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Analysis of SUMOylated proteins using SUMO-traps

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SUMO-modified proteins are recognized by SUMO interacting motifs (SIMs), thus triggering diverse cellular responses. Here SIMs were used to develop SUMO-traps to capture endogenous SUMOylated proteins. Our results show that these small peptides are transferable motifs that maintain their SUMO binding capacity when fused to the heterologous carrier protein GST. The tandem disposition of SIMs increases the binding capacity of SUMO-traps to specifically interact with polySUMO but not poly-Ubiquitin chains. We demonstrate that this SUMO capturing system purifies SUMOylated proteins such as IκBα, PTEN, PML or p53 *in vitro* and *in vivo*. These properties can be used to explore the many critical functions regulated by protein SUMOylation.

SUMO (Small Ubiquitin MOdifier) is an ubiquitin-like protein involved in post- translational modification of critical cellular factors controlling protein localisation, transcription, DNA repair or cell cycle progression among other functions[1,2]. Protein SUMOylation is defined as the covalent modification of target proteins with SUMO molecules on the lysine of the canonical consensus sequence ψKxE (where ψ is a large hydrophobic aminoacid and x any aminoacid)[3]. Generally, this process is carried out by an enzymatic cascade involving three enzymes, E1 (SUMO-activating enzyme (SAE)), E2 (SUMO-conjugating enzyme (Ubc9)) and E3 (SUMO ligating enzyme)[2]. The reverse reaction known as protein deSUMOylation, is carried out by SENPs or SUSPs (SUMO specific proteases) implicated in the regulation of important cellular responses[4–6]. In mammals three different SUMO molecules are involved in protein SUMOylation: SUMO-1, SUMO-2 and SUMO-3[2]. In response to general cellular insults, such as heat shock, oxidative stress or the use of proteasome inhibitors, the level of protein SUMOylation notably increases[7,8]. Certain viral infections or arsenic trioxide treatment are also able to induce SUMOylation on specific proteins such as p53 and PML (personal communication Rivas C)[9,10].

Non-covalent interactions with SUMO can also occur through SIMs (SUMO interacting motifs). SIMs are often present within SUMO substrates or enzymes regulating the levels of SUMOylated proteins[11–13]. SIMs were classified in 2000 by Miteva and collaborators in three categories according to their aminoacid residue conservation SIM-a [PILVM]-[ILVM]-x-[ILVM]-[DSE](3); SIM-b [PILVM]-[ILVM]-D-L-T and SIM-r [DSE](3)-[ILVM]-x-[ILVMF](2)[14,15]. Generally SIMs contain a hydrophobic core with 3–4 aliphatic residues, adjacent to a negatively charged cluster of acidic amino acids[13,16]. SIMs are present in many key cellular mediators and therefore the identification of new proteins containing SIMs can be important to further understand SUMO-dependent regulatory mechanisms[12,17]. Particular attention has been given to proteins comprising multiple SIMs, such as CASP8AP2 (CASP8-associated protein 2), RNF4 and RNF111 among others[18,15]. Sun and Hunter (2012) demonstrated that mutation of SIM1 and SIM3 in RNF111 results in the loss of its polySUMO binding capacity. RNF4, the best characterised multiple SIM-containing protein shows SUMO-dependent ubiquitin ligase activity[15]. Among the 4 SIM motifs of RNF4, SIM2 and SIM3 play a more important role in the interaction with poly-SUMO chains[18]. These findings highlight the importance of clustered SIMs, which efficiently interact with polySUMO chains by increasing the binding capacity for SUMO, since the affinity of an isolated SIM for SUMO is usually low (2–3 μM range)[16,13].

Here, we engineered a GST-fusion protein carrying tandem repeats of SIM2 and SIM3 motifs of RNF4 to generate a system to capture SUMOylated proteins. We demonstrate that our SUMO-traps are potent affinity purification tools to isolate total and specific SUMOylated proteins, such as PTEN, IκBα, PML and p53 *in vivo* and/or *in vitro*.

Results

Designing SUMO-traps to capture SUMOylated proteins. SUMO-traps, also named SUMO binding entities (SUBEs), were engineered by introducing RNF4-derived SIM2 and SIM3 motifs in tandem, into a GST vector (Fig. 1A). GST itself was modified by introducing an N-terminal His-6 tag and a C-terminal SV5 epitope (Fig. 1A), allowing to assess the integrity of the constructs (Fig. 1B). The GST system was chosen to obtain a format comparable to the one we used for tandem ubiquitin binding entities (TUBEs)[19]. Two types of SUMO-traps were designed: SUBE-l (SUBE-long), containing four inserts (eight SIMs), and SUBE-s (SUBE-short), containing only one insert (two SIMs) (Fig. 1A). SUBE-l was able to pulldown polySUMOylated proteins from HeLa cells challenged at 43°C for 1h (Fig. 1C)[7]. However, under the same experimental conditions, the efficiency of polySUMOylated protein pulldown by SUBE-s was poor (Fig. 1D). These results support the notion of cooperativity between tandem-repeated SIMs. It has been reported that the acidic aminoacids that flank each SIM core, are important for SUMO recognition[11]. We thus replaced the natural acidic linker between the hydrophobic cores of SIM2 and SIM3 by seven glycine residues. The resulting mutant (SUBE-l mut) is unable to interact with SUMOylated proteins (Fig. 1E), underlining the importance of the flanking acidic aminoacids in the recognition of polySUMO chains.

In addition to their capacity to interact specifically and purify modified proteins, molecular traps such as TUBEs hinder the action of cysteine proteases[19]. To ascertain if this was the case for SUBEs, we tested the capacity of the traps to interfere with the SUMO deconjugation process in a cell free system. Compared to popular cysteine protease inhibitors, such as N-ethylmaleimide (NEM), Iodoacetamide (IAA) and cell-permeable deubiquitylating enzyme (DUB) inhibitor PR-619, SUBEs showed only a modest protection of high molecular weight forms of SUMO-2/-3 compared to the GST control (Supplementary Fig. 1). Although NEM was the most efficient SUMO protease inhibitor (on the basis of free SUMO-2/-3 accumulation), it interferes with GST binding to the glutathione beads (unpublished observations), so we rather used PR-619 in cell media or lysis buffers.

Interaction of SUMO-traps with polySUMO chains. To further demonstrate the cooperativity of tandem SIMs within SUBE-l when interacting with polySUMO chains, Surface Plasmon Resonance (SPR) experiments were performed using the previously reported tetra-SUMO-2 fusion protein (4xS2)[20] (Fig. 2). As shown in Fig. 2A, SUBE-l interacted stronger with 4xS2 than SUBE-s. GST pulldowns using either SUBE-1 or SUBE-s yielded similar results (Fig. 2B). Of note, no binding of free SUMO-2 (S2) was observed in these assays (Fig. 2B). No significant interaction could be detected between the ubiquitin-trap TUBE-HR23 and 4xS2, both by SPR (Fig. 2A right panel) and GST pulldown approaches (Fig. 2C),

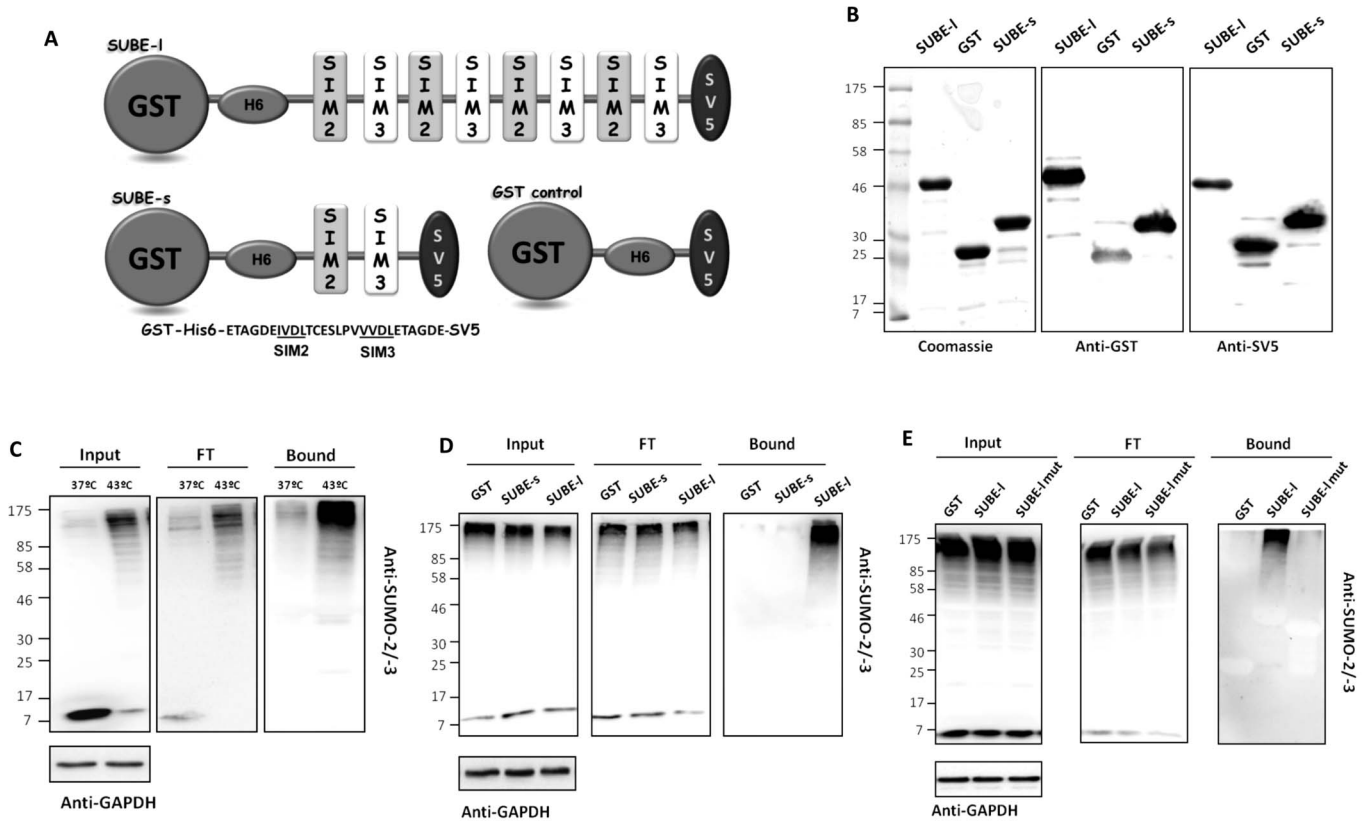

Figure 1 | SUMO-binding entities (SUBEs) present the capacity to bind SUMOylated proteins. (A) Cartoon illustrating the design of SUMO-binding entities (SUBEs). SUBE-l and SUBE-s contain 8 and 2 SIMs respectively. The SIM2 and SIM3 region cloned is the natural sequence of RNF4 protein. As a control we used the same GST vector without any insert (GST control). (B) Purified SUBEs can readily be detected by Coomassie, anti-GST and anti-SV5 antibodies. (C) Capture of SUMOylated proteins from heat-shock induced HeLa cells using SUBE-l. (D) SUBE-s do not capture polySUMO proteins from heat-shock induced HeLa cells with the same efficiency as SUBE-l. (E) Contribution of the linker region of SUBE-l to the capture of polySUMO chains from heat-shock induced HeLa cells. In the SUBE-l mutant (SUBE-l mut), the linker region was mutated to a poly-glycine sequence preserving the length of the natural linker. Western-blot detection with anti-SUMO-2/-3 or GAPDH antibodies.

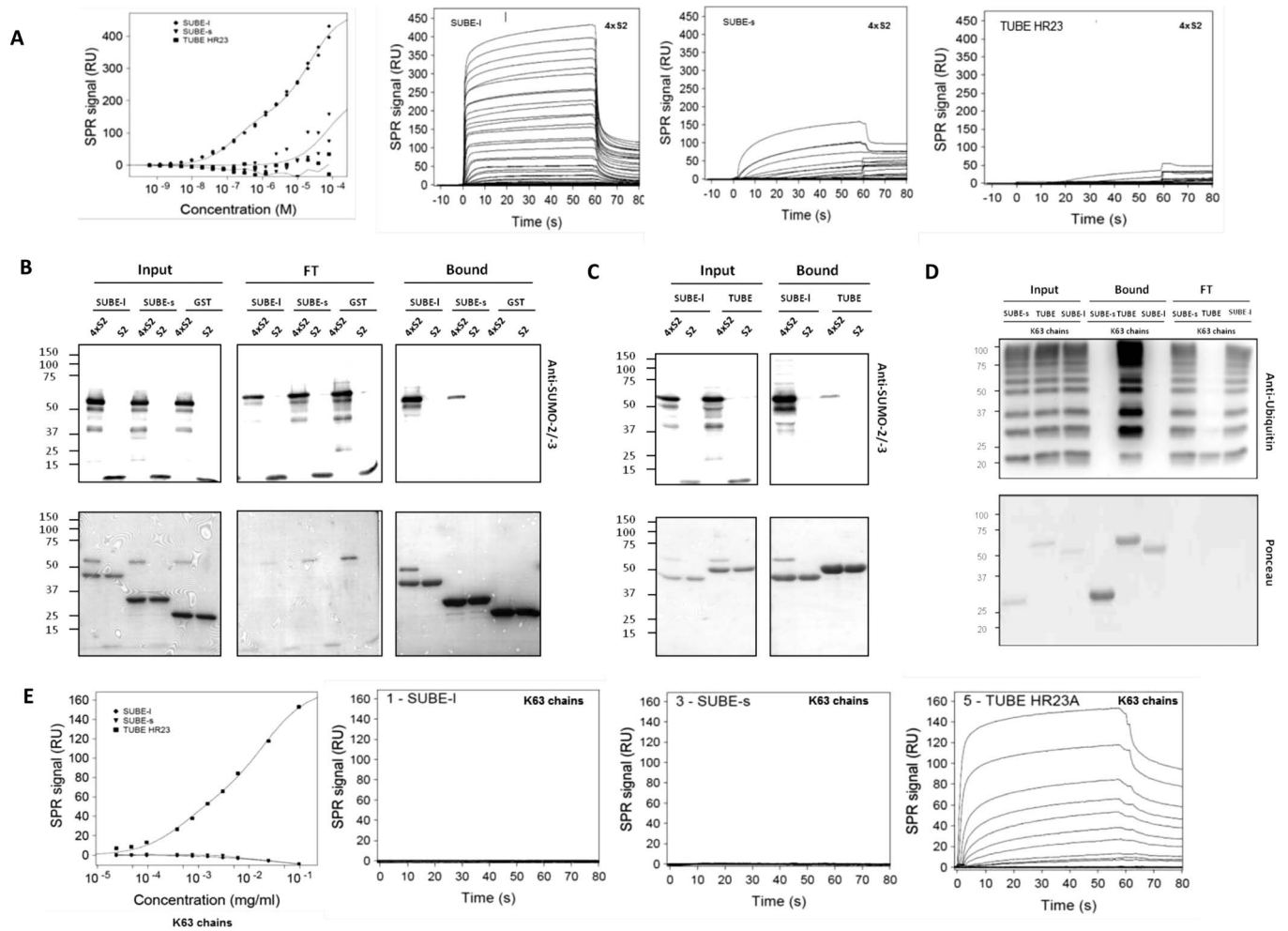

Figure 2 | SUBE-1 interacts with polySUMO chains. (A) The interaction of SUMO chains containing four SUMO-2 molecules (4xS2) with SUBE-l, SUBE-s or TUBE-HR23 was monitored by real-time SPR. SUBEs or TUBE-HR23 were captured on an anti-GST surface through their GST moiety, to a level of 100 RU, and 4xS2 (0–150 μM) was injected in a randomized order. (B) SUBE-l interacts better with 4xS2 chains than SUBE-s. A GST pulldown was performed with both SUBEs to test the interaction with polySUMO chains. Input, flow-through (FT) and bound fractions were analysed by Western-blot against anti-SUMO-2/-3 antibody. (C–D) Specific interaction of SUBE-l with 4xS2 chains. (C) A similar approach than (B) was used to investigate if the 4xS2 was also able to bind other molecular traps such as TUBEs. (D) Conversely it was also tested if SUBEs were able to bind a collection of K63 ubiquitin chains. Western-blot detection with the indicated antibodies. (E) The absence of binding of both SUBE-l and SUBE-s to K63 ubiquitin chains was verified by SPR in the same conditions as (A). TUBE-HR23 was used as positive control.

showing that polySUMO chains are recognized specifically by our SUBE-l trap. The specificity of SUBEs was further investigated using linear K63 poly-ubiquitin chains (and TUBE-HR23 as a positive control). GST pulldowns (Fig. 2D) and SPR assays (Fig. 2E) showed that none of the SUBEs could interact with K63 chains, unlike TUBE-HR23. Altogether, these experiments demonstrate that SUBEs specifically interact with polySUMO chains and do not bind to K63 poly-ubiquitin chains and free SUMO moieties.

As the SUMO-2 moieties of 4xS2 construct are linked by peptide bonds, the capacity of SUBE-l to interact with polySUMO chains linked by isopeptide bonds was tested using an *in vitro* SUMOylation assay (Supplementary Fig. 2). This assay, with no added substrate and SUMO E3 enzyme, resulted in a modest production of polySUMO chains. However, it can be seen that chains containing multiple molecules of SUMO-2/3 are efficiently depleted from the extract by SUBE-l and recovered in the bound fraction (Supplementary Fig. 2). Chains comprising a combination of SUMO-2 and SUMO-3 molecules were more efficiently retained than those containing only SUMO-1 (Supplementary Fig. 2). Thus, these results indicate that SUBE-l binds to isopeptide bond linked polySUMO chains in a similar way than to the 4xS2 construct.

Capture of multiple SUMO substrates using SUMO-traps. To verify that SUBE-l could be used to purify SUMOylated substrates, 4 different subtrates: PML[10], IκBα[21,22], p53[9] and PTEN[23] were SUMOylated *in vitro* using SUMO-1 or SUMO-2/-3 (Fig. 3 A–D). PolySUMOylated forms of all these substrates were efficiently retained by SUBE-l only when SUMO-2/-3 was used in the reaction, SUMO-1 conjugated forms being captured much less efficiently (Fig. 3 A–D). In our experimental conditions, the GST control bound to unmodified sticky forms of proteins such as IκBα or p53 in different proportions (Supplementary Fig. 3). Given that SUMO-1 and SUMO-2/-3 poly-SUMOylated chains were present in similar quantities in the input of each experimental set (Fig. 3 A–D) we can conclude that SUBE-l preferentially captures substrates comprising polySUMO-2/-3 chains.

SUMO-traps captures SUMOylated PML and p53 *in vivo*. To investigate the capacity of the SUMO-trap SUBE-l to interact with naturally SUMOylated PML or p53, SUMOylation was induced by challenging/treating cells with arsenic trioxide (ATO), (interferon-α) or vesicular stomatitis virus (VSV) infection as indicated (Fig. 4 A–D). Conditions were set to capture SUMOylated PML from NB4

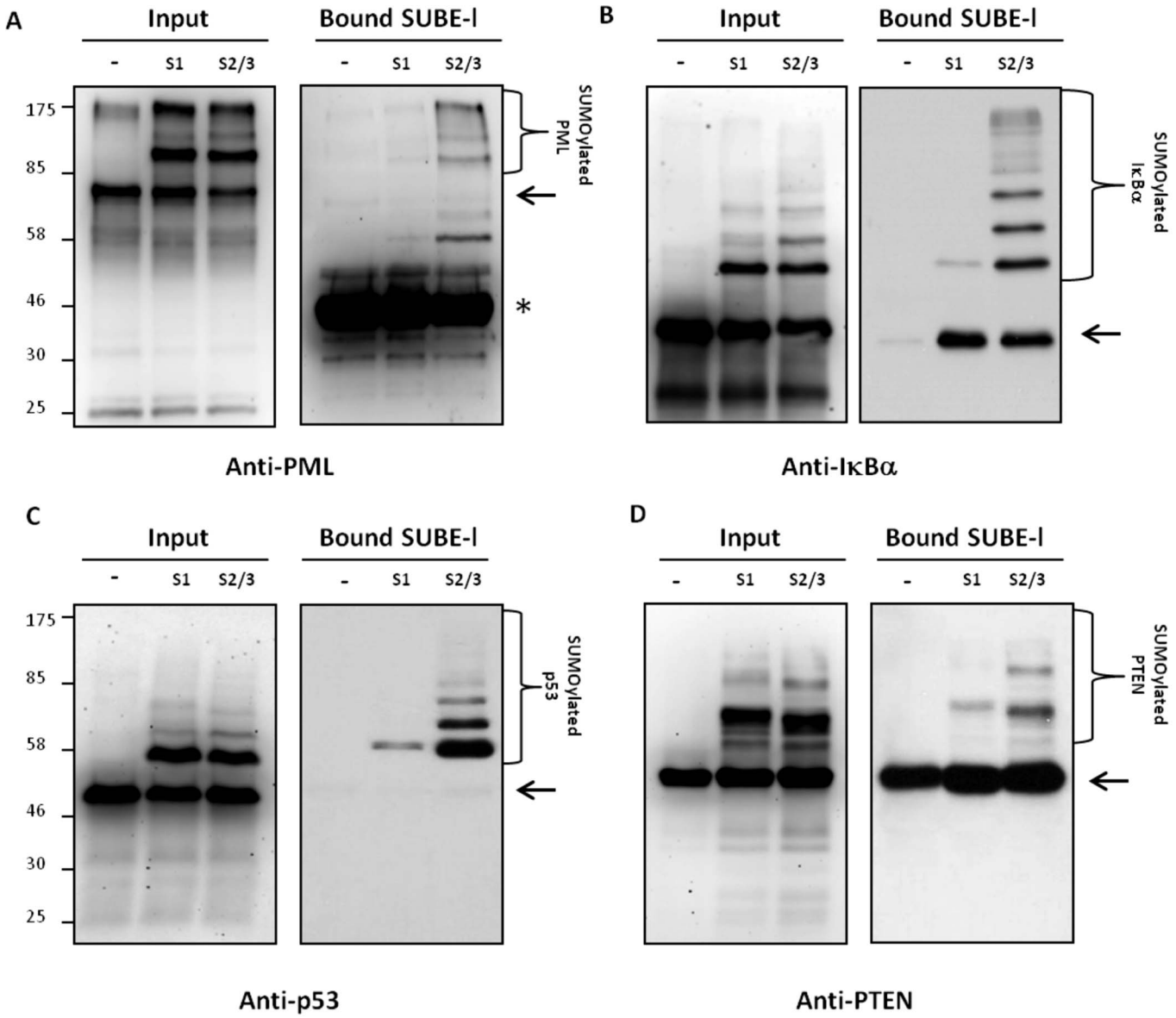

Figure 3 | Multiple SUMO substrates can be captured by SUBE-I. SUMOylated (A) PML, (B) IκBα, (C) p53 and (D) PTEN were SUMOylated *in vitro* with SUMO-1 (S1) or SUMO-2/-3 (S2/3) to generate modified proteins (input). Control reactions (-) do not contain SUMO activating enzyme. SUBE-I captured proteins are present in the bound fraction. Analysis by Western-blot using the indicated antibodies. The arrow shows unmodified proteins. * non specific signal.

(acute promyelocytic leukemia cell line, Fig. 4A) and MCF7 cells (breast cancer cell line, Fig. 4B), and to evaluate the co-localization of SUMO-2/-3 and PML in the nuclear bodies of NB4 cells (Fig. 4C). One hour pre-treatment with proteasome inhibitor MG-132 followed by one hour of stimulation with ATO resulted in the optimal capture of SUMOylated PML (Fig. 4A and B). Basal levels of SUMOylated PML were not captured indicating that this is an inducible process[10]. To capture SUMOylated p53, HEK-293 transfected with His6-SUMO-2 were treated with IFN-α for 24 hours or infected with VSV for 4 h (Fig. 4D). Here again, SUMOylated forms of p53 were captured and detected by Western blot only when cells were induced. The importance of SUMOylated p53 induced by IFN-α or VSV infection was further studied by Marcos-Villar et al (Rivas C. personal communication).

Discussion

As SIMs are very short peptide sequences, their efficiency for SUMOylated protein purification could be questioned, especially in terms of affinity and specificity. The multiplication of SIM motifs to increase the capacity to interact with polySUMOylated proteins is an approach naturally used by the cell in proteins such as RNF4 and RNF111[18,15]. Here we show that small SIM sequences can be transferred to non-related proteins, resulting in SUMO-binding entities (SUBEs) that have the capacity to bind and even purify SUMOylated proteins. Our finding opens the door to the design of multiple traps using different SIMs to study SUMO-regulated events. More studies are warranted to ascertain whether the nature of SIM sequences will determine the type of SUMOylated proteins captured. However, it is tempting to speculate that the flanking acidic regions around the hydrophobic core in the SUMO-binding domain could provide sufficient sequence elements to distinguish between different SUMO-chain types. In our experimental conditions, not all SUMOylated proteins were captured from cell extracts using SUBE-I (Fig. 1C–E and Fig. 4 B, C). Increasing the concentration of SUBE-I did not widen its selectivity towards other SUMOylated proteins, indicating that the non-captured material had SUMO chains with a different

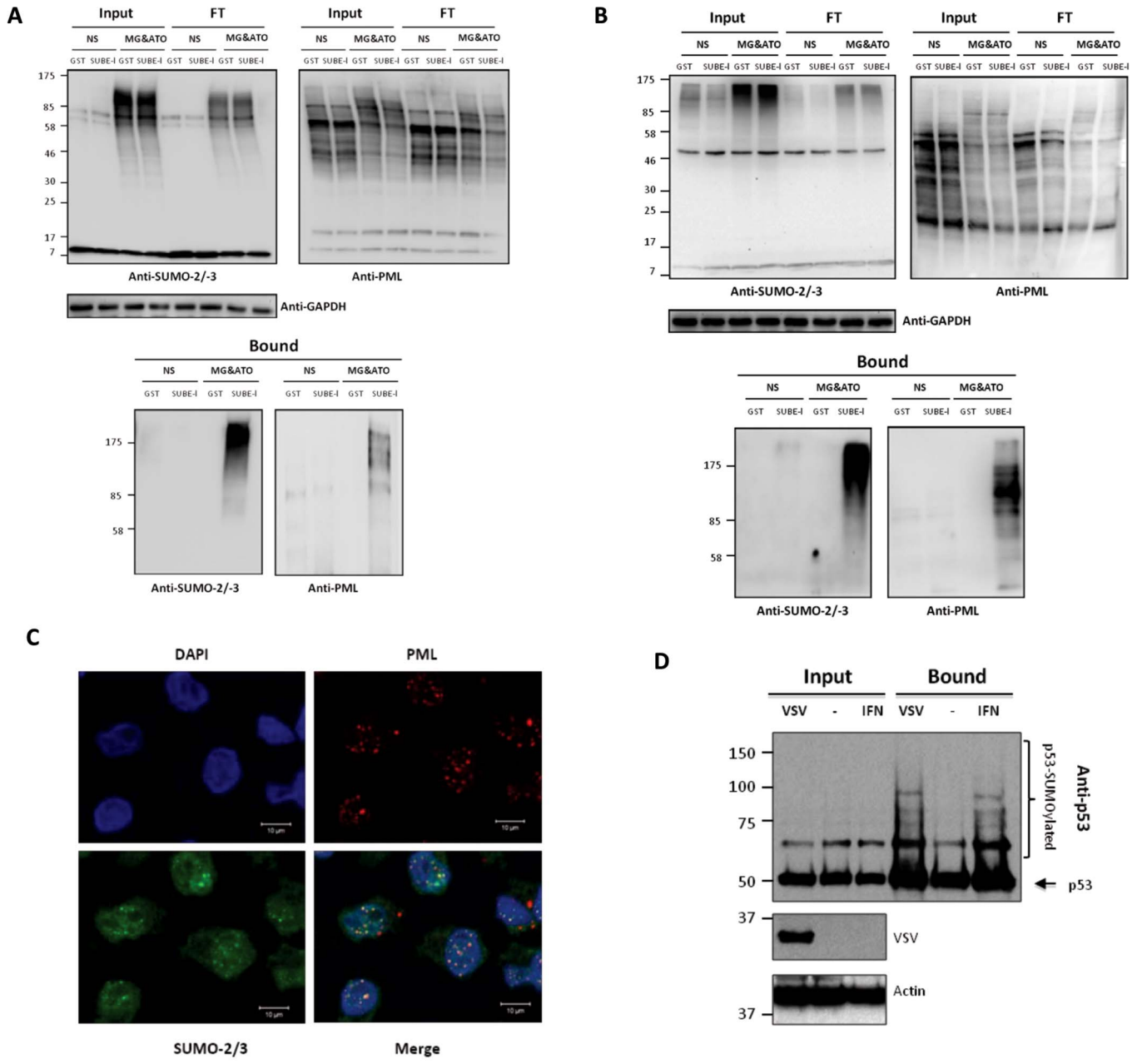

Figure 4 | Capture of SUMOylated PML and p53 using SUBE-l. NB4 cells (A) or MCF7 (B) were pre-treated with MG-132 before 1 h of ATO treatment. (C) SUMO and PML can be co-localized in the nuclear bodies (NB) of NB4 cells under the same conditions than (A or B). SUMOylated p53 can be captured by SUBE-l (D). HEK-293 were untreated or treated with IFN-α or infected with VSV. Input, flow-through (FT) and bound fractions were analysed with anti-SUMO-2/-3, PML or p53 antibodies. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as loading control.

architecture. The natural domains of RNF4 have already been used as affinity traps to purify SUMOylated proteins or to identify putative SUMOylated substrates by mass spectrometry[20]. By extending the number of SIMs, our SUBEs show an improved capacity to purify SUMOylated proteins compared to the natural RNF4 SIM sequences (Supplementary Fig. 4). The use of molecular traps such as SUBEs to analyse endogenous SUMOylated proteins will be critical to understand the role of this post translational modification in the control of diverse fundamental molecular events.

Methods

Cloning, protein expression and purification. Synthetic oligonucleotides encoding the SIM2 and SIM3 regions of RNF4 (P78317) (41 to 63:ETAGDEIVDLTCESLEPV VVDL) were cloned in tandem into the PGEX-6P1vector (Amersham). This vector was modified in frame by the introduction, of a His6 tag and a SV5 epitope upstream and downstream of the Multi-Cloning Site, respectively[19]. Such modifications allow the recognition of the construct by the respective anti-His6 and anti-SV5 antibodies. SUBEs (produced by Inbiomed Pharma) were expressed in *Escherichia coli* BL-21 strain and purified by affinity chromatography using GST agarose beads (Glutathione S-transferase, Biontex), and ion exchange chromatography, using Sepharose beads (Sigma), according to manufacturers' instructions.

Protein pulldown. In order to use SUBEs as affinity traps for total and specific SUMOylated protein pulldown, different cell lines such as NB4, HeLa and MCF-7 were used. Cells were grown at 37°C in RPMI (NB4 cells) or DMEM (HeLa, MCF-7 and HEK-293) media (Gibco) both supplemented with 10% FBS. In the case of HeLa, 2×10^6 cells were treated 30 minute with 20 μM MG-132, (Sigma) and stressed for 60 minutes at 43°C (to induce SUMOylation)[7]. For NB4 and MCF-7, 5×10^6 and 2×10^6 cells, respectively, were plated and treated next day for 1 hour with 20 μM MG-132, and stressed for 1 hour by 0,15 μM Arsenic Trioxide (ATO) (Sigma). HEK-293 were transfected with His6-SUMO-2 protein and were treated with 500 U/ml of IFN-α (GenScript) for 24 h, infected with Indiana strain VSV at an MOI of 1 PFU/ml for 4 h, or left untreated. After all treatments, cells were sonicated twice for

15 seconds with 10% amplitude (Branson digital sonifier) in 500 µl of lysis buffer (50mM Tris pH 8.5; 150 mM NaCl, 5 mM EDTA, 1% Igepal, supplemented with 1× protease inhibitor cocktail (Roche) and 50 µM of PR-619 (ubiquitin and ubiquitin-like isopeptidases inhibitor, LifeSensors).

Lysates were centrifuged at 14000xg (Beckman Coulter Microfuge 22R) and the supernatant was incubated with 50 µl of GST-agarose beads containing 50 µg of SUBEs or GST and 1 mM DTT (Dithiothreitol) for 2 h at 4°C. Beads were then pulled down by centrifugation, 1000xg for 5 minutes (Beckman Coulter Microfuge 22R), and 1/10 of the unbound fraction was saved for western blot analysis (flow through-FT). Washes were carried out using 30 column volumes of wash buffer (50 mM Tris pH 8.5; 50 mM NaCl, 5 mM EDTA and 1% Igepal). Elutions were performed in one column volume of 2× Laemmli Buffer.

For western blot analysis, samples were separated in 10% polyacrylamide gels and membranes were incubated with anti-PML (Bethyl Laboratories Inc), anti-p53 (DO1, Santa Cruz), anti-IκBα (Cell signalling), anti-PTEN (Cell signalling) and anti-SUMO-1 or anti-SUMO-2/-3 (Eurogentec) antibodies.

Immunofluorescence assays. The day before the experiment, 3×10^5 MCF-7 cells and 1×10^6 NB4 cells were plated in a 24 well plate. Cells were treated with 20 µM MG-132 for 1 h, and 0,15 µM ATO for an additional hour. Cells were then washed with PBS 1X, fixed with 1% paraformaldehyde and permeabilized in PBS 1X/Triton 0,1%. Direct immunofluorescence measurements were performed as previously reported[24]. Monoclonal SUMO-1 and SUMO-2 antibodies (generously provided by C. Gwizdek and M. Matunis) were used at a final dilution of 1/50 and rabbit polyclonal PML (Bethyl Laboratories Inc) antibody was used at 1/500. Texas Red and Alexa 288 were respectively used as secondary antibodies, at 1/1000 dilution.

In vitro SUMOylation assay. For the SUMOylation assays, *in vitro* transcribed/translated IκBα, PML or p53, recombinant PTEN (50 ng/pt) were incubated in a buffer containing an ATP regenerating system [(50 mM Tris pH 7.5, 10 mM MgCl2, 2 mM ATP, 10 mM creatine phosphate (Sigma), 3.5 U/ml of creatine kinase (Sigma), and 0.6 U/ml of inorganic pyrophosphatase (Sigma)], 10 µg of SUMO-1, -2 or -3 or 5 µg (if used in combination with SUMO-2 and SUMO-3), Ubc9 (0.325 µg) and purified SAE1/2 (0.8 µg, ENZO Life Sciences). For the formation of SUMO-1 or -2/-3 chains (Supplementary fig. 2), no protein substrate was added to the previously described assay. Reactions were incubated at 30°C for 2 hours and stopped by addition of SDS sample buffer. For pulldown assays, 1/10 of input was saved and the rest of the reaction was incubated with 50 µl of GST-agarose beads containing 50 µg of SUBEs or GST and 1 mM DTT (Dithiothreitol) for 2 h, at 4°C. After incubation, beads were pulled down by centrifugation, as previously described, and 1/10 of the unbound fraction (FT) was kept for western analysis. Subsequently, the same beads were washed with 30 column volumes of binding buffer (50 mM Tris pH 8.5; 50 mM NaCl, 5 mM EDTA and 1% Igepal) and were resuspended in one column volume of 2× Laemmli Buffer.

Surface Plasmon Resonance experiments. SPR experiments were performed on a Biacore 3000 system, equilibrated at 25°C in HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) (GE Healthcare), using a CM5 sensor chip with a density of around 6000 resonance units (RU) of covalently immobilized anti-GST antibody (GE Healthcare). Approximately 100 RU of SUBEs [SUBE-l (l-long) and SUBE-s (s-short)] were captured by the antibody. The 4xS2 (4xSUMO-2) molecule (generously provided by R.T. Hay[20], and the multi-ubiquitin chains (Ub2-7, K63 chains, Enzo) were injected for 60s at a flow rate of 30 µl/min. All injections were carried out in duplicate and in randomized order.

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Author contributions

E.D.S.-F., W.X., V.L., F.A., I.M., F.L.-O., C.F.C.H. and M.S.R. carried out experiments; A.C., S.J.G., C.R., P.E. provided material, technical support and know-how; E.D.S.-F., M.S.R. wrote the manuscript. All authors designed experiments, discussed results and commented on the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: This work is part of a patent application by CIC bioGUNE.

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