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Ubiquitin-directed AAA+ ATPase p97/VCP unfolds stable proteins crosslinked to DNA for proteolysis by SPRTN

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The protease SPRTN degrades DNA-protein crosslinks (DPCs) that threaten genome stability. SPRTN has been connected to the ubiquitin-directed protein unfoldase p97 (also called VCP or Cdc48), but a functional cooperation has not been demonstrated directly. Here, we biochemically reconstituted p97-assisted proteolysis with purified proteins and showed that p97 targets ubiquitin-modified DPCs and unfolds them to prepare them for proteolysis by SPRTN. We demonstrate that purified SPRTN alone was unable to degrade a tightly-folded Eos fluorescent reporter protein even when Eos was crosslinked to DNA (Eos-DPC). However, when present, p97 unfolded poly-ubiquitinated Eos-DPC in a manner requiring its ubiquitin adapter, Ufd1-Npl4. Notably, we show that, in cooperation with p97 and Ufd1-Npl4, SPRTN proteolyzed unfolded Eos-DPC, which relied on recognition of the DNA-crosslink by SPRTN. In a simplified unfolding assay, we further demonstrate that p97, while unfolding a protein substrate, can surmount the obstacle of a DNA crosslink site in the substrate. Thus, our data demonstrate that p97, in conjunction with Ufd1-Npl4, assists SPRTN-mediated proteolysis of tightly-folded proteins crosslinked to DNA, even threading bulky protein-DNA adducts. These findings will be relevant for understanding how cells handle DPCs to ensure genome stability and for designing strategies that target p97 in combination cancer therapy.

Chromatin-associated proteins commonly form covalent crosslinks with the DNA, termed DNA-protein crosslinks (DPCs) that are induced by physical or chemical crosslinkers and can even occur naturally (1, 2). DPCs represent bulky barriers that interfere with transcription, DNA replication and repair, and therefore threaten cell survival and genome stability.

DPCs can be removed by dedicated metalloproteases such as SPRTN (SprT-like N-terminal domain, also known as Spartan or DVC1), its yeast counterpart Wss1, or related proteases (1–7). DPC proteases do not have any defined substrate sequence preference consistent with the diversity of proteins that form DPCs. However, proteolytic activity of SPRTN requires recognition of the crosslinked DNA by a zinc-binding domain (ZBD) and a basic region (BR) in SPRTN to ensure specificity for DPCs (8, 9). Consistent with its central role, SPRTN is essential in mice while mutations in SPRTN cause severe genetic disorders in humans (10, 11).

In vitro assays that directly monitor proteolysis of DPCs by SPRTN have relied on structurally unstable reporter proteins (9), raising the question if and how tightly folded proteins crosslinked to DNA are proteolyzed by SPRTN. SPRTN and Wss1 have been connected in cells to the AAA+ ATPase p97 (also called VCP or Cdc48 in yeast) (3, 12–14). Of note, p97 is a protein unfolding machine and therefore may assist SPRTN by unfolding tightly folded DPCs for proteolysis.

The hexameric p97 is best known for unfolding ubiquitylated proteins and preparing them for proteolysis in the proteasome (15, 16). Notably, p97 unfolds even tightly folded proteins lacking any disordered regions, which are otherwise required for the proteasome in the absence of p97 (17). For ubiquitin-directed unfolding by p97, substrate-conjugated ubiquitin serves as a universal unfolding tag that is recognized by the ubiquitin adapter complex Ufd1-Npl4 bound to p97 (18, 19). However, Ufd1-Npl4 not only recruits the ubiquitylated substrate to p97 but also inserts one of the ubiquitin moieties into the central pore of the p97 hexamer (19). Upon ATP hydrolysis, p97 threads ubiquitin and the attached substrate through its central channel, thereby unfolding the substrate for degradation in the proteasome (20, 21).

In this study, we asked whether p97-mediated unfolding can directly assist also SPRTN-catalyzed proteolysis of tightly folded DPCs. We hypothesized that, in this case, unfolding by p97 may be directed by ubiquitin and, possibly, Ufd1-Npl4. Alternatively, given that SPRTN harbors both substrate-binding elements and a p97-interacting suppressor of high copy PP1 (SHP) box motif (1, 2), SPRTN itself could in principle serve as a substrate adapter for p97.

Using biochemical reconstitution with purified proteins, we show here directly that p97-mediated unfolding prepares DPCs for proteolysis by SPRTN. Importantly, we find that p97 and SPRTN alone do not suffice but require the Ufd1-Npl4 adapter that recognizes substrate ubiquitylation. Thus, p97 supports proteolysis of DPCs by assisting SPRTN in an ubiquitin and Ufd1-Npl4–dependent manner, and this can occur independently of the proteasome.

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Results

Generation of an ubiquitylated DPC reporter protein

To enable investigation of the cooperation between p97 and SPRTN, we first aimed to generate a substrate reporter protein that combines a DNA crosslink required for targeting by SPRTN with ubiquitylation for targeting by p97, as well as providing reliable readouts for unfolding and proteolysis.

The majority of p97-mediated unfolding events is directed by ubiquitylation of the substrate, and cell-based assays suggested ubiquitylation also involved in DPC proteolysis (14). Ubiquitin-dependent unfolding by p97 can be conveniently examined with a di-ubiquitin fusion to the mEos3.2 fluorescent reporter moiety (Ub-Eos) that is subsequently enzymatically polyubiquitinated (17, 20, 22). Unfolding of the reporter protein can be directly monitored by the loss of Eos fluorescence using fluorescence spectrometry. A backbone break in Eos induced by UV irradiation results in a fluorescence shift from green to red. Crucially, this photoconversion conveniently prevents refolding and thus regaining of fluorescence of Eos after p97-mediated unfolding. In contrast, the remaining unbroken green Eos refolds after unfolding. Loss of green fluorescence therefore strictly reports on proteolysis. As a first step, we generated the Ub-Eos reporter protein in bacteria (Fig. 1, *A* and *B*). The UV-induced backbone break occurs typically in 30% of the protein fraction and therefore resulted in two fragments in addition to the full length Ub-Eos in denaturing SDS-gels (Fig. 1, *A* and *B*).

To allow targeting by SPRTN, we crosslinked a DNA oligonucleotide to cysteines in Ub-Eos. The oligonucleotide was hybridized with a complementary strand resulting in a 5 base overhang which is specifically recognized by SPRTN and activates its protease activity (9). Cysteines are only present in the C-terminal part downstream of the backbone break site of the reporter. Therefore, the DNA crosslink occurred to the nonfragmented full-length Ub-Eos-DNA as well as to the smaller C-terminal fragment of Eos, leading to a gel shift in

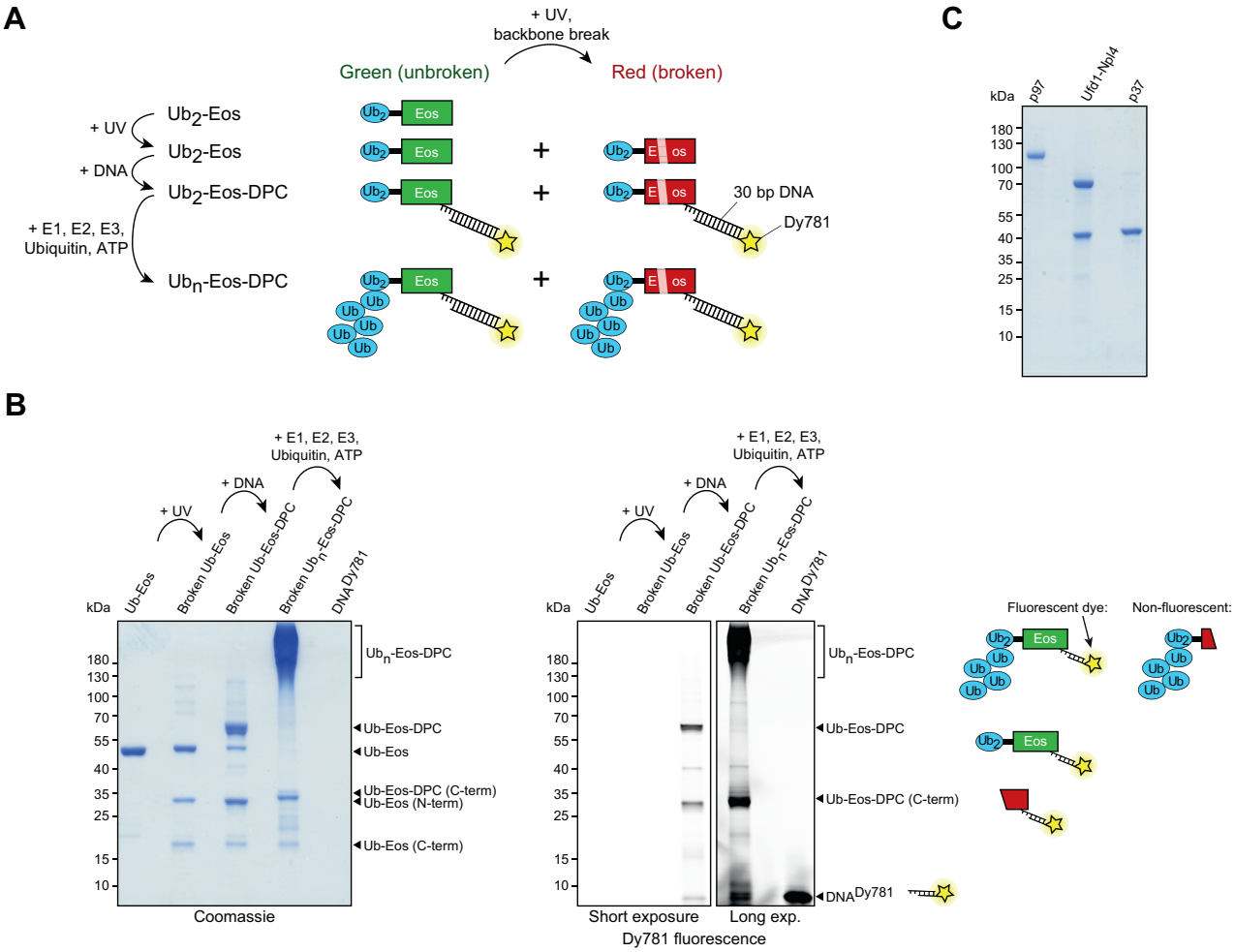

Figure 1. Generation of a poly-ubiquitylated DNA-protein-crosslink reporter substrate protein. *A*, steps of the substrate reporter generation. A di-ubiquitin-Eos fusion (Ub2-Eos) was generated in bacteria. Ub2-Eos was first irradiated with UV light to introduce a backbone break in Eos in roughly 30% of the protein population, which entails a conversion from the green to the red fluorescent form of Eos and facilitates monitoring unfolding reactions. A double-strand DNA oligonucleotide with a 5′ overhang was crosslinked to a cysteine in the Eos moiety, yielding Ub2-Eos-DPC. The oligonucleotide was coupled to Dy781 to facilitate visualization of the DPC and its fragments in SDS-PAGE. The reporter substrate was subsequently polyubiquitylated on the N-terminal ubiquitin moieties yielding Ubn-Eos-DPC. *B*, the steps described in (A) analyzed in SDS-PAGE and visualized by Coomassie staining (*left*) and infrared Dy781 fluorescence scan (*right*). Note that N- and C-terminal Eos fragments remain associated in the native protein but are separated in the denaturing gel. *C*, Coomassie gel of purified protein components. DPC, DNA-protein crosslink.

both cases (Fig. 1, *A*–*C*). For easier detection, we used a DNA oligonucleotide coupled to the Dy781 chromophore. Consistent with the DNA crosslink to Ub-Eos, we detected fluorescence exclusively of the crosslinked bands in fluorescence scans of the SDS-gel (Fig. 1, *A* and *B*).

In the next step, we enzymatically polyubiquitylated the Ub-Eos-DPC reporter substrate with long ubiquitin chains to allow efficient targeting by p97. Ubiquitylation was mediated with a previously established gp78-Ubc7 fusion protein that efficiently ubiquitylates the di-ubiquitin acceptor site in the Ub-Eos reporter (21). Ubiquitylation resulted in a quantitative shift of Ub-Eos-DPC reporter to a high molecular weight smear in the SDS-gel that was positive for the fluorescence probe on the DNA oligo, as expected (Fig. 1, *A* and *B*). Because the C-terminal photoconversion fragment of Ub-Eos lacked the ubiquitylation site, but was labeled with the DNA-chromophore, it was detected in the fluorescence scans in a nonubiquitylated form migrating with an apparent molecular weight of ∼35 kDa (Fig. 1*B*).

SPRTN and the ubiquitin adapter Ufd1-Npl4 bind the p97 hexamer simultaneously

SPRTN and p97 have been functionally linked in cells, but whether SPRTN can act as a substrate ubiquitin adapter for p97 or needs to cooperate with the canonical ubiquitin adapter Ufd1-Npl4 of p97 is unclear. Intriguingly, both SPRTN and Ufd1-Npl4 contain an SHP box motif to interact with p97 (11, 13) raising the question as to whether SPRTN and Ufd1-Npl4 can bind p97 at the same time. To clarify this question, we performed a competition experiment with purified proteins, in which we incubated p97 and SPRTN with increasing concentrations of Ufd1-Npl4. We then immunoisolated SPRTN and analyzed associated proteins. Importantly, SPRTN-bound p97 did not decrease in the presence of Ufd1-Npl4 showing that Ufd1-Npl4 did not compete with SPRTN for p97 binding (Fig. 2*A*). Instead, Ufd1-Npl4 coisolated with the SPRTN–p97 complex but not SPRTN alone (Fig. 2*A*), demonstrating that Ufd1-Npl4 and SPRTN bind simultaneously to p97, most likely by interacting with different N-domains within the p97 hexamer. Thus, simultaneous binding is consistent with both factors cooperating during DPC proteolysis by SPRTN assisted by p97.

p97-assisted proteolysis by SPRTN

SPRTN activity can be monitored by detecting the generation of proteolytic fragments of its DPC substrate (9). We therefore asked whether SPRTN could generate proteolytic fragments of our poly-Ubiquitin-Eos-DNA reporter substrate protein and whether that was stimulated by p97. We incubated our substrate alone or with SPRTN and p97-Ufd1-Npl4 either in the absence of presence of ATP to compare the contribution of ATP-driven unfolding activity by p97. The reactions were stopped at different time points during the course of 30 min. Samples were loaded on denaturing SDS-gels and fluorescence scanned to detect DNA-chromophore–crosslinked protein and fragments (Fig. 2, *B* and *D*).

In the absence of SPRTN, only the polyubiquitylated species of the substrate and the C-terminal nonubiquitylated ∼35 kDa fragment was detected, as in the input (Fig. 2*B*). In the presence of SPRTN without ATP, a cleavage product was generated migrating with an apparent molecular weight of ∼40 kDa (Fig. 2*B*). This ∼40 kDa band is consistent with DNA-crosslinked Eos core, whose N-terminal flexible ubiquitylation acceptor moiety along with the ubiquitin chain was clipped by SPRTN. Western blot confirmed that the band was derived from Eos (Fig. S1). The fact that no smaller fragments were detected indicated that the tightly folded Eos core was resistant to cleavage by SPRTN. Of note, in the presence of ATP (and thus of p97 unfolding activity), a smaller fragment was generated over time (Fig. 2, *B* and *C*). The fact that the smaller fragment migrated at an apparent molecular weight of only ∼20 kDa and harbored the DNA crosslink indicated that it was a fragment derived from the Eos core. The ∼20 kDa fragment was not generated when either SPRTN or p97 was omitted (Fig. 2, *D* and *E*), showing that both factors cooperated. Moreover, proteolysis was largely reduced by the p97 inhibitors CB-5083 or NMS-873 (Fig. 2, *D* and *E*), indicating that p97 unfolding activity was required for the generation of proteolytic fragments. Of note, SPRTN-mediated Eos proteolysis was dependent on the p97 ubiquitin adapter Ufd1-Npl4 (Fig. 2, *D* and *E*), showing that substrate protein ubiquitylation and binding was required for proteolysis to occur and that SPRTN did not suffice as a substrate adapter of p97.

Both ATP requirement and sensitivity to p97 inhibitors indicated that p97 unfolding activity was required and that this was coupled to SPRTN-mediated proteolysis. To further substantiate the involvement of substrate unfolding, we monitored loss of Eos fluorescence during the reaction. We profited from the fact that our substrate fraction contained both “unbroken” as well as “broken” Eos with green and red fluorescence, respectively (see Fig. 3*A* for cartoon depiction). Unfolding of red “broken” Eos by p97 leads to a net loss of fluorescence already, because “broken” Eos cannot refold. In contrast, green “unbroken” Eos refolds after p97-mediated unfolding leading to only an initial drop in fluorescence followed by a steady state of unfolding and refolding. We confirmed this notion for our substrate reporter by adding the GroEL^{D87K} mutant that traps the unfolded state (23) (Fig. S2). If a protease is added instead, any additional loss of fluorescence can therefore be attributed to proteolysis (17).

Consistent with that prediction, in a reaction containing the polyubiquitylated Ub-Eos-DPC as well as p97, Ufd1-Npl4, and ATP, we observed a loss of fluorescence in the red channel demonstrating that p97-Ufd1-Npl4 was able to efficiently unfold our DNA-crosslinked reporter protein (Fig. 3*B*). Of note, loss of fluorescence was not increased by addition of SPRTN (Fig. 3*B*), consistent with unfolding being sufficient for red fluorescence loss and indicating that p97 unfolding was rate determining.

In contrast, for green “unbroken” Ub-Eos-DNA, p97 alone only caused a minor net decrease in fluorescence, as expected, because refolding was not prevented by a backbone break. Importantly, in this case, addition of SPRTN to the unfolding reaction led to a continuous decline of fluorescence (Fig. 3*B*) demonstrating that the substrate was proteolyzed after

unfolding. These experiments demonstrate that, for tightly folded protein moieties, proteolysis by SPRTN is coupled to p97-mediated protein unfolding and requires the recognition of the ubiquitin chain by Ufd1-Npl4.

Functional elements in SPRTN required for p97-assisted proteolysis

SPRTN obtains specificity for DPCs by binding of the DNA moiety *via* its ZBD and BR domains (9). To ask whether substrate proteolysis by SPRTN was still dependent on DNA recognition by SPRTN after p97-mediated unfolding, we performed Ub-Eos-DPC degradation assays with SPRTN lacking the ZBD and BR domains, and compared it to wild type SPRTN. Notably, proteolysis was abolished by the mutations (Fig. 4, *A–D*) demonstrating that DNA recognition was essential and that SPRTN retained its selectivity for DPCs even for proteins unfolded by p97. For comparison, the reaction was also performed with SPRTN harboring the E112Q mutation in the catalytic center (4) which abolished proteolysis, as expected (Fig. 4, *A–D*).

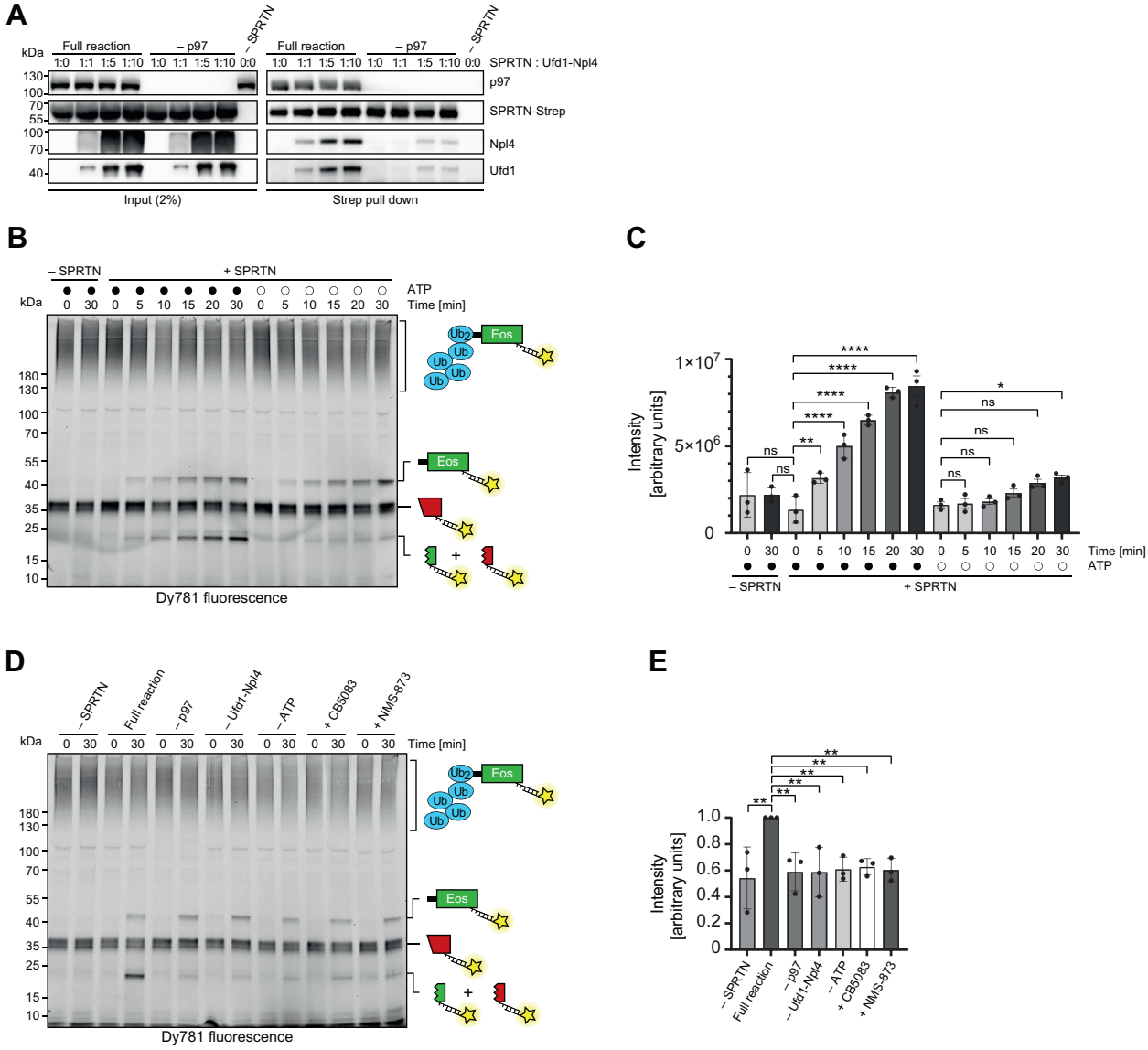

Figure 2. Proteolysis of the DPC substrate by SPRTN is dependent on p97- and Ufd1-Npl4–mediated unfolding. *A*, Ufd1-Npl4 and SPRTN bind p97 at the same time. Binding assay containing Strep-tagged SPRTN, p97, and increasing concentrations of Ufd1-Npl4 followed by pull-down of SPRTN. Proteins were visualized by Western blot. Note that Ufd1-Npl4 did not compete with SPRTN for p97, but instead that Ufd1-Npl4 coisolated with SPRTN in the presence of p97. *B*, the Ubn-Eos-DPC substrate was incubated with p97 and Ufd1-Npl4, and without or with SPRTN for 0 to 30 min at 37 °C, as indicated. ATP was added as indicated to support p97 unfolding activity. Samples were separated by SDS-PAGE followed by a fluorescence scan detecting Dy781. Note that while generation of the ∼40 kDa Eos-containing fragment is ATP independent, the generation of an internal ∼20 kDa Eos proteolysis fragment requires ATP-driven p97 activity. *C*, quantification of the chromophore intensity associated with the ∼20 kDa band in (B) using a fluorescence scanner. n = 3; the error bars represent mean ± s.d. One-way ANOVA, *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. *D*, assay as in (A). Individual components were omitted or p97 activity was inhibited by CB5083 or NMS-873, as indicated. Note that generation of the ∼20 kDa proteolysis product consistently requires p97 activity. *E*, quantification of the chromophore intensity associated with the ∼20 kDa band in (D) using a fluorescence scanner. Values were normalized to signal in full reaction condition. n = 3; the error bars represent mean ± s.d. One-way ANOVA, **$p < 0.01$. DPC, DNA-protein crosslink; SPRTN, SprT-like N-terminal domain.

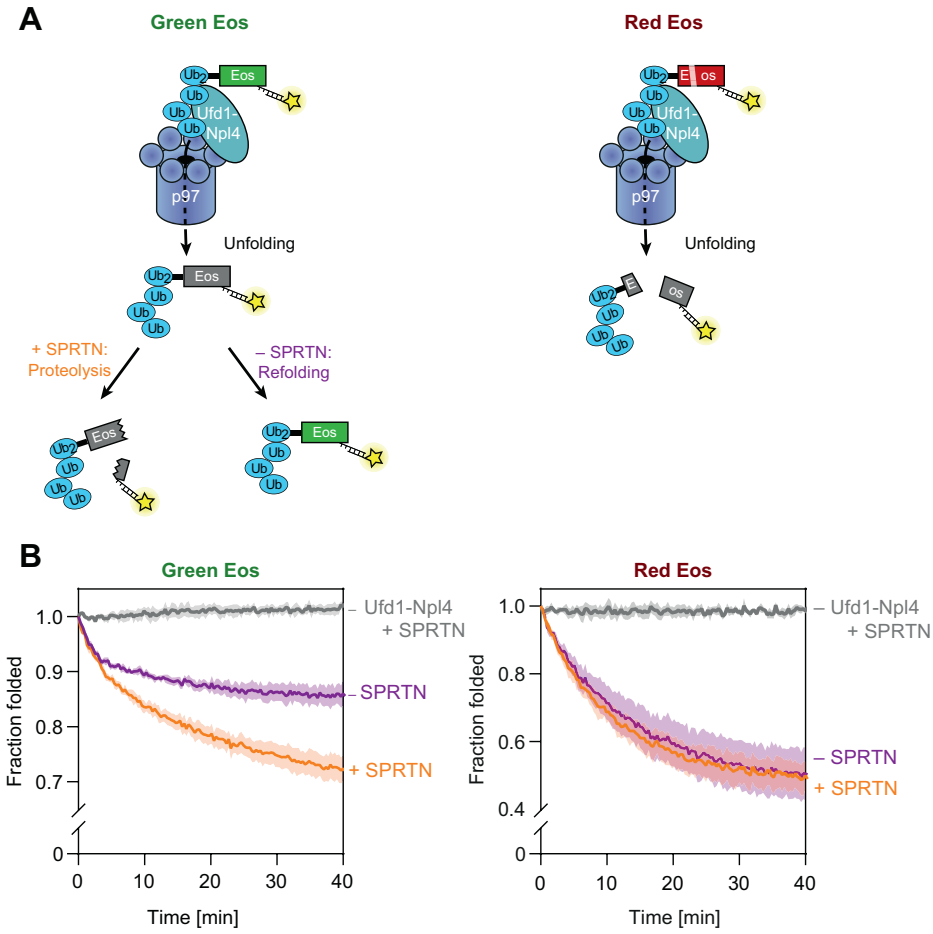

Figure 3. Real-time monitoring of DPC substrate unfolding and proteolysis by p97-Ufd1-Npl4 and SPRTN. *A*, depiction of the Eos fluorescence-based substrate unfolding assay. *Green* (unbroken) and *red* (broken) Ubn-Eos-DPC are recruited to p97 by Ufd1-Npl4. Unfolding of *red* Eos leads to net loss of Eos fluorescence due to the backbone break that prevents refolding. Unfolding of *green* Eos results only in an initial loss of fluorescence that plateaus due to refolding of the reporter. Additional loss of fluorescence therefore reports on proteolysis of the reporter. *B*, assay as described in (A). *Green* and *red* Eos fluorescence was monitored over time in the presence and absence of SPRTN, as indicated. Note that loss of red fluorescence is not affected by SPRTN, as expected. Instead, loss of green fluorescence is accelerated indicating proteolysis of the reporter by SPRTN following p97-mediated unfolding. n = 3; mean ± s.e.m. DPC, DNA-protein crosslink; SPRTN, SprT-like N-terminal domain.

SPRTN contains two additional elements relevant for this analysis, a linear SHP box that mediates binding to p97 and a ubiquitin-binding zinc-finger (UBZ) domain that binds ubiquitin (4, 24). Deletion of the UBZ domain had only a minor effect (Fig. 4, *A-D*). Notably, mutations of the SHP box significantly affected proteolysis, but only partially, and even the combination with the UBZ deletion did not further decrease proteolytic activity (Fig. 4, *A–D*). We confirmed the effect of SHP box mutation on SPRTN binding to p97 by *in vitro* interaction reactions followed by coimmunoprecipitation (Fig. 4*E*). This indicates that, while p97-Ufd1-Npl4 is required to bind the ubiquitin moieties of the substrate, ubiquitin and p97 binding by SPRTN contributes to efficiency of the reaction, but is not essential in the *in vitro* setting.

p97 surmounts DNA crosslink sites during protein unfolding

p97 unfolds client proteins by threading them through the central channel of the p97 hexamer (20, 22). The fact that p97 can unfold DPCs, as shown above, raised the question as to how p97 processes substrates when it encounters the DNA crosslink site in a protein. To address this question, we turned to a simplified unfolding reaction that does not rely on ubiquitylation but initiates at a defined internal site within a substrate protein (Fig. 5*A*). In this reaction, p97, in concert with an alternative substrate adapter p37 (encoded by UBXN2B), targets inhibitor-3 (I3, encoded by PPP1R11) bound to a complex of protein phosphatase-1 catalytic subunit (PP1) and SDS22 (encoded by PPP1R7) (22). By threading I3 through its central channel, p97 strips I3 off PP1-SDS22. I3 threading is initiated at an internal recognition site in I3 and then progresses to the Eos-unfolding reporter fused to the N-terminus of I3 (25). To test the effect of DNA crosslinks, we introduced a SNAP domain between Eos and I3 and expressed the resulting Eos-SNAP-I3 fusion protein as a complex with PP1 and SDS22 for efficient targeting by p97-p37 (Fig. 5*B*). A fraction of the purified protein was further modified by linking the SNAP domain to a 30 bp DNA oligonucleotide which resulted in a quantitative shift of the Eos-SNAP-I3 proteins in SDS-gels (Fig. 5*B*).

We next compared unfolding of unmodified Eos-SNAP-I3 with DNA-linked Eos-SNAP-I3 by fluorescence spectrometry as above. The position of the SNAP domain linked to DNA

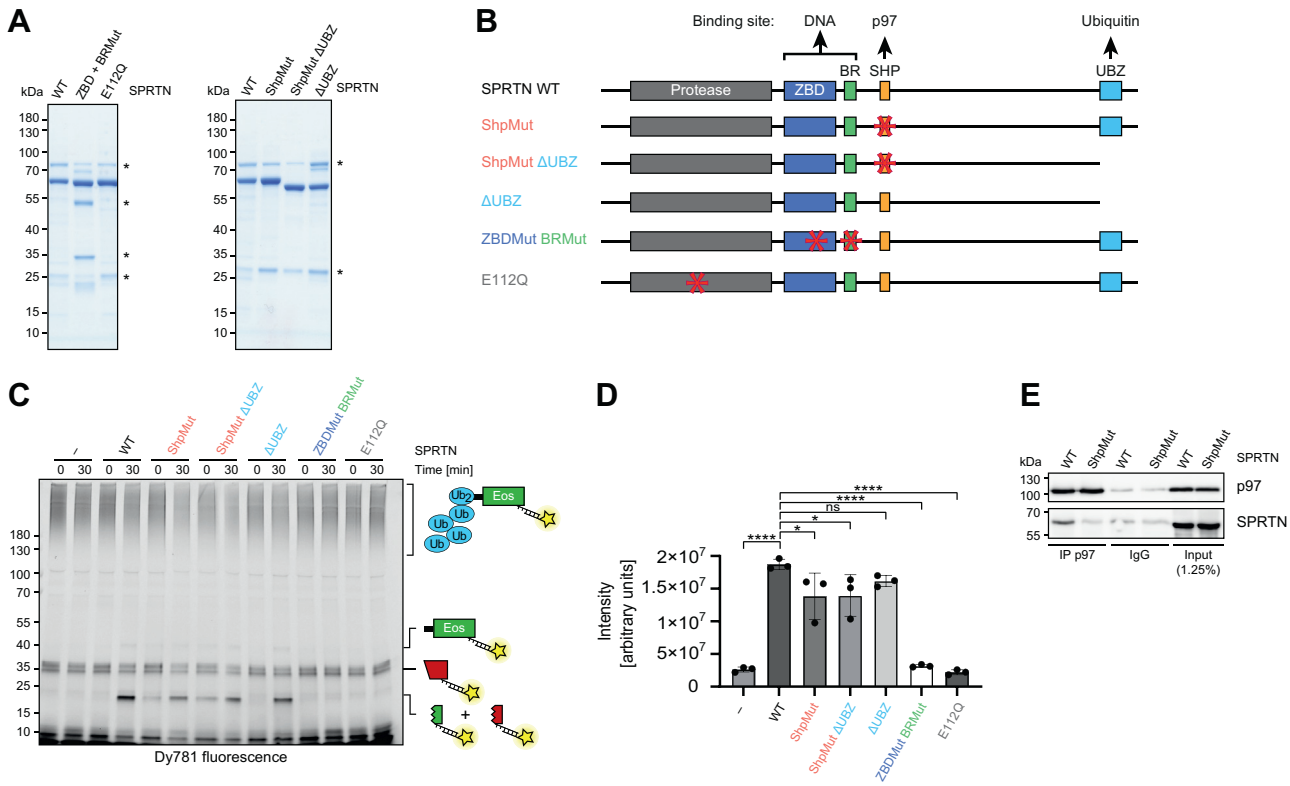

Figure 4. p97-assisted DPC reporter proteolysis depends on DNA binding by SPRTN and is stimulated by SPRTN-p97 interaction. *A*, Coomassie gels of indicated SPRTN mutant proteins used. *Asterisks* indicate impurities. *B*, domain structure of WT SPRTN and mutants. E112Q mutation abolishes protease activity. *Red asterisk* indicates mutations. *C*, DPC reporter proteolysis assay as in Figure 2*A* with WT SPRTN and indicated mutants. *D*, quantification of chromophore intensity associated with the ∼20 kDa band in (C) using a fluorescence scanner. n = 3; the error bars represent mean ± s.d. One-way ANOVA, $*p < 0.05$; $****p < 0.0001$. *E*, mutation of the SHP box in SPRTN abolishes p97 binding. p97 was incubated with WT SPRTN or SPRTN with mutated SHP box (ShpMut) before immunoprecipitation of p97. p97 and coimmunoprecipitated SPRTN was detected by Western blot. BR, basic region; DPC, DNA-protein crosslink; SHP, suppressor of high copy PP1; SPRTN, SprT-like N-terminal domain; UBZ, ubiquitin-binding zinc-finger; ZBD, zinc-binding domain.

between the unfolding initiation site in I3 and the fluorescence reporter demands that protein processing by p97 needs to pass the DNA crosslink before unfolding of Eos can be detected. Importantly, both substrate proteins were unfolded at nearly identical rates irrespective of whether linked to DNA or not (Fig. 5*C*). This result demonstrates that p97 can efficiently surmount a DNA crosslink site while processing substrate proteins.

Discussion

In this study, we provide direct biochemical evidence that SPRTN cooperates with the p97 unfolding machine to proteolyze tightly folded proteins crosslinked to DNA that SPRTN alone is unable to degrade (Fig. 6). In doing so, our biochemical dissection of the reaction establishes the key hallmarks of this cooperation.

By extending an only recently established approach to recapitulate protein unfolding by p97 and combining it with proteolysis analysis, we demonstrate that p97-assisted proteolysis by SPRTN relies on substrate unfolding by p97 and on targeting of the substrate by the Ufd1-Npl4 ubiquitin adapter. This strongly suggests that SPRTN alone cannot serve as a ubiquitin substrate adapter for p97, despite the fact that SPRTN harbors both a ubiquitin-binding UBZ domain as well as a SHP box motif that could bridge the substrate and p97. This can be explained by the fact that substrate adapters such as Ufd1-Npl4 not only link p97 to the ubiquitylated substrate but also have specific structural features to guide the substrate into the central channel of p97 for unfolding (19, 26). Consistent with a role of Ufd1-Npl4 in SPRTN-mediated proteolysis, Ufd1-Npl4 was shown to associate with SPRTN along with p97 in cells (14). The fact that the Ufd1-Npl4 ubiquitin adapter is required also implies that SPRTN cannot simply recruit DPC substrates to p97 by binding to the DNA moiety of DPCs. Therefore, if a tightly folded protein is crosslinked to DNA, ubiquitylation can serve to attract the p97–Ufd1-Npl4 complex and trigger unfolding to assist SPRTN in proteolysis. Recently, the protein TEX264 has been proposed as a ubiquitin-independent p97 substrate adapter in SPRTN-mediated proteolysis of the TOP1 cleavage complex (27), which is a special form of DPC. However, TEX264 is specific for the TOP1 cleavage complex only (27) and therefore cannot account for the large diversity of possible DPCs.

We find that the SHP box that links SPRTN to the p97 N-domain, although not essential, significantly contributes to p97-assisted proteolysis by SPRTN. This suggests that physical interaction of SPRTN with p97 is critical for the efficient coupling of protein unfolding by p97 with proteolysis by SPRTN. In contrast, the UBZ domain in SPRTN is not

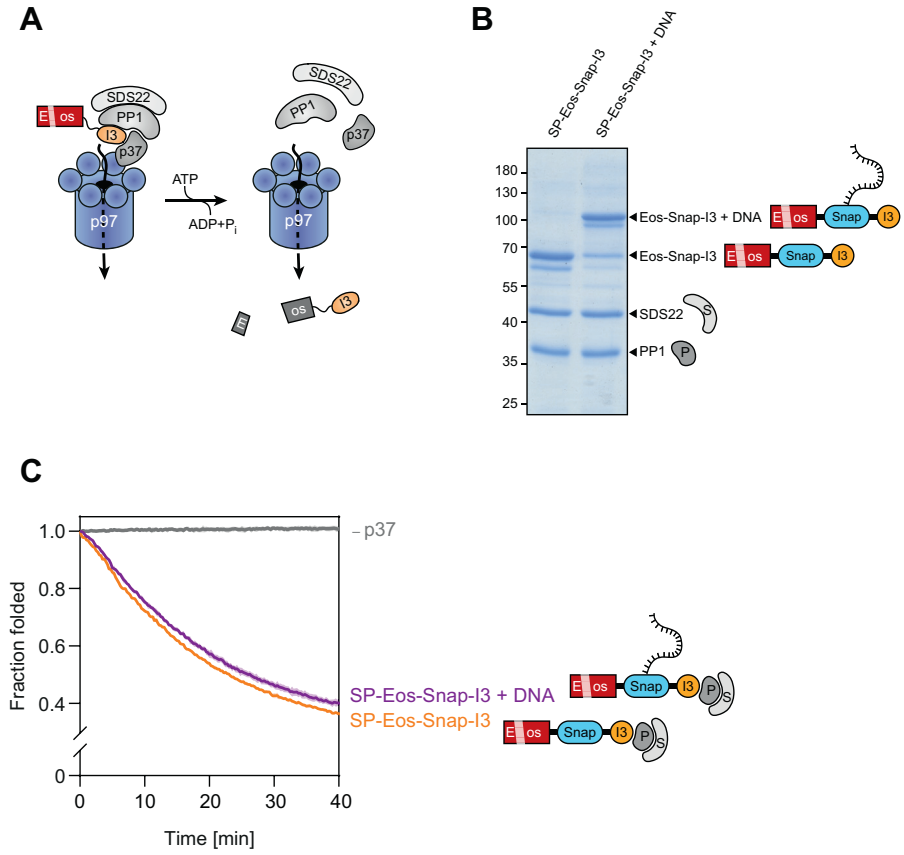

Figure 5. p97 can surmount DNA crosslink sites. *A*, an alternative, ubiquitin-independent p97-mediated unfolding reaction with defined unfolding initiation site relative to the Eos unfolding reporter moiety. A protein complex of PP1γ catalytic subunit, SDS22 and Inhibitor-3 (I3), is disassembled by threading of I3 through the p97 channel. Unfolding is initiated at I3 by binding to the p37 adapter and progresses to the Eos fluorescent unfolding sensor. *B*, a reporter substrate was generated with a SNAP-tag between the I3 and Eos moieties and purified with PP1 γ (P) and SDS22 (S) as a complex (SP–Eos-Snap-I3). A 30 nt DNA oligonucleotide was conjugated to the SNAP-tag. Coomassie-stained SDS-PAGE of free and DNA-conjugated SP–Eos-Snap-I3. *C*, unfolding assay with SP–Eos-Snap-I3 substrate conjugated to DNA or not, as indicated, p97 and the I3-specific adapter p37. p37 was omitted as a negative control (-p37). Note that unfolding kinetics are similar irrespective of the conjugated DNA. I3, inhibitor 3; PP1, protein phosphatase-1 catalytic subunit.

essential, further speaking against a role of SPRTN as a ubiquitin adapter. This is consistent with the fact that ubiquitin binding of the UBZ domain in SPRTN has been connected to intramolecular regulation of SPRTN by mono-ubiquitylation (24, 28), which does not occur in our simplified system.

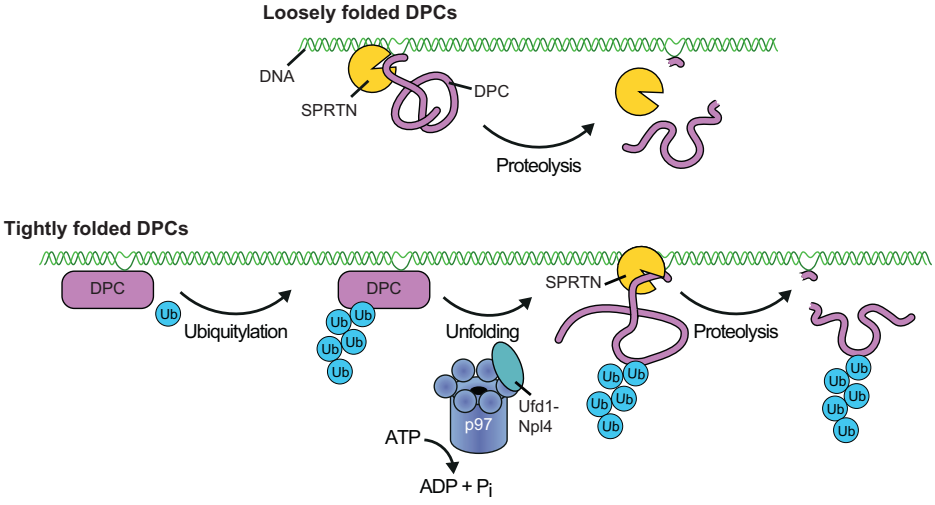

Figure 6. Model for p97-assisted DPC proteolysis by SPRTN. Loosely folded DPCs can be directly accessed by SPRTN, resulting in proteolysis of the DPC allowing subsequent removal of the remaining fragment (*upper panel*). More tightly folded DPCs require ubiquitylation and assistance by p97. p97 is recruited by Ufd1-Npl4 to the ubiquitylated DPC and unfolds the crosslinked protein to allow proteolysis by SPRTN (*lower panel*). DPC, DNA-protein crosslink; SPRTN, SprT-like N-terminal domain.

The unfolding of DPCs shown here raises the question as to how DPCs are processed by p97. Of note, by placing the DNA crosslink between a well-defined internal unfolding initiation site and the unfolding reporter moiety Eos, we demonstrate that p97 can surmount the protein crosslink site during substrate protein processing. Based on these data, it is likely that p97 threads the protein along with the DNA through the central pore of the p97 hexamer. Threading of elements wider than a single peptide strand is not unheard of because p97 can thread protein loops through its pore and even processes ubiquitin chains (19, 25). Alternatively, p97 has been proposed to open the hexamer sideways to let pass bulky moieties while the peptide chain is translocated in the channel (29). Importantly, we demonstrate that efficient proteolysis by SPRTN of the unfolded substrate still requires the DNA recognition domains of SPRTN showing that SPRTN maintains selectivity for DPCs even when cooperating with p97. Together, our data establish the hallmarks of p97-assisted proteolysis of DPC by SPRTN and, in turn, show that p97 cooperates with proteases other than the proteasome for protein degradation.

Experimental procedures

Plasmids

The cDNA of human SPRTN (NM_032018) was cloned into pGEX-6P-1 vector. A C-terminal His-tag or TwinStrep-tag was added to SPRTN by site-directed mutagenesis. Deletion and point mutants of SPRTN were generated by site-directed mutagenesis. His-diUb-mEos3.2 was generated by replacing GFP in pET28a His-diUB-GFP (21) with PCR-amplified mEos3.2 (Addgene plasmid #54550, (30)), using BamHI and XhoI restriction sites. In the plasmid encoding the E1 enzyme His-mUbe1 (Addgene plasmid # 32534, (31)), the thrombin site was replaced by a PreScission site and His-tag by a Strep-tag by consecutive site-directed mutageneses. In the recombinant E3-E2 enzyme His-gp78-ubc7 (21), the tobacco etch virus site was replaced by a PreScission site *via* site-directed mutagenesis. All constructs generated in this study were confirmed by DNA sequencing. See supporting information Table S1 for a complete list of plasmids and Table S2 for a complete list of primers used for site-directed mutagenesis.

SPRTN purification

For protein expression, plasmids were transformed into BL21(DE3) or Rosetta (DE3) *Escherichia coli* cells and grown at 37 °C in Terrific broth medium until they reached an OD_{600} 0.8. Protein expression was induced by addition of 0.5 mM IPTG overnight at 18 °C. Cells were harvested and resuspended in buffer A1 (50 mM Hepes, 500 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, pH 7.5) supplemented with 25 mM imidazole (Sigma-Aldrich, 56750). Resuspended cells were incubated for 30 min with lysozyme (PanReac AppliChem, A3711), lysed by sonication (5× 30 s at 60% intensity) and centrifuged at 20,000*g* for 45 min. Supernatant was filtered, loaded onto a HisTrap FF 5 ml column (Cytiva), and washed with 40 column volumes of buffer A1. Proteins were eluted with NiNTA elution buffer A1 supplemented with 300 mM imidazole directly onto a GSTrap High Performance 5 ml (Cytiva) and washed with 15 column volumes of buffer A1. SPRTN was eluted by cleaving off the GST-tag using PreScission protease. SPRTN was further purified by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 pg column equilibrated in buffer A1 supplemented with 1 mM DTT. For purification of SPRTN-TwinStrep, the NiNTA purification step was replaced by a StrepTag purification as follows. Cleared lysate was loaded onto a StrepTrap HP 5 ml column (Cytiva) and eluted with buffer A1 supplemented with 2.5 mM desthiobiotin. All purification steps were carried out at 4 °C and proteins were concentrated using 10 kDa centrifugal concentrator (Vivaspin Turbo 15) before snap freezing in liquid nitrogen and stored at −80 °C.

Purification of recombinant Ub_2-Eos and ubiquitylation enzymes

Proteins were expressed as described above. Cells were resuspended in buffer A2 (50 mM Hepes, 150 mM KCl, 2 mM $MgCl_2$, 5% glycerol) or buffer A2 supplemented with 25 mM imidazole for Strep- and His-tagged proteins. Lysis, centrifugation, and purification steps were performed as described for SPRTN using buffer A2. His-tag of recombinant gp78-ubc7 was cleaved off by GST-PreScission. His-diUb-mEos3.2 was converted to the red form by irradiation at 365 nm wavelength for 1 h on ice using a longwave UV lamp (B-100AP, Blak-Ray) as previously described (22).

Ubiquitin purification

Untagged ubiquitin was expressed in bacteria and lysates resuspended in buffer A2 (50 mM Hepes, 150 mM KCl, 2 mM $MgCl_2$, 5% Glycerol, pH 7.4). Cleared supernatant was stirred at 4 °C for 10 min after addition of 70% perchloric acid and centrifuged at 20,000*g* for 1 h. The supernatant was dialyzed overnight in ubiquitin wash buffer A3 (25 mM ammonium acetate pH 4.5) and loaded onto a 5 ml HiTrap SP HP cation exchange column (Cytiva). Protein was gradually eluted using buffer A4 (250 mM ammonium acetate 200 mM NaCl pH 7.6). Buffer was exchanged to buffer A2 and ubiquitin was concentrated with 5 kDa centrifugal concentrator (Vivaspin Turbo 15) before snap freezing in liquid nitrogen and stored at −80 °C.

p97, p37, Ufd1-Npl4, and GroEL purification

Expression and purification of His-p97, p37, His-Ufd1-Npl4 and $GroEL^{D87K}$ was performed as previously described (22, 25)

Generation of DNA-Protein–Crosslink substrate

Amide-tagged 30 nucleotide X30 oligo (Microsynth, Table S2) was hybridized in hybridization buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 8) with 25 nucleotide oligos X30_G_5_rv or X30_G_5_rv_Dy781 (Table S2) with six-fold excess of Dy781-labeled oligonucleotides. Hybridized primers were purified with Zeba Spin desalting column (Thermo Scientific, 89890) in crosslink buffer (100 mM NaH_2PO_4, 150 mM NaCl, 5 mM EDTA, pH 7.3) and incubated with SMCC

(succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate; Thermo Scientific, 22360) crosslinker for 2 h at room temperature. After purification *via* Zeba Spin column in crosslink buffer, maleimide crosslink reaction was performed at 4 °C overnight using a 10-fold excess of DNA over recombinant Eos protein. Crosslinked protein was separated by size-exclusion chromatography using a HiLoad 16/600 Superdex 75 pg column in buffer A2 (50 mM Hepes, 150 mM KCl, 2 mM $MgCl_2$, 5% glycerol, pH 7.4).

For DNA crosslinking with SP-Eos-SnapTag-I3, amide-tagged 30 bp S30 oligo (IDT, Table S2) was incubated with ten-fold excess of *N*-hydroxysuccinimide (NHS)-reactive SnapTag (BG-GLA-NHS, New England Biolabs) overnight at room temperature in buffer A2. Excess NHS-reactive SnapTag was quenched by addition of 20 mM Tris pH 8.0 and subsequently removed by ethanol precipitation, and the oligonucleotide was resuspended in buffer A2. Crosslinking with SP-Eos-SnapTag-I3 was performed for 90 min at room temperature with ten-fold excess of DNA oligonucleotide.

Enzymatic poly-ubiquitylation reaction and purification

DPC substrate was enzymatically polyubiquitylated as previously described (21). Briefly, DPC-substrate was incubated with mUbe1 (2 μM), gp78-ubc7 (20 μM), ubiquitin (400 μM), and ATP (10 mM) overnight at 37 °C before the poly-ubiquitylated substrate was purified from free ubiquitin chains by NiNTA affinity chromatography (HisTrap FF, 1 ml; Cytiva) and size-exclusion chromatography (Superdex 10/300,200 Gl; Cytiva), as described above.

Fluorescence-based DPC unfolding and proteolysis assay

p97 unfolding activity and SPRTN proteolytic activity was assessed by decrease in Ub_n-Eos-DPC fluorescence. Reactions with DPC substrate (40 nM), p97 hexamer (125 nM), and Ufd1-Npl4 (250 nM) were incubated in reaction buffer (25 mM Hepes pH 7.4, 100 mM NaOAc, 5 mM MgCl2, 1 mM DTT) for 5 min at 37 °C before the reaction was started by addition of SPRTN (80 nM) and ATP (3 mM). Fluorescence decay was measured every 15 s over 45 min (green excitation: 500 nm; green emission: 520 nm; red excitation: 540 nm; red emission: 580 nm) in a Cary Eclipse fluorescence spectrophotometer (Varian). Results were normalized to the first data point after addition of SPRTN and ATP. Background was subtracted using control samples without ATP.

DPC proteolysis assay

The proteolytic activity of SPRTN was visualized by Dy781 detection after SDS-PAGE. DPC model substrate (80 nM) was incubated with p97 hexamer (250 nM), Ufd1-Npl4 (500 nM), SPRTN (20 nM), and ATP (3 mM) in reaction buffer at 37 °C for indicated times before the reaction was stopped by addition of Laemmli sample buffer. Samples were boiled for 5 min at 95 °C and slowly cooled down. After SDS-PAGE, Dy781 infrared fluorescence was scanned (Odyssey DLx, LI-COR). Images were analyzed using ImageJ.

Immunoprecipitation of p97

Reactions with p97 (200 nM hexamer), SPRTN-His (400 nM WT or ShpMut), ATPγS (5 mM), and bovine serum albumin (BSA) (1%), were incubated with anti p97/VCP antibody (see below) or IgG control and loaded onto Protein G beads (ProteinG Sepharose 4 Fast Flow, GE Healthcare) for 3 h at 4 °C in buffer A5 (50 mM Hepes, 150 mM KCl, 5 mM $MgCl_2$, 5% Glycerol, pH 8, 1% Triton) supplemented with 1% BSA. Beads were washed with buffer A5 before proteins were eluted by boiling in Laemmli sample buffer.

Antibodies

The following antibodies were used for Western blot analysis: mouse monoclonal anti-VCP (Santa Cruz Biotechnology, sc-57492); rabbit polyclonal anti-SPRTN (Atlas Antibodies, HPA025073); rabbit polyclonal anti-C1orf124/SPRTN (Origene, TA333626); rabbit polyclonal anti-NPLOC4/Npl4 (Atlas Antibodies, HPA021560); mouse monoclonal anti-Ufd1L (BD Transduction Laboratories, 611642); and rabbit polyclonal anti-mEos2 (Badrilla, A010-mEOS2).

Pull down of SPRTN-Twin-Strep

Reaction of a final concentration of p97 (200 nM hexamer), SPRTN-TwinStrep (400 nM), BSA (2%), and Ufd1-Npl4 (0–4 μM as indicated) were incubated with Strep beads (Strep-Tactin Sepharose, iba) for 1 h at 4 °C in buffer A5 supplemented with 2% BSA. Beads were washed with buffer A5 and eluted by boiling in Laemmli sample buffer at 95 °C.

Data availability

All data are contained within the article and supporting information.

Supporting information—This article contains supporting information.

Author contributions—A. K., J. vd. B., M. K., and A. F. K. investigation; J. vd. B. and H. M. supervision; A. K., J. vd. B., M. K., A. F. K., and H. M. formal analysis; A. K., J. vd. B., M. K., A. F. K., and H. M. data curation; H. M. conceptualization; H. M. writing–original draft; A. K., J. vd. B., M. K., A. F. K., and H. M. writing–review and editing; J. vd. B. visualization; M. K. resources; H. M. funding acquisition.

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Abbreviations—The abbreviations used are: BSA, bovine serum albumin; BR, basic region; DPC, DNA-protein crosslink; I3, inhibitor-3; PP1, protein phosphatase-1 catalytic subunit; SHP, suppressor of

high copy PP1; SPRTN, SprT-like N-terminal domain; UBZ, ubiquitin-binding zinc-finger; ZBD, zinc binding domain.

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