Exploring Transcriptional Regulation in *Saccharomyces cerevisiae*: from Binding Motifs to ncRNA

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Abstract

For a transcription factor to be able to bind DNA, it must recognize a specific nucleotide sequence called a binding motif. Looking at a specific transcription factor and to where it binds across many strains of *Saccharomyces cerevisiae* allows for insight as to how effective a gene's motif is, and how the site may be regulated. Understanding this basic level of transcriptional regulation is key to understanding how gene expression is controlled.

Another factor affecting gene expression is antisense transcription, which was previously considered as mostly transcriptional noise, but has recently been found to act as a transcriptional regulator (Gelfand, B., et al., 2011). It can affect gene expression either from its own transcription or from the noncoding RNA (ncRNA) that it produces. The *FRE6* gene-- a gene that curates ferric-chelate reductase activity-- has an antisense transcript associated with it. The expression of the ncRNA produced correlates with the activity of *AQY2,* which shares an intragenic and promoter region with *FRE6*.

The goal of the following experiments is to understand the relationship between a DNA sequence and transcription factor binding by examining which genomic contexts are important for the binding of Reb1 and the transcription of *FRE6* ncRNA by analyzing many yeast strains, as well as examining the role *FRE6* ncRNA in co-regulation of *AQY2.*

Introduction

Understanding the relationship between DNA sequence, Reb1 binding and ncRNA transcription

The *FRE6* gene in S. cerevisiae is a ferric reductase that is expressed in low iron conditions. Fre6p is found in the cell's vacuoles, and also plays a role in copper and iron ion import and sequestering. One known regulator of *FRE6* expression is Reb1. Reb1 is a transcription factor to *FRE6* that is a RNA Polymerase I enhancer binding protein. The binding motif for Reb1 in the 3' end of *FRE6* is fairly well conserved, and analysis of Reb1 binding throughout the genome produced an average binding motif. The motif at *FRE6* follows the average with the exception of a single nucleotide that differs amongst yeast strains. ChIP-seq data show that Reb1 binds in the Σ1278b strain of *S. cerevisiae* but not the S288c strain (Figure 1). A single nucleotide polymorphism (SNP) exists between the two binding motifs. ChIP-seq also shows the presence of a *FRE6* ncRNA in Σ1278b, but, again, not in S288c (Figure 1). This suggests a possible relationship between Reb1 binding and ncRNA production. Comparing the differences between multiple strains could shed light on the *cis* and *trans* contexts affecting regulation at this locus.

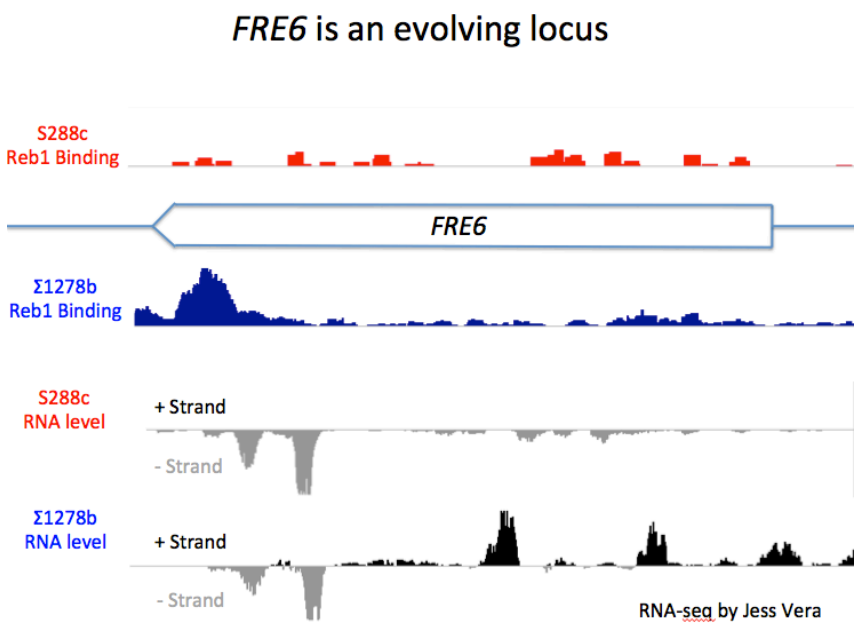

Figure 1: ChIP-seq and RNA-seq data in *FRE6* region (chromosome 12: 37,250-39,500). The top track (red) shows the ChIP-seq binding signal in S288c for myc-tagged Reb1. The second track (blue) shows the ChIP-seq binding in Σ1278b for myc-tagged Reb1. The third track (light gray) shows RNA-seq data for RNA levels at locus in S288c. The bottom track (black and light gray) shows RNA-seq data for RNA levels at locus in Σ1278b. RNA-seq is by Jess Vera and ChIP-seq is by Tim Read.

Background of AQY2 *in* S. cerevisiae

As a member of the aquaporin family, the yeast gene *AQY2* encodes for a water channel that mediates water transport across cell membranes and is only expressed in proliferating cells. As a result, its expression is controlled in part by osmotic signals, and it is thought to potentially be involved in freeze tolerance. *AQY2* and the *FRE6* ncRNA are arranged in divergent orientation, separated by 950 base pairs. Many lab yeast strains, including S288c, have a non-functional *AQY2* as the sequence is disrupted by a stop codon. However, in the strains that have a functional

AQY2, its expression is seen to spike at the beginning of mid-log phase, then is seen dropping in levels throughout mid-log phase (Figure 2).

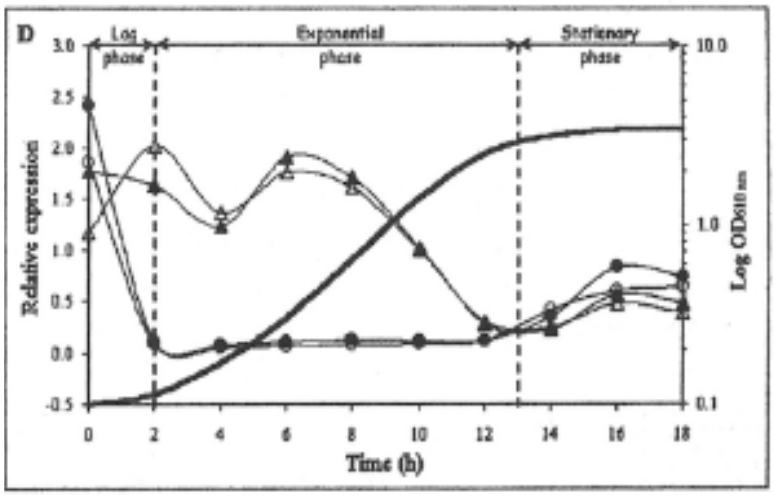

Figure 2: Expression of yeast aquaporins during growth in YPD medium. A) Strain Σ1278b B) *aqy1*Δ mutant C) *aqy2*Δ mutant D) time-course relative expression of AQY1 (circles) and AQY2 (triangles) in Σ1278b (filled symbols) or in deletion mutant strains (open symbols).
A1=AQY1, A2=AQY2, I1=IPP1

Figure reproduced from: Laize, V., Celeste, Ferreria, M.C.D.J., & Hohmann, S. (2000) Aquaporins in *Saccharomyces cerevisiae. Molecular Biology and Physiology of Water and Solute Transport.* 416-421. Springer

Tim Read, a graduate student in the Dowell lab, shows that the RNA levels of both the *FRE6* ncRNA and *AQY2* are almost identical in several yeast strains (Figure 3), suggesting that they are connected in their regulation. Yeast strains TR018 and TR023 are strains created in the Dowell lab to explore the importance of the intragenic region between *FRE6* and *AQY2.* TR018 is Σ1278b with its whole intragenic region replaced with that of S288c (Figure 4). This swap shows that the loss of Σ1278b's intragenic region leads to a substantial decrease in the levels of both *AQY2* and the *FRE6* ncRNA transcripts. There are 28 SNPs existing in this region between Σ1278b and S288c and TR023 replaces 14 of Σ1278b's with S288c's

(or, replaces half of the intragenic region). This strain also shows a decrease, though it is not as extreme as that of TR018's, of both the *AQY2* and *FRE6* ncRNA transcripts. This figure not only shows that levels of both transcripts are closely related, but also shows that the region between the two transcription start sites is important to their regulation, meaning they have common regulatory elements.

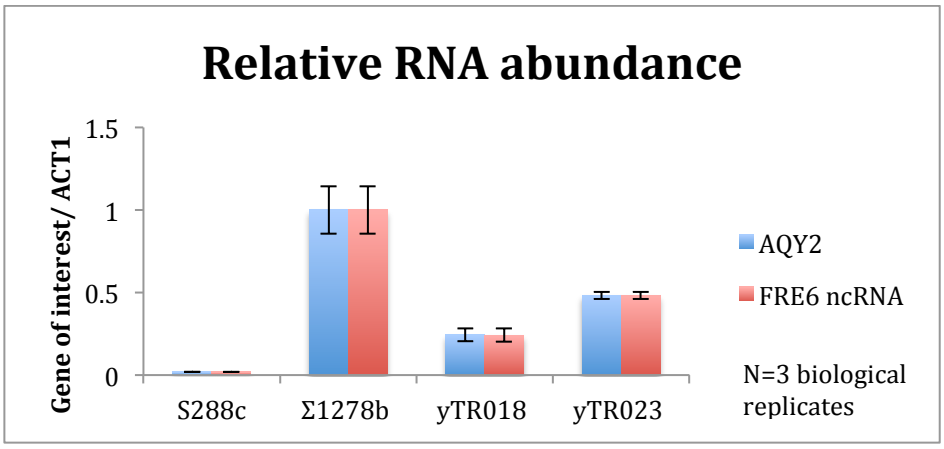

Figure 3: qPCR data of relative RNA abundance of both FRE6 ncRNA and AQY2, normalized to ACT1 RNA in S288c, Σ1278b, yTR018, and yTR023 yeast strains. Courtesy of: Tim Read of the Dowell lab.

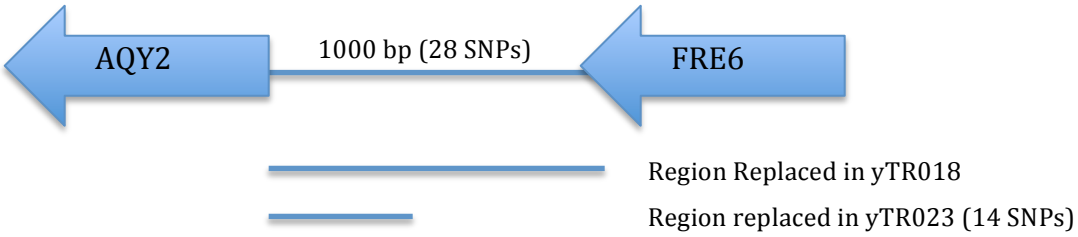

Figure 4: Orientation of *AQY2* and *FRE6* with 1000bp intragenic region. In yTR018, this whole region (with all 28 SNPs) of Σ1278b was replaced with S288c's. yTR023 replaced only the *AQY2* proximal half of Σ1278b's intragenic region (containing 14 SNPs) with S288c's.

An overview of noncoding RNAs

The role of RNA itself is much better understood now as more than simply just the mechanism to translate nucleotides to protein, but its other cellular roles are still not completely clear. Antisense transcription is present throughout many organisms, especially in laboratory *S. cerevisiae* strains, and produces ncRNAs. ncRNAs certainly are highly pervasive throughout the cell and throughout nature's genomes. Focus has been increasingly placed on ncRNA as more than 30% of annotated transcripts in the human genome have been discovered to have antisense transcription (Ozsolak, F. et al., 2010). Given this link to understanding our own genetics, it is desirable to understand ncRNAs role in a model organism, such as yeast.

The characteristics of ncRNA, like its function, can vary a great deal. Classified first as short or long ncRNA, short ncRNAs are less than 200 nucleotides in length while long ncRNAs are longer than 200 nucleotides in length. Many short ncRNAs have already been discovered to be components in genetic regulation. Some classified short ncRNAs include small interfering RNAs and microRNAs, which play roles in post-transcriptional regulation, mRNA targeting, and gene repression, all of which can affect what the cell will eventually translate into functional proteins. Long ncRNAs, which include antisense transcripts, are, for the most part, not as well understood. Though the structure of antisense transcripts is not biochemically unique from other transcripts, they often lack a proper open reading frame (ORF), making them unable to code for proteins. Their functions are beginning to be

studied, and, because of their proximity to protein-coding and non-protein-coding genes, their function is hypothesized to involve gene regulation. This role may be aided by the fact that, unlike protein-coding RNA, most ncRNA is localized in the nucleus of the cell (Derrien, T., et al 2012) where it is able to influence components of transcription.

Identifying antisense transcripts can be difficult from sequence alone. However, once transcribed, several patterns and traits of theirs become apparent. Independent, bi-directional, or cryptic promoters can initiate transcription, but the rate of their transcription is generally ten times lower than that of coding genes in the cell, and seems to be linked to the transcription of neighboring genes (Ozsolak F., et al. 2010). In yeast, it seems that bi-directional promoters regulate most antisense transcripts (Xu, Z., et al. 2009), which may shed light why expression levels of several transcripts (sense and antisense) are linked. Using these traits, experiments can be designed and adjusted to better explore the role ncRNAs might play in the cell.

Antisense transcripts and their effects on the cell

Even though it does not code for genes, the DNA coding for ncRNA does play a role in regulation, so it requires conservation to remain functional. However, the resulting antisense transcript has a flexible nature and can play several roles because it does not code for proteins. This may give it an advantage as a genetic regulator, allowing the gene to potentially have multiple regulatory signals, on-off switches, as well as giving antisense transcripts the ability to affect regulatory

networks. The function of the antisense transcript can be mediated by surrounding cellular events or by the transcription events that produce the ncRNA transcript.

The regulatory effects of antisense transcripts can act either in *cis* or in *trans*, giving antisense transcripts a wide range of influence over transcription of protein coding genes. Antisense transcripts also have many opportunities to affect transcription, from transcription initiation to post transcriptionally. The domains of the transcript itself can interact with DNA, RNA and proteins and also have the potential to form functional complexes. Because they can interact with so many cellular elements, they can affect transcription as early as the initiation stage. Antisense transcripts can potentially bind transcription factors and could inhibit them, or delay their activation. Transcription interference and promoter competition can occur, negatively affecting the sense transcript, acting in *cis* (Shearwin K. E., et al, 2005). Antisense expression has also been shown to affect the methylation of DNA (Lister, R., et al., 2009) that can lead to long-term repression of promoters, especially if the promoter regions are cytosine rich.

As well as modifying the DNA itself, antisense transcripts have been shown to modify chromatin structure, a well-known example of which is the mammalian *XIST* antisense transcript that is essential to X chromosome inactivation. *XIST*, when present on one copy of the X chromosome can then recruit chromatin-remodeling complexes that repress transcription. Another example of repression, although not of whole chromosome silencing, is histone modification. It has been shown that an antisense transcript, *ANRIL*, can recruit in *cis* a complex that induces histone methylation, repressing the locus' ability to be transcribed (Yap, K., et al., 2010). The

act of transcription of the antisense transcript can also cause modifications to the chromosome. Cryptic promoters, particularly in *S. cerevisiae*, as they initiate antisense transcription can cause a modification to the chromatin of the correlating sense genes that significantly delays transcription initiation (Pinskaya, M., et al., 2009).

Transcriptional interference can also occur co-transcriptionally, with *S. cerevisiae* studies showing that antisense transcription can block polymerase elongation of its sense transcript (Gelfand, B., et al., 2011). Post-transcriptionally, antisense transcripts can act on the stability of the sense mRNA. In yeast, this type of effect is rare as generally genes on average expressed as one mRNA per gene per cell with even lower levels of antisense transcription (Miura, F., et al., 2008). However, this type of effect may play an important role in more complex organisms, from mice to humans.

Overall, ncRNA and antisense transcripts have far reaching effects on transcriptional regulation in the cell. Antisense transcripts can potentially signal other genes, allowing for complex regulatory networks, and also have the potential to influence genome organization by affecting the roles of transcription factors as either activators or repressors (Kim, T., et al., 2012). Understanding when and how antisense transcripts, sense transcripts, neighboring transcripts function, and when transcription factors are able to bind, is essential to understanding the interactions that occur between all these elements to regulate transcription as a whole.

Understanding binding events then could shed light on the relationship between transcription factor binding and the produced transcript. The relationship

between the expression of *FRE6* ncRNA and *AQY2* is being explored to understand whether *FRE6* ncRNA and *AQY2* expression always correlates with hopes of understanding how their regulation may differ in other yeast strains. Examining the relationship between an antisense transcript and the *AQY2* transcript could shed light on how the ncRNA may act as a regulator, and allow us to better understand the role of ncRNAs in the cell.

Materials and Methods

Yeast Strain Construction

The construction of the myc-tagged Reb1 strains of yeast was done using standard yeast transformation procedures. Using both pFA6a and pYM20 (courtesy of the Odorizzi Lab), primers were designed to amplify off the myc-KanMX sequence to use on non-G418 resistant yeast strains (CliB215, CliB324, JAY270, JAY291, JAY297, YJM789, YPS163, S288C, and Σ1278b). Primers contained twenty base pair sequences of the beginning and end of the desired section of plasmid, and also contained forty nucleotide sequences that flank the stop codon of the *REB1* gene. Primers were designed individually to account for nucleotide differences between the strains. Selection of transformed cells was done using G418 media plates (50uL/mL). (For those strains with G418 resistance (RM11-1a), pAG32 specific primers were used to amplify off a hygromycin tag, designed to flip out the *TRP1* gene first. The successful transformants were selected for on both –trp and hph (50uL/mL) plates.) Keeping the transformed cells under G418 selection, PCR was

performed after genomic DNA isolation for preliminary verification of integration of the tag.

Western Blotting

To prepare the protein extract, cells were grown under selection to an OD of 1.0, put in 10mM NaN3 to stop growth and kill the cells. Volumes of cells were adjusted according to OD, the cells were then boiled for 10 minutes, and beaten before the supernatant was collected from the debris.

To run the protein gel, a SDS Page gel was used (8% acrylamide resolving gel, 4% stacking gel) and run at 90V for an hour at room temperature. The transfer to nitrocellulose was done overnight at 20V, 4°C.

After transferring, membrane was blocked with 5% Milk in TBST for one hour, then incubated with a 1:250 dilution of primary antibody (IgG) overnight at room temperature. Incubation with secondary antibody (hrp) diluted 1:5000 in TBST for 90 minutes followed. Membrane was then developed using SuperSignal kit.

To strip membrane to run control of β-tubulin detection, membrane was incubated at room temperature for 10 minutes in mild stripping buffer, followed by PBS and TBST washes before re-blocking with different antibodies. Detection was done on membrane using alkaline phosphatase.

DNA Sequencing

To verify the correct integration of the tags, strain specific primers were designed flanking the insertion at the 3' end of *REB1*. PCR products from the

verification PCR were purified, and the concentrations were adjusted to 50 ng/uL before being mixed with the primers. The pre-mixed volumes were then sent to GENEWIZ to be sequenced.

Time Course Cell Cultures

To gather data on gene expression over a growth period, two colonies of SAV273 (a Σ1278b derivative) were grown as biological replicates. Beginning with a saturated culture, cells were spiked into fresh media (YPD) to achieve a 0.2OD. A sample was then taken to isolate RNA from. A sample was taken every two hours for 16 hours, and OD was recorded.

RT-qPCR

Cell cultures of all strains of interest were grown to 0.8-1.2 OD to ensure they had entered mid-log phase. Cells were removed from media, disrupted and RNA was isolated using several phenol chloroform and chloroform extractions. To remove all DNA from the samples, a DNase reaction was completed before cleaning up the RNA. RNA was diluted so that 2ug RNA existed in each sample before adding buffer, dNTPs, gene specific or random hexamer primers and reverse transcriptase. A thermal cycler was used to maintain reaction conditions to obtain cDNA.

For samples where *ACT1* was being measured for normalization purposes, cDNA was diluted 1:100. For samples measuring *AQY2, FRE6* and *FRE6* ncRNA, samples were diluted 1:10. Technical triplicates of each sample were included, and a

standard curve was created using cDNA of known concentration to allow for proper analysis of the data.

Results

Creating C-terminally myc-tagged Reb1 yeast strains

To assay for the presence or absence of a Reb1 binding event proximal to the *FRE6* 3' end, a myc-tagged Reb1 was introduced into the YJM789 and JAY291 backgrounds. Using PCR to amplify the 9XMyc-KanMX portion of the pFA6a plasmid, products were produced with a length of approximately 1600 base pairs (Figure 5a), which is consistent with the estimated length for the 9Xmyc sequence and KanMX sequence. After transformation of tag into strains YJM789 and JAY291, plated cultures with the transformation grew on G418 plates, while wild type strains did not. Colonies of "transformed" cells were then analyzed using PCR, where "transformed" strains showed a product size of approximately 2500 base pairs, whereas the wild type strains showed a product size of approximately 950 base pairs (Figure 5b). These sizes are consistent with the expected sizes for wild type products and transformed products.

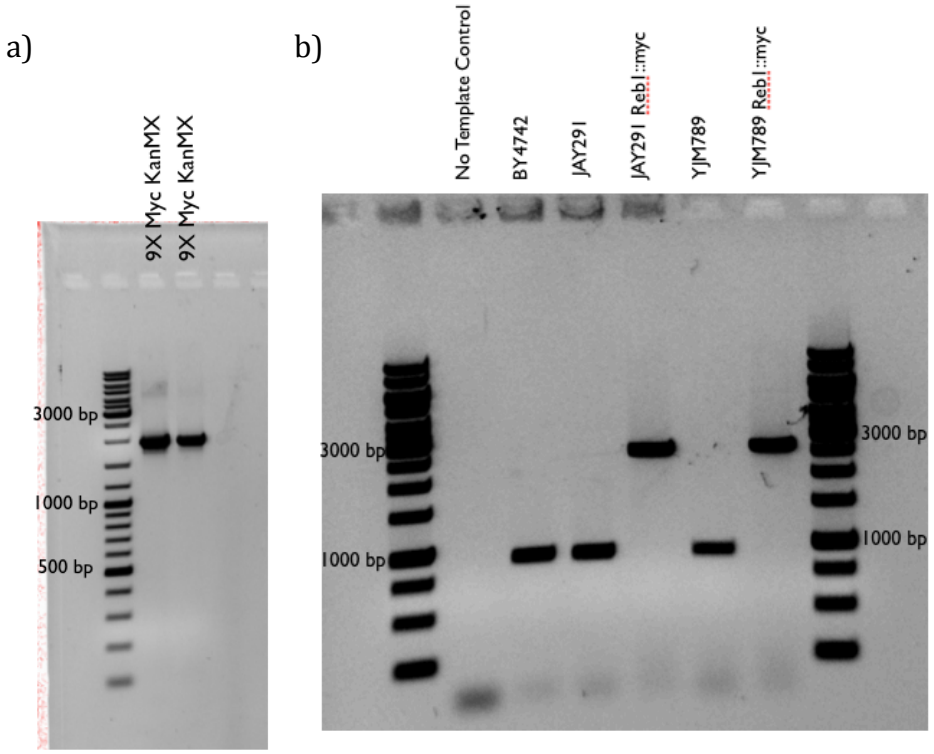

Figure 5: a) PCR gel showing products of amplifying off primers. Two products shown to have length of ~1600 base pairs, while the no-template control had no product (not shown). b) PCR gel of transformed and wild-type (WT) strains. Showing no-template control, wild-type control, WT JAY291, transformed JAY291, WT YJM789, and transformed YJM789. WTs show product size of ~950 base pairs, while transformed strains show product of ~2600 base pairs.

To confirm that the transformations were successful, both nucleotide sequencing and protein Western blots were used. Sequencing showed, for both JAY291 Reb1::myc and YJM789 Reb1::myc, that the stop codon for *REB1* had been replaced by transformation with the beginning of the tag sequence (Figure 6).

WT JAY291
5'-AAGAGCTAGTTGATTATTTTAGCTCCAATATTTCAATGAAAACAGAAAATTAATTCCGGA
AAATATAATTGGTGAAAGCGAAAAACTCAATAATGTTTGATAGTACGCGTTATTTTTCAT-3'

WT JAY291 Stop Codon
5'-AAGAGCTAGTTGATTATTTTAGCTCCAATATTTCAATGAAAACAGAAAATTAATTCCGGA
AAATATAATTGGTGAAAGCGAAAAACTCAATAATGTTTGATAGTACGCGTTATTTTTCAT-3'

WT JAY291 Primers
5'-AAGAGCTAGTTGATTATTTTAGCTCCAATATTTCAATGAAAACAGAAAATTAATTCCGGA
AAATATAATTGGTGAAAGCGAAAAACTCAATAATGTTTGATAGTACGCGTTATTTTTCAT-3'

JAY291 Reb1::myc
5'_AAGAGCTAGTTGATTATTTTAGCTCCAATATTTCAATGAAAACAGAAAATGCTGCTAGTG
GTGAACAAAAGTTGATTTCTGAAGAAGATTTGAACGGTGAACAAAAGCTAATCTCCGAGG-3'

Figure 6: Sequencing results of the 3' end of REB1 in JAY291. First sequence shows WT JAY291 sequence. Second sequence shows WT JAY291 stop codon (red). Sequence three shows primer tails (blue) flanking stop codon (red). Sequence four shows the sequencing results received, showing primer tails (blue) reading into tag sequence (green) and absence of stop codon.

The western blot was used to confirm that translation was not interrupted by the transformation and showed protein band sizes of approximately 130kDa and smaller. These sizes are close to the expected band size estimates of the Reb1 myc-tagged protein (106kDa) but do differ. The beta tubulin (used as a control and as a loading control) stained membrane shows bands of protein at 50kDa in all samples, including mouse control MEF (Figure 7).

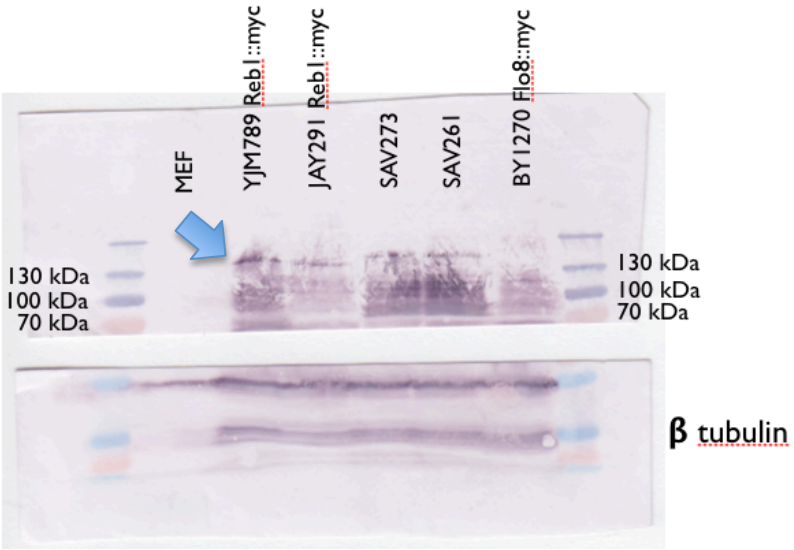

Figure 7: Western blot exposed using alkaline phosphatase to develop on nitrocellulose membrane. Top half showing secondary antibody to myc-tag, bottom half showing secondary antibody to β-tubulin. Detection shows Myc-tagged protein at ~130kDa (blue arrow) and smaller, in YJM789 Reb1::myc, JAY291 Reb1::myc, SAV273 (myc-tagged Σ1278b), and SAV261 (myc-tagged S288c). BY1270 has bands of ~115kDa and smaller, while mouse control MEF has no bands responding to anti-myc antibody. β-tubulin shows a band at 50kDa in all samples, as well as smaller bands in all but MEF

Exploring a link between AQY2 *and* FRE6 *ncRNA expression*

To analyze the transcription levels of both the *FRE6* ncRNA and of *AQY*, a time course experiment on a Σ1278b derivative, SAV271. The samples taken to create the time course were harvested with a range of 0.19-1.82 OD. Over 16 hours, the following ODs were recorded for the biological replicates of SAV273:

Hours from Start	OD Replicate 1	OD Replicate 2
0	0.2	0.19
2	0.22	0.21
4	0.25	0.22
6	0.49	0.44
8	0.90	0.84
10	1.31	1.27
12	1.64	1.63
14	1.81	1.81
16	1.82	1.81

After completing RNA isolation and clean-up on the above samples, a reverse transcriptase reaction was preformed to produced cDNA in order to complete qPCR to create a time curve of *AQY2* (Figure 8) and *FRE6* ncRNA expression. The expression levels seen in the *AQY2* data is comparable to the data produced and shown in Figure 2.

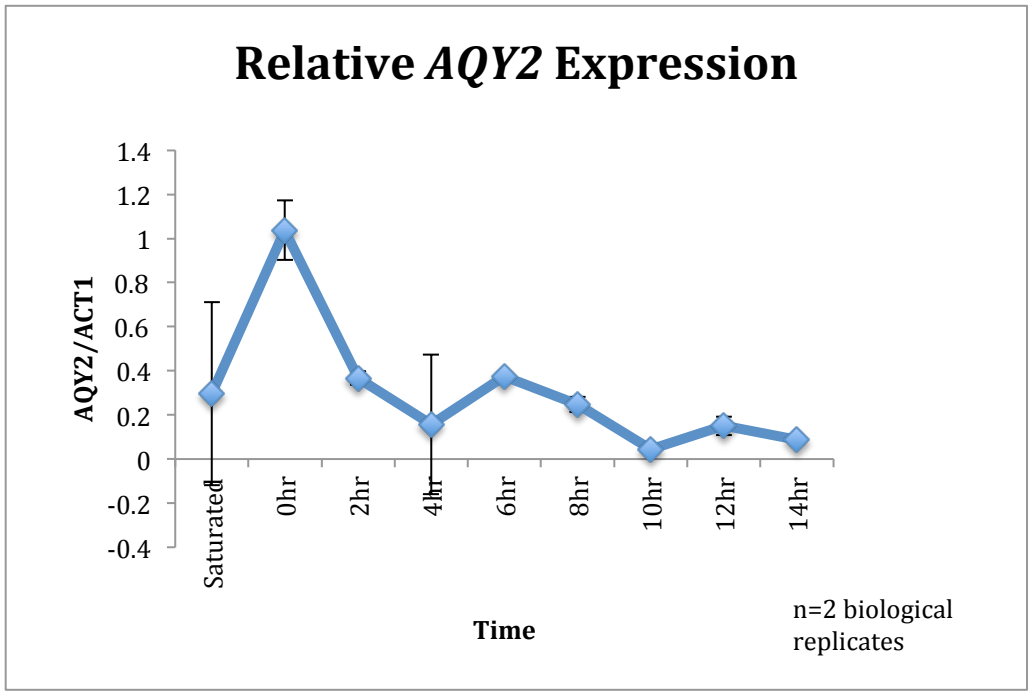

Figure 8: Relative *AQY2* expression in two Σ1278b biological replicates. *AQY2* expression normalized to housekeeping gene *ACT1*. Expression data from time points beginning from saturated overnight culture, to 14 hours after saturated cells were spiked into fresh media to 0.2OD.

Discussion

Creating C-terminally myc-tagged strains

In order to be able to compare multiple strains of *S. cerevisiae* to determine which strains had Reb1 binding in the *FRE6* locus, all strains used for the comparison had to be created. The size of the product created from amplifying off

the pFA6a plasmid showed the correct size of 1625 base pairs. Once transformed, yeast strains JAY291 and YJM789 succeeded in growing on G418 plates, indicating that they had received the resistance marker (KanMX) from the DNA transformed in. When PCR was completed on these strains, the samples that had undergone the transformation had product sizes of 2568 base pairs, where as the WT controls had product sizes of 943 base pairs, showing that the length of the tag had been transformed into these strains. Sequencing was used to make sure the transformation had occurred in the correct place of each genome, and verified that the *REB1* stop codon had been replaced with the beginning of the desired tag.

Western blots were used to verify that translation of Reb1 was not interrupted by the transformation. The size of the 9Xmyc tag was expected to be approximately 106 kDa based on the myc sequence. However, the Western showed a size greater than 130 kDa, as well as multiple smaller bands. The larger than expected band size could be caused by post-transcriptional modification of Reb1 causing a change in the charge and therefor how far the protein would run on the gel. The presence of multiple bands could be due to several things; it could be that multiple isoforms exist of Reb1, but this is highly unlikely in yeast, made even more unlikely by the fact that it also occurs in the β-tubulin. It is more likely that these smaller bands were due to proteases and re-freezing and heating causing protein degradation. This is the likely case as the MEF control does not have these bands and was prepared by another lab.

Given that the controls showed the expected sizes of flo8 tag and β-tubulin, questions arose as to whether the myc-tag was indeed correct. The beginning of the

tag was known to be correct from the sequencing done to verify the transformation, but once sequencing was completed using primers designed inside the tag region, repeats of only three myc sequences was discovered. This plasmid was modified by multiple labs, which led to changes of what the plasmid actually contained. However, these constructed strains can still be used in ChIP(chromatin immunoprecipitation)-seq analysis even though the tag does not contain as many Myc repeats as expected.

A new plasmid of correct Myc repeats and known sequence, pYM18, yielded expected band sizes when amplifying off the plasmid, and after the transformation procedure. However, sequencing after using this plasmid showed that the *REB1* stop codon had not been replaced. This occurred in the strains in which a hygromycin myc-tag was used as well. The presence of the stop codon suggests that the transformation occurred somewhere else in the genome. Despite many rounds of optimization of conditions and newly designed primers, the transformation never yielded correct sequencing results.

Had these transformations worked correctly, and been confirmed by sequencing and Western blot, then ChIP-seq would have been used to identify where Reb1 was bound in the genome, and to which sequences. With multiple strains, this would have allowed for more information on whether this *cis* context is required throughout evolution of strains for binding. We would predict that the strains that have the same SNP as Σ1278b would have had binding of Reb1, whereas those strains with the same SNP as S288c would be predicted to not have binding, given the current data. To expand on this experiment, transformations could be

done to change the SNP in strains with the S288c motif to that of the Σ1278b motif, to see if that rescues binding of Reb1. If it did, that would mean the *cis* context was sufficient for the rescue. However, unpublished data from the Dowell lab show that in S288c the SNP change is not sufficient for binding, and there is a *trans* factor that is influencing the binding of Reb1 as well. We would also complete transformations to change the Σ1278b SNP in these strains to that of S288c's. From previous observations, we would expect this SNP change to cause a loss of the Reb1 binding event. These experiments could shed more light on what has previously been observed in the S288c and Σ1278b strains, and add more information to the conclusion that the Σ1278b motif is required but not sufficient for a Reb1 binding event. Based on previous data, we would also expect to see that those strains that had Reb1 binding would also have expression of the *FRE6* ncRNA.

Exploring a link between AYQ2 *and* FRE6 *ncRNA expression*

It is well known that *cis* and *trans* contexts of the genome are important to regulation, however, we are now discovering the wide variety of factors that are included in these categories- especially that of *trans.* With ncRNAs now recognized as regulators of the genome, understanding how and when they function is the next step towards understanding their roles. With the observed correlation between *AQY2* and the *FRE6* ncRNA, preforming a time course of their expression may shed some light on whether the two transcripts are co-regulated.

Using biological replicates of Σ1278b cultures were grown from 0.2OD to saturation. This ensured that the cells were growing through their mid-log phase

(~.8-1.2OD), a phase of cell proliferation, or the period in which *AQY2* is known to be highly expressed. From the ODs recorded, both biological replicates were observed to grow through this phase. From previous observations, it was expected that when *AQY2* is expressed, the *FRE6* ncRNA would also be expressed. This might indicate that the antisense transcript of *FRE6* plays a role in the regulation of *AQY2*. From the data shown in Figure 2, it was expected that *AQY2* would be expressed with spikes at about two and six hours after beginning growth at 0.2OD. As shown in Figure 8, the data from this time course experiment also shows spikes at two and six hours. Given that the *AQY2* time course data was reproduced accurately, and now that RT-qPCR conditions have been optimized, expression levels of the *FRE6* ncRNA can now be analyzed. It would also be expected that these spikes of expression would be mirrored in the *FRE6* ncRNA. The next step would also be to use the cDNA from this experiment to run an RT-qPCR for expression of *FRE6*, which would allow insight as to how the *FRE6* ncRNA may affect the regulation of its sense transcript, *FRE6*, in *cis.*

Continuing in this vein of exploration, this experiment could be repeated using all strains that have a functional *AQY2* and *FRE6* ncRNA. Using conditions, like changes in temperature, that change expression of *AQY2* could result in a clearer picture of how the two genes may be regulated, and also how *FRE6* itself is regulated. Putting laboratory strains through freeze-thaw cycles should show some change in *AQY2* expression (Tanghe, A., *et al.,* 2002) and would make for an interesting experiment.

The implications for understanding how gene regulation work, both in terms of *cis* and *trans* factors, are both simple and great. One aspect, especially in *S. cerevisiae,* would be the ability to see how regulatory elements have evolved through the strains, giving clues to what actually drives changes at various gene loci and which "rules" for regulation are conserved. Understanding regulatory elements and how the whole genome interacts would also allow us to potentially predict which genes may be expressed by only looking at DNA. This of course is a complicated goal, especially because we are now realizing that elements we once thought of as "transcriptional noise" are in fact regulatory elements, and that is only in a simple organism like yeast. However, if this could be accomplished, it would be a huge advance towards personalized medicine.

Understanding ncRNAs and how they affect expression also has many implications. From how their presence may activate transcription, to how it may repress it, ncRNAs could be used as a therapeutic tool. For example, antisense transcripts are known to repress their sense genes, so if the antisense transcripts themselves were repressed, it could increase the expression of the sense gene. Experiments in yeast are ideal for working towards this goal because of the simplicity of the organism, yet how easily knowledge from its genome can be translated to the human genome. Another advantage is the quick lifecycle of yeast, which allows for studies on evolution that would be very time consuming in humans. Simply studying whether a transcription factor is bound in multiple yeast strains, or whether two transcripts expression levels are linked, are small but hopefully in some way add to the understanding of the genome of a whole.

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