Mouse Suppressor of fused is a negative regulator of Sonic hedgehog signaling and alters the subcellular distribution of Gli1

Q. Ding*[†¶], S-i. Fukami[‡¶], X. Meng*, Y. Nishizaki[§], X. Zhang*, H. Sasaki[§], A. Dlugosz[†], M. Nakafuku[‡] and C-c. Hui*

The Hedgehog (Hh) signaling pathway has critical functions during embryogenesis of both invertebrate and vertebrate species [1]; defects in this pathway in humans can cause developmental disorders as well as neoplasia [2]. Although the Gli1, Gli2, and Gli3 zinc finger proteins are known to be effectors of Hh signaling in vertebrates, the mechanisms regulating activity of these transcription factors remain poorly understood [3,4]. In *Drosophila*, activity of the Gli homolog Cubitus interruptus (Ci) is likely to be modulated by its interaction with a cytoplasmic complex containing several other proteins [5,6], including Costal2, Fused (Fu), and Suppressor of fused (Su(fu)), the last of which has been shown to interact directly with Ci [7]. We have cloned mouse Suppressor of fused (mSu(fu)) and detected its 4.5 kb transcript throughout embryogenesis and in several adult tissues. In cultured cells, mSu(fu) overexpression inhibited transcriptional activation mediated by Sonic hedgehog (Shh), Gli1 and Gli2. Co-immunoprecipitation of epitope-tagged proteins indicated that mSu(fu) interacts with Gli1, Gli2, and Gli3, and that the inhibitory effects of mSu(fu) on Gli1's transcriptional activity were mediated through interactions with both amino- and carboxy-terminal regions of Gli1. Gli1 was localized primarily to the nucleus of both HeLa cells and the Shh-responsive cell line MNS-70; co-expression with mSu(fu) resulted in a striking increase in cytoplasmic Gli1 immunostaining. Our findings indicate that mSu(fu) can function as a negative regulator of Shh signaling and suggest that this effect is mediated by interaction with Gli transcription factors.

Addresses: *Program in Developmental Biology, The Hospital for Sick Children, and Department of Molecular and Medical Genetics, University of Toronto, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada. [†]Department of Dermatology and Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan 48109, USA. [‡]Division of Neurobiology, Graduate School of Medicine, The University of Tokyo, Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. [§]Laboratory of Developmental Biology, Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 569-0871, Japan.

[¶]Q.D. and S-i. F. contributed equally to this work.

Correspondence: C-c. Hui
E-mail: cchui@sickkids.on.ca

Results and discussion

Expressed sequence tag (EST) clone number 513730, identified by searching a database for sequences homologous to *Drosophila Su(fu)*, was used to identify a mouse homolog of *Su(fu)*, termed *mSu(fu)*, from an embryonic day 16.5 (E16.5) cDNA library. The open reading frame of *mSu(fu)* is predicted to encode a protein with 485 amino acid residues and a calculated molecular weight of 54 kDa. The *Drosophila* and mouse proteins are 39% identical overall, and both contain a PEST sequence, although at

Figure 1
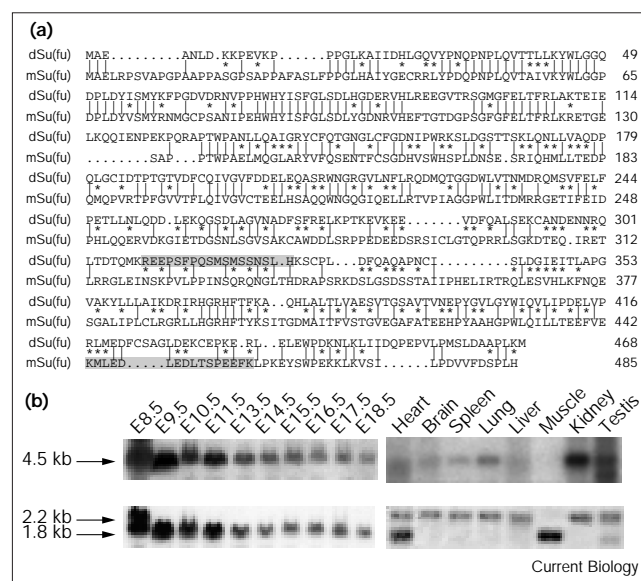
Sequence and expression of mSu(fu). **(a)** Amino acid sequence alignment of *Drosophila* Su(fu) (dSu(fu)) and mSu(fu). Identical and conserved residues are indicated by vertical lines and asterisks, respectively. Shaded boxes represent potential PEST sequences. A mouse E16.5 embryonic cDNA library was screened with an EST clone homologous to *dSu(fu)*. The positive mouse clone was sequenced on both strands. **(b)** Northern-blot analysis of RNA from mouse embryos and various adult tissues revealed a 4.5 kb *mSu(fu)* transcript, detected using a 5′ *Eco*RI fragment of the *mSu(fu)* cDNA labeled with [^{32}P]ATP by random priming. Total RNA was isolated from mouse embryos ranging between E8.5 and E18.5 following extraction in guanidine-isothiocyanate-based buffer. Samples of total RNA (40 µg each) were separated by electrophoresis and transferred to a nylon membrane. The adult mouse multi-tissue RNA blot (containing 2 µg poly-A$^+$ RNA per lane) was purchased from Clontech. Filters were probed for actin as a control for loading (lower panel).

Figure 2
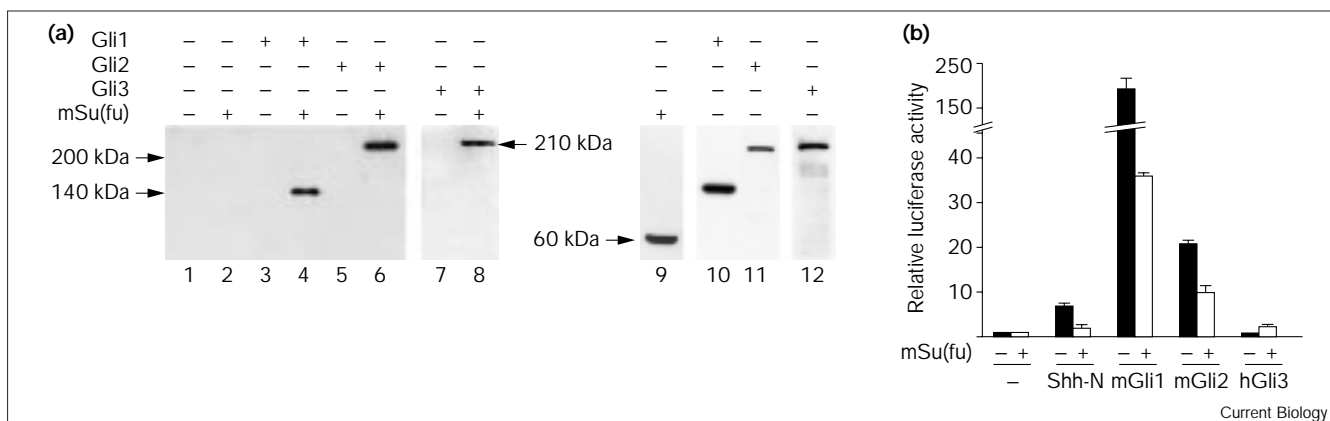
The mSu(fu) protein interacts with vertebrate Gli proteins and inhibits Shh signaling. **(a)** Co-immunoprecipitation of mSu(fu) and Gli proteins. COS cells were transfected with Myc–mSu(fu) and FLAG–mGli1, FLAG–mGli2 or His–hGli3, and harvested as previously described [14], with minor modifications. Immunoprecipitations (lanes 1–8) were carried out with rabbit anti-Myc antibody A14 (Santa Cruz) and protein-G–Sepharose (Pharmacia). Proteins were eluted from beads, separated by SDS–PAGE, and transferred onto nitrocellulose membranes. Immunoblotting was performed using monoclonal anti-FLAG (M2) antibodies (Sigma; lanes 1–6) or anti-His antibodies (InVitrogen; lanes 7,8), with horseradish-peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) visualized using enhanced chemiluminescence (ECL) reagents (Pierce). The expression level of epitope-tagged proteins was determined in cell lysates using antibodies against Myc (lane 9), FLAG (lanes 10,11) and His epitopes (lane 12). The ratio of protein used for IP and western lanes was 300:1. Expression constructs were generated as follows: the open reading frame of *mSu(fu)* was cloned into Myc-pCMV5β (kindly provided by J. Wrana); open reading frames of mouse *Gli1* and *Gli2* were cloned into pCMV/SV-FLAG1 (kindly provided by Y. Kamachi); and the open reading frame of human *GLI3* (kindly provided by K. Kinzler and B. Vogelstein) was cloned into pcDNA-3.1-HIS (InVitrogen). **(b)** Transcriptional activity of Shh-N, Gli1, and Gli2 is inhibited by mSu(fu). MNS-70 cells were seeded at a density of 3×10^5 cells per well in six-well dishes. For transfection, each well received 1 μg of the p8x3′Gli-BS-Luc reporter [8] in combination with various amounts of cDNA expression plasmids, as indicated below. The plasmid pEF-BOS-β-gal (2 μg) [15], which drives expression of β-galactosidase, was also included to equalize the results for transfection efficiency. Transfection and measurements of luciferase and β-galactosidase activities were performed as described [16]. Standard deviations are indicated by the error bars. Plasmids and amounts of DNA per well used for transfection were as follows: pEF-BOS-Shh-N, encoding mouse Shh-N (amino acids 1–198), 2.5 μg; pcDNA3-mGli1, encoding full-length mGli1, 0.75 μg; pcDNA3-mGli2, encoding full-length mGli2, 0.75 μg; pcDSRα-hGli3, encoding full-length hGli3, 0.75 μg; pCMV5β-Myc-mSu(fu), encoding full-length mSu(fu) with an amino-terminal Myc-tag, 3.75 μg.

different sites within the molecule (Figure 1a). A 4.5 kb *mSu(fu)* transcript was detected by northern-blot analysis of RNA isolated from E8.5–E18.5 mouse embryos and several adult tissues, with particularly high levels detected in kidney and testis (Figure 1b).

Monnier *et al.* [7] have recently shown that *Drosophila* Su(fu) interacts directly with Ci and Fu and is likely to be part of a large cytoplasmic protein complex that is tethered to microtubules by Costal2 (Cos2) [5,6]. To determine whether mSu(fu) can interact with mammalian Gli proteins, plasmids encoding epitope-tagged proteins were transfected into COS cells either alone or in various combinations. When Myc-tagged mSu(fu) (Myc–mSu(fu)) was expressed with either FLAG-tagged mouse Gli1 (FLAG–mGli1), FLAG-tagged mouse Gli2 (FLAG–mGli2) or His-tagged human Gli3 (His–hGli3), each of the three Gli proteins co-immunoprecipitated with mSu(fu) (Figure 2a). To examine the functional relevance of mSu(fu)–Gli protein interactions, Shh signaling activity was assayed in a Shh-responsive cell type cotransfected with a luciferase Gli reporter construct [8]. Gli reporter activity was stimulated in MNS-70 cells cotransfected with plasmids expressing mGli1, mGli2 or the active amino-terminal fragment of Shh (Shh-N); in all cases, cotransfection of mSu(fu) greatly inhibited this stimulation (Figure 2b) These results suggest that mSu(fu) negatively regulates Shh signaling by inhibiting the function of Gli1 and Gli2. Note that hGli3 did not activate the Gli–luciferase reporter used in these studies, consistent with earlier findings [8].

As *Gli1* gene expression is consistently upregulated in cells in which the Shh pathway is active [9–11], additional experiments focused on interaction of this molecule with mSu(fu). A series of mutants was generated to broadly define Gli1 domains capable of interacting with mSu(fu) (Figure 3a). Expression of full-length Gli1 and each of the four mutants was confirmed by western-blot analysis (Figure 3b, left panel). The shortest mutant, containing only the zinc-finger domain of Gli1 (Zn), failed to co-immunoprecipitate when expressed with mSu(fu). In contrast, two mutants lacking the carboxyl terminus

Figure 3

Interactions between Gli1 and mSu(fu) mediate inhibition of transcription. **(a)** Schematic representation of mouse Gli1 deletion mutants N, Zn, ΔC and C. For construction of N+VP16 and Zn+VP16 mutants, the cDNA fragment derived from the transactivation domain of VP16 (78 residues) was joined in-frame to the carboxy-terminal ends of the N and Zn mutants, respectively. Three tandem copies of the influenza haemagglutinin (HA) tag were further attached in-frame to the amino-terminal ends of the full-length and mutant fragments of mGli1. All of the above constructs were cloned into the mammalian expression vector pcDSRα [17]. **(b)** Expression of full-length Gli1 and mutants and their interaction with mSu(fu). The HA-tagged full-length mGli1 and various deletion mutants of mGli1 were expressed in COS cells, and their physical interactions with FLAG–mSu(fu) were examined by co-immunoprecipitation assay. COS cells were seeded at a density of 5×10^5 cells onto 60 mm dishes, and expression plasmids for tagged proteins were cotransfected in various combinations by the DEAE–dextran method. Minus and plus signs indicate transfection with the control and corresponding expression plasmids, respectively. The cells were harvested 48 h after transfection and lysed in a buffer containing 50 mM Hepes–NaOH (pH 7.5), 100 mM NaCl, 0.5% (w/v) Nonidet P-40, 2.5 mM EGTA, 2.5 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 1 mM p-aminophenyl methanesulfonyl fluoride hydrochloride, 10 μg/ml leupeptin and 10 μg/ml aprotinin to yield whole cell lysates (WCL). Mouse Gli1 proteins detected in equal volumes of WCL are shown in the left panel. Arrowheads indicate the expected location of full-length Gli1 and mutants. FLAG-tagged mSu(fu) proteins in WCL were recovered by immunoprecipitation with anti-FLAG M2 antibody (Kodak) coupled with protein-A–sepharose CL4B (Pharmacia), and the resultant immune complexes subjected to PAGE and western blot analysis with anti-HA 12CA5 antibody (Boehringer Mannheim/Roche; right panel). **(c)** Effects of mSu(fu) on transcriptional activity of mGli1 and its derivatives in MNS-70 cells. Transient transfection and reporter assays were performed as described in Figure 2. The plasmids and amounts of DNA per well used for transfection were as follows: pCMV5β-Myc-mSu(fu), 4.25 μg; pCDSRα-HA-mGli1, 0.25 μg; pCDSRα-HA-mGli1-N, pCDSRα-HA-mGli1-N+VP16, pCDSRα-HA-mGli1-Zn, pCDSRα-HA-mGli1-Zn+VP16, pCDSRα-HA-mGli1-C, pCDSRα-HA-mGli1-ΔC, all 0.25 μg.


(N and ΔC) and a mutant lacking the amino terminus (C) co-immunoprecipitated with mSu(fu), as did full-length Gli1 (Figure 3b). These data indicate that both amino- and carboxy-terminal domains of Gli1 can interact with mSu(fu). We next performed luciferase reporter assays to examine the relationship between Gli1–mSu(fu) interaction and transcriptional repression. Cotransfection of mSu(fu) inhibited transactivation by full-length Gli1 and the Gli1 deletion mutant lacking the amino terminus (C; Figure 3c), indicating that mSu(fu) can mediate its repression through interaction with the carboxy-terminal region of Gli1. Reporter assays using the N, Zn, and ΔC mutants revealed no transcriptional activity (Figure 3c), consistent with the localization of a transactivation domain to the distal carboxyl terminus of hGLI1 [12] and mGli1 (our unpublished observations). To restore transcriptional activity to carboxy-terminally deleted Gli1, a VP16 activation domain was fused in-frame to the N mutant of Gli1 to produce N+VP16. Transactivation by N+VP16 was inhibited by mSu(fu), indicating that mSu(fu) can mediate repression through the amino-terminal region of Gli1 (Figure 3c). In contrast, mSu(fu) did
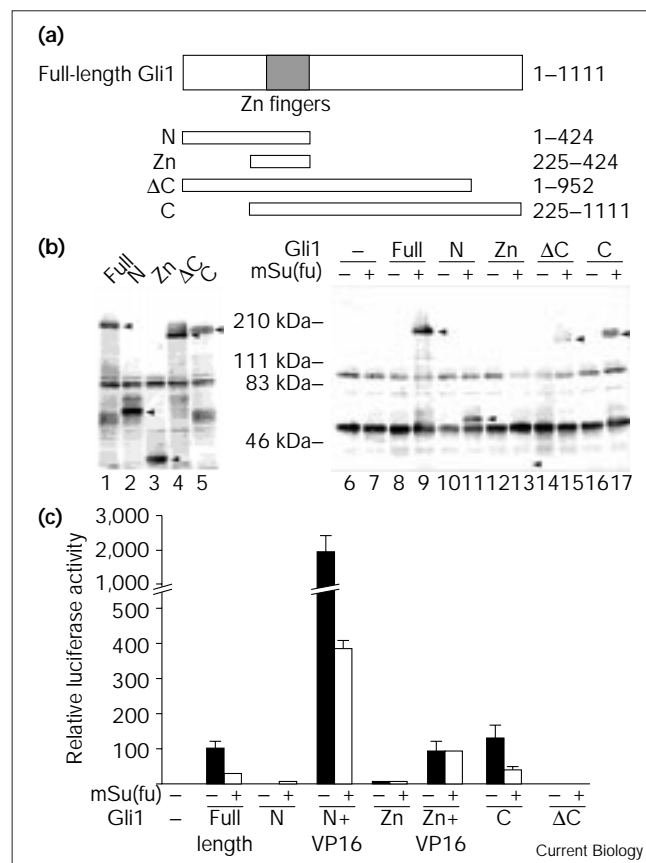
not inhibit transactivation by the VP16 fusion construct designated Zn+VP16, which lacks both the amino- and carboxy-terminal regions of Gli1 (Figure 3c). Taken together, these results suggest that inhibition of Gli1 transcriptional activity by mSu(fu) requires interaction with either the amino or carboxyl terminus of Gli1.

To further explore the effects of mSu(fu) on Gli1, we performed immunolocalization studies using cells transfected with mSu(fu) and mGli1, alone and in combination. We detected mGli1 primarily in the nucleus of HeLa cells (Figure 4), COS cells (data not shown), and MNS-70 cells (see Supplementary material). These findings are in contrast to those described for certain other cell types, in which Gli1 is clearly found outside the nucleus [13]. Prominent expression of mSu(fu) was detected throughout the cytoplasm as well as in the nucleus (Figure 4 and see Supplementary material). Interestingly, cotransfection of mSu(fu) with Gli1 was associated with a striking alteration of Gli1 staining — cytoplasmic staining became clearly detectable (Figure 4). Similar results were obtained in immunofluorescence experiments using COS cells (data not shown) and MNS-70 cells (see Supplementary material). In all cases, mSu(fu) and mGli1 colocalized, suggesting that these two proteins interact *in vivo* as they do *in vitro*. In an effort to quantify the subcellular distribution of Gli1, western-blot analysis was performed following cell

Figure 4
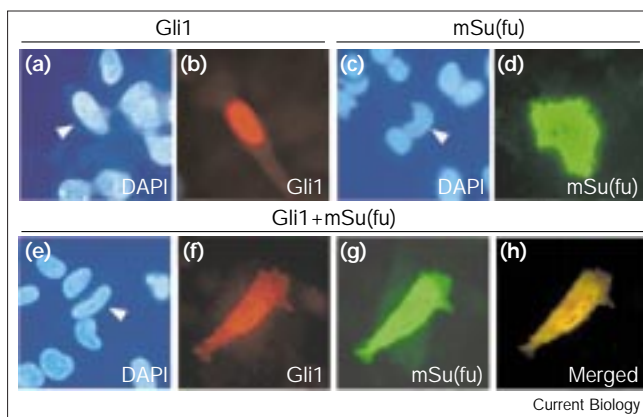
The subcellular distribution of Gli1 in HeLa cells is altered by mSu(fu). Cells were transfected with **(a,b)** FLAG–mGli1 alone, **(c,d)** Myc–mSu(fu) alone or **(e–h)** both. Cells were fixed 42 h after transfection in 4% paraformaldehyde for 10 min and permeabilized in methanol for 2 min. **(b,f)** A monoclonal M2 anti-FLAG antibody followed by Texas-Red-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) was used to detect mGli1. **(d,g)** A rabbit anti-Myc antibody A14 and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) was used to detect mSu(fu). **(a,c,e)** Arrowheads indicate nuclei of transfected cells stained with 4',6-diamidino-2-phenylindole (DAPI). **(h)** The images seen in (f,g), merged. Transfection of mGli1 alone shows localization to nuclei in over 90% of Gli1-positive cells. Cotransfection with mSu(fu) resulted in cytoplasmic Gli1 localization in 86% of double-labeled cells.

fractionation of HeLa cells transfected with single or multiple expression vectors. Although mSu(fu) reduced the transcriptional activity of Gli1, overexpression of mSu(fu) did not appreciably reduce nuclear Gli1 levels (see Supplementary material). Moreover, although immunofluorescence experiments clearly indicated an increase in cytoplasmic Gli1 staining upon overexpression of mSu(fu), Gli1 could not be detected by western blotting in any of the cytoplasmic fractions (see Supplementary material). This discrepancy is probably due to a higher sensitivity of the immunostaining *in situ* of a relatively small population of doubly transfected cells.

In summary, we have cloned a cDNA encoding mSu(fu) and demonstrated that this protein can function as a negative regulator of the Shh signaling pathway, based on its ability to suppress Gli reporter activity. Taken together, our data suggest that mSu(fu) inhibits Shh signaling by interacting with and modulating Gli protein function. Additional loss-of-function studies will be required to ascertain the biological relevance of our findings.

Supplementary material
Supplementary material including immunostaining showing the distribution of Gli1 in MNS-70 cells and immunoblots showing the distribution of Gli1 with or without mSu(fu) in HeLa cells is available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

Mouse Suppressor of fused is a negative regulator of Sonic hedgehog signaling and alters the subcellular distribution of Gli1

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Figure S1

The subcellular distribution of Gli1 in MNS-70 cells is altered by mSu(fu). Plasmids expressing **(a,b)** Gli1, **(c,d)** mSu(fu) or **(e–g)** both were transfected as follows. Plasmid DNAs (0.5 µg each of pCMV-FLAG-mGli1 and pCMV5b-HA-Su(fu) or the corresponding empty vectors) were transfected using Fugene 6 (Boehringer Mannheim/Roche) following the manufacturer's protocol. Cells were fixed using 4% paraformaldehyde 2 days after addition of DNA. (b,f) The FLAG–mGli1 and (d,g) the HA–mSu(fu) proteins were detected by anti-FLAG monoclonal Ab M5 (Kodak) and anti-HA polyclonal antibody Y11 (Santa Cruz), in combination with rhodamine-conjugated anti-mouse IgG (TAGO) and FITC-conjugated anti-rabbit IgG (Cappel), respectively. (a,c,e) Nuclei were stained with DAPI.
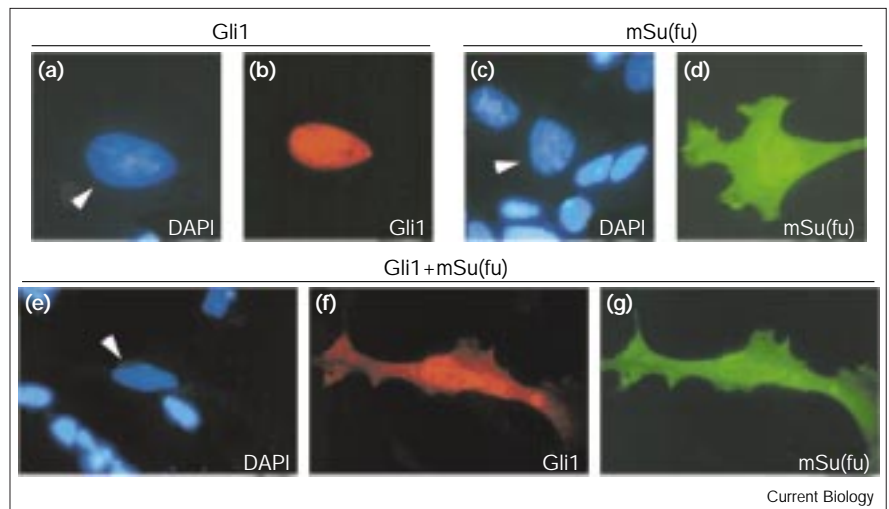
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Figure S2

Subcellular distribution of Gli1 in the presence and absence of mSu(fu) shown by immunoblot analysis. HeLa cells were transfected with control, Myc–mSu(fu), FLAG–mGli1, or Myc–mSu(fu) + FLAG–mGli1 expression vectors as indicated. Cells were harvested 45 h after transfection and cytoplasmic (lanes 5–8) and nuclear (lanes 1–4) proteins were extracted according to a previously described protocol [S1]. Protein extract (55 µg) from each fraction was denatured, separated on an SDS–PAGE gel, and transferred onto a nitrocellulose membrane. The mGli1 (band indicated by arrowhead) was detected with anti-FLAG (M2) antibodies (Sigma) and the mSu(fu) (band indicated by arrow) was detected with anti-Myc (A14) antibodies (Santa Cruz). Anti-lamin A [S2] and anti-tubulin (Sigma) antibodies were used to detect nuclear and cytoplasmic markers, respectively.