Ube2j2 ubiquitinates hydroxylated amino acids on ER-associated degradation substrates

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Ubiquitin (Ub) modification of proteins plays a prominent role in the regulation of multiple cell processes, including endoplasmic reticulum–associated degradation (ERAD). Until recently, ubiquitination of substrates was thought to occur only via isopeptide bonds, typically to lysine residues. Several recent studies suggest that Ub can also be coupled to nonlysine residues by ester/thiolester bonds; however, the molecular basis for these novel modifications remains elusive. To probe the mechanism and importance of nonlysine ubiquitination, we have studied the viral ligase murine K3 (mK3), which facilitates the polyubiquitination of hydroxylated amino acids serine/threonine on its ERAD substrate. In this paper, we identify Ube2j2 as the primary cellular E2 recruited by the mK3 ligase, and this E2–E3 pair is capable of conjugating Ub on lysine or serine residues of substrates. However, surprisingly, Ube2j2–mK3 preferentially promotes ubiquitination of hydroxylated amino acids via ester bonds even when lysine residues are present on wild-type substrates, thus establishing physiological relevance of this novel ubiquitination strategy.

Introduction

Modification of proteins with ubiquitin (Ub) is involved in almost all fundamental cellular functions, including regulation of the cell cycle, DNA repair, apoptosis, gene expression, and signal transduction (Hershko and Ciechanover, 1998; Fang and Weissman, 2004). In addition, ubiquitination plays a central role in the homeostasis and quality control of proteins expressed by the secretory pathway. The latter function of Ub is largely performed by ER-associated degradation (ERAD), which targets aberrant proteins for proteasome-dependent degradation (Hampton, 2002; Kostova and Wolf, 2003; Meusser et al., 2005; Sayeed and Ng, 2005). Not surprisingly, abnormalities in the ubiquitination system cause numerous human diseases ranging from neurodegenerative diseases to cancers (Glickman and Ciechanover, 2002). Using an evolutionarily conserved mechanism, ubiquitination is performed by a cascade of three types of enzymes: activating enzyme E1, conjugating enzyme E2, and ligase E3. Ub is first activated by E1, which forms a thiolester with the C-terminal Gly of Ub in an ATP-dependent manner. The activated Ub is then transiently transferred to a conserved cysteine (C) residue of an E2. Finally, the charged E2 interacts with an E3 and facilitates the transfer of the Ub moiety to lysine (K) residues of the substrate or less commonly to the α-amino group of the N-terminal amino acid of a substrate (Ciechanover and Ben-Saadon, 2004). The interaction of E2–E3 is also responsible for the assembly of poly-Ub chains on substrates (Pickart, 2001). All seven K residues of Ub have been found to participate in the formation of poly-Ub chains in vivo. However, the most abundant chains detected by mass spectrometry analyses of modified yeast proteins are linked through K48, K63, and K11 residues (Peng et al., 2003; Xu et al., 2009). K48-linked poly-Ub chains typically target the substrate for proteasomal degradation in the cytosol, whereas K63-linked poly-Ub chains are usually involved in endocytosis, DNA repair, and signal transduction (Pickart and Fushman, 2004; Chen and Sun, 2009). More recently K11-linked chains were also found to target ERAD substrates for proteasome degradation (Jin et al., 2008;

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Abbreviations used in this paper: DUB, deubiquitinating enzyme; Endo H, endoglycosidase H; ERAD, ER-associated degradation; FII, fraction II; HC, major histocompatibility complex class I heavy chain; KSHV, Kaposi's sarcoma-associated herpesvirus; mK3, murine K3; RING, really interesting new gene; shRNA, short hairpin RNA; TMD, transmembrane domain; Ub, ubiquitin; Ub-Al, Ub aldehyde; WT, wild type.

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Xu et al., 2009). Despite the fact that subtle differences in the ubiquitination of substrates dictate their fate, the mechanisms governing substrate residue selection and Ub chain assembly remain poorly understood. A major hindrance for addressing this critical issue in mammals is the lack of defined E2–E3 interactions for physiological substrates.

Surprisingly, recent discoveries indicated that Ub moieties can be linked to substrates by thiolester or ester bonds. These two types of conjugations were found to be mediated by related viral E3 ligases that share a highly homologous RING (really interesting new gene)-CH domain, transmembrane topology, and the ability to target major histocompatibility complex class I heavy chains (HCs) as an immune evasion strategy. The kK3 and kK5 ligases of Kaposi's sarcoma-associated herpesvirus (KSHV) promote ubiquitination of C or K residues on HC substrates, resulting in their rapid endocytosis and degradation in the lysosome (Cadwell and Coscoy, 2005, 2008). In contrast, the murine K3 (mK3) ligase of murine γ-HV68 promotes the ubiquitination of hydroxylated amino acids (serine [S] or threonine [T]) or K residues on HCs, resulting in their ERAD (Wang et al., 2007; Herr et al., 2009). Whether the same E2s or specialized E2s are responsible for interacting with kK3/kK5 or mK3 to form thiolester or ester versus isopeptide linkages has not been reported. Thus, questions remain about the molecular mechanisms of these novel forms of non-K ubiquitination. However, there is increasing indirect evidence for the physiological relevance of substrate ubiquitination via thiolester or ester bonds. For example, an extensive mutagenesis study by Tait et al. (2007) showed that apoptosis induction by Bid requires the ubiquitination of S and C residues on its N-terminal fragment. Furthermore, in yeast and mammals, it was revealed that export of Pex5p, a peroxisomal import receptor, requires ubiquitination of a conserved C residue of Pex5p (Carvalho et al., 2007; Williams et al., 2007). Thus, non-K ubiquitination appears to be a conserved mechanism in yeast and mammals, suggesting that it is a physiologically important posttranslational modification.

In yeast, there are three E2s, Ubc6p, Ubc7p, and Ubc1p, that are known to be involved in the ERAD of misfolded/unassembled lumenal or ER membrane proteins (Chen et al., 1993; Sommer and Jentsch, 1993; Hiller et al., 1996; Biederer et al., 1997; Friedlander et al., 2000; Bays et al., 2001). Both Ubc6p and Ubc7p are ER membrane associated. Interestingly, the ERAD of different substrates mediated by the E3 ligase Hrd1/Der3 requires predominantly Ubc7p or, less frequently, Ubc1p (Bays et al., 2001). However, ERAD mediated by the E3 ligase Doa10 depends exclusively on Ubc6p and Ubc7p (Swanson et al., 2001; Ravid et al., 2006), suggesting that these E2s may preferentially interact with E3 ligases to induce the ERAD of a different subset of substrates (Kikkert et al., 2005; Kostova et al., 2007). In mammals, there are two clear homologues of yeast Ubc6p and Ubc7p named Ube2j2 and Ube2g2, respectively (Tiwari and Weissman, 2001). In addition, homologues with less sequence identity to yeast Ubc6p and Ubc7p have been identified in mammals and named Ube2j1 (also called Ubc6e) and Ube2g1 (Lester et al., 2000; Lenk et al., 2002; Kostova et al., 2007). Of the four of these homologues, Ube2g2, Ube2j1, and Ube2j2 have been implicated in ERAD (Kostova et al., 2007). In addition, cytosolic E2s such as Ube2k (also called E2-25k; homologue of yeast Ubc1p) and Ube2d1 (homologue of yeast Ubc5a) were reported to be involved in ERAD in mammals (Younger et al., 2004; Flierman et al., 2006). In higher eukaryotes, including mammals, there is also a growing list of ER membrane–associated E3 ligases (Fang et al., 2001; Didier et al., 2003; Nadav et al., 2003; Kikkert et al., 2004; Hassink et al., 2005; Kreft et al., 2006; Brauweiler et al., 2007; Lerner et al., 2007). However, with the exception of the recently characterized Ube2g2–gp78 complex (Li et al., 2007, 2009; Das et al., 2009), the functional interaction of these E2s with their cognate E3s has not been well characterized. Genetic studies of yeast suggested that Ubc6p interacts with ligase Doa10 to induce ubiquitination and proteasome degradation of model ERAD substrates (Swanson et al., 2001; Ravid et al., 2006). Interestingly, Doa10 is a RING-CH–type ER membrane–bound E3 ligase similar to mK3. However, in mammals, no cognate E3 ligases of Ube2j2 (Ubc6p homologue) have been identified.

In this study, permeabilized cells were used to show that Ub-conjugating enzymes Ube2j2 and Ube2d1 can interact with mK3 to promote the ubiquitination of HC substrates, whereas other potential ERAD-associated E2s cannot interact with mK3. Using permeabilized cells expressing mK3 and HC mutants having only 1K or 1S Ub acceptor site, Ube2d1 only ubiquitinated K residues on HC substrates, whereas Ube2j2 could ubiquitinate either S or K residues. Interestingly, using wild-type (WT) HC substrates, inhibition by short hairpin RNA (shRNA) of only Ube2j2 resulted in impaired ubiquitination and induced stabilization of HCs, demonstrating that Ube2j2 is the primary E2 interacting with mK3 ligase in vivo. Furthermore, the Ub conjugates on the WT HC mediated by Ube2j2–mK3 were sensitive to mild alkaline treatment, indicating that this E2–E3 interaction strongly favors the ubiquitination of hydroxylated residues on HC substrates even in the presence of K residues. Importantly, the preferential ubiquitination of hydroxylated residues of HC by Ube2j2–mK3 was not the result of differential deubiquitination by C proteases. Thus, these findings demonstrate for the first time that the same E2–E3 complex can catalyze the coupling of Ub to substrates via either an isopeptide bond or an ester bond and that this latter form of nonconventional ubiquitination can be preferentially used to induce ERAD.

Results

Membrane-bound E2s support the ubiquitination of the HC by mK3 ligase

To test whether the cellular E2 supporting mK3 ubiquitination of HC is membrane associated, a permeabilized cell system was used (Shamu et al., 1999). WT3 cells expressing mK3 ligase and its substrate L^d HCs were treated briefly with digitonin and then centrifuged to separate cytosolic proteins in solution from membrane proteins in the pellet. Next, pellets of permeabilized cells were resuspended with an energy-regenerating system (Feldman et al., 1997) and buffer containing E1, E1 + HA-Ub, or fraction II (FII; which contains E1, most E2s, some E3s, and

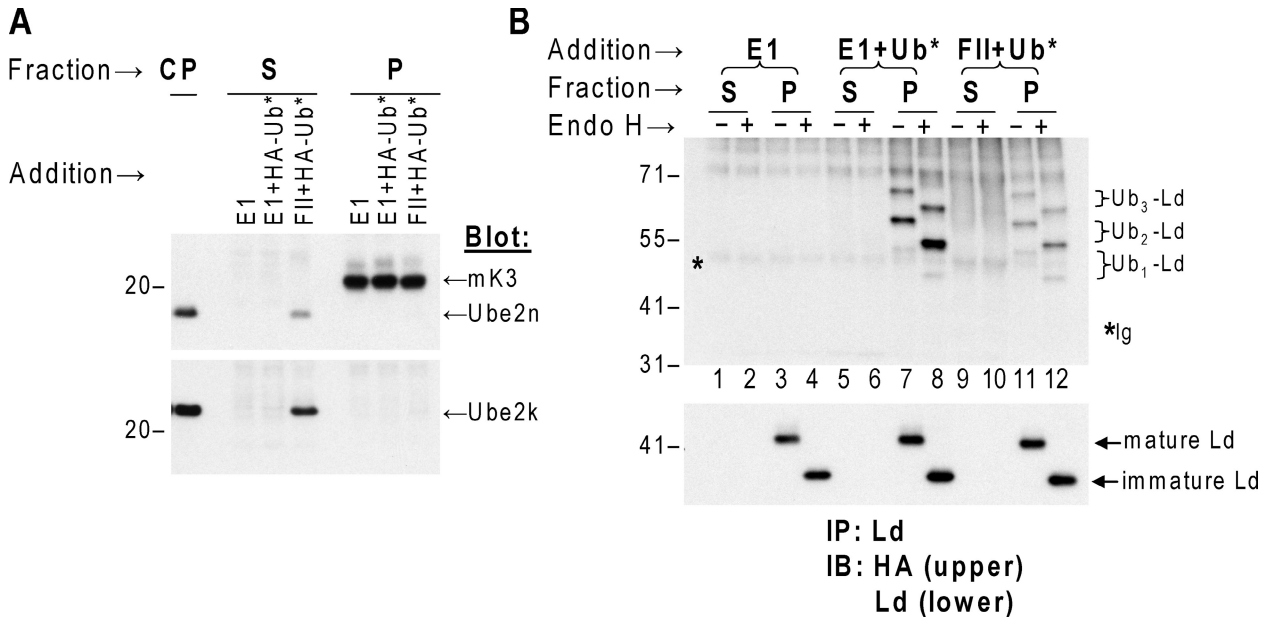

Figure 1. **Membrane-bound E2s support the ubiquitination of the HC by mK3.** (A) Mouse WT3 cells coexpressing WT L^d and mK3 were treated briefly with digitonin. The cytosolic proteins (CP) in solution and membrane proteins in the pellet were separated by centrifugation. The pellet was then suspended with reaction buffer containing an ATP-regenerating system. Three aliquots were made from this suspension and incubated with E1 only, E1 + HA-Ub (Ub*), or rabbit reticulocyte FII + Ub*, respectively, and followed by centrifugation. Samples from supernatant (S) and pellet (P) of each group as well as the cytosolic protein fraction were blotted by the antibodies indicated to verify the depletion of the cytosolic proteins and the maintenance of membrane proteins in the pellet. It should be noted that FII likely contains most soluble E2s; thus, the supernatant fraction with FII added is Ube2k and Ube2n positive. (B) Precipitations of L^d HCs from the supernatant or the pellet fractions of three reaction groups described in A were performed using anti-L^d mAbs. The precipitates were blotted for newly formed Ub-L^d conjugates with anti-HA. An anti-L^d blot was included to show similar input in each group and as an Endo H treatment control. Data shown are representative of three independent experiments. (A and B) Molecular mass is indicated in kilodaltons. IB, immunoblot; IP, immunoprecipitation.

the proteasome) + HA-Ub. After incubation, the reaction mixture was separated into the supernatant and pellet fractions. Samples from the supernatant and pellet fractions of these three experimental groups as well as samples from the cytosolic protein fraction were tested by immunoblot to verify the elimination of cytosolic proteins (Ube2n and Ube2k) and the retention of membrane proteins such as mK3 (Fig. 1 A). The de novo Ub conjugates on the HCs in these supernatant and pellet fractions were identified by immunoprecipitation of the HC and blotting for HA. To clearly distinguish the ubiquitinated HC from nonspecific bands, half of the HC precipitates were treated with endoglycosidase H (Endo H). This treatment cleaves the high mannose glycan moieties from immature HCs (nonubiquitinated as well as ubiquitinated), resulting in a down shift of corresponding HC bands in the gel. From the experiment shown in Fig. 1 B, four important conclusions can be drawn. First, Endo H–sensitive ubiquitinated HC bands were only observed in the samples incubated with HA-tagged Ub (lanes 7, 8, 11, and 12) but not in the samples without added HA-Ub (lanes 1–4). Thus, only newly ubiquitinated HCs were detected in the HA blots. Second, the pattern of the de novo ubiquitinated HCs in the permeabilized cells is the same as previously seen with nonpermeabilized cells (Wang et al., 2007), demonstrating recapitulation of what is observed in vivo. Third, the ubiquitinated HCs were only detected in the pellet, indicating that they were membrane bound, which is consistent with in vivo observations (Wang et al., 2007). Fourth, profound ubiquitination of HC was detected in the samples with only E1 (lanes 7 and 8), supporting the hypothesis that a cognate E2 of mK3 is membrane bound. Consistent with this conclusion, samples with added FII fraction (likely containing most soluble E2s) gave less robust ubiquitination of HC (lanes 11 and 12). Furthermore, by using the cells expressing a catalytically inactive RING mutant of mK3, we confirmed that ubiquitination in the permeabilized system was indeed mediated by mK3 (Fig. S1). Collectively, these data validate the approach and further suggest that a membrane-bound E2 can support mK3-mediated ubiquitination of HC.

Ube2j2 supports both ester- and amide-linked ubiquitination of the HC by mK3

We next wanted to determine whether the cognate E2s of mK3 are the same or different for ubiquitination of hydroxylated versus K residues. For this purpose, an L^d HC mutant with one S in its native position 329 (L^d 1S) was made on a template lacking C, K, S, and T residues on the tail (L^d ΔCKST; Fig. 2 A). To exclude possible position effects, a similar L^d mutant was also made by replacing L^d 329S with a K residue (L^d 1K). Cells expressing either the 1S or 1K HC mutants and mK3 were permeabilized, and the membrane portions were incubated with E1 and E2 in the presence of ATP and HA-Ub. All known ER membrane–associated E2s and their homologues in the mouse (Ube2g1, Ube2g2, Ube2j1, and Ube2j2) and two mouse cytosolic E2s, Ube2k and Ube2d1, implicated in ERAD were tested. Ube2n was also included as a negative control. Each of these seven E2s was expressed as a soluble recombinant protein in *Escherichia coli* and normalized for enzymatic

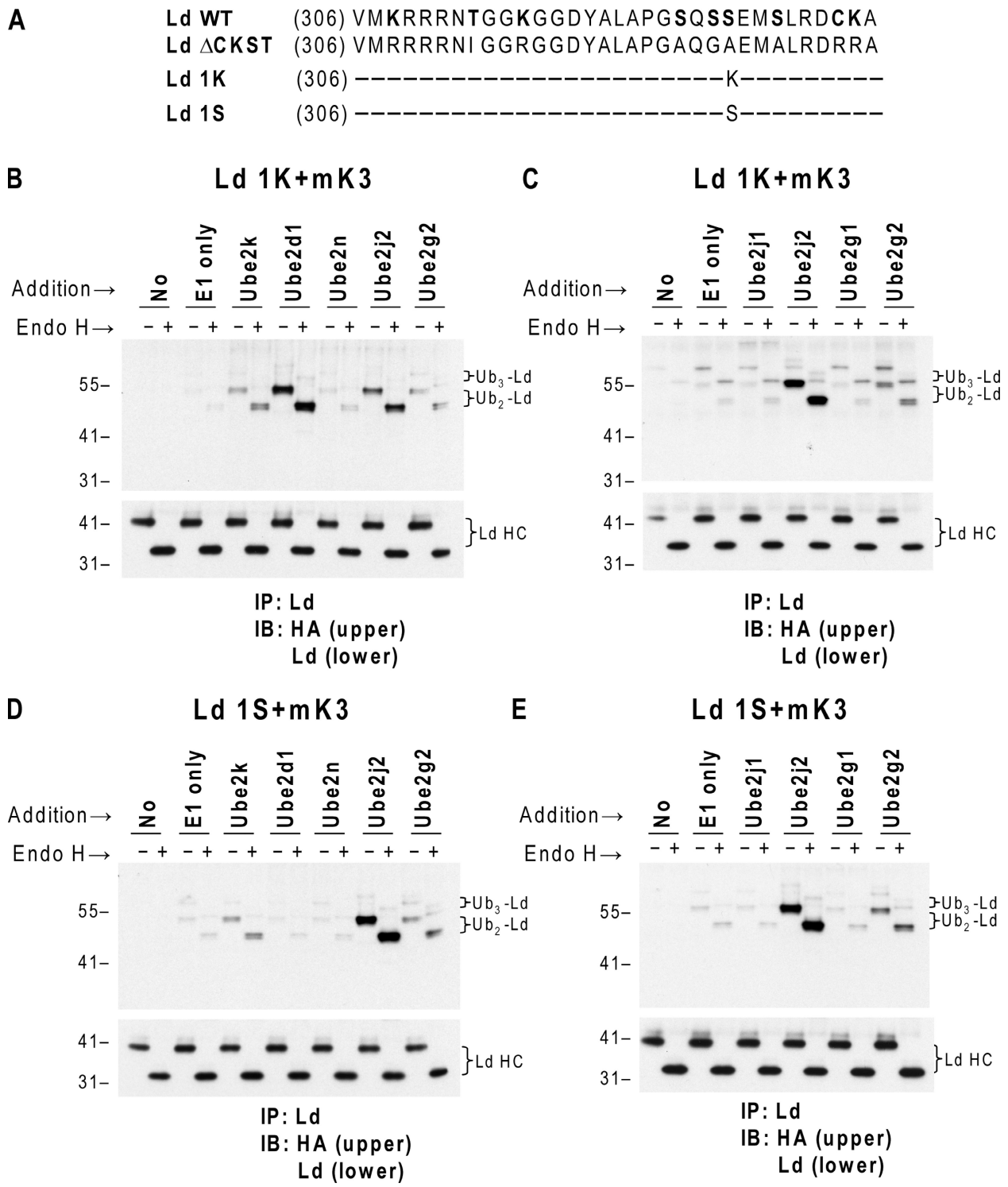

Figure 2. **Ube2j2 interacts with mK3 to promote the Ub conjugation of S or K residues, whereas Ube2d1 interacts with mK3 to promote only ubiquitination of K residues.** (A) Nomenclature and sequence alignment of the cytoplasmic tails of L^d mutants are shown with original WT residues in bold. (B) WT3 cells coexpressing L^d 329K (1K) and mK3 were permeabilized, cytosol depleted, and incubated with E1 and selective mouse E2 in the presence of ATP and HA-Ub. Precipitated L^d molecules (−/+ Endo H) were resolved by SDS-PAGE and blotted for newly ubiquitinated L^d by anti-HA. An anti-L^d blot was included to show similar input in each group and as an Endo H treatment control. (C) After permeabilization and centrifugation, cells used in B were incubated with one of the four ER-associated E2s or homologues. Precipitates of L^d HCs were blotted for HA-Ub and L^d. (D and E) The same experiments were conducted as in B and C, respectively, using cells stably expressing L^d 329S (1S) and mK3. (B–E) Data are representative of at least two independent experiments. Molecular mass is indicated in kilodaltons. IB, immunoblot; IP, immunoprecipitation.

activity (Fig. S2, A and B). Of the seven E2s tested, Ube2d1 and Ube2j2 were found to greatly enhance the Ub conjugation of L^d 1K (Fig. 2, B and C). Surprisingly, Ube2j2 but not Ube2d1 greatly enhanced the ubiquitination on L^d 1S molecules (Fig. 2, D and E). In contrast, the other four E2s did not appreciably change the extent of ubiquitination of the HCs in comparison with samples with Ube2n or samples without added E2 (E1 only). Thus, Ube2j2 can cooperate with mK3 to promote ubiquitination of either S or K residues on HC substrates, whereas Ube2d1 can only support the ubiquitination on K and not S residues on HC substrates.

Ube2j2 is required for ubiquitination and ERAD of HC substrates by the mK3 ligase

To determine whether Ube2j2 and/or Ube2d1 is required for mK3-mediated HC ubiquitination and degradation, shRNAs targeting specific E2s were introduced into WT3-L^d-mK3 cells individually. Target gene expression was evaluated by quantitative RT-PCR (Fig. S3). shRNAs that yielded >70% inhibition were selected for comparisons (Table S1). Compared with controls, the extent of ubiquitination of WT L^d in cells expressing Ube2j2 shRNA (S#1) was lower, especially in the HC bands containing more than two Ub moieties (Fig. 3 A, left). The specificity of this effect was confirmed by another Ube2j2 shRNA (S#2) that targets a different sequence on Ube2j2 (Fig. 3 B, left). In contrast to Ube2j2, inhibiting Ube2g1, Ube2g2, Ube2j1, Ube2k, and Ube2n had no effect on the levels of L^d ubiquitination (Fig. 3, A [left] and B [left]; and not depicted), although comparable levels of mK3 were expressed in each cell line (Fig. 3, A [middle] and B [middle]). Concordant with the reduced level of ubiquitination, the half-life of the L^d molecules was dramatically increased in cells with depleted levels of Ube2j2 (Fig. 3, A [right] and B [right]). Thus, selective inhibition of Ube2j2 by shRNA resulted in impaired Ub conjugation and degradation of the HC substrates by mK3. In contrast, suppression of Ube2d1 by shRNA had no obvious effect on ubiquitination and stability of HCs (Fig. 3 A). Also, there was no additive effect observed when Ube2d1 shRNA was coexpressed with Ube2j2 shRNA (unpublished data). Thus, Ube2j2 is the uniquely required E2 supporting mK3 function in vivo.

As noted in the previous paragraph, inhibition of Ube2j2 impaired polyubiquitination of HC substrates, yet Ub_2-L^d forms were still detected. This finding suggests that either another E2 besides Ube2j2 added the initial Ub moieties to HC substrates or that the residual levels of Ube2j2 after inhibition were sufficient for initial ubiquitination but not chain assembly. To determine whether Ube2j2 is the primary E2 responsible for initial ubiquitination as well as chain assembly on HC substrates, WT3-L^d-mK3 cells expressing shRNA to Ube2j2 or Ube2d1 were permeabilized. The membrane fraction of these cells was then incubated with ATP, HA-Ub, and E1. The ubiquitination of L^d HCs, including Ub_1-L^d and Ub_2-L^d, was severely impaired when Ube2j2 but not Ube2d1 was inhibited (Fig. 3 C), and this impaired ubiquitination was completely reversed by adding recombinant Ube2j2 to the reaction (Fig. 3 C). Furthermore, the same result was observed with cells expressing the 1S L^d mutant and mK3 (Fig. 3 D). Thus, these data definitively demonstrate that Ube2j2 is the primary E2 that is required for mK3-induced initial Ub conjugation and chain assembly on S residues of HC substrates.

mK3 mediates dominant S/T ubiquitination on the tail of L^d while K residues are available

To determine whether mK3–Ube2j2 preferentially promotes the formation of ester bonds, we monitored the sensitivity of ubiquitinated HCs to mild alkaline treatment (Hershko et al., 1980). WT3 cells expressing mK3 and L^d (WT, 1S, or 1K mutants or ΔCKST as shown in Fig. 2 A) were permeabilized and incubated with reaction buffer containing ATP, E1, and Ube2j2. L^d HCs were then precipitated and treated with 0.1 M NaOH. As shown in Fig. 4 A, L^d ΔCKST molecules were not ubiquitinated by Ube2j2 in the presence of mK3, confirming that the tail is indeed the site of ubiquitination. Furthermore, Ub conjugated by Ube2j2 on L^d 1S tails and not L^d 1K tails was found to be labile in 0.1 M NaOH, which is consistent with their respective ester versus isopeptide bonds. Thus, these findings provided additional evidence that Ube2j2 has the capacity to cooperate with the mK3 ligase to form ester or isopeptide bonds on the tails of HC substrates. Strikingly, the newly formed Ub conjugates on WT L^d molecules (as detected in HA blots) were also sensitive to the mild alkaline treatment, suggesting that mK3–Ube2j2 preferentially ubiquitinates S residues on WT HC tails even though K residues are available. To extend these observations to nonpermeabilized cells, L^d HCs were precipitated from the lysates of the same intact cell lines, NaOH treated and blotted for Ub-HCs. Again, the Ub conjugates formed on WT L^d and L^d 1S were labile in mild alkaline conditions (Fig. 4 B). Collectively, these findings demonstrate that mK3 primarily interacts with Ube2j2 in vivo to preferentially ubiquitinate hydroxylated amino acids on WT HC even when K residues are available.

Contribution of deubiquitinating enzymes (DUBs) to mK3-mediated Ub conjugation of HC

DUBs could potentially contribute to the dominant ubiquitination of hydroxylated amino acids on the WT HCs by mK3 if they preferentially cleave K-linked Ub conjugates rather than S/T-linked Ub conjugates. To test this possibility, a similar experiment as in Fig. 4 A was conducted in the presence or absence of Ub aldehyde (Ub-Al), a potent inhibitor for all C protease families of DUBs (Pickart and Rose, 1986; Hershko and Rose, 1987). It should be noted that a few known DUBs belong to the metalloprotease family and thus are not inhibited by Ub-Al, although the majority of DUBs discovered so far are C proteases (Amerik and Hochstrasser, 2004). The results show that both S- and K-linked ubiquitination on either 1S or 1K L^d molecules conjugated by Ube2j2 and mK3 in a permeabilized cell system were enhanced by the inhibition of DUB activity with Ub-Al (Fig. 5 A), suggesting that the DUBs retained in permeabilized cells cleave both K Ub and S Ub conjugates. Although the extent of enhancement of ubiquitination after DUB inhibition was greater on 1K than 1S mutant HC substrates, the signal of 1S

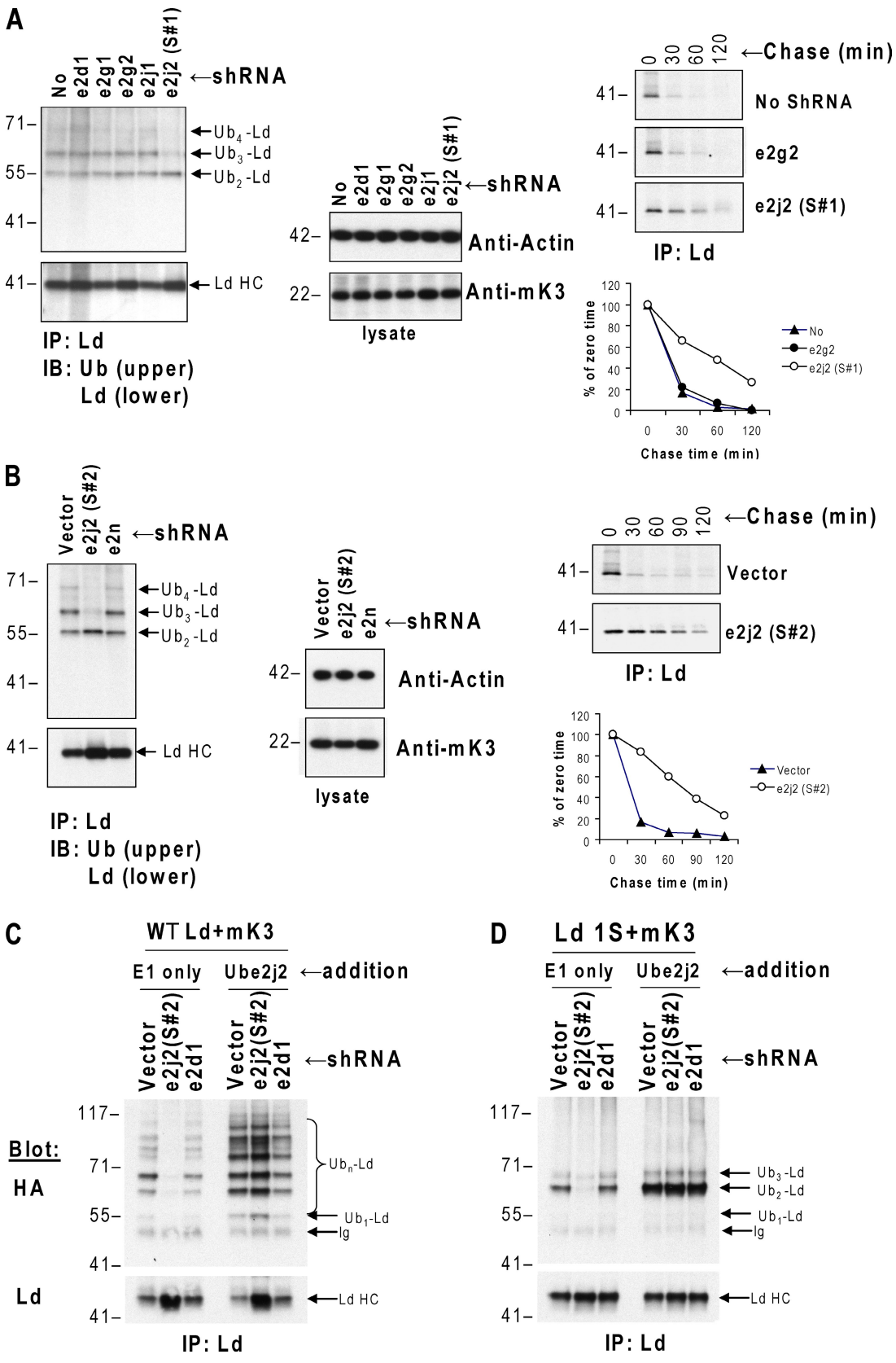

Figure 3. **Ube2j2 is required for mK3-induced HC ubiquitination and degradation.** (A) WT3 cells expressing WT L^d and mK3 were transduced by lentivirus-based shRNA targeting one of the following Ub-conjugating enzymes: Ube2d1, Ube2g1, Ube2g2, Ube2j1, or Ube2j2 (S#1). After incubation with 50 μM MG132 for 2 h, L^d HCs were precipitated from these cells and followed by SDS-PAGE and blotting with anti-Ub and anti-L^d antibodies. The β-actin and mK3 blots in the middle panel were included to show a similar amount of input lysate and mK3 in each cell line. In the right panel, selected cell lines from the left panel were incubated for 24 h with 125 U/ml IFN-γ, pulse labeled with [^{35}S]Cys/Met, and chased for the indicated times with unlabeled Cys/Met.

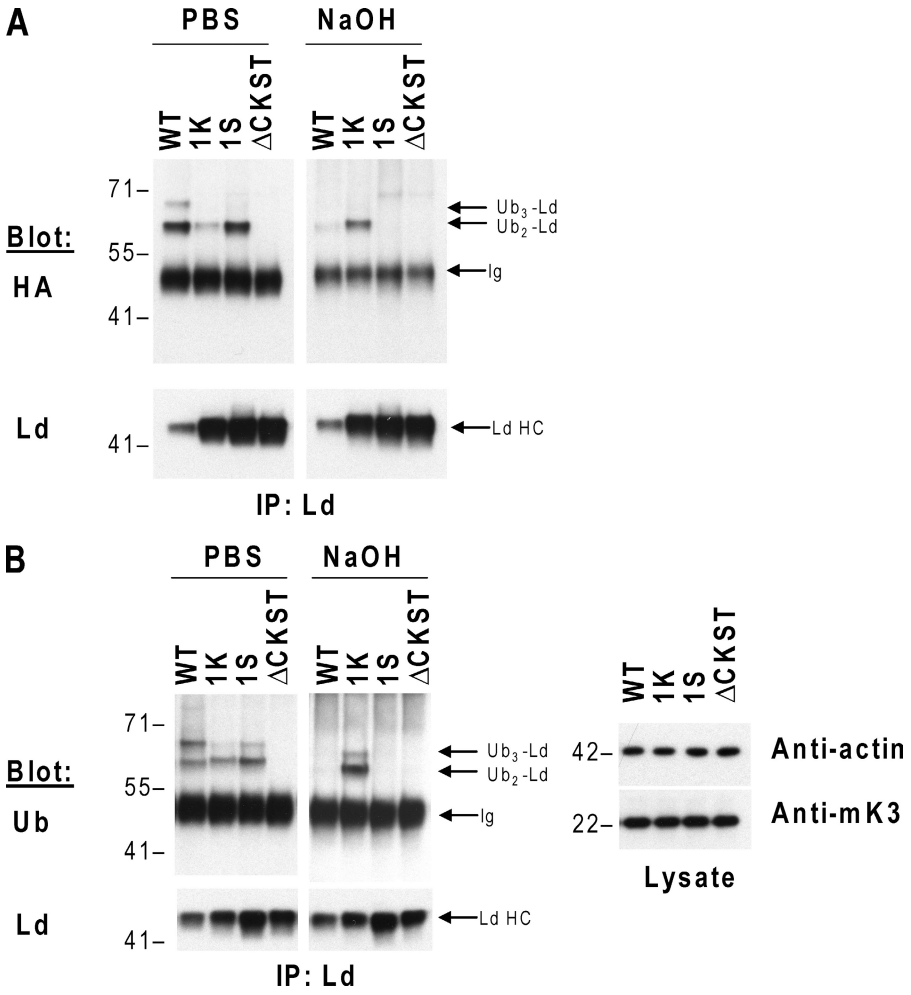

Figure 4. **Ub conjugates on WT L^d molecules induced by mK3 are sensitive to mild alkaline treatment.** (A) WT3 cells expressing mK3 and WT L^d or one of the mutants L^d 1K, L^d 1S, or L^d ΔCKST were permeabilized, cytosol depleted, and incubated with E1, Ube2j2, and HA-Ub. After treatment with PBS or 0.1 M NaOH for 1 h at 37°C, L^d HC precipitates from these cells were neutralized and resolved by reducing SDS-PAGE and then blotted with anti-HA and anti-L^d. A background Ig heavy chain band can be seen in the blot. Data are representative of two independent experiments. (B) L^d HCs were precipitated from cells used in A without permeabilization. After NaOH treatment, SDS-PAGE and immunoblotting were conducted as in A, except using anti-Ub to visualize ubiquitinated L^d HC. The β-actin and mK3 blots on the right were included to show a similar amount of input lysate and mK3 in each cell line. Data are representative of three independent experiments. (A and B) Molecular mass is indicated in kilodaltons. IP, immunoprecipitation.

ubiquitination was consistently stronger than that of 1K molecules regardless of whether DUB inhibitor was present or not (Fig. 5 A, lane 3 vs. lane 1 or lane 4 vs. lane 2). This finding further suggests that Ube2j2–mK3 favors the ubiquitination of S/T residues on HCs. Importantly, this preference can neither be attributed to the position of acceptor residue because the same position was used for 1K and 1S mutants nor by differential susceptibility of hydroxylated versus amide-linked Ub conjugates to DUB activity. It is also notable that there was a marked accumulation of Ub conjugates on WT L^d molecules in the presence of Ub-Al (Fig. 5 B, lane 2 vs. lane 1), and these conjugates remained sensitive to NaOH treatment (Fig. 5 B, lane 6 vs. lane 2). This observation demonstrates that dominant S/T-linked ubiquitination on WT L^d molecules is not the consequence of differential DUB activity but rather preferential S/T ubiquitination by Ube2j2–mK3. This conclusion is further supported by direct comparison of alkaline sensitivity of ubiquitinated WT HCs catalyzed by Ube2j2 with Ube2d1. In the presence of Ub-Al, in contrast to Ube2j2–mK3-catalyzed Ub-L^d conjugates, which were sensitive to NaOH treatment, Ub-L^d molecules conjugated by Ube2d1–mK3 were stable under the same conditions (Fig. 5 C, lane 3 vs. lane 4), thus clearly demonstrating that these two E2s interact with mK3 to ubiquitinate different residues on the WT HCs. These findings show that Ube2j2 is directly responsible for the preferential ubiquitination of S/T residues on WT HC substrates. In addition, it is worthy to note that S/T Ub conjugates, once formed, may be more resistant to DUB-mediated cleavage than K Ub conjugates. This is indicated by the greater enhancement of K Ub conjugates than S Ub conjugates upon DUB inhibition (Fig. 5 A, compare lane 2 vs. lane 1 with lane 4 vs. lane 3). Preferential ubiquitination of S/T residues on WT HCs may be a strategy developed by γ-HV68 to effectively down-regulate major histocompatibility complex I molecules, thus evading CD8 cell detection.

L^d precipitates were resolved by SDS-PAGE and visualized by autoradiography. Relative band intensities from the gels are plotted as a percentage of the intensity at time 0 for each cell line. Data are representative of three independent experiments. (B) WT3 cells expressing WT L^d and mK3 were transduced by retrovirus-based shRNA targeting Ube2j2 (S#2) or Ube2n or by an empty vector. The ubiquitination and degradation of L^d HCs were determined by immunoblotting and pulse–chase experiments as in A. Data are representative of three independent experiments. (C) Selected cells used in A and B were permeabilized, cytosol depleted, and incubated with E1 or E1 + Ube2j2 in the presence of ATP, HA-Ub, and Ub-Al. Precipitated L^d molecules were resolved by SDS-PAGE and blotted for newly ubiquitinated L^d by anti-HA. An anti-L^d blot was included to show the level of L^d molecules in each cell line. (D) Same as in C except that WT3 cells expressing 1S L^d and mK3 were used. (A–D) Molecular mass is indicated in kilodaltons. IB, immunoblot; IP, immunoprecipitation.

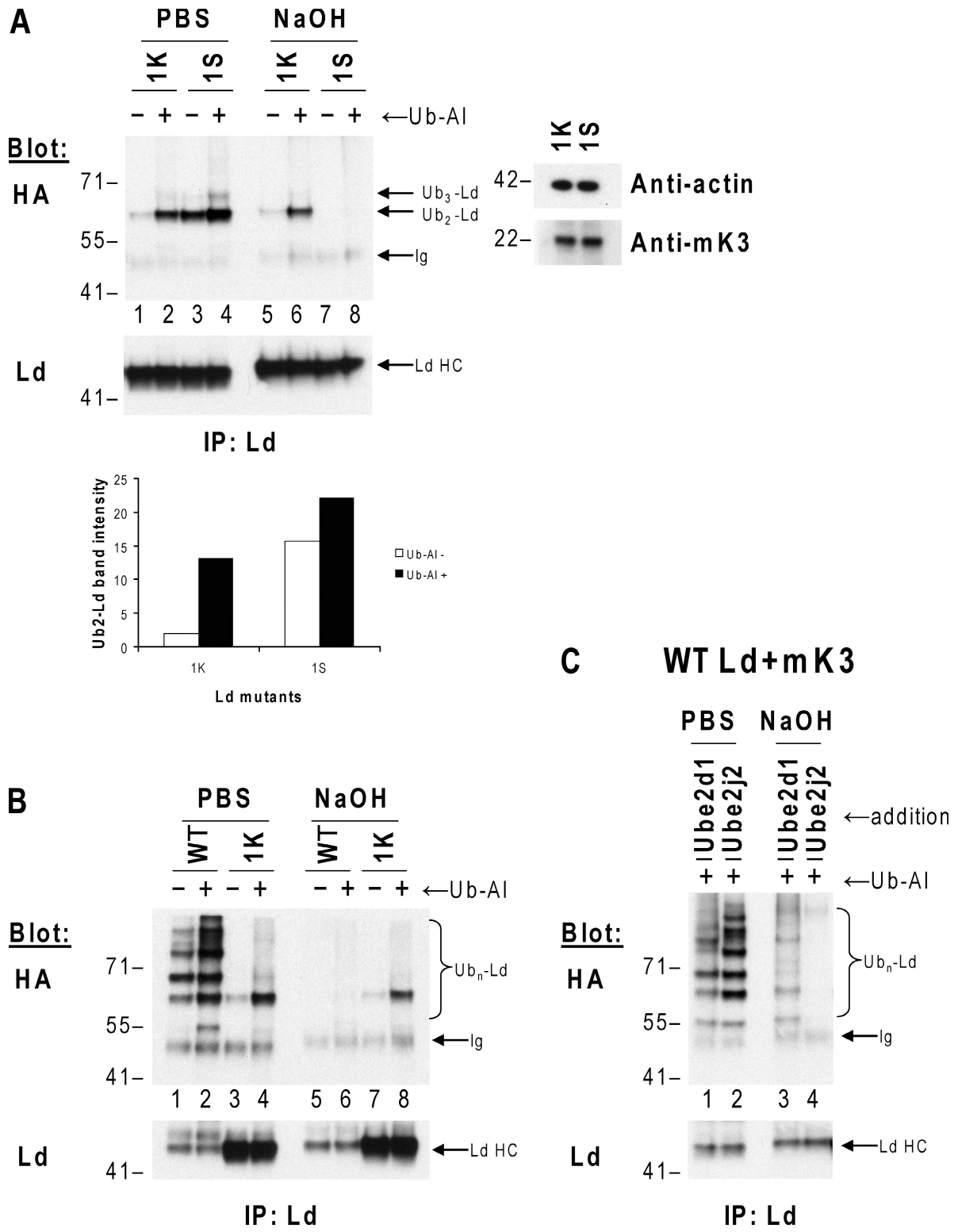

Figure 5. **Ube2j2–mK3 favors ubiquitination of S residues over K residues on the tail of HC substrates.** (A) WT3 cells expressing mK3 and L^d 1K (329K) or L^d 1S (329S) were permeabilized, cytosol depleted, and incubated with E1, Ube2j2, and HA-Ub in the presence or absence of 2 µM Ub-Al, a DUB inhibitor. After treatment with PBS or NaOH for 45 min, L^d HC precipitates from these cells were neutralized and resolved by reducing SDS-PAGE and then blotted with anti-HA and anti-L^d. Background Ig heavy chain bands can be seen in the blot. Quantification of Ub_2-L^d bands from PBS treatment in the gel is shown in the graph. Samples from cytosolic proteins and pellets of each line were blotted to verify an equal amount of mK3 expression (right). (B) WT3 cells expressing mK3 and WT L^d or L^d 1K were permeabilized and incubated with E1, Ube2j2, and HA-Ub in the presence or absence of Ub-Al. All other steps were performed as in A. (C) Same as in A, except permeabilized cells expressing mK3 and WT L^d were incubated with either Ube2d1 or Ube2j2 in the presence of Ub-Al. Data shown are representative of two independent experiments. (A–C) Molecular mass is indicated in kilodaltons. IP, immunoprecipitation.

Sequences in addition to the RING-CH domain of the ligase are critical for ubiquitination of substrates via nonisopeptide bonds

Unlike mK3, kK3 and kK5 ligases ubiquitinate HCs in a post-ER compartment, resulting in their rapid endocytosis and lysosomal degradation (Lehner et al., 2005). The cognate E2s of kK3 have been identified as Ubc5b/c (Ube2d2/3) and Ubc13 (Ube2n), which catalyze a K63-linked poly-Ub chain on the tail of the HCs (Duncan et al., 2006). Moreover, both kK3 and kK5 can facilitate Ub conjugation of C and K residues (Cadwell and Coscoy, 2005, 2008) and not S/T residues like mK3. Whether the same or different E2s facilitate a C ubiquitination versus a K ubiquitination by kK3 and kK5 has not yet been reported. Nevertheless, the fact that mK3 and kK3/kK5 have similar overall structures with highly homologous RING-CH domains make them valuable probes for understanding the determinants and consequences of specific E2–E3 interactions with each other and with substrates. To determine the importance of the RING-CH domain in E2 selection and the potential to ubiquitinate hydroxylated residues, the RING domains of kK3 and kK5 were each swapped into mK3. Each of these RING swap constructs was introduced into cells expressing WT L^d or L^d mutants with only C, K, S, or T residues available in the tail. As expected, both kK3 and kK5 RINGs in the context of mK3-mediated robust ubiquitination of K, S, or T but not C in tails of the HC in these cells (unpublished data). Their lack of ability to efficiently conjugate C residues is not caused by the position of the C residue because other positions were tested, including sites proximal to the C terminus of the tail, as favored by intact kK3 ligase (Herr et al., 2009). Thus, in the context of mK3, the RING domains of kK3 or kK5 no longer supported ubiquitination of C residues like their intact ligases but facilitated S or T ubiquitination like intact mK3. Furthermore, in our permeabilized cell system, Ube2j2 and not Ube2n interacted with kK3 or kK5 RING swaps to greatly enhance the ubiquitination on an S residue of the tail of HC (Fig. 6 A, left and right, respectively). Accordingly, the Ub chains formed on the tail of L^d 1S molecules in kK3 or kK5 swap–expressing cells were Ub K48 and not K63 dependent (Fig. 6 B), which is the same as what was observed with WT mK3 (Fig. S4). Collectively, these data demonstrate that sequences outside the RING domain of mK3 ligase are critical determinants for which E2 is recruited, which non-K residues can be ubiquitinated, and which linkage is used for poly-Ub chain assembly.

Discussion

Nonisopeptide bond–linked substrate ubiquitination was initially shown with homologous RING-CH–type viral ligases from KSHV and γ-HV68. More specifically, the kK3 and kK5 ligases of KSHV were shown to ubiquitinate C residues on HC, thereby inducing their rapid endocytosis and degradation in the lysosome (Cadwell and Coscoy, 2005, 2008). In addition, we demonstrated that ubiquitination of HCs by γ-HV68 ligase mK3 can occur via an ester bond to hydroxylated amino acids (S or T), resulting in ERAD of the HCs (Wang et al., 2007). These findings have raised considerable interest in understanding the molecular basis and physiological importance of this nonconventional ubiquitination. In this study, we show that Ube2j2 can promote the ubiquitination on HC mutants having only 1S or only 1K on the tail. Consistent with their ester linkage, Ub conjugates on the 1S mutant were substantially more labile to the mild alkaline treatment than Ub conjugates on 1K mutants. Furthermore, regardless of whether S or K residues of substrate HCs were ubiquitinated, the poly-Ub chain was assembled with K48 linkages (Fig. S4). Thus, these findings demonstrate that a specialized Ub-conjugating enzyme is not required; instead, the same E2 cooperates with mK3 to promote either ester bonds or isopeptide bonds between Ub and substrate. We previously found that there are no sequence constraints around either K or S/T ubiquitination sites on HC substrates but that a certain proximity from the ER membrane is required (Wang et al., 2007; Herr et al., 2009). These combined findings suggest a model whereby the correct position or orientation of Ube2j2 relative to the substrate is imposed by mK3 and that this is a common requirement for both K and S/T ubiquitination. Surprisingly we find that Ub conjugates on the WT L^d molecules mediated by mK3–Ube2j2 are mainly linked via hydroxylated residues even though WT L^d contains multiple Ks in the tail. This S/T ubiquitination preference by Ube2j2 is observed when C protease DUB activity is inhibited and thus cannot be explained by DUB more efficiently cleaving K-linked Ub conjugates. Given the fact that there has been increasing evidence showing that nonconventional ubiquitination may occur on multiple substrates in different cellular pathways, it is very likely an underappreciated mechanism. The flexibility of the selection of conjugation residues may lead to more efficient Ub modification and would benefit protein quality control for proteins having no K residues accessible for ubiquitination. Furthermore, because S or T residues are also sites of phosphorylation, it is attractive to speculate that there is cross talk between two major posttranslational modifications, ubiquitination and phosphorylation.

The mechanistic basis for the preferential S/T ubiquitination of substrates by Ube2j2–mK3 remains undefined. However, the transmembrane domain (TMD) of Ubc6p has been shown to play a dominant role in its ER localization and is required for its in vivo function (Chen et al., 1993; Yang et al., 1997). Interestingly, in this study, we show that soluble Ube2j2 in which the TMD is deleted is capable of restoring the Ub conjugation of S residues on HC substrates after inhibition of native Ube2j2 by shRNA. Thus, the TMD of Ube2j2 is not required for non-K ubiquitination and is not likely to be involved in its interaction with mK3. However, the TMD may facilitate the localization of Ube2j2 in the ER, which is compensated by the overexpression of soluble Ube2j2 in a permeabilized cell system. It should be noted that we were not able to coprecipitate Ube2j2 with mK3, suggesting that their interaction may be transient. Such a transient interaction would contrast with the strong binding of the E3 ligase gp78 to Ube2g2, as mediated by its G2BR domain (Chen et al., 2006). Nevertheless, mK3 is the first E3 ligase in mammals found to interact with Ube2j2 and promote the ubiquitination and ERAD of its substrate. This defined

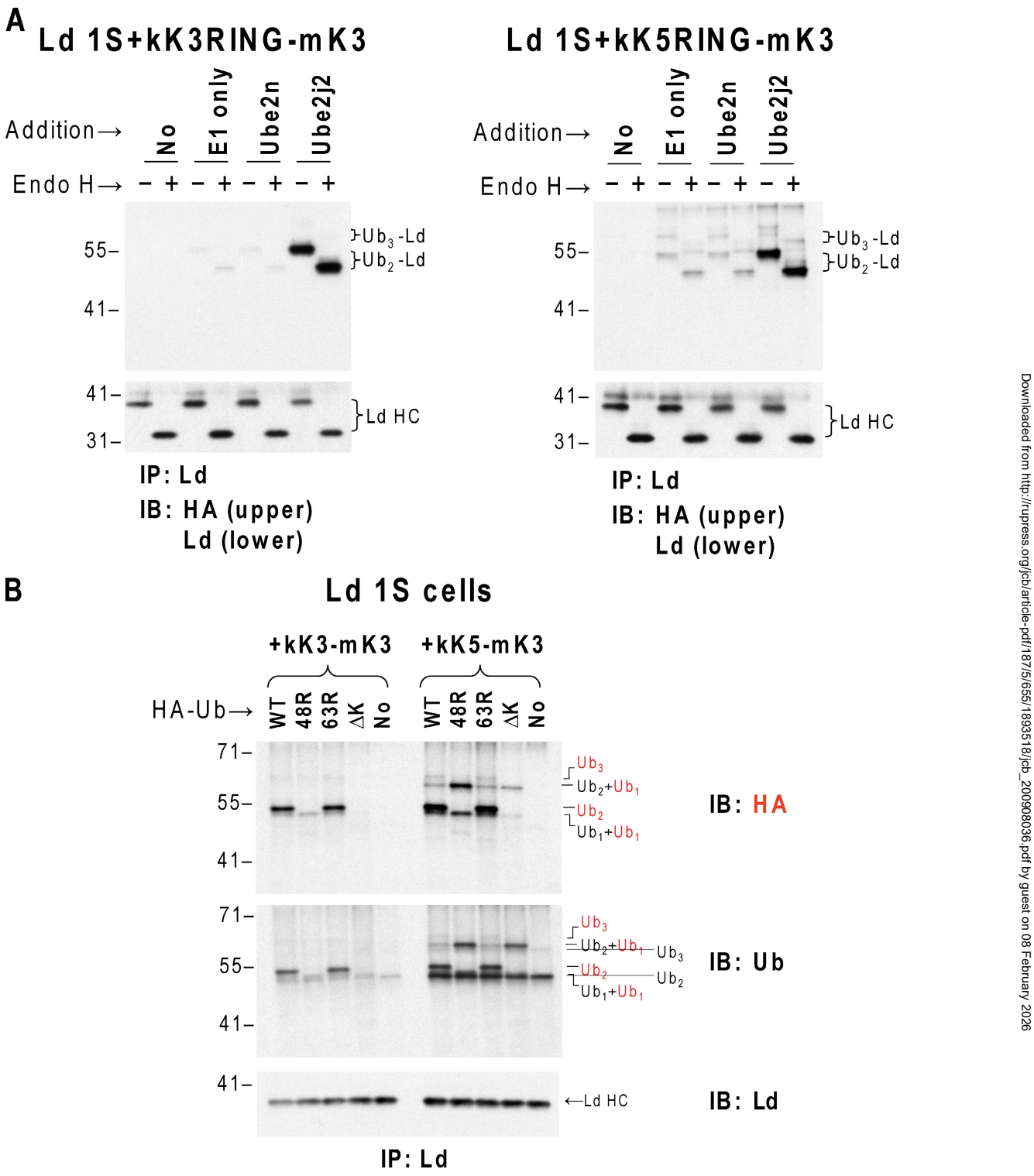

Figure 6. **The kK3 and kK5 RINGs in the context of mK3 interact with Ube2j2 to promote the formation of K48-linked Ub chain on S residues of the HCs.** (A) kK3 or kK5 RING-mK3 chimeric molecules were expressed in WT3–L^d 1S cells. After permeabilization and depletion of cytosolic proteins, aliquots of these cells were incubated with no enzyme, E1 only, or E1 plus one of the two E2s in the presence of ATP and HA-Ub. L^d HCs were immunoprecipitated and blotted as indicated. (B) Aliquots of the cells used in A were incubated with ATP, E1, and WT or mutant HA-Ub in an ubiquitination assay of permeabilized cells as described in A. Immunoprecipitation (IP) of L^d and blotting by anti-HA (top) or anti-Ub (middle) antibodies shows newly formed Ub-L^d HCs and total Ub-L^d HCs, respectively. The conjugates formed by HA-Ub are in red. (A and B) Molecular mass is indicated in kilodaltons. IB, immunoblot.

E2–E3–substrate interaction will be a foundation for future functional characterization of Ube2j2 as well as dissection of the mechanism of S/T ubiquitination.

Interestingly, in our permeabilized cell assays, a second E2, Ube2d1, supported mK3-induced ubiquitination of HC. However, in contrast to Ube2j2, Ube2d1 facilitated ubiquitination

on L^d 1K molecules but not on L^d 1S molecules in the presence of mK3. In addition, Ube2d1–mK3-promoted Ub conjugates on the WT HCs were stable in mild alkaline conditions, confirming an isopeptide bond linkage. These results indicate that mK3-recruited Ube2d1 can only catalyze the formation of isopeptide bonds between Ub and substrate but not ester bonds. Different from Ube2j2, which has a C-terminal extension and a TMD, Ube2d1 is comprised exclusively of the core catalytic domain (Scheffner et al., 1994). Ube2d1 has been shown to have broad E3-interacting activity, including HECT (homologous to the E6-AP C terminus)-, RING-, and U box–type E3s, to promote Ub conjugation on K residues of substrates (Lorick et al., 2005). In addition, Grou et al. (2008) recently reported that all three closely related Ube2d family members, Ube2d1, -2, and -3, can support ubiquitination of a C residue on Pex5p, a peroxisomal import receptor. In yeast, this process is mediated by a specialized Ub-conjugating enzyme, Pex4p (Williams et al., 2007). These findings suggest that Ube2d members have the potential to catalyze both K and non-K ubiquitination. However, in the Grou et al. (2008) study, Ube2d members were only shown to mediate the ubiquitination on C residues of Pex5p, and the E3 ligase has not been defined. Therefore, it is not clear whether these Ube2d members can catalyze both types of ubiquitinations when interacting with the same E3. Furthermore, thiolesteration between C residues and Ub is a standard reaction used during charging of E1, E2, or HECT-type E3 with Ub. Thus, the question remains whether an E2 capable of promoting thiolesteration can also catalyze the addition of Ub to hydroxylated amino acids. In this study, we clearly demonstrate that Ube2d1 is capable of mK3-induced HC ubiquitination on K residues but lacks the ability to ubiquitinate S residues. The mechanistic implication of these findings is that not all E2s capable of interacting with mK3 can catalyze both K and S/T ubiquitination, implying that non-K ubiquitination requires a specific E2–E3 interaction. It should be noted that among seven E2s tested in our system, Ube2d1 shares more predicted E3 interaction sites with Ube2j2 than the other E2s tested (4/5 vs. ≤3 conserved residues). Furthermore, comparison of available structures of homologues of mouse Ube2d1 and Ube2j2 shows a similar E3 interaction surface, but they vary around their catalytic C residues.

A single E3 ligase can interact with more than one E2, as demonstrated in several in vitro and a few in vivo studies (Chen et al., 1993; Christensen et al., 2007; Rodrigo-Brenni and Morgan, 2007; Windheim et al., 2008). This redundancy adds another apparent layer of regulation in Ub modification. However, precise consequences of two or more E2s cooperating with a particular E3 remains largely undefined. Recently proposed models have speculated that certain E2s can primarily catalyze the conjugation of the initial Ub moiety to the substrate, whereas other E2s assemble the poly-Ub chain with either K48 or K63 linkages (Christensen et al., 2007; Windheim et al., 2008). Whether this sequentially acting E2 model also functions in ERAD is currently unknown. Although ERAD-implicated E3 ligases have been shown to interact with more than one E2, it is unclear whether these distinct E2s combine to polyubiquitinate substrates (Bays et al., 2001; Kikkert et al., 2004; Arteaga et al., 2006; Mueller et al., 2008). Furthermore, it was recently found that Ube2g2 alone can interact with gp78 in vitro to transfer a preassembled K48-linked Ub chain to a substrate (Li et al., 2007). Our study shows that both Ube2d1 and Ube2j2 are capable of interacting with mK3 to facilitate K48-linked poly-Ub conjugation to the HCs (Fig. S4 B). However, in vivo, only Ube2j2 is primarily required for mK3 function because inhibition of Ube2j2 alone impaired HC ubiquitination and degradation, whereas inhibition of Ube2d1 in vivo had no such effect. Given the fact that Ube2d family members have never been found in association with an ER membrane E3, it is possible that the subcellular localization of Ube2d1 prevents its participation in ERAD. Alternatively, because S residues are the primary sites of ubiquitination on WT L^d tails, only depletion of Ube2j2 and not Ube2d1 would be expected to impair mK3-mediated ubiquitination and ERAD of HC substrates. In support of this model, inhibition of Ube2j2 strikingly impairs HC ubiquitination, including Ub_1-L^d forms on the WT HC and Ub conjugation on the 1S HC mutant. All of these Ub conjugates were found to be sensitive to mild alkaline treatment, which is consistent with ester linkage. Thus, our data demonstrate that Ube2j2 is primarily responsible for initial ubiquitination and Ub chain assembly on HC substrates.

Our findings also have implications on the mechanism of mK3-induced Ub chain assembly. Interestingly, although Ube2j2–mK3 primarily ubiquitinates hydroxylated amino acids on L^d substrates, it promotes a K48-linked poly-Ub chain. This suggests that the determinants for attachment of the initial Ub and assembly of the Ub chain are different. Curiously, in this and other studies of mK3 ubiquitination by us and others (Boname and Stevenson, 2001; Wang et al., 2007), Ub_1 modifications were only weakly detected relative to predominant Ub_2 and Ub_3 polychains on HC substrates. However, Ub_1-L^d forms were clearly observed along with the overall increase in ubiquitination seen after inhibition of C protease DUBs. Thus, Ub_1-HC forms may be transient, reflecting rapid chain elongation which is retarded in the presence of DUBs. This observation is similar to a study with the yeast cullin-RING Ub ligase SCF (Skp1–Cul1–F box protein) and Ub-conjugating enzyme Cdc34 (Petroski and Deshaies, 2005). SCF interacts with a single E2, Cdc34, to catalyze both the attachment of the first Ub and elongation of a specific Ub chain on substrate. However, these two reactions occur with strikingly different kinetics in which the first step is relatively slow followed by rapid chain elongation (Petroski and Deshaies, 2005). Alternatively, Ub chain assembly by mK3 may preferentially initiate with the addition of two Ub moieties. Such a strategy would be similar to that of gp78–Ube2g2 (Li et al., 2007, 2009). It was recently shown that ER-associated E3 ligase gp78 interacting with Ube2g2 mediated the attachment of preassembled K48-linked Ub chain to substrate by the formation of a gp78–Ube2g2 hetero-oligomer (Li et al., 2009). Although the precise molecular strategy used by mK3–Ube2j2 for Ub assembly is currently unknown, the predominance and specificity by which mK3–Ube2j2 interacts with HC substrates will allow us to further dissect this process. In summary, we identify Ube2j2 as the primary E2 required for mK3 function. Mechanistically, our findings demonstrate that Ube2j2

in the context of mK3 adds the initial Ub primarily to S residues of the HCs and then assembles a K48-linked poly-Ub chain, which leads to the ERAD of the HCs.

Materials and methods

Cell lines

Murine cell line B6/WT3 (WT3; *H-2*^{b}) was described previously (Wang et al., 2004). The Vpack vector system (Agilent Technologies) and vectors pHR'8.2ΔR and pCMV-VSV-G (provided by S. Stewart, Washington University School of Medicine, St. Louis, MO) were used to produce retrovirus and lentivirus in 293T cells (DuBridge et al., 1987). Cells transduced by pMSCV.IRES.neo (pMIN)-, pSRPU6-, or pLKO.1-based constructs were selected by corresponding antibiotics, whereas GFP^{+} cells from pMSCV.IRES.GFP (pMIG)-transduced lines were enriched by cell sorting. Where indicated, cells were cultured for 24 h with 125 U/ml of mouse IFN-γ (Invitrogen) and for 2–3 h with 50–60 μM of proteasome inhibitor MG132 (Boston Biochem) before harvesting with trypsin-EDTA.

DNA constructs

Two retroviral expression vectors, pMIG and pMIN (Wang et al., 2004), were used to express mK3 and L^{d} constructs, respectively. The retroviral shRNA expression vector pSRPU6 was modified from pSUPER.retro.puro (Oligoengine) by replacing the H1 promoter with the human U6 promoter. The lentiviral shRNA expression vector pLKO.1 was provided by S. Stewart. mK3 sequence from a γ-HV68 subclone (Virgin et al., 1997), kK5 sequence from a KSHV subclone (provided by H.W. Virgin IV, Washington University School of Medicine), and kK3 sequence from BCBL-1 (provided by J.U. Jung, Harvard Medical School, Southborough, MA) were obtained by PCR amplification. The kK5 or kK3 RING-mK3 chimeric constructs were made by replacing the RING-CH of mK3 (1–51 aa) in pMIG.mK3 with the RING-CH of kK5 (1–59 aa) or kK3 (1–53 aa) using overlap PCR. The L^{d} mutants were generated by site-directed mutagenesis (Agilent Technologies) as described previously (Wang et al., 2007). The sequences for all of the constructs were confirmed by DNA sequencing. The shRNA sequences and sources used in this study are listed in Table S1.

Antibodies

Antibodies to mK3 and L^{d} (30-5-7 and 64-3-7) were previously described (Smith et al., 1992; Lybarger et al., 2003). Antibodies to Ub (P4D1), β-actin (AC-74), Ube2k, Ube2n, and HA.11 (clone 16B12) were purchased from Santa Cruz Biotechnology, Inc., Sigma-Aldrich, Cell Signaling Technology, AnaSpec, and Covance, respectively.

Immunoprecipitation and immunoblots

Cells were lysed in PBS buffer containing 1% NP-40, 20 mM iodoacetamide (Sigma-Aldrich), and protease inhibitors (complete mini [Roche] and 0.4 mM PMSF [Sigma-Aldrich]). Postnuclear lysates were incubated with protein A–Sepharose 4B beads (Sigma-Aldrich) and antibodies. After washing beads four times with PBS/iodoacetamide buffer containing 0.15% NP-40, immunoprecipitates were eluted from protein A by boiling 3–5 min in lithium dodecyl sulfate sample buffer (Invitrogen). For Endo H treatment, immunoprecipitates were eluted in 10 mM Tris-HCl, pH 6.8, with 0.5% SDS; the eluates then were mixed with an equal volume of 100 mM Na acetate, pH 5.4, and incubated with 1 μU Endo H at 37°C for 2 h. For alkaline treatment, immunoprecipitates were boiled in 0.5% SDS for 3 min followed by incubation in 0.1 M NaOH for 1 h at 37°C. Immunoblotting was performed after SDS-PAGE separation of precipitated proteins or postnuclear cell lysates on Immobilon-P transfer membranes (Millipore). Specific proteins were visualized by chemiluminescence using the ECL system (Thermo Fisher Scientific).

Metabolic labeling and pulse–chase

After 30 min of preincubation in Cys- and Met-free medium (MEM-Earle's with 5% dialyzed FCS), cells were pulse labeled with Express [^{35}S]Cys/Met labeling mix (PerkinElmer) at 200 μCi/ml for 15 min. Chase was initiated by the addition of an excess of unlabeled Cys/Met (5 mM each). Immunoprecipitation was performed as described in the previous section. Immunoprecipitates were separated by SDS-PAGE and revealed by autoradiography. Densities of the specific bands were analyzed using ImageJ software (National Institutes of Health).

Cell permeabilization and ubiquitination

A method described by Shamu et al. (1999) was adopted with modification. In brief, after detachment by trypsin-EDTA and washing with PBS twice, the cells were resuspended in potassium acetate buffer (25 mM Hepes, pH 7.4, 115 mM KOAc, 5 mM NaOAc, 2.5 mM $MgCl_2$, and 0.5 mM EGTA) containing 0.02% digitonin (Wako Chemicals USA, Inc.) at 0.5–1.0 × 10^{7}/ml and incubated on ice for 20 min. Soluble cytosolic proteins were squeezed out by centrifugation of digitonin-treated cells at 18,000 *g* and 4°C for 20 min and washed once with potassium acetate buffer. These soluble protein–depleted cells were then incubated for 45 min at 37°C with 50 nM E1 (human Ub-activating enzyme; Boston Biochem) or 2.5 mg/ml FII from rabbit reticulocyte extract (Boston Biochem), 20 μM HA-Ub, and ∼1.25 μM candidate E2 in 25 mM Tris-HCl, pH 7.4, buffers containing an ATP-regenerating system (10 mM MgOAc, 300 mM phosphocreatine, 0.6 mg/ml creatine phosphokinase, and 2 mM ATP), 1 mM PMSF, and 50 μM MG132. 2 μM Ub-Al (Boston Biochem) was included when indicated. After incubation, ubiquitinated HCs were visualized by immunoprecipitation of L^{d} molecules and then blotting for tagged Ub by anti-HA antibody.

Recombinant E2s and HA-Ub proteins

Recombinant proteins used in this study, including mouse Ube2g1, Ube2g2, soluble Ube2j1 (1–285 aa), soluble Ube2j2 (1–226 aa), Ube2k, HA-Ub, and its mutants, were expressed as GST fusion proteins in *E. coli* (BL21-CODONPLUS-RIL; Agilent Technologies) by pGEX4T (GE Healthcare). The GST fusion proteins were purified with glutathione beads (GE Healthcare) from the bacteria lysates according to the manufacturer's instructions. The recombinant proteins were cleaved from the beads using thrombin (Sigma-Aldrich). The activity of purified recombinant E2s was assessed using a thiolester formation assay and compared with the commercial E2s UbcH5a and His_6-UbcH13–Uev1a complex (Boston Biochem), which have the same amino acid sequence as mouse Ube2d1 and Ube2n. Purified recombinant HA-Ub proteins possessing two vector-derived amino acid remains (GS) at the N terminus were compared with commercial HA-Ub (Boston Biochem) in a thiolester formation assay with functional E2. Aliquots of the aforementioned purified proteins were stored at −80°C in 50 mM Tris-HCl, pH 8.0, 150 mM KCl, 2 mM $MgCl_2$, and 5% glycerol.

Online supplemental material

Fig. S1 shows that ubiquitination of HCs in permeabilized cells is dependent on the RING domain of mK3. Fig. S2 shows that the recombinant E2s used in this study had similar enzymatic activity and protein level, as determined by in vitro thiolester formation and silver stain. Fig. S3 shows the inhibition by shRNA of targeted E2s, as determined by quantitative RT-PCR. Fig. S4 shows that mK3-facilitated poly-Ub chains on either S or K residues of HCs are dependent on K48 of Ub. Table S1 shows the shRNA sequences used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200908036/DC1.

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