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The HOG Pathway Dictates the Short-Term Translational Response after Hyperosmotic Shock

Jonas Warringer,*† Malin Hult,*†‡ Sergi Regot,§ Francesc Posas,§ and Per Sunnerhagen*

*Department of Cell and Molecular Biology, Lundberg Laboratory, University of Gothenburg, S-405 30 Göteborg, Sweden; and §Cell Signaling Unit, Department de Ciènces Experimentals i de la Salut, Universitat Pompeu Fabra (UPF), Parc de Recerca Biomèdica de Barcelona (PRBB), E-08003 Barcelona, Spain

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Cellular responses to environmental changes occur on different levels. We investigated the translational response of yeast cells after mild hyperosmotic shock by isolating mRNA associated with multiple ribosomes (polysomes) followed by array analysis. Globally, recruitment of preexisting mRNAs to ribosomes (translational response) is faster than the transcriptional response. Specific functional groups of mRNAs are recruited to ribosomes without any corresponding increase in total mRNA. Among mRNAs under strong translational up-regulation upon shock, transcripts encoding membrane-bound proteins including hexose transporters were enriched. Similarly, numerous mRNAs encoding cytoplasmic ribosomal proteins run counter to the overall trend of down-regulation and are instead translationally mobilized late in the response. Surprisingly, certain transcriptionally induced mRNAs were excluded from ribosomal association after shock. Importantly, we verify, using constructs with intact 5′ and 3′ untranslated regions, that the observed changes in polysomal mRNA are reflected in protein levels, including cases with only translational up-regulation. Interestingly, the translational regulation of the most highly osmostress-regulated mRNAs was more strongly dependent on the stress-activated protein kinases Hog1 and Rck2 than the transcriptional regulation. Our results show the importance of translational control for fine tuning of the adaptive responses.

INTRODUCTION

A central feature of all living systems is the maintenance of cellular functionality in the face of fluctuations in the intracellular or extracellular milieu, i.e., stress. The resilience of genetic networks lets the cell tolerate a certain level of environmental or mutational perturbation with little loss of fitness. A key feature of this robustness is the activation of cellular adaptation mechanisms dedicated to the protection against the perturbation at hand (Stelling *et al.,* 2004). The most immediate, but also least enduring, of these responses is the activation or stabilization of proteins already present in the cell by posttranslational modification. More powerful, as it is intrinsically repeatable, but also more costly in terms of energy and resource expenditure, is the translational mobilization of specific mRNA transcripts. An even more long-lasting potential lies in the transcription factor–mediated activation of a distinct subset of gene promoters, resulting in the increased production of stress-related mRNA transcripts. Of these three main adaptation mechanisms, transcriptional regulation has been the most extensively studied, primarily due to the ease and speed with which changes in global mRNA levels can be quantified through DNA microarray technology. Unfortunately, this focus on total mRNA abundance has left severe gaps in our understanding of cellular adaptation mechanisms because only about 70% of the observed variance in protein abundance is caused by variation in mRNA abundance (Lu *et al.,* 2007).

Recently, the means to study translational mobilization of specific mRNA has emerged in the form of a combination of the DNA microarray technology with a preceding biochemical isolation of transcripts physically associated with actively translating ribosomes (polysomes). This technique has previously been used to study the global impact on translating mRNAs in yeast of various external stresses including carbon source shift (Kuhn *et al.,* 2001), oxidative stress (Shenton *et al.,* 2006; Swaminathan *et al.,* 2006), amino acid starvation (Smirnova *et al.,* 2005), as well as that of ionizing radiation (Lü *et al.,* 2006) or mitogenic signaling in mammalian cells (Rajasekhar *et al.,* 2003). Here, we utilize this approach to perform a time-resolved differentiation between the translational and transcriptional responses to hyperosmosis in *Saccharomyces cerevisiae*.

Adaptation of yeast cells to osmostress is one of the best known responses in any experimental system and has been the targeted focus of numerous scientific publications (reviewed in Hohmann, 2002). The most important signal transduction system in the yeast hyperosmosis response is

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† These authors contributed equally to this work.

‡ Present address: Karolinska University Laboratory, Huddinge, S-141 86 Stockholm, Sweden.

Address correspondence to: Per Sunnerhagen (Per.Sunnerhagen@cmb.gu.se).

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the HOG (high-osmolarity glycerol) pathway (Hohmann, 2002). Activation of the HOG pathway by increases in extracellular osmolarity elicits a wide range of responses, notably increased transcription of the glycerol dehydrogenase gene *GPD1*, resulting in accumulation of the compatible solute glycerol and restoration of turgor. An active HOG pathway leads through a three-layered kinase cascade to phosphorylation of the mitogen-activated protein kinase (MAPK) Hog1 (Maeda *et al.*, 1994). Phosphorylated Hog1 transiently accumulates in the nucleus where it activates transcription factors such as Hot1, Sko1, and Smp1 as well as recruits histone deacetylase components to osmoresponsive promoter sites for transcription (de Nadal *et al.*, 2004). Apart from its role as an activator of transcription factors in the nucleus, phosphorylated Hog1 also has other, less well-defined functions. One of these is phosphorylation and activation of the cytoplasmic kinase Rck2, which acts on translation elongation factor 2 mediating a transient repression of protein synthesis after osmotic shock (Bilsland-Marchesan *et al.*, 2000; Teige *et al.*, 2001). Defects in the stress-activated MAPK (SAPK) Hog1, the downstream cytoplasmic kinase Rck2, or their homologues in fission yeast, compromise translational recovery after stress (Teige *et al.*, 2001; Uesono and Toh, 2002; Dunand-Sauthier *et al.*, 2005; Swaminathan *et al.*, 2006; Asp *et al.*, 2008).

In this article, we have probed the cellular adaptation to 0.4 M NaCl, a concentration with limited impact on cellular fitness, but sufficient to fully induce the HOG pathway and hence relevant for normal yeast physiology. We measured changes in the pool of total mRNA ("transcriptional regulation"), resulting from changes in transcription and mRNA degradation rates, and in the pool of actively translated mRNA, defined as mRNA bound by two or more ribosomes (polysomal mRNA; "translational regulation"). We found that for most transcripts, their transcriptional regulation was matched by translational regulation, as may be expected ("homodirectional changes"; Preiss *et al.*, 2003). However, many transcripts not previously recognized as stress-induced were recruited to polysomes without a concomitant increase in the total mRNA pool ("translation-activated osmostress genes"). On the other hand, some transcripts whose total amount increases during hyperosmotic stress unexpectedly decreased their association to polysomes, leading to a net decrease in representation among translationally active transcripts ("translation-deactivated osmostress genes"). These unforeseen regulatory patterns were confirmed by Western analysis for selected cases.

Additionally, the importance of the HOG pathway components *HOG1* and *RCK2* for the regulatory response to osmostress was also investigated, both on the level of total mRNA and polysomal mRNA. The importance of the translational regulation level was underscored by our finding that its Hog1 dependency was even stronger than for the transcriptional level.

MATERIALS AND METHODS

Strains

S. cerevisiae strains used were in the W303-1A background (Table 1). To create strains expressing epitope-tagged proteins from chromosomally integrated constructs with conserved 5′- and 3′-untranslated regions (UTR), W303-1A cells were transformed with the integrative cassettes derived from pOM22 (Gauss *et al.*, 2005), introducing recombination sequences inside the open reading frame (ORF) of the indicated gene. The recombination is designed to conserve the first five amino acid residues of the protein, in order to maintain the physiological AUG context. The cassette introduces the *LoxP*-flanked *URA3* marker and nine Myc tags in frame with the rest of the protein. Integration was confirmed by PCR. Then cells were transformed with the plasmid pSH62 (Cheng *et al.*, 2000), which expresses the CRE recombinase under the *GAL1* promoter. Cre-mediated recombination was induced in 2% galactose for 5 h, and cells were plated on 5-fluoroorotic acid to select for loss of the *LoxP*-flanked *URA3* marker. Finally, positive colonies were confirmed by sequence and proteins detected by Western blot.

Table 1. *S. cerevisiae* strains

Strain	Genotype	Source or reference
W303-1A	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	S. Hohmann
*hog1*Δ	W303-1A *hog1::TRP1*	S. Hohmann
*rck2*Δ	W303-1A *rck2::TRP1*	Dahlkvist and Sunnerhagen (1994)
YSR115	W303-1A *GDH3:myc$_{(9)}$::loxP*	This work
YSR118	W303-1A *HXT9:myc$_{(9)}$::loxP*	This work
YSR120	W303-1A *TPO4:myc$_{(9)}$::loxP*	This work
YSR114	W303-1A *HSP31:myc$_{(9)}$::loxP*	This work
YSR93	W303-1A *SSA1:myc$_{(9)}$::loxP*	This work

Cultivation and Harvest for Array Experiments

After overnight (18–24 h) precultivation in 10 ml YEPD, cells were inoculated (in triplicates) to an OD_{610} of 0.05–0.1 in 1 l of fresh YEPD and cultivated for 6–10 h until midexponential phase (OD_{610} = 0.35–0.45), whereupon cells were subjected to 0.4 M NaCl stress. Samples for polysomal analysis were prepared essentially as described (Swaminathan *et al.*, 2006). Briefly, 80-ml samples (two times for polysomal samples and one time for total RNA samples) were harvested after 0, 2, 6, and 30 min of NaCl stress, into cooled containers containing 0.1 mg/ml cycloheximide. Cells were immediately pelleted by 4-min centrifugation at 4000 × *g* at 4°C, washed (two time , 2.5 ml) in lysis buffer (20 mM Tris-HCl, 140 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 0.1 mg/ml cycloheximide, 0.5 mg/ml heparin, 1% Triton X-100), and lysed in 0.7 ml lysis buffer, with 0.5 ml acid-washed glass beads using repeated disruption (four times for 30S at 4°C) in a FastPrep (Bio-101, Carlsbad, CA). Cell debris was discarded after repeated centrifugation (one time at 2600 × g and one time at 7200 × *g* at 4°C). Extracts were stored frozen at −70°C.

Polysomal Profiling and RNA Extraction

Ribosomal light subunit (40S), heavy subunit (60S), monosomes (80S), and polysomes (≥2 ribosomes) were separated in analytical or preparative mode by ultracentrifugation of cycloheximide-treated extracts, followed by online UV monitoring and fractionation as described (Swaminathan *et al.*, 2006). Polysomal RNA was purified from fractions corresponding to ≥2 ribosomes by extraction with phenol and repeated ethanol and LiCl precipitations, as detailed previously (Swaminathan *et al.*, 2006). Quantity and quality of RNA isolations was checked on 1% agarose gels and by measurement of the $A_{260/240}$ ratio.

Array Hybridization and Analysis

Labeling of samples and array hybridization were performed essentially as previously described (Bilsland *et al.*, 2007). Briefly, cDNA was reverse-transcribed from 15 to 20 μg of RNA using a 1:1 mixture of random primers and anchored oligo dT (Invitrogen, Carlsbad, CA). Samples were labeled with equal amounts of Cy5-dUTP (experimental sample) and Cy3-dUTP (control sample, total RNA from unstressed wt cells; GE Healthcare, Waukesha, WI). After labeling, RNA was hydrolyzed with NaOH, and labeled cDNA probes were purified using a postlabeling CyScribe GFX purification kit (GE Healthcare) according to instructions from the supplier. Probes were dissolved in 80 μl of DIG Easy hybridization buffer (Roche, Indianapolis, IN) and hybridized onto Yeast 6.4K microarray slides (Microarray Center, University Health Network, Toronto, ON, Canada) at 42°C overnight and sequentially washed in 2× SSC, 0.1% SDS, 1× SSC, and 0.1× SSC before scanning in a VersArray Chipreader (Bio-Rad, Richmond, CA) at 10-μm resolution. Spot intensities were quantified using ImaGenes software (Carlsbad, CA), and raw data were normalized using the LIMMA Microarray add-in to the open-source Conductor R. Each slide was normalized by printtip lowess normalization of $\log_{(2)}$ ratios (http://www.bioconductor.org/) without background subtraction. The biological triplicates were fitted to a linear model where the flagged genes were down-weighted to a factor of 0.1. The change in $\log_{(2)}$ ratios (Cy5/Cy3) in each time series, using the 0-min sample (no stress) as a reference point, was calculated to obtain the $\log_{(2)}$ n-fold NaCl induction for each gene and each time point.

Defining a Core Set of NaCl-induced Genes

We extracted a set of NaCl-induced genes from five previous studies (Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale and Bohnert, 2001; Krantz *et al.*, 2004) that were induced ≥2-fold in at least two time points in ≥3 of the five

studies, as described earlier (Molin *et al.*, 2009). In total, 66 genes passed these criteria.

Statistical Test of Functional Overrepresentation among Induced and Repressed Genes

Functional over- and underrepresentations among the top 200 most induced and the top 200 most repressed genes in each class was tested using biological process information from the Munich Institute of Protein Science (MIPS) *S. cerevisiae* genome database (http://mips.helmholtz-muenchen.de/genre/proj/yeast/). Only biological processes with at least 15 members present in the tested gene set, corresponding to a total of 225 processes, were included. Statistically robust ($p < 10^{-4}$) features of over- and underrepresentation were determined using a hypergeometric distribution assumption (Fisher's exact test).

RESULTS

NaCl Shock Causes a Transient Depression of Translationally Active Ribosomes

To investigate the effects of hyperosmotic shock on general translation activity, polysomes were isolated and quantified at defined time points during the first 30 min of adaptation to 0.4 M NaCl. In wild-type (wt) cells, the polysome fraction decreased rapidly, starting as early as 0.5 min to reach a minimum at 4–6 min (Figure 1, A and B). The translational depression was similar to wt in *rck2* mutants, but more severe in *hog1* mutants (Figure 1C). This reduction in polysomes was accompanied by a corresponding increase in free 40S, 60S, and 80S ribosomal material. The translational activity started to recover after 6 min of NaCl exposure, to reach preshock levels after ~30 min (Figure 1D). In *hog1* mutants, the recovery was impaired and did not reach preshock levels in the course of the experiment. These findings are in line with earlier observations in *S. cerevisiae* (Teige *et al.*, 2001; Uesono and Toh, 2002) or *Schizosaccharomyces pombe* (Asp *et al.*, 2008) cells, with or without functional SAPK, after hyperosmotic shock. The *rck2* mutants recovered from shock essentially as quickly as wt cells (Figure 1D).

We noted, however, that *rck2* mutants consistently displayed a significantly increased fraction of free 40S and 60S ribosomal subunits, relative to intact 80S monosomes (Figure 1E); this defect was present already before stress. Such an altered balance was not observed in *hog1* mutants (Figure 1E). This is consistent with the previously inferred role for Rck2 in translation (Melcher and Thorner, 1996; Teige *et al.*, 2001; Swaminathan *et al.*, 2006).

Hence, Hog1 is required for translational recovery after salt stress, whereas Rck2 has a function in monosome assembly, but not noticeably in translational recovery.

Translational Mobilization Is Faster Than the Transcriptional Response during Adaptation to High Osmolarity

To compare the timing of stress-induced translational and transcriptional changes, total as well as polysome-bound mRNA was isolated after 0, 2, 6, and 30 min of osmostress, hybridized on genome-wide ORF arrays, and the respective levels of induction were calculated for each mRNA. Frequency distributions of the salt induction of all 5500 transcripts show that at 2 and 6 min, the fraction of salt-regulated transcripts is higher in the polysome-bound pool than in the total pool, both in terms of salt induction and in terms of salt repression (Figure 2A). Quantifying the differences between the distributions, we found that after 2 min of NaCl exposure, there are three times as many transcripts that were strongly (>2-fold) mobilized by polysomes than that were transcriptionally induced to that level (Figure 2B). However, after 30 min the roles were reversed; the transcriptional changes were then substantially stronger than the translational changes. It is worth noting that after 2 min, many of the translationally mobilized transcripts correspond to proteins with well-known functions in salt stress, such as the Na^+ pumps encoded by *ENA1* and *ENA5* and the trehalose phospho-synthase Tps2; however, transcriptionally up-regulated mRNAs at this time point have no such functional link (Figure 2C, top panel). Hence, the very early translational response is not only stronger but also more biologically relevant than the transcriptional response. After 6 min of adaptation, there was a weak positive overall correlation (linear correlation, $r^2 = 0.05$) between transcriptional and translational induction that was substantially stronger for the most induced genes but completely absent for the most repressed genes (Figure 2C, middle panel). The vast majority of genes that were mobilized on the polysomal level, notably *ENA1*, *ENA2*, *ENA5*, *TPS2*, and several uncharacterized ORFs such as *YLR108c* and *YJL107c*, were also induced, albeit to a lower extent, as total mRNAs. After 30 min, essentially the same genes were transcriptionally induced as after 6 min. However, at this time of the response, there is an inverse correlation between transcriptional induction and translational mobilization (Figure 2C, bottom panel), in line with that transcriptional and translational regulation operate with different time scales with transcriptional regulation lagging behind.

Taken together, these patterns are consistent with an early mobilization of existing transcripts to the ribosome, preceding the de novo production of mRNA. In contrast, de novo synthesis of transcripts persists as a longer term stress response and is in full swing even after 30 min of stress exposure when the translational mobilization of transcripts has already wound down.

A Subgroup of Osmostress-Responsive Transcripts Is Translationally Down-Regulated in a Hog1-dependent Manner

Transcriptional regulation in yeast cells under osmostress has received considerable scientific interest, and from that perspective the translational fate of well-known salt-induced transcripts deserves particular attention. On the basis of a meta-analysis of earlier published data sets, we defined a core set of transcriptional NaCl responders (see *Materials and Methods*). Compared with the strong transcriptional induction of these genes, the translational mobilization of the corresponding transcripts was substantially lower (Figure 3A). This is not surprising given that these genes were selected on the basis of their strong transcriptional induction.

Clustering of the individual strong salt responders revealed a distinct subgroup of 18 transcripts that accounted for the bulk of the observed difference between total mRNA regulation and translational mobilization (Figure 3B, indicated with green lines). Surprisingly, these transcripts were selectively rejected from polysomes at all time points during the adaptation period ("counterdirectional changes"; Preiss *et al.*, 2003). This suggests that they are either not translated at all or that they have a role in long-term adaptation and will be translated later than the here-studied 30-min time point. Many of these gene products do not have described functions in osmoprotection, such as the heat-shock proteins Hsp26, Hsp31, Hsp78, and Ssa1, and the poorly known aldoketo reductases Ald2 and Ald3. However, also the trehalose cycle components Nth1 and Tps1 that are necessary for generation of the carbohydrate trehalose, assumed to have osmoprotective properties in stationary phase cells (Hounsa *et al.*, 1998), belong to this group. The selective translational repression of some transcriptionally induced genes after salt stress was completely dependent on both *HOG1* and *RCK2*, suggesting that the presence of Hog1 and Rck2 blocks translation of these transcripts (Figure 3B). Thus, many of these genes

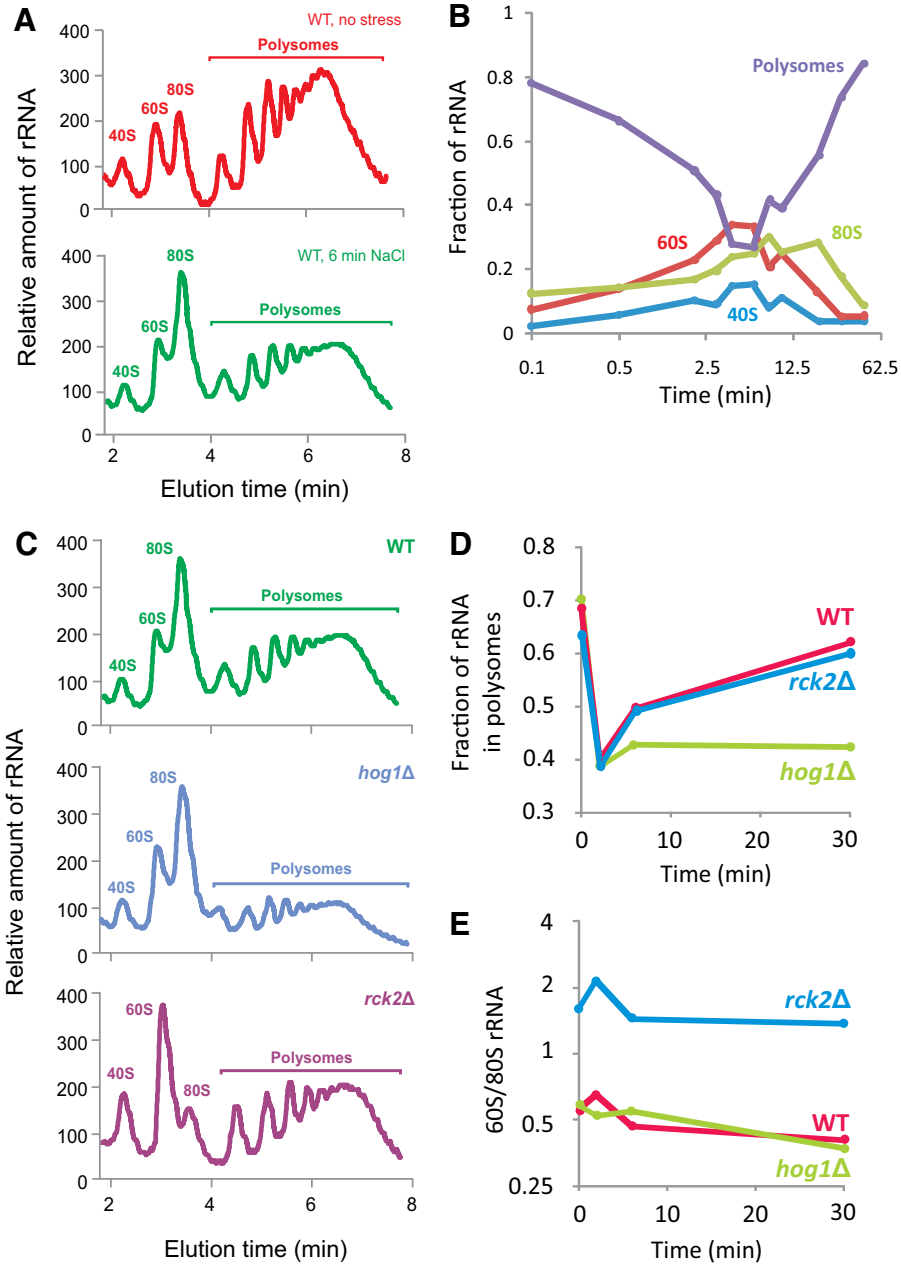

Figure 1. Changes in translational activity during adaptation to mild osmostress. (A and B) The fraction of mRNA bound ribosomes (polysomes) decreases temporarily after exposure to 0.4 M NaCl but rebounds to preshock levels after 30 min. (A) Ribosomal light subunits (40S), heavy subunits (60S), monosomes (80S), and polysomes (>80S) from samples collected before or after salt shock were separated using a linear 10–50% sucrose density gradient, and the relative amount of rRNA in each fraction was measured online by UV spectrometry after gradient elution. Representative absorbance curves are displayed. (B) Fraction of rRNA located in 40S, 60S, monosomes, and polysomes at different time points after a 0.4 M NaCl shock (n = 3–5) calculated from profiles as shown in A. (C–E) Formation of translating ribosomes is hampered in *hog1*Δ and *rck2*Δ mutants, albeit in different ways. (C) 40S, 60S, monosomes (80S), and polysomes from samples collected before or 6 min after salt shock in wt, *hog1*Δ, and *rck2*Δ were separated using a linear 10–50% sucrose density gradient. The relative amount of rRNA in each fraction was measured by spectrometry after gradient elution; representative absorbance curves are displayed. (D) Fraction of rRNA found in polysomes before and after salt exposure in wt and *hog1*Δ cells (n = 6–10), calculated from online profiles as shown in C. No or delayed recovery is observed in *hog1*Δ cells. (E) Relative proportion 60S/80S rRNA in wt and *rck2*Δ cells before and after salt exposure in wt and *hog1*Δ (n = 6–10) calculated from online profiles as shown in C. Assembly of monosomes is partially impeded in *rck2*Δ mutants.

were both induced, on a transcriptional level, by the HOG pathway activity, and repressed, in terms of translation mobilization, by the same pathway.

Figure 3C shows the mRNAs that were most up-regulated in polysomal mRNA at the peak of the translational response after 6 min; these are in principle predicted to result in the most highly increased protein levels. These highest increases in polysomal mRNA result from three different combinations of factors: 1) elevated total mRNA with no additional polysomal association (e.g., *GPD1*, *PIL1*), 2) elevated total mRNA, increased polysomal association (e.g., *ENA1*, *ENA5*, *HXT1*; "homodirectional changes"; Preiss *et al.*, 2003), and 3) no increase in total mRNA, increased representation in polysomal mRNA entirely dependent on higher association to polysomes (e.g., *HXT2*, *TOR2*, *GDH3*). In this top class, roughly 50% of transcripts belonged to the third category (indicated with blue lines in Figure 3C). This last group obviously goes undetected in standard DNA array studies using total mRNA only and thus potentially represents "translation-activated osmostress genes."

Translational Regulation Is More Strongly Hog1 Dependent Than Transcriptional Regulation after Hyperosmotic Shock

To assess the overall role of Hog1 and Rck2 in translational and transcriptional salt regulation, we investigated the effect of *hog1*Δ and *rck2*Δ mutations on the top 200 genes most transcriptionally induced by NaCl. In *hog1*Δ mutants, induction levels were strongly diminished, both in terms of the total mRNA pool and in terms of the polysome association of mRNAs (Figure 4A). Notably, the Hog1 dependency was significantly (Student's *t* test, $p = 3 \times 10^{-10}$) larger in terms of the polysome association of transcripts; in fact salt induction was close to abolished in *hog1*Δ in terms of the poly-

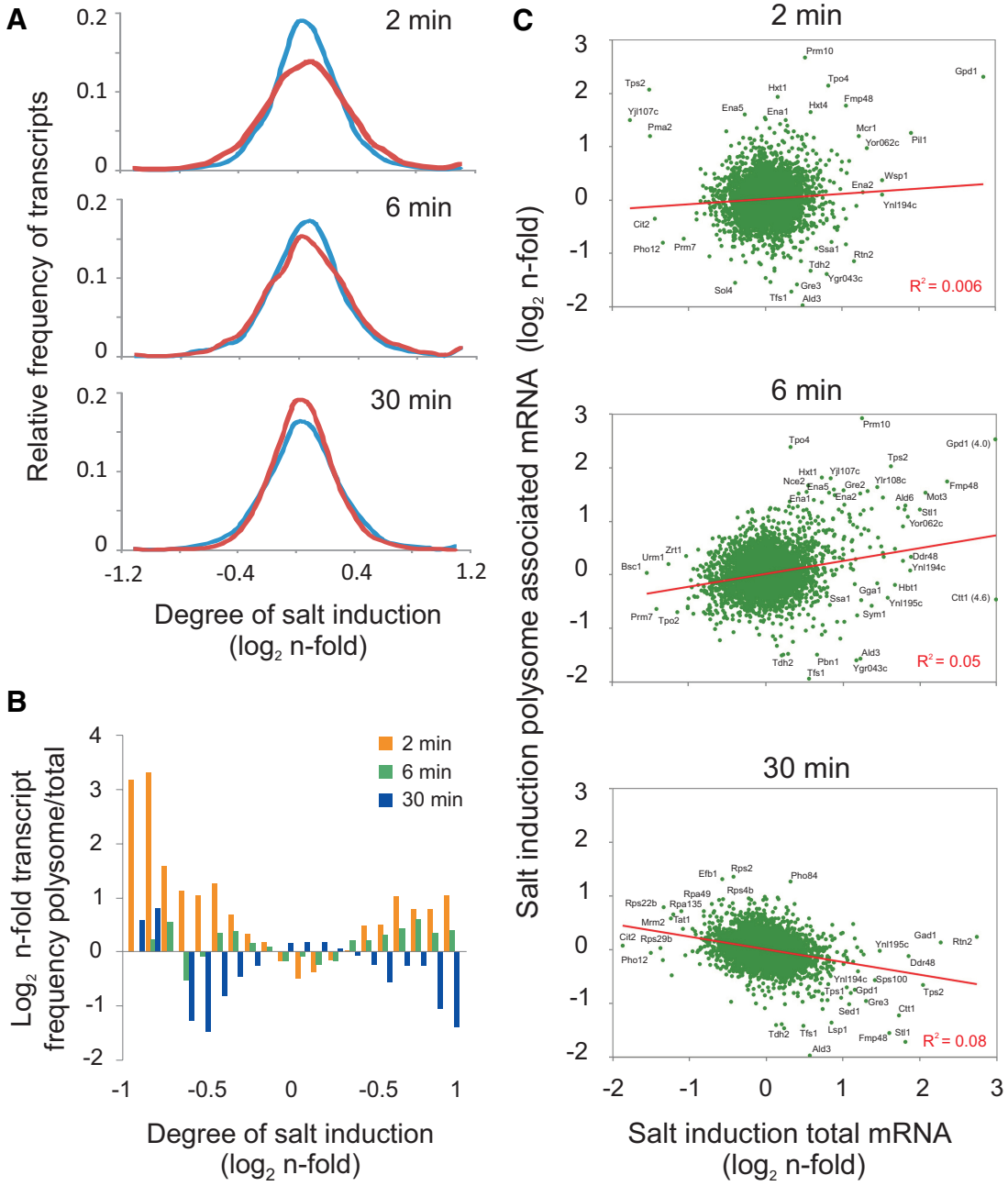

Figure 2. Translational regulation is faster than transcriptional regulation during adaptation to osmostress. (A and B) Regulation of the polysome association of mRNAs precedes changes in total mRNA. (A) The regulation of each of ~5500 transcript after 2, 6, and 30 min of exposure to 0.4 M NaCl on the levels of total mRNA, and polysome association was determined by mRNA microarrays. The number of regulated transcripts at different levels of salt induction/repression (grouped in 22 bins) was plotted in frequency histograms. Red line, polysome associated mRNA; blue line, total mRNA. (B) At each level of salt induction and salt repression, the number of regulated mRNAs in the polysome-bound pool and in the total mRNA pool was compared, and $\log_2$ of the ratio between them was plotted ($\log_2$ [polysome/total]). Zero is the number of salt regulated transcripts at the indicated level of regulation is the same in the polysome-bound pool and in the total mRNA pool. Frequency data were derived from A, with similar bins. (C) Scatter plots portraying the correlation between polysomal association regulation and total mRNA regulation for all individual ~5500 transcripts at 2, 6, and 30 min after exposure to 0.4 M NaCl. Red line, the overall linear correlation; linear regression coefficients (r^2) are indicated.

some association of transcripts. The stronger Hog1 dependency of translational than transcriptional regulation was observed also for the most repressed transcripts (Student's *t* test, $p = 2 \times 10^{-8}$; Figure 4B). Regulation of both the most salt-induced and the most salt-repressed transcripts was also dependent on Rck2, albeit not to the same extent as on Hog1 (Figure 4, A and B). Transcriptional induction of the most induced genes was ~50% lower in *rck2*Δ than in the wt. However, no further effect on translational mobilization was observed in *rck2*Δ mutants as there was no significant difference between the changes in total and polysomal mRNA for these mutants (Student's *t* test, $p > 0.16$), suggesting that the role of Rck2 is mainly in transcriptional regulation. Interestingly, the correlation between induction levels in

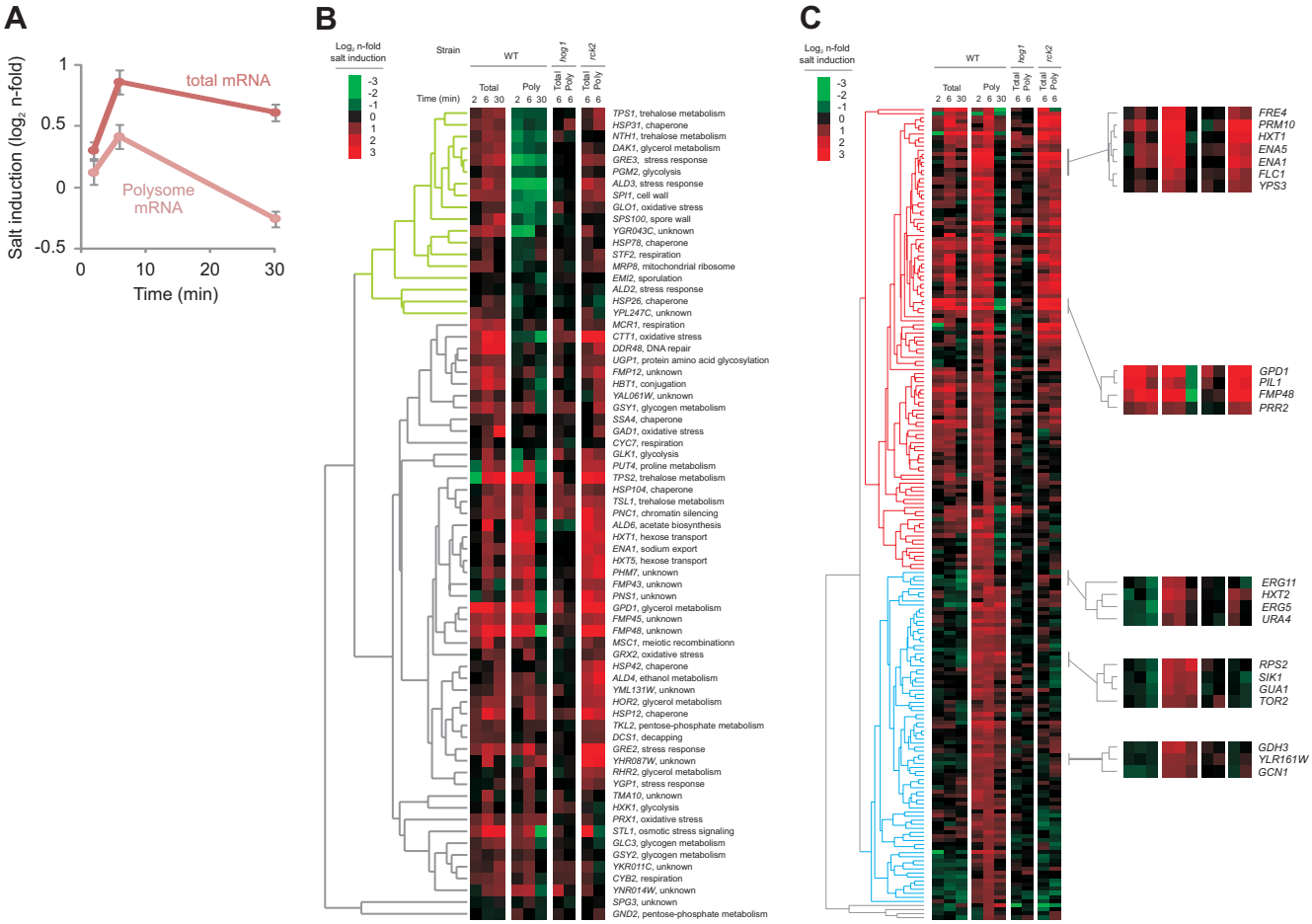

Figure 3. Translational regulation of translation-activated and translation-deactivated osmostress genes is Hog1 and Rck2 dependent. (A) The 66 core transcriptional NaCl responders identified by meta-analysis of published salt induction data sets (Molin *et al.*, 2009; see *Materials and Methods*) are unequally mobilized by the translational apparatus. Average induction of the core transcriptional set responders considering the total and polysomal mRNA levels. Error bars, SE. (B) A subset of the core salt-induced transcripts is selectively rejected by the translational apparatus after NaCl shock. The core transcriptional salt responders were hierarchically clustered (uncentered Pearson similarity metric, average linkage mapping) on the basis of total mRNA and polysomally associated mRNA salt induction (log_2 n-fold induction) in wt, *hog1*Δ, and *rck2*Δ cells. A subcluster of genes that are selectively rejected by the translational apparatus is indicated with color (green lines). (C) Many translationally mobilized transcripts are not transcriptionally induced during salt stress (blue lines). Average (n = 3) polysomal and transcriptional induction of the top 200 polysomally most induced genes during salt stress.

*rck2*Δ and wt for the most induced transcripts was good (linear regression, $r^2 = 0.48$), reflecting that the same transcripts are regulated in *rck2*Δ and wt, albeit to a lower level. This clearly suggests a general rather than a transcript-specific role for Rck2 in transcriptional regulation. However, Rck2 was of specific importance for the translational mobilization of cytoplasmic ribosomal proteins (RPs; see below). Taken together, our results demonstrated a strong role for Hog1 in adaption to osmostress, both on the transcriptional and translational regulation levels. They also demonstrated a general role for Rck2 in transcriptional regulation.

The Majority of the Stress Response Is Homodirectional

To investigate the overall pattern of activated and deactivated biological processes, we quantified degrees of functional enrichments among the top 200 most induced transcripts at each respective time point both in total and polysomal RNA. The degree of overrepresentation of a specific functional category among these mRNAs compared with the expectation, the n-fold enrichment, was expressed in a heat map where yellow indicates overrepresentation and purple indicates underrepresentation (Figure 5A). Enriched categories were generally similar for the polysome-bound mRNA pool and the total mRNA pool, reflecting that the same biological processes, although not always the same specific transcripts, are induced translationally and transcriptionally (Figure 5A; homodirectional changes). For both levels, there was a disproportional induction of genes involved in cell defense and rescue (stress- and heat-shock response), cellular transport (ion and carbohydrate transport), and interaction with the environment (cellular sensing and chemoperception), as well as of components of energy metabolism. There was also a corresponding repression of genes involved in cell cycle progression, translation (rRNA and tRNA synthesis, processing and modification), and in cytoskeletal biogenesis. Taken together, these responses reflect the cellular shift in focus from growth promotion to general stress protection, as seen in several previous array studies on total mRNA (Gasch *et al.*, 2000; Posas *et al.*, 2000; Causton *et al.*, 2001; Yale and Bohnert, 2001), that is also largely reflected in polysome-associated mRNA.

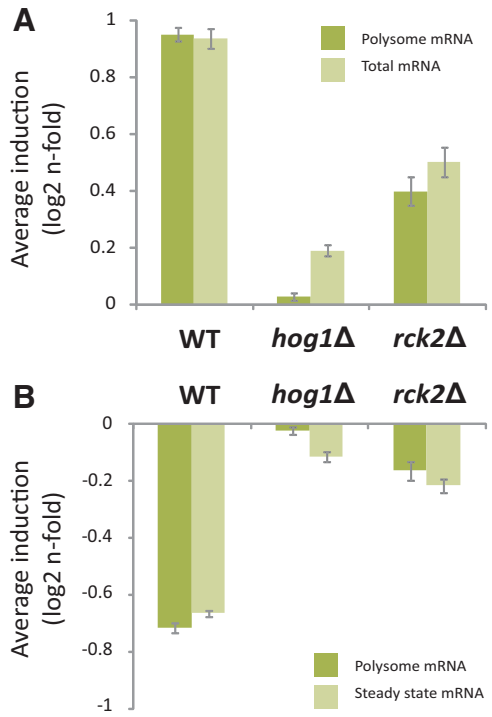

Figure 4. Stress mRNA regulation dependence on Hog1 and Rck2 is stronger on the translational level. (A) Average induction at 6 min after NaCl exposure of the 200 most salt-induced genes (in wt), considering either polysomal or total mRNA levels. Induction is strongly reduced in *hog1*Δ mutants and moderately reduced in *rck2*Δ mutants, and particularly for *hog1*Δ mutants, reduction is stronger on the level of polysomal mRNA. Error bars, SE. (B) Average induction at 6 min after NaCl exposure of the 200 most salt repressed genes (in the wt), considering either polysomal or total mRNA levels. Repression is strongly impeded in both *hog1*Δ and *rck2*Δ mutants, and particularly for *hog1*Δ cells, the effect is stronger on the level of polysomal mRNA. Error bars, SE.

Interestingly, although individual gene responses were highly dependent on the HOG pathway (e.g., *HXT1*, *ENA1*, *ENA5*, *TOR1*, *HXT2*; Figures 3C and 5D), the overall changes for entire functional categories were not (Figure 5A). There are only two functional categories heavily dependent on Hog1 and/or Rck2 for NaCl induction on the transcriptional and translational levels: genes involved in ion and carbohydrate transport (Figure 5 A, red text). This demonstrates a role for Rck2 in the transcriptional regulation of the abundance of a functionally distinct subset of osmostress-induced mRNAs. These observations are somewhat surprising, since the best described role of phosphorylated Hog1 is direct regulation of transcription factors in a nuclear context (Alepuz *et al.*, 2001, 2003; Proft *et al.*, 2001; de Nadal *et al.*, 2003, 2004). Also, Rck2 is understood as a cytoplasmic kinase with no previously demonstrated direct role in transcriptional regulation (Teige *et al.*, 2001; Swaminathan *et al.*, 2006).

A Large Group of Transcripts Encoding RPs Display Counterdirectional Changes and Are Translationally Up-Regulated

The total levels of RP-encoding mRNAs were disproportionably repressed at all time points and independently of Hog1 or Rck2 (Figure 5A), in agreement with many earlier observations from different stress conditions including hyperosmotic shock (e.g., Posas *et al.*, 2000; Krantz *et al.*, 2004; Swaminathan *et al.*, 2006; Thorsen *et al.*, 2007; Molin *et al.*, 2009). However, a disproportionably large subgroup of cytoplasmic RPs (cRPs) displayed a strong polysomal recruitment mRNA after 30 min of salt adaptation (Figure 5A, marked in blue text). This was the case for e.g., RPS2, RPS22B, and RPS4B (Figures 3C and 5B). These were heavily repressed on both levels at early time points, reflecting a diversion of resources from growth to stress adaptation. However, although the repression persisted on the level of total mRNA also after 30 min of stress, they were by then mobilized by the translational machinery. Hence, although the transcriptional machinery still strives to produce stress-related transcripts after 30 min of salt stress exposure; the translational machinery has refocused on growth-related transcripts and has initialized growth recovery.

Comparing induction levels in term of polysome association and total mRNA, we also found that it was predominantly cRPs that had a higher induction as polysomally associated mRNAs than as total mRNAs, whereas mitochondrial RPs (mRPs) were essentially unchanged or slightly more induced as total mRNAs (Figure 5B). Most genes in this cRP subset were dependent on Rck2 but not on Hog1 for their regulation, suggesting a HOG-independent, cRP-specific, function of Rck2 (Figure 5B, bottom cluster).

Translational Up-Regulation of Transcripts Encoding Membrane-bound Proteins

We also saw a disproportionate translational mobilization of transcripts encoding membrane proteins after salt shock, and the higher the induction level the stronger the enrichment (Figure 5C). Scrutinizing the translationally induced membrane-bound proteins, we found that they for the most part encode ion or sugar transporters (Figure 5A; e.g., ENA1, *ENA5*, *GAL2* and *HXT9*; Figures 3C and 5D).

Clustering of all sugar transporters on the ratio of polysome association and total mRNA salt induction revealed that the enrichment is accounted for by six hexose transporters *HXT1*, *HXT2*, *HXT4*, *HXT5*, *HXT9*, and *GAL2* (Figures 3C and 5, C and D). Of these, only *HXT1*, *HXT2*, and *HXT4* have been functionally characterized, and they all encode low-affinity transporters (Özcan and Johnston, 1999). The translational induction of hexose transporters contrasts to the behavior of genes involved in sugar catabolism that tended to be more salt-induced on the total mRNA level than on the polysomal mRNA level (Figure 5A).

Changes in Polysomal mRNA upon Stress Are Reflected in Protein Abundance

Discrepancies between total mRNA amounts measured from array studies and protein levels have been identified numerous times earlier (e.g., Gygi *et al.*, 1999; Ghaemmaghami *et al.*, 2003; Washburn *et al.*, 2003), and it has been speculated that changes in polysomal mRNA amounts would correspond more closely to protein levels (Laloo *et al.*, 2009). To illustrate this point, we chose examples where the changes in polysomal and total mRNA diverged. We examined protein expression from chromosomally integrated constructs where both 5′- and 3′-UTRs had been preserved (see *Materials and Methods*). In Figure 6, A–C, we show a Western blot time series after hyperosmotic shock of proteins predicted by array analysis of polysomal mRNA to be up-regulated in stress, but for which no increase of total mRNA was detected. For Gdh3 (an $NADP^+$-dependent glutamate dehydrogenase), Tpo4 (a polyamine transporter), and Hxt9 (a hexose transporter), there was in each case a clear-cut increase in protein levels by 30 and 40 min after onset of osmostress. In all three cases, a marked increase of

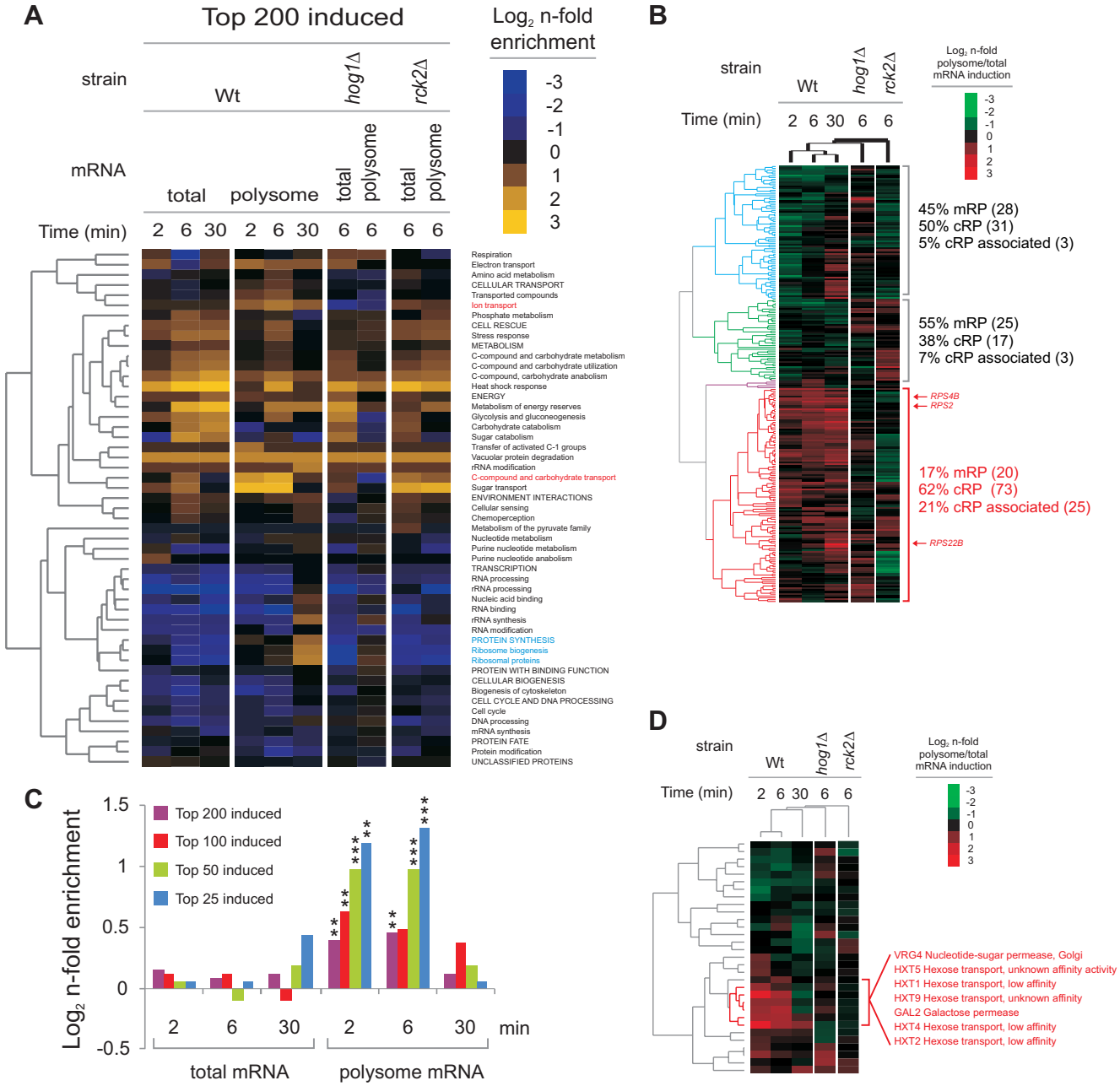

Figure 5. Divergent translational and transcriptional regulation for cRPs and hexose during adaptation to high NaCl. (A–D) A subset of genes are differently regulated on the level of total mRNA and on the level of polysome association. (A) To identify major functional differences between polysomal and the total mRNA salt regulation, the overrepresentation of known cellular functions among the top 200 most salt-induced (left panel) genes in each of wt (2, 6, and 30 min), *hog1*Δ (6 min), and *rck2*Δ cells (6 min). Colors indicate $\log_{(2)}$ fold enrichment in each class for functional categories that deviated significantly from the chance expectation at $p < 10^{-4}$ (Fisher's exact test) in at least one class. Yellow, functional overrepresentation; blue, functional underrepresentation. Functional annotations were taken from the MIPS *S. cerevisiae* genome database, including only categories with at least 15 members present in the screen. All enriched categories were hierarchically clustered according to degree of enrichment in each class using an uncentered Pearson similarity metric and average linkage mapping. Blue text, counterdirectional functional enrichments for total and polysome-associated mRNA; red text, Hog1 dependent functional enrichments. (B and D) The difference in the degree of salt induction on the polysomal association level and the total mRNA level ($\log_2$ [polysome NaCl induction/total mRNA NaCl induction]) was calculated for each individual transcript. (B) All genes annotated as associated with ribosomes were hierarchically clustered (as above) according to the difference in degree of translational and transcriptional induction ($\log_2$ [polysome NaCl induction/total mRNA NaCl induction]). Red squares, higher translational than transcriptional induction and green squares, the converse. Major clustering groups and the relative proportions (absolute numbers within brackets) of gene products in each clustering group that are resident members of the mitochondrial ribosome, the cytoplasmic ribosome, or more loosely associated with the cytoplasmic ribosome are indicated with color. A subset of mRNAs encoding cRPs is more strongly induced on the translational level (red lines). (C) Membrane protein–encoding transcripts are more frequent among the polysome association salt-induced transcripts than among the total mRNA salt-induced transcripts. Degree of enrichment of membrane proteins (GO annotations) among the top 25, 50, 100, and 200 most salt-induced transcripts considering either polysomal association induced or total mRNA–induced transcripts in wt cells. Enrichments are significantly stronger on the level of polysomal association among the very highly salt-induced genes (Student's *t* test; $^{**}p < 0.01$, $^{***}p < 0.001$). (D) All genes annotated (MIPS FunCat) as possessing sugar transport activity was

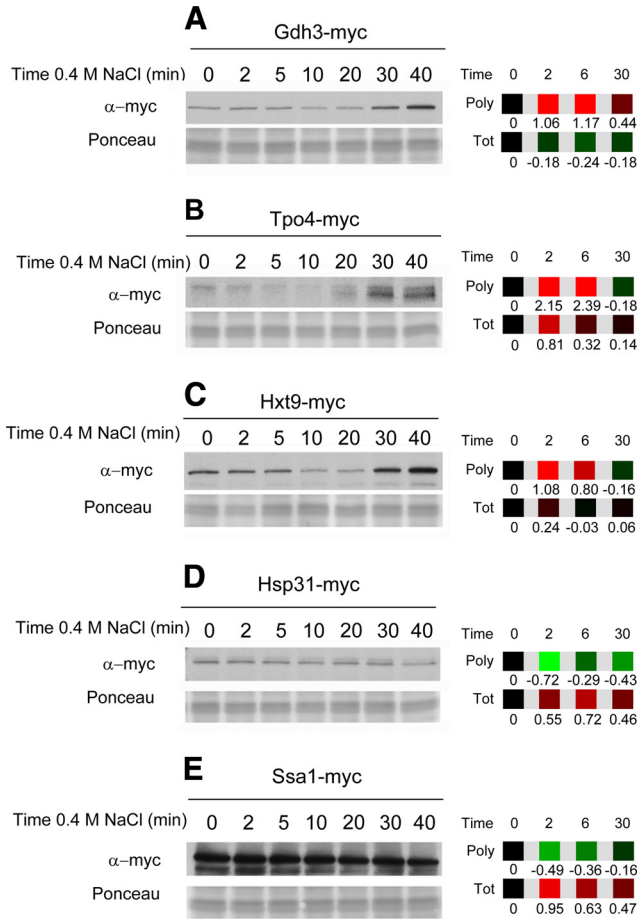

Figure 6. Protein levels after shock parallel the levels of the corresponding polysomal mRNAs. (A–E) Left, Western blots of Myc-tagged proteins at the indicated number of min after shock with 0.4 M NaCl. Strains express the tagged proteins from constructs integrated at the native chromosomal site and preserving the original 5′- and 3′-UTRs (see *Materials and Methods*). Ponceau staining of total protein was used as a loading control. Right, changes in total and polysomal mRNA abundance ($\log_2$ values) for the cognate transcripts at three different time points after NaCl shock in array measurements. For Gdh3, Tpo4, and Hxt9 (A–C), a transient increase is seen in the polysomal, but not the total, mRNA pool. For Hsp31 and Ssa1 (D and E), total mRNA but not polysomal mRNA is increased.

polysomal mRNA was seen at 2 and 6 min after stress, followed by a decline at 30 min (Figure 6, A–C, bottom panels); this is characteristic of a large group of stress-related mRNAs (see above). By contrast, the total amount of these mRNAs did not increase (Figure 6, A–C). We also examined cases where no increase of the mRNA was seen in the polysomal pool, despite an increase in total mRNA. This indicates that these mRNAs produced by transcriptional induction upon stress were not recruited to ribosomes and translated ("translation-deactivated osmostress genes"). Figure 6, E and F, show that for Hsp31 (a heat-shock protein and chaperone) and Ssa1 (a Hsp70-related chaperone), protein levels did not go up throughout the 40 min tested. This is despite the increase the total mRNA pool seen for the corresponding transcripts, but in accordance with their diminished representation in polysomal RNA.

By contrast, when protein expression after stress was tested from epitope-tagged constructs where the tag had been inserted in the standard manner, not preserving the 3′-UTR, in all cases protein levels reflected transcript abundance in the total mRNA pool, rather than in polysomal mRNA (data not shown). This clearly demonstrates the importance of 3′-UTRs for proper regulation of mRNA translation.

Figure 5 (cont). hierarchically clustered (as above) according to the difference in degree of salt induction on the polysomal association level and the total mRNA level ($\log_2$ [polysome salt induction/total mRNA salt induction]). Major clustering groups are indicated with color. A subset of sugar transport genes with primarily low affinity hexose transport activity is more strongly salt-induced on the polysomal level (red lines).

DISCUSSION

Polysomal mRNA Profiling Yields Novel "Osmostress Genes" and Modifies the View on Preexisting Ones

The transcriptional response to hyperosmotic and other stress agents in yeast has been thoroughly investigated in array studies using total mRNA (Gasch *et al.*, 2000; Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale and Bohnert, 2001; Krantz *et al.*, 2004; Watson *et al.*, 2004; Thorsen *et al.*, 2007), leading to well-defined sets of stress-induced and stress-repressed genes. Combined analyses of stress-activated transcripts, promoter elements, and mutants deficient in specific transcription factors have formed the concept of a core environmental stress response (CESR; Chen *et al.*, 2003; Segal *et al.*, 2003), activated by a wide range of stress agents. At the same time, global data indicate substantial discrepancies between steady-state mRNA and protein levels, pointing at the necessity to invoke posttranscriptional and posttranslational regulation (Ghaemmaghami *et al.*, 2003; Washburn *et al.*, 2003; Brockmann *et al.*, 2007; Laloo *et al.*, 2009). Our work demonstrates that not only do such static discrepancies exist, but that stress adaptation occurs differently on the transcriptional and translational levels.

This work now puts forward a revised list of osmostress genes, taking translational regulation into account. Some of the most highly induced mRNAs in the polysomal pool achieve this solely through enhanced ribosomal attachment, and so represent potential translation-activated osmostress genes. This translational induction is strongly dependent on Hog1 and Rck2. Earlier global array investigations have pointed at the possibility that polysomal recruitment on its own may lead to expression changes. Importantly, we demonstrate in this work, with several examples, that protein level changes do match changes in polysomal mRNA better than those in total mRNA (Figure 6, A–C). It is essential to make this point, because increased mRNA association with polysomes, rather than reflecting active translation, could also be due to e.g., stalled ribosomes. A possible concern would be that some mRNA could be associated with processing bodies or stress granules, leading to potential misinterpretation. However, these RNA granules do not accumulate under moderate osmostress, as studied here, but at NaCl concentrations upward of 0.8 M (Teixeira *et al.*, 2005; Buchan *et al.*, 2008). Recently, it was shown that a number of mammalian DNA repair proteins are translationally upregulated, without transcriptional induction, after UV irradiation (Powley *et al.*, 2009). This provides a case that is parallel to our findings and strengthens the notion that for some responses, translation is the most important regulatory level.

For another group of genes, the transcriptional and translational responses after NaCl shock are counterdirectional (translation-deactivated osmostress genes). Significantly, we show in selected cases that this behavior is indeed reflected on the protein level: for mRNAs that are produced in higher quantities but fail to engage ribosomes, no corresponding protein increase is observed (Figure 6, D and E). To understand the seemingly paradoxical behavior of this subset, we need to consider the performance of these mRNAs under other stress conditions. In cells exposed to tertbutyl hydroperoxide (Swaminathan *et al.*, 2006), most of the mRNAs in this subset, which are likewise transcriptionally induced, are highly associated with polysomes (Supplementary Figure 2). Our interpretation is that many cellular stress conditions trigger the generic CESR based on the presence of STRE elements in gene promoters and stress-specific transcription factors. Elevated levels of the protein products of the CESR are appropriate for most stress conditions, but a subset may be inadequate for hyperosmosis. The translational level is then used to modulate the response of this subset, repressing energy-demanding protein production. Interestingly, the translational suppression of this subset is fully dependent on both Hog1 and Rck2 (Figure 3B), indicating that this is an actively regulated process.

Translational Mobilization Is Faster Than the Transcriptional Response

The multireplicated data from this work at several time points over the first 30 min of adaptation allows time-resolved comparisons of transcriptional versus translational regulation. The translational response should be faster as it allows preferential use of preexisting transcripts and circumvents the need for time-consuming gene transcription. The transcriptional response should be longer lasting. Indeed, previous studies on translational responses to environmental and hormonal cues in mammalian and yeast cells estimated that its magnitude exceeded that of the transcriptional response, early after the stimulus (Preiss *et al.*, 2003; Rajasekhar *et al.*, 2003; Lü *et al.*, 2006; Halbeisen and Gerber, 2009). In our work, we included very early time points after shock (2 and 6 min), allowing us to capture rapid shifts in the translatome. We found the translational response to be functionally coordinated already at 2 min, in that it comprises many gene products with a recognized role in the stress response. This is in contrast to the transcriptional response, which at this time point appears like noise from a functional standpoint. The entire translational mobilization thus precedes transcriptional induction: late in the response, while total mRNA levels of induced genes are still elevated, the translational response has actually reversed its direction, preparing for the recovery phase (Figure 2C). This is in line with the time courses of mRNA turnover and transcription after hyperosmotic shock (Molin *et al.*, 2009; Romero-Santacreu *et al.*, 2009). The delay between the peak of polysome-associated mRNA, between 6 and 30 min, and the appearance of induced protein at 30–40 min (Figure 6, A and C) is consistent with the delay observed for the mRNA and protein peaks for NaCl stress-induced genes (Supplementary Figure 1). It is conspicuous, however, that in all cases of translation-activated osmostress genes examined, the rise in protein levels occurs only after 30 min, even though the polysomal association for the corresponding mRNAs peaks significantly earlier, at 6 min after osmoshock. It is not to be excluded that a common mechanism operates on these mRNAs that are subject to translational regulation only. Given that a large proportion of the translation-activated osmostress proteins are membrane-bound, we speculate that the ribosome pausing described for the synthesis of proteins destined for cell membranes or secretion (Wolin and Walter, 1989) may provide a partial explanation for the delay observed. The purpose of such delayed translation could be to deliver newly made proteins required for the stress recovery phase exactly at the required place and time.

Specific Translational Regulation of Membrane Proteins and Selected cRPs

We have found two large protein classes to be enriched among translationally regulated transcripts: membrane proteins and cRPs. For membrane proteins, two principally different arguments are possible. First, they could be regulated coordinately because they are translated at a distinct location: at endoplasmic reticulum–bound ribosomes. As an alternative explanation, membrane-bound proteins, such as ion, sugar, and amino compound channels and transporters, might be needed more quickly after hyperosmotic shock and would be produced faster using translational mobilization as the main mechanism than with enhanced transcription. Arguing for the latter, functional, interpretation, we observe most of the sugar catabolic genes to be transcriptionally induced, but translationally repressed (Figure 5A). The joint observations of an early, strong translational induction of low-affinity sugar uptake genes and a strong translational repression of sugar catabolism genes suggest an early, transient intracellular accumulation of glucose, which may provide immediate osmoprotection, giving the cell time to ramp up more long-lasting defenses in the form of glycerol. From an ecological perspective, glucose may be a frequent inducer of osmotic stress, and a rapid glucose uptake by translational mobilization of low-affinity transporters is likely to be an efficient protection mechanism. Translational up-regulation of mRNAs encoding transport proteins, including *HXT2* and *MAL31*, was also observed under hyperosmotic stress using 1 M sorbitol (Halbeisen and Gerber, 2009). The multidrug transporter Tpo4 transports the polyamines spermine and spermidine (Tomitori *et al.*, 2001; Albertsen *et al.*, 2003). *TPO4* has not previously been acknowledged as an osmoresponsive gene; it is noteworthy that *tpo4* mutants were found to be sensitive to osmostress in high-throughput screens (Giaever *et al.*, 2002; Fernandez-Ricaud *et al.*, 2005).

We also find in this work a markedly divergent behavior of cRPs. Although the entire translational apparatus is strongly repressed (in a HOG-independent way) at multiple levels quickly after application of environmental stress, as reported by many groups previously, a subgroup consisting of about one-third of the mRNAs encoding cRPs is translationally up-regulated shortly after hyperosmotic shock. This continues up to 30 min, in spite of transcriptional repression. There is not enough distinguishing functional information about this subset to allow hypotheses on the specific roles of these cRPs in adaptation to stress; however, it is reasonable to assume that the cell is mobilizing existing ribosomal transcripts to resume growth when the most immediate needs with regard to protection systems has been satisfied. By contrast, there is no corresponding increase in polysomal RNA during oxidative stress for this subgroup, nor for any other group of RPs (Swaminathan *et al.*, 2006), presumably because there is no distinct recovery phase during continuous oxidative stress. The behavior of this cRP subgroup is dependent on Rck2, but not Hog1, in agreement with the previously observed general deregulation of cRPs in *rck2* mutants (Swaminathan *et al.*, 2006).

Potential Regulatory Mechanisms and HOG Pathway Dependency

Regulatory elements for posttranscriptional control are predominantly located in the 5′- and 3′-UTRs, although some are found in the coding region (Hogan *et al.*, 2008). In yeast, the use of epitope tags is commonplace. Routine constructs for epitope tagging interfere with untranslated regions (most commonly the 3′-UTR), and this may have led investigators to overlook the global extent of translational regulation. Analyzing array data representing total and polysome-attached mRNA from stress-exposed yeast cells, the authors arrived at the conclusion that long 5′-UTRs correlate with the propensity to be regulated on the translational level (Lawless *et al.*, 2009).

We have found the translational regulation after mild hyperosmotic shock (0.4 M NaCl) to be extensively dependent on the MAPK Hog1 and the downstream kinase Rck2. In a previous study (Melamed *et al.*, 2008), a much smaller dependence of translational activity of mRNAs on Hog1 was found than in this work. This has three probable causes. First, Melamed *et al.* (2008) used high osmotic stress (1 M NaCl). High or severe hyperosmotic stress is known to activate other signal transduction chains beside the HOG pathway and functions outside of the HOG pathway are required for survival under such conditions (Yang *et al.*, 2006). Second, activation of stress responses is slower and less distinct under high osmostress than under moderate hyperosmotic shock as in our work (Van Wuytswinkel *et al.*, 2000). On the basis of our quantification of the translational response after hyperosmotic shock, we chose early time points (2 and 6 min) where the effect on translation is maximal. We are thus likely to cover much more of the HOG-dependent posttranscriptional regulation events. Third, it was recently found, in a direct comparison within the same study, that mild hyperosmotic stress produces mainly a translational response, whereas the transcriptional component is more pronounced under severe stress (Halbeisen and Gerber, 2009).

Interestingly, we found regulation to be dependent on both Hog1 and Rck2. New observations, however, question the importance of transcriptional regulation by Hog1 for survival. A Hog1 construct that is anchored in the plasma membrane, and thus seems unable to enter the nucleus, regardless of phosphorylation status does not confer the severe growth defects under hyperosmosis observed for *hog1*Δ mutants (Westfall *et al.*, 2008). Indeed, it has been shown that Hog1 phosphorylates ion channels and transporters in the plasma membrane very early after hyperosmotic shock, facilitating adaptation (Proft and Struhl, 2004). Another possibility, interesting for the interpretation of this work, is that posttranscriptional regulation exerted by cytoplasmic Hog1 is important for survival under hyperosmosis. We also show here a role for Rck2 in transcriptional regulation, which is surprising because this is a cytoplasmic kinase. This could be explained by Rck2 bound to cytoplasmic Hog1, regulating its activity or location. In line with this, *rck2* mutants display increased nuclear Hog1 accumulation (Bilsland *et al.*, 2004), raising the possibility that Rck2 negatively influences Hog1 nuclear localization. Srk1, a fission yeast homolog of Rck2, is constitutively bound to Sty1, the Hog1 homolog (Asp and Sunnerhagen, 2003). Similarly, there are indications that Srk1 dissociates from Sty1 upon activation of the pathway, and that this in conjunction with transient Srk1 degradation is required for full Sty1 activity (Lopez-Aviles *et al.*, 2008). Even though a marked dependency on Hog1 and Rck2 is found for individual or small groups of mRNAs, the regulatory adaptive process overall is remarkably robust to genetic perturbations, in the sense that the responses, seen collectively for larger functional groups, are maintained in *hog1* or *rck2* mutants (Figure 5A). This is achieved by compensatory changes within each group on the transcriptional and translational levels, both regarding induction and suppression.

The translational response is ultimately governed by signaling pathways acting on RNA-binding proteins that recognize motifs in transcripts. The search for the key RNA-protein interactions required for this regulation will be aided by screening mutants lacking RNA-binding proteins for defects in translational regulation, and by direct isolation of RNA sequences attached to candidate binding proteins, as recently demonstrated for ribosome-protected RNA fragments (Ingolia *et al.*, 2009). We have experimentally demonstrated translational regulation in the stress response only in a handful of cases; to finally resolve the global degree of translational regulation, careful simultaneous comparisons will be needed with samples during identical experimental setups assessing changes in protein (e.g., SILAC mass spectrometry), total, and polysomal mRNA levels. Such results will determine how we should modify our view of stress adaptation physiology.

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