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Suppressor of fused and Spop regulate the stability, processing and function of Gli2 and Gli3 full-length activators but not their repressors

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SUMMARY

Gli2 and Gli3 are primary transcriptional regulators that mediate hedgehog (Hh) signaling. Mechanisms that stabilize and destabilize Gli2 and Gli3 are essential for the proteins to promptly respond to Hh signaling or to be inactivated following the activation. In this study, we show that loss of suppressor of fused (Sufu; an inhibitory effector for Gli proteins) results in destabilization of Gli2 and Gli3 full-length activators but not of their C-terminally processed repressors, whereas overexpression of Sufu stabilizes them. By contrast, RNAi knockdown of Spop (a substrate-binding adaptor for the cullin3-based ubiquitin E3 ligase) in Sufu mutant mouse embryonic fibroblasts (MEFs) can restore the levels of Gli2 and Gli3 full-length proteins, but not those of their repressors, whereas introducing Sufu into the MEFs stabilizes Gli2 and Gli3 full-length proteins and rescues Gli3 processing. Consistent with these findings, forced Spop expression promotes Gli2 and Gli3 degradation and Gli3 processing. The functions of Sufu and Spop oppose each other through their competitive binding to the N- and C-terminal regions of Gli3 or the C-terminal region of Gli2. More importantly, the Gli3 repressor expressed by a Gli3 mutant allele (*Gli3*$^{\Delta 699}$) can mostly rescue the ventralized neural tube phenotypes of Sufu mutant embryos, indicating that the Gli3 repressor can function independently of Sufu. Our study provides a new insight into the regulation of Gli2 and Gli3 stability and processing by Sufu and Spop, and reveals the unexpected Sufu-independent Gli3 repressor function.

KEY WORDS: Gli2, Gli3, Hedgehog, Sufu, Spop

INTRODUCTION

The hedgehog (Hh) family of secreted signaling molecules plays an important role in the patterning of embryonic structures in species ranging from *Drosophila* to human. In *Drosophila*, the Hh signal is mediated by the Cubitus Interruptus (Ci) transcription factor. The Ci function is positively regulated by Fused (Fu) serine/threonine kinase, but suppressed by Suppressor of Fused (Sufu) and the kinesin-like molecule Costal2 (Cos2) (Hooper and Scott, 2005). In the absence of Hh signaling, the full-length Ci protein (Ci^{FL}) is phosphorylated first by protein kinase A (PKA) and subsequently by glycogen synthase kinase 3 (Gsk3; Shaggy – FlyBase) and casein kinase 1 (CK1) at multiple sites of the Ci C-terminus. The hyperphosphorylated Ci^{FL} then undergoes proteasome-dependent proteolytic processing to generate a C-terminally truncated transcriptional repressor, Ci^{Rep} (Jia et al., 2002; Jia et al., 2005; Price and Kalderon, 2002; Smelkinson and Kalderon, 2006). Ci^{Rep} is exclusively localized to the nucleus and suppresses Hh target gene expression (Aza-Blanc et al., 1997). Hh signaling inhibits Ci processing through an unknown mechanism and activates a fraction of Ci^{FL}, which translocates to the nucleus and activates the expression of target genes (Chen et al., 1999; Wang and Holmgren, 1999).

In vertebrates, the bipartite functions of Ci have been expanded into three Gli proteins: Gli1, Gli2 and Gli3. In mice, Gli2 and Gli3 collectively mediate Hh signaling and regulate *Gli1* RNA transcription (Bai et al., 2004). Gli2 acts mainly as a strong activator with a weak repressor function, whereas Gli3 serves largely as a strong repressor and has weak activator activity (Buttitta et al., 2003; Ding et al., 1998; Hui and Joyner, 1993; Matise et al., 1998; McDermott et al., 2005; Pan et al., 2008; Pan et al., 2009; Wang et al., 2007). Consistent with their roles in vivo, in the absence of sonic hedgehog (Shh) signaling, the majority of endogenous full-length Gli3 ($Gli3^{FL}$) protein but only a small fraction of $Gli2^{FL}$ is proteolytically processed to generate transcriptional repressors, $Gli3^{Rep}$ and $Gli2^{Rep}$, respectively (Pan et al., 2006; Wang et al., 2000). Similar to that of Ci, Gli2 and Gli3 processing is initiated by the phosphorylation of the first four of six PKA sites in their C-termini, followed by the phosphorylation of the adjacent Gsk3 and CK1 sites. Hyperphosphorylated Gli2 and Gli3 proteins are recognized and ubiquitylated by the $SCF^{\beta TrCP}$ ubiquitin E3 ligase and degraded by the proteasome in a limited and site-specific manner (Pan et al., 2006; Tempe et al., 2006; Wang and Li, 2006). The different levels of Gli2 and Gli3 processing are determined by the processing determinant domain (PDD) located C-terminal to the zinc finger DNA-binding domain (Pan and Wang, 2007). Shh stimulation inhibits the processing of both $Gli2^{FL}$ and $Gli3^{FL}$ and converts them into activators, $Gli2^{Act}$ and $Gli3^{Act}$ (Pan et al., 2006; Wang et al., 2000).

Like Ci, Gli transcriptional activities are also inhibited by Sufu. However, unlike the fly Sufu, the vertebrate Sufu is indispensable, as mice deficient for Sufu die at mid-gestation with ventralized spinal cord (Cooper et al., 2005; Svard et al., 2006). Sufu is localized to both the nucleus and the cytoplasm. The nuclear Sufu is thought to suppress Gli transcriptional activity by recruiting

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corepressors (Cheng and Bishop, 2002), whereas the cytoplasmic Sufu sequesters Gli proteins in the cytoplasm (Barnfield et al., 2005; Ding et al., 1999; Kogerman et al., 1999; Merchant et al., 2004; Murone et al., 2000).

In addition to proteasome-mediated Ci processing, levels of the Ci^{FL} protein are also controlled by HIB (Hh-induced MATH and BTB domain protein) or Rdx, a substrate-binding adaptor for cullin3 (Cul3)-based E3 ubiquitin ligase (Kent et al., 2006; Zhang et al., 2006). Overexpression of HIB reduces Ci^{FL} levels and blocks Hh signaling, whereas loss of *Hib* leads to an excessive accumulation of Ci. HIB binds the N- and C-terminal regions of Ci, and both binding regions are required for HIB-mediated Ci degradation. Thus, HIB targets both Ci^{FL} and Ci^{Rep} for degradation. Interestingly, the HIB function in the regulation of Ci stability is antagonized by Sufu, which also binds the N- and C-terminal regions of Ci. Loss of Sufu function has been shown to destabilize Ci (Ohlmeyer and Kalderon, 1998), whereas its overexpression stabilizes Ci (Zhang et al., 2006). As it can bind both Ci^{FL} and Ci^{Rep}, Sufu presumably regulates the functions of both Ci^{FL} and Ci^{Rep}, although this has not been experimentally determined.

The vertebrate homolog of HIB is speckle-type POZ protein or Spop (Kwon et al., 2006). While this manuscript was being prepared, a study showed that vertebrate Sufu and Spop also antagonistically regulate Gli2 and Gli3 protein stability (Chen et al., 2009). Similarly, another recent study has reported that the Gli3 protein level is significantly reduced in Sufu mutant embryos, although the link between Gli3 degradation and Spop has not been investigated (Jia et al., 2009). Nevertheless, several important questions remain unanswered. First, it is not clear whether Spop targets only $Gli2^{FL}$/$Gli3^{FL}$ or both $Gli2^{FL}$/$Gli3^{FL}$ and $Gli2^{Rep}$/$Gli3^{Rep}$ for degradation. Second, do Spop and Sufu regulate Gli3 processing to generate $Gli3^{Rep}$? Third, does Spop degrade Gli2 and Gli3 proteins through its binding to the N- and C-terminal regions of Gli2 and Gli3? Last, it is thought that Sufu inhibits Gli2 and Gli3 through the recruitment of Sap18, a component of the mouse Sin3 and histone deacetylase complex (Cheng and Bishop, 2002). The fact that Sufu can bind both the N- and C-terminal regions of Gli2 and Gli3 raises an important question: are the $Gli2^{Rep}$ and $Gli3^{Rep}$ functions dependent on Sufu?

In the present study, we showed that Sufu is essential for the stabilization of $Gli2^{FL}$ and $Gli3^{FL}$ but not of $Gli2^{Rep}$ and $Gli3^{Rep}$, whereas Spop promotes $Gli2^{FL}$ and $Gli3^{FL}$ degradation. Spop can also promote Gli3 processing in a Sufu-dependent manner. This opposing action of Sufu and Spop is dependent on their direct interaction with the N- and C-terminal regions of Gli3 or with the Gli2 C-terminus. More importantly, we found that $Gli3^{Rep}$ can largely rescue Sufu mutant phenotypes, indicating that $Gli3^{Rep}$, and most likely $Gli2^{Rep}$ as well, can function independently of Sufu. Our study provides a new insight into the regulation of Gli2 and Gli3 stability, as well as Gli3 processing by Sufu and Spop, and uncovers an unexpected Sufu-independent Gli3 repressor function.

MATERIALS AND METHODS

Mouse strains and the generation of the *Sufu* mutant allele

A PAC clone containing mouse Sufu genomic DNA sequences (Geneservices, UK) was used to create a $Sufu^{flox}$ targeting construct. The $Sufu^{flox}$ construct was engineered by inserting the neomycin cassette flanked by loxP sites into the intron immediately before and a loxP site immediately after the first coding exon of the *Sufu* gene (see Fig. S1A in the supplementary material). The linearized construct was electroporated into W4 ES cells, and targeted ES cell clones were identified by restriction enzyme digestion, followed by a Southern blot analysis of ES cell DNA using 5′ and 3′ probes (see Fig. S1B in the supplementary material). One Sufu-targeted ES cell clone was injected into C57BL/6 blastocysts to generate chimeric founders, which were then bred with C57BL/6 to establish F1 heterozygotes. A Sufu mutant allele was created by crossing the F1 heterozygotes with Act-Cre to delete the *neo* cassette and the first coding exon. PCR analysis was used for routine genotyping with the following primers: BW406 (forward, 5′-CCTTGATGGATGACAACA-TCCA-3′) and BW407 (reverse, 5′-AGAAGCGCTTAATATTGTTTTAC-3′) for the wild-type allele, which produced a 230 bp fragment; and BW406 and BW486 (reverse, 5′-ACCAGGAAGCTCCTGGTTCCCC-3′) for the targeted Sufu allele after the removal of the *neo* cassette, which produced a 230 bp fragment. $Gli3^{\Delta 699}$ mutant mice were obtained from Dr Uli Ruther in Germany (Bose et al., 2002) and genotyped as described previously (Wang et al., 2007). Mice used in this study were in a 129sve, C57BL/6, and CD1 mixed background.

Reporter assay

Reporter assay was performed by transfecting $Gli2^{-/-}$; $Gli3^{xt/xt}$ mouse embryonic fibroblasts (MEFs) with a luciferase reporter driven by 8×Gli-binding sites (Sasaki et al., 1997), a TK-renillar control plasmid, and Gli2 or Gli3 expression constructs, together with or without a Spop expression construct using lipofectamine reagent (Invitrogen). Thirty-six hours post-transfection, luciferase activity was assayed by a dual luciferase assay kit (Promega). Luciferase activity was normalized against the renillar control. Data presented in this study were compiled from three representative experiments.

Immunoblotting and co-immunoprecipitation

Three mouse Spop shRNA constructs were created by cloning the following three double-stranded oligonucleotides into pSuper.retro vector:

shRNA1, 5′-GATCCCCAGC AGTTGATTTC ATCAACTATT AGTGAAGCCA CAGATGTAAT AGTTGATGAA ATCAACTGCC TTTTTA-3′ and 5′-AGCTTAAAAA GGCAGTTGAT TTCATCAACT ATTACATCTG TGGCTTCACT AATAGTTGAT GAAATCAACT GCTGGG-3′;

shRNA2, 5′-GATCCCCACG TCTGAAGGTC ATGTGTGAGT AGTGAAGCCA CAGATGTACT CACACATGAC CTTCAGACGC TTTTTA-3′ and 5′-AGCTTAAAAA GCGTCTGAAG GTCATGTGTG AGTACATCTG TGGCTTCACT ACTCACACAT GACCTTCAGA CGTGGG-3′;

shRNA3, 5′-GATCCCCCGG AGATGATGTG CTTCATCTAT AGTGAAGCCA CAGATGTATA GATGAAGCAC ATCATCTCCT TTTTTA-3′ and 5′-AGCTTAAAAA AGGAGATGAT GTGCTTCATC TATACATCTG TGGCTTCACT ATAGATGAAG CACATCATCT CCGGGG-3′.

Retrovirus for the pSuper.retro Spop shRNA and Sufu was prepared as described (Low et al., 2008). MEFs were infected with the virus particles and lysed for immunoblotting analysis 36 hours later. C3H10T1/2 cells in 6-well plates were transfected with pMIWII-Gli2, pRK-Gli3, pCDNA-HA-Spop, CB6-Sufu or empty vector DNA (1.5 μg DNA/well) using lipofectamine reagent (Invitrogen). HEK293 cells were transfected using the calcium phosphate precipitation method. To inhibit the proteasome, MEFs and 10T1/2 cells were incubated with MG132 (25 μM) for 6 hours, and HEK293 cells with MG132 (50 μM) for 30 minutes prior to lysis of the cells.

Precipitation of Gli proteins by Gli-binding oligonucleotides and co-immunoprecipitation were described previously (Pan et al., 2006). RNA preparation and semi-quantitative RT-PCR were carried out as described (Low et al., 2008). Endogenous Gli2 and Gli3 proteins were detected by antibodies previously described (Pan et al., 2006; Wang et al., 2000). Sufu was detected using an antibody raised against GST-hSufu-271-351aa in rabbits. Antibodies against HA and Myc epitopes and GST were purchased from Covance, Santa Cruz Biotechnogy, and Sigma, respectively.

Immunohistochemistry and in situ hybridization

For immunohistochemistry, mouse embryos at 9.5 days post-coitus (E9.5) were dissected, fixed in 4% paraformaldehyde/PBS for 20-30 minutes at 4°C, equilibrated in 30% sucrose/PBS overnight at 4°C, and embedded in OCT. The frozen embryos were transversely cryosectioned at forelimb

areas (10 μm). Tissue sections prepared for in situ hybridization were used for immunostaining with Nkx2.2 and Pax6 antibodies following antigen retrieval (Kawase-Koga et al., 2010). Tissue sections were immunostained using antibodies against Foxa2, Nkx2.2, Hb9, Nkx6.1, Pax7 [Developmental Studies Hybridoma Bank (DSHB), Iowa, USA] and Pax6 (Covance), as described (Pan et al., 2009). The secondary antibodies (Jackson ImmunoResearch Laboratories) were Cy3a-conjugated donkey anti-mouse IgG for Nkx2.2 and Foxa2, Dylight 488-conjugated donkey anti-rabbit IgG for Pax6, and rhodamine-conjugated donkey anti-mouse IgG for others. In situ hybridization of embryonic sections was performed as described (Pan et al., 2009). A digoxigenin-labeled Olig2 cRNA probe was kindly provided by Tao Sun (Weill Medical College, New York, USA). The mouse Ptc probe was described previously (Goodrich et al., 1996).

RESULTS

Sufu and Spop antagonistically regulate Gli2 and Gli3 protein stability and Gli3 processing

To elucidate the role of Sufu in Hh signaling, its first coding exon was deleted to generate a mutant *Sufu* allele (*Sufu*$^{-}$) in the mouse (see Fig. S1 in the supplementary material). This allele is the same as the one that has been reported previously (Svard et al., 2006). Sufu$^{-/-}$ homozygous embryos died around E9.5 and exhibited ventralized neural tube (Fig. 1A and below), confirming previous observations.

As loss of Sufu results in destabilization of Ci protein in fly embryos (Ohlmeyer and Kalderon, 1998), we wanted to know whether this Sufu function is conserved in vertebrates. Immunoblotting and quantification analysis showed that levels of both Gli2^{FL} and Gli3^{FL} were reduced about six times, but levels of Gli3^{Rep} were decreased around 20 times in Sufu$^{-/-}$ embryos compared with those in wild-type embryos (Fig. 1B). Consistent with this observation, overexpression of Sufu resulted in an increase in Gli2 and Gli3 protein levels (Fig. 1C). Together, these data indicate that Sufu is required for stabilization of Gli2^{FL} and Gli3^{FL} proteins and for Gli3 processing in vivo.

To elucidate the molecular mechanism of Gli2 and Gli3 degradation in the Sufu mutant, we first investigated whether the reduction of Gli2 and Gli3 proteins was dependent on the proteasome. Treatment of Sufu mutant MEFs with MG132, a proteasome inhibitor, restored Gli2 and Gli3 expression levels (Fig. 2A), indicating that Gli2 and Gli3 degradation in the absence of Sufu is dependent on the proteasome. We then examined Gli1, Gli2 and Gli3 RNA levels by semi-quantitative RT-PCR to determine whether their transcription was affected by the Sufu mutation. In the Sufu mutant MEFs, *Gli1* RNA levels were increased, and *Gli2* RNA expression was not affected, whereas *Gli3* RNA levels were reduced, but not by as much its protein levels were (0.8- versus 20-fold, $P \leq 0.0005$, Student's *t*-test; see Fig. S2 in the supplementary material). Thus, we conclude that a loss of Sufu results in the destabilization of Gli2 and Gli3 proteins. Changes in *Gli1* and *Gli3* RNA levels are likely to be the consequence of activation of Gli2 and Gli3 in the absence of Sufu, as *Gli1* and *Gli3* RNA expression is up- and downregulated, respectively, by Hh signaling (Bai et al., 2004; Wang et al., 2000).

Because Gli2 and Gli3 have been shown to be degraded by the HIB-Cul3 and proteasome pathway in transgenic fly embryos (Zhang et al., 2006), we speculated that the degradation of Gli2 and Gli3 proteins resulting from a loss-of-Sufu function was probably mediated by Spop. To test this prediction, we created three short-hairpin RNA (shRNA) constructs against mouse Spop. All three shRNAs were capable of knocking down Spop expression with shRNA3 being the most effective (see Fig. S3 in the supplementary material). Thus, the mouse Spop shRNA3 was used to knock down

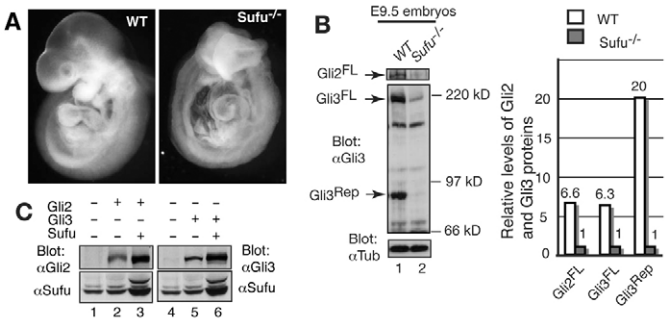

Fig. 1. Sufu is required for the stabilization of Gli2 and Gli3 proteins in vivo. (**A**) Gross morphology of wild-type and *Sufu*$^{-/-}$ embryos at E9.5. (**B**) Immunoblot showing that levels of Gli2 and Gli3 proteins are significantly reduced in *Sufu*$^{-/-}$ embryos. The relative intensity of Gli2^{FL}, Gli3^{FL} and Gli3^{Rep} was quantified by NIH image software and is shown above each bar in the righthand graph. (**C**) Overexpression of Sufu stabilizes Gli2 and Gli3 proteins in HEK293 cells.

endogenous Spop in Sufu$^{-/-}$ MEFs. Immunoblotting analysis revealed that Spop shRNA knockdown, but not GFP shRNA knockdown, restored the levels of both Gli2^{FL} and Gli3^{FL}, but not those of Gli3^{Rep} (Fig. 2B, compare lane 4 with lane 1). Consistent with this observation, forced expression of Sufu in Sufu$^{-/-}$ MEFs partially restored levels of both Gli2^{FL}/Gli3^{FL} and Gli3^{Rep} (Fig. 2C, compare lane 4 with lanes 1-3; $P \leq 0.0073$, Student's *t*-test). The incomplete restoration is most likely due to lower levels of exogenous Sufu expression. In addition, coexpression of Spop induced degradation of both Gli2^{FL} and Gli3^{FL} proteins and Gli3 processing in both HEK293 cells and Hh-responsive C3H10T1/2 cells (Fig. 2D-E). This Spop-induced Gli3 processing was still dependent on the phosphorylation of the PKA sites in the Gli3 C-terminus, as the Gli3P1-6 mutant, which lacks the six PKA sites, remained unprocessed (Fig. 2E, compare lane 3 with lane 5). Taken together, these data indicate that Spop is responsible for Gli2 and Gli3 degradation in Sufu mutant cells and that Spop can facilitate Gli3 processing only in the presence of Sufu.

As Spop is a substrate-binding adaptor for Cul3-based E3 ubiquitin ligase, we next determined whether Spop-mediated Gli2^{FL} and Gli3^{FL} degradation was dependent upon the proteasome and the ubiquitylation of the proteins. Treatment with the proteasome inhibitor MG132 inhibited Spop-mediated Gli2^{FL} and Gli3^{FL} degradation (Fig. 2D, compare lane 4 with lanes 2-3, and lane 8 with lanes 6-7); coexpression of Spop and Myc-tagged ubiquitin with either Gli2 or Gli3 resulted in increased levels of ubiquitylated forms of both Gli2 and Gli3 proteins (Fig. 2F). It should be noted that the extent of ubiquitylation between the two proteins was different. This difference is likely to be due to the difference in their expression levels and/or to their subcellular localization, as overexpressed Gli2 was primarily found in the nucleus, whereas Gli3 was mainly in the cytoplasm.

To determine the effect of Spop on Gli2 and Gli3 transcriptional activity, we carried out a reporter assay using a Gli-dependent luciferase reporter in *Gli2*$^{-/-}$; *Gli3*$^{xt/xt}$ cells. Overexpression of Gli2 or Gli3 activated the luciferase reporter about seven or more than two times, respectively. However, coexpression of Spop with Gli2 or Gli3 brought the reporter activity nearly to the basal level (Fig. 3), indicating that Spop-induced Gli2 and Gli3 degradation reduces Gli2 and Gli3 transcriptional activity.

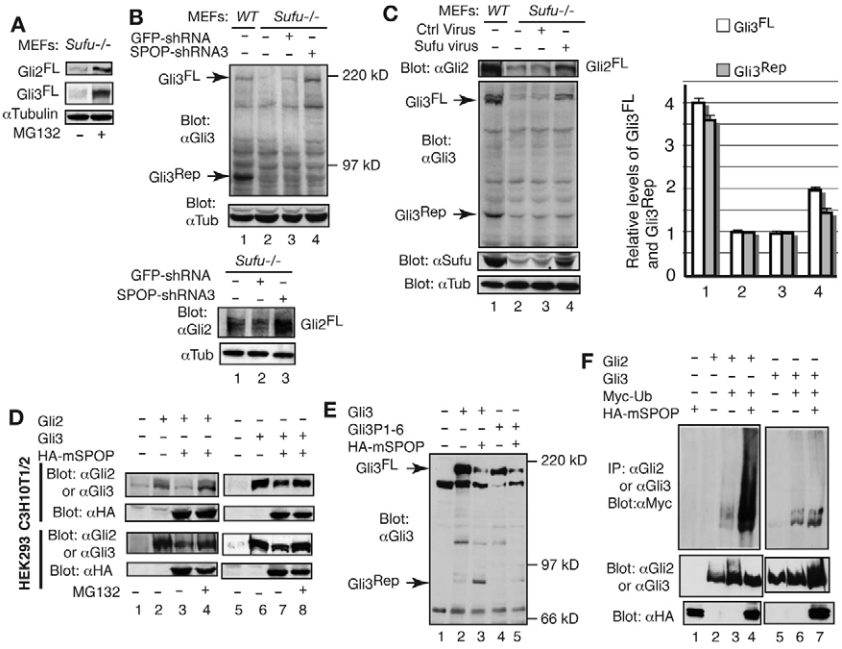

Fig. 2. Spop promotes Gli2 and Gli3 degradation and Gli3 processing only in the presence of Sufu. All panels are immunoblots. (**A**) Inhibition of the proteasome activity with MG132 restores Gli2 and Gli3 protein levels in Sufu mutant MEFs. (**B**) *Spop* shRNA knockdown rescues levels of $Gli2^{FL}$ and $Gli3^{FL}$, but not those of $Gli3^{Rep}$ in $Sufu^{-/-}$ MEFs (compare lane 1 with lane 4). (**C**) Introducing Sufu into $Sufu^{-/-}$ MEFs rescues both Gli2 and Gli3 stability and Gli3 processing. $Sufu^{-/-}$ MEFs were infected with control or Sufu retrovirus. After cells were lysed, Gli2, Gli3, Sufu and αtubulin expression was determined by western blot with the indicated antibodies. Relative levels of $Gli3^{FL}$ and $Gli3^{Rep}$ from three exposures are plotted on the right. $P \leq 0.0073$, Student's *t*-test. (**D**) Overexpression of Spop promotes Gli2 and Gli3 degradation in a proteasome-dependent manner in both HEK293 and C3H10T1/2 cells. Compare lane 4 with lanes 2 and 3, and lane 8 with lanes 6 and 7 for Gli2 and Gli3 levels. Expression constructs are shown at the top, antibodies for western blot and cell lines to the left. (**E**) Overexpression of Spop facilitates the processing of Gli3 but not the Gli3P1-6 mutant in transfected HEK293 cells (compare the $Gli3^{Rep}$ levels in lane 3 with those in lane 5). (**F**) Gli2 and Gli3 are ubiquitylated by Spop. HEK293 cells were transfected with constructs as indicated. After cells were treated with MG132 and lysed, the lysates were subjected to immunoprecipitation with anti-Gli2 or anti-Gli3 antibodies, then immunoblotting with an anti-Myc antibody.

Spop competes with Sufu to bind the C-terminal region of Gli2 and both the N- and C-terminal regions of Gli3

To determine the molecular basis of Gli2 and Gli3 degradation by Spop, we first examined the interaction of Spop with Gli2 and Gli3 proteins. Sepharose beads conjugated with Gli-binding oligonucleotide, to which Gli proteins specifically bind (Pan et al., 2006), could readily pull down Spop when it was coexpressed with Gli2 or Gli3 proteins, but not when Spop was expressed alone (Fig. 4A, compare lane 1 with lane 3, and lane 5 with lane 7). Thus, Gli2 and Gli3 interact with Spop.

We next mapped the Spop-binding regions in Gli2 and Gli3 proteins by coexpressing Spop and various Gli2 or Gli3 fragments and examining their interactions by co-immunoprecipitation or GST (glutathione S-transferase) pull-down methods. Spop bound only the C-terminal region of Gli2, and did not interact with the Gli2 N-terminal region. GST pull-down results showed that there were two Spop-binding regions in the second half of the Gli2 C-terminus (Fig. 4B-D, lanes 14 and 16). Unlike those of Gli2, both the N- and C-terminal regions of Gli3 interacted with Spop. One was located in the second half of the N-terminus, the other in the second half of the C-terminus (Fig. 4E,F, lanes 4, 12, 14 and 18). The Spop-binding region in the Gli3 C-terminus is equivalent to the first Spop-binding region in the Gli2 C-terminus.

It has been shown that vertebrate Sufu binds both N- and C-terminal regions of Gli2 and Gli3 (Dunaeva et al., 2003; Merchant et al., 2004). The fact that Spop also interacts with Gli2 and Gli3 raises the possibility that Spop and Sufu might regulate Gli2 and Gli3 stability through a competitive binding to the Gli proteins. To test this possibility, Gli2 or Gli3 was coexpressed with either Sufu or Spop alone or with both, and the levels of Gli2 or Gli3 proteins were determined by immunoblotting. As expected, coexpression with Spop alone significantly reduced levels of Gli2 and Gli3 proteins, whereas coexpression with both Sufu and Spop, or with Sufu alone, restored the levels of Gli2 and Gli3 proteins (Fig. 4G). To directly test the hypothesis that Spop competes with Sufu for Gli2 or Gli3 binding, a fixed amount of Sufu and Gli2 or Gli3 were coexpressed with various amount of the MATH domain of Spop, which is responsible for substrate binding. The MATH domain was chosen because the full-length Spop could induce Gli2 and Gli3 degradation, thus influencing the competitive interaction of Sufu and Spop with Gli2 or Gli3 proteins. Co-immunoprecipitation results showed that the amount of Sufu that bound Gli2 or Gli3 decreased as Spop-MATH expression levels increased (Fig. 4H). Therefore, Spop and Sufu regulate the Gli2 and Gli3 protein stability through a competitive interaction with Gli2 and Gli3 proteins.

Spop and Sufu regulate the stability of $Gli2^{FL}$ and $Gli3^{FL}$, but not their repressors or C-terminal fragments

The fact that Spop interacts with both the N- and C-terminal regions of Gli3 raised the question of whether both or only one of the regions are necessary for Spop-mediated Gli3 degradation. The

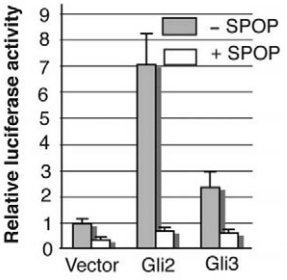

Fig. 3. Overexpression of Spop inhibits Gli2 and Gli3 transcriptional activity. *Gli2*$^{-/-}$; *Gli3*$^{xt/xt}$ MEFs were transfected with 8×Gli-binding site-luciferase, TK-renillar luciferase, and a control vector, Gli2, or Gli3 expression constructs, together with or without the HA-Spop expression construct as indicated. Gli2 and Gli3 transcription activity is significantly reduced by coexpression of Spop.

answer to this question has important implications, as it would address the outstanding question of whether Spop regulates only Gli3^{FL}, or both Gli3^{FL} and Gli3^{Rep}. To address this question, Gli3^{FL}, Gli3-1-700 (mimicking Gli3^{Rep}), or Myc-Gli3CT (the C-terminal region alone) were expressed individually or coexpressed with Spop in C3H10T1/2 cells. Immunoblotting analysis revealed that Gli3^{FL}, but not Gli3-1-700 or Myc-Gli3CT, was degraded by Spop (Fig. 5A, compare lane 1 with lanes 3, 2 and 4, and lane 5 with lane 6). Similarly, Spop targeted Gli2^{FL}, but not Gli2-1-676 or Gli2CT, for degradation (Fig. 5B, compare lane 2 with lane 3, lane 4 with lane 5, and lane 6 with lane 7). It should be noted that the only small effect of Spop overexpression on endogenous Gli2 and Gli3 degradation was likely to be due to the fact that only a small fraction of C3H10T1/2 cells were usually transfected with Spop.

If Spop indeed targets Gli2^{FL} and Gli3^{FL}, but not Gli2^{Rep} and Gli3^{Rep}, one would predict that Gli2^{Rep} and Gli3^{Rep} should be stable in Sufu mutant embryos. However, because Gli2^{Rep} and Gli3^{Rep} are processed from Gli2^{FL} and Gli3^{FL}, the dramatic decrease in Gli3 repressor levels observed in Sufu mutant embryos could result from an overall decrease of Gli3^{FL} levels or from degradation of both Gli3^{FL} and Gli3^{Rep}. To distinguish between these two mechanisms in vivo, we examined the stability of Gli3$^{\Delta 699}$ protein in *Gli3*$^{\Delta 699/\Delta 699}$; *Sufu*$^{-/-}$ double mutant embryos. The *Gli3*$^{\Delta 699}$ mutant allele expressed a C-terminally truncated Gli3 that resembles the naturally processed Gli3^{Rep} (Bose et al., 2002; Wang et al., 2007). Unexpectedly, Gli3$^{\Delta 699}$ protein levels in the wild type and in a mutant Sufu background were similar, even though Gli3$^{\Delta 699}$ retained its N-terminal Spop-binding site (Fig. 6, compare lane 1 with lane 3 in the upper panel). Thus, Spop does not target Gli3^{Rep} for degradation.

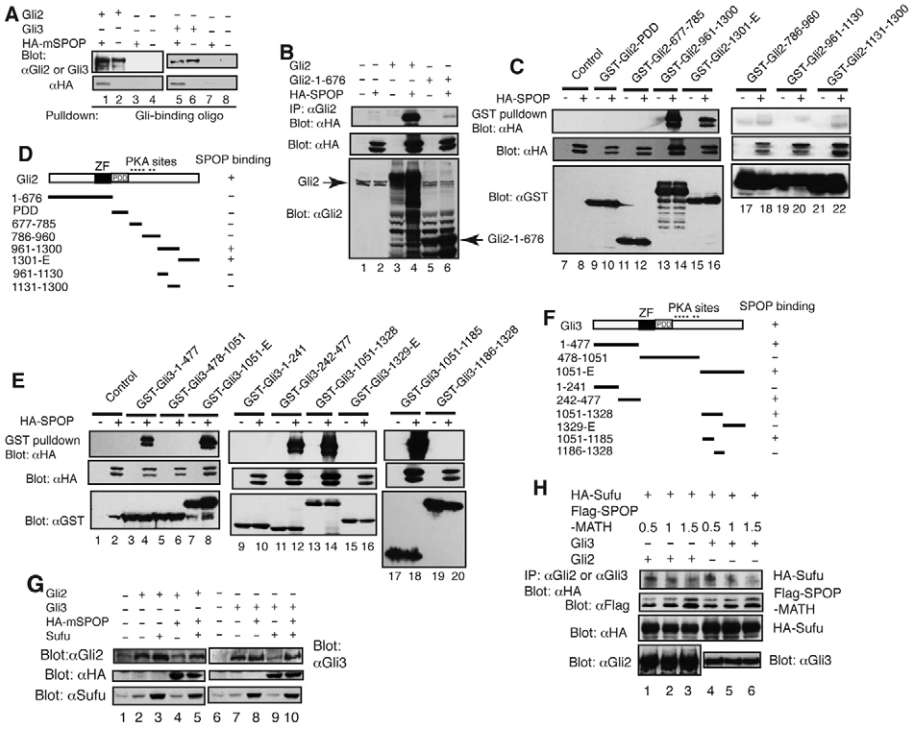

Fig. 4. Spop interacts with the N- and C-terminal regions of Gli3 and the N-terminal region of Gli2. (**A**) Gli-binding oligonucleotide beads pulled down Spop when Spop was coexpressed with Gli2 or Gli3 in HEK293 cells treated with MG132. (**B-F**) Mapping of Spop-binding sites to the Gli2 C-terminus (B-D) and the N- and C-termini of Gli3 (E,F). Shown at the top are proteins expressed in HEK293 cells. Co-immunoprecipitation and GST pull-down were performed (upper panels in B, C and F). Shown in the middle and lower panels are immunoblots of protein lysates with indicated antibodies. Mapping results are summarized in D and F. (**G**) Immunoblot showing that Sufu and Spop antagonistically regulate Gli2 and Gli3 protein stability. Gli2 and Gli3 protein levels were reduced by coexpression with Spop, but increased or restored by coexpression of Sufu or both. (**H**) Sufu and Flag-Spop-MATH competitively bind to Gli2 and Gli3. HEK293 cells were transfected with HA-Sufu, various amount (μg) of Flag-Spop-MATH, and Gli2 or Gli3 expression constructs. Cell lysates were subjected to immunoprecipitation with Gli2 or Gli3 antibodies, then immunoblotting with an HA antibody (upper panel). Shown in other panels are immunoblots of cell lysates with αFlag, αHA, αGli2 or αGli3 antibodies.

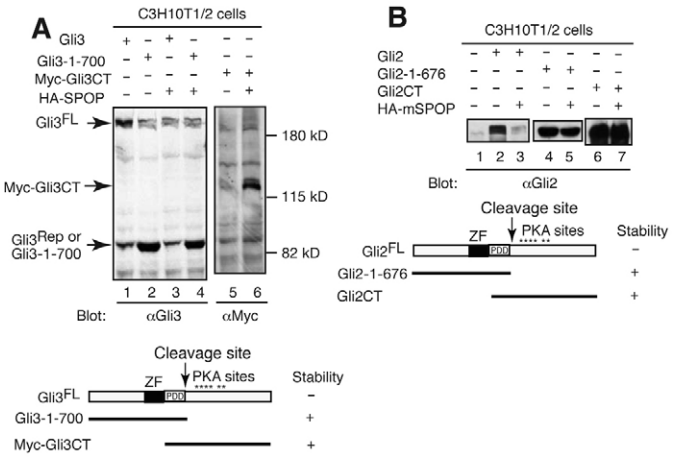

Fig. 5. Spop promotes the degradation of Gli2 and Gli3 full-length proteins but not their repressors or C-terminal fragments. (**A**,**B**) Immunoblots showing that overexpression of Spop degraded $Gli2^{FL}$ and $Gli3^{FL}$ but did not affect the stability of Gli2 and Gli3 repressors or their C-terminal fragments (compare lanes with Spop to those without it). The endogenous Gli2 and Gli3 proteins were clearly detected. As only a small fraction of cells were transfected, the decrease in endogenous Gli2 and Gli3 levels in cells transfected with Spop was not obvious.

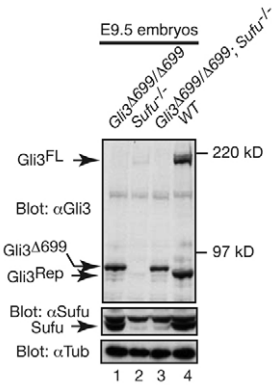

Fig. 6. Immunoblot showing that both $Gli3^{FL}$ and $Gli3^{Rep}$, but not the $Gli3^{\Delta 699}$ repressor, were degraded in *Sufu* mutant mouse embryos. Compare lane 1 with lane 3, and lane 2 with lane 4 in the upper panel. Immunoblot in middle panel confirms the loss of Sufu expression in the mutant. The lower panel shows tubulin expression for loading controls.

The Gli3 repressor functions independently of Sufu

Sufu binds both the N- and C-terminal regions of all three Gli proteins (Dunaeva et al., 2003; Merchant et al., 2004; Pearse et al., 1999). Because the $Gli3^{Rep}$ still contains a Sufu-binding site in its N-terminus, but its stability is not affected by a loss of Sufu, we wanted to know whether the activity of $Gli3^{Rep}$ is dependent on Sufu. To address this question, we examined the neural tube patterning of $Sufu^{-/-}$, $Gli3^{\Delta 699/\Delta 699}$ and $Sufu^{-/-}$; $Gli3^{\Delta 699/\Delta 699}$ embryos, as Hh signaling is necessary for the specification of all ventral cell types and the suppression of dorsal neural cell types (Briscoe and Ericson, 2001). In the wild-type neural tube, Foxa2 expression occupied the floor plate. Nkx2.2 was expressed in the ventral-most area juxtaposed to Foxa2 and marked Vp3 progenitors. The Hb9 motoneuron marker was expressed more laterally, whereas Olig2 marked motoneuron progenitors, and Nkx6.1 was expressed throughout Vp1-Vp3 progenitors. Unlike the ventral markers whose expression is induced by Hh signaling, Pax7 expression was restricted to the dorsal region of the neural tube by low levels of Hh signaling, whereas Pax6 expression was inhibited by high levels of Hh signaling (Fig. 7A-E,U).

In the $Gli3^{\Delta 699/\Delta 699}$ neural tube, the Hb9+ domain was significantly smaller than that in the wild-type embryo, while the expression pattern of the rest of the markers examined appeared to be very similar to that in wild-type embryos (Fig. 7, compare F-J with A-E), suggesting that $Gli3^{\Delta 699}$ exhibited a slightly more repressive activity than did wild-type Gli3. By contrast, loss of Sufu resulted in open neural tubes and an ectopic expansion of Foxa2+, Nkx2.2+, Olig2+ and Nkx6.1+ domains. The presumptive Hb9 expressing domain was largely replaced by Nkx2.2+ cells, leading to fewer mispatterned Hb9+ motoneurons. Expression of both Pax6 and Pax7 was almost completely suppressed (Fig. 7K-O,W). Together, these data indicate that Gli2 and Gli3 are activated in $Sufu^{-/-}$ embryos even though the protein levels are reduced. However, the combined Gli2 and Gli3 activity in $Sufu^{-/-}$ neural tube lacks a gradient to properly specify and pattern neural progenitors.

Interestingly, the $Gli3^{\Delta 699/\Delta 699}$; $Sufu^{-/-}$ double mutant neural tube was closed and showed only a residual Foxa2 expression compared with that in the wild-type neural tube (Fig. 7, compare P with A). Only sparsely localized Nkx2.2+ cells were found in the double mutant (Fig. 7Q, arrowheads). In agreement with this observation, most of the lateral ventral neural tube was occupied by Hb9+ motoneurons, albeit mispatterned (Fig. 7R). Unlike its expression throughout the entire $Sufu^{-/-}$ open neural tube, Olig2 expression was found in the right region as being slightly dorsally expanded, as compared with that in the wild-type neural tube (Fig. 7, compare X with W and U). In addition, the Nkx6.1 expression pattern was largely restored. The Pax6 expression pattern was also mostly rescued, although not in a graded fashion. The dorsal Pax7 expression was partially rescued as well (Fig. 7Q,S,T). Taken together, these results indicate that the expression of the Gli3 repressor can significantly rescue neural tube patterning caused by the loss-of-Sufu function, and thus that Gli3 functions independently of Sufu activity.

To directly determine the Gli3 repressor activity in the absence of Sufu function, the expression of Ptc (a direct transcriptional target of Gli proteins) in wild-type and different mutant neural tubes was examined by in situ hybridization. In the wild-type neural tube, a graded Ptc expression (highest in the ventral-most area and gradually decreasing towards the dorsal area) was detected; a similar pattern of its expression was also found in the $Gli3^{\Delta 699/\Delta 699}$ mutant (Fig. 8A,B). In $Sufu^{-/-}$ neural tube, Ptc was expressed throughout the entire open neural tube without a gradient, indicating that Gli2 and Gli3 are activated, but their activity in the ventral-most area was lower than that in the wild type (Fig. 8C). By contrast, the normal pattern of Ptc expression was largely restored in $Gli3^{\Delta 699/\Delta 699}$; $Sufu^{-/-}$ double mutant neural tube except for its levels remaining low in the ventral-most area compared with that of the wild-type neural tube (Fig. 8D). These results further indicate that the Gli3 repressor can suppress transcriptional targets of Hh signaling independently of Sufu function.

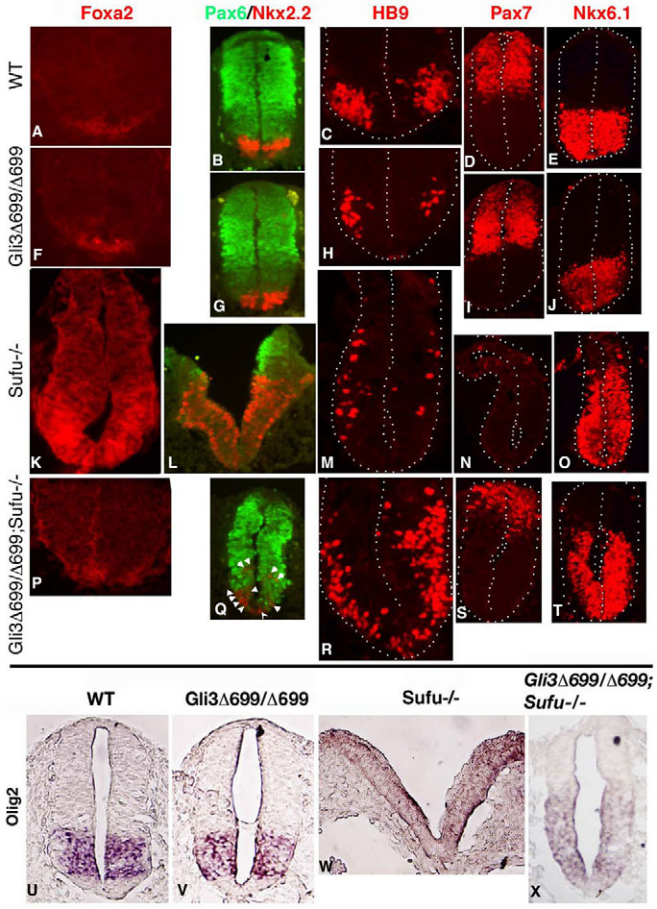

Fig. 7. Expression of the Gli3$^{\Delta 699}$ repressor rescues Sufu mutant neural tube phenotypes. (**A-T**) Neural tube sections prepared from E9.5 embryos with indicated genotypes (left) were immunostained with antibodies against the transcription factors shown at the top. In the Gli3$^{\Delta 699}$ mutant, only the Hb9 expression domain is much smaller than that in wild type; the patterns of the rest of the markers examined are similar (compare A-E with F-J). The difference between the left and right lateral Nkx2.2+ domains in B and G does not represent actual differences, rather it is the result of the position of the embryos while sectioning. Gli3$^{\Delta 699}$ rescues Sufu mutant neural tube phenotypes (compare K-O with P-T). Arrowheads in Q indicate Nkx2.2+ cells. (**U-X**) In situ hybridization of neural tube sections of E9.5 embryos (genotypes at the top) with the Olig2 probe.

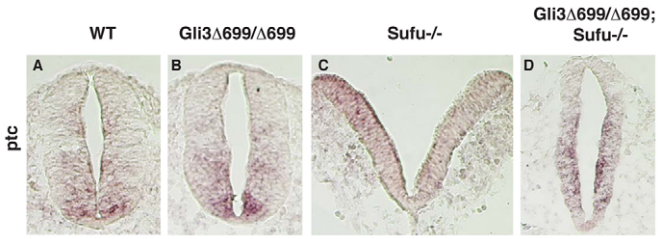

Fig. 8. Gli3$^{\Delta 699}$ expression largely restores the pattern of Ptc expression in the Sufu mutant neural tube. (**A-D**) E9.5 neural tube sections with the indicated genotypes were hybridized with the Ptc probe.

DISCUSSION

In this study, we investigated the role of and the relationship between Sufu and Spop in the regulation of Gli2 and Gli3 protein stability, processing and function. We show that a loss of Sufu results in the destabilization of the full-length Gli2 and Gli3 proteins, but not of their C-terminally truncated repressors or C-termini, whereas shRNA knockdown of Spop can restore Gli2 and Gli3 protein stability. Consistent with this, overexpression of Spop promotes the degradation of the full-length Gli2 and Gli3 proteins, but not of their repressors or C-termini, whereas overexpression of Sufu stabilizes the proteins. The functions of Sufu and Spop oppose each other through their competitive binding to the Gli3 N- and C-terminal regions or the Gli2 C-terminal region. Overexpression of Spop inhibits Gli2 and Gli3 transcriptional activity. More importantly, expression of the Gli3 repressor in Sufu mutant mouse embryos can mostly rescue the ventralized neural tube phenotypes resulting from the loss-of-Sufu function. Therefore, the Gli3 repressor can function independently of Sufu. Together, our study elucidates the molecular mechanism of the regulation of Gli2 and Gli3 protein stability and function by Sufu and Spop, and reveals the unexpected Sufu-independent Gli3 repressor activity.

In addition to its role in the degradation of Gli2 and Gli3 proteins, Spop can also enhance Gli3 processing in transfected cells. The ability of Spop to facilitate Gli3 processing is dependent on the presence of Sufu, as Spop shRNA knockdown fails to rescue Gli3^{Rep} levels (Fig. 2B), but expression of exogenous Sufu in *Sufu*$^{-/-}$ MEFs can restore levels of both Gli3^{FL} and Gli3 repressor (Fig. 2C). The fact that the extent of reduction in the levels of Gli3^{FL} and Gli3^{Rep} in Sufu mutant embryos is very different (Fig. 1B) also indicates the role of Sufu and Spop in the regulation of Gli3 processing. However, Spop- and Sufu-regulated Gli3 degradation and processing appear to be two separable processes, as Spop can induce only Gli3P1-6 degradation and not processing (Fig. 2E). The failure in the rescue of Gli3 processing in Sufu mutant MEFs by Spop shRNA knockdown is probably caused by an inaccessibility of Gsk3 to the Gli3 protein, as Gli3 phosphorylation by Gsk3 is essential for Gli3 processing (Tempe et al., 2006; Wang and Li, 2006) and a recent report showed that Sufu mediates the interaction between Gsk3 and Gli3 (Kise et al., 2009).

A recent study reported that both Gli3^{FL} and Gli3^{Rep} levels are significantly reduced in the Sufu mutant (Jia et al., 2009). Another study showed that besides Gli3, Gli2 is also unstable in the Sufu mutant (Chen et al., 2009). Because Gli2^{Rep} and Gli3^{Rep} are generated from their full-length forms, it is not clear from these studies whether Sufu and Spop target both Gli2^{FL}/Gli3^{FL} and Gli2^{Rep}/Gli3^{Rep} or Gli2^{FL}/Gli3^{FL} alone for degradation. Also unknown is the molecular basis of Gli2/Gli3 degradation by Spop. In the present study, we mapped Spop-binding sites to both the N- and C-terminal regions of Gli3 and to the C-terminal region of Gli2, the latter of which overlaps with Serine/Threonine-rich degrons that were very recently identified to be the Spop-binding sites (Zhang et al., 2009). Coincidently, Sufu has also been shown to interact with both the N- and C-terminal regions of all three Gli proteins (Dunaeva et al., 2003; Merchant et al., 2004; Pearse et al., 1999). Although the minimal Sufu-binding region in the Gli3 (or Gli1 and Gli2) C-terminal region has not been defined, the Sufu-binding site in the Gli3 N-terminal region also mapped to the second half of its N-terminus where Spop binds. This might explain why Sufu and Spop competitively interact with Gli2 and Gli3 proteins. Given the fact that levels of the Gli2 and Gli3 proteins are well maintained under normal physiological conditions

when both Sufu and Spop are present, Sufu is likely to exhibit a higher binding affinity than Spop to Gli2 and Gli3. This allows Gli2 and Gli3 proteins to be maintained at certain levels to promptly respond to Hh signaling. Conversely, when Hh signaling activates Gli2 and Gli3 proteins by inducing Sufu degradation (Zhang et al., 2009), $Gli2^{FL}$ and $Gli3^{FL}$ activators are exposed to Spop for degradation so that they are deactivated following their activation. Therefore, the opposing functions of Sufu and Spop ensure the prompt activation and deactivation of $Gli2^{FL}$ and $Gli3^{FL}$ proteins in response to Hh signaling.

The fact that Sufu and Spop can bind both the N- and C-terminal regions of Gli3 raises the possibility that they may target both $Gli3^{FL}$ and $Gli3^{Rep}$. Unexpectedly, both our in vitro and in vivo results indicate that both Sufu and Spop regulate the stability of $Gli3^{FL}$, but not that of $Gli3^{Rep}$ (Figs 5, 6). Thus, the binding alone of Spop and Sufu to the N- or C-terminal regions of Gli3 is not sufficient to regulate $Gli3^{FL}$ stability. In the case of Gli2, even though Spop does not bind its N-terminal region, its N-terminal truncation can still prevent the remaining protein fragment from being degraded by Spop. These results are in contrast to findings in the fly, where the binding of HIB to either the N- or the C-terminal region of Ci is sufficient to degrade the Ci protein (Zhang et al., 2009; Zhang et al., 2006). Two possible mechanisms might explain why Spop and Sufu do not regulate the stabilities of $Gli2^{Rep}$ and $Gli3^{Rep}$. First, the binding affinity with one binding site could be too low for Sufu or Spop to effectively interact with Gli2 and Gli3 under physiological levels. Second, the ubiquitylation sites might spread to both the N- and C-terminal regions of Gli2 and Gli3, and the truncation of either one of the regions could reduce or abolish the ubiquitylation of Gli2 and Gli3 so that $Gli2^{Rep}$ and $Gli3^{Rep}$ are spared from degradation.

Sufu is an essential inhibitory regulator for all three Gli proteins. It is localized to both the cytoplasm and the nucleus. Cytoplasmic Sufu sequesters Gli proteins in the cytoplasm, whereas nuclear Sufu serves as a corepressor for Gli proteins (Barnfield et al., 2005; Ding et al., 1999; Kogerman et al., 1999; Merchant et al., 2004; Murone et al., 2000). The reason that the nuclear Sufu can inhibit Gli protein activity is thought to be because it can recruit Sap18, a component of the mouse Sin3 and histone deacetylase complex (Cheng and Bishop, 2002). Because Sufu can bind both $Gli2^{FL}$/$Gli3^{FL}$ and $Gli2^{Rep}$/$Gli3^{Rep}$, one important question is whether the $Gli2^{Rep}$ and $Gli3^{Rep}$ functions are dependent on Sufu activity. Analysis of $Gli3^{\Delta699/\Delta699}$; $Sufu^{-/-}$ neural tube patterning clearly indicates that expression of the $Gli3^{\Delta699}$ repressor can largely rescue Sufu mutant phenotypes, although the rescue is incomplete (Figs 7, 8). For example, compared with that in wild-type and $Gli3^{\Delta699/\Delta699}$ neural tube, Ptc expression in the ventral-most area of the double mutant is significantly reduced, some Nkx2.2+ cells are scattered, the Olig2+ domain is slightly expanded dorsally and is weaker, and Hb9 expression occupies a larger area. The partial rescue of the Sufu mutant neural tube phenotype in $Gli3^{\Delta699/\Delta699}$; $Sufu^{-/-}$ mutants can be explained by the fact that the $Gli3^{\Delta699}$ allele expresses only the Gli3 repressor that is presumably no longer regulated by Shh signaling and thus lacks a gradient, and that the $Gli2^{FL}$ activator levels in the absence of Sufu are only one-sixth of those in wild type (Fig. 1B). This view is further supported by the observation that the expression of both Ptc and Olig2 in Sufu single mutants is also lower than that in wild type or in the $Gli3^{\Delta699/\Delta699}$ mutant (Fig. 7, compare W with U-V; Fig. 8, compare C with A,B). Thus, the combined activity of the increased Gli3 repressor and significantly reduced $Gli2^{FL}$ activator levels appears to lack a gradient along the dorsoventral axis of the neural tube, and it cannot properly specify and pattern every cell type. Interestingly, the neural tube phenotype of the $Sufu^{-/-}$; $Gli3^{\Delta699/\Delta699}$ mutant is very similar to that of the *Arl13b* mutant, in which the highest Gli2 activator function is lost, but the low Gli2 activator activity and the Gli3 repressor function remain unaffected (Caspary et al., 2007). It will be interesting to see whether the loss of the highest Gli2 activator function in the *Arl13b* mutant is the result of reduced $Gli2^{FL}$ levels, or changes in protein modification and/or subcellular localization. Nevertheless, because loss of Sufu affects both Gli2 and Gli3 protein stability and processing, our findings that *Sufu* mutant neural tube phenotypes are significantly restored by expression of the $Gli3^{\Delta699}$ repressor strongly indicate that $Gli3^{Rep}$ functions independently of Sufu in vivo.

This study then raises an important question: how do $Gli3^{Rep}$ and $Gli2^{Rep}$ suppress their target gene expression? One possible answer to this question is that $Gli2^{Rep}$ and $Gli3^{Rep}$ simply lack the activation domain, and that they can effectively compete with $Gli2^{FL}$ and $Gli3^{FL}$ activators for Gli-binding sites in the target genes, as $Gli2^{Rep}$ and $Gli3^{Rep}$ are exclusively localized to the nucleus, whereas $Gli2^{FL}$ and $Gli3^{FL}$ are predominantly found in the cytoplasm (Svard et al., 2006). The other possible answer is that $Gli2^{Rep}$ and $Gli3^{Rep}$ might be able to recruit corepressors(s) other than Sufu to suppress their target genes. More studies are needed to distinguish between these two possibilities.

Note added in proof

While this manuscript was being reviewed, a report was published that showed that Sufu could facilitate Gli3 processing and stabilize $Gli3^{FL}$ not $Gli3^{Rep}$ in cultured cells (Humke et al., 2010).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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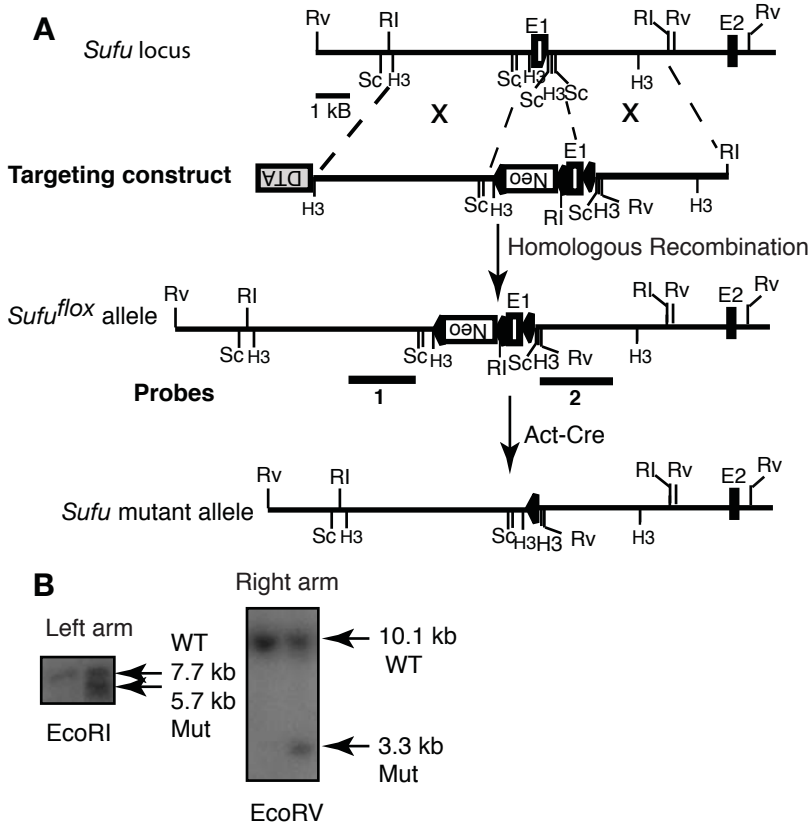
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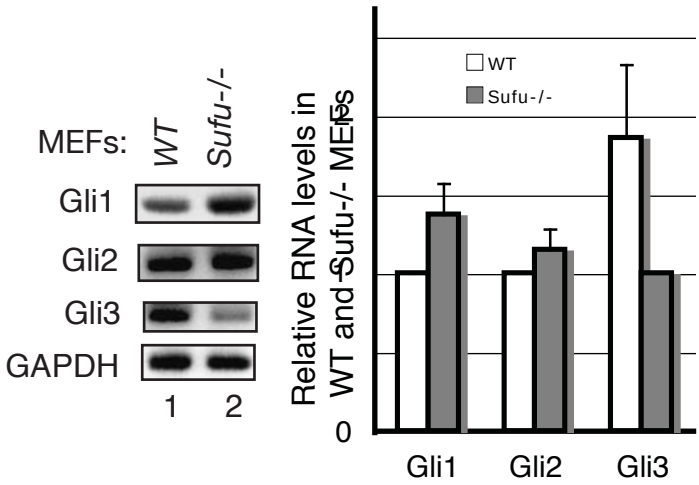
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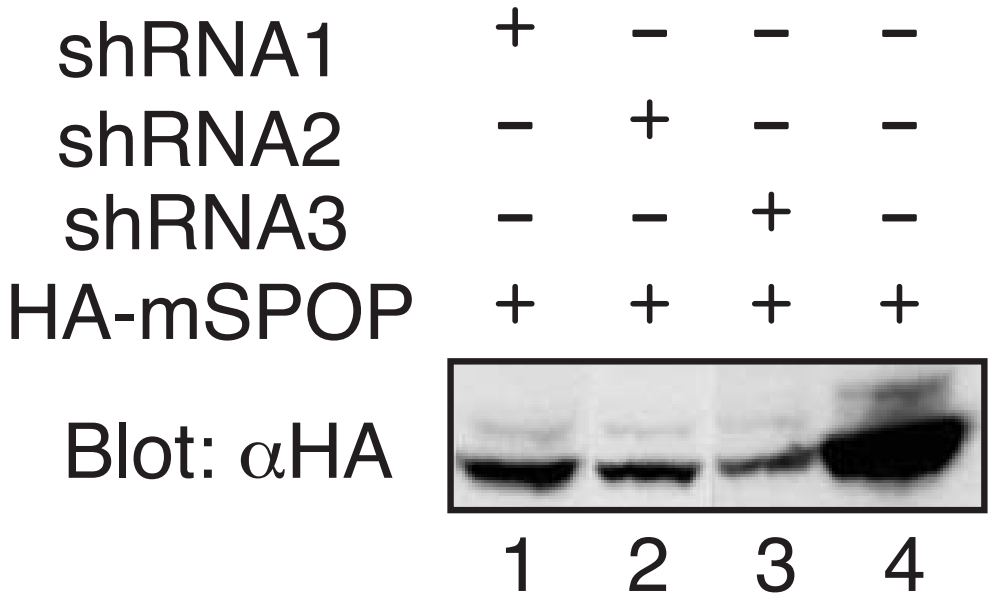
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