Post-transcriptional coordination by an RNA-binding protein

By

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ABSTRACT

RNA-binding proteins can regulate the stability, localization, and translation of their target mRNAs. Post-transcriptional regulation can orchestrate dynamic changes in gene expression, and can coordinate multiple cellular processes in response to various stimuli. Filamentous growth in *Saccharomyces cerevisiae* is a morphogenetic switch that occurs in response to nitrogen starvation and requires alterations in cell growth, cell cycle, and cell wall functions. Ty1 element retrotransposition is also induced under conditions of nitrogen starvation. I describe a role for the RNA-binding protein Khd1 in regulating these two responses to environmental stress through its mRNA targets. I identified the RNA targets of Khd1 using *in vivo* crosslinking and immunoprecipitation (CLIP), combined with deep sequencing. This produced a high-resolution map of Khd1 binding sites across the transcriptome, and provided unprecedented insight into its biological functions. Khd1 regulates multiple post-transcriptional regulatory loops to coordinate the components of filamentous growth and Ty1 retrotransposition. Although similar mechanisms were known to transcriptionally regulate these processes, the post-transcriptional coordination is a novel discovery. The feed-forward regulation that Khd1 confers on *FLO11,* which encodes a protein required for filamentous growth, enables asymmetric expression between mother and daughter cells to switch between filamentous and yeast form growth. In this thesis, I describe regulation of gene expression by RNA-binding proteins, methods to identify their target transcripts and recognition sequences, the KH domain, known functions of Khd1, and the phenotypes it coordinates. My work represents the first application of CLIP to budding yeast, and the growing understanding of RNA-binding proteins in this organism facilitated the placement of Khd1 into its post-transcriptional regulatory network. While many questions remain regarding the role Khd1 plays in regulating cellular activities, this thesis addresses its direct role in key processes.

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Chapter 1: Introduction

The phenotype of a cell is determined by the ensemble of processes that control gene expression. Much of experimental biology focuses on understanding gene expression to discover the underlying mechanisms of these phenotypes. Many efforts have sought to understand the basis of phenotypic outputs through studies of whole genome transcript levels. The steady state mRNA level of a gene is often used as a proxy for its expression because mRNA levels are easily assayed on a genome-wide scale using microarray technology or high throughput sequencing. Although this approach provides important information about mRNA accumulation, it ignores subsequent steps of regulation in the gene expression pathway. Steady state mRNA levels frequently fail to correlate with protein levels (GYGI *et al.* 1999) because of ensuing regulatory processes including translational regulation and differences in protein stability. Understanding translational regulation is an important step towards understanding gene expression.

Methods that measure events downstream of mRNA accumulation in the gene expression pathway more accurately represent expression levels. Proteomic methods (DE GODOY *et al.* 2006) and techniques designed to profile the translational status of mRNAs on a genome-wide scale using either microarray technology (HALBEISEN *et al.* 2009) or high throughput sequencing (INGOLIA *et al.* 2009) provide additional metrics of global gene expression patterns. Although these approaches successfully identify variations between mRNA levels and protein levels or ribosome occupancies, they do not provide a mechanistic explanation for these differences. Understanding the discrepancies between mRNA levels and protein production requires specific characterization of post-transcriptional regulatory interactions by other methods.

In this thesis, I identify and characterize post-transcriptional regulation by the RNA-binding protein (RBP) Khd1 in *Saccharomyces cerevisiae* to understand its role in regulating gene expression. I used the crosslinking immunoprecipitation (CLIP) method (ULE *et al.* 2005) and deep sequencing to identify the direct RNA targets of this protein, and investigated its regulation of prominent targets. These experiments identified new regulatory roles for Khd1 in coordinating gene expression at the post-transcriptional level. The information gained by identifying the target transcripts provides important information about the role of Khd1 in regulating cellular activities and allowed me to identify two phenotypes it controls.

This introduction contains two disparate sections bridged by their relation to Khd1. The first section addresses functions and properties of RBPs that are relevant to my work. I begin by considering the role of RBPs in regulating gene expression, followed by a discussion of methodologies for identifying their RNA targets and specific recognition sequences. A discussion of the K homology RNA binding domain, which is found three times in Khd1, and a summary of known Khd1 activities follows. Next follows discussion of filamentous growth and Ty retrotransposition, two phenotypes that my work identifies as being regulated by Khd1. The genes that dictate these phenotypes encode the most frequent target transcripts I identify for Khd1, *FLO11* mRNA and Ty1 mRNA, and known mechanisms coordinate their transcription. My work demonstrates that Khd1 coordinates their activity post-transcriptionally in the context of a broader regulatory network.

RNA-binding proteins and gene expression

RBPs associate with an mRNA molecule throughout its lifespan, forming a ribonucleoprotein complex (RNP) that dictates the behavior of the transcript (MOORE

2005). The protein composition of the RNP is dynamic, changing throughout the lifespan of the RNA (DREYFUSS 1986), and the proteins associated with a transcript at any given stage of gene expression play important roles in regulating its splicing, polyadenylation, nuclear export, stability, localization and translation (GLISOVIC *et al.* 2008). These processes are frequently coupled through interactions between the RBPs involved, lending a high degree of interconnectivity to the gene expression pathway (MANIATIS and REED 2002). RBPs play essential roles in eukaryotes, and determination of their function is key to understanding overall gene expression.

RBPs initially bind transcripts as they emerge from the transcription machinery, forming heterogeneous nuclear RNP (hnRNP) complexes (DREYFUSS 1986). The hnRNP protein components were identified based on their purification with heterogeneous nuclear RNA (DREYFUSS 1986). Immunoprecipitation using antibodies raised against a number of individual hnRNP proteins reproducibly enriched a set of proteins, suggesting the existence of a single hnRNP complex (DREYFUSS *et al.* 1984). Around 20 proteins can be purified in this process, many of which remain complexed when heparin is added to compete nonspecific interactions with the RNA (PINOL-ROMA *et al.* 1988). Additionally, many hnRNP components can be crosslinked to RNA using UV light, which requires direct contact, supporting their role as hnRNP components (DREYFUSS *et al.* 1984). These proteins have been shown to regulate the splicing and polyadenylation of pre-mRNAs in the nucleus, and mRNA nuclear export (DREYFUSS *et al.* 1993).

Whereas some hnRNP proteins are exclusively nuclear, others shuttle between the nucleus and cytoplasm (PINOL-ROMA and DREYFUSS 1992), and can remain associated with specific mRNAs after export (DREYFUSS *et al.* 1993). Other RBPs that bind a transcript in the nucleus and remain associated during nuclear export, such as the exon junction

complex (LE HIR *et al.* 2000a; LE HIR *et al.* 2000b), impart a nuclear history to an mRNA RNP (mRNP). These nuclearly-derived components can significantly effect mRNA expression in the cytoplasm (LE HIR and SERAPHIN 2008).

Additional cytoplasmic RBPs are recruited to the transcript following nuclear export, further modulating mRNP function. These proteins control the stability, localization and translational activity of their target transcripts (DREYFUSS *et al.* 2002; LE HIR and SERAPHIN 2008). The components of the exon junction complex associate with spliced mRNAs and play a role in translation and quality control (LE HIR and SERAPHIN 2008). The major mRNP components, poly(A) binding protein (PABP) and YB-1, both associate with most or even all cytoplasmic mRNAs. PABP preferentially binds poly(A) sequences, while YB-1 binds mRNAs through both sequence specific and nonspecific interactions (EVDOKIMOVA and OVCHINNIKOV 1999). Whereas some proteins bind mRNAs nonspecifically or in a position dependent manner, most appear to recognize specific sequences frequently found in the 3' untranslated region (GEBAUER and HENTZE 2004).

RBPs in this context influence a number of biological events, including developmental patterning (KUERSTEN and GOODWIN 2003) and responses to environmental cues (SIOMI and DREYFUSS 1997). RBPs allow for rapid changes in gene expression by regulating the ability of mRNAs to serve as templates for translation. In response to appropriate cellular signals, RBPs can act to affect polyadenylation, translation initiation, or mRNA stability to bring about changes in gene expression independent of new transcription (MACDONALD and SMIBERT 1996). Under the appropriate conditions (i.e. developmental stage, spatial localization, extracellular signals) post-translational modifications of the RBPs can alter their activity, allowing for changes in expression of

their target transcripts (HUTTELMAIER *et al.* 2005; LUKONG *et al.* 2008; PAQUIN *et al.* 2007). These regulatory dynamics play critical roles in determining cellular activities.

Multiple studies have shown that RBPs associate with mRNAs encoding functionally related proteins. This feature forms the basis of the concept of the "post-transcriptional operon" whereby genes can be co-regulated at the post-transcriptional level through interactions with a common RBP (KEENE and TENENBAUM 2002). A corollary of this phenomenon is that transcripts bound by the same RBP often encode functionally related proteins. Identifying RBP targets provides important insight into its cellular function.

Methods that identify targets of RNA-binding proteins

Discovering interactions between RBPs and their target mRNAs provides the framework in which to study RBP activity. Characterizing the effects of binding enables assignment of regulatory functions to RBPs. Functional interactions between RBPs contribute to the overall regulation of an mRNA (GLISOVIC *et al.* 2008). Comprehensive analysis must identify the RNAs an RBP binds, and the proteins with which it interacts to regulate these transcripts.

The use of microarray technology to discover RBP targets has contributed significantly to the understanding of post-transcriptional regulation. This approach identifies enriched transcripts following RBP immunoprecipitation and has been applied to a number of systems (KEENE 2007). Applied to *S. cerevisiae,* this approach revealed the potential for widespread post-transcriptional regulation. A survey that looked at 36 of the ~600 RBPs in budding yeast found extensive protein-mRNA associations. Of the 36 RBPs studied, 33 enrich a reproducible set of transcripts, and over 70% of mRNAs associate with at least one RBP (HOGAN *et al.* 2008). Many transcripts associate with multiple RBPs,

suggesting that a complex interaction network regulates gene expression at the post-transcriptional level (HOGAN *et al.* 2008).

Identification of the sequence constraints that dictate protein-RNA interactions provide insight into RBP function. Some RBPs bind RNA motifs in a sequence or structure specific manner, whereas others bind non-specifically or are deposited through a position dependent mechanism (MOORE 2005). RBPs that recognize primary or secondary structures may interact with targets closer to, or more degenerate than, a consensus motif based on global or local concentrations (DREYFUSS *et al.* 1993). Both RBP binding specificity and affinity can be altered by post-translational modifications or protein-protein interactions (DEJGAARD *et al.* 1994; KEENE 2007; OSTROWSKI *et al.* 2001; OSTROWSKI *et al.* 2000). Computational approaches may be able to identify additional mRNA targets of an RBP based on a well-characterized motif, but whether these represent *in vivo* interactions requires experimental validation. Compartmentalization, accessibility, and lack of expression can all prevent an RBP from interacting with a predicted target *in vivo*. While microarray studies provide insight into the RNAs bound by a given protein, they do not pinpoint the binding site within the transcript. In some cases, computational analysis can identify potential binding motifs in target mRNAs, but in many instances the recognition site remains unclear (HOGAN *et al.* 2008). Identifying the sequence constraints for recognition by an RBP requires an experimental approach that pinpoints the binding interaction with higher resolution.

Two methods identify the sequence determinants of recognition by RBPs by screening RNA libraries for molecules that bind a protein of interest. By identifying common sequences or structures, they can pinpoint features that contribute to, or detract from, protein-RNA interactions. Systematic evolution of ligands by exponential

enrichment (SELEX) takes an *in vitro* approach to identifying RBP target sites. A randomized RNA library is enriched through successive rounds of binding to a purified RBP in order to identify molecules recognized by the protein (SCHNEIDER *et al.* 1993). Yeast three-hybrid analysis can generate a similar set of sequences recognized by an RBP in a cellular environment. Expressing the RBP fused to the activation domain of a transcription factor, and tethering an RNA library to a DNA-binding domain leads to activation of reporter genes only when the two interact, allowing for selection of desired sequences and a measurement of the interaction strength (SENGUPTA *et al.* 1996). The comparison of multiple sequences identified using either of these approaches can highlight motifs that mediate specific interactions with the protein. Although these screening approaches provide a useful tool for identifying sequences bound by RBPs, they have limitations. Both techniques can sample broad sequence space but neither targets of RBPs in their natural contexts, and the protein may not recognize transcripts containing a consensus motif because of cellular constraints.

The cross-linking immunoprecipitation (CLIP) method identifies direct protein-RNA interactions, enabling the identification of *in vivo* RBP targets and localizing the binding sites within these transcripts (ULE *et al.* 2003). This approach uses UV radiation to crosslink RBPs to their direct RNA targets *in vivo,* providing a snapshot of binding interactions across the transcriptome. The bond formed between the species permits stringent protein purification while maintaining association with the RNA. This crosslinking overcomes a drawback of microarray analysis following immunoprecipitation. The low stringency purification used to preserve protein-RNA associations can lead to false positives because of *in vitro* protein-RNA associations that occur after cell lysis (MILI and STEITZ 2004). CLIP removes this obstacle. CLIP also pinpoints biologically relevant

interactions through sequencing of the target transcripts. A short sequence is sufficient to identify the transcript from which it derived, and RNAse treatment can produce a library of target sites within 60-100 nucleotide RNA fragments (ULE *et al.* 2003).

The CLIP method was recently developed to identify targets of Nova, a splicing factor expressed in mouse brains. CLIP showed that Nova preferentially binds neuronal transcripts to regulate alternative splicing (ULE *et al.* 2003). CLIP has subsequently been adapted for use with deep sequencing, which expanded the list of targets for Nova (LICATALOSI *et al.* 2008), as well as the splicing factors Fox2 in human embryonic stem cells (YEO *et al.* 2009) and SFRS1 in human embryonic kidney cells (SANFORD *et al.* 2009). Analysis of these proteins' global interactions increased understanding of their biological roles by cataloging their binding sites across the transcriptome.

The specificity and resolution of CLIP revealed unexpected complexity in post-transcriptional regulation. Nova, Fox2, and RFRS2 were known to interact with specific sequence motifs near alternative exons to regulate their inclusion in mRNA. However, CLIP demonstrated additional binding and regulatory activities for these proteins. CLIP identified 34 of 39 previously validated alternatively spliced transcripts regulated by Nova, and extended its activity to 46 additional transcripts shown to undergo Nova-dependent alternative splicing (LICATALOSI *et al.* 2008). 23% of the CLIP sequences, however, map to intergenic regions, and cluster near polyadenylation sites. These sites contain the canonical Nova binding motif. *Nova* knockout mice display alternative polyadenylation profiles for these transcripts, establishing a new role for Nova in RNA processing.

The binding sites identified using CLIP indicate an imperfect understanding of RBP specificity. The GCAUG motif required for Fox2-mediated alternative splicing appears in only 33% of Fox2 binding clusters CLIP identifies (YEO *et al.* 2009). The

majority of Fox2 binding sites do not contain its canonical binding motif and the authors do not report a common sequence that explains these binding events. The number of SFRS1 motifs in predicted binding sites range from 0 to 16 (SANFORD *et al.* 2009), so in some instances SFRS1 binds in the absence of its known recognition site. CLIP offers unprecedented insight into protein-RNA interactions, which adds important information to the understanding of RBP function.

The K homology RNA-binding domain

RBPs contain RNA-binding domains that dictate their RNA specificities. Families of RNA-binding domains can be grouped based on protein folds to highlight common features in their interactions with RNA. Examples include the RRM motif, K homology (KH) domains, and PUF domains. These domains are widely conserved, and studies of their functions are possible in a range of organisms. Protein domains within these families adopt similar folds and share many properties in their interactions with RNA (MESSIAS and SATTLER 2004). Characterization of individual domains can provide important information about the activities of the families of proteins in which they are found.

KH domains are evolutionary conserved RNA-binding motifs that bind RNA, and in some cases single stranded DNA (ssDNA), to promote a number of regulatory activities (BOMSZTYK *et al.* 2004). The domain was originally identified in the human RBP hnRNP K (MATUNIS *et al.* 1992), from which it derives its name. KH domains frequently occur multiple times in a protein, and can be present as many as 15 times in a single polypeptide (LEWIS *et al.* 1999). Proteins containing KH domains exist in archaea, bacteria, and eukaryotes, and have been studied in various systems (VALVERDE *et al.* 2008). These proteins regulate mRNA splicing, stability, translational repression, and translational

activation, as well as transcriptional activation and repression through interactions with ssDNA (BOMSZTYK *et al.* 2004). KH domains consist of a core αββα motif linked to an additional βα N-terminal (type I) or αβ C-terminal (type II) to the core by a variable region, which can range in size (Figure 1A) (VALVERDE *et al.* 2008). The KH domains found in eukaryotes are typically type I, whereas those found in bacteria and archaea are type II (VALVERDE *et al.* 2007).

Studies of the KH domain have largely focused on a group of proteins in mammals that contain three such domains. The members of this group, the poly(C) binding proteins hnRNP K and PCBP 1-4 and the neural splicing regulators Nova1 and 2, share many structural similarities but have distinct functional properties (MAKEYEV and LIEBHABER 2000). These proteins all contain a central KH domain located closer to the N-terminus than the C-terminus. A region of variable length connects the two C-terminal KH domains. In this context KH domains share higher homology with the corresponding domains in similar positions of other proteins than with other domains within the same protein, even between species (THISTED *et al.* 2001). The Nova proteins preferentially bind poly(rG) *in vitro,* and bind poly(rU) as well (BUCKANOVICH *et al.* 1996). hnRNP K, E1 (PCBP1), and E2 (PCBP2) constitute the major cellular poly(rC) binding proteins (LEFFERS *et al.* 1995) and also bind poly(rG) (DEJGAARD and LEFFERS 1996). In different contexts, they can stabilize mRNA, and either enhance or repress translation (MAKEYEV and LIEBHABER 2002). hnRNP K also binds ssDNA and can both activate and repress transcription (BOMSZTYK *et al.* 2004).

The binding specificities of these proteins have been interrogated using SELEX and yeast three-hybrid analysis of the target sites recognized by various domains, global identification of mRNA targets using microarray analysis following immunoprecipitation,

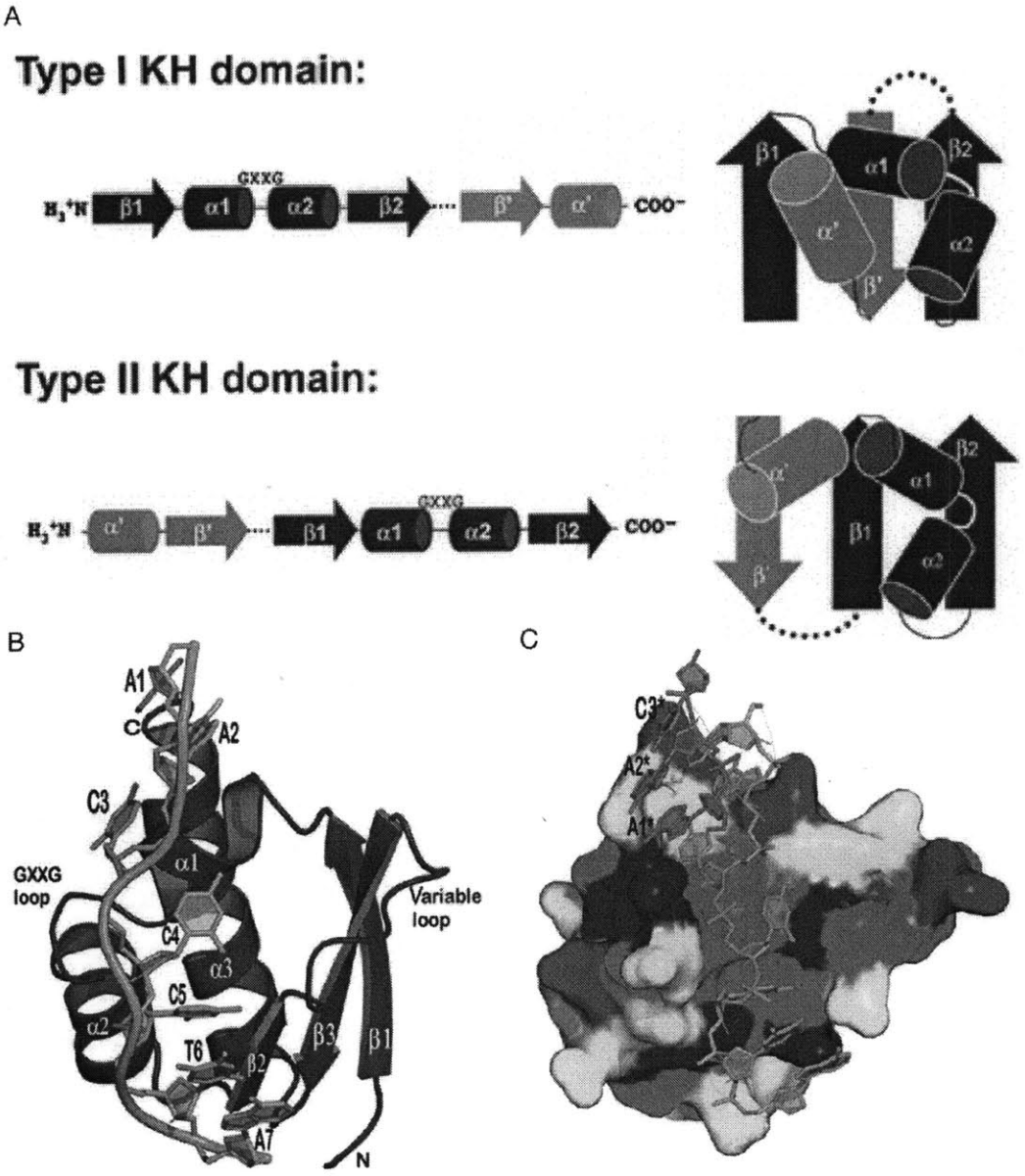

Figure 1.—KH domain secondary and tertiary structures. (A) Secondary structure arrangements and tertiary conformations of type I and type II KH domain folds (VALVERDE *et al.* 2008). (B) Crystal structure of the third KH domain of hnRNP E2 in complex with telomeric DNA shown as a ribbon diagram and a surface representation with positively charged, negatively charged, uncharged hydrophilic and hydrophobic residues colored blue, red, yellow and green, respectively (BACKE *et al.* 2005). The binding groove contacts bases C3 through T6.

and CLIP. Structural studies on protein fragments containing one, two, or all three KH domains with and without nucleic acid targets provide further insight into their binding properties. KH domain-containing proteins interact with single stranded nucleic acids

through a common platform to regulate biological processes, but the specifics of the interactions can vary between individual domains, which results in different binding activities for the full-length proteins.

Although KH domains recognize different sequences, there are many features common to their nucleic acid interactions. The β strands of a KH domain form a three-stranded β sheet, over which the α helices reside. Type I domains contain an antiparallel β sheet with the three successive strands alternating polarity, whereas the type II β sheet contains two adjacent parallel strands (Figure 1A) (VALVERDE *et al.* 2008). The adjacent α helices are connected by a G-X-X-G loop where X is usually arginine, lysine or glycine (LEWIS *et al.* 1999). The invariant G-X-X-G loop faces the loop formed by the variable region, forming a binding groove across a platform formed by the α helices and β sheets (Figures 1B) (BACKE *et al.* 2005). Conserved hydrophobic residues line the floor of the groove creating a channel for nucleic acid binding (Figure 1C) (FENN *et al.* 2007). These common structural features define the basis of nucleic acid recognition by KH domains.

The binding groove in KH domains accommodates four nucleic acids (Figure 1B, 1C). Interaction with four bases is seen in the all crystal structure of KH domains in complex with ssDNA or RNA (VALVERDE *et al.* 2008) and is consistent with the short consensus sequences identified experimentally (see below). The four bases in contact with a KH domain adopt a common conformation across the binding surface (VALVERDE *et al.* 2008). Hydrogen bonding, van der Waals contacts, and shape complementarities specify the core of the binding sequence (DU *et al.* 2005). There may be some flexibility in the identity of the outer bases, although they are frequently pyrimidines (BACKE *et al.* 2005). The consensus motif for Nova is YCAY (where Y is a pyrimidine) (ULE *et al.* 2003) and the target site of the first KH domain of hnRNP E2 shifts by one base when provided one

versus two repeats of the C-rich strand of human telomeric DNA, altering the outer but not the core bases it recognizes (DU *et al.* 2007). Some domains form additional contacts to bases outside of the binding channel (FENN *et al.* 2007). Unlike many RNA binding domains, the KH domain does not stabilize bases through stacking interactions with aromatic side chains (VALVERDE *et al.* 2008). Another unique feature of RNA recognition by some KH domains is the lack of interaction with 2'-OH groups, which allows these domains to interact with ssDNA molecules as well as RNA (BACKE *et al.* 2005). Although the overall fold of a KH domain dictates its general nucleic acid binding properties, unique contacts dictate the specific interactions.

A degenerate four base target sequence is fairly indiscriminate, and interactions between KH domains may provide additional binding specificity. The asymmetric units in crystal structures of Nova 2 KH3 (LEWIS *et al.* 1999) and hnRNP E2 KH1 (DU *et al.* 2005) contain dimeric protein complexes that suggest the potential for intermolecular interactions between KH domains. The dimerization of hnRNP E2 KH1 occurs opposite the nucleic acid binding groove where hydrophobic side chains interact between an α helix and β sheet from each monomer. The interactions produce a six-stranded β sheet, resembling intramolecular folding interactions. The hnRNP E2 KH3 domain does not form dimers during crystallization, suggesting this is a property of specific KH domains (FENN *et al.* 2007). The lack of dimerization from KH3 may result from the absence of the hydrophobic surface where interaction occurs between KH1 domains. Attempts to identify intermolecular interactions between KH domains in solution have been unsuccessful (DU *et al.* 2008) and some authors caution that the available data does not support a biological function for their homodimerization (VALVERDE *et al.* 2008).

Intramolecular interactions can have important effects on nucleic acid binding. NMR and X-ray crystallographic studies of the first two domains of hnRNP E2 indicate that KH1 interacts with KH2 in a manner analogous to the KH1 dimers seen in the crystal structure (DU *et al.* 2008; DU *et al.* 2007). This interaction contrasts with crystal structures of other multi KH-domain protein fragments that do not involve interactions between domains, though it should be noted that these structures were obtained from proteins with either two or four KH domains. The pseudodimer between KH1 and KH2 is not compatible with nucleic acid binding because of steric and energetic limitations (DU *et al.* 2008). Both domains do bind nucleic acid, however, and the three KH domains bind target RNAs cooperatively. Interactions with nucleic acid may dissociate the pseudodimer, freeing the dimerization domains for interactions with other proteins. This potential regulatory mechanism could coordinate KH domain proteins with other cellular proteins and RBPs (DU *et al.* 2008). The stabilization of α globin mRNA by hnRNP E1 and E2 requires PABP, and the protein-protein interactions require RNA binding by hnRNP E1 and E2 (WANG *et al.* 1999). The interaction of domains within a multi-KH domain protein can vary the nucleic acid binding properties of segments of the protein and can coordinate binding with other proteins. The *in vivo* binding activity of an RBP is determined through a combination of these factors.

Target identification experiments reinforce many of the binding features identified by KH domain structural data. Most target identification experiments suggest that proteins with multiple KH domains bind cooperatively to RNAs through short, repeated, pyrimidine-rich sequences. However, some identify single motifs of similar composition that are nevertheless sufficient for binding. Although the motifs are often consistent between SELEX and yeast three-hybrid screens, they can vary. Limited *in vivo*

analysis may support a simple binding interaction, but as with Fox2, binding interactions may prove more complex when extended to the entire transcriptome.

SELEX identifies short sequence motifs targeted by proteins containing multiple KH domains that can occur multiple times within a target RNA. Analysis performed using full-length hnRNP E1 identified targets containing three C-rich patches of 3-5 cytosines separated by 2-6 adenines or uracils. Guanines were largely excluded from the region spanning the C-rich patches (THISTED *et al.* 2001). The presence of three patches in all 26 sequenced targets suggests the three KH domains of hnRNP E1 bind cooperatively to RNA *in vitro*. Mutational analysis validated the importance of the cytosines and that hnRNP E1 binds cooperatively, as mutation of multiple patches have a synergistic effect on binding affinity (THISTED *et al.* 2001). *In vitro* selection of Nova-1 targets produces three UCAU tetranucleotide repeats suggesting the three domains may bind cooperatively to identical motifs (BUCKANOVICH and DARNELL 1997). The same analysis on the third KH domain identifies a single UCAY tetranucleotide (JENSEN *et al.* 2000). Since KH3 is both necessary and sufficient for binding, multimerization may facilitate binding by this single domain rather than intramolecular cooperative binding between domains.

In contrast to the evidence supporting cooperative binding by KH domains, SELEX identifies a single target site for hnRNP K that enriches RNAs with a single stretch of 3-4 cytosines (THISTED *et al.* 2001). This result is analogous to the selection of targets with a single KH domain from Nova, which suggests a single domain is responsible for the interaction. Although some of the sequences enriched with hnRNP K contain additional C-patches, most do not. This result implies that either only one of the KH domains binds RNAs, or that all three domains recognize identical sequences and a single interaction provides maximum binding affinity. The DICE motif, found 10 times in LOX15 mRNA, a

natural target of hnRNP K (OSTARECK-LEDERER *et al.* 1994), contains a similar C-rich sequence. Multiple target sites may modulate hnRNP K binding *in vivo*.

A yeast three-hybrid study suggested the KH domains in hnRNP K bind RNA cooperatively, but also identified targets lacking this feature. The screen identified 17 target transcripts from a human RNA library that bind hnRNP K. 13 of these transcripts contain three C-rich clusters (PAZIEWSKA *et al.* 2004). Three additional transcripts from the screen contain only one C-patch, similar to the targets obtained in the SELEX experiment, and one sequence has no discernible target element. Fragments of hnRNP K containing one, two, or three KH domains were used in the three-hybrid setup to measure expression levels of a β–galactosidase reporter to assess RNA binding strength. Both RNAs used in this experiment contained three C-rich clusters. Constructs containing one or two KH domains are able to bind some transcripts, but produce low β–galactosidase levels. The complete protein generates much higher activity from the reporter, suggesting that cooperativity between the domains increases affinity for RNA. Cooperative binding between KH domains within hnRNP K may increase affinity and specificity for *in vivo* targets, although this has only been tested for targets with three C-rich clusters.

The sequence requirements identified for hnRNP K binding differ depending on whether the results support cooperative binding. Yeast three-hybrid analysis revealed additional sequence requirements for recognition beyond those identified by SELEX, in accordance with supporting cooperative binding by the domains in hnRNP K. In some targets, two C-rich clusters flank a central C-patch. While the central patch is invariant in its cytosine composition, the external clusters can contain one or two adenines or uracils. Guanine is underrepresented in hnRNP K targets (OSTROWSKI *et al.* 2002). Although the data suggests that cooperative binding between KH domains occurs in the context of

these extended recognition motifs, the same yeast-three hybrid screen identified targets that contain a single C-patch as in the SELEX experiment. Target recognition cannot be simply described for even a single protein with multiple KH domains.

Many techniques have been used to identify KH domain target motifs, but considerable ambiguity remains. All reports agree that the domains interact with short, pyrimidine-rich sequence motifs. The nature of the interactions between domains is unclear, with some studies pointing to direct physical interaction, some supporting cooperative RNA binding interactions, and others refuting both of these properties. Additional constraints can lead to unexpected effects for well-defined target sites. An endogenous hnRNP E1 target, α–globin mRNA, contains the same C-rich patches as the target transcripts identified using SELEX, but has 20-fold lower affinity for the protein *in vitro.* This discrepancy likely occurs because it is embedded in secondary structure, whereas the targets identified using SELEX existed in single stranded regions (THISTED *et al.* 2001). The presence of a well-defined target sequence does not necessitate binding by an RBP. Cellular constraints such as secondary structure, competitive binding, compartmentalization, protein-protein interactions, and post-translational modifications can affect the interactions between an RBP and its biologically relevant targets. Binding of RBPs to RNAs is an intricate interaction that depends on many factors within the cell, which complicates target prediction and binding site characterization.

The *Saccharomyces cerevisiae* RNA-binding protein Khd1

The RBP Khd1 is *Saccharomyces cerevisiae* contains a domain structure similar to hnRNP K. It contains three KH domains with 58%, 56%, and 61% homology to the analogous domains in hnRNP K (Figure 2) (DENISENKO and BOMSZTYK 2002). Eight

additional proteins in yeast contain KH domains. Five proteins contain one KH domain, one protein contains two KH domains, one protein contains 14 KH domains, and one other protein contains three KH domains (CURRIE and BROWN 1999). Pbp2, the other yeast protein with three KH domains has lower homology with K, at 45%, 47% and 59% for each respective domain. The second and third KH domains are separated by only 33 amino acids in Khd1, compared with 172 in hnRNP K and 112 in Pbp2 (DENISENKO and BOMSZTYK 2002). The shortened region in Khd1 lacks the SH3 binding domain known to facilitate many of the regulatory functions of hnRNP K (Figure 2) (DENISENKO and BOMSZTYK 2002). Despite these differences, Khd1 is an attractive model for the study of RBPs with three KH domains.

Studies have begun to describe a role for Khd1, but many aspects of its function remain unknown. Khd1 localizes at the bud tip with seven mRNAs, and is reported to interact with many more transcripts (HASEGAWA *et al.* 2008). It is phosphorylated *in vivo,* and phosphorylation affects its affinity for RNA *in vitro* (PAQUIN *et al.* 2007). The RNA binding properties of Khd1 have been probed using yeast three-hybrid analysis and functional studies, but its specificity remains uncertain. A Khd1-GFP fusion shows cytoplasmic localization (NEWMAN *et al.* 2006), a result supported by immunofluoresence of epitope tagged Khd1, which also localizes to the bud tip with a subset of its mRNA targets (HASEGAWA *et al.* 2008; IRIE *et al.* 2002). Chromatin immunoprecipitation (ChIP) experiments indicate that Khd1 is present in the nucleus, where it plays a role in telomere maintenance and chromatin structure (DENISENKO and BOMSZTYK 2002; DENISENKO and BOMSZTYK 2008). Little is known about the overall role of Khd1 in regulating global cellular activity through its target nucleic acids or how it responds to cellular signals.

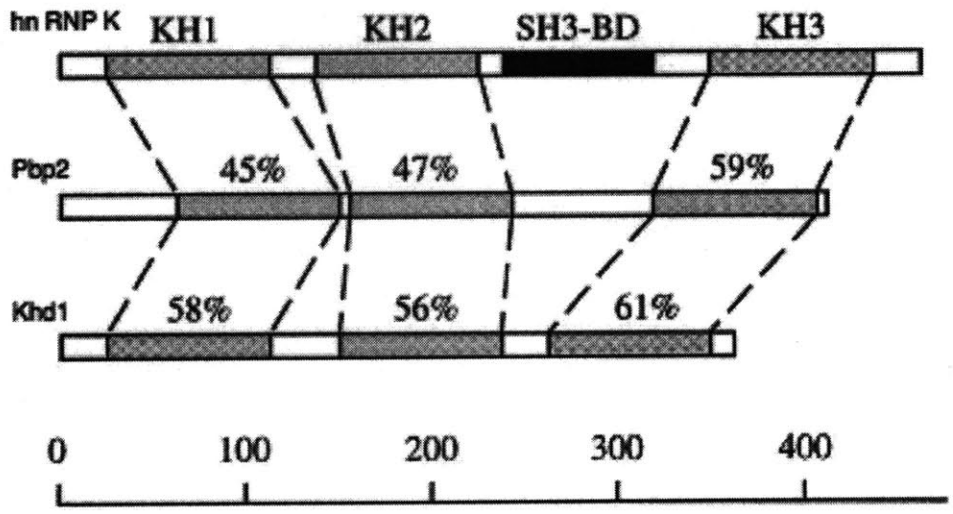

Figure 2.—Comparison of human hnRNP K with yeast proteins containing 3 KH domains. Percentages represent similarities between amino acid sequences in domains of the yeast proteins relative to hnRNP K (DENISENKO and BOMSZTYK 2002). The scale depicts length in amino acids.

Khd1 plays a key role in *ASH1* mRNA localization. *ASH1* mRNA encodes a transcription factor that is asymmetrically localized after cell division. Ash1 protein accumulates specifically in the daughter nucleus where it acts to repress expression of *HO* endonuclease (BOBOLA *et al.* 1996; SIL and HERSKOWITZ 1996). *HO* expression initiates mating type switching (HICKS and HERSKOWITZ 1976), and its asymmetric expression in the mother but not the daughter leaves only mother cells capable of switching mating type (NASMYTH 1993). Ash1 protein asymmetry is established by localization of *ASH1* mRNA to the bud tip (Figure 3) (LONG *et al.* 1997). *ASH1* mRNA is bound by the RBP She2, which is tethered to the motor protein Myo4 by the adaptor protein She3 (BOHL *et al.* 2000). Recent evidence suggests a She2-independent role for She3 in binding *ASH1* mRNA as well (LANDERS *et al.* 2009). Myo4 moves directionally along actin filaments to localize *ASH1* mRNA to the bud tip (TAKIZAWA *et al.* 1997).

The asymmetric distribution of Ash1 protein requires asymmetric translation *ASH1* mRNA. Translational repression of *ASH1* mRNA by Khd1 and the RBP Puf6 prevent

synthesis of Ash1 during transport (GU *et al.* 2004; IRIE *et al.* 2002). At the bud tip, phosphorylation of Khd1 and Puf6 reduces their affinity for *ASH1* mRNA, relieving repression and allowing translation to occur (DENG *et al.* 2008; PAQUIN *et al.* 2007). Ash1 protein contains a nuclear localization sequence, and preferentially enters the daughter nucleus after being produced at the bud tip. The repression conferred by Khd1 is required for establishing the asymmetric distribution. In the *khd1*Δ mutant, both mother and daughter nuclei accumulate Ash1 protein although *ASH1* mRNA localization is unaffected (IRIE *et al.* 2002).

Khd1 may regulate *ASH1* mRNA translation by interacting with the translation initiation factor eIF4G1 (PAQUIN *et al.* 2007). Khd1 from a cellular extract is retained by immobilized eIF4G1 in an RNA-dependent manner. The purified proteins also interact *in vitro,* although this reaction does not contain RNA. The interaction between purified components occurs through the C-terminal domain of eIF4G1. When this domain is removed *in vivo,* expression of an *ASH1* reporter construct increases in a manner similar to the increase observed in a *khd1*Δ background, implying the repressive function is lost even though Khd1 is present. Technical issues, however, such as the changing requirement for RNA, make the conclusions from these experiments suspect.

Khd1-mediated repression must be relieved for efficient translation of *ASH1* mRNA. Post-translational modifications of RBPs can affect their affinity for RNAs, and consequently their regulatory activity. The kinase Yck1 phosphorylates Khd1 *in vitro* (PAQUIN *et al.* 2007; PTACEK *et al.* 2005), decreasing its affinity for the E1 hairpin in *ASH1* mRNA (PAQUIN *et al.* 2007). A Split-Venus complementation assay shows that Yck1 and Khd1 physically interact at the cell periphery *in vivo.* This observation led to the model

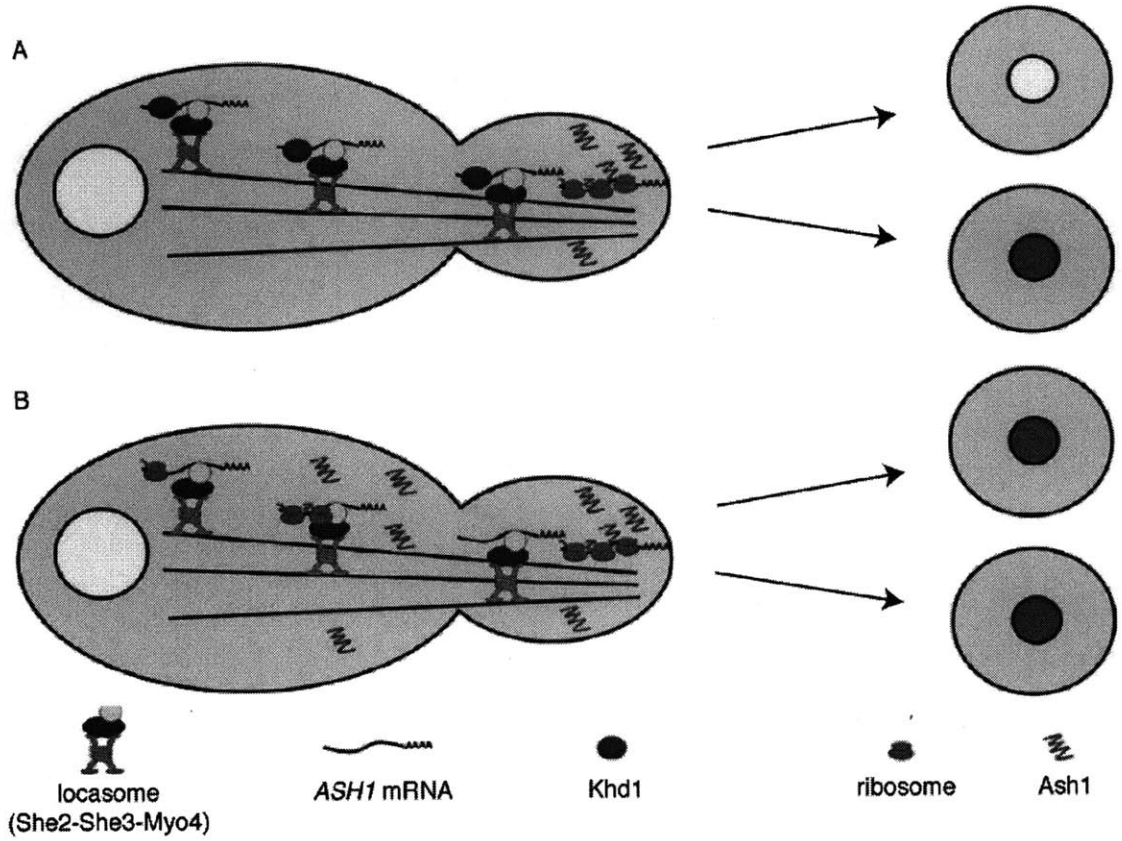

Figure 3.—Translational regulation of *ASH1* mRNA enables asymmetric Ash1 protein segregation. (a) Khd1 represses translation of *ASH1* mRNA during transport to the bud tip, producing accumulation of Ash1 specifically in the daughter nucleus. (b) In *khd1*Δ strains, premature expression leads to expression of Ash1 in the mother cell and symmetric distribution.

that Khd1 represses translation of *ASH1* mRNA during its transport to the bud tip, where phosphorylation by Yck1 reduces its affinity for the transcript, relieving repression and allowing translation to occur. In support of this, a luciferase reporter containing a localization element of *ASH1* mRNA shows higher activity in the *khd1*Δ mutant and decreased activity in the *yck1*Δ mutant. The *khd1*Δ *yck1*Δ double mutant displays the same activity as the *khd1*Δ mutant, confirming that the repression in the *yck1*Δ mutant requires Khd1 (PAQUIN *et al.* 2007). Interestingly, the Split-Venus complementation assay indicates that Khd1 and Yck1 interact all along the cell periphery and not only at the bud tip, suggesting potential interactions that may regulate other Khd1 activities.

Khd1 interacts with a number of mRNAs in addition to *ASH1* mRNA. Immunoprecipitation of Khd1 followed by microarray analysis of the enriched transcripts identified 1,210 mRNAs associated with the protein (HASEGAWA *et al.* 2008). Six of these mRNAs - *MID2, MTL1, WSC2, SRL1, EGT2,* and *CLB2* - are localized in a manner similar to *ASH1* mRNA (SHEPARD *et al.* 2003), and Khd1 co-localizes with them at the bud tip (HASEGAWA *et al.* 2008). For *MID2, MTL1,* and *WSC2* mRNAs, this co-localization occurs through sequences within their open reading frames (ORFs), similar to the colocalization of Khd1 with *ASH1* mRNA (HASEGAWA *et al.* 2008). One obvious model is that Khd1 represses translation of these mRNAs in a manner analogous to its function in localization of *ASH1* mRNA. However, of the proteins encoded by these mRNAs, only Srl1 levels are reduced by Khd1 overexpression in a manner similar to Ash1 (HASEGAWA *et al.* 2008). Mtl1 protein levels increase almost two-fold, and the levels of the other four proteins do not change upon Khd1 overexpression. The increase in Mtl1 protein levels results from stabilization of *MTL1* mRNA, an effect that is reversed in *khd1*Δ cells (HASEGAWA *et al.* 2008). Khd1 overexpression was induced for only two hours before assaying proteins levels in this experiment, and Khd1 could in fact repress production of the proteins that showed no change if they have lower turnover rates than Ash1. Regardless, the effect of Khd1 binding on protein expression varies for a small subset of co-localized transcripts. N Although the transcripts that enrich with Khd1 show decreased ribosome occupancy (HOGAN *et al.* 2008), little is known about how Khd1 affects expression of non-localized mRNAs, or the mechanisms that regulate its activity in other contexts.

A yeast three-hybrid screen designed to identify targets of Khd1produced very different results from the microarray-based approach. The screen identified an interaction between Khd1 and a transcript antisense to 25S rRNA represented by 13 clones. One

clone corresponded to an 18S rRNA antisense transcript, and one to 25S rRNA itself (PAZIEWSKA *et al.* 2005). No other clones were identified, suggesting Khd1 may have different binding activity based on its compartmentalization.

The RNA motif recognized by Khd1 is not fully understood. Like hnRNP K, Khd1 binds poly(rC) and poly(rU) specifically *in vitro* (DENISENKO and BOMSZTYK 2002). Analysis of the top 35 targets from the microarray study identified CNN repeats as an enriched motif. Although this motif is overrepresented in the list of mRNAs bound by Khd1, it is not found in every target transcript and occurs in 908 mRNAs that were not called as targets of Khd1. Khd1 associates with regions that contain small stretches of these repeats within the ORFs of the localized mRNA it targets. Gel shift assays show that Khd1 binds RNA containing CNN repeats *in vitro.* The binding can be competed strongly by poly(rC) and weakly by poly(rU) but not by poly(rA) or poly(rG) (HASEGAWA *et al.* 2008). Yeast three-hybrid analysis of Khd1 binding using both human and yeast RNA libraries identified a number of transcripts that contain C-rich patches typical of hnRNP K binding. Whereas most transcripts contained three of these patches, some contained a single element corresponding to the SELEX-identified hnRNP K target or none at all (PAZIEWSKA *et al.* 2004; PAZIEWSKA *et al.* 2005). However, these studies were performed using cellular RNA libraries and did not sample the same sequence space as randomized libraries.

A separate study found that Khd1 interacts with the E1 hairpin element in the coding sequence of *ASH1* mRNA to repress its translation (PAQUIN *et al.* 2007). The E1 element interacts with She2 to promote *ASH1* mRNA localization (CHARTRAND *et al.* 1999). A translational fusion with the *ASH1* ORF was shown to confer Khd1-mediated repression on a luciferase reporter construct only when the 250 bp E1 sequence was

included (PAQUIN *et al.* 2007). However, this structure only has micromolar affinity for Khd1 *in vitro* and the authors did not use mutational analysis to determine its role in binding. E1 does not overlap with the CNN-containing region with which Khd1 co-localizes *in vivo*. The sequence determinants for Khd1 binding and the effects of the interaction on target mRNA expression remain uncertain.

Khd1 has at least one nuclear function in addition to its cytoplasmic role in mRNA regulation. The finding that hnRNPs K and E1 bind telomeric DNA prompted studies of the role of KH domain proteins in telomere maintenance in *S. cerevisiae*. Khd1 affects silencing by heterochromatin at telomeric loci (DENISENKO and BOMSZTYK 2002). Compared to WT, *khd1*Δ shows decreased expression of telomeric reporter constructs. This effect is more dramatic when *PBP2,* which encodes the other yeast protein that contains three KH domains, is also deleted. Khd1 levels do not effect a telomere-distal reporter. The decreased silencing at the telomeres may result from the increased telomeric lengths seen in *khd1*Δ strains. Only the telomere proximal to the marker increases in length, regardless of which telomere it borders, raising questions about the experimental setup. However, ChIP reveals a physical association between Khd1 and subtelomeric sequences, supporting its possible role in telomere regulation.

Further exploration of this regulation identified a genetic interaction between *KHD1* and *SIR1* in regulating chromatin at telomeres and the mating loci (DENISENKO and BOMSZTYK 2008). In *sir1*Δ, expression from the telomeric reporter decreases relative to wild type, similar to *khd1*Δ. Expression from this reporter in the *sir1*Δ *khd1*Δ double mutant is equivalent to either individual single mutant, suggesting both genes act in the same pathway. Additional lines of evidence support this possibility. The *khd1*Δ mutation rescues the mating deficiency of a *sir1*Δ mutation that results from derepression of the

silenced mating loci. This derepression is also seen in the enhanced expression of a reporter construct at the MAT locus in *khd1*Δ. ChIP of Sir2 and Sir3 showed reduced occupancy at *HMR* in the *sir1*Δ background, but WT levels in the *sir1*Δ *khd1*Δ double mutant. Khd1 also localizes to *HMR* by ChIP, suggesting a direct role in *HMR* silencing and an effect on local chromatin composition. Khd1 therefore regulates chromatin structure at both the silent mating cassettes and telomeres.

Despite the progress made in understanding the role of Khd1, many questions remain. What are the sequence determinants for Khd1 recognition? How does binding effect expression of its target transcripts? And how do these properties combine to regulate cellular activities? To better understand the global role of Khd1 in regulating cellular activity, I used CLIP to identify its *in vivo* RNA targets. The goal was to identify all RNA targets of Khd1, and the specific sequences that mediate the interactions. This effort identified 1,163 transcripts that Khd1 binds *in vivo.* Khd1 appears to bind a C-rich motif similar to the CNN repeats previously identified, but this sequence does not explain all of its interactions with RNAs. To understand the regulation conferred by Khd1, I investigated the effects of binding on its most prominent targets, *FLO11* mRNA and Ty1 RNA. Khd1 represses *FLO11*-dependent filamentous growth and Ty1 retrotransposition by repressing translation of these two transcripts. A discussion of these phenotypes follows.

FLO11 and filamentous growth

FLO11 encodes a cell surface protein in *S. cerevisiae* similar in structure to mammalian mucin proteins (Lo and Dranginis 1996). Flo11 is approximately 1,200 amino acids in length, and contains a repetitive element rich in serine and threonine residues. Efficient transcription of the DNA encoding the repetitive element requires the

THO complex (VOYNOV *et al.* 2006). Flo11 protein is highly glycosylated in its repetitive element, and is covalently linked to the cell wall through a C-terminal glycosylphosphatidylinisotol anchor (LO and DRANGINIS 1996). There are two different repeated units in the repetitive element, and their respective contributions, as well as the overall length of the allele, differentially effect the *FLO11*-dependent phenotypes of diploid filamentous growth, haploid invasive growth, and biofilm formation (FIDALGO *et al.* 2008). Recombination between individual repeat units can produce new alleles with phenotypic consequences in as many as 1 in 250 cells under non-selective conditions (FIDALGO *et al.* 2008).

Diploid cells respond to low nitrogen environments by switching from yeast form to filamentous growth (GIMENO *et al.* 1992). Filamentation can also be stimulated by other environmental stresses such as heat, osmotic shock, and cell wall damaging agents (ZARAGOZA and GANCEDO 2000). The morphogenetic switch produces elongated cells that bud in a unipolar manner in contrast with the round cells and bipolar budding pattern seen in high nitrogen (Figure 4A) (KRON *et al.* 1994), and results in the unidirectional outgrowth of filaments from the colony thought to act as a foraging mechanism (Figure 4B) (GIMENO *et al.* 1992). This growth pattern results from an altered cell cycle that includes a delay in division that allows mother and daughter cells to synchronously re-enter mitosis (KRON *et al.* 1994). The developmental transition requires the coordinated alteration of multiple cellular processes, including the cell cycle and cell wall maintenance (GAGIANO *et al.* 2002), The first daughter cell under nitrogen starvation displays these properties (AHN *et al.* 1999), requiring rapid and coordinated alterations in multiple cellular processes.

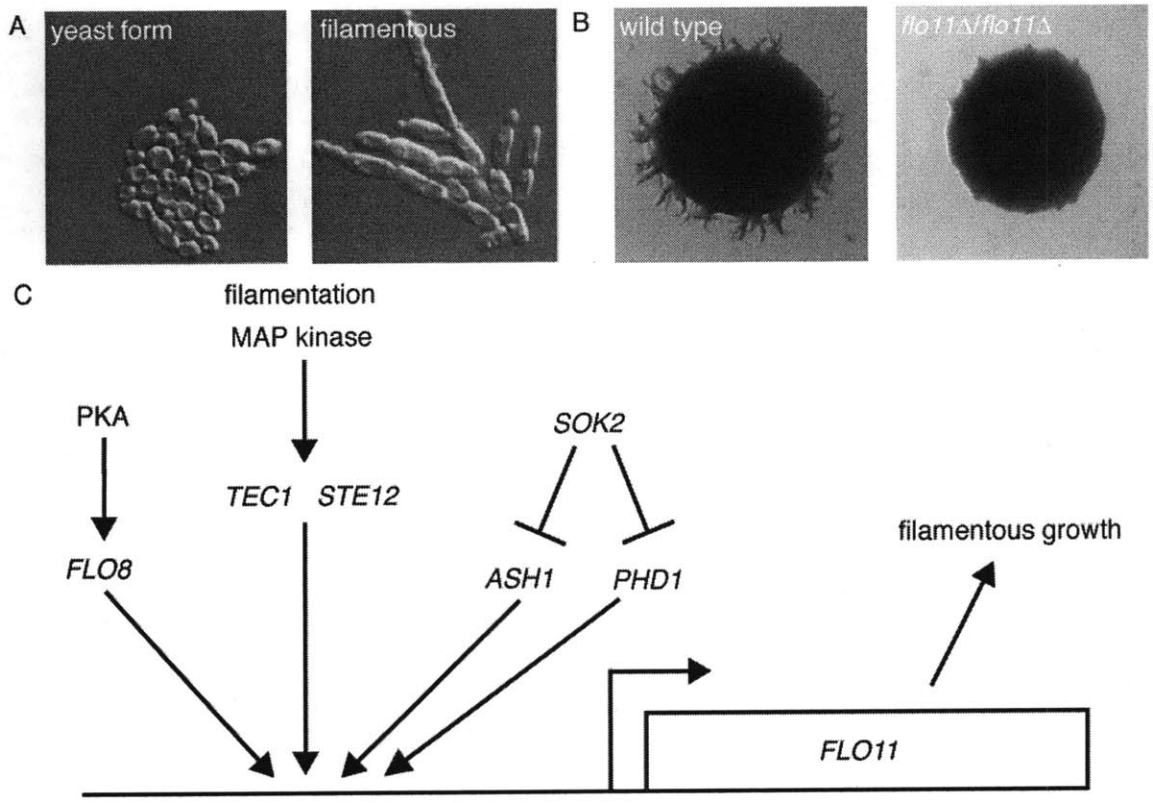

Figure 4.— *FLO11* expression and filamentous growth. (A) Yeast form and filamentous cells shown at high magnification (KRON *et al.* 1994). (B) Colony morphologies of wild type and *flo11Δ/flo11Δ* cells under low nitrogen conditions. Filamentous growth requires *FLO11*. (C) An overview of known pathways that regulate *FLO11* expression and filamentous growth.

Filamentous growth in *S. cerevisiae* serves as a model for filamentous growth of pathogenic fungi. Infectious fungi such as *Candida albicans* undergo a transition to filamentous growth inside the host (MITCHELL 1998). Mutants defective in this growth form show decreased virulence (LO *et al.* 1997). *C. albicans* biology hinders attempts to study filamentation in this pathogen. Cells only exist as diploids, making genetic screens for recessive traits impractical. Homologous recombination is inefficient relative to budding yeast, limiting genetic studies (NOBLE and JOHNSON 2007). The similarities between filamentous growth in *S. cerevisiae* and *C. albicans* facilitate the study of this disease-causing growth form in a model system. Similar to *FLO11,* members of the *C.*

albicans ALS gene family encode cell surface proteins with repetitive elements rich in serine and threonine. The *ALS* genes mediate adhesion to surfaces (HOYER *et al.* 2008) and contribute to filamentous growth (CHEN and CHEN 2000), but their high level of redundancy complicates functional studies. *FLO11* plays a role in *S. cerevisiae* analogous to the *ALS* genes in *C. albicans,* providing a tractable genetic system to investigate this developmental switch. Studies in *S. cerevisiae* have identified a number of pathways that regulate *FLO11* and filamentous growth.

Multiple pathways regulate *FLO11* expression (Figure 4C). The transcription factors Ste12 and Tec1, activated by the filamentation MAP kinase signaling pathway, and Flo8, responding to protein kinase A (PKA) signaling, act through multiple regions of the 3.5 kb *FLO11* promoter, which is large compared to the average yeast promoter (RUPP *et al.* 1999). A transcription factor cascade involving Sok2, Phd1, and Ash1 (PAN and HEITMAN 2000) also activates *FLO11. FLO11* expression requires each of these pathways, and the common laboratory strain S288C does not express *FLO11* because of a nonsense mutation in *FLO8* (LIU et al. 1996). Multiple chromatin remodeling factors regulate *FLO11* transcription epigenetically (HALME *et al.* 2004). In addition to this transcriptional regulation, *FLO11* is regulated post-transcriptionally. Translational repression through the *FLO11* 5' UTR is relieved under conditions of amino acid starvation, enabling haploid invasive growth in mutants with diminished *FLO11* mRNA levels (FISCHER *et al.* 2008). Some of these regulatory mechanisms may also contribute to ploidy control of *FLO11,* which is repressed to a greater extent in higher ploidy cells through unknown mechanisms (GALITSKI *et al.* 1999).

The filamentation MAP kinase and PKA signaling pathways regulate distinct features of filamentous growth in addition to *FLO11* expression. Cells in which PKA

signaling has been disrupted still elongate when starved for nitrogen, but do not adopt the unipolar budding pattern. Addition of cAMP activates PKA signaling and causes cells to form chains of yeast form filaments without elongation (PAN and HEITMAN 1999). Loss of function mutations in the MAP kinase pathway prevent cells from elongating, but do not alter the unipolar budding pattern (PAN and HEITMAN 1999) and activation of the pathway produces elongated buds in rich media and a hyperfilamentous phenotype under nitrogen starvation (AHN *et al.* 1999).

Cells within a clonal population express different levels of *FLO11,* with important phenotypic consequences. Two *FLO11* reporter constructs best demonstrate its transcriptional variegation. A clonal population of cells containing the P_{flo11}*::GFP* transcriptional fusion contains both 'on' and 'off' cells. Time-lapse microscopy reveals that cells can switch between the two states, and that daughter cells can enter either state regardless of the state of the mother cell (BUMGARNER and FINK unpublished data). A P_{flo11}*::URA3* transcriptional fusion also demonstrates the ability of cells to switch between these states. Cells can be serially passaged under conditions that select fist for, and then against *URA3* expression, demonstrating the ability of the cell to switch its expression on and off through variegation of the *FLO11* promoter (HALME *et al.* 2004).

FLO11 variegation leads to phenotypic differences within a clonal population. When grown into colonies in low nitrogen, only a subset of cells undergoes the transition to filamentous growth. Immunofluoresent staining of cells containing a Flo11::HA allele shows that these cells express Flo11 at their surface, whereas cells that remain in yeast form do not. Mutations that bias cells towards the 'on' or 'off' state modulate the expression of Flo11 and enhance or diminish filamentation respectively (HALME *et al.* 2004). Regulation of *FLO11* has important consequences for filamentous growth.

Ty element retrotransposition

Ty elements are retrotransposons found in budding yeast (BOEKE *et al.* 1985). There are five families of Ty elements classified by the homology of their reverse transcriptases. Ty3 is a Metaviridiae (gypsy-like element), whereas Ty1, Ty2, Ty4, and Ty5 are Pseudoviridiae (copia/Ty elements) (LESAGE and TODESCHINI 2005). Ty elements are 5-7 kb in length depending on the type, and are flanked by long terminal direct repeats (LTRs) of approximately 300 bp. Two overlapping ORFs, TYA and TYB, encode the proteins required for retrotransposition in all families except Ty5, which contains a single ORF encoding both polypeptides. TYA encodes a structural capsid protein and TYB encodes protease, integrase, and reverse transcriptase (LESAGE and TODESCHINI 2005). The Ty1 and Ty2 elements are highly homologous to each other, differing primarily in a 1.8 kb region spanning TYA, a .9 kb region in TYB (CURCIO and GARFINKEL 1991a) and by a single base indel in their LTRs (KIM *et al.* 1998).

Ty elements require a programmed frameshift followed by extensive post-translational processing to generate the mature proteins required for transposition. A +1 frameshift that occurs at a frequency of 35% enables TyB expression by generating a TyA-TyB fusion protein (KAWAKAMI *et al.* 1993). The fusion protein must be post-translationally processed to generate functional reverse transcriptase, integrase, and protease enzymes from TyB (GARFINKEL *et al.* 1991). The TyA primary translation product also undergoes programmed proteolysis to achieve its functional form (MERKULOV *et al.* 1996). In the absence of frameshifting, translation terminates after completing TyA synthesis, and TyA cannot be processed to its mature form.

The structural and functional features of Ty elements make them excellent models for studying retroviruses (CURCIO and GARFINKEL 1991a). Although Ty elements never

leave the cell and are not infectious, their transposition through an RNA intermediate, overlapping ORFs flanked by LTRs, and programmed frameshift are reminiscent of retroviral replication. TYA corresponds to the viral *gag* gene, and TYB, *pol*. Mature TyA proteins assemble into virus-like particles (VLPs) that contain the components required retrotransposition, similar to retroviral preintegration complexes (LESAGE and TODESCHINI 2005). The frameshift mechanism differs between Ty elements and retroviruses. In retroviruses, ribosome stalling at RNA secondary structures enables the simultaneous slippage of adjacent tRNAs into the -1 reading frame at specific heptamers (BALVAY *et al.* 2007). In Ty element translation, the availability of the low abundance tRNA R(CCU) regulates ribosome pausing and subsequent shift into the +1 reading frame due to a promiscuous anticodon (BELCOURT and FARABAUGH 1990). Changes in frameshifting efficiencies dramatically affect transposition rates. Increasing the ratio of Gag-Pol to Gag for HIV, analogous to increasing frameshifting, decreases infectivity up to 1,000-fold (SHEHU-XHILAGA *et al.* 2001), and either increasing or decreasing frameshifting by modulating tRNA R(CCU) expression decreases Ty1 transposition (KAWAKAMI *et al.* 1993; XU and BOEKE 1990).

Ty element retrotransposition can be mutagenic, and is regulated by the host to maintain its genomic integrity. Recombination between Ty elements can lead to inversions, deletions, and translocations, and the proliferation of these sequences in the genome increases the chances of deleterious events (GARFINKEL 2005). Insertion of a Ty element in a promoter or ORF can produce a null allele by preventing normal gene expression, but certain insertions activate gene expression. Although Ty1 elements usually insert near Pol III transcribed genes, they will infrequently insert near Pol II transcribed genes. If an insertion occurs upstream of a gene, within 175 bp of its initiation codon and

in the opposite orientation, sequences within TYA act as upstream activation sequences to promote transcription. These ROAM alleles adopt novel and cell type dependent regulation that may provide adaptive benefits (LESAGE and TODESCHINI 2005).

Expression of Ty1, the best-characterized family of Ty element, is regulated at multiple levels. In addition to the programmed frameshift and proteolytic cleavage previously mentioned, Ty1 elements are subject to transcriptional, post-transcriptional, and post-translational controls that contribute to its overall transposition activity. Ty1 transcription is dependent on host factors including *SPT3* (WINSTON *et al.* 1984) and *TEC1* (LALOUX *et al.* 1990). All Ty1 elements in a cell are either transcribed or silenced simultaneously, and can switch between the two states. Within a clonal population, at any given moment some cells express Ty1 while others do not. This is seen in *Ty1-URA3* fusions that can grow in both media lacking uracil and media containing 5-FOA. Ura^+ cells enriched in media lacking uracil contain abundant Ty1 RNA as assessed by Northern blot, while Ura^- cells enriched in 5-FOA express very low levels of Ty1 (JIANG 2002). Ty1 is also subject to post-transcriptional co-suppression. Inserting Ty1 elements into strains that lack endogenous transposons revealed that increasing the copy number decreased transposition of a marked element without affecting its RNA levels (GARFINKEL *et al.* 2003). A Ty1 antisense RNA represses transcriptional activity through histone modifications at the promoter, further modulating transposition activity (BERRETTA *et al.* 2008).

Differences between expression patterns of the Ty families necessitate different modes of regulation to limit their activities. For Ty1 and Ty2, the most highly transcribed elements, regulation is primarily post-transcriptional. Although Ty1 RNA can account for 1% of total cellular RNA, any given element undergoes transposition in only one of every

10^7 cell divisions (CURCIO and GARFINKEL 1991b). Nevertheless, transcription factors specifically activate one family or the other. Tec1 enhances Ty1 transcription without affecting Ty2 (LALOUX *et al.* 1990). In contrast to the highly transcribed Ty1 and Ty2 elements, Ty3 and Ty5 RNAs are barely detectable under normal conditions and require transcriptional activation for transposition activity. The addition of mating pheromones to haploid cells activates transcription and subsequent transposition of Ty3 and Ty5 elements while repressing Ty1 at a post-transcriptional level (LESAGE and TODESCHINI 2005).

Coordination of filamentous growth and retrotransposition

Similar regulatory mechanisms control Ty1 and *FLO11*. The variegated expression of Ty1 and *FLO11,* where cells within a clonal population differ in their expression profiles for these genes, has already been described. In addition, both are highly expressed in haploids but silenced in diploids under high nitrogen conditions. Low nitrogen activates their expression and their dependent phenotypes, filamentous growth and Ty1 transposition, in this cell type (LO and DRANGINIS 1998; MORILLON *et al.* 2000). The filamentation MAP kinase pathway regulates expression of *FLO11* and Ty1. Loss of function mutations in the pathway disable both filamentous growth and Ty1 transposition, and hyperactive alleles augment the phenotypes (MORILLON *et al.* 2000; MOSCH and FINK 1997; ROBERTS and FINK 1994). This common regulation is thought to coordinate responses to environmental stress. Under low nitrogen conditions, filamentous growth allows cells to forage for better nutritional environments, and transposition can generate new ROAM alleles that may provide a selective advantage (MORILLON *et al.* 2000).

Multiple MAP kinase pathways affect both transposition and filamentous growth. Fus3, the mating pheromone MAP kinase, suppresses transposition of Ty1 elements by

destabilizing VLPs (CONTE *et al.* 1998). It also plays a role in signaling specificity between the filamentation and pheromone response MAP kinase pathways (MADHANI and FINK 1998).The ability of *FUS3* to regulate Ty1 retrotransposition depends on the transcription factors Ste12 and Tec1 that operate downstream of the filamentation MAP kinase Kss1, but not Far1 which acts downstream of mating pheromone response MAP kinase signaling (CONTE *et al.* 1998). Deletion of *HOG1,* which leads to an increase in filamentation, shows a similar increase in Ty1 transposition but the molecular mechanisms have not been characterized (MADHANI *et al.* 1997).

My thesis demonstrates that Khd1 regulates filamentous growth and Ty1 transposition post-transcriptionally. Khd1 binds *FLO11* mRNA and Ty1 RNA and represses translation of these transcripts. Khd1 has an additional function in controlling *FLO11* transcription through its repression of *ASH1* mRNA. The resulting feed-forward regulation enables asymmetric *FLO11* expression and switching between yeast form and filamentous growth. Khd1 overexpression represses both filamentation and Ty1 retrotransposition. Deletion of *KHD1* produces hyperfilamentous cells, but Ty1 transposition is unaffected suggesting that additional mechanisms may repress Ty1 translation. Taken together, these data demonstrate novel coordination for Ty1 transposition and filamentous growth.

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Chapter 2: Feed-forward regulation of a cell fate determinant by an RNA-binding protein generates asymmetry in yeast

ABSTRACT

Saccharomyces cerevisiae can divide asymmetrically so that the mother and daughter cells have different fates. We show that the RNA-binding protein Khd1 regulates asymmetric expression of *FLO11* to determine daughter cell fate during filamentous growth. Khd1 represses transcription of *FLO11* indirectly through its regulation of *ASH1* mRNA. Khd1 also represses *FLO11* through a post-transcriptional mechanism independent of *ASH1.* Cross-linking immunoprecipitation (CLIP) coupled with high-throughput sequencing shows that Khd1 directly binds repetitive sequences in *FLO11* mRNA. Khd1 inhibits translation through this interaction, establishing feed-forward repression of *FLO11.* This regulation enables changes in *FLO11* expression between mother and daughter cells, which establishes the asymmetry required for the developmental transition between yeast form and filamentous growth.

INTRODUCTION

Asymmetric cell division produces two cells with different developmental fates (HORVITZ and HERSKOWITZ 1992). The unequal inheritance of cell fate determinants establishes this asymmetry in many systems through diverse mechanisms that ultimately produce asymmetric gene expression between cells (MACARA and MILI 2008). In multicellular eukaryotes, this process directs a cell lineage down a developmental path. In *Saccharomyces cerevisiae,* each mitotic division requires a new decision to determine the fate of the daughter cell, providing a tractable model to study the underlying mechanisms of asymmetric cell division.

The RNA-binding protein Khd1 (KH-domain protein 1) regulates asymmetric expression of *ASH1* in budding yeast to control mating type switching, a key developmental event in haploid cells (HASEGAWA *et al.* 2008; IRIE *et al.* 2002; PAQUIN *et al.* 2007). Ash1 protein accumulates specifically in the nuclei of daughter cells (BOBOLA *et al.* 1996; SIL and HERSKOWITZ 1996). Genetic and biochemical analysis led to the model that Khd1 represses translation of *ASH1* mRNA during transport to the bud tip, where phosphorylation by Yck1 reduces the affinity of Khd1 for the transcript, relieving repression and allowing translation to occur (CHARTRAND *et al.* 2002; IRIE *et al.* 2002; LONG *et al.* 1997; PAQUIN *et al.* 2007). As Ash1 is a transcription factor that represses mating type switching, translational repression of *ASH1* mRNA in the mother but not the daughter leads to asymmetry - the mother can switch mating type, but the daughter can not (CHARTRAND *et al.* 2002; PAQUIN and CHARTRAND 2008; STRATHERN and HERSKOWITZ 1979).

ASH1 has also been implicated in the regulation of filamentous growth, another developmental event in *S. cerevisiae* (CHANDARLAPATY and ERREDE 1998). Under conditions of nitrogen starvation, diploid cells enact a specialized growth program characterized by an elongated morphology and unipolar budding that leads to the formation of filaments (GIMENO *et al.* 1992). The transition to filamentous growth requires an asymmetric cell division, as a yeast form mother cell produces a filamentous daughter cell. *ASH1* regulates filamentous growth by activating expression of *FLO11* (PAN and HEITMAN 2000), which encodes a cell wall protein required for this growth form (LAMBRECHTS *et al.* 1996; LO and DRANGINIS 1996; LO and DRANGINIS 1998). Cells induce *FLO11* expression to activate filamentation in response to nitrogen starvation (LO and

DRANGINIS 1998). Deletion of *ASH1* prevents both *FLO11* expression (PAN and HEITMAN 2000) and the transition to filamentous growth (CHANDARLAPATY and ERREDE 1998).

Khd1 has no known role in regulating filamentous growth. However, since Khd1 represses *ASH1* in the context of mating type switching, it may regulate *ASH1* during filamentation as well. Given that RNA-binding proteins can coordinate the expression of mRNAs encoding functionally related proteins (KEENE 2007), Khd1 may regulate additional genes in the filamentation pathway. Microarray analysis following immunoprecipitation of Khd1 has been used to identify its mRNA targets (HASEGAWA *et al.* 2008; HOGAN *et al.* 2008), but the strains used do not transcribe *FLO11* mRNA (LIU *et al.* 1996) and the binding of Khd1 to mRNAs of the filamentation pathway such as *FLO11* would not have been detected.

The ability to comprehensively define post-transcriptional regulatory networks has been enormously advanced by the cross-linking immunoprecipitation (CLIP) method. CLIP utilizes UV radiation to crosslink an RNA-binding protein to its direct RNA targets *in vivo,* providing a snapshot of binding interactions. Direct sequencing of the RNAs following RNAse treatment localizes binding sites to a 60-100 nucleotide region within target transcripts (ULE *et al.* 2003). CLIP has been used in combination with high-throughput sequencing to comprehensively identify RNA targets of mammalian RNA-binding proteins (LICATALOSI *et al.* 2008; SANFORD *et al.* 2009; YEO *et al.* 2009), but has not been previously applied to yeast.

In this report, we use genetic analysis and CLIP coupled with high-throughput sequencing to determine the role of Khd1 in regulating filamentous growth. We find that Khd1 regulates both transcription and translation of *FLO11* to repress filamentation. Khd1 represses *FLO11* at the transcriptional level through its inhibition of *ASH1*, as we

predicted based on published regulatory interactions (CHANDARLAPATY and ERREDE 1998; HASEGAWA *et al.* 2008; IRIE *et al.* 2002; PAN and HEITMAN 2000; PAQUIN *et al.* 2007), and at the post-transcriptional level by directly repressing translation of *FLO11* mRNA. The feed-forward regulation of *FLO11* by Khd1 provides a dynamic mechanism to generate asymmetric expression and determine daughter cell fate following cell division. *FLO11* mRNA is the predominant unique transcript bound by Khd1, indicating this regulation is a primary function of the protein. Khd1 binds to repeated sequences in the coding region of *FLO11* mRNA and mRNAs encoding many other cell surface proteins, suggesting that this RNA binding protein may coordinate the synthesis of many disparate proteins that assemble into the cell wall.

MATERIALS AND METHODS

Yeast strains, media and growth conditions

All yeast strains used in this study are derived from Σ1278b and listed in Table S1. Standard yeast media, yeast transformations and genetic manipulations were performed as previously described (GUTHRIE and FINK 1991). To induce filamentation, strains were grown on nitrogen-poor SLAD media (GIMENO *et al.* 1992). Approximately 20 cells per strain were spotted onto a SLAD plate in 50 μL of water to compare filamentation under comparable conditions. To assay agar adhesion, 10^6 cells were spotted onto a YPD plate in 5 μL and grown for 3 days at 30°C prior to washing. Yeast strains carrying gene deletions were constructed by PCR amplification of kanamycin-resistance gene cassettes from the yeast deletion library (WINZELER *et al.* 1999) with approximately 200 bases of flanking sequence and transformation into Σ1278b. Yeast strains carrying TAP-tagged Khd1 were similarly constructed by amplification of the *KHD1-TAP::HIS3* construct from the TAP-tag

library (GHAEMMAGHAMI *et al.* 2003) and transformation into Σ1278b. Strains carrying P_{ADH} or P_{CYC1} (JANKE *et al.* 2004) were constructed by PCR amplification with primers containing 50bp of homology to the target locus and transformation into Σ1278b. Strains carrying *GFP::ADH 3' UTR::URA3* or *ADH 3' UTR::URA3* were similarly constructed using a plasmid provided by Sherwin Chan. See Table S2 for primer sequences.

Plasmid construction

The Khd1 overexpression construct was made by amplifying the gene using PCR, with oligonucleotides that added restriction sites (*NotI* at the 5' end, *XhoI* at the 3' end) to the final product (Table S2). Amplified DNA was digested using *NotI* and *XhoI* and cloned into p413TEF (MUMBERG *et al.* 1995).

Flow cytometry and immunofluoresence

Single colonies were picked after 2 days of growth on YPD plates and resuspended in 1.5 mL liquid YPD. Cells were inoculated into 10mL liquid YPD and grown for 18 hours to OD_{600} 0.13-0.16, washed twice with PBS, and resuspended in 50μL PBS containing $^1/_4$ μL Alexafluor 488-conjugated anti-hemaglutinin antibody (Molecular Probes A-21287). Cells were incubated 30 minutes at 4°C and washed three times in PBS prior to flow cytometry using the BD FACSCalibur, or imaging with the Nikon Eclipse TE2000-S.

qPCR

Total RNA was obtained by standard acid phenol extraction from 1 ml of cultures grown to OD_{600} 0.9-1.1 in YPD. The Qiagen QuantiTect Reverse Transcription Kit was used to remove residual genomic DNA and reverse transcribe the RNA templates to

generate cDNAs. Aliquots of cDNA were used in Real Time PCR analyses with reagents from Applied Biosystems and the ABI 7500 real-time PCR system.

Immunoprecipitation for measuring RNA enrichment

TAP tag immunoprecipitation and RNA isolation was performed as previously described (GERBER *et al.* 2004), using 200mL of starting culture rather than 1L and proportionately fewer reagents.

Cross-linking immunoprecipitation

Khd1-TAP was purified from 1L of cells grown to an OD_{600} of 2.5 and UV-crosslinked three times at 400 mJ/cm^2. Purification using calmodulin sepharose was followed by binding to magnetic IgG beads (File S1). The CLIP protocol was then followed as previously described (ULE *et al.* 2005). The resulting cDNA was amplified using PCR with oligonucleotides containing sequences for hybridization to the Illumina flow cell (Table S2).

Illumina sequencing

Samples were sequenced using Illumina sequencing with a custom primer (Table S2), returning 16,026,920 thirty-six nucleotide long reads. Reads containing unresolved bases (N) were ignored. The complete set of reads contained 6,324,854 unique sequences. All reads were mapped to the ∑1278b genome (DOWELL *et al.* 2010) using Novoalign (v1.05; 2nd September 2008) with default settings. All mappings are included, weighted inversely by the number of genomic locations to which a read maps. The reads have been deposited in the Sequence Read Archive under accession number SRA012416.

Peak calling

The peak caller uses a rolling window approach (10 base windows; 5 base offset) to compare the observed reads to those expected from a Poisson background model. Adjacent enriched windows are combined into peaks. Peaks are assigned to genes based on overlap with existing annotation, extending 500 nucleotides in each direction (unless the extension overlaps adjacent annotation) to account for UTRs.

A local (5 kb) window is used to parameterize the background model. A visual examination of the read mappings relative to available tiled expression data (DANFORD *et al.* 2010) indicates reads are strand specific and show perfect correspondence with expressed segments, indicating the background of possible RNA binding sites is the transcriptome, not the genome. A weak correlation is observed between the expression levels of a transcript and the number of observed reads.

We set a peak cutoff by maximizing the correspondence of gene targets predicted relative to the targets reported by Hasegawa (HASEGAWA *et al.* 2008). The peaks are weighted by the corresponding expression level of each transcript, as determined from tiled expression data (DANFORD *et al.* 2010). Only peaks containing at least 50% of the reads of the transcript's maximal peak size are considered.

Motif discovery

Three methods were utilized to identify the motif recognized by Khd1. First, MEME (v4.1; (BAILEY and ELKAN 1994)) was utilized on the sequences under the peaks, filtering to remove highly identical sequences (80% identity). Second, all k-mers were evaluated (for k=1,2-,3,4) to identify over-represented sequences under the peaks. Random non-peak windows of matching length were selected from the same set of

transcripts as the peaks to calculate the distribution of background k-mers. Finally, RNApromo (RABANI et al. 2008) and CMfinder (YAO et al. 2006) were applied to the peaks to search for potential secondary structure. The structure motifs returned were single stranded loops with sequence patterns consistent with the primary sequence motif identified by MEME. Presence of the discovered MEME motif within the peak list was determined using MAST (v4.1; (BAILEY and GRIBSKOV 1998)) with default parameters.

Western blot analysis

Protein was prepared using TCA precipitation from 3 mL of culture grown to OD_{600} of .9-1.1, resuspended in 150 µL SDS loading buffer, and boiled for 10 minutes. 10 µL were run on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose filter paper. Blotting against GFP was performed with mouse anti-GFP primary antibody (Roche 11814460001) and HRP-conjugated sheep anti-mouse secondary antibody (Amersham NA931V), and against tubulin using rat anti-tubulin (Accurate Chemicals MCA77G) and HRP-conjugated goat anti-rat antibody (Jackson ImmunoResearch 112-035-062). Blots were detected using SuperSignal West Femto Substrate (Thermo Scientific 34095).

RESULTS

Khd1 has *ASH1*-dependent and *ASH1*-independent functions in repressing *FLO11*

Given that *ASH1* promotes filamentous growth (CHANDARLAPATY and ERREDE 1998) by activating transcription of *FLO11* (PAN and HEITMAN 2000), and that Khd1 represses *ASH1* in the context of mating type switching (HASEGAWA *et al.* 2008; IRIE *et al.* 2002; PAQUIN *et al.* 2007), we hypothesized that Khd1 regulates filamentous growth. Genetic analysis shows that Khd1 represses filamentation. The *khd1*Δ/*khd1*Δ mutant is

hyperfilamentous relative to wild type, and cells fail to filament when Khd1 is overexpressed (Figure 1A). The hyperfilamentation phenotype of the *khd1Δ/khd1Δ* mutant requires *FLO11*. As is the case with the *flo11Δ/flo11Δ* mutant, the *khd1Δ/khd1Δ flo11Δ/flo11Δ* mutant is nonfilamentous (Figure 1B). These findings are consistent with our prediction that Khd1 regulates filamentation by repressing transcription of *FLO11* indirectly through its translational repression of *ASH1* mRNA.

However, Khd1 represses filamentation at least in part through an *ASH1*-independent pathway. The *khd1Δ/khd1Δ ash1Δ/ash1Δ* double mutant is filamentous,

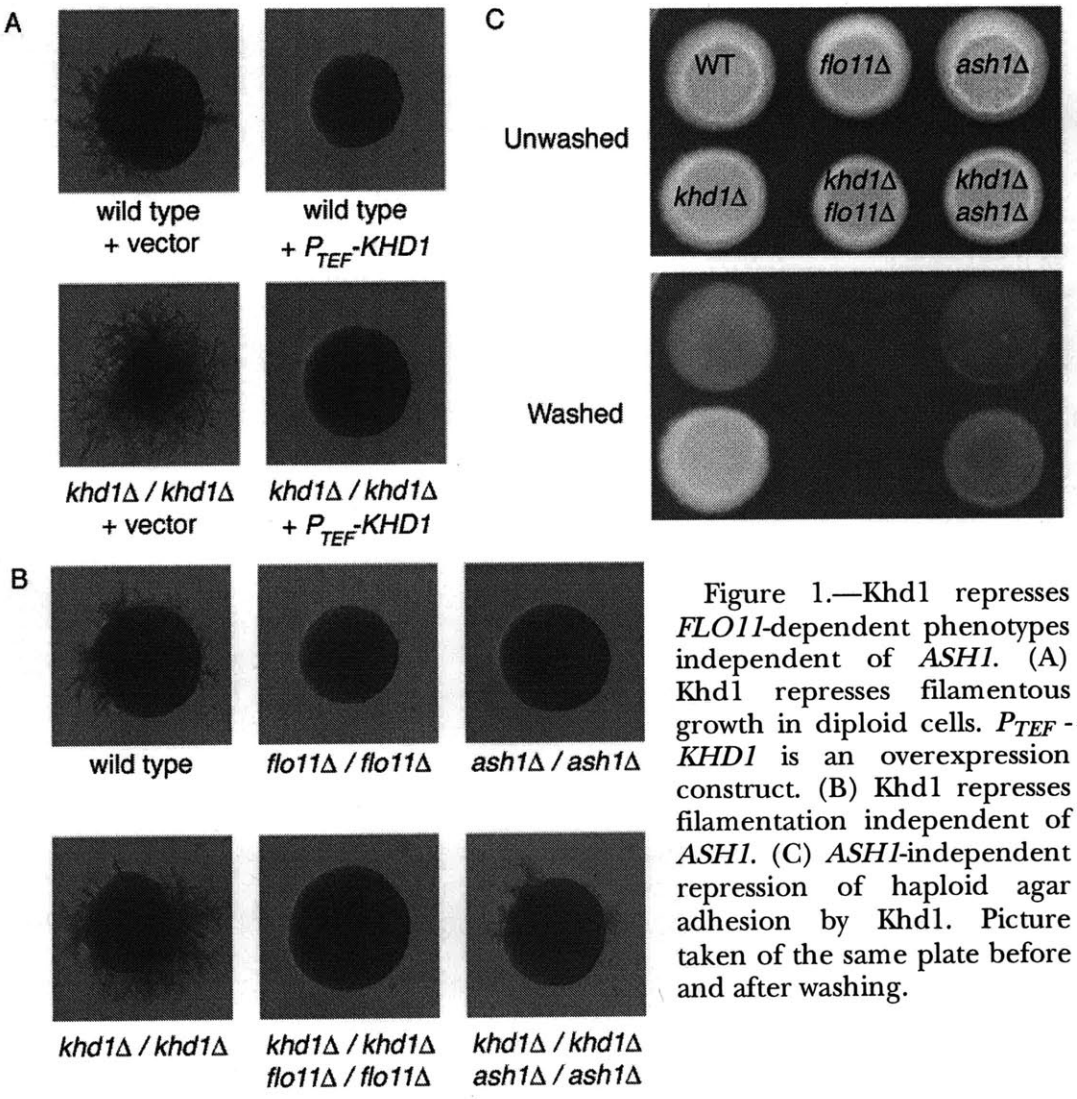

Figure 1.—Khd1 represses *FLO11*-dependent phenotypes independent of *ASH1*. (A) Khd1 represses filamentous growth in diploid cells. P_{TEF}-*KHD1* is an overexpression construct. (B) Khd1 represses filamentation independent of *ASH1*. (C) *ASH1*-independent repression of haploid agar adhesion by Khd1. Picture taken of the same plate before and after washing.

unlike the *ash1Δ/ash1Δ* single mutant, indicating that Khd1 represses filamentation independent of *ASH1* (Figure 1B). This finding extends to haploid agar adhesion, another *FLO11*-dependent phenotype. Cells deleted for *KHD1* adhere more than wild type cells, and *khd1Δ ash1Δ* double mutants adhere more than *ash1Δ* single mutants (Figure 1C). As is the case for filamentation, adhesion of both wild type and *khd1Δ* cells requires *FLO11* (Figure 1C; LAMBRECHTS *et al.* 1996, LO and DRANGINIS 1998). These data show that Khd1 represses *FLO11*-dependent phenotypes independent of *ASH1*.

Given the repression of *FLO11*-dependent phenotypes by Khd1, we tested whether Khd1 regulates *FLO11* expression. To quantify *FLO11* expression, we employed a *FLO11::HA* allele that permits the measurement of Flo11 protein in individual cells (GUO *et al.* 2000). Flo11 protein is expressed in a subset of cells in a clonal population because of variegating transcription from the *FLO11* promoter (BUMGARNER *et al.* 2009; HALME *et al.* 2004). Mutations that affect *FLO11* mRNA levels and filamentation show a corresponding change in the number of cells containing the *FLO11::HA* allele that stain positive using an anti-HA antibody (HALME et al. 2004).

Flow cytometry shows that Khd1 represses expression of Flo11 protein. Deletion of *KHD1* increases the percentage of diploid cells expressing Flo11 protein (Table 1). In addition, the *khd1Δ/khd1Δ* cells that express Flo11 protein do so at a higher level than wild type cells expressing Flo11.

Table 1

Khd1 represses Flo11 protein expression independent of *ASH1*

Strain	% Cells Expressing Flo11	Mean Expression in Flo11 Positive Cells
wild type	58 ± 6	100 ± 13
khd1Δ/khd1Δ	80 ± 4	153 ± 18
ash1Δ/ash1Δ	14 ± 2	56 ± 3
khd1Δ/khd1Δ ash1Δ/ash1Δ	32 ± 5	61 ± 5

Values are average of four independent trials. Error reported as standard deviation.

Similar to its regulation of filamentous growth, Khd1 represses Flo11 protein expression independent of *ASH1*. Although the populations of *khd1Δ/khd1Δ ash1Δ/ash1Δ* and *ash1Δ/ash1Δ* cells that express Flo11 display similar levels of the protein, a higher percentage of *khd1Δ/khd1Δ ash1Δ/ash1Δ* cells express Flo11 (Table 1). The Flo11 expression data, together with the filamentation and agar adhesion phenotypes, point to an *ASH1*-independent function for Khd1 in repressing *FLO11*.

To explore the regulation of *FLO11* by Khd1, we used qPCR to measure *FLO11* mRNA levels. *khd1Δ/khd1Δ* mutants have increased *FLO11* mRNA levels relative to wild type (Figure 2), which indicates that Khd1 represses *FLO11* mRNA accumulation. In contrast to its *ASH1*-independent repression of filamentation and Flo11 protein expression, Khd1 represses *FLO11* mRNA levels exclusively through its regulation of *ASH1*. *khd1Δ/khd1Δ ash1Δ/ash1Δ* double mutants display the same *FLO11* mRNA levels as *ash1Δ/ash1Δ* single mutants, which are below that of wild type (Figure 2). We conclude that Khd1 represses transcription of *FLO11* mRNA through its regulation of *ASH1*. The restoration of filamentation and increased Flo11 protein expression in *khd1Δ/khd1Δ ash1Δ/ash1Δ* relative to *ash1Δ/ash1Δ*, without a concomitant increase in *FLO11* mRNA levels, suggests that Khd1 represses *FLO11* through a post-transcriptional mechanism as well.

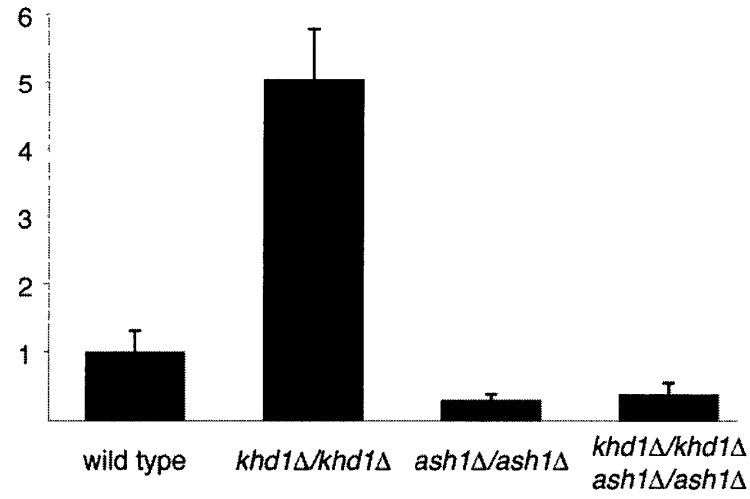

Figure 2—Khd1 represses *FLO11* mRNA levels through *ASH1*. *FLO11* mRNA levels normalized to *ACT1* mRNA. Values are average of four independent experiments. Error reported as standard deviation.

Khd1 binds repeated sequences in the *FLO11* open reading

frame

The post-transcriptional regulation of *FLO11* by Khd1 suggested that Khd1 might interact with *FLO11* mRNA. To address this possibility, we tested whether *FLO11* mRNA co-immunoprecipitates with a TAP-tagged version of Khd1. qPCR shows that immunoprecipitation of Khd1-TAP enriches *FLO11* mRNA more than 50-fold (Figure 3A). The same immunoprecipitation does not enrich *FLO11* mRNA when Khd1 is untagged. Immunoprecipitations testing for an interaction between Khd1 and constructs containing different combinations of the *FLO11* open reading frame and untranslated regions indicate that Khd1 interacts with the *FLO11* coding sequence (Figure S1).

To examine the interaction between Khd1 and *FLO11* mRNA further, we identified RNA binding sites for Khd1 using CLIP in conjunction with high-throughput sequencing (File S2, Figure S2, Table S3). The CLIP analysis shows that Khd1 interacts directly with repetitive sequences in *FLO11* mRNA *in vivo* (Figure 3B). *FLO11* mRNA is the most frequently represented unique mRNA in the data set; of the 16 million sequences we generated, 1.97 million derive from Khd1 binding to *FLO11* mRNA.

To determine whether the repeated sequences in *FLO11* mRNA are sufficient for recognition by Khd1, we generated a construct that isolates the *FLO11* repetitive element. Immunoprecipitation of Khd1-TAP enriches a transcript with the *FLO11* repeats fused to *GFP* driven by the *ADH* promoter (Figure 3C). Because the repeats cause a ten-fold decrease in *GFP* mRNA levels relative to the *ADH* promoter driving *GFP* alone (Figure S3), we used the weaker *CYC1* promoter to express comparable levels of *GFP* without the repeated sequences. *GFP* mRNA does not enrich in the Khd1-TAP immunoprecipitation when driven by either promoter in the absence of the *FLO11* repetitive element (Figure

3C). We conclude that the repeated sequences in *FLO11* mRNA are sufficient for recognition by Khd1.

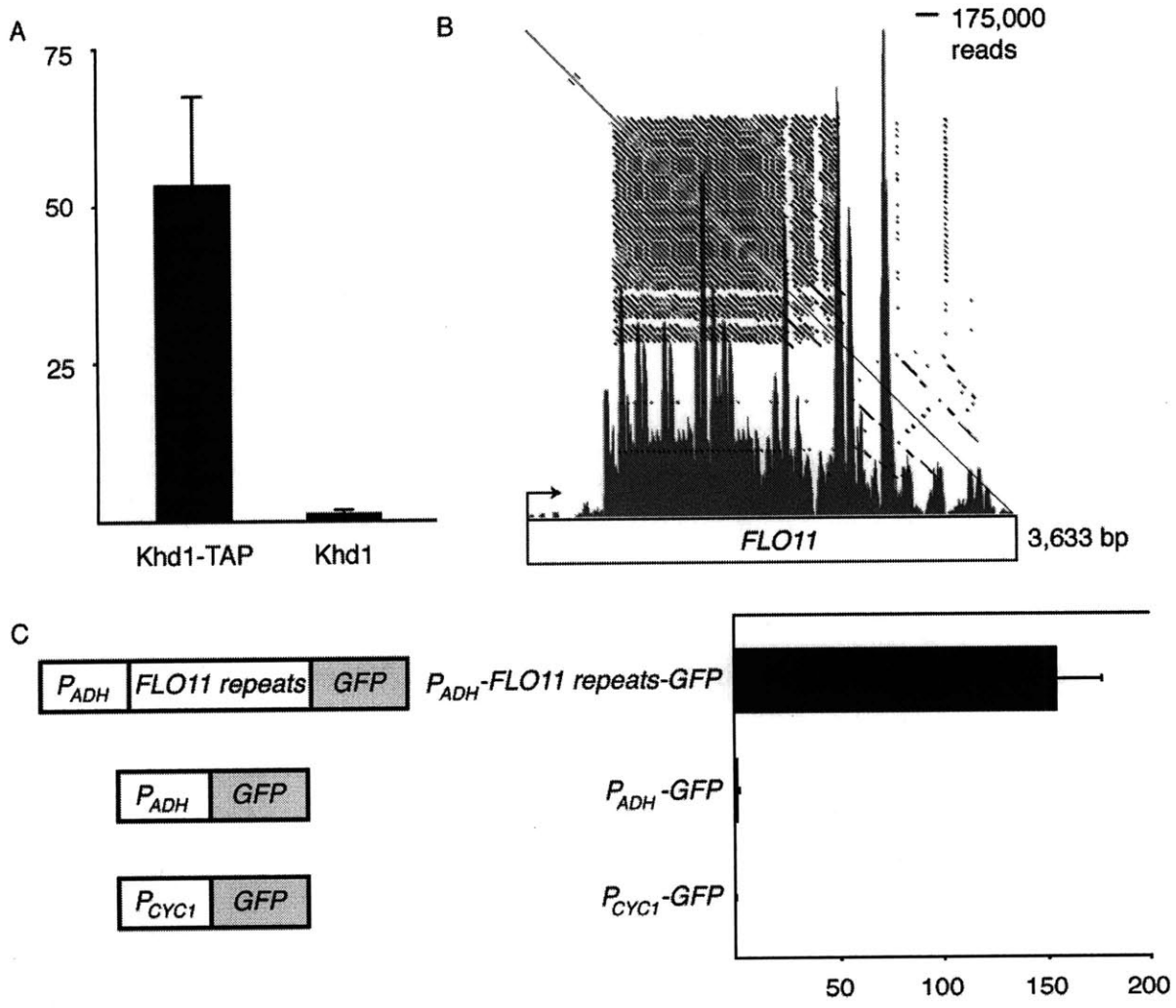

Figure 3.—Khd1 binds repetitive sequences in the *FLO11* open reading frame. (A) Enrichment of *FLO11* mRNA following immunoprecipitation from cells expressing either Khd1-TAP or untagged Khd1. (B) Khd1 target sequences from CLIP map to the *FLO11* repetitive element. Histogram of read mappings overlaid on a dot plot highlighting the repetitive region of the *FLO11* open reading frame from the ∑1278b genome (http://www.vivo.colostate.edu/molkit/dnadot/ window size = 11, mismatch limit = 1). (C) Enrichment of constructs following immunoprecipitation of Khd1-TAP. Enrichments expressed as the level of the transcript relative to *ACT1* mRNA in the immunoprecipitate divided by the level of the transcript relative to *ACT1* mRNA in the input. Values are average of four independent experiments. Error reported as standard deviation.

Khd1 represses translation through the *FLO11* repetitive element

We used the construct with *GFP* fused to the *FLO11* repetitive element to test the effect of Khd1 binding to this region. Western blotting shows that GFP protein levels from this fusion construct increase 12-fold in *khd1*Δ relative to wild type (Figure 4A compare lanes 1 and 2). qPCR measurements show that Khd1 expression causes a two-fold decrease in mRNA levels from this construct (Figure 4B compare lanes 1 and 2). We attribute the remaining six-fold difference in GFP protein levels relative to *GFP* mRNA levels between wild type and *khd1*Δ to translational repression that results from Khd1 binding the *FLO11* repetitive element. Khd1 overexpression further represses the construct with the *FLO11* repeats fused to *GFP*, reducing the amount of GFP protein below that seen with the empty vector, without affecting *GFP* mRNA (Figure 4A, 4B, compare lanes 1 and 3).

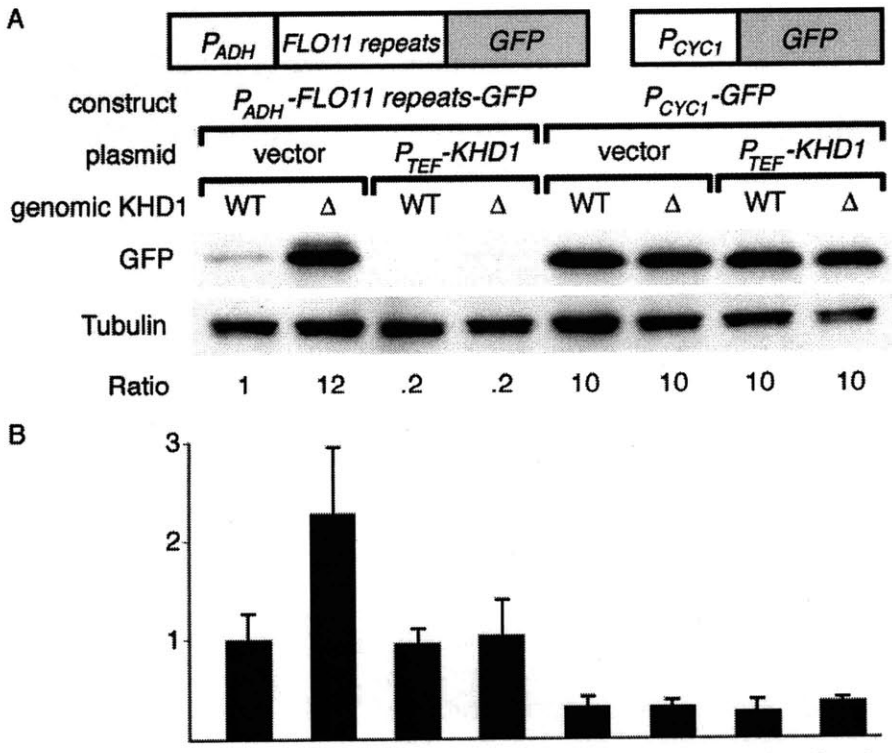

Figure 4.—Khd1 represses translation through the *FLO11* repeats. (A) Western blot analysis of GFP protein levels from constructs expressing *GFP* alone, or *GFP* fused to the *FLO11* repetitive sequences. P_{TEF}-*KHD1* is an overexpression construct. The only visible band detected from wild type, and the predominant band from the *khd1*Δ mutant, migrate at the same molecular weight as GFP alone, suggesting that translation initiated at the *GFP* start codon. The higher migrating band from *khd1*Δ may result from low levels of translation initiation inside the repetitive element that become visible after derepression. (B) *GFP* mRNA levels normalized to *TUB1* mRNA levels for the strains shown in (A). Values are average of four independent experiments. Error reported as standard deviation.

Neither deletion or overexpression of Khd1 affects protein or mRNA levels from constructs lacking the *FLO11* repetitive element (Figure 4A, 4B lanes 5-8, Figure S4). In addition to repressing transcription of *FLO11* by regulating *ASH1* expression, Khd1 represses translation through its interaction with repeated sequences in *FLO11* mRNA.

Translational repression of the fusion construct is consistent with the post-transcriptional repression of Flo11 protein expression by Khd1. Although Khd1 does not appear to regulate endogenous *FLO11* mRNA levels independent of *ASH1* (Figure 2), mRNA levels from the construct with the *FLO11* repeats fused to *GFP* increase in the *khd1*Δ mutant (Figure 4B). The fusion transcript may be subject to different regulation than *FLO11* mRNA independent of Khd1. Alternatively, low levels of *FLO11* mRNA in the *ash1*Δ/*ash1*Δ mutant may preclude detection of small changes in stability. To test *FLO11* mRNA stability, we used the *ADH* promoter to transcribe full-length *FLO11* mRNA and measured its steady-state levels, similar to our measurement of mRNA from the fusion construct. In the *khd1*Δ mutant, *FLO11* mRNA levels from this construct are 63% of those in wild type. Changes in mRNA stability alone do not explain the differences between mRNA and protein levels for either the fusion construct or endogenous *FLO11* in the absence of Khd1. Therefore, translational repression through the repeats is the predominant post-transcriptional regulation of *FLO11* mRNA by Khd1.

Khd1 regulates Flo11 asymmetry

Flo11 protein expression determines daughter cell fate during filamentous growth. To determine whether the transcriptional and translational regulation by Khd1 affects Flo11 expression between mother and daughter cells, we scored Flo11 expression patterns using the *FLO11::HA* allele and fluorescence microscopy. The four possible expression patterns

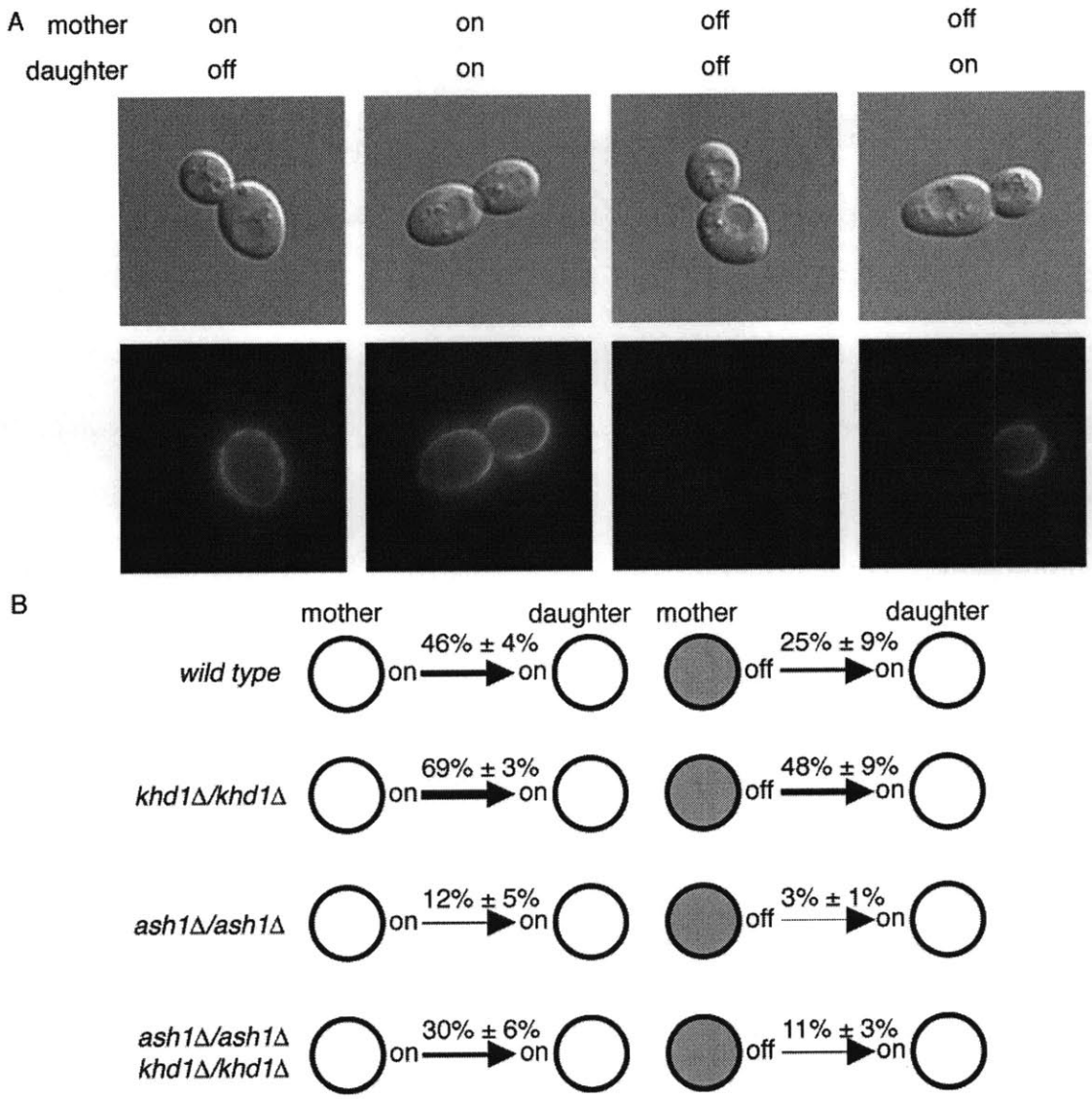

Figure 5.—Khd1 regulates mother-daughter Flo11 expression. Fluorescence microscopy was used to visualize Flo11 protein expression from the *FLO11::HA* allele. (A) Flo11 expression patterns in mother-daughter pairs. (B) Khd1 affects the frequency at which daughter cells express Flo11 protein. The chance that a mother cell gives rise to a daughter cell expressing Flo11 protein increases when *KHD1* is deleted, independent of *ASH1* and whether or not the mother cell expresses Flo11 protein. The frequency of a daughter cell expressing Flo11 protein being produced from a mother cell that expresses Flo11 protein was determined by dividing the number of these mother-daughter pairs by the total number of pairs in which the mother expresses Flo11 protein. The frequency of a daughter cell expressing Flo11 protein being produced from a mother cell that does not express Flo11 protein was determined by dividing the number of these mother-daughter pairs by the total number of pairs in which the mother does not express Flo11. 250 mother-daughter pairs were analyzed per genotype in each of nine separate trials. Error reported as standard deviation.

between mother and daughter cells were each observed (Figure 5A). Mother cells that express Flo11 can give rise to daughter cells that also express the protein, or those that

switch Flo11 expression off. Reciprocally, mother cells that do not express Flo11 can produce daughter cells that similarly do not express the protein, or those that switch Flo11 expression on. We calculated probabilities for daughter cell Flo11 expression given the Flo11 expression of the mother cell based on the frequencies of these expression patterns.

Repression by Khd1 reduces the frequency of Flo11 expression in daughter cells. Compared to wild type daughter cells, *khd1Δ/khd1Δ* daughter cells are more likely to express Flo11 protein whether or not it is expressed in the mother (Figure 5B). These increases result from the loss of the combined transcriptional and translational repression of *FLO11* by Khd1. More *khd1Δ/khd1Δ ash1Δ/ash1Δ* daughter cells than *ash1Δ/ash1Δ* daughter cells also express Flo11 protein whether or not it is expressed in the mother (Figure 5B). These increases result solely from the loss of translational repression by Khd1, since the deletion of *ASH1* inactivates the transcriptional regulation. Although the loss of Khd1-mediated translational repression of *FLO11* mRNA increases the expression of Flo11 protein in daughter cells, maximal induction of Flo11 expression in daughter cells, seen in the *khd1Δ/khd1Δ* mutant, requires the dual relief of both the transcriptional and translational repression of *FLO11* by Khd1.

Khd1 binds many mRNAs that encode cell wall proteins

Khd1 binds a number of mRNAs encoding cell wall proteins in addition to *FLO11* mRNA. 54 of the Khd1 target mRNAs we identify using CLIP (Table S4) encode proteins that play a role in cell wall function, nearly half of the 114 genes with this annotation ($p = 5.85 \times 10^{-15}$) (BEISSBARTH and SPEED 2004). Similar to *FLO11* mRNA, many of the Khd1 targets that encode cell surface proteins contain repeated sequences. When target genes

are sorted by the number of sequences that map to their binding sites, nine of the top ten – *FLO11, SED1, YIL169C, AGA1, SCW10, MSB2, RPO21, CRH1,* and *YNL190W*- contain repeats (reported in VERSTREPEN *et al.* 2005 or determined by visual inspection) and eight of these nine encode cell surface proteins, with the lone exception being *RPO21.* With the exception of *CRH1* mRNA, Khd1 binds these nine transcripts through their repetitive elements (Figure S5, Figure 3B), implying that Khd1 frequently binds repeated sequences. Khd1 appears to have a bias for messages with repeated sequences as it binds mRNAs transcribed from 32 of the 44 *S. cerevisiae* genes previously reported to contain intragenic repeats (VERSTREPEN *et al.* 2005).

However, the presence of repeats is not the only determinant of Khd1 binding. First, not all mRNAs bound by Khd1 have repeated sequences. Second, in some cases where Khd1 binds to messages with repeated sequences, the binding is not in the region of repeats (Figure S3, *CRH1*). Third, Khd1 does not bind all mRNAs that contain repeated sequences.

To understand the determinants of recognition by Khd1, we analyzed the sequences within its binding sites. MEME analysis (BAILEY and ELKAN 1994) produces a degenerate octamer motif (Figure 6) that occurs in 12% of the Khd1 binding sites. This result is consistent with the CNN repeats found to mediate Khd1 binding in a previous study (HASEGAWA *et al.* 2008). Examination of our motif reveals additional features that may contribute to the interaction between Khd1 and its target RNAs. The repeating CA pattern is similar to the one found in RNAs

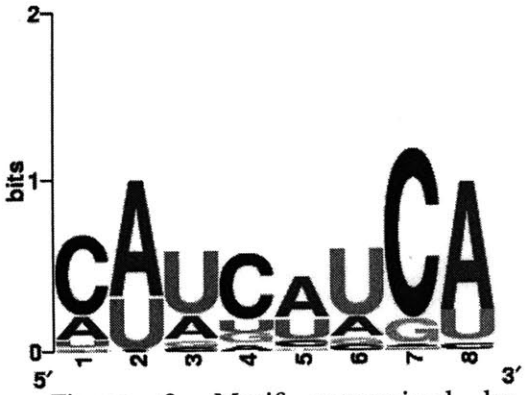

Figure 6.—Motif recognized by Khd1. MEME result from the sequences within the binding sites identified by CLIP.

recognized by the mammalian RNA-binding protein Nova (BUCKANOVICH and DARNELL 1997; JENSEN *et al.* 2000; LICATALOSI *et al.* 2008; ULE *et al.* 2003). Khd1 and Nova both contain three K-homology RNA-binding domains (BUCKANOVICH *et al.* 1993; CURRIE and BROWN 1999), and structural studies indicate that the third KH domain in Nova makes specific contacts with the internal CA in a YCAY (where Y indicates a pyrimidine, U or C) tetramer (LEWIS *et al.* 2000). CA is the most enriched dinucleotide (1.8-fold relative to background) in the Khd1 binding sites. Two of the four tetranucleotides with the highest enrichments relative to background - CAAC, CUCC, CAUC, and CUAC are enriched 3.3-, 3.0-, 2.9-, and 2.6-fold respectively - contain CA in the first and second position, but not internally as in the YCAY motif. All four contain C in the first and last position. This analysis identifies new possible determinants of recognition by Khd1, but despite our high-resolution detection of *in vivo* binding sites, we do not find a motif to explain the specificity of Khd1 for all of its RNA targets.

DISCUSSION

Our genetic and biochemical studies show that Khd1 acts post-transcriptionally on two mRNAs to repress *FLO11* expression and filamentation. Previous studies showed that *ASH1* activates *FLO11* expression (PAN and HEITMAN 2000) and filamentous growth (CHANDARLAPATY and ERREDE 1998), and that Khd1 represses translation of *ASH1* mRNA in the context of mating type switching (HASEGAWA *et al.* 2008; IRIE *et al.* 2002; PAQUIN *et al.* 2007). Our results demonstrate that Khd1 represses *FLO11* expression both through its regulation of *ASH1,* and by directly inhibiting translation of *FLO11* mRNA through repetitive sequences in the open reading frame. This dual inhibition places Khd1 at the

head of a feed-forward loop regulating *FLO11* (Figure 7) and raises the question of why cells employ this regulatory architecture.

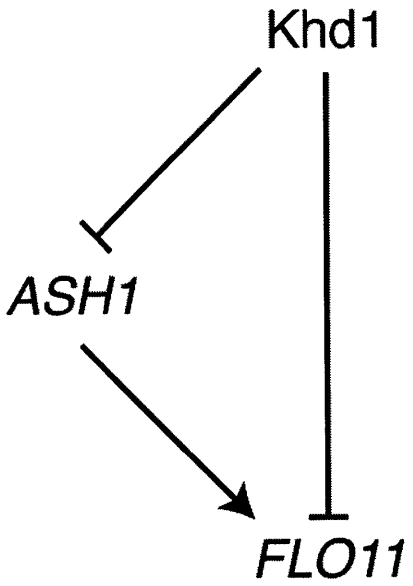

Figure 7.—Feed-forward regulation of *FLO11* by Khd1. Khd1 regulates transcription of *FLO11* through its repression of *ASH1* mRNA, and directly represses translation by binding repeated sequences in the open reading frame of *FLO11* mRNA.

The answer may reside in the biology of *FLO11*, whose function is required to switch from the yeast form to the filamentous form (HALME *et al.* 2004; LAMBRECHTS *et al.* 1996; LO and DRANGINIS 1998). In the first cell cycle under conditions of nitrogen starvation, over 90% of yeast form cells produce a filamentous bud (AHN *et al.* 1999). The immediate relief of Khd1-mediated translational repression on an existing pool of *FLO11* mRNA would allow for the rapid production of Flo11 protein in the first daughter cell even if the mother cell did not express the protein during yeast form growth. This effect is seen comparing Flo11 protein expression between *ash1Δ/ash1Δ* and *khd1Δ/khd1Δ ash1Δ/ash1Δ*. More *khd1Δ/khd1Δ ash1Δ/ash1Δ* cells express Flo11 protein than *ash1Δ/ash1Δ* cells (Table 1), resulting from the higher likelihood that a daughter cell expresses Flo11 protein whether or not it is expressed in the mother cell (Figure 5B). Given that there is not a concomitant increase in *FLO11* mRNA levels (Figure 2), this change represents increased translation of *FLO11* mRNA upon the loss of Khd1-mediated repression. The rapid inductive response leading to filamentation in the daughter suggests that repression by Khd1 may be quickly relieved under conditions of nitrogen starvation.

A filamentous cell expressing Flo11 protein can divide to produce a yeast form cell that does not express Flo11 protein (HALME *et al.* 2004). Such a rapid transition may require inhibition of both transcription and translation of *FLO11* mRNA. This dual control would repress preexisting *FLO11* mRNA from the mother, and prevent the daughter from transcribing new *FLO11* mRNA. Khd1 can execute both of these functions to produce asymmetric Flo11 protein expression. Since Flo11 protein is required in the daughter cell to maintain filamentous growth, the increase in Flo11 protein expression when repression by Khd1 is lost in the *khd1Δ/khd1Δ* mutant (Table 1, Figure 5C) likely explains its hyperfilamentation phenotype (Figure 1A).

This model for asymmetric *FLO11* expression and developmental switching posits differential Khd1 activity between cells. This heterogeneity would explain a surprising aspect of the changes in Flo11 protein expression between the *ash1Δ/ash1Δ* and *khd1Δ/khd1Δ ash1Δ/ash1Δ* mutants. In the absence of *ASH1,* the loss of Khd1 enables a higher percentage of cells to express Flo11 protein, but not more of it (Table 1). Individual cells can therefore express Flo11 protein at the same level whether or not they can express Khd1. Because *ASH1* is deleted, deletion of *KHD1* relieves translational repression on *FLO11* mRNA, but does not affect *FLO11* transcription (Figure 7). If Khd1 repressed translation of *FLO11* mRNA uniformly across all cells, its absence in *khd1Δ/khd1Δ ash1Δ/ash1Δ* cells would result in increased levels of Flo11 protein. Instead, it appears that some cells containing Khd1 fail to repress translation of *FLO11* mRNA, and deletion of *KHD1* simply expands this population. Phosphorylation of Khd1 by Yck1 regulates its repression of *ASH1* mRNA during mating type switching (PAQUIN *et al.* 2007). Although deletion of *YCK1* does not affect filamentous growth (data not shown), post-translational modifications may regulate Khd1 to generate heterogeneous

activity and enable the rapid changes in *FLO11* expression that underlie asymmetry during filamentous growth.

The asymmetry that arises when a yeast form mother cell produces a filamentous daughter cell has similarities to the asymmetry of mothers and daughters with respect to mating type switching. In both morphogenetic events, the mother and daughter have different developmental outcomes dependent on asymmetric gene expression. The two processes also have some differences. One striking difference is that Ash1 activates filamentation but represses mating type switching, which could reflect the different potentials of the mother and daughter cells between the two processes. The asymmetric expression of *ASH1* allows the mother to switch mating type, but prevents the daughter from doing so (CHARTRAND *et al.* 2002; PAQUIN and CHARTRAND 2008; STRATHERN and HERSKOWITZ 1979). However, an elliptical yeast form mother cell already encased in a cell wall of defined structure does not elongate. Instead it is the daughter cell that must express Flo11 protein to develop into a filamentous cell.

The developmental potential of the mother cell is constrained because filamentous growth requires a different program for construction of the cell wall. In this context it may be significant that Khd1 binds 54 mRNAs that encode proteins annotated to function in this macromolecular structure. Post-transcriptional regulation of these genes by Khd1 could provide a unifying mechanism for constructing this organelle. One mechanism for coordinating translational control of these messages would be to have a signature binding site in the mRNAs dedicated to this function. Although we observe a motif consistent with a previous report that used other methods to identify Khd1 binding sites (HASEGAWA *et al.* 2008), we do not identify a sequence that comprehensively explains recognition by Khd1.

These data suggest that although the motif we identify contributes to target recognition by Khd1, there must be additional recognition determinants.

Our studies identify a new biological role for Khd1. Its bipartite repression of *FLO11* provides dynamic regulation that controls the expression of a cell fate determinant in the daughter cell. Given the prevalence of sequences derived from *FLO11* in the CLIP experiment, this likely represents a major function for Khd1. Khd1 binds a number transcripts that encode cell wall proteins through repetitive sequences in addition to *FLO11* mRNA, and Khd1 may regulate the synthesis of many proteins that play a role in this structure. The documented expansion and contraction of the repeats bound by Khd1 (VERSTREPEN *et al.* 2005) would generate target sequences of diverse lengths that could be bound differentially, and as a consequence produce altered levels of these cell surface proteins. These changes could have important consequences for the structure and function of the yeast cell wall.

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SUPPORTING INFORMATION

File S1

Supporting Methods

Cross-linking Immunoprecipitation

1 L of cells containing the *KHD1-TAP* allele were harvested at OD 2.5, washed twice with water and resuspended in 10X pellet volume. UV crosslinking was performed by irradiating shallow layers of this suspension three times at 400 mJ/cm^2. Cells were washed in calmodulin binding buffer (CBB) with .1% NP-40 (RIGAUT *et al.* 1999) and protease inhibitors (leupeptin, pepstatin, PMSF), resuspended in 10 mL and separated and into FastPrep tubes. 600 micron glass beads were added to ~1/3 volume and lysis performed three times, 45 seconds at maximum speed in a FastPrep 3000.

The lysate was collected by puncturing the bottom of the tube and spinning the sample into a 15 mL tube. Samples were spun at 3,000 rpm for 5 minutes and the supernatant was collected. The pellet was resuspended in 5 mL CBB with .1% NP-40 and protease inhibitors, spun again at 3,000 rpm for 5 minutes, and the supernatant collected. This process was repeated one more time. RNAse A (USB 70194Y) was added to the pooled supernatants at a dilution of 1:50,000 or 1:1,000 and incubated at 37 Celsius for 10 minutes. The sample was split into microcentrifuge tubes and spun at 9,500 rpm for 5 minutes. The supernatant was transferred to fresh tubes and spun at 12,000 rpm for 5 minutes. The supernatant was used for immunoprecipitation.

300 µL calmodulin-agarose beads (GE Healthcare 17-0529-01) were equilibrated for ten minutes in CBB with .1% NP-40 three times. The equilibrated beads were added to the supernatant and incubated for 2 hours at 4 Celsius on a rocking platform. The beads were collected through a column and washed twice in 5 mL CBB with .1% NP-40 and

twice in 5 mL CBB with .02% NP-40. Protein was incubated in 3 mL calmodulin elution buffer (CEB) (RIGAUT *et al.* 1999) for 45 minutes at 4 Celsius on a rocking platform and then collected in a 15 mL tube. The beads were twice washed with 1 mL CEB which was pooled with the initial eluate.

800 μL Dynalbeads (Invitrogen 112-010) were equilibrated in CEB three times for ten minutes, added to the eluate, and incubated for 1 hour at 4° Celsius on a rocking platform. The beads were collected using a magnet and transferred to a microcentrifuge tube. They were washed with Nelson stringent buffer (5 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% Na-DOC, 0.1% SDS, 120 mM NaCl, 25 mM KCl) followed by Nelson high salt buffer (15 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% Na-DOC, 0.1% SDS, 1 M NaCl) for 10 minutes each at 4° Celsius on a rocking platform and transferred to a new tube where they were washed twice in Nelson low salt buffer (15 mM Tris, pH 7.5, 5 mM EDTA).

After this immunoprecipitation, the CLIP protocol was followed as previously described to obtain DNA molecules for sequencing (ULE *et al.* 2005), with the exception of the primers used for cDNA amplification as noted in the main text.

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File S2

Supporting Results

CLIP specifically identifies *in vivo* RNA targets of Khd1

Multiple control experiments were conducted to ensure that CLIP specifically identified *in vivo* RNA targets of Khd1. As part of this analysis, Khd1-RNA complex formation was visualized by end labeling RNAs following immunoprecipitation of Khd1-TAP and treatment with RNAse A as previously described (ULE *et al.* 2005). Non-specific RNA contaminants migrate at a much lower molecular weight than protein-RNA complexes, and can be separated using SDS-PAGE. Additionally, transfer to nitrocellulose membrane retains the protein-RNA complexes, but not free RNA (SANFORD *et al.* 2008; ULE *et al.* 2003). Khd1-RNA complexes were then visualized using autoradiography.

Khd1-RNA complex formation requires UV crosslinking (Figure S2) as has been previously demonstrated using mammalian RNA-binding proteins (SANFORD *et al.* 2008; ULE *et al.* 2003). Khd1-TAP has a predicted molecular weight of approximately 63 kilodaltons (kDa), but Western blot analysis shows the protein migrates at slightly less than 75 kDa during SDS-PAGE (data not shown). When a high concentration of RNAse A is used, Khd1-RNA complexes migrate slightly higher than 75 kDa (Figure S2). With lower RNAse A concentrations, longer RNA molecules are maintained leading to an increase in the molecular weight of the complexes (Figure S2).

Immunoprecipitation of Khd1-TAP from un-crosslinked cells was used to determine whether pure samples of Khd1 were obtained. Mass spectrometry of the band at about 75 kDa revealed no major protein species co-migrating with Khd1-TAP (data not shown), suggesting the signal on the autoradiogram derives specifically from Khd1-RNA complexes.

Immunoprecipitation of other RNA-binding proteins following crosslinking resulted in the formation of protein-RNA complexes of an expected size based on the molecular weight of the protein, but no complexes were seen when proteins without RNA-binding domains were used (data not shown).

Based on the above results, we conclude the sequences we obtained derive specifically from interactions between Khd1 and its endogenous RNA targets.

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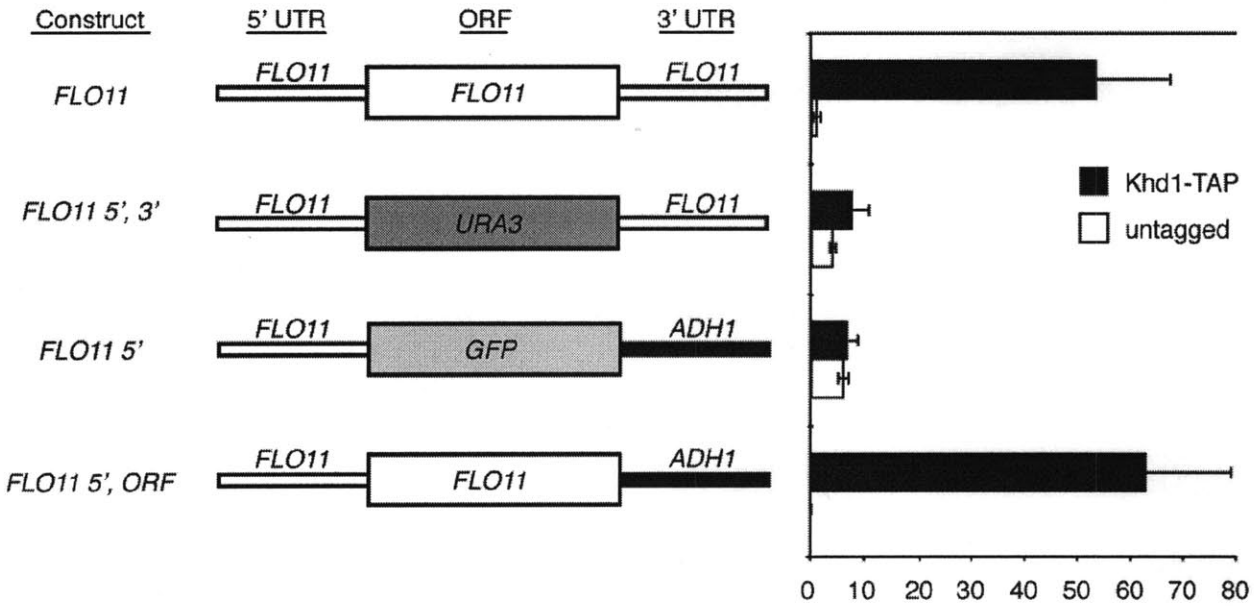

Figure S1.—Khd1 Interaction with *FLO11* mRNA requires the ORF. Enrichment following immunoprecipitation from cells expressing Khd1-TAP or untagged Khd1 calculated as in Figure 3. The *FLO11 5', 3'* construct contains a complete replacement of the *FLO11* ORF with *URA3*. The *FLO11* 5' and 3' UTRs remain intact in this construct. The *FLO11 5'* construct retains the *FLO11* 5' UTR but replaces everything downstream of the start codon with *GFP* followed by the *ADH1* 3' UTR. The *FLO11 5', ORF* construct maintains the *FLO11* 5' UTR and ORF but substitutes the *ADH1* 3' UTR for the *FLO11* 3' UTR. Neither *FLO11* UTR is sufficient for the interaction with Khd1; immunoprecipitation of Khd1-TAP only enriches transcripts containing the *FLO11* ORF.

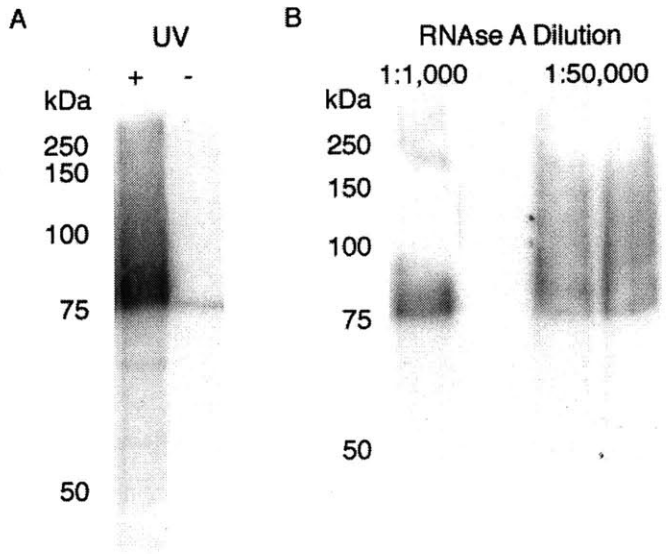

Figure S2.— CLIP identifies RNA targets of Khd1. Following immunoprecipitation of Khd1-TAP, protein RNA complexes were labeled as previously described (ULE et al. 2005), separated by size using SDS PAGE, and visualized using autoradiography. (A) Khd1-RNA complex formation is dependent on UV crosslinking. (B) Increased digestion with RNAse A increases complex mobility. A band from the sample with the 1:50,000 RNAse A dilution was isolated for sequencing as previously described (ULE et al. 2005).

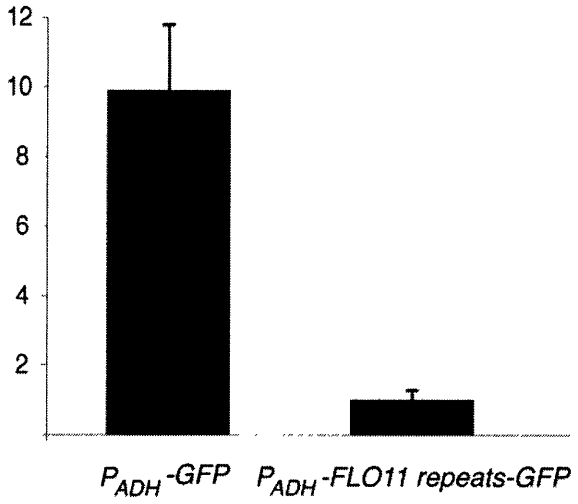

Figure S3.—Fusion to the *FLO11* repeats decreases *GFP* mRNA levels. *GFP* mRNA normalized to *TUB1* mRNA. Constructs diagrammed in Figure 4. Values are average of four independent experiments. Error reported as standard deviation.

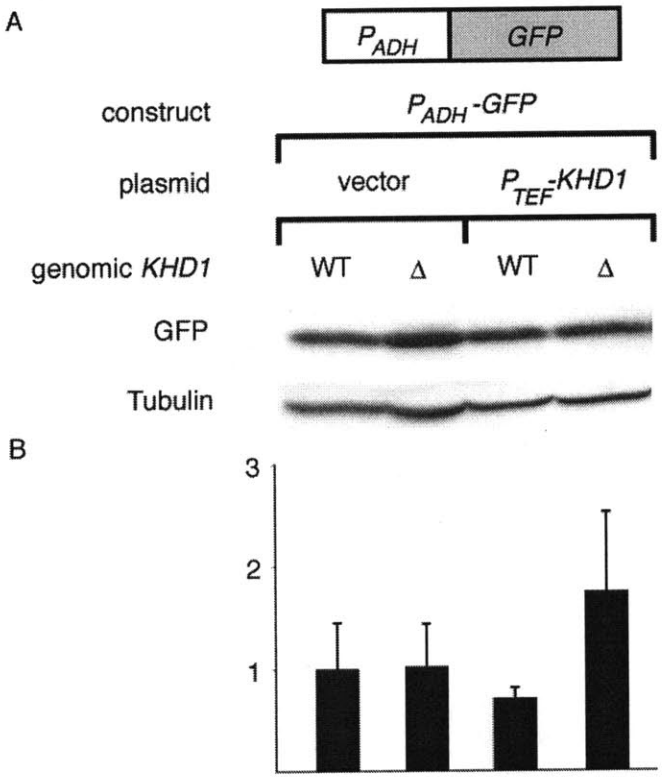

Figure S4.—*KHD1* does not affect expression from the *ADH* promoter. (A) Western blot analysis of P_{ADH} – *GFP* reporter construct. (B) *GFP* mRNA normalized to *TUB1* mRNA for the strains shown in (A). Values are average of four independent experiments. Error reported as standard deviation.

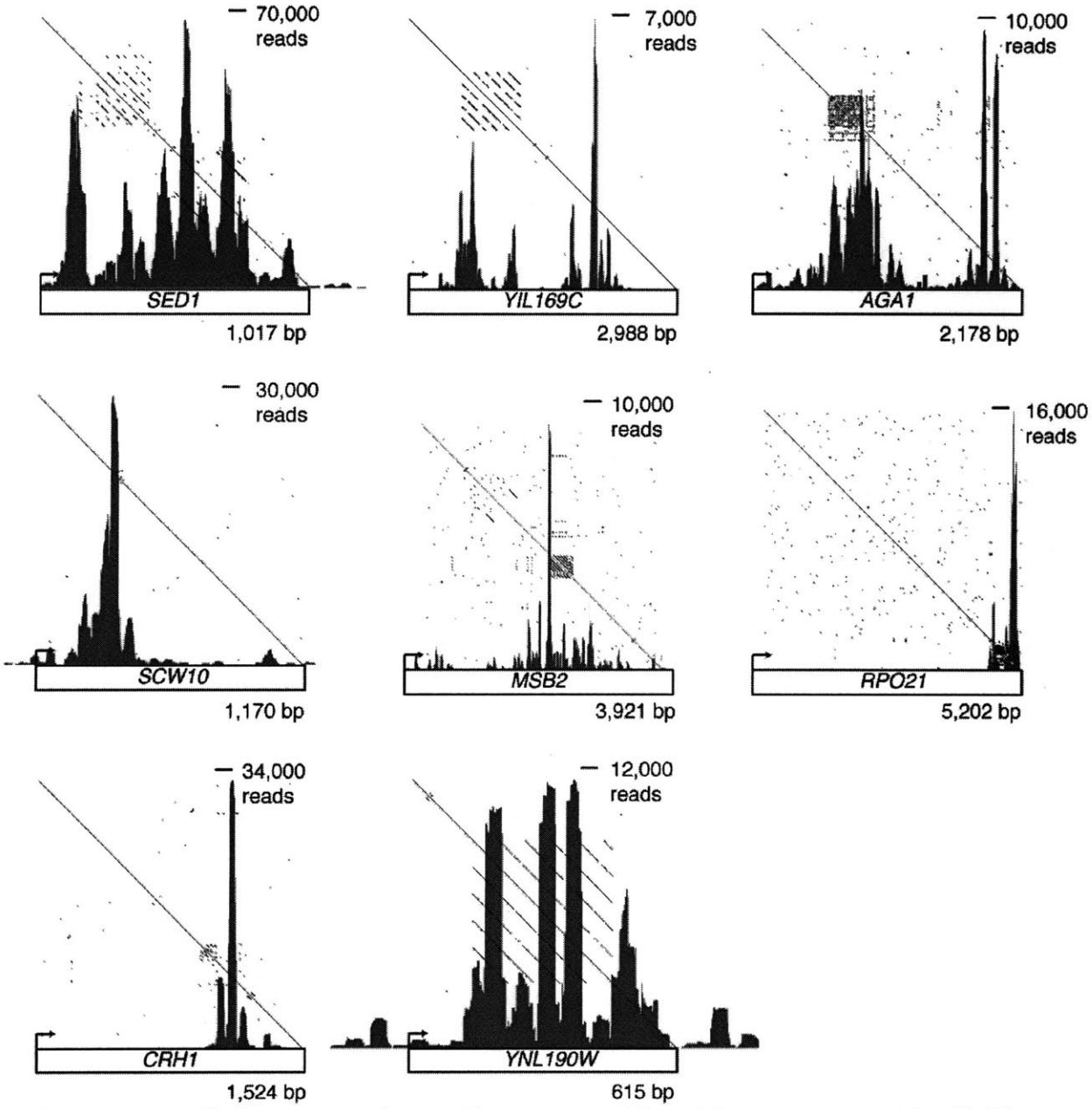

Figure S5.—Top Khd1 targets contain repetitive sequences. Nine of the top ten targets identified by CLIP contain internal repeats (*FLO11* depicted in Figure 3B). Histogram of read density from CLIP experiment overlaid on a dot plot highlighting the repetitive region of each ORF. For each ORF, histogram scale shown at top right, ORF length shown at bottom right. See Figure 4 for dot plot specifications.

TABLE S1

Yeast strains and plasmids used in this study.

Strain	Genotype	Source
JW971	*MATa/α his3::hisG/his3::hisG ura3-52/ura3-52*	This study
JW1064	*MATa/α his3::hisG/his3::hisG ura3-52/ura3-52 khd1::kanMX4/khd1::kanMX4*	This study
JW1066	*MATa/α his3::hisG/his3::hisG ura3-52/ura3-52 ash1::kanMX4/ash1::kanMX4*	This study
JW1068	*MATa/α his3::hisG/his3::hisG ura3-52/ura3-52 flo11::kanMX4/flo11::kanMX4*	This study
JW1033	*MATa/α his3::hisG/his3::hisG ura3-52/ura3-52 ash1::kanMX4/ash1::kanMX4 khd1::kanMX4/khd1::kanMX4*	This study
JW1027	*MATa/α his3::hisG/his3::hisG ura3-52/ura3-52 flo11::kanMX4/flo11::kanMX4 khd1::kanMX4/khd1::kanMX4*	This study
JW928	*MATα his3::hisG ura3-52*	This study
JW994	*MATα his3::hisG ura3-52 khd1::kanMX4*	This study
JW1052	*MATα his3::hisG ura3-52 ash1::kanMX4*	This study
JW1055	*MATα his3::hisG ura3-52 flo11::kanMX4*	This study
JW1032	*MATα his3::hisG ura3-52 ash1::kanMX4 khd1::kanMX4*	This study
JW1026	*MATα his3::hisG ura3-52 flo11::kanMX4 khd1::kanMX4*	This study
L6902	*MATa/α FLO11::HA/FLO11::HA his3::hisG/his3::hisG ura3-52/ura3-52*	Fink laboratory collection
JW214	*MATa/α FLO11::HA/FLO11::HA his3::hisG/his3::hisG ura3-52/ura3-52 khd1::kanMX4/khd1::kanMX4*	This study
JW364	*MATa/α FLO11::HA/FLO11::HA his3::hisG/his3::hisG ura3-52/ura3-52 ash1::kanMX4/ash1::kanMX4*	This study
JW1045	*MATa/α FLO11::HA/FLO11::HA his3::hisG/his3::hisG ura3-52/ura3-52 ash1::kanMX4/ash1::kanMX4 khd1::kanMX4/khd1::kanMX4*	This study
JW295	*MATα his3::hisG trp1::hisG leu2::hisG ura3-52 KHD1-TAP::HIS3*	This study
JW700	*MATα his3::hisG trp1::hisG leu2::hisG ura3-52 KHD1-TAP::HIS3 P_{FLO11}::URA3*	This study
JW248	*MATα his3::hisG trp1::hisG leu2::hisG ura3-52 KHD1-TAP::HIS3 P_{FLO11}::GFP::ADH1 3' UTR::URA3*	This study
JW715	*MATα his3::hisG trp1::hisG leu2::hisG ura3-52 KHD1-TAP::HIS3 FLO11::ADH1 3' UTR::URA3*	This study
JW1239	*MATa his3::hisG ura3-52 KHD1-TAP::HIS3 flo11::natNT2::P_{ADH}::FLO11 repeats::GFP::ADH1 3' UTR::URA3*	This study
JW1255	*MATa his3::hisG ura3-52 KHD1-TAP::HIS3 flo11::natNT2::P_{ADH}::GFP::ADH1 3' UTR::URA3*	This study
JW1778	*MATa his3::hisG ura3-52 KHD1-TAP::HIS3 flo11::natNT2::P_{CYC1}::GFP::ADH1 3' UTR::URA3*	This study
JW1226	*MATa his3::hisG ura3-52 flo11::natNT2::P_{ADH}::FLO11 repeats::GFP::ADH1 3' UTR::URA3*	This study
JW1245	*MATa his3::hisG ura3-52 khd1::kanMX4 flo11::natNT2::P_{ADH}::FLO11 repeats::GFP::ADH1 3' UTR::URA3*	This study
JW1547	*MATa his3::hisG ura3-52 flo11::natNT2::P_{ADH}::GFP::ADH1 3' UTR::URA3*	This study
JW1564	*MATa his3::hisG ura3-52 flo11::natNT2::P_{ADH}::GFP::ADH1 3' UTR::URA3 khd1::kanMX4*	This study
JW1549	*MATa his3::hisG ura3-52 flo11::natNT2::P_{CYC1}::GFP::ADH1 3' UTR::URA3*	This study
JW1566	*MATa his3::hisG ura3-52 flo11::natNT2::P_{CYC1}::GFP::ADH1 3' UTR::URA3 khd1::kanMX4*	This study
JW1330	*MATα his3::hisG ura3-52 natNT2::P_{ADH}::FLO11*	This study

JW1430	*MATα his3::hisG ura3-52 natNT2::P_{ADH}::FLO11 khd1::kanMX4*	This study

Plasmid	Insert	Source
p413TEF		Mumberg et al. 1995
p413TEF-*KHD1*	*KHD1*	This study
yEGFP3::ADH1 3' UTR::*URA3*		Sherwin Chan

TABLE S2

Oligonucleotides used in this study

Name	Sequence (5' to 3')	Description
JW4	caaacttgctgagtccatgc	amplify *KHD1-TAP::HIS3*
JW5	cgcgaaatgtttaaagcaag	amplify *KHD1-TAP::HIS3*
JW17	tggtcatcctgtaggtttgttg	amplify *khd1::kanMX4*
JW18	cagttctgccgggatacagt	amplify *khd1::kanMX4*
JW39	cgtgcgtctgatttctacga	amplify *ash1::kanMX4*
JW44	aagcaggttccgctatttca	amplify *ash1::kanMX4*
JW46	aattgggattcaaggcatca	amplify *flo11::kanMX4*
JW47	aattgggattcaaggcatca	amplify *flo11::kanMX4*
JW48	aaggaaaaaagcggccgctggtcatcctgtaggtttgttg	clone *KHD1*
JW49	atatatccgctcgagcgttgtattgttgttcggattg	clone *KHD1*
JW176	taattaagaatatacttttgtaggcctcaaaaatccatatacgcacactatgcgtacgctgcaggtcgac	P_{ADH}/P_{CYC1} amplification
JW186	caattgttgtcacaatctatgttccaatagaagcctgggaaatctgtttgcatcgatgaattctctgtcg	P_{ADH} fused to *FLO11* repeats
JW218	tcaaccaaaattgggacaacaccagtgaataattcttcacctttagacat-catcgatgaattctctgtcg	P_{CYC1} fused to *GFP*
JW228	aatgatacggcgaccaccgacagagggaggacgatgcgg	CLIP RT-PCR
JW229	caagcagaagacggcatacgaccgctggaagtgactgacac	CLIP RT-PCR
JW230	cgacagagggaggacgatgcgg	Illumina sequencing
FLO11 FW	cacttttgaagtttatgccacacaag	*FLO11* qPCR
FLO11 RV	cttgcatattgagcggcactac	*FLO11* qPCR
ACT1 FW	ctccaccactgctgaaagagaa	*ACT1* qPCR
ACT1 RV	ccaaggcgacgtaacatagtttt	*ACT1* qPCR
Vla292	cactggtgttgtcccaattttg	*GFP* qPCR
Vla293	caccggagacagaaaatttgtg	*GFP* qPCR
JW348	aggaggacgcggctaataatta	*TUB1* qPCR
JW349	tcgcccaaaatttctctacca	*TUB1* qPCR

TABLE S3

CLIP peaks

Chromosome:coordinates;strand		
1:7210-7274;-	2:59535-59589;+	2:376455-376499;+
1:7285-7459;-	2:60080-60129;+	2:377350-377469;-
1:30355-30399;+	2:61855-61909;+	2:381115-381154;+
1:43565-43610;-	2:62000-62044;+	2:390895-390944;-
1:48375-48444;+	2:72660-72704;-	2:393155-393197;-
1:49860-49989;-	2:80970-81014;-	2:393325-393374;-
1:55540-55639;+	2:83805-83889;-	2:421215-421294;+
1:55745-56004;+	2:89001-89037;+	2:431970-432074;-
1:56740-56869;+	2:93110-93146;+	2:432255-432454;-
1:68925-68969;-	2:102595-102644;+	2:432500-432629;-
1:78430-78475;+	2:107115-107594;-	2:446155-446199;+
1:91340-91429;-	2:114730-114994;-	2:446455-446504;+
1:94620-94654;-	2:115025-115119;-	2:447210-447284;+
1:94675-94774;-	2:129405-129449;+	2:457395-457499;-
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TABLE S4

Khd1 target RNAs

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BAL002C		235-299
BAR009W	*BIO1A*	1313-1357
BIL038C		-107-22
BIR039C		-310–251, -190–111, -75–41
BMR058C		909-953
BNL060C		4285-4334
BPR065W	*FLO16*	2300-2394, 2690-2829
BPR066W		55-144
LSR1		86-135
RDN37-1		2446-2495, 5771-5820, 6606-6650
RDN37-2		2448-2497, 5768-5822, 6603-6647
RDN58-1		117-161
RDN58-2		119-163
SNR11		226-264
SNR128		91-135
SNR14		70-111
SNR189		145-188
SNR37		166-220
SNR6		49-88
SNR71		34-92
telomeric-14L		234-268
telomeric-5L		402-445
tH(GUG)G2		100-140
tK(CUU)D1		32-79
tK(CUU)D2		25-74
tK(CUU)E1		26-75
tK(CUU)E2		27-71
tK(CUU)F		25-74
tK(CUU)G1		33-77
tK(CUU)G2		24-73
tK(CUU)G3		32-79
tK(CUU)I		25-69
tK(CUU)J		33-77
tK(CUU)K		33-77
tK(CUU)M		27-76
tK(CUU)P		34-78
tL(CAA)K		82-120
TLC1		445-487
tR(CCU)J		33-74
tR(UCU)B		32-76

tR(UCU)D		31-75
tR(UCU)E		33-76
tR(UCU)G1		34-76
tR(UCU)G2		30-73
tR(UCU)G3		32-76
tR(UCU)J1		34-78
tR(UCU)J2		29-73
tR(UCU)K		30-72
tR(UCU)M1		26-70
tR(UCU)M2		30-70
tT(UGU)G1		33-77
tT(UGU)G2		33-76
tT(UGU)P		30-79
tW(CCA)P		31-70
YAL003W	*EFB1*	451-525
YAL005C	*SSA1*	340-384
YAL012W	*CYS3*	103-157
YAL021C	*CCR4*	266-320, 2506-2605
YAL022C	*FUN26*	-300–266
YAL023C	*PMT2*	1047-1136
YAL027W		-27-18
YAL031C	*GIP4*	1834-1878
YAL038W	*CDC19*	-23-76, 182-441, 1177-1306
YAL040C	*CLN3*	1323-1452
YAL041W	*CDC24*	1748-1817
YAL043C	*PTA1*	1234-1279
YAL053W	*FLC2*	669-713
YAL063C	*FLO9*	4325-4499
YAR014C	*BUD14*	1934-2012
YAR019C	*CDC15*	1741-1822
YAR042W	*SWH1*	2-51
YAR050W	*FLO1*	1338-1462, 1768-1867, 2088-2212
YAR066W		1199-1288
YAR068W		53-207, 233-322
YAR071W	*PHO11*	1343-1379
YBL002W	*HTB2*	367-416
YBL004W	*UTP20*	4444-4481
YBL007C	*SLA1*	2879-2928
YBL014C	*RRN6*	2437-2486
YBL016W	*FUS3*	1066-1116
YBL030C	*PET9*	-21-28
YBL032W	*HEK2*	13-57, 168-212
YBL038W	*MRPL16*	-289–245
YBL047C	*EDE1*	1085-1179, 1210-1474
YBL051C	*PIN4*	1329-1808
YBL054W		845-894
YBL060W		986-1022
YBL063W	*KIP1*	2923-2959
YBL066C	*SEF1*	421-505, 3296-3340

YBL072C	*RPS8A*	615-659
YBL079W	*NUP170*	80-134, 625-674, 2400-2454, 2545-2589
YBL081W		403-947
YBL084C	*CDC27*	815-859, 955-1004
YBL085W	*BOI1*	1499-1558
YBL096C		349-383
YBL099W	*ATP1*	-153–69
YBL101C	*ECM21*	175-214, 1305-1344, 1625-1654
YBL103C	*RTG3*	554-568, 614-668
YBR007C	*DSF2*	916-960
YBR011C	*IPP1*	130-274, 570-674, 720-904
YBR016W		7-146
YBR029C	*CDS1*	873-917
YBR031W	*RPL4A*	95-144, 820-924
YBR048W	*RPS11B*	552-611, 747-836
YBR054W	*YRO2*	-203–159
YBR057C	*MUM2*	257-301
YBR059C	*AKL1*	775-824
YBR078W	*ECM33*	394-463, 539-623, 634-838, 849-938, 1034-1178, 1409-1473
YBR079C	*RPG1*	954-1017
YBR082C	*UBC4*	247-366
YBR083W	*TEC1*	1402-1441
YBR086C	*IST2*	210-259, 387-429, 2640-2689
YBR106W	*PHO88*	60-139
YBR112C	*CYC8*	1641-1770, 1816-2015, 2196-2300
YBR118W	*TEF2*	-13-31, 287-336, 1042-1116
YBR126C	*TPS1*	1394-1498
YBR136W	*MEC1*	-341–297
YBR140C	*IRA1*	1077-1186
YBR149W	*ARA1*	1468-1557
YBR162C	*TOS1*	581-730
YBR169C	*SSE2*	1284-1322
YBR172C	*SMY2*	514-563, 1729-1778
YBR189W	*RPS9B*	735-776, 852-891
YBR191W-A		-27-27
YBR195C	*MSI1*	-336–292
YBR196C	*PGI1*	1152-1326
YBR198C	*TAF5*	250-359
YBR212W	*NGR1*	948-997, 1078-1127
YBR214W	*SDS24*	-36-98, 1299-1398
YBR218C	*PYC2*	1404-1452
YBR221C	*PDB1*	-23-26, 832-931
YBR222C	*PCS60*	905-949
YBR225W		1063-1112, 1658-1752
YBR230C	*OM14*	461-500
YBR238C		264-418
YBR260C	*RGD1*	960-1004
YBR263W	*SHM1*	-51-38, 179-217, 1153-1189
YBR283C	*SSH1*	931-1000

YBR286W	*APE3*	48-122, 1545-1592
YBR287W	*ZSP1*	927-971
YBR289W	*SNF5*	159-343
YBR302C	*COS2*	1223-1267
YCL001W	*RER1*	-279–239
YCL004W	*PGS1*	897-941
YCL009C	*ILV6*	465-509
YCL025C	*AGP1*	1224-1273
YCL028W	*RNQ1*	739-998
YCL031C	*RRP7*	878-928
YCL037C	*SRO9*	629-768, 1159-1243
YCL039W	*GID7*	493-537
YCL040W	*GLK1*	-18-26, 1177-1221
YCL043C	*PDI1*	491-555; 601-650
YCL044C	*MGR1*	784-893, 1098-1153
YCL045C		1780-1849
YCL049C		438-482
YCR008W	*SAT4*	350-499
YCR012W	*PGK1*	-6-88, 104-173, 664-738
YCR024C-A	*PMP1*	-124-30
YCR030C	*SYP1*	1050-1169, 1200-1249, 1310-1499
YCR034W	*FEN1*	515-568
YCR052W	*RSC6*	1421-1465
YCR053W	*THR4*	-36-13, 24-218, 339-383
YCR065W	*HCM1*	1214-1268, 1359-1403, 1489-1533, 1644-1688
YCR067C	*SED4*	1751-1935
YCR081W	*SRB8*	3846-3880
YCR084C	*TUP1*	474-633, 2264-2343
YCR088W	*ABP1*	1124-1188
YCR089W	*FIG2*	3778-3917, 4048-4207
YCR093W	*CDC39*	5524-5563
YDL005C	*MED2*	538-582
YDL007C-A		609-633
YDL012C		199-241
YDL019C	*OSH2*	-16-23
YDL020C	*RPN4*	112-156
YDL022W	*GPD1*	1019-1073, 1109-1153
YDL025C		0-64, 105-179, 317-494, 735-839
YDL035C	*GPR1*	1461-1505
YDL037C	*BSC1*	481-595
YDL038C		1701-1744
YDL039C	*PRM7*	152-256
YDL048C	*STP4*	196-300, 316-380, 466-630
YDL054C	*MCH1*	135-181
YDL055C	*PSA1*	-145–6
YDL056W	*MBP1*	834-883
YDL060W	*TSR1*	1616-1655
YDL061C	*RPS29B*	39-83, 139-163
YDL070W	*BDF2*	251-290

YDL072C	*YET3*	1-90
YDL073W		459-468
YDL082W	*RPL13A*	970-1014
YDL083C	*RPS16B*	579-623
YDL088C	*ASM4*	330-366
YDL095W	*PMT1*	1152-1249
YDL106C	*PHO2*	95-169
YDL109C		1484-1526
YDL130W-A	*STF1*	-42-3
YDL132W	*CDC53*	2001-2045
YDL133C-A	*RPL41B*	-8-38
YDL140C	*RPO21*	-142–88, 4878-5182
YDL159W	*STE7*	329-366
YDL160C	*DHH1*	1284-1413
YDL161W	*ENT1*	698-742
YDL167C	*NRP1*	1591-1635
YDL171C	*GLT1*	807-856
YDL173W		733-769
YDL184C	*RPL41A*	-7-33
YDL185C-A		275-299
YDL185W	*TFP1*	37-81
YDL189W	*RBS1*	117-136, 957-1001
YDL191W	*RPL35A*	869-918
YDL195W	*SEC31*	-45–1, 2865-3144
YDL197C	*ASF2*	1355-1364
YDL203C	*ACK1*	199-238
YDL211C		377-421, 522-536
YDL223C	*HBT1*	581-620
YDL224C	*WHI4*	1075-1214, 1310-1374
YDL229W	*SSB1*	1203-1352, 1483-1557
YDL231C	*BRE4*	1975-2019
YDL232W	*OST4*	331-369
YDL233W		171-225
YDL234C	*GYP7*	112-157
YDR006C	*SOK1*	706-955
YDR012W	*RPL4B*	101-145
YDR017C	*KCS1*	789-823, 1984-2013
YDR023W	*SES1*	612-681
YDR025W	*RPS11A*	544-638
YDR028C	*REG1*	1297-1376, 1562-1601, 1617-1661, 1682-1721
YDR033W	*MRH1*	-58-6
YDR043C	*NRG1*	-69–55, 206-225, 311-320, 368-407
YDR044W	*HEM13*	-3-41
YDR051C		-342–298
YDR055W	*PST1*	722-766, 855-891, 1047-1111
YDR060W	*MAK21*	1740-1789, 2080-2120, 2328-2369
YDR069C	*DOA4*	1548-1617
YDR072C	*IPT1*	255-334
YDR073W	*SNF11*	344-388

YDR074W	*TPS2*	595-629, 785-829, 850-899, 975-1019, 1795-1852
YDR077W	*SED1*	70-179, 230-799
YDR091C	*RLI1*	1983-2062
YDR096W	*GIS1*	767-816, 1977-2036, 2242-2310
YDR099W	*BMH2*	695-784
YDR103W	*STE5*	267-296, 372-411, 792-836, 907-946
YDR123C	*INO2*	236-280, 496-605
YDR133C		318-392
YDR134C		-52-101, 177-281
YDR135C	*YCF1*	815-859
YDR142C	*PEX7*	850-894
YDR143C	*SAN1*	1159-1208, 1224-1288
YDR144C	*MKC7*	1636-1740
YDR145W	*TAF12*	306-390
YDR150W	*NUM1*	8227-8269
YDR151C	*CTH1*	366-402, 499-533
YDR153C	*ENT5*	1050-1094, 1220-1269
YDR155C	*CPR1*	-46-43
YDR166C	*SEC5*	2121-2167
YDR169C	*STB3*	1206-1315
YDR170C	*SEC7*	2025-2214
YDR172W	*SUP35*	87-156
YDR184C	*ATC1*	446-530
YDR186C		550-644, 1020-1109, 1920-1959, 1995-2044
YDR189W	*SLY1*	-253–199
YDR205W	*MSC2*	236-280
YDR207C	*UME6*	1252-1301, 1462-1511
YDR208W	*MSS4*	805-854, 875-944
YDR213W	*UPC2*	876-930, 1096-1144
YDR224C	*HTB1*	125-174
YDR227W	*SIR4*	120-164, 230-319, 1730-1769
YDR228C	*PCF11*	1019-1068
YDR232W	*HEM1*	1183-1227
YDR233C	*RTN1*	334-453, 529-668
YDR239C		370-409
YDR240C	*SNU56*	957-991
YDR246W	*TRS23*	545-594
YDR251W	*PAM1*	49-58, 84-93
YDR255C	*RMD5*	1135-1149
YDR259C	*YAP6*	275-309, 335-364
YDR266C		1272-1311
YDR288W	*NSE3*	1459-1498
YDR292C	*SRP101*	675-715
YDR293C	*SSD1*	767-886
YDR295C	*HDA2*	247-281
YDR297W	*SUR2*	828-872
YDR303C	*RSC3*	291-334
YDR309C	*GIC2*	543-592, 738-787, 978-1097
YDR310C	*SUM1*	1085-1164

YDR322W	*MRPL35*	13-62
YDR326C	*YSP2*	898-962, 983-1027, 2498-2547, 3728-3822
YDR334W	*SWR1*	969-1013
YDR335W	*MSN5*	2358-2407
YDR349C	*YPS7*	1356-1416
YDR351W	*SBE2*	729-848
YDR379W	*RGA2*	1315-1359
YDR385W	*EFT2*	-19-35, 2096-2225
YDR389W	*SAC7*	-72–23
YDR390C	*UBA2*	1256-1275
YDR416W	*SYF1*	1788-1822
YDR418W	*RPL12B*	157-246
YDR420W	*HKR1*	1347-1401, 1437-1681, 1847-1941, 2377-2426, 3132-3256
YDR425W	*SNX41*	602-646
YDR427W	*RPN9*	536-590
YDR432W	*NPL3*	288-337
YDR436W	*PPZ2*	41-79
YDR443C	*SSN2*	2830-2873
YDR449C	*UTP6*	1382-1426
YDR450W	*RPS18A*	656-725
YDR457W	*TOM1*	3325-3359
YDR463W	*STP1*	1442-1486
YDR471W	*RPL27B*	573-637
YDR475C	*JIP4*	2275-2314
YDR484W	*VPS52*	722-766
YDR485C	*VPS72*	1691-1735
YDR497C	*ITR1*	228-271
YDR505C	*PSP1*	714-798
YDR508C	*GNP1*	2005-2114
YDR518W	*EUG1*	586-630
YDR524C	*AGE1*	355-419
YDR524C-B		-47-37
YDR527W	*RBA50*	801-850
YDR528W	*HLR1*	33-78
YEL007W		1838-1917
YEL008C-A		482-626
YEL009C	*GCN4*	-69–15
YEL015W	*EDC3*	661-702
YEL016C	*NPP2*	379-418
YEL017C-A	*PMP2*	196-310
YEL017W	*GTT3*	715-759, 770-824
YEL026W	*SNU13*	-9-40
YEL027W	*CUP5*	440-524
YEL031W	*SPF1*	2736-2785
YEL033W		0-84
YEL034W	*HYP2*	-22-62, 203-277
YEL036C	*ANP1*	1297-1396
YEL040W	*UTR2*	1071-1320
YEL043W		1791-1845, 2471-2620

YEL045C		379-448
YEL046C	*GLY1*	184-283
YEL060C	*PRB1*	618-672, 748-797, 1808-1852
YEL063C	*CAN1*	1036-1090
YEL074W		173-218
YER020W	*GPA2*	754-788
YER021W	*RPN3*	1012-1061, 1199-1240
YER025W	*GCD11*	1626-1663
YER033C	*ZRG8*	101-155, 296-355
YER036C	*ARB1*	1449-1498
YER040W	*GLN3*	605-704
YER043C	*SAH1*	860-904, 1070-1154, 1340-1459
YER044C	*ERG28*	77-126
YER045C	*ACA1*	224-268, 404-448, 759-808
YER047C	*SAP1*	1232-1286
YER061C	*CEM1*	219-263, 354-373
YER064C		1460-1554
YER068W	*MOT2*	1147-1241
YER069W	*ARG5%2C6*	57-101
YER070W	*RNR1*	2425-2504
YER075C	*PTP3*	828-872
YER079W		443-492
YER086W	*ILV1*	1080-1124
YER087C-B	*SBH1*	-43-41
YER088C	*DOT6*	669-783, 1879-2028
YER102W	*RPS8B*	643-682
YER110C	*KAP123*	354-423
YER111C	*SWI4*	613-662
YER112W	*LSM4*	439-453
YER114C	*BOI2*	1490-1533
YER118C	*SHO1*	368-417
YER123W	*YCK3*	1353-1397, 1418-1452
YER125W	*RSP5*	434-483, 494-613, 1234-1278
YER129W	*SAK1*	2240-2279
YER131W	*RPS26B*	209-263
YER132C	*PMD1*	1917-1956, 2712-2756
YER133W	*GLC7*	1601-1655
YER143W	*DDI1*	770-812
YER144C	*UBP5*	1063-1192
YER150W	*SPI1*	216-260
YER151C	*UBP3*	172-251, 362-446, 602-656
YER154W	*OXA1*	811-855, 1026-1075, 1151-1205
YER155C	*BEM2*	1096-1175, 1436-1505
YER158C		-175–121, 235-279, 1245-1289, 1315-1364
YER165W	*PAB1*	1670-1819
YER167W	*BCK2*	1506-1560
YER169W	*RPH1*	208-287
YER177W	*BMH1*	516-605, 646-735
YER188C-A		-275–161, -135-29

YFL021W	*GAT1*	1350-1509
YFL022C	*FRS2*	637-687
YFL024C	*EPL1*	2708-2750
YFL026W	*STE2*	920-956
YFL031W	*HAC1*	626-671
YFL033C	*RIM15*	1426-1500, 1984-2060
YFL034C-B	*MOB2*	275-289, 315-359
YFL036W	*RPO41*	-46–2
YFL037W	*TUB2*	1487-1536
YFL039C	*ACT1*	522-721
YFL051C		1720-1839
YFR017C		-55-24
YFR019W	*FAB1*	1413-1552
YFR029W	*PTR3*	475-521, 1350-1394
YFR031C-A	*RPL2A*	935-1004
YFR044C	*DUG1*	52-111
YFR051C	*RET2*	184-288
YFR052W	*RPN12*	273-317
YFR053C	*HXK1*	453-494
YGL006W	*PMC1*	712-761
YGL008C	*PMA1*	-67-67, 3123-3227
YGL009C	*LEU1*	194-263, 1913-2008
YGL013C	*PDR1*	3012-3071
YGL014W	*PUF4*	1078-1142
YGL021W	*ALK1*	336-360
YGL023C	*PIB2*	1572-1591
YGL025C	*PGD1*	965-1009
YGL028C	*SCW11*	204-253, 449-498
YGL030W	*RPL30*	242-311
YGL031C	*RPL24A*	69-138
YGL035C	*MIG1*	766-815, 1141-1255
YGL036W		2142-2186
YGL038C	*OCH1*	648-697
YGL049C	*TIF4632*	108-157, 348-485
YGL052W		108-153
YGL053W	*PRM8*	-8-11
YGL055W	*OLE1*	774-953, 1349-1398
YGL056C	*SDS23*	1166-1225
YGL066W	*SGF73*	1618-1697
YGL071W	*AFT1*	259-329
YGL076C	*RPL7A*	1474-1523
YGL083W	*SCY1*	1988-2032
YGL092W	*NUP145*	286-330
YGL122C	*NAB2*	355-524
YGL123W	*RPS2*	355-564
YGL125W	*MET13*	1287-1331
YGL128C	*CWC23*	361-455, 496-570
YGL129C	*RSM23*	410-469
YGL131C	*SNT2*	4796-4840, 4851-4880

YGL135W	*RPL1B*	-4-50
YGL139W	*FLC3*	-74–5
YGL142C	*GPI10*	1696-1739
YGL147C	*RPL9A*	186-235
YGL150C	*INO80*	1317-1358, 3769-3818
YGL151W	*NUT1*	761-865, 1131-1185
YGL167C	*PMR1*	-154–105
YGL172W	*NUP49*	677-713
YGL178W	*MPT5*	705-784, 2525-2644, 2675-2819
YGL197W	*MDS3*	2425-2474, 2885-2949
YGL206C	*CHC1*	1181-1225
YGL207W	*SPT16*	1363-1403
YGL208W	*SIP2*	-306–262
YGL209W	*MIG2*	762-806, 1087-1123
YGL215W	*CLG1*	173-412, 1163-1347
YGL219C	*MDM34*	464-493, 854-903, 1314-1323
YGL222C	*EDC1*	141-270, 346-395
YGL223C	*COG1*	230-309
YGL225W	*VRG4*	-55-19, 80-125, 210-314
YGL234W	*ADE5%2C7*	-48-56, 1322-1376
YGL237C	*HAP2*	321-380
YGL238W	*CSE1*	871-915, 2029-2070
YGL245W	*GUS1*	1460-1549
YGL253W	*HXK2*	450-569, 930-1014
YGR002C	*SWC4*	864-913, 1224-1268
YGR014W	*MSB2*	-79–35, 2046-2365
YGR018C		106-145
YGR019W	*UGA1*	613-652
YGR020C	*VMA7*	283-324
YGR023W	*MTL1*	546-820
YGR027C	*RPS25A*	80-124
YGR032W	*GSC2*	665-709, 2540-2584
YGR034W	*RPL26B*	169-218
YGR040W	*KSS1*	875-911
YGR054W		547-596
YGR056W	*RSC1*	1847-1936, 1972-2026
YGR060W	*ERG25*	669-743
YGR070W	*ROM1*	405-519
YGR077C	*PEX8*	1591-1640
YGR080W	*TWF1*	51-95
YGR085C	*RPL11B*	423-497
YGR086C	*PIL1*	733-784
YGR089W	*NNF2*	988-1087
YGR097W	*ASK10*	2663-2727
YGR116W	*SPT6*	1614-1651
YGR124W	*ASN2*	564-618
YGR138C	*TPO2*	-57-132
YGR146C		14-63, 249-338
YGR155W	*CYS4*	287-339, 1550-1604

YGR161C	*RTS3*	539-663
YGR162W	*TIF4631*	175-219, 530-579
YGR166W	*KRE11*	822-864
YGR180C	*RNR4*	-209–157, 10-53, 719-768
YGR184C	*UBR1*	1435-1489
YGR189C	*CRH1*	1043-1242
YGR191W	*HIP1*	-141–107
YGR192C	*TDH3*	218-587
YGR198W	*YPP1*	676-712
YGR204W	*ADE3*	547-588
YGR214W	*RPS0A*	850-919
YGR218W	*CRM1*	188-237
YGR221C	*TOS2*	1380-1414, 1620-1694
YGR227W	*DIE2*	1141-1280
YGR233C	*PHO81*	929-972
YGR237C		2144-2223
YGR240C	*PFK1*	-27-107, 428-482
YGR241C	*YAP1802*	1390-1484
YGR249W	*MGA1*	209-252, 820-864, 920-964, 1080-1129
YGR254W	*ENO1*	-18-41, 787-876
YGR274C	*TAF1*	1154-1196
YGR279C	*SCW4*	215-414
YGR285C	*ZUO1*	445-484
YHL001W	*RPL14B*	599-608
YHL004W	*MRP4*	507-551
YHL007C	*STE20*	-74–30, 1456-1510
YHL015W	*RPS20*	-18-256
YHL021C	*FMP12*	1020-1074
YHL023C	*RMD11*	545-589, 761-804
YHL027W	*RIM101*	893-1032
YHL028W	*WSC4*	666-885
YHL029C	*OCA5*	208-257, 474-522
YHL030W	*ECM29*	1112-1164
YHL033C	*RPL8A*	195-214
YHL050C		1696-1745, 1771-1800
YHR007C	*ERG11*	1612-1666
YHR007C-A		-112–33
YHR010W	*RPL27A*	968-1008
YHR017W	*YSC83*	1051-1088
YHR021C	*RPS27B*	689-738
YHR042W	*NCP1*	1314-1443
YHR048W		713-722
YHR056C	*RSC30*	1168-1217
YHR064C	*SSZ1*	172-236
YHR071W	*PCL5*	-56–7
YHR072W	*ERG7*	252-300
YHR073W	*OSH3*	1310-1354, 1800-1844
YHR074W	*QNS1*	1870-1906
YHR076W	*PTC7*	1771-1805

YHR079C	*IRE1*	-94–40
YHR082C	*KSP1*	1251-1294, 1461-1505, 1896-1945, 2256-2310, 2331-2385
YHR084W	*STE12*	1338-1382, 1503-1542, 1765-1807
YHR086W	*NAM8*	755-799
YHR092C	*HXT4*	578-612
YHR097C		842-886
YHR098C	*SFB3*	577-631
YHR099W	*TRA1*	5411-5454
YHR102W	*KIC1*	2479-2528
YHR108W	*GGA2*	1018-1062, 1288-1337
YHR115C	*DMA1*	-13-51
YHR127W		789-834
YHR135C	*YCK1*	1286-1565
YHR141C	*RPL42B*	401-445
YHR143W	*DSE2*	509-628
YHR149C	*SKG6*	1580-1669
YHR152W	*SPO12*	54-118
YHR158C	*KEL1*	1790-1834, 1893-1939
YHR161C	*YAP1801*	1631-1705
YHR165C	*PRP8*	4783-4797
YHR174W	*ENO2*	386-440, 496-545
YHR175W	*CTR2*	-192–145
YHR179W	*OYE2*	21-125
YHR182W		2234-2273, 2309-2353
YHR188C	*GPI16*	1271-1370
YHR203C	*RPS4B*	1054-1143
YHR205W	*SCH9*	417-501
YHR206W	*SKN7*	1388-1452, 1698-1741, 1798-1922
YHR208W	*BAT1*	5-139
YHR211W	*FLO5*	925-1009, 1890-1954, 2040-2159, 2215-2334
YHR214W-A		-172–38, 58-152
YIL018W	*RPL2B*	1170-1210
YIL034C	*CAP2*	430-474
YIL038C	*NOT3*	1042-1106, 1272-1316
YIL041W	*GVP36*	320-374
YIL051C	*MMF1*	-8-56, 342-406
YIL052C	*RPL34B*	-10-24
YIL053W	*RHR2*	12-131
YIL055C		743-787
YIL056W	*VHR1*	695-744, 1020-1063, 1145-1229, 1781-1824
YIL069C	*RPS24B*	856-925
YIL075C	*RPN2*	936-985
YIL078W	*THS1*	1072-1110
YIL083C		18-37
YIL088C	*AVT7*	824-918
YIL091C		1342-1379
YIL095W	*PRK1*	1462-1521
YIL101C	*XBP1*	1679-1748
YIL105C	*SLM1*	425-454

YIL109C	*SEC24*	984-1028
YIL119C	*RPI1*	940-1019
YIL122W	*POG1*	778-807, 1093-1127
YIL123W	*SIM1*	271-630
YIL128W	*MET18*	220-258
YIL129C	*TAO3*	855-898
YIL130W	*ASG1*	2437-2481, 2527-2576
YIL135C	*VHS2*	650-699, 1205-1304
YIL137C	*TMA108*	808-857, 873-952
YIL140W	*AXL2*	1418-1467
YIL142W	*CCT2*	582-626, 824-866
YIL146C	*ECM37*	1627-1676
YIL148W	*RPL40A*	462-511, 627-636, 667-711, 762-771, 867-904
YIL154C	*IMP2'*	875-912
YIL162W	*SUC2*	858-902
YIL169C		577-876, 2147-2241
YIR006C	*PAN1*	579-673, 1304-1393
YIR010W	*DSN1*	592-631
YIR018C-A		28-67
YIR019C	*MUC1*	720-1779, 1825-2019, 2335-2419, 2430-2489, 2690-2789
YIR023W	*DAL81*	2543-2597
YIR033W	*MGA2*	1193-1332
YJL005W	*CYR1*	425-466, 794-841, 3822-3859
YJL016W		-115–81, -1-44, 188-229
YJL020C	*BBC1*	2354-2603, 2651-2738
YJL029C	*VPS53*	895-943
YJL041W	*NSP1*	139-188, 199-288
YJL042W	*MHP1*	959-1008
YJL050W	*MTR4*	2099-2135
YJL052W	*TDH1*	286-590
YJL054W	*TIM54*	542-586
YJL060W	*BNA3*	1009-1018
YJL062W	*LAS21*	760-796, 2000-2044
YJL073W	*JEM1*	1531-1575
YJL076W	*NET1*	1051-1095
YJL078C	*PRY3*	718-782, 2288-2367
YJL079C	*PRY1*	361-410
YJL080C	*SCP160*	-117-157, 223-412
YJL083W	*TAX4*	1164-1213, 1224-1263
YJL084C	*ALY2*	756-815
YJL090C	*DPB11*	889-933, 989-1033
YJL091C	*GWT1*	1825-1889
YJL095W	*BCK1*	1417-1476
YJL097W	*PHS1*	1-90
YJL109C	*UTP10*	339-443
YJL110C	*GZF3*	1012-1061
YJL129C	*TRK1*	4466-4530
YJL130C	*URA2*	-72-45
YJL136C	*RPS21B*	496-540

YJL138C	*TIF2*	534-623
YJL139C	*YUR1*	1687-1776
YJL141C	*YAK1*	154-341, 1274-1338, 1384-1448
YJL158C	*CIS3*	77-381
YJL159W	*HSP150*	153-292, 318-567, 598-677, 713-877
YJL168C	*SET2*	2093-2132
YJL172W	*CPS1*	175-357, 530-572
YJL174W	*KRE9*	444-508, 829-898
YJL187C	*SWE1*	1051-1110
YJL189W	*RPL39*	564-613
YJL201W	*ECM25*	1294-1348
YJR003C		-307–278
YJR004C	*SAG1*	1296-1430
YJR009C	*TDH2*	210-554
YJR016C	*ILV3*	1787-1832
YJR041C	*URB2*	1856-1960
YJR044C	*VPS55*	418-467
YJR045C	*SSC1*	486-527, 919-963
YJR047C	*ANB1*	-12-17
YJR054W		1217-1256
YJR059W	*PTK2*	1341-1377, 1498-1543, 2329-2338
YJR064W	*CCT5*	-318–264
YJR066W	*TOR1*	2754-2808
YJR091C	*JSN1*	760-839
YJR094W-A	*RPL43B*	550-599
YJR106W	*ECM27*	416-454
YJR107W		-196–155
YJR117W	*STE24*	943-1007
YJR121W	*ATP2*	-88–44, -8-66, 1072-1116
YJR123W	*RPS5*	322-451
YJR127C	*RSF2*	1242-1291, 1637-1686, 1862-1966, 2007-2046
YJR143C	*PMT4*	1020-1064
YJR145C	*RPS4A*	277-318, 869-913
YJR147W	*HMS2*	851-910
YJR151C	*DAN4*	452-713
YKL014C	*URB1*	1272-1331, 1547-1621
YKL020C	*SPT23*	1143-1230
YKL025C	*PAN3*	397-556
YKL029C	*MAE1*	1436-1515
YKL032C	*IXR1*	907-1136
YKL035W	*UGP1*	-1-43, 1007-1068, 1454-1521
YKL043W	*PHD1*	236-340
YKL044W		231-330
YKL051W	*SFK1*	1142-1191
YKL054C	*DEF1*	1203-1447, 1948-2002
YKL055C	*OAR1*	-299–255
YKL060C	*FBA1*	-22-59, 100-189
YKL062W	*MSN4*	1521-1570
YKL067W	*YNK1*	-17-21

YKL068W	*NUP100*	2558-2612
YKL081W	*TEF4*	1166-1210
YKL084W	*HOT13*	263-292
YKL096W-A	*CWP2*	-16-238
YKL101W	*HSL1*	1689-1843
YKL103C	*LAP4*	1122-1171
YKL109W	*HAP4*	240-285, 630-679, 695-729
YKL121W		902-938, 1079-1115
YKL125W	*RRN3*	351-387
YKL129C	*MYO3*	3110-3147
YKL152C	*GPM1*	580-714
YKL159C	*RCN1*	911-920
YKL164C	*PIR1*	387-736
YKL175W	*ZRT3*	1003-1037
YKL180W	*RPL17A*	657-751
YKL182W	*FAS1*	285-334, 1180-1364
YKL183W	*LOT5*	1500-1544
YKL185W	*ASH1*	287-451
YKL193C	*SDS22*	702-739
YKL204W	*EAP1*	1341-1440
YKL216W	*URA1*	-27-17
YKL217W	*JEN1*	-191–157
YKR008W	*RSC4*	1539-1582
YKR013W	*PRY2*	322-601
YKR016W	*FMP13*	446-500, 981-1025
YKR021W	*ALY1*	1951-2000
YKR042W	*UTH1*	314-378, 634-738
YKR051W		277-316
YKR052C	*MRS4*	453-489
YKR059W	*TIF1*	540-629
YKR060W	*UTP30*	-326–282
YKR075C		853-877
YKR077W		682-786
YKR090W	*PXL1*	2132-2161
YKR093W	*PTR2*	1432-1481
YKR094C	*RPL40B*	392-471
YKR098C	*UBP11*	665-674
YKR102W	*FLO10*	1452-1506, 1582-1801, 2147-2191
YKR103W,YKR104W	*NFT1*	997-1041
YLL010C	*PSR1*	40-54, 70-84, 470-514
YLL013C	*PUF3*	886-940, 1111-1385, 1411-1545
YLL017W,YLL016W	*SDC25*	3635-3674
YLL019C	*KNS1*	267-305, 756-840
YLL021W	*SPA2*	1982-2011, 2162-2211
YLL024C	*SSA2*	339-383, 1249-1308
YLL043W	*FPS1*	83-172
YLL045C	*RPL8B*	61-105, 196-215, 474-515, 736-780
YLL048C	*YBT1*	577-621
YLR006C	*SSK1*	726-768, 1516-1559, 1780-1819

YLR017W	*MEU1*	43-81
YLR019W	*PSR2*	20-105
YLR023C	*IZH3*	359-408
YLR024C	*UBR2*	5135-5154
YLR026C	*SED5*	-56-10, 601-660
YLR029C	*RPL15A*	499-573
YLR039C	*RIC1*	-297–253
YLR040C		204-498
YLR042C		158-202
YLR044C	*PDC1*	383-497, 683-777, 1178-1232
YLR048W	*RPS0B*	576-630, 671-730
YLR050C		66-115
YLR052W	*IES3*	115-159
YLR056W	*ERG3*	503-602, 678-972
YLR057W		-325–281
YLR058C	*SHM2*	1456-1545
YLR060W	*FRS1*	460-519
YLR064W		116-165
YLR079W	*SIC1*	113-153
YLR089C	*ALT1*	63-147
YLR091W		119-163
YLR095C	*IOC2*	2109-2153, 2312-2366, 2387-2441
YLR096W	*KIN2*	1988-2028
YLR106C	*MDN1*	8386-8465
YLR110C	*CCW12*	-44-40
YLR116W	*MSL5*	1118-1172
YLR120C	*YPS1*	1514-1563, 1709-1758
YLR121C	*YPS3*	1464-1523, 1599-1693
YLR131C	*ACE2*	1409-1517
YLR134W	*PDC5*	380-419
YLR139C	*SLS1*	447-551, 587-636, 792-841, 967-1051, 1252-1316
YLR153C	*ACS2*	1640-1694
YLR167W	*RPS31*	60-109
YLR176C	*RFX1*	401-445
YLR177W		410-464
YLR180W	*SAM1*	15-59, 1060-1209
YLR187W	*SKG3*	2908-3082
YLR194C		391-660
YLR202C		333-342
YLR203C	*MSS51*	-33-6
YLR206W	*ENT2*	437-481, 522-571
YLR212C	*TUB4*	1018-1062
YLR228C	*ECM22*	759-818, 1379-1458
YLR249W	*YEF3*	1977-2056
YLR256W	*HAP1*	1107-1356, 1562-1716
YLR257W		455-498
YLR270W	*DCS1*	-330–271
YLR274W	*CDC46*	433-471

YLR278C		-55–16, 155-197, 1020-1059, 1190-1234, 3105-3149, 3170-3249, 3400-3444, 3655-3694
YLR285C-A		-60–16
YLR286C	*CTS1*	953-1147
YLR290C		-315–269
YLR293C	*GSP1*	572-621
YLR294C		82-131
YLR304C	*ACO1*	1400-1449
YLR305C	*STT4*	2170-2218
YLR310C	*CDC25*	86-165, 1366-1407
YLR327C	*TMA10*	-29-10
YLR328W	*NMA1*	157-226
YLR332W	*MID2*	694-793
YLR335W	*NUP2*	969-1093, 1559-1633
YLR337C	*VRP1*	875-1074
YLR342W	*FKS1*	-208–164, 5482-5711
YLR347C	*KAP95*	499-543
YLR350W	*ORM2*	57-101
YLR354C	*TAL1*	119-179, 556-598
YLR355C	*ILV5*	1195-1264
YLR357W	*RSC2*	1639-1680
YLR359W	*ADE13*	1200-1238, 1385-1444
YLR370C	*ARC18*	282-323
YLR371W	*ROM2*	960-1074
YLR373C	*VID22*	832-876, 947-1006, 1217-1266
YLR375W	*STP3*	817-861
YLR378C	*SEC61*	-196–22, 799-958
YLR389C	*STE23*	37-96, 1747-1791
YLR390W-A	*CCW14*	274-578
YLR403W	*SFP1*	754-978
YLR413W		283-372, 1273-1417
YLR414C		-43–2
YLR420W	*URA4*	169-218, 901-938
YLR436C	*ECM30*	145-189, 865-914, 1275-1319, 1415-1489, 1505-1549, 1996-2044, 3190-3232
YLR438W	*CAR2*	-9-40, 191-228
YLR439W	*MRPL4*	66-185, 211-265
YLR441C	*RPS1A*	397-486
YLR446W		879-917
YLR450W	*HMG2*	1502-1551
YLR452C	*SST2*	1555-1669
YLR454W	*FMP27*	2949-2985
YLR455W		1025-1074
YLR459W	*GAB1*	877-926
YLR463C		940-981
YML006C	*GIS4*	277-371
YML015C	*TAF11*	819-853
YML016C	*PPZ1*	113-437
YML026C	*RPS18B*	545-674

YML034C-A		-83–39
YML035C	*AMD1*	1353-1397
YML053C		221-315
YML054C	*CYB2*	1847-1890
YML056C	*IMD4*	139-238
YML058W	*SML1*	429-473
YML059C	*NTE1*	93-142, 1168-1197, 1738-1777
YML073C	*RPL6A*	776-820
YML081W		1004-1053, 1179-1233, 1264-1318, 1469-1513, 1979-2028
YML091C	*RPM2*	192-311
YML092C	*PRE8*	699-763
YML100W	*TSL1*	564-613, 3009-3083
YML103C	*NUP188*	4-58
YML111W	*BUL2*	11-54
YML120C	*NDI1*	414-458
YML123C	*PHO84*	1710-1761
YML129C	*COX14*	147-189
YMR002W	*MIC17*	110-149
YMR006C	*PLB2*	1882-2011
YMR008C	*PLB1*	510-564, 1130-1174, 2021-2059
YMR009W	*ADI1*	456-525
YMR012W	*CLU1*	604-673
YMR016C	*SOK2*	1920-2014
YMR022W	*QRI8*	207-251
YMR037C	*MSN2*	1620-1699
YMR038C	*CCS1*	153-202
YMR043W	*MCM1*	490-869
YMR047C	*NUP116*	172-211, 3202-3244
YMR054W	*STV1*	1117-1156
YMR067C	*UBX4*	954-1003
YMR070W	*MOT3*	807-921, 987-1076
YMR079W	*SEC14*	778-827
YMR081C	*ISF1*	573-617, 678-722, 738-782, 852-889, 973-1022
YMR086W		-34-10, 2600-2645
YMR093W	*UTP15*	133-177
YMR102C		1176-1220, 1661-1704
YMR104C	*YPK2*	412-451
YMR108W	*ILV2*	-149–100, 771-890
YMR116C	*ASC1*	931-1020
YMR120C	*ADE17*	-16-93, 1174-1238
YMR122W-A		-15-259
YMR124W		934-1078, 1119-1173
YMR129W	*POM152*	961-1006
YMR136W	*GAT2*	296-405, 446-500, 931-980
YMR140W	*SIP5*	979-1021
YMR142C	*RPL13B*	547-586
YMR145C	*NDE1*	1510-1554
YMR146C	*TIF34*	288-328, 364-413
YMR162C	*DNF3*	868-904

YMR164C	*MSS11*	1352-1403, 1729-1773
YMR173W-A		169-178
YMR181C		-27-42, 58-107, 263-312
YMR182C	*RGM1*	405-449, 475-564, 585-664
YMR186W	*HSC82*	16-125, 1101-1139, 1366-1415, 1701-1740
YMR189W	*GCV2*	1787-1831
YMR192W	*GYL1*	364-403
YMR199W	*CLN1*	1193-1247, 1498-1552
YMR202W	*ERG2*	2-56
YMR203W	*TOM40*	-8-156
YMR205C	*PFK2*	156-365, 401-535
YMR215W	*GAS3*	1082-1128, 1152-1196, 1382-1431, 1507-1547
YMR217W	*GUA1*	1033-1112
YMR221C	*FMP42*	1360-1405
YMR226C	*TMA29*	225-269
YMR230W	*RPS10B*	631-730
YMR241W	*YHM2*	-30-14
YMR242C	*RPL20A*	800-989
YMR246W	*FAA4*	-56–7, 2144-2188
YMR250W	*GAD1*	1362-1396
YMR266W	*RSN1*	966-1020, 1046-1105, 2245-2294
YMR273C	*ZDS1*	2184-2278, 2724-2763
YMR275C	*BUL1*	2166-2205
YMR276W	*DSK2*	750-794, 860-903, 935-1009
YMR289W	*ABZ2*	694-730
YMR290C	*HAS1*	1352-1406
YMR291W		1409-1458, 1474-1493
YMR296C	*LCB1*	361-440
YMR303C	*ADH2*	313-362
YMR305C	*SCW10*	159-468
YMR307W	*GAS1*	929-1073, 1519-1673
YMR309C	*NIP1*	1525-1669, 1715-1809
YMR310C		13-57, 333-377
YMR312W	*ELP6*	85-159
YMR314W	*PRE5*	436-472
YMR316W	*DIA1*	932-968
YMR317W		446-460
YNL009W	*IDP3*	1812-1856
YNL016W	*PUB1*	0-59, 940-1034
YNL020C	*ARK1*	991-1035, 1486-1520
YNL025C	*SSN8*	643-682
YNL027W	*CRZ1*	744-806
YNL042W	*BOP3*	-302–258, 683-767
YNL044W	*YIP3*	216-259
YNL045W		115-159
YNL047C	*SLM2*	347-386
YNL054W	*VAC7*	382-436
YNL055C	*POR1*	-33-21, 47-91, 207-291
YNL066W	*SUN4*	278-322

YNL067W	*RPL9B*	-3-11
YNL068C	*FKH2*	890-964, 1730-1749
YNL071W	*LAT1*	810-904
YNL074C	*MLF3*	800-899
YNL085W	*MKT1*	-227–188, 1638-1682, 2283-2332
YNL091W	*NST1*	2993-3042, 3608-3652
YNL094W	*APP1*	120-164, 611-654
YNL096C	*RPS7B*	10-59, 835-924
YNL101W	*AVT4*	-90-29
YNL103W	*MET4*	930-974, 1220-1284
YNL106C	*INP52*	2998-3041
YNL118C	*DCP2*	979-1023
YNL123W	*NMA111*	2920-2956
YNL124W	*NAF1*	1058-1167, 1328-1437
YNL137C	*NAM9*	1394-1438
YNL138W	*SRV2*	989-1038
YNL142W	*MEP2*	1349-1373
YNL143C		138-177
YNL152W		852-906, 962-991, 1102-1146
YNL153C	*GIM3*	432-481
YNL154C	*YCK2*	1180-1274
YNL158W	*PGA1*	328-375
YNL160W	*YGP1*	225-319, 405-509, 1020-1079
YNL161W	*CBK1*	628-747
YNL167C	*SKO1*	1462-1536
YNL172W	*APC1*	656-692
YNL176C		-79–40, 301-465, 521-685, 731-774, 1016-1065, 1271-1320
YNL180C	*RHO5*	897-941
YNL183C	*NPR1*	-89–45, 806-860
YNL186W	*UBP10*	1459-1508
YNL190W		-94–45, 116-565
YNL192W	*CHS1*	121-220, 391-460
YNL197C	*WHI3*	-115–73, 713-977
YNL209W	*SSB2*	1210-1359, 1495-1644
YNL219C	*ALG9*	733-777
YNL230C	*ELA1*	936-980
YNL238W	*KEX2*	-22-22
YNL239W	*LAP3*	107-146
YNL241C	*ZWF1*	-137–68
YNL255C	*GIS2*	471-515
YNL271C	*BNI1*	3815-3869
YNL278W	*CAF120*	2766-2805, 2971-3020, 3116-3165
YNL281W	*HCH1*	535-579
YNL283C	*WSC2*	453-677
YNL287W	*SEC21*	424-538
YNL288W	*CAF40*	635-679
YNL297C	*MON2*	1232-1256, 3422-3459
YNL298W	*CLA4*	845-889, 990-1134, 1145-1229, 1240-1424
YNL300W		-6-228

YNL301C	*RPL18B*	-12-37
YNL307C	*MCK1*	1275-1319
YNL309W	*STB1*	740-784
YNL311C	*SKP2*	1512-1553
YNL322C	*KRE1*	564-663
YNL327W	*EGT2*	1204-1263, 1294-1593
YNL329C	*PEX6*	386-429
YNR006W	*VPS27*	1377-1539
YNR009W	*NRM1*	638-687
YNR010W	*CSE2*	-216–182
YNR013C	*PHO91*	1439-1488, 1734-1788
YNR014W		181-195, 266-310
YNR016C	*ACC1*	-317–268, 2986-3032
YNR021W		743-787
YNR026C	*SEC12*	1053-1107
YNR030W	*ALG12*	985-1028, 1420-1534
YNR031C	*SSK2*	513-527
YNR033W	*ABZ1*	-335–291
YNR035C	*ARC35*	11-55
YNR038W	*DBP6*	1249-1292, 1889-1926
YNR044W	*AGA1*	570-1014, 1825-1919, 1960-2024
YNR047W		532-576, 1327-1411
YNR052C	*POP2*	1027-1101
YNR067C	*DSE4*	829-888, 974-1058
YNR075W	*COS10*	277-321, 702-721, 732-746
YOL004W	*SIN3*	176-220, 1086-1206, 1401-1439
YOL007C	*CSI2*	225-269
YOL011W	*PLB3*	2205-2249
YOL019W		1336-1500, 1596-1645
YOL020W	*TAT2*	82-161
YOL030W	*GAS5*	1237-1311
YOL036W		1792-1830
YOL038W	*PRE6*	1096-1140
YOL040C	*RPS15*	335-384
YOL051W	*GAL11*	430-509, 825-959, 1130-1234
YOL060C	*MAM3*	1503-1642
YOL072W	*THP1*	348-397
YOL081W	*IRA2*	1270-1559
YOL086C	*ADH1*	-52-167
YOL087C		1062-1120, 2211-2275
YOL100W	*PKH2*	2870-2992
YOL104C	*NDJ1*	438-474
YOL105C	*WSC3*	456-530
YOL126C	*MDH2*	972-1016
YOL129W	*VPS68*	-18-31
YOL130W	*ALR1*	7-41
YOL135C	*MED7*	321-365
YOL136C	*PFK27*	948-995
YOL155C	*HPF1*	329-373, 2339-2378, 2409-2443

YOR002W	*ALG6*	964-1098, 1114-1233
YOR008C	*SLG1*	390-499
YOR014W	*RTS1*	240-479
YOR018W	*ROD1*	1781-1817
YOR023C	*AHC1*	1104-1208
YOR047C	*STD1*	69-113, 174-233
YOR052C		465-524
YOR063W	*RPL3*	518-562
YOR066W		1376-1565
YOR081C	*TGL5*	1463-1512, 1633-1667, 1738-1777
YOR085W	*OST3*	384-718
YOR086C	*TCB1*	841-881
YOR098C	*NUP1*	1956-2101, 2372-2421
YOR107W	*RGS2*	-200–186, 910-954
YOR109W	*INP53*	2940-2989
YOR113W	*AZF1*	1073-1117, 2498-2587
YOR118W		2301-2344
YOR127W	*RGA1*	1385-1429
YOR132W	*VPS17*	254-298
YOR133W	*EFT1*	850-894, 1710-1764, 2095-2224
YOR134W	*BAG7*	943-1008
YOR138C	*RUP1*	1587-1636
YOR140W	*SFL1*	1395-1479, 1945-1989, 2595-2699
YOR149C	*SMP3*	369-413
YOR153W	*PDR5*	656-700, 1821-1867
YOR156C	*NFI1*	1554-1588
YOR178C	*GAC1*	1578-1587
YOR181W	*LAS17*	804-903, 1224-1408, 1524-1623
YOR188W	*MSB1*	2757-2806
YOR197W	*MCA1*	626-675
YOR198C	*BFR1*	-123–29, 167-226
YOR204W	*DED1*	424-468, 2419-2455
YOR207C	*RET1*	2041-2111
YOR208W	*PTP2*	1423-1467
YOR219C	*STE13*	407-476
YOR227W		745-769, 1400-1443
YOR248W		-49-170, 351-480
YOR254C	*SEC63*	243-285
YOR260W	*GCD1*	825-871
YOR267C	*HRK1*	95-529
YOR270C	*VPH1*	1088-1152
YOR271C	*FSF1*	482-526
YOR275C	*RIM20*	869-923
YOR290C	*SNF2*	1062-1111
YOR296W		199-240
YOR303W	*CPA1*	-50-59
YOR310C	*NOP58*	60-114
YOR312C	*RPL20B*	848-945
YOR315W	*SFG1*	1174-1263

YOR321W	*PMT3*	1075-1117
YOR322C	*LDB19*	2261-2305
YOR329C	*SCD5*	2126-2415
YOR333C		456-500
YOR343C		11-55
YOR344C	*TYE7*	1063-1107
YOR347C	*PYK2*	2178-2217
YOR353C	*SOG2*	612-653
YOR354C	*MSC6*	1345-1384
YOR355W	*GDS1*	807-856, 867-916, 987-1101, 1247-1291
YOR359W	*VTS1*	950-1173
YOR363C	*PIP2*	2490-2529
YOR370C	*MRS6*	899-983
YOR372C	*NDD1*	1472-1526
YOR373W	*NUD1*	2095-2133
YOR385W		640-794
YOR394C-A		-115–31, 115-164
YPL011C	*TAF3*	643-687
YPL014W		642-706, 837-1031
YPL016W	*SWI1*	505-569, 615-699
YPL026C	*SKS1*	-27–8, 1098-1182
YPL032C	*SVL3*	-91–37, 444-493, 1069-1123, 1244-1318, 1914-1968, 2009-2078, 2109-2208
YPL036W	*PMA2*	2996-3035, 3056-3100
YPL037C	*EGD1*	-19-30
YPL049C	*DIG1*	1292-1466
YPL054W	*LEE1*	458-494
YPL057C	*SUR1*	-38-1, 1442-1496
YPL063W	*TIM50*	-64–15, 1546-1590
YPL066W		561-615
YPL070W	*MUK1*	354-398
YPL075W	*GCR1*	677-751, 2017-2066
YPL076W	*GPI2*	379-408
YPL079W	*RPL21B*	826-870
YPL085W	*SEC16*	2145-2224, 2830-2893, 6400-6454
YPL089C	*RLM1*	1552-1766
YPL106C	*SSE1*	1240-1374
YPL115C	*BEM3*	-94–46, 651-925, 976-1050, 1231-1390
YPL126W	*NAN1*	1189-1285
YPL128C	*TBF1*	1059-1103
YPL131W	*RPL5*	-30-244
YPL135W	*ISU1*	593-637
YPL141C		1569-1618
YPL149W	*ATG5*	381-421
YPL154C	*PEP4*	269-340
YPL163C	*SVS1*	163-337
YPL164C	*MLH3*	-330–252
YPL166W	*ATG29*	1124-1166
YPL179W	*PPQ1*	375-589

YPL184C	*MRN1*	-230–206, -55–11
YPL187W	*MF(ALPHA)1*	579-623
YPL190C	*NAB3*	1132-1186
YPL198W	*RPL7B*	1374-1418
YPL202C	*AFT2*	1006-1052
YPL204W	*HRR25*	1325-1374, 1385-1464
YPL210C	*SRP72*	571-615, 901-970
YPL220W	*RPL1A*	-3-41
YPL226W	*NEW1*	439-530, 569-668
YPL231W	*FAS2*	685-784
YPL240C	*HSP82*	1818-1862
YPL256C	*CLN2*	1076-1135
YPL259C	*APM1*	747-796
YPL262W	*FUM1*	483-527
YPR013C		342-371, 422-466
YPR022C		406-485, 506-560
YPR024W	*YME1*	289-339, 377-433
YPR032W	*SRO7*	185-227, 1178-1222
YPR035W	*GLN1*	1203-1280
YPR036W	*VMA13*	1245-1289
YPR036W-A		306-355
YPR040W	*TIP41*	171-230
YPR042C	*PUF2*	1240-1284, 1520-1569
YPR065W	*ROX1*	611-685, 946-1050
YPR072W	*NOT5*	971-1015, 1221-1255
YPR080W	*TEF1*	286-335, 1041-1115
YPR083W	*MDM36*	1431-1445
YPR089W		1212-1256
YPR095C	*SYT1*	667-711
YPR102C	*RPL11A*	423-497
YPR106W	*ISR1*	641-678
YPR117W		-92–43
YPR119W	*CLB2*	1582-1611
YPR122W	*AXL1*	385-429, 460-504, 530-539
YPR129W	*SCD6*	266-350
YPR132W	*RPS23B*	858-916
YPR144C	*NOC4*	1132-1161
YPR149W	*NCE102*	-47–4
YPR150W		-294–250
YPR154W	*PIN3*	377-626
YPR161C	*SGV1*	1806-1825
YPR164W	*MMS1*	1547-1556
YPR171W	*BSP1*	898-952, 1204-1241
YPR181C	*SEC23*	1370-1514
YPR183W	*DPM1*	726-780
YPR184W	*GDB1*	-153–113

173 binding sites map to Ty elements

Chapter 3: High resolution identification of *in vivo* binding sites reveals post-transcriptional coordination by Khd1

ABSTRACT

RNA-binding proteins can interact with mRNAs encoding functionally related proteins to coordinate their expression. Our analysis of the RNA-binding protein Khd1 in *Saccharomyces cerevisiae* using cross-linking immunoprecipitation (CLIP) identified a post-transcriptional network that coordinates cellular responses to environmental stress. We previously showed that Khd1 binds *FLO11* and *ASH1* mRNAs to regulate filamentous growth, which cells enact under conditions of nitrogen starvation. Here we show that Khd1 binds additional transcripts that encode regulators of *FLO11* and filamentation. Khd1 also represses retrotransposition of Ty1 elements, another cellular response to low nitrogen conditions, by repressing translation of Ty1 mRNA. Through interactions with its target transcripts, Khd1 coordinates two responses to environmental stress.

INTRODUCTION

Many cellular processes require coordinated expression of multiple genes. The organization of related genes into polycistronic operons permits translation of multiple genes from a single mRNA to coordinate gene expression in bacteria. Transcriptional operons are rare in eukaryotic organisms (BLUMENTHAL 1998), but growing evidence points to the widespread use of post-transcriptional regulons in eukaryotic systems (KEENE and LAGER 2005). RNA-binding proteins coordinately affect the localization, stability, and translation of mRNAs encoding functionally related proteins by recognizing specific sequences or structures in target transcripts (KEENE 2007). Genes subject to coordinated post-transcriptional regulation are likely to share related functions (KEENE 2007; KEENE

and LAGER 2005; KEENE and TENENBAUM 2002). RNA-binding proteins can change their binding patterns upon cellular differentiation, suggesting they play a role in altering cellular processes (TENENBAUM *et al.* 2000).

Under conditions of nitrogen starvation, diploid *Saccharomyces cerevisiae* cells undergo a morphogenetic transition to filamentous growth that requires the coordinated alteration of multiple cellular processes (GAGIANO *et al.* 2002). In rich nitrogen, diploid cells have an elliptical morphology and undergo bipolar divisions. The filamentous growth program employs a modified cell cycle to generate elongated cells that undergo unipolar divisions (GIMENO *et al.* 1992; KRON *et al.* 1994). This change initiates with the first cell division (AHN *et al.* 1999), and leads to the directional outgrowth of filaments from the colony, which is thought of as a foraging mechanism (GIMENO *et al.* 1992).

Filamentous growth requires expression of *FLO11* (LAMBRECHTS et al. 1996; LO and DRANGINIS 1998), which encodes a cell surface protein (LO and DRANGINIS 1996). Multiple signaling pathways converge on the *FLO11* promoter, which at 3.5kb is unusually large for a yeast promoter, to control transcription (Figure 1). A mitogen-activated protein (MAP) kinase pathway and a protein kinase A (PKA) pathway each regulate transcription through multiple regions of the *FLO11* promoter (RUPP *et al.* 1999). Activated Kss1, the filamentation MAP kinase, phosphorylates the transcription factors Tec1 and Ste12, which bind in tandem to the *FLO11* promoter to activate transcription (MADHANI and FINK 1997). PKA signaling activates the transcription factor Flo8, which promotes *FLO11* transcription (PAN and HEITMAN 1999). In a parallel transcription factor cascade, Sok2 regulates Phd1 and Ash1 to activate *FLO11* transcription (PAN *et al.* 2000). In addition to activating *FLO11,* regulators of filamentous growth control other aspects of the developmental program (Figure 1). The filamentation MAP kinase pathway

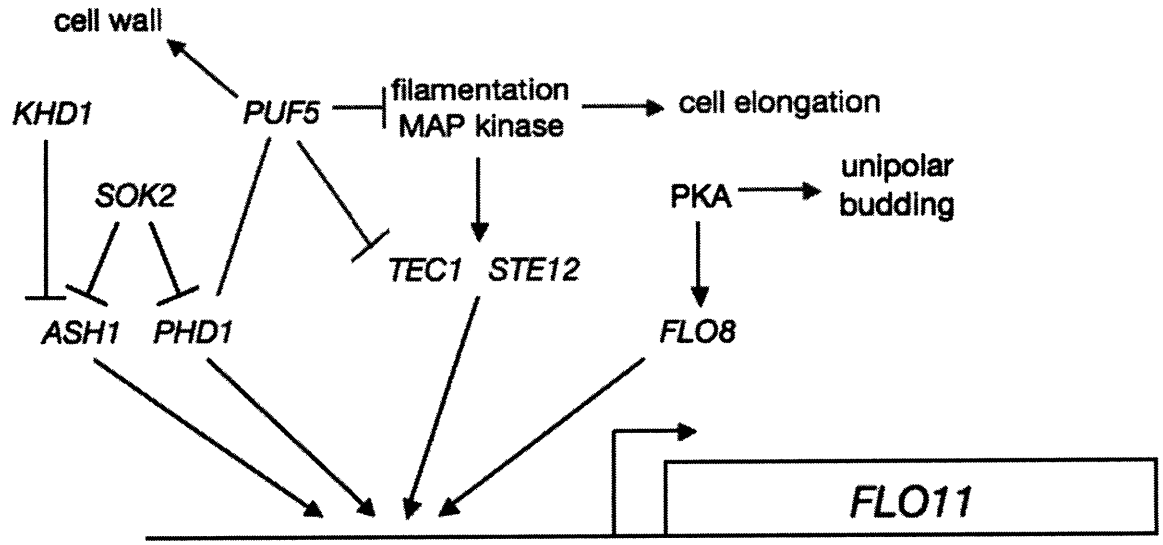

Figure 1.—Coordinate regulation of processes required for filamentous growth. Genetic interactions represented in black control *FLO11* transcription. Genetic interactions in red coordinate *FLO11* transcription with other processes required for filamentous growth. Khd1 binds mRNAs shown in blue.

alters the cell cycle to control cell elongation (AHN *et al.* 1999), and PKA signaling promotes the unipolar budding pattern (PAN and HEITMAN 1999). The RNA-binding protein Puf5 inhibits the filamentation MAP kinase pathway by repressing translation of *STE7* and *TEC1* mRNAs (PRINZ *et al.* 2006), and also binds *PHD1* mRNA (GERBER *et al.* 2004), although the effect of this interaction is not known. Puf5 also plays a role in maintaining cell wall structure (KAEBERLEIN and GUARENTE 2002), which undergoes changes during the transition to filamentous growth (CID *et al.* 1995). These networks coordinate the cellular processes that contribute to filamentous growth.

In addition to coordinating components of filamentous growth, the filamentation MAP kinase pathway coordinates the morphogenetic switch with retrotransposition. In diploid cells, Ty1 retrotransposition, like filamentation, occurs only in low nitrogen. Both Tec1 and Ste12, the downstream transcription factors of the filamentation MAP kinase pathway, are required for Ty1 transcription (LALOUX *et al.* 1990; MORILLON *et al.* 2000). Filamentation allows cells to search out more habitable environments, and transposition

has the potential to generate mutations that provide advantages in the existing conditions (MORILLON *et al.* 2000). Through this regulation, the filamentation MAP kinase pathway transcriptionally enables two cellular responses to environmental stress. However, post-transcriptional regulation enables the rapid switch to filamentous growth under conditions of nitrogen starvation (Chapter 2) and a similar mechanism may regulate retrotransposition as well.

We report post-transcriptional coordination of retrotransposition and filamentation by the RNA-binding protein Khd1. Identification of the RNA targets of Khd1 revealed its role in regulating asymmetric expression of *FLO11* during filamentous growth (Chapter 2). Khd1 binds *ASH1* and *FLO11* mRNAs to regulate *FLO11* expression, but also binds additional transcripts, a number of which affect processes involved in filamentous growth. These mRNA targets relate to the previously described role for Khd1 in regulating filamentous growth. We expand this role to include repression of retrotransposition. Khd1 represses retrotransposition by repressing translation of Ty1 RNA, coordinating filamentation and retrotransposition through a common post-transcriptional regulator. Interactions between Khd1 and its RNA targets, including mRNAs encoding other post-transcriptional regulators, coordinate related processes within the cell.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains used in this study (Table 1) are derived from Σ1278b 10560-2B. Standard yeast media, yeast transformations and genetic manipulations were performed as previously described (GUTHRIE and FINK 1991). Yeast strains carrying gene deletions were

constructed by PCR amplification of kanamycin-resistance gene cassettes from the yeast deletion library (WINZELER *et al.* 1999) and transformation into Σ1278b.

Plasmid construction

The Khd1 overexpression construct was made by amplifying the gene using PCR, with oligonucleotides that added restriction sites (*NotI* at the 5' end, *XhoI* at the 3' end) to the final product. Amplified DNA was digested using *NotI* and *XhoI* and cloned into p415TEF (MUMBERG *et al.* 1995).

Western Blot Analysis

Protein was prepared using TCA precipitation from 3 mL of strains carrying either P_{TEF}-*KHD1* construct or the empty vector grown to OD_{600} of .9-1.1 and resuspended in 150 mL SDS loading buffer. 10 mL were run on 10% SDS-polyacrylamide gels and transferred to nitrocellulose filter paper. Blotting was performed against TyA with rabbit anti-VLP antiserum generously provided by David Garfinkel and HRP-conjugated donkey anti-rabbit secondary antibody (Amersham NA934V), and against tubulin using rat anti-tubulin (Accurate Chemicals MCA77G) and HRP-conjugated goat anti-rat antibody (Jackson ImmunoResearch 112-035-062). Blots were detected using SuperSignal West Femto Substrate (Thermo Scientific 34095).

Northern Blot Analysis

Ty1 mRNA was detected as previously described (DRINNENBERG *et al.* 2009).

Frequency of Ty1*his3-AI* mobility

Ty1*his3-AI* mobility was measured as previously described (NYSWANER *et al.* 2008). In brief, ~10^3 cells carrying P_{TEF}-*KHD1* or empty vector were inoculated into 1 mL of SC – Leu -Ura, grown to saturation, and spread on both SC –Leu –Ura and SC – Leu –Ura –His plates. The frequency of Ty1*his3-AI* mobility was calculated by dividing the average number of Leu$^+$ His$^+$ Ura$^+$ cells per milliliter by the average number of Leu$^+$ Ura$^+$ cells per milliliter. To adjust for the slow growth caused by Khd1 overexpression, an equal number of cells were inoculated into 10 mL of media and aliquots from the 1 mL culture were plated when the 10 mL culture had grown to an OD_{600} of 1.

Table 1. Yeast strains and plasmids used in this study

Strain	Genotype	Source
JW1552	*MATa his3::natNT2 ura3-52 leu2::hisG* Ty1*his 3-AI*	This study
JW1571	*MATa his3::natNT2 ura3-52 leu2::hisG* Ty1*his3-AI khd1::kanMX4*	This study

Plasmid	Insert	Source
pBJC573	Ty1 *his3-AId1*	Nyswaner et al. 2008
p415TEF		Mumberg et al. 1995
p415TEF-*KHD1*	*KHD1*	This study

RESULTS

Khd1 binds related RNA targets *in vivo*

We identified the transcriptome-wide targets of Khd1 using the cross-linking immunoprecipitation (CLIP) method (Chapter 2) to provide comprehensive understanding of its role in regulating cellular activities. Khd1 binds 1,114 unique mRNAs, as well as mRNAs transcribed from approximately 30 homologous Ty1 and Ty2 loci. Khd1 binds ten mRNAs encoded in the Σ1287b genome that are not present in the S288c reference genome. The Σ1287b strain was used because, unlike S288c, it is competent for filamentous growth. Khd1 also binds 47 noncoding RNAs, including rRNA, tRNAs, snRNAs, snoRNAs, and telomerase RNA. Khd1 binds a subset of tRNAs: tK(CUU),

tR(UCU), tR(CCU), tT(UGU), tH(GUG), tL(CAA), and tW(CCA). Khd1 binds only a single version of tH(GUG) (1/8 genomic copies), tL(CAA) (1/35) and tW(CCA) (1/13), but 3 of 12 tT(UGU), 11 of 26 tK(CUU) and 12 of 21 tR(UCU). These tRNAs all appear to be valid targets of Khd1, since non-specific interactions would produce an even distribution across all tRNAs. With the exception of Ty elements, Khd1 binds a single site in most of its target RNAs. Khd1 binds Ty1 and Ty2 RNAs through sites that are highly homologous between individual elements within each family, as well as between the two families, precluding identification of the specific elements it targets.

CLIP identified known binding sites for Khd1. Khd1 binds *ASH1* mRNA, its best-studied target transcript, within a 165 nucleotide region in the open reading frame (Figure 2). These sequences fall within a region of the open reading frame previously shown to be necessary and sufficient for Khd1 recognition of *ASH1* mRNA (HASEGAWA *et al.* 2008). Similar to its interaction with *ASH1* mRNA, Khd1 binds 87% of its target mRNAs within the open reading frame. We identified Khd1 binding sites within regions of three other transcripts previously shown to be sufficient for recognition, although Khd1 binds two of these transcripts through additional regions as well (Figure 2). The successful identification of known *in vivo* Khd1 binding sites supports the biological relevance of the previously unknown binding sites CLIP identified.

We looked for enrichments within the functional annotations of Khd1 target mRNAs to provide insight into its biological function. The target list is enriched for multiple functional annotations, including the cell wall (54 out of 114, $p < 3.9e\text{-}16$), filamentous growth (46 out of 96, $p < 5.0e\text{-}14$), and mRNA catabolic processes, deadenylation-dependent decay (16 of 24, 4.4e-6) (BEISSBARTH and SPEED 2004). Ty elements are the most abundant Khd1 target in the data set, but we cannot determine the

exact number of elements that Khd1 binds. Khd1 also binds the single copy of tR(CCU), which regulates the frequency of programmed frameshifting in Ty expression (KAWAKAMI *et al.* 1993).

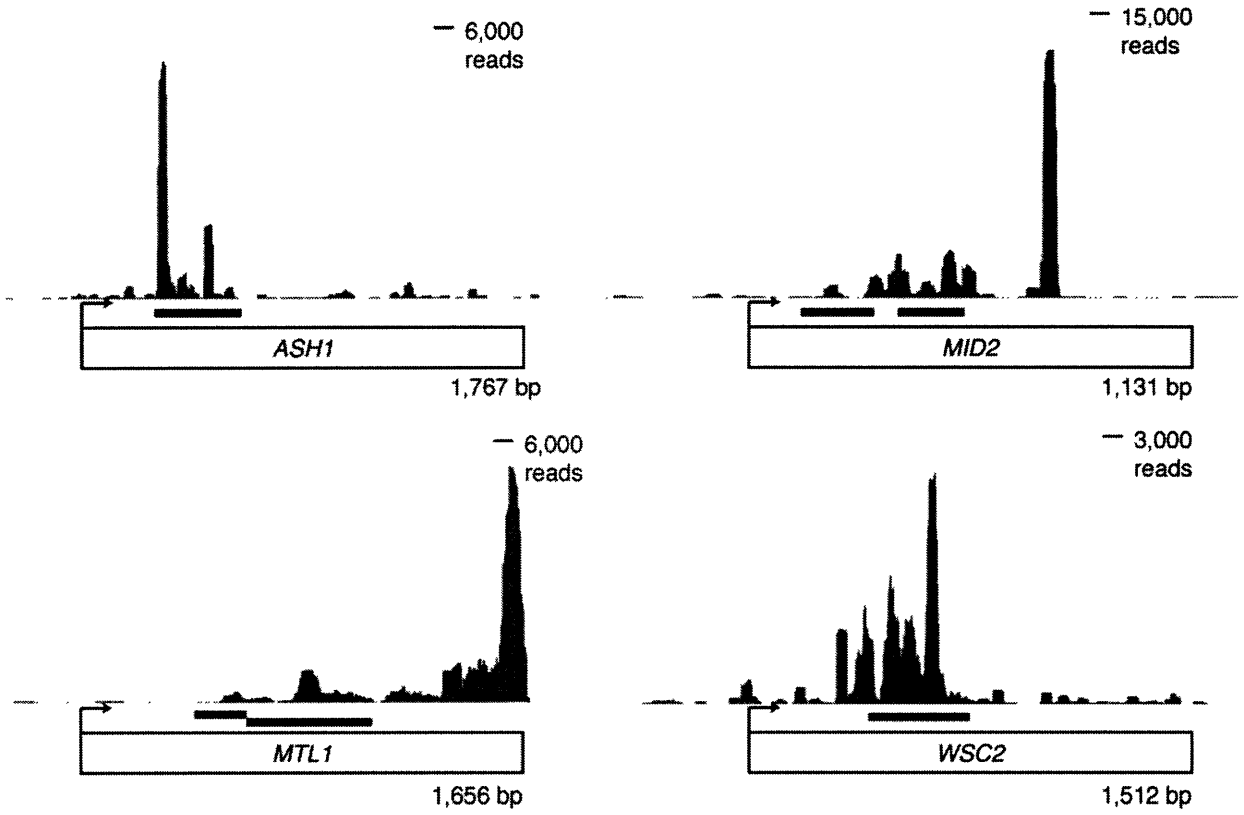

Figure 2.—CLIP identifies known Khd1 targets. Histograms of sequences from CLIP mapped to the four Khd1 target mRNAs with previously defined binding sites. Black bars represent regions shown to be sufficient for Khd1 binding (HASEGAWA *et al.* 2008).

The mRNAs bound by Khd1 affect multiple aspects of filamentous growth (Figure 1). Khd1 binds *FLO11* mRNA, and a number of mRNAs encoding proteins that regulate *FLO11* transcription. These transcripts include mRNAs encoding Phd1 and Sok2, which act in a transcription factor cascade with Ash1, and components of the filamentation MAP kinase signaling pathway. In addition, Khd1 binds a number of mRNAs that encode components of the cell wall, and may play a role in its reconfiguration during filamentous growth. Some of the Khd1 target mRNAs involved in filamentation, such as *KSS1* and

CLB2 mRNA, localize to the bud tip similarly to *ASH1* mRNA (SHEPARD *et al.* 2003). *CLB2* encodes a mitotic cyclin that acts to promote cell elongation during filamentation in conjunction with the filamentation MAP kinase pathway. *clb2Δ/clb2Δ* mutants filament in rich nitrogen, and *CLB2* overexpression represses filamentous growth (AHN *et al.* 1999). Khd1 binds *CLN1, CLN2,* and *CLN3* mRNAs, which encode G1 cyclins that play a role during filamentous growth as well (LOEB *et al.* 1999; OEHLEN and CROSS 1998).

Khd1 binds a number of mRNAs encoding other RNA-binding proteins. These targets are contained in the enriched functional category mRNA catabolic processes, deadenylation-dependent decay. In addition to binding transcripts encoding these post-transcriptional regulators, Khd1 binds many of their target mRNAs as well. It binds within the coding sequences of two transcripts encoding components of the filamentation MAP kinase pathway, *STE7* and *TEC1* mRNAs, that Puf5 represses through interactions with their 3' untranslated regions (PRINZ *et al.* 2006). Comparison with a study that identified the genome-wide targets of five Puf proteins using microarray analysis following immunoprecipitation (GERBER *et al.* 2004) shows that Khd1 shares 50 additional targets with Puf5, including *PHD1* mRNA. Khd1 also binds *PUF5* mRNA, conferring feed-forward regulation on their common targets. Khd1 displays this network motif with the RNA-binding proteins Puf1, Puf2, Puf3, and Puf4 as well, binding to both their mRNAs as well as a subset of their target transcripts (Figure 3). Khd1 binds 143 mRNA targets of the Puf proteins. The overlaps vary, with Khd1 binding over 40% of the targets identified for Puf1 and Puf2, but less than 10% of those for Puf3. Khd1 binds mRNAs encoding decapping and deadenylation enzymes, as well as its own mRNA, adding an autoregulatory feed back loop to its post-transcriptional network.

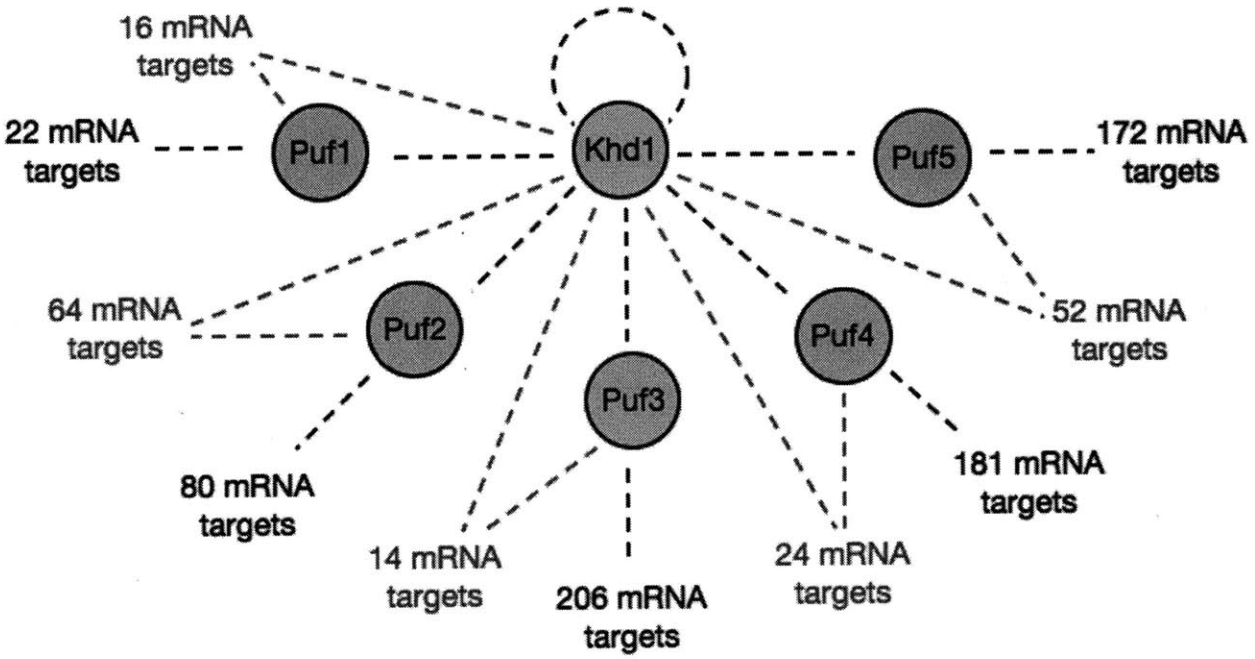

Figure 3.—Khd1 confers feed-forward regulation on many mRNAs within the cell. Khd1 binds mRNAs encoding the Puf family of RNA-binding proteins, and a subset of their target mRNAs. The specific effect of protein binding on the expression of most target mRNAs is not known.

Khd1 represses Ty1 translation and retrotransposition

Sequences mapping to Ty RNAs are the most abundant category in our CLIP data set. Because Ty RNA comprises up to 1% of total RNA in yeast (CURCIO *et al.* 1990), its prevalence could be a consequence of non-specific binding. However, the clustering of sequences into discrete peaks (Fig. 4A) argues that Khd1 binds specific sites within Ty1 and Ty2 RNAs *in vivo*. Regulation of retrotransposition by Khd1 would support this interaction.

We measured mobility of the Ty1*his3-AI* construct (NYSWANER *et al.* 2008) to test regulation of retrotransposition by Khd1. The parent strain containing this construct cannot grow on media lacking histidine. An artificial intron interrupts the *HIS3* coding sequence in the antisense orientation, and the splicing machinery does not recognize the intron when *HIS3* is transcribed from its own promoter. However, the intron lies in the sense orientation relative to the Ty element, and if the entire element is transcribed,

Table 2. Khd1 represses Ty1 retrotransposition

Strain	Ty1*his3-AI* mobility x 10^{-5} (SD)	Fold change
wild type	8.4 (1.4)	1
khd1Δ	10 (1.8)	1.2
P_{TEF}-*KHD1*	.64 (.08)	0.08

Values calculated as described in Nyswaner, et. al, 2008. Error represented as standard deviation.

spliced, reverse transcribed, and reintegrated into the genome, the cell can grow without histidine. Reintegration of Ty cDNA can occur via transposition or gene conversion, but transposition is more frequent.

Mobility of the Ty1*his3-AI* construct in *khd1Δ* is not significantly different from wild type, but Khd1 overexpression reduces its mobility 13-fold (Table 2). To investigate the mechanism of this repression, we performed Western blotting using antiserum against Ty virus-like particles (VLPs). TYA encodes the structural component of the VLPs and corresponds to the retroviral *gag* gene. The initial translation product is post-translationally processed by a C-terminal proteolytic cleavage to its mature form by a product of TYB.

TyA levels upon *KHD1* deletion or overexpression mirror the changes in Ty1*his3-AI* mobility. TyA levels are unchanged in *khd1Δ*, and Khd1 overexpression reduces TyA protein levels 8-fold (Fig. 4B). The levels of both the primary product of translation and mature TyA decrease upon Khd1 overexpression, and the ratio between them does not change. Northern blotting shows that Ty1 RNA levels remain constant in all three strains (Fig. 4C). Given the CLIP results indicating that Khd1 binds Ty1 RNA, these experiments suggest that Khd1 represses Ty1 retrotransposition by repressing translation of Ty1 RNA.

Given that Khd1 overexpression, but not deletion, decreases TyA levels and Ty1*his3-AI* mobility, these results do not prove a role for Khd1 in regulating retrotransposition under physiological conditions. We attempted to use a sensitized assay

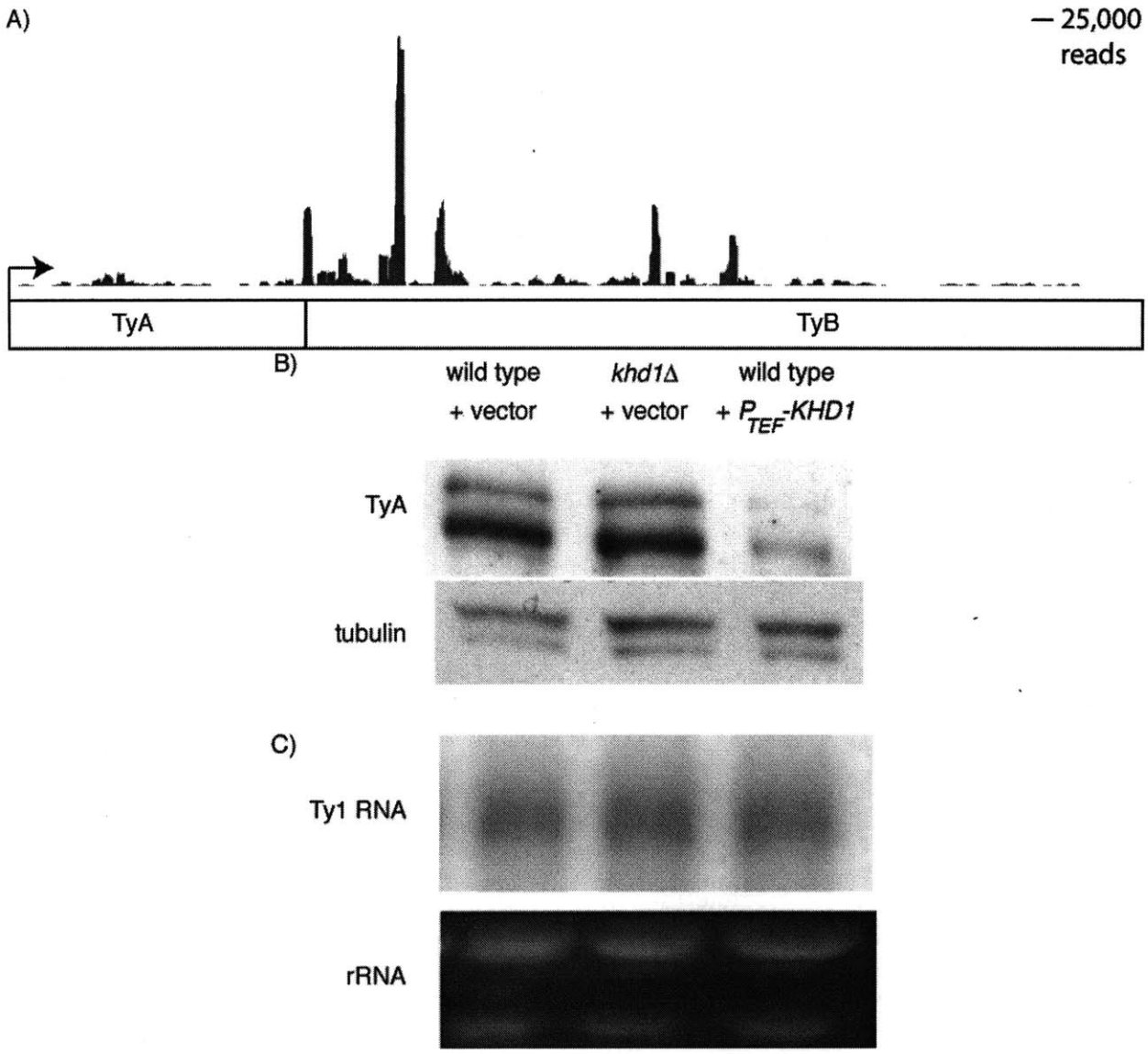

Figure 4.—Khd1 binds Ty1 RNA to repress translation. (A) Histograms of sequences from CLIP mapped to a representative Ty1 element. (B) Western blot analysis of TyA protein levels. P_{TEF}-*KHD1* is an overexpression construct. (C) Northern blot analysis of Ty1 RNA for the strains shown in (B). Ethidium bromide staining of rRNA demonstrates equal loading. Khd1 overexpression reduces TyA protein levels without affecting Ty1 mRNA levels.

to uncover a physiological role. A previous study reported that immunoprecipitation of the RNA-binding protein She2 enriches Ty RNA (SHEPARD *et al.* 2003). She2 binds both *ASH1* mRNA and the localization machinery, leading to the localization of *ASH1* mRNA (BOHL *et al.* 2000), but an attempt to visulize localized Ty RNA elements using *in situ* hybridization was unsuccessful (SHEPARD *et al.* 2003). Low levels of Ty RNA localization,

able to escape visual detection, could potentially be detected functionally using the Ty1*his 3-AI* allele and mutations that affect localization. However, retrotransposition frequencies are similar for wild type, *she2*Δ, and *she2*Δ *khd1*Δ (data not shown). The specific role for Khd1 in regulating transposition remains to be determined.

DISCUSSION

Rapid changes in cellular processes require regulation that cannot be accomplished solely at the transcriptional level (MANSFIELD and KEENE 2009). The transition from yeast form to filamentous growth, which occurs with the first cell division under low nitrogen conditions (AHN *et al.* 1999), is enabled by regulation of *FLO11* at both the transcriptional and translation level (Chapter 2). The change in growth form requires the coordinated alteration of multiple cellular processes in addition to *FLO11* expression, including the cell cycle, and cell wall maintenance (GANCEDO 2001). Khd1 regulates asymmetric *FLO11* expression (Chapter 2), and binds mRNAs encoding cell cycle regulators and components of the cell wall. Khd1 may regulate these processes during filamentation as well. Studies have shown that RNA-binding proteins can alter the complement of mRNAs they bind upon differentiation (TENENBAUM *et al.* 2000). Khd1 could undergo a similar transition in response to nitrogen starvation to control filamentous growth.

The effects of Khd1 binding need not be the same on expression of all of these genes. Khd1 can differentially affect its target genes (HASEGAWA *et al.* 2008), though the details that determine the specific regulation remain unknown. The binding sites we identify in *MTL1* and *MID2* in addition to those previously shown to be sufficient for co-localization with Khd1 may contribute to this differential regulation.

Khd1 binds a number of mRNAs encoding RNA-binding proteins, making it a hub in the broader post-transcriptional regulatory network in yeast. By controlling expression of these post-transcriptional regulators, Khd1 can impart dynamic regulation on many transcripts in addition to its direct targets. Many RNA-binding proteins target mRNAs encoding other RNA-binding proteins, forming interconnected post-transcriptional regulatory networks (MANSFIELD and KEENE 2009). Studies in mammalian cells identified such interactions between six RNA-binding proteins, where each bound its own transcript as well as transcripts encoding other members of the study (PULLMANN *et al.* 2007). Khd1 has similar properties, binding within its own coding sequence and those of other post-transcriptional regulators. Khd1 also binds some of the same target mRNAs as the RNA-binding proteins whose mRNAs it targets. In addition, Khd1 binds mRNAs encoding decapping and deadenylation enzymes, and may regulate the stability of a number of transcripts, including but not limited to those it binds directly.

Repression of both transposition and filamentous growth by Khd1 is an example of its ability to coordinate cellular events. We identify Khd1 as a repressor of Ty1 transposition, similar to the role we identified for its repression of filamentation (Chapter 2). A previous study found that similar to filamentous growth, Ty1 retrotransposition is induced in diploid cells by nitrogen starvation, and requires the filamentation MAP kinase pathway for transcription (MORILLON *et al.* 2000). The study identified a larger increase in transposition frequency than in Ty1 mRNA levels following genetic modulation of signaling through the filamentation MAP kinase pathway, suggestive of post-transcriptional regulation. Khd1 may contribute to this post-transcriptional regulation.

Khd1 overexpression reduces both TyA protein levels and mobility of the Ty1*his 3-AI* allele. Although Khd1 binds near the site of the programmed frameshift between TYA

and TYB, and binds the tR(CCU) tRNA whose availability regulates frameshifting efficiency (KAWAKAMI *et al.* 1993), the symmetrical decline in both primary translation product and mature TyA implicates Khd1 in translational repression. Increased frameshifting resulting from loss of tR(CCU) elevates protease levels derived from TYB to an extent that quickly processes TyA to its mature form (KAWAKAMI *et al.* 1993). The primary TyA translation product is not detectable by Western blot in a mutant lacking tR(CCU) (data not shown). The upper band in Figure 4 blots implies that Khd1 overexpression does not affect Ty1 transposition by regulating the frameshift.

Our experiments did not reveal any regulation of Ty1 retrotransposition by Khd1 under physiological conditions. Although Khd1 overexpression has a clear phenotype related to Ty1, deletion of *KHD1* does not affect TyA levels or mobility of the Ty1*his 3-AI* allele. One hypothesis is that other mechanisms repress Ty1 translation in parallel to Khd1 to maintain wild type TyA levels and transposition rates in the *khd1*Δ background. Another is that Khd1 overexpression produces regulation that does not occur under physiological conditions. Given that the interaction between Khd1 and Ty mRNA was identified using Khd1 expressed from its endogenous promoter, we believe that the interaction is physiologically relevant and other factors act in parallel to repress retrotransposition in the absence of Khd1. The cell must carefully regulate transposition to avoid deleterious effects, and additional post-transcriptional repressors would ensure this protection.

Khd1 binds a number of mRNAs *in vivo* to regulate cellular processes. The functions of these targets point to post-transcriptional coordination of various aspects of filamentous growth with Ty1 retrotransposition. The integration of multiple post-transcriptional regulatory networks through Khd1 binding suggests an even broader role

for Khd1 in coordinating cellular activities. Genome-wide approaches have identified widespread networks of these interactions in other systems, but the extent of their effect on cellular activity remains an open question. Future studies will be needed to determine the global effects of regulation by Khd1.

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Chapter 4: Conclusions and Future Directions

CONCLUSIONS

I pursued a role for the RBP Khd1 in *S. cerevisiae* based on interactions described in the literature. Once I identified Khd1 as a repressor of filamentous growth, my goal was to identify cellular functions for Khd1 by characterizing its *in vivo* interactions with RNAs. The application of CLIP and deep sequencing to this pursuit was incredibly successful, and identified a number of targets for Khd1 that relate to its biological function. I experimentally confirmed a role for Khd1 in regulating the two most prevalent target mRNAs in the CLIP data, but the global details of Khd1 mRNA recognition and regulation remain unknown. The pioneering studies of RBPs using CLIP and deep sequencing have shown that RBP binding specificity is not easily described. My work supports some aspects of our understanding of post-transcriptional regulation by RBPs, but shows limitations in our understanding of these regulators.

Khd1 associates with mRNAs encoding functionally related proteins. The identification of target transcripts encoding proteins involved in filamentous growth was of particular interest because the regulation of filamentation by Khd1 initiated the CLIP study. My work describing Khd1 as a repressor of both filamentous growth and retrotransposition nicely fits the model for coordinated regulation by RBPs, but this study cannot be used to draw conclusions about the consequences of Khd1 binding for other transcripts. Khd1 differentially regulates its target mRNAs that localize to the bud tip (HASEGAWA *et al.* 2008), and could have opposing effects on the expression of even functionally related genes.

I did not anticipate the difficulty we encountered identifying a target motif for Khd1. I believe the lack of a consensus target sequence or structure represents the biology

of Khd1 rather than experimental limitations. Although studies using microarray analysis following immunoprecipitation rarely report high percentages of targets that contain a consensus motif, there are errors inherent in this approach for RNA target identification. Although I conceptually believed we had a superior approach, there is little precedent for the use of CLIP with deep sequencing to identify protein-RNA interactions. KH domain proteins interact with varied sequences *in vitro.* Published reports tend to focus on the common features in their experiments to fit a simple model, but the data sets as a whole do not conform to this assumption. With our transcriptome-wide identification of Khd1 binding sites, we revealed the complex nature of its binding interactions. Other applications of CLIP in conjunction with deep sequencing also identify many target mRNAs that lack the consensus binding motif (YEO *et al.* 2009), which necessitates modified thinking about protein-RNA interactions.

Orthogonal binding data would prove extremely useful in characterizing RNA-binding protein specificities. I am convinced that we identified a number of biologically relevant targets of Khd1. This belief comes from our ability to identify with high precision known interactions shown to have *in vivo* relevance, and my work testing two of the new targets we identify. However, I was unable to identify a complementary assay to test candidate binding sites in a high throughput manner.

The existing literature is contradictory with respect to the known Khd1 target, *ASH1* mRNA. Two non-overlapping regions of *ASH1* mRNA, each approximately 250 bases in length, are reported to mediate the interaction with Khd1. One study identifies a region that is sufficient for Khd1-mediated repression of a luciferase reporter construct – expression of both endogenous Ash1 protein and the reporter increases in *khd1*Δ cells (PAQUIN *et al.* 2007). Another study identifies a distinct region that is both necessary and

sufficient for binding to Khd1, and uses a co-localization reporter construct to assay binding because the authors see no change in Ash1 protein levels in *khd1*Δ cells (HASEGAWA *et al.* 2008). These discrepancies could result from the use of different strain backgrounds. However, the identification of mutually exclusive binding sites in a highly conserved transcript is unlikely to reflect the actual biological function of Khd1. In the strain where I conducted my study, deletion of *KHD1* does not affect Ash1 protein levels. Khd1 overexpression also has no effect on Ash1 protein levels in this strain. This contrasts with the study that used co-localization to monitor Khd1 binding that reports a reduction in Ash1 protein levels upon Khd1 overexpression, even though Ash1 protein levels are unaffected by the *KHD1* deletion (HASEGAWA *et al.* 2008).

These findings limit options for experimental validation of Khd1 target sites. Changes in protein levels are a poor assay in a reporter system since Khd1 expression differentially affects its targets (HASEGAWA *et al.* 2008). Colocalization of Khd1 with reporter constructs containing its binding sites within target mRNAs may not identify sites from endogenous transcripts that do not undergo localization. CLIP identified the regions of two mRNAs, *MTL1* and *MID2,* that were shown to be both necessary and sufficient for colocalization with Khd1, but also identified much stronger binding interactions in other regions of the transcripts. A high-throughput assay would allow for more rapid testing of candidate target sequences or, ideally, screens enabling their identification. The best option may be yeast-3 hybrid studies, which would enable rapid testing of potential target sequences (HOOK *et al.* 2005). However, this approach establishes an unnatural context in which to study protein-RNA interactions and may not accurately represent native activities.

The most broadly applicable finding from this study may be the high level of interconnectivity of the post-transcriptional regulatory network. There are many previous reports of RBPs that bind mRNAs encoding other RBPs (MANSFIELD and KEENE 2009). The best insight into this phenomenon has come from combining deep sequencing with CLIP because of the previously discussed capabilities of the technique. The real advantage of this study is the second-level understanding of the interactions. Khd1 binds not only mRNAs encoding Puf RBPs, but a number of mRNAs bound by the Puf proteins as well. This understanding is enabled by previous studies of the Puf proteins in yeast (GERBER *et al.* 2004). Interactions between post-transcriptional regulators has emerged as an important component of cellular activities (MANSFIELD and KEENE 2009). I expect that this network topology is not unique to Khd1 and that future studies will add to our understanding of these functional relationships.

FUTURE DIRECTIONS

The functional characterization of RBPs through the transcriptome-wide discovery of their binding targets and the downstream effects of their interactions is an expanding field with many opportunities for further study. My work revealed new regulatory functions for Khd1, but I believe the majority of its functions remain unknown. Other global analyses can reveal additional phenotypes regulated by this RBP through its effects on other genes. How Khd1 participates in the broader post-transcriptional regulatory network is also an interesting question. Finally, the potential for functional conservation between possible Khd1 orthologs in pathogenic fungi is an area with important medical implications. My work has elucidated aspects of post-transcriptional regulation by Khd1, and generated additional avenues for future study.

The global role Khd1 plays in regulating cellular activities remains unknown. I characterized the effect of Khd1 binding on two of its targets because of their prominence in the data set and their relationship to interesting phenotypes. Little is known about the effect of Khd1 binding on its additional targets or its indirect effects through interactions with mRNAs encoding other biological regulators. Proteins containing KH-domains regulate mRNAs in numerous ways, and individual proteins can have different effects on different targets. hnRNP K has been implicated in both translational silencing and translational activation of its target mRNAs (EVANS *et al.* 2003; OSTARECK *et al.* 1997). I have shown a role for Khd1 in translational silencing, but it may have different effects on the expression of its other targets. Its interactions with transcripts encoding other post-transcriptional regulators add even more potential functions to its regulatory repertoire. It would be interesting to apply the recently developed ribosome profiling technique to characterize the global role Khd1 plays in regulating gene expression. This approach identifies the positions of cycloheximide-stalled ribosomes using deep sequencing to quantify ribosome-protected mRNA fragments, providing a method to simultaneously interrogate the translational state of every transcript in the cell (INGOLIA *et al.* 2009). Sequencing counts are normalized to transcript levels similarly evaluated using deep sequencing, which would address both changes in mRNA levels and ribosome loads for genes regulated both directly and indirectly by Khd1. Applying this technique to wild type cells and those either overexpressing or deleted for *KHD1* would identify the global effects of Khd1 and point to additional phenotypes it regulates.

Khd1 acts as part of a larger post-transcriptional regulatory network. It associates with seven mRNAs that are localized through their interaction with the RBP She2 (HASEGAWA *et al.* 2008). Although microarray technology has been used to identify mRNA

targets of She2, CLIP identified a number of RNA targets of Khd1 that were not identified by microarray analysis, and some mRNAs localized through their interaction with She2 may have been similarly missed. It would be interesting to apply CLIP to She2 in order to gain a deeper understanding of its mRNA targets. This approach could expand the list of localized mRNAs in yeast, and enable the comparison of regulation of targets that are bound by both proteins to those targeted by one or the other. The transcriptome-wide identification of additional RBPs that target a subset of the mRNAs bound by Khd1 would continue to expand understanding of the post-transcriptional regulatory network in yeast.

Nothing is known about the mechanisms that regulate Khd1 in contexts other than mRNA localization to the bud tip. Given that so few of its targets appear to be localized in this manner, it is likely that additional pathways regulate its activities in other contexts. I generated a construct that fuses the *FLO11* repetitive element to *URA3,* similar to the GFP reporter I used to test the effect of Khd1 binding to the *FLO11* repetitive element. The ability of cells with the *URA3* construct to grow under selection and counterselection against *URA3* expression is altered by changes in Khd1 expression. A variety of genetic selections could identify pathways that regulate the activity of Khd1 binding to the *FLO11* repetitive element, and possibly other targets as well. This would expand understanding of the mechanisms that control this important regulator.

Finally, it would be interesting to see how these findings translate to other systems. Many fungal pathogens undergo dimorphic switches inside their hosts similar to the transition to pseudohyphal growth regulated by Khd1 (Gow *et al.* 2002). The pathogenic fungus *Candida albicans* can adopt two filamentous growth forms, and mutants that cannot filament are avirulent in a mouse model (Lo *et al.* 1997). This species contains a putative RBP with homology to Khd1. Given the role Khd1 plays in regulating

pseudohyphal growth in *S. cerevisiae,* it would be interesting to test the role of this putative ortholog in *C. albicans.* The *ALS* gene family in *C. albicans* encodes cell surface proteins similar to Flo11. The Khd1 homolog could potentially bind *ALS* mRNAs through their repetitive elements. If this homolg regulates filamentous growth in *C. albicans,* the protein would offer a potential drug target in treating the most frequently isolated fungal pathogen from humans (EDWARDS, 1990).

There are many potential avenues for research based on the studies presented in this thesis. My work has provided a strong foundation for investigating the role of Khd1 in regulating cellular activities, and points to future directions that could reveal interesting biological regulatory mechanisms relevant to all eukaryotic systems.

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