The Substrate Binding Domains of Human SIAH E3 Ubiquitin Ligases are Now Crystal Clear

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Running title: Substrate binding domains of SIAH E3 ligases

Abstract

Background: Seven *in absentia* homologs (SIAHs) comprise a family of highly conserved E3 ubiquitin ligases that play an important role in regulating signalling pathways in tumorigenesis, including the DNA damage repair and hypoxia response pathways. SIAH1 and SIAH2 have been found to function as a tumour repressor and a proto-oncogene, respectively, despite the high sequence identity of their substrate binding domains (SBDs). Ubiquitin-specific protease USP19 is a deubiquitinase that forms a complex with SIAHs and counteracts the ligase function. Much effort has been made to find selective inhibitors of the SIAHs E3 ligases. Menadione was reported to inhibit SIAH2 specifically.

Methods: We used X-ray crystallography, peptide array, bioinformatic analysis, and biophysical techniques to characterize the structure and interaction of SIAHs with deubiquitinases and literature reported compounds.

Results: We solved the crystal structures of SIAH1 in complex with a USP19 peptide and of the apo form SIAH2. Phylogenetic analysis revealed the SIAH/USP19 complex is conserved in evolution. We demonstrated that menadione destabilizes both SIAH1 and SIAH2 non-specifically through covalent modification.

Conclusions: The SBDs of SIAH E3 ligases are structurally similar with a subtle stability difference. USP19 is the only deubiquitinase that directly binds to SIAHs through the substrate binding pocket. Menadione is not a specific inhibitor for SIAH2.

General Significance: The crystallographic models provide structural insights into the substrate binding of the SIAH family E3 ubiquitin ligases that are critically involved in regulating cancer-related pathways. Our results suggest caution should be taken when using menadione as a specific SIAH2 inhibitor.

Keywords: crystallography; deubiquitinase; E3 ubiquitin ligase; menadione; small molecule inhibitors; protein-protein interaction

Abbreviations: DUB, deubiquitinase; ERAD, endoplasmic-reticulum associated degradation; ESI, electrospray ionization; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ITC, isothermal titration calorimetry; ML, maximum-likelihood; RING, really-interesting new genes; SBD, substrate binding domain; SIAH, seven *in absentia* homolog; T_{agg} , temperature of aggregation; TCEP, tris(2-carboxyethyl)phosphine; TEV, tobacco etch virus; USP, ubiquitin-specific protease; ZnF, zinc finger.

1. Introduction

Protein homeostasis, or proteostasis, plays a critical role in many biological processes including cell development, stress responses, and aging. Dysregulation of proteostasis may lead to tumorigenesis, obesity or neuronal degenerative diseases. Post-translational modification of proteins, by covalent attachment of ubiquitin, plays a central role in proteostasis. Ubiquitylation is mediated by a three-enzyme cascade comprised of a ubiquitin activating enzyme (E1), a conjugating enzyme (E2) and a substrate-specific ubiquitin ligase (E3). The covalently attached ubiquitin or ubiquitin chain can be removed by a family of enzymes called deubiquitinases. The Seven *In Absentia* Homolog (SIAH) family of proteins are Really Interesting New Genes (RING) type E3 ubiquitin ligases, targeting substrate proteins for proteasomal degradation. It was first discovered in *Drosophila* and found to regulate the degradation of transcriptional repressor Tramtrack, which determines cell fate in *Drosophila* eye development [1]. The human genome encodes three *SIAH* genes: *SIAH1*, *SIAH2* and *SIAH3*. *SIAH1* and *SIAH2* share overlapping but non-redundant functions, both playing critical roles in cancer-related signalling pathways including the Ras/Raf [2], estrogen [3], DNA damage repair [4,5], inflammation [6], unfolded protein response [7], and hypoxia response pathways [8,9]. Recent knock-out studies in mouse models and proof-of-concept experiments using substrate-competitive covalent peptidomimetic inhibitor revealed that inhibition of *SIAH* function could impair tumour growth and metastasis [10], which suggests *SIAH* proteins may be promising therapeutic targets in cancer treatment [11–13]. *SIAH3*, however, does not contain a RING domain and is localized predominantly on mitochondria and regulates mitophagy [14].

SIAHs were reported to interact with ubiquitin-specific protease 19 (USP19) directly and target USP19 for proteasomal degradation based on yeast two-hybrid and co-immunoprecipitation analyses [15]. USP19 is an endoplasmic-reticulum (ER)-resident deubiquitinase that participates in the unfolded protein response and rescues ER-associated degradation (ERAD) substrates. Both *SIAHs* and USP19 interact with a variety of proteins involved in different signalling pathways, especially those involved in stress response [15–17]. They also have overlapping expression spectra in different tissues [15].

Given the importance of *SIAH* E3 ligases in tumorigenesis, extensive efforts have been made to find selective small molecule inhibitors for the *SIAH* E3 ligases using high-throughput screening [18], virtual screening [19,20] or structure-based design [10]. Menadione, commonly known as vitamin K3, was discovered, based on a ubiquitylation activity assay, to specifically inhibit *SIAH2* ligase activity at an IC_{50} of 20 μ M, attenuating hypoxia/MAPK signalling and blocking melanoma tumorigenesis [18]. Menadione has since been used in several studies to show the effect of inhibiting *SIAH2* ligase on downstream signalling [13,21–24]. However, given the high sequence similarity between *SIAH1* and *SIAH2*, it is unclear how menadione affects the two enzymes differently, and it was also not known whether *SIAH1* and *SIAH2* both interact with USP19 using the canonical substrate binding site.

The crystal structure of the substrate binding domain of mouse and human *SIAH1* in apo form [25,26] and in complex with peptides from adaptors Siah-interaction protein (SIP)[27], phyllopod (PHYL) [28] and a co-valent peptidomimetic inhibitors [10] have been reported previously. We report here the crystal structures of *SIAH2* in the apo form, and *SIAH1* in complex with a peptide from deubiquitinase USP19. We found that USP19 is the only deubiquitinase that interacts with *SIAH* E3 ligases directly through the substrate binding domain (SBD). We also characterized the interaction of *SIAH* proteins with several compounds reported in the literature, and found menadione destabilizes *SIAH1* and *SIAH2* through covalent modification of the proteins, suggesting caution should be exercised to use menadione as an inhibitor for *SIAH2*.

2. Materials and methods

2.1. Cloning, Expression, and Purification

DNAs encoding human SIAH1 (a.a. 91–282) and SIAH2 (a.a. 131–321) were subcloned into pET28-MHL (EF456735, for TEV protease cleavable N-terminal His6-tag) and pET28-MKH8SUMO (for TEV protease cleavable N-terminal His8-SUMO tag) vectors, respectively. Site-directed mutagenesis was carried out using the PrimeSTAR HS DNA polymerase (Takara) and verified by DNA sequencing. All proteins were over-expressed in a phage-resistant BL21(DE3) *E. coli* strain harbouring a pRARE2 plasmid or in the BL21-CodonPlus-RIL strain (Agilent) using Terrific Broth cultured in LEX Bioreactors (Harbinger Biotech). Protein production was induced using 0.5 mM IPTG for overnight growth at 18 °C. All proteins were first purified using nickel-nitrilotriacetic acid (NTA) agarose resin (Qiagen), and the His6- or His8-SUMO tag was then removed by TEV protease (1:30 w/w) when necessary. Uncleaved proteins and TEV protease were removed by another pass through the nickel-NTA resin. The proteins were further purified using anion-exchange chromatography (HiTrap Q HP 5mL, GE Healthcare) and size-exclusion chromatography (HighLoad 16/60 Superdex 200, GE Healthcare). The purified proteins were concentrated to a final concentration of 10–20 mg/mL, and stored at -80°C. All protein concentrations were determined by measuring UV absorbance at 280 nm using NanoDrop 2000 (Thermo Scientific). The peptides used for crystallization were chemically synthesized (Peptide 2.0, 95% purity) and contained N-terminal acetylation and C-terminal amidation.

2.2. Crystallization, Data Collection and Structure Determination, Refinement and Analysis

All crystals were grown at 20°C using the sitting drop vaporization method by mixing 0.5 µL protein solution with 0.5 µL well solution. For the SIAH1/USP19 peptide complex, SIAH1 protein was first mixed with a 2-fold molar excess of USP19 peptide and incubated on ice for 30 min before setting up crystallization. SIAH1/USP19 complex crystal was grown in 20% PEG 6000, 0.1 M bicine, pH 9.0. SIAH2 crystal was grown in 25% PEG 8000, 0.2 M NaCl, 0.1 M HEPES, pH 7.5. All harvested crystals were cryo-protected by immersion in a drop containing the well solution plus 15% ethylene glycol, then in Paratone-N (Hampton Research), before flash-frozen in liquid nitrogen. X-ray diffraction data were collected at beamline 19-ID of Advanced Photon Source, Argonne National Laboratory. All datasets were processed with the HKL-3000 suite[29]. The structures were solved by molecular replacement using PHASER[30] with PDB ID: 4CA1 as the search template. COOT[31] was used for model building and visualization, REFMAC[32] for restrained refinement. The final models were validated by MOLPROBITY[33].

2.3. Isothermal Titration Calorimetry (ITC)

Proteins were dialyzed overnight with a buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM TCEP and adjusted to a final concentration of 50 µM. Peptides were dissolved and diluted using the dialysis buffer to a final concentration of 1 mM. All ITC measurements were performed at 25 °C on a VP-ITC Micro Calorimeter (Malvern). A total of 25 injections, each of 10 µL peptide solution, were delivered into a sample cell of 1.4 mL containing proteins. The data were analysed using the Origin for ITC software supplied by the manufacturer and fitted to a one-site binding model.

2.4. Peptide Array

Peptides were synthesized directly on a modified cellulose membrane with a polyethylglycol linker using the peptide synthesizer MultiPep (Intavis). The binding assay was performed as described previously[34]. Briefly, the

membrane was extensively blocked with 5% skim milk, incubated overnight with 1 μ M His6-tagged SIAH1 or His8-SUMO tagged SIAH2 proteins, washed with phosphate buffered saline with Tween-20 (8 mM sodium phosphate, 150 mM NaCl, 2 mM potassium phosphate, 3 mM KCl, 0.05% Tween-20, pH 7.4) and bound SIAH proteins were visualized via Western blot analysis with an horseradish peroxidase-conjugated anti-His monoclonal antibody (EMD Millipore).

2.5. *Compounds and Thermostability Analysis*

Compounds were purchased from Fisher Scientific (purity of >90–95%) and dissolved in DMSO to a final concentration of 50 mM. All proteins concentrations were adjusted to 0.2 mg/mL in a buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP. Thermostability of the proteins was measured by differential static light scattering using Stargazer-384^{TM} (Harbinger). Measurement of each sample was repeated 3 times. Briefly, 45 μ L protein sample in the presence or absence of 0.5 mM compounds was added to a clear-bottom Nunc^{TM} 384-well black plate (Thermo Scientific) and then covered with the same volume of mineral oil to prevent evaporation. The plate was heated from 25 °C to 85 °C at 1 °C/min. Protein aggregation was monitored by tracing the change of scattered light detected by a CCD camera for every 0.5 °C temperature increase. The pixel intensities of each well at different temperatures were quantified and integrated using the image analysis software provided by the manufacturer. The aggregation temperature, T_{agg} , was obtained by fitting the data to a Boltzmann-distribution curve using the BioActive software (Integrated Proteomics Applications, Inc.)

2.6. *Phylogenetic analysis*

Sequences of well-annotated SIAH family proteins and USP19 from typical organisms were extracted from the Uniprot database[35] on February 22, 2016, then loaded into Geneious software (version 8.1.8) [36] for analysis. Multiple sequences alignments were finished by MUSCLE[37]. Eventually, gene trees were constructed using both neighbour-joining and maximum-likelihood methods with bootstrapping test and Shimodaira-Hasegawa test[38], respectively.

2.7. *Mass spectrum*

Protein samples were prepared by transferring 5 ng protein to 150 μ L 0.1% formic acid. The molecular mass analyses were carried out on an Agilent 1100 LC/MSD Time-of-Flight (TOF) mass spectrometer (Agilent Technologies) equipped with an electrospray ionization (ESI) source. Proteins were passed through a reverse phase HPLC on an Agilent Poroshell 300SB-C3 column to remove salt and buffer. The molecular weights or target proteins were calculated by the Agilent BioConfirm software.

3. **Results:**

3.1. *Crystal structure of SIAH2 reveals a nearly identical fold as that of SIAH1*

The SIAH family proteins feature a C-terminal TNF-receptor associated factor (TRAF)-like SBD (Fig. 1A) known to bind substrates and to self-associate. Both SIAH1 and SIAH2 contain an N-terminal 40–80 a.a. unstructured region, a typical RING-domain that conforms to consensus sequence CX₂CX₍₉₋₃₉₎CX₍₁₋₃₎HX₍₂₋₃₎CX₂CX₍₄₋₄₈₎CX₂C [39], followed by two zinc fingers (ZnFs) and one SBD. SIAH3 does not have the RING domain but contains a histidine-rich motif and one ZnF instead of two. The SBD is highly conserved, where SIAH2 has 90% sequence identity with SIAH1 and 54% with SIAH3 (Fig. 1A). We solved the crystal structure of a SIAH2 construct comprised of the two ZnFs and the SBD at 1.76 Å resolution (PDB ID: 5H9M, Fig. 1B, Table 1). The SIAH2 protein was over-expressed in *E. coli*, solubilized with a SUMO tag, and purified using Ni-NTA immobilized

metal affinity chromatography. The tag was removed before crystallization. Each asymmetric unit contains two SIAH2 molecules that dimerize through the SBD. ZnF1 coordinates one zinc ion using C138, C145, H157, and C161, whereas ZnF2 uses C168, C175, H187, and H192. Both ZnFs contain two short antiparallel β -strands and one α -helix. This type of ZnFs is most often seen in DNA-binding transcription factors but may also mediate protein-protein interaction [40]. The SBD comprises two anti-parallel β -sheets, each containing four strands, with one additional strand, β 0, linking ZnF2 with the SBD (Fig. 1B). Whereas ZnF2 packs on the SBD and has a buried surface area of 626 Å², the ZnF1 has limited contacts with the SBD. The overall structure of the SIAH2 is similar to that of SIAH1 (PDB ID: 4CA1) [25,26] with a slight orientation change of ZnF1 relative to the rest part of the molecule. To identify whether there is a subdomain movement or a global conformational rearrangement, we used Rapido (Rapid Alignment of Protein structures In the presence of Domain mOvements) analysis [41] to compare the structure of SIAH2 with that of SIAH1. The two structures have an r.m.s.d. of 2.29 Å over 181 residues using rigid body alignment, whereas a flexible alignment gives an r.m.s.d. of 0.65 Å for the same residues. When the two structures are aligned against the SBD, the ZnF1 is rotated 26° to 39°, depending on the chain selected for comparison, around an axis cross-sectioning the linker between ZnF1 and ZnF2. This suggests ZnF2 and SBD forms a tightly packed subdomain, and ZnF1 is a relatively independent subdomain. The movement of ZnF1 may have been caused by crystal packing (Fig. S1A). However, the moderate B-factors of the linker region between ZnF1 and ZnF2 (Fig. S1B) indicates that the domain movement of ZnF1 is not completely flexible. Such movement is probably limited by a conserved proline, P165, in the linker region, which is known to cause structural rigidity of domain linkers [42]. Similar to the structure of SIAH1[25–27], SIAH2 also forms a dimer in the asymmetric unit. This is consistent with the elution profile of the protein on size exclusion chromatography (data not shown). Each monomer has a buried surface area of 1016 Å². The dimerization is mediated mainly by residues on β 6, β 7, and α 3 (Fig. S1A).

Figure 1. Structural characterization of SIAHs. (A) Domain architecture of human SIAH1–3 and USP19 proteins and the sequence identity of different domains of SIAHs. The catalytic domain of USP19 is split by insertions of ubiquitin-like (UBL), zinc-finger (ZnF) domains, and a disordered region. TM stands for trans-membrane. (B) Ribbon representation of the structure of the apo form SIAH2 (wheat) and the complex structure of SIAH1 (cyan) and USP19 peptide (magenta) complex. Zn ions of SIAH2 are shown in spheres (wheat) and those of SIAH1 are in grey.

Table 1. Crystallography data and refinement statistics

	SIAH1/USP19	SIAH2
PDB ID	4X3G	5H9M
Data collection		
Space group	$P2_1$	$P2_12_12_1$
Cell dimensions		
a, b, c (Å)	41.3, 88.1, 59.6	53.6, 68.8, 102.9
α, β, γ (°)	90.0, 103.2, 90.0	90.0, 90.0, 90.0
Resolution (Å)	50.00–2.33(2.33–2.37)*	50.00–1.76(1.79–1.76)
Measured reflections	36140	352371
Unique reflections	15818	38381
R_{merge} (%)	8.9 (44.0)	10.1(87.9)

$I/\sigma I$	9.6 (1.0)	25.7(1.7)
Completeness (%)	90.9(58.9)	100.0(99.9)
Redundancy	2.3(1.5)	9.2(6.5)
Refinement		
Resolution (Å)	48.4-2.34	50.0-1.76
No. reflections (test set)	15802(780)	37374(945)
$R_{\text{work}}/R_{\text{free}}$ (%)	22.2/25.1	17.8/22.7
No. atoms		
Protein (SIAH)	2958	3083
Peptide (USP19)	153	
Zn	4	4
Water	56	312
B-factors (Å ²)		
Protein (SIAH)	82.8	21.5
Peptide (USP19)	82.4	
Zn	92.6	19.8
Water	69.0	30.5
RMSD		
Bond lengths (Å)	0.008	0.010
Bond angles (°)	0.95	1.506
Ramachandran plot % residues		
Favored	98.0	97.2
Additional allowed	2.0	2.8
Generously allowed	0	0
Disallowed	0	0

* Values in parentheses are for highest-resolution shell unless otherwise indicated.

We measured the stability of SIAH1 and SIAH2 proteins using differential static light scattering (DSLS) [43] and found the aggregation temperature, T_{agg} , of the two proteins are higher than 65 °C, suggesting both proteins are very stable. The T_{agg} of SIAH1 is 3.7 °C higher than that of SIAH2 (Table 2). We predicted the effect of swapping residues between SIAH1 and SIAH2 on the protein stability using network enthalpic modelling [44] and found L250 of SIAH2 has highest impact on the protein stability if changed to the corresponding glutamine residue (Q210) in SIAH1 (Table S1). L250 is completely buried at the interface between ZnF2 and SBD, forming a hydrophobic core with neighbouring residues M230, V232, and V287 (Fig. S1C). This hydrophobic pocket could be potentially involved in compound binding [19]. When we mutated L250 of SIAH2 to a glutamine, the T_{agg} of the mutant is reduced by 1.7 °C. On the contrary, when we mutated Q210 of SIAH1 to a leucine, the T_{agg} of the mutant is increased 1.4 °C compared to that of the wild type SIAH1. These results suggest that although the sequences of the ZnFs and SBD of SIAH1 and SIAH2 are highly homologous and the structures nearly identical, the stability of the two proteins are subtly different. The higher stability of SIAH1 over SIAH2 is probably caused by an overall contribution from residues that are different between the two proteins.

Table 2. Characterization of the effect of compounds on the thermal stability of SIAH1 and SIAH2 using DSLS.

Proteins	Compounds	T_{agg} (°C) [*]	ΔT_{agg} (°C) [†]
SIAH1 ⁹¹⁻²⁸²	-	69.14±0.26 [*]	-
SIAH1 ⁹¹⁻²⁸²	SCR00073	69.62±0.20	0.48

SIAH1 ⁹¹⁻²⁸²	SB01122 (vitamin k3)	50.49±0.27	-18.64
SIAH1 ⁹¹⁻²⁸²	DP01471	68.45±0.68	-0.69
SIAH1 ⁹¹⁻²⁸²	DSHS00977	68.44±0.25	-0.70
SIAH1 ⁹¹⁻²⁸²	HTS09269	68.96±0.20	-0.18
SIAH1 ⁹¹⁻²⁸²	STK386864	68.68±0.09	-0.46
SIAH2 ¹³¹⁻³²¹	-	65.38±0.21	-
SIAH2 ¹³¹⁻³²¹	SCR00073	65.91±0.24	0.53
SIAH2 ¹³¹⁻³²¹	SB01122 (vitamin k3)	42.80±0.85	-22.58
SIAH2 ¹³¹⁻³²¹	DP01471	65.05±0.17	-0.33
SIAH2 ¹³¹⁻³²¹	DSHS00977	65.45±0.19	0.07
SIAH2 ¹³¹⁻³²¹	HTS09269	65.54±0.19	0.16
SIAH2 ¹³¹⁻³²¹	STK386864	65.07±0.05	-0.31
RC3H1 ¹⁻⁴⁴⁵	-	58.87±0.09	-
RC3H1 ¹⁻⁴⁴⁵	SB01122 (vitamin k3)	51.65±0.65	-7.22
UHRF2 ¹⁰⁹⁻³⁹⁵	-	59.60±0.21	-
UHRF2 ¹⁰⁹⁻³⁹⁵	SB01122 (vitamin k3)	48.57±0.13	-11.03
SIAH1 ⁹¹⁻²⁸² Q210L	-	70.56±0.49	1.42
SIAH2 ¹³¹⁻³²¹ L250Q	-	63.65±0.31	-1.73

*: T_{agg} is the average of three independent measurements.

†: ΔT_{agg} is the difference of the T_{agg} of the measured sample to the T_{agg} of the corresponding wild type in the absence of compound.

3.2. USP19 is the only DUB that interacts with the SBD of SIAHs

USP19 contains three SIAH-interaction consensus PxxxVxP motifs (Fig. 2A). Two of them are located at the N-terminus of the USP catalytic domain (a.a. 497–1214). We searched the sequences of all human deubiquitinases (DUBs), plus the putative MCPIP deubiquitinase family [45], and found 15 other sequences that conform to the PxxxVxP pattern (Table S2). We used Fmoc-based SPOT technique [46] and synthesized a peptide array to test the binding of these motifs (Fig. 2B, spots A1–C4) with SIAH1. USP19 peptide S⁴⁶¹PKPTCMVPPMPHS⁴⁷⁴ (hereinafter referred to as pepS461) is the only one that binds to SIAH1 (Fig. 2B, spot C2). We further synthesized the three USP19 peptides and measured the binding affinity using isothermal titration calorimetry (ITC) and confirmed that only pepS461 interacts with SIAH1 at a dissociation constant (K_D) of 2.7 μ M, and a K_D of 2.2 μ M with SIAH2 (Fig. 2C). An alanine scan array (Fig. 2B, spots C5–E6) revealed K463 and V468 of USP19 to be the critical residues involved in interaction with SIAH1. Mutation of either residue abolishes the binding.

To better understand the atomic details of the interaction between SIAHs and USP19, we solved the crystal structure of the SIAH1/pepS461 complex at 2.3 Å resolution (Fig. 1B, 2D, Table 1). The side chains of most residues of the peptide are clearly visible in the electron density map (Fig. S2A). The USP19 peptide adopts an

extended β -strand conformation and binds to the cleft between the two β -sheets of the SIAH1 SBD, which comprises of residues from strands $\beta 0$, $\beta 1$, $\beta 2$ (Fig. 2D). This cleft has previously been shown to bind other consensus motifs [10,27,28]. The conserved residues of the consensus motif, V468 and P470, point to a hydrophobic groove formed by a set of hydrophobic residues including F165, V179, V176, A167, W178 and M180 from SIAH1 (Fig. 2D and Fig. S2B). The side chain of another critical residue K463 latches onto a negatively charged patch centred on residue D162 of SIAH1 and forms charged interactions with D162 and N276 (Fig. S2C). Conserved residue P464 forms a hydrophobic cluster with the side chains of L158 and I163 to maintain the preceding K463 in a conformation favourable for interaction.

We also tried to co-crystallize the SIAH2/pepS461 complex but ended up with a crystal that contained only SIAH2. Inspection of the apo-structure of SIAH2 revealed a penta-ethylene glycol molecule binds to the VxP motif binding site of SIAH2 (Fig. S2D). The four ether oxygen atoms of the glycol molecule coordinate a water molecule that binds to the main chain carboxyl group of D217, which may explain the failure to obtain a crystal of the SIAH2/USP19 complex.

Figure 2. Interaction between SIAHs and DUBs. (A) Sequence alignment of SIP, Phyllopod (PHYL) peptides with the three PxxxVxP motifs of USP19 (referred to as pepR445, pepS461, and pepG1266). The sequences of SIP and PHYL were extracted from corresponding SIAH1/peptide structures (PDB IDs: 1K2F

and 2AN6). (B) Peptide array analysis of SIAH1 with deubiquitinases (DUBs) peptides. Plus sign + indicates poly-His peptide positive control. Spots A1–C4 are the PxxxVxP motifs from all the DUBs. USP19 pepS461 (spot C2) are indicated with a red ×. Spots C5–E6 are alanine scan array of USP19 pepS461. Spots E7 and E8 are positive control OBF1 and SIP[27] peptides respectively. SIP peptide has a low affinity of $K_D=10\text{ }\mu\text{M}$ with SIAH1 and the intensity of the blot is relatively weak. (C) ITC measurements of SIAHs with USP19 peptides. (D) Interaction details of pepS461 (magenta) with SIAH1 (cyan).

Residues involved in USP19 peptide binding are completely conserved between SIAH1 and SIAH2 (Fig. S3). These results implicate that the function of the substrate binding domains of SIAH1 and SIAH2 are highly similar if not identical, and the functional differences between the two proteins are probably caused by the N-terminal extension and the RING domain that are less conserved.

3.3. Phylogenetic analysis of the SIAH family of proteins and USP19

To understand how SIAHs evolved in different species, we did phylogenetic analysis of the SIAH family of proteins. The maximum-likelihood (ML) phylogenetic tree of SIAH proteins (Fig. 3) is consistent with that built using neighbour-joining method and used for further analysis. The ML tree shows a clear distinction between the plant and animal SIAH proteins. The model plant, *Arabidopsis thaliana* genome encodes five SIAH paralogs that have only 14–16% sequence identity to human SIAH1, whereas the worm (*C. elegans*) SIAH-1 has 73% sequence identity. In vertebrate genomes, there are generally three types of SIAH homologs with high support values (>0.94) for clustering. In contrast, the invertebrate SIAH homologs can be classified into two types. One clusters with vertebrate SIAH1, and the other clusters with the whole animal branch of SIAH homologs. This suggests SIAH2 was the most recent common ancestor of the SIAH family proteins. Interestingly, SIAH2 and SIAH3 show a closer relation in evolution despite higher overall sequence similarity between SIAH1 and SIAH2. The phylogenetic analysis suggests *SIAH3* was derived from the duplication of the *SIAH2* gene at around a time when amniotes (reptiles, birds and mammals) appeared. The loss of the RING and ZnF1 domains and the acquisition of a histidine-rich region (Fig. 1A) suggested SIAH3 may harbour novel functions.

Figure 3. The maximum-likelihood phylogenetic tree of the SIAH family protein of different organisms. SIAH genes from the following organisms were included in the analysis: human (*Homo sapiens*), monkey (*Chlorocebus sabaeus*), chimp (*Pan troglodytes*), chicken (*Gallus gallus*), mouse (*Mus musculus*), turtle (*Trionyx sinensis*), lizard (*Anolis carolinensis*), oyster (*Crassostrea gigas*), fish (*Danio rerio*), rat (*Rattus norvegicus*), worm (*Caenorhabditis elegans*), bug (*Lygus Hesperus*), fly (*Drosophila melanogaster*), cress (*Arabidopsis thaliana*). Vertebrate SIAH1, SIAH2, and SIAH3 were coloured in green, blue, and red respectively. The split of SIAH1 and SIAH2 branches was indicated with a line in magenta. SIAH3 was derived from a gene duplication of the SIAH2 gene.

USP19 gene can be found in the genomes from worm to human (Fig. 4A). Interestingly, the consensus motif PxxxVxP is conserved in the USP19 sequences of all the species checked (Fig. 4B), suggesting the SIAH/USP19 complex is functionally conserved during evolution.

Figure 4. Evolution of USP19. (A) The maximum-likelihood phylogenetic tree of USP19 homologs from different species. Uniprot IDs are used. (B) The PxxxVxP motif is conserved in all the USP19 sequences examined except in *Arabidopsis*, where the valine is replaced by a leucine of similar size and hydrophobicity. The motif is N-terminal to the catalytic cysteine, which is indicated with a filled triangle.

3.4. Menadione is not a specific inhibitor for SIAH2 E3 ligase

Although the sequences of the SBD of SIAH1 and SIAH2 are highly similar, the two proteins are differently regulated at transcription and post-translational modification levels and play different functional roles in tumorigenesis [11–13,47]. Great efforts have been made to find selective small molecule inhibitors. Menadione (vitamin K3, Fig. S4A) was found to inhibit the E3 ligase activity of SIAH2 specifically with an IC₅₀ of 20 μM based on an electro-chemiluminescent ubiquitination assay[18]. Several other compounds have also been postulated to bind the SBD of SIAH2 based on virtual screening [19]. We evaluated the interaction of these compounds with the ZnFs and SBD of both SIAH1 and SIAH2 using DSLs and ITC. We were only able to see

insignificant stabilization of both proteins by SCR00073 but not any other compounds (Fig. S4B, Table 2). We also measured the binding of SCR00073 using ITC but could not observe significant interaction with SIAHs (Fig. S4C). Quite unexpectedly, we observed that menadione significantly destabilizes both SIAH1 and SIAH2 by 18 °C and 22 °C using DSLS, respectively (Fig. 5A, Table 2). We measured the mass of the proteins after incubation with 0.5 mM menadione in a time course, and found both proteins were covalently modified by menadione (Fig. 5B). The SIAH1 construct used contained four free cysteines, all of which can be modified. The SIAH2 construct used contained three free cysteines, whereas at least four sites of the protein were modified, suggesting a cysteine from a ZnF may also be modified. To confirm that cysteine modification by menadione is non-specific and destabilizes target proteins, we incubated menadione with two other ZnF containing proteins RC3H1 and UHRF2 and found the T_{agg} of these two proteins were reduced by 7 and 11 degrees, respectively (Table 2). It is noteworthy that all the free cysteines in SIAH1 and SIAH2 except C282 are mostly buried (Table S1), modification by menadione will create spatial clash with near-by residues and thus destabilize the proteins. We thus concluded menadione is not a specific inhibitor for SIAH2 E3 ligase but a covalent modifier that reduces the E3 activity of SIAHs by destabilizing the proteins.

A

B

Figure 5. Interaction of menadione with SIAH proteins. (A) The aggregation temperature of SIAHs in the presence or absence of menadione. Experiments were repeated 3 times. (B) Mass spectrometry of SIAH1 and SIAH2 upon overnight incubation with 10 times molar excess of menadione on ice.

4. Discussion

4.1. The Structures of the Substrate Binding Domain of SIAH Family Proteins are Highly Similar

About 95% of human E3 ligases belong to the RING family. Most RING-type E3s act as adaptor proteins that bring together the substrate and an E2~ubiquitin conjugate. The RING domain binds to the E2~ubiquitin conjugate and facilitates the transfer of the ubiquitin cargo to the substrate, which is recognized by the substrate binding domain of the E3 ligase or an E3 ligase complex [48]. Our crystal structures of SIAH2 and the complex of SIAH1 bound with a USP19 peptide suggest that despite some difference in the thermal stability of the two proteins, the substrate recognition pocket and the residues involved in substrate binding are identical between the two proteins. This is confirmed by the similar binding affinities of the USP19 peptide with the two proteins, as measured by ITC. We also built a structural model of the SIAH3 SBD using the SWISS-MODEL webserver [49] (Fig. 6A). The ZnF and SBD domains of SIAH3 also form a compact structure and has an r.m.s.d of 0.38 Å for 118 Cα atoms compared to that of SIAH1, suggesting a highly similar conformation. It is noteworthy that most residues on the substrate binding groove involved in substrate binding are also highly conserved in SIAH3 (Fig. S3). However a small charge difference was observed. Notably, residue T156 of SIAH1 that forms a hydrogen bond with C466 of the USP19 peptide, the second x residue of the PxxxVxP motif, was replaced by a negatively charged aspartic acid in SIAH3 (Fig. 6B). Residue N276 of SIAH1 in the negatively charge patch that interacts with the positively charge K/R preceding this first proline is replaced by a glycine in SIAH3 (Fig. 6C). This suggests SIAH3 could probably also bind to PxxxVxP consensus motif, but may have difference preference for the residues in positions x compared to SIAH1 and SIAH2.

Figure 6. Structural model of SIAH3 and comparison with SIAH1. (A) Homology model of SIAH3 built with SWISS-MODEL using SIAH2 as a template. (B) Surface charge map of the substrate peptide binding groove revealed the groove of SIAH3 is more negatively charged due to the presence of an aspartic acid D134. In SIAH1, the less charge pocket accommodates the side chain of C466. (C) The patch binds to the critical K463 preceding the conserved proline is less charged in SIAH3.

Both SIAH1 and SIAH2 exhibit high thermal stability with a T_{agg} over 65 °C. The SIAH1 protein is slightly more stable than SIAH2. The most significant difference between the two proteins is a buried Q210 residue of SIAH1, which corresponds to a hydrophobic leucine residue in SIAH2. Q210 resides inside a hydrophobic core between the ZnF2 and SBD. Mutation of Q210 to a leucine further increases the thermal stability of SIAH1 protein. The higher thermal stability could probably be caused by small contributions from each residue that is different between SIAH1 and SIAH2, as SIAH1 has a lower content of alanine, histidine, glutamine, and threonine residues that are less preferred in thermostable than in mesostable proteins[50].

4.2. *Complex between E3/DUB and Evolution of SIAH and USP19*

Many deubiquitinases are known to form complexes with E3 ubiquitin ligases [51], for example, USP7 with MDM2 [52], USP8 with RNF128 [53] or RNF41 [54]. Such interactions may transregulate the two binding partners by each other or may fine tune the ubiquitin chain linkages deposited on the substrate to enforce proper downstream signalling [55,56]. Two deubiquitinases are currently known to interact with the SIAH proteins, USP13 and USP19. USP13 does not contain a SIAH binding consensus motif but only bind to the polyubiquitin chain of an auto-ubiquitinated SIAH2 through its UBA domain [57]. We have over-expressed and purified full length USP13 and verified that USP13 does not bind to the ZnF1-ZnF2-SBD domains of SIAH2 using size-exclusion chromatography (data not shown). In contrast, USP19, based on our peptide array data, is the only deubiquitinase that binds to SIAH1 and SIAH2 through a PxxxVxP consensus motif. Out of the three PxxxVxP motifs of USP19, only pepS461 binds to both SIAH1 and SIAH2. A further inspection of the crystal structure of SIAH1/USP19 and peptide sequences suggests that in the other two peptides, a negative charged residue, a glutamic acid residue for pepR445 or an aspartic acid residue for pepG1226 (Fig. 2A), respectively, replaces a cysteine in the second x position of pepS461. In the SIAH1/pepS461 structure, the side chain of cysteine C466, sticks into a hydrophobic pocket that comprises of residues L158, I163, F165, and M180 (Fig. S2B), whereas such an interaction would be abolished by a negatively charged residue. For comparison, in the complex structures of SIAH1 with Siah-interaction protein (SIP) [27] and phyllopod [28] peptides, this position is occupied by an alanine that maintains such a hydrophobic interaction. Furthermore, alanine scanning experiments suggest a lysine preceding the first proline in the motif is critical for the interaction, which is absent in the two other motifs of USP19. Not surprisingly, the SIP and Phyllopod peptides also [10,27,28] contain a positively charged residue (either a lysine or an arginine) preceding the first proline in the consensus motif (Fig. 2A).

The SIAH-interacting peptide of USP19, pepS461, is located close to the catalytic cysteine (C506) of USP19 (Fig. 1A, Fig. 4B), suggesting that the SIAH/USP19 interaction may regulate the activity of USP19. Both USP19 and SIAHs are important regulators of hypoxia-inducible factor 1 α (HIF-1 α) during hypoxia [16,58,59]. Formation of the USP19 and SIAH complexes suggests that the ubiquitination of HIF-1 α is under fine control, which is essential for HIF-1 α mediated cell signalling [60] and cell survival under hypoxic conditions [61]. The substrate binding pocket of SIAHs and the SIAH-interaction peptide of USP19 are highly conserved during evolution. This implicates regulation of hypoxia pathway by the SIAH/USP19 complex may also have been conserved during evolution.

It is also worth noting that several E2 conjugating enzymes, namely TSG101, UBE2O, BIRC6, UBE2J2, UBE2Z and AKTIP, contain the PxxxVxP SIAH-interacting consensus motif. Knock-down of TSG101 has been found to associate with reduced level of HIF-1 α in ovarian cancer [62]. Further experiments are needed to explore whether these E2s participate in the SIAH E3 ligases-mediated ubiquitination pathway besides those previously demonstrated, such as UBE2L6[63–65], UBE2D2[66].

4.3. *Targeting SIAHs in tumorigenesis*

An increasing body of evidence suggests SIAH1 and SIAH2 play critical functional roles in tumorigenesis, metastasis, and chemo-/radio-sensitivity in cancer treatment [2,11–13,67,68]. Many studies found the expression level of SIAH1 is down-regulated in cancers [63,69,70] and demonstrated a tumour suppression and reversion capacity of SIAH1 [71]. In contrast, the role of SIAH2 in tumorigenesis is less well defined. Whereas the expression level of SIAH2 correlates with pro-tumorigenic features in prostate cancer [47,72] lung cancer [73], and ovarian cancer [74], SIAH2 also targets several oncogenes for ubiquitin-proteasomal degradation. For example, SIAH2 suppresses oncogenic signalling mediated by tyrosine kinase-2 (TYK2) and transcription factor STAT3 in lung cancer [75], by activated Cdc42-associated kinase 1 (ACK1) in breast cancer [76], and by mutant receptor tyrosine kinase FLT3 with an internal tandem duplication (FLT3-ITD) in leukaemia [65]. The link between SIAH2 and Ras-ERK signalling pathway and the oncogenic activities of SIAH2 were recently called into question in several reports [12,67]. It was actually proposed that SIAH2 may have stage-specific function in tumorigenesis and progression [77].

Given that the sequences and structures of SIAH1 and SIAH2 are highly conserved and they share a number of substrates, a selective inhibitor that targets SIAH2 but not SIAH1 would be highly desirable to untangle the functional roles of the two close orthologs in tumorigenesis and for cancer treatment. Menadione was used in several recent studies as a selective SIAH2 inhibitor to study the function of SIAH2 [13,21–24]. Our thermal stability assay and mass spectrometry, however, suggests that the inhibitory effect of menadione on SIAH2 is actually caused by non-specific covalent modification of free cysteines in the target protein. A further inspection of the RNF5 E3 ubiquitin ligase, which was used as a control E3 ligase in the initial compound screening study [18], reveals that all cysteines residues coordinate with zinc ions and there are no free cysteines that can be readily modified by menadione. Indeed, menadione is a cysteine alkylator [78] and could destabilize many cysteine-containing proteins, such as RC3H1[79] and UHRF2, both of which we tested. Our results caution the use of menadione as a selective SIAH2 inhibitor. A peptidomimetic inhibitor for SIAHs was also reported based on structure-guided design [10], but it does not distinguish SIAH1 and SIAH2. Our structural analysis of SIAH proteins suggests the substrate binding domain of SIAHs may not be a good target for selective inhibitor development.

However, SIAH1 and SIAH2 E3 ligases have highly dissimilar N-terminal extensions and are differentially regulated at transcription and post-translational modification levels. For example, whereas transcription factor p53 regulates the expression of both SIAH1 [80] and SIAH2 [75], only the expression of SIAH2 is regulated by sex hormone [3,81] and cytokine signalling [67,82]. SIAH1 is phosphorylated by ATM/ATR checkpoint kinases[4], mitogen-activated protein kinase MLK1 [83], and apoptosis-regulating kinase 1 (ASK1) [84]. In contrast, SIAH2 is phosphorylated by dual specificity tyrosine phosphorylation regulated kinase 2 (DYRK2) [85], and check-point kinase 2 (CHK2) [86]. Specifically, S19 of SIAH1 is phosphorylated by ATM kinase, which disrupts its interaction with HIPK2 [4]; whilst the equivalent S16 of SIAH2 is phosphorylated by DYRK2 kinase in addition to four other threonine/serine residues spanning the N-terminal extension and the RING domain [85]. Targeting regulators up-stream of SIAHs proteins, such as kinases and epigenetic modifiers, or protein-protein complex involving the N-terminal extension of SIAHs, may be a more viable strategy to regulate SIAHs selectively. For example, histone lysine methyltransferase EHMT2 directly regulates the transcription of SIAH1 and a specific inhibitor for EHMT2 induce the expression of SIAH1 and halts the growth of bladder and lung cancer cells [87]. Inhibitors of histone deacetylases (HDACs) have been shown to enhance SIAH-mediated proteasomal

degradation in difference cancer cells[63,88]. Future studies should focus on the differential regulation of SIAH1 and SIAH2 to find druggable therapeutic targets of the SIAHs-mediated ubiquitination pathway.

Author contribution

Q.Z. and Y.T. conceived and designed the experiments. Q.Z. performed most of the experiments. X.H. crystallized the SIAH1/USP19 complex. F.H. characterized SIAHs/DUB interactions. Z.W. did the bioinformatics analysis. A.D. and J.R.W collected X-ray dataset and solved the structures. Y.T., Q.Z. and R.H wrote the manuscript.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgements

The Structural Genomics Consortium (SGC) is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada through Ontario Genomics Institute, Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, Merck & Co., Novartis Pharma AG, Ontario Ministry of Economic Development and Innovation, Pfizer, São Paulo Research Foundation-FAPESP, Takeda, and the Wellcome Trust. X-ray diffraction data were collected at Argonne National Laboratory, Structural Biology Center at the Advanced Photon Source, which is operated by UChicago Argonne, LLC, for the U.S. Department of Energy, Office of Biological and Environmental Research under contract DE-AC02-06CH11357. Z.W is a visiting scholar at the SGC sponsored by the National Natural Science Foundation of China (31201996), and Excellent Young Teachers Program of Guangdong Province (Yq2013091 and Yq2013089). We are grateful for Guillermo Senisterra and Albina Bolotokova for technical support.

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