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A ubiquitin ligase complex assembles linear polyubiquitin chains

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The ubiquitin system plays important roles in the regulation of numerous cellular processes by conjugating ubiquitin to target proteins. In most cases, conjugation of polyubiquitin to target proteins regulates their function. In the polyubiquitin chains reported to date, ubiquitin monomers are linked via isopeptide bonds between an internal Lys and a C-terminal Gly. Here, we report that a protein complex consisting of two RING finger proteins, HOIL-1L and HOIP, exhibits ubiquitin polymerization activity by recognizing ubiquitin moieties of proteins. The polyubiquitin chain generated by the complex is not formed by Lys linkages, but by linkages between the C- and N-termini of ubiquitin, indicating that the ligase complex possesses a unique feature to assemble a novel head-to-tail linear polyubiquitin chain. Moreover, the complex regulates the stability of Ub-GFP (a GFP fusion protein with an N-terminal ubiquitin). The linear polyubiquitin chain generated post-translationally may function as a new modulator of proteins.

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Introduction

The ubiquitin system plays an important role in the regulation of a broad array of cellular functions, including cell cycle progression, DNA repair, signal transduction and membrane protein transport (Hershko and Ciechanover, 1998). Defects in the system have been shown to cause diseases such as cancer and neurodegenerative disorders (Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002). Ubiquitination is carried out by a cascade of reactions catalyzed by three classes of enzymes: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3). Ubiquitin is activated first by E1 and ATP to form a high-energy thioester intermediate with E1. The activated ubiquitin is then transferred from E1 to one of several E2s. Finally, E2 transfers the activated ubiquitin to a target protein recognized by an E3. In most cases, isopeptide bonds are formed between the ε-amino groups of an internal Lys (K) in the substrate protein and the carboxyl group of the ubiquitin C-terminus.

Conjugation of polyubiquitin to proteins plays a crucial role in cell functions, although monoubiquitination has recently been shown to have a signaling function in the endocytic pathway (Hicke and Dunn, 2003). In the polyubiquitin chains reported to date, ubiquitins are linked via isopeptide bonds between an internal Lys and the C-terminal Gly. The K48-linked polyubiquitin chain serves as a signal for proteasomal degradation (Chau *et al*, 1989), whereas the K63-linked polyubiquitin chain functions in signal transduction and DNA repair without functioning as a degradation signal (Hofmann and Pickart, 1999; Deng *et al*, 2000). Although most polyubiquitin chains are supposed to be assembled by repeated cycles of activity by E1, E2 and E3 (Hershko and Ciechanover, 1998), several models for polyubiquitin conjugation have been proposed recently (Hochstrasser, 2006).

We have reported that HOIL-1 is a RING finger ubiquitin ligase for heme-oxidized IRP2 (Yamanaka *et al*, 2003). HOIL-1 belongs to the RING-IBR (in between ring)-RING (RBR) protein family (Marin *et al*, 2004). In characterizing HOIL-1, we identified a longer form of HOIL-1, designated HOIL-1L, which is the predominant form of HOIL-1 and forms an ~600 kDa (600 K) complex with another RBR protein, HOIP (HOIL-1L Interacting Protein, see below for details). The ubiquitin associated (UBA) domain of HOIP binds directly to the ubiquitin like (UBL) domain of HOIL-1L, but not to ubiquitin, and the interaction is critical for stable complex formation. Moreover, the 600 K complex exhibits ubiquitin ligase activity *in vitro,* and elongates polyubiquitin chains via recognition of ubiquitin moieties conjugated to proteins. Interestingly, the polyubiquitin chains generated by the complex were not Lys-linked, but consisted of a head-to-tail linear polyubiquitin chain in which the C-terminal Gly of one ubiquitin monomer was conjugated to the N-terminal Met of the next ubiquitin monomer. Moreover, the ligase complex induced the proteasomal degradation of Ub-GFP (a GFP fusion protein with an N-terminal ubiquitin) in HeLa cells. To our knowledge, this is the first demonstration of a ubiquitin ligase that assembles linear polyubiquitin chains.

Results

HOIL-1L, the predominant isoform of HOIL-1, forms a large protein complex with HOIP

Two types of HOIL-1 transcript can be generated by alternative splicing (Tokunaga *et al*, 1998; Yamanaka *et al*, 2003)

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(Supplementary Figure 1A). Anti-HOIL-1 immunoblotting of several human cell lines revealed that the endogenous HOIL-1 migrated more slowly than exogenously expressed HOIL-1 (Supplementary Figure 1B). Since HOIL-1 is translated from the shorter transcript lacking exon-2, the endogenous HOIL-1 appeared to be derived from the longer transcript (Supplementary Figure 1A). RT–PCR analysis revealed that the longer mRNA was more abundant than the shorter, although both transcripts were expressed in all cell lines tested (Supplementary Figure 1C). Although the longer mRNA has been reported to encode RBCK1 (Tokunaga *et al*, 1998), our careful examination of the longer mRNA revealed that another AUG (shaded black) can be found 10 codons upstream of the initiation codon (underlined) of the previously reported RBCK1 (Supplementary Figure 1D). The HOIL-1L synthesized from the upstream AUG migrated to almost the same position as the endogenous HOIL-1 (Supplementary Figure 1B, compare lanes 5 with 1–3 and 6), implying that the majority of the endogenous HOIL-1 in human cells is HOIL-1L.

RING finger ubiquitin ligases are subdivided into monomers such as Cbl and multimers such as the SCF complex (Joazeiro and Weissman, 2000). We thus tested whether HOIL-1L exists as a monomer or a multimer in cells, using gel filtration analysis of S100 lysates from various human cell lines. In HeLa and SH-SY5Y cells, HOIL-1L was collected almost exclusively in fractions 4 and 5, which contain ~600 kDa proteins (Figure 1A), indicating that HOIL-1L exists primarily in a 600 kDa protein complex. The complex is likely to be hetero-multimeric, since overexpressed HOIL-1L fractionates into the ~200 kDa protein fraction (Figure 1F). Thus, we attempted to identify additional component(s) of the 600 K complex, as described in Figure 1B. Among the proteins that were co-purified with HOIL-1L (Figure 1C), one protein, designated HOIP (accession number: AB265810), was demonstrated to bind to HOIL-1L by co-immunoprecipitation (Figure 1D) and to form an ~600 kDa protein complex by the gel filtration assay (Figure 1E). When exogenously co-expressed in HeLa cells, both HOIP and HOIL-1L were found in fractions 3–6 (~600 kDa) of the gel filtration eluate (Figure 1G). HOIP and HOIL-1L also formed an ~600 kDa complex when co-expressed in baculovirus-infected insect cells or bacteria (Supplementary Figures 2A and B). As was observed with HOIL-1L, when expressed alone, HOIP was unable to form an ~600 kDa complex and appeared in fractions 7–8 (~350 kDa) (Figure 1H). These results indicate that HOIP is a component of the 600 K complex, and that HOIL-1L and HOIP are sufficient to form the complex. Since the molecular masses of HOIL-1L and HOIP are 58 and 123 kDa, respectively, it is likely that several molecules of each are present in each 600 K complex.

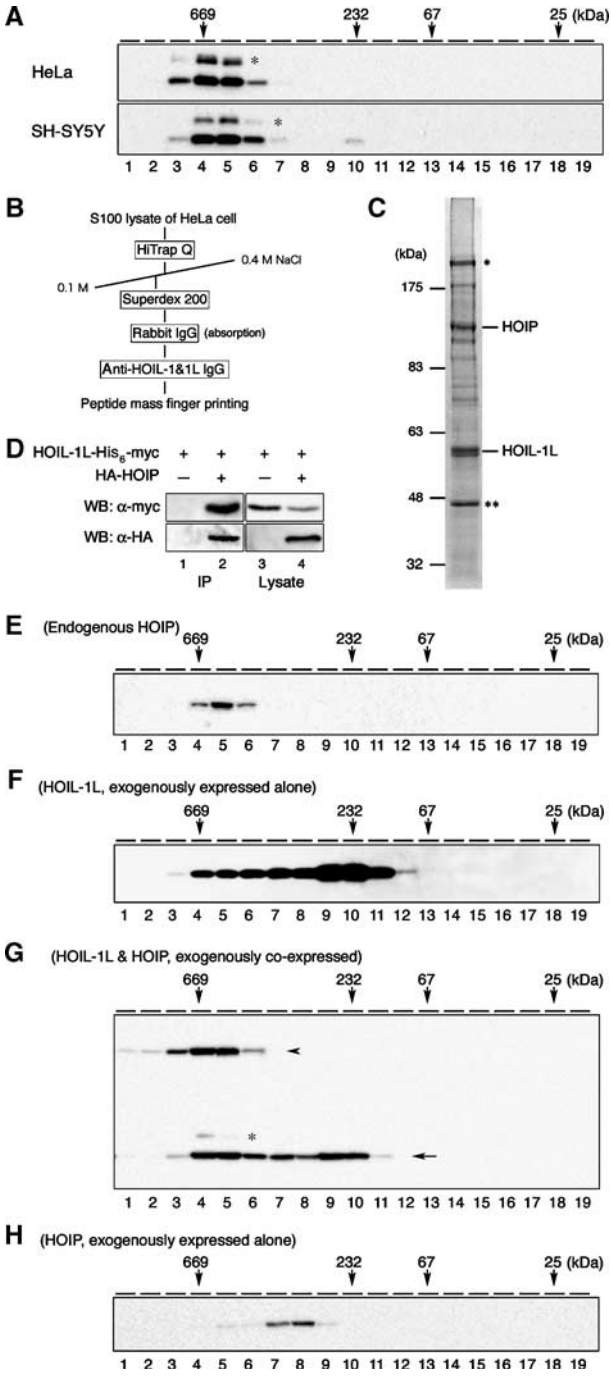

Figure 1 Identification of HOIP, a component of the 600 K complex. (**A**) HOIL-1L forms an ~600 kDa complex in HeLa and SH-SY5Y cells. Fractions from S100 lysates separated by Superdex 200 HR were subjected to SDS–PAGE, followed by immunoblotting with anti-HOIL-1. * indicates an unidentified modified form of HOIL-1L. (**B**) Procedure for isolation of the 600 K complex and identification of HOIP. Details are described in Materials and methods. (**C**) Coomassie staining of the proteins in the final fraction collected using the procedure outlined in (B). * represents a protein which crossreacted with the antibody used for purification and ** is actin. (**D**) HOIP binds to HOIL-1L. Anti-HA immunoprecipitates (IP) and cell lysates from U2OS cells expressing HOIL-1L-His_6-myc alone (lanes 1 and 3) or those of U2OS cells co-expressing HOIL-1L-His_6-myc and HA-HOIP (lanes 2 and 4) were separated by SDS–PAGE, followed by immunoblotting with rabbit anti-myc (α-myc) or mouse anti-HA (α-HA). (**E**) HOIP has a distribution similar to HOIL-1L. Fractionated S100 lysates of HeLa cells were probed with anti-HOIP. (**F**) HOIL-1L-His_6-myc forms the 600 K complex poorly when expressed alone in HeLa cells. Fractions from S100 lysates of HeLa cells expressing HOIL-1L-His_6-myc were probed by immunoblotting using anti-myc. (**G**) Co-expression of HA-HOIP (arrowhead) and HOIL-1L-His_6-myc (arrow) is sufficient for formation of the 600 K complex in HeLa cells. Fractionated S100 lysates of HeLa cells expressing both HOIL-1L-His_6-myc and HA-HOIP were probed with anti-HA or anti-myc. * indicates an unidentified modified form of HOIL-1L. (**H**) HA-HOIP forms the 600 K complex poorly when expressed alone in HeLa cells. Fractionated S100 lysates of HeLa cells expressing HA-HOIP were probed with anti-HA.

Database analysis revealed that the splice isoform and the mouse homolog of HOIP have been reported previously as Zibra and PAUL, respectively (Bromann *et al*, 2004; Thompson *et al*, 2004).

Specific interaction between the UBA of HOIP and the UBL of HOIL-1L is crucial for the 600 K complex formation

HOIP also belongs to the RBR family, a subgroup of the RING finger protein family (Marin *et al*, 2004) (Figure 2A). In addition to RBR domains, HOIP contains a UBA domain (Hicke *et al*, 2005) and three Ran-BP2-type zinc fingers (ZF) and HOIL-1L carries a UBL domain and a Ran-BP2 ZF. Two of the three ZFs of HOIP and the ZF of HOIL-1L are the Npl4-type ZF (NZF).

We probed the domains of HOIL-1L and HOIP involved in the formation of the 600 K complex by co-immunoprecipitation experiments. HOIL-1L and its mutants shown in Figure 2B were co-expressed with HOIP. The HOIL-1L mutant lacking the UBL did not co-immunoprecipitate with HOIP, although HOIL-1L, or its mutants lacking the NZF or RBR, did co-immunoprecipitate with HOIP. When HOIP or its mutants depicted in Figure 2C were co-expressed with HOIL-1L, the UBA-deleted HOIP failed to co-precipitate with HOIL-1L. UBA is a ubiquitin-binding motif (Bertolaet *et al*, 2001), and we therefore examined the binding spectrum of the HOIP UBA domain using a Ni^{2+}-agarose pull down assay with various recombinant proteins. The UBA of HOIP could bind to the UBL of HOIL-1L, but not to ubiquitin (Figure 2D). These results indicate that the UBA domain of HOIP interacts directly with the UBL domain of HOIL-1L and the specific binding between these two domains is critical for the 600 K complex formation.

The 600 K complex assembles polyubiquitin chains

NZF has been reported to function as a ubiquitin-binding motif (Meyer *et al*, 2002; Kanayama *et al*, 2004; Hicke *et al*, 2005). Thus, the 600 K complex might exhibit ubiquitin-binding as well as E2-binding activities (the RING finger motif is an E2-binding motif), which prompted us to hypothesize that the 600 K complex exhibits ubiquitin conjugation activity towards ubiquitinated proteins through ubiquitin recognition. We examined this possibility with an *in vitro* ubiquitination assay using Ub-GST (GST fused to the C-terminus of an uncleavable form of ubiquitin) as a model substrate. Ub-GST, but not GST, was ubiquitinated in the presence of E1, E2 and the baculovirus-prepared 600 K complex (Figure 3A). The complex also ubiquitinated Ub-GFP (GFP with an N-terminal ubiquitin), but not GFP (Figure 3B). During this analysis, in addition to ubiquitination of the substrates, we noticed, by Coomassie staining, that the ladder-shaped signals included bands that migrated more

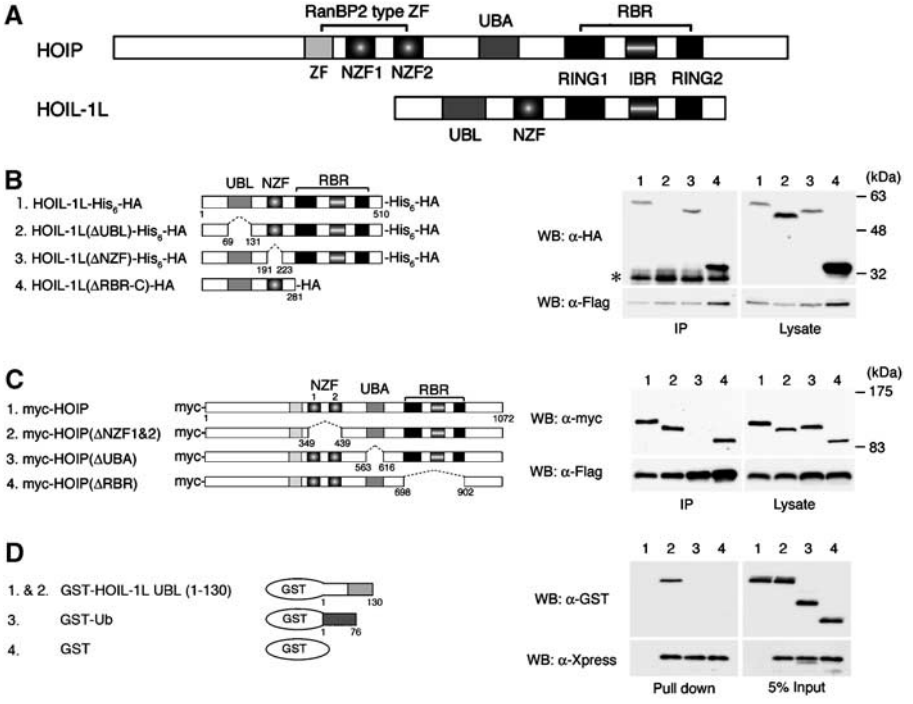

Figure 2 Specific interaction between the UBA of HOIP and the UBL of HOIL-1L is crucial for the 600 K complex formation. (**A**) Schematic representation of HOIP and HOIL-1L. ZF, zinc-finger domain; NZF, Npl4 type ZF domain; UBA, ubiquitin associated domain; UBL, ubiquitin like domain; IBR, in between ring domain; and RBR, RING-IBR-RING domain. (**B**) The UBL of HOIL-1L is required for the binding between HOIL-1L and HOIP. Anti-Flag immunoprecipitates (IP) and cell lysates from U2OS cells co-expressing Flag-HOIP and C-terminally HA-tagged HOIL-1L (1), or co-expressing Flag-HOIP and the HA-tagged HOIL-1L domain-deleted mutants (2, 3 or 4) were separated by SDS–PAGE and subjected to immunoblotting with anti-HA (α-HA) or anti-Flag (α-Flag). * represents a nonspecific signal. (**C**) The UBA of HOIP is required for binding HOIL-1L. Anti-Flag immunoprecipitates (IP) and cell lysates from U2OS cells co-expressing HOIL-1L-His_6-Flag and myc-HOIP (1) or co-expressing HOIL-1L-His_6-Flag and myc-HOIP domain-deleted mutants (2, 3 or 4) were separated by SDS–PAGE, followed by immunoblotting with anti-myc (α-myc) or anti-Flag (α-Flag). (**D**) The UBA of HOIP directly recognizes the UBL of HOIL-1L. Mixtures of bacterially purified His_6-Xpress-tagged HOIP UBA (aa 556–636) and GST-HOIL-1L UBL (aa 1–130) (2), GST-ubiquitin (3), GST (4), and GST-HOIL-1L UBL (aa 1–130) alone (1) were incubated with Ni^{2+}-affinity gel. Proteins bound to the Ni^{2+}-affinity gel and 5% input samples were separated by SDS–PAGE and subjected to immunoblotting with anti-GST (α-GST) or anti-Xpress (α-Xpress).

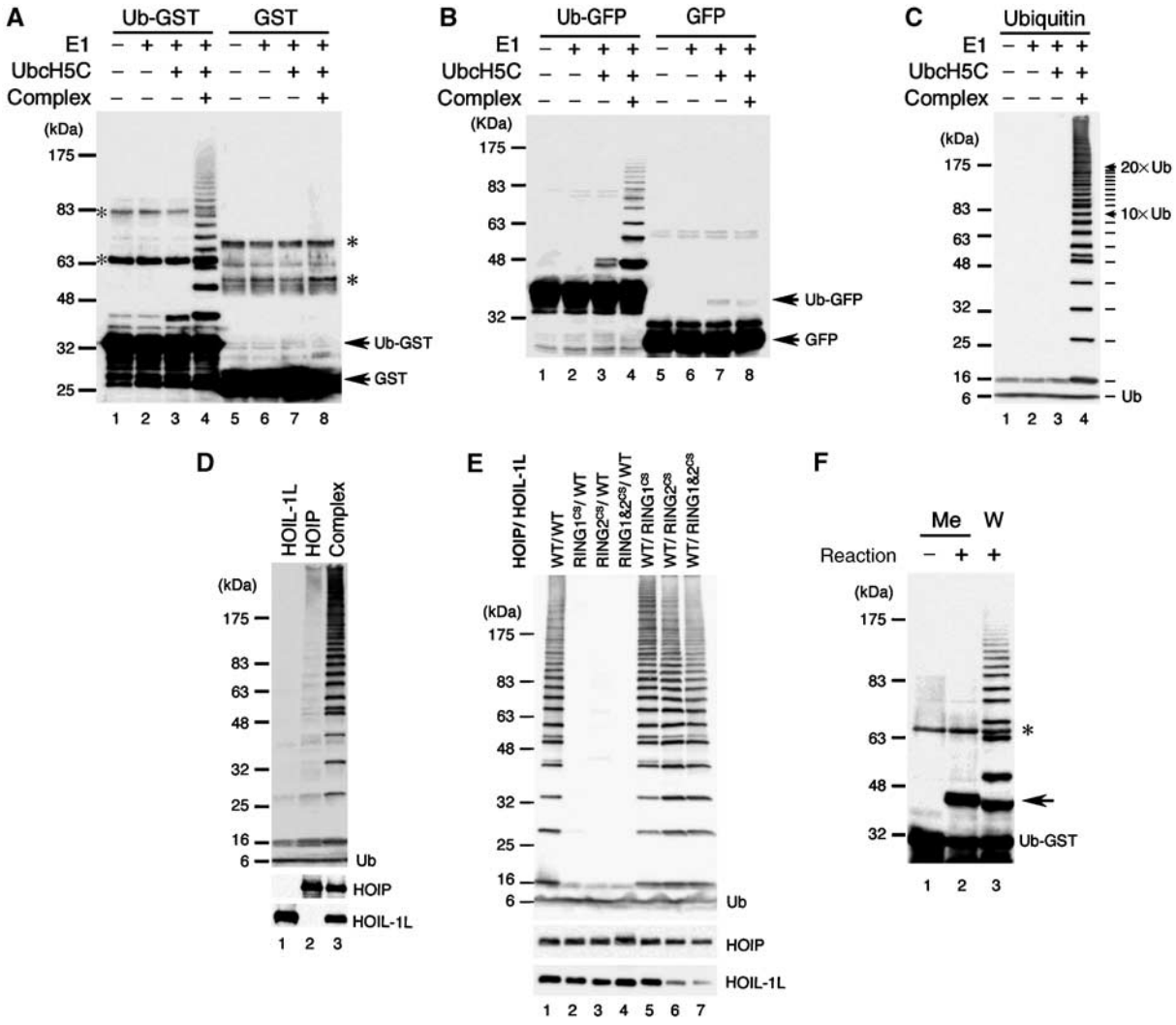

Figure 3 The 600 K complex assembles polyubiquitin chains *in vitro*. (**A**) The 600 K complex from insect cells (Complex) ubiquitinates Ub-GST. Ub-GST or GST were incubated with ubiquitin and ATP, together with the indicated components at 37°C for 2 h, followed by immunoblotting with anti-GST. Asterisks indicate nonspecific signals. (**B**) The 600 K complex ubiquitinates Ub-GFP. Ub-GFP or GFP were incubated as described in (A), followed by immunoblotting with anti-GFP. (**C**) The 600 K complex polymerizes ubiquitin. Ubiquitin was incubated, as indicated, together with ATP at 37°C for 2 h, followed by immunoblotting with anti-ubiquitin. (**D**) The 600 K complex exhibits much greater ubiquitination activity than either HOIL-1L or HOIP alone. Ubiquitin was incubated as in (C) together with HOIL-1L (lane 1), HOIP (lane 2) or the 600 K complex (lane 3), followed (by immunoblotting with) anti-ubiquitin, anti-HOIP or anti-HOIL-1. (**E**) RING finger domains of HOIP but not those of HOIL-1L are critical for the ubiquitin conjugation activity of the 600 K complex. Ubiquitin was incubated as in (C) in the presence of the WT 600 K complex, or the mutant 600 K complex containing a RING finger mutant of either HOIL-1L or HOIP, as indicated, at 37°C for 2 h, followed by immunoblotting as described in (D). (**F**) The 600 K complex conjugates only one methylated ubiquitin to Ub-GST. Ub-GST was incubated with methylated ubiquitin (Me) at 4°C (lane 1) or 37°C (lane 2), or with WT ubiquitin (W) at 37°C (lane 3) in the presence of E1, UbcH5C, ATP and the 600 K complex from insect cells, followed by immunoblotting as described in (A). The arrow and * indicate mono-ubiquitinated Ub-GST and a nonspecific signal, respectively.

rapidly than the ubiquitin fusion proteins, suggesting that other protein(s) were also ubiquitinated. An *in vitro* ubiquitination assay revealed that E1, E2, ubiquitin and the 600 K complex were sufficient to generate the ladder-shaped ubiquitination signal (Figure 3C). These results strongly indicated that the 600 K complex recognizes not only the ubiquitin moiety of the ubiquitin fusion proteins but also ubiquitin itself, and conjugates additional ubiquitins to these moieties. HOIL-1L or HOIP exhibited much weaker ubiquitination activity towards ubiquitin than did the complex (Figure 3D). The 600 K complex prepared from bacteria also exhibited ubiquitination activity (Supplementary Figure 2C). These results demonstrated clearly that HOIL-1L and HOIP are necessary and sufficient for the ubiquitin conjugation activity of the complex.

Replacement of the first two consensus cysteines in the two RING finger domains of HOIP with serines diminished ubiquitin conjugation activity, whereas the same mutations in the RING fingers of HOIL-1L had little effect on ubiquitin conjugation (Figures 3E), implying that both RING fingers of HOIP but not those of HOIL-1L function as E2-binding sites for this ubiquitin conjugation activity.

Methylated ubiquitin cannot form polyubiquitin chains because methylation of ubiquitin blocks all the acceptor sites. In an *in vitro* ubiquitination assay using methylated ubiquitin, Ub-GST was mono-ubiquitinated, but could not be

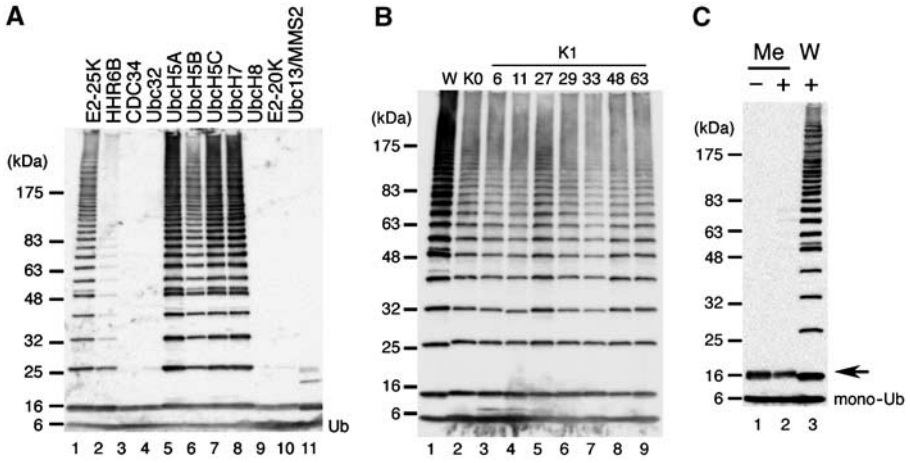

Figure 4 The 600 K complex assembled the N-terminal Met-linked polyubiquitin chain with a broad spectrum of E2s. (**A**) E2-25K, HHR6B, UbcH5s and UbcH7 could function as E2s for the 600 K complex to generate polyubiquitin chains. Ubiquitin was incubated with the indicated E2s in the presence of E1, the 600 K complex and ATP, followed by immunoblotting with anti-ubiquitin. (**B**) The 600 K complex polymerized Lys-less and K1 ubiquitin. WT (lane 1), the Lys-less (K0, lane 2) and the K1 ubiquitin mutants (six out of seven Lys are mutated to arginines and the number represents the remaining Lys residue) (lanes 3–9) were incubated with E1, UbcH5C, the 600 K complex and ATP, followed by immunoblotting as described in (A). (**C**) The 600 K complex failed to polymerize methylated ubiquitin. Methylated (Me) and WT ubiquitin (W) were incubated as in (B) at 4°C (lane 1) or 37°C (lanes 2 and 3), followed by immunoblotting as in (A). The arrow represents possible methylated di-ubiquitin contamination in the methylated ubiquitin preparation.

multiubiquitinated (Figure 3F), indicating that the 600 K complex generates polyubiquitin chains, but does not mono-ubiquitinate the substrate at multiple sites (multiubiquitination). Collectively, these results indicate that the 600 K complex recognizes ubiquitin moieties, and exhibits polyubiquitin chain elongation activity.

The 600 K complex assembles linear polyubiquitin chains

The 600 K complex could generate polyubiquitin chains, as detected by the laddered-signal on gels, in conjunction with a broad spectrum of E2s, such as E2-25K, HHR6B, UbcH5s and UbcH7 (Figure 4A), suggesting that the 600 K complex but not the E2s determine the type of linkage in the polyubiquitin chain. To probe the linkage of polyubiquitins generated by the 600 K complex, *in vitro* ubiquitination assays were performed in the presence of ubiquitin mutants, which possess either no Lys (K0) or one Lys (K1) (Figure 4B). The 600 K complex generated polyubiquitin chains from either ubiquitin mutant, but the complex failed to polymerize methylated ubiquitin (Figure 4C). Since methylation of ubiquitin blocks all the acceptor sites of ubiquitin including Lys and the α-amino group of the N-terminal Met, these results indicate that the 600 K complex conjugates ubiquitin onto the α-amino group of the N-terminus of ubiquitin.

To confirm that the 600 K complex catalyzes head-to-tail polyubiquitin conjugation, the linkage of polyubiquitin chains generated by the 600 K complex and UbcH5C *in vitro* (2–8 × ubiquitin and a mixture of higher polymer ubiquitin chains (polyUb)) (Figure 5A, lane 4) were analyzed using a method combining trypsin digestion and mass spectrometry (Peng *et al*, 2003; Saeki *et al*, 2004b). Although signals of the peptide masses expected from Lys-linked polyubiquitin chains could barely be detected (the calculated masses are given in Table I), a strong signal was detected for the polymerized ubiquitins, but not mono-ubiquitin (Figure 5B and Supplementary Figure 3). The peptide fragment mass of M1 ($m/z^+ = 879.48$–879.58) coincided with the sequence GG-MQIFVK, which is generated specifically from linkage between the α-amino group of the N-terminal Met of one ubiquitin and the carboxyl group of the C-terminal Gly of another (Table I, M1). The amino-acid sequence of the peptide was confirmed to be GG-MQIFVK, using MS/MS analysis (Figure 5C). The same results were obtained from the polyubiquitin chains generated by the 600 K complex with UbcH5A or UbcH7 (data not shown). These results clearly demonstrate that the 600 K complex assembles N-terminally linked linear polyubiquitin chains.

E2-25K can generate K48-linked polyubiquitin chains without E3 (Chen and Pickart, 1990). We confirmed that E2-25K could assemble polyubiquitin chains in the absence of E3, although at much lower efficiency (Figure 5A, lanes 1, 2 and 6), and the polymer was indeed a K48-linked chain (Figure 5D). However, mass spectrometric analysis proved that, in addition to the K48 chain, a linear ubiquitin chain was generated in the presence of the 600 K complex and E2-25K (Figure 5E), indicating that the 600 K complex can catalyze linear ubiquitin conjugation, even with E2-25K. Although the signal of the M1 peptide was not as strong as that of the K48 peptide in our mass spectrometry analysis, it is likely that the 600 K complex primarily catalyzes linear polyubiquitination with E2-25K, because the ubiquitin ladder generated by the complex and E2-25K was similar to that of the linear polyubiquitin chain rather than that of the K48-linked chain (Figure 5A).

The 600 K complex exhibited ubiquitin conjugation activity towards Ub-GST, but not towards GST-Ub (GST with unconjugatable ubiquitin fused to the C-terminus) although the complex could bind to both proteins (Supplement Figures 4A and B). Together, these results clearly indicate that the 600 K complex almost exclusively conjugates ubiquitin to the N-terminal Met residue of ubiquitin. Considering the nature of the 600 K complex, we have named the complex LUBAC (linear ubiquitin chain assembly complex).

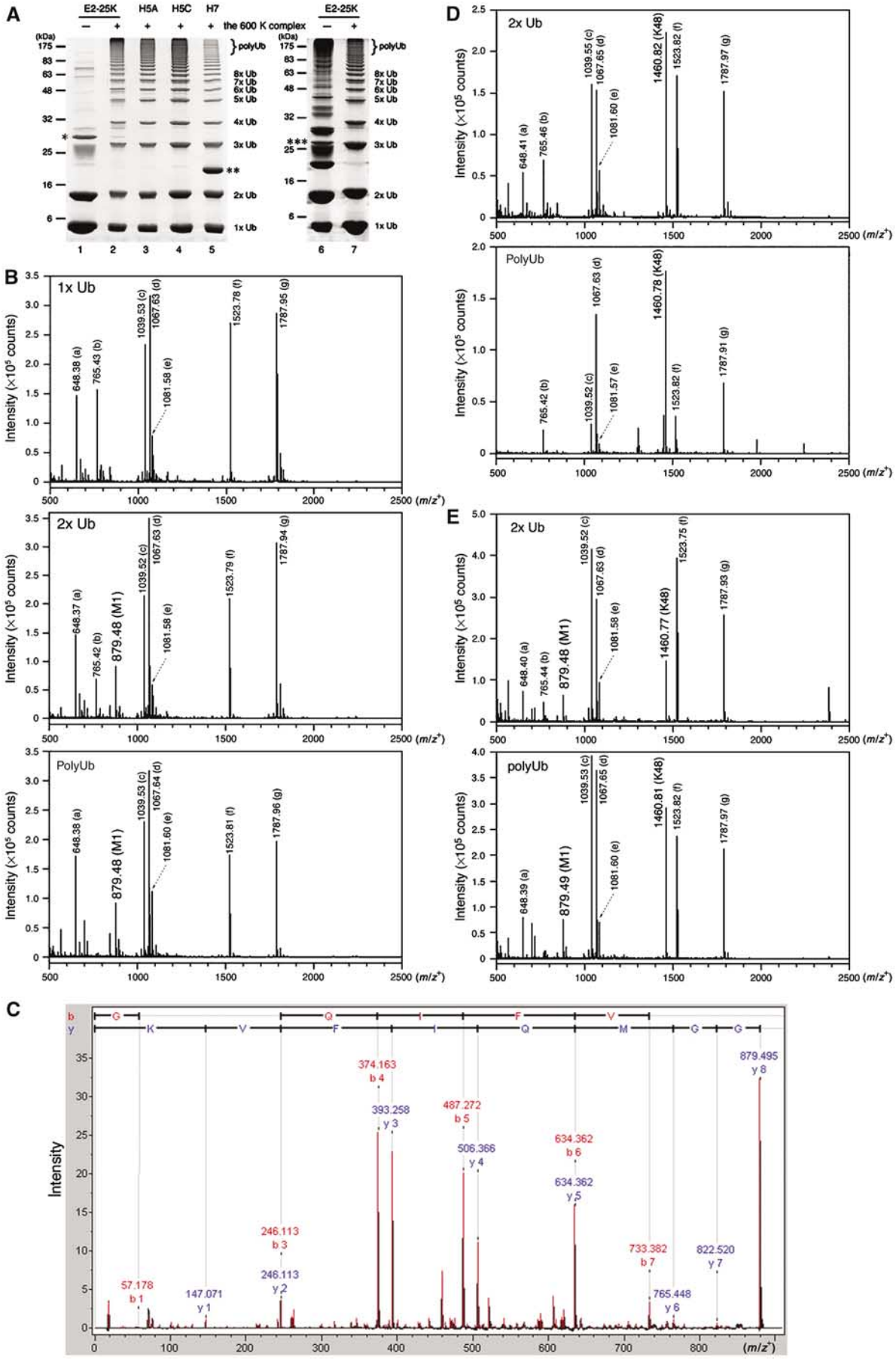

Table I Masses of the specific peptides generated from linear and Lys-linked polyubiquitin chains using tryptic digestion

Chain type	Mass of the specific peptide $[M+H]^+$
K6	1379.7715
K11	2402.2660
K27	2101.1022
K29	816.4574
K33	1637.8241
K48	1460.7855
K63	2244.1982
M1	879.4757

LUBAC destabilizes Ub-GFP in HeLa cells

We have shown that Ub-GFP is effectively polyubiquitinated by LUBAC *in vitro* (Figure 3B). The effect of LUBAC on the amount of the protein in HeLa cells was analyzed to probe the physiological role of linear polyubiquitin chains. As shown in Figure 6A, the amount of Ub-GFP decreased in the presence of HOIL-1L and HOIP (LUBAC-WT), but introduction of HOIL-1L and HOIP RING1&2^{CS} (LUBAC-CS), which are unable to generate linear polyubiquitin chains, increased Ub-GFP levels. The amount of Ub-GFP increased in control or LUBAC-WT transfected cells when treated with MG132, an inhibitor of the proteasome (Figure 6B). These results suggest that LUBAC enhances the proteasomal degradation of Ub-GFP.

Ub-GFP is a short-lived protein because of its degradation by the ubiqutin-fusion degradation (UFD) pathway (Dantuma *et al*, 2000). In this pathway, the N-terminal ubiquitin moiety of a substrate is ubiquitinated on K29 and K48 of the N-terminal ubiquitin moiety by reactions catalyzed by UFD4 and UFD2 ligases, which targets the substrate for degradation by the proteasome (Johnson *et al*, 1995). To examine whether or not linear polyubiquitination is indeed critical for LUBAC mediated-degradation of Ub-GFP, the effect of LUBAC on the stability of K0-Ub-GFP, in which all the Lys residues including K29 and K48 of the N-terminal ubiquitin moiety of the protein were replaced by Arg, was examined. K0-Ub-GFP decreased in the presence of LUBAC-WT, although the efficiency was reduced compared to Ub-GFP, whereas K0-Ub-GFP increased in the presence of LUBAC-CS (Figure 6C). Treatment with MG132 increased the amount of K0-Ub-GFP protein in cells co-expressing LUBAC-WT (Figure 6D). Knockdown of HOIP by siRNA increased expression of both Ub-GFP and K0-Ub-GFP, but not GFP, indicating that the N-terminal ubiquitin moiety of Ub-GFP is crucial for LUBAC-induced degradation of the protein (Figure 6E). Collectively, these results clearly indicate that the linear polyubiquitination activity is critical for the degradation of Ub-GFP in HeLa cells.

Discussion

Our results revealed that the predominant form of HOIL-1, HOIL-1L, interacts with HOIP to form LUBAC. However, Tatematsu *et al* (2005) recently reported that RBCK1 cycles between the nucleus and cytoplasm, exhibiting transcriptional activity and Bayle *et al* (2006) reported that HOIL-1 induced ubiquitin-mediated degradation of SOCS6-associated proteins. Some HOIL-1L and a faster migrating band were found in the ~200 kDa protein fraction in pVHL-deficient UOK111 cells (Supplementary Figure 1E). Kidney cancer cell lines lacking pVHL, a renal cell tumor suppressor gene product which is a substrate recognition subunit of a ubiquitin ligase (Kaelin, 2005), have been shown to express HOIL-1/HOIL-1L mRNA more abundantly than pVHL expressing cells (Staller *et al*, 2003). Thus, HOIL-1 and/or HOIL-1L may possess a specific function, which plays a role in VHL tumorigenesis. However, since HOIL-1L exclusively forms LUBAC in most cell lines (Figure 1A), it seems likely that HOIL-1L becomes physiologically active when complexed with HOIP.

We showed that the UBA of HOIP specifically interacts with the UBL of HOIL-1L, but not ubiquitin, and that the interaction between the two domains was crucial for stable LUBAC complex formation (Figure 2). Most UBA domains have been shown to interact directly with ubiquitin (Bertolaet *et al*, 2001); additionally, some have been reported to bind to ubiquitin-like proteins (Dieckmann *et al*, 1998; Tanaka *et al*, 2003). UBL domains have been implicated in binding to the proteasome (Elsasser and Finley, 2005). The roles of the UBL and UBA domains are best characterized in UBL-UBA proteins such as Rad23. These proteins are hypothesized to function as ubiquitin receptors, transferring ubiquitinated substrates to the proteasome (Wilkinson *et al*, 2001), and suggest that binding of UBL-UBA proteins to ubiquitinated proteins or the proteasome is transient (Elsasser and Finley, 2005). However, the interaction between the UBA of HOIP and the UBL of HOIL-1L appears essential for stable complex formation (Figures 3). Upadhya and Hegde (2003) reported that the UBL of HOIL-1L is structurally distinct from UBLs harboring the proteasome interacting motif. This unique

Figure 5 Mass spectrometric analyses of the N-terminally-linked head-to-tail polyubiquitin chains generated by the 600 K complex. (**A**) Coomassie staining of polyubiquitin chains. Ubiquitin was incubated with E1, ATP, the indicated E2s in the presence (lanes 2–5) or absence (lane 1) of the 600 K complex at 37°C for 2 h. Ubiquitin was also incubated with E1, ATP, non-tagged E2-25K in the absence of the 600 K complex at 37°C for 12 h (lane 6) or in the presence of the 600 K complex at 37°C for 2 h (lane 7). The reactions were stopped by heating at 60°C for 15 min, followed by centrifugation at 20 000 *g* for 20 min. The resulting supernatants were separated by SDS–PAGE and stained with Coomassie. *, ** and *** indicate His_6-E2-25K, His_6-UbcH7 and non-tagged E2-25K, respectively. (**B**) Mass spectrometry analysis of the polyubiquitin chains generated by the 600 K complex and UbcH5C. 1–2 × Ub and the polyubiquitin chain mixtures (polyUb) generated by UbcH5C and the 600 K complex as depicted in (A, lane 4) were digested with trypsin in the gels at 37°C for 12 h, followed by MALDI-TOF/MS. (M1) indicates the fragment specifically generated from the N-terminally linked ubiquitin chain. (a–g) represent the ubiquitin fragments of amino acids 43–48, 1–6, 34–42, 64–72, 55–63, 30–42 and 12–27, respectively. (**C**) MS/MS spectrum acquired from the M1 peptide ($m/z^+ = 879.48$–879.58). The spectrum was obtained by the Lift analysis according to the manufacturer's instructions using ultraflex TOF/TOF and interpreted by the Mascot Search Program. The amino-acid sequences from the C- and N-terminus (the starting point is on the left) were shown on the top based on y_m and b_i (where m and l denote positions counted from C- and N-terminus) that were produced by cleavage of peptide bonds during MS/MS. (**D, E**) Mass spectrometry analysis of polyubiquitin chains generated by E2-25K in the absence (D) or presence (E) of the 600 K complex. 2 × Ub and polyUb generated by E2-25K in the presence or absence of the 600 K complex depicted in (A, lanes 6 and 7) were subjected to MALDI-TOF/MS as described in (B). (K48) indicates the fragment specifically generated from the K48-linked ubiquitin chain. (M1) and (a–g) are the same as (B).

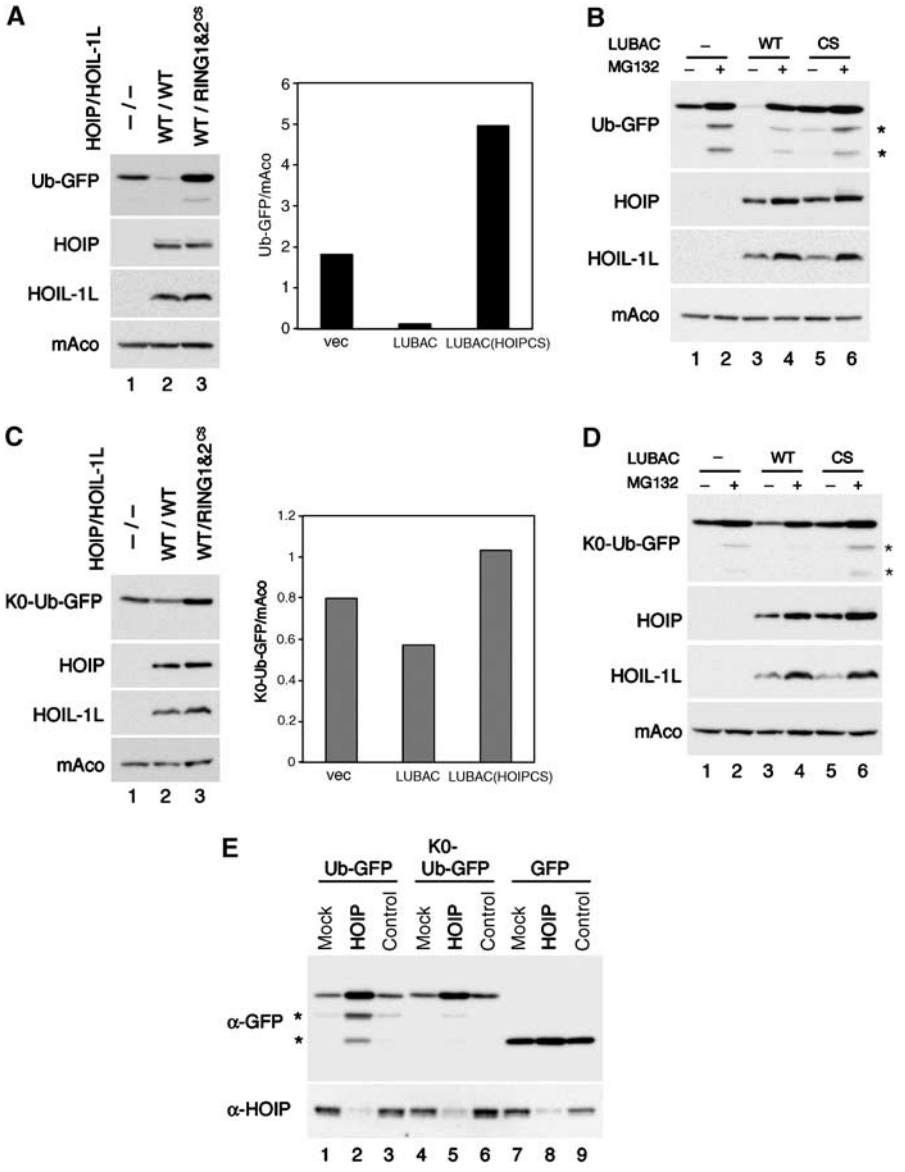

Figure 6 *In vivo* roles of LUBAC. (**A**) LUBAC induces the decrease of Ub-GFP. HeLa cells were co-transfected with expression plasmids encoding Ub-GFP together with mitochondrial aconitase-myc (mAco: transfection control), HOIL-1L-WT and HOIP-WT or HOIP-RING1&2^{CS} as indicated. Total cell lysates were probed with anti-GFP (Ub-GFP), anti-FLAG (HOIP) anti-HA (HOIL-1L) or anti-myc (mAco). Relative amounts of Ub-GFP shown in the bar graph were quantified by LAS3000. (**B**) LUBAC enhances the proteasomal degradation of Ub-GFP. HeLa cells were co-transfected with expression plasmids encoding Ub-GFP and mAco together with control plasmids (vec: lanes 1 and 2), HOIL-1L-WT and HOIP-WT (WT: lanes 3 and 4) or HOIL-1L-WT and HOIP-RING1&2^{CS} (CS: lanes 5 and 6). MG132 was added for 8 h before harvesting cells (lanes 2, 4 and 6). Total cell lysates were probed as in (A). (**C**) LUBAC induces a decrease in K0-Ub-GFP. HeLa cells were co-transfected as indicated in (A), with the exception that the expression plasmid for K0-Ub-GFP was transfected instead of the Ub-GFP plasmid. Total cell lysates were probed as in (A). (**D**) LUBAC enhances the proteasomal degradation of K0-Ub-GFP. HeLa cells were co-transfected as indicated in (B) except that the expression plasmid for K0-Ub-GFP was transfected instead of the Ub-GFP plasmid. Total cell lysates were probed as in (A). (**E**) Knockdown of HOIP increased the amount of Ub-GFP and K0-Ub-GFP. HeLa cells were transfected with si-RNA for HOIP (lane 2, 5 and 8), scrambled contol siRNA (lanes 3, 6 and 9) or mock (lanes 1, 4 and 7) followed by transfection of expression plasmids encoding Ub-GFP (lanes 1–3), K0-Ub-GFP (lanes 4–6) or GFP (lanes 7–9). Total cell lysates were probed with anti-GFP (Ub-GFP, K0-Ub-GFP or GFP) or anti-HOIP. Asterisks indicate possible degradation products of (K0-)Ub-GFP.

feature of the UBL of HOIL-1L may enable the stable interaction between HOIL-1L and HOIP. Raasi *et al* (2005) proposed that UBA domains can be sorted into four groups. The UBA of HOIP may belong to one of the groups that cannot bind to ubiquitin, and, therefore, may instead bind specifically to the UBL of HOIL-1L.

The most interesting feature of LUBAC is that it conjugates ubiquitin to the α-amino group of the N-terminal Met of ubiquitin by recognizing the ubiquitin moieties of the substrates. At first, we hypothesized that the NZFs in LUBAC might function as substrate recognition motifs for linear polyubiquitin formation. However, not only NZFs but also ZFs of the 600 K complex seemed dispensable for the recognition of ubiquitin moieties because the ubiquitin polymerizing activity of LUBAC was not affected by deletion of all NZFs (Supplementary Figure 5A) or all ZFs (data not shown). These observations imply that LUBAC possesses another ubiquitin binding domain that recognizes ubiquitin moieties

for conjugation. At least one of the LUBAC NZFs is likely to function as a ubiquitin-binding motif because deletion of all NZFs from LUBAC drastically reduced its ability to bind to ubiquitinated proteins (Supplementary Figure 5B). Therefore, although we could not completely rule out the possibility that NZFs might be involved in linear ubiquitin conjugation, NZFs of LUBAC seem to play a role distinct from that of target recognition, possibly protection of the linear polyubiquitin chains from processing by deubiquitinating enzymes.

The N-terminal α-amino group of substrate proteins has been shown to function as an acceptor site for ubiquitin (N-terminal ubiquitination) (Breitschopf *et al*, 1998; Bloom *et al*, 2003; Ben-Saadon *et al*, 2004), and, although not proven directly, AIP4 and WWP2 have been proposed as E3s for the N-terminal ubiquitination of EBV LMP2A (Ikeda *et al*, 2002). Here, we provide clear evidence that LUBAC can transfer ubiquitin to the α-amino group of the N-terminal Met of a protein, ubiquitin itself. To our knowledge, this is the first report of a ligase complex that specifically generates a head-to-tail linear polyubiquitin chain, although N-terminal Met-linked ubiquitination has been suggested to be catalyzed by a mutant UBC1, a yeast homolog of E2-25K (Hodgins *et al*, 1996). The residue ubiquitinated by LUBAC seems to be strictly restricted to the N-terminal Met of ubiquitin because (i) LUBAC could bind to GST-Ub, but failed to ubiquitinate the protein (Supplemental Figures 4A and B) and (ii) LUBAC is able to assemble the head-to-tail polyubiquitin chain using a broad spectrum of E2s (Figure 4A). These data suggest that the position of the N-terminal Met is important for polyubiquitination by LUBAC. Since LUBAC recognizes the ubiquitin moieties of substrates, the spatial distance between the amino-acid residues to be conjugated with ubiquitin and E2 may be constant during polyubiquitin chain elongation. Namely, it is possible that LUBAC recognizes the distal ubiquitin moiety of ubiquitinated proteins, and conjugates ubiquitin to the tip of the polyubiquitin tree. Identification of critical domain(s) of LUBAC for the recognition of ubiquitin moieties for linear polyubiquitination is needed to understand the mechanism of polyubiquitin chain elongation by LUBAC.

Our present observations provide insight into the function of the linear polyubiquitin chain. We have shown that LUBAC enhances degradation of K0-Ub-GFP as well as Ub-GFP, but not GFP in HeLa cells (Figure 6). In *in vitro* experiments, LUBAC also conjugated linear polyubiquitin chains onto heme-loaded IRP2 (Supplementary Figure 6), which has been shown to be ubiquitinated by HOIL-1 leading to the degradation of the protein (Yamanaka *et al*, 2003). These data indicate that LUBAC can conjugate the linear polyubiquitin chain onto an authentic substrate. Moreover, Saeki *et al* (2004a) demonstrated that uncleavable linear polyubiquitin chains bind to the 26S proteasome. Thus, the linear polyubiquitin chains assembled by LUBAC may function as a degradation signal. Ub-GFP is known to be a substrate of the UFD pathway. Thus, we cannot rule out the possibility that ubiquitin conjugated N-terminally by LUBAC provides acceptor Lys residues for ubiquitnation by the UFD pathway, which has been shown to be mediated by ubiquitination of K29 and K48 of the N-terminal ubiquitin moiety of UFD substrates (Johnson *et al*, 1995).

Linear polyubiquitin genes exist in the genome; however, translated linear polyubiquitins appear to be cleaved into ubiquitin monomers co-translationally by deubiquitinating enzymes (Turner and Varshavsky, 2000). We suspect that translated 'free' linear polyubiquitin chains must be cleaved to avoid competition between 'free' and conjugated linear polyubiquitin chains. Further analyses will be needed to clarify the importance and precise roles of the head-to-tail polyubiquitin chains.

Materials and methods

Plasmid construction and RT–PCR

Open reading frames (ORFs) encoding HOIL-1L, HOIP and mAco were amplified by RT–PCR from HeLa cell mRNA. Mutants of the proteins were constructed using two-step PCR. Two cysteine residues in each RING finger were substituted with serines to generate the RING finger mutants of HOIL-1L and HOIP. The mutants generated were, HOIL-1L RING1^{CS} (C282,285S), HOIL-1L RING2^{CS} (C447,450S), HOIL-1L RING1&2^{CS} (C282,285S, C447,450S), HOIP RING1^{CS} (C699,702S), HOIP RING2^{CS} (C871,874S) and HOIP RING1&2^{CS} (C699,702S, C871,874S). The wild-type (WT) and the mutant ORFs, linked to the appropriate tags, were cloned into pcDNA3.1, pVL1393 (Invitrogen), pTRCHis A (Invitrogen), pGEX-2T (Amersham) or pT7-7 (Tabor and Richardson, 1985). ORFs encoding mAco, GFP, Ub-GFP and K0-Ub-GFP were cloned into pcDNA 3.1. Ub-GST, GST-Ub, Ub-GFP-His$_6$ and GFP-His$_6$ were cloned into pT7-7 for bacterial expression. pT7-7-His$_6$-E2s have been described previously (Iwai *et al*, 1999). Both ORFs of His$_6$-HOIP and HOIL-1L were cloned into pETDuet-1 (Novagen) for bacterial expression of LUBAC. DNA fragments encoding the C-terminal 61 amino acids of HOIL-1L, the N-terminal 349 amino acids of HOIP or the C-terminal 146 amino acids of HOIP were cloned into pGEX-2T to generate GST-HOIL-1L-C, GST-HOIP-N or GST-HOIP-C, respectively, as antigens for antibody production. ORFs of UbcH8, HHR6B, Ubc13 and MMS2 were amplified by RT–PCR. The ORFs encoding UbcH8 and HHR6B were cloned into pT7-7-His$_6$. His$_6$-Ubc13 and MMS2 (Ubc13/MMS2) were connected via a ribosome loading sequence, and cloned into pT7-7.

Antibodies

Mouse monoclonal antibodies recognizing HOIL-1 and HOIL-1L (2E2) or HOIP (2NE12) were generated by three intra-peritoneal immunizations of BALB/c mice using GST-HOIL-1L-C or GST-HOIP-N, respectively, followed by fusion of the spleen cells with X63.Ag8.653 cells. Rabbit anti-HOIP antiserum was raised against GST-HOIP-C. Anti-ubiquitin antibodies were a gift from Dr Aaron Ciechanover. Anti-Flag (mouse (M2) and rabbit), anti-HA (16B12) and anti-Xpress antibodies were purchased from Sigma, Covance and Invitrogen, respectively. Anti-myc (mouse (9E10) and rabbit), anti-GST, anti-GFP and rabbit anti-HA antibodies were purchased from Santa Cruz Biotechnology.

Cell culture, transfection and immunoprecipitation

HeLa, SH-SY5Y, U2OS and UOK111 cells were cultured in DMEM plus 10% fetal bovine serum, 100 IU/ml penicillin G and 100 μg/ml streptomycin. Transient transfection was performed using FuGENE6 (Roche). siRNA transfection was performed using Lipofectamine RNAi MAX (Invitrogen). The nucleotide sequences of double-stranded stealth-siRNAs for HOIP and its scrambled control RNA (Invitrogen) were CCAGGCACACUACAAAGAGUAUCUU for HOIP and CCACACUCAACAGAAUGAUAGGCUU for scrambled control RNA. Cells were lysed 36–48 h after transfection of expression plasmids using 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 containing 2 mM PMSF or SDS sample buffer. For immunoprecipitation, Triton X-100 lysates were incubated with the appropriate antibodies for 2 h on ice, followed by precipitation with protein A Sepharose (Amersham).

Immunoblotting

Samples were separated by SDS–PAGE and transferred to PVDF membranes. After blocking in PBS containing 0.1% Tween-20 and 5% skim-milk, the membrane was incubated with the appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham). The

membrane was visualized using enhanced chemiluminescence and analyzed with LAS3000 (Fujifilm).

Gel filtration

Cells were lysed in a lysis buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and a protease inhibitor cocktail (Complete EDTA-free, Roche), by passing through polycarbonate filters (Whatman) (Kihara *et al*, 2001). After adding an equal volume of lysis buffer containing 300 mM NaCl, lysates were centrifuged at 100 000 *g* for 30 min to obtain S100 lysates. S100 lysates were injected onto a Superdex 200 HR (10/30) column (Amersham) and fractionated in 50 mM Tris–HCl pH 7.5, containing 150 mM NaCl using an ÄKTA chromatography system (Amersham).

Isolation of LUBAC

HeLa S100 lysates were separated using a HiTrap Q anion exchange column (Amersham) with a 0.1–0.4 M NaCl linear gradient in 50 mM Tris–HCl, pH 7.2. The HOIL-1L enriched fractions, confirmed by immunoblotting with anti-HOIL-1, were separated by gel filtration as described in the preceding section to obtain LUBAC-enriched fractions. After preclearing with rabbit-IgG-protein A beads, the fractions containing LUBAC were incubated anti-HOIL-1 polyclonal antibody-protein A beads (Yamanaka *et al*, 2003). Complexes were eluted with 0.1 M Glycine–HCl, pH 2.5, for mass spectrometry.

Preparation of recombinant proteins

Recombinant baculoviruses encoding His_6-HOIP or its mutants, HOIL-1L or its mutants, and HOIL-1L-His_6 were generated using the Bac-PAK6 baculovirus expression system (Clonetech). His_6-E1 and IRP2-myc baculoviruses were described (Iwai *et al*, 1999; Ishikawa *et al*, 2005). Purification of recombinant proteins (expressed in bacteria or in the baculovirus system) was performed as described previously (Iwai *et al*, 1999). LUBAC and its mutants were prepared from Hi Five cells infected with the appropriate combinations of baculoviruses encoding His_6-HOIP or its mutants, and HOIL-1L or its mutants, followed by purification using an Ni^{2+}-affinity gel. HOIL-1L or HOIP was prepared from insect cells infected with HOIL-1L-His_6 or His_6-HOIP, respectively. Bacterially expressed LUBAC was prepared as follows. Bacterial lysates were incubated with the Ni^{2+}-affinity gel and the eluate was then separated on a HiTrap Q anion-exchange column. The LUBAC enriched fraction was then isolated by gel filtration, as described above. The non-tagged E2-25K was generated from His_6-E2-25K, by removal of the His_6-tag with TEV protease (Invitrogen).

Recombinant protein binding assays

Recombinant proteins (1 μg) were mixed in 50 μl of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Triton X-100 for 2 h on ice followed by precipitation with either Glutathione Sepharose or Ni^{2+}-affinity gel.

In vitro ubiquitination assays

Reaction mixtures contained 5 μg/ml of E1, 2.5 or 10 μg/ml of the appropriate E2, 50 μg/ml of the 600 K complex or its mutants, and the appropriate substrate, as described in the figure legends, and were incubated with 250 μg/ml of ubiquitin (Sigma), methylated ubiquitin (BioMol) or ubiquitin mutants (Boston Biochem) at 37°C for 2 h in 20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ and 2 mM DTT in the presence of ATP and an ATP regeneration system (0.5 mM ATP, 10 mM creatine phosphate and 50 μg/ml creatine phosphate kinase). For preparation of polyubiquitin chains for mass spectrometry analysis, ubiquitin was incubated with E1, E2s and the recombinant 600 K complex, as described above, with the exception that 2 mM ATP alone was added, rather than ATP and the ATP regeneration system. For reactions without the complex, a two-fold concentration of E2-25K was used.

Mass spectrometry analysis

Samples were separated by SDS–PAGE and stained with Coomassie. Gel slices containing the proteins to be identified by mass spectrometry were treated with trypsin (Sigma) for 12 h at 37°C, as described previously (Peng *et al*, 2003; Saeki *et al*, 2004b). The peptides were extracted from the gel slices using 0.1% trifluoroacetic acid and 50% acetonitrile, followed by concentration using vacuum centrifugation. The resulting peptide solution was deionized using a ZipTip (Millipore), mixed with a matrix solution (10 mg/ml α-cyano-4-hydroxycinnnamic acid, 0.1% trifluoroacetic acid and 50% acetonitrile) and dried on a stainless steel plate. Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS) was conducted using an ultraflex TOF/TOF (Burker Daltonics). Peptide mass finger printing was performed using the Mascot Search program (Matrix Science).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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