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Sufu recruits GSK3β for efficient processing of Gli3

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ABSTRACT

Hedgehog (Hh) signaling activates the transcription factor Gli by suppressing the function of the suppressor of fused (Sufu) protein in mammals. Here, a novel role of mammalian Sufu is identified where it mediates the phosphorylation of Gli3 by GSK3β, essential for Gli3 processing to generate a transcriptional repressor for Hh-target genes. Studies using *Sufu*(−/−) mouse embryonic fibroblasts and siRNA targeting Sufu demonstrate the requirement of Sufu for Gli3 processing. In addition, Sufu can bind to GSK3β as well as Gli3, and mediates formation of the trimolecular complex Gli3/Sufu/GSK3β. Thus, Sufu stimulates Gli3 phosphorylation by GSK3β and Gli3 processing. Furthermore, Sonic Hh stimulation dissociates the Sufu/GSK3β complex from Gli3, resulting in the blockade of Gli3 processing. Collectively, Sufu presumably functions as a GSK3β recruiter for Hh-dependent regulation of Gli3 processing. Such a function is very similar to that of Costal2 in *Drosophila*, suggesting a functional complementation through evolution.

Introduction

The Hedgehog (Hh) signaling pathway is evolutionarily conserved between *Drosophila* and mammals, and plays an important role not only in the patterning of various tissues through regulation of cell proliferation and differentiation, but also in human tumorigenesis [1]. Hh directly binds to its receptor Patched (Ptc) and ultimately induces the expression of Hh-target genes via activation of the transcription factors Ci/Gli. In the absence of Hh, Ci/Gli are kept inactive by the concerted action of various Ci/Gli-interacting proteins, the action of which is canceled by Hh stimulation.

In *Drosophila*, the kinesin-related protein Costal2 (Cos2) interacts with Ci to inhibit its nuclear translocation [2]. Cos2 also promotes proteolytic processing of Ci to generate a transcriptional repressor form of Ci [3]. This processing of Ci requires a primary phosphorylation by PKA, and successive phosphorylation by GSK3β and CK1. The protein Slimb then ubiquitinates such hyperphosphorylated Ci and induces proteolytic processing by the proteasome [4–6]. Cos2 recruits these three kinases to Ci, thus promoting efficient phosphorylation of Ci, and Hh stimulation induces the dissociation of the three kinases from Cos2 [7].

Gli3, which is one of the Ci homologs in mammals, is also phosphorylated by PKA, GSK3β, and CK1, and then processed to become a transcriptional repressor, as is Ci in *Drosophila* [8–10]. However, the regulatory mechanism of Gli3 phosphorylation remains unknown because Kif7 and Kif27, believed to be mammalian orthologs of *Drosophila* Cos2, appear to not be involved in Sonic hedgehog (Shh) signaling [11].

Suppressor of fused (Sufu) protein, originally identified as a genetic suppressor of *fused* mutant flies, appears to play a minor role in *Drosophila* Hh signaling because *sufu* mutant flies show no appreciable phenotype [12]. In contrast, Sufu is believed to be a major negative regulator of Shh signaling in mammals because *Sufu*(−/−) mice show embryonic lethality and mouse embryonic fibroblasts (MEF) obtained from *Sufu*(−/−) mice show constitutive activation of the Shh signaling [13]. It has been reported that Sufu suppresses Gli activity by sequestering Gli in the cytoplasm [14], but its precise role is not understood.

Materials and methods

Materials. GSK3β inhibitor Bio was purchased from Calbiochem. PKA inhibitor H89 was from D. Western Therapeutics Institute. PKA activator forskolin was from Sigma. Proteasome inhibitor MG132 was from Peptide Institute. Anti-flag M2 antibody-conjugated beads was from Sigma. Protein G Sepharose was from Thermo scientific. Glutathione Sepharose was from GE Healthcare. Ni–NTA agarose was from Qiagen. Normal rabbit IgG was from Santa Cruz. Anti-Gli3 rabbit polyclonal antibody (H-280, sc-20688), anti-myc rabbit polyclonal antibody (A-14, sc-789), and anti-glutathione *S*-transferase (GST) rabbit polyclonal antibody (Z-5, sc-459) were from Santa Cruz. Anti-GSK3β mouse monoclonal

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antibody (610201) was from BD Transduction Laboratories. Anti-flag rabbit polyclonal antibody (F7425) was from Sigma. Anti-Sufu rabbit polyclonal antibody was raised by immunizing rabbits with recombinant full-length Sufu protein and then affinity-purified with GST-tagged Sufu-conjugated CNBr-activated Sepharose 4B (GE Healthcare). CIAP was from TOYOBO.

Plasmids. Mouse Sufu cDNA (Sufu(WT)) [15] was inserted into pGEX6p, pCMV-myc, pEF-BOS-myc, and pMX-IRES-GFP vectors. Sufu deletion mutant cDNAs were generated by using appropriate restriction enzymes or by PCR. They were inserted into pGEX6p vectors. Sufu(ΔGSK) cDNA, which lacks amino acid residues 304–315, was inserted into pGEX6p, pCMV-myc, and pEF-BOS-myc vectors. RNAi-resistant Sufu(WT) and mutant Sufu(ΔGSK) cDNAs were generated with Pfu turbo following the manufacturer's instructions (Stratagene). Following primers were used, fw: 5′-cccttggactaCgt ATCcatgtacagg-3′; rv: 5′-gttcctgtacatgGATacGtagtccaaggg-3′. Human GSK3β cDNA inserted into pEF-BOS-flag and pFastbac-GST vectors have been described [15]. Human Gli3 expression vector (Omni-Gli3-pcDNA3.1) was kindly provided by Dr. Hiroshi Sasaki (RIKEN Center for Developmental Biology, Japan). Human Gli3 cDNA was inserted into pEF-BOS-flag vector. Human Gli3(amino acids 18–1028) cDNA was inserted into pFastbac-GST vector. S861A, S873A, S903A Gli3(18–1028) mutants were generated with Pfu turbo as described above and were inserted into pFastbac-GST vector. Following primers were used, S861A-fw: 5′-ggcctacctgagcG Cccgccgctcctcag-3′; S861A-rv: 5′-ctgaggagcggcggGCgctcaggtaggcc-3′; S873A-fw: 5′-gccctgcttctccGCccgccgctccagc-3′; S873A-rv: 5′-gc tggagcggcggGCggagaagcagggc-3′; S903A-fw: 5′-ctccaccgacgccGcgc gccgctccag-3′; S903A-rv: 5′-ctggagcggcgcgCggcgtcggtggag-3′. Human CK1ε cDNA was from IMAGE clone (ID: 10398). CK1εΔ319 (amino acids 1–319) cDNA was generated by PCR and inserted into pGEX6p. cDNA of mouse PKA catalytic subunit was cloned by RT-PCR and inserted into pFastbac-His vector. The expression vector for N-terminal fragment of mouse Shh (ShhN, amino acids 1–198) has been described [15].

Cell culture. *Sufu*(−/−) MEFs were established by a 3T3-like protocol in the previous study [13]. Wild type MEFs were derived from B16 mouse strain and also established by a 3T3-like protocol. MEFs, C3H10T1/2 cells, COS7 cells, and BOSC23 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum.

RNA interference of Sufu. C3H10T1/2 cells were transfected twice with stealth RNA against Sufu (Invitrogen) by Lipofectamine 2000 (Invitrogen) every 2 days. Stealth RNA sequences are as follows, fw: 5′-CCCUUGGACUAUGUUAGCAUGUACA-3′; rv: 5′-UGUACAU GCUAACAUAGUCCAAGGG-3′ (used in Figs. 1A and 3E), and fw: 5′-CAGAGUCCAUGAGUUUACAGGAACA-3′; rv: 5′-UGUUCCUGUAA ACUCAUGGACUCUG-3′ (used in Supplementary Fig. S1). Two days after the second transfection, cell lysates were collected, separated by SDS–PAGE, and immunoblotted with anti-Gli3 or anti-Sufu antibodies. The amount of Gli3 was quantified with ImageJ.

In vitro kinase assay. For examining the effect of Sufu on Gli3 phosphorylation by GSK3β, 200 ng GST-Gli3(18–1028) immobilized on glutathione Sepharose was incubated with 50 ng PKA and cold ATP in kinase buffer (50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM DTT) for 1 h at room temperature, and then with 40 ng/μl Sufu(WT) or Sufu(ΔGSK), 5 ng/μl GSK3β, γ-^{32}P-ATP, and 5 μM H89 for 30 min at 30 °C in kinase buffer. For analyzing the phosphorylation site of Gli3 promoted by Sufu, wild type and mutant GST-Gli3(18–1028) were subjected to *in vitro* kinase assay. For analyzing the effect of Sufu on Gli3 phosphorylation by CK1εΔ319 or PKA, 200 ng GST-Gli3(18–1028) was incubated with 40 ng/μl Sufu(WT), 5 ng/μl CK1εΔ319 or PKA, and γ-^{32}P-ATP for 30 min at 30 °C in kinase buffer. For analyzing the kinase activity of Sufu-IPs, 200 ng GST-Gli3(18–1028) was prephosphorylated by PKA as described above, and incubated with Sufu- or normal IgG-IPs in the presence of γ-^{32}P-ATP and 5 μM H89 for 30 min at 30 °C in kinase buffer.

All samples were separated by SDS–PAGE and detected by autoradiography, immunoblotting, or Coomassie Brilliant Blue (CBB) stain. The radioactivity of Gli3 was quantified with the BAS2000 imaging analyzer (Fuji film).

Analysis of Gli3 phosphorylation state. For the analysis of Gli3 hyperphosphorylation, COS7 cells were transfected with expression plasmids. Next day, cells were pretreated with DMSO or 1 μM Bio for 3 h and further treated with 50 μM forskolin and 10 μM MG132 for 4 h. Cell lysates were collected with lysis buffer supplemented with 10 mM NaF and immunoprecipitated with anti-flag M2 beads for 1 h at 4 °C. Alkaline phosphatase treatment was done with CIAP in CIAP buffer (TOYOBO) for 1 h at 37 °C.

Additional description of methods are provided in the Supplementary information online.

Results and discussion

Sufu is required for processing of Gli3

It has been reported that constitutive activation of Shh signaling in *Sufu*(−/−) MEFs was only partially suppressed by PKA

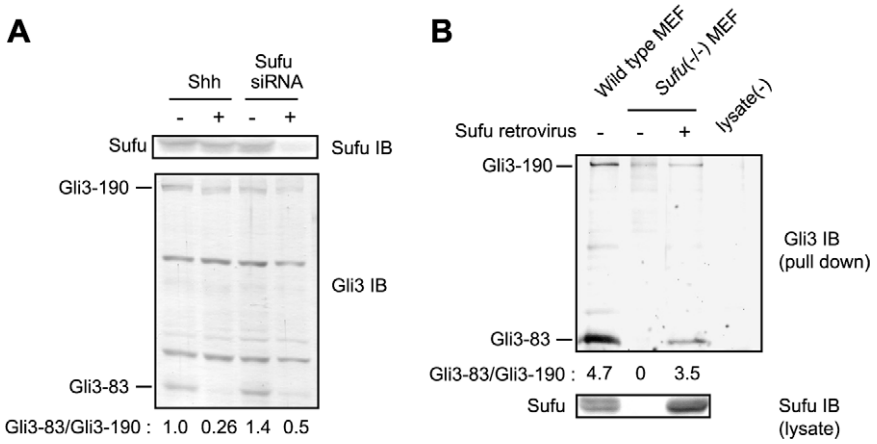

Fig. 1. Sufu is required for Gli3 processing. (A) C3H10T1/2 cells were treated with or without Shh-conditioned medium, or transfected with control or Sufu siRNA. Cell lysates were subjected to immunoblotting analyses with anti-Sufu or anti-Gli3 antibodies. (B) Lysates of wild type MEFs, *Sufu*(−/−) MEFs, or *Sufu*(−/−) MEFs that express Sufu by retroviral infection were incubated with GST-Sufu beads to concentrate Gli3. The precipitated proteins were examined as in (A). The Gli3–83/Gli3–190 ratio was quantified and presented in both (A) and (B).