein consisting of tubulin, the Gli3 PDD and an unrelated degron gave that construct the ability to be partially processed, suggesting that the Gli3 PDD may be sufficient to mediate partial proteolysis (Pan and Wang, 2007). However, a simple swapping of the Gli3 PDD into the corresponding region of Gli1 was not sufficient to induce partial processing (Schrader et al., 2011). It was determined that three distinct regions are required: the PDD, a degron and a linker sequence consisting of a stretch of simple sequences. These simple sequences are defined as a stretch of few represented amino acids such as repeating glycines. Gli1 lacked these two latter features (Schrader et al., 2011).

Earlier studies suggest a mechanical model whereby partial processing occurs when the proteasome encounters a stretch of simple sequences immediately followed by a tightly-folded domain. Proteins targeted for destruction normally lead to complete degradation, with the polypeptide chain being processively hydrolyzed as the proteasome runs along it (Lee et al., 2001). However, when the proteasome runs into a tightly folded domain, it spends more time unfolding it giving the rest of the protein a chance to escape degradation. If the amino acid sequence adjacent to this folded domain consists of simple sequences, the amount of partial proteolysis increases (Zhang and Coffino, 2004; Tian et al., 2005; Hoyt et al., 2006). It is not clear how simple sequences promote processing but one hypothesis is that they may weaken proteasome binding and thus allow escape of the substrate. For Ci/Gli proteins, this tightly folded domain is represented by the zinc fingers. The simple sequence in Ci is a stretch of asparagines, serines and glutamines, while the simple sequence is less defined for Gli.

Besides Gli/Ci proteins only three other proteins are known to be partially proteolyzed by the proteasome: NFκB and the yeast proteins Spt23 and Mga2 – all transcription factors. NFκB is produced as a larger precursor p150 protein. Partial proteolysis removes an inhibitory C-terminal domain and is converted into p50. p50 is now able to enter the nucleus and activate target gene transcription (Fan and Maniatis, 1991; Palombella et al., 1994; Sears et al., 1998). Spt2 and Mga2 are integral membrane yeast proteins related to NFκB. They are initially tethered to the endoplasmic reticulum. After partial proteolysis, the N-terminal region is freed and enters the nucleus to activate genes involved in unsaturated fatty acid synthesis (Zhang et al. 1999; Rape and Jentsch, 2002). In NFκB, the tightly folded domain is represented by the Rel-homology domain while the simple sequence is represented by a glycine-rich region (Lin and Ghosh, 1996; Orian et al., 1999; Lin et al., 2000; Lee et al., 2001; Tian et al., 2005).

Transcriptional Gli targets and feedback regulation

The transcriptional program initiated by the Hh signal occurs in two waves. During the first wave, Gli2 and Gli3 activator forms induce the transcription of the *Gli1* gene for positive feedback and signal amplification. Accumulating Gli1 proteins then carry out a second wave of transcription of target genes. Gli1 is therefore regulated primarily at the transcriptional level (Ruiz i Altaba, 1998; Dai et al., 1999). However, Gli1 is still bound and inhibited by SuFu and trafficked to cilia (Merchant et al., 2004; Chen et al., 2009; Zeng et al., 2010). In cells lacking Gli2 and Gli3, Gli1 expression cannot be induced by Hh stimulation (Lipinski et al., 2008).

As part of the first wave, *Patched1* (*Ptch1*) gene transcription is also induced, this time as part of a negative feedback loop that poises the cell to accumulate Ptc at cell membranes and

restrict Hh signaling. The accumulation *Gli1* and *Ptch1* mRNA transcripts are therefore the most robust markers of pathway activation during normal as well as deregulated Hh signaling.

The list of Gli targets genes is incomplete but continually growing. However, it is becoming clear that the identification of specific Gli targets are dependent on the cellular context that takes into consideration the potency and duration of the Hh signal as well as the transcriptional interplay of all available Gli activator and repressor forms in a cell (reviewed in Stecca and Ruiz i Altaba, 2010). Gli target genes include those involved in positive (*Gli1*) and negative feedback (*Ptch1*, *Hip1*), as well as genes implicated in cancer such as those involved in proliferation and differentiation (*CyclinD1*, *N-Myc*, *Hes1*), survival (*Bcl2*), self-renewal (*Bmi1*, *Nanog*) and invasiveness (*Osteopontin*).

Dissertation Summary

Genetic studies in model animals such as the fly and mouse have identified the core members of Hh pathway and have delineated positive (Hh, Smo and Gli activator forms) and negative regulators (Ptc, SuFu and Gli repressor forms). Epistasis analyses have placed Gli transcription factors as the most downstream component, the effectors of the pathway. All modulation of the pathway by upstream components ultimately leads to changes in the regulation of Gli. In this dissertation, I identified mechanisms that add to our understanding of the step-wise nature of Gli activation.

In Chapter 2, I show that in response to the Hh signal, Gli/SuFu complexes are rapidly recruited into cilia and that these complexes are rapidly dissociated. I also uncover a novel mechanism for negative regulation of the Hh pathway by PKA: PKA blocks the recruitment of Gli/SuFu complexes to cilia, keeping Gli activity repressed.

In Chapter 3, I show that proteasome inhibitors are potent inhibitors of Gli proteins despite accumulation of Gli activator forms. I determine that proteasome inhibition blocks Gli binding to target gene promoters. I show that a functioning proteasome is needed for Gli's transcriptional activity. I then provide preliminary evidence and rationale for the use of proteasome inhibitors as a therapeutic agent for Gli-activated cancers.

In Chapter 4, I discuss the significance of my findings and propose future studies.

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CHAPTER TWO:

THE REGULATION OF GLI PROTEINS THROUGH THE PRIMARY CILIUM

The following section contains previously published material from:

Tukachinsky H, Lopez LV, Salic A. A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. *Journal of Cell Biology*. **191**(2):415-28 (2010).

Author contributions:

H Tukachinsky and I contributed to the manuscript equally. We performed the immunofluorescence experiments studying SuFu and Gli recruitment together. I performed the RT-PCR experiments studying effects on downstream signaling. H Tukachinsky carried out the biochemical experiments showing complex dissociation upon signal activation.

A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes.

ABSTRACT

In vertebrates, Hedgehog (Hh) signaling initiated in primary cilia activates the membrane protein Smoothened (Smo) and leads to activation of Gli proteins, the transcriptional effectors of the pathway. In the absence of signaling, Gli proteins are inhibited by the cytoplasmic protein Suppressor of Fused (SuFu). It is unclear how Hh activates Gli and whether it directly regulates SuFu. We find that Hh stimulation quickly recruits endogenous SuFu-Gli complexes to cilia, suggesting a model in which Smo activates Gli by relieving inhibition by SuFu. In support of this model, we find that Hh causes rapid dissociation of the SuFu-Gli complex, thus allowing Gli to enter the nucleus and activate transcription. Activation of protein kinase A (PKA), an inhibitor of Hh signaling, blocks ciliary localization of SuFu-Gli complexes, which in turn prevents their dissociation by signaling. Our results support a simple mechanism in which Hh signals at vertebrate cilia cause dissociation of inactive SuFu-Gli complexes, a process inhibited by PKA.

INTRODUCTION

The Hedgehog (Hh) cell-cell signaling pathway is conserved in animals and has critical roles in embryonic development, in the maintenance of adult stem cells and in cancer (Huangfu and Anderson, 2006; Kalderon, 2005; Lum and Beachy, 2004; Rohatgi and Scott, 2007). In the resting state of Hh signaling, the transcriptional output of the pathway is kept off by the membrane protein Patched (Ptc), which inhibits the seven-spanner Smoothened (Smo) (Alcedo et al., 1996). The Hh pathway is activated when the secreted protein Hh binds and inactivates Ptc

(Marigo et al., 1996; Stone et al., 1996), thus relieving the inhibition exerted on Smo, which becomes active. Active Smo signals to the cytoplasm, leading to the activation of the zinc finger transcription factors that control the output of the Hh pathway, Cubitus interruptus (Ci) in Drosophila (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998) and the Gli proteins (Gli1, 2, and 3) in vertebrates.

A unique feature of vertebrate Hh pathway is that primary cilia are essential for signal transduction (Huangfu and Anderson, 2005), and the initial membrane events occur at cilia. Ptc is located at the base of the primary cilium (Rohatgi et al., 2007), and binding of Hh to Ptc leads to activation and recruitment of Smo to the cilium (Corbit et al., 2005; Rohatgi et al., 2007). Through an unknown mechanism, active Smo at the cilium relays Hh signals to the cytoplasm, resulting in the activation of Gli2 and Gli3 (Lipinski et al., 2006; Ohlmeyer and Kalderon, 1998; Wang et al., 2000), which control transcription of Hh target genes (Alexandre et al., 1996; Dai et al., 1999; Ruiz i Altaba, 1998). Since the discovery that Ptc and Smo function at the vertebrate primary cilium, an important question has been to understand how signaling through these upstream components of the Hh pathway couples to activation of the downstream Gli proteins.

An early study showed that Gli proteins localize to cilia in vertebrate limb bud cells (Haycraft et al., 2005); however, the relationship between ciliary localization and the state of Hh signaling was not investigated. Recently, Gli2 and Gli3 were shown to be recruited to the tip of primary cilia upon Hh stimulation (Chen et al., 2009; Kim et al., 2009; Wen et al., 2010), consistent with the idea that activation of Gli2 and Gli3 by Hh signaling occurs at cilia; however, the mechanism by which Gli proteins are activated at cilia has not been clarified.

In the cytoplasm of unstimulated cells, two major negative regulators ensure that the vertebrate Hh pathway is kept off. The first negative regulator is the Gli-binding protein

Suppressor of Fused (SuFu), which in vertebrates is essential for repressing Hh signaling: in cells lacking SuFu, the Hh pathway is maximally activated in a ligand-independent manner (Cooper et al., 2005; Svard et al., 2006). SuFu is thought to inhibit Gli proteins by preventing their nuclear translocation (Ding et al., 1999; Kogerman et al., 1999; Methot and Basler, 2000). Interestingly, constitutive activation of the Hh pathway in the absence of SuFu is independent of cilia (Jia et al., 2009), suggesting that Hh signaling at cilia may activate Gli proteins by inhibiting SuFu.

The second major negative regulator of Hh signaling is protein kinase A (PKA). In Drosophila, PKA phosphorylates Ci and loss of PKA leads to Hh pathway activation (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Price and Kalderon, 1999), while overexpression of PKA inhibits Hh signaling (Li et al., 1995). The inhibitory effect of PKA is conserved in vertebrate Hh signaling (Concordet et al., 1996; Epstein et al., 1996) and, interestingly, depends on SuFu (Chen et al., 2009; Svard et al., 2006), suggesting that PKA might inhibit Gli proteins by modulating their interaction with SuFu.

Although SuFu is essential for inhibiting Gli in unstimulated cells, it is unclear if Hh signaling regulates SuFu. In one model, SuFu is a simple buffer for Gli, and is not regulated by Hh signaling. This model is consistent with a recent study (Chen et al., 2009), which found that Hh stimulation does not affect the interaction between overexpressed Gli2 and Gli3, and SuFu; however, the relevance of this result for normal Hh signaling is unclear, given the non-physiological levels of Gli and SuFu proteins produced by transient transfection. In another model, Hh signaling at cilia activates Gli proteins by relieving SuFu inhibition, resulting in Gli nuclear translocation and transcriptional activation. This simple model is consistent with at least two findings: 1) the Hh pathway is constitutively active in SuFu-/- cells independent of cilia (Chen et al., 2009; Jia et al., 2009), suggesting that active Smo at cilia might signal by inhibiting

SuFu; and 2) activation of PKA by forskolin inhibits signaling by active Smo in cells that have SuFu (Wu et al., 2004), but cannot block constitutive signaling caused by loss of SuFu (Chen et al., 2009; Svard et al., 2006), suggesting that Smo and PKA might exert their opposing effects on Hh signaling through SuFu.

To begin deciphering how active Smo at the cilium activates Gli proteins, we examined the behavior of endogenous SuFu, Gli2, and full-length Gli3 (Gli3-FL) in Hh-responsive mammalian cultured cells. Focusing on endogenous proteins avoided problems associated with misregulation of overexpressed proteins. Furthermore, we analyzed biochemically the effect of Hh signaling on endogenous SuFu-Gli protein complexes, after brief Hh pathway stimulation, to avoid any confounding secondary effects due to prolonged pathway stimulation. Our results complement and extend the findings of a recent study (Humke et al., 2010) that described how Hh signaling leads to the dissociation of SuFu from Gli. Specifically, our study demonstrates that Hh stimulation through active Smo leads to the recruitment of endogenous SuFu-Gli complexes to cilia, and causes the rapid dissociation of a defined SuFu-Gli complex. Activation of PKA blocks localization of SuFu-Gli complexes to cilia and inhibits their dissociation by Smo, providing an explanation for how PKA inhibits Hh signaling: by uncoupling Smo activation from dissociation of SuFu-Gli complexes. We propose that vertebrate Hh signals are transduced by active Smo at the primary cilium by dissociating inhibitory SuFu from Gli, and that a protein complex that likely contains only SuFu and Gli forms the core of vertebrate Hh signal transduction downstream of Smo.

RESULTS

Hedgehog stimulation quickly recruits endogenous SuFu and Gli proteins to the cilium

Tagged SuFu and Gli proteins localize to primary cilia in vertebrate cells (Haycraft et al., 2005). To study the subcellular dynamics of SuFu and Gli during Hh signaling and to avoid expressing proteins at non-physiological levels, we raised polyclonal antibodies that specifically detect endogenous mouse SuFu, Gli2, and Gli3 in Hh-responsive cells (Supplemental Figure 2.S1). We first used these antibodies to examine how Sonic hedgehog (Shh) stimulation affects subcellular localization of endogenous SuFu, Gli2 and Gli3-FL (Figure 2.1A). Without Shh stimulation, low levels of SuFu, Gli2, and Gli3-FL were detected at cilia in NIH-3T3 cells and in MEFs; in contrast, Smo was absent from cilia in the absence of Shh stimulation (Figure 2.1A, B; see also Supplementary Table 2.1 for SuFu, Gli, and Smo behavior in all cell lines used in this study). Hh stimulation led to the dramatic increase in the localization of SuFu, Gli2, and Gli3-FL to cilia (Figure 2.1A), similar to that recently reported for endogenous or overexpressed Gli2 and Gli3 (Chen et al., 2009; Kim et al., 2009; Wen et al., 2010) and paralleling the recruitment of Smo to cilia (Rohatgi et al., 2007). Previous studies (Chen et al., 2009) failed to detect a signal-dependent recruitment of SuFu to cilia; one reason for this discrepancy might be that our antibodies are more sensitive than the commercial antibodies used for SuFu detection. Our other findings (that SuFu and Gli form a complex and that SuFu localization to cilia is strictly dependent on Gli – see below) are consistent with the Hh-stimulated recruitment of SuFu to cilia that we observed.

Recruitment of SuFu, Gli2, Gli3-FL and Smo was very rapid: strong ciliary localization of all these proteins was seen in as little as 30 minutes after addition of Shh to cells. The number of cilia positive for SuFu, Gli2, Gli3-FL and Smo continued to increase with time (Figure 2.1B).

We conclude that, although low amounts of SuFu and Gli proteins are present at cilia in unstimulated cells, the ciliary levels of these proteins quickly rise upon Hh stimulation.

SuFu, Gli2, and Gli3-FL show very similar "comet tail" patterns at the cilium, with the highest accumulation at the distal tip (Figure 2.1C). This pattern is different from that of Smo, which localizes along the entire length of the cilium, often at higher level towards its base (Figures 2.1A and C). Identical results were obtained when the Hh pathway was activated by the oxysterols 20-hydroxycholesterol (20-OHC) and 25-hydroxycholesterol (25-OHC) (Corcoran and Scott, 2006; Dwyer et al., 2007) (Supplemental Figure 2.S2A), as well as by the synthetic Smo activator, SAG (Chen et al., 2002; Frank-Kamenetsky et al., 2002) (Figure 2.1D).

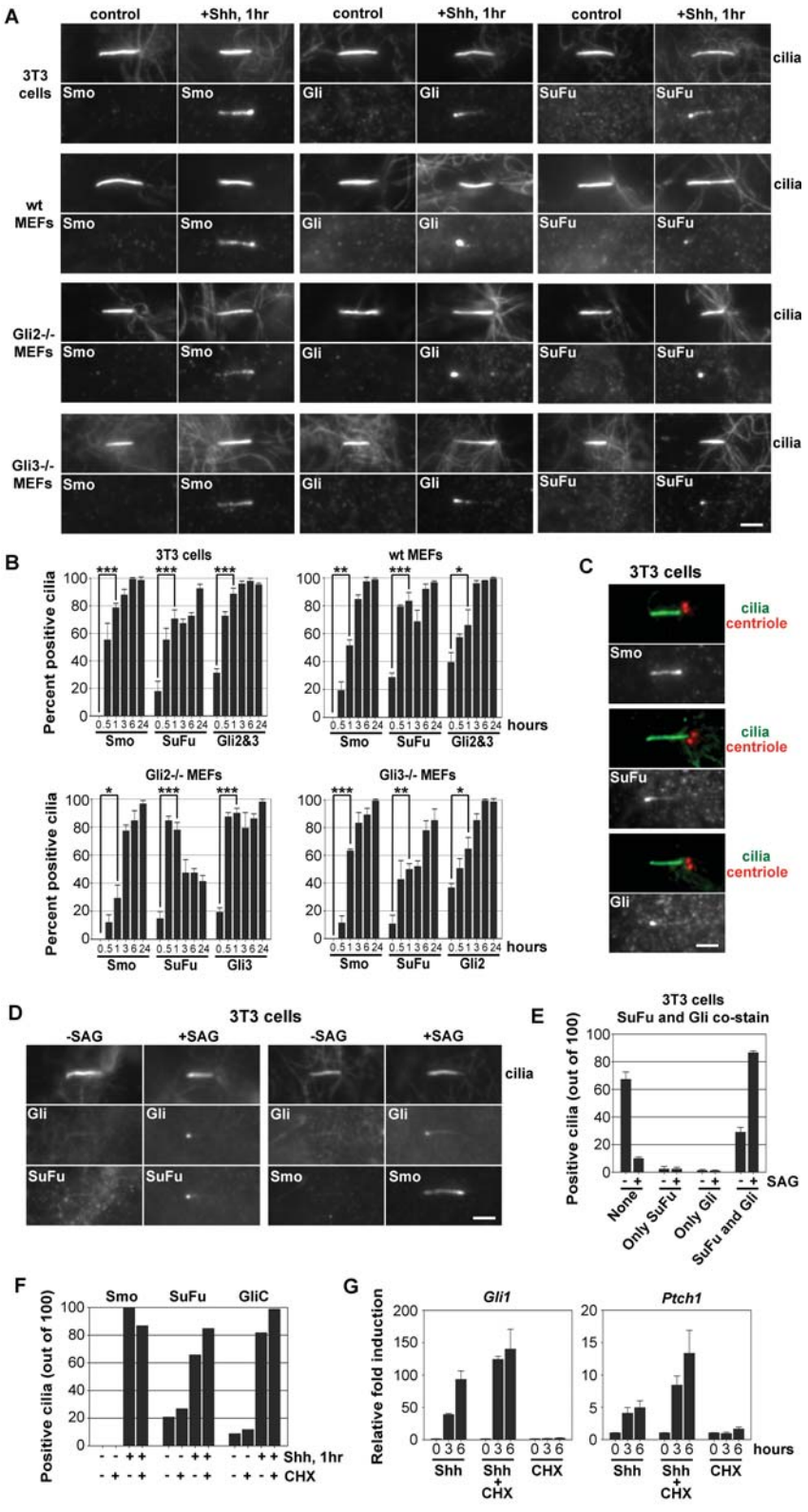

Figure 2.1 Endogenous SuFu is rapidly recruited to primary cilia by Hh signaling, paralleling recruitment of endogenous Smo, Gli2 and full-length Gli3 (Gli3-FL).

Figure 2.1 (Continued)
A) Fluorescence micrographs of cilia from untreated cells or cells treated with Shh. Cilia were detected by staining against acetylated tubulin. Since the anti-GliC antibody detects both Gli2 and Gli3-FL, Gli2-/- and Gli3-/- mouse embryonic fibroblasts (MEFs) are shown to demonstrate ciliary recruitment of Gli2 and Gli3-FL separately. In all panels, the tip of the cilium points to the left. Scale bar is 2 μm.
B) Cells were treated with Shh for varying amounts of time, and ciliary recruitment of SuFu, Smo, Gli2 and Gli3-FL was determined. Data shown are mean ± SD for three independent counts. Asterisks indicate P value for ciliary recruitment at one hour, compared to t=0 (one asterisk $P<0.05$, two asterisks $P<0.01$, three asterisks $P<0.001$). $P<0.05$ for all later time points.
C) In NIH-3T3 cells stimulated with Shh for 1 hr, SuFu and Gli proteins localize at the tip, while Smo localizes along the length of cilia. Cilia were stained as in (A) and centrioles were stained with anti-γ-tubulin.
D) Endogenous SuFu and Gli proteins co-localize at the tips of primary cilia in SAG-treated NIH-3T3 cells. Left panels: cilia co-stained for endogenous SuFu (rabbit antibody) and Gli (goat antibody). Right panels: cilia co-stained for Smo (rabbit antibody) and Gli (goat antibody).
E) Cilia counts for the experiment in (D), left panels. Endogenous SuFu and Gli co-localize, both in the resting and the stimulated states of the Hh pathway.
F) Recruitment of SuFu, Smo, and Gli to cilia in response to Hh stimulation does not require new protein synthesis. Ciliary localization was determined in NIH-3T3 cells treated or not with Shh, in the presence or absence of cycloheximide (CHX).
G) Inhibition of protein synthesis does not block the transcriptional output of the Hh pathway. Transcription of the direct transcriptional targets, Gli1 and Ptch1 was assayed by Q-PCR after 3 and 6 hours of stimulation with Shh, in the presence or absence of CHX.

The similar localization pattern of SuFu and Gli at the tips of cilia, and the fact that SuFu binds Gli proteins (Pearse et al., 1999) suggests that SuFu and Gli likely localize to the cilium as a complex. Co-staining for endogenous SuFu and Gli (using a goat anti-Gli antibody, Supplemental Figure 2.S1E) shows identical patterns at cilia (Figure 4.1D); furthermore, SuFu and Gli always appear together in cilia, both in the unstimulated and stimulated states of Hh signaling (Figure 4.1E). We thus propose that Hh stimulation quickly recruits SuFu-Gli complexes to cilia, suggesting that the molecular species to which the signal from active Smo is relayed might be the SuFu-Gli complex.

Recruitment of endogenous SuFu and Gli proteins to the cilium does not require new protein synthesis

Although the rapid recruitment of SuFu, Gli and Smo suggests that it represents an immediate response to Hh activation, results from Drosophila cultured cells showed that protein synthesis is required for certain aspects of Hh signal transduction (Lum et al., 2003). In contrast to Drosophila cells, we find that in Shh-stimulated NIH-3T3 cells, inhibiting protein synthesis does not block the recruitment of endogenous SuFu, Gli, and Smo to cilia (Figure 2.1F and Supplemental Figures 2.S2B and C) or the transcriptional activation of Hh target genes (Figure 2.1G). Also in contrast to Drosophila cells, we did not observe any change in the electrophoretic mobility of SuFu or SuFu levels upon stimulation of the Hh pathway in NIH-3T3 cells or in MEFs (Supplemental Figures 2.S4A and B). Recruitment of SuFu and Gli protein to cilia is thus an immediate response to Hh stimulation.

Uncoupling ciliary recruitment of SuFu and Gli from the transcriptional response to Hh signaling: the role of dynamic microtubules

Recruitment of SuFu, Gli and Smo to cilia upon Shh stimulation is not affected when microtubules (MTs) are depolymerized with nocodazole (Noc, Supplemental Figures 2.S2D and E), suggesting that these proteins do not need dynamic MTs to arrive at the ciliary base. Noc does not disrupt the stable MTs of primary cilia (Supplemental Figure 2.S2F), suggesting that in the presence of Noc, motors such as Kif3a (Kovacs et al., 2008) and Kif7 (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009), which were implicated in Hh signaling, can still move along ciliary MTs, explaining the proper SuFu, Gli and Smo localization to cilia. Interestingly, Noc inhibits Hh transcriptional responses in a dose-dependent manner

(Supplemental Figure 2.S2G). Thus dynamic MTs are not required for recruitment of SuFu, Gli and Smo to cilia, but are required for the transcriptional output of the pathway. We speculate that dynamic MTs are required downstream of ciliary events, such as the transport of Gli from cilia to the nucleus (Humke et al., 2010; Kim et al., 2009).

Active Smo is required for the recruitment and continued maintenance of SuFu and Gli to cilia

Low levels of SuFu and Gli localize to cilia even in unstimulated cells, and do not require Smo, as seen in Smo-/- MEFs (Supplemental Figure 2.S3A). Shh stimulation of Smo-/- MEFs does not increase ciliary SuFu and Gli, indicating that signal-dependent recruitment of SuFu and Gli requires Smo.

Active Smo translocates to cilia during normal Hh signaling, but inactive Smo can be pharmacologically forced to localize to cilia with the Smo inhibitor cyclopamine (Cyc) (Rohatgi et al., 2009; Wang et al., 2009; Wilson et al., 2009). Thus Smo might recruit SuFu and Gli to cilia irrespective of its activation state; alternatively, only active Smo recruits SuFu and Gli. To distinguish between these two alternatives, we compared SuFu, Gli and Smo localization in cells treated with SAG (Chen et al., 2002; Frank-Kamenetsky et al., 2002) or Cyc (Taipale et al., 2000). While both SAG and Cyc recruited Smo to cilia, SuFu and Gli were recruited only by SAG but not by Cyc (Figure 2.2A–C), demonstrating that only active Smo recruits SuFu and Gli to cilia.

We next asked if maintaining high levels of SuFu and Gli in cilia is continuously dependent on active Smo. We first activated Hh signaling by addition of Shh, followed by Smo inhibition with Cyc; in this manner, Smo is inactivated without changing its ciliary localization.

When Smo, SuFu, and Gli were recruited to cilia by Shh stimulation, addition of Cyc caused the levels of SuFu and Gli at the cilium to drop, while levels of Smo continued to rise (Figure 2.2D). Similar kinetics for the exit of SuFu and Gli from cilia were seen when cells were first stimulated with Shh, followed by Smo inhibition with the small molecule inhibitor, SANT-1 (Figure 2.2E). Smo inhibited by SANT-1 exited cilia more rapidly than SuFu and Gli proteins. Taken together, these experiments demonstrate that active Smo at cilia is required for maintaining high levels of SuFu and Gli at cilia during Hh signaling.

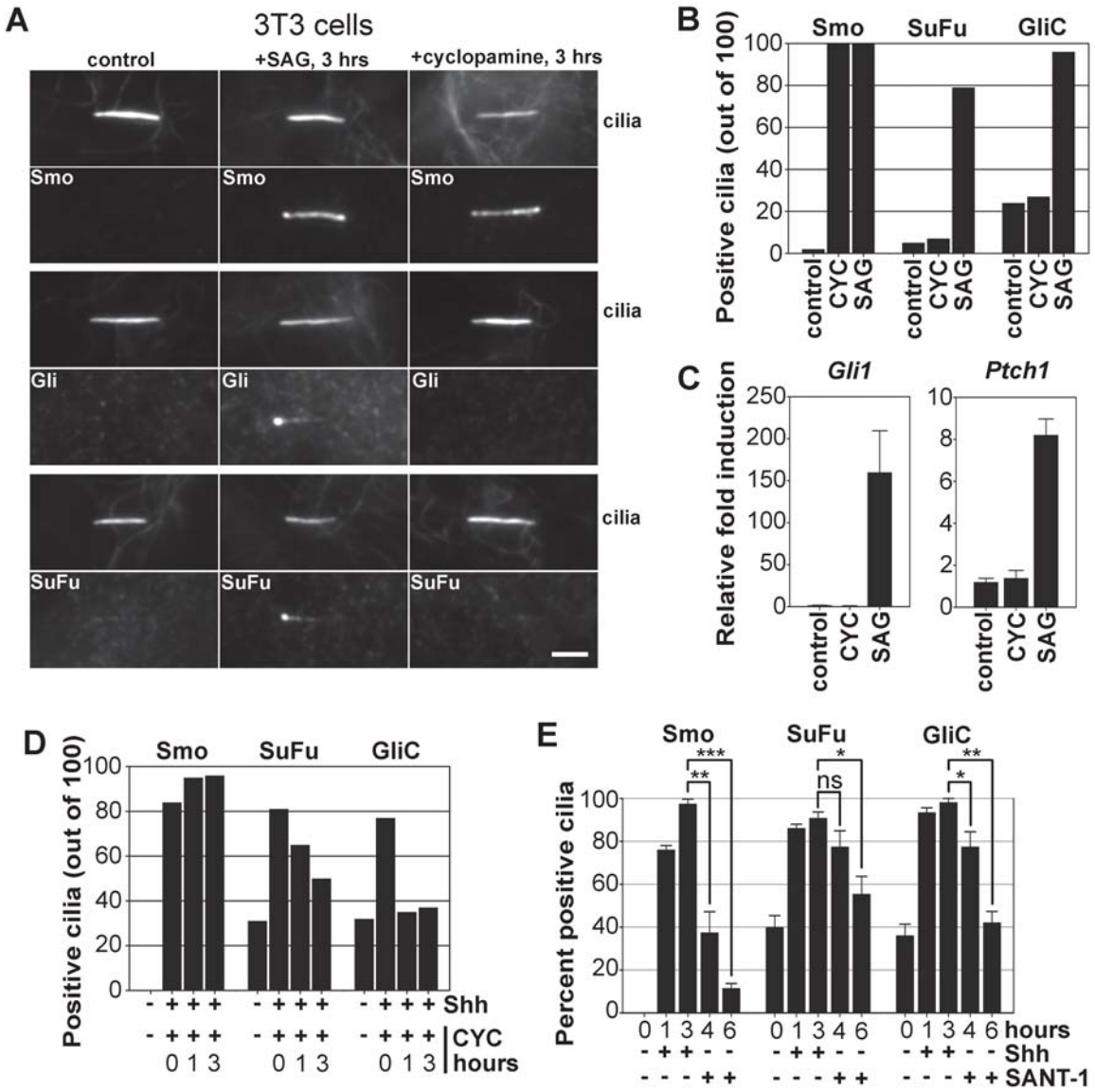

Figure 2.2 Hh-dependent recruitment of SuFu and Gli proteins to cilia requires active Smo.
A) NIH-3T3 cells were treated with the Smo agonist, SAG, or with the antagonist cyclopamine (Cyc). SuFu and Gli are recruited to cilia by SAG but not by Cyc, although both SAG and Cyc recruit Smo to cilia. In all panels, the tips of cilia point to the left. Scale bar is 2 μm.
B) Cilia counts for the experiment in (A).
C) Q-PCR assay of Hh pathway target genes for the experiment in (A).
D) Maintaining increased levels of SuFu and Gli at cilia is continuously dependent on active Smo. Cyc was added in the presence of Shh to NIH-3T3 cells, pre-stimulated with Shh for 3 hours. Ciliary localization was determined before and after 3 hours of Shh stimulation, and after 1 and 3 hours following Cyc addition.
E) NIH-3T3 cells were stimulated with Shh for 3 hrs, followed by incubation with the Smo antagonist, SANT-1 for 3 hrs. Ciliary localization of SuFu, Gli and Smo was measured at the indicated times. P values were all less than 0.002 for the recruitment of Smo, SuFu and Gli by Shh stimulation. P values for exit from the cilium were calculated relative to ciliary localization after 3 hrs of Hh stimulation. Asterisks indicate the P value for ciliary exit (one asterisk $P<0.05$, two asterisks $P<0.01$, three asterisks $P<0.001$, ns – not significant).

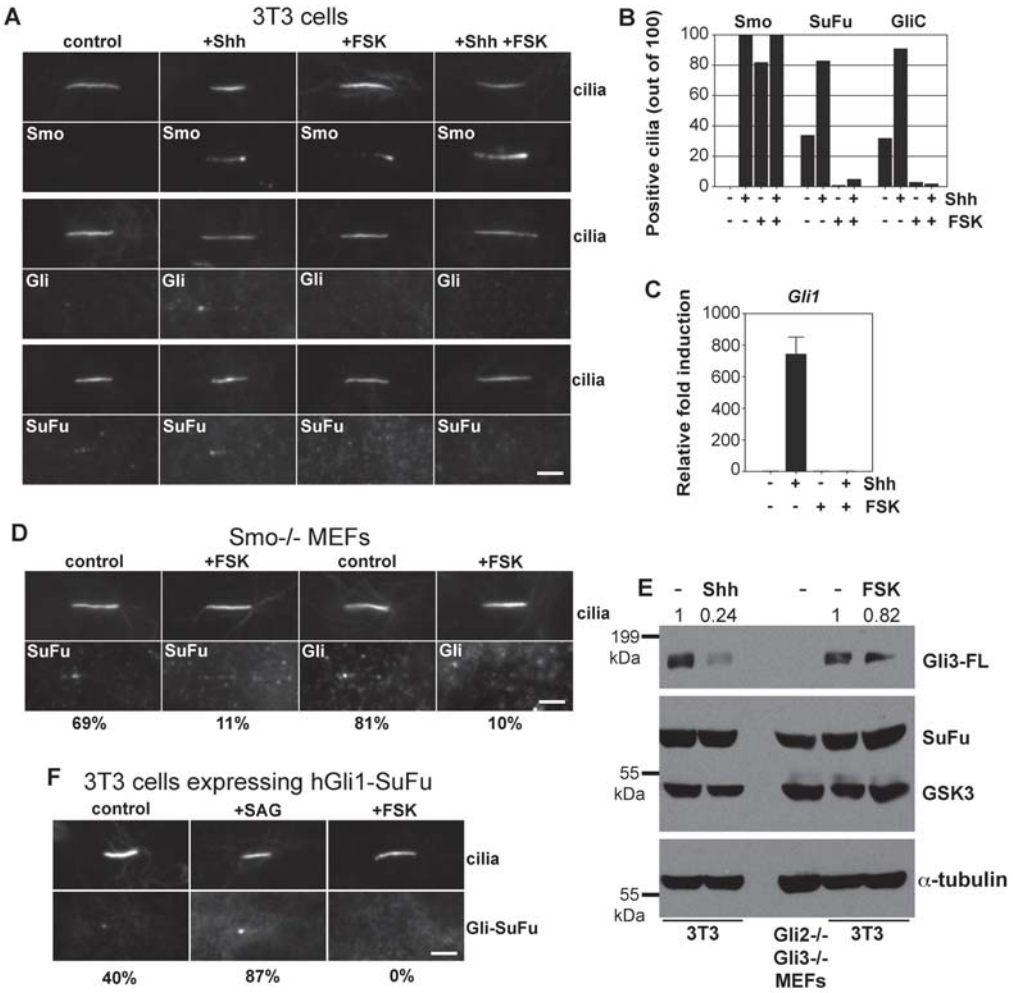

Figure 2.3 Localization of endogenous SuFu and Gli to cilia is antagonized by protein kinase A (PKA).

A) Activation of PKA by forskolin (FSK) blocks localization of endogenous SuFu and Gli proteins to cilia. NIH-3T3 cells were treated with or without Shh and FSK. Shh, FSK, or Shh and FSK recruit Smo to the cilium; in contrast, endogenous SuFu and Gli are removed from cilia by FSK, both in the presence and absence of Shh stimulation. Scale bar is 2 μm.

B) Cilia counts for the experiment in (A).

C) Q-PCR analysis of the experiment in (A). Inhibition of SuFu and Gli ciliary localization by FSK correlates with complete inhibition of the transcriptional output of the Hh pathway.

D) FSK inhibits localization of SuFu and Gli to primary cilia in Smo-/- MEFs. The percentages under the bottom panels indicate corresponding ciliary counts.

E) NIH-3T3 cells were incubated with or without Shh (the two left-most lanes), or with or without FSK (the two right-most lanes), followed by immunoblotting for SuFu, Gli3-FL, GSK3 and α-tubulin. The numbers above the top panel indicate levels of Gli3-FL in each lane, relative to α-tubulin. FSK treatment causes only a slight reduction in Gli3-FL, much smaller than the decrease caused by Shh.

F) NIH-3T3 cells stably expressing a Gli1-SuFu fusion were incubated with control media, SAG, or FSK. The Gli1-SuFu fusion localizes to cilia in unstimulated cells and its localization is increased by SAG. FSK treatment completely blocks ciliary localization of the Gli1-SuFu fusion. Percentages below the lower panels indicate ciliary localization of the fusion.

Activation of protein kinase A (PKA) blocks ciliary trafficking of endogenous SuFu and Gli

PKA is a negative regulator of the Hh pathway and forskolin (FSK), which activates PKA, is a potent inhibitor of Hh signaling. Recently, FSK was shown to recruit Smo to the cilium without activation of Hh signaling (Wilson et al., 2009). Interestingly, FSK treatment abolishes the ciliary localization of SuFu and Gli in both unstimulated and Shh-stimulated cells (Figure 2.3A and B), correlating with a complete inhibition of the transcriptional response to Hh stimulation (Figure 2.3C). We next asked if the effect of FSK on SuFu and Gli localization to cilia depends on Smo. SuFu and Gli localize to the tips of cilia in Smo-/- MEFs (Supplemental Figure 2.S3A), and FSK causes a strong decrease in ciliary SuFu and Gli (Figure 2.3D), demonstrating that FSK prevents SuFu-Gli ciliary localization independently of Smo.

One possible explanation for the dramatic inhibition of SuFu-Gli localization to cilia by FSK is an increased degradation of Gli proteins; indeed, FSK promotes partial proteolysis of overexpressed Gli2 and Gli3-FL (Pan et al., 2006; Wang and Li, 2006). In cells treated with FSK, endogenous SuFu levels do not change, and Gli3-FL levels decrease only modestly (much less than the decrease caused by Shh stimulation, Figure 2.3E), demonstrating that absence of SuFu-Gli from cilia in the presence of FSK is not due to degradation of SuFu or Gli proteins. Another explanation is that FSK blocks ciliary localization of the SuFu-Gli complex by promoting its dissociation. We excluded this possibility using 3T3 cells stably expressing a direct fusion between Gli1 and SuFu, in which FSK completely abolishes ciliary localization of the fusion (Figure 2.3F), without significantly affecting its expression level (Supplemental Figure 2.S3B). This effect of FSK is mediated by PKA, as it is reversed by the small molecule inhibitor of PKA, H-89 (Supplemental Figure 2.S3C). Furthermore, in FSK-treated cells, binding between endogenous SuFu and Gli3-FL is unaffected (Figure 2.5I). We conclude that activation of PKA

by FSK blocks ciliary trafficking of the SuFu-Gli complex, providing a pharmacological means for uncoupling recruitment of Smo to cilia from that of the SuFu-Gli complex.

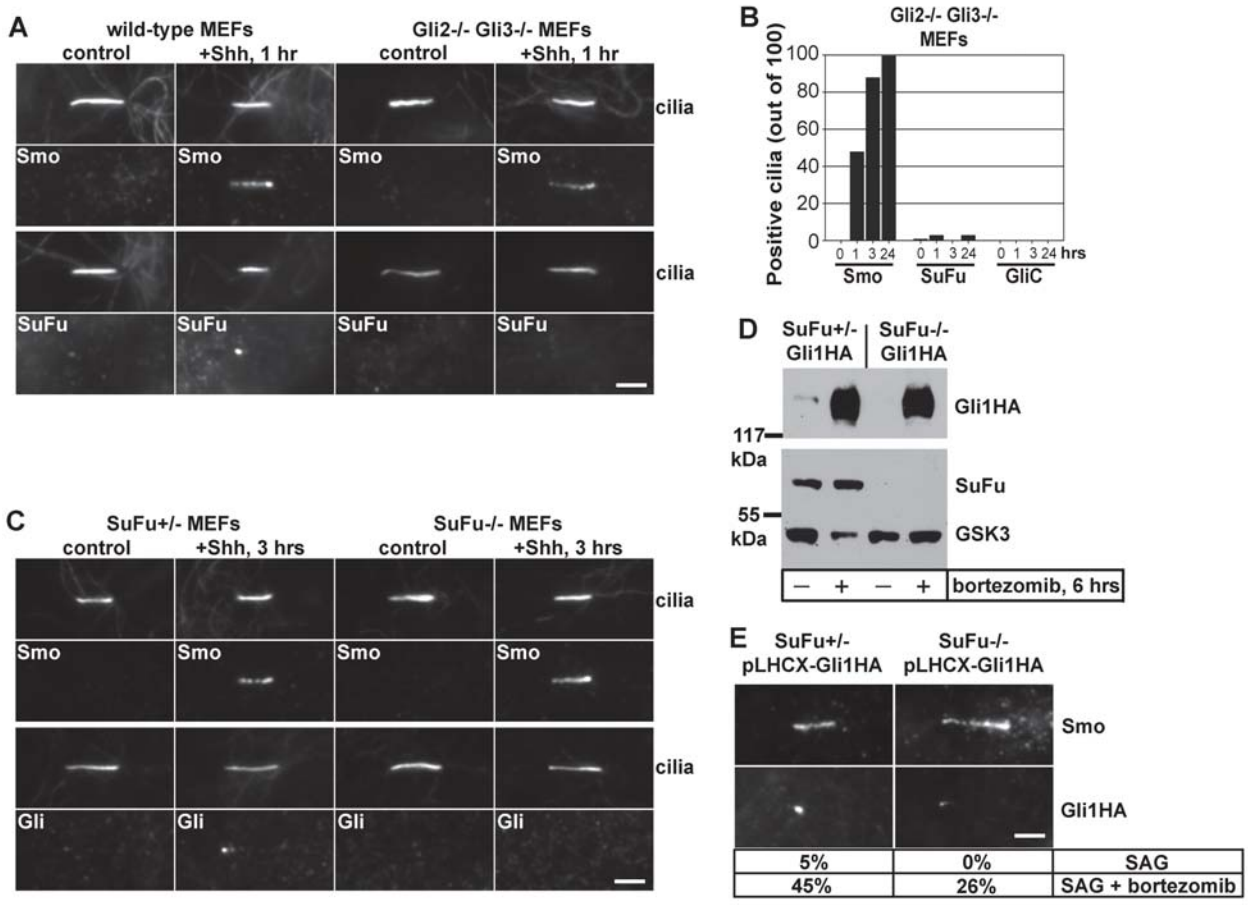

Figure 2.4 Gli proteins are required to localize SuFu to cilia but Gli proteins can localize to cilia in the absence of SuFu.

A) Wild-type and Gli2-/- Gli3-/- MEFs were incubated with or without Shh. SuFu does not localize to cilia, with or without Shh stimulation in Gli2-/- Gli3-/- MEFs, while Smo recruitment is normal. Scale bar is 2 μm.

B) Cilia counts for a time course of ciliary recruitment of Smo, SuFu, and Gli in Gli2-/- Gli3-/- MEFs stimulated with Shh.

C) SuFu+/- and SuFu-/- MEFs were stimulated or not with Shh. Endogenous Gli proteins do not localize to cilia, with or without Shh stimulation, in the absence of SuFu. Recruitment of Smo is normal.

D) Immunoblot of SuFu-/- and SuFu+/- MEFs, stably expressing HA-tagged Gli1 (Gli1HA) and treated with the proteasome inhibitor bortezomib. Proteasome inhibition allows SuFu-/- cells to accumulate Gli1HA to levels similar to those in the control SuFu+/- cells.

E) Stably expressed Gli1HA localizes to ciliary tips in SuFu-/- MEFs stimulated with SAG, in the presence of bortezomib. Percentages below the lower panels indicate corresponding ciliary counts.

Gli proteins are required to recruit SuFu to cilia but Gli proteins can localize to cilia in the absence of SuFu

Since SuFu and Gli interact, we asked if they require each other for ciliary localization, by examining localization of Gli and SuFu in MEFs lacking SuFu and Gli proteins, respectively. Gli proteins are necessary for SuFu localization to cilia: in Gli2-/- Gli3-/- MEFs (Lipinski et al., 2006), SuFu is completely absent from cilia, with or without Shh stimulation (Figure 2.4A and B), although SuFu levels are normal (Supplemental Figure 2.S4A); this excludes SuFu degradation as causing its absence from cilia in cells without Gli2 and 3. Importantly, Smo recruitment to cilia was normal in Gli2-/- Gli3-/- MEFs (Figure 2.4A and B), showing that ciliary transport and upstream Hh signaling were intact in these cells, and that localization of Smo to cilia does not depend on SuFu and Gli proteins. Either Gli2 or Gli3 is sufficient to localize SuFu to cilia, as seen in Gli2-/- and Gli3-/- MEFs (Figure 2.1A and B). Taken together, these findings argue in favor of the recruitment of SuFu-Gli2 and SuFu-Gli3 complexes to cilia.

We next asked if, conversely, SuFu is required for localizing Gli proteins to cilia. In SuFu-/- MEFs, Gli proteins do not localize to cilia, with or without Shh stimulation, although Smo recruitment is normal (Figure 2.4C and Supplemental Figure 2.S3D). Localization of Gli to cilia was restored by stable expression of SuFu in SuFu-/- MEFs (Supplemental Figure 2.S3E). One explanation for the absence of Gli proteins from cilia in SuFu-/- cells is the dramatically reduced Gli levels in the absence of SuFu (Chen et al., 2009; Ohlmeyer and Kalderon, 1998). Indeed, in SuFu-/- MEFs, Gli3-FL is dramatically decreased compared to SuFu+/- MEFs (Supplemental Figure 2.S3F), and pharmacological inhibition of the proteasome only partially rescues Gli3-FL levels. To overcome the instability of Gli proteins, we generated SuFu-/- cells stably overexpressing HA-tagged Gli1 (Gli1HA), which we stabilized by proteasomal inhibition

with bortezomib. This treatment allowed Gli1HA to accumulate in SuFu-/- MEFs to levels similar to those in the SuFu+/- MEFs (Figure 2.4D). Under these conditions, some Gli1HA can be detected in cilia of SuFu-/- MEFs (Figure 2.4E), demonstrating that at least Gli1 can localize to cilia in the absence of SuFu, as demonstrated for transiently transfected Gli proteins (Chen et al., 2009). In SuFu-/- cells, Gli1HA was concentrated in the nucleus, while in SuFu+/- cells it was excluded from the nucleus (Supplemental Figure 2.S3G), consistent with the proposed mechanism of SuFu inhibition by sequestering Gli proteins in the cytoplasm (Ding et al., 1999; Kogerman et al., 1999; Methot and Basler, 2000). Nuclear accumulation of Gli1 in the absence of SuFu might also explain why ciliary levels of Gli1HA in SuFu-/- cells were lower than in SuFu+/- cells expressing comparable amounts of Gli1HA (Figure 2.4E).

Hh stimulation causes the rapid disappearance of a defined SuFu-Gli complex

Our cellular studies of endogenous SuFu and Gli proteins suggested that active Smo at cilia relays the signal to cytoplasmic SuFu-Gli complexes. As SuFu blocks nuclear import of Gli proteins, the major mechanistic question is how active Smo at the cilium modifies the SuFu-Gli complex to allow Gli activation and nuclear entry. Since Hh signaling can occur in the absence of new protein synthesis (Figures 2.1F and G), we hypothesized that signaling must regulate SuFu-Gli complexes posttranslationally. To identify possible changes in endogenous SuFu-Gli complexes caused by Hh stimulation, we turned to measuring the size of native protein complexes by sucrose gradient centrifugation (Martin and Ames, 1961) of cellular lysates. Since prolonged Hh signaling causes a decrease in the level of Gli proteins (Supplemental Figures 2.S4A–C), we examined the effect of brief Hh stimulation (1-1.5 hours). Given that SuFu and

Gli proteins are recruited to cilia within 30 minutes or less, we reasoned that such a brief period of pathway activation should be sufficient to observe changes in SuFu-Gli complexes.

NIH-3T3 cells were stimulated or not with Shh for 1 hour, after which they were lysed and SuFu was analyzed by sucrose gradient centrifugation. The majority of endogenous SuFu (MW=54 kDa) migrates as a small molecular weight peak (Figure 2.5A), similar in size and shape to the peak of glycogen synthase kinase 3 (GSK3, MW=47 kDa). This hydrodynamic behavior indicates that most SuFu in cells is present as a monomer.

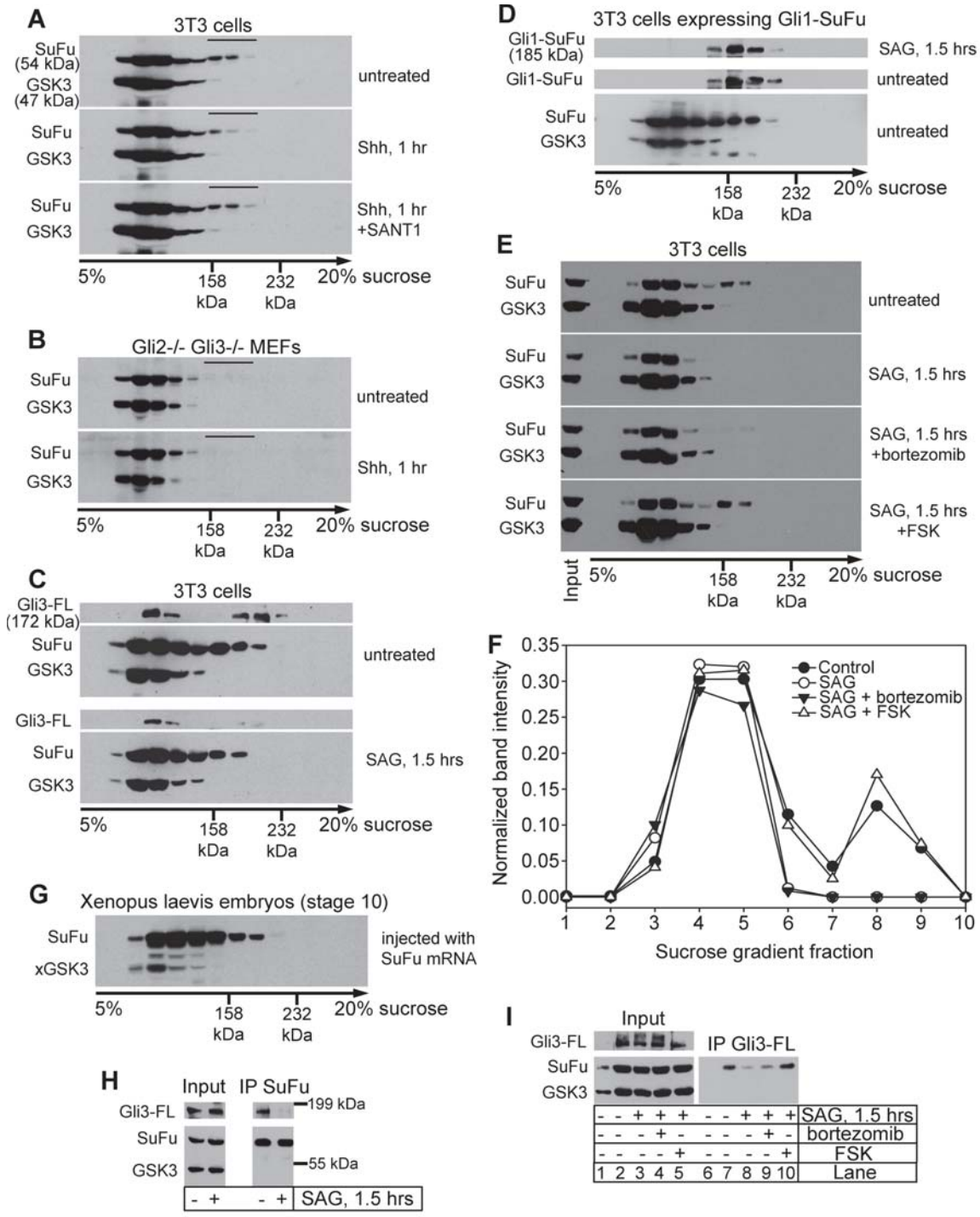

Figure 2.5 Biochemical evidence that Hh pathway activation causes rapid dissociation of endogenous SuFu-Gli complexes.

Endogenous SuFu-Gli complexes were analyzed by sucrose gradient centrifugation (A–G) and by immunoprecipitation (H, I).

Figure 2.5 (Continued)
A) In untreated NIH-3T3 cells, the majority of endogenous SuFu (MW=54 kDa) exists as a monomer, of similar size as the kinase GSK3β (MW=47 kDa). A small fraction of SuFu from untreated cells forms a higher molecular weight complex (top panel, overlined in black), the level of which quickly drops in cells treated with Shh for 1 hour (middle panel), an effect completely blocked if Smo is inhibited with SANT-1 (200 nM, bottom panel). The position in the gradient of two size markers run in parallel is shown below the Western blots: aldolase, MW= 158 kDa, Stokes radius=48.1 Angstrom; and catalase, MW=232 kDa, Stokes radius=52.2 Angstrom.
B) In Gli2-/- Gli3-/- MEFs, only the monomeric SuFu peak is seen by sucrose gradient centrifugation. Hh stimulation of Gli2-/- Gli3-/- MEFs does not change the size of the SuFu peak, although Smo is recruited to the cilia normally in these cells.
C) As in (A) but cells were stimulated or not with SAG and sucrose gradient fractions were immunoblotted for endogenous SuFu, GSK3, and Gli3-FL. The higher molecular weight SuFu peak overlaps with endogenous Gli3-FL in unstimulated cells. Acute Hh pathway stimulation causes the simultaneous disappearance of the overlapping, higher molecular weight SuFu and Gli3-FL peaks.
D) To prevent dissociation of SuFu from Gli, a direct fusion of Gli1 to SuFu was generated. NIH-3T3 cells stably expressing this Gli1-SuFu fusion were stimulated or not with SAG. The apparent size of the Gli1-SuFu fusion peak does not change upon Hh pathway activation.
E) Treatment of NIH-3T3 cells with SAG causes complete disappearance of the SuFu-Gli complex, which is not reversed by inhibition of the proteasome with bortezomib. In contrast, activation of PKA with forskolin (FSK) completely blocks SuFu-Gli dissociation induced by SAG stimulation.
F) Quantification of the experiment in (E). The amount of SuFu in each fraction was measured relative to the amount of SuFu in the input lane. The first fraction represents the top of the sucrose gradient.
G) Mouse SuFu expressed in Xenopus embryos shows the same size distribution as endogenous SuFu in mammalian cultured cells, suggesting that SuFu forms a similar complex with endogenous Gli proteins in Xenopus embryos.
H) NIH-3T3 cells were incubated with or without SAG, followed by immunoprecipitation with anti-SuFu antibodies. The level of Gli3-FL is similar in SAG-treated and in untreated cells (left panels). Gli3-FL co-immunoprecipitates with SuFu only in untreated cells but not in SAG-stimulated cells (right panels), indicating that acute Hh pathway activation dissociates endogenous Gli3-FL from SuFu.
I) NIH-3T3 cells were incubated with control media, SAG, SAG and bortezomib, and SAG and FSK, followed by immunoprecipitation with anti-Gli3-FL antibodies. Gli2-/-Gli3-/- MEFs were used as negative control (lanes 1 and 6). Endogenous SuFu does not co-immunoprecipitate with Gli3-FL in cells stimulated with SAG, although levels of Gli3-FL decrease only slightly. Proteasome inhibition by bortezomib (sufficient to abolish any decrease in the level of Gli3-FL) does not block dissociation of endogenous SuFu from Gli3-FL. In contrast, SAG-induced dissociation of SuFu from Gli3-FL is completely blocked by FSK.

In untreated cells, a small fraction of SuFu appears in fractions of higher Stokes radius (Figure 2.5A, top panel), consistent with SuFu associating with other proteins. Stimulating cells with Shh for 1 hour causes the dramatic decrease of the higher molecular weight SuFu (Figure 2.5A, middle panel), an effect that is completely reversed by the small molecule Smo inhibitor, SANT1 (Figure 2.5A, bottom panel). In another experiment, a 1.5-hour stimulation of NIH-3T3 cells

with the Smo agonist, SAG, causes the complete disappearance of the high molecular weight SuFu complex (Figure 2.5E and F).

Two lines of evidence demonstrate that the high molecular weight SuFu species is a SuFu-Gli complex: 1) The SuFu complex is absent from Gli2-/- Gli3-/- MEFs (Lipinski et al., 2006), in which only monomeric SuFu is seen on sucrose gradients (Figure 2.5B, top panel). This also indicates that SuFu is dedicated to binding Gli proteins and, in their absence, SuFu does not stably associate with other proteins. Additionally, the size of endogenous SuFu in Gli2-/- Gli3-/- cells does not change upon Hh pathway stimulation (Figure 2.5B, bottom panel), indicating that signaling specifically couples to SuFu-Gli complexes and not to monomeric SuFu. 2) The high molecular weight SuFu complex overlaps with a Gli3-FL peak (Figure 2.5C, top panel), and Hh stimulation causes the simultaneous disappearance of the high molecular weight SuFu and Gli3-FL peaks (Figure 2.5C, bottom panel). Taken together, these data demonstrate that Hh stimulation causes the quick disappearance of the SuFu-Gli complex.

Although we do not know the shape of the SuFu-Gli complex and thus cannot determine its exact size, its migration on sucrose gradients is consistent with the calculated size of a 1:1 complex (54+172=226 kDa for a mouse SuFu-Gli3-FL complex), suggesting that the complex might contain only one molecule of SuFu and Gli3-FL. To examine if SuFu behavior is conserved in other vertebrate systems, we determined the sucrose gradient profile of SuFu expressed in Xenopus embryos (Figure 2.5G), and found it very similar to that in NIH-3T3 cells, suggesting that SuFu forms complexes of a similar size with endogenous Gli proteins in Xenopus embryos.

The SuFu-Gli complex dissociates in response to Hh signaling

We considered two possibilities for the mechanism underlying the disappearance of the SuFu-Gli complex in response to Hh stimulation: 1) the SuFu-Gli complex disappears through proteolysis, either of SuFu or Gli; and 2) the SuFu-Gli complex disappears due to dissociation. Our results support the idea that Hh stimulation causes the dissociation of the SuFu-Gli complex.

A recent study suggested that Hh signaling triggers the proteasomal degradation of SuFu in certain cancer cells (Yue et al., 2009). We find that in NIH-3T3 cells, neither the steady-state level nor the half-life of SuFu changes upon Shh stimulation (Supplemental Figures 2.S4A–E), suggesting that Hh signaling does not affect bulk SuFu levels or stability. It is, however conceivable that Hh signaling might stimulate degradation of the small fraction of SuFu in SuFu-Gli complexes, but that the size of this pool is too small to detect. We excluded this possibility by blocking proteasomal degradation with the small molecule, bortezomib (see below).

The levels of both Gli3-FL and Gli3-R (Supplemental Figures 2.S4A–C), and the half-life of Gli3-FL (Supplemental Figures 2.S4D and E) decrease following Hh pathway activation; it is thus possible that the disappearance of the SuFu-Gli complex reflects the increased turnover of Gli caused by Hh signaling. The following results show that SuFu-Gli dissociation and not Gli degradation is responsible for the disappearance of the SuFu-Gli complex: 1) the SuFu-Gli complex disappears after as little as 1.5 hours of SAG stimulation, which has little or no effect on Gli3-FL levels (Figures 2.5H and I); 2) the SuFu-Gli complex disappears even when the proteasome is blocked with high levels of bortezomib (Figures 2.5E, F and I), which are sufficient to completely block Gli3-FL degradation (see also Supplemental Figure 2.S4B); and 3) if dissociation is prevented by fusing SuFu and Gli1, the size of the stably expressed covalent SuFu-Gli1 complex no longer changes in response to Hh stimulation (Figure 2.5D).

Finally, we used immunoprecipitation of endogenous SuFu and Gli3-FL from 3T3 cells to demonstrate dissociation of SuFu-Gli3-FL by Hh stimulation. The amount of Gli3-FL immunoprecipitated with SuFu from stimulated cells is dramatically reduced compared to untreated cells, although total Gli3-FL levels do not change appreciably during the 1.5 hour stimulation time (Figure 2.5H). Conversely, the amount of SuFu immunoprecipitated with Gli3-FL is greatly decreased following acute Hh stimulation, an effect that is not reversed if Gli3-FL levels are stabilized by inhibition of the proteasome (Figure 2.5I).

In summary, Hh signaling causes the rapid dissociation of SuFu from Gli, suggesting a simple mechanism for relieving the inhibition of Gli by SuFu. We also conclude that Gli3-FL degradation during Hh signaling is not a cause but a consequence of dissociation from SuFu, consistent with the pronounced instability of Gli in cells lacking SuFu, in spite of maximal activation of Gli target genes.

PKA inhibits SuFu-Gli complex dissociation: evidence that dissociation occurs at cilia

Activation of PKA by FSK potently inhibits Hh signaling, and we found that FSK completely blocks the localization of the SuFu-Gli complex to cilia. Since FSK does not prevent recruitment of Smo to cilia by Hh stimulation, we used FSK to uncouple activation and recruitment of Smo to cilia, from ciliary recruitment of SuFu-Gli. We then asked if FSK affects dissociation of the SuFu-Gli complex caused by Hh stimulation. In cells treated with FSK, dissociation of endogenous SuFu-Gli3-FL by acute Hh stimulation is completely blocked (Figures 2.5E, F and I). This result is consistent with a model in which dissociation of SuFu-Gli complexes by active Smo occurs at cilia; alternatively, FSK might independently inhibit both SuFu-Gli ciliary localization and dissociation. We favor the first model because it is consistent

with inhibition of SuFu-Gli dissociation in Kif3a-/- cells, in which ciliary localization of Smo is inhibited (Humke et al., 2010). Our findings also provide a new mechanism explaining the inhibition of Hh signaling by FSK, and its strict dependence on SuFu (Chen et al., 2009; Svard et al., 2006).

DISCUSSION

A unique feature of the vertebrate Hh pathway is that primary cilia are critical for signal transduction (Huangfu and Anderson, 2005). The Hh ligand binds its receptor, Patched (Ptc), localized at the primary cilium (Rohatgi et al., 2007), leading to activation and recruitment of the seven-spanner Smo to the cilium (Corbit et al., 2005; Rohatgi et al., 2007), from where it signals to the cytoplasm to activate Gli proteins. In unstimulated cells, Gli proteins are kept inactive by the cytoplasmic protein SuFu. In vertebrate cells lacking SuFu, the Hh pathway is maximally active, independent of Smo (Cooper et al., 2005; Svard et al., 2006) and independent of cilia (Jia et al., 2009). A simple model for vertebrate Hh signaling is that active Smo at the cilium inhibits SuFu, to allow Gli activation; however, a major unanswered question has been if and how SuFu is regulated by Hh signaling.

We found that the endogenous complex formed by SuFu and Gli proteins localizes to cilia, and that this ciliary localization is strongly increased by Hh signaling through active Smo. This suggested that the Hh signal is transmitted from active Smo to the SuFu-Gli complex, leading to Gli activation. To determine the mechanism that activates Gli, we searched for biochemical changes of SuFu-Gli complexes caused by acute Hh stimulation. SuFu is an abundant protein (we estimated its concentration in 3T3 cells at about 100 nM) and a small fraction of SuFu forms a complex with Gli in unstimulated cells, while most SuFu is monomeric.

Hh stimulation leads to the rapid dissociation of the SuFu-Gli complex (Humke et al., 2010), suggesting a simple mechanism in which Gli activation is the consequence of relieving its inhibition by SuFu, which allows Gli to enter the nucleus (Figure 2.6). We do not yet know if SuFu dissociation from Gli is sufficient to activate Gli or if posttranslational changes are also required (Ohlmeyer and Kalderon, 1998), such as Gli phosphorylation (Humke et al., 2010). We also do not know if all dissociation of the SuFu-Gli complex takes place at cilia or if it also occurs in other parts of the cell. We propose that SuFu-Gli dissociation is the first step in a series of molecular events through which Gli proteins are activated by Hh signaling. This mechanism of vertebrate Hh signaling is reminiscent of Hh signal transduction in Drosophila, in which Hh stimulation causes the release of Ci complexes by decreasing the affinity of the atypical kinesin Costal-2 for microtubules (Robbins et al., 1997).

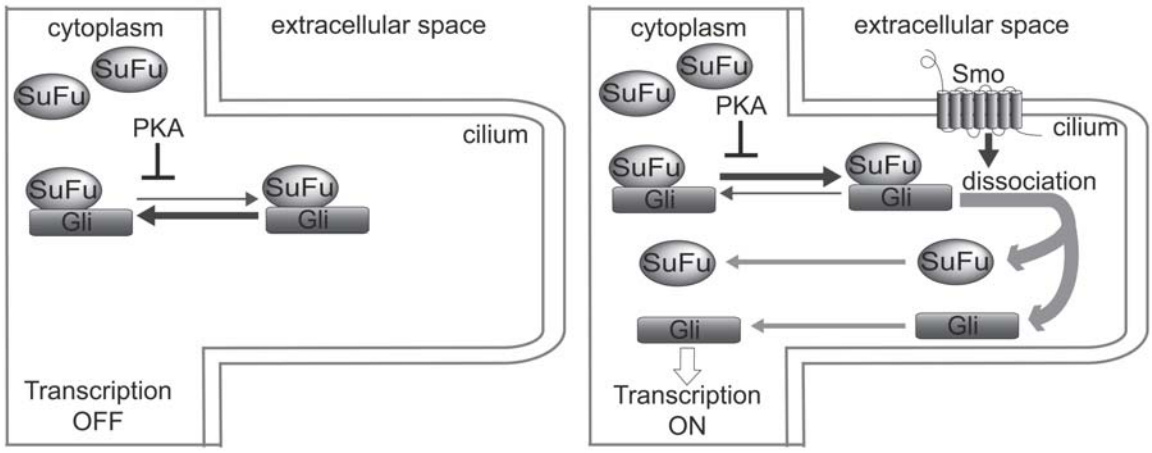

Figure 2.6 A model for activation of Gli proteins during vertebrate Hh signaling.
In the resting state of the Hh pathway (left panel), SuFu forms inactive complexes with Gli2 and Gli3-FL, which are sequestered in the cytoplasm. Without Hh stimulation, SuFu-Gli complexes traffic to the primary cilium at low level, independently of Smo; this basal ciliary trafficking is antagonized by PKA. Hh pathway stimulation (right panel) leads to the translocation of active Smo to the cilium, which, in turn, recruits SuFu-Gli complexes. Active Smo at cilia causes the dissociation of SuFu from Gli. Monomeric SuFu and Gli leave the cilium, followed by Gli nuclear translocation and activation of the transcriptional program of the Hh pathway. PKA antagonizes Hh signaling by blocking ciliary localization of SuFu-Gli complexes, thus preventing coupling between active Smo and dissociation of SuFu-Gli complexes.

Whether "active" Gli moves to the nucleus by itself or in complex with SuFu has been a matter of debate. We favor a model in which Gli enters the nucleus without SuFu, for the following reasons: 1) SuFu blocks nuclear localization of overexpressed Gli (Barnfield et al., 2005), while Gli proteins are nuclear in the absence of SuFu ((Humke et al., 2010) and the present study); 2) Hh stimulation causes the rapid dissociation of SuFu-Gli complexes, indicating that a critical step in generating active Gli is the removal of bound SuFu; and 3) SuFu is not required in the nucleus, as the transcriptional output of the Hh pathway is maximal in SuFu-/- cells (Svard et al., 2006).

Recently, the BTB domain protein SPOP was suggested to antagonize the interaction between SuFu and Gli (Chen et al., 2009). However, SPOP does not localize to cilia (Chen et al., 2009), and loss of SPOP causes only a modest increase in the unstimulated transcription of Hh target genes (Wen et al., 2010), suggesting that while SPOP might play a role in Gli turnover, it likely does not regulate the SuFu-Gli complex during the initial Hh signaling events at the ciliary membrane.

The compartmentalization of vertebrate Hh signaling in primary cilia is accomplished through at least three, largely independent ciliary localization events: 1) localization of Ptc, which is independent of Smo (Rohatgi et al., 2007); 2) localization of Smo, which can be uncoupled from upstream components (Ptc and Hh), is independent of downstream components (SuFu and Gli), and is stimulated by PKA; and 3) localization of SuFu-Gli complexes, which is inhibited by PKA. We speculate that recruitment of SuFu-Gli complexes to cilia ensures that the signal from active Smo is channeled to Gli molecules inhibited by SuFu. If SuFu were recruited to cilia alone, it would compete with SuFu-Gli complexes and inhibit signaling because monomeric SuFu is present in a large excess over SuFu-Gli. Gli-SuFu complexes thus serve not

only to keep Gli proteins inactive and stable but also to make them activatable by Hh signaling at the cilium.

Based on the size of the endogenous SuFu-Gli complex, we estimate it might consist of only these two proteins. Thus an unexpectedly simple protein complex lies at the core of vertebrate Hh signal transduction downstream of Smo. It will be important to understand how the integrity of the SuFu-Gli complex is maintained, how signaling stimulates its dissociation, and whether the posttranslational control of SuFu-Gli dissociation occurs at the levels of SuFu, Gli, or both. Additionally, it will be important to determine how SuFu-Gli complexes localize to cilia, and how active Smo increases their ciliary localization.

The PKA activator, forskolin (FSK), blocks the transcriptional output of the Hh pathway, although only in the presence of SuFu. We found that FSK abolishes the localization of the SuFu-Gli complex to cilia, and its dissociation by Hh stimulation. We interpret these findings as follows: 1) dissociation of SuFu-Gli occurs at cilia during Hh signaling, and is inhibited if SuFu-Gli cannot travel to the cilium, similar to inhibition of SuFu-Gli dissociation observed in Kif3a-/- cells (Humke et al., 2010); and 2) PKA controls trafficking of SuFu-Gli complexes to cilia, independent of Smo, suggesting a novel mechanism for Hh inhibition by PKA. Although PKA localizes to the base of cilia (Barzi et al., 2010), whether Hh signaling regulates PKA remains unclear; one possibility is that local inhibition of PKA might allow coupling between active Smo and SuFu-Gli complexes at cilia. It is likely, however, that additional events are required to transmit the signal from active Smo to the SuFu-Gli complex, since pharmacological inhibition of PKA blocks, rather than activates, Hh signaling (not shown).

Of the three members of the Gli family of transcription factors, our study focused only on Gli2 and Gli3, which mediate the initial response to Hh stimulation. Gli1 is synthesized in response to Hh signaling (Dai et al., 1999; Ruiz i Altaba, 1998) and is part of a positive feedback loop that amplifies the output of the pathway. Gli1 binds to and is inhibited by SuFu (Chen et al., 2009; Merchant et al., 2004). We envision that another role of SuFu is to inhibit newly synthesized Gli1, and that the SuFu-Gli1 complex has to pass through the cilium in the presence of active Smo in order for Gli1 to become active. This would ensure that the Hh pathway remains signal-dependent even after prolonged stimulation and accumulation of Gli1 protein, avoiding runaway transcriptional activation.

MATERIALS AND METHODS

Cell culture and Hh pathway assays

NIH-3T3 cells were grown in DMEM supplemented with 10% bovine calf serum, penicillin and streptomycin. Mouse embryonic fibroblasts (MEFs) were grown in DMEM supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids, penicillin and streptomycin. To assay Hh signaling, confluent cell cultures were starved for 24-48 hours in starvation media (DMEM without serum for NIH-3T3 cells or with 0.2% fetal bovine serum for MEFs). The media was then replaced with starvation media supplemented with the appropriate Hh pathway agonist, antagonist or control vehicle. After incubation for the desired amount of time, the cultures were processed for immunofluorescence or were harvested for real-time PCR, Western blotting, immunoprecipitation or sucrose gradient centrifugation.

Antibodies

Polyclonal antibodies against mouse Smo, SuFu and Gli were generated in rabbits or goats (Cocalico Biologicals, Reamstown, PA), and were affinity-purified. The antibodies were tested for specificity by immunoblot (on either overexpressed or endogenous proteins) and by immunofluorescence on cells (against endogenous proteins) – see Supplemental Figure 2.S1.

For the anti-Smo antibody, a fragment of the intracellular C-terminal domain of mouse Smo (amino acids 683-794) was expressed in bacteria as a soluble fusion with the maltose-binding protein (MBP). Serum from rabbits immunized with this recombinant protein was depleted of anti-MBP antibodies, after which anti-Smo antibodies were affinity purified against the antigen immobilized on Affigel 15 beads (BioRad). To generate anti-SuFu antibodies, full-length mouse SuFu (mSuFu) was expressed and purified from bacteria as an MBP fusion. The serum was affinity purified against a 6His-tagged eGFP fusion of mSuFu covalently attached to Affigel 15. To generate anti-Gli antibodies, two fragments of the human Gli3 protein (an N-terminal fragment consisting of amino acids 1-799 and a C-terminal fragment consisting of amino acids 1061-1599) were expressed in bacteria as insoluble 6His-MBP-tagged fusions. Inclusion bodies were isolated, separated by SDS-PAGE and gel slices were used to immunize rabbits or goats (Cocalico Biologicals, Reamstown, PA). The serum from rabbits immunized with the mixed recombinant Gli3 fragments was affinity purified successively against 6His-hGli3(1-799) and 6His-hGli3(1061-1599), to generate the anti-GliN and anti-GliC antibodies. On immunoblots, anti-GliN detects both full-length and processed Gli3, while anti-GliC only detects full-length Gli3. By immunofluorescence, anti-GliC detects strongly both Gli2 and full-length Gli3, while anti-GliN detects Gli3 strongly and Gli2 only weakly (Supplemental Figure 2.S1 and

data not shown). Anti-GliN and anti-GliC do not detect human or mouse Gli1 by either immunoblotting or immunofluorescence.

Real-time PCR assays of Hh pathway activity

Total cellular RNA was treated with DNase (Promega), purified, and cDNA was generated from 1 microgram of total RNA using Transcriptor reverse transcriptase (Roche) and random hexamers. *Gli1* and *Ptch1* gene expression was assayed by Quantitative Real Time PCR using FastStart SYBR Green (Roche) on a Rotor-Gene 6000 (Corbett Robotics). Relative gene expression was calculated using a Two Standard Curve method in which each gene-of-interest was normalized to the *Ribosomal Protein L27* gene. The sequences for gene-specific primers are: *L27*: 5'-GTCGAGATGGGCAAGTTCAT-3' and 5'-GCTTGGCGATCTTCTTCTTG-3', *Gli1*: 5'-GGCCAATCACAAGTCAAGGT-3' and 5'-TTCAGGAGGAGGGTACAACG -3', *Ptch1*: 5'-ACTGTCCAGCTACCCCAATG-3' and 5'-CATCATGCCAAAGAGCTCAA-3'. Error bars represent standard error of the mean for 3 independent experiments.

Effect of protein synthesis inhibition on Hh signaling in NIH-3T3 cells

To determine if ciliary recruitment and transcription activation by the Hh pathway require new protein synthesis, NIH-3T3 cells were starved overnight and then were incubated for 30 minutes in starvation media supplemented or not with cycloheximide (CHX, 50 μg/mL final). CHX-treated cells or controls were then incubated with Shh, in the presence or absence of CHX, respectively. Recruitment of Smo, SuFu and Gli to cilia was assayed by immunofluorescence following 3 hours of Shh stimulation. Expression of Gli1 and Ptch genes was assayed by Q-PCR after 0, 3 and 6 hours of stimulation. To determine the degree of protein synthesis inhibition by

CHX, cell cultures were starved for methionine by incubation for 2 hours in Met-starvation media (DMEM without methionine). The cells were then incubated for 30 minutes in Met-starvation media with or without 50 microgram/mL CHX, followed by incubation with or without CHX for 3 hours in Met-starvation media supplemented with 35S-methionine (50 microCi/mL final). The cells were harvested and 35S-labeled proteins were detected by SDS-PAGE and autoradiography. Protein synthesis was also measured by scintillation counting of 35S incorporated into TCA-insoluble material during the 3-hour incubation period.

Requirement of active Smo for ciliary recruitment of SuFu and Gli proteins

Starved, confluent NIH-3T3 cells were incubated with or without 200 nM SAG or 10 microM cyclopamine. After 3 hours, parallel cell cultures were either processed for immunofluorescence (to assay Smo, SuFu and Gli recruitment to cilia) or for Q-PCR (to assay Gli1 and Ptch1 transcription). To determine if continued localization of SuFu and Gli proteins to cilia requires active Smo, confluent NIH-3T3 cells were first incubated in the absence or presence of Shh for 3 hours, to recruit Smo, SuFu and Gli to cilia. Cyclopamine (10 microM) was then added to Shh-stimulated cells, and ciliary localization of Smo, SuFu and Gli was determined, after the desired incubation time. To determine the effects of forskolin (FSK), starved, confluent NIH-3T3 cells were treated overnight with control vehicle, Shh, FSK (10 microM, from Sigma) or FSK (10microM) and Shh. Parallel cell cultures were processed for immunofluorescence or analyzed by Q-PCR. To reverse the effects of FSK, the small molecule PKA inhibitor, H-89 (Calbiochem), was used at 10 microM.

Immunoprecipitation

Affinity-purified anti-Gli3 and anti-SuFu antibodies were covalently attached to AffiPrep Protein A beads (Bio-Rad), by crosslinking with dimethyl-pimelimidate (Pierce). Confluent cell cultures were starved for 48 hours, followed by treatment for 1.5 hours with or without SAG (100 nM), bortezomib (2 microM) or FSK (20 microM). The cells were lysed on ice in lysis buffer (20 mM HEPES pH 7.5, 50 mM potassium chloride, 1 mM magnesium chloride) with 0.5% digitonin, in the presence of protease inhibitors (Complete, Roche). The lysate was clarified by centrifugation at 20,000g and the supernatant was incubated with antibody beads for 1.5 hours at 4C. The beads were washed in lysis buffer with 0.1% digitonin before elution in SDS-PAGE sample buffer and analysis by SDS-PAGE followed by immunoblotting.

Sucrose gradient centrifugation

Linear sucrose gradients (5-20% sucrose, 12.8 mLs) in XB buffer (10 mM HEPES pH 7.5, 100 mM potassium chloride, 1 mM magnesium chloride, 100 microM calcium chloride, supplemented with protease inhibitors) were prepared using a gradient maker (BioComp), and were cooled to 4C. Cells were treated and lysed as described for immunoprecipitation experiments and a volume of 150 microL of clarified lysate was layered on the top of the gradient. Gradients were centrifuged for 20 hours at 4 C at 38,000 RPM in a SW-40 rotor (Beckman). The sucrose gradients were fractionated and each fraction was precipitated with trichloracetic acid (TCA). The TCA-precipitated proteins were analyzed by SDS-PAGE followed by immunoblotting for endogenous SuFu, Gli3 and GSK3. The sucrose gradients were calibrated using the molecular weight markers ovalbumin (MW=44 kDa, Stokes radius=30.5A), aldolase (MW=158 kDa, Stokes radius=48.1A), catalase (MW=232 kDa, Stokes radius=52.2A),

ferritin (MW=440 kDa, Stokes radius=61A) and thyroglobulin (MW=669 kDa, Stokes radius=85A).

Immunofluorescence

Cells grown on glass coverslips were fixed for 30 minutes at room temperature in PBS with 4% formaldehyde. The coverslips were rinsed with TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.2% Triton X-100) and then non-specific binding sites were blocked by incubation in TBST supplemented with 25 mg/mL bovine serum albumin (TBST-BSA). The coverslips were incubated with primary antibodies diluted in TBST-BSA, for one hour at room temperature. Coverslips were then washed with TBST, blocked again with TBST-BSA and incubated with the appropriate secondary antibodies in TBST-BSA. After washing, the coverslips were mounted on glass slides in mounting media (0.5% p-phenylenediamine, 20 mM Tris pH 8.8, 90% glycerol). Affinity-purified primary antibodies against Smo, Gli3 and SuFu were used at a final concentration of 1-2 microgram/mL. Mouse anti-acetylated tubulin, mouse anti-gamma-tubulin, and mouse anti-FLAG antibodies were purchased from Sigma. Alexa dye-conjugated secondary antibodies (Invitrogen) were used at a final concentration of 1 microgram/mL. The immunostained cells were imaged by epi-fluorescence microscopy on an inverted Nikon TE2000U microscope equipped with an OrcaER digital camera (Hammamatsu) and a 100x PlanApo 1.4NA oil objective (Nikon). Images were collected using Metamorph image acquisition software (Applied Precision). To measure ciliary localization of SuFu, Smo, and Gli, 150 cilia for each coverslip were identified by anti-acetylated tubulin staining and were scored visually for the presence or absence of SuFu, Smo, or Gli at the cilium. Error bars represent the standard deviation for groups of 50 cilia counted on different visual fields, on the same coverslip.

P values for cilia counts were calculated using an unpaired two-tailed T test, comparing each time point to t=0.

All experiments showing ciliary counts were repeated independently at least twice. Quantification of a representative experiment is shown in the panels where error bars are not provided.

Immunoblotting

Cells were resuspended in TBS with protease inhibitors, and were lysed with 1% Triton X-100 on ice for 20-30 minutes. The cell lysate was clarified by centrifugation for 30 minutes in a refrigerated microfuge at 20,000g. The supernatant was collected, mixed with DTT (50 mM final) and 5x SDS-PAGE sample buffer, and separated by SDS-PAGE on 5-15% polyacrylamide gradient gels, followed by transfer to nitrocellulose membranes. For immunoblotting, antibodies were used at a final concentration of 1 microgram/mL in TBST with 5% non-fat dry milk.

Measurement of the half-life of endogenous SuFu by CHX chase

To determine if activation of Hh signaling affects the half-life of endogenous SuFu, confluent, starved NIH-3T3 cells were pre-treated for 15 minutes in DMEM with CHX (50 microgram/mL). Parallel cultures were then incubated with CHX, in the presence or absence of 200 nM SAG in DMEM. At the indicated times, the cells were harvested and endogenous SuFu protein was detected by immunoblotting.

Nocodazole treatment

To test if microtubules (MTs) are required for recruitment of Smo, SuFu and Gli to cilia and for the transcriptional responses of Hh signaling, confluent, starved NIH-3T3 cells were pre-incubated for 1 hour with 0.25-5 microM nocodazole (Noc) or with control vehicle. The cells were then stimulated or not with Shh or with 200 nM SAG, in the presence of the same Noc concentration as during pre-incubation. After 1 hour, the cells were processed for immunofluorescence against Smo, SuFu and Gli. Cilia were stained with the mouse anti-acetylated tubulin monoclonal antibody. To determine MT depolymerization, cells treated in parallel were stained with a mouse anti-α-tubulin antibody (DM1α, Sigma). For Q-PCR analysis, cells were harvested after 2 hours of incubation with or without Shh (or SAG), and in the absence or presence of the indicated concentration of Noc.

Shh, chemical agonists and antagonists of the Hh pathway

Shh was produced in 293T cells by transient transfection of an expression plasmid encoding amino acids 1-198 of human Sonic Hedgehog. Shh-conditioned media was harvested after 48 hours, pooled, filter sterilized and used in cellular assays, usually diluted 1:4 in starvation media. Media conditioned by mock-transfected 293T cells was used as control; it had no effect on ciliary recruitment of Smo, Gli or SuFu. The Smo agonist SAG was from Axxora, the Smo antagonists SANT-1 was from Calbiochem, cyclopamine was from LC Laboratories, 20-hydroxycholesterol (20-OHC) and 25-hydroxycholesterol (25-OHC) were from Steraloids Inc.

Pharmacological inhibition of the proteasome

To block ubiquitin-dependent proteolysis, confluent cells were starved for 24-48 hours and were then pretreated with or without 2 microM bortezomib for 0.5-3 hours. The cells were then incubated with or without Hh pathway agonist, in the presence or absence of 2 microM bortezomib for the desired amount of time. Parallel cultures were processed for immunofluorescent detection, Western blotting, Q-PCR or sucrose gradient centrifugation.

Generation of stable cell lines

Constructs were generated in the retroviral vector pLHCX (Clontech), and retroviruses produced in 293T cells were used to infect NIH-3T3 cells or MEFs. Stably transduced lines were generated by hygromycin selection. Expression of the desired protein was confirmed by Western blotting and immunofluorescence. The retroviral constructs used in this study were: 1) full-length mouse SuFu tagged at the C-terminus with 3 copies of the FLAG epitope; 2) full-length human Gli1 tagged at the C-terminus with one copy of the HA epitope; 3) a fusion between N-terminally Myc-tagged human Gli1 and mouse SuFu, which incorporates a flexible, 24 amino acid linker between Gli1 and SuFu.

Quantitation of endogenous SuFu levels in NIH-3T3 cells

The concentration of endogenous SuFu protein in NIH-3T3 cells was estimated by immunoblotting, against serial dilutions of recombinant mouse SuFu expressed and purified from baculovirus-infected Sf9 cells.

Xenopus embryo injections

Capped messenger RNA for mouse SuFu was generated in vitro using the Message Machine kit (Ambion). One hundred picograms of SuFu mRNA in 10 nL of water were injected per blastomere, into both blastomeres of a two cell stage Xenopus embryo. Twenty-five injected embryos were harvested at stage 10-11 (staged according to Nieuwkoop and Faber) and were homogenized on ice in 150 microliters of XB buffer supplemented with 10 micrograms/mL cytochalasin B and protease inhibitors. The homogenate was clarified by centrifugation for 15 minutes at 20,000g, at 4 Celsius. The supernatant was harvested and subjected to sucrose gradient centrifugation, as described above for lysates from cultured cells.

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ABBREVIATIONS USED IN THIS PAPER

CHX (cycloheximide), Cyc (cyclopamine), FSK (forskolin), Gli1HA (HA-tagged Gli1), Gli3-FL (full-length Gli3), GSK3 (glycogen synthase kinase 3), Hh (Hedgehog), Shh (Sonic hedgehog), MT (microtubules), Noc (Nocodazole), MEF (mouse embryonic fibroblast), PKA (protein kinase A), Ptc (Patched), Smo (Smoothened), SuFu (Suppressor of Fused).

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CHAPTER THREE:

THE REGULATION OF GLI PROTEINS BY THE PROTEASOME

Proteasome inhibition blocks Gli-mediated Hedgehog pathway activation

ABSTRACT

Gli transcription factors are the ultimate effectors of the vertebrate Hedgehog signaling pathway. As such, all modulation of the Hedgehog pathway converges on Gli transcriptional activity. In the absence of Hedgehog signaling, Gli proteins are negatively regulated by the proteasome in two ways: first, Gli proteins are completely degraded; and second, full-length Gli-activator forms are partially proteolyzed into shorter forms of Gli that act as repressors of Hedgehog target genes. In response to the Hedgehog signal, these proteolytic events are somehow inhibited, allowing Gli-activator forms to accumulate and activate target gene transcription. Unexpectedly, we find that pharmacologic proteasome inhibitors potently abrogate Gli-mediated transcription in response to Hedgehog signals or in cases of constitutive pathway activation. This transcriptional block is due to the reduction of Gli protein occupancy and RNA polymerase II recruitment to target gene promoters. We uncover a positive role for the proteasome in regulating Gli and suggest the use of proteasome inhibitors as a potential therapeutic for Hedgehog-activated cancers.

INTRODUCTION

The Hedgehog (Hh) signaling pathway is essential for patterning during embryogenesis and organogenesis across many species (Ingham and McMahon, 2001). Moreover, the Hh pathway plays an important post-embryonic role in adult stem cell homeostasis and injury response (Trowbridge et al., 2006; Beachy et al., 2004). Loss of Hh signaling during embryogenesis leads to developmental abnormalities (Ingham and McMahon, 2001); while

unchecked Hh activation is implicated in a number of cancers (Taipale and Beachy, 2001; Barakat et al., 2010).

The Hh ligand binds its receptor Patched (Ptc) on the surface of the cell. This relieves Ptc inhibition of another transmembrane protein, Smoothened (Smo). Once activated, Smo translocates to primary cilia in vertebrate cells (Corbit et al., 2005; Rohatgi et al., 2007) and initiates a series of events that leads to the upregulation of Hh target genes by the transcription factor Glioma-associated oncogene (Gli). Ultimately, any modulation of Hh signaling by upstream pathway components leads to activation or inhibition of Gli transcriptional activity.

There are three Gli homologs in mammals (Gli1, Gli2 and Gli3) that can either function as a transcriptional activator in its full-length form (Gli-A) or as a repressor in its shorter, processed form (Gli-R). Gli-R processing is initiated by sequential phosphorylation events carried out by Protein Kinase A (PKA), Casein Kinase 1 (CK1), and Glycogen Synthase Kinase 3 (GSK3) (Pan et al., 2006; Tempe et al., 2006; Wang et al., 2000; Wang and Li, 2006). These phosphorylated sites are recognized by the E3 ubiquitin ligase complex SCF-βTRCP, which ubiquitinates and targets Gli to the proteasome for incomplete proteolysis (Pan et al., 2006; Tempe et al., 2006; Wang and Li, 2006). Gli3-R is robustly processed in the absence of Hh signal and is the major Gli repressor form (Wang et al., 2000). In contrast, Gli2-R is processed very inefficiently (Pan et al., 2006), while Gli1 is not processed at all (Dai et al., 1999; Kaesler et al., 2000; Park et al., 2000).

When the Hh ligand is absent, Ptc inactivates Smo and transcription of Hh target genes is kept low. At the level of Gli proteins, this is accomplished by at least three mechanisms. First, a fraction of Gli2 and Gli3 proteins are completely degraded via the ubiquitin-proteasome pathway (Bhatia et al., 2006; Di Marcotullio et al., 2011; Zhang et al., 2006; Zhang et al., 2009). Second,

a fraction of full-length Gli is bound and repressed by the small cytoplasmic protein Suppressor of Fused (SuFu) (Tukachinsky et al., 2010; Wang et al. 2010). Third, full-length Gli3 is partially proteolyzed into the Gli3-R form to repress target gene expression (Wang et al., 2000). Gli3-R itself is completely degraded in a constitutive manner and it is not known whether Gli3-R turnover is regulated (Humke et al., 2010). Gli1 is a direct Hh target gene and is not expressed in the absence of Hh pathway activity.

In the presence of Hh signal, a complex regulatory program impacting Gli proteolysis is initiated. Full length Gli2 accumulates likely reflecting an inhibition of its complete degradation (Chen et al., 2009; Pan et al., 2006). Paradoxically, full-length Gli3 becomes unstable despite a concurrent increase in its transcriptional activity. Its instability is partially due to dissociation from its binding and stabilizing partner, SuFu (Humke et al., 2010). Gli3-R processing is also inhibited, allowing for the accumulation of activator forms (Humke et al., 2010). Finally, the *Gli1* gene begins to be transcribed and Gli1 protein accumulates.

The proteasome plays an important role in keeping the Hh pathway off to prevent unregulated pathway activation. The Hh signal somehow leads to the inhibition of these proteolytic events. In this study, we show that proteasome inhibitors are in fact potent inhibitors of Hh target gene transcription. Our results suggest that intact proteasome function is important not only for Hh pathway repression by regulating the stability of Gli-A and processing of Gli-R forms, but also for pathway activation by regulating Gli-A localization to promoters, recruitment of the transcriptional machinery and induction of target gene expression.

RESULTS

Proteasome inhibition blocks Hh target gene transcription

The simplest model for Gli activation is that Hh signaling inhibits Gli proteolysis resulting in the accumulation of Gli activator forms. Therefore, we asked whether proteasome inhibition synergized with Hh pathway activation to increase target gene expression. We stimulated NIH3T3 cells with Sonic Hedgehog (Shh) in the presence of the proteasome inhibitor bortezomib and quantified the accumulation of *Gli1* and *Ptch1* transcripts, the best indicators of pathway activation. While both transcripts levels increase upon Shh stimulation, we unexpectedly found that the addition of bortezomib blocks their accumulation in a dose-dependent manner (Figure 3.1A). Similarly, *Gli1* and *Ptch1* transcription is blocked by bortezomib after Smo activation by the synthetic agonist SAG (Figure 3.1A; Chen et al., 2002; Frank-Kamenetsky et al., 2002). This transcriptional block is specific to proteasome inhibition since we observe identical results using other selective proteasome inhibitors MG132 and epoxomicin (Figure 3.1B). Furthermore, we observe a significant drop in target gene transcription by as little as 20 nM (Figure 3.1A) and as early as 3 hours (Figure 3.1C) following bortezomib treatment. Bortezomib treatment alone did not induce target gene transcription (Supplemental Figure 3.S1A). These results indicate that proteasome inhibition did not synergize with but rather prevented a robust transcriptional response to Hh pathway activation.

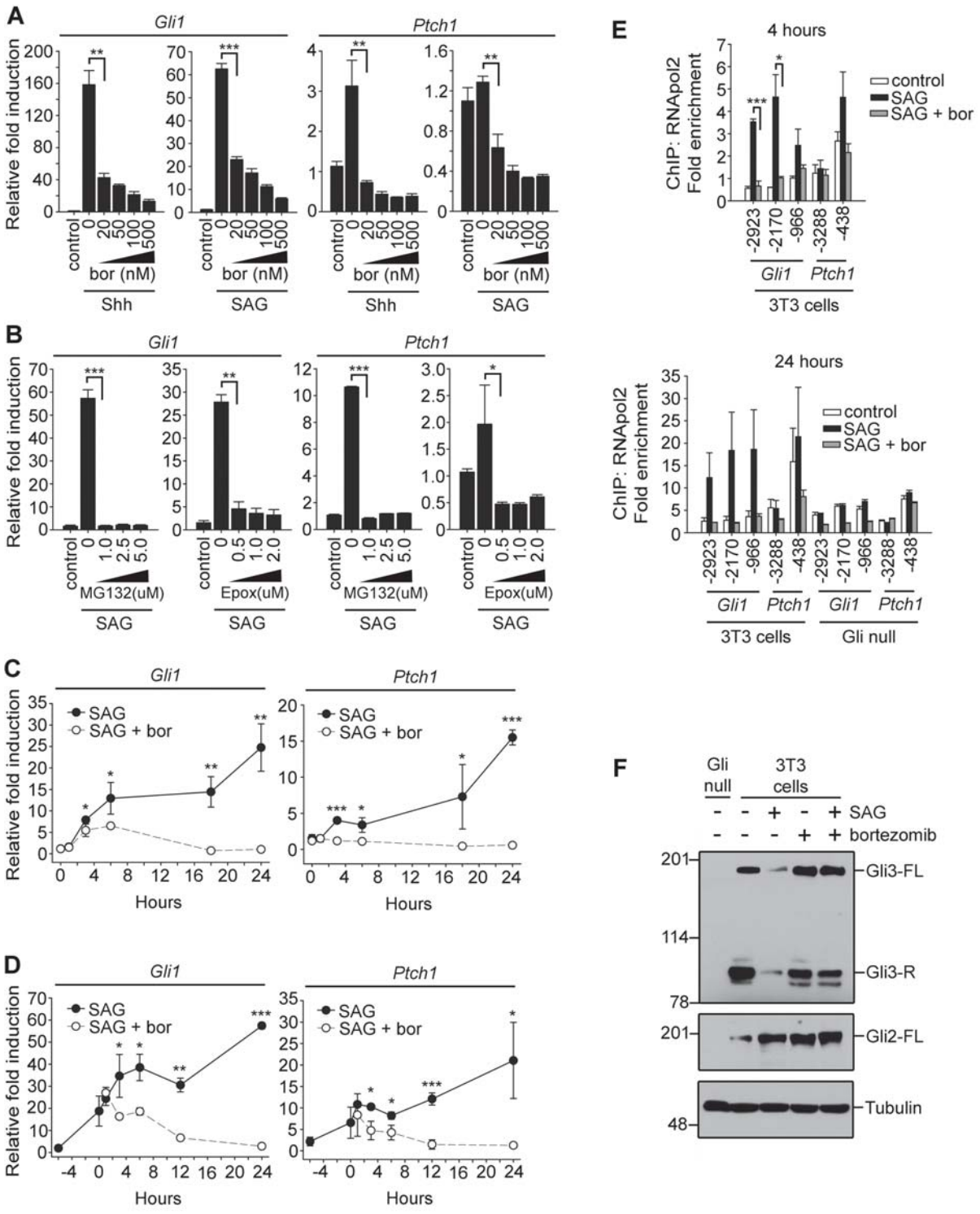

Figure 3.1 Proteasome inhibition blocks transcription of Hh target genes despite accumulation of Gli proteins in NIH3T3 cells.

Figure 3.1 (Continued)
A) Transcription of direct transcriptional targets *Gli1* and *Ptch1*was assayed by Q-PCR after 6 hours of stimulation with Shh-conditioned media or 100 nM SAG in the absence and presence on increasing concentrations of bortezomib (bor). Target gene transcript levels were normalized to the reference gene *L27*. Error bars indicate mean ± SD of three replicates. Asterisks indicate p-values for combined SAG and bortezomib treatment compared with SAG alone (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
B) Effect of treatment with epoxomicin (Epox) and MG132 on target gene transcription.
C) Time course of target gene transcription following treatment with 100 nM SAG with or without 500 nM bortezomib.
D) Time course of target gene transcription in cells pre-stimulated for 6 hours with 100 nM SAG, then refreshed with 100 nM SAG in the presence or absence of 500 nM bortezomib.
E) Chromatin immunoprecipitation (ChIP) analysis for RNA polymerase II occupancy at known Gli-binding sites (-2923, -2170 and -966 from the transcriptional start site (TSS) of *Gli1*; and, -3288 and -438 from the *Ptch1* TSS) reported as fold enrichment over a non-binding site (-5627 from the *Gli1* TSS). Cells were incubated with vehicle or 100 nM SAG with or without 500 nM bortezomib for 4 hours; or with 100 nM SAG with or without 100 nM bortezomib for 24 hours. ChIP on Gli null cells (Gli2-/-, Gli3-/- MEFs) was performed to show background signal. Error bars indicate mean ± SEM of three replicates.
F) Cells were incubated with or without 100 nM SAG in the presence or absence of 100 nM bortezomib for 24 hours followed by immunoblotting for Gli3, Gli2 and Tubulin.

We next asked whether proteasome inhibition can inhibit ongoing transcription of Hh target genes. We pre-stimulated cells with SAG or Shh for 6 hours, allowing target gene transcripts to accumulate then added bortezomib under continued treatment with SAG or Shh. Both *Gli1* and *Ptch1* transcripts stopped accumulating within 3 hours after addition of bortezomib and declined for several hours thereafter (Figure 3.1D and Supplemental Figure 3.S1B). *Ptch1* transcripts are expressed at some basal level (~10 femtograms per nanogram of total RNA, Supplemental Figure 3.S1A), since Hh-responsive cells require Ptch1 protein to receive the Hh signal. Bortezomib treatment decreased *Ptch1* transcripts to below basal levels in unstimulated cells (Supplemental Figure 3.S1A). These results demonstrate that bortezomib blocks both induced transcription of Hh target genes in response to pathway activation as well as the maintenance of transcription as in continued presence of the Hh signal and in basal *Ptch1* transcription in the absence of signal. These changes in transcript levels correlated with RNA polymerase II occupancy of *Gli1* and *Ptch1* promoters indicating that recruitment of the

transcriptional machinery is perturbed (Figure 3.1E). In cells treated acutely (4 hours) or overnight (24 hours), RNA polymerase II occupancy increased with SAG treatment while bortezomib blocked recruitment. We conclude that Hh target gene transcription is potently blocked by proteasome inhibition.

We verified the effects of proteasome inhibition on Gli protein levels by Western blotting (Figure 3.1F). Full-length Gli2 increases upon Hh pathway activation and is further stabilized by proteasome inhibition. In contrast, Gli3 proteins are destabilized upon pathway activation. This is consistent with the idea that Gli3 proteins function mainly as repressors. Bortezomib treatment stabilizes both Gli3 activator and repressor forms regardless of pathway activation state. Therefore, the transcriptional block by proteasome inhibition occurs despite the accumulation of Gli-A forms. It is possible that accumulation of Gli-R overrides transcriptional activation by Gli-A. We explore this possibility in a later section below.

Proteasome inhibition does not block general transcription.

Bortezomib is toxic at high doses and may have pleiotropic effects on many cellular processes as a result of proteasome inhibition (Chen et al., 2011). Indeed, bortezomib inhibited the growth of proliferating NIH3T3 cells at concentrations greater than 100 nM (Supplemental Figures 3.S2A and B). In order to promote primary cilia assembly, we utilized confluent, serum-starved cells. These conditions are known to induce quiescence. Under these conditions bortezomib was not cytotoxic and the redox capacity of cells was unaffected (Supplemental Figures 3.S2C and D). Furthermore, the observed transcriptional block is reversible, as *Gli1* and *Ptch1* transcription can recover after washoff of bortezomib and the subsequent induction with SAG (Supplemental Figure 3.S1C).

We next asked whether general transcription is inhibited by proteasome inhibition. We first looked at the transcription of housekeeping genes like the ribosomal protein *L27* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). *L27* transcript levels are unaffected by bortezomib treatment (Figure 3.2A). Furthermore, we found that RNA polymerase II occupancy of the *GAPDH* promoter is not significantly affected by bortezomib (Figure 3.2B). Next, we asked whether induced transcription in an unrelated signaling pathway is still able to occur in the presence of proteasome inhibitors. We looked at transcriptional response of *cJun* to growth factor signaling in the presence of proteasome inhibition. In response to Epidermal Growth Factor (EGF) ligand, the transcription of positive feedback effectors and direct target genes *cJun* and *cFos* is quickly induced. Since maximal induction of these immediate-early genes occur within minutes, we first pre-treated cells with bortezomib for 4 hours, then added EGF ligand and incubated for an additional 30 minutes. We detected a modest induction of *cJun* in response to EGF treatment alone, however, the combination with bortezomib pre-treatment greatly increased *cJun* transcription (Figure 3.2C, top right panel). In contrast, EGF-induced *cFos* transcription was inhibited with bortezomib pre-treatment (Figure 3.2C, bottom right panel). Interestingly, bortezomib treatment in the absence of EGF induced both *cJun* and *cFos* transcription (Figure 3.2C, top left and bottom left panels). To demonstrate the differential effects of proteasome inhibition to induced transcription in the same cells, we first incubated cells with SAG and bortezomib for 4 hours then added EGF for an additional 30 minutes. In these doubly induced cells, transcription of *Gli1*, *Ptch1*, and *cFos* was inhibited by bortezomib while transcription of *cJun* was enhanced (Figure 3.2D). We conclude that the transcriptional block by proteasome inhibition of the Hh pathway is not due to a global deficiency in transcription, or to cytotoxic effects.

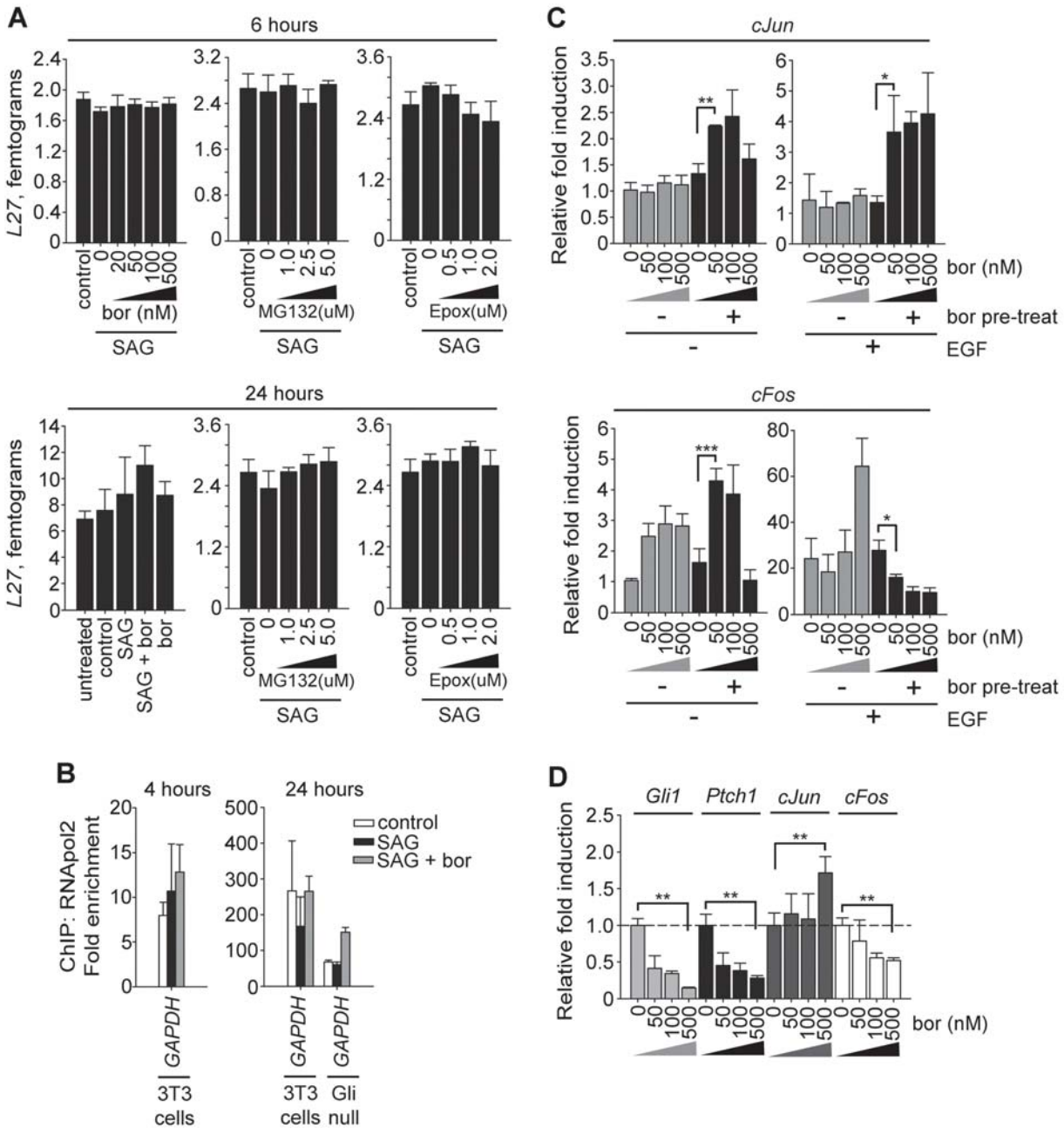

Figure 3.2 Proteasome inhibition does not block general transcription in NIH3T3 cells.
A) Transcription of the ribosomal protein *L27* gene reported as absolute values in femtograms per nanogram of total RNA determined from a plasmid standard dilution series by Q-PCR. Error bars indicate mean ± SD of three replicates.
Top panel: *L27* absolute values used for normalization of *Gli1* and *Ptch1* transcription for the experiment in Figures 3.1A and B.
Bottom panel: *L27* absolute values for new experiments after 24 hour incubation with 100 nM SAG with or without 100 nM bortezomib or increasing amounts of MG132 or epoxomicin.

Figure 3.2 (Continued)
B) ChIP analysis for RNA polymerase II occupancy at the *GAPDH* promoter. Error bars indicate mean ± SEM of three replicates. Left panel: treatment with 100 nM SAG with or without 500 nM bortezomib for 4 hours. Right panel: treatment with 100 nM SAG with or without 100 nM bortezomib for 24 hours.
C) Transcription of *cJun* and *cFos* in response to EGF stimulation in the presence of bortezomib. Cells were pre-treated or not with increasing amounts of bortezomib for 4 hours, with the addition or absence of 50 ng/ml EGF ligand for an additional 30 min. Error bars indicate mean ± SD of three replicates. Asterisks indicate p-values for bortezomib-treated compared with untreated samples (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
D) Differential transcriptional response to Hh and EGF stimulation in the absence or presence of increasing amounts of bortezomib. Cells were treated with 100 nM SAG and increasing amounts of bortezomib for 4 hours followed by addition of 50 ng/ml EGF ligand and incubation for an additional 30 min. Transcription of *Gli1*, *Ptch1*, *cJun* and *cFos* are relative to Hh and EGF pathway stimulation in the absence of bortezomib.

Transcriptional block by proteasome inhibition is not mediated by Hh pathway components upstream of Gli

We used knockouts of Hh pathway components to probe whether proteasome inhibition might affect a protein upstream of Gli. Ptc is a membrane receptor and negative regulator, its loss resulting in constitutive pathway activation (Goodrich et al., 1997). Bortezomib treatment in Ptc-/- MEFs eliminates constitutive target gene transcription (Figure 3.3A). Smo is the major positive regulator of Hh signaling at the cell membrane. To determine whether the transcriptional block by bortezomib can overcome constitutively activated Smo, we created cell lines expressing low levels of either wildtype (SmoWT) or a gain of function mutant (SmoM2, Taipale et al., 2000), in a Smo-/- background (Figure 3.3B, Nedelcu et al., 2013). In SmoM2 MEFs, constitutively high basal target gene transcription does not change in presence of SAG indicating maximal pathway activation. This is in contrast to MEFs expressing SmoWT, which require pathway activation by SAG reflecting rescue of endogenous regulation. Proteasome inhibition dramatically reduces transcription in both SAG-stimulated SmoWT and constitutively active SmoM2 MEFs (Figure 3.3B).

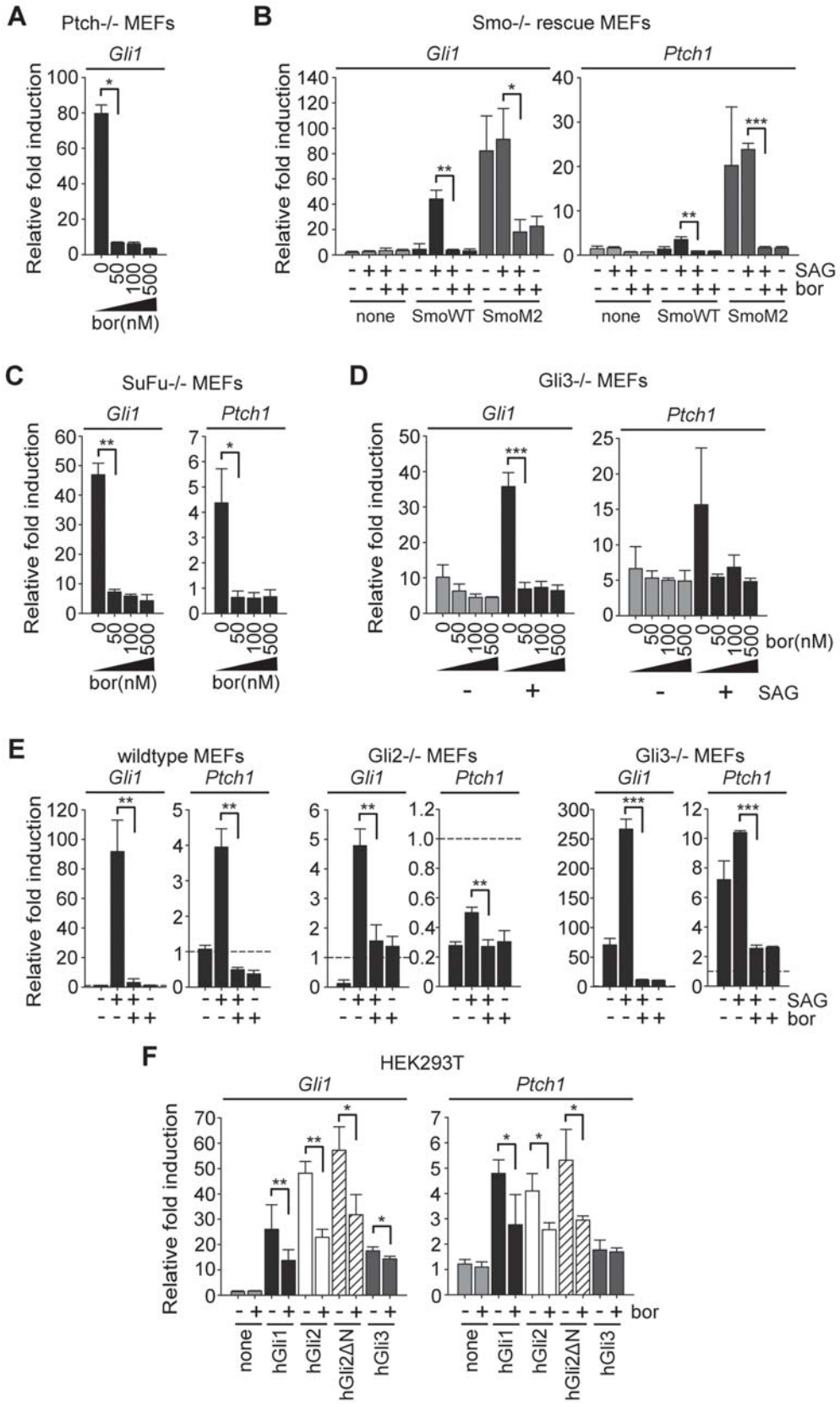

Figure 3.3 The transcriptional block by proteasome inhibition is mediated by Gli proteins.

Figure 3.3 (Continued)
A) Transcription of *Gli1* in Ptc-/- MEFs to increasing amounts of bortezomib (bor) after 24 hours assayed by Q-PCR. Target gene transcript levels were normalized to the reference gene *L27*. Error bars indicate mean ± SD of three replicates. Asterisks indicate p-values for bortezomib-treated compared with untreated samples (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
B) Transcription of *Gli1* and *Ptch1* in Smo-/- MEFs rescued with wildtype Smo (SmoWT), constitutively active Smo mutant (SmoM2) or control (none) in response to 24 hour treatment of 100 nM SAG with or without 100 nM bortezomib. Asterisks indicate p-values for combined SAG and bortezomib treatment compared with SAG alone (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
C) Transcriptional response of SuFu-/- MEFs to increasing amounts of bortezomib for 24 hours.
D) Transcriptional response of Gli3-/- MEFs to increasing amounts of bortezomib in the absence or presence of 100 nM SAG. Values were normalized to unstimulated NIH3T3 cells.
E) Comparison of the transcriptional response to treatment with 100 nM SAG with or without 100 nM bortezomib in Gli2-/- MEFs, Gli3-/- MEFs and littermate wildtype MEFs. Values were normalized to unstimulated littermate wildtype MEFs.
F) Transcription of endogenous *Gli1* and *Ptch1* in HEK293T cells transiently transfected with hGli1, hGli2, hGli2ΔN, hGli3 or control (none) in the absence or presence of 500 nM bortezomib for 24 hours.

Cytoplasmic protein SuFu forms an inhibitory complex with Gli proteins keeping Gli activator forms sequestered outside the nucleus and promoting Gli repressor formation (Ding et al., 1999; Kogerman et al., 1999; Methot and Basler, 2000). Loss of SuFu leads to constitutive pathway activation (Cooper et al., 2005; Svard et al., 2006). In SuFu-/- MEFs, constitutive target gene transcription is also blocked by proteasome inhibition (Figure 3.3C). We conclude that the transcriptional block by proteasome inhibition is acting downstream of SuFu and most likely at the level of Gli proteins to inhibit target gene transcription. Next, we were interested in whether this effect relies on any one Gli homolog in particular.

Proteasome inhibition blocks transcription by all Gli species

Gli proteins are incompletely proteolyzed from full-length Gli-A forms to shorter Gli-R forms. In unstimulated cells, Gli3 is very efficiently processed into Gli3-R and is thus a major contributor to the repression of Hh target genes in the absence of signal. One explanation for how proteasome inhibition shuts down the Hh pathway is by stabilization of Gli3-R. We therefore analyzed Gli3-/- MEFs to exclude the contribution of Gli3-R. Loss of Gli3 resulted in

higher basal Hh target gene transcription (Figure 3.3D). However, bortezomib treatment reduced basal transcription and eliminated any transcriptional induction by SAG. Thus proteasome inhibition does not rely on Gli3 to block target gene expression.

Gli2 and Gli3 are the principal effectors of the Hh pathway at early times immediately following pathway activation; whereas Gli1 is a direct target of the Gli2 and Gli3 proteins that serve as a positive feedback and signal amplification. To look at the contribution of Gli2 and Gli3 separately, we assayed Gli3-/- MEFs and Gli2-/- MEFs, respectively, and compared target gene transcription with litter-mate wildtype MEFs following activation with SAG and bortezomib treatment. In all cases SAG induced gene transcription, whereas the addition of bortezomib blocked it (Figure 3.3E).

Finally, we looked directly at the specific contribution of individual Gli protein homologs in isolation. We expressed human Gli1, Gli2 or Gli3 by transient transfection in HEK293T cells, a cell line that is unresponsive to Hh signaling (Supplemental Figure 3.S3A). Expression of each Gli protein resulted in transcription of endogenous Hh target genes, whereas bortezomib blocked it (Figure 3.3F). Of the three Gli proteins, Gli2 has the most potent transcriptional activity. This activity can be further enhanced by a deletion on an N-terminal repressor domain (hGli2ΔN, Roessler et al., 2005). Expression of hGli2ΔN resulted in higher Hh target gene transcription compared to wildtype Gli2 and bortezomib blocked this increased transcriptional activity. In contrast, Gli3 is weakly activating in this system, owning to its weak transcriptional activity and to the larger contribution of Gli3-R forms in repressing gene expression.

These results suggest that proteasome inhibition does not block target gene transcription by disrupting a delicate balance of Gli species but rather inhibits the transcriptional activity of all

three species whether repressor or activator. However, these results do not exclude the possibility of another as yet unknown negative regulator acting downstream of SuFu and upstream of Gli.

Proteasome inhibition does not disrupt Gli protein nuclear entry

We interrogated each step of Gli protein regulation to determine at which stage proteasomal inhibition is affecting Gli transcriptional activity. An event leading to Gli activation is the recruitment and accumulation of Gli-SuFu complexes to primary cilia (Tukachinsky et al., 2010). In the presence of bortezomib, accumulation of Gli at ciliary tips occurred as previously described (Figure 3.4A). Following their recruitment to cilia, Gli-SuFu inhibitory complexes dissociate via an unknown Smo-dependent mechanism. We demonstrated in a previous study that the dissociation of Gli from SuFu in response to the Hh signal still occurs in the presence of bortezomib indicating that Gli-SuFu complex dissociation is not mediated by proteasomal degradation (Figure 2.5E, Tukachinsky et al., 2010).

Once free from SuFu, full length Gli enters the nucleus to activate target gene transcription. Several lines of evidence indicate that nuclear import of Gli is not affected by proteasome inhibition. Immunoblot analysis of fractionated cell lysates demonstrates an accumulation of Gli2 and Gli3 proteins in the nuclear fraction following combined treatment with agonist and bortezomib (Figure 3.4B). In addition, we visualized nuclear localization of Gli proteins by immunofluorescence. As accumulation of endogenous Gli proteins is not detectable by immunofluorescence, we ectopically expressed Gli in HEK293T cells. In these cells, we observe an increase in nuclear localization of N-terminally Myc tagged full-length Gli2 in the presence of bortezomib (Figure 3.4C).

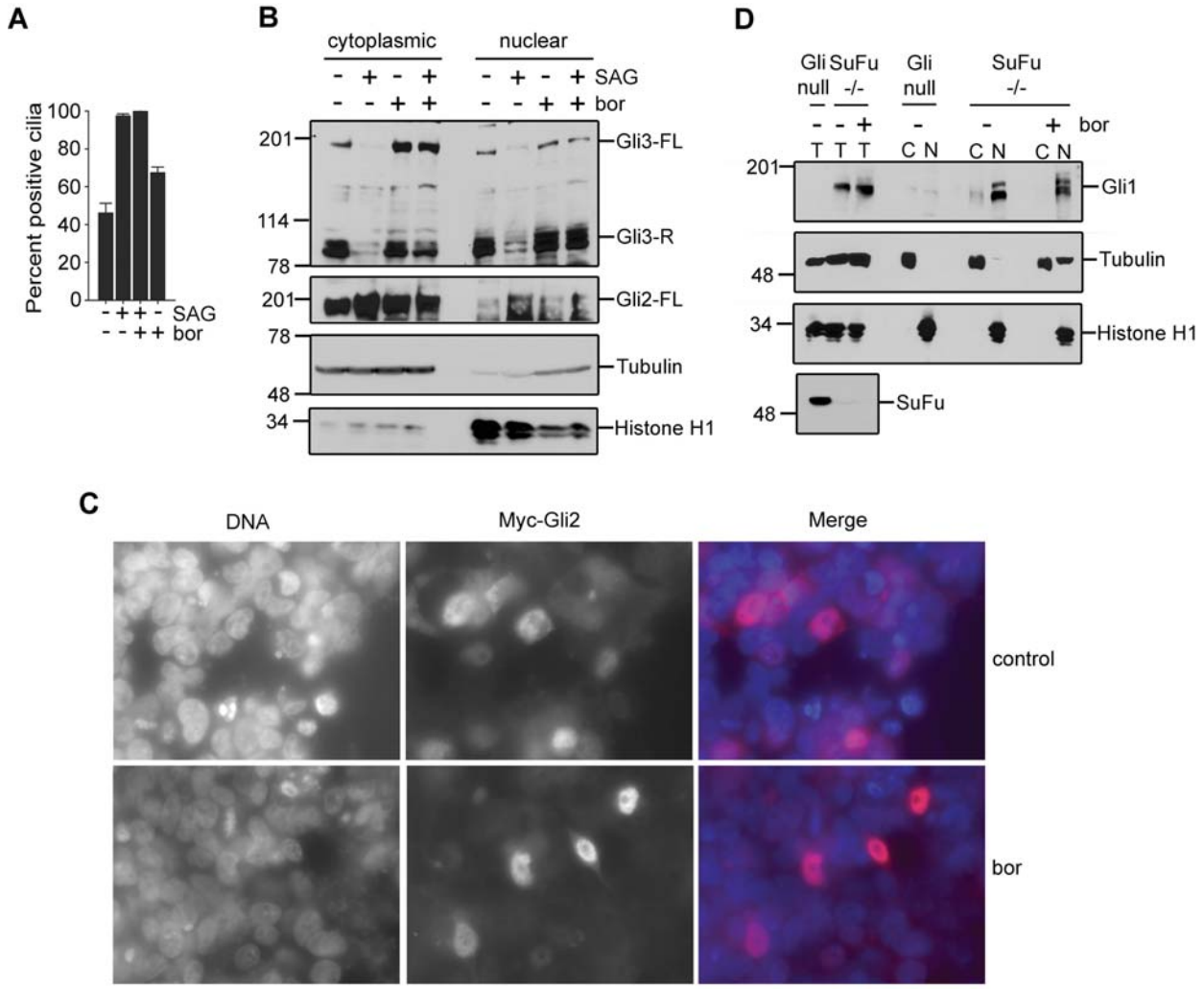

Figure 3.4 Proteasome inhibition does not impair Gli ciliary recruitment and stabilizes Gli proteins in the cytoplasm and nucleus.

A) NIH3T3 cells were treated with 100 nM SAG and/or 100 nM bortezomib (bor) for 6 hours and ciliary recruitment of Gli2-A and Gli3-A determined using an antibody that recognizes the C-terminus of both Gli2 and Gli3 proteins. Error bars indicate mean ± SD of three independent counts.

B) NIH3T3 cells were incubated with or without 100 nM SAG in the presence or absence of 100 nM bortezomib for 24 hours followed by nuclear-cytoplasmic fractionation and immunoblotting for Gli3, Gli2, Tubulin and Histone H1.

C) Fluorescence micrographs of HEK293T cells transiently transfected with Myc-tagged hGli2, treated with or without 500 nM bortezomib for 16 hours, showing nuclear localization of Gli2 stained with anti-Myc antibody (9E10) and counterstained with DNA dye Hoechst 33258 to visualize the nucleus using a 60 x objective.

D) SuFu-/- MEFs were incubated with or without 100 nM SAG in the presence or absence of 100 nM bortezomib for 24 hours followed by nuclear-cytoplasmic fractionation. Total cell lysate (T), cytoplasmic fraction (C) and nuclear fraction (N) were immunoblotted for Gli1, SuFu, Tubulin, and Histone H1. Gli null cells (Gli2-/- Gli3-/- MEFs) are included to show antibody specificity.

In the absence of the Hh signal, SuFu binds and sequesters Gli proteins away from the nucleus (Ding et al., 1999; Kogerman et al., 1999). In SuFu-/- MEFs Gli1 is the major Gli-A form and localizes constitutively to the nucleus (Figure 3.4D). Bortezomib treatment stabilizes the levels of Gli1 in these cells while simultaneously inhibiting target gene transcription (Figures 3.4D and 3.3C). Moreover, in a previous study, we visualized nuclear staining of overexpressed HA-tagged Gli1 in the presence of bortezomib in SuFu-/- MEFs but not in SuFu+/- MEFs (Supplemental Figure 2.S3G, Tukachinsky et al., 2010). Taken together, these results suggest that proteasome inhibition does not disrupt the regulatory events on Gli leading to nuclear entry. However, with these analyses we cannot rule out the possibility that nuclear Gli proteins accumulate due to protein stabilization even with partially impaired nuclear import.

Transcriptional repression upon proteasome inhibition is due to loss of Gli promoter occupancy

We wondered whether the Gli proteins that accumulate in the nucleus are competent to bind target gene promoters. Expression of Hh target genes correlated with recruitment of RNA polymerase II in the absence and presence of proteasome inhibition (Figures 3.1C and E). To determine whether the occupancy of endogenous Gli proteins at promoters is altered, we performed chromatin immunoprecipitation (ChIP) assays in NIH3T3 cells. We included Gli2-/- Gli3-/- MEFs as a control for specificity of the antibodies used for immunoprecipitation and to measure the level of non-specific background signal. These double knockout MEFs are functionally null for all three Gli proteins (Gli null) since *Gli1* is not expressed in the absence of Gli2 and Gli3 and these cells do not respond to pathway stimulation (Supplemental Figure 3.S3B).

Gli1 occupancy at promoters is increased after 24 hours treatment with SAG but is decreased to basal levels in the presence of bortezomib (Figure 3.5A). More strikingly, using an antibody against the Gli2 N-terminus (Gli2N-ChIP), Gli2 occupancy is increased at promoters after 24 hours SAG treatment but this response is reduced in the presence of bortezomib (Figure 3.5B).

To look specifically at Gli2 and Gli3 Gli activator forms, we used an antibody that recognizes the C-terminus of both Gli2 and Gli3. We observe similar results with Gli2/3C-ChIP, that is, Gli2-A and Gli3-A occupancy increased with SAG treatment and is decreased in combination with bortezomib in wildtype cells (Figure 3.5C). To probe Gli3-specific activity we performed ChIP-Gli3N in the absence of Gli2. Gli3 is a weak transcriptional activator (Figure 3.3F). Nevertheless, Gli3 is still able to mediate a transcriptional response in response to SAG in Gli2-/- MEFs concurrent with increased Gli3-A promoter occupancy (Figures 3.3E and 3.5E). Bortezomib treatment reduced Gli3-mediated transcription and promoter occupancy in these cells (Figures 3.3E and 3.5E).

Since Gli3-FL and Gli3-R forms are equally abundant in wildtype cells, we used an antibody that recognizes its N-terminus so we can interrogate both full-length and processed forms by ChIP. There is increased basal Gli3 occupancy by Gli3N-ChIP at promoters of unstimulated wildtype cells compared to Gli null MEFs (Figure 3.5D). This likely represents the occupancy of Gli3-R keeping target gene transcription repressed. SAG treatment increased, while addition of bortezomib reduced, Gli3 occupancy back to basal levels. Since Gli3 occupancy does not drop below basal levels, the transcriptional block by bortezomib can be explained by the stabilization and continued occupancy of Gli3-R at promoters.

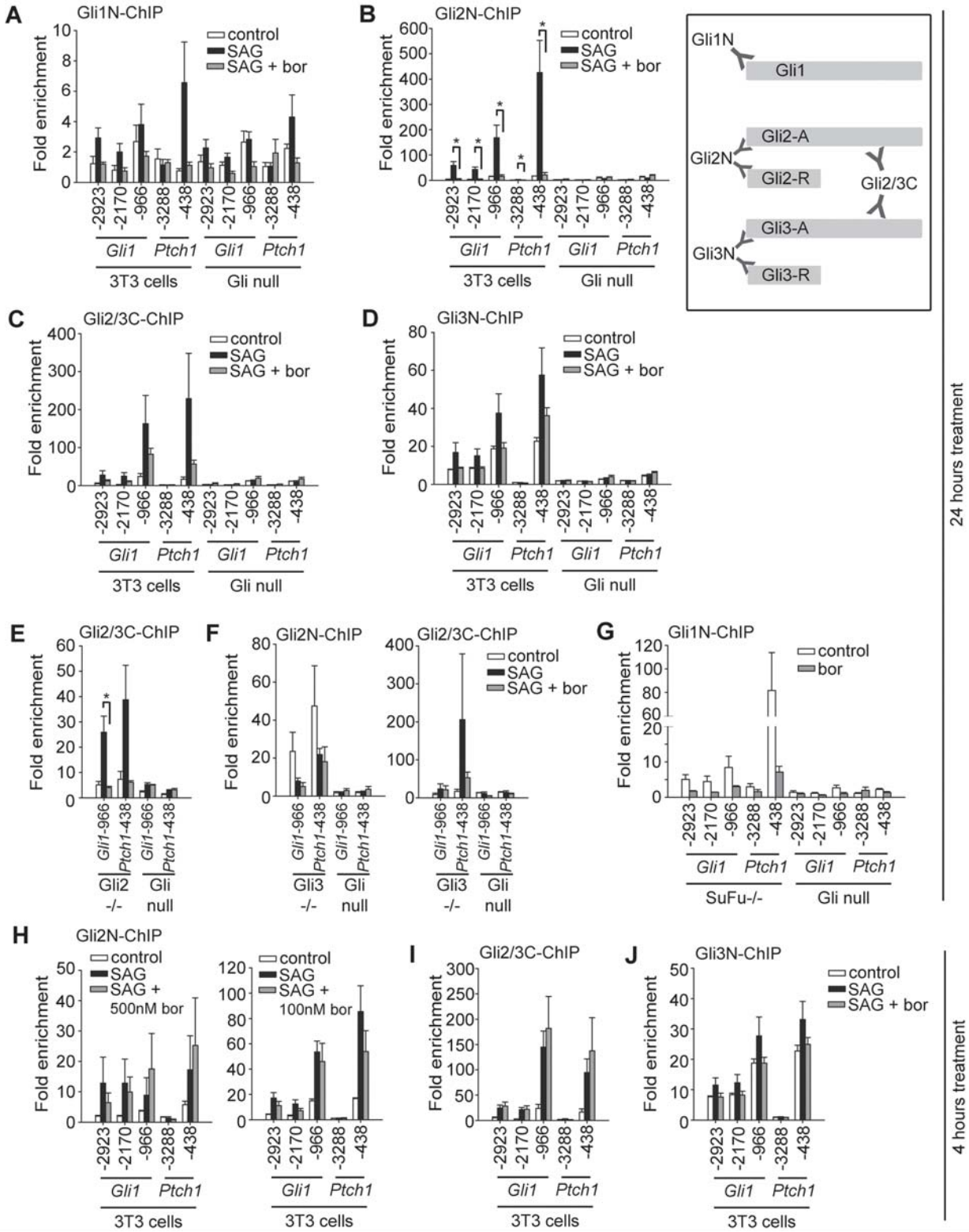

Figure 3.5 Gli protein occupancy on target gene promoters is maintained after 4 hours of proteasome inhibition but is reduced after 24 hours.

Figure 3.5 (Continued)
Gli occupancy was measured by chromatin immunoprecipitation (ChIP) analysis at known Gli-binding sites (-2923, -2170 and -966 from the transcriptional start site (TSS) of *Gli1*; and, -3288 and -438 from the *Ptch1* TSS) reported as fold enrichment over a non-binding site (-5627 from the *Gli1* TSS). Inset shows a pictorial summary of antibody specificities used for ChIP experiments. Where shown, ChIP on Gli null cells (Gli2-/-, Gli3-/- MEFs) was performed to determine background signal. Error bars indicate mean ± SEM of three replicates. Asterisks indicate p-values for combined SAG and bortezomib treatment compared with SAG alone (*, $P < 0.05$). ChIP was performed on indicated cells treated with vehicle (control) or with 100 nM SAG with or without 100 nM bortezomib (bor) for 24 hours (A-G). ChIP on NIH3T3 cells after 4 hours of indicated treatment (H-J).
A) ChIP in NIH3T3 cells probing for Gli1-A using a Gli1 N-terminus antibody.
B) ChIP in NIH3T3 cells for Gli2-A using a Gli2 N-terminus antibody.
C) ChIP in NIH3T3 cells for Gli2-A and Gli3-A using an antibody that recognizes both Gli2 and Gli3 C-terminus (Gli2/3C).
D) ChIP in NIH3T3 cells for Gli3-A and Gli3-R using a Gli3 N-terminus antibody.
E) ChIP in Gli2-/- MEFs probing for Gli2-A and Gli3-A using a Gli2/3C antibody.
F) ChIP in Gli3-/- MEFs.
Left panel: Probing for Gli2-A using a Gli2 N-terminus antibody.
Right panel: Probing for Gli2-A and Gli3-A using a Gli2/3C antibody.
G) ChIP in SuFu -/- MEFs probing for Gli1 using a Gli1 N-terminus antibody.
H) Comparison between vehicle (control) or treatment with 100 nM SAG with or without either high (500 nM, left panel) and low (100 nM, right panel) doses of bortezomib and probing for Gli2-A using a Gli2 N-terminus antibody.
I) ChIP for Gli2-A and Gli3-A using a Gli2/3C antibody. Cells were treated as in (A-G).
J) ChIP for Gli3-A using a Gli3 N-terminus antibody. Cells were treated as in (A-G).

While this mechanism remains possible, it does not completely explain why bortezomib blocks transcription mediated by Gli1 in HEK293Ts, which is not known to be processed to a repressor form (Figure 3.3F), or why transcription is inhibited in the absence of Gli3-R forms as in Gli3-/- MEFs (Figure 3.3E). Indeed, in cells lacking Gli3 (Gli3-/- MEFs), high basal transcription of target genes correlates with high basal promoter occupancy of Gli2 by Gli2N-ChIP (Figures 3.3E and 3.5F). Similar to the results in wildtype cells (Figure 3.5B), Gli2 promoter occupancy decreased in the presence of bortezomib by Gli2N-ChIP or by Gli2/3C-ChIP in Gli3-/- MEFs (Figure 3.5F).

Next we looked specifically in a cellular context where Gli1 transactivation plays a major role in Hh pathway activation. Knockdown of *Gli1* abrogated luciferase reporter activity in

SuFu-/- MEFs suggesting that Gli1 is primarily responsible for constitutive target gene transcription in these cells (Chen et al., 2009). Indeed, SuFu-/- MEFs exhibits high constitutive Hh target gene transcription and Gli1 nuclear localization (Figures 3.3C and 3.4D). Basal Gli1 promoter occupancy was also high and bortezomib reduced Gli1 promoter occupancy (Figure 3.5G), mirroring transcriptional inhibition (Figure 3.3C).

We observed a reduction of promoter occupancy of all Gli protein forms despite their nuclear accumulation after 24 hours of bortezomib treatment. We therefore conclude that the transcriptional block by proteasome inhibition is due to loss of binding of Gli activator proteins to target gene promoters.

We also investigated Gli protein promoter occupancy at an earlier time after pathway activation. We see transcriptional inhibition as early as 4 hours with proteasomal inhibition (Figures 3.1C and D) concurrent with decreased RNA polymerase 2 recruitment to target gene promoters (Figure 3.1E). At this early time point Gli2 and Gli3 proteins provide the greatest contribution to transcription, thus we performed ChIP assays on these proteins in wildtype cells. Intriguingly, after 4 hours in bortezomib, we do not see a significant loss of occupancy of Gli proteins despite already reduced transcription of target genes (Figures 3.5H–J). Gli2 occupancy assessed by Gli2N-ChIP is not reduced after SAG stimulation in the presence of bortezomib even at the highest concentration tested (Figure 3.5H). Likewise, occupancy of Gli2-A and Gli3-A forms by Gli2/3C-ChIP is maintained (Figure 3.5I). On the other hand, Gli3-A and Gli3-R occupancy is reduced to basal levels in the presence of bortezomib as assessed by Gli3N-ChIP (Figure 3.5J). These results may represent a mixture of Gli-A and Gli-R forms vying for promoter occupancy whereby Gli-R forms are having a greater influence on target gene transcription. Alternatively, these results hint at another factor in play besides Gli repressors. In

support of this, we noticed a significant reduction in *Ptch1* transcription in Gli null cells treated with bortezomib suggesting that transcriptional inhibition is occurring in the absence of Gli repressors (Supplemental Figure 3.S3B).

New protein synthesis is required for the transcriptional block by proteasome inhibition.

To determine whether a protein factor other than Gli is responsible for the transcriptional block of Gli target genes, we pre-treated NIH3T3 cells with protein synthesis inhibitor cycloheximide (CHX), then stimulated with SAG in the absence or presence of bortezomib. Inhibition of protein synthesis prevented the transcriptional block by proteasome inhibition (Figure 3.6A).

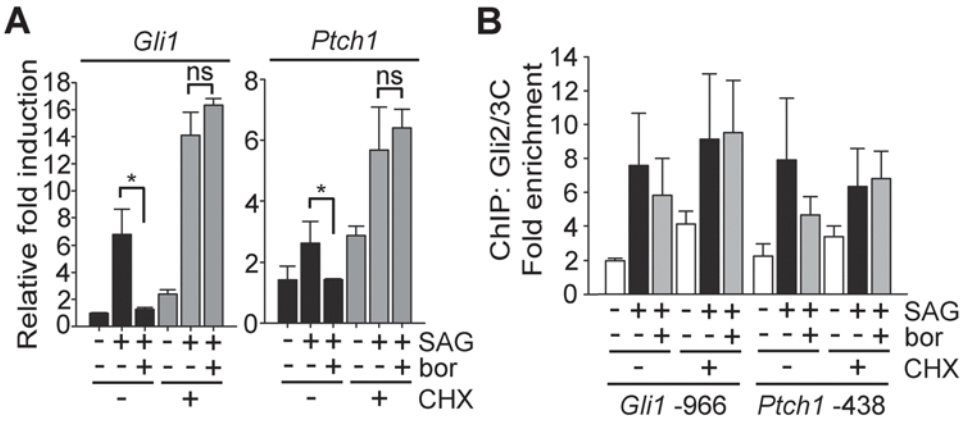

Figure 3.6 The transcriptional block by proteasome inhibition requires new protein synthesis.
A) NIH3T3 cells pre-treated or not with protein synthesis inhibitor cycloheximide (CHX, 20 μg/ml) for 30 min, then treated with 500 nM SAG with or without 500 nM bortezomib (bor) for an additional 6 hours in the continued presence or absence of CHX. Transcription of *Gli1* and *Ptch1* was assayed by Q-PCR and normalized to the reference gene *L27*. Error bars indicate mean ± SD of three replicates. Asterisks indicate p-values for combined SAG and bortezomib treatment compared with SAG alone (*, $P < 0.05$).
B) Chromatin immunoprecipitation (ChIP) analysis in NIH3T3 cells probing for Gli2-A and Gli3-A using a Gli2/3 C-terminus antibody at known Gli-binding sites (-966 from the transcriptional start site (TSS) of *Gli1*; and -438 from the *Ptch1* TSS) reported as fold enrichment over a non-binding site (-5627 from the *Gli1* TSS). Treatments were performed as in (A). Error bars indicate mean ± SEM of three replicates. To alleviate cytotoxicity due to prolonged exposure to CHX, confluent cells were not serum-starved and the treatments above were performed in complete media. Q-PCR and ChIP signals are therefore muted.

In addition, in the absence of bortezomib, basal as well as induced transcription of target genes increased after protein synthesis inhibition. Translational inhibitors have been shown to stabilize certain mRNAs by preventing their degradation (Ross, 1995; Jacobson and Peltz, 1996). It is therefore possible that *Gli1* and *Ptch1* transcripts are stabilized and accumulate in the presence of cycloheximide, masking the transcriptional block by bortezomib. However, consistent with our transcription results, Gli2-A and Gli3-A still localized to target gene promoters in SAG-treated cells with or without bortezomib when protein synthesis is inhibited (Figure 3.6B). Furthermore, there is an increase in Gli activator occupancy in cycloheximide-treated cells in the absence of Hh stimulation mirroring increased basal Hh target gene transcription. These results suggest that transcription of target genes is on going when protein translation is inhibited and that the transcriptional block after proteasome inhibition requires new protein synthesis. We surmise that an as yet unknown protein factor with a half-life of minutes to hours is stabilized by proteasome inhibition and acts to repress transcription by preventing Gli binding to DNA.

Proteasome inhibition as a potential therapeutic for Hh pathway activated cancers

Since we see a striking transcriptional block upon proteasome inhibition of Hh target genes in Ptc-/- MEFs (Figure 3.3A), we asked whether the same block occurs in cancer cells. We turned to a mouse model for medulloblastoma where one *Ptch1* allele is inactivated in a p53 null background (Ptc+/-, p53-/-). In these mice, spontaneous tumors arise due to a loss of heterozygosity in *Ptch1*. We took three independent neurosphere lines that arose from isolated tumors and determined whether the Hh pathway was activated in these cells by treatment with the Smo inhibitor SANT-1 for 12 hours. *Gli1* transcription was inhibited by SANT-1 treatment in all three cell lines verifying that loss of Ptc led to subsequent constitutive activation of Smo

(Figure 3.7A). Next, we asked whether proteasome inhibition also blocked Hh signaling in these cells. Treatment with bortezomib for 12 hours potently inhibited *Gli1* transcription in all three cell lines (Figure 3.7A).

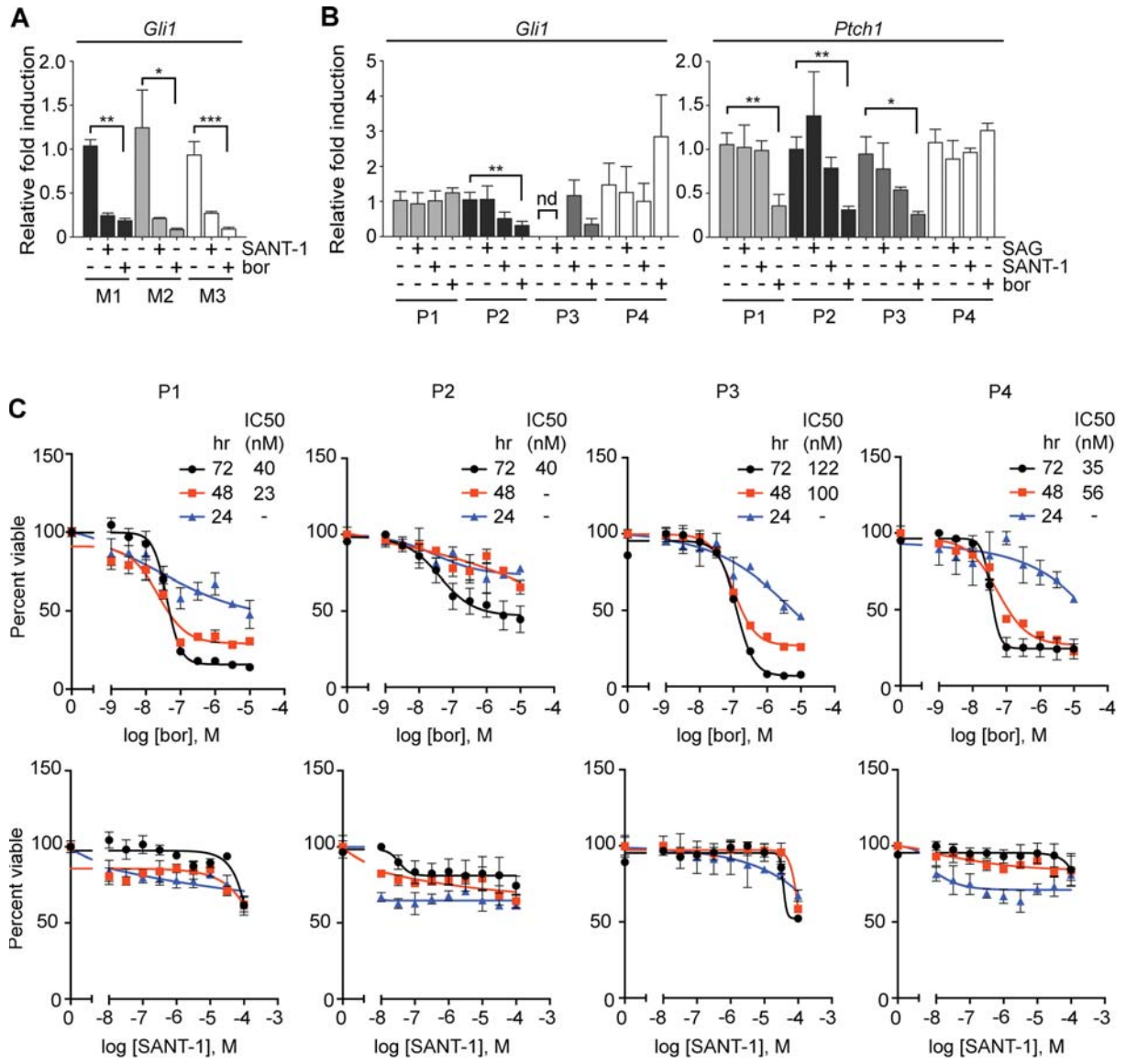

Figure 3.7 Bortezomib inhibits Hedgehog target gene transcription and growth in cancer cells.
A) Transcriptional response to 12-hour treatment with 500 nM SANT-1 or 100 nM bortezomib (bor) of three independent neurosphere cell lines derived from a mouse model of medulloblastoma. Error bars indicate mean ± SD of three replicates. Asterisks indicate p-values for bortezomib-treated compared with untreated samples (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
B) Transcriptional response to 24-hour treatment with 1μM SAG, 1 μM SANT-1 or 100 nM bortezomib (bor) of four independent cell lines derived from human pancreatic adenocarcinoma tumors. Transcription of *Gli1* and *Ptch1* was assayed by Q-PCR and normalized to the reference gene *L27*. Non-detectable transcripts are indicated by nd.

Figure 3.7 (Continued)
C) Growth inhibition curves on pancreatic cancer cell lines in (B) after 24, 48 and 74-hour exposure to increasing concentrations of bortezomib (bor) or SANT-1 determined using Resazurin Cell Viability Kit (Biotium) and reported as percent remaining viable cells relative to vehicle-treated cells. Absolute IC50 values were estimated for 48 and 72-hour treatments. Error bars indicate mean ± SD of three replicates.

We then turned to human cancers which are suspected to exhibit Gli activation, but where the dependence on Hh pathway signaling is less defined. We took three independent cell lines established from human pancreatic adenocarcinomas with suspect Gli activation (P1, P2 and P3) and a control pancreatic cancer line that showed no Hh pathway activity (P4). We determined the status of the Hh pathway in these four cell lines by asking whether they are responsive to Smo activation by SAG or inhibition by SANT-1. We also asked whether proteasome inhibition blocked any Hh pathway activity (Figure 3.7B). Looking at *Gli1*transcripts, SAG treatment did not increase transcription in any cell line and P2 showed transcriptional inhibition with both SANT-1 and bortezomib. Cell lines P1, P3 and P4 showed no changes in *Gli1* with SANT-1 or bortezomib. Looking at *Ptch1* transcripts, only P2 showed a slight but insignificant increase with SAG treatment, whereas no changes were observed in all other samples. Two out of three Gli-suspect cell lines (P2 and P3) exhibited slight inhibition of *Ptch1* transcription with SANT-1 treatment, while the other cell line (P1) was insensitive to SANT-1. Interestingly, in these three samples *Ptch1* expression was inhibited by bortezomib. The control line (P4) showed no changes in *Ptch1* with all treatments. The lack of transcriptional changes to some of the treatments above was not due to the quality of the primers used since these performed robustly by Q-PCR in human embryonic palatal mesenchyme (HEPM) cells, a normal human cell line that is responsive to Hh signaling (Supplemental Figure 3.S4; Yauch et al., 2008). These results suggest that, except for line P2, any Hh pathway activity in these cells are not mediated by Smo. These

results also suggest that bortezomib blocks Hh target gene transcription in these cells independent of Smo activity.

Bortezomib (trade name Velcade®) is an FDA approved drug for multiple myeloma and relapsed mantle cell lymphoma that kills these cancer cells with surprising selectivity. We therefore performed a growth inhibition assay to determine whether bortezomib affects the growth of these pancreatic cancer cell lines (Figure 3.7C, top panels). Samples P1, P3 and P4 showed growth inhibition after 48 hour incubation with bortezomib with an estimated absolute IC50 in range of 23 to 100 nM. Sample P2, on the other hand, showed growth inhibition only after 72 hours with an estimated absolute IC50 of 40 nM. In contrast, SANT-1 did not inhibit growth in these cells (Figure 3.7C, bottom panels) suggesting that Smo activity is not required for growth. These results are preliminary since the growth inhibition seen in these cells can be the result of a proliferative block and cytotoxicity of bortezomib by other means such as the induction of ER stress (McConkey and Zhu, 2008). It is yet to be determined whether Gli proteins are involved in the transcriptional block of *Ptch1* in these cancer cells as in mouse fibroblasts. A careful molecular characterization of the role of Gli proteins is yet to be performed.

DISCUSSION

Gli proteins are the effectors of the Hh signaling pathway and signals from upstream components ultimately impinge on the regulation of Gli transcriptional activity. In the absence of the Hh signal, Gli is kept in an inhibitory complex with SuFu. In addition, the proteasome plays a major role in keeping the Hh pathway silent through the complete degradation of Gli proteins and their partial proteolysis into repressor forms. In response to Hh signal, Gli is released from

SuFu and its transcriptional activity is promoted. In addition, Gli-A forms accumulate and Gli-R forms are reduced, perhaps reflecting an inhibition of both complete and partial degradation of Gli by the proteasome. How the Hh signal is transduced into the inhibition of these Gli proteolytic events is unknown. We therefore explored a simple model where the Hh signal leads to inhibition of the proteasome. In using pharmacological inhibitors to probe for the effect of partial loss of proteasome function, we have uncovered an unexpected positive role for the proteasome in the activation of Gli proteins.

We found that proteasome inhibitors quickly and potently blocked the transcription of Hh target genes in fibroblasts and in cancer cells. This transcriptional block by proteasome inhibition occurred in cells where the Hh signal was added exogenously as in cells responding to the Hh ligand or to SAG. This block also occurred in cells where Hh signaling was constitutively activated as in the loss of Ptc or SuFu as well as in the expression of an oncogenic Smo mutant. These observations indicate the transcriptional block is mediated primarily at the level of Gli proteins.

We investigated how proteasome inhibition affected Gli transcriptional activity and found that the regulation of Gli proteins leading to nuclear entry is normal. We then looked at Gli promoter occupancy and elucidated three possible non-mutually exclusive mechanistic explanations for this transcriptional block: (1) Gli-R forms accumulate and compete with Gli-A forms for binding to promoters. This was evident in ChIP analysis using a Gli3 N-terminus antibody (which recognizes both Gli3-A and Gli3-R) whereby Gli3 occupancy is maintained at basal levels perhaps indicating that continued presence of Gli3-R mediated transcriptional repression. It was previously shown that engineered Gli3 truncations that mimic endogenous Gli3-R display dominant transcriptional repression over Gli activators in chick and mouse

models (Wang et al., 2000; Vokes et al., 2008; Rajurkar et al., 2012). It is also possible that Gli2-R forms exist, but at levels undetectable by our antibodies. However, since Gli2-R production is highly inefficient (Pan et al. 2006), we believe that its contribution to the Gli-R pool is low. (2) Gli-A forms are unable to bind promoters upon proteasome inhibition despite their accumulation in the nucleus. All Gli-A forms (Gli1, Gli2-A and Gli3-A) probed using ChIP analysis showed a loss of promoter occupancy. However, displacement of Gli-A by Gli-R does not solely mediate this transcriptional block since it occurs even in the absence of Gli3, the main contributor to the Gli-R pool. Further, this block occurs with isolated expression in HEK293T cells of either Gli2, which is poorly processed (Pan et al., 2006) or of Gli1, which is not processed (Dai et al. 1999; Kaesler et al., 2000; Park et al., 2000). (3) Stabilization of an unknown protein interferes with the promoter occupancy of Gli proteins. We found that new protein synthesis is required for the transcriptional block by proteasome inhibition. Furthermore, promoter occupancy of Gli-A forms is restored when protein synthesis is first inhibited. These results suggest that a rapidly turned over protein (or proteins) are stabilized by proteasome inhibition and act to prevent Gli binding to DNA leading to transcriptional repression. We conclude that a fully functional proteasome plays an important role, not only in keeping Hh signaling off in the absence of signal, but also in pathway activation.

Cancers that exhibit deregulated Hh signaling lead to the activation of Gli. Drug resistance mutations to Smo inhibitors have been reported in some cancers, requiring an alternative form of therapy (Dijkgraaf et al., 2011). In addition, a number of cancers show upregulation of Gli activity independent of upstream Hh pathway components (Blotta et al., 2012; Li et al., 2012; Rajurkar et al., 2012; Atwood et al., 2013). Thus, direct inhibition of Gli proteins is an attractive avenue for cancer therapy. The small molecule antagonist GANT-61

shows anti-tumorigenic activity by preventing the DNA binding of Gli (Lauth et al., 2007). Also, arsenic trioxide has been shown to inhibit tumor growth by directly binding and inactivating Gli1 (Beauchamp et al., 2011). Our results suggest the use of proteasome inhibitors as indirect Gli antagonists due to its potency in repressing Gli-mediated transcription and its promising anti-growth activity in pancreatic cancer cell lines.

MATERIALS AND METHODS

Cell culture, Hh pathway assays, and proteasome inhibition

NIH3T3 cells were grown in DME supplemented with 10% bovine calf serum, penicillin and streptomycin. MEFs, HEK293Ts, pancreatic cancer cell lines and HEPMs were grown in DME supplemented with 10% fetal calf serum, penicillin and streptomycin. To assay Hh signaling, confluent cell cultures were starved for 16-24 hours in starvation media (DME without serum for NIH3T3 cells or with 0.2% fetal bovine serum for MEFs). The media were replaced with starvation media supplemented with the appropriate combinations of pathway agonist, antagonist, proteasome inhibitor, or control vehicle. After incubation for the desired amount of time, cultures were harvested for real-time Q-PCR and Western blotting or processed for chromatin immunoprecipitation (ChIP). The following compounds were used: SAG (Axxora), SANT-1 (EMD), bortezomib (LC Labs), MG132 (Enzo), epoxomicin (Enzo), cycloheximide (Sigma) and EGF (Sigma).

Transient expression of Gli proteins

HEK293T cells grown to 30-50% confluency in a 12-well plate were transfected in triplicate with either 0.1 µg hGli1, hGli2, hGli2ΔN, or hGli3-containing plasmid DNA or water supplemented with pBluescript SK+ empty vector up to a total DNA amount of 1 µg. A transfection mix consisting of 1 µg DNA, 7 µg polyethylenimine (Polysciences) and 85 µl Optimem (Life Technologies) was assembled at room temperature for 15 min, resuspended in 1 ml complete media, added to each well, and incubated overnight. The next day, complete media was replaced and cells were incubated again overnight. Cells were then treated with 500 nM bortezomib or mock treated for 24 hours then harvested for real time Q-PCR analysis.

Real-time Q-PCR assays of transcription

Total cellular RNA was isolated using RNA-Bee (Tel-Test), treated with DNase (Promega), and purified. Complimentary DNA (cDNA) was generated from 500 ng total RNA using reverse transcriptase and random hexamers (Transcriptor; Roche). Gene expression from 50 ng cDNA was assayed by quantitative real-time PCR using SYBR green (FastStart; Roche) on a Rotor-Gene 6000 (Corbett Robotics). Relative gene expression was calculated using a two standard curve method in which each gene of interest was normalized to the ribosomal protein *L27* gene. The following sequences for gene-specific primers were used: mouse *L27*: 5'-GCTTGGCGATCTTCTTCTTG-3' and 5'-GTCGAGATGGGCAAGTTCAT-3'; mouse *Gli1,* 5'-GCTGGAGGTCTGCGTGGTA-3' and 5'-GGTGGAGTCATTGGATTGAACA-3'; mouse *Ptch1,* 5'-CATCATGCCAAAGAGCTCAA-3' and 5'-ACTGTCCAGCTACCCCAATG-3'; mouse *cFos,* 5'-CGGGTTTCAACGCCGACTA-3' and 5'-TTGGCACTAGAGACGGACAGA-3'; mouse *cJun,* 5'-CCTTCTACGACGATGCCCTC-3' and 5'-

GGTTCAAGGTCATGCTCTGTTT-3'; human *L27,* 5'-GGAAGACCCGGAAACTTAGGG-3' and 5'-GCCTGGGTGGTATTTGTCGAA-3'; human *Gli1,* 5'-TCTGGACATACCCCACCTCCCTCTG-3' and 5'-ACTGCAGCTCCCCCAATTTTTCTGG-3'; and human *Ptch1,* 5'-CCACAGAAGCGCTCCTACA-3' and 5'-CTGTAATTTCGCCCCTTCC-3'. Data represent mean ± SD from three independent experiments. P-values were calculated using a 1-tailed Student's *t* test with unequal variance.

Antibodies

Goat anti-Gli3 N-terminus, goat anti-Gli2 N-terminus, and goat anti-Gli1 N-terminus was purchased from R&D Systems. Rabbit anti-SuFu and rabbit anti-Gli2 and Gli3 C-terminus were generated as previously described in (Tukachinsky et al., 2010). Mouse anti-Tubulin was purchased from Sigma. Mouse anti-Histone H1 was purchased from Thermo Scientific. Mouse anti-RNA polymerase II (8WG16) was purchased from Covance. Alexa-594- and Alexa-488-conjugated antibodies (Invitrogen) were used at a final concentration of 1 microgram/mL. HRP-conjugated anti-rabbit, anti-mouse secondary antibodies were purchased from GE Healthcare. HRP-conjugated anti-goat secondary antibody was purchased from Thermo Fisher.

Immunoblotting

For total cell lysate immunoblots, cells were resuspended in TBS with protease inhibitors and were lysed with 1% SDS at room temperature for 20-30 min in the presence of benzonase (Novagen) supplemented with 5mM $MgCl_2$. The cell lysate was clarified by centrifugation for 10 min in a microfuge at 20,000 *g*. For nuclear and cytoplasmic fractionation, cells were resuspended in 5 times pellet volume of lysis buffer (150 mM NaCl, 20 mM TBS pH 7.5) with

protease inhibitors. Cells were permeabilized with 0.5% digitonin (EMD) on ice for 15 min, centrifuged for 5 min at 3000 *g*, and the supernatant collected. The supernatant, representing the cytoplasmic fraction, was centrifuged again to remove any nuclear remnants. The pellet, representing the nuclear fraction, was washed twice in lysis buffer supplemented with 0.1% digitonin followed by centrifugation for 5 min at 3000 *g*. The pellet was resuspended in 2 times pellet volume in lysis buffer supplemented with benzonase and solubilized in 1% SDS for 15 min at room temperature. The solubilized nuclear fraction was clarified by centrifugation for 5 min at 20,000 *g*. Total protein concentrations were measured by BCA assay and equal amounts of protein were calculated, mixed with DTT (50 mM final) and 5x SDS-PAGE sample buffer, and separated by SDS-PAGE on 5-15% polyacrylamide gradient gels followed by transfer to nitrocellulose membrane. Antibodies were used at a final concentration of 1 μg/ml in TBST with 5% nonfat dry milk.

Chromatin immunoprecipitation

Twenty million starved, confluent NIH3T3 cells or MEFs were incubated with appropriate treatments for the desired times. Cells were then washed with DME, and cross-linked with 1% formaldehyde in DME at 37°C for 10 min, then quenched with DME supplemented with glycine (0.1375 M final). Cells then were rinsed twice with ice-cold PBS supplemented with PMSF (0.5 mM final concentration, PBS-PMSF), collected into PBS-PMSF, and centrifuged for 5 min. The pellets were then resuspended and incubated for 10 min in 10 pellet volumes of ice-cold swelling buffer (25 mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10mM KCl, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, and 1x protease inhibitors [Roche]). Cells were dounced 20 times and centrifuged for 5 min to pellet nuclei. Nuclei were resuspended in 1 ml sonication

buffer (0.3% SDS, 50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.5 mM PMSF, and1x protease inhibitor cocktail), and sonicated with repeating cycles of 30 s sonication followed by a 30 s pause for a total of 30 min using Bioruptor Plus (Diagenode) to generate DNA fragment sizes of 0.5-1 kb. Solubilized chromatin was then centrifuged for 15 min; supernatant was collected and diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1 and1x protease inhibitors) to bring SDS concentration down to 0.1%. Immunoprecipitation was performed by tumbling overnight at 4°C with 2-4 μg specific antibodies bound to Protein A or Protein G Dynabeads (Invitrogen). After immunoprecipitation, Dynabeads were washed three times for 5 min each in RIPA buffer (1% NP-40, 0.7% sodium deoxycholate, 1 mM EDTA, 500mM LiCl, 50 mM HEPES, pH 7.6 and 1x protease inhibitors), and washed two times in TE buffer. Antibody-protein complexes were eluted from Dynabeads and formaldehyde crosslinks were reversed by incubation in elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 16 hours at 65°C. DNA was isolated by phenol/chloroform/isoamyl alcohol extraction, precipitated in ethanol and sodium acetate supplemented with glycogen, and resuspended in 10 mM Tris-HCl, pH 7.5. For quantitative real-time PCR, 2 μl out of 50 μl DNA was used in 21–25 cycles of amplification using SYBR green (FastStart; Roche) on a Rotor-Gene 6000 (Corbett Robotics). The following sequences for mouse gene promoter-specific primers were used: *Gli1* -966, 5'-GTTCCGTTCCCCATTTTACC-3' and 5'-TCCACTCCAGGTTTTTCAGC-3'; *Gli1* -2170, 5'-CCCGCTCTGAATCCTCTTTC-3' and 5'-CCTTTCCTTGATGCTGTTCC-3'; *Gli1* -2923, 5'-TATGGGGTTGGGAGAGTTTG-3' and 5'-AAAGAGACCTGGGACAGACAC-3'; *Gli1* -5627, 5'-CACTGGGAAGACAGAAGCAAG-3' and 5'-GCCCCTGATTGGATGATTG-3'; *Ptch1* -438, 5'-TGGGTGGTCTCTCTACTTTGG-3' and 5'-TGTCAGATGGCTTGGGTTTC-3'; *Ptch1* -3288, 5'-ACTGGCTCCTCTTCCCTTTC-

3' and 5'-GCTTCCCCTGTGGTCTGC-3'; and *GAPDH*, 5'-CCGCATCTTCTTGTGCAGT-3' and 5'-TCCCTAGACCCGTACAGTGC-3'. Data represent mean ± SEM from three independent experiments. P-values were calculated using a 1-tailed Student's *t* test with unequal variance.

Immunofluorescence and cilia counts

Cells grown on glass coverslips were fixed for 30 min at room temperature in PBS with 4% formaldehyde. The coverslips were rinsed with TBST (10mM Tris, pH 7.5, 150mM NaCl, and 0.2% Triton X-100), and non-specific binding sites were blocked by incubation in TBST supplemented with 25 mg/mL BSA (TBST-BSA). The coverslips were incubated with primary antibodies diluted in TBST-BSA for 1 hour at room temperature. Coverslips were then washed with TBST, blocked again with TBST-BSA and incubated with the appropriate secondary antibodies in TBST-BSA. After washing, the coverslips were mounted on glass slides in mounting media (0.5% p-phenylenediamine, 20 mM Tris pH 8.8, 90% glycerol). Affinity-purified primary antibodies against C-term Gli2 and Gli3 and mouse monoclonal anti-Myc (9E10) were used at a final concentration of 1-2 µg/mL. Mouse anti-acetylated tubulin was purchased from Sigma. Alexa dye-conjugated secondary antibodies (Invitrogen) were used at a final concentration of 1 µg/mL. The DNA stain Hoechst 33258 was purchased from Invitrogen. The immunostained cells were imaged by epi-fluorescence microscopy on an inverted Nikon TE2000U microscope equipped with an OrcaER digital camera (Hammamatsu). Images were collected using Metamorph image acquisition software (Applied Precision). To measure ciliary localization of Gli, 300 cilia for each coverslip were identified by anti-acetylated tubulin staining and were scored visually for the presence or absence of Gli at the cilium using a 100x PlanApo 1.4NA oil objective (Nikon). Error bars represent the standard deviation for groups of 100 cilia

counted on different visual fields, on the same coverslip. To visualize nuclear staining of Gli, micrographs of three visual fields were taken using a 60x PlanFluor 0.85NA oil objective (Nikon).

Cell viability assay

Cell viability was measured using Resazurin Cell Viability Assay Kit (Biotium) according to the manufacturer's instructions. For viability of proliferating cells, cells were seeded in a 96-well plate at a density of 10,000 cells/well in a volume of 100 µl and were treated as needed the next day in complete media. For viability of quiescent cells, cells were seeded in a 96-well plate at a density of 40,000 cells/well in a volume of 100 µl. The next day, media was replaced with starvation media (DME without serum) and incubated for 16 hours. Cells were treated with different concentrations of bortezomib ranging from 0 to 10 µM or SANT-1 ranging from 0 to 100 µM in complete or starvation media. Experiments were carried out in quadruplicate for each drug concentration. After treatment for the desired time, media was exchanged with 100 µl of a 1:10 mixture of Resazurin and complete or starvation media. After 2 h of incubation at 37°C, cell viability was monitored by measuring absorbance at 570 nm subtracted by background absorbance at 600 nm using the Epoch Microplate Spectrophotometer (Biotek). The colorimetric signal generated from the assay is proportional to the number of living cells in the sample. The percentage of survival of drug-treated cells was calculated relative to control vehicle-treated cells and plotted as mean ± SD. Absolute IC50 values were estimated for 48 and 72-hour treatments using four-parameter dose-response curve fit using Prism (GraphPad).

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ABBREVIATIONS USED IN THIS PAPER

Bor (bortezomib), CHX (cycloheximide), ChIP (chromatin immunoprecipitation), ChIP-Gli1N (ChIP for Gli1 N-terminus), ChIP-Gli2N (ChIP for Gli2 N-terminus), ChIP-Gli2/3C (ChIP for Gli2 and Gli3 C-terminus), ChIP-Gli3N (ChIP for Gli3 N-terminus), EGF (epidermal growth factor), Epox (epoxomicin), hGli1 (human Gli1), hGli2 (human Gli2), hGli2ΔN (human Gli2 N-terminal repressor domain deletion), hGli3 (human Gli3), Gli-A (Gli activator), Gli-R (Gli repressor), Gli2-A (Gli2 activator), Gli2-R (Gli2 repressor), Gli3-A (Gli3 activator), Gli3-R (Gli3 repressor), Hh (Hedgehog), Q-PCR (quantitative reverse transcription polymerase chain reaction), MEF (mouse embryonic fibroblast), Ptc (Patched), Shh (Sonic Hedgehog), Smo (Smoothened), SmoWT (Smoothened wildtype), SmoM2 (Smoothened oncogenic mutant M2), SuFu (Suppressor of Fused), TSS (transcriptional start site).

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CHAPTER FOUR:

CONCLUSIONS AND PERSPECTIVES

An overarching question in investigating the mechanism of Hh regulation is how Gli proteins are activated. It has been proposed that there are at least three general species of Gli proteins: full-length Gli, repressors (the shorter processed Gli form), and "activated" Gli. The functional definition of Gli activation is the ability to induce Hh target gene transcription. From the many mechanisms that regulate Gli discussed throughout this dissertation the process of Gli activation is complex and involves many steps. In this dissertation, I investigated early steps of Gli ciliary recruitment and dissociation from SuFu, and the late steps of promoter occupancy and the active role of proteasomes in gene transcription.

Gli activation occurs within primary cilia

In chapter 2, I provide one of the first evidence for the recruitment of endogenous Gli proteins to cilia. Previous studies that demonstrate Gli ciliary localization made use of overexpressed and tagged constructs due to the paucity of sensitive antibodies. The importance of generating reagents such as antibodies should therefore not be overlooked.

I provide evidence of Gli activation within cilia. Gli proteins localize to cilia at some basal level and rapidly accumulate upon pathway activation. This puts ciliary recruitment as one of the earliest events towards Gli activation. This recruitment is dependent on the activity of Smo. While Smo can move into cilia in an inactive form, when using the Smo inhibitor cyclopamine or with PKA activation using forskolin, Gli does not. This suggests that a signal originating from Smo is required for Gli ciliary accumulation. The nature of the signal bridging Smo and Gli remains one of the biggest unanswered questions in the field. The timescale with which this earliest of events occurs limits the prospects to rapid mechanisms such as a post-

translational modification, a protein-binding event, or an action of a secondary messenger-like molecule.

I also demonstrate that Gli is recruited to cilia as a complex with SuFu. How, then, are Gli/SuFu complexes selectively brought to cilia? A ciliary localization sequence has not yet been identified for Gli or SuFu. Overexpressed Gli can localize to cilia in the absence of SuFu, while SuFu requires the presence of Gli. This suggests that if a ciliary localization motif exists, it lies within Gli. In support of this, a recent study identified a region C-terminal to the last zinc finger that is important for ciliary localization and may represent the location of a ciliary localization sequence (Zeng et al., 2010).

It is possible that the Smo-dependent accumulation of Gli/SuFu in ciliary tips represents a change in the observed rates of transport in and out of cilia mediated by IFT proteins. However, analysis of IFT mutants is complicated by the general ciliary defects that result from these mutations. It is not likely that signals from Smo affect the activities of kinesin and dynein motors themselves but rather their coordination with cargo. For instance, there could be more availability of Gli/SuFu at the ciliary base for transport into cilia; Gli/SuFu association could be increased for kinesins and decreased for dyneins; or Gli/SuFu proteins could experience a greater delay at ciliary tips before retrograde transport. It is also possible that dissociation of the Gli/SuFu complex occurs at ciliary tips and individual proteins are transported out less efficiently.

Gli activation by dissociation from SuFu

In chapter 2, I provide evidence for another essential feature of Gli activation - the dissociation of Gli from SuFu. Using biochemical approaches, I show that a defined complex

composed of Gli and SuFu rapidly dissociates in response to the Hh signal. Gli/SuFu complex dissociation occurred synchronously with their ciliary accumulation. Complex dissociation also required the activity of Smo and coincided with Smo ciliary recruitment. These results suggest that Gli/SuFu complexes and activated Smo meet within cilia where Gli activation is initiated. In addition, PKA activation by forskolin blocked both the recruitment to cilia and dissociation of Gli/SuFu complexes suggesting that recruitment to cilia is a prerequisite for their dissociation.

The biochemical evidence I presented, however, does not exclude dissociation occurring outside of cilia. Current biochemical fractionation of cilia from mammalian cells in culture has not been amenable to our studies. Mechanical methods such as cilia shearing or chemical methods such as calcium shock or chloral hydrate may disrupt Hh signaling or cause soluble proteins such as Gli and SuFu to leak out. Methods of chemical fixation prior to cilia fractionation will need to be explored. New methods to purify contents of cilia may also need to be devised, such as the enzymatic or chemical tagging of cilia-localized proteins. Alternatively, concrete evidence of Gli/SuFu dissociation within cilia may be visualized by using microscopy-based approaches such as fluorescent resonance energy transfer or bimolecular fluorescence complementation.

SuFu is made in great excess over Gli in cells. Once Gli dissociates from SuFu, what prevents it from re-binding with free SuFu in the cytosol? This remains an unanswered question though it is likely modification on Gli such as a post-translational modification, a conformational change, or a binding partner that prevents SuFu interaction. Differential phosphorylation of Gli after Hh pathway activation has been reported (Humke et al., 2010). It was suggested that phosphorylation of full-length Gli3 by an unknown kinase converts it into the “activated” form, which prevented both SuFu binding and processing into repressor forms.

It is worth noting that Gli activation and transcription of Hh target genes occurs constitutively in SuFu null cells. In this case, trafficking to cilia and the activity of Smo is not required. This gives a relatively simple role for cilia in vertebrate cells that are diverged from *Drosophila* – that is, primary cilia are required to free Gli from SuFu inhibition.

PKA antagonizes Gli activation by blocking ciliary recruitment

Protein kinase A (PKA) is a known negative regulator of Hh signaling. Its inhibitory role is ascribed largely to promotion of Gli repressor processing (Wang et al., 2000; Pan et al., 2006; Tempe et al., 2006; Wang and Li, 2006). In chapter 2, I provide evidence for a new mode of PKA action - its activation blocked both ciliary recruitment and dissociation of Gli/SuFu complexes. However, this finding is based on the use of forskolin, an agent that activates adenylyl cyclase, which catalyzes the production of cAMP leading to the indirect activation of PKA. Other means of PKA activation, such as cAMP analogs (Van Haastert et al., 1984; Schwede et al., 2000), can be tested to rule out any PKA-independent effects of forskolin. A recent study found that PKA localizes to the ciliary base and the authors propose an alternative mechanism whereby PKA acts on Gli/SuFu complexes after they have entered the cilium (Tuson et al., 2011). In this model, Gli/SuFu complexes are modified within the cilium in the absence of the Hh signal. This modification is yet undefined and could be in the form of a post-translational modification or a change in protein complex composition. This modified Gli/SuFu complex is then recognized by PKA at the base of cilia as it exits, to promote Gli3 processing as well as to keep Gli2 in tight association with SuFu and therefore inhibited (Tuson et al., 2011).

Proteasome inhibition blocks Hh target gene transcription

In chapter 3, I report the first observation that proteasome inhibition leads to a block of Hh target gene transcription. To avoid the confounding effects of proteasome inhibition on translated reporter genes, I follow the accumulation of endogenous *Gli1* and *Ptch1* transcripts after proteasome inhibition. I found that their transcription is blocked in all cases of pathway activation – whether induced, as with using the Hh ligand, Smo agonist (SAG) or overexpression of individual Gli proteins; or constitutive, as with the loss of Ptc, loss of SuFu, or with basal *Ptch1* transcription in the absence of signal. An immediate question is whether other Hh target genes are also repressed by proteasome inhibition. This can be determined by an analysis of gene expression by microarray or RNA-seq. I also provided specific examples of genes whose transcription is unaffected (*L27*) or induced (*cJun*) by proteasome inhibition. However, since proteasome inhibition affects the stability of a multitude of proteins, a crucial concern is whether there is a trend toward general transcriptional inhibition or activation. Again, an analysis of changes in the transcriptome will prove useful.

The proteasome has six catalytic active sites, consisting of a pair each of chymotrypsin-like, trypsin-like, and caspase-like sites, based on the type of residues they prefer to cleave (Dick et al., 1998; Nussbaum et al., 1998; Kisselev et al., 2003). Bortezomib (also known as PS-341, or Velcade) and epoxomicin selectively inhibit the chymotrypsin-like sites at low concentrations and do not fully inactivate the proteasome (Kisselev et al., 2006). In contrast, MG132 inhibits all three of these catalytic activities (Taggart et al., 2002). The robustness of transcriptional inhibition by bortezomib suggests that Gli proteins are particularly sensitive to the chymotrypsin-like activity of the proteasome. It will be interesting to determine how specific inhibition of the other proteolytic sites in the proteasome affects Hh target gene expression.

Hh pathway activation leads to accumulation of Gli activator forms in order to induce target gene transcription. Thus a naïve model of Hh pathway activation is the inhibition of both the complete and partial proteolysis of Gli. Using proteasome inhibitors, I determined that this is not the case because inhibiting Gli proteolysis is not sufficient to generate transcriptionally active Gli. It is unlikely that the Hh signal simply inhibits the proteolytic actions of the proteasome the way that bortezomib does. It is likely more selective than that: the Hh signal must allow stabilization of Gli activators (Gli-A) perhaps concurrent with degradation of Gli repressors (Gli-R). The requirements for selectivity mentioned above can be accomplished by regulating E3 ubiquitin ligases. The activity of one E3 ligase can promote elimination of Gli-R while another E3 ligase that targets Gli-A for degradation is inhibited. In addition, deubiquinating enzymes (DUBs) can selectively remove the polyubiquitin degradation signal of Gli-A, thus stabilizing them. It is currently not known whether DUBs participate in Gli regulation. It is also not known whether the degradation of Gli-R is promoted. A study tracking the stability of Gli3 repressors suggests that it is degraded constitutively and not dependent on pathway activation (Humke et al., 2010).

Proteasome inhibition reduces Gli promoter occupancy

Proteasome inhibition results in a transcriptional block of Hh signaling. I found that this is due to loss of promoter occupancy of Gli-A and the possible continued occupancy of Gli-R after 4 hours, and a loss of promoter occupancy of all Gli forms after 24 hours.

A simple explanation for this observed transcriptional inhibition is the stabilization of Gli-R. Particularly at early time points, Gli-R accumulates and may compete with Gli-A for promoter occupancy. Expression in mice of a truncated Gli3 construct that mimics Gli3-R

antagonizes Hh signaling suggesting that Gli-R is dominant over Gli-A (Wang et al., 2000; Vokes et al., 2008; Rajurkar et al., 2012). It will be interesting to see whether the transcriptional targets that are repressed by the Gli3 truncated construct overlaps with the list of Hh target genes repressed by proteasome inhibition. It is also possible that Gli2-R forms exist and contribute to transcriptional inhibition, but at levels undetectable by our antibodies. Another rather simple explanation for this transcriptional block is the loss of promoter occupancy of all Gli forms, particularly after 24 hours – the absence of Gli at promoters precludes transcription of downstream genes. Although not mutually exclusive, these two possible mechanisms can be distinguished by looking at Gli activators in the absence of repressors. I showed that this transcriptional block still occurs with in cells lacking Gli3, the major source of Gli-R. I also showed that this block occurs in HEK293T made to overexpress Gli1, which is not processed. Similar analyses can be done with processing-deficient mutants of Gli2 and Gli3 (Wang et al., 2007, Pan et al., 2009).

An intriguing implication of these results is that proteasome inhibition hinders Gli promoter occupancy by directly or indirectly interfering with Gli DNA binding. I proposed that an unknown protein (or proteins) might mediate this effect because inhibition of protein synthesis rescued this transcriptional block by proteasome inhibition. This unknown protein likely plays a repressive role in the Hh pathway and affects Gli's DNA binding activity. There are a number of possibilities for how this protein factor may work. It may be a DNA binding protein and directly compete with binding to Gli responsive elements. It may interact with Gli to prevent DNA binding, similar to the action of SuFu. It may modify Gli with a post-translational mark leading to a loss DNA binding competency, such as a phosphorylation by a nuclear kinase.

This unidentified protein may also work indirectly, such as the silencing of chromatin regions by histone deacetylases.

A positive role for the proteasome in transcription

An important implication from this study is that proteasome function is required for Gli's transcriptional activity. In general, how might the proteasome positively regulate transcription? Consistent with its roles as a destruction machine, the proteasome degrades proteins that promote or inhibit transcription such as transcription factors, transcriptional co-activators, co-repressors, or chromatin remodelers, thus affecting the expression of genes. Degradation by the proteasome may also regulate the localization of certain transcription factors. Such is the case with NFκB. Full-length NFκB is not transcriptionally active and proteasome degradation promotes the formation of a shorter, transcriptional activator. In addition, the proteasome regulates the stability of an inhibitory interacting protein (IκB) that keeps NFκB in the cytoplasm. There is also evidence for the direct involvement of proteasomes in transcriptional upregulation. The proteasome is found to co-precipitate with RNA polymerase II and is recruited directly on DNA to resolve stalled transcriptional complexes (Gillette et al., 2004). Genome-wide chromatin immunoprecipitation studies in yeast reveal that the proteasomal subunits can occupy DNA. These studies showed that proteasome promoter occupancy correlated with RNA polymerase II occupancy and increased gene transcription (Auld et al., 2006; Sikder et al., 2006).

The proteasome may also directly remove transcription factors off of DNA. It has been proposed for the nuclear receptor ERα that an initial round of transcription correlates with promoter occupancy by ERα, RNA polymerase II and co-activators. This is then followed by occupancy of proteasomal subunits correlating to the clearance of the promoter (Reid et al.,

2003). This will have the effect of silencing that locus. An intriguing hypothesis has been proposed that has the positive effect: proteasomes clear transcription factors off DNA to *promote* transcription (discussed in Lipford and Deschaies, 2003; Collins and Tansey, 2006). According to this model, a transcription factor induces transcription in a limited round of firing, after which it becomes inert (or even inhibitory). The proteasome is then required to remove this "spent" transcription factor to allow "fresh" ones to promote another round of transcription. Proteasome inhibition will then be predicted to increased promoter occupancy of the transcription factor, concurrent with reduced gene expression. This model, however, may not apply to Gli since I detect a loss of Gli activator occupancy of promoters.

Proteasome inhibitors as Gli antagonists for cancer treatment

A common feature in Hh dependent cancers is the overexpression of *Gli1*, an effector as well as a direct target gene of the pathway. *Gli1* upregulation occurs as a consequence of mutations in upstream components, as in the oncogenic activation of Smo or the loss of Ptc function. Targeting Smo using small molecule inhibitors has recently been successful, however refractory mutations in *Smo* are known to occur (Rudin et al., 2009; Dijkgraaf et al., 2010). In addition, activation of Gli can also occur independently of the Hh pathway (Blotta et al., 2012; Li et al., 2012; Atwood et al., 2013; Rajurkar et al., 2012). Direct targeting of Gli proteins is therefore an attractive mode of therapy in Gli-activated cancers. The critical assumption in this strategy is that the expression of genes mediated by Gli is required for the initiation and/or maintenance of the cancer being treated.

Transcription factors are typically considered to be "undruggable" as they do not possess enzymatic activity or catalytic clefts that can be inhibited. Despite this, several compounds have

been identified that inhibit Gli. Two small molecule antagonists were found to inhibit and reduce Gli's oncogenicity: GANT61, by interfering with DNA binding; and GANT58, by an unknown mechanism (Lauth et al., 2007). A series of small molecule Hedgehog pathway inhibitors (HPI) were also found to inhibit Gli through different ways such as by disrupting Gli stability, repressor processing or trafficking to cilia (Hyman et al., 2009). Arsenic trioxide was shown to directly bind and inhibit Gli1 (Beauchamp et al., 2011). More recently, lithium was shown to indirectly reduce Gli1 protein levels and downregulate target gene expression (Peng et al., 2013). In this study, I provided a rationale and the first evidence for the use of proteasome inhibitors as Gli antagonists.

The best example of the use of proteasome inhibitors in cancer treatment is with bortezomib. Bortezomib (Velcade) is an FDA approved as a single agent for multiple myeloma and mantle cell lymphoma, and in combination with certain relapsed or refractory cancers (reviewed in Richardson et al., 2006). However, certain observations suggest that proteasome inhibitors may be used as a more general cancer therapeutic. Increases in both expression of proteasomal subunits as well as activity of the 26 proteasome is observed in some cancers (Kumatori et al., 1990; Chen and Madura, 2005; Bazzaro et al., 2006). This gives rise to the notion that cancer cells have a heightened dependence on the proteasome and therefore increased sensitivity to proteasome inhibition than normal cells. Early pre-clinical studies showed increased cancer cell death compared to normal cells with bortezomib (PS-341), implying its efficacy in a variety of cancers (Adams et al., 1999; Teicher et al., 1999). Cell death in these cancer cells have been attributed to the inhibition of NFκB, activation of unfolded protein response, ER stress, upregulation of apoptosis, and stabilization of tumor suppressor proteins (Adams et al., 1999; Teicher et al., 1999; Hideshima et al., 2001; Obeng et al., 2006; reviewed in

McConkey and Zhu, 2008). Despite promising pre-clinical efficacy, the results from trials in human solid tumors have been disappointing (Yang et al., 2006; Friday et al., 2012). Although this therapeutic strategy has been effective in blood cancers, resistance to proteasome inhibitors does arise (Orlowski et al., 2002; Oerlemans et al., 2008). Second generation proteasome inhibitors based on bortezomib are now being developed (reviewed in Mitsiades et al., 2012). Also, recent studies show that proteasome inhibition may sensitize cancer cells to traditional therapeutics such as radiation and chemotherapy (Davies et al., 2007; Awada et al., 2008).

The effect of proteasome inhibition on downregulation of Hh target genes is clear. However, further studies need to establish whether this transcriptional inhibition translates to a reduction of tumorigenicity and/or induction cell death in Gli-activated cancers. The cells lines I tested that were derived from mouse medulloblastoma have defined genetic alterations, the loss of two tumor suppressors *p53* and *Ptch1,* and a dependency on Hh signaling. Cell viability and tumorigenicity assays can be done to test the efficacy of proteasome inhibitors in comparison to Smo inhibitors in these cells. While this will establish proof of concept, it is worth noting that current proteasome inhibitors do not appear to penetrate the brain (Singh et al., 2010). Their benefit will therefore be limited to those tumors that compromise the blood-brain barrier as observed in certain malignant gliomas (Phuphanich et al., 2010).

The human pancreatic cancer cell lines I tested showed varying transcriptional responses to Hh pathway agonist (SAG), antagonist (SANT-1), and proteasome inhibitor (bortezomib). Cell viability assay results indicate that these cancer cells are insensitive to Smo inhibition by SANT-1, suggesting a non-canonical activation of Gli. A search for other transcriptional targets, besides *Gli1* and *Ptch1*, may yield better indicators of Gli activation in these cells. Indeed, a careful molecular characterization of the role of Gli proteins in these cells is needed. Are Gli

proteins expressed? Are they mutated? Are they, in fact, activated? Are Gli1 or Gli2 activators required for cell proliferation and/or survival?

Cell line P2 gave the best canonical transcriptional response to Smo activation and inhibition. However, these cells were also the most resistant to bortezomib, showing reduced cell viability only after 72 hours. These results may indicate that the effect of bortezomib may largely be on inhibition of cell proliferation rather than the induction of cell death in these cells. This cytostatic effect may be brought about by the induction of autophagy, the lysosomal-mediated degradation pathway. Increasing evidence suggest that ER stress brought about by proteasome inhibition leads to an increase in autophagy to relieve such stress (Ding et al., 2007; Zhu et al., 2009; Suraweera et al., 2012; Selimovic et al., 2013). Interestingly, Gli2 has been implicated in inhibiting autophagy by repressing pro-autophagy factors (Jimenez-Sanchez et al., 2012). Furthermore, in hepatocellular carcinoma cells, Hh pathway activation prevented autophagy induction while treatment with GANT61, a small molecule Gli inhibitor, promoted it (Wang et al., 2013). It is therefore worth testing whether Gli antagonism by proteasome inhibition increases markers of autophagy. It can then be tested whether the inhibition of both proteasome activity and autophagy can lead to increased cancer cell death.

Ultimately, a better understanding and molecular characterization of cancers that are dependent on Hh signaling is important for individualized cancer therapies. The hope is that this and succeeding studies may validate the use of proteasome inhibitors as a successful part of that arsenal.

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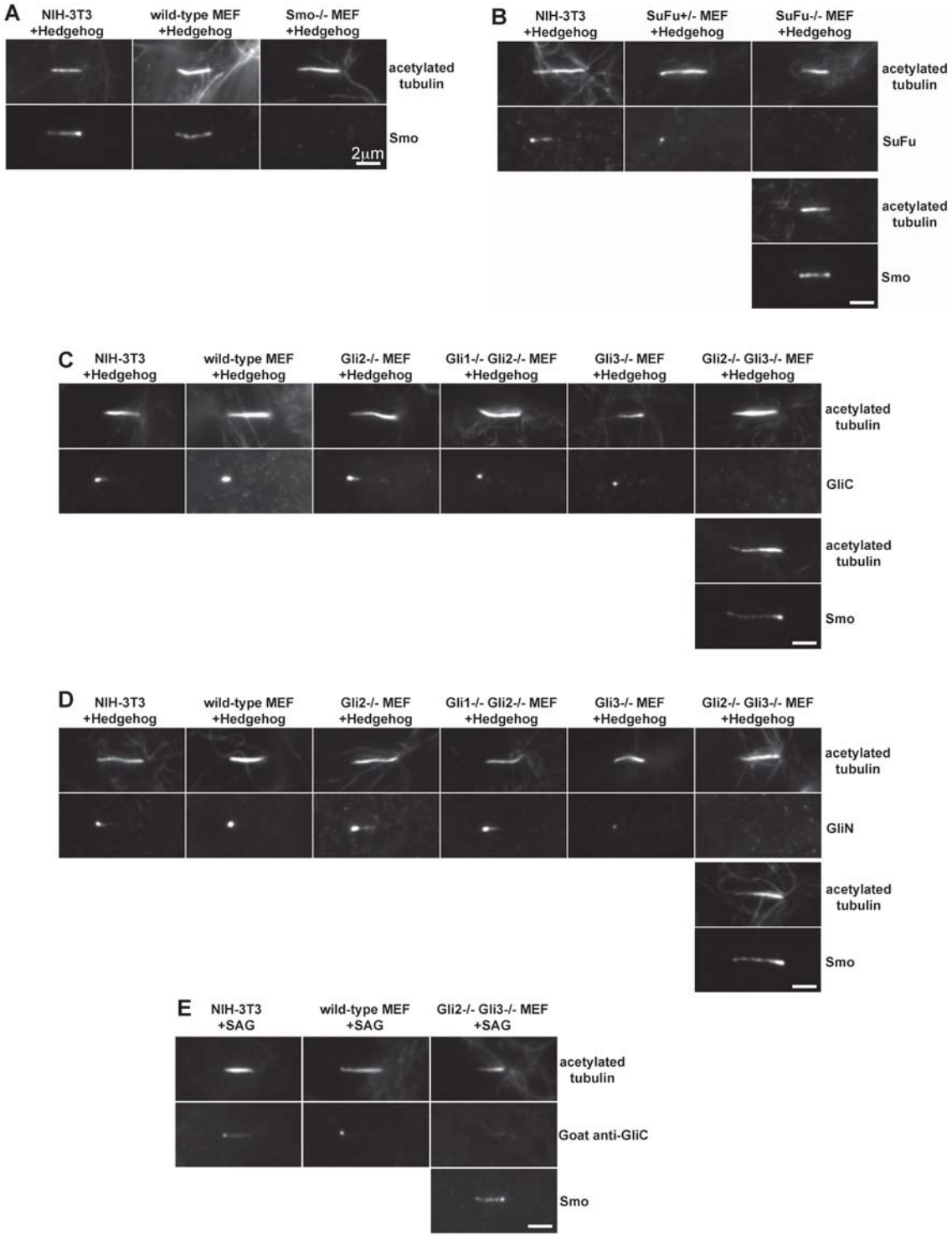
APPENDIX

SUPPLEMENTARY MATERIAL (CHAPTER TWO)



Supplemental Figure 2.S1 Specificity of the novel polyclonal antibodies used for immunofluorescence staining in this study.

Supplemental Figure 2.S1 (Continued)

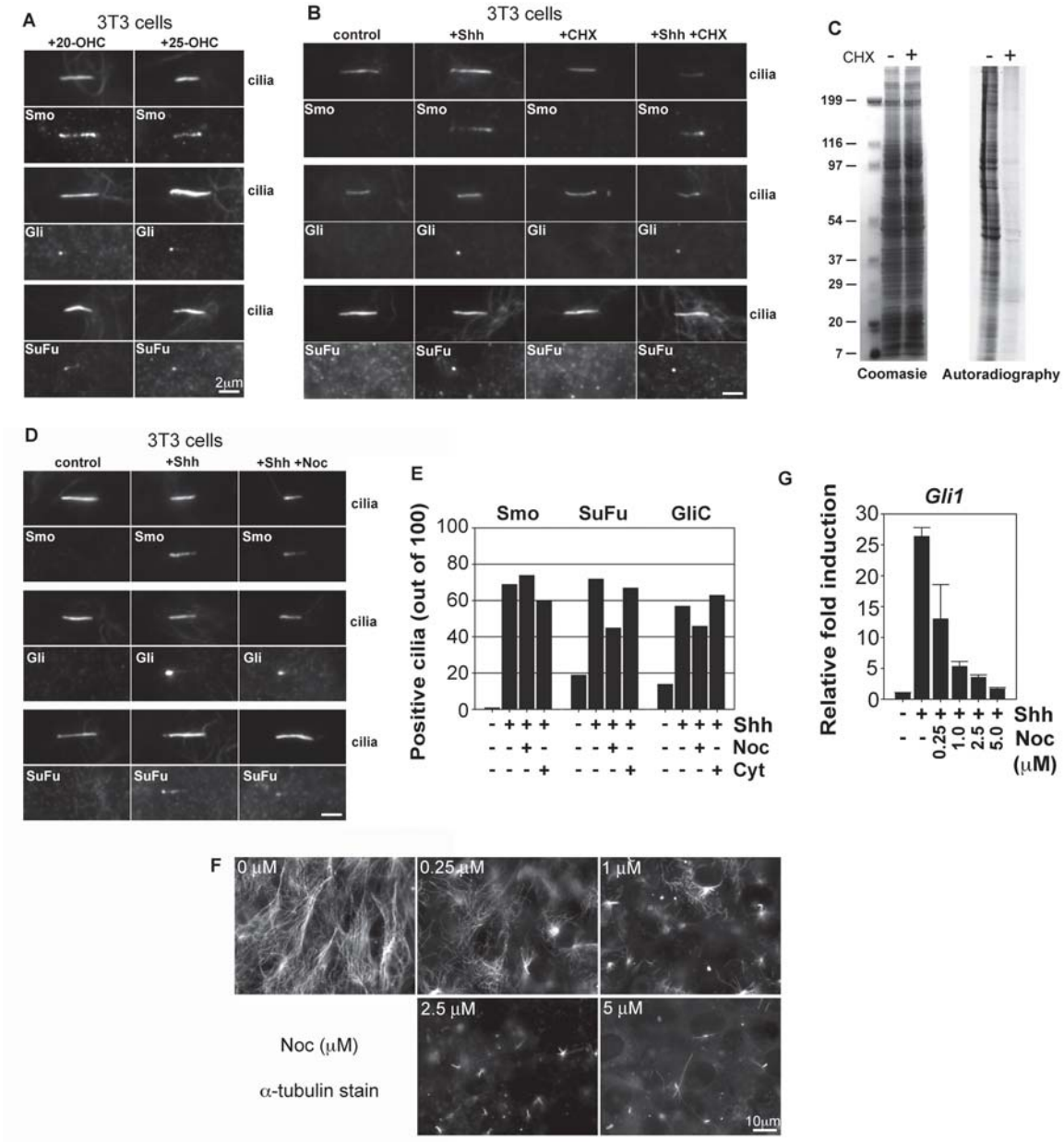
A) Specificity of the rabbit anti-mSmo antibody in immunofluorescence staining
NIH-3T3 cells, wild-type mouse embryonic fibroblasts (MEFs) and Smo-/- MEFs were grown to confluence, starved and stimulated with Shh for 6 hours. The cells were stained with mouse anti-acetylated tubulin antibody (to reveal primary cilia) and affinity-purified rabbit anti-mSmo antibody. Strong staining of cilia is seen in NIH-3T3 cells, wild-type MEFs but not in Smo-/- MEFs. Scale bar is 2 μm.

B) Specificity of the rabbit anti-mSuFu antibody in immunofluorescence staining
NIH-3T3 cells, SuFu+/- MEFs and SuFu-/- MEFs were treated as in A). The cells were stained with mouse anti-acetylated tubulin antibody and affinity-purified rabbit anti-mSuFu antibody. Specific staining of cilia is seen in NIH-3T3 cells and SuFu+/- MEFs, while staining is absent in SuFu-/- MEFs. SuFu-/- MEFs respond to Hh stimulation as shown by Smo recruitment to cilia (bottom panels), demonstrating that the lack of SuFu staining is not due to a defect in signaling at the level of Smo. Scale bar is 2 μm.

C) Specificity of the rabbit anti-GliC antibody in immunofluorescence staining
NIH-3T3 cells, wild-type MEFs, Gli2-/- MEFs, Gli1-/- Gli2-/- MEFs, Gli3-/- MEFs and Gli2-/- Gli3-/- MEFs were treated as in A). The cells were stained with mouse anti-acetylated tubulin antibody and rabbit anti-Gli antibody affinity purified against a C-terminal fragment of human Gli3 (amino acids 1061-1599). Specific staining of cilia is seen in NIH-3T3 cells, wild-type MEFs, Gli2-/- MEFs, Gli1-/- Gli2-/- MEFs and Gli3-/- MEFs. Gli staining is absent in Gli2-/- Gli3-/- MEFs, although Smo is recruited to cilia normally upon Shh stimulation of Gli2-/- Gli3-/- MEFs (bottom panels), demonstrating that the lack of Gli staining is not due to a defect in signaling at the level of Smo. Data in this figure demonstrate that the rabbit anti-GliC antibody recognizes both mouse Gli2 and Gli3 proteins. Scale bar is 2 μm.

D) Specificity of the rabbit anti-GliN antibody in immunofluorescence staining
NIH-3T3 cells, wild-type MEFs, Gli2-/- MEFs, Gli1-/- Gli2-/- MEFs, Gli3-/- MEFs and Gli2-/- Gli3-/- MEFs were treated as in A). The cells were stained with mouse anti-acetylated tubulin antibody and rabbit anti-Gli antibody affinity purified against an N-terminal fragment of human Gli3 (amino acids 1-799). Strong specific staining of cilia is seen in NIH-3T3 cells, wild-type MEFs, Gli2-/- MEFs and Gli1-/- Gli2-/- MEFs. Weak but specific staining is seen in Gli3-/- MEFs. The anti-GliN antibody does not stain Gli2-/- Gli3-/- MEFs, although Smo is recruited to cilia normally upon Hh stimulation of Gli2-/- Gli3-/- MEFs (bottom panels), demonstrating that the lack of Gli staining is not due to a defect in signaling at the level of Smo. These data demonstrate that the anti-GliN antibody recognizes endogenous mouse Gli2 only poorly and most of the signal corresponds to endogenous mouse Gli3 protein, consistent with the staining of overexpressed Gli proteins (not shown). Scale bar is 2 μm.

E) Specificity of the goat anti-GliC antiserum in immunofluorescence staining
NIH-3T3 cells, wild-type MEFs, and Gli2-/- Gli3-/- MEFs were treated as in A). The cells were stained with mouse anti-acetylated tubulin, rabbit anti-Smo, and goat anti-human Gli3C antibodies. Strong specific staining of cilia tips is seen in NIH-3T3 cells and wild-type MEFs, as well as a faint non-specific staining of the cilium shaft. The goat anti-GliC antibody does not stain Gli2-/- Gli3-/- MEFs, although Smo is recruited to cilia normally upon Shh stimulation of Gli2-/- Gli3-/- MEFs (bottom panel). Scale bar is 2 μm.



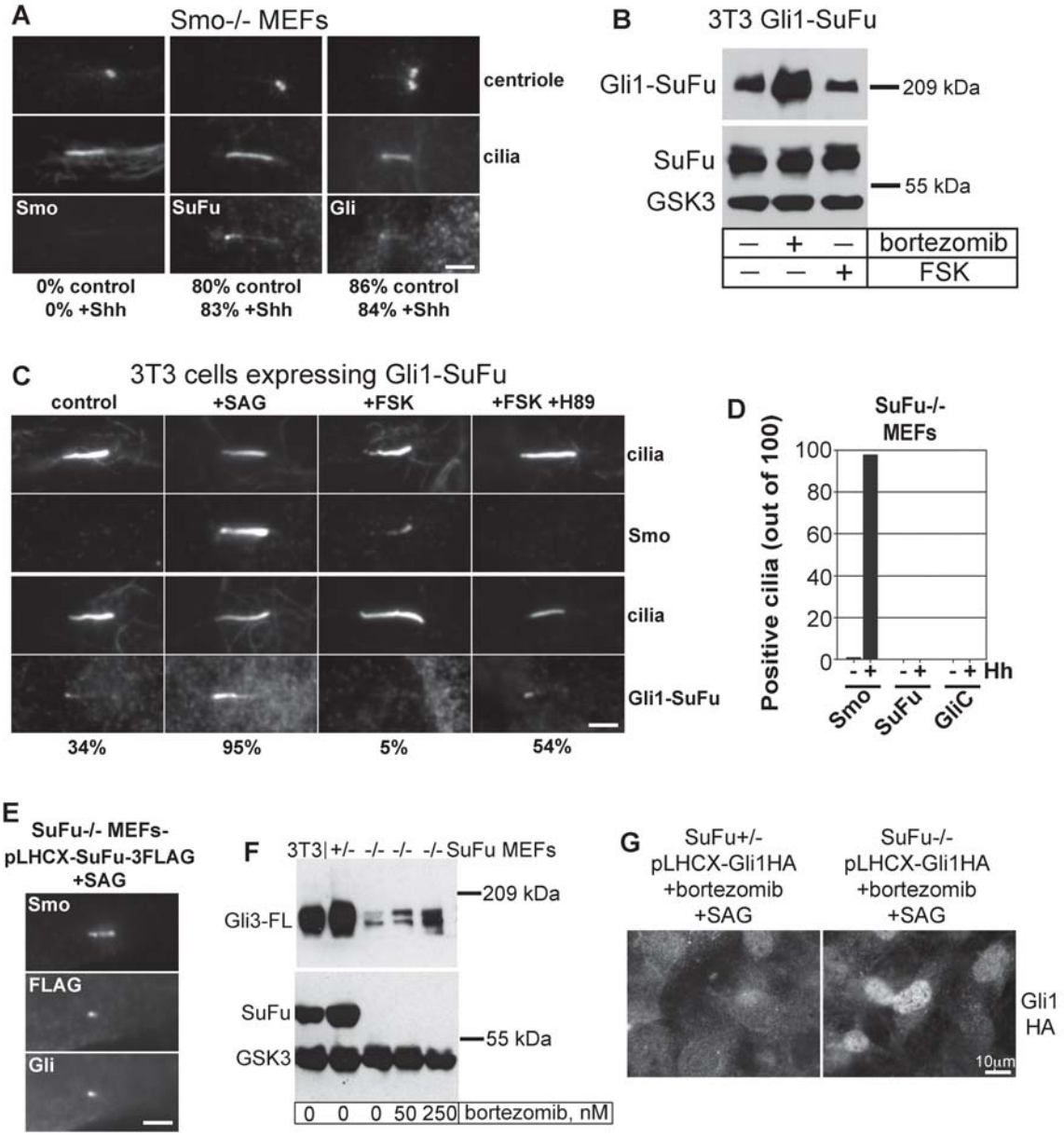
Supplemental Figure 2.S2 The effects of oxysterols, protein synthesis inhibition, and microtubule depolymerization.

A) NIH-3T3 cells were treated with 10 microM of either 20-hydroxycholesterol or 25-hydroxycholesterol, for 3 hours. Immunofluorescence micrographs show recruitment of SuFu and Gli to cilia. The oxysterol 7-hydroxycholesterol, which does not activate the Hh pathway, does not recruit SuFu and Gli to cilia above basal levels (not shown). Scale bar is 2 μm.

B) Inhibition of protein synthesis by cycloheximide (CHX) does not block recruitment of Smo, Gli and SuFu to cilia. NIH-3T3 cells were treated or not with Shh for 1 hour, in the presence or absence of CHX (50 micrograms/mL). Scale bar is 2 μm.

C) Inhibition of proteins synthesis by CHX in the experiment in B). NIH-3T3 cells were incubated with or without CHX (50 micrograms/mL), in the presence of 35S-methionine. Total cell lysates were separated by SDS-PAGE and either stained by Coomassie for total protein or autoradiographed to reveal new protein synthesis.

Supplemental Figure 2.S2 (Continued)
D) Microtubule (MT) depolymerization does not affect recruitment of SuFu, Gli and Smo to cilia. NIH-3T3 cells were pre-treated or not with nocodazole (Noc, 2.5 microM) for 1 hour, then stimulated or not with Shh for 3 hours, in the continued presence or absence of Noc. Recruitment of Smo, SuFu and Gli is not affected by MT depolymerization. Scale bar is 2 μm.
E) Cilia counts for the experiment in (D).
F) MT depolymerization by Noc in the experiment in D). NIH-3T3 cells were treated with various concentrations of Noc for 4 hours, and were immunostained for α-tubulin. Even the highest concentration of Noc does not affect the stable MTs in cilia, which are visible against the diffuse cytoplasmic staining due to depolymerized tubulin. Disappearance of cytoplasmic MTs in the presence of increased Noc concentration correlates with the degree of inhibition of Hh signaling by Noc. Scale bar is 10 μm.
G) MT depolymerization by Noc inhibits the transcriptional output of the Hh pathway in a dose-dependent manner. NIH-3T3 cells pre-incubated for 1 hour with the indicated Noc concentrations, were then treated for 3 hours with Shh, in the continued presence of Noc. Transcription of the Gli1 gene was measured by Q-PCR relative to the RPL27 transcript. Error bars represent standard error of three independent experiments.

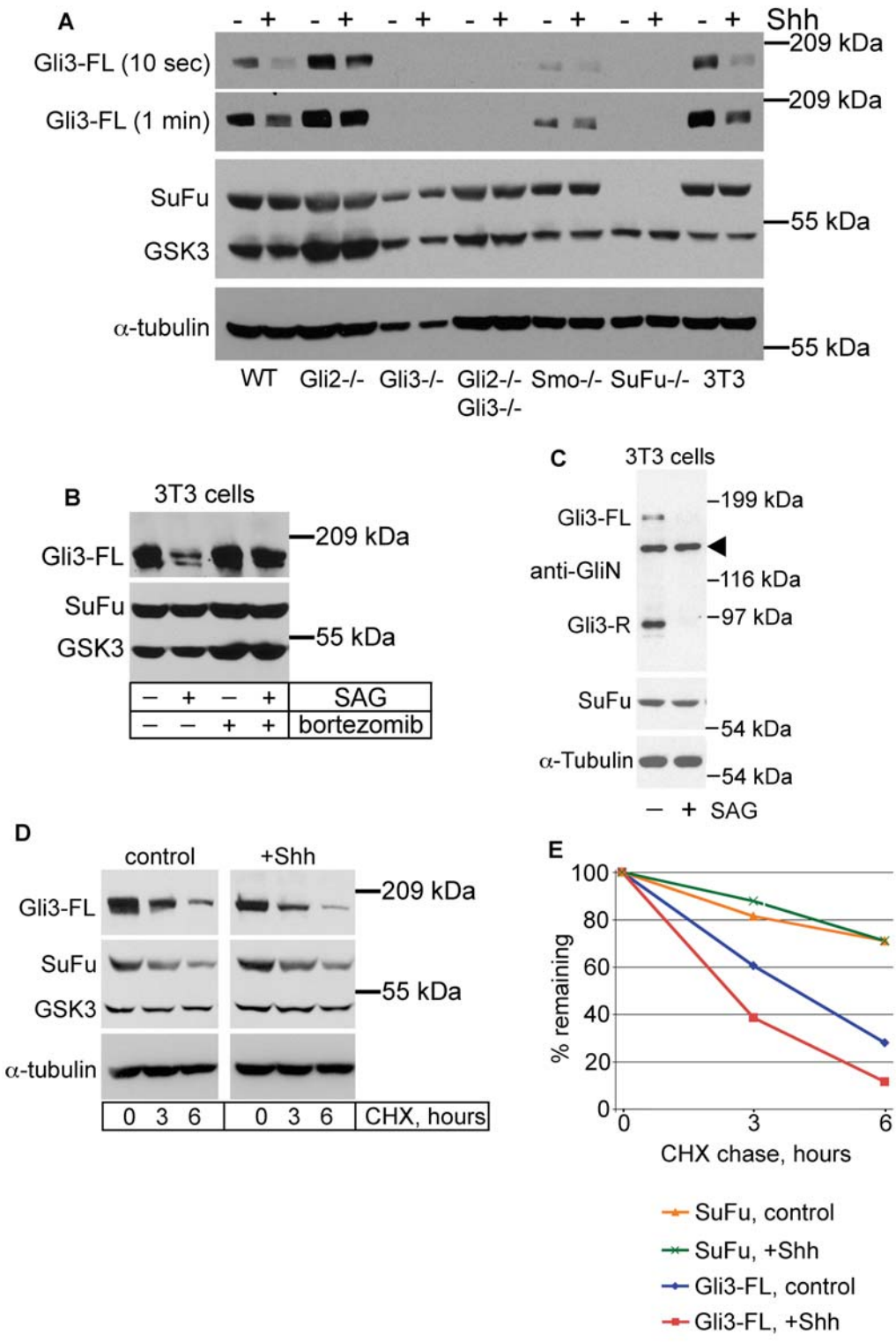


Supplemental Figure 2.S3 Experiments characterizing Smo-/- MEFs, SuFu-/- MEFs, and 3T3 cells expressing Gli1-SuFu fusion.

A) SuFu and Gli localize to the tips of cilia in Smo-/- MEFs. Cilia were stained with anti-acetylated tubulin and basal bodies were stained with anti-gamma tubulin. Percentages shown under the bottom panels indicate ciliary localization of Smo, SuFu and Gli, in untreated cells and in cells stimulated overnight with Shh. SuFu and Gli are not recruited to cilia above basal levels following Shh stimulation of Smo-/- MEFs. Scale bar is 2 μm.

B) The effect of forskolin (FSK) on the levels of Gli1-SuFu fusion. NIH-3T3 cells stably expressing Gli1 directly fused to SuFu, were treated overnight with control vehicle, the proteasome inhibitor bortezomib (100 nM), or FSK (10 microM). Expression levels of Gli1-SuFu fusion, endogenous SuFu and GSK3 (loading control) were determined by immunoblotting.

Supplemental Figure 2.S3 (Continued)
C) The effect of FSK on localization of Smo and Gli1-SuFu fusion to cilia is reversed by the small molecule PKA inhibitor, H-89. NIH-3T3 cells stably expressing Gli1-SuFu fusion were treated overnight with control vehicle, SAG (200 nM), FSK (5 microM), or FSK and H-89 (5 and 10 microM, respectively). Recruitment of endogenous Smo to cilia by FSK is reversed by H-89. Ciliary localization of Gli1-SuFu, which is abolished by FSK, is rescued by H-89. Percentages shown under the bottom panels show ciliary localization of the Gli1-SuFu fusion for the various treatments. Scale bar is 2 μm.
D) No Gli signal is present at cilia in SuFu-/- MEFs, while recruitment of Smo to cilia by Shh is normal in SuFu-/- MEFs. The graph shows cilia counts for the experiment shown in Figure 2.4C.
E) Expression of FLAG-tagged SuFu in SuFu-/- MEFs rescues ciliary localization of Gli. SuFu-/- MEFs stably expressing mouse SuFu tagged with 3 FLAG epitopes were stimulated with 100 nM SAG for 6 hours. Endogenous Smo was detected with a rabbit antibody, SuFu-3FLAG was detected with a mouse anti-FLAG antibody and endogenous Gli was detected with a goat antibody. Scale bar is 2 μm.
F) Gli3-FL levels are greatly decreased in SuFu-/- MEFs, compared to SuFu+/- MEFs and to NIH-3T3 cells. Gli3-FL levels are partially rescued by inhibition of the proteasome with bortezomib. Cell cultures were incubated for 6 hours in the absence or presence of bortezomib, and probed with antibodies against GliC, SuFu and GSK3. Lane 1: NIH-3T3 cells, lane 2: SuFu+/- MEFs, lanes 3-5: SuFu-/- MEFs treated with 0, 50, or 250 nM bortezomib, respectively.
G) Overexpressed Gli1 accumulates in the nucleus in SuFu-/- MEFs, but not in SuFu+/- MEFs. The cells shown are from the same experiment as the one in Figure 2.4E. Gli1-HA was overexpressed in both SuFu+/- and SuFu-/- MEFs by stable retroviral transduction, followed by treatment with 2 microM bortezomib for 6 hours. Scale bar is 10 μm.



Supplemental Figure 2.S4 Levels of SuFu and Gli3 in the cell lines used in this study.

Supplemental Figure 2.S4 (Continued)
A) Effect of Shh stimulation on SuFu and Gli3-FL levels in various cell lines used in this study. Starved, confluent cultures of wild-type MEFs, Gli2-/- MEFs, Gli3-/- MEFs, Gli2-/-Gli3-/- MEFs, Smo-/- MEFs, SuFu-/- MEFs, and NIH-3T3 cells were incubated overnight in the absence or presence of Shh. SuFu, Gli3-FL and the loading controls GSK3 and α-tubulin, were detected by immunoblotting. Two different exposures of the immunoblot for Gli3-FL are shown. Gli3-FL levels decrease during prolonged Shh stimulation in wild-type MEFs, Gli2-/- MEFs, and in 3T3 cells. Gli3-FL levels are not affected by Shh stimulation in Smo-/- MEFs. Gli3-FL is not detectable in MEFs that lack Gli3, as well as in SuFu-/- MEFs, in which Gli proteins are very unstable. SuFu levels and its electrophoretic mobility are not affected by Shh stimulation in any of the cell lines in this panel. Blotting against GSK3 and α-tubulin was used to control for loading.
B) Endogenous Gli3-FL levels are decreased following stimulation of 3T3 cells with 100 nM SAG for 6 hours. Gli3-FL was detected by immunoblotting with anti-GliC antibodies. The decrease in Gli3-FL levels can be reversed by incubation with 2 microM of the proteasome inhibitor bortezomib.
C) The levels of both Gli3-FL and Gli3 repressor (Gli3-R) are decreased following prolonged Hh pathway stimulation. Serum-starved, confluent cultures of 3T3 cells were incubated for 12 hours in starvation media in the absence or presence of 100 nM SAG. Arrowhead indicates a non-specific band.
D) Hh pathway stimulation does not change the half-life of endogenous SuFu but reduces the half-life of Gli3-FL. Confluent, starved 3T3 cells were pre-incubated for 10 minutes with CHX (100 micrograms/mL), followed by incubation with CHX in the absence or presence of Shh, for the indicated amount of time. Endogenous levels of SuFu and Gli3-FL were determined by immunoblotting. Immunoblotting against GSK3 and α-tubulin was used to control loading.
E) Quantification of the experiment in D). The plot shows the percentage of endogenous SuFu and Gli3-FL remaining during the CHX chase, measured relative to the level of α-tubulin.

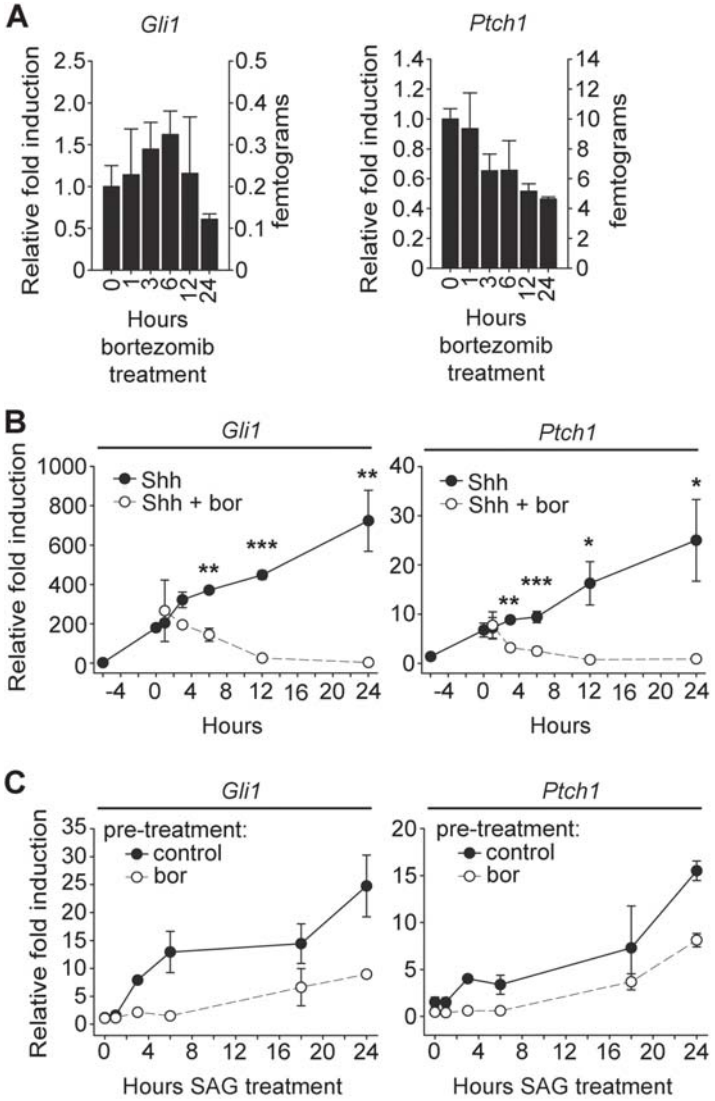
Supplementary Table 2.S1

Recruitment of Smo, SuFu, Gli2 and Gli3 to primary cilia in NIH-3T3 cells and in various mouse embryonic fibroblast lines

	Smo	SuFu	Gli2 & 3
Cell Line			
WT 3T3	-, I	+, I	+, I
WT 3T3 + cyclopamine	+	-, N	-, N
WT 3T3 + SANT-1	-, N	-, N	-, N
WT 3T3 + forskolin	+, I	-, N	-, N
WT MEFs	-, I	+, I	+, I
SuFu-/-	-, I	-	-, N
SuFu+/-	-, I	+, I	+, I
Smo-/-	-	+, N	+, N
Smo-/- + forskolin	-	-, N	-, N
Gli2-/-	-, I	+, I	+, I
Gli3-/-	-, I	+, I	+, I
Gli1-/- Gli2-/-	-, I	+, I	+, I
Gli2-/- Gli3-/-	-, I	-, N	-

- = not detectable at cilia without Shh treatment
+ = detectable at cilia without Shh treatment
N = not inducible by Shh
I = inducible by Shh

SUPPLEMENTARY MATERIAL (CHAPTER THREE)

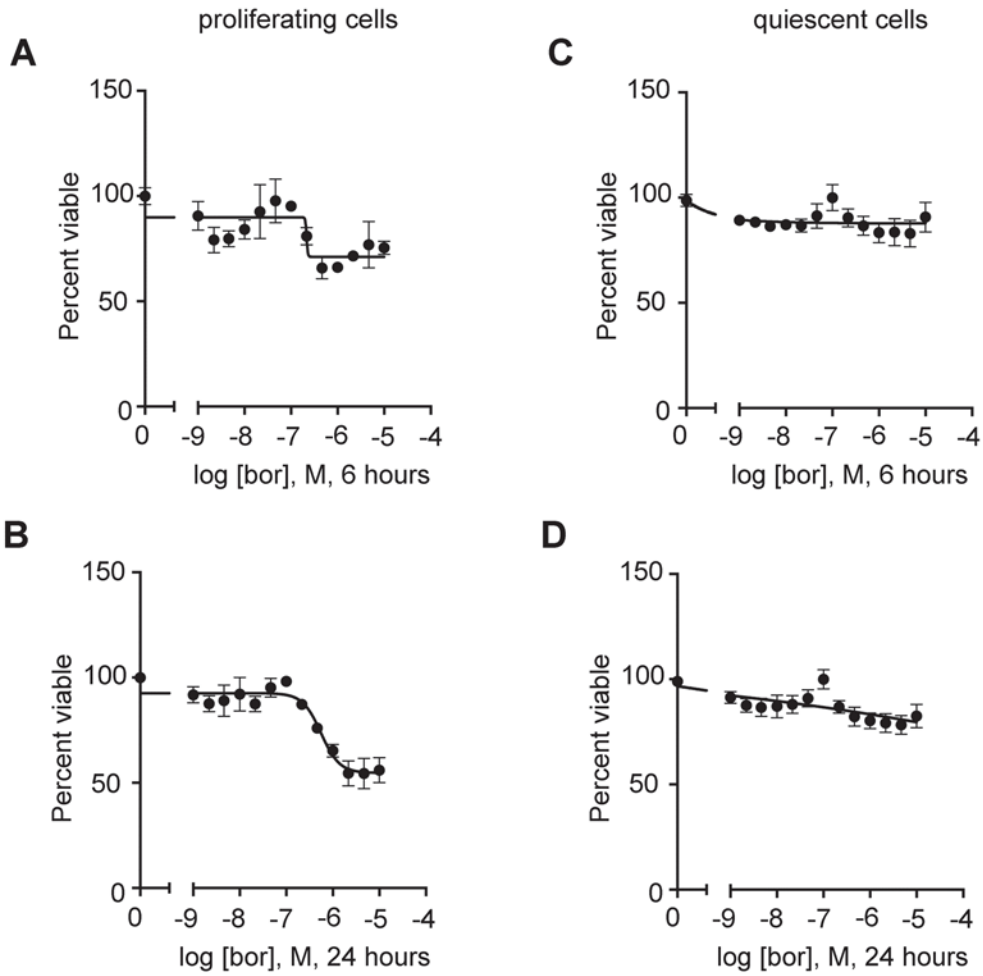


Supplemental Figure 3.S1 Hedgehog target gene transcriptional response of NIH3T3 cells to proteasome inhibition by bortezomib.

A) Time course of target gene transcription assayed by Q-PCR in unstimulated cells treated with 500 nM bortezomib. Transcript levels are reported as relative fold induction over the reference gene *L27* (left vertical axis) and as absolute values in femtograms per nanogram of total RNA determined from a plasmid standard dilution series (right vertical axis). Error bars indicate mean ± SD of three replicates.
Left panel: transcription of *Gli1*.
Right panel: transcription of *Ptch1*.

B) Time course of target gene transcription in cells pre-stimulated for 6 hours with Shh condition media, then refreshed with Shh in the presence or absence of 500 nM bortezomib (bor). Asterisks indicate p-values for combined Shh and bortezomib treatment compared with Shh alone (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

C) Time course of recovery of Hh target gene transcription after washoff of bortezomib. Cells were pre-treated with vehicle (control) or 500 nM bortezomib (bor) for 6 hours, washed with PBS, then stimulated with 100 nM SAG. *Gli1* and *Ptch1* transcript levels in (B and C) were normalized to the reference gene *L27*.



Supplemental Figure 3.S2 Bortezomib inhibits growth in proliferating cells but not in quiescent cells.

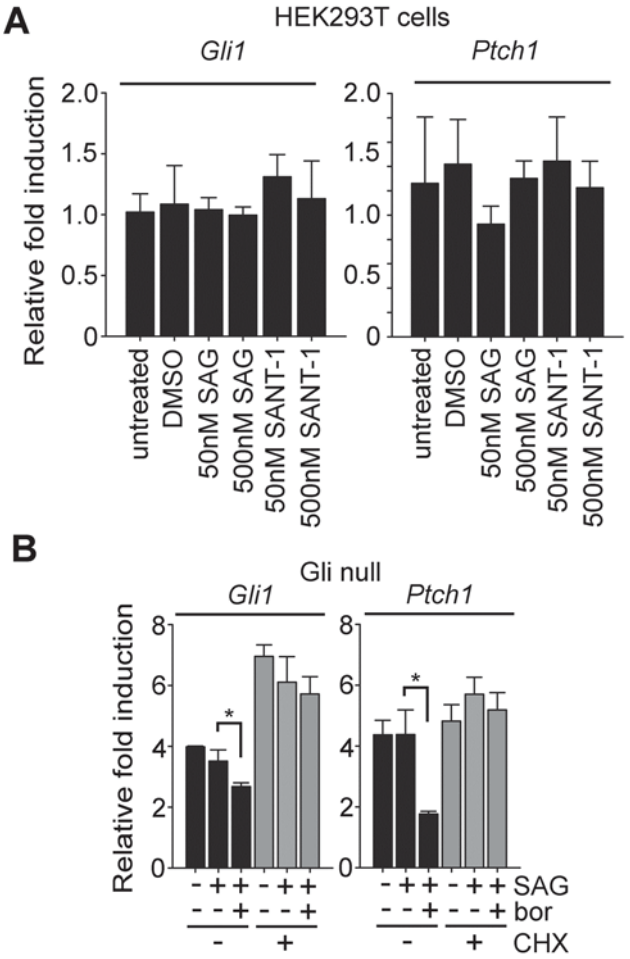
Growth inhibition curves of NIH3T3 cells in increasing amounts of bortezomib assayed using Resazurin Cell Viability Kit (Biotium). Error bars indicate mean ± SD of four replicates.

A) Ten thousand cells were seeded per well in a 96-well plate in quadruplicate and incubated with increasing amounts of bortezomib for 6 hours.

B) Cells were assayed as in (A) but for 24 hours.

C) Forty thousand cells were seeded per well in a 96-well plate, serum starved for 16 hours and incubated with increasing amounts of bortezomib for 6 hours.

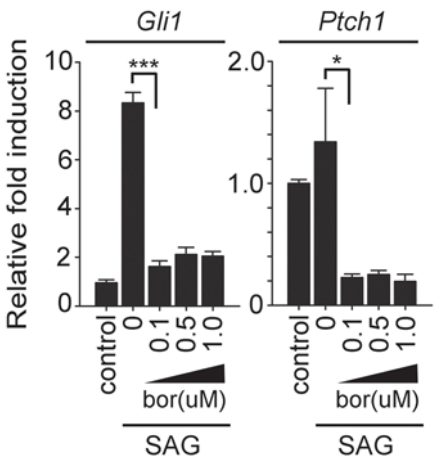
D) Cells were assayed as in (C) but for 24 hours.



Supplemental Figure 3.S3 HEK293T cells and Gli null cells (Gli2-/- Gli3-/- MEFs) do not respond to Hedgehog pathway stimulation.

A) *Gli1* and *Ptch1* transcription does not change with indicated treatments with Smo agonist (SAG) or Smo antagonist (SANT)-1 in HEK293T cells. Error bars indicate mean ± SD of three replicates.

B) *Gli1* and *Ptch1* transcription does not change with 500 nM SAG treatment but is inhibited with 500 nM bortezomib treatment in Gli null cells (Gli2-/- Gli3-/- MEFs). Target gene transcript levels were normalized to the reference gene *L27* and reported relative to unstimulated NIH3T3 cells. Error bars indicate mean ± SD of three replicates. Asterisk indicate p-values for combined SAG and bortezomib treatment compared with SAG alone (*, $P < 0.05$).



Supplemental Figure 3.S4 Human embryonic palatal mesenchyme (HEPM) cells respond to Hh pathway stimulation and transcriptional inhibition by bortezomib.
Transcription of *Gli1* and *Ptch1*was assayed by Q-PCR after 24 hours of stimulation with vehicle (control) 100 nM SAG in the absence and presence on increasing concentrations of bortezomib (bor). Target gene transcript levels were normalized to the reference gene *L27*. Error bars indicate mean ± SD of three replicates. Asterisks indicate p-values for combined SAG and bortezomib treatment compared with SAG alone (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).