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An N-terminal SIAH-interacting motif regulates the stability of the ubiquitin specific protease (USP)-19

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ABSTRACT

The Ubiquitin Specific Protease-19 (USP19) regulates cell cycle progression and is involved in the cellular response to different types of stress, including the unfolded protein response (UPR), hypoxia and muscle atrophy. Using the unique N-terminal domain as bait in a yeast-two hybrid screen we have identified the ubiquitin ligases Seven In Absentia Homolog (SIAH)-1 and SIAH2 as binding partners of USP19. The interaction is mediated by a SIAH-consensus binding motif and promotes USP19 ubiquitylation and proteasome-dependent degradation. These findings identify USP19 as a common substrate of the SIAH ubiquitin ligases.

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1. Introduction

Post-translational modification of proteins by covalent attachment of ubiquitin regulates their stability, traffic and function in fundamental biological processes such as the cell cycle, DNA repair, transcription, and signal transduction. Ubiquitylation is mediated by the sequential action of three enzymes: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a substrate specific ubiquitin ligase (E3) that recognizes its targets via unique amino acid sequences known as degrons [1]. The conjugation reaction is reversed by deconjugases, known as de-ubiquitylating enzymes (DUBs) that, like the E3s, specifically recognize their substrates. The DUBs process ubiquitin precursors and edit or remove ubiquitin chains generating free ubiquitin, thus contributing to ubiquitin homeostasis and regulating the fate of ubiquitylated proteins [2]. The human genome encodes approximately 98 putative DUBs that have been grouped into six families according to the sequence similarities of their ubiquitin protease domains [3]. The members of these families are highly diverse, which underlies their specificity for different substrates and participation in several cellular functions. Efforts towards the identification of DUB binding partners have revealed numerous substrates, modifiers and scaffolds, and have provided insights on their functions and mode of regulation [2,4,5]. Several DUBs are found in association with E3 ligases that have an intrinsic capacity for self-ubiquitylation. Hence, the de-ubiquitylation and stabilization of E3 ligases is likely to be a major aspect of DUB physiology. The E3s may, in turn, destabilize their cognate DUB through ubiquitylation, or the two enzymes may act together in fine-tuning the ubiquitylation of common substrates [2,6].

USP19 is a 150 kDa enzyme that belongs to the largest family of DUBs, the ubiquitin-specific proteases (USPs). In vitro, it shows preference towards Lys63-linked ubiquitin chains over Lys48-linked chains, which may indicate the involvement in non-proteolytic signaling events [7]. USP19 is strongly induced during muscle atrophy but the mode of regulation and substrates targeted in this process remain unknown [8]. The DUB activity has been implicated in the regulation of diverse cellular functions. The active enzyme stabilizes the E3 ligase KPC1, which promotes the proliferation of rat fibroblasts [9]. During hepatitis C virus (HCV) infection, re-localization of USP19 to the virus replication compartments hampers its ability to rescue proteasomal substrates. This is dependent on the interaction of USP19 with the non-structural proteins NS5A and is likely to mediate the cell-proliferation-inhibitory properties of NS5A since infection with a recombinant virus lacking the USP19 interaction site failed to decrease cell growth [10]. In addition, USP19 has been implicated in several non-catalytic functions. The inactive enzyme stabilizes the inhibitors of apoptosis c-IAP1 and c-IAP2 [11]. Furthermore, we have previously described a non-catalytic effect of USP19 in the rescue of endoplasmic reticulum

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associated degradation (ERAD) substrates [12] and in the stabilization of components of the hypoxia pathway, including HIF-1α, which may explain the failure of cells lacking USP19 to mount an appropriate response to hypoxia [13]. Collectively, these findings highlight the participation of this DUB in the regulation of a broad variety of diverse physiological and pathological processes.

Similar to many DUBs, USP19 can regulate its own ubiquitylation [11], but additional mechanisms involved in the transcriptional and post-translational regulation remain largely unknown. Splice variants of the human USP19 gene code for at least four isoforms, some of which lack the C-terminal transmembrane domain that anchors the protein to the ER membrane [12]. In addition, USP19 is phosphorylated by ATM or ATR in response to DNA damage [14].

In an effort to gain insight in the function or regulation of USP19 we have performed a yeast-two hybrid (Y2H) screen using as bait its unique N-terminal domain. Here we report that USP19 interacts with the ubiquitin ligases SIAH1 and SIAH2, which promote USP19 ubiquitylation and degradation by the proteasome. Bioinformatics and biochemical analysis revealed the USP19 contains a SIAH-specific degron that is found in several SIAH substrates. Thus, the interaction between SIAHs and USP19 may reciprocally regulate the activity and specificity of the enzymes.

2. Materials and methods

2.1. Cell culture and reagents

The cell lines HEK293T and HeLa (American Type Culture Collection ATCC, Teddington, UK) were maintained at 37 °C and 5% CO_2 in Dulbecco's modified eagles medium supplemented with 10% FCS (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich, St. Louis, MO, USA), and 2 mM glutamine. Transient transfections of plasmid DNA were performed using the Jet-PEI kit (Polyplus Transfection, Illkirch, France) according to manufacturer's protocol, or by calcium phosphate precipitation. When indicated, proteasomal degradation was blocked by treating the cells for indicated time with 10 μM of the proteasome inhibitor MG132 (Enzo Life Sciences, Lausen, Switzerland).

2.2. Plasmids and yeast two-hybrid screen

Plasmids expressing Myc-USP19, Myc-USP19^{C506S} and USP19-GFP have been previously described [12]. A deletion of the predicted SIAH interacting motif in USP19 was generated using the primers: (anti-sense) 5′GAT**GAATTC**ACCATGGAACAAAAACTCA3′ (EcoRI site in bold), (sense) 5′AACTGGGCT-CGAGGCCAGGTGTGTCTC3′, (anti-sense) 5′GCCC**TCTAGA**CTTCATCTCCAGCGACTCTGGGATAC3′ (XbaI site in bold), (sense) 5′CTGGCCTCG-AGCCCAGTTAGTGGAGACAG3′. The PCR product was cloned in the EcoRI-HF (High-Fidelity) (New England Biolabs NEB) and XbaI (NEB) sites of pCDNA4B generating pCDNA4B-Myc-USP19 (Δ462–473). The QuickChange II site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA) was used to generate Ala mutations of the Val468 and Pro470 residues in the predicted SIAH interacting motif using the primers (sense) 5′AGCCTACATGCATGGCGCCTGCC ATGCCCCACAGCC and (anti-sense) 5′GGCTGTGGGGCATGGCAGGCGCC ATGCATGTAGGCT. Plasmids encoding HA-SIAH1 and HA-SIAH1ΔRING were gifts from Solomon H. Snyder (Johns Hopkins University School of Medicine, Baltimore, USA). Plasmid encoding HA-SIAH2 was provided by Ze'ev Ronai (Burham Institute for Medical Research, USA). Plasmids encoding FLAG-SIAH2 and FLAG-SIAH2ΔRING was provided by Chris Pugh (University of Oxford, UK). The yeast two-hybrid bait construct pGBKT7-GAL4(DBD)-USP19 (1–495aa)-Myc that contains the first 1485 nucleotides of USP19 cloned in-frame with the GAL4 DNA binding domain (DBD) and Myc tag in the yeast expression vector pGBKT7 (Clontech) was described previously [13]. The yeast two-hybrid screen was performed using the Matchmarker™ pre-transformed HeLa library (Clontech, Mountain View, CA, USA) according to manufacturer's protocol.

2.3. Immunoprecipitations and immunoblotting

The procedures for co-immunoprecipitations and Western blot analysis were performed as previously described [13]. The following antibodies were used: anti-FLAG (M2, Sigma), anti-β-actin (AC-15, Sigma), anti-Myc (9E10 or A14, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-USP19 (A301-586A or A301-587A, Bethyl Laboratories Inc., Montgomery, TX, USA), anti-HA tag (12CA5, Abcam, Cambridge, MA, USA for immunoblotting; H-6908, Sigma for immunoprecipitation), anti-GFP (FL) (sc-8334, Santa Cruz Biotechnology Inc.), anti-Ubiquitin (Z0458, Dako, Denmark), donkey anti-rabbit and sheep anti-mouse horse radish peroxidase (HRP)-conjugated (Zymed Inc., South San Francisco, CA, USA). Immunocomplexes were detected by enhanced chemiluminescence (Amersham ECL Prime, GE Healthcare) or Super Signal West Dura Extended Duration Substrate (Thermo Scientific Inc., Waltham, MA, USA).

2.4. Bioinformatics analysis

Sequence patterns and hidden Markov model (HMM) profiles were generated using the 12 amino acids long SIAH binding motifs of the known interacting partners: SIAH interacting protein (SIP), disable homolog-1 (DAB-1), kinesin like DNA binding protein (Kid), Numb, paternally express-10 (PEG-10), VAV1 guanine nucleotide exchange factor, deleted in colorectal carcinoma (DCC), BOB.1/OBF.1 coactivator, TGFβ-inducible early gene-1 (TIEG1), elongation factor-1 delta subunit, EF1-Δ, nuclear receptor corepressor (N-CoR), SIAHBP1/FIR peptide, apoptosis-related protein in the TGFβ signaling pathway (ARTS) and the infected cell polypeptide-0 (ICP0) from herpes simplex virus (HSV)-1 and HSV2. HMM was constructed using the HMMER suite of programs [15] to generate a statistical profile of SIAH binding sequences.

3. Results

3.1. USP19 interacts with SIAH ubiquitin ligases

A yeast-two-hybrid screen was performed to gain insight in the molecular interactions that regulate the functions of USP19. As bait we used the unique 495 amino acids long N-terminal region, which excludes the conserved USP domain (Fig. 1). The only defined domain in this region is a bipartite CHORD-containing proteins and SGT1 (CS) domain that is found in co-chaperones of the heat shock protein HSP90 [16] and mediates protein–protein interactions [17]. The screen identified 10 putative USP19 interacting proteins, of which three, SIAH1, SIAH2, and RING2, are E3 ubiquitin ligases (Table 1).

SIAH1 and SIAH2 are highly conserved RING-type ubiquitin ligases that have been implicated in a variety of signaling pathways and stress-related cellular events, including mitosis [18], the p53 responses [19], the response to hypoxia [20] and cancer [21]. Although SIAH1 and SIAH2 share some interacting partners, they are functionally distinct, which is probably due to their divergent N-terminal region [22]. To assess whether USP19 interacts with SIAH1 and SIAH2 in eukaryotic cells, HEK293T cells were co-transfected with plasmids expressing Myc-USP19 and HA-SIAH1 or HA-SIAH2 and cell lysates were subjected to immunoprecipitation with an anti-Myc antibody. Since the SIAH ligases are highly

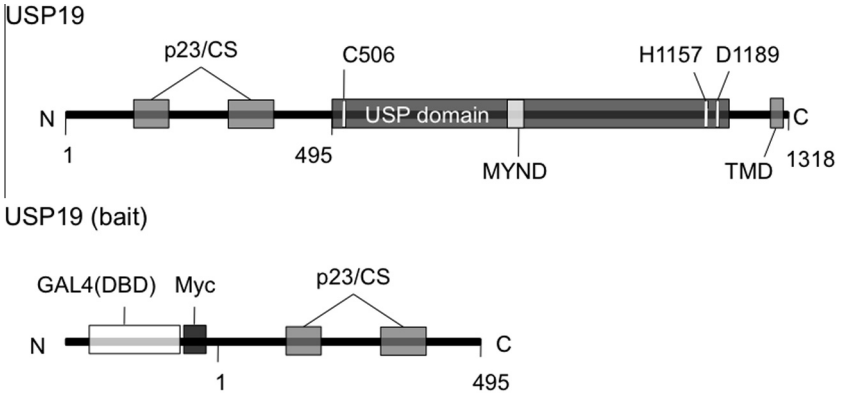

Fig. 1. Schematic illustration of the known protein domains of USP19 and of the N-terminal fragment used as bait in the yeast two-hybrid screen. The N-terminal region of USP19 harbors two p23-like CHORD and Sgt1 (p23/CS) domains. A MYND domain (myeloid translocation protein 8, Nervy, and DEAF-1) and the catalytic triad formed by the C506, H1157 and D1189 residues are found in the C-terminal USP domain. The transmembrane domain (TMD) is located close to the C-terminus.

unstable due to auto-ubiquitylation mediated by the RING domains [23], their accumulation was induced by treating the transfected cells with 10 μM of the proteasome inhibitor MG132 for 6 h before harvesting (Fig. 2A). Probing Western blots of anti-Myc immunoprecipitates with an anti-HA antibody confirmed that USP19 interacts with both SIAH1 and SIAH2 (Fig. 2B). Similar results were obtained when the SIAH ligases were stabilized by removal of the RING domain (Fig. 2C). Furthermore, comparable levels of co-immunoprecipitations were observed in cells expressing the USP19 catalytic mutant Myc-USP19^{C506S}, indicating that the E3 ligase and DUB activities are not required for the interaction (Fig. 2C).

Next, we investigated whether the interaction occurs at physiological levels of USP19 expression. Due to the insufficient levels of endogenous SIAH1 and SIAH2, immunoprecipitation experiments were performed in HEK293T cells expressing the stable RING-deletion mutants HA-SIAH1ΔRING or FLAG-SIAH2ΔRING. A specific interaction between the two SIAH ligases and endogenous USP19 was detected in immunoprecipitates performed with either the anti-HA or anti-FLAG antibodies (Fig. 2D). It is noteworthy that the endogenous USP19 appears as multiple bands in Western blots, which reflects the expression of different splice variants of this protein. One USP19 species of approximately 100 kDa, was highly enriched in immunoprecipitates containing HA-SIAH1ΔRING or FLAG-SIAH2ΔRING. Taken together, these results identify USP19 as a binding partner of both the SIAH1 and SIAH2 ligases and point to a specific splice variant of USP19 as the preferential partner in the interaction.

Table 1
USP19 interacting partners identified in the yeast-2-hybrid screen.

Gene	Protein name	Hits
NM_003031	E3 ubiquitin-protein ligase SIAH1	16
NM_001456	Filamin-A (FLN-A)	9
NM_005067	E3 ubiquitin-protein ligase SIAH2	5
NM_001008395	UPF0539 protein C7orf59	4
NM_014756	Cytoskeleton-associated protein 5 (Ch-TOG)	2
NM_004826	Endothelin-converting enzyme-like 1 (Xce protein)	2
NM_007212	E3 ubiquitin-protein ligase RING2	1
NM_014014	U5 small nuclear ribonucleoprotein 200 kDa helicase	1
NM_031266	Heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B)	1
NW_001838958	Homo sapiens chromosome 5 genomic contig, alternate assembly HuRef SCAF_1103279179917	1
	Total	42

3.2. SIAH regulates the stability of USP19

Numerous examples of interactions between E3 ligases and DUBs have been reported. Functionally, the enzymes may reciprocally regulate their activities, with subsequent effects on their specific targets, or they can oppose each other's activity on common substrates [2,6,9,11]. To investigate whether the interaction with the SIAHs target USP19 for proteasomal degradation, HEK293T cells were co-transfected with Myc-USP19 and HA-SIAH1 or HA-SIAH2 expressing plasmids in the presence or absence of the proteasome inhibitor MG132. Co-expression of functional SIAH1 or SIAH2 dramatically decreased the expression levels of Myc-USP19, while the expression was rescued by in treatment with MG132, suggesting that both SIAHs target USP19 for proteasomal degradation (Fig. 3A). In line with this possibility, the reduction of Myc-USP19 was not observed in cells co-transfected with the inactive HA-SIAH1ΔRING or FLAG-SIAH2ΔRING mutants, confirming that the effect is dependent on the ligase activity (Fig. 3B). To directly probe the capacity of SIAHs to ubiquitylate USP19, immunoprecipitation of USP19-GFP was performed from cell extracts co-expressing either the wild-type or RING-mutants and Western blots were probed with ubiquitin specific antibodies. High molecular weight species corresponding to poly-ubiquitylated USP19-GFP were readily detected in the immunoprecipitates of cells expressing the wild-type E3s but not the RING mutants (Fig. 3C), indicating that USP19 is a bona fide SIAH substrate. To test whether the regulation of USP19 by SIAH is true also for the endogenous proteins, SIAH1 or SIAH2 were over-expressed in HEK293T cells and western blots of total cell lysates were probed with the USP19 specific antibody (Fig. 3D). As described previously (Fig. 2A), the HA-SIAHs were barely detectable. Nevertheless, the endogenous USP19 was dramatically reduced in the presence of both SIAH1 and SIAH2 supporting the conclusion that USP19 is a true target of SIAHs.

3.3. USP19 contains a SIAH degron

Several SIAH-interacting proteins contain a conserved PxAxVxP motif that may function as a specific degron [24]. To investigate whether the motif is present in USP19, we performed a HMM search based on the sequence of the binding domains of known SIAH interacting partners. This analysis revealed that USP19 contains a putative SIAH-binding domain characterized by the presence of the conserved core VxP motif (USP19-Val468 and Pro470) and other key residues, such as a Pro residue in position 464 and positively charged Lys and His residues in position 463 and 473, respectively (Fig. 4A).

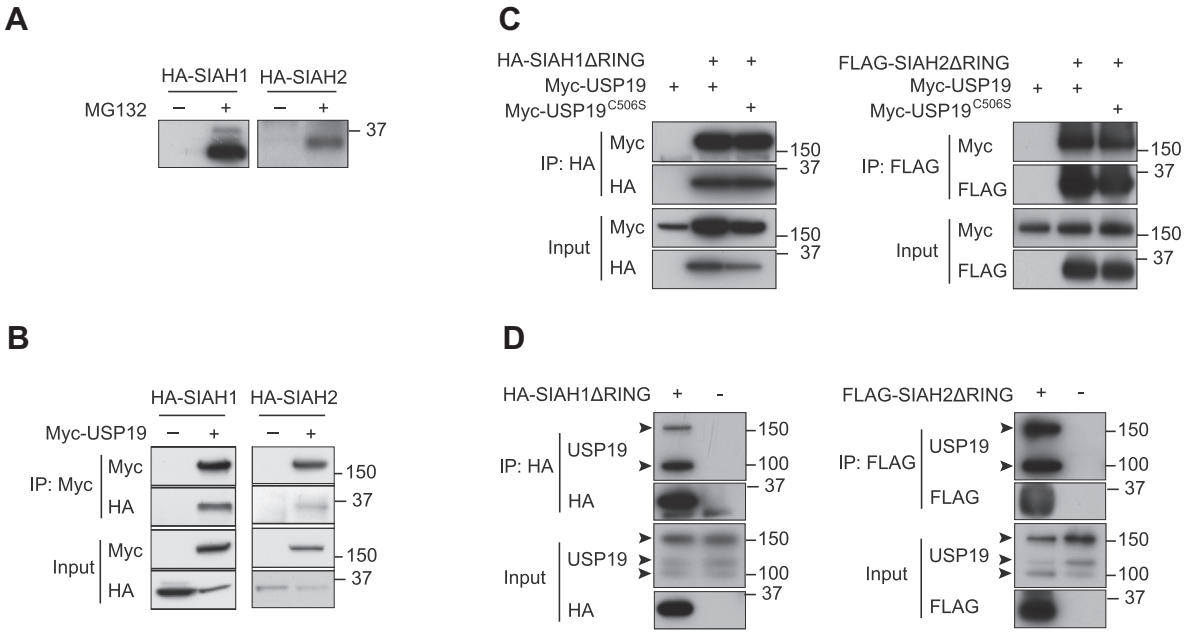

Fig. 2. USP19 interacts with SIAH1 and SIAH2. (A) SIAH1 and SIAH2 are degraded by the proteasome. HEK293T cells expressing HA-SIAH1 or HA-SIAH2 were treated with 10 μM MG132 (overnight), subjected to western blot and probed as indicated. (B) USP19 interacts with SIAH1 and SIAH2. Lysates of HEK293T cells expressing Myc-USP19 and HA-SIAH1 (left panel) or HA-SIAH2 (right panel) were immunoprecipitated with anti-Myc antibodies. The cells were pretreated overnight with 10 μM of MG132. One representative experiment out of three is shown. (C) The catalytic activity of USP19 is not required for interaction with SIAH. Myc-USP19 or the catalytic mutant Myc-USP19^{C506S}, were co-expressed in HEK293T cells with HA-SIAH1ΔRING (right panel) or FLAG-SIAH2ΔRING (left panel). Co-immunoprecipitation and western blots were performed as indicated. One representative experiment out of three is shown. (D) Endogenous USP19 interacts with SIAH. HA-SIAH1ΔRING (left panel) or FLAG-SIAH2ΔRING (right panel), were transiently expressed in HEK293T cells and immunoprecipitation was performed with anti-HA or anti-FLAG antibodies, respectively. The co-immunoprecipitated USP19 was detected using a USP19 specific antibody. Multiple USP19 isoforms detected by the antibody are indicated.

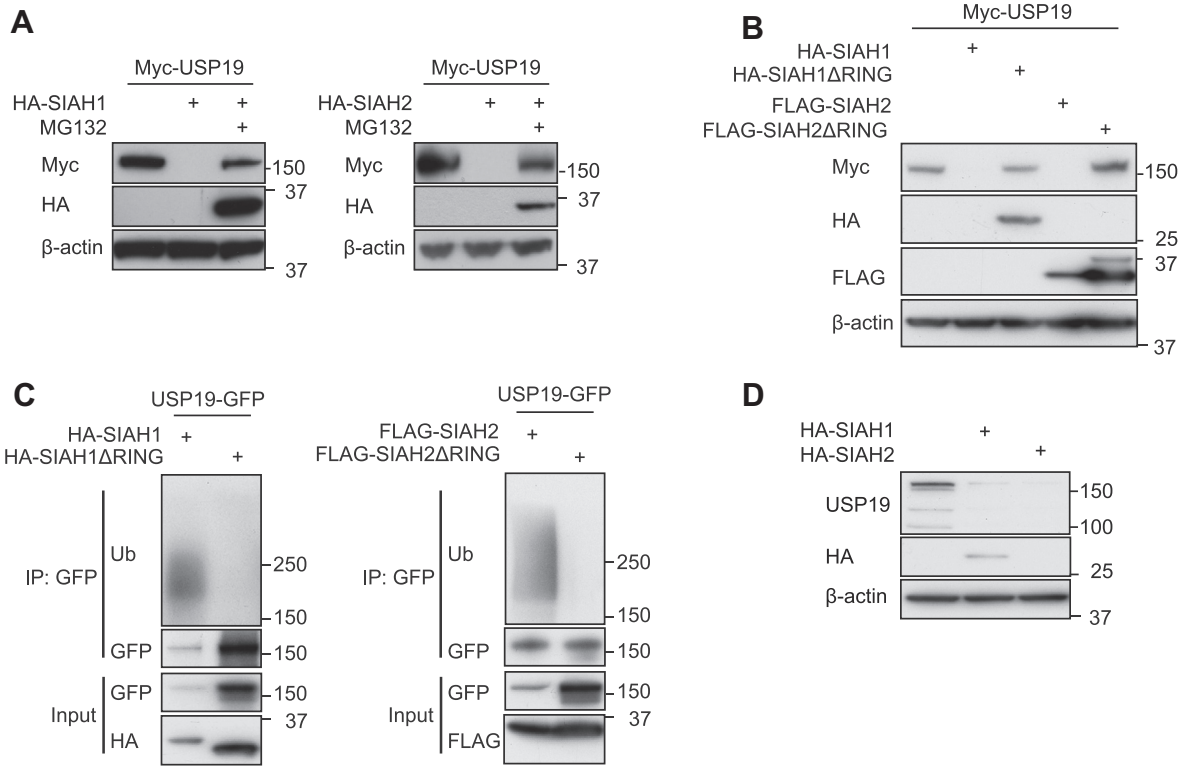

Fig. 3. SIAH1 and SIAH2 target USP19 for proteasomal degradation. (A) SIAH promotes the degradation of USP19 by the proteasome. Western blot of HEK293T cells co-expressing Myc-USP19 and HA-SIAH1 (left panel) or HA-SIAH2 (right panel) in the presence or absence of 10 μM of MG132. (B) The RING domain of SIAH is required for USP19 degradation. Western blot of HEK293T cells expressing Myc-USP19 and the wild-type or RING-deletion mutant SIAH1 and SIAH2. (C) USP19 is a SIAH substrate. USP19-GFP was immunoprecipitated from HEK293T cells co-expressing the wild-type or RING-deleted mutant SIAH1 (left panel) or SIAH2 (right panel). The cells were pretreated for 4 h with 10 μM of MG132. Western blots were probed with antibodies against ubiquitin, GFP, FLAG and HA as indicated. (D) Endogenous USP19 is degraded in SIAH overexpressing cells. Western blots HEK293T cells expressing HA-SIAH1 or HA-SIAH2 were probed with the USP19 specific antibody.

To test whether the predicted SIAH-binding motif mediates the interaction, the region was deleted from wild-type USP19 generating the Myc-USP19$^{\Delta 462-473}$ mutant. Anti-FLAG immunoprecipitation of lysates of HEK293T cells co-transfected with plasmids expressing FLAG-SIAH2ΔRING and Myc-USP19 or Myc-USP19$^{\Delta 462-473}$, revealed that the Myc-USP19$^{\Delta 462-473}$ mutant does not co-precipitate with SIAH2, thus confirming the location of the interacting domain within amino acids 462–473 of USP19 (Fig. 4B). Co-precipitation was also abolished by mutation of Val468 and Pro470 to Ala, which further supports the involvement of the conserved residues in mediating the interaction (Fig. 4C).

We finally tested whether the SIAH-binding motif regulates the stability of USP19. To this end, the expression levels of Myc-USP19 and Myc-USP19$^{\Delta 462-473}$ were compared in Western blots of HEK293T cells co-transfected with plasmids expressing the wild type HA-SIAH1 or HA-SIAH2 (Fig. 4D). The expression levels of Myc-USP19$^{\Delta 462-473}$ remained constant in spite of SIAH overexpression while Myc-USP19 was efficiently degraded, confirming that the SIAH-binding motif is required for USP19 ubiquitylation and subsequent degradation.

4. Discussion

We have shown that the E3 ubiquitin ligases SIAH1 and SIAH2 promote the ubiquitylation and proteasomal degradation of USP19 and we have identified a putative SIAH-specific degron in USP19 that is conserved in many SIAH substrates.

The capacity of certain DUBs to specifically interact with ubiquitin ligases is well documented [2,6]. Depending on the interacting partners, the outcome varies from a reciprocal or unidirectional regulation of protein stability, to fine-tuning of the stability or function of common substrates [6]. We have documented the capacity of SIAHs to target USP19 for proteasomal degradation but we did not observe stabilization of SIAHs by USP19. This does not exclude that a reciprocal regulation might occur in particular physiologic or pathologic conditions, for example during the response to hypoxia or in muscle atrophy where the involvement of both USP19 and SIAH was documented.

The interaction with SIAH is likely to have important effects on the downstream targets of USP19. Indeed, both SIAHs and USP19 can associate with a large variety of proteins that participate in different signaling pathways [8–10,12,13,21], are involved in the response to different types of stress [12,13,25], and are expressed in many tissues, with coincidental higher levels of expression in skeletal muscle and testis [8,26]. It is noteworthy that, in our experiments, SIAH1 and SIAH2 appeared to preferentially interact with a 100 kDa isoforms of USP19 (Fig. 2D). Very little is known about the function, cellular expression and regulation of different USP19 isoforms but all of them are likely to include the N-terminal SIAH-interaction motif. Thus, the interaction with SIAHs may have different consequences depending on the cell type, subcellular localization or post-translation modification of the two proteins [27].

Although SIAH and USP19 act on common substrates and pathways, their effects are often different. For example, both regulate the stability of the cyclin-dependent kinase inhibitor p27^{Kip1}. However, while USP19 regulates the function of p27^{Kip1} in the cell cycle [9], SIAH1 modulates the effect of p27^{Kip1} on cell migration [28], suggesting that they may act on different pools of the protein. Both

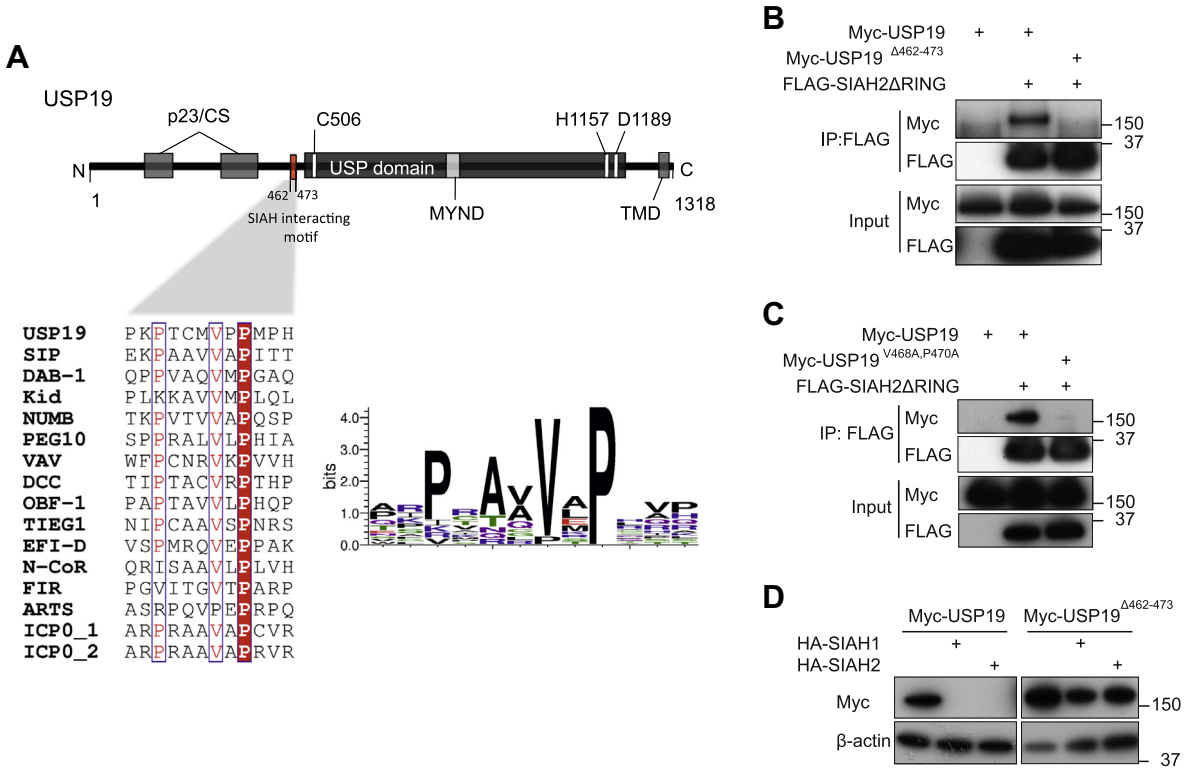

Fig. 4. USP19 contains a SIAH interacting motif. (A) The N-terminus of USP19 contains a putative SIAH-interacting motif. Sequence alignment of USP19 with a selection of previously identified SIAH interacting partners and sequence logo of the conserved region. Key residues of the consensus SIAH-binding motif PxAxVxP are highlighted in red. (B) FLAG immunoprecipitation of HEK293T cells expressing FLAG-SIAH2ΔRING and Myc-USP19 or the Myc-USP19$^{\Delta 462-473}$ mutant. Western blots were probed as indicated. (C) Point mutations of the conserved Val468 and Pro470 residues in the SIAH-interacting motif abrogate the interaction of USP19 with SIAH. FLAG immunoprecipitation on HEK293T cells expressing FLAG-SIAH2ΔRING and Myc-USP19 or the Myc-USP19^{V468A,P470A} mutant. Western blots were probed as indicated. (D) Deletion of the SIAH-interacting motif stabilizes USP19. HEK293T cells expressing indicated combinations of Myc-USP19, Myc-USP19$^{\Delta 462-473}$ HA-SIAH1 and HA-SIAH2 were analyzed in Western blots probed with antibodies to the HA or Myc tags as indicated. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

USP19 and SIAH1 interact with the inhibitors of apoptosis (IAPs) but SIAH1 targets XIAP for destruction [29], while USP19 stabilizes c-IAPs and to a minor extent XIAP [11]. Furthermore, both stabilize the key player of the hypoxic response HIF-1α [13]. SIAH2 promotes the degradation prolyl-hydroxylases (PHDs) that target HIF-1α for ubiquitination by VHL [27] whereas the targets of USP19 in this pathway are unknown. Interestingly, while the effect of SIAH on HIF-1α was observed under mild hypoxic conditions [27], the effect of USP19 was demonstrated during severe hypoxia when the regulation of response is likely to be different [13]. Thus, the destabilizing effect of SIAHs on USP19 may produce different outcomes dependent on the cell type and function involved.

It is noteworthy that, in addition to SIAH1 and SIAH2, our yeast-two hybrid screen identified a third E3 ligase, RING2, as a potential interacting partner of USP19. RING2 is a subunit of the Polycomb group (PcG) multi-protein PRC1-like complex that monoubiquitylates histone H2A and regulates thereby DNA replication, transcription and repair [30]. Thus, the involvement of USP19 in a multitude of diverse cellular processes may be explained by its capacity to interact with many different E3 ligases.

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