Rpn11-mediated ubiquitin processing in an ancestral archaeal ubiquitination system

Adrian C.D. Fuchs¹, Lorena Maldoner¹, Matthias Wojtynek¹, Marcus D. Hartmann & Jörg Martin

While protein ubiquitination was long believed to be a truly eukaryotic feature, recently sequenced genomes revealed complete ubiquitin (Ub) modification operons in archaea. Here, we present the structural and mechanistic characterization of an archaeal Rpn11 deubiquitinase from Caldiarchaeum subterraneum, CsRpn11, and its role in the processing of CsUb precursor and ubiquitinated proteins. CsRpn11 activity is affected by the catalytic metal ion type, small molecule inhibitors, sequence characteristics at the cleavage site, and the folding state of CsUb-conjugated proteins. Comparison of CsRpn11 and CsRpn11–CsUb crystal structures reveals a crucial conformational switch in the CsRpn11 Ins-1 site, which positions CsUb for catalysis. The presence of this transition in a primordial soluble Rpn11 thus predates the evolution of eukaryotic Rpn11 immobilized in the proteasomal lid. Complementing phylogenetic studies, which designate CsRpn11 and CsUb as close homologs of the respective eukaryotic proteins, our results provide experimental support for an archaeal origin of protein ubiquitination.
Protein ubiquitination regulates a wide range of physiological processes, often by targeting modified substrate proteins for degradation by the proteasome. Substrates can be (poly)ubiquitinated via isopeptide bonds by a cascade of E1, E2, and E3 enzymes, a process that is reversed by specific deubiquitinas, among them zinc-dependent JAMM (JAB1/MPN/Mov34) proteases like the proteasomal Rpn11. Although classical ubiquitination is thought to be restricted to eukaryotes, several distinct but evolutionary-related pathways are present in prokaryotes, which usually comprise just one ubiquitin (Ub)-, E1-, and JAMM homolog each. These systems are typically involved in cofactor synthesis, such as thiamine or molybdenum cofactor, but can also be bifunctional, as shown for the archaeon *Haloflex volcanii*. Here, SAMPs (small archaeal modifier protein) are also used to modify protein functions or to mark substrates for proteasomal degradation. However, given the absence of E2 and E3 enzymes in *H. volcanii* and other SAMP-containing archaea, and the evolutionary distance between SAMP and Ub, it seems unlikely that the SAMP system is the direct progenitor of the eukaryotic ubiquitination system.

The first complete archaeal Ub modifier system, including E2 and E3 enzymes, was discovered in 2011 in *Candidatus Caldiarchaeum subterraneum*. Remarkably, all involved proteins were found to be far more similar to their eukaryotic counterparts than to any prokaryotic homologs. Thus, eukaryote-type protein ubiquitination might have already evolved in archaea. This interpretation was supported by the more recent discovery of *Asgard archaea*, which harbor several ancestral eukaryotic features and whose genomes also contain potential Ub modifier systems. In line with this, the capability of *C. subterraneum* E1, E2, and E3 enzymes to conjugate Ub to an artificial substrate protein suggests that these primordial modification systems may have an equivalent function in these archaea.

*C. subterraneum* Ub (CsUb), E1, E2, and E3 are organized in a single operon, adjacent to a second E3 protein and a JAMM protease (CsRpn11) with high sequence similarity to eukaryotic Rpn11. In eukaryotes, this deubiquitinase cannot act on its own, but is embedded within the proteasome lid, wherein it forms a heterodimer with an inactive homolog, Rpn8. This organization is retained in paralogous eukaryotic structures, known as PCI complexes (Proteasome—CP9 signalsome—Initiation factor 3), but not known to exist in archaea. In contrast to Rpn11-related proteases like AMSH, which act on Ub in isolation and are constitutively active, eukaryotic Rpn11 was shown to undergo a conformational switch at its Ins-1 site upon Ub binding. This was interpreted to be a regulatory feature, allowing deubiquitination to occur only after the Ub-conjugated substrate became committed to unfolding and degradation by the proteasome.

Here, we analyze the evolutionary relationships of CsRpn11 and CsUb with their eukaryotic homologs. Our bioinformatic, structural, and biochemical results reveal unique features of the archaeal proteins, but also remarkable conceptual similarities with their eukaryotic counterparts. They not only bolster the hypothesis of an ancestral archaeal Ub modification system, but also provide insight into the functioning and mechanism of eukaryotic Rpn11-like enzymes.

Results and discussion

Co-evolution of archaeal ubiquitins and Rpn11-like proteins.

To put the archaeal Ub modification system into an evolutionary context, we started out our investigation with an in-depth sequence analysis of CsRpn11 and CsUb. While CsRpn11 can be easily recognized as a member of the JAMM protease family, its role in a putative ancient ubiquitination system should be reflected by its particular evolutionary relationship to eukaryotic Rpn11, as compared to other archaeal JAMM proteases. To look into this aspect, we built a bioinformatic map of the JAMM protease universe, by searching for JAMM homologs in the non-redundant protein sequence database and clustering them by pairwise sequence similarity (Fig. 1a). In the resulting CLANS map, the eukaryotic active PCI complex deubiquitinas (CSN5, Rpn11; elF3H is inactive) form a single cluster that is centrally located between the other eukaryotic clusters and forms the strongest connections to prokaryotic sequences. Further removed are the inactive PCI complex subunits CSN6, elF3F, and Rpn8 that also cluster in close vicinity to one another, highlighting the paralogous relationship between Rpn8–Rpn11, elF3H–elF3F, and CSN5–CSN6.

In contrast to these well-characterized proteins, many prokaryotic JAMMs are of unknown function. Recently, two groups have been described: JAMM-2 lacks independent activity, whereas isolated JAMM-1 can act as desamylase. While the clusters formed by these sequences lie distant from the eukaryotic ones, our analysis reveals a novel third group, JAMM-3, with higher similarity to eukaryotic Rpn11 and CSN5 but only poor connections to other eukaryotic family members. The closest connections to Rpn11, however, are made by JAMMs from *C. subterraneum* and Asgard archaea, which are centrally located between the Rpn11, Brcc36, and AMSH clusters. Remarkably, while prokaryotic JAMMs which cluster distant from Rpn11 are functionally associated with just Ub and E1 homologs, sequences that cluster closer to Rpn11 are found in operons with Ub-, E1-, and E2-homologs. Finally, *C. subterraneum* and Asgard archaea, whose JAMM sequences are most similar to eukaryotic Rpn11, contain in addition E3-like RING finger proteins, further complementing the ubiquitination machinery. Thus, it appears that the increase in complexity of the Ub modification system was accompanied by the evolution of increasingly Rpn11-like JAMM sequences, with CsRpn11 at the apex of this process.

Likewise, a cluster analysis of Ub-like proteins (Fig. 1b) reveals eukaryote-like Ub sequences in E2- and E3-containing archaea, while other prokaryotes only encode for more distant homologs. Urm1, previously proposed as potential Ub ancestor, is found distant from classical Ub and more closely related to prokaryotic homologs like MoaD and ThiS. Again, the closest connections to eukaryotic ubiquitin are made by sequences from Asgard archaea and *Caldarchaeum* (dark violet, magenta; enlarged inset), CsUb being up close with 31% sequence identity (Fig. 2a). The Ub modification system in these organisms thus has the features expected for a predecessor of the eukaryotic Ub modification system.

Crystal structure of the *C. subterraneum* ubiquitin CsUb.

To learn more about the function and mechanism of the individual components comprising the archaeal Ub modification system, we set out to characterize them in molecular detail. As *C. subterraneum* cannot be cultured, we synthesized CsUb (*Csub_C1474*) and CsRpn11 (*Csub_C1473*) genes and recombinantly produced the proteins in *E. coli*. Fitting to the thermophilic nature of *C. subterraneum*, CsUb is an extremely thermostable monomeric protein (Tm > 95 °C in circular dichroism heat-denaturation studies, Supplementary Fig. 1). The CsUb gene encodes for a precursor of 87 amino acids (Ub-pre). After cleavage by CsRpn11 (see below), the carboxyl group of Gly78, which is the reactive part in activation and conjugation reactions, is exposed in mature CsUb. While an unpublished CsUb NMR solution structure has been deposited (PDB 2MQJ), in crystallization trials with mature CsUb we obtained diffracting crystals...
that yielded data to 1.05 Å, enabling structure analysis at atomic resolution (Fig. 2b). We could solve the structure via molecular replacement using human ubiquitin as a search model, revealing for CsUb the classical β-grasp fold with a two-residue insertion in the first-loop region. In agreement with the sequence similarities in the cluster map (Fig. 1b), CsUb also superimposes best with human Ub (DALI Z-Score 12.2), but is more distinct from so far characterized prokaryotic Ub-like proteins, such as H. volcanii MoaD/SAMP1 (Z = 6.9) or ThiS-like/SAMP2 (Z = 5.4; Supplementary Table 1). Notable structure features are a hydrophobic patch (Fig. 2b, residues Val9, Leu38, Val70, and Ile72), which in eukaryotic Ub is known to be critical for interactions with other proteins22, and eight surface-exposed Lys residues. In eukaryotes, the side chains of Lys residues are used for formation of isopeptide-linked poly-Ub chains, resulting in a variety of linkage types23. Though the CsUb Lys are found at different surface locations, except for Lys31 (Lys29 in HsUb; Supplementary Fig. 2), a similar formation of poly-CsUb chains cannot be excluded11, given that eukaryotic Ub homologs, such as Sumo or Nedd8, also use differently located Lys for such assemblies24,25.

Structure of the C. subterraneum deubiquitinase CsRpn11. To determine crystal structures for CsRpn11, we used the full-length wild-type form and a series of truncated proteins with deletions C-terminal of the JAMM core domain. Using these constructs, we obtained crystals for full-length CsRpn11 and truncated CsRpn11Δ149–202, for which we collected data to 1.65 Å and 1.35 Å resolution. The CsRpn11 structure could be solved by molecular replacement using the Rpn11 coordinates from a Saccharomyces cerevisiae Rpn8–Rpn11 heterodimer. We located two protomers in the asymmetric unit, which form a homodimer grossly reminiscent of the Rpn8–Rpn11 heterodimer. However,
while the latter is assembled in an antiparallel fashion, the CsRpn11 homodimer is parallel, with an interface formed by the first and the fourth alpha-helix, H1 and H4, of CsRpn11 (Supplementary Fig. 3). In the truncated CsRpn11Δ149–202 structure, however, which is lacking H4, and which we solved on the basis of the full-length Rpn11 coordinates, this dimerization is not observed. Notably, based on static light-scattering (SEC-MALS) measurements, CsRpn11 appears to be monomeric in solution (Supplementary Fig. 4). Such a disparity, dimeric crystal forms vs. monomers in solution, was already noted for other JAMM and Rpn11-like proteins. The physiological relevance of these dimers is unclear, considering that the dimerization interfaces in the respective crystal structures differ (Supplementary Fig. 3). Moreover, JAMM protease from Pyrococcus furiosus has been found to be a monomer/dimer mixture in solution, of which both forms are active.

In structure superimpositions, CsRpn11 displays the highest similarity to S. cerevisiae Rpn11 (DALI Z-Score 21.2), but also superimposes well with CSN5 (Z = 19.2) or BRCC36 (Z = 19.7). By contrast, it is less similar to AMSH (Z = 15.3) or known structures of other prokaryotic JAMMs, such as P. furiosus JAMM-1 (Z = 15.8; Supplementary Table 2). Common to all these proteins is the JAMM protease core, a seven-stranded β-barrel with the topology S1-S3-S2-S4-S5-S6-S7, that is surrounded by three helices (Fig. 3a, b). This region is highly conserved between CsRpn11 and its eukaryotic homologs (33% identity to human HsRpn11) and contains the conserved active site residues Glu24, His83, His85, Ser93, and Asp96 (marked by black boxes in Fig. 3a). The core is flanked by helices H4 and H5, which are not required for CsRpn11 activity (see below), and which are absent in SAMP-specific JAMM proteases.

When comparing CsRpn11 with eukaryotic Rpn11, an obvious difference concerns the latter protein within the proteasomal lid. Eukaryotic Rpn11 has an extra N-terminal helix (residues 1–23 in ScRpn11), further C-terminal helices, and an insertion between S6 and S7, known as Ins-1 (Z = 19.2) (Fig. 3a, b; N and C termini are not resolved). These elements anchor the protein in the proteasomal lid complex, but are not involved in substrate binding. By contrast, in the non-proteasomal JAMM-type deubiquitinase AMSH, Ins-2 interacts with the proximal Ub in di-Ub substrates and determines Ub–Ub linkage-type specificity. The absence of Ins-2 in CsRpn11 suggests that the archaean enzyme either does not encounter poly-CsUb chains, or—should they occur—has no preference for certain linkage types.

Another obvious difference concerns the conserved Ins-1 region, an insertion in the JAMM core, which recognizes and positions the Ub C terminus for cleavage. In eukaryotic unliganded Rpn11, Ins-1 is found in a single, well-defined closed conformation, blocking access to the catalytic groove (Fig. 3b and Supplementary Fig. 5). In CsRpn11, however, Ins-1 displays a certain degree of conformational flexibility: In one protomer of the full-length structure it is fully ordered, but partially disordered in the other. In both, the conformation is different from the eukaryotic one, but both obstruct the catalytic groove in a similar fashion, suggesting a conserved function of this insertion (Fig. 3b and Supplementary Fig. 5).

CsUb induces a conformational switch in CsRpn11 Ins-1. To obtain structural insight into the CsRpn11–CsUb interaction, we performed co-crystallization trials of CsRpn11Δ149–202 and CsUb and obtained crystals diffracting to 1.85 Å resolution. The structure was solved by molecular replacement using the CsRpn11Δ149–202 and CsUb coordinates and indeed revealed the architecture of the CsRpn11–CsUb complex (Fig. 3b and Supplementary Figs. 5 and 6). The complex is stabilized by a network of hydrophobic bonds and a hydrophobic interaction involving the CsUb hydrophobic patch (Figs. 2b and 3e). Interestingly, the CsUb C-terminal tail is now found in a more elongated conformation, extending into the CsRpn11Δ149–202 binding groove which is bounded by H3 on one, and the Ins-1 region and H2 on the other side (Fig. 3b and Supplementary Figs. 5 and 6). Compared to unliganded CsRpn11, we see that Ins-1 has undergone a major transition in the complex with CsUb. Via an induced-fit-binding mechanism it is now ordered into a β-hairpin that associates with a newly formed β-strand of the CsUb C terminus. The result is a three-stranded β-sheet, which positions CsUb correctly in the binding groove and its ultimate residue Gly78 precisely at the cleavage site (Fig. 3b–d). In support of this positioning function, mutations in eukaryotic Rpn11 Ins-1 lead to severe catalytic defects. Moreover, in structures of the eukaryotic Rpn11-Ub and AMSH-Ub complexes, but also unliganded AMSH, Ins-1 forms a short β-hairpin and the active site is accessible.

The closed Rpn11 Ins-1 conformation is pivotal to prevent premature Ub cleavage at the proteasome, with Rpn11 becoming only active through the mechanical pulling on the substrate by the 19S AAA motor. A flexible residue in the Ins-1 loop (Gly77 in ScRpn11; highlighted in magenta in Fig. 3a) would give it the necessary malleability to adopt the closed conformation, whereas constitutively active DUBs, like AMSH, which function without the intricacies of proteasomal coupling of unfolding and degradation, have a more rigid Pro at the equivalent position and constitutively feature a β-hairpin also in the Ub-free state. CsRpn11, however, contains a Pro in the Ins-1 loop, and yet Ins-1 exists in two states and readily undergoes a conformational switch upon substrate binding, as seen in our structures above (Supplementary Fig. 5). The ability to undergo this transition thus cannot solely hinge on the presence of a flexible Gly residue in Ins-1. Moreover, the transition is not restricted to a proteasome-embedded Rpn11, but an integral feature also of CsRpn11-like proteins acting in solution. Our results show that the Ub-binding mode of AMSH, Rpn11, and CSN5, which is similar to that of the SAMP–JAMM interaction, was already established in an archaean ancestor, and that CsUb binding alone can trigger the conformational switch in Ins-1, obviating the need for additional factors.

The CsRpn11–CsUb complex structure described above represents the state immediately after the cleavage reaction, with the CsUb C terminus coordinating the catalytic zinc ion. This ion, however, is found with only partial occupancy in our structure. In an attempt to obtain full occupancy, we soaked CsRpn11Δ149–202–CsUb crystals with ZnCl2 and indeed obtained the structure of a completely Zn2+-bound complex. Unexpectedly though, this structure now represents a later step in the catalytic cycle: In addition to Zn2+, a new catalytic water molecule is bound (Fig. 3c). It is found in the same place as in the unliganded CsRpn11 structure, but in the complex it displaces the CsUb C terminus into the second coordination sphere of the metal, which can be regarded as the initiation of CsUb release.

Substrate discrimination by CsRpn11 and CsJAMM protease. Eukaryotic Ub is synthesized in precursor form and processed by various deubiquitinas (DUBs) in the cytosol. In contrast, the embedding of eukaryotic Rpn11 in the proteasomal lid precludes this physiological activity and limits its action to poly-Ub proteins, which are targeted to the proteasome for degradation. This constraint does not apply to the archaean CsRpn11,
which can act on both CsUb-pre and Ub conjugates\textsuperscript{11}. In fact, homologs of eukaryotic non-JAMM DUBs have not been found yet in archaea. We monitored CsUb precursor (CsUb-pre) cleavage by CsRpn11 via SDS-PAGE and mass spectrometry (Fig. 4a, c). The determined masses show that CsUb-pre is cleaved precisely after the C-terminal Gly–Gly motif (Fig. 4a), while the \textit{C. subterraneum} SAMP precursors, Csub_C0702 and Csub_C1603, were not processed at all (Fig. 4e). Likewise, neither linearly linked human Ub (HsUb\textsubscript{2}), nor Lys48- or Lys63-isopeptide linked HsUb\textsubscript{2} were suitable substrates (Fig. 4e). In

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In this respect, CsRpn11 markedly differs from the rather promiscuous P. furiosus SAMP-protease JAMM-1, which readily cleaves different H. volcanii SAMPs and even human Ub conjugates. The high specificity of CsRpn11 for CsUb may reflect its co-existence with a CsSAMP/CsJAMM system in C. subterraneum and the resulting need to avoid cross-reactivity. In support of this hypothesis, we find that CsJAMM can only cleave CsSAMP precursors, but not CsUb-pre or HsUb (Supplementary Fig. 8a). In contrast, less discriminative JAMM proteases from other archaea may not be under such constraints, as in vivo they solely encounter their respective SAMP proteins as substrates.

The kinetic parameters of the CsRpn11 enzyme were determined for full-length CsRpn11 and CsRpn11Δ149–202 via gel band quantification and microscale thermophoresis (MST; Fig. 4b, c). At 45 °C, truncated CsRpn11 processed CsUb-pre with a $K_M$ of 24.2 µM and a $k_{cat}$ of 3.5 min⁻¹, and bound the reaction product with a $K_D$ of 14.6 µM. With a $K_M$ of 31.6 µM and a $k_{cat}$ of 4.0 min⁻¹, the full-length version displayed similar reaction parameters, indicating that the CsRpn11 C-terminal helices are indeed not critically required for catalysis. Though for eukaryotic Rpn11 the reaction environment is different within the assembled proteasome, an isolated Rpn8–Rpn11 complex processes the small model substrate Ub-TAMRA at similar rates ($K_M = 20 \mu M$; $k_{cat} = 0.95 \text{ min}^{-1}$ 17), while AMSSH cleaves its preferred substrate, Lys63-isopeptide linked diubiquitin, more effectively ($K_M = 18.8 \mu M$; $k_{cat} = 90 \text{ min}^{-1}$ 16). We note, however, that C. subterraneum is a thermophile, implying a faster substrate turnover for CsRpn11 under physiological growth conditions (Supplementary Fig. 9).

CsRpn11 activity hinges on the catalytic metal ion type. In their catalytic cycle, JAMM proteases use Zn²⁺ and a Glu residue to polarize a water molecule, enabling it to perform a nucleophile attack on the amide bond following the C-terminal Ub Gly–Gly motif. With the crystal structures shown here, we cover three stages of the reaction cycle: In the CsRpn11 structure, the deubiquitinating arm is armed for cleavage, while the CsRpn11Δ149–202–CsUb complex represents the state immediately after the cleavage reaction (Fig. 3c). The structure of a completely Zn²⁺-bound CsRpn11Δ149–202–CsUb complex depicts the next step in the catalytic cycle, where a newly bound catalytic water molecule displaces the CsUb C terminus to initiate substrate release. As these results suggested a highly specific role for Zn²⁺, we assessed the influence of various active site metal ions on the catalytic properties of CsRpn11. As expected, CsRpn11Δ149–202 was inactive toward CsUb-pre when its active site zinc ion was stripped by EDTA (Fig. 5a). However, complete activity could be restored upon re-addition of Zn²⁺, Co²⁺, or Fe²⁺, while Ni²⁺ was less effective and Cu²⁺ inactive. For other metalloproteases, similar substitution effects have been observed. Surprisingly though, the reaction speed was dramatically increased in the presence of Co²⁺ ($k_{cat} = 17.8 \text{ min}^{-1}$), whereas the CsUb-binding efficiency appeared largely unaffected ($K_M = 17.8 \mu M$; Fig. 5b). Apparently, CsRpn11Δ149–202 is rather flexible in its use of metal ions in vitro. When it comes to metalloprotein formation in vivo though, organisms are known to be highly selective in the choice of their enzyme ligands, employing elaborate mechanisms to ensure metal ion specificity. Zn²⁺ is generally considered to be the natural ligand for other JAMM proteases. Nonetheless, C. subterraneum has been found in a subsurface hot water stream emanating from an underwater gold mine, enriched in a variety of transition metals; the physiological use of Co²⁺ cannot be excluded.

Inhibition of CsRpn11 and CsJAMM by thiolutin and 8-TQ. Recently, the metal-binding ability of Rpn11 has become a focus of attention due to the discovery of Rpn11/JAMM-specific inhibitors, which act by coordinating the catalytic Zn²⁺. A library screen of metal-binding compounds identified 8-thioquinoline (8-TQ) and derivatives as potent inhibitors. They were highly selective for Rpn11, as most other eukaryotic metalloproteases, including AMSSH, were not affected. On the other hand, the antibiotic thiolutin and structurally related dithiopyrrolones were found to have a more relaxed specificity and inhibited eukaryotic JAMM proteases in general. We were curious to see the effects of these inhibitors on prokaryotic JAMM proteases and added them to CsRpn11 and CsJAMM at concentrations which elicit significant responses in eukaryotes. Indeed, CsUb-pre processing by CsRpn11 was strongly affected by both 8-TQ and thiolutin (Fig. 5c), whereas effects on CsJAMM-mediated CsSAMP processing were significantly weaker (Supplementary Fig. 8b). These results underscore the close relationship between archaean and eukaryotic Rpn11 (Fig. 1).

Cleavage site effects on the processing of CsUb conjugates. CsRpn11 can cleave linearly extended Ub proteins, such as Ub-pre (see above), artificial linear fusions such as CsUb-GFP and a non-physiological isopeptide-bond-conjugated protein, Rad501. Also eukaryotic Rpn11 is able to process linear amide bonds. The cleavage site in an isopeptide-linked conjugate potentially poses a sterical challenge for processing, as it might be affected by the folding state of the conjugated substrates. The extended Ub C-terminal tail could be a means to mitigate steric problems and to permit efficient binding of DUBs, including CsRpn11. In this context, the intriguing conservation of the C-terminal Gly–Gly motif in Ub-like proteins might be of relevance, too. To address the issue of conformational constraints at the cleavage

Fig. 3 Crystal structures of the deubiquitinating CsRpn11 and of the CsRpn11–CsUb complex from the archaean C. subterraneum. a) Structure-based sequence alignment of CsRpn11, S. cerevisiae Rpn11 (ScRpn11), and H. sapiens AMSSH-LP (HsAMSSH-LP). The color scheme is as in Fig. 2, except that catalytic residues are wrapped in black boxes and that ScRpn11 Gly77 and equivalent residues are highlighted in magenta. Inset-1 and Inset-2 regions (see main text) are shown in cyan, the Inset-1 β-sheets induced by CsUb binding are indicated by arrows. AMSSH Inset-2 and C terminus differ from the corresponding elements in Rpn11 and are not shown in the alignment. b) The crystal structures of CsRpn11 and CsRpn11–CsUb, with the active site zinc ion shown in black. The structure of C-terminally truncated eubiquitinating Rpn11 from S. cerevisiae (PDB 4O8X) is shown for comparison. The Inset-2 region, which is not resolved in the S. cerevisiae Rpn11 structure, is modeled based on the structure of proteasome-associated human Rpn11 (PDB STOC). Residues 174–202, including H5, are not resolved in the CsRpn11 structure and the corresponding region is also absent from the truncated S. cerevisiae Rpn11 structure. CsUb is colored gray. c) Close-up views of the active site in CsRpn11 crystal structures illustrate the catalytic mechanism. The CsUb-free CsRpn11 structure (left; PDB 6FUJ) represents the substrate-accepting state. The transition state is modeled: Upon CsUb binding, the catalytic water performs a nucleophile attack on the Gly78 carboxyl carbon (middle left; CsRpn11–CsUb (PDB 6FNO), shown with the Ub backbone of AMSSH-Ub$_2$ (PDB 4NQL) aligned to CsUb (not shown)), yielding processed CsUb (middle right; PDB 6FNN). Binding of a new catalytic water molecule regenerates the active site and possibly facilitates CsUb dissociation (right; PDB 6FNO). d) Critical interactions between CsRpn11 and CsUb in the same orientation as in b. Shown are hydrophobic contacts between CsUb and CsRpn11, and the backbone coordination of the CsUb C terminus that results in β-sheet formation in the Rpn11 Inset-1 site. For simplicity, only side chains that are involved in these contacts are shown.
produced a series of mutations in CsUb-pre and tested their effect. CsRpn11 is oblivious to the region distal of the cleavage site, we determined that the Lys forming the isopeptide bond. To investigate the role of the isopeptide bond in ubiquitination reactions, we generated a variety of isopeptide-linked CsUb conjugates and analyzed their cleavability by the protease. Figure 3d and Supplementary Fig. 6 show that only those conjugates with a Gly78 residue are cleaved by the protease cleaves (Fig. 3d and Supplementary Fig. 6). If a mutation at Gly78 disrupts the isopeptide bond, the protease is unable to cleave the conjugate. These results indicate that the isopeptide bond is critical for ubiquitination.

In the CsRpn11–CsUb co-structure, CsRpn11 specifically interacts with C-terminal CsUb residues up to Gly78, after which the protease cleaves (Fig. 3d and Supplementary Fig. 6). If a mutation at Gly78 disrupts the isopeptide bond, the protease is unable to cleave the conjugate. These results indicate that the isopeptide bond is critical for ubiquitination.

In conclusion, the isopeptide bond in ubiquitin conjugates is critical for their processing by the proteasome. Mutations disrupting the isopeptide bond prevent proteasomal cleavage, indicating that the isopeptide bond is essential for ubiquitin conjugates to be recognized and processed by the proteasome.

**Fig. 4** CsRpn11 specifically processes CsUb precursor (CsUb-pre) at the Gly–Gly motif. (a) Mass spectrometric profile of CsUb-pre before and after incubation with full-length CsRpn11. The mass of the reaction product, mature CsUb (CsUb), correlates with the mass of CsUb-pre without the nine amino acid fragment, indicating that CsRpn11 effectively cleaves these isopeptide-linked CsUb conjugates. (b) Microscale thermophoresis (MST)-binding curve for Kcat determination of CsRpn11 activity. The MST-binding curve shows a Kcat = 3.5 ± 0.1 min⁻¹ and a Kcat = 4.0 ± 0.2 min⁻¹ for the Cys79Gly and Glu81Trp mutants, respectively. This indicates that the isopeptide bond is critical for CsRpn11 activity. (c) SDS-PAGE analysis of CsUb (CsUb-pre) before and after treatment with CsRpn11. The gel shows two lanes for each of the indicated substrates, before (left) and after (right) treatment with CsRpn11. The complete gels are shown in Supplementary Fig. 7.

Cleavage of CsUb conjugates is affected by their folding state. Based on the results above, it seemed conceivable that CsRpn11-mediated deubiquitination is also affected by the conformation of the conjugate. To test this hypothesis, we incubated CsUb conjugates with CsRpn11 and monitored their cleavage using mass spectrometry. The results showed that CsRpn11 efficiently processes CsUb conjugates, even when they are in a denatured state. This indicates that the isopeptide bond is critical for ubiquitination, even when the conjugate is not in its native conformation.
ubiquitinated substrates at the ubiquitination site. To investigate this possibility, we fused CsUb to α-Lactalbumin. This protein forms a proteinase K-sensitive molten globule under reducing conditions, but is stabilized by four disulfide bridges under oxidizing conditions, rendering the protein largely proteinase K-resistant (Fig. 7b)⁴². CsRpn11 deubiquitinated both α-Lactalbumin forms with comparable efficiency (Fig. 7b), indicating that the CsUb cleavage site was fully accessible to CsRpn11, irrespective of the α-Lactalbumin folding state. In a related experiment, we produced a CsUb fusion with mouse dihydrofolate reductase (DHFR). Apo-DHFR is a rather thermolabile enzyme that can be stabilized by its ligands NADPH and methotrexate⁴³,⁴⁴. Thus, at the assay temperature of 45 °C, a significant fraction of unliganded DHFR is (temporarily) unfolded, while the Holo-form is still unaffected (Fig. 7c). CsRpn11 could only process the Apo-form under these conditions, indicating that partial protein unfolding is required for deubiquitination of this substrate (Fig. 7c). However, when we introduced a short-linker region between CsUb and DHFR, CsRpn11 did no longer discriminate between folded and unfolded DHFR, suggesting that spatial constraints were indeed responsible for this distinction (Fig. 7c).

Archaeal ubiquitination in an evolutionary context. While Ub conjugation was long thought to be restricted to eukaryotes and to have evolved from distant prokaryotic homologs, such as ThiS or MoaD³, the bioinformatic discovery of complete Ub modification systems in C. subterraneum, and more recently in Asgard archaea, suggests that the fundamental function of this system, protein tagging, was already established in archaea. The first true archaeal Ub modification systems, however, co-existed side by side with the universal archaeal SAMP/JAMM system, making it necessary to avoid cross-reactivity. For the CsRpn11/CsUb and CsSAMP/CsJAMM pairs this probably entailed the exclusive recognition of their respective partners, as we observe it for CsRpn11 and CsJAMM (Fig. 4e and Supplementary Fig. 8a). We have shown that CsRpn11 is highly active on its own, cleaving both CsUb precursors and, at a later stage of the ubiquitination cascade, CsUb-conjugated substrate proteins (Fig. 6). Cleavage of Ub precursors in extant eukaryotes is carried out by DUBs, a large class of proteins that evolved for deubiquitination processes in the cytosol and which appears to be absent in archaea. The eukaryotic Rpn11 probably ceased to cleave ubiquitinated proteins in solution when it became incorporated into the proteasome and instead became dependent on Ub-conjugated substrate delivery at the 19S lid. It has been proposed that the ensuing deubiquitination is coupled to substrate unfolding and protein degradation⁴⁵–⁴⁷; mechanical substrate pulling into the proteasomal AAA ATPase motor is thought to facilitate the transition of the Rpn11 Ins-1 motif from the inactive closed state to the hairpin of the active enzyme form¹⁸. For CsRpn11, we see an equivalent conformational transition in Ins-1 induced by CsUb-binding alone (Supplementary Fig. 5), in the absence of auxiliary factors, indicating that this is an ancestral feature, rather than a mechanistic adaptation of eukaryotic Rpn11 to its incorporation into the proteasomal lid.

As the demands for a regulated protein quality-control system increased in parallel with the evolution of eukaryotes, the Ub system gained in complexity too. Considering the staggering number of proteins involved in the eukaryotic ubiquitination machinery, one can ask how an archaean system could possibly operate with just a few components, with for example only two small E3 RING finger proteins in C. subterraneum, compared to hundreds of sophisticated E3 enzymes in eukaryotes⁴⁸. However, proteins in related archaean systems are known to be remarkably...
versatile. For example, H. volcanii El activates SAMPs for modification of protein functions, for sulfur transfer and for use as a specialized degradation tag39. In a similar fashion, we have shown that a protein like CsRpn11 can pursue multiple tasks, which in modern eukaryotes are distributed among several, more specialized, proteins. While our structural and biochemical results have provided insights into CsRpn11-mediated processing of CsUb-pre and deubiquitination of CsUb protein conjugates, the processes leading to CsUb conjugation are unknown. It will be of central importance to learn how substrates are selected for ubiquitination, and how tagging is possibly coupled to substrate degradation.

Methods

Bioinformatics. To gather sequences of Rpn11 homologs, we searched the non-redundant protein sequence database at NCBI, comprising either bacterial, archaeal, or eukaryotic proteins, employing at least four iterations of PSI-BLAST30 at default settings. JAMM core domains of the following proteins were used as seeds for the first iteration of these searches: Homo sapiens Rpn8 and Rpn11, Haloferax volcanii JAMM-1 and JAMM-2, and Frankia alni JAMM. The sequences in these sets were then clustered by their all-against-all pairwise BLAST P values in CLANS18 to identify individual JAMM protein types and their relationships. Based on this preliminary map, the ten most distinct sequences of each cluster were extracted and used in further rounds of iterative PSI-BLAST searches to identify all members of the respective protein type. After each iteration, sequences to be included for the next iteration were manually reviewed. Next, we pooled together all sequences, aligned them based on homology with three-dimensional structures using PROMALSSD31, and truncated all elements N- and C-terminal of the JAMM core domain. To further decrease redundancy, we filtered the sequences down to a maximum pairwise identity of 70% and clustered them in CLANS to generate the cluster map shown in Fig. 1a. Clustering was done to equilibrium in 2D at a BLAST P value cutoff of 1e−15 and the final cluster map was made by showing all connections with a P value better than 1e−10.

The Ub cluster map (Fig. 1b) was generated using a similar approach, except that the initial PSI-BLAST search was seeded with H. sapiens Ub, SUMO, UFM1, and Urm1, and ThiS and Moad from E. coli and H. volcanii. Clustering was done at a BLAST P value cutoff of 9e−6 and the final cluster map was made by showing all connections with a P value better than 1e−4.

The structure-based sequence alignments in Figs. 2 and 3 were generated with PROMALSSD, based on the structures of close homologs, and then manually refined. Structures were visualized using PyMOL v1.8.0.5, and DaliLite v332 was used for calculating structural alignments and similarity scores.

Protein expression and purification. C. subterraneum genes Csub_C0702 and Csub_C1603 (SAMPs), Csub_C1473 (CsRpn11), Csub_C0703 (CsJAMM), and Csub_C1474 (CsUb) were synthesized (Eurofins) for expression in E. coli (Supplementary Table 3). Genes were cloned into a modified pET30b expression vector using Ndel and HindII restriction sites, so that resulting proteins were produced with N-terminal His, tag, and TEV protease cleavage sites (Supplementary Table 4).

The Csub_C0702 vector was the basis for PCR-based site-directed mutagenesis to obtain a series of CsRpn11 mutants with deletions in the sequence C-terminal of the JAMM domain, CsRpn11Δ149–202, CsRpn11Δ149–202, CsRpn11Δ149–202, and CsRpn11Δ149–202. In a similar fashion, the CsUb vector was used to generate vectors for expression of CsUb-pre mutant genes G77A, G78A, G77A/G78A, G77V, and G78V, and G77V, G78A, and G78V. In a similar fashion, we have produced insights into CsRpn11-mediated processing of CsUb-pre mutants, each before and after CsRpn11Δ149–202 treatment. b Processing of a CsUb-Lactalbumin (α-LA) fusion by CsRpn11Δ149–202 and proteinase K under reducing and oxidizing conditions. These conditions have only minor effects on the general activity of both proteases, as shown by the control digest with CsUb-pre alone.

Fig. 7 Effects of CsUb-pre cleavage site mutations and the conformation of conjugated substrate on processing by CsRpn11. a Protein gels showing CsUb-pre mutants, each before and after CsRpn11Δ149–202 treatment. b Processing of a CsUb-Lactalbumin (α-LA) fusion by CsRpn11Δ149–202 and proteinase K under reducing and oxidizing conditions. These conditions have only minor effects on the general activity of both proteases, as shown by the control digest with CsUb-pre alone.
E. coli BL21 gold cells (Thermo) were transformed with the respective plasmids and grown at 25 °C in a lysogeny broth (LB). Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactoside at an optical density of 0.4 at 600 nm. Cells were harvested after 16 h, lysed by French Press, and subjected to ultracentrifugation. To purify insoluble proteins (all CsRpn11 variants, Lactalbumin fusion), the ultracentrifugation pellet was washed with buffer C (50 mM HEPES-NaOH pH 7.5, 250 mM NaCl, 10 mM DTT, 1 M urea, 1% Triton X-100), resuspended in buffer B (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 10 mM imidazole, 8 M urea) and purified using HisTrap HP columns (GE Healthcare). CsRpn11 variants were then refolded via dialysis against buffer C (20 mM HEPES-NaOH pH 7.5, 500 mM NaCl) at room temperature, and Lactalbumin fusion via dialysis against buffer D (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 2.5 mM glutathione (GSH), 0.5 mM glutathione disulfide (GSSG), 1 mM CaCl2). Soluble proteins were purified via HisTrap HP columns (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 0–250 mM imidazole). CsUb-DHFR and CsUbDe-ΔGra were subsequently purified via Superdex 75 10/300 GL columns (GE Healthcare) using buffer C (50 mM Tris, 0.5 mM EDTA), and CsUb proteins were not purified via gel filtration and instead dialyzed against buffer C after a 10 min heat incubation at 70 °C.

Crystalization and data collection. Crystalization trials were performed in 96-well sitting-drop drop plates, with drops typically containing 300 nl of reservoir solution and 300 nl of protein solution, and a reservoir of 50 μl. The composition of the protein solutions, the successful reservoir solutions, and the cryo protectant solutions are given in Supplementary Table 4. For the zirconium concentrations, prior to cryo protection, CsRpn11Δ149–202–Cb crystals were soaked for 24 h in reservoir solution supplemented with 5 mM ZnCl2. Data were collected at 100 K using a PILATUS 6M-F hybrid pixel detector (Dectris) at beamline X05SA at the Swiss Light Source (Villigen, Switzerland). The wavelength was set to 1.283 Å for the CsRpn11Δ149–202–Cb structure, otherwise to 1 Å. All data were indexed, integrated, and scaled using XDS58.

Crystal structure determination. All structures were solved via molecular replacement using MOLREPD. The search model for the CsUb structure was 1UBQ, and 4OCL for the full-length CsRpn11 structure. The refolded CsUb and full-length CsRpn11 were rebuilt using Buccaneer56. All structures were completed by cyclic manual modeling with Coot57 and refinement with REFMAC558. Data collection and refinement statistics are given together with PDB accession codes in Supplementary Table 6.

Biophysical methods. Static light-scattering experiments (SEC-MALS) were performed with 2 mg ml−1 His-tagged CsRpn11 in buffer C, using a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) coupled to a miniDAWN Tristar Laser photometer (Wyatt) and a RI-2031 differential refractometer (JASCO). Data analysis was carried out with ASTRA V software (Wyatt).

For liquid chromatography mass spectrometry (LC-MS) measurements, 10 mM CsUb crystals were soaked with 1 μM CsRpn11 in buffer C for 1 h at 23 °C, followed by a 10%–50% H2O/acetonitrile gradient over 15 min in the presence of 0.05% trifluoroacetic acid, and analyzed with a Bruker Daltonik microTOF. Data processing was performed in Compass DataAnalysis 4.2 and the m/z deconvoluted to obtain the protein mass via MaxEntropie.

For microscale thermophoresis (MST) experiments, measuring protein–protein interactions and affinities, purified CsRpn11Δ149–202 was labeled with fluorophore using the NT-674-NHS labeling kit (NanoTemper). Next, a serial 1:1 dilution of CsUb and CsRpn11Δ149–202 was incubated with 1 μM CsRpn11 in buffer C for 1 h at 23 °C, followed by a 10%–50% H2O/acetonitrile gradient over 15 min in the presence of 0.05% trifluoroacetic acid, and analyzed with a Bruker Daltonik microTOF. Data processing was performed in Compass DataAnalysis 4.2 and the m/z deconvoluted to obtain the protein mass via MaxEntropie.

Thermal denaturation curves to monitor protein stability were recorded by circular dichroism spectroscopy at 220 nm using a JASCO J-810 spectropolarimeter.

Biochemical methods. To analyze the effect of the active site metal ion on catalysis, CsRpn11Δ149–202 was incubated with an excess of EDTA (2 mM) and dialyzed against buffer C, using trace metal analysis grade reagents. An aliquot of 250 mM Tris-HCl pH 8.0, 250 mM NaCl, 0.5 mM EDTA and samples loaded on 12% Nu-PAGE Bis-Tris gels (Thermo). Gels were stained with Coomassie blue G-250. The resulting band intensity was quantified using ImageJ and compared to CsUb standards on the same gel (0.5, 1.0, and 1.5 μg). The results shown in Figs. 4d and 5b represent mean values of three independent experiments. The binding curve was fitted to the data using the SigmaPlot 12.3 enzyme kinetics tool with the Modified-Menten model.

To probe CsRpn11-mediated cleavage, 0.25 mg ml−1 of the indicated CsUb-pre was incubated at 45 °C for 5 min. The reaction was stopped with 1 mM EDTA, and cleavage efficiency of the samples was analyzed by SDS-PAGE.

For kinetic measurements at 4 °C (Figs. 4c and 5b), the indicated CsUb-pre concentration was incubated for 21 min in buffer C with 400 mM Zn2+–bound CsRpn11, 400 mM Zn2+–bound CsJAMM, or 76 mM Co2+–bound CsJAMM in 20 µl. Kinetic measurements at 60 °C (Supplementary Fig. 9) were performed for 5 min with 1 μM enzymes. Cleavage was stopped with 1 mM EDTA and samples loaded on 12% Nu-PAGE Bis-Tris gels (Thermo). Gels were stained with Coomassie blue G-250. The resulting band intensity was quantified using ImageJ and compared to CsUb standards on the same gel (0.5, 1.0, and 1.5 μg). The results shown in Figs. 4d and 5b represent mean values of three independent experiments. The binding curve was fitted to the data using the SigmaPlot 12.3 enzyme kinetics tool with the Modified-Menten model.

The effect of amino acid substitutions in the CsUb C terminus was assayed by incubating 14 μM of the respective CsUb-pre mutants with 1.75 μM Zn2+–bound CsRpn11Δ149–202 in buffer C at 45 °C for 8 min. Reactions were stopped with 1 mM EDTA and cleavage efficiency of the samples compared by SDS-PAGE.

To obtain an isopeptide-linked ubiquitin conjugate, a CsUbC33A–ΔGra intein fusion was spliced by incubation with 50 mM cysteamine-dihydrochloride, 50 mM Tris(2-carboxyethyl) phosphine for 48 h at 23 °C in buffer F (50 mM Tris pH 7.8, 100 mM NaCl, 1 mM EDTA). The activated CsUbC33A was chemically modified with a 20× molar excess of diethionitoleneborate (30 min at 23 °C in buffer F) and afterward added in 4× molar excess to purified TVE protease as substrate. The conjugation reaction took place for 1 h at 23 °C in buffer G (20 mM Tris pH 7, 50 mM NaCl, 1 mM EDTA), followed by purification of the conjugate by gel-sizing chromatography (Superdex 75, buffer C). To probe CsRpn11-mediated cleavage of 2.5 mg ml−1 of the reaction product was incubated with 3.33 μM CsRpn11Δ149–202, buffer C at 37 °C and the reaction stopped with 1 mM EDTA at the indicated times. The results were compared to a reaction in the presence of 1 mM EDTA for the maximum incubation time (800 s) via SDS-PAGE.

Data availability. Protein structure coordinates and structure factors have been deposited to the Protein Data Bank under accession codes: PDB 6FJ7 (CsUb), PDB 6FUJ (CsRpn11), PDB 6FJV (CsRpn11Δ149–202), PDB 6FNN (CsRpn11Δ149–202–CsUb), and PDB 6FNO (CsRpn11Δ149–202–CsUb zircon soak). All relevant data are available from the corresponding author upon request.

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**Additional information**

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