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RESEARCH ARTICLE

Btn3 regulates the endosomal sorting function of the yeast Ent3 epsin, an adaptor for SNARE proteins

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ABSTRACT

Ent3 and Ent5 are yeast epsin N-terminal homology (ENTH) domain-containing proteins involved in protein trafficking between the Golgi and late endosomes. They interact with clathrin, clathrin adaptors at the Golgi (AP-1 and GGA) and different SNAREs (Vti1, Snc1, Pep12 and Syn8) required for vesicular transport at the Golgi and endosomes. To better understand the role of these epsins in membrane trafficking, we performed a protein–protein interaction screen. We identified Btn3 (also known as Tda3), a putative oxidoreductase, as a new partner of both Ent3 and Ent5. Btn3 is a negative regulator of the Batten-disease-linked protein Btn2 involved in the retrieval of specific SNAREs (Vti1, Snc1, Tlg1 and Tlg2) from the late endosome to the Golgi. We show that Btn3 endosomal localization depends on the epsins Ent3 and Ent5. We demonstrated that in *btn3Δ* mutant cells, endosomal sorting of ubiquitylated cargos and endosomal recycling of the Snc1 SNARE are delayed. We thus propose that Btn3 regulates the sorting function of two adaptors for SNARE proteins, the epsin Ent3 and the Batten-disease-linked protein Btn2.

KEY WORDS: Epsin, Multivesicular body, Ubiquitin, ESCRT, Yeast

INTRODUCTION

Endosomes are key compartments where endocytic, biosynthetic and exocytic pathways intersect. At the late endosome or multivesicular body (MVB), proteins undergo crucial sorting (Hurley, 2008). Membrane proteins destined to the vacuolar or lysosomal lumen are sorted in MVB intraluminal vesicles and are subsequently delivered to the vacuolar lumen upon fusion of the MVB with the vacuole. Proteins remaining at the limiting membrane of the MVB are either delivered to the vacuolar membrane or recycled to the Golgi or the plasma membrane. Several studies have shown that cargo modification by ubiquitin serves as a signal that causes cargo to enter the vesicles of the MVB (Hicke and Dunn, 2003; Lauwers et al., 2010).

In the yeast *Saccharomyces cerevisiae*, several screens have led to the identification of *vps* (vacuolar protein sorting) mutants grouped in six classes, A to F, based on their phenotypes (Raymond et al., 1992). The class E *vps* mutants are defective in MVB sorting and accumulate an aberrant endosomal structure (the class E compartment) in which cargos and endosomal proteins are trapped (Odorizzi et al., 1998). Some class E Vps

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proteins are associated in complexes termed ESCRT (endosomal sorting complex required for transport) (Henne et al., 2011). In yeast, MVB sorting is regulated by two types of endosomal lipids, phosphatidylinositol 3-phosphate (PtdIns3*P*) and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2]. PtdIns3*P*, synthesized by the Vps34 lipid kinase, mediates the specific recruitment of Vps27 through its FYVE domain (Katzmann et al., 2003). PtdIns(3,5)P_2, synthesized by the Fab1 lipid kinase from PtdIns3*P*, controls the MVB sorting of ubiquitylated cargos like the carboxypeptidase S (Cps1) or the endopolyphosphatase (Phm5) (Odorizzi et al., 1998; Reggiori and Pelham, 2002). Yeast epsins Ent3 and Ent5 are PtdIns(3,5)P_2 effectors participating in MVB sorting (Eugster et al., 2004; Friant et al., 2003).

In yeast there are five epsins named Ent1 to Ent5 (Duncan et al., 2003; Wendland et al., 1999). Epsins are cytoplasmic proteins, recruited to membranes through their ENTH (epsin N-terminal homology) domain (De Camilli et al., 2002; Kay et al., 1999). Ent3 and Ent5 were first identified as interactors of the Golgi-localized clathrin adaptors the AP-1 complex and GGA (Golgi-localizing, gamma-adaptin ear homology domain, ARF-binding) proteins (Duncan et al., 2003). However, they have broad functions at the trans Golgi network (TGN) and endosome level. Indeed, *ent3Δ ent5Δ* double-mutant cells display several trafficking defects: (1) in Golgi-to-endosome trafficking of some endosomal SNAREs [SNAP (soluble NSF attachment protein) receptors] and vacuolar carboxypeptidases, (2) in MVB sorting of ubiquitylated cargos and (3) in endosome-to-Golgi retrograde transport of specific cargos like the SNARE Snc1 or the casein kinase Yck2 (Chidambaram et al., 2004; Chidambaram et al., 2008; Copic et al., 2007; Duncan et al., 2003; Eugster et al., 2004; Zimmermann et al., 2010). However, Ent3 and Ent5 proteins also display some specific functions. Ent3 acts principally in the GGA-mediated TGN sorting (Copic et al., 2007; Costaguta et al., 2006) and only the ENTH domain of Ent3 binds to the endosomal SNAREs Vti1, Pep12 and Syn8 (Chidambaram et al., 2004; Chidambaram et al., 2008; Copic et al., 2007). Ent5 (but not Ent3) binds to the chitin synthase Chs3, but Ent3 and Ent5 are both required for its trafficking from the Golgi (Copic et al., 2007). All these results show that Ent3 and Ent5 are key players in membrane trafficking at the TGN and endosomal levels; it is therefore important to better understand their regulation. Indeed, *in vitro* quantitative studies have shown using surface plasmon resonance (Biacore) that the ENTH domain of both Ent3 and Ent5 binds to PtdIns(3,5)P_2 as efficiently as to PtdIns(4,5)P_2 (Narayan and Lemmon, 2006), suggesting that some additional protein interactions are required to mediate their PtdIns(3,5)P_2-dependent specific functions in MVB sorting (Eugster et al., 2004; Friant et al., 2003). Here, we performed a yeast proteome microarray screen to identify new Ent3 and Ent5 binding partners. Among

the different proteins identified, we chose to further characterize Btn3. Btn3 was previously linked to endosomal trafficking and identified as a regulator of the Batten disease protein Btn2, which interacts with different SNAREs (Kama et al., 2007; Kanneganti et al., 2011). We show that the epsins Ent3 and Ent5 are required for the endosomal localization of Btn3. Moreover, in *btn3Δ* mutant cells, endosomal sorting of ubiquitylated cargos and recycling of the Snc1 SNARE are delayed. Our results suggest that Btn3 is a cytoplasmic protein regulating two adaptors for SNARE proteins, the epsin Ent3 and the Batten-disease-linked protein Btn2.

RESULTS

Identification of Btn3 as a new direct Ent3 and Ent5 interactor

To identify new direct interactors of Ent3 and/or Ent5, we carried out a protein–protein interaction screen using yeast protein microarrays (Yeast ProtoArray® Invitrogen) (Zhu et al., 2001). Ent3 and Ent5 were N- or C-terminally tagged with V5-6xHis to avoid missing interactions owing to the tag position and reduce the number of false-positive hits. The fusion proteins were produced in *Escherichia coli* and purified using Ni-NTA–agarose beads. Yeast ProtoArray® chips were incubated in the presence of purified recombinant V5-6xHis-tagged Ent3 and Ent5 proteins and the interactions were revealed using fluorescently tagged anti-V5 antibody. A Z-score, representing the number of standard deviations away from the mean fluorescence of the entire array, was calculated for each signal (ProtoArray® Prospector; Invitrogen). A significant hit was characterized by a Z-score of ≥ 3 and a coefficient of variance for signals from the two replicates (each protein is spotted in duplicate on the chip) of <0.5. This resulted in the identification of 53 direct hits for N- and C-terminally tagged Ent3, and 31 for N- and C-terminally tagged Ent5 (supplementary material Table S1). Among the significant hits, 17 were common to Ent3 and Ent5 (supplementary material Table S1), including the gamma subunit of the AP-1 adaptor complex Apl4 (also known as Ypr029c), previously identified by yeast two-hybrid (Duncan et al., 2003), thus validating our approach (Fig. 1A). Btn3 was also found as a common hit and was of particular interest. Indeed, Btn3 (also known as Tda3 or Yhr009c) was shown to interact *in vitro* with the ubiquitin ligase Rsp5 (Hesselberth et al., 2006) required for MVB sorting (Katzmann et al., 2004; Morvan et al., 2004). Btn3 was recently identified as a binding partner of the Btn2 protein (Kanneganti et al., 2011). Btn2, a Batten-disease-related protein, binds to endocytic SNARE complexes (Snc1, Snc2, Tlg1, Tlg2 and Vti1) and regulates late endosomal sorting of some specific cargos (Kama et al., 2007). Btn3 negatively regulates Btn2 sorting function by sequestering Btn2 away from its cargos (Kanneganti et al., 2011). This was of particular interest for our study, as Ent3 is also involved in SNARE (Vti1, Pep12 or Snc1) binding and trafficking. Surprisingly, the *BTN3* gene was identified in a screen for deletion mutants sensitive to the overexpression of the DNA topoisomerase I *top1*-T_{722}A mutant, and was thus named *TDA3* (topoisomerase I damage affected) (Reid et al., 2011). Interestingly, the largest class of mutants identified in this screen affects the endosomal trafficking, including ESCRT subunits or the Fab1 lipid kinase. In conclusion, all these results point towards a functional link between Btn3 and Ent3.

To confirm the interactions between Btn3 and Ent3 and Ent5, we performed GST (gluthatione-S-transferase) pull-down experiments using GST, GST–Ent3 or GST–Ent5 recombinant proteins and a protein extract from cells expressing chromosomally GFP-tagged Btn3 (Fig. 1B). Btn3–GFP was not retained on GST beads, but specifically interacted with GST–Ent3 and GST–Ent5, with a higher affinity for Ent5. Similar results were obtained for the protein microarray interactions showing a better Z-score for the Btn3–Ent5 interaction (Fig. 1A).

To determine whether Ent3 could be associated with Btn3 in yeast protein extracts, we carried out a co-immunoprecipitation (co-IP) assay. Ent3–HA was expressed in a wild-type yeast strain expressing or not expressing Btn3–GFP at the locus. Ent3–HA proteins were immunoprecipitated with anti-HA antibodies on γ-bind Sepharose beads and pull down of Btn3–GFP was assessed by immunoblotting (Fig. 1C). Under these conditions, Btn3–GFP was co-immunoprecipitated by Ent3–HA (Fig. 1C), showing that Btn3 interacts with Ent3 in wild-type cells. As a control, we also tested the non-specific binding of Btn3–GFP to the beads in the absence of Ent3–HA [Fig. 1C, lane 'WT Btn3-GFP+pHAC' (pHAC, empty vector)], the results show that Btn3–GFP was not retained on the beads. The microarray interaction screen suggests that Btn3 binds to Ent5 with a higher affinity than that its affinity for Ent3 (Fig. 1A). To determine whether Ent5 competes with Ent3 for Btn3 binding, we performed co-IP from an *ent5Δ* cell extract. We observed similar binding efficiency of Btn3–GFP to Ent3–HA, showing that Ent5 is neither required nor competing with Ent3 for Btn3 binding (Fig. 1C). We next determined whether Btn2 competes with Ent3 for Btn3 binding by analyzing their interaction in *btn2Δ* cells, because Btn3 has been shown to interact with Btn2 (Kanneganti et al., 2011). Interestingly, in a *btn2Δ* cell extract, about two times more Btn3–GFP co-immunoprecipitated with Ent3–HA (Fig. 1C); indeed, the mean percentage of Btn3–GFP input protein co-immunoprecipitated by Ent3–HA (two or three different experiments) was 1.18% for the wild-type strain, 2.12% for *btn2Δ* strain and 1.06% for the *vps27Δ* strain. This suggests that Btn2 competes with Ent3 for its interaction with Btn3. We also tested whether the absence of Vps27 interacting with Ent3 and Ent5 (Eugster et al., 2004) affected the interaction between Btn3–GFP and Ent3–HA. In *vps27Δ* mutant cells, Btn3–GFP also co-immunoprecipitated with Ent3–HA (Fig. 1C; 1.06% of coIP), showing that Vps27 is not required for this interaction. Taken together, these results show that Btn3 is a direct interactor of the Ent3 epsin and that this interaction does not require Vps27 and competes with the Btn2 interaction.

Although Btn2 was not found as a hit in our microarray screen for Ent3 interactors, it could be associated with the Btn3–Ent3 protein complex. We thus analyzed the co-IP between Ent3–HA and Btn2–GFP using Pgk1 as a negative control (Fig. 1D). Compared to the co-IP performed on protein extracts from cells lacking Ent3–HA (Fig. 1D, lane 'WT+pHAC') that was used as a negative control, Btn2–GFP was not retained on the Ent3–HA beads, regardless of the presence of Btn3 in the extract (from wild-type or *btn3Δ* cells), showing that Btn2 was not associated with the Ent3 protein.

We then asked whether the Ent3 and Ent5 epsins are required for the Btn2–Btn3 interaction described previously (Kanneganti et al., 2011). We performed a co-IP between Btn3–HA and Btn2–GFP in extracts from wild-type or *ent3Δ ent5Δ* mutant cells (Fig. 1E). In both cell lysates, Btn2–GFP similarly interacts with Btn3–HA. Given that Btn2 facilitates the recruitment of Btn3 to endosomal structures (Kanneganti et al., 2011), we also analyzed the co-IP between Btn3–HA and Btn2–GFP in the *vps27Δ*
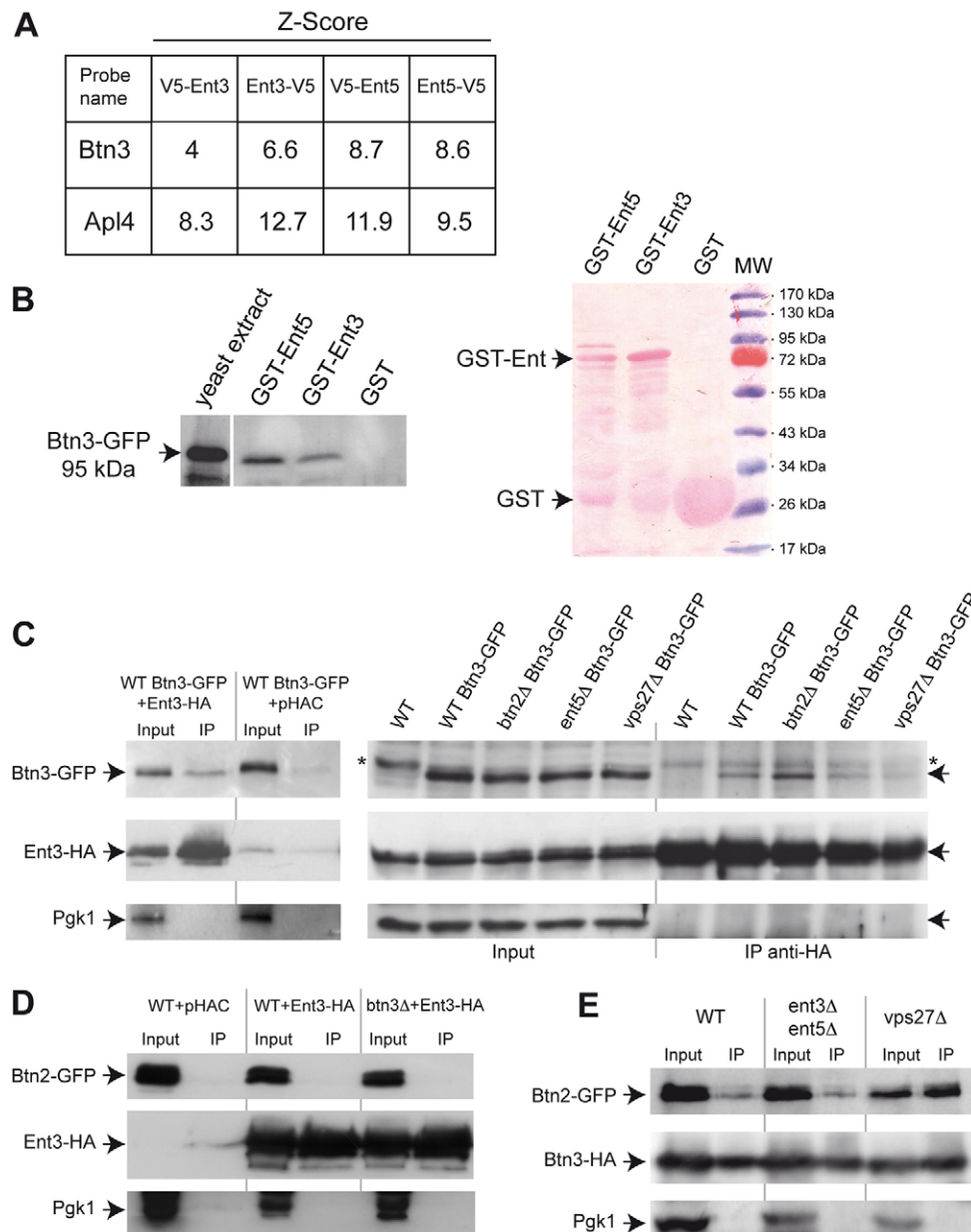
Fig. 1. Btn3 interacts with Ent5 and Ent3 *in vitro* and *in vivo*. (A) Interactions between V5-6xHis-tagged Ent3 or Ent5 and the proteins spotted on the arrays were determined by a *Z*-score calculated by using the ProtoArray® Prospector (Invitrogen) software. A *Z*-Score >3 identifies the statistically significant interactors. (B) Yeast lysate from BY4741 expressing Btn3–GFP was incubated with GST alone, GST–Ent3 or GST–Ent5 on gluthatione-Sepharose beads. Beads were pelleted and the supernatant removed. After washing, proteins attached to beads were eluted with SDS buffer and analyzed by SDS-PAGE. Total yeast extract represents 5% of the protein amount used for the GST-pull down. Btn3–GFP was revealed with anti-GFP antibodies. Ponceau staining of the nitrocellulose membrane shows the amount of GST fusion protein present on the beads. MW indicates the molecular mass marker lane. (C) Yeast cytosol from wild-type (WT) Btn3–GFP (BY4741 Btn3–GFP) cells transformed with either the Ent3–HA expression vector (pFL575) or the empty vector (pHAC, negative control) was immunoprecipitated with anti-HA antibodies and subjected to western blotting with anti-GFP (to detect Btn3–GFP) and anti-Pgk1 phosphoglycerate kinase (loading control). The same co-IP experiment was performed on wild-type (BY4741), wild-type Btn3-GFP (BY4741 Btn3-GFP), *btn2Δ* Btn3-GFP (SFY127), *ent3Δ ent5Δ* Btn3-GFP (SFY23) or *vps27Δ* Btn3-GFP (SFY25) strains expressing HA-tagged Ent3 (pFL575). Input represents 2.5% of the cytosol used for immunoprecipitation. The asterisk shows a non-specific band detected with the anti-GFP antibodies. (D) Coimmunoprecipitation experiments between Btn2–GFP (pAD6-Btn2-GFP) and Ent3–HA (pFL575) expressed from a wild-type or *btn3Δ* strain. The wild-type strain bearing Btn2–GFP (pAD6-Btn2-GFP) and the empty vector (pHAC) was used to control for the non-specific binding of Btn2–GFP to the beads. Pgk1 was used as a negative control. (E) The interaction between Btn2–GFP (pAD6-Btn2-GFP) and Btn3–HA (pSF191) was monitored by co-IP with anti-HA antibodies in different yeast strains, wild type, *ent3Δ ent5Δ* (SFY2) and *vps27Δ*.

mutant. We observed that more Btn2–GFP was retained on the Btn3–HA beads, as expected if the interaction occurred at the endosomal membrane (Fig. 1E). This shows that the Btn3 interaction with Btn2 is not mediated by the Ent3 and Ent5 epsins.
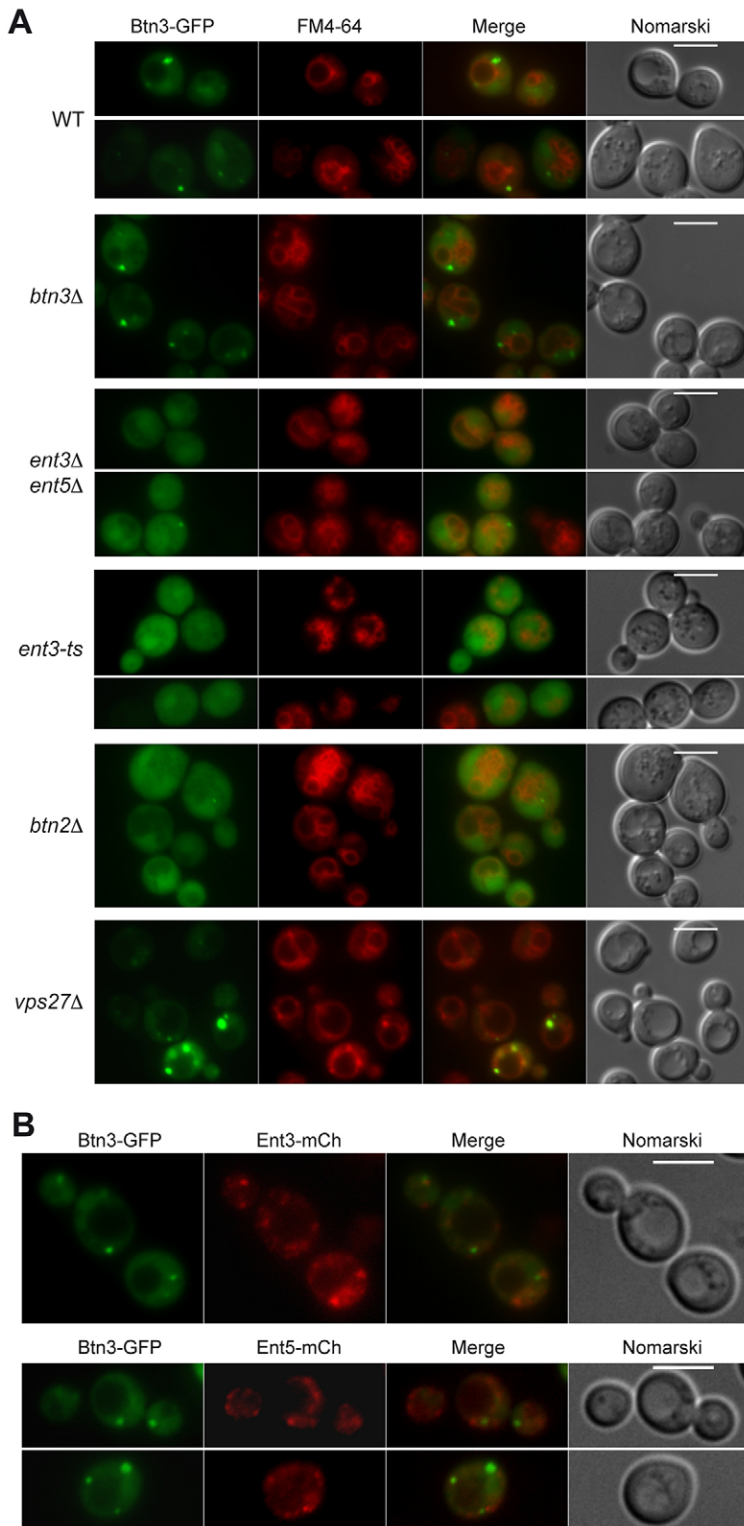
Btn3 endosomal localization depends on the Ent3 and Ent5 epsins

It was shown that Btn3 expressed from the *ADH1* (alcohol dehydrogenase) promoter localizes to endosomes in a Btn2-dependent manner (Kanneganti et al., 2011). To determine

whether Ent3 and Ent5 were also required for this endosomal localization, we expressed Btn3–GFP under the control of the constitutive *GPD1* (glyceraldehyde-3-phosphate dehydrogenase) promoter in wild-type and *btn3Δ*, *ent3Δ ent5Δ*, *ent3-ts*, *btn2Δ* and *vps27Δ* mutant cells (Fig. 2A). In wild-type and *btn3Δ* cells, Btn3–GFP clearly localized to punctae corresponding to endosomes based on their colocalization with the FM4-64-stained class E compartment in *vps27Δ* cells (Fig. 2A). In *btn2Δ*

cells, Btn3–GFP was mainly cytoplasmic, and this was also observed in the *ent3Δ ent5Δ* and *ent3-ts* mutant cells. In *ent3Δ* and *ent5Δ* single-deletion mutants, Btn3–GFP localized to endosomes (data not shown), showing that the *ent3-ts* mutation has a dominant-negative effect. This shows that the endosomal localization of Btn3 depends on Ent3 and Ent5 epsins.

Btn3–GFP expressed from the *ADH1* promoter colocalized perfectly with endogenous Btn2–RFP (Kanneganti et al., 2011).

Fig. 2. Btn3 endosomal localization depends on Ent3 and Ent5.
(A) Btn3–GFP expressed from the constitutive promoter of *GPD1* (glyceraldehyde-3-phosphate dehydrogenase) on a low-copy-number plasmid (pSF170) was transformed into wild-type (WT), *btn3Δ*, *ent3Δ ent5Δ*, *ent3-ts*, *btn2Δ* and *vps27Δ* cells. Transformants grown in selective medium to the exponential phase of growth were stained with the lipophilic fluorescent dye FM4-64 to label the vacuolar membrane, prior to observation by fluorescence microscopy. (B) Fluorescence microscopy of Ent3–mCherry (Ent3-mCh) or Ent5–mCherry (Ent5-mCh) chromosomally tagged strains transformed with a plasmid expressing Btn3–GFP (pSF170) under the control of a constitutive promoter. Scale bars: 5 µm.

To determine whether the epsins Ent3 and Ent5 behave similarly to Btn2, we analyzed the localization of Btn3–GFP expressed from the constitutive *GPD1* promoter in a yeast strain bearing endogenous mCherry (mCh)-tagged Ent3 or Ent5 proteins (Fig. 2B). Ent3–mCh and Ent5–mCh did not colocalize with Btn3–GFP, even though some Ent3–mCh and Ent5–mCh are localized next to Btn3–GFP positive structures (Fig. 2B). This shows that Ent3 does not behave like Btn2, despite the two proteins being able to bind to Btn3 and SNAREs (Vti1 and Snc1).

The endosomal localization of Btn2 does not require Btn3 (Kanneganti et al., 2011), but could be dependent on the Ent3 and Ent5 epsins. We analyzed the intracellular localization of Btn2–GFP expressed under the control of the constitutive *ADH1* promoter in wild-type and *btn2Δ*, *btn3Δ*, *ent3-ts*, *ent3Δ ent5Δ* and *vps27Δ* mutant cells (supplementary material Fig. S1A). In all these cells, Btn2–GFP was localized to the endosomes. However, in *ent3-ts* and *ent3Δ ent5Δ* mutants, the Btn2–GFP-labeled punctate structures were smaller (supplementary material Fig. S1A). This was previously observed for other mutants, such as the *snc1Δ snc2Δ* cells that are defective in endocytosis and secretion and also have fragmented vacuoles (Kama et al., 2007). To determine whether Btn2–GFP colocalizes with Ent3 or Ent5, we expressed Btn2–GFP in yeast strains bearing locus mCh-tagged Ent3 and Ent5 (supplementary material Fig. S1B). Btn2–GFP did not colocalize with Ent3–mCh nor with Ent5–mCh, showing that constitutively expressed Btn2 does not recruit Ent3 or Ent5 to endosomes and supporting our findings that these proteins belong to different complexes (Fig. 1). Moreover, in *btn3Δ* cells, the Ent3–mCh and Ent5–mCh localization to intracellular punctae (Golgi and endosomes) was similar to that observed in wild-type cells (supplementary material Fig. S1B). Thus neither Btn2, nor Ent3 and Ent5 intracellular localizations depend on their interaction with Btn3 (supplementary material Fig. S1B; Kanneganti et al., 2011).

The endosomal sorting of Ent3-dependent cargos requires Btn3

Ent3 and Ent5 play multiple roles in membrane trafficking, one at the Golgi level in coordination with AP-1 and GGA adaptors and one at the endosomes for either retrograde Snc1 trafficking, SNARE (Vti1, Pep12 and Syn8) sorting or MVB sorting of Cps1 (Chidambaram et al., 2008; Copic et al., 2007; Duncan et al., 2003; Eugster et al., 2004; Zimmermann et al., 2010). We tested whether Btn3 was involved in the same trafficking steps as Ent3 and Ent5. We analyzed the vacuolar trafficking of the dipeptidyl aminopeptidase B (DPAP-B or Dap2) and, as a control, the vacuolar SNARE Vam3, which is transported from the Golgi to the vacuolar membrane through the AP-3 adaptor complex (along the endosome-independent alkaline phosphatase pathway) (Darsow et al., 1998). In *btn3Δ* mutant cells, GFP–DPAP-B and GFP–Vam3 are localized at the vacuolar membrane as in wild-type cells, showing that there is no defect in their trafficking (supplementary material Fig. S2A). We tested the VPS pathway by analyzing the secretion of carboxypeptidase Y (CPY) into the extracellular medium, and could not observe any significant CPY accumulation in the *btn3Δ* mutant cells, contrary to what was observed for *ent3Δent5Δ*, *fab1Δ* or *vps27Δ* mutants (supplementary material Fig. S2B). Thus, Btn3 is not required for GGA-dependent CPY trafficking.

The endosomal sorting and recycling of the exocytic v-SNARE Snc1 depends on Ent3 and Ent5 (Zimmermann et al., 2010). In wild-type cells, Snc1 is transported from the Golgi to the plasma membrane, from where it is internalized and sorted at endosomes

for retrograde transport to the Golgi for another round of exocytosis (Lewis et al., 2000). In *ent3Δ ent5Δ* and *ent3-ts* mutant cells GFP–Snc1 was mainly localized intracellularly, whereas in wild-type cells GFP–Snc1 stained the plasma membrane and some intracellular punctate structures corresponding to the Golgi and endosomes (Fig. 3A). In the *btn3Δ*, *btn2Δ* and *vps27Δ* mutant cells, GFP–Snc1 was distributed between the plasma membrane and intracellular structures (Fig. 3A). These results are in agreement with a previous study showing no defect in GFP–Snc1 trafficking in *btn3Δ* and *btn2Δ* mutant cells, even though Btn2 binds to Snc1 (Kama et al., 2007). The *ent3-ts* mutant phenocopies the GFP–Snc1 recycling defect observed in *ent3Δ ent5Δ* double-mutant cells, whereas the *ent3Δ* deletion-mutant strain has no defect in GFP–Snc1 recycling (Zimmermann et al., 2010). This shows that the *ent3-ts* allele displays a dominant-negative effect. It was shown previously that the phosphorylation status of GFP–Snc1 (thus its molecular mass) correlates with its intracellular localization, the hyperphosphorylated form being localized at the plasma membrane (Galan et al., 2001). To quantify the rate of GFP–Snc1 recycling, we analyzed the ratio of hyperphosphorylated (GFP–Snc1-P) to hypophosphorylated GFP–Snc1 in the different yeast strains – wild type, *btn3Δ*, *btn2Δ*, *vps27Δ*, *ent3Δ ent5Δ* and *ent3-ts* (Fig. 3B,C). In wild-type and *btn2Δ* cells, ~60% of GFP–Snc1 was hyperphosphorylated and thus efficiently recycled. In the *ent3Δ ent5Δ* and *ent3-ts* mutant cells, GFP–Snc1 recycling was defective, because only ~20% of GFP–Snc1 was hyperphosphorylated. Interestingly, in *btn3Δ* and *vps27Δ* mutant cells, only ~50% of GFP–Snc1 was hyperphosphorylated, revealing a delay in Snc1 recycling. The data between the wild-type or *btn2Δ* mutant and the *btn3Δ* strain were compared by using Student's *t*-test (Fig. 3C). The results of the Student's test were as follows: $P=0.000017$ for the wild-type cells compared with *btn3Δ* cells, $P=0.00033$ for *btn2Δ* compared with *btn3Δ* and no significant differences were observed between the wild-type cells and the *btn2Δ* mutant ($P=0.444$). Thus, the delay in GFP–Snc1 recycling observed for the *btn3Δ* cells was significant when compared to the results of the wild-type or *btn2Δ* cells. This shows that Ent3 and Ent5 are necessary for Snc1 recycling, as is their interacting partner Btn3.

Ent3 and Ent5 play a role in the MVB sorting of ubiquitylated cargo (Eugster et al., 2004); thus, we investigated whether Btn3 could also be involved in this sorting step. The MVB sorting of both Cps1 (membrane bound pre-carboxypeptidase 1) and Sna3 (an integral membrane protein) depends on the ubiquitin-ligase Rsp5, but whereas Cps1 sorting is Fab1-dependent, Sna3 sorting is not (Katzmann et al., 2004; Morvan et al., 2004; Odorizzi et al., 1998; Oestreich et al., 2007; Reggiori and Pelham, 2001; Reggiori and Pelham, 2002; Stawiecka-Mirota et al., 2007). We analyzed the intracellular localization of these GFP-tagged Cps1 and Sna3 cargos in *ent3-ts*, *ent3Δ ent5Δ* and *btn3Δ* cells, as well as in wild-type, *fab1Δ* and *vps27Δ* cells (Fig. 4). In wild-type cells, GFP–Cps1 was detected in the vacuolar lumen, whereas in *fab1Δ* cells presenting enlarged vacuoles the fluorescence was mainly in the vacuolar membrane, and in the *vps27Δ* cells the fluorescence accumulated both in the class E structure and in the vacuolar membrane. In the *ent3-ts* and *ent3Δ ent5Δ* mutant cells, Cps1–GFP was also mainly localized to the vacuolar membrane (Eugster et al., 2004; Friant et al., 2003). In *btn3Δ* mutant cells, Cps1–GFP was localized to both the vacuolar lumen and membrane, suggesting some delay in MVB sorting (Fig. 4). Next we analyzed the intracellular localization of Sna3–GFP, which does not depend on
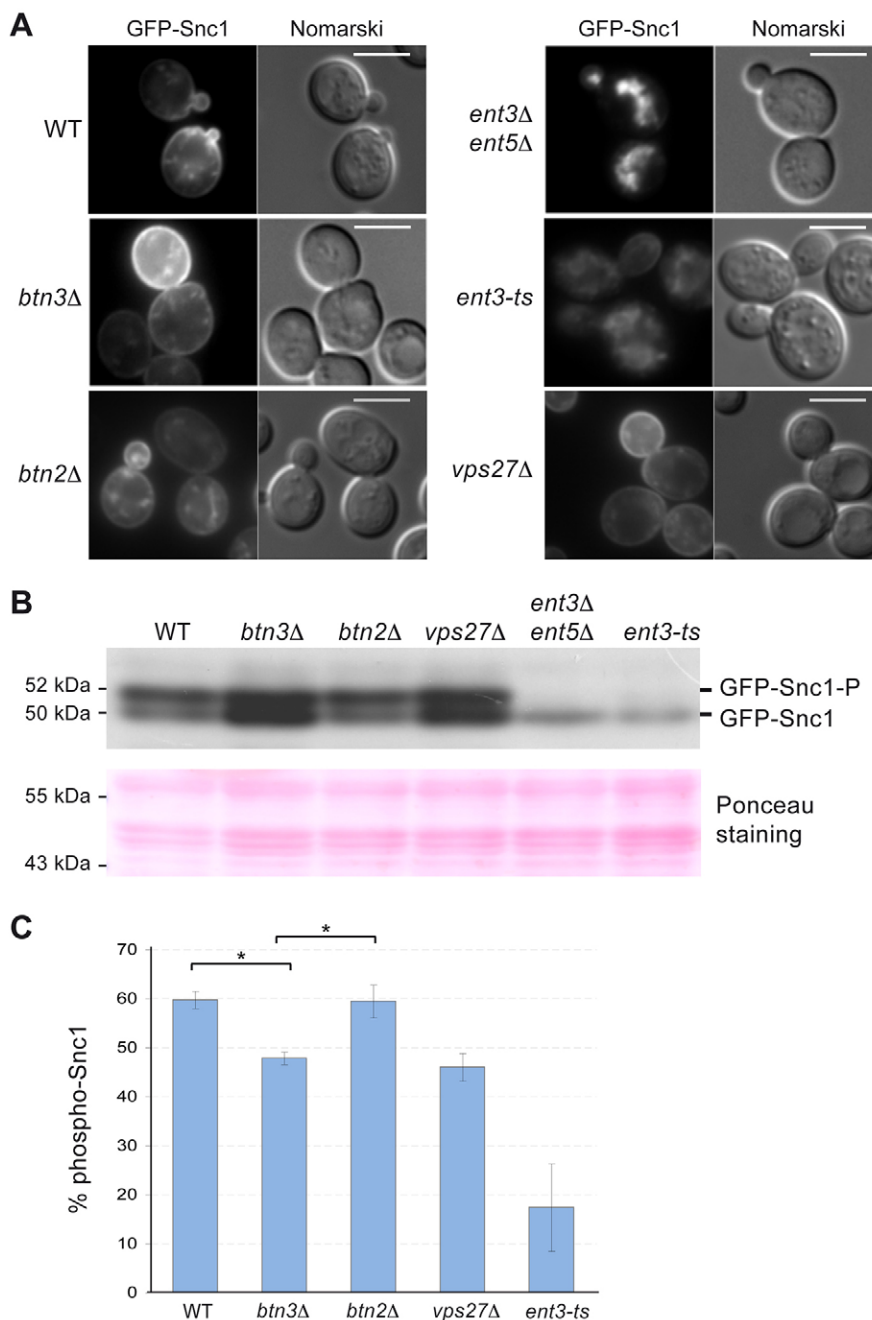
Fig. 3. In the absence of Btn3, endosomal recycling of the Snc1 SNARE is delayed. (A) Wild-type (WT) BY4742, *btn3Δ*, *btn2Δ*, *ent3Δ ent5Δ*, *ent3-ts* and *vps27Δ* mutant cells expressing GFP–Snc1 were visualized by fluorescence (GFP filter) and DIC (Nomarski) microscopy. Scale bars: 5 μm. (B) Total extracts from the cells visualized in A were analyzed by immunoblot using anti-GFP antibodies. Ponceau staining of the western blot nitrocellulose membrane shows the protein quantities loaded on the gel. The hyperphosphorylated form of GFP–Snc1 (GFP-Snc1-P) is indicated. (C) The percentage of hyperphosphorylated GFP–Snc1 was quantified in the different strains (*n*=4 experiments). The data show the mean±s.d., and the wild-type or *btn2Δ* cells and the *btn3Δ* cells were compared by using Student's *t*-test (**P*<0.001).

PtdIns(3,5)P_2 synthesis by Fab1 for its MVB sorting (Reggiori and Pelham, 2002). Sna3–GFP was properly localized to the vacuolar lumen in all strains, including *fab1Δ*, *ent3-ts* and *ent3Δ ent5Δ* mutant cells, except for *vps27Δ*, in which it was localized at the Class E compartment and vacuolar membrane (Fig. 4). This shows that the MVB sorting of Sna3, which does not depend on PtdIns(3,5)P_2 or epsins Ent3 and Ent5, does not require the Btn3 protein. Thus, Btn3 is specifically involved in the MVB sorting of PtdIns(3,5)P_2-dependent biosynthetic cargos, which requires its binding partners Ent3 and Ent5.

Btn3 is required for endosomal sorting of internalized endocytic cargos

The Fab1 kinase, through its PtdIns(3,5)P_2 synthesis activity, is required for the endosomal sorting and trafficking of the

endocytic cargo Ste3, the a-factor receptor (Shaw et al., 2003). The *ent3Δ ent5Δ* double-mutant cells are also defective in the endosomal sorting of the internalized α-factor receptor Ste2 (Eugster et al., 2004). We analyzed the internalization and vacuolar delivery of the uracil permease Fur4–GFP in *btn3Δ* and *ent3Δ ent5Δ* mutant cells (Fig. 5A). The Fur4 permease relies on ubiquitylation by Rsp5 for both its internalization and endosomal sorting in MVB intraluminal vesicles prior to its vacuolar lumen delivery (Blondel et al., 2004). To study the effects of *btn3Δ* and *ent3Δ ent5Δ* mutations on the vacuolar delivery of Fur4–GFP from the plasma membrane, its production was induced by galactose, then stopped by addition of glucose and chased for 30 min so that all Fur4–GFP was at the plasma membrane. Its internalization was then triggered by the addition of cycloheximide. In all strains assayed, Fur4–GFP was localized
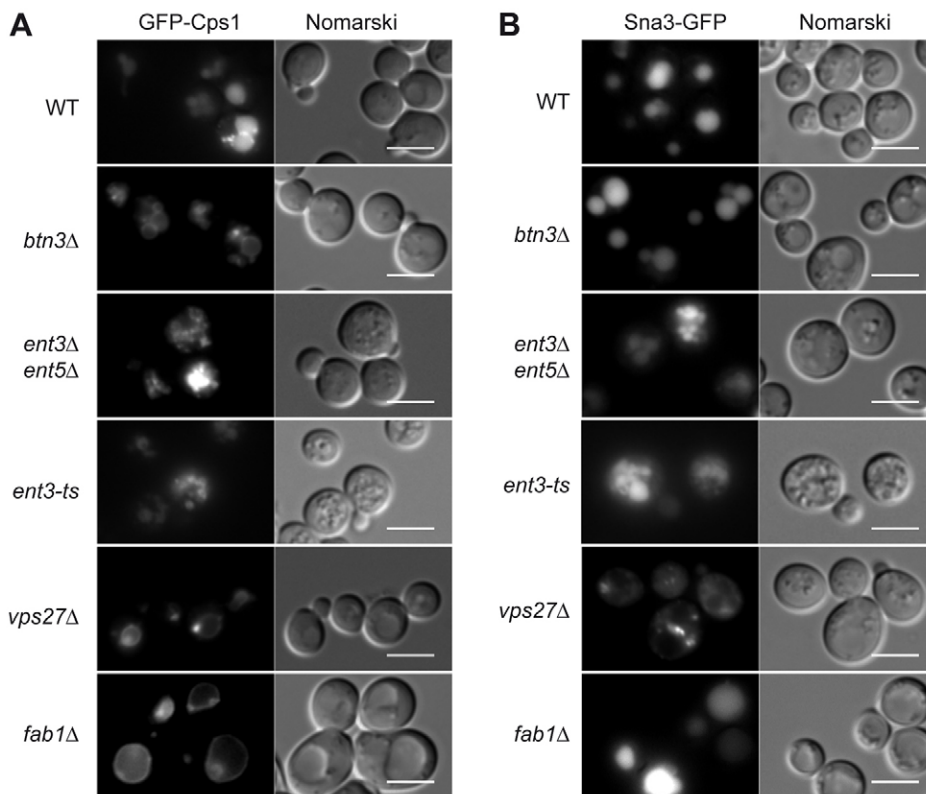
Fig. 4. Btn3 regulates the endosomal sorting of ubiquitylated biosynthetic cargos. Wild-type (WT) BY4742, *btn3Δ*, *ent3Δ ent5Δ*, *ent3-ts*, *vps27Δ* and *fab1Δ* mutant cells expressing GFP–Cps1 and Sna3–GFP fusion proteins were visualized by fluorescence (GFP filter) and DIC (Nomarski) microscopy. Scale bars: 5 μm.

at the plasma membrane at time 0 min. At 30 min after cycloheximide addition, Fur4–GFP was residually at the plasma membrane and mainly in intracellular punctae, showing no defect in internalization (Fig. 5A). At 60 min, Fur4–GFP staining was localized to the vacuolar lumen for wild-type cells, whereas in *btn3Δ* and *ent3Δ ent5Δ* mutant cells, Fur4–GFP fluorescence appeared mainly as bright dots close to the vacuoles, corresponding to the endosomes. At later time-points after internalization (120 min), Fur4–GFP was delivered to the vacuolar lumen in *btn3Δ* cells, whereas it remained at the endosomes in the *ent3Δ ent5Δ* mutant cells (Fig. 5A). This shows that the epsins Ent3 and Ent5 are required for the endosomal sorting of the internalized Fur4 permease and that Btn3 regulates this process. To determine whether this defect was observed for another internalized cargo relying on ubiquitylation for its endosomal MVB sorting, we analyzed the endocytosis trafficking of the general amino acid permease Gap1 (Lauwers et al., 2009). In cells growing on poor nitrogen sources like proline, Gap1–GFP accumulated at the plasma membrane (Fig. 5B, time 0), and upon ammonium addition Gap1–GFP was internalized (Fig. 5B, time 30 min and 60 min). In wild-type cells, 60 min after induction of internalization, Gap1–GFP stained the vacuolar lumen, whereas in *btn3Δ* cells, Gap1–GFP accumulated in intracellular endosomal structures, as observed for the *bro1Δ* class E *VPS* mutant used as negative control. However, 120 min after internalization, some Gap1–GFP was localized to the vacuolar lumen in *btn3Δ* cells, whereas, in the *bro1Δ* mutant, the fluorescence remained in the class E compartment. These different results show that the epsins Ent3 and Ent5 are required for the endosomal sorting of internalized ubiquitylated cargos and that Btn3 is also involved in this sorting step, maybe by regulating the cellular function of the epsins through direct interaction.

DISCUSSION

The yeast epsins Ent3 and Ent5 have many different functions in protein sorting at the Golgi and endosomal levels. They are required for the Golgi-to-endosome transport of the SNARE Pep12, the retrograde endosome-to-Golgi trafficking of the SNARE Snc1 and the kinase Yck2, the TGN or endosome retention of the chitin synthase Chs3 and the endosomal sorting of ubiquitylated membrane proteins (Copic et al., 2007; Chidambaram et al., 2008; Eugster et al., 2004). These functions require the finely tuned regulation and specific recruitment of the cytoplasmic epsins to the appropriate membrane at the site of action. This recruitment is ensured by protein–protein and protein–lipid interactions, some mediated through the ENTH domain of the epsins (Friant et al., 2003; Eugster et al., 2004; Zimmermann et al., 2010). Here, we identified the Btn3 protein as a direct binding partner of both Ent5 and Ent3 in a yeast proteome microarray interaction screen. Our screen was validated by the identification of known binding partners of Ent3 and Ent5 as the large subunit of the clathrin-associated protein AP-1 complex Apl4 (Duncan et al., 2003). However, some previously characterized Ent3 or Ent5 binding partners were not found in this screen, which can be explained by the low concentration or the absence of some *S. cerevisiae* proteins spotted on the proteome chip (Zhu et al., 2001). For example, the Apl4 concentration is 140 nM, whereas the Gga2 concentration is 60 nM and the clathrin heavy chain Chc1 is absent from the microarray. Furthermore, this screening method allows only the identification of proteins interacting directly with the probes. Therefore, some interactions previously characterized by co-immunoprecipitation experiments are probably not direct. This is certainly the case for the interactions of Ent3 and Ent5 with the ESCRT-0 protein Vps27 (Eugster et al., 2004), as Vps27 is spotted at high concentration on the protein microarray but was
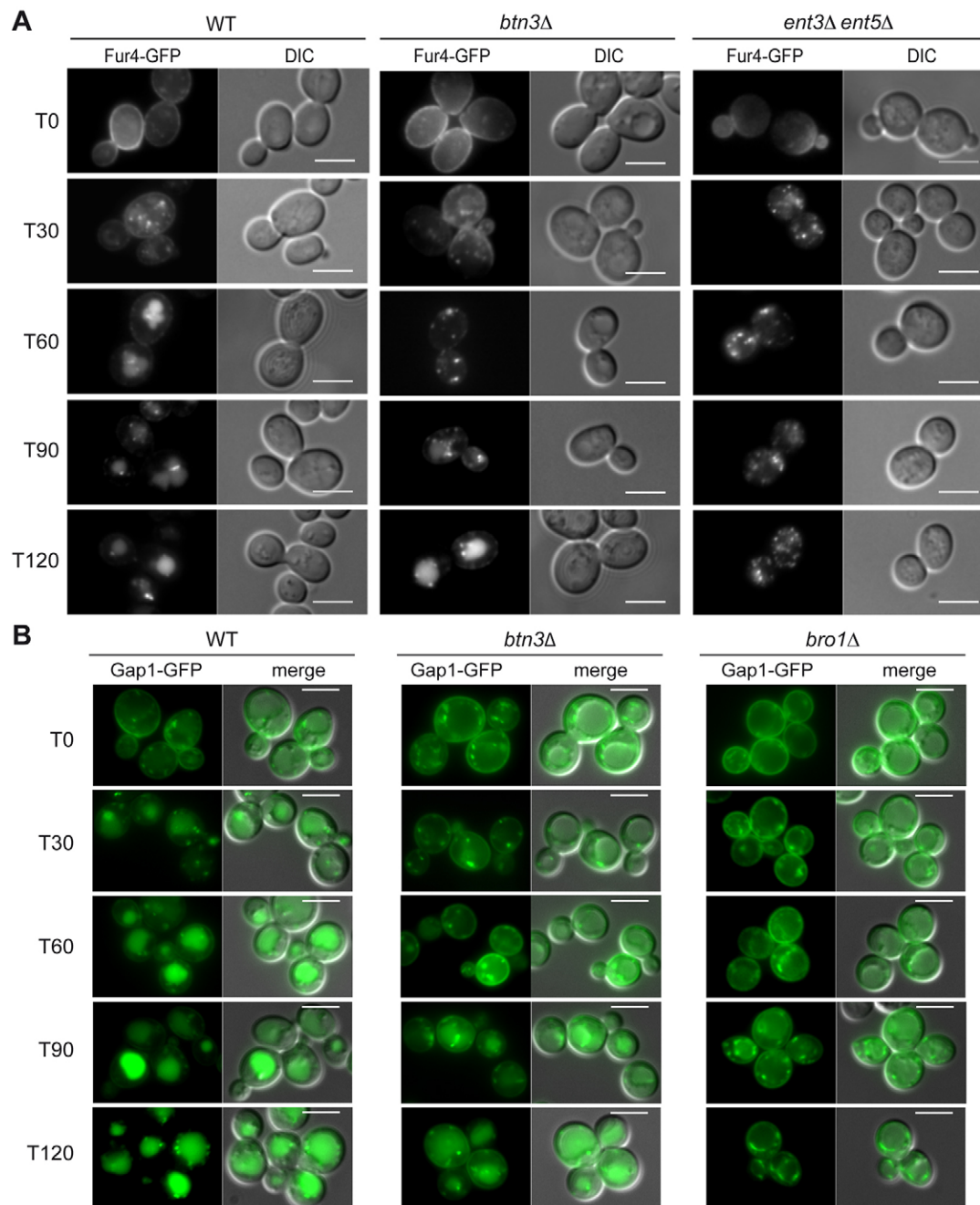
Fig. 5. Btn3 is required for the endosomal sorting of endocytic cargos. (A) Wild-type (WT), btn3Δ and ent3Δ ent5Δ cells transformed with pFL38-GAL-FUR4-GFP were grown exponentially in sucrose medium, then Fur4–GFP expression was induced by addition of galactose for 2 h before being chased for 30 min with glucose. Internalization was induced by the addition of cycloheximide (T0) and the timecourse (30, 60, 90 and 120 min) of Fur4–GFP endocytosis was followed by fluorescence microscopy. (B) Wild-type, btn3Δ and bro1Δ cells transformed with GAP1-GFP plasmid were grown exponentially on Proline medium to accumulate Gap1–GFP at the plasma membrane (T0), and ammonium was added to the culture to induce Gap1–GFP endocytosis that was examined at different time-points (30, 60 and 120 min) by fluorescence microscopy. Merge indicates an image resulting from superimposition of the GFP and DIC images. Scale bars: 5 μm.

not revealed by Ent3 or Ent5. This was already suggested based on tandem affinity purification (TAP) assays of Ent3 and Ent5 (Copic et al., 2007).

Two recent studies pointed to a role of Btn3 in membrane trafficking. First, Btn3 was identified as interacting with Btn2 (Kanneganti et al., 2011), a protein linked to Batten disease and required for the endosome-to-Golgi recycling of some specific cargos like Snc1 (Kama et al., 2007). Second, Btn3 was also identified in a screen for deletion mutants sensitive to DNA topoisomerase I $top1$-T_{722}A mutant overexpression and was thus named Tda3 (Reid et al., 2011). In this study, the authors could not assign a function to Tda3/Btn3 but, interestingly, the largest class of mutants identified in this screen affects MVB sorting, with genes encoding ESCRT subunits or the Fab1 lipid kinase. Their isolation in this screen was linked to their defect in the processing of the

small ubiquitin-like modifier (SUMO)-modified form of Top1 topoisomerase, because mutation of the three lysine residues in $top1$-T_{722}A mutant suppressed the ESCRT mutant sensitivity phenotype. Interestingly, SUMOylation and ubiquitylation both occur on lysine residues and recent studies have shown that these two modifications intersect and can even be found on the same protein (Denuc and Marfany, 2010). Here, we show that in the absence of Btn3/Tda3, the endosomal sorting of Fab1-, ubiquitin- and ESCRT-dependent cargos like Cps1, Fur4 or Gap1 is delayed. Moreover, Btn3 is localized to the Class E compartment in $vps27Δ$ cells and its endosomal localization requires the Ent3 and Ent5 epsins (Fig. 2A). However, the Btn3 protein is not required for the Ent3 or Ent5 endosomal and Golgi localization (supplementary material Fig. S1B). Taken together, these results point towards a regulatory role for Btn3 in ubiquitin-dependent endosomal sorting.

Btn3 might regulate the endosomal sorting function of Ent3 and Ent5 epsins through direct protein–protein interactions.

Interestingly, another direct interactor of Btn3, the Btn2 protein, shares cellular functions with Ent3 (Kanneganti et al., 2011). Indeed, Ent3 and Btn2 bind to the Vti1 and Snc1 SNAREs. Here, we confirm that Ent3 and Ent5 epsins are required for the endosome-to-Golgi recycling of the SNARE Snc1 (Zimmermann et al., 2010). Btn2 is required for the endosome-to-Golgi recycling of the Yif1 protein (Kama et al., 2007). Despite sharing similar cellular functions and interacting directly with Btn3, Btn2 and the epsins Ent3 and Ent5 are certainly not in the same protein complex because they do not interact by co-immunoprecipitation (Fig. 1D) and they do not colocalize (supplementary material Fig. S2B). Btn3 interacts with Btn2 and colocalizes with Btn2 at the endosomes; however, this endosomal localization of Btn3 does not depend on its direct interaction with Btn2, as Btn3 mutants lacking the Btn2-interaction site colocalize with Btn2 (Kanneganti et al., 2011). We show that Btn3 interacts with the Golgi-endosomal epsins Ent3 and Ent5 (Fig. 1). Btn3 is also interacting genetically with the SNARE Vti1 that requires the epsin Ent3 for its trafficking (Kanneganti et al., 2011; Chidambaram et al., 2004). Indeed, *BTN3* overexpression inhibits the growth of the temperature-sensitive *vti1-11* mutant cells, and this suggests that Btn3 is acting as an inhibitor in endosome-to-Golgi transport (Kanneganti et al., 2011). We show that the endosome-to-Golgi recycling of the Snc1 SNARE is delayed in *btn3Δ* mutant cells (Fig. 3). Moreover, overexpression of Btn3 results in mislocalization into the vacuole and the late endosomes of two different Golgi cargos (Yif1 and Kex2), indicating that Btn3 is involved in protein retrieval from the late endosomes to the Golgi (Kanneganti et al., 2011). Thus, Btn3 overexpression mimics the trafficking phenotypes observed for the *btn2Δ* deletion strain, indicating that Btn3 is a negative regulator of the Btn2 protein (Kama et al., 2007; Kanneganti et al., 2011). Moreover, Btn3 clusters Btn2 away from its cargos (Kanneganti et al., 2011). These results suggest that Btn3 might regulate the endosomal sorting function of two SNARE-interacting proteins, the Batten-disease-related protein Btn2 and the epsins Ent3 and Ent5. This also suggests that Batten disease might be linked to defects in the endosomal recycling of proteins involved in the vesicular fusion.

MATERIALS AND METHODS
Plasmids, yeast Strains, media and genetic manipulations
pGO45 (pRS426-GFP-CPS1), pGO89 (pRS426-GFP-DPAP-B) and pRS426-GFP-VAM3 were a gift from Scott D. Emr (Darsow et al., 1998; Odorizzi et al., 1998), pRS416-TPI1-GFP-PHM5 pRS416-TPI1-SNA3-GFP and pRS416-TPI1-GFP-SNC1 were a gift from Hugh R. Pelham (Lewis et al., 2000; Reggiori and Pelham, 2001), pFL38-Gal-Fur4-GFP was a gift from Rosine Haguenauer-Tsapis (Blondel et al., 2004), pFL38-GAP1-GFP was a gift from Bruno André (Lauwers et al., 2009), pAD6-Btn2-GFP was a gift from Jeffrey E. Gerst (Kama et al., 2007) and pHAC (YCplac33-3XHA) and pFL575 (YCplac33-promENT3-ENT3-3XHA) were as described previously (Friant et al., 2003).

BTN3, *ENT3* and *ENT5* were amplified from wild-type BY4742 *S. cerevisiae* genomic DNA using the polymerase chain reaction (PCR) with Phusion High-Fidelity DNA polymerase (Thermo Scientific). *ENT3* and *ENT5* were cloned into pET101/D-TOPO and pET151/D-TOPO vector (Invitrogen) for V5-6xHis tagging to obtain the pSF37 (pET101-ENT3-6xHIS-V5), pSF38 (pET151-6xHIS-V5-ENT3), pSF39 (pET101-ENT5-6xHIS-V5) and pSF40 (pET151-6xHIS-V5-ENT5) plasmids. *BTN3* was cloned into the pDONR207 entry vector by Gateway® BP reaction (Invitrogen), followed by recombination using the Gateway® LR reaction (Invitrogen) into yeast destination vectors (Addgene plasmid numbers 14196 and 14252; Alberti et al., 2007) to obtain the pSF170

(pAG416-promGPD-BTN3-eGFP) and pSF191 (pAG426-promGPD-BTN3-3xHA) plasmids.

S. cerevisiae strains used in this study are BY4742 WT (*MATα leu2Δ0 ura3Δ0 his3Δ0 lys2Δ0*), *btn3Δ* (BY4742 *btn3::kanMX*), *btn2Δ* (BY4742 *btn2::kanMX*), *vps4Δ* (BY4742 *vps4::kanMX*), *vps27Δ* (BY4742 *vps27::kanMX*), *fab1Δ* (BY4742 *fab1::kanMX*), SFY2 ent3Δ ent5Δ (*MATa his3 leu2 ura3 ent3::HIS3MX ent5::KanMX*), *ent5Δ* (BY4742 *ent5::kanMX*) and FLY639 *ent3-ts* (*MATα bar1 ura3 leu2 lys2 ent3-1*); the wild-type 27061 (*MATa ura3 trp1*) and its isogenic *npi1/rsp5* mutant strains were a gift from Bruno André (Université Libre de Bruxelles, Belgium). The Ent3-mCherry (LRY4-YVD236, *MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 ENT3-mCherry:HIS3MX*) and Ent5-mCherry (LRY6-YVD247, *MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 ENT5-mCherry:HIS3MX*) strains were constructed in the BY4741 background using standard techniques (Sherman, 1991) and were a kind gift from Vincent Dalibard, Gladys Mirey and Barbara Winsor (University of Strasbourg, France). The strains deleted for *BTN3* and expressing Ent3–mCherry or Ent5–mCherry were constructed by crossing the *btn3Δ* deletion strain in the BY4742 background with BY4741 Ent3-mCh (LRY4-YVD236) or Ent5-mCh (LRY6-YVD247), followed by sporulation of the resulting diploid and tetrad dissection to obtain haploid strains (SFY153 *btn3Δ* Ent3-mCh and SFY154 *btn3Δ* Ent5-mCh) using standard techniques (Sherman, 1991). The chromosomally *GFP*-tagged *BTN3* BY4741 yeast strain used in this study (*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 BTN3-GFP:HIS3MX*) (Huh et al., 2003) was termed Btn3-GFP and was a gift from Sébastien Léon (Institut Jacques Monod, France). Strains deleted for *BTN2*, *ENT5*, *VPS27* or *VPS4* and expressing Btn3–GFP were constructed by using standard techniques (Sherman, 1991).

The indicated yeast strains were grown at 30°C to mid-exponential growth phase in rich medium (YPD: 1% yeast extract, 2% peptone, 2% glucose) or in synthetic medium (SD: 0.67% yeast nitrogen base without amino acids, 2% glucose and the appropriate dropout mix). Yeast cells were transformed using the modified lithium acetate method (Gietz et al., 1992).

Expression and purification of V5-6xHis-tagged proteins
The production of V5-6xHis-tagged Ent3 and Ent5 proteins was induced in *E. coli* BL21 (DE3) (Invitrogen) at 30°C for 4 h with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation and lysed by sonication in PBS buffer (three times for 30 s each). Recombinant Ent3 and Ent5 were purified using Ni-NTA–agarose beads (Qiagen, Hilden, Germany). Purification was assessed by Coomassie staining after PAGE. Protein concentration was determined by the Bradford method using Bio-Rad Protein Assay (Bio-Rad).

Binding assay using protein microarrays
The ProtoArray® Yeast Proteome Microarray was manufactured by Invitrogen. It contains 4088 *S. cerevisiae* open reading frames expressed as 5′-GST fusions, purified and spotted in duplicate on a 1×3 inch nitrocellulose-coated slide.

Microarray experiments were all carried out in a cold room (4°C) as described by the manufacturer (Invitrogen). The probing procedures and the binding specificity of the N- or C-terminally tagged Ent3 or Ent5 probes were validated by the ProtoArray® Control Protein microarrays, before assaying the Yeast Proteome microarrays. The arrays were blocked for 1 h in PBS-Tween-BSA 1%. Each array was incubated for 2 h with 6 μg of purified N- or C-terminally V5-6xHis-tagged Ent3 or Ent5. After several washes, the microarrays were incubated with anti-V5–Alexa-Fluor-647® antibodies for 30 min, and after several washes the arrays were dried before being scanned. Fluorescent scans of each protein microarray were obtained using an Axon GenePix® scanner, data were acquired using GenePix® Pro (Molecular Devices, Sunnyvale, CA) and analyzed with ProtoArray® Prospector (Invitrogen).

GST pull-down experiments
Yeast cells (Btn3-GFP BY4741) were grown in YPD medium to an OD$_{600}$ of 0.6, harvested and resuspended in cold PBS, 10 mM EDTA and protease inhibitor cocktail (Roche Diagnostics) prior to lysis with glass beads (Sigma). Production of glutathione-S-transferase (GST)-fused Ent3

and Ent5 protein was induced at 37℃ in *E. Coli* Rosetta II BL21 (Novagen, Merck Chemicals Ltd, Nottingham, UK) for 2 h with 0.1 mM IPTG. Cells were harvested, resuspended in PBS with protease inhibitor cocktail (Roche Diagnostics) and lysed on ice by sonication. A total of 250 µg of GST fusion proteins pre-bound to 50 µl of glutathione–Sepharose beads (GE Healthcare) were incubated with 300 µg of total yeast extract in PBS buffer containing protease inhibitors. Reactions were incubated at 4℃ overnight under gentle agitation and then washed four times with PBS-protease inhibitors. Proteins bound to the Sepharose beads were eluted in 100 µl of Laemmli sample buffer and boiled for 5 min. Proteins were separated on 10% SDS-PAGE prior to immunoblotting with mouse monoclonal anti-GFP (Roche Diagnostic).

Coimmunoprecipitation

Whole-cell lysates were prepared by glass bead lysis with FastPrep (MP Biomedicals, Illkirch, France) in cold PBS, 0.25 M sorbitol, 1 mM PMSF and protease inhibitor cocktail (Roche Diagnostics) and subjected to clarification by a 10 min spin at 13,000 *g*. The resulting yeast cytosols (1 mg) were immunoprecipitated overnight at 4℃ with rat monoclonal anti-HA antibodies (Roche Diagnostics) bound to Gamma-bind Sepharose beads (GE Healthcare) and then subjected to immunoblotting with mouse monoclonal anti-GFP (Roche Diagnostics), anti-HA (Roche Diagnostics) and anti-Pgk1 (Invitrogen) as indicated in the figure legends. The percentage of co-IP was determined by quantification of the resulting western blot using the ImageJ software (Rasband W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, http://imagej.nih.gov/ij/).

Membrane trafficking assays and fluorescence microscopy

Living cells expressing Btn3–GFP, GFP–Cps1, Sna3–GFP, GFP–Phm5, GFP–DPAP-B, GFP–Snc1 or GFP–Vam3 were harvested at an OD_{600} of 0.5–1 and resuspended in synthetic complete yeast medium before visualization. For Fur4–GFP and Gap1–GFP internalization assays, the experiments were performed as described previously (Blondel et al., 2004; Lauwers et al., 2009). For FM4-64 (Invitrogen) staining, the indicated yeast strain was harvested by a 500 *g* centrifugation for 1 min, resuspended in 50 µl of YPD medium and stained with 2 µl of FM4-64 (200 µM) for 15 min at 30℃, prior to washing with 900 µl of YPD and chasing by incubation at 30℃ for 10 min followed by a second washing step in PBS. The stained cells were then observed by fluorescence microscopy. Observation was performed with 100× objective (Zeiss, 1.45 oil, ∞/0.17) on a fluorescence Axiovert200 microscope (Zeiss) equipped with GFP, DAPI and Rhodamine filters, and DIC optics. Images were captured with a CoolSnap HQ2 photometrix camera (Roper Scientific, Evry, France) and analyzed by using ImageJ. The CPY secretion assay was performed as described previously using anti-CPY antibodies (gift of Howard Riezman, University of Geneva, Switzerland) (Morvan et al., 2012). For analysis of the phosphorylation state of GFP–Snc1, total yeast extracts were obtained by NaOH lysis followed by TCA precipitation as described previously (Volland et al., 1994). The equivalent of 1.5 OD_{600} units of yeast cells was resuspended in 50 µl of 2× Laemmli buffer plus Tris Base. Samples were incubated for 5 min at 37℃ and analyzed by 10% SDS-PAGE followed by immunoblotting with anti-GFP (Roche Diagnostics) using standard procedures. The percentage of hyperphosphorylated GFP–Snc1 was determined by quantification of the resulting western blot using the ImageJ software; the statistical analysis of the data (obtained for four independent experiments) was performed with the Microsoft Excel software.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Experiments were carried out by V.A., J.M., B.R., C.M. and J.-O.D.C. Results were analyzed by J.M., J.-O.D.C. and S.F. The article was written by J.M., J.-O.D.C. and S.F.

Supplementary material
Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.159699/-/DC1

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