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

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Summary

Ent3 and Ent5 are yeast epsin N-terminal homology (ENTH) domain containing proteins involved in protein trafficking between the Golgi and late endosomes (LE). They interact with clathrin, clathrin adaptor 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/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 LE to the Golgi. We show that Btn3 endosomal localization depends on epsins Ent3 and Ent5. We demonstrated that in btn3Δ mutant cells, endosomal sorting of ubiquitinated 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.
Introduction

Endosomes are key compartments where endocytic, biosynthetic and exocytic pathways intersect. At the late endosome (LE) or multivesicular body (MVB), proteins undergo critical sorting (Hurley, 2008). Membrane proteins destined to the vacuolar/lysosomal lumen are sorted in MVB intraluminal vesicles and 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 MVB vesicles entry signal (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 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 (PtdIns3P) and PtdIns 3,5-bisphosphate (PtdIns(3,5)P2). PtdIns3P, synthesized by the Vps34 lipid kinase, mediates the specific recruitment of Vps27 through its FYVE domain (Katzmann et al., 2003). PtdIns(3,5)P2, synthesized by the Fab1 lipid kinase from PtdIns3P, controls the MVB sorting of ubiquitinated 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)P2 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 via their ENTH (Epsin N-Terminal Homology) domain (De Camilli et al., 2002; Kay et al., 1999). Ent3 and Ent5 were first identified as interactor of the Golgi localized clathrin adaptors 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: (i) in Golgi to endosome trafficking of some endosomal SNAREs (SNAP (Soluble NSF Attachment Protein) REceptor) and vacuolar carboxypeptidases, (ii) in MVB sorting of ubiquitinated cargos, (iii) 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.,
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 of 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 PtdIns(3,5)P2 as efficiently as PtdIns(4,5)P2 (Narayan and Lemmon, 2006), suggesting that some additional protein interactions are required to mediate their PtdIns(3,5)P2-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 ubiquitinated 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 due to the tag position and reduce the number of false positive hits. The fusion proteins were produced in E. 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
A significant hit was characterized by a Z-score of at least 3 and a coefficient of variance (CV) for signals from the two replicates (each protein is spotted in duplicate on the chip) of less than 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 (Table S1). Among the significant hits, 17 were common to Ent3 and Ent5 (Table S1), including the gamma subunit of the AP-1 adaptor complex Apl4 (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/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 YHR009c/BTN3 gene was identified in a screen for deletion mutants sensitive to the overexpression of the DNA topoisomerase I top1-T722A mutant, and 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/Ent5, we performed GST (Gluthatione-S-transferase) pull-down experiments using GST, GST-Ent3 or GST-Ent5 recombinant proteins and a protein extract of cells expressing chromosomally GFP (green fluorescent protein) 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 (WT) expressing or not 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 immunobloting (Fig. 1C). In 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 absence of Ent3-HA (Fig. 1C, lane WT Btn3-GFP+pHAC (pHAC, empty vector)), the results show that Btn3-GFP was not retain on the beads. The microarray interaction screen suggests that Btn3 binds Ent5 with a higher affinity than Ent3 (Fig. 1A). To determine whether Ent5 competes with Ent3 for Btn3 binding, we performed the 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 since Btn3 was 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 coimmunoprecipitated by Ent3-HA (2-3 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/Ent5 (Eugster et al., 2004), affects 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. All 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 to the Btn3-Ent3 protein complex. We thus analyzed the co-IP between Ent3-HA and Btn2-GFP using Pgk1 as negative control (Fig. 1D). Compared to the co-IP done on protein extracts from cells lacking Ent3-HA (Fig. 1D, lane WT+pHAC) that was used as negative control, Btn2-GFP was not retained on the Ent3-HA beads and this regardless of the presence of Btn3 in the extract (from WT or btn3Δ ent5Δ), showing that Btn2 was not associated to the Ent3 protein.

We then asked whether the Ent3 and Ent5 epsins are required for the previously described Btn2-Btn3 interaction (Kanneganti et al., 2011). We did a co-IP between Btn3-HA and Btn2-GFP in extracts from wild type (WT) or ent3Δ ent5Δ mutant cells (Fig. 1E). In both cell lysates, Btn2-GFP similarly interacts with Btn3-HA. Since 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 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 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 depended 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, *btn3Δ*, *ent3Δ ent5Δ*, *ent3-ts*, *btn2Δ* and *vps27Δ* mutant cells (Fig. 2A). In wild type and *btn3Δ* cells, Btn3-GFP is 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 is mainly cytoplasmic and this is also observed in the *ent3Δ ent5Δ* and *ent3-ts* mutant cells. In *ent3Δ* and *ent5Δ* single deletion mutants Btn3-GFP localizes to endosomes (our unpublished observations), 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 colocalizes perfectly with endogeneous Btn2-RFP (Kanneganti et al., 2011). To determine whether the epsins Ent3/Ent5 behave as Btn2, we analyzed the localization of Btn3-GFP expressed from the constitutive *GPD1* promoter in a yeast strain bearing endogenous mCherry-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 as Btn2, despite the two proteins being able to bind 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, *btn2Δ*, *btn3Δ*, *ent3-ts*, *ent3Δ ent5Δ* and *vps27Δ* mutant cells (Supplementary Fig. 1A). 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 Fig. 1A), this was previously observed for other mutants such as the *snc1Δ snc2Δ* cells that are defective in endocytosis and secretion and have also 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 mCherry-tagged Ent3 and Ent5 (Supplementary Fig. 1B). 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 the one observed in wild type cells (Supplementary Fig. 1B). Thus neither Btn2, nor Ent3/Ent5 intracellular localizations depend on their interaction with Btn3 (Supplementary Fig. 1B and (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 via the AP-3 adaptor complex (along the endosome independent ALP (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 Fig. 2A). We tested the VPS pathway by analyzing the secretion of CPY in the extracellular medium, and could not observe any significant CPY accumulation in the btn3Δ mutant cells, contrary to what was observed for ent3Δ ent5Δ, fab1Δ or vps27Δ mutants (Supplementary Fig. 2B). 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 (WT) GFP-Snc1 stained the plasma membrane and some intracellular punctate structures corresponding to Golgi and endosomes (Fig. 3A). In the btn3Δ, btn2Δ and vps27Δ mutant cells, GFP-Snc1 is distributed between the PM and intracellular structures (Fig. 3A).
results are in agreement with the previous study showing no defect of GFP-Snc1 trafficking in \textit{btn3}\textgreek{a} and \textit{btn2}\textgreek{a} mutant cells, even though Btn2 binds to Snc1 (Kama et al., 2007). The \textit{ent3-ts} mutant phenocopies the GFP-Snc1 recycling defect observed in \textit{ent3}\textgreek{a} \textit{ent5}\textgreek{a} double mutant cells, whereas the \textit{ent3}\textgreek{a} deletion mutant strain has no defect in GFP-Snc1 recycling (Zimmermann et al., 2010). This shows that the \textit{ent3-ts} allele displays a dominant negative effect. It was previously shown that the phosphorylation status of GFP-Snc1 (thus its molecular weight) correlates with its intracellular localization, the hyper-phosphorylated form being PM localized (Galan et al., 2001). To quantify the rate of GFP-Snc1 recycling, we analyzed the ratio of hyper-phosphorylated (GFP-Snc1-P) to hypo-phosphorylated GFP-Snc1 in the different yeast strains, wild type, \textit{btn3}\textgreek{a}, \textit{btn2}\textgreek{a}, \textit{vps27}\textgreek{a}, \textit{ent3}\textgreek{a} \textit{ent5}\textgreek{a} and \textit{ent3-ts} (Fig. 3C). In wild type and \textit{btn2}\textgreek{a} cells, about 60\% of GFP-Snc1 was hyper-phosphorylated thus efficiently recycled. In the \textit{ent3}\textgreek{a} \textit{ent5}\textgreek{a} and \textit{ent3-ts} mutant cells, GFP-Snc1 recycling was defective since only about 20\% of GFP-Snc1 was hyper-phosphorylated. Interestingly, in \textit{btn3}\textgreek{a} and \textit{vps27}\textgreek{a} mutant cells only about 50\% of GFP-Snc1 was hyper-phosphorylated, revealing a delay in Snc1 recycling. The data between the wild-type or \textit{btn2}\textgreek{a} mutant and the \textit{btn3}\textgreek{a} strain were compared by using the Student’s t-test, and interpreted using the p-value, the * means that p<0.001 (Fig. 3C). The results of the Student’s test were: p=0,000017 for the data of the wild-type compared with the \textit{btn3}\textgreek{a}; p=0,00033 for the data of the \textit{btn2}\textgreek{a} compared with the \textit{btn3}\textgreek{a}; no significant differences were observed between the data of the wild-type compared with the \textit{btn2}\textgreek{a}, p=0,444. This shows that the delay in GFP-Snc1 recycling observed for the \textit{btn3}\textgreek{a} cells is significant when compared to the results of the wild-type or \textit{btn2}\textgreek{a} cells. This shows that Ent3 and Ent5 are necessary for Snc1 recycling as well as their interacting partner Btn3.

Ent3 and Ent5 play a role in the MVB sorting of ubiquitinated cargo (Eugster et al., 2004), thus we investigated if 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-Mirotta et al., 2007). We analyzed the intracellular localization of these GFP-tagged Cps1 and Sna3 cargos in \textit{ent3-ts}, \textit{ent3}\textgreek{a} \textit{ent5}\textgreek{a}, \textit{btn3}\textgreek{a} as well as in wild type, \textit{fab1}\textgreek{a} and \textit{vps27}\textgreek{a} cells (Fig. 4). In wild type cells, GFP-Cps1 was detected in the vacuolar lumen, whereas in \textit{fab1}\textgreek{a} 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 in 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 that does not depend on PtdIns(3,5)P2 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 vps27Δ in which it is 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)P2 or epsins Ent3 and Ent5, does not require the Btn3 protein. Thus, Btn3 is specifically involved in the MVB sorting of PtdIns(3,5)P2-dependent biosynthetic cargos which requires its binding partner Ent3 and Ent5.

**Btn3 is required for endosomal sorting of internalized endocytic cargos**

The Fab1 kinase via its PtdIns(3,5)P2 synthesis activity is required for the endosomal sorting and trafficking of the endocytic cargo Ste3, the α-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 ubiquitination by Rsp5 for both its internalization and endosomal sorting in MVB intralumenal 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 is induced by galactose, then stopped by addition of glucose and chased for 30 min so that all Fur4-GFP is at the plasma membrane. Its internalization is then triggered by the addition of cycloheximide. In all strains assayed, Fur4-GFP is localized at the plasma membrane at time 0 min; 30 min after cycloheximide addition Fur4-GFP is 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 ubiquitination 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 source like proline, Gap1-GFP accumulates at the plasma membrane (Fig. 5B, time 0), and upon ammonium addition Gap1-GFP is 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 ubiquitinated-cargos and that Btn3 is also involved in this sorting step, maybe by regulating the cellular function of the epsins via 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 endosomal transport of the SNARE Pep12, the retrograde endosome to Golgi trafficking of the SNARE Snc1 and the kinase Yck2, the TGN/endosome retention of the chitin synthase Chs3 and the endosomal sorting of ubiquitinated membrane proteins (Copic and Schekman, 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 via the epsins ENTH domain (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 while 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 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 the Batten disease 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-T722A 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 SUMO (small ubiquitin-like modifier) modified form of Top1 topoisomerase, since mutation of the three lysine residues in top1-T722A mutant suppressed the ESCRT mutant sensitivity phenotype. Interestingly, SUMOylation and ubiquitination occur both 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 Fig. 1B). Altogether 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 via 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 Ent3/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 Fig. 2B). 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 vtii-11 mutant cells and this suggests that Btn3 is acting as an inhibitor in endosomal-Golgi transport (Kanneganti et al., 2011). We show that the endosomal to Golgi recycling of the Snc1 SNARE is delayed in btn3Δ mutant cells (Fig. 3). Moreover, overexpression of Btn3 results into 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 the Batten disease might be linked to defect 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), pRS426-GFP-VAM3 are a gift from S. Emr (Darsow et al., 1998; Odorizzi et al., 1998), pRS416-TPI1-GFP-PHM5 pRS416-TPI1-SNA3-GFP and pRS416-TPI1-GFP-SNC1 a gift from H. Pelham (Lewis et al., 2000; Reggiori and Pelham, 2001), pFL38-Gal-Fur4-GFP a gift from R. Haguenauer-Tsapis (Blondel et al., 2004), pFL38-GAP1-GFP a gift from B. André (Lauwers et al., 2009), pAD6-Btn2-GFP a gift from G.E. Gerst (Kama et al., 2007), pHAC (YCplac33-3XHA) and pFL575 (YCplac33-promENT3-ENT3-3XHA) were previously described (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-I); the wild type 27061 (MATa ura3 trpl) and its isogenic npi1/rsp5 mutant strains were a gift from B. André. 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 V. Dalibard, G. Mirey and B. 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. Strains deleted for *BTN2*, *ENT5*, *VPS27* or *VPS4* and expressing Btn3-GFP were constructed by using standard techniques (Sherman, 1991).

The indicated yeast strain 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 V5-6His tagged proteins**
The production of V5-6xHis tagged Ent3 and Ent5 proteins was induced in E. coli BL21 (DE3) (Invitrogen) at 30°C for 4h with 0.5mM isopropyl-beta-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation and lysed by sonication in PBS buffer (3 times for 30 sec each). Recombinant Ent3 and Ent5 were purified using Ni-NTA Agarose beads (Qiagen, Hilden, Germany). Purification was assessed by Coomassie staining after polyacrylamide gel electrophoresis. Protein concentration was determined by 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 4,088 S. cerevisiae open reading frames expressed as 5'-GST fusions, purified and spotted in duplicate on a 1 inch x 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-terminal tagged Ent3 or Ent5 probes were validated by the ProtoArray® Control Protein microarrays, before assaying the Yeast Proteome microarrays. The arrays were blocked for one hour in PBS-Tween-BSA 1%. Each array was incubated for 2h with 6 µg purified N- or C-terminal 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 microarrays were obtained using an Axon GenePix® scanner, data were acquired using GenePix® Pro (Molecular Devices, Sunnyvale, CA, USA) and analyzed with ProtoArray® Prospector (Invitrogen).

**GST pull down experiments**

Yeast cells (Bnt3-GFP BY4741) were grown in YPD medium to OD$_{600}$=0.6, harvested and resuspended in cold PBS, 10mM EDTA, protease inhibitor cocktail (Roche Diagnostics) prior to lysis with glass beads (Sigma). Glutathione-S-transferase (GST) fusion Ent3 and Ent5 proteins production was induced at 37°C in E. Coli Rosetta II BL21 (Novagen, Merck Chemicals Ltd., Nottingham, UK) for 2h, with 0.1mM IPTG. Cells were harvested, resuspended in PBS with protease inhibitor cocktail (Roche Diagnostics), and lysed on ice by sonication. 250µg GST fusion proteins pre-bound to 50µl glutathione-Sepharose beads (GE Healthcare) were incubated with 300µg of total yeast extract in PBS buffer containing
protease inhibitors. Reactions were incubated at 4°C overnight under gentle agitation and then washed 4 times with PBS-protease inhibitors. Proteins bound to the Sepharose beads were eluted in 100μl Laemmli sample buffer and boiled for 5min. Proteins were separated on 10% SDS PAGE, prior to immunoblotting with mouse monoclonal anti-GFP (Roche Diagnostic).

Coimmunoprecipitations
Whole cell lysates were prepared by glass beads lysis with FastPrep (MP Biomedicals, Illkirch, France) in cold PBS, Sorbitol 0.25M, PMSF 1mM and protease inhibitor cocktail (Roche Diagnostics) and subjected to clarification by a 10min spin at 13 000xg. The resulting yeast cytosols (1 mg) were immunoprecipitated over-night at 4°C 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), 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, USA, 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 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 previously described (Blondel et al., 2004; Lauwers et al., 2009). For FM4-64 (Invitrogen) staining, the indicated yeast strain was harvested by a 500xg centrifugation for 1min, resuspended in 50μl YPD medium and stained with 2μl FM4-64 (200 μM) for 15min at 30°C, prior washing with 900μl YPD and chasing by incubation at 30°C for 10 min followed by a second washing step in PBS, the stained cells were observed by fluorescent microscopy. Observation was performed with 100x objectif (Zeiss, 1,45 oil, ∞/0,17) on 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 treated by ImageJ. The CPY secretion assay was done as previously described (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 previously described (Volland et al., 1994). The equivalent of 1,5 OD600nm
unit of yeast cells were resuspended in 50μl of 2X Laemmli buffer plus Tris Base. Samples were incubated 5 min at 37°C and analyzed on 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 4 independent experiments) was done with the Microsoft Excel software.

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References


Figure legends

Figure 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-scores calculated by the ProtoArray® Prospector (Invitrogen) software. A Z-Score higher than 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 proteins 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. (C) Yeast cytosol from 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 a western-blot anti-GFP (to detect Btn3-GFP) and anti-Pgk1 phosphoglycerate kinase (loading control). The same co-IP experiment was done on WT (BY4741), WT 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. (D) Coimmunoprecipitation experiments between Btn2-GFP (pAD6-Btn2-GFP) and Ent3-HA (pFL575) expressed from a wild type (WT) or btn3Δ strain. The wild-type strain (WT) bearing Btn2-GFP (pAD6-Btn2-GFP) and the empty vector (pHAC) was used to control 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 coimmunoprecipitation with anti-HA antibodies into different yeast strains, wild type (WT), ent3Δ ent5Δ (SFY2) and vps27Δ.

Figure 2: Btn3 endosomal localization depends on Ent3 and Ent5.

(A) Btn3-GFP expressed from a constitutive promoter 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 exponential phase of growth were stained with the lipophilic fluorescent dye FM4-64 to label the vacuolar membrane, prior observation by fluorescent 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.

Figure 3: In the absence of Btn3, endosomal recycling of the Snc1 SNARE is delayed.

(A) Wild type BY4742, btn3Δ, btn2Δ, ent3Δ ent5Δ, ent3-ts and vps27Δ mutant cells expressing GFP-Snc1 were visualized by fluorescence (GFP filter) and DIC (Nomarski) microscopy. (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 hyper-phosphorylated form of GFP-Snc1 (GFP-Snc1-P) is indicated. (C) The percentage of hyper-phosphorylated GFP-Snc1 was quantified in the different strains (n=4 experiments). The data between the wild-type (WT) or btn2Δ cells and the btn3Δ cells were compared by using the Student’s t-test, and interpreted using the p-value, the * means that p<0.001.

Figure 4: Btn3 regulates the endosomal sorting of ubiquitinated biosynthetic cargos
Wild type 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.

Figure 5: Btn3 is required for the endosomal sorting of endocytic cargos
(A) Wild type, 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 addition of cycloheximide (T0) and the time-course (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.
### Table A

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### Figures

#### Figure B

- **Diagram**: Protein gel showing bands for Btn3-GFP and GST-Ent.
- **Legend**: Yeast extract, GST-Ent5, GST-Ent3, GST, MW (molecular weight markers).

#### Figure C

- **Western Blot**: Comparison of WT Btn3-GFP, WT Btn3-GFP + Ent3-HA, WT Btn3-GFP + pHAC. Bands for Btn3-GFP, Ent3-HA, Pjk1.
- **Legend**: Input, IP anti-HA.

#### Figure D

- **Western Blot**: Comparison of WT-pHAC, WT+Ent3-HA, btm3Δ+Ent3-HA. Bands for Btm2-GFP, Ent3-HA, Pjk1.
- **Legend**: Input, IP.

#### Figure E

- **Western Blot**: Comparison of WT, ent3Δ, ent5Δ, vps27Δ. Bands for Btn2-GFP, Btn3-HA, Pjk1.