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RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation

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In acute promyelocytic leukaemia (APL), promyelocytic leukaemia (PML) protein is fused to the retinoic acid receptor α (RAR). This disease can be treated effectively with arsenic, which induces PML modification by small ubiquitin-like modifiers (SUMO) and proteasomal degradation. Here we demonstrate that the RING-domain-containing ubiquitin E3 ligase, RNF4 (SNURF), targets poly-SUMO-modified proteins for degradation mediated by ubiquitin. RNF4 depletion or proteasome inhibition led to accumulation of mixed, polyubiquitinated, poly-SUMO chains. In RNF4-depleted cells, PML protein accumulated and was ubiquitinated by RNF4 in a SUMO-dependent fashion *in vitro*. In the absence of RNF4, arsenic failed to induce degradation of PML and SUMO-modified PML accumulated in the nucleus. These results demonstrate that poly-SUMO chains can act as discrete signals from mono-SUMOylation, in this case targeting a poly-SUMOylated substrate for ubiquitin-mediated proteolysis.

Modification of proteins with ubiquitin-like proteins (Ubls) can have very different outcomes, depending on whether the substrate is conjugated to a single Ubl or a polymeric chain of Ubls[1]. It is well established that monoubiquitination can have a regulatory function, whereas addition of a polyubiquitin chain can target proteins for proteasomal degradation. SUMO modification regulates a wide variety of cellular processes by covalent attachment to a diverse array of proteins. In most cases modification by SUMO takes place within a consensus modification site (ψKxD/E)[2], although modification at non-consensus sites has also been documented. In lower eukaryotes a single *SUMO* gene is expressed (*Smt3* in *Saccharomyces cerevisiae*), whereas in vertebrates three paralogues designated SUMO-1, SUMO-2 and SUMO-3 are expressed. The conjugated forms of SUMO-2 and SUMO-3 only differ from one another by three amino-terminal residues and form a distinct subfamily known as SUMO-2/-3 that is 50% identical in sequence to SUMO-1. As with ubiquitin, SUMO-2 and SUMO-3 can form polymeric chains through a conserved lysine (Lys 11) embedded in a consensus modification site[3]. As SUMO-1 lacks such a consensus modification site, it is not thought to be modified to form a polymeric chain, but recently, it has been shown that SUMO-1 can be linked to the end of a poly-SUMO-2/-3 chain, effectively terminating chain growth[4].

The physiological consequences of SUMO modification are typically mediated by effector proteins that recognize SUMO through SUMO interaction motifs (SIMs), the best-studied of which contains three hydrophobic residues (typically valine, leucine or isoleucine) in a sequence of four amino acids (V/L/I, V/L/I, X, V/L/I or V/L/I, X, V/L/I, V/L/I)[5,6]. Recently the identification in yeast, of RING-domain-containing proteins bearing either one or two SIMs (Slx5 and Slx8 in *S. cerevisiae* and Rfp1 and Rfp2 in *Schizosaccharomyces pombe*) led to the suggestion that these proteins could target SUMO-modified proteins for ubiquitin-mediated proteolysis[7–10]. However no such targets for degradation were detected and ubiquitination of the mono-SUMO-modified proteins *in vitro* was limited.

Using RNA interference, we demonstrate that the mammalian homologue of Slx5/8 and Rfp1/2, RNF4 (SNURF)[11] is required for arsenic-induced degradation of PML. *In vitro* ubiquitination assays showed that RNF4 only ubiquitinates PML when conjugated by SUMO-2, and binding analyses reveal that RNF4 shows a significantly higher affinity for poly-SUMO chains over monomeric SUMO. Mutational analysis of RNF4 showed that four N-terminal SIMs are required for poly-SUMO binding and ubiquitination, thus identifying RNF4 as the first poly-SUMO-specific effector protein.

RESULTS

RNF4 preferentially binds to and ubiquitinates SUMO-2 polymers over SUMO-2 monomers *in vitro*

Although it contains the highly conserved carboxy-terminal RING domain, inspection of the N-terminal region of RNF4 reveals that it also contains multiple distinct sequences that conform to SIMs. Human and rat RNF4s each contain four putative SIMs (Fig. 1a), suggesting that they
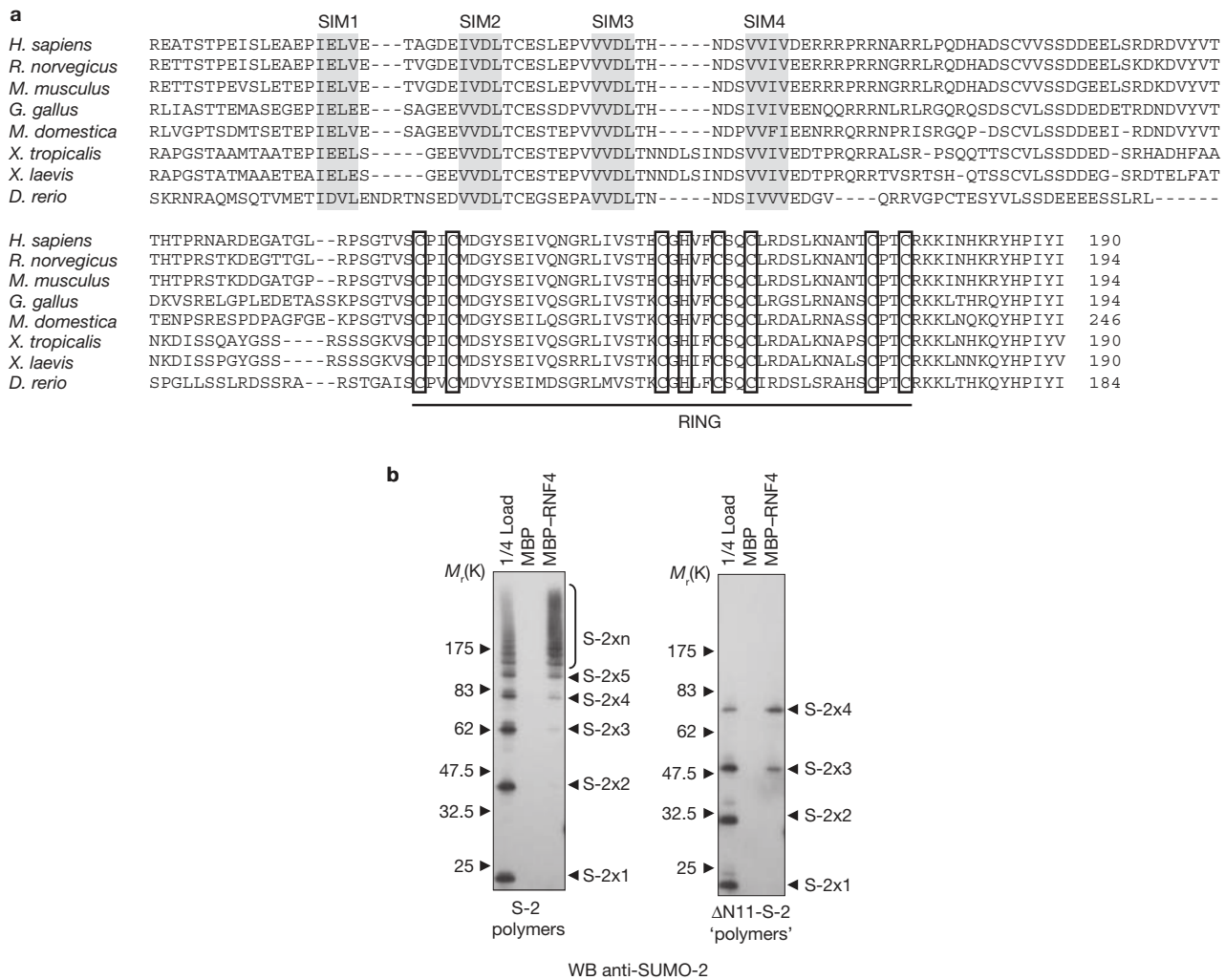
a

b

WB anti-SUMO-2

Figure 1 RNF4 preferentially binds poly-SUMO-2 chains. (**a**) Sequence alignment of RNF4 orthologues from vertebrate species (indicated). The four putative SIM domains are shaded and zinc coordinating residues from the RING domain are boxed. (**b**) Interaction between RNF4 and SUMO-2 polymeric forms was determined by amylose affinity chromatography purification of 1.6 μM MBP and MBP–RNF4 after incubation *in vitro* with 0.4 mg ml^{-1} SUMO-2 multimers. Western blot of SDS–PAGE fractionated samples using an anti-SUMO-2 antibody shows the extent of interaction between RNF4 and native SUMO-2 polymers left and artificial ΔN11–SUMO-2 'polymers' in an 8:4:2:1 ratio of mono-, di-, tri- and tetra-ΔN11–SUMO-2 proteins (right). Loading controls (1/4) are included.

could recognize poly-SUMO chains. To establish the SUMO-binding properties of RNF4, a preparation containing various lengths of recombinant poly-SUMO-2 chains was analysed for binding to RNF4. Binding to mono- and di-SUMO-2 was not detected; however, poly-SUMO-2 species were efficiently bound by RNF4 (Fig. 1b, left panel). As the poly-SUMO-2 preparation is heterogeneous, we generated 'head to tail' fusions of ΔN11–SUMO-2 and analysed their binding to RNF4. Again, binding of mono- and di-SUMO to RNF4 was not detected, whereas tri-SUMO-2 bound weakly and tetra-SUMO-2 bound strongly to RNF4 (Fig. 1b, right panel). To establish the RNF4 sequence determinants of poly-SUMO-2 binding, we generated a series of deletion mutants and a set of SIM mutants in which the conserved V/L/I residues were changed to Ala, which is known to disrupt SUMO–SIM interactions[6] (Fig. 2a, b). Deletion of either the 20 N-terminal residues [**AU: OK?**] (ΔN, 1–20) or the region between the SIMs and RING (Δ3, 99–118) did not affect binding to poly-SUMO-2 or tetra-ΔN11–SUMO-2. However, deletion of 3 of the SIMs (Δ1, 31–65) severely compromised poly-SUMO-2 binding

(Fig. 2c, d). Apart from SIM2, which seems to be critical for binding, mutation of single SIMs did not markedly reduce poly-SUMO-2 binding. Increasing the number of SIM mutations led to a progressive loss of poly-SUMO-2 binding. RNF4 with all of the SIMs mutated (mtSIM 1, 2, 3, 4) did not show detectable poly-SUMO-2 binding, whereas RING-domain mutants (CS1, CS2) had wild type levels of binding [**AU: OK?**] (Fig. 2c, d). Thus RNF4 shows high selectivity for poly-SUMO-2 chains, with relatively weak binding to mono- or di-SUMO.

RNF4 undergoes auto-ubiquitination[12], but its strong binding preference for poly-SUMO-2 chains suggests that such chains may be substrates for RNF4-dependent ubiquitination. We therefore incubated a range of 6His–SUMO substrates with ^{125}I ubiquitin, Uba1, Ubc5a and RNF4. Modified SUMO substrates were collected on nickel beads and ^{125}I ubiquitin conjugation determined. Although 6His–SUMO-2 is a poor substrate, 6His–poly-SUMO-2 was efficiently ubiquitinated (Fig. 3a, b). To establish the SUMO-paralogue specificity of RNF4, polymers of SUMO-1 were generated using a mutant form (D15V)

Table 1 Summary of mass spectrometry analysis to identify sites of Lys ubiquitination			
	SUMO-2 purified from MG132 treated cells (sequence coverage)	*In vitro* ubiquitination of unlinked poly-SUMO-2 chains (sequence coverage)	*In vitro* ubiquitination of GST–PML conjugated by poly-SUMO-2 chains (sequence coverage)
Ubiquitin	6, **11**, **48**, **63** (97%)	6, **11**, **48**, **63** (97%)	6, **11**, **48**, **63** (97%)
SUMO-2	**11**, **32**, 41 (98%)	**11**, **32**, 34, **41**, 44 (95%)	**11**, **32**, **41**, 44 (95%)
PML	None detected (17%)	-	337, **380**, 394, **400**, **401**, **476**, 515 (68%)

Samples are derived from experiments described in Figs 3d, 4b and a poly-SUMO *in vitro* ubiquitination reaction (data not shown). Residues in bold represent the modified Lys most abundantly detected in the samples tested.

of SUMO-1 with a consensus modification motif. Poly-SUMO-1 was efficiently ubiquitinated, whereas mono-SUMO-1 was poorly ubiquitinated (Supplementary Information, Fig. S1). Thus RNF4 does not discriminate between SUMO-1 and SUMO-2 but has a clear preference for polymeric chains over monomeric SUMO. To explore the chain length requirement for RNF4-dependent ubiquintination, the series of ΔN11–SUMO-2 'head to tail fusions' (Figs 1, 2) was tested as substrates. Mono- and di-SUMO-2 were not appreciably ubiquitinated. Although tri-SUMO-2 ubiquitination was detected, it was a poor substrate when compared to tetra-SUMO-2, which was ubiquitinated almost as effectively as 'natural' poly-SUMO-2 chains (Fig. 3a, b). To establish the role of the RING and SIM domains in RNF4 activity, the mutants described previously (Fig. 2a, b) were tested for activity using 6His–poly-SUMO-2 chains and ^{125}I ubiquitin as substrates. These assays are RNF4-dependent and deletion of sequences N-terminal to the SIMs (ΔN, 1–20) and between the SIMs and RING (Δ3, 99–188) is without consequence for RNF4 activity. As expected, activity requires the integrity of the RING domain, as mutation of zinc-coordinating cysteine residues abolishes activity (CS1, CS2). Mutation of individual SIMs caused a modest reduction of ubiquitination activity, but deletion of three SIMs (Δ1, 31–65) or mutation of multiple SIMs (mtSIM 1, 2, 3, 4) abolished ubiquitination to background levels (Fig. 3c). Apart from the RING mutants (CS1, CS2), all of these RNF4 mutants were competent for auto-ubiquitination (Fig. 3d). Thus, multiple SIMs and the RING domain are required for RNF4 dependent ubiquitination of poly-SUMO-2 chains.

RNA interference of RNF4 causes the accumulation of high molecular weight SUMO conjugates *in vivo*.

To establish the role of RNF4 in SUMO metabolism *in vivo*, short interfering (si) RNA was used to ablate RNF4 expression. Using a pool of siRNAs in HeLa cells, RNF4 expression was reduced to undetectable levels (Fig. 4a). Analysis of the same cell extracts indicated that high molecular weight SUMO-2 species accumulated in the absence of RNF4. Although less apparent, RNF4 depletion also led to an increase in high molecular weight forms of SUMO-1 (Fig. 4a). In addition, analysis of HeLa cells stably expressing a short hairpin (sh) RNA for RNF4 (Hela Cl6) indicated that RNF4 depletion resulted in the accumulation of SUMO-1, SUMO-2 and PML in subnuclear bodies (Fig. 4b; Supplementary Information, Fig. S2a, b).

As RNF4 was capable of ubiquitinating poly-SUMO chains, it seemed likely that such modified material would be destined for ubiquitin-mediated proteosomal degradation. Thus, a HeLa cell line stably expressing a TAP-tagged version of SUMO-2 was treated with the proteasome inhibitor MG132 and the SUMO-2-modified forms revealed by western blotting. Proteasome inhibition caused a marked accumulation of high molecular weight SUMO-modified species. To determine the nature

of the high molecular weight SUMO-modified species, we used stable isotope labelling with amino acids in cell culture (SILAC) coupled to mass spectrometry[13]. SUMO-1 and SUMO-2 increased markedly after MG132 treatment, consistent with the immunofluorescence microscopy experiment (Fig. 5a). SUMO-2-modified PML also accumulated, although RanGAP1, which is stably modified by SUMO, was unchanged by MG132 treatment (Fig. 5b). Proteasome inhibition also causes a large increase in the amount of ubiquitin associated with SUMO-2 (Fig. 5b). Sites of ubiquitination in SUMO-2 peptides were identified by the presence of an isopeptide-linked diglycine on Lys residues that were resistant to trypsin cleavage. Ubiquitin-modified Lys 11, 32 and 41 in SUMO-2 were detected in the presence of MG132 (Table 1). To determine whether the same Lys residues were ubiquitinated by RNF4, poly-SUMO-2 chains were subjected to RNF4-dependent ubiquitination *in vitro* (data not shown) and the sites of ubiquitination determined by mass spectrometry, as described above. The same residues shown to be ubiquitinated *in vivo* were also sites of RNF4-dependent ubiquitination *in vitro* (Table 1). Furthermore, the ubiquitin associated with SUMO-2 or SUMO-2 conjugates was found to be polyubiquitinated (mostly by Lys 11, 48 and 63 linkages, and to a lesser degree, Lys 6) both *in vivo* and *in vitro* (Table 1). Thus it seems likely that RNF4 is involved in the ubiquitination of poly-SUMO-2 chains destined for proteasome-mediated degradation *in vivo*.

RNF4 only ubiquitinates PML when conjugated by SUMO-2

As cells with reduced levels of RNF4 showed increased levels of PML (Fig. 4b), and SUMO-2-modified PML accumulated after MG132 treatment (Fig. 5b), it seemed likely that SUMO-modified PML was a substrate for RNF4-dependent ubiquitination. Thus, GST–PML bound to glutathione agarose was modified with poly-SUMO-2 (Fig. 6a) and incubated with Uba1, Ubc5, RNF4 and ^{125}I ubiquitin. In the absence of RNF4 neither GST–PML nor SUMO-2-modified GST–PML was ubiquitinated. Unmodified GST–PML was not a substrate for RNF4-dependent ubiquitination. However, SUMO-2-modified GST–PML was ubiquitinated efficiently in the presence of RNF4 (Fig. 6b, c). To identify the sites of RNF4-dependent ubiquitination, GST–PML was modified sequentially with SUMO-2 and ubiquitin, as described in Fig. 6a, b, before purification, SDS–PAGE fractionation, tryptic digestion and mass spectrometry analysis, as described above. As expected, we detected PML peptides in an isopeptide linkage with the C-terminal peptide from SUMO-2 and the SUMO-2–SUMO-2 linkage, indicative of Lys 11-linked poly-SUMO-2 chains[4]. The diglycine-linked signature of ubiquitination was also detected on SUMO Lys 11, 32 and 41 (with minor amounts on Lys 44) and on PML Lys 380, 400, 401 and 476 (with minor amounts on Lys 337, 394 and 515). The ubiquitin associated with SUMO-2-modified PML was found to be polyubiquitinated, mostly by Lys 11, 48 and 63 linkages and to a lesser
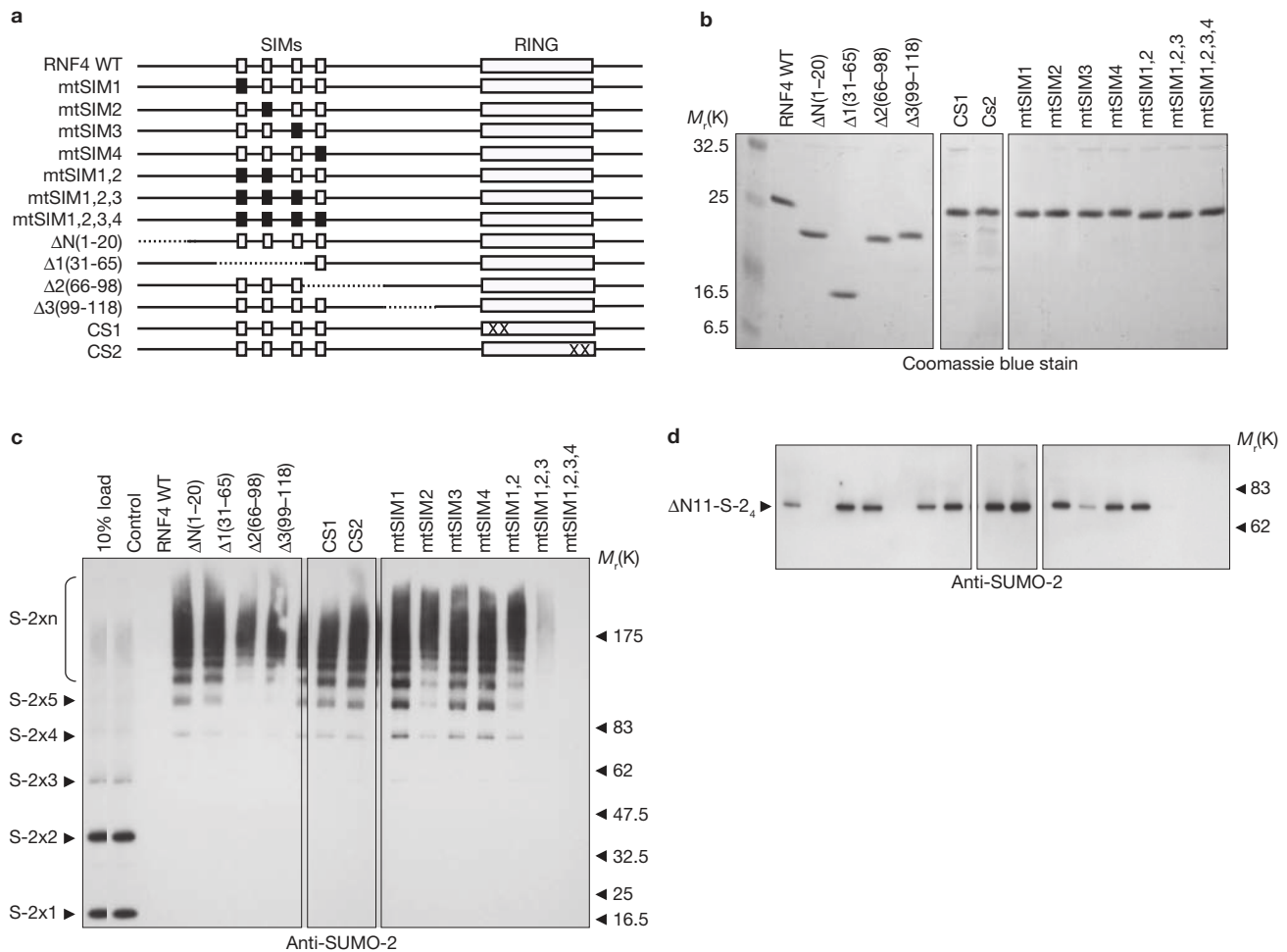
Figure 2 Four SIMs in RNF4 are required to bind SUMO polymers. (**a**) Schematic representation of RNF4 and the mutant constructs used in this study. Dashed lines indicate deleted regions, black boxes represent mutated SIM domains and X denotes a mutated cysteine in the RING domain. (**b**) Coomassie blue-stained SDS–PAGE gel of 500 ng of wild-type and each of the 13 mutants of RNF4 used in this study. (**c**, **d**) Comparison of the SUMO-2-binding activity of wild-type and mutant RNF4 proteins using the same assay described in Fig. 1b. Binding to both 'native' SUMO-2 polymers (**c**) and the artificial $\Delta N11$–SUMO-2 $\times$ 4 polymer (**d**) was tested. loading control (10%) is shown.

degree, Lys 6 (Table 1). The GST attached to PML was not appreciably ubiquitinated. Thus, poly-SUMO chains on PML recruit RNF4 which catalyses ubiquitination of the bound poly-SUMO chains and a defined region of PML.

RNF4 is required for arsenic induced degradation of PML

In APL, the PML protein is fused to RAR and arsenic treatment induces SUMO modification and proteasomal degradation of PML and the PML–RAR fusion[14–19]. Given the involvement of SUMO modification , we hypothesized that RNF4 was the ubiquitin ligase responsible for arsenic-induced, SUMO-dependent PML degradation. HeLa cells treated with either control siRNA or siRNAs specific for *RNF4* were exposed to arsenic and the fate of PML followed by immunofluorescence microscopy and western blotting. In cells treated with control siRNA, arsenic treatment caused a dramatic reduction in PML nuclear fluorescence (Fig. 7a). Twenty-four h after arsenic treatment, the only remaining PML was found in the cytoplasm (Fig. 7a). In contrast, arsenic treatment of cells RNF4-depleted caused the accumulation of PML in large subnuclear bodies (Fig. 7a). Similar results were obtained for HeLa–Cl6 cells with stable knockdown of *RNF4* expression (Supplementary

Information, Fig. S2b). In HeLa cells, arsenic treatment caused the rapid recruitment (60 min) of SUMO-1 and SUMO-2 into PML bodies, but this staining was lost as arsenic-induced degradation of PML proceeded. In HeLa-Cl6 cells, SUMO-1 and SUMO-2 were already prominent in PML bodies and this localization remained during arsenic treatment (Supplementary Information, Figs S3, S4). Western blot analysis of control siRNA-treated cells exposed to arsenic indicates that high molecular weight forms of PML accumulate before PML degradation. When RNF4 expression had been ablated, arsenic treatment caused the accumulation of what seemed to be SUMO-modified forms of PML and under these conditions, PML did not undergo arsenic-induced degradation (Fig. 7b). Thus, RNF4 seems to be required for arsenic-induced degradation of PML. To control for potential off-target effects of the siRNA, a rescue experiment was carried out on Hela-Cl6 cells. As depletion of RNF4 causes an increase in PML expression, a cDNA expressing the rat homologue of RNF4, which is not recognized by the human RNF4 shRNA, was introduced into HeLa-Cl6 cells. In cells expressing wild-type RNF4, but not an inactive RING mutant, PML expression was reduced (Fig. 7c). Thus, the accumulation of PML observed when cells are depleted of RNF4 with siRNA is due to reduction in RNF4 levels.
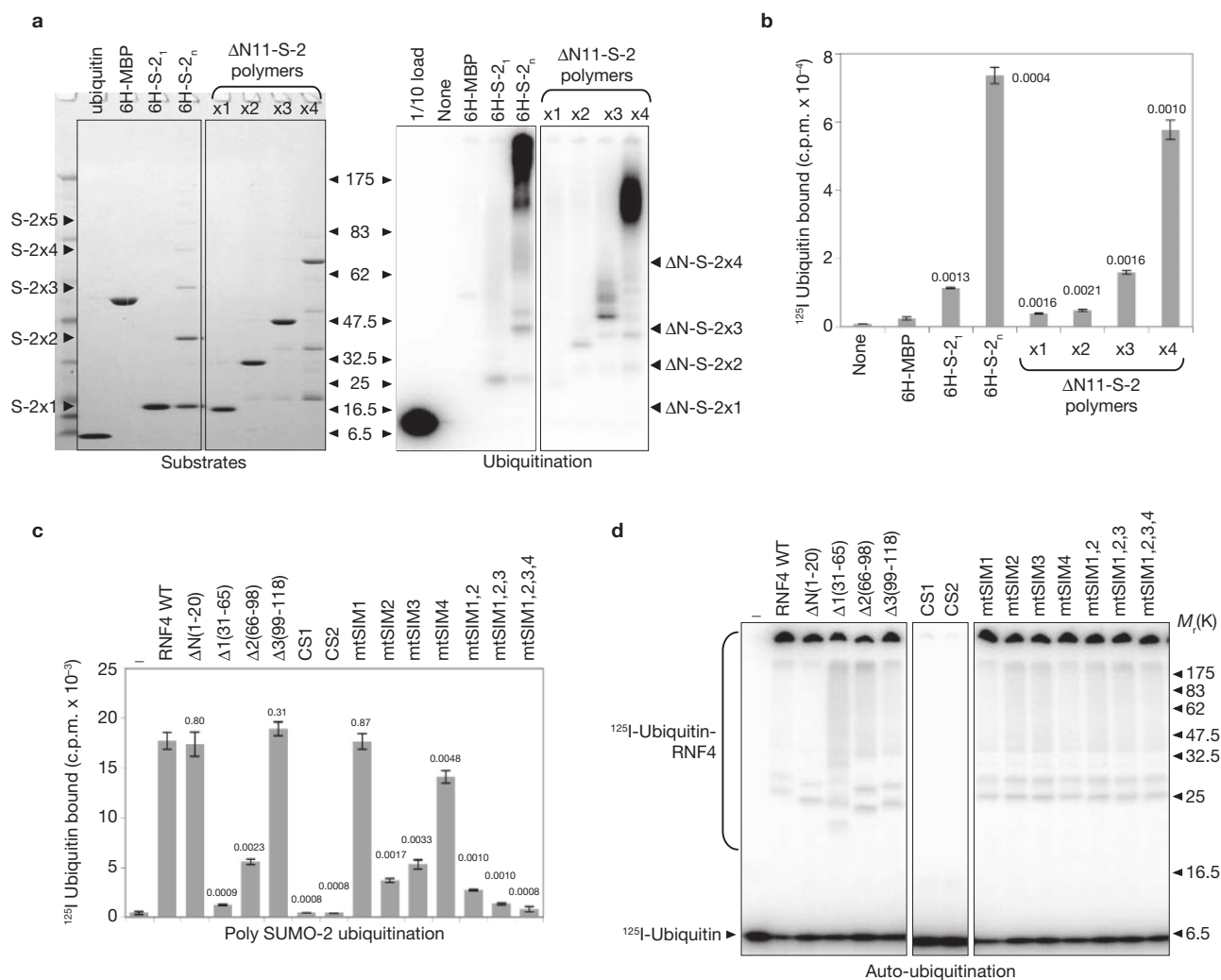
Figure 3 RNF4 preferentially ubiquitinates poly-SUMO-2 chains. (**a**) Coomassie blue stained SDS–PAGE gel (left panel) of 1 μg ubiquitin and the 6His-substrates used in the *in vitro* ubiquitination assay (right panel). Phosphorimager scan of a dried SDS–PAGE gel fractioning *in vitro* ^{125}I-ubiquitination assays, using 20 μg each of the indicated proteins as substrates in the presence of 0.5 μM RNF4 and 0.7 μM UbcH5a (right panel; see Methods for further details). 6His–SUMO-2 proteins were purified from the assay mixture by nickel affinity chromatography before analysis. (**b**) γ-counting of purified 6His–SUMO-2 proteins from *in vitro* ubiquitination assays before fractionation, as shown in **a** (right panel). (**c**) γ-counting of purified 6His–SUMO-2 proteins from *in vitro* ubiquitination assays using 6His–poly-SUMO-2 as a substrate and each of the RNF4 variants, as indicated. (**d**) Phosphorimager scan of a dried SDS–PAGE gel fractionating the components of *in vitro* RNF4 self ^{125}I-ubiquitination assays, using each RNF4 variant indicated. In assays lacking substrate, active RNF4 conjugates ubiquitin to internal Lys residues. For **b** and **c**, data are mean ± s. d. for triplicate reactions. *P* values relative to 6His–MBP (**b**) and RNF4 WT (**c**) are shown for each condition **[AU: please include statistical analysis used]**.

DISCUSSION

The data presented here demonstrate that RNF4 is a poly-SUMO-specific E3 ubiquitin ligase that controls PML stability both under normal conditions and after arsenic treatment. It is established that PML is modified by SUMO under normal growth conditions[20,21] and is inducibly SUMOylated after arsenic treatment[15,16,18]. Thus, if RNF4 is required for PML degradation and the substrate for RNF4 is a poly-SUMO chain, then it follows that PML must be modified by a poly-SUMO chain, probably on Lys 160, as this residue is required for arsenic-induced degradation of PML[15]. Although poly-SUMO chains are predominantly SUMO-2/-3 (ref. 3), it has been demonstrated recently that chains can be terminated by SUMO-1 (ref. 4). As RNF4 does not seem to discriminate between SUMO paralogues (Supplementary Information, Fig. S1), it is possible that mixed poly-SUMO chains could function in the PML degradation pathway.

In *S. cerevisiae* **[AU: OK? If not, please specify which species]**, homologues of RNF4 are involved in DNA-damage responses and provide a genetic link between the SUMO and ubiquitin pathways[22–24]. The yeast proteins were reported to target monomeric SUMO for ubiquitination, although poly-SUMO chains were not tested as substrates[7–10]. In the work reported here RNF4-dependent ubiquitination of monomeric SUMO is detectable, but is relatively inefficient and results only in monoubiquitination (Fig. 3a), whereas ubiquitination of poly-SUMO chains is 8–10-fold more efficient and results in formation of the polyubiquitin signal known to be required for proteasomal degradation (Fig. 3a; Table 1). We therefore believe that modification with a single SUMO is unlikely to target proteins for RNF4-dependent ubiquitination and that RNF4 is an E3 ligase that specifically targets poly-SUMO-modified proteins for ubiquitin modification. This being the case, regulation of the poly-SUMO signal is likely to occur both at the level of conjugation (by
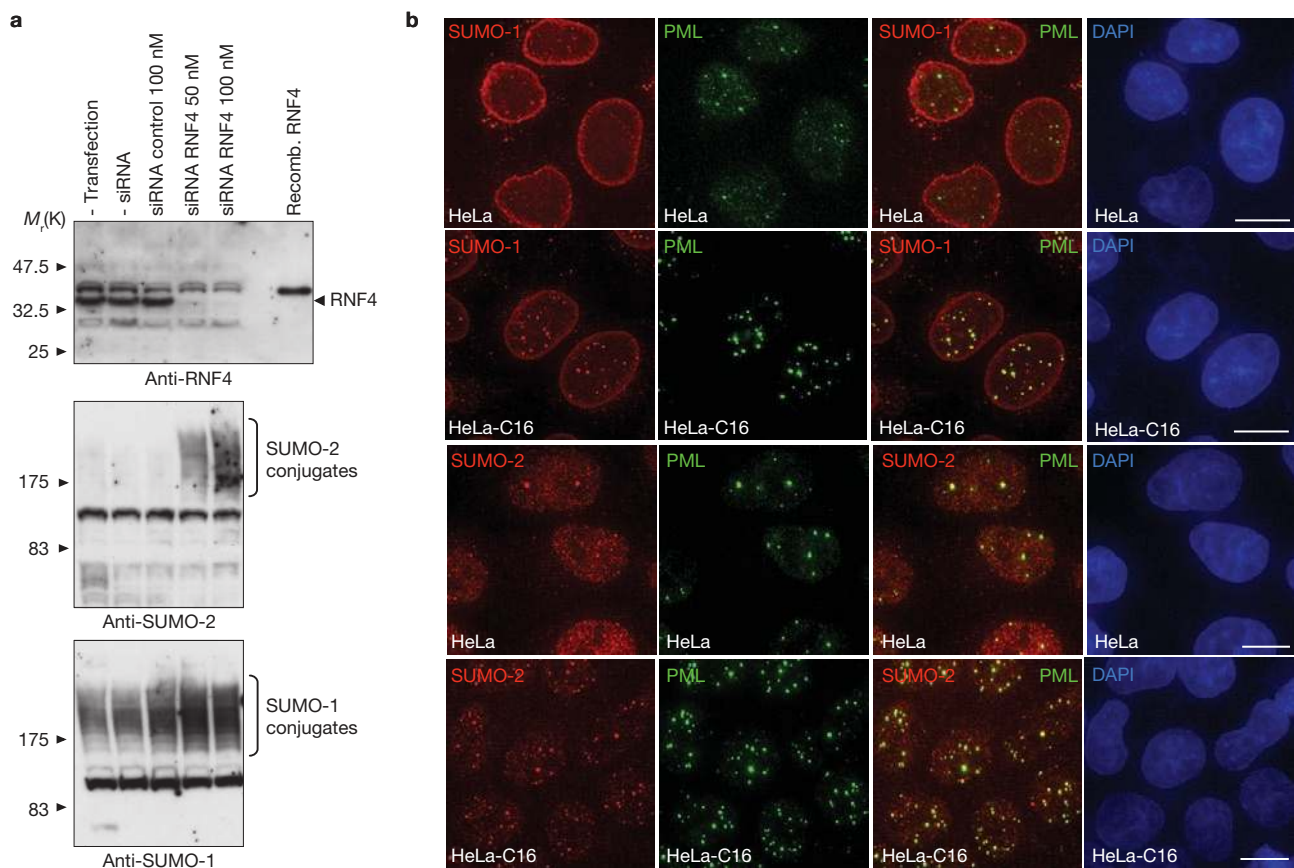
Figure 4 *RNF4* knockdown causes accumulation of SUMO-conjugated PML in ND10 nuclear domains. (**a**) HeLa cells were treated with siRNA to *RNF4* or a control siRNA for 48 h and cell extracts analysed by western blotting with antibodies to RNF4, SUMO-2 and SUMO-1. (**b**) HeLa cells or HeLa Clone 6 cells in which *RNF4* expression has been stably knocked down (Supplementary Information, Fig. S2 for details) were analysed by immunofluorescence microscopy with antibodies to SUMO-1 (red), SUMO-2 (red) and PML (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm. See Supplementary Information, Fig. S4 for full blot images.

SAE1/2, Ubc9 and SUMO E3s), as well as at the level of depolymerization (by the specialized depolymerizing proteases Senp6 and putatively, Senp7; Supplementary Information, Fig. S5).

Although the existence of poly-SUMO chains has been known for some time[3], RNF4 is the first example of a poly-SUMO-chain-specific binding protein, and its requirement for arsenic-induced degradation of PML suggests an important role for poly-SUMO chains *in vivo*. It therefore appears that, as in the case of ubiquitin, monomeric and polymeric chains of SUMO can have distinct biological functions. Efforts in the future will concentrate on investigating the molecular details of poly-SUMO recognition by RNF4, and determining whether poly-SUMO recognition is a feature unique to RNF4 or a widely recognized signal important in other intracellular signalling pathways.

METHODS

Antibodies. Antigen affinity-purified sheep anti-SUMO-1 and anti-SUMO-2 antibodies were prepared in-house. Mouse anti-TAP (Open Biosystems), mouse anti-β-actin (Sigma), mouse anti-FLAG M2 (Sigma) and rabbit [**AU: move OK?**] anti-SUMO-2 (Zymed) antibodies were obtained from commercial sources. Rabbit anti-RNF4 was as described previously[25]. Chicken anti-PML antibody was a gift from Valérie Lallemand-Breitenbach and Hugues de Thé ([**AU: please include Institute or affiliation**] Paris) and mouse monoclonal 5E10 antibody was a gift from Roel van Driel ([**AU: please include Institute or affiliation**] Amsterdam). [**AU: please include antibody dilutions**]

cDNA cloning and protein expression and purification. *Rattus* [**AU: please include species**] RNF4 (accession number NM_019182) and mutant (Δ1, Δ2, Δ3, CS1 and CS2) cDNAs were amplified by PCR from pcDNA3.1 and subcloned into a bacterial expression vector pLou3 (a gift from J. H. Naismith, University of St. Andrews, UK). N-terminal 6His-tagged MBP fusion proteins were expressed in *Escherichia coli* Rosetta (DE3) cells (Novagen) and purified using nickel affinity chromatography (Ni-NTA agarose, Qiagen). 6His-tagged MBP was cleaved using 6His-tagged TEV protease in 50 mM Tris-HCl (pH 7.5), 500 mM NaCl and 0.5 mM β-mercaptoethanol and removed from RNF4 using Ni-NTA agarose. PCR-based mutagenesis[26] was used to generate the following mutations: RNF4ΔN (deleting residues 1–20), RNF4 mtSIM1 (I40A, L42A and V43A), mtSIM2 (I50A, V51A and L53A), RNF4 mtSIM3 (V61A, V62A and V63A), RNF4mtSIM4 (V71A, V72A, I73A and V74A), RNF4 mtSIM1, 2, RNF4 mtSIM1, 2, 3 and RNF4 mtSIM1, 2, 3, 4. Recombinant PML 560 in pGEX-2T (a gift from D. Bailey and P. O'Hare, [**AU: please include Institute or affiliation**] Oxted) was expressed in *E. coli* Rosetta (DE3) (Novagen) and purified using glutathione sepharose 4B (Pharmacia).

cDNA encoding 'artificial' SUMO-2 'polymers' was generated by ligating PCR products encoding ΔN11–SUMO-2 while simultaneously digesting with restriction enzymes specific for *Bam*HI (N-terminal site) and *Bgl*II (C-terminal site) in T4 DNA ligase buffer, 150 mM NaCl, 0.1 mg ml^{−1} BSA at 22 °C for 5 h (enzymes and buffers from New England Biolabs). This created a range of cDNAs encoding ΔN11–SUMO-2 cDNA 'monomers' and 'multimers' that were separated by agarose gel electrophoresis and individually extracted into solution. These were then ligated into the *Bam*HI site in pHis–TEV plasmid[27] which had the *Eco*RI restriction site mutated into a stop codon. Expression from these plasmids gives N-terminal 6His-tagged proteins containing one, two, three or four copies of
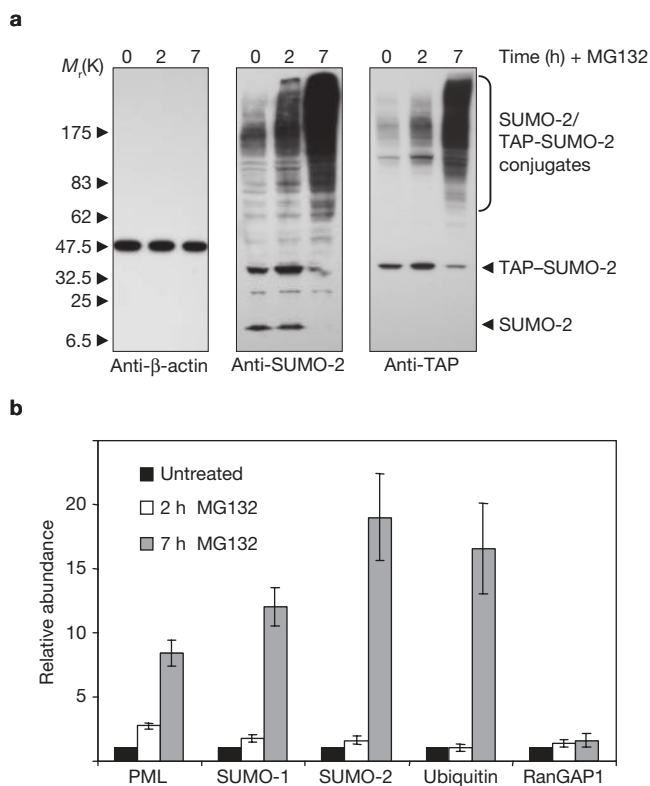
Figure 5 Proteasome inhibition causes accumulation of PML, SUMO-1 and ubiquitin covalently associated with SUMO-2 in HeLa cells. (**a**) Western blot analysis of SDS–PAGE fractionated cell lysates from TAP–SUMO-2-expressing HeLa cells either untreated (0) or treated for 2 or 7 h with 20 μM MG132, using β-actin, SUMO-2 and TAP specific antibodies. (**b**) Quantitative comparison of the relative abundance of PML, SUMO-1, SUMO-2, ubiquitin and RanGAP1 proteins in TAP-purified TAP–SUMO-2 HeLa cell extracts either untreated or treated for 2 or 7 h with 20 μM MG132. The relative abundance in the untreated cells is standardized to 1 so data are internally comparable for each protein, but not across proteins. Data are mean ± s. d., obtained for peptides extracted from the >150 kDa region of the gel (SUMO-1, SUMO-2 and ubiquitin) or 80–150 kDa region (PML and RanGAP1). In each case at least two peptides were quantified per protein **[AU: Please include exact n for each protein and if less than 3, error bars should be removed]**

ΔN11–SUMO-2. Each SUMO-2 'monomer' is separated from the other by Arg–Ser, which is also present at the C-terminus before the stop codon. Details of oligonucleotides and PCR protocol are available in the Supplementary Information [AU: please provide this in the Supplementary Information].

Recombinant 6His-tagged proteins were expressed and purified as described above. Proteins were stored at –80°C in 50 mM Tris/HCl (pH 7.5), 200 mM NaCl, 5 mM β-mercaptoethanol, 0.1% NP40.

Generation of recombinant SUMO-2 protein conjugates. For 6His–WT–SUMO-2 polymers, a 3 ml *in vitro* conjugation reaction was prepared containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM DTT, 5 mM MgCl₂, 2 mM ATP, 0.2 μM SAE2/SAE1 (ref. 3), 4 μM Ubc9 (ref. 28) and 100 μM 6His–SUMO-2 (ref. 29) and incubated at 37°C for 4 h. 6His–SUMO-2 species were purified from the assay mix using nickel NTA sepharose affinity chromatography incorporating a 1 M NaCl wash to dissociate any proteins non-covalently bound to SUMO-2. Eluted proteins were stored at –80°C in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM β-mercaptoethanol.

SUMO-2 modified GST–PML was prepared in a similar manner except that the assay was carried out in the presence of 7 mg GST–PML bound to 4 ml glutathione agarose beads. The GST–PML–SUMO-2 beads were washed with buffer containing 1 M NaCl before storage at 4°C in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM β-mercaptoethanol.
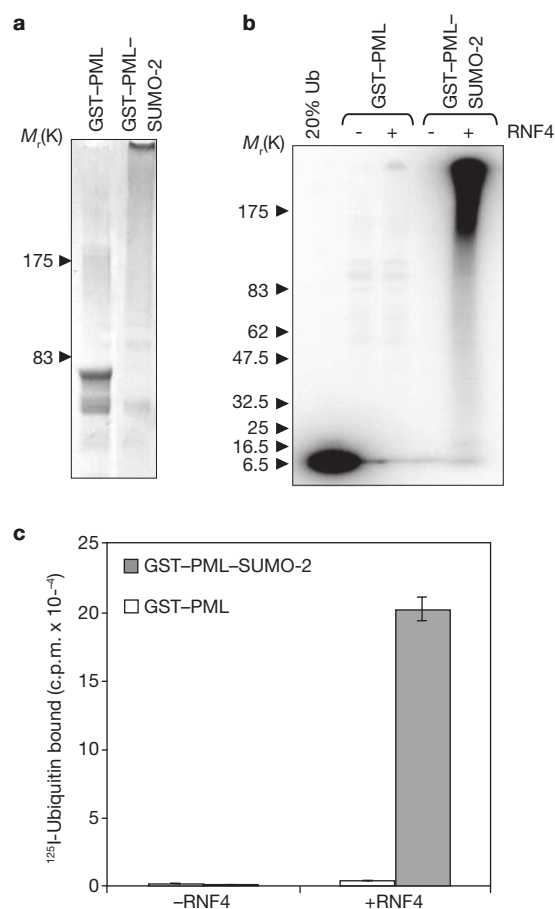

Figure 6 RNF4 ubiquitinates PML only when conjugated by SUMO-2. (**a**) Coomassie blue-stained SDS–PAGE gel of unmodified and SUMO-2-modified GST–PML prepared for analysis in *in vitro* ubiquitination assays. (**b**) Phosphorimager analysis of a dried SDS–PAGE gel fractioning *in vitro* ¹²⁵I-ubiquitination assays using the GST–PML and GST–PML–SUMO-2 as substrates, both with and without RNF4. *In vitro* ¹²⁵I-ubiquitination assays were carried out with GST–PML and GST–PML–SUMO-2 immobilized on glutathione agarose beads, which were subsequently washed before analysis. (**c**) γ-counting of *in vitro* ubiquitination assays before analysis shown in panel **b**. Data are mean ± s. d. for triplicate reactions. The *P* value for '+RNF4' compared with '–RNF4' for GST–PML–SUMO-2 is 0.0006. The *P* value for the '+RNF4' data for GST–PML compared with GST–PML–SUMO-2 is 0.0006 **[AU: please include statistical analysis]**.

***In vitro* interaction studies between 6His–MBP–RNF4 and SUMO-2 proteins.** Analysis of binding between RNF4 and the different SUMO protein forms was performed *in vitro* using 6His–MBP fusion forms of RNF4. In 50 μl reactions, 1.6 μM 6His–MBP–RNF4 was incubated with 20 μg 6His–SUMO-2 protein in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 0.1% NP-40, 2 mM DTT, 5% glycerol, for 1 h at 22°C. Equilibrated amylose beads (10 μl; New England Biolabs) were added to each assay and agitated for 60 min. Beads were washed once with binding buffer containing 1 M NaCl, and twice with binding buffer alone before bound proteins were eluted by addition of Laemmli's sample buffer. SUMO-2 species bound by 6His–MBP–RNF4 were detected by western blotting of the eluate fractionated on 4–12% NuPAGE precast Bis-Tris gels (Invitrogen).

***In vitro* ubiquitination assays.** A preliminary screen of 15 E2 enzymes showed that ubiquitination of SUMO-2 polymers by RNF4 was most efficient in the presence of either Ubc4 or one of the UbcH5 isoforms (data not shown). Ubiquitination assays were prepared using 8 μM ¹²⁵I-ubiquitin, 40 nM Uba1, 0.7 μM UbcH5a in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM MgCl₂, 2 mM ATP, 0.1% NP-40. In assays containing RNF4, the ligase was used at 0.5 μM. For substrate ubiquitination analysis, 1 μg of each substrate protein was used in 50 μl
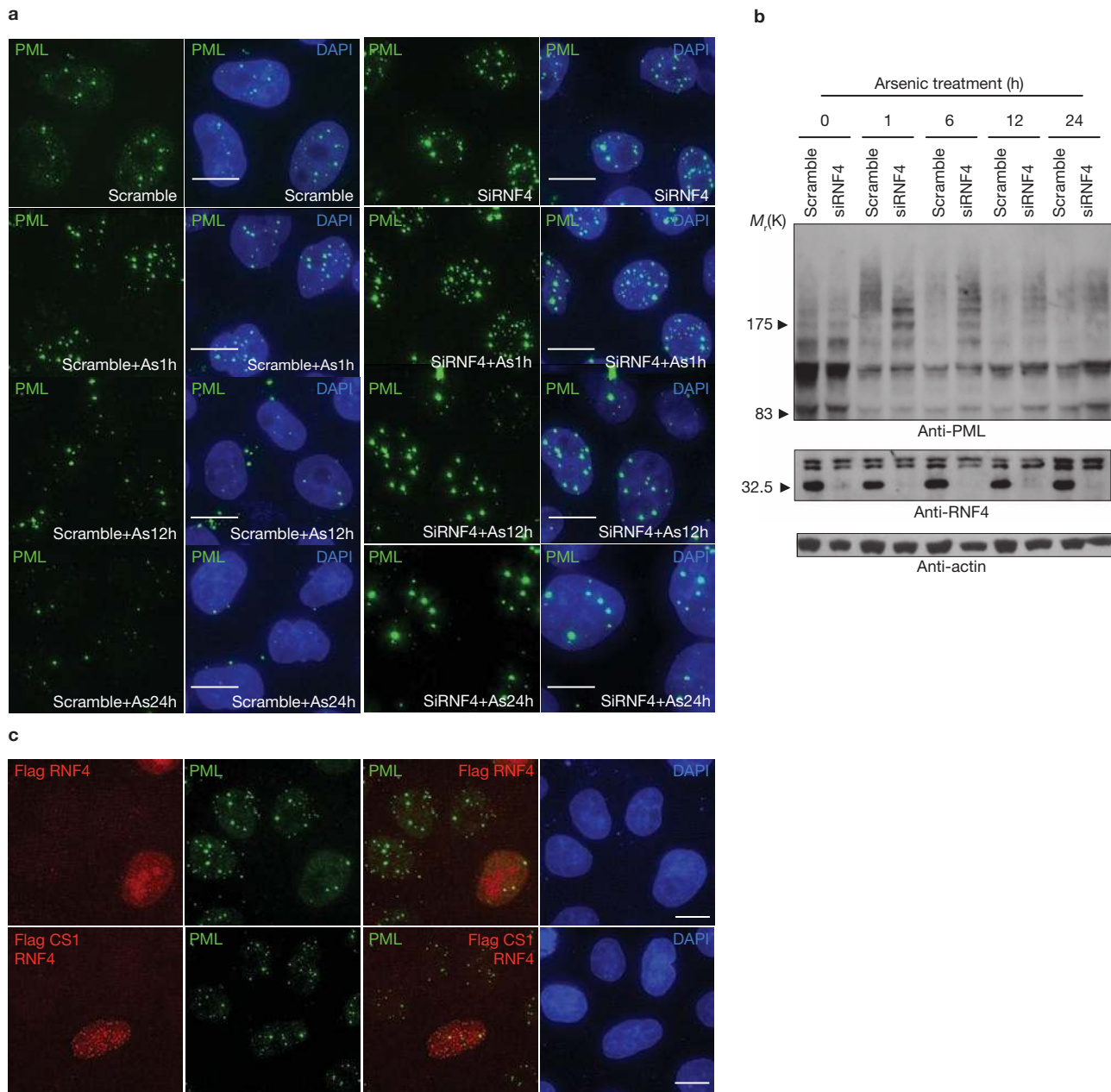
Figure 7 Arsenic-mediated degradation of PML is RNF4-dependent. (**a**) HeLa cells treated with a pool of control siRNAs (scramble) or a pool of siRNAs specific for RNF4 were exposed to 1 μM arsenic trioxide for either 1, 12 or 24 h. Cells were analysed by immunofluorescence microscopy using the 5E10 PML specific monoclonal antibody (green). DNA was stained with DAPI (blue). Scale bar represents 10 μm. (**b**) HeLa cells treated as in **d** [AU: there is no panel d. Please clarify] were analysed by western blotting for PML, RNF4 and actin. (**c**) HeLa Cl6 cells were transfected with a Flag tagged version of rat wild-type RNF4 or the CS1 mutant and stained with anti-Flag (red) and anti-PML (green) antibodies. DNA was stained with DAPI (blue). Scale bar represents 10 μm. See Supplementary Information, Fig. S7 for full blot images.

assays, therefore keeping the number of SUMO-2 moieties the same for each reaction. Assays were incubated at 37°C for 3–4 h. Substrates were purified from reactions by nickel affinity chromatography (10 μl beads), before γ-counting and elution by Laemmli's sample buffer, followed by phosphorimaging analysis. RNF4 self-ubiquitination assays were prepared as described above in 10 μl volumes and stopped by addition of Laemmli's sample buffer, followed by phosphorimaging analysis. To analyse the sites of ubiquitination of poly-SUMO-2 chains by mass spectrometry 6His [AU: OK?]–SUMO-2 chains (fraction consisting mainly of monomeric, dimeric, trimeric and tetrameric SUMO-2) were ubiquitinated in a reaction containing 160 nM Uba1, 5 μM UbcH5a, 0.7 μM RNF4, 40 μM ubiquitin, 3 mM ATP, 5 mM MgCl$_2$, 1 mM DTT, complete protease inhibitors, EDTA-free (Roche) and 50 mM Tris-HCl, pH 7.5. After an incubation of 3.5 h at 37°C, the

reaction was terminated by the addition of Laemmli sample buffer and analysed by SDS–PAGE. Coomassie-stained gel was cut into slices and subjected to in-gel trypsin digestion, followed by LC-Ms Ms analysis on a Thermo Orbitrap mass spectrometer (University of Dundee MS facility). The individual peak lists were searched in the international protein index (IPI) database (http://www.ebi.ac.uk/IPI/IPIhelp.html) using the MASCOT program (Matrix Science). MASCOT search was performed not only to identify proteins, but also those peptides with the diglycine signature peptide found on lysine residues modified with ubiquitin. A similar method was also used to identify sites of ubiquitination in GST–PML modified with poly SUMO-2.

siRNA methods. RNF4 expression was ablated with either 4 individual siR-NAs (sense strand sequence: RNF4-1 GCUAAUACUUGCCCAACUUUU;

RNF4-2 GAAUGGACGUCUCAUCGUUUU; RNF4-3´ GACAGAGACGUAUAUGUGAUU; RNF4-4 GCAAUAAAUUCUAGACAAGUU) or a pool containing equal amounts of each double-stranded oligoribonucleotide purchased from Dharmacon (On-target Plus). SiRNAs (50 nM final concentration) were introduced into HeLa cells using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Cells were analysed after 48 h. Stable knockdown of *RNF4* in cells was achieved by constructing a plasmid encoding an shRNA based on *RNF4-2*. Complementary oligonucleotides were inserted into the pLKO.1 lentiviral vector (Addgene) and after transfection into 293T cells with lentiviral packaging and VSV G expression plasmids, the cellular supernatant was used to transduce HeLa cells to puromycin resistance.

Note: Supplementary Information is available on the Nature Cell Biology website.

AUTHOR CONTRIBUTIONS
[AU: Authors are encouraged to include a statement called 'Author Contributions' to specify the contributions of each co-author, such as experimental work, project planning, data analysis, etc. The statement should be short, and refer to authors by their initials.]

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests. [AU: O.K.?]

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