**btn1** affects cytokinesis and cell-wall deposition by independent mechanisms, one of which is linked to dysregulation of vacuole pH

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Summary
btn1, the *Schizosaccharomyces pombe* orthologue of the human Batten-disease gene *CLN3*, is involved in vacuole pH homeostasis. We show that loss of *btn1* also results in a defective cell wall marked by sensitivity to zymolyase, a β-glucanase. The defect can be rescued by expression of Btn1p or CLN3, and the extent of the defect correlates with disease severity. The vacuole and cell-wall defects are linked by a common pH-dependent mechanism, because they are suppressed by growth in acidic pH and a similar glucan defect is also apparent in the V-type H⁺ ATPase (v-ATPase) mutants *vma1Δ* and *vma3Δ*. Significantly, Btn1p acts as a multicopy suppressor of the cell-wall and other vacuole-related defects of these v-ATPase-null cells. In addition, Btn1p is required in a second, pH-independent, process that affects sites of polarised growth and of cell-wall deposition, particularly at the septum, causing cytokinesis problems under normal growth conditions and eventual cell lysis at 37°C. Thus, Btn1p impacts two independent processes, which suggests that Batten disease is more than a pH-related lysosome disorder.

Key words: *CLN3*, *btn1*, Batten disease, Neuronal ceroid lipofuscinosis, V-type H⁺ ATPase (v-ATPase), *vma1*, *vma3*, Vacuole, Neurodegeneration, *Schizosaccharomyces pombe*

Introduction
Juvenile-onset neuronal ceroid lipofuscinosis (JNCL), or Batten disease, is a severe neurodegenerative lysosomal-storage disorder of childhood that is characterised by accumulation of lipofuscin-like material and is caused specifically by an intragenic mutation in *CLN3* (The International Batten Disease Consortium, 1995; Kitzmüller et al., 2008; Santavuori, 1988). *CLN3*, a transmembrane protein (Kyttäälä et al., 2004; Nugent et al., 2008), has no homology with other proteins or functional domains and its function is unknown. In mammalian cells, this protein has been connected with lysosome homeostasis (Holopainen et al., 2001; Ramirez-Montaleagre and Pearce, 2005). Intriguingly, several CLC (chloride channel) chloride transporters that are thought to neutralise the membrane potential generated by V-type H⁺ ATPase (v-ATPase) proton pumping (Jentsch, 2007) are mutated in mice that have NCL-like disease (Kasper et al., 2005; Lange et al., 2006; Poët et al., 2006; Stobrawa et al., 2001; Yoshikawa et al., 2002) and in human NCL patients (Poët et al., 2006), confirming a pH contribution to disease pathophysiology. However, *CLN3* is also associated with a variety of apparently unconnected processes (Brass et al., 2008; Cao et al., 2006; Chang et al., 2007; Fossale et al., 2004; Hobert and Dawson, 2007; Lutro et al., 2004; Narayan et al., 2006; Persaud-Sawin et al., 2002). Disease caused by complete lack of *CLN3* function has not yet been described (Kitzmüller et al., 2008).

*CLN3* is conserved in yeasts, suggesting that it performs a basic cellular function. To help understand the role of *CLN3*, we use the fission yeast *Schizosaccharomyces pombe* as a model system because, similar to mammalian cells, this yeast has many small vacuoles (approximately 50-80), the equivalent organelle to the lysosome (Bone et al., 1998; Moreno et al., 1991). We have previously shown that deletion of *btn1*, the functional orthologue of *CLN3* in fission yeast affects vacuole homeostasis, causing larger and less-acidic vacuoles (Gachet et al., 2005; Kitzmüller et al., 2008), as in patient cells (Holopainen et al., 2001). In *S. pombe*, Bnt1p traffics slowly to the vacuolar membrane via the endomembrane system and has a functional role prior to reaching the vacuole that affects vacuole function (Gachet et al., 2005). Ectopic expression of N-terminally fused GFP-Bnt1p and GFP-CLN3 constructs in *btn1Δ* cells complement the defects in vacuole size and pH (Gachet et al., 2005), confirming that Bnt1p and CLN3 are functional homologues. Also, the effect that the mutations have on the severity of the disease corresponds to their effect on vacuole pH.

Cells lacking *btn1* have other pleiotropic phenotypes. These include an increased number of septated cells under normal growth conditions (Gachet et al., 2005) – suggesting a defect in cytokinesis – and depolarised growth at 37°C (Codlin et al., 2008). In this study, we explored these defects and whether they were linked to defective vacuole acidification. We discovered that *btn1Δ* cells have an aberrant cell-wall structure under normal growth conditions that worsens when grown at higher temperature, and that this is a consequence of defects in two pathways. In one pathway, deletion of *btn1* affects the glucan composition of the cell wall and is a direct consequence of a loss of endocytic and/or vacuole pH homeostasis. This is phenocopied in cells deleted for the v-ATPase, mutants *vma1* and *vma3*. A second pathway, which is independent of vacuole acidification, leads to defective deposition of the cell wall, particularly at the septum, causing cytokinesis defects at the...
We used electron microscopy (EM) to examine defects in cells deleted for Btn1p and further confirms the potential of this model system in understanding the function of CLN3.

**Results**

**Cells deleted for btn1 have a cytokinesis defect**

We have previously noted that, at 25°C, a higher proportion of btn1Δ cells had a division septum (Gachet et al., 2005), suggesting defects in the final stages of cytokinesis. We monitored the septation index of cells grown in yeast extract with supplements (YES) at 25°C, and found that 22% of btn1Δ cells were in the process of septation, compared with 8.6% for wild-type cells (Fig. 1A). We also monitored septa in cells lacking btn1 at 37°C, because we recently identified these conditions promote a severe growth phenotype (slow and progressive depolariisation growth, swelling and eventual lysis) (Codlin et al., 2008). We found that, after 18 hours at 37°C, the majority of the intact swollen btn1Δ cells were septated, with a septation index of 61%, compared with 8% for wild-type cells (Fig. 1A). In addition, 12% of these btn1Δ cells displayed multiple septa, suggesting a severe defect in the final stages of cytokinesis (Fig. 1B). Thus, Btn1p is important for completion of cytokinesis at 25°C, but particularly at 37°C.

**Cells deleted for btn1 have aberrant septa as well as cell-wall defects**

We used electron microscopy (EM) to examine btn1Δ cells for septa defects at both 25°C and 37°C. In btn1Δ cells grown at 25°C, the septum of dividing btn1Δ cells was significantly thicker than that of wild-type cells (193 nm compared with 127 nm) (Fig. 2A). On closer inspection, the primary septum appeared normally formed (Fig. 2Ai, arrowhead). However, the secondary septa, regions of new cell-wall deposition that will become the new cell ends, were significantly thickened (Fig. 2Ai, arrow). btn1Δ cells also had a slight thickening of cell-wall regions on the sides of cells when compared with wild-type cells (Fig. 2B), with longer and less-densely packed fibrillar extensions that were difficult to quantify. Similar, but more excessive, defects were also apparent in btn1Δ cells grown for 18 hours at 37°C. Septated btn1Δ cells displayed a grossly thickened septum (Fig. 2Ci), with a thickness of 732 nm compared with 242 nm for wild-type cells grown under the same conditions (Fig. 2Ci). We noted that swollen interphase cells also had severe cell-wall defects with excessive amounts of cell-wall material (Fig. 2Cii), often asymmetrically located at swollen regions of cells. Btn1p, therefore, is important for the correct deposition of new cell-wall material at the septum and for normal cell-wall structure, and this role is crucial following heat stress.

Growth of mutant strains in which the cell wall is defective can typically be rescued by incorporating 1 M sorbitol into the growth medium, which is reported to osmotically stabilise these cells (Cortes et al., 2005). Lysis and death of btn1Δ cells at 37°C was completely prevented by the addition of 1 M sorbitol to YES (Fig. 2Di), and growth was restored (Fig. 2Dii). This suggests either that sorbitol, as an osmoregulatory agent, is able to rescue the underlying defect of btn1Δ cells, or that sorbitol stabilises the cell and permits growth in the absence of a functional cell wall. The number of cell cycles completed for btn1Δ cells grown over 18 hours at 37°C in the presence of sorbitol was similar to that of wild-type cells (see later), suggesting a relief of the cytokinesis block. We explored whether there was a connection between the cytokinesis defect and the cell-wall defect. We found that the septation index of btn1Δ cells was rescued to near wild-type levels when grown at 25°C in the presence of 1 M sorbitol (Fig. 2E). Thus, the cell-wall defect appears to be a major cause of the delay in cytokinesis in btn1Δ cells at both temperatures, and of the additional cell death and lysis at 37°C. Presumably the increased thickness of the secondary septa in these cells causes a significant delay in digestion of the primary septum and in cell separation, and contributes to the more-severe growth defect at 37°C.

We considered the basis for the cell-wall defect further. The major portion of the cell wall of *S. pombe* consists of α- and β-glucans, which are synthesised by specific glucan synthases (Cortes et al., 2005). We tested btn1Δ cells for sensitivity to the glucanase zymolyase-20T, which specifically digests β-glucans. btn1Δ cells were found to be more sensitive to zymolyase than wild-type cells when grown at 25°C. At 37°C, btn1Δ cells were even more sensitive to zymolyase digestion (Fig. 3B). Thus, btn1Δ cells appear to have an inherent defect in the glucan composition of the cell wall, and this defect appears to be exacerbated upon growth at 37°C. Together, these data support the existence of a defect in the cell wall that contributes to the severe growth phenotype at 37°C, rather than a defect in osmoregulation.

**Defective acidification of the endocytic system can cause a cell-wall defect**

Given that, at 25°C, cells deleted for btn1 have an aberrant cell wall and cytokinesis defects, as well as defective vacuole acidification (Gachet et al., 2005), we explored whether, in *S. pombe*, an increased endocytic and/or vacuole pH might be the cause of the described defects in the cell wall. We monitored cells lacking v-ATPase function, the major contributor to the acidic pH of the vacuole (Iwaki et al., 2004) and endocytic pathway (Sun-Wada et al., 2004), for sensitivity to zymolyase and other cell-wall defects. We found that cells deleted for the *vma1* gene (human orthologue *ATP6V0A1*), which encodes subunit A of the soluble V0 domain, or for the *vma3* gene (human orthologue *ATP6V0C*), which encodes subunit c of the membrane V0δ domain, were, similar to btn1Δ cells, sensitive to zymolyase (Fig. 4A). Thus, cell mutants with increased endocytic and/or vacuole pH appear to have a similar glucan defect to btn1Δ cells. Interestingly, Fig. 1. btn1Δ cells have an increased septation index compared with wild-type at 25°C, and this is increased even further at 37°C. (A) Graph of septation indices of wild-type 972 and btn1Δ cells grown at 25°C, or for 18 hours at 37°C. n=300 (three replicates). (B) btn1Δ cells have multiple septa at 37°C. Calcofluor-stained septa of wild-type 972 and btn1Δ cells grown for 18 hours at 37°C. Scale bars: 10 μm.
after 90 minutes exposure to zymolyase, btn1Δ cells and vma1Δ cells exhibited very similar sensitivities and were more sensitive to zymolyase than were vma3Δ cells (Fig. 4A), despite vma3 encoding the proton-pore component of the v-ATPase V0 complex.

To confirm whether Btn1p was affecting glucan composition and/or deposition by an effect on vacuole pH, and possibly by a direct effect on the v-ATPase itself, we also tested the double-deletion strain vma1Δbtn1Δ (Gachet et al., 2005) for zymolyase sensitivity. (We also crossed btn1Δ cells with vma3 cells but were unable to successfully derive viable spores, which might indicate synthetic lethality between these genes.) Interestingly, we found that the vma1Δbtn1Δ strain was not significantly more sensitive to zymolyase than cells deleted for either gene alone (Fig. 4A). This indicates that Btn1p might indeed affect cell-wall structure via modulation of vacuole pH, and could possibly have a direct impact on v-ATPase activity.

Previous work showed that the vacuole pH of btn1Δ cells was less acidic than that of wild-type cells, by approximately 1 pH unit, and the defects in vacuole pH and size of these cells were linked and could be rescued by growth in acidic medium (pH 4) (Gachet et al., 2005), presumably because of compensatory acidification of the vacuole by an unknown mechanism. To explore the connection between defects in the vacuole pH and cell wall, we tested btn1Δ cells grown in YES at pH 4 for altered sensitivity to zymolyase, as well as monitoring acidification of vacuoles using the proton-sensitive probe carboxy-dichlorofluorescein diacetate (CDCFDA), the fluorescence intensity of which correlates with vacuole acidification (Pringle et al., 1989). We found that btn1Δ cells grown in YES at pH 4 were no longer sensitive to zymolyase digestion (Fig. 4Bi); we also observed an increase in CDCFDA fluorescence (Fig. 4Bii), indicating that vacuoles were acidified. Thus, artificial acidification of the vacuole, and presumably the endomembrane...
Defective vacuole acidification does not underlie all cell-wall defects. The data prompted us to examine whether the defects in the vacuole pH and cell wall of v-ATPase mutants were similarly affected following growth in medium of low pH. Cells deleted for vma1 or vma3, and grown in normal medium at ~pH 6, exhibited minimal CDCFDA vacuole fluorescence (Fig. 4C, bottom panel), as expected because v-ATPase-null cells are defective in vacuole acidification (Iwaki et al., 2004). However, we observed a dramatic increase in vacuolar CDCFDA fluorescence in vma1Δ, btn1Δvma1Δ and vma3Δ cells grown in low-pH medium (Fig. 4C), consistent with medium-driven vacuole acidification, as previously reported for Saccharomyces cerevisiae (Plant et al., 1999), in S. pombe, acidification of the vacuole can occur in cells lacking functional v-ATPase when grown in medium of low pH. We went on to test whether growth in acidic medium could rescue the zymolyase sensitivity of v-ATPase mutants, as is the case for btn1Δ cells. The zymolyase sensitivity of cells deleted for vma3 was partially rescued by growth in acidic medium, as was the strain btn1Δvma1Δ (Fig. 4D), demonstrating that a link does exist between vacuole acidification and cell-wall composition. By contrast, the zymolyase sensitivity of vma1Δ cells was not rescued by growth at pH 4 but was further exacerbated at this pH, particularly during early stages of zymolyase incubation (Fig. 4Di), which is suggestive of a more-severe cell-wall defect under these acidic conditions. Thus, deletion of different components of either V1 or V0 domains of the v-ATPase results in differential phenotypes, which was unexpected.

Defective vacuole acidification does not underlie all btn1Δ cell-wall defects. We explored whether a defect in vacuole acidification contributed to all described phenotypes of btn1Δ cells. First, we investigated whether growth in acidic medium could rescue the increased septation index of btn1Δ cells. However, there was no rescue of the cytokinesis defect in acidic medium, with btn1Δ cells having the same increased septation index (22%) when grown at 25°C in YES at pH 4 or pH 6 (Fig. 5A) and, by EM, there was also no rescue of the thicker septum (Fig. 5B). Next, we investigated whether acidic medium could rescue the defective growth of btn1Δ cells at 37°C (Codlin et al., 2008). Growth in low-pH medium did not prevent cell swelling or lysis (Fig. 5Ci) and cells exhibited a similar reduced number of cell cycles to those grown in pH 6 medium (Fig. 5Cii). So, although growth in acidic medium rescued the vacuole defects (Gachet et al., 2005) and zymolyase sensitivity (Fig. 4B,C) of btn1Δ cells, growth in the same medium was not able to rescue all defects. There was little difference in sensitivity to zymolyase between cells deleted for vma1, btn1 or both genes (Fig. 4A). However, cells lacking vma1 did not exhibit a swelling and lysis phenotype, even after 3 days of slow growth at 37°C (Fig. 5D), confirming that cell-wall defects marked by high zymolyase sensitivity do not necessarily cause cell lethality or cell lysis, and that this phenotype of btn1Δ cells is not due to defective endocytic and/or vacuole acidification. We have previously reported that cells deleted for both vma1 and btn1 exhibited a severe conditional synthetic lethality at 30°C (Gachet et al., 2005). We investigated the cause of cell death of these btn1Δvma1Δ cells and found that they have a severe cytokinesis defect at high temperature (Fig. 5E) that was also evident at 25°C. At 25°C, btn1Δvma1Δ cells had a septation index of 38%, compared with 13% for vma1Δ cells (Fig. 5Ei). After 4 hours at 37°C, their septation index was increased to 49%, compared with 12% for vma1Δ cells (Fig. 5Eii), with aberrant accumulation of multiple septa, often more than five per cell, which was not observed in vma1Δ cells alone (Fig. 5Eiii). Thus, in btn1Δ cells, the underlying cytokinesis defect is exacerbated by loss of v-ATPase function, but failure in endocytic and/or vacuole acidification alone does not cause cytokinesis problems. We considered whether the cytokinesis defect of btn1Δ cells and their depolarised growth defect at 37°C were connected. Supporting this, we found that the cytokinesis defect at 25°C was rescued by growth in YES plus 1 M sorbitol (Fig. 5A, Fig. 2A). In addition, the cytokinesis defect, swollen growth and lysis phenotype of btn1Δ cells at 37°C was rescued by growing in YES containing 1 M sorbitol (Fig. 5Ci,ii and Fig. 2D). Thus, these pH-independent btn1-associated defects also appear to be linked by a common mechanism (Codlin et al., 2008).

Severity of JNCL disease might correlate with sensitivity to zymolyase. We have previously shown that expression of Bt1p rescues the increased vacuole size of btn1Δ cells grown in minimal medium (MM), as well as their defect in vacuole pH (Gachet et al., 2005) and depolarised growth defect (Codlin et al., 2008). We tested whether expression of GFP-Bt1p could rescue the zymolyase sensitivity of btn1Δ cells and found that expression of this fusion protein rescued the zymolyase sensitivity of these cells grown in MM (Fig. 6A). In addition, expression of human GFP-CLN3 was able to partially rescue the defect. Next, we examined whether there was a correlation between the severity of JNCL disease and zymolyase sensitivity, as previously shown for vacuole pH (Gachet et al., 2005). We compared the sensitivity to zymolyase of btn1Δ cells containing constructs expressing mutant GFP-Bt1p proteins. Expression of GFP-Bt1p carrying the mutation Glu295Lys (GFP-Bt1pE295K) (conferring mild disease), which we previously showed could partially rescue vacuole pH (Gachet et al., 2005), was able to rescue zymolyase sensitivity (Fig. 6A). However, expression of GFP-Bt1p carrying the mutation Gly187Ala (GFP-Bt1pG187A) (conferring classic disease), which was unable to rescue vacuole pH (Gachet et al., 2005), was also unable to rescue sensitivity to zymolyase (Fig. 6A). Thus, vacuole pH and zymolyase sensitivity appear to be coupled and correlate with the severity of JNCL disease.
Fig. 4. Defective vacuole and/or endocytic acidification causes a cell-wall defect. (A) vma1Δ, vma3Δ, btn1ΔΔ and btn1Δvma1Δ cells are sensitive to zymolyase. Enzyme sensitivity over time (minutes) of the indicated cells grown at 25°C. Cell lysis was monitored by measuring OD600 nm. (B) Acidic medium rescues zymolyase sensitivity and vacuole pH of btn1Δ cells. (i) Zymolyase sensitivity of wild-type cells or btn1Δ cells grown at 25°C in medium at pH 6 or pH 4. (ii) Vacuole pH as monitored by CDCFDA fluorescence. btn1Δ cells grown at 25°C in a medium of pH 6 are marked by post-staining with DAPI, those grown at pH 4 are unmarked. (C) Growth in acidic medium acidifies the vacuoles but differentially affects the zymolyase sensitivity of btn1Δvma1Δ cells and those deleted for vma1 or vma3. (i) Zymolyase sensitivity after 90 minutes and (ii) zymolyase sensitivity over time of indicated cells grown in medium of pH 4 or pH 6 at 25°C. Unpaired Student’s t-tests were performed (n=3, *P<0.05, **P<0.01, ***P<0.001). (D) Vacuole pH as monitored by CDCFDA fluorescence of the indicated cells grown in a medium of pH 4 or pH 6 at 25°C. Typical results are presented for A, Bi and Cii. Scale bars: 10 μm.
Btn1p is a multicopy suppressor of v-ATPase-null phenotypes

Finally, we considered whether the role of Btn1p at the vacuole was linked to that of v-ATPase. We investigated whether ectopic expression of Btn1p had any effect on cells deleted for vma1 or vma3. Interestingly, we found that expression of Btn1p rescued the zymolyase sensitivity of these cells grown in MM (Fig. 6Bi), suggesting a rescue of the glucan imbalance. We considered whether this was linked to the simultaneous rescue of vacuole pH, i.e. whether Btn1p expression promoted acidification of the vacuole despite an inactive v-ATPase. We examined vacuole pH using CDCFDA. As before, cells that were deleted for vma1 or vma3 exhibited minimal CDCFDA vacuole fluorescence (Fig. 6Ci, bottom panel), whereas vma1 or vma3 cells expressing GFP-Btn1p exhibited CDCFDA vacuole fluorescence (Fig. 6Ci, top panel). This increased CDCFDA fluorescence was not observed in all cells, consistent with the variable expression levels of GFP-Btn1p from the multicopy expression plasmid pREP41 (Fig. 6Cii). We then explored the location of GFP-Btn1p in vma3Δ cells. Btn1p localised to cytoplasmic vesicles and the vacuole-perimeter membrane, and was absent from the vacuole lumen (Fig. 6Cii), the site of CDCFDA fluorescence (Fig. 6Ciii). A link between vacuole pH and vacuole size is known (Gachet et al., 2005; Iwaki et al., 2004). We therefore monitored the size of vacuoles of vma3Δ cells expressing GFP-Btn1p. Normally, the dye FM4-64 is used to label vacuoles. However, because FM4-64 is unable to traffic to the vacuole in v-ATPase-null cells (Iwaki et al., 2004), we monitored vacuole size by phase contrast, because the large vacuoles are clearly visible. We found a striking reduction in vacuole size when Btn1p was ectopically expressed (Fig. 6Di). Together, these data are consistent with overexpression of Btn1p promoting vacuole acidification of v-ATPase-null cells, thereby rescuing defects in vacuole size and the cell wall.

Btn1p is able to rescue phenotypes of v-ATPase-null cells, so we determined whether expression of Btn1p was able to rescue the growth defect of v-ATPase-null cells on high-pH medium. We found that expression of Btn1p could rescue growth of both vma1Δ and, particularly, vma3Δ cells on MM plates at pH 6.5 (Fig. 6Dii). It could not rescue growth at a higher pH of 7 (data not shown), indicating that, although Btn1p can suppress some v-ATPase-null phenotypes, it cannot fully rescue all defects. Together, these data show that Btn1p acts as a multicopy suppressor of at least some v-ATPase-null phenotypes.
Discussion

CLN3 was first identified as the gene underlying JNCL in 1995 (The International Batten Disease Consortium, 1995). Despite much effort, its mechanism of action is still unknown, although its conservation suggests that it has a basic cellular role that affects lysosome homeostasis. Our results using the fission yeast S. pombe shed light on CLN3 function. In this study, we further examined the effects of complete absence of CLN3 by gene deletion of its S. pombe orthologue btn1. There are multiple phenotypes associated with loss of btn1, and further investigation revealed that these could be divided into those that were pH dependent and those that were not (Table 1).

This study revealed that btn1 affects more than one process that is involved in the maintenance of a normal cell-wall structure. Cell-wall defects of S. pombe btn1Δ cells were indicated by: (1) thicker cell walls and secondary septa visualised by EM; (2) a sensitivity to zymolyase (a β-glucanase); (3) a defect in cytokinesis; (4) depolarised and slow growth at high temperatures that lead to cell lysis (Codlin et al., 2008); and (5) rescue of the cytokinesis defect, cell death and lysis at 37°C by incorporation of 1 M sorbitol, an osmolyte that is able to restore growth of mutants bearing aberrant cell walls, into the medium. The composition of the S. pombe cell wall is complex, consisting of a variety of linear and branched α- and β-glucans as well as...
glycoproteins, and its laying down is a highly coordinated process that is not fully understood (Garcia et al., 2006; Humbel et al., 2001). Many genes, including mok1, that encode members of the family of α-glucan synthases or the Bgs family of β-glucan synthases are required for the coordinated synthesis of the different gluca ns, with some made in situ and others possibly made intracellularly and transported to the cell surface (Cortes et al., 2005; Humbel et al., 2001; Konomi et al., 2003).

One aspect of the cell-wall defect of btn1Δ cells, as monitored by zymolyase sensitivity, correlated with the defective vacuole pH. Both these defects were rescued by growth in acidic medium. We also showed, for the first time in S. pombe, that a defect in v-ATPase activity can influence cell-wall composition in a similar manner to that of btn1Δ cells, because cells lacking vma1 or vma3 (which are defective in vacuole acidification) are similarly sensitive to zymolyase. A cell-wall defect had previously been implicated in Vma mutants of S. cerevisiae (Davis-Kaplan et al., 2004; Lussier et al., 1997; Ram et al., 1994). The mechanism for rescue of vacuole acidification and zymolyase sensitivity of btn1Δ cells is unknown. It is unlikely to depend on the endocytic pathway, because the vacuole pH of cells lacking functional v-ATPase, which are severely defective in endocytosis, is acidified during growth in low-pH medium (Iwaki et al., 2004; Plant et al., 1999). It might be that other plasma-membrane and vacuole H⁺ transporters, antiporters or co-transporters are mislocalised and support vacuole acidification following an increased H⁺ concentration gradient driven by the external medium in such cells. Alternatively, the transport to and protonation of ammonia in the vacuole from the ammonia and/or ammonium present in the medium might drive vacuole acidification (Plant et al., 1999).

It remains to be determined how defects in vacuole pH affect cell-wall synthesis. A defect in v-ATPase function is known to differentially affect protein trafficking to the vacuole in S. pombe (Iwaki et al., 2004), but the effects on other trafficking routes have not been studied. The sensitivity of btn1Δ cells and v-ATPase mutants to zymolyase suggests that the defect is in the delivery or synthesis of a particular glucan. Cells mutated in mok1, which encodes an α-glucan synthase, or deleted for pck2, which encodes a kinase controlling Mok1p delivery, are also sensitive to zymolyase digestion (Sengar et al., 1997; Win et al., 2001). Cells lacking btn1 or v-ATPase activity might therefore have a defect in a pH-dependent membrane-trafficking pathway required for correct delivery of Mok1p, or other catalysts of α-glucan synthesis. In the case of btn1, this can be fully rescued by artificial acidification of vacuole pH. We cannot, however, rule out a direct link between vacuole function and cell-wall synthesis. Interestingly, the subcellular location of Mok1p is dependent on the integrity of the F-actin cytoskeleton (Katayama et al., 1999), and F-actin dynamics are defective in btn1Δ cells (Codlin et al., 2008).

The cell-wall defect of btn1Δ cells is more complex than an effect arising only from defective endocytic and/or vacuole acidification. Cells lacking btn1Δ also had thicker septa and cytokinesis defects at 25°C, and a more severe cytokinesis defect and cell swelling and lysis phenotypes at 37°C, which were not rescued by growth in acidic medium, nor observed in cells fully compromised for v-ATPase function. In addition, btn1Δ showed conditional synthetic lethality with vma1 (Gachet et al., 2005), with cells displaying severe cytokinetic defects. Significantly, the cytokinesis, swelling and lysis defects of btn1Δ cells appear linked by a common mechanism that affects the cell wall, because these phenotypes were rescued by growth in 1 M sorbitol, but not by growth in acidic medium. Rescue by sorbitol, although consistent with a defect in the cell wall, might also, or additionally, reflect a defect in osmoregulation that causes increased lysis because of increased osmotic pressure. Cytokinesis or cell-wall defects are often caused by an aberrant secretory and/or exocyst pathway (Cheng et al., 2002; Craighead et al., 1993; Wang et al., 2003; Wang et al., 2002), and it will be interesting to determine whether this or related pathways are affected when btn1Δ is deleted.

Ectopic expression of Btn1p or CLN3 rescued the vacuole defects (Gachet et al., 2005) and zymolyase sensitivity of btn1Δ cells. Using mutant Btn1p proteins that modelled CLN3 disease mutations, we showed that zymolyase sensitivity correlated with changes in vacuole pH and disease severity. This confirms that the molecular basis underlying these defects in S. pombe is directly relevant to the disease. The correlation between vacuole pH and disease severity suggests that future JNCL therapies might include a component that promotes lysosome acidification.

Significantly, Btn1p acted as a multicopy suppressor of v-ATPase-null cells, and was able to rescue their zymolyase sensitivity. In addition, Btn1p was able to relieve vacuole defects of v-ATPase-null cells (vacuole pH and size), and restore growth at pH 6.5. It is probable that these activities are linked, and that they are linked to the effect of Btn1p on vacuole pH (Gachet et al., 2005). Because Btn1p is unlikely to enable functioning of v-ATPase in the absence of major subunits such as Vma1p, which contributes to the catalytic site for ATP hydrolysis, or Vma3p (subunit c), which is part of the Vₐ proton pore, it must be contributing to vacuole acidification in some other way. This could be directly, if btn1 encodes some type

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### Table 1. The pH-dependent and -independent phenotypes of btn1Δ cells compared with wild type grown at 25°C or 37°C

| Phenotype                          | 25°C                           | 37°C                           | Reference                      |
|------------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Vacuole size                       | Increased                      | nd                             | (Gachet et al., 2005)          |
| Vacuole pH                         | Less acidic                    | nd                             | (Gachet et al., 2005)          |
| Sensitivity to zymolyase           | Increased                      | Further increased              | This study                     |
| Septation index                    | Increased                      | Further increased and some     | (Gachet et al., 2005), this study |
| Septum thickness                   | Increased                      | Further increased              | This study                     |
| Cell-wall thickness                | Increased                      | Further increased with asymmetric deposition of cell-wall material | This study                     |
| Number of cell cycles completed in 18 hours in YES | 6 (as for wild type) | 2-3                            | (Codlin et al., 2008)          |
| Cell swelling                      | None                           | After completion of first cell cycle | (Codlin et al., 2008)          |
| Cell lysis                         | None                           | After completion of second cell cycle | (Codlin et al., 2008)          |

nd, not determined.
of ion transporter or channel, as previously postulated from distant sequence homology (Baldwin et al., 2004; Finn et al., 2006; Lobley et al., 2007). More likely is that Bnt1p affects the activity or trafficking of other transporters or channels. Some types of NCL are caused by defective transporters (Sintola et al., 2007) or channels (Kasper et al., 2005; Lange et al., 2006; Poét et al., 2006; Stobrawa et al., 2001; Yoshikawa et al., 2002) that have homologues in S. pombe (SPCC330.07c, SPBC16A3.17c, SPAC1399.02 and SPAC31H1.06c; SPBC19C7.11 and SPBC887.02), making them good candidates for the v-ATPase-independent rescue of pH by Bnt1p. Indeed, in S. cerevisiae, loss of the gene GEF1/CLC, encoding the single voltage-gated chloride channel, is similar to the loss of GEF2/VMA3, which encodes subunit c of the v-ATPase (Greene et al., 1993; Schwappach et al., 1998). Bnt1p could also be contributing to vacuole acidification by promoting protonation of ammonia in the vacuole (Plant et al., 1999), either by driving its uptake from the medium or its transport into the vacuole. Finally, our data contrasts with a recent report suggesting that, in S. cerevisiae, Bnt1p directly affects the activity of v-ATPase (Padilla-Lopez and Pearce, 2006).

We additionally report differing phenotypic effects resulting from deletion of essential components of the V0 or V1 domains of v-ATPase, at least in S. pombe, indicating a hitherto unsuspected complexity of function. Cells deleted for vma3 or vma1 had different sensitivities to zymolyase treatment, and the zymolyase sensitivity of vma3Δ cells could be partially rescued by growth in low-pH medium, but that of vma1Δ cells could not. This might reflect the importance of the V1 domain, and particularly Vma1p (subunit A), in the integration of intracellular signals that regulate v-ATPase activity (Breton and Brown, 2007; Inoue et al., 2005), or in other intracellular processes. Certainly, the balance of v-ATPase subunits can be important in supporting vacuole acidification, and in silencing cytosolic activities of the V1 domain in S. cerevisiae (Rizzo et al., 2007). Alternatively, these differences might be accounted for by a gain of function associated with a partially assembled v-ATPase lacking Vma1p or Vma3p, as originally reported for the F-type H+ ATPase (ATP synthase) (Lai-Zhang et al., 1999). However, the lack of CDCFDA fluorescence under normal growth conditions indicates the loss of vacuole acidification in both strains.

In conclusion, we have shown that the multiple phenotypes that arise from total loss of bnt1 are consistent with CLN3 impacting on many intracellular processes. We have now been able to ascribe some of these apparently unrelated phenotypes in fission yeast to one of two defective processes, and are currently investigating how Bnt1p connects these. We recently reported that the depolarized growth and cell lysis of bnt1Δ cells at 37°C is caused by failure to correctly polarize sterol-rich domains at the plasma membrane, due to loss of Myo1p localization, and is accompanied by defective formation and polarisation of F-actin patches and disruption of endocytosis (Codlin et al., 2008). Strikingly, the intracellular location of Bnt1p is altered at high temperature, becoming more polarized, and this location is not vacuolar. This is consistent with earlier studies that indicate that Bnt1p had a functional role in a pre-vacuolar compartment (Gachet et al., 2005). We therefore hypothesise that Bnt1p acts in a pre-vacuolar compartment to affect the trafficking of key proteins, so that its absence causes these striking growth defects. It could also be acting from this same upstream compartment to affect the trafficking of proteins important for vacuole homeostasis.

In the same way, CLN3 might be affecting equivalent mammalian pathways, resulting in the many phenotypes described. This suggests that disease caused by defective CLN3 might not merely be a pH-related lysosome disorder. It is tempting to speculate that pH-related defects underlie the characteristic storage of JNCL, and that neuronal cell death arises from the particular sensitivity of neuronal cells to defects in a second pathway affecting polarized growth, possibly in response to external stress. Given that we have recently shown that JNCL is a mutation-specific phenotype arising from partial loss of function of CLN3 (Kitzmüller et al., 2008), it will be necessary to use a total-loss-of-function system such as ours to further define the mechanism of action of CLN3 and correlate this with the JNCL disease phenotype.

### Materials and Methods

#### Yeast strains and general techniques

Strains used in this study are listed in Table 2. Medium, growth, maintenance of strains and genetic methods were as described (Moreno et al., 1991). Cells were grown in rich medium (YES) or synthetic ‘minimal’ medium (MM) containing appropriate supplements. Sorbitol was incorporated at 1 M. Low-pH medium (pH 4) consisted of YES containing 40 mM potassium hydrogen phthalate pH 4. High-pH plates consisted of MM + agar containing 40 mM potassium phosphate pH 6.5 or pH 7. For protein expression, cells were grown overnight in MM plus thiamine (4 μM). Cells were washed three times in MM lacking thiamine and grown for 18 hours in the same medium.

#### Enzymatic cell lysis

Lysis experiments were carried out on log-phase cells (usually 4 × 10^6 cells/ml) 10 ml of cells were washed once in H_2O and resuspended in 10 ml H_2O. OD600 nm

| Table 2. Strains |
|-----------------|-----------------|------------------|
| **Strain** | **Genotype** | **Source** |
| 972 | h* | Laboratory stock |
| ED665 | h* , ura4-D18, leu1-32, his2 | Laboratory stock |
| YG660 (bnt1Δ) | h*, bnt1::ura4, bnt1::leu2, bnt1::leu2, his2 | Laboratory stock |
| SC2D (bnt1Δ) | h*, bnt1::ura4, bnt1::leu2 | Laboratory stock |
| SC1A (YG660 + pREP42GFP) | h*, ura4-D18, leu1-32, his2 | Laboratory stock |
| SC4A (YG660 + pREP42GFPBn1) | h*, ura4-D18, leu1-32, his2 | Laboratory stock |
| SC522D (YG660 + pREP42GFPBn1::G136A) | h*, ura4-D18, leu1-32, his2 | Laboratory stock |
| SC17A (YG660 + pREP42GFPBn1::G136A) | h*, ura4-D18, leu1-32, his2 | Laboratory stock |
| SC23D (bnt1Δvma3Δ) | h*, vma3::ura4, vma3::leu2, vma3::his2 | Laboratory stock |
| SC93D (vma1Δ) | h*, vma1::ura4, vma1::leu2, vma1::his2 | Laboratory stock |
| SC93D (vma1Δ + pREP41GFP) | h*, vma1::ura4, vma1::leu2, vma1::his2 | Laboratory stock |
| SC93D (vma1Δ + pREP41GFPBn1) | h*, vma1::ura4, vma1::leu2, vma1::his2 | Laboratory stock |
| SC22D (vma3Δ + pREP41GFP) | h*, vma3::ura4, vma3::leu2, vma3::his2 | Laboratory stock |
| SC22D (vma3Δ + pREP41GFPBn1) | h*, vma3::ura4, vma3::leu2, vma3::his2 | Laboratory stock |
readings were obtained before addition of glucanase. At time 0 hours, 100 μg/ml zymolyase-20T (MP Biomedicals) was added, and cells were incubated at 30°C with vigorous shaking (225 rpm). OD600 nm readings to assess cell integrity versus lysis were taken at 15-minute intervals over 90 minutes. Results were expressed as percentages of OD600 nm reading taken prior to addition of enzyme. Unpaired Student’s t-tests were performed using GraphPad Prism version 4.0c.

Fluorescence staining and microscopy
For cell fixation, cells in log-phase growth were fixed in 10% formaldehyde for 15 minutes, washed three times in 1× PBS and stored at 4°C. Calcofluor (50 μg/ml; Polysciences, Warrington, UK) was used to visualise septa and cell-wall deposition (Moreno et al., 1991). Cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for DNA. To monitor intravacuolar pH, 1 ml of equivalent numbers of log-phase cells was incubated with 1 μl CDCFDA (47 mM in DMSO) (Molecular Probes) for 30 minutes. Cells were washed three times in medium and viewed immediately. Note that the ability of this dye to fluoresce is dependent on correct trafficking of its activating esterase to the vacuole, where its fluorescence correlates with increased vacuolar acidification (Pringle et al., 1989). Images were visualized using a Hamamatsu digital camera. C4742-95 fitted to a Zeiss Axiostar microscope with plan-achromat 63×1.25 oil objective and were recorded using OpenLab 3.0 software (Improvision, UK). Digital images were uploaded to either Microsoft Excel for analysis or to Adobe Photoshop 7 for assembly into montages. A DAPI filter was used for DAPI and calcofluor fluorescence and a FITC filter for GFP detection. CDCFDA images were captured at 0.1 seconds, an exposure time for which the GFP signal was not detectable.

High-pressure freezing EM using EMPACT system
Samples in log phase or after overnight growth at the indicated temperatures (Fig. 2) were collected by filtration and immediately loaded into 1.5-mm hats and frozen under high pressure using the Leica EMPACT system (Leica, Vienna, Austria) according to the manufacturer’s instructions. Freeze substitution was carried out in a Leica AF5 freeze substitution unit with the following protocol: –90°C for 26 hours in 2% osmium tetroxide and 0.5% uranyl acetate in acetone, then –60°C for 8 hours, –30°C for 8°C, 0°C for 1 hour, 20°C for 2 hours. Samples were embedded in epoxy resin (EPON; TAAB Laboratories). Sections were cut with a Leica Ultracut UCT microtome onto formvar-coated slot grids, stained with lead citrate and viewed with a transmission electron microscope (TECNAl2; Philips, Eindhoven, The Netherlands). Images were acquired using a Morada digital camera (Olympus-SIS).

Septum thickness measurements
Measurements were made using ImageJ software. In each septated cell, three individual septum measurements were taken that spanned the septum at a position that was between the centre of the septum and the adjacent outer cell wall. Unpaired Student’s t-tests were performed using GraphPad Prism version 4.0c.

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