Université de Montréal

The Na^+/H^+ exchanger Nhx1 of *Saccharomyces cerevisiae* is essential to limit drug toxicity

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Ce mémoire intitulé:

The Na^+/H^+ exchanger Nhx1 of *Saccharomyces cerevisiae* is essential to limit drug toxicity

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Résumé

Nhx1 est un antiport vacuolaire de Na^+/H^+ chez la levure *Saccharomyces cerevisiae*. Nhx1 joue un rôle important dans le maintien de l'homéostasie ionique du cytoplasme de la cellule. En effet, la mutation du gène *NHX1* chez la levure *nhx1Δ* entraîne une perte de l'homéostasie cellulaire quand les cellules sont cultivées dans un milieu de faible osmolarité.

Ce travail rapporte pour la première fois, et contrairement à la cellule parentale, que la mutation du gène *NHX1* a pour effet une sensibilité du mutant *nhx1Δ* à une variété des drogues et des agents cationiques et anioniques lorsque les cellules sont cultivées dans un milieu riche. En outre, dans ces conditions de culture, aucune sensibilité n'a été observée chez le mutant *nhx1Δ* quand les cellules sont traitées avec différentes concentrations de sel. Nous avons aussi démontré que la sensibilité du mutant *nhx1Δ* aux différents agents ainsi que la sécrétion de l'enzyme carboxypeptidase Y observé chez ce mutant n'ont pas été restauré lorsque les cellules sont cultivées dans des milieux avec différents pH ou avec différentes concentrations de sel.

Enfin, une analyse génétique a révélé que le mutant *nhx1Δ* montre un phénotype distinct d'autres mutants qui ont un défaut dans le trafic entre le compartiment pré-vacuolaire et l'appareil de Golgi quand ces cellules sont traitées avec différents agents. Cette analyse prouve que la sensibilité de *nhx1Δ* aux différents agents n'est pas liée au trafic entre le compartiment pré-vacuolaire et l'appareil de Golgi.

Mots-clés: Bléomycine, Processus d'endocytose, Compartiment pré-vacuolaire, Appareil de Golgi.

Abstract

Nhx1 is an intracellular Na^+/H^+ exchanger localized to the late endosome in *Saccharomyces cerevisiae*. It is believed that Nhx1 plays a major role in pH-mediated vesicle trafficking, as *nhx1Δ* mutant is defective in maintaining the intracellular pH in the vacuoles and cytoplasm when grown in low osmolarity media.

In this work, we reported novel drug sensitivities of the *nhx1Δ* mutant to a range of cationic and anionic agents when cells are grown in rich media. Unlike the low osmolarity media, the *nhx1Δ* mutant showed no sensitivity to salt. Furthermore, we showed that the drug phenotypes of the *nhx1Δ* mutant, as well as the secretion of the vacuolar protein carboxypeptidase Y, were not rescued by either altering the pH or salt concentration. Although, amino acid substitution of the phylogenetically conserved residue Glu355 for Ala (E355A) in Nhx1 resulted in sensitivity to genotoxic drug bleomycin, it was not observed for the non-conserved residue Glu371Ala (E371A). Moreover, genetic analysis revealed that the *nhx1Δ* mutant displayed distinct drug phenotypes in comparison to mutants that are defective in retrograde trafficking from the prevacuole to the late Golgi, excluding the possibility that the drug sensitivity of the *nhx1Δ* mutant is related to retrograde trafficking.

Keywords: Bleomycin, Endocytic pathway, Prevacuolar compartment, Golgi.

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List of Abbreviations

Ala: Alanin

ALP: Alkaline phosphatase

APG: Arginine/phosphate/glucose

AP site: Apurinic/apyrimidinic site

ATP: Adenosine-5'-triphosphate

ATPase: Adenosine-5'-triphosphatase

BLM: Bleomycin

$CaCl_2$: Calcium chloride

cDNA: complementary DNA

CPY: Carboxypeptidase Y

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

DSB: Double-stranded breaks

DTT: Dithiothreitol

EDTA: Ethylene diamine tetraacetic acid

EE: Early endosome

ESCRT: Endosomal sorting complexes required for transport

Fe II: Iron (reduced form)

Glu: Glutamic acid

GTP: Guanosine-5'-triphosphate

GTPase: Guanosine 5'-Triphosphatase

H^+: Hydrogen ion

HOPS: Homotypic fusion and protein sorting complex

K^+: Potassium ion

KCl: Potassium chloride

KDa: Kilo Dalton

LB: Luria-Bertani

LE: Late endosome

LiCl: Lithium chloride

M_1G: MDA-deoxyguanosine

$MgCl_2$: Magnesium chloride

mRNA: Messenger ribonucleic acid

MVB: Multivesicular body

Na^+: Sodium ion

NaCl: Sodium chloride

Na_2HPO_4: DiSodium hydrogen phosphate

NaOH: Sodium hydroxide

O.D.: Optical density

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

PI3P: Phosphatidyl inositol 3-phosphate

PMSF: Phenylmethylsulphonyl fluoride

ProCPY: Precursor Carboxypeptidase Y

RNA: Ribonucleic acid

rpm: Rotations per minute

rRNA: Ribosomal RNA

RT: Reverse transcriptase

S. cerevisiae: Saccharomyces cerevisiae

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPD: Spermidine

SPM: Spermine

7-TMS: 7-transmembrane segments

TGN: *trans*-Golgi network

3'-PG: 3'-phosphoglycolate

Vam: vacuolar abnormal morphology

Vps: vacuolar protein sorting

UV: Ultraviolet

YPD: Yeast peptone dextrose

Dedication:

To my family for their love and support

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I am in the fortunate position of being able to thank my research director Dr. Dindial Ramotar for his valuable guidance, advise, support and encouragement throughout the course of this study.

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Words cannot express my deepest love and appreciation for my family. I am deeply indebted to my father and my mother for their understanding, encouragement and untiring support throughout my life.

Chapter 1 Introduction

1.1 Bleomycin

Bleomycin is a chemotherapeutic glycopeptide antibiotic isolated from *Streptomyces verticillis* (Umezawa, 1965; Umezawa *et al.*, 1966) and is believed to reduce the growth of the tumors in mice, rats and human (Ichikawa *et al.*, 1969; Kanno *et al.*, 1969; Oka *et al.*, 1970; Suzuki *et al.*, 1968; Terasima *et al.*, 1970). It has been shown that bleomycin acts as a chemotherapeutic agent by directly damaging the DNA and causing cell death (Burger *et al.*, 1981a; Burger *et al.*, 1982; Hecht, 1986; Kane & Hecht, 1994). Further analysis revealed that bleomycin is involved in micronuclei formation as well as chromosome aberrations in human lymphocytes. It also induces mitotic recombination in many organisms (Hoffmann *et al.*, 1993; Ramotar & Wang, 2003). Since the drug creates some lesions similar to those created by ionizing radiation (Burger, 1998; Worth *et al.*, 1993), it is known as radiomimetic agent (Absalon *et al.*, 1995a; Dedon & Goldberg, 1992; Hoehn *et al.*, 2001; Steighner & Povirk, 1990a; Steighner & Povirk, 1990b).

Bleomycin is efficient against lymphomas, testicular carcinomas, squamous cell carcinomas of the cervix, head, and neck (Lazo *et al.*, 1996; Povirk, 1996). It is often used in combination therapy with other agents such as cisplatin (Einhorn, 2002; Jani *et al.*, 1992; Umezawa, 1971; Wharam *et al.*, 1973). Although bleomycin treats testicular carcinomas with high cure rate (~80%) (Einhorn, 2002; Hecht, 2000; Jani *et al.*, 1992; Umezawa, 1971; Wharam *et al.*, 1973), it is not effective for other types of cancer including colon, breast, and ovarian (Lazo *et al.*, 1996; Povirk & Austin, 1991). Therefore, it is likely that a specific mechanism is involved in responding to bleomycin

treatment in some tumors but not in the others. While the mechanism(s) describing the reason for resistance of some tumors towards bleomycin treatment has not yet been found, the possible explanations include: (1) increased repair of bleomycin-induced DNA lesions, (2) decreased bleomycin uptake, (3) increased bleomycin efflux, and (4) inactivation or degradation of bleomycin by bleomycin-hydrolase (Akiyama *et al.*, 1981; Jani *et al.*, 1992; Mir *et al.*, 1996; Miyaki *et al.*, 1975; Morris *et al.*, 1992; Sanz *et al.*, 2002; Sebti *et al.*, 1991; Urade *et al.*, 1992).

1.1.2 Structure of bleomycin

Bleomycin consists of three functional domains, the N-terminal metal-binding domain, the C-terminal DNA-binding domain, and the carbohydrate moiety (Fig. 1). The DNA binding domain contains a polyamine-like region (Abraham, 1999; Hecht, 1986; Hoehn *et al.*, 2001; Leitheiser *et al.*, 2000; Petering *et al.*, 1996).

The N-terminal domain is essentially required for the antitumor effect of bleomycin. This domain interacts with molecular oxygen as well as both redox-active and non-redox-active metal ions such as iron and cobalt respectively (Ehrenfeld *et al.*, 1985; Ehrenfeld *et al.*, 1987; Hoehn *et al.*, 2001; Levy & Hecht, 1988; Oppenheimer *et al.*, 1980; Petering *et al.*, 1996). The metal ion has crucial role in: (1) interaction between bleomycin and DNA, and (2) oxygen activation resulting in generation of a reactive radical species (Burger *et al.*, 1981a; Ehrenfeld *et al.*, 1985; Ehrenfeld *et al.*, 1987; Oppenheimer *et al.*, 1981; Sausville *et al.*, 1976; Sausville *et al.*, 1978).

Although the C-terminal is required to bind DNA (Abraham, 1999; Hecht, 1986), its chemical modification does not prevent DNA cleavage (Abraham, 1999).

Whereas the role of carbohydrate moiety is unknown, bleomycin lacking this domain has less effect on DNA cleavage (Leitheiser *et al.*, 2000).

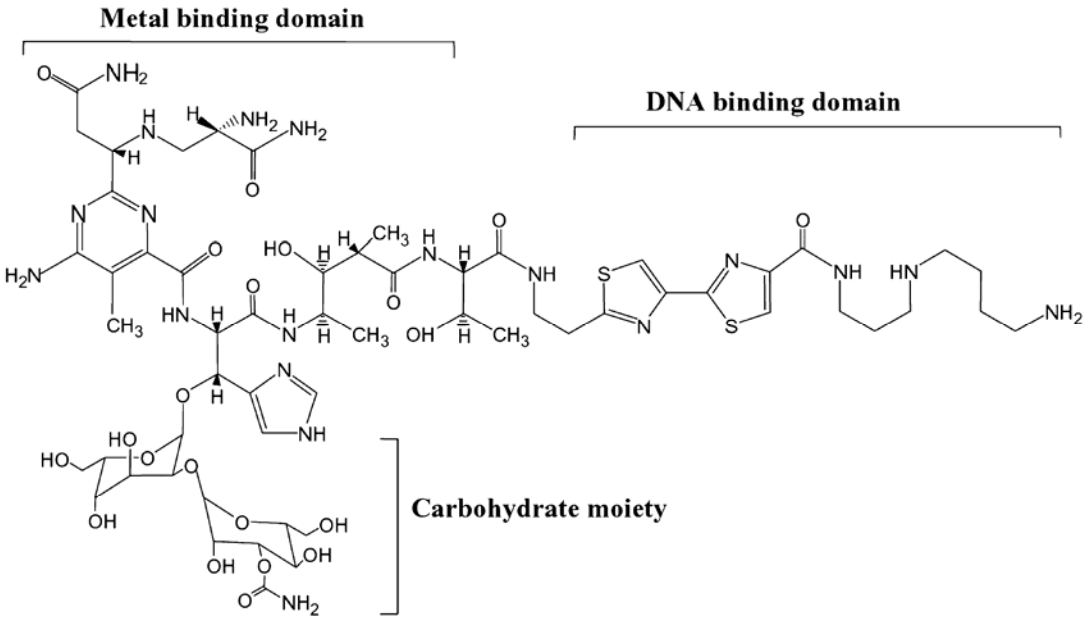


Figure 1: Structure of bleomycin
Bleomycin possesses three functional domains, the N-terminal metal-binding domain, the C-terminal DNA-binding domain, and the carbohydrate moiety (Ramotar & Wang, 2003).

1.1.3 Bleomycin-inducted DNA lesions

Bleomycin induces DNA lesions through a free radical-driven process (Burger, 1998). First, reduced form of iron (Fe II) binds to metal domain of bleomycin and in the

presence of oxygen, the bleomycin (Blm)-Fe(II)-O_2 complex is formed (Burger *et al.*, 1979; Burger *et al.*, 1981a). This activated complex is able to attack DNA to generate at least 4 types of DNA lesions. Several factors such as presence or absence of oxygen influence the type of DNA lesion generated by bleomycin (Burger, 1998; Suzuki *et al.*, 1970; Terasima & Umezawa, 1970; Tounekti *et al.*, 2001; Umezawa *et al.*, 1966).

In the absence of molecular oxygen, bleomycin abstracts a hydrogen atom from the 4' carbon of deoxyribose to make an unstable ring (Burger *et al.*, 1980; Burger *et al.*, 1981b; Ekimoto *et al.*, 1980; Povirk *et al.*, 1977) (Fig. 2). The unstable sugar forms an oxidized (ketoaldehyde) apurinic/apyrimidinic (AP) site, where the DNA strand is missing a base (Burger, 1998; Worth *et al.*, 1993).

In the presence of oxygen, bleomycin can damage the sugar to create a single-strand break where 3'-terminus is blocked by 3'-phosphoglycolate (3'-PG). This lesion blocks DNA synthesis (Burger, 1998; Giloni *et al.*, 1981; Worth *et al.*, 1993). Remarkably, the remaining portion of the fragmented sugar (base propenal), which is able to react with DNA to create base adducts, can interact with guanine to generate M_1G lesion, 3-(2'-deoxy-β-D-erythro-pentofuranosyl)-pyrimido[1,2-a]purin-10(3H)-one (Dedon *et al.*, 1998) (Fig. 2).

Bleomycin creates bi-stranded DNA lesions at CGCC sequences as well. This lesion is generated when the bleomycin makes an AP site on one strand and a single-strand break on the complementary strand (Absalon *et al.*, 1995b; Dedon & Goldberg, 1992; Hoehn *et al.*, 2001; Steighner & Povirk, 1990a).

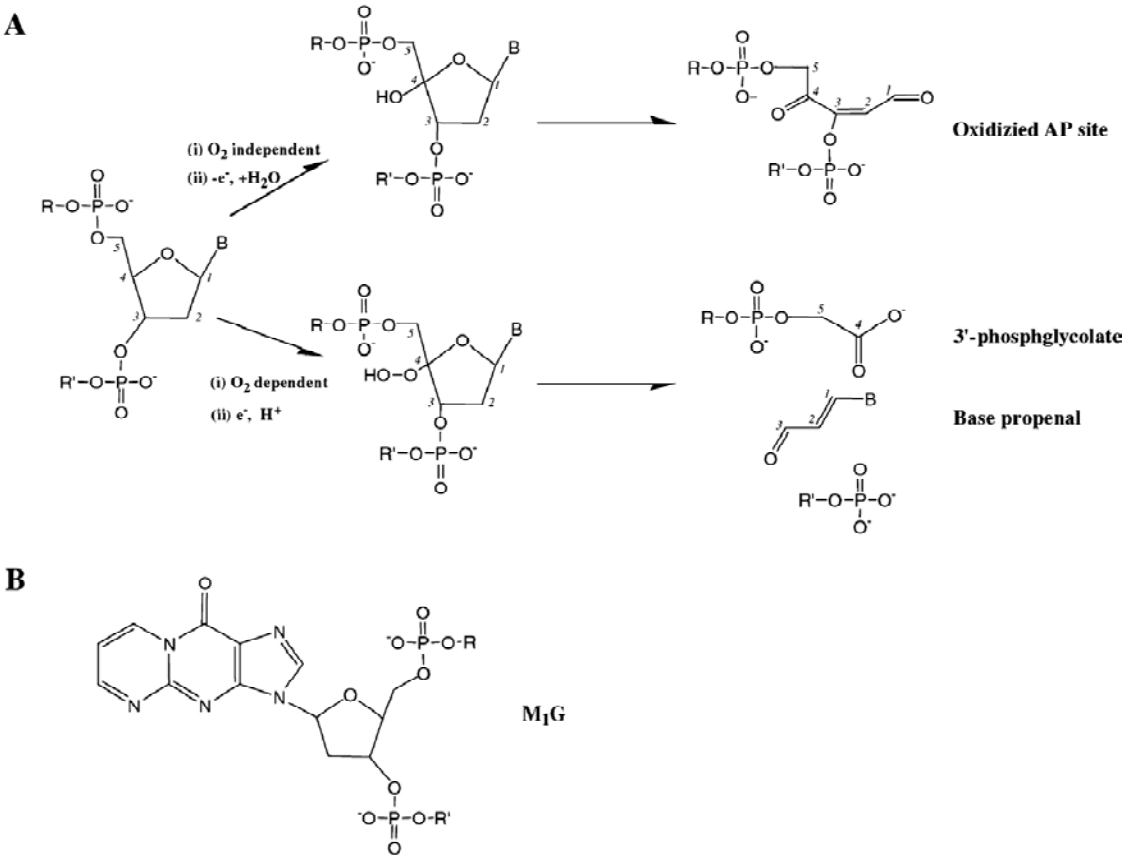


Figure 2: Structure of DNA lesions

A, in the absence of molecular oxygen, bleomycin creates an unstable ring resulting in formation of an oxidized apurinic/apyrimidinic (AP) site. In the presence of oxygen, bleomycin generates a single-strand break where 3'-terminus is blocked by 3'-phosphoglycolate (3'-PG). **B**, the M_1G lesion is generated by interaction between base propenal and guanine (Ramotar & Wang, 2003).

Bleomycin is also able to cleave mRNA, tRNA as well as rRNA at 5'-GU-3' sequences through an oxidative pathway similar to the mechanism involved in DNA cleavage (Carter *et al.*, 1990; Hecht, 1994; Holmes *et al.*, 1993; Huttenhofer *et al.*, 1992; Keck & Hecht, 1995; Morgan & Hecht, 1994). Therefore, bleomycin-induced cell death might result from the destruction of more than one target. However, it is unlikely that RNA is the main target of the bleomycin (Carter *et al.*, 1990; Holmes & Hecht, 1993; Huttenhofer *et al.*, 1992).

1.1.4 Mechanisms involved in repairing the bleomycin-induced DNA lesions

Since bleomycin induces formation of DNA lesion leading to cell death, cells exposed to this drug must activate the mechanism(s) involved in repairing the damaging DNA. In yeast, the mechanisms required to remove specifically bleomycin-induced DNA lesions have not yet been well characterized. However, Apn1, Apn2 and Tpp1 which are known as enzymes required for the base-excision repair, have been demonstrated to be involved in removing the lesions induced by bleomycin (Leduc *et al.*, 2003; Vance & Wilson, 2001a; Vance & Wilson, 2001b). Apn1 and Apn2 are able to cleave DNA at AP site as well as to remove the blocked 3'-terminus at strand breaks. These enzymatic activity leads to regeneration of 3'-hydroxyl group which results in DNA repair synthesis (Demple *et al.*, 1986; Levin *et al.*, 1988; Ramotar *et al.*, 1991; Ramotar & Wang, 2003). Tpp1 functions only on 3'-phosphate at the single-strand breaks (Ramotar & Wang, 2003; Vance & Wilson, 2001a). It has been documented that the mutants lacking all three enzymes (*apn1Δ apn2Δ tpp1Δ*) are extremely

hypersensitive to bleomycin, while single mutants are not significantly sensitive to the drug (Leduc *et al.*, 2003; Vance & Wilson, 2001b). Moreover, recombination and post-replication DNA-repair pathways are also involved in the repair of DNA lesions induced by bleomycin (Ramotar & Wang, 2003).

1.2 Genome-wide screen

As it was pointed out above, it is unclear how certain cancers such as colon carcinoma do not respond to bleomycin. Our group has performed a genome-wide screen with the *Saccharomyces cerevisiae* strains, each deleted for a single non-essential gene in order to identify mutants that exhibit resistance or hypersensitivity to the drug when grown in rich media (Aouida *et al.*, 2004b). The screens detected only five mutants (*agp2Δ, sky1Δ, ptk2Δ, brp1Δ* and *fes1Δ*) that were resistant to bleomycin. Among these mutants, *agp2Δ* mutant revealed the greatest resistance (Aouida *et al.*, 2004b). Agp2 is very well characterized as transporter of L-carnitine in the cells (Lee *et al.*, 2002; van Roermund *et al.*, 1999). Our group has demonstrated that Agp2 acts as transporter of both bleomycin (Aouida *et al.*, 2004b) and polyamine (Aouida *et al.*, 2005). The screens also identified 231 hypersensitive mutants (4- to 20-fold more sensitive as compared to the parent). The corresponding genes encode the proteins that are involved in many biological processes including DNA repair, chromatin remodeling and the proper functional maintenance of the mitochondria and vacuoles (Aouida *et al.*, 2004b). It is noteworthy that amongst the hypersensitive mutants, the group consisting of the largest number of bleomycin-sensitive genes belongs to the endosomal pathway (Aouida *et al.*, 2004b). Deletion of any one of these genes resulted in varying

sensitivity to bleomycin implying that this pathway plays a crucial role in preventing the toxicity of the drug (Aouida *et al.*, 2004a; Aouida *et al.*, 2004b).

1.3 Endocytic pathway

Endocytosis is a trafficking mechanism involved in internalization of the extracellular and plasma membrane components which either are recycled to the plasma membrane or delivered to the vacuole (Henkel *et al.*, 1999; Samaj *et al.*, 2004; Shaw *et al.*, 2001). The yeast vacuole, equivalent to lysosome in higher eukaryotes, is an organelle involved in degradation of cytoplasmic proteins (Chiang & Schekman, 1991). Endocytosis has been detected in all the eukaryotic cells (Geli & Riezman, 1998). It has been documented that some processes such as actin regulation, ubiquitylation, lipid modification, and signal transduction are involved in the endocytic pathway (Shaw *et al.*, 2001). Remarkably, several screens have identified the mutants such as vacuolar protein sorting (*vps*) and vacuolar abnormal morphology (*vam*) which are defective in protein trafficking to the vacuole. The vacuolar protein sorting (*vps*) mutants are divided to six classes (A-F) depending on the phenotype (Banta *et al.*, 1988; Bowers & Stevens, 2005; Raymond *et al.*, 1992). The mutants in the same class are often defective in the same step of protein trafficking.

Two types of endocytosis have been characterized based on cargo: fluid endocyosis and receptor-mediated endocytosis. In the fluid endocytosis, cargos consist of the bulk solutes which do not bind to the membrane. They are engulfed in a vesicle that is subsequently delivered into the cell (Ferris *et al.*, 1987; Swanson *et al.*, 1985). In the receptor-mediated endocytosis, internalization requires specific proteins on the cell

surface which function as receptor to bind their ligands (macromolecular cargos) (Lamaze & Schmid, 1995; Penalver *et al.*, 1997). In contrast to receptor-mediated endocytosis, fluid endocyosis is not saturable with respect to the external concentration (Riezman, 1985; Swanson *et al.*, 1985). Fluid endocyosis and receptor-mediated endocytosis share some features.

Since the receptors involved in endocytosis of bleomycin and the other drugs used in this study are not yet known, α-factor endocytosis is explained to provide a general description of the receptor-mediated endocytosis. The receptor-mediated endocytosis for bleomycin remains to be detected.

Initially, the specific plasma membrane proteins, eisosomes, act as organizing sites (Walther *et al.*, 2006). Clathrin, a multimeric protein, is consequently recruited (Newpher *et al.*, 2005). Clathrin is required for recruitment of the later proteins, but does not play significant roles in yeast endocytosis (Kaksonen *et al.*, 2005). In fact, it has been documented that the clathrin-deficient cells resulted from deletion of *CHC1* gene retain the ability to deliver the cargos to the vacuole (Payne *et al.*, 1988). α-factor which is a small peptide secreted by *MATα* cells binds to its receptor, Ste2, at the endocytic site of the *MATa* cells (Baggett & Wendland, 2001). Ste2 belongs to a group of transporters that possess 7-transmembrane segments (7-TMS) with trimeric G-proteins-dependent function (Singer & Riezman, 1990; Tan *et al.*, 1993). Ligand-receptor binding leads to Ste2 modification such as phosphorylation and ubiquitylation which act as signals for internalization (Engqvist-Goldstein & Drubin, 2003; Hicke *et al.*, 1998; Howard *et al.*, 2002). The recruitment of a subset of proteins involved in actin assembly such as Arp2/3 and class I myosin result in initiation of the actin

assembly. While, mutations in many genes which encode the proteins such as tropomyosin and myosins V cause significant defects in the actin cytoskeleton, endocytosis is not affected in these mutants (Riezman *et al.*, 1996). Therefore, it is likely that the proteins involved in actin assembly are very specific. The assembling actin and myosin activity lead to plasma membrane inward (Sun *et al.*, 2006). The internalization is followed by releasing of the vesicle and consequently coat and actin disassembly. The coat components is recycled in order to generate more vesicle (Toshima *et al.*, 2005). The endocytic vesicles connection with actin cable lead to efficient association of the vesicle to the early endosome (Huckaba *et al.*, 2004; Toshima *et al.*, 2006).

The internalized proteins have two distinct destinations, either they are returned to the plasma membrane or delivered to the vacuole for degradation. The first step of the sorting occurs at the early endosome where the proteins for recycling to the plasma membrane are transported to the *trans*-Golgi network (TGN). These proteins are consequently returned to the cell surface through the exocytic secretory pathway. The proteins which are not recycled enter the late endosome/prevacuolar compartment (Shaw *et al.*, 2001). It is noteworthy that some proteins could be either recycled to the plasma membrane or delivered to the vacuole. While, the Ste3 known as a-factor receptor is recycled in a ligand dependent manner, it is targeted to vacuole in absence of a-factor (Shaw *et al.*, 2001). Progressive maturation of the early endosome leads to generation of the late endosome (Russell *et al.*, 2006).

The invagination of vesicles from the membrane of the late endosome/prevacuolar compartment into the endosomal lumen generates a

multivesicular body (MVB). The proteins which will be degraded in the vacuole (e.g., endocytic cargo) or the proteins which need to undergo special processing in the vacuole (e.g., pCPY) are sorted into these vesicles. It has been demonstrated that MVB sorting requires ubiquitylation of the cargo. Ubiquitin acts as recognition signal for entry of the cargo to MVB (Katzmann *et al.*, 2001; Shaw *et al.*, 2001). Four endosomal sorting complexes required for transport (ESCRT) are involved in transport of the cargo to MVB vesicle in *Saccharomyces cerevisiae*. These complexes (ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III) comprise the soluble "class E" vacuolar sorting proteins (Vps) which selectively bind the cargos and direct sorting of these cargos to MVB vesicles (Bowers & Stevens, 2005).

Protein sorting initiates via generating Phosphatidyl inositol 3-phosphate (PI3P) at the late endosome membrane where ESCRT-0 recruitment results in ubiquitylation of the non-ubiquitylated cargos by Rsp5. ESCRT-I is consequently recruited to form a complex with ESCRT-0 and ubiquitylated cargos. ESCRT-I functions to recruit ESCRT-II and ESCRT-III. While, ubiquitylated cargos bind to ESCRT-I and ESCRT-II, ESCRT-III interacts with lipids and ESCRT-III-associated proteins. Cargos deubiquitylation requires deubiquitylating enzyme Doa4 which is known as crucial protein in removing ubiquitin from ubiquitylated cargos. A protein which belongs to ESCRT-III-associated proteins is involved in Doa4 recruitment. ESCRT-III-associated proteins and the ESCRT complexes dissociate from the membrane and cargos are sorted to invaginating MVB vesicle (Fig. 3) (Bowers & Stevens, 2005; Zhang *et al.*, 2008). The cargos such as Ste2, which are previously ubiquitylated for internalization, use the same ubiquitin modification during MVB sorting. Furthermore, the endocytosed

proteins could be deubiquitylated after internalization and the reubiquitylated for MVB sorting (Shaw *et al.*, 2001).

Remarkably, the proteins destined to remain intact in the vacuolar membrane, without undergoing degradation, are not sorted from the late endosome into the multivesicular body. These proteins are directly incorporated into the limiting vacuolar membrane (Shaw *et al.*, 2001).

The multivesicular body eventually fuses with the vacuole and releases its intralumenal vesicles into the vacuole where the cargos are degraded or undergo special processing (Odorizzi *et al.*, 1998; Shaw *et al.*, 2001). Fusion with the vacuole requires homotypic fusion and protein sorting complex (HOPS), Rab GTPase Ypt7 as well as SNARE complex (Bowers & Stevens, 2005). The HOPS complex consists of several proteins in "class C" of vacuolar protein sorting (Vps). This complex is involved in activation of Ypt7 and acts as Ypt7 effector. The activated Ypt7 is believed to tether vacuole and late endosome membrane compartment. The tethering leads to membrane fusion via the integral membrane proteins (SNARE) (Bowers & Stevens, 2005; Wurmser *et al.*, 2000).

Vacuolar delivery of the cargos could be affected in the mutants defective in the "class E" of vacuolar protein sorting (Vps). In these mutants, late endosome is unable to mature into the multivesicular body resulting in accumulation of the cargos in an aberrant and enlarged late endosome (Katzmann *et al.*, 2001; Odorizzi *et al.*, 1998; Shaw *et al.*, 2001).

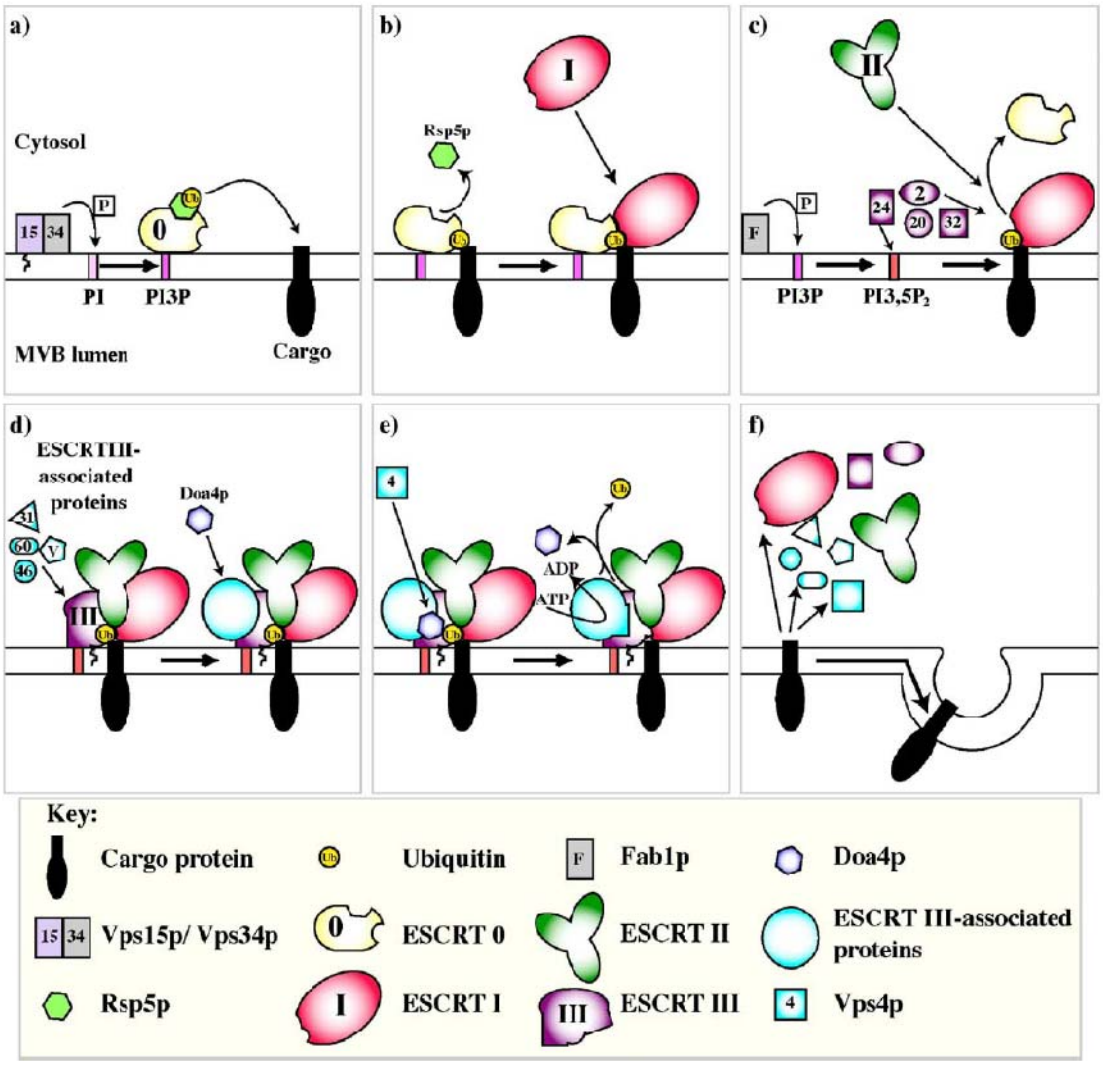


Figure 3: Model for protein sorting into the MVB vesicles

ESCRT-0 is required for cargo ubiquitylation. Ubiquitylated cargo is recognized by ESCRT-I, which initiates cargo entry into MVB vesicles. Function of some additional class E Vps proteins result in recruitment of the deubiquitylating enzyme Doa4 to remove ubiquitin from cargo prior to its entry into invaginating vesicles (Bowers & Stevens, 2005).

1.4 Nhx1

The Na^+/H^+ antiporters (NHE proteins) are required for the exchange of Na^+ for H^+ across membranes and play a role in regulation of internal pH, cell volume, and sodium level in the cytoplasm (Wells & Rao, 2001). In *Escherichia coli*, three antiporters (NhaA, NhaB, and ChaA) (Ohyama *et al.*, 1994); in mammals, nine members of Na^+/H^+ antiporters (exchangers) (NHE1-9) (Mukherjee *et al.*, 2006); in *Saccharomyces cerevisiae*, two plasma membrane transporters (Nha1& Ena1-4) (Kinclova-Zimmermannova *et al.*, 2006) and one intracellular transporters (Nhx1) (Nass *et al.*, 1997; Nass & Rao, 1998); in plants, Na^+/H^+ antiporters isolated from some species such as *Arabidopsis thaliana* (AtNhx1) (Gaxiola *et al.*, 1999) have been reported.

In yeast, the *NHX1* gene which has been identified as *VPS44* (Bowers *et al.*, 2000) encodes an intracellular Na^+/H^+ exchanger, which is located in the late endosomes/prevacuolar compartment (Nass & Rao, 1998). It is involved in regulating vesicle trafficking out of the prevacuolar compartment to the vacuoles (Bowers *et al.*, 2000). Nhx1 is also required to mediate vacuolar sequestration of Na^+ and K^+ by coupling their transport to the H^+ gradient created by the vacuolar H^+-ATPase (Nass *et al.*, 1997). Thus, mutant lacking Nhx1 is reported to be sensitive to NaCl in media with low ionic strength (Brett *et al.*, 2005; Wells & Rao, 2001). It has been shown that the role of Nhx1 in cellular physiology is not limited to ion homeostasis, as *nhx1Δ* mutant reveal a phenotype of the "class E" vacuolar protein sorting (*vps*) mutants resulting in enlargement of the late endosomal or prevacuolar compartment (Bowers *et al.*, 2000).

Nhx1 contains 12 transmembrane segments, as well as a C-terminal hydrophilic domain that is involved in protein–protein interactions (Fig. 4) (Mukherjee *et al.*, 2006). The C-terminal tail of Nhx1 was shown to interact with C-terminal region of Gyp6, a GTPase-activating protein involved in Ypt6-mediated retrograde traffic to the Golgi (Ali *et al.*, 2004). Evidence suggests that Gyp6 negatively regulates Nhx1 function and that Nhx1 may exert a role to allow trafficking from the prevacuole to the late Golgi via Ypt6 (Ali *et al.*, 2004).

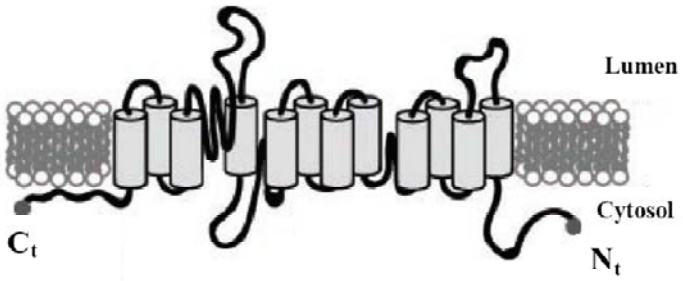


Figure 4: Topology model of Nhx1
Nhx1 consists of 12 transmembrane domains with C-terminal and N-terminal tails located in cytoplasmic side (Ali *et al.*, 2004).

To date, the phenotypes reported for *nhx1Δ* mutant include sensitivity to NaCl (Brett *et al.*, 2005; Wells & Rao, 2001) hygromycin B (Ali *et al.*, 2004; Brett *et al.*, 2005), bleomycin (Aouida *et al.*, 2004b) and gentamicin, an aminoglycoside antibiotic used for killing Gram-negative bacteria (Wagner *et al.*, 2006). In addition, *nhx1Δ* mutant displayed missorting of carboxypeptidase Y (CPY) causing its secretion into the growth media (Ali *et al.*, 2004).

1.5 CPY secretion

In *Saccharomyces cerevisiae, PRC1* encodes carboxypeptidase Y (CPY) (Ohi *et al.*, 1996) which is required for the proteolytic function of the vacuole (Van Den Hazel *et al.*, 1996). It has been recently demonstrated that CPY is also required in the synthesis of phytochelatins (Wunschmann *et al.*, 2007). In eukaryotes, the phytochelatins consist of the cysteine-rich peptides with low molecular weight which sequester excess heavy metal ions and maintain metal ions homeostasis (Cobbett & Goldsbrough, 2002; Rea *et al.*, 2004; Zenk, 1996).

CPY is synthesized as an initial precursor (preproCPY) contains a 20 amino acid signal peptide in N-terminal which is followed by a 91 amino acid propeptide. The initial precursor then enters the lumen of the endoplasmic reticulum where removal of signal peptide resulting in formation of the inactive precursor (proCPY). The inactive precursor rapidly becomes N-glycosylated and is transported to the Golgi where the elongation of the carbohydrate side chains results in the 69 KDa proCPY. The proCPY is channeled to the late Golgi by its receptor. Vps10p, the CPY receptor, binds proCPY in the late Golgi and moves to the late endosome where proCPY is released. Vps10p is then returned to the late Golgi to capture and carry more proCPY (Fig. 5) (Bowers *et al.*, 2000; Cereghino *et al.*, 1995; Cooper & Stevens, 1996; Marcusson *et al.*, 1994). The vacuole is the final destination for proCPY. Cleavage of the propeptide by proteinase B (Prb1p) leads to formation of the active 61 kDa CPY, which is the last step in the maturation of CPY (Sorensen *et al.*, 1994).

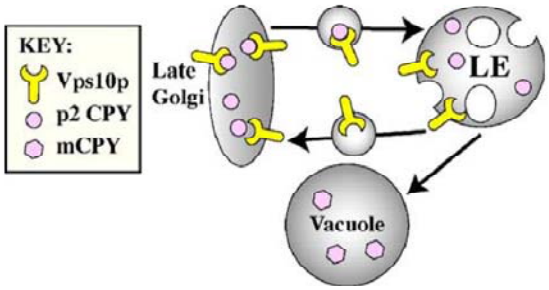


Figure 5: Vps10 is involved in trafficking of CPY

Vps10 acts as transporter to carry CPY to the late endosome (LE) where CPY is released from Vps10. Then, CPY is transported to the vacuole to form mature CPY (mCPY). Vps10 is recycled back to the Golgi (Bowers & Stevens, 2005).

Propeptide acts as a chaperone to ensure the proper folding of the CPY during its maturation. It also inhibits the hydrolytic activity until the protein reaches its final destination (Winther & Sorensen, 1991). It is noteworthy that Tfs1 has been characterized as high-affinity inhibitor of yeast CPY (Bruun *et al.*, 1998).

There are also two other pathways for the newly synthesized proteins which are destined for the vacuole. (i) The vacuolar proteins such as alkaline phosphatase (ALP) enter the vacuole directly from late Golgi, by-passing late endosome (Bowers *et al.*, 2000; Cowles *et al.*, 1997; Piper *et al.*, 1997), and (ii) The proteins which have been carried to the cell surface enter the vacuole through endocytosis (Fig. 6) (Bowers *et al.*, 2000).

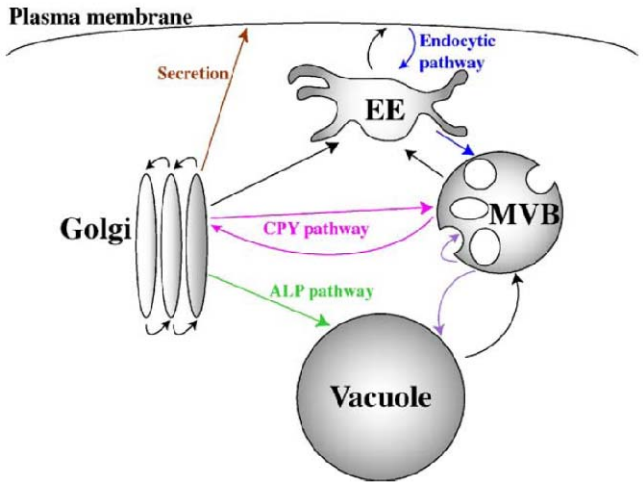


Figure 6: **Protein trafficking pathways to the vacuole in yeast**

The newly synthesized proteins are secreted or transported to early endosomes (EE). The proteins can also be taken to the vacuole via the late endosome/multivesicular body (MVB) (the CPY pathway). The ALP pathway sorts proteins to the vacuole from the late Golgi via an alternative route, bypassing the endosomal network (Bowers & Stevens, 2005).

Since the endocytosis pathway converge with CPY route at the late endosome (Davis *et al.*, 1993; Piper *et al.*, 1995; Rieder *et al.*, 1996), CPY is extensively used to monitor the proteins transport pathway to the vacuole as well as the function of the endosome and the vacuole (Bryant & Stevens, 1998; Gabriely *et al.*, 2007; Losev *et al.*, 2006). For example, the vacuolar protein sorting (*vps*) mutants including the "class E" *vps* mutants secrete abnormally CPY and other vacuolar hydrolases into the

extracellular medium (Raymond *et al.*, 1992; Rothman & Stevens, 1986). In "class E" *vps* mutants cells, Vps10p accumulates in the large and aberrant late endosome and is not able to recycle to the late Golgi to transport more CPY (Cereghino *et al.*, 1995; Piper *et al.*, 1995; Rieder *et al.*, 1996). This defect is the main reason of CPY secretion in the "class E" compartment. The collected CPY in the late Golgi is then secreted into the extracellular medium. Vps10p accumulated in the late endosome cleaves to a smaller form (Vps10p*) via an abnormal proteolytic cleavage (Bowers *et al.*, 2000; Cereghino *et al.*, 1995; Piper *et al.*, 1995). Since ALP directly enters the vacuole via the Golgi, by-passing the late endosome, transport through the ALP pathway is not affected in "class E" *vps* mutants (Bowers *et al.*, 2000).

The last step in CPY and ALP pathways is the fusion of the late endosome with the vacuole. This step is controlled by a complex of the proteins including Vam3p, Vti1p, Vps43p/Vam7p, Ykt6p, Rab GTPase Ypt7p as well as a complex of "class C" vacuolar protein sorting (Vps) (Kweon *et al.*, 2003; Mayer *et al.*, 1996).

1.6 Rationale

As genome-wide screen revealed that the mutants defective in endocytic pathway show hypersensitivity to bleomycin, we decided to conduct additional characterization of one of the endosomal pathway genes, namely *NHX1*, as deletion of this gene resulted in mutant with severe sensitivity to bleomycin (unpublished data). It has been shown that *nhx1Δ* mutant is sensitive to NaCl and hygromycin B but these sensitivities were reported under conditions of a special growth media (APG) containing low salt where the investigators suggested that these phenotypes could be

correlated with a defect in the mutant ability to maintain salt and pH homeostasis (Brett *et al.*, 2005). In contrast, the sensitivity to bleomycin and gentamicin were observed with rich growth media (Aouida *et al.*, 2004b; Wagner *et al.*, 2006). These combined studies raised the possibility that *nhx1Δ* mutant phenotypes may depend upon the growth media. In this study, we examined the *nhx1Δ* mutant for sensitivity to cationic and anionic drugs that enter the cell via both receptor mediated and fluid endocytosis, as well as to various DNA damaging agents with different mode of action, when the cells were cultured only in rich growth media. We showed that *nhx1Δ* mutant is sensitive to several of these agents, but the sensitivity was not influence either by pH or NaCl. We further showed that *nhx1Δ* mutant secretes CPY, but which was also not prevented by changes in pH or NaCl concentrations. We concluded that Nhx1 plays a general role to detoxify those drugs that are channeled to the vacuole via the endosomal pathway, and that on its absence there is likely a leak from the prevacuole into the cytosol resulting in the observed toxicity.

Chapter 2 Materials and methods

2.1 Yeast strains, media and plasmid

The *S. cerevisiae* strains used in this study are listed in Table 1. Cells were grown at 30ºC in either yeast peptone dextrose (YPD) (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) or minimal synthetic (SD: 0.65% yeast nitrogen base without amino acids, 2% dextrose, 0.17% dropout mix) used for transformation (Guthrie & Fink, 1991). pRin73, a 2μ plasmid harbouring *NHX1* tagged with a C-terminal triple hemagglutinin epitope (*NHX1-HA*), under control of its endogenous promoter, was kindly provided by Dr. Rajini Rao (Johns Hopkins, USA).

Table 1: Strains

Strain	Genotype
BY4741 (parent)	*MATa his3*Δ *leu2*Δ *met15*Δ *ura3*Δ
BY4741 (*nhx1Δ*)	Isogenic to BY4741, except *nhx1::KAN*
BY4741 (*agp2Δ*)	Isogenic to BY4741, except *agp2::URA3*
BY4741 (*vma12Δ*)	Isogenic to BY4741, except *vma12::KAN*
BY4741 (*vps27Δ*)	Isogenic to BY4741, except *vps27::KAN*
BY4741 (*ypt6Δ*)	Isogenic to BY4741, except *ypt6::KAN*
BY4741 (*ypt7Δ*)	Isogenic to BY4741, except *ypt7::KAN*
BY4741 (*nhx1Δ ypt6Δ*)	Isogenic to BY4741, except *nhx1::KAN ypt6::LEU2*
W303 (parent)	*MATa ade2*Δ *leu2*Δ *trp1*Δ *ura3*Δ *his3*Δ

2.2 Plasmid amplification in *E.coli*

E.coli incubation for 30 minutes on ice was followed by a heat chock at 42ºC for a precise time (90 seconds). The sample was incubated at 37ºC for 1 h after adding 1 ml LB media (0.5% (w/v) yeast extract, 1% (w/v) tryptone, and 1% (w/v) NaCl).

Finally, cells were spun down (10,000 rpm, 2 minutes), resuspanded in 100 µl LB media and plated in LB-Agar 1.5% containing 100 µg/ml Ampicilin. The plates were incubated at 37ºC overnight.

2.3 Plasmid purification from *E.coli*

One colony grown on LB media was inoculated in 5 ml LB containing 100 µg/ml Ampicilin and incubated at 37ºC over night. The next day, the sample was spun down (12,000 rpm, 5 minutes) and 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8) were added to the supernatant. After homogenization, 200 µl of solution II (0.2 M NaOH, 1% SDS) were added and the contents thoroughly were mixed by gently inverting the tube several times. Five minutes incubation at room temperature was followed by adding 150 µl solution III (5 M potassium acetate, 11.5% glacial acetic acid). After ice incubation for 10 minutes, the sample was spun down (10,000 rpm, 5 minutes). The next step is to add 500 µl phenol and 500 µl chloroform to the supernatant. After centrifugation, the sample which consisted of the supernatant and 1 ml of 100% ethanol was kept at -20ºC for 10 minutes and then was spun down for 10 minutes at high speed (14,000 rpm). The supernatant was decanted and the pellet was rinsed by 500 µl of 70% ethanol. The ethanol was drained off and the pellet was dissolved in 50 µl TE (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8).

2.4 Transformation

Wild-type and *nhx1Δ* mutant were grown in 2 ml YPD media overnight and subcultured in fresh YPD at 30ºC for 4 h. Cells were washed twice with 5 ml of

sterilized water and spun down for 5 minutes at 1,000 rpm. The pellet was washed in 800 µl of TE/LiAc (10 mM Tris-HCl, 1 mM EDTA, 100 mM LiAc pH 7.5) and resuspended in 50 µl of TE/LiAc. The following samples (table 2) were then prepared and incubated at 30ºC for 45 minutes.

Table 2: Transformation Protocol

	BY4741			**BY4741 (*nhx1Δ*)**		
Sample	1	2	3	4	5	6
Cell	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl
Carrier DNA	5µl	5 µl	5 µl	5µl	5 µl	5 µl
Plasmid	-	5 µl	-	-	5 µl	-
Vector YCplac111	**-**	**-**	5 µl	**-**	**-**	5 µl
PEG solution*	300 µl	300 µl	300 µl	300 µl	300 µl	300 µl

* PEG solution: TE/LiAc 1 X and 40% PEG (polyethylene glycol).

Cells were then incubated at 42ºC for 15 minutes. The pellet was collected by centrifugation for 2 minutes at 10,000 rpm and resuspended in 100 µl of sterilized water and plated on selective media plates. The plates were incubated at 30ºC for 2 days. The single colonies from plates of samples 2, 3, 5 and 6 were purified by patching on the new selective media.

2.5 Spot tests

Cells were grown overnight at 30ºC in 2 ml YPD media and were subcultured in 3 ml fresh YPD media for 4 h. Exponentially growing cells were diluted to an A_{600} of 0.6. Cell suspensions were then serially diluted as indicated and 5 µl were spotted onto YPD agar containing described amount of the drugs. Plates were photographed after 48 h of incubation at 30°C.

2.6 Spermidine uptake analysis

Prior to the polyamine uptake assay, cells were grown to the mid logarithmic phase, washed three times with uptake buffer A (50 mM sodium citrate, pH 5.5, 2% D-glucose), and resuspended in 100 µl of the same buffer at 2 X 10^7 cells/ml. The uptake assay was initiated by the addition of 2.5 µM [^{14}C] spermidine followed by incubation at 30ºC with shaking. The reaction was stopped at predetermined intervals by adding 1 ml of ice-cold uptake buffer. Cells were washed three times with uptake buffer and resuspended in 100 µl of the this buffer. Five ml of scintillation mixture (Amersham Bioscience) were added to each sample, and the retained radioactivity was determined by liquid scintillation spectrometry.

2.7 Western blot

Immunoblot analysis was performed as previously described (Vongsamphanh *et al.*, 2001). Briefly, yeast cells were grown overnight in selective media, washed twice with sterilized water and centrifuged at 4ºC at 4000rpm. The pellet was resuspended in yeast extraction buffer (50 mM Tris pH 7.0, 30 mM KCl, 10% glycerol, 1 µg/ml leupeptin, 1µg/ml aprotinin, 1 mM PMSF and 1 mM DTT) followed by homogenizing

via a MINI bead beater for 20 seconds at 5000rpm. Cells were beat for 6 rounds with 1 minute intervals on ice. Protein was collected after removal of cellular debris via centrifugation at 9000 g for 10 minutes. A Bradford assay was used for protein quantification. 150 μg of whole cell lysate per lane was loaded onto 10% SDS-PAGE gel. The gel was run for approximately 1 h at 120 V/ 20 mA until the dye reached close to the end of the gel. The running buffer condition was as follows: 25 mM Tris pH 7.0, 192 mM Glycine and 0.1% SDS. The proteins were then transferred to a nitrocellulose membrane at 100V/250 mA for 1 h. Transfer buffer condition: 25 mM Tris pH 7.0, 192 mM Glycine, 0.05% SDS and 20% methanol. The blot was blocked with a blocking buffer (10 mM Tris, 0.15 M NaCl, 1.26 mM EDTA, 0.001% Tween 20 and 5% nonfat dried milk). The blot was then incubated with anti-HA monoclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:5000, and the secondary antibodies were anti-mouse (Amersham Bioscience). Protein bands were revealed by using PerkinElmer Chemiluminescne Reagent Plus followed by exposure to Kodak double emulsion film.

2.8 Coomassie staining

Samples were resolved by SDS-PAGE gels. SDS–polyacrylamide gels were stained with coomassie for 30 min in 4.6% methanol (v/v), 9.2% acetic acid, and 0.25% (w/v) coomassie Brilliant Blue R-25 and then destained in 7.5% acetic acid and 12% methanol.

2.9 CPY plate assay

Exponentially growing cells OD_{600} ~0.6 were serially diluted. Five µl aliquots were spotted on a nitrocellulose membrane (Amersham Biosciences) which was placed on the surface of YPD or SC plates and incubated at 30ºC for 12-18 h. The membrane was lifted and washed with deionized and distilled water to remove all cells. Proteins absorbed on the membrane were detected by immunoblotting using monoclonal CPY antibody (molecular Probes; 1:2500 dilution). The data represent results from three independent experiments.

2.10 Quinacrine vacuole staining

Vacuolar accumulation of quinacrine was examined as described previously (Roberts *et al.*, 1991). Briefly, 3 X 10^7 log-phase yeast cells were harvested and resuspended in 500 µl of YPD buffered with 50 mM Na_2HPO_4, pH 7.6, and containing 200 mM quinacrine. After incubation at the room temperature for 5 min, cells were sedimented at 10,000 g for 5 s, washed once with 500 µl of 2% glucose buffered with 50 mM Na_2HPO_4, pH 7.6, and resuspended in 100 µl of the same solution. Samples were applied to a microscope slide and photographed by a fluorescent microscope.

2.11 UVC treatment

Overnight cultures were sub-cultured into 4 ml YPD and cells at $O.D_{600}$ ~1.0 were harvested and resuspended in 4 ml of 20 mM PBS buffer. The cells were diluted 10^{-4} and 100 µl plated on YPD agar. Plates were irradiated with 254nm UVC (0-90 J/cm^2) and scored for survival after 2 days of growth at 30°C.

2.12 RT-PCR

2.12.1 RNA extraction

Total RNA was prepared using the RiboPure-Yeast extraction kit (Ambion). Briefly, 3 ml overnight cultures were spun down and the pellet was resuspended in lysis reagents consisting of lysis buffer, 10% SDS and phenol:Chloroform:IAA. The mixture of the cells were transferred to the tubes containing 750 µl cold Zirconia Beads. Cells were beat on the vortex adapter for 10 minutes at maximum speed. After centrifugation at 16,000 X g at room temperature, the aqueous phase of each sample was transferred to the new tube. 1.9 ml binding buffer and 1.25 ml of 100% ethanol were then added to each sample. The samples were drawn through a filter cartridge. The filters were washed with wash solutions 1 and 2/3 and RNA was eluted by applying RNA elution buffer. Isolated RNA contains chromosomal DNA. To remove it, DNase digestion reaction at room temperature was assembled: 50-100 µl RNA sample, 1/10th volume of 10X DNase 1 buffer and 4 µl DNase 1 (8U). The sample was incubated 30 minutes at 37°C. Total RNA was quantified by absorption at 260 nm.

2.12.2 cDNA Synthesis

To synthesize cDNA, the following components were added to a nuclease-free tube: 5 µg RNA, 1 µl of 10 mM dNTP, 250 ng random primers (Invitrogen) and sterile water to total volume of 12 µl. The mixture was heated to 65°C for 5 minutes. Then, 4 µl of 5 X first-strand buffer, 2 µl of 0.1 M DTT and 1 µl RNaseOUT^{TM} were added and the sample was incubated at 37°C for 2 minutes. One µl of M-MLV RT (Reverse transcriptase) (200 units) was gently mixed with the sample and the following

incubations were done: 10 minutes at 25°C, 50 minutes at 37°C and 15 minutes at 70°C. The cDNA can now be used as template for amplification in PCR.

2.12.3 PCR Amplification

To detect *NHX1* and *ACT1* expression the following PCR primers were chosen (Table 3).

Table 3: PCR Primers

Name	Sequence
RT-PCR-NHX1-F1	(5'-GTTATTGGCTTGATAATAAGGATGTCCCCCGGGCA-3')
RT-PCR-NHX1-R1	(5'-CAATTTGAGGATAGCGCCTTATGTGAGTGTGTTTCA ACAG-3')
RT-PCR-ACT1-F1	(5'- GTTTTGCCGGTGACGACGCTCCTCGTGCTG-3')
RT-PCR-ACT1-R1	(5'-CGGCTTGGATGGAAACGTAGAAGGCTGGAACG-3')

PCR machine: PTC-100^{TM} Programmable Thermal Controller MJ research INC.

Master Mixture: 2 µl of cDNA, 2.5 µl of 10 X taq buffer, 1 µl of upstream primer (1µg/µl), 1 µl of downstream primer (1µg/µl), 1 µl of 10 mM dNTP, 1.5 µl of 25 mM MgCl2, 0.5 µl of Taq DNA polymerase (5U/µl) and add sterilized water up to 25µl.

PCR designed program:

1- 95ºC for 2 minutes, 2- 94ºC for 1 minute, 3- 55ºC for 1 minute, 4- 72ºC for 4 minutes, 5- 22 times repeat of step 2-4, 6- 72ºC for 7 minutes, and 7- 4ºC.

Chapter 3 Results

3.1 Deletion of the *NHX1* gene confers hypersensitivity to bleomycin-A5.

Genome-wide screen revealed that *nhx1*Δ mutant is sensitive to bleomycin-A5 (Aouida *et al.*, 2004b). We confirmed the sensitivity of *nhx1*Δ mutant to this drug by using an independent approach. Spot test analysis revealed that the mutant was extremely sensitive to bleomycin-A5, when compared to the parent or *agp2*Δ mutant (Fig. 7). Since Agp2 has been demonstrated as bleomycin-A5 transporter, the cells show resistance to the drug when *AGP2* gene is deleted (Aouida *et al.*, 2004b). Therefore, in this experiment we used *agp2*Δ mutant as control for the spot test analysis.

Furthermore, deletion of the *NHX1* gene in another wild-type strain W303 also resulted in *nhx1Δ* mutant displaying hypersensitivity to bleomycin-A5 (data not shown). The *nhx1/NHX1* diploid retained parental resistance to bleomycin-A5, suggesting that the *nhx1* allele is recessive.

We next examined whether enhanced expression of *NHX1* would restore bleomycin-A5 resistance to the *nhx1Δ* mutant. A multicopy plasmid (pNHX1-HA) was used to overproduce Nhx1p as a HA fusion protein (Nhx1-HA) under the control of its own promoter. Since latter plasmid has been tagged with the triple HA, Nhx1-HA_3 is the fusion protein used in our study but for simplicity it has been called Nhx1-HA. The expression of the *NHX1* gene in the following six strains (i) the parent, (ii) the parent carrying the empty vector, (iii) the parent carrying the plasmid pNHX1-HA, (iv) the *nhx1Δ* mutant, (v) the *nhx1Δ* mutant carrying the empty vector, and (vi) the *nhx1Δ* mutant carrying the plasmid pNHX1-HA were assessed by performing RT-PCR. The

results indicated that *NHX1* gene can be overexpressed in parent and mutant strains carrying the plasmid pNHX1-HA (Fig. 8A). Western blot analysis revealed that a total cell extract prepared from the *nhx1Δ* mutant strain carrying the plasmid pNHX1-HA expressed the expected 73.5 kDa fusion protein (Fig. 8B). The overexpressed Nhx1-HA conferred nearly full resistance to the *nhx1Δ* mutant towards bleomycin-A5 at the lower drug concentration, while at a 2-fold higher concentration (0.5 μg/ml) only partial resistance was observed (Fig. 7). These findings demonstrated that the *NHX1* gene product is required to protect cells against the toxicity caused by bleomycin-A5. We noted that the overexpression of Nhx1-HA showed no additional bleomycin-A5 resistance to the parent strain (Fig. 7), suggesting that Nhx1 function is not limiting in the cells.

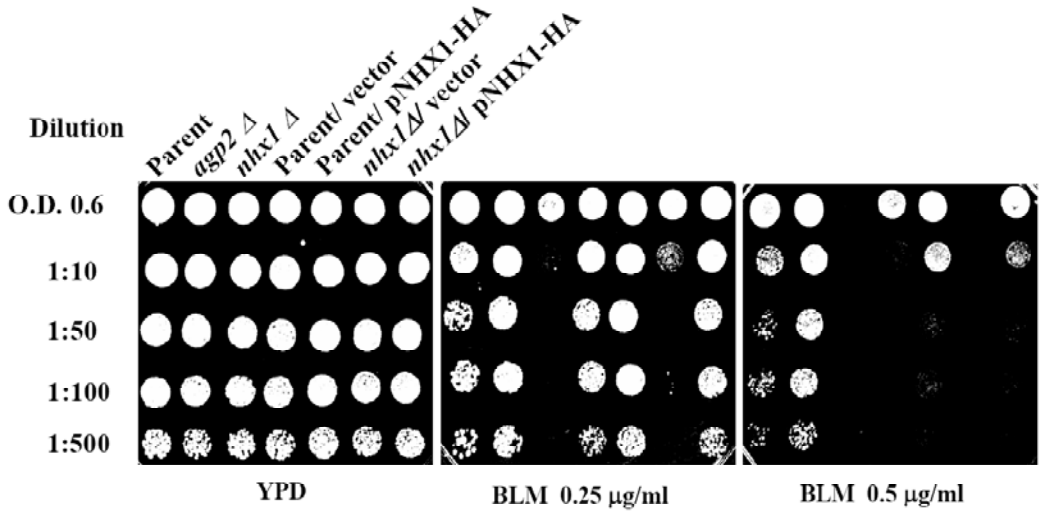


Figure 7: Effect of the *NHX1* gene deletion and Nhx1-HA expression on sensitivity of the indicated strains towards bleomycin.

Exponentially growing cells were diluted to an A_{600} of 0.6. Cell suspensions were then serially diluted as indicated and 5 µl were spotted onto YPD solid media without and with indicated concentration of BLM (0.25 µg/ml and 0.5 µg/ml). Plates were photographed after 48 h of incubation at 30°C. *agp2Δ* mutant displayed resistance to the drug as previously described. The data is representative of two independent analyses.

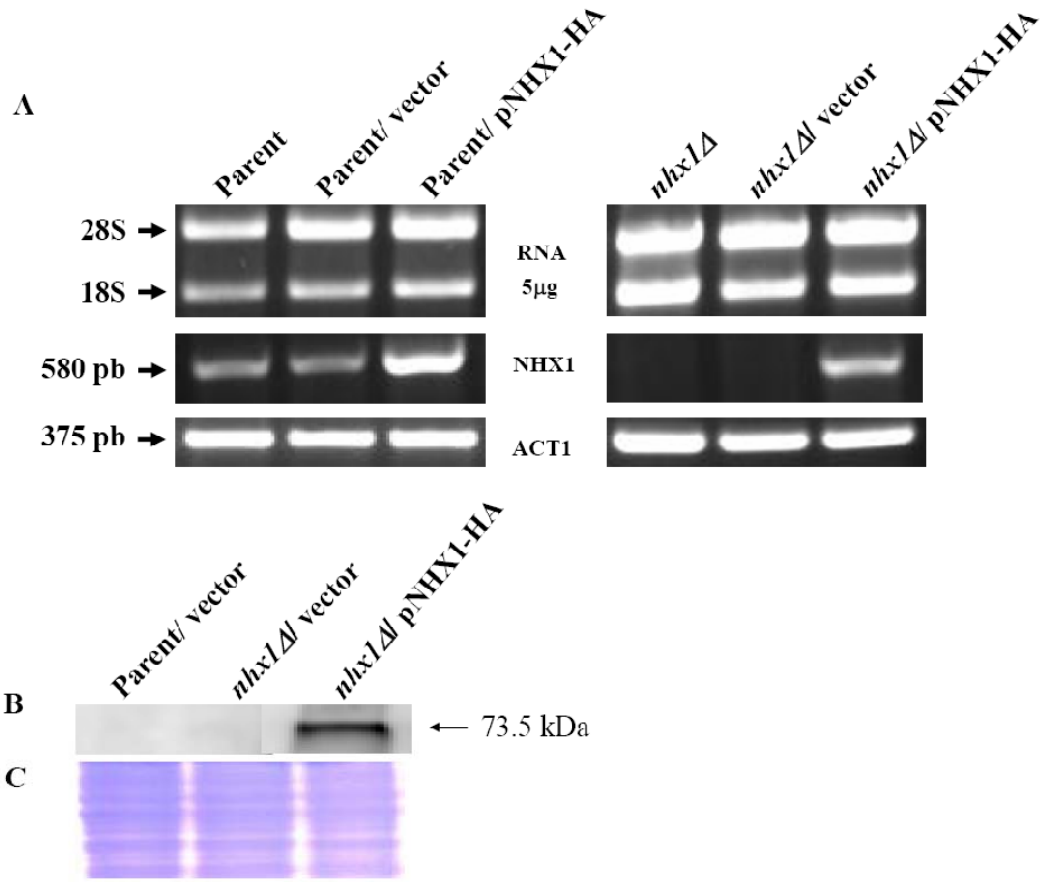


Figure 8: Expression of *NHX1* gene and Nhx1-HA fusion protein in the parent and *nhx1Δ* mutant.

A, RT-PCR analysis to detect *NHX1* and *ACT1* expression in the indicated strains. **B**, Expression of Nhx1-HA fusion by Western blot analysis. Total extracts (150 µg) were subjected to Western blot analysis and probed with anti-HA monoclonal antibodies. **C**, Coomassie staining to monitor protein loading.

3.2 *nhx1Δ* mutant is hypersensitive to polyamines

Since bleomycin possesses a chemical composition similar to polyamine structure (Aouida *et al.*, 2004a), it is likely that bleomycin could act as anticancer polyamine analogue. We speculated that *nhx1Δ* mutant sensitivity to bleomycin might be a result of this specific structure. Therefore, we tested if the *nhx1Δ* mutant would display cross-sensitivity to polyamines. Although polyamines such as spermine (SPM) and spermidine (SPD) play multiple functions including the regulation of cell proliferation and cell differentiation (Heby, 1981; Stabellini *et al.*, 1995; Stabellini *et al.*, 1997), an excess of polyamines is toxic for the cells.

Spot test analysis revealed that the *nhx1Δ* mutant was hypersensitive to both SPM and SPD. These phenotypes were rescued by the introduction of the plasmid pNHX1 (Fig. 9). Thus, it appears that Nhx1 function is also required to prevent toxicities caused by polyamines. Polyamine sensitivity can be explained by an increase accumulation in some mutants such as *end3Δ* mutant. While End3 is required for initial phase of endocytosis, internalization of plasma membrane proteins, mutant lacking End3 showed increased uptake of polyamine (Aouida *et al.*, 2005). Our group demonstrated that this phenotype is due to enhanced abundance of Agp2, transporter of polyamine, at the plasma membrane of *end3Δ* mutant (Aouida *et al.*, 2005).

Polyamine hypersensitivity could be result of an increase accumulation in *nhx1Δ* mutant. Alternatively, *NHX1* deletion could result in polyamine accumulation in the cells due to polyamine transporter activation as shown in *end3Δ* mutant. To detect the intracellular amount of radiolabeled polyamine, we monitored the uptake of ^{14}C-labeled spermidine in the *nhx1Δ* mutant. In comparison to the parent, the *nhx1Δ* mutant

showed only a modest increase in the accumulation of the labelled SPD (Fig. 10). These data exclude the possibility that the sensitivity of the *nhx1Δ* mutant is due to enhanced polyamine uptake.

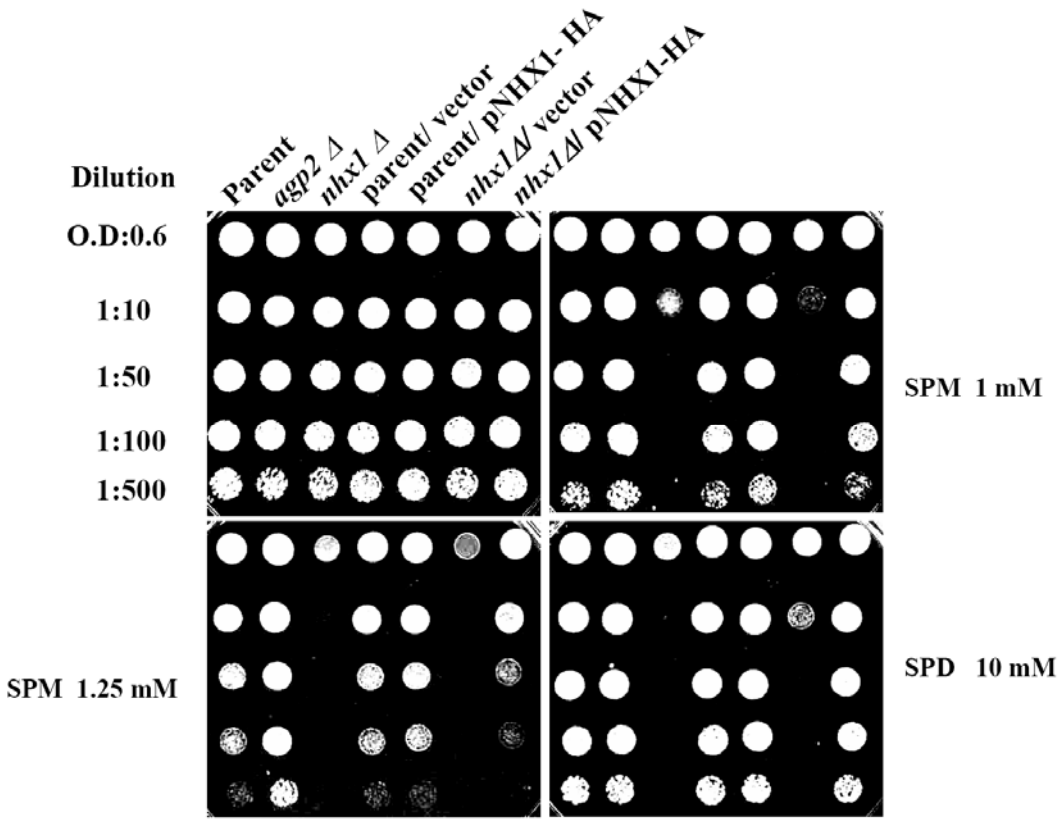


Figure 9: *nhx1Δ* mutant displays sensitivity towards polyamines
Cells were spotted on YPD plates without and with the indicated concentration of SPM and SPD and photographed as in Fig. 7 after 48 h of incubation at 30ºC. The data is representative of three independent analyses.

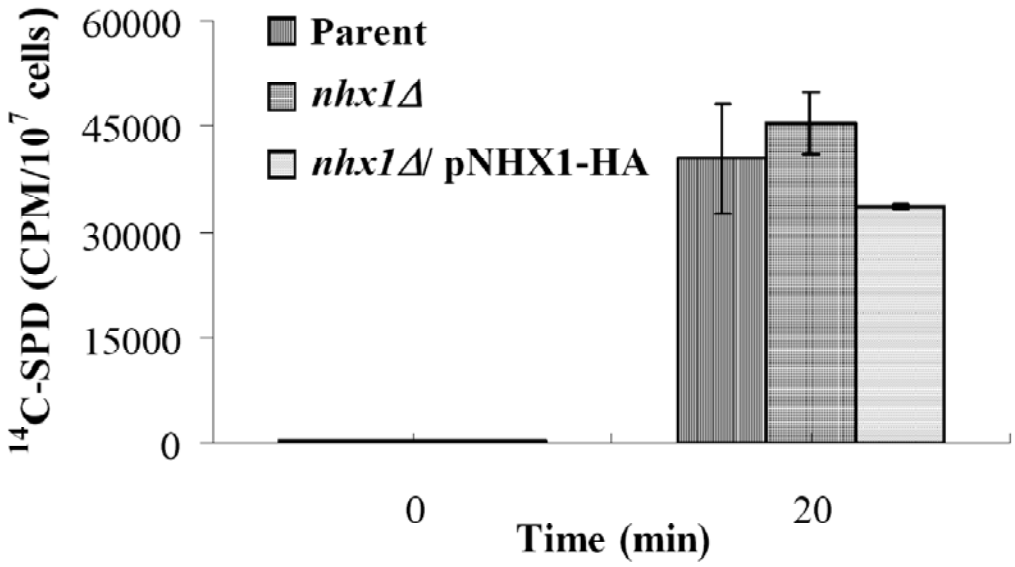


Figure 10. [^{14}C] spermidine uptake in *nhx1Δ* mutant.
Exponentially growing cells were incubated with [^{14}C] spermidine and processed for the uptake as described in "Materials and methods". The data represent results from three independent experiments.

3.3 Hypersensitivity of *nhx1Δ* mutant is not limited to bleomycin-A5 or polyamines.

Previous studies documented that *nhx1Δ* mutant show sensitivity towards hygromycin B (Gaxiola *et al.*, 1999). Hygromycin B is a cationic amino glycoside (Perlin *et al.*, 1988) and it has been documented that mutants defective in the "class E" of vacuolar protein sorting (Vps) show common phenotype of sensitivity towards hygromycin B (Mukherjee *et al.*, 2006). It is believed that the *nhx1Δ* mutant hypersensitivity to hygromycin B results from the defect in sequestration of this cation into intracellular compartments (Cagnac *et al.*, 2007; Kinclova-Zimmermannova *et al.*, 2006).

Since bleomycin-A5 and polyamines are cations as well, we therefore tested if the mutant sensitivity was related to the charge by examining a panel of drugs that are both cationic and anionic. We indeed reproduced *nhx1Δ* mutant sensitivity to hygromycin B in YPD media (Fig. 11). Interestingly, the mutant was hypersensitive to the anionic drug deoxycholine, as well as to calcofluor white, a related anionic agent which interferes with the construction and stress response of the cell wall (Fig. 11) (Ram & Klis, 2006). These data suggest that the Nhx1 function is required to prevent toxicity caused by a range of charged compounds and not limited to either cationic or anionic agents.

The *nhx1Δ* mutant was also hypersensitive to two other species of bleomycin, bleomycin-A2 and bleomycin-B2, which lack the polyamine moiety (Fig. 11). Thus, it is unlikely that the initial sensitivity observed for *nhx1Δ* mutant towards bleomycin is related to the polyamine moiety.

Taken together, these data suggest that *nhx1Δ* mutant shows sensitivity to the drugs which are detoxified via endocytic pathway. At this point, the structure or charges of the drugs do not play a crucial role.

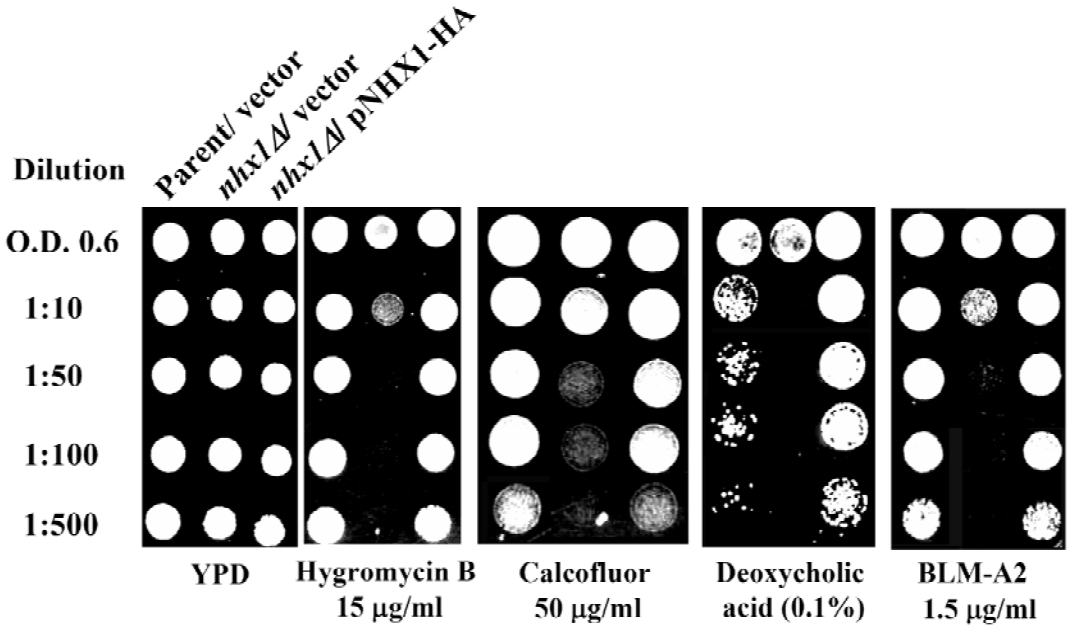


Figure 11. ***nhx1Δ*** **mutant displays wide sensitivity towards various drugs.**
Cells were spotted on YPD plates without and with the indicated concentration of hygromycin B, calcofluor white, deoxycholic acid or BLM-A2 and photographed as in Fig. 7 after 48 h of incubation at 30ºC. The data is representative of three independent analyses.

3.4 *nhx1Δ* mutant exhibits no sensitivity to UV irradiation.

Since bleomycin is known to damage the DNA and to be detoxified via the endocytic machinery (Aouida *et al.*, 2004b), we checked if the *nhx1Δ* mutant would be sensitive to the other DNA damaging agents which are not detoxified through the endosomal pathway. Alternatively, we were interested to detect whether the increased sensitivity of *nhx1Δ* mutant to the DNA damaging agents could be partially due to a defect in mechanism(s) apart from those involved in vacuolar sequestration of the cytotoxic agents. In comparison to the parent, the *nhx1Δ* mutant showed no additional sensitivity to 254-nm ultraviolet light, which creates pyrimidine dimers (Fig. 12) (Gale *et al.*, 1987). Thus, it would appear that the drugs which are known to be detoxified via the endosomal pathway can cause sensitivity to the *nhx1Δ* mutant, consistent with the identification of Nhx1 as a prevacuolar protein involved in endocytic pathway (Nass & Rao, 1998). It has been documented that the mutants defective in V-ATPase activity of the vacuole (*vma6Δ*, *vma8Δ*) show sensitivity to UV irradiation (Liao *et al.*, 2007). Although V-ATPase activity of the vacuole is required to protect the cells against UV irradiation, our data showed that Nhx1 is not involved in limiting the effect of UV irradiation. We concluded that the *nhx1Δ* mutant exhibits sensitivity to DNA damaging agents which are detoxified only via endocytic pathway.

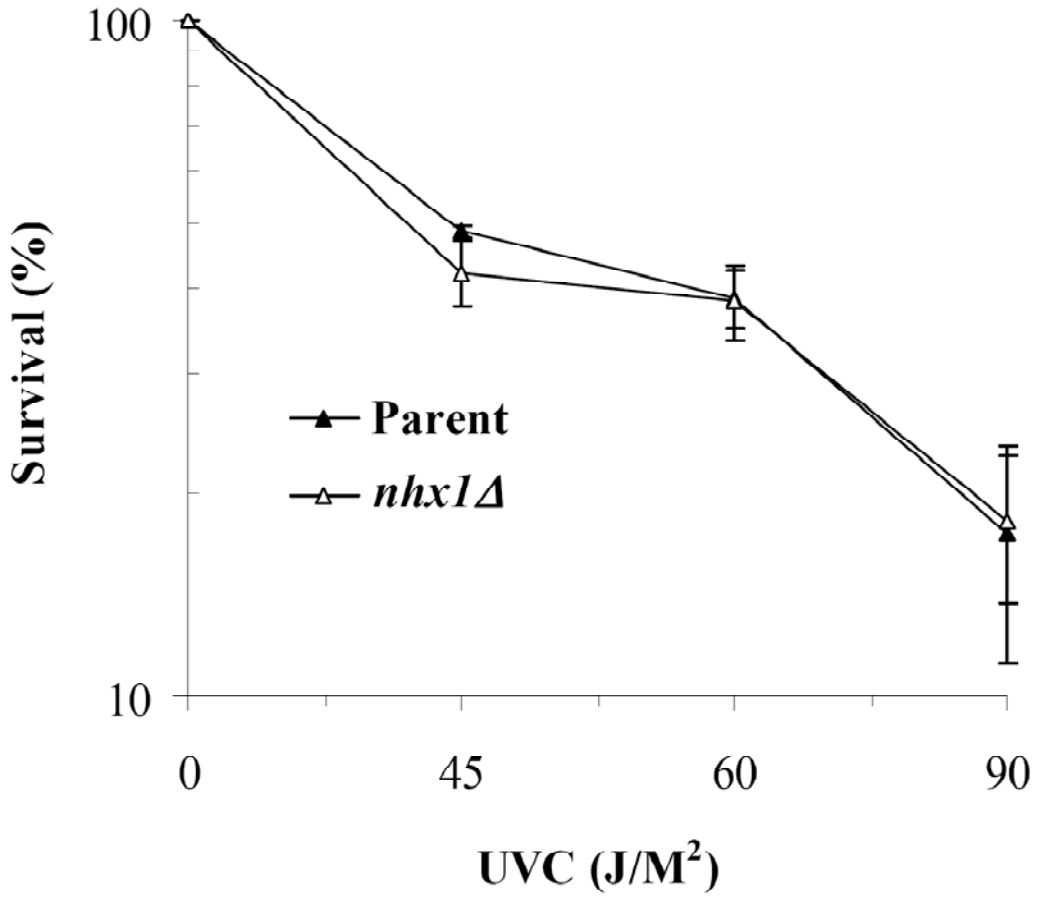


Figure 12. *nhx1Δ* mutant exhibits parental sensitivity towards UVC.
Cells were treated as described in "Materials and methods". The data are the average of three independent experiments.

3.5 *nhx1Δ* mutant is not sensitive to pH and NaCl when grown in YPD media

Mutants defective in vacuolar membrane H^+-ATPase activity such as *vma12Δ* are unable to grow on solid YPD media with increasing pH (Hirata *et al.*, 1993). Since it has been reported that *nhx1Δ* mutant has a defect in maintaining the intracellular pH in the vacuole and cytoplasm under the special growth media APG, which has a low osmolarity and acidic pH (Brett *et al.*, 2005), we therefore tested if the mutant can grow on YPD media with varying pH. Like the parent, the *nhx1Δ* mutant was able to grow and tolerate a wide range of pH (pH 4 to 8) (Fig. 13). In contrast, the vacuolar defective mutant *vma12Δ* was unable to grow efficiently at the higher pH (Fig. 13) (Hirata *et al.*, 1993). Thus, under this growth condition Nhx1 does not appear to play a key role in maintaining the vacuolar pH, although the previous report showing *nhx1Δ* mutant sensitivity to pH may be related to the specific growth media, APG (Brett *et al.*, 2005).

nhx1Δ mutant was reported to be sensitive to NaCl when grown on APG media (Brett *et al.*, 2005; Wells & Rao, 2001). However, we found no sensitivity of the *nhx1Δ* mutant to NaCl when grown on YPD (Fig. 14). In fact, the *nhx1Δ* mutant exhibited sensitivity to $CaCl_2$, but not to LiCl or NaCl, when grown on solid YPD media (Fig. 14).

We did not perform any analysis for drug sensitivity with the APG media, as the mutant exhibited sensitivity to hygromycin B whether grown in APG or YPD media. Thus, the reported NaCl sensitivity of the *nhx1Δ* mutant is specifically related to the APG media.

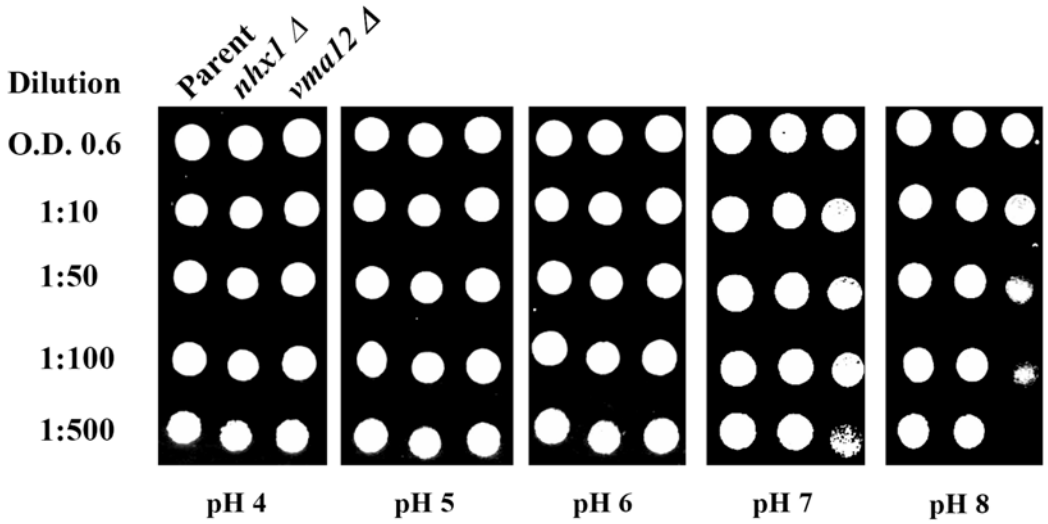


Figure 13. Effect of pH on *nhx1Δ* mutant sensitivity
Exponentially growing cultures of the strains were serially diluted and 5 µl spotted onto YPD solid media adjusted to the indicated pH. Plates were photographed after 48 h incubation at 30°C. The data is representative of two independent analyses.

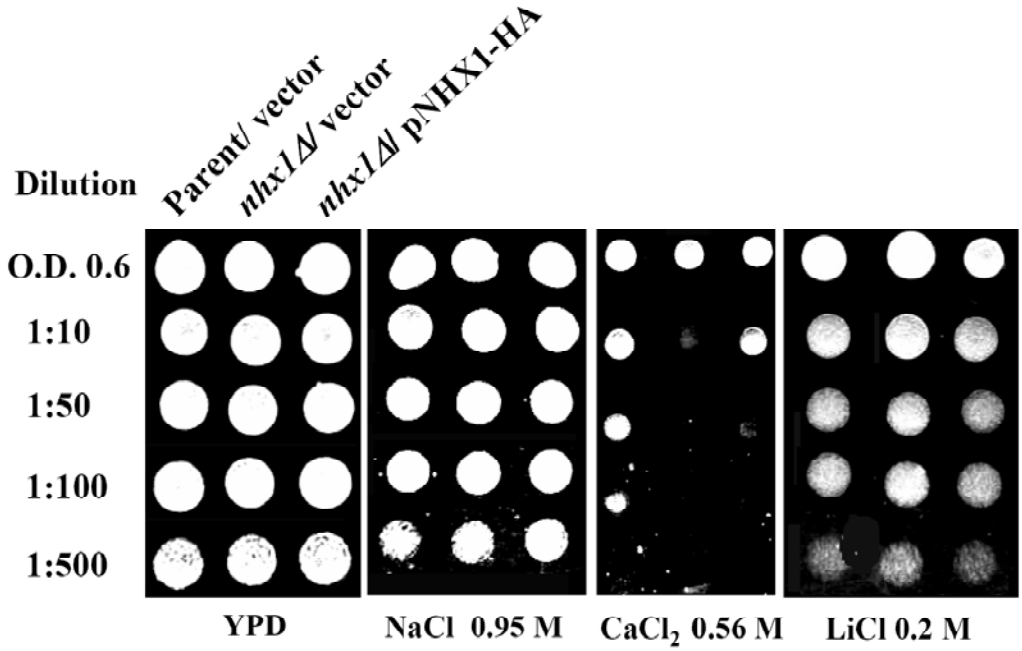


Figure 14. *nhx1Δ* mutant displays sensitivity towards $CaCl_2$ but not to NaCl. NaCl, $CaCl_2$ and LiCl sensitivity of wild-type or mutant strains were assayed as described for Fig. 7. The data is representative of two independent analyses.

3.6 Deletion of *NHX1* gene does not affect vacuolar acidification.

To further examine if the *nhx1Δ* mutant has a defect in vacuolar acidification, we tested if the vital dye quinacrine can accumulate in the vacuole. Quinacrine is a basic dye that accumulates in acidic compartments in living yeast cells and is used to rapidly monitor vacuolar acidification (Weisman *et al.*, 1987). No defect was observed in the accumulation of quinacrine into the vacuoles of the *nhx1Δ* mutant, as compared to the parent (Fig. 15). These data confirm that the vacuole was acidified and functional. Consistent with this observation it has been shown that blocking endocytosis in *end4Δ* mutant does not affect vacuolar acidification (Plant *et al.*, 1999). Thus, it would appear that the sensitivity of the *nhx1Δ* mutant to the drugs is likely unrelated to the maintenance of vacuolar pH.

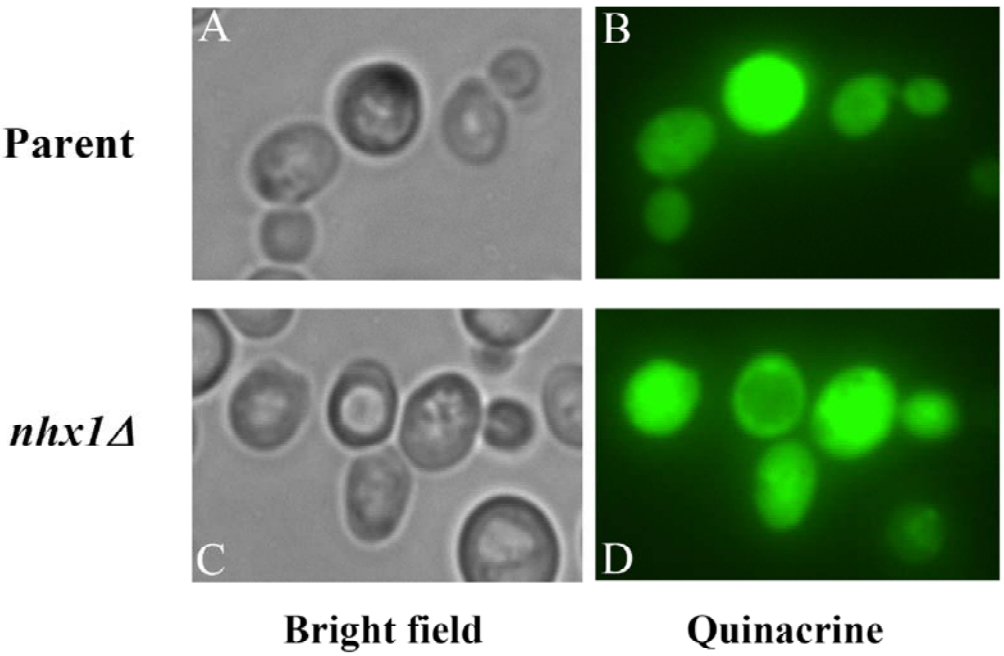


Figure 15. Positive staining of the vital dye quinacrine in the *nhx1Δ* mutant. Vacuolar acidification in living cells; parent strain (A and B) and *nhx1Δ* mutant (C and D) were assessed by visualizing quinacrine accumulation in this organelle as described in "Materials and methods". Figure showed both the bright field (A and C) and fluorescent (B and D) images. The data is representative of three independent analyses.

3.7 NaCl does not prevent the toxic effect of bleomycin in *nhx1Δ* mutant.

Since Nhx1 is a Na^+ antiporter involved in sequestration of this ion (Nass *et al.*, 1997; Nass & Rao, 1998), we tested if *nhx1*Δ mutant sensitivity to bleomycin could be suppressed by adding NaCl to the media. Spot test analysis revealed that addition of NaCl does not rescue *nhx1Δ* mutant sensitivity to bleomycin (Fig. 16). *rad52Δ* mutant which is defective in repair of double-stranded breaks (DSBs) (Symington, 2002) was used as control for two reasons: (i) It is sensitive to bleomycin (Keszenman *et al.*, 1992), and (ii) It is not affected by varying in concentration of NaCl. It is noteworthy that bleomycin loses its genotoxic effect in higher concentration of NaCl (150 mM) (Fig. 16). The same results were obtained when NaCl was added to Hygromycin B (data not shown). These data suggest that drug-phenotype displayed by the *nhx1*Δ mutant is not due to Na^+ deficiency.

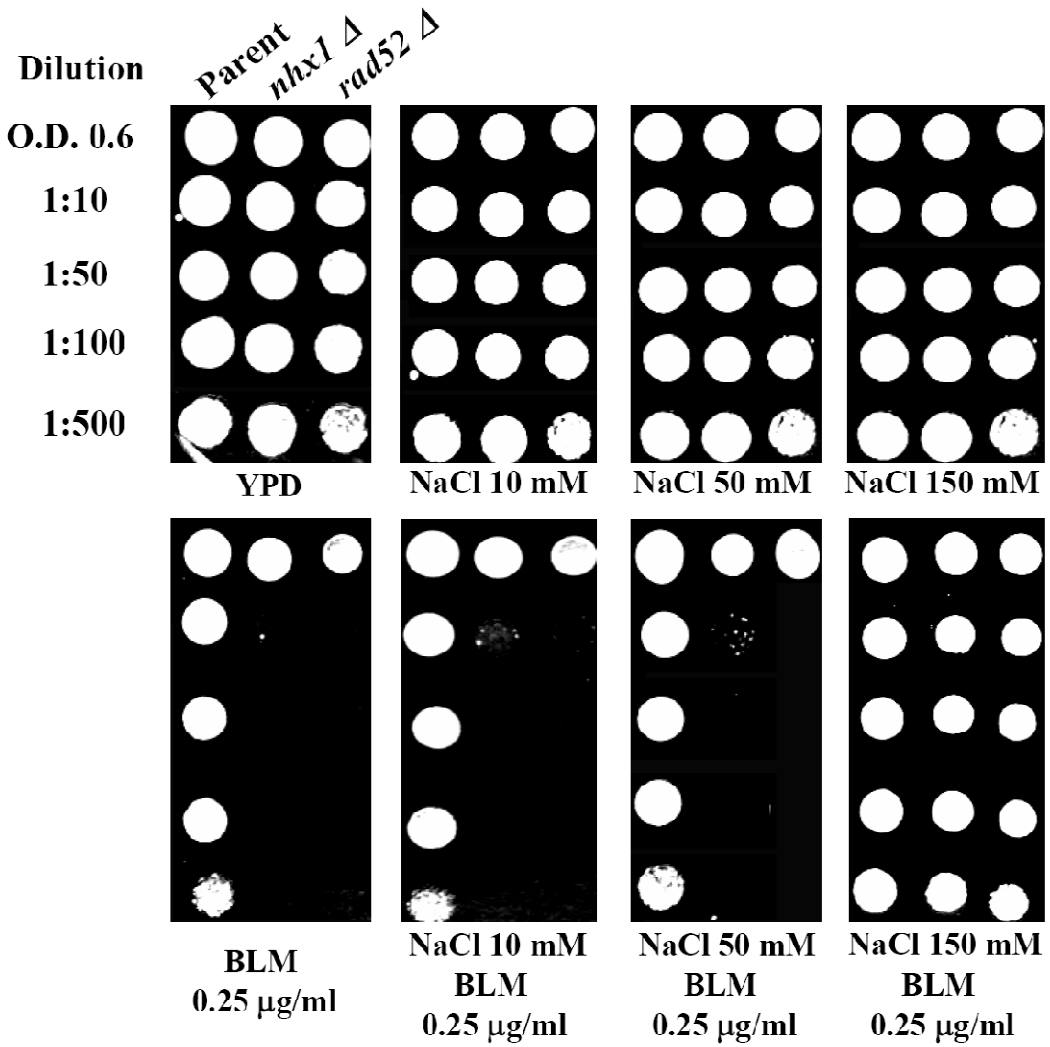


Figure 16: Bleomycin sensitivity of *nhx1*Δ mutant is not inhibited by NaCl.
Exponentially cultures were serially diluted and spotted in absence or presence of indicated concentration of NaCl and BLM. *rad52Δ* mutant was used as control.

3.8 CPY secretion by the *nhx1Δ* mutant is not blocked by varying the pH or NaCl concentrations.

nhx1Δ mutant has been documented to secrete carboxypeptidase Y (CPY) (Ali *et al.*, 2004). If *nhx1Δ* mutant phenotypes such as CPY secretion are associated with a defect in maintaining the pH of the prevacuolar compartment, then culturing cells in low pH is likely to block CPY secretion. In this experiment, the cells were grown in a range of pH under minimal media and tested for CPY secretion using nitrocellulose lift that was probed with anti-CPY antibodies. Interestingly, the low pH did not prevent CPY secretion by the *nhx1Δ* mutant, while introduction of the pNHX1 plasmid into the mutant inhibited the secretion (Fig. 17A).

We also checked if the CPY secretion could be blocked by increasing NaCl concentration. However, we found that increasing NaCl concentrations also did not suppress CPY secretion (Fig. 17B). These data raise the possibility that Nhx1 may execute an additional function that is crucial to prevent CPY secretion.

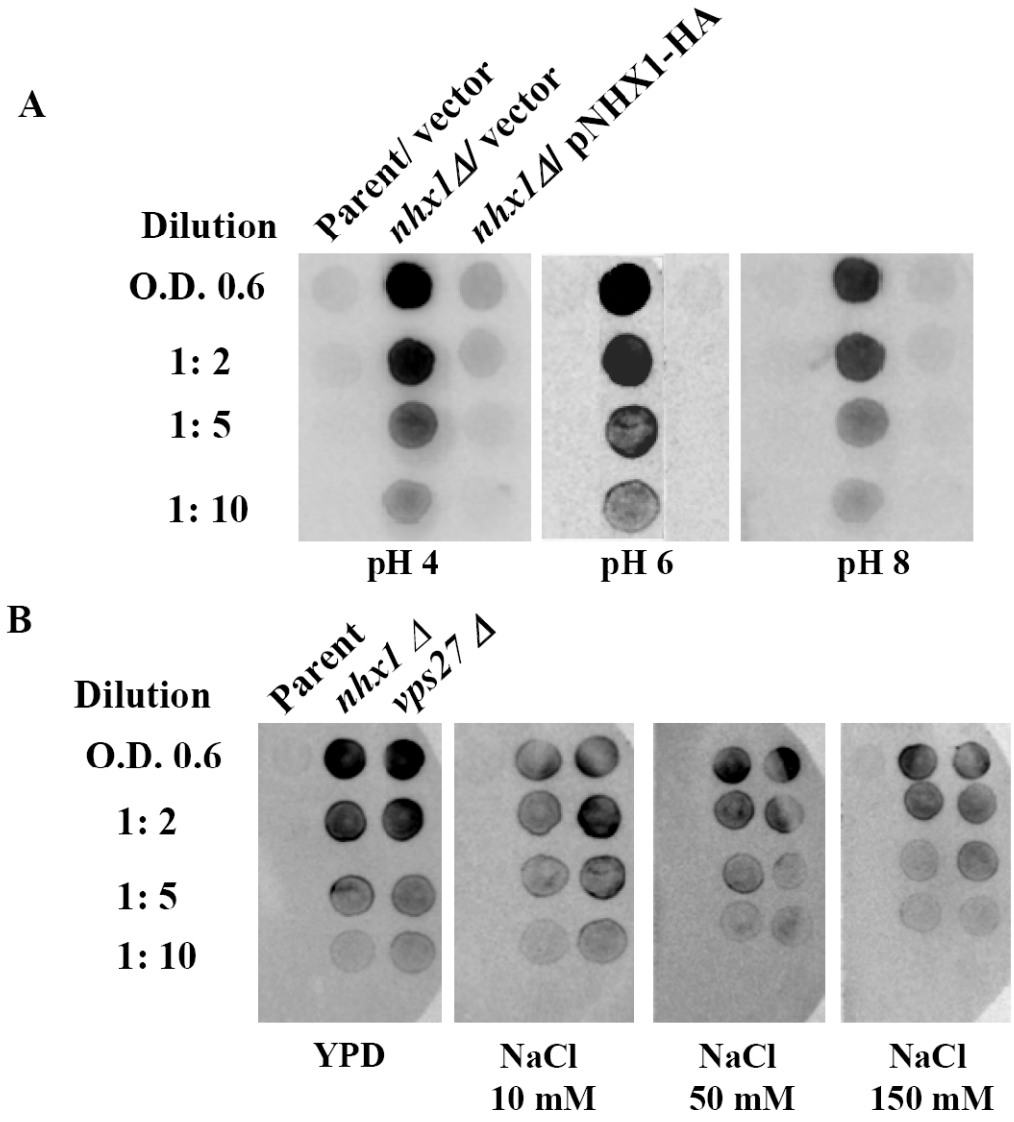


Figure 17: CPY secretion by the *nhx1Δ* mutant is not inhibited by varying either the pH or NaCl concentrations.
Exponentially growing cells OD600 ~0.6 were serially diluted, spotted onto nitrocellulose membrane followed by incubation of the membrane on top of: **A**, SD media with varying pH, **B**, YPD media with increasing NaCl concentrations. After incubating the plates at 30°C for 12-18 h, the membranes were subjected to immunoblotting with mouse anti-CPY. The data is representative of three independent analyses. CPY secretion is blocked by introduction of the pNHX1 plasmid into the mutant. *vps27Δ* mutant was used as control.

3.9 The Nhx1 variant E355A, but not E371A, is unable to rescue the drug phenotype of *nhx1Δ* mutant.

To examine if Nhx1 plays a functional or structural role in protecting cells against drug toxicity, we introduced into the *nhx1Δ* mutant a plasmid expressing either Glu355Ala (E355A) or Glu371Ala (E371A) variant of Nhx1. Both variants are located on an extracellular loop (H10) that is flanked by two transmembrane helices predicted to be inserted into the membrane (Fig. 18) (Mukherjee *et al.*, 2006). Remarkably, the mutants were produced and the expression levels of mutant protein were similar to the parent strain. Moreover, it has been demonstrated that mutant proteins are localized to the late endosome (Mukherjee *et al.*, 2006). While Glu355, but not Glu371, was shown to be essential for Nhx1 function in tolerating growth in APG media at low pH (Mukherjee *et al.*, 2006), spot test analysis revealed that neither E355A variant nor E371A shows sensitivity to varying pH when grown in YPD media (Fig. 19). Furthermore, as shown in Fig. 20, the E355A variant did not rescue the *nhx1Δ* sensitivity to either bleomycin or hygromycin B. In contrast, the E371A variant complemented these defects to the same extent as the native Nhx1. Taken together, these data confirmed that the sensitivity of *nhx1Δ* mutant or its variants E355A to bleomycin or hygromycin B is not due to defect in maintaining intracellular pH under the growth on YPD media. Since replacement of the Glu355 residue with neutral alanine residue in Nhx1 resulted in sensitivity to bleomycin or hygromycin B as almost the same extent as *nhx1Δ* mutant, it is possible that function rather than structure of the exchanger would be critical for drug tolerance. Protein crystallography would figure out structural change in E355A and E371A. It is noteworthy that Glu355 residue is

invariant among all NHEs (Na^+/H^+ exchanger) sequences (Mukherjee *et al.*, 2006). Therefore, we speculated that E355A substitution in the other NHEs would result in sensitivity to bleomycin.

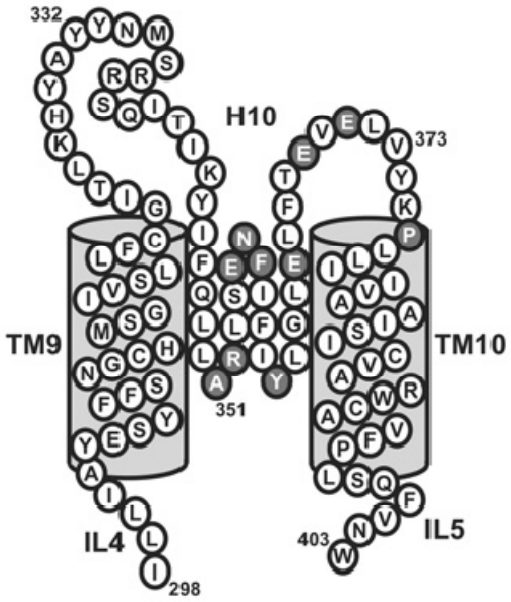


Figure 18: Predicted topology of Nhx1
Glu355 and Glu371 in the intramembranous H10 loop of Nhx1 between transmembrane segments 9 and 10 are replaced with Ala (Mukherjee *et al.*, 2006).

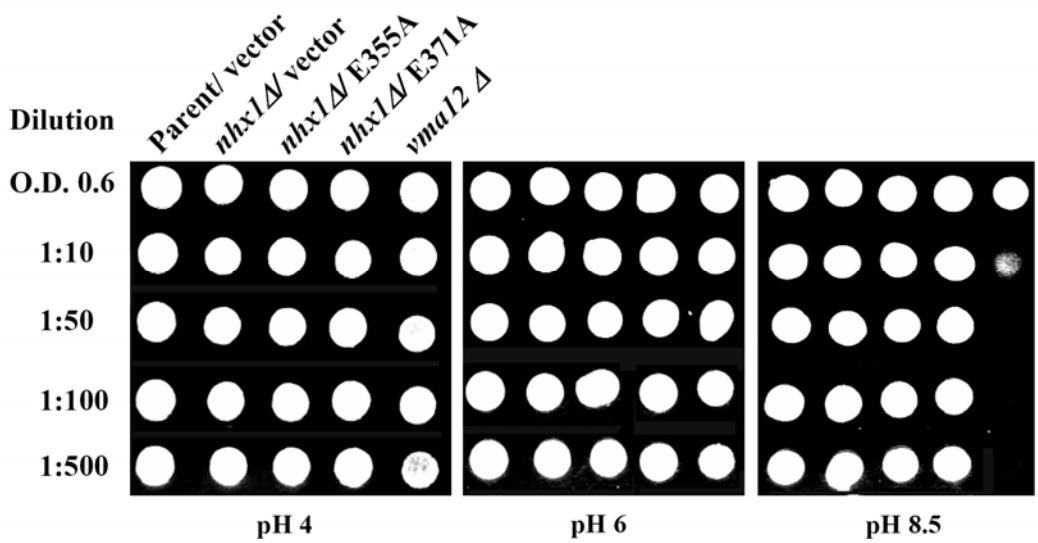


Figure 19: Replacement of the Glu355 or Glu371 residue with neutral Ala residue in Nhx1 has no effect on pH sensitivity.
Exponentially cultures were serially diluted and spotted as in Fig. 13.

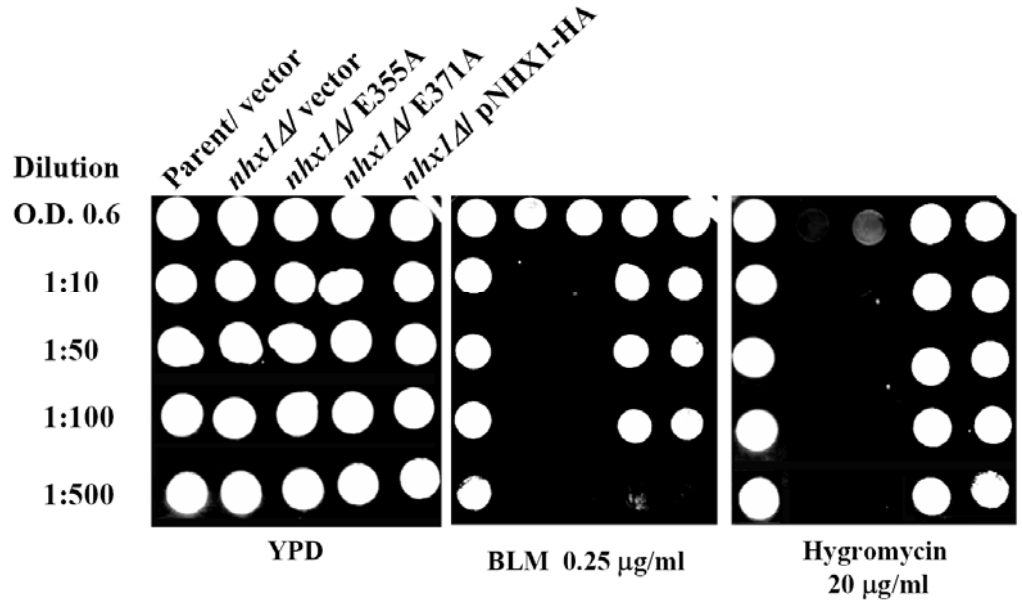


Figure 20: The variant E355A, but not E371A, is defective in rescuing *nhx1Δ* mutant from drug toxicities.
Exponentially cultures were serially diluted and spotted as in Fig. 7.

3.10 Nhx1 acts independently of Ypt6 and Ypt7 in drug resistance

Previous study suggests that Nhx1 and Ypt6 may function along the same trafficking pathway as they showed similar sensitivity to hygromycin B in the APG media (Ali *et al.*, 2004). As such, we tested if the *ypt6Δ* mutant would display similar sensitivities as *nhx1Δ* mutant towards various drugs. Surprisingly, although *ypt6Δ* mutant showed hypersensitivity to SPM and calcofluor white, it did not reveal any significant sensitivity to bleomycin or hygromycin B on the YPD media (Fig. 21). On the basis of these findings, it is unlikely that Nhx1 and Ypt6 are functioning along the same pathway in drug detoxification, at least for bleomycin. Interestingly, deletion of the *YPT6* gene in *nhx1Δ* mutant resulted in the *nhx1Δ ypt6Δ* double mutant that was more resistant to bleomycin, hygromycin B and SPM (Fig. 22), suggesting that accumulation of these drugs in the prevacuole may leak into the Golgi via functional Ypt6 which consequently leads to increased cell killing.

It is noteworthy that the *ypt7Δ* mutant lacking a protein involved in fusion of the vesicles derived from endosomes with the vacuoles (Ali *et al.*, 2004) also did not show any significant sensitivity towards either bleomycin or hygromycin B (Fig. 21). This is also in contrast to the hygromycin B sensitivity observed for *ypt7Δ* mutant when tested on APG media, suggesting that the type of media might influence the phenotypes displayed by these mutants.

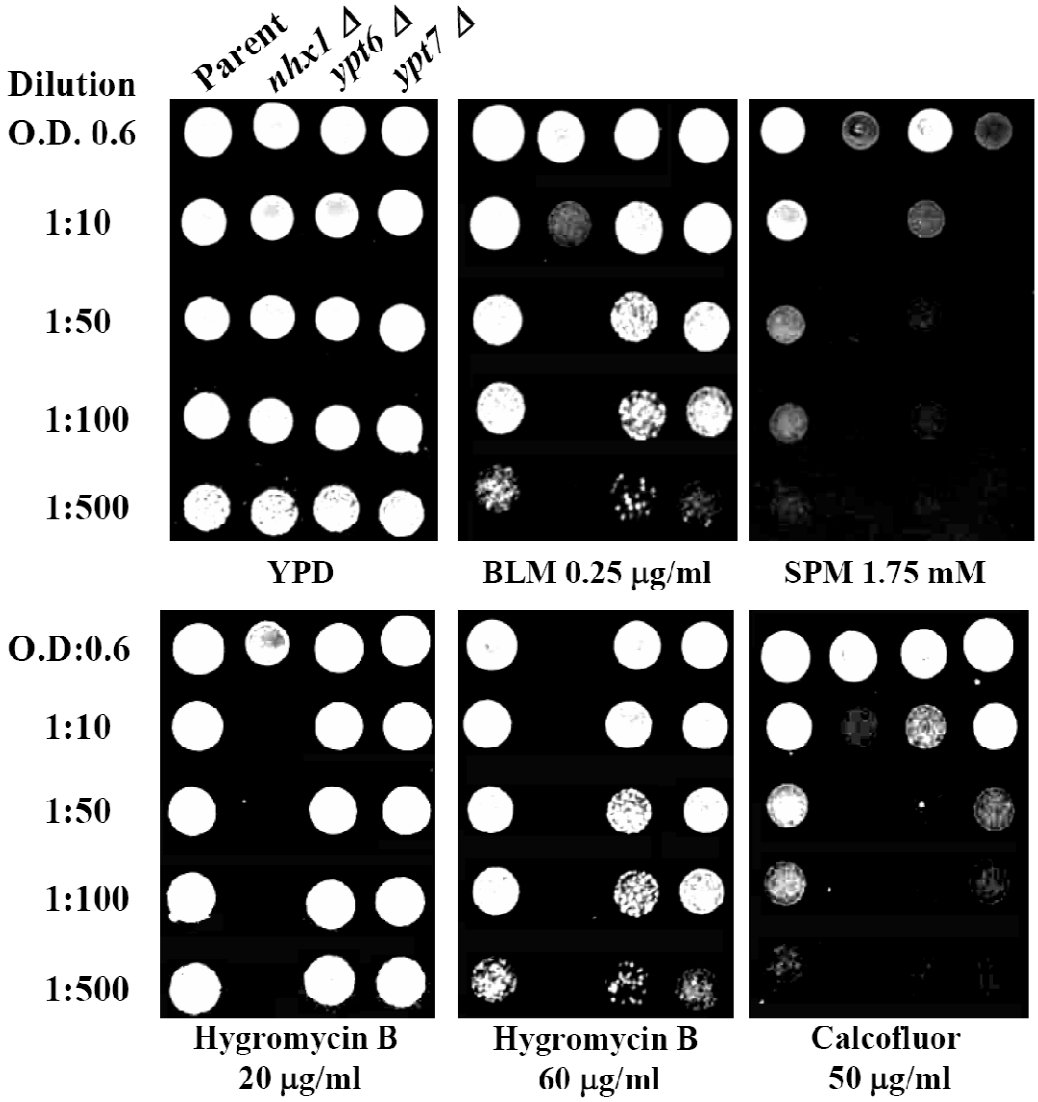


Figure 21. Comparison of the sensitivities of the parent and the indicated mutants towards various drugs.
Exponentially cultures were serially diluted and spotted as in Fig. 7

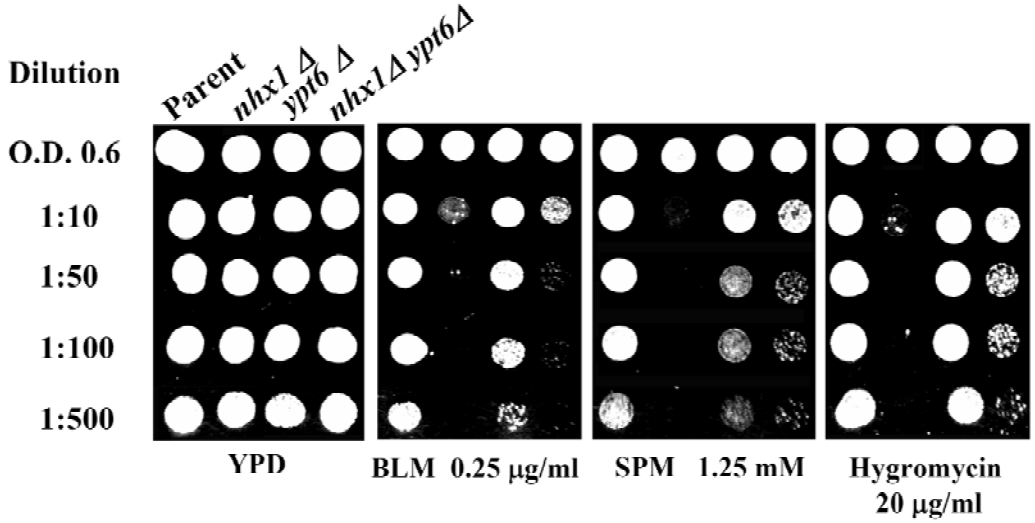


Figure 22. *nhx1Δ ypt6Δ* double mutant shows more resistance towards indicated drugs when compared to *nhx1Δ* mutant.
Exponentially cultures were serially diluted and spotted as in Fig. 7

Discussion

We report novel phenotypes of the *nhx1Δ* mutant that raise additional interesting questions regarding the function of Nhx1. The initial experiments done to characterize the physiological role of Nhx1 were performed mainly in APG medium with low osmolarity, which is essential in order to reveal the osmotolerance defect and to accurately measure the intracellular pH of *nhx1Δ* mutant (Brett *et al.*, 2005). This media was also used to demonstrate that the *nhx1Δ* mutant is sensitive to NaCl (Brett *et al.*, 2005; Wells & Rao, 2001). In accordance with the previous studies, we show that the *nhx1Δ* mutant revealed hypersensitivity to hygromycin B, as well as displayed the CPY missorting phenotype even when grown in the rich media YPD. However, under the YPD growth media, the *nhx1Δ* mutant no longer retained sensitivity to NaCl even at high concentration (1 M in YPD). Moreover, addition of NaCl to the YPD media did not suppress the *nhx1Δ* sensitivities to any of the drugs (Fig 16). These observations clearly uncoupled the drug sensitivities, as well as CPY missorting, from a need to maintain osmolarity, suggesting that any deficiency of Na^+ in the late endosome can not account for the drug phenotypes displayed by the *nhx1Δ* mutant.

Another unique observation reported in this study is that neither *ypt6Δ* mutant nor *ypt7Δ* mutant was sensitive to hygromycin B when grown in YPD media as compare to the sensitivity seen with the APG media (Ali *et al.*, 2004). If indeed Nhx1 and Ypt6 function along the same pathway as proposed (Ali *et al.*, 2004), it is expected that *ypt6Δ* mutant should show at least some level of sensitivity to hygromycin B particularly since *nhx1Δ* mutant is dramatically sensitive to the drug. One explanation

for this observation is that under YPD media, an alternative member of the Ypt proteins, of which there are at least eleven (Lazar *et al.*, 1997), could be expressed to substitute for Ypt6. Likewise, a similar explanation may hold true for the lack of sensitivity of *ypt7Δ* mutant to hygromycin B in YPD media.

As pointed out above, one of our main interests is to understand how bleomycin is detoxified in yeast cells. Our data to date suggest that the key detoxification pathway involves the endosomal process, as mutants defective in this pathway are sensitive to bleomycin (Aouida *et al.*, 2004b). However, we do not know if the main reason for cytotoxicity or genotoxicity is a result of leak from the endosomal process. The data presented here would indicate that defect in the late endosome is a major factor leading to toxicity. It is possible that the toxicity could arise as a result of drug leak from the late endosome to the cytosol or the leak is via retrograde trafficking from the late endosome to the nucleus through the Golgi and the endoplasmic reticulum. In the latter situation, we would expect that blocking Ypt6 function (involved in recycling of cargoes from the late endosome to the Golgi) would cause elevated resistance to bleomycin. Instead, we observed that *ypt6Δ* mutant was only weakly sensitive to bleomycin. Therefore, since the endocytic pathway is functional in the *ypt6Δ* mutant, it is unlikely that leak from the late endosome to Golgi is a major contributing factor for severe toxicity caused by bleomycin. The modestly increased resistance to bleomycin observed when *YPT6* is deleted in the *nhx1Δ* mutant may be due to lack of cargoes exchange from prevacuolar compartment to the Golgi. On the other hand, in the absence of Nhx1, Ypt6 could play an important role in transporting bleomycin to the Golgi which causes bleomycin sensitivity.

What could be the role of Nhx1, if it is not involved in osmotolerance or pH homeostasis in YPD media? Nhx1 could play a role in mediating the tethering of the late endosome to the vacuoles for efficient drug delivery. For example, the HOPS complex presented on the vacuoles consists of several proteins and some of which could participate in the fusion process with the late endosome, as reported for Ypt7 which is required for fusion of vesicles derived from endosome with the vacuole, through an interaction with the HOPS complex (Ungermann *et al.*, 2000). In this manner, the fusion process might occur by tethering of the HOPS protein directly or indirectly with Nhx1. Consistent with this notion, Nhx1 has been shown to participate in protein-protein interaction whereby its C-terminal tail interacts with Gyp6, an activator of the Ypt6 GTPase activity (Ali *et al.*, 2004). However, further studies would be needed to clarify if this is the actual mechanism by which Nhx1 functions in drug tolerance when cells are grown in YDP media.

Conclusion

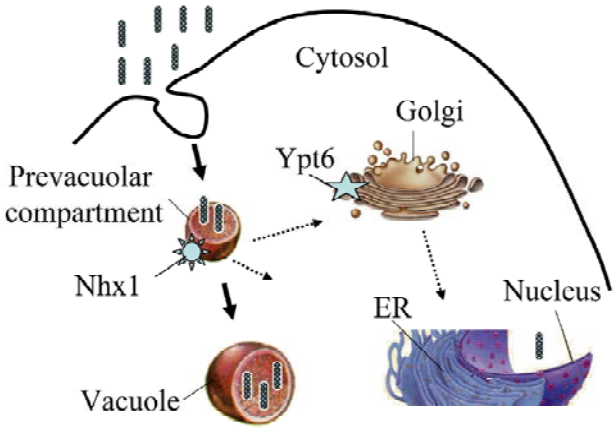
On the basis of our study, it is clear that additional studies are needed to characterize the role of Nhx1 on drug detoxification, in particular bleomycin, which could lead to improve chemotherapeutic response. We draw a model to describe how Nhx1 could be involved in protecting the cells against harmful agents. Nhx1 is located in prevacuolar compartment which fuses with vacuole to deliver the cargoes for degradation. Defect in prevacuolar compartment resulted from *NHX1* deletion leads to cargoes accumulation in prevacuolar compartment. Since latter compartment does not contain the essential enzymes required for cargoes degradation, accumulated cargoes might reach to nucleus either directly through cytosol or indirectly via the Golgi.

Our data showed that when *YPT6* is deleted in the *nhx1Δ* mutant, *nhx1Δ ypt6Δ* double mutant shows more resistance towards bleomycin as compared to *nhx1Δ* mutant. These data could indicate the effect of Ypt6 on bleomycin-induced cells killing in the *nhx1Δ* mutant. If indeed Ypt6 is a major contributing factor in the absence of *NHX1*, we expect that *YPT6* overexpression in the *nhx1Δ* mutant causes more sensitivity. Althernativly, *YPT6* overexpression would lead to more leaking from prevacuolar compartment to the Golgi resulting in increased sensitivity in the *nhx1Δ* mutant.

Saccharomyces cerevisiae possesses two plasma membrane Na^+/H^+ antiporters (Ena1-4 & Nha1). If drug-phenotype in *nhx1Δ* mutant is not due to Na^+ deficiency, we expect that the single *ena1-4Δ* mutant which is sensitive to NaCl (Kinclova-Zimmermannova *et al.*, 2006) to show parental sensitivity to bleomycin. In addition, we expect that the *nhx1Δ ena1-4Δ* double mutants to be hypersensitive to NaCl, but retain

the same level of sensitivity to bleomycin as the *nhx1Δ* single mutant. The same expectations would be speculated for the single *nha1Δ* mutant and the *nhx1Δ nha1Δ* double mutants.

It is noteworthy that Nhx1-related proteins, NHE6, 7, 8 and 9 are present in human cells (Nakamura *et al.*, 2005), although it is not known if any of these, when knockdown separately, would cause sensitivity to the same class of drugs as the *nhx1Δ* mutant. Perhaps targeting this unique isoform of the human NHEs would provide a way of improving the chemotherapeutic benefits of bleomycin.



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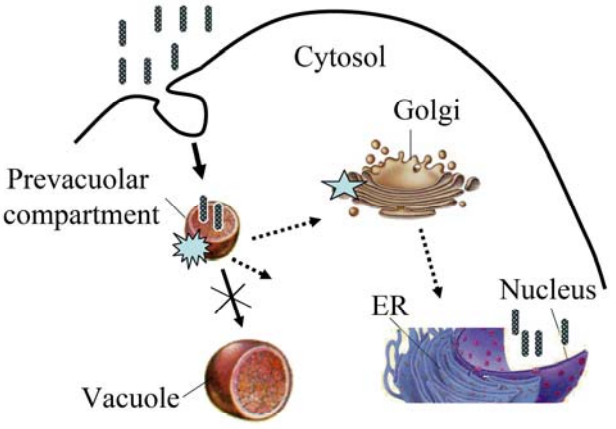
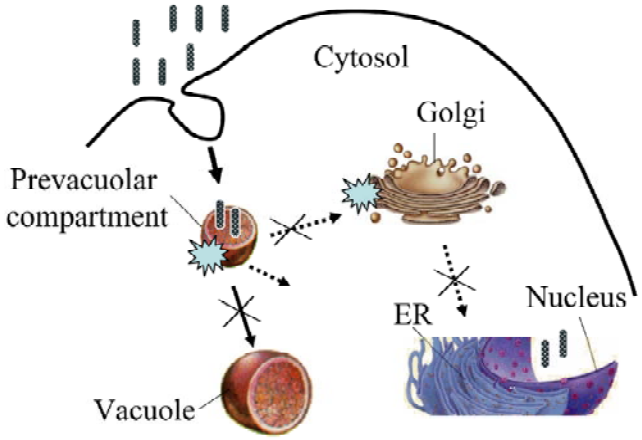
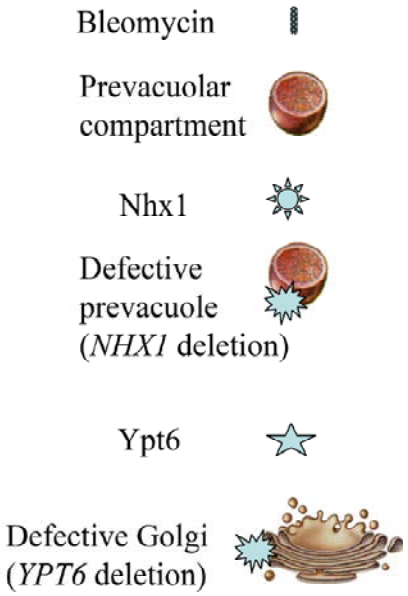


nhx1Δ


Cytosol
Golgi
Prevacuolar compartment
ER
Nucleus
Vacuole

nhx1Δ ypt6Δ


Bleomycin
Prevacuolar compartment
Nhx1
Defective prevacuole (*NHX1* deletion)
Ypt6
Defective Golgi (*YPT6* deletion)

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