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SEN1 participates in the dynamic regulation of Elk-1 sumoylation.

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Abstract

The modification of proteins with SUMO plays an important role in determining their functional properties. Importantly though, sumoylation is a highly dynamic process enabling transient responses to be elicited. This dynamism is controlled by two competing conjugating and deconjugating activities. The latter activity is mediated by the SENP family of SUMO-specific proteases. The transcription factor Elk-1 undergoes rapid desumoylation following cellular stimulation with growth factors, and this contributes to its conversion from a SUMO-dependent repressor to a potent transcriptional activator. Here we demonstrate an important role for SENP1 in the desumoylation of Elk-1 and therefore an integral role in determining the Elk-1-dependent transcriptional programme. Amongst the SENPs, Elk-1 preferentially forms a complex with SENP1. This preferential binding is reflected by the higher efficiency of SENP1 in promoting Elk-1 transactivation. Moreover, depletion of SENP1 causes a reciprocal effect and reduces the transactivation properties of Elk-1. Partial redundancy of function with SENP2 is revealed by combinatorial knockdown studies. Importantly, depletion of SENP1 also reduces the activation of the Elk-1 target gene, *c-FOS*. Together, these results therefore reveal an important role of SENP1 in the regulation of Elk-1-mediated gene expression in response to mitogenic signaling cues.

Introduction

Protein sumoylation is being increasingly recognised as an important post-translational modification. Both cytoplasmic and nuclear proteins have been shown to be SUMO substrates but the majority of substrates fall into the latter class, with one major function of SUMO being in transcriptional control [reviewed in 1-3]. Importantly, protein sumoylation is a dynamic process. SUMO is added to proteins through the action of an enzymatic cascade involving an E1 SUMO activating enzyme, and the E2 SUMO conjugating enzyme Ubc9 which transfers the SUMO to substrates. In several cases, E3 ligases can act as molecular bridges to facilitate the action of Ubc9 and promote substrate sumoylation [reviewed in 2, 4]. Conversely, sumoylation can be reversed through the activity of SUMO-specific proteases (SENPs) which cleave SUMO from substrates [reviewed in 5-6]. There are currently six known SENPs in humans, which are thought to act in part in distinct subnuclear structures with SENP1 and 2 localised to the nuclear pore complex and nucleoplasm, whereas SENP3 and SENP5 are localised to the nucleolus, at least under normal conditions [reviewed in 5-6].

Protein sumoylation can be controlled through the activity of protein kinase cascades in response to extracellular signals [reviewed in 7]. For example, HSF-1 sumoylation status is enhanced following heat shock-mediated phosphorylation [8]. Similarly, sumoylation is promoted in several other proteins following signal-dependent phosphorylation [reviewed in 9-10]. In contrast, following mitogen-dependent activation of the ERK MAP kinase pathway, reduced sumoylation of the ETS-domain transcription factor Elk-1 occurs [11]. Desumoylation of Elk-1 is a component of the transcriptional activation process that is orchestrated by this transcription factor in response to ERK pathway signalling. On the contrary, anisomycin-dependent activation of the p38 MAP kinase cascade does not result in Elk-1 desumoylation, although the activation component of the signalling pathway still occurs [13]. Thus Elk-1 desumoylation is an important mechanism that dictates the outcome of MAP kinase signalling through Elk-1 and its target genes and helps set the amplitude of the resulting transcriptional response.

In this study, we further investigated how mitogenic signalling promotes Elk-1 desumoylation and hence the functional outcome in terms of target gene expression. We sought to identify which SENP is involved in the desumoylation process. Through a combination of overexpression and loss of function approaches, we have identified SENP1 as the predominant SENP acting on Elk-1 and hence placing SENP1 as a key player in determining the transcriptional outcomes to mitogenic signalling.

Materials and Methods

Plasmid constructs

The following plasmids were used in mammalian cell transfections. pRL-TK-Renilla (Promega), pG5-E1B-Luc, pAS1561 [encoding GAL-Elk(1-428)^{WT}], pAS2058 [encoding GAL-Elk(1-428)^{K2R}], pAS383 [encoding Flag-His-tagged Elk(1-428)^{WT}] [11] and pCDNA3-HA-SUMO-2 [14] have been described previously.

pAS1138 (pCDNA3-FlagB-SEN1^{WT}), pAS1140 (pCDNA3-FlagB-SEN2^{WT}), pAS1139 (pCDNA3-FlagB-SEN1^{C602S}), and pAS1141 (pCDNA3-FlagB-SEN2^{C547S}) were constructed by inserting EcoRI-NotI fragments from pAS1134, pAS1136, pAS1135, and pAS1137 respectively, into pCDNA3-FlagB (pAS2236). pAS1134, pAS1135, pAS1136, and pAS1137 were constructed by inserting EcoRI-SalI fragments from pCMV-Flag-SEN1^{WT}, pCMV-Flag-SEN1^{C602S}, pCMV-Flag-SEN2^{WT} and pCMV-Flag-SEN2^{C547S} respectively (WT versions kindly provided by Edward Yeh; [15]) into the same sites in pGEX4T3. pCMV-Flag-SEN1^{C602S} and pCMV-Flag-SEN2^{C547S} were constructed by Quikchange mutagenesis (Stratagene) using the template and the primer pair combinations; pCMV-Flag-SEN1^{WT} & ADS2518/2519 and pCMV-Flag-SEN2^{WT} & ADS2520/2521 respectively. pAS1142 (pCDNA3-FlagC-SEN3^{WT}), pAS1143 (pCDNA3-FlagC-SEN3^{C532S}), were constructed by inserting BamHI-XhoI fragments from pCDNA3-RGS-SEN3^{WT} and pCDNA3-RGS-SEN3^{C532S} respectively (kindly provided by Edward Yeh; [15]), into pCDNA3-FlagC (pAS2237).

For bacterial expression, GST (pAS2751) and GST-Elk-1 (205-428) (pAS407) were used [17].

Tissue culture, cell transfections, siRNA, reporter gene assays, and RT-PCR.

HEK-293T and HeLa cells were grown in DMEM supplemented with 10% foetal bovine serum. Transfections were performed using polyfectTM (Qiagen) according to the manufacturer's instructions. Where indicated, cells were serum starved for 24 hours, then treated with PMA (10 nM) or anisomycin (250 ng/ml) prior to luciferase assays (6 hrs) or RT-PCR analysis (40 mins).

Transfections of siRNAs were achieved using Lipofectamine siRNAMax (Invitrogen) according to the manufacturer's instructions. ON-TARGETplus SMARTpool siRNAs (Dharmacon) against SENP1, SENP2, SENP3 and GAPDH were used.

For reporter gene assays, typically 0.25 µg of reporter plasmid and 50 ng of pRL-TK-Renilla were co-transfected with 0.1-1 µg of expression plasmids. Cell extracts were prepared and equal amounts of protein were used in luciferase assays using the Dual-Luciferase kit (Promega). Data were normalised against the expression of the Renilla Luciferase.

Real time RT-PCR was carried out using the QuantiTect SYBR Green RT-PCR mix (Qiagen). All data were normalized to the levels of 18S rRNA. The following primer-pairs were used for RT-PCR experiments. 18S rRNA: ADS4005 (5'-TCAAGAACGAAAGTCGGAGGTT-3') and ADS4006 (5'-GGACATCTAAGGGCATCACAG-3'), *c-FOS*: ADS1690 (5'-AGAATCCGAAGGGAAAGGAA-3') and ADS1691 (5'-

CTTCTCCTTCAGCAGGTTGG-3'),
 TTAACCTAACCAGGAACAGCTG-3')
 GAGTCTGATCCTTCAGATTGTG-3'),
 GAACTTACAGAGGACATGGA-3')
 CTGAATACATGAAGTGCTGG-3'),
 GAGCATCTTGGACGAATTCC-3')
 GTTCATCACCTGGTCATTGAG-3'). Three independent RT-PCR reactions were run on the same RNA samples to reduce variability and produce a mean value for each data point.

<i>SEN1</i> :	ADS2502	(5'-
and	ADS2503	(5'-
<i>SEN2</i> :	ADS2506	(5'-
and	ADS2507	(5'-
<i>SEN3</i> :	ADS2512	(5'-
and	ADS2513	(5'-

In vivo sumoylation and *in vitro* desumoylation assays

In vivo sumoylation of overexpressed Elk-1 was detected by co-transfection of His-tagged Elk-1 and HA-tagged SUMO-2 proteins, followed by purification of the conjugates under denaturing conditions as described previously [11].

In vitro desumoylation assays were performed using SUMO modified recombinant GST-Elk-1 fusion proteins and immunoprecipitated Flag-tagged SENP proteins. To prepare sumoylated recombinant Elk-1, a reconstituted sumoylation system in *E. coli* was used [18]. To prepare SENPs, 293T cells were transfected with 30 µg of flag-SENP1 DNA. 48 h post-transfection, cells were resuspended in Buffer II (25 mM Tris pH8, 150 mM NaCl, 0.1% tween 20, 2mM DTT, complete 1:100) and lysed by passing them through a 25 G needle. The SENP protein was purified using anti-flag antibody conjugated to agarose beads. Beads containing the protein were washed twice in Buffer II containing 1M NaCl and twice in Buffer II. Protein was eluted by adding flag-peptide. Assays were carried out as described previously [18]. Sumoylated Elk-1 from 50 ml cultures was purified and kept bound to the GSH-agarose. 50 µl reactions in Buffer II were incubated at 37°C, shaking at 700 rpm, for the indicated times. The samples were spun down, the supernatant was removed and proteins were eluted by adding 1X SDS-PAGE loading buffer. Gels were stained overnight using SYPRO-Ruby and visualised using a Biorad gel doc.

GST Pulldown assays

GST pulldown assays were carried out using recombinant GST-Elk-1 and cell lysates of 293T transfected with constructs encoding each of the catalytically dead Flag-tagged SENP1-3 enzymes, essentially as described previously [19].

Immunoprecipitation and Western blot analysis

Western blotting and immunoprecipitations were carried out using SuperSignal™ West Pico or Dura (Pierce) chemiluminescent substrates with the primary antibodies; Flag (Sigma), Elk-1 (Santa Cruz), or HA (Roche) according to the manufacturer's instructions. Data were analysed using Quantity-One software (Biorad). Co-immunoprecipitation assays were performed in 50 mM potassium phosphate pH 7.8, 0.1% Triton X-100, 150 mM NaCl, 10 nM N-ethylmaleimide, 10 nM E64 (Sigma) and 1/100 protease inhibitor cocktail (Roche), followed by three washes in the same solution.

Results

Elk-1 is preferentially activated by SENP1.

Elk-1 is SUMO modified and this dampens down its transactivation activity and promotes active transcriptional repression. Sumoylation is lost during the transition to a transcriptional activating state [11]. To begin to probe which SUMO protease(s) is involved in reversing Elk-1 sumoylation we compared the abilities of different SUMO proteases (SENPs) to activate Elk-1 through desumoylation. We first compared the expression levels of the SENPs. SENP1 and 3 were expressed to similar levels with SENP2 being expressed to a slightly lesser extent (Fig. 1B). Next, we compared the abilities of the SENPs to activate a GAL-Elk-1(1-428) fusion protein in a luciferase reporter assay (Fig. 1A). This fusion protein is regulated by SUMO pathway in an analogous manner to full-length Elk-1 [11, 12]. SENP1 had the biggest effect on Elk-1 activation whereas SENP3 barely affected Elk-1 activity. SENP2 had an intermediary effect (Fig. 1C). Due to the reduced expression levels of SENP2, we performed a dose response experiment to compare the ability of SENP1 and SENP2 to activate Elk-1. However, even at 10 fold higher levels, SENP2 still activated Elk-1 to a lesser extent than SENP1 (Fig. 1D, compare column 2, black bars to column 6, grey bars).

The effects of the SENPs could be indirect and/or independent of their SUMO deconjugating activities. We therefore first established whether the Elk-1 sumoylation sites are required for SENP-mediated activation. Again we used a luciferase reporter assay but instead of the wild-type protein, we tested GAL-Elk-1(1-428)(K2R), which lacks the two major sumoylation sites at K230 and K249 [11]. In comparison to wild-type Elk-1, Elk-1(K2R) showed much reduced activation by the SENPs (Fig. 1C), consistent with a role for SENPs in desumoylating Elk-1. Conversely, we tested the activity of catalytically inactive mutant versions of the SENPs which had their active site cysteine residues mutated. The ability of SENP1 to activate Elk-1 was much reduced and a similar effect was seen on the smaller activation caused by SENP3 (Fig. 1E). In contrast, the mutant version of SENP2 was able to activate Elk-1 to a similar extent as the wild-type version (Fig. 1E), demonstrating that the activating effect of SENP2 on Elk-1 occurs by a mechanism that is independent from its SUMO deconjugating activity.

Together, these results demonstrate that SENP1 is the most efficient SUMO protease acting on Elk-1 and that SENP3 has little effect on Elk-1. SENP2 has an intermediate effect but its ability to activate Elk-1 is independent from its SUMO deconjugating activity.

SENP1 binds to Elk-1.

As SENP1 was established as the most efficient SUMO protease acting on Elk-1, we investigated whether we could detect complexes between these two proteins. Initial attempts at detecting interactions between Elk-1 and wild-type SENP1 were unsuccessful, either at endogenous or overexpressed levels. We reasoned that this might be due to the transient interactions between the enzymatic SENPs and their substrate, Elk-1. Therefore to potentially trap these interactions, we used a catalytically dead version of SENP1 (SENP1^{CS}) in co-immunoprecipitation assays. In contrast to wild-type SENP1 where no complex could be detected, a complex could be detected between Elk-1 and the mutant SENP1^{CS} (Fig. 2A, lanes 2 and 3). Next we compared the ability of

SEN1^{CS} and the equivalent catalytically dead mutant version of SEN2 (SEN2^{CS}) to bind Elk-1. Preferential binding of SEN1 was observed in a co-immunoprecipitation assay (Fig. 2B). To further probe the specificity of these interactions we performed GST pulldown assays with recombinant GST-Elk-1 and 293T cell lysates containing each of the catalytically dead SEN1-3 enzymes. Preferential interactions were observed with SEN1 (Fig. 2C).

Collectively, these results demonstrate that SEN1 can form complexes with Elk-1 and this interaction appears to be specific amongst the SENPs.

SEN1 desumoylates Elk-1.

The ability of SEN1 to activate Elk-1 in a SUMO-dependent manner suggests that SEN1 can desumoylate Elk-1. To prove that this is the case, we performed *in vitro* desumoylation assays using recombinant SUMO-modified Elk-1, and Flag-tagged SEN1 or SEN3 immunoprecipitated from 293T cells. Elk-1 was modified by SUMO using an *E. coli* system containing a reconstituted SUMO pathway (Fig. 3A; [18]) and equal amounts of immunoprecipitated SEN1 and SEN3 (Fig. 3B) were incubated with this recombinant protein. The addition of SEN1 resulted in loss of SUMO conjugation to Elk-1 (Fig. 3C, lanes 1-3), demonstrating that Elk-1 is a direct target of the SUMO proteolytic activity of SEN1. In contrast, little desumoylation was seen upon incubation with SEN3 (Fig. 3C, lanes 4-6). Thus, in agreement with the reporter and binding assays, SEN1 and SEN3 show different activities towards Elk-1 with SEN1 more efficiently desumoylating Elk-1 *in vitro*.

Endogenous SEN1 is important for Elk-1 activation.

We have established that amongst SENPs, Elk-1 is preferentially activated by overexpressed SEN1 and binds specifically to SEN1. However, to determine whether endogenous SEN1 is important for Elk-1 activation, we compared the effect of siRNA-mediated depletion of SEN1 with depletion of other SENPs on Elk-1 activity. First we determined the efficacy and specificity of each siRNA in depleting SENP mRNA levels. Each of the siRNA pools specifically depleted the corresponding SENP mRNA with high efficiency, with negligible effects on the other SENPs (Fig. 4B), thereby demonstrating their utility for subsequent functional assays. Next, we compared the activity of Elk-1 in a luciferase reporter assay under serum starved conditions or upon PMA treatment. PMA treatment leads to activation of the ERK pathway and loss of SUMO on Elk-1 and hence loss of its repressive properties (Fig. 4A; [11]). In the absence of PMA, a 10% reduction in Elk-1 activity was observed following SEN1 depletion, but little effect was seen upon depleting the other SENPs (Fig. 4C, grey bars). This effect was even more pronounced in the presence of PMA, with >20% reduction in Elk-1 activity seen following SEN1 depletion. Again, depletion of the other SENPs had a negligible effect (Fig. 4C, black bars). We also probed the effect of SENP depletion on the activity of the Elk-1(K2R) mutant protein that could not be SUMO modified. In contrast to the effects seen with the wild-type protein, depletion of any one of the SENPs had no effect on the activity of Elk-1(K2R), either in the presence or absence of PMA treatment (Fig. 4D). This is consistent with a role for SEN1 in directly acting to desumoylate Elk-1.

Although significant, the depletion of SEN1 caused relatively small changes in the activity of Elk-1. This could be due to functional redundancy whereby in the absence

of SENP1, another SENP can at least partially substitute for its activity. To test such a possibility, a combinatorial knockdown approach was undertaken. SENP1 siRNAs were used in combination with either SENP2 or SENP3 siRNAs. Again, SENP1 depletion caused a reduction in the activity of Elk-1 following PMA treatment, but this effect was amplified by the simultaneous depletion of SENP2, with Elk-1 activity reduced by nearly 40% (Fig. 4E, grey bars). In contrast, co-depletion of SENP3 did not further enhance the activity of Elk-1, suggesting that at the level of functional redundancy, there is still specificity of action. Again, neither the depletion of SENP1 alone nor in combination with other SENPs led to a reduction in the activity of the Elk-1(K2R) mutant (Fig. 4E, black bars).

Together these results therefore demonstrate that SENP1 is the predominant SENP acting to promote Elk-1 activation in a SUMO-dependent manner, although there is a degree of functional redundancy with SENP2.

SENP1 plays a role in Elk-1 target gene activation in response to mitogenic signalling.

One of the best characterised Elk-1 target genes is *c-FOS*. Elk-1 orchestrates the activation of *c-FOS* in response to both mitogenic and stress signals such as PMA and anisomycin. However, while PMA treatment leads to loss of sumoylation, anisomycin treatment does not (Fig. 5A; [13]) although both treatments lead to Elk-1 phosphorylation and *c-FOS* activation. We therefore probed the roles of SENPs in signal-dependent *c-FOS* activation. First we demonstrated that PMA and anisomycin had the predicted effects on Elk-1 sumoylation, with only PMA promoting SUMO loss (Fig. 5B). Next, we depleted either SENP1, SENP2 or SENP3, and investigated *c-FOS* activation. The loss of SENP2 or SENP3 had little effect but SENP1 loss caused a reduction in *c-FOS* activation following PMA treatment (Fig. 5D). In contrast, little reduction was seen in *c-FOS* activation by anisomycin upon depletion of individual SENPs, including SENP1 (Fig. 5D).

Studies using reporter gene analysis suggested that a degree of functional redundancy exists between SENP1 and SENP2 which might mask the effect of SENP1 depletion (Fig. 4). We therefore compared the effect of depleting both SENP1 and SENP2 with depletion of SENP1 alone. Again, SENP1 depletion caused a reduction in *c-FOS* activation following PMA stimulation but this effect was enhanced upon co-depletion of SENP2 (Fig. 5E). In contrast, anisomycin-dependent *c-FOS* activation was unaffected by SENP1 depletion, either alone or in combination with SENP2 (Fig. 5F). The lack of involvement of SENPs in *c-FOS* activation by anisomycin is consistent with the lack of requirement for SUMO loss in the activation of Elk-1 by this treatment.

These data therefore demonstrate that SENP1 is the predominant SENP acting to promote activation of the Elk-1 target gene *c-FOS* following mitogenic stimulation and consistent with the reporter gene assays, there is a degree of functional redundancy with SENP2. In contrast, under signalling conditions where Elk-1 sumoylation is not required (ie anisomycin treatment), SENP1 is not required for *c-FOS* induction.

Discussion

Sumoylation plays an important physiological role in determining the outcome of numerous processes in the cell. Importantly, sumoylation is a dynamic process, enabling transient responses to be elicited, to processes such as cellular signalling events [reviewed in 7, 21]. However, in most cases, it is unclear how SUMO conjugation is either promoted or subsequently lost in response to signalling cues. SUMO loss is promoted by the SENPs [reviewed in 5-6]. Here, we have focussed on Elk-1 and investigated the roles of SENPs in the desumoylation process. Elk-1 makes an attractive model as sumoylation promotes its transcriptional repressive properties, and depending on the stimulus received, sumoylation is either retained (upon stress signalling) or lost (upon mitogenic signalling). Here, we identified SENP1 as a key player in the loss of SUMO from Elk-1 in response to ERK pathway signalling (Fig. 6).

Several lines of evidence establish SENP1 as the predominant SENP involved in Elk-1 desumoylation; (i) SENP1 is the most efficient activator of Elk-1 in overexpression studies, (ii) Elk-1 binds preferentially to SENP1, (iii) SUMO-modified Elk-1 is a substrate for SENP1 *in vitro*, (iv) SENP1 depletion reduces the activation of Elk-1 by PMA, and (v) SENP1 depletion reduces the activation of the Elk-1 target gene *c-FOS* following mitogenic stimulation. Importantly, we compared the effect of several SENPs in each of the assays to demonstrate the specificity of SENP1 action. Moreover, we also verified that the effect of SENP1 was direct by determining the SUMO-dependence of its effects. SENP1-dependent activation of Elk-1 depended on its catalytic activity and the presence of sumoylation sites in Elk-1 (Fig. 1). Similarly, depletion of SENP1 only had an effect on Elk-1 activity when its sumoylation sites were intact (Fig. 4). Finally, SENP1 depletion only affected *c-FOS* activation under signaling conditions where SUMO loss is known to occur (Fig. 5).

There are six known SENPs in humans and it is possible that some degree of functional redundancy occurs. Here we only tested the effects of three different SENPs although several assays were performed to test the role of SENP5 but this had little effect on the activity of Elk-1 or its target gene *c-FOS* (data not shown). It therefore remains possible that the additional SENPs, SENP6 and SENP7, play a role in Elk-1 activation, maybe in a redundant manner. Nevertheless, it is clear that SENP1 plays a predominant role in Elk-1 desumoylation. We do however observe functional redundancy in SENP depletion assays where SENP2 specifically substitutes for SENP1 upon SENP loss (Figs. 4 and 5). In this scenario though, the primary role of SENP1 is probably dictated due to its stronger binding to Elk-1 than SENP2 (Fig. 2), thereby occluding SENP2 access to the sumoylation sites. Only when SENP1 is lost will SENP2 be able to work on Elk-1.

Binding experiments demonstrated a specific interaction between SENP1 and Elk-1 (Fig. 2). However, to perform such experiments *in vivo*, we had to use catalytically inactive versions of SENPs. The rationale behind this was that such mutants would stay associated for longer rather than merely transiently binding and dissociating following SUMO cleavage. Precedents for such behavior exists in other enzyme-substrate systems, where for example, p38 kinase binding to MEF2 could only be strongly detected using catalytically dead p38 [22]. Unfortunately, this observation precluded the possibility of detecting interactions between endogenous SENP1 and Elk-1, and likely will make any such substrate-SENP pairs difficult to detect at endogenous levels.

While SENP1 is the predominant SENP involved, overexpression of SENPs has varying effects on Elk-1 activity (Fig. 1). For example, SENP2 and SENP3 caused smaller, yet significant increases in Elk-1 activity. This might merely be due to overexpression and displacement of endogenous SENP1 but might also have an important role. For example, SENP3 activates Elk-1 to a small extent, and this activation might be through p300 as this was recently shown to be a physiologically relevant SENP-substrate pairing [23]. In contrast, SENP2 can activate Elk-1, but this activation appears to be largely independent from its desumoylation function as the catalytically dead version activates Elk-1 as efficiently as the wild-type version (Fig. 2E). It is not clear what this desumoylation-independent activity of SENP2 might be, although a similar phenomenon has previously been described for the SENP2-dependent activation of c-Jun [24]. The observation that SENP2 functions in a manner independent from its desumoylation activity apparently contradicts its ability to substitute for SENP1 in regulating Elk-1 in a SUMO-site dependent manner. However, it is possible that in the case of overexpression, additional effects are triggered which would not be seen at endogenous levels, therefore interpretation of the results of these type of overexpression assays need treating with caution. Nevertheless, the assays do demonstrate a more potent activity of SENP1 towards Elk-1 as a SUMO protease, especially considering the alternative mode of action of SENP2 in these overexpression assays. Knockdown studies further support the role of SENP1 and more realistically delineate the potential functional redundancy of SENP2 at endogenous levels where its effects are at least in part elicited through its SUMO-protease activity.

Initially, it was thought that desumoylation would be a fairly non-specific process. However, our demonstration that Elk-1 is preferentially targeted by SENP1 provides another member of a growing list of specific SENP-substrate interactions. Notable examples include SENP3 and p300 in response to mild oxidative stress [23], and the specific role of SENP1 in targeting HIF1 α [25] and HDAC-1 [14]. At least part of the functional specificity of SENPs appears to result in their subnuclear localisation, with SENP3 being localised to the nucleolus and thereby desumoylating nucleolar substrates such as nucleophosmin [26], although recent results demonstrate that nucleolar SENPs such as SENP3 can be released into the nucleoplasm following mild oxidative stress [23]. In contrast, SENP1 and SENP2 are thought to be the major SENPs in the nucleoplasm, which is consistent with a role for SENP1 in controlling Elk-1 activity. It appears likely therefore that each SENP will have a specific spectrum of substrates, although some degree of functional redundancy is likely built in as a potential fail safe mechanism and subcellular localization will to some degree dictate the availability of substrates. It is however clear that the SENPs play a positive role in determining the dynamics and functional outcome of protein sumoylation.

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Figure legends

Figure 1. SENP1 preferentially activates Elk-1. (A) Schematic of the GAL-driven reporter system used to assay Elk-1-mediated transactivation. (B) IP-western analysis of the expression of the indicated Flag-tagged SENP constructs. 293T cells were transfected with different SENP expression vectors followed by immunoprecipitation with anti-Flag antibody and detection by western analysis with the same antibody. Asterisk represents a band corresponding to the antibody heavy chain. (C-D) Luciferase reporter analysis of the activity of GAL-Elk-1(1-428) constructs in the presence of co-transfected SENP expression constructs. (C) Wild-type (black bars) and K230R/K249R(K2R) mutant (grey bars) Elk-1 constructs were transfected in the absence and presence of the indicated SENP constructs. (D) Wild-type GAL-Elk-1 constructs were transfected in the presence of increasing amounts (0 ng, 125 ng, 250 ng, 500 ng, 1 μ g) of the indicated SENP constructs. (E) Wild-type GAL-Elk-1 constructs were transfected in the presence of 1 μ g of the indicated wild-type (WT; black bars) or catalytically dead (mut; grey bars) SENP constructs. In all cases data are presented relative to the activity of either WT or K2R versions of GAL-Elk-1(1-428) in the presence of control empty vector (taken as 1). Data are the average of two independent experiments, each with triplicate samples.

Figure 2. Elk-1 interacts with SENP1. (A and B) Co-immunoprecipitation analysis of Elk-1(1-428) and SENPs. (A) Elk-1 was transfected in the absence and presence of the indicated wild-type (WT) and catalytically inactive (CS) versions of Flag-tagged SENP1. Following immunoprecipitation of Elk-1 (IP), bound SENPs were detected by immunoblotting (IB) with anti-Flag antibody. Immunoprecipitated proteins are shown in the top two panels and input proteins in the bottom two panels. (B) Elk-1 was transfected in the absence and presence of the indicated catalytically inactive (CS) versions of Flag-tagged SENPs. Following immunoprecipitation of Elk-1 (IP), bound SENPs were detected by immunoblotting (IB) with anti-Flag antibody. Immunoprecipitated proteins are shown in the top two panels and input proteins in the bottom panel. Asterisks indicate non-specific cross-reacting bands in the IP samples. (C) GST pulldown analysis of GST-Elk-1(205-428) binding to SENPs. The indicated catalytically inactive SENPs were expressed in 293T cells and the lysates bound to recombinant GST or GST-Elk-1 and detected by IB with anti-Flag antibody (top panel). A coomassie stained gel of the input bait proteins is shown in the bottom panel.

Figure 3. SENP1 desumoylates Elk-1 *in vitro*. (A) Coomassie stained gel showing the non-sumoylated and sumoylated recombinant GST-Elk-1 fusion proteins. (B) Western blot of Flag-tagged SENP1 and SENP3 purified by IP from transfected 293T cells. The broken line indicates where irrelevant lanes were removed. (C) Sumoylated recombinant GST-Elk-1(205-428) was incubated in the presence or absence of Flag-tagged SENP1 or SENP3 for the indicated times and samples detected by SYPRO-Ruby staining after SDS-PAGE. The positions of the recombinant Elk-1 protein and its sumoylated forms are indicated. The asterisks indicate the position of a co-purifying contaminant.

Figure 4. Depletion of SENP1 decreases the transactivation capacity of Elk-1. (A) Schematic of the GAL-driven reporter system used to assay Elk-1-mediated

transactivation following ERK pathway activation. (B) RT-PCR analysis of the expression of the indicated *SENPs* in HeLa cells in the presence of control siRNAs or siRNA duplexes directed against the indicated *SENPs*. Data are representative of two independent experiments and are the average of two samples, presented relative to the transcript levels in the presence of control siRNA duplexes (taken as 1). (C-E) Luciferase reporter analysis of the activity of GAL-Elk-1(1-428) constructs in 293T cells in the presence of co-transfected siRNA duplexes against *SENPs*. All data are the average of two experiments carried out in duplicate. Note that the axis begins does not begin at zero to emphasise the changes we observe. (C and D) Individual siRNA duplexes (25 pmol) were transfected in the presence of either wild-type (WT)(E) or mutant K230R/K249R(K2R)(E) forms of Elk-1. Cells were either untreated (grey bars) or treated with PMA for 6 hrs (black bars). (E) Cells were treated with PMA after transfection with vectors encoding WT (grey bars) or mutant (K2R)(black bars) versions of GAL-Elk-1(1-428) and with siRNA targeting *SEN1* (12.5 pmol) and the indicated additional siRNA constructs (12.5 pmol) against different *SENPs*.

Figure 5. Depletion of *SEN1* decreases the activation of the Elk-1 target gene *c-FOS*. (A) Schematic showing the different molecular events occurring on the *c-FOS* promoter after PMA (SUMO loss) or anisomycin (SUMO retention) stimulation. In both cases, *c-FOS* promoter activation is still observed. (B) Western blot analysis of Elk-1 sumoylation levels. 293T cells were transfected with expression vectors for His-Flag-tagged Elk-1 and HA-tagged SUMO-2 and His-tagged Elk-1 was pulled down (PD) from lysates from 293T cells treated with PMA or anisomycin for 40 mins. Total and sumoylated Elk-1 were detected by immunoblotting (IB) with anti-Flag and anti-HA antibodies respectively. (C-F) RT-PCR analysis of the expression of *c-FOS* in HeLa cells in the presence of control *GAPDH* siRNAs or siRNA duplexes directed against the indicated *SENPs*. Either an siRNA duplex against a single *SEN1* (25 pmol) or two siRNA duplexes against distinct *SENPs* (12.5 pmol each) were transfected and cells were either serum starved (grey bars) or treated with PMA (B and D) or anisomycin (C and E) for 40 mins (black bars). Data are the average of duplicate samples and are presented relative to the transcript levels in serum starved cells in the presence of control *GAPDH* siRNA duplexes (taken as 1).

Figure 6. Model for the role of *SEN1* in Elk-1 regulation. In the absence of ERK pathway signaling, Elk-1 is kept in a repressive state by SUMO-mediated HDAC-2 recruitment. Following ERK activation, Elk-1 is desumoylated, leading to enhanced transactivation capacity and *SEN1* plays a pivotal role in this process.

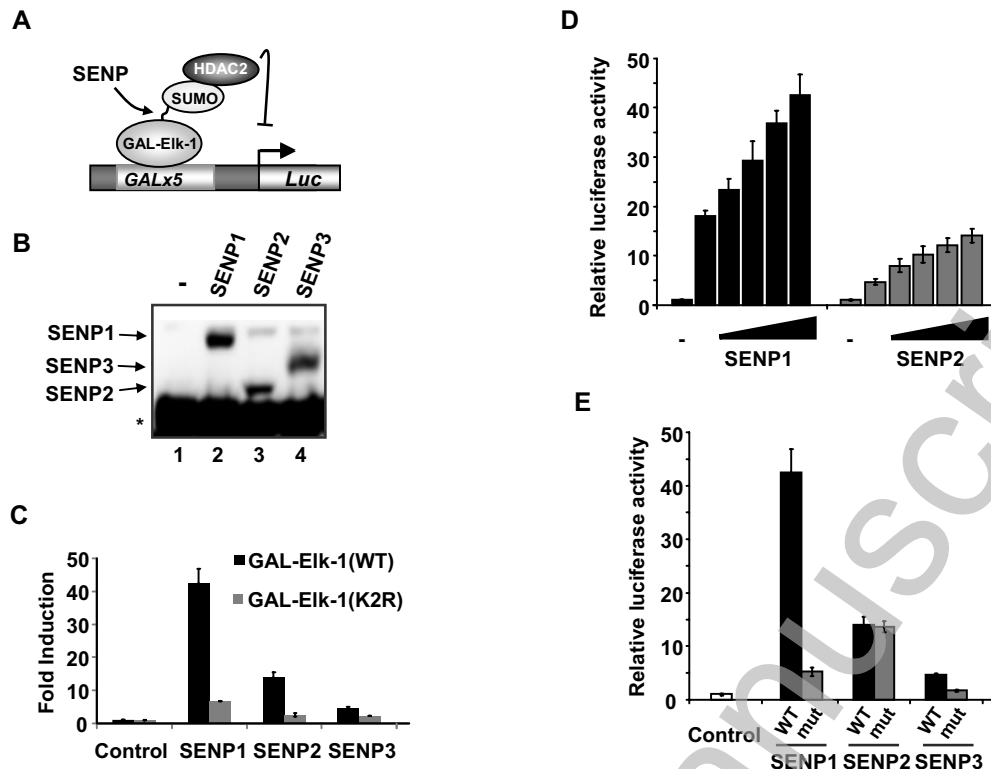


Fig. 1

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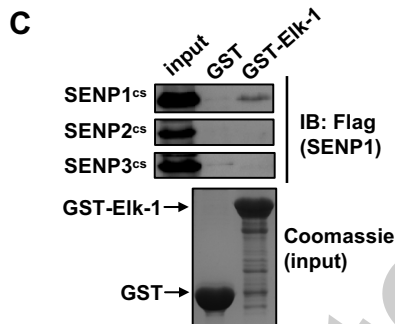
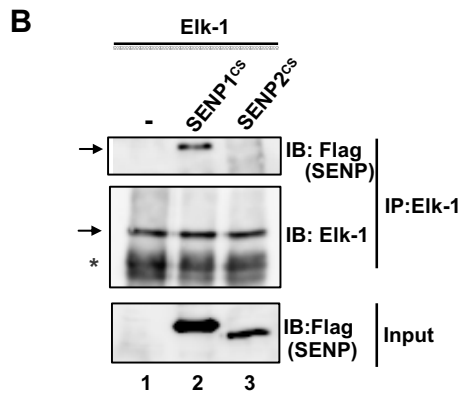
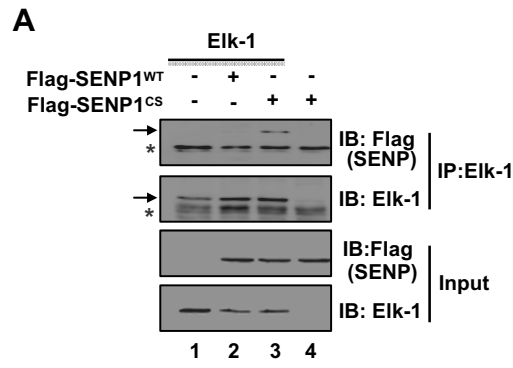


Fig. 2

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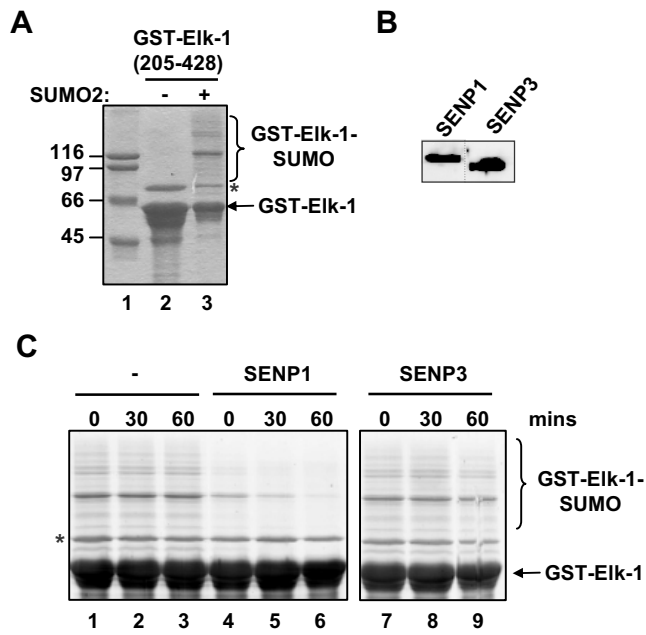


Fig. 3

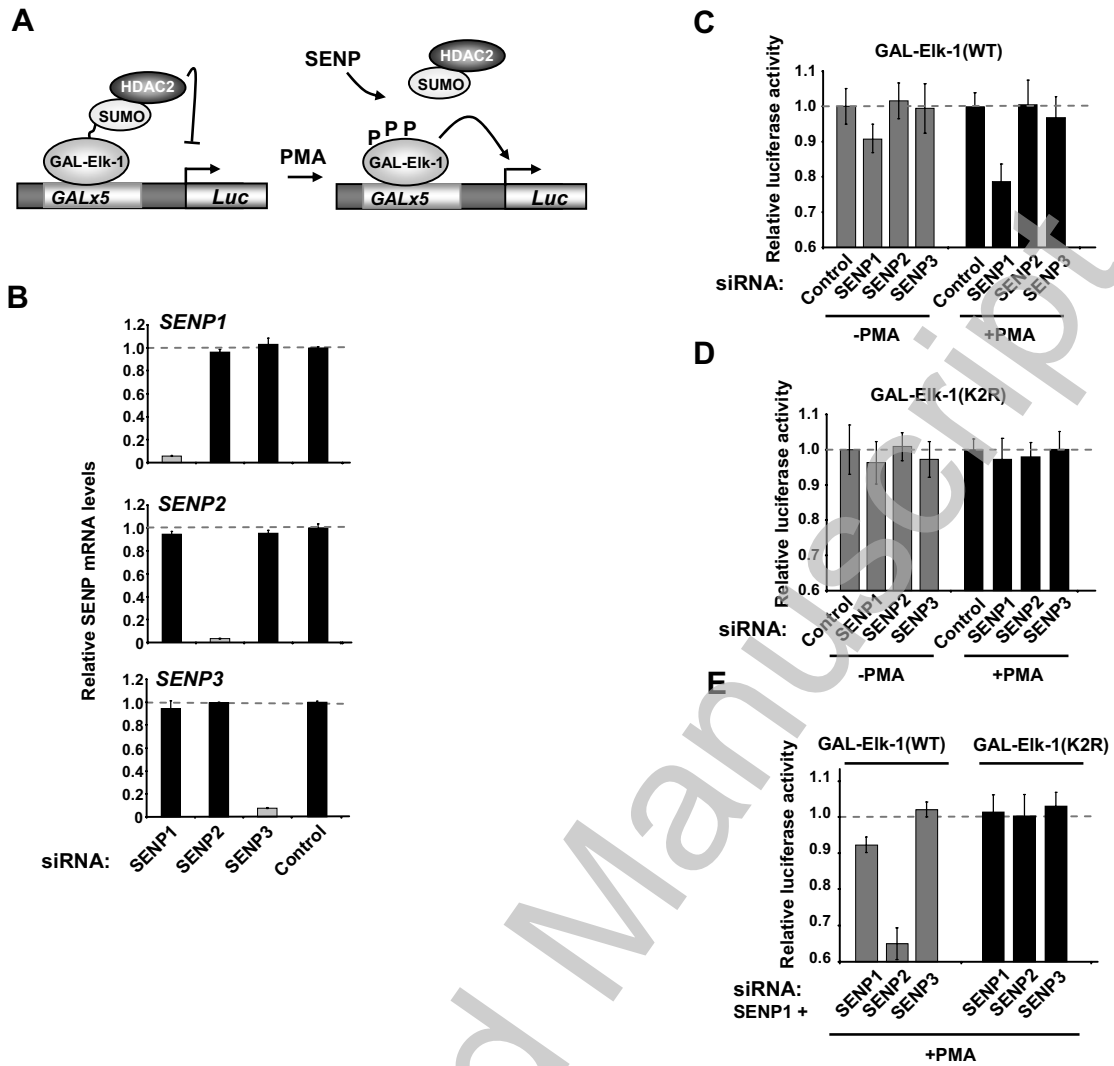


Fig. 4

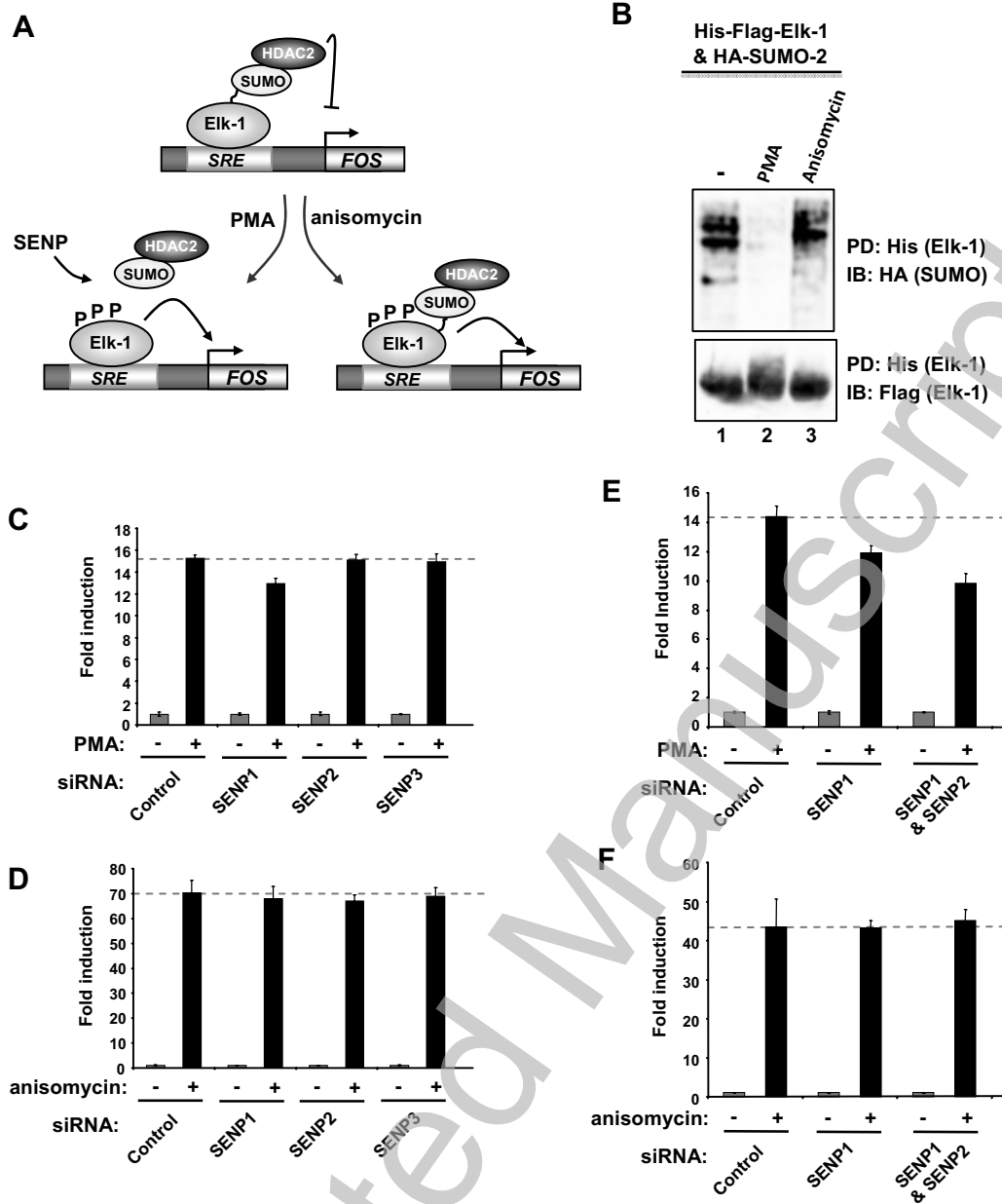


Fig. 5

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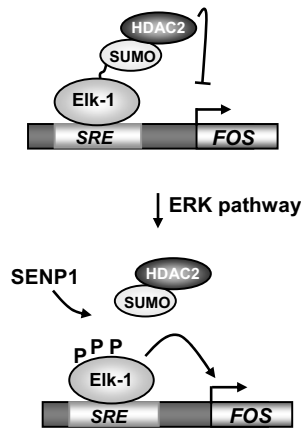


Fig. 6

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