Bro1 coordinates deubiquitination in the multivesicular body pathway by recruiting Doa4 to endosomes

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Ubiquitination directs the sorting of cell surface receptors and other integral membrane proteins into the multivesicular body (MVB) pathway. Cargo proteins are subsequently deubiquitinated before their enclosure within MVB vesicles. In Saccharomyces cerevisiae, Bro1 functions at a late step of MVB sorting and is required for cargo protein deubiquitination. We show that the loss of Bro1 function is suppressed by the overexpression of DOA4, which encodes the ubiquitin thiolesterase required for the removal of ubiquitin from MVB cargoes. Overexpression of DOA4 restores cargo protein deubiquitination and sorting via the MVB pathway and reverses the abnormal endosomal morphology typical of bro1 mutant cells, resulting in the restoration of multivesicular endosomes. We further demonstrate that Doa4 interacts with Bro1 on endosomal membranes and that the recruitment of Doa4 to endosomes requires Bro1. Thus, our results point to a key role for Bro1 in coordinating the timing and location of deubiquitination by Doa4 in the MVB pathway.

Introduction

Multivesicular bodies (MVBs) are late endosomes containing lumenal vesicles formed by invagination of the limiting endosomal membrane. The MVB vesicles are delivered into the hydrolytic lumen of the lysosome/vacuole upon fusion of the limiting MVB membrane with the lysosomal/vacuolar membrane. A variety of cell surface receptors down-regulated from the plasma membrane are sorted into MVB vesicles en route to being degraded, including many growth factor receptor tyrosine kinases in animals and G protein-coupled pheromone receptors in the budding yeast Saccharomyces cerevisiae. In yeast, several biosynthetic enzymes are also sorted into MVB vesicles during their transport from the Golgi to the vacuole (for review see Hicke and Dunn, 2003).

Ubiquitination mediates the sorting of integral membrane proteins into the MVB pathway. Ubiquitin (Ub) is a highly conserved 76-aa polypeptide that is covalently linked to specific protein substrates by a cascade of Ub-conjugation enzymes. Ubiquitination was first characterized to occur on soluble proteins that are polyubiquitinated via the attachment of a chain of four or more Ub subunits, which targets these substrates for degradation by the proteasome (Weissman, 2001). In contrast, MVB cargo proteins are monoubiquitinated via the linkage of a single Ub subunit (or a chain of two to three subunits) to their cytoplasmic domains (Hicke and Dunn, 2003). Ub is removed from both polyubiquitinated and monoubiquitinated proteins by deubiquitinating enzymes, thereby enabling cells to maintain a constant pool of Ub (Weissman, 2001; Wing, 2003).

The sorting of ubiquitinated MVB cargoes is controlled by class E Vps proteins, a set of conserved cytoplasmic proteins that associate with endosomal membranes. Several class E Vps proteins coassemble into distinct complexes that bind to ubiquitinated cargo proteins and guide them into the MVB pathway. In yeast, cargoes initially bind the Vps27–Hse1 complex and subsequently interact with the ESCRT-I complex (Vps23, Vps28, and Vps37; Bilodeau et al., 2003; Katzmann et al., 2003). The ESCRT-II (Vps22, Vps25, and Vps36) and ESCRT-III (Vps2, Vps20, Vps24, and Snf7) complexes function downstream of ESCRT-I (Babst et al., 2002a, b), but their precise roles are not known. In mammalian cells, class E Vps orthologues also mediate the sorting of MVB cargo proteins (Hicke and Dunn, 2003). Furthermore, this machinery is exploited by certain enveloped viruses in order to escape from host cells. For example, Tsg101, the mammalian orthologue of yeast Vps23, is recruited to HIV-1 budding sites at the plasma membrane by directly interacting with the HIV-1 Gag protein (Huang et al., 2000).

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Abbreviations used in this paper: CPS, carboxypeptidase S; CPY, carboxypeptidase Y; CPY-Inv, CPY-invertase; DIC, differential interference contrast; MVB, multivesicular body; Ub, ubiquitin; UBP, ubiquitin-specific processing protease.
with ubiquitated viral Gag proteins (for review see Pornillos et al., 2002).

Additional class E Vps proteins function in the MVB pathway in yeast, including Vps4, an AAA-type ATPase that catalyzes the dissociation of ESCRT complexes from endosomal membranes (Babst et al., 2002a,b). Vps4 also functions in the endosomal dissociation of Bro1, a class E Vps protein required for the deubiquitination of cargo proteins at a late stage of the MVB pathway downstream of the ESCRT-III complex (Nikko et al., 2003; Odorizzi et al., 2003). The association of Bro1 with endosomes requires Snf7 (Odorizzi et al., 2003), and the mammalian Bro1 orthologue, Alix (also known as Aip1), has been shown to bind to the mammalian Snf7 orthologue, CHMP4b (Katoh et al., 2003; Martin-Serrano et al., 2003; Peck et al., 2004; Stack et al., 2003; von Schwedler et al., 2003). Studies of HIV-1–infected cells have revealed that Alix/Aip1 also binds Tsg101 during viral budding from the plasma membrane (Strack et al., 2003; von Schwedler et al., 2003), suggesting that Alix/Aip1 interacts with the unconventional phospholipid to promote membrane invagination (Matsuo et al., 2004), but the role of Alix/Aip1 in controlling membrane dynamics is not clear.

Here, we report that the defects in MVB sorting caused by the loss of Bro1 function in yeast are suppressed by overexpression of the DOA4 gene, which encodes the Ub thiolesterase required for the removal of Ub from MVG cargo proteins (Dupre and Haguenauer-Tsapis, 2001; Katzmann et al., 2001; Losko et al., 2001). Specifically, our data demonstrate that high-copy expression of DOA4 restores cargo protein deubiquitination and subsequent transport via the MVB pathway in bro1 mutant cells. The catalytic activity of Doa4 is essential for its role as a suppressor, indicating that the mechanism of suppression involves substrate deubiquitination. Furthermore, the multivesicular morphology of late endosomes, which is abnormal in bro1 mutant cells, is restored by the overexpression of DOA4. The ability of high-copy DOA4 to suppress the loss of Bro1 function is explained by our finding that Bro1 is essential for the localization of Doa4 to endosomes and that Bro1 and Doa4 physically interact on endosomal membranes. Therefore, Bro1 has a crucial role in coordinating substrate deubiquitination in the MVB pathway by recruiting Doa4 to endosomes.

**Results**

**The coiled-coil domain of Bro1 is essential for MVB sorting**

Many class E Vps proteins have one or more coiled-coil domains, which are structural elements that typically mediate protein–protein interactions. Bro1 has a central coiled-coil domain spanning residues 543-583 (Fig. 1 A). We constructed a mutant allele (bro1ΔCC) in which the sequence encoding this domain had been deleted, and then integrated the bro1ΔCC allele by homologous recombination into the genome in place of the wild-type BRO1 gene. To determine whether MVB sorting was functional in bro1ΔCC cells, we examined the intracellular localization of a fusion protein in which GFP was attached to the cytoplasmic domain of carboxypeptidase S (CPS), a biosynthetic enzyme that is sorted via the MVB pathway during its transport from the Golgi to the vacuole (Odorizzi et al., 1998). CPS was found entirely within the vacuole lumen of wild-type cells (Fig. 1 B). In contrast, GFP-CPS in bro1ΔCC cells was observed at the vacuole membrane (Fig. 1 B), which is indicative of a defect in sorting of the fusion protein via the MVB pathway (Odorizzi et al., 1998). CPS was also mislocalized to the vacuole membrane and to a structure that resembles the vacuole lumen (unpublished data). Thus, the coiled-coil domain of Bro1 is essential for the function of Bro1 in the MVB pathway.

Interestingly, when we overexpressed the bro1ΔCC mutant allele in wild-type cells, GFP-CPS was mislocalized to the vacuole membrane and to a structure that resembles the class E compartment (Fig. 1 C). In contrast, overexpression of the wild-type BRO1 gene had no deleterious effect on the localization of GFP-CPS (Fig. 1 C). The overexpression of bro1ΔCC, therefore, causes a dominant-negative phenotype, possibly because the mutant gene product interferes with the function of the wild-type Bro1 protein.

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**Figure 1. The coiled-coil domain of Bro1 is essential for CPS transport via the MVB pathway.** (A) Schematic diagram of Bro1 indicating the locations of the Bro1 domain (BOD), the coiled-coil domain (CC), and the proline-rich domain (PRD). (B and C) Fluorescence and DIC microscopy of HIV-1–infected cells expressing a GFP-CPS fusion. In C, cells were transformed with a high-copy (2 µ) plasmid encoding bro1ΔCC or wild-type BRO1. Arrowheads indicate class E compartments (B) and class E compartment-like structures (C). Bars, 2.5 µm.
The coiled-coil domain is not required for Bro1 to associate with endosomes

We previously demonstrated that Bro1 is a cytoplasmic protein which associates with endosomal compartments and that the dissociation of Bro1 from endosomes requires the ATPase activity of Vps4 (Odorizzi et al., 2003). Thus, when we replaced the wild-type BRO1 gene with a genomically integrated BRO1-GFP gene fusion in cells that have normal Vps4 function (VPS4+), we observed multiple fluorescent punctate structures in addition to diffuse cytoplasmic fluorescence (Fig. 2 A). Upon deletion of the VPS4 gene (vps4Δ), the GFP fluorescence was concentrated at class E compartments, which had formed in these cells due to the absence of Vps4 activity (Odorizzi et al., 2003). Thus, the secretion of CPY by Bro1-coated vesicles (Vps4-dependent) was blocked, whereas CPY is transported further toward the vacuole (Cereghino et al., 1995; Cooper and Stevens, 1996). The class E compartments that are formed in vps4Δ mutant cells prevent CPY from efficiently recycling to the Golgi (Fig. 2 B, C), resulting in a significant portion of newly synthesized CPY entering by default into the secretory pathway (Odorizzi et al., 2003). Thus, the secretion of CPY by vps4Δ mutant cells is likely to be an indirect consequence of the aberrant endosomal morphology that occurs upon the loss of Bro1 function.

The colorimetric assay for invertase activity can also be used to detect the secretion of CPY-Inv by cells growing on solid medium (Darsow et al., 2000). We used this assay in a genetic screen to identify any gene that, when overexpressed in vps4Δ cells, reduced the amount of secreted CPY-Inv. We reasoned that such a high-copy suppressor would encode a protein that interacts with Bro1 and would be able to restore normal function of the MVB pathway when over-produced in the vps4Δ mutant strain. One clone contained a plasmid that could reproduc-
The catalytic region of UBP enzymes have a conserved three-domain architecture that includes a critical cysteine residue, which forms a thiolester bond with Ub (Hu et al., 2002). We constructed a point mutant allele (doa4ΔC/S) in which the corresponding cysteine in Doa4 (Cys571) had been changed to a serine residue. The doa4ΔC/S allele failed to suppress CPY-Inv secretion when overexpressed in either bro1ΔCC or bro1Δ cells. Instead, doa4ΔC/S overexpression enhanced the mutant phenotype in both strains and caused a stronger dominant-negative phenotype in wild-type cells (Fig. 3 E). Catalytic activity, therefore, is essential for high-copy DOA4 to suppress CPY-Inv secretion caused by the loss of Bro1 function.

Because high-copy DOA4 partially suppressed the bro1Δ phenotype, we determined whether the loss of any class E Vps protein could be bypassed by DOA4 overexpression. We transformed either the empty library vector or the library plasmid containing DOA4 into strains in which individual class E Vps genes had been deleted, and then measured the percentage of secreted CPY-Inv (Table I). Suppression failed to occur in strains in which components of the ESCRT-I, -II, or -III complexes had been deleted (Table I). High-copy DOA4 also could not suppress the loss of other class E Vps proteins, including Vps27 (Table I), which functions upstream of the ESCRT-I complex in the recognition of ubiquitinated MVB cargo proteins (Bilodeau et al., 2003; Katzmann et al., 2003). Interestingly, DOA4 overexpression suppressed the secretion of CPY-Inv by vps4Δ cells but could not suppress either bro1Δ vps4Δ or bro1ΔCC vps4Δ double mutant cells (Table I). These observations suggest that high-copy DOA4 suppresses the CPY sorting defects in bro1 and vps4 mutants by different mechanisms.

**High-copy DOA4 suppresses CPS deubiquitination and sorting defects in bro1ΔCC mutant cells**

We had hypothesized that a high-copy suppressor of the aberrant secretion of CPY-Inv by bro1Δ mutant cells would restore normal function of the MVB pathway. Indeed, when we examined the localization of GFP-CPS in bro1ΔCC cells overexpressing DOA4, most of the fusion protein was seen inside the vacuole lumen, with only a small amount detected on the vacuole membrane (Fig. 4 A). Fluorescent punctate structures were not evident in these cells, suggesting that class E compartment formation was significantly reduced. We also observed more GFP-CPS within the vacuole lumen of bro1Δ cells overexpressing DOA4 (Fig. 4 B) compared with bro1Δ cells alone (Fig. 1 B). However, compared with bro1ΔCC cells that overexpress DOA4 (Fig. 4 A), much more GFP-CPS in bro1Δ cells overexpressing DOA4 was seen at the vacuole membrane and at structures that resembled class E compartments (Fig. 4 B). Thus, high-copy DOA4 is much less efficient at suppressing the defect in GFP-CPS sorting in bro1Δ compared with bro1ΔCC cells.

Although the overexpression of DOA4 in vps4Δ cells suppressed CPY-Inv secretion (Table I), it did not restore the sorting of GFP-CPS into the vacuole lumen. As shown in Fig. 4 D, GFP-CPS was mislocalized to the vacuole mem-

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**Figure 3.** **DOA4 overexpression in bro1 mutant cells suppresses CPY-Inv secretion.** Schematic diagram of the trafficking of CPY in wild-type cells (A) and in bro1 mutant cells (B). Not depicted in A is the fusion of the endosome/MVB with the vacuole. PM, plasma membrane. C–E) Quantitation of CPY-Inv secretion by BHY10, CC57, and KGY1 cells transformed either with the empty high-copy (2μ) plasmid (C), the 2μ plasmid containing the wild-type DOA4 gene (D), or the 2μ plasmid containing the mutant doa4ΔC/S allele (E). The bar graph depicts the mean ± standard error from multiple independent experiments (Table I).
brane and the class E compartment in \textit{vps4}\Delta cells overexpressing \textit{DOA4}, which is identical to the mislocalization of GFP-
CPS in \textit{vps4}\Delta cells (Odorizzi et al., 1998). High-copy \textit{DOA4}
also failed to restore the sorting of GFP-CPS via the MVB
pathway in other class E \textit{vps} mutants (Table I).

In \textit{doa4} mutant cells, CPS and other MVB cargoes accumu-
late in their ubiquitinated forms (Dupre and Haguena ure-
Tsapis, 2001; Katzm ann et al., 2001; Losko et al., 2001). We
recently showed that ubiquitinated CPS (Ub-CPS) also accumu-
lates in \textit{bro1}\Delta cells (Odorizzi et al., 2003). Therefore, we
investigated whether \textit{DOA4} overexpression could alleviate
the defect in CPS deubiquitination that occurs upon loss of
Bro1 function. Because the deubiquitination of CPS is nor-
mally efficient, Ub-CPS was difficult to detect in wild-type
cells, but a significant amount of Ub-CPS was observed in
\textit{bro1}\Delta cells (Fig. 4 E). As shown in Fig. 4 (E and F), overex-
pression of \textit{DOA4} completely restored CPS deubiquitination
in \textit{bro1}\Delta cells, which is consistent with a role for Doa4 in
deubiquitinating MVB cargo proteins.

Interestingly, much more Ub-CPS was observed in \textit{bro1}\Delta
cells compared with the level of Ub-CPS seen in \textit{bro1}\Delta\Delta
cells (Fig. 4 E), indicating that the deubiquitination of CPS
is more severely compromised if the Bro1 protein is absent.
Furthermore, the amount of Ub-CPS in \textit{bro1}\Delta mutants

### Table I. Effect of \textit{DOA4} over-expression on CPY-Inv secretion and GFP-CPS sorting

<table>
<thead>
<tr>
<th>Strain</th>
<th>(-/+ 2 \mu ) \textit{DOA4}(^a)</th>
<th>Percentage of secreted CPY-Inv(^b)</th>
<th>GFP-CPS localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>–</td>
<td>1.0 ± 2.1(^c) ((5)^d)</td>
<td>VL(^e)</td>
</tr>
<tr>
<td>\textit{bro1}\Delta</td>
<td>–</td>
<td>11.5 ± 4.7 ((24))</td>
<td>VM + EC(^f)</td>
</tr>
<tr>
<td>\textit{vps4}\Delta</td>
<td>–</td>
<td>13.0 ± 3.8 ((6))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>\textit{bro1}\Delta \textit{vps4}\Delta</td>
<td>–</td>
<td>35.2 ± 2.9 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>ESCRT-I mutants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{vps23}\Delta</td>
<td>–</td>
<td>20.0 ± 5.9 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>\textit{vps28}\Delta</td>
<td>–</td>
<td>13.4 ± 1.2 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>\textit{vps37}\Delta</td>
<td>–</td>
<td>16.3 ± 1.7 ((3))</td>
<td>VL(^g)</td>
</tr>
<tr>
<td>ESCRT-II mutants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{vps22}\Delta</td>
<td>–</td>
<td>20.1 ± 4.5 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>\textit{vps25}\Delta</td>
<td>–</td>
<td>23.1 ± 1.0 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>\textit{vps36}\Delta</td>
<td>–</td>
<td>24.2 ± 6.3 ((3))</td>
<td>VM + EC</td>
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<tr>
<td>ESCRT-III mutants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{vps2}\Delta</td>
<td>–</td>
<td>25.2 ± 5.0 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>\textit{vps24}\Delta</td>
<td>–</td>
<td>29.1 ± 4.3 ((3))</td>
<td>VM + EC</td>
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<tr>
<td>\textit{vps20}\Delta</td>
<td>–</td>
<td>24.2 ± 2.9 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>\textit{snf7}\Delta</td>
<td>–</td>
<td>25.8 ± 5.0 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>Other class E \textit{vps} mutants tested</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{vps27}\Delta</td>
<td>–</td>
<td>28.0 ± 1.5 ((3))</td>
<td>VM + EC</td>
</tr>
<tr>
<td>\textit{nhx1}\Delta</td>
<td>–</td>
<td>41.8 ± 13.0 ((3))</td>
<td>VL(^g)</td>
</tr>
<tr>
<td>\textit{vps60}\Delta</td>
<td>–</td>
<td>17.7 ± 1.3 ((3))</td>
<td>VM + EC</td>
</tr>
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</table>

\(^a\)Strains were transformed with the empty \(2 \mu\) vector (−) or the \(2 \mu\) vector containing \textit{DOA4} (+).

\(^b\)The percentage of total cellular CPY-Inv secreted by cells.

\(^c\)Mean ± standard error.

\(^d\)Number of independent experiments.

\(^e\)Vacuole lumen.

\(^f\)Vacuole membrane and class E compartment.

\(^g\)There was no noticeable defect in GFP-CPS sorting in \textit{vps37}\Delta and \textit{nhx1}\Delta cells.
was much less significantly reduced upon overexpression of DOA4, in contrast with the complete restoration of CPS deubiquitination that occurred in bro1ΔCC cells. This result is consistent with our observation that high-copy DOA4 was much more effective at suppressing the GFP-CPS sorting defect in bro1ΔCC cells (Fig. 4 A) versus bro1Δ cells (Fig. 4 B). Thus, excess amounts of Doa4 effectively alleviate the mutant phenotype caused by deletion of the Bro1 coiled-coil domain but cannot substitute for deletion of the entire Bro1 protein.

High-copy DOA4 restores MVB morphology in bro1 mutant cells

By fluorescence microscopy, it appeared that the overexpression of DOA4 caused a reduction in the number of class E compartments in bro1 mutant cells. Therefore, we used EM to examine the effect that high-copy DOA4 has on endosomal ultrastructure. In wild-type cells, we readily observed MVBs, with each compartment consisting of a limiting
membrane bilayer surrounding numerous vesicular profiles (Fig. 5, A and B). In contrast, MVBs were never evident in \( \text{bro}1^{\Delta \text{CC}} \) cells, which, instead, contained numerous class E compartments. An example of a class E compartment seen in \( \text{bro}1^{\Delta \text{CC}} \) cells is shown in Fig. 5 C. Similar structures were also seen in \( \text{bro}1^{\Delta} \) cells (not depicted).

High-copy \( \text{DOA}4 \) caused a striking change in endosomal morphology in \( \text{bro}1^{\Delta \text{CC}} \) mutant cells. Rather than class E compartments, we observed numerous multivesicular structures, examples of which are shown in Fig. 5 D. These compartments consisted of a limiting membrane bilayer that encircled multiple vesicular profiles within the compartment lumen, which is similar to MVBs in wild-type cells (Fig. 5 B). Quantitative analysis indicated that \( \text{DOA}4 \) overexpression in \( \text{bro}1^{\Delta \text{CC}} \) cells caused a dramatic reduction in the number of class E compartments and resulted in almost as many MVBs as we had observed in wild-type cells (Fig. 5 E). The apparent reformation of MVBs and the concomitant disappearance of class E compartments upon \( \text{DOA}4 \) overexpression occurred to a lesser extent in \( \text{bro}1^{\Delta} \) cells (Fig. 5 E), again indicating that high-copy \( \text{DOA}4 \) is not as efficient at suppressing the phenotype caused by a total loss of Bro1 compared with its ability to suppress the phenotype caused by a deletion of only the Bro1 coiled-coil domain.

Interestingly, the overexpression of \( \text{DOA}4 \) in \( \text{bro}1 \) mutant cells occasionally resulted in multivesicular compartments having tubules projecting toward the cytoplasm that resemble the cisterna-like structures of class E compartments (Fig. 5 D, arrowhead). Although further studies are needed in order to establish the identity of these unusual structures, they may correspond to intermediate MVB/class E compartments.

The localization of \( \text{Doa4} \) to endosomes requires \( \text{Bro1} \)

\( \text{Doa4} \) had previously been shown to associate with endosomal compartments (Amerik et al., 2000). Indeed, when we integrated a \( \text{DOA}4\)-GFP gene fusion in place of the wild-type \( \text{DOA}4 \) gene in cells that have normal Vps4 function (\( \text{VPS}4^{+} \)), we observed multiple fluorescent punctate structures in addition to diffuse cytoplasmic fluorescence (Fig. 6 A). This pattern of fluorescence resembles the intracellular localization of \( \text{Bro1-GFP} \) in \( \text{VPS}4^{+} \) cells (Fig. 2 A). Also like \( \text{Bro1-GFP} \), \( \text{Doa4-GFP} \) was concentrated at class E compartments in \( \text{vps}4^{\Delta} \) cells (Fig. 6 B). \( \text{Doa4-GFP} \) was also localized to class E compartments in \( \text{bro}1^{\Delta \text{CC}} \) and \( \text{bro}1^{\Delta \text{CC}} \text{vps}4^{\Delta} \) cells (Fig. 6, C and D, closed arrowheads), indicating that the Bro1 coiled-coil domain is not required for the association of \( \text{Doa4} \) with late endosomes. Interestingly, \( \text{Doa4-GFP} \) was localized to additional punctate structures that were not labeled by FM 4-64. In E and F, open arrowheads indicate FM 4-64-positive class E compartments at which \( \text{Doa4-GFP} \) is not localized. Bars, 2.5 \( \mu \text{m} \).

A dramatic change in the localization of \( \text{Doa4-GFP} \) occurred in cells in which the \( \text{BRO1} \) gene had been deleted. Both in \( \text{bro}1^{\Delta} \) cells (Fig. 6 E) and \( \text{bro}1^{\Delta} \text{vps}4^{\Delta} \) double mutant cells (Fig. 6 F), \( \text{Doa4-GFP} \) failed to localize to class E compartments and was, instead, diffusely distributed. Some faintly fluorescent structures were evident at the periphery of these cells but are unlikely to be endosomes, as they were never stained when FM 4-64 was incubated continuously with cells (unpublished data), a procedure that results in staining of all compartments of the endocytic pathway (Vida and Emr, 1995). Western blot analysis confirmed that the expression of full-length \( \text{Doa4-GFP} \) in \( \text{bro}1^{\Delta} \) and \( \text{bro}1^{\Delta} \text{vps}4^{\Delta} \) double mutant cells was equivalent to its expression in wild-type and \( \text{vps}4^{\Delta} \) cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200403139/DC1), indicating that the loss of punctate localization of \( \text{Doa4-GFP} \) in the
absence of Bro1 was not due to aberrant cleavage of the fusion protein. Furthermore, transformation of a plasmid encoding the wild-type BRO1 gene into bro1Δ and bro1Δ vps4Δ cells restored the localization of Doa4-GFP to endosomal compartments (unpublished data). Thus, Bro1 is essential for the localization of Doa4 to endosomes.

To test whether the endosomal localization of Bro1 is likewise dependent on Doa4, we examined the localization of Bro1-GFP in cells in which the DOA4 gene had been deleted. In doa4Δ VPS4Δ cells, we observed punctate fluorescent structures in addition to diffuse cytoplasmic fluorescence (Fig. 7 A), whereas in doa4Δ vps4Δ double mutant cells, the GFP fluorescence was concentrated at class E compartments (Fig. 7 B). This pattern of fluorescence was virtually identical to the intracellular localization of Bro1-GFP in VPS4Δ and vps4Δ cells (Fig. 2). Therefore, Doa4 is not required for the endosomal localization of Bro1.

**Doa4 interacts with Bro1**

Because Bro1 is required for the endosomal localization of Doa4, we investigated whether Doa4 physically interacts with Bro1 by immunoprecipitating the Doa4-GFP fusion protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions. As shown in Fig. 8 A, protein from detergent-solubilized extracts of yeast cell lysates under native conditions.

**Endosomal localization of Doa4 in ESCRT-III mutant cells**

A previous study had suggested that the association of Doa4 with endosomes was dependent on two other class E Vps proteins, Vps24 and Snf7 (Amerik et al., 2000). Both Vps24 and Snf7 are components of the ESCRT-III complex that oligomerizes on endosomal membranes (Babst et al., 2002a). We recently showed that the endosomal localization of Bro1 requires Snf7 but not Vps24, suggesting that Snf7 has a role in the recruitment or stabilization of Bro1 at endosomes (Odorizzi et al., 2003). Therefore, we examined the localization of Doa4-GFP in vps4Δ cells in which either VPS4 or SNF7 had been deleted. In contrast with the previous analysis of Doa4 localization (Amerik et al., 2000), we observed Doa4-GFP at class E compartments in vps24Δ vps4Δ double mutant cells (Fig. 9 A). However, Doa4-GFP was diffusely dis-
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Figure 9. Localization of Doa4-GFP in ESCRT-III mutants. (A and B) Fluorescence and DIC microscopy of GOY113 (A) and GOY114 (B) cells stained with FM 4-64. Closed arrowheads indicate colocalization of Doa4-GFP and FM 4-64 at class E compartments. Open arrowheads indicate class E compartments at which Doa4-GFP is not localized. Bars, 2.5 μm. (C) Native immunoprecipitations from detergent extracts of total lysates of GOY115 and GOY116 cells.

Recruitment of Doa4 by Bro1

Both Doa4-GFP and Bro1-GFP were associated with endosomal compartments, but upon deletion of the BRO1 gene, the localization of Doa4-GFP shifted to the cytoplasm. Consistent with this model, the association of Doa4-GFP with endosomes was dependent on Snf7, a component of the ESCRT-III complex. We recently showed that Snf7 is essential for the endosomal localization of Bro1 (Odorizzi et al., 2003), and in mammalian cells, the orthologue of Bro1, Alix/Aip1, binds Chmp4b, which is an orthologue of Snf7 (Katoh et al., 2003; Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003; Peck et al., 2004). Thus, it is likely that Snf7 has an indirect role in the endosomal localization of Doa4 by functioning to recruit Bro1 to endosomes or to stabilize the association of Bro1 with endosomal membranes. Indeed, we were unable to detect a physical interaction between Doa4 and Bro1, which could reflect direct binding of Doa4 to Bro1 or, alternatively, could be mediated by another component that binds both proteins. In either case, the association of Doa4 with Bro1 occurred predominantly in subcellular fractions containing late endosomal membranes, which is consistent with the ordered recruitment of Bro1 to endosomes followed by the recruitment of Doa4 (Fig. 10).

Discussion

Protein sorting in the MVB pathway requires Bro1, a cytoplasmic protein that associates with endosomal compartments (Odorizzi et al., 2003). Here, we show that DOA4 overexpression suppresses the defects in MVB transport and restores the multivesicular morphology of endosomes in bro1 mutant cells. These observations can be explained by our finding that Bro1 associates with Doa4 on endosomal membranes and that the recruitment of Doa4 to endosomes requires Bro1. Thus, our results indicate a key role for Bro1 in regulating the timing and location of Doa4 activity in the MVB pathway.
transport of GFP-CPS was blocked in bro1<sup>Δcc</sup> mutant cells as effectively as it was in bro1<sup>Δ</sup> cells. Therefore, the recruitment of Doa4 to endosomes is only one aspect of the function of Bro1 in the MVB pathway. The role of the Bro1 coiled-coil domain is not yet clear. Thus far, we have been unable to identify another protein that interacts with this region of Bro1, and we have been unable to detect homo-oligomerization of Bro1 that could be mediated by the coiled-coil domain (unpublished data).

**Suppression by high-copy DOA4**

The restoration of multivesicular structures upon the overexpression of DOA4 coincided with the disappearance of class E compartments, which presumably enables the CPY receptor, Vps10, to recycle to the Golgi where it binds newly synthesized CPY-Inv in order to transport this soluble cargo protein to the endosome (Cereghino et al., 1995; Cooper and Stevens, 1996). Accordingly, we have observed that a Vps10-GFP fusion protein was not concentrated at class E compartments in bro1<sup>Δ</sup> mutant cells overexpressing DOA4 but was, instead, localized to multiple punctate structures (unpublished data), which is similar to its localization in wild-type cells (Burda et al., 2002; Odorizzi et al., 2003).

Interestingly, although high-copy DOA4 restored the multivesicular morphology of late endosomes and suppressed CPY-Inv secretion by both bro1<sup>Δcc</sup> and bro1<sup>Δ</sup> cells, the transport of GFP-CPS via the MVB pathway was efficiently restored by DOA4 overexpression only in bro1<sup>Δcc</sup> and not bro1<sup>Δ</sup> cells. Furthermore, the overexpression of DOA4 in bro1<sup>Δ</sup> cells did not significantly enhance the deubiquitination of CPS, whereas the minor amount of Ub-CPS seen in the bro1<sup>Δcc</sup> strain was completely eliminated by DOA4 overexpression. These observations are consistent with the ability of Doa4 to be recruited to endosomes in cells expressing the mutant bro1<sup>Δcc</sup> protein but not in cells in which Bro1 is completely absent. Moreover, these findings suggest that restoring MVB morphology alone by DOA4 overexpression is not sufficient for a complete restoration of the MVB pathway. The overexpression of DOA4 in bro1<sup>Δ</sup> cells may result in excess amounts of Doa4 that could deubiquitinate proteins which regulate the formation of MVB vesicles, but the sorting of cargo into MVB vesicles per se may be critically dependent on the coordination of Doa4 on endosomal membranes by Bro1 as well as other aspects of Bro1 function in the MVB pathway.

**Deubiquitination in the MVB pathway**

Deubiquitination is tightly controlled by Ub-conjugation enzymes and is counterbalanced by deubiquitination. Both poly- and monoubiquitinated proteins undergo deubiquitination, but few Ub thiolesterases have been assigned to specific substrates (Wing, 2003). There are 16 UBPs in S. cerevisiae, suggesting that many of these enzymes have highly specific and regulated functions. However, Doa4 probably has a wide variety of substrates. In addition to its role in the removal of monoubiquitin from MVB cargo proteins, Doa4 is likely to function in the removal of poly-ubiquitin from proteasomal substrates, as Doa4 copurifies with proteasomes that have been isolated from yeast cell extracts (Papa et al., 1999).

Consistent with a role for Bro1 in coordinating Doa4 function are the previous observations that Bro1 is required for the deubiquitination of CPS (Odorizzi et al., 2003) as well as Gap1, an amino acid permease at the plasma membrane that is down-regulated by endocytosis and sorted via the MVB pathway (Nikko et al., 2003). Interestingly, in bro1<sup>Δ</sup> mutant cells, Gap1 is recycled to the plasma membrane but, unlike CPS, does not accumulate in its ubiquitinated form. However, ubiquitinated Gap1 is readily detected in bro1<sup>Δ</sup> mutant cells if recycling is blocked, suggesting that in the absence of Bro1, the deubiquitination of Gap1 (and possibly other endocytic cargoes) occurs elsewhere within the cell and may be catalyzed by one of the 15 other UBPs in yeast (Nikko et al., 2003).

The suppressor activity of high-copy DOA4 was abolished if the putative active-site cysteine residue was altered to serine, indicating that suppression most likely occurred through substrate deubiquitination. However, the deubiquitination of cargoes is not essential for MVB sorting because chimeric cargo proteins that are expressed as transmembrane fusions to Ub cannot be deubiquitinated yet are transported efficiently via the MVB pathway (Reggiori and Pelham, 2001; Urbanowski and Piper, 2001; Bilodeau et al., 2003). Nevertheless, Doa4 is required for the MVB pathway, as the sorting of MVB cargo proteins is blocked in doa4<sup>Δ</sup> mutant cells (Losko et al., 2001; Reggiori and Pelham, 2001). In addition to MVB cargoes, Doa4 could deubiquitinate a component of the MVB sorting machinery in order to regulate its activity. It is not known whether class E Vps proteins in yeast are regulated by ubiquitination. In mammalian cells, however, several cytoplasmic proteins that control receptor down-regulation are monoubiquitinated, including Hrs, which is the orthologue of the yeast class E Vps protein, Vps27 (Polo et al., 2002). CIN85 and endophilin are also monoubiquitinated cytoplasmic components in mammalian cells that associate with endosomes (Haglund et al., 2002; Angers et al., 2004), and both proteins interact with Alix/Aip1 (Vito et al., 1996; Chatellard-Causse et al., 2002). Like Bro1 in yeast, Alix/Aip1 could recruit a UBP that deubiquitinates CIN85 and endophilin in order to regulate their function and/or localization.

Our results suggest a model in which Bro1 recruits Doa4 to endosomal membranes, thereby controlling the timing and location of Doa4 activity in the MVB pathway (Fig. 10). The association of Bro1 itself with endosomes occurs after the assembly of the ESCRT-III complex on endosomes (Odorizzi et al., 2003). Similarly, the deubiquitination of MVB cargo proteins occurs downstream of the functions of the ESCRT-I, -II, and -III complexes (Nikko et al., 2003) but before enclosure of cargoes within MVB vesicles (Hicke and Dunn, 2003). Thus, the Bro1-dependent coordination of Doa4 on endosomal membranes and the subsequent deubiquitination of cargo proteins (and possibly MVB sorting components) is likely to be one of the last steps in the MVB pathway, ensuring that cargo proteins are concentrated at regions of the endosomal membrane where invagination occurs. In mammalian cells, the assembly/budding of HIV-1 depends on ubiquitination of the viral Gag protein (for review see Pornillos et al.,
2002), but a role for deubiquitination in this process has not been described. However, recent studies indicate that Alix/Aip1 interacts with both the ESCRT-I and -III complexes and is involved in viral escape from host cells (Strack et al., 2003; von Schwedler et al., 2003). Future studies may determine whether Alix/Aip1 has a role analogous to Bro1 and functions in the recruitment of a specific UBP to the site of viral budding.

**Materials and methods**

**Yeast strains and plasmid constructions**

Standard protocols were used for culturing *S. cerevisiae*, cellular transformations, and spheroplast preparations (Guthrie and Fink, 2002). See Table II for the genotypes of yeast strains used in this work. Gene deletions were constructed by homologous recombination using site-specific deletion cassettes (Guthrie and Fink, 2002).

**Microscopy**

GFP and FM 4-64 fluorescence and differential interference contrast (DIC) microscopy was performed using a DMRA microscope (Leica) equipped with a Cooke Sensicam digital camera (Applied Scientific Instruments). Images were deconvolved using Slidebook® software (Intelligent Imaging Innovations) and processed using Adobe Photoshop 7.0® software (Adobe Systems Inc.). Cells were stained with FM 4-64 using a pulse-chase proce-
dure at 30°C as described previously (Odorizzi et al., 2003). To observe the localization of GFP-CP5, cells were transformed with pGO47, which contains the GFP-CP5 fusion (Odorizzi et al., 1998). For EM, cells growing logarithmically at 30°C were high pressure frozen, freeze substituted, sectioned, and stained as described in Giddings (2003). Thin sections were viewed with a CM10 electron microscope (Philips) and images were captured on film. For quantitation, the number of multivesicular structures and class E compartments were counted in ~200 randomly chosen cells measuring at least 2.5 μm in diameter.

Genetic screen for high-copy suppressors of bro1

GOY57 cells were transformed with a high-copy library of yeast genomic DNA in plasmid pRS202 (2 μURA3). Transformants were screened for secreted invertase activity using the agar overlay assay described in Darsow et al. (2000). Library plasmids that reliably displayed suppression were recovered and transformed into naive GOY57 cells to confirm that the suppression was plasmid linked. One plasmid (designated pGO289) identified as a suppressor of the bro1

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were rotated at 4°C for 2 h. Immune complexes were recovered by centrifugation at 4°C, pelleted the detergent-insoluble material. Five A600 equivalents of the detergent-insoluble extract, which was rotated at 4°C for 2 h. Immune complexes were recovered by centrifugation at 4°C, washed twice with ice-cold lysis buffer plus 0.5% Triton X-100, and twice with ice-cold lysis buffer. Bound antigen was eluted by boiling the beads in SDS-PAGE sample buffer. Four A600 equivalents of immunoprecipitates and 0.5 A600 equivalents of total lysate were resolved by SDS-PAGE, transferred to nitrocellulose, and examined by Western blotting using anti-GFP mAbs (Zymed Laboratories), anti-HA mAbs (Roche), or polyclonal antisera against Snf7 and Vps24 (Babst et al., 1998).

Online supplemental material

Fig. S1 shows that there is not a significant difference in the expression levels of full-length Doa4-GFP in the strains shown in Fig. 6. Fig. S2 shows the results from a negative control for Fig. 8 in which Bro1-HA is not immunoprecipitated under native conditions by anti-GFP antisem in the absence of Doa4-GFP expression. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200403139/DC1.

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