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Subunit S5a of the 26*S* proteasome is regulated by antiapoptotic signals

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We performed a functional genetic screen to find novel antiapoptotic genes that are under the regulation of the oncoprotein c-Src. Several clones were identified, including subunit S5a of the 26*S* proteasome. We found that S5a rescued Saos-2 cells from apoptosis induced by Src inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP1). S5a mRNA and protein levels were downregulated as a result of Src inhibition, either by siRNA or PP1. In cell lines that possess high activity of Src S5a levels were elevated. Cloning of the S5a promoter region showed that S5a transcription responds to several stimuli. Analysis of the promoter sequence revealed a binding site for Tcf/Lef-1 transcription factor. Indeed, β-catenin significantly induced transcription from the S5a promoter, whereas EMSA studies showed that Lef-1 binds the S5a promoter-binding site. Furthermore, we also found that PP1 and LY294002, but not PD98059 inhibit the S5a promoter activity. These results suggest that S5a is regulated during apoptosis at the transcriptional level and that S5a upregulation by antiapoptotic signals can contribute to cell survival.

Src family kinases are involved in numerous cellular processes such as cell differentiation, proliferation, cell-cycle control, receptor signaling and transformation [1–3]. The persistently activated (unmutated) form of c-Src, as well as its abnormally high level of expression, seems to contribute to the development, progression and metastasis of various cancers [4–10], most probably because of its persistent activation by the upstream signaling of cell-surface receptors [11–16]. An important aspect of Src activity is in the role it plays in the control of cell-survival pathways. Src induces expression of the antiapoptotic Bcl-X_L protein, through the activation of STAT3 transcription factor [17–19]. Also, stimulation of Src activates the phosphatidylinositol 3-kinase (PtdIns3K)/Akt module [20], a key player in antiapoptotic signaling.

To search for novel antiapoptotic target genes regulated by Src, we performed a functional genetic screen to identify genes whose overexpression inhibits apoptosis of Saos-2 cells, induced by the Src inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP1) [21]. To this end, a HeLa cDNA expression library was screened in Saos-2 cells, which undergo apoptosis as a result of Src kinase inhibitor, PP1. Among the 20-cDNA clones that surfaced in our screen, most prominent was a clone identical to subunit S5a of the 26*S* proteasome, the subject of this study.

The ubiquitin–proteasome system is a key pathway responsible for protein turnover. The multiubiquitin chain is recognized by the 26*S* proteasome, a 2.5 MDa complex that catalyzes the degradation of multiubiquitin conjugated proteins [22,23].

A subunit of the regulatory particle, S5a, Rpn10, Pus1 in human [24], budding yeast [25] and fission yeast [26], respectively, was first identified as the multiubiquitin binding subunit of the proteasome. This subunit shows a distinct preference for the binding of

Abbreviations
GFP, green fluorescent protein; NH_2-Mec, 7-amino-4-methylcoumarin; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine; PtdIns3K, phosphatidylinositol 3-kinase; UIM, ubiquitin interacting motif.

tetra- or more multiubiquitin chains, via a ubiquitin interacting motif (UIM) localized to its C-terminus [27,28]. Surprisingly, deletion of S5a in yeast is not lethal but results in growth defects, and in difficulties in protein degradation [25]. This phenotype is mediated by the N-terminus of S5a [26,27,29], which is highly conserved among all known S5a homologs. The N-terminus contains a VWA domain that is critical to the association of the lid and the base of the regulatory particle of the proteasome [30,31]. In mice, there are five different transcripts that result from developmentally regulated alternative splicing [32]. Human S5a is expressed in all tissues [33] and has two splice variants that contain either one or two UIMs. Although S5a incorporated into 26*S* particles does not cross-link to tetraubiquitin chains [34], proteasomes purified from S5a null yeast strain (Δrpn10) showed partial defects in ubiquitin chain recognition [35], and the UIM of yeast S5a was shown to be necessary for ubiquitin chain binding by the proteasome [36]. Finally, it appears that, along with S5a, several multiubiquitin-binding proteins exist, and may exhibit specificity in binding their multiubiquitinated substrates [37,38]. Few reports connect S5a to apoptosis. As Sun *et al*. [39] showed, S5a and two other proteasome subunits are targets for caspase proteolysis during apoptosis in Jurkat cells.

Here, we report on regulation of the expression of proteasomal subunit S5a by Src and the possible role of S5a regulation in apoptosis.

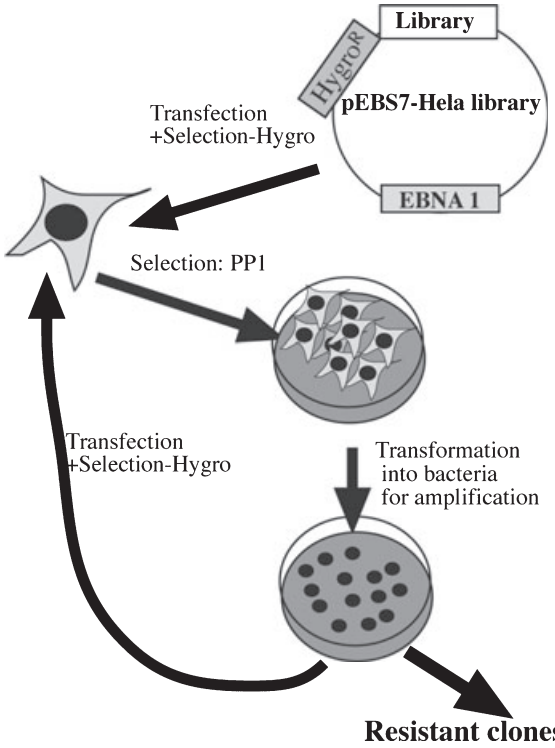
Results

Screen for antiapoptotic genes that are under the regulation of Src

In order to find novel antiapoptotic genes that are regulated by Src, a HeLa cDNA library was screened in Saos-2 cells, to identify genes that confer resistance to the Src inhibitor PP1 (Scheme 1). Saos-2 is a human osteosarcoma line. It has been shown that, in Src knockout mice, the only phenotype was osteopetrosis, which is caused by defects in osteoclast function [40]. These cells are highly sensitive to the Src inhibitor PP1, undergoing massive apoptosis upon exposure to the inhibitor as seen by elevated sub-G1 fraction as a result of PP1 treatment (Fig. 1A). Moreover, PP1 treatment induced activation of caspase 3 in Saos-2 cells (Fig. 1B), as seen by its cleavage to 19 kDa form and further into the 17 kDa subunit. The mature caspase 3 enzyme is formed from 17 and 12 kDa subunits [41]. Figure 1B shows that as PP1 treatment progresses over time, further cleavage into 17 kDa subunit occurs. The data indicate a role for Src in the survival of Saos-2 cells. The library was cloned into vector pEBS7, which enables episomal replication in mammalian cells. DNA was transfected into Saos-2 cells and PP1 resistant clones were selected as described in Experimental procedures (Scheme 1). After two rounds of selection with PP1, plasmid DNA from resistant clones underwent electroporation into *Escherichia coli* DH5α cells in order to analyze the clones. About 20 library cDNAs were identified and subjected to DNA sequencing. Each cDNA sequence was compared with known sequences in the database using the NCBI-BLAST program.

S5a rescues Saos-2 cells from PP1-induced apoptosis

The ability of each of the library cDNA clones to rescue cells from PP1-induced apoptosis was tested in



Scheme 1. Screening for antiapoptotic targets of Src kinase. A HeLa cDNA library was transfected into Saos-2 cells. Transfected cells were selected for by Hygromycin (Hygro). After selection the cells underwent two rounds of treatment with 40 μM of Src inhibitor PP1. Surviving cells were pooled and cDNA was extracted and amplified in *E. coli*, then transfected back into Saos-2 cells for a second round of PP1 treatment. Library cDNAs extracted from pooled resistant Saos-2 colonies were amplified in *E. coli* and were analyzed and sequenced as described in Experimental procedures. Several cDNAs were identified including S5a.

rescue assays in two cell lines: Saos-2 and HeLa cells. HeLa cells, like Saos-2 cells, also undergo PP1-induced apoptosis (not shown).

Cells were cotransfected with one of the library cDNAs and a plasmid encoding green fluorescent protein (GFP), to monitor transfection efficiency, treated with PP1 for 72 h (Saos-2) or 48 h (HeLa), until massive cell death was visible by GFP fluorescence. After PP1 treatment, adherent cells were harvested and green cells were counted. Among the few clones that consistently showed 1.5–2-fold rescue from 20 or 40 μM PP1 (data not shown) was a clone coding for the S5a proteasome subunit to which this study is devoted.

Initial experiments indicated that the overexpression of subunit S5a can rescue Saos-2 cells from PP1-induced apoptosis. We confirmed the ability of S5a to rescue Saos-2 cells from PP1-induced death by expressing $(HA)_3$S5a (Experimental procedures). Saos-2 cells were cotransfected with $(HA)_3$S5a and GFP-encoding plasmids, in a 3 : 1 ratio to maximize the likelihood that every cell carrying the GFP plasmid also harbored the $(HA)_3$S5a plasmid. Twenty-four hours after transfection, cells were treated with PP1 for 72 h. Adherent cells were harvested as described and subjected to FACS analysis as follows: green cells were counted in each sample, for a fixed time, so that the volume that was counted remained identical (as in the initial rescue experiments). As shown in Fig. 1C, S5a rescues 1.8-fold (relative to empty plasmid) at 20 μM PP1, and twofold at 40 μM in Saos-2 cells (Fig. 1C). These results provide further support for the initial rescue experiments. Expression of $(HA)_3$S5a was confirmed in these rescue experiments (Fig. 1D). As before, we do not observe rescue in HeLa cells (data not shown).

S5a expression is regulated by Src activity

We next examined whether the S5a mRNA level is affected by the inhibition of Src activity by PP1. S5a mRNA levels decreased after 24 h of PP1 (40 μM) treatment (not shown) and after 48 h, levels decreased over threefold (Fig. 1E). We also measured S5a protein levels in Saos-2 cells subsequent to treatment with 40 μM PP1 up to 6 days (Fig. 1B and data not shown). As shown in Fig. 1B, S5a protein levels decrease after 72 h of PP1 treatment and after 6 days the protein declines to levels barely observable using western analysis (data not shown). We observed over 90% apoptosis after 96 h of PP1 treatment (40 μM) (Fig. 1A). The activity of the 20*S* particle of the proteasome was measured in PP1-treated Saos-2 cells, at times when S5a protein was downregulated. Figure 1F shows that 20*S* activity was reduced at 72 and 96 h of PP1 treatment. However, the decrease in 20*S* activity cannot be accounted for solely by S5a downregulation. It has been shown [39] that the proteasomal subunits S5a, S6′ and S1 are downregulated because of caspase cleavage during apoptosis, leading to inhibition of proteasomal degradation. However, we found no correlation between caspase activation and reduction in S5a protein level during PP1 or cisplatin-induced apoptosis (data not shown).

In addition, we sought to inhibit Src levels by means of siRNA. As shown in Fig. 2A, S5a protein levels decreased about twofold in Saos-2 cells transfected with siRNA against Src for 48 h.

We further examined the effect of Src activity on the expression of S5a by analyzing the expression of S5a in cells that exhibit high Src activity. S5a mRNA levels were measured in NIH3T3, NIH3T3 transformed with active Src (SrcNIH) and CSH12 cells, which are NIH3T3 that overexpress the chimeric receptor $EGFR_{out}/HER\text{-}2_{in}$, and possess constitutive Src activity [11,42]. S5a transcript (Fig. 2B) and protein levels (Fig. 2C) in SrcNIH and CSH12 were found to be significantly higher than in NIH3T3.

Overexpression of S5a results in the accumulation of IκB

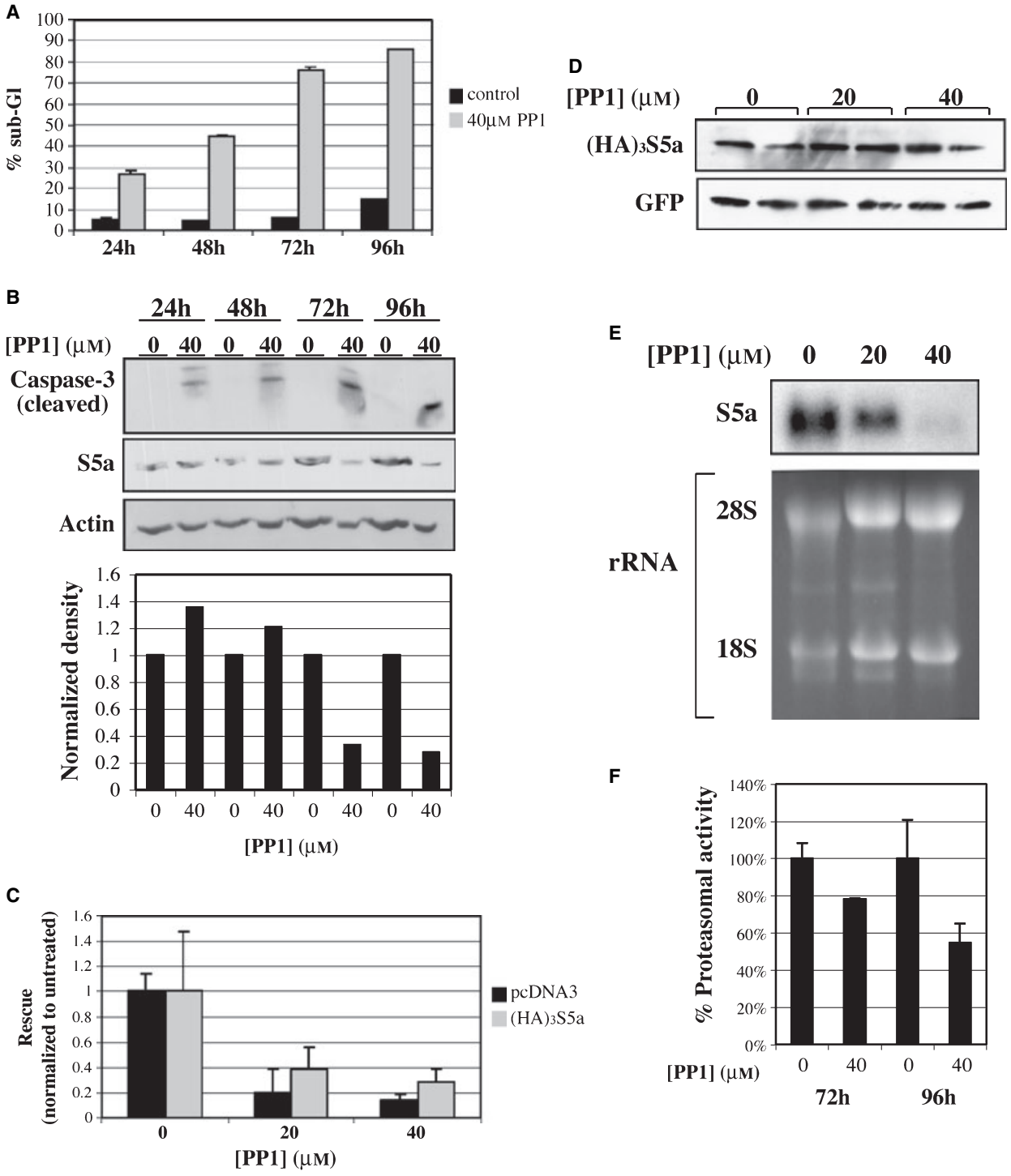
It has been reported previously that an excess of S5a and other ubiquitin-binding proteins, such as Rad23, inhibits proteasomal degradation, resulting in the stabilization of proteasomal substrates in cell-free systems [37,43].

We measured the levels of a model proteasomal substrate, IκB, in cells transiently overexpressing the library S5a clone. As shown in Fig. 3A, the level of HA–IκB was higher when S5a was transiently overexpressed. HA–IκB levels were almost threefold higher when S5a was overexpressed compared with empty plasmid, and nearly as high as the levels when active Src was coexpressed with HA–IκB (Fig. 3B). Thus, high S5a levels induce the accumulation of IκB. Because human S5a was found to be stochiometrically incorporated into 26*S* particles, overexpression of S5a may cause stabilization due to free forms of S5a, that are not incorporated into the proteasome, and competitively inhibit 26*S* proteasome function [43,44]. We did not note a general increase in multiubiquitinated conjugates under these conditions (data not shown). It is possible that a much greater excess of S5a protein is needed to observe a general accumulation effect. Also, it was reported that yeast S5a exhibits specificity for ubiquitinated substrates, which account for 10% of all

multiubiquitinated proteins in yeast [38]. An open question remains whether the same is true for human S5a. Assuming so, S5a overexpression will induce a very minor change in the total amount of multiubiquitin conjugates.

The S5a promoter responds to signaling proteins

To investigate transcriptional regulation of the human *S5a* gene, we used PCR to isolate 1.2 kb of HeLa genomic DNA upstream of the first exon of the



S5a gene. This sequence contains the start codon, as well as the first exon for the *S5a* gene (Fig. 4A, supplementary Fig. S1). We constructed a luciferase reporter plasmid containing this genomic sequence in vector pGL2-basic. The activity from the S5a reporter plasmid was tested in HEK 293 cells, in comparison with empty pGL2 plasmid. A significant signal from the S5a reporter plasmid was observed (Fig. 4B). We then investigated whether the S5a promoter is regulated by Src and other pathways that are enhanced by Src activity, such as the PtdIns3K/mTOR and the Ras/Raf/ERK pathways [4,45]. As can be seen in Fig. 4C, transcription from the S5a promoter region decreased in response to the Src inhibitor PP1 and the PtdIns3K inhibitor LY294002, whereas the ERK inhibitor, PD98059 had no effect. An effect of PP1 on S5a promoter region was also observed when expressed in HEK 293 cells, but to a lesser extent (Fig. 4D). The results suggest that S5a expression is regulated by Src, the PtdIns3K-mediated pathways, but not by Erk-mediated signaling.

We also compared the transcriptional activity from the S5a promoter region in NIH3T3 versus SrcNIH. As shown in Fig. 4E, transcription from S5a promoter is higher in SrcNIH than in NIH3T3 cells, further supporting the notion that Src upregulates S5a expression.

Analysis of the sequence for transcription factors binding sites (using MATINSPECTOR, at Genomatix-Suite, http://www.genomatix.de/products/MatInspector/MatInspector2.html) led to the identification of a binding site for Tcf/Lef-1 transcription factor, which is involved in the Wnt/β-catenin signal transduction pathway [46] at position −234 to −250 (Fig. 4A). We therefore transfected HEK293 cells with pGL2–S5a-p and myc–β-catenin. As shown in Fig. 5A, myc–β-catenin upregulated transcription from S5a promoter region, 15-fold. To determine whether the Lef-1 binding sequence in the S5a promoter can bind Lef-1, we conducted EMSAs. *In vitro* synthesized Lef-1 bound the Lef-1 sequence in the S5a promoter (Fig. 5B). Two mutated sequences (S5aFOP and Mut, see Experimental procedures) failed to bind Lef-1 (data not shown). Binding of Lef-1 to the S5a promoter was blocked by the addition of excess unlabeled S5aWT probe, but not by the mutant S5aFOP or Mut probes (Fig. 5B and data not shown). Furthermore, the Lef-1 and S5a promoter complex was shifted with a Lef-1 antibody, although Flag antibody did not generate a shift (Fig. 5B). Deletion of the Lef-1 binding site resulted in 25% reduction in β-catenin activation (Fig. 5C). The results suggest that S5a may be a new target gene for the Lef-1/β-catenin pathway. However, it seems that the majority of β-catenin upregulation of S5a promoter

Fig. 1. S5a expression and rescue from PP1-induced apoptosis in Saos-2 cells. (A) Saos-2 cells were seeded on 10 cm plates (1.2×10^6 cells per plate) and treated with 40 μM PP1 for the indicated times. At each time point, adherent and suspended cells were collected and fixed with methanol. After an overnight incubation in −20 °C, cells were resuspended in 1 mL NaCl/P_i, and RNAse A was added to a final concentration of 100 μg·mL^{-1}. Cells were then incubated for 20 min in the dark at room temperature, and propidium iodide solution was added to a final concentration of 50 μg·mL^{-1} (Sigma). FACS analysis was performed to establish the size of sub-G1 population out of 10 000 cells that were counted. Plot indicates percentage of sub-G1 population. Black bars indicate control (untreated); gray bars indicate PP1-treated cells. Average of duplicates is shown. (B) Saos-2 cells were grown on 10 cm plates (1.8×10^8 cells per plate). After 24 h cells were treated with 40 μM PP1 for the indicated times. Cells were lyzed with sample buffer at each time point, boiled and subjected to western blotting. Cleavage of caspase 3 (1 : 1000, Santa Cruz) and S5a (1 : 5000, gift from K. Hendil, August Krogh Institute, Denmark) levels were examined. Actin (1 : 1000, Santa-Cruz) was used as loading control. The graph shows quantification of S5a protein levels normalized to actin. (C) Saos-2 cells were grown on 10 cm plates (1.7×10^6 cells per plate) and 1 day later were transfected with 2.5 μg GFP and 7.5 μg of either $(HA)_3$S5a or empty vector (pcDNA3). Twenty-four hours after transfection cells were treated with PP1 at the indicated concentrations for 72 h. Adherent cells were then trypsinized and subjected to FACS analysis. Rescue was assessed by normalizing the number of green cells at each concentration to the number of green cells in untreated samples (for each plasmid separately). Shown is the average of duplicates from one of two independent experiments. (D) Expression of $(HA)_3$S5a was examined simultaneously in another set of Saos-2 cells. Cells were grown on six-well plates (200 000 cells per well) and were transfected 1 day later with 1.5 μg·well^{-1} $(HA)_3$S5a and 0.5 μg·well^{-1} GFP. Twenty-four hours later cells were treated (in duplicate) with the indicated concentrations of PP1 for 72 h, and then lyzed and subjected to western analysis. The expression of $(HA)_3$S5a and GFP levels were examined. (E) Saos-2 cells were grown on 10 cm plates (1×10^6 cells per plate) for 24 h and later treated with PP1 at the indicated concentrations for 48 h. Cells were harvested and S5a mRNA transcript levels were determined by northern analysis. Ribosomal RNA is shown as control. (F) Saos-2 cells were grown on 10 cm plates (1.8×10^6 cells per plate). Twenty-four hours later cells were treated with 40 μM PP1 for the indicated times. Cells were lyzed and 10 μg protein was used to determine 20*S* proteasome activity, by detecting the cleavage of the fluorescent proteasomal substrate Suc-LLVY-NH_2-Mec, using Chemicon's 20*S* Proteasome Activity Assay Kit. Proteasome-dependent activity was determined by subtracting cleavage of Suc-LLVY-NH_2-Mec in the presence of 25 μM lactacystin from total cleavage (without lactacystin, see Experimental procedures). Proteasome-dependent cleavage of Suc-LLVY-NH_2-Mec constituted up to 95% of total cleavage in untreated cells, and 90 and 70% of total cleavage in cells treated with PP1 for 72 and 96 h, respectively. Proteasome-dependent activity in cells not treated with PP1 was then set as 100% activity. The average of duplicates is shown.

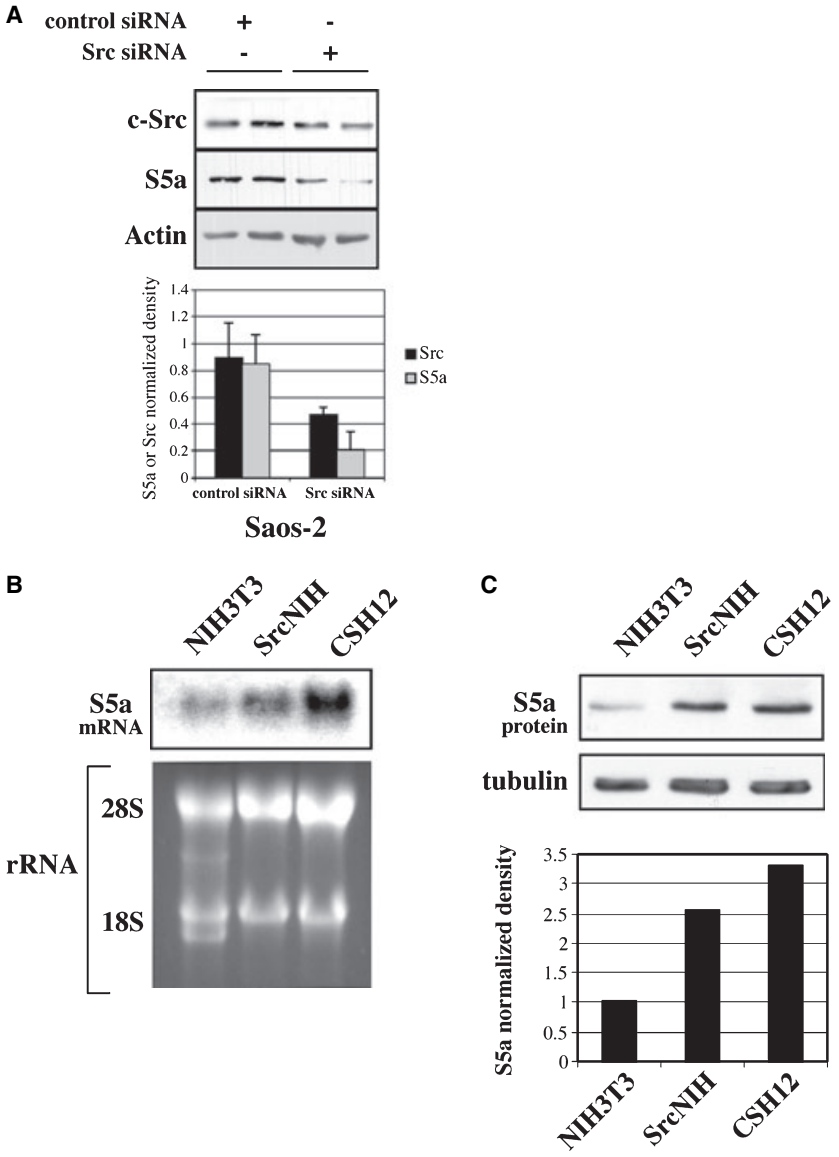


Fig. 2. S5a expression is regulated by Src activity. (A) Saos-2 cells were grown on 24-well plates (80 000 cells per well) in DMEM (without antibiotics). Twenty-four hours later duplicates were transfected with 200 ng GFP and 50 nM of either siRNA against Src (SMARTpool, Dharmacon Inc.) of control siRNA (nontargeting siRNA #1, Dharmacon Inc.), for 48 h. Cells were then lyzed with sample buffer, boiled and subjected to western blotting with S5a antibody, Src antibody and actin antibody as loading control. Quantification of S5a or Src band intensity normalized to actin was performed. The average of duplicates with standard deviation is shown. (B) NIH3T3, SrcNIH (NIH3T3 transformed with active Src Y530F) and CSH12 (NIH3T3 that overexpress the chimeric receptor EGFR/HER-2) cells were grown on 10 cm plates for 24 h (1.5×10^6 cells per plate). Cells were later harvested and S5a mRNA transcript levels were determined by northern analysis. Ribosomal RNA is shown as control for RNA integrity. (C) NIH3T3, SrcNIH and CSH12 cells were grown on 10 cm plates for 24 h (1.5×10^6 cells per plate). Cells were lyzed with sample buffer, boiled, and S5a protein levels were examined by western analysis (1 : 1000, Santa Cruz). Quantification of S5a band intensity normalized to control protein α-tubulin (1 : 20 000, Santa Cruz) is shown. Results are normalized to the level of expression in NIH3T3.

is mediated by mechanisms other than the Lef-1 binding site, which makes a small but significant contribution to this activation.

Binding sites for Hif-1alpha and p53 were also identified on the S5a promoter (Fig. 4A). The effect of Hif-1alpha on transcription from the S5a promoter is threefold (Fig. 6A) with a smaller effect of p53 (~ 1.8-fold) (Fig. 6B). Expression of all of these constructs was confirmed by measuring the ability of each to activate transcription from their own responsive elements (Figs 5,6).

Discussion

In this study, we undertook the identification of potential novel antiapoptotic signaling elements downstream of Src. Towards this end, we performed a genetic screen, to identify genes whose overexpression would rescue cells from apoptosis induced by the Src kinase inhibitor, PP1. A HeLa cDNA expression library was screened in Saos-2 cells. Saos-2 cells are highly sensitive to Src inhibition and undergo massive apoptosis upon treatment with the Src kinase inhibitor PP1 (Fig. 1A,B). Twenty cDNAs surfaced in the screen. These cDNAs were then tested for their ability to rescue from PP1-induced apoptosis, in both Saos-2 and HeLa cells. The purpose of this initial screen was to establish which of the cDNAs had the highest potency to rescue reproducibly from PP1-induced Saos-2 cell death, and study them further. Most prominent was the S5a clone that consistently rescued Saos-2 cells from PP1-induced apoptosis

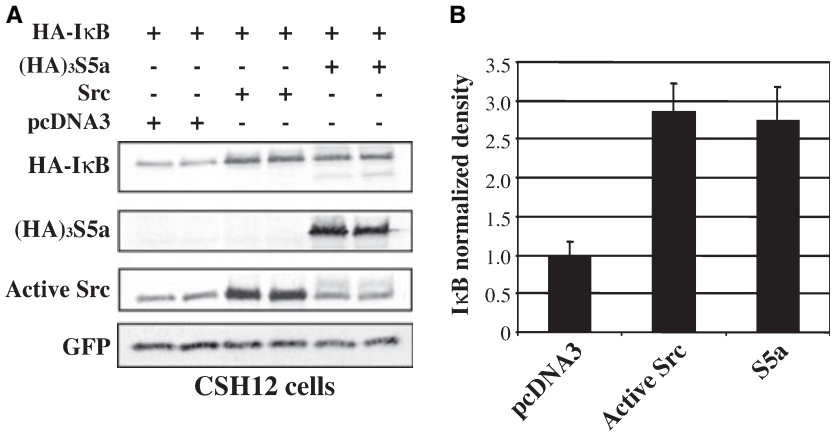


Fig. 3. Overexpression of S5a induces the accumulation of IκB. (A) CSH12 cells were seeded on six-well plates (70 000 cells per well) and 24 h later were transfected with 50 ng HA–IκB cDNA, together with 1 μg $(HA)_3$S5a cDNA, 1 μg active Src cDNA or 1 μg pcDNA3, as indicated. pEGFP cDNA (0.5 μg) was added to each cotransfection mix. Forty-eight hours after transfection cells were lyzed with hot sample buffer and subjected to western analysis. The membrane was probed with antibodies to HA (1 : 1000, Roche), Src (mAb.327), and to GFP as control for transfection efficiencies (1 : 3000, Santa Cruz). (B) Quantification of HA–IκB band intensity normalized to GFP is shown. The average of duplicates is shown; results are normalized to pcDNA3 lanes.

(Fig. 1), suggesting a role in mediating a Src-dependent antiapoptotic pathway. It is probably reasonable to expect that in cells where S5a decreases as a result of an apoptotic stimulus, the forced expression of S5a would be able to compensate for the decreased levels, and therefore rescue these cells from apoptosis. It is likely therefore that Src and perhaps other antiapoptotic proteins, confer their antiapoptotic effect by inducing the elevation in S5a concomitantly to their other antiapoptotic effects.

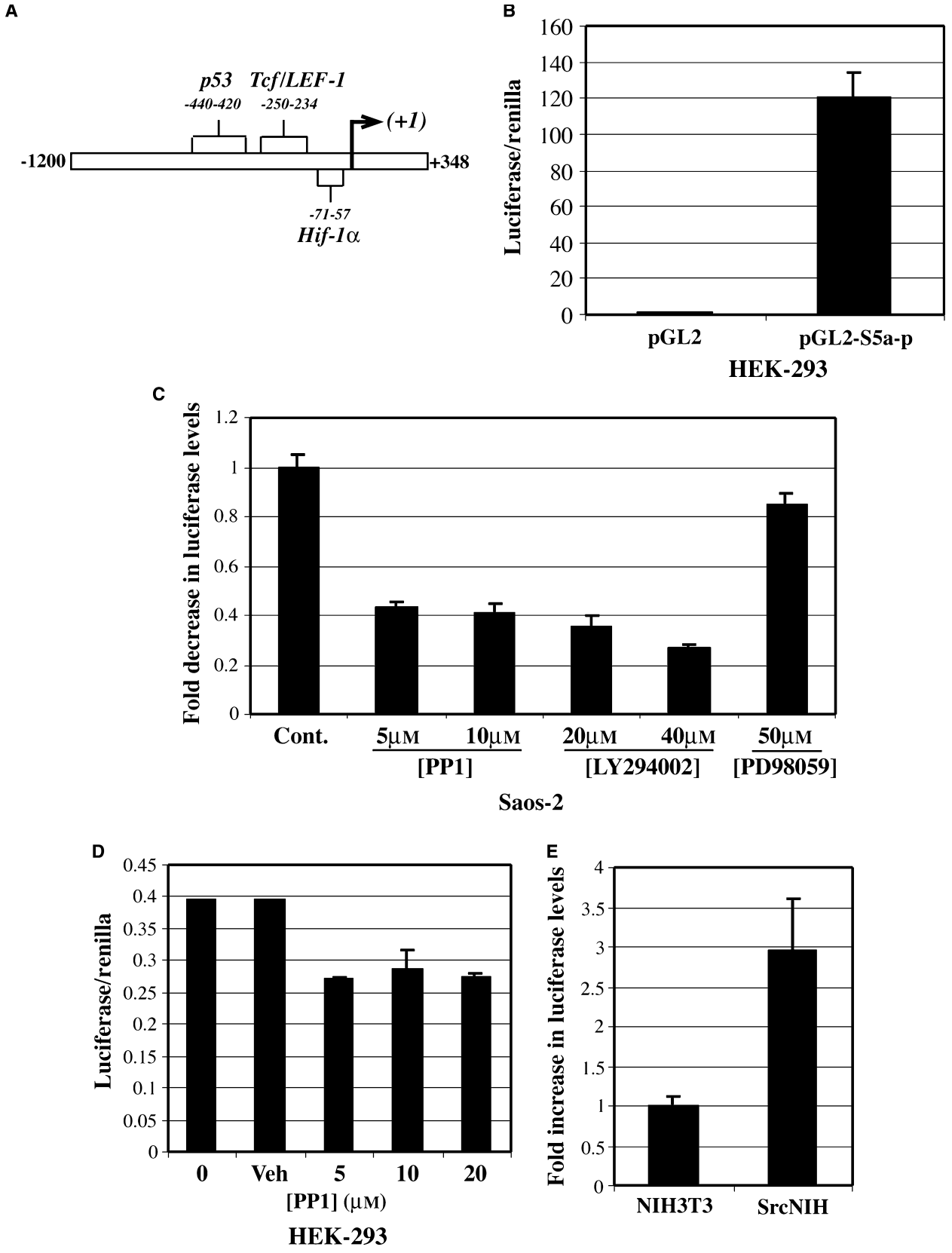
Regulation of S5a expression

We found that the mRNA level of S5a, was consistently decreased in Saos-2 cells treated with PP1 (Fig. 1E). Furthermore, the mRNA levels of S5a were higher in cell lines that express highly active Src as in SrcNIH and CSH12 cells (Fig. 2B). S5a protein expression decreased in PP1-treated Saos-2 cells (Fig. 1B), probably as a result of the decrease in mRNA levels. siRNA against Src also induced a small but reproducible decrease in S5a (Fig. 2A). These results suggest that Src positively regulates S5a at the transcriptional level. Downregulation in S5a levels during PP1-induced apoptosis may result in inhibition of proteasomal degradation, leading to the accumulation of proteins that may further facilitate the apoptotic process. We show that 20*S* activity was reduced in PP1-treated Saos-2 cells at times when S5a was downregulated (Fig. 1F).

Overexpression of S5a resulted in the accumulation of IκB (Fig. 3), suggesting a role for S5a in the turnover of this well-known proteasomal substrate. Indeed, while this study was in preparation, Arlt *et al.* found that IEX-1, a stress-induced proapoptotic protein, which attenuates the NFκB pathway by interfering with IκB turnover, inhibits S5a expression [47]. The physiological relevance of IκB stabilization by overexpression of S5a and the implications for the apoptotic process remain to be investigated.

In order to gain more insight into the regulation of S5a expression we cloned the promoter of S5a. Cloning of the S5a promoter region revealed that S5a is under the transcriptional regulation of components of various pathways affecting apoptosis. Transcriptional activity from the S5a promoter decreased in response to Src inhibitor, PP1. Apparently, the extent of this decrease is cell-type specific (Fig. 4C,D). Our finding that the PtdIns3K inhibitor, LY294002, inhibits the S5a promoter suggests that the antiapoptotic pathway mediated by PtdIns3K /PKB (Akt)/mTor, which is partially regulated by Src activity, may also play an important role in regulating S5a. In addition, transcription from S5a promoter was higher in cells in which Src is overexpressed (Fig. 4E).

While trying to identify specific elements that confer this regulation, we found that a binding site for the Tcf/Lef-1 transcription factor, a key factor of the Wnt signaling cascade, was present at position −244 of the promoter. The Wnt pathway regulates the ability of the proto-oncogene β-catenin to activate the transcription of specific target genes like c-*myc* and cyclin D1. β-Catenin is a multifunction protein, having important roles in both signaling and cell–cell adhesion. It is


A
p53
Tcf/LEF-1
-440-420
-250-234
(+1)
-1200
+348
-71-57
Hif-1α
B
Luciferase/renilla
160
140
120
100
80
60
40
20
0
pGL2
pGL2-S5a-p
HEK-293
C
Fold decrease in luciferase levels
1.2
1
0.8
0.6
0.4
0.2
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Cont.
5μM
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[PP1]
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Saos-2
D
Luciferase/renilla
0.45
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[PP1] (μM)
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4
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2
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0.5
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NIH3T3
SrcNIH

found either bound to cadherins, or as a very small free pool, which is transcriptionaly competent [48,49]. The free form of β-catenin has a very short half-life, because of its phosphorylation by the APC/Axin/GSK3 complex that signals its ubiquitination and degradation by the proteasome. Upon Wnt signaling, β-catenin is stabilized, and a transcriptionaly active complex of Tcf/Lef and β-catenin is formed, leading to transcription of its target genes [46]. Nevertheless, the cadherin-bound fraction, which represents the majority of the β-catenin protein in the cell, is highly stable [48]. Therefore, the regulation of β-catenin signaling is due to changes in subpools of this protein, rather then overall change in its total level.

We tested the effect of β-catenin on transcription from the S5a promoter region. We found that β-catenin induced a 15-fold upregulation of transcription from the S5a promoter (Fig. 5A). Furthermore, we have shown that the Lef-1-binding consensus sequence in the S5a promoter can bind Lef-1 *in vitro* (Fig. 5B). Deletion of the Tcf/Lef-1 binding site resulted in up to 25% decrease of β-catenin-induced activation of the promoter (Fig. 5C). Two other potential sites, for Hif-1α and p53, were found using sequence analysis and tested. Hif-1α had a smaller effect than β-catenin on S5a promoter, whereas p53 had a small effect on the promoter activity (Fig. 6). Therefore, we conclude that S5a is new target gene for β-catenin/Lef-1 pathway, although the majority of S5a activation by β-catenin probably occurs through mechanisms other than Lef-1 binding with the Tcf/Lef binding site. We have recently found [50] that Src activity enhances rates of protein synthesis, leading to elevated levels of expression of β-catenin. Src activates the eIF4E translation machinery by PtdIns3K/mTOR and MEK/ERK^{MAPK} pathways. The result is elevation in cap-dependent translation, which causes enhanced synthesis of β-catenin. The enhancement in β-catenin synthesis leads to its nuclear accumulation and elevation in its transcriptional activity, inducing target genes such as cyclin D1 and c-*myc*. Inhibition of Src by PP1 resulted in decreased protein translation. As a result, nuclear β-catenin levels decreased and β-catenin transcriptional activity was inhibited [50].

We suggest that this same mechanism may contribute to the regulation of S5a by β-catenin.

A role for S5a in apoptosis?

In this study, we have shown that a subunit of the proteasome is under the direct regulation of signaling pathways. Because the proteasome is involved in numerous cellular pathways, it is not surprising that certain signaling pathways regulate its subunits, at different stages during the life course of the cell. The subunit composition of the proteasome seems to vary depending on specific conditions [33,51,52], perhaps reflecting a mechanism for adjusting proteasomal degradation in response to a changing environment. We show that S5a levels decrease during Src inhibitor-induced apoptosis, and that Src in fact regulates this subunit mediated pathways. A possible outcome for S5a downregulation could be in selective accumulation of proteasomal substrates, which enhance apoptosis. Therefore, restoring S5a levels by Src and other antiapoptotic signals may lead to renewed degradation by the proteasome. Evidence from the literature suggests that S5a is one of several ubiquitin receptors, each selective towards certain proteasomal substrates. This concept stems from the confusion that surrounded the

Fig. 4. Transcription from S5a promoter is affected by pro- and antiapoptotic stimuli. (A) 1.2 kB of the 5′ region of the *S5a* gene was cloned using PCR on HeLa genomic DNA. Diagram represents the cloned sequence (not to scale). Transcription start site is indicated by arrow and designated as +1. The cloned sequence contains the first exon of the *S5a* gene. Putative binding sites positions for Tcf/Lef-1, p53 and Hif-1α transcription factors are indicated. These binding sites were identified using MATINSPECTOR at GenomatixSuite (http://www.genomatix.de/products/MatInspector/MatInspector2.html). For nucleotide sequence see Fig. S2. (B) HEK 293 cells were seeded on six-well plates (220 000 cells per well) and 24 h later were transfected with 1.8 μg·well^{-1} of either S5a reporter plasmid (pGL2–S5a-p) or empty pGL2 and 0.2 μg·well^{-1} of CMV–Renilla as internal control vector. Cells were lyzed after 48 h and luciferase activity was measured. Results are normalized to Renilla activity. The average of duplicates is shown. (C) Saos-2 cells were seeded on six-well plates (150 000 cells per well) and 24 h later were transfected with 2 μg·well^{-1} of S5a reporter plasmid and 0.5 μg·well^{-1} of CMV–β-galactosidase as internal control vector. Twenty-four hours after transfection cells were treated for 24 h with PP1 (5, 10 μM), LY294002 (20, 40 μM), and PD98059 (50 μM). Cells were lyzed and luciferase activity was measured and normalized to β-galactosidase activity. Fold decrease in luciferase levels relative to untreated is shown (average of duplicates). Shown is a representative of at least three independent experiments. (D) HEK 293 cells were seeded on six-well plates (200 000 cells per well) and 24 h later were transfected with 0.15 μg·well^{-1} of CMV–Renilla and 1.85 μg·well^{-1} of S5a reporter plasmid. 24 h after transfection cells were treated with PP1 at the indicated concentrations for 24 h. Cells were lyzed and luciferase activity was measured. Results are normalized to Renilla activity. Average of duplicates is shown. Shown is a representative of two independent experiments. (E) Activity from S5a promoter in NIH3T3, SrcNIH was measured. Cells were seeded on six-well plates (70 000 cells per well) and 1 day later were transfected with 1.85 μg·well^{-1} of S5a reporter plasmid (pGL2–S5a-p) and 0.15 μg·well^{-1} of CMV–Renilla (the internal control vector). Cells were lyzed and luciferase activity was measured. Results are normalized to Renilla activity. Fold increase in luciferase levels relative to NIH3T3 is shown (average of duplicates). Shown is a representative of three independent experiments.

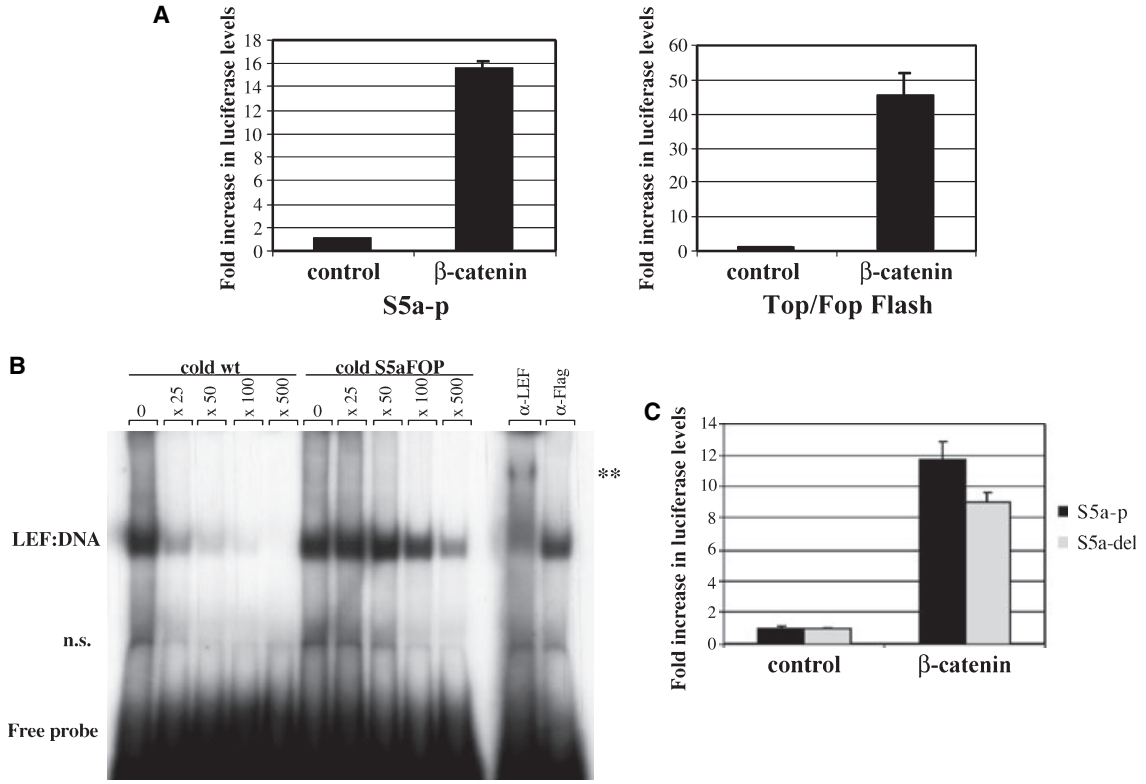

Fig. 5. S5a is a target of β-catenin/Lef-1 pathway. (A) HEK 293 cells were seeded on six-well plates (200 000 cells per well) and 24 h later were transfected as follows: 0.15 μg·well^{-1} of CMV–Renilla (internal control), 0.37 μg·well^{-1} of S5a reporter plasmid and 1.48 μg·well^{-1} of either β-galactosidase (empty vector-control) or myc–β-catenin. As control for β-catenin expression, a set of cells was transfected with 1 μg·well^{-1} of either TOPFlash or FOPFlash cDNA, and 1 μg·well^{-1} of myc–β-catenin or 1.48 μg·well^{-1} of β-galactosidase (empty vector control). Cells were lyzed after 48 h and luciferase activity was measured. Results are normalized to Renilla activity. TOPFlash activity was normalized to FOPFlash activity. Fold increase in luciferase levels relative to control is shown (average of triplicates). Shown is a representative of three independent experiments. (B) EMSA of the S5a promoter. Duplex oligonucleotides (probes) containing the Lef-1 binding sequence of the S5a promoter (WT) were labeled with [^{32}P-dCTP] and incubated with *in vitro* translated Lef-1 and increasing amounts of unlabeled WT of S5aFOP (contains mutated Lef-1 binding site) probes were used as competitors. Also, wild-type labeled probe was incubated with *in vitro* translated Lef-1 and either anti-Lef-1 or anti-Flag serum. Protein–DNA complexes were analyzed by electrophoresis and visualized by exposing the gel to film. Asterisks indicate Lef-1/anti-Lef-1 complex; ns, nonspecific band. (C) Tcf/Lef-1 core binding sequence (5′-TTCAAAG-3′) was deleted using QuickChange site-directed mutagenesis kit (Stratagene), using primers as described in Experimental procedures. HEK 293 cells were seeded in six-well plates (200 000 cells per well). One day later cells were cotransfected with 0.37 μg·well^{-1} of either S5a promoter containing the deletion (S5a-del), or S5a-p, together with 0.15 μg·well^{-1} of CMV–Renilla (internal control) and 1.48 μg·well^{-1} of myc–β-catenin or β-galactosidase (control). Forty-eight hours after transfection, cells were lyzed and luciferase activity was measured as described. Results are normalized to Renilla activity (average of triplicates). Shown is a representative of two independent experiments.

role of S5a as the multiubiquitin-binding subunit of the proteasome. Whereas most proteasomal genes are essential, yeast with mutant S5a is viable, with mild phenotype [25]. Indeed, we have also found that knockdown of S5a in Saos-2 cells by siRNA to 70% of its initial level, did not have an effect on cell viability or sensitivity to PP1-induced apoptosis (supplementary Fig. S2). Furthermore, Rpn10 does not cross-link to tetraubiquitin chains while incorporated into 26*S* proteasomes [34]. Thus, it was suggested that S5a shares its role in multiubiquitin recognition with other proteins. Such proteins were indeed discovered. Rad23 and Dsk2 were shown to bind both ubiquitin chains and the proteasome, through distinct domains [53,54]. Triple deletion of S5a, Rad23 and Dsk2 in yeast showed greater accumulation of multiubiquitinated proteins then either single or double mutants [54–56]. Accumulation of proteasomal substrates in single Rad23 or Dsk2 mutants is exacerbated by further deletion of Rpn10-UIM [36]. Therefore, it seems that in yeast, S5a, Rad23 and Dsk2 are partially redundant [57,58]. In addition, S5a deletion in other

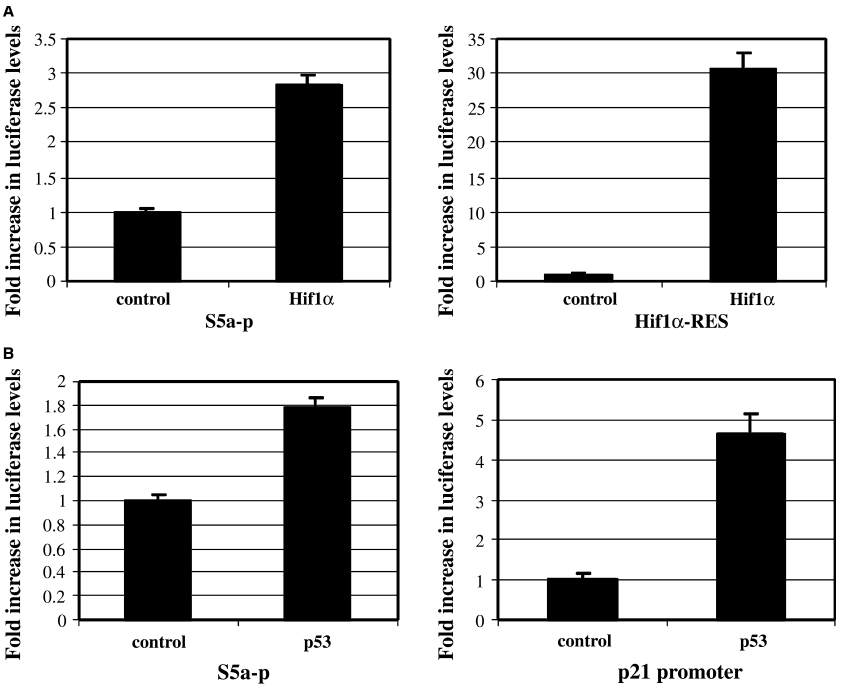

Fig. 6. Hif-1α and p53 affect S5a promoter. HEK 293 cells were seeded on six-well plates (200 000 cells per well) and 24 h later were transfected as follows: 0.15 μg·well^{-1} of CMV–Renilla (internal control), 0.37 μg·well^{-1} of S5a reporter (A, B left), Hif-1α responsive element (A, right), p21 promoter (promoter of p21 upstream of luciferase) (B, right), and 1.48 μg·well^{-1} of either β-galactosidase (empty vector control), Hif-1α (A) or p53 (B). Cells were lyzed after 48 h and luciferase activity was measured. Results are normalized to Renilla activity. Fold increase in luciferase levels relative to control is shown (average of triplicates). Shown is a representative of three independent experiments.

organisms resulted in stabilization of specific groups of proteins [25,59–61], and overexpression of S5a induced accumulation of certain ubiquitinated proteins [43,62], most probably due to the competition for proteasome binding site by the free S5a [43]. It has been shown [37,38] that yeast S5a is selective towards several ubiquitinated substrates that were not degraded by Rad23, Dsk2 and Cdc48. Thus it seems that in the ubiquitin–proteasome pathway, selectivity can be determined by a group of multiubiquitin-binding proteins.

Based on these findings and our results, we would like to purpose that during the course of apoptosis, downregulation of S5a levels can be counteracted by signaling from antiapoptotic proteins, such as Src and the Lef-1/β-catenin pathway. This restoration in S5a levels may ensure continued proteasomal activity during apoptosis, and lead to degradation of specific S5a substrates that would otherwise accumulate. The continued search for signals that regulate S5a, as well as identifying its repertoire of substrates, is essential to better understand its precise role in proteasomal degradation and cell survival.

Experimental procedures

Cell culture and materials

Saos-2 cells were grown in McCoy-5A medium supplemented with 10% fetal bovine serum. NIH3T3, SrcNIH (NIH3T3 transformed with active Src-Y530F) [63] and CSH12 (NIH3T3 which overexpress the chimeric receptor $EGFR_{out}$/$HER\text{-}2_{in}$) [11,42] cells, as well as HeLa and HEK 293 cells were grown in DMEM supplemented with 10% fetal bovine serum. All media were supplemented with penicillin and streptomycin. PP1 was from Synthos (Rechovot, Israel) and synthesized as described previously [64].

Transient transfections

Transient transfections of Saos-2 and HeLa cells were performed with poly(ethylenimine) reagent (Sigma, St Louis, MO) as described previously [65], or with Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transfections of CSH12, NIH3T3 and SrcNIH cells were performed with Fugene 6 (Roche, Mannheim, Germany), according to the manufacturer's instructions. Transfections of HEK 293 cells were performed with $CaPO_4$ as described previously [66].

Identification of antiapoptotic target genes of Src by a genetic screen

A HeLa cDNA library was screened in Saos-2 cells. The cDNA library was cloned into vector pEBS7 [67]. The library was transfected into Saos-2 cells and transfected cells were selected for by growth in the presence of Hygromycin. Cells underwent two rounds of treatment with 40 μM PP1 for 72 h and were allowed to recover for 3 days in between treatments. After this first round of selection with PP1, surviving clones were pooled and plasmid DNA was extracted [68]. DNA was amplified in electrocompetent *E. coli DH5a*

cells, and transected back into Saos-2 cells for another round of selection with 40 μM PP1, in order to enrich for the resistant clones. After the second round of selection, plasmid DNA was extracted from each clone separately and amplified in electrocompetent *E. coli* cells. Bacteria were grown on Luria–Bertani + Ampicillin. Colonies, which contained the library plasmid, were identified. Library cDNAs were pulled out of vector pEBS7 and ligated into the *Xba*I site in Bluescript plasmid pBSKSII. The library cDNA on the subclone was sequenced using T7 and T3 primers, and results were compared with known sequences using the NCBI-BLAST program.

HA-S5a rescue assays

Library S5a was cloned in frame with a N-terminus $(HA)_3$ tag in vector pcDNA3. Expression of this construct was measured in Saos-2 and HeLa tet-off cells. Saos-2 (1.7×10^6 cells per plate) and HeLa tet-off cells (2×10^6 cells per plate) were seeded on 10 cm plates. Twenty-four hours later cells were cotransfected with 2.5 μg of pEGFP and either 7.5 μg HA_3S5a or pcDNA3 (empty vector) DNA, using poly(ethylenimine) reagent. Twenty-four hours after transfection, cells were treated with 20 or 40 μM of the Src kinase inhibitor PP1, for 72 (Saos-2) or 48 h (HeLa tet-off). Cells were washed with NaCl/P_i (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 0.77 M NaCl) and adherent cells were harvested using 1 mL of trypsin-EDTA. Medium (5 mL) was added and cells were centrifuged for 5 min at 1200 ***g*** (Sigma 4K10 centrifuge with Nr11140 rotor). Cells were resuspended in 600 μL NaCl/P_i and were analyzed by Fluorescence-Activated Cell Sorter (FACS Calibu Becton-Dickinson, Franklin Lakes, NJ). Green cells from each sample were counted for 3 min at low speed. The number of green cells for each concentration was normalized to the number of green untreated cells for each plasmid.

Northern blotting

Cells were grown on 10 cm plates and were treated with PP1 for 24 or 48 h as indicated in the results. RNA was prepared using trizol reagent (Sigma). Total RNA (10 μg) was denatured and loaded on a 1% agarose gel containing 2 M formaldehyde and ethidium bromide. After electrophoresis the gel was photographed to verify equal loading and quality of RNA. Following capillary blotting onto nylon membrane (Hybond XL, Amersham, Little Chalfont, UK) the membrane was again photographed to ensure equal transfer, and RNA was cross-linked to the membrane. The blot was hybridized for 16 h at 42 °C with ^{32}P-labeled DNA probe, prepared with the Rediprime™ II kit (Amersham). The blot was later washed three times with 1 × NaCl/Cit, 0.1% SDS at 50 °C, and was exposed to MS sensitive film (Kodak). An *Xba*I fragment from pcDNA3 containing S5a was used to detect S5a transcript.

Immunoblotting

Cells were washed twice with NaCl/P_i, then lyzed with sample buffer (40% glycerol, 0.2 M Tris pH 6.8, 20% β-mercaptoethanol, 12% SDS, Bromophenol Blue) and boiled for 5 min. Lysates were loaded on Whatman no. 3M paper clips. The paper clips were stained with Coomassie Brilliant Blue and washed five times for 6 min with destain solution (20% methanol, 7% acetic acid). Stain was extracted from the paper clips with 3% SDS and protein amounts were determined using a BSA calibration curve, reading the absorbance at 590 nm. Equal amounts of protein were then subjected to SDS/PAGE [69], and transferred to nitrocellulose. Membranes were blocked with NaCl/TrisT (170 mM NaCl, 10 mM Tris pH 7.5, 0.2% Tween-20) containing 5% low fat (1%) milk for 30 min, followed by overnight incubation with primary antibodies (indicated in figure legends). Membranes were then washed with NaCl/TrisT and immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated secondary antibodies.

Proteins were visualized using ECL. Quantification of band intensity was performed using NIH IMAGE software. Antibodies: human S5a antibody was a gift from K. Hendil (August Krogh Institute, Copenhagen, Denmark). Mouse S5a (pUbR-2) antibody was from Santa Cruz (Santa Cruz, CA), β-catenin from Transduction Laboratories (Lexington, MA), and HA antibody from Roche. Antibodies to actin, tubulin and GFP were from Santa Cruz. Antibody to Src was obtained from mAb 3.27 hybridoma. All secondary antibodies were from Jackson Immuno-Research (West Grove, PA).

siRNA experiments

RNA interference experiments were performed using commercial siRNA against c-Src (Src SMARTpool siRNA reagent, Dharmacon Inc., Lafayette, CO), and siRNA against S5a (OnTargetPlus S5a siRNA, Dharmacon Inc.). As a control, nontargeting siRNA was used (siCONTROL, Non-targeting siRNA #1, Dharmacon Inc.).

Saos-2 cells were seeded in 24-well plate (80 000 cells per well) in DMEM without penicillin/streptomycin. Twenty-four hours later cells were transfected according to the manufacturer's instructions with 50 nM of either Src or control siRNA, or 100 nM of S5a or control siRNA, together with 200 ng GFP as transfection control. Transfections were performed using Lipofectamine 2000 (Invitrogen).

In S5a siRNA experiments where cell sensitivity to PP1 was measured, Saos-2 cells were seeded in 96-well plates, in quadruplicates(10 000 cells per well). Cells were transfected 1 day later with 100 nM S5a or control siRNA. Twenty-four hours later, cells were treated with the indicated concentrations of PP1. Viability of cells was assessed using

methylene blue assay, at the indicated times of PP1 treatment. The cells were fixated in 0.5% formaldehyde for 10 min at room temperature, washed with 0.1 M sodium borate, pH 8.5, stained with 1% methylene blue for 1 h then washed with double-distilled water. Two hundred microliters of 0.1 M HCl was used to dilute the cell-bound dye. Absorbance was measured at 630 nm. Experiments were performed three times, with at least four replicates in each experiment.

20*S* proteasomal activity assay

Saos-2 cells were grown for 24 h then treated with 40 μM PP1 for 72 and 96 h. Cells were lyzed using 1% Triton lysis buffer (50 mM Hepes pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100) at 4 °C. Lysates were centrifuged at maximum speed for 15 min, 4 °C. Sup was collected and protein concentration was determined. The 20*S* Proteasome Activity Assay Kit (Chemicon, Temecula, CA) was used to measure the 20S proteasome activity. The assay is based on detection of the fluorophore 7-amino-4-methylcoumarin (NH_2-Mec) after cleavage from the labeled substrate Suc-LLVY-NH_2-Mec. Ten micrograms of protein was incubated with 25 μM Suc-LLVY-NH_2-Mec, in 100 μL of reaction mixture for 1 h at 37 °C. The free NH_2-Mec fluorescence was quantified using 380/460-nm filter set in a fluorometer (Tecan-Safire, Neotec, Austria). To determine the fraction of cleavage that is proteasomal dependent, a different set of samples (10 μg) was simultaneously incubated for 15 min at room temperature with 25 μM of the proteasome inhibitor, lactacystin, prior to incubation with Suc-LLVY-NH_2-Mec. In untreated cells, proteasome-dependent cleavage of Suc-LLVY-NH_2-Mec constituted up to 95% of total cleavage. In PP1-treated cells, proteasome-dependent cleavage of Suc-LLVY-NH_2-Mec constituted 90 and 70% of total cleavage in 72 and 96 h, respectively. Proteasomal-dependent activity was calculated by subtracting the readings in the presence of lactacystin from readings without the inhibitor. Proteasome-dependent activity in cells not treated with PP1 was then set to 100%.

Cloning of the S5a promoter region

A QIAamp DNA minikit (Qiagen, Hilden, Germany) was used to isolate whole genomic DNA from HeLa cells. Oligonucleotide primers are shown in supplementary Fig. S2. PCR was performed using these two primers, to obtain a 1.2 kb product. The product was ligated into vector pDrive using the Qiagen Cloning Kit and sequenced using T7 and SP6 primers. Results were compared with known sequences in the database using NCBI-BLAST program. A *Sac*1/*Mlu*1 restriction fragment from vector pDrive was ligated into vector pGL2 basic (Promega, Madison, WI).

Reporter assays

Saos-2 cells were seeded on six-well plates (150 000 cells per plate) and were transfected 1 day later. Total DNA for transfection was 2.5 μg·well^{-1}, comprising 2 μg of S5a reporter plasmid and 0.5 μg of β-galactosidase as an internal control vector. One day after transfection cells were treated for 24 h with PP1, LY294002 or PD98059 using concentrations as indicated. In the experiments utilizing NIH3T3 and SrcNIH cells, the cells were seeded (70 000 cells per well) on six-well plates, and were transfected 1 day later. Total DNA for transfection was 2 μg·well^{-1}, comprising of 1.85 μg of S5a reporter plasmid (pGL2–S5a-p) and 0.15 μg of CMV–Renilla as an internal control vector. Cells were lyzed 48–72 h after transfection. HEK 293 cells were seeded on six-well plates (200 000 cells per plate) and were transfected 1 day later. Total DNA for transfection was 2 μg·well^{-1}, comprising of 0.15 μg CMV–Renilla (internal control), 0.37 μg S5a reporter plasmid and 1.48 μg of either β-galactosidase (as control vector) p53 (gift from Y. Haupt, Hebrew University, Israel), Hif-1alpha or myc–β-catenin cDNAs. As control, a different set of HEK 293 cells was transfected with the same ratios of known target promoters of these cDNAs: p21 promoter upstream to luciferase (as control for p53 expression, gift from Y. Haupt), Hif-1alpha responsive element. As control for β-catenin expression, the TOP/FOP Flash system was used (1 μg·well^{-1} of TOP/FOP, and 1 μg·well^{-1} of myc–β-catenin or 1.48 μg·well^{-1} of β-galactosidase cDNAs). The TOPFlash reporter contains three Tcf/Lef/β-catenin DNA-binding sites and a minimal promoter, upstream of luciferase. In the FOPFlash reporter these three sites are mutated. Normalization of transcription from the TOPFlash reporter to that from the FOPFlash reporter gives the net effect of the Wnt/β-catenin pathway on transcription [70]. Cells were lyzed after 48 h. In all reporter experiments, cells were lyzed with passive lysis buffer (Promega) and luciferase activity was measured using the Dual luciferase reporter assay system kit (Promega). β-Galactosidase activity was separately measured where it was used as internal control. Results were normalized either to β-galactosidase or Renilla activities.

Deletion of the Tcf/Lef-1 core-binding site (5′-TTC AAAG-3′) was performed using QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA), with the following primers: Sense, 5'-GAATCGACACAGCACTGTT CCTCCATGGCTCC-3'; antisense, 5'-GGAGCCATGGA GGAACAGTGCTGTGTCGATTC-3'. After sequencing, the S5a promoter containing the deletion was re-cloned into pGL2-basic, and activation of this construct (S5a-del) by β-catenin was measured in HEK 293 cells as described above.

In vitro translation and DNA-binding analysis

In vitro translated Lef-1 was prepared by using a coupled transcription and translation kit (Promega). Lef-1 was

expressed from pcDNA3–Lef-1 construct (gift from U. Gat, Hebrew University, Jerusalem, Israel). Efficiency of translation was determined by a reaction containing [^{35}S]methionine, followed by SDS/PAGE and western blotting.

For EMSA, either 1 or 3 μL of *in vitro* translated Lef-1 was incubated with ^{32}P-labeled duplex oligonucleotide probes: S5aWT, sequence containing −263 to −230 of the promoter (sense 5′-CCGGAATCGACACAGCACTTTCAAAGGTTCCTCC-3′; antisense 5′-AGCTATGGAGGAACCTTTGAAAGTGCTGTGTCG-3′), S5aFOP (sense 5'-CCGGAATCGACACAGGCCAAAGGGTCCTCC-3', antisense 5'-CATGGAGGACCCTTTGGCCTGTGTCGATTCC-3') substitution of consensus Lef-1 site with mutant site based on FOPFlash sequence.

Mut (sense 5′-CCGGAATCGACACAGCACTTTGCTAGGTTCCTCC-3′, antisense 5′-AGCTATGGAGGAACCTAGCAAAGTGCTGTGTCG-3′) substitution of nucleotides −242 to −240 CAA to GCT.

The wild-type and mutated Lef-1 binding sites are underlined.

The binding reactions were performed as described previously [71]. Briefly, 2 ng duplex oligonucleotide were labeled with [^{32}P-dCTP], then incubated for 30min with the *in vitro* translated Lef-1 in 20 mM Hepes, pH 7.9, 75 mM NaCl, 1 mM dithiotreitol, 10% glycerol, 0.1 mg BSA, 10 μg·mL^{-1} salmon sperm DNA. DNA–protein complexes were electrophoresed in 5% native polyacrylamide gels and visualized by exposing the dried gel to film. Binding reactions were also carried out with 1 μg of anti-Lef-1 (Upstate) or anti-Flag (Sigma) sera.

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Supplementary material

The following supplementary material is available online:

Fig. S1. Cloning of the S5a promoter region.

Fig. S2. Effect of S5a siRNA on viability and sensitivity to PP1 in Saos-2 cells.

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