The negative regulator of Gli, Suppressor of fused (Sufu), interacts with SAP18, Galectin3 and other nuclear proteins

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Sufu (Suppressor of fused) is a negative regulator of the Hedgehog signal-transduction pathway, interacting directly with the Gli family of transcription factors. However, its function remains poorly understood. In the present study, we determined the expression, tissue distribution and biochemical properties of mSufu (mouse Sufu) protein. We identified several mSufu variants of which some were phosphorylated. A yeast two-hybrid screen with mSufu as bait allowed us to identify several nuclear proteins as potential partners of mSufu. Most of these partners, such as SAP18 (Sin3-associated polypeptide 18), pCIP (p300/CBP-co-integrator protein) and PIAS1 (protein inhibitor of activated signal transduction and activators of transcription 1), are involved in either repression or activation of transcription and two of them, Galectin3 and hnRNPA1 (heterogeneous nuclear ribonucleoprotein A1), have a nuclear function in pre-mRNA splicing. We confirmed the mSufu–SAP18 and mSufu–Galectin3 interactions by independent biochemical assays. Using a cell transfection assay, we also demonstrated that mSufu protein (484 amino acids) is predominantly cytoplasmic but becomes mostly nuclear when a putative nuclear export signal is mutated or after treatment of the cells with leptomycin B. Moreover, mSufu is translocated to the nucleus when co-expressed with SAP18, which is normally found in this compartment. In contrast, Galectin3 is translocated to the cytoplasm when it is co-expressed with mSufu. Our findings indicate that mSufu is a shuttle protein that appears to be extremely versatile in its ability to bind different proteins in both the cytoplasm and nucleus.

Key words: Galectin3, Gli, SAP18, Suppressor of fused (Sufu).

INTRODUCTION

During development, a wide variety of cellular decisions are taken based on specific cell–cell interactions. Most of these decisions are performed through activation or repression of gene expression. The Hh (Hedgehog) signalling pathway is crucial for the development of metazoans and is regulated at multiple levels. Hh exerts its effect via the transcriptional factors Ci (Cubitus interruptus) in *Drosophila* and Glis (Gli1, Gli2 and Gli3) in vertebrates. Phosphorylation by PKA (protein kinase A) and Shaggy/GSK3 (glycogen synthase kinase 3), proteolysis via the proteasome and cytoplasmic retention are three of the mechanisms that regulate Ci/Gli activity.

Sufu (Suppressor of fused) has been characterized in *Drosophila* as a negative regulator of the Hh pathway [1], and is normally found in a cytoplasmic tetrameric complex with the serine/threonine kinase Fused, the kinesin-like Costal-2 and Ci [2–4]. dSufu (*Drosophila* Sufu) is thought to control the nuclear distribution of Ci by tethering it in the cytoplasm [5]. A unique homologue of the *dSufu* gene has been described for several vertebrate species [6–8], which also seem to have a negative effect on Hh signalling. Sufu is known to interact physically with the Gli transcription factors [7–10]. When Sufu is overexpressed in cells, its negative effect on Hh target gene expression is exerted through inhibition of the transcriptional activities of Gli1 and Gli2. This effect is correlated with an increase in Gli1 immunostaining in the cytoplasm [8,11] although no modification of the nuclear level of Gli1 has been observed [9]. Moreover, the interaction between Sufu and Gli1 does not compromise the ability of Gli1 to associate with DNA [8] and chick Sufu protein has even been shown to enhance DNA binding by Gli1 and Gli3 [7]. Thus the role of Sufu in the regulation of the Hh pathway probably involves both cytoplasmic and nuclear effects on the Gli transcription factors. Moreover, another study demonstrates that mSufu (mouse Sufu) is also involved in Wnt signalling in mouse where it functions as a tumour suppressor [12]. Finally, mutations in hSufu (human Sufu) predispose to medulloblastoma [13].

In the present study, we characterized mSufu biochemically and examined the protein to detect any possible post-translational modification, such as phosphorylation. We also used a yeast two-hybrid approach to identify possible mSufu interacting proteins. Some of the identified proteins, such as CBF1 (CCAAT-binding

Abbreviations used: AD, activation domain; CBF1, CCAAT-binding factor 1; Ci, Cubitus interruptus; CBP, cAMP-response-element-binding protein; dpc, day post-coïtum; Sufu, Suppressor of fused; dSufu, *Drosophila* Sufu; mSufu, mouse Sufu; GFP, green fluorescent protein; GSK, glycogen synthase kinase; GST, glutathione S-transferase; Hh, Hedgehog; HMGiy, high mobility group; hnRNP, heterogeneous nuclear ribonucleoprotein; LMB, leptomycin B; MALDI, matrix-assisted laser-desorption ionization; NES, nuclear export signal; pCIP, p300/CBP-co-integrator protein; PIAS1, protein inhibitor of activated STAT1; PKA, protein kinase A; SAP18, Sin3-associated polypeptide 18; STAT, signal transduction and activators of transcription; SUMO, small ubiquitin-related modifier; X-Gal, 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside.

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factor 1), are involved in the basal transcriptional regulation machinery. Others, namely SAP18 (Sin3-associated polypeptide 18), pCIP [p300/CBP-co-integrator protein, where p300 is a transcriptional activator protein required to drive p53 expression and CBP stands for cAMP-response-element-binding protein (CREB)-binding protein] and PIAS1 [protein inhibitor of activated STAT1 (signal transduction and activators of transcription)], are associated with transcriptional co-regulator complexes, and Galectin3 and hnRNP (heterogeneous nuclear ribonucleoprotein) A1 are implicated in pre-mRNA splicing. We confirmed the existence of mSufu–SAP18 and mSufu–Galectin3 interactions using independent methods and we demonstrated that mSufu is a nucleocytoplasmic shuttle protein with a predominantly cytoplasmic localization. An NES (nuclear export signal) was shown to be required for nuclear export of mSufu. Moreover, the intracellular localization of mSufu and some of its partners can be modified through their co-expression.

EXPERIMENTAL

Antibodies and immunodepletion of anti-Sufu antibody

A rabbit polyclonal antiserum was raised against residues 13–484 of mSufu. Recombinant mSufu cDNA was used to generate a GST (glutathione S-transferase)–Sufu-fusion construct and the GST-fusion protein was used by BioAtlantic SARL (Nantes, France) to raise a rabbit polyclonal antibody using their standard procedures. Specific anti-Sufu antibody was purified by affinity chromatography using GST–Sufu immobilized on nitrocellulose filters and eluted by 0.2 M glycine (pH 2.8) and 1 mM EGTA [14]. Anti-Xpress (Invitrogen, Carlsbad, CA, U.S.A.) is a mouse monoclonal antibody directed against the N-terminal tag Xpress encoded by pcDNA3.1/HisC (Invitrogen). Anti-GFP (green fluorescent protein; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) is a rabbit polyclonal antibody. Anti-α tubulin is a mouse monoclonal antibody (DSHB, Iowa City, IA, U.S.A.). Anti-lamin B is a goat polyclonal antibody (Santa Cruz Biotechnology). For immunodepletion, the purified anti-Sufu antibody (1:500) was incubated for 1 h at 4 °C with 56 μg of mSufu peptide (amino acids 13–484).

Protein extracts, Western blotting, co-immunoprecipitations and GST pull-down

Total protein extracts were prepared from 8.5 to 19.5 dpc (day post-coïtum) whole mouse embryos (strain Swiss Webster/NIH, Bethesda, MD, U.S.A.). Gravid females were anaesthetized and dissected at 4 °C and embryos were crushed with a Dounce homogenizer in detergent buffer [50 mM Tris, pH 8/150 mM NaCl/0.1 % (w/v) SDS/1 mM PMSF/1 μg/ml aprotinin/1 μg/ml leupeptin/10 μg/ml pepstatin]. Lysates were centrifuged at 12 000 ***g*** for 10 min at 4 °C. Protein concentration of each supernatant was determined using the Bradford reagent system (Bio-Rad Life Science Group, Marnes-La-Coquette, France). Aliquots of 100 μg of proteins were checked with 5× loading buffer [62.5 mM Tris, pH 6.8/10 % (v/v) glycerol/2 % SDS/0.7 M 2-mercaptoethanol/0.1 % Bromophenol Blue], separated by 8 or 10 % one-dimensional PAGE [15] and electrotransferred on to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) [16]. The blots were saturated in TBS-T [20 mM Tris, pH 8/137 mM NaCl/0.1 % (v/v) Tween 20], containing 5 % (w/v) low-fat milk. Western-blot analysis was performed with purified anti-Sufu or immunodepleted anti-Sufu at a dilution of 1:500 in 5 % (w/v) BSA–TBS-T overnight at 4 °C, followed by a secondary anti-rabbit horseradish-peroxidase-conjugated antibody (Vector) at a dilution of 1:10 000. ECL® (enhanced chemiluminescence) detection (Amersham Biosciences U.K. Limited, England) was performed according to the manufacturer's instructions for 30 s to 15 min.

Co-immunoprecipitations were performed using an anti-GFP rabbit polyclonal antibody (Santa Cruz Biotechnology) or anti-Xpress mouse monoclonal antibody (Invitrogen) coupled with Protein A–Sepharose and Protein G–Sepharose (Sigma) respectively in immunoprecipitation buffer [50 mM Tris, pH 8/150 mM NaCl/2.5 mM $MgCl_2$/1 mM EDTA] at 4 °C for 2 h.

Transfected cells were lysed 48 h after transfection in immunoprecipitation buffer with detergent [1 % (v/v) Triton X-100], protease inhibitors (aprotinin, leupeptin, pepstatin and PMSF, see above) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM β-glycerophosphate and 0.1 mM sodium orthovanadate). Lysates (200 μg of proteins) were incubated, for 1 h, with Sepharose beads (Sigma) to preclear the protein extract. Then, precleared lysates were incubated overnight with antibodies coupled to Sepharose beads. The pellets were washed three times with immunoprecipitation buffer and the immunoprecipitates were run on 7.5–10 % one-dimensional PAGE followed by electrotransfer on to a nitrocellulose membrane (Schleicher and Schuell). Filters were then treated, incubated and revealed as described above. The same membranes were stripped to incubate with anti-Xpress mouse monoclonal antibody (Invitrogen) and then with an anti-mouse secondary antibody conjugated with peroxidase (Jackson ImmunoResearch, Stratech Scientific Ltd., Soham, U.K.) used at a dilution of 1:10 000 and revealed with the ECL® detection system.

GST pull-down assay was performed using GST–Sufu-fusion protein as described previously [10]. ^{35}S-labelled Galectin3 was produced *in vitro* with TNT®-coupled reticulocyte lysate systems (Promega, Madison, WI, U.S.A.).

Two-dimensional-PAGE and MS analysis

Samples containing 300 μg of total proteins were separated by two-dimensional PAGE as described previously [17] except that ampholines 5–7 (Amersham Biosciences) were used in the isoelectric focusing dimension. A Coomassie Blue stain was used to detect proteins. Western-blot analysis was performed as described above.

Spots from two-dimensional PAGE or regions corresponding to the Sufu signal were excised from the gel, reduced, alkylated and digested in-gel with trypsin. Digest aliquots were removed from eight gels, pooled and subjected to a desalting/concentration step on μZTipχ18 (Millipore, Bedford, MA, U.S.A.) before MALDI (matrix-assisted laser-desorption ionization)-MS analysis. Peptide mixtures were analysed by using Voyager-DE PRO mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.). ProFound software package was used to identify spots from independent non-redundant sequence databases. Candidates from peptide-matching analysis were evaluated further by comparing their calculated molecular mass and pI.

De-phosphorylation assay

Protein extracts from 12.5 dpc embryos (150 μg) were treated at 37 °C with 3.75 units of alkaline phosphatase type III (Sigma) for 48 h, in a phosphatase buffer (1 M Tris, pH 8.5/1 mM PMSF/1 μg/ml aprotinin/1 μg/ml leupeptin/10 μg/ml pepstatin) with or without phosphatase inhibitors (100 mM sodium fluoride, 10 mM β-glycerophosphate and 0.1 mM sodium orthovanadate). The enzyme was inactivated by addition of phosphatase inhibitors. Each sample, corresponding to 150 μg of proteins, was then

treated as follows: 5× loading buffer was added to 50 μg of proteins, which were boiled and run on one-dimensional PAGE; the remainder of the sample (100 μg) was treated to perform two-dimensional PAGE separation. Western-blot analysis with anti-Sufu antibody was then performed.

Yeast two-hybrid library screening and interaction assay

Bait plasmid for yeast two-hybrid screening (LexA-Sufu) was constructed by subcloning the m*Sufu* gene encoding the region from amino acids 13 to 484 in-frame with the LexA DNA-binding domain encoded by the yeast vector pEG202 (as described in [6]). All experiments were performed using the *Saccharomyces cerevisiae* reporter strain RFY231 [*Mat* α, *his*3, *trp*1, *ura*3, LexAop(×6)-*LEU*2]. This strain was successively transfected by *LacZ* reporter plasmid pSH18-34 (provided by R. Brent, The Molecular Sciences Institute, Berkeley, CA, U.S.A.), the bait plasmid expressing LexA-Sufu and pB42AD plasmid expressing the AD (activation domain) of *Escherichia coli* B42 polypeptide fused to proteins corresponding to a *Mus musculus* embryonic fibroblast cDNA library (LexA Matchmaker library; ClonTech Laboratories, Palo Alto, CA, U.S.A.). All transfections were performed using the lithium acetate procedure as described by the manufacturer. Approx. 10^7 clones were screened. Co-transfectants were plated on synthetic galactose/raffinose induction medium lacking uracil, histidine, tryptophan and leucine. Positive LEU2 clones were tested for *LacZ* reporter gene activity on synthetic galactose/raffinose induction medium lacking uracil, histidine and tryptophan and with an overlayer of X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) solution [0.25 M Na_2HPO_4, pH 7.5/0.5 % (w/v) Bactoagar/0.1 % SDS/7 % (v/v) dimethylformamide/0.04 % (w/v) X-Gal] and incubated at 30 °C for 1 h. PCR amplification was performed on each blue isolated clone by using primers located on both sides of the insert (5′-CTCTTGCTGAGTGGAGATGCC-3′ and 5′-GACTTGACCAAACCTCTGGCG-3′). The clones with an insert smaller than 0.6 kb were eliminated because they may not probably include more than the 3′-untranslated region and the polyadenylated tail. Plasmids were isolated from β-galactosidase-positive colonies, introduced into the *E. coli* KC8 strain by electroporation and transformed clones were selected for tryptophan prototrophy on M8 medium minus tryptophan. Interacting cDNA clones were sequenced with the primers mentioned above.

The specificity of the interactions was tested by mating the *S. cerevisiae* strain RFY206 [*Mat* a, *his*3, *trp*1, *ura*3, LexAop(×6)-*LEU*2] transfected by preys in pB42AD or pJG45 constructs, with the RFY231 yeast strain transfected by Sufu (mSufu or dSufu) or mSufu-deleted mutants (amino acids 13–73, 13–109 and 13–325, described in [10]) into pEG202 or other preys (SAP18, PIAS1, Bifurcated1, Galectin3, Rnf19, ZNF219 and pCIP) previously excised from pB42AD and cloned into pEG202.

Cell culture and transfections

The mSufu-coding region from amino acids 2 to 484 was amplified by PCR and inserted in-frame with GFP into the pEGFPC2 vector (ClonTech Laboratories). Mutagenesis in mSufu of leucine codons 383 and 385 into alanine was performed by the Quik Change site-directed mutagenesis kit (Stratagene) using mutagenic primers 5′-GCCCTCATCCCTGCCTGCGCAAGGGGCAGACTC-3′ and 5′-GAGTCTGCCCCTTGCGCAGGCAGGGATGAGGGC-3′.

Two of the mSufu partners isolated from the two-hybrid screen, Galectin3 and SAP18, were excised by *Eco*RI/*Xho*I digestion from pB42AD and inserted into the mammalian expression vector pcDNA3.1/HisC (Invitrogen), which adds an N-terminal His_6/Xpress tag to the coding sequence. Human HeLa cells were cultured routinely in RPMI 1640 medium supplemented with 5 % (v/v) foetal bovine serum (Perbio Science, Bezons Cedex, France) and antibiotics at 37 °C in 5 % CO_2. The day before transfection, 5×10^5 freshly passaged cells were seeded on to glass coverslips in sterile cell culture 6-well plates (Costar, Bethesda, MD, U.S.A.). Transfection experiments were performed with 1–2 μg DNA added to FuGENE 6 reagent (Roche, Basel, Switzerland) prediluted in serum-free RPMI 1640 medium as indicated by the manufacturer. The medium was replaced, 3 h after transfection, by a medium containing 5 % foetal bovine serum and antibiotics. Cells were lysed or fixed 48 h after transfection. LMB (leptomycin B), generously provided by Dr M. Yoshida (University of Tokyo, Japan), was added at a final concentration of 50 ng/ml for 3 h before fixation.

Cell fractionation experiments

HeLa cells were transfected with Sufu, $SufuNES^{mut}$ or pEGFP vector. To isolate nuclear and cytoplasmic fractions, cells were washed twice with cold PBS and resuspended in buffer A (10 mM Hepes, pH 8.0/1.5 mM $MgCl_2$/10 mM KCl/1 mM dithiothreitol) plus 1× complete protease inhibitor cocktail (Roche). After incubation for 20 min at 4 °C, cells were gently lysed by 20 strokes in a Dounce homogenizer and centrifuged at 110 ***g*** for 10 min. After recovery, the supernatants representing cytoplasmic fractions, the nuclei were resuspended in buffer A, layered over buffer A plus 30 % sucrose and pelleted by centrifugation at 420 ***g*** for 10 min. The nuclei were then washed with buffer A and lysed with buffer C (10 mM Hepes, pH 8.0/25 % glycerol/420 mM NaCl/1.5 mM $MgCl_2$/0.4 mM EDTA/0.5 mM dithiothreitol) plus 1× complete protease inhibitor cocktail (Roche). Cytosolic and nuclear fractions were separated by SDS/PAGE and analysed by Western immunoblot using Sufu (1:750; Santa Cruz Biotechnology), tubulin (DSHB, 1:1000) and lamin B (1:1000; Santa Cruz Biotechnology) antibodies. Images were captured using a ChemiDoc (Bio-Rad Life Science Group) and bands were semi-quantified using the Quantity One Quantification software (Bio-Rad Life Science Group).

Immunofluorescence experiments and subcellular localization

Subconfluent-transfected HeLa cells, grown on glass coverslips, were fixed 48 h after transfection by 4 % (w/v) paraformaldehyde in PBS for 15 min at room temperature (23 °C) and washed twice with PBS. Non-specific binding sites were blocked with 5 % (v/v) normal goat serum, 1 % BSA and 0.5 % Triton X-100 in PBS for 30 min. Cells were incubated overnight with primary mouse monoclonal antibody anti-Xpress (Invitrogen) at a dilution of 1:200 in 2 % normal goat serum, 1 % BSA, 0.1 % Tween 20 and 1× PBS. Cells were washed five times in 0.1 % Tween 20 and 1× PBS. Diluted (1:200 in 0.1 % Tween 20, 1× PBS) CyTM3 fluorochrome-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) was then added for 1 h. Cells were washed in 0.1 % Tween 20, 1× PBS for 2 h and then mounted in CitiFluor (CitiFluor, London, U.K.), and observed on a Leica optical microscope using the ×63 lens. We used cells stained only with secondary antibody as controls.

For protein localization studies, 45–201 transfected cells were analysed by two or three independent experiments. A standard χ^2 test was performed for data analysis.

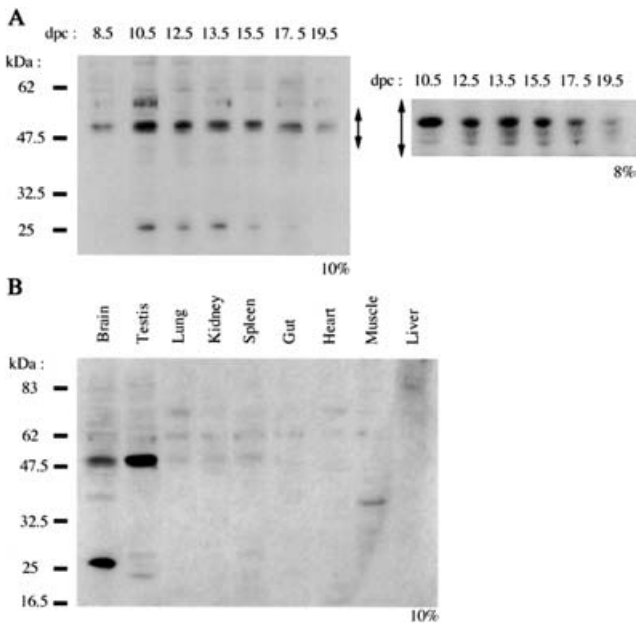

Figure 1 Expression of different mSufu forms

mSufu distribution in embryos (**A**) and various adult tissues (**B**) by Western-blot analysis using an anti-Sufu antibody. Samples of total proteins (100 μg), isolated from mouse embryos ranging between 8.5 and 19.5 dpc or from adult tissues, were separated on 8 % (right panel) and 10 % (left panel) one-dimensional PAGE. Molecular masses are indicated on the left.

RESULTS

mSufu expression during mouse development and in adult tissues

We used Western-blot analysis to determine the expression of mSufu protein in mouse both during development and in the adult. As shown in Figure 1(A), mSufu expression is very dynamic during development. Several polypeptides reacted with the purified antibody directed against the mSufu protein of 484 amino acids (= 54 kDa). Three forms exhibit an apparent molecular mass comprised between 50 and 54 kDa (Figure 1A, right panel) and the fourth a molecular mass of approx. 30 kDa (Figure 1A, left panel). Present at low levels at 8.5 dpc, these different forms are strongly expressed by 10.5 dpc and then progressively decrease to 19.5 dpc.

In adult tissues, the 54 kDa isoform of mSufu is present at high levels in testis and brain, at lower levels in lung, kidney and spleen and was not detected in the other tissues tested (Figure 1B). Surprisingly, the most representative mSufu variant in brain is the shorter 30 kDa form. In muscle, one band, approx. 40 kDa, is presumably actin (see two-dimensional PAGE). Our results indicate that various mSufu proteins are present in both embryos and adult organs. The different relative amounts of these various forms in different tissues suggest tissue specialization of mSufu function.

Biochemical characterization of mSufu

Since the 54 kDa form of mSufu protein predominates during development, we decided to focus our analysis on this form. We separated proteins using two-dimensional PAGE. For this analysis, we used protein extracts from 12.5 dpc embryos because mSufu is strongly expressed at this stage. Figure 2(A) shows a typical two-dimensional separation pattern of mSufu variants after immunodetection with anti-Sufu antibody. The various mSufu forms, approx. 50–54 kDa, were resolved into three major spots at a pI ranging from approx. 5.8 to 5.5. This horizontal trail of spots suggests changes of protein mSufu charge. These spots disappeared on a duplicated immunoblot using immuno-depleted anti-Sufu antibody (Figure 2B), whereas actin and α-tubulin (identified by MALDI-MS) appeared as background proteins because of the long time exposure. On a Coomassie Blue-stained gel (Figure 2C), no spots were visible in the region where mSufu is immunodetected (see rectangle) suggesting that mSufu is present in very small amounts in the total embryonic extracts. Consequently, mSufu mass spectrometric identification was unsuccessful.

Phosphorylation of mSufu

Since both mammalian and fly Sufu proteins physically interact with the Fused kinase and the primary sequence of mSufu has many putative phosphorylation sites, we tested whether the different 50–54 kDa forms of mSufu arise due to the addition of phosphate residues. Therefore we performed a de-phosphorylation assay on 12.5 dpc mouse embryonic protein extracts in the presence or absence of phosphatase inhibitors. As shown by one-dimensional PAGE and Western-blot analysis (Figure 3A), alkaline phosphatase treatment for 48 h drastically reduced the amount of the 54 kDa mSufu band (lane 6 versus lane 3). In contrast, in the presence of phosphatase inhibitors, the alkaline phosphatase had no effect on this form of mSufu (lane 4 versus lane 6). Interestingly, two-dimensional-PAGE analysis

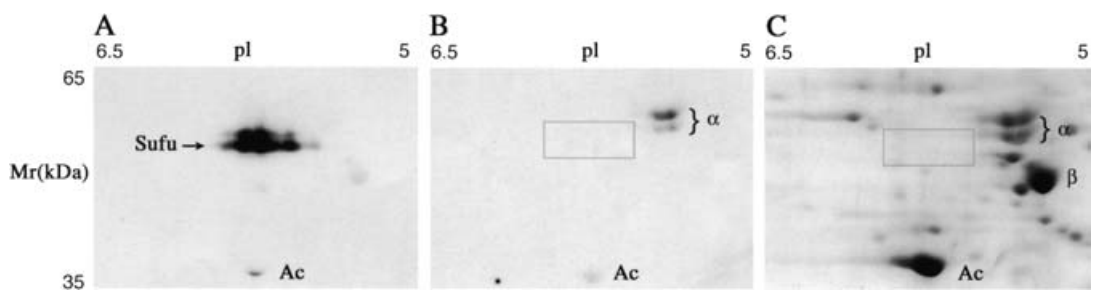

Figure 2 Two-dimensional-PAGE analysis of mSufu from 12.5 dpc embryonic extracts

(**A**) Immunoblot of total protein extracts (300 μg) stained with anti-Sufu antibody. Three major spots in the range of 50–54 kDa are resolved with a pI between 5.5 and 5.8. (film exposure time, 30 s). (**B**) Duplicate immunoblot using immuno-depleted anti-Sufu antibody. α-Tubulin and actin appear as background proteins (exposure time, 5 min). (**C**) Coomassie Blue-stained two-dimensional gel of total protein extracts. No spots were visible in the region of mSufu protein (grey rectangle) indicating that a very small amount of this protein is present in total embryonic extracts. Highly expressed proteins were subjected to in-gel digestion and identified by MALDI-MS as α, α-tubulin; β, β-tubulin; Ac, actin.

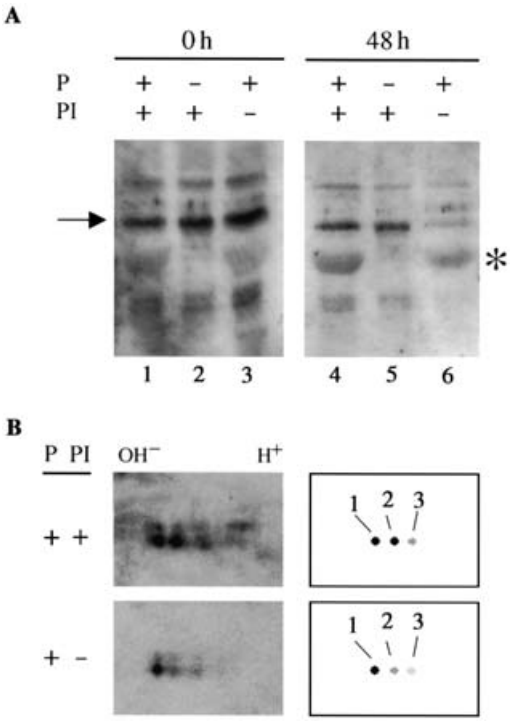

Figure 3 Analysis of phosphorylation of mSufu

(**A**) Total protein extracts (150 μg) from 12.5 dpc embryos were treated with alkaline phosphatase (P) (lanes 3 and 6), phosphatase inhibitors (PI) (lanes 2 and 5) or both (lanes 1 and 4) for 48 h at 37 °C. As controls, protein extracts were treated in the same manner but treatment was immediately arrested. Only 50 μg of each sample was separated on one-dimensional PAGE. The arrow indicates the 50–54 kDa mSufu forms and *, the position of the phosphatase. (**B**) The same extracts (100 μg), treated with alkaline phosphatase or with alkaline phosphatase and phosphatase inhibitors for 48 h at 37 °C, were separated on two-dimensional PAGE. Spots 2 and 3 decreased in intensity without modification of spot 1 as indicated by the scheme.

of the same samples (Figure 3B) showed changes in the relative proportions of the various mSufu forms after phosphatase treatment: spots 2 and 3 decreased in intensity (Figure 3B, lower panel). Thus, these results, observed in one- and two-dimensional PAGE, suggest that mSufu is phosphorylated. Moreover, the modifications observed in two-dimensional PAGE without an enrichment of spot 1 (Figure 3B, lower panel) suggest a specific degradation of de-phosphorylated forms. Alternatively, some de-phosphorylated epitopes of mSufu are perhaps no longer recognized by the antibody.

Identification of several partners of mSufu

To find new partners for mSufu protein in mouse, and with an aim to isolate the mouse homologue of the *dfu* (*Drosophila fused*) gene, we screened a mouse embryonic fibroblast cDNA library with the fusion protein LexA–Sufu as bait using the two-hybrid system in yeast. From 10^7 independent clones, 150 positive clones were isolated, based on their ability to activate the reporter genes that allow yeast to grow on leucine-deficient medium and produce blue staining in the presence of X-Gal substrate. A specificity assay was performed. A total of 69 clones reproducibly interacted with mSufu but not with control baits (results not shown). Among them, 30 clones, encoding 13 different proteins, were kept to go further into their analysis because of their redundancy in our screen and their common nuclear function (Table 1). In our screen, we did not find any proteins known to interact with mSufu, such as Gli transcription factors or the Fused kinase, but we found probable new mSufu partners: most of them have been suggested to be involved in basal transcription machinery such as CBF1, histone macroH2A, ZNF219, HMGiy (high mobility group) or Rnf19, to play a role in regulating gene transcription such as SAP18, pCIP, MP1, PIAS1, Bifurcated1 [restricted to SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain], or to be involved in pre-mRNA splicing such as Galectin3 and hnRNP A1.

To determine which domain of mSufu interacts with its partners, we used a series of deleted forms of mSufu (Figure 4). The region from amino acids 13 to 73, in the N-terminal half of mSufu, bound only Galectin3. The domain from amino acids 13 to 325 was sufficient to interact with most other partners including human Gli (HGli1 and HGli3). In contrast, full-length mSufu was necessary to bind histone H2A, SAP18 and HMGiy. Lastly, mSufu (amino acids 13–325) associated with itself (amino acids 13–484) and with its *Drosophila* homologue.

Table 1 Identification of potential partners of Sufu in mouse

Thirty positive clones that activated both the *LEU2* and the *LacZ* reporter genes are listed. DNA sequences selected from the *Mus musculus* embryo cDNA library (3T3 fibroblast) were identified by their homology with related genes in the GenBank database, and the accession number of the related gene was reported. 'Occurrence' indicates the number of related clones identified in this screen. 'Domains of proteins' indicates the region of the putative protein partner encoded by positive clones in frame with B42AD. For each protein, different clones were obtained and are noted in parentheses. 'References' are the citations that refer to the first identification of related genes or their homologues in mammals. aa, amino acid.

Identity of the putative protein partners	Accession no.	Occurrence	Domains of proteins	Reference
CBF1	GI:790574	3	From aa 775 (or aa 797, 886) to the end (aa 997)	[18]
Galectin3 (Mac-2)	GI:52986	1	From aa 1 to the end (aa 264)	[19]
SAP18	GI:13879301	6	From aa 1 to the end (aa 153)	[20]
pCIP (NcoA3, nuclear receptor co-activator protein 3)	GI:6679025	1	From aa 17 to 989	[21]
RNP A1 (Hnrpa1; Fli-2)	GI:6754219	4	From aa 17 (or aa 27) to the end (aa 320)	[22]
Ring finger protein 19 (Rnf19; GEG-154, XY body protein)	GI: 7305436	1	From aa 490 to the end (aa 840), containing C3HC4 domains	[23]
Bifurcated 1 (ets-related gene-associated protein; SET domain)	GI:9256534	4	From aa 1002 (or aa 1072, 1099, 1119) to the end (aa 1307), containing SET domain	[24]
Krüppel-related zinc finger (ZNF219)	GI:14549185	1	From aa 430 to the end (aa 726), containing C2H2 zinc finger	[25]
PIAS1	GI:3643104	1	From aa 81 to the end (aa 651), containing Ring domain	[26]
Histone macroH2A1.2	GI:15799252	3	From aa 15 to the end (aa 372)	[27]
HMGiy (AT hook)	GI:14531289	1	From aa 1 to the end (aa 106)	[28]
Peroxiredoxin 5 (antioxidant protein 2)	GI:6671548	2	From aa 1 to the end (aa 223), containing Ahpc/TSA	[29]
MEK partner 1 (MP1)	GI:3549604	1	From aa 1 to 34 in frame with the end from aa 79 to the end (aa 124)	[30]

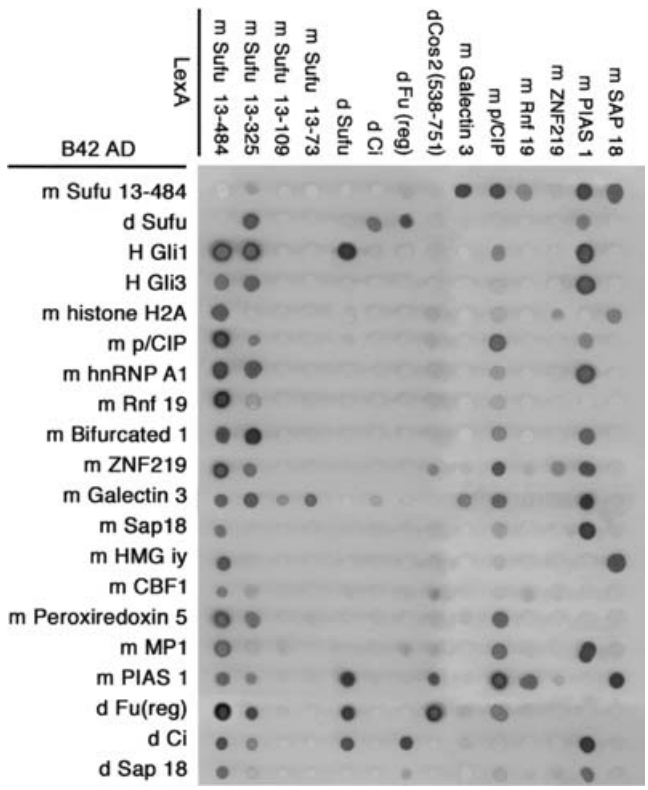

Figure 4 Yeast two-hybrid assay (β-galactosidase reporter expression)

Direct specific interactions between mSufu and its partners. Each dot corresponds to a diploïd yeast co-expressing the LexA DNA-binding domain fused to the protein (or protein domain) named on the top of the panel, and the B42 activation domain (B42AD) fused to the protein named on the left of the panel. The longest clone encoding each mSufu partner was tested. The nomenclature and accession number for each protein are indicated in Table 1. Physical interaction between two proteins was considered to occur when the LacZ reporter was activated and revealed after overlaid X-Gal mix by blue cells. m-protein, *Mus musculus* proteins identified in the two-hybrid screen (except for mSufu); d-protein, *Drosophila melanogaster* proteins; H-protein, human proteins.

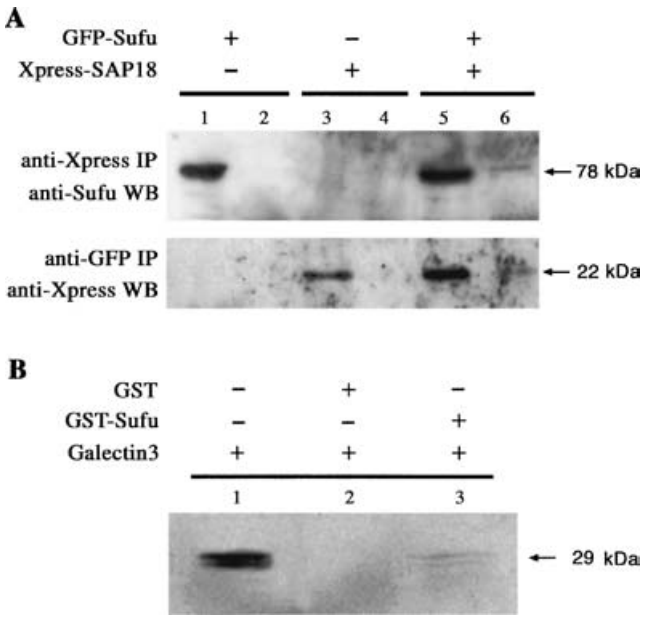

Figure 5 Physical interactions between mSufu and SAP18 or mSufu and Galectin3

(**A**) Association of mSufu with SAP18 in mammalian HeLa cells analysed by co-immunoprecipitation. Cells were transfected with GFP-tagged mSufu (lanes 1 and 2), Xpress-tagged SAP18 (lanes 3 and 4) or both (lanes 5 and 6). Total cell lysates (lanes 1, 3 and 5) and pellets from immunoprecipitation (lanes 2, 4 and 6) were separated by one-dimensional PAGE, which was followed by Western-blot analysis. GFP–Sufu migrated as a 78 kDa protein (IP, immunoprecipitation; WB, Western-blot analysis). (**B**) *In vitro* association of mSufu with Galectin3 by GST pull-down assay. *In vitro*-translated ^{35}S-labelled Galectin3 input was incubated with recombinant GST alone (lane 2) or GST–Sufu (lane 3) immobilized on glutathione–Sepharose beads. The pellet obtained from interaction between radiolabelled protein and recombinant protein, bound on beads, was separated by one-dimensional PAGE and observed by autoradiography. The ^{35}S-labelled Galectin3 input (100 %) was loaded on to lane 1.

mSufu is involved in new protein complexes

Because many of the potential mSufu partners are involved in different transcriptional co-regulator complexes, we tested for their possible interactions with one another by the two-hybrid assay. Many potential interactions were revealed (Figure 4), e.g. (i) SAP18, the protein most frequently found in our screen and which is a component of the Sin3/SMRT (silencing mediator for retinoic acid receptor and thyroid hormone receptor) nuclear receptor co-repressor, is able to bind histone macroH2, HMGiy and PIAS1; (ii) pCIP interacts with HGli1, ZNF219, Galectin3 and PIAS1; and (iii) Galectin3 also binds pCIP and PIAS1. Finally, in our test, Galectin3 (as demonstrated previously), pCIP and ZNF219 interact with themselves.

To test conservation of these interactions during evolution, we also tested the mouse proteins with some of the *Drosophila* proteins involved in Hh signalling. It is interesting to note that none of the mSufu partners was able to interact with the dSufu homologue except HGli1 and PIAS1 (Figure 4). PIAS1 interacted with many other proteins in our assay. In particular, it was able to bind fly and mammalian proteins, such as Sufu (both dSufu and mSufu), human Gli (HGli1, HGli3), *Drosophila* Ci (Ci) and *Drosophila* Costal2 (Cos2, amino acids 538–751), which are all known to be involved in the Hh pathway. Galectin3 interacted weakly with Ci and with the regulatory domain of *Drosophila* Fused [dFu(reg)]. We also tested whether interaction between Sufu and SAP18 is conserved in fly. Surprisingly, dSufu does not bind dSAP18. Since both mouse and fly SAP18 (mSAP18 and dSAP18) are able to interact with mSufu and mPIAS1, we supposed that some SAP18 domains are conserved during evolution.

The two-hybrid screen, combined with the specificity assay, shows that mSufu can bind some of the nuclear proteins involved in transcription regulation and that most of these partners, except PIAS1, are specific to mSufu protein.

mSufu binds Galectin3 and SAP18 directly

To investigate further two of these interactions, mSufu–SAP18 and mSufu–Galectin3, we used complementary interaction assays *in vitro*. We confirmed the mSufu–SAP18 interaction by a co-immunoprecipitation assay from lysates of HeLa cells expressing one (Figure 5A, lanes 1 and 3) or both proteins (lane 5). The 78 kDa GFP–Sufu co-precipitated with Xpress-SAP18 when Xpress-SAP18 was immunoprecipitated with an anti-Xpress antibody (upper panel, lane 6 versus lane 2). Reciprocally, Xpress-SAP18 (22 kDa) was also present with GFP–Sufu immunoprecipitated with anti-GFP antibody (lower panel, lane 6 versus lane 4).

A GST pull-down assay was performed to confirm the mSufu–Galectin3 interaction. As shown in Figure 5(B), the GST–Sufu-fusion protein and radiolabelled Galectin3 (lane 3) revealed a direct specific interaction between these two proteins, compared

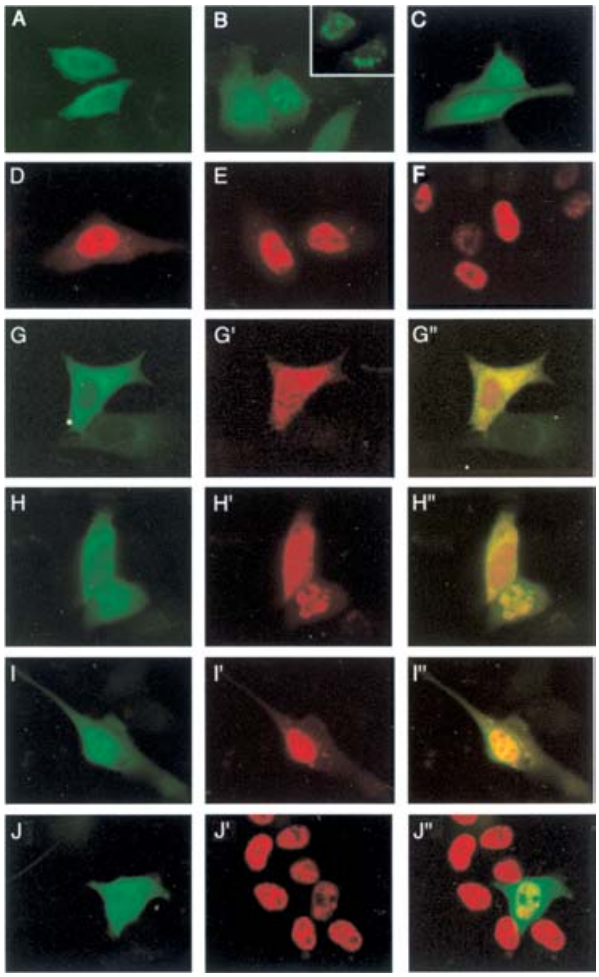

Figure 6 Nucleocytoplasmic distribution of mSufu and its partners

Wild-type and mutated mSufu, Galectin3 and SAP18 were overexpressed in transiently transfected HeLa cells. The most representative subcellular localizations of the different expressed proteins are shown. (**A**–**F**) HeLa cells were singly transfected. (**A**) mSufu was mostly located in the cytoplasm. (**B**) SufuNES^{mut} became constitutively nuclear [Note the different staining patterns observed for this mutated mSufu protein, uniform or punctuate (inset) nuclear labelling]. (**C**) mSufu accumulated in the nucleus when nuclear export was inhibited by the addition of LMB. Galectin3 was mostly located in the nucleus in untreated cells (**D**) or cells treated (**E**) by LMB. (**F**) SAP18 was localized exclusively in the nucleus. Cells were co-transfected with Galectin3 and mSufu (**G** and **I**) or mSufuNES^{mut} (**H**). Galectin3 could accumulate in the cytoplasm in cells co-expressing mSufu (**G′** versus **D**) or SufuNES^{mut} (**H′** versus **B**). LMB treatment restored a high level of Galectin3 in the nucleus (**I′**) and mSufu was also found in this compartment (**I**). The merged views (**G′′**, **H′′**, **I′′**) show co-localization of the two overexpressed proteins. (**J**, **J′**, **J′′**) Cells were co-transfected with GFP–Sufu and SAP18. mSufu was translocated in the nucleus (**J** versus **A**) where it co-localized with SAP18 (**J′**, **J′′**). mSufu was detected by direct GFP fluorescence (**A**–**C**, **G**–**J**). Galectin3 (**D**, **E**, **G′**, **H′** and **I′**) and SAP18 (**F** and **J′**) were revealed by immunofluorescence.

with the control performed with GST protein alone (lane 2). Therefore these assays demonstrate that mSufu interacts directly with Galectin3 and SAP18.

mSufu protein contains a functional NES

Since mSufu bound some nuclear proteins, we tested the subcellular distribution of the 54 kDa mSufu protein in cultured cells. An *mSufu* cDNA encoding the 484-amino-acid variant was cloned into an eukaryotic GFP-expression vector and was then transfected transiently into HeLa cells. GFP–Sufu was largely confined to the cytoplasm (Figure 6A), 84 % of transfected cells showing this cytoplasmic staining ($n = 201$). *mSufu* exon

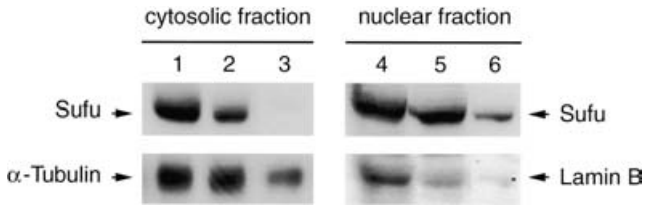

Figure 7 Subcellular localization of wild-type or mutated Sufu

Western-blot analysis with anti-Sufu of cytoplasmic and nuclear proteins from HeLa cells transfected with wild-type Sufu untreated (lanes 1 and 4) or treated with LMB (24 h; lanes 3 and 6) and SufuNES^{mut} (lanes 2 and 5). Sufu signal (lane 3) appeared after a long exposure. Western-blot analysis with α-tubulin and lamin B antibodies is shown as a loading control (lower panels). No signal was observed with α-tubulin and lamin B antibodies in nuclear and cytoplasmic fractions respectively (results not shown).

9 encodes a leucine-rich motif, which resembles the consensus sequence for an NES starting at amino acid 380 (LIPLCLRGRL versus LXXLXLXL). To demonstrate that this motif is involved in nuclear export of mSufu protein, we overexpressed a mutated mSufu protein in which the two critical leucine residues (amino acids at positions 383 and 385) were replaced by alanine residues. This protein with mutated NES (GFP-SufuNES^{mut}) was mostly detected in the nucleus in 62 % of stained cells ($n = 139$), with different distributions, uniform or punctuate (Figure 6B). To understand this specific behaviour, we observed some living cells expressing GFP-SufuNES^{mut} protein from 24 to 34 h post-transfection (time interval, 10 min). Surprisingly, these different distributions correspond to temporal changes of the mutated Sufu protein in stained cells. We observed a biphasic time course consisting of a first phase with a uniform staining both in nucleus and cytoplasm (as in Figure 6B) and a second phase with a punctuate staining confined to the nucleus (as in Figure 6B, inset). The second phase might be observed as soon as we started the dynamic observation with no change during all the analysis. In contrast, the signal corresponding to wild-type GFP–Sufu had remained uniform in the cytoplasm during the entire dynamic study (as in Figure 6A) or alternatively, it was partitioned between nucleus and cytoplasm (E. Petit, A. Troullier and M.F. Blanchet-Tournier, unpublished work).

To determine whether mSufu is predominantly cytoplasmic because of the activity of nuclear export sequences, we tested its subcellular localization in the presence of the antibiotic LMB, a specific inhibitor of CRM1-mediated nuclear export [31]. In the presence of LMB, GFP–Sufu was mostly nuclear in 88 % of stained cells ($n = 70$) (Figure 6C).

This microscopic analysis was followed by Western-blot analysis to detect Sufu protein levels in cytosolic and nuclear fractions (Figure 7). The purity of the fractions was determined by α-tubulin antibody as a marker of cytoplasmic fraction and Lamin B antibody as a marker of nuclear fractions. The results are consistent with the microscopic observations. Four independent experiments showed a 2-fold increase in the nuclear level of SufuNES^{mut} versus wild-type Sufu. LMB treatment led also to an increase in Sufu protein in the nuclear fraction (3.7-fold).

Taken together, these results demonstrate that mutation of a putative NES and blocking nuclear export by LMB lead to the accumulation of overexpressed Sufu in the nucleus, suggesting that endogenous Sufu shuttles between the nucleus and cytoplasm.

Subcellular co-localization of mSufu and its partners in HeLa cells

To explore mSufu function in relation to its newly discovered interactions with SAP18 and Galectin3, we analysed the

subcellular localization of these proteins in transiently co-transfected HeLa cells. We have described above that GFP–Sufu alone was predominantly located in the cytoplasm (Figure 6A) despite its transient nuclear localization (Figures 6B and 6C). In contrast, Xpress-tagged Galectin3 was predominantly located in the nucleus in 81 % of stained cells ($n = 68$; Figure 6D). After treatment of cells with LMB, Galectin3 was nuclear in 100 % of stained cells ($n = 107$; Figure 6E). Xpress-tagged SAP18 was exclusively nuclear in 100 % of transfected cells ($n = 82$; Figure 6F).

When Galectin3 was co-expressed with mSufu (Figures 6G, 6G′ and 6G″), nuclear Galectin3 levels decreased and the protein accumulated in the cytoplasm in 61 % of double-labelling cells ($n = 118$; Figure 6G′) where it co-localized with mSufu (Figure 6G″). Unexpectedly, a co-localization of Galectin3 and SufuNES^{mut} was found in the nucleus in only 41 % of cells ($n = 120$; Figures 6H, 6H′ and 6H″). This new result suggests that SufuNES^{mut} and Galectin3 are probably prevented from entering the nucleus. Moreover, the fact that both wild-type mSufu and mutated mSufu overexpression can decrease Galectin3 nuclear levels indicates that these two proteins have the ability to tether Galectin3 in the cytoplasm probably through association with other component(s). In contrast, LMB treatment restored a high level of Galectin3 staining in the nucleus of mSufu overexpressing cells (75 % of cells, $n = 126$) and mSufu is nuclear in only 63 % of these cells (Figures 6I, 6I′ and 6I″). The proportion of cells with nuclear accumulation of mSufu is significantly lower than that for cells expressing mSufu alone and treated with LMB (see above). These results indicate that LMB treatment can block the effect of mSufu on the cytoplasmic localization of Galectin3 and confirm that the nuclear export of mSufu is partially CRM1-dependent.

Finally, mSufu was shifted partially to the nucleus in 67 % of cells ($n = 45$; Figure 6J) when it was co-expressed with SAP18, which remained in this compartment (Figures 6J′ and 6J″). SufuNES^{mut} was more effectively found in the nucleus (96 % of cells, $n = 54$) when co-expressed with SAP18 (results not shown) than in cells expressing only SufuNES^{mut} (see above). Thus the nuclear import of wild-type mSufu and mutated mSufu seems to be dependent on SAP18.

Overall, these results are consistent with the physical interactions found between mSufu and Galectin3 or mSufu and SAP18, which suggest a functional co-operation between these proteins as a function of their subcellular localization.

DISCUSSION

In the present study, we found that the mouse *Sufu* gene encodes multiple protein isoforms. Previous work [11] had identified three different protein products encoded by the human *Sufu* gene: 433, 481 and 484 amino acids. Two additional splice variants encoding 359 and 388 amino acids respectively were found in human [32]. All these proteins share a common N-terminal region of 340 residues, corresponding to the first eight exons, and variable C-terminal regions. The three mSufu forms of approx. 50–54 kDa and the shorter one of approx. 30 kDa that we observed by one-dimensional PAGE suggest that the same alternative splice variants are produced from the different *mSufu* RNAs described in mouse [7–9]. The developmental expression profile of mSufu proteins is in accordance with *mSufu* gene expression [10]. Moreover, the presence of variable amounts of the different mSufu isoforms, in different adult tissues, might reflect specific mSufu function in multiple processes.

Our biochemical study in one-dimensional PAGE shows that mSufu protein band disappears after treatment with alkaline phosphatase, suggesting that mSufu is phosphorylated and that this modification probably prevents its degradation. Moreover, two-dimensional analysis shows a horizontal trail of mSufu spots probably owing to phosphorylation changes of the mSufu protein. We aim to purify a sufficient amount of mSufu protein to identify its modifications.

Although hSUFU interacts directly with the kinase human FUSED [33], no evidence has been reported so far regarding the phosphorylation of Sufu by this kinase, either in fly or vertebrates. Primary sequence analysis of mSufu indicates three putative phosphorylation sites by PKA (starting at amino acids 125, 298 and 343). The first is also a putative Shaggy (SGG/GSK3)-specific site (amino acids 124–128). We postulate that mSufu, repressor of Hh signalling, may be protected from degradation by addition of phosphate residues by PKA and/or GSK3, which also negatively regulate the Hh pathway [34]. Moreover, mSufu has a consensus sequence (LKRE; amino acids 124–127) for a SUMOylation (where SUMO stands for small ubiquitin-related modifier) [35]. It is interesting to stress that one of the mSufu partners, PIAS1, is known to act as a specific E3-like ligase that promotes SUMOylation of several proteins such as p53 [36]. Possible SUMOylation of mSufu could be performed by this factor.

Detected in both cytoplasm and nucleus, Sufu was assumed to be a nucleocytoplasmic shuttle protein [8,9]. Other results have suggested that the intracellular distribution of Sufu is regulated by CRM1-mediated nuclear export [12]. However, in NIH-3T3 cells, LMB treatment had no effect on the subcellular distribution of endogenous Sufu present mostly in the cytosolic fraction [36a]. Our results show that inhibition of the CRM1-dependent nuclear export pathway by LMB caused a shift in the distribution of Sufu in HeLa cells from predominantly cytoplasmic to nuclear and cytoplasmic, indicating that Sufu shuttles between both compartments. Moreover, the candidate export signal (amino acids 380–389) seems to be a functional NES that contributes to exclusion of mSufu from the nucleus. Interestingly, the nuclear accumulation of wild-type Sufu was also observed in response to the co-expression of partners of Sufu such as Galectin3 and SAP18. Thus the shuttle of Sufu is probably subject to the co-existence of partners that can modify the relative rates of Sufu in each cellular compartment. Additional works are now necessary to identify all import and export signals and other regulations involved in Sufu subcellular distribution. Finally, if the identified Sufu NES motif in mouse is not conserved in dSufu protein, it is interesting to note that a similar leucine-rich motif (amino acids 409–418) is present in dSufu suggesting a similar nuclear export of this protein in fly.

Using the yeast two-hybrid screening strategy, we have identified a plethora of potential partners for mSufu. Intriguingly, most of them exhibit nuclear function and are components of various co-regulator complexes involved in repression or activation of transcription. For example, SAP18 is involved in the SMRT/Sin3/HDAC (histone deacetylase) co-repressor complex. A recent biochemical study demonstrated that mSufu functionally co-operates with SAP18 to repress transcription by recruiting this SAP18–mSin3 complex to promoters containing the Gli-binding elements [37]. Using another approach, we also show a functional interaction between mSufu and SAP18. Our experiments in HeLa cells clearly indicate an increase in nuclear mSufu staining after co-expression of mSufu and SAP18 suggesting a direct nuclear repressive function for mSufu.

We also show that mSufu specifically binds pCIP, which belongs to the p160 family of nuclear receptor co-activators [38]. pCIP provides acetyl transferase activity for nuclear receptors in concert with other factors, resulting in the activation of target gene transcription. pCIP is able to interact with CBP and p300 cofactors. CBP itself is known to interact with Ci [39], Gli3 [40]

and β-catenin [41] to promote target gene activation. mSufu is also able to interact with two pCIP partners, namely PIAS1 and Galectin3. PIAS1, defined by an unusual RING-finger-like motif [42], binds STAT1 in response to interferon-stimulation, blocking the DNA-binding activity of STAT1 and thus inhibiting STAT1-mediated gene activation [26,43]. Moreover, mSufu specifically binds the SET domain of Bifurcated1. This SET motif, involved in protein–protein interaction, is usually found in proteins that harbour histone methyltransferase activity and function as either transcriptional activators or repressors [44]. Our findings suggest that mSufu is extremely versatile in its capacity to bind multiple proteins. Since several partners of mSufu interact with each other, we postulate that mSufu might contribute to the assembly of different multicomponent complexes as reported for the complex Gli1–Sufu–SAP18–mSin3–HDAC [37].

Galectin3, first isolated as a macrophage surface antigen (Mac-2), is a protein involved in many developmental processes such as cartilage formation and skin differentiation [45]. It is found in the cytoplasm or in the nucleus depending on its state of phosphorylation and depending on the cell cycle [46]. In the nucleus, Galectin3, associated with RNP complexes, acts as a pre-mRNA splicing factor [47]. It may also be exported from the nucleus to take part in the biogenesis of the small nuclear RNP complexes [48]. Another partner of mSufu, hnRNP A1, appears to bear a functional similarity to Galectin3. Since we observed a subcellular redistribution of Galectin3, which is translocated in the cytoplasm when co-expressed with mSufu, we postulate that mSufu might also be indirectly involved in global mRNA maturation.

The same redistribution of proteins, from the nucleus to cytoplasm, has been observed for Gli1 [8,11] and for β-catenin [12] when co-expressed with mSufu. Thus mSufu seems to be involved in the intracellular repartition of, at least, three of its partners: Gli1, mediator of Hh signalling, β-catenin, a key mediator of Wnt signalling and Galectin3, probably involved in Ihh (Indian hedgehog) regulation since Ihh is slightly up-regulated in a Galectin3 null mutant in mouse [49]. Together, these results indicate that mSufu, in each cellular compartment, emerges as an important effector in the regulation of the cellular response to Hh and Wnt signals.

In conclusion, our report shows that mSufu is a nucleocytoplasmic shuttle protein and suggests that it might contribute to the assembly of complexes involved in transcriptional gene regulation and other processes. It also reveals post-translational modifications of mSufu such as phosphorylation and possible SUMOylation. These modifications might modulate mSufu function by influencing mSufu stability or by changing the subcellular localization of the modified mSufu protein or its ability to interact with different ligands in each cellular compartment.

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