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Modification of proteins by SUMO (Small Ubiqutin-like Modifer) plays important roles in regulating the activity, stability and cellular localization of target proteins. Like ubiquitination, SUMO modification is a dynamic process that can be reversed by SUMO-specific proteases (SENPs). So far, six SENPs have been discovered in humans although knowledge of their regulation, specificity and biological functions is limited. Here, we report that SENP7 has a restricted substrate specificity being unable to process SUMO precursors and displaying paralogue specific isopeptidase activity. The C-terminal catalytic domain of SENP7 efficiently depolymerised polySUMO-2 chains but had undetectable activity against polySUMO-1 chains. SENP7 also displayed isopeptidase activity against di-SUMO-2 and SUMO-2 modified RanGAP1, but had limited activity against SUMO-1 modified RanGAP1. *In vivo*, full-length SENP7 was localized to the nucleoplasm and preferentially reduced the accumulation of high molecular weight conjugates of SUMO-2 and SUMO-3 compared to SUMO-1. siRNA mediated ablation of SENP7 expression led to the accumulation of high molecular weight SUMO-2 species and to the accumulation of promyelocytic leukaemia protein in sub-nuclear bodies. These findings suggest that SENP7 acts as a SUMO-2/-3 specific protease that is likely to regulate the metabolism of polySUMO-2 /-3 rather than SUMO-1 conjugation *in vivo* .

Key words: small-ubiquitin-related modifier (SUMO), ubiquitin, sentrin-specific protease7 (SENP7), SUMO specific protease, isopeptidase, conjugation, deconjugation.

Abbreviations: SUMO, small ubiquitin-like modifier; MBP, maltose-binding protein; HA, haemagglutinin; PML, promyelocytic leukemia; IPTG, isopropyl β-D-thiogalactoside; Ni-NTA, Ni^{2+}-nitriloacetate; RanGAP1, Ran GTPase-activating protein 1; SENP, sentrin-specific protease; SMT3, suppressor of MIF2 (mitotic fidelity protein 2); SMT3IP, SMT3-specific isopeptidase; TEV, tobacco etch virus; Ubc9, ubiquitin-conjugating enzyme 9; Ubl, ubiquitin-like protein, Ulp1, Ubl-specific protease 1.

INTRODUCTION

SUMOs (small ubiquitin-like modifiers) are covalently linked to lysine side chains in target proteins and confer altered properties on the modified proteins that are

involved in a variety of processes such as nuclear transport, transcription, recombination and chromosome segregation [1-4]. Three members of the SUMO family have been identified in vertebrates: SUMO-1 (also known in humans as Smt3c, PIC1, GMP1, Sentrin and Ubl1), SUMO-2 (also known as Smt3a and Sentrin3) and SUMO-3 (also known as Smt3b and Sentrin2). The mature form of SUMO-2 and SUMO-3 are 97% identical to each other, but only 50% identical in sequence to SUMO-1 [5, 6]. SUMOs are conjugated to target proteins through a cascade of reactions that typically involved three enzymes: an activating enzyme (E1), a conjugating enzyme (E2) and, usually, a SUMO ligase (E3) [7]. In the first step, SUMO activating enzyme E1 (a heterodimer containing SAE1/SAE2 subunits) utilises ATP to adenylate the C-terminal glycine of SUMO. Formation of a thioester bond between the C-terminal glycine of SUMO and a cysteine in SAE2 is accompanied by release of AMP. The second step is a transesterification reaction, in which SUMO is transferred from the E1 to a cysteine residue within the SUMO –specific E2 conjugating enzyme (Ubc9). Finally, the thioester linked Ubc9-SUMO conjugate catalyses formation of an isopeptide linkage between the C-terminal carboxyl group of SUMO and the ε-amino group of lysine in the substrate protein. Many sumoylation sites are found within a consensus motif ΨKXE/D, (where Ψ is a large hydrophobic amino acid, K is a lysine, X is any amino acid and E/D is glutamic or aspartic acid), although modification at non-consensus sites has been reported [8]. A number of SUMO E3 enzymes such as RanBP-2 and the PIAS family of proteins have been identified [review 9]. These enzymes can enhance the conjugation reaction and are likely to provide substrate specificity in the transfer of the SUMO to the lysine residue of target proteins. SUMO-2 and SUMO-3 like Smt3p, contain SUMO consensus motifs at their N-termini that can be utilised to form polymeric SUMO chains *in vitro* and in *vivo* [10-12]. There is clear evidence that SUMO-2/-3 chain formation might be important for regulation of some target proteins *in vivo* [13-14]. In mammalian cells, CENP-E was found to be modified specifically by SUMO-2/-3 and to possess polySUMO-2/-3 chain binding activity essential for kinetochore localization [15]

Sumoylation is a highly dynamic and fully reversible modification. The family of Sentrin-specific proteases (SENPs) are cysteine proteases that cleave the isopeptide bond between SUMO and the bound proteins. These proteases are also responsible for processing of the SUMO precursors to reveal the di-glycine motif that is conjugated to acceptor lysines in target proteins [see review16]. In yeast, there are two SUMO- specific proteases, Ulp1 and Ulp2 have been characterised and detected at the nuclear pore and nuleoplasm respectively [17-19]. In humans, six SENPs have been reported. All share a conserved C-terminal sequence of ~200 residues within which are the conserved amino acids (cysteine, histidine and aspartic acid) that form the catalytic triad. SENP1 (also designated SuPr-2), SENP2 (also designated AXAM2 or SMT3IP2), SENP3 (also designated SMT3IP1) and SENP5 have been shown to function as SUMO specific proteases. SENP1 is a nuclear protease that deconjugates a large number of sumoylated proteins [20]. SENP2 is a nuclear envelope-associated protease, although differential splicing generates SENP2 proteins can be

cytoplasmic nuclear pore localised and nuclear body localized [21,22]. SENP3 and SENP5 are nucleolar SENPs with preference for SUMO-2/-3 [23]. SENP6 is nuclear protease and appears to be specific for deconjugation of isopeptide-linked SUMO-2 and SUMO-3, but is unable to deconjugate isopeptide-linked SUMO-1 and does not process SUMO-1, SUMO-2 or SUMO-3 precursors [24].

Previous studies have shown that SENP7 failed to process SUMO precursors and implied that its natural substrates may not be SUMO conjugates [25, 26]. Here, we report the characterization of SENP7. We found that SENP7 does not process the precursor forms of SUMO-1, SUMO-2, SUMO-3; but SENP7 can remove SUMO-2 from RanGAP-1-SUMO-2 and efficiently deconjugate polySUMO-2 chains. However, SENP7 only has negligible activity against RanGAP-1-SUMO-1 and polySUMO-1 chains *in vitro*. *In vivo*, SENP7 localised to the nucleoplasm and displayed preferential isopeptidase activity against SUMO-2/-3 compared to SUMO-1 conjugates. Ablation of SENP7 expression with siRNA resulted in the accumulation of high molecular weight SUMO-2 forms and to the increased accumulation of promyelocytic leukaemia protein in sub-nuclear bodies. These findings suggest that SENP7 acts as a SUMO-2/-3 specific protease that is likely to regulate the metabolism of polySUMO-2 /-3 rather than SUMO-1 in *vivo* .

EXPERIMENTAL

Plasmid constructions

The cDNAs for SUMO-1, SUMO-2 and SUMO-3 were subcloned into a pcDNA3-HA vector as described previously [10]. Full-length human SENP7 cDNA was amplified from a human fetal brain cDNA library by PCR. The PCR product was then subcloned into pcDNA3 vector (Invitrogen) and pEGFP-C1 vector (Clontech) using standard techniques. The above plasmids were transfected into COS-7 cells using FuGENE6 (Roche Applied Science) according to the manufacture's instructions.

Full-length SUMO-1 (residue1-101), SUMO-2 (residue1-103) and SUMO-3 (residue1-95) were cloned into pHisTEV vector between BamHI and HindIII. pLous3-SENP7C (residue 733-1050) for the expression of SENP7 catalytic domain in bacteria was constructed by PCR amplification of SENP7 cDNA. The PCR product was cloned into an expression vector pLous3 (a kind gift from Dr. Jim Naismith, University of St.Andrews) between EcoRI and SalI. For clarity, we will refer to catalytic domain of SENP7 as SENP7C throughout the text.

All clones were verified by automated DNA sequence analysis and shown to be identical with those previously reported for SENP7, SUMO-1, SUMO-2 and SUMO-3 (GeneBank accession nos. NP_065705, AAH53528, NP_008867 and AAH68465, respectively). SENP7 and SENP7C mutants (C992S) were generated using a PCR-based mutagenesis [27] and verified by DNA sequence analysis (Dundee University DNA Sequencing Unit).

Protein expression and purification

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SUMO-1, SUMO-2 and SUMO-3 precursors were expressed from pHisTEV vector in *E.coli* BL21 (DE3) and purified by Ni-NTA-affinity chromatography and gel filtration (Superdex75, Amersham-Pharmacia) as described previously [28]. Recombinant SENP7C was expressed as a 6HisMBP tagged fusion protein in *E.coli* BL21 (DE3) by induction with 0.1 mM IPTG at 20°C for 12 hours. 6HisMBP tagged SENP7C was purified by Ni-NTA affinity chromatography. The 6HisMBP tag was cleaved from the fusion protein by 6His –tagged TEV protease and SENP7C was further purified by Ni-NTA affinity chromatography and gel filtration (Superdex200, Amersham-Pharmacia). The expression and purification of SENP7C mutant was as described above. The purity of proteins was evaluated by SDS-PAGE and Coomassie blue staining. Concentrations of the purified proteins were determined by Bradford method.

In vivo analysis of SENP7 deconjugation activity

COS7 cells were maintained at 37°C in Dulbecco's Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. COS7 cells were cultured to 50%-60% confluency and transfected with expression constructs for SENP7 or SENP7Mut and either HA-SUMO-1, HA-SUMO-2 or HA-SUMO-3 using FuGENE 6 reagent according to the manufacturer's instructions (Roche). Controls were performed with identical conditions and empty plasmids. The total amount of DNA was equalized to 3 μg with pcDNA 3.

At 48 hours after transfection, cell extracts were separated by SDS-PAGE, proteins were transferred to nitrocellulose membranes, which were blocked with 5% non-fat milk in TPBS (PBS with 0.1%Tween-20) for one hour. Membranes were incubated for another hour with first antibody in TPBS containing 5% non-fat milk, washed three times with TPBS and incubated for a further hour in TPBS plus 5% non-fat milk containing the appropriate secondary antibody. After three washes with PBS, membranes were processed with ECL chemiluminescence detection reagents (Amersham Biosciences). Primary antibodies used in Western blotting were: mouse monoclonal anti HA antibody 12CA5 (1:2000, BabCO); antigen affinity purified sheep polyclonal antiSENP7 antibody (1:3000 produced in house); mouse monoclonal anti ß-actin antibody (1:30000 Sigma)

The SENP7 specific polyclonal antibody was generated by immunizing sheep with MBP-SENP7C. The third bleed was diluted with PBS and initially passed over a column of MBP-SENP6 coupled agarose beads to remove cross reacting antibodies. The unbound material was then passed over a second column with MBP-SENP7C coupled agarose beads. The MBP-SENP7C column was washed with 40ml of 0.5 M NaCl, 10mM Tris-HCl (pH7.5) and the SENP7 antibody eluted with 0.1M glycine-HCl pH2.25. 500 μl fractions were collected into tubes containing 50 μl 1M Tris.Cl (pH8.0). Fractions containing antibody were pooled and BSA was added to 1mg/ml, glycerol to 10% (W/V), and azide to 0.1%.

In vitro SUMO processing assay

To assay SUMO processing activity, full length SUMO-1, SUMO-2 and SUMO-3 precursors (25 μM) were incubated either in the absence (negative control), or in

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the presence of purified SENP7C (5 μM) or SENP1C (0.5 μM) (positive control) at 37°C for 1 hour in a reaction mixture containing 50 mM Tris-HCl (pH7.5), 150 mM NaCl and 5 mM ß-mercaptoethanol. Reactions are terminated by adding 2x protein sample buffer and reaction products were fractionated by SDS-PAGE followed by Coomassie brilliant blue R250 staining.

Isopeptidase assay in vitro with RanGAP1-SUMO-1/-2, SUMO-2 dimer, PolySUMO-1/-2.

All isopeptidase assays were performed in a buffer containing 50 mM Tris.Cl (pH7.5), 2 mM $MgCl_2$, 150 mM NaCl and 5 mM ß-mercaptoenthanol.

The C-terminal fragment of RanGAP1 (residues 418-587) modified by SUMO-1, -2 and -3 was prepared as described previously [29]. The isopeptide-linked diSUMO-2 (for clarity, we will refer to the isopeptide-linked SUMO-2 dimer as diSUMO-2) was made by self conjugation in vitro and purified by gel filtration (Superdex75 Amersham-Pharmacia). To make polySUMO-1, SUMO-1 mutant (D15V) was generated by PCR based mutagenesis. PolySUMO-1 (D15V) and polySUMO-2 were prepared as described previously [10].

Isopeptidase assays were performed in 50 μl reactions containing substrates at the indicated concentration and a range of concentration of SENP7C (7.5 nM - 2 μM) for 1 hour at 37ºC. Reactions were terminated by adding 2x protein sample buffer. Samples were fractionated by SDS-PAGE (4-12%) followed by Coomassie Blue staining.

RESULTS

Expression and purification of SENP7C

On the basis of sequence alignment SENP7 is a member of SUMO specific proteases. All SENPs share a conserved catalytic domain (~200 residues) that contains a characteristic catalytic triad of cysteine, histidine and aspartate [see review16]. Interestingly 50-200 amino acid insertions split the SENP7 and SENP6 catalytic domains into two halves (Figure1 A). To establish biochemical activity of SENP7, We cloned the SENP7 gene and expressed and purified the SENP7 catalytic domain. SENP7C (residue733-1050) was expressed in bacteria as a 6xHisMBP fusion protein and purified by Ni-NTA affinity chromatography. The 6xHisMBP tag was cleaved by TEV protease and SENP7C was purified by exclusion from Ni-NTA agarose and gel filtration. Coomasie Blue staining revealed that the purified SENP7C was essentially homogenous (Figure 1 B).

SENP7 does not act as a SUMO C-terminal hydrolase.

SUMO-1, SUMO-2 and SUMO-3 are all expressed as inactive precursors that need to be precisely cleaved by SUMO proteases to reveal the C-terminal di-glycine motif. Previous research has demonstrated that SENP1, SENP2, SENP3 and SENP5 possess hydrolase activity and can discriminate between SUMO paralogs as processing substrates [see review 16]. To examine SENP7

processing activity, recombinant, purified SUMO-1, SUMO-2 and SUMO-3 precursors (25μM) were incubated with purified SENP7C (5μM) and SENP1C (0.5μM) and reaction products analysed by SDS-PAGE followed by staining with Coomassie Blue. Consistent with the earlier findings, we observed efficient SENP1 mediated cleavage of SUMO-1 and SUMO-3 precursors, with less activity towards SUMO-2 precursor. However, we found that even at a 10 fold higher concentration SENP7C does not detectably cleave SUMO-1, SUMO-2 and SUMO-3 precursors during the course of one hour incubation at 37ºC (Figure 2A). These results suggested that like SENP6, SENP7 does not function as a general SUMO processing enzyme.

SENP7 is an isopeptidase with preference for SUMO-2 conjugates *in vitro* .
To assess the isopeptidase activity of SENP7, model substrates were generated by conjugating the C-terminal fragment of RanGAP1 (residue 418-587) to SUMO-1 or SUMO-2. The RanGAP1-SUMO conjugates were purified by affinity chromatography and gel filtration and the purified proteins were used as substrates (20μM) to evaluate isopeptidase activity. Consistent with earlier findings [29], SENP1 (10nM) can efficiently remove both SUMO-1 and SUMO-2 from RanGAP-1 (Figure 2B). While SENP7C (100nM) did not deconjugate SUMO-1 from RanGAP-1, it could partially deconjugate SUMO-2 from RanGAP-1–SUMO-2 (Figure 2B). To evaluate the deconjugating activity of SENP7C, 20 μM RanGAP1-SUMO-1 or RanGAP1-SUMO-2 was incubated with a range of concentrations of SENP7C. Even at high concentration of SENP7C (2 μM), only limited deconjugation of RanGAP1-SUMO-1 was observed (Figure2C). Although SENP7C was capable of cleaving RanGAP1-SUMO-2, the reaction did not appear to proceed very efficiently as 120 nM SENP7C was required to cleave 50% of the substrate (Figure 2C). As an alternative isopeptidase substrate diSUMO-2 was generated by conjugation in vitro and purification by gel filtration. SENP7C robustly cleaved diSUMO-2 (20 μM) achieving 50% cleavage with 15 nM SENP7C (Figure 2C). Thus SENP7C has limited isopeptidase activity against RanGAP1-SUMO-1, increased activity against RanGAP1-SUMO-2, but efficiently cleaves diSUMO-2.

SENP7 deconjugates polymeric chains of SUMO-2 but not SUMO-1.
It has been reported that, like Ub, SUMO-2/-3 but not SUMO-1 can form polymeric chains, either as homopolymers or conjugated to protein substrates [10]. The apparent preference of diSUMO-2 compared to RanGAP1-SUMO-2 as a substrate for the isopeptidase activity of SENP7C suggested that the preferred substrates for SENP7 might be polySUMO chains. Although SUMO-1 does not usually form poly SUMO-1 chains, we generated a D15V version of SUMO-1 that is capable of forming polySUMO-1 chains. This allowed us to directly compare the ability of SENP7 to depolymerize polySUMO-1 and polySUMO-2 chains. Equal amounts of polySUMO-1 or polySUMO-2 chains were incubated with either SENP7C, a catalytically inactive form of SENP7 or SENP1C. It is clear that SENP7C is capable of deconjugating polySUMO-2 chains, but has no detectable activity against polySUMO-1 chains. As expected SENP1 efficiently

deconjugated both polySUMO-1 and polySUMO-2 chains (Figure 3A). To allow a direct comparison of the substrate preference of SENP7, 0.85 μg/μl polySUMO-1 (D15V) or polySUMO-2 chains were incubated with a range of concentrations of SENP7 and the reaction products were analysed by SDS-PAGE followed by Coomassie Blue staining. Even at high concentration, SENP7C had no detectable activity against polySUMO-1 chains but is very active in depolymerising polySUMO-2 chains (Figure 3B and C). At high concentrations, SENP7 is capable of completely depolymerising polySUMO-2 chains to monomeric SUMO-2. Thus SENP7 discriminates between SUMO-1 and SUMO-2 *in vitro*, raising the possibility that SENP7 may differentially regulate SUMO-2/-3 versus SUMO-1 conjugates *in vivo*.

SENP7 has SUMO-2/-3 isopeptidase activity *in vivo*

To establish that SENP7 is active *in vivo*, the cDNA encoding full-length SENP7 was inserted in to the eukaryotic expression vector pCDNA3 and transfected into COS7 cells along with expression constructs for either HA-SUMO-1, HA-SUMO-2 or HA-SUMO-3. As a control, the HA versions of SUMO were also cotransfected with empty expression construct or a construct encoding SENP7 mutant (C992S) where the catalytic cysteine residue is mutated to serine. After 48 hours, SUMO modified substrates in cell extracts were revealed by Western blotting with an anti-HA monoclonal antibody. Equal expression levels of SENP7 and its mutant were verified by anti-SENP7 Western blotting (Figure 4A). Transfection of HA-SUMO-1, HA-SUMO-2 or HA-SUMO-3 led to the accumulation of a number of high molecular weight SUMO modified species. Cotransfection with wild type SENP7 led to a reduction in the level of high-molecular weight SUMO-2 and SUMO-3 conjugates, but not SUMO-1 conjugates. As expected, cysteine 992 is required for desumoylation since expression of the SENP7 mutant (C992S) failed to promote the reduction of either SUMO-2 or SUMO-3 conjugates (Figure 4A).

As most SUMO conjugates are known to be nuclear it was of interest to determine the sub-cellular localisation of SENP7. We therefore fused the cDNA for SENP7 in-frame with the cDNA for EGFP and an expression construct for the EGFP-SENP7 fusion protein was transfected into COS7 cells. The localisation of the fusion protein was determined by fluorescence microscopy and shown to be diffusely nuclear with some subnuclear punctuate staining (Figure 4B). Cells were also stained with an antibody to visualise PML sub-nuclear bodies. Although the bulk of SENP7 was not co-localized to PML bodies, a small portion of SENP7 appeared to localized with PML in sub-nuclear bodies. The distribution of the catalytically inactive C992S SENP7 fused to EGFP was indistinguishable from that of wild type SENP7 (Figure 4B).

SENP7 deletion cause accumulation of SUMO-2 conjugates, not SUMO-1 conjugates *in vivo*

To examine the biological function of SENP7 *in vivo*, its expression was suppressed by siRNA mediated gene silencing in human Hela cells. 48 hr after siRNA treatment with a pool of siRNAs that target SENP7 mRNA or a non-targeting pool of siRNAs, cell extracts were prepared and analysed by Western blotting. SENP7 expression was efficiently reduced in cells treated with the targeting siRNA, but not the non-targeting siRNA (Figure 5A). The same cell extracts were also analysed by Western blotting with antibodies to SUMO-1 and SUMO-2/-3. Ablation of SENP7 expression resulted in the accumulation of high molecular weight SUMO-2 species with only a small increase in SUMO-1 staining species. As SUMO modification is known to contribute to the metabolism and localisation of PML subnuclear bodies, cells treated with either a siRNA against SENP7, or a non-targeting siRNA were stained with an antibody to PML and PML localisation determined by immunofluorescence. Cells treated with the SENP7 siRNA displayed both an increase in the number of PML bodies (Figure 5C) and an increase in the intensity of PML sub-nuclear body staining (Figure 5B). Thus SENP7 appears to regulate both SUMO-2/3 and PML metabolism *in vivo*.

DISCUSSION

Here, we present an analysis of the biochemical and biological activities of the SUMO specific protease SENP7. We find that SENP7 appears to function primarily as an isopeptidase and has no detectable capacity to endoproteolytically process the natural precursors of SUMO-1, SUMO-2 and SUMO-3. In surveying the biochemical activities of the SENPs the inability of SENP7 to process SUMO precursors had been noted [26]. As an isopeptidase the preferred substrate of SENP7 appears to be polymeric chains of SUMO-2/3, although it is capable of deconjugating SUMO-2/3 from model substrates such as RanGAP1, albeit with reduced efficiency. It appears to have undetectable activity against either polySUMO-1 chains or against SUMO-1 conjugated to model substrates. Thus SENP7 is a SUMO-2/3 specific protease with a distinct preference to cleave the isopeptide bond that links two SUMO-2/3 molecules, While this manuscript was in preparation the structure of SENP7 was reported [31]. This revealed that SENP7 was structurally related to previously characterised SENP1 and SENP2, but had a number of unique structural features. This study also concluded that SENP7 had a preference for polySUMO chains, but the structural basis for this preference was not evident.

In *vivo* co-expression of SENP7 with HA-SUMO-2 or HA-SUMO-3 leads to the reduction of high molecular weight SUMO-2/3 species. This effect is highly specific as it is not observed when a catalytically inactive version of SENP7 is co-expressed with HA-SUMO-2 or HA-SUMO-3. Co-expressed SENP7 does not influence the modification profile of co-transfected HA-SUMO-1, consistent with the lack of activity of SENP7 against SUMO-1 modified substrates or polySUMO-1 in *vitro*. Consistent with these over-expression studies siRNA mediated ablation of SENP7 expression leads to a substantial accumulation of high

molecular weight SUMO-2/3 modified species, with a small accumulation of high molecular weight SUMO-1 modified species. While the in *vitro* analysis indicated that SENP7 was highly selective for SUMO-2/3 with little activity against SUMO-1, the in *vivo* observations can be explained by the presence in *vivo* of polySUMO-2/3 chains that are terminated by SUMO-1 [32]. If the polySUMO-2/3 component of this mixed chain is cleaved by SENP7 then this would account for the apparent loss of the high molecular weight SUMO-1 material. Analysis of PML protein expression by immunofluorescence indicates that the intensity of PML staining increases, as does the number of PML bodies per nucleus, in the absence of SENP7. As SUMO-2/3 modification of PML influences the metabolism of PML protein by recruitment of the SUMO-specific ubiquitin E3 ligase Rnf4 [33, 34] this indicates that SENP7 is likely to be a key regulator of PML turnover. SENP6 has a similar substrate specificity and effect on PML bodies to that observed above for SENP7 [24, 31] suggests that the two SUMO proteases do not have redundant functions and ablation of expression of one of these proteases cannot be complemented by the other.

Figure Legends

Figure 1 (A) Sequence alignment of catalytic domain of SENP7 with other members of SUMO specific protease family. Sequences were aligned using ClustalW (Thompson et al, 1994). Identical residues are indicated by (*), Highly Conserved residues (:), loosely conserved residues (.) and residues of the catalytic triad are boxed. (B) Expression and purification of catalytic domain of SENP7. Samples were taken from various steps during purification of SENP7C. Molecular weight markers (Kd) are shown on the left, with sample loading as follows: lane1, total bacterial extract from non-induced cells; lane2, total cell lysate from induced cells; lane3, proteins in pellet; lane4, proteins in supernatant; lane5, purified 6HisMBP–SENP7C from Ni-NTA column; lane6, Cleavage of fusion protein by TEV protease overnight at 4ºC; Lane7, purified SENP7C.

Figure 2. processing and deconjugation activities for SENP7. (A) Assays for SUMO processing activities by SENP7C (5 μM) and SENP1C (0. 5 μM) using SUMO-1, SUMO-2 and SUMO-3 precursors (25μM). SENP7C does not have C-terminal hydrolase activity against SUMO-1, SUMO-2 and SUMO-3 precursors, whereas SENP1C is active for all SUMO precursors. (B) Deconjugation activity assays for SENP7C (100nM) and SENP1C (10nM) using RanGAP-1-SUMO-1/2 (20μM). SENP7C does not remove SUMO-1 from RanGAP1-SUMO-1; however it does remove SUMO-2 from RanGAP-1-SUMO-2 with less efficiency compared to SENP1C. (C) Deconjugation of RanGAP1-SUMO-1/2 and SUMO-2 dimer with a range of concentration of SENP7C (7.5nM -2μM) at 37ºC. Reactions were stopped after 1 hour with loading buffer, analyzed by SDS-PAGE followed by Coomassie blue staining.

Figure 3. Comparison of substrate preference of SENP7 against poly SUMO-1 and poly SUMO-2 chains. (A) Equal amounts of polySUMO-1 or polySUMO-2 chains (0.85μg/μl) were incubated with SENP7C (100nM) or SENP1C (10nM) . SENP1 effectively deconjugates both poly SUMO-1 and poly SUMO-2. However, SENP7C (100nM) is less active, only partially deconjugates polySUMO-2 chains. SENP7C has no detectable activity against polySUMO-1 chains. (B) Deconjugation of polySUMO-1 chains with different concentration of SENP7C (7.5nM -2μM). (C) Same reaction as described in (B), but substituting polySUMO-2 chains for polySUMO-1chains.

Figure 4. (A) SENP7 acts as a SUMO-2/3 specific protease *in vivo*.
COS7 cells were cotransfected with either empty pCDNA3 (-), or plasmids expressing HA-SUMO-1, HA-SUMO-2, HA-SUM-3, SENP7 or SENP7Mutant (C992S) as indicated. Anti-HA, anti-SENP7 and anti-ß-actin Western blots were performed on the cell lysates as detailed under "Experimental".
(B) Subcellular localization of SENP7. Full-length of human SENP7 cDNA or its inactive mutant (C992S) was subcloned into pEGFP-C1 vector (Clontech) and transfected into COS7 cells. The localisation of the fusion protein was determined by fluorescence microscopy. Cells were also stained with an antibody to visualise PML subnuclear bodies.

Figure 5. (A) Knockdown of SENP7 leads to accumulation of SUMO-2 conjugates *in vivo*. HeLa cells were transfected with a pool of siRNAs that target SENP7 mRNA or control siRNAs. 48 hours after siRNA treatment, cell lysates were analysed by western blotting with antiSENP7, antiSUMO-1 and antiSUMO-2 antibody.
(B) and (C) SENP7 regulates PML metabolism *in vivo*. Cells treated with either siRNAs against SENP7 or control siRNAs were stained with a mouse anti-PML 5E10 antibody and PML localisation was determined by immunofluorescence microscopy with antibody to PML (green). DNA was stained with DAPI (blue).

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                424 .         .                :   *:  *   .:*** ::::* . ::::   .:     * *.:**.  *
SENP1 ------TEEMEKEIKNVFRNGNQDEVLSEAFRLTITRKDIQTLNHLNWLNDEIINFYMN-MLMERSKEKGLPSVHAFNTFFFTKLK----------------TA
SENP2 ------TEDMEKEISNALGHGPQDEILSSAFKLRITRGDIQTLKNYHWLNDEVINFYMN-LLVERNKKQGYPALHVFSTFFYPKLK----------------SG
SENP3 -DIGSVLQLIQ-SYQRMPGNAMVRGFRVAYKRHVLTMDDLGTLYGQNWLNDQVMNMYGD-LVMDTVPEK----VHFFNSFFYDKLR----------------TK
SENP5 EDFSNRKPFINREITNYRARHQKCNFRIFYNKHMLDMDDLATLDGQNWLNDQVINMYGE-LIMDAVPDK----VHFFNSFFHRQLV----------------TK
SENP6 -DEEEETGENHTIFIGPVEKLIVYPPPPAKGGISVTNEDLHCLNEGEFLNDVIIDFYLKYLVLEKLKKEDADRIHIFSSFFYKRLNQRER-RNHETTNLSIQQK
SENP7 --DEEWREVRHT---GLVQKLIVYPPPPTKGGLGVTNEDLECLEEGEFLNDVIIDFYLKYLILEKASDELVERSHIFSSFFYKCLTRKENNLTEDSPNLSMAQR
      733

       :  *: **: :: *. . :::*::   ** *  : .
SENP1 GYQAVKRWTKKVD-FSVDILLVPIHLGVHWCLAVVDFR----------------------------------------------------------------
SENP2 GYQAVKRWTKGVNLFEQEIILVPIHRKVHWSLVVIDLR----------------------------------------------------------------
SENP3 GYDGVKRWTKNVDIFNKELLLIPIHLEVHWSLISVDVR----------------------------------------------------------------
SENP5 GYNGVKRWTKKVDLFKKSLLLIPIHLEVHWSLITVTLS----------------------------------------------------------------
SENP6 RHGRVKTWTRHVDIFEKDFIFVPLNEAAHWFLAVVCFPGLEKPKYEPNPHYHENAVIQKCSTVEDSCISSSASEMESCSQNSSAKPVIKKMLNKKHCIAVIDSN
SENP7 RHKRVRTWTRHINIFNKDYIFVPVNESSHWYLAVICFPWLKEAVYEDFP-------------------------QTVSQQSQAQ--------------------


SENP1 ----------------------------------------------------------------------------------------------------
SENP2 ----------------------------------------------------------------------------------------------------
SENP3 ----------------------------------------------------------------------------------------------------
SENP5 ----------------------------------------------------------------------------------------------------
SENP6 PGQEESDPRYKRNICSVKYSVKKINHTASENEEFNKGESTSQKVADRTKSENGLQNESLSSTHHTDGLSKIRLNYSDESPEAGKMLEDELVDFSEDQDNQDDSS
SENP7 ----------------------------QSQN--------------DNKTIDNDLRTTST-------------LSLSAE--------------------DSQSTES


                              .  :   **          . : :**  *   *   .                  :.:* * **** : .:* . :   .  .
SENP1 ------------------------KKNITYYDSM-GGINNEACRILLQYLKQESIDKKRKEFDTNGWQLFSKKSQEIPQQMNGSDCGMFACKYADCITKDRPI
SENP2 ------------------------KKCLKYLDSM-GQKGHRICEILLQYLQDESKTKRNSDLNLLEWTHHSMKPHEIPQQLNGSDCGMFTCKYADYISRDKPI
SENP3 ------------------------RRTITYFDSQ-RTLNRRCPKHIAKYLQAEAVKKDRLDF-HQGWKGYFKMN--VARQNNDSDCGAFVLQYCKHLALSQPF
SENP5 ------------------------NRIISFYDSQ-GIHFKFCVENIRKYLLTEAREKNRPEF-LQGWQTAVTKC--IPQQKNDSDCGVFVLQYCKCLALEQPF
SENP6 DDGFLADDNCSSEIGQWHLKPTICKQPCILLNDSLRGPSRSNVVKILREYLEVEWEVKKGSKR-SFSKDVMKGSNPKVPQQNNFSDCGVYVLQYVESFFENPIL
SENP7 N--------------MSVPKKMCKRPCILILDSLKAASVQNTVQNLREYLEVEWEVKLKTHR-QFSKTNMVDLCPKVPKQDNSSDCGVYLLQYVESFFKDPIV

       *      . :*    :   *  .  :643
SENP1 NFTQ-----QHMPYFRKRMVWEILHRKLL-----------------------------------
SENP2 TFTQ-----HQMPLFRKKMVWEILHQQLL-----------------------------------
SENP3 SFTQ-----QDMPKLRRQIYKELCHCKLT-----------------------------------
SENP5 QFSQ-----EDMPRVRKRIYKELCECRLMD----------------------------------
SENP6 SFELPMNLANWFPPPRMRTKREEIRNIILKLQEDQSKEKRKHKDTYSTEAPLGEGTEQCVNSISD
SENP7 NFELPIHLEKWFPRHVIKTKREDIRELILKLHLQQQKGSSS-----------------------
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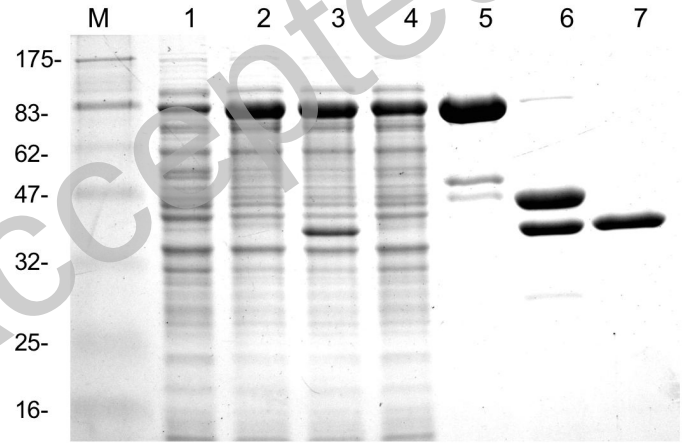

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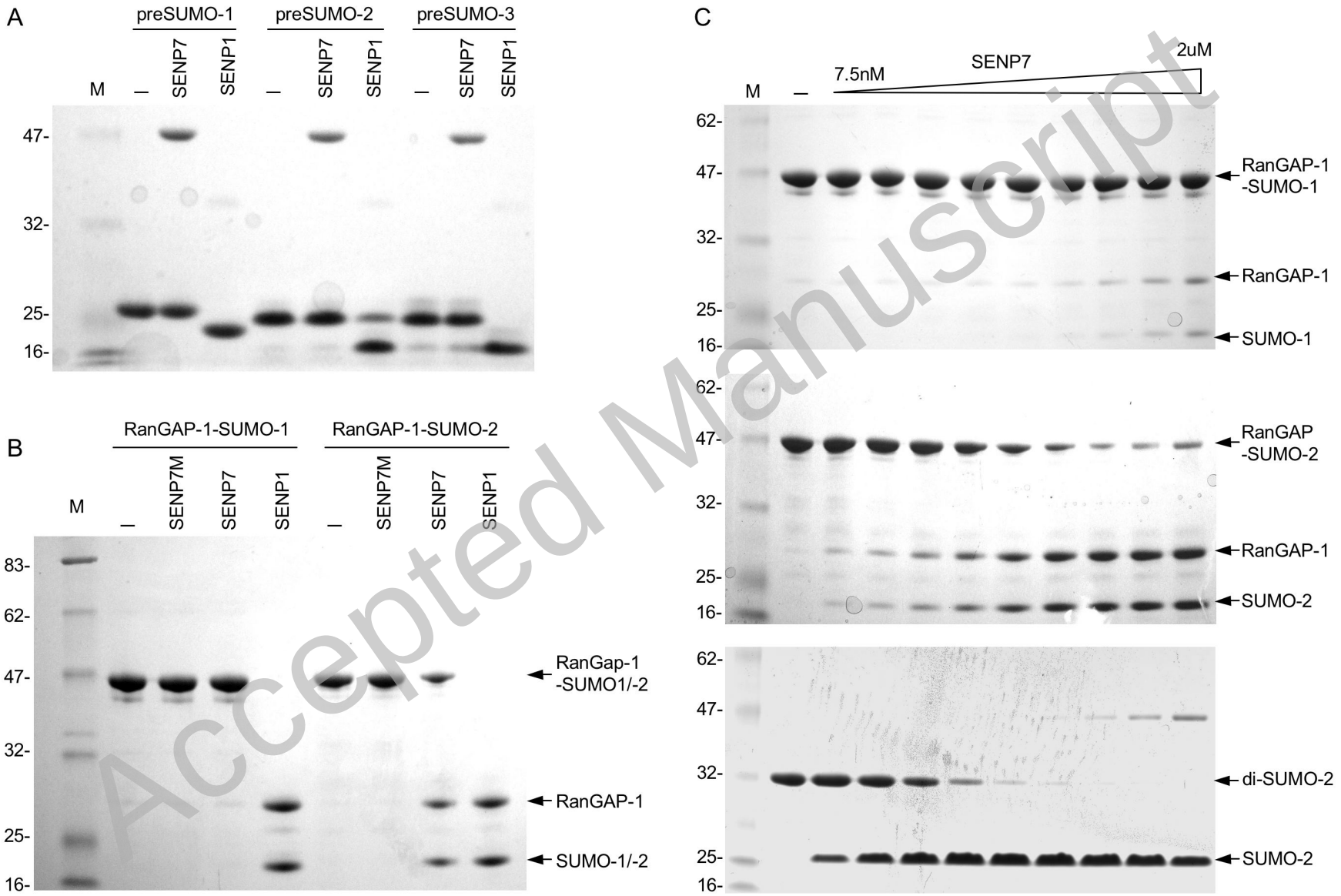

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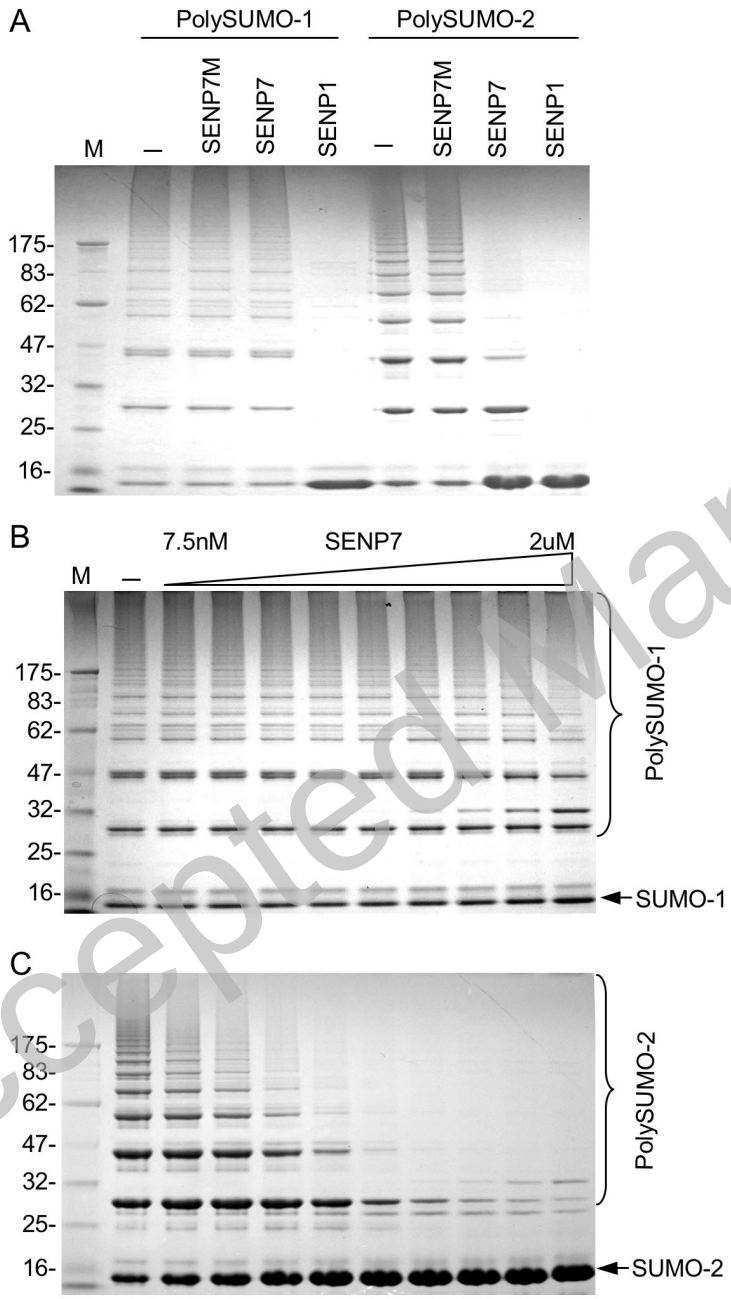

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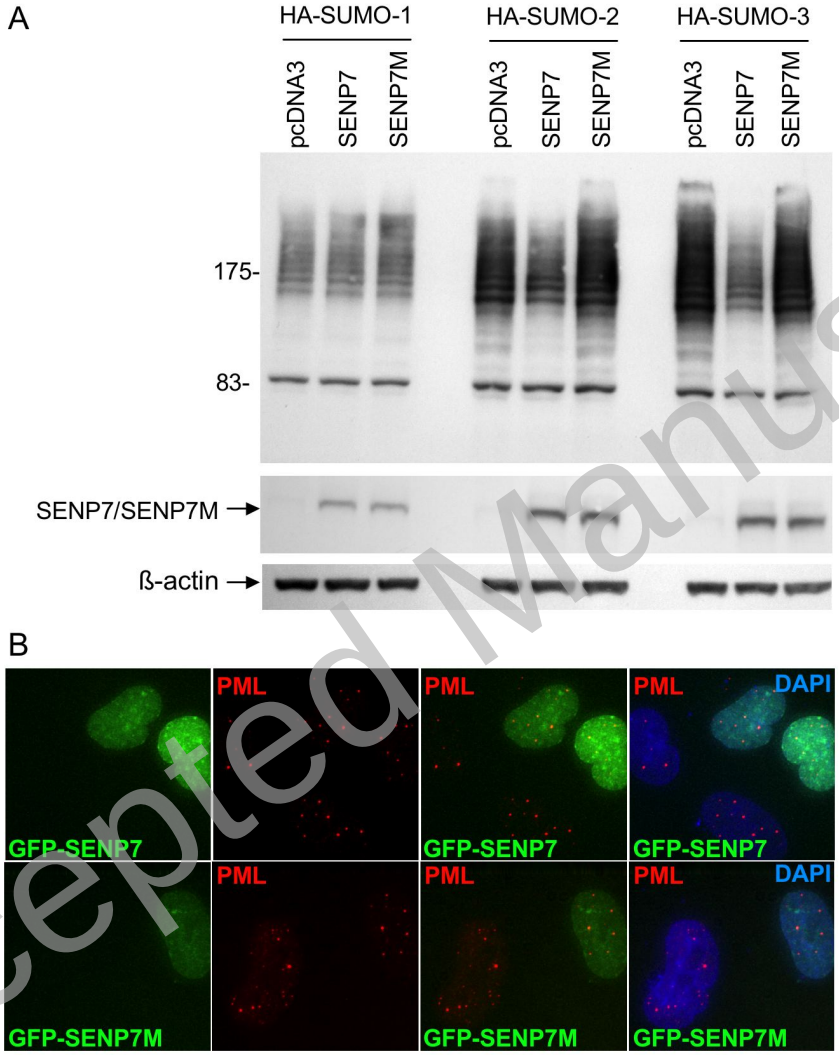

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