THE ROLE OF THE RCK1 AND RCK2 KINASES IN THE OSMOTIC STRESS RESPONSE OF BUDDING YEAST

By

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ABSTRACT

In response to high extracellular salt levels, *Saccharomyces cerevisiae* activates the HOG1 mitogen-activated protein kinase (MAPK) cascade, which signals to decrease cell growth and provide a method of survival in osmotic stress. Studies have shown that this occurrence can be attributed to an interaction between Hog1 and TOR (target of rapamycin); however, the method of interaction is unknown. Here, we use microarrays to investigate Rck2, a direct downstream substrate of Hog1, and its close homologue Rck1, as possible intermediates connecting Hog1 and TOR in the osmotic stress response. Our findings demonstrated that Rck1 and Rck2 were not acting in a salt dependent manner; however knocking out these kinases did cause significant changes in gene expression at 2452 genes. Rather, using a gene ontology search program, we found that Rck1 and Rck2 are involved in regulating cell growth, and that they interact with 7 transcription factors: Fhl1, Rap1, Ino2, Hir1, Hir2, Spt2 and YML081W.

INTRODUCTION

In order to survive in stressful conditions, cells conserve nutrients and energy by inhibiting cell growth. In eukaryotes the TOR (target of rapamycin) kinase serves as a control center for growth control. In a nutrient rich environment, TOR activates growth through the regulation of processes like ribosome biogenesis, transcription, translation, and repression of autophagy and nutrient transport (Cherry et al.). When the cell is under stress, however, TOR is blocked and cell growth is inhibited. Complete understanding of the TOR pathway circuitry has yet to be exposed. This pathway is of great interest due to the significance of cell growth in cancer, aging and diabetes. Understanding exactly how the cell decides to grow or arrest growth could ultimately lead to methods for control and prevent these diseases.

The Hog1 mitogen-activated protein kinase (MAPK) is activated by high osmolarity (Bilsland-Marchesan et al.). When the cell is in a high salt environment, active Hog1 promotes an osmotic stress response which allows the cell to adapt to its environment and survive under the harsh conditions (Bilsland-Marchensan et al.; Capaldi et al.). One study showed that mRNA translation is transiently inhibited in osmotic stress, and that the Hog1 MAPK pathway is required for this adaptation (Uesono and Toh-e 2002). This suggests that Hog1 may interact with TOR to pause cell growth during times of osmotic stress. The theory of interaction between Hog1 and TOR has never been proven, yet it does seem plausible. This repression could be due to direct phosphorylation of TOR by Hog1, or alternatively through an intermediate protein that connects the two pathways.

Further understanding of the osmotic stress response is reliant upon deciphering the circuitry of the HOG (high-osmolarity glycerol response) pathway. Hog1, as a MAP kinase, works using a signaling cascade, and one known downstream substrate of this pathway is Rck2 (Bilsland-Marchesan et al.). Phosphorylation by Hog1 is, to date, the only known regulator of Rck2. When the cell is under osmotic stress, Hog1 binds to the C-terminal regulatory domain of Rck2 and phosphorylates it at serine 519 (Bilsland-Marchesan et al.) Therefore, as the kinases Rck1 and Rck2 are regulated by Hog1, it is possible that they serve as an intermediate between Hog1 and TOR.

A first step in determining whether or not Rck1 and Rck2 are truly signaling between Hog1 and TOR, is to obtain a general understanding of their function within the cell. Here we use DNA microarrays to achieve this goal. A DNA microarray is a 1’’x2’’ glass microscope slide covered with thousands of distinct short (in our case 60 base) nucleotide oligimers, each immobilized at a high concentration in a distinct “spot.” The sequence of each oligonucleotide is

complementary to that of a single gene in the genome. We use this complementary binding to our advantage by isolating mRNA from a cell, using it as a template to synthesize cDNA, and allowing this cDNA to bind to its matching sequence on the array. Before the hybridization takes place, a fluorescent tag (Cy3 or Cy5) is added to the cDNA so that a laser scanner can detect the amount of cDNA bound to each array spot.

Microarrays are typically used to compare gene expression between cells which differ genomically, or which have been grown in different conditions. The cDNA extracted from each sample is labeled with a different fluorescent tag, and the samples are mixed together and hybridized to the array. The amount of cDNA that binds to each spot is directly related to the corresponding gene's expression in the cell. Because Cy3 and Cy 5 fluoresce different colors, the scanner can compare the amount of each sample at every fixed spot, showing the differences in gene expression between the two cellular conditions.

We hypothesized that Rck1 and Rck2 act as a link between Hog1 and the TOR pathway. Following phosphorylation by Hog1 under osmotic stress, it is possible that Rck1 and Rck2 phosphorylate TOR. This link transmits stress signals to TOR, causing a halt in mRNA translation and, therefore, cell growth. We tested his hypothesis using a Rck1/Rck2 double knock out strain of *Saccharomyces cerevisiae* and microarrays.

Our findings show that Rck1 and Rck2 do not act in a salt dependent manner, confirming that these kinases are not acting as the exclusive link between Hog1 and TOR. However, our data does illustrate that Rck1 and Rck2 are involved in the regulation of ribosomes, ion transport, carbohydrate metabolism, the nuclear lumen and the nucleolus. All of these are involved in cell growth, suggesting that Rck1 and Rck2 may somehow act as indirect regulators in the TOR pathway, or as regulators of only a small part of the TOR pathway. Our data also highlights

specific transcription factors (TF) that appear to be interacting with Rck1 and Rck2. These include Fhl1, Rap1, Ino2, Hir1, Hir2, SPT2 and YML081W.

MATERIALS AND METHODS

Yeast Strains:

The *Δ rck1/ Δ rck2* double mutant strain, which was constructed, started with the *Saccharomyces cerevisiae* diploid strain ACY138. We began by using the polymerase chain reaction (PCR) to amplify a gene marker (his3 or leu2), which was flanked on either side by the same 40 nucleotides upstream or downstream of the target gene (rck1 or rck2) (figure explaining PCR). The resulting PCR product, containing the marker gene (his3 or leu2) was then transformed into ACY138 using a standard protocol (Appendix A). Cells where the PCR product containing the marker gene has replaced the gene target through recombination (Fig. 1) were then isolated by plating the transformation mix on plates without histidine or leucine supplements (his- or leu-). Colonies from these plates were then sporulated to create 4 progeny haploid cells. The spores from each individual diploid cell were then separated using tetrad dissection (Fig. 2a), grown, and the haploid cells containing rck1::his3 or rck2::leu2 were identified by looking for growth on his- and leu- plates (Fig. 2b). Haploid *rck1Δ* and *rck2Δ* cells of opposite mating type were then mated in liquid culture, and haploid progeny containing both the rck1::his3 or rck2::leu2 mutations were isolated using the same procedures described above.

PCR: PCR was used to amplify the his3 or leu2 markers from the ACY042 and 43 plasmids and incorporate the appropriate 40bp flanking regions to generate the gene markers which replaced rck1 or rck2 in the cell genome during transformation. To do this, primers were designed by taking 40 nucleotides on one side of the rck1 or rck2 gene, and adding 20

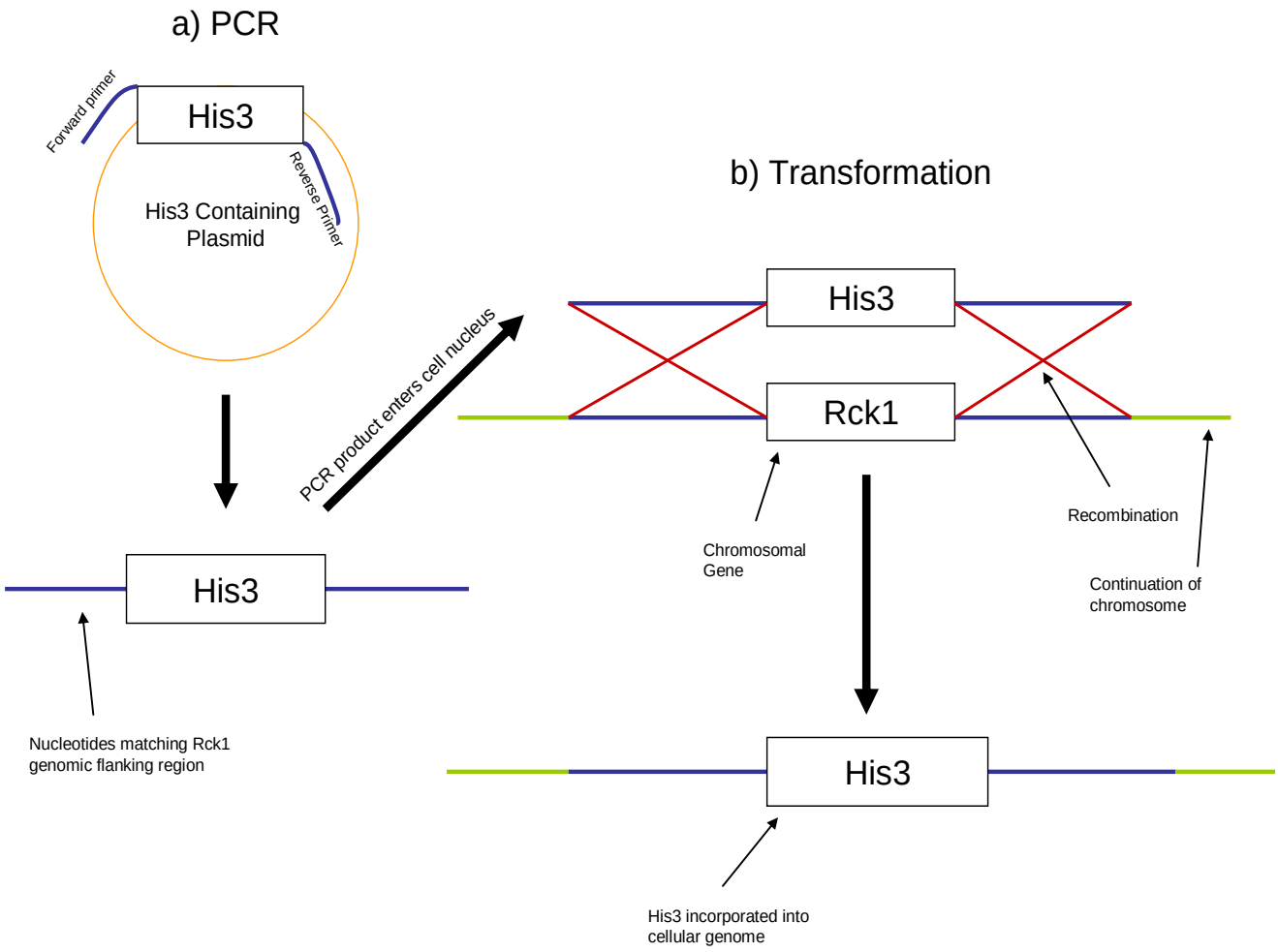

Fig. 1

a) To produce a selective marker gene that would recombine with, and therefore knock out, Rck1 or Rck2, the polymerase chain reaction (PCR) was used. The marker gene was amplified using primers, which included a sequence at each end of the gene that matched the nucleotide sequence flanking Rck1, or Rck2, in the genome.

b) Through heat shock transformation, the PCR product containing the marker gene enters the cell. Here, the complementary regions flanking each gene line up and recombination takes place, resulting in a gene knock out.

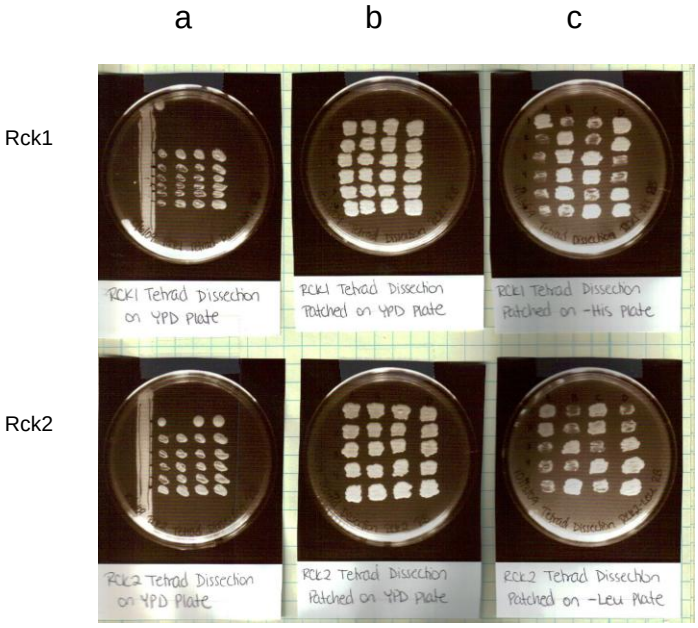

Fig. 2

a) Tetrad dissection of haploid cells (progeny from sporulation of diploid knock out cells) where Rck1 has been replaced by His3, or Rck2 has been replaced by Leu2, through recombination.

b) Haploid progeny cells of tetrad dissection, patched on YPD plate

c) Haploid cells patched onto selective marker plates. As expected, 2 out of every 4 progeny haploid cells have acquired the Rck1 or Rck2 knock out mutation.

nucleotides that matched the region flanking the his3 or leu2 gene on the corresponding plasmid. In total, 4 primers were ordered from Operon (forward and reverse for rck1, and forward and reverse for rck2) and each one totaled 60 nucleotides in length. Two 50µL PCR reactions were carried out. The first contained rck1KOf, rck1KOr and ACB043, while the second contained rck2KOf, rck2KOr and ACB044. PCR resulted in the amplification of the desired his3 and leu2

gene markers. The length of the products was checked by running the samples on a 1% agarose gel and comparing the bands to a 1KB ladder (Fig. 3).

Transformation: The his3 and leu2 gene containing PCR products were transformed into the cell using heat shock. First the diploid strain was grown overnight in 100ml of YEDP medium (95ml YPD plus 5ml 40% dextrose) at 30°C shaking at 200rpm. The following day, a new 100ml YPD + dextrose mixture was inoculated with the overnight culture to an optical density (OD_{600}) of 0.5. The culture was then grown at 30°C for two additional cell divisions (~5 hours). The cells were then harvested by centrifugation and rinsed multiple times with ddH2O, before being suspended in the transformation mix (Appendix A) the appropriate PCR product or ddH20 (negative control). The cells then underwent heat shock for 40 minutes at 40°C, then the whole transformation was plated on selective marker plates (his- for *rck1Δ* and leu- for *rck2Δ*). Through transformation, the PCR product gains entry into the cell, where the 40 nucleotides flanking the his3 and leu 2 marker genes can line up with the matching 40 nucleotides on either side of the rck1 or rck2 gene, respectively. This promotes recombination, where the chromosomal rck1 gene is replaced with the his3 marker, and the chromosomal rck2 gene is replaced with the leu2 marker (Fig. 1b). Incorporation of the marker gene into the genome gives the cell the ability to generate its own histidine or leucine, thereby allowing it to grow on a histidine or leucine deficient plate. Thus, we can use cell growth on a selective marker plate as an indicator of a successful gene knock out. After two days, growing, single colonies were patched onto new selective marker plates.

Sporulation: Every diploid eukaryotic cell contains two copies of each chromosome, yet growth on a selective marker plate requires recombination to occur on just one chromosome. Using sporulation, we were able to induce the division of each diploid cell to produce 4 progeny

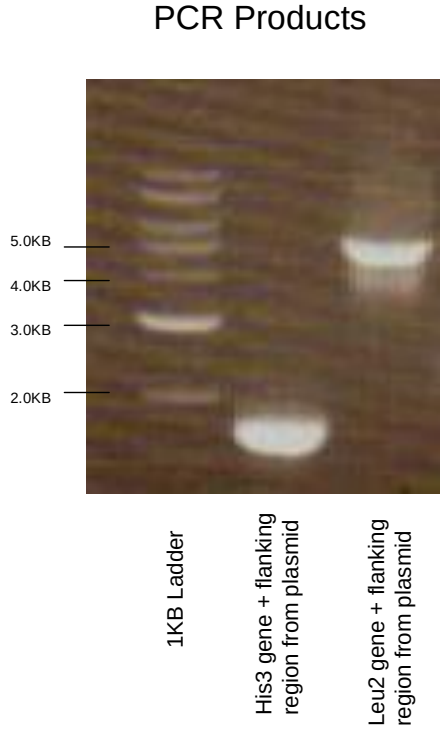

Fig. 3

PCR products were run on a 5% agarose gel and compared to a 1KB ladder to estimate the product length. The expected length corresponds to the length of the His3 or Leu2 gene plus the 60 nucleotides flanking each gene on the plasmid. This gel indicates that His3 is ~1.5KB and Leu2 is ~4.5KB, which were the expected products.

haploid cells. Because haploid cells contain only one chromosome, we knew that two of these cells would acquire the mutation and the other two would retain the wild type genome. By separating the cells with a tetrad dissection, we were able to select for haploid mutant cells of a specific mating type (Fig. 2). To induce sporulation of diploid cells, the cells from the selective marker plates were transfered onto sporulation plates, and left at room temperature for 5 days. Following sporulation, cells were picked using a pipette tip and were added to 20ul 0.5mg/ml Zymolayse (an enzyme that degrades the cell wall) in 1M sorbitol. 5 minutes later, 200ul 1M sorbitol was added, and 20ul of this mixture was run down a thin YPD plate (Appendix B). This effectively stops the zymolyase activity. 5-6 tetrads were then picked under a microscope using

a glass needle, and the four haploid cells were separated and spread across the YPD plate. These haploid cells were left to grow for 2 days at room temperature. Tetrads showing equal growth across all four cells at this time were then transferred onto YPD plates to create patches (squares of confluent cell growth) and were then replica plated onto separate lawn plates containing the mating test strains ACY026 (A mating type) and ACY027 (α mating type). Diploid cells were produced when haploid cells mated with test strain cells of the opposite mating type. The mixture of mated and diploid cells was then transferred onto minimal media plates (no amino acid supplement). The alpha cells grow only after mating to the a-type test strain and the a-cells grow only after mating to the alpha-type test strain.

Check PCR: Although we had obtained our *rck1Δ* and *rck2Δ* strains, we wanted to ensure that the selective markers had recombined into the correct genomic locations. For this, we used PCR to amplify a nucleotide region using one primer at the 5' end of the marker gene and one primer in a region 500bp upstream of the loci of the target gene (i.e. rck1 or rck2). In each case we identified a 500bp PCR product in our mutant, but not a control wild-type strain, confirming that our single knock out *rck1Δ* (ACY187) and *rck2Δ* (ACY188) strains had been successfully formed (Fig. 4).

Microarray:

The microarray is a glass slide on which thousands of gene sequences are fixed at specific locations. As a result of complimentary base pairing, cellular mRNA will find its complementary DNA sequence on the microarray and bind. Therefore, before running the microarray we needed to extract the mRNA from the cell. Instead of incubating the microarray with the mRNA, however, we used the mRNA as a template to synthesize cDNA, a double stranded and more stable form of the mRNA sequence. To acquire data comparing cells in

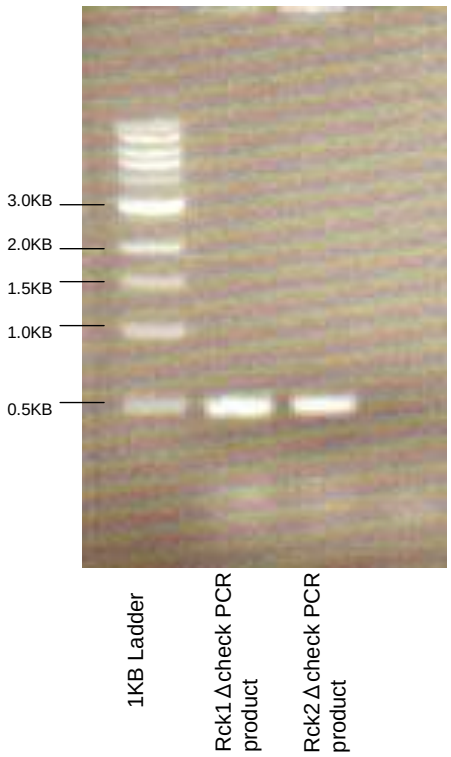

Fig. 4

Products of check PCR run on 5% agarose gel to determine whether recombination of His3 for Rck1, or Leu2 for Rck2, took place at the correct genomic location. The PCR primers were located at the 5' end of the marker gene (complementary to the reverse primer) and 500bp upstream of the Rck1 or Rck2 start codon. The gel shows that both products were roughly 500bp, indicating that His3 and Leu2 gene insertion occurred at the appropriate loci.

different conditions, we set up 4 cell cultures: *rck1Δ/rck2Δ*, wild type, *rck1Δ/rck2Δ* + KCl, and wild type + KCl. Each culture was grown for an equal amount of time allowing the same number of cell divisions to take place. Once the cDNA was isolated, each sample was tagged with either Cy3 or Cy5 fluorescent dye. Then the samples for comparison were mixed together and incubated with the microarray, so that the cDNA could bind to its matching gene sequence. We used a laser scanner to detect the amount of fluorescence at each gene spot on the array.

Cell Growth: The *rck1Δ/rck2Δ* double knock out cells used for the microarray were of the A mating type (Fig. 5). This mutant strain and the wild type strain ACY026 were each struck out onto YPD plates and left to grow for 2 days at 30°C. Following cell growth, 3 overnight

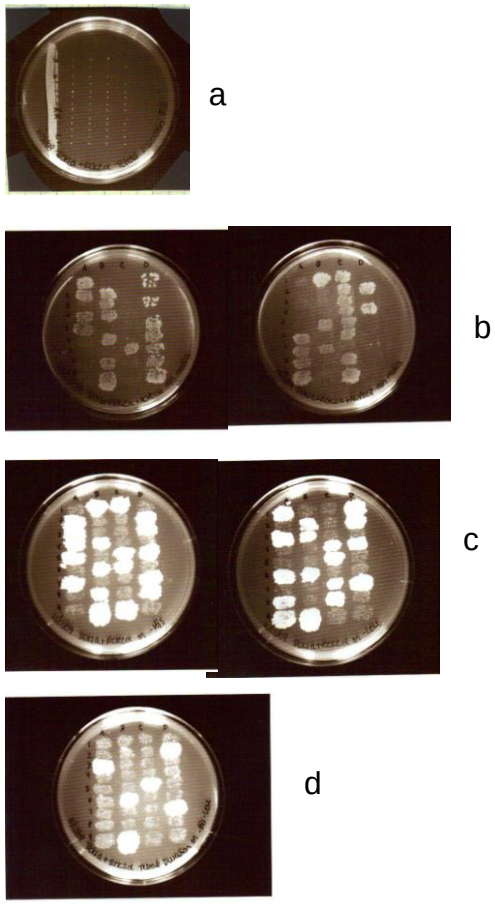

Fig. 5

a) Tetrad dissection of Rck1a/Rck2 αmated cells

b) Replica plate mating test of Rck1a/Rck2 αmated progeny cells. Left side is on ACY026 (a mating type) lawn, so cells growing on plate are αmating type. Right side is on ACY027 (αmating type) lawn, so only a mating type cells grow. This data was used to select for an a mating type cell.

c) Haploid progeny cells from Rck1a/Rck2 αmating are plated on His3 (left) and Leu2 (right) selective marker plates. These plates were used to identify spores with the appropriate genotype.

d) Haploid progeny cells from Rck1a/Rck2 αmated cells, on a –His –Leu selective marker plate. This allows for selection of a double mutant haploid cell.

cultures were set up in 250ml flasks. Each flask contained 47.5ml YPD and 2.5mL dextrose. *rck1Δ/rck2Δ* cells were added to the first flask, ACY026 cells were added to the second flask and the third flask was used as a control. The cultures were left to grow overnight on a shaker at 30°C. In addition, a 2.5L media was made in a large plastic beaker. This buffer contained 1% yeast extract, 2% peptone and 950ml of H_2O per liter. The buffer was separated into 2 flasks of 500ml, 2 flasks of 400ml, 2 beakers of 100ml, and the extra 500ml was poured into a beaker and KCl was added to a concentration of 1.875M. For sterilization, all solutions were autoclaved for 80 minutes and left to cool overnight. The following day a preliminary OD_{600} of the cell cultures was taken, using the control solution as the base point. Initial OD_{600} was staggered to allow for time separation in preparation for filtering cells. The ACY026 cell culture was added to a 500ml

flask with buffer to an OD_{600} of 0.1, and to a 400ml flask to an OD_{600} of 0.06. The *rck1Δ/rck2Δ* cell culture was added to 500ml of buffer to an OD_{600} of 0.08, and to 400ml of buffer to an OD_{600} of 0.04. The OD_{600} was read after 3 hours, 3 hours and 50 minutes, and every subsequent 10-15 minutes until an OD_{600} of 0.6 was reached. To create the various growth conditions, when each of the 400ml solutions reached OD_{600} 0.6 it was inoculated with 100ml of KCl containing buffer solution. For all solutions, reaching OD_{600} 0.6 marked the beginning of a 20 minute period of cell growth. After exactly 20 minutes, the cells were collected using vacuum filtration. The filter paper covered with cells was rolled, placed in a Falcon tube, and instantly frozen in liquid nitrogen. The cells were stored at -80°C. All subsequent steps were carried out using a protocol that can be found in Appendix C.

Total RNA Isolation: Now that the cells had been harvested, we needed to lyse them and isolate the RNA. The cells were taken from the -80°C freezer, gently warmed and dislodged from the filter paper using a 12ml AE buffer, and siphoned into RNAse-free centrifuge tubes. The cells were lysed by adding 800ul of 25% SDS and 12ml of pre-warmed acid phenol, then incubating at 65°C for 10 minutes while vortexing continuously. The mixture was incubated on ice for 5 minutes, followed by centrifugation at 10000rpm for 20 min at 8°C. The supernatant was then transferred into pre-spun Phase Lock tubes, and 13ml of chloroform was added to each tube. Tubes were then centrifuged for 10 minutes at 3000rpm and 4°C, and the supernatant was poured into an RNAse free tube. 1ml of 3M sodium acetate and 10ml of isoproponol were added to each tube, and the RNA precipitate was spun down for 45 minutes at 12000 rpm and 10°C. The pellet was gently rinsed with 10ml of 70% ethanol and was once again spun down. Remaining ethanol was removed with a pipette and the pellet was left to dry until the edges became clear (approx. 45 minutes). We then resuspended the pellet in 800ul of DPEC dH2O and

transferred it to an RNAse-free eppendorf. The sample was nanodropped at 1:10 dilution. The total RNA could now be stored at -80°C.

PolyA RNA Isolation: After RNA is transcribed from DNA in the cell, the RNA is cleaved by an endonuclease complex to form mRNA. Once the mRNA is formed, a polyA tail (~250 adenosine residues) is added to the 3' end of the nucleotide strand to protect it from further degradation. The mRNA now holds the sequence that will be translated for protein synthesis. This is the sequence that we utilized for the microarray by isolating the polyA RNA from the total RNA. All necessary materials and buffers can be found in the microarray protocol (Appendix C). Prior to starting polyA RNA isolation, 60mg of oligo dT cellulose was weighed into 2ml Sarstedt screw tubes and 4mg of total RNA was brought to 750ul with DEPC water. In addition, fresh 1xNETS, 2xNETS and 1xETS buffers were made. The 2xNETS buffer was heated to 65°C in a water bath and the 1xNETS buffer was heated gently to prevent precipitation. Oligo dT cellulose was mixed 3 times with 750ul of 1xNETS buffer. After each wash, the mixture was spun down at 3000rpm for 1 minute and the wash was removed with a pipette. The start time of the final wash also marked the start time of the 10 minute, 65°C RNA incubation. The oligo dT cellulose was resuspended with 750ul of 2xNETS buffer, then the RNA was added and the whole mixture was mixed on a rotor for 1 hour at room temperature. After 30 minutes, the 1xETS buffer was heated to 65°C. The chromatography column was primed with a 750ul 1xNETS wash. Then the RNA/cellulose mix was poured into the column and settled by gravity. The column was gently washed 3 times with 750ul 1xNETS buffer. For this, the buffer was released down the side of the column so as to avoid disturbing the cellulose. The RNA was forcefully eluted into a fresh eppendorf tube with 650ul of 65°C 1xETS buffer. This step was repeated into a second tube. 65ul of 3M Sodium Acetate was added to each tube, followed by

700ul of Isopropanol, and the samples were mixed gently by inversion. By spinning the sample at full speed and 4°C for 30-60 minutes, we were able to precipitate the RNA. The supernatant was removed and the RNA was washed with 250ul of 70% ethanol. Then the RNA was once again spun down at full speed for 5 minutes. The ethanol was removed and the pellet was left to air dry completely. The pellet was then resuspended in 40ul of DPEC dH2O and any residual cellulose was removed by spinning at full speed for 30 seconds. The supernatant containing the polyA RNA was transferred to a new eppendorf and nanodropped at 1:10 dilution. The samples were stored at -80°C.

RT PCR and aa-dUTP Incorporation: For increased stability, we chose to use cDNA for the microarray. We synthesized cDNA from the polyA RNA using reverse transcriptase (RT) PCR. Setup included a 50x dNTP and aa-dUTP mixure, a priming reaction and a cDNA synthesis mix (see Appendix C for details). The samples were taken from the -80°C freezer and centrifuged at 13,000 rpm for 1 min. The priming reaction was incubated at 70°C for 8 minutes, followed by incubation on ice. Then 14.5ul of cDNA synthesis mix was added to the priming reaction and the mixture was incubated at 42°C for 2 hours. To terminate the reaction and degrade RNA, 8ul of 50mM EDTA and 4ul of 1M NaOH were added, and the mixture was incubated at 65°C for 10 minutes. The cDNA was purified and concentrated using zymo kit columns. The samples were mixed with 600ul of binding buffer, washed twice (500ul and 200ul), and eluted with 12ul ddH_2O. Finally, the products were nanodropped and the cDNA containing aa-UTP was stored at -20°C.

Dye Labeling: The cDNA from each sample was labeled with either NHS Ester Cy3 or Cy5 fluorescent dye via coupling to the aa-UTP continued within each cDNA molecule so that we could detect the cDNA concentration on the array. Cy3 emits green light and Cy5 emits red

light. By tagging each sample for comparison with a different dye, we could measure the ratio of gene expression between the two test conditions. The Cy dyes were suspended in (n+1)ul DMSO. Then 1ul of 1M sodium bicarbonate was added to the cDNA samples, followed by the addition of dye. The *rck1Δ/rck2Δ* samples were labeled with Cy5 and the wild type samples were labeled with Cy3. The samples were mixed by pipetting, spun down, and stored in the dark for 4-6 hours.

Array Loading: Before loading the array, the cDNA needed to be purified. We did this with a zymo-kit using a standard protocol. The concentration of each sample was then determined using a nanodrop spectrophotometer, and diluted to 10ng/ul with ddH2O. 2 new eppendorf tubes were set up with 12.5ul of each sample for comparison. By mixing the samples prior to loading, the fluorescently labeled cDNA from each test condition was given an equal chance to bind to the gene spots on the array. In one eppendorf, we added *rck1Δ/rck2Δ* with no salt and the wild type with no salt, while in the other we added *rck1Δ/rck2Δ* + KCl and the wild type + KCl. The mixed samples were heated at 98°C for 2 minutes and spun down to cool. Then 25ul of 2x hybridization buffer (containing urea and detergents to ensure stringent binding conditions and limited background binding to the glass) was added and mixed by pippetting gently to avoid introducing bubbles. 40ul of sample was loaded onto the gasket side of the array, being careful to avoid introducing bubbles or touching the gaskets. The active side of the slide was then lowered down and the slide was sealed and incubated at 65°C for 17 hours in a rotating incubator. This gave the cDNA sufficient time to hybridize with the fixed DNA on the slide.

Array Wash: The next step was to wash all debris off of the slide so that only the tightly bound cDNA would remain. To begin the array wash, the microarray was removed from the cage while keeping both sides together. It was then opened while completely submerged in wash

buffer 1. The slide was quickly transferred to a rack in wash buffer 1 and was incubated for 1 minute. This first wash is low stringency, due to a high salt concentration. The rack holding the slide was then moved from wash bath 1 to wash buffer 2. This stringent wash only allowed highly homologous sequences to remain bound to the array. The slide remained in this was for a 1 minute incubation time, at which point the rack was moved from wash bath 2 to the drying buffer, and was incubated for exactly 1 minute.

Data Analysis: The microarray was read using a laser scanner, which measured the amount of Cy3 and Cy5 fluorescent dye at each spot on the array. The resulting data was then quantified using GenePix Pro 6.1 (Molecular Devices). The ratio of Cy3 to Cy5 intensity was calculated for each gene, and then uploaded into the Stanford Microarray Database (Hubble et al.). In order to eliminate background noise from the data, we used this program to remove any data where the signal from both Cy3 and Cy5 is less than 2-fold the background signal. Then the data was uploaded into Gene Cluster 3.0 (Eisen Lab). Only genes where the $\log_2$ of the intensity ratio was greater than 1 or less than -1 were clustered. The three microarray datasets that we compared were (*rck1Δ/rck2Δ* / wildtypte) and (*rck1Δ/rck2Δ* +KCl / wild type + KCl) from this experiment, and (wild type + KCl / wild type) which had been measured at an earlier time (Capaldi Lab). We used a single linkage cluster to organize the data because it gave the groups when compared to other clustering methods (Fig.6). Clusters were visualized using Java Treeview (Eisen Lab).

RESULTS

Regulation by Rck1 and Rck2 is not salt dependent. Following osmotic stress, Rck2 is phosphorylated in a Hog1-dependent manner (Bilsland-Marchesan et al.), suggesting that the

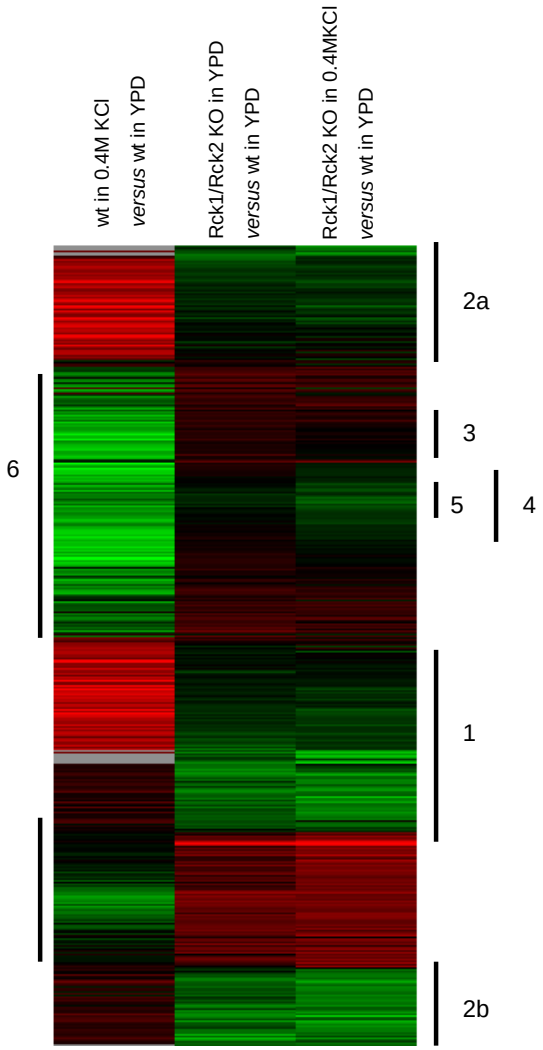

Fig. 6

Microarray Cluster (single linkage)

Each column represents a single microarray experiment comparing gene expression in two conditions (Cy5 labeled *versus* Cy3 labeled). Each row shows the data for a single gene. Genes are placed into groups (1-6) based on their expression behavior. Red indicates higher expression in the experiment than the control (higher Cy5 than Cy3) and green indicates a higher signal in the control than the experiment. Saturated red or green signals represent X fold up or down regulation respectively.

activity of Rck2, and its close homolog Rck1, is Hog1 and osmotic stress dependent. To test this hypothesis and determine the function of Rck1/2, we performed two microarray experiments: one comparing gene expression in a *rck1Δ/rck2Δ* strain to that in a wild-type strain, both in low salt conditions, and the other comparing gene expression in *rck1Δ/rck2Δ* to that in the wild-type strain, both in high salt conditions. In both cases the cDNA from Rck1/2 was labeled with Cy5 and the wild-type labeled with Cy3 (see Materials and Methods). To analyze and visualize this data, the Cy5/Cy3 ratio for each gene was log2 transformed and the microarray data was clustered (see Materials and Methods) to identify genes with similar behavior. This analysis

revealed that there is little difference between Rck1/2 in low and high salt conditions and therefore Rck1/Rck2 activity changes little in response to phosphorylation by Hog1. However knocking out Rck1 and Rck2 causes dramatic changes in gene expression at 2452 genes (Fig. 6). Therefore, Rck1 and Rck2 are important for gene expression, this function just does not change dramatically in stress.

Rck1 and Rck2 regulate processes involved in cell growth. From our data, we knew that Rck1 and Rck2 played a role in cell regulation (see above results), but their function was unknown. To determine the role of Rck1 and Rck2, we separated the microarray clusters into groups of genes with similar expression (Fig. 6). The groups compared the same genes across all three clusters to see how expression was changing in each condition. We then analyzed the list of genes from each group using the online program GOstat (Beisbarth). This program identifies statistically over-represented Gene Ontology (GO) terms in any list of terms. We selected a GO annotation with a p-value <0.005 as considered significant. By matching the GO annotation with the pattern of gene expression levels presented by the three microarray clusters, we were able to determine whether the presence of Rck1/Rck2 increased or decreased the activity of each process. We found that the presence of Rck1/Rck2 resulted in higher levels of ribonucleoprotein complex (p=1.07e-14), carbohydrate metabolism (p=9.95e-18), transmembrane ion transport (p=2.69e-21) and oxidoreductase activity (p=1.81e-14) (Fig. 7). We also found that the presence of Rck1/Rck2 lowered the expression of genes involved in ribosome biogenesis and assembly (p=0), the nucleosome (p=3.47e-13), the nuclear lumen (p=1.68e-21), the nucleolus (p=8.85e-21) and RNA processing (p=1.21e-16).

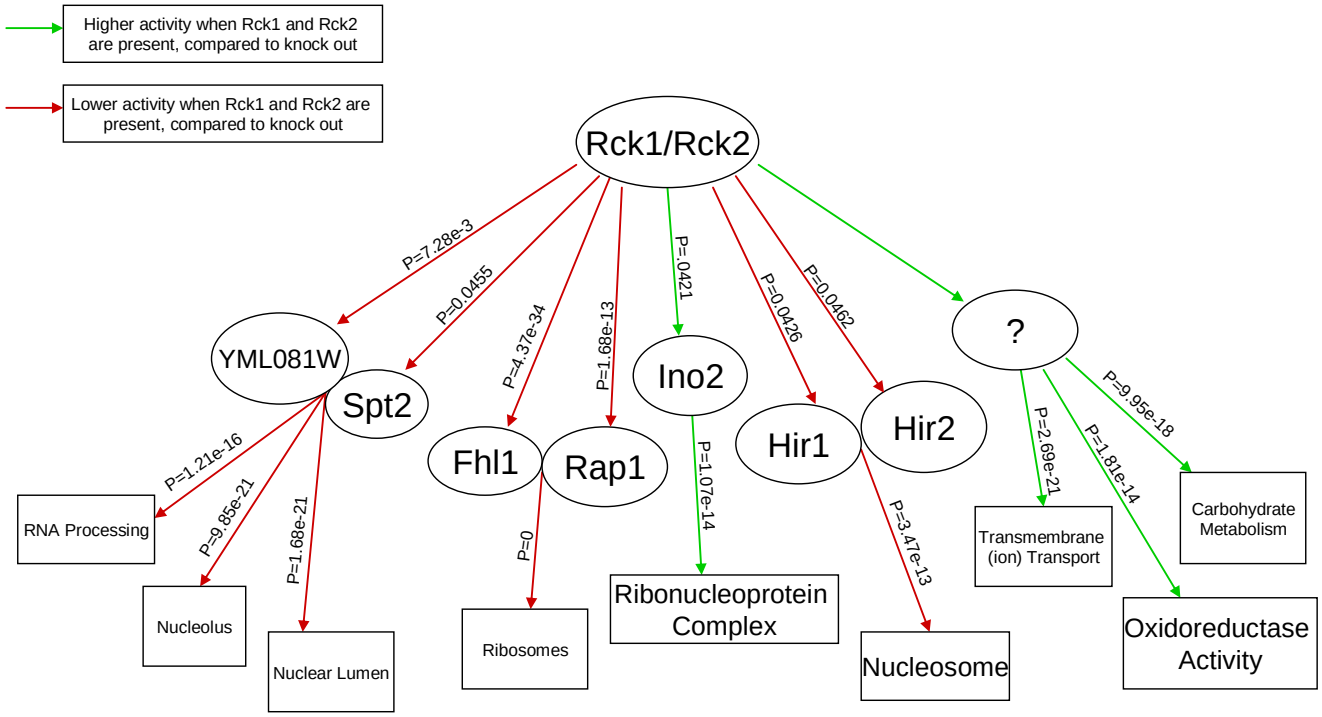

Fig. 7

Circuit diagram representing regulation by Rck1/Rck2. P<0.05 was considered significant. Rck1 and Rck2 together regulate cell growth processes, through interactions with 7 different transcription factors. The TF through which Rck1/Rck2 regulate carbohydrate metabolism was not found in our data.

Rck1 and Rck2 regulate 7 transcription factors: Fhl1, Rap1, Ino2, Hir1, Hir2, Spt2 and YML081W. From our data, we were aware that Rck1 and Rck2 were involved in the regulation of specific cell processes; however we did not know how this regulation was taking place. Thus far, we knew that Hog1 was activated in osmotic stress and that Hog1 activated Rck2 (Bilsland-Marchesan et al.). The question as to what activity was occurring downstream of Rck1 and Rck2 still remained. We sought to find the answer through transcription factors (TFs), because TFs directly control gene expression in the cell. We acquired a list of 204 eukaryotic TFs from a Young Lab publication, in which researchers determined the genes that were regulated by each TF (Harbison et al.). By comparing the genes regulated by each TF, to the

genes present in each microarray cluster group (see above results), we again looked for statistical over-representation within our cluster sets, but this time of TF binding not GO. Any group with p-value<0.05 was considered to be significantly linked to the corresponding transcription factor. From this, we found that SPT2 (p=0.0455) and YML081W (p=7.28e-3) had significant connections to Group 3, INO2 (p=0.0421) had a significant connection to Group 5, and Fhl1 (p=4.37e-34), Rap1 (p=1.68e-13), Hir1 (p=0.0426) and Hir2 (p=0.0462) all had significant connections to Group 6. This indicates that Rck1 and Rck2 are likely to be regulating these TFs, either directly or indirectly (Fig.7).

DISCUSSION

Increases in exracellular osmolarity cause activation of the HOG pathway. The HOG pathway responds to osmotic stress though a MAPK signaling cascade, which helps the cell adapt to the environmental changes (Bilsland-Marchesan et al.). When Hog1 is turned on due to high salt levels, the TOR pathway, which controls cell growth, is subsequently turned off (Capaldi et al.). While it is understood that this event is caused by an interaction between Hog1 and Tor, the method of interaction is unknown. It is possible that Tor is directly phosphorylated by Hog1, however, it is equally feasible that there is an intermediate acting between them. One known downstream substrate of Hog1 is Rck2, a protein kinase of which little information is known (Bilsland-Marchesan et al.). To investigate the role of Rck1 and Rck2 in the cell, and to determine if these kinases are acting as a link between Hog1 and the TOR pathway, we performed two microarrays comparing gene expression between Rck1/Rck2 knock out cells and wild type cells, in both high and low osmolarity environments. We demonstrate here that Rck1

and Rck2 are not operating as the sole link between Hog1 and TOR, however, they are regulating processes involved in cell growth through 7 identified transcription factors.

This study reveals that Rck1 and Rck2 do not affect the cellular response to osmotic stress. This finding, however, seems inconsistent with the fact that Rck2 is regulated by Hog1 and that these kinases are involved in regulating cell growth, a function of the TOR pathway. This leaves us to speculate on where Rck1 and Rck2 fall in the overall osmotic stress response. Through use of the online gene ontology search program GOstat, we discovered that Rck1 and Rck2 regulate 7 transcription factors: Fhl1, Rap1, Hir1, Hir2, Ino2, Spt2 and YML081W. Each of these transcription factors is involved in processes of cell growth (Fig. 7).

Interestingly, Rck1/Rck2 was most strongly correlated with Fhl1 and Rap1, two TFs that are known to interact and control ribosomal protein (RP) gene transcription (Martin et al.). A previous study showed that TOR controls Fhl1 through a Ras-PKA-Yak1 effector pathway (Martin et al.). This study suggests that active TOR turns on PKA which blocks Yak1, thereby allowing RP transcription via Fhl1 to occur. However, in unfavorable growth conditions TOR does not activate PKA, allowing active Yak1 to promote localization of Crf1 into the nucleus, where it binds Fhl1 and turns off RP transcription (Martin et al.). Although we have not yet considered the role of PKA in the osmotic stress response, and our two microarray experiments lack sufficient evidence to confirm any intermediate regulation between Rck1/Rck2 and Fhl1, it is possible that Rck1 and Rck2 may play a role in this pathway to either directly or indirectly regulate Fhl1 and Rap1. This connection, however, still fails to explain why Rck1 and Rck2 do not seem to impact the osmotic stress response. Furthermore, the involvement of Rck1 and Rck2 in this pathway is merely speculation and would require further research for verification.

Another study showed that the transcription factor Spt2 genetically interacts with members of the HIR/HPC complex, which includes Hir1 and Hir2. The HIR/HPC complex is involved in transcription elongation (Nourani et al.), which is consistent with our finding that Hir1 and Hir2 play a role in nucleosome regulation (Fig. 7). This study also showed that Spt2 plays an important role in genomic stability by protecting regions from hyperrecombination (Nourani et al.). This too is consistent with our findings that Spt2 is involved in RNA processing. Our data indicates that all of these transcription factors are less active in the presence of Rck1 and Rck2, suggesting that Rck1 and Rck2 negatively regulate Spt2, Hir1 and Hir2 together to cause a decrease in cell growth.

Other transcription factors that appear to be regulated by Rck1 and Rck2 are Ino2 and YML081W. Ino2 is involved in ribonucleoprotein complex regulation and appears to be upregulated in the presence of Rck1 and Rck2 (Fig. 7). YML081W is involved in regulation of the nucleolus, the nuclear lumen and RNA processing (Fig. 7). This makes sense because it is less active in the presence of Rck1 and Rck2, causing cell growth to decline. Although both of these TFs appear to act downstream of Rck1 and Rck2, little outside information could be found on either one to suggest how regulation might be taking place.

In this study, we also reported that Rck1 and Rck2 are involved in the positive regulation of carbohydrate metabolism, transmembrane ion transport and oxidoreductase activity (Fig. 7). However, we were not able to identify any transcription factors acting downstream of Rck1 and Rck2 that would explain these results.

CONCLUSION

Although the experiment did not give the results we expected, it was successful in many ways. In our search to understand the method of Hog1 and TOR interaction, this study allowed us to rule out one hypothesis. Now that we know Rck1 and Rck2 are not acting in the response to osmotic stress, we can focus our efforts on exploring other ideas, such as the direct phosphorylation of TOR by Hog1, or the possibility of a more complex chain of intermediate interactions. In addition, the two microarray experiments that we performed left us with a large amount of data regarding the functions of Rck1 and Rck2 in the cell. Although independent of the osmotic stress response, gene expression levels helped us find 7 transcription factors which all appear to be regulated by Rck1 and Rck2. Even though these finding were not what we anticipated, this project did make a novel discovery, which adds to our understanding of stress responses and growth control pathways.

Through this project I not only expanded my understanding of the intricate processes that contribute to cell survival, but I also acquired a great appreciation for the thought process, time, money, and work that goes into scientific research and data analysis. Even this seemingly simple, two microarray experiment was a two semester project, split between data collection and data analysis. Although frustrating at times, this project was an incredible learning experience because it allowed me to formulate hypotheses, reason the use of specific scientific tests, perform lab experiments, troubleshoot, and analyze large amounts of data to deduce logical results; none of which can be learned by sitting in a classroom. Through the process of completing this project, I have come to the conclusion that research is less about definitive answers and more about connecting small pieces of information to reach a higher level of understanding.

Therefore, despite the disproval of our hypothesis, I am pleased with our results and hope that they can be of use for future research into understanding the complex workings of the cell.

FUTURE WORK

Our microarray experiments left us with a large amount of data, but few definitive conclusions. These experiments only looked into the effects of knocking out both Rck1 and Rck2 together to determine whether these kinases were linking Hog1 to the TOR pathway. We now know that this is not the case, but we can still use the data we collected to understand the function of Rck1 and Rck2. Therefore, repeating the experiment with single knock outs would be useful in learning more about each kinase's individual function. An important fact to note is that unlike Rck2, Rck1 is not necessarily regulated by Hog1. Thus, it may be valuable to investigate the upstream regulation of Rck1 to determine if it likely has any involvement in the osmotic stress pathway, or if perhaps it functions elsewhere.

We also collected data on transcription factors that appear to be regulated by Rck1 and Rck2. To gain a better understanding of these interactions, experiments could be done to determine if Rck1 and Rck2 are directly phosphorylating these TFs, or if there are intermediates involved. Other future work could include repeating the experiment using oxidative stress because both kinases are known to act in this response pathway. Therefore, observing gene expression changes caused by knocking out Rck1 and Rck2 in oxidative stress may provide better insight into their general functions.

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APPENDIX A

Transformation

Inoculate 5ml YPD with yeast strain of interest in tube and grow in shaking incubator at 30^{o}C overnight.

Re-inoculate 95ml YPD + 5ml 40% dextrose with overnight culture to an OD ~0.5 and allow to grow for a further 2 cell divisions (OD~2, ~5hrs). 100ml culture will provide enough cells for 20 transformations (Can scale down to 50 ml)

Prepare salmon sperm carrier DNA with 5min boil

Split culture into 2x 50ml Falcon tubes and pellet cells with 5min 3000rpm room temperature spin

Remove supernatant and resuspend cells in 25ml ddH2O

Pellet cells with 5min 3000rpm room temperature spin

Remove supernatant, resuspend in 1ml ddH2O, and transfer to eppendorf tube

Pellet cells once more at 8000rpm for 30s and remove supernatant

Bring up cell volume to 1ml with ddH2O

Transfer 100ul cells to new eppendorf for each transformation

Pellet cells once more at 8000rpm for 30s and remove supernatant

Resuspend cells by vortexing with 316ul transformation mix and 44ul PCR DNA or ddH2O (negative control)

Heat shock cells at 42^{o}C for 40 mins

Pellet cells with 30s 6000 rpm spin and remove supernatant
If PCR product for tagging or knock out, **Gently** resuspend cells in 150ul ddH2O – mix with pipette tip, do not aspirate or vortex. Plate whole transformations on selective marker plates and incuabate for 2 days at 30^{o}C. For plasmid transformations, add 1ml of water instead of 150ul. Then plate 100ul of a 1:100 dilution for a single plasmid, 1:50 dilution for loop in-loop out, and 1:10 dilution for 2 plasmids.

Transformation Mix	**x1**
50% w/v PEG3350	240ul
1M LiAC	36ul
Salmon Sperm carrier DNA	20ul

ddH2O	20ul
total	316ul

Raised white colonies should be visible (top down view) after 36-48hrs. These can be picked and restreaked onto selective media plates, and incubated as before for a further day.

Pick single large colony and patch onto selective media. The remainder may be used for colony PCR check.

APPENDIX B

Tetrad Dissection

PATCH diploid cells on YEPD, so they are growing happily
Day 2: Patch diploid cells on SPORULATION plates, leave at room temperature for ~5 days
Pick cells with pipette tip/matchstick and add to 20ul 0.5mg/ml Zymolayse in 1M sorbitol.
Leave at room temperature for up to 5mins before adding 200ul 1M sorbitol.
Run down 20ul down thin YPD plate
Pick tetrads
Leave to grow for ~2days (room temp or 30°C is fine)
Compare tetrad growths. Avoid single mutants (single small or large tetrads) and pay attention for differences between tetrad pairs.

Mating Test

Patch tetrads onto YPD plate ensuring sufficient spacing between patches. Replica plate mating test strains (ACY026 and ACY027) onto separate YPD plates. Grow Plates overnight.
Replica plate mating test strains onto separate YPD media plates. Repeat with tetrad patches onto both plates (use separate velvet cutouts). Incubate overnight, opposing mating types will form diploids.
Replica plate mated prints onto separate MINIMAL media plates. As the mating strains are auxotrophic only for lys1 but the EY0690 base strain is *lys1+*, only mated cells will be able to grow. Ensure two opposite pairs of tetrads grow on the plates.

APPENDIX C

Total RNA Isolation from S. *cerevisiae*

Materials (all solutions in DEPC H20), solutions CAREFULLY pH'd

YOU MUST BE METICULOUS WITH TUBE LABELLING ALL THE WAY THROUGH THE PROTOCOL!!!

50ml size RNase-free 50ml Nalgene centrifuge tube
50ml 5-primephase lock tubes, heavy gel
25% SDS (w/v)
AE Buffer (AE=50mM NaAcetate pH 5.2, 10mM EDTA)
This 8.33ml 3M NaOAc, 10ml 0.5M EDTA in 500ml
Acid Phenol (Fluka 77608) in fridge
Chloroform (fume cupboard)
3M NaAcetate (pH 5.2)
Isopropanol (fume cupboard)
70% EtOH

Protocol

1.) Collect cells on filter (approx 250ml at OD600=0.6). Place filter in 50ml Falcon tube in -80 freezer.

2.) Thaw phenol in 65C water bath, and set centrifuges to chill

3.) Add 12ml of AE buffer to filter, unroll and shake, transfer to 50ml Nalgene

4.) Add 800ul 25% SDS and 12ml 65C acid phenol

5.) Incubate 10' in 65C water bath, vortexing every 20s

6.) Incubate 5' on ice, and find balance for each tube

7.) Spin down for 20' at 10K rpm on the SS34 rotor (floor standing centrifuge) at 8C

8.) During the above spin, prepare a phase lock tube by spinning at 1500rpm for 5' on the Legend RT benchtop 4C

9.) Decant the supernatant into the pre-spun phase lock tubes. Do not transfer pellet. Add 13ml of chloroform, shake to mix.

10.) Spin down for 10' at 3000 rpm on legend at 4C

11.) Pour supernatant into new Nalgene centrifuge tube. Add 1m 3M NaAC and 10ml of room temperature isopropanol.

12.) Spin for 45' at 12K rpm in SS34 rotor at 8C

13.) Decant supernatant and add 10ml of 70% EtOH, spin again 20' at 12K

14.) Decant EtOH and spin again for 2'. Remove remaining EtOH with pipette

15.) Resuspend pellet in 800ul of DEPC H2O

16.) Add 5ul RNA to 45ul DEPC H2O and analyze on nanodrop

For a good prep yield should be greater than 2mg total
260/280 ratio is approximately 2.1-2.2

polyARNA Isolation from S. *cerevisiae*

Materials (all solutions in DEPC H20), solutions CAREFULLY pH'd

YOU MUST BE METICULOUS WITH TUBE LABELLING ALL THE WAY THROUGH THE PROTOCOL!!

2ml screwcap tubes
Bio-Rad disposible columns
Oligo-dt cellulose (in freezer)
5.0M NaCl
0.5M EDTA
1.0M TrisHCl pH 7.4
10% SDS
70% EtOH

Protocol

1.) Measure 60mg of oligo dT cellulose into a 2ml tube, one per RNA prep

2.) Make fresh buffers

	1X NETS	2X NETS	1X ETS	
RNase free H2O	5.2ml	0.67ml	1.425ml	
5M NaCl	0.75ml	240ul	-	
0.5M EDTA	125ul	40ul	30ul	
1M Tris pH7.4	62.5ul	20ul	15ul	
10% SDS	125ul	31.25ul	30ul	

This is for one prep scale up as necessary. 1X and 2XNETS need to be heated to 65C. Watch 1X NETS for ppt. If SDS starts to crash out, warm gently 1-2 min in 65C water bath and cool back to room temp before use. DO NOT wash column in warm NETS you will lose your RNA

3.) Dilute RNA to 4mg in 800ul RNase free water. If you do not have enough >2mg or so will still work

4.) Wash oligo dT cellulose 3X with 750ul 1XNETS. Spin down gently, 3000rpm 1min, between washes and aspirate) Spin down an extra time at the end and remove most buffer with a yellow tip

5.) Incubate RNA samples at 65C for 10min

6.) Add 750ul of RNA sample and the 750ul of 2XNETS to each oligo dt tube

7.) Mix the rubes on a rotator for 1hr

8.) Prepare column by washing with 750ul 1X NETS

9.) Gently pour RNA/cellulose mix into column and allow it to settle by gravity.

10.) Gently wash column three times with 1X NETS (pipette down the side of the column)

11.) Elute the RNA into a fresh eppendorf by pipetting 650ul for 65C 1XETS directly and forcibly into the cellulose

12.) Add 65ul 3M NaOAc and 700ul of Isopropanol to each tube. Mix well by inverting the tubes several times and leave at -20 overnight

13.) Spin sample at 4C in a microfuge at max speed for 1hr.

14.) Aspirate the supernatant and wash pellet (but do not disturb) with 500ul of 70% EtOH and spin down at full speed room temp for 5'

15.) Remove EtOH, spin again and remove remaining EtOH. Dry on the bench, pellet should be white and powdery, around an hour.

16.) Add 40ul of DEPC water (20ul if the strain you are using is sick and grows slowly), dissolve RNA, and then spin down again, transfer supernatant to a fresh tube to remove oligo dt cellulose fines by centrifugation at full speed for 1min.

17.) Check the concentration (2ul mRNA+ 18ul DEPC H2O) on nanodrop.

Sample should be around 260/280 2.1 and 80ug of RNA

Reverse Transcription

Materials (all solutions in DEPC H20), solutions CAREFULLY pH'd

YOU MUST BE METICULOUS WITH TUBE LABELLING ALL THE WAY THROUGH THE PROTOCOL!!!

DO NOT REFREEZE ALIQUOTS

50X aa-DNTP mix

50ul of 100mM dATP
50ul of 100mM dCTP
50ul of 100mM dGTP
30ul of 100mM dTTP
20ul of H2O

add all of this to 1mg of aa-dUTP powder. Make 6ul aliquots

Protocol

1.) Set up priming reaction for each RT: 1ul of oligoT primer/N9 mix, 5ug/ul each and 4ug of polyA RNA in 14.5 ul.
2.) Incubate at 70C for 8min, cool on ice

3.) Setup master mix

Reagent	
10X RT Buffer	3 ul
aa-dNTP mix	0.6 ul
100mM DTT	3 ul
Reverse Transcriptase	1 ul
Water	6.9 ul

4.) Add 14.5ul of mastermix to each priming reaction

5.) Incubate at 42C for 2 hours

6.) Add 8ul of 50mM EDTA and 4ul of 4ul of 1M NaOH to each tube. Incubate at 65C for 10'

7.) Add 40ul of 1M HEPES pH 7.0 to each tube to neutralize.

8.) Purify using Clean and Concentrator Kit (ZYMO)

Add 1ml of binding buffer to each tube and load onto column, will take two steps.

Wash with 500ul of wash buffer and then again with 200ul of wash buffer

Add 12ul of H2O incubate 1min, and spin down.

Check cDNA on nanodrop

Yield is around 1.5-2ug total

260/280 ratio=1.8

Freeze cDNA in -20