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DDI2 Is a Ubiquitin-Directed Endoprotease Responsible for Cleavage of Transcription Factor NRF1

Graphical Abstract

Highlights

- DDI2 KO cells accumulate highly ubiquitylated proteins that are slowly degraded
- DDI2 KO cells are hypersensitive to pharmacological proteasome inhibition
- Purified DDI2 protein can directly cleave the highly ubiquitylated proteins in vitro
- DDI2 site-specifically cuts NRF1 in vitro but only if NRF1 is poly-ubiquitylated

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In Brief
Dirac-Svejstrup et al. show that DDI2 knockout cells accumulate highly ubiquitylated proteins that are difficult to degrade and that these cells are hypersensitive to pharmacological proteasome inhibition. The DDI2 protein can directly cleave the highly ubiquitylated proteins in vitro and does this site specifically in the ubiquitylated transcription factor NRF1.
DDI2 Is a Ubiquitin-Directed Endoprotease Responsible for Cleavage of Transcription Factor NRF1

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SUMMARY

The Ddi1/DDI2 proteins are ubiquitin shuttling factors, implicated in a variety of cellular functions. In addition to ubiquitin-binding and ubiquitin-like domains, they contain a conserved region with similarity to retroviral proteases, but whether and how DDI2 functions as a protease has remained unknown. Here, we show that DDI2 knockout cells are sensitive to proteasome inhibition and accumulate high-molecular weight, ubiquitylated proteins that are poorly degraded by the proteasome. These proteins are targets for the protease activity of purified DDI2. No evidence for DDI2 acting as a de-ubiquitylating enzyme was uncovered, which could suggest that it cleaves the ubiquitylated protein itself. In support of this idea, cleavage of transcription factor NRF1 is known to require DDI2 activity in vivo. We show that DDI2 is indeed capable of cleaving NRF1 in vitro but only when NRF1 protein is highly poly-ubiquitylated. Together, these data suggest that DDI2 is a ubiquitin-directed endoprotease.

INTRODUCTION

The ubiquitin-proteasome system (UPS) plays a crucial role in the regulated degradation of proteins (Varshavsky, 2017). As a general rule, UPS targets are poly-ubiquitylated, typically via a lysine 48-linked ubiquitin chain and recognized by ubiquitin receptors on the proteasome that guide them to the proteasome barrel for degradation, during which mono-ubiquitin is released by the action of ubiquitin proteases (also called deubiquitylating enzymes [DUBs]). In some cases, however, so-called ubiquitin shuttling factors are involved in directing proteins to the proteasome as well (Saeki, 2017; Zientara-Rytter and Subramani, 2019). The function of these important UPS factors remains poorly understood.

The main ubiquitin shuttling factors are HRAD23 (Rad23 in budding yeast), Ubiquilin (Dsk2), and DDI2 (Ddi1). In yeast, the ubiquitin shuttling factors all contain a ubiquitin-associated (UBA) domain that binds ubiquitin (Bertolaet al., 2001b; Nowicka et al., 2015), and a ubiquitin-like (UBL) domain enabling them to bring ubiquitylated substrates to the proteasome (Gabriely et al., 2008; Gomez et al., 2011; Ivantsiv et al., 2006; Kaplan et al., 2005; Zientara-Rytter and Subramani, 2019). Interestingly, the UBA domain is missing in human DDI2, which instead contains a domain with homology to ubiquitin-interacting motifs (UIMs) (Sivá et al., 2016). Moreover, the UBL domains of both yeast Ddi1 and human DDI2 are capable of binding ubiquitin as well (Nowicka et al., 2015; Sivá et al., 2016).

While the functional importance of DDI2 in particular remains relatively poorly understood in metazoans, genetic experiments have indicated an involvement of budding yeast Ddi1 in a variety of cellular functions, including cell-cycle progression, mating type switching, maintenance of genome stability, and protein secretion (e.g., Gabriely et al., 2008; Ivantsiv et al., 2006; Kaplan et al., 2005; Svoboda et al., 2019; Voloshin et al., 2012; White et al., 2011; Zhu and Xiao, 1998, 2001). Some yeast ddi1Δ phenotypes are exacerbated by concomitant deletion of RAD23 and DSK2 (Diaz-Martinez et al., 2006). This and other evidence support the idea that the shuttling factors functionally overlap (Diaz-Martinez et al., 2006; Saeki et al., 2002). Indeed, the yeast Ddi1 and Rad23 proteins are capable of forming a complex (Bertolaet al., 2001a).
DDI2/Ddi1 is unique among the ubiquitin shuttling factors in also containing a domain with structural similarity to the active site domain of retroviral aspartyl proteases (Sirkes et al., 2006). Remarkably, however, only a single publication has reported DDI2/Ddi1 protease activity in vitro, with the purified Leishmania major enzyme showing activity at low pH against BSA and some peptide substrates (Perteguer et al., 2013). There has thus generally been a failure to detect DDI2/Ddi1 protease activity in vivo (see, for example, Gabriely et al., 2008; Koizumi et al., 2016; Lehrbach and Ruvkun, 2016; Svoboda et al., 2019; White et al., 2011).

Recent results in human cells point to a role for DDI proteins in suppressing replication stress through an effect on the stability of replication fork substrates (Perteguer et al., 2013). There has thus generally been a failure to detect DDI2/Ddi1 protease activity in vivo (see, for example, Gabriely et al., 2008; Koizumi et al., 2016; Lehrbach and Ruvkun, 2016; Svoboda et al., 2019; White et al., 2011).

Intriguingly, however, attempts to reconstitute DDI2-mediated NRF1 cleavage reaction in vitro with purified proteins were unsuccessful (Koizumi et al., 2016). So, whether the effect of DDI2 mutation on NRF1 cleavage is direct and, if so, how DDI2 acts as a protease has remained unclear.

Here, we provide evidence that DDI2 is required for the timely degradation of a subset of ubiquitylated proteins. Indeed, in the absence of DDI2, human cells accumulate slowly migrating ubiquitylated proteins, which are substrates for the proteasome of DDI2 protein in vitro. Importantly, we show that DDI2 can also cleave its well-known target NRF1, but only when NRF1 is isolated in a highly poly-ubiquitylated form. Together, our data indicate that DDI2 is a ubiquitin-directed endoprotease.

Results and Discussion

In order to study the function of DDI2, we used CRISPR technology to generate MRC5VA cells in which the DDI2 gene was knocked out (DDI2 KO) (Figure 1A). These cells grew normally and showed no outward signs of cellular stress. To study the effect of DDI2 KO on ubiquitin biology, ubiquitylated proteins were analyzed by western blot analysis, initially after isolation via GST-DSK2 affinity chromatography (Anindya et al., 2007; Tufegdzic Vidakovic et al., 2019). Such proteins migrated as smears upon SDS-PAGE and western blot analysis using anti-ubiquitin antibodies (Figure 1B). Intriguingly, we noticed that ubiquitylated proteins migrated markedly more slowly when isolated from DDI2 KO cells. Importantly, although comparing the heterogeneous smears observed upon anti-ubiquitin western blotting can sometimes be challenging, the differences were highly consistent and independent of the level of protein loading (Figure 1B, compare lanes 2 and 3, for example). Quantification of the differences confirmed the validity of these observations (Figures S1A–S1C). Moreover, we noticed that electrophoresis on BioRad 3%–8% Tricine (or TGX) SDS-PAGE gels gave rise to a more clear-cut readout for these differences, with slowly migrating ubiquitylated proteins from DDI2 KO cells observed in an area at the top of the gel that was often largely free of signal in wild-type (WT) cells (Figure 1C). These data suggest a role for DDI2 in the processing of a subset of ubiquitylated proteins.

To investigate the possibility that DDI2 affects the degradation of some ubiquitylated proteins, WT and DDI2 KO cells were treated with the translation inhibitor cycloheximide, and the fate of ubiquitylated proteins was analyzed at different times over the next several hours. While, as expected, ubiquitylated proteins were generally rapidly degraded in WT cells, the high molecular weight (MW) ubiquitylated species were more persistent in DDI2 KO cells (Figure 1D; see also the quantification in Figures S1D and S1E), indicating that these proteins are more slowly removed by the UPS.

Recognizing the potential importance of DDI2 in the context of proteasome inhibitor therapy in cancer treatment, we also investigated the effect of DDI2 KO in the myeloma cell line U266B, which was shown to be sensitive to the first generation of proteasome inhibitors (Hideshima et al., 2001). For this purpose, we generated a U266B DDI2 KO cell line (Figure 1E). Although this DDI2 KO showed no significant growth defects under normal conditions, it had significant problems recovering from treatment with proteasome inhibitor PS-341 compared to parental cells (Figure 1F). Similar results were observed in MRC5VA cells, although the return to growth in these cells was slightly faster (Figure S2). Interestingly, compared to WT, the DDI2 KO cells showed evidence of even higher MW ubiquitylated proteins after proteasome inhibition (Figure 1G, compare lanes 2–4 with 6–8). Moreover, also in multiple myeloma cells, DDI2 KO has the effect of perturbing the processing of ubiquitylated proteins (Figure 1G, compare lane 1 to lane 5).

Together, these results suggest that human DDI2 assists the UPS and that, in its absence, a cohort of slowly migrating ubiquitylated proteins accumulate. They also suggest an important role for DDI2 in the survival and re-growth of cells after treatment with proteasome inhibitor.

DDI2 Protease Activity In Vitro

To investigate whether the slowly migrating ubiquitylated proteins in DDI2 KO cells are substrates for DDI2 protease activity, we incubated extracts from these cells with purified DDI proteins from different sources (see Figures S3A and S3B for purified proteins). Yeast Rad23 and Ddi1 were previously shown to form a complex (Bertolaet et al., 2001a), but we failed to find evidence for a stable human DDI2-HRAD23 (RAD23) interaction (Figure S3C). For most in vitro protease experiments, we separated the ubiquitylated species by Tricine or TGX SDS-PAGE because this analysis method generally provided a clearer, more “binary” readout of the ubiquitin smears at the top of the gel than other gel types (compare Figures 1B and 1C).

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Upon incubation of extracts with human or *Leishmania major* DDI proteins, the most clearly visible effect was that the slowly migrating ubiquitylated proteins from *DDI2* KO cells disappeared or greatly diminished (Figure 2A). In support of the functional connection between RAD23 and DDI2 reported by others (Bertolaet et al., 2001a), we found that RAD23 stimulated DDI2-mediated cleavage (Figure 2A, lane 6 versus lane 4; see also Figure 4) but had no effect on its own (lane 5). RAD23 was therefore added

**Figure 1. **DDI2 KO Cells Accumulate Slow-Migrating Ubiquitylated Proteins

(A) DDI2 western blot analysis of MRC5VA WT and *DDI2* KO (Δ) cells. Asterisks denote non-specific bands.
(B) Western blot analysis of ubiquitylated proteins after DSK2 chromatography of extracts from WT and *DDI2* KO (Δ) cells. For ease of comparison between WT and Δ, increasing amounts of protein was loaded. See quantification in Figures S1A–S1C.
(C) As in (B), but using another SDS-PAGE gel type, as indicated.
(D) Stability of ubiquitylated protein species in WT and *DDI2* KO (Δ) extracts after cycloheximide inhibition of new protein production at time = 0. Gel as in (B). See quantification in Figures S1D and S1E.
(E) As in (A), but in U266B cells.
(F) Incucyte Live-Cell Analysis of U266B multiple myeloma re-growth after a 16 h treatment with 5 nM PS-341 (Bortezomib) and control (DMSO). Confluence analyzed in Prism. Experiments were performed in triplicate, and numbers represent mean ± SD. See Figure S2 for MRC5VA cells.
(G) Western blot analysis of ubiquitylated proteins after treatment of U266B multiple myeloma cells with different concentrations of Bortezomib/PS-341. Cell extracts were analyzed directly, on 4%-15% Criterion TGX gels. See also Figures S1 and S2.
\section*{short article}

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\section*{LEGEND (continued)}

\begin{itemize}
\item **A**: Western blot analysis of DDI1 and DDI2 expression in WT and DDI2 KO cell extracts. 
\item **B**: Western blot analysis of ubiquitinated proteins in WT and DDI2 KO cell extracts. 
\item **C**: Western blot analysis of ubiquitinated proteins in WT and DDI2 KO cell extracts. 
\item **D**: Western blot analysis of ubiquitinated proteins in WT and DDI2 KO cell extracts. 
\item **E**: Western blot analysis of ubiquitinated proteins in WT and DDI2 KO cell extracts. 
\end{itemize}

\section*{RESULTS}

\subsection*{DDI2 KO affects ubiquitination and SUMOylation}

In WT cells, DDI2, DDI1, and R23 are expressed at similar levels. In DDI2 KO cells, the levels of DDI2, DDI1, and R23 are reduced, indicating a role for DDI2 in the regulation of these proteins. 

\subsection*{DDI2 KO affects SUMOylation}

In WT cells, DDI2 is efficiently SUMOylated, whereas in DDI2 KO cells, the levels of SUMOylation are reduced, indicating a role for DDI2 in the regulation of SUMOylation.
to most subsequent experiments. Given that no ATP was added to these reactions, the DD1 proteins did not work indirectly by stimulating ATP-dependent degradation by the proteasome in these assays. Indeed, addition of a proteasome inhibitor had little or no effect on the removal of ubiquitylated proteins (Figure 2B, left and right panels). Scanning and quantification of the gel suggested a small, general decrease in ubiquitin signal upon DD12 addition (Figure S4B), but ubiquitylated proteins of high MW were clearly preferentially affected (Figure S4C). Importantly, highly purified human DD12 from E. coli removed the high MW ubiquitylated proteins as well, and this required the DD12 active site (Figure 2C, compare WT in lanes 3 and 4 with DD12-ΔN mutant in lane 5; DD12-ΔN has the catalytic aspartate252 in the retropepsin domain mutated to asparagine), further indicating that the effect is direct.

Ubiquitylated proteins are strongly bound to GST-DSK2 resin, allowing their isolation under stringent conditions, including wash in high-salt buffer (Tufegdzic Vidakovic et al., 2019; see also STAR Methods). As in extracts, when such beads containing ubiquitylated proteins were incubated with WT DD12, a clear reduction in the amount of high MW species was again observed, while DD12-ΔN or RAD23 alone had little or no effect (Figure 2D, compare lane 2 with lanes 3 and 4; see also quantification of an independent experiment in Figures S5A–S5C). Other experiments were separated by 4%–12% Bis-Tris PAGE (as in Figure 1B), with similar results (Figure 2E, compare DD12 treatment in lane 4 with the controls in lanes 2 and 5).

Together, these results indicate that DD12 is an active protease, capable of digesting the slowly migrating ubiquitylated proteins accumulating in DD12 KO cells.

**Lack of Evidence for a Specific Ubiquitin Chain Type Being Recognized by DD12**

Although DD12 digestion of ubiquitylated targets did not appear to generate mono-ubiquitin or ubiquitin multimers (Figure S5D; data not shown), we also tested whether our active, purified DD12 protein is capable of cleaving a range of commercially available ubiquitin chains. DD12-RAD23 showed no activity against such peptides, including K29- and K33-linked tetra-ubiquitin, as well as K48- and K63-linked tetra- or poly-ubiquitin, even when added in large amounts relative to substrate, while the non-specific catalytic domain of the DUB USP2 (USP2CD, here called USP2) efficiently digested the same substrates, mostly to mono-ubiquitin (Figure 3A; data not shown). These results mirror and extend previously reported negative results using the yeast Ddi1 protein (see, for example, Nowicka et al., 2015). Moreover, addition of a large excess of ubiquitin monomer or the different ubiquitin tetramers failed to inhibit the removal of slow migrating ubiquitylated proteins by DD12 (data not shown).

We also investigated the potential role of SUMOylation. For this purpose, the membrane from the experiment of Figure 2E was also probed with anti-SUMO antibodies (Figure 2E, lower panel). SUMOylation is not a notable characteristic of the slowly migrating ubiquitylated proteins that characterize DD12 KO cells: in contrast to ubiquitylation (upper panel), there was no marked difference between WT and DD12 KO cells (lower panel, compare lanes 1 and 2). Moreover, while treatment with SUMO protease Ulp1 completely removed the SUMO signal (Figure 2E, lower panel, lane 3), it had no specific effect on the slowly migrating ubiquitylated proteins but instead appeared to generally decrease the signal across the entire cohort of ubiquitylated proteins (upper panel, lane 3). Together, this suggests that DD12 does not specifically recognize mixed ubiquitin-SUMO chains.

The experiments above focused on the ability of DD12 activity to remove slowly migrating ubiquitylated proteins from DD12 KO extracts. We now performed experiments, outlined in Figure 3B, in which the binding and cleavage substrates of DD12 were investigated. In order to first look at DD12 binding without cleavage, we incubated DD12 KO cell extract with FLAG-DD12 in which the active site had been chemically in-activated (DD12<sub>inact</sub>) and then immobilized this material on M2 beads (Figure 3C, lanes with uneven numbers); empty M2 beads were used as control (even numbers). Elution for western blot analysis was achieved by boiling in loading buffer. Strikingly, the most slowly migrating ubiquitylated proteins were preferentially bound by DD12<sub>inact</sub> (Figure 3C, compare bound proteins in lanes 5 and 7 with those in the supernatants of lanes 1–4). Indeed, with a relatively large volume of extract used compared to the volume of DD12<sub>inact</sub>-affinity beads, the bound fractions were highly concentrated in DD12-interacting proteins when compared to the extracts in lanes 1–4. A strong signal for high MW proteins was thus found associated with beads (lanes 5 and 7), while these proteins were depleted from the supernatants (compare lanes 1 and 3 with empty beads in lanes 2 and 4). By contrast, faster-migrating species were barely bound at all but were clearly detected in the supernatants. These data support the idea that DD12 preferentially binds slowly migrating ubiquitylated proteins.
Figure 3. DDI2 Fails to Cleave Purified Ubiquitin Chains but Preferentially Binds and Cleaves Slowly Migrating Ubiquitylated Species

(A) Coomassie-stained gels of different commercially available ubiquitin chains before and after incubation with DDI2, RAD23, or the catalytic domain of USP2, as indicated. Untreated substrates are indicated by stippled boxes. Note that USP2 cleaves all these chains to mono- or di-ubiquitin. See also lack of detectable mono-/di-ubiquitin in the blot of Figure S5 D.

(B) Schematic of experiments in (C) and (D).

(C) Western blot analysis of ubiquitylated proteins in bound and unbound (supernatant) fractions after incubation with chemically inactivated DDI2 protein (note that two biological replicates of the same experiment are shown; lanes 1 and 2/5 and 6 and lanes 3 and 4/7 and 8). Note the specific depletion of slowly migrating ubiquitylated proteins in lanes 1 and 3 and the enrichment of the same in lanes 5 and 7.

(D) As in (C), but ubiquitylated proteins on FLAG-DDI2DN beads, incubated with DDI2 or DDI2D/N (non-FLAG tagged to avoid non-specific “displacement” from the beads), as indicated. Note the disappearance of ubiquitylated proteins from beads after incubation with WT DDI2 (above black stippled line, lane 3) and concomitant release of faster-migrating ubiquitylated proteins into the supernatant (below red stippled line, lane 9). See also ImageJ scanning traces below, with position of stippled lines indicated for reference. Please note that the exposure time of the blot on the right is longer than that on the left; only small amounts DDI2-bound material can be eluted.

See also Figure S5.
We now investigated whether we could detect cleavage of DDII2-binding proteins. For this purpose, we treated proteins bound to immobilized FLAG-DDII2D→N beads with WT DDII2 in the hope of releasing cleaved proteins into the supernatant, using the inactive DDII2D→N as control (both were non-FLAG tagged) (Figure 3D). Importantly, both western blots and scanning profiles from such experiments consistently showed that the ubiquitylated proteins eluted with active DDII2 migrated significantly faster than those displaced by simple competition with the DDII2D→N control (Figure 3D, compare lane 9 with lane 11 [blot and scan]), indicating that they were indeed cleaved.

Frustratingly, using experiments such as those in Figures 3C and 3D as a starting point, a variety of mass spectrometry approaches failed to uncover convincing and consistent DDII2 binding partners/targets or specific binding determinants of the DDII2-associating, ubiquitylated material (an example is shown in Table S1). However, ubiquitin was consistently detected in such experiments. Moreover, although mass spectrometry did detect the release of ubiquitin, we failed to detect ubiquitin peptides with new amino acid termini that would be indicative of site-specific ubiquitin cleavage after DDII2-mediated digestion.

To investigate the possibility that DDII2 recognizes chains of a specific linkage, or perhaps even branched/forked ubiquitin chains, DDII2-associated material was also subjected to analysis via the UbiSite approach, which allows comprehensive mapping of lysine and N-terminal ubiquitylation sites, including ubiquitin-ubiquitin linkages (Akimov et al., 2018a, 2018b). Here, ubiquitylated proteins from DDII2 KO cells purified via immobilized DDII2D→N were compared with the total “ubiquitylome” in WT cells. No significant enrichment in any particular ubiquitin-ubiquitin linkage was observed in the DDII2D→N-associated material (Table S2), suggesting that ubiquitin chain composition might not be the major factor in DDII2 substrate recognition.

**DDII2 Cleaves NRF1 in a Ubiquitylation-Dependent Fashion**

We now investigated the hypothesis that DDII2 recognizes ubiquitylated proteins and then sometimes cleaves the ubiquitylated protein itself. Upon proteasome inhibition, DDII2-dependent processing allows NRF1 to enter the nucleus and upregulate a subset of genes, including proteasome genes (reviewed by Motosugi and Murata, 2019), but whether DDII2 is directly responsible was unclear. Indeed, previous attempts at cleaving NRF1 in *vivo* were unsuccessful (Koizumi et al., 2016).

Interestingly, when cell extracts were probed using anti-NRF1 antibody, it appears as if NRF1 is not ubiquitylated (Figure 4A, lanes 1 and 2). However, after first enriching for ubiquitylated proteins on DSK2 beads, a smear of very-slow-migrating NRF1 forms becomes evident (lanes 5 and 6). To the best of our knowledge, these forms of NRF1 have not previously been observed, but they must represent poly-ubiquitylated NRF1 as they migrate like the main NRF1 form upon de-ubiquitylation by USP2CD (compare lanes 5 and 6 with 10 and 11). It seems reasonable to speculate that the ubiquitin chains on NRF1 must be very long; indeed, the apparent size difference between the de-ubiquitylated protein and the ubiquitylated species is substantial, with the most slowly migrating forms detected far above the 250 kDa marker (compare lanes 10 and 11 with lanes 5 and 6).

As previously reported by others (Koizumi et al., 2016), WT, but not DDII2 KO, cells contained the faster-migrating DDII2-dependent cleavage in *vivo* (Figure 4A, compare lane 1 [arrow] with lane 2). When fractionated by GST-DSK2 chromatography, these un-ubiquitylated NRF1 species were detected exclusively in the flow-through (FT) fractions (Figure 4A, compare lanes 1 and 2 and 3 and 4 with lanes 5 and 6). Although WT cells generally contained less of the slowly migrating ubiquitylated protein species than DDII2 KO cells, they contained more ubiquitylated NRF1 protein (compare lanes 5 and 6 between Figures 4A and 4B). The underlying reasons remain to be investigated, but USP2CD digestion of the same samples suggests that a majority of the more abundant, ubiquitylated NRF1 protein in WT extract may actually already be cleaved (Figure 4A, compare lane 10 [arrow] with 11 and with lanes 5 and 6).

No obvious effect was observed after incubation of ubiquitylated NRF1 with DDII2-RAD23 (Figure 4A, lanes 6–9). Importantly, however, NRF1 cleavage products were clearly visible when the reactions were subsequently treated with USP2 to remove all ubiquitylation (Figure 4A, lane 14, compare with lanes 11–13; see also zoomed-in panel), Cleavage required the catalytic activity of DDII2, as DDII2D→N failed to digest NRF1 (Figure 4C, compare lanes 4 and 5). So, the substrate for DDII2 appears to be the ubiquitylated NRF1, with its activity generating endo-proteolytic NRF1 cleavage products reminiscent of those in WT extracts upon ubiquitin removal (Figure 4A, compare lane 14 with lane 15; see also Figure S6A). In agreement with this interpretation, no cleavage was detected if USP2 was allowed to remove ubiquitin chains prior to DDII2-RAD23 incubation, showing that DDII2 requires the ubiquitin chain yet cleaves in the body of the NRF1 protein (Figure 4A, compare lanes 17–20 with 11–14). Similar results were obtained with ubiquitylated NRF1 protein isolated via FLAG-DDII2D→N chromatography (Figure S6A), confirming that ubiquitylated NRF1 protein is indeed the substrate for DDII2 activity *in vitro*.

As noted above, the NRF1 cleavage products produced by DDII2-RAD23 were reminiscent of those observed in WT extracts. However, in order to investigate whether NRF1 cleavage requires the same site as previously mapped in *vivo* (Radhakrishnan et al., 2014), transgenes expressing C-terminally Myc-tagged versions of NRF1 containing either the normal (N) or mutant (m) version of the cleavage site (NAWLVH106AAAAA “m1” in Radhakrishnan et al., 2014) were transiently expressed in DDII2 KO cells and treated as above. Significantly, normal NRF1, but not the mutated version, was cleaved by DDII2 *in vitro* (Figure 4D, lower panel, compare lanes 2 and 6 and independent biological replicates in Figure S6B, lanes 2 and 6). This further indicates that the DDII2-mediated NRF1 cleavage reaction *in vitro* faithfully reconstitutes that occurring inside cells. Note that although cleavage of transfected WT NRF1 by DDII2 was very weak in these experiments, digestion of the slowly migrating ubiquitylated species was similar in efficiency to that observed previously (compare Figure 4D, upper panel [ubiquitin blot], lane 2 and lane 6, with Figure 4B, lane 8). The weak cleavage of NRF1 thus likely reflects the fact that the exogenously expressed Myc-tagged version has to compete with endogenous NRF1 in these experiments.

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**OPEN ACCESS**
Together, the findings described here can potentially explain the activation of DDI2 and NRF1 upon proteasome inhibition: when proteasome activity is limiting, ubiquitin chains grow very long due to the delay in their degradation. DDI2 recognizes this signal and cuts proteins containing them, such as NRF1, which can then activate proteasome genes. Our results on NRF1 also shed light on the mechanism underlying its activation. Indeed, although it has previously been shown that DDI2 activity is somehow required for cleavage of NRF1 in vivo, successful reconstitution of the reaction in vitro has not previously been reported.

The slowly migrating ubiquitylated proteins seem to represent a small fraction of total ubiquitylated proteins in DDI2 KO cells: the fact that these proteins can be detected by western blot analysis suggests that the abundance of ubiquitin-antigens in long chains. Indeed, mass spectrometry analysis of the ubiquitylated proteins bound by DDI2 has proven extremely challenging, with even the analysis of their long ubiquitin chains being difficult. The scarcity of ubiquitylated proteins targeted by DDI2 has also hampered our attempts to identify and perform experiments with other targets than NRF1. It is indeed only a very small proportion of the NRF1 pool that carries what must be a very long ubiquitin chain, recognized by DDI2. To the best of our knowledge, this NRF1 form has not previously been visualized, but it is clearly pivotal for DDI2-mediated cleavage and NRF1 function.

We note that our work does not exclude the possibility that DDI2 cuts very long ubiquitin chains as well, for example, in one or a few places rather than at every ubiquitin along the chain. Indeed, it would in many ways make sense in the context of the role of Ddi1/DDI2 as ubiquitin shuttles if infrequent cleavage of long ubiquitin chains facilitated the loading into the proteasome of a large variety of proteins with long ubiquitin chains. Interestingly, while this manuscript was in revision, Rapoport and co-workers reported complementary data that showed that the yeast Ddi1 protein preferentially (or exclusively) recognizes an artificial target protein when it carries a long ubiquitin chain and that it may even weakly cut the chain itself (Yip et al., 2020). Importantly, our results clearly indicate that proteins with very long ubiquitin chains are slowly degraded by the UPS and require DDI2 activity for their timely removal in vivo.

In conclusion, the results reported here indicate that DDI2 represents a highly unusual enzyme in the UPS, namely a ubiquitin-directed endoprotease, the only other being the proteasome itself. DDI2 may even be unique in that it appears to also
be a ubiquitin-directed, site-specific endoprotease, at least in the case of NRF1 outlined here. Finally, our results, including those on multiple myeloma cells lacking DDI2, support the idea that inhibition of DDI2 protease might have clinical benefit in cancer therapy, alone or in conjunction with proteasome inhibition.

**AUTHORS CONTRIBUTIONS**

The CRISPR knockouts and the bortezomib therapy, alone or in conjunction with proteasome inhibition.

**STAR METHODS**

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  - Mass spectrometry analysis
  - Analysis of ubiquitylation (UbiSite)
- **SUPPLEMENTAL INFORMATION**
  - Supplemental Information can be found online at https://doi.org/10.1016/j.molcel.2020.05.035.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-Ubiquitin mouse mAb (P4D1) | ENZO Life Sciences | Cat# BML-PW0930; RRID: AB_10998070 |
| Anti-Ubiquitin mouse mAb (P4G7) | ENZO Life Sciences | Cat# ENZ-ABS142; RRID: AB_2331077 |
| Anti-TCF11/NRF1 rabbit mAb (D5B10) | Cell signaling technology Europe BV | Cat# 6052S; RRID: AB_11178947 |
| Anti-mouse secondary (HRP) | Santa Cruz | Cat# sc-516102; RRID: AB_2687626 |
| Anti-rabbit secondary (HRP) | Jackson ImmunoResearch | Cat# 711-035-152; RRID: AB_10015282 |
| Anti-mouse secondary (HRP) | VWR | Cat# NA9310; RRID: AB_772193 |
| Anti-Sumo 2 + Sumo 3 rabbit polyclonal | Abcam | Cat# ab32058; RRID: AB_778173 |
| Anti-Sumo 1 (Y299) rabbit mAb | Abcam | Cat# ab32058; RRID: AB_778173 |
| Anti-DDI2 rabbit polyclonal | Bethyl Laboratories | Cat# A304-629A |
| Anti-Myc mouse monoclonal (9E10) | Crick laboratories | Evan et al. (1985) |
| **Bacterial and Virus Strains** |        |            |
| NEB 5-alpha Competent E. coli (High Efficiency) | New England Biolabs | Cat# C2987H |
| BL21-CodonPlus (DE3)-RIL Competent Cells | Agilent technologies LDA UK LTD | Cat# 230245 |
| XL10-Gold ultracompetent cells | Agilent technologies LDA UK LTD | Cat# 200315 |
| BL21-CodonPlus (DE3)-RIL Competent Cells | Agilent technologies LDA UK LTD | Cat# 230245 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| PS-341 (Bortezomib) | Enzo Life Sciences | Cat# BV-1846-1 |
| Recombinant Human USP2 Catalytic Domain, CF | BIO-TECHNE | Cat# E-504-050 |
| Recombinant Human USP2 Catalytic Domain, CF His tagged | CALTAG Medsystems LTD | Cat# AG-40T-0539-C050 |
| Human Rad23 from E. coli | MYBIOSOURCE INC | Cat# MBS717584 |
| N-Ethylmaleimide (NEM) | Sigma-Aldrich | Cat# 04260 |
| Ampicillin | Cambridge Bioscience limited | Cat# 2484 |
| MG-132 | Sigma-Aldrich | Cat# M7449 |
| L-Glutamine | Fisher Scientific UK LTD | Cat# 15410314 |
| Medium, Basal; Gibco; DMEM (for SILAC) | Fisher Scientific UK LTD | Cat# 12817552 |
| Sodium Pyruvate, Gibco | Fisher Scientific UK LTD | Cat# 12539059 |
| L-Poline for SILAC | Life Technologies LTD | Cat# 8840 |
| Dimethyl Sulfoxide (DMSO) | Sigma-Aldrich | Cat# D2650 |
| Cycloheximide | 2BSCIENTIFIC | Cat# C084-10x1ml |
| Blasticidin | Thermo-Fisher (Gibco) | Cat# R21001 |
| Polyornithine | Sigma-Aldrich | Cat# P4957 |
| EPNP (1,2-Epoxy-3-(4-nitrophenoxy)propane: sc. 1,2-Epoxy-3-(4-nitrophenoxy)propane) | Santa Cruz | Cat# sc-258906 |
| Tetra Ubiquitin K48 linked | Boston Biochem | Cat# UC-210B |
| Tetra Ubiquitin K29 linked | Boston Biochem | Cat# UC-83 |
| Tetra Ubiquitin K63 linked | Boston Biochem | Cat# UC-310B |
| Tetra Ubiquitin K33 linked | Boston Biochem | Cat# UC-103 |
| Octa Ubiquitin K63 linked | Boston Biochem | Cat# UC-318B |
| Poly-ubiquitin (Ub2-16) K48 linked | ENZO Life Sciences | Cat# BML-UW0670 |
| **Critical Commercial Assays** |        |            |
| XT-sample buffer | BioRad Laboratories | Cat# 1610791 |
| Precision PLUS pre-stained markers | BioRad Laboratories | Cat# 1610393 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Fetal Bovine Serum Dialysed | Labtech international limited | Cat# FB-1001D |
| DMEM/F12 | Sigma-Aldrich | Cat# D6421 |
| FBS (Australian Origin) | Thermo-Fisher (GIBCO) | Cat# 10099-141 |
| GlutaMAX | Thermo-Fisher (GIBCO) | Cat# 35050-038 |
| Lipofectamine 2000 | Thermo-Fisher Scientific | Cat# 1668019 |
| Lipofectamine 3000 | Thermo-Fisher Scientific | Cat# L3000015 |
| QIAamp DNA Mini Kit | QIAGEN | Cat# 51306 |
| Q5® High-Fidelity 2X Master Mix | New England BioLabs | Cat# M0492 |
| 4-15% TGX gels | BioRad Laboratories | Cat# 5671084 |
| NuPAGE 10% Bis-Tris protein gels | Life Technologies LTD | Cat# NP03031BOX |
| Criterion XT Tris-Acetate Gel 3-8% | BioRad laboratories | Cat# 3450130 |
| Criterion XT Bis-Tris 4-12% | BioRad Laboratories | Cat# 3450124; Cat# 3450125 |
| Nitrocellulose membrane 0.45 uM | GE Healthcare Life Sciences | Cat# 10600002 |
| Nitrocellulose membrane 0.2 uM | GE Healthcare Life Sciences | Cat# 10600019 |
| Hyperfilm ECL | VWR international | Cat# 29-9068-37 |
| SuperSignal West Pico PLUS ECL reagent | Thermo Scientific | Cat# 34580 |
| DMEM media | Thermo Scientific | Cat# 41666029 |
| RPMI-1640 media | Sigma-Aldrich | Cat# R8758 |
| Radiance ECL HRP substrate | Azure Biosystems | Cat# AC2101 |
| Radiance plus femtogram HRP substrate | Azure Biosystems | Cat# AC2103 |
| Ni-NTA Superflow | QIAGEN LTD | Cat# 30410 |
| Glutathione Sepharose 4B | Sigma-Aldrich | Cat# GE17-0756-01 |
| ANTI-FLAG M2 Affinity Gel | Sigma-Aldrich | Cat# A2220 |
| InstantBlue | Expedeon | Cat# IS81L |
| HiTrap Q FF | Fisher scientific UK LTD | Cat# 10607275 |
| HiLoad 16/60 superdex 200 | GE health care | Cat# 28-9893-35 |
| XT MOPS running Buffer | BIO-RAD Laboratories | Cat# 1610788 |
| XT Tricine running Buffer | BIO-RAD Laboratories | Cat# 1610790 |
| XT MES running buffer | BIO-RAD Laboratories | Cat# 1610789 |

| Deposited Data |
|----------------|
| Proteomics (Mass Spec DDI2 interaction) | This study | PRIDE: PXD018215 |
| Proteomics (Mass Spec DDI2 interaction and UbiSite) | This study | PRIDE: PXD019152 |
| Source data (Western blots, etc.) | This study | Mendeley: https://doi.org/10.17632/cz336hbwy8.1 |

| Experimental Models: Cell Lines |
|--------------------------------|
| Human lung fibroblast cell line MRC5VA | Francis Crick Institute cell depository | N/A |
| Human lung fibroblast cell line MRC5VA DDI2 KO | This study | N/A |
| Human myeloma cell line U266B | Francis Crick Institute cell depository | N/A |
| Human myeloma cell line U266B DDI2 KO | This study | N/A |

| Oligonucleotides |
|------------------|
| DDI2 gRNA | CGAATAGATTTCAGTAGTAT | N/A |
| DDI2 Forward PCR Primer | ACTACCATCACCTCCCCCTA | N/A |
| DDI2 Reverse PCR Primer | TTGGCAGCAGATAACCTAGGT | N/A |
| DDI2 Sequencing primer | CTCATTGTJTGGGCAGCA | N/A |

| Recombinant DNA |
|-----------------|
| pLent6/V5-DEST™ Gateway™ Vector | Invitrogen | V49610 |
| pLent6-DDI2-FLAG | Life Technologies Custom Services | This study |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead Contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jesper Svejstrup (jesper.svejstrup@crick.ac.uk).

**Materials Availability**

Plasmids will be deposited with and distributed by the non-profit distributor Addgene. There are restrictions to the availability of pLenti6-DDI2-FLAG for human cell expression as it was generated by Life Technologies (now ThermoFisher) through a custom service agreement with GSK, which limits its distribution to third-parties. However, active human DDI2 is now generated in much large quantities using bacterial expression.

**Data and Code Availability**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD018215 and PXD019152.

Original/source data for the figures in the paper is available [Mendeley Data https://doi.org/10.17632/cz336hbwy8.1].

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell lines and culture conditions**

MRC5VA-derived cells were cultured in high glucose DMEM (Thermo Fisher Scientific, 41966029) supplemented with 10% v/v FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C with 5% CO₂ and routinely passaged 2 times a week. All cell lines were confirmed to be mycoplasma-free. For SILAC Mass Spec experiments, cells were grown in the same manner, but with SILAC media (Basal GIBCO, DMEM Fisher Scientific 12817552), heavy Arginine and/or Lysine with dialysed serum (Labtech international FB-1001D). U266B multiple myeloma cells were cultured in RPMI-1640 (Sigma Aldrich R8758) supplemented with 10% v/v FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, in upright flasks at 37°C with 5% CO₂ and routinely passaged 2 times a week. 293T cells for overexpression of DDI1 and DDI2 were grown in DMEM/F-12 supplemented with 10% FBS and 2mM GlutaMAX in a 5% CO₂ incubator at 37°C.
**METHOD DETAILS**

**Plasmid and lentivirus construction**

His-, SUMO- and FLAG-tags were appended to the ORFs for human DDI2 and HRAD23A to generate His-Sumo-Rad23A_pET11a, His-Sumo-DDI2_pET11a, His-Sumo-DDI2_pET11a, His-Sumo-flag-DDI2, His-Sumo-flag-DDI2_pET11a, for protein production and purification. The pET11a cloning sites were Ndel-BamHI, and all plasmids were produced by GenScript. For generation of DDI2-FLAG Lentivirus, a codon-optimized cDNA was first synthesized by GeneArt (Life Technologies), encoding the DDI2 protein with an appended flexible glycine-rich linker and FLAG epitope at the C terminus (GGGSGGGGSSGYKDDDDK), and subcloned into pLent6/V5-DEST Gateway Vector to generate pLent6-DDI2-FLAG. Generation of a Lentivirus to enable expression of FLAG-tagged DDI2 was carried out using Thermo Fisher Scientific’s ViraPower system through their custom Lentivirus generation service (formerly Life Technologies). pLent6-DDI2-FLAG was transfected into 293FT cells (Life Technologies) to generate DDI2-FLAG Lentivirus with a titer of $4.9 \times 10^6$ (Titer Units/mL as determined by the number of colonies obtained per dilution in a blasticidin resistance assay). For transient transfections of NRF1, the human NRF1 ORF was inserted into pcDNA3.1(+) -C-Myc plasmid according to the manufacturer’s instructions. After 48 h, the MRC5VA-GFP-negative cells were selected by confocal microscopy based on GFP fluorescence. U266B cells were transfected using the Neon electroporation system (Thermo Fisher Scientific) and GFP-positive cells were collected by FACS as a pool. Cells were allowed to recover and grow for 2 weeks, then sorted into 96-well plates. After growth, cells were lysed in sample buffer and examined by Western Blot using a DDI2 antibody (Bethyl Laboratories). For DDI2-FLAG purification, 1.75 x 10^8 cells were seeded into a 10-chamber Cell-Stack (Corning), expressed by IPTG induction at 1 mM for 4 h at 30°C. All proteins contained a His-Sumo-tag; but some also His-Sumo-FLAG, allowing non-tagged and FLAG-tagged versions to be generated by cleavage with the SUMO protease, Ulp1.

**Generation of cell lines**

DDI2 knock-out MRC5VA and U266B cells were created using the plasmid pSpCas9(BB)-2A-GFP (PX458 Addgene plasmid 48138). The gRNA sequence is listed in Key Resources. MRC5VA cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. After 48 h, MRC5VA GFP-positive cells were selected into 96 well plates by FACS. U266B cells were transfected using the Neon electroporation system (Thermo Fisher Scientific) and GFP-positive cells were collected by FACS as a pool. Cells were allowed to recover and grow for 2 weeks, then sorted into 96-well plates. After growth, cells were lysed in sample buffer and examined by Western Blot using a DDI2 antibody (Abcam). Genomic DNA from potential knock-out cells was isolated using a Qiaamp Kit (QIAGEN) and the region around the target site amplified by PCR. The PCR fragment was sequenced and analyzed using the TIDE program (Deskgen) to identify DDI2 KO cells.

**Growth analysis after Proteasome Inhibitor Treatment**

U266B parental and DDI2 KO cells were seeded in a poly-ornithine coated 96 well plate and treated with 5 nM PS-341 (Bortezomib) in RPMI-1640 medium (Sigma Aldrich R8758) for 16 h. The medium was then carefully removed and cells were washed twice with PBS, then 150 μl RPMI-1640 carefully added to the cells. The plate was incubated in an Incucyte Live Cell Analysis incubator for 6 days and the confluence analyzed in Prism. Likewise, the MRC5VA parental and DDI2 KO cells were seeded in a 96 well plate at $2 \times 10^8$ cells per well and treated with either DMSO or 5nM PS-341 for 16 h, washed twice in PBS and 150μl of DMEM (Life Technologies Ltd) added. The plate was incubated in an Incucyte Live Cell Analysis incubator for 6 days and the confluence analyzed in Prism.

**Myc-NRF1 expression**

Plasmids expressing C-terminally Myc-tagged normal or mutant NRF1 were transiently transfected into DDI2 KO MRC5VA cells, using Invitrogen Lipofectamine 3000. Briefly, 80% confluent cells in 15 cm dishes were incubated with 40 μg WT- or mutant NRF1 pcDNA3.1 (+) -C-Myc plasmid according to the manufacturer’s instruction using 60 μl Lipofectamine 3000 Reagent per ml Opti-MEM Medium. After 24 h, extracts were generated as described below.

**Protein purification**

**DDI2 expression in human cells, and its purification**

293T cells were infected with DDI2-FLAG Lentivirus at a multiplicity of infection (MOI) of 1 in two wells of a 24 well plate. Following overnight incubation, infected cells were selected in media containing 10 μg/mL blasticidin and expanded onto T175 flasks over the course of 10 days. Expression of DDI2 in the resulting non-clonal pool of DDI2-FLAG-expressing cells was confirmed by immunoblot using an anti-DDI2 antibody (Bethyl Laboratories). For DDI2-FLAG purification, 1.75 x 10⁸ cells were seeded into a 10-chamber Cell-Stack (Corning), cultured for 4 days following which the cell pellet was harvested and resuspended in ice-cold TBS (150 mM NaCl, 50 mM Tris, pH 7.4). The resuspended cells were lysed by sonication on ice, and insoluble material removed following centrifugation at 100,000 g for 1 h. The FLAG-tagged DDI2 protein was purified on a HiTrap column loaded with anti-FLAG M2 affinity gel using an Äkta purifier (GE Healthcare). The FLAG-tagged DDI2 protein was then purified using an anti-DDI2 antibody (Bethyl Laboratories). For DDI2-FLAG purification, 1.75 x 10⁸ cells were seeded into a 10-chamber Cell-Stack (Corning), cultured for 4 days following which the cell pellet was harvested and resuspended in ice-cold TBS (150 mM NaCl, 50 mM Tris, pH 7.4). The resuspended cells were lysed by sonication on ice, and insoluble material removed following centrifugation at 100,000 g for 1 h. The FLAG-tagged DDI2 protein was purified on a HiTrap column loaded with anti-FLAG M2 affinity gel using an Äkta purifier (GE Healthcare). Fractions containing the eluted protein were identified by UV absorbance, collected, run on 4%–20% Tris-Glycine Mini gels (Novex WedgeWell), and Coomassie stained using Instant Blue (Expedeon). Identity of the purified DDI2-FLAG protein was also confirmed by Protein Mass Fingerprinting.

**E.coli expression and protein purification**

Recombinant DDI2, DDI2p, and HRAD23 proteins with various tags were generated from plasmids transformed into BL21 E.coli (Agilent Technologies 130245), expressed by IPTG induction at 1 mM for 4 h at 30°C. All proteins contained a His-Sumo-tag; but some also His-Sumo-FLAG, allowing non-tagged and FLAG-tagged versions to be generated by cleavage with the SUMO protease, Ulp1. Bacteria were harvested by centrifugation, washed in PBS and resuspended in PBS with 10 mM Imidazole, Protease inhibitors and 0.1 mg/mL lysozyme followed by sonication (2 min on, 15 s on 20 s off, 20% amplitude Branson sonicator). Cleared lysates were
added to Ni-agarose and incubated at 4°C overnight; the beads were washed in 50 mM Tris, pH 8, 10 mM Imidazole, 500 mM NaCl, 10% Glycerol (high salt buffer), then in the same buffer at 150 mM NaCl (low salt buffer), and the His-tagged protein was eluted with low salt buffer containing 300 mM Imidazole. The nickel-purified protein was diluted 1:1 with water and further purified on HiTrap Q FF (Fisher Scientific 10207275), using a 10-column volume gradient from 150 mM (Buffer A) to 1 M NaCl (Buffer B) in 25 mM Tris pH 8, 1% Glycerol, 0.1 mM DTT and Protease inhibitors (2.2 mM PMSF, 2 mM Benzamidine HCL, 2 μM Leupeptin, 1 μg/mL Pep statin A). For removal of the His-SUMO tag, the purified proteins were treated with Ulp1 (a kind gift from Peter Cherepanov) in buffer A for 30 min at 30°C. Proteins were further purified by gel filtration on HiLoad 16/60 Superdex 200 (GE health care 28-9893-35) in buffer A. No marked difference in the activity of His-SUMO-DDI2, His-SUMO-FLAG-DDI2, FLAG-DDI2 (His-SUMO tag removed) and DDI2 (no tag) was detected (Figure 2C, and data not shown).

Human cell extract preparation

For preparing whole cell extracts, WT or DDI2 KO MRC5VA grown on 15 cm dishes to 80%–90% confluency, were rinsed twice with ice-cold PBS containing Protease Inhibitors (2.2 mM PMSF, 2 mM Benzamidine HCL, 2 μM Leupeptin, 1 μg/mL Pep statin A) and 2 mM N-ethylmaleimide (NEM). Cells were then lysed on the plate in 1.5 mL TENT lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100), containing 2 mM NEM and Protease inhibitors, for 5 min at room temperature (RT). The lysates were collected in 15 mL Falcon tubes and kept on ice for 20 min followed by a brief sonication; 10 s at 20% amplitude in a Branson sonicator. Debris was removed by centrifugation 5 min at 20,000 RCF in an Eppendorf microfuge. The extracts were either used directly or snap-frozen in liquid nitrogen. Extracts were used for affinity purifications or directly in biochemical assays (see below).

Western blot analysis

Approximately, 50 μg protein/lane was separated on 4%–12% Bis-Tris Criterion XT (BioRad 3450124/5), 3%–8% Tris-Acetate Criterion XT (BioRad, 3450130), or 4%–15% TGX gels (BioRad, 56711084/5), and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, 10600002, 10600019). Membranes were stained with Ponceau S, scanned and the membranes blocked in 6% (w/v) skimmed milk in PBS for 1 h at room temperature and incubated with primary antibody (in 6% (w/v) skimmed milk in PBS-T or 5% BSA) for 1 h at RT or overnight at 4°C. Primary antibodies are listed in Key Recourses Table. For anti-ubiquitin blots, an equal mixture of P4D1 and P4G7 were used. For SUMO, an equal mixture of Anti-Sumo2 + Sumo3 and Anti-Sumo1 were used. All antibodies were used at 1:1000 dilution, except anti-DDI2 (1:5000). Membranes were washed several times in PBS-T (PBS, 0.1% Tween20 (w/v)), incubated with HRP-conjugated secondary antibody (key resources) in 6% (w/v) skimmed milk in PBS-T for 1 h at RT, washed again, and visualized using SuperSignal West Pico PLUS or Radiance ECL reagents (Azure Biosystems, AC2101,3). Blots were visualized on Amersham hyperfilm ECL or by AI600 Amersham Imager chemiluminescence. Quantification was performed using ImageJ.

GST-DSK2 and FLAG-DDI2O-N affinity chromatography

Human cell extracts were generated as described above. GST-DSK2 beads were generated as described (Tufegdzic Vidakovic et al., 2019). Typically, 1 mL cell extract at 1 mg/mL was incubated with 50 μl DSK2 beads rotating overnight at 4°C. Unbound material was saved and the beads were washed three times in 1 mL TENT buffer. Where beads were to be treated with DDI2, they were also washed with high salt TENT buffer (500 mM NaCl) and then washed back into TENT buffer. Beads were then used for biochemical reactions.

Two methods of Flag-DDI2 inactive affinity binding were carried out, either using chemically inactivated DDI2 (see below) or DDI2O-N; in both cases, the extracts were pre-incubated for 2 h at 4°C with M2 anti-flag agarose (Sigma-Aldrich 30410) to remove non-specific M2-resin binding proteins. The pre-cleared extracts were then incubated with Flag-DDI2 inactive protein, and fresh M2-anti-flag agarose. For some biochemical experiments, an excess of Flag-DDI2O-N was first bound to the beads, the beads washed several times; and then incubated with the cell extracts in TENT buffer. For their use in DDI2 cleavage assays, these beads were treated as described above for DSB-extracts, except extract binding to the Flag-DDI2O-N affinity beads was for 4°C for 4 h, and the resin washed in TENT buffer, then with TENT buffer with 500 mM NaCl, then twice with TENT buffer before being used for cleavage assays.

Chemical inactivation of flag-hDDI2

The DDI2 protease domain comprises a highly conserved retropepsin domain, otherwise best exemplified by HIV protease, which is structurally highly similar to DDI2 (Siva’ et al., 2016). 1,2-Epoxy-3-(4-nitrophenoxy) propane (EPNP) is a widely used, mechanistic inhibitor of retropepsins and other aspartic proteases, and potently inactivates HIV protease (Meek et al., 1989). To similarly inactivate DDI2, 100 μL of 1 mg/mL DDI2 was incubated with 10 mM EPNP for 4 h at room temperature, subsequently diluted into PBS (10 mL), and re-concentrated using VIVAspin 500 column (GE Healthcare) with a 10 kDa molecular weight cut-off. A control active DDI2-FLAG preparation without EPNP treatment was processed alongside. Final protein concentrations were determined by bicinchoninic acid (BCA) assay. The chemically inactivated protein was used for the experiments in Figure 3C.
DDI2 protease assays

Human cell extracts, ubiquitin peptides, and ubiquitylated proteins isolated by affinity chromatography (see above), were treated with enzymes as described below.

For Ddi1/DDI2 treatment of extracts, approximately 100 μg of human cell extract in TENT buffer was incubated directly with 1-3 μg DDI2 protein in the presence or absence of the same amount of HRAD23 protein at 26, 30, or 37 °C; for 30-60 min. Reactions were stopped by adding SDS-PAGE loading buffer. The results were analyzed by SDS-PAGE followed by western blotting.

Ubiquitin tetramers K29, K33, K48, K63 (Boston Biochem UC-83, UC-103, UC-210B, UC-318B) (2μg), K48 polymers Ub2-16 (8 μg), and K63 octamers (4 μg) were treated with 2-4 μg DDI2 and/or 2-4 μg HRAD23 or Usp2 (Bio-Techne E-504-050) for 45 min at 37 °C in cleavage reaction buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% (v/v) Triton X-100, 5 mM DTT). Reactions were stopped by adding SDS-PAGE loading buffer (LB: XT sample buffer BioRad (1610791) with 0.1M DTT and 10% V/V BME), and the samples analyzed by SDS-PAGE followed by Coomassie (instant blue) staining.

For affinity-purified substrates on beads, incubations were carried out in shaking thermomixers. Beads were incubated in the presence or absence of DDI2 and HRAD23. Beads were also treated with USP2 (USP2) pre- and post-cleavage. Substrates were also treated with Ulp1 protein Figures 2E and 4C. For cleavage reactions on DSK2 beads, ubiquitylated proteins were typically 20-fold concentrated onto the beads, which were stringently washed, as described above. 20 μl of beads were treated with 3 μg of DDI2 and/or the same amount of HRAD23 in bead cleavage buffer (25 mM Tris-HCl pH 7.4, 120 mM NaCl, 0.5 mM EDTA, 0.25% (v/v) Triton X-100, 5 mM DTT) at 30 °C for 1 h. USP2 digests were for 30 min at 37 °C. Reactions were stopped adding SDS-PAGE loading buffer, and the results analyzed by SDS-PAGE followed by western blot.

Mass spectrometry analysis

For mass-spectrometry of ubiquitylated proteins associated with DDI2, proteins bound to FLAG-DDI2 were subjected to mass spectrometry (data from proteins in Figure 3C, lanes 5-8 shown in Table S1). Briefly, DDI2 KO cells were labeled either heavy or light using stable isotope labeling with amino acids in cell culture (SILAC; Ong et al., 2002). Cell extracts were then subjected to affinity chromatography using immobiLysed, catalytically inactive DDI2 protein. Bound proteins were eluted with SDS-loading buffer or by proteolysis with DDI2. Forward and reverse SILAC mixtures were created by mixing the eluates and subjecting them to SDS-PAGE. Next, the gel lanes were cut from the top to the bottom into multiple different molecular weight regions and prepared for mass spectrometry analysis using an in-gel trypsin digestion protocol. Mass spectrometry data were acquired using an Ultimate3000 HPLC connected to Lumos- Tribrid Orbitrap operated in Data Dependent Acquisition mode. Heavy to light protein ratios for each gel slice were determined using Maxquant software version 1.6.0.1 whereby a shift in heavy/light ratio indicates DDI2 dependent proteolysis (Table S1; see also source data at PRIDE PXD018215). In a second round of searches using the same raw data files, the enzyme specificity setting was changed from tryptic to semi-tryptic to search for novel proteolytic cleavage sites.

Analysis of ubiquitylation (UbiSite)

Ubiquitylated proteins purified from DDI2 KO cells and the total ubiquitylome in WT cells were compared. Ubiquitylated proteins from FLAG-DDI2 beads or GST-DSK2 resin were eluted with 8M Guanidine-HCl and subjected to reduction, alkylation and digestion with Lysyl Endopeptidase (LysC) (Wako) followed by enrichment of ubiquitinated peptides using the UbiSite approach exactly as described (Akimov et al., 2018a). 90% of the samples were used for the high pH reversed-phase fractionation (HpH) to reduce samples' complexity by a stepwise elution of peptides with 1.75, 3.5, 7, 8, 9, 10.5, 14 and 50% of acetonitrile. The remaining 10% of each sample was subjected to parallel reaction monitoring (PRM) for targeted quantitative analyses.

Tryptic peptides from each HpH fraction were injected into a 20-cm fused silica column with an inner diameter of 75 μm packed in house with C18 resin (1.9-μm beads, Reprosil, Dr. Maisch) using an EASY-nLC 1000 chromatography system (Thermo Fisher Scientific) connected online to a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) assembled with a nano-electrospray ion source (Thermo Fisher Scientific). Peptides were loaded in solvent A (0.5% acetic acid) and eluted with a gradient of solvent B (80% ACN, 0.5% acetic acid) from 7% to 12% solvent B over 8 min, from 12% to 33% over 90 min, followed by increasing solvent B to 45% for 10 min and finished by a run with 98% for 6 min at 250 nL/min. The Q Exactive HF-X mass spectrometer was operated in positive polarity mode with spray voltage set to 2.3 kV and heated capillary temperature at 275 °C. MS data were acquired using a data-dependent method switching between full scan events and the top 12 MS/MS scans. An automatic gain control (AGC) target value was set to 3 × 106 and resolution was set to 60,000 for full MS scan events with a scan range of 300–1,700 m/z and a maximum ion injection time (IT) of 15 ms. Precursors were fragmented by higher-energy collisional dissociation (HCD) with a normalized collisional energy (NCE) of 28. MS/MS scans were acquired with a resolution of 30,000, maximum IT of 45ms, 1.2 m/z isolation window. Repeat sequencing of peptides was minimized by setting the dynamic exclusion window to 60 s.

Raw MS data were searched using MaxQuant software v 1.5.3.17 with Andromeda search engine and FASTA file from UniProt released July 2015 (42 127 reviewed sequences), supplemented with commonly observed contaminants. The following search parameters were used: the enzyme used for digestion was specified as trypsin with up to three missed cleavages. Fixed modification was cysteine carbamidomethylation and variable modifications were oxidation of methionine and di-Glycine on lysine residues, excluding lysines on the C-terminal end of peptides. Spectra were searched with a mass accuracy of 4.5 ppm for precursors and 20 ppm for fragment ions. False discovery rate (FDR) was set to 0.01, both at protein and peptide levels, using a reverse database as a decoy.
PRM analyses and quantitation of ubiquitin chains were performed as described previously (Akimov et al., 2018b) with minor modifications, including the specific monitoring of the three peptide sequences TLTGK(gg)TITLEVEPSDTIENVK(gg)AK, TITLEVEPSDTIENVK(gg)AK(gg)IQDK and AK(gg)IQDK(gg)EGIPPDQQR, corresponding to the branched ubiquitin chains at positions K11+K27, K27+K29 and K29+K33, respectively (Table S2; see also source data at PRIDE: PXD019152).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical details of experiments can be found in the relevant figure legends.