RESEARCH NOTE

REVISED RNF4 interacts with multiSUMOylated ETV4 [version 2; referees: 2 approved]

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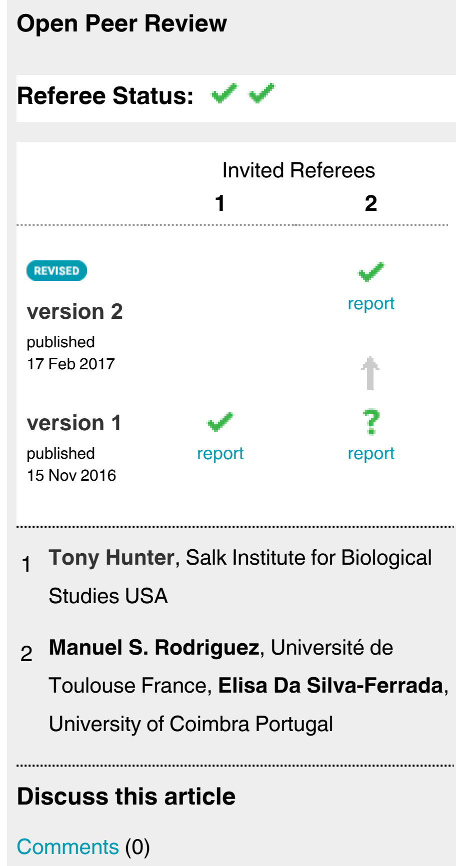
v2 **First published:** 15 Nov 2016, **1**:3 (doi: 10.12688/wellcomeopenres.9935.1)
Latest published: 17 Feb 2017, **1**:3 (doi: 10.12688/wellcomeopenres.9935.2)

Abstract

Protein SUMOylation represents an important regulatory event that changes the activities of numerous proteins. Recent evidence demonstrates that polySUMO chains can act as a trigger to direct the ubiquitin ligase RNF4 to substrates to cause their turnover through the ubiquitin pathway. RNF4 uses multiple SUMO interaction motifs (SIMs) to bind to these chains. However, in addition to polySUMO chains, a multimeric binding surface created by the simultaneous SUMOylation of multiple residues on a protein or complex could also provide a platform for the recruitment of multi-SIM proteins like RNF4. Here we demonstrate that multiSUMOylated ETV4 can bind to RNF4 and that a unique combination of SIMs is required for RNF4 to interact with this multiSUMOylated platform. Thus RNF4 can bind to proteins that are either polySUMOylated through a single site or multiSUMOylated on several sites and raises the possibility that such multiSIM-multiSUMO interactions might be more widespread.



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How to cite this article: Aguilar-Martinez E, Guo B and Sharrocks AD. **RNF4 interacts with multiSUMOylated ETV4 [version 2; referees: 2 approved]** Wellcome Open Research 2017, **1**:3 (doi: 10.12688/wellcomeopenres.9935.2)

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Grant information: This work was supported by the Wellcome Trust [093052]; and a Royal Society-Wolfson award to ADS.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 15 Nov 2016, **1**:3 (doi: 10.12688/wellcomeopenres.9935.1)

REVISED **Amendments from Version 1**

Both of the referees made some valuable suggestions to increase the clarity of this paper. We have made a number of textual changes and alterations to the figures to address the issues raised.

Changes to figures:

Two additional lanes have been added to Figure 1C which demonstrate that the levels of multi-SUMOylation we observe are sufficient to promote binding of RNF4 to ETV4 (as no binding is observed to the non-SUMOylatable ETV4(K1234R) mutant).

An additional figure panel (1F) has been added to demonstrate that ETV4 modified with SUMO(K11R) (which cannot form chains) still binds to RNF4 and hence binding is mediated by multi-SUMO.

Molecular weight markers have been added throughout.

The schematics have been modified to show the correct number of SIMs and SUMO modification sites of the proteins.

Changes to text:

Further discussion has been added regarding the structural studies on RNF4-polySUMO interactions. In addition, the implications for SUMO presentation by structural changes in the ETV4 N-terminal region are discussed.

Several methodological clarifications have been added and more precision used in describing the various RNF4 and ETV4 constructs that were used.

Discussion of the new data shown in the figures has been added.

See referee reports

Introduction

SUMOylation is a process whereby proteins are modified by SUMO (small ubiquitin-like modifier) through conjugation to specific lysine residues. This type of posttranslational modification has been increasingly recognised as an important regulatory event in many cellular processes and is often deregulated in disease situations (reviewed in Yang & Chiang, 2013). Much of the focus has been on the role of SUMO in regulating nuclear processes such as DNA repair, chromatin structural changes and transcriptional regulation (reviewed in Cubeñas-Potts & Matunis, 2013; Jackson & Durocher, 2013). The addition of SUMO to a protein constitutes a large change in overall protein size and as such, one major mode of action is through providing an additional protein binding surface which changes the repertoire of potential interacting proteins (reviewed in Hay, 2013). Binding partners are attracted to SUMO through short hydrophobic regions known as SIMs (SUMO interacting motifs) (Hecker *et al.*, 2006; Song *et al.*, 2004). Proteins are typically modified by a single SUMO molecule by conjugation to specific lysine residues. However while this is the case for SUMO1, both SUMO2 and SUMO3 can develop SUMO chains through conjugation of additional SUMO moieties through a lysine residue located within the N-terminal region of these proteins (reviewed in Ulrich, 2008). This leads to the deposition of SUMO chains on substrates which provide a polymeric binding surface for targeting new protein-protein interactions. This type of interaction has been exploited by proteins like RNF4 which has multiple SIMs which can simultaneously interact with several SUMO moieties in polymeric SUMO chains (Keusekotten *et al.*, 2014; Kung *et al.*, 2014; Tatham *et al.*, 2008; Xu *et al.*, 2014).

RNF4 is a SUMO targeted ubiquitin ligase (STUbL) which is recruited by polySUMOylated substrates like PML and subsequently targets PML for degradation (Lallemand-Breitenbach *et al.*, 2008; Tatham *et al.*, 2008; reviewed in Gärtner & Muller, 2014; Praefcke *et al.*, 2012). Since this initial discovery, RNF4 has been shown to play an essential role in DNA repair (Galanty *et al.*, 2012; Yin *et al.*, 2012) and further RNF4 substrates have been identified such as FANCI and FANCD2 which function in the DNA repair pathways (Gibbs-Seymour *et al.*, 2015). The role of RNF4 has been broadened by the identification of substrates involved in chromatin and transcription regulation such as TRIM28/KAP1 (Kuo *et al.*, 2014) and ETV4 (Guo & Sharrocks, 2009). In these contexts, it is assumed that RNF4 elicits its functions through polySUMO chains. However, it is possible that other multivalent interaction modalities might be employed. Indeed, rather than one modification of individual component, efficient DNA repair requires SUMOylation of many different proteins involved in homologous recombination (Psakhye & Jentsch, 2012). It is generally assumed that polySUMOylation is the driving factor but this finding raises the possibility that multiSUMOylation of many components of a single protein complex might instead present a binding surface for recruiting RNF4. Alternatively, RNF4 might be recruited to substrates by binding to a multi-SUMO platform created by the simultaneous SUMOylation of multiple lysine residues in the same proteins. Indeed many proteins have the potential to be multiSUMOylated due to having multiple consensus sites for SUMOylation (Yang *et al.*, 2006), and proteome-wide studies have shown that large numbers of proteins contain two or more SUMO2 modified lysine residues (Hendriks *et al.*, 2014; Tammsalu *et al.*, 2014). Conversely, other proteins could potentially recognise multi- or polySUMO platforms through utilising multiple SIMs in an analogous manner to RNF4. Recently, through the use of an artificial multi-SUMO scaffold we identified dozens of multiSUMO binding proteins, one of which, the transcriptional regulator ZMYM2, was investigated in detail and shown to use multiple SIMs to interact with this scaffold (Aguilar-Martinez *et al.*, 2015).

To further our understanding of multimeric SUMO interactions, we investigated the requirements for RNF4 binding to SUMOylated ETV4 (otherwise known as PEA3 and E1AF). ETV4 is an ETS transcription factor and represents an example of a protein with multiple SUMO modification sites which forms high molecular weight SUMOylated species (Guo & Sharrocks, 2009; Nishida *et al.*, 2007). SUMOylation was previously shown to be important for ETV4 transactivation activity but also triggers its degradation in a temporally delayed manner following growth factor-mediated signalling through the ERK pathway (Guo & Sharrocks, 2009). The STUbL RNF4 was shown to play an important role in controlling both ETV4-mediated target gene activation and ETV4 degradation through the ubiquitin pathway. Here, we investigated whether multiSUMOylation could provide a means for driving RNF4 recruitment to ETV4. Using a series of binding assays we show that multiSUMOylation can promote the binding of the multi-SIM containing protein RNF4 to ETV4.

Materials and methods

Plasmid constructs

The following plasmids were used in mammalian cell transfections; pSG5-PIAS4 (encoding Myc-tagged PIAS4)(kindly provided by Frances Fuller-Pace; Jacobs *et al.*, 2007), pCDNA3-Ubc9/UBE2I, pCDNA3-His-SUMO-3 and pCDNA3-His-SUMO-3(K11R) (kindly provided by Ron Hay; Tatham *et al.*, 2001). pAS981 (encodes full-length flag-tagged zebrafish ETV4/PEA3 cloned in the XbaI/KpnI sites of pCDNA3; constructed by Amanda Greenall).

The following plasmids were used for bacterial expression. MBP-RNF4(WT), MBP-RNF4(SIM1mut), MBP-RNF4(SIM2mut), MBP-RNF4(SIM3mut), MBP-RNF4(SIM4mut), MBP-RNF4(SIM1,2mut), MBP-RNF4(SIM1,2,3mut), and MBP-RNF4(SIM1,2,3,4mut) all in pLou3 (kindly provided by Ron Hay; Tatham *et al.*, 2008) have been described previously. MBP-RNF4(SIM1,4mut) (pAS2760) and MBP-RNF4(SIM2,3mut) (pAS2761) were constructed by Quikchange mutagenesis (Stratagene), using the templates pLou-MBP-RNF4(SIM1mut) (pAS2753) and pLou-MBP-RNF4(SIM2mut) (pAS2754) and the primer pair combinations ADS2587/ADS2588 and ADS2583/ADS2584 respectively. pAS2502 [encoding GST-ETV4 (1-335)(WT)] (Guo *et al.*, 2011), has been described elsewhere. His-SUMO3-K11R (pAS2767) was constructed by inserting a NcoI-EcoRI-cleaved PCR fragment (generated using primer pair ADS2582/ADS2581 and the template pCDNA3-HA-SUMO2K11R; Tatham *et al.*, 2001) into the same sites in pET30b. pAS2501 [encoding GST-ETV4(1-480)(WT)] was constructed by Niki Panagiotaki by ligating a BamHI/EcoRI-cleaved PCR product (primers ADS1580/ADS1584 and template pAS1801) into the same sites in pGEXKG. pAS1801 contains the full-length mouse cDNA cloned into the HindIII and SalI sites of pCDNA3. pAS4159 [encoding GST-ETV4(1-480)(K12R)], pAS4160 [encoding GST-ETV4(1-480)(K34R)], pAS4161 [encoding GST-ETV4 (1-480) (K1234R)] were constructed ligating BamHI/EcoRI-cleaved PCR products (primers ADS1580/ADS1584 and templates pAS1034, pAS1040 and pAS1037; Guo & Sharrocks, 2009) into the same sites in pGEXKG. Details of PCR primers are given in Table 1.

Cell culture, co-immunoprecipitation analysis and western blotting

HEK293T cells were grown in DMEM supplemented with 10% foetal bovine serum and where indicated, cells were treated with phorbol 12-myristate (PMA)(Sigma)(10 nM) for 6 hours. Plasmid transfection was performed using Polyfect (Qiagen). Western blotting and immunoprecipitation were carried out with the primary antibodies; rabbit polyclonal anti-RNF4 (1:5,000 gift from Jorma Palvino; Häkli *et al.*, 2005), mouse monoclonal anti-Flag M2 (1:2,000 Sigma; F3165), and mouse monoclonal anti-MBP (1:1,000 Abcam, ab49923 {Figure 2A} and 1:1,000 Cell Signaling mouse monoclonal cat no 2396 {Figure 2B}). The proteins were detected as described previously (Aguilar-Martinez *et al.*, 2015).

Protein purification and GST pulldown assays

Recombinant proteins were expressed in *Escherichia coli* BL21 or BL21-CodonPlus(DE3)-RIL (Stratagene). To prepare SUMOylated recombinant GST-ETV4, a reconstituted SUMOylation system in *E. coli* was used (Mencía & de Lorenzo, 2004). Recombinant GST-fusion proteins were purified and GST pulldown assays were carried out as described previously (Aguilar-Martinez *et al.*, 2015).

Results

ETV4 contains five evolutionarily conserved sites which fit the core SUMO consensus sequence ψKxE. We previously showed that the three most N-terminally located sites in mouse ETV4 (K96, K222 and K256) are the major sites for modification with SUMO (Guo & Sharrocks, 2009). It is assumed that RNF4 binds to proteins containing polySUMO chains, through its multiple SIM motifs. However, it is equally plausible that RNF4 might be able to recognise multiple single SUMO moieties conjugated to different sites in ETV4 (Figure 1A). Indeed, the latter possibility is supported by the observation that RNF4-mediated polyubiquitination *in vivo* is diminished as the number of SUMO conjugation sites in ETV4 are reduced (Guo & Sharrocks, 2009).

We first examined whether we could find evidence for multi- or polySUMOylation of ETV4 *in vivo*. We examined ETV4 SUMOylation status following co-transfection of cells with UBE2I/UBC9 and PIAS4 (to maximise SUMOylation levels) and either wild-type SUMO3 or the SUMO3(K11R) mutant. This latter form of SUMO cannot form polySUMO chains (Tatham *et al.*, 2001). Cells were treated with PMA to enhance the levels of SUMOylation (Guo *et al.*, 2009). Multiple high molecular weight SUMO conjugates were observed in the presence of wild-type SUMO3 (Figure 1B, lanes 3 and 4) and an identical pattern of conjugation was observed in the presence of SUMO3(K11R) (Figure 1B, lanes 5 and 6). The multiple bands arising are likely due to a

Table 1. PCR primers used in this study.

Primer	Sequence (5'-3')
ADS1580	ATCGGGATCCATGGAGCGGAGGATGAAAGGC
ADS1584	ATCGGAATTCAGTAAGAATATCCACCTCTGTG
ADS2581	GCGGGGAATTCGGTAGTGGTAGTGGTAGTATGTCCGAGGAGAAGCCCAAG
ADS2582	GCGGGCCATGGCTATGTCCGAGGAGAAGCCCAAG
ADS2583	GTGAATCTTTAGAGCCTGCGGCTGCGGACCTGACTCACAATGA
ADS2584	TCATTGTGAGTCAGGTCCGCAGCCGCAGGCTCTAAAGATTCAC
ADS2587	GACTCACAATGACTCTGCTGCGGCTGCTGAAGAAAGGAGAAGGC
ADS2588	GCCTTCTCCTTTCTTCAGCAGCCGCAGCAGAGTCATTGTGAGTC

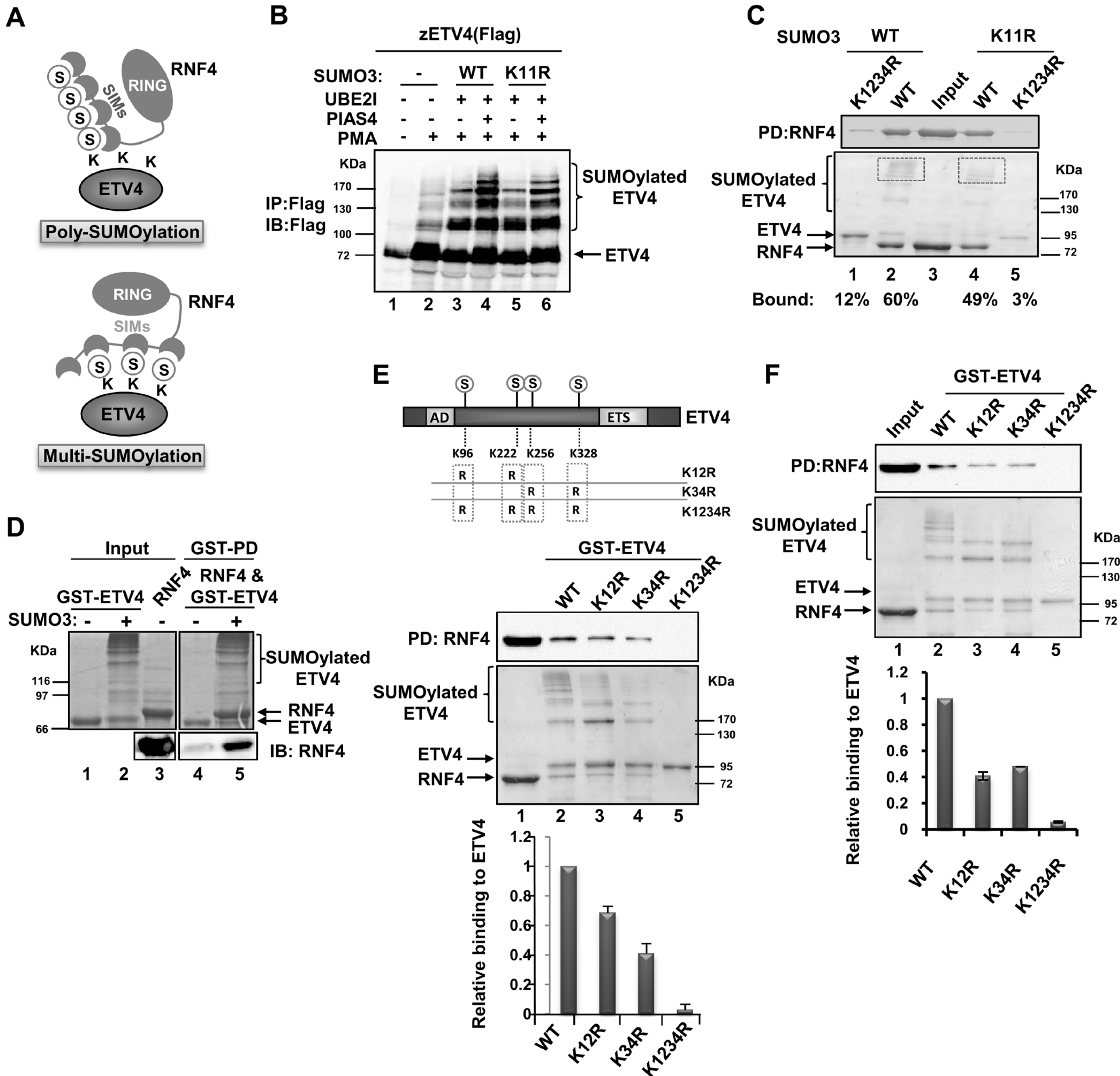

Figure 1. RNF4 binds to multiSUMOylated ETV4. (**A**) Schematic representation of RNF4 interacting with a poly- or multiSUMOylated form of ETV4 through its SIMs. (**B**) Flag-tagged zETV4 was immunoprecipitated (IP) with anti-Flag antibody from HEK293T cells co-transfected with wild-type (WT) SUMO3 or SUMO3(K11R), UBC9/UBE2I and PIAS4 where indicated. ETV4 was detected by immunoblotting (IB) with anti-Flag antibody. Where indicated, cells were treated with PMA for 6 h. (**C**) GSTpulldown analysis of the interaction of recombinant MBP-RNF4 with GST-ETV4(full-length; WT or K1234R mutant) modified with SUMO3(WT) or SUMO3(K11R). Pulled down RNF4 was detected by immunoblotting with anti-RNF4 antibody (top panel; pulldown [PD]). Bottom panel: Ponceau stained nitrocellulose membrane. Boxes highlight the presence or absence of high molecular weight SUMO chains. Percentages of binding indicate the binding of RNF4 relative to input. (**D**) GST pulldown (GST-PD) analysis of MBP-RNF4 binding to non-SUMOylated or SUMOylated recombinant GST-ETV4(1-355). Top: Coomassie stained gel of the input proteins (lanes 1–3) and results of the pulldown (lanes 4–5). Bottom: immunoblot of the pulldown (PD) using anti-RNF4 antibody. (**E** and **F**) GST pulldown (PD) analysis of the interaction of recombinant MBP-RNF4 with the indicated WT and mutant forms of GST-ETV4(full-length) modified with SUMO3(WT)(**E**) or SUMO3(K11R)(**F**). Top: schematic of ETV4 showing its SUMOylation sites and locations of the amino acid substitutions in the ETV4 mutants. Middle: GST pulldown assay with RNF4 binding shown in the top panel using an anti-RNF4 antibody (pulldown [PD]) and a Ponceau stained membrane at the bottom showing the GST bait and input proteins. Bottom: Quantification of RNF4 binding to the different SUMOylated (WT) forms of ETV4 (relative to input, taken as 1). The SUMOylated GST-ETV4 species in (**C**–**F**) were generated using an *in vivo* recombinant protein bacterial expression system (Mencia & Lorenzo, 2004). Molecular weight markers are shown on coomassie and Ponceau stained gels.

combination of single site SUMOylation events and multi-site SUMOylation to produce the higher molecular weight lower mobility species. Thus, as SUMO3(K11R) gives the same banding pattern as wild-type SUMO3, the multiple species that we observe in cells likely represent different combinations of multiSUMOylated ETV4 rather than polySUMOylated conjugates.

Next we explored whether multiSUMOylation is sufficient for binding of RNF4 to ETV4. To produce SUMOylated protein, full-length ETV4 was co-expressed in bacteria as a GST fusion protein along with the SUMO conjugation machinery and either wild-type SUMO3 or SUMO3(K11R) and in both cases, multiple SUMOylated species were observed, with wild-type SUMO3 generating substantially more of the higher molecular weight conjugates, indicative of polySUMOylation (Figure 1C, lower panel). Note that the banding pattern was not identical to Flag-tagged ETV4 in mammalian cells (Figure 1B), likely due to the differing mobilities of the branched chain SUMO conjugates associated with differentially tagged ETV4 constructs (N-terminal large GST tag versus a short C-terminal Flag tag). We then tested the binding of recombinant RNF4 to these SUMOylated forms of GST-ETV4 and found that both forms of SUMOylated ETV4 were able to efficiently bind to recombinant RNF4 in GST pulldown assays (Figure 1C, lanes 2 and 4; top panel) indicating that polySUMOylation is not required for efficient RNF4 binding. Although the levels of multi-SUMOylation are low, this is sufficient for RNF4 binding as mutant forms of ETV4 that are defective in SUMOylation cannot recruit RNF4 (Figure 1C, lanes 1 and 5). Importantly, we further verified that ETV4 SUMOylation is required for efficient RNF4 binding (Figure 1D) ruling out SUMO-independent binding of RNF4 to ETV4. To further extend these findings, we removed pairs of SUMOylation sites in ETV4 to reduce the potential for multi-SUMOylation. Loss of these SUMO modification sites reduced the levels of ETV4 multiSUMOylation, and provoked a concomitant reduction in RNF4 binding (Figure 1E). A virtually identical effect was observed for binding to ETV4 mutants modified with SUMO3(K11R) further demonstrating that SUMO chain formation is not required for RNF4 binding (Figure 1F). Although we have not determined the sites of SUMO modification on ETV4 in the bacterially generated SUMOylated ETV4, the loss of SUMOylation in the ETV4(K1234R) mutant that lacks all of the mapped SUMO conjugation sites (Guo *et al.*, 2009) demonstrates that SUMOylation is taking place at the correct lysine residues rather than at cryptic sites (Figure 1E and F, bottom panels). Thus RNF4 binding is diminished by reducing the potential for ETV4 multi-SUMOylation by either removing SUMO conjugation sites or using a chain forming mutant version of SUMO.

RNF4 has previously been shown to require multiple SIM motifs for binding to polySUMO chains. We therefore asked whether the multiple SIMs in RNF4 are required for binding to SUMO conjugated ETV4. We created multi-SUMOylated ETV4 by expressing GST-ETV4 along with the SUMO3 (Figure 2A and B). We then tested the interaction of multi-SUMOylated ETV4 in GST pulldown assays with RNF4 proteins containing mutations in one or more of its SIMs in RNF4. Mutating different combinations of two or more SIMs reduced binding to multiSUMOylated ETV4 (Figure 2A and B), with the simultaneous mutation of SIMs 2 and 3 eliminating detectable binding (Figure 2B, lane 10). We then asked whether any of the individual SIMs are important for binding to multiSUMOylated ETV4 and found that mutation of either SIM2 or SIM3 substantially reduced RNF4-ETV4 interactions while minimal effects were seen after mutating SIMs 1 or 4 (Figure 2B, lanes 3–6). These findings are broadly in keeping with the requirements determined for RNF4 binding to polySUMO chains, where SIM2 plays the most important role (Tatham *et al.*, 2008). However, mutation of SIM3 alone in RNF4 had little impact on RNF4 binding to polySUMO chains (Tatham *et al.*, 2008) indicating that SIM3 appears to play a more prominent role in binding to multiSUMOylated ETV4. Importantly, we got virtually identical results when wild-type SUMO3 was replaced with the mutant SUMO3(K11R) that is defective in polySUMO chain formation (Figure 2C). Thus, RNF4 can make differential use of its SIMs to potentially bind to both multi- and polySUMOylated proteins (Figure 2D).

Collectively, these findings on ETV4-RNF4 interactions establish that multiSUMOylation can act as a platform to recruit a multi-SIM domain containing protein.

Raw data for Figures 1 and 2

1 Data File

https://doi.org/10.6084/m9.figshare.4641067.v1

Discussion

One of the major mechanisms through which SUMOylation affects target protein activity is through providing a binding platform for other proteins. This can be elicited through the conjugation of single SUMO moieties, or through the subsequent formation of polySUMO chains on top of these initial conjugation events. These polySUMO chains act as a binding platform for STUbLs such as RNF4, which subsequently target the SUMOylated protein for ubiquitination and degradation (Boutell *et al.*, 2011; Lescasse *et al.*, 2013; Sun & Hunter, 2012; Tatham *et al.*, 2008).

However, in addition to presenting SUMO in the form of chains, multimeric SUMO platforms can be created through the simultaneous modification of several residues at the same time, either on a single protein or several proteins in a complex. We recently provided evidence for this possibility through the use of an artificial multiSUMO scaffold and identified dozens of proteins which are recruited by this scaffold (Aguilar-Martinez *et al.*, 2015). Here we have approached the problem from the other direction and asked whether a multiSUMOylated substrate can act as a binding platform for RNF4. Using multiSUMOylated ETV4 as a binding platform, we demonstrate that RNF4 can be recruited in a multiSUMO-dependent manner. Thus RNF4 can potentially be recruited to multimeric SUMO scaffolds created through polySUMO chains, multiSUMO platforms (Figure 2D), or even a combination of both. Indeed, the prospect of a combination of multi- and polySUMOylation involvement in STUbL targeting, is attractive as the availability of more SUMO modification sites increases the probability of chain formation.

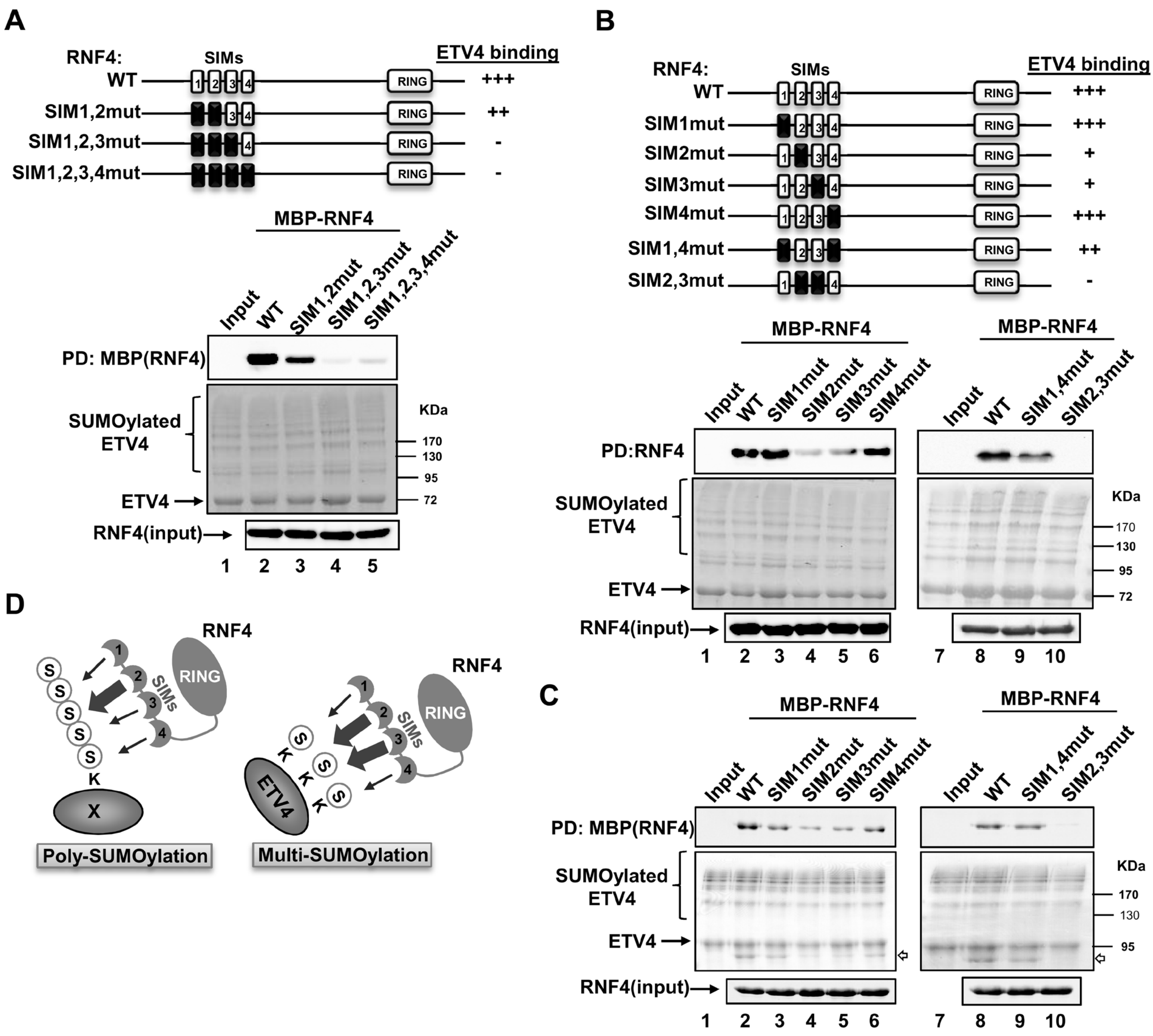

Figure 2. Mapping the SIM motifs in RNF4 for binding to multiSUMOylated ETV4. (**A**–**C**) GST pulldown assays of the indicated wild-type and mutant forms of MBP-RNF4 binding to ETV4(1-355) SUMOylated with wild-type SUMO3 (**A** and **B**) or full length ETV4 with SUMO3(K11R)(**C**). Schematic representations of wild type (WT) and mutant forms of RNF4 are shown at the top. Symbols on the right show the binding of the different RNF4 forms relative to RNF4(WT) (-, ≤10%), (+, <40%), (++, ≥50%) and (+++, ≥80%). RNF4 binding (top panels; pulldown [PD]) and input proteins (bottom panels) were detected by immunoblotting using an anti-MBP antibody. SUMOylated GST-ETV4 is shown by Ponceau staining (middle panels). Open arrows in (**C**) indicate the band corresponding to MBP-RNF4. Molecular weight markers are shown on Ponceau stained gels. (**D**) Schematic representation of RNF4 interacting with a polySUMOylated protein (X) or multiSUMOylated form of ETV4 through its SIMs. Arrows represent SIM-SUMO interactions, with larger arrows denoting the dominant SIM-SUMO interactions.

RNF4 contains multiple SIMs for binding to polySUMO chains (Keusekotten *et al.*, 2014; Kung *et al.*, 2014; Tatham *et al.*, 2008). Of the four SIM motifs, SIM2 is the most important and is thought to nucleate the binding of the other SIMs to the polySUMO chains. This type of behaviour has led to the concept of one or a subset of SIMs being designated as "dominant" (Sun & Hunter, 2012). More recently, a structural study confirmed an important role for SIM2 in polySUMO binding by RNF4 but also provided evidence for a supporting substantive role for SIM3 (Xu *et al.*, 2014). Here we show that both SIM2 and SIM3 play dominant roles in the case of RNF4 binding to multiSUMOylated ETV4. This is unsurprising given the likely different stereospecific presentation of the multimeric SUMO binding platforms in the form of polySUMO chains and through the presentation of

multiple SUMO moieties on the surface of ETV4. Thus for RNF4 the linear series of closely spaced SIMs might be optimally configured to interact with polySUMO chains and a select subset of multiSUMO platforms while other proteins might have different binding modes. Indeed, we demonstrated that the multiple SIMs in ZMYM2 are not linearly arranged in the protein but are spread throughout a large region (Aguilar-Martinez *et al.*, 2015). Again one of these SIMs is "dominant" and presumably helps nucleate a multi-SIM scaffold that recognises a unique subset of multiSUMO substrates. Conversely from the substrate perspective, the presentation of the conjugated SUMO moieties following multiSUMOylation is unlikely to be ordered in a linear fashion as seen in polySUMO chains. Indeed, the SUMOylation sites in ETV4 are spread across a 330 amino acid region in its N-terminal region. This means that to provide a clustered array that can be simultaneously recognised by the tandem SIMs RNF4, the N-terminal region of ETV4 must exhibit considerable flexibility. The lack of recognisable ordered domains in this region would facilitate such flexibility.

SUMOylation of ETV4 triggers both its activation and subsequent degradation, and we have also shown that RNF4 is also important for both processes (Guo & Sharrocks, 2009). Thus, multiSUMOylation could provide an important link in this sequence of events. In addition, multiSUMOylated ETV4 might also recruit other multi-SIM domain containing proteins during the activation process allowing the sequential binding of a coactivator followed by exchange for RNF4. This would provide an attractive mechanism for the coordinated activation and subsequent inactivation of ETV4. The requirement for mutiSUMOylation would effectively set a threshold which triggers progression along the activation-inactivation pathway and enable temporal control over ETV4-mediated transcriptional activation activity.

In summary, we have provided evidence that in addition to its documented polySUMO binding activities, RNF4 has the potential to bind to multiSUMOylated substrates such as ETV4. This has broader significance as it opens up the possibilities of more widespread regulatory interactions between multiSUMOylated proteins and multi-SIM domain containing protein partners.

Data availability

Figshare: Raw data for Figures 1 and 2. doi: https://doi.org/10.6084/m9.figshare.4641067.v1 (Aguilar-Martinez *et al.*, 2016).

Author contributions

ADS conceived the study. EAM, BG and ADS designed the experiments. EAM and BG carried out the experiments. EAM, BG and ADS wrote and edited the manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by the Wellcome Trust [093052]; and a Royal Society-Wolfson award to ADS.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank Karren Palmer for excellent technical assistance; Catherine Millar, Shen-Hsi Yang and other members of our laboratory for comments on the manuscript and stimulating discussions; Senturan Soundrasritharan for carrying out the coomassie gel Fig.S1D; Richard Kammerer, Frances Fuller-Pace, and Ron Hay for reagents.

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Data Source

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Open Peer Review

Current Referee Status:

Version 2

Referee Report 17 February 2017

doi:10.21956/wellcomeopenres.11629.r20342

Manuel S. Rodriguez
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Authors have well addressed most comments and suggestions. I am happy to accept this article for indexing.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 09 December 2016

doi:10.21956/wellcomeopenres.10706.r18378

Manuel S. Rodriguez[1], **Elisa Da Silva-Ferrada**[2]
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The manuscript RNF4 interacts with multiSUMOylated ETV4 by Aguilar-Martinez *et al* describes that multiSUMOylation of a target protein (ETV4) can be equally well recognised by RNF4, offering an alternative recruitment mechanism to the well known polySUMOylation signal that is recognised by this SUMO-dependent ubiquitin ligase. Authors propose this polySUMO/multiSUMO RNF4 recruitment as a widespread mechanism for this ubiquitin ligase. Further experiments will be required to conclude on the possible molecular and biological differences between the recruitment of RNF4 through poly vs multiple SUMOylation signals.

The authors present a clear set of data indicating that SUMOylation of ETV4 is required for the interaction of RNF4. In this interaction the SUMO-interacting motifs (SIM) 2 and 3 of RNF4 play a critical role. However, one of the main data supporting the role of the multi-SUMOylation (Figure 1C) lacks quality. In fact, its difficult to observe multi-SUMOylated material in the condition with SUMO3 (K11R). Since the “in

bacteria" modification system is not very efficient in terms of production of SUMOylated proteins, perhaps an "in vitro" all recombinant SUMOylation system can be used instead to pull down RNF4 WT. In such experiment ETV4 modified with SUMO3 vs SUMO3(K11R) can be compared. Alternatively, authors can use the same conditions than Figure 1B to pull-down recombinant RNF4. This data will support the actual information.

Minor comments and suggestions:

- Molecular weight markers should be included in each Western-blot to estimate the size of the poly or mono-SUMOylated forms of ETV4.
- Please justify the use of PMA, including its full name and distributor
- It would be nice to try other STUBL and check if the interaction with multiSUMOylated ETV4 is similar to RNF4.

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 26 Jan 2017

Andrew D Sharrocks, Faculty of Life Sciences, University of Manchester, UK

Our point by point response is indicated below:

(1) The authors present a clear set of data indicating that SUMOylation of ETV4 is required for the interaction of RNF4. In this interaction the SUMO-interacting motifs (SIM) 2 and 3 of RNF4 play a critical role. However, one of the main data supporting the role of the multi-SUMOylation (Figure 1C) lacks quality. In fact, its difficult to observe multi-SUMOylated material in the condition with SUMO3 (K11R). Since the "in bacteria" modification system is not very efficient in terms of production of SUMOylated proteins, perhaps an "in vitro" all recombinant SUMOylation system can be used instead to pull down RNF4 WT. In such experiment ETV4 modified with SUMO3 vs SUMO3(K11R) can be compared. Alternatively, authors can use the same conditions than Figure 1B to pull-down recombinant RNF4. This data will support the actual information.

Response: The referee indicates that the amount of SUMOylation present from the bacterial expression system is low, and therefore provides low stoichiometry which could be improved. However, the critical thing is whether this level of SUMOylation is sufficient to mediate RNF4 binding. We have now included two additional lanes from the original gel that proves that this is the case and we have sufficient SUMOylation. These lanes include a non-SUMOylatable ETV4 derivative that lacks four consensus SUMOylation motifs, and therefore lacks evidence of any multi- and poly-SUMOylation. This mutant ETV4 binds very little RNF4, thereby demonstrating the importance of multi-SUMOylation in this binding event.

Minor comments and suggestions:
• Molecular weight markers should be included in each Western-blot to estimate the size of the poly or mono-SUMOylated forms of ETV4.

Response: These have now been added throughout the paper and help clarify the nature of the bands we observe.

• Please justify the use of PMA, including its full name and distributor

Response: We have used this as we previously showed that this enhances ETV4 SUMOylation (now indicated in the text). The definition of the acronym has now been added to the materials and methods section.

• It would be nice to try other STUBL and check if the interaction with multiSUMOylated ETV4 is similar to RNF4.

Response: We agree with this and is something that will be of interest for others to pursue in the future.

Competing Interests: No competing interests were disclosed.

Referee Report 28 November 2016

doi:10.21956/wellcomeopenres.10706.r17628

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Here the authors have extended their studies of sumoylation of the ETS family transcription factor, ETV4. They have previously reported that ETV4 K96, K222 and K256, which lie in sumoylation consensus sequences, are sumoylated, and shown that sumoylated ETV4 is a target for the RNF4 SUMO-targeted E3 ubiquitin ligase (STUbL). Here, they demonstrated that ETV4 is multi-sumoylated when co-expressed in HEK293T cells together with UBE2/UBC9, PIAS4 and WT SUMO3 or K11R SUMO3. Since K11R SUMO3 cannot form SUMO chains, they deduced that the slowly migrating sumoylated forms of ETV4 detected contain mono-SUMO3 adducts at multiple sites rather than SUMO chains. Bead-bound sumoylated GST-ETV4 brought down RNF4 efficiently, whereas mutant GST-ETV4 lacking pairs of the identified sumoylation sites bound less RNF4, and mutant GST-ETV4 lacking 4 SUMO sites (including K328) brought down no detectable RNF4. Next they assessed the importance of the individual SIMs in the RNF4 tetra-SIM array for binding multi-sumoylated ETV4, by mutating SIM consensuses singly and in combination, concluding that the central SIM2 and SIM3 motifs contribute most significantly to the binding of RNF4 to multi-sumoylated ETV4, as others have found for RNF4 binding to poly-SUMO chains. They conclude that RNF4 can recognize multi-monosumoylated proteins through its SIM array, in addition to proteins with poly-SUMO chains.

Overall, the experiments are described in sufficient detail and the data are of good quality, providing strong experimental support for their conclusion that RNF4 can bind to multi-monosumoylated proteins through its array of four SIMs. This theoretical possibility had been proposed by others (e.g. Sun and Hunter (2012)), but it is important to have direct experimental validation of this concept, since the ability of RNF4 to bind to multi-monosumoylated proteins expands the range of possible targets for RNF4 STUbL-mediated ubiquitylation. One point that needs further discussion is the likely topology of binding of the RNF4 SIMs to a multi-monosumoylated protein such as ETV4. For instance, the schematic in panel D is not accurate in terms of the relative size of the sumoylated region in ETV4 and the size of the SIM

domain in RNF4. Presumably, this region of ETV4 is flexible, but there would still need to be looping out of sequences between two adjacent SUMO residues for them to be able to bind simultaneously to SIMs 2 and 3 in a single RNF4 molecule. Some discussion of this point is merited.

Other points:

1. Figure 1: The overall stoichiometry of ETV4 sumoylation in transfected HEK293 cells shown in panel B was low even under conditions designed to maximize sumoylation, i.e. there was a lot of unmodified ETV4. This might suggest that ETV4 sumoylation is cooperative, in the sense that sumoylated ETV4 molecules may be more susceptible than unmodified ETV4 to further sumoylation at additional sites. The patterns of sumoylated ETV4 bands in panels C and D which were obtained by co-expression of GST-ETV4 and the sumoylation machinery in bacteria are quite different from the pattern in panel B obtained in mammalian cells, and given the apparent size of the shift of the bands near the top of the gel in the WT SUMO3 sample it seems likely that SUMO3 chains were attached, as the authors suggest. Unfortunately, the Ponceau stain is rather faint and it is hard to tell what bands are present in the K11R SUMO3 sample. Although the data in panel D suggest that ETV4 sumoylation site specificity is similar in bacterial and mammalian cells, one would like to see bacterial samples run side by side with sumoylated GST-ETV4 from mammalian cells (is it clear that there are no SUMO sites in GST?), and some discussion of sumoylation specificity in this bacterial system is desirable (the stoichiometry seems significantly higher than in mammalian cells), and in some ways it is surprising that adventitious sumoylation did not occur at nonconsensus sites in ETV4, particularly in the absence of SUMO proteases in the system. Did the authors confirm which Lys were sumoylated in the bacterial system using mass spec? Ideally, the experiments in panels D and E should be repeated with K11R SUMO3 to formally establish that RNF4 binding is not dependent to any extent on SUMO chain formation.

2. Figure 2: The patterns of sumoylated ETV4 look different from those in Figure 1C-E. The patterns with WT SUMO3 could be due to polysumoylation, but the bands seem to be rather discrete for this, and there are more than 4 bands even with K11R SUMO3, as seen in panel C. Admittedly this could be because a single SUMO at different sites in ETV4 results in a different gel mobility, but this issue ought to be discussed.

3. With regard to the topology of the binding of the RNF4 SIM array to SUMO chains or to multiple single SUMOs, further discussion of the structural insights into RNF4 SIM/SUMO interactions reported in Xu et al. (2014), and the importance of SIMs 2 and 3 in this interaction, is warranted.

Minor points:

1. In Figure 1A RNF4 is depicted with only 3 SIMs, possibly because ETV4 is shown with three SUMO sites or a SUMO chain with three residues. In contrast, RNF4 in Figure 2D is depicted with 4 SIMs in Figure 2D, which is the correct number of SIMs. I recommend changing Figure 1A to be consistent with Figure 2D. In addition, the legend to Figure 1 should indicate very clearly that the samples in panels C-E were obtained with ETV4 sumoylated in bacteria.

2. In the experiments in Figure 2, MBP-RNF4 was used. Was MBP-RNF4 also used in the experiments in Figure 1C-E. If so this should be indicated? If not, how was recombinant RNF4 purified for this experiment - only MBP-RNF4 is described in the Methods.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 26 Jan 2017

Andrew D Sharrocks, Faculty of Life Sciences, University of Manchester, UK

Our point by point response to the issues raised is provided below:

One point that needs further discussion is the likely topology of binding of the RNF4 SIMs to a multi-monosumoylated protein such as ETV4. For instance, the schematic in panel D is not accurate in terms of the relative size of the sumoylated region in ETV4 and the size of the SIM domain in RNF4. Presumably, this region of ETV4 is flexible, but there would still need to be looping out of sequences between two adjacent SUMO residues for them to be able to bind simultaneously to SIMs 2 and 3 in a single RNF4 molecule. Some discussion of this point is merited.

Response: This is an important point and we have now added brief discussion of this in the manuscript.

Other points:
1. Figure 1: The overall stoichiometry of ETV4 sumoylation in transfected HEK293 cells shown in panel B was low even under conditions designed to maximize sumoylation, i.e. there was a lot of unmodified ETV4. This might suggest that ETV4 sumoylation is cooperative, in the sense that sumoylated ETV4 molecules may be more susceptible than unmodified ETV4 to further sumoylation at additional sites. The patterns of sumoylated ETV4 bands in panels C and D which were obtained by co-expression of GST-ETV4 and the sumoylation machinery in bacteria are quite different from the pattern in panel B obtained in mammalian cells, and given the apparent size of the shift of the bands near the top of the gel in the WT SUMO3 sample it seems likely that SUMO3 chains were attached, as the authors suggest. Unfortunately, the Ponceau stain is rather faint and it is hard to tell what bands are present in the K11R SUMO3 sample. Although the data in panel D suggest that ETV4 sumoylation site specificity is similar in bacterial and mammalian cells, one would like to see bacterial samples run side by side with sumoylated GST-ETV4 from mammalian cells (is it clear that there are no SUMO sites in GST?), and some discussion of sumoylation specificity in this bacterial system is desirable (the stoichiometry seems significantly higher than in mammalian cells), and in some ways it is surprising that adventitious sumoylation did not occur at nonconsensus sites in ETV4, particularly in the absence of SUMO proteases in the system. Did the authors confirm which Lys were sumoylated in the bacterial system using mass spec? Ideally, the experiments in panels D and E should be repeated with K11R SUMO3 to formally establish that RNF4 binding is not dependent to any extent on SUMO chain formation.

Response: We added an additional panel to Fig.1 to compare binding to WT and K11R versions of SUMO. This confirms that SUMO chain formation is not needed for RNF4 binding (new Fig. 1F). We have not performed mass spectrometry on the bacterially generated SUMOylated ETV4. However, Fig. 1E and F demonstrate that removal of the mapped SUMOylation sites in ETV4 eliminates ETV4 SUMOylation in the bacterial expression system and this loss of SUMOylation results in the loss of the associated RNF4 binding ability. We altered the text of the paper to emphasise this point.

2. Figure 2: The patterns of sumoylated ETV4 look different from those in Figure 1C-E. The patterns with WT SUMO3 could be due to polysumoylation, but the bands seem to be rather discrete for this, and there are more than 4 bands even with K11R SUMO3, as seen in panel C. Admittedly this could be because a single SUMO at different sites in ETV4 results in a different gel mobility, but this issue ought to be discussed.

Response: The differences in mobility likely result from differences in the mobility of branched chain SUMO conjugates, as the size of the ETV4 construct varies. Fig. 1B contains Flag staged ETV4 but Fig. 1D contains GST-ETV4(1-335) and Fig. 1C, E and F contain GST-ETV4(full-length). As the referee points out, the different bands can be the result of multiple different combinations of SUMO modifications where either single or multiple SUMO molecules are attached. We have now discussed this more in the paper.

3. With regard to the topology of the binding of the RNF4 SIM array to SUMO chains or to multiple single SUMOs, further discussion of the structural insights into RNF4 SIM/SUMO interactions reported in Xu et al. (2014), and the importance of SIMs 2 and 3 in this interaction, is warranted.

Response: We have now added further discussion of this and how the structure reveals a more substantive role for SIM3 than previously appreciated.

Minor points:
1. In Figure 1A RNF4 is depicted with only 3 SIMs, possibly because ETV4 is shown with three SUMO sites or a SUMO chain with three residues. In contrast, RNF4 in Figure 2D is depicted with 4 SIMs in Figure 2D, which is the correct number of SIMs. I recommend changing Figure 1A to be consistent with Figure 2D. In addition, the legend to Figure 1 should indicate very clearly that the samples in panels C-E were obtained with ETV4 sumoylated in bacteria.

Response: We have now harmonised the two schematics to more accurately depict the interactions and protein structures under investigation. The legend to Fig. 1 has been modified to indicate the source of theSUMOylated GST-ETV4 proteins.

2. In the experiments in Figure 2, MBP-RNF4 was used. Was MBP-RNF4 also used in the experiments in Figure 1C-E. If so this should be indicated? If not, how was recombinant RNF4 purified for this experiment - only MBP-RNF4 is described in the Methods.

Response: MBP-RNF4 was used throughout and this is now clearly indicated in the legend to Fig.1.

Competing Interests: No competing interests were disclosed.