Structural basis for the specificity of USP18 towards ISG15

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Abstract

Protein modification by ubiquitin and ubiquitin-like modifiers (Ubls) is counteracted by ubiquitin- and Ubl-proteases collectively called DUBs. In contrast to other proteases of the ubiquitin-specific protease (USP) family, USP18 shows no reactivity towards ubiquitin but specifically deconjugates the interferon induced Ubl ISG15. To identify molecular determinants for this specificity, we solved the crystal structures of mouse USP18 and of mouse USP18 in complex with mouse ISG15. USP18 was crystallized in an open and a closed conformation revealing high flexibility of the enzyme. Structural data, biochemical and mutational analysis showed that only the C-terminal ubiquitin-like domain of ISG15 is recognized and essential for USP18 activity. A critical hydrophobic patch in USP18 interacts with a hydrophobic region unique to ISG15 providing evidence that ISG15 specificity of USP18 is mediated by a small interaction interface. Our results may provide the structural basis for the development of new drugs modulating ISGylation.

Posttranslational protein modifications by ubiquitin (Ub) and ubiquitin-like proteins (Ubls) such as SUMO, NEDD8, FAT10 or Interferon-stimulated gene 15 (ISG15) are involved in...
the regulation of a wide variety of cellular processes including protein stability, DNA repair, cell cycle control, intracellular trafficking and antiviral defense. ISG15 was the first Ub identified and is composed of two ubiquitin-like domains connected by a short linker region. Covalent linkage of ISG15 (ISGylation) is mediated by the consecutive action of the E1 activating enzyme Ube1L3, the E2 conjugating enzyme UbCH84,5 and a few E3 ligases, including mouse HERC6 and human HERC56–9, which account for the conjugation of most substrates in mice and humans, respectively. All components of the ligation process are strongly induced by type I IFN.

ISGylation represents one of the major antiviral effector systems and is essential to counteract various pathogens including influenza-, herpes-, noro-, and coronavirus11,12. ISGylation can occur at the ribosome in a co-translational manner, favoring the modification of viral proteins in infected cells13. Concordantly, ISGylated viral capsid proteins fail to assemble properly, thereby inhibiting the propagation of the virus13. However, also multiple cellular proteins14,15 including the ubiquitin E2 enzymes Ubc1316 and UbcH617 as well as the transcription factor IRF318,19 are modified by ISG15. Moreover, free ISG15 is released from cells and acts in a cytokine-like manner on NK cells to stimulate IFN-γ production20. Concordantly, humans with inherited ISG15 deficiency showed impaired IFN-γ responses and failed to effectively counteract mycobacterial infection21. Besides the function of ISG15 in immunity, ISGylation is involved in DNA repair22 and tumorigenesis23,24.

Analogous to other posttranslational control mechanisms, covalent linkage of Ub and Ubls to substrate proteins is a reversible process antagonized by deubiquitinating enzymes and Ubl-proteases (DUBs). Ubiquitin-specific proteases (USPs) represent the largest subclass of DUBs25. Most of the more than 50 known USPs cleave ubiquitin chains with different types of linkage, and some like USP2, USP14 or USP21 were reported to recognize not only Ub but also ISG1526,27. In contrast to these cross-reactive isopeptidases, USP18 does not deconjugate ubiquitin from target substrates and represents the only ISG15-specific protease known so far28,29. Yet, there are reports that in a specific context USP18 is associated with ubiquitin protease activity30,31. USP18 cleaves ISG15 from target proteins with high catalytic activity28 and constitutes the major ISG15 isopeptidase in vivo. Accordingly, Knock-In mice selectively lacking USP18 enzymatic activity exhibit strongly enhanced and prolonged ISGylation32. Intriguingly, enhanced ISGylation in these mice mediated increased resistance against influenza and vaccinia virus infections and diminished myocarditis upon coxsackie virus B3 infection, thus qualifying USP18 protease inhibition as a potential antiviral strategy33,34. Irrespective of its enzymatic function, USP18 is a major negative regulator of type I IFN signaling by interacting with the interferon receptor (IFNAR)35,36. Recent studies have shown that depletion of USP18 causes interferonopathies characterized by aberrant microglia activation35,37. Furthermore, ISG15 deficiency in humans correlated with destabilization of USP18, thereby enhancing the IFN response38.

Despite increasing knowledge about these physiological functions of USP18, the molecular structure and determinants for the unique specificity of USP18 towards ISG15 have remained unidentified. The sequence of the catalytic region of USP18 is closely related to that of ubiquitin deconjugating USPs. In contrast to most other USPs, USP18 lacks...
additional typical interaction domains, putting forward the question, how the unique specificity towards ISG15 is mediated on the molecular level. To gain insights into the mechanism of recognition and catalysis by USP18 we have determined structures of murine USP18 and of a complex of murine USP18 with murine ISG15 by X-ray crystallography. Analysis of these structures, biochemical investigations and characterization of several site-specific variants of USP18 reveal insights into molecular determinants of ISG15 recognition and cleavage.

Results

Overall structure of USP18

For crystallization experiments we used the catalytic core of murine USP18 that lacks the N-terminus which is predicted to be unstructured and not required for enzymatic activity, ISG15 binding or interaction with IFNAR. The unbound USP18 protein crystallized as thin needles that diffracted to a resolution of 2.8 Å (Table 1). There are two molecules in the asymmetric unit, forming an extended crystal contact covering a surface area of 900 Å². A single Zn²⁺ stabilizes the crystal contact (Supplementary Fig. 1a) and is coordinated by residues His334 and Cys336 of both molecules (Supplementary Fig. 1c).

The USP18 structure adopts the typical fold of the catalytic core of USPs resembling a right hand (Fig. 1, Supplementary Fig. 2). The three major domains are therefore termed as finger, palm and thumb domain (Supplementary Fig. 2). The finger domain encompasses a Zn²⁺ coordinated by four cysteine residues stabilizing the extended loop of the finger domain (Supplementary Fig. 1a,b).

USP18 adopts two different conformations in the crystal

Structural alignment of the two different USP18 molecules present in the asymmetric unit revealed some remarkable differences. Whereas the palm and thumb domains of the two chains A and B align well, the orientation of the finger domain differs in both molecules. The finger domain of chain B exerts a closing movement towards the thumb domain, reorienting β-sheets 4 and 5 and the connecting loop by 10 Å (Fig. 1). Furthermore, the loop located between residues 126 and 136 exhibits major differences. This loop corresponds to the previously described switching loop of USP7 and was shown to influence the catalytic activity. Whereas in one USP18 molecule (chain B) this loop exhibits no secondary structure, the residues 131-135 of the second molecule (chain A) fold into a short α-helix. Minor differences were e.g. observed for a loop that comprises residues 307-314 and corresponds to the so called blocking loop 2 described in several USPs like USP14, USP8, and USP7. With respect to the USP18 finger domain, the two conformations were assigned to an “open” and a “closed” state. The structure of the USP18–ISG15 complex described below, revealed that the closed state of USP18 is not compatible with ISG15 binding. Residues Cys61, His314 and Asn331 form the catalytic triad of USP18. The distances between these residues within the two molecules of unbound USP18 are very similar: the Cys61 Sγ and the His314 Nδ2 atom are separated by 4.0 Å - 4.2 Å and the carbonyl group of the Asn331 side chain resides in a distance of 3.5 Å - 3.7 Å from the Ne2 atom of His314 (Fig. 1c,d) (Supplementary Fig. 3a,b). The observed distances suggest that
the catalytic triad is not in catalytic configuration in unbound USP18. In summary, the conformations observed for both USP18 molecules in the asymmetric unit represent two different states that are both enzymatically inactive.

Overall structure of the USP18–ISG15 complex

The covalent complex of murine USP18 with murine ISG15 was prepared using ISG15 modified with a C-terminal propargylamide (PA) reactive group (ISG15-PA). Crystals diffracted to a resolution of 3.0 Å (Table 1). There are two USP18–ISG15 complexes in the asymmetric unit. In contrast to the un-complexed enzyme, the USP18 molecules adopt virtually identical conformations with an r.m.s.d. of 0.4 Å over 300 Cα positions (Fig. 1). These observations put forward that unbound USP18 exhibits pronounced conformational flexibility but is arrested in a distinct conformation once ISG15 is bound.

ISG15 consists of two Ubl domains connected by a flexible linker region. In both complexes ISG15 interacts extensively via its C-terminal Ubl domain with the palm and thumb domain of USP18 (Fig. 2a) covering an area of 1870 Å². This is similar in size to the contact area formed between Ub and USP740 or Ub and USP2127. The C-terminal tail of ISG15 containing the LRLRGG motif also present in ubiquitin binds into the active site cleft of USP18 (Fig. 2a; Supplementary Fig. 3c,d).

An extensive network of hydrogen bonds mediate the interaction between ISG15 and USP18. In addition, a hydrophobic pocket of the USP18 palm domain accommodates the residues Leu150 and Leu152 of ISG15. This mode of binding closely resembles the interaction of ubiquitin with other USPs like USP740.

The C-terminal Ubl domain of ISG15 is essential and sufficient for USP18 binding and activity

The comparison of both USP18–ISG15 complexes in the asymmetric unit revealed that the C-terminal Ubl domains of ISG15 align with high similarity (r.m.s.d. of 0.5 Å over 73 Cα positions). In contrast, the N-terminal domain shows no or only marginal interaction with USP18 and its orientation differs largely between both complexes (Fig. 2a): Whereas in one complex the N-terminal Ubl domain approaches the thumb domain with the closest distance between Cα positions of 8 Å (Fig. 2a), the N-terminal domain in the second complex resides at a distance of 18 Å from USP18 (Fig. 2a). This conformational difference corresponds to a rotational movement of approximately 10 degrees around the flexible linker region of ISG15.

Taken together, the structural data suggest that only the C-terminal domain of ISG15 is critical for interaction with USP18. This is in contrast to the structures of di-ubiquitin in complex with USP2127 or with SARS coronavirus PLpro46, in which both domains of di-ubiquitin show noticeable contacts with the protease. Based on the structure of USP21 in complex with di-ubiquitin it had been suggested that USP18 binds ISG15 in a similar mode. In order to prove the observations from the structure described here, we tested the reactivity of USP18 towards the isolated C-terminal Ubl domain of ISG15 (ctodified with a propargylamide (PA) as an active site probe and compared it with full-length ISG15-PA. Both, full-length ISG15-PA and ct-ISG15-PA reacted readily with the active site cysteine of...
USP18 and formed a covalent complex. In contrast, no binding of USP18 to the respective Ub probe was observed (Fig. 2b). Likewise, the kinetic analysis of USP18 reactivity towards a fluorescent ct-ISG15-FP substrate revealed highly efficient cleavage (Table 2, Supplementary Fig. 4a). Moreover, USP18 cleaved ct-ISG15 as effectively as full-length ISG15 from cellular substrates (Fig. 2c). Thus, our results demonstrate that only the C-terminal Ubl domain of ISG15 is essential and sufficient for USP18 binding and activity.

**ISG15 binding induces conformational changes in USP18**

Alignment of the unbound USP18 molecules with the ISG15-bound USP18 revealed substantial structural differences. The most obvious changes occur at the finger domain that - after binding to ISG15 - adopts an orientation right between the two different conformations of unbound USP18 (Fig. 1a).

Moreover, the region comprising residues 255-267, which is disordered in unbound USP18, folds into a short stable antiparallel \(\beta\)-sheet (Fig. 3a). This part corresponds to the blocking loop 1 in other USPs like e.g. USP1442.

Another major structural change occurs in the switching loop. In ISG15-bound USP18, residues 129-135 of the thumb domain form an extended loop. This conformation enables access of the C-terminal LRLRGG tail of ISG15 into the catalytic cleft. In contrast, in unbound USP18 residues 131-135 fold into a short \(\alpha\)-helix. A structural overlay of unbound USP18 and the USP18–ISG15 complex reveals that residues Arg151 and Arg153 of the LRLRGG motif clash with this short \(\alpha\)-helix of unbound USP18 (Fig. 3b). Therefore, the \(\alpha\)-helix must unwind and the loop needs to change its conformation to allow for ISG15 binding.

Finally, the catalytic triad in ISG15-bound USP18 is formed by small movements of all three residues (His314, Asn331, Cys61) involved (Fig. 1b-d).

**Comparison with ubiquitin-binding USPs**

The overall structures of USP–Ub complexes and the USP18–ISG15 complex are remarkably similar (Fig. 4a,b) and as shown here only the C-terminal Ubl domain of ISG15 is required for binding. This puts forward the question how the exceptional specificity of USP18 is achieved and which residues in particular are involved. In order to identify critical residues, we performed a Dali search47 with the structure of ISG15-bound USP18 defining USP2, USP4, USP5, USP7, USP8, USP14 and USP21 as closest homologues. These proteases align with r.m.s.d. values between 1.8 Å and 2.7 Å with USP18. The highest similarity is shared between USP18 and USP7 (r.m.s.d. of 1.8 Å over 290 C\(\alpha\)). Although the SARS coronavirus PLpro shows high deISGylation activity46, the structural alignment reveals only an r.m.s.d. of 3.5 Å over 196 C\(\alpha\).

Intriguingly, the residues required for recognition of the C-terminal tail of ISG15 or Ub are strictly conserved between USP18, ubiquitin-processing USPs and the SARS CoV enzyme PLpro. Thus, other areas of USP18 must confer the specificity for ISG15. A structure-based sequence alignment of these proteases revealed a number of residues important for ISG15 binding and at the same time unique to USP18 (Supplementary Note 1). Two areas in the
USP18 binding surface were identified as candidates to confer specificity towards ISG15. The first area comprises residues Ala138, Leu142, Ser192 and His251 of USP18, which form a contiguous patch (Fig. 4a, Supplementary Note 1). The second area encompasses residues 256-263 located in the blocking loop 1 (Fig. 4a, Supplementary Note 1). We defined these areas as ISG15-binding box 1 and 2 (IBB-1 and IBB-2).

**ISG15-binding box 1 and 2 (IBB-1 and IBB-2)**

IBB-1 provides exclusively hydrophobic contacts with ISG15. The side chains of His149 and Trp121 in ISG15 are in close proximity forming a \( \pi-\pi \) contact that stabilizes the orientation of these residues and form a hydrophobic bulb. In USP18, residues His251, Ala138 and Leu142 are part of a hydrophobic pocket that perfectly accommodates the hydrophobic bulb of ISG15 (Fig. 4c). This characteristic hydrophobic contact is further stabilized by the side chain of Pro128 of ISG15 and Leu142 of USP18 (Fig. 4c). The corresponding regions of USP7 largely differ from USP18. In particular, the residues in USP7 corresponding to IBB-1 are more bulky and polar. Ser192, His251 and Ala138 of USP18 are all substituted by Gln residues in USP7. The hydrophobic Leu142 is replaced by an Arg. In particular, replacement of the Ala138 in USP18 by a polar residue in other USPs might block the access of the bulky and hydrophobic Trp121 side chain of ISG15 (Fig. 4d). In contrast to IBB-1, IBB-2 located on blocking loop 1 mediates mainly hydrogen bond binding of ISG15 (Fig. 4e). The corresponding blocking loop in USP7 is extended by two residues and adopts a conformation incompatible with ISG15 or ubiquitin binding. No hydrogen bonds are formed between USP7 and ubiquitin in this region (Fig. 4f).

**IBB-1 but not IBB-2 is critical for ISG15 binding and activity of USP18**

In order to evaluate the role of IBB-1 and IBB-2, we created USP18 variants carrying the respective residues of USP7. In USP18^{IBB-1-USP7} residues Ala138, Leu142, His251 and Ser192 of IBB-1 were replaced, in USP18^{IBB-2-USP7} the blocking loop 1 of USP7 was inserted, and USP18^{IBB-1-USP7—IBB-2-USP7} combined both modifications. Likewise, we generated an ISG15 variant that carries an Arg residue instead of Trp121 (ISG15-W121R), as well as a further variant where Trp121, Pro128 and His149 of ISG15 were changed to the corresponding residues of ubiquitin (ISG15-W121R-P128G-H149V). The interaction of murine USP18 and ISG15 as well as of several variants was analyzed by surface plasmon resonance (SPR). The binding curves and parameters are given in Supplementary Fig. 5. ISG15 bound to immobilized USP18 with high affinity characterized by a fast association and dissociation phase. The \( K_d \) of 1.49 ± 0.03 \( \mu \)M is in good agreement with the \( K_d \) of 1.3 ± 0.2 \( \mu \)M determined previously by microscale thermophoresis. Changing the residues in IBB-1 to the corresponding residues of USP7 (variant USP18^{IBB-1-USP7}) or in IBB-2 (variant USP18^{IBB-2-USP7}) resulted in a clear decrease in affinity towards ISG15. A more drastic drop in affinity was observed for USP18^{IBB-1-USP7—IBB-2-USP7} that combines both changes. In order to validate these results, we also analyzed variants of ISG15 with changes in the residues interacting with IBB-1 of USP18. The exchange of Trp121 in ISG15 to the corresponding Arg of Ub (variant ISG15-W121R) caused a clear drop in affinity and mutation of all three interacting residues Trp121, Pro128 and His149 (variant ISG15-W121R-P128G-H149V) almost abolished the interaction with USP18. Likewise, the observed binding ratio of ISG15 per immobilized USP18 dropped to 0.4 if Trp121 was
mutated and no clear stoichiometry of binding could be obtained for the variant ISG15-W121R-P128G-H149V, indicating that the observed binding might be due to some residual unspecific interaction. The observations for the ISG15 variants corroborated the structural data showing that IBB-1 represents an important determinant for the interaction between USP18 and ISG15.

To analyze the effect of changes in IBB-1 and IBB-2 on the catalytic activity of the enzyme, all three variants of murine USP18 were tested for covalent adduct formation with ISG15-PA, ct-ISG15-PA, and Ub-PA45. Wildtype USP18 reacted readily with ISG15-PA and ct-ISG15-PA within one minute. In contrast, USP18IBB-1-USP7 formed no adduct even after 5 minutes. The covalent adduct was observed only after prolonged incubation (Fig. 5a). USP18IBB-2-USP7 reacted almost as fast as wildtype but completion of the reaction was impaired as indicated by the presence of unreacted USP18. Strikingly, mutation of both IBBs in USP18IBB-1-USP7—IBB-2-USP7 completely abolished covalent complex formation (Fig. 5a). Of note, neither wildtype USP18 nor the USP18 variants reacted with Ub-PA even after prolonged incubation (Supplementary Fig. 6a). As shown by multiple sequence alignments IBB-1 and IBB-2 are well conserved among mammalians (Supplementary Fig. 7). To evaluate whether human USP18 recognizes ISG15 like murine USP18, we built a 3D model of the human USP18–ISG15 complex based on the structure of the murine USP18–ISG15 complex. As expected, the interaction surfaces are complementary and the model puts forward that IBB-1 of human USP18 is crucial for reactivity (data not shown). To evaluate these observations, we expressed and purified human USP18 and generated a human ISG15-PA probe. Since the interaction surfaces in the USP18–ISG15 complex are well conserved between human and murine proteins, we tested whether the ISG15-PA active site probes label USP18 across species. As predicted by the model, human USP18 reacted readily with murine ISG15-PA and vice versa (Supplementary Fig. 6b).

Next, we expressed human USP18 and USP18 variants carrying mutations for the active site cysteine, IBB-1 or IBB-2 in HEK 293T cells and tested those for reactivity (Supplementary Fig. 6c,d). Wildtype murine and human USP18 were readily labeled by human ISG15-PA, whereas the variants of USP18 lacking the active site cysteine (murine US18-C61A, human USP18-C64A) showed no reactivity (Supplementary Fig. 6c,d). Changing the residues of IBB-1 in human USP18 to the corresponding residues of USP7 abolished reactivity of human USP18 towards human ISG15-PA. This fully confirmed the results obtained for the murine enzyme. Changing the residues of IBB-2 in human USP18 had no noticeable effect, corroborating the results obtained for murine USP18. Since we introduced the residues of ubiquitin-cleaving USP7, we tested whether human USP18 wildtype or the different human USP18 variants are reactive towards Ub-PA. Neither human USP18 nor the variants were labeled by Ub-PA (Supplementary Fig. S6d).

To further characterize the importance of IBB-1 and -2, we tested the three murine USP18 variants for ISG15 deconjugation activity using the ISG15-FP28 and ct-ISG15-FP as substrates in fluorescence polarization assay. The kinetic analysis corroborated the previous results (Fig. 5b). For USP18IBB-1-USP7 only minor residual catalytic activity was observed (0.5% of wt activity), whereas USP18IBB-2-USP7 still cleaved the model substrate but with lower efficiency compared to USP18 wildtype (65% of wt activity).
USP18^{IBB-1-USP7—IBB-2-USP7} entirely lost enzymatic activity (kinetic parameters in Table 2). Neither wildtype USP18 nor the USP18 variants showed activity towards the Ub-FP substrate (Supplementary Fig 4b-e).

Next, we addressed the contribution of IBB-1 and IBB-2 on the recognition of endogenous cellular substrates of USP18. USP18-deficient cells were stimulated with interferon β to induce ISGylation of cellular proteins serving as substrates for recombinant USP18. As expected, wildtype USP18 added to cell lysates readily deconjugated ISG15 (Fig. 5c, lane 4). USP18^{IBB-1-USP7} showed no apparent cleavage (Fig. 5c, lane 6), whereas USP18^{IBB-2-USP7} still cleaved ISG15 conjugates (Fig. 5c, lane 8). In agreement with all previous experiments USP18^{IBB-1-USP7—IBB-2-USP7} did not cleave endogenous substrates. In summary, our results show that proper accommodation of the ISG15 hydrophobic patch is critical for both human and murine USP18 activity as even minor changes in this region have large impact on enzyme activity.

**The hydrophobic nature of IBB-1 is critical for ISG15 specificity**

Multiple sequence alignments of USP18 show that the hydrophobic character of IBB-1 is highly conserved among mammals and marsupialia (Supplementary Fig. 7). Likewise, the corresponding counterpart in ISG15, which carries the bulky tryptophan protruding from the surface, is also highly conserved (Supplementary Fig. 8). Thus, the hydrophobic interaction described here appears to be strictly conserved among different species. In particular, IBB-1 of USP18 and the hydrophobic patch of ISG15 represent one of the most conserved regions of these proteins. A similar degree of conservation is only observed for the catalytic cleft of USP18 and the C-terminal tail of ISG15 (Supplementary Fig. 7b and 8b). In contrast, instead of the two hydrophobic residues Ala138 and Leu142 (numbering *Mus musculus*), fish USP18 carries an Asp and a His residue at the respective positions (Supplementary Fig. 7a). Most interestingly, in fish ISG15 these variations are compensated by an exchange of the hydrophobic Trp121 and Pro128 (numbering *M. musculus*) to the polar residues Arg and Gln, respectively (Supplementary Fig. 8a). A molecular model of a USP18–ISG15 complex carrying these mutations suggested that the side chains of these polar residues can form several hydrogen bonds. This type of interaction is reminiscent of the respective interaction between other USPs and ubiquitin. Thus, we speculated that fish USP18 might not be restricted to ISG15 but also capable to recognize ubiquitin. To test this hypothesis we expressed USP18 from *Danio rerio* in HEK 293T cells and analyzed the cell lysates for reactivity with ISG15- and Ub-reactive probes (Fig. 6a). USP18 from *D. rerio* reacted readily with murine ISG15-PA (Fig. 6b). Most interestingly, USP18 from *D. rerio* reacted not only with ISG15-PA, but also with mammalian Ub-PA, whereas murine USP18 showed no cross-reactivity with Ub (Fig. 6c) 28. Although the portion of covalent modification of USP18 from *D. rerio* with Ub appeared to be less than with ISG15, it is clearly visible and not seen when the active site cysteine is mutated to alanine (Fig. 6c). These data further support that the IBB-1 region is critical for ISG15 specificity.
Discussion

Most members of the USP family of deubiquitinating enzymes cleave different kinds of ubiquitin chains from substrates. In addition, some USPs show cross-reactivity towards ISG15. To ensure specific functions of the different USPs, most enzymes of the USP family comprise further large domains mediating interaction with other protein complexes, specific organelles, or show temporal distribution. In contrast, USP18 represents a remarkably small member, which comprises only the USP core domain. Nevertheless, USP18 is specific for ISG15 and not cross-reactive towards ubiquitin or any other member of the Ubl family. Thus, the unique specificity must arise entirely from the USP core domain itself. Models based on structures of the isopeptidases USP21 and the viral SARS CoV PLpro suggested that also USP18 might recognize both Ubl domains of ISG15. In the USP21-diubiquitin complex the N-terminal Ub-domain is in contact with the finger domain. In the SARS CoV PLpro binding of the N-terminal Ubl domain to the thumb domain was observed. However, so far no structure of a complex of full-length ISG15 with an ISG15 protease was available. Our analysis demonstrates that only the C-terminal ubiquitin-like domain of ISG15 is sufficient for USP18 binding and activity. Moreover, we identified the structural determinants essential for the extraordinary substrate specificity of USP18. We have mapped two regions, namely IBB-1 and IBB-2, which are unique for USP18 and recognize regions of in the C-terminal Ubl domain of ISG15, which are not present in ubiquitin. Analysis of site-specific variants showed that IBB-1 represents the major determinant for ISG15 cleavage whereas IBB-2 has only minor contributions. This is also reflected in the conservation of IBB-1 across different species. While there is strict conservation of the residues in IBB-1 in mammals, fish USP18 shows no conservation within IBB-1. Our results suggest that fish USP18 constitutes a DUB cross-reactive to ubiquitin and ISG15. Furthermore, the IBB-1 region in fish USP18 has characteristics of ubiquitin-specific DUBs and lacks the two hydrophobic amino acids characteristic for IBB-1 of USP18.

Molecular modelling and interaction studies using several variants of murine and human USP18 with ISG15 showed that the conclusions drawn from the structure hold true for the human homologues. In contrast, in a recent study it was concluded that the mode of USP18–ISG15 interaction might differ between murine and human proteins. Yet, two recent studies provide evidence that USP18 is involved in de-ubiquitination of the TAK1-TAB1 complex or NEMO thereby inhibiting activation of NF-κB. The authors provide evidence that USP18 influences the ubiquitination status of NEMO independent of its protease activity. In contrast, they suggest that USP18 inhibits ubiquitination of the TAK1/TAB1 complex in a protease dependent manner. Our results clearly show that USP18 exhibits no reactivity towards ubiquitin in vitro indicating/demonstrating that the described inhibition of the NF-κB pathway by USP18 is rather an indirect effect and not mediated by direct interaction between USP18 and ubiquitin.

Besides ISG15, FAT10 constitutes another member of the Ubl family harboring two ubiquitin-like domains. So far no protease specific for FAT10 was discovered. It is likely that...
such an enzyme exists and it will be interesting to see whether specificity is achieved via specific detection of a particular patch within one Ubl domain as shown in this study for USP18 recognition of ISG15 or by contact to both Ubl domains.

Besides its function as an ISG15-protease USP18 constitutes a major negative regulator of type I interferon signaling. This property is clearly independent of the catalytic activity as IFN signaling in mice selectively lacking the active site cysteine is unaffected32. Based on mutational studies it was suggested that USP18 binds to the intracellular region of type I IFN receptor subunit IFNAR2 and outcompetes the downstream kinase JAK1 thereby abrogating IFN signaling36. The binding of primary signaling molecules to their cognate receptors in IFN signaling (JAK1 to IFNAR2 and Tyk2 to IFNAR1) is suggested to be highly conserved54. In IFNAR2 the interaction region consists of two motifs termed Box1 and Box2, with Box1 described to be critical for USP18 binding36. The hydrophobic nature of Box1 and of the USP18–ISG15 interaction plane suggests that a similar mode of interaction might occur between USP18 and IFNAR2.

In summary, here we unravel the molecular properties that determine the unique specificity of USP18. By identifying a distinct hydrophobic patch critical for ISG15–USP18 recognition, this study shows that even small changes on the surface are sufficient to generate a highly specific enzyme capable to distinguish between ubiquitin and the structurally related Ubl domain of ISG15.

**Online Methods**

**Cloning**

Sequences for all primers are listed in Supplementary Table 1. The vector pKL-His-3C-USP18 was generated as described previously28. Briefly, the cDNA of murine USP18 encoding residues 46-368 was amplified from vector pTriEx2-mUSP1832 with primers BstBI-3C-USP18-for and HindIII-USP18-rev and cloned into pKL vector55. In a second step, a His6-tag was introduced at the N-terminus using primers BstBI-His-3C-for and HindIII-USP18-rev. For generation of vectors encoding His6-tagged USP18 variants, synthetic cDNAs comprising the respective cDNA flanked by BstBI and HindIII restriction sites were purchased from Life technologies, digested with BstBI and HindIII and ligated into pKL vector.

The sequence of human USP18 (hUSP18) cDNA encoding residues 16-372 was PCR amplified using as primers attB1-3C-hUSP18-for and attB2-hUSP18-rev and as template the vector pCMV5a-hUSP18-Flag containing the full-length hUSP18 cDNA (kindly provided by Prof. Claudio Brancolini, University of Udine). The PCR fragment was cloned into pVL1393-His6-GST vector (in house modified) using the Gateway system (Invitrogen). The resulting sequence, coding for His6-GST-3C-hUSP18(16-372), was sequence verified by an external service (BMR Genomics, Italy).

To generate vector pTXB1-m(ouse)ISG15-C76S, the cDNA encoding for amino acid residues 1-154 of murine ISG15 was amplified from vector pACE-ISG15-C76S28 using primers NdeI-ISG15-for and SpeI-ISG15-rev. The PCR product was digested with restriction
enzymes NdeI and SpeI and ligated into pTXB1 vector. Vector pTXB1-h(uman)ISG15-C78S was generated cloning a synthetic cDNA with codons optimized for expression in *E. coli* encoding for residues 1-156 of human ISG15 with restriction sites NdeI and SpeI into pTXB1 (New England Biolabs). The resulting vector contains a serine instead of a cysteine residue at position 78 of ISG15.

The expression vector for mouse ISG15 pACE-m(ouse)ISG15-C76S was generated as described previously. Briefly, a synthetic cDNA encoding residues 1-155 with codons optimized for expression in *E. coli* fused to an N-terminal His<sub>6</sub>-tag and a HRV 3C protease cleavage site was cloned via NdeI and XhoI into vector pACE. The coding sequence contains a serine instead of a cysteine residue at position 76. Expression vectors pACE-mISG15-C76S-W121 and pACE-mISG15-C76S-W121-P128G-H149V were generated likewise using synthetic cDNAs. In the coding sequences tryptophan 121 or tryptophan 121, proline 128 and histidine 149 were replaced by the corresponding residues of ubiquitin, respectively.

For cloning of pcDNA3.1-HA-ISG15-C, a synthetic cDNA encoding the C-terminal domain of murine ISG15 (amino acid residues 77-155) in frame with an N-terminal HA-tag was purchased from Life technologies. The synthetic construct was digested with restriction enzymes NheI and BamHI and cloned into vector pcDNA3.1 (Invitrogen). Plasmids encoding human Ube1l and human UbcH8 as well as full-length murine ISG15 and murine Herc6 were described previously.

To generate an expression construct for USP18 of *Danio rerio* (zebrafish), cDNA isolated from the intestine of the organism (kindly provided by Pierre Boudinot, French National Institute for Agricultural Research) was used as a template for amplification with primers KpnI-drUSP18-for and XhoI-drUSP18-rev. The PCR product was cloned into vector pCR2.1 using the TA cloning kit (Life technologies). The resulting vector was digested with restriction enzymes KpnI and XhoI and cloned into vector pTriEx2 (Novagen). Vector pTriEx2-drUSP18-C38A was generated using the QuikChange II kit (Stratagene) and primers drUSP18-C38A-for and drUSP18-C38A-rev.

Vector pTriEx2-USP21cd encoding residues 197-565 of human USP21 was generated by amplification of USP21 cDNA from vector Flag-HA-USP21 (Addgene) with primers KpnI-USP21cd-for and XhoI-USP21cd-rev. The PCR product was digested with restriction enzymes KpnI and XhoI and cloned into vector pTriEx2 (Novagen).

**Protein expression and purification**

Mouse wildtype His<sub>6</sub>-tagged USP18 as well as the mouse USP18 variants were expressed in *Sf21* cells using the MultiBac system as described in28. For large scale expression, 200 ml of *Sf21* cells at a density between 0.5 and 1 × 10<sup>6</sup> cells·ml<sup>-1</sup> were infected with 200-600 µl of the respective virus. The cells were kept at the same density until proliferation arrest. Subsequently, expression of YFP was monitored every 24 h to determine the time point for cell harvesting. The cells were resuspended in Buffer A (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 500 mM NaCl, 4 mM DTT, pH 7.9) supplemented with 0.1% Tween20, 5 mM MgCl<sub>2</sub> and traces of DNAse and lysed by two steps of freezing and thawing. The cleared lysate was applied to
a 1 ml HisTrapFF column (GE Healthcare). The bound protein was eluted in Buffer B (50 mM Na$_2$HPO$_4$, 500 mM NaCl, 500 mM imidazole, 4 mM DTT, pH 7.9).

His$_6$-GST-h(uman)USP18(16-372) was expressed in Sf9 cells using BaculoGold expression system (BD Biosciences) following the standard protocol given by the producer. The Sf9 cells were co-transfected with linearized BaculoGold DNA and pVL-His$_6$-GST-3C-USP18. The high titre viral stock was generated by four rounds of amplification and used for large scale protein expression. For large scale expression, 2 l of Sf9 cells at density of $1.5 \times 10^6$ cells ml$^{-1}$ were infected with 15 ml of virus stock and harvested by centrifugation after 3 days cultivation at 27°C. The cells were lysed by homogenization (Emulsiflex - Avestin) in 150 ml of lysis buffer (50 mM Na$_2$HPO$_4$, 500 mM NaCl, 10% glycerol, 20 mM DTT, pH 8.0) supplemented with 20 µg ml$^{-1}$ DNase I, 1 mM MgCl$_2$, 0.01% Tween and protease inhibitor cocktail. The lysate was cleared by centrifugation at 20,000 $g$ for 1 h at 4°C and the supernatant was applied to a 12 ml column of GST Sepharose 4FF (GE Healthcare). The column was washed with lysis buffer equilibrated with 3C protease buffer (50 mM Na$_2$HPO$_4$, 500 mM NaCl, 10% glycerol, 2 mM DTT, 1 mM EDTA, pH 8.9). hUSP18 without tag was obtained by the 3C protease cleavage on the GST column at 4°C overnight and elution in the same buffer. The protein was further purified by size exclusion chromatography on a Superdex 200 (10/300) column (GE Healthcare) in a buffer containing 25 mM Hepes-NaOH, 500 mM NaCl, 10% glycerol, 2 mM DTT, pH 8.0.

Mouse ISG15 variants were expressed and purified as described previously for ISG15 wildtype28.

**Generation of ISG15-PA, ct-ISG15-PA, and Ub-PA probes**

**ISG15-PA**—Mouse and human ISG15-PA was prepared using intein chemistry via a method similar to that reported in literature58. Expression of mouse and human ISG15-intein fused to intein and chitin-binding domains was performed in E. coli BL21 (DE3) cells using ZYP-5052 autoinduction media59 supplemented with 50 µg ml$^{-1}$ carbenicillin at 37°C. After 3 to 4 h at an OD$_{600nm}$ = 0.6, the temperature was lowered to 18°C and the cells were allowed to grow an additional 16 h after which they were harvested by centrifugation. The cell pellet from a 2.5 l culture was resuspended in 80 ml lysis buffer (50 mM HEPES, 100 mM sodium acetate, pH 6.5 with protease inhibitor cocktail (Complete, Roche) and lysed by sonication. The lysate was clarified by centrifugation and the supernatant was loaded onto a 30 ml chitin bead column (New England Biolabs) at a flow rate of 0.5 ml min$^{-1}$. The column was washed with 120 ml lysis buffer, followed by 60 ml lysis buffer containing 50 mM β-mercaptoethanesulfonic acid sodium salt (MESNa). Another 30 ml of lysis buffer containing 50 mM MESNa was added to the beads, which were then incubated overnight at 37°C. The ISG15-MESNa thioester was eluted with 25 ml lysis buffer. The combined fractions were concentrated by ultrafiltration and applied to size exclusion chromatography column Superdex 75 (16/600) equilibrated in 50 mM MES, 100 mM sodium acetate, pH 6.5 and eluted in the same buffer. Fractions containing ISG15-MESNa were combined concentrated by ultrafiltration (Millipore, Amicon Ultra-15 centrifugal filter unit, 3,000 Da cut-off) to a concentration of 5 mg ml$^{-1}$. The ISG15-MESNa thioester was converted into the corresponding propargylamide (PA) by addition of propargylamine at a final concentration.
of 225 mM. LC-MS analysis indicated a complete conversion after 90 minutes. The mixture was acidified by addition of acetic acid to pH 4.5 and directly purified by RP-HPLC on a Waters HPLC system equipped with a Waters XBridge Prep C18 5µm OBD column (30 x 150 mm). Column mobile phases: A= MQ, B= CH₃CN and C= 1% TFA in MQ. Flow rate was 37.5 ml min⁻¹ applying a gradient of 20% to 60% B with 10% C over 15 min. Pure fractions containing ISG15-PA were combined and lyophilized. This yielded 22 mg of the pure protein probe. The construct was dissolved in DMSO (10 mM) and reconstituted into a buffer containing 50 mM MES and 100 mM NaCl, pH 6.5.

**Ac-ct-ISG15-PA**—The C-terminal domain of mouse ISG15 comprising residues 80-154 (ct-ISG15) was synthesized by solid phase peptide chemistry (SPPS), via a method similar to that reported in literature60. The peptide sequence Ac-LSILVR NERGHNSIYE VFLTQTVDTL KKKVSEQEQV HEDQFWLSFE GRPMEDKELL GEYGLKPQCT VIKHRLRPG (corresponding to mouse ISG15 residues 80-154) was synthesized in one linear automated (Prelude, Protein Technologies, inc.) Fmoc-based SPPS using a pre-loaded Fmoc-glycine-trityl-resin (TentaGel R Trt resin, RAPP Polymere, 0.19 mmol g⁻¹) on a 50 µmol scale. The underlined residues indicate the positions where pseudoproline dipeptides were used. 12.5 µmol of the fully protected polypeptide was selectively cleaved from the resin by treatment with 1,1,1,3,3,3-hexafluoroisopropanol (20% v/v in DCM, 2 x 20 min.) after which all solvents were removed by evaporation under reduced pressure. The residue was dissolved in 2 ml DCM and to this were added propargylamine (10 eq., 125 µmol, 8.0 µl), Pybop (4 eq., 50 µmol, 26 µg) and triethylamine (4 eq., 50 µmol, 8.5 µl). After overnight stirring the mixture was concentrated to dryness and co-evaporated three times with 1,2-dichloroethane. The polypeptide was fully deprotected by treatment with TFA/H₂O/phenol/iPr₃SiH (90.5/5/2.5/2, v/v/v/v) for 3 h. After washing the resin with 3x1 ml TFA, the crude protein was precipitated with cold Et₂O/n-pentane 3:1 v/v. The precipitated protein was washed 3x with Et₂O, the pellet was dissolved in a mixture of H₂O/CH₃CN/AcOH (65/25/10 v/v/v) and finally lyophilized. The final product was purified by RP-HPLC on a Shimadzu LC-20AD/T equipped with a C8 Vydac column (Grace Davison Discovery SciencesTM). Column mobile phases: A= 0.05% aq. TFA and B= 0.05% TFA in CH₃CN. T= 40°C. Flow rate= 5 ml min⁻¹. Gradient: 25 to 60%B over 18 min. The pure product containing fractions were pooled and lyophilized. The Ac-ct-ISG15-PA probe was finally purified by size exclusion chromatography using a NGC Chromatography System (Biorad) equipped with a Superdex 75 (16/600) column (GE Healthcare) in a buffer containing 50 mM MES and 100 mM NaCl at a flow rate of 1 ml min⁻¹.

**Ub-PA**—Ub-PA was synthesized by the method reported in literature45.

**Enzyme kinetics and ISG15 FP and Ub FP assays**

The fluorescence polarization (FP) assays were performed in non-binding surface flat bottom low flange black 384-well plates (Corning) at room temperature in a buffer containing 50 mM Tris-HCl pH 7.5, 2 mM DTT, 100 mM NaCl, 1 mg ml⁻¹ 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid (CHAPS) and 0.5 mg ml⁻¹ bovine gamma globulin (BGG). Two-fold serial dilutions (200 nM to 0.4 nM) of the enzyme solutions (USP18 wt, USP18IBB-1-USP7, USP18IBB-2-USP7 and USP18 IBB-1—IBB-2-USP7)
were made. 10 μl of each of the dilution steps was added to the empty wells of the plate. The reaction was started by addition of 10 μl of the ISG15-FP substrate or the Ub-FP substrate (200 nM final concentration)28. The fluorescence intensities in the S (parallel) and P (perpendicular) directions were recorded in intervals of 1 min on a BMG Labtech Pherastar plate reader (excitation 540 nm, emission 590 nm). From these S and P values the FP values (in mP) were calculated by adjusting the FP value (L) of the tracer molecule TAMRA-KG to 50 mP:

\[
Polarization\ (mP) = \frac{S - (G \cdot P)}{S + (G \cdot P)} \cdot 1000 \quad \text{where} \quad G = \frac{\text{average} S}{\text{average} P} \cdot \frac{1 - L/1000}{1 + L/1000}
\]

The kinetic parameters were determined with a fixed USP18 final concentration of 6 nM for USP18 wt, 500 nM for USP18BB-1-USP7, 6 nM for USP18BB-2-USP7 and 500 nM for USP18-1—2-USP7 and a serial dilution series of the substrate (0.2 µM to 5 µM). Kinetic data were collected in intervals of 90 s. From the obtained polarization values \( P_t \) the amount of processed substrate \( S_t \) was calculated with the following equation:

\[
S_t = S_0 - S_0 \times \frac{P_t - P_{\text{min}}}{P_{\text{max}} - P_{\text{min}}}
\]

\( P_t \) is the polarization measured (in mP); \( P_{\text{max}} \) is the polarization of 100% unprocessed substrate (determined for every reagent at all used substrate concentrations); \( P_{\text{min}} \) is the polarization of 100% processed substrate; \( S_0 \) is the amount of substrate added to the reaction. From the obtained \( P_t \) values the values for initial velocities \( v_i \) were calculated, which were used to determine the Michaelis-Menten constants \( (K_m, V_{\text{max}} \text{ and } k_{\text{cat}}) \) by fitting the data according to the formula below (where \( k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \)). All experimental data was processed using Ms Excel and Prism 7.00 (GraphPad Software, Inc.).

\[
v_i = \frac{V_{\text{max}} \times S_0}{K_m + S_0}
\]

Analysis of USP18/ISG15 interaction with surface plasmon resonance

Surface plasmon resonance binding analysis was performed on a Biacore X100 machine (GE Healthcare). Wildtype mouse USP18 and mouse USP18 variants were immobilized via the N-terminal His\(_6\)-tag on a NID200M Ni-NTA-chip (XanTec bioanalytics). Protein binding analysis was performed at 25°C in 50 mM HEPES, 150 mM NaCl, 0.05 mM EDTA, 0.5 mM DTT, 0.01% P20, pH 7.5 with a flow-rate of 20 µl min\(^{-1}\). Stock solutions of mouse ISG15 and mouse ISG15 variants were diluted in running buffer. Binding traces were analyzed with BIAevaluation software (GE Healthcare) and fitted with a 1:1 binding model including a drift of the baseline.

Reaction of USP18 and USP18 variants with ISG15-PA, ct-ISG15-PA and Ub-PA probes

USP18 wildtype and USP18 variants were diluted as pure proteins in 50 mM Na\(_2\)HPO\(_4\), 500 mM NaCl, 10 mM DTT pH 7.9 or in 50 mM Tris-HCl, 100 mM NaCl, 0.5 mg ml\(^{-1}\) CHAPS.
and 5 mM DTT, pH 7.6 to a final concentration of 5 µM. Pure ISG15-PA, ct-ISG15-PA or Ub-PA probes were added at a 1:1 molar ratio and incubated for up to 60 min at room temperature.

For time course experiments, samples were taken at 1 min, 5 min, and 40 min and the reaction was stopped by addition of 4x loading buffer buffer (250 mM Tris-Cl pH 6.8, 40% (w/v) glycerol, 5% (w/v) SDS, bromophenol blue, 400 mM DTT). Complex formation was visualized by Tricine SDS-PAGE.

Generation of USP18–ISG15 complex for crystallization

For crystallization, 4 mg of His$_6$-tagged USP18 was mixed with ISG15-PA at a 1:1 molar ratio and incubated for 30 min at 21°C. The complex was purified by size exclusion chromatography in 20 mM Tris-Cl pH 7.9, 300 mM NaCl, 5 mM DTT, 0.1 mM EDTA using a Superdex 200 (16/600) column (GE Healthcare). The purified complex was supplemented with 50 mM DTT (final concentration) and directly used for crystallization.

Reaction of USPs expressed in HEK 293T cells with ISG15-PA and Ub-PA probes

HEK 293T cells were transfected with plasmids encoding for full-length USP18 from different species (Mus musculus, Homo sapiens, Danio rerio) and USP21 catalytic domain (residues 197-565) (Homo sapiens) using Xtreme Gene (Roche) according to the manufacturer’s instructions. Following variants of USP18 were obtained by gene synthesis: murine USP18$^{ribb-1-usp7}$ with A138Q-L142R-H251Q, murine USP18$^{ribb-2-usp7}$ with residues 256-263 (S-A-R-N-S-R-T-E) replaced by the corresponding residues 411-420 of USP7 (M-Y-D-P-Q-T-D-Q-N-I), murine USP18$^{ibb-1-usp7-ibb-2-usp7}$ comprising both changes, human USP18$^{ribb-1-usp7}$ with A141Q-L145R-H255Q, human USP18$^{ribb-2-usp7}$ residues 260-267 (S-I-R-N-S-Q-T-R) replaced by the corresponding residues 411-420 of USP7 (M-Y-D-P-Q-T-D-Q-N-I), and human USP18$^{ibb-1-usp7-ibb-2-usp7}$ comprising both changes. All proteins were expressed with an N-terminal S-tag. Cells were lysed 48 h after transfection in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton-X100, 10 mM DTT. To monitor reactivity and specificity of the different USPs, 10 µg of each lysate was combined with 1-2 µg of mISG15-PA, hISG15-PA or Ub-PA probes, respectively. The reaction mixture in 50 mM Na$_2$HPO$_4$, 500 mM NaCl, 10 mM DTT, pH 7.9 was incubated for 2 h at 37°C and stopped by addition of sample buffer and boiling. The samples were analyzed by Western Blot with antibodies directed against the S-tag (Novagen) and GAPDH (Millipore).

Generation of ISGylated substrates and deISGylation assay

USP18-deficient mouse embryonic fibroblasts were stimulated with 250 U/ml IFNβ for 24h to induce ISGylation. To generate lysates with HA-ISG15- or HA-ct-ISG15-conjugated substrates, HEK 293T cells were transfected with vectors encoding hUbe1L, hUbcH8 and mHerc6 together with the plasmid encoding HA-ISG15 or HA-ct-ISG15. The cells were lysed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton-X100. 20 µg of the lysate was incubated with 20 µg of USP18 or the USP18 variants. The reaction was performed in 50 mM Na$_2$HPO$_4$, 500 mM NaCl, 10 mM DTT pH 7.9 for 0 and 2 h at 37°C. The samples were analyzed by Western blot with antibodies directed against ISG1562, HA
(Y-11, Santa Cruz, Santa Cruz, USA), β-Actin (I-19, Santa Cruz, Santa Cruz, USA) and GAPDH (Millipore, Billerica, USA).

**Crystallization**

Crystallization was performed in sitting-drop vapor diffusion experiments at 20°C. His₆-tagged USP18 was used at a concentration of 2.7 mg ml⁻¹ in 20 mM Na₂HPO₄, 300 mM NaCl, 50 mM DTT, pH 7.9 for crystallization. 300 nl of the protein was mixed with 300 nl crystallization solution and equilibrated against 50 µl of the same solution. Initial spherulithes were obtained with 0.2 M MgCl₂, 0.1 M Hepes, pH 7.5, 25% (w/v) PEG 3350 as crystallization solution and used to generate seedstocks. To this end, the spherulites were diluted and crushed in 50 µl crystallization solution. In subsequent crystallization trials, 300 nl protein was mixed with 200 nl crystallization solution and 100 nl seedstock and crystals grew in 0.09 M succinate, pH 7.0, 13.5% (w/v) PEG 3350, 10 mM KBr. Prior to vitrification in liquid nitrogen, the crystals were incubated for 1 min in crystallization solution supplemented with 20% (v/v) glycerol. The USP18–ISG15 complex was used at a concentration of 5 mg ml⁻¹ for crystallization. Spherulithes grew in 12.5% (w/v) PEG 1000, 12.5% (w/v) PEG 3350, 12.5% (v/v) MPD, 0.03 M MgCl₂, 0.03 M CaCl₂, 0.1 M MES/Imidazole, pH 6.5 and were used to generate seedstocks. The seedstock was added in further crystallization trials and crystals were obtained using a crystallization solution with 12.5% (w/v) PEG 1000, 12.5% (w/v) PEG 3350, 12.5% (v/v) MPD, 0.03 M NaNO₃, 0.03 M Na₂HPO₄, 0.03 M (NH₄)₂SO₄, 0.1 M MOPS-Hepes-NaOH, pH 7.5. The crystals were flash frozen in liquid nitrogen.

**Data collection, structure determination and refinement**

For USP18, data were collected at 100 K with a wavelength of 1.0 Å at beamline X06SA at the Swiss Light Source (Villigen, Switzerland) using a microfocus setup and a Mar225 CCD detector. Data for the USP18–ISG15 were collected at 100 K with a wavelength of 1.0 Å at beamline X06DA, Swiss Light Source, equipped with a PILATUS 2M detector. All diffraction data were integrated with XDS63. The structure of unbound USP18 was solved by molecular replacement with Phaser64 using USP7 (PDB code 1NB8) as search model. For the structure of the USP18–ISG15 complex the structures of unbound USP18 and murine ISG15 were used as search model in molecular replacement trials with Phaser. Subsequent rounds of model building and refinement were done with Coot65, Refmac566, and phenix.refine67. Data collection and refinement statistics are given in Table 1. Figures of the structures were prepared with Pymol68.

**Structure and sequence analysis**

Multiple structural alignment was performed using the Dali server47 and the coordinates of the USP18–ISG15 complex as search model. The structures of human USPs were chosen from the list of structural neighbors and the structure-based sequence alignment was visualized with Jalview69. The following structures were compared with USP18: USP7 (PDB code 1NBF, chain B)40, USP21 (PDB code 2Y5B, chain A)27, USP2 (PDB code 2IBI, chain A), USP14 (PDB code 2AYN, chain A)42, USP8 (PDB code 3N3K, chain A)70, USP4 (PDB code 2Y6E, chain B)71, USP5 (PDB code 3IHP, chain A)72, SARS CoV PLpro (PDB code 5E6J, chain A)46.
Data Availability Statement

Coordinates and structure factors for USP18 and USP18–ISG15 complex were deposited at the protein database under accession code 5CHT and 5CHV, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


44. Molland K, Zhou Q, Mesecar AD. A 2.2 Å resolution structure of the USP7 catalytic domain in a new space group elaborates upon structural rearrangements resulting from ubiquitin binding. Acta Crystallographica Section F, Structural Biology Communications. 2014; 70:283–287. [PubMed: 24598911]

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Figure 1. Structure of USP18 in the unbound and ISG15-bound state

(a) USP18 crystallized with two molecules in the asymmetric unit. Superposition of the two molecules in the asymmetric unit (chain A dark blue, chain B, green). For comparison ISG15-bound USP18 (light blue) is structurally aligned. The Zn$^{2+}$ in the finger domain are shown as spheres and illustrate the extent of the movement. Residues 255-267 corresponding to blocking loop 1 are unordered in unbound USP18 and not resolved in the electron density, whereas they form a short antiparallel $\beta$ sheet in ISG15-bound USP18. (b-d) Close-up view of the catalytic triad in unbound and ISG15-bound USP18. In all structures of USP18 the residues Cys61, His314, Asn331 forming the catalytic triad are in close proximity. (b) Only in ISG15-bound USP18 the side chains of the three residues exhibit the correct orientation and distances to stabilize a thiolate state of the $S\gamma$ of Cys61. (c) (d). In both molecules of unbound USP18 the imidazole of His314 is slightly shifted away from Cys61 weakening the interaction of the two side chains.
Figure 2. The C-terminal Ubl domain of ISG15 is sufficient for USP18 binding and activity.
(a) Structural alignment of both USP18–ISG15 complexes present in the asymmetric unit. Just one USP18 molecule (blue) is shown for clarity. Left hand side: surface representation of USP18 (blue) with ISG15 aligned from both complexes (red, orange). Right hand side: the molecules are rotated by 180° around the y-axis. USP18 is shown as cartoon with outline of the surface. The C-terminal Ubl domain of ISG15 is embedded between the finger and the thumb domain. In contrast the N-terminal Ubl domain of ISG15 exhibits no contacts to USP18. In both complexes the N-terminal domain adopts a different position with regard to the C-terminal domain and resides in a distance of 8 Å or 18 Å from USP18 thumb domain (distance between the Cα atoms of ISG15 Ser22 and USP18 Glu91). (b) USP18 binds both, full-length ISG15 and the C-terminal domain of ISG15 (ct-ISG15) with the same efficiency. The reaction products of stoichiometric amounts of USP18 with full-length ISG15-PA, ct-
ISG15-PA, or ubiquitin-PA (Ub-PA) were visualized by a shift in molecular mass on Coomassie-stained SDS-PAGE. (e) USP18 cleaves ct-ISG15 from cellular substrates. Cell lysates with cellular substrates modified by HA-tagged ct-ISG15 or HA-tagged-full-length ISG15 were incubated with recombinant USP18. Deconjugation of full-length ISG15 or ct-ISG15 from cellular proteins was monitored by immunoblotting with an anti HA antibody (lane 7 and lane 11).
Figure 3. Conformational changes in USP18 upon ISG15 binding
Structural alignment of USP18 (light blue) bound to ISG15 (orange) and unbound USP18 (dark blue) reveals a conformational change in the finger domain as well as in the blocking and switching loops. (a) Residues 255-267 (blocking loop 1) that are unordered in unbound USP18 fold into a short β-sheet that interacts with ISG15. (b) The switching loop connects helix 4 and helix 5 of the thumb domain and comprises residues 129-135. In unbound USP18 residues 131-135 fold into a short α-helix that blocks the access of the C-terminal LRLRGG motif of ISG15 to the catalytic site of USP18. In ISG15-bound USP18 the α-helix unwinds and the entire loop comprising these residues is displaced.
Figure 4. ISG15-binding boxes 1 and 2 in USP18.

(a) Structure of the USP18–ISG15 complex. USP18 is shown in blue and ISG15 in orange. The ISG15-binding boxes 1 (IBB-1) and 2 (IBB-2) of USP18 are depicted in green and purple, respectively. (b) Structural alignment of the USP18–ISG15 complex with the USP7–Ub complex (PDB code 1NBF). USP18 and ISG15 are shown in blue and orange, USP7 is shown in grey and Ub in yellow. The orientation of Ub bound to USP7 closely resembles the orientation of the ISG15 C-terminal domain in the USP18–ISG15 complex. (c) Close-up view of ISG15-binding box 1 (IBB-1). The residues of USP18 forming IBB-1 are shown in
green and form a hydrophobic pocket to accommodate the bulky aromatic side chain of Trp121 from ISG15 shown in orange. (d) Superposition of IBB-1 with the respective region of the USP7–Ub complex. The labeling refers to residues in USP18 and ISG15. The interaction between USP18 and ISG15 is mediated by hydrophobic residues, whereas USP7 and ubiquitin display polar residues in this region. (e) Close-up view of IBB-2 (purple). Several residues of a short antiparallel β-sheet of USP18 from hydrogen bonds (dotted lines) with ISG15. For clarity, the side chains of the interacting residues are omitted. (f) Superposition of IBB-2 with the respective region of the USP7–Ub complex. The distances between ubiquitin and USP7 are larger than in the USP18–ISG15 complex.
Figure 5. ISG15-binding box 1 but not ISG15-binding box 2 is critical for USP18 activity.
(a) Wildtype USP18 and the different USP18 variants were incubated with ISG15- and ct-ISG15-PA probes for the indicated times. Complex formation was visualized on Coomassie-stained SDS-PAGE. The figure shown is representative for three independent experiments.
(b) Catalytic activity of wildtype USP18 and USP18 variants towards the ISG15-FP substrate. Different amounts of USP18 proteins were incubated with ISG15-FP and the cleavage of the substrate was monitored by the change in fluorescence polarization. mP, millipolarization unit. (c) DeISGylation of endogenous substrates by wildtype USP18 or USP18 variants. ISG15 cleavage by USP18 is monitored by Western blot with an antibody directed against ISG15.
Figure 6. Zebrafish USP18 recognizes ISG15 and ubiquitin
(a) HEK 293T cells were transfected with S-tagged versions of mouse USP18 (mUSP18), mouse USP18 with the active site cysteine replaced by alanine (mUSP18-C61A), human USP21 (hUSP21), zebrafish USP18 (drUSP18) and zebrafish USP18 with the active site cysteine replaced by an alanine (drUSP18-C38A) or left untransfected (control). Protein expression was visualized on Western blot with an antibody directed against the S-tag. (b) Protein lysates from cells transfected with indicated expression constructs were incubated with the active site-directed probe ISG15-PA and complex formation was monitored by a size shift detectable upon immunoblotting using an anti-S-Tag antibody. (c) Protein lysates from cells transfected with indicated expression constructs were incubated with the active site-directed probe Ub-PA and complex formation was monitored by a size shift detectable upon immunoblotting using an anti-S-Tag antibody. USP21, which is cross-reactive for ISG15 and ubiquitin served as a positive control for Ub binding. Results shown in panel a-c are representative for three independent experiments.
Table 1
Data collection and refinement statistics (molecular replacement)

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<tr>
<td>a, b, c (Å)</td>
<td>53.96, 89.75, 149.40</td>
<td>64.04, 72.80, 217.26</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 90, 90</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>28-2.8 (2.95-2.8)</td>
<td>37-3.0 (3.16-3.0)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>30.1 (277.1)</td>
<td>62.7 (293.4)</td>
</tr>
<tr>
<td>Rpim (%)</td>
<td>30.1 (134.5)</td>
<td>18.0 (84.4)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>6.29 (0.79)</td>
<td>3.53 (0.86)</td>
</tr>
<tr>
<td>CC₁/₂</td>
<td>98.3 (23.9)</td>
<td>97.1 (34.8)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.7 (99.9)</td>
<td>99.9 (99.9)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.86 (4.95)</td>
<td>13.0 (12.9)</td>
</tr>
</tbody>
</table>

Refinement

| Resolution (Å)  | 28-2.8 | 37-3.0 |
| No. reflections | 18487  | 20936  |
| Rwork / Rfree (%) | 22.5 / 28.3 (39.0 / 40.8) | 22.5 / 28.6 (32.6 / 36.9) |
| No. atoms       | 4943   | 7445   |
| Protein         | 4885   | 7426   |
| Ion             | 3      | 14     |
| Water           | 55     | 5      |
| B factors       |        |
| Protein         | 74.1   | 59.3   |
| Ligand/ion      | 78.4   | 66.0   |
| Water           | 51.8   | 18.2   |
| R.m.s. deviations |       |        |
| Bond lengths (Å) | 0.009  | 0.003  |
| Bond angles (°) | 1.059  | 0.736  |

Data were collected from one crystal, respectively.

*Values in parentheses are for highest-resolution shell.*
Table 2
Kinetic parameters of murine USP18 and USP variants

<table>
<thead>
<tr>
<th>protein</th>
<th>reagent</th>
<th>$K_M$ / µM</th>
<th>$k_{cat}$ / sec$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>USP18</td>
<td>ISG15-FP</td>
<td>4.8 ± 0.3</td>
<td>0.23 ± 0.3</td>
</tr>
<tr>
<td>USP18$^{#1}$</td>
<td>ISG15-FP</td>
<td>n/a</td>
<td>$1.2 \times 10^{-4} \pm 0.6 \times 10^{-4}$ $^{*2}$</td>
</tr>
<tr>
<td>USP18$^{#2}$</td>
<td>ISG15-FP</td>
<td>39 ± 11</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>USP18</td>
<td>ISG15-FP</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>USP18$^{#2}$</td>
<td>ct-ISG15-FP</td>
<td>0.48 ± 0.03</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

$^{*1}$values from Basters et al.28  

$^{*2}$no saturation was observed at the concentrations that can be used in the assay. The highest activity observed is reported. No $K_M$ value could be extracted from the data.