ABSTRACT

Polyubiquitin chains linked via lysine (K) 63 play an important role in endocytosis and membrane trafficking. Their primary source is the ubiquitin protein ligase (E3) Rsp5/NEDD4, which acts as a key regulator of membrane protein sorting. The heterodimeric ubiquitin-conjugating enzyme (E2), Ubc13–Mms2, catalyses K63-specific polyubiquitylation in genome maintenance and inflammatory signalling. In budding yeast, the only E3 proteins known to cooperate with Ubc13–Mms2 so far is a nuclear RING finger protein, Rad5, involved in the replication of damaged DNA. Here, we report a contribution of Ubc13–Mms2 to the sorting of membrane proteins to the yeast vacuole via the multivesicular body (MVB) pathway. In this context, Ubc13–Mms2 cooperates with Pib1, a FYVE-RING finger protein associated with internal membranes. Moreover, we identified a family of membrane-associated FYVE-(type)-RING finger proteins as cognate E3 proteins for Ubc13–Mms2 in several species, and genetic analysis indicates that the contribution of Ubc13–Mms2 to membrane trafficking in budding yeast goes beyond its cooperation with Pib1. Thus, our results widely implicate Ubc13–Mms2 as an Rsp5-independent source of K63-linked polyubiquitin chains in the regulation of membrane protein sorting.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: FYVE domain, K63-polyubiquitylation, Membrane protein sorting, RING finger, Ubiquitin protein ligase, Ubiquitin-conjugating enzyme

INTRODUCTION

Post-translational modification with ubiquitin serves as a versatile means to modulate protein function, not least because of the diversity of ubiquitin signals (Kwon and Ciechanover, 2017).

Ubiquitin is usually attached to its substrates via an isopeptide bond between its C-terminus and an internal lysine (K) residue of the target protein. Modification is accomplished by the successive action of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin protein ligase (E3). Ubiquitin itself can serve as a target for further modification. Depending on which of its seven lysine residues is used as an acceptor for conjugation, polyubiquitin chains are formed with varying topology (Komander and Rape, 2012). Downstream effector proteins that recognise the modification via dedicated ubiquitin-binding domains mediate the biological effects (Husnjak and Dikic, 2012). As a consequence, ubiquitylation can impinge on the conformation, localisation or interactions of the modified protein. Polyubiquitin chain linkage is thought to play an important role in specifying the consequences of ubiquitylation. Whereas K48- and K11- and K29-linked polyubiquitylation have been implicated in proteasomal degradation, the K63-linkage is involved in the regulation of numerous non-proteasomal pathways ranging from the DNA damage response to inflammatory signalling, endocytosis, intracellular membrane trafficking and lysosomal targeting (Erpapazoglou et al., 2014; García-Rodríguez et al., 2016; Panier and Durocher, 2009; Wu and Karin, 2015).

The topology of a polyubiquitin chain is determined by the enzymes involved in its assembly (Suryadinata et al., 2014). Among the characterised E2s, Ubc13 is unique in exclusively generating K63-linked chains. Its linkage specificity arises from an obligatory interaction partner, the catalytically inactive E2 variant Mms2 (Hofmann and Pickart, 1999). Ubc13 cooperates with a number of E3s, such as human TRAF6, CHFR, RNF8, RNF168 and CHIP (Hodge et al., 2016). In the budding yeast, Saccharomyces cerevisiae, the only E3 known to function with Ubc13 is Rad5, which is involved in DNA damage bypass (Ulrich and Jentsch, 2000). All these E3s belong to the RING finger or the related U-box families and, in each case, Ubc13 apparently dictates linkage specificity (Zheng and Shabek, 2017). When combined with other E2s, some RING E3s, such as CHIP, can assemble polyubiquitin chains of alternative or even mixed linkages (Kim et al., 2007). In contrast to the RING and U-box proteins, E3s of the HECT and RBR families directly determine chain linkage. For example, the budding yeast HECT E3 Rsp5 predominantly catalyses ubiquitin polymerisation via K63 (Erpapazoglou et al., 2014). Most probably, the influence of these E3 families on linkage selectivity is due to their direct participation in the thioester-mediated transfer of ubiquitin to the acceptor lysine.

Together, Rsp5 (or its mammalian homologue NEDD4) and Ubc13 appear to be responsible for the bulk of K63-polyubiquitylation in yeast and vertebrate cells. Rsp5/NEDD4 is mainly involved in membrane trafficking, mediating ubiquitylation of various plasma membrane proteins and components of the endocytic machinery (Erpapazoglou et al., 2014). Human UBC13 (also calledUBE2N) cooperates with the E2 variant UBE2V2 (also called MMS2) in genome maintenance-related functions in the
nucleus, while in the cytoplasm it associates with UBE2V1 (also called UEV1A) for inflammatory signalling in the NF-κB pathway (Andersen et al., 2005). Surprisingly, in budding yeast, which lacks an NF-κB pathway, Ubc13–Mms2 also localises mainly to the cytoplasm and accumulates in the nucleus only in response to DNA damage (Ulrich and Jentsch, 2000), thus raising the question of its cytoplasmic function.

Here, we report that Ubc13–Mms2 from S. cerevisiae can act as a cognate E2 for a family of RING E3s associated with membranes of the endocytic and lysosomal compartments. Our findings are based on the identification of a poorly characterised RING E3, Pib1, as an interactor of Ubc13 and Mms2 in a two-hybrid screen. Pib1 localises to endosomal and vacuolar membranes via a lipid-binding FYVE domain (Burd and Emr, 1998; Shin et al., 2001) and plays a role in the multivesicular body (MVB) pathway where it acts redundantly with an Rsp5 adaptor, Bsd2, in delivering plasma membrane proteins to the vacuole (Nikko and Pelham, 2009). We found that Pib1 is highly selective for Ubc13–Mms2 in terms of both physical interaction and activation of ubiquitin transfer, and that the proteins cooperate in vivo as a K63-specific E2–E3 pair. Moreover, we observed a comparable activity with Ubc13–Mms2 in a series of other RING finger proteins associated with membranes of the endocytic and lysosomal compartments. Some of these share the FYVE-RING domain arrangement of Pib1, such as the postulated Pib1 homologue from Schizosaccharomyces pombe (SpPib1, also known as Putative E3 ubiquitin-protein ligase C36B7.05c) (Kampmeyer et al., 2017), a protein from Ustilago maydis involved in mRNP particle transport (Upa1, also known as UMA1_12183) (Pohlmann et al., 2015) and human Rififylin (RFFL, also known as CARP2 or Sakura), which harbours a FYVE-like domain (Coumaillou et al., 2004). Consistent with these interactions, Ubc13–Mms2 functionally contributes to the delivery of membrane proteins to the vacuole via the MVB pathway, where it cooperates with Pib1, but also exhibits additional Pib1-independent activity. In combination with an analysis of genetic interactions, our data widely implicate Ubc13–Mms2 as a significant Rsp5-independent source of K63-polyubiquitin chains in the regulation of intracellular membrane protein sorting and turnover.

RESULTS

Ubc13 interacts with the dimeric RING finger protein Pib1

In an effort to identify potential regulators of the heterodimeric E2 complex Ubc13–Mms2, we performed a two-hybrid screen using the S. cerevisiae E2 variant Mms2 as a bait. From a budding yeast genomic library of ∼5×10⁶ clones (James et al., 1996), we obtained at least five independent isolates encoding the full sequence of Pib1 as the only verifiable interactor. This 286 amino acid protein harbours an N-terminal FYVE domain and a C-terminal RING finger (Fig. 1A) (Burd and Emr, 1998). When analysed individually in two-hybrid assays, Pib1 exhibited a moderate interaction with Ubc13, Mms2 and itself in the two-hybrid assay (AD, Gal4 activation domain; BD, Gal4 DNA-binding domain). Growth on -His medium indicates positive interactions; growth on -His-Ado medium indicates strong associations. Growth on control medium containing His and Ade is also shown (ctrl.). Images shown are representative of n=3 experiments. (C) Pib1–Mms2 interaction is mediated by Ubc13. Two-hybrid analysis was performed as in B using mms2Δ or ubc13Δ mutant reporter strains. Images shown are representative of n=2 experiments. (D) Mms2 requires Ubc13 for interaction with Pib1 in vitro. Association of recombinant GST-Pib1FYVE with HisUbc13 and HisMms2 was assessed by pre-loading GST-Pib1FYVE or GST onto glutathione Sepharose before incubation with 1 µM recombinant HisUbc13, HisMms2 or HisUbc13–HisMms2 complex. Bound material was detected by western blotting with an anti-His6-tag antibody and Ponceau staining of the membrane. Blots shown are representative of n=4 experiments.

![Fig. 1. Pib1 interacts with Ubc13 in a manner that is enhanced by Mms2.](https://example.com/figure1)

(A) Schematic representation of Pib1 domain structure. (B) Interactions of Pib1 with Ubc13, Mms2 and itself in the two-hybrid assay (AD, Gal4 activation domain; BD, Gal4 DNA-binding domain). Growth on -His medium indicates positive interactions; growth on -His-Ado medium indicates strong associations. Growth on control medium containing His and Ade is also shown (ctrl.). Images shown are representative of n=3 experiments. (C) Pib1–Mms2 interaction is mediated by Ubc13. Two-hybrid analysis was performed as in B using mms2Δ or ubc13Δ mutant reporter strains. Images shown are representative of n=2 experiments. (D) Mms2 requires Ubc13 for interaction with Pib1 in vitro. Association of recombinant GST-Pib1FYVE with HisUbc13 and HisMms2 was assessed by pre-loading GST-Pib1FYVE or GST onto glutathione Sepharose before incubation with 1 µM recombinant HisUbc13, HisMms2 or HisUbc13–HisMms2 complex. Bound material was detected by western blotting with an anti-His6-tag antibody and Ponceau staining of the membrane. Blots shown are representative of n=4 experiments.

The robust interaction between Pib1 and Ubc13 pointed to a direct association, whereas the weak interaction between Pib1 and Mms2 might reflect an indirect contact mediated by endogenous Ubc13 in the two-hybrid assay. We therefore deleted either UBC13 or MMS2 in the reporter strain (Fig. 1C). Consistent with an Ubc13-mediated indirect interaction between Pib1 and Mms2, the two-hybrid signal was abolished in the ubc13Δ background. Surprisingly, deletion of MMS2 also weakened the interaction between Pib1 and Ubc13 in one orientation, suggesting a contribution of Mms2 to the interaction between Pib1 and the E2. Mutations in Ubc13 corresponding to positions that generally mediate RING binding in E2s, such as K6, K10, M64 and S96, also

Fig. 1. Pib1 interacts with Ubc13 in a manner that is enhanced by Mms2. (A) Schematic representation of Pib1 domain structure. (B) Interactions of Pib1 with Ubc13, Mms2 and itself in the two-hybrid assay (AD, Gal4 activation domain; BD, Gal4 DNA-binding domain). Growth on -His medium indicates positive interactions; growth on -His-Ado medium indicates strong associations. Growth on control medium containing His and Ade is also shown (ctrl.). Images shown are representative of n=3 experiments. (C) Pib1–Mms2 interaction is mediated by Ubc13. Two-hybrid analysis was performed as in B using mms2Δ or ubc13Δ mutant reporter strains. Images shown are representative of n=2 experiments. (D) Mms2 requires Ubc13 for interaction with Pib1 in vitro. Association of recombinant GST-Pib1FYVE with HisUbc13 and HisMms2 was assessed by pre-loading GST-Pib1FYVE or GST onto glutathione Sepharose before incubation with 1 µM recombinant HisUbc13, HisMms2 or HisUbc13–HisMms2 complex. Bound material was detected by western blotting with an anti-His6-tag antibody and Ponceau staining of the membrane. Blots shown are representative of n=4 experiments.
affected the Pib1–Ubc13 interaction (Fig. S1B). This indicates that Ubc13 contacts the Pib1 RING domain in a ‘standard’ orientation that is comparable to its interaction with other E3s, including Rad5 (Metzger et al., 2014; Ulrich, 2003).

We confirmed the direct interaction of Pib1 with Ubc13–Mms2 in vitro by immobilising recombinant, GST-tagged Pib1ΔFYVE on glutathione Sepharose and examining retention of the E2. Consistent with the two-hybrid data, Ubc13 alone or in complex with Mms2 bound to GST-Pib1ΔFYVE, whereas Mms2 failed to interact with GST-Pib1ΔFYVE in the absence of Ubc13 (Fig. 1D). Again, the presence of Mms2 enhanced the interaction between Ubc13 and Pib1.

In the two-hybrid assay, association of Pib1 with itself was also noted (Fig. 1B). Size-exclusion chromatography analysis of a series of recombinant Pib1 truncation constructs revealed a running behaviour consistent with dimerisation, mediated by a sequence N-terminal to, and including, the RING finger. The minimal region adjacent to the RING domain required for robust dimerisation was found to be between 50 and 100 amino acids in length (Fig. S1C).

Pib1 is an E3 with high selectivity for Ubc13–Mms2

Our results suggested that Pib1 acts as a cognate E3 for Ubc13–Mms2. Indeed, in vitro ubiquitin polymerisation by Ubc13–Mms2 was strongly stimulated by the addition of Pib1ΔFYVE in a concentration-dependent manner (Fig. 2A). Chain formation proceeded exclusively via K63, as mutant ubiquitin, Ub(K63R), was not polymerised at all (Fig. 2B), and the K63-specific ubiquitin isopeptidase AMSH (McCullough et al., 2004) efficiently disassembled the products of the Pib1-stimulated reaction (Fig. 2C). Comparison of the in vitro activities of Pib1 truncation constructs showed a correlation between stimulation of polyubiquitin chain synthesis and Pib1 dimerisation, indicating that Pib1 is more effective as a dimer than as a monomer, but its FYVE domain is not required for catalytic activity (Fig. S2A).

As some E3s can function with multiple E2s, we asked how specific the Pib1–Ubc13–Mms2 interaction was. In fact, Pib1 has previously been reported to cooperate with one of the most non-selective human E2s, UBCH5A (also known as UBE2D1), in vitro (Shin et al., 2001); however, these observations were made using GST-tagged versions of both Pib1 and the E2, which might have induced inappropriate E2–E3 pairing. In a systematic two-hybrid assay with all budding yeast E2s, Pib1 exclusively interacted with Ubc13 and Mms2 (Fig. S2B–D). In vitro interaction assays with GST-Pib1ΔFYVE showed no significant binding to a range of recombinant yeast E2s, including Rad6, Cdc34, Ubc7 and two highly promiscuous UBCH5A homologues, Ubc4 and Ubc5 (Fig. 2D). Moreover, and consistent with a high selectivity for Ubc13–Mms2, Pib1ΔFYVE did not stimulate ubiquitin polymerisation by Ubc4 or Ubc7 (Fig. 2E), even though both E2s were active in combination with appropriate partners, Rsp5 and Cue1 (Bazirgan and Hampton, 2008), respectively (Fig. 2F,G). Notably, Rsp5 and Cue1 activated only their cognate E2, but not Ubc13–Mms2, thus indicating selectivity on the side of the E2s as well.

In order to exclude the possibility that the failure of Pib1 to activate Ubc4 was due to the lack of a suitable substrate, we performed thioester discharge assays with Ubc13 and Ubc4. Here, the ability of an E3 to stimulate E2 activity is measured by its effect on a substrate-independent discharge of the ubiquitin thioester from the E2 to free lysine (Branigan et al., 2015). In order to prevent an intramolecular attack on the catalytic cysteine in Ubc13 or Ubc4, we used E2 mutants, Ubc13(K92R) and Ubc4(K91R), which are catalytically active but resistant to autoubiquitylation (McKenna et al., 2001). Consistent with its high selectivity for Ubc13, Pib1 promoted the discharge of the Ubc13 thioester (Ubc13–Ub), but not the Ubc4 thioester (Ubc4–Ub), whereas effective discharge of Ubc4–Ub was observed with Rsp5 (Fig. 2H, I).

Based on these observations, we conclude that Pib1 acts as an E3 with high selectivity for Ubc13–Mms2, and – as was observed with other RING E3s – the E2 dictates the linkage specificity of the reaction.

Ubc13–Mms2 cooperates with multiple RING E3s associated with internal membranes

Pib1 shares its domain arrangement with a number of proteins from other organisms (Fig. 3A). The FYVE domain in association with a FYVE-like domain was found to be between 50 and 100 amino acids in length (Fig. S1C).

Systematic two-hybrid assays (Fig. 3B and Fig. S3) revealed interactions of the fungal FYVE-RING proteins with budding yeast Ubc13 and with its human homologue (UBC13, also known as UBE2N) and weaker interactions with the Ubc13 cofactors, Mms2 and the human E2 variants, UBE2V1 and UBE2V2. In addition, SpPib1 displayed robust interactions with a range of human E2s, among them members of the UBCH5 family (UBE2D1, UBE2D2 and UBE2D3). A similar, possibly even less selective interaction pattern was observed for ZNR2F. In contrast, RFFL did not show any strong interactions in the two-hybrid assay. The cytoplasmic domain of Tu1 displayed weak interaction with Ubc13, but also with Ubc6 and a number of human E2s, including the UBCH5 family and UBE2V1. A C-terminal construct of Hrd1 did not exhibit measurable interactions with any yeast E2s, but with some human E2s, including UBC13 (UBE2N).

Stable associations between E2 and RING finger proteins do not necessarily correlate with efficient cooperation in ubiquitin transfer (Lorick et al., 1999). Therefore, we performed in vitro ubiquitin polymerisation assays using the purified E3s in combination with budding yeast Ubc4, Ubc7 and Ubc13–Mms2. All of the RING finger proteins strongly stimulated Ubc13–Mms2 in K63-polyubiquitylation (Fig. 3C–H), indicating that robust interactions are not required for productive cooperation. In contrast, only Hrd1 stimulated Ubc7 activity but only in the presence of Cue1 (Fig. 3H and Fig. S4A). Hrd1 and RFFL also promoted the assembly of ubiquitin conjugates by Ubc4. For the latter E2–E3 pair, use of a series of ubiquitin mutants indicated a diverse set of linkages and attachment sites among the conjugates, as only a lysine-less ubiquitin variant significantly interfered with their assembly (Fig. S4B).

In order to exclude the possibility that the observed selectivities were due to species-specific features, we repeated the ubiquitin polymerisation assays with the recombinant human E2s, UBC13–UBE2V2 and UBC5A. As expected, human
UBC13–UBE2V2 was active with both human E3s (Fig. S4C). Human UBCH5A proved even less selective than its yeast homologue, Ubc4, cooperating at least to some degree with all the constructs tested (Fig. S4C). However, stimulation of Pib1 by UBCH5A was marginal compared to Ubc13–Mms2 or UBC13–UBE2V2.

Taken together, these observations suggest that K63-specific polyubiquitylation in cooperation with Ubc13–Mms2 may be a salient feature of an entire group of membrane-associated RING E3s. Although some of them also appear to be able to cooperate with other E2s, possibly resulting in alternative linkages, Pib1 is one of the most highly selective E3s.
Fig. 2. Pib1 is an E3 with high selectivity for Ubc13–Mms2. (A) Pib1 stimulates in vitro polyubiquitin chain synthesis by Ubc13–Mms2. Recombinant GSTPib1-FYVE, H13Ubc3 and untagged Mms2, as well as ATP, were added as indicated to reactions containing E1 and ubiquitin and incubated at 30°C for the indicated times. Products were analysed by western blotting with an anti-ubiquitin antibody. (B) Pib1 selectively stimulates the synthesis of K63-linked polyubiquitin chains. In vitro ubiquitination reactions were set up as in A, using WT or a K63R mutant ubiquitin, and incubated at 30°C for 30 min. (C) Pib1-catalysed chains are disassembled by the K63-specific ubiquitin isopeptidase AMSH. Conjugation reactions like those shown in B were terminated by addition of apyrase, followed by further incubation in the presence or absence of GST-AMSH at 37°C for the indicated times. (D) Pib1 selectively interacts with Ubc13–Mms2. Interaction of GSTPib1-FYVE with HisRad6, HisCdc34, HisUbc4, HisUbc13, HisMms2 and HisUbc7 was analysed as described in Fig. 1D. (E–G) Pib1, Rsp5 and Cue1 exhibit distinct E2 and linkage selectivities in polyubiquitin chain synthesis. In vitro ubiquitination assays were carried out at 30°C for 60 min as described in A, using HisUbc4, Ubc7His and H13Ubc13–Mms2 as E2s and GSTPib1-FYVE, GSTRsp5 and GSTCue1His as E3s, respectively. Use of mutant ubiquitin is indicated by K63R or K48R, respectively. (H) Discharge of the Ubc13-thioester is enhanced by Pib1. Ubc13 (K92R) was charged with ubiquitin in the presence of E1, ATP and ubiquitin for 60 min at 30°C. Reactions were stopped by apyrase treatment, and discharge of Ubc13–Ub was initiated by the addition of lysine (for all conditions), Mms2 and Pib1-RING+100aa as indicated. Reactions were incubated at 30°C for the indicated times and analysed under non-reducing conditions unless indicated (+DTT, addition of dithiothreitol). (I) Ubc4-thioester discharge is stimulated by Rsp5, but not by Pib1. Ubc4(K91R) was charged with ubiquitin for 10 min at 30°C, and discharge of Ubc4–Ub was analysed in the presence of lysine (in all conditions) and Rsp5 or Pib1-RING+100aa as indicated, using non-reducing conditions unless otherwise noted (+DTT). Blots and gels are representative of n=2 (A–E,H,I) or n=2 (F,G) experiments performed under identical or similar conditions.

Ubc13–Mms2 colocalises with Pib1 at internal membranes

Pib1 localises to membranes of the endocytic compartment via its lipid-binding FYVE domain and accumulates at the vacuolar periphery (Burd and Emr, 1998; Shin et al., 2001). As expected, the fungal E3s featuring a genuine FYVE domain localised in a Pib1-like pattern when expressed as fusions to GFP in budding yeast (Fig. 4A). In contrast, RFFL was targeted predominantly to the plasma membrane. This is consistent with the situation in mammalian cells, where RFFL has been reported to associate with both the plasma membrane and the endocytic compartment (Counailleau et al., 2004; McDonald and El-Deiry, 2004), and its targeting was described to be in part governed by palmitoylation (Araki et al., 2003).

Although the Ubc13–Mms2 complex is mostly distributed in the cytoplasm in a diffuse pattern, sporadic vacuole-associated Ubc13GFP foci colocalising with Pib1mCherry were detectable in some of the cells, consistent with an interaction of the proteins in vivo (Fig. 4B). Upon overexpression of Pib1mCherry by means of a copper-inducible promoter, localisation of Ubc13GFP to the vacuolar membrane was strongly enhanced, indicating that Pib1 is able to recruit the E2 to internal membranes (Fig. 4B,C). Consistent with the mode of E2–E3 interaction defined above (Fig. S1A), recruitment of Ubc13 required an intact Pib1 RING finger, as mutation of I227 to alanine abolished the colocalisation without affecting membrane association of Pib1 itself (Fig. 4C,D). In addition, Ubc13 recruitment was independent of Mms2 (Fig. 4E), but membrane association of Mms2 in turn required the presence of Ubc13 in this assay (Fig. 4F).

In order to evaluate the specificity of Ubc13–Mms2 recruitment, we also monitored the localisation of Ubc13GFP upon overexpression of mCherry-tagged Hrd1 (Fig. 4C). Although ER association of Hrd1mCherry was clearly detectable, Ubc13GFP did not accumulate at this compartment (Fig. 4G). These data indicate that the complex of Ubc13–Mms2 and Pib1 is characterised in the two-hybrid screen and verified biochemically is also identifiable in the physiological environment of a cell, and that recruitment of the E2 is specific for Pib1 and the endocytic compartment.

Genetic interactions implicate Ubc13–Mms2 in membrane protein sorting

The cooperation of Ubc13–Mms2 with the membrane-associated E3s in vitro and their colocalisation in cells strongly suggested a functional involvement of Ubc13–Mms2 in the endocytic pathway. In order to test this hypothesis, we used analysis of genetic interaction profiles as an unbiased approach to identify gene functions (Costanzo et al., 2011). We mined a previously published genome-scale genetic interaction map (Costanzo et al., 2016) for genes whose interaction patterns resembled those of UBC13 and MMS2 (Table S1). This map comprises fitness measurements based on colony size for ~23 million double-mutant combinations, covering ~90% of budding yeast genes. In this data set, the genetic interaction profiles of UBC13 and MMS2 correlated not only with each other, but also – to a comparable extent – with those of various genes involved in vacuolar transport and the MVB pathway (Fig. 5A,B). Based on gene ontology (GO) term analysis, these pathways were strongly overrepresented among the most highly correlated genes for both UBC13 and MMS2 (Fig. 5C). In particular, numerous genes encoding components of all ESCRT complexes correlated at the top of the list (these were HSE1 and YNR0005C, a dubious predicted ORF overlapping with VPS27, for ESCRT-0; SNR2 and MVB12 for ESCRT-I; VPS25 for ESCRT-II and the ESCRT-III-associated VPS4). Similarly, the UBC4 interaction profile correlated with those of MVB pathway-associated genes, as expected from Ubc4’s cooperation with Rsp5 (Fig. 5C). In contrast, such correlations were not found for genes encoding other E2s like RAD6, UBCC7 or UBC5, suggesting that those observed for UBC13, MMS2 and UBC4 are specific. Surprisingly, the genetic interaction profile of PIB1 did not correlate with those of UBC13 and MMS2, and although more than 20% of the top PIB1-correlating genes (>0.13) encoded proteins associated with either the plasma membrane or the vacuolar compartment, no single GO term was significantly overrepresented.

Analysis of individual genetic relationships (Table S2) revealed significant negative interactions of both UBC13Δ and MMS2Δ mutants with candidates that were expected based on their contribution to genome maintenance (such as rev1Δ, rev3Δ, rev7Δ and pol32Δ), but also with three different rps5 alleles and (for UBC13Δ) with tul1Δ (Fig. 5D,E). Thus, both genetic interaction profiles and individual genetic interactions suggested a functional role for Ubc13–Mms2 in membrane protein sorting. Although individual genetic interaction data obtained by high-throughput assays should be evaluated with care due to the potential to generate false positives, the statistical analysis as well as the overlap between UBC13 and MMS2 provide additional confidence, consistent with the well-established cooperation between the two proteins.

Ubc13–Mms2 contributes to membrane protein turnover via the MVB pathway

In order to directly evaluate a potential role of Ubc13–Mms2 in the endocytic pathway, we focussed on a set of membrane proteins whose trafficking has been reported to involve K63-polyubiquitylation. We first asked whether Ubc13 might cooperate with Pib1 in the modification of the v-SNARE Snc1, which was previously found to be subject to K63-linked polyubiquitylation (Xu et al., 2017). This modification was reported to mediate interactions with the COPII complex, thus...
contributing to the recycling of Snc1 to its donor membranes (Xu et al., 2017). Based on the impairment of a pib1Δ tul1Δ double mutant in Snc1 recycling, Pib1 and Tul1 were postulated to act redundantly as E3s modifying the v-SNARE (Xu et al., 2017). Ubc13–Mms2 could thus contribute to the modification as an E2. However, although Snc1 ubiquitylation was detectable in our hands, we were unable to detect any modification in the Snc1 Δpib1 Δtul1 double mutant using mass spectrometry or Western blotting, suggesting that the modification occurs prior to the binding of the E3s. To further investigate the role of the E3s in Snc1 ubiquitylation, we performed a series of experiments using a range of membrane-associated RING E3s.

Fig. 3. Other membrane-associated RING E3s share Pib1’s selectivity for Ubc13–Mms2. (A) Domain structures of the indicated membrane-associated RING E3s (TM: transmembrane domain). (B) Membrane-associated RING E3s interact with a range of budding yeast and/or human E2s in the two-hybrid assay. Strong interactions are indicated in bold. Original plates are shown in Fig. S3. (C–H) Selected RING E3s cooperate with Ubc13–Mms2 in K63-polyubiquitylation. Recombinant SpPib1(123–279) (C), GSTUpa1(1131–1287) (D), RFFL(97–363) (E), Tul1(655–758) (F), GSTZNRF2 (G) and Hrd1(325–551) (H) were analysed in ubiquitin chain formation assays with the indicated recombinant E2s (see Fig. 2E–G) as described in Fig. 2A. All reactions contained E1, ATP and ubiquitin (WT or K63R) and were carried out at 30°C for 60 min. Blots shown are representative of n=2 experiments.
the modification pattern was not altered in \textit{ubc13Δ, pib1Δ, tul1Δ} or \textit{pib1Δ tul1Δ} mutants (Fig. S5A). Snc1 has also been identified as a target of K63-ubiquitylation upon treatment of yeast with hydrogen peroxide in a proteomic screen (Silva and Vogel, 2015). Although Snc1 ubiquitylation levels were enhanced by addition of hydrogen peroxide, deletion of \textit{UBC13, PIB1} and/or \textit{TUL1} did not diminish them (Fig. S5B). However, the modification was completely abolished in an \textit{rsp5} mutant (Fig. S5C). These results argue against Ubc13 acting with Pib1 and Tul1 directly on Snc1, but rather imply an indirect effect of the two E3s on Snc1 recycling or turnover.

K63-polyubiquitylation is also involved in the endocytosis of plasma membrane transporters such as the uracil permease Fur4 (Galan and Haguenuer-Tsapis, 1997). Modification of Fur4 itself...
is mediated by Rsp5 (Galán et al., 1996), but other ligases, including Pib1, contribute to cargo delivery to the vacuole via the MVB pathway (Nikko and Pelham, 2009). When we monitored endocytosis of Fur4 by uracil uptake assays after cycloheximide treatment (Volland et al., 1994), we observed delays in both ubc13Δ and mms2Δ mutants (Fig. 6A). Fur4 ubiquitylation was unaffected in ubc13Δ and pib1Δ mutants, suggesting that the permease is not a direct target of this E2–E3 pair (Fig. 6B). In the MVB pathway, Pib1 acts redundantly with an endosomal Rsp5 adaptor, Bsd2, which facilitates transport of endocytic vesicles from the Golgi to the vacuole (Nikko and Pelham, 2009). In consequence, Fur4 accumulates in endosomes and fails to be degraded in the vacuole. In order to determine the step at which Ubc13–Mms2 participates in Fur4 turnover, we employed fluorescence microscopy to monitor the localisation of GFP-tagged Fur4 after inducing endocytosis by means of cycloheximide treatment in a bsd2Δ background. We found that deletion of UBC13, like deletion of PIB1, did not prevent Fur4 internalisation, but caused its accumulation in internal vesicles (Fig. 6C). Colocalisation with mCherry-tagged Vph1, a subunit of the vacuolar ATPase, confirmed that these vesicles were predominantly situated at the vacuolar periphery (Fig. S5D). Residual GFP signal in the vacuole of bsd2Δ pib1Δ mutants, but not bsd2Δ ubc13Δ, suggested a stronger defect in Ubc13—compared to Pib1-deficient cells.

If Ubc13–Mms2 cooperates with Pib1 in the MVB pathway, then ubc13Δ and pib1Δ mutations should behave epistatically with respect to Fur4 degradation in a bsd2Δ background. In order to assess this quantitatively, we made use of the fact that the GFP moiety of Fur4GFP is resistant to proteolysis and transiently accumulates in the vacuole (Nikko and Pelham, 2009). This allows for the monitoring of free GFP as a reporter for vacuolar delivery. A doa4Δ mutant was used as a control because it was previously shown to be completely defective in Fur4 internalisation and vacuolar targeting (Galán and Hagenauer-Tsaps, 1997). As reported previously (Nikko and Pelham, 2009), deletion of PIB1 alone had no effect, but did impede Fur4GFP degradation in a bsd2Δ background (Fig. 6D–E, Fig. S5E). The ubc13Δ mutant exhibited a phenotype similar and additive to that of bsd2Δ. Importantly, pib1Δ did not significantly enhance the ubc13Δ phenotype, either alone or in combination with bsd2Δ. This implies that Ubc13–Mms2 cooperates with Pib1 in vacuolar delivery of Fur4. The defect of the ubc13Δ single mutant suggests that the E2 fulfils additional roles in membrane trafficking not shared by Pib1 or Bsd2. The tul1Δ mutant or combinations of tul1Δ with bsd2Δ or pib1Δ were not impaired in Fur4 degradation, indicating that Tul1 does not functionally overlap with Pib1 in Fur4 turnover (Fig. S5F).

In order to assess whether the activity of Ubc13–Mms2 extended to other endocytic cargo proteins, we characterised the cadmium-induced vacuolar delivery of the metal transporter Smf1, which was reported to also involve Bsd2 and Pib1 (Nikko and Pelham, 2009). Consistent with a contribution of Ubc13–Mms2, GFP-Smf1 accumulated in vesicles and at the vacuolar periphery in bsd2Δ ubc13Δ and bsd2Δ mms2Δ double mutants (Fig. 7).

Thus, consistent with the genetic interaction data (Fig. 5), Ubc13-mediated ubiquitylation appears to exert an effect on the endocytic system at the stage of vacuolar delivery of endocytic vesicles via the MVB pathway. In this context the E2 cooperates with Pib1, but our data suggest additional, Pib1-independent activities that might reflect a cooperation with other E3s. This function of Ubc13–Mms2 is distinct from that of Rsp5, but is important for the turnover of plasma membrane proteins like Fur4 and Smf1. In conclusion, our observations suggest that K63-polyubiquitylation in the endocytic compartment is mediated not exclusively by Rsp5, but also by Ubc13–Mms2.

**DISCUSSION**

**Contribution of Ubc13–Mms2 to K63-polyubiquitylation at internal membranes**

Polyubiquitin chains of K63-linkage have been implicated in many aspects of membrane trafficking (Erpapazoglou et al., 2014). In budding yeast, Rsp5 has so far been the only verified source of that chain type within the cytoplasmic compartment. Our results show that Ubc13–Mms2, aided by the RING E3 Pib1 and potentially other membrane-associated E3s, contributes to protein sorting in the MVB pathway, thus expanding the scope of this E2 beyond its known nuclear function with Rad5 in genome maintenance.

Recombinant Pib1 was previously reported to cooperate *in vitro* with a highly non-selective E2, UBCH5A (Shin et al., 2001), which is not known for a particular bias towards the K63-linkage. Our results indicate a clear preference of Pib1 for Ubc13–Mms2, thus strongly implicating this E2–E3 pair in the formation of K63-chains at internal membranes. An earlier claim regarding K63-specificity of Pib1 was made by Xu et al. (2017), who observed a redundant function of Pib1 and Tul1 in COPI-mediated vesicle sorting. They found that an interaction of the COPI complex with a ubiquitylated v-SNARE, Snc1, mediated by K63-selective ubiquitin binding, drives the recycling of the v-SNARE to its donor membranes. Deletion of PIB1 and TUL1 inhibited Snc1 recycling, and fusion of a non-specific deubiquitylating enzyme (DUB) to Pib1 or Tul1 interfered with Snc1 ubiquitylation and trafficking (Xu et al., 2017). From these indirect data they concluded that Pib1 – together with Tul1 – acts as a cognate E3 in K63-ubiquitylation of Snc1. However, by direct observation of its modification pattern, we found no defect in Snc1 ubiquitylation in the pib1Δ tul1Δ mutant, which suggests an indirect effect of the ligases on vesicle trafficking rather than an involvement in ubiquitin conjugation to the v-SNARE. The observed dominant negative effects exerted by the E3–DUB fusions could be attributable to a physical proximity of the ligases to Snc1,
mediated by the membrane association of the E3s, but they do not prove an enzyme-substrate relationship.

Our findings provide conclusive evidence for a cooperation of Ubc13–Mms2 with Pib1, and possibly with additional E3s, in K63-polyubiquitylation at endosomal and vacuolar membranes. However, several attempts using proteomic analysis and two-hybrid screens have failed to reveal any direct physiological substrates of Ubc13–Mms2 or Pib1 in our hands (C.R., V.T., T.K.A. and H.D.U. unpublished). One possible reason for this failure might be a high degree of redundancy between the ubiquitin conjugation factors.

Fig. 5. Genetic interactions implicate UBC13 and MMS2 in membrane protein sorting. (A) Functional similarity between UBC13, MMS2 and components of membrane protein sorting pathways. Histograms of Pearson correlation coefficients calculated between the genetic interaction profiles of UBC13 and MMS2 and ∼90% of all yeast genes are shown, obtained from a previously published genome-scale genetic interaction map (Costanzo et al., 2016). All interactions included in the analysis are shown in Table S1. (B,C) Genes involved in vacuolar transport and the MVB pathway are overrepresented among those whose interaction profiles most strongly correlate with UBC13 and MMS2 (Pearson correlation coefficient >0.13). The network of genes correlating with UBC13 or MMS2 is shown in B. All correlations within the group above the 0.13 threshold are shown. Genes involved in vacuolar transport (gene ontology term GO: 0007034) and the MVB sorting pathway (GO: 0071985) are highlighted. Note that YNR005C is a dubious ORF overlapping with VPS27. The fraction of genes with relevant GO terms among those depicted in B versus the total fraction of genes with these GO terms in the data set is shown in C. Significance is indicated by P-values. A corresponding analysis for UBC4 is shown for comparison. (D,E) UBC13 and MMS2 exhibit negative genetic interactions with genes involved in translesion synthesis and membrane protein sorting. Genetic interaction scores of UBC13 and MMS2 with relevant genes are shown in D, obtained from the same genetic interaction map as in A (Table S2). Significance is indicated by P-values (ns, not significant; P > 0.05). Note that two interaction scores are provided for some combinations, based on the availability of query and array alleles in the library. Plots of all genetic interaction scores for UBC13 and MMS2 are shown in E. Symbols indicate significant interactors from D.
involved in the endocytic pathway, as emerging information about E3s such as Rsp5, Pib1 and Tul1 suggests that they might be able to compensate for each other to some degree (Nikko and Pelham, 2009). Moreover, it is well established that delivery of membrane proteins to the vacuole requires multiple ubiquitylation events at various stages and on many different targets, not limited to the cargo proteins themselves. Redundancy between membrane-associated E3s also provides a likely explanation for the lack of a detectable phenotype of the pib1Δ single mutant and thus the absence of any GO term enrichment in its genetic interaction profile.

Finally, the marked preference of Ubc13–Mms2 for K63 of ubiquitin could indicate a predominant function in chain elongation rather than de novo modification of specific substrates. This is the case for Ubc13–Mms2 in cooperation with Rad5 (Hoege et al., 2002; Ulrich and Jentsch, 2000), and the exquisite specificity of Pib1 for Ubc13–Mms2 could indicate a similar phenomenon.

**Selectivity of E2–E3 pairing in membrane protein sorting**

Our identification of budding yeast Pib1 as an E3 with an unusually high preference for the K63-specific Ubc13–Mms2 complex raises...
Within the endocytic compartment, the reported colocalisation and redundancy between Pib1 and Tul1, along with our in vitro activity assays, suggest that Tul1 – like Pib1 – cooperates with Ubc13–Mms2 in K63-ubiquitylation. Yet, the protein was originally described to interact with Ubc4 (Reggiori and Pelham, 2002). Although we were unable to reproduce this finding or detect any catalytic activity with yeast Ubc4, our interaction and activity assays suggest that Tul1 might be less selective than Pib1.

Ubc13–Mms2 was also found to be active with a family of related E3s from other species, all harbouring a lipid-binding FYVE- or FYVE-like domain in addition to the RING finger. Interestingly, while they all supported Ubc13–Mms2 activity, the scope of their interactions and activities with other E2s varies considerably. The putative Pib1 homologue from S. pombe, SpPib1, displayed an identical localisation and a very similar activity pattern, although its interactions in the two-hybrid system were less selective than those of budding yeast Pib1. Given its preference for Ubc13, it is surprising that SpPib1 was reported to target a subunit of the exocyst, Sec3, for proteasomal degradation (Kampmeyer et al., 2017), a process usually associated with K48-linked polyubiquitylation. However, a degree of redundancy between Pib1 and other E3s (including Rsp5) was noted in that study, and a contribution of vacuolar degradation was not rigorously excluded, thus leaving open the possibility that multiple degradation systems act on Sec3. U. maydis Up1 closely resembled budding yeast Pib1 with respect to localisation, E2 binding and E2 activation, implying a similarly Ubc13-selective function. Interestingly, the protein bears a large N-terminal extension, including a motif for interaction with the key RNA transport protein Rrm4, which implicates Up1 in vesicle-mediated long-distance transport of mRNP particles (Pohlmann et al., 2015). Thus, the protein appears to couple endosome dynamics with RNA transport.

RFFL (also known as CARP2 or Sakura) displayed the lowest selectivity among the FYVE-type E3s. Accordingly, the protein has been reported to modify a diverse set of substrates, many of them localising to the plasma membrane or endocytic vesicles (Araki et al., 2003; Liao et al., 2008; McDonald and El-Deiry, 2004; Okiyoneda et al., 2018). RFFL was often observed to act redundantly with a closely related protein, RNF34 (also called CARP1 or Momo) (Araki et al., 2003; Liao et al., 2008). Whereas most instances of RFFL- and RNF34-mediated ubiquitylation appear to involve proteasomal degradation (Araki et al., 2003; Liao et al., 2009, 2008; McDonald and El-Deiry, 2004; Wei et al., 2012; Yang et al., 2007; Zhang et al., 2014), there are cases where a contribution of lysosomal proteolysis has been demonstrated (Jin et al., 2014; Okiyoneda et al., 2018). For example, in a recent report, RFFL was shown to mediate retrieval from the plasma membrane and subsequent lysosomal degradation of a non-native CFTR mutant via interaction and K63-polyubiquitylation in a post-Golgi compartment (Okiyoneda et al., 2018). In this context, cooperation with UBC13 appears plausible.

In conclusion, our data point to the existence of a conserved group of membrane-associated RING E3s that can cooperate with Ubc13–Mms2 in the generation of K63-linked polyubiquitin chains and contribute to protein sorting in the endocytic and MVB pathways. The molecular features determining their selectivity, as well as the relevant targets of ubiquitylation, remain to be explored.

**MATERIALS AND METHODS**

**Yeast strains, plasmids and antibodies**

All yeast strains used in this study are listed in Table S3. Single gene deletions in the BY4741 background were obtained from the yeast knockout collection (Dharmacon). Other gene deletions were generated by PCR-based
methods (Jank et al., 2004) or genetic crosses. The np1 strain, carrying a KanMX cassette within the RSP5 promoter, was a gift from Bruno André (Université Libre de Bruxelles, Faculté des Sciences, Belgium). Tagging of Pib1, Ubc13, Mms2 and Vph1 at their endogenous loci was achieved by recombination-mediated integration of PCR-amplified cassettes (Jank et al., 2004). Yeast strains were grown inYPD or, if selecting for maintenance of centromeric or 2 µ plasmids, synthetic complete (SC) medium lacking the appropriate amino acids [2% glucose unless otherwise noted, 6.7 g l⁻¹ yeast nitrogen base without amino acids (Sigma Aldrich), 86 mg l⁻¹ myo-inositol, 86 mg l⁻¹ uracil, 21 mg l⁻¹ adenine, 6.8 mg l⁻¹ p-aminobenzoic acid and 86 mg l⁻¹ of each of the 20 biogenic amino acids except lysine (172 mg l⁻¹)] and those specified for selection.

All plasmids are listed in Table S4. For Fur4 endocytosis assays, Fur4GFP was expressed under control of a GAL1 promoter from a centromeric vector based on YCpC3aR (Gietz and Sugino, 1988). For uracil uptake assays and visualisation of Fur4 ubiquitylation, myc-tagged Fur4 was expressed under control of its own promoter from a 2 µ vector, YEplac195 (Gietz and Sugino, 1988). Two-hybrid plasmids were based on pGT97 (Clontech) or the pGAD-C- and pGBD-C-series, expressing candidates as fusions to the Gal4 activation domain (GAD) or DNA-binding domain (GBD) and carrying the LEU2 and TRP1 markers, respectively (James et al., 1996). For localisation studies of Pib1 orthologues, the corresponding genes were cloned in frame with yeGFP under control of a CUP1 promoter into a YCpC3a3-based centromeric plasmid. Overexpression of mCherry-tagged HRD1, Pib1 and its RING finger mutant was achieved in the same manner. Plasmids for expression of HIS1-SC1 and HUS1-SC1(KR) under control of the TP11 promoter in budding yeast were kindly provided by Todd Graham (Xu et al., 2017). Point mutations were created by PCR-based mutagenesis. All antibodies are listed with their sources and dilutions where appropriate in the following sections. All have been extensively validated in this and other labs.

**Yeast two-hybrid assays**

The initial two-hybrid screen for interactors of Mms2 was performed with a histidine- and adenine-responsive reporter strain, PJ69-4a, and a set of three yeast genomic libraries kindly provided by Philip James (James et al., 1996). These libraries, constructed in the pGAD-C vector series representing all 3 reading frames, were amplified in E. coli, yielding between 80 and 200 million clones, and used in parallel for transformation of PJ69-4a expressing GBD-Mms2 from a bait plasmid carrying the URA3 marker. Approximately 5 million clones per library were screened on SC Ura− markers, respectively (James et al., 1996). For analysing interactions between Pib1 and the yeast E2s, this screen was repeated in the opposite direction, swapping GAD and GBD.

**Protein purification**

Recombinant proteins were produced in E. coli. Expression and purification conditions for all recombinant proteins are listed in Table S5. Briefly, Hiss-, and GST-tagged proteins were affinity-purified in batch on Ni-NTA agarose (Qiangen) and glutathione Sepharose 4 Fast Flow (GE Healthcare), respectively. Protease cleavage (PreScission, GE Healthcare; TEV-Protease, Biomol) was performed overnight at 4°C. Subsequent purification steps were carried out on an NGC chromatography system (Bio-Rad). Bovine ubiquitin (Ub) was purchased from Sigma-Aldrich and Ub(K6R) from Boston Biochem. His6-Ub (from mouse) and other ubiquitin variants were purified as described previously (Carvalho et al., 2012; Pickart and Raasi, 2005). GST-Cue1His and Hrd1 were purified as described before (Bogala et al., 2013) with some modifications.

**Ubiquitin chain formation assays**

Free ubiquitin chain formation was assayed in reactions containing 40 mM HEPES, pH 7.4, 8 mM magnesium acetate, 50 mM NaCl, 5 µM purified bovine ubiquitin (Sigma) or ubiquitin variants, 50 nM HisUba1, 30 µM ATP, 2 µM E2 and 0.5 µM E3. Reactions were incubated for 30 min (Pib1) or 60 min (other E2s) at 30°C (yeast E3s) or 37°C (human E3s), stopped by addition of NuPage LDS sample buffer (Thermo Fisher) supplemented with 25 mM DTT for 10 min at 95°C, and the eluate was analysed by SDS–PAGE and western blotting with anti-His-tag antibody (Sigma H1029, 1:5000).

**E2 thioester discharge assays**

Ubc13(K92R) was charged with ubiquitin for 60 min at 30°C in a reaction containing 40 mM HEPES, pH 7.4, 2 mM MgCl2, 50 mM NaCl, 240 nM His6-E1, 30 µM Ubc13(K92R), 24 µM ubiquitin and 200 µM ATP. The reaction was stopped by addition of 1 unit of apyrase (New England Biolabs) per 100 µl reaction and incubation at 30°C for 15 min. This reaction was then further incubated in the presence or absence of 2 µM GST-ASM at 37°C for up to 60 min.

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**E2 thioester discharge assays**

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**E2 and E3 localisation by fluorescence microscopy**

Fluorescence microscopy images were acquired with a DeltaVision Elite system (softWoRX 6.5.2) equipped with a 60× objective (NA 1.42) and a DV Elite sCMOS camera (GE Healthcare). Cells were grown to exponential conditions at 30°C for 10 min, and lysine discharge assays were performed with 1.5 µM Pib1-RING+100aa or Rsp5 at a final concentration of 5 mM L-lysine.

**Endocytosis and vacuolar delivery assays**

Fur4 internalisation was measured by uracil uptake assays as described previously (Galán and Haguenauer-Tsapis, 1997; Volland et al., 1994). Briefly, exponential cultures of the relevant strains expressing Fur4myc from
a multicopy plasmid, grown in SC Ura− medium, were treated with 100 µg ml−1 cycloheximide. At the indicated time points, 1 ml aliquots were incubated with 5 µM [14C]uracil for 20 s at 30°C and filtered immediately through Whatman GF/C filters. Filters were then washed twice with ice-cold water and subjected to scintillation counting. The amount of radiation taken up at each time point was plotted relative to the value at t=0. No technical replicates were performed, but the entire assay was repeated independently at least three times with comparable results, and a representative data set is shown in Fig. 6A.

Microscopic analysis of Fur4 endocytosis was performed by growing relevant strains harbouring YCplac33-GAL-Fur4-GFP overnight in SC Ura− medium containing 2% raffinose and 0.2% glucose, then shifted to medium containing 2% galactose and 0.1% glucose. After 2.5 h, 2% glucose was added, and incubation was continued for 30 min. Endocytosis was triggered by addition of 100 µg ml−1 cycloheximide, and aliquots were collected for microscopy at the specified times. Smf1 endocytosis was analysed in strains harbouring plasmid pRS416-ADH-GFP-Smf1 by growing cultures in SC Ura− medium to exponential phase and addition of CdSO4 for 30 min. Cells were mounted in SC medium and observed at room temperature with a motorised fluorescence microscope (model BX-61; Olympus) equipped with a Plan-Apochromat 100× oil-immersion objective (1.40 NA; Olympus), a Spot 4.05 charge-coupled device camera, and the MetaVue acquisition software (Molecular Devices). Images were processed using ImageJ (NIH, Bethesda, MD).

Delivery of Fur4 to the vacuole was assayed as described previously (Nikko and Pelham, 2009). Briefly, yeast cells harbouring YCplac33-GAL-Fur4-GFP were grown to exponential phase, treated with 2% galactose. After 2 h, 2% glucose was added, and incubation was continued for 30 min. Endocytosis was triggered by addition of 100 µg ml−1 cycloheximide, and aliquots were collected for microscopy at the indicated time points and flash-frozen on dry ice. Total cell extracts were prepared by trichloroacetic acid (TCA) precipitation as described previously (Morawska and Ulrich, 2013) with some modifications: precipitation was performed directly in SC medium without pelleting the cells, and the final pellet was resuspended in NuPage LDS sample buffer supplemented with 25 mM DTT and incubated for 20 min at 30°C. Samples were analysed by SDS–PAGE followed by western blotting with mouse anti-GFP (Roche #11814460001, 1:2000) and rabbit anti-α-tubulin antibodies (Abcam ab184970, 1:20,000). For Fig. 7B, Stain-Free technology (Bio-Rad) was used as a loading control.

Western blots were imaged with an Odyssey CLx system (LI-COR) using near-infrared fluorophore-labelled secondary antibodies. Signals were quantified (background correction: average) with Image Studio Version 3.1 (LI-COR). For quantification of vacuolar degradation of Fur4, three independent biological replicates with two technical replicates each were performed. Based on inspection of the blots, this number was deemed sufficient to account for variabilities in experimental conditions and detection of western blot signals. For each sample, the intensity of free GFP (800 nm channel) was normalised by division by the intensity of the tubulin loading control (700 nm channel). To account for variations in the amount of free GFP at t=0 in a given strain, these values were corrected by subtracting the normalised intensity at t=0. The resulting corrected signal of GFP at 4 h in the bsd2Δ strain was arbitrarily set to 1 for each individual western blot, and the other values were calculated relative to this reference point. For statistical analysis, unpaired two-tailed Student’s t-tests were performed with GraphPad Prism 8.3.0, using all six values collected for each sample and time point.

Detection of Fur4 and Snc1 ubiquitylation

Fur4 ubiquitylation was detected by expression of Fur4myc from a multicopy plasmid in the relevant yeast strains, preparation of a membrane-enriched lysate from exponential cultures as described previously (Galán et al., 1996) and SDS–PAGE followed by western blotting with an anti-myc antibody (9E10, produced in-house, 0.2 µg ml−1). For detection of Snc1 ubiquitylation, relevant yeast strains expressing wild-type 3HA-tagged Snc1 (pRS416-3xHA-Snc1) or a mutant lacking lysine residues [pRS416-3xHA-Snc1(8KR)] were grown to exponential phase, treated – where indicated – with 2.4 mM H2O2 for 45 min at 30°C and subjected to total cell extraction with TCA (see above), followed by SDS–PAGE and western blot analysis using an anti-HA antibody (Santa Cruz sc-805, 1:5000).

Analysis of genetic interaction data

Genetic interaction profile similarities of relevant E2 and E3 genes (Table S1) and genetic interaction scores of abc13 and mms2 (Table S2) were downloaded from http://www.thecellmap.org (Table S2) and used for genetic interaction profile similarities of relevant E2 and E3 genes (Table S2) were downloaded from http://www.thecellmap.org (Table S2) and used for genetic interaction profile similarities was performed using goTermFinder (https://www.yeastgenome.org/goTermFinder), considering all genes with a score above 0.13 after removing duplicates.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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