Synthetic biology approach to reconstituting the ubiquitylation cascade in bacteria

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Covalent modification of proteins with ubiquitin (Ub) is widely implicated in the control of protein function and fate. Over 100 deubiquitylating enzymes rapidly reverse this modification, posing challenges to the biochemical and biophysical characterization of ubiquitylated proteins. We circumvented this limitation with a synthetic biology approach of reconstructing the entire eukaryotic Ub cascade in bacteria. Co-expression of affinity-tagged substrates and Ub with E1, E2 and E3 enzymes allows efficient purification of ubiquitylated proteins in milligram quantity. Contrary to *in-vitro* assays that lead to spurious modification of several lysine residues of Rpn10 (regulatory proteasomal non-ATPase subunit), the reconstituted system faithfully recapitulates its monoubiquitylation on lysine 84 that is observed *in vivo*. Mass spectrometry revealed the ubiquitylation sites on the Mind bomb E3 ligase and the Ub receptors Rpn10 and Vps9. Förster resonance energy transfer (FRET) analyses of ubiquitylated Vps9 purified from bacteria revealed that although ubiquitylation occurs on the Vps9-GEF domain, it does not affect the guanine nucleotide exchanging factor (GEF) activity *in vitro*. Finally, we demonstrated that ubiquitylated Vps9 assumes a closed structure, which blocks additional Ub binding. Characterization of several ubiquitylated proteins demonstrated the integrity, specificity and fidelity of the system, and revealed new biological findings.

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Introduction

Ubiquitin (Ub) is a key post-translational modifier in all eukaryotes. The process of ubiquitylation, in which Ub attaches to a lysine residue of a substrate protein, regulates the function of thousands of proteins and is involved in numerous cellular processes such as protein degradation, DNA remodelling and repair, and protein trafficking. Malfunctions in the Ub system underlie various human disorders, including cancer and neurodegenerative, metabolic and infectious diseases (Ciechanover and Brundin, 2003; Nalepa *et al*, 2006; Mizushima *et al*, 2008). Ubiquitylation involves the concerted action of Ub-activating, Ub-conjugating, and Ub-ligase enzymes (E1, E2 and E3, respectively); the latter conclude the process by promoting the covalent attachment of Ub to a lysine residue of a specific target protein (Hershko *et al*, 1980; Wilkinson *et al*, 1980; Bachmair *et al*, 1986; Li *et al*, 2008; Deshaies and Joazeiro, 2009). Some forms of ubiquitylation serve as canonical signals for rapid degradation by proteasomes and lysosomes, while others perform various signalling functions.

Ubiquitylated proteins are recognized by a large set of cognate Ub receptors (Hicke *et al*, 2005; Hurley *et al*, 2006). These Ub receptors decrypt the Ub signal by tethering a Ub-binding domain (UBD) to a functional domain, thus linking the ubiquitylated target to a specific function *in trans*. Many Ub receptors undergo coupled monoubiquitylation themselves, presumably in order to impose a closed (*cis*) conformation that prevents binding of ubiquitylated targets (Di Fiore *et al*, 2003; Prag *et al*, 2003; Shih *et al*, 2003; Hoeller *et al*, 2006). Deubiquitylating enzymes (DUBs) are able to rapidly reverse this auto-regulatory signal by opening the *cis* conformation. Hence, the current models suggest that Ub receptors may exist in three states: (i) in the free, unmodified *apo* form, (ii) in the ubiquitylated and closed *cis* form, which is inactive due to intramolecular Ub-UBD interactions and (iii) in the *trans* form, which binds ubiquitylated target proteins through the UBD. The rapid dynamics of ubiquitylation/deubiquitylation has impeded the structure–function analysis of Ub receptors. Indeed, of the >100 UBD structures in the Protein Data Bank (PDB; January 2011), none is of a ubiquitylated *cis* form.

To bypass this obstacle, we reconstructed the entire eukaryotic ubiquitylation system in *Escherichia coli*. The system is built modularly, facilitating the straightforward production of ubiquitylated proteins. Two affinity tags, one on Ub and the other on the substrate, enable the ubiquitylated proteins to be purified in quantities and homogeneity suitable for biochemical, biophysical and crystallographic analyses. This system will facilitate the determination of the structures of ubiquitylated proteins, and will enable high-resolution insight into cellular mechanisms for interpreting Ub signals. Moreover, permutations of the system can be devised to screen for targets of E3 enzymes and UBDs or to study in detail the ubiquitylation of human disease proteins, including the facile identification of their modified residues.

In this work, we demonstrate the utility of this expression system by reporting several new biological findings. First, we demonstrate E2-specific auto-ubiquitylation of the E3 enzyme, Mind bomb. Next, we show that Epsin proteins undergo E3-independent ubiquitylation. Finally, we focus on the ubiquitylation of the yeast Ub receptor Vps9, in which a UBD is tethered to a guanine nucleotide exchanging factor (GEF) domain. We characterize *in vitro* the GEF activity and Ub-binding functions of this Ub receptor in its *apo*, *trans* and *cis* forms. A cycle of Ub binding and monoubiquitylation was postulated to regulate the association of the human orthologue of Vps9, Rabex-5, with endosomes (Mattera and Bonifacino, 2008). Our study provides a direct explanation for the regulatory function of Ub in controlling the cellular localization of Vps9.

Results

Construction of an integrated recombinant ubiquitylation system in bacteria

We developed a ubiquitylation system in bacteria that permits the purification of stably modified ubiquitylated proteins. The system consists of two compatible expression vectors. One is a generic plasmid (pGEN) that harbours His₆-Ub, E1-activating enzyme and E2-conjugating enzyme (Figure 1). We cloned these genes into a modified pHis₆-parallel2 vector in which the β -lactamase gene was replaced with a kanamycin resistance cassette. A second plasmid (pCOG) encodes a selected substrate for ubiquitylation and its cognate E3 ligase. In this vector, the substrate is typically fused to glutathione S-transferase (GST) or to maltose-binding protein (MBP) affinity tags. We constructed each type of vector such that it expressed its corresponding genes from a single promoter (pT7 or pTac), giving rise to a polycistronic mRNA. We produced a large collection of pGEN and pCOG vectors expressing different E2s, E3s, substrates, and their mutants (Supplementary Table SIV). Co-expression of the proteins encoded by these vectors yields large quantities of ubiquitylated substrates (0.5–1.0 mg of purified ubiquitylated Vps9 or Rpn10 per 1 l culture). Affinity tags such as His₆ fused to Ub, and MBP or GST fused to the substrate facilitate separation of the ubiquitylated from the unmodified substrate (as demonstrated for Rpn10 and Vps9, respectively). Moreover, tobacco etch virus (TEV) protease or rhinovirus protease recognition sites located between the tags and the ubiquitylated substrate enable tag removal, which, upon further affinity chromatography, facilitates the separation of the ubiquitylated product from the non-modified form. Using this method, the ubiquitylated protein is highly purified and ready for biochemical and biophysical studies.

To assess the functionality of the system, we first produced pGEN vectors co-expressing Ub and wheat Uba1 with either human UbcH5b or yeast Ubc5, the most promiscuous E2s with regards to association with E3s. Since most E3 ligases undergo auto-ubiquitylation in the absence of a specific substrate (Lorick *et al*, 1999), we simplified the system by first testing whether E3 ligases would undergo auto-ubiquitylation when expressed in bacteria from pCOG. E3 ligases fall into two large groups, U-Box/RING (really interesting new gene) and HECT (homologous to E6-AP carboxyl terminus) domain enzymes (Lorick *et al*, 1999; Hatakeyama *et al*, 2001; Hagglund and Roizman, 2002). To assess the general

Figure 1 Bacterial system for expression and purification of ubiquitylated proteins. A scheme describing the ubiquitylation system and the steps for purifying ubiquitylated proteins from *E. coli* (see also Supplementary Table SIV).

applicability of the reconstituted system, we first asked whether RING- and HECT-domain E3 ligases undergo auto-ubiquitylation in bacteria (Figure 2).

The RING-containing E3 Mind bomb (Mib) of *Danio rerio* (zebrafish), which is known to promote endocytosis in the Delta/Notch pathway (Itoh *et al*, 2003), was chosen as a representative protein. A GST-Mib construct containing its three RING domains (a.a. 738–1030) was co-expressed with His₆-Ub, E1 and UbcH5b, sequentially purified on reduced glutathione- and Ni²⁺-affinity matrices and resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein displayed a ladder pattern that entirely depended on the co-expression of Ub, indicating that Mib underwent multi-monoubiquitylation or polyubiquitylation (Figure 2A). A similar pattern was observed by western blot analysis with an anti-His antibody recognizing the His₆-Ub fusion (Figure 2A). The ubiquitylation pattern of Mib observed here was consistent with the pattern previously found to occur *in vitro* (Itoh *et al*, 2003), confirming the reliability of the system.

We next tested whether the ubiquitylation signal on Mib is K48- or K63-polyUb, the most abundant polyUb modifications (Peng *et al*, 2003). We found that replacing the wild-type Ub with a point mutant in which lysines 48 and 63 were replaced by arginine residues (K48R, K63R double mutant) did not affect the ubiquitylation pattern, suggesting that Mib undergoes either multi-monoubiquitylation or polyubiquitylation through lysines other than the K48 or K63. We then used Ub K0 (in which all Lys residues were mutated to Arg). The ladder pattern was retained, demonstrating that Mib is multi-monoubiquitylated in the bacterial system. Using mass spectrometry analysis, we identified five distinct lysine residues that underwent ubiquitylation (Figure 2D; Supplementary Table SI; Supplementary Figure S1).

Since Mib is expressed as GST fusion, we tested whether ubiquitylation takes place on Mib or on the GST moiety. The linker between the fusion partners was cleaved and the proteins were separated by chromatography on a GSH-affinity matrix. As shown in Figure 2B and C, the flow through (FT) contained heavily ubiquitylated Mib. Using anti-GST

Figure 2 Reconstituted E3 auto-ubiquitylation activity in bacteria. Auto-ubiquitylation of the RING and HECT E3-ligase protein families is shown. (A–C) Bacterial lysates co-expressing of His₆-Ub (or the indicated mutants), Uba1, UbcH5b (pGEN1), and GST-Mib (pCOG9) were purified on Ni²⁺ and/or GSH beads as indicated. (A) On the left, a Coomassie blue-stained SDS-PAGE of auto-ubiquitylated *D. rerio* (zebrafish) Mib, a representative RING-containing E3s ligase is shown. Wild-type Ub (lane 1 from the left), Ub K48R, K63R double mutant (lane 2), Ub K0 (lane 3) or without pGEN (lane 4). On the right, anti-His tag western blot of GSH purified Mib is shown, without or with pGEN1 (lanes 1 and 2, respectively). (B, C) Ubiquitylated Mib was purified on GSH (uncut), then GST tag was cleaved and the sample was bound to GSH beads. Flow through (FT) and elution (Elution) were analysed using western blot with anti-GST tag (B) or with anti-His tag (C). (D) Scheme of Mib ubiquitylation sites detected by mass spectrometry (see Supplementary Figure S1 and Supplementary Table S1). (E–G) Auto-ubiquitylation of yeast Rsp5, a representative HECT-containing E3s. Bacterial lysates co-expressing of His₆-Ub, Uba1, Ubc5 (pGEN5), and MBP-Rsp5 WT (pCOG3) or C777A mutant (pCOG7) were purified on amylose beads. (E) Input expression levels are shown. (F) Coomassie blue-stained SDS-PAGE of bound fraction is shown. (G) Western blot using anti-His tag of bound fraction is shown.

antibody, we found no evidence for ubiquitylation of GST (Figure 2B, Elution). However, blotting with anti-His antibody detected a faint band at ~39 kDa which may represent a minor fraction of monoubiquitylated GST (see Elution in Figure 2C). Thus, the majority of the Ub modification was attached to Mib not to GST. To determine whether HECT-containing E3 ligases are also active in the reconstituted ubiquitylation system, we cloned a representative HECT protein, yeast Rsp5, as MBP fusion, into pCOG. We found that, like Mib, Rsp5 underwent auto-ubiquitylation when co-expressed with its cognate E2s yeast Ubc4 or Ubc5 (Figure 2E–G). HECT proteins contain a catalytic cysteine

that transfers the Ub to the substrate. When we substituted the predicted catalytic cysteine by alanine (C777A), the ligase failed to charge with Ub (Figure 2G), although the expression levels of ubiquitylation components were equal in both bacterial lysates (Figure 2E; Supplementary Figure S4). Interestingly, the level of Ub-charged E2 was very low apparently because Ub is rapidly transferred to the E3 ligase (Figure 2G). However, in the presence of catalytically inactive Rsp5, accumulation of Ub-charged E2 was apparent (compare WT and C777A lanes in Figure 2F). Nevertheless, Ub-charged E3 did not accumulate when a substrate such as Rpn10 was added to the reconstituted system, ostensibly because of

Figure 3 Purification of K84-monoubiquitylated Rpn10. (A, B) Rpn10 ubiquitylation in bacteria is shown. Bacterial lysates co-expressing of His₆-Ub, Uba1, yeast Ubc5 (expressed from pGEN5) and GST-Rpn10 and MBP-Rsp5 (expressed from pCOG5) were purified on GSH beads. Systematic deletions of the ubiquitylation enzymes (-E1, -E2 and -E3) show that all are required for ubiquitylation. Only when all components are co-expressed, ubiquitylation is observed (All). (A) Coomassie blue-stained SDS-PAGE of GSH beads bound fraction is shown. Input expression levels of His₆-Ub, MBP-Rsp5 and GST-Rpn10 are shown below. Typical expression levels of E1 and E2 are shown in Supplementary Figure S4. (B) Western blot analysis of same samples shown in (A) using anti-His tag antibody is shown. (C) Coomassie blue-stained SDS-PAGE of the purification steps of ubiquitylated Rpn10 is shown. Bacterial lysates co-expressing pGEN24, Rsp5 and MBP-Rpn10 expressed from pCOG30 and pCOG31, respectively, were purified on amylose-affinity matrix (amylose), followed by Ni²⁺ beads (Ni²⁺). The apo Rpn10 was removed by wash (FT-Ni²⁺), Rpn10 was eluted (elution); His₆ and MBP tags were cleaved and removed (cleaved). The UV₂₈₀ absorbance chromatogram output of the size exclusion column (SEC) is shown (right of the gel). (D) A scheme of Rpn10 ubiquitylation site detected by mass spectrometry (see Supplementary Figure S2 and Supplementary Table SII) is shown.

rapid Ub transfer to the substrate (Supplementary Figure S4), since Ub was transferred to it. Taken together, this series of experiments demonstrates our successful reconstitution of the entire ubiquitylation cascade for RING and HECT E3 ligases in *E. coli*.

The reconstituted system faithfully recapitulates Rpn10 ubiquitylation

It has recently been shown that Rpn10 (regulatory proteasomal non-ATPase subunit) is ubiquitylated by Rsp5 *in vivo* at a single lysine residue (K84) (Isasa *et al*, 2010). However, *in-vitro* assays performed by the same team and others showed that Rpn10 and its human orthologue S5a undergo multi-monoubiquitylation or polyubiquitylation (Lu *et al*, 2008; Uchiki *et al*, 2009; Isasa *et al*, 2010). To test whether, unlike an *in-vitro* ubiquitylation assay, the reconstituted bacterial system would faithfully recapitulate Rpn10 ubiquitylation observed in *S. cerevisiae in vivo*, we studied the dependence of Rpn10 ubiquitylation on the concerted action of E1, E2 and E3 enzymes. As shown in Figure 3A, co-expression of His₆-Ub, Uba1, Ubc5 and Rsp5 was necessary and sufficient for Rpn10 monoubiquitylation. To assess the requirement of individual components, we systematically omitted each of the enzymes and the substrate (Figure 3A and B; for typical expression levels of E1, E2 and E3, see Supplementary Figure S4). As expected, Rpn10 monoubiquitylation was only observed when all components were present. Applying the scheme depicted in Figure 1, we purified ubiquitylated Rpn10. Sequential purification via the two affinity tags, MBP on Rpn10 and His₆ on Ub, efficiently isolated an apparently homogenous species corresponding to monoubiquitylated Rpn10 (Figure 3C). Size-exclusion chromatography upon enzymatic removal of the MBP and the His₆ tags resulted in a highly symmetric peak at the elution volume expected for correctly folded Ub-Rpn10

(Figure 3C). The yield of pure Ub-Rpn10 obtained from 1 L of bacterial culture was ~1.0 mg.

Using an *in-vitro* ubiquitylation assay followed by mass spectroscopy analysis, Crosas and co-workers identified four lysine residues in Rpn10 that were modified by Ub (K71, K99, K84 and K268), although K84 was found as the major site (52%). Using tandem mass spectrometry, we only identified K84 as ubiquitylated in Ub-Rpn10 produced in the bacterial system (Figure 3D; Supplementary Figure S2; Supplementary Table SII). Although the mass spectrometry on an LTQ-Orbitrap instrument employed here is exquisitely sensitive, we cannot entirely exclude the possibility of minor modifications of other lysine residues in Rpn10.

Rpn10 contains a Ub interaction motif (UIM), a domain that in some cases can mediate the ubiquitylation of a fusion partner such as GST (Oldham *et al*, 2002). Our mass spectrometry data revealed a similar phenomenon as we identified ubiquitylation of MBP within the context of the MBP-Rpn10 fusion protein (Supplementary Table SII). This finding corroborates the suggestion that UIMs can function to direct protein ubiquitylation.

Specificity and fidelity of the bacterial reconstituted ubiquitylation system

In the ubiquitylation process, particular protein-protein interactions govern several levels of specificity, including the interactions of E1:Ub-like protein (UbL), E1:E2, E2:E3 and E3:substrate. For example, the E1 Ub-activating enzyme (Uba1) associates with Ub but not with the UbL SUMO; whereas the E1-like enzyme of the autophagy system, ATG7 cooperates with two different UbLs, ATG12 and LC3 (Schulman and Harper, 2009). *E. coli* possesses at least two UbLs that serve as sulphur donors in the thiamine (vitamin B1) and the molybdenum cofactor biosynthesis pathways (Gutzke *et al*, 2001; Lake *et al*, 2001; Wang *et al*, 2001). We,

Figure 4 Specificity and fidelity of the ubiquitylation system in the bacteria. Specificity to cognate E2s by RING-containing (A and B) and HECT-containing (C and D) E3 ligases is shown. (A, B) Bacterial lysates co-expressing GST-Mib (pCOG9) and His₆-Ub, Uba1, and UbcH5b or Cdc34 (pGEN1 or pGEN8, respectively), were purified on Ni²⁺ and/or GSH beads as indicated. The purified proteins were resolved on SDS-PAGE and subjected to western blot analysis with anti-His tag or anti-GST antibodies as indicated. (A) Shows that Mib is ubiquitylated by UbcH5b as indicated in both purifications (Ni²⁺ and GSH). Free polyUb chains are shown in the Cdc34 lane that was purified on Ni²⁺, but not seen in the GSH purification. (B) The ubiquitylation of Mib by UbcH5b; Cdc34 does not ubiquitylates Mib (only *apo* Mib was detected) in the GSH purification is shown. (C, D) Bacterial lysates co-expressing MBP-Rsp5 and GST-Rpn10 (pCOG5) and His₆-Ub, Uba1, and Ubc4 or Cdc34 (pGEN4 or pGEN8, respectively), were purified on Ni²⁺ beads. The purified proteins were resolved on SDS-PAGE and subjected to western blot with anti-His tag or anti-GST antibodies as indicated. (C) Shows that Rpn10 is ubiquitylated by Rsp5 in the presence of Ubc4. Free polyUb chains are shown in the Cdc34 lane blotted with anti-His. (D) Rsp5-dependent ubiquitylation of Rpn10 is clearly evident in the Ubc4 lane. In the Cdc34 lane, a faint band of ubiquitylated Rpn10 is seen. (E) E3:substrate specificity is shown. Rsp5-dependent ubiquitylation of Cps1 (N-terminal residues PVEKAPR) fused to GST (lane 1) versus GST alone (lane 2). (F-H) Fidelity of the polyUb linkage is shown in free polyUb chains. Bacterial lysates co-expressing Ub, Uba1 and the specified E2s were subjected to western blot with the indicated antibodies. (F, G) Formation of K48-polyUb by Cdc34 or E2-25K, respectively and (H) the formation of K63-polyUb by UBC13/UEV1a are shown. (I, J) Coomassie blue-stained SDS-PAGE of fractions derived from size-exclusion chromatography of purified K48 and K63 diUb produced in the bacterial system is shown; proteins were purified using the Pickart's protocol (Pickart and Raasi, 2005) followed by gel filtration.

therefore, asked whether bacterial E1-like enzymes interact with the eukaryotic factors in the context of the reconstituted system. The lack of Rpn10 ubiquitylation in the absence of Uba1 (Figure 3A) suggests that, assuming their constitutive expression under our culture conditions, *E. coli* E1-like proteins do not participate in reactions observed with the reconstituted ubiquitylation system.

We demonstrated the E2:E3 specificity of the system by showing that Mib auto-ubiquitylation activity occurred in conjunction with UbcH5b, a cognate E2 (Itoh *et al*, 2003),

but not in conjunction with Cdc34, a non-cognate E2, known to be involved in cell-cycle control (Goebel *et al*, 1988; Figure 4A and B). The functionality of Cdc34 is supported by the formation of free polyUb chains (Gazdoui *et al*, 2005). Similar E2 specificity was found for the Rsp5-dependent ubiquitylation of Rpn10, which was clearly observed with the cognate E2 Ubc4 (Huibregtse *et al*, 1995), but barely with Cdc34 (Figure 4C and D). This indicates that Rps5 can associate with Cdc34, but with a significant lower efficiency compared with its cognate E2, Ubc4.

To examine E3:substrate specificity, we used the yeast biosynthetic multi-vesicular body (MVB) cargo, Carboxypeptidase-S (Cps1p), as the substrate. Cps1p is a type II transmembrane protein in which a short N-terminal tail is facing the cytosol. Monoubiquitylation of the cytosolic tail was demonstrated to serve as crucial signal for its MVB sorting (Katzmann *et al*, 2001). Emr and co-workers identified Rsp5 as the cognate E3 ligase for Cps1p (Katzmann *et al*, 2004). Recently, Katzmann and co-workers established that a C-terminal GST fusion of the Cps1p tail mimics its membrane localization and identified residues that contribute to the specific interaction with Rsp5 (Oestreich *et al*, 2007; Lee *et al*, 2009). We found that co-expressing Ub, Uba1, Ubc4, Rsp5 and the Cps1p^{Tail}-GST fusion in the bacterial system was sufficient for the ubiquitylation of Cps1p (Figure 4E). In contrast, GST alone was not ubiquitylated (Figure 4E, lane 2).

PolyUb chains linked through different Ub lysine residues encode distinct cellular signals (Peng *et al*, 2003; Fushman and Walker, 2010). It seems that E2s determine the lysine specificity of these polyUb chains. For example, Cdc34 or E2-25K form free polyUb chains conjugated via lysine 48 (K48 polyUb; Gazdaru *et al*, 2005), whereas UBC13/UEV complex forms K63 polyUb (Hofmann and Pickart, 1999). Co-expression of His₆-Ub, E1 and Cdc34 specifically yielded K48 polyUb (Figure 4F). We purified the resultant His₆-tagged proteins on Ni²⁺ beads and subjected them to western blot analysis using anti-His antibody. Bands corresponding to di-, tri-, tetra- and penta-Ub were evident (Figure 4F). Replacing wild-type Ub with the K48R mutant abolished this ubiquitylation. Similar results were obtained by replacing Cdc34 with E2-25K (Figure 4G). Moreover, mass spectrometry analysis of trypsin-digested peptides confirmed that these bands contained Ub. Similarly, co-expression of UBC13 together with its partner UEV1a, along with Ub and E1, resulted in free chains of K63 polyUb (Figure 4H). The reconstituted system allowed the purification 15–22 mg of K48 or K63 diUb chains from 1 L of *E. coli* culture. Additional steps of purification (Pickart and Raasi, 2005) resulted in the isolation of apparently homogenous K48- and K63-linked diUb from the polyUb chains as shown by Coomassie blue staining (Figure 4I and J). In conclusion, the reconstituted bacterial ubiquitylation system fully maintains the lysine linkage specificity of all E2 enzymes tested here.

E3-independent auto-ubiquitylation of Ub receptors

Many Ub receptors are regulated by auto-monoubiquitylation, presumably to promote intramolecular binding of the UBD to the attached Ub moiety thus leading to a closed, inactive conformation of the receptor (Shih *et al*, 2000; Prag *et al*, 2003; Hoeller *et al*, 2006; Mosesson *et al*, 2009). Dikic and co-workers showed that several Ub receptors, including signal-transducing adaptor molecule (STAM), undergo auto-ubiquitylation in an E3-independent manner (Hoeller *et al*, 2007). Mouse STAM2 was chosen to test whether the reconstituted ubiquitylation system maintains this E3-independent auto-ubiquitylation. GST-STAM was co-expressed in *E. coli* with its cognate E2s (UbcH5b or C). Upon purification on GSH-affinity resin, the protein was subjected to western blot analysis using anti-His or anti-GST antibodies. In agreement with a previous report (Hoeller *et al*, 2007), STAM underwent auto-monoubiquitylation in the presence of either UbcH5b or C (Figure 5A).

Figure 5 Yeast Epsin-1 (Ent1p) undergoes E3-independent ubiquitylation. (A) The bacterial system maintains recently reported (Hoeller *et al*, 2007) E3-independent ubiquitylation of mouse STAM2 with the UbcH5b or UbcH5c E2s. Bacterial lysates co-expressing of His₆-Ub, Uba1, UbcH5b or UbcH5c (pGEN1 or pGEN3, respectively), and GST-STAM2 (pCOG11) were purified on GSH beads and subjected to western blot analysis with anti-GST (left) and anti-His tag (right) antibodies. (B) Western blot analysis showing that the yeast Epsin-1 (Ent1p) undergoes E3-independent ubiquitylation. Bacterial lysates co-expressing of His₆-Ub, Uba1, yeast Ubc5 or yeast Cdc34 (pGEN6 or pGEN8, respectively), and His₆-MBP-Ent1p (pCOG21) were purified on Ni²⁺ beads. Bound proteins were subjected to western blot analysis with anti-His tag antibody. Without pGEN, no ubiquitylation is observed (–Ub). With Ubc5, Ent1p ubiquitylation was observed (Ubc5). With Cdc34, no Ent1p ubiquitylation was detected (Cdc34). Nevertheless, formation of polyUb chains by Cdc34 was clearly observed, indicating it is active.

We next examined auto-ubiquitylation of an additional Ub receptor. Some Ub receptors, including Sts1/2, Hrs and STAM, undergo E3-independent auto-ubiquitylation, whereas others such as Vps9, Rabex5, GGA3, Rpn10 and human Epsin were shown to require E3 ligases (Shih *et al*, 2003; Timsit *et al*, 2005; Mattera *et al*, 2006; Yogosawa *et al*, 2006; Isasa *et al*, 2010). The reconstituted system provides a straightforward assay to test the E3 dependency of Ub receptor auto-ubiquitylation. We addressed this for yeast and *D. rerio* (zebrafish) Epsin proteins (Eps15 interacting protein). Epsin tethers UBDs, a membrane-binding domain and a clathrin-binding motif through flexible hinges. By binding to ubiquitylated transmembrane proteins such as receptor tyrosine kinases, Epsin acts as sensor that binds Ub thereby mediating endocytosis. A His₆-MBP fusion of the yeast Epsin, Ent1p was co-expressed along with pGEN vectors harbouring either yeast Ubc5 or Cdc34 (Supplementary Table SIV). The proteins were enriched on Ni²⁺-affinity matrix, resolved on SDS-PAGE and subjected to western blot analysis using anti-His tag antibody, which recognized both His₆-MBP-Ent1 and His₆-Ub. We found that yeast Ubc5 was sufficient to promote Ent1p auto-ubiquitylation (Figure 5B), whereas Cdc34 did not (Figure 5B). Thus, unlike human Epsin, Ent1 underwent auto-ubiquitylation in an E3-independent manner. We also found that a yet uncharacterized *D. rerio* Epsin undergoes auto-ubiquitylation in an E3-independent manner (manuscript in preparation). As yeast and zebrafish are evolutionarily distant, E3-independent ubiquitylation may be a general mechanism common to the Epsin protein family.

Mapping the ubiquitylation sites on Vps9

Vps9 (vacuolar protein sorting) is a Ub receptor that promotes the trafficking of ubiquitylated transmembrane cargo

Figure 6 Identification of the ubiquitylation sites on yeast Vps9. Bacterial lysates co-expressing His₆-Ub, Uba1, Ubc5 (pGEN5), Rsp5 and GST-Vps9 (pCOG2) were purified as described in Figure 1. (A) A Coomassie blue-stained SDS-PAGE of two sequential affinity chromatography purification steps (Ni²⁺ and GSH) of ubiquitylated Vps9 is shown. Since Vps9 is a dimer, the monoubiquitylated complex is composed of stoichiometry equal amounts of modified and non-modified proteins. (B) Western blot analysis of the purified protein with an anti-His-tag antibody is shown. (C) Identification of the ubiquitylation sites on Vps9 as found in mass spectrometry analysis (see Supplementary Table SIII and Supplementary Figure S3). (D) The ubiquitylation sites are projected on the structural model of Vps9. Vps9-GEF domain was modelled based on the structure of the human orthologue Rabex5 GEF domain (Delprato *et al*, 2004). The structure of the hinge region linking the GEF and the CUE domain was not modelled and is illustrated as dashed lines. The CUE:Ub complex (Prag *et al*, 2003) is shown where the C-terminus of Ub is conjugated to K328, one of the lysine residues that were found to be ubiquitylated. (E) Mutational analyses of specific lysine residues found to undergo ubiquitylation by mass spectrometry. Vps9 mutants were expressed as His₆-MBP fusions (pCOG23–pCOG29) with Uba1, Ubc4 and Rsp5 (pGEN24 and pCOG30, respectively) and purified on Ni²⁺ beads. Western blot analysis with anti-His tag antibody of purified ubiquitylated Vps9 wild-type and mutant proteins is shown. Five single lysine to arginine mutants and a combined mutant harbours all five substitutions (5Xmut) are shown. All mutants underwent ubiquitylation with various levels compared with wild-type Vps9 (WT). Coomassie blue-stained SDS-PAGE with the indicated inputs (0.2%) is shown below.

on early endosomes. Vps9 contains a GEF domain and a UBD named CUE (conjugation of Ub to endoplasmic-reticulum degradation; Figure 6). In Vps9, Ub binding and intramolecular monoubiquitylation are coupled (Shih *et al*, 2003). To test if Vps9 is ubiquitylated in the bacterial reconstituted ubiquitylation system, we co-expressed GST-Vps9 with its cognate ubiquitylation proteins (Ub, Uba1, Ubc4/5 and Rsp5). The system yielded large quantities of monoubiquitylated Vps9 (Figure 6A and B). Comprehensive mass spectrometry analysis of purified ubiquitylated Vps9 identified at least five modified lysine residues (K83, K109, K144, K204 and K328), all but K328, within the GEF domain (Figure 6C and D; Supplementary Figure S3 and Supplementary Table SIII in Supplementary data show the fragmentation spectra). The ubiquitylated peptides of Vps9 carried the GG or LRGG tags that characteristically result from trypsin digestion (Peng *et al*, 2003). We mutated these lysine residues to arginine residues, either individually or all five residues together. Neither of the single mutants showed a significant decrease in the amount of ubiquitylation (Figure 6E). In fact, the quintuple mutant still underwent ubiquitylation, although to a lesser degree, suggesting that other lysine residues become modified in the absence of the major sites we identified by mass spectrometry. A similar observation has been reported for the ubiquitylation of β -Arrestin2 where only after substitution of all 31 lysine residues ubiquitylation was abolished (Shenoy *et al*, 2007).

Does ubiquitylation regulate the GEF activity of Vps9?

How auto-ubiquitylation regulates the activity of Ub receptors remains to be explored. In an attempt to shed light onto this question, we chose Vps9 as a model Ub receptor. As shown

above, most ubiquitylation takes place in the Vps9-GEF domain. We found that a point mutation (M419D) in the CUE domain, which is known to abolish Ub binding and Vps9 ubiquitylation (Prag *et al*, 2003), elicits higher GEF activity (Figure 7A and B). Interestingly, the human orthologue of Vps9, Rabex5, harbours a coiled-coil element that confers auto-inhibitory activity on its GEF domain (Mattera *et al*, 2006; Delprato and Lambright, 2007). Moreover, interaction of this element with Rabaptin5 reverses the inhibition. To date, no Rabaptin5 orthologue has been identified in yeast. Taking together the higher GEF activity of the M419D mutant and the apparent lack of a Rabaptin5 orthologue in yeast, we investigated the possibility that Vps9-GEF activity is downregulated by auto-ubiquitylation. Purified ubiquitylated Vps9 enabled us to test this question directly. Using Förster resonance energy transfer (FRET) between the yeast Rab5 GTPase Vps21 and mantGDP as the nucleotide probe (Davies *et al*, 2005), we monitored the nucleotide exchange rate (GEF activity) of the unmodified *apo* and the ubiquitylated *cis* forms of Vps9 (Figure 7A and B and see also model in Figure 8). To our surprise, *apo*-Vps9 and *cis*-Ub-Vps9 presented similar levels of GEF activity that were significantly lower than the maximal activity measured with the M419D mutant. These results indicate that ubiquitylation of Vps9 does neither impair nor enhance its GEF activity *in vitro*, suggesting that another factor, such as a yet undefined Rabaptin5 orthologue, regulates the Vps9-GEF.

Direct evidence for *cis* and *trans* Ub binding by Vps9

Previous work has postulated that the activity of a ubiquitylated Ub receptor to bind ubiquitylated target proteins is inhibited owing to *cis* interaction of the UBD with the

Figure 7 Functionality of ubiquitylated Vps9. Purified ubiquitylated Vps9 derived from reconstituted ubiquitylation system in *E. coli* was used for GEF activity and Ub-binding assays. **(A)** Crude data of GEF activity analyses of Vps9, ubiquitylated Vps9, GEF:Rab interface double mutant (D251A/E288A) and CUE:Ub interface mutant (M419E) are shown (colour code as indicated in **B**). The GEF activity of ubiquitylated Vps9 was compared with that of wild type, D251A/E288A and M419D Vps9 in assays monitoring mantGDP release from Vps21 in the presence of excess GTP (1 mM). The concentrations of Vps9 and Vps21 were 100 nM and 2.5 μ M, respectively. FRET between Vps21 and mantGDP (ex. 290 nm, em. 440 nm) was carried out to continuously assess Vps21 $\bullet$ mantGDP binding over a 15-min incubation period (1 μ M Vps9^{M419D}-stimulated release is presented to indicate the range of Vps21 $\bullet$ mantGDP release that could be achieved ($\times 10$)). The rate of Vps9-stimulated mantGDP release relative to Vps21 alone is shown. **(B)** Averaged data of at least three independent experiments (as shown in **A**) are shown, and error bars indicate standard deviations. **(C–E)** Direct evidences for close structure of ubiquitylated Vps9 are shown. Pull-down and crosslinking assays of Vps9 and ubiquitylated Vps9 with immobilized or free Ub are shown. **(C)** Ub-agarose beads pull-down assay with purified proteins expressed as MBP fusions (see Materials and methods). Only the *apo*-Vps9 binds Ub agarose while the Ub-MBP-Vps9 does not. **(D)** Ub agarose was used in the pull-down assay with *E. coli* cell extracts that expressed GST, GST-Vps9 or ubiquitylated GST-Vps9 (as indicated). Purified proteins from the same cell extracts (input) presented that all three are highly expressed. Only the *apo*-Vps9 binds Ub agarose. **(E)** Crosslinking assay of purified Ub, Vps9 and ubiquitylated Vps9 proteins is shown. A mild crosslinker (DSS) was used. Vps9 and ubiquitylated Vps9 do not show self-association (lanes 1 and 2 from the left). Vps9:Ub association represents formation of trans complex (lane 3). Ubiquitylated Vps9 does not bind free Ub (lane 4). No band of the predicted migration distance of Ub bound to ubiquitylated Vps9 is seen (as represented by *).

conjugated Ub moiety (Prag *et al*, 2003; Hurley *et al*, 2006). The bacterial purification system for ubiquitylated proteins established here allowed us to directly address this proposition for Vps9.

Vps9 is a homodimer that undergoes coupled monoubiquitylation (Prag *et al*, 2003). In this process, Ub binding to the CUE domains is a prerequisite of ubiquitylation (Shih *et al*, 2003). In the ubiquitylated Vps9 dimer, only one Vps9 protomer is ubiquitylated, as is evident by the two bands in Figure 6A. Nevertheless, according to the model proposed here, both CUE domains are occupied with the same Ub moiety. To assess this hypothesis, we performed pull-down and crosslinking assays with Ub and Vps9 (both the *apo* form and the ubiquitylated Vps9 form). This was made feasible using the bacterial reconstituted ubiquitylation system that

produced large amounts of purified ubiquitylated Vps9. Purified *apo* and ubiquitylated MBP-Vps9 proteins were incubated with Ub-agarose beads and washed extensively before loading onto SDS-PAGE. The Coomassie blue-stained gel clearly showed that the unmodified Vps9 (*apo*) bound to the Ub agarose, whereas the ubiquitylated MBP-Vps9 (*cis*) did not (Figure 7C). To verify if the cellular environment affects the binding, we performed the same assay but in bacterial cell extracts rather than with purified proteins (Figure 7D). Furthermore, to confirm that the fusion partner does not affect the interactions, we replaced MBP-Vps9 by GST-Vps9. *E. coli* lysates containing GST-Vps9, ubiquitylated GST-Vps9 or GST were incubated with Ub-agarose beads and washed extensively followed by separation on SDS-PAGE. We found that the *apo*-GST-Vps9 bound to the

Figure 8 Model for the regulatory function of UBD-GEF proteins auto-ubiquitylation. A comparison is shown between models of yeast and of human GEF-containing Ub receptors of the endocytic pathway. The localization of human Rabex5 and of yeast Vps9 proteins is regulated by auto-ubiquitylation. *Trans* complexes with ubiquitylated transmembrane cargo are on the membrane of early endosomes. This *trans* interaction promotes the nucleotide exchange of the Rab5 protein family members that are anchored to the same membrane through a geranylgeranyl group. Auto-ubiquitylation of the GEF-containing Ub receptors blocks their UBDs in *cis*, and therefore sequesters them from the membrane. It is believed that a specific deubiquitylation enzyme (DUB) stimulates the cycle by reversing the ubiquitylation signal.

Ub agarose, but that the ubiquitylated GST-Vps9 did not (Figure 7D). Only a faint band of Vps9 was visible, a finding that is consistent with an excess amount of *apo*-Vps9 that is present in the bacteria. To verify that ubiquitylated Vps9 was indeed present in the lysate, we further purified a fraction of the same lysates and showed that similar amounts of proteins were expressed and modified in the tested cultures (see input in Figure 7D).

While Ub agarose may mimic ubiquitylated transmembrane conjugates, we also devised a crosslinking assay with free, soluble Ub (that mimics cytosolic Ub). Moreover, here we used purified *apo* and ubiquitylated Vps9 proteins whose tags were removed (as shown in the purification scheme of Figure 1). Equal amounts of *apo* and ubiquitylated *cis*-Vps9 proteins were incubated with excess Ub, followed by crosslinking with disuccinimidyl suberate (DSS). Figure 7E shows that *apo*-Vps9 bound Ub, whereas the ubiquitylated *cis*-Vps9 did not. Interestingly, in the ubiquitylated sample we observed a substantial shift in the stoichiometric *apo*:*cis* ratio after crosslinking, reflecting the dynamic nature of the complex ubiquitylated Vps9 dimer. The latter observation corroborates our earlier findings that the CUE domain can bind Ub as a monomer with low affinity and as a dimer with high affinity (Prag *et al*, 2003). While it appears that some of the ubiquitylated Vps9 undergoes limited degradation in the crosslinking buffer, thus complicating the experiment, the data clearly show that monoubiquitylation of Vps9 inhibits its Ub-binding function.

Discussion

E. coli is the established work-horse for producing large quantities of recombinant proteins. Despite the availability of advanced cloning and purification techniques, relatively few eukaryotic post-translational modification systems have been implemented in *E. coli* (Cronan, 1990; Mencia and de Lorenzo, 2004; Baba *et al*, 2005; Su *et al*, 2006); and as a result, the utility of this expression system has been limited. Two studies used *E. coli* expression of HA-tagged Ub, Uba1, E2 and E3 to detect a specific substrate for a given E3 ligase (Su *et al*, 2006; Rosenbaum *et al*, 2011). Here, we present a modular bacterial system not just for identification, but also

for the purification and characterization of ubiquitylated proteins. A main advantage of *E. coli* is the absence of DUBs, which renders the modified product fully stable. As a result, the system yields large amounts of ubiquitylated proteins that can be easily purified. It provides tools for identifying new substrates and for swiftly deciphering the enzymatic requirements and the specific amino-acid residues subjected to modification.

Due to the close physical coupling of mRNA transcription and translation, bacterial polycistronic systems may facilitate protein-protein interactions of the nascent products. Therefore, the reconstituted ubiquitylation system described here used polycistronic vectors. To allow for different combinations of its various components, we designed the system as sets of two modular plasmids, pGEN and pCOG. Currently, the system includes over 20 plasmids harbouring different E2s and Ub mutants, and >30 vectors harbouring cognate substrates and E3s. The advantage of this system lies in the ease and swiftness of combining and transforming the experimental vectors into bacteria.

Two studies reported a chemical biology approach for Ub modification of H2B and PCNA by mutating target lysine into cysteine residues that enabled disulphide exchange and intein chemistry (Chatterjee *et al*, 2010; Chen *et al*, 2010). However, the limitation of this chemical approach is that the modified protein is not in its natural form, since a short disulphide bond is formed rather than an isopeptide bond. Indeed, in the case of H2B, the authors showed that shortening the Ub~H2B linker reduces the signal decoding by 40% (in terms of the resulting methyltransferase activity) (Chatterjee *et al*, 2010). Several other chemical biology approaches to generate Ub chains were recently reported (El Oualid *et al*, 2010; Kumar *et al*, 2010; Virdee *et al*, 2010; Fushman *et al*, 2011). These applications are currently feasible for small well-folded proteins (such as Ub chains) that could be synthesized *in vitro*. A genetic incorporation of δ -thio-lysine combined with native chemical ligation and followed by desulphurization was demonstrated to yield diUb and Ub-SUMO conjugates (Virdee *et al*, 2011). While these chemical approaches produced only free Ub or UbL chains, using the reconstituted system we also obtained conjugated E1, E2, E3 and ubiquitylated substrates. Our

system ensures production of the native form of ubiquitylated proteins, as it relies exclusively on an enzymatic cascade.

The reconstitution of Rpn10 monoubiquitylation is one example that showcases this authenticity. Isasa *et al* (2010) recently showed that Rpn10 is monoubiquitylated *in vivo* in yeast. *In-vitro* ubiquitylation of Rpn10, however, results in multi-monoubiquitylated or polyubiquitylated substrate, as reported by several groups, including Isasa *et al* (Lu *et al*, 2008; Uchiki *et al*, 2009; Isasa *et al*, 2010). Why is the bacterial system more successful in recapitulating the eukaryotic process than an *in-vitro* assay? We suggest that the high protein concentration in the cytosol of *E. coli* (~500 mg/ml; Luria, 1960) better mimics the crowded eukaryotic cell environment than the dilute conditions commonly used for *in-vitro* ubiquitylation assays.

While purifying ubiquitylated proteins using the reconstituted system, we could not detect background of endogenous ubiquitylated proteins from *E. coli* (Supplementary Figure S4). The latter finding demonstrates that the high specificity of the eukaryotic ubiquitylation system is fully maintained upon reconstitution in *E. coli*.

Many Ub receptors undergo auto-monoubiquitylation. A parsimony model suggests that this mechanism serves to block the UBD in *cis* (Figure 8; Hurley *et al*, 2006). However, many Ub receptors, including the human orthologue of Vps9, Rabex5, harbour multiple UBDs that bind the same patch on the Ub surface. Although only one of the UBDs will be blocked by auto-monoubiquitylation of Rabex5, the modification is sufficient to promote the dissociation of Rabex5 from early endosome membranes (Figure 8). Vps9 differs from its mammalian orthologue in several important aspects. A conserved GEF domain present in both Rabex5 and Vps9 acts on the Rab5 protein family. However, in Vps9 a C-terminal CUE domain functions as a single UBD, while in Rabex5 two different UBDs (A20-like ZnF and IUIM) are located at the N-terminus (Figure 8; Prag *et al*, 2003; Lee *et al*, 2006; Penengo *et al*, 2006). Whereas in yeast the CUE domain is responsible both for binding ubiquitylated cargo and for auto-ubiquitylation, in humans these functions are divided between the ZnF and IUIM domains (Lee *et al*, 2006). The combined results for both the human and the yeast GEF-Ub receptors, Rabex5 and Vps9 suggest a mechanism of convergent evolution. The principle that ubiquitylation acts to sequester membrane-associated proteins from their targets appears to be conserved in evolutionarily unrelated Ub receptors (Mattera and Bonifacino, 2008; Mosesson *et al*, 2009).

Previous studies have provided indirect evidence that ubiquitylated Ub receptors form a closed structure (*cis*) (Hoeller *et al*, 2006; Mattera and Bonifacino, 2008). The reconstituted system has enabled us to demonstrate that auto-monoubiquitylation of Vps9 reduces its ability to bind Ub in *trans*. The fact that human Rabex5 and yeast Vps9 developed virtually the same mechanism to regulate GEF localization, notwithstanding their unrelated UBDs, suggests a general paradigm by which the cell interprets the Ub signal. Given the huge spectrum of ubiquitylation signalling, it will be interesting to explore the possibility that different mechanisms decoded by Ub receptors (other than shown here for Vps9) govern the association of proteins with membranes.

In conclusion, we have reconstituted in bacteria a pathway that specifically and accurately generates ubiquitylated

eukaryotic proteins. The availability of the bacterial system, its ease of use, and its large yields should greatly facilitate future crystallographic, biophysical and biochemical analyses of ubiquitylated proteins.

Materials and methods

Plasmid construction

cDNAs were PCR amplified, digested and subcloned into the parallel system vectors (Sheffield *et al*, 1999). To create the pGEN plasmid, the β -lactamase resistance gene in the pHIS-parallel2 vector was replaced with a kanamycin resistance cassette. Then, the cDNA of human Ub was subcloned into the *Bam*HI and *Eco*RI sites to create His₆-Ub. The cDNA of wheat Uba1 was subcloned between *Eco*RI and *Bst*BI sites. Finally, the cDNAs of the different E2s were subcloned into the *Eco*RI and *Asc*I endonuclease recognition sites (the *Asc*I site was inserted by PCR). Shine-Dalgarno sequences derived from the Lac operon of the *E. coli* strain K12/W3110 (accession #AP009048) were for the E2 5'-AGGAAATCC ATTATG-3' and for E1 5'-AGGAAACAGCTATG-3. Ub was cloned downstream to the Shine-Dalgarno sequence that already existed in the pHIS-parallel vector. These sequences were added to the forward primer of E2 and E1 to form a polycistronic expression vector.

The His-Ub-E2/Ubc5-E1 segment was PCR amplified, digested and subcloned into the pCDF-duet vector (Novagen) between *Avr*II sites.

To create the pCOG plasmid, the ORF of yeast Vps9 encoding amino acids 55–451 was PCR amplified, digested and subcloned into a pMBP-Rsp5 vector (Davies *et al*, 2003) between *Spe*I and *Sph*I sites. The same was done for yeast Rpn10. Mind bomb and mSTAM were subcloned into the pGST-parallel2 vector between *Bam*HI and *Eco*RI endonuclease recognition sites. The ORF of MBP was inserted into the pCDF-duet vector between *Eco*RI and *Asc*I to create pCDF-His₆-MBP. Ent1p and Vps9 were subcloned into the pCDF-duet vector as His₆-MBP fusions. UEV1A was subcloned into the pHIS-parallel2 vector, UBC13 was subcloned downstream of the UEV1A ORF with a preceding Shine-Dalgarno sequence.

Site-directed mutagenesis

Point mutations were introduced using the ExSite or QuickChange (Stratagene) protocols. All constructs were verified by DNA sequencing to ensure introduction of the desired mutations and that no other mutations were introduced.

Protein expression and purification

Rosetta2(λ DE3)BL21 *E. coli* cells were co-transformed with pGEN and pCOG vectors. Cultures were grown in Terrific Broth medium at 37°C. Each culture was induced by addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and was further grown at 16°C for 16–20 h. Cells were harvested by centrifugation and were resuspended in buffer with lysozyme and AEBSF (4-(2-Aminoethyl) benzenesulphonyl fluoride hydrochloride). Complete lysis and DNA shearing were achieved by sonication following centrifugation to isolate the soluble fraction. Proteins were affinity purified using reduced glutathione, nickel, (GE-Amersham Biosciences) or amylose-affinity matrixes (New England Biolabs) according to the manufacturer's instructions. Affinity tags were removed with His₆-tagged TEV proteases and with GST-tagged rhinovirus proteases. Size-exclusion chromatography was used for further purification of the affinity-purified proteins.

Western blotting

Samples were transferred onto a nitrocellulose membrane and incubated with rabbit anti-His₆ epitope tag antibody (1:20 000 dilution, Rockland), mouse anti-Ub antibody, or mouse anti-GST antibody (1:200, Santa Cruz), and infrared dye coupled goat anti-mouse secondary antibody (1:12 000, LI-COR). Scanning was performed with the Odyssey infrared imaging system (LI-COR Biosciences) in accordance with the manufacturer's instructions at 700 and 800 nm.

Pull-down experiments

In all, 100 ml of *E. coli* BL21(λ DE3) cultures expressing GST, GST-Vps9_(55–451) and GST-Vps9_(55–451) with E1, Ubc4 Rps5 and His₆-Ub was harvested and prepared described. In all, 10% of the lysates

were used for binding assays and the rest were further purified. For binding assays, equal protein concentrations were used diluted with binding buffer containing 25 mM Hepes-Na pH 7.4, 75 mM NaCl and 0.1% Triton X-100 to a final volume of 0.5 ml. In all, 20 µl of activated Ub-agarose beads (Sigma) was incubated with the lysates for 2 h at 4°C. Unbound fraction was removed by centrifugation and beads were washed three times with 1 ml of binding buffer. The bound and unbound fractions were analysed by SDS-PAGE. The remaining lysate fractions were batch purified using nickel beads followed by GSH beads (for Ub-Vps9) or GSH beads only (for *apo*-Vps9 or GST tag). Equal amounts were analysed by SDS-PAGE. For the pull down with purified proteins, purified MBP, MBP-Vps9 and ubiquitylated MBP-Vps9 were used. Buffer was exchanged with AmiconUltra Centricon (Millipore) to binding buffer (25 mM Hepes-Na pH 7.4, 75 mM NaCl, 1 mM DTT). Samples were concentrated to ~15 mg/ml and 20 µl was used with 80 µl of binding buffer supplemented with 0.1% BSA and 0.25% Triton X-100. The mixture was incubated with 30 µl Ub agarose, washed and analysed by SDS-PAGE.

Crosslinking

Crosslinking assays with purified proteins were carried out according to the method described by Azem *et al* (1998). Reactions contained 0.5 mM DSS in 25 mM Hepes buffer pH 7.4, 150 mM NaCl and 10 µM of the indicated proteins. The Vps9 to Ub or ubiquitylated Vps9 to Ub ratios were 1:1. The reaction was stopped after 10 min by boiling the solution in SDS-loading buffer and samples were analysed by SDS-PAGE.

Mapping ubiquitylation sites by RP/LTQ-OrbiTrap mass spectrometry

Mass spectroscopy protocol is described in detail in Supplementary data. Briefly, gel bands containing ubiquitylated GST-Vps9, GST-Mib or MBP-Rpn10 were excised and subjected to reduction and alkylation. Samples were digested with either trypsin or Asp-N (and in the case of Mib also with GluC). Protease-digested samples were extracted, concentrated and desalted. Eluates were vacuum dried and redissolved in LC/MS loading buffer (2% acetonitrile in 0.1% formic acid in water). ADVANCE low-flow electrospray ionization source was used to load the digested samples onto a Magic C18 AQ reversed-phase column (Michrom Bioresources Inc.) with a 2-h LC gradient coupled to the OrbiTrap mass spectrometer. To avoid the iodoacetamide artifact that may mimic ubiquitylation, cysteine residues were alkylated at low temperature and concentration (40°C, 15 mM iodoacetamide) for 40 min (Nielsen *et al*, 2008). Search results were viewed, sorted, filtered and analysed using comprehensive proteomics data analysis softwares.

GEF activity analysis

GEF activity analysis was carried out according to a published protocol (Davies *et al*, 2005). Briefly, wild-type proteins and the D25A1/E288A and M419D mutants were expressed as His₆-MBP

fusions. Proteins were purified by Ni²⁺ chromatography, cleaved with TEV protease and resubjected to Ni²⁺ chromatography, and then fractionated by size-exclusion chromatography on a Superdex 75 column. Vps21 was purified by a similar procedure. Vps21 was then loaded with mantGDP, and excess nucleotide was removed via size-exclusion chromatography. Vps21 release assays containing 2.5 µM Vps21●mantGDP and 100 nM Vps9 (wild-type, mutant or ubiquitylated Vps9) in release assay buffer (20 mM Hepes pH 7.5, 5 mM MgSO₄) were initiated by addition of GTP to 1 mM. FRET (ex. 290 nm, em. 440 nm) was measured continuously in a Photon Technology International Quanta Master 2001 fluorometer with 1 per second time points for 15 min. Data were graphed in Prism (GraphPad Software, Inc.) and rates of release determined by linear regression.

Supplementary data

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

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Author contributions: TKK designed, performed, analysed and implemented the ubiquitylation system, including the Rpn10, Rsp5 and Vps9 functionality experiments and participated in writing the manuscript. IA performed and analysed the Mib and Cps1 experiments. BD and BH designed, performed and carried out data analysis for the Vps9-GEF activity. NT performed the studies on yeast Epsin. YR performed the studies with Cdc34 and zebrafish Epsin. OLK and EL carried out the studies on STAM. OK and MG performed the mass spectrometry analysis of Rpn10. MK subcloned the Ubc13/UEV. KM and DAW designed, performed and analysed the mass spectrometry of Mib and Vps9; GP conceived the idea of the reconstituted ubiquitylation system, designed and planned experiments, analysed data and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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