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-Sam68 (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 Mg^{2+}-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 hybridome culture supernatant, provided by Adrián Krainer, Cold Spring Harbor Laboratory, NY), anti-HA (Covance), anti-T7 (Bethyl Laboratories and Novagen), anti-T7 coupled to agarose beads (Novagen), anti-FLAG (Sigma), anti-Myc, anti-β-actin, anti-p53, anti-GFP, and anti-Sam68 (Santa Cruz Biotechnology), anti-SUMO1 (Zymed/Invitrogen), anti-SUMO2/3 (IMGENEX), anti-Ubc9 (BD Biosciences), anti-Topo I (LAE Biotech), anti-GST (Abcam), anti-GAPDH (Abcam), and anti-Nop58 (Human Protein Atlas).

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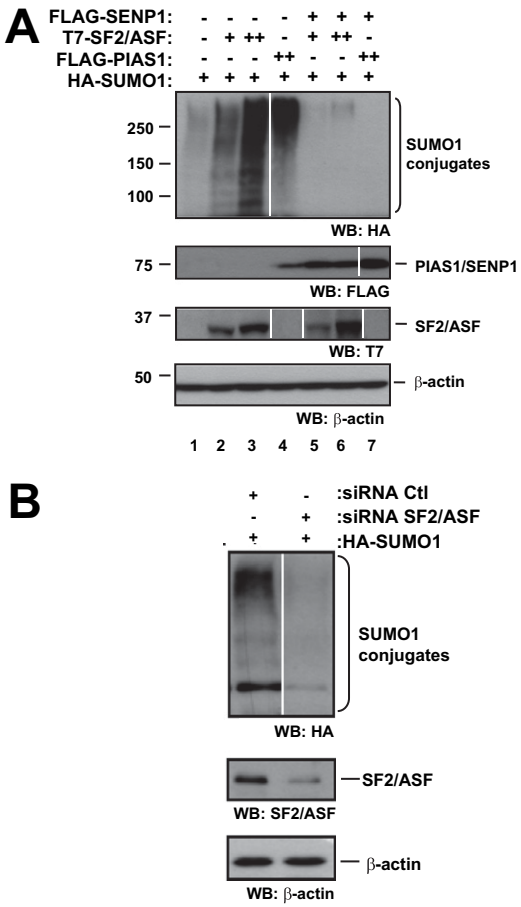

Fig. S1. (*A*) HEK 293T cells were transfected with HA-SUMO1, FLAG-PIAS1 (protein inhibitor of activated STAT-1) and FLAG-SENP1 (SUMO-specific proteases-1) (500 ng each, 2 μg total amount of DNA) and 100 ng (+) or 500 ng (++) of T7-SF2/ASF, as indicated. Cells were lysed 48 h post transfection in Laemmli 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.

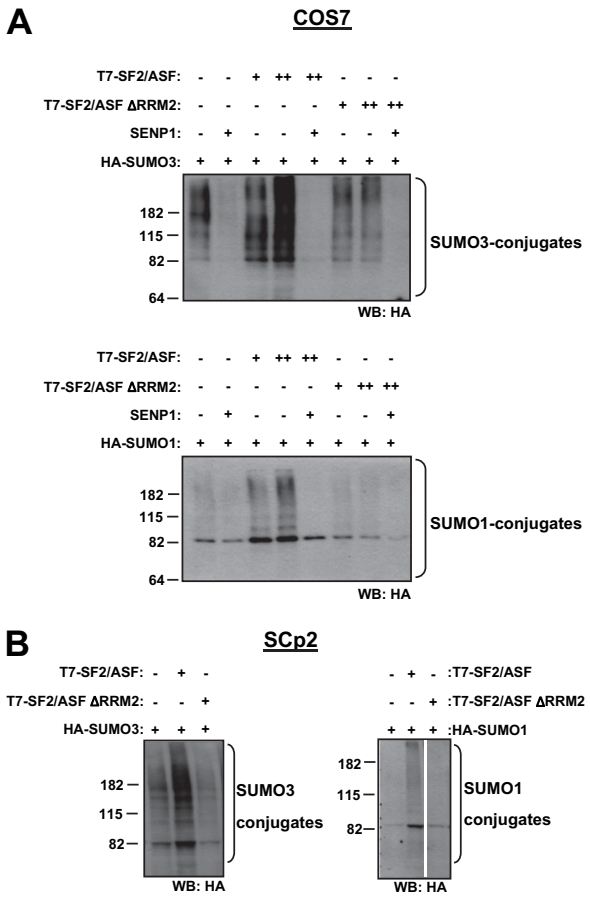

Fig. S2. (*A*) COS7 cells were transfected with HA-SUMO (1 or 3) and FLAG-SENP1 as indicated (500 ng each), either with or without T7-SF2/ASF or T7-SF2/ASF ΔRRM2 [250 (+) or 500 (++) ng]. Total amount of DNA was brought up to 2 μg. After 48 h, cells were lysed in 2× Laemmli sample buffer and subject 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*.

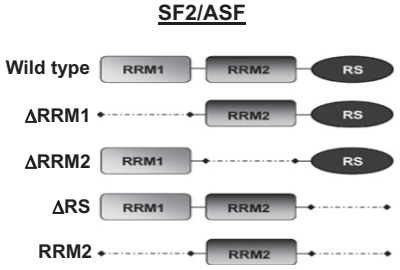

Fig. S3. The scheme represents the modular structure of SF2/ASF consisting of two RRMs and one C-terminal domain rich in arginine and serine dipeptides (RS domain), and the deletion mutants used in this study.

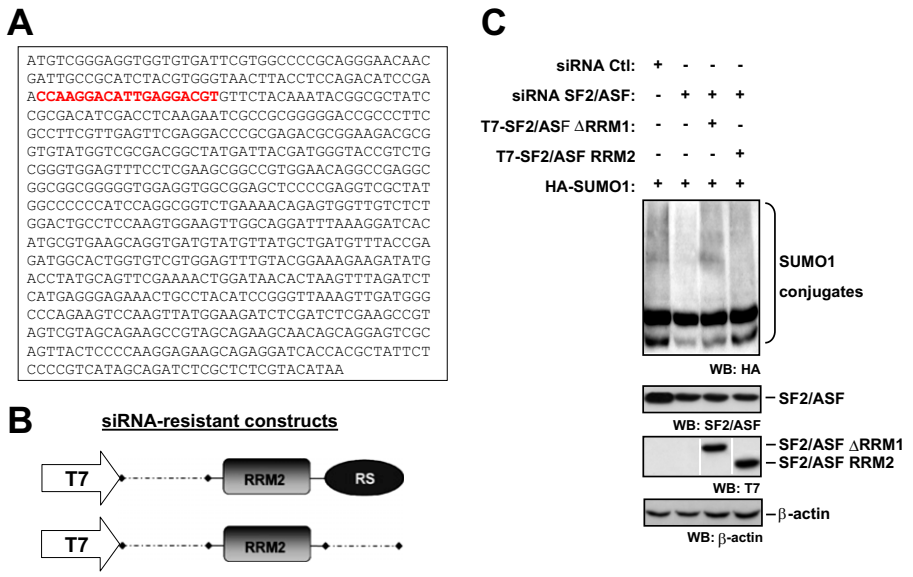

Fig. S4. (*A*) SF2/ASF cDNA showing (in red) the sequence targeted by the siRNA used to knock-down SF2/ASF expression. (*B*) SF2/ASF deletion constructs refractory to SF2/ASF siRNA because of the lack of the RRM1 domain and therefore the sequence targeted by the siRNA. (*C*) Cells were transfected with 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 consturcst 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.

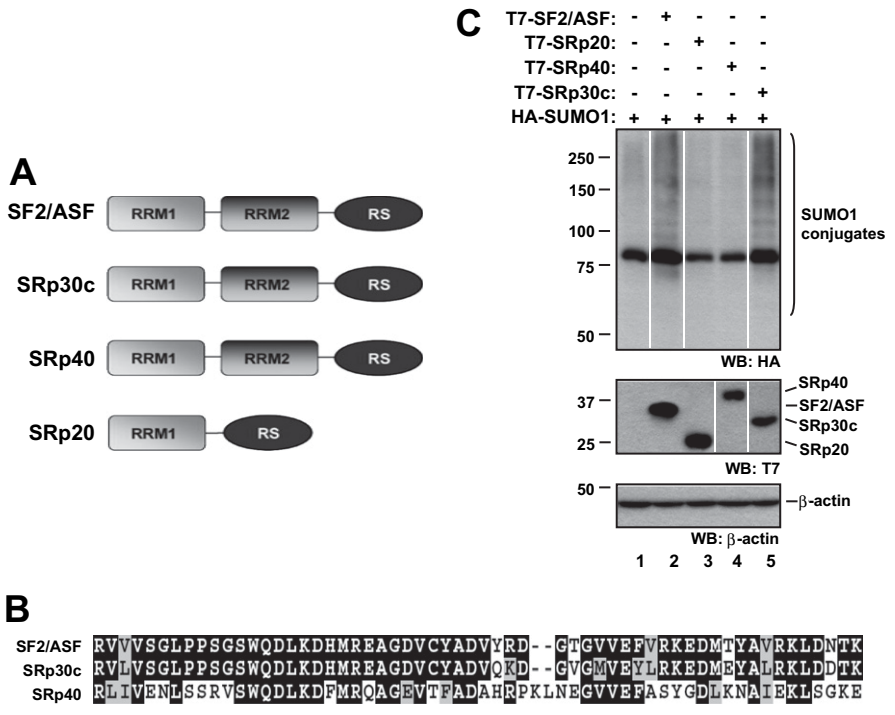

Fig. S5. (*A*) Scheme of SF2/ASF and other members of the Ser/Arg-rich (SR) family of proteins used to compare their sumoylation stimulatory activity. (*B*) Alignment of the RRM2 domains of SF2/ASF, SRp30c, and SRp40 according to ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html) and BOXSHADE 3.21 (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.

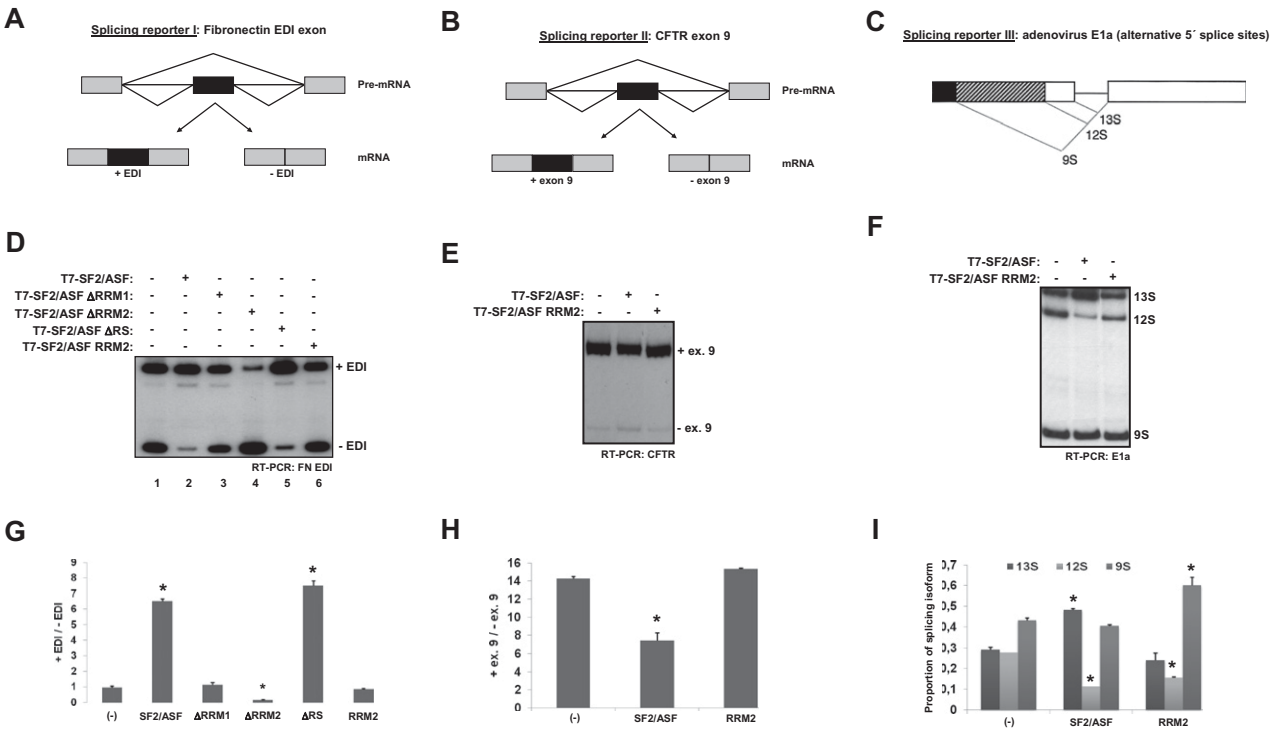

Fig. S6. Three SF2/ASF alternative splicing targets ("splicing reporter minigenes") were used to analyze the effect of over-expressing wild type SF2/ASF or its deletion mutants: fibronectin EDI exon (*A*), CFTR exon 9 (*B*), and adenovirus E1a (*C*) (1–3). (*D–I*) Cells were transfected with 500 ng of 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).

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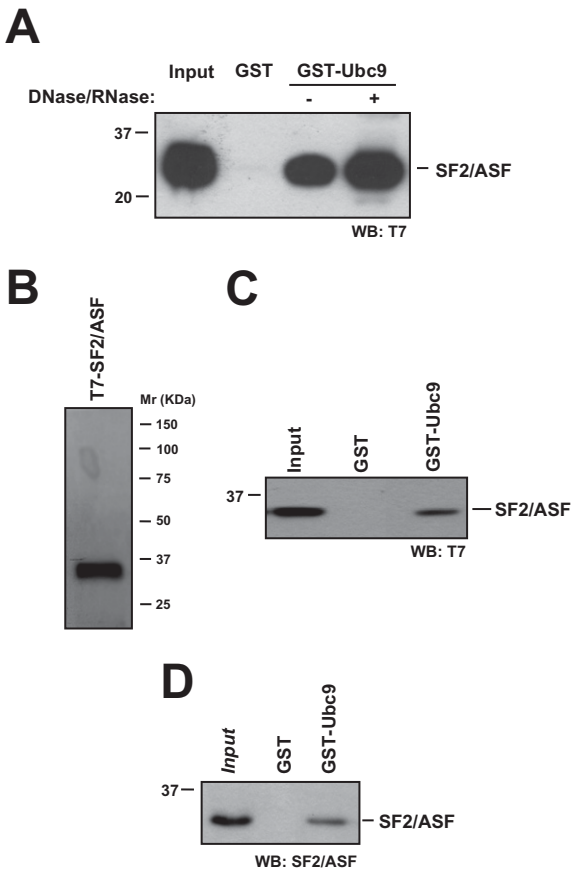

Fig. S7. (*A*) Pull-down assay was performed essentially as in Fig. 2*A*. Before addition of the recombinant GST fusion protein, the lysate was divided in two aliquots: one of them was mock treated and the other was treated with DNase and RNase. (*B*) T7-SF2/ASF was purified from transfected HEK 293T 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.

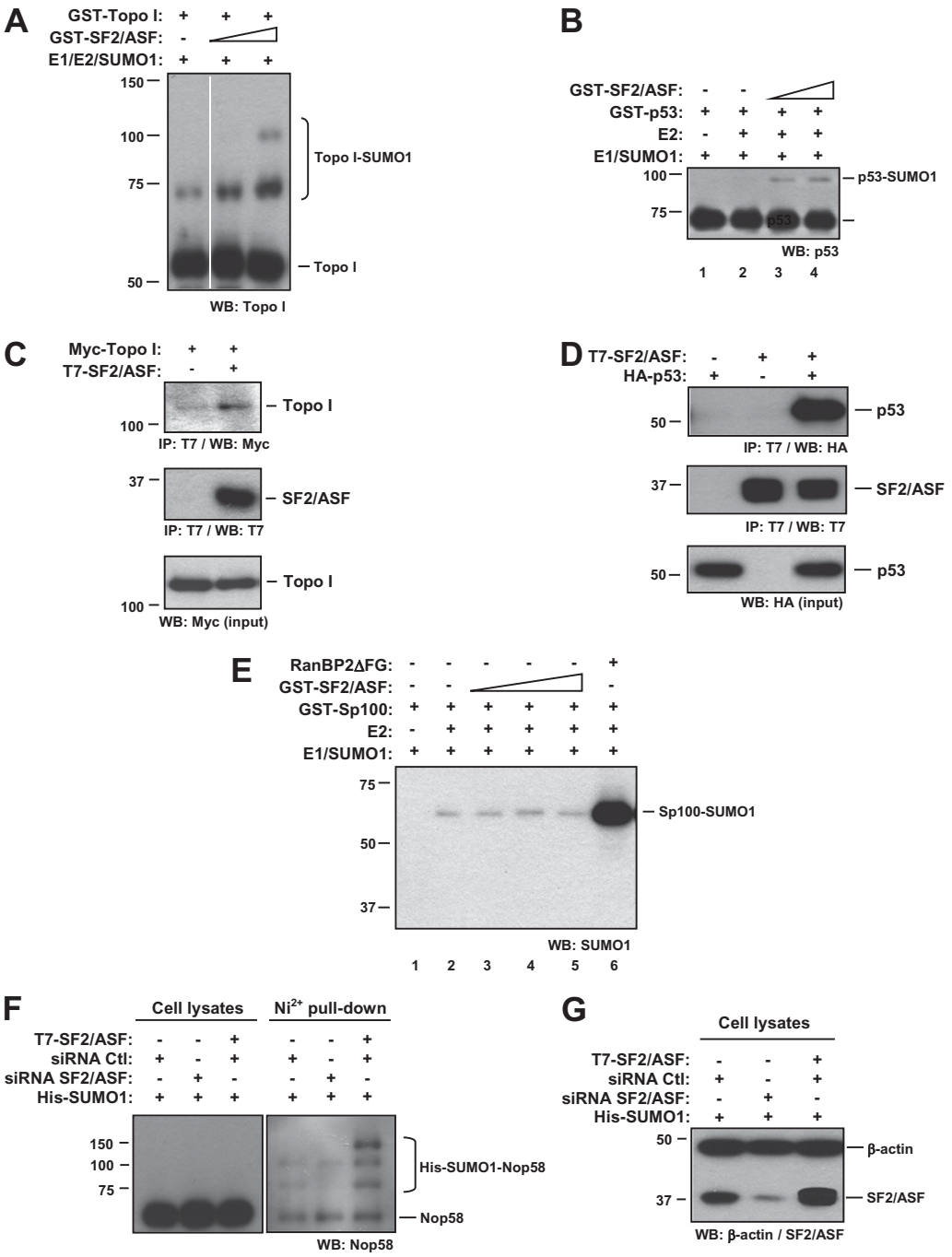

Fig. S8. (*A*) SF2/ASF stimulates SUMO 1 conjugation to Topo I in vitro. GST-Topoisomerase I ("Topo I," 1 μg) was incubated with E1 (150 ng), E2 (30 ng), and SUMO1 (1 μg), either with or without GST-SF2/ASF (150 ng or 300 ng) for 60 min in sumoylation assay buffer. Reactions were stopped by addition of an equal volume of 2× Laemmli sample buffer, and analyzed by SDS/PAGE followed by Western blotting with an anti-Topo I antibody. (*B*) SF2/ASF stimulates SUMO 1 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 2× 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 Myc-Topo I 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 2× 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 coimmunoprecipitation. HEK 293T cells were transfected with HA-p53 and T7-SF2/ASF as indicated, and coimmunoprecipitation 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 (200 ng, 500 ng, 1 μg) or GST-RanBP2ΔFG (10 ng) for 30 min in sumoylation assay buffer. Reactions were stopped by addition of an equal volume of 2× Laemmli sample buffer, and analyzed by SDS/PAGE, followed by Western blotting with an anti-SUMO1 antibody. (*F*, *G*) SF2/ASF regulates SUMO 1 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 2× 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.

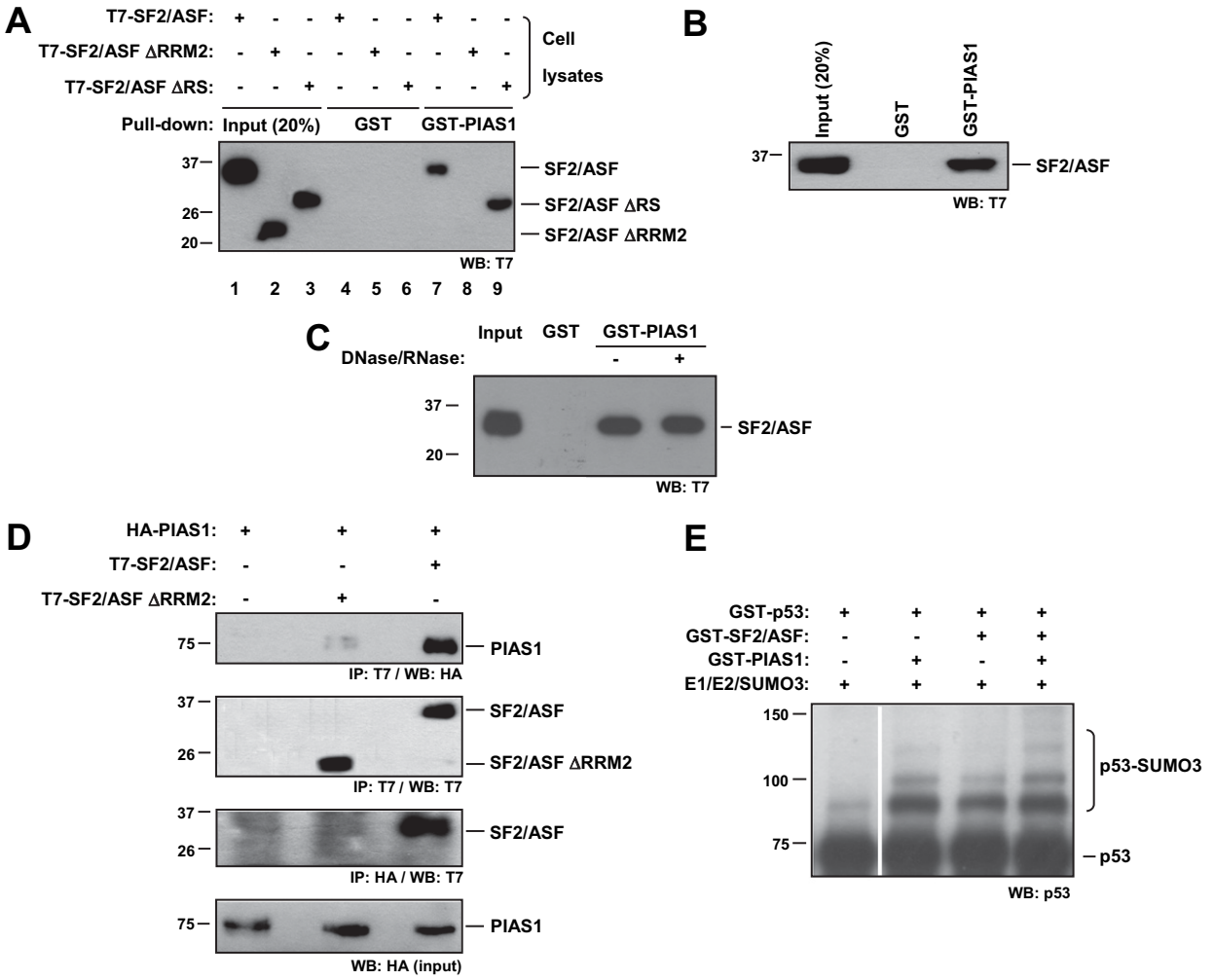

Fig. S9. (*A*) HEK 293T cells were transfected either with wild type T7-SF2/ASF ΔRRM2, or ΔRS, and lysates were prepared as described in *Materials and Methods*. Cleared lysates were incubated with 2 μg GST or GST-PIAS1 and pulled-down with glutathione sepharose beads. SF2/ASF binding was analyzed by 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.