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# Fyziologické úlohy $Na^+/H^+$ antiporterů v kvasinkách

# Physiological role of $Na^+/H^+$ antiporters in yeast cells

Disertační práce

Vedoucí práce: RNDr. Hana Sychrová, DrSc.

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**Prohlášení:**

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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**Abstrakt**

Kvasinky *Saccharomyces cerevisiae* patří k důležitým modelům při studiu homeostase iontů alkalických kovů. Stejně jako v jiných buňkách je pro *S. cerevisiae* nezbytné udržení určité koncentrace $K^+$ uvnitř buněk, na druhou stranu $Na^+$ a další ionty alkalických kovů jsou pro buňky toxické. $K^+$ je akumulován pomocí uniporterů Trk1 a Trk2, zatímco export toxických iontů alkalických kovů, a také nadbytečného $K^+$, je zajištěn ATPasami Ena, $Na^+(K^+)/H^+$ antiporterem Nha1 a $K^+$ selektivním kanálem Tok1. Ačkoli jsou jednotlivé transportery poměrně dobře prozkoumány, není dosud mnoho známo o celkové regulaci homeostase iontů alkalických kovů ani o vzájemné interakci a regulaci mezi jednotlivými transportery. V rámci této práce byl studován antiporter Nha1 a jeho fyziologické úlohy v kontextu ostatních transporterů iontů alkalických kovů. Ukázalo se, že nejen Nha1p, ale také další exportery, ATPasy Ena a kanál Tok1, jsou, přes jejich výraznou odlišnost v mechanismu transportu i způsobu regulace, regulovány společně změnou aktivity importerů $K^+$ a to prostřednictví membránového potenciálu. Vzájemná regulace a funkční propojení importerů a exporterů $K^+$, ale také další výsledky poprvé ukázaly, jak velice důležitá je neustálá cirkulace $K^+$, tedy současný vstup a výstup $K^+$, pro udržování homeostase iontů alkalických kovů. Ačkoli bylo prokázáno, že homeostáze iontů alkalických kovů a související fyziologické parametry (např. membránový potenciál, velikost buněk či tolerance k solím) mohou být výrazně ovlivněny volbou konkrétního kmene *S. cerevisiae*, byla potvrzena nezastupitelná úloha Nha1p pro přežití buněk v přirozeném prostředí, kde může koncentrace solí výrazně kolísat. V této práci byly znalosti o regulaci Nha1p ještě dále rozšířeny o dva nově nalezené pozitivní regulátory aktivity Nha1p a to 1) proteiny 14-3-3 fyzicky interagující s Nha1p na více místech a 2) kinasu Cka1, která byla dosud známa pouze jako regulátor exprese *ENA1*. Znalosti získané studiem Nha1p byly využity ke studiu lidského $Na^+/H^+$ antiporteru NHAoc/NHA2 v buňkách *S. cerevisiae* postrádajících vlastní transportery a byly identifikovány aminokyselinové zbytky, jejichž mutací dochází k disfunkci transporteru vedoucí často k závažné chorobě tvorby kostí, osteopetrose. Práce tedy přispěla mnoha novými poznatky k lepšímu pochopení úlohy Nha1p a dalších transporterů v udržování homeostase iontů alkalických kovů a během práce došlo také k přínosu v oblasti metodiky (především měření vnitrobuněčného pH), který v budoucnu umožní další posun v řešené problematice.

**Abstract**

Yeast *Saccharomyces cerevisiae* belongs to important models for alkali-metal-cation homeostasis research. As other cells, certain intracellular content of $K^+$ is necessary for *S. cerevisiae*, but $Na^+$ or other alkali metal cations ($Li^+$, $Rb^+$) are toxic for yeast cells. Uniporters Trk1 and Trk2 are responsible for $K^+$ accumulation, while efflux of $Na^+$, $Li^+$, $Rb^+$ and $K^+$ is ensured by Ena ATPases, $Na^+(K^+)/H^+$ antiporter Nha1 and $K^+$ specific channel Tok1. Several regulators of $K^+$ ($Na^+$) transporters are already known, but reciprocal regulation between transporters and overall picture of the maintenance of alkali-metal-cation homeostasis is still unclear. In this work, $K^+$ circulation (simultaneous uptake and export of $K^+$) was shown to be important in alkali-metal-cation homeostasis maintenance. $K^+$ circulation is maintained using reciprocal regulation and interactions between $K^+$ exporters and importers. Though obtained results showed that the alkali-metal-cation homeostasis and associated physiological parameters (e.g. membrane potential, cell size, salt sensitivity) are strain specific, Nha1p was verified to be important for cell survival in ever-changing natural environment. Furthermore, two novel positive regulators of Nha1p activity were found, 14-3-3 proteins and Cka1 kinase. 14-3-3 proteins interact physically with multiple parts of Nha1p. Cka1 kinase was previously known as *ENA1* expression regulator, moreover, regulation of Nha1p activity by Cka1p was observed in this work. Using knowledge and skills obtained during this work, *S. cerevisiae* strain lacking its own alkali-metal-cation exporters was used for characterization of human Nha1p homologue, $Na^+/H^+$ antiporter NHAoc/NHA2. Residues, whose mutation could be one of crucial points in the development of serious bone disease (osteopetrosis) in human, were identified in the NHAoc/NHA2 sequence. Altogether, results obtained in this work helped for better understanding the role of Nha1p and other transporters in maintenance of alkali-metal-cation homeostasis and its regulation, furthermore, an important progress has been made in methodology (especially in intracellular pH measurement) which will help in future studies.

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**O B S A H**

# 1 Úvod

Kvasinky patří mezi nejdéle studované organismy. Jedním z důvodů je to, že jsou lidmi využívány již tisíce let např. při výrobě chleba a fermentovaných nápojů. Nejznámějším zástupcem jsou kvasinky *Saccharomyces cerevisiae*, které jsou v současné době jedním z nejdůležitějších modelových organismů eukaryotních buněk pro základní i aplikovaný výzkum a hojně se využívají také v biotechnologické a potravinářské praxi. Z pohledu základního výzkumu byl význam *S. cerevisiae* jako modelového organismu v posledních dekádách velmi posílen tím, že byl genom této kvasinky kompletně sekvenován jako první genom eukaryotního organismu [1]. K popularitě tohoto modelu dále přispívá fakt, že se jedná o jednoduchý, snadno a rychle rostoucí mikroorganismus, pro který bylo postupně vytvořeno mnoho nástrojů molekulární biologie a genetiky umožňující vnášení mutací, delece, nebo vkládání genů nejen vlastních, ale i genů z jiných organismů [2, 3]. Díky relativně jednoduchému genomu, vysoké sekvenční homologii a funkční podobnosti s proteiny vyšších organismů je *S. cerevisiae* dobrým nástrojem využívaným při rozsáhlých studiích proteinů z jiných eukaryotních organismů [3]. Kromě biomedicínských studií se na základě společenské poptávky po nových technologiích do popředí stále častěji dostává také výzkum možností využití těchto buněk např. při produkci biopaliv, organických plastů, vitamínů, nebo rekombinantních proteinů a peptidů.

Udržování homeostase iontů alkalických kovů je důležitým předpokladem přežití organismů v přirozeném prostředí. Stejně jako pro jiné organismy, také pro *S. cerevisiae* platí, že $K^+$ je důležitým vnitrobuněčným kationtem, který je uvnitř buněk udržován v poměrně vysoké koncentraci a podílí se např. na udržování osmolarity, iontové síly a pH cytoplasmy nebo na udržování membránového potenciálu. Na rozdíl od $K^+$ působí $Na^+$ uvnitř buněk toxicky i ve velmi nízkých koncentracích a buňky využívají několik mechanismů, aby vstupu $Na^+$ zabránily [4-6].

Modelový organismus *S. cerevisiae* je s úspěchem používán také při výzkumu homeostase iontů alkalických kovů [6, 7]. Ačkoli je udržování homeostase $K^+$ a $Na^+$ důležité pro přežívání buněk v přirozeném prostředí, nejsou geny kódující transportery těchto iontů v *S. cerevisiae* esenciální. Při optimálním přísunu $K^+$ v růstovém médiu (cca 200 mM $K^+$; [6]) a absenci toxických iontů alkalických kovů nejsou růstové schopnosti buněk ovlivněny ani v případě současné delece genů všech známých transporterů $K^+$ a $Na^+$ (delece *trk1 trk2 tok1 nha1 ena1*; viz dále) v plasmatické membráně (PM). Tento fakt a jednoduchost genetických manipulací dělá z *S. cerevisiae* velice účinný a velmi citlivý nástroj pro studium transporterů iontů alkalických kovů, ať už pocházejících z této kvasinky nebo z jiných organismů.

# 2 Literární přehled

## 2.1 Transport iontů alkalických kovů

Prvky označované jako alkalické kovy nalezneme v 1. skupině periodické tabulky a jedná se o lithium (Li), sodík (Na), draslík (K), rubidium (Rb), cesium (Cs) a francium (Fr). Jelikož jde o velmi reaktivní kovy, vyskytují se v přírodě prakticky pouze ve sloučeninách nebo po disociaci solí ve formě jednomocných kationtů. Ve větším množství nalézáme pouze dva z nich, ionty $Na^+$ a $K^+$, které zaujímají 5. a 6. místo ve výskytu prvků na zemi ($Na^+$ tvoří 2,89 % a $K^+$ 2,80 % kontinentální zemské kůry; [8]). Ostatní zástupci alkalických kovů se v přírodě vyskytují v mnohem menším množství (Rb 112 ppm[1], Cs 4,8 ppm, Li 20 ppm), v případě radioaktivního Fr je zastoupení mizivé (cca $10^{-18}$ ppm; [8]). Kromě vysoké reaktivity elementárních prvků je alkalickým kovům společná nízká elektronegativita (0,86 -1,00) a související tvorba solí, které se ve vodě zpravidla dobře rozpouštějí. Z hlediska transportu přes buněčné membrány je důležitějším fyzikálním ukazatelem iontový poloměr, který je považován za faktor zásadně ovlivňující selektivitu iontových transporterů a kanálů [4, 9]. V případě iontů alkalických kovů byly jednotlivé iontové poloměry stanoveny jako: $Li^+$ 0,76 Å, $Na^+$ 1,02 Å, $K^+$ 1,38 Å, $Rb^+$ 1,52 Å, $Cs^+$ 1,67 Å a $Fr^+$ 1,8 Å [10]. Relativní podobnost v iontovém poloměru a zároveň nízké zastoupení prvku v přírodě (vysoký poměr signálu a šumu a vysoká citlivost experimentů) patří k důvodům, proč je $Li^+$ využíván jako analog při studiu transportu $Na^+$ a $Rb^+$ při studiu transportu $K^+$ [4, 7, 9]. Vzhledem k velmi nízkému zastoupení a také malému významu $Fr^+$ a $Cs^+$ ve výzkumu transportních mechanismů iontů alkalických kovů v buňkách se tato práce dále věnuje pouze $Li^+$, $Na^+$, $K^+$ a $Rb^+$.

Přítomnost iontů alkalických kovů vně i uvnitř buněk zásadně ovlivňuje buněčnou fyziologii, a proto je udržování vnitrobuněčné homeostase iontů alkalických kovů nezbytným předpokladem přežívání buněk v přirozeném prostředí. Kvasinka *S. cerevisiae* se s úspěchem používá jako modelový organismus pro studium homeostase iontů alkalických kovů po mnoho desetiletí [4-6], výsledky těchto studií pomáhají k pochopení, jak je rovnováha iontů v buňkách udržována a k čemu slouží. Výsledky pocházející z tzv. základního výzkumu homeostase iontů alkalických kovů v *S. cerevisiae* lze často přímo využít např. v biotechnologiích, nebo zobecnit a využít pro pochopení fungování jiných buněk a buněčných systémů vyšších organismů. Získané poznatky pak mohou být využity pro

[1] 1 ppm (particles per milion) odpovídá 0,0001 %

pochopení jednotlivých aspektů tak složitých dějů, jako je např. funkce ledvin, nervové nebo svalové soustavy, kde hrají ionty $K^+$ a $Na^+$ důležitou roli [11].

Homeostase iontů alkalických kovů u buněk *S. cerevisiae* se příliš neliší od jiných eukaryotních buněk. Pro přežití buněk je nutné, aby byl $K^+$ udržován v buňkách v relativně vysoké koncentraci [7]. Při růstu v laboratorních podmínkách ve standardním médiu a bez přídavku dalších solí byla tato koncentrace stanovena v rozmezí 200-300 mM [6] v závislosti na kmenu a použitém médiu. Ionty $K^+$ tvoří hlavní vnitrobuněčný kationt zajišťující elektroneutralitu buňky, zatímco mezi intracelulární anionty patří hlavně fosfáty, polyfosfáty a také karboxylové skupiny. Relativně vysoký obsah $K^+$ uvnitř buněk je udržován aktivitou vstupních transporterů $K^+$, ale předpokládá se, že v rámci dynamické rovnováhy jsou mírně aktivní také výstupní transportery a dochází tak k obousměrnému transportu, cirkulaci $K^+$ [6]. Narušením vstupu $K^+$ do buněk dochází k hyperpolarisaci PM [12] a stejný efekt má také nadexprese exporterů $K^+$ (prokázáno u antiporteru Nha1 a predikováno pro ATPasu Ena1; [13, 14]) vedoucí k urychlení výstupu $K^+$ z buněk, zatímco po odstranění exporterů $K^+$ (zpomalení výstupu $K^+$) je PM depolarisována [15]. Kromě rolí, jako je např. zajištění elektroneutrality systému, udržování membránového potenciálu (ΔΨ) a vytvoření vhodného prostředí pro správnou funkci enzymů, jsou ionty $K^+$ důležité pro regulaci vnitrobuněčného turgoru, vnitrobuněčného pH a objemu buňky [5, 6, 15].

Za snížené dostupnosti $K^+$ dochází v buňkách k částečnému nahrazení tohoto iontu protony, okyselování cytoplasmy a k narušení osmotické rovnováhy uvnitř buňky. Tyto změny dále způsobují inhibici syntézy proteinů a ovlivňují aktivitu enzymů. Za specifických podmínek, při hladovění na $K^+$ mohou tento iont nahradit také ionty $Na^+$ [5]. Běžně ani při stonásobně vyšší koncentraci $Na^+$ než $K^+$ v médiu nenalézáme zvýšené množství $Na^+$ uvnitř buněk, k tomu dochází až při překročení extracelulárního poměru koncentrací $Na^+/K^+$ cca 700:1 [16], kdy část $Na^+$ vstupuje do buněk a zůstává uvnitř, zatímco adekvátní množství $K^+$ je z buňky vyloučeno z důvodu zachování elektroneutrality [17]. Poměr vstupujících iontů $Na^+/K^+$ je výrazně ovlivněn vnějším pH [4].

Zatímco přítomnost $K^+$ je pro přežití buněk nezbytná, jiné ionty alkalických kovů, $Na^+$, $Li^+$ a $Rb^+$ jsou pro buňky toxické. Ionty $Na^+$ obecně patří mezi hojně zastoupené v prostředí (viz výše), ale v cytoplasmě buněk působí toxicky i v mírně zvýšeném množství. Toxicita $Na^+$ je tvořena více faktory. Stejně jako u jiných iontů dochází při zvýšené koncentraci $Na^+$ v prostředí k hyperosmotickému stresu [19] a inhibici aktivity enzymů vlivem změny iontové síly cytoplasmy (již při vnějších koncentracích anorganických solí okolo 0,4 M; [18]). Kromě výše zmíněného, působí $Na^+$ také mnohem specifičtěji na úrovni proteinů. Toxické účinky $Na^+$ uvnitř buňky *S. cerevisiae* nejsou na molekulární úrovni dostatečně prozkoumány, ale je známo již mnoho specifických cílů účinku $Na^+$ v buňce, díky kterým

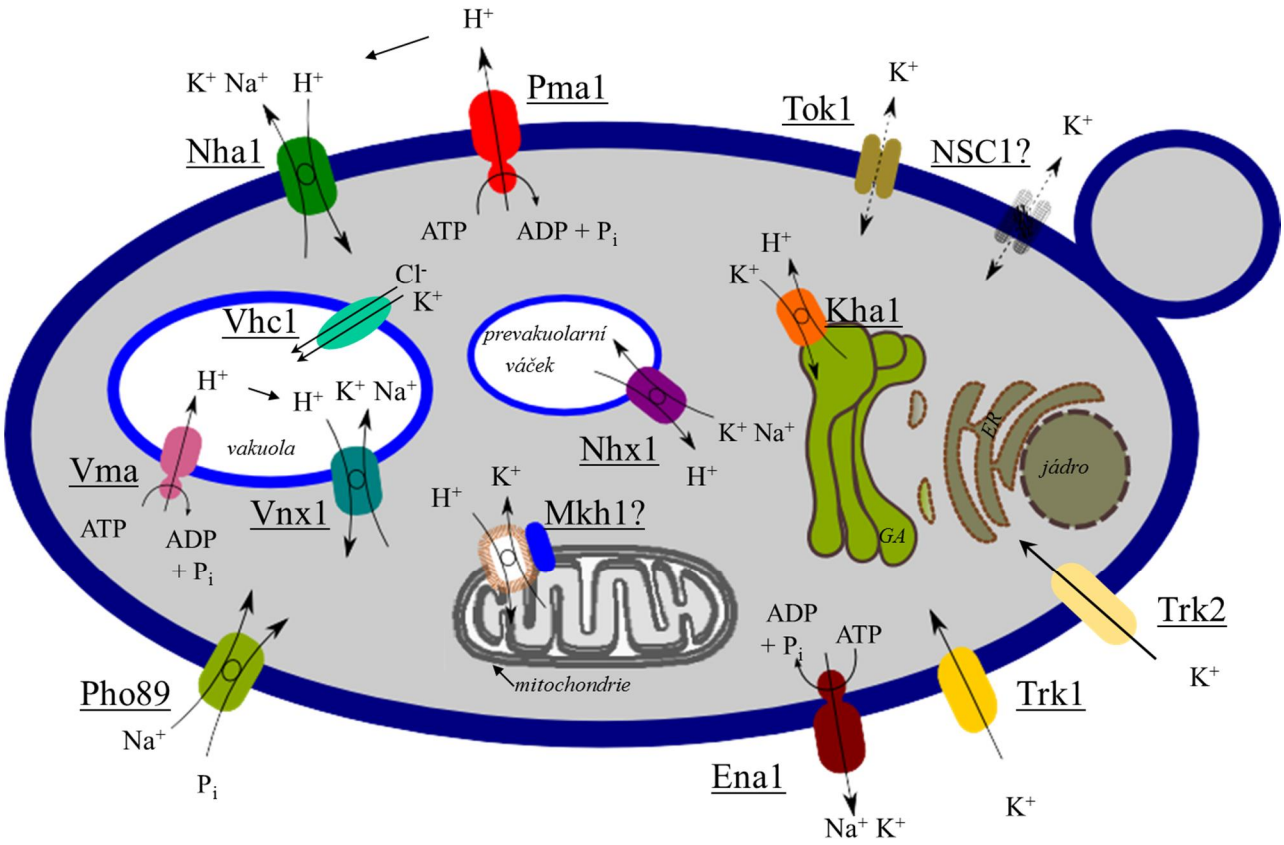
působí $Na^+$ toxicky i v mnohem nižších koncentracích (cca 0,1 M [20]). Mezi konkrétní příklady účinku iontů $Na^+$ patří jejich působení na vazebná místa $K^+$, $Mg^{2+}$ či $Ca^{2+}$ některých enzymů, např. Hal2p (viz dále). Ionty $Na^+$ vytěsňují ostatní kationty z jejich vazebných pozic a tak inhibují aktivitu enzymů, které tyto kationty potřebují [18, 21]. Fosfatasa Hal2, jenž katalyzuje přeměnu 3´-fosfoadenosin-5´-fosfátu (PAP) na adenosinmonofosfát, patří mezi známé molekulární cíle toxického působení $Na^+$ v *S. cerevisiae*. Zvýšená koncentrace $Na^+$ nebo $Li^+$ vede k vytěsňování jednoho ze dvou iontů $Mg^{2+}$ v aktivním centru enzymu a inhibici aktivity enzymu. Následné hromadění PAP v buňce může být příčinou inhibice sulfotransferas a enzymů upravujících RNA [18, 22].

Ionty $Li^+$ a $Rb^+$ se v přirozeném prostředí téměř nevyskytují, ale s úspěchem se díky podobným vlastnostem používají k výzkumu transportních systémů $Na^+$ a $K^+$ [4, 9]. Pro studium transporterů specifických pro $K^+$ se často využívá vlastnostmi velmi podobný, ale mnohem toxičtější $Rb^+$, který je zpravidla přenášen stejnými transportery jako $K^+$ a díky zanedbatelné přirozené koncentraci jsou měření s $Rb^+$ velmi přesná a vhodná např. při studiu regulace importerů $K^+$ [5, 23-25]. Z podobných důvodů je používán $Li^+$ pro studium transportu $Na^+$. $Li^+$ je mnohem toxičtější než $Na^+$ a díky tomu je možné při studiu transportu a působení $Na^+$ rozlišit, jaký podíl na toxicitě má osmotický tlak způsobený zvýšenou koncentrací solí a jaký podíl připadá specifickému působení iontu samotného [18]. Mechanismus toxicity $Li^+$ bývá v *S. cerevisiae* studován také jako model působení $Li^+$ v lidských buňkách, jelikož je tento iont farmakologicky využíván např. k léčbě deprese nebo bipolární poruchy [26, 27]. Mezi sledované cíle toxicity $Li^+$ patří např. fosfoglukomutasa z metabolismů cukrů [26] nebo glutamátové receptory NMDA (N-metyl-D-aspartát) lokalisované v neuronech [28].

Při studiu homeostase iontů alkalických kovů jsou často využívány (např. [25, 29]) toxické organické kationty, jako je hygromycin B, spermin a tetrametylamonné kationty. Zvýšená senzitivita současně ke všem těmto kladně nabitým látkám (každá z látek působí na jiný molekulární cíl) je často spojena s hyperpolarisací PM, která může upozorňovat např. na poruchu importu $K^+$, nebo jiný defekt v homeostasi iontů alkalických kovů [12, 30].

### 2.1.1 Transportery zajišťující import $K^+$ do buněk

Udržovat vnitrobuněčnou koncentraci $K^+$ v přípustných mezích je pro buňku životně důležité. Kvasinkové buňky jsou schopny růst ve velmi širokém spektru vnějších koncentrací $K^+$ (cca 2 μM – 2 M), ale pro růst a dělení buněk je nutné, aby vždy převažoval vstup $K^+$ nad výstupem a $K^+$ byl kontinuálně přijímán a předáván dceřiným buňkám [31, 32]. Proto buňky využívají dva režimy importu $K^+$ do buněk [5]. Nízkoafinitní režim ($K_T$ v řádu mM) je důležitý pro růst buněk v prostředí s dostatkem $K^+$, zatímco vysokoafinitní režim ($K_T$ v řádu μM) umožňuje růst za velmi nízkých koncentrací $K^+$ v prostředí nebo např. za zvýšené extracelulární koncentrace $Na^+$ [5].




**Obr. 2.1.1 Transportery iontů alkalických kovů**
*zdroj: převzato a upraveno [33]*

Akumulace $K^+$ uvnitř buněk je zajištěna aktivními transportery kódovanými geny *TRK1* [34] a *TRK2* [35], jejichž aktivita je řízena membránovým potenciálem [36] udržovaným pomocí ATPasy Pma1 ([37]; obr. 2.1.1, str. 12). Ačkoli jsou tyto transportery považovány za uniportery a výsledky mnoha experimentů toto potvrzují, lze předpokládat, že mechanismus uniportu nemůže plně dostačovat k tak vysokému stupni akumulace $K^+$ jaký byl dříve pozorován [4, 6, 34] v prostředí s nízkým extracelulárním $K^+$. Přes intenzivní výzkum probíhající po několik dekád není mechanismus akumulace $K^+$ prostřednictvím Trk1p a Trk2p plně objasněn [6, 38].

Dominantní úlohu pro vstup $K^+$ do buněk hraje Trk1p (obr. 2.1.1, str. 12), jenž tvoří 1235 amino-kyselinových (AA) zbytků. Topologie proteinu byla predikována na základě zastoupení hydrofobních AA v sekvenci a bylo identifikováno 12 transmembránových segmentů (TMS), tedy předpokládaných úseků prostupujících z jedné strany PM na druhou [34]. Mezi fyziologické úlohy tohoto transporteru patří udržování homeostase $K^+$ a pH [12, 39], turgoru [40] a $\Delta\Psi$ [12, 41]. Kromě transportu $K^+$ byla před několika lety objevena schopnost Trk1p exportovat $Cl^-$ [42]. Předpokládá se, že chloridový kanál je tvořen symetrickou agregací 4 monomerů Trk1p v PM, nicméně kinetické parametry přenosu $Cl^-$ tímto kanálem dosud nebyly stanoveny. Ačkoli se této roli Trk1p věnovala pouze jedna práce [42],

bylo ukázáno, že výstup $Cl^-$ prostřednictvím Trk1p by mohl být důležitým faktorem regulujícím import $K^+$ a udržování ΔΨ.

Trk2p je homolog Trk1p s vysokým stupněm identity (55%). Jedná se o 889 AA dlouhý protein pravděpodobně obsahující 12 TMS [43]. Za běžných podmínek i během hladovění buněk na $K^+$ se předpokládá, že téměř všechen $K^+$ je transportován prostřednictvím Trk1p [6, 30, 38], proto se nejdříve předpokládalo, že Trk2p je odpovědný za nízkoafinitní import $K^+$ [35]. Brzy se však ukázalo, že tomu tak není a že Trk2p je transporterem s afinitou střední až vysokou a s velmi nízkou $V_{max}$ [32], jeho úloha je proto minoritní díky velmi nízkému přirozenému množství proteinu v buňkách [44].

Ačkoli není ani jeden z importerů $K^+$ esenciální pro život buňky, mutanty postrádající gen *TRK1* nejsou schopné růstu v prostředí s nízkou koncentrací $K^+$ (cca 0.1 - 1 mM v závislosti na kmeni a médiu; [30, 34, 38]), import $K^+$ a $Rb^+$ [30, 34] je u nich omezen, vykazují zvýšený ΔΨ [12, 30] a zvýšenou citlivost k $Li^+$, $Na^+$ a jiným toxickým (např. organickým) kationtům [30, 38]. Delece *trk1* dále zamezuje přechodnému zmenšení buněk, jenž bylo pozorováno u kmenů exprimujících *TRK1* ihned po přenosu do média s nízkou koncentrací $K^+$ [38].

Samotná delece *trk2* nemá téměř žádný pozorovatelný fenotyp [32, 38], ale situace je odlišná při deleci obou transporterů, *trk1 trk2*. Do nedávna byla publikována pouze jediná práce prokazující fyziologickou úlohu Trk2p v homeostasi iontů alkalických kovů a to při nedostatku $K^+$ v médiu a za nízkého pH, ale pouze při současné deleci genu *SIN3*, jehož produkt je součástí komplexu Sin3p-Rpd3p odpovědného za deacetylaci histonů [44]. Za této situace je *TRK2* mnohem více exprimován a Trk2p je pak schopen zastoupit roli chybějícího Trk1p. V nedávno publikované práci [38] byl detailně popsán fenotyp delece *trk2* na pozadí kmenu postrádajícího gen *TRK1*. Kmen s delecí *trk1 trk2* vykazuje mnohem vyšší citlivost k nízkým koncentracím $K^+$ (s limitem růstu cca 10 mM) než kmen postrádající pouze *TRK1* a také další fenotypy delece *trk1* jsou následnou delecí *trk2* akcentovány (nárůst æCl a citlivost k organickým kationtům). Experimentální zvýšení exprese *TRK2* kompenzuje fenotyp delece *trk1*, což potvrzuje hypotézu velmi podobné fyziologické úlohy, ale rozdílného množství proteinů Trk1 a Trk2 v buňkách [38].

#### 2.1.1.1 Nespecifický a nízkoafinitní import $K^+$

Vztah mezi existencí dvou režimů importu $K^+$, nízkoafinitním a vysokoafinitním, a importery samotnými byl po dlouhou dobu kontroverzní. V současné době se odborníci shodují, že převážná většina $K^+$ vstupuje do buněk pomocí Trk1p a to jak v režimu nízkoafinitním za dostatku $K^+$ v okolí, tak v režimu vysokoafinitním v médiu s nízkou koncentrací $K^+$ (nebo s vyšší koncentrací $Na^+$). Trk2p přitom hraje minoritní roli díky nízké expresi za běžných podmínek, přestože je schopen importovat

$K^+$ se střední až vysokou afinitu [6]. Nízkoafinitní transport $K^+$ (označován jako zbytkový, nebo ektopický) byl pozorován v případě dvojité delece *trk1 trk2* ($K_T$ v řádu mM) [44]. Za tento druh transportu je odpovědný nespecifický transport $K^+$ pomocí jiných transporterů, jako je např. NCS1 (obr. 2.1.1, str. 12), nespecifický kationtový kanál jednomocných a dvoumocných kationtů, jehož gen dosud nebyl nalezen a jehož existence byla prokázána pouze pomocí elektrofyziologických experimentů zaměřených na toky iontů přes membránu [30, 45, 46]. Import $K^+$ může za specifických podmínek probíhat také prostřednictvím transporterů cukrů kódovaných geny *HXT* a *GAL2* [47].

### 2.1.2 Transportery zajišťující import $Na^+$ do buněk

Na rozdíl od $K^+$ je přítomnost $Na^+$ v buňkách *S. cerevisiae* nežádoucí, proto neexistují specifické transportní systémy pro vstup $Na^+$ do buněk (kromě Pho89p se specifickou funkcí, viz dále). Zvýšené koncentrace $Na^+$ v okolí buněk ovlivňují kinetiku transportu $K^+$ formou kompetitivní inhibice [9], ale za běžných podmínek je díky vysokoafinitnímu importu $K^+$ pomocí Trk1p a Trk2p potlačen nespecifický import $Na^+$. U kmenů postrádajícím oba importery $K^+$ (delece *trk1 trk2*) je akumulace $K^+$ narušena a zvyšuje se import $Na^+$ [16], což potvrzuje nespecifickou povahu ektopického transportu iontů alkalických kovů [45].

Jediným transporterem, který je schopen specificky přenášet $Na^+$ do buněk (obr. 2.1.1, str. 12), je transporter kódovaný genem *PHO89* [48], symporter anorganických fosforečných aniontů ($P_i$) a $Na^+$. Aktivita tohoto transporteru je důležitá v prostředí s nedostatkem $P_i$ ($K_T$ pro $P_i$ je 0,5 μM), za vysokého pH (pH optimum 9,5) a v přítomnosti $Na^+$ v okolí buňky. Aktivace importu $P_i$ je maximální při 25 mM $Na^+$ v prostředí [49], tedy koncentraci, která pro buňky není ani zdaleka toxická. Potřebný $P_i$ není za těchto podmínek možné získávat z prostředí běžnou cestou, pomocí vysokoafinitního $P_i$-$H^+$ symporteru kódovaného genem *PHO84*, který je aktivní pouze za nízkého externího pH [50]. Transporter Pho89 je silně regulován několika faktory na úrovni exprese, je silně specifický pro $Na^+$ a je-li přítomen v PM za nízkého pH, není aktivní. Z uvedených důvodů je jeho aktivita za běžných růstových podmínek zastoupena aktivitou Pho84p [50]. V *S. cerevisiae* je Pho89p jediný známý sekundární transporter využívající gradient $Na^+$ místo $H^+$, buňkami je však využíván jen za mimořádných podmínek (nedostatek $P_i$ a vysoké vnější pH).

### 2.1.3 Exportní systémy pro ionty alkalických kovů

Transportery zajišťující export iontů alkalických kovů z buňky mají několik úloh, z nichž nejdůležitější je vypuzování toxických kationtů ($Na^+$, $Li^+$, $Rb^+$) a přebytečných iontů $K^+$ z buněk. Přítomnost exporterů umožňuje také již zmíněnou kontinuální cirkulaci $K^+$, která je považována za důležitý faktor regulace ovlivňující řadu fyziologických ukazatelů. Důležitost těchto exporterů v *S. cerevisiae*

potvrzuje to, že se zde vyskytují tři systémy s rozdílným mechanismem transportu a regulace. Jedná se o kanál Tok1, ATPasy kódované geny *ENA* a antiporter Nha1 (obr. 2.1.1, str. 12).

### 2.1.3.1 $K^+$ kanál Tok1

Gen *TOK1* kóduje jediný $K^+$ specifický kanál v PM *S. cerevisiae* umožňující výstup $K^+$ z buněk [51, 52]. Tok1p je tvořen 691 AA s predikovanou topologií obsahující 8 TMS [6, 52] a dvě tzv. P domény, které jsou běžné také u $K^+$ kanálů z jiných organismů. Na základě elektrofyziologických experimentů bylo zjištěno, že úkolem Tok1p je napěťově řízený export $K^+$ tak, že k otevření kanálu dochází při depolarisaci PM [30, 53]. Vnitrobuněčný $K^+$ uvolněný do okolí buňky má pravděpodobně zajistit regeneraci æ [54]. Tomu nasvědčuje fakt, že aktivita Tok1p může být modulována také externí koncentrací $K^+$ [55]. Zajímavé je, že za určitých podmínek je zřejmě možný také transport $K^+$ do buňky přes Tok1p [56]. Jedná se pouze o případ, kdy jsou odstraněny importery Trk1p a Trk2p, v prostředí je $K^+$ přítomen ve větším množství (80 mM) a při zvýšené expresi Tok1p. Import $K^+$ přes Tok1p může za těchto okolností přispět k lepšímu růstu buněk [56].

Delece *tok1* vede k poklesu æ , zatímco zvýšená exprese způsobí hyperpolarisaci PM [15]. Mimo účinku na æ a přímo měřené toky iontů přes kanál pomocí elektrofyziologických metod [53, 54] nebyly pro samotnou deleci *tok1* objeveny změny v akumulaci nebo exportu $K^+$ a jediným nalezeným fenotypem byla zvýšená tolerance k $Cs^+$ [30].

### 2.1.3.2 $Na^+$-ATPasy Ena

Za aktivní export iontů alkalických kovů jsou z velké části odpovědné ATPasy typu P kódované tandemovou repeticí genů *ENA* [57]. Počet genů *ENA* je pro různé kmeny *S. cerevisiae* různý (1-5 kopií; [6, 58, 59]), příkladem může být porovnání dvou běžně užívaných laboratorních kmenů W303 a BY4741, které byly použity také v této práci. Zatímco první kmen W303 [60] obsahuje čtyři geny *ENA* (*ENA1* až *ENA4*), v případě kmenu BY4741 jsou přítomny pouze tři (*ENA1*, *ENA2* a *ENA5*; [6, 58, 61]. Sekvenčně si jsou jednotlivé repetice velmi podobné a za běžných podmínek se v buňce vyskytuje jen malé množství těchto proteinů (cca 600 molekul na buňku; [62]). Dominantní roli u zmíněných kmenů *S. cerevisiae* hraje *ENA1*, jelikož jeho exprese může být v případě potřeby silně indukována (např. v přítomnosti $Na^+$, viz dále). Předpokládá se, že role ostatních proteinů Ena je stejná jako v případě Ena1p, ale jejich význam je minoritní. Lokalizace v PM byla zatím prokázána pouze pro Ena1p a Ena2p, jejichž sekvence jsou až na 13 AA shodné. Zajímavé je, že takto malý rozdíl způsobí to, že Ena1p je více specifický pro $Na^+$, zatímco Ena2p pro $Li^+$ [58].

Ena1p, nejdůležitější a nejstudovanější z těchto ATPas, tvoří 1091 AA uspořádaných pravděpodobně do 10 TMS [6, 57]. Ačkoli byl tento protein nejdříve označován jako $Ca^{2+}$ specifická ATPasa, brzy se

ukázalo, že energie uvolněná hydrolýzou ATP je využita pro export iontů $Na^+$, $Li^+$ a $K^+$ [17, 57, 63]. ATPasa Ena1 je považována za transporter determinující citlivost buněk k vnějším vysokým koncentracím $Na^+$ a $Li^+$ a její role je klíčová v prostředí s vysokým pH, kde je omezena funkce antiporteru Nha1 (viz kapitola 2.1.3.3). Ve výzkumných studiích je zpravidla z genomu odstraňován celý segment repetice genů *ENA*, což vede k silné citlivosti vůči $Na^+$ a $Li^+$ [6, 57, 58] a k růstovému defektu v prostředí s vysokým pH [57, 64].

#### 2.1.3.3 $Na^+/H^+$ antiporter Nha1

Dalším ze systémů zajišťujících export iontů alkalických kovů z buněk je $Na^+/H^+$ antiporter kódovaný genem *NHA1* [65]. Nha1 je protein dlouhý 985 AA uspořádaných pravděpodobně do 12 TMS [66, 67], který se v PM vyskytuje ve formě funkčního dimeru [68]. Dimerizace probíhá pravděpodobně již v endoplasmatickém retikulu a vede ke konformační změně ve struktuře monomerů, která je nutná pro správnou funkci antiporteru Nha1 [68]. Vznik dimeru umožňuje správný průchod proteinu sekreční dráhou až do PM a v případě, že je dimerizace narušena (např. mutací D145N), tak se protein do PM nedostane [68]. Na základě sekvence byla predikována topologie Nha1p tak, že po krátkém hydrofilním N-konci (13 AA) následuje hydrofobní transmembránová část (AA zbytky 14 – 439 tvořící již zmíněných 12 TMS a spojujících hydrofilních smyček) a hydrofilní C-koncová část (zbylých 546 AA; [66]).

Porovnáme-li sekvenci Nha1p z *S. cerevisiae* s homology z jiných organismů (viz kapitola 2.1.6, str. 20), je první polovina proteinu (hlavně transmembránová část) vysoce konzervována nejen mezi homology Nha1p z jiných druhů kvasinek (např. Sod2p z *Schizosaccharomyces pombe*) ale také s bakteriálními (např. NhaA z *Escherichia coli*), rostlinnými (např. *SOS1* z *Arabidopsis thaliana*) a živočišnými (např. lidskými NHE1-9, nebo NHA2) homology patřícími společně s Nha1p do „superrodiny“ CPA (cation proton antiporter) [66, 69-73]. Vysoký stupeň identity vykazují nejen jednotlivé predikované TMS, ale také některé smyčky, jež je spojují. Díky cílené i náhodné mutagenesi Nha1p v *S. cerevisiae* i homologů jiných kvasinek bylo identifikováno mnoho AA zbytků v transmembránové části důležitých pro transport iontů alkalických kovů a protonů [66, 74-78]. Jedním z dlouhodobých cílů při výzkumu Nha1p je vznik modelu na základě získaných dat a také dříve publikované struktury bakteriálního homologu NhaA (jediná struktura proteinu ze „superrodiny“ CPA v dostatečném rozlišení získaná na základě rentgenové difrakce; [79]). Jde hlavně o vytvoření modelu struktury (konzervované) hydrofobní transmembránové části Nha1p, jež by pomohla osvětlit detaily mechanismu transportu, aktivity a substrátové specifity Nha1p a dalších homologních antiporterů.

Oproti transmembránové části je C-konec Nha1p (55% celého proteinu) konzervovaný pouze částečně, a to jen v rámci kvasinkových homologů. V C-koncích těchto antiporterů bylo nalezeno

6 konzervovaných sekvencí (C1-C6; [66, 80]). Role těchto sekvencí je různá. Zatímco některé části (C2-C3) transport iontů aktivují, jiné (C4-C6) ho inhibují [80]. Z celého C-konce je pro transport nezbytná pouze doména C1 (AA zbytky 434-449), která pravděpodobně přímo navazuje na transmembránovou část, obsahuje sekvenci umožňující průchod Nha1p sekreční dráhou a další AA regulující transportní aktivitu antiporteru [80, 81]. Odstranění zbylé části C-konce (od AA zbytku 473) nemá vliv na lokalizaci
a neovlivňuje transport $K^+$, zatímco transport $Na^+$ a $Li^+$ je snížen [82]. Je-li exprimována samotná C-koncová část Nha1p (AA zbytky 473-985), je vzniklý protein směrován do buněčného jádra, jelikož obsahuje motiv jaderné lokalizační sekvence, to indikuje dosud neprozkoumané regulační úlohy této části proteinu [83].

Z hlediska homeostase iontů alkalických kovů je nejdůležitější funkcí antiporteru Nha1p export $K^+$ a $Na^+$ [63, 84], jenž je poháněn gradientem $H^+$ vytvořeným přes PM pomocí $H^+$-ATPasy Pma1 (viz kapitola 2.1.5). Na izolovaných váčcích PM bylo prokázáno, že v případě Nha1p se jedná o elektrogenní transport, kdy je jeden $Na^+$ exportován při současném importu dvou protonů [85]. Na rozdíl od ATPas Ena (viz kapitola 2.1.3.2) je Nha1 aktivní při nižším vnějším pH, kde zajišťuje udržení homeostase $K^+$ a export toxických iontů. Dále se podílí na regulaci vnitrobuněčného pH, $\Delta\Psi$ a účastní se také regulace buněčného objemu [7, 13, 15, 63, 67]. V případě náhlého nárůstu vnitrobuněčného pH mohou být pomocí antiporteru Nha1 importovány potřebné protony umožňující dočasné přežití buněk, energii tomuto transportu dodává gradient $K^+$ orientovaný směrem ven z buňky [82].

Na základě dřívějších prací je zřejmé, že transport iontů alkalických kovů je zprostředkován transmembránovou částí Nha1p a role hydrofilního C-konce je spíše regulační [82, 83]. Kromě změny v transportní aktivitě $Na^+$ a $Li^+$ zhoršuje absence C-konce přežívání a růst buněk vystavených hyperosmotickému šoku [82, 83, 86], protože nemůže dojít k inaktivaci Nha1p zabraňující úniku $K^+$ a vody typickém při osmotickém stresu. Běžně je Nha1p při osmotickém šoku inaktivován pomocí fosforylace AA zbytků T765 a T876 v C-konci kinasou Hog1 (viz kapitola 2.2.2; [86]). Mimo tuto regulační úlohu Nha1p byla pozorována i funkce nesouvisející s homeostasí iontů alkalických kovů, jedná se schopnost ovlivnění buněčného cyklu. Zvýšená exprese Nha1p umožní buňkám překonat blok mezi fázemi $G_1$ a S, jež byl způsoben současnou delecí *sit4* a *hal3* [77, 87].

### 2.1.4 Vnitrobuněčné transportery $Na^+$ a $K^+$

Celková koncentrace iontů alkalických kovů v buňce je určována činností výše popsaných transporterů v PM, ale zdaleka není distribuce iontů uvnitř buněk rovnoměrná. Mezi důležité fyziologické parametry patří iontová síla, pH a koncentrace $K^+$ v cytosolu, ale také v jednotlivých

organelách, kde tyto parametry zásadně ovlivňují funkčnost organel. Vnitrobuněčné transportery, které nejčastěji fungují jako antiportery, obecně pomáhají zajistit správné rozložení $K^+$ v buňkách a sekvestraci $Na^+$ (také $Li^+$ a $Rb^+$), dále se účastní regulace objemu a pH organel [6]. Přísná regulace pH uvnitř váčků zapojených do endocytické nebo sekreční dráhy je důležitá pro správné směrování váčků [6, 88-90], které může být narušeno např. pouze malou změnou pH ve váčcích. V regulaci směřování váčků proto mají nezastupitelnou úlohu právě antiportery kationtů alkalických kovů a protonů. Při studiu vnitrobuněčných přenašečů je často nejdříve nutné odstranit výše zmíněné transportery umístěné v PM, aby bylo např. možné sledovat případné fenotypy delece genů pro transportery v organelách [89-91], které nemusí být běžně pozorovatelní díky funkci transporterů PM.

Dosud bylo identifikováno celkem pět systémů (obr. 2.1.1, str. 12), jež se podílejí na transportu iontů alkalických kovů mezi jednotlivými kompartmenty v buňkách *S. cerevisiae*. Tři z těchto transporterů, Nhx1 [92], Kha1 [93] a Vnx1 [94], patří mezi antiportery a transport zprostředkovaný těmito přenašeči je řízen protonovým gradientem vytvořeným $H^+$-ATPasou Vma (viz kapitola 2.1.5, str. 19). První dva antiportery (Nhx1p a Kha1p) jsou navíc sekvenčně velmi podobné antiporteru Nha1. Dalšími účastníky regulace vnitrobuněčného homeostase iontů alkalických kovů v organelách jsou proteiny Mkh1 [95], Mrs7, nebo Yld183 [96] a nově objevený $K^+$-$Cl^-$ kotransporter Vhc1 [91].

Nejlépe charakterisovaným intracelulárním transporterem je $Na^+(K^+)/H^+$-antiporter Nhx1 [92] lokalisovaný v prevakuolárních váčcích odpovídajících pozdnímu endosomu v živočišných buňkách. Mezi úlohy tohoto proteinu patří sekvestrace iontů alkalických kovů ($K^+$, $Na^+$, $Li^+$; [97]) do prevakuolárních váčků a do vakuoly [90, 98], regulace vnitrobuněčného pH a vesikulárního transportu. Aktivita Nhx1p dále ovlivňuje také sensitivitu k jiným chemickým látkám (např. k organickým kationtům) a změnám teploty [88-90]. Nhx1p se podílí rovněž na časné fázi adaptace buněk vystavených osmotickému stresu (viz kapitola 2.2, str. 22; [99]).

Podobnou úlohu jako Nhx1p hraje i antiporter Kha1, který byl lokalisován v membráně Golgiho aparátu [93, 100]. Jeho úloha v homeostasi iontů alkalických kovů ($K^+$, $Na^+$ a $Li^+$), regulaci pH a také vesikulárního transportu byla prokázána teprve nedávno [89, 93, 100]. Shodně s Nhx1p byla dále pozorována jistá úloha Kha1p v toleranci k dalším chemickým látkám a teplotním změnám. Ukazuje se, že antiportery Nhx1 a Kha1 jsou společně s malými GTPasami (Ypt6 a Arl1) důležité pro udržení a regulaci obousměrného vesikulárního transportu mezi Golgiho aparátem a prevakuolárními váčky, tento transport samotný může determinovat toleranci nejen k vysokým koncentracím solí, ale k řadě dalších stresů [89, 90]. Nově byla identifikována úloha vesikulárního transportu (a řady proteinů důležitých pro vesikulární transport včetně Nhx1p, Ypt6p a Arl1p) také v regulaci vstupu $K^+$ do buněk [25].

Za transporter zajišťující sekvestraci $K^+$ a $Na^+$ do vakuol byl po dlouho dobu považován prevakuolární Nhx1, dokud nebyl nalezen $Na^+(K^+)/H^+$ antiporter Vnx1, jež je svou sekvencí mnohem podobnější $Ca^{2+}/H^+$ antiporterům než typickým zástupcům $Na^+/H^+$ antiporterů . Přesto je specifický pro $Na^+$ a $K^+$ a netransportuje $Ca^{2+}$ [94]. Vnx1p využívá stejně jako Nhx1p a Kha1p protonový gradient vytvořený ATPasou Vma (kapitola 2.1.5, str. 19). Kromě transportu iontů, patří k jeho úlohám také regulace vnitrobuněčného pH a po jeho deleci (*vnx1*) byla ve vakuolách pozorována ještě zbytková (nespecifická) $Na^+$ transportní aktivita, za kterou je zřejmě odpovědný $Ca^{2+}/H^+$ antiporter Vcx1 [101], který je ale označován jako $Ca^{2+}$ specifický. Nedávno byl objeven další vakuolární transporter schopný ovlivňovat homeostasi iontů alkalických kovů. Jedná se o dosud jediný známý kotransporter $K^+$ a $Cl^-$ (homolog k těmto transporterům známým v jiných organismech) v *S. cerevisiae*, který dostal název Vhc1 [91]. Bylo prokázáno, že se Vhc1p skutečně účastní sekvestrace $K^+$ do vakuol. Aktivita Vhc1p ovlivňuje celkovou homeostasi $K^+$ a morfologii vakuol během časné fáze adaptace buněk na osmotický stres.

Existence systému zajišťujícího výměnu iontů $K^+$ a protonů přes mitochondriální membránu byla predikována již před více než 50 lety [102] a později byla také prokázána při měření toků iontů na izolovaných mitochondriích mnoha organismů [6]. Má se za to, že na rozdíl od vnější mitochondriální membrány je průchod malých nabitých molekul a iontů přes vnitřní membránu striktně řízen. Přesto dochází k neselektivnímu nízkoafinitnímu importu $K^+$ do mitochondrií přes jiné přenašeče, který je umocněn vnitřním záporným $\Delta\Psi$. Tento přísun $K^+$ (zvyšování osmotického tlaku) je pro mitochondrie nežádoucí a může vést k nasávání vody, bobtnání a až prasknutí mitochondrie. Tomu se mitochondrie brání $K^+/H^+$ antiportním transportem, kterého se účastní proteiny Mkh1 [95], Mrs7 a Yld183 [96]. Tyto proteiny samotné pravděpodobně netvoří antiporter, ale jsou jeho důležitými a navzájem nezávislými regulátory, mezi kterými nedochází k přímému kontaktu [96]. Antiporter odpovědný za aktivní export $K^+$ z mitochondrií je tedy stále neznámý a je označován zkratkou KHE ($K^+/H^+$ exchanger), protože se pravděpodobně jedná o komplex mnoha proteinů [95, 96].

### 2.1.5 Úloha $H^+$-ATPas pro udržení homeostase $K^+$ a $Na^+$

$H^+$-ATPasy jsou odpovědné za vytváření protonového gradientu přes PM i membrány organel, regulaci pH uvnitř i vně buněk a organel. Vzniklý gradient je využíván sekundárními aktivními transportery k přenosu důležitých živin, metabolitů a dalších látek ve směru (symport), nebo proti směru (antiport) gradientu protonů (kromě iontů alkalických kovů se jedná např. o transport aminokyselin, vitamínů, stopových prvků, glycerolu nebo $P_i$ [50, 103, 104]). Gradient protonů, rozdíl v koncentracích uvnitř a vně buněk, je tak jednou z důležitých součástí buněčné fyziologie umožňující

přežívání buněk. V *S. cerevisiae* jsou činností $H^+$-ATPas řízeny všechny známé antiportery, tedy Nha1p a dále intracelulárních Nhx1p, Kha1p a Vnx1p.

Esenciální gen *PMA1 [36]* kóduje $H^+$-ATPasu typu P tvořenou jedním proteinem lokalisovanou v PM. Mezi její úlohy patří export $H^+$ z buněk, tím reguluje vnitrobuněčné pH, okyseluje médium okolo buněk, tvoří protonový gradient a vytváří ΔΨ [37]. Pma1p je nejvíce zastoupeným proteinem v PM, je velice stabilní a spotřebovává až 20 % buňkou vyprodukovaného ATP [6, 105]. Díky svému vysokému zastoupení v PM je Pma1p využívána při studiích zaměřených na průchod nově vzniklých membránových proteinů sekreční drahou [105]. Kromě *PMA1* byl v genomu *S. cerevisiae* nalezen gen *PMA2* kódující také $H^+$-ATPasu sekvenčně velmi podobnou Pma1p. V porovnání s *PMA1* je exprese *PMA2* za standartních podmínek velice nízká, což snižuje význam *PMA2* pro tvorbu protonového gradientu přes PM [106].

Na rozdíl od Pma1p je vakuolární $H^+$-ATPasa Vma ($H^+$-ATPasu typu V) tvořena třinácti proteiny uspořádanými do dvou domén [107], transmembránové $V_0$ a periferní $V_1$. Sekvenčně i strukturně je ATPasa Vma velice podobná mitochondriálním ATP synthasam typu F [108]. Vma zajišťuje okyselování vnitřního prostoru vakuol, všech váčků a organel včetně endoplasmatického retikula a Golgiho aparátu, podílí se na regulaci transportu sekrečních váčků a je odpovědná za tvorbu protonového gradientu na vnitrobuněčných membránách [109-111]. Tento gradient pak umožňuje fungování výše pospaných vnitrobuněčných antiporterů Nhx1, Kha1 a Vnx1.

### 2.1.6 Studium antiporterů z jiných organismů v *S. cerevisiae*

Antiportery sekvenčně homologní k Nha1p z *S. cerevisiae* patří do již zmíněné „superrodiny" CPA [69]. Jedná se o jak prokaryontní, tak eukaryotní transportery umístěné v PM, nebo v membránách organel. Společným znakem členů rodiny CPA je to, že se predikovaná transmembránová část přenašeče vždy skládá z 10-12 hydrofobních α-helixů vykazujících silnou sekvenční podobnost. Jednotlivé proteiny se mohou lišit v řadě parametrů: substrátovou specifitou, délkou hydrofilních konců, regulací, nebo tím, jestli jsou elektrogenní či elektroneutrální [69, 71, 84]. Studium těchto antiporterů je také v jiných organismech stále velice aktuální a atraktivní. Jedná se např. o studie zaměřené na osmotoleranci nekonvenčních kvasinek potenciálně využitelných pro biotechnologické aplikace [84, 112], studium chování patogenních kvasinek rodu *Candida* [113, 114], výzkum rostlinných antiporterů ovlivňujících osmotoleranci rostlin [115, 116], ale také antiporterů savčích a lidských, které hrají důležité role např. v diferenciaci buněk, ve funkci ledvin, ale také v regulaci buněk nádorových [73, 117, 118, 70, 119, 120]. Mezi nejstudovanější patří savčí/ lidské antiportery NHE, které se v organismu účastní regulace celé řady důležitých procesů, jako je např. angiogenese, migrace buněk, růst a proliferace, apoptosa, syntesa DNA nebo glykolysa [121]. Zkoumají se také

antiportery specifické pro jednotlivé tkáně. Jedním z takových je lidský $Na^+/H^+$ antiporter NHAoc/NHA2. Tento antiporter je exprimován ve vysokém množství pouze v osteoklastech, kde zajišťuje správnou diferenciaci buněk [118]. Porucha v jeho funkci může mít vážné následky ve formě závažných onemocnění tvorby kostní tkáně (*osteopetrosa*) a vede až ke smrti jedince.

Při výzkumu $Na^+/H^+$ antiporterů jiných organismů (ale také např. iontových kanálů [122]) bývá s úspěchem využíván modelový organismus *S. cerevisiae* [7, 84, 112, 114, 115, 119]. Výhodou využití heterologní exprese v kvasinkách je mimo jiné jasně definované a dobře popsané genetické pozadí, ale také jednoduchost a finanční i časová nenáročnost (např. v porovnání se savčími buněčnými kulturami) vnášení genů, mutací a jiných genových manipulací [123]. Při výzkumu aktivity a vlastností transporterů iontů alkalických kovů heterologní expresí lze navíc využít upravených kmenů *S. cerevisiae*, které postrádají geny svých vlastních transporterů [6, 7]. Tak je možné studovat izolovaně také transportery, jejichž aktivita je v původním organismu velmi nízká nebo překrytá existencí řady dalších transporterů pro tytéž substráty.

## 2.2 Regulace homeostase iontů alkalických kovů

V předchozí části (kapitola 2.1, str. 9) byly pospány jednotlivé přenašeče zajišťující přenos iontů alkalických kovů. V přirozeném prostředí jsou buňky vystaveny často velmi proměnlivým podmínkám a udržení homeostase iontů alkalických kovů v buňkách je důležitým předpokladem pro přežití, růst a dělení buněk. Aktivita jednotlivých transporterů je proto silně regulována, a to na úrovni regulace exprese genů nebo na úrovni post-translačních modifikací. Dále lze aktivitu transporterů ovlivnit také např. složením membrány nebo změnou vnějšího pH [6, 124-127]. Dalším kritériem dělení regulátorů homeostase iontů alkalických kovů je také, jestli se jedná obecně o regulaci časné, nebo adaptivní odpovědi např. na osmotický stres. Zatímco časná odpověď zahrnuje rychlý souběh dějů, který se odehraje ihned po působení stresu a umožnuje buňkám krátkodobé přežití ve změněných podmínkách (selektivní změny v expresi genu nejsou zpravidla patrné), adaptivní odpověď zajišťuje dlouhodobější adaptaci zahrnující změnu v úrovni exprese genů [6, 64, 128]. Hyperosmotický stres obecně způsobený zvýšenou koncentrací osmolytu (např. NaCl) v prostředí vede ke ztrátě vody společně s $K^+$ a dochází ke zmenšování buněk [19]. Časná buněčná odpověď na tento stres se skládá mimo jiné z post-translační regulace proteinů, konkrétně zastavení výstupu $K^+$ (fosforylací Nha1p) a zastavení výstupu glycerolu (důležitého buněčného osmolytu; [19]). Adaptivní fáze odpovědi obsahuje změny v expresi genů, např. indukci exprese *ENA1* a genů pro tvorbu glycerolu [19].

Regulace na úrovni exprese genů je jedním z nejznámějších a nejdéle studovaných regulačních dějů [125, 126] a díky pokročilým metodám se v současné době v kvasinkách dostávají do popředí studie na úrovni celého genomu [62] zachycující celkový obraz regulace exprese genů v organismu za daných podmínek. Ukazuje se, že kromě přítomnosti transkripčních faktorů, aktivátorů a represorů, se na této regulaci výrazně podílí také řada dalších faktorů, jako je např. struktura chromatinu nebo tvorba smyček umožňujících klastrování (a společnou regulaci) „spřízněných“ genů [126, 129]. Exprese proteinu může být ovlivněna také při přepisu genetické informace do molekuly mRNA, jejíž stabilita (rychlost degradace) zásadně ovlivňuje množství vzniklého proteinu. Degradace mRNA obecně probíhá buď specificky pro určitou mRNA, nebo nespecificky a existuje několik možných cest degradace, jako např. deadenylace 3´ konce mRNA a rozštěpení za pomoci exosomu, degradace pomocí endonukleas, nebo odštěpení čepičky z 5´ koncové části mRNA [124].

Oba výše uvedené děje (regulace exprese genů a stabilita mRNA) jsou zastoupeny také při reakci buněk na osmotický stres [127]. V tomto případě dochází po účinku osmotického stresu obecně k destabilizaci mRNA a utlumení exprese, ale množství mRNA pro proteiny nutné k adaptaci buněk na osmotický stres je zvýšeno vlivem jak růstu exprese příslušných genů, tak specifickým zvýšením stability těchto mRNA. Společným výsledkem je zvýšení množství proteinů nutných pro adaptaci na

osmotický stres [127], jako hlavní regulační článek v tomto ději byla identifikována stresem aktivovaná kinasa Hog1 ([130]; viz dále).

Na rozdíl od většiny organismů a také jiných druhů kvasinek [131], nebyla u *S. cerevisiae* nalezena dráha RNA interference [132] zajišťující post-transkripční umlčování genů pomocí siRNA (silencing). Tento stupeň regulace exprese genů v *S. cerevisiae* zcela chybí.

Post-translační modifikace obecně umožňují řízení funkce, lokalizace nebo degradace již syntetizovaných proteinů. V homeostasi iontů alkalických kovů je nejdůležitější modifikací fosforylace [133]. Fosforylovány mohou být nejen samotné transportery iontů alkalických kovů, ale také transkripční faktory ovlivňující míru exprese některých z nich (viz dále).

Kromě výše zmíněných možností existují ještě další cesty, kterými lze ovlivnit činnost transporterů iontů alkalických kovů. Jednou z nich je změna ΔΨ, jež může vést např. k otevření napěťově řízených kanálů Tok1 [30, 53] nebo ke změnám v nespecifickém importu kationtů (včetně kationtů alkalických kovů). Umístění transporterů v tzv. raftech patří mezi další možnosti regulace aktivity [134] a uplatňuje se pravděpodobně také v transporterů $K^+$ a $Na^+$. Jak bylo dříve ukázáno, patří transportery Nha1 [135] a Trk1 [25, 136] mezi proteiny, jež se v raftech vyskytují, a lze předpokládat, že jejich aktivita bude do značné míry regulována správným složením raftů stejně, jako tomu je u Pma1p. Je-li porušena tvorba a sekrece nasycených sfingolipidů do PM (a tím i do raftů), může dojít k destabilizaci a nesprávnému průchodu Pma1p sekreční drahou. Protein je následně transportován do vakuol [105] a nemůže plnit svou úlohu v PM.

### 2.2.1 Regulace importu $K^+$

Jak bylo uvedeno výše, vstup $K^+$ do buněk zajišťují transportery Trk1 a Trk2, které jsou schopné pracovat jak ve vysokoafinitním (za nedostatku $K^+$ nebo v přítomnost $Na^+$), tak v nízkoafinitním (při dostatku $K^+$ v okolí) režimu [137]. Exprese obou importerů není podle všeho ovlivňována odchylkami v homeostasi iontů alkalických kovů [6], ačkoli některé případy byly pozorovány. Exprese *TRK1* a *TRK2* U *TRK1* dochází k inhibici exprese během stacionární fáze růstu [138] a jako inhibitor exprese *TKR2* byl nalezen komplex proteinů Rpd3 a Sin3, který byl dříve identifikován jako komplex odpovědný za deacetylaci histonů. [44, 139].

Přestože je regulace Trk1p a Trk2p na post-translační úrovni již dlouho studována, není dosud mechanismus regulace plně objasněn. Již dříve byla identifikována a následně potvrzena aktivace Trk1p pomocí glukosy a dalších fermentovatelných zdrojů uhlíku [6, 24]. Pro dosažení plné aktivity transporterů zajišťujících vstup $K^+$ do buněk je důležitá i kinasa Snf1, jež se také účastní metabolismu

cukrů [140]. Její účinek na transportery Trk1 a Trk2 je spíše nepřímý, zřejmě prostřednictvím transkripčního faktoru Sip4 [140].

Mezi identifikované aktivátory transporterů Trk1 a Trk2 patří kinasy kódované geny *HAL4* a *HAL5*, jejichž substrát zatím není znám. Tyto kinasy stabilizují transportery v membráně (kromě Trk1p a Trk2p také např. přenašeče živin Hxt1 a Fur4) v prostředí nízké koncentrace $K^+$ a zabraňují endocytose přenašečů a jejich degradaci ve vakuole [141, 142]. Ukazuje se, že účinek kinasy Hal5 na Trk1p je závislý na aktivitě $Ca^{2+}$ aktivované fosfatasy kalcineurinu [143]. Kalcineurin byl již dříve identifikován jako nezbytná součást procesu přechodu od nízkoafinitního k vysokoafinitnímu režimu importu $K^+$, který je aktivován zvýšenou koncentrací $Na^+$ v prostředí a slouží k zvýšení selektivity importu ve prospěch $K^+$ [137]. Nově byla důležitost kalcineurinu pro vysokoafinitní import $K^+$ prokázána také bez přítomnosti $Na^+$ [143]. Kalcineurin také pravděpodobně tvoří spojovací prvek mezi Trk1p a proteinem Ypi1, jeho nedávno objeveným regulátorem. Ypi1 je regulační podjednotkou proteinfosfatasy Glc7, jenž reguluje další buněčné děje, jako je mitosa, metabolismus glykogenu, nebo sporulace [144].

Nepřímý účinek na aktivitu Trk1p má také Hal3p regulující nejen vstup $K^+$ do buněk, ale také výstup $Na^+$ a $Li^+$ z buněk [145]. Hal3p byl identifikován jako negativní regulátor fosfatasy Ppz1, která je sama negativním regulátorem vstupních (Trk1p) i výstupních (Ena1p) transporterů iontů alkalických kovů (viz dále), účinek Hal3p je tedy na aktivitu transporteru Trk1p positivní. Fosfatasa Ppz1 se společně Trk1p vyskytuje v membránových raftech, kde dochází k jejich fyzické interakci a ačkoli nebyla defosforylace Trk1p pomocí Ppz1p přímo prokázána, byla v kmenech postrádajících *PPZ1* stanovena nižší úroveň fosforylace Trk1p než je běžné [136]. Účinek Hal3p na Ppz1p je závislý na vnitrobuněčném pH, proto je systém regulace Hal3-Ppz1 považován za vnitrobuněčný senzor pH, který kromě vnitrobuněčného pH ovlivňuje i koncentraci $K^+$ [6, 136].

Kinasa Sky1 se také účastní homeostase iontů alkalických kovů jako negativní regulátor a při její absenci dochází ke zvýšení tolerance buněk k organickým i anorganickým kationtům včetně $Na^+$ a $Li^+$ [146]. Ačkoli některé práce potvrzují možnost ovlivňování homeostase kinasou Sky1 prostřednictvím transporterů Nha1, Kha1, a Tok1 [147], jiné práce ukazují, že je pravděpodobnější regulace prostřednictvím přímého nebo nepřímého ovlivňování aktivity Trk1p [6, 146].

Již dříve bylo nalezeno několik proteinů známých svou úlohou v regulaci vesikulárního transportu, které inhibují import $K^+$ do buněk (např. [148]), ale nejnovější práce naznačuje, že spíš než jednotlivé proteiny ovlivňuje vstup $K^+$ do buněk (měřeno vstupem $Rb^+$) správná funkce vesikulárního transportu jako taková [25]. Lze předpokládat, že vesikulární transport ovlivňuje biogenesi a degradaci

membránových proteinů, ale přesný mechanismus, jakým je import $K^+$ ovlivňován, znám není. Podle všeho se jedná o obecnější způsob regulace, protože aktivita Trk1p může být ovlivněna velkým množstvím proteinů důležitých pro vesikulární transport (včetně Nhx1p; viz kapitola 2.1.4, str. 17), ale také kinasami fosforylujícími inositol a fosfatydilinositol (geny *KCS1*, *ARG82* a *FAB1*). Zapojení fosfatidilinositolových drah do regulace Trk1p bylo předpovězeno již před mnoha lety [24], ale možnost ovlivnění homeostase iontů alkalických kovů prostřednictvím změn ve složení raftů ještě čeká na prozkoumání.

V neposlední řadě byla identifikována možnost regulace vstupu $K^+$ pomocí antiporteru Nha1 [149]. Absence *NHA1* vedla podle této publikace [149] ke snížení afinity Trk1p ke $K^+$. Touto prací bylo dokázáno, že existuje funkční spojení mezi importery a exportery iontů alkalických kovů.

### 2.2.2 Regulace exportu iontů alkalických kovů

Systém exporterů iontů alkalických kovů je regulován mnoha cestami [6]. Nalézáme tu jak regulaci post-translační (Nha1p, Tok1p), tak regulaci na úrovni exprese (*ENA1*). Zatímco první cesta upravuje kinetické parametry transporterů (hlavně Nha1) a patří proto do časné odpovědi na stres způsobený $Na^+$ a $Li^+$, druhý typ regulace, regulace exprese, určuje množství transporteru v PM a kvůli časové prodlevě nutné k syntéze nových proteinů patří do odpovědí zpožděných, tzv. adaptivních.

Přes značnou rozdílnost hlavních exporterů iontů alkalických kovů (Ena1p a Nha1p) v sekvenci, mechanismu transportu (antiporter vs. ATPasa) i filosofii regulace (časná vs. adaptivní) nalézáme několik sdílených komponent v jejich regulaci. Podstatným prvkem řízení obou systémů (ale také kanálu Tok1) je dráha kinasy Hog1 [6, 86, 150]. Už při lehce zvýšeném množství $Na^+$ v okolí buňky (0,2-0,4 M) dochází k nastartování kinasové signální kaskády (označována jako dráha HOG; „High Osmolarity Glycerol“) vedoucí k fosforylaci Hog1p [150, 151]. Fosforylovaná kinasa Hog1 na jedné straně vstupuje do jádra a aktivuje expresi *ENA1* [151, 152] a dalších genů, na straně druhé sama fosforyluje transportery Nha1 a Tok1 v PM [86]. Hog1p se účastní odpovědi na osmotický stres, regulace metabolismu glycerolu a kromě zvýšené koncentrace $Na^+$ může být aktivován řadou dalších stresů, jako například teplotním šokem, hypoxií, nebo kyselinou octovou [128].

Mezi kinasy regulující homeostasi iontů alkalických kovů patří rovněž kaseinkinasa 2 (CK2). Jedním ze substrátů CK2 je polyfunkční transkripční faktor Nrg1p (viz také kapitola 2.2.2.1, str. 26), který mimo jiné inhibuje expresi genu *ENA1* [153, 154]. CK2 se skládá ze čtyř podjednotek, dvou katalytických (kinasových), kódovaných *CKA1* a *CKA2*, a dvou regulačních, kódovaných *CKB1* a *CKB2* [155], tvořících společně funkční heterotetramer. Na rozdíl od regulačních podjednotek se mohou Cka1p a Cka2p v tetrameru vzájemně zastoupit, nicméně delece genů pro obě kinasy je letální. Delece každé

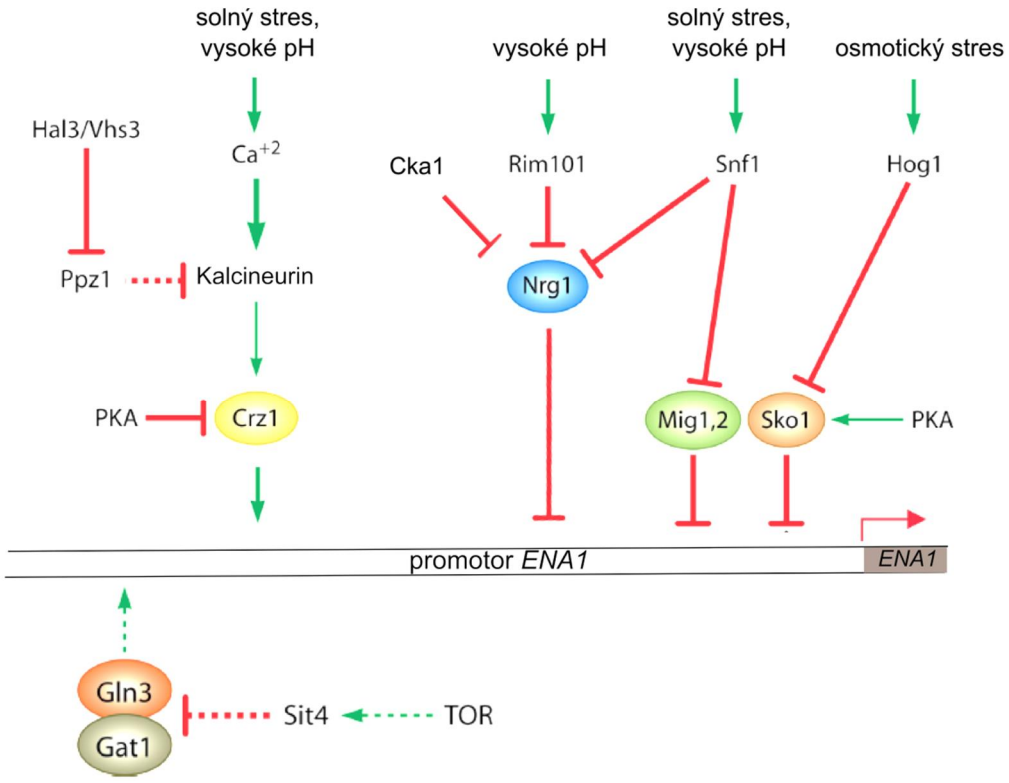
z podjednotek (regulační, nebo katalytické) vede k nárůstu citlivosti buněk k NaCl, v případě delece regulačních podjednotek (*ckb1*, nebo *ckb2*) je tato citlivost mnohem vyšší [153]. Má se za to, že absence jedné z regulačních podjednotek znemožňuje tvorbu tetrameru a kinasové podjednotky jsou pak mnohem méně aktivní, zatímco při deleci pouze jedné z kinasových podjednotek je její pozice v tetrameru a aktivita částečně zastoupena druhou podjednotkou. Ačkoli Cka1p a Cka2p vykazují vysokou sekvenční podobnost, bylo zjištěno, že kinasová aktivita Cka1p je *in vitro* mnohem citlivější k zvýšeným koncentracím NaCl, heparinu a sperminu než aktivita Cka2p [156]. Dále bylo zjištěno, že pouze Cka1p je schopna fosforylovat transkripční faktor Nrg1 a tím omezit jeho působení na represi *ENA1* [153, 154]. Ve výsledku tedy působí zvýšená aktivita Cka1p aktivačně a delece *cka1* inhibičně na expresi *ENA1* (viz dále; [153]). Specifická pro Cka1p (nikoli Cka2p) je také fosforylace Yfc1p, vakuolárního transporteru umožňujícího eliminaci toxických látek (iontů $Cd^{2+}$ a dalších těžkých kovů), která může být ovlivněna přítomností NaCl v médiu [157]. Kromě výše uvedených funkcí se CK2 a její podjednotky účastní regulace řady dalších důležitých fyziologických dějů, jako je např. flokulace, proliferace, odpověď buňky na poškození DNA, nebo regulace exprese RNA polymerasy I a III [153, 155, 156].

Na rozdíl od dalších exporterů (Nha1p a Ena1p), není v případě kanálu Tok1 mnoho známo o mechanismu regulace jak na úrovni exprese, tak post-translační regulace aktivity. Jak již bylo uvedeno, je transport kanálem Tok1 řízen napěťově (tedy $\Delta\Psi$) tak, že k otevření kanálu dochází při depolarisaci membrány [30]. Jediným dosud známým regulačním prvkem je fosforylace Tok1p kinasou Hog1, která byla prokázána, ale dosud nebyl prokázán žádný vliv této modifikace na aktivitu kanálu [86]. Obecně je Tok1p považován za transporter potřebný pro udržení dlouhodobé homeostase a podle dostupných dat je exprese *TOK1* konstitutivní [158]. Na základě nedávno publikovaného modelu homeostase iontů alkalických kovů [14] bylo predikováno, že dříve prokázaná fosforylace Tok1p pomocí Hog1p vede pravděpodobně k poklesu aktivity Tok1p.

#### 2.2.2.1 Regulace ATPasy Ena1

ATPasa Ena1 je kódována prvním z řady genů *ENA* umístěných v tandemové repetici. Jak již bylo uvedeno, z genů *ENA* je *ENA1* nejlépe prozkoumaný a je považován za nejdůležitější prvek determinující toleranci buněk k vysokým koncentracím solí alkalických kovů. Na rozdíl od ostatních genů v klastru, ale také na rozdíl od genů ostatních transporterů iontů alkalických kovů, je gen *ENA1* silně regulován na úrovni exprese. Ke značnému nárůstu exprese *ENA1* dochází při zvýšených koncentracích $Na^+$, $K^+$ nebo $Li^+$ v médiu, při osmotickém stresu a při vyšším extracelulárním pH [57, 58, 152, 159]. Ačkoli se *de facto* jedná pouze o několik málo stimulů aktivujících expresi genu, vstupuje do regulace exprese *ENA1* několik fyziologicky velmi důležitých signálních drah, jejichž

působení se integruje do signálu regulace promotoru *ENA1* (obr. 2.2.2.1, str. 27; [6, 152]). Možnost regulace Ena1p na post-transkripční úrovni byla předpokládána dosud pouze v jediné práci [58], podle které se na regulaci podílí jeden z regulátorů mající zároveň svou úlohu v regulaci exprese *ENA1*, konkrétně se jedná o kalcineurin (viz výše).




**Obr. 22.2.2.1 Regulace exprese *ENA1***
Obrázek zachycuje známé regulátory exprese *ENA1* a ukazuje, jestli se jedná o inhibici (červená čára s tupým koncem) nebo aktivaci (zelená šipka). Interakce, které nejsou plně prokázány, jsou vyznačeny nespojitou čárou.

*zdroj: převzato a upraveno podle [6]*

První z důležitých faktorů regulujících expresi *ENA1* je již zmíněná signální dráha kinasy Hog1 (obr. 2.2.2.1, str. 27). Už při mírně zvýšených koncentracích $Na^+$ v prostředí (0,2-0,4 M; [151]) je Hog1p aktivována fosforylací a následně se podílí nejen na časné fázi odpovědi na tento stres (fosforylace Nha1p, viz dále), ale i na odpovědi adaptivní tak, že aktivovaná kinasa Hog1 fosforyluje transkripční faktory aktivující expresi *ENA1* [152]. Aktivace Hog1p a přenos signálu signální kaskádou vede ve finále k fosforylaci transkripčního faktoru Sko1 [160], který v nefosforylované formě tvoří společně s dalšími faktory komplex inhibující expresi *ENA1* tak, že se tento komplex represorů váže na specifické místo v promotoru *ENA1* označované jako CRE (cyclic-AMP response element; [161]). Tvorba komplexu represorů obsahujících Sko1p je po aktivaci signální kaskády HOG (po fosforylaci Sko1p) inhibována a exprese *ENA1* je tím posílena. Zajímavé je, že Sko1p může být fosforylován také pomocí proteinkinasy A (PKA), jejíž aktivita závisí na přítomnosti cyklického adenosinmonofosfátu

(cAMP), známého druhého posla mezibuněčné signalizace [160]. Na rozdíl od dráhy HOG vede fosforylace Sko1p pomocí PKA ke zvýšené inhibici exprese *ENA1*. Kromě fosforylace Sko1p („de-represe“ *ENA1*) vstupuje aktivovaný Hog1p do regulace exprese *ENA1* ještě v dalším bodě, a to aktivací tvorby komplexu Rpd3-Sin3, jenž vede k deacetylaci histonů, nasednutí RNA polymerasy II a aktivaci exprese *ENA1* [162]. Dráha HOG je důležitá v prostředí se zvýšenou koncentrací $Na^+$, zatímco v přítomnosti $Li^+$ (50 – 100 mM) se Hog1p účastní aktivace exprese *ENA1* jen z menší části [163]. Nárůst exprese *ENA1* za vysokého pH je řízen spíše bez účasti Hog1p, účastní se další dráhy, které jsou popsány dále [6].

Druhou důležitou dráhou, jež se regulace exprese *ENA1* účastní, je signální dráha fosfatasy kalcineurinu (obr. 2.2.2.1, str. 27). V *S. cerevisiae* je kalcineurin tvořen dimerem obsahujícím kromě jedné fosfatasové (redundantně kódované geny *CNA1* a *CNA2*) jednou regulační (*CNB1*) podjednotku, jejíž delece (*cnb1*) vede ke zvýšené citlivosti k $Na^+$, $Li^+$ a zásaditému vnějšímu pH [137]. Kalcineurin je aktivován přítomností volného $Ca^{2+}$ v cytoplasmě, k nárůstu vnitrobuněčné koncentrace $Ca^{2+}$ dochází vlivem solného stresu [164, 165], a následně dochází k aktivaci řady genů způsobené hlavně defosforylací transkripčního faktoru Crz1 (obr. 2.2.2.1, str. 27) a jeho vstupem do jádra [6]. Dvě vazebná místa pro Crz1p zajišťující aktivaci exprese se nacházejí také v promotoru *ENA1* [166]. Do regulačního účinku kalcineurinu vstupuje několik dalších faktorů. Prostřednictvím kalcineurinu působí na homeostasi iontů alkalických kovů již zmíněný Ypi1p (viz kapitola 2.2.1, str. 23), zvýšená exprese *YPI1* vede k aktivaci kalcineurinové dráhy a ke zvýšení tolerance buněk k $Li^+$, zatímco jeho delece (*ypi1*) toleranci k $Li^+$ snižuje [144]. Prostřednictvím kalcineurinu působí také inhibitory exprese *ENA1*, jako např. PKA [152], která je schopna fosforylovat Crz1p a tím inhibovat signál kalcineurinu a snížit expresi *ENA1* (obr. 2.2.2.1, str. 27; [6]). Negativní účinek jako PKA má také výše zmíněná fosfatasa Ppz1p (viz kapitola 2.2.1, str. 23), která se podílí na regulaci importu $K^+$ (Trk1p) a která je společně s Hal3p označována jako vnitrobuněčný senzor pH [6, 136]. Mechanismus účinku fosfatasy Ppz1 není plně objasněn, ale pravděpodobně se účastní deaktivace dráhy kalcineurinu, protože u mutantů postrádajících Ppz1 je tato dráha konstitutivně aktivována [167]. Represivní působení Ppz1p na expresi *ENA1* může být inhibováno Hal3p [6, 168], stejně jako v případě Trk1p (viz kapitola 2.2.1, str. 23) je tedy Hal3p také pro *ENA1* positivním regulátorem, bez kterého není možné dosáhnout plné indukce *ENA1*. Jak se ukazuje, je kalcineurinová dráha důležitým spojovacím prvkem mezi homeostasí iontů alkalických kovů a mnoha dalšími důležitými buněčnými pochody jako je např. regulace buněčného cyklu, nebo tvorba acetylkoenzymu A. Celkově se kalcineurin podílí na asi 40 % transkripční odpovědi *ENA1* za vysokého pH [6].

Podobně jako u kalcineurinu a Crz1p dochází k integraci signálů více signálních drah u transkripčního faktoru Nrg1 (obr. 2.2.2.1, str. 27). V rámci promotoru *ENA1* byla predikována 2 - 3 vazebná místa pro aktivovaný Nrg1p, který vazbou na promotor způsobuje inhibici exprese [6, 154, 169]. S ohledem na homeostasi iontů alkalických kovů jsou dosud známy tři faktory omezení represivního působení Nrg1p (jde tedy o aktivátory exprese *ENA1*), a to transkripční represor Rim101 a kinasy CK2 a Snf1. CK2 byla podrobněji popsána v předchozí kapitole (kapitola 2.2.2, str. 25). Pouze jedna z podjednotek CK2, Cka1 je schopna fosforylovat Nrg1p a tím jeho represivní působení na promotor *ENA1* omezit [153]. Druhou regulační drahou zasahující do exprese *ENA1* prostřednictvím represoru Nrg1 je dráha pojmenovaná podle transkripčního faktoru Rim101 ovlivňujícího hlavně meiosu a sporulaci [6, 170]. Na rozdíl od Cka1p, Rim101p inhibuje expresi *NRG1* (a tím se podílí na regulaci exprese *ENA1*; obr. 2.2.2.1, str. 27) za vysokého vnějšího pH, zatímco při vysokých koncentracích $Na^+$($Li^+$) není tato dráha příliš aktivní [6, 170]. Delece *rim101* způsobuje citlivost buněk jak k vysokému pH, tak k vysokým koncentracím $Na^+$ [169, 170]. Rim101p byl nedávno identifikován také jako důležitý faktor ovlivňující přežívání buněk ve slabých kyselinách [171], proto je zřejmé, že úloha Rim101p může být především udržování vhodného vnitrobuněčného pH a adaptace na změny v pH prostředí.

Prostřednictvím Nrg1p působí také další regulátor exprese *ENA1*, kinasa Snf1 (obr. 2.2.2.1, str. 27). Při změně zdroje uhlíku z glukosy na galaktosu, nebo rafinosu dochází k mnoha změnám v metabolismu *S. cerevisiae*. Mimo jiné byla na jmenovaných zdrojích uhlíku pozorována zvýšená exprese *ENA1* [172], kterou způsobuje kinasa Snf1 aktivovaná při nedostatku glukosy. Kromě glukosy, může být Snf1p aktivována také pomocí zásaditého pH, a tím se tato kinasa výrazně podílí na indukci exprese *ENA1* v bazickém prostředí [64, 172] nezávisle na dráze HOG a kalcineurinu. Dosud byly identifikovány dva transkripční faktory zajišťující přenos signálu po aktivaci Snf1p na promotor *ENA1*, a to Mig1p (alternativně Mig2p) a již zmíněný Nrg1p [6]. Nedostatkem glukosy aktivovaný Snf1p vstupuje do jádra a zabraňuje Mig1p ve tvorbě komplexu s dalšími transkripčními faktory a nedochází tak k inhibici exprese *ENA1*, zatímco v případě aktivace Snf1p v prostředí se zvýšenou koncentrací solí alkalických kovů je exprese *ENA1* ovlivňována spíše přes dráhu Nrg1p (Snf1p nevstupuje do jádra) [64, 172]. V bazickém prostředí je pomocí aktivovaného Snf1p exprese *ENA1* indukována oběma cestami, tj. deaktivací represoru Nrg1p i Mig1p (obr. 2.2.2.1, str. 27; [64]).

Regulace exprese *ENA1* probíhá ještě pomocí dráhy TOR pojmenované podle kinasy Tor1 [173], jejíž aktivita je inhibována v přítomnosti imunosupresiva rapamycinu (TOR je zkratkou „Target Of Rapamycine“) a v prostředí s nedostatkem nebo metabolicky špatně dostupným zdrojem dusíku. Dráha TOR je považována za hlavní regulační mechanismus zajišťující regulaci růstu v závislosti na dostupnosti živin [174]. Přechodná indukce *ENA1* byla pozorována po inhibici dráhy TOR působením

rapamycinu na buňky [173]. Indukce je podmíněna přítomností transkripčních faktorů Gat1 a Gln3, jenž se váží na specifické sekvence GATA v promotoru (v promotoru *ENA1* jich je 6) a aktivují expresi [173, 175]. Mezičlánkem je pravděpodobně ještě fosfatasa Sit4 (viz kapitola 2.2.2.2, str. 30) odpovědná za přenos signálu tak, že aktivovaný Tor1p aktivuje Sit4p, který následně inhibuje působení Gln3p a Gat1p [6, 172, 176]. Ve výsledku je tedy Tor1p negativním regulátorem exprese *ENA1* (obr. 2.2.2.1, str. 27).

Kromě zmíněných regulátorů jsou s regulací exprese *ENA1* spojeny také produkty genů *HAL1* a *REF2* a některé další faktory se na této regulaci mohou podílet nepřímou formou [6]. Řada z těchto regulací byla v rámci výzkumných prací zatím spíše pouze naznačena a jejich úloha v celém obrazu regulace exprese *ENA1* čeká na objasnění.

V nedávno zveřejněné práci bylo na základě počítačové simulace predikováno, že růst exprese *ENA1* může být důležitým krokem pro obnovení ΔΨ po přenesení buněk do prostředí s vysokou koncentrací $K^+$, nebo při vysokém pH [14]. Obnovení ΔΨ pokleslého díky popsanému stresu může pomáhat k přežívání buněk. Zajímavé je, že podle této predikce by měl být vliv změn exprese *ENA1* na ΔΨ poměrně velký, ale zatím nebyl prokázán experimentálně, ačkoli transportery Ena byly v minulosti tématem mnoha studií [6].

### 2.2.2.2 Regulace antiporteru Nha1

Na rozdíl od *ENA1* byla u *NHA1* pozorována stabilní exprese, která se neměnila vlivem vnější koncentrace $Na^+$ nebo pH [63] a dosud nebyly nalezeny ani jiné podmínky, které by vedly k signifikantním změnám v jeho expresi. Antiporter Nha1 je tak považován za protein důležitý pro permanentní udržování homeostase iontů alkalických kovů, který je v buňkách stále přítomen v téměř konstantním množství, a jehož regulace je řízena na úrovni post-translační. Ačkoli je Nha1p intenzivně studován již mnoho let, je jedinou prokázanou modifikací fosforylace dvou Thr zbytků (T765 a T876) aktivovanou kinasou Hog1 [86]. Tato fosforylace je důležitou součástí včasné odpovědi buněk na hyperosmotický šok (vyvolaný NaCl), jež pomáhá k přežívání buněk do té doby, než se nastartují další adaptivní mechanismy (např. indukce exprese *ENA1*). Později bylo zjištěno, že osmotický šok způsobený sorbitolem vede po aktivaci Hog1p ke snížení exportu $K^+$ přes Nha1p [83]. Omezení výstupu $K^+$ vede v prostředí s vysokou osmolaritou ke snížení úniku vody a v prostředí s $Na^+$ nedochází k tak snadné výměně vnitrobuněčných iontů $K^+$ za toxické $Na^+$.

Výstup $K^+$ může být při solném stresu také aktivován zvýšenou expresí již zmíněné fosfatasy Sit4 indukovanou zvýšenou koncentrací $Na^+$, $K^+$ a $Li^+$ [176]. Dále bylo zjištěno, že výstup $K^+$ může být prostřednictvím Sit4p (a pouze za přítomnosti Nha1p) positivně regulován produktem genu *SAP185*

(při zvýšené expresi *SAP185* je výstup $K^+$ aktivován) a negativně regulován produktem genu *SAP155* (jehož zvýšená exprese vede k inhibici exportu $K^+$) [177]. Fosfatasa Sit4 tedy pravděpodobně hraje roli nejen při regulaci *ENA1* (viz předchozí kapitola), ale také při regulaci Nha1p. Jako důkaz o vzájemné interakci mezi proteiny Sit4 a Nha1 se uvádí již zmíněný fakt, že blok mezi fázemi $G_1$ a S způsobený současnou delecí *sit4* a *hal3* může být překonán zvýšenou expresí *NHA1* [77, 87].

Kromě výše uvedených interakcí bylo na základě více studií identifikováno ještě několik dalších proteinů, které s Nha1p přímo interagují, jako je Cos3p, který svou přítomností zvyšuje toleranci buněk k solím [178], nebo např. proteiny Ppz1, Hsp30 a další [179], úloha interakcí však ještě nebyla prostudována. Jak bylo uvedeno, jedinou dosud známou post-translační modifikací je fosforylace pomocí Hog1p, ačkoli metodou hmotnostní spektroskopie bylo v minulosti identifikováno ještě alespoň 12 dalších AA zbytků fosforylovaných za různých podmínek dosud neznámými kinasami (přehled viz publikace č. 4, str. 41). Dále byla v rámci této práce predikována ještě celá řada dalších potencionálních míst fosforylace, která jsou sekvenčně i na základě předpokládané topologie pro fosforylaci vhodná.

### 2.2.3 Další proteiny podílející se na homeostasi iontů alkalických kovů, proteiny 14-3-3

Mezi potenciální regulátory homeostase iontů alkalických kovů byly identifikovány také proteiny 14-3-3 tvořící rodinu vysoce konzervovaných, malých, kyselých proteinů, které se vyskytují ve formě funkčního dimeru ve všech dosud zkoumaných eukaryotních buňkách z pravidla ve více isoformách. Jedná se o rozpustné proteiny, které interagují s velkým množstvím (stovkami) partnerů a účastní se tak řady důležitých procesů. Ačkoli dosud nebyla role těchto proteinů plně objasněna, dosavadní studie ukazují, že vytvoření vazby mezi proteinem z rodiny 14-3-3 a jeho interakčním partnerem hraje důležitou roli např. při aktivaci, nebo inaktivaci některých enzymů, ovlivňuje subcelulární lokalizaci některých transkripčních faktorů, může stimulovat interakci jiných proteinů, nebo může působit jako chránící skupina specifického aktivního místa některých enzymů [180-183]. Ačkoli se nejedná o kinasy, ani o fosfatasy, hraje fosforylace interakčního partnera důležitou úlohu v tom, jestli k vazbě s proteinem z rodiny 14-3-3 dojde či nikoli.

V *S. cerevisiae* existují dvě isoformy proteinů 14-3-3 kódované geny *BMH1* a *BMH2*. Dominantní úlohu hraje protein Bmh1 (267 AA) s 80% zastoupením, zatímco Bmh2p (273 AA) tvoří v buňkách pouze zbylých 20 % [184]. O důležitosti těchto proteinů svědčí to, že vykazují tzv. syntetickou letalitu, tedy delece obou genů zároveň nebo nefunkčnost obou proteinů vede ke smrti buňky ve většině studovaných kmenů *S. cerevisiae*. Na základě proteomické analysy bylo identifikováno v *S. cerevisiae* asi 270 interakčních partnerů a dalšími pracemi byla potvrzena regulační úloha proteinů Bmh1 a Bmh2 v řadě signálních drah [185].

Z experimentálně potvrzených interakčních partnerů Bmh1p a Bmh2p uveďme např. Snf1p, Hal4p, nebo Ppz1p, tedy proteiny účastnící se regulace homeostase iontů alkalických kovů, $\Delta\Psi$ a vnitrobuněčného pH (viz kapitola 2.2, str. 22). Účel těchto interakcí je zatím neznámý. Možnost zapojení proteinů 14-3-3 do regulace homeostase iontů alkalických kovů také naznačuje zvýšená citlivost kmene postrádajícího gen *BMH1* k $Na^+$ a k některým organickým kationtům (např. hygromycin B; [29, 186]), ale také silná indukce exprese *BMH1* při zvýšených koncentracích NaCl [159]. Je třeba dodat, že u jiných organismů bylo již dříve provedeno několik studií ukazujících funkční propojení mezi transportery iontů alkalických kovů a proteiny 14-3-3, které byly identifikovány např. jako regulátory savčích $Na^+/H^+$ antiporterů [187] a rostlinného $K^+$ kanálu Tpk1 [188].

# 3 Cíle práce

Hlavním cílem předkládané disertační práce byla charakterisace fyziologických úloh $Na^+/H^+$ antiporterů v kvasinkách. Práce vznikla v prostředí intenzivní mezinárodní spolupráce při výzkumu homeostase iontů alkalických kovů v kvasinkách v rámci projektu ERA SysMo Translucent I a II.

Pro zjištění fyziologických úloh $Na^+/H^+$ antiporterů v buňkách kvasinek bylo třeba připravit celou sérii mutantních kmenů postrádajících v různých kombinacích geny kódující jednotlivé transportery iontů alkalických kovů, charakterisovat základní vlastnosti a fyziologické parametry těchto kmenů, a nakonec tyto kmeny cíleně využít pro studium úloh $Na^+/H^+$ antiporterů plasmatické membrány

K hlavním cílům této disertační práce patří:

1) Zjistit, zda existují rozdíly v obsahu iontů alkalických kovů mezi různými kmeny *S. cerevisiae* a od nich odvozenými mutanty.
2) Objasnit vzájemné interakce mezi vstupními a výstupními transportery iontů alkalických kovů a zjistit, jakým způsobem je zajištěno přežívání buněk v prostředí s extrémně nízkými nebo vysokými koncentracemi $K^+$.
3) Najít dosud neznámé regulátory exporterů iontů alkalických kovů a studovat, jakým způsobem regulace probíhá.
4) Využít získané znalosti při studiu heterologně exprimovaných transporterů iontů alkalických kovů z vyšších eukaryot a při vývoji nových metod.

# 4 Výsledky

Výsledky této disertační práce jsou shrnuty celkem v pěti publikovaných pracích (kapitola 4.1, 4.2, 4.3, 4.4 a 4.6), v jednom rukopise (kapitola 4.5) a v jedné další kapitole obsahující stručný popis výsledků projektu aplikovaného výzkumu, o jejichž publikaci se zatím neuvažuje (kapitola 4.7). Materiály a metody použité v rámci disertační práce jsou uvedeny přímo v jednotlivých publikacích.

První publikace **„*Saccharomyces cerevisiae* BY4741 and W303-1A laboratory strains differ in salt tolerance“** (kapitola 4.1, str. 37) se zabývá studiem dvou (ve výzkumu) nejvíce používaných laboratorních kmenů *S. cerevisiae*, BY4741 a W303. Bylo pozorováno, jak se v těchto kmenech vzájemně liší jednotlivé parametry homeostase iontů alkalických kovů a ukázalo se, že vykazují značné odchylky v toleranci k jednotlivým iontům alkalických kovů a organickým kationtům (např. spermin) nebo v relativním membránovém potenciálu. Dále byly nalezeny rozdíly ve velikosti buněk a stanovené suché váze. Výsledky této práce ukázaly, že i rozdíly v rámci dvou kmenů *S. cerevisiae* mohou v některých parametrech značně ovlivnit regulaci homeostase iontů alkalických kovů. Výsledky z této práce (např. nově stanovené hodnoty suché váhy nebo velikosti buněk) byly dále využity při přípravě a vyhodnocování experimentů, jejichž výsledky byly publikovány v dalších studiích (např. kapitola 4.2 a 4.3).

V rámci druhé publikace **„Lack of main $K^+$ uptake systems in *Saccharomyces cerevisiae* cells affects yeast performance in both potassium-sufficient and potassium-limiting conditions“** (kapitola 4.2, str. 38) byla detailně studována role transporterů Trk1 a Trk2 ve fyziologii buňky v prostředí s dostatkem i s nedostatkem $K^+$ a bylo zjištěno, že přes velkou řadu sledovaných parametrů se v prostředí s dostatečným množstvím $K^+$ delece obou vstupních systémů (*trk1 trk2*) projeví hlavně ve sníženém intracelulárním pH a růstu $\Delta\Psi$, zatímco za nedostatku $K^+$ bylo pozorováno smršťování buněk, objevil se růst rozdílu v $\Delta\Psi$, pokles intracelulárního pH a změny v citlivosti k organickým kationtům. V této publikaci byla poprvé detailně popsána časová závislost pozorovaných dějů a změny kinetických parametrů importu $K^+$ po přenesení buněk do média bez draslíku. Poprvé zde byla také objevena inhibice exportu $K^+$ po přenesení buněk do média bez $K^+$ (hladovění buněk na $K^+$). Inhibice exportu $K^+$ ukazovala na to, že absence vstupních transporterů inhibuje exportery, toto funkční propojení dosud nebylo popsáno a jev byl proto zkoumán detailněji v rámci třetí publikace **„Plasma-membrane hyperpolarization diminishes the cation efflux via Nha1 antiporter and Ena ATPase under potassium limiting conditions“** (kapitola 4.3, str. 40). Nejdříve bylo zjištěno, že v případě absence Trk1p a Trk2p je export $K^+$ po přenesení buněk do média bez $K^+$ plně zastaven podobně, jako když jsou exportery odstraněny. Dále se ukázalo, že exportu $K^+$ (při hladovění na $K^+$)

se účastní hlavně Nha1p a Ena1p a částečně také Tok1p. Po deleci *TRK1* a *TRK2* jsou tedy inhibovány všechny exportery. Jako regulační mechanismus byl identifikován zvýšený ΔΨ kmenů postrádajících Trk1p a Trk2p, který stojí za inhibicí aktivity všech tří exportních systémů. Objevený regulační mechanismus výrazně přispívá k objasnění obecných zásad regulace homeostase iontů alkalických kovů.

V rámci čtvrté publikace **„Yeast 14-3-3 proteins participate in the regulation of cell cation homeostasis via interaction with Nha1 alkali-metal-cation/proton antiporter“** (kapitola 4.4, str. 41) byl poprvé popsána regulace homeostase iontů alkalických kovů pomocí proteinů 14-3-3 u *S. cerevisiae*. Pomocí studia genetických i fyzických interakcí bylo zjištěno, že proteiny 14-3-3 jsou specifickými positivními regulátory (aktivátory) antiporteru Nha1 a že s ním také fyzicky interagují. Bylo prokázáno, že existuje pravděpodobně více míst interakce, protože proteiny 14-3-3 interagují jak s transmembránovou polovinou, tak C-koncovou hydrofilní polovinou Nha1p. Druhým nově identifikovaným regulátorem Nha1p je jedna z podjednotek kaseinkinasy 2 (CK2), katalytická podjednotka Cka1p. Genetická interakce mezi *CKA1* a *NHA1* byla objevena v rámci páté publikace **„Pleiotropic role of *CKA1* in salt tolerance and cation homeostasis“** (kapitola 4.5, str. 42). Ukázalo se, že již dříve popsaná schopnost Cka1p regulovat expresi *ENA1* není jedinou cestou, kterou Cka1p ovlivňuje homeostasi iontů alkalických kovů. Kinasa Cka1 byla v rámci publikace poprvé pospána jako positivní regulátor (aktivátor) Nha1p. Hledání místa interakce (fosforylace) Cka1p v sekvenci Nha1p bylo (i přes použití *in silico* predikce) neúspěšné, ale bylo prokázáno, že pro interakci není nutná C-koncová hydrofilní část proteinu. Kromě regulace exprese *ENA1* a regulace aktivity Nha1p byla poprvé identifikovaná také další účast Cka1p v homeostasi iontů alkalických kovů nezávislá na přítomnosti exporterů Nha1 a Ena. V rámci čtvrté a páté publikace tedy byly identifikovány dva dosud neznámé positivní regulátory aktivity Nha1p.

$Na^+/H^+$ antiporter byl studován rovněž v šesté publikaci **„Mutational analysis of NHAoc/NHA2 in *Saccharomyces cerevisiae*“** (kapitola 4.6, str. 44), která na rozdíl od ostatních popisuje studii zaměřenou na lidský $Na^+/H^+$ antiporter NHAoc/NHA2, homolog *ScNHA1*. NHAoc/NHA2 je nutný pro správnou diferenciaci lidských osteoklastů. cDNA savčího genu byla v rámci této studie úspěšně exprimována v mutantním kmeni *S. cerevisiae* postrádajícím vlastní exportery iontů alkalických kovů a byla testována aktivita exprimovaného proteinu a vliv specifických mutací na aktivitu. Na základě studie byly identifikovány důležité aminokyseliny, po jejichž mutaci (nebo kombinací více mutací) docházelo k výraznému poklesu transportní aktivity proteinu. Tímto přístupem bylo odhaleno, že i samotná mutace v jednom ze tří identifikovaných AA zbytků může zásadně ovlivnit aktivitu transporteru a v lidském organismu může taková mutace stát za poruchami tvorby kostí.

Součástí této disertační práce jsou také nepublikované výsledky projektu aplikovaného výzkumu (kapitola 4.7, str. 45). V rámci získaného grantu probíhala spolupráce s firmou DEL, a.s. zaměřenou na vývoj software pro průmyslové podniky. Výsledkem spolupráce byl vznik funkční aplikace Ocellaris (www.ocellaris.cz) pro automatickou analysu mikroskopických snímků, která poskytuje výpočetní podporu a *de facto* umožňuje měření pH v mikroskopu v jednotlivých buňkách kvasinek exprimujících pH sensitivní verzi zeleného fluorescenčního proteinu (pHluorin).

## 4.1 Publikace č. 1 – *Saccharomyces cerevisiae* BY4741 and W303-1A laboratory strains differ in salt tolerance

V oblasti základního výzkumu je celosvětově standardně používáno pouze několik tzv. laboratorních kmenů *S. cerevisiae*. Také v oblasti výzkumu homeostase iontů alkalických kovů je skupina standardně používaných laboratorních kmenů poměrně ustálená, přesto jsou v rámci jednotlivých laboratoří používány ke stejným pokusům různé kmeny. Tento fakt často nemusí být významný, ale v řadě případů se v minulosti ukázalo, že není vždy možné srovnávat výsledky získané s různými kmeny. V naší laboratoři byl po dlouhá léta k výzkumu homeostase iontů alkalických kovů využíván kmen W303-1A (viz např. [63]) a od něj odvozené mutanty. Na základě vývoje ve vědecké komunitě, výběru kmenů, u kterých byla v relevantní dobu již známa sekvence genomu, a také na základě nově vznikajících projektů a spoluprací se objevila potřeba vyměnit dosud používaný kmen W303 za kmen BY4741. Pro zachování kontinuity a možnosti využití dřívějších výsledků laboratoře bylo potřeba provést intenzivní porovnání základních fyziologických parametrů obou kmenů, a také srovnat jejich chování (a chování odvozených mutantů) v přítomnosti extrémně vysokých nebo nízkých koncentracích iontů alkalických kovů.

Základní fyziologické parametry obou kmenů (a příslušných odvozených mutantních kmenů) byly současně charakterisovány a bylo zjištěno, že buňky obou kmenů se vzájemně liší ve velikosti, objemu, váze sušiny, obsahu $K^+$, toleranci k iontům alkalických kovů, amonným a organickým kationtům (např. hygromycin B) a pravděpodobně také v $\Delta\Psi$. Mimo vzájemné srovnání obou kmenů byl v této práci poprvé odhalen negativní vztah mezi růstovou fází a vnitrobuněčným obsahem $K^+$. Buňky ve velmi časné růstové fázi měly v obou zkoumaných kmenech mnohem vyšší obsah $K^+$, než buňky v pozdější fázi růstu buněčné kultury.

Práce tedy ukázala na značné rozdíly v absolutních hodnotách výsledků, které je možné nalézt u dvou běžně používaných kmenů *S. cerevisiae*, poukázala na fakt, že variabilita v rámci jednoho druhu může být významná, a přispěla do diskuse o tom, jestli je při dalším výzkumu důležité pozorované fenotypy ověřovat ve více kmenech.

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# *Saccharomyces cerevisiae* BY4741 and W303-1A laboratory strains differ in salt tolerance

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ABSTRACT

*Saccharomyces cerevisiae* yeast cells serve as a model to elucidate the bases of salt tolerance and potassium homeostasis regulation in eukaryotic cells. In this study, we show that two widely used laboratory strains, BY4741 and W303-1A, differ not only in cell size and volume but also in their relative plasma-membrane potential (estimated with a potentiometric fluorescent dye diS-$C_3$(3) and as Hygromycin B sensitivity) and tolerance to alkali-metal cations. W303-1A cells and their mutant derivatives lacking either uptake (*trk1 trk2*) or efflux (*nha1*) systems for alkali-metal cations are more tolerant to toxic sodium and lithium cations but also more sensitive to higher external concentrations of potassium than BY4741 cells and their mutants. Moreover, our results suggest that though the two strains do not differ in the total potassium content, the regulation of intracellular potassium homeostasis is probably not the same in BY4741 and W303-1A cells.



## Introduction

Ion and pH homeostases are fundamental to the physiology of all living cells, including yeast. As far as the homeostasis of alkali-metal cations is concerned, cells usually expend a substantial proportion of their energy to accumulate high amounts of potassium and maintain low cytosolic concentrations of toxic sodium or lithium cations. To ensure an optimal cytoplasmic $K^+/Na^+$ ratio, cells employ several systems transporting cations with differing affinities, capacities, substrate specificities and diverse mechanisms, both across the plasma membrane, and in eukaryotic cells also across the membranes of intracellular organelles. *Saccharomyces cerevisiae* cells have at least five $K^+$ transport systems at their disposal at their plasma membrane. For $K^+$ uptake, there are Trk1p and Trk2p as high-affinity specific transporters (Gaber *et al.* 1988; Ramos *et al.* 1994), and its efflux is mediated *via* the voltage-gated $K^+$-specific channel Tok1 (Ketchum *et al.* 1995), and two efflux systems that in addition to $K^+$, also transport $Na^+$ and $Li^+$: P-type ATPase (encoded by *ENA/PMR2* locus) (Haro *et al.* 1991) and the Nha1 antiporter (Prior *et al.* 1996).

A high intracellular $K^+/Na^+$ ratio is important for many cellular processes, such as the activation of enzymatic reactions, cell volume regulation, response to osmotic shock or buffering the intracellular pH (Sychrova 2004). The uptake and efflux of potassium cations is also indispensable for the preservation of a constant level of membrane potential ($\Delta\psi$). Membrane potential across the plasma membrane (negative inside) is built up by the activity of the $H^+$-ATPase Pma1 (Serrano *et al.* 1986), and consumed by the electrophoretic uptake of $K^+$ through Trk transporters and by many other secondary active systems (symporters and antiporters) including Nha1p. Plasma-membrane $H^+$-ATPase and potassium systems for influx and efflux act in synergy to fulfill their roles and damage to any of them is reflected in changes in intracellular pH and potassium content and in increased sensitivity to toxic alkali-metal cations or cationic drugs in general.

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If yeast cells are grown in the presence of high NaCl concentrations (salt stress) they must cope both with an elevated external osmotic pressure and an increasing amount of $Na^+$ entering the cells. Inside the cells, sodium replaces potassium, thus the cytosolic $K^+/Na^+$ ratio decreases with increasing external $Na^+$ and the cells must actively pump sodium out or sequestrate it in organelles (mainly the vacuole). To ensure this, *S. cerevisiae* cells mainly use Ena ATPases at the plasma membrane and the Nhx1, Vnx1 antiporters localized to the membranes of their late endosomes and vacuoles, respectively.

Numerous laboratory strains are used in yeast research, which in some cases results in discrepancies for comparable data. Within the framework of the exploration of salt tolerance and cation homeostasis in *S. cerevisiae*, we have observed specific differences among alkali-metal cation-transport deficient mutants derived from different parental strains. To elucidate the observed inconsistency, we employed two commonly used laboratory strains: BY4741 and W303-1A. While BY4741 is a derivative of S288C (Brachmann *et al.* 1998), used in the systematic sequencing of the *S. cerevisiae* genome (Goffeau *et al.* 1996), strains with a W303 background have served in many physiological and biochemical studies. The aim of the present study was to compare and describe the differences in salt-tolerance-associated phenotypes between the two strains and to find at least some of the factors determining the observed differences.

## Experimental/materials and methods

### *Strains and growth conditions*

The yeast *Saccharomyces cerevisiae* strains W303-1A (*MAT*a *leu2*-3/112 *ura3*-1 *trp*1-1 *his3*-11/15 *ade2*-1 *can*1-100) (Wallis *et al.* 1989) and BY4741 (*MAT*a *his*3Δ1 *leu*2Δ *met*15Δ *ura*3Δ; EUROSCARF, Germany) were used in this work, together with their derivatives lacking either the Trk systems for potassium uptake or the Nha1 antiporter for sodium and potassium efflux. W303-1A derivatives were W12 (*trk1*::*LEU2 trk2*::*HIS3* (Madrid *et al.* 1998), kindly provided by Dr. M.A. Bañuelos) and CW25 (*nha1*::*LEU2*; Kinclova-Zimmermannova *et al.* 2006). The BY4741 derivatives BYT12 (*trk1*::*loxP trk2*::*loxP*) and BYT4 (*nha1*::*loxP*) were constructed in this work by homologous recombination using the KanMX marker gene and Cre-*loxP* system (Guldener *et al.* 1996). The oligonucleotides used for deletion cassette amplifications and recombination diagnosis are listed in Table 1.

Cells were grown in YPD (1 % yeast extract, 2 % bacto peptone, 2 % glucose, 0.015 g $L^{-1}$ adenine) or YNB (0.17 % yeast nitrogen base without amino acids, 0.5 % ammonium sulphate, 2 % glucose) with appropriate auxotrophic supplements added after autoclaving. For optimal growth of *trk1*Δ*trk2*Δ mutant strains (BYT12 and W12), KCl was added to the media (as indicated in the text, at least 50 mM). Solid media were supplemented with 2 % agar.

### *Salt-tolerance drop test*

Growth phenotypes of strains in the presence of salts were tested on solid media. Yeast strains growing overnight in liquid medium without salts were washed and resuspended in sterile water to the same initial $OD_{600}$ (approx. 1). Tenfold serial dilutions were prepared and 3 μL aliquots spotted on a series of plates containing increasing concentrations of salts (1.0–2.0 M KCl, 1.0–2.0 M NaCl, 0.1–0.5 M LiCl). Plates were incubated at 30 °C for 3–4 d. Representative results are shown.

### *Gradient plate assay*

A gradient plate assay was performed as previously described (Maresova & Sychrova 2005). Briefly, a Hygromycin B gradient

**Table 1 – List of oligonucleotides.**

| | |
|---|---|
| *Gene deletion primers* | |
| NHA1-kan-F | 5′GTACATTATAAAAAAAAATCCTGAACTTAGCTAGATATTAttcgtacgctgcaggtcgac-3′ |
| NHA1-kan-R | 5′ATATACTAAAATAATATATCTTTGTGTATTAATAAATTACgcataggccactagtgga-3′ |
| TRK1-kan-F | 5′TCAAGGAAGTCATTCCTATCCATTTTACTTAAAGTTATTACCTTTTTTTGATAACTAACAttcgtacgctgcaggtcgac-3′ |
| TRK1-kan-R | 5′TTGAGTACGAAAACCTATTTCTAAAGAATGAGTATATATGgcataggccactagtggatctg-3′ |
| TRK2-kan-F | 5′GATGAGAAAAGAGGCTATTTTGTACTATTCACCGACGATAAGAGGCTGTAAGAACCACTCttcgtacgctgcaggtcgac-3′ |
| TRK2-kan-R | 5′ACGTTGGCTCTTATGTAGGTAAAGAGGGGTAAACTTGATTTTgcataggccactagtggatctg-3′ |
| *Diagnostic primers – deletion cassettes* | |
| KANX-R1 | 5′CTCTGGCGCATCGGGC-3′ |
| KANX-F1 | 5′CATTTGATGCTCGATGA-3′ |
| *Diagnostic primers – target gene* | |
| NHA1-UP | 5′CAACTCTGTGTGATATAG-3′ |
| NHA1-DR | 5′CAATGTGAACCCCAGTG-3′ |
| NHA1-20 | 5′CCTGCGGCCACCGAGAGGAG-3′ |
| TRK1-UP | 5′GAGAGTAGATGTGGAGT-3′ |
| TRK1-1 | 5′GATCAGAGGCAAGAATAG-3′ |
| TRK1-DR | 5′GGAGAACACCGGCGGG-3′ |
| TRK2-UP | 5′GATGCAAGCTGCCATG-3′ |
| TRK2-1 | 5′GTTTGAAGTTGTTAGCGC-3′ |
| TRK2-DR | 5′CGTAGGGGACAAAATGC-3′ |

Lower case letters indicate nucleotides complementary to sequences of the template.

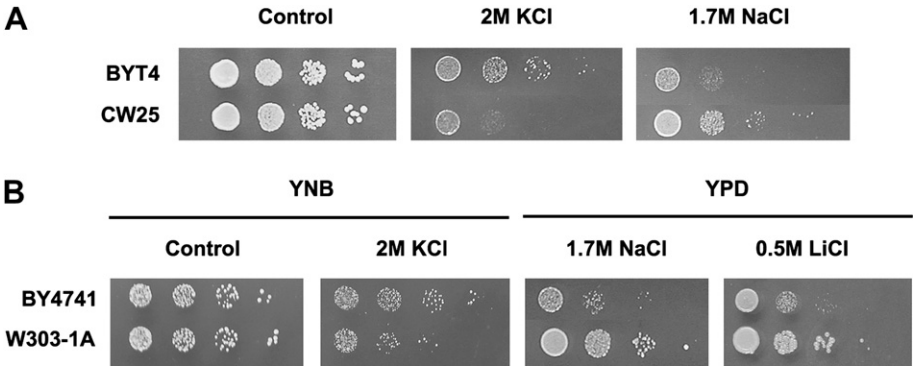


**Fig 1 – (A) Growth of *nha1*Δ mutants derived from BY4741 (BYT4) and W303-1A (CW25) on YPD media in the presence of high KCl and NaCl concentrations. (B) Growth comparison of parental strains under KCl, NaCl and LiCl stress conditions. Representative images from YNB or YPD media are shown.**

was formed by pouring a bottom layer of medium supplemented with 0.5 g $L^{-1}$ Hygromycin B in a tilted square Petri dish. After agar solidification, the Petri dish was turned flat and a top layer of medium (without Hygromycin B) was added. Lines of 3 μL aliquots of cell suspensions (the same initial $OD_{600}$, approx. 1) were spotted on the plate across the gradient. Plates were incubated at 30 °C for 2–3 d. Each experiment was repeated at least three times.

### *Potassium content measurement*

To estimate intracellular potassium content, 50 mL of YNB (+50 mM KCl) medium was inoculated and cultivated in 30 °C. 6 mL samples were withdrawn every 2 h during the beginning of the exponential phase of growth (~8 h; $OD_{600}$ 0.05–0.8). For each sample, the $OD_{600}$ was measured and 5 mL of cell suspension was washed with deionized water, resuspended in 10 mM Tris, 0.1 mM $MgCl_2$, pH 4.5 (adjusted by citric acid and $Ca(OH)_2$) and quickly harvested by filtration (Millipore filters, 0.8 μm). Filters with cells were rapidly washed twice with 5 mL of 20 mM $MgCl_2$ (in deionized water). Acid extraction of cations and atomic-absorption measurements were carried out as in Kinclova *et al.*, 2001.

### *Fluorescence measurement of relative Δψ (diS-$C_3$(3) assay)*

The fluorescence assay of relative Δψ was performed according to Maresova *et al.*, 2009. Strains were grown overnight in YNB or YPD to the exponential phase ($OD_{600}$ 0.5–0.6), harvested, washed twice with 10 mM $Na_2HPO_4$ (pH 6.0, adjusted by citric acid) and resuspended in the same buffer to final $OD_{600}$ 0.2. The potential-sensitive dye diS-$C_3$(3) (3,3′-dipropylthiacarbocyanide iodide; 0.1 mM stock solution in ethanol) was added to 3 mL of cell suspension to a final probe concentration of 0.2 μM. Fluorescence emission spectra were measured on an ISS PC1 spectrofluorometer. The excitation wavelength was 531 nm, emission intensities were measured at 560 and 580 nm. The staining curves (*i.e.* the dependence of the emission intensity ratio ($I_{580}/I_{560}$) on the duration of staining *t*) were fitted as described in (Malac *et al.* 2005), and the value of the intensity ratio at equilibrium was estimated.

### *Cell volume measurements*

Overnight cultures were grown in YNB medium; cells were harvested, washed twice and resuspended in the same volume of fresh YNB medium. Cell volume was measured using

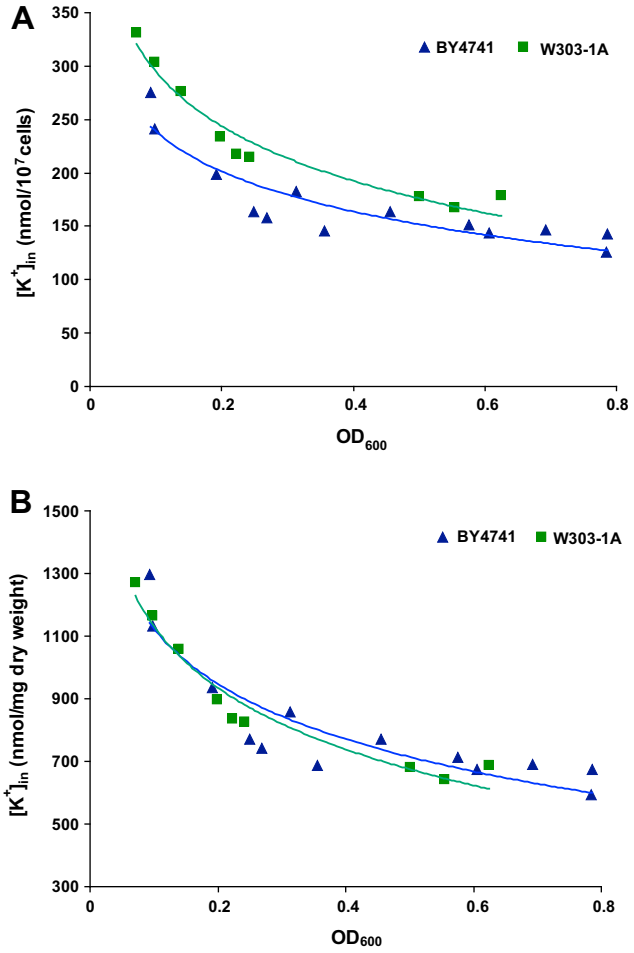


**Fig 2 – Intracellular potassium content of BY4741 and W303-1A cells estimated during exponential growth phase in YNB + 50 mM KCl and calculated (A) in nmol per $10^7$ cells or (B) in nmol per mg of dry weight. Results obtained from three independent experiments are presented.**

the Z2 Coulter® Particle Count and Size Analyzer and Coulter® Isoton II® dilution buffer. The experiment was repeated three times, each time approx. $10^6$ cells were analyzed for each strain.

### *Light microscopy*

Strains were grown overnight in YNB or YPD to the exponential phase ($OD_{600}$ 0.5–0.6) and cells were examined under an Olympus AX70 microscope with an Olympus DP70 digital camera using a 100× oil-immersion objective, equipped for Nomarski optics.

## Results and discussion

Numerous *Saccharomyces cerevisiae* strains lacking the genes encoding various cation-transport sytems have been used in our laboratory over the last 10 y. Most of the mutants have been derived from the two widely used laboratory strains BY4741 and W303-1A. During our work on $Na^+$ and $Li^+$ toxicity, we surprisingly noticed some differences in salt tolerance between mutants harboring the same deletions but originating from different parental strains. For example, deletion of the *NHA1* gene encoding a plasma-membrane alkali-metal cation/$H^+$ antiporter resulted in different growth characteristics in the presence of high concentrations of KCl or NaCl in YPD medium (Fig 1A). In the W303-1A background (CW25), the deletion brought about a higher sensitivity to potassium than the same deletion in BY4741 (BYT4), whereas with high external concentrations of sodium the situation was the other way round, CW25 grew better than BYT4. To determine whether this difference is also reflected on other media and if it is dependent on the genetic background, we performed a series of drop tests for both the parental BY4741 and W303-1A strains on YPD and YNB media with three different salts. Fig 1B shows a representative result with visible differences in salt tolerance. Similar differences were observed on both media. The BY4741 cells support higher concentrations of external potassium but much lower concentrations of toxic $Na^+$ and $Li^+$ cations compared to W303-1A. This difference in growth cannot be due to the differing auxotrophies of both strains, as they grew the same on control plates without salts (Fig 1B) and they exhibited the same growth curves when grown in liquid YPD and YNB (containing a complete mix of supplements) media (not shown).

One possible reason for the differing alkali-metal cation tolerance of the two strains could be a difference in the intracellular concentration of potassium. As mentioned in the Introduction, the salt tolerance of *S. cerevisiae* cells depends on the ability to maintain an optimal intracellular $K^+/Na^+$ ratio (Rodriguez-Navarro 2000). To know whether the two strains differ in their intracellular potassium content, $[K^+]_{in}$ was measured over 8 h of the exponential growth phase (from $OD_{600}$ 0.05 to 0.8). Surprisingly, a rapid decrease in $[K^+]_{in}$, up to 50 %, was observed for both strains during the experiment (Fig 2) though potassium was not the limiting compound in

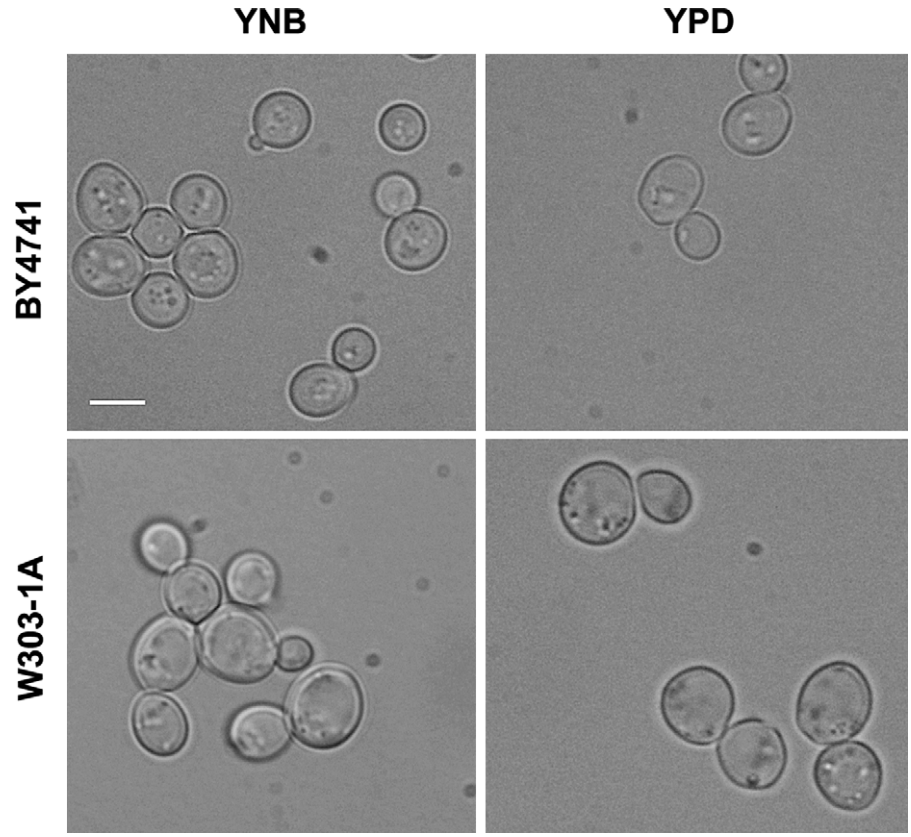


**Fig 3 – Cell images (taken with Nomarski optics) of BY4741 and W303-1A strains grown in YNB and YPD media to exponential growth phase. Bar, 5 μm.**

**Table 2 – Comparison of median volumes of cells grown in YNB.**

| Strain | Median cell volume (fL) |
|---|---|
| BY4741 | 45.54 ± 0.9 |
| W303 | 49.66 ± 1.3 |

Median values with the standard deviations of three independent experiments are presented.

the medium (50 mM KCl was added into the medium, *cf.* Experimental/materials and methods) and the exponential phase of growth was longer than the surveyed 8 h. Interestingly, if the intracellular potassium content was calculated in nmol per $10^7$ cells, the intracellular level of $K^+$ in W303-1A cells was significantly higher than in BY4741 strain (Fig 2A) but if the content was expressed in nmol per mg of dry weight, it was comparable in both strains (Fig 2B). It suggested that the apparent discrepancy between potassium content in the two strains can be due to differences in cell size and volume. This presumption was confirmed in three independent experiments. Differences in size were observed under the microscope for cells growing both in YNB and YPD media (Fig 3), and confirmed in Coulter counter measurements which revealed that the relative cell volume of W303-1A cells is ~10 % higher than that of BY4741 cells (Table 2). Finally, an estimation of cell number (colony-forming units, cfu) and dry weight (μg) showed that there are ~10 % more BY4741 than W303 cells in 1 mL of cultures with $OD_{600}$ 1.0 (Table 3), and they have a significantly lower weight. BY4741 cells were smaller and lighter than W303-1A cells both in YPD and YNB media (Table 3). This difference in the number of cells and their size explains the dissimilarity in potassium content observed in Fig 2A. Taken together, these results led to the conclusion that the intracellular concentration of potassium is rather similar in both strains and the observed differing sensitivities to high external salts is not based on differences in the total content of intracellular potassium. We conclude that $[K^+]_{in}$ has no effect on the differences between BY4741- and W303-derived strains in salt tolerance.

If potassium content is not responsible for the differing salt tolerances of BY4741 and W303-1A, a higher uptake of and/or lower efflux capacities for surplus alkali-metal cations can result in differing levels of salt tolerance. Plasma-membrane potential is one of the factors determining cation fluxes. The relative $\Delta\psi$ of BY4741 and W303-1A cells grown in YNB or YPD was estimated using the potential-sensitive diS-$C_3$(3) florescent probe (Fig 4). BY4741 cells showed a higher uptake of the potentiometric probe, which could indicate an elevated $\Delta\psi$ compared to W303-1A cells. Cells of both strains grown in YPD showed a higher content of the probe than cells from YNB, which suggests a higher $\Delta\psi$ across the plasma membrane of cells grown in rich medium. However, in both strain and media comparisons, the observed difference might also be caused by the differing activities of PDR systems that actively expel the diS-$C_3$(3) dye (Gaskova *et al.* 2002) or by distinct plasma-membrane compositions.

Sensitivity to Hygromycin B has been previously described as a useful tool for the determination of mutants with a hyperpolarized plasma membrane (*e.g.* Madrid *et al.* 1998), as the level of Hygromycin B uptake is driven by $\Delta\psi$. Therefore, we tested the Hygromycin B sensitivity of parental strains and their *trk1*Δ*trk2*Δ derivatives (whose plasma membrane is hyperpolarized due to the absence of potassium influx). Gradient plate assay experiments (Fig 5) confirmed the higher $\Delta\psi$ of BY4741 cells. Differences between BYT12 and W12 cells (*trk1*Δ*trk2*Δ mutants) and between both parental strains were clearly evident on YPD medium. On Hygromycin B-containing YNB media (with the same concentration of the drug as in YPD), the BY4741 and W303-1A strains were both highly tolerant, probably due to the lower $\Delta\psi$ of cells grown in YNB (Fig 4) but the difference in sensitivity was still visible for the relatively hyperpolarized *trk1*Δ*trk2*Δ mutants. This higher $\Delta\psi$ of BY4741 cells, indicated by the fluorescence measurements with a potentiometric probe and by a higher sensitivity to Hygromycin B, might be responsible for the observed increased sensitivity of this strain to $Na^+$ and $Li^+$ salts (Fig 1B), which enter *S. cerevisiae* cells by non-specific transport pathways and their uptake is driven by the actual level of $\Delta\psi$.

On the other hand, a higher $\Delta\psi$ cannot explain the fact that BY4741 cells exhibited an increased tolerance to high external $K^+$ compared to W303-1A cells (Fig 1) despite their similar $[K^+]_{in}$ under standard conditions. When testing the sensitivity of *trk1*Δ*trk2*Δ mutants to low concentrations of external $K^+$, we did not observe any significant differences, and mutants of both genetic backgrounds grew only slowly with 50 mM KCl in the medium and both strains achieved the 'normal'

**Table 3 – Comparison of number of viable cells (cfu) and their dry weight (μg) in 1 mL of cultures $OD_{600}$ 1.0.**

| Strain | Medium | cfu | Dry weight (μg mL$^{-1}$) |
|---|---|---|---|
| BY4741 | YPD | $3.2 \times 10^7$ | 789 ± 16 |
| | YNB | $3.2 \times 10^7$ | 679 ± 8 |
| W303 | YPD | $2.8 \times 10^7$ | 874 ± 21 |
| | YNB | $2.8 \times 10^7$ | 726 ± 13 |

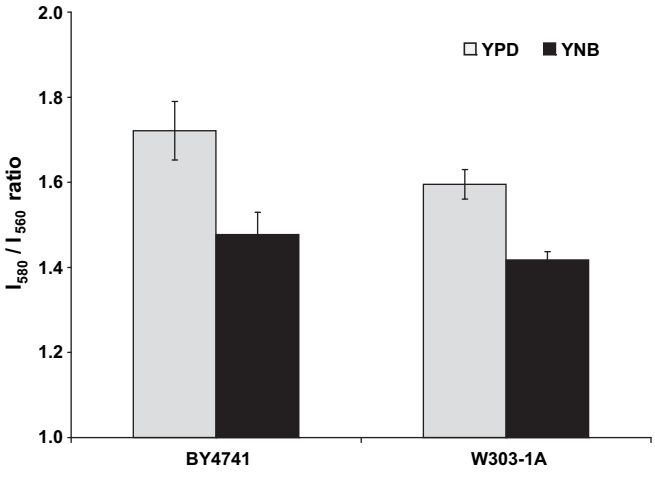


**Fig 4 – Comparison of relative plasma-membrane potential of BY4741 and W303-1A cells grown in YNB and YPD.**

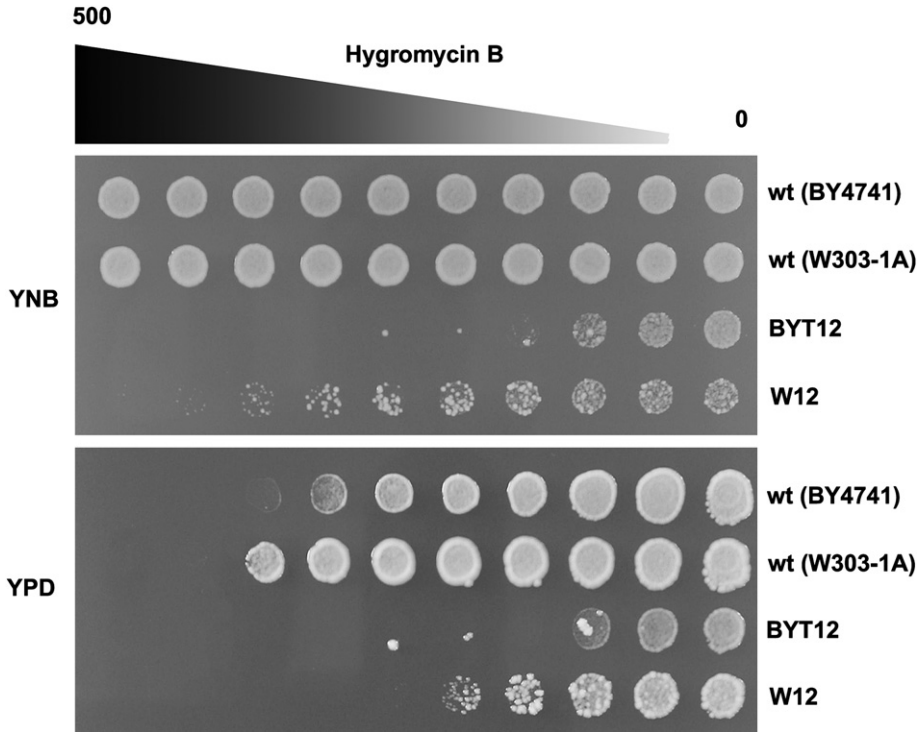


**Fig 5 – Growth of BY4741 and W303-1A cells on YPD or YNB plates containing increasing concentration of Hygromycin B (0.5 g $L^{-1}$ on the left and 0 g $L^{-1}$ on the right).**

rate of growth at $K^+$ concentrations higher than 100 mM (not shown). Nevertheless, when we tested the toxicity of ammonium cations on cells starved of potassium (*trk1*Δ*trk2*Δ mutants grown in the presence of 50 mM KCl), another discrepancy between BY4741 and W303-1A strains in internal $K^+$ level control was observed. Under potassium-limiting conditions, an increased influx of ammonium cations occurs when ammonium is present in abundant external concentrations and it becomes toxic to cells (Hess *et al.* 2006). We compared the ability of our BYT12 and W12 mutants (and their respective parental strains) to tolerate low and high levels of ammonium salts in potassium-limiting conditions (Fig 6). Indeed, the impaired growth of mutants lacking high-affinity $K^+$ transporters was observed in the presence of 2 % $(NH_4)_2SO_4$ (~150 mM) and 50 mM KCl, whereas an increase in potassium concentration (to 100 mM) or decreased concentration of ammonium salts (to 0.4 %) weakened this growth inhibition. Interestingly, the W303-derived W12 strain was more sensitive to ammonium under potassium limitation than the BY4741 derivative BYT12 (Fig 6) and it was similarly more sensitive to high levels of potassium (Fig 1B). These results also verified the importance of Trk1p and Trk2p in high ammonium tolerance under the tested conditions.

Our comparative study of two popular *S. cerevisiae* laboratory strains shows that the genetic background is a relevant characteristic in the interpretation of results from salt-tolerance studies. A few other studies have shown that different *S. cerevisiae* genetic backgrounds result in significant

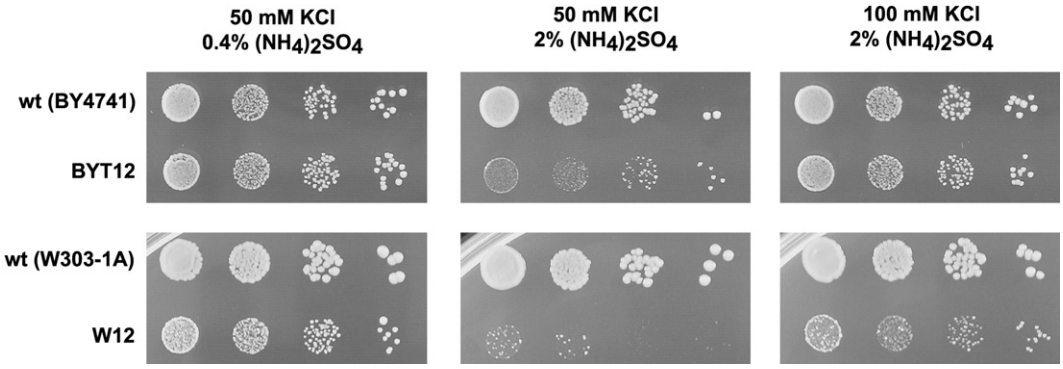


**Fig 6 – High ammonium sensitivity of BY4741 and W303-1A-derived mutants (BYT12 and W12) lacking Trk1 and Trk2 transporters under potassium-limiting conditions.**

physiological differences, *e.g.* in lipid metabolism (Daum *et al.* 1999) or protein expression (Rogowska-Wrzesinska *et al.* 2001). Another good example is the comparison of *ENA*/*PMR2* loci encoding the alkali-metal cation ATPase (Wieland *et al.* 1995; Daran-Lapujade *et al.* 2003). The W303-1A strain contains four tandem repeats of the *ENA* gene, whereas the BY4741 genome only harbors three copies. Thus, the higher number of *ENA* gene repeats in W303-1A could be responsible for the increased $Na^+$ and $Li^+$ tolerance which results from an increased sodium and/or lithium efflux mediated by Ena ATPases.

In conclusion, we were able to show that the two widely used *S. cerevisiae* laboratory strains, BY4741 and W303-1A, differ in their tolerance to toxic sodium/lithium cations, which is most probably connected to differences in their level of membrane potential on the one hand and in their toxic cation efflux capacity on the other, and that the two strains differ in their regulation of $K^+$ homeostasis.

## Acknowledgement

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## 4.2 Publikace č. 2 – Lack of main $K^+$ uptake systems in *Saccharomyces cerevisiae* cells affects yeast performance in both potassium-sufficient and potassium-limiting conditions

Při studiu homeostase iontů alkalických kovů byla nejprve věnována pozornost importerům $K^+$ Trk1 a Trk2. Mezi fenotypy delece *trk1* a *trk2* patří neschopnost růstu při snížené koncentraci $K^+$, zvýšení relativního ΔΨ a vyšší citlivost k organickým kationtům (např. spermin), $Li^+$ a $Na^+$ [12, 30, 34]. V rámci dříve publikovaných studií však dosud nebyl podrobně studován přechod buněk z prostředí s nelimitující koncentrací $K^+$ do prostředí s nízkým obsahem $K^+$, dalším problémem dřívějších studií byla často relativně vysoká přirozená koncentrace $K^+$ v médiu. Aby bylo možné proces hladovění na $K^+$ studovat, bylo v rámci této práce navrženo a na objednávku vyrobeno růstové médium, jehož použití umožnovalo pozorovat chování buněk v přítomnosti dostatečného množství všech živin kromě $K^+$.

V první části práce byl sledován růst buněk (BY4741 a kmene postrádajícího *TRK1* a/nebo *TRK2*) v přítomnosti různých koncentrací $K^+$ a za různého pH. Zároveň byl měřen relativní ΔΨ, vnitrobuněčné pH, míra okyselování okolí buňkami, ale také citlivost na $Na^+$, $Li^+$ a některé organické kationty. Při mnohem lépe definované koncentraci $K^+$ v médiu byly potvrzeny výsledky předchozích studií tak, že kmeny postrádající *TRK1* (platí stejně pro kmen postrádající současně *TRK1* a *TRK2*) jsou hyperpolarisované, citlivé ke kationtům ($Na^+$, $Li^+$ a organickým kationtům) a také ke snížené koncentraci $K^+$. Dále bylo pozorováno snížené vnitrobuněčné pH a snížená schopnost okyselování prostředí jako důsledek delece *trk1*. Vliv delece *trk1* byl pozorován také v prostředí s nelimitujícím (cca 50 – 100 mM) množstvím $K^+$ (např. snížení vnitrobuněčného pH, okyselování okolí buněk a rozdíl v ΔΨ).

V druhé části byla studována časová závislost dějů, které probíhají po přenesení buněk do prostředí bez $K^+$. Také zde byly porovnávány charakteristiky buněk BY4741 s derivátem tohoto kmene postrádajícím *TRK1* a *TRK2*. Po přenesení buněk do média bez $K^+$ byl pozorován intenzivní pokles vnitrobuněčného obsahu $K^+$ u buněk BY4741 v čase, zatímco u buněk bez *TRK1* a *TRK2* byl pokles mnohem pomalejší. Současně se ztrátou $K^+$ docházelo k poklesu velikosti buněk BY4741 a tento pokles byl opět mnohem slabší u kmenů postrádajících *TRK1* a *TRK2.* Rozdíl ΔΨ mezi oběma kmeny (hyperpolarisace kmene bez importerů $K^+$), který je značný i při dostatku $K^+$, se v prostředí bez $K^+$ zvětšil. Překvapivě, nebyla pozorována zásadní změna vnitrobuněčného pH v čase u obou kmenů. pH se během hladovění buněk na $K^+$ jen velmi pozvolna zvyšovalo (rovnoměrně) u obou kmenů. V práci byly také stanoveny kinetické parametry vstupu ($K_T$ a $V_{max}$; měřením vstupu $Rb^+$ jako analoga $K^+$) u obou kmenů v závislosti na obsahu $K^+$ v prostředí. Kinetické parametry se v závislosti na koncentraci

$K^+$ měnily pouze u kmene BY4741, zatímco u kmene postrádajícího *TRK1* a *TRK2* byly konstantní, což potvrzuje, že po deleci importerů nedochází ke specifickému importu $K^+$.

Výsledky z této práce, a to především pozorované zastavení výstupu $K^+$ během hladovění buněk na $K^+$ u kmene bez *TRK1* a *TRK2*, se staly východiskem pro další práci (kapitola 4.3, str. 40), ve které bylo zastavení exportu vlivem delece importerů podrobněji studováno.

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RESEARCH ARTICLE

# Lack of main $K^+$ uptake systems in *Saccharomyces cerevisiae* cells affects yeast performance in both potassium-sufficient and potassium-limiting conditions

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## Abstract

A new YNB medium containing very low concentrations of alkali metal cations has been developed to carry out experiments to study potassium homoeostasis. Physiological characterization of *Saccharomyces cerevisiae* BY4741 strain and the corresponding mutant lacking the main potassium uptake systems (*trk1 trk2*) under potassium nonlimiting and limiting concentrations was performed, and novel important differences between both strains were found. At nonlimiting concentrations of KCl, the two strains had a comparable cell size and potassium content. Nevertheless, mutants were hyperpolarized, had lower pH and extruded fewer protons compared with the BY4741 strain. Upon transfer to $K^+$-limiting conditions, cells of both strains became hyperpolarized and their cell volume and $K^+$ content diminished; however, the decrease was more relevant in BY4741. In low potassium, *trk1 trk2* cells were not able to accomplish the cell cycle to the same extent as in BY4741. Moreover, $K^+$ limitation triggered a high-affinity $K^+/Rb^+$ uptake process only in BY4741, with the highest affinity being reached as soon as 30 min after transfer to potassium-limiting conditions. By establishing basic cellular parameters under standard growth conditions, this work aims to establish a basis for the investigation of potassium homoeostasis at the system level.

## Introduction

Ion homoeostasis is a fundamental characteristic of living cells. The concentration of $H^+$, $K^+$ and $Na^+$ cations must be tightly regulated because, on the one hand, $H^+$ and $K^+$ are involved in important processes and activities of many cellular systems and, on the other hand, too high $Na^+$ concentrations are toxic for cells (Hoffman, 1964; Serrano *et al.*, 1999; Rodríguez-Navarro, 2000; Ariño *et al.*, 2010). The asymmetric distribution of potassium across the plasma membrane has been a constant during the evolution of most organisms. As $K^+$ concentration in yeast cells contributes to the cell volume, turgor, intracellular pH, electrical membrane potential and ionic strength (Yenush *et al.*, 2005), $K^+$ uptake and efflux across *Saccharomyces cerevisiae* plasma membrane have been studied during the last 50 years. $K^+$ concentration in the cytoplasm is approximately 200–300 mM, depending on the strain and growth conditions. *Saccharomyces cerevisiae* cells can grow in media with $K^+$ concentrations ranging from *c.* 2 μM to 2 M, and the activity of $K^+$ uptake systems usually prevails over the efflux systems, thus providing almost constant net $K^+$ uptake, which allows cell growth and division (Ramos *et al.*, 1994; Haro & Rodríguez-Navarro, 2002).

Two different modes of $K^+$ transport exist in *S. cerevisiae* (Ramos & Rodríguez-Navarro, 1986; Rodríguez-Navarro, 2000). The low-affinity mode has a $K_m$ in the millimolar range and is observed in cells growing without $K^+$ limitations, whereas the high-affinity mode with the $K_m$ in the micromolar range is in operation in $K^+$-starved cells or in cells growing in the presence of $Na^+$. The active $K^+$ uptake is mediated by the plasma-membrane Trk1 and Trk2 transporters, with Trk1 being the most important (Ko *et al.*, 1990; Ramos *et al.*, 1994; Vidal *et al.*, 1995). Deletion of *TRK1* and

*TRK2* genes in strains with different genetic backgrounds and under various experimental conditions (Ko *et al*., 1990; Ramos *et al*., 1994; Madrid *et al*., 1998; Bertl *et al*., 2003) always results in inhibited growth at low $K^+$ concentrations, certain hyperpolarization of the plasma membrane and the observation of residual ectopic potassium transport. Whereas the phenotypes listed above are mainly connected to the deletion of *TRK1*, the effect of *TRK2* absence is much smaller, being almost negligible in some experimental conditions (Madrid *et al*., 1998), or observable only in specific mutants (Michel *et al*., 2006) and in certain reference strains (Bertl *et al*., 2003; Petrezselyova *et al*., 2010a).

Besides potassium, protons are also important players in cell physiology. In yeasts, the plasma-membrane $H^+$-ATPase encoded by the *PMA1* gene has an essential role in ion homoeostasis (Morsomme *et al*., 2000). Its activity creates the electrochemical potential of protons across the plasma membrane (negative inside), which in turn allows any type of positively charged substrate (including $K^+$ or $Na^+$) to be drawn into the cell, and it is also indispensable for other secondary active transport processes. The activity of Pma1 is regulated by metabolic and physiological conditions of cells, for example it is positively regulated in response to a decrease in $pH_{in}$ or to an increase in potassium uptake (Serrano *et al*., 1986; Serrano, 1991).

A full understanding of processes involved in the maintenance of cell homoeostasis from a systems biology point of view implies the combination of coherent pieces of data from very diverse sources. To this end, many experiments using various techniques must be performed under identical or very similar conditions. Characterization of the regulation of cation (particularly potassium) homoeostasis in yeast cells has been hampered till now by the fact that very different growth media and yeast strains have been employed for specific purposes. Because of the high and varying concentration of $K^+$ (mM range) in the commercial growth media (YNB, YPD), it has been difficult to standardize methods and procedures to study cation requirements, cell behaviour during $K^+$ starvation, and $K^+$ transport parameters in *S. cerevisiae*. To deal with these problems, various fully synthetic media and approaches have been used (e.g. Ramos *et al*., 1994; Bertl *et al*., 2003; Michel *et al*., 2006). Nevertheless, it is extremely difficult to compare diverse pieces of data obtained in different laboratories, and to combine them as a starting point for plausible modelling aimed at achieving a complete understanding of potassium homoeostasis in yeast cells.

In this paper we describe the formulation of a new standard YNB-based medium that contains negligible $K^+$ and $Na^+$ amounts, and the use of a series of isogenic BY4741 mutants lacking various combinations of genes encoding potassium uptake and/or efflux systems. With these new tools, we have performed a detailed physiological characterization of the BY4741 strain and *trk1 trk2* mutant under potassium nonlimiting and limiting concentrations and found important, previously unnoticed differences between the two strains under both conditions tested.

## Materials and methods

### Strains, media and growth conditions

The *S. cerevisiae* reference strain BY4741 (*MAT*a *his3*Δ1 *leu2*Δ*met*15Δ*ura3*Δ; EUROSCARF, Germany) and its derivatives lacking one (BYT1, *trk1*Δ::*loxP*; BYT2, *trk2*Δ::*loxP*) or both (BYT12, *trk1*Δ::*loxP trk2*Δ::*loxP*) *TRK1* and *TRK2* genes were used in this work (Petrezselyova *et al*., 2010b). Further, *NHA1*, *ENA1-5* and *TOK1* genes were deleted in this work by homologous recombination using the KanMX marker gene and Cre-*loxP* system, producing strains BYT45 (*nha1*Δ::*loxP ena1-5*Δ::*loxP*), BYT1245 (*trk1*Δ::*loxP trk2*Δ::*loxP nha1*Δ::*loxP ena1-5*Δ::*loxP*) and BYT12345 (*trk1*Δ::*loxP trk2*Δ::*loxP nha1*Δ::*loxP ena1-5*Δ::*loxP tok1*Δ::*loxP*). Yeast cultures were routinely grown at 28 °C in standard YPD or in synthetic $K^+$-free medium (YNB without amino acids, ammonium sulphate or potassium, ForMedium™ UK, CYN7505), from now on, Translucent $K^+$-free medium with appropriate auxotrophic supplements, and the indicated amount of KCl. Before autoclaving, the pH of the YNB-based medium was adjusted to 5.8 with ammonium hydroxide solution. Solid media were prepared by adding 2% (w/v) agar. To estimate the dependence of growth rate on $K^+$ concentrations, liquid $K^+$-free YNB medium supplemented with various amounts of KCl was inoculated with yeast cells ($OD_{600\,nm}$ 0.05), and growth was monitored for 48 h. The effect of low pH on potassium requirements was estimated on plates adjusted with citric acid to pH 4.5 or 3.0.

For $K^+$ starvation experiments, cell cultures were grown at 28 °C in Translucent YNB medium containing 50 mM KCl to $OD_{600\,nm}$ 0.6, washed with $K^+$-free YNB medium and resuspended in the same medium without KCl. At indicated times, cell samples were withdrawn and used for various measurements.

To estimate the cell survival upon $K^+$ starvation, aliquots of cells withdrawn at indicated times were appropriately diluted and plated on YPD media. The number of colonies formed (CFU) was counted after growth at 28 °C for 2 days. Budding index was estimated according to Clotet *et al*. (1999). Briefly, random micrographs of the samples were taken in a Nikon Eclipse E800 microscope and the cell number determined with the help of WASABI software (Hamamatsu). Budding cells were assigned visually in parallel by two independent individuals. A minimum of 600–700 cells were evaluated for each condition. Statistical differences were estimated by the paired Student's *t*-test.

### Determination of $K^+$ content and $Rb^+$ uptake

To estimate internal $K^+$ content, cell samples were collected at various times on Millipore filters, which were rapidly washed with 20 mM $MgCl_2$. The cells were then extracted with acid and the extracts analysed by atomic emission spectrophotometry (Ramos *et al.*, 1990). The experiments were repeated at least five times and the SDs calculated.

To study the characteristics of $Rb^+$ uptake, cell samples were washed and suspended ($OD_{600\,nm}$ 0.3) in the uptake buffer [MES 10 mM supplemented with 2% glucose, $MgCl_2$ 0.1 mM at pH 5.8 adjusted with $Ca(OH)_2$]. The required amount of RbCl was added to the buffer at time zero and aliquots were withdrawn at various times. Cells were treated as described above. Potassium and rubidium values are expressed as nanomoles per milligram dry weight of cells. All experiments were repeated at least three times and the SDs calculated.

### Cell volume determination

Cell volume was analysed in a Cell Counter Z2 (Beckman-Coulter) and expressed in femtolitres (fL) (Merchan *et al.*, 2004). The experiment was repeated at least three times, each time *c.* $6 \times 10^4$ cells were analysed for each strain and each condition.

### Assessment of changes in plasma-membrane potential

Relative values of plasma-membrane potential ($\Delta\psi$) were measured with the fluorescent dye diS-$C_3$(3) (3,3′-dipropylthiacarbocyanide iodide; 0.1 mM stock solution in ethanol) as described previously (Maresova *et al.*, 2009; Petrezselyova *et al.*, 2010b). Harvested cells were washed twice with 10 mM $Na_2HPO_4$ (pH 6.0, adjusted with citric acid) and resuspended in the same buffer to a final $OD_{600\,nm}$ of 0.2. The potentiometric probe (final concentration 0.2 μM) was added to 3 mL of cell suspension and the sample was immediately analysed on an ISS PC1 spectrofluorometer (excitation: 531 nm, emission: 560 and 580 nm). Staining curves were fitted and the value of the intensity ratio at equilibrium estimated as in Malac *et al.* (2005). The assay was repeated at least three times with consistent results.

### Measurement of cytosolic pH

Cytosolic pH estimations and calibration curves were performed using pHluorin as described previously (Brett *et al.*, 2005) with some modifications (Maresova *et al.*, 2010). Harvested cells expressing pHluorin gene from a multicopy plasmid (Maresova *et al.*, 2010) were washed twice with 30 mM $Na_2HPO_4$ buffer (pH 6.0, adjusted with citric acid) and resuspended in the same buffer to $OD_{600\,nm}$∼0.5. Aliquots 100 μL of cell sample were transferred to a clear-bottomed, polystyrene 96-well microtitre plate and the fluorescence (excitation: 400 and 485 nm, emission: 516 nm) of each sample was determined in a Synergy HT spectrofluorometer equipped with GEN5 software (Biotek) at 28 °C.

### Proton efflux

Proton extrusion was measured by recording extracellular pH change after glucose addition as described previously (Serrano, 1980). Briefly, cells were grown to mid-log phase ($OD_{600\,nm}$ 0.6), washed three times with distilled water and stored on ice for at least 1 h. Cells were pelleted by centrifugation and resuspended in 2 mL of a 10-mM glycyl-glycine solution (pH 4.5 adjusted with HCl) containing 50 mM KCl. Glycyl-glycine solution 6 mL were added to 1.8 mL of cell suspension and pH was monitored with constant stirring until a stable baseline was reached. Glucose was then added to a final concentration of 20 mM. The pH was recorded every 10 s with a pH meter GLP21 (CRISON) and the slope was calculated. Values are expressed as nmol $mg^{-1}$ wet weight of cells $min^{-1}$. All experiments were repeated at least three times and the SDs calculated.

## Results

### Deletion of *TRK1* and *TRK2* genes in the BY4741 background results in strong phenotypes noticeable even under potassium nonlimiting conditions

As mentioned above, the study of potassium transport and homoeostasis is often hampered by the relatively high concentration of alkali metal cations in the medium. To avoid this problem, we sought to develop a synthetic minimal growth medium that would allow any experiment aimed at studying potassium homoeostasis. This $K^+$-free YNB medium has the following properties. Firstly, it carries very low amounts of potassium or sodium (15 μM $K^+$ and 1.3 mM $Na^+$) so it can be brought to any desired concentration of these cations by simple addition of the appropriate salt. Secondly, it sustains much more vigorous growth of yeast strains than synthetic media reported previously (Rodríguez-Navarro & Ramos, 1984) when properly complemented. Whereas in previous synthetic media, cells entered stationary phase at $OD_{600\,nm}$ 0.6–0.8, cells grown in the new designed medium were still mid-exponential at these densities and finally reached $OD_{600\,nm}$ of 7–8 (not shown). Thirdly, it is very similar in composition to the widely used standard YNB medium, so reasonable comparison with previously reported data can be made. In that medium, whose detailed composition is described in Supporting Information, although it is also commercially

available (ForMedium$^{TM}$), ammonium phosphate substitutes for potassium phosphate. BY4741 background, very similar to the one whose genome was sequenced, was used to characterize potassium requirements of the wild-type and *trk1 trk2* cells. We estimated first the growth rate in liquid Translucent YNB supplemented with KCl concentrations ranging from 1 to 50 mM. The double mutant showed defective growth at low potassium concentrations and only when external amounts of the cation were as high as 20–50 mM, were the growth rates of both strains comparable (Fig. 1a). Similar results were obtained when cells were grown on solid media, where low $K^+$ (15 μM in YNB plus an extra amount from the agar) was sufficient to sustain BY4741 growth but not growth of strains lacking *TRK1* and *TRK2* genes (Fig. 1b). We also observed that the increased $K^+$ requirements of the *trk1 trk2* mutant were partially suppressed by additional deletions of genes encoding $K^+$ efflux systems. An illustration is shown in Fig. 1b: mutant cells lacking Trk transporters and also the Ena ATPases and Nha1 antiporter efflux systems could grow on lower $K^+$ concentrations slightly better than the *trk1 trk2* cells, and this advantage was clearly enhanced by the additional deletion of *TOK1* gene, encoding the potassium outward channel (cf. Fig. 1b; 10 mM KCl). On the other hand, the deletion of the efflux systems abolished the growth of *trk1 trk2* mutants (and the BY4741 strain) on high extracellular KCl (Fig. 1b).

Strains lacking Trk1 and Trk2 transporters have been proposed to be hypersensitive to low pH (Gaber *et al.*, 1988); therefore we decided to compare potassium requirements of both BY4741 and *trk1 trk2* strains at pHs 4.5, 3.0 and 5.8. However, our results (Fig. 1c) indicate that acidic pH only slightly increases $K^+$ requirements of the mutant strain, but does not abolish its growth.

As the growth rates of BY4741 and *trk1 trk2* strains were similar at $K^+$ nonlimiting concentrations (50 mM KCl), potassium content, cell size, plasma-membrane potential, intracellular pH and the capacity to extrude protons were analysed to elucidate whether these physiological parameters differ between the two strains under $K^+$ nonlimiting conditions. There were no differences between the two strains in internal $K^+$ concentration ($392 \pm 18$ and $424 \pm 40$ nmol mg$^{-1}$ in BY4741 and BYT12, respectively) or cell volume ($48.22 \pm 0.07$ fL in both strains) but deletion of both *TRK* genes results in a significant hyperpolarization of the plasma membrane, as well as in a decrease of internal pH and in the capacity to extrude protons (Fig. 2a–c). It is worth noting that a similar difference in internal pH and proton extrusion capacity between the BY4741 and the *trk1 trk2* mutant was observed when cells were grown in YPD (not shown).

According to the hyperpolarization observed for the *trk1 trk2* mutant, these cells should be more sensitive to cationic

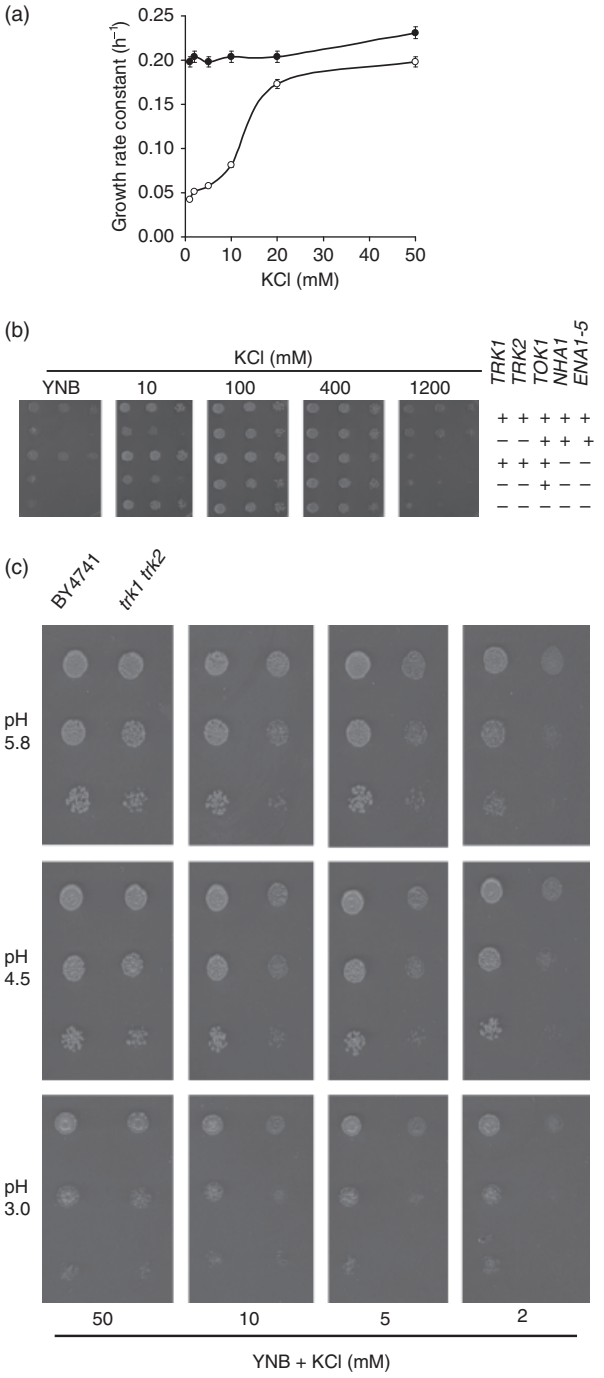


**Fig. 1.** Growth of *Saccharomyces cerevisiae* strains in Translucent $K^+$-free YNB supplemented with various KCl concentrations. (a) Specific growth rates of BY4741 (filled symbols) and *trk1 trk2* (empty symbols) cells in liquid medium. Growth rate constant values are the mean of three independent experiments ± SEM. (b) Growth of BY4741 and mutants affected in $K^+$ uptake and/or efflux systems on solid media. The right-hand column indicates the genes deleted (–) in used strains. (c) Effect of acidic pH on potassium requirements of BY4741 and *trk1 trk2* cells. Serial dilutions of each strain were spotted on plates containing the indicated amount of KCl (b, c) and growth was recorded after 2–3 days.

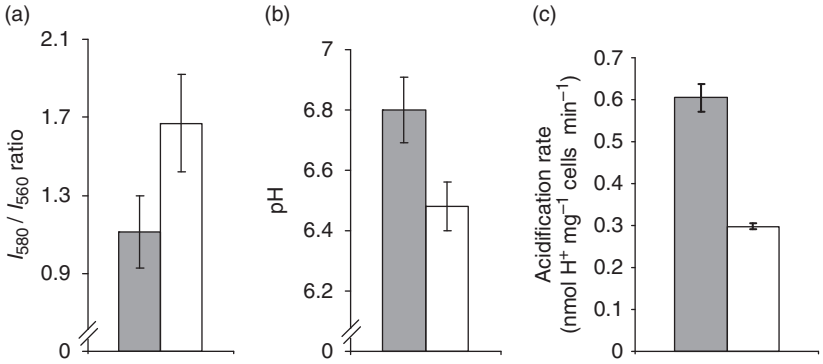


**Fig. 2.** Physiological parameters of BY4741 (grey bars) and *trk1 trk2* (open bars) cells grown under potassium nonlimiting conditions. Cells were grown in liquid Translucent YNB supplemented with 50 mM KCl, samples were taken during the exponential growth phase and relative membrane potential (a), internal pH (b) and proton efflux (c) were determined. Values are mean of three to six independent experiments ± SEM.

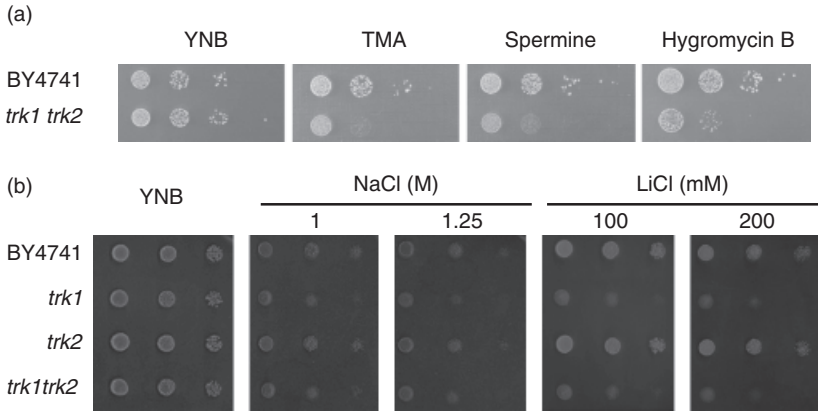


**Fig. 3.** Effect of toxic compounds on growth of mutants lacking potassium uptake systems. Serial dilutions of each strain were spotted on Translucent YNB plus 50 mM KCl plates containing 0.4 M tetramethylammonium, 1 mM spermine or 150 μg $mL^{-1}$ hygromycin B (a), and the indicated amount of NaCl or LiCl (b). Growth was recorded after 2–3 days.

drugs such as spermine, tetramethylammonium (TMA) and hygromycin B (Madrid *et al.*, 1998), and our results (Fig. 3a) confirmed this presumption. The relative hyperpolarization can also explain the observed increased sensitivity of *trk1 trk2* mutant to the toxic $Na^+$ or $Li^+$ cations (Fig. 3b).

## BY4741 cells undergo remarkable changes upon potassium limitation and these changes are dependent on the activity of Trk systems

In a subsequent set of experiments, the response of cells to potassium limitation was studied. Cells grown in YNB containing 50 mM KCl were starved for potassium in $K^+$-free YNB medium as described in Materials and methods and various physiological parameters were estimated. Figure 4 shows the changes in potassium content in BY4741 and *trk1 trk2* cells during 5 h of starvation. Both strains decreased internal potassium within the time but the decrease was much higher in the reference strain. This could be due either to potassium efflux or to the 'dilution' of intracellular potassium in dividing cells. To distinguish which *TRK* gene is responsible for the observed decrease in intracellular potassium in the BY4741 strain, and to distinguish among the two possibilities mentioned above, two types of experiments were performed.

Starvation of single mutants lacking either Trk1 or Trk2 system showed clearly that the observed decrease of intracellular potassium in the BY4741 cells depends on the presence of functional Trk1, as deletion of *TRK2* did not bring about any significant change compared with the loss of potassium from the wild-type cells (not shown).

Number of viable cells, estimated as CFU during 3 h of starvation, revealed that about 50% of BY4741 cells were able to divide, but only about 17% of the *trk1 trk2* mutant cells accomplished the cell division (not shown). This difference was observed in spite of the fact that the starting intracellular concentration of potassium in the two strains was almost identical (Fig. 4) and it suggests that, under conditions of potassium limitation, the presence of Trk proteins is important to accomplish the cell cycle. Microscopic observation revealed a decrease in the percentage of actively budding cells in the potassium-starved *trk1 trk2* strain ($28.3 \pm 1.8$ vs. $44.3 \pm 1.6$, $P < 0.005$ in the same strain grown in 50 mM potassium). Detailed analysis of the contribution of the two Trk transporters showed that the activity of both systems is involved in cell proliferation upon potassium starvation. About 31% of *trk1* and 34% of *trk2*

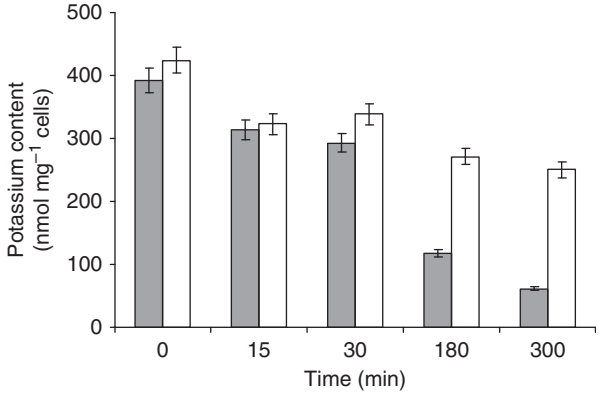


**Fig. 4.** Changes in potassium content in BY4741 (grey bars) and *trk1 trk2* (open bars) cells upon potassium limitation. Cells were grown at 28 °C in liquid Translucent YNB supplemented with 50 mM KCl to $OD_{600\,nm}$ 0.6, washed with $K^+$-free YNB medium and resuspended in the same medium w/o KCl. Samples were taken at various times and internal potassium concentration was measured. Values are mean of six independent experiments ± SEM.

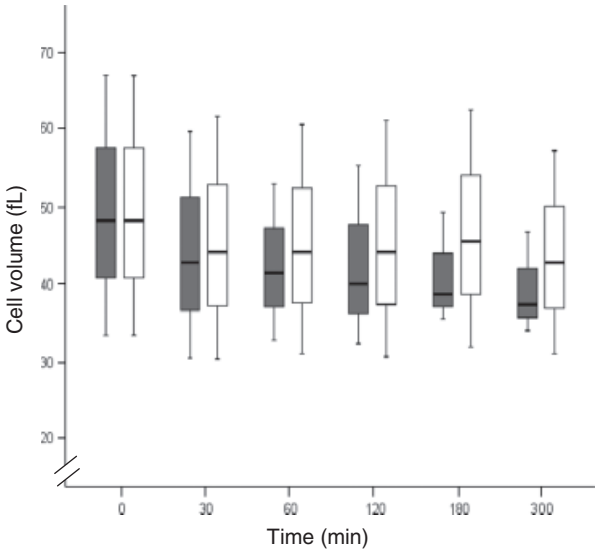


**Fig. 5.** Changes in cell volume of BY4741 (grey bars) and *trk1 trk2* (open bars) cells upon potassium limitation. Cells were grown in liquid Translucent YNB supplemented with 50 mM KCl and resuspended in $K^+$-free YNB. Samples were taken at various times and cell volume was determined. Data were obtained by analysing approximately $6 \times 10^4$ cells in each sample and they were represented in a box plot diagram.

single mutants were able to divide during 3 h under these conditions (not shown).

As only 50% of BY4741 cells divided during 3 h in $K^+$-free medium (division rate in our YNB supplemented with 50 mM KCl is approximately 3 h), but about 70% of the intracellular $K^+$ content was lost (Fig. 4), it is evident that a net potassium efflux occurred in the wild-type cells, and that this efflux was significantly diminished by deletion of the *TRK* genes (Fig. 4).

We have already mentioned that during growth in the presence of 50 mM KCl, BY4741 and the *trk1 trk2* strains showed the same cell volume. In a subsequent experiment, changes in volume were followed during $K^+$ starvation (Fig. 5). The cell size was the same for both strains at the beginning of starvation. Similarly as for $K^+$ content, a decrease in cell volume during starvation was observed. Again, the change was more remarkable for the BY4741 strain (median values from 48 to 37 or 43 fL in wild type and double mutant, respectively, after 5 h of starvation). Surprisingly, potassium starvation did not bring any significant changes in the intracellular pH of either BY4741 or *trk1 trk2* cells (Fig. 6a). In both strains, the intracellular pH increased only slightly during the 3 h of starvation, and the difference between the reference and double mutant strains was preserved, the intracellular pH of BY4741 being higher during the whole experiment. On the other hand, potassium starvation brought about significant hyperpolarization of the plasma membrane, and this hyperpolarization was more pronounced in the *trk1 trk2* cells than in the BY4741 strain (Fig. 6b). This difference may result from the re-uptake of lost $K^+$ by the wild-type cells. Detailed analysis revealed that deletion of both *TRK1* and *TRK2* contributed to the

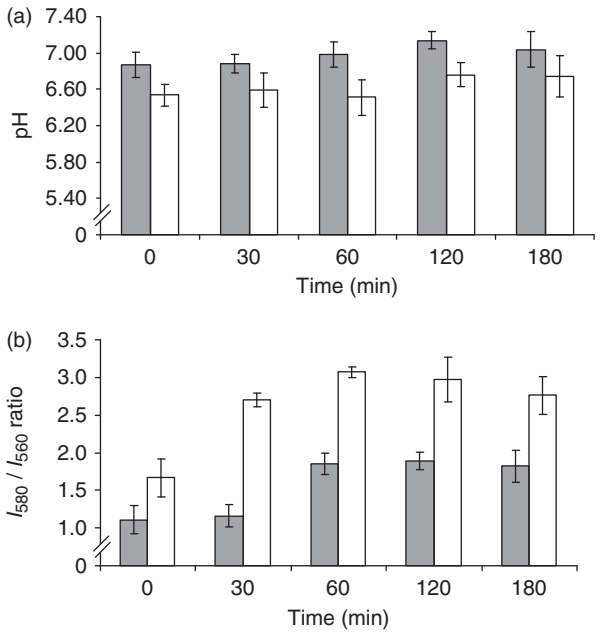


**Fig. 6.** Changes in intracellular pH (a) and relative membrane potential (b) in BY4741 (grey bars) and *trk1 trk2* (open bars) cells upon potassium limitation. Cells were grown in liquid Translucent YNB supplemented with 50 mM KCl and resuspended in $K^+$-free YNB. Samples were taken at various times and internal pH and membrane potential were measured. Values are the mean of at least three independent experiments ± SEM.

**Table 1.** Kinetic constants for rubidium transport at different starvation times (min)

| | $K_m$ (mM) | | | | | | | $V_{max}$ (nmol mg$^{-1}$ min$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | 0 | 15 | 30 | 60 | 120 | 180 | 300 | 0 | 15 | 30 | 60 | 120 | 180 | 300 |
| BY4741 | 6.21 | 0.31 | 0.14 | 0.13 | 0.13 | 0.14 | 0.16 | 5.12 | 8.91 | 9.64 | 15.4 | 26.8 | 24.9 | 27.2 |
| *trk1 trk2* | 20.1 | – | 20.1 | – | – | 20.4 | – | 5.23 | – | 6.03 | – | – | 5.27 | – |

Values are the average of at least three independent experiments. SEM was always $< 10\%$.
–, not done.

hyperpolarization (both single mutants being more hyperpolarized than BY4741 but less hyperpolarized than the double mutant; Petrezselyova *et al.*, 2010a), suggesting that both systems are active and contribute to re-uptake of lost potassium during starvation.

As mentioned in the Introduction, the adaptation of cells to low potassium conditions involves changes in the kinetic parameters of $K^+$ uptake via Trk systems. $Rb^+$ (used as a $K^+$ analogue in transport experiments) uptake was measured and the kinetic parameters of its transport before and during the potassium starvation were calculated (Table 1). The initial parameters of transport were quite similar, with low $V_{max}$ and $K_m$ in the millimolar range for both strains. $K_m$ values of 6.2 mM for BY4741 and 20.1 mM for the *trk1 trk2* mutant show the low activity of Trk proteins in the presence of 50 mM KCl. $K^+$ limitation induced the appearance of a high affinity (50-fold increase) and high-velocity process only in the BY4741 strain, indicating an enormous increase in the activity of the Trk proteins. Interestingly, the highest affinity was reached after 30 min of starvation, whereas the highest $V_{max}$ was only observed after 2 h of incubation without potassium.

Our results show that the *trk1 trk2* deletion in the BY4741 background results in changes of cell physiological parameters even in the presence of a sufficient amount of potassium and these changes become more pronounced when potassium is the limiting factor.

## Discussion

Potassium requirements, transport characteristics and even the genes encoding the main potassium transporters in *S. cerevisiae* have been known for more than 20 years (Ramos & Rodríguez-Navarro, 1986; Gaber *et al.*, 1988). However, a number of constraints, such as (i) the use of different yeast genetic backgrounds for the diverse studies performed, (ii) the lack of an appropriate culture medium that, mimicking the commonly used standard synthetic media, would enable experiments to be carried out under potassium-limiting conditions and (iii) the different standard conditions used for growth and treatment of cells, have made difficult to reach general conclusions and to establish user-friendly methodology to characterize potassium homoeostasis in yeast cells in detail. In addition, most, if not all, studies with *trk1 trk2* mutants have been performed under potassium-limiting conditions in which growth of these strains is severely impaired. In this paper we report a new $K^+$-free YNB medium, containing very low amounts of $K^+$ (15 μM) that allows comprehensive and straightforward characterization of potassium requirements and $K^+$ transport activities.

We have characterized both BY4741 cells and potassium uptake-deficient mutants grown under potassium nonlimiting concentrations and during starvation. Our results confirm what was known previously, in the sense of increased potassium requirements, defective high-affinity transport and higher sensitivity to toxic cations in the mutant lacking the main potassium uptake systems Trk1 and Trk2. Moreover, we show that under nonlimiting conditions both strains maintain a similar internal $K^+$ content and cell volume; however, even under these conditions, intracellular pH is lower in the double mutant and the mutant cells are hyperpolarized compared with BY4741. Remarkably, the previously reported hypersensitivity of *trk1 trk2* cells to acidic pH was not confirmed in our experiments. At low pH, the potassium requirements of both wild-type and mutant strains were only slightly increased compared to pH 5.8, indicating that this phenotype is most probably a side effect of the depolarization occurring at acidic pH. On the other hand, our observations confirmed previous results (obtained in the W303 genetic background) showing that the deletion of *TRK1* and *TRK2* genes results in significant hyperpolarization (Madrid *et al.*, 1998; Maresova *et al.*, 2006), and showed for the first time, surprisingly, that lack of Trk systems brings about significant decrease of the intracellular pH. The observed decrease of the intracellular pH probably results from a decreased activity of the Pma1 $H^+$-ATPase, as the proton extrusion from the *trk1 trk2* mutant is much lower than from the BY4741 strain (even at potassium nonlimiting conditions; Fig. 2b and c). A likely explanation for these observations is that the absence of the high-affinity potassium uptake in the *trk1 trk2* mutant entails less potassium influx, a decrease in the consumption of the membrane potential, a consequent downregulation of the Pma1 ATPase activity and, as a final result, a slight decrease of intracellular pH.

Upon potassium starvation, cell volume and internal potassium decreased in both strains, but whereas in the double mutant this decrease was only marginal, BY4741 cells

lost about 70% of internal $K^+$ and diminished their volume by about 23% after 5 h of potassium starvation. It is evident that potassium efflux systems are active in potassium-limiting concentrations (Figs 1b and 4). Preliminary results (not shown) obtained by our groups suggest that all three efflux systems (Tok1, Nha1, Ena1) are probably involved in the loss of potassium from BY4741 upon potassium starvation. Why they are not functional to the same level in the *trk1 trk2* mutant remains an open question, which we plan to address in the future. During potassium starvation, both strains are hyperpolarized, *trk1 trk2* mutant to a higher extent than the wild type. The observed hyperpolarization of membranes is not connected to any significant change of the intracellular pH and the pH difference between the strains observed under potassium nonlimiting conditions remained practically unchanged during 3 h of potassium starvation.

The difference in potassium content in the BY4741 and *trk1 trk2* strains during potassium starvation is striking, as the initial internal potassium content in the two strains is almost identical and both exponentially growing cells transferred to $K^+$-free YNB medium contain the same percentage of cells with buds (not shown). We presume that BY4741 cells that had reached the critical point of cell division before the transfer to the $K^+$-free medium, finished the cell cycle and divided, but the next division was slowed down due to the low intracellular potassium concentration. However, in contrast with the *trk1 trk2* strain, no difference in the budding index was observed after 3 h of culture in the presence or the absence of potassium. The decrease in the percentage of budding cells in the potassium-starved *trk1 trk2* strain has to be interpreted with caution, as it has been shown that budding index could be affected by numerous processes not always directly related to cell cycle progression (Zettel *et al.*, 2003). In any case, our results pose an important question that remains to be solved: why the double mutant lacking the Trk potassium uptake systems seems to be affected in accomplishing cell division more than the BY4741 strain, although it retains relatively stable high intracellular $K^+$ concentration. It is conceivable that, independently of their function as $K^+$ transporters, TRK proteins might have additional roles, for instance in cell division. If so, this would be a situation similar to that observed for Nha antiporters, for which independent potassium export and cell cycle regulatory capacities have been reported (Simón *et al.*, 2001).

The study of rubidium uptake revealed several aspects worth being mentioned. When growing in 50 mM KCl, the $V_{max}$ of transport was very similar in BY4741 and double mutant strains and the $K_m$s differed only slightly (Table 1). It is evident that in the double mutant, the low-affinity ectopic transport systems (Madrid *et al.*, 1998) support cell growth at 50 mM KCl. The absence of changes in affinity ($K_m$) and in rubidium uptake velocity ($V_{max}$) in the double mutant upon potassium starvation suggest that the residual ectopic potassium transport existing in these cells is poorly regulated and cannot adapt to changes of environmental conditions.

On the other hand, BY4741 cells adapt to potassium limitation by increasing both their $V_{max}$ and affinity for the cation very quickly. At this respect it is very interesting that the highest affinity for rubidium was reached within 30 min after cell transfer to potassium-limiting conditions, whereas previously reported data (Ramos & Rodríguez-Navarro, 1986; Ramos *et al.*, 1994) indicated a much slower kinetics (4–5 h). This could be due to the use of a different strain background (XT300.3A, DBY746) or, more likely, to different experimental conditions (i.e. composition of the medium). This dramatic difference emphasizes the need for standardized protocols and reagents that allow determination and comparison of diverse cellular parameters under equivalent settings. Altogether, the observed changes in kinetic parameters suggest that the adaptation of BY4741 cells to limiting concentrations of potassium consist of two phases; first, very rapidly, the affinity of Trk proteins (already existing in the plasma membrane) dramatically increases, and secondly, more slowly, the capacity increases due to an increased synthesis of Trk proteins, prolongation of Trk proteins half life, or changes in the maintenance of the protein at the cell membrane. The nature of the signal leading to the rapid increase of Trk affinity for the substrate and the molecular mechanism underlying this process remain to be elucidated.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Yeast nitrogen base w/o ammonium sulphate and w/o potassium (YNB-AS-K-AA; CYN7505) (Translucent $K^+$-free medium):

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**Supplementary material.**

**Yeast Nitrogen Base w/o Ammonium Sulfate and w/o Potassium (YNB-AS-K-AA; CYN7505) (**Translucent $K^+$-free medium)**:**

For one Liter:

| **Vitamins** | **$K^+$ free** |
|---|---|
| Biotin | 2 µg |
| Calcium Pantothenate | 400 µg |
| Folic Acid | 2 µg |
| Inositol | 2,000 µg |
| Niacin | 400 µg |
| p-Aminobenzoic Acid | 200 µg |
| Pyridoxine Hydrochloride | 400 µg |
| Riboflavin | 200 µg |
| Thiamine Hydrochloride | 400 µg |
| | |
| **Compounds Supplying Trace Elements** | |
| Boric Acid | 500 µg |
| Copper Sulfate | 40 µg |
| Ferric Chloride | 200 µg |
| Manganese Sulfate | 400 µg |
| Potassium Iodide | 50 µg |
| Sodium Molybdate | 200 µg |
| Zinc Sulfate | 400 µg |
| | |
| **Salts** | |
| Magnesium Sulfate | 0.5 g |
| Sodium Chloride | 0.1 g |

| | |
|---|---|
| Calcium Chloride | 0.1 g |
| Ammonium Phosphate (Monobasic) | 0.92 g |

**For Potassium-free Medium:**

| | |
|---|---|
| 20 g | Glucose |
| 4 g | $NH_4SO_4$ |
| 1.63 g | YNB-AS-K-AA |
| 1.285 g | Brent Supplement Mix ï Austrian modification (DBSM225) |
| | Supplements as desired |

## 4.3 Publikace č. 3 – Plasma-membrane hyperpolarization diminishes the cation efflux via Nha1 antiporter and Ena ATPase under potassium limiting conditions

Na základě výsledků získaných v rámci předchozí publikace (kapitola 4.2, str. 38) vyvstala řada otázek. Výstup $K^+$ z buněk během hladovění na $K^+$ byl pozorovnám u kmene BY4741, ale po deleci genů *TRK1* a *TRK2* se tento výstup zastavil. To naznačovalo, že s odstraněním *TRK1* a *TRK2* je snížený výstup, takže u některého ze tří exporterů došlo ke snížení jeho aktivity. Pro objasnění toho, který z exporterů se výstupu $K^+$ při hladovění účastní, byly použity kmeny postrádající jednotlivě geny *ENA*, *NHA1* a *TOK1*, dále kombinace dvojic a také kmen postrádající všechny tři exportní systémy. Výsledky z předchozí publikace (kapitola 4.2; výstup $K^+$ z buněk v čase) byly opakovány a porovnány s kmenem postrádajícím všechny důležité transportery $K^+$ v PM (delece *trk1*, *trk2*, *tok1*, *nha1* a geny *ena1-5*). Na základě těchto experimentů bylo zřejmé, že pozorovaný výstup $K^+$ probíhá částečně přes všechny exportní systémy a že všechny tyto exportery (Ena, Tok1 a Nha1) mají po deleci *trk1* a *trk2* výrazně nižší aktivitu.

Současná nefunkčnost všech tří exporterů majících odlišný mechanismus transportu (kanál, antiporter, ATPasa) a také rozdílnou regulaci (regulace exprese, či post-translační modifikace) naznačovala, že se jedná o obecný regulační jev, který byl schopen inhibovat aktivitu všech exporterů. Bylo zjištěno, že zvýšený $\Delta\Psi$ je tím regulačním prvkem upravujícím aktivitu všech exporterů. Pomocí delece *tok1* v kmeni postrádajícím *TRK1* a *TRK2* byla tato skutečnost prokázána, protože následně došlo k poklesu $\Delta\Psi$, opětovnému odblokování exportu $K^+$ a zmenšování buněk při hladovění na $K^+$ jako v kmeni rodičovském.

V rámci této publikace bylo poprvé objeveno, že se všechny exportery podílejí na exportu $K^+$ při sníženém obsahu $K^+$ v okolí a všechny mohou být společně inhibovány pomocí zvýšeného $\Delta\Psi$.

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RESEARCH ARTICLE

# Plasma-membrane hyperpolarization diminishes the cation efflux via Nha1 antiporter and Ena ATPase under potassium-limiting conditions

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**Keywords**
potassium homeostasis; potassium efflux; plasma-membrane potential; yeast.

## Abstract

*Saccharomyces cerevisiae* extrudes $K^+$ cations even when potassium is only present in scarce amounts in the environment. Lost potassium is taken up by the Trk1 and Trk2 uptake systems. If the Trk transporters are absent or nonfunctional, the efflux of potassium is significantly diminished. A series of experiments with strains lacking various combinations of potassium efflux and uptake systems revealed that all three potassium-exporting systems the Nha1 antiporter, Ena ATPase and Tok1 channel contribute to potassium homeostasis and are active upon potassium limitation in wild-type cells. In *trk1*Δ *trk2*Δ mutants, the potassium efflux via potassium exporters Nha1 and Ena1 is diminished and can be restored either by the expression of *TRK1* or deletion of *TOK1*. In both cases, the relative hyperpolarization of *trk1Δ trk2Δ* cells is decreased. Thus, it is the plasma-membrane potential which serves as the common mechanism regulating the activity of $K^+$ exporting systems. There is a continuous uptake and efflux of potassium in yeast cells to regulate their membrane potential and thereby other physiological parameters, and the cells are able to quickly and efficiently compensate for a malfunction of potassium transport in one direction by diminishing the transport in the other direction.

## Introduction

The maintenance of intracellular alkali-metal-cation homeostasis is a complex process which is important for the survival of all organisms. Yeast cells usually spend more energy to accumulate and maintain the high intracellular concentration of potassium that is required for many physiological processes (e.g. protein synthesis, enzyme activation, cell volume, membrane potential and intracellular pH regulation) (Rodriguez-Navarro, 2000), and to maintain low cytosolic concentrations of toxic cations such as sodium or lithium (Arino *et al.*, 2010). The tolerance of yeast cells to high external concentrations of alkali-metal-cation salts is determined by several factors, the most important being the presence and activity of plasma-membrane and organellar cation transporters. Yeast species vary significantly in their ability to grow in either the presence of high concentration of salts or in external potassium-limiting conditions. This difference is based on the presence/absence of distinct transporters and on the specific ways they are regulated [for a review, see (Arino *et al.*, 2010; Ramos *et al.*, 2011)]. Significant differences in salt sensitivity/tolerance and cation homeostasis were not only found among different yeast species but were even observed between two laboratory strains of *Saccharomyces cerevisiae* [BY4741 and W303 (Petrezselyova *et al.*, 2010)].

In the *S. cerevisiae* plasma membrane, two transport systems are employed to accumulate the necessary amount of potassium and three transporters serve to eliminate surplus intracellular alkali metal cations and maintain the optimum concentration of potassium. The uptake and accumulation of $K^+$ is mediated by the Trk1 (Gaber *et al.*, 1988) and Trk2 (Ko & Gaber, 1991; Ramos *et al.*, 1994) uniporters whose activity is driven by the membrane potential generated by the Pma1 $H^+$-ATPase (Arino *et al.*, 2010). The *TRK1* gene encodes the main potassium uptake system, a high-affinity transporter whose activity influences $K^+$ and pH homeostases, cell turgor and plasma-membrane potential [for a review, see

(Arino *et al.*, 2010)]. The transporter encoded by the *TRK2* gene is also involved in $K^+$ accumulation and in the regulation of membrane potential; however, its expression level is rather low and the effect of its absence is much less pronounced (Navarrete *et al.*, 2010; Petrezselyova *et al.*, 2011). The presence of the *TRK1* and *TRK2* genes enables *S. cerevisiae* cells to grow even in very limited (micromolar) concentrations of potassium, and their absence (*trk1Δ trk2Δ* strains) strongly reduces the ability of cells to grow if the external potassium concentration is not at least 50 mM (Navarrete *et al.*, 2010).

Three systems differing in their transport mechanisms and activity regulation mechanism were found to mediate the export of $K^+$ from *S. cerevisiae* cells. Tok1 is a voltage-gated outward rectifying $K^+$-specific channel (Gustin *et al.*, 1986) that opens upon plasma-membrane depolarization (Bertl *et al.*, 2003) and serves for fine tuning plasma-membrane potential (Bertl *et al.*, 2003; Maresova *et al.*, 2006). The other two $K^+$ exporters are active, have broader substrate specificity and serve to export surplus potassium and eliminate toxic sodium and lithium cations from the cytosol. The Nha1 $Na^+(K^+)/H^+$ antiporter (Prior *et al.*, 1996) is a constitutively expressed housekeeping protein that uses the inward gradient of $H^+$ (created by the Pma1 $H^+$-ATPase) as a driving force to export $Na^+$, $K^+$, $Li^+$ and $Rb^+$ (Banuelos *et al.*, 1998; Kinclova *et al.*, 2001). The activity of Nha1 also plays a role in the maintenance of plasma-membrane potential and regulation of cell volume and internal pH (Sychrova *et al.*, 1999; Kinclova-Zimmermannova *et al.*, 2006; Arino *et al.*, 2010). The Tok1 and Nha1 proteins are expressed at rather low levels and their activity is regulated at the post-translational level, for example by Hog1-mediated phosphorylation upon osmotic stress (Proft & Struhl, 2004).

On the other hand, the third system exporting alkali metal cations, Ena $Na^+(K^+)$-ATPase (Haro *et al.*, 1991), is mainly regulated at the expression level. It is strongly induced by osmotic stress or alkaline external pH (Ruiz & Arino, 2007; Arino *et al.*, 2010). Ena ATPase is the main sodium and lithium detoxifying system in *S. cerevisiae*, but it also contributes significantly to high potassium tolerance (Banuelos *et al.*, 1998). The number of *ENA* gene copies varies among the different *S. cerevisiae* strains (e.g. *ENA1*, *ENA2* and *ENA5* in BY4741), but the dominant role in $Na^+$ and $K^+$ export is played by *ENA1* (Wieland *et al.*, 1995; Arino *et al.*, 2010).

Surprisingly, *S. cerevisiae* cells can survive without the five plasma-membrane potassium transporters. The BY4741-derived quintuple mutant strain (BYT12345, *trk1Δ trk2Δ tok1Δ nha1Δ ena1-5Δ*) is viable and grows well if it is supplemented with an appropriate (not too low and not too high) external potassium concentration (Navarrete *et al.*, 2010). In fact, these cells survive potassium-limiting concentrations better than double mutants lacking only the Trk1 and Trk2 $K^+$ uptake systems (Navarrete *et al.*, 2010). A detailed study of the phenotypes and physiological parameters of wild-type BY4741 and Trk-deficient strains showed several surprising results, not only under potassium-limiting, but also under potassium-sufficient conditions. Even in the presence of sufficient amounts of potassium, the deletion of *TRK1* and *TRK2* resulted in a significant plasma-membrane hyperpolarization, a decrease in intracellular pH and in a diminished ability to acidify external media. Furthermore, *trk1Δ trk2Δ* cells were more sensitive to high concentrations of NaCl, LiCl and cationic drugs such as spermine, hygromycin B and tetramethylammonium (Navarrete *et al.*, 2010). Additional phenotypes of *trk1Δ trk2Δ* mutants were observed during $K^+$ starvation. In media with micromolar potassium concentrations, *trk1Δ trk2Δ* cells did not divide and their relative hyperpolarization became more evident. Interestingly, at nonlimiting concentrations of KCl (above 50 mM), the wild-type and *trk1Δ trk2Δ* strains had a comparable cell size and potassium content, whereas upon transfer to potassium-limiting conditions, the wild-type cells released more potassium and diminished their size to a higher extent than the *trk1Δ trk2Δ* cells (Navarrete *et al.*, 2010). This data indicated that a net potassium efflux occurs during potassium starvation in wild-type cells but not in cells lacking the *TRK1* and *TRK2* genes.

In this study, we examined the contribution of individual $K^+$ export systems (Tok1, Nha1, Ena1) to the observed $K^+$ loss during potassium starvation of wild-type cells, and we analysed the physiological basis of their relative inactivity in the *trk1Δ trk2Δ* strain.

## Materials and methods

### Strains, media and plasmids

The *S. cerevisiae* BY4741 (*MAT*a *his3Δ1 leu2Δ met15Δ ura3Δ*; EUROSCARF) strain and its derivatives lacking one or various combinations of the *TRK1, TRK2, TOK1, NHA1* and *ENA1-5* genes were used in this study. All mutant strains were prepared by homologous recombination using the Cre-*loxP* system (Gueldener *et al.*, 2002) and their genotypes are listed in Table 1.

Yeast strains were grown either in standard YPD, in standard YNB or in $K^+$-free YNB-F [0.175% YNB-F w/o amino acids, ammonium sulphate and potassium (Formedium) adjusted to pH 5.8 and supplemented with 0.4% ammonium sulphate and various concentrations of KCl (Navarrete *et al.*, 2010)] at 30 °C. All media contained 2% glucose, and both YNB and YNB-F were

**Table 1.** Strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| BY4741 | *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | EUROSCARF |
| BYT12 | *trk1Δ::loxP trk2Δ::loxP* | Petrezselyova *et al.* (2010) |
| BYT123 | *trk1Δ::loxP trk2Δ::loxP tok1Δ::loxP* | This work |
| BYT124 | *trk1Δ::loxP trk2Δ::loxP nha1Δ::loxP* | This work |
| BYT125 | *trk1Δ::loxP trk2Δ::loxP ena1-5Δ::loxP* | This work |
| BYT3 | *tok1Δ::loxP* | This work |
| BYT4 | *nha1Δ::loxP* | Petrezselyova *et al.* (2010) |
| BYT5 | *ena1-5Δ::loxP* | This work |
| BYT45 | *nha1Δ::loxP ena1-5Δ::loxP* | Navarrete *et al.* (2010) |
| BYT345 | *tok1Δ::loxP nha1Δ::loxP ena1-5Δ::loxP* | This work |
| BYT1245 | *trk1Δ::loxP trk2Δ::loxP nha1Δ::loxP ena1-5Δ::loxP* | Navarrete *et al.* (2010) |
| BYT12345 | *trk1Δ::loxP trk2Δ::loxP tok1Δ::loxP nha1Δ::loxP ena1-5Δ::loxP* | Navarrete *et al.* (2010) |

supplemented either with Brand Supplement Mix (BSM) or BSM w/o uracil. Solid media were supplemented with 2% agar.

YEp352-based plasmid pTRK1 was used for *TRK1* expression under the control of its own promoter (≈ 500 bp). It was constructed and kindly provided by S. Petrezselyova.

## Potassium tolerance tests

To compare the growth of yeast cells at various concentrations of KCl, drop tests on solid YNB-F media were performed according to (Krauke & Sychrova, 2011). Growth was tested either at limited potassium concentrations (approximately 15 μM to 50 mM), at standard potassium concentration (200 mM) or in the presence of high KCl concentrations (up to 2.0 M KCl). Plates were incubated at 30°°C and the growth of cells was recorded for 3–5 days.

## Estimation of $K^+$ content changes upon potassium limitation

Cells were grown overnight in YNB-F + 200 mM KCl to $OD_{600} \approx 0.5$, washed, resuspended ($t = 0$ min) in $K^+$-free YNB-F and incubated for 60 min. Samples were withdrawn at $t = 0$, 30 and 60 min, washed, resuspended in the incubation buffer [10 mM Tris, 0.1 mM $MgCl_2$, pH adjusted to 4.4 with citric acid and then increased to 4.5 with $Ca(OH)_2$] and rapidly collected by filtration using Millipore filters. Cells on filters were washed immediately with 20 mM $MgCl_2$, acid-extracted and the intracellular $K^+$ concentration was estimated by atomic absorbtion spectrophotometry (Kinclova *et al.*, 2001). Samples were taken in triplicate for each time point and the average results of three independent experiments are presented.

## Measurement of $K^+$ efflux rate

Cells grown overnight in YNB-F + 200 mM KCl ($OD_{600} \approx 0.5$) were harvested, washed, resuspended ($t = 0$ min) in the incubation buffer [10 mM Tris, 0.1 mM $MgCl_2$, 10 mM RbCl, 2% glucose, pH adjusted to 4.4 with citric acid and then increased to 4.5 with Ca $(OH)_2$] and incubated for 60 min. Samples were withdrawn at 1, 5, 10, 25, 30, 35, 45 and 60 min, collected by filtration using Millipore filters and processed as described above. The experiment was repeated three times and a representative result is shown.

## Estimation of relative plasma-membrane potential

Relative changes in plasma-membrane potential ($\Delta\psi$) were measured using the fluorescent dye diS-$C_3(3)$ (3,3′-dipropylthiacarbocyanide iodide; 0.1 mM stock solution in ethanol). Cells were grown overnight in YNB-F + 200 mM KCl to $OD_{600} \approx 0.5$, washed, resuspended ($t = 0$ min) in $K^+$-free YNB-F and incubated for 60 min. Cells were harvested, washed twice with 10 mM MES (pH 6.0, adjusted with triethanolamin), resuspended in the same buffer to a final $OD_{600}$ of 0.1 and the potentiometric probe was added to a final concentration of 0.2 μM. The ratio of 560/580 nm emission intensities was immediately measured with an ISS PC1 spectrofluorometer (excitation: 531 nm) and processed as described previously (Maresova *et al.*, 2009; Navarrete *et al.*, 2010).

## Estimation of cell size

Cell diameter was estimated for cells growing in YNB-F + 200 mM KCl and for cells incubated in $K^+$-free YNB-F for 60 min. A cell counter (CASYTM model TT; Innovatis) with a 60 μm capillary was used. The experiment was

repeated twice, each time $2 \times 10^4$ cells were analysed for each strain and each set of conditions. Intervals containing most typical 60% of the cell population were visualized using a box plot diagram with the mean diameter from the observed interval (3–9 μm) inside the box.

## Results and discussion

### $K^+$ exporters are not functional in a *trk1*Δ *trk2*Δ strain in potassium-limiting conditions

When *S. cerevisiae* cells were grown in standard YNB or YPD media (containing approximately 20 mM $K^+$), their intracellular potassium concentration was approximately 600 nmol per mg dry wt, that is roughly 0.3 M, depending on the strain and media pH (data not shown). When the growth medium contained a higher potassium level, for example as in our standardly used YNB-F supplemented with 200 millimoles KCl/l, the intracellular concentration of potassium in cells having all plasma-membrane potassium transporters was around 650 nmol per mg dry wt, and those of cells lacking all five $K^+$ transporters (BYT12345; *trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1-5*Δ) was slightly higher (700 nmol per mg dry wt; Fig. 1). The small increase in potassium content (approximately 10%) when the cells grew in the presence of tenfold higher concentration of KCl (20 vs. 200 mM) suggested a very efficient regulation of potassium influx and efflux. If the cells were grown in the presence of 50 or 200 mM KCl and subsequently incubated in YNB-F without the addition of KCl (i.e. in the presence of only 15 μM $K^+$), their intracellular concentration of potassium decreased [Fig. 1 and (Navarrete *et al.*, 2010)]. Surprisingly, the decrease in intracellular potassium content was more pronounced in the wild-type cells (BY4741; about 50% of intracellular $K^+$ lost in 60 min) than in cells without potassium uptake systems (BYT12, *trk1*Δ *trk2*Δ; 15% lost in 60 min). This result suggested that the potassium efflux via export system(s) was prominent in the BY4741 wild type but not in the mutant lacking the Trk1 and Trk2 uptake systems. Comparison of the data obtained for the BYT12 (*trk1*Δ *trk2*Δ) and BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1-5*Δ) cells showed the same level of $K^+$ loss and confirmed the hypothesis that the three systems exporting potassium are not functional in BYT12 (*trk1*Δ *trk2*Δ) cells upon potassium starvation (Fig. 1). This observation led to two main questions: Which of the three potassium exporters was responsible for the potassium efflux in BY4741 cells, and what mechanism was responsible for the reduced potassium efflux in *trk1*Δ *trk2*Δ cells?

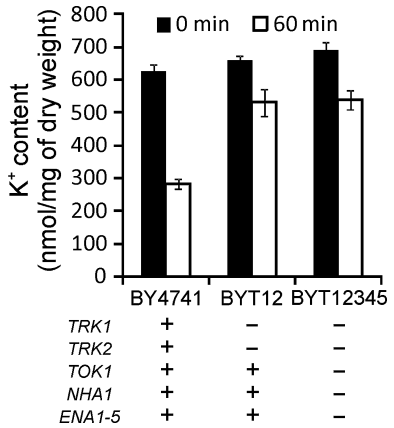


**Fig. 1.** Potassium exporters Nha1, Ena1-5 and Tok1 are not functional in *trk1*Δ *trk2*Δ cells during potassium starvation. $K^+$ content was estimated in wild-type (BY4741), BYT12 (*trk1*Δ *trk2*Δ) and BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1-5*Δ) cells grown in YNB-F supplemented with 200 mM KCl immediately after the transfer of cells into $K^+$-free YNB-F medium (black bars) and after 60 min of incubation (open bars). Average results of three independent experiments are shown ± SD.

### All three potassium-exporting systems participate in $K^+$ loss observed under potassium-limiting conditions

To elucidate which of the three $K^+$ export systems is responsible for the observed efflux of $K^+$ from BY4741 cells during potassium starvation, the changes in intracellular $K^+$ content were followed in a series of mutants lacking one or two or all three $K^+$ exporter genes (*TOK1*, *NHA1* and *ENA*) (Fig. 2). Surprisingly, values obtained for single mutants were similar to those obtained for the wild-type BY4741 cells. Within one hour under potas-

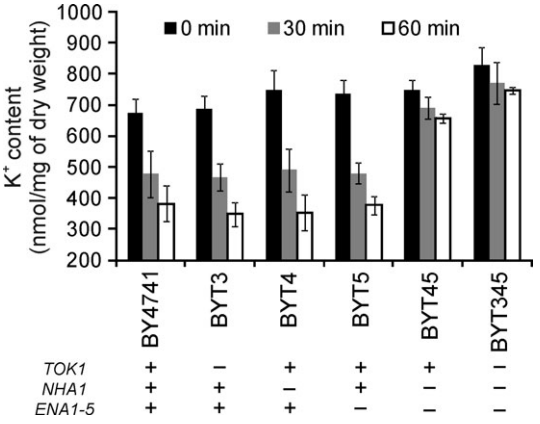


**Fig. 2.** All potassium exporters (Nha1, Ena1-5 and Tok1) contribute to the $K^+$ loss observed under potassium-limiting conditions. Cells were grown in YNB-F supplemented with 200 mM KCl, transferred into $K^+$-free YNB-F, and the intracellular concentration of $K^+$ was estimated immediately (black bars), after 30 min (grey bars) and 60 min (open bars). Average results of three independent measurements of each strain are shown ± SD.

sium-limiting conditions, the intracellular content of potassium in these strains dropped from the initial concentration of about 700 nmol $K^+$ per mg dry weight to approximately 350 nmol $K^+$ per mg dry weight. A similar loss of $K^+$ in all single deletion mutants and the wild type indicated that the remaining two exporters were able to substitute for the activity of the missing exporter. The initial $K^+$ concentration in BYT45 strain, lacking both active export systems (*nha1*Δ *ena1-5*Δ), was slightly higher than in the wild type, but the loss of $K^+$ during starvation was strongly reduced (Fig. 2), which corresponded to the stable intracellular potassium concentration observed when the rate of potassium efflux from cells was measured (cf. Fig. 5a; cells BYT45 [YEp352]). This result suggested that the Nha1 and Ena systems are the two main systems involved in potassium efflux. The observed significant role of Ena ATPase was surprising, as under our experimental conditions (pH 5.8, 200 mM KCl) the expression of Ena1 should not be very high (Ruiz & Arino, 2007; Arino *et al.*, 2010).

The role of the Tok1 channel became evident when potassium content and efflux were measured in the BYT345 (*tok1*Δ *nha1*Δ *ena1-5*Δ) strain. The potassium concentration in BYT345 grown in YNB-F + 200 mM KCl was significantly higher (850 nmol $K^+$ per mg dry weight; Fig. 2) than in BYT45 (*nha1*Δ *ena1-5*Δ) or the wild type (BY4741), but the loss of $K^+$ from BYT345 cells was slightly smaller than from BYT45 cells (approximately 9.1 ± 1.7% vs. 12.2 ± 2.2% of internal $K^+$ lost in 60 min; Fig. 2). Our results clearly showed that the activity of the Tok1 channel significantly influences the potassium content in cells grown at standard potassium levels and that its contribution to potassium export in potassium-limiting conditions is lower than those of the active exporters Nha1 and Ena1.

## Deletion of *TOK1* in BYT12 (*trk1*Δ *trk2*Δ) strain results in hypersensitivity to low $K^+$ concentrations

To study the contribution of the activity of $K^+$ efflux system to cell growth under potassium-limiting conditions, we used strains derived from BYT12 (*trk1*Δ *trk2*Δ) bearing additional deletions of *TOK1* (BYT123), *NHA1* (BYT124), *ENA1-5* (BYT125) or the double deletion of *NHA1* and *ENA1-5* (BYT1245). First, the growth of these strains was characterized and compared with the wild type (BY4741) and BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1*Δ) in either high or limiting concentrations of KCl (Fig. 3). Similar growth of all tested strains was only observed on plates containing 0.2 M KCl, that is under conditions closest to the optimal intracellular concentration of potassium. The tested strains also grew similarly well on 50 mM KCl, with the exception of BYT123 (*trk1*Δ *trk2*Δ *tok1*Δ), which grew slightly slower (Fig. 3). As expected, the growth of strains lacking more than one potassium exporter (BYT1245 and BYT12345) was inhibited at high external potassium concentrations. Surprisingly, the inability of the BYT124 strain (*trk1*Δ *trk2*Δ *nha1*Δ) to grow on high KCl (e.g. 2 M, Fig. 3) indicated a major role of Nha1p in eliminating surplus $K^+$. Although the *ENA1* gene is highly expressed under these high-osmolarity conditions (Ruiz & Arino, 2007; Arino *et al.*, 2010), it is the antiporter and not the ATPase that is crucial for maintaining the optimum intracellular concentration of potassium.

The growth of all tested strains lacking *TRK1* and *TRK2* genes was strongly reduced under limiting potassium conditions. No significant difference in growth was observed between BYT12 (*trk1*Δ *trk2*Δ), BYT124 (*trk1*Δ *trk2*Δ *nha1*Δ), BYT125 (*trk1*Δ *trk2*Δ ena*1-5*Δ) and BYT1245 (*trk1*Δ *trk2*Δ *nha1*Δ *ena1-5*Δ) in 20 and 30 mM KCl. In agreement with previously published results (Navarrete *et al.*, 2010), we observed a slightly increased growth ability of strains lacking the three $K^+$ exporters (BYT12345) at limiting external potassium concentrations (Fig. 3). This advantage is most probably due to the fact that these cells do not lose internal $K^+$ during cell growth and thus need less time to accumulate the necessary amount of potassium to establish cell turgor and accomplish cell division. The phenotype of reduced growth at suboptimal potassium concentrations observed for BYT123 at 50 mM KCl became even more evident at

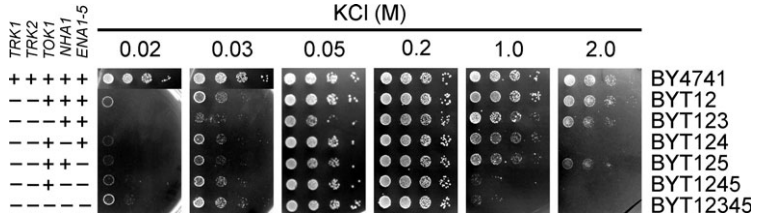


**Fig. 3.** Deletion of *TOK1* increases sensitivity of *trk1*Δ *trk2*Δ cells to limited potassium concentrations. Growth of wild-type (BY4741), BYT12 (*trk1*Δ *trk2*Δ), BYT123 (*trk1*Δ *trk2*Δ *tok1*Δ), BYT124 (*trk1*Δ *trk2*Δ *nha1*Δ), BYT125 (*trk1*Δ *trk2*Δ *ena1-5*Δ), BYT1245 (*trk1*Δ *trk2*Δ *nha1*Δ *ena1-5*Δ) and BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1-5*Δ) cells was compared on YNB-F media supplemented with various concentrations of KCl. Images shown were obtained after 3 days of growth.

lower KCl concentrations (Fig. 3). This observation suggested that the efflux activity of the Nha1 antiporter and Ena ATPases was restored in the strain lacking Trk uptake systems and the Tok1 channel. As the absence of Trk systems leads to a hyperpolarization (Madrid *et al.*, 1998; Navarrete *et al.*, 2010) and deletion of *TOK1* to a depolarization (Maresova *et al.*, 2006) of the plasma membrane, we hypothesized that the observed phenotype of *TOK1* deletion (reduced growth of cells with *tok1*Δ in the *trk1*Δ *trk2*Δ background at low KCl) might result from a plasma-membrane depolarization.

## Efflux of $K^+$ during potassium starvation is regulated by membrane potential

To verify this hypothesis, we estimated three basic physiological parameters (potassium content, relative membrane potential and cell size) of BYT123 (*trk1*Δ *trk2*Δ *tok1*Δ) cells under standard (i.e. in YNB-F with 200 mM KCl) or limiting (60 min in YNB-F without added KCl) potassium conditions and compared them with those of the BY4741, BYT12 (*trk1*Δ *trk2*Δ) and BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1*Δ) strains. While almost 90% of intracellular $K^+$ remained in BYT12 cells after 60 min in YNB-F (similarly as in BYT12345, Fig. 4a), 25% of $K^+$ was exported from BYT123 and 50% from the wild type (Fig. 4a). The results showed that deletion of the *TOK1* gene restored the efflux of potassium from cells lacking Trk1 and Trk2 systems and this efflux depended on the presence of the Nha1 and Ena1-5 efflux systems (Fig. 4a). The deletion of *TOK1* also counteracted the relative hyperpolarization of BYT12 cells (Fig. 4b) and resulted in a 'wild-type' size of BYT123 cells (Fig. 4c). During 60 min of $K^+$ starvation, the mean diameter of cells dropped by about 11%, similar to wild-type and BYT123 cells, but the mean diameter of BYT12 and BYT12345 decreased only slightly (approximately 5%; Fig. 4c). From all these results, we concluded that the phenotypes observed upon deletion of *TOK1* in the BYT12 (*trk1*Δ *trk2*Δ) background resulted from the changed membrane potential (relative depolarization of BYT123 compared to BYT12) and that it was the relative hyperpolarization that affected the efflux of potassium via exporters when cells were exposed to low potassium concentrations and simultaneously could not exploit the high-affinity potassium uptake systems. Due to this hyperpolarization, cells contained more potassium cations and were consequently bigger.

Our data (very low growth rate of *trk1*Δ *trk2*Δ *tok1*Δ cells in media with low KCl) revealed the importance of the Tok1 channel for the growth of cells in media with potassium-limiting concentrations. The positive role of overexpressed Tok1 in the growth of *trk1 trk2* mutants

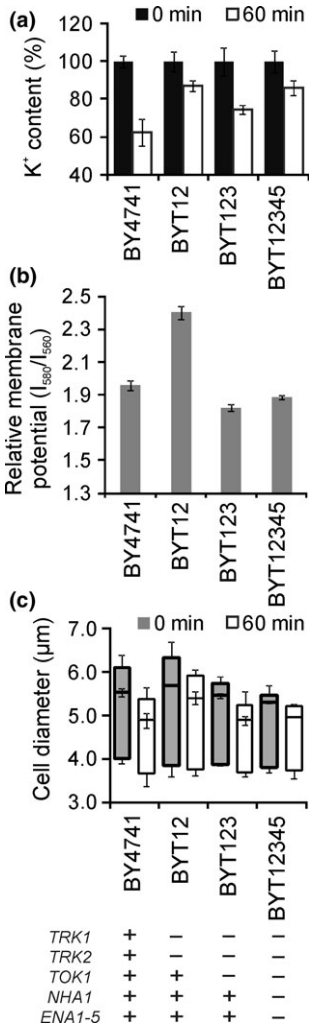


**Fig. 4.** Deletion of *TOK1* in *trk1*Δ *trk2*Δ background restores wild-type potassium content, membrane potential and cell size. Cells were grown in YNB-F supplemented with 200 mM KCl, transferred into $K^+$-free YNB-F, and (a), the intracellular $K^+$ concentration was measured immediately (black bars) and after 60 min (open bars); (b), the relative membrane potential was estimated; and (c), cell diameter was measured. For cell diameter assessment, intervals containing the most typical 60% of cell populations are shown as a grey box (initial diameter) or open box (after 60 min in $K^+$-free YNB-F). The mean diameter is represented by a line inside each box. Data were obtained in two independent experiments and represent the analysis of approximately $2 \times 10^4$ cells in each sample. Average results are shown ± SD.

under potassium-limiting conditions was observed earlier (Fairman *et al.*, 1999) and the uptake of $K^+$ via this channel was suggested but not experimentally proved. Based on our data, we believe that the *TOK1* overexpression results in a hyperpolarization of the cell membrane [as shown in (Maresova *et al.*, 2006)] which increases the ectopic potassium uptake (Madrid *et al.*, 1998).

### Expression of Trk1 restores efflux of potassium via Nha1 and Ena1-5 exporters

To confirm our hypothesis, we tried to depolarize cells by the overexpression of *TRK1*. When the potassium efflux rate was measured (Fig. 5a), it was evident that the efflux of potassium depended on the presence of both potassium uptake (Trk1) and potassium efflux (Nha1, Ena1-5) systems. Cells lacking Trk systems did not release potassium similarly to cells lacking the Nha1 antiporter and Ena ATPases (Fig. 5a; BYT12[YEp352] and BYT45 [YEp352]). A $K^+$ efflux comparable to that observed for the wild-type (BY4741[YEp352]) cells was restored by overexpressing of *TRK1* (using pTRK1) in the BYT12 (*trk1*Δ *trk2*Δ) strain (Fig. 5a). That the observed efflux was not mediated by the Trk1 transporter itself was evident from the absence of potassium efflux in cells expressing Trk1 but lacking the Nha1 and Ena1-5 systems (BYT 45[YEp352]; Fig. 5a). The estimation of relative membrane potential in cells transformed either with an empty vector (YEp352) or with pTRK1 (Fig. 5b) confirmed that the overexpression of the *TRK1* gene resulted in a significant depolarization of the cell membrane.

Taken together, our results showed that, under potassium-limiting conditions, the three $K^+$ export systems (Nha1, Ena1 and Tok1) do not mediate potassium efflux in the *trk1*Δ *trk2*Δ mutant but all of them are active in the wild type and participate in continuous potassium efflux. As the three export systems use distinct transport mechanisms and are regulated differently, the absence of potassium efflux in the *trk1*Δ *trk2*Δ mutant must be caused by a general mechanism. We show that it is the plasma-membrane potential that regulates not only the activity of the voltage-gated Tok1 channel [as has been shown earlier (Bertl *et al.*, 2003)] but surprisingly, the hyperpolarization also diminishes the potassium efflux via Nha1 cation/$H^+$ antiporter and Ena ATPases. The interconnection of potassium uptake and efflux systems was already shown in a study in which the absence of a potassium-exporting system (Nha1) was compensated for by a decrease in Trk1 affinity for potassium (Banuelos *et al.*, 2002). Here we show the opposite effect: the absence of $K^+$ uptake systems leads to the changes of potassium efflux rate of the active exporters. Our results confirm that *S. cerevisiae* cells need a continuous uptake and efflux of potassium to regulate their membrane potential and thereby other physiological parameters such as cell size and that they are able to quickly and efficiently compensate for an insufficiency in potassium transport in one direction by diminishing the transport in the other direction.

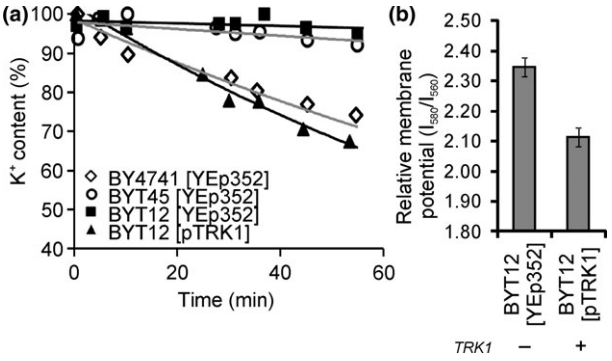


**Fig. 5.** Overexpression of *TRK1* in *trk1*Δ *trk2*Δ cells restores $K^+$ efflux and depolarizes plasma membrane. (a) $K^+$ efflux rate was measured in wild type [BY4741 (YEp352); open diamonds], in *trk1*Δ *trk2*Δ cells transformed with an empty vector [BYT12 (YEp352); black squares] or with a plasmid for *TRK1* expression [BYT12 (pTRK1); black triangles] and in *nha1*Δ *ena1-5*Δ cells [BYT45 (YEp352); open circles]. Cells grown overnight in YNB-F + 200 mM KCl were resuspended ($t$ = 0 min) in the incubation buffer and samples were withdrawn regularly over 55 min. Representative results of three independent experiments are shown. (b) Relative membrane potential was estimated in *trk1*Δ *trk2*Δ cells transformed either with an empty vector (YEp352) or with a plasmid for *TRK1* expression (pTRK1). Cells were grown in YNB-F supplemented with 200 mM KCl, and incubated in $K^+$-free YNB-F for 60 min before the measurement.

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## 4.4 Publikace č. 4 – Yeast 14-3-3 proteins participate in the regulation of cell cation homeostasis via interaction with Nha1 alkali-metal-cation/proton antiporter

Vzájemný regulační vztah mezi importery a exportery iontů alkalických kovů byl na základě publikací č. 2 a 3 objeven a prokázán (kapitola 4.2 a 4.3). Dalším úkolem této disertační práce tedy bylo pokusit se identifikovat proteiny, které by se na regulaci aktivity studovaných transporterů mohly podílet.

Na základě nedávno publikovaných dat [29, 186-188] byly proteiny 14-3-3 (kódované v *S. cerevisiae* geny *BMH1* a *BMH2*) identifikovány jako potenciální regulátory homeostase iontů alkalických kovů. Systematickou přípravou a testováním kmenů s různými kombinacemi delecí transporterů iontů alkalických kovů v kombinaci s delecí genu *BMH1* či *BMH2* bylo zjištěno, že jediným interakčním partnerem proteinů 14-3-3 je antiporter Nha1. Nepřítomnost *BMH1* vedla u buněk nesoucích gen *NHA1* ke snížení tolerance k iontům alkalických kovů a také k organickým kationtům (např. spermin). Dále bylo zjištěno, že vlivem delece *bmh1* nedochází ke změnám membránového potenciálu a že citlivost k organickým kationtům nesouvisí s objevenou interakcí proteinů 14-3-3 s Nha1p, ale jde pravděpodobně o regulaci intracelulární detoxifikace organických kationtů (např. sekvestrace do vakuol). Dále bylo prokázáno, že při absenci *BMH1*, může být role Bmh1p zastoupena umělým zvýšením exprese *BHM2*, oba geny tedy hrají v homeostasi iontů alkalických kovů stejnou úlohu, ale liší se úrovní exprese.

V druhé části práce bylo objasněno, že nejde pouze o interakci genů, ale také o přímou fyzickou interakci antiporteru Nha1 s proteiny 14-3-3 (na úrovni proteinů). Při hledání místa interakce v sekvenci Nha1p bylo zjištěno, že interakce probíhá na více místech, alespoň jednom v C-koncové cytosolické části Nha1p a alespoň jednom v části transmembránové (např. na jednom z konců poblíž zanoření proteinu do membrány nebo v cytosolicky orientovaných smyčkách; [81]). Na základě průzkumu specializovaných databází bylo (kromě AA zbytků fosforylovaných Hog1p; [86]) nalezeno dvanáct nových míst fosforylace (potenciálních míst vazby proteinů 14-3-3) v sekvenci Nha1p (viz Supplementary table 1 v této publikaci). Vzhledem k vysoké pravděpodobnosti, že by bylo nutné otestovat ještě více potenciálních míst interakce, bylo rozhodnuto výsledky publikovat a identifikaci místa interakce v budoucnu řešit jako samostatný projekt.

V rámci této práce došlo k odhalení nového regulátoru homeostase iontů alkalických kovů v kvasinkách, proteinů 14-3-3, které se účastní regulace prostřednictvím více přímých interakcí s Nha1p. Proteiny 14-3-3 působí jako positivní regulátory Nha1p odpovědné za plnou aktivaci antiporteru.

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# Yeast 14-3-3 proteins participate in the regulation of cell cation homeostasis via interaction with Nha1 alkali-metal-cation/proton antiporter

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ABSTRACT

*Background:* In yeast, 14-3-3 proteins bind to hundreds of phosphorylated proteins and play a role in the regulation of many processes including tolerance to NaCl. However, the mechanism of 14-3-3 involvement in the cell answer to salt or osmotic stresses is weakly understood.
*Methods:* We studied the role of the *Saccharomyces cerevisiae* 14-3-3 homologs Bmh1 and Bmh2 in the regulation of alkali-metal-cation homeostasis using the genetic-interaction approach. Obtained results were confirmed with the Bimolecular-Fluorescence-Complementation method.
*Results:* Deletion of *BMH1*, encoding the major 14-3-3 isoform, resulted in an increased sensitivity to $Na^+$, $Li^+$ and $K^+$ and to cationic drugs but did not affect membrane potential. This *bmh1Δ* phenotype was complemented by overexpression of *BMH2*. Testing the genetic interaction between *BMH* genes and genes encoding plasma-membrane cation transporters revealed, that 14-3-3 proteins neither interact with the potassium uptake systems, nor with the potassium-specific channel nor with the $Na^+(K^+)$-ATPases. Instead, a genetic interaction was identified between *BMH1* and *NHA1* which encodes an $Na^+(K^+)/H^+$ antiporter. In addition, a physical interaction between 14-3-3 proteins and the Nha1 antiporter was shown. This interaction does not depend on the phosphorylation of the Nha1 antiporter by Hog1 kinase. Our results uncovered a previously unknown interaction partner of yeast 14-3-3 proteins and provided evidence for the previously hypothesized involvement of Bmh proteins in yeast salt tolerance.
*General significance:* Our results showed for the first time that the yeast 14-3-3 proteins and an alkali-metal-cation efflux system interact and that this interaction enhances the cell survival upon salt stress.


## 1. Introduction

The 14-3-3 proteins form a family of highly conserved acidic dimeric proteins that are present (often in multiple isoforms) in eukaryotic cells including the yeast *Saccharomyces cerevisiae*. They bind to hundreds of different phosphorylated proteins and play a role in the regulation of many important cellular processes. Although the exact function of the 14-3-3 proteins is still not completely understood, the consequences of binding to 14-3-3 proteins are diverse. First, 14-3-3 proteins positively or negatively regulate the activity of enzymes; second, 14-3-3 proteins may act as localization anchors, controlling the subcellular localization of proteins; third, 14-3-3 proteins can function as adaptor molecules or scaffolds, thus stimulating protein-protein interactions; and fourth, 14-3-3 proteins may shield protein interaction domains (for reviews, see [1–4]). Although, 14-3-3 proteins are neither kinases nor phosphatases, the phosphorylation status of their interaction partner is often crucial for proper binding.

In the model yeast *S. cerevisiae*, two genes (*BMH1* and *BMH2*) encode 14-3-3 proteins. Bmh1 is the major form, representing about 80% of the total 14-3-3 proteins, and Bmh2 is the minor one [5]. The characterization of mutants lacking *BMH* genes revealed a synthetic lethality phenotype in most genetic backgrounds, indicating the importance of Bmh proteins for cell viability. Detailed proteomic analysis identified approximately 270 interaction partners of 14-3-3 proteins in *S. cerevisiae* cells [6] and confirmed the role of 14-3-3 proteins as regulators of various signaling pathways. Among the interaction partners, several proteins (e.g. Snf1, Hal4, Ppz1, Ptk2) have been found that are known to be regulators of the activity of plasma-membrane transporters involved in intracellular cation and pH homeostases [7]. A role of 14-3-3 proteins in cation homeostasis is further supported by the observations that a mutant strain lacking *bmh1* is NaCl sensitive [8] and the expression of *BMH1* is strongly induced by an increased external concentration of NaCl [9]. Moreover, a genome-wide screen for tolerance to cationic drugs revealed an increased sensitivity of a *bmh1Δ* mutant to Hygromycin B, Spermine

*Abbreviations:* BiFC, bimolecular fluorescence complementation; diS-$C_3(3)$, 3,3′-dipropylthiacarbocyanide iodide; YFP, yellow fluorescent protein

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**Table 1**
Strains used in this study. All strains were derived from BY4741.

| Strain | Relevant genotype | Source |
|---|---|---|
| BY4741 | *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | EUROSCARF |
| BYT-B1 | *bmh1Δ::loxP* | W. Hendriksen and G.P.H. van Heusden, unpublished results. |
| BYT-B2 | *bmh2Δ::loxP* | W. Hendriksen and G.P.H. van Heusden, unpublished results. |
| BYT12 | *trk1Δ::loxP trk2Δ::loxP* | [31] |
| BYT12-B1 | *trk1Δ::loxP trk2Δ::loxP bmh1Δ::loxP* | This work |
| BYT12-B2 | *trk1Δ::loxP trk2Δ::loxP bmh2Δ::loxP* | This work |
| BYT123 | *trk1Δ::loxP trk2Δ::loxP tok1Δ::loxP* | This work |
| BYT123-B1 | *trk1Δ::loxP trk2Δ::loxP tok1Δ::loxP bmh1Δ::loxP* | This work |
| BYT124 | *trk1Δ::loxP trk2Δ::loxP nha1Δ::loxP* | This work |
| BYT124-B1 | *trk1Δ::loxP trk2Δ::loxP nha1Δ::loxP bmh1Δ::loxP* | This work |
| BYT125 | *trk1Δ::loxP trk2Δ::loxP ena1-5Δ::loxP* | This work |
| BYT125-B1 | *trk1Δ::loxP trk2Δ::loxP ena1-5Δ::loxP bmh1Δ::loxP* | This work |
| BYT35 | *tok1Δ::loxP ena1-5Δ::loxP* | This work |
| BYT35-B1 | *tok1Δ::loxP ena1-5Δ::loxP bmh1Δ::loxP* | This work |
| BYT45 | *nha1Δ::loxP ena1-5Δ::loxP* | [18] |
| BYT45-B1 | *nha1Δ::loxP ena1-5Δ::loxP bmh1Δ::loxP* | This work |
| BYT345 | *tok1Δ::loxP nha1Δ::loxP ena1-5Δ::loxP* | This work |
| BYT12345 | *trk1Δ::loxP trk2Δ::loxP tok1Δ::loxP nha1Δ::loxP ena1-5Δ::loxP* | [18] |
| BYT12345-B1 | *trk1Δ::loxP trk2Δ::loxP tok1Δ::loxP nha1Δ::loxP ena1-5Δ::loxP bmh1Δ::loxP* | This work |
| BYT12345-B2 | *trk1Δ::loxP trk2Δ::loxP tok1Δ::loxP nha1Δ::loxP ena1-5Δ::loxP bmh2Δ::loxP* | This work |
| BYT-H1 | *hog1Δ::loxP-KanMX-loxP* | L. Maresova and H. Sychrova, unpublished results. |

and tetramethylammonium (TMA) [10], toxic cationic drugs that enter cells in amounts that depend on the plasma-membrane potential [11]. Simultaneously, 14-3-3 proteins are known as regulators of multiple mammalian and plant plasma-membrane ion transporters including mammalian $Na^+/H^+$-exchangers [12] and the *Arabidopsis thaliana* potassium channel Tpk1 [13].

*S. cerevisiae* cells maintain their intracellular cation and pH homeostases with the help of several plasma-membrane transporters — an $H^+$-ATPase, $K^+$ uptake uniporter, $K^+$ efflux channel, $Na^+(K^+)/H^+$ antiporter and $Na^+(K^+)$-ATPase [7]. Accumulation of the required potassium is mediated by two independent transporters encoded by the *TRK1* and *TRK2* genes [14,15]. The uptake of $K^+$ via both high-affinity transporters is driven by the electrochemical $H^+$ gradient generated across the plasma membrane by the essential $H^+$-ATPase Pma1 [16]. Deletion of the *TRK1* gene results in a membrane hyperpolarization, which is further increased by subsequent deletion of *TRK2* [11,17,18]. Potassium can be released via a potassium-specific channel (Tok1) that serves for fine tuning of the plasma-membrane potential. It only opens upon plasma-membrane depolarization [19] and its absence results in significant cell depolarization [20]. In addition, $K^+$ is actively exported from cells by two less-specific systems, the Nha1 antiporter and the Ena1 ATPase, which also mediate the efflux of toxic sodium ions. *ENA1* expression is strongly induced by osmotic stress or alkaline external pH. The Ena1 ATPase exports $Na^+$, $K^+$ and their analogs $Rb^+$ and $Li^+$ using ATP hydrolysis as a source of energy [21,22]. The second system is encoded by the *NHA1* gene and belongs to the CPA (Cation-proton antiporters) family. Similarly to Ena1, the Nha1 antiporter is able to export all four alkali metal cations and it uses the inward gradient of $H^+$ generated by the Pma1 $H^+$-ATPase as a driving force [23,24]. *NHA1* is believed to be a housekeeping gene whose product is important not only for cation homeostasis, but also for the regulation of cell volume, the cell cycle, and the maintenance of plasma-membrane potential and intracellular pH [7,23].

Despite intensive studies being done on yeast alkali-metal-cation homeostasis for several decades, a comprehensive regulatory network for the activity of all the transporters has not been elucidated yet. In addition, little is known on its regulation at the transcriptional level [7]. The uptake of $K^+$ via Trk1 is regulated by protein phosphatases Ppz1 and Calcineurin, protein kinases Snf1, Hal4 and Hal5 [25] and the Hal3 protein [26]. The expression of *ENA1* is regulated by protein kinases Hog1 and Snf1, protein phosphatases Ppz1 and Calcineurin, the Rim101 transcription factor [7,27], and the proteins Hal3, Nrg1 and Nrg2 [26,28]. Hog1p is the only protein kinase identified so far to be responsible for the phosphorylation and regulation of the Nha1 and Tok1 transporters [29,30].

In this study, we investigated the role of 14-3-3 proteins in yeast alkali-metal-cation homeostasis using a genetic approach, i.e. characterizing the genetic interactions between the *BMH* genes and genes encoding alkali-metal-cation transporters. A genetic interaction between the *BMH1* and *NHA1* genes was found. This interaction was confirmed by the observation that Nha1 interacts with 14-3-3 proteins at the protein level.

## 2. Materials and methods

### 2.1. Yeast strains and growth media

All *S. cerevisiae* strains used in this study were derived from BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (EUROSCARF) and are listed in Table 1. Mutant strains lacking potassium uptake transporters BYT12 (*trk1Δ trk2Δ*), potassium and sodium efflux transporters BYT45 (*nha1Δ ena1-5Δ*), or all plasma membrane alkali-metal-cation transporters BYT12345 (*trk1Δ trk2Δ tok1Δ nha1Δ ena1-5Δ*) were described previously [18,31]. Five other strains with deletions of alkali-metal-cation-transporter genes have been constructed in this work; BYT35 (*tok1Δ ena1-5Δ*), BYT123 (*trk1Δ trk2Δ tok1Δ*), BYT124 (*trk1Δ trk2Δ nha1Δ*), BYT125 (*trk1Δ trk2Δ ena1-5Δ*) and BYT345 (*tok1Δ nha1Δ ena1-5Δ*). Lastly, *BMH1* or *BMH2* were deleted in mutants lacking cation transporters. All strains were generated using homologous recombination and the Cre-loxP system [32]. For deleting of transporters' genes, the same oligonucleotides and deletion cassettes were used as in [18,31]. Integration cassettes for *BMH1* and *BMH2* deletions were amplified by PCR using the primers listed in Table 2. Proper incorporation and subsequent excision of the deletion cassette was confirmed by PCR using diagnostic oligonucleotides (Table 2).

Yeast strains were grown either in YPD (1% yeast extract, 2% peptone, and 2% glucose), YNB (0.17% YNB w/o amino acids, 0.5% ammonium sulphate, and 2% glucose) or $K^+$-free YNB-F (0.175% YNB-F w/o amino acids, ammonium sulphate and potassium (ForMedium™ UK), adjusted to pH 5.8 and supplemented with 0.4% ammonium sulphate, 2% glucose and various concentrations of KCl [18]) at 30 °C. YNB and YNB-F were supplemented with auxotrophic supplements either with Brand Supplement Mix (BSM) or BSM w/o uracil or with individual supplements (each to a final concentration of 15 mg/l). Solid media were supplemented with 2% agar.

**Table 2**
Oligonucleotides used in this study.

| Name | Nucleotide sequence |
|---|---|
| BMH1-WH01 | gcaagtgagaagaaaaagcaagttaaagataaactaaagataaaacagctgaagcttcgtacgc |
| BMH1-WH02 | agatttatcagaatacttactttggtgcttcaccttcggcggcagcgcataggccactagtggatctg |
| BMH2-WH33 | gaaaaattatcaaatcaacaaaaagtacccgttacaacaaaaaaacagctgaagcttcgtacgc |
| BMH2-WH34 | gcaagaaaactggagtggtaaatcttcatttccccttgtatttctgcataggccactagtggatctg |
| BMH1-WH25 | acgaggcaaaaagcaaagaa |
| BMH1-WH29 | cgtgctagactcccacttaatt |
| BMH1-rev1 | catacacgtcaaaacagtata |
| BMH2-a | catcatcaaaggttacgaaatc |
| BMH2-WT | gtcttctattacgaaattcaaaac |
| BMH2-revAB | caactctgaacgatagccaaa |
| NHA1-VC/N-60F | tccatactctagaactagtggatcccccgggctgcaggaatatggctatctgggagcaact |
| NHA1-VC-60R | tctgtttcaggtcgttcgggatttgcacgccggacggttcttattgagaccaagcgttt |
| NHA1-VN-60R | gctcctcgcccttgctcaccatggtggcgatggatctgttcttattgagaccaagcgttt |
| Nha1-Cter-VN-60F | tccatactctagaactagtggatcccccgggctgcaggaatatgcaaaggttgccatcgtt |
| Nha1-472-VN-60R | gctcctcgcccttgctcaccatggtggcgatggatctgttcgatggcaaccttgcatcc |

### 2.2. Plasmids

The plasmids used in this study are listed in Table 3. pUG34-VC was created by replacing the sequences encoding GFP in pUG34 with the C-terminal part of Venus, a variant of yellow fluorescent protein, obtained by PCR on plasmid pFA6a-VC-His3MX6 [33]. pUG35-VN was created by replacing of the sequences encoding GFP in pUG35 with the N-terminal part of Venus obtained by PCR on plasmids pFA6a-VN-His3MX6 [33] (P.A. Sakalis and G.P.H. van Heusden, unpublished results). pUG34-VC-BMH2 was created by inserting an *Eco*RI–*Sal*I fragment with *BMH2* from pGBDK-BMH2 [34] into pUG34-VC digested with *Eco*RI and *Xho*I. pUG35-NHA1-VN, pUG35-NHA1-472-VN and pUG35-NHA1-Cterm-VN were created by inserting DNA fragments encoding complete Nha1 (aa residues 1–985) or truncated versions encoding the N-terminal part of Nha1 (aa residues 1–472) and C-terminal part (aa residues 467–985) into *Eco*RI-digested pUG35-VN by homologous recombination. DNA fragments were amplified by PCR using pNHA1-985 as a template [23] and the oligonucleotides listed in Table 2.

### 2.3. Growth tests

The growth of yeast cells was monitored in drop tests on solid media or in liquid media. Drop tests were performed according to [35]. Growth differences observed on plates were usually confirmed by growth measurements in liquid media using a microplate reader [36]. The growth of mutant strains was tested in the presence of high concentrations of various alkali-metal-cation salts (KCl, 1.8–2.2 M; NaCl, 0.5–1.5 M; LiCl, 20–200 mM) or at limiting potassium concentration (3–10 mM) in YNB-F media. The effect of the cationic drugs Spermine (1–3 mM), Hygromycin B (10–80 mg/l), and tetramethylammonium (TMA, 0.1–0.9 M) was tested using YPD media. The experiments were repeated three times; representative results are shown.

### 2.4. Measurement of relative membrane potential

Relative plasma-membrane potential was measured with the fluorescent dye diS-$C_3$(3) (3,3′-dipropylthiacarbocyanide iodide) as previously described [37]. Cells were cultivated in YNB-F + 0.2 M KCl to the early exponential phase ($OD_{600} \approx 0.4$). Harvested cells were washed twice and resuspended in 10 mM citrate–phosphate buffer (pH 6.0) and the probe was added to a final concentration of 0.2 μM. Intensity of fluorescence emission was measured at 560 and 580 nm with an excitation wavelength of 531 nm using an ISS PC1 spectrofluorometer. The 560/580 nm intensity ratio at equilibrium was estimated and compared among the studied strains [37].

### 2.5. Confocal microscopy and flow cytometry

Yeast cells were grown in YNB-F medium supplemented with 0.05 M KCl. Bimolecular fluorescence complementation (BiFC) [33] was analyzed using a Zeiss LSM5 Exciter confocal microscope (excitation at 514 nm; emission at 530–600 nm). Microscopic images were analyzed using ImageJ software [38]. For flow cytometry, a Millipore Guava EasyCyte 5 flow cytometer was used. Fluorescence was determined after excitation at 488 nm and using the standard green emission filter (525/30 nm). For each analysis 3000 cells were used.

### 2.6. Measurement of $Na^+$ efflux and $Li^+$ uptake

To estimate the sodium efflux capacity, cells were grown overnight in YNB to $OD_{600} \approx 0.5$, resuspended in YNB + 0.1 M NaCl or 1.0 M NaCl, pH 7.0 (adjusted with $NH_4OH$) and incubated for 1 h. $Na^+$-preloaded cells were washed, resuspended (t = 0 min) and incubated in the incubation buffer (10 mM Tris, 0.1 mM $MgCl_2$, 2% glucose, and 10 mM KCl, pH adjusted to 4.4 with citric acid and then brought up to 4.5 with $Ca(OH)_2$). Samples were withdrawn and processed at distinct time

**Table 3**
Plasmids used in this study.

| Plasmid | Features | Source |
|---|---|---|
| YCplac33-BMH2 | Derived from YCplac33, *BMH2*, own promoter | [45] |
| YEplac195 | *URA3*, 2μ, *AmpR*, *ori* | [46] |
| YEplac195-BMH1 | Derived from YEplac195, *BMH1*, own promoter | [47] |
| YEplac195-BMH2 | Derived from YEplac195, *BMH2*, own promoter | [45] |
| pUG34-VC and pUG34-VN | Derived from pUG34, *HIS3*, *CEN*, *AmpR*, *ori*, expressing C-terminus of YFP Venus (VC), or N-terminus of YFP Venus (VN), *MET25* promoter | P.A. Sakalis and G.P.H. van Heusden, unpublished results |
| pUG34-VC-BMH2 | Derived from pUG34-VC, expressing *BMH2* N-terminally tagged with VC | S. Arslan and G.P.H. van Heusden, unpublished results |
| pUG34-VN-BMH2 | Derived from pUG34-VN, expressing *BMH2* N-terminally tagged with VN | S. Arslan and G.P.H. van Heusden, unpublished results |
| pUG35-NHA1-VN | Derived from pUG35-VN, expressing *NHA1* C-terminally tagged with VN | This work |
| pUG35-NHA1-VC | Derived from pUG35-VC, expressing *NHA1* C-terminally tagged with VC | This work |
| pUG35-NHA1-472-VN | Derived from pUG35-VN, expressing truncated *NHA1* sequence starting M1 to S472 C-terminally tagged with VN | This work |
| pUG35-NHA1-Cterm-VN | Derived from pUG35-VN, expressing C-terminal part of *NHA1* sequence starting M467 to K985 C-terminally tagged with VN | This work |
| YEp352 | *URA3*, 2μ, *AmpR*, *ori* | [48] |
| pNHA1-985 | Derived from YEp352, *NHA1*, own promoter | [23] |

points within 75 min and extracted as described previously [35]. The intracellular concentration of $Na^+$ was estimated by atomic absorption spectroscopy [23].

To measure $Li^+$ uptake, cells were grown overnight in YNB-F + 0.2 M KCl to $OD_{600} \approx 0.3$, resuspended (t = 0 min) in YNB-F + 0.2 M KCl + 0.2 M LiCl and incubated for 30 min. Samples were withdrawn at t = 0 and after 30 min, washed, resuspended in the incubation buffer (as with $Na^+$ efflux) and processed in the same way as the $Na^+$ efflux samples.

### 2.7. Survival rate estimation

Cells were grown overnight in YNB to $OD_{600} \approx 0.5$, resuspended in fresh YNB and incubated in the presence or absence of 1.0 M NaCl for 60 min. Aliquots of 10 μl were withdrawn, 1000-fold diluted and plated on YPD in triplicate. The number of colonies was counted after 2 days of growth.

## 3. Results

### 3.1. Deletion of BMH1 results in cation and drug sensitivity

In order to study the involvement of 14-3-3 proteins in *S. cerevisiae* cation homeostasis, deletion strains lacking *BMH1* or *BMH2* in the BY4741 background were constructed. Characterization of these *bmh* mutant strains showed *bmh1Δ* cells to have a decreased tolerance to high concentrations of NaCl (1.5 M), KCl (2 M) and LiCl (0.2 M), as well as to the cationic drugs Hygromycin B, spermine and TMA that are thought to enter the cells in direct proportion to the plasma-membrane potential. On the other hand, deletion of the *BMH2* gene had no phenotype. Fig. 1A shows the differences in growth on plates containing a high concentration of KCl, NaCl, Spermine and TMA. Similar differences were observed when the growth of strains was compared in liquid media (data not shown).

### 3.2. Deletion of BMH1 does not influence plasma-membrane potential

The increased sensitivity of a *bmh1Δ* strain to NaCl, KCl and cationic drugs suggested that the Bmh1 protein might influence the membrane potential ($\Delta\Psi$) of yeast cells, possibly via interaction with alkali-metal-cation transporters or the Pma1 $H^+$-ATPase. The observed phenotypes would correspond to a membrane hyperpolarization resulting in a higher uptake of alkali metal cations and cationic drugs. To investigate this hypothesis, the effect of *BMH1* deletion on relative membrane potential was estimated using the fluorescent dye diS-$C_3(3)$ in both wild-type (BY4741) cells and in cells lacking all potassium plasma-membrane transporters (BYT12345; *trk1Δ trk2Δ tok1Δ nha1Δ ena1-5Δ*). The measurement of relative $\Delta\Psi$ showed no statistically significant variations in this parameter in pairs of strains differing in the presence/absence of *BMH1* (Fig. 2A and B). Based on these results, we can conclude that *BMH1* is not involved in the generation/maintenance of plasma-membrane potential and thus the increased sensitivity of cells lacking *BMH1* to toxic alkali metal cations and cationic drugs is not due to an increased potential-driven uptake of these compounds.

### 3.3. Deletion of BMH1 in a strain lacking plasma-membrane cation transporters reveals the role of 14-3-3 proteins in intracellular detoxification of cationic drugs

To determine whether the *bmh1* deletion phenotype depends on the presence of active cation transporters, *BMH1* and *BMH2* genes were deleted in a strain lacking all potassium transporters (strain BYT12345) resulting in strains BYT12345-B1 and BYT12345-B2, respectively. Due to the absence of appropriate transporters, BYT12345 is highly sensitive to both low and high concentrations of KCl, and to a high concentration of NaCl or LiCl in the media. A series of drop tests revealed the same growth rate for all three strains (BYT12345, BYT12345-B1 and BYT12345-B2) in the presence of 0.8 M KCl, 0.02 M LiCl (Fig. 1B) and 0.5 M NaCl (not shown). On the other hand, cells with *BMH1* deletion were more sensitive to 3.0 mM Spermine, 15 mg/l Hygromycin B (Fig. 1B) and 0.9 M TMA (not shown). In summary, the data obtained with the BYT12345 strain and its *bmh1Δ* derivative show that the absence of the major 14-3-3 protein only affects the growth in the presence of cationic drugs but not the membrane potential (Fig. 2) or the salt tolerance of cells lacking cation transporters (Fig. 1B). Obtained results suggest that 14-3-3 proteins participate in the intracellular detoxification of cationic drugs (e.g. sequestration to vacuoles) and do not influence the level of the drugs' uptake. The loss of the effect of the *bmh1* deletion on the sensitivity to high salt concentrations after deletion of the five cation transporters suggests, on the other hand, a genetic interaction between *BMH1* and one (or more) of the five genes encoding these transporters.

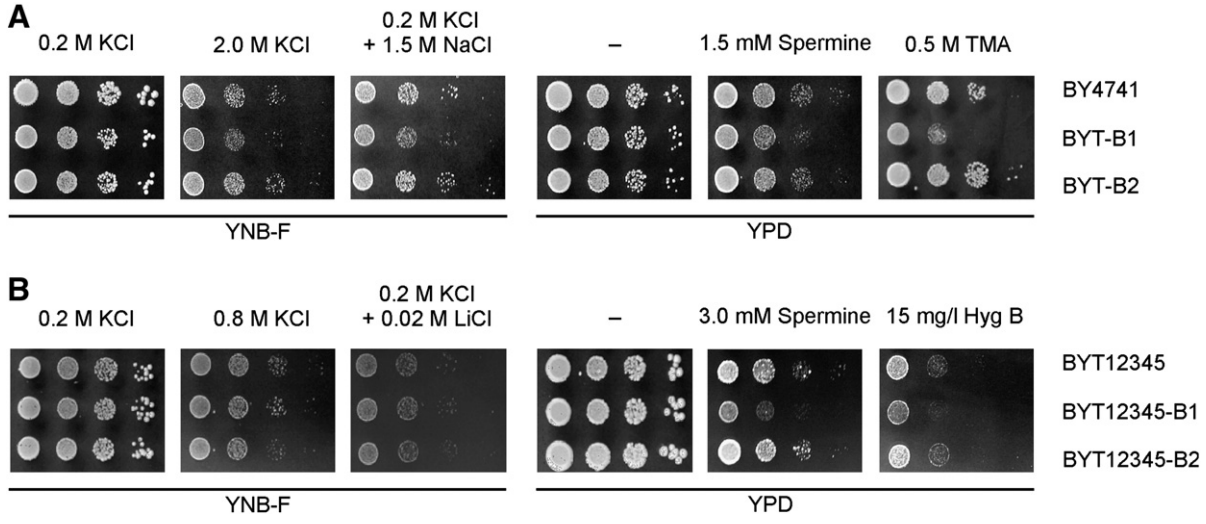


**Fig. 1.** Deletion of *BMH1* results in salt and drug sensitivity. A. Growth of wild-type (BY4741) and mutant strains lacking *BMH1* (BYT-B1) or *BMH2* (BYT-B2) was compared on YNB-F media supplemented with salts or on YPD media supplemented with Spermine or TMA. B. Growth of mutant strains lacking all five plasma-membrane cation transporters (BYT12345, *trk1Δ trk2Δ tok1Δ nha1Δ ena1-5Δ*) and *BMH1* (-B1 strain) or *BMH2* (-B2 strain) was compared on YNB-F media supplemented with salts or on YPD media supplemented with Spermine or Hygromycin B (Hyg B).

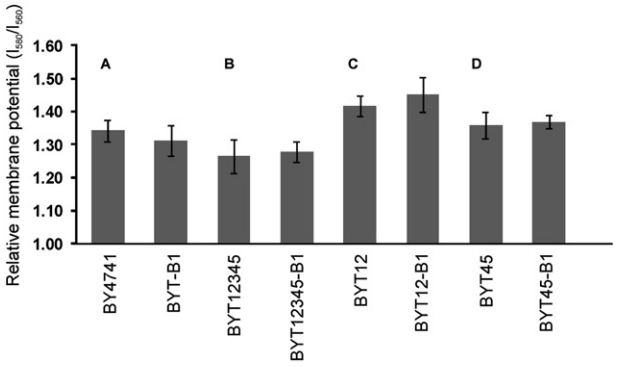


**Fig. 2.** Deletion of *BMH1* does not change plasma-membrane potential. The relative plasma-membrane potential was estimated with the diS-$C_3(3)$ fluorescent probe in (A) wild-type (BY4741), (B) BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1-5*Δ), (C) BYT12 (*trk1*Δ *trk2*Δ) and (D) BYT45 (*nha1*Δ *ena1-5*Δ) cells and their derivatives lacking *BMH1* (-B1 strains). Average results of four independent experiments are shown ± SD.

### 3.4. Deletion of BMH1 does not affect the activity of potassium uptake systems

To distinguish whether Bmh1 interacts with potassium uptake (Trk1 and Trk2) or efflux (Tok1, Nha1, and Ena1-5) systems, the genetic interaction between *BMH1* and transporter-encoding genes was studied. At limiting low concentrations of KCl (5 mM), the growth of the BY4741 and BYT-B1 (*bmh1*Δ) strains was similar, indicating that Bmh1 is not involved in active intracellular potassium accumulation (Fig. 3B). Construction of the BYT12-B1 strain (*trk1*Δ *trk2*Δ *bmh1*Δ) revealed that this strain is more sensitive to three tested cationic drugs and to high salt levels than the parental BYT12 strain (Fig. 3A shows the growth in the presence of high KCl or Spermine as an example). As in BY4741, the deletion of *BMH2* did not result in any higher-sensitivity phenotypes, neither at low KCl (not shown), nor at high KCl/cationic drug concentrations (Fig. 3). Due to the absence of high-affinity potassium uptake systems, the BYT12 strain is hyperpolarized [11] and its higher membrane potential is not changed upon *BMH1* deletion (Fig. 2C). To confirm the estimate of the relative membrane potential using a fluorescence probe, the accumulation of lithium was measured in four strains (BY4741, BYT-B1, BYT12 and BYT12-B1). There is no specific $Li^+$ transporter in *S. cerevisiae*, and this toxic cation enters the cells in amounts proportional to the membrane potential. The measured accumulation of $Li^+$ in cells showed convincingly that the absence of Trk transporters leads to a hyperpolarization of the plasma membrane. Within 30 min, BYT12 cells accumulated almost three times more $Li^+$ than BY7471 (30 vs. 13 nmol/mg dry wt). On the other hand cells lacking the *BMH1* gene accumulated the same amount as their parental strains, BY4741 and BYT12, respectively. These results clearly showed that the Bmh1 protein does not influence the activity of the Trk1 and Trk2 potassium transporters under the tested conditions.

### 3.5. BMH1 genetically interacts with NHA1

To determine which of the cation exporters might be affected by the absence of *BMH1*, a series of mutants lacking (in addition to Trk1 and Trk2) one of the three potassium exporters was constructed and phenotypically tested. As shown in Fig. 4A, *BMH1* deletion increased the high-salt sensitivity in strains lacking either the Tok1 channel (BYT123, *trk1*Δ *trk2*Δ *tok1*Δ) or the Ena1-5 ATPases (BYT125, *trk1*Δ *trk2*Δ *ena1-5*Δ). On the other hand, the deletion of *BMH1* in a strain already lacking the *NHA1* gene encoding the alkali-metal-cation/proton antiporter (BYT124, *trk1*Δ *trk2*Δ *nha1*Δ) had no cumulative effect on salt sensitivity. These results, suggesting an interaction between *BMH1* and *NHA1*, were confirmed by the construction of a strain possessing only the Nha1 antiporter (BYT35; *tok1*Δ *ena1-5*Δ). The deletion of *BMH1* in this background resulted in a very high sensitivity to the three tested salts (NaCl, LiCl and KCl), similar to that observed in a strain where all three efflux systems are missing (Fig. 4B).

Overall, the estimation of possible genetic interactions between genes encoding 14-3-3 proteins and five genes for alkali-metal-cation transporters only revealed a genetic interaction between *BMH1* and *NHA1* and suggested the Nha1 $Na^+(K^+)/H^+$ antiporter to be a putative Bmh-binding partner.

### 3.6. Overexpression of BMH2 complements the bmh1Δ phenotypes

All the observed phenotypes were only related to the absence of *BMH1*, the deletion of *BMH2* did not produce any significant effect. To distinguish whether this is due to protein specificity, i.e. Bmh1 having other binding partners during the cell response to high salt or cationic-drug stresses than Bmh2, or to the fact that Bmh1 is present in cells in much higher amounts than Bmh2 [5], we tried to complement *bmh1*Δ with *BMH2* on the plasmid. As shown in Fig. 5, overexpression of both *BMH1* and *BMH2* restores the tolerance of cells to salts and cationic drugs. In addition, the effect of *BMH2* depends on its dose. The growth of cells expressing *BMH2* from a multicopy plasmid is much better than the growth of cells transformed with a centromeric plasmid harboring

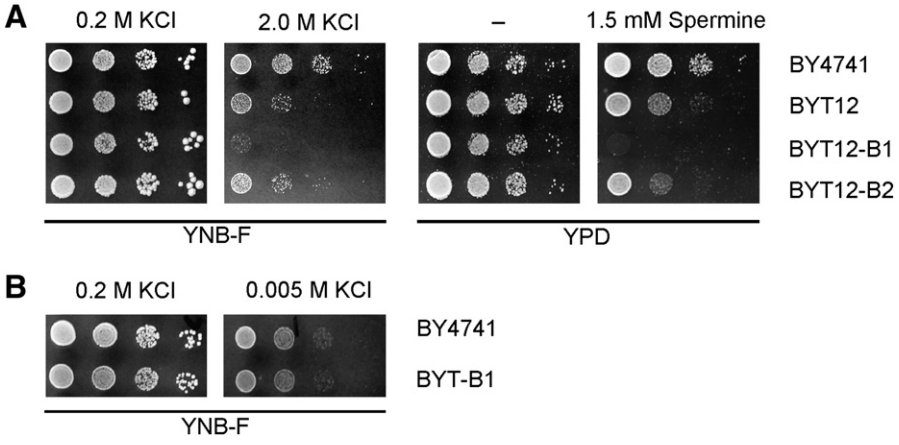


**Fig. 3.** Deletion of *BMH1* results in salt and drug sensitivity in the absence of Trk potassium-uptake systems. A. Growth of wild type (BY4741), BYT12 (*trk1 trk2*) and its derivatives lacking either *BMH1* (BYT12-B1) or *BMH2* (BYT12-B2) was compared on YNB-F media supplemented with 0.2 M or 2.0 M KCl or on YPD media supplemented with 1.5 mM Spermine. B. Growth of wild type (BY4741) and mutant strain lacking *BMH1* (BYT-B1) was compared on potassium free YNB-F media supplemented with 0.2 M or 0.005 M KCl.

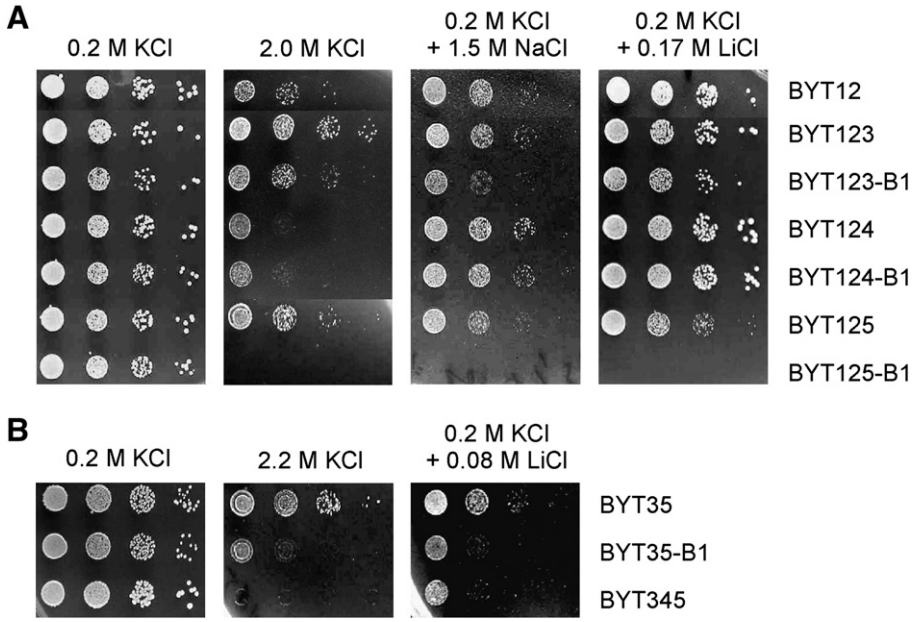


**Fig. 4.** Deletion of *BMH1* does not change the potassium tolerance of strains lacking Nha1 antiporter. A. Growth of pairs of strains lacking in addition to *TRK1* and *TRK2* (BYT12) one of the three genes encoding cation-efflux systems (*TOK1* in BYT123; *NHA1* in BYT124; *ENA1-5* in BYT125) and *BMH1* (-B1 strains) was compared on YNB-F media supplemented with salts. B. Growth of pairs of strains lacking either two (*TOK1* and *ENA1-5* in BYT35) or three (*TOK1, NHA1* and *ENA1-5* in BYT345) genes encoding cation-efflux systems and *BMH1* (-B1 strain) was compared on YNB-F media supplemented with salts.

the *BMH2* gene (Fig. 5). These results indicate that the difference in phenotype between *bmh1* and *bmh2* deletion mutants can be explained by the lower expression level of *BMH2* rather than by a difference in the interaction partners of the two 14-3-3 isoforms.

### 3.7. The Bmh1–Nha1 interaction is important for cell survival upon salt stress

To further study the interaction between *BMH1* and *NHA1*, we measured the efflux of sodium from the BYT4741 wild type, BYT345 lacking all cation exporters (*tok1*Δ *nha1*Δ *ena1-5*Δ), and from the BYT35 (*tok1*Δ *ena1-5*Δ) and BYT35-B1 (*tok1*Δ *ena1-5*Δ *bmh1*Δ) strains, i.e. cells having only the Nha1 cation-efflux system at their disposal and differing in the presence or absence of *BMH1*. Cells were either preloaded with 0.1 or 1.0 M NaCl. These two concentrations were chosen to be a low concentration where the deletion of *BMH1* has no effect on BYT35 cells, and a high salt concentration at which the growth of BYT35-B1 strain is significantly inhibited (Fig. 6A). The measurements of sodium efflux from cells preloaded with 0.1 M NaCl confirmed a high efflux capacity of wild-type cells and no measurable efflux from cells lacking all three exporters (Fig. 6B). As expected, preloading of wild-type cells with 0.1 M NaCl at pH 7.0 resulted in a much lower sodium content (approx. 90 nmol/mg dry wt) than in the three mutants (approx. 200 nmol/mg dry wt). This difference is due to the induction of Ena ATPase in the BY4741 strain during the preloading. Ena1 activity is responsible for the much faster efflux of $Na^+$ from the BY4741 cells than from the mutants lacking the *ENA* genes (almost all intracellular $Na^+$ left the cells in 40 min, cf. Fig. 6B). As expected, the export of sodium from BYT35 (*tok1*Δ *ena1-5*Δ) cells, having only Nha1 at their disposal, was lower than from the wild-type cells. Under these conditions, BYT35-B1 (*tok1*Δ *ena1-5*Δ *bmh1*Δ) exported sodium similarly to the BYT35 cells, confirming the observation from the drop tests that under mild sodium stress (0.1 M) the presence/absence of Bmh1 has no effect. On the other hand, our attempts to preload the BYT35-B1 cells with 1.0 M NaCl and subsequently measure the sodium efflux failed. With BYT35 cells, the intracellular concentration of sodium was approximately 260 nmol/mg dry wt after 60 min of preloading and the efflux was measurable (not shown). With BYT35-B1, the concentrations of sodium after the preloading varied significantly in independent experiments and the measurement of intracellular sodium content over 75 min did not show the typical efflux curve (not shown). When the salt-stress survival rate of both strains was estimated, it revealed that whereas more than 80% of BYT35 cells were able to form colonies after 60-min preloading with 1.0 M NaCl, the majority (more than 60%) of BYT35-B1 cells died. The high number of dead cells explains the low reproducibility in the NaCl loading experiments and the impossibility of following the sodium efflux from cells lacking Bmh1.

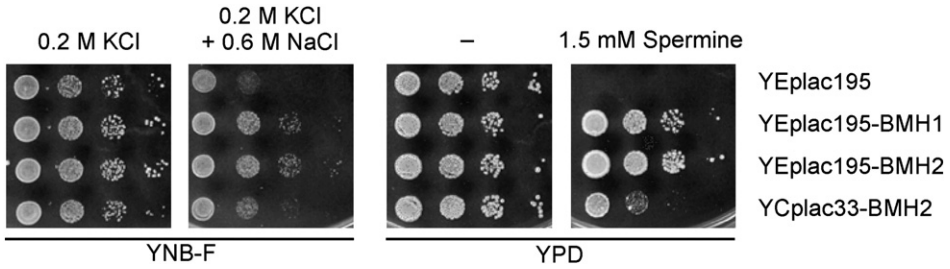


**Fig. 5.** Overexpression of *BMH2* complements *bmh1*Δ phenotypes. Growth of BYT12-B1 (*trk1*Δ *trk2*Δ *bmh1*Δ) cells expressing *BMH2* from centromeric (YCplac33-BMH2) or multicopy (YEplac195-BMH2) vectors was followed on YNB-F media supplemented with KCl and NaCl or on YPD media supplemented with 1.5 mM Spermine. Cells with the empty YEplac195 vector were used as negative, and cells expressing *BMH1* from YEplac195 as positive controls, respectively.

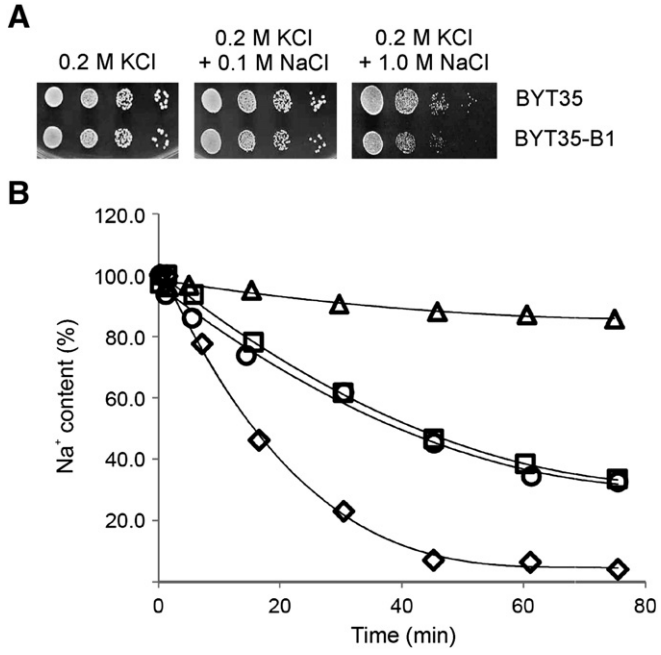


**Fig. 6.** Deletion of *BMH1* only influences the activity of Nha1 in the presence of high salt concentrations. A. Growth of BYT35 (*tok1Δ ena1-5Δ*) and BYT35-B1 (*tok1Δ ena1-5Δ bmh1Δ*) cells on YNB-F with 0.2 M KCl and 0.1 or 1.0 M NaCl. B. $Na^+$ efflux from BYT35 (squares) and BYT35-B1 (circles) cells preloaded with 0.1 M NaCl. Cells possessing (BYT4741; diamonds) or lacking (BYT345; triangles) all three cation exporters were used as positive and negative controls, respectively.

### 3.8. Bmh1 protein interacts with Nha1 antiporter

The Bimolecular fluorescence complementation (BiFC) method was used to analyze the interaction between 14-3-3 and Nha1 at the protein level. This approach allows visualization of protein–protein interactions in the living cell and is based on the association of fluorescent protein fragments attached to sequences of two putative interaction-partner proteins. The fluorescence signal only appears if the studied proteins physically interact and the two fragments of the fluorescent protein, the YFP variant Venus in our case, come into close proximity to each other [33,39]. From our previous work, we know that attaching GFP to the C-terminus of Nha1 has no effect on its activity [29], thus for the BiFC experiment, two plasmid constructs with *NHA1* extended at its 3′ with either the N-terminal (VN) or C-terminal (VC) sequence of YFP were prepared and the activity of the resulting chimeras tested. As shown in Supplementary Fig. 1, the expression of Nha1 tagged with VN was able to complement the sodium sensitivity of the BYT45 (*nha1Δ ena1-5Δ*) cells similarly to the expression of the non-tagged version. Then, various combinations of plasmids containing VC or VN fragments attached to Nha1 or Bmh2 (listed in Table 3) were co-expressed in the BY4741 and BYT45-B1 strains. Confocal microscopy of cells expressing Nha1-VN and VC-Bmh2 shows a clear fluorescent signal at the cell membrane in many cells, indicating an interaction between Bmh2 and Nha1 (Fig. 7A). This fluorescence was not observed in cells carrying the empty plasmids pUG34-VC and pUG35-VN, or in cells expressing VC-Bmh2 in combination with free VN. A very weak fluorescence at the cell membrane was observed in cells expressing Nha1-VN in combination with free VC when a high laser power was used during confocal microscopy. To quantify fluorescence, cells were also analyzed by flow cytometry (Fig. 7B). A substantial number of cells expressing Nha1-VN and VC-Bmh2 were highly fluorescent. This was not observed for control cells (Fig. 7B). Similar results were obtained in the BY4741 and BYT45B1 strains. It is worth noting that the co-expression of Nha1-VC and VN-Bmh2 did not result in any fluorescence signal, which suggests a specific and oriented binding between the two proteins. Overall, the results obtained showed a physical interaction between the Nha1 and Bmh2 proteins; in line with the observed genetic interaction (Figs. 4 and 6).

### 3.9. Hog1-mediated phosphorylation of Nha1 is not crucial for Bmh binding

Hog1 is the only protein kinase identified so far to phosphorylate and regulate the Nha1 antiporter [29,30]. Residues T765 and T876 of the Nha1 hydrophilic C-terminus were identified as NaCl-stress-dependent Hog1 kinase substrates by an *in vitro* phosphorylation assay [30]. As the phosphorylation/dephosphorylation status of the binding partner protein is crucial for its interaction with 14-3-3 proteins [1–4], we used the BiFC method to visualize the interaction between the Nha1 and Bmh2 proteins in a BY4741-derived mutant lacking *HOG1*. As shown in Fig. 8A, the fluorescence signal observed both under the microscope and in flow-cytometry analysis was at least similar to or even slightly higher than that observed in the wild-type cells. This result indicated that the phosphorylation of Nha1 by Hog1 kinase is not crucial for the recognition and binding of the Bmh proteins.

### 3.10. Both the N-terminal and C-terminal parts of Nha1 interact with Bmh2

The Nha1 protein (985 amino acids long) consists of a hydrophobic N-terminal part (approx. 430 amino acids, 12 transmembrane domains) and a long hydrophilic (555 amino acids) C-terminal part that is believed to regulate its transport activity. A truncated Nha1 antiporter (amino acid residues 1–472) is targeted to the plasma membrane and is partially functional whereas a protein consisting of the Nha1 C-terminal part (amino acid residues 467–985) is targeted to the cell nucleus [29]. We amplified DNA fragments corresponding to Nha1 amino acid residues 1–472 and to amino acid residues 467–985 (C-terminal hydrophilic part), fused them to VN and analyzed the interaction with VC-Bmh2. Microscopy and flow-cytometry analyses revealed that fluorescence was observed both in cells harboring the N- and C-terminal VN-tagged parts of Nha1 and VC-tagged Bmh2 (Fig. 8B and C). All control combinations resulted in a much lower fluorescence signal. The fluorescence corresponding to the interaction with Bmh2 was observed at the plasma membrane (and perinuclear ER) for the N-terminal part of Nha1 (amino acids 1–472) and in the nucleus for the Nha1 C-terminal protein corresponding to the localizations of the two parts of Nha1 observed previously [29]. These results suggested that Bmh2 recognizes and binds both to the hydrophobic “transporting” and to the hydrophilic “regulatory” parts of the Nha1 protein.

## 4. Discussion

Our results confirmed the previously observed role of 14-3-3 proteins in yeast tolerance to NaCl and cationic drugs [8,10], and moreover, revealed the importance of Bmh1 in cell tolerance to high salts in general (both toxic NaCl, LiCl, and non-toxic KCl). The deletion of *BMH1* has a stronger effect than that of *BMH2*. Nevertheless, the observed phenotypes of *bmh1Δ* are complemented by the overexpression of *BMH2* (Fig. 5). The results suggest that it is the difference in quantity of Bmh proteins in cells and not their specificity that is reflected in the salt- and drug-sensitivity phenotypes observed upon deletion of the two genes. The major role of Bmh1 in overall cell fitness was also observed when the growth of cell cultures in liquid media was examined. In all pairs of tested strains, *BMH1* deletion resulted in a very slight but reproducible increase in doubling time (usually 5–10%) in standard growth media without added salts or cationic drugs (not shown). This difference was not observable for *bmh2Δ* strains.

The increased sensitivity of cells to alkali metal cations and cationic drugs may be caused by hyperpolarization of the cell plasma membrane, as the inside-negative membrane potential is the driving force controlling the amounts of cations and drugs entering the cells. Our results showed that the deletion of *BMH1* does not change the relative membrane potential in any of the pairs of compared strains

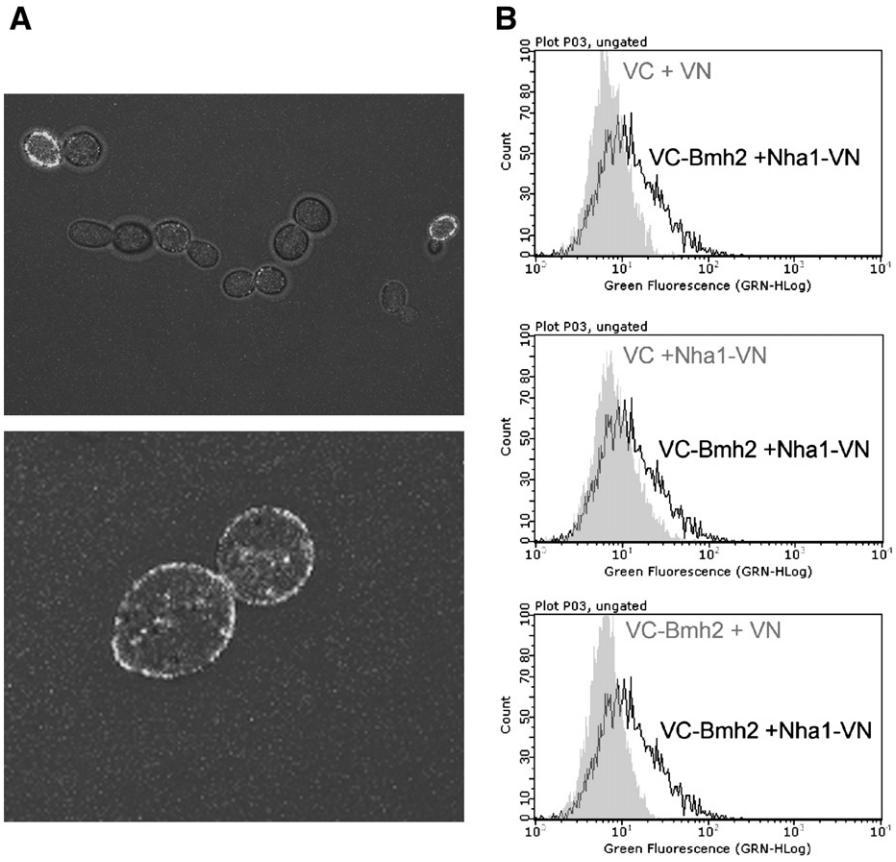


**Fig. 7.** Bimolecular fluorescence complementation analysis shows a physical interaction between Bmh2 and Nha1. A. Confocal microscopy of BY4741 cells co-transformed with pUG35-NHA1-VN and pUG34-VC-BMH2. B. Flow cytometry of BY4741 cells co-transformed with pUG35-NHA1-VN and pUG34-VC-BMH2 (black lines), of BY4741 cells co-transformed with pUG35-VN and pUG34-VC (upper panel, gray area), of BY4741 cells co-transformed with pUG35-NHA1-VN and pUG34-VC (middle panel, gray area) and of BY4741 cells co-transformed with pUG35-VN and pUG34-VC-BMH2 (lower panel, gray area). For each strain, 3000 cells were analyzed. The results of a typical experiment are shown.

(Fig. 2). The absence of the increased-salt-sensitivity phenotype of *bmh1*Δ in the strain lacking all five cation transporters (BYT12345; Fig. 1B) suggested that Bmh1 influences the activity of one or more cation transporters. On the other hand, the phenotype of increased drug sensitivity of BYT12345 upon *BMH1* deletion (Fig. 1B) and the absence of a *bmh1*Δ effect on the membrane potential suggest that the observed increased sensitivity to cationic drugs is not due to an enhanced influx of the drugs. It seems probable that Bmh1 and Bmh2 participate in cationic-drug detoxification (Fig. 5), and this role is independent of their function in the regulation of intracellular alkali-metal-cation homeostasis.

Using a series of mutants lacking various combinations of alkali-metal-cation uptake and efflux systems, we showed that 14-3-3 proteins are not involved in the regulation of potassium uptake via the Trk1 and Trk2 transporters (Fig. 3). When the genetic interaction between *BMH1* and genes encoding efflux systems was examined, it revealed the Nha1 $Na^+(K^+)/H^+$ antiporter as the interaction partner of 14-3-3 proteins (Fig. 4). Neither the potassium-specific channel Tok1 nor the Ena $Na^+(K^+)$-ATPases seemed to be affected by the *BMH1* deletion. Further analysis confirmed the interaction of Bmh1 and Nha1 under a salt stress (Fig. 6). The presence of both is crucial for cells lacking Ena ATPases to survive 1 M NaCl but is not important for growth under a mild (0.1 M NaCl) stress. In wild-type cells, the relatively small phenotype of *BMH1* deletion (compared to the phenotype of BYT35 or BYT125 cells) upon salt stress may be explained by the high activity of Ena ATPases in the export of surplus sodium or potassium (Figs. 1 and 4). The visualization of the interaction of 14-3-3 and Nha1 proteins using the BiFC technique (Fig. 7) unambiguously showed that Bmh proteins interact with Nha1, that this interaction is specific and it is only visible when tested using Nha1 tagged with the N-terminal half of YFP and Bmh2 tagged with the C-terminal YFP part. Attachment of the YFP C-terminal part to Nha1 and N-terminal part to Bmh2 did not result in any fluorescence signal under any of the tested conditions (not shown).

Phosphorylation of the binding partner proteins is crucial for the majority of interactions with 14-3-3 proteins [1–4]. It has been shown that the Nha1 protein is phosphorylated by Hog1 kinase upon salt stress [30] but this phosphorylation is not a prerequisite for the binding of 14-3-3 proteins, as we observed the same level of interaction in the wild type and in the *hog1Δ* mutant. Several other phosphorylated Ser/Thr residues were identified in the Nha1 transporter by Mass spectrometry in recent large-scale studies (Supplementary Table 1). All these sites are located in the hydrophilic C-terminus of Nha1. Most of these phosphorylations were observed in yeast cells grown under standard conditions (YNB or YPD media) [40,41], but several were identified in α-factor-arrested cells [42], or after treatment with methylmethanesulfonate [43,44]. The corresponding kinases and/or phosphatases have not yet been identified, and neither has the relevance of phosphorylation status for the Nha1 activity and functionality been characterized.

The N- and C-terminal Nha1 constructs used in this study (Fig. 8B and C) share only one amino acid residue that is phosphorylated in YNB-grown cells, i.e. Ser472 (Supplementary Table 1). It might be that phosphorylation of this seryl residue is crucial for the interaction of Nha1 with 14-3-3 proteins. Our future work will aim to verify this hypothesis and to identify the corresponding kinase and conditions for phosphorylation/dephosphorylation.

In summary, our results showed for the first time that the 14-3-3 proteins and a yeast alkali-metal-cation efflux system interact and that this interaction enhances the cell survival upon salt stress. The expression of *BMH1* is induced by the presence of salt [9]. On the other hand, the *NHA1* gene is expressed constitutively. Salt stress has no

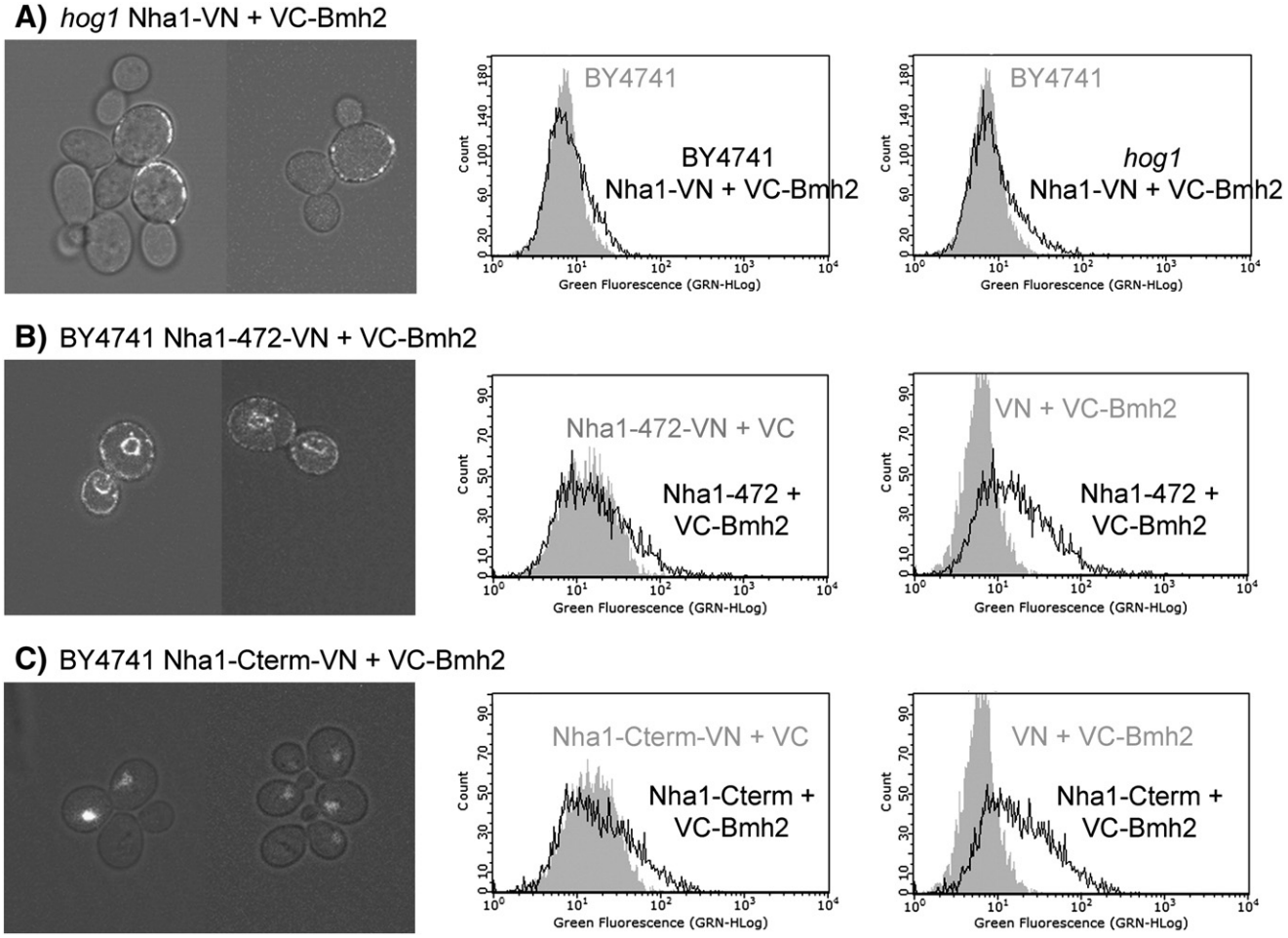


**Fig. 8.** Bmh2 interacts with Nha1 independent of *HOG1* and interacts with both the N- and C-terminal parts of Nha1. A. Confocal microscopy and flow cytometry of BY4741-derived *hog1Δ* cells co-transformed with pUG35-NHA1-VN and pUG34-VC-BMH2. Flow cytometry of transformed and untransformed BY4741 is shown as a control. B. Confocal microscopy and flow cytometry of BY4741cells co-transformed with pUG35-NHA1-472-VN and pUG34-VC-BMH2. Flow cytometry of BY4741 cells co-transformed with pUG35-NHA1-472-VN and pUG34-VC and BY4741 cells co-transformed with pUG35-VN and pUG34-VC-BMH2 is shown as a control. C. Confocal microscopy and flow cytometry of BY4741cells co-transformed with pUG35-NHA1-Cterm-VN and pUG34-VC-BMH2. Flow cytometry of BY4741 cells co-transformed with pUG35-NHA1-Cterm-VN and pUG34-VC and BY4741 cells co-transformed with pUG35-VN and pUG34-VC-BMH2 is shown as a control. For each strain, 3000 cells were analyzed. The results of a typical experiment are shown.

effect on its expression [24] but the C-terminus of the Nha1 protein is phosphorylated by Hog1 kinase at two sites upon NaCl stress [30]. These phosphorylation events are not crucial for the interaction between Bmh and Nha1, and thus a detailed characterization of the physical interaction together with identifying the kinase(s) and phosphatase(s) involved will be the focus of our next studies.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbagen.2012.03.013.

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**Supplementary Table 1.** Phosphorylated residues of the Nha1 antiporter

| P-site | Kinase | Proved by | Experimental conditions | Source |
|---|---|---|---|---|
| T447 | -[b] | Mass Spectrometry | YNB grown cells<br>MMS[a] treatment | [1-3] |
| T449 | - | Mass Spectrometry | YNB grown cells | [1] |
| S472 | - | Mass Spectrometry | YNB grown cells | [4] |
| S492 | - | Mass Spectrometry | MMS[a] treatment | [2] |
| S568 | - | Mass Spectrometry | YPD grown cells<br>Ŭfactor arrested cells | [5, 6] |
| S669 | - | Mass Spectrometry | YNB grown cells | [1] |
| S683 | - | Mass Spectrometry | YNB grown cells | [1] |
| S684 | - | Mass Spectrometry | YNB grown cells | [1] |
| S736 | - | Mass Spectrometry | YNB grown cells | [1] |
| T765 | Hog1 | Mass Spectrometry | Ŭfactor arrested cells<br>YNB grown cells<br>MMS[a] treatment | [1-3, 6, 7] |
| | | *In vitro* phosphorylation | NaCl stress (0.4 M) | |
| S768 | - | Mass Spectrometry | Ŭfactor arrested cells<br>YNB grown cells<br>MMS[a] treatment | [1, 3, 6] |
| S774 | - | Mass Spectrometry | Ŭfactor arrested cells | [6] |
| S918 | - | Mass Spectrometry | MMS[a] treatment | [3] |
| T876 | Hog1 | *In vitro* phosphorylation | NaCl stress (0.4 M) | [7] |

[a] MMS, methyl methanesulfonate, 0.05%
[b] -, not identified

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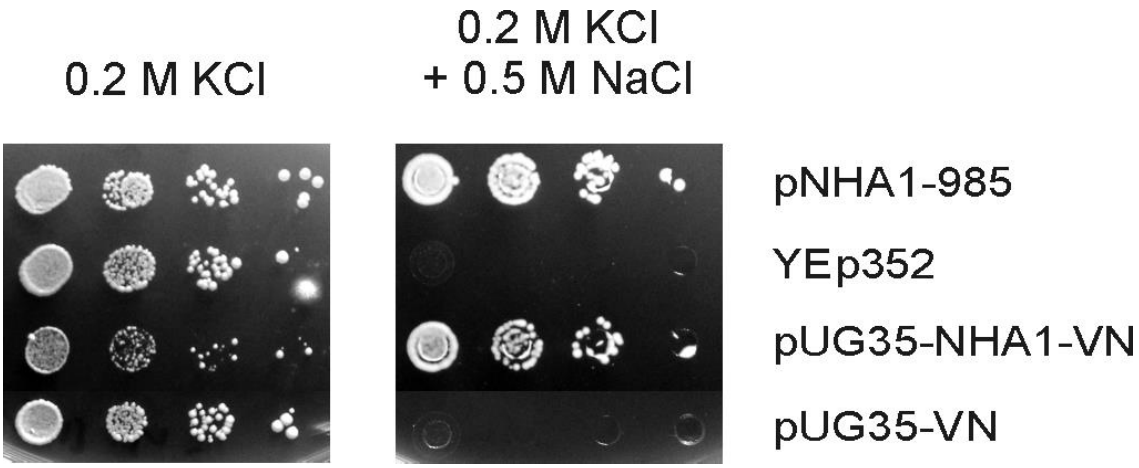


**Supplementary Figure 1.** Nha1p activity is not affected by VN-tagging.

Growth of BYT45 (*nha1*æ *ena1-5*æ) cells expressing *NHA1* (pNHA1-985) or *NHA1* tagged C-terminally with N-terminus of YFP Venus (pUG35-NHA1-VN) was compared on YNB-F medium supplemented with KCl and NaCl. Cells with an empty vector (YEp352) or with plasmid harboring only N-terminus of YFP Venus (pUG35-VN) were used as negative controls.

## 4.5 Rukopis č. 5 – Pleiotropic role of *CKA1* in salt tolerance and cation homeostasis

Stejně jako předchozí publikace se také tato zabývá identifikací nových interakčních partnerů a proteinů schopných regulovat transportery iontů alkalických kovů. Jak již bylo uvedeno v předchozí publikaci (kapitola 4.4, str. 41), ve specializovaných databázích bylo možné nalézt celkem čtrnáct míst fosforylace Nha1p, u kterých byla fosforylace prokázána metodou hmotnostní spektroskopie, ale pouze u dvou z nich byla prokázána fosforylace kinasou Hog1 [86]. U ostatních dvanácti AA zbytků byla fosforylace za určitých podmínek prokázána, ale odpovědná kinasa nebyla nalezena. V rámci této práce bylo provedeno hledání charakteristických sekvenčních motivů kvasinkových kinas pro identifikovaná místa fosforylace a po odborné konsultaci (Dr. O. Schmidt, Freiburg, Německo) byla jako odpovědná kinasa pro fosforylaci zbytků S669 a S683 identifikována kaseinkinasa 2 (CK2). Jelikož byla jedna z katalytických podjednotek CK2, Cka1p již dříve identifikována jako positivní regulátor exprese *ENA1* [153], bylo rozhodnuto se touto podjednotkou zabývat detailněji a zjistit, jestli je regulace exprese *ENA1* jedinou cestu, kterou Cka1p může do homeostase iontů alkalických kovů zasáhnout.

V první části práce byla připravena sada kmenů postrádajících jednotlivé geny, nebo různé kombinace genů *NHA1*, *ENA* a *CKA1*. Testy citlivosti k iontům alkalických kovů a měření rychlosti exportu $K^+$ a $Na^+$ odhalily, že Cka1p hraje důležitou roli v regulaci homeostase iontů alkalických kovů nejen prostřednictvím regulace *ENA1*, ale také regulací aktivity Nha1p a dále ještě alespoň jedním mechanismem nezávislým na exporterech Ena a Nha1, který je pravděpodobně spojen s regulací $\Delta\Psi$. Dále bylo prokázáno, že při konstitutivní expresi *ENA1* není regulována aktivita Ena1p pomocí Cka1p (pomocí Cka1p je tedy regulována výhradně exprese *ENA1*). Na druhou stranu, Nha1p je regulován i při uměle zvýšené stabilní expresi a zdá se, že regulace probíhá (na rozdíl od Ena1p) právě na úrovni regulace aktivity transporteru pomocí Cka1p.

V druhé části bylo hledáno místo interakce (pravděpodobně fosforylace některého AA zbytku Nha1p) mezi Nha1p a Cka1p. Na základě již zmíněné analysy dat z databází byla provedena cílené mutace v Nha1p tak, že zbytky S669 a S683 (a několik okolních S a T) byly nahrazeny alaninem. Po charakterisaci fenotypů bylo zřejmé, že žádný z testovaných AA zbytků nebyl odpovědným za regulaci Nha1p pomocí Cka1p. Vzhledem k velkému množství možností, které by bylo nutné otestovat v sekvenci Nha1p, byla testována nejdříve zkrácená verze Nha1-472p, které obsahovala pouze transmembránovou část Nha1p (prvních 472 AA; [82]) a téměř celý cytosolický C-konec chyběl. Překvapivě bylo zjištěno, že AA zbytek (nebo více zbytků) odpovědný za interakci Nha1p s Cka1p se pravděpodobně nachází v některé z intracelulárních smyček, nebo krátkých, cytosolicky

orientovaných koncích (N-konec a začátek C-konce) v rámci prvních 472 AA. Na základě dříve publikovaného modelu topologie Nha1p [82], dat dostupných ve specializovaných databázích [189-191] a *in silico* predikčních nástrojů [190] bylo identifikováno celkem 13 předpokládaných míst fosforylace Nha1p kinasou Cka1, které je nutno v budoucnu otestovat.

V rámci této práce byly identifikovány dosud neznámé cesty, kterými je podjednotka CK2, kinasa Cka1 schopna regulovat homeostasi iontů alkalických kovů. Kromě dříve popsané regulace exprese *ENA1* byla poprvé popsána schopnost Cka1p regulovat transportní aktivitu Nha1p (jako positivní regulátor). Poprvé byla popsána také schopnost Cka1p zapojit se do regulace homeostase iontů alkalických kovů bez přítomnosti exporterů Ena a Nha1, v tomto případě se pravděpodobně jedná o regulaci prostřednictvím změn v ΔΨ.

Tato publikace byla zařazena ve formě rukopisu, který je připravován k zaslání do časopisu Microbiology (SGM).

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# Pleiotropic role of *CKA1* in salt tolerance and cation homeostasis

The Contents Category:

Physiology and Biochemistry (*metabolic pathways and their regulation, bioenergetics and transport, synthesis of macromolecules, metabolomics*)

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Abbreviations:

CK2 , casein kinase II

TMA, tetramethylammonium

SUMMARY

Casein kinase (CK2) is constitutively active and highly conserved kinase in eukaryotes with pleiotropic roles in many different cellular processes. Tetrameric structure of CK2 is composed of two catalytic and two regulatory subunits; catalytic subunits isoforms are encoded by *CKA1* and *CKA2*, whereas regulatory subunits isoforms are encoded by *CKB1* and *CKB2* in *Saccharomyces cerevisiae*. Strong, but not complete function overlap of catalytic subunits was observed and presence of at least one catalytic subunit isoform (Cka1, or Cka2) is essential for cells survival. Recently, Cka1p was found to be able to influence alkali-metal-cation homeostasis, because Cka1p was identified as a positive regulator of $Na^+$-ATPase Ena1 expression. Ena1p determines $Na^+$ and $Li^+$ tolerance of cells and its expression is regulated via Nrg1 transcription factor, a downstream target of Cka1p. Alkali-metal-cations homeostasis is maintained using several transporters in *S. cerevisiae.* Uptake of required potassium is mediated by high-affinity active transporters Trk1 and Trk2; the efflux of surplus potassium or toxic sodium is ensured by Ena ATPases, Nha1 $Na^+/H^+$-antiporter and Tok1 potassium channel. In this work, we verified, that Ena1p is regulated by Cka1p only on the level of expression. We showed for the first time, that presence of Cka1p is necessary for proper transport activity of Nha1 antiporter; Cka1p was identified as positive regulator of Nha1p. Besides that, Cka1p participates in alkali-metal-cation homeostasis regulation also by some third mechanism, most probably by regulation of plasma membrane potential. In total, a pleiotropic role of Cka1p was uncovered in regulation of salt tolerance and cation homeostasis.

INTRODUCTION

The casein kinase II (CK2) is an essential serine/threonine kinase that is highly conserved and constitutively active in eukaryotic organisms (Berkey & Carlson, 2006; Hermosilla *et al.*, 2005). Enzymatic activity of CK2 is important for regulation of proliferation, flocculation, DNA damage response, cell polarity, or mitochondrial protein import (Berkey & Carlson, 2006; Litchfield, 2003; Olsten & Litchfield, 2004; Rethinaswamy *et al.*, 1998; Sajnaga *et al.*, 2008; Schmidt *et al.*, 2011). A pleiotropic effect of CK2 is represented in phosphorylation of more than 300 proteins which was observed *in vitro* and for many targets validated also *in vivo* (Meggio & Pinna, 2003).

Tetrameric structure of CK2 in *S. cerevisiae* is composed of two regulatory subunit isoforms, encoded by *CKB1* and *CKB2*, and two catalytic subunit isoforms, *CKA1* and *CKA2* (Glover, 1998). Though mutants with single deletion of any subunit or deletion of both regulatory subunits (*ckb1Δ ckb2Δ*) are viable; deletion of both catalytic subunits (*cka1Δ cka2Δ*) is lethal (Padmanabha *et al.*, 1990). Strong, but not complete function overlap of catalytic subunits was observed using temperature sensitive *cka1Δ* or *cka2Δ* mutants and testing various regulatory functions in cells, where either Cka1p or Cka2p was identified as the major subunit (Glover, 1998; Rethinaswamy *et al.*, 1998).

Alkali-metal-cation homeostasis has been studied in *S. cerevisiae* cells for decades (Arino *et al.*, 2010; Gaber *et al.*, 1988). Potassium is the major intracellular cation, which has important roles in cell physiology such as maintenance of suitable conditions for protein synthesis and enzymatic reactions, maintenance of membrane potential, regulation of cell volume, osmotic pressure and internal pH (Rodriguez-Navarro, 2000). Since limited concentration of $K^+$ is usually present in natural environment, $K^+$ needs to be accumulated via high-affinity transporters Trk1 and Trk2 (Gaber *et al.*, 1988; Ko & Gaber, 1991). The active import of $K^+$ via Trk1p and Trk2p is driven by the electrochemical $H^+$ gradient generated across the plasma membrane by the essential $H^+$-ATPase Pma1 (Serrano *et al.*, 1986). Cells lacking *TRK1* are hyperpolarized and highly sensitive to limited $K^+$ (Madrid *et al.*, 1998; Navarrete *et al.*, 2010). Subsequent deletion of *TRK2* (*trk1Δ trk2Δ* strain) enhances both described phenotypes of *trk1Δ* mutant (sensitivity to low $K^+$ and hyperpolarization), while single deletion (*trk2Δ*) is without significant effect (Petrezselyova *et al.*, 2011).

Fine tuning of plasma membrane potential is thought to be important role of Tok1, only known $K^+$ specific outward-rectifying channel in *S. cerevisiae* (Arino *et al.*, 2010; Ketchum *et al.*, 1995). Plasma-membrane depolarization is the opening signal for Tok1p and upon *TOK1* deletion, strong depolarization occurs (Bertl *et al.*, 2003; Maresova *et al.*, 2006). Alkali metal cations are actively exported from cells via two different systems, Nha1 antiporter (Banuelos *et al.*, 1998; Kinclova *et al.*, 2001; Prior *et al.*, 1996) and Ena ATPase (Haro *et al.*, 1991; Ruiz & Arino, 2007). Both transport

systems have specificity not only for $K^+$, but also for toxic alkali metal cations, $Na^+$, $Li^+$ and $Rb^+$ (Kinclova *et al.*, 2001; Ruiz & Arino, 2007). The Ena1 ATPase mediating export of cations is encoded by the *ENA1* gene, the firs gene in tandem repetition which in various *S. cerevisiae* strains consists of 2 to 4 genes (*ENA2* to *ENA5*; Arino *et al.*, 2010; Wieland *et al.*, 1995). Export of alkali metal cations via Ena1p is energized by ATP hydrolysis (Haro *et al.*, 1991) and it is considered to determine $Na^+$ and $Li^+$ tolerance (Arino *et al.*, 2010). *ENA1* expression is strongly induced by increased $K^+$, $Na^+$ and $Li^+$ concentration, or in alkaline pH, and thus the sensitivity to $K^+$, $Na^+$ and $Li^+$ and survival of cells in alkaline pH is dramatically increased in absence of *ENA1* (Haro *et al.*, 1991; Platara *et al.*, 2006). Recently, an important regulative effect of Ena1p on plasma membrane potential was predicted (Ke *et al.*, 2013), *ENA1* upregulation was suggested to be important for re-estabilishing plasma membrane potential during KCl and alkaline pH stresses.

Secondary active exporter, $Na^+(K^+)/H^+$-antiporter Nha1 uses Pma1p-generated inward $H^+$ gradient as a source of energy for cation efflux (Banuelos *et al.*, 1998). *NHA1* is constitutively expressed house-keeping gene which is important for immediate osmotic stress response and survival of cells in increased concentration of $K^+$ and $Na^+$ at acidic external pH (Arino *et al.*, 2010; Banuelos *et al.*, 1998; Kinclova *et al.*, 2001). Beside cation homeostasis, Nha1p is responsible for several physiological functions such as regulation of internal pH, cell volume, cell cycle, or membrane potential (Arino *et al.*, 2010; Kinclova-Zimmermannova *et al.*, 2006; Kinclova-Zimmermannova & Sychrova, 2006; Sychrova, 2004).

Though important plasma-membrane alkali-metal-cation transporters have been characterized, the network of alkali-metal-cation homeostasis regulators has not been fully elucidated yet. Among proteins which have been identified as Trk1p-mediated $K^+$ uptake regulators belong kinases Snf1, Hal4 and Hal5, phosphatases Ppz1 and Calcineurin, and protein Hal3 (Ferrando *et al.*, 1995; Portillo *et al.*, 2005). Uptake of $K^+$ is regulated also by proteins responsible for proper vesicular transport and inositol kinases encoded by *KCS1*, *ARG82* and *FAB1* (Fell *et al.*, 2011).

Recently, an inhibition of efflux activity of all alkali-metal-cation exporters (Nha1p, Ena1p and Tok1p) by membrane potential changes after *TRK1* and *TRK2* deletion was observed (Zahradka & Sychrova, 2012) which uncovered complexity of alkali-metal-cation homeostasis regulation. Besides that, *ENA1* expression is regulated by complex network of enzymes and factors, in detail, kinases Snf1 and Hog1, phosphatases Calcineurin and Ppz1, or transcription factors Rim101, Hal3, Nrg1 and Nrg2 (for review, see Arino *et al.*, 2010). Catalytic subunit of CK2, Cka1 was recently identified as an activator (derepressor) of *ENA1* expression via phosphorylation of transcription factor Nrg1 (*ENA1* repressor; Berkey & Carlson, 2006; Vyas *et al.*, 2005). Increase in $Na^+$ sensitivity after *CKA1* deletion was fully

complemented by subsequent deletion of *NRG1*. Unlike Cka1p, the second CK2 catalytic subunit, Cka2p was not able to phosphorylate Nrg1p (Berkey & Carlson, 2006).

Regulation of Nha1p and Tok1p activity is poorly understood and beside Hog1 kinase, just a little is known about Nha1p and Tok1p regulators. Hog1p is responsible for phosphorylation of both, Tok1p and Nha1p (residues T765 and T876) in presence of $Na^+$ resulting in Nha1p deactivation (Proft & Struhl, 2004). Decreased $K^+$ efflux via Nha1p inhibits uptake of $Na^+$ which resulting in increased $Na^+$ tolerance (Kinclova-Zimmermannova & Sychrova, 2006). Deactivation of Nha1p and Tok1p by Hog1p phosphorylation was also predicted by recently published integrative model of alkali-metal-cation homeostasis regulation in yeast (Ke *et al.*, 2013).

Nha1p consists of 985 residues, but only about first 440 form (including very short N-terminus) the transmembrane part responsible for transport itself (Kinclova *et al.*, 2001) and about 540 residues from hydrophilic C-terminus which is considered to have a regulatory role (Kinclova-Zimmermannova & Sychrova, 2006). Similarly as in mammalian cells (Sweeney *et al.*, 1995), phosphorylation of Nha1p seems to be preferred way of regulation. Beside the two Hog1p targets, other 12 phosphorylated residues were identified using mass spectroscopy in various conditions, but responsible kinases for phosphorylation of these 12 residues has not been identified yet (for summary, see Zahradka *et al.*, 2012 and Tab. 2). In our recent study, 14-3-3 proteins were identified as regulators of Nha1p (Zahradka *et al.*, 2012). 14-3-3 proteins are able to bind multiple targets, but usually only after a target phosphorylation, which is usually needed e.g. for stabilization of phosphorylation.

In this work, the role of *CKA1* in alkali-metal-cation homeostasis was studied. Previously observed regulation of *ENA1* expression by Cka1p was confirmed, moreover, regulation of Nha1p activity by Cka1p has been shown for the first time. Finally, other, yet-unidentified role of *CKA1* in alkali-metal-cation homeostasis and membrane potential maintenance was uncovered.

## MATERIALS AND METHODS

### Strains and media

*S. cerevisiae* strains derived from BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (EUROSCARF) were used in this study. Mutant strains lacking Nha1 antiporter BYT4 (*nha1Δ*), *ENA* genes BYT5 (ena*1-5Δ*), or both systems BYT45 (*nha1Δ ena1-5Δ*) were described previously (Navarrete *et al.*, 2010; Petrezselyova *et al.*, 2010; Zahradka & Sychrova, 2012) and using this parental strains, appropriate

*cka1Δ* strains were prepared, in detail: BY-C1 (*cka1Δ*), BYT4-C1 (*nha1Δ cka1Δ*), BYT5-C1 (*ena1-5Δ cka1Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*). Novel strains were constructed by homologous recombination using the Cre-loxP system (Guldener *et al.*, 1996). Integration cassettes for *CKA1* deletion were prepared by PCR using oligonucleotides Cka1-kanMX-F and Cka1-kanMX-R; proper integration of the cassette was tested by PCR using diagnostic oligonucleotides Cka1-p1F, Cka1-inF, Cka1-inR and Cka1-t1R (oligonucleotides are listed in Tab. 1).

Yeast cells were cultivated in complete YPD (1% yeast extract, 2% peptone, 2% glucose), or minimal YNB (0.17% YNB w/o amino acids, 0.5% ammonium sulphate, 2% glucose) media at 30 °C. YNB media were supplemented either with Brand Supplement Mix (BSM) or BSM w/o uracil (ForMedium$^{TM}$ UK). Solid media were supplemented with 2 % agar.

**Plasmids**

Plasmids used in this study were derived from YEp352 (Hill *et al.*, 1986). pNHA1 plasmid contains *NHA1* behind its own promoter (Kinclova *et al.*, 2001) and pENA1 was constructed by the exchange of the *NHA1* ORF by the *ENA1* coding sequence in pNHA1 by P. Herynková using oligonucleotides YEpN-ScENA1-F and YEpN-ScENA1-R. Proper incorporation *ENA1* into the plasmid was tested using Ena1-1R and pNHA1-1 oligonucleotides (listed in Tab. 1). To identify Cka1p target residues, point mutations were introduced in *NHA1* sequence in pNHA1 using PCR based QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) and oligonucleotides listed in Tab. 1 resulting in plasmids pNHA1-S669A, pNHA1-T663A-S665A-S669A, pNHA1-S683A-S684A and pNHA1-S669A-S683A-S684A. Finally, plasmid bearing truncated *NHA1*, pNHA1-472 was used in this study (Kinclova *et al.*, 2001).

**Growth tests**

Tolerance of cells to salts was tested using drop test on solid media as described by (Krauke & Sychrova, 2010). The growth of mutant strains was tested in YNB in presence of KCl (1.0 – 2.2 M), NaCl (0.1 – 1.7 M), or LiCl (0.02 – 0.2 M). Growth of cells was recorded for 5 days, representative results of at least three independent experiments after 3 days of growth (if not stated differently) are shown.

**Measurement of cation efflux and accumulation**

Atomic absorption spectroscopy was used to estimate intracellular concentration of $K^+$, $Na^+$ and $Li^+$ as described previously (Krauke & Sychrova, 2010; Zahradka *et al.*, 2012) with slight modifications described below. Cells were grown in YNB media to $OD_{600} \approx 0.5$ for all experiments, every washing step consist of harvesting, washing and resuspending and all samples were extracted and processed

according to (Krauke & Sychrova, 2010). Averages of at least three independent experiments consist of three paralels for each point (nine values for each point) were counted and plotted including standard deviation.

To follow changes in $K^+$ content, cells were washed (t = 0 min) and incubated in the $K^+$- free incubation buffer (10 mM Tris, 0.1 mM $MgCl_2$, 2% glucose, 10 mM RbCl, pH adjusted to 4.4 with citric acid and then brought up to 4.5 with $Ca(OH)_2$). Samples were collected in three parallels at t = 0 and 60 min.

To estimate changes in $Na^+$ content, cells were washed and incubated in YNB + 0.5 M NaCl, pH 7.0 (adjusted with $NH_4OH$) for 60 min ($Na^+$ preloading). Then, cells were washed (t = 0 min) and incubated in the $Na^+$-free incubation buffer (10 mM Tris, 0.1 mM $MgCl_2$, 2% glucose, 10 mM KCl, pH adjusted to 4.4 with citric acid and then brought up to 4.5 with $Ca(OH)_2$). Three parallels of samples were collected at t = 0 and 45 min.

To estimate $Li^+$ accumulation, cells were washed (t = 0 min) and incubated in YNB + 0.1 M LiCl, pH 7.0 (adjusted with $NH_4OH$). Three parallels of samples were collected at t = 0 and 30 min, cells were washed, resuspended to the $Li^+$-free incubation buffer (10 mM Tris, 0.1 mM $MgCl_2$, 2% glucose, 10 mM RbCl, pH adjusted to 4.4 with citric acid and then brought up to 4.5 with $Ca(OH)_2$) and immediately collected.

**Fluorescence measurement of relative membrane potential**

Relative membrane potential was estimated by fluorescence assay based on the redistribution of the fluorescence probe diS-$C_3$(3) (3,3'-dipropylthiacarbocyanine iodide) in cells as described earlier (Maresova *et al.*, 2006) with following modifications. Cells were grown in YNB media to $OD_{600} \approx 0.5$, harvested, washed twice with distilled water and resuspended in 10 mM MES buffer (pH 6.0 adjusted by triethanolamine) to final $OD_{600} = 0.08$. The probe was added to a final concentration of 0.02 µM. Fluorescence emission spectra of the cell suspension were measured every 2 – 4 min on ISS PC1 spectrofluorimeter. Samples were kept at room temperature and occasionally gently stirred. Excitation wavelength was 531 nm, emission range 560 – 590 nm and duration of one spectral scan 20 s. Scattered light was eliminated by an emission filter with a cutoff wavelength at 540 nm. The fluorescence emission maximum ($\lambda_{max}$) was followed for 60 min. For each sample, the signal reached equilibrium (stabilized signal) circa after 30 min and average $\lambda_{max}$ between 45 and 60 min was plotted. Averages of at least three independent experiments were show using standard deviation for error bars.

RESULTS

**_CKA1_ influences alkali-metal-cation homeostasis via regulation of Ena1p and Nha1p**

In this study, novel regulators of Nha1p were searched. Using *in silico* prediction tools (Gnad *et al.*, 2007), two putative CK2 phosphorylation targets, Ser669 and Ser683 were recognized (CK2 sequence motif S/T-X-X-E) in Nha1p C-terminal part among known phosphorylation sites (see Tab. 2). It implicated a hypothesis that Cka1p could influence alkali-metal-cation homeostasis not only via regulation of *ENA1* expression, but may be also via other interactions.

First, previously published interaction (Berkey & Carlson, 2006) of *ENA1* and *CKA1* was studied. To confirm observed interaction of *ENA1* and *CKA1* (Berkey & Carlson, 2006), single mutant *cka1Δ* (BY-C1), mutant lacking Nha1 antiporter simultaneously with *CKA1* (BYT4-C1; *nha1Δ cka1Δ*) and their parental strains, BY4741 (WT) and BYT4 (*nha1Δ*) were used. Growth of cells in presence of various concentrations of alkali-metal-cation was tested (Fig. 1a). Cells lacking *CKA1* (BY-C1 and BYT4-C1) were more sensitive to NaCl and LiCl than their parental strains. These observation was in accordance with previously published results (Berkey & Carlson, 2006) describing inhibition of *ENA1* expression in absence of *CKA1*. Slightly lower tolerance to KCl after *CKA1* deletion was observed in this work for the first time, but only in BYT4-C1 strain, in absence of *NHA1*, which could indicate that in case of $K^+$ (but not $Na^+$ and $Li^+$) Nha1p present in cells was sufficient to compensate inhibition of *ENA1* expression in absence of *CKA1*, in BY4741 strain. To observe changes in transporters' activity, cation efflux was measured. Efflux of $K^+$ (Fig. 1c) and $Na^+$ (not shown) was not affected by *CKA1* deletion neither in BY4741, nor BYT4 strain. These results showed that absence of *CKA1* did not affect affinity to cations or transport activity of Ena1p, regulation of *ENA1* expression by Cka1p is most probably the only interaction between ATPase Ena1 and Cka1p.

To determine whether *CKA1* regulates alkali-metal-cation homeostasis via *ENA1*-independent way, *CKA1* was deleted in strains lacking *ENA* genes (BYT5-C1; *ena1-5Δ cka1Δ*), or lacking both $Na^+$ ($K^+$) active export systems, *NHA1* and *ENA* genes (BYT45-C1; *nha1Δ ena1-5Δ cka1Δ*). Growth of cells in presence of salts was estimated and compared with appropriate parental strains BYT5 (*ena1-5Δ*), or BYT45 (*nha1Δ ena1-5Δ*) respectively. For both parental strains, deletion of *CKA1* brought about increased sensitivity to $Li^+$ and $Na^+$ (Fig. 1b). Sensitivity to $K^+$ was slightly higher in case of BYT45-C1 compared to BYT45, but growth of BYT5-C1 and BYT5 was similar on media high $K^+$ concentration (Fig. 1b). Significantly increased sensitivity to $Na^+$ and $Li^+$ in case of BYT5 (absence of *ENA* genes) due to *CKA1* deletion indicated, that the activity of the only present active transporter, Nha1p, could be regulated by Cka1. Furthermore, other *NHA1*- and *ENA1*-independent way, how salt tolerance was

influenced by *CKA1*, was identified using BYT45 strain and its increased sensitivity to cations after *CKA1* deletion.

For better understanding of *ENA1*-independant *CKA1* deletion phenotypes, we measured efflux of $K^+$ and $Na^+$ using BYT5-C1, BY45-C1 and appropriate parental strains. Due to *CKA1* deletion, efflux of $K^+$ (Fig. 1c) was significantly lower in BYT5-C1 strain (*ena1-5Δ cka1Δ*; 66 % of initial $[K^+]_{in}$) in comparison to BYT5 (*ena1-5Δ*; 58 % of initial $[K^+]_{in}$ ) showing reduced Nha1p activity. Similarly to $K^+$, a decrease in $Na^+$ efflux capacity after *CKA1* deletion was observed comparing BYT5 and BYT5-C1 strains (13 % difference; not shown). Efflux of $K^+$ (Fig. 1c) and $Na^+$ (not shown) was not affected by *CKA1* deletion in all other tested strains (BY4741, BYT4, BYT45). Thus, regulation of Nha1p transport activity by *CKA1* was observed only in absence of *ENA1*.

**Cka1p regulates overexpressed Nha1p, but not Ena1p**

BYT45 (*nha1Δ ena1-5Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*) strains overexpressing *NHA1*, or *ENA1* from a multicopy plasmid (pNHA1, or pENA1) were used to study post-translational regulation of Nha1p or Ena1p by Cka1p. Comparing BYT45 and BYT45-C1 background, $Na^+$ tolerance was affected only in case of overexpressed *NHA1*, but not *ENA1* (Fig. 2a). Furthermore, decrease in $Na^+$ efflux activity was observed comparing BYT45 (34 % of initial $[Na^+]_{in}$ after 45 min in $Na^+$-free buffer), and BYT45-C1 (47 % of initial $[Na^+]_{in}$) cells overexpressing *NHA1* (Fig. 2b). Analogically, $K^+$ efflux via Nha1p in the same cells was significantly reduced comparing BYT45 and BYT45-C1 background (20 % reduction; not shown). On the other hand, in case of overexpressed *ENA1*, no significant difference in BYT45 or BYT45-C1 cells was observed neither by $Na^+$ efflux assay (Fig 2b), nor by $K^+$ efflux assay (not shown). Results obtained using plasmids showed that activity of Nha1p was regulated on post-translational level by Cka1p, but activity of Ena1p was not.

**Deletion of *CKA1* influences relative membrane potential and $Li^+$ accumulation**

Surprisingly, *CKA1* deletion affected $Na^+$, $Li^+$ and $K^+$ tolerance even after deletion of active alkali-metal-cation exporters Ena and Nha1, in parental strain BYT45 (Fig. 1b), where no changes in active ion export were expected. Changes in membrane potential were suggested as possible explanation of observed salt sensitivity phenotype brought about by *CKA1* deletion in BYT45 strain, because increased membrane potential should increase unspecific accumulation of cations resulting in increased salt sensitivity. Relative membrane potential was estimated in whole set of mutants using fluorescence probe (Fig. 3a). Except BY4741 and BY-C1, the membrane potential was changed due to *CKA1* deletion in all parental strains. While BYT4 (*nha1Δ*) and BYT45 (*nha1Δ ena1-5Δ*) were in comparison to BY4741 depolarized and *CKA1* deletion in these strains (BYT4-C1 and BYT45-C1)

resulted in repolarization to BY4741 level, membrane potential of BYT5 (*ena1-5Δ*) was very similar to BY4741, but subsequent deletion of *CKA1* (BYT5-C1) resulted in depolarization. In presence of at least one active export system (strains BYT4 and BYT5), changes in membrane potential due to *CKA1* deletion were probably not crucial for alkali-metal-cations sensitivity, but relatively increased membrane potential after *CKA1* deletion in BYT45 (BYT45-C1 strain) lacking *NHA1* and *ENA* genes was very likely the reason of increased salt sensitivity (Fig. 1b) due to increased nonspecific uptake of cations. Interestingly, depolarization of BYT5-C1 (*ena1-5Δcka1Δ*) in comparison with BYT5 (*ena1-5Δ*) probably reduced nonspecific uptake of cations, but increased salt sensitivity of BYT5-C1 strain (Fig. 1b) was observed because of significant Nha1p transport activity inhibition.

Since increased $Li^+$ accumulation could be a hallmark of increased membrane potential (Arino *et al.*, 2010), the accumulation assay was used to validate relative membrane potential measurements. Incubation of BYT5, BYT5-C1, BYT45 and BYT45-C1 cells in media with LiCl (100 mM) increased $[Li^+]_{in}$ significantly (circa 80 - 100 nmol $Li^+$ per mg of dry weight), but only limited concentration of $Li^+$ was accumulated into cells expressing *ENA1*, BY4741 and BYT4 (circa 30 nmol $Li^+$ per dry weight; Fig. 3b), may be due to effective $Li^+$ efflux by Ena exporters. Furthermore, no significant differences after *CKA1* deletion were observed in BY4741 and BYT4 (*nha1Δ*). Corresponding to relative membrane potential assay results, absence of *CKA1* in BYT5 (*ena1-5Δ*) resulted in lower accumulation of $Li^+$, while $[Li^+]_{in}$ in BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*) was after incubation with LiCl significantly higher than in BYT45 (*nha1Δ ena1-5Δ*). Changes in $Li^+$ accumulation observed after *CKA1* deletion in strains lacking *ENA* genes confirmed results of membrane potential measurement (Fig. 3a).

**Residues S669, S683 and their neighbors in Nha1p are not involved in Nha1p regulation via Cka1**

Phosphorylation of Nha1p by Cka1p is, to our knowledge, the most probable way of Nha1p activity regulation by Cka1p. Predicted Cka1p perspective sites were searched among previously identified Nha1p phosphorylation sites found by mass spectrometry (see Tab. 2; Gnad *et al.*, 2009; Zahradka *et al.*, 2012). Residues Ser669 and Ser683 were identified as putative targets of Cka1 kinase, because CK2 sequence motif S/T-X-X-E was found for both residues and, simultaneously with residues in close proximity (Thr663, Ser665 and Ser684), site-directed mutagenesis approach was used to verify phosphorylation by Cka1p. A set of pNHA1 plasmids with one or more point mutations changing specific Ser(or Thr) to Ala were prepared and expressed in BYT45 (*nha1Δ ena1-5Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*). Four plasmids pNHA1-S669A, pNHA1-T663A-S665A-S669A, pNHA1-S683A-S684A and pNHA1-S669A-S683A-S684A were used to follow changes in Nha1p activity due to selected mutations. Surprisingly, no changes in tolerance to NaCl (Fig. 4), nor KCl (not shown) were brought about by any prepared mutation, or combination of mutations of Nha1p expressed in BYT45

and BYT45-C1 strains. Results uncovered that that predicted Cka1p targets in Nha1p sequence, Ser669 and Ser683, and residues in close proximity, Thr663, Ser 665 and Ser684, were not important for observed regulation of Nha1p activity by Cka1p.

Since most probable Cka1p targets in Nha1p sequence were disproved, broader *in silico* analysis of Nha1p phosphorylation sites was performed. Beside two previously confirmed targets of Hog1 kinase (Thr765 and Thr876; see Tab. 2a and Proft & Struhl, 2004) and 12 phosphorylated sites with unknown kinase (including Ser669, Ser683 and Ser684 which were tested in this study; see Tab. 2b and Gnad *et al.*, 2009; Zahradka *et al.*, 2012), 48 unique sites were predicted using Phosida database prediction tool (Gnad *et al.*, 2007) in cytosolic C-terminal part of Nha1p and other 6 sites were predicted in internal loops of transmembrane part (loops were selected according to Nha1p topology model published previously Kinclova *et al.*, 2001). Since more than 50 residues in Nha1p were selected for additional testing, a reduction of number of putative Cka1p phosphorylation was desirable, thus, previously prepared plasmid (Kinclova *et al.*, 2001) bearing truncated version of Nha1p, pNHA1-472, was expressed in BYT45 and BYT45-C1 and tested. Surprisingly, decrease in salt tolerance was brought about by *CKA1* deletion also in strains overexpressing only first 472 residues of Nha1p (pNHA1-472). BYT45 cells bearing pNHA1-472 were more tolerant to $Na^+$, $Li^+$ (Fig. 5) and $K^+$ (not shown) than BYT45-C1 cells with the same plasmid. pNHA1-472 consist of Nha1p transmembrane part with circa first 440 residues and other circa 30 residues of hydrophilic C-terminus which forms C1 conserved region (Mitsui *et al.*, 2004) important for Nha1p dimerization and passage through the secretory pathway. In total, ten putative phosphorylation sites (Ser68, Ser70, Ser134, Ser291, Ser358, Ser388, Thr456, Thr457, Ser464, Ser465) were predicted in pNHA1-472 sequence (Tab. 2c). Additionally, three of previously identified phosphorylation sites belong to pNHA1-472 sequence as well (Thr447, Thr449 and Ser472; see Tab. 2b). Taken together, the use of pNHA-472 helped to preselect 13 residues for future studies of Cka1p target in Nha1p sequence.

## DISCUSSION

Previous work of other groups identified Cka1 catalytic subunit of CK2 kinase as activator (derepresor) of *ENA1* expression via phosphorylation of *ENA1* repressor, Nrg1p (Berkey & Carlson, 2006; Vyas *et al.*, 2005), beside other results, down regulation of *ENA1* expression due to absence of *CKA1* resulted in $Na^+$ sensitivity tests. Our work confirmed previously published results, furthermore, cells lacking only *CKA1* (BY-C1) were beside $Na^+$ sensitive to $Li^+$. In accordance with down-regulation of *ENA1* expression, drop in $Na^+$ tolerance due to *CKA1* deletion was observe also in strain lacking *NHA1* (BYT4 and BYT4-C1; Fig. 1a). In this case, $K^+$ sensitivity was observed simultaneously with $Na^+$

and $Li^+$ sensitivity indicating that there was no change in ion specificity, which fits together with regulation of *ENA1* expression, but not Ena1p activity. Interestingly, $K^+$ sensitivity was not observed in BY-C1 (ck*a1Δ*) strain most probably because present Nha1p was able to compensate inhibition of *ENA1* expression, or changes of $K^+$ sensitivity were below detection limit of growth tests (Fig. 1a and 1b).

Additionally, no changes in $Na^+$ and $K^+$ efflux were observed in case of *ENA1* was natively expressed using its ORF in genome (compare BYT4 and BYT4-C1; Fig. 1c) or it was overexpressed using multicopy plasmid and constitutive promoter (compare BYT45 [pENA1] and BYT45-C1 [pENA1]; Fig 2B). Since expression of *ENA1* was not induced before efflux assay (BYT4 and BYT4-C1; Fig. 1c) and efflux experiments were rather too short to expect changes in amounts of Ena1p present in plasma membrane, only basal level, which existence was indicated recently (Zahradka & Sychrova, 2012), of Ena transporters was present in plasma membrane during efflux assay. In case of BYT4 and BYT4-C1 cells, export activity of basally expressed Ena transporters was observed and it was not affected by *CKA1* deletion confirming results with overexpressed Ena1p. In sum, we showed that, contrary to *ENA1* expression, activity of Ena1p is not regulated by Cka1p.

Increased salt sensitivity observed in *cka1Δ* mutants derived from cells lacking either *ENA* genes (BYT5), or *NHA1* and *ENA* genes (BYT45), uncovered novel *ENA1*-independent way of alkali-metal-cation-homeostasis regulation by Cka1p (Fig. 1b). Regulation of Nha1p by Cka1p was showed for the first time. Reduced tolerance to salts due to Nha1p inhibition was observed in absence of *CKA1*, furthermore, Nha1p mediated efflux of $Na^+$ and $K^+$ was significantly affected by *CKA1* deletion in cells natively expressing Nha1p from its ORF in genome (BYT5 vs. BYT5-C1; Fig. 1c), or in cells with overexpressed *NHA1* using multicopy plasmid and constitutive promoter (BYT45 [pNHA1] vs. BYT45-C1 [pNHA1]; Fig. 2b).

Comparing BYT5 (*ena1-5Δ*) and BYT5-C1 (*ena1-5Δ cka1Δ*) strains, inconsistency of results of KCl tolerance and $K^+$ efflux was found. Though $K^+$ efflux was in BYT5 strain significantly reduced after *CKA1* deletion (Fig. 1c) uncovering changes in Nha1p activity, there was no difference between BYT5 and BYT5-C1 strains in KCl tolerance (but $Na^+$ and $Li^+$ tolerance was affected; Fig. 1b). Observed inconsistency may result from very different setup of both experiments (growth tests and cation efflux; see materials and methods) and related divers physiological parameters of tested cells such as internal pH, membrane or surface potential (Arino *et al.*, 2010; Maresova *et al.*, 2006; Navarrete *et al.*, 2010). Interestingly, despite significant drop in NaCl tolerance observed in single *cka1Δ* mutant (BY-C1, Fig. 1a), no defect in cation efflux was observed for BY-C1 (Fig. 1c). It indicated, that the basal level of Ena transporters (efflux experiments were too short to see regulation on level of expression)

was sufficient to compensate Nha1p activity drop in BY-C1 cells during efflux experiments. All these results led us to conclusion that absence of *CKA1* reduced the Nha1p activity.

Surprisingly, salt tolerance (NaCl, KCl and LiCl) was decreased after *CKA1* deletion also in BYT45 strain lacking export systems, *NHA1* and *ENA* genes (Fig. 1b). Due to absence of both active exporters, BYT45 strain (and derivated strains) is not able to export toxic cations or surplus of $K^+$ and cells are thus very sensitive even to very low concentrations of salts (Navarrete *et al.*, 2010; Zahradka *et al.*, 2012). Increased membrane potential of BYT45-C1 in comparison with BYT45 may be explanation of slight decrease in salt tolerance; results of relative membrane potential measurement were partially confirmed using $Li^+$ accumulation assay (Fig. 3a and b). In absence of cation export systems, increased non-selective uptake of cations (due to increased membrane potential) affects salt sensitivity of cells (see BYT45 and BYT45-C1, Fig. 1b), but in presence of exporters (*NHA1* or *ENA* genes), increased uptake of cations could be compensated by exporters activation (Arino *et al.*, 2010).

Relative membrane potential was changed also in other sets of strains, except BY4741, after *CKA1* deletion (Fig. 3a). Strains lacking *NHA1*, BYT4 (*nha1Δ*) and BYT45 (*nha1Δ ena1-5Δ*) were in accordance to previously published results (Maresova *et al.*, 2006) depolarized, but in both strains, deletion of *CKA1* (BYT4-C1 and BYT45-C1) resulted in repolarization to the values of BY4741 strain. Membrane potential of BYT5 (*ena1-5Δ*) was similar to BY4741, but subsequent deletion of *CKA1* (BYT5-C1) resulted in depolarization (Fig. 3a). This may be due to inhibition of Nha1p activity after *CKA1* deletion (Fig. 1c), because plasma membrane depolarization after *NHA1* deletion was described previously (Maresova *et al.*, 2006) and similar depolarization could probably occurred even after Nha1p deactivation. Depolarization of plasma membrane after Nha1p inhibition corresponds to recently published model (Ke *et al.*, 2013), where increased activity of Nha1p (simultaneous uptake of $H^+$ and efflux of $Na^+$ or $K^+$) could activate Pma1 ATPase. Since Pma1p is crucial for membrane potential maintenance, decrease in $H^+$ export via Pma1p after inhibition of Nha1p activity probably stay behind depolarization of BYT5-C1 strain observed in this work. In conclusion, plasma membrane potential was influenced by *CKA1* only when at least one of exporters was missing and it was identified as novel (third) way, how *CKA1* could influence alkali-meta-cation homeostasis showing a complex regulatory role of Cka1p. Interestingly, results of membrane potential measurements were confirmed by $Li^+$ uptake only in case of strains lacking *ENA* genes (BYT5 and BYT45), because presence of basal Ena ATPases level was most probably enough to prevent $Li^+$ accumulation during assay regardless membrane potential of cells. Additionally to membrane potential, other interactions could stay behind *NHA1*- and *ENA1*-independent effects of *CKA1* deletion to alkali-metal-cation homeostasis e.g. regulation of intracellular transporters such as Nhx1p or Vnx1p, which are

responsible for sequestration of ions into cell compartments (Arino *et al.*, 2010), but just a little is so far known about regulation of intracellular transporters and to study a role of Cka1p in regulation of these transporters remains for future studies.

Since Cka1p was identified as so far unknown regulator of Nha1p (Fig. 1b) and phosphorylation was previously considered to be general mechanism of regulation by Cka1p (Meggio & Pinna, 2003), CK2 sequence motif S/T-X-X-E was used to identify the most likely targets in Nha1p (Gnad *et al.*, 2007). Two residues were identified as putative targets of CK2 phosphorylation among 12 known phosphorylated Nha1p residue with unknown responsible kinase (see Tab. 2). Unfortunately, neither Ser669, nor Ser683 was confirmed to be crucial for interaction between Nha1p and Cka1p. Additional mutations in Ser/Thr residues in neighborhood (Ser684, Thr663 and Ser665) were prepared to prevent alternative phosphorylation in mutated *NHA1*. According to our results, no effect in comparison to native Nha1p was observed even after triple site-directed mutation (e.g. T663A S665A S669A or S669A S683A S684A) indicating that tested residues in Nha1p sequence are not targets of Cka1p. Using truncated version of Nha1p (plasmid pNHA1-472; Fig. 5), it was indicated, that putative Cka1p targets should be searched among firs 472 reside of Nha1p, because the activity of truncated version of Nha1p was affected by *CKA1* deletion similarly to complete Nha1p (compare Fig. 5 and Fig. 2a). After very short N-terminus, Nha1p was predicted (Kinclova *et al.*, 2001) to consist of 12 transmembrane segments forming 5 internal loops, additionally, N-terminus and C- terminus were predicted to be oriented into cytosol. Ten candidates of phosphorylation sites were identified using *in silico* prediction tools in internal loops (6 residues) and initial C-terminal part (4 residues in sequence between residues 440 to 472), furthermore, 3 previously found phosphorylated sites with unknown responsible kinase belong to the initial C-terminal part (see Tab. 2 and Gnad *et al.*, 2009). In total 13 residues were preselected for future studies.

Mammalian homologs of Nha1p (NHE3 and NHE5) were previously identified among targets of CK2, their CK2 phosphorylated residues belonged to cytosolic C-terminal part (Lukashova *et al.*, 2011; Sarker *et al.*, 2008). Previously identified CK2 targets were, in general either in yeast or in mammals, soluble cytosolic proteins, or cytosolic parts of transmembrane proteins (Hermosilla *et al.*, 2005; Lukashova *et al.*, 2011; Meggio & Pinna, 2003; Sarker *et al.*, 2008; Schmidt *et al.*, 2011). Surprisingly, our results indicated that targets of CK2 phosphorylation in Nha1p sequence are very likely residues localized in internal loops of transmembrane part of Nha1p, or in closer proximity to plasma membrane.

In our work, complex regulatory role of Cka1p in alkali-metal-cation homeostasis was uncovered. Similarly to Hog1 kinase, Cka1p influence not only long-term adaptation (*ENA1*), but also early

response (Nha1p) to salt stress and it could influence membrane potential. Simultaneous regulation of multiple systems and fine-tuning of their cooperation was studied using integrative model of ion homeostasis (Ke *et al.*, 2013). It was concluded, that use of multiple systems help cells to survive in environment and cooperation could partially compensate disadvantages of individual exporters, such as increased energy needs of Ena ATPases, or changes in internal pH after Nha1p activation (Ke *et al.*, 2013). According to our results, Cka1p enters the regulation alkali-metal-cation homeostasis in multiple points similarly to Hog1 kinase and partial functional overlap of Cka1p and Hog1p could be expected, but unfortunately, we have not enough data to consider if and how effectively could be lack of one of regulatory pathways compensated by the other. Furthermore, Hog1p is thought to be tightly regulated stress response kinase with specific targets, but Cka1 (CK2 respectively) is considered to be a housekeeping kinase with pleiotropic effect, which could indicate (analogically to *NHA1* and *ENA1*) a strategy of two independent systems with diverse behavior to increase cell survival in ever-changing environment.

Taken together, our results uncovered novel roles of CK2 catalytic subunit Cka1p and showed for the first time that (beside regulation of *ENA1* expression) Cka1p positively regulates transport activity of $Na^+/H^+$ antiporter Nha1 and thus participates also on early response to $Na^+$ ($Li^+$, $K^+$) stress. Furthermore, another *NHA1*- and *ENA1*-independent mechanism of alkali-metal-cation homeostasis regulation by Cka1p was found. Though regulation of Nha1p by Cka1p was identified, predicted putative targets of phosphorylation were disproved and nature of interaction between Nha1p and Cka1p remains to be discovered in future.

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**Tab. 1.** Oligonucleotides used in this study

| Name | Oligonucleotide sequence |
|---|---|
| Cka1-kanMX-F | caaaaataggggttgtagaaggaatatttgattcgaactttcgtacgctgcaggtcgac |
| Cka1-kanMX-R | agatggtaaaaaaaagtaatcgttatatcgtttgtcagtggcataggccactagtggatctg |
| Cka1-p1F | cgtaatgattgatcataag |
| Cka1-inF | cctaatttcgagatcgg |
| Cka1-inR | catatactgttgatactcgg |
| Ena1-1R | gcaaacgagattatcatgg |
| Cka1-t1R | ctgttgtatggaggaagccc |
| Nha1-S669A-F | gacagaaagcgagattgaagccgaagacgagatggaaaatg |
| Nha1-S669A-R | cattttccatctcgtcttcggcttcaatctcgctttctgtc |
| Nha1-T663A-S665A-F | ccgttacgatggagaagaggcagaagccgagattgaagccg |
| Nha1-T663A-S665A-R | cggcttcaatctcggcttctgcctcttctccatcgtaacgg |
| Nha1-SS683-4AA-F | gaaagtgaaagatcaatggccgctgctgaagagaggagaattcg |
| Nha1-SS683-4AA-F | cgaattctcctctcttcagcagcggccattgatctttcactttc |
| pNHA1-1 | caactctgtgtgatatag |
| YEpN-ScENA1-F | gtacattataaaaaaaaatcctgaacttagctagatattatgggcgaaggaactactaag |
| YEpN-ScENA1-R | cacgacgttgtaaaacgacggccagtgccaagcttgcatgtcattgtttaataccaata |

**Tab. 2.** Phosphorylation sites of the Nha1 antiporter

a. Sites phosphorylated by known kinase

| P-site | Kinase | Proved by | Experimental conditions | Source |
|---|---|---|---|---|
| T765 | Hog1 | Mass Spectrometry | α factor arrested cells<br>YNB grown cells<br>MMS* treatment | (Albuquerque *et al.*, 2008; Gnad *et al.*, 2009; Li *et al.*, 2007; Proft & Struhl, 2004; Smolka *et al.*, 2007) |
| | | *In vitro* phosphorylation | NaCl stress (0.4 M) | |
| T876 | Hog1 | *In vitro* phosphorylation | NaCl stress (0.4 M) | (Proft & Struhl, 2004) |

* MMS, methyl methanesulfonate, 0.05%

b. Mass spectrometry proved phosphorylated sites of the Nha1p with unknown kinase

| P-site | Predicted kinase (specific motif) | Experimental conditions | Source |
|---|---|---|---|
| T447 | -# | YNB grown cells<br>MMS* treatment | (Albuquerque *et al.*, 2008; Gnad *et al.*, 2009; Smolka *et al.*, 2007) |
| T449 | - | YNB grown cells | (Gnad *et al.*, 2009) |
| S472 | - | YNB grown cells | (Gruhler *et al.*, 2005) |
| S492 | - | MMS* treatment | (Smolka *et al.*, 2007) |
| S568 | - | YPD grown cells<br>α factor arrested cells | (Chi *et al.*, 2007; Li *et al.*, 2007) |
| S669 | CK1, CK2 | YNB grown cells | (Gnad *et al.*, 2009) |
| S683 | CK1, CK2 | YNB grown cells | (Gnad *et al.*, 2009) |
| S684 | CK1 | YNB grown cells | (Gnad *et al.*, 2009) |
| S736 | PLK1 | YNB grown cells | (Gnad *et al.*, 2009) |
| S768 | - | α factor arrested cells<br>YNB grown cells<br>MMS* treatment | (Albuquerque *et al.*, 2008; Gnad *et al.*, 2009; Li *et al.*, 2007) |
| S774 | - | α factor arrested cells | (Li *et al.*, 2007) |
| S918 | - | MMS* treatment | (Albuquerque *et al.*, 2008) |

* MMS, methyl methanesulfonate, 0.05%

# -, not identified

c. Predicted phosphorylation sites of internal loops and beginning of C-terminal part of Nha1p (among first 472 residues; Phosida database tool)

| Nha1- part | | Source |
|---|---|---|
| Internal loops | S68, S70, S134, S291, S358[†], S388 | (Gnad *et al.*, 2007) |
| Beginning of C-terminus | T456, T457, S464, S465, | (Gnad *et al.*, 2007) |

[†] CK2 sequence motif was found (S/T-X-X-E )

FIGURE LEGENDS

**Fig. 1.** Salt tolerance and changes in $K^+$ content in cells lacking *CKA1*. Growth of BY4741 and BYT4 (*nha1Δ*) cells was compared with appropriate mutants lacking *CKA1*, BY-C1 (cka*1Δ*) and BYT4-C1 (*nha1Δ cka1Δ*), on YNB supplemented with salts (a). Growth of BYT5 (*ena1-5Δ*) and BYT45 (*nha1Δ ena1-5Δ*) cells was compared with appropriate mutants lacking *CKA1*, BYT5-C1 (*ena1-5Δ cka1Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*), on YNB supplemented with salts (b). Changes in $K^+$ content (c) in BY4741, BYT4 (*nha1Δ*), BYT5 (*ena1-5Δ*) and BYT45 (*nha1Δ ena1-5Δ*) were measured and compared with their *cka1Δ* mutants, BY-C1 (cka*1Δ*), BYT4-C1 (*nha1Δ* cka1Δ), BYT5-C1 (*ena1-5Δ cka1Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*). Samples were taken at t = 0 min (100 %; black bars), and after 60 min (white bars).

**Fig. 2.** Activity of overexpressed Nha1p and Ena1p. Growth of BYT45 (*nha1Δ ena1-5Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*) cells bearing pNHA1 or pENA1 was tested on YNB with NaCl (a). Changes in $Na^+$ content (b) in BYT45 (*nha1Δ ena1-5Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*) cells with pNHA1 and pENA1 plasmids preloaded with 0.5 M NaCl were estimated. To see relative efflux, values at t = 0 (100 %, black bars) and at t = 45 min (white bars) were compared. Cells bearing YEp352 were used as control for all tests.

**Fig. 3.** Estimation of relative membrane potential and $Li^+$ accumulation. The relative plasma-membrane potential (a) was estimated in BY4741, BYT4 (*nha1Δ*), BYT5 (*ena1-5Δ*) and BYT45 (*nha1Δ ena1-5Δ*) cells and compared with their *cka1Δ* mutants, BY-C1 (cka*1Δ*), BYT4-C1 (*nha1Δ* cka1Δ), BYT5-C1 (*ena1-5Δ cka1Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*). Using similar cells, $Li^+$ content was measured (b) at t = 0 min (black bars) and after 30 min (white bars) of incubation with 0.1 M LiCl.

**Fig. 4.** Phenotype Characterization of selected Nha1p mutations. Growth of BYT45 (*nha1Δ ena1-5Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*) strains transformed with plasmids pNHA1; pNHA1-S669A, pNHA1-T663A-S665A-S669A, pNHA1-S683A-S684A and pNHA1-S669A-S683A-S684A was tested on YNB supplemented with NaCl. Cells bearing pNHA1 and YEp352 were used as controls.

**Fig. 5.** Estimation of truncated Nha1p activity after CKA1 deletion. Growth of BYT45 (*nha1Δ ena1-5Δ*) and BYT45-C1 (*nha1Δ ena1-5Δ cka1Δ*) strains transformed with plasmids pNHA1-472 and YEp352 was tested on YNB supplemented with NaCl or LiCl.

Figure 1

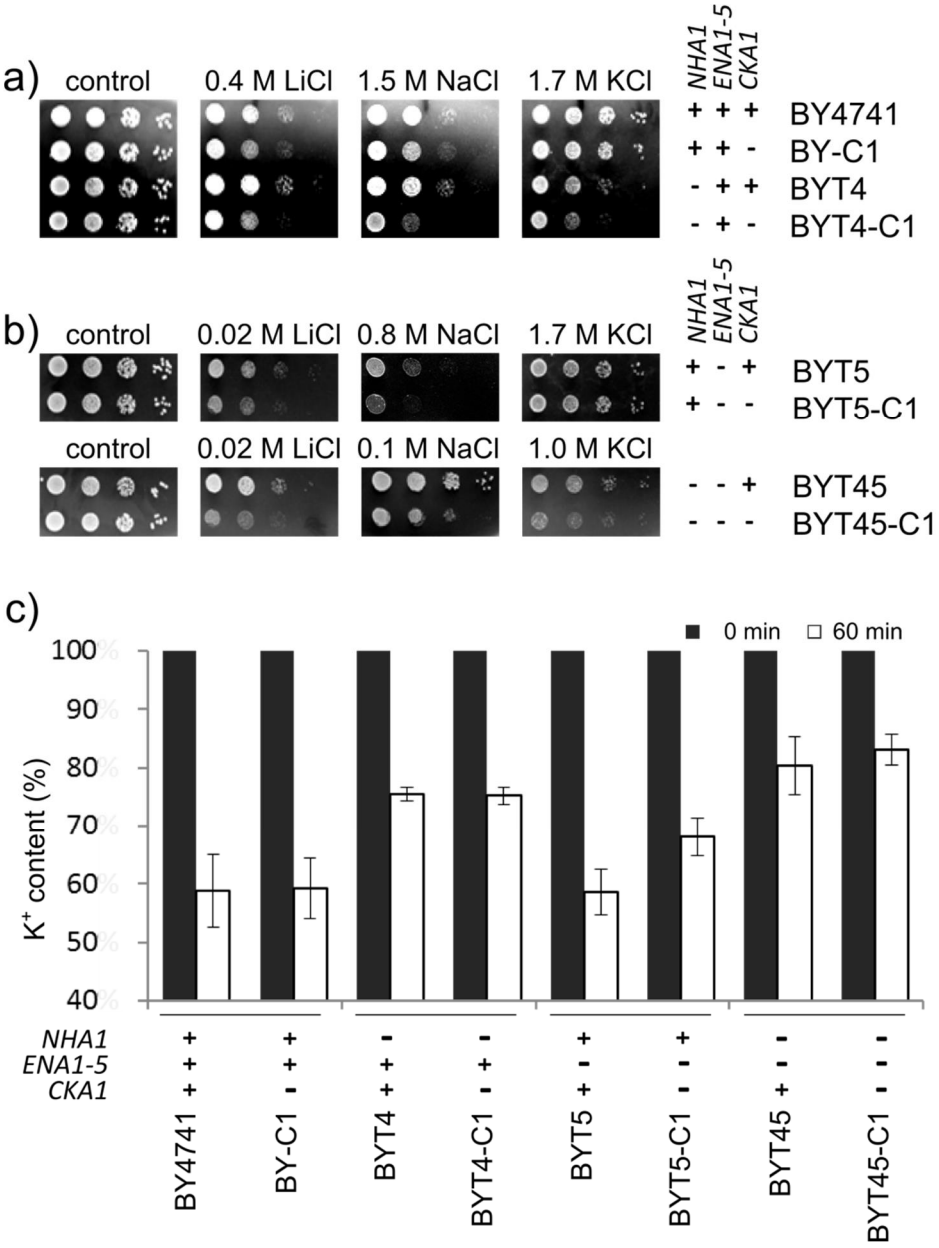

a)
control
0.4 M LiCl
1.5 M NaCl
1.7 M KCl
NHA1
ENA1-5
CKA1
+ + + BY4741
+ + - BY-C1
- + + BYT4
- + - BYT4-C1
b)
control
0.02 M LiCl
0.8 M NaCl
1.7 M KCl
+ - + BYT5
+ - - BYT5-C1
control
0.02 M LiCl
0.1 M NaCl
1.0 M KCl
- - + BYT45
- - - BYT45-C1
c)
0 min
60 min
K+ content (%)
100
90
80
70
60
50
40
NHA1
ENA1-5
CKA1
BY4741 + + +
BY-C1 - + +
BYT4 + + -
BYT4-C1 - + -
BYT5 + - +
BYT5-C1 - - +
BYT45 + - -
BYT45-C1 - - -

Figure 2

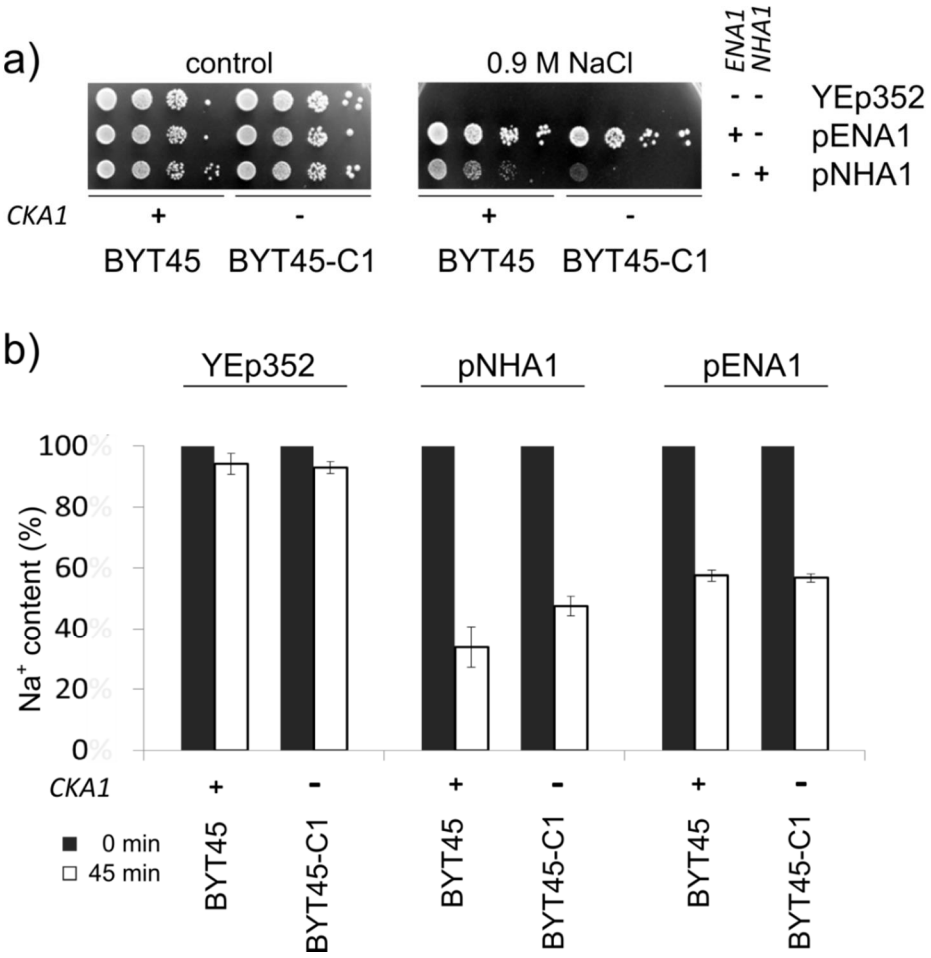

a)
control
0.9 M NaCl
ENA1
NHA1
- - YEp352
+ - pENA1
- + pNHA1
CKA1
+
-
+
-
BYT45
BYT45-C1
BYT45
BYT45-C1
b)
YEp352
pNHA1
pENA1
100 %
80 %
60 %
40 %
20 %
0 %
Na+ content (%)
CKA1
+
-
+
-
+
-
0 min
45 min
BYT45
BYT45-C1
BYT45
BYT45-C1
BYT45
BYT45-C1

Figure 3

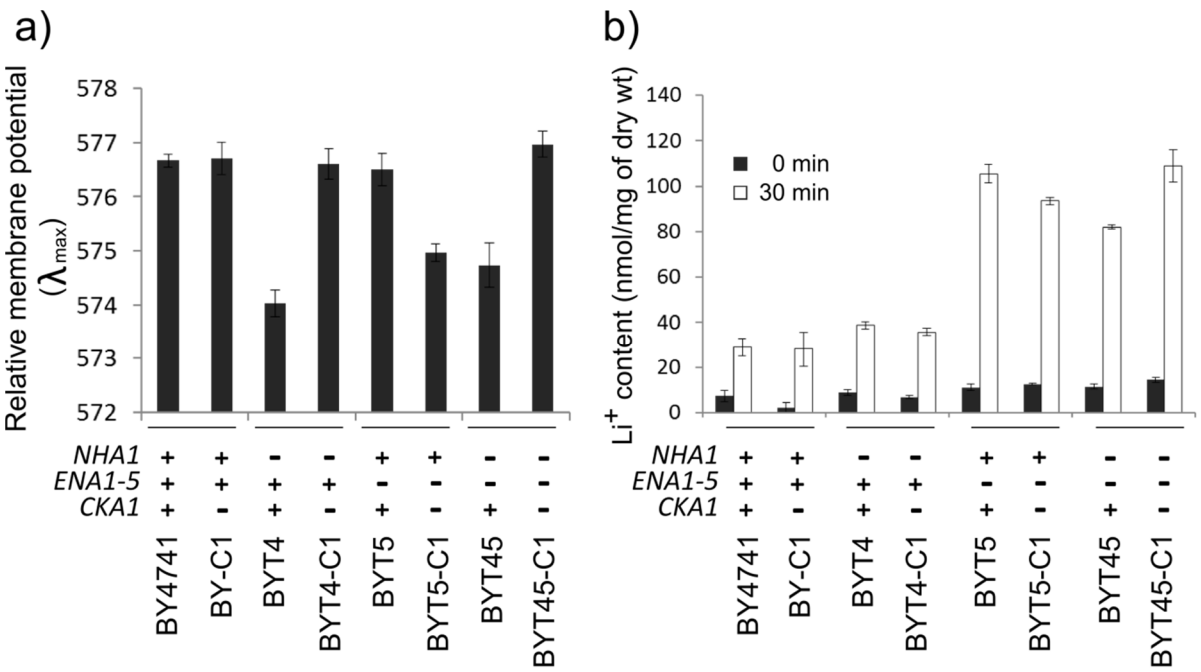

a)
Relative membrane potential
($\lambda_{max}$)
578
577
576
575
574
573
572
NHA1
ENA1-5
CKA1
BY4741
BY-C1
BYT4
BYT4-C1
BYT5
BYT5-C1
BYT45
BYT45-C1
b)
Li+ content (nmol/mg of dry wt)
140
120
100
80
60
40
20
0
0 min
30 min

Figure 4

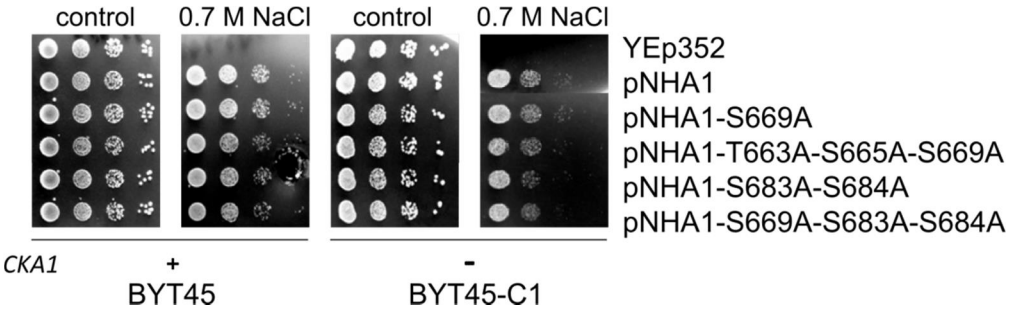
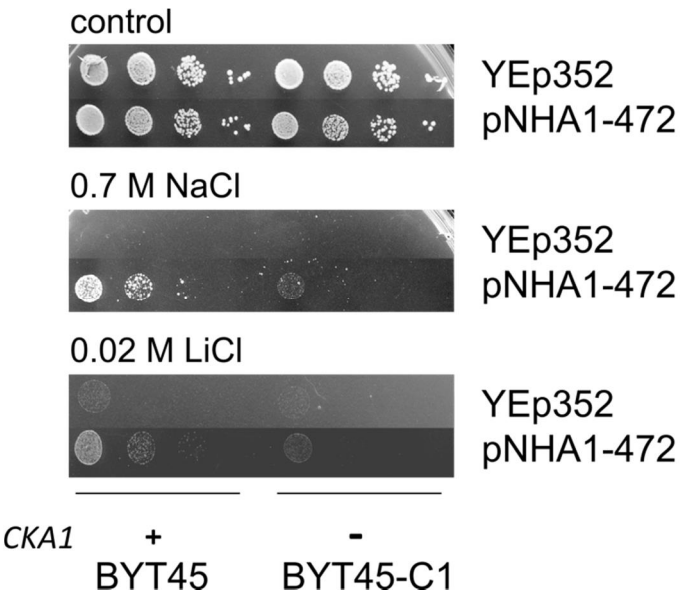

control
0.7 M NaCl
control
0.7 M NaCl
YEp352
pNHA1
pNHA1-S669A
pNHA1-T663A-S665A-S669A
pNHA1-S683A-S684A
pNHA1-S669A-S683A-S684A
CKA1
+
-
BYT45
BYT45-C1

Figure 5


control
YEp352
pNHA1-472
0.7 M NaCl
YEp352
pNHA1-472
0.02 M LiCl
YEp352
pNHA1-472
CKA1
+
-
BYT45
BYT45-C1

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## 4.6 Publikace č. 6 – Mutational analysis of NHAoc/NHA2 in *Saccharomyces cerevisiae*

V předchozích publikacích byla homeostase iontů alkalických kovů studována z mnoha podhledů. V této práci bylo využito získaných znalostí a existence řady vhodných mutantů *S. cerevisiae* byla využita jako nástroj pro studium lidského antiporteru. Díky relativně nízkým nárokům a jednoduchosti práce s kvasinkovými buňkami a relativně vysokému stupni sekvenční podobnosti s transportery jiných eukaryotních organismů mohou být buňky *S. cerevisiae* s výhodou využity pro studium transporterů jiných organismů (viz kapitola 2.1.6, str. 20). Jedním z nedávno objevených antiporterů, který je sekvenčně podobný Nha1p z *S. cerevisiae* je lidský $Na^+/H^+$-antiporter NHAoc/NHA2, který je exprimován ve vysokém množství pouze v osteoklastech (viz kapitola 2.1.6, str. 20; [118, 192]). Ačkoli byl tento gen objeven teprve nedávno, bylo zjištěno, že je důležitý pro správnou diferenciaci osteoklastů a mutace v tomto antiporteru by mohly stát za mnohdy smrtelným onemocněním tvorby kostní tkáně (o*steopetrosa*).

V rámci této práce byla cDNA lidského NHAoc/NHA2 exprimována v kvasinkových buňkách postrádajících kromě *NHA1* také geny *ENA*. Aktivita antiporteru byla následně testována jak pomocí měření tolerance buněk k NaCl, tak experimenty sledujícími v čase výstup $Na^+$ z buněk. Na základě informací uveřejněných v souhrnných databázích [193] byly identifikovány tři AA zbytky v sekvenci NHAoc/NHA2 (I159, V161 a F357), k jejichž záměně může v lidských buňkách docházet vlivem jednonukleotidových polymorfismů (SNP; single nucleotide polymorphism). SNP jsou takové nukleotidy v sekvenci DNA, u kterých často dochází k záměně nukleotidů, proto jsou považovány za zdroj genetické charakterisace jednotlivce. SNP v NHAoc/NHA2 byly v minulosti označeny jako důležité faktory vzniku a rozvoje onemocnění tvorby kostní tkáně. Ve AA zbytcích vybraných na základě SNP byla provedena cílená mutace (vždy pouze jedna z mutací I159T, V161A a F357C) a aktivita mutovaného proteinu byla sledována. Zavedením každé z mutací došlo k poklesu aktivity antiporteru a s tím spojeným zvýšením citlivosti k NaCl. S podobným výsledkem byly testovány také další vybrané evolučně konservované AA zbytky.

Na základě získaných dat bylo zřejmé, že mutace v evolučně konservovaných zbytcích a ve zbytcích, které mohou být mutovány díky SNP, zásadně ovlivňují aktivitu lidského antiporteru NHAoc/NHA2 a tím mohou pravděpodobně ovlivňovat správnou diferenciaci kostních buněk a stát v pozadí rozvoje poruch tvorby kostí. Místa SNP vedoucí v lidském organismu k mutacím ve sledovaných AA zbytcích tak pravděpodobně jsou důležitým faktorem určujícím predispozici k těmto poruchám.

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# Mutational analysis of NHAoc/NHA2 in *Saccharomyces cerevisiae*

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ABSTRACT

*Background: NHAoc/NHA2* is highly and selectively expressed in osteoclasts and plays a role(s) in normal osteoclast differentiation, apoptosis and bone resorptive function *in vitro*. Extensive mutational analysis of a bacterial homologue, NhaA, has revealed a number of amino acid residues essential for its activity. Some of these residues are evolutionarily conserved and have been shown to be essential not only for activity of NhaA in bacteria, but also of NHAoc/NHA2 in eukaryotes.
*Methods:* The salt-sensitive *Saccharomyces cerevisiae* strain BW31a was used for heterologous expression of mutants of NHAoc/NHA2. Membrane expression of NHAoc/NHA2 was confirmed by confocal microscopy. Intracellular concentration of Na+ (a measure of Na+ antiporter activity) was estimated by atomic absorption spectroscopy. The growth phenotypes of cells expressing NHAoc/NHA2 mutants were studied on YNB agar supplemented with NaCl and by growth curves in YNB broth.
*Results:* Mutations in amino acid residues V161 and F357 reduced the ability of transfected BW31a cells to remove intracellular sodium and to grow in NaCl-containing medium. Yeast expressing the double mutant F357 F437 cannot grow in 0.4 M NaCl, suggesting that these residues are also essential for antiporter activity.
*Conclusions:* Evolutionarily conserved amino acids are required for full antiporter function.
*General Significance:* Mutations in these amino acid residues may impact NHAoc activity and therefore osteoclast function *in vitro* and *in vivo*.



## 1. Introduction

The exchange of $Na^+$ or $K^+$ and $H^+$ down their concentration gradients (antiport or exchange activity) occurs in cells in all phyla and kingdoms. This activity is essential to control intracellular pH, cell volume and reuptake of $Na^+$, and cellular events such as migration, adhesion, proliferation and apoptosis [1–3].

We identified a mouse gene, *nhaoc/NHA2*, which is induced by RANKL stimulation of osteoclast precursors *in vitro* and *in vivo* [4,5]. Orthologues of *nhaoc/NHA2* are found in all metazoans studied and define a newly recognized subfamily of metazoan proteins within the CPA2 family of antiporters [1,4,6,7]. Members of this family share a conserved N-terminus (~500 amino acids) predicted to have 10 to 12 transmembrane segments, and a short C-terminal tail (~50–100 amino acids). Together they form a novel family of antiporters which share a common ancestor with NhaA, the main antiporter of *Escherichia coli* [8].

NhaA has been extensively studied and is the only antiporter for which the 3D crystal structure is known. It is an *electrogenic* antiporter with a stoichiometry of $2H^+/1Na^+$ whose activity is strongly pH-dependent [8]. Data from genetic-complementation, biochemical pull-down experiments, intermolecular cross-linking and cryo-electron microscopy of 2D crystals studies reveal that NhaA exists as a *dimer* in the native membrane.

Despite the evolutionary distance, NhaA and its eukaryotic orthologues show remarkable sequence similarity, suggesting that these proteins also have a similar structural architecture, characterized by 10 to 12 predicted transmembrane segments (TMS), depending on the software used [9]. Many amino acid residues are conserved, notably, a pair of adjacent aspartic acid residues, which are essential for antiporter activity in NhaA [10] as well as in HsNHA2 [7]. NhaA has been also subjected to extensive mutational studies. An analysis of the NhaA E241–F267 segment revealed several roles for this region of the protein: i.e. amino acids located in it participate in the "pH sensor", have effects on the determination of the $H^+/Na^+$ stoichiometry, form part of the cytoplasmic funnel leading to the cation binding sites and contribute to the NhaA dimer interface. Finally, a F267C mutation reduces the $H^+/Na^+$ stoichiometry of NhaA and a double mutation F267C/F344C inactivates the antiporter activity [8].

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NHAoc/NHA2 is unique in that it is predominantly expressed in osteoclasts and is required for osteoclast differentiation *in vitro* and *in vivo* [4,5]. In addition, *nhaoc/NHA2* silencing inhibits osteoclast formation *in vitro*. Mutations in genes that affect osteoclast activity and function in mice usually result in osteopetrotic phenotypes *in vivo*. Likewise, based on our preliminary characterization of NHAoc/NHA2 expression and function, we predict that mutations in this gene that affect activity of the protein will also result in reduced osteoclast differentiation, activity and/or survival.

Nine isoforms of $Na^+/H^+$ antiporters have been described in humans. Most cells usually express several of them simultaneously [11], which hinders the proper biochemical and physiological characterization of a particular transporter, due to the presence of other molecules with similar transport velocity and ion specificity in the same cell. These difficulties can be overcome by expressing a given transporter in a host lacking ion transport systems. One such organism employed for heterologous expression is the yeast *Saccharomyces cerevisiae*.

*S. cerevisiae* is one of the best characterized eukaryotic organisms. It is a unicellular fungus and yet yeast cells are very similar to higher eukaryotes with regards to cell structure and physiology. Because of this, yeasts have been utilized to study a variety of cell functions, including ion transport mechanisms [12–14]. Two different types of membrane transporters mediate $Na^+$ efflux: $Na^+$-ATPases and $Na^+/H^+$ antiporters. The *ENA* genes (*ENA1* to *ENA4*, this number can vary according to the strain) encode $Na^+$-ATPases. The *NHA1* gene encodes the $H^+$/cation antiporter.

The *S. cerevisiae* BW31a mutant strain (*ena1-4Δnha1Δ*) is very sensitive to salt because it lacks the main sodium and potassium extrusion systems. For this reason, phenotype complementation of BW31a cells (the ability of a heterologously expressed gene to rescue the salt-sensitive growth phenotype) is a powerful approach for the functional characterization of novel antiporters.

The goal of this study is to characterize the antiporter activity of NHAoc/NHA2 mutants in a yeast model. We hypothesize that evolutionarily conserved amino acids that are essential for NhaA antiporter activity will have a similar role in NHAoc/NHA2. We also hypothesize that mutations in those amino acid residues will impact NHAoc/NHA2 activity, and because of its restricted expression pattern, osteoclast function.

To test these hypotheses, we expressed mutants of NHAoc/NHA2 in *S. cerevisiae* BW31a cells and assessed the ability of the expressed mutants to rescue the salt sensitive phenotype. We have introduced several mutations in the NHAoc/NHA2 molecule: these are a) mutations in Aspartic Acid residues 278 and 279 to Cysteine individually. A double D278C–D279C mutant, which has previously been shown to abolish activity [7,18] was used as negative control b) three point mutations of hydrophobic amino-acid residues: Isoleucine 159, Valine 161 and Phenylalanine 357, which are amino acids that can be substituted in humans as a result of single nucleotide polymorphisms (SNP) (I159T, V161A and F357C) and whose conservation throughout evolution suggests that each may be an important determinant of NHAoc/NHA2 activity, and c) a mutation of F437 to Cysteine alone or F357C–F437C double mutant. F437 is homologous to NhaA F344.

## 2. Materials and methods

### 2.1. Yeast strains, media and growth conditions

For heterologous expression of NHAoc/NHA2 we used the salt-sensitive *S. cerevisiae* strain BW31a (*ena1-4Δ, nha1Δ*) [12]. Yeast cells were cultured in YPD (2% peptone, 1% yeast extract, 2% glucose) or YNB media (0.67% yeast nitrogen base without amino acids, 2% glucose or galactose, and appropriate supplements).

### 2.2. DNA manipulations

DNA manipulation, including the isolation of plasmids and cell transformations, were performed according to standard protocols.

### 2.3. Plasmid construction

For expression of NHAoc/NHA2 we used the plasmid pYES2.1-NHAoc/NHA2, which harbors the ORF of human NHAoc/NHA2 in the pYES2.1/V5-His-TOPO vector (Invitrogen) for galactose-inducible expression in yeast. The human NHAoc/NHA2 cassette was generated by Taq PCR amplification from plasmid SC128249 (OriGene Technologies, Inc., Rockville, MD) using the following primers:

Sense: 5′-GCCATGGGGGATGAAGATAAAAG-3′
Antisense: 5′-AACTTGCACAGAAGTCTCTCC-3′

Mutations of pYES2.1-NHAoc were done using the NEB Phusion Site-directed Mutagenesis Kit (F-541S), according to the manufacturer's instructions, using the following primers. All mutations were confirmed by sequencing.

D278C single mutation:
Sense: Phos-TGCATTCTGGCCATCACTGGCTTC, Antisense: Phos-ATCGAAGCT GCCAGCTGCCATGAG
D279C single mutation:
Sense: Phos-GACATTCTGGCCATCACTGGCTTC, Antisense: Phos-ACAGAA GCTGCCAGCTGCCATGAG
D278C D279C double mutation:
Sense: Phos-TGCATTCTGGCCATCACTGGCTTC, Antisense: Phos-ACAGAA GCTGCCAGCTGCCATGAG
F357C single mutation
Sense: Phos-TGTGGTTTCCCTGGATCAGGAGGA, Antisense: Phos-ATGCAC ACTGCTGAACACAGCTAG
F437C single mutation
Sense: Phos-TGTCTGATGGTGTGTTTTGCTGG, Antisense: Phos-TGTAGTCAA AATTCGTATCAATAC
F357 F437–C357 F437 double mutation
Using F357C mutated vector as template, introduce the second (F437C) mutation
I159T
Sense: Phos-TTTCTCATCAGAAATACCCCAGTCATCAACGAT, Antisense: Phos-CCCTGCAAGCAGCATGCCAAGAAGAGA
V161A
Sense: Phos-GCCATCAACGATAATGTGCAGATC, Antisense: Phos-TGGGA TATTTCTGATGAGAAACCC

### 2.4. Yeast electroporation

BW31a cells were prepared for electroporation as follows: One vial of BW31a cells was inoculated into 50 ml YPD and cultured overnight at 30 °C with shaking. Cells were harvested in a 50 ml sterile conical tube, centrifuged at 1500 rpm (4 °C) for 5 min and kept on ice. Cells were next serially washed with 40 ml then 20 ml ice-cold sterile water, resuspended in 5 ml 1 M ice-cold Sorbitol and pelleted at 2000 rpm for 5 min at 4 °C. Finally, the cells were resuspended in 200 μl ice-cold 1 M Sorbitol. 40 μl of yeast suspension were mixed with 1 μl (~0.5 μg) DNA in a pre-chilled electroporation cuvette (Cell Projects Ltd. Cat #: EP-102) and electroporated with one pulse: V = 1.5 kV, 25 μF, 200 Ω (time constant ~4.8 s). Two-hundred-microliter 1 M Sorbitol was immediately added to the cells and 100 μl of electroporated cells was spread onto 2% glucose YNB plates. The plates were incubated at 30 °C. Colonies appeared usually after 3 days. Individual colonies were picked up, tested for expression of NHAoc/NHA2 by Western blot analysis, and selected for phenotypic tests.

### 2.5. Western blot analysis

For expression studies, yeast protein extracts were subjected to Western blot analysis. Briefly, cultures were grown for 48 h at 30 °C in YNB medium supplemented with 2% Galactose to induce expression. Cells (100 μl aliquot) were centrifuged, washed with water, resuspended in 12.5 μl lysis buffer (ZYMO RESEARCH, Orange, CA) plus 0.5 μl Zymolase and incubated for 1 h at 37 °C. The lysates were further diluted with 25 μl 2× SDS sample buffer and used for Western blot analysis using anti-V5 antibody (Invitrogen dilution: 1/2000) as the primary antibody and an HRP-conjugated secondary antibody (Cell Signaling Technology, Inc., 1:1000). Blots were washed in TBST to remove unbound antibodies, incubated with 10 ml LumiGLO with gentle agitation for 1 min at room temperature and exposed to X-ray film.

### 2.6. Confocal microscopy

BW31a cells transformed with the indicated plasmids were grown in YNB supplemented with Glucose or Galactose (to induce expression of NHAoc/NHA2) as indicated. Cells were fixed by adding 37% formaldehyde to the culture followed by incubation at 30 °C for 30 min. Cells were then pelleted, washed in 0.1 M potassium phosphate pH 6.5 and treated with 2 μl Zymolase (5 unit, Zymo Research, E1004) and 5 μl of B-mercaptoethanol for 60 min at 37 °C to digest the cell wall. Cover slips (No. 1.5 thickness—Fisher 12-545-81) were then coated with poly-lysine solution (Sigma P8920) for several minutes, washed once with water, and allowed to air dry. Cells (25 μl) were then placed on the cover slips and allowed to attach for several minutes. The excess was aspirated after several minutes and the slides were immersed successively in ice-cold methanol for 6 min, then ice-cold acetone for 30 s, and allowed to air dry. Cells were blocked once in PBS-BSA (5 mg/ml powdered bovine serum albumin in 1× PBS) and incubated in primary antibody (anti-V5 from Invitrogen, R960-25) (diluted in PBS-BSA) in a moist chamber overnight at 4 °C. Cells were then washed four times in the wells with PBS-BSA and incubated in secondary antibody (Alexa Fluor® 488, Invitrogen A-11017 diluted in PBS-BSA) in a moist chamber for 2 h. Cells were washed three times with PBS-BSA, once with PBS and then incubated in 1× PBS-DAPI (SIGMA D9542, final concentration 0.1 μg/ml) for at least 30 s. Cells were then washed in PBS and mounted onto microscope slides. Images were captured with a Leica SP5X confocal microscope.

### 2.7. Phenotypic tests: Solid and liquid media

The growth phenotypes of cells expressing NHAoc/NHA2 mutants were estimated by drop test technique on YNB agar supplemented with NaCl. Briefly, freshly grown cells of each tested strain were resuspended in water and adjusted to the same initial $OD_{600}$ (about 1). Aliquots (3 μl) of 10-fold serial dilutions of yeast suspensions were spotted on agar plates. Plates were incubated at 30 °C (usually for 4 days) and images were obtained using a digital camera. To estimate the growth of cells in liquid YNB media with or without NaCl, we prepared a pre-culture by growing the previously electroporated yeast in 2 ml YPD plus adenine (20 μg/ml) overnight at 30 °C. The cells were centrifuged, washed once in YNB and resuspended in YNB/2% galactose at $OD_{600} \approx 0.05$. The growth was followed by measuring $OD_{600}$ every 12 h for 72 h. YNB was supplemented with varying NaCl concentrations (indicated in the figures).

### 2.8. Determination of cell Na+ content

Fresh BW31a cells expressing NHAoc/NHA2 wild-type antiporter or its mutant versions were inoculated in 50 ml of YNB media and grown overnight (30 °C; 160 rpm) to $OD_{600} \sim 0.6$. Fresh YNB medium with 1 M NaCl was added to reach a final concentration of 100 mM NaCl in the cell suspensions. Forty milliliters of suspension was incubated at 30 °C for 30 min, then 5-ml aliquots (three for each strain) were withdrawn, washed with cold deionized water and resuspended in 5 ml of 10 mM TRIS, 0.1 mM MgCl pH 4.5 (adjusted with solid $Ca(OH)_2$). Immediately, cells were collected on Millipore membrane filters, washed, acid extracted, and the intracellular concentration of $Na^+$ was estimated by atomic absorption spectroscopy [15]. The experiment was repeated three times, i.e. the sodium content measured in nine samples for each strain. The intracellular content of $Na^+$ was expressed in nmol of $Na^+$ per mg of cell dry weight. The intracellular $Na^+$ content in cells growing in the absence of NaCl was below 15 nmol/mg of dry weight. Cells expressing *ScNHA1* were used as positive controls and cells with the empty vector were used as negative controls.

## 3. Results

### 3.1. Bacterial NhaA and mammalian NHAoc/NHA2 have extensive sequence similarity

SIM [16,17] is a program which finds a user-defined number of best non-intersecting alignments between two protein sequences or within a sequence. The alignments are reported in order of decreasing similarity score and share no aligned pairs. We aligned NhaA and NHAoc/NHA2 using the BLOSUM30 comparison matrix with the following parameters: Number of alignments to be computed: 20; Gap open penalty: 12, Gap extension penalty: 4 (Fig. 1). Two of the returned alignments indicate several potentially conserved amino acids, which are indicated with an asterisk "*". Also highlighted (with a red asterisk) are: NHAoc/NHA2 D278–D279, I159, V161, F357 and F437 as well as their matching NhaA residues. D278–D279 are homologous to D163–D164 of bacterial NhaA. These two amino acids have been found to be essential for antiport activity in bacterial, yeast and mammalian antiporters. I159, V161 and F357 are amino acids that are substituted as a result of human SNPs. F357 and F437 are homologous to F267 and F344 of NhaA, respectively. F267 regulates the stoichiometry of NhaA and a double F267C F344C mutation can abolish NhaA antiporter activity [8].

### 3.2. Heterologous expression of human NHAoc/NHA2 can rescue the salt-sensitive phenotype of BW31a yeast

For expression in yeast, we cloned the cDNA of NHAoc/NHA2 into pYES2.1/V5-His TOPO TA under the control of the inducible GAL1 promoter. This plasmid allows for the production of a V5 tag/fusion protein that can be detected using an anti-V5 antibody. We used the vector pNHA1-985GFP (expressing *S. cerevisiae*'s own antiporter, *NHA1*) [12] and the empty pYES2.1/V5-His TOPO TA vector as positive and negative controls, respectively. Plasmids were electroporated into the salt-sensitive BW31a (*ena1-4Δ nha1Δ)* yeast strain. Western blot analysis of independent transformants (Fig. 2, *bottom*) shows galactose-inducible expression of a ~60 kDa protein in lanes 2–7, corresponding to the NHAoc/NHA2–V5 fusion protein. To study NHAoc/NHA2 function, we tested the ability of high-expressing transformants 2–5 to grow on YNB plates supplemented with varying concentrations of NaCl (0–0.4 M) and galactose (Fig. 2, *top*). Glucose containing plates were used as a control for galactose inducible expression. All strains grew equally well on YNB plates without any added salt, plus glucose or galactose, indicating that over-expressed NHAoc/NHA2 was not toxic to cells (0 M NaCl). When plated on YNB media supplemented with 0.4 M NaCl, cells expressing the NHAoc/NHA2 exchanger grew significantly better than the negative control cells, confirming that NHAoc/NHA2 has antiporter activity and can rescue the salt sensitivity of BW31a cells, as previously reported [1,4,6,7]. NHAoc/NHA2 expression did not result in the same sodium tolerance as that achieved by expression of the yeast Nha1 antiporter (lane +). NHAoc/NHA2 expressing cells can grow in the presence of NaCl only in galactose-containing plates, confirming that the salt

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17.5% identity in 399 residues overlap; Score: 114.0; Gap frequency: 6.5%

NhaA,          7 FFSSDASGGIILIIAAILAMIMANSGATSGWYHDFLETPVQLRVGSLEINKNMLLWINDA
NHAoc,       121 LFYCAIIGGKLLGLIK-LPTLPPLPSLLGMLLAGFLIRNIPVINDNVQIKHKWSSSLRSI

NhaA,         67 LMAVFFLLVGLEVKRE-LMQGSLASLRQAAFPVIAAIGGMIVPALLYLAFNYADPITREG
NHAoc,       180 ALSIILVRAGLGLDSKALKKLKGVCVRLSMGPCIVEACTSALLAHYLLGLPWQWGFI-LG

NhaA,        126 WAIPAATDIAFALGVLALLGSR--VPLALKIFLMALAIIDDLGAIIIIALFYTNDLSMAS
NHAoc,       239 FVLGAVSPAVVVPSMLLLQGGGYGVEKGVPTLLMAAGSFDDILAITGFNTCLGIAFSTGS

NhaA,        184 L------GVAAVAIAVLA------VLNLCGARRTGVYIL--VGVVLWTAVLK--SGVHAT
NHAoc,       299 TVFNVLRGVLEVVIGVATGSVLGFFIQYFPSRDQDKLVCKRTFLVLGLSVLAVFSSVHFG

NhaA,        228 LAGV--IVGFFIPLKEKHGRSPAKRLEHVLHPWVAYLILPLFAFANAGVSLQGVTLDGLT
NHAoc,       359 FPGSGGLCTLVMAFLAGMGWTSEKAEVEKIIA-VAWDIFQPLLFGLIGAEVSIASLRPET

NhaA,        286 SILPLGIIAGLLIGKPLGISL-FCWLALRLKLAHLPEGTTYQQIMVVGILCGIGFTMSIF
NHAoc,       418 VGLCVATVGIAVLIRILTTFLMVCFAGFNLKEKIFISFAWLPKATVQAAIGSVALDTARS

NhaA,        345 IASLAFGSVDPELINWAKLGILVGS-ISSAVIGYSWLRV
NHAoc,       478 HGEKQLEDYGMDVLTVAFLSILITAPIGSLLIGLLGPRL


18.8% identity in 367 residues overlap; Score: 106.0; Gap frequency: 4.9%

NhaA,         32 GATSGWYHDFLETPVQLRV-GSLEINKNMLLWINDALMAVFFLLVGLEVKRELMQGSL--
NHAoc,       113 GNLFGIIILFYCAIIGGKLLGLIKLPTLPPLPSLLGMLLAGFLIRNIPVINDNVQIKHKW

NhaA,         89 -ASLRQAAFPVIAAIGGMIVPALLYLAFNYADPITREGWAIPAATDIAFALGVLALLGSR
NHAoc,       173 SSSLRSIALSIILVRAGLGLDSKALKKLKGVCVRLSMGPCIVEACTSALLAHYLLGLPWQ

NhaA,        148 VPLALKIFLMALAIIDDLGAIIIIALF-YTNDLSMASLGVAAVA---IAVLAVLNLCGAR
NHAoc,       233 WGFILGFVLGAVSPAVVVPSMLLLQGGGYGVEKGVPTLLMAAGSFDDILAITGFNTCLGI

NhaA,        204 RTGVYILVGVVLWTAVLKSGVHATLAGVIVGFFIPLKEKHGRSPA--KRLEHVLHPWV-A
NHAoc,       293 AFSTGSTVFNVLR-GVLEV-VIGVATGSVLGFFIQYFPSRDQDKLVCKRTFLVLGLSVLA

NhaA,        261 YLILPLFAFANAGVSLQGVT--LDGLTSILPLGIIAGLLI--GKPLGISLFCWLALRLKL
NHAoc,       351 VFSSVHFGFPGSGGLCTLVMAFLAGMGWTSEKAEVEKIIAVAWDIFQPLLFGLIGAEVSI

NhaA,        317 AHLPEGTTYQQIMVVGILCGIGFTMSIFIASLAFGSVDPELINWAKLGILVGSISSAVIG
NHAoc,       411 ASLRPETVGLCVATVGIAVLI-RILTTFLMVCFAGFNLKEKIFISFAWLPKATVQAAIGS

NhaA,        377 YSWLRVR
NHAoc,       470 VALDTAR
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**Fig. 1.** NhaA–NHAoc/NHA2 sequence alignment. The alignments are reported in order of decreasing similarity score and share no aligned pairs. We aligned NhaA and NHAoc/NHA2 using the BLOSUM30 comparison matrix with the following parameters: Number of alignments to be computed: 20; Gap open penalty: 12, Gap extension penalty: 4. Above are two of the returned alignments indicating with a "*" the conserved amino acids. Also highlighted (in red): NHAoc/NHA2 Asp278–Asp279, I159, V161, F357 and F437 as well as their matching NhaA residues. Asp278–Asp279 are homologous to Asp163–Asp164 of NhaA, which are essential for antiport activity. I159, V161 and F357 are amino acids that are substituted as a result of human SNPs. F357 is homologous to F267 of NhaA and amino acid that regulates the stoichiometry of NhaA.

tolerance is only improved when the heterologous protein is expressed and that cells grown on glucose remain salt-sensitive.

### *3.3. Phenotypes of mutants of NHAoc/NHA2: Growth curves in liquid media*

To characterize the growth and NaCl tolerance of cells expressing mutants of NHAoc/NHA2 in detail, we performed growth tests in liquid media. All mutants of NHAoc/NHA2 expressed at equivalent levels (Fig. 3B). We did not observe any difference in growth rates between cells overexpressing any version of NHAoc/NHA2 exchanger and control cells in liquid YNB media without any sodium added, confirming that the expression of the NHAoc/NHA2 exchanger (wild type or mutant) was not toxic to the cells. In medium containing 0.4 and 0.6 M NaCl the growth rates changed significantly (Fig. 3A). Cells transfected with the empty vector (negative control) grew very

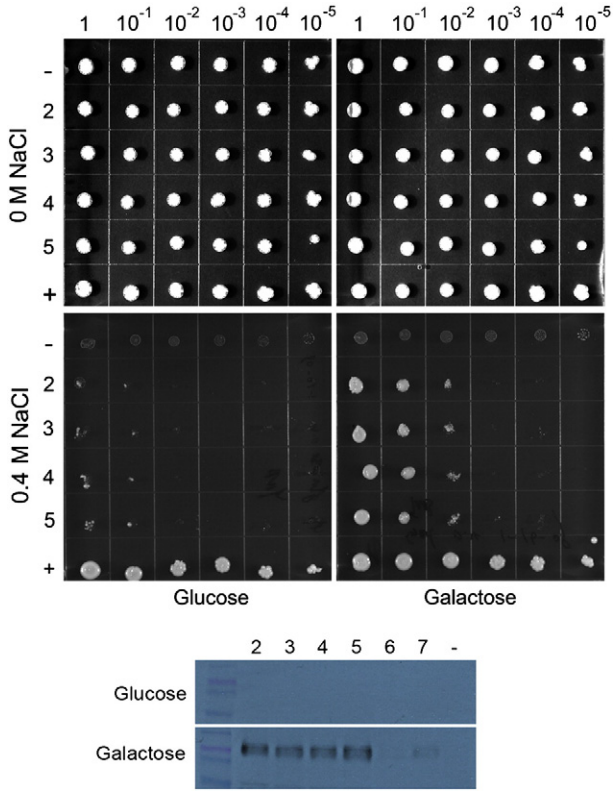


**Fig. 2.** Solid agar growth of the alkali–metal–cation sensitive BW31a yeast strain expressing human NHAoc/NHA2. (*top*) Ten-fold serial dilutions of yeast suspensions ($1–10^{-5}$) were spotted on YNB plates supplemented with NaCl and 2% glucose or 2% galactose, as indicated. Cells transformed with the four independent plasmids that were high expressers of NHAoc/NHA2 from the GAL1 promoters were tested (rows 2, 3, 4 and 5). Cells transformed with empty vector (row −) and cells expressing their own NHA1 antiporter (pNHA1–985GFP, row +) were used as negative and positive controls, respectively. (*bottom*) Western blot analysis of BW31a cells transformed with NHAoc/NHA2 expression plasmids (lanes 2–7). Blots were incubated with a V5 antibody. Clones 2–5 were high expressers of NHAoc/NHA2 and were therefore used in the subsequent experiment seen in the top panel of this figure. Cells transformed with an empty vector (lane −) were used as negative control. A ~60 kDa protein, corresponding to NHAoc/NHA2–V5 fusion protein, can be detected in lanes 2–5.

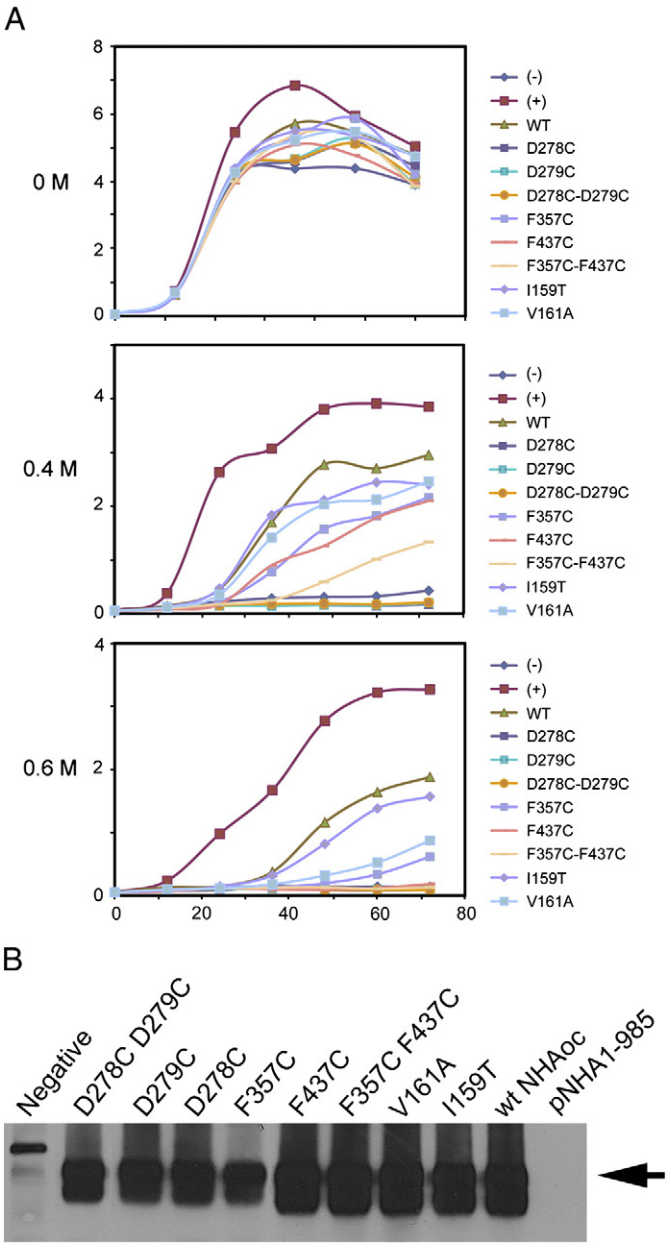


**Fig. 3.** (A, B) Growth curves of BW31a cells in liquid media. Cells were transformed with a plasmid expressing wild type or different mutants of NHAoc/NHA2 and grown in YNB broth and 2% galactose and without NaCl or with 0.4 and 0.6 M NaCl. Cells transformed with an empty vector (−) and cells transformed with *S. cerevisiae* NHA1 antiporter (+) were used as negative and positive controls, respectively. (B) Western blot analysis of BW31a cells transformed with a plasmid expressing wild type or different mutants of NHAoc/NHA2 using a V5-specific antibody, show equivalent protein expression of NHAoc/NHA2 (60 kDa band, black arrow).

slowly. Cells expressing wild type NHAoc/NHA2 exchanger showed improved ability to grow, though they grew more slowly than cells expressing the yeast NHA1 antiporter. These results confirmed the observation from drop tests in solid agar and indicated that NHAoc is functional in BW31a cells.

Mutations in aspartic acid residues in positions 278 and 279 completely abolished the ability of BW31a cells to grow in NaCl, in agreement with previous results [7,18]. In addition, we also mutated D278 and D279 *separately* and observed that *each* residue is essential for activity. Mutation I159T did not affect the ability of BW31a cells to grow in NaCl, whereas mutations V161A and F357C resulted in reduced growth (~50% inhibition compared to WT NHAoc/NHA2). Finally, yeast expressing the double mutant F357 F437 cannot grow in 0.6 M NaCl (93% inhibition of growth), suggesting that these residues are indeed homologues of NhaA F267 F344 which are essential for antiporter activity [8].

### 3.4. NHAoc/NHA2 is expressed in the membrane of BW31a cells

The results shown in the previous section indicate that mutations V161A, F357C, F357C–F437C as well as D278C and D279C diminished the ability of BW31a cells to grow in NaCl to different extents, or failed to complement the salt-sensitive phenotype. However, inhibition of growth in the presence of NaCl could also be due to mis-localization of the mutant NHAoc/NHA2 rather than a loss of activity. To determine whether the mutations affected membrane localization of NHAoc/NHA2 in BW31a cells, we performed confocal image analysis of BW31a cells expressing wild-type antiporter or mutant versions. The results (Fig. 4) indicate that all mutants tested localized to the plasma membrane. These results demonstrate that the mutations did not interfere with membrane localization of NHAoc/NHA2 and suggest that the mutations affected antiporter activity.

### 3.5. Mutations V161A and F357C inhibit Na+ antiport activity of NHAoc/NHA2

In order to test the ability of WT and mutant versions of NHAoc/NHA2 to mediate Na+ exchange, we performed an analysis of cell Na+

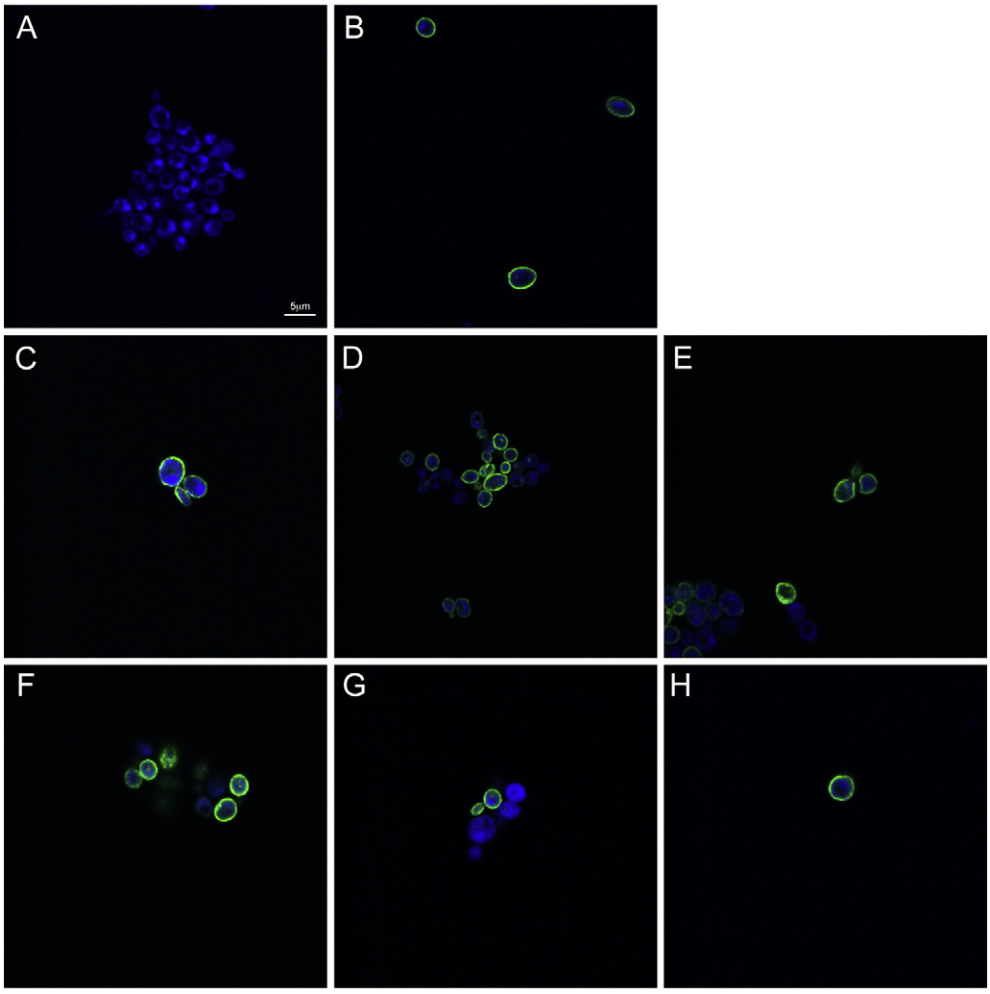


**Fig. 4.** WT and mutant versions of NHAoc/NHA2 localize to the plasma membrane. BW31a cells expressing different versions of NHAoc/NHA2 were fixed, permeabilized and stained using a primary anti-V5 antibody and an Alexa 488-conjugated fluorescent secondary antibody to detect the NHAoc/NHA2–V5 fusion protein. Confocal imaging of the cells shows anti-V5 reactivity in the membrane, indicating that none of these mutations interfere with protein localization. A) WT NHAoc/NHA2 in medium supplemented with glucose (no expression), B) WT NHAoc/NHA2 in medium supplemented with galactose (Induction), C) I159T, D) V161A, E) F357C, F) D278C, G) D279C, H) F357C–F437C.

content. The results (Fig. 5) indicate that there is no Na+/H+ exchange activity in negative control cells (−) as these cells have neither antiporter nor Na+/ATPase to pump Na+ out of the cells. Therefore, they contain more intracellular Na+ (relative intracellular content of Na+ is 70.58 nmol/mg dry weight or 100%). When BW31a cells express an active exchanger (e.g. cells expressing the yeast ScNHA1, (+)), the antiporter transports Na+ out of the cells, resulting in a lower intracellular Na+ content (37.13 nmol/mg dry weight or 52.6% of the negative control). In cells expressing the WT NHAoc/NHA2, the intracellular Na+ content is 55.69 nmol/mg dry weight or 78.9% of the negative control. These results conclusively show that NHAoc/NHA2 has Na+ antiporter activity. These findings are in complete agreement with the phenotypic complementation results since cells expressing wild type NHAoc/NHA2 exchanger showed improved Na+ antiporter activity compared to cells expressing no antiporter, though the antiport activity is less than that of the yeast ScNHA1 antiporter (Fig. 5).

We also tested mutants I159T, V161A and F357C. In cells expressing I159T the intracellular Na+ content was 56.18 nmol/mg dry weight or 79.6% of the negative control. This value is statistically undistinguishable from the WT NHAoc/NHA2, which indicates that this mutation has no effect on antiporter activity. V161A and F357C, on the other hand, resulted in Na+ contents of 61.75 (or 87.5% of the negative control) and 60.29 nmol/mg dry weight (85.4% of the negative control), respectively. These values show a 10% (V161A) and 8% (F357C) increase in the intracellular Na+, compared to the WT NHAoc/NHA2 which demonstrates that these mutations have an inhibitory effect on antiporter activity.

## 4. Discussion

We have performed a mutational analysis of the osteoclast-specific Sodium-Proton antiporter NHAoc/NHA2 by phenotypic complementation in yeasts and found that evolutionarily conserved amino acids are required for full antiporter function.

Na+/H+ antiporters are ubiquitous throughout the biological kingdom. The main Na+/H+ antiporter of *E. coli*, NhaA, has eukaryotic orthologues. Among these are the murine NHAoc/NHA2 [4] and the human HsNHA2 [7], which are part of a newly recognized family of *metazoan* CPA2 antiporters [1]. To date, the structure of these metazoan CPA2 antiporters have not been solved, however the primary sequence predicts a structure with 10 to 12 transmembrane segments (TMS) comparable to that of NhaA [10], suggesting that these proteins have a similar structural architecture. In addition to their structural similarity and despite the enormous evolutionary distance, NhaA and its eukaryotic homologues also have some sequence similarity. Many amino acid residues are conserved, notably, a pair of adjacent Aspartic acid residues, which are essential for antiporter activity in NhaA [10] as well as in NHAoc/NHA2 and *Hs*NHA2 [7] and in antiporters from other organisms. In this report we

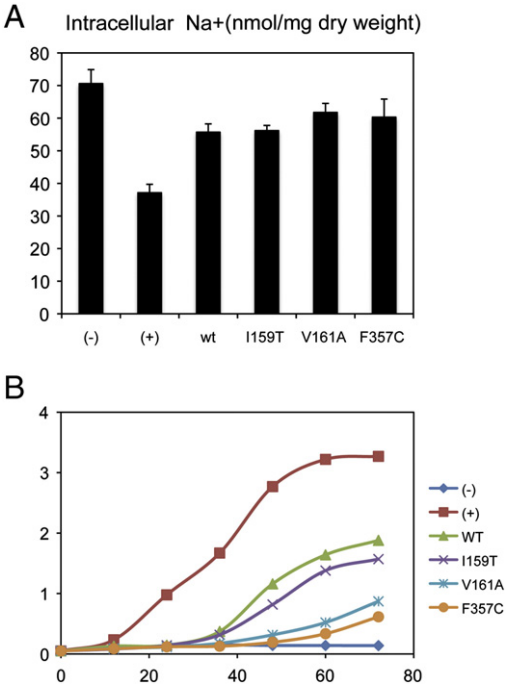


**Fig. 5.** Mutations V161A and F357C inhibit NHAoc/NHA2 Na+ antiport activity and fail to complement the salt-sensitive phenotype of BW31a cells. (−) cells transfected with an empty vector, (+) yeast ScNha1, (WT) WT NHAoc/NHA2, I159T, V161A and F357C: three mutants of NHAoc/NHA2. A) Intracellular Na+ content after culturing BW31a cells for 30 min in 100 mM NaCl. B) Growth curves in YNB/0.6 M NaCl of BW31a cells expressing different mutants of NHAoc/NHA2.

have confirmed those findings and we have described new mutations that affect NHAoc/NHA2 antiporter activity: Valine 161 to Alanine, Phenylalanine 357 to Cysteine and the double mutant Phenylalanine 357–437 to Cysteine. These mutations correspond to amino acid residues that are conserved between NhaA and NHAoc/NHA2 and are required for proper function.

The pathophysiology of many skeletal diseases is associated with either increased (osteoporosis, metastatic bone disease and Paget's disease) or decreased (various types of osteopetrosis) bone resorption by osteoclasts. In normal osteoclasts, all resorption activity may be experimentally abolished by combined pharmacological inhibition of proton pumping and sodium/proton antiport [19] .This observation, together with our previous reports [4] suggest that a molecule responsible for Sodium/Proton Antiport, like NHAoc/NHA2, is likely to be intimately involved in the regulation of osteoclast function and bone resorption, which will ultimately have an impact in bone homeostasis.

Single nucleotide polymorphisms have recently been recognized as an important factor in the development of human osteoporosis. Review of the online GeneCards database (Genecards.org, Weizmann Institute of Science, Israel) reveals the existence of three human SNPs (non-synonymous) in the coding region of NHAoc/NHA2 result in three amino acid substitutions: I159T, V161A and F357C. These amino acid residues are evolutionarily conserved. In addition, one of them (F357) is a putative homolog to NhaA F267, an amino acid essential for stoichiometry of NhaA [8]. Our data supports a role for two of these amino acids (V161 and F357) in the activity of NHAoc/NHA2. Measured as the ability to sustain yeast growth in 0.6 M NaCl, the V161A and F357C mutants result in loss of antiporter activity of 54% and 68%, respectively. Measured as the ability to extrude intracellular Na+, V161A and F357C cause a loss of activity of 10% and 8%, respectively. Finally, we found that the double mutant F357C–F437C abolishes 93% of the antiporter activity. These results are in agreement with the phenotypic complementation results as mutation I159T did not inhibit cell growth in NaCl, whereas mutations V161A and F357C did inhibit cell growth in NaCl, reflecting a reduced capacity of those mutants to remove Na+ from cells. Our findings suggest that the three human NHAoc/NHA2 SNPs may impact bone health and alter fracture risk for those carrying these mutations due to altered NHAoc/NHA2 activity.

The functional characterization of NHAoc/NHA2 will provide a better understanding of the mechanisms responsible for altered bone resorption that characterizes many bone diseases. Knowing which domains of NHAoc/NHA2 are critical for antiporter function will aid us in the design of appropriate new therapies, based on pharmacological agents that may interfere with NHAoc/NHA2 activity and ultimately help maintain bone mass. These studies provide the groundwork and context of our efforts to understand the molecular mechanisms of osteoclast differentiation and function as they affect bone mass in health and disease.

## Acknowledgements

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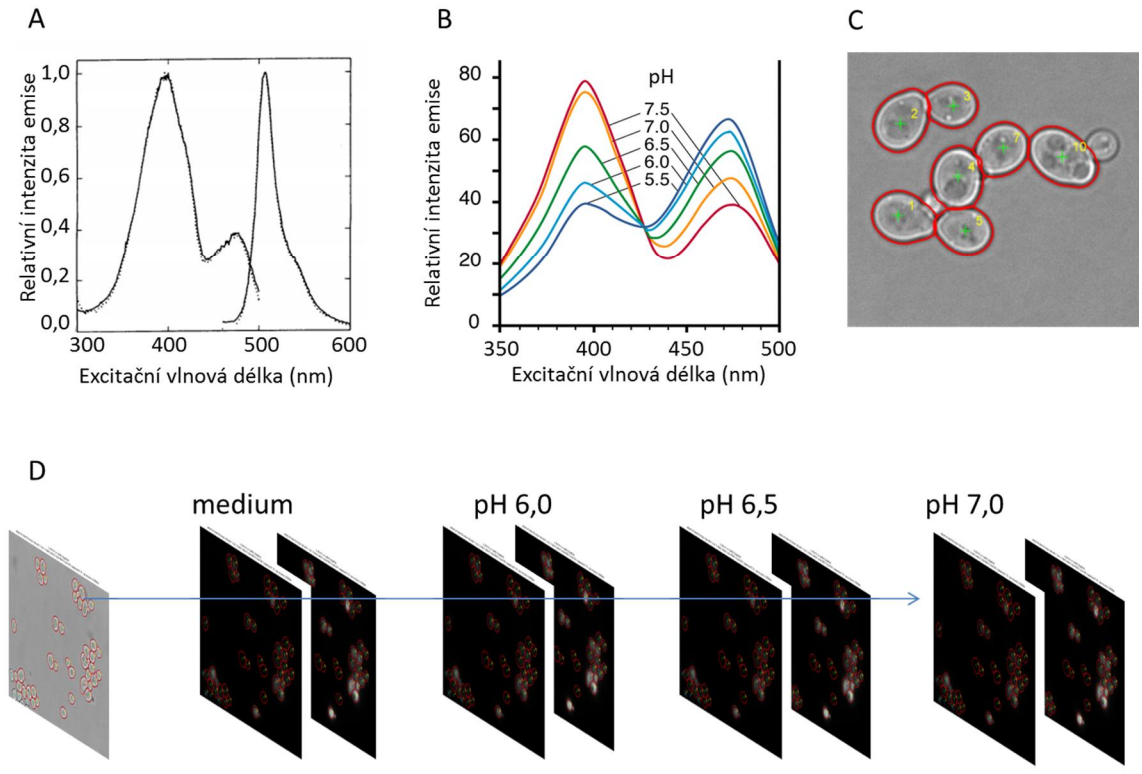
## 4.7 Další nepublikované výsledky, metodika měření vnitrobuněčného pH

Při výzkumu prováděném v rámci této disertační práce byla využita řada pokročilých metod z oblasti molekulární biologie, biochemie a fyzikální chemie. Mezi nejpokročilejší z nich patří měření vnitrobuněčného pH *in vivo* pomocí pH sensitivní verze zeleného fluorescenčního proteinu (GFP), pHluorinu, který je v buňkách heterologně exprimován [194, 195]. Metoda využívá změny v excitačním spektru pHluorinu způsobené změnou pH. Dva výrazné vrcholy, 398 nm a 477 nm, které byly nalezen již v excitačním spektru původního GFP (obr. 4.7.1A; [196]) jsou přítomny také u pHluorinu a s klesajícím pH dochází k poklesu intenzity u původně dominantního vrcholu 398 nm a roste intenzita vrcholu 477 nm (obr. 4.7.1B; [195]). S využitím vhodných kalibračních pufrů a vhodného poměru excitačních vlnových délek (např. 410/470 nm) získáme po vynesené do grafu v závislosti na pH téměř lineární kalibrační křivku, kterou je možné využít na stanovení absolutní hodnoty pH v buňkách na základě naměřených poměrů excitačních vlnových délek [195].




**Obr. 4.7.1 pHluorin a jeho využití v měření pH v mikroskopu**
Obrázek zachycuje excitační a emisní spektrum GFP (A), změnu intenzity emise pHluorinu v závislosti na excitační vlnové délce v různém pH (B), ukázka identifikace středů a obrysů buněk aplikací Ocellaris na obrázku z mikroskopu ve viditelném světle (C) a schéma měření pH v jednotlivých buňkách s využitím aplikace Ocellaris a systému CellASIC (D).

*zdroj: upraveno podle [195, 196] (A-B) a vlastní tvorba (C-D)*

Měření vnitrobuněčného pH je v laboratoři obvykle prováděno ve formátu 96 jamkových destiček s buněčnou suspenzí, který umožňuje měření většího množství vzorků současně a měření ve více

paralelách, dále vede k úsporám jak časovým, tak materiálním [194]. Ačkoli je metoda široce využívána, vyskytuje se řada problémů a limitací způsobených např. nehomogenitou signálu z buněčné suspenze nebo fluorescencí růstového média či buněk samotných.

Ve snaze posunout možnosti metody, získat přesnější data, ale také rozšířit množství údajů, které je možné z měření získat, byla v rámci této práce vyvíjena nová metoda pro měření pH v jednotlivých buňkách v mikroskopu. Pro vývoj metody byla získána podpora v letech 2011 – 2012 pro řešení projektů aplikovaného výzkumu financovaná Technologickou Agenturou ČR jako projekt Alfa s názvem: „SW analysa změn v mikroskopických obrazech buněčných indikátorů“. Cílem tohoto projektu bylo ve spolupráci s Mikrobiologickým ústavem AV ČR, v.v.i. a firmou DEL a.s. vytvořit software pro automatické vyhodnocení mikroskopického obrazu, který umožní měření vnitrobuněčného pH s použitím pHluorinu.

Pro potřeby tohoto projektu bylo vytvořeno několik plasmidů umožňujících stabilní a co nejvíce homogenní expresi pHluorinu v buňkách *S. cerevisiae*. Tyto plasmidy byly exprimovány v buňkách BY4741 a v odvozeném mutantním kmeni postrádajícím *TRK1* a *TRK2*, protože rozdíl v pH těchto dvou kmenů byl znám již z předchozích měření (viz kapitola 4.2, str. 38). S využitím těchto kmenů byla optimalizována metoda ve dvou provedeních. Nejdříve bylo využito klasických podložních a krycích sklíček tak, že byla buněčná suspenze (s kalibračním pufrem nebo bez) aplikována na sklíčko a bylo pořízeno několik trojic mikroskopických snímků (viditelné světlo, excitace 410 nm a excitace 470 nm). Snímky byly následně vyhodnoceny s použitím vyvíjené aplikace tak, že nejdříve byl odečten obrys buněk z první fotografii z trojice (viditelné světlo; obr. 4.7.1C) a tento obrys byl využit pro odečet fluorescence v jednotlivých buňkách na dalších fotografiích z trojice. V tomto nastavení vznikl každý kalibrační bod i hodnoty vzorku na jiném sklíčku (a tedy na jiných buňkách).

Druhou variantou měření bylo využití mikrokapilárového systému CellASIC (CellASIC Corporation), který umožňuje fixaci živých buněk pod objektivem a zároveň plynulé promývání zvolenými roztoky a médii. Bez ohledu na vysokou cenu nabízí druhá varianta řadu výhod, protože buňky ležící ve vybraném zorném poli bylo možné sledovat za různých podmínek. Zpravidla byly vybrané buňky nejdříve promývány růstovým médiem a následně promývány jednotlivými pufry. Pro každé médium i kalibrační pufr byla pořízena již zmíněná trojice fotografií stále stejného zorného pole. Díky tomu bylo následně možné sledování změn pH ve stejných buňkách, v jakých byl provedena kalibrace (obr. 4.7.1D). Každá jednotlivá buňka tak měla vlastní kalibrační křivku, která byla využita ke stanovení vnitrobuněčného pH příslušné buňky za různých podmínek (např. v různých médiích). Ke každé buňce bylo navíc možné přiřadit její parametry získané analysou fotografie ve viditelném světle, jako je velikost, obvod a tvar buňky, případně, jestli se buňka dělí, a bylo možné sledovat, jestli rozdílné pH

jednotlivých buněk nezávisí na některém z těchto parametrů. Po vyhodnocení bylo možné např. pozorovat individuální odchylky buněk, vyřadit buňky s nedostatečnou intenzitou signál, identifikovat přítomnost subpopulací se stejnými vlastnostmi nebo specifické chování buněk ve stejném fyziologickém stavu (např. dělících se buněk).

Nově vytvořenou metodou za použití destičky CellASIC byly nejdříve ověřeny výsledky publikované v rámci druhé publikace (kapitola 4.2, str. 38), které prokázaly, že buňky postrádající *TRK1* a *TRK2* mají signifikantně nižší pH než buňky BY4741. Následně byla prověřena schopnost metody identifikovat více subpopulací z hlediska pH a dalších parametrů. Jednou z výhod této metody bylo také to, že z měření bylo možné odstranit buňky s nedostatečnou expresí pHluorinu, nebo např. takové, jejichž kalibrační křivka byla nestandartní (to bylo pozorováno často u starých a zjevně narušených buněk s vysokou autofluorescencí). Odstranění těchto buněk z analysy bylo zpravidla dosaženo mnohem přesnějších výsledků, než které bylo možné získat dříve používanou metodou s 96 jamkovou destičkou.

Výstupem projektu byl vznik software Ocellaris (www.ocellaris.cz), který je v současné době ve formě funkční testovací verze, a na jehož dokončení a komercializaci se v současnosti dále pracuje.

# 5 Souhrnná diskuse

Předkládaná disertační práce svým tématem volně navazuje na diplomovou práci s názvem „Příprava a charakterisace plasmidu YEX-GFP-Nha1 a jeho využití pro studium proteinu Nha1 ze *Saccharomyces cerevisiae*“ obhájenou v roce 2008 na Katedře biochemie Přírodovědecké fakulty Univerzity Karlovy [33]. V diplomové práci byla, mimo jiné, studována role vybraných aminokyselinových zbytků z transmembránové oblasti Nha1p na aktivitu a substrátovou specifitu tohoto velice důležitého transporteru a bylo vytvořeno několik nástrojů (nové plasmidy a mutantní kmen), které byly využity pro další studium úlohy a regulace Nha1p v homeostasi iontů alkalických kovů. V rámci této disertační práce nebyla věnována pozornost pouze Nha1p, ale problematika udržování homeostase iontů alkalických kovů a regulace transporterů $Na^+$ a $K^+$ byla studována mnohem komplexněji. Práce vedla k objevu dosud neznámé dráhy regulace exportních systémů pomocí změn v membránovém potenciálu a byly objeveny dva nové regulátory antiporteru Nha1, které jsou nezbytné pro jeho správnou funkci. Získané zkušenosti a znalosti byly dále využity ke studiu lidského $Na^+/H^+$ antiporteru NHAoc/NHA2 a objasnění, které mutace by mohly stát za závažným onemocněním. V neposlední řadě byla v rámci práce vyvinuta nová metoda a softwarová aplikace, která má v budoucnu posunout možnosti dalších studií homeostase kationtů a pH v jednotlivých bukách.

Výsledky zahrnuté do této práce přinášejí mnoho nového. Z hlediska vědeckého přínosu do problematiky je zásadní objev vzájemné regulace vstupních a výstupních transporterů iontů alkalických kovů. Již delší dobu se předpokládalo, že neustálá cirkulace $K^+$ přes plasmatickou membránu je u kvasinek důležitá pro udržování optimálního membránového potenciálu a pro mnoho fyziologických dějů. Až na základě této práce (kapitoly 4.2 a 4.3) bylo překvapivě prokázáno, že k neustálému výstupu $K^+$ dochází také v prostředí s nedostatkem $K^+$. Navíc bylo poprvé ukázáno, že ATPasy Ena jsou přítomné a funkční také bez indukce jejich exprese a v případě absence antiporteru Nha1 jsou schopny Nha1p zastoupit při udržování cirkulace $K^+$. Zjištění jsou o to překvapivější, že v prostředí s nedostatkem $K^+$ by se mohlo zdát logičtější to, že budou exportery nefunkční proto, aby nedocházelo k zbytečnému úniky potřebného $K^+$ z buněk. Důležitost udržení cirkulace je potvrzena také tím, že výstup $K^+$ neprobíhá pouze jedním exportním systémem, ale je jištěn tak, že v případě poruchy (delece) jednoho exporteru zastoupí jeho úlohu exporter další a rychlost celkového výstupu $K^+$ není ovlivněna. Předpokladem pro neustálou aktivitu exportních systémů je, že zároveň s exportem probíhá také mnohem účinnější vysokoafinitní import $K^+$, který umožňuje nejen získávat exportovaný $K^+$ zpět, ale také efektivně akumulovat další $K^+$ z prostředí, aby mohly buňky růst a dělit se. Z výsledků je zřejmé, že výstup a cirkulace $K^+$ probíhá i v případech, kdy to je pro buňku zjevně energeticky náročné, což svědčí o mimořádné důležitosti tohoto děje pro buněčnou fyziologii. Na

narušení vstupu $K^+$ (deleci genů pro transportery *TRK1* a *TRK2*) proto buňky reagují významným snížením výstupu $K^+$ tak, aby nedocházelo k přílišným ztrátám $K^+$, obdobně jako v případě narušení výstupu (např. delece *nha1*) se sníží afinita transporterů Trk ke $K^+$ [149]. Až na základě studií zahrnutých v této disertační práci bylo prokázáno, že aktivita importerů a exporterů je synchronizována, navzájem se ovlivňují a že jedním ze spojujících prvků je regulace membránového potenciálu. Výsledky uvedené v této práci umožnily důležitý posun v chápání komplexnosti systému regulace homeostase iontů alkalických kovů, bez těchto výsledků bylo pravděpodobně nemohl vzniknout ani nedávno publikovaný komplexní model homeostase $K^+$ v kvasinkových buňkách [14].

Až do vzniku této práce byl dosud detailněji prostudován pouze jediný regulátor antiporteru Nha1, kinasa Hog1, která je schopna fosforylovat dva AA zbytky v C-koncové části Nha1p, tím omezit výstup $K^+$ z buněk a následně zlepšit toleranci k $Na^+$ a sorbitolu [83, 86]. V této disertační práci byly objeveny dva další regulátory Nha1p, a to kinasa Cka1 a proteiny 14-3-3. Přítomnost obou těchto positivních regulátorů je důležitá pro zajištění plné aktivity Nha1p hlavně za podmínek solného stresu. Zajímavé je, že Cka1p působí na několika úrovních najednou a svou úlohou připomíná Hog1p, protože obě kinasy regulují jak expresi genu *ENA1*, tak aktivitu proteinu Nha1, ale do homeostase iontů alkalických kovů mohou zasáhnout ještě dalšími mechanismy (účast Hog1p v regulaci osmotického stresu a regulace $\Delta\Psi$ pomocí Cka1p). Přestože předkládaná práce přinesla celou řadu nových poznatků o úlohách Nha1p a regulaci jeho aktivity, téma ještě není zdaleka vyčerpáno. Nejméně 12 dalších AA zbytků Nha1p je fosforylováno avšak ani odpovědná kinasa, ani k čemu tato fosforylace slouží (viz kapitola 4.4, str. 41), není známo. S velkou pravděpodobností je také možno říci, že v předcházejících studiích (např. [189]) nebyla identifikována všechna místa fosforylace, a to především taková, která mohou být fosforylována/ defosforylována pouze za specifických podmínek a která by právě proto mohla být z hlediska regulace homeostase iontů alkalických kovů klíčová.

Porovnání dvou laboratorních kmenů, které bylo provedeno v první publikaci (kapitola 4.1, str. 37) naznačilo, že výsledky mohou být značně ovlivněny výběrem použitého kmene a že rozdíly v rámci jednoho kmene mohou být z hlediska fenotypu velmi důležité. Vznik této práce nepřímo navázal na vývoj ve vědecké komunitě, kde se s příchodem vysokokapacitních studií zkoumajících rozsáhlé knihovny mutantních kmenů objevila potřeba verifikovat získaná data ve více genetických pozadích. Na základě této filosofie musí být pozorovaný fenotyp nezávislý na určitém kmeni *S. cerevisiae* a výsledky jsou považovány za relevantní až po ověření ve více kmenech (viz např. [29]). Jelikož se v oblasti homeostase iontů alkalických kovů této otázce dříve nikdo systematicky nevěnoval, jsou získané výsledky pro dotčenou vědeckou komunitu dosti zásadní.

Získané zkušenosti a poznatky byly také využity při studiu vlastností savčího antiporteru a při vývoji nové metody. Studie zaměřená na lidský NHAoc/NHA2 (kapitola 4.6, str. 44) ukazuje mimo jiné to, že využití heterologní exprese v *S. cerevisiae* může přinést zajímavé a relevantní výsledky při studiu proteinů vyšších eukaryot, zároveň jsou zachovány přednosti práce s kvasinkami, jako je např. relativní jednoduchost a rychlost práce, přesně definované a dobře prozkoumané genetické pozadí, dobře fungující nástroje molekulární biologie a nižší náklady. Je zřejmé, že i v dnešní době může *S. cerevisiae* nabídnout mnoho možností ve vědecké práci a zdaleka se nejedná o přežitý modelový organismus. To potvrzuje také to, že k rychlému vývoji dochází i na poli metodologie a sledování nových trendů a zavádění nových metod je nezbytné pro udržení vysoké kvality prováděného výzkumu. Nová metoda měření vnitrobuněčného pH v mikroskopu, která byla vyvinuta v rámci této práce (kapitola 4.7, str. 45), byla navržena na základě identifikované poptávky vědecké komunity po tzv. „single cell“ metodách a měření individuálních rozdílů u jednotlivých buněk. Při vývoji byl využit pokročilý mikrokapilární systém CellASIC v kombinaci s moderní technikou pro fluorescenční mikroskopii a pokročilým matematickým aparátem tak, že vznikla unikátní metoda umožňující získávat ve srovnání s dříve používanými metodami mnohem podrobnější data. Zároveň bylo vyvinuto řešení cenově mnohem dostupnější než metody průtokové cytometrie, které jako jediné umožnují získání podobných dat na úrovni jednotlivých buněk. Vytvořená aplikace, která popsané měření umožňuje, je v současné době ve fázi testování (www.Ocellaris.cz) a přípravy na fázi komercializace, nicméně lze předpokládat, že by se měla v blízké budoucnosti začít používat v laboratorní praxi, protože již dnes existuje konkrétní poptávka ze strany zahraničních pracovišť.

# 6 Závěr

Výsledky uvedené v této disertační práci byly publikovány v pěti článcích a jeden další rukopis byl připraven pro odeslání k recenzi. Z těchto šesti publikací je u třech z nich uveden autor této disertační práce na prvním místě. Pro experimentální práci bylo připraveno a použito více než 20 nových mutantních kmenů *S. cerevisiae* a více než 10 nových plasmidů a byla využita celá řada pokročilých metod z oblasti molekulární biologie, biochemie, buněčné fyziologie a biofyzikální chemie. Na základě krátké zahraniční stáže byla převzata a v naší laboratoři zavedena metoda pro měření interakcí proteinů *in vivo* (metoda BiFC viz kapitola 4.4, str. 41), dále byla zavedena nová metoda na měření velikosti buněk pomocí přístroje (CASY TT, Roche) a byla vytvořena aplikace pro automatické a snadné vyhodnocení naměřených dat, která byla využita při měření výsledků uvedených v této práci (např. kapitola 4.3, str. 40). V neposlední řadě byla v rámci spolupráce s komerční firmou navržena a vytvořena softwarová aplikace umožňující unikátní sledování změn vnitrobuněčného pH *in vivo* v reálném čase v jednotlivých buňkách v různém prostředí.

V rámci této práce:

1) Bylo zjištěno, že mezi dvěma laboratorními kmeny *S. cerevisiae* a jejich mutanty existují značné rozdíly nejen v obsahu iontů alkalických kovů, ale také např. ve velikosti buněk a citlivosti k solím. Práce upozornila na fakt, že rozdíly mezi kmeny v rámci jednoho druhu mohou být významné a proto lze předpokládat pouze omezenou možnost ztotožňování výsledků z různých kmenů *S. cerevisiae*.
2) Vznikly výsledky, které pomohly lépe pochopit problematiku vzájemných interakcí vstupních a výstupních transporterů $K^+$, přinesly nové světlo do regulace transporterů odpovědných za export iontů alkalických kovů, antiporteru Nha1 a ATPas Ena, a jejich zapojení do funkčního celku společně s importery $K^+$.
3) Byly nalezeny dva dosud neznámé regulátory antiporteru Nha1, proteiny 14-3-3 a kinasa Cka1p. V obou případech se jedná o positivní regulátory aktivity Nha1p a jejich přítomnost je nezbytná pro plnou aktivaci Nha1p v prostředí solného stresu.
4) Získané znalosti byly využity při studiu lidského antiporteru NHAoc/NHA2 heterologně exprimovaného v kvasinkách. Byla studována závislost aktivity transporteru na mutacích specificky zavedených do jeho cDNA a bylo nalezeno několik AA zbytků, jejichž mutace mohou pravděpodobně ovlivnit vznik a rozvoj závažné choroby u lidí.

Výsledky práce byly publikovány v renomovaných mezinárodních časopisech a prezentovány na zahraničních kongresech a konferencích. Vytýčené cíle této disertační práce tak byly v plné šíři splněny.

# 7 Seznam zkratek

| | |
|---|---|
| ΔΨ | membránový potenciál |
| AA | aminokyselina/y |
| CK2 | kaseinkinasa 2 |
| GFP | zelený fluorescenční protein |
| PKA | proteinkinasa A |
| PM | plasmatická membrána |
| ppm | particles per milion; označení pro 0,0001% |
| SNP | single nucleotide polymorphism; jednonukleotidový polymorfismus |
| TMS | transmembránový segment |

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