The serine/arginine-rich protein SF2/ASF regulates protein sumoylation

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Protein modification by conjugation of small ubiquitin-related modifier (SUMO) is involved in diverse biological functions, such as transcription regulation, subcellular partitioning, stress response, DNA damage repair, and chromatin remodeling. Here, we show that the serine/arginine-rich protein SF2/ASF, a factor involved in splicing regulation and other RNA metabolism-related processes, is a regulator of the sumoylation pathway. The overexpression of this protein stimulates but its knockdown inhibits SUMO conjugation. SF2/ASF interacts with Ubc9 and enhances sumoylation of specific substrates, sharing characteristics with already described SUMO E3 ligases. In addition, SF2/ASF interacts with the SUMO E3 ligase PIAS1 (protein inhibitor of activated STAT-1), regulating PIAS1-induced overall protein sumoylation. The RNA recognition motif 2 of SF2/ASF is necessary and sufficient for sumoylation enhancement. Moreover, SF2/ASF has a role in heat shock-induced sumoylation and promotes SUMO conjugation to RNA processing factors. These results add a component to the sumoylation pathway and a previously unexplored role for the multifunctional SR protein SF2/ASF.


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SF2/ASF also enhances SUMO3 conjugation and both effects are observed in different cell lines (Fig. S2). We then addressed the role of endogenous SF2/ASF in this process. SUMO3 and SUMO1 conjugation was drastically impaired in SF2/ASF-depleted cells (Fig. 1B and Fig. S1B, respectively).

We assessed the impact of SF2/ASF expression levels on endogenous SUMO2/3 conjugation. Diminishing SF2/ASF expression by siRNA reduces (Fig. 1C), but its overexpression stimulates SUMO2/3 conjugation in a dose-dependent manner (Fig. 1D).

These results demonstrate that SF2/ASF is a hitherto unexplored regulator of the sumoylation pathway. Its physiological expression is required for maintaining normal overall sumoylation levels, as shown by the RNAi strategy, and reminiscent of the effect of the yeast E3 ligases Siz1 and Siz2 gene disruption that abolishes modification of most targets (38).

RRM2 Is Necessary and Sufficient for SF2/ASF-Stimulated Sumoylation. We used deletion mutants (Fig. S3) to analyze the role of each SF2/ASF domain on SUMO conjugation. SF2/ASF mutants lacking the RRM1 (ΔRRM1) or the RS domain (ΔRS) show similar sumoylation-enhancing activity to wild-type SF2/ASF. In contrast, the mutant lacking the RRM2 (ΔRRM2) is unable to stimulate sumoylation (Fig. 1E and F and Fig. S2). Remarkably, expression of the RRM2 by itself is sufficient to stimulate sumoylation (Fig. 1F).

We took advantage of the fact that the ΔRRM1 and RRM2 constructs are siRNA-resistant (Fig. S4 A and B) and the effect of SF2/ASF knockdown on SUMO conjugation was rescued by expression of either one of these constructs, ruling out any nonspecific effect of this siRNA (Fig. S4C).

The effect of other members of the SR protein family was analyzed (Fig. S5). Srp20 [recently renamed SRSF3 (14)], which does not contain an RRM2, failed to stimulate sumoylation. We then tested two SR proteins that harbor RRM2s differing in their degree of identity with SF2/ASF RRM2, Srp30c [recently renamed SRSF9 (14)], and Srp40 [recently renamed SRSF5 (14)] (72 and 37% identity, respectively) (Fig. S5B). Srp30c but not Srp40 is able to stimulate protein sumoylation (Fig. S5C).

These results point to the RRM2 as the major determinant of SF2/ASF sumoylation-stimulatory function, and also indicate that the mere presence of an RRM2 is not sufficient to confer sumoylation-enhancing activity. A structural and mutational analysis of different RRM2 domains should provide insightful information in this respect.

To compare SF2/ASF ability to regulate sumoylation with its activity as an alternative splicing regulator, we analyzed three well-characterized SF2/ASF-responsive splicing events (fibronectin EDI exon, CFTR exon 9, and adenovirus E1a) (Fig. S6 A–C) upon overexpression of SF2/ASF or its deletion mutants. The RRM2 domain alone, although sufficient to enhance sumoylation, does fail to alter splicing patterns of FN EDI exon and CFTR exon 9, or alters the splicing pattern in a different fashion than SF2/ASF, as shown for adenovirus E1a transcripts (Fig. S6 D–F). This lack of correlation between SF2/ASF effects on sumoylation and splicing suggest that the role for SF2/ASF in the sumoylation process cannot be explained exclusively by its splicing activity, and point to a splicing-independent function for SF2/ASF in the sumoylation process.

SF2/ASF Interacts with Ubc9 and Stimulates Sumoylation of Specific Substrates. To test whether SF2/ASF directly interacts with the sumoylation machinery, we performed GST pull-down assays by incubating GST-Ubc9 with cell extracts expressing full-length SF2/ASF or its deletion mutants. T7-SF2/ASF is pulled down by GST-Ubc9 but not by GST alone. The ΔRRM2 mutant is unable to bind Ubc9, but the ΔRS mutant that is as efficient as wild-type SF2/ASF in stimulating sumoylation does interact with Ubc9 (Fig. 2A). SF2/ASF-Ubc9 interaction is independent from RNA or DNA, as it is not disturbed by the addition of RNase/DNase to the reaction mixture (Fig. S7A), and appears to be direct because T7-SF2/ASF purified from HEK 293T cells (Fig. S7B) could be pulled down by GST-Ubc9 (Fig. S7C). GST-Ubc9 also pulled down endogenous SF2/ASF (Fig. S7D).

To address whether the sumoylation-stimulatory function of SF2/ASF can take place independently of its other known regulators, we tested the effect of SF2/ASF in cell-free in vitro sumoylation reactions with well-known sumoylation substrates. Sumoylation of Topoisomerase I (hereafter Topo I) is weakly seen upon incubation with E1, E2, and SUMO1 (Fig. 2B). Addition of purified T7-SF2/ASF increased sumoylation of Topo I. However, although the active fragment of RanBP2, RanBP2AFG, displays marginal stimulation of Topo I sumoylation compared with SF2/ASF, the SUMO E3 ligsate Topors exerts a stronger effect, enhancing the formation of high molecular-weight SUMO1-Topo I conjugates, as previously reported (39). SF2/ASF is unable to conjugate SUMO in the absence of Ubc9, ruling out a role as an E2-conjugating enzyme (Fig. 2B).
GST-SF2/ASF purified from bacteria also enhances in vitro sumoylation of Topo I (Fig. S8A). In agreement with these results, knockdown of endogenous SF2/ASF by siRNA inhibits Topo I sumoylation in living cells (Fig. 2C).

In vitro sumoylation reactions were also performed with p53 as a substrate (40, 41). Limiting amounts of Ubc9 were used and SUMO3 conjugation to p53 was hardly detectable at early time-points. Addition of purified GST-SF2/ASF increased the amount of SUMO-conjugated p53 in a time-dependent manner (Fig. 2D). Similar results were obtained with SUMO1 (Fig. S8B).

Consistently, depletion of SF2/ASF by siRNA diminishes SUMO conjugation to p53 in living cells (Fig. 2E).

SF2/ASF interacts with Topo I, as previously described (42, 43), and with p53 (Fig. S8 C and D). The nature and biological relevance of this latter interaction await further investigation. Furthermore, SF2/ASF has no effect on sumoylation of Sp100, a substrate of the E3 ligase RanBP2 (Fig. S8E) (28).

Thus, SF2/ASF directly affects the sumoylation machinery by interacting with the E2 conjugating enzyme and promoting the sumoylation of specific substrates, Topo I and p53, both in vitro and in vivo.

Our initial finding that depletion of SF2/ASF drastically impairs sumoylation in living cells could be due in part to the reported effects of SF2/ASF depletion on cell cycle and apoptosis (13). However, a direct action of SF2/ASF on the SUMO pathway is evident from the results obtained with in vitro sumoylation assays.

**SF2/ASF Stimulates SUMO Transfer from Ubc9 to the Substrate.** Based on the results described above, SF2/ASF could be acting at two nonmutually exclusive levels: promotion of Ubc9-SUMO thioester linkage formation or stimulation of SUMO transfer from Ubc9 to the substrate. Although Ubc9 loading increased with time, SF2/ASF failed to enhance the rate of SUMO-Ubc9 thioester bond formation (Fig. 3A). SUMO transfer from Ubc9 to p53 was then measured in single turnover reactions, as previously described (44). SF2/ASF clearly stimulates this step in the SUMO conjugation pathway (Fig. 3B), indicating that SF2/ASF action takes place downstream of Ubc9.

E3 ligases are known to facilitate the transfer of ubiquitin or Ubl proteins from an E2 enzyme to a substrate protein and to display substrate specificity. Except for ubiquitin HECT E3 ligases, all other known E3 ligases form complexes with the SUMO-charged E2 and the target (19). Thus, SF2/ASF shares some characteristics with E3 ligases. Further biochemical and
structural characterization is needed to determine the precise mechanism of action of SF2/ASF at the SUMO transfer step.

**SF2/ASF Interacts with PIAS1 and Regulates its E3 Activity.** Given that PIAS1 copurifies with the spliceosome (34) and resembles scaffold-attachment factors known to interact with SR proteins (33), we tested if SF2/ASF could interact with PIAS1. GST pull-down assays demonstrate this interaction, which is dependent on SF2/ASF RRM2 (Fig. S9A) and seems to be direct, as purified T7-SF2/ASF could be pulled down by GST-PIAS1 (Fig. S9B). SF2/ASF-PIAS1 interaction takes place in a DNA/RNA-independent manner (Fig. S9C). Coimmunoprecipitation assays show that PIAS1 interacts with wild-type SF2/ASF but not with the mutant lacking the RRM2 in whole-cell lysates (Fig. S9D).

As expected, PIAS1 enhances SUMO conjugation. Coexpression of PIAS1 and SF2/ASF leads to a synergistic effect on overall protein sumoylation (Fig. 4A). The mutant SF2/ASF lacking the RRM2 was unable to enhance PIAS-mediated sumoylation (Fig. 4A). When a suboptimal amount of PIAS1 was transfected, it still enhanced the ability of wild-type SF2/ASF, ∆RRM1, or ∆RS mutants to stimulate sumoylation (Fig. 4B). Furthermore, PIAS-enhanced SUMO conjugation is impaired when SF2/ASF is depleted by siRNA (Fig. 4C), indicating that the SUMO E3 ligase activity of PIAS1 depends on the presence of SF2/ASF. These experiments show an RRM2-dependent functional synergism between PIAS1 and SF2/ASF that stimulate protein sumoylation in living cells. It is worth noting that the functional interaction between PIAS1 and SF2/ASF appears to involve yet unidentified cellular proteins because it could not be recapitulated with in vitro sumoylation assays (Fig. S9E). The results presented here are not enough to rule out an effect of SF2/ASF on PIAS1 translation, as suggested by Fig. 4B (lanes 6–10). However, under this hypothesis, depletion of SF2/ASF by siRNA should reduce PIAS protein levels, which does not seem to be the case. Keeping in mind the already described activity of SF2/ASF in the translation process (5, 11, 36, 37), this potential additional level of control remains to be explored. Considering that SF2/ASF has no SUMO E2 activity, its role as a coregulator of a SUMO E3 ligase adds a further level of regulation to the sumoylation pathway, resembling the case of ubiquitin E3 ligase complexes (31).

**SF2/ASF Has a Role in Heat Shock-Stimulated Sumoylation.** We decided to study SF2/ASF role in the heat-shock response because this treatment enhances SUMO conjugation (45) (Fig. 5A). Furthermore, SUMO2 and SUMO3 are required for cells to survive to hyperthermic stress (46). Depletion of SF2/ASF by siRNA greatly inhibits heat shock-induced sumoylation, pointing to SF2/ASF as a key factor in this regulatory phenomenon. Moreover, overexpression of SF2/ASF ΔRRM2 exerts similar effects to SF2/ASF depletion (Fig. 5A), suggesting this mutant could be acting in a dominant-negative manner.

Considering that (i) SF2/ASF shifts its subnuclear localization in response to heat shock from splicing speckles to nSBS in an RRM2-dependent manner (17, 18), and (ii) nSBS are known to recruit heat-shock transcription factors, as well as a subset of pre-mRNA processing factors (47) leading to changes in splicing patterns (48), we asked whether nSBS are sites of SUMO-conjugated proteins. Upon heat shock, Sam68, an RNA processing factor and a hallmark of nSBS (47–49), colocalized with wild-type GFP-SUMO1 but not with a mutant that is unable to conjugate to target proteins [GFP-SUMO1(GA)] (50) (Fig. 5B and C). These results indicate that nSBS colocalized with SUMO-conjugated proteins but not with the free SUMO pool. Thus, upon heat shock, sumoylated proteins localize in nSBS, where SF2/ASF as well as other pre-mRNA processing factors reside. It is tempting to speculate that SF2/ASF could be part of a regulatory network accounting not only for splicing regulation but also for the regulation of sumoylation-dependent protein activity required for cell recovery upon hyperthermic stress.

**SF2/ASF and Sumoylation of RNA Processing Factors.** Taking into account that (i) many RNA binding proteins have been reported to be sumoylated (51–54) and (ii) recent studies have shown an enrichment in the fraction of RNA processing- and RNA binding-proteins upon purification of SUMO substrates in vivo (55, 56), we wondered whether SF2/ASF could enhance sumoylation of RNA processing factors.

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**Figure 4.** PIAS1 activity is regulated by SF2/ASF. (A) SF2/ASF enhances PIAS1 activity on SUMO conjugation. HEK 293T cells were transfected with HA-SUMO3 (500 ng) and FLAG-PIAS1, T7-SF2/ASF or T7-SF2/ASF ΔRRM2 (500 ng each) as indicated, together with 50 ng of pECFP for transfection efficiency and loading control (total amount of DNA, 2 μg). Cells were lysed in Laemmli sample buffer after 48 h. Western blotting was performed with the antibodies indicated at the bottom of each panel. (B) HEK 293T cells were transfected with HA-SUMO1 (500 ng), FLAG-PIAS1 (100 ng), T7-SF2/ASF, or its deletion mutants (500 ng each) as indicated (total amount of DNA, 2 μg). Cells were lysed in Laemmli sample buffer after 48 h. Western blotting was performed with the antibodies indicated at the bottom of each panel. (C) SF2/ASF is critical for PIAS1-mediated SUMO conjugation. HEK 293T cells were transfected either with a control siRNA (siRNA Ctl) or an SF2/ASF specific siRNA. After 24 h, cells were retransfected with the plasmids expressing HA-SUMO3 and FLAG-PIAS1 (500 ng). Forty-eight hours later, cells were lysed in Laemmli sample buffer and subject to Western blot, as indicated at the bottom of each panel.
Cell extracts from HEK 293T transfected with His-SUMO1 were subject to Ni-NTA purification to enrich His-tagged sumoylated proteins. Pulled-down proteins were analyzed by Western blot with specific antibodies against RNA metabolism-related factors known to be sumoylation substrates (57–59). SF2/ASF overexpression stimulates, but its knockdown diminishes SUMO conjugation to the nucleolar protein Nop58 (Fig. S8 F and G) and to Sam68 (Fig. 5D).

An effect of sumoylation on the activity of different RNA-binding proteins has been reported (57, 60, 61), and sumoylation regulates its nucleolar delocalization upon cell treatment with anticancer drugs (63). SENP3-dependent sumoylation status of nucleophosmin (NPM1) is involved in the regulation of rRNA processing and ribosome synthesis (64). Our finding that SF2/ASF exerts its effect at least at two different levels: it interacts with Ubc9 promoting the sumoylation of specific substrates and it regulates the SUMO E3 ligase activity of PIA1. We have identified specific targets of this activity and deciphered SF2/ASF mechanism of action. Finding additional targets for SF2/ASF sumoylation-regulatory task and deepening into its physiological relevance is our immediate future challenge.

Materials and Methods

Cell Lines. HEK 293T cells were grown in DMEM supplemented with 10% FBS, 4.5 g/L glucose, and 110 mg/L sodium pyruvate.

Plasmids, siRNAs, and Transfection. HEK 293T cells were transfected with Lipofectamine 2000 (Invitrogen). The list of expression vectors used is available in SI Materials and Methods. For knockdown experiments, siRNA duplexes were transfected at the concentration indicated in each figure. Small interfering RNA targeting luciferase mRNA was used as a control.

Immunofluorescence of Heat-Shocked Cells. HEK 293T cells were transfected with GFP-SUMO1 or the GFP-SUMO1(GA) mutant 24 h after plating. The next day, cells were transferred to a 42 °C water bath for 1 h and then allowed to recover for 1 h at 37 °C. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton X-100 in PBS. More details of the protocol are available in SI Materials and Methods. Sam68 was used as an nSBs marker, as described (47–49).

Recombinant Proteins. GST, GST-Ubc9, and GST-SUMO3 were expressed in Escherichia coli M15(pREP4) cells and GST-SF2/ASF, GST-Topors (268/644), GST-PIAS1 in E. coli BL21(DE3) Rosetta strain by induction with 1 mM IPTG and purified with glutathione Sepharose beads (GE Healthcare). T7-SF2/ASF and T7-SF2/ASF ΔRRM2 were purified from transfected HEK 293T lysates exactly as described (65). Proteins were analyzed by SDS/PAGE and Coomassie staining for quantification and purity. Recombinant GST-Topo I, SUMO1, and SUMO3 were purchased from LAE Biotech. SUMO E1, SUMO E2, GST-p53, GST-RanBP2, and GST-Sp100 were from BIOLOM/Enzo Life Sciences.

Immunoprecipitation. HEK 293T cells were lysed in 1 mL of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 10% glycerol, 1× Complete Protease Inhibitor (Roche)) and incubated for 30 min at 4 °C. After centrifugation for 20 min at 4 °C, supernatants were used immediately for coimmunoprecipitation or kept at −80 °C. Details of this protocol are available in SI Materials and Methods.

GST Pull-Down Assays. Lysates from HEK 293T cells expressing the indicated proteins or purified recombinant proteins were used for pull-down experiments, as described (30).

Ni2+ Pull Down. HEK 293T cells were transfected in 6-cm dishes with the indicated siRNAs (10 nM) and 24 h later with the indicated plasmids. After 48 h, His-SUMO1 conjugated proteins were purified under denaturing conditions using Ni-NTA agarose beads according to the manufacturer’s instructions (QIAGEN).

In Vitro Sumoylation Reactions. In vitro sumoylation reactions were performed as described (66). Recombinant proteins were added at the concentrations indicated in each figure legend.
Thioester Formation Assay. Assays were carried out essentially as described (30), with 100 ng E1, 100 ng E2, 200 ng SUMO1, and 200 ng recombinant SF2/ASF when indicated.

SUMO Transfer Reactions. Recombinant E1 (150 ng), E2 (300 ng), SUMO1 (200 ng), GST–p53 (200 ng), and GST–SF2/ASF (200 ng) were used. A detailed protocol is available in SI Materials and Methods.

Western Blot and Antibodies. Protein samples were resolved by SDS/PAGE and transferred to Hybond-P membranes (GE Healthcare). Membranes were blocked and incubated with the primary antibody. After washing, mem-

branes were incubated with HRP-conjugated secondary antibodies (Biorad). Membranes were developed using ECL plus reagent (GE Healthcare). The list of antibodies used is available in SI Materials and Methods.

The experiments were performed at least three times, and representative images are shown in each case.

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Supporting Information

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SI Materials and Methods

Plasmids. The expression vectors used were: pcDNA3.1-HA-SUMO1 and pcDNA3.1-HA-SUMO3 (1, 2), pcDNA3.1-FLAG-HA-SENP1 (3, 4), pcDNA3-HA-PIAS1 and pcDNA3-FLAG-PIAS1 (5), GFP-SUMO and GFP-SUMO(GA) (6), Myc-Topo I and GST-Topors (7), HA-Samt68 (8).

Immunofluorescence. Fixed and permeabilized cells were blocked with 3% BSA in PBS. Afterward, cells were incubated with the primary antibody for 1 h in blocking buffer. After washing with PBS, cells were incubated with Alexa 637-conjugated secondary antibody for another hour. Cells were extensively washed with PBS and mounted.

Immunoprecipitation. For coimmunoprecipitation experiments, 30 μL of anti-T7-agarose beads (Novagen) or 1 μg of anti-HA (Covance) plus 30 μL of Protein A/G Plus agarose (Santa Cruz Biotechnology) were used. After incubation for 1 h at 4 °C, beads were pelleted, and washed three times with lysis buffer and once with PBS. Immunoprecipitates were resuspended in 2× Laemmli sample buffer.

SUMO Transfer Reactions. Recombinant E1 (150 ng), E2 (300 ng), and SUMO1 (small ubiquitin-related modifier-1) (200 ng) were incubated for 30 min at 30 °C in sumoylation assay buffer (9) supplemented with 0.5 mM ATP. Reactions were then diluted 3-fold in the same buffer but lacking ATP and containing EDTA (10 mM final concentration) to prevent further loading of the E2 by inhibiting the Mg2+-dependent activation by the E1. The SUMO-loaded E2 was then incubated with GST-p53 (200 ng) either with or without 200 ng GST-SF2/ASF for the indicated time points. Reactions were stopped by addition of an equal volume of 2× Laemmli sample buffer.

Western Blot and Antibodies. The antibodies used were: anti-SF2/ASF (mAb 103), hybridoma culture supernatant, provided by Adrián Pelisch et al.


Pelisch et al. 2006. SUMO modification of Sam68 enhances its ability to repress cyclin D1 expression and inhibits its ability to induce apoptosis. Oncogene 25: 4955–4964.
Fig. S1. (A) HEK 293T cells were transfected with HA-SUMO1, FLAG-PIAS1 (protein inhibitor of activated STAT-1) and FLAG-SENP1 (SUMO-specific proteases-1) (500 ng each, 2 μg total amount of DNA) and 100 ng (+) or 500 ng (++) of T7-SF2/ASF, as indicated. Cells were lysed 48 h posttransfection in Laemmli sample buffer and proteins were separated by SDS/PAGE and subject to Western blot as indicated at the bottom of each panel. (B) Cells were transfected with a control siRNA (siRNA Ctl) or with an SF2/ASF-specific siRNA (25 nM). After 24 h, cells were retransfected with HA-SUMO1. Cells were lysed 48 h posttransfection in Laemmli sample buffer, proteins were separated by SDS/PAGE, and subject to Western blot, as indicated at the bottom of each panel.
**Fig. S2.** (A) COS7 cells were transfected with HA-SUMO (1 or 3) and FLAG-SENP1 as indicated (500 ng each), either with or without T7-SF2/ASF or T7-SF2/ASF ΔRRM2 [250 (+) or 500 (++) ng]. Total amount of DNA was brought up to 2 μg. After 48 h, cells were lysed in 2× Laemmli sample buffer and subject to Western blot with an anti-HA antibody. (B) Functionally normal mouse mammary epithelial cells (SCp2) were transfected with HA-SUMO (1 or 3, 500 ng), either with or without T7-SF2/ASF or T7-SF2/ASF ΔRRM2 (RNA-recognition motif-2) (500 ng). Total amount of DNA was brought up to 2 μg. After 48 h, cells were lysed in Laemmli sample buffer and subject to Western blot as in A.

**Fig. S3.** The scheme represents the modular structure of SF2/ASF consisting of two RRMs and one C-terminal domain rich in arginine and serine dipeptides (RS domain), and the deletion mutants used in this study.
Fig. S4. (A) SF2/ASF cDNA showing (in red) the sequence targeted by the siRNA used to knock-down SF2/ASF expression. (B) SF2/ASF deletion constructs refractory to SF2/ASF siRNA because of the lack of the RRM1 domain and therefore the sequence targeted by the siRNA. (C) Cells were transfected with a control siRNA (siRNA Ctl) or with an SF2/ASF-specific siRNA (40 nM). After 24 h, cells were retransfected with HA-SUMO1 (500 ng) and either one of the siRNA-resistant construct shown in B (300 ng). After 48 h from the second transfection, cells were lysed in Laemmli buffer and subject to Western blot analysis with the antibodies indicated at the bottom of each panel.

Fig. S5. (A) Scheme of SF2/ASF and other members of the SerlArg-rich (SR) family of proteins used to compare their sumoylation stimulatory activity. (B) Alignment of the RRM2 domains of SF2/ASF, SRp30c, and SRp40 according to ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html) and BOXSHADE 3.21 (www.ch.embnet.org/software/BOX_form.html) software. Identical amino acids are shown in black and similar ones in gray. (C) HEK 293T cells were transfected with 500 ng HA-SUMO1 and 100 ng of T7-SF2/ASF, T7-SRp20, T7-SRp40, or T7-SRp30c. Cells were lysed 48 h posttransfection in Laemmli sample buffer; proteins were separated by SDS/PAGE and subject to Western blot as indicated at the bottom of each panel.
Fig. S6. Three SF2/ASF alternative splicing targets ("splicing reporter minigenes") were used to analyze the effect of over-expressing wild type SF2/ASF or its deletion mutants: fibronectin EDI exon (A), CFTR exon 9 (B), and adenovirus E1a (C) (1–3). (D–I) Cells were transfected with 500 ng of each splicing reporter minigene shown in A to C, and 50 ng of the indicated SF2/ASF construct. Forty-eight hours later, RNA was extracted and subject to RT-PCR as described (4). Radioactive samples were run on native 6% polyacrilamide gels, which were subsequently dried and exposed to X-ray films (Agfa).

Fig. S7.  (A) Pull-down assay was performed essentially as in Fig. 2A. Before addition of the recombinant GST fusion protein, the lysate was divided in two aliquots: one of them was mock treated and the other was treated with DNase and RNase. (B) T7-SF2/ASF was purified from transfected HEK 293T lysates as indicated in Materials and Methods. An aliquot of the purified protein (∼2 μg) was analyzed by SDS/PAGE and Coomassie staining. (C) Purified recombinant T7-SF2/ASF (500 ng) was incubated with 2 μg GST or GST-Ubc9 and pulled down with glutathione Sepharose beads. SF2/ASF binding was analyzed by Western blotting with an anti-T7 antibody. (D) GST pull-down assay was performed as in A but from nontransfected HEK 293T cell lysates. Samples were analyzed by Western blotting with an anti-SF2/ASF antibody.
(A) SF2/ASF stimulates SUMO1 conjugation to Topo I in vitro. GST-Topoisomerase I ("Topo I," 1 μg) was incubated with E1 (150 ng), E2 (30 ng), and SUMO1 (1 μg), either with or without GST-SF2/ASF (150 ng or 300 ng) for 60 min in sumoylation assay buffer. Reactions were stopped by addition of an equal volume of 2x Laemmli sample buffer, and analyzed by SDS/PAGE followed by Western blotting with an anti-Topo I antibody. (B) SF2/ASF stimulates SUMO1 conjugation to p53 in vitro. GST-p53 (250 ng) was incubated with E1 (150 ng), E2 (30 ng), and SUMO1 (1 μg), either with or without GST-SF2/ASF (100 ng or 200 ng) for 60 min in sumoylation assay buffer. Reactions were stopped by addition of an equal volume of 2x Laemmli sample buffer, and analyzed by SDS/PAGE, followed by Western blotting with an anti-p53 antibody. (C) SF2/ASF interacts with Topo I in whole-cell lysates as assessed by coimmunoprecipitation. HEK 293T cells were transfected with HA-p53 and T7-SF2/ASF when indicated (500 ng each). Cells were lysed as described in Material and Methods and lysates were immunoprecipitated with anti-T7 agarose beads. After washing, precipitated proteins were resuspended in 2x Laemmli sample buffer and subject to Western blot, as indicated at the bottom of each panel. (D) SF2/ASF interacts with p53 in whole-cell lysates as assessed by commounoprecipitation. HEK 293T cells were transfected with HA-p53 and T7-SF2/ASF as indicated, and commounprecipitation assay was performed as in C. (E) SF2/ASF does not stimulate Sp100 sumoylation. A GST-Sp100 fragment encompassing amino acids 241 to 360 (Lys-297 is the physiological SUMO1 attachment site, 200 ng) was incubated with E1 (150 ng), E2 (30 ng), and SUMO1 (1 μg), either with or without GST-SF2/ASF (100 ng or 200 ng) for 60 min in sumoylation assay buffer. Reactions were stopped by addition of an equal volume of 2x Laemmli sample buffer, and analyzed by SDS/PAGE, followed by Western blotting with an anti-Sp100 antibody. (F) SF2/ASF regulates SUMO1 conjugation to Nop58 in living cells. HEK 293T cells were transfected with the indicated siRNAs and 24 h later with the indicated plasmids. After 48 h, cells were harvested and lysates were subject to Ni-NTA agarose purification under denaturing conditions. His-tagged sumoylated proteins were eluted in buffer containing 100 mM imidazole and 2% SDS, mixed with an equal volume of 2x Laemmli sample buffer and subject to Western blot using Nop58 antibodies (F). A fraction of each cell lysate (3%) was run in parallel as input control. (G) Lysates were analyzed for SF2/ASF expression levels and β-actin as a control.

Fig. S8. (A) SF2/ASF stimulates SUMO1 conjugation to Topo I in vitro. GST-Topoisomerase I ("Topo I," 1 μg) was incubated with E1 (150 ng), E2 (30 ng), and SUMO1 (1 μg), either with or without GST-SF2/ASF (150 ng or 300 ng) for 60 min in sumoylation assay buffer. Reactions were stopped by addition of an equal volume of 2x Laemmli sample buffer, and analyzed by SDS/PAGE followed by Western blotting with an anti-Topo I antibody. (B) SF2/ASF stimulates SUMO1 conjugation to p53 in vitro. GST-p53 (250 ng) was incubated with E1 (150 ng), E2 (30 ng), and SUMO1 (1 μg), either with or without GST-SF2/ASF (100 ng or 200 ng) for 60 min in sumoylation assay buffer. Reactions were stopped by addition of an equal volume of 2x Laemmli sample buffer, and analyzed by SDS/PAGE, followed by Western blotting with an anti-p53 antibody. (C) SF2/ASF interacts with Topo I in whole-cell lysates as assessed by coimmunoprecipitation. HEK 293T cells were transfected with HA-p53 and T7-SF2/ASF when indicated (500 ng each). Cells were lysed as described in Material and Methods and lysates were immunoprecipitated with anti-T7 agarose beads. After washing, precipitated proteins were resuspended in 2x Laemmli sample buffer and subject to Western blot, as indicated at the bottom of each panel. (D) SF2/ASF interacts with p53 in whole-cell lysates as assessed by commounprecipitation. HEK 293T cells were transfected with HA-p53 and T7-SF2/ASF as indicated, and commounprecipitation assay was performed as in C. (E) SF2/ASF does not stimulate Sp100 sumoylation. A GST-Sp100 fragment encompassing amino acids 241 to 360 (Lys-297 is the physiological SUMO1 attachment site, 200 ng) was incubated with E1 (150 ng), E2 (30 ng), and SUMO1 (1 μg), either with or without GST-SF2/ASF (100 ng or 200 ng) for 60 min in sumoylation assay buffer. Reactions were stopped by addition of an equal volume of 2x Laemmli sample buffer, and analyzed by SDS/PAGE, followed by Western blotting with an anti-Sp100 antibody. (F) SF2/ASF regulates SUMO1 conjugation to Nop58 in living cells. HEK 293T cells were transfected with the indicated siRNAs and 24 h later with the indicated plasmids. After 48 h, cells were harvested and lysates were subject to Ni-NTA agarose purification under denaturing conditions. His-tagged sumoylated proteins were eluted in buffer containing 100 mM imidazole and 2% SDS, mixed with an equal volume of 2x Laemmli sample buffer and subject to Western blot using Nop58 antibodies (F). A fraction of each cell lysate (3%) was run in parallel as input control. (G) Lysates were analyzed for SF2/ASF expression levels and β-actin as a control.
Fig. S9. (A) HEK 293T cells were transfected either with wild type T7-SF2/ASF ΔRRM2, or ΔRS, and lysates were prepared as described in Materials and Methods. Cleared lysates were incubated with 2 μg GST or GST-PIAS1 and pulled-down with glutathione sepharose beads. SF2/ASF binding was analyzed by Western blotting with an anti-T7 antibody. (B) Purified recombinant T7-SF2/ASF (500 ng) was incubated with 2 μg GST or GST-PIAS1 and pulled down with glutathione Sepharose beads. SF2/ASF binding was analyzed by Western blotting with an anti-T7 antibody. (C) Pull-down assay was performed essentially as in A. Before addition of the recombinant GST fusion protein, the lysate was divided in two aliquots: one of them was mock-treated and the other was treated with DNase and RNase. (D) SF2/ASF interacts with PIAS1 in whole-cell lysates as assessed by coimmunoprecipitation. HEK 293T cells were transfected with HA-PIAS1 alone or with either wild-type T7-SF2/ASF or its ΔRRM2 mutant. Cells were lysed as described in Materials and Methods and lysates were immunoprecipitated with anti-T7 agarose beads or an anti-HA antibody together with protein A/G plus agarose beads. After washing, precipitated proteins were resuspended in Laemmli sample buffer and subject to Western blot, as indicated at the bottom of each panel. (E) GST-p53 (250 ng) was incubated with E1 (150 ng), E2 (30 ng), and SUMO3 (1 μg), either with or without GST-SF2/ASF (400 ng) and GST-PIAS1 (100 ng) in sumoylation assay buffer for 30 min. Reactions were stopped by addition of one volume of Laemmli sample buffer and analyzed by Western blotting with an anti-p53 antibody.