THI73 Mediates Regulation of *CLN3* G1 Cyclin Activity in *Saccharomyces cerevisiae*.

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THE G1 CYCLIN *CLN3*

The cell cycle is responsible for proper growth and division of proliferating cells. It consists of four phases: G1, S, G2, and M. The cell cycle has positive regulatory complexes that push the cell onward, and negative regulatory complexes that halt its progress. These must act in a balanced manner for the cell to commit to replicating its DNA and undergoing cytokinesis during M phase (Figure 1). The positive complexes are composed of specific cyclin proteins and an associated cyclin-dependent-kinase (CDK). Positive regulators of the cell cycle are counteracted by various checkpoints. Checkpoints mark specific timepoints when machinery within the cell must determine if it is prepared to engage in the cycle (1).

During G1, the cell monitors available nutrients and prepares to reach "Start," i.e. the "restriction point" in mammalian cells. After this point, the cell is fully committed to undergoing a replication cycle (2). One gene encoding a G1 cyclin responsible for this step is *CLN3* (1,3). In yeast, the genes necessary for other cyclins and proteins involved in S phase (DNA replication) are found downstream of an SCB element that is repressed by Whi5 (an analog to mammalian Rb) (3,4). Functional Cln3 binds and activates the CDK Cdc28, which phosphorylates the Whi5 repressor, releasing it from the SCB element, allowing proficient transcription of replication enzymes (1). If the Whi5 repressor, proteins of the SCB element, and DNA itself all have specific regulators, what is regulating Cln3 and the Cln3/Cdc28 complex?

In order for Cln3 to direct transcription of genes needed for the G1/S phase transition, it must localize to the nucleus (5). Nuclear localization of Cln3 requires the presence of its C-terminal bipartite nuclear localization sequence (NLS). The NLS of Cln3 supports movement to the nucleus via Ran dependent hydrolysis of GTP (Figure 2) (5). An analysis of 68 targeted genes showed twenty with defects in Cln3 localization when knocked out. Of these twenty genes, nine showed functionally relevant growth defects in the context of full length Cln3 activity. *THI73* was identified as the only gene to demonstrate specificity for a Cln3 NLS in comparison to the monopartite NLS from the SV40 Large T protein and a bipartite NLS from the nucleoplasmin (NP) of *Homo sapiens* (Figure 3,4). Thus, we postulate that *THI73* maintains a role in Cln3 nuclear import.

Figure 1: The cell cycle

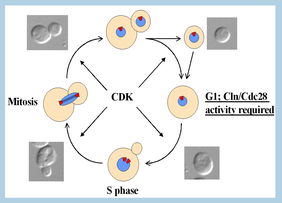
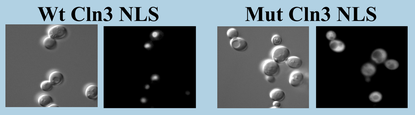


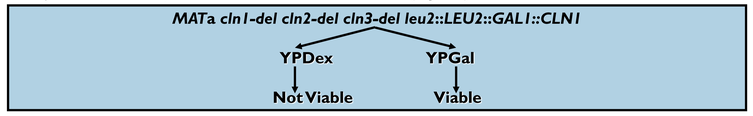
Figure 2: Cln3 NLS is sufficient to confer nuclear localization to GFP



Wild type Cln3 NLS directs GFP localization to the nucleus (left). Mutating residues of the bipartite Cln3 NLS disrupts nuclear localization, shown by diffuse GFP signal (right).

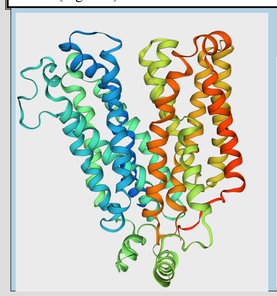
EXPERIMENTAL DESIGN

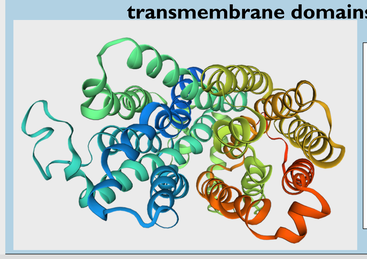
Assay for *CLN* function: The *MAT***a** *cln1-del cln2-del cln3-del leu2::LEU2::GAL1::CLN1* yeast strain (1254-14D) with or without specific gene deletions indicated in the figures were transformed with plasmids that allow expression of the Cln2 or Cln3 protein. Cln-dependent viability was assayed by spotting 10-fold serial dilutions on YPDex, YPGal, & minimal media. All media containing thiamine had a final concentration of 1μM thiamine. Transformants were also grown in liquid YPDex media to log phase, formaldehyde fixed, sonicated, and % unbudded cells were determined (Figure 6).



Cln2, a G1/S cyclin, is able to support progression through G1 in the absence of Cln3. However, Cln2 and Cln3 use distinct mechanisms to support viability (6). Growth with Cln2 was not hindered by *THI73* deletion at high temperature, thus serving as a control (Figure 5).

THI73 encodes a putative protein with 12 transmembrane domains

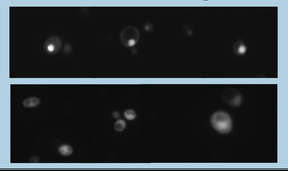




Ribbon model of Thi73 protein structure. The twelve transmembrane domains of the protein are displayed, suggesting membrane-associated function (13). Side view (left) and top view (right). Generated by Swiss Institute of Bioinformatics.

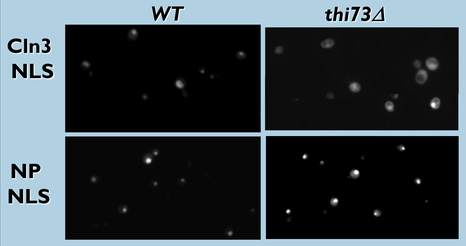
THI73

Figure 3: *THI73* is important for Cln3 NLS activity



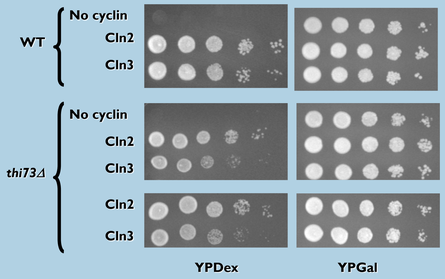
Cln3 NLS-dependent nuclear accumulation of GFP is impaired in *thi73* deletion strains

Figure 4: *THI73* function is specific to the bipartite Cln3 NLS



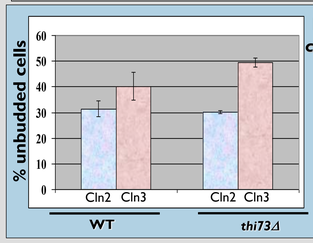
Cln3 NLS: KKRSTSSVDCDFNDSSNLKKTR
NP NLS: KRPAATKKAGQAKKKK

Figure 5: Cln-dependent Viability in the *cln- GAL::CLN1* Deletion Strains



Reduction of Cln3 activity is observed in the absence of *THI73* at 38°C. Ten-fold serial dilutions showing that Cln3 and Cln2 are each able to support viability to similar levels in the wildtype strain, while Cln3 shows reduced activity relative to Cln2 in the *thi73* deletion mutant.

Figure 6: % Unbudded Cells in *cln- GAL::CLN1* Deletion Strains



Reduction of Cln3 activity correlates to accumulation of unbudded cells in *thi73* deletion strains. Cln3 dependent viability results in an increase in % unbudded cells in log phase cultures when compared to Cln2 dependent viability when *thi73* is deleted.

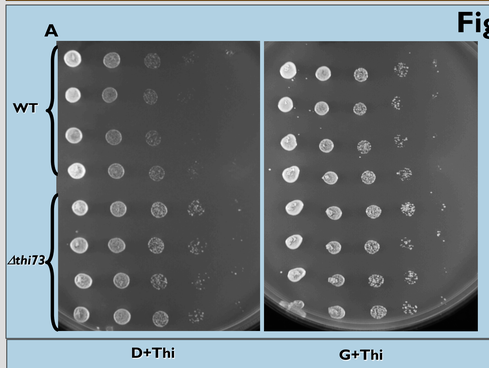
Saccharomyces cerevisiae provides a thorough model organism for identifying targets of thiamine synthesis and regulation. Yeast produce thiamine (Vitamin B_1) *de novo* from various *THI* genes. *THI* genes are regulated by three primary transcription factors (*THI2, THI3,* and *PDC2)* that contain individual and mutual responsibilities (7). Research has demonstrated that *THI* gene products, including *THI73* are modulated by the relative amount of thiamine (7,8). Homology characterization and sequence analysis of Thi73 suggests a role as a membrane protein of the anion:cation symporter subfamily within the major facilitator superfamily (MFS) (9). Given that Thi73 is induced in the absence of thiamine, it may facilitate the transport of some biosynthetic precursors, though its role in transport is unclear (9,10). Yeast are still viable after deletion of *THI73*, even in the absence of thiamine, suggesting that Thi73 does not perform transport of thiamine precursors (7). Moreover, this study indicates that the protein localizes to the ER, which is consistent with the physical interactions of Thi73 and other ER proteins (7) (Genemania). We are unable to confirm or refute, but our data suggest that Thi73 localizes to the periphery and membrane-bound organelles within the cell (Figure 8).

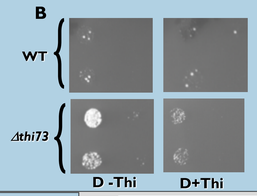
Our data demonstrate the Thi73 is important for Cln3 NLS activity (Figure 3) and has a reproducible effect in Cln3-dependent growth (Figure 5,6,7). Deletion of *THI73* results in a loss of function of the Cln3 NLS, thus we find that the absence of Thi73 contributes to defects in Cln3-dependent viability.

As one of the *THI*-associated genes, *THI73* expression is sensitive to relative levels of thiamine (7,8). Experiments in this lab have addressed disruptions to growth patterns when Cln3 is the only cyclin activated in the presence and absence of thiamine (Figure 7). Our data demonstrate some interplay between Cln3 and Thi73 in regulating growth patterns in yeast, but more research is necessary to establish the precise role of putative transporters that genetically interact with Cln3.

Minimal Media Nutrient Analysis

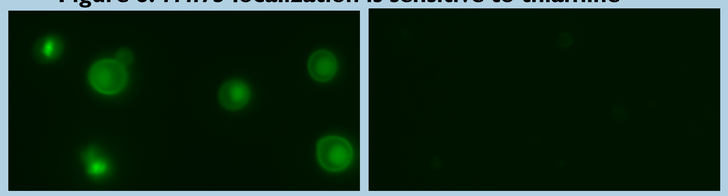
Figure 7: Thiamine modulates Cln-dependent Viability in the *cln- GAL::CLN1 thi73* Deletion Strains.





***THI73* deletion strains show increased Cln3 activity on minimal media compared to wild type.** Ten-fold serial dilutions of Cln3 (A,B) strains exhibit more growth on minimal media when *THI73* is deleted. (A) Cln3 gain of function is observed when *THI73* is deleted compared to WT. (B) The Cln3 gain of function is intensified in the absence of thiamine. Cln3 growth phenotypes display variable yet reproducible intensities. Control strains with no cyclin present did not produce colonies (data not shown).

Figure 8: *THI73* localization is sensitive to thiamine



***THI73* fused to GFP localizes to cell periphery and membrane-bound organelles.** GFP tagged *THI73* signal in the absence (left) and presence (right) of thiamine.

CONCLUSIONS

- **Thi73 is required for Cln3 NLS activity**
- **Thi73 is not required for NP NLS activity, suggesting specificity for the Cln3 NLS**
- **Cln3-dependent viability is disrupted in the absence of *THI73***
- ***THI73* deletion confers a gain of function or loss of function to Cln3 during stress**

Acknowledgements

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