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The Auto-Generated Fragment of the Usp1 Deubiquitylase Is a Physiological Substrate of the N-End Rule Pathway

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SUMMARY

Deamidation of N-terminal Gln by the Ntaq1 Nt^{Q}-amidase is a part of the Arg/N-end rule pathway, a ubiquitin-dependent proteolytic system. Here we identify *Gln*-$Usp1^{Ct}$, the C-terminal fragment of the autocleaved Usp1 deubiquitylase, as the first physiological Arg/N-end rule substrate that is targeted for degradation through deamidation of N-terminal Gln. Usp1 regulates genomic stability, in part through the deubiquitylation of monoubiquitylated PCNA, a DNA polymerase processivity factor. The autocleaved Usp1 remains a deubiquitylase because its fragments remain associated with Uaf1, an enhancer of Usp1 activity, until the *Gln*-$Usp1^{Ct}$ fragment is selectively destroyed by the Arg/N-end rule pathway. We also show that metabolic stabilization of *Gln*-$Usp1^{Ct}$ results in a decreased monoubiquitylation of PCNA and in a hypersensitivity of cells to ultraviolet irradiation. Thus, in addition to its other functions in DNA repair and chromosome segregation, the Arg/N-end rule pathway regulates genomic stability through the degradation-mediated control of the autocleaved Usp1 deubiquitylase.

INTRODUCTION

The ubiquitin (Ub) system plays major roles in responses of cells to DNA damage (Ciccia and Elledge, 2010; Huang and D'Andrea, 2006; Jentsch et al., 1987; Kim and D'Andrea, 2012; Ulrich, 2011). Some of these responses involve cycles of monoubiquitylation-deubiquitylation of specific proteins. The functions of the Usp1 deubiquitylase (DUB) in the control of genomic stability include the Usp1-mediated deubiquitylation of the monoubiquitylated FancD2, FancI, and PCNA (Cohn et al., 2007; Cotto-Rios et al., 2011a; Huang et al., 2006; Jones et al., 2012; Kim and D'Andrea, 2012; Moldovan et al., 2007; Ulrich, 2011). The FancD2-FancI heterodimer is a component of the Fanconi anemia (FA) pathway, whose major function is repair of DNA interstrand crosslinks (Kim and D'Andrea, 2012; Ulrich, 2011). PCNA is the DNA polymerase processivity factor that forms a sliding homotrimeric ring around double-stranded DNA and specifically associates with different types of DNA polymerases either in S phase replication forks or during episodes of localized DNA synthesis that mediate DNA repair (Kim et al., 2009; Lange et al., 2011; Moldovan et al., 2007; Murai et al., 2011; Ulrich, 2011). Upon DNA damage and/or perturbations of DNA replication, PCNA is monoubiquitylated at a specific lysine by the Rad6-Rad18 E3-E2

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SUPPLEMENTAL INFORMATION

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Ub ligase (Alpi and Patel, 2009; Fox et al., 2011; Hendel et al., 2011; Huang et al., 2006; Kim and D'Andrea, 2012). Monoubiquitylated PCNA binds to damage-tolerant, error-prone DNA polymerases of the Y family (Jones et al., 2012; Lange et al., 2011; Ulrich, 2011). These polymerases contain Ub-binding domains that recognize monoubiquitylated PCNA.

Usp1 forms a heterodimeric complex with Uaf1 (*U*sp1-*a*ssociated *f*actor *1*) (Wdr48), a protein containing WD40-type repeats (Cohn et al., 2007, 2009; Villamil et al., 2012). The binding of Uaf1 to Usp1 DUB strongly increases its deubiquitylating activity (Cohn et al., 2007; Villamil et al., 2012). Usp1 is a conditionally short-lived protein. We have shown that the APC/C^{Cdh1} E3 Ub ligase targets Usp1 for the proteasome-mediated degradation in the G1 phase of the cell cycle. Low levels of Usp1 in G1 allow an efficacious monoubiquitylation of PCNA in response to UV-mediated DNA damage before DNA replication (Cotto-Rios et al., 2011a, 2011b). During mitosis, phosphorylation of Usp1 by cyclin-dependent kinases partially protects it against degradation via the APC/C^{Cdh1} Ub ligase (Cotto-Rios et al., 2011a).

Remarkably, Usp1 can also cleave itself, in a reaction accelerated by UV irradiation of mammalian cells (Huang et al., 2006). Usp1 autocleaves immediately after the internal Gly-Gly sequence (Figure 1A), which is identical to the C-terminal sequence of Ub. The C-terminal fragment of Usp1, denoted as *Gln*-$Usp1^{Ct}$, bears N-terminal Gln and contains a part of the sequence ("His box") that encompasses the active site of Usp1 (Figure 1A). The DUB activity of autocleaved Usp1 can apparently be retained if the N-terminal fragment (denoted as $Usp1^{Nt}$) and *Gln*-$Usp1^{Ct}$ are held together within the cleaved Usp1-Uaf1 complex (Cohn et al., 2007). Because Gln is a destabilizing residue in the N-end rule, the evolutionary conservation of N-terminal Gln in *Gln*-$Usp1^{Ct}$ suggested that this fragment might be targeted for degradation by the Arg/N-end rule pathway (Figures 1A and 1B and Figure S1 available online).

The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal residue. Regulated degradation of proteins by the Ub/proteasome-dependent Arg/N-end rule pathway mediates a strikingly broad range of biological functions (Dougan et al., 2011; Graciet and Wellmer, 2010; Mogk et al., 2007; Piatkov et al., 2012; Tasaki et al., 2012; Varshavsky, 2011) (Figure S1). The N-end rule pathway polyubiquitylates proteins that contain specific degradation signals (degrons), either internal or N-terminal ones (N-degrons) (Varshavsky, 2011). The main determinant of an N-degron is a destabilizing N-terminal residue of a protein. Recognition components of the N-end rule pathway, called N-recognins, are specific E3 Ub ligases that can target N-degrons. The N-end rule pathway consists of two branches, the Ac/N-end rule and the Arg/N-end rule pathways. The Ac/N-end rule pathway recognizes proteins with N-terminally acetylated residues (Hwang et al., 2010b). The Arg/N-end rule pathway targets specific unacetylated N-terminal residues (Hwang et al., 2010a; Varshavsky, 2011) (Figure S1). In eukaryotes, the primary destabilizing N-terminal residues Arg, Lys, His, Leu, Phe, Tyr, Trp, and Ile are directly recognized by E3 N-recognins. In contrast, N-terminal Asp, Glu, Asn, Gln, and Cys function as destabilizing residues through their preliminary enzymatic modifications, which include N-terminal deamidation (Nt-deamidation) of Asn and Gln, as well as Nt-arginylation of Asp, Glu, and (oxidized) Cys (Figure S1) (Brower and Varshavsky, 2009; Tasaki et al., 2012; Varshavsky, 2011; Wang et al., 2009).

Here we identify the *Gln*-$Usp1^{Ct}$ fragment of Usp1 as the first physiological substrate of the Arg/N-end rule pathway that is targeted for degradation through deamidation of N-terminal Gln, a reaction mediated by the *Ntaq1*-encoded Nt^{Q}-amidase (Wang et al., 2009) (Figure S1). We also show that a metabolic stabilization of *Gln*-$Usp1^{Ct}$, a change that prolongs DUB

activity of the autocleaved Usp1 in the Usp1-Uaf1 complex, makes cells hypersensitive to UV irradiation.

RESULTS AND DISCUSSION

The C-Terminal Fragment of the Autocleaved Usp1 Deubiquitylase Is Deamidated and Degraded by the Arg/N-End Rule Pathway

To determine whether the *Gln*-Usp1^{Ct} fragment could be degraded by the Arg/N-end rule pathway, we used the *U*b *r*eference *t*echnique (URT), derived from the Ub fusion technique (Varshavsky, 2005, 2011). Cotranslational cleavage of a URT-based Ub fusion by DUBs produces, at initially equimolar ratio, a test protein with a desired N-terminal residue and a "reference" $_{f}$DHFR-Ub^{R48}, a flag-tagged derivative of the mouse dihydrofolate reductase (Figure 1C). In URT-based pulse-chase assays, the labeled test protein is quantified by measuring its levels relative to the levels of a stable reference at the same time point during a chase. In addition to being more accurate than pulse chases without a "built-in" stable reference, URT makes it possible to measure the degradation of the test protein before the chase, i.e., during the pulse (Hwang et al., 2010b; Piatkov et al., 2012; Varshavsky, 2005).

URT-based ^{35}S pulse chases with $_{f}\mathrm{DHFR}-\mathrm{Ub}^{R48}-\mathbf{X}-\mathrm{Usp}\,1_{f}^{Ct}$ (**X** = Gln, Glu, Val) were performed in a transcription-enabled rabbit reticulocyte extract, which contains the Arg/N-end rule pathway and has been extensively used to analyze this pathway (Varshavsky, 2011). $_{f}\mathrm{DHFR}-\mathrm{Ub}^{R48}-\mathbf{X}-\mathrm{Usp}\,1_{f}^{Ct}$ fusions (**X** = Gln, Glu, Val) were labeled with ^{35}S-Met/Cys for 10 min at 30°C, followed by a chase, immunoprecipitation with anti-flag antibody, SDS-PAGE, autoradiography, and quantification (Figures 1D and 1E). The logic of these assays (Piatkov et al., 2012) involves a comparison between the degradation rates of a protein bearing a destabilizing N-terminal residue and an otherwise identical protein with an N-terminal residue such as Val, which is not recognized by the Arg/N-end rule pathway (Figure S1). $Gln-\mathrm{Usp}\,1_{f}^{Ct}$ and $Glu-\mathrm{Usp}\,1_{f}^{Ct}$ were equally short-lived in reticulocyte extract ($t_{1/2}$ of ~20 min) (Figures 1D and 1E). Moreover, ~40% of pulse-labeled $Gln-\mathrm{Usp}\,1_{f}^{Ct}$ and $Glu-\mathrm{Usp}\,1_{f}^{Ct}$ proteins were degraded during the pulse, before the chase, in comparison to $Val-\mathrm{Usp}\,1_{f}^{Ct}$, which was stable (Figures 1D and 1E).

Cells Lacking Ntaq1 or Ate1 Contain Metabolically Stabilized *Gln*-Usp1^{Ct}, the C-Terminal Fragment of Autocleaved Usp1

To ascertain whether the Arg/N-end rule pathway mediated the degradation of the auto-generated $Gln-\mathrm{Usp}\,1_{f}^{Ct}$ fragment, we constructed *Ntaq1*$^{-/-}$ mouse strains and established *Ntaq1*$^{-/-}$ embryonic fibroblast (EF) cell lines lacking the Gln-specific Ntaq1 Nt^{Q}-amidase (see the Supplemental Experimental Procedures). Using a coupled enzymatic assay for Nt-deamidation that utilized Nt-deamidation-dependent Nt-arginylation (Wang et al., 2009), we found that extracts from *Ntaq1*$^{-}$/$^{-}$ EFs were unable to deamidate N-terminal Gln of a protein reporter, in contrast to extracts from wild-type EFs (Figure S2B). We concluded that Ntaq1 is the sole Nt^{Q}-amidase in mouse cells, and that *Ntaq1*$^{-/-}$ mice (and EF cells) lacked the Gln-specific Nt^{Q}- amidase activity (see the Supplemental Experimental Procedures for additional details). *Ntaq1*$^{-/-}$ mice were anatomically normal and fertile but behaviorally abnormal, as will be described elsewhere (K.I.P. and AV., unpublished data). Degradation of the autogenerated *Gln*-Usp1^{Ct} fragment was examined using a cycloheximide (CHX) chase assay (Hwang et al., 2010b) with wild-type EFs, *Ntaq1*$^{-/-}$ EFs (produced in the present work), and previously produced *Ate1*$^{-/-}$ EFs (Kwon et al., 2002). *Ate1*$^{-/-}$ EF cell lines lack the Ate1 R-transferase and therefore lack the arginylation branch of the Arg/N-end rule pathway, downstream of its deamidation branch (Piatkov et al., 2012) (Figure S1).

Full-length mouse Usp1 was a short-lived protein in all three EF cell lines (Figure 2A), in agreement with the degradation of Usp1 through an internal degron (upstream of the autocleavage site) that is targeted by the APC/C^{Cdh1} Ub ligase (Cotto-Rios et al., 2011b), and also, as shown below, through the ongoing autocleavage of Usp1 that generates a Gln-specific N-degron. Although the *Gln*-$Usp1^{Ct}$ fragment (detected by the previously characterized antibody [Huang et al., 2006] against C-terminal region of mouse or human USP1 and termed anti-$USP1^{Cterm}$) was present in all examined EF cellss, this fragment was short lived in wild-type cells but long lived in both *Ntaq1*$^{-/-}$ and *Ate1*$^{-/-}$ EF cells (Figure 2A, middle panel), in agreement with the necessity of both Nt-deamidation (by Ntaq1) and Nt-arginylation (by Ate1) for the targeting of N-terminal Gln (Figure 1B).

In other assays with wild-type, *Ntaq1*$^{-/-}$, and *Ate1*$^{-/-}$ EF cells, they were transfected with plasmids that expressed the C-terminally myc-tagged, full-length mouse Usp1 (Usp1-myc), which yielded the $Gln-\mathrm{Usp}\,1^{\mathrm{Ct}}_{\mathrm{myc}}$ fragment upon autocleavage, or, alternatively, the otherwise identical $\mathrm{Usp}\,1^{\mathrm{G669A}}_{\mathrm{myc}}$ mutant, which could not be autocleaved, owing to the Gly-to-Ala mutation of the P1 residue in the autocleavage site (Huang et al., 2006) (Figure 1A). In agreement with *Gln*-$Usp1^{Ct}$ being a short-lived Arg/N-end substrate (Figures 1D, 1E, and 2A), the C-terminally myc-tagged $Gln-\mathrm{Usp}\,1^{\mathrm{Ct}}_{\mathrm{myc}}$ fragment was short lived in wild-type EF cells but long lived in both *Ntaq1*$^{-/-}$ EFs (Figure 2B) and *Ate1*$^{-/-}$ EF cells (Figure 2C, lanes 4–6, and Figure 2D, lanes 4–6). As expected (Huang et al., 2006), the $\mathrm{Usp}\,1^{\mathrm{G669A}}_{\mathrm{myc}}$ mutant did not yield the $Gln-\mathrm{Usp}\,1^{\mathrm{Ct}}_{\mathrm{myc}}$ fragment (Figure 2C, lanes 7–9; compare with Figure 2D, lanes 7–9).

In further CHX chases, human U2OS cells were transfected with plasmids that expressed either C-terminally myc-tagged mouse Usp1 (Usp1-myc) and its mutants $Usp1^{G669A}$ and $Usp1^{Q670A}$ (Figure 2E) or N-terminally myc-tagged human USP1 (myc-USP1) and its mutant $USP1^{Q672A}$ (Figure S2C). In agreement with the data about mouse *Gln*-$Usp1^{Ct}$ in mouse cells (Figures 1D, 1E, and 2A–2D), wild-type mouse Usp1-myc gave rise to the short-lived $Gln-\mathrm{Usp}\,1^{\mathrm{Ct}}_{\mathrm{myc}}$ fragment in human cells (Figure 2E, lanes 4–6). In contrast, the otherwise identical full-length $\mathrm{Usp}\,1^{\mathrm{Q670A}}_{\mathrm{myc}}$ mutant yielded much higher initial (and subsequent) levels of the long-lived $Ala-\mathrm{Usp}\,1^{\mathrm{Ct}}_{\mathrm{myc}}$ fragment, whose (mutant) N-terminal Ala is not recognized by the Arg/N-end rule pathway (Figure 2E, lanes 10–12; compare with lanes 4–6). As expected, $\mathrm{Usp}\,1^{\mathrm{G669A}}_{\mathrm{myc}}$, which is resistant to autocleavage (Huang et al., 2006), did not produce the $Gln-\mathrm{Usp}\,1^{\mathrm{Ct}}_{\mathrm{myc}}$ fragment in human cells (Figure 2E, lanes 7–9; compare with lanes 4–6 and lanes 10–12).

The results of CHX-chases with the N-terminally myc-tagged human USP1 and its myc-$USP1^{Q672A}$ mutant were in agreement with other findings about *Gln*-$Usp1^{Ct}$ and the dependence of its degradation on N-terminal Gln (Figure S2C). In conjunction with anti-$USP1^{Cterm}$ antibody, the N-terminal myc tag in myc-USP1 made it possible to detect the formation of both the N-terminal USP1 fragment (myc-$USP1^{Nt}$) and its C-terminal (untagged) counterpart (*X*-$Usp1^{Ct}$; either *Gln*-$Usp1^{Ct}$ or *Ala*-$Usp1^{Ct}$). The metabolic stability of the N-terminal myc-$USP1^{Nt}$ fragment (assessed by its levels after 3 hr CHX chase) was significantly higher in the case of myc-$USP1^{Q672A}$ (it yielded long-lived *Ala*-$Usp1^{Ct}$) than in the case of wild-type myc-USP1 (it yielded short-lived *Gln*-$Usp1^{Ct}$) (Figure S2C). Because the bulk of USP1 in vivo is in the complex with UAF1 (see the Introduction), a parsimonious interpretation of this result is that a metabolic stabilization of the C-terminal USP1 fragment (in this experiment, through a change of its N-terminal residue from Gln to Ala) stabilizes the N-terminal fragment as well. This would happen because both fragments

of the autocleaved USP1 are bound to UAF1, thereby retaining the DUB activity of USP1 within this complex. In this interpretation, the selective destruction of *Gln*-Usp1^{Ct} by the Arg/N-end rule pathway also destabilizes the N-terminal USP1 fragment, possibly by facilitating its dissociation from UAF1.

Metabolic Stabilization of *Gln*-Usp1^{Ct} Results in a Decreased Monoubiquitylation of PCNA and Hypersensitivity of Cells to UV Damage

In our working model, the targeting of the *Gln*-Usp1^{Ct} fragment for degradation via its Arg/N-degron is mechanistically coupled to fragment's dissociation from Uaf1. If so, the Arg/N-end rule pathway acts, in this setting, as a regulator that determines the duration of DUB activity of the autocleaved Usp1 in the Usp1-Uaf1 complex. By selectively destroying *Gln*-Usp1^{Ct}, the Arg/N-end rule pathway would abrogate the DUB activity of the autocleaved Usp1 and at the same time (according to this model) metabolically destabilize the N-terminal Usp1 fragment. This "resetting" function of the Arg/N-end rule pathway recurs in previously analyzed circuits, for example, during chromosome segregation in *Saccharomyces cerevisiae*. Scc1, a subunit of yeast cohesin that holds together sister chromatids, is cleaved by separase during mitosis, yielding the C-terminal fragment that bears N-terminal Arg, a destabilizing residue (Figure S1), and remains associated with the rest of cohesin (Rao et al., 2001). The selective destruction of the C-terminal fragment of Scc1 by the Arg/N-end rule pathway has been shown to be required for the high fidelity of chromosome segregation (Rao et al., 2001).

The Usp1-mediated deubiquitylation of monoubiquitylated PCNA makes Usp1 a negative regulator of the UV-induced translesion DNA synthesis, mediated by damage-bypassing DNA polymerases bound to monoubiquitylated PCNA (Lange et al., 2011; Moldovan et al., 2007; Ulrich, 2011). We have previously shown that the autocleavage and inactivation of Usp1 were augmented by UV irradiation of cells, thus accounting for an increase in both steady-state levels of monoubiquitylated PCNA and damage-bypassing DNA synthesis upon UV irradiation (Huang et al., 2006). For the reasons above, the demonstrated degradation of *Gln*-Usp1^{Ct} by the Arg/N-end rule pathway (Figures 1B–1D and 2) at least contributes to (and possibly entirely mediates) the abrogation of DUB activity of the autocleaved Usp1. If so, does the Arg/N-end rule pathway also play a role in the UV-induced DNA damage response?

To address this question, we compared wild-type EF cells with mutant EFs that lacked subsets of the Arg/N-end rule pathway. The levels of monoubiquitylated PCNA were greatly reduced in *Ate1*$^{-/-}$ EF cells after UV irradiation, in comparison to wild-type EFs (Figure 3A, middle panel, lane 2; compare with lane 4), consistent with higher levels of the active Usp1 DUB in these cells. These data indicated that the metabolic stabilization of *Gln*-Usp1^{Ct} in the absence of Nt-arginylation) was required for the DUB activity of autocleaved Usp1, since both *Ate1*$^{-/-}$ and wild-type EFs had comparable levels of full-length Usp1 (Figure 3A, lanes 2 and 4). In agreement with these results, both *Ntaq1*$^{-/-}$ EF cells and *Ate1*$^{-/-}$ EF cells were significantly more sensitive to UV than were wild-type EF cells (Figure 3B).

In another approach, we replaced the destabilizing Gln^{672} P1' residue (it becomes N-terminal upon the autocleavage of USP1) with Met, a residue that is not recognized by the Arg/N-end rule pathway (Figure S1). In addition, the open reading frame encoding the USP1^{Q672M} mutant was made resistant, through synonymous codon replacements, to RNA interference (RNAi)-mediated knockdown of the wild-type endogenous USP1 (see the Supplemental Experimental Procedures). Using this strategy (it included an RNAi-resistant control messenger RNA [mRNA] encoding wild-type USP1), we examined the effect of stabilizing the C-terminal fragment of the autocleaved USP1 on the in vivo levels of monoubiquitylated PCNA and on the resistance of cells to UV. As expected (given the

results in Figure 3A), mutant USP1^{Q672M} (it yields the long-lived *Met*-Usp1^{Ct} fragment) decreased the levels of monoubiquitylated PCNA, implying higher levels of DUB activity of the autocleaved USP1 (Figure 3C). In agreement with these results, human U2OS cells expressing the USP1^{Q672M} mutant were reproducibly more sensitive to UV irradiation over a range of UV doses than were identically treated cells expressing wild-type USP1 (Figure 3D).

Although there is still much to learn about specific mechanisms involved, our findings (Figures 1, 2, 3, and S2) indicate a role of the Arg/N-end rule pathway in modulating, through the degradation of the *Gln*-Usp1^{Ct} fragment, the DUB activity of the autocleaved Usp1, and thereby in modulating the levels of monoubiquitylated PCNA. Because monoubiquitylated PCNA makes possible the lesion-bypassing, error-prone DNA synthesis, the Arg/N-end rule pathway is also a regulator of DNA damage responses that involve error-prone DNA replication. Other known functions of the Arg/N-end rule pathway in DNA damage responses include the regulation, through selective degradation, of the *S. cerevisiae* Mgt1 alkylguanine transferase, a DNA repair enzyme (Hwang et al., 2009). In sum, it is likely that the Arg/N-end rule pathway mediates the regulated degradation not only of the Gln-Usp1 fragment of Usp1 but also of other proteins that control the sensitivity of cells to UV irradiation. This interpretation is consistent with the fact that genetic ablation of specific branches of the Arg/N-end rule pathway in *Ate1*$^{-/-}$ and *Ntaq1*$^{-/-}$ EF cells studied in the present work would make them even more UV sensitive that of the metabolic stabilization of a specific UV-relevant Arg/N-end rule substrate such as Gln-Usp1.

Gln-Usp1^{Ct} is the first physiological substrate of the Arg/N-end rule pathway that involves the Ntaq1 Nt^{Q}-amidase and Gln-specific Nt-deamidation (Figures 1B and S1). A number of questions remain to be addressed. For example, how does UV irradiation of cells increase the autocleavage of Usp1? Is this autocleavage followed (or accompanied) by other physiologically relevant modifications of this DUB, e.g., its phosphorylation or acetylation? Such modifications might modulate the kinetics of targeting and degradation of the newly formed *Gln*-Usp1^{Ct} fragment, for example, by altering the steric accessibility of the newly exposed N-terminal region of *Gln*-Usp1^{Ct} to the Ntaq1 Nt^{Q}-amidase and downstream components of the Arg/N-end rule pathway. Setting up and exploring an Arg/N-end rule-based in vitro ubiquitylation/degradation system, with the Usp1-Uaf1 complex as its autocleaved substrate, is likely to be a particularly informative approach to mechanistic questions in this arena, and to some physiological questions as well.

EXPERIMENTAL PROCEDURES

Transfection and Immunoprecipitation

Mouse and human cell lines were transiently transfected with Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen). Cells were lysed in Triton lysis buffer (1% Triton X-100, 50 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl [pH 7.4]), and immunoprecipitations were carried out with specific antibodies, as described in the Supplemental Experimental Procedures.

In Vitro Transcription-Translation-Degradation Assay

Pulse-chase assays were carried out with the TNT T7 Coupled Transcription/Translation System (Promega). Newly formed proteins were pulse-labeled with L-^{35}S-methionine (MP Biomedicals) for 10 min at 30°C. The labeling was quenched by the addition of cycloheximide and unlabeled methionine. Reactions were terminated at indicated time points, and flag-tagged proteins were immunoprecipitated with anti-flag antibody, followed

by SDS-PAGE electrophoresis, autoradiography, and quantification, as described in the Supplemental Experimental Procedures.

Construction of *Ntaq1*$^{-/-}$ Mouse Strains

Mouse embryonic stem cells containing an insert of a gene-trapping vector in the first intron of the *Ntaq1* gene (clone IST14542A4) were injected into C57BL/6J blastocysts and implanted into pseudopregnant mouse females. The resulting chimeric male offspring were mated with C57BL/6J females. Standard mating techniques (Joyner, 2000; Nagy et al., 2003) were used to produce *Ntaq1*$^{-/-}$ mice. See the Supplemental Experimental Procedures.

UV Sensitivity Assay

Cells were trypsinized and plated at 300 cells per 35 mm tissue culture dish in triplicate. After 12 hr, cells were treated with specified doses of 254 nm UV irradiation (Stratalinker 2400; Agilent Technologies), followed by recovery in complete medium. UV sensitivity was scored 10 days later with the Syto-60 (Invitrogen) DNA staining kit. See the Supplemental Experimental Procedures for details.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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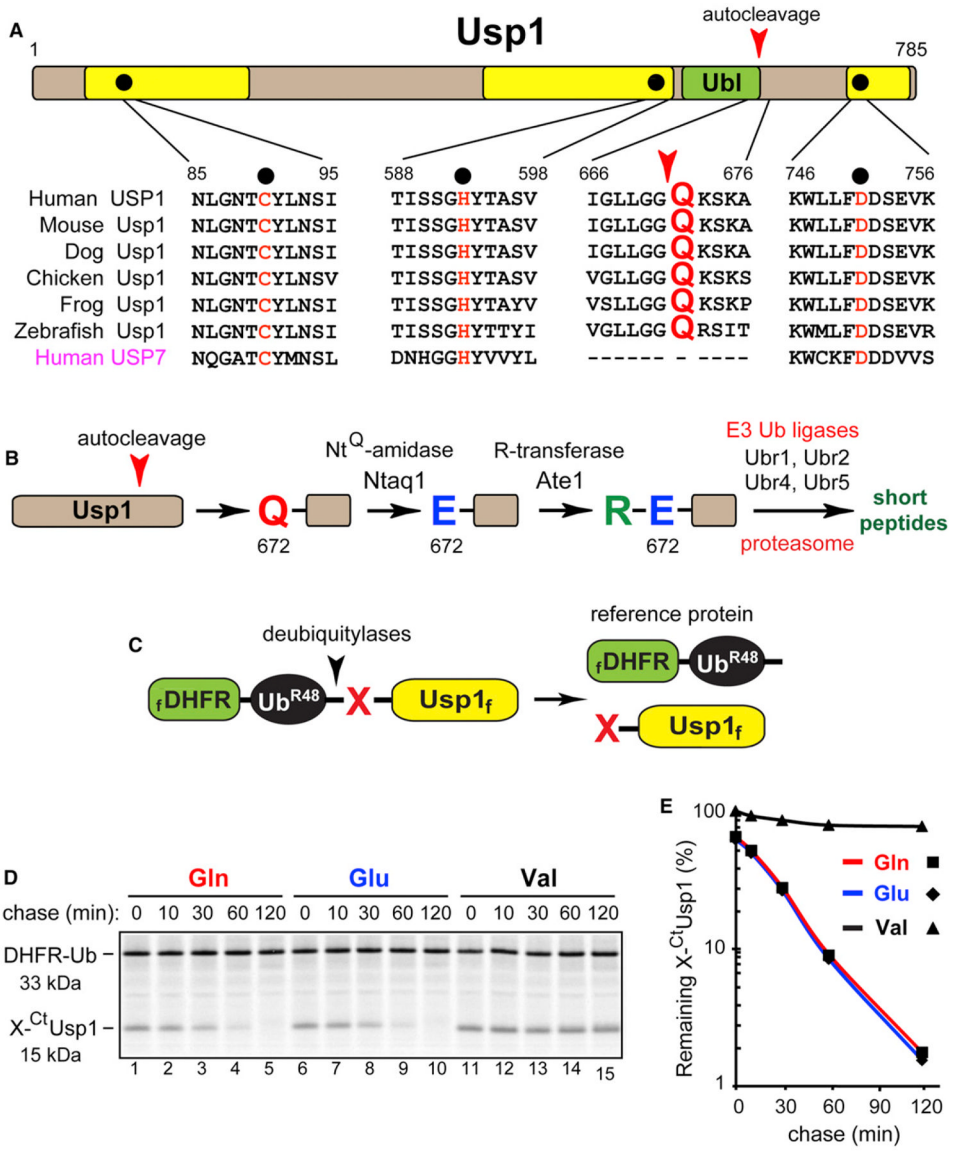

Figure 1. *Gln*-Usp1^{Ct}, the Autogenerated C-Terminal Fragment of the Usp1 Deubiquitylase, Is a Short-Lived Substrate of the Arg/N-End Rule Pathway

(A) Sequence alignments, among vertebrates, of the catalytic domains of Usp1 and its autocleavage site, with amino acid residues numbered as in human USP1. The corresponding domains of the structurally characterized human USP7, a USP-type deubiquitylase (DUB) that lacks the autocleavage site (Hu et al., 2002), are also shown. Key residues of the catalytic triad are in red, and are also marked by black circles. The Ub-like domain (Ubl, in green) abuts the autocleavage site of Usp1. The Gln (Q) P1' residue of the autocleavage site is in red and a larger font size. This residue, #672 in human USP1 and #670 in mouse Usp1, becomes N-terminal after the autocleavage.

(B) The enzymatic cascade that includes the Arg/N-end rule pathway, from the autocleavage of Usp1 and modifications of the resulting *Gln*-Usp1^{Ct} fragment by the Ntaq1 Nt^{Q}-amidase, the Ate1 R-transferase, and UBR-type E3 Ub ligases (N-recognins) to the degradation of the resulting *Arg-Glu*-Usp1^{Ct} by the 26S proteasome.

(C) The Ub reference technique (URT) assay (Piatkov et al., 2012; Varshavsky, 2005), with $_{f}$DHFR-Ub^{R48}, a flag-tagged derivative of the mouse dihydrofolate reductase, as the reference protein.
(D) *Gln*-Usp1^{Ct}, *Glu*-Usp1^{Ct}, and *Val*-Usp1^{Ct} (produced in reticulocyte extract from the corresponding URT fusions) were labeled with ^{35}S-Met/Cys for 10 min at 30°C, followed by a chase, immunoprecipitation with anti-flag antibody, SDS-PAGE, and autoradiography.
(F) Quantification of data in E, using the $_{f}$DHFR-Ub^{R48} reference protein. Squares, *Gln*-Usp1^{Ct}; rhombs, *Glu*-Usp1^{Ct}; triangles, *Val*-Usp1^{Ct}.
See also Figure S1.

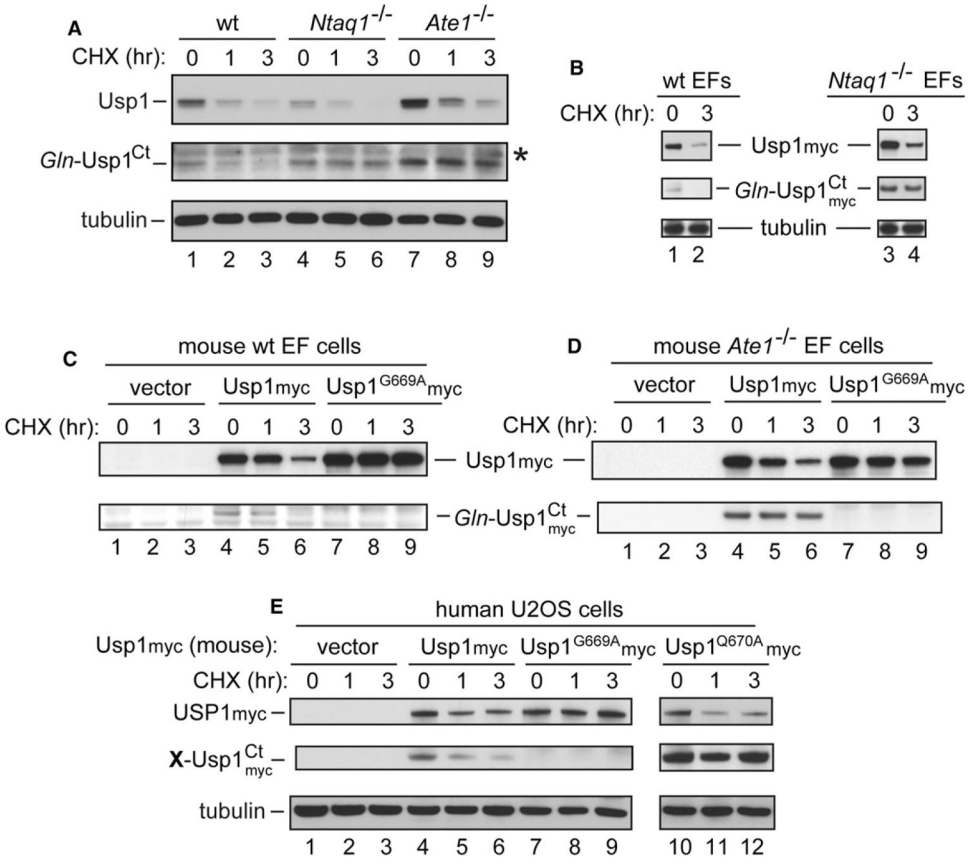

Figure 2. In Vivo Degradation of the Endogenous Gln-Usp1^{Ct} Fragment, Its Mutant, and Effects of Mutations in the Arg/N-End Rule Pathway

(A) Cycloheximide (CHX) chase assays with endogenous Usp1 in wild-type, *Ntaq1*$^{-/-}$, and *Ate1*$^{-/-}$ mouse embryonic fibroblast (EF) cell lines. Full-length Usp1 and the *Gln*-Usp1^{Ct} fragment were detected by immunoblotting with an antibody (Huang et al., 2006), termed anti-USP1^{Cterm}, that recognizes a C-terminal region of mouse Usp1 or human USP1. Immunoblotting with anti-tubulin antibody was used for loading controls.

(B) CHX chase for 0 and 3 hr with wild-type and *Ntaq1*$^{-/-}$ EF cell lines that had been transiently transfected with a plasmid expressing mouse Usp1-myc. A monoclonal anti-myc antibody was used to detect, by immunoblotting, full-length Usp1-myc and its autogenerated *Gln*-Usp1$^{Ct\ myc}$ C-terminal fragment.

(C) CHX-chase for 0, 1, and 3 hr with a wild-type mouse EF cell line that had been transiently transfected with plasmids expressing mouse Usp1-myc or Usp1^{G669A}-myc. Lanes 1–3, vector alone; lanes 4–6, Usp1-myc; lanes 7–9, Usp1^{G669A}-myc.

(D) Same as in (C) but with *Ate1*$^{-/-}$ EF cells. Lanes 1–3, vector alone; lanes 4–6, Usp1-myc; lanes 7–9, Usp1^{G669A}-myc.

(E) Human U2OS cells were transiently transfected with plasmids expressing mouse Usp1-myc (C-terminally tagged with the myc epitope), USP1^{G669A}-myc (it cannot autocleave), or USP1^{Q670A}-myc (its autocleavage yields $Ala-\mathrm{Usp}\,1^{\mathrm{Ct}}_{\mathrm{myc}}$, whose N-terminal Ala is not recognized by the Arg/N-end rule pathway). CHX chases were carried via immunoblotting with anti-myc antibody. See also Figures S1 and S2.

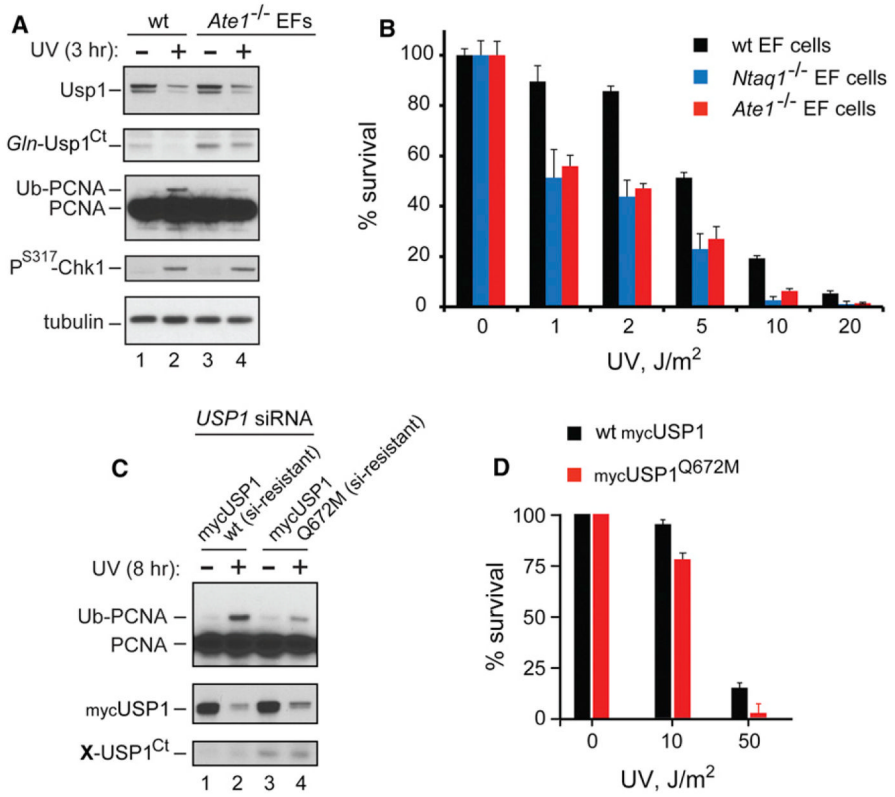

Figure 3. Metabolic Stabilization of the C-Terminal Fragment of Autocleaved Usp1 Decreases Monoubiquitylation of PCNA and Increases UV Sensitivity of Cells

(A) Wild-type and *Ate1*$^{-/-}$ EF cells were either untreated or UV irradiated (254 nm; 50 J/m^2) and incubated for 3 hr, followed by SDS-PAGE of cell extracts and immunoblotting to detect Usp1, *Gln*-Usp1^{Ct}, PCNA, Chk1 (phosphorylated on Ser^{317}), and tubulin. Phosphorylated Chk1 was detected with the phospho-S317 Chk1 antibody (Abcam), to verify that activation of the UV-induced DNA damage checkpoint was not significantly affected by the absence of Ate1.

(B) Wild-type, *Ntaq1*$^{-/-}$, and *Ate1*$^{-/-}$ mouse EF cell lines were either untreated or UV irradiated at 2, 5, 10, and 50 J/m^2, followed by incubation for 5 days and measurements of the fraction of surviving cells using the SYTO-60 DNA staining technique (Invitrogen).

(C) Human U2OS cells were transfected at first with USP1-decreasing small interfering RNA (siRNA) to deplete the endogenous USP1. Twenty-four hours later, cells were transfected with plasmids that expressed siRNA-resistant mRNAs encoding either myc-USP1 or myc-Usp1^{Q672M}. The latter would yield, upon the autocleavage, the *Met*-Usp1^{Ct} C-terminal fragment whose N-terminal Met is not recognized by the Arg/N-end rule pathway, in contrast to N-terminal Gln of wildtype *Gln*-Usp1^{Ct} (Figure S1). Forty-eight hours after the second transfection, cells were either left untreated or UV irradiated (254 nm, at 50 J/m^2), followed by a further incubation for 8 hr, SDS-PAGE of cell extracts, and immunoblotting with both anti-myc and anti-USP1$^{C\text{-}term}$ antibodies, the latter detecting both full-length USP1 and its C-terminal fragment.

(D) Same as in (C) but U2OS cells were either untreated or UV irradiated at 10 and 50 J/m^2, followed by incubation for 5 days and measurements of the fraction of surviving cells.

All assays in (B) and (D) were carried out in triplicate with standard deviations shown. See also Figures S1 and S2.