Deubiquitinating enzymes Ubp2 and Ubp15 regulate endocytosis by limiting ubiquitination and degradation of ARTs

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ABSTRACT **Endocytic down-regulation of cell-surface proteins is a fundamental cellular process for cell survival and adaptation to environmental stimuli. Ubiquitination of cargo proteins serves as the sorting signal for downstream trafficking and relies on the arrestin-related trafficking adaptor (ART)-Rsp5 ubiquitin ligase adaptor network in yeast. Hence proper regulation of the abundance and activity of these ligase–adaptor complexes is critical for maintenance of optimal plasma membrane protein composition. Here we report that the stability of ARTs is regulated by the deubiquitinating enzymes (DUBs) Ubp2 and Ubp15. By counteracting the E3 ubiquitin ligase Rsp5, Ubp2 and Ubp15 prevent hyperubiquitination and proteasomal degradation of ARTs. Specifically, we show that loss of both Ubp2 and Ubp15 results in a defect in Hxt6 endocytosis associated with Art4 instability. Our results uncover a novel function for DUBs in the endocytic pathway by which Ubp2 and Ubp15 positively regulate the ART-Rsp5 network.**

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INTRODUCTION

Ubiquitination is a reversible posttranslational modification involved in numerous cellular processes, including protein degradation, membrane trafficking, and signaling. Conjugation of ubiquitin to substrates is achieved by an enzymatic cascade involving E1 activating enzymes, E2 conjugating enzymes, and E3 ubiquitin ligases. Deubiquitinating enzymes (DUBs) remove ubiquitin from substrates and serve many cellular functions, including processing ubiquitin precursors, editing/rescuing ubiquitin conjugates, facilitating proteasomal degradation, and maintaining free ubiquitin pools (Amerik and Hochstrasser, 2004). Many DUBs and E3 ligases interact with each other and work in concert. Such DUB–E3 interactions can be used to fine-tune substrate ubiquitination, as well as regulate their own stability or activity (Nijman *et al.*, 2005).

Proper remodeling of cell-surface receptors and transporters is critical for cell survival in response to environmental stimuli or changing nutrient conditions. Endocytic down-regulation is a major mechanism to regulate the homeostasis and composition of cell-surface proteins. Ubiquitin has emerged as a critical component of endocytosis; ubiquitin conjugation to plasma membrane (PM) proteins, or cargoes, promotes their internalization, followed by sorting into multivesicular bodies (MVBs) and delivery to the lysosome/vacuole (Henne *et al.*, 2011; MacGurn *et al.*, 2012; Piper *et al.*, 2014). Failure to properly down-regulate PM receptors is associated with many human diseases. For example, inability to attenuate growth signaling by endocytosis can lead to abnormal cell proliferation and cancer (Mosesson *et al.*, 2008).

In budding yeast, arrestin-related trafficking adaptors (ARTs) recruit the Nedd4-like ubiquitin ligase Rsp5 to the PM and mediate cargo ubiquitination (Lin *et al.*, 2008; Nikko and Pelham, 2009; Hatakeyama *et al.*, 2010). ARTs have similarity to human arrestin domain-containing (ARRDC) proteins, with both having N-terminal arrestin-like domains and C-terminal PY (PPXY/LPXY) motifs (Lin *et al.*, 2008; Becuwe *et al.*, 2012a), and they promote PM protein remodeling through endocytic down-regulation in response to nutrient availability, environmental stress, and proteotoxic stress (Lin *et al.*, 2008; MacGurn *et al.*, 2011; Merhi and Andre, 2012;

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Abbreviations used: ART, arrestin-related trafficking adaptor; DUB, deubiquitinating enzyme; ESCRT, endosomal sorting complexes required for transport; MVB, multivesicular body; PM, plasma membrane.

Becuwe *et al.*, 2012b; Zhao *et al.*, 2013; Alvaro *et al.*, 2014; Crapeau *et al.*, 2014). The ART-Rsp5 network is highly regulated and influenced by intracellular signaling, such as phosphorylation/dephosphorylation (MacGurn *et al.*, 2011; Becuwe *et al.*, 2012b; O'Donnell *et al.*, 2013; Herrador *et al.*, 2015). For example, Art1/Ldb19 is phosphorylated and inhibited by Npr1, which in turn is inhibited by TORC1 (MacGurn *et al.*, 2011). Similarly, Art4/Rod1 is subjected to inhibitory phosphorylation by yeast AMPK Snf1 (Becuwe *et al.*, 2012b). Therefore ARTs are important regulatory hubs for ubiquitin-mediated endocytosis of PM proteins.

Of interest, ARTs are also Rsp5 substrates. In some cases, these ubiquitination events are critical for ART function in promoting PM protein turnover (Lin *et al.*, 2008; Herrador *et al.*, 2010; Becuwe *et al.*, 2012b), whereas in other cases, the role of ART ubiquitination is unclear (Kee *et al.*, 2006; Alvaro *et al.*, 2014). Similarly, ubiquitination of endocytic machinery and endosomal sorting complexes required for transport (ESCRTs) has been reported (Stamenova *et al.*, 2004; Gupta *et al.*, 2007; Lu *et al.*, 2008; Dores *et al.*, 2010; Lauwers *et al.*, 2010; Erpapazoglou *et al.*, 2012; Weinberg and Drubin, 2012, 2014), but the functional significance remains to be elucidated.

The human genome encodes ~90 DUBs with diverse functions (Nijman *et al.*, 2005; Ye *et al.*, 2009). Given the broad involvement of ubiquitination in endocytosis, as well as in other protein-sorting pathways, it is not surprising that DUBs have been implicated in many aspects of membrane protein trafficking (Millard and Wood, 2006; Clague *et al.*, 2012a,b). Although DUBs are clearly important for endocytic down-regulation of PM proteins in yeast (Amerik *et al.*, 2000b; Dupre and Haguenauer-Tsapis, 2001; Ren *et al.*, 2007; Lam *et al.*, 2009; Erpapazoglou *et al.*, 2012; Tardiff *et al.*, 2013; Weinberg and Drubin, 2014), precisely how they coordinate and interact with the ART-Rsp5 network remains unclear. Here we describe a novel function of the DUBs Ubp2 and Ubp15 in the endocytic pathway by which Ubp2 and Ubp15 protect ARTs from proteasomal degradation, and we propose deubiquitination of ARTs as a critical regulatory mechanism that contributes to the fine-tuning of ART-Rsp5 network activity.

RESULTS

Identifying potential ART-Rsp5–regulating DUBs

To investigate whether ART function is regulated by DUBs, we set out to look for changes of ART protein modifications and/or levels in strains with deletions of DUBs. We initially focused on Art1 and Art4, two of the well-characterized ARTs that regulate the ubiquitin-mediated endocytosis of several PM cargoes (Lin *et al.*, 2008; Nikko and Pelham, 2009; Becuwe *et al.*, 2012b; Becuwe and Leon, 2014; Alvaro *et al.*, 2014; O'Donnell *et al.*, 2015; Prosser *et al.*, 2015). In addition, both Art1 and Art4 are regulated by the phosphorylation/dephosphorylation cycle (MacGurn *et al.*, 2011; Becuwe *et al.*, 2012b; Alvaro *et al.*, 2014, 2016; Llopis-Torregrosa *et al.*, 2016), yet it is unclear whether Art1 and Art4 are subject to DUB-mediated regulation despite both of them being ubiquitinated by Rsp5 (Lin *et al.*, 2008; Becuwe *et al.*, 2012b).

To identify DUBs that potentially regulate Art1 and Art4, we took a candidate gene/protein approach and investigated the effects of DUBs with known genetic and/or physical interactions with the ART-Rsp5 network (Supplemental Figure S1A): Ubp2 has been shown to physically/genetically interact with the E3 ligase Rsp5 (Kee *et al.*, 2005, 2006; Lam *et al.*, 2009), as well as to deubiquitinate Art2/Ecm21 and Art8/Csr2 in vitro and influence their ubiquitination in vivo (Kee *et al.*, 2006). Ubp7 also physically interacts with Rsp5 (Ren *et al.*, 2007), and overexpression of Ubp7 and its paralogue Ubp11 affects Rsp5-mediated endosomal trafficking (Tardiff *et al.*, 2013). Ubp9 (and its paralogue Ubp13) and Ubp15 were also included in our initial consideration because they exhibit similar cellular localizations (peripheral punta or cytoplasmic punta; Huh *et al.*, 2003) to ARTs (Lin *et al.*, 2008; Herrador *et al.*, 2010; O'Donnell *et al.*, 2010; MacGurn *et al.*, 2011; Becuwe and Leon, 2014).

Plasmid-encoded Art4 and Art1 were expressed ectopically in yeast strains with deletions of these DUBs, and protein extracts were analyzed by immunoblot. We observed an apparent decrease in steady-state Art4 (and less obviously Art1) protein level in *ubp2*Δ (Figure 1A and Supplemental Figure S1B), a phenotype consistent with hyperubiquitination and enhanced protein turnover. To test whether any of these DUBs play a redundant role with Ubp2, we also examined Art4 and Art1 levels in *ubp2*Δ *ubp15*Δ, *ubp2*Δ *ubp7*Δ *ubp11*Δ, and *ubp2*Δ *ubp9*Δ *ubp13*Δ mutants. We found whereas *ubp2*Δ *ubp7*Δ *ubp11*Δ and *ubp2*Δ *ubp9*Δ *ubp13*Δ exhibited similar Art4 and Art1 levels to *ubp2*Δ, *ubp2*Δ *ubp15*Δ showed a more prominent decrease in Art4 and Art1 levels than with *ubp2*Δ (Figure 1A and Supplemental Figure S1B).

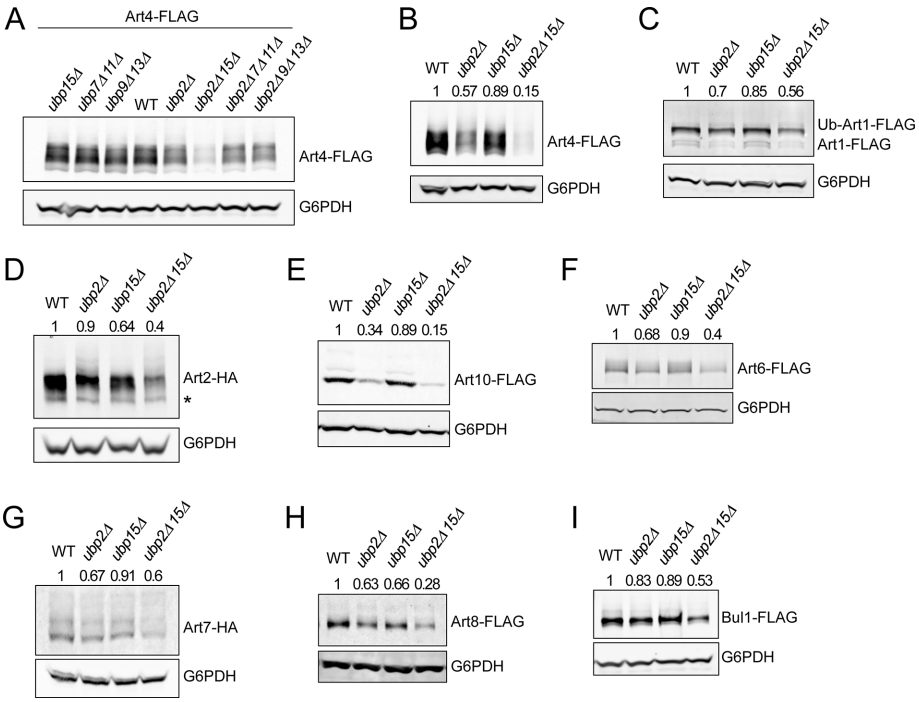

FIGURE 1: Ubp2 and Ubp15 maintain the abundance of Rsp5 adaptor proteins. (A) Protein extracts of wild-type (WT) or the indicated yeast mutant strains expressing plasmid-encoded Art4-FLAG were analyzed by SDS–PAGE and immunoblot. (B–I) Protein extracts of WT, *ubp2*Δ, *ubp15*Δ, and *ubp2*Δ *ubp15*Δ yeast cells expressing chromosomally tagged ARTs were analyzed by SDS–PAGE and immunoblot. Ub-Art1, K486-dependent ubiquitinated form of Art1 (Lin *et al.*, 2008). *Nonspecific band. The numbers above each lane indicate the protein abundance of ARTs determined by the LI-COR quantification and normalized with the loading control G6PDH.

Given its broad involvement in the endocytic pathway, we also tested Doa4, whose primary functions include deubiquitination in the MVB pathway, as well as maintaining cellular ubiquitin levels (Swaminathan *et al.*, 1999; Amerik *et al.*, 2000b). We found that *doa4*Δ completely suppressed the reduced abundance of Art4 and Art1 in *ubp2*Δ *ubp15*Δ (Supplemental Figure S1, C and D), indicating that Doa4 does not share a similar role to Ubp2 and Ubp15. Recently Ubp3 (as well as Ubp2) was shown to interact with Rsp5 upon heat stress and regulate the Rsp5-mediated cytosolic protein quality control pathway (Fang *et al.*, 2016). We therefore examined the effect of *ubp3*Δ, and found that *ubp3*Δ also partially suppressed the reduced abundance of Art4 in *ubp2*Δ *ubp15*Δ (Supplemental Figure S1E). Although the reduced Art4 and Art1 protein levels in *ubp2*Δ *ubp15*Δ cells are in line with increased ubiquitination and protein degradation in which Ubp2 and Ubp15 may play a direct role, the reverse phenotypes caused by *doa4*Δ and *ubp3*Δ are likely due to indirect effects, including reduced free ubiquitin levels (see *Discussion*). We therefore decided to focus on investigating Ubp2 and Ubp15 as potential regulators of Rsp5 adaptor proteins.

Ubp2 and Ubp15 regulate the abundance of Rsp5 adaptor proteins, the ARTs

We first asked whether the abundance of other ART proteins is regulated by Ubp2 and Ubp15 in a similar manner. To examine the levels of ARTs more precisely, we generated WT, *ubp2*Δ, *ubp15*Δ, and *ubp2*Δ *ubp15*Δ strains with epitope-tagged ARTs at their endogenous chromosome loci. We confirmed that steady-state levels of Art4 and Art1 were indeed reduced in both *ubp2*Δ cells and *ubp2*Δ *ubp15*Δ cells, with *ubp2*Δ *ubp15*Δ cells showing a greater reduction (Figure 1, B and C). These results suggest that Ubp2 is the major regulator for ARTs, and Ubp15 may be partially redundant with Ubp2.

Furthermore, protein levels of many other Rsp5 adaptors, including Art2, Art6/Aly1, Art7/Rog3, Art8, Art10, and Bul1 (Figure 1, D–I), were also significantly decreased. Among these adaptors, Art4 and Art10 showed the most drastic reduction in *ubp2*Δ *ubp15*Δ. By contrast, Art3/Aly2 and Art9/Rim8 protein levels exhibited little or no decrease in *ubp2*Δ *ubp15*Δ (Supplemental Figure S1, F and G). Of importance, the abundance of Rsp5 was not decreased (Supplemental Figure S1H). These data indicate that Ubp2 and Ubp15 regulate the abundance of several Rsp5 adaptor proteins.

Ubp15 physically interacts with ARTs

Ubp2 was previously reported to physically interact with Rsp5 (Kee *et al.*, 2005), providing a mechanism for regulation of ARTs by Ubp2. How Ubp15 is integrated into the ART-Rsp5 network, on the other hand, is not clear. To explore how Ubp15 regulates Rsp5 adaptor proteins, we performed stable isotope labeling with amino acid in culture (SILAC) analysis to identify Ubp15-interacting proteins and found Art2 as one of the top hits (Supplemental Figure S2A). We then performed coimmunoprecipitation (CoIP) experiment and confirmed the physical interaction between Art2-FLAG and GFP-Ubp15 (Supplemental Figure S2B). Consistent with the physical interactions (Rsp5-Ubp2 and Art2-Ubp15), Art2 abundance was also regulated by Ubp2 and Ubp15 (Figure 1D). The interaction between Ubp15 and Art2 raises an interesting possibility that Ubp15 might regulate other ARTs by physically associating with them as well. These interactions may not be stable enough to be detected under our experimental conditions for the Ubp15 interactome. To test directly whether Ubp15 interacts with Art4, we performed another CoIP experiment and found that indeed GFP-Ubp15 was coprecipitated with Art4-FLAG (Supplemental Figure S2C). Together these findings suggest that the genetic interaction we observed for *ubp2*Δ and *ubp15*Δ is likely to be physiologically relevant to the ART-Rsp5 network. Ubp2 and Ubp15 may regulate the ART-Rsp5 network through physical interactions: Ubp2 interacts with Rsp5, and Ubp15 interacts with ARTs. Given the greater reduction of ART protein levels in the *ubp2*Δ *ubp15*Δ double mutant compared with either single mutant, we conducted the remainder of this study by characterizing the double mutant in more detail.

Reduced abundance of ARTs in *ubp2*Δ *ubp15*Δ is due to accelerated protein turnover

To test whether the reduced abundance of ARTs in *ubp2*Δ *ubp15*Δ results from lack of deubiquitination on ARTs, we used a DUB catalytic domain (UL36) that renders its fusion proteins resistant to ubiquitination (Stringer and Piper, 2011) and assessed whether restoring deubiquitination artificially can suppress Art4 protein reduction in *ubp2*Δ *ubp15*Δ. Indeed, we found the Art4-UL36 fusion was able to maintain its abundance in *ubp2*Δ *ubp15*Δ cells, whereas fusion with catalytic-inactive UL36^{C40S} had no effect (Figure 2A). Furthermore, expressing functional Ubp2 or Ubp15, but not catalytically inactive Ubp2^{C745V} or Ubp15^{C214V}, restored Art4 levels in *ubp2*Δ *ubp15*Δ cells (Figure 2B), suggesting maintaining Art4 levels requires the catalytic activity of Ubp2 and Ubp15.

Defects in deubiquitination can lead to increased ubiquitination and subsequent protein degradation. To investigate whether the reduced abundance of ARTs in *ubp2*Δ *ubp15*Δ cells is due to protein degradation, we monitored protein turnover of Art4, as well as of Art1, in wild-type versus *ubp2*Δ *ubp15*Δ cells. The turnover of Art4 and Art1 in the presence of cycloheximide (CHX), which inhibits nascent protein synthesis, was accelerated in *ubp2*Δ *ubp15*Δ cells compared with WT cells (Figure 2, C–F). Given the drastic reduction in steady-state Art4 levels in *ubp2*Δ *ubp15*Δ cells, we further analyzed Art4 turnover after overexpression in this background and found the rate of Art4 turnover to be similar (Figure 2, C and D). These data indicate that decreased Art1 and Art4 levels are likely caused by protein degradation but not protein synthesis defects. Together our results demonstrate that the DUB activities of Ubp2 and Ubp15 prevent the degradation of ARTs.

ARTs are hyperubiquitinated and degraded by the proteasome in *ubp2*Δ *ubp15*Δ

In eukaryotic cells, ubiquitin-dependent protein degradation occurs in either the vacuole/lysosome or the proteasome. Proteins are delivered to the vacuole for degradation through either vesicle trafficking (the MVB pathway) or autophagy. Although ARTs are not transmembrane proteins and thus trafficking through the MVB pathway is unlikely, in principle they could still be degraded in the vacuole through autophagy. To test this possibility, we examined whether ART degradation in *ubp2*Δ *ubp15*Δ requires Pep4 and Prb1, two major vacuolar proteases (Hemmings *et al.*, 1981). The degradation of Art4, Art10, and Art1 in *ubp2*Δ *ubp15*Δ was not affected by *pep4*Δ *prb1*Δ (Figure 3, A–C), suggesting that their degradation does not occur in the vacuole.

We therefore investigated whether proteasomal degradation is involved. To sensitize yeast cells to proteasome inhibitor MG132, we used strains containing a deletion of the multidrug transporter gene *PDR5* (Fleming *et al.*, 2002). We found that MG132 substantially inhibited the degradation of Art4, Art10, and Art1 in *pdr5*Δ *ubp2*Δ *ubp15*Δ cells (Figure 3, D–F). Together with the observation that *PEP4* and *PRB1* are dispensable for ART degradation (Figure 3, A–C), these findings indicate that ARTs are targeted to the proteasome for degradation in the absence of Ubp2 and Ubp15.

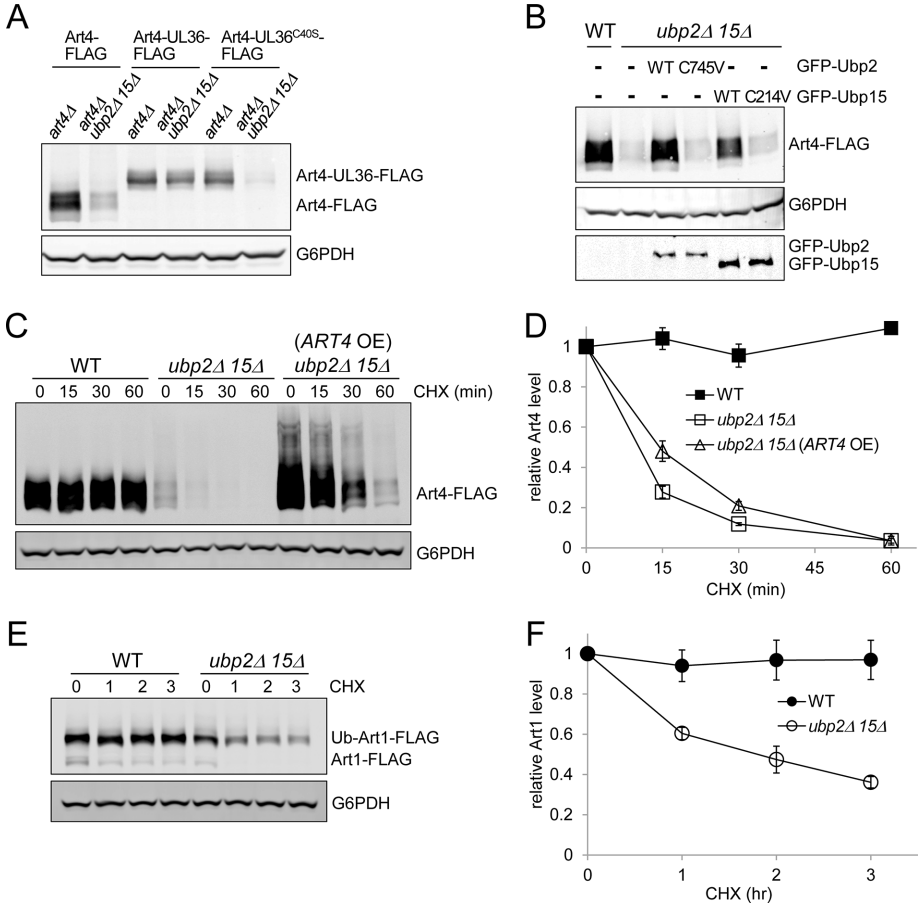

FIGURE 2: Ubp2 and Ubp15 regulate the degradation of ARTs. (A) *art4*Δ and *art4*Δ *ubp2*Δ *ubp15*Δ yeast cells carrying Art4-FLAG, Art4-UL36-FLAG, or Art4-UL36^{C40S}-FLAG expression vector. Protein extracts were analyzed by SDS–PAGE and immunoblot. (B) Immunoblot analysis of chromosomal Art4-FLAG in WT and *ubp2*Δ *ubp15*Δ yeast cells carrying functional or catalytically dead Ubp2/Ubp15 expression vector. (C) WT and *ubp2*Δ *ubp15*Δ yeast cells expressing chromosomal Art4-FLAG or *ubp2*Δ *ubp15*Δ cells overexpressing (OE) Art4-FLAG (driven by the *CYC1* promoter inserted into *ART4* locus) were treated with 100 μg/ml CHX to stop protein synthesis. Protein extracts from the indicated time points were analyzed by SDS–PAGE and immunoblot. (D) Art4 levels (relative to $t = 0$) as in C were quantified by the LI-COR system. Data from three independent experiments are presented as mean ± SD. (E, F) similar to C and D, except that yeast strains with chromosomal Art1-FLAG were examined.

Collectively our data demonstrate that Ubp2 and Ubp15 maintain the protein stability of ARTs by preventing their proteasomal degradation, possibly by physically interacting with Rsp5 and/or ARTs, and counteracting ubiquitination of ARTs. To explore further how loss of Ubp2 and Ubp15 affects the global ubiquitination landscape, we compared the ubiquitin-modified proteome of WT and *ubp2*Δ *ubp15*Δ cells using SILAC followed by tandem purification of ubiquitin conjugates and quantitative mass spectrometry (Supplemental Figure S3A). To improve sensitivity as well as obtain better understanding of mechanisms for Ubp2 and Ubp15 functions, we prefractionated lysates into membrane (P13) and soluble (S13) fractions for separate analysis. Our analysis revealed that several Rsp5 adaptor proteins, including Art2, Art3, Bul1, and Bul2 in the P13 fraction, as well as Art2 and Art3 in the S13 fraction, exhibited increased ubiquitination in *ubp2*Δ *ubp15*Δ cells, based on multiple peptides resolved (Supplemental Figure S3B), further implicating Ubp2 and Ubp15 in limiting ubiquitination of Rsp5 adaptor proteins, regardless of whether ARTs associate with membrane compartments.

The fact that we did not recover other ARTs such as Art4 and Art10 in this analysis may reflect the relatively low abundance of these ubiquitinated adaptors, perhaps due to their higher susceptibility to proteasomal degradation. To examine directly the ubiquitination status of Art4 in *ubp2*Δ *ubp15*Δ, we overexpressed myc-ubiquitin in *pdr5*Δ and *pdr5*Δ *ubp2*Δ *ubp15*Δ cells. After inhibition of the proteasome by MG132, Art4-FLAG was immunoprecipitated under denaturing conditions, and ubiquitin conjugates were detected by immunoblotting using anti-myc antibody. Increased high–molecular weight (MW) signals were evident in *pdr5*Δ *ubp2*Δ *ubp15*Δ cells compared with *pdr5*Δ cells (Figure 3G), indicating that Art4 is hyperubiquitinated in the absence of Ubp2 and Ubp15. Similar hyperubiquitination was observed for Art2 and Art1 in the absence of Ubp2 and Ubp15 (Figure 3, H and I). Together these results provide strong evidence that Ubp2 and Ubp15 protect at least a subset of ARTs against proteasomal degradation by preventing their hyperubiquitination.

E3 ubiquitin ligase Rsp5 mediates ART degradation in *ubp2*Δ *ubp15*Δ

Because ARTs associate with the E3 ubiquitin ligase Rsp5, we tested whether ART degradation in *ubp2*Δ *ubp15*Δ requires Rsp5 function. Rsp5 belongs to the Nedd4 ubiquitin ligase family and contains a C-terminal HECT catalytic domain and three WW domains that interact with PY motifs (PPXY or LPXY) in substrates or adaptor proteins, including ARTs. We found that disruption of Rsp5–Art4 interaction by Art4 PY-motif mutations completely suppressed Art4 degradation in *ubp2*Δ *ubp15*Δ (Figure 4A), indicating that Art4 degradation in *ubp2*Δ *ubp15*Δ requires the Art4–Rsp5 interaction. Similarly, Art1 degradation in *ubp2*Δ *ubp15*Δ was suppressed by Art1 PY-motif mutations (Figure 4B). To test whether the Rsp5 ligase activity is required, we used a hypomorphic allele of *RSP5* with a mutation in the HECT domain (G747E; Oestreich *et al.*, 2007) to monitor ART levels in *ubp2*Δ *ubp15*Δ. We found that the *rsp5*^{G747E} allele suppressed the instability of both Art4 and Art1 (Figure 4, C and D), indicating that Rsp5 ligase activity is required for ART degradation in *ubp2*Δ *ubp15*Δ cells. These results suggest that Ubp2 and Ubp15 function to counteract Rsp5-mediated hyperubiquitination of ARTs.

It was shown previously that during heat stress, Rsp5 associates with the PY motif–containing Hsp40 cochaperone Ydj1 to promote ubiquitination of misfolded proteins and target them for proteasomal degradation (Lee *et al.*, 1996; Fang *et al.*, 2014). Given their similarity as Rsp5-dependent proteasomal degradation processes, we tested whether ART degradation in *ubp2*Δ *ubp15*Δ is also mediated by Ydj1. We found, however, that ART degradation in *ubp2*Δ *ubp15*Δ did not require Ydj1 (Supplemental Figure S4A), indicating that it is a distinct process.

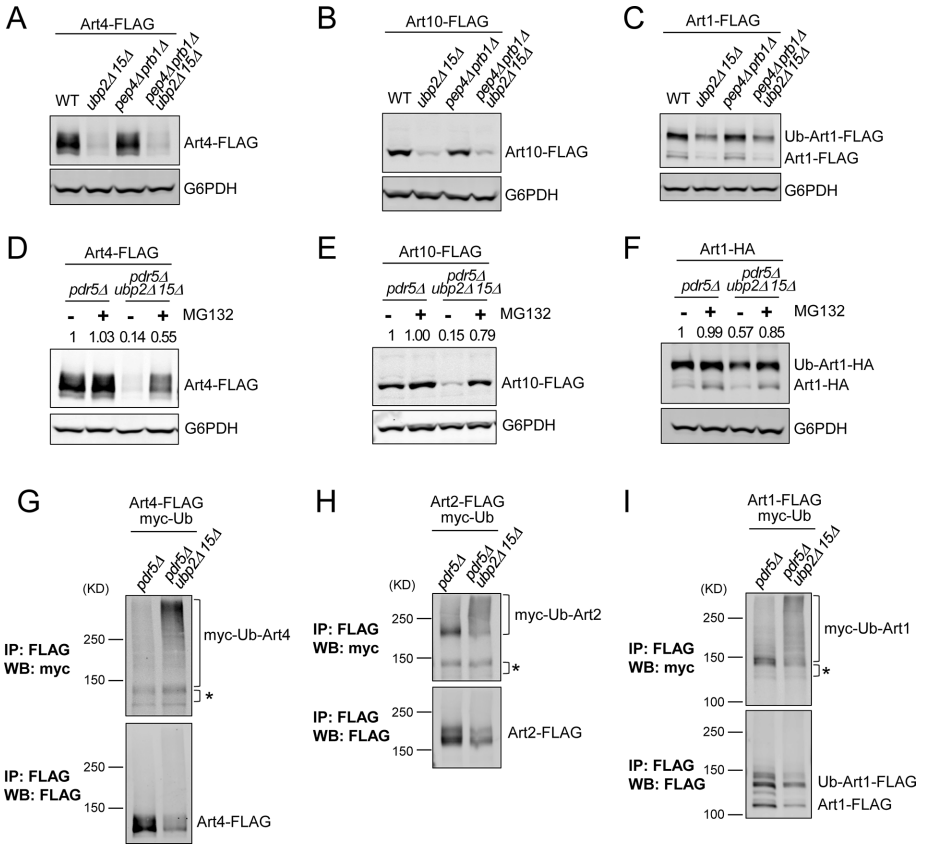

FIGURE 3: Ubp2 and Ubp15 prevent hyperubiquitination and proteasomal degradation of ARTs. (A–C) Protein extracts of indicated yeast strains expressing plasmid-encoded Art4-FLAG (A), Art10-FLAG (B), or Art1-FLAG (C) were analyzed by SDS–PAGE and immunoblot. (D–F) *pdr5*Δ and *pdr5*Δ *ubp2*Δ *ubp15*Δ cells expressing plasmid-encoded Art4-FLAG (D), Art10-FLAG (E), or Art1-HA (F) were mock-treated (dimethyl sulfoxide [DMSO]) or treated with MG132 (25 μg/ml) for 60 min, and protein extracts were analyzed by SDS–PAGE and immunoblot. The numbers above each lane indicate the protein abundance of ARTs determined by the LI-COR quantification and normalized with the loading control G6PDH. (G–I) *pdr5*Δ and *pdr5*Δ *ubp2*Δ *ubp15*Δ cells expressing plasmid-encoded Art4-FLAG (G), Art2-FLAG (H), or Art1-FLAG (I) and myc-ubiquitin (overexpression controlled by copper-inducible promoter) were treated with MG132 (25 μg/ml) for 60 min. Samples were immunoprecipitated using anti-FLAG antibody under denaturing conditions and analyzed by SDS–PAGE and immunoblot. *Nonspecific.

We further investigated whether other ubiquitin ligases, in addition to Rsp5, are required for ART degradation in *ubp2*Δ *ubp15*Δ by further enhancing polyubiquitination of ARTs. These ligases are sometimes referred as E4 ubiquitin chain extension enzymes (Koegl *et al.*, 1999). Among them, Hul5 and Cul3 are particularly interesting. Hul5 has been shown to target prion-like protein Pin3/Lsb2 for proteasomal degradation (Fang *et al.*, 2011). Pin3, like ARTs, is also a PY motif–containing protein and is ubiquitinated by Rsp5 (Chernova *et al.*, 2011). Moreover, Rsp5 and Cul3 act sequentially to promote polyubiquitination of RNA polymerase II (Harreman *et al.*, 2009). We therefore assessed the involvement of these E4 enzymes and found Art4 degradation in *ubp2*Δ *ubp15*Δ was largely unperturbed (Supplemental Figure S4B).

In addition, we tested other ubiquitin ligases functioning in cytoplasmic protein quality control pathways, including San1 and Ubr1 (Heck *et al.*, 2010; Prasad *et al.*, 2010; Khosrow-Khavar *et al.*, 2012), as well as Doa10 (Carvalho *et al.*, 2006; Ravid *et al.*, 2006; Ast *et al.*, 2014). We found none of these E3 ligases altered Art4 degradation in *ubp2*Δ *ubp15*Δ cells (Supplemental Figure S4, C and D). We cannot rule out the possibility that other ubiquitin ligases or E4 enzymes are involved in the ART degradation we observed here. Nevertheless, our results revealed a novel Rsp5-dependent mechanism targeting cytosolic proteins for proteasomal degradation.

Art4 hyperubiquitination and degradation require the formation of K63-linked polyubiquitin chains

Proteasomal degradation is usually mediated by K48-linked polyubiquitination (Chau *et al.*, 1989; Finley *et al.*, 1994), although K63-linked chains have also been reported to be the targeting signal for the proteasome (Kirkpatrick *et al.*, 2006; Saeki *et al.*, 2009). Although Rsp5 prefers to conjugate monoubiquitin or K63-linked polyubiquitin chains (Kee *et al.*, 2005; Kim and Huibregtse, 2009), K48-linked polyubiquitination by Rsp5 is also evident both in vitro and in vivo (French *et al.*, 2009; Fang *et al.*, 2014, 2016). Whereas Ubp2 appears to be K63 specific in vitro (Kee *et al.*, 2005, 2006), evidence from in vivo studies suggests that Ubp2 may act on both K48 and K63 polyubiquitin chains (Xu *et al.*, 2009; Anton *et al.*, 2013). Ubp15 can target both K48 and K63 linkages in vitro, but long K63 chains are cleaved more rapidly by Ubp15 than long K48 chains (Schaefer and Morgan, 2011). To explore the linkage specificity/preference of Ubp2 and Ubp15, we used our SILAC ubiquitin proteome experiment (Supplemental Figure S3A) and analyzed linkage-specific ubiquitin peptides resolved. We found that in both membrane and soluble fractions, K63 ubiquitin linkages were increased in *ubp2*Δ *ubp15*Δ cells more prominently than with K48 linkages (Supplemental Figure S5A), consistent with the in vitro preference of both Ubp2 and Ubp15 for K63-linked polyubiquitin chains.

To determine directly the involvement of K63 versus K48 linkages in ART hyperubiquitination, we examined the effect of overexpressing myc-ubiquitin with K48R, K63R, or K48R K63R mutations on Art4 high-MW conjugates as described earlier (Figure 3G). We found that expressing myc-ubiquitin^{K63R} substantially reduced Art4 hyperubiquitination in *ubp2*Δ *ubp15*Δ cells, whereas myc-ubiquitin^{K48R} had only a mild effect (Figure 5A). In addition, the effect of the K48R K63R double mutations was similar to that of the K63R single mutation. These results suggest Art4 hyperubiquitination in *ubp2*Δ *ubp15*Δ is mediated mainly by K63-linked polyubiquitin chains, although it is possible that other linkage types, including K48, may still be involved.

To investigate further whether one or both ubiquitin linkages are responsible for Art4 degradation in *ubp2*Δ *ubp15*Δ, we used a set of mammalian DUBs exhibiting linkage specificity/preference (McCullough *et al.*, 2006; Edelmann *et al.*, 2009; Wang *et al.*, 2009; Mevissen *et al.*, 2013; Hospenthal *et al.*, 2015), including OTUB1 (highly K48 specific), OTUD1 (prefers K63 over K48), and AMSH (highly K63 specific). We fused these DUBs to Art4 and examined their relative abundance in *ubp2*Δ *ubp15*Δ cells compared with WT

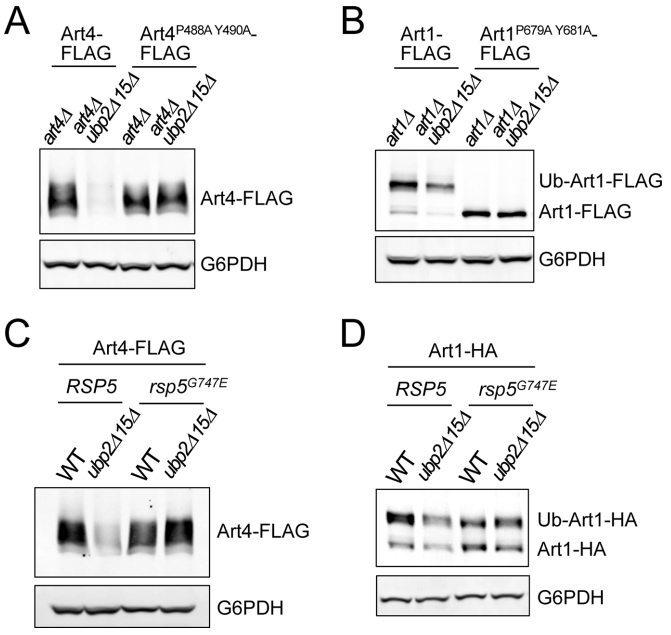

FIGURE 4: ART degradation in *ubp2*Δ *ubp15*Δ is mediated by E3 ubiquitin ligase Rsp5. (A) Protein extracts of *art4*Δ and *art4*Δ *ubp2*Δ *ubp15*Δ yeast cells expressing plasmid-encoded Art4-FLAG or Art4$^{P488A\ Y490A}$-FLAG were analyzed by SDS–PAGE and immunoblot. (B) Protein extracts of *art1*Δ and *art1*Δ *ubp2*Δ *ubp15*Δ yeast cells expressing plasmid-encoded Art1-FLAG or Art1$^{P679A\ Y681A}$-FLAG were analyzed by SDS–PAGE and immunoblot. (C, D) Protein extracts of *RSP5*, *RSP5 ubp2*Δ *ubp15*Δ, *rsp5*^{G747E}, and *rsp5*^{G747E} *ubp2*Δ *ubp15*Δ cells expressing plasmid-encoded Art4-FLAG (C) or Art1-HA (D) were analyzed by SDS–PAGE and immunoblot.

cells. Similar to WT Art4, the Art4-OTUB1 fusion showed reduced abundance in *ubp2*Δ *ubp15*Δ cells (Figure 5, B, lanes 1–4, and C), suggesting that OTUB1 fusion does not affect Art4 hyperubiquitination. The level of Art4-OTUD1 fusion in *ubp2*Δ *ubp15*Δ cells, however, was nearly identical to that in WT cells (Figure 5, B, lanes 5 and 6, and C), suggesting that OTUD1 efficiently prevents Art4 hyperubiquitination. In addition, Art4-AMSH exhibited an intermediate reduction in *ubp2*Δ *ubp15*Δ cells (Figure 5, B, lanes 7 and 8, and C), suggesting partial inhibition of Art4 hyperubiquitination by AMSH. To examine whether OTUB1 and OTUD1 fusion constructs are functionally active toward K48-linked polyubiquitin chains, we also compared the effect of OTUB1 and OTUD1 fusions on CPY* degradation, which is mediated by K48 polyubiquitination (Hiller *et al.*, 1996). We found both OTUB1 and OTUD1 delayed the turnover of CPY* (Supplemental Figure S5B), indicating that these DUB fusion constructs are indeed capable of cleaving K48-linked chains. Together these data suggest that although Art4 hyperubiquitination might be decorated by K48-linked chains in addition to K63-linked chains, K48-linked chains alone are not sufficient for Art4 degradation in *ubp2*Δ *ubp15*Δ. Instead, our results revealed that Art4 degradation in *ubp2*Δ *ubp15*Δ requires the formation of K63-linked chains. The fact that the Art4-OTUD1 fusion has a stronger effect than the Art4-AMSH fusion may suggest that either Art4 degradation in *ubp2*Δ *ubp15*Δ cells is mediated by heterotypic chains containing both K48 and K63 linkages, which are removed more efficiently by OTUD1 that cleaves both, or the degradation is mediated by very long K63 chains, which are poor substrates for AMSH without its binding partner STAM (McCullough *et al.*, 2006; Baiady *et al.*, 2016).

In *ubp2*Δ *ubp15*Δ, ARTs are hyperubiquitinated at residues different from the activating lysines

Next we investigated whether ART degradation in *ubp2*Δ *ubp15*Δ cells is caused by hyperubiquitination at previously identified lysine residues. Art1 K486 and Art4 4K (K235/245/264/267) are important for ubiquitination and endocytosis of several PM cargoes (Lin *et al.*, 2008; Becuwe *et al.*, 2012b), although they are not required for Ste2 endocytosis (Alvaro *et al.*, 2014). To test whether the same lysine residues are also involved in ART hyperubiquitination and degradation in *ubp2*Δ *ubp15*Δ, we examined the levels of Art1^{K486R} and Art4^{4KR} in WT versus *ubp2*Δ *ubp15*Δ cells. Note that Art1 migrates as two major species (Ub-Art1 and Art1) on the SDS gel, and the mobility shift is dependent on K486 (Lin *et al.*, 2008). Whereas Art1^{K486R} migrated predominantly as the fast-moving/nonubiquitinated band, the level of Art1^{K486R} was still reduced in *ubp2*Δ *ubp15*Δ cells compared with *UBP2 UBP15* cells (Figure 6A). Similarly, Art4^{4KR} did not suppress Art4 degradation in *ubp2*Δ *ubp15*Δ (Figure 6B). These results suggest that ART degradation in *ubp2*Δ *ubp15*Δ is mediated by functionally distinct ubiquitination events different from the activating ubiquitination.

The prediction of Art4 ubiquitination sites using the online resource UbPred (Radivojac *et al.*, 2010) revealed a cluster of lysine residues at the C-terminus and a few N-terminal lysine residues as potential sites of ubiquitination. To test whether Art4 degradation in *ubp2*Δ *ubp15*Δ is caused by ubiquitination at these sites, we generated Art4 mutants with different combinations of lysine-to-arginine (KR) substitutions (Supplemental Figure S6A). We found that the mutants with most of the N-terminal and C-terminal lysine sites mutated (27KR/30KR/44KR) prominently restored Art4 levels in *ubp2*Δ *ubp15*Δ cells (Figure 6, C and D, and Supplemental Figure S6B). We infer from these findings that ARTs can be regulated by two different types of ubiquitination: activating ubiquitination (such as at Art1 K486 or Art4 4K) and degradative ubiquitination, which is counteracted by Ubp2 and Ubp15 (Figure 6E).

Ubp2 and Ubp15 ensure efficient Hxt6 endocytosis by stabilizing Art4

Art4 promotes endocytic trafficking of the PM hexose transporter Hxt6 in response to high concentrations of glucose (Nikko and Pelham, 2009; Llopis-Torregrosa *et al.*, 2016). Given the prominent proteasomal degradation of Art4 in *ubp2*Δ *ubp15*Δ, we reasoned that Hxt6 endocytosis would be affected as well. Indeed, we found that *ubp2*Δ *ubp15*Δ cells exhibited a strong defect in glucose-induced Hxt6 endocytosis (Figure 7, A–C). The accumulation of Hxt6 at the PM in *ubp2*Δ *ubp15*Δ cells (Figure 7C) suggests that Ubp2 and Ubp15 affect Hxt6 internalization, likely the ubiquitination step mediated by Rsp5-ARTs. Endocytic trafficking of Jen1, another Art4-dependent PM cargo (Becuwe *et al.*, 2012b), was also impaired in *ubp2*Δ *ubp15*Δ cells (Supplemental Figure S7A). To confirm that the Hxt6 endocytosis defect in *ubp2*Δ *ubp15*Δ is caused by Art4 hyperubiquitination and degradation, we examined the effect of Art4^{27KR} on Hxt6 endocytosis in *ubp2*Δ *ubp15*Δ cells. We used CHX as an additional inducer for Hxt6 endocytosis (Nikko and Pelham, 2009), as well as to inhibit protein synthesis. Therefore only the preexisting, PM-localized pool of Hxt6 can be delivered to the vacuole. We found that Art4^{27KR} substantially suppressed the Hxt6 trafficking defect in *ubp2*Δ *ubp15*Δ (Figure 7, D and E). We also tested whether MG132 treatment, which partially suppressed Art4 degradation (Figure 3D), can also alleviate the Hxt6 trafficking defect. We found that MG132

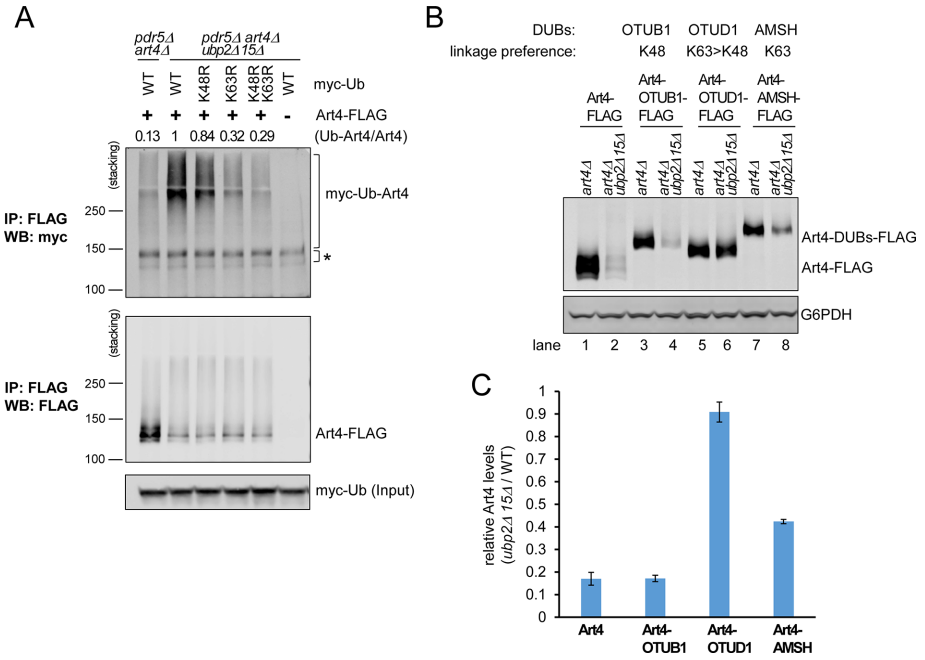

FIGURE 5: Art4 hyperubiquitination and degradation in *ubp2*Δ *ubp15*Δ depends on the formation of K63-linked polyubiquitin chains. (A) *pdr5*Δ *art4*Δ and *pdr5*Δ *art4*Δ *ubp2*Δ *ubp15*Δ yeast cells carrying Art4-FLAG or empty vector, as well as WT, K48R, K63R, or K48R K63R myc-ubiquitin expression vector. Cells were treated with MG132 (25 μg/ml) for 60 min. Samples were immunoprecipitated using anti-FLAG antibody under denaturing conditions and analyzed by SDS–PAGE and immunoblot. The numbers above each lane indicate the relative ratio of myc-Ub-Art4 vs. total Art4 determined by the LI-COR quantification. To compare more accurately the levels of myc-Ub conjugated Art4, stacking gel was included for the analysis. *Nonspecific. (B) *art4*Δ and *art4*Δ *ubp2*Δ *ubp15*Δ yeast cells carrying Art4-FLAG, Art4-OTUB1-FLAG, Art4-OTUD1-FLAG, or Art4-AMSH-FLAG expression vector. Protein extracts were analyzed by SDS–PAGE and immunoblot. (C) Relative Art4/Art4-OTUB1, Art4-OTUD1, and Art4-AMSH levels (*art4*Δ *ubp2*Δ *ubp15*Δ cells versus *art4*Δ cells) as in B were quantified using the LI-COR system. Data from at least three independent experiments are presented as mean ± SD.

DISCUSSION

Endocytic down-regulation is crucial for maintaining PM protein homeostasis. By regulating the composition of integral membrane proteins at the PM, cells are able to adapt to various environmental stimuli and challenges, such as nutrient availability and heat stress. The ART-Rsp5 network ubiquitinates cargoes and promotes their internalization at the PM. Therefore proper regulation of ART-Rsp5 is of great importance for cells to survive in response to the changing environment. In this study, we identified two deubiquitinating enzymes, Ubp2 and Ubp15, as novel regulators of the ART-Rsp5 network. We demonstrate that Ubp2 and Ubp15 maintain the stability of several ARTs by counteracting Rsp5-mediated hyperubiquitination and subsequent proteasomal degradation of ARTs. Loss of Ubp2 and Ubp15 causes decreased steady-state levels of ARTs, leading to defects in endocytic turnover of PM cargoes (Figure 8). Our results revealed another layer of regulatory complexity in the ART-Rsp5 network: besides inhibitory phosphorylation/activating dephosphorylation by kinases/phosphatases, ARTs are also subject to ubiquitination/deubiquitination regulation imposed by E3/DUBs so that cells can maintain appropriate levels of Rsp5 adaptor proteins for proper control of endocytosis and PM composition.

treatment considerably restored Hxt6 endocytic degradation in *ubp2*Δ *ubp15*Δ cells (Figure 7, F and G). Furthermore, Hxt6 endocytic degradation upon MG132 treatment was still dependent on Art4 and its paralogue Art7 (Figure 7, F and G), suggesting that upon proteasome inhibition, Hxt6 still undergoes physiological ubiquitination mediated by the ART-Rsp5 network.

To further confirm that impaired Hxt6 trafficking in *ubp2*Δ *ubp15*Δ cells is not caused by other processes such as defects in MVB sorting, we used a single-ubiquitin fusion that bypasses Rsp5/ART-dependent ubiquitination and constitutively sorts cargo proteins into the vacuole (Stringer and Piper, 2011). We found that Hxt6-GFP-Ub was sorted to the vacuole and degraded in both WT and *ubp2*Δ *ubp15*Δ cells (Figure 7, H and I), suggesting that there is no gross defect in the ESCRT machinery or MVB formation. Moreover, we found that whereas Art2-dependent Lyp1 endocytosis (Lin *et al.*, 2008) is partially defective (Supplemental Figure S7B), Art3-dependent Dip5 endocytosis (Hatakeyama *et al.*, 2010) was nearly unchanged in *ubp2*Δ *ubp15*Δ cells (Supplemental Figure S7C). These trafficking phenotypes are consistent with the reduced abundance of Art2 (Figure 1D) and unaltered levels of Art3 (Supplemental Figure S1F) in *ubp2*Δ *ubp15*Δ. Of importance, these data also demonstrate that endocytic processes are not generally impaired. We therefore conclude that Ubp2 and Ubp15 ensure efficient endocytic down-regulation of Hxt6, at least in part by preventing Art4 hyperubiquitination and subsequent proteasomal degradation.

The ART-Rsp5 network is regulated by the ubiquitin system

It is evident that many components of the ubiquitin system are also regulated by ubiquitination (Weissman *et al.*, 2011). Several ARTs are ubiquitinated, but functions of these modifications are not always clear (Kee *et al.*, 2006; Lin *et al.*, 2008; Herrador *et al.*, 2010; Becuwe *et al.*, 2012b; Alvaro *et al.*, 2014). Here we show Ubp2 and Ubp15 counteract ART hyperubiquitination, thereby preventing degradation of several ARTs. On the basis of our findings and those from other studies, we propose that Ubp2 and Ubp15 are crucial regulators for the ART-Rsp5 network. Ubp2 operates on ARTs by binding to Rsp5 (Kee *et al.*, 2005), and Ubp15 may engage ARTs directly or indirectly through other interacting partners (Supplemental Figure S2, A–C, and Figure 8). Additional work is required to determine mechanistic details of ART-Ubp15 interactions.

In contrast to the *ubp2*Δ *ubp15*Δ mutant, deletion of *DOA4* or *UBP3* inhibited the degradation of ARTs (Supplemental Figure S1, C–E). It is known that Doa4 maintains free ubiquitin levels by recycling ubiquitin from the MVB pathway (Swaminathan *et al.*, 1999; Amerik *et al.*, 2000b). Hence ubiquitin deficiency in *doa4*Δ mutants may in turn limit ART degradation in the absence of Ubp2 and Ubp15. How *ubp3*Δ affects ART degradation in *ubp2*Δ *ubp15*Δ is less clear, as there is no obvious defect in free ubiquitin levels in *ubp3*Δ (Amerik *et al.*, 2000a). Recently Ubp3 and Ubp2 were shown to regulate the Rsp5-mediated cytosolic protein quality control pathway upon heat stress (Fang *et al.*, 2016). In this pathway, Ubp3 and Ubp2 cleave Rsp5-conjugated K63 chains, which, together with

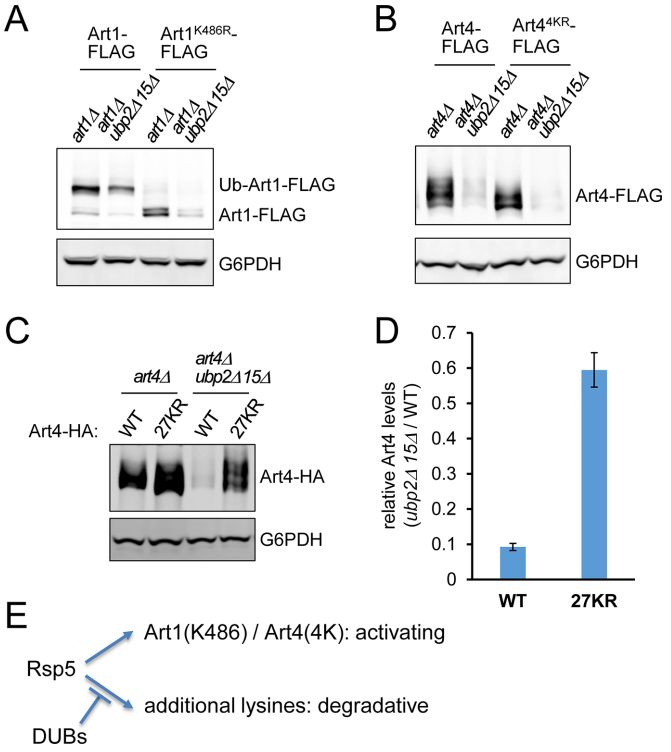

FIGURE 6: ART degradation in *ubp2*Δ *ubp15*Δ is not mediated by ubiquitination at the activating lysine residues. (A) Protein extracts of *art1*Δ and *art1*Δ *ubp2*Δ *ubp15*Δ yeast cells expressing plasmid-encoded Art1-FLAG or Art1^{K486R}-FLAG were analyzed by SDS–PAGE and immunoblot. (B) Protein extracts of *art4*Δ and *art4*Δ *ubp2*Δ *ubp15*Δ yeast cells expressing plasmid-encoded Art4-FLAG or Art4$^{K(235/245/264/267)R}$-FLAG were analyzed by SDS–PAGE and immunoblot. (C) Protein extracts of *art4*Δ and *art4*Δ *ubp2*Δ *ubp15*Δ cells expressing plasmid-encoded Art4-HA or Art4^{27KR}-HA were analyzed by SDS–PAGE and immunoblot. (D) Relative Art4 levels (*art4*Δ *ubp2*Δ *ubp15*Δ cells vs. *art4*Δ cells) as in C were quantified using the LI-COR system. Data from three independent experiments are presented as mean ± SD. (E) Model for functionally distinct ubiquitinations of ARTs. Rsp5 ubiquitinates Art1 at K486 and Art4 at K235/K245/K264/K267, which are required for their activation. In the absence of Ubp2 and Ubp15, additional lysines on ARTs are ubiquitinated, causing their degradation.

the enhanced K48-catalyzing activity of Rsp5 upon heat shock, promotes proteasomal degradation of cytosolic misfolded proteins. Although this function of Ubp3 could potentially explain the suppression of the ART degradation observed here, we suspect that it is unlikely for the following reasons: 1) Ubp2 plays an opposite role and inhibits the degradation of ARTs; 2) the degradation of ARTs in *ubp2*Δ *ubp15*Δ cells does not require heat shock; and 3) K48-linked polyubiquitination does not play a major role in ART degradation in *ubp2*Δ *ubp15*Δ.

Proteasomal degradation of Art4 requires K63-linked polyubiquitin chains

We show in this study that Art4 hyperubiquitination in *ubp2*Δ *ubp15*Δ is mediated mainly by K63-linked chains (Figure 5A), consistent with the preferences of Rsp5, Ubp2, and Ubp15 toward K63 linkages (Kee *et al.*, 2005, 2006; Kim and Huibregtse, 2009; Schaefer and Morgan, 2011). Given that we find no evidence that Art4 (as well as Art10 and Art1) is degraded in the vacuole (Figure 3, A–C), and the proteasome inhibitor MG132 can significantly suppress ART degradation in *ubp2*Δ *ubp15*Δ cells (Figure 3, D–F), it is likely that hyperubiquitination of ARTs leads to protein degradation by the 26S proteasome. Collectively these findings suggest that ART degradation in *ubp2*Δ *ubp15*Δ cells is via unconventional K63-mediated proteasomal targeting, because proteasomal degradation is widely considered to be mediated by non-K63 linkages (Chau *et al.*, 1989; Finley *et al.*, 1994; Hershko and Ciechanover, 1998; Pickart and Fushman, 2004; Xu *et al.*, 2009). It is also evident, however, that K63-linked polyubiquitin chains can be used as the proteasome targeting signal in certain circumstances (Saeki *et al.*, 2009; Isasa *et al.*, 2016), and longer chain lengths can potentially increase the affinity with the proteasome (Saeki *et al.*, 2009). Furthermore, proteasome recruitment of a human E3 ligase, TRIM21, involved in virus degradation is preceded by the formation of K63-linked polyubiquitination (Fletcher *et al.*, 2015). Hyperubiquitinated ARTs may therefore represent another example of the complexity of the ubiquitin-proteasome pathway and highlight the importance of K63-specific DUBs in preserving nonproteolytic ubiquitinated proteins.

Alternatively, the degradation of ARTs in *ubp2*Δ *ubp15*Δ can be the consequence of heterotypic ubiquitin chains containing both K63 and K48 linkages, which could have higher affinity for the proteasome, as described previously for K11/K48 mixed ubiquitin chains (Grice *et al.*, 2015). We demonstrate here that the OTUB1 fusion can delay CPY* turnover mediated by K48-linked polyubiquitination (Supplemental Figure S5B) but has no effect on Art4 degradation in *ubp2*Δ *ubp15*Δ (Figure 5, B and C). These data suggest that Art4 degradation in *ubp2*Δ *ubp15*Δ is unlikely caused solely by K48 linkages. We cannot, however, rule out the possibility that in addition to K63 linkages, some K48-linked chains are present and enhance the delivery to the proteasome. Such K48 linkages could potentially be catalyzed by Rsp5 itself, as evident by the considerable (though weaker than K63) K48-conjugating activity of Rsp5 in vitro (French *et al.*, 2009; Kim and Huibregtse, 2009), or they could result from activities of other E3 ligases or E4 enzymes that require ubiquitination events primed by Rsp5. Although we show here that several E3s/E4s, including San1, Ubr1, Doa10, Cul3, and Hul5, are not involved (Supplemental Figure S4, A and D), it remains possible that there are other ligase activities conjugating these K48 linkages.

Modulation of the ART-Rsp5 network by deubiquitinating enzymes Ubp2 and Ubp15

Of the 10 Rsp5 adaptors we tested, steady-state levels of 8—Art1, Art2, Art4, Art6, Art7, Art8, Art10, and Bul1—are reduced in *ubp2*Δ *ubp15*Δ cells (Figure 1, B–I). It is intriguing that Ubp2 and Ubp15 have little or no effect on the levels of Art3 and Art9 (Supplemental Figure S1, F and G), and yet Art3 ubiquitination is still increased in the absence of Ubp2 and Ubp15 (Supplemental Figure S3C). It therefore seems that Ubp2 and Ubp15 are general regulators for preventing hyperubiquitination of most, if not all, Rsp5 adaptor proteins, but the outcomes (degraded or not) of hyperubiquitination are further dictated by other factors, such as protein–protein interactions, posttranslational modifications, or subcellular localizations. Further studies are required to elucidate mechanisms determining the fates of individual Rsp5 adaptor proteins upon hyperubiquitination.

It is an emerging theme that many DUBs physically interact with E3 ubiquitin ligases and in many cases protect E3s from autoubiquitination and degradation. Whereas Rsp5 remains stable in the absence of Ubp2 and Ubp15 (Supplemental Figure S1H), we found in this study that ART adaptor proteins are susceptible to Rsp5-mediated hyperubiquitination and degradation, and therefore require

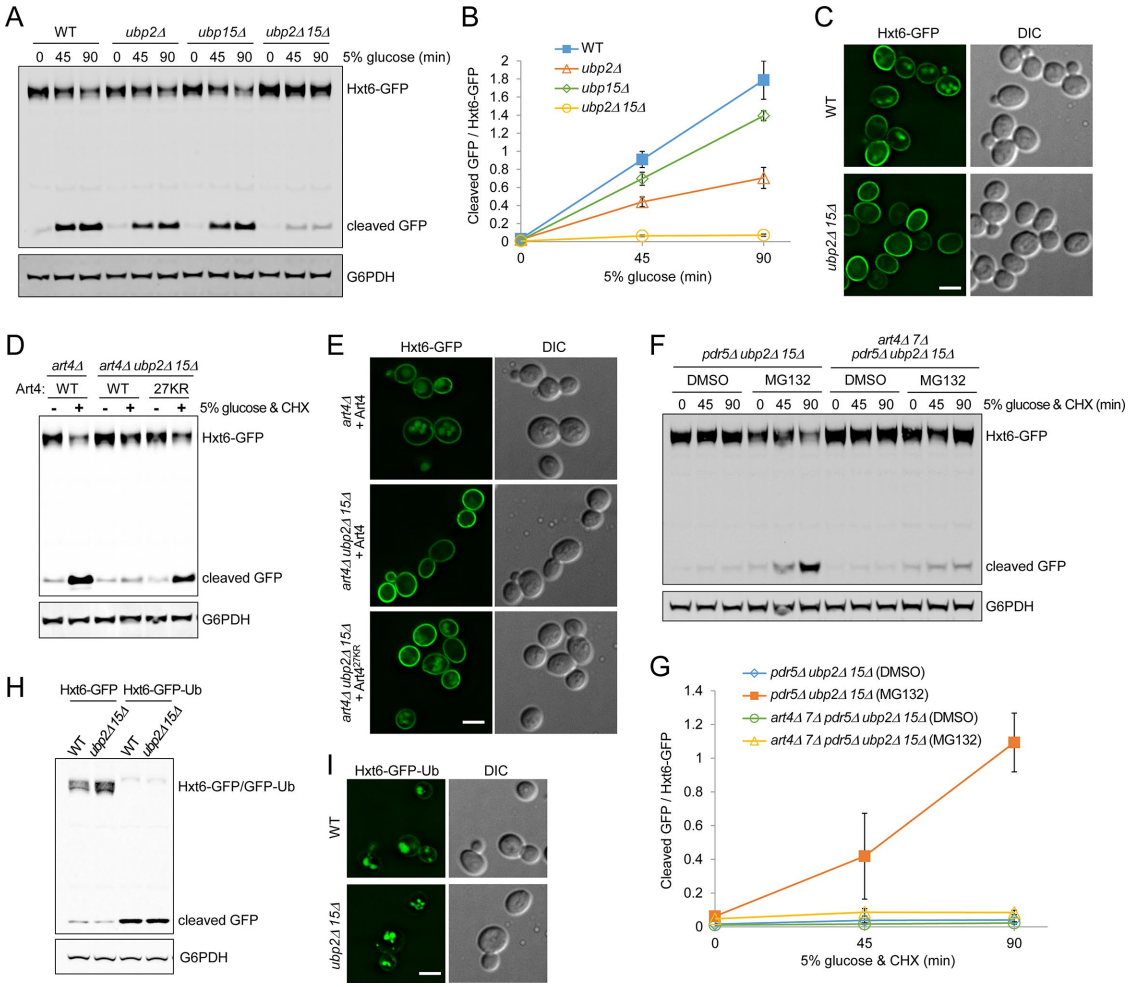

FIGURE 7: Ubp2 and Ubp15 promote Hxt6 endocytic down-regulation by stabilizing Art4. (A–C) The indicated strains expressing chromosomal Hxt6-GFP were grown to mid log phase in medium containing 0.05% glucose. Hxt6 endocytosis was stimulated with 5% glucose. (A) Protein extracts at the indicated time points were analyzed by SDS–PAGE and immunoblot. The cleaved GFP signals indicate the product of Hxt6-GFP degradation in the vacuole. (B) The ratio of cleaved GFP vs. full-length Hxt6-GFP as in A was determined by the quantification using the LI-COR system. Data from three independent experiments are presented as mean ± SD. (C) Samples were analyzed by fluorescence microscopy after 90 min of stimulation. Scale bar, 2.5 µm. (D, E) *art4*Δ and *art4*Δ *ubp2*Δ *ubp15*Δ cells expressing chromosomal Hxt6-GFP and plasmid-encoded Art4 or Art4^{27KR} were grown in medium containing 0.05% glucose. Hxt6 endocytosis was stimulated with 5% glucose and 50 µg/ml CHX. (D) Protein extracts after 3.5 h of stimulation were analyzed by SDS–PAGE and immunoblot. (E) Samples were analyzed by fluorescence microscopy after 3 h of stimulation. Scale bar, 2.5 µm. (F) *pdr5*Δ *ubp2*Δ *ubp15*Δ and *art4*Δ *art7*Δ *pdr5*Δ *ubp2*Δ *ubp15*Δ yeast cells expressing chromosomal Hxt6-GFP were grown to early log phase in medium containing 0.05% glucose and then mock-treated (DMSO) or treated with MG132 (25 µg/ml) for 90 min. Hxt6 endocytosis was stimulated with 5% glucose and 50 µg/ml CHX. Protein extracts from the indicated time points were analyzed by SDS–PAGE and immunoblot. (G) The ratio of cleaved GFP vs. full-length Hxt6-GFP as in F was determined by the quantification using the LI-COR system. Data from three independent experiments are presented as mean ± SD. (H) WT and *ubp2*Δ *ubp15*Δ cells expressing plasmid-encoded Hxt6-GFP or Hxt6-GFP-Ub were grown to mid log phase in medium containing 0.05% glucose. Protein extracts were analyzed by SDS–PAGE and immunoblot. (I) Similar to H, WT and *ubp2*Δ *ubp15*Δ cells expressing plasmid-encoded Hxt6-GFP-Ub were analyzed by fluorescence microscopy. Scale bar, 2.5 µm.

DUB activities to maintain their stability and trafficking function. ARRDC1, one of the mammalian counterparts of ARTs, has been shown to form heterodimers with β-arrestins and recruit the Nedd4 ubiquitin ligase Itch to down-regulate Notch receptor at the PM (Puca *et al.*, 2013). Of interest, the mammalian DUB USP9X associates with Itch and protects Itch from proteasomal degradation (Mouchantaf *et al.*, 2006). It is conceivable that USP9X or other DUBs may also function in receptor down-regulation by protecting ARRDCs or other ligase–adaptor complexes. The human orthologue of Ubp15, USP7, is an integral component of an endosomal E3 ligase complex and promotes endosomal protein recycling by preventing autoubiquitination and degradation of the ligase (Hao *et al.*, 2015). It remains to be seen whether USP7 is also involved in regulating PM receptors by associating with other E3 ligases and/or adaptors.

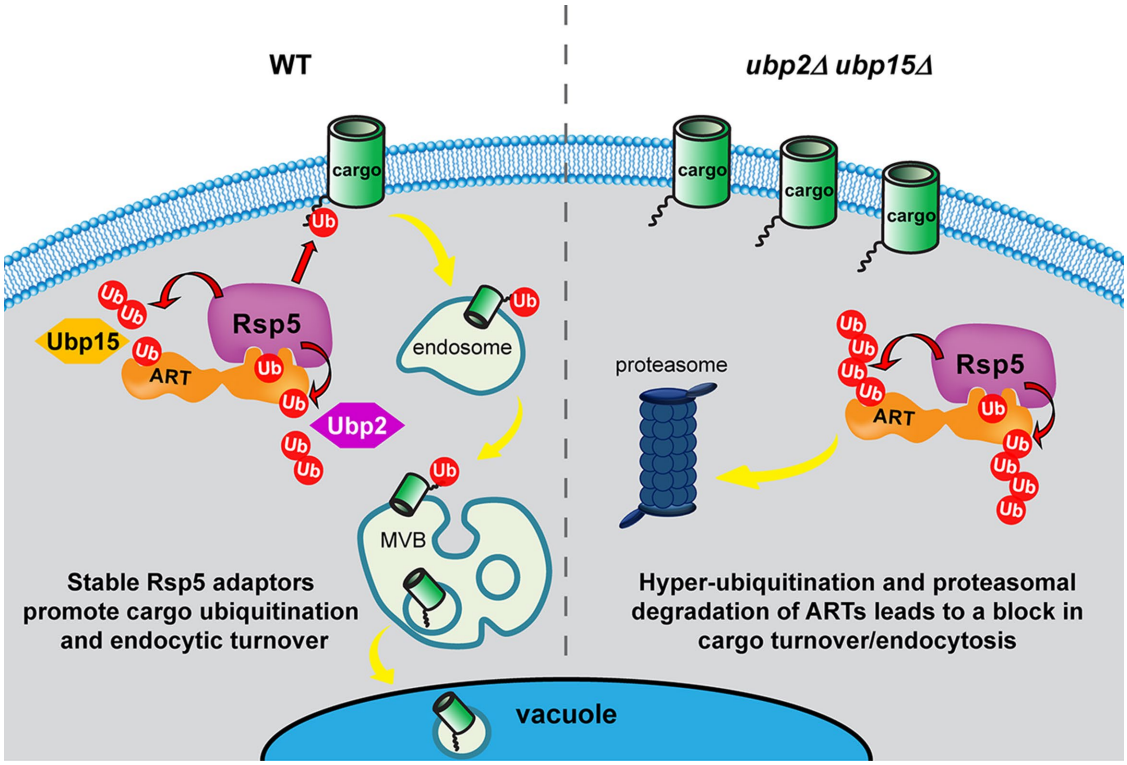

FIGURE 8: Model for Ubp2 and Ubp15 preventing Rsp5-mediated ART hyperubiquitination. In WT cells, Ubp2 and Ubp15 counteract Rsp5 activity and prevent ART hyperubiquitination. This is achieved most likely by physical interactions between Ubp2 and Rsp5, as well as between Ubp15 and ARTs. We propose that ART-Rsp5 complexes are direct targets of Ubp2 and/or Ubp15. Ubp2 and Ubp15 may regulate different steps of an ART-Rsp5 loading cycle, with Ubp2 engaging ART-Rsp5 complexes by binding to Rsp5, and Ubp15 engaging ARTs. Whether Ubp15 interacts with ARTs in complexes with Rsp5 or with ARTs that have dissociated from Rsp5 is unclear. ART-Rsp5 promotes PM cargo ubiquitination, leading to endocytic down-regulation. In *ubp2*Δ *ubp15*Δ cells, ARTs are hyperubiquitinated by Rsp5. At least several ARTs exhibit enhanced protein degradation through the proteasome, leading to reduced ART abundance and defective cargo turnover. The degradative hyperubiquitination of Art4 occurs most likely at N- and C-terminal regions. Regions of degradative ubiquitination on other ARTs have not been determined. Although the model illustrates that Ubp2 and Ubp15 target different residues/regions, it is unknown how these DUBs recognize substrates or whether motif specificity exists. Ub, ubiquitin; red arrow, Rsp5 E3 ligase activity.

Ubp2 and Ubp15 affect cargo trafficking by regulating ART stability

This work unveiled a novel role for Ubp2 and Ubp15 in maintaining the stability of ARTs by preventing their hyperubiquitination and subsequent proteasomal degradation. Given that ARTs recognize PM cargoes and promote their endocytic turnover, Ubp2 and Ubp15 may modulate the endocytic pathway by stabilizing ARTs. We demonstrate that *ubp2*Δ *ubp15*Δ cells exhibit defects in Hxt6 and Jen1 endocytic turnover (Figure 7, A–C, and Supplemental Figure S7A), consistent with the instability of Art4 (Figure 1B). These findings imply that the endocytic phenotypes in *ubp2*Δ *ubp15*Δ cells are likely caused by the degradation of ARTs. In support of this idea, suppressing Art4 degradation by either proteasome inhibition (Figure 3D) or 27KR mutations (Figure 6, C and D) considerably restores Hxt6 trafficking to the vacuole in *ubp2*Δ *ubp15*Δ (Figure 7, D–G).

Of note, partial suppression of the Hxt6 trafficking defect by proteasome inhibition reveals that ARTs reside at a point of cross-talk between vacuolar and proteasomal protein degradation pathways. Recently expression of nonpolymerizable (lysine-less/K0-Ub) ubiquitin has been used to identify polyubiquitination-dependent proteasomal substrates (stabilized upon K0-Ub expression) and mono-ubiquitination-dependent proteasomal substrates (further degraded upon K0-Ub expression; Braten *et al.*, 2016). Jen1 and Hxt6 are among the proteins exhibiting enhanced degradation upon expression of K0-Ub. Although it is possible that Jen1 and Hxt6 are direct substrates of the proteasome under certain conditions, as shown by the increased proteasomal targeting of other PM transporters at low temperature (Isasa *et al.*, 2016), this finding can also be explained by increased vacuolar degradation due to stabilization of Art4 upon K0 expression, consistent with the present observations that Art4 is a proteasomal substrate and proteasome inhibition improves Hxt6 trafficking to the vacuole in *ubp2*Δ *ubp15*Δ.

Although our findings strongly support a model in which Ubp2 and Ubp15 affect endocytic trafficking by regulating ART stability, they do not exclude the possibility that Ubp2 and Ubp15 play additional roles in the endocytic pathway. Ubp2 has been implicated in efficient MVB sorting of Fur4 and Cps1 (Ren *et al.*, 2007; Lam *et al.*, 2009). Ubp2, along with Ubp7, has also been shown to regulate formation and stability of the endocytic coat by deubiquitinating the early endocytic protein Ede1 (Weinberg and Drubin, 2014). It remains to be investigated how certain DUBs, such as Ubp2, can coordinate various steps, including cargo ubiquitination, internalization, and MVB sorting, to achieve successful endocytic down-regulation of PM cargoes.

MATERIALS AND METHODS

Yeast strains, plasmids, and growth conditions

All *Saccharomyces cerevisiae* strains and plasmids used in this study are listed in Supplemental Tables S1 and S2. Standard procedures were used for manipulation of yeast. Homologous recombination was used to tag or delete genes (Longtine *et al.*, 1998). All integrations and deletions were verified by PCR analysis. For protein extractions and fluorescence microscopy, yeast liquid cultures were grown to mid log phase and analyzed. All yeast cultures were grown at 30°C unless otherwise indicated. To induce Hxt6 expression and PM localization, synthetic medium with 0.05% glucose (instead of regular 2% glucose) was used. Under this condition, the culture density is able to reach $OD_{600} \approx 1.2$. For the Hxt6-GFP degradation assay, yeast cells in medium with 0.05% glucose were grown to $OD_{600} \approx 0.4–0.5$ before addition of 5% glucose, or 5% glucose plus 50 µg/ml CHX to induce Hxt6 endocytic turnover.

Cellular protein levels and antibodies

Denaturing whole-cell lysates were prepared by pelleting three to five OD_{600} equivalents of cells and precipitating with 10% trichloroacetic acid (TCA). Samples were then washed in acetone, dried, resuspended in cracking buffer (6 M urea, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% SDS), and mechanically disrupted by bead beating. Urea sample buffer (6 M urea, 150 mM Tris, pH 6.8, 6% SDS, and 10% β-mercaptoethanol [2-ME] with bromophenol blue) was added, and extracts were analyzed by SDS–PAGE and immunoblotting. For analysis of PM cargoes (Hxt6-GFP, Lyp1-GFP, Dip5-GFP), 8 M urea and 8% SDS were included in both cracking buffer and sample buffer, with replacement of 2-ME with 100 mM dithiothreitol in sample buffer. Standard SDS gels were used (6% for ARTs; 9.5% for PM cargoes). For Hxt6-GFP analysis, stacking gel was omitted to reduce protein aggregation. Quantitative fluorescence imaging of immunoblots was performed using an Odyssey infrared imaging system (LI-COR Biosciences). Protein levels were quantified by fluorescence intensity of immunoblots using the Odyssey software or Image Studio Lite software. For presentations, immunoblots were linearly adjusted for brightness and contrast in Odyssey or Image Studio Lite (which does not affect their quantifications) and cropped in Photoshop (Adobe). Antibodies used in this study include anti-FLAG (M2; Sigma-Aldrich), anti-hemagglutinin (12CA5; Roche), anti-GFP (B2; Santa Cruz Biotechnology), anti–glucose-6-phosphate dehydrogenase (G6PDH; Sigma-Aldrich), anti-myc (9E10; Sigma-Aldrich), anti-GFP (TP401; Torrey Pines Biolabs), and anti-FLAG (F7425; Sigma-Aldrich).

Immunoprecipitation and detection of polyubiquitinated ARTs

To facilitate the detection of proteasome-targeting polyubiquitin conjugates, MG132 (25 µg/ml; ApexBio) was used to inhibit the proteasome in the *pdr5*Δ strain background. Myc-ubiquitin was overexpressed under the control of the copper-inducible *CUP1* promoter. Cells were grown to early log phase, treated with 100 µM $CuSO_4$ for 2–3 h, and then treated with MG132 for 1 h. Between 20 and 25 OD_{600} equivalents of cells were harvested and precipitated with 10% TCA. Acetone-washed samples were disrupted by bead beating in 200 µl of cracking buffer (with 6 M urea), cleared by centrifugation at 21,130 × *g* for 3 min (room temperature), and then 10-times diluted in IP buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2% NP-40, 5 mM EDTA, protease inhibitors, phosphatase inhibitors, 2.5 mM *N*-ethylmaleimide, and 25 µg/ml bortezomib). Art1/2/4-FLAG was immunoprecipitated with M2 anti-FLAG beads (Sigma-Aldrich) at 4°C for 2.5 h. Beads were then washed three times with IP buffer and resuspended in IP buffer plus urea sample buffer (1:1 ratio). Elutes were analyzed by SDS–PAGE and immunoblotting. To enhance the transfer efficiency of high-MW polyubiquitinated proteins, methanol was completely omitted from the transfer buffer. Ubiquitinated ARTs were detected by α-myc (9E10) antibody.

Microscopy

All microscopy was performed using the DeltaVision RT (Applied Precision, Issaquah, WA) equipped with a 100× objective and fluorescein isothiocyanate and rhodamine filters. Images were captured with a digital camera (CoolSNAP HQ; Photometrics) and deconvolved using the softWoRx software (Applied Precision). Images were linearly adjusted for brightness and contrast in softWoRx and cropped in Photoshop (Adobe).

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