LETTERS

A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate

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In eukaryotic cells, many short-lived proteins are conjugated with Lys 48-linked ubiquitin chains and degraded by the proteasome[1]. Ubiquitination requires an activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3)[2]. Most ubiquitin ligases use either a HECT (homologous to E6-associated protein C terminus) or a RING (really interesting new gene) domain to catalyse polyubiquitination[3], but the mechanism of E3 catalysis is poorly defined[4]. Here we dissect this process using mouse Ube2g2 (E2; identical at the amino acid level to human Ube2g2) and human gp78 (E3), an endoplasmic reticulum (ER)-associated conjugating system essential for the degradation of misfolded ER proteins[5,6]. We demonstrate by expressing recombinant proteins in *Escherichia coli* that Ube2g2/gp78-mediated polyubiquitination involves preassembly of Lys 48-linked ubiquitin chains at the catalytic cysteine of Ube2g2. The growth of Ube2g2-anchored ubiquitin chains seems to be mediated by an aminolysis-based transfer reaction between two Ube2g2 molecules that each carries a ubiquitin moiety in its active site. Intriguingly, polyubiquitination of a substrate can be achieved by transferring preassembled ubiquitin chains from Ube2g2 to a lysine residue in a substrate.

To understand how Lys 48-linked ubiquitin chains are synthesized, we characterized a simple conjugating system consisting of a conserved E2 (Ube2g2) and gp78, a RING domain ubiquitin ligase. Because the amino-terminal transmembrane segments of gp78 are not required for its polyubiquitination function, we used its carboxy-terminal cytosolic domain (gp78c)[5,7]. Purified Ube2g2 and gp78c rapidly assembled Lys 48-linked polyubiquitin chains in a RING-dependent manner (Fig. 1a; Supplementary Fig. 1). Surprisingly, these chains were attached to a 20 kD protein via a thiol linkage, and were converted to unanchored species on addition of a reducing reagent, dithiothreitol (DTT) (Fig. 1a; Supplementary Fig. 2). Indeed, these ubiquitin chains could also be detected by immunoblotting with antibodies against Ube2g2, the only 20 kD protein in the reaction (Fig. 1b; Supplementary Fig. 3). The Ube2g2-linked ubiquitin chains were similarly assembled by full-length gp78 purified from mammalian cells (Supplementary Fig. 4). These chains were attached to the catalytic cysteine (Cys 89) of Ube2g2, because mutating the Cys 89, but not the other two cysteines in Ube2g2, abolished chain assembly (Fig. 1c; data not shown). The formation of Ube2g2-linked ubiquitin chains also required a conserved acidic loop of Ube2g2, which was located near its catalytic cysteine[8]. A loop mutant (2D2E) containing substitutions of four acidic residues by alanine failed to polymerize ubiquitin chains on itself (Fig. 1c). Because a similar loop has been found to be essential for the polyubiquitination function of a cell cycle specific E2, Cdc34 (ref. 9), the loop-dependent assembly of Ube2g2-anchored ubiquitin chains may be important for Ube2g2 and gp78-mediated polyubiquitination.

To understand how gp78c assembles ubiquitin chains on Ube2g2, we dissected the simplest step of this reaction—the formation of a di-ubiquitin on Ube2g2. One possible mechanism to generate Ube2g2-linked di-ubiquitin is by an aminolysis-based transfer reaction between two Ube2g2~ubiquitins, in which the Lys 48 in one Ube2g2-bound ubiquitin molecule attacks a donor ubiquitin that is conjugated to a neighbouring Ube2g2 (Fig. 2a). To test this idea, Ube2g2 was charged with either untagged wild-type ubiquitin or Flag-tagged K48R ubiquitin mutant (F–UbK48R). The reactions were quenched to block recharging of Ube2g2 (ref. 9). The two charging reactions were combined, and incubated in the presence of gp78c (Fig. 2b). If a transfer reaction between two Ube2g2~ubiquitins was feasible, addition of Ube2g2~wild-type-ubiquitin to Ube2g2~F–UbK48R should lead to the formation of Ube2g2-linked hybrid di-ubiquitin consisting of one wild-type ubiquitin and one F–UbK48R mutant, because Ube2g2~wild-type-ubiquitin could serve as an acceptor to receive the donor F–UbK48R (Fig. 2b). Indeed, addition of Ube2g2~wild-type-ubiquitin, but not a charging reaction containing either no Ube2g2 (mock) or the Ube2g2 C89A mutant, to Ube2g2~F–UbK48R resulted in the formation of Ube2g2-anchored hybrid di-ubiquitin (Fig. 2b, c). When more Ube2g2~F–UbK48R and Ube2g2~wild-type-ubiquitin molecules were used (by increasing the precharging time), a small fraction of Ube2g2 conjugated with two wild-type ubiquitins and one F–UbK48R was formed (Fig. 2c, lanes 2, 3). These results validate the proposed transfer reaction. They also indicate that longer ubiquitin chains may be formed on Ube2g2 by multiple rounds of such transfer reactions.

Because the Ube2g2 loop mutant (2D2E) failed to assemble ubiquitin chains on itself (Fig. 1c), the ubiquitin transfer reaction probably involves the acid loop. Indeed, when Ube2g2 2D2E~ubiquitin was used as acceptor, the rate of F–UbK48R transfer from wild-type Ube2g2 was dramatically reduced (Fig. 2d, lanes 5–8 versus lanes 1–4). When the 2D2E mutant conjugated with F–UbK48R was used as donor, the transfer reaction was completely abolished (lanes 9–16). Together, these data suggest that the acidic loop of Ube2g2 has a critical role with the donor ubiquitin during the assembly of Ube2g2-linked ubiquitin chains.

To determine whether Ube2g2-linked ubiquitin chains are involved in substrate polyubiquitination, we used an ER-associated protein termed HERP because it is a short-lived protein that interacts with several E3 ligases including gp78 (refs 10–12). We purified the cytosolic domain of human HERP (HERPc) that contained the E3 binding site[13], and found that it was rapidly polyubiquitinated by Ube2g2 and gp78c (Supplementary Fig. 5). Polyubiquitination of HERPc required the gp78 RING domain (Fig. 3a) and the Lys 48 of ubiquitin (Supplementary Fig. 5). Mass spectrometry analysis identified Lys 61 in HERPc as the site of polyubiquitination (data not shown). Indeed, a HERPc K61R mutant failed to undergo

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polyubiquitination by gp78c (Fig. 3b). Thus, gp78c and Ube2g2 conjugate a single Lys 48-linked ubiquitin chain to Lys 61 in HERPc. We next compared the rate of chain assembly on Ube2g2 with that of HERPc polyubiquitination (Supplementary Fig. 6). The formation of Ube2g2-linked chains slightly preceded the appearance of polyubiquitinated HERPc, raising the possibility that ubiquitin chains may be first assembled on Ube2g2 before being transferred to HERPc. Consistent with this idea, fewer Ube2g2-linked ubiquitin chains were generated when wild-type HERPc was present compared with those formed in the presence of HERPc K61R (Supplementary Fig. 7). Presumably, this was owing to the simultaneous transfer of ubiquitin chains from Ube2g2 to HERPc.

To test further the preassembly idea, we analysed HERPc polyubiquitination using the Ube2g2 loop mutant (2D2E) because it was defective in assembling Ube2g2-linked ubiquitin chains. Indeed, this mutant was able to conjugate one ubiquitin to HERPc, but was inactive in conjugating longer chains to HERPc (Fig. 3c). To obtain further evidence, we mutated the highly conserved Tyr 83 and His 94 of Ube2g2, respectively, because these amino acids seemed to communicate with the catalytic cysteine, and were located near the acid loop (Supplementary Fig. 8a–c). The Y83A mutant was able to assemble Ube2g2-linked ubiquitin chains, albeit with reduced activity; whereas the H94A mutant was almost completely inactive (Supplementary Fig. 8d). Consistent with our interpretation, the Y83A mutant exhibited similarly reduced activity towards ubiquitination of HERPc, whereas the H94A mutant was only capable of conjugating shorter ubiquitin oligomers ($n < 3$) to HERPc (Supplementary Fig. 8e). Furthermore, when similar mutations were introduced in the yeast homologue Ubc7, the mutant proteins were defective in rescuing the growth defect of a mutant yeast strain that lacked both the ER degradation and the unfolded protein response pathways (Δ*Ubc7* and Δ*Ire1* double deletion strain) (Supplementary Fig. 8f). Together, these data indicate that preassembly of Ube2g2-linked ubiquitin chains is essential for the function of Ube2g2 *in vitro* and *in vivo*.

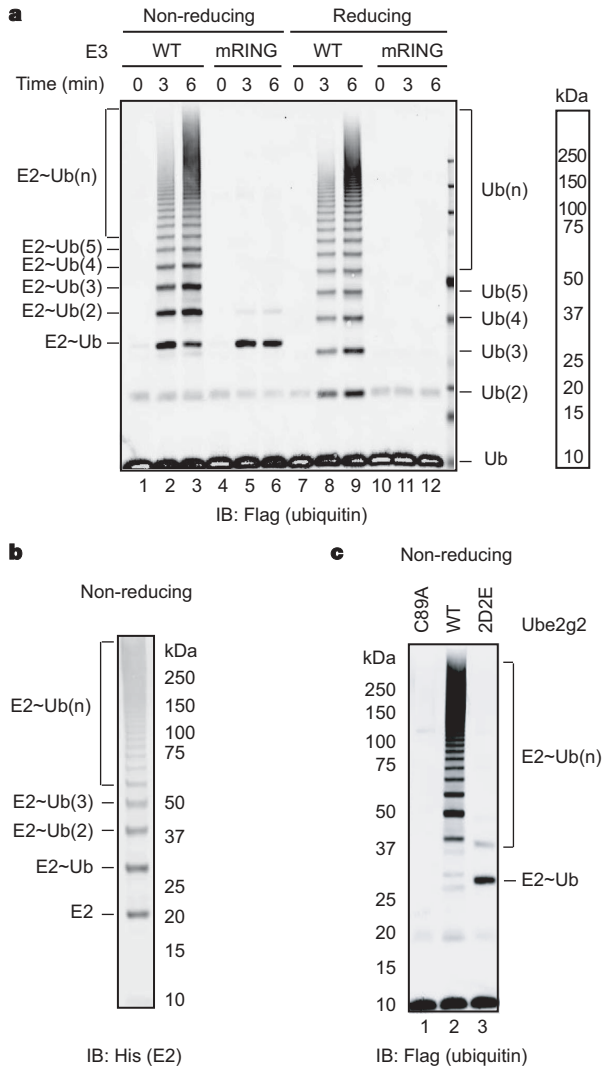

Figure 1 | Formation of Ube2g2-linked ubiquitin chains. a, gp78c (E3) assembles ubiquitin chains in a RING-dependent manner. Ubiquitination reactions performed with Flag-tagged ubiquitin in the presence of either wild-type (WT) gp78c or its RING mutant (mRING) were analysed by immunoblotting (IB) with anti-Flag antibodies. E2~Ub(n), E2-linked ubiquitin (Ub) chains consisting of a certain number (n) of ubiquitin molecules. Ub(n), unanchored ubiquitin chains. **b**, Ubiquitin chains are linked to Ube2g2 (E2). Ubiquitin chains assembled by Ube2g2 and gp78c were analysed by immunoblotting with anti-His antibodies. **c**, Formation of Ube2g2-linked ubiquitin chains requires the catalytic cysteine (C89) and the acidic loop of Ube2g2. Ubiquitination reactions were performed as in **a**, except that the indicated E2 variants were used.

To prove directly that Ube2g2-linked ubiquitin chains are transferable, we developed a two-step ubiquitination assay (Fig. 3d). First, Ube2g2-anchored ubiquitin chains were assembled in the absence of HERPc using a low concentration of ubiquitin. This ensured that most ubiquitin molecules were incorporated into Ube2g2-linked chains (Fig. 3d, lane 1; Supplementary Fig. 9). The reaction was stopped by dialysis, which removed ATP. Ube2g2-linked ubiquitin chains were then incubated with either HERPc or HERPc K61R. After incubation, ubiquitin chains were indeed transferred to HERPc, but not to HERPc K61R (Fig. 3d, lanes 2–7). These chains could not be generated from Ube2g2 that carried only one ubiquitin moiety because few such molecules were present in the preassembly reaction. In addition, incubation of HERPc with Ube2g2 precharged with one ubiquitin moiety formed only shorter ubiquitin chains on HERPc (Supplementary Fig. 9). Presumably, removal of ATP prevented recharging of Ube2g2, which rendered the chain assembly process less efficient. Together, these data demonstrate that ubiquitin chains assembled on Ube2g2 can be transferred 'en bloc' to a lysine residue in a substrate. They also indicate that the processivity of gp78-dependent polyubiquitination (the number of ubiquitin molecules transferred in one binding event) may be regulated by controlling the rate of chain transfer to substrate versus that of chain assembly on Ube2g2.

To prove further the 'chain transfer' idea, we tested whether chemically synthesized ubiquitin polymers can be directly transferred to HERPc. It was shown previously that ubiquitin chains consisting of up to four ubiquitins (Ub4) could be charged onto Ubc4 and Ubc7 (ref. 14). We confirmed that chemically synthesized Ub2, Ub3 and Ub4 could also be conjugated to the catalytic cysteine of Ube2g2 (Supplementary Fig. 10). When ubiquitination of HERPc was performed with Ub2, Ub3, or Ub4, a set of ubiquitinated HERPc species was generated, apparently by transfer of the corresponding ubiquitin oligomers from Ube2g2 to HERPc (Fig. 4a). The transfer of preassembled ubiquitin chains to substrates still required the gp78 RING domain, because the RING mutant gp78c was unable to conjugate Ub2 and Ub3 to HERPc (Fig. 4b). Together, these data confirm that ubiquitin chains can be transferred 'en bloc' from a conjugating enzyme to a substrate in the presence of a functional RING ligase.

To see whether assembly of Ube2g2-linked ubiquitin chains occurs *in vivo*, we expressed Flag-tagged Ube2g2 together with HA-tagged ubiquitin[15] in 293T cells. Because Ube2g2-linked ubiquitin chains are probably quite labile in cells (either as a result of simultaneous transfer to substrates or rapid turn-over by the proteasome), cells were treated with a proteasome inhibitor to accumulate such species. As expected, only a small fraction of Ube2g2 carried polyubiquitin conjugates (Fig. 4c, lanes 2, 5), and these conjugates could be removed from Ube2g2 by treatment with DTT (lanes 3). Thus, ubiquitin

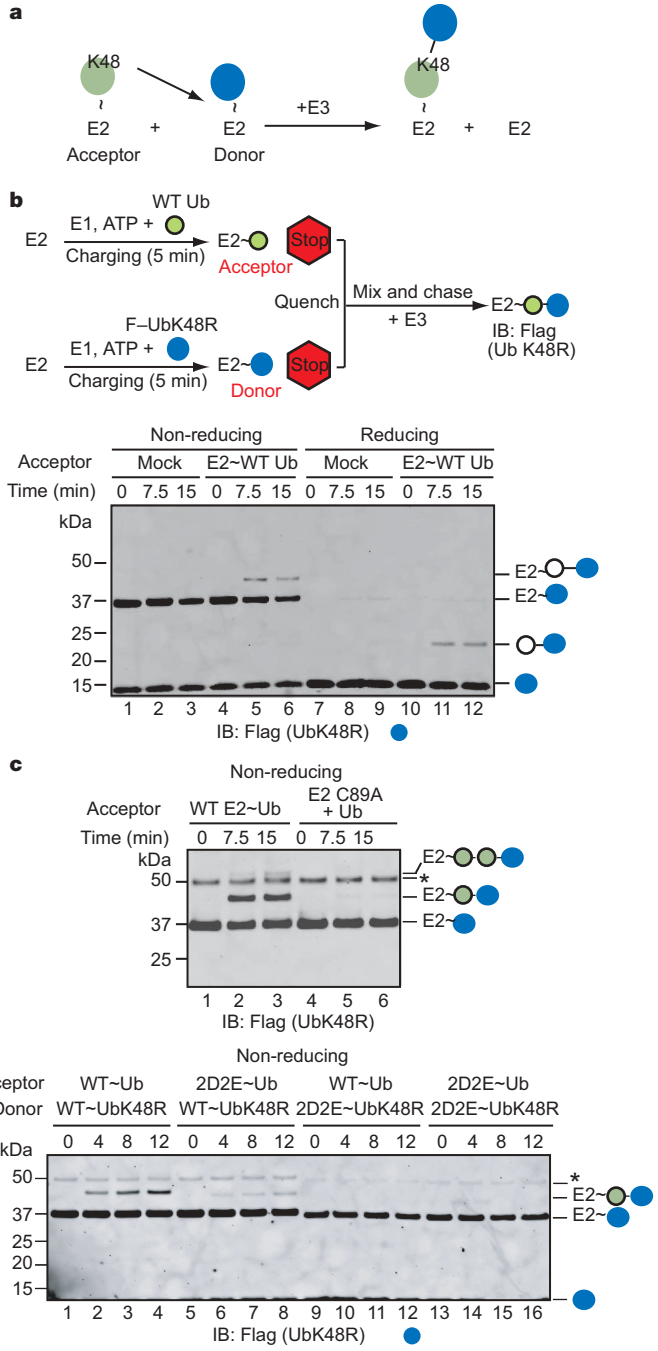

Figure 2 | Formation of Ube2g2-linked di-ubiquitin by an aminolysis-based transfer reaction. **a**, Outline of the proposed transfer reaction between two E2~Ubs. (K48, Lys48; ~ indicates thioester bond). **b**, Formation of Ube2g2-linked hybrid di-ubiquitin. Ube2g2 (E2) charged with Flag-tagged UbK48R (F–UbK48R, blue circle) was treated with EDTA/NEM (stop), and mixed with equal molar concentration of EDTA/NEM-quenched Ube2g2 precharged with untagged wild-type ubiquitin (green circle). The chase reaction was conducted in the presence of gp78c (E3). The reaction was analysed by immunoblotting with anti-Flag antibodies to detect products containing F–UbK48R. Where indicated, Ube2g2 charged with F–UbK48R was mixed with a mock charging reaction containing no Ube2g2 (mock). **c**, As **b**, except that the charging of Ube2g2 with either F–UbK48R or wild-type ubiquitin was conducted for a longer period of time (15 min). Where indicated, charging reaction containing the Ube2g2 C89A mutant (E2 C89A + Ub) was used as the acceptor. The asterisk indicates Ube2g2 conjugated with two F–UbK48Rs that was probably generated by transfer of one F–UbK48R molecule to a lysine residue in Ube2g2 during the charging reaction. **d**, As in **c**, except that the indicated E2s precharged with the indicated ubiquitin variants were used.

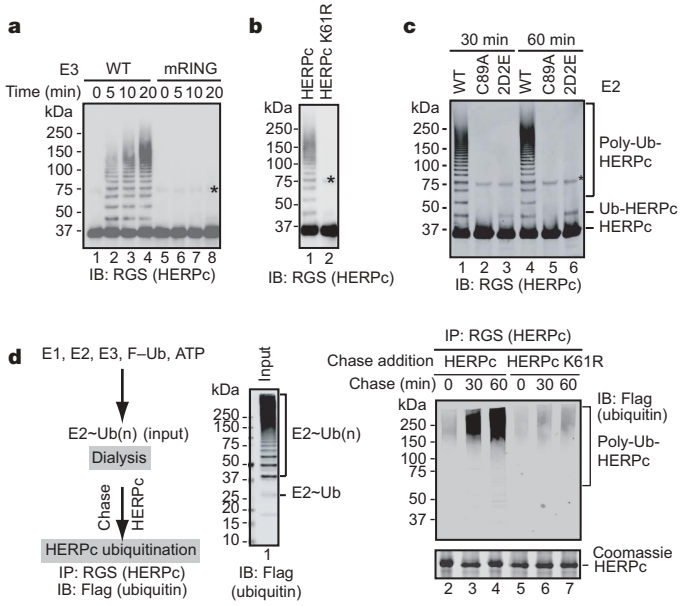

Figure 3 | Transfer of ubiquitin chains from Ube2g2 to HERPc. **a**, Ubiquitination of HERPc by gp78c. Ubiquitination of RGS-His-tagged HERPc was performed in the presence of either wild-type gp78c (E3) or its RING mutant (mRING). The asterisk marks a fraction of aggregated HERPc species. Unless otherwise specified, asterisks in the following panels refer to the same. **b**, Lys 61 of HERPc is required for its polyubiquitination. HERPc or a HERPc mutant bearing a Lys 61 to Arg (K61R) substitution was subjected to ubiquitination as in **a**. **c**, Polyubiquitination of HERPc requires the acidic loop of Ube2g2. Ubiquitination of HERPc was performed with the indicated Ube2g2 (E2) variants. **d**, Ube2g2-linked ubiquitin chains (E2~Ub(n)) were first synthesized in the presence of E1, E2, E3, Flag-tagged ubiquitin (F–Ub) and ATP. A fraction of the reaction was directly analysed by immunoblotting with anti-Flag antibodies (input), whereas the rest of the reaction was subjected to dialysis to remove ATP. The dialysed product was incubated with either HERPc or the HERPc K61R mutant for the indicated time points (chase). Ubiquitinated HERPc was detected by immunoblotting the immunoprecipitated HERPc with anti-Flag antibodies. A fraction of the immunoprecipitated HERPc was analysed by SDS–PAGE and Coomassie blue staining.

chains can be assembled on Ube2g2 in a thiol-dependent manner in cells.

In this report, we demonstrate that ubiquitin chains can be pre-assembled on the catalytic cysteine of an E2 (Ube2g2) before being transferred to a substrate (Fig. 4d). The proposed 'preassembly' model is distinct from the generally presumed 'sequential addition' model, in which ubiquitin molecules are added one at a time, first to a lysine residue in a substrate, and then to the Lys 48 in the distal ubiquitin of a growing chain. Our data indicate that Ube2g2-linked ubiquitin chains are assembled by multiple rounds of transfer reactions between two Ube2g2s bound by ubiquitin. To efficiently transfer ubiquitin, Ube2g2 may need to form a dimer. Indeed, a small fraction (~5%) of the purified Ube2g2 does exist as dimer. Moreover, gp78 forms homo-oligomers using its cytosolic domain in cells (Supplementary Fig. 11). Thus, gp78 may promote the efficient transfer of ubiquitin between two Ube2g2~ubiquitins by facilitating their dimerization. Dimerization of E2 has been observed for other conjugating enzymes including Cdc34 (refs 16–19). In fact, the activity of Cdc34 is significantly enhanced under conditions that favour its dimerization[20]. These observations together with the fact that Cdc34 also contains a similar acidic loop indicate that it may operate by a similar mechanism. Intriguingly, like Ube2g2, many E2 enzymes are able to assemble ubiquitin chains independent of substrates[21–26]. Further characterization of these reactions may reveal additional examples of thiol-linked ubiquitin chains on E2 enzymes.

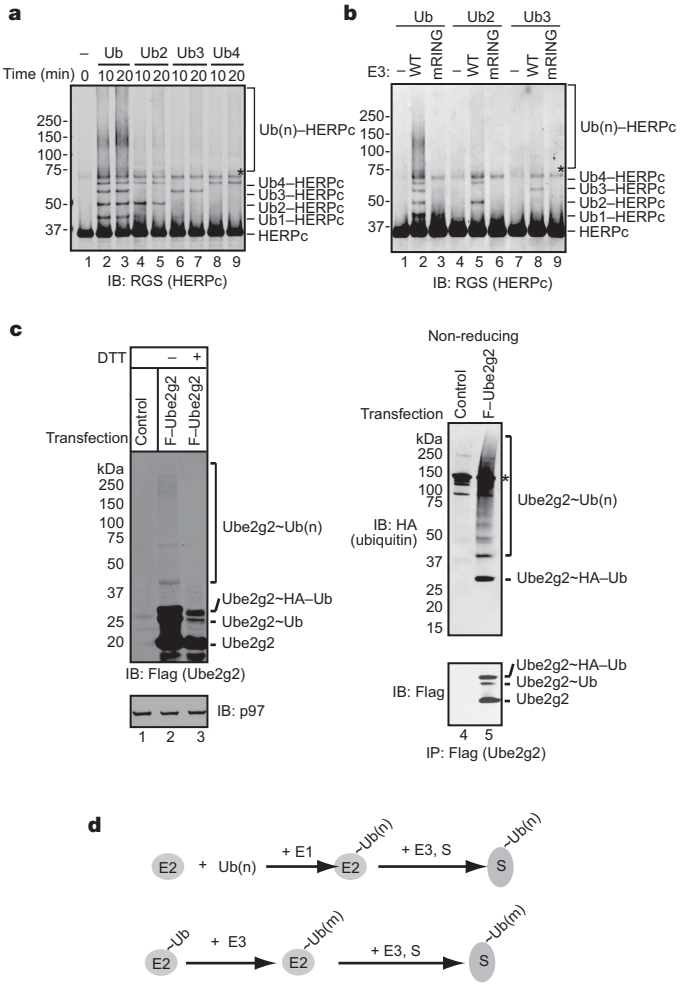

Figure 4 | Ubiquitination of HERPc by chemically synthesized ubiquitin oligomers. a, Ubiquitin oligomers can be directly transferred from Ube2g2 to HERPc. Ubiquitination of HERPc performed in the presence of ubiquitin or the indicated ubiquitin oligomers was analysed by immunoblotting (IB) with anti-RGS-His antibodies. **b**, Transfer of ubiquitin oligomers from Ube2g2 to HERPc requires the gp78c RING domain. Ubiquitination of HERPc was conducted with the indicated ubiquitin oligomers in the absence of (−) or in the presence of wild-type gp78c (E3) or its RING mutant (mRING). **c**, Formation of thiol-linked ubiquitin chains on Ube2g2 in cells. 293T cells transfected with a construct expressing haemagglutinin (HA)-tagged ubiquitin together with either an empty vector (control) or a plasmid expressing Flag-tagged Ube2g2 were treated with a proteasome inhibitor MG132. Cell extracts were analysed by immunoblotting with the indicated antibodies. A fraction of the extracts was subjected to immunoprecipitation with anti-Flag antibodies before immunoblotting analysis. Asterisk, IgG. **d**, A model for Ube2g2 (E2)- and gp78 (E3)-mediated polyubiquitination. Upper panel, ubiquitin chains present in cells[13] can be directly charged onto an E2 and then transferred to a substrate (S) in the presence of an E3. Alternatively (lower panel), longer chains can be assembled on E2 before being transferred to substrates. Ub(m), Ubiquitin chains containing a certain number (m) of ubiquitin molecules.

METHODS

Detailed methods are presented in Supplementary Information.

Antibodies and proteins. Anti-Flag and anti-RGS-His antibodies (antibodies recognizing Arg-Gly-Ser plus 4 consecutive His residues) were from Sigma and Qiagen, respectively.

Flag-tagged ubiquitin, Myc-tagged ubiquitin, untagged bovine ubiquitin, untagged UbK48R, methylated ubiquitin, E1, and chemically synthesized ubiquitin oligomers were purchased from Boston Biochem.

Ubiquitination assay. Unless specified in the figure legends, ubiquitination experiments were performed as described previously[7]. Briefly, E1 (60 nM), Ube2g2 (200 nM), gp78c (300 nM) were incubated with Flag-tagged ubiquitin (10 μM) at 37 °C in buffers containing 25 mM Tris HCl, pH 7.4, 2 mM magnesium/ATP, and 0.1 mM DTT. For reducing conditions, samples were treated with 100 mM DTT before SDS–polyacrylamide gel electrophoresis analyses. Ubiquitination of HERPc was conducted using the same conditions described above with the addition of HERPc (500 nM). Ubiquitinated HERPc was detected by immunoblotting with anti-RGS-His antibody.

Ubiquitin transfer assay. To monitor the transfer of ubiquitin between two Ube2g2s, Ube2g2 (1 μM) was incubated with E1 (60 nM), Flag-tagged UbK48R or untagged wild-type ubiquitin (1 μM) at 25 °C in buffers containing 25 mM Tris HCl, pH 7.4, and 2 mM magnesium/ATP. The reactions were treated with 50 mM EDTA and 10 mM NEM for 15 min at 25 °C to prevent further rounds of charging. The two reactions were then combined and incubated (chase) at 37 °C in the presence of gp78c (300 nM). The reaction was stopped by Laemmli buffer and analysed by immunoblotting with anti-Flag antibodies.

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1. Chau, V. *et al.* A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* **243,** 1576–1583 (1989).
2. Scheffner, M., Nuber, U. & Huibregtse, J. M. Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade. *Nature* **373,** 81–83 (1995).
3. Pickart, C. M. & Eddins, M. J. Ubiquitin: structures, functions, mechanisms. *Biochim. Biophys. Acta* **1695,** 55–72 (2004).
4. Hochstrasser, M. Lingering mysteries of ubiquitin-chain assembly. *Cell* **124,** 27–34 (2006).
5. Fang, S. *et al.* The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc. Natl Acad. Sci. USA* **98,** 14422–14427 (2001).
6. Chen, B. *et al.* The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. *Proc. Natl Acad. Sci. USA* **103,** 341–346 (2006).
7. Ye, Y., Meyer, H. H. & Rapoport, T. A. Function of the p97–Ufd1–Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *J. Cell Biol.* **162,** 71–84 (2003).
8. Arai, R. *et al.* Structure of human ubiquitin-conjugating enzyme E2 G2 (UBE2G2/UBC7). *Acta Crystallogr. F* **62,** 330–334 (2006).
9. Petroski, M. D. & Deshaies, R. J. Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF–Cdc34. *Cell* **123,** 1107–1120 (2005).
10. Sai, X. *et al.* The ubiquitin-like domain of Herp is involved in Herp degradation, but not necessary for its enhancement of amyloid β-protein generation. *FEBS Lett.* **553,** 151–156 (2003).
11. Hori, O. *et al.* Role of Herp in the endoplasmic reticulum stress response. *Genes Cells* **9,** 457–469 (2004).
12. Schulze, A. *et al.* The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. *J. Mol. Biol.* **354,** 1021–1027 (2005).
13. Kokame, K., Agarwala, K. L., Kato, H. & Miyata, T. Herp, a new ubiquitin-like membrane protein induced by endoplasmic reticulum stress. *J. Biol. Chem.* **275,** 32846–32853 (2000).
14. van Nocker, S. & Vierstra, R. D. Multiubiquitin chains linked through lysine 48 are abundant *in vivo* and are competent intermediates in the ubiquitin proteolytic pathway. *J. Biol. Chem.* **268,** 24766–24773 (1993).
15. Vong, Q. P., Cao, K., Li, H. Y., Iglesias, P. A. & Zheng, Y. Chromosome alignment and segregation regulated by ubiquitination of survivin. *Science* **310,** 1499–1504 (2005).
16. Silver, E. T., Gwozd, T. J., Ptak, C., Goebl, M. & Ellison, M. J. A chimeric ubiquitin conjugating enzyme that combines the cell cycle properties of CDC34 (UBC3) and the DNA repair properties of RAD6 (UBC2): implications for the structure, function and evolution of the E2s. *EMBO J.* **11,** 3091–3098 (1992).
17. Chen, P., Johnson, P., Sommer, T., Jentsch, S. & Hochstrasser, M. Multiple ubiquitin-conjugating enzymes participate in the *in vivo* degradation of the yeast MATα2 repressor. *Cell* **74,** 357–369 (1993).
18. Varelas, X., Ptak, C. & Ellison, M. J. Cdc34 self-association is facilitated by ubiquitin thiolester formation and is required for its catalytic activity. *Mol. Cell. Biol.* **23,** 5388–5400 (2003).
19. Eddins, M. J., Carlile, C. M., Gomez, K. M., Pickart, C. M. & Wolberger, C. Mms2–Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. *Nature Struct. Mol. Biol.* **13,** 915–920 (2006).
20. Gazdoiu, S. *et al.* Proximity-induced activation of human Cdc34 through heterologous dimerization. *Proc. Natl Acad. Sci. USA* **102,** 15053–15058 (2005).
21. Lorick, K. L. *et al.* RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl Acad. Sci. USA* **96,** 11364–11369 (1999).

22. Joazeiro, C. A. *et al.* The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286,** 309–312 (1999).
23. Tan, P. *et al.* Recruitment of a ROC1–CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of IκBα. *Mol. Cell* **3,** 527–533 (1999).
24. Huang, H. *et al.* The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes *in vitro* monoubiquitination of caspases 3 and 7. *J. Biol. Chem.* **275,** 26661–26664 (2000).
25. Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A. & Hampton, R. Y. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nature Cell Biol.* **3,** 24–29 (2001).
26. Lu, Z., Xu, S., Joazeiro, C., Cobb, M. H. & Hunter, T. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. *Mol. Cell* **9,** 945–956 (2002).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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