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Dosage Suppression of Mutations in Casein Kinase II by *RIM11*, a Glycogen Synthase Kinase-3 Homolog

(Under the direction of CLAIBORNE V. C. GLOVER, III)

In this report, a glycogen synthase kinase-3 homolog, *RIM11*, was isolated in a multicopy screen for suppressors of the salt-sensitive phenotype of casein kinase II (CKII) regulatory subunit mutants. Previously known as a gene involved in regulating the initiation of meiosis, *RIM11* is now characterized as a determinant of salt-tolerance in cells growing on media containing galactose as the primary carbon source. Consistent with this, *rim11*Δ mutants are shown to be salt-sensitive on galactose media, but not on glucose media. The salt-sensitivity of *rim11*Δ mutants is found to be additive with that of CKII mutants, suggesting that the Rim11 and CKII kinases do not operate in the same biochemical pathway. *RIM11* overexpression is found to improve the salt-tolerance of the wild-type strain, as well as CKII and calcineurin mutants, and the catalytic activity of Rim11 is determined to be necessary for this effect. Finally, Rim11 protein levels are shown to be higher when cells are grown on galactose-containing compared to glucose-containing media, and also to be induced upon exposure to NaCl. *RIM11* is proposed to be a galactose-specific determinant of salt-tolerance in *Saccharomyces cerevisiae*.

INDEX WORDS: *S. cerevisiae*, CKII, *RIM11*, GSK3, salt sensitivity

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by

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B.A., Carleton College, 1997

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2001

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December 2001

Dedication

This work is dedicated to my parents, James J. Dowd and Laura A. Dowd, for their constant support over the years and for always encouraging me down the right path even if I wasn't so sure at the time.

Acknowledgements

I would like to thank Dr. Claiborne Glover for being there to help me when I needed him, for providing me with insightful advice and comments, for trying to keep me exposed to new technologies in the field of genomics, and for being always understanding and patient.

I would like to thank the members of my committee, Dr. Scott Gold and Dr. Kelley Moremen, for their advice, helpful suggestions, and support.

I must thank Sricharan Bandhakavi for the guidance he has given me in performing the physical side of the experiments described here. When I first joined the Glover lab, and even throughout the time I spent there, Sri was a mentor to me at the bench, and this work would not have been possible without his help.

All the members of the Glover lab have been invaluable to me at one time or another, and I thank them for their help, support, and for all the fun we had together. Wenfan Zhao, George Hinkal, Giovanna Lomolina, Raymond Evans, and Timothy Poore—thank you.

The members of the Gold lab have been good friends as well, and I thank them all. I will certainly miss our joint lab meetings, although not the weeks when I was the speaker.

I would like to thank Kirsten Tenney, a member of the Glover lab whose time did not quite overlap with mine. I used several of the strains she created, as well as her *ENA1-lacZ* constructs.

Finally, I would like to thank Dr. Stephan Zweifel and Dr. John Tymoczko, who first sparked my interest in biochemistry, genetics, yeast, and molecular biology with their excellent, enthusiastic teaching.

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Introduction

With the exception of halophiles, the growth of unicellular organisms is generally inhibited by high concentrations of any salt. This is due in part to the loss of turgor pressure caused by osmosis and in part to interference with the chemical reactions of a normal cellular physiology. For example, high intracellular ion concentrations can detrimentally affect the electrostatic and hydrophobic forces that are involved in creating and stabilizing protein structure (Serrano, 1996). Sodium and lithium are a pair of similar ions that are toxic in their own manner, in addition to the general effects just mentioned. Unlike ions such as potassium, which is innocuous unless present at high levels, sodium can inhibit cell growth even at low media concentrations (~100 mM). From this, it seems likely that these two ions poison their own unique set of intracellular targets[1] (Serrano et al., 1999). Many plants, including economically important ones, are also susceptible to high salt concentrations which can be a significant problem in arid regions (Robinson and Downton, 1984). It is the hope of researchers in this field that *Saccharomyces cerevisiae* will prove to be an effective model system to allow understanding of sodium tolerance, and that this understanding can be applied to improve the growth of crops in saline conditions worldwide.

Mechanisms of salt-tolerance in *Saccharomyces cerevisiae*

There are generally three challenges that a cell must overcome when faced with a high external concentration of salt. Osmotic pressure is the first, and this occurs if any solute is present in a high enough external concentration. The struggle to keep the

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[1] Currently only Hal2 and the RNase MRP are known *in vivo* targets of sodium and lithium in *Saccharomyces cerevisiae* (Serrano et al., 1999).

internal concentration of toxic ions at low levels, even in the presence of a high external concentration, is the second challenge. Ion homeostasis involves mechanisms for both influx and efflux of ions, the details of which can become quite complicated. The third challenge involves the sensitivity of internal cellular metabolism to salt; these essential processes must be stable enough to withstand at least a slight, temporary increase in the intracellular ion concentration. These three problems will be discussed in order, along with the methods that *S. cerevisiae* uses to solve them.

Maintaining life in a high-osmolarity environment often requires the production and accumulation of organic solutes known as osmolytes. The primary osmolyte in *S. cerevisiae* is glycerol, which is produced by the High Osmolarity Glycerol (HOG) pathway. Other osmolytes utilized are sucrose, trehalose, and proline (Serrano et al., 1999). The HOG pathway is a mitogen-activated protein (MAP) kinase cascade activated by two different osmosensors, Sln1 and Sho1, which indirectly activate the MAPK Hog1. Although some of the downstream effectors are unknown, *HOG1* is required for growth under hyperosmotic conditions because it activates *GPD1*, which codes for glycerol phosphate dehydrogenase. *GPD1* is only one of several genes activated by this pathway, yet it is primarily responsible for the resulting accumulation of glycerol (Marquez et al., 1998; Siderius et al., 1997). Thus, *GPD1* allows *S. cerevisiae* to survive hyperosmotic stress by producing glycerol to balance the concentration of solutes across the plasma membrane. Although not discussed further in this work, low external osmolarily is also a problem. This is dealt with by expelling glycerol from the cell through the glycerol transporter Fps1 (Tamas et al., 1999).

Ion transport and homeostasis are important functions of all living cells, not just to provide resistance to high salt concentrations, but for several necessary physiological tasks. Various mechanisms of homeostasis maintain the intracellular environment near the optimum concentration for each ion, which is higher for K^+ than Na^+, and higher for Mg^{++} than Ca^{++} (Serrano et al., 1999). Ion transport also allows for the creation of

electrochemical gradients across the cell membrane and the secondary influx of nutrients made possible thereby. However, it is necessary that ion transport be tightly controlled to prevent accidental accumulation of an unwanted ion, such as Na^+; and when this does occur, the poisonous ion must be pumped out of the cell efficiently. In the context of salt-tolerance, a discussion of sodium influx and efflux mechanisms follows.

Saccharomyces cerevisiae has no mechanisms specifically designed for sodium influx; to the contrary, *S. cerevisiae* is simply unable to completely block the passage of this ion through its cell membrane. Various channels and transporters used for other purposes allow sodium, especially when present in high extracellular concentrations, to leak through the membrane down its electrochemical gradient. One of the most important of these is the Trk1 potassium channel. Since potassium is larger than sodium, but in other ways physically similar, it is a difficult engineering problem to create a channel that only allows potassium to pass through (Doyle et al., 1998). The Trk1 system is able to do this amazingly well, but not perfectly; some sodium can still leak past it. Strangely, though, sodium and lithium influx is actually greater in a *trk1*Δ mutant strain than in wild-type (Rios et al., 1997). One theory used to explain this phenomenon relies on the hyperpolarization of the plasma membrane seen in these mutants (Madrid et al., 1998). *S. cerevisiae* uses protons to form an electrochemical gradient in order to drive secondary transport, making the external medium positively charged with H^+ ions. Apparently, Trk1 is a major factor involved in dispersing this gradient, by allowing K^+ ions inside the cell. Thus, the result in a *trk1*Δ mutant is a hyperpolarized cell membrane. This additional force is enough to push any positively charged ion or molecule inside the cell through any route possible, including nearly every channel, transporter, and permease on the cell surface. Therefore, *trk1*Δ mutants are actually more sensitive to salt than wild-type, even though a route of sodium influx has been taken away (Madrid et al., 1998). In *S. cerevisiae*, then, it appears that the electrical and concentration gradients

pushing sodium into the cell are at least as important to consider as the routes that the ions actually take to get there.

Most salt tolerance pathways in *S. cerevisiae* focus on improving efflux of sodium once it manages to leak inside. Ena1 is an ATP-dependent Na^+ pump and is by far the most important sodium efflux mechanism in the organism, although there are others[2]. There are four genes in the *ENA* tandem array, *ENA1* being the most highly expressed and the most important for salt-tolerance (Haro et al., 1991). The promoter of *ENA1* is extemely large, and has been divided into two regions based on lacZ fusions: a proximal region extending from –751 to +1, and a distal region extending from –1384 to –752 (Garciadeblas et al., 1993). *ENA1* is tightly regulated at the transcriptional level by several different pathways that respond to environmental signals. Osmotic stress acts through the HOG pathway to relieve *ENA1* from repression mediated by a *CRE*, or cAMP response element (Marquez and Serrano, 1996), whereas the phosphatase calcineurin activates *ENA1* in response to salt stress through the transcription factor Crz1 (Mendizabal et al., 2001). Somewhat suprisingly, another pathway impinging on the *ENA1* promoter includes glucose repression, mediated by Mig1 and the Ssn6/Tup1 general repressor complex, and relieved by the Snf1 kinase (Alepuz et al., 1997). Without the Ena1 sodium pump, *S. cerevisiae* would be extremely vulnerable to changes in salt concentration as shown by the profound sensitivity of *ena1-4*Δ mutants (Haro et al., 1991).

When salt is allowed to pass through the cell membrane, and before it can be expelled, it is clear that tolerance must come from the resistance of internal cellular physiology to such a perturbation. Even in the same species, some genotypes may be more or less tolerant to a brief rise in intracellular salt concentration. An example is seen in a strain overexpressing one of the two targets of sodium toxicity known in *S.*

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[2] The Nha1 Na^+/H^+ antiporter is one example (Prior et al., 1996).

cerevisiae, Hal2 (Glaser et al., 1993). *HAL2*, also known as *MET22*, is required to convert two toxic intermediates of the sulfate assimilation pathway, 3'-phosphoadenosine-5'-phosphate (PAP) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS), into adenosine-5'-phosphate (AMP). This enzyme is highly Na^+-and Li^+-sensitive, and a rise in the concentration of either of these ions can inhibit it greatly, causing PAP and PAPS to accumulate, which eventually results in cell death (Murguia et al., 1996). When *HAL2* is overexpressed, the same increase in intracellular salt concentration may not be enough to completely eliminate all Hal2 activity, consequently preventing PAP and PAPS from accumulating to a large degree. It could be said that increased expression of *HAL2* makes the physiology of *S. cerevisiae* more robust to changes in intracellular salt concentration. Thus, in order to protect against sodium toxicity once the ion has entered the cell, the targets need to be increased in expression, or perhaps sequestered into a secure compartment. This might be somewhat difficult, or unsatisfactory, which could be why most salt tolerance mechanisms in *S. cerevisiae* rely on simply expelling the offending ion.

There are many other *S. cerevisiae* genes involved in salt tolerance in addition to those listed above. A large group of halotolerance (HAL) genes contains a few kinases that, when combined with other kinases such as Snf1, Hog1, casein kinase I, casein kinase II, and Gcn2, as well as a group of phosphatases such as calcineurin, Ppz1, and Sit4, form a vast network of phosphorylation/dephosphorlyation that regulates salt resistance (Glover, 1998; Goossens et al., 2001; Serrano et al., 1999). Previous work in the Glover lab has focused on casein kinase II (CKII), and that enzyme is discussed in detail below.

Casein Kinase II

CKII is an evolutionarily conserved Ser/Thr protein kinase[3] that is essential for viability in *S. cerevisiae* (Padmanabha et al., 1990). Its consensus sequence for phosphorylation consists of a serine or threonine in an acidic context (Meggio et al., 1994). Basic residues tend to disrupt activity when present from –2 to +5, relative to the target Ser/Thr, and proline will prevent phosphorylation if present at the +1 position (Meggio et al., 1994). Although CKII is activated by polycations (such as spermidine) and inhibited by polyanions (such as heparin) *in vitro*, its activity is not regulated physiologically in any known way. In fact, it may be possible that differences in compartmental localization of the enzyme with respect to its substrates may be the only way in which it is regulated at all. Since the CKII consensus sequence is very hydrophilic, it is likely that many of these stretches in the *S. cerevisiae* genome encode for amino acids that are located on the surfaces of their respective proteins, and therefore exposed to the cytoplasm. If this is true, one would expect CKII to phosphorylate any protein present in the same intracellular compartment that has such a sequence—a large number of proteins, indeed (Glover, 1998). However, only a small number of substrates have actually been identified in *S. cerevisiae*. These include topoisomerase II, eIF-2α, Srp40, and Fpr3 (for references, see Glover, 1998).

The holoenzyme of CKII in *S. cerevisiae* is composed of four polypeptides encoded by four separate genes: *CKA1*, *CKA2*, *CKB1*, and *CKB2*. The CKA genes code for the catalytic α subunits, and the *CKB* genes encode the regulatory β subunits. The available evidence suggests that these four polypeptides form a heterotetramer in which the two β subunits (β and β') form a stable dimer, making inter-β chain contact and contact with each α subunit (Glover et al., 1994), as is the case for the human enzyme

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[3] CKII typically phosphorylates only serine and threonine, but on one particular substrate, Fpr3, it can phosphorylate a specific tyrosine residue (Marin et al., 1999).

(Niefind et al., 2001). The α subunits do not make physical contact and exist as monomers when isolated from the β subunits. *CKA1* is similar, but not identical, to *CKA2*; in fact these two genes are as divergent from one another as they are from the CKII catalytic subunits in other organisms (Glover, 1998). This suggests that they may have slightly different functions, but nevertheless it is not essential for a cell to possess both genes to be viable. Even the *triple* deletion mutants[4] of CKII are viable—all necessary CKII functions can be performed by either *CKA1* or *CKA2* alone (Glover, 1998). Phenotypes of a single *CKA* deletion are mild, but include slight salt-sensitivity and increased flocculation, which is a Ca^{++}-dependent, lectin-mediated, cell-cell aggregation (Teunissen and Steensma, 1995). As stated above, the double *CKA* deletion is lethal. A deletion of either *CKB* gene causes strong salt-sensitivity, but these strains show no obvious phenotype on normal media without salt (Bidwai et al., 1995). The double *ckb1*Δ, *ckb2*Δ mutant shows the same level of salt-sensitivity as either of the single mutants, and causes no additional phenotype. This is consistent with the idea that the regulatory subunits form a heterodimer, the function of which is necessary for *CKB* function in general. Removal of either β subunit would destroy the possibility of forming the heterodimer and would be as damaging as the deletion of either gene (Glover, 1998).

The salt-sensitivity of *ckb*Δ mutants points to a role for CKII in ion homeostasis, specifically for the sodium ion. CKII has been found to increase expression of *ENA1*, which encodes the primary sodium pump in *S. cerevisiae*, by about three- to four-fold under both basal conditions and when induced with salt or high pH (Tenney and Glover, 1999). However, this was disputed in a subsequent study which found *no* significant increase of *ENA1* expression by CKII (de Nadal et al., 1999). The controversy has not yet been resolved, but nevertheless it is clear that CKII is involved in sodium tolerance in

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[4] The genotypes of the two triple deletion mutants of CKII are: *cka1*Δ, *ckb1*Δ, *ckb*2Δ and *cka*2Δ, *ckb1*Δ, *ckb2*Δ.

some manner. CKII mutants are not osmosensitive; their sodium sensitivity is in fact suppressable by adding a similar concentration of KCl to the sodium-containing medium (Bidwai et al., 1995). Since the role for CKII in sodium homeostasis is currently in question, it seems very possible that CKII could be involved in strengthening the intracellular resistance to sodium ions, rather than encouraging their departure from the cell. A certain CKII substrate (or substrates) could be inefficiently phosphorylated in the *ckb*Δ mutants, and become partially destabilized as a result, although not to the point of causing a phenotype under normal conditions. If this destabilized substrate were sensitive to increased intracellular concentrations of sodium, this could cause critical problems when the cell is subjected to salt stress (de Nadal et al., 1999). In order to test this hypothesis, and hopefully to find the theorized substrate(s), a multicopy suppression screen of genomic DNA was performed in a *ckb1*Δ, *ckb2*Δ background. This screen revealed only one gene as a suppressor of the salt-sensitive phenotype of *ckb*Δ mutants: *RIM11*, which encodes a member of the glyogen synthase kinase-3 family in *S. cerevisiae*.

Rim11, a Glycogen Synthase Kinase-3

Glycogen synthase kinase-3 (GSK3) was originally identified as a kinase that phosphorylates glycogen synthase (Embi et al., 1980), but recent studies have expanded its physiological role to include such processes as cellular differentiation, insulin regulation, and gene expression in a variety of eukaryotic organisms, including mammals (Nikolakaki et al., 1993; Ruel et al., 1993; Welsh and Proud, 1993). There are four members of the GSK3 family in *S. cerevisiae*: *MCK1*, *RIM11*, *MRK1*, and *YOL128c* (Zhan et al., 2000). Very little is known about MRK1 and open-reading frame *YOL128c*, but *MCK1* has been implicated in meiosis and sporulation as well as chromosomal stability (Neigeborn and Mitchell, 1991; Shero and Hieter, 1991). *RIM11* (Regulator of Inducer of Meiosis) was originally identified as a suppressor, upon deletion, of the toxic

nature of *IME1* (Inducer of Meiosis) overexpression in haploid cells (Mitchell and Bowdish, 1992). It was later determined that Rim11 is necessary to activate Ime1 for initiation of meiosis, and thus *RIM11* was found to be required for sporulation of *S. cerevisiae* in general (Bowdish and Mitchell, 1993). At the start of meiosis, Ime1 activates the transcription of *IME2*, which proceeds to activate the other genes necessary for meiosis and sporulation (Burgess et al., 1999). During vegetative growth, Ume6 binds to a *URS1* sequence present in the 5' untranscribed region of *IME2*, preventing Ime1 from activating *IME2* expression (Rubin-Bejerano et al., 1996). Under conditions encouraging sporulation, Rim11 binds and phosphorylates both Ime1 and Ume6, promoting an Ime1-Ume6 complex, and thereby converting Ume6 from a transcriptional repressor into a transcriptional activator (Malathi et al., 1999; Zhan et al., 2000). Hence, *IME2* is transcribed, and so thereafter are the other genes required during the early phases of meiosis.

In addition to its role in meiosis and as a suppressor upon deletion of *IME1* overexpression toxicity, *RIM11* (also called *MDS1*, for *MCK1 Dosage Suppressor*) was isolated as a multicopy suppressor of the various phenotypes of *mck1*Δ mutants: cold sensitivity, temperature sensitivity, and sensitivity to the microtubule-destabilizing drug benomyl (Puziss et al., 1994). Several years later, *mck1*Δ mutants were also found to be salt-sensitive, marking the first time a yeast GSK3 homolog was shown to be involved in salt-stress resistance in *S. cerevisiae* (Piao et al., 1999). In the same 1999 study, a *rim11*Δ mutant was also tested for salt-sensitivity, but it was found to be as resistant to NaCl as the wild-type control on YPD. It is interesting to note that the strain was not tested on YPGal. In this work, *RIM11* is isolated as a multicopy suppressor of the salt-sensitivity of CKII regulatory subunit mutants when grown on media with galactose as the sole carbon source. It is also shown here that *rim11*Δ mutants are salt-sensitive, specifically on YPGal and not YPD. The salt-sensitivity of *rim11*Δ mutants is found to be additive with that of *ckb*Δ mutants, suggesting that the Rim11 and CKII kinases do not

operate in the same biochemical pathway. *RIM11* overexpression is found to improve the salt-tolerance of the wild-type strain, as well as CKII and calcineurin mutants. Finally, Rim11 protein levels are shown to be induced upon exposure to NaCl and diminished when cells are grown on glucose media; this is offered as at least a partial explanation of the peculiar function of *RIM11* as a galactose-specific promoter of salt-tolerance.

Materials and Methods

Strains, Plasmids, and Media

Escherichia coli strain DH5α (Clonetech) was used to propagate yeast shuttle plasmids (harvested using the QIA prep kit by Qiagen) and in routine molecular cloning. *E. coli* was grown in Luria Broth at 37°C with 50 μg/ml ampicilin as needed (Ausubel et al., 1987). The *Saccharomyces cerevisiae* strains used in this work are listed in Table 1, along with their relevant chromosomal and plasmid genotypes. The plasmids used in this work are listed and described in Table 2. Yeast strains were routinely grown in rich glucose (YPD) or galactose (YPGal) media at 30°C. Minimal glucose medium lacking the appropriate component(s) was used for selection of transformed yeast strains (Ausubel et al., 1987).

Multicopy Suppressor Screen

A yeast genomic library prepared in the multicopy plasmid YEp24 (Carlson and Botstein, 1982) was screened for plasmids that would suppress the salt-sensitivity of YAPB10-2C, a yeast strain with null mutations in *CKB1* and *CKB2*. Due to a high number of spontaneous salt-resistant revertants, a two-step selection process was used to isolate suppressor plasmids. After transformation of YAPB10-2C with the genomic library, cells were plated on minimal medium lacking uracil to select for YEp24, which contains the *URA3* gene. The cells were allowed to grow for four days at 30°C, at which time those colonies containing YEp24 had grown to an appreciable size. The transformants were plated on forty such plates, and grew to a density of a few hundred colonies per plate. These colonies were pooled in 50 ml of liquid medium lacking uracil, diluted and washed with water, and replated on twenty plates containing rich galactose medium supplemented with 500 mM NaCl at a density of 1-2 million cells per plate.

Galactose medium was used because the salt-sensitive phenotype of YAPB10-2C is more evident on these plates than on plates that have glucose as the carbon source. Plates were incubated for four days at 30^{o}C, at which time roughly 10-20 colonies of various sizes were visible per plate, a number that does not include thousands of tiny colonies that were also visible. Fifty-seven colonies of various sizes were picked, and the plasmids from twenty of the fifty-seven were isolated and transformed into *E. coli* strain DH5α. These twenty plasmids were transformed back into YAPB10-2C and into the isogenic wild-type strain, YPH499. The salt-resistant phenotype provided by all twenty plasmids was then confirmed by a salt-sensitivity assay (see below). Two sequencing runs were performed on the twenty plasmids by the Molecular Genetics Research Facility at the University of Georgia, one at the front and one at the back of each insert. The identity and complete sequence of each suppressor insert was revealed using a BLAST search against the *S. cerevisiae* genome, using the two sequencing runs as starting and ending points. The part of each insert responsible for suppression was determined through a series of restriction digests and ligation reactions. The primers used for sequencing were named YEp24-Up (5' AGTCACTATGGCGTGCTGCT 3') and YEp24-Down (5' ATACCCACGCCGAAACAGC 3').

Salt Sensitivity Assays

Cells were grown overnight in the appropriate rich medium (typically YPGal) without added salt to an OD_{600} of around 1, or mid-log phase. At this point, cells were diluted in water in an attempt to get an equal number of cells in an equal volume for all the strains being tested. A standard curve to convert OD to cell number was created using a hemacytometer, and the equation of this curve ($y = 4*10^{-5}x$, where y is OD_{600} and x is number of cells per μl) was used in all subsequent assays. Cells were spotted on the appropriate rich medium supplemented with salt (typically YPGal + 500 mM NaCl) in three dilutions per strain. This resulted in three spots containing roughly 500, 200, and

100 cells per spot in 12-25 μl of water per strain. Cells were grown at 30°C for a varying length of time, typically four days, although for as little as three days or as many as seven. This was necessary because the growth rate of *S. cerevisiae* can change greatly depending on media conditions, including carbon source and concentration of salt.

Plasmid Construction

Plasmids pCD1 and pCD2 were isolated in a search for multicopy suppressors of the salt sensitivity of CKB mutants, which is described later. pCD3, 4, and 5 were created from pCD2 (and pRS424, in the case of pCD5) in an attempt to identify the suppressor. This process is illustrated in Figure 1. Briefly, pCD3 was created by cutting pCD2 with BsiWI, excising the resulting fragment, and religating the plasmid with T4 DNA ligase. pCD4 was constructed similarly using SalI instead of BsiWI. pCD5 was created by cutting pCD2 with both SacI and SalI, and subcloning the RIM11-containing fragment into pRS424. pCD6 and pCD7 were created during the same process, but using a different method. The *RIM11* and *YMR140w* genes on pCD2 were amplified by PCR and given KpnI and BamHI sites on their ends. These fragments were then subcloned into pRS424, creating pCD6 and pCD7, respectively. The enzymes utilized in plasmid construction are all from Promega or New England Biolabs. Enzymes and nucleotides were removed from DNA preparations using the Wizard DNA Clean-Up system (Promega). The Geneclean II kit (Bio101) was used to isolate DNA fragments from agarose gels.

Strain Construction

In general, the strains created in this study were constructed by transformation of a parent strain with the plasmid indicated. These transformations were performed by the lithium acetate method (Gietz et al., 1995). Yeast strains YCD15-16 and YCD17-18 were constructed by PCR-based disruption from start to stop of the *RIM11* and *MIG1*

genes, respectively. The *KanMX4* module was used for both disruptions (Wach et al., 1994). The primers used to construct YCD15-16 were named RIM11KO-Up (5' TTTTCTTTCTGGCGCATTGCATTTTAACTTTTTTTCCCGTACGCT GCAGGTCGAC 3') and RIM11KO-Down (5' ATATATGTTCCTTCCTTCTCCCATT ATTCTTGCCTGGGCTATCGATGAATTCGAGCTC 3'). The primers used to consctuct YCD17-18 were named MIG1-FOR-KO (5' CGAGAGTTGAGTATAGT GGAGACGACATACTACCATAGCCCGTACGCCTGCAGGTCGAC 3') and MIG1-REV-KO (5' TCTTTTGATTTATCTGCACCGCCAAAAACTTGTCAGCGTAATCG ATGAATTCGAGCTCG 3').

β-Galactosidase Assay

Strains were grown overnight in 4 ml of YPGal at 30^{o}C. When the cultures reached mid-log phase (OD_{660} of 0.5-1.0), each was divided into two 2 ml aliquots. 5 M NaCl was added to one of the aliquots to a final concentration of 400 mM, and sterile water was added to the other. After a 30 minute incubation at 30^{o}C, β-galactosidase activity was determined using the Yeast β-Galactosidase Assay Kit from Pierce. Briefly, aliquots of the cell cultures were added to an equal volume of the working reagent (including the Y-PER™ protein extraction reagent) and allowed to incubate at room temperature for 30 minutes. The reaction was stopped by addition of 1 M Na_2CO_3, and cell debris was removed by centrifugation. The OD_{420} of the supernatant was recorded, and β-galactosidase activity in Miller units was determined using the following formula: $1000 \times OD_{420} / (t \times V \times OD_{660})$, where t = time in minutes of the reaction, and V = volume of cells used.

Western Blot Analysis of RIM11 Expression

A single colony of the yeast strain YCD13 (YAPB10-2C transformed with the multicopy vector pKB166) was grown overnight in 5 ml of URA^{-} medium at 30^{o}C. The

next day this culture was split into four aliquots and diluted with 25 ml of YPD, YPGal, YPD + 300 mM NaCl, and YPGal + 300 mM NaCl. The cultures were then grown to early log phase before an aliquot (5 A_{600} units) from each was collected by centrifugation and resuspended in sterile deionized water to a final volume of approximately 100 µl. Each cell suspension was mixed rapidly with 100 µl of 2x SDS-PAGE sample buffer that had been heated to 100^{o}C, vortexed, and incubated at 100^{o}C for five minutes longer. After heat treatment, the samples were centrifuged at 11,000 x g for 10 minutes, and the clarified supernatants were stored at -20^{o}C. Protein concentrations were determined with the Bio-Rad Protein Assay Kit. Following SDS-PAGE (10%) and transfer to a PVDF membrane, immunodetection was performed using the Bio-Rad Immun-Star Goat Anti-Mouse IgG Detection Kit. The primary antibody used was Anti-HA mouse monoclonal antibody, clone 12CA5 (Boehringer Mannheim).

Table 1. *S. cerevisiae* strains

Strain	Relevant Chromosomal Genotype	Plasmid	Source
YPH499	*MAT***a**	—	(Sikorski and Hieter, 1989)
YAPB10-2C	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	—	(Bidwai et al., 1995)
MCY3-1C	*MAT***a** *cnb1Δ1::LEU2*	—	(Cyert and Thorner, 1992)
YKAT1	*MAT***a** *ura3-52::ENA1-lacZ::URA3*	—	(Tenney and Glover, 1999)
YKAT2	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2 ura3-52::ENA1-lacZ::URA3*	—	(Tenney and Glover, 1999)
YKAT5	*MAT***a** *cnb1Δ1::LEU2 ura3-52::ENA1-lacZ::URA3*	—	(Tenney and Glover, 1999)
YKAT1-1	*MAT***a** *ura3-52::ENA1-lacZ::URA3*	pRS424	This study
YKAT2-1	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2 ura3-52::ENA1-lacZ::URA3*	pRS424	This study
YKAT5-1	*MAT***a** *cnb1Δ1::LEU2 ura3-52::ENA1-lacZ::URA3*	pRS424	This study
YKAT1-2	*MAT***a** *ura3-52::ENA1-lacZ::URA3*	pCD6	This study
YKAT2-2	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2 ura3-52::ENA1-lacZ::URA3*	pCD6	This study
YKAT5-2	*MAT***a** *cnb1Δ1::LEU2 ura3-52::ENA1-lacZ::URA3*	pCD6	This study
YCD1	*MAT***a**	YEp24	This study
YCD2	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	YEp24	This study
YCD3	*MAT***a** *cnb1Δ1::LEU2*	YEp24	This study
YCD4	*MAT***a**	pCD1	This study
YCD5	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	pCD1	This study
YCD6	*MAT***a** *cnb1Δ1::LEU2*	pCD1	This study
YCD7	*MAT***a**	pRS-1067	This study
YCD8	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	pRS-1067	This study
YCD9	*MAT***a** *cnb1Δ1::LEU2*	pRS-1067	This study
YCD10	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	pRS424	This study
YCD11	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	pCD6	This study

YCD12	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	pCD7	This study
YCD13	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	pKB166	This study
YCD14	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2*	pKB199	This study
YCD15	*MAT***a** *rim11Δ1::KanMX4*	—	This study
YCD16	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2 rim11Δ1::KanMX4*	—	This study
YCD17	*MAT***a** *mig1Δ1::KanMX4*	—	This study
YCD18	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2 mig1Δ1::KanMX4*	—	This study
YCD19	*MAT***a** *mig1Δ1::KanMX4*	YEp24	This study
YCD20	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2 mig1Δ1::KanMX4*	YEp24	This study
YCD21	*MAT***a** *mig1Δ1::KanMX4*	pCD1	This study
YCD22	*MAT***a** *ckb1Δ1::HIS3 ckb2Δ1::LEU2 mig1Δ1::KanMX4*	pCD1	This study

Table 2. Plasmids

Plasmid	Backbone	Description	Source
YEp24	—	2μ *URA3*	(Christianson et al., 1992)
pRS424	—	2μ *TRP1*	(Christianson et al., 1992)
pCD1	YEp24	2μ *URA3* 6861 bp insert from Chrom. XIII including *RIM11*	This study
pCD2	YEp24	2μ *URA3* 7958 bp insert from Chrom. XIII including *RIM11*	This study
pCD3	YEp24	2μ U*RA3* BsiWI fragment excised from pCD2	This study
pCD4	YEp24	2μ *URA3* SalI fragment excised from pCD2	This study
pCD5	pRS424	2μ *TRP1* SacI-SalI fragment kept from pCD2	This study
pCD6	pRS424	2μ *TRP1 RIM11*	This study
pCD7	pRS424	2μ *TRP1 YMR140w*	This study
pKB166	pRS426	2μ *URA3* P_{RIM11}-HA-*RIM11*	(Bowdish et al., 1994)
pKB199	pRS426	2μ *URA3* P_{RIM11}-HA-*rim11K68A*	(Bowdish et al., 1994)
pRS-1067	YEp352	2μ *URA3 HAL3*	(Ferrando et al., 1995)

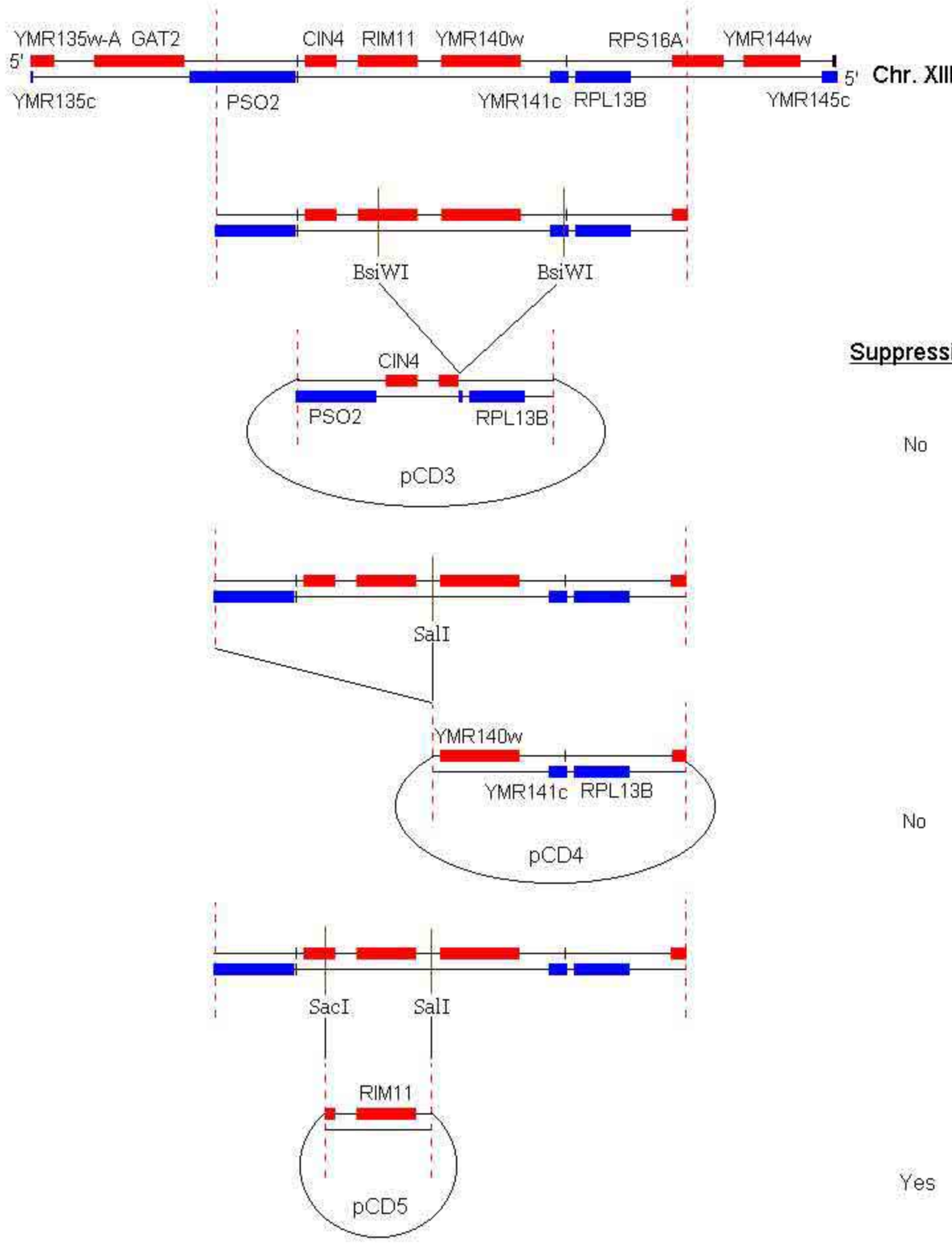
Results

Multicopy Suppressor Screen

In a search for suppressors of the salt-sensitive phenotype of CKII regulatory subunit mutants, YAPB10-2C, a *ckb1*Δ, *ckb2*Δ mutant, was transformed with a yeast multicopy genomic library and screened in a two-step selection process. The transformants were first grown on minimal media lacking uracil to select for the library-containing plasmid, pooled, and later examined for salt resistance while growing on rich galactose media supplemented with 500 mM NaCl. Twenty plasmids were isolated from separate colonies exhibiting rapid growth on this medium, although due to the pooling process it is unlikely that all of these represent independent transformants. Two sequencing runs were performed on the twenty suppressor plasmids by the Molecular Genetics Research Facility at the University of Georgia, one at the front and one at the back of each insert. The entire sequence of each insert was revealed using a BLAST search against the *S. cerevisiae* genome, using the two sequencing runs as starting and ending points. Nineteen of the twenty suppressor plasmids were shown to carry the same 6861 nucleotide stretch of DNA from chromosome XIII, including the genes *PSO2*, *CIN4*, *RIM11*, *RPL13B*, and the unknown ORF's *YMR140w* and *YMR141c*. The first of these plasmids was named pCD1. The last suppressor insert to be sequenced was somewhat larger (7958 nt), containing the same six genes as on pCD1, and part of *RPS16A* as well. This plasmid was named pCD2. Since these plasmids have nearly identical sequences, the search for new suppressors was ended so that analysis of the suppressor(s) already found could begin.

pCD2 was subjected to a series of restriction digests and ligation reactions (see Figure 1) in an attempt to determine the minimum length of DNA required for the suppression event. First, pCD2 was digested with BsiWI to remove most of *RIM11*,

Figure 1. Identification of *RIM11* as the suppressor gene on pCD2. The original suppressor clone is derived from chromosome XIII between the red dashed lines. This clone was cut with BsiWI, SalI, and SalI-SacI to create the plasmids pCD3, pCD4, and pCD5 respectively. Only pCD5 exhibited the same ability to suppress salt-sensitive mutations as the original clone. The red and blue rectangles represent ORF's present on the Watson and Crick DNA strands.


YMR135w-A
GAT2
CIN4
RIM11
YMR140w
RPS16A
YMR144w
5'
5'
Chr. XIII
YMR135c
PSO2
YMR141c
RPL13B
YMR145c
BsiWI
BsiWI
CIN4
PSO2
RPL13B
pCD3
Suppression
No
SalI
YMR140w
YMR141c
RPL13B
pCD4
No
SacI
SalI
RIM11
pCD5
Yes

YMR140w, and *YMR141c*. This construct, when retransformed into YAPB10-2C, failed to confer salt tolerance on YPGal + 500mM NaCl plates. Next, pCD2 was cut with SalI to remove all of *PSO2*, *CIN4*, and *RIM11*, leaving only the two unknown ORF's, *RPL13B*, and a small fragment of *RPS16A*. The resulting plasmid, pCD4, also failed to suppress the salt-sensitive phenotype of YAPB10-2C. Finally, pCD2 was cut with both SacI and SalI to remove a fragment which included only *RIM11* and a tiny portion of *CIN4*. This fragment was subcloned into pRS424, and the resulting plasmid was named pCD5. Upon retransformation into YAPB10-2C, pCD5 conferred as much resistance to salt as the entire suppressor plasmid pCD2. However, it was still somewhat unclear if *RIM11* was indeed the gene responsible for the suppression effect because the construct used to separate *YMR140w* from *RIM11* (pCD4) unfortunately removed part of the assumed promoter region for *YMR140w*. In order to solve this problem, both potential suppressors were amplified by PCR and subcloned into pRS424 to create plasmids pCD6 and pCD7. The plasmid with *RIM11* (pCD6) showed suppression activity on YPGal + 500 mM NaCl plates, whereas the plasmid with *YMR140w* (pCD7) did not. In a final experiment to comfirm these results, pKB166 and pKB199 were kindly given to us by Dr. Aaron Mitchell, and they were transformed into YAPB10-2C. pKB166 contains the wild-type allele of *RIM11*, and pKB199 contains the K68A allele of RIM11, a catalytically-inactive mutant (Bowdish et al., 1994). pKB166 suppressed the salt-sensitivity of YAPB10-2C, as expected, but pKB199 did not, therefore confirming that *RIM11* is the gene responsible for the suppression conferred by pCD1 and pCD2 (data not shown). Another important result from this experiment is that the catalytic activity of *RIM11* is required for the suppression event, not just its physical presence.

Characterization of *RIM11* as a Salt-Tolerance Gene

RIM11 provides YAPB10-2C with a strong resistance to salt on galactose media when overexpressed, elevating this strain's tolerance to sodium to nearly wild-type

levels. However, *RIM11* is barely able to provide any noticable salt-resistance at all on glucose media, as is shown in Figure 2. Although surprising, this result is consistent with previous literature on the subject, in which experiments were performed on media with glucose as the carbon source. For example, *RIM11* was assumed to be uninvolved in salt-tolerance after a *rim11*Δ mutant strain was found to be as salt-resistant as the isogenic wild-type on YPD (Piao et al., 1999). Once *RIM11* was connected to salt-tolerance, several questions arose regarding the nature of the tolerance it gives, and the focus of this work began to shift towards characterizing *RIM11* in its new role as a halotolerance gene.

RIM11 confers salt-tolerance on galactose media exceeding that provided by a previously known halotolerance gene, *HAL3*, when both are present in multicopy. On glucose media, the situation is reversed; *HAL3* now provides a high degree of resistance, whereas *RIM11* is almost completely ineffective. Although *RIM11* was isolated as a multicopy suppressor of *ckb*Δ mutants, the suppression effect can also be seen in MCY3-1C, a *cnb1*Δ strain, and it even confers the wild-type some additional resistance to salt as well (see Figure 2). Certainly, *RIM11* cannot be thought of as a CKII-specific suppressor, although it will suppress the salt-sensitivity of CKII mutants.

Null alleles of *RIM11* were generated by PCR-based gene disruption in a *ckb1*Δ, *ckb2*Δ strain as well as the isogenic wild-type strain. As shown in Figure 3, both resulting strains were found to be sensitive to sodium on galactose, but not glucose, media; correlating well with the overexpression data decribed above. This effect was small, but reproducible. The *ckb1*Δ, *ckb2*Δ, *rim11*Δ strain is very sensitive, and is recognizably more sensitive than the parent *ckb1*Δ, *ckb2*Δ strain at salt concentrations as low as 250 mM. The wild-type strain's general salt-resistance masks the effect of the *rim11*Δ mutation until salt concentrations reach about 700 mM, at which point the difference in sensitivity between wild-type and *rim11*Δ become obvious.

Although the *in vivo* relevance of overexpression data alone seems tenuous, when combined with the deletion results it becomes clear that *RIM11* is directly involved in a

Figure 2. *RIM11* is a suppressor of salt-sensitive mutations that is unable to function on glucose-containing media. YPH499 (wild-type) and two salt-sensitive mutants were grown on glucose or galactose media in the presence or absence of NaCl. All three strains had been previously transformed with either an empty plasmid or a halotolerance gene on a multicopy vector. The halotolerance gene was either *HAL3* or *RIM11*, each under the control of their own promoters. All strains were grown at 30^{o}C for the amount of time indicated.

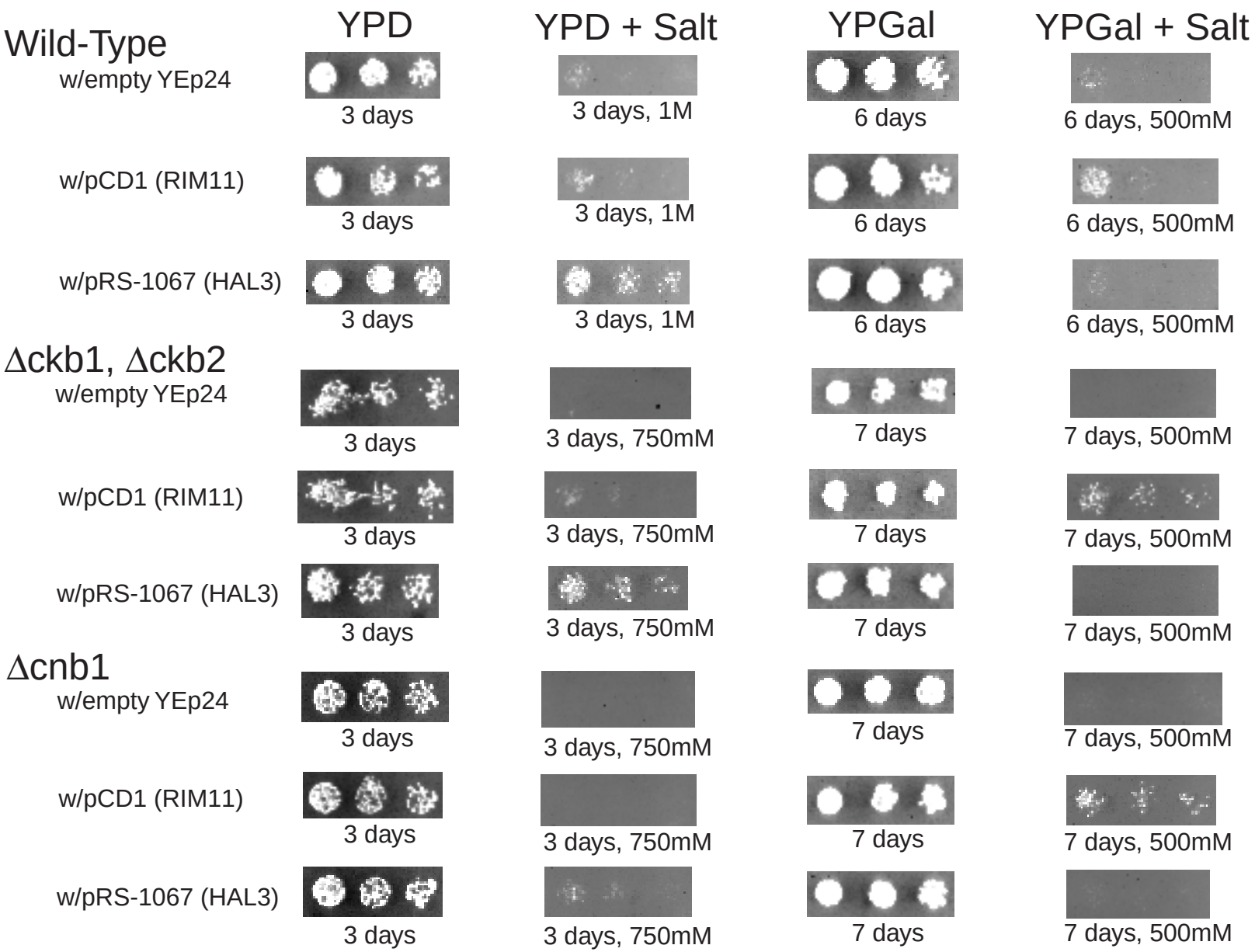
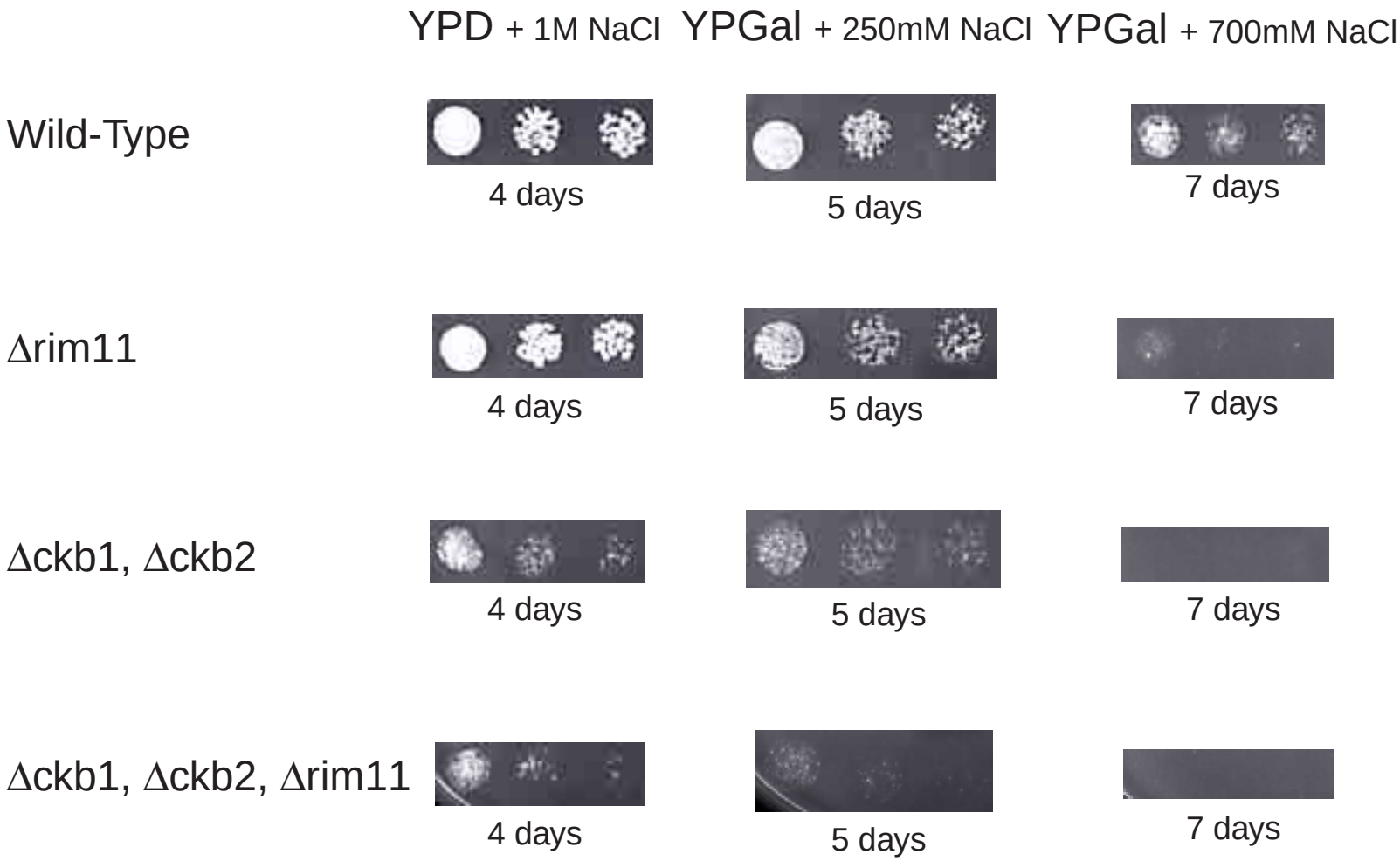

YPD
YPD + Salt
YPGal
YPGal + Salt
Wild-Type
w/empty YEp24
3 days
3 days, 1M
6 days
6 days, 500mM
w/pCD1 (RIM11)
3 days
3 days, 1M
6 days
6 days, 500mM
w/pRS-1067 (HAL3)
3 days
3 days, 1M
6 days
6 days, 500mM
Δckb1, Δckb2
w/empty YEp24
3 days
3 days, 750mM
7 days
7 days, 500mM
w/pCD1 (RIM11)
3 days
3 days, 750mM
7 days
7 days, 500mM
w/pRS-1067 (HAL3)
3 days
3 days, 750mM
7 days
7 days, 500mM
Δcnb1
w/empty YEp24
3 days
3 days, 750mM
7 days
7 days, 500mM
w/pCD1 (RIM11)
3 days
3 days, 750mM
7 days
7 days, 500mM
w/pRS-1067 (HAL3)
3 days
3 days, 750mM
7 days
7 days, 500mM

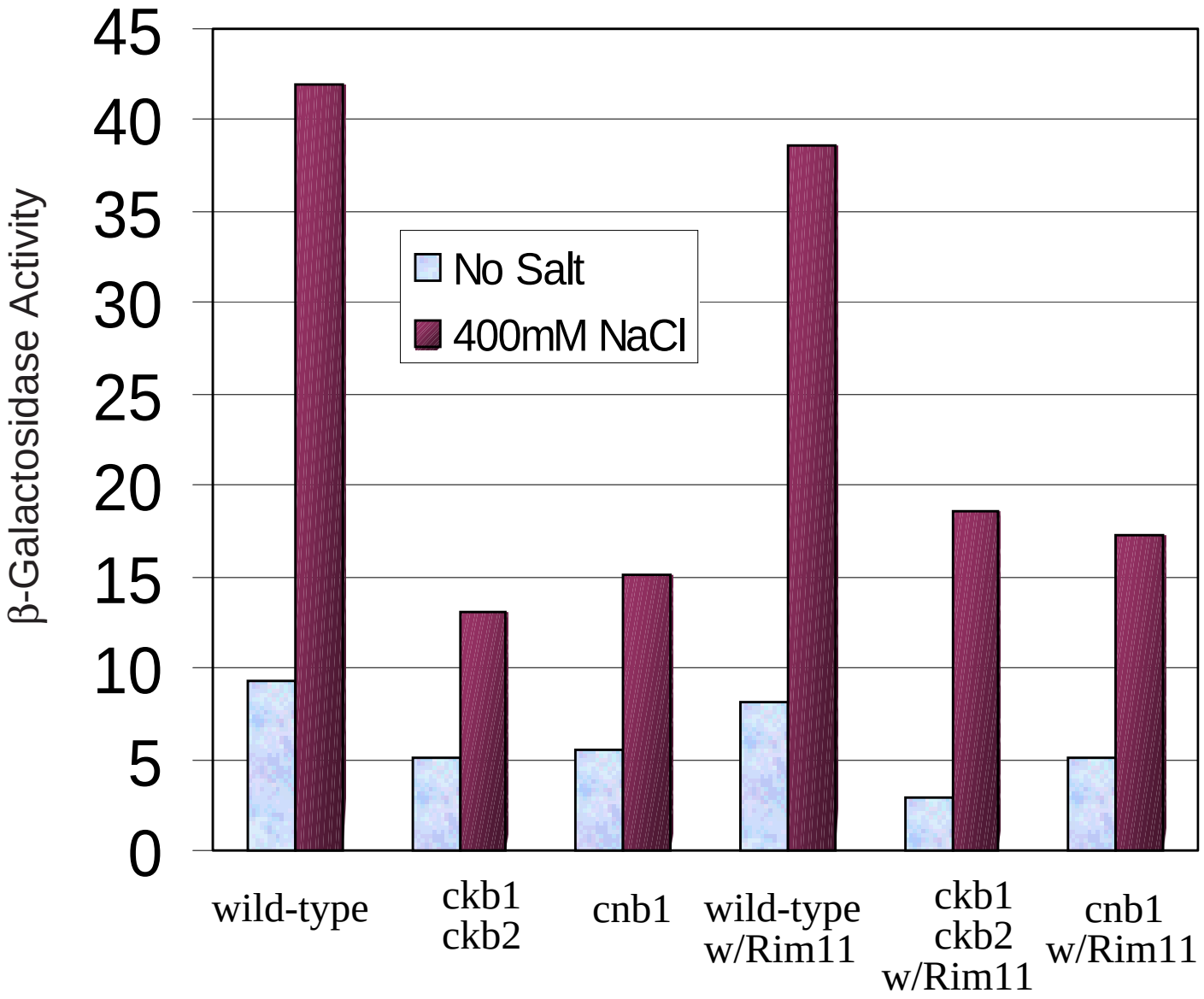
Figure 3. *RIM11* disruption mutants are salt-sensitive on galactose media, but not glucose media. Two different *rim11*Δ mutants and their parent strains (YPH499 and YAPB10-2C, a *ckb1*Δ, *ckb2*Δ strain) were grown on glucose or galactose media at various salt concentrations. All strains were grown at 30^{o}C for the amount of time indicated.


YPD + 1M NaCl
YPGal + 250mM NaCl
YPGal + 700mM NaCl
Wild-Type
4 days
5 days
7 days
Δrim11
4 days
5 days
7 days
Δckb1, Δckb2
4 days
5 days
7 days
Δckb1, Δckb2, Δrim11
4 days
5 days
7 days

salt-tolerance pathway of some kind. The sodium pump encoded by *ENA1* is tightly regulated at the transcriptional level by a number of different salt and osmotic stress resistance pathways and is one of the most important determinants of salt-resistance in *Saccharomyces*. Since a gene closely related to *RIM11, MCK1,* has been previously shown to regulate *ENA1* expression levels (Piao et al., 1999), it seemed plausible that *RIM11* could be involved in the same process. This hypothesis was tested by use of plasmid pFR70i, which has the promoter of *ENA1* linked to a *lacZ* reporter gene, so that *lacZ* expression is completely controlled by *ENA1* signals (Mendoza et al., 1994). Yeast strains containing one integrated copy of pFR70i (YKAT1, YKAT2, and YKAT5; Tenney and Glover, 1999), were transformed with *RIM11* on an overexpression plasmid (pCD6) or with the empty vector (pRS424). This allowed the detection of *ENA1* transcription levels in these strains, as well as any potential alterations caused by addition of salt or overexpression of *RIM11*, as seen in Figure 4. Consistent with previous results, both the *ckb1*Δ, *ckb2*Δ, and *cnb1*Δ strains are deficient in basal and salt-induced *ENA1* transcription levels, although *ENA1* levels are still inducible to some degree. However, *RIM11* overexpression does not seem to affect β-galactosidase activity significantly at all, for any of the three strains. Apparently, neither basal nor salt-induced *ENA1* transcription levels are changed by the volume of *RIM11* expression. This provides support for the idea that *RIM11* is involved in a salt-tolerance pathway that does not regulate *ENA1* at the transcriptional level.

In addition to NaCl, yeast cells with varying levels of *RIM11* expression were tested with other salts and stresses to futher characterize the physiological role of *RIM11*. A *ckb*Δ mutant strain (YAPB10-2C) transformed with empty vector (YEp24) or with a *RIM11*-overexpression plasmid (pCD1), along with the appropriate wild-type strain (YCD1), were exposed to media containing either 1.5 M sorbitol, 1 M KCl, or 100 mM $CaCl_2$, as well as media at pH 4 through pH 9 in separate experiments. On glucose or galactose media with the above additives, *RIM11*-overexpression was unable to prevent

Figure 4. *RIM11* expression level does not affect *ENA1* expression level. Three yeast strains (YPH499, YAPB10-2C, and MYC3-1C) with an integrated *ENA1-lacZ* reporter construct were transformed with pCD1, thereby increasing the copy number of *RIM11* in these strains. All six resulting strains were used in a β-galactosidase assay to determine the level of expression of *ENA1*. Before the assay, all strains were grown overnight in 4 ml of YPGal at 30^{o}C and split into two aliquots. One aliquot of each strain was induced by raising the NaCl concentration to 400 mM NaCl for another thirty minutes.


β-Galactosidase Activity
45
40
35
30
25
20
15
10
5
0
No Salt
400mM NaCl
wild-type
ckb1 ckb2
cnb1
wild-type w/Rim11
ckb1 ckb2 w/Rim11
cnb1 w/Rim11

any reduction in growth rate that may have occured in the CKII-mutant strain. Similarly, deletion of *RIM11* did not hinder growth any further under these conditions in either strain background (data not shown). Thus, the suppression conferred by overexpressing *RIM11* is probably not due to osmotic stabilization, or to a generic method of resisting toxic cations.

Thus far, the salt-resistance conferred by overexpressing *RIM11* seems specific to sodium, but not to any particular strain background. When overexpressed, *RIM11* is able to improve the salt tolerance of CKII mutants, calcineurin mutants, and also the corresponding wild-type strain. Another characteristic feature of *RIM11* in this context is its inability to function on glucose media, a peculiarity that merited further research. It was not clear at this point whether the effect caused by *RIM11* was glucose-repressed, galactose-activated, or perhaps some combination of the two. In order to distinguish between these possibilities, several salt sensitivity assays were performed with different sugars as the carbon source in each. The sugars chosen in the first set of these experiments were glucose (2%), galactose (2%), and glucose/galactose (2% of each, 4% total). As Figure 5 illustrates, the wild-type strain grows well on all three types of media, even when added salt is present, though taking a few days longer to reach the same colony size with galactose as the sole carbon-source[5]. The *ckb1*Δ, *ckb2*Δ strain grows very poorly on each type of media which contains added salt, but is rescued by *RIM11* on an overexpression plasmid (pCD1) when glucose is not present. These data suggest that the suppression effect caused by *RIM11* is glucose-repressed, not galactose-activated.

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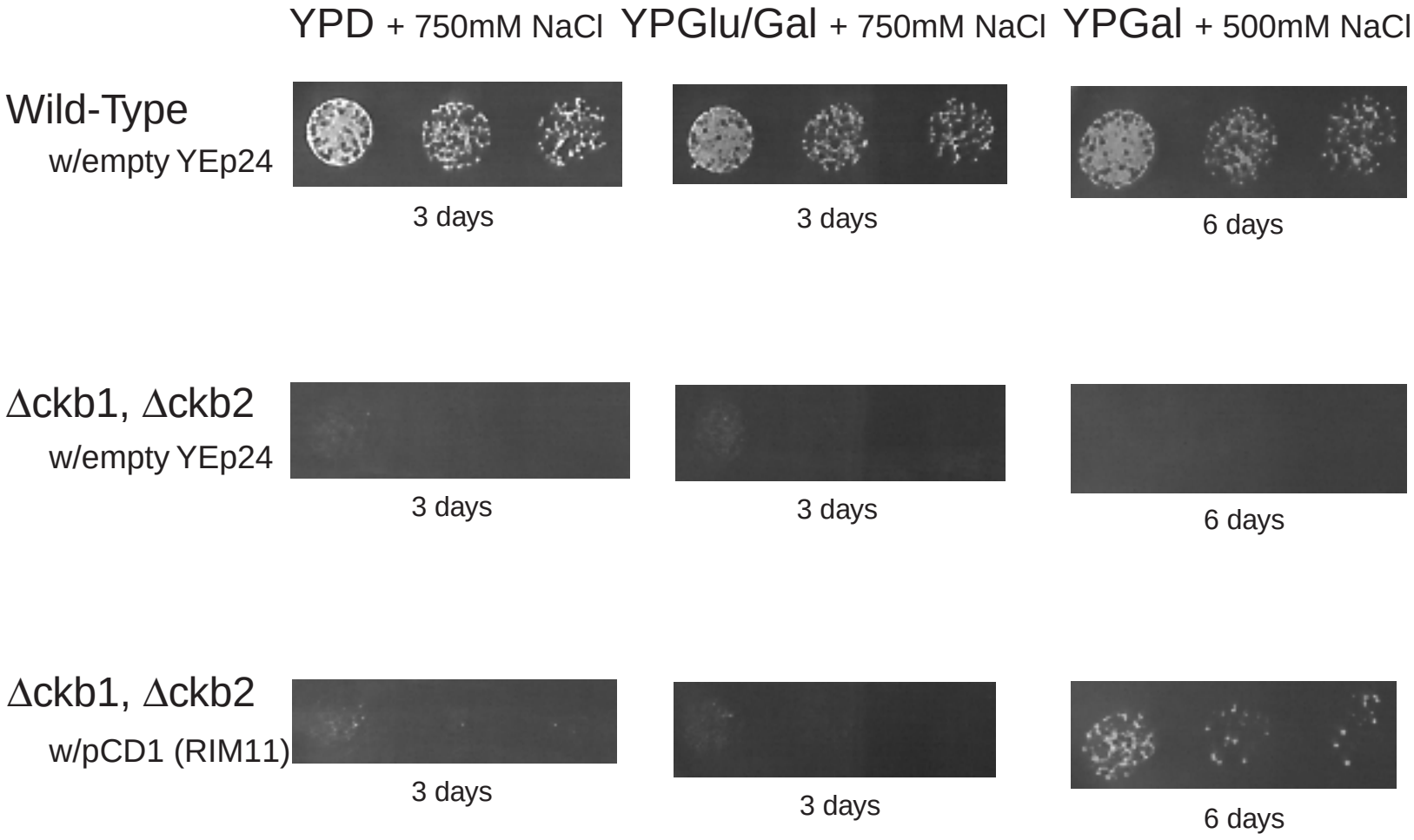
[5] A change in the number of days the strains were allowed to grow on different types of media was required in order to keep colony size roughly constant. In the same way, a change in salt concentration was required to maintain the apparent difference in sensitivity between the wild-type and *ckb1*Δ, *ckb2*Δ mutant on the different carbon sources. For example, the strains used in this study are more sensitive to salt when using galactose as a carbon source than when they utilize glucose.

Figure 5. The effect of *RIM11* on salt tolerance appears to be glucose-repressed. YAPB10-2C, a salt-sensitive *ckb1*Δ, *ckb2*Δ strain, was transformed with empty YEp24 or pCD1 and grown together with wild-type (also possessing empty YEp24) on 2% glucose media, 2% galactose media, or media containing 2% of both sugars. All strains were grown at 30^{o}C for the amount of time indicated at the salt concentration indicated.


YPD + 750mM NaCl
YPGlu/Gal + 750mM NaCl
YPGal + 500mM NaCl
Wild-Type
w/empty YEp24
3 days
3 days
6 days
Δckb1, Δckb2
w/empty YEp24
3 days
3 days
6 days
Δckb1, Δckb2
w/pCD1 (RIM11)
3 days
3 days
6 days

If *RIM11* is glucose-repressed, then by definition it should be able to function on any media in which glucose is not present. In order to clarify the situation, *RIM11* was tested on several different carbon-sources to see if it could still suppress the salt sensitivity of a *ckb1*Δ, *ckb2*Δ strain. The carbon-sources tested include glycerol (3%), raffinose[6] (2%), mannose (2%), and a combination of glycerol and galactose (3%/2%). As shown in Figure 6, *RIM11* overexpression is unable to suppress the salt sensitivity of a *ckb1*Δ, *ckb2*Δ mutant on glycerol medium, although the strain's poor growth rate on this medium even without salt (not shown) makes any conclusion difficult. If 2% galactose is added to this same medium, *RIM11* overexpression is again able to confer salt resistance, suggesting that *RIM11* may indeed be activated by galactose. On mannose medium, *RIM11* does seem to be able to provide some resistance to salt, but this effect is small compared to that seen on galactose-containing media. Together with those from the previous paragraph, these results demonstrate that *RIM11* is both galactose-activated and glucose-repressed.

Mig1 is a Cys_2-His_2 zinc finger protein that binds to the promoters of glucose-repressible genes, and is partially responsible for that repression (Vallier and Carlson, 1994). Under low-glucose conditions Mig1 is phosphorylated by Snf1, thereby expelling it from the nucleus and allowing transcription of the formerly repressed genes (Treitel et al., 1998). As has been discussed previously, overexpression of *RIM11* is unable to suppress salt-sensitive mutants on glucose-containing media. If this inability is caused by glucose-repression of *RIM11* or its downstream targets, it seems reasonable that it might be removed by deletion of *MIG1*, therefore allowing *RIM11* to provide salt resistance even on glucose media. *MIG1* was deleted by replacement with the *KanMX4*

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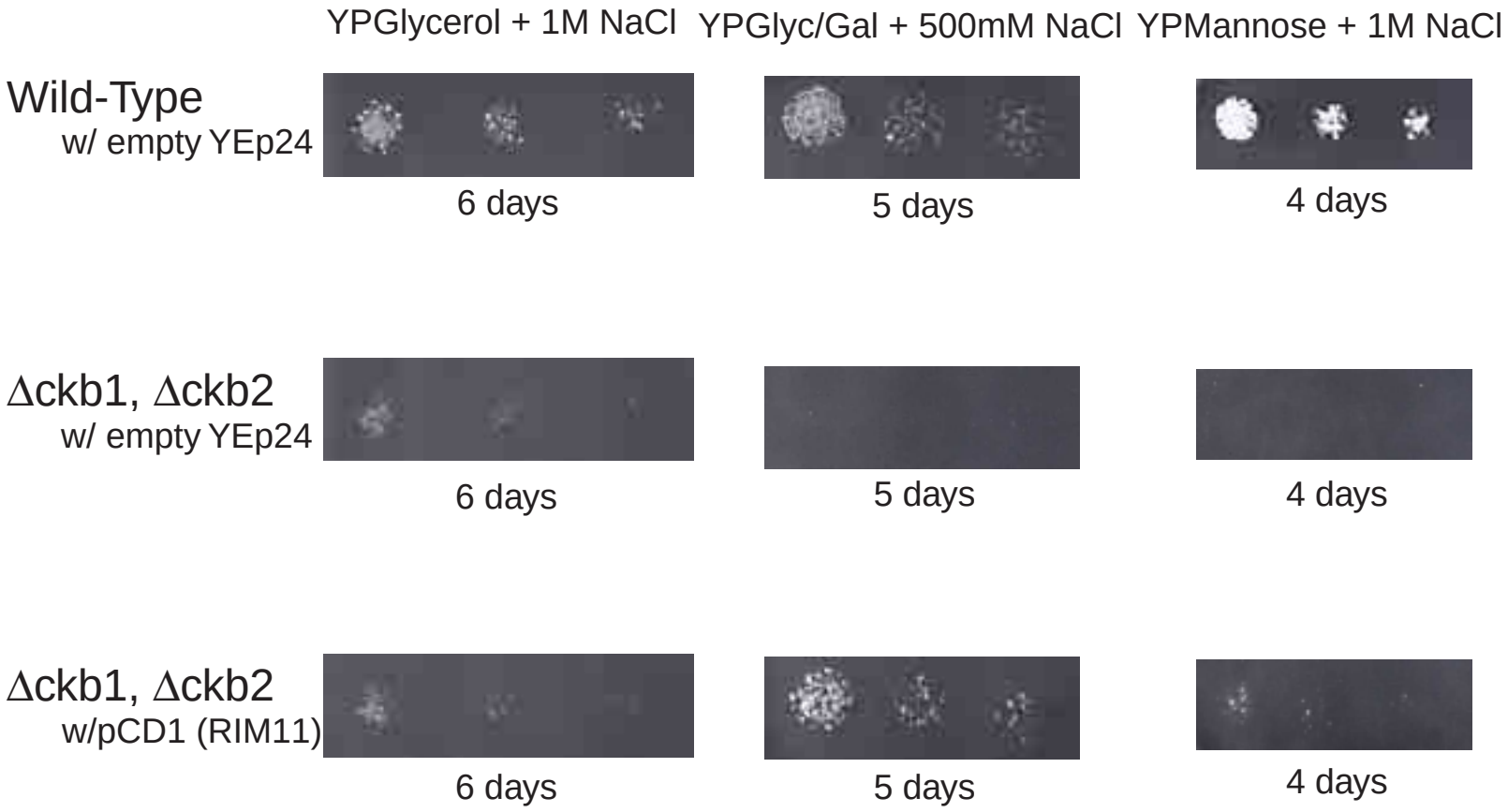
[6] The data for raffinose-containing media are not shown because it was difficult to demonstrate a difference in salt sensitivity between a *ckb*Δ mutant and the wild-type strain; both strains seemed to grow equally well on this type of medium.

Figure 6. The effect of *RIM11* on salt tolerance appears to be galactose-activated. YAPB10-2C, a salt-sensitive *ckb1*Δ, *ckb2*Δ strain, was transformed with empty YEp24 or pCD1 and grown, together with wild-type (also possessing empty YEp24), on 3% glycerol medium, 3% glycerol/2% galactose medium, or 2% mannose medium. All strains were grown at 30^{o}C for the amount of time indicated at the salt concentration indicated.


YPGlycerol + 1M NaCl
YPGlyc/Gal + 500mM NaCl
YPMannose + 1M NaCl
Wild-Type
w/ empty YEp24
6 days
5 days
4 days
Δckb1, Δckb2
w/ empty YEp24
6 days
5 days
4 days
Δckb1, Δckb2
w/pCD1 (RIM11)
6 days
5 days
4 days

module through homologous recombination in a *ckb1*Δ, *ckb2*Δ strain, and in the wild-type. The resulting triple mutant (*ckb1*Δ, *ckb2*Δ, and *mig1*Δ) was checked for salt sensitivity on YPD and YPGal media containing 1M and 500 mM NaCl, respectively. The cells were allowed to grow for six days in both cases, and the results are shown in Figure 7. *RIM11* overexpression suppresses the salt sensitivity of the triple mutant on YPGal, as expected, but on YPD *RIM11* is still unable to suppress, even though a major glucose repression pathway is not completely active. Therefore, in addition to the absence of glucose repression, *RIM11* may require another factor for its activity which results in salt tolerance. Although *MIG1* is only responsible for part of the glucose repression in *Saccharomyces*, these results provide support for the idea that *RIM11* is activated by galactose as well as being glucose-repressed.

Most sporulation genes are inactive during normal growth and only become transcribed under certain unfavorable conditions, such as the lack of an efficiently utilized carbon or nitrogen source. Although not sufficient by itself, yeast cells growing on galactose media will meet the criteria for sporulation more closely than cells growing on a better carbon source such as glucose, for example. It seems possible that a gene responsible for sporulation, if transcribed in the presence of glucose at all, would be transcribed at a higher rate when the cell is utilizing galactose, although undoubtedly at a higher rate still when growing in a true sporulation medium. Thus, it could be that the inability of *RIM11* to suppress a salt-sensitive mutant on YPD, even when overexpressed, is due to a simple lack of the enzyme itself under these conditions. In order to check this, the overexpression plasmid pKB166 (Bowdish et al., 1994) containing a wild-type *RIM11* gene fused to an HA tag was transformed into the yeast strain YAPB10-2C. The addition of this plasmid suppressed the strains' sensitivity to salt on galactose media, appearing nearly identical to the suppression given by pCD1 (data not shown). A Western blot was performed to check Rim11 protein levels after growth on glucose, glucose + 300mM NaCl, galactose, and galactose + 300mM NaCl. The results are shown

in Figure 8, where it is evident that Rim11 protein levels are affected by both salt concentration and carbon source. Rim11 protein levels are induced by salt on both carbon sources, but are higher in the case of galactose, both with and without salt.

Figure 7. *RIM11* is unable to suppress the salt-sensitive phenotype of *ckb1*Δ, *ckb2*Δ mutants on glucose media, even in the absence of *MIG1*. *MIG1* was disrupted in a *ckb1*Δ, *ckb2*Δ strain background, and the resulting strain was transformed with either empty YEp24 or pCD1. These two strains were grown alongside their parent strains (which still possess a wild-type *MIG1* gene) and the wild-type control on galactose and glucose media with varying NaCl concentrations. All strains were grown at 30^{o}C for 6 days.

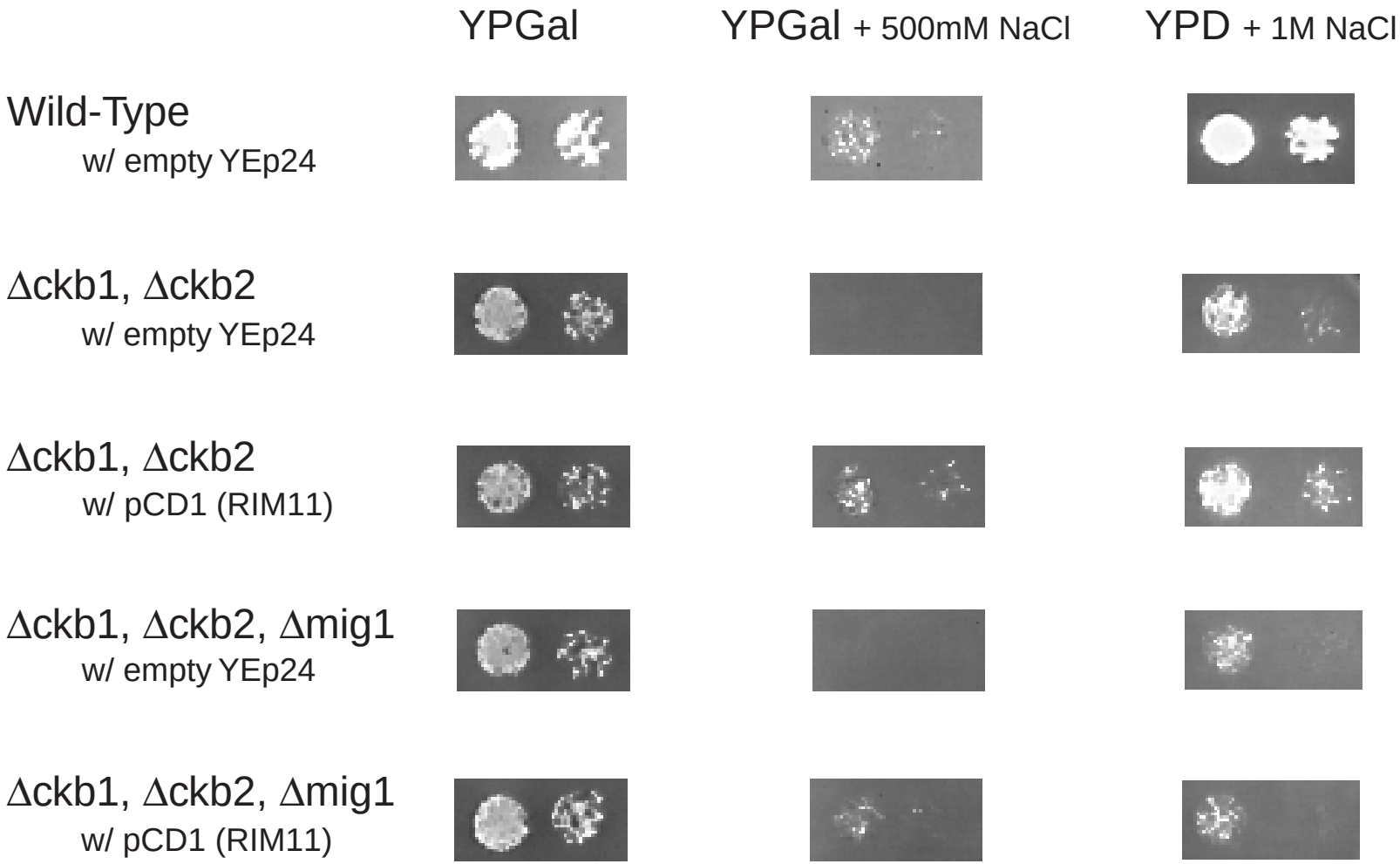
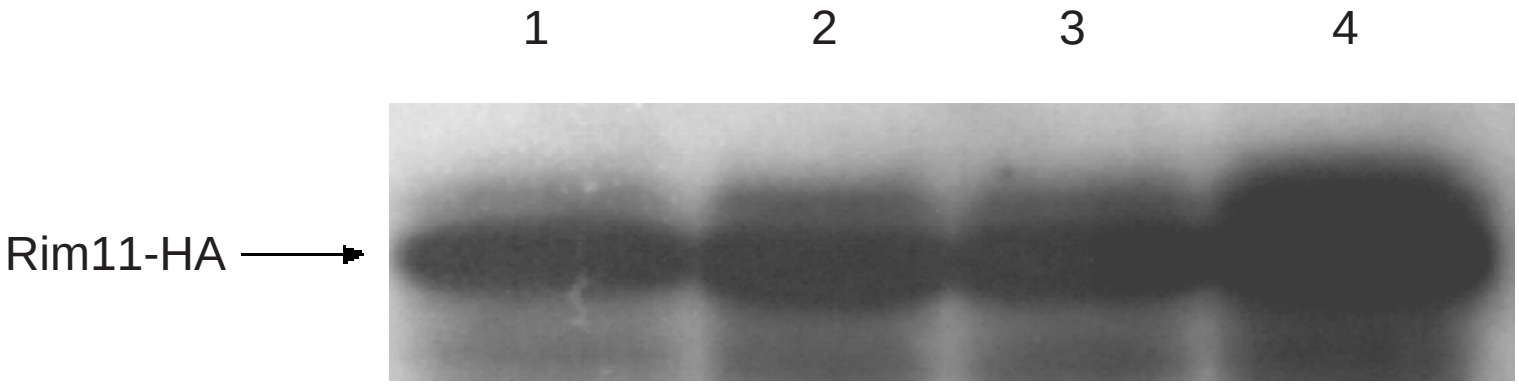

YPGal
YPGal + 500mM NaCl
YPD + 1M NaCl
Wild-Type
w/ empty YEp24
Δckb1, Δckb2
w/ empty YEp24
Δckb1, Δckb2
w/ pCD1 (RIM11)
Δckb1, Δckb2, Δmig1
w/ empty YEp24
Δckb1, Δckb2, Δmig1
w/ pCD1 (RIM11)

Figure 8. Rim11 protein levels are induced by NaCl and galactose. Yeast strain YAPB10-2C was transformed with pKB166 (containing *RIM11* fused to an HA epitope tag) to create YCD13, and this strain was subjected to western blot analysis. After being grown overnight, the cells were divided into four aliquots and grown to early log phase, each in a different medium. Lane 1 shows cells grown in YPD. Lane 2 shows cells grown in YPD + 300mM NaCl. Lane 3 shows cells grown in YPGal. Lane 4 shows cells grown in YPGal + 300mM NaCl. Anti-HA mouse monoclonal antibody clone 12CA5 was used to detect the Rim11-HA fusion protein.


1
2
3
4
Rim11-HA

Discussion

RIM11: A galactose-specific determinant of salt-tolerance

Glycogen synthase kinase-3 homologs are not generally considered to play an important role in ion homeostasis in any organism; cell fate determination and transcriptional regulation are instead considered to be their primary functions (Woodgett, 1994). Even in *S. cerevisiae*, most members of the GSK3 family are ignored in the literature discussing mechanisms of salt tolerance. Although little is known about the GSK3 homologs *MRK1* and *YOL128c*, a *mck1*Δ mutant was found to have a salt-sensitive phenotype as well as reduced *ENA1* expression levels in one study (Piao et al., 1999). Unfortunately, this observation has not yet been explored further. In this work, another GSK3 homolog in *S. cerevisiae, RIM11*, is shown to have an important role in salt tolerance when cells are grown with galactose as the main carbon source. When overexpressed, *RIM11* is able to suppress null alleles in CKII and calcineurin regulatory subunits, both being mutations that result in a high degree of salt sensitivity. The suppression conferred by *RIM11* is superior to that provided by *HAL3* on galactose media, although the situation is reversed on YPD.

Several experiments were performed in an attempt to distinguish between glucose repression and galactose activation of *RIM11*, as an explanation of the inability of *RIM11* to suppress on glucose media. Unfortunately, the results of these experiments were sometimes confusing. In general, the presence of galactose in the culture medium was necessary, but not sufficient for the suppression effect; no medium allowed for a large degree of suppression without galactose as a component, but at least one type of medium (possessing glucose and galactose combined) did not allow for suppression even though galactose was a component. Therefore, the salt-tolerance effect conferred by overexpression of *RIM11* seems to be both glucose-repressed and galactose-activated. Furthermore, it is interesting to ponder the meaning of the *MIG1* deletion experiment

shown in Figure 7. Since *RIM11* is still unable to suppress on glucose media when an important part of the machinery responsible for most glucose repression in *S. cerevisiae* was deleted, the factors causing the repression in this case become unclear. The repression of the ability of *RIM11* to confer salt tolerance by glucose may not be transcriptional in nature, or it may involve a downstream target of this kinase. Further work is necessary to resolve this issue.

One possible objection that can be raised against the conclusions of this study regards the usefulness of overexpression results in general. *RIM11* has not been previously shown to have a physiological role involving salt-tolerance, but the closely-related *MCK1* gene has been characterized in this manner (Piao et al., 1999). It is possible that, when overexpressed, Rim11 is able to phosphorylate Mck1 targets involved in salt-resistance. Thus, *RIM11* would not be involved in a salt tolerance pathway itself under physiological conditions, and the data presented here would become nothing but interesting artifacts resulting from overexpression. This scenario seems unlikely for two reasons. First, *rim11*Δ mutants are found to be salt-sensitive only when grown on galactose media, in agreement with the overexpression results. This observation shows that *RIM11* is important for salt-tolerance even at normal expression levels. Secondly, *mck1*Δ mutants were shown to be salt-sensitive when grown on glucose media, quite unlike *rim11*Δ mutants. In addition, *mck1*Δ mutants were found to have reduced *ENA1* mRNA levels, whereas overexpression of *RIM11* does not seem to affect *ENA1* transcript levels. Therefore, both GSK3 homologs appear to be involved in separate salt tolerance pathways, one which functions on glucose media by modulating the expression of *ENA1*, the other which functions on galactose media through an unknown mechanism.

Originally, the genetic screen described here was designed as a method to isolate specific suppressors of mutations in casein kinase II, a gene also involved in salt tolerance in *S. cerevisiae*. Not a single previously identified halotolerance gene was isolated in this work, and so it is clear that the screen was not saturating. *CKB1* and

CKB2 themselves were prevented from being isolated by design; either single *ckb*Δ mutant is as sensitive to salt as the double deletion mutant, and as a result the presence of one of these genes on a multicopy plasmid would not ameliorate the sensitivity of the double mutant used in this screen. The poor saturation and lack of isolation of a specific suppressor of salt-sensitive *ckb*Δ mutants is somewhat disappointing, although a novel determinant of salt tolerance in *S. cerevisiae* was discovered. *RIM11* does not appear to be a specific suppressor of *ckb*Δ mutants, as it can suppress other salt-sensitive mutants[7] as well as improve the resistance of the wild-type strain. This does not mean that CKII and *RIM11* cannot work in the same salt tolerance pathway, but the possibility seems unlikely given results regarding the *ckb1*Δ, *ckb2*Δ, *rim11*Δ triple mutant. On galactose media, the triple mutant had a higher degree of salt-sensitivity than either the *ckb1*Δ, *ckb2*Δ double or *rim11*Δ single mutants. If only one pathway were involved, then eliminating any combination of components should result in the same degree of sensitivity. This was not observed. Moreover, *RIM11* does not appear to be connected to the *HOG1* or *ENA1* pathways, because of the irrelevence of the *rim11*Δ mutation to osmotolerance and the inability of *RIM11* in multicopy to activate *ENA1* expression, respectively. Therefore, evidence linking *RIM11* to any known salt tolerance pathway is currently lacking. Considering the vast salt-resistance network present in *S. cerevisiae*, speculation regarding the correct place of *RIM11* is probably premature. Yet, since GSK3 homologs are involved in transcriptional regulation in other organisms (Woodgett, 1994), it is likely that *RIM11* promotes salt tolerance in yeast by activating the expression of other stress-resistance genes. Transcriptional profiling or another genome-wide approach may be the best way to test this hypothesis.

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[7] Overexpression of *RIM11* suppresses *cnb*Δ (calcineurin regulatory subunit) mutants, as illustrated by Figure 2.

The GSK3 family: Regulators of ion homeostasis in *S. cerevisiae*?

MCK1 and *RIM11* are the only GSK3 homologs in *S. cerevisiae* that have been studied extensively, and both have been found recently to contribute in some manner to salt-tolerance. This may be a general feature of the GSK3 family in yeast, a possibility that should be explored in the future by examining *MRK1* and *YOL128c* more closely, as well as GSK3 homologs in other species, such as *Schizosaccharomyces pombe*. A search of this type may reveal the general function of the GSK3 family in unicellular organisms, and may provide clues for researchers working with them in more complex organisms as well.

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