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The ubiquitin specific protease-4 (USP4) interacts with the S9/Rpn6 subunit of the proteasome

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ABSTRACT

The proteasome is the major non-lysosomal proteolytic machine in cells that, through degradation of ubiquitylated substrates, regulates virtually all cellular functions. Numerous accessory proteins influence the activity of the proteasome by recruiting or deubiquitylating proteasomal substrates, or by maintaining the integrity of the complex. Here we show that the ubiquitin specific protease (USP)-4, a deubiquitylating enzyme with specificity for both Lys48 and Lys63 ubiquitin chains, interacts with the S9/Rpn6 subunit of the proteasome via an internal ubiquitin-like (UBL) domain. S9/Rpn6 acts as a molecular clamp that holds together the proteasomal core and regulatory sub-complexes. Thus, the interaction with USP4 may regulate the structure and function of the proteasome or the turnover of specific proteasomal substrates.

1. Introduction

The active pool of a given protein is controlled by its rates of synthesis and degradation. The balance between these events is highly regulated and essential for the successful progression of cellular functions such as the cell cycle, signal transduction and gene expression [1]. The typical signal for targeting intracellular proteins for degradation is ubiquitylation [1]. The covalent linkage of ubiquitin to target proteins is performed by an enzyme cascade that includes a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a substrate-specific ubiquitin ligase (E3) that mediates the transfer of ubiquitin to the substrate [1].

Ubiquitylation is reversed by a family of cysteine- or metalloproteases collectively known as deubiquitylating enzymes (DUBs), of which approximately 80 members have been identified in the human genome [2]. DUBs have two main functions: they cleave ubiquitin monomers from ubiquitin precursors or ubiquitin chain conjugates, thus maintaining the pool of free ubiquitin available for conjugation, and they regulate the turnover of ubiquitin chains, thus controlling the fate of specific substrates and signaling pathways [3]. Compelling evidence indicates that at least part of the latter function occurs at the proteasome where proteasome-associated DUBs determine the time of residence and rate of substrate degradation by cleaving or trimming the ubiquitin chains [4].

The proteasome is a highly conserved multi-catalytic protease complex composed of a barrel-shaped 20S catalytic core with the proteolytic activities buried in the inside, and a 19S regulatory particle that serves as a gatekeeper for entry into the catalytic chamber. The 19S can be further divided into the 'base' and 'lid' sub-complexes. The base, located in closest proximity to the opening of the 20S cylinder, comprises six AAA-ATPases that mediate the unfolding and threading of substrates into the catalytic chamber, two scaffolding subunits S1 and S2 (known as Rpn1 and Rpn2 in yeast) and the ubiquitin receptors S5a/Rpn10 and ADRM1/Rpn13. The composition and function of the lid are less understood but it is believed to play key roles in the recognition of ubiquitylated substrates and regulation of the rate of proteolysis since it exerts both ubiquitin ligase and DUB activities [1].

Three DUBs are known to be associated with the mammalian proteasome: the constitutive lid subunit S13/Rpn11 and the transiently associated USP14/Ubp6 and UCH37, also known as UCHL5 [5]. These proteasome-associated DUBs have opposite effects on substrate degradation, while the S13 subunit promotes the degradation of ubiquitylated substrates [6,7], USP14 and UCH37 delay proteolysis [8,9].

Here we identify USP4, a multifunctional DUB with potential roles in cancer, as a proteasome interacting partner. We show that

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USP4 directly binds to the S9 subunit of the 19S regulatory particle through an N-terminal domain that resembles but is functionally distinct from the UBL domains of hHR23a/b and Ubiquilin-1. S9 is an essential subunit of the proteasome [10,11] and was recently shown to form a molecular clamp that holds together the 20S core and 19S, regulating thereby the structural integrity of the 26S proteasome [12–14]. Thus, USP4 may play a role in proteasome assembly or in the dynamics of substrate ubiquitylation at the proteasome.

2. Materials and methods

2.1. Plasmids and yeast-two hybrid screen

The N-terminal region of USP4 was cloned in-frame with the GAL4 DNA binding domain (DBD) and Myc tag in the yeast expression vector pGBKT7 (Clontech, Mountain View, CA, USA), generating the yeast two-hybrid bait pGBKT7–GAL4(DBD)–USP4(1–318)-Myc. The screen was performed using the Matchmaker™ pretransformed HeLa library (Clontech) according to manufacturer's protocol. GST-S9, GST-S9(1–142), GST-S9(143–422) and USP4-GST cloned in the pGEX-5X-1 plasmid (Amersham Biosciences, Buckinghamshire, UK) have been described previously [15]. His(6×)-USP4 cloned in pDEST17 plasmid was kindly provided by Rohan Baker (The Australian National University, Canberra, Australia). The USP4 mammalian expression has been previously described [15] and USP4 truncations USP4(1–318), USP4(1–220), USP4(1–120), USP4(1–963)(ΔUBL), USP4(UBL) were cloned in frame with a C-terminal Myc-tag in pCDNA4 (Invitrogen, Carlsbad, CA, USA). Myc-Ubiquilin-1 [16] and mHR23A-YFP were kindly provided by Nico Dantuma (Karolinska Institutet, Stockholm, Sweden).

2.2. Recombinant protein purification

GST or GST-fusion proteins were expressed in the *Escherichia coli* strain BL21. Bacteria cultured to an OD of 0.7 were induced for 5 h with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 27 °C. The cells were washed in ice-cold PBS, resuspended in buffer containing 50 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 0.5% IGEPAL supplemented with phenylmethylsulfonylfluorid (PMSF) (0.18 mg/ml) (Sigma–Aldrich, MO, USA) and protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland) and lysed by three cycles of freeze–thaw and sonication. The proteins were purified on glutathione sepharose according to manufacturer's protocol (Amersham Biosciences). USP4-His was expressed in *E. coli* strain BL21 and purified using His-trap columns (GE Healthcare, Little Chalfont, UK).

2.3. Antibodies and tissue culture

The following primary antibodies were used: anti-β-actin (AC-15), anti-GFP (sc-8334), anti-Myc (A14) and anti-Myc (9E10) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-S9 (A302–750A) from Bethyl Laboratories (Montgomery, USA), anti-S8 (Rpt6) and anti-20S core from Biomol (Exeter, UK), anti-His (Ab-1) from Oncogene (La Jolla, CA, USA), anti-USP4(188–200) from Sigma. Secondary antibodies for Western blot were donkey anti-rabbit or sheep anti-mouse conjugates (Zymed Laboratories Inc., San Francisco, CA, USA). The human cervical cancer cell line HeLa and the human embryonic kidney cell line HEK293T were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (v/v), 2 mM glutamine, and 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma). Transfections were performed using jetPEI (Polyplus Transfection, Illkirch, France) according to manufacturer's protocol or by the calcium phosphate method [17].

2.4. Immunoprecipitation, GST pull-down and in vitro binding assays

Co-immunoprecipitations were performed in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.05 mM EDTA, and 1% IGEPAL (Sigma) supplemented with protease inhibitor mixture cocktail (Roche Applied Science). For immunoprecipitations of 19S or 26S, the lysis buffer was complemented with 2 mM ATP (Sigma). The immunoprecipitation of FLAG-tagged proteins was performed with FLAG (M2) affinity gel (Sigma). For GST-pull-down, purified proteins were incubated with cell lysates for 3 h at 4 °C with gentle rotation and for *in vitro* binding studies purified proteins were mixed in buffer containing 50 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 0.5% IGEPAL. The unbound protein was washed away and specific interaction was detected by western blotting.

3. Results

3.1. USP4 interacts with the S9 subunit of the proteasome

USP4 is a multifunctional DUB that cleaves both Lys48 and Lys63 ubiquitin chains [15,18,19] and regulates various cellular functions including NF-κB and Wnt signaling, the activity of the tumor suppressor p53 and correct mRNA splicing [15,20–22]. In order to learn more about the activities of USP4 we endeavor to identify its interacting partners. To this end, the N-terminal domain of USP4 (amino acid 1–318) that precedes the conserved DUB-homology domain was used as bait in a yeast-2 hybrid (Y2H) screen (Supplementary Table S1). This region contains a DUSP (Domain present in Ubiquitin Specific Proteases) domain juxtaposed to an ubiquitin-like (UBL)-domain and a nuclear export signal (NES). The function of the DUSP and UBL domains are unknown while the NES works in concert with a C-terminal nuclear localization (NLS), enabling the nuclear-cytoplasmic shuttling of USP4 [23].

A total of 90 interactions were identified in the Y2H screen (Supplementary Table S1). The most frequent interaction was with LGALS1 but this was excluded from further analysis since it is often detected as a non-specific binding partner in this type of screen. The next most frequent interaction was observed with the S9 subunit of the proteasome. In order to validate the interaction, pull-down experiments were performed using purified recombinant GST-S9 and lysates of HEK293T cells transiently transfected with a USP4-Myc expressing plasmid. A robust interaction between USP4-Myc and GST-S9 was observed in western blots probed with the anti-Myc antibody (Fig. 1A) and similar results were obtained with lysates of cells overexpressing USP4-GFP (not shown). The finding was further supported by immunoprecipitation of the endogenous S9 in USP4-Myc transfected HE293T cells, which revealed efficient co-precipitation (Fig. 1B). The interaction was not dependent on the enzymatic activity of USP4 since the catalytic site mutant USP4 (C311S)-Myc co-immunoprecipitated with endogenous S9 with equal efficiency (not shown). To investigate whether the interaction is direct or dependent on accessory proteins that may be present in the cell lysates, *in vitro* binding assays were performed using purified GST, GST-S9 and His(6×)-USP4. As illustrated by the representative experiment shown in Fig. 1C, purified His(6×)-USP4 exclusively co-precipitated with GST-S9, confirming that the interaction is specific and direct.

3.2. USP4 interacts with the 26S proteasome

We next tested whether USP4 could interact with the assembled 26S proteasome. To this end, proteasomes were

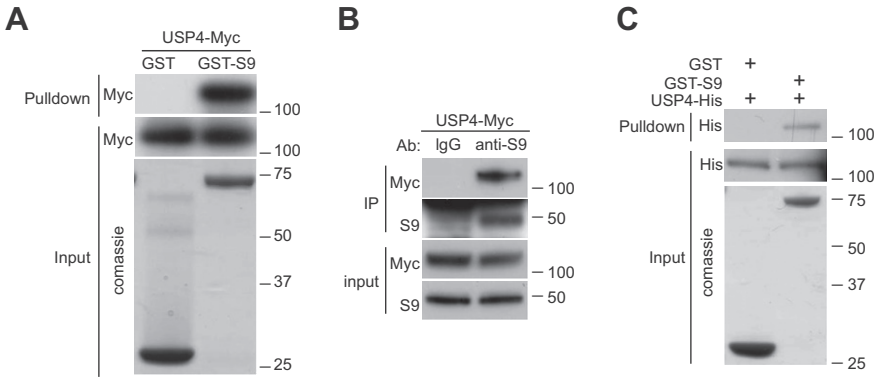

Fig. 1. USP4 interacts with the S9 subunit of the proteasome. (A) GST pull-down experiments showing the interaction between S9 and USP4. Purified GST and GST-S9 were used together with lysates of HEK293T cells overexpressing USP4-Myc. Input equals 5% of the total cell lysate. (B) Endogenous S9 interacts with USP4. S9 was precipitated from lysates of HEK293T cells overexpressing USP4-Myc and western blots were probed with S9 and Myc antibodies. (C) *In vitro* binding experiment showing direct interaction of USP4 with S9. Purified GST-S9 and USP4-His were mixed at 1:1 ratio. USP4-His(6×) was detected with an anti-His antibody.

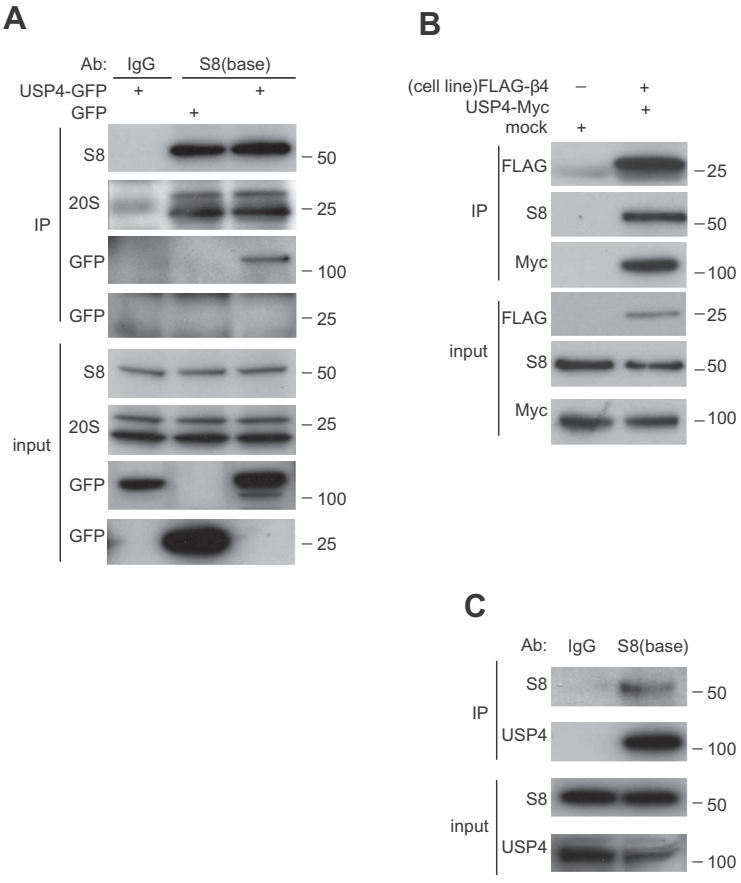

Fig. 2. USP4 associates with the 26S proteasome. (A) Proteasomes were immunoprecipitated from HEK293T cells expressing USP4-GFP or GFP with an antibody to the S8 subunit. Western blots were probed as indicated. Detection of the 20S core particle confirms the immunoprecipitation of intact proteasomes. (B) HeLa-S3 cells either stably expressing a FLAG-β4 subunit of the 20S, were transiently transfected with USP4-Myc and immunoprecipitation was performed using FLAG-affinity gel. The lysates were analyzed by western blot and probed as indicated. (C) Endogenous USP4 associates with the proteasome. The proteasome was immunoprecipitated with the S8 antibody and blots were probed with antibody specific for S8 and USP4.

immunoprecipitated form HEK293T cells using antibodies to the S8 ATPase that is localized in the base of the 19S. Immunoprecipitation of the entire proteasomes was confirmed by probing the blots with antibodies specific for the 20S core complex. Co-precipitation of transfected USP4-GFP was readily detected (Fig. 2A), whereas GFP alone was not present in the IP. In order to further confirm that USP4 interacts with the assembled 26S proteasome the immunoprecipitations were repeated in the HeLa-S3 cell line that stably expresses a FLAG-tagged β4 subunit of the 20S core. The cells were transiently transfected with USP4-Myc and immunoprecipitation was performed using FLAG affinity gel. Western blots of FLAG-IPs were probed with antibodies to the S8 subunit, which confirmed immunoprecipitation of the intact 26S (Fig. 2B). USP4-Myc was also readily detected in the blots confirming that USP4 interacts with the assembled proteasome. Finally we tested whether the interaction of USP4 with the proteasome also occurs under

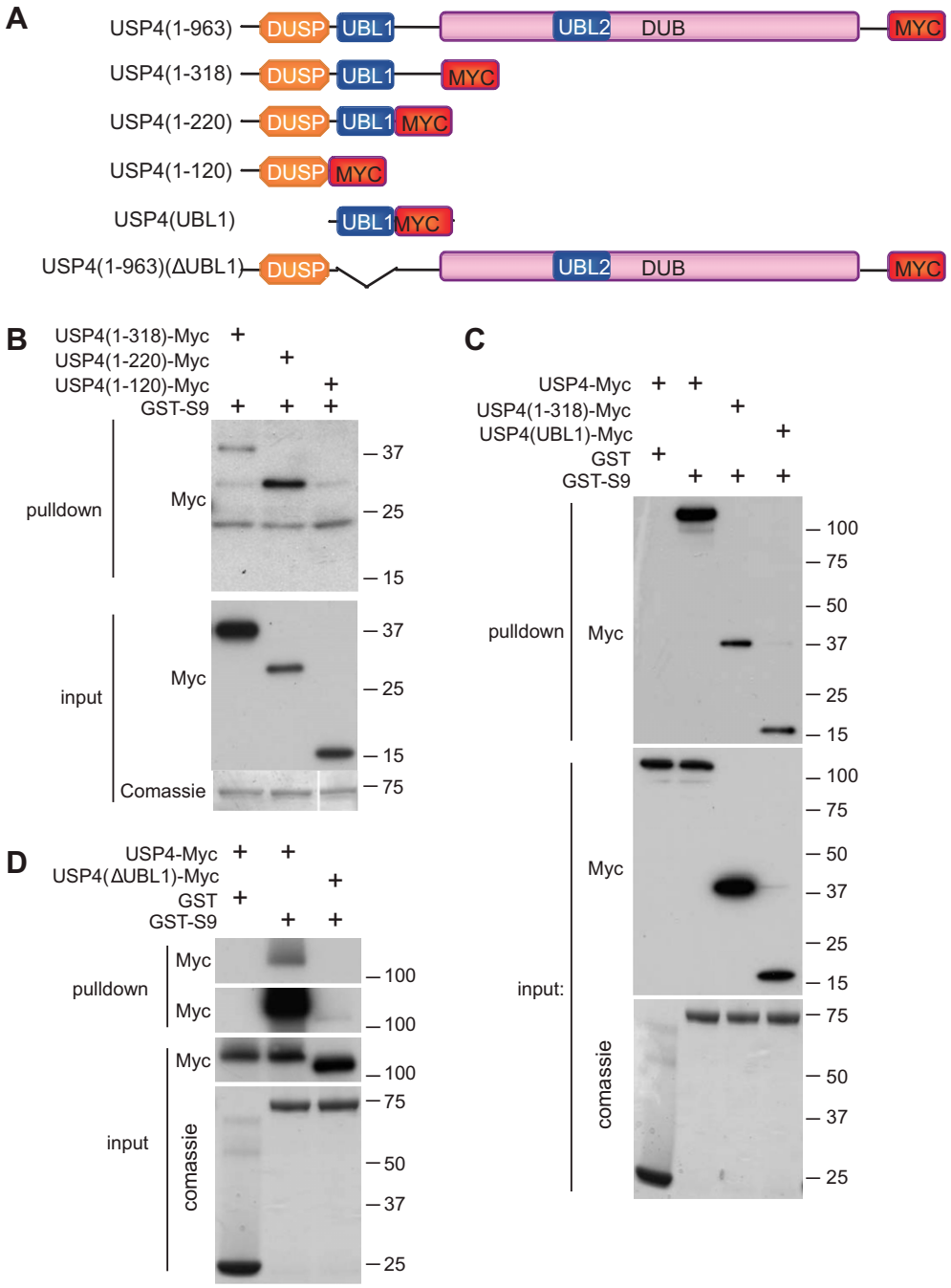

Fig. 3. The N-terminal UBL1 domain of USP4 interacts with S9. (A) Schematic illustration of the USP4 constructs used to identify the domain responsible for interaction with S9. The numbers in brackets indicate the amino acid position within USP4. (B) Deletion of the UBL1 domain abolishes binding to S9. Purified GST-S9 was used in pull-down assays together with lysates of HEK293T cells overexpressing sequential truncated USP4. Western blots were probed with an anti-Myc antibody. A Comassie stained gel confirming equal input of GST and GST-S9 is shown in the lower panel. (C) GST-pull-down experiments illustrating the interaction of the UBL1 domain of USP4 with GST-S9. The cells were pretreated over night with 10 μM of MG132 in order to accumulate detectable levels of USP4 (UBL1)-Myc. Western blots of input and pull-down samples were probed with an anti-Myc antibody. (D) GST-pull-down experiment illustrating requirement of the UBL1 domain for binding to S9. Purified GST and GST-S9 were used in pull-down experiment together with lysates of HEK293T cells overexpressing UBL1 deleted USP4. Western blots were probed with an anti-Myc antibody.

physiological levels of expression. As shown in Fig. 2C, endogenous USP4 was detected in complex with proteasomes precipitated from HEK293T cells using antibodies to the S8 subunit, supporting the physiological relevance of the interaction.

3.3. The UBL1 domain of USP4 specifically interacts with the N-terminus of S9

In order to map the region of USP4 involved in the interaction with S9, a series of Myc tagged USP4 deletion mutants were expressed in HEK239T and cell lysates were used in pull-down assays with purified GST-S9 or, as control, GST alone. USP4 contains two UBL domains. The C-terminal UBL2 domain is embedded in the catalytic domain and has autoregulatory functions [19], while the function of the N-terminal UBL1 domain is unknown. In the first set of experiments we tested the interaction of S9 with the N-terminus of USP4 that was used in the Y2H assays, USP4(1–318)-Myc, and with two sequentially truncated forms removing either the region downstream of the UBL1 domain, USP4(1–220)-Myc, or this region together with the UBL1 domain, USP4(1–120)-Myc,

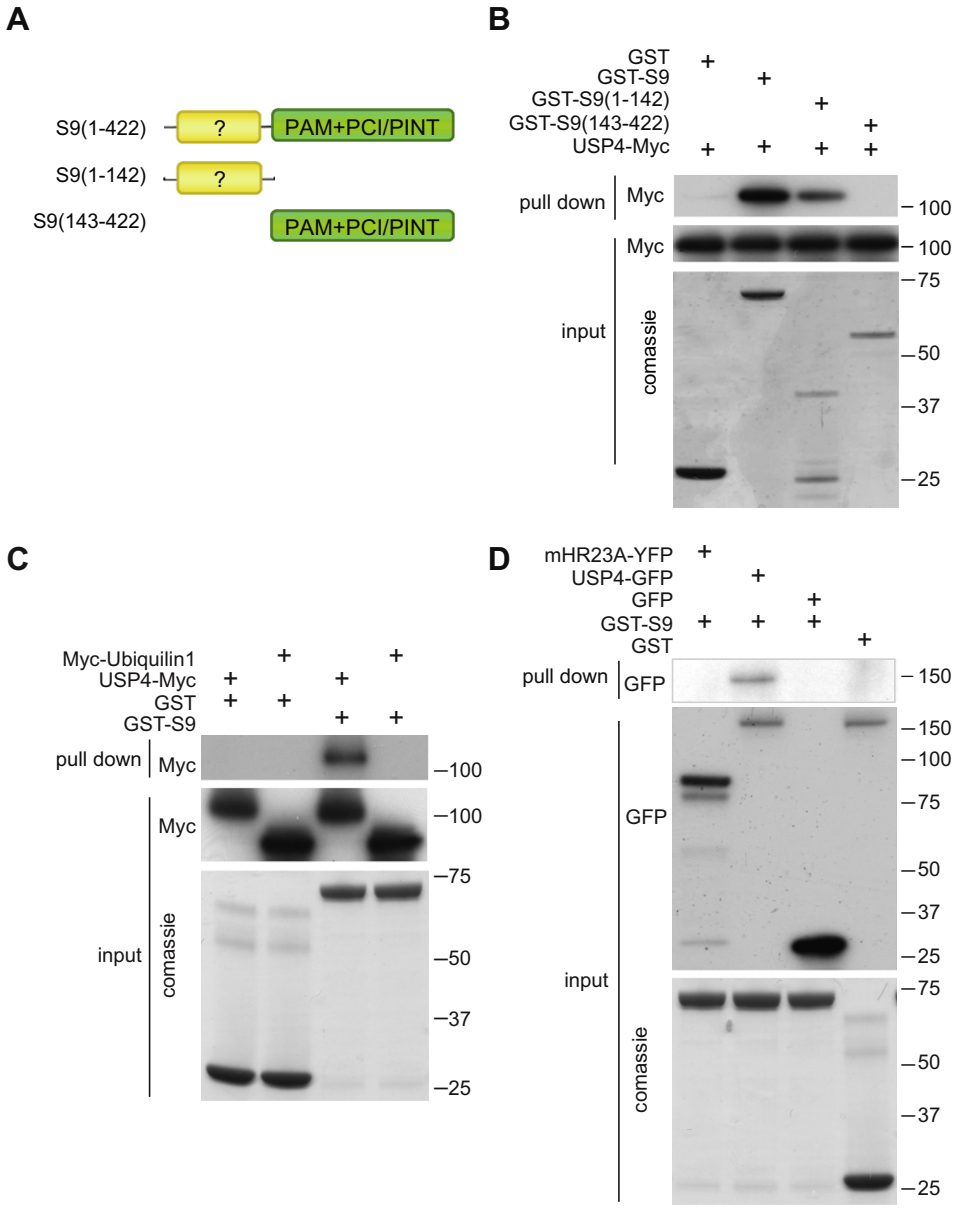

Fig. 4. USP4 interacts with the N-terminal region of S9. (A) Schematic illustration of the domain structure of S9 and the constructs generated in order to identify the region interacting with USP4. The PCI/PINT and PAM domain is indicated. (B) GST pull-down experiment demonstrating the interaction of USP4 with the N-terminus of S9. Purified GST, GST-S9, GST-S9(1–142) and GST-S9(143–422) were incubated with lysates of HEK293T cells overexpressing USP4-Myc. (C and D) The N-terminus of S9 does not interact with known UBL-containing proteins. Lysates of HEK293T cells transiently overexpressing USP4-Myc and Myc-Ubiquilin1 (C) or USP4-Myc and mHR23A-YFP (D) were incubated with purified GST or GST-S9. Western blots were probed with anti-Myc antibodies and the input GST and GST-S9 was detected in coomassie stained gels.

which leave intact the DUSP domain (Fig. 3A). As expected, interaction of GST-S9 with USP4(1–318) was readily detected. Deletion of the C-terminal 102 amino acids had no effect while the interaction was lost upon further deletion of the UBL1 domain (Fig. 3B). To further investigate the contribution of the UBL1 domain, pull-down assays were performed with either the Myc-tagged domain alone (Fig. 3C) or with an USP4 mutant lacking the UBL1 domain (Fig. 3D) alongside appropriate control. The transfected USP4(UBL1)-Myc polypeptide was highly unstable in cells (not shown) but interaction with GST-S9 was readily detected when the cells were pretreated overnight with 10 μM of the proteasome inhibitor MG132 (Fig. 3C). Furthermore, internal deletion of the UBL1 domain (USP4ΔUBL-Myc) prevented the interaction of USP4 with GST-S9 (Fig. 3D), indicating that the UBL1 is the only region of USP4 required for interaction with the proteasome.

The C-terminus of S9 contains a PCI-domain, also referred to as PINT motif (Proteasome, Int-6, Nip-1 and TRIP-15) [24] and a PAM domain (PCI associated module) (Fig. 4A). The PAM and PCI/PINT regions mediate protein–protein interactions and are present in six of the non-ATPase subunits of the 19S. The PCI/PINT motifs connect the six subunits in a horseshoe-like structure that sits slightly tilted on the top of the proteasome. To narrow down the region of interaction with USP4, the GST-S9 construct was divided in two parts containing either the N-terminal 142 amino, GST-S9(1–142), or the C-terminal PAM and PCI/PINT domains, GST-S9(143–422) (Fig. 4A). Pull-down assays performed with purified GST-S9 and lysates of USP4-Myc transfected HEK293T cells indicated that USP4 exclusively interacts with the N-terminus of S9 (Fig. 4B).

Since an UBL domain mediates the interaction of USP4 with S9, we surmised that the N-terminus of S9 might contain an UBL-binding domain. The UBL-binding domains of proteasome subunits S5a/Rpn10 or S2/Rpn1 act as docking sites for substrate chaperones such as Rad23 (also known as HR23A) and Ubiquilin-1 (also known

as Dsk2 or PLIC-1) [18]. Thus, although the N-terminus of S9 does not contain a canonical UBL-binding motif, we tested whether these UBL-containing proteins also interact with S9. To this end, purified GST-S9 was used in pull-down assays together with lysates of HEK293T cells overexpressing paired USP4-Myc and Myc-Ubiquilin-1 (Fig. 4C) or USP4-GFP and mHR23A-YFP (Fig. 4D) alongside appropriate controls. In both cases, co-precipitation was exclusively observed between S9 and USP4, which confirms the specificity of the interaction and suggests dependence on distinct features of the UBL1 domain of USP4 and the N-terminus of S9.

4. Discussion

The proteasome is a dynamic complex where numerous transiently interacting proteins contribute to the overall function of protein degradation. The identity and number of interacting partners vary depending on the method used for analysis [25]. Here we identify USP4 as a third proteasome-interacting DUB. The best-characterized proteasome-interaction motif is the UBL domain. While the amino acid composition of different UBL domains vary substantially, they typically share the β-grasp fold of ubiquitin [26]. Compelling evidence support a role of UBL domains in regulating the activity, specificity or interactions of several DUBs [27,28]. We have found the N-terminal UBL domain of USP4 binds to the N-terminus of the 19S proteasome subunit S9. This interaction is specific for USP4 and does not involve a conventional UBL-binding domain in S9.

The functional significance of the interaction between USP4 and S9 remains to be elucidated. The ubiquitylation status of proteasomal substrates is dynamic and it remains unclear how, and in which order, the substrate binds, is deubiquitylated and unfolded prior to degradation. Previously identified proteasome interacting DUBs, USP14/Ubp6 and UCH37, dock to the S1/Rpn1 and ADRM1/Rpn13 respectively and are strategically positioned in close proximity to the incoming ubiquitylated substrates [13]. Furthermore, their enzymatic activity is dramatically increased upon interaction with the proteasome [29,30], which is likely to restrict their function to the complex. Unlike these DUBs, the enzymatic activity of USP4 against fluorogenic substrates was not affected by the interaction with S9 (Supplementary Fig. S2), suggesting that USP4 is active also in the absence of the proteasome. This is well in line with previous studies that have documented the capacity of USP4 to control the activity of specific substrates and signaling pathways [15,20,21,31]. The site of interaction at the proteasome also suggests a different function compared to other proteasome associated DUBs. The Rpn6 subunit of the *Saccharomyces cerevisiae* and *Drosophila melanogaster* proteasome was recently shown to form a flexible finger that touches the α2 subunit of the 20S, facing away from the entrance of the catalytic chamber [12,13]. In agreement with this structure, functional studies suggest that Rpn6 stabilizes the 26S holocomplex [14,32]. It is tempting to speculate that the interaction of USP4 with this region of the proteasome could play a role in the maturation or stability of the 26S complex. Since binding to S9 was not dependent on the catalytic activity of USP4, this effect may not require the DUB function. It is also possible that the proteasome docking and DUB activities of USP4 may cooperate in regulating the turnover of specific substrates, or in view of the double specificity of USP4 for both Lys48 and Lys63 chains, the stability or function of larger protein complexes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.075.

References

[1] D. Finley, Recognition and processing of ubiquitin–protein conjugates by the proteasome, Annu. Rev. Biochem. 78 (2009) 477–513.
[2] S.M. Nijman, M.P. Luna-Vargas, A. Velds, T.R. Brummelkamp, A.M. Dirac, T.K. Sixma, R. Bernards, A genomic and functional inventory of deubiquitinating enzymes, Cell 123 (2005) 773–786.
[3] D. Komander, The emerging complexity of protein ubiquitination, Biochem. Soc. Trans. 37 (2009) 937–953.
[4] M.J. Lee, B.H. Lee, J. Hanna, R.W. King, D. Finley, Trimming of ubiquitin chains by proteasome-associated deubiquitinating enzymes, Mol. Cell Proteomics 10 (2011). R110 003871.
[5] A. Borodovsky, B.M. Kessler, R. Casagrande, H.S. Overkleeft, K.D. Wilkinson, H.L. Ploegh, A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14, EMBO J. 20 (2001) 5187–5196.
[6] T. Yao, R.E. Cohen, A cryptic protease couples deubiquitination and degradation by the proteasome, Nature 419 (2002) 403–407.
[7] R. Verma, L. Aravind, R. Oania, W.H. McDonald, J.R. Yates 3rd, E.V. Koonin, R.J. Deshaies, Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome, Science 298 (2002) 611–615.
[8] B.H. Lee, M.J. Lee, S. Park, D.C. Oh, S. Elsasser, P.C. Chen, C. Gartner, N. Dimova, J. Hanna, S.P. Gygi, S.M. Wilson, R.W. King, D. Finley, Enhancement of proteasome activity by a small-molecule inhibitor of USP14, Nature 467 (2010) 179–184.
[9] E. Koulich, X. Li, G.N. DeMartino, Relative structural and functional roles of multiple deubiquitylating proteins associated with mammalian 26S proteasome, Mol. Biol. Cell 19 (2008) 1072–1082.
[10] S. Lier, A. Paululat, The proteasome regulatory particle subunit Rpn6 is required for Drosophila development and interacts physically with signalosome subunit Alien/CSN2, Gene 298 (2002) 109–119.
[11] A. Saito, T.K. Watanabe, Y. Shimada, T. Fujiwara, C.A. Slaughter, G.N. DeMartino, N. Tanahashi, K. Tanaka, cDNA cloning and functional analysis of p44.5 and p55, two regulatory subunits of the 26S proteasome, Gene 203 (1997) 241–250.
[12] G.R. Pathare, I. Nagy, S. Bohn, P. Unverdorben, A. Hubert, R. Korner, S. Nickell, K. Lasker, A. Sali, T. Tamura, T. Nishioka, F. Forster, W. Baumeister, A. Bracher, The proteasomal subunit Rpn6 is a molecular clamp holding the core and regulatory subcomplexes together, Proc. Natl. Acad. Sci. USA 109 (2012) 149–154.
[13] G.C. Lander, E. Estrin, M.E. Matyskiela, C. Bashore, E. Nogales, A. Martin, Complete subunit architecture of the proteasome regulatory particle, Nature 482 (2012) 186–191.
[14] P.G. Santamaria, D. Finley, J.P. Ballesta, M. Remacha, Rpn6p, a proteasome subunit from Saccharomyces cerevisiae, is essential for the assembly and activity of the 26S proteasome, J. Biol. Chem. 278 (2003) 6687–6695.
[15] B. Zhao, C. Schlesiger, M.G. Masucci, K. Lindsten, The ubiquitin specific protease 4 (USP4) is a new player in the Wnt signalling pathway, J. Cell Mol. Med. 13 (2009) 1886–1895.
[16] J. Viswanathan, A. Haapasalo, C. Bottcher, R. Miettinen, K.M. Kurkinen, A. Lu, A. Thomas, C.J. Maynard, D. Romano, B.T. Hyman, O. Berezovska, L. Bertram, H. Soininen, N.P. Dantuma, R.E. Tanzi, M. Hiltunen, Alzheimer's disease-associated ubiquilin-1 regulates presenilin-1 accumulation and aggresome formation, Traffic 12 (2011) 330–348.
[17] R.E. Kingston, C.A. Chen, H. Okayama, Calcium phosphate transfection, Curr. Protoc. Immunol. (2001). Chapter 10:Unit 10.13.
[18] F. Zhou, X. Zhang, H.V. Dam, P.T. Dijke, H. Huang, L. Zhang, Ubiquitin-specific protease 4 mitigates Toll-like/interleukin-1 receptor signaling and regulates innate immune activation, J. Biol. Chem. (2012).
[19] M.P. Luna-Vargas, A.C. Faesen, W.J. van Dijk, M. Rape, A. Fish, T.K. Sixma, Ubiquitin-specific protease 4 is inhibited by its ubiquitin-like domain, EMBO Rep. 12 (2011) 365–372.
[20] X. Zhang, F.G. Berger, J. Yang, X. Lu, USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1, EMBO J. 30 (2011) 2177–2189.
[21] E.J. Song, S.L. Werner, J. Neubauer, F. Stegmeier, J. Aspden, D. Rio, J.W. Harper, S.J. Elledge, M.W. Kirschner, M. Rape, The Prp19 complex and the Usp4Sart3 deubiquitinating enzyme control reversible ubiquitination at the spliceosome, Genes Dev. 24 (2010) 1434–1447.
[22] N. Xiao, H. Li, J. Luo, R. Wang, H. Chen, J. Chen, P. Wang, Ubiquitin-specific protease 4 (USP4) targets TRAF2 and TRAF6 for deubiquitination and inhibits TNFalpha-induced cancer cell migration, Biochem. J. 441 (2012) 979–986.

[23] T.A. Soboleva, D.A. Jans, M. Johnson-Saliba, R.T. Baker, Nuclear-cytoplasmic shuttling of the oncogenic mouse UNP/USP4 deubiquitylating enzyme, J. Biol. Chem. 280 (2005) 745–752.
[24] K. Hofmann, P. Bucher, The PCI domain: a common theme in three multiprotein complexes, Trends Biochem. Sci. 23 (1998) 204–205.
[25] H.C. Besche, W. Haas, S.P. Gygi, A.L. Goldberg, Isolation of mammalian 26S proteasomes and p97/VCP complexes using the ubiquitin-like domain from HHR23B reveals novel proteasome-associated proteins, Biochemistry 48 (2009) 2538–2549.
[26] C. Grabbe, I. Dikic, Functional roles of ubiquitin-like domain (ULD) and ubiquitin-binding domain (UBD) containing proteins, Chem. Rev. 109 (2009) 1481–1494.
[27] X. Zhu, R. Menard, T. Sulea, High incidence of ubiquitin-like domains in human ubiquitin-specific proteases, Proteins 69 (2007) 1–7.
[28] A.C. Faesen, M.P. Luna-Vargas, T.K. Sixma, The role of UBL domains in ubiquitin-specific proteases, Biochem. Soc. Trans. 40 (2012) 539–545.
[29] D.S. Leggett, J. Hanna, A. Borodovsky, B. Crosas, M. Schmidt, R.T. Baker, T. Walz, H. Ploegh, D. Finley, Multiple associated proteins regulate proteasome structure and function, Mol. Cell 10 (2002) 495–507.
[30] T. Yao, L. Song, W. Xu, G.N. DeMartino, L. Florens, S.K. Swanson, M.P. Washburn, R.C. Conaway, J.W. Conaway, R.E. Cohen, Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1, Nat. Cell Biol. 8 (2006) 994–1002.
[31] I.Z. Uras, T. List, S.M. Nijman, Ubiquitin-specific protease 4 inhibits mono-ubiquitination of the master growth factor signaling kinase PDK1, PLoS One 7 (2012) e31003.
[32] E. Isono, N. Saito, N. Kamata, Y. Saeki, E.A. Toh, Functional analysis of Rpn6p, a lid component of the 26 S Proteasome, using temperature-sensitive Rpn6 mutants of the yeast Saccharomyces cerevisiae, J. Biol. Chem. 280 (2005) 6537–6547.