The Effects of Stress on Expression of Pin3 in *Candida albicans*

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In host cell environments *C.albicans* often face numerous stress conditions, which prompt specific stress responses. Stress responses often involve the expression of heat shock proteins. Previous studies on a model organism, *Saccharomyces cerevisiae,* have shown that a protein often involved in stress responses is Pin3. In *S.cerevisiae*, Pin3 regulates protein aggregation and stabilizes prions. This study aimed to determine if Pin3 in *C.albicans* serves a similar role in stress responses. In this study *C.albicans* were placed under various stress and growth conditions, including oxidative stress, thermal stress, and induction of hyphal growth. After *C.albicans* were exposed to a given stressor, Pin3 levels were analyzed with a Western Blot. By comparing *C.albicans* strains with and without Pin3, the results could indicate if exposure to a stressor affected Pin3 levels and if Pin3 was involved in a stress response. The study did not find that Pin3 in *C.albicans* had the same role as in *S.cerevisiae*. Pin3 did not affect responses to oxidative stress and Pin3 levels did not change as a result of heat shock. However, experiments on hyphae induction found that growth in fetal calf serum media increased Pin3 levels. These findings may be related to the various signaling pathways that can be activated when *C.albicans* switch from a budding yeast morphology to a hyphal morphology (Feng et al., 1999) (Sato et al.,2020). This change in morphology is often attributed to *C.albicans'* virulence, thus these findings can be considered to expand on the role of Pin3 during filamentous growth.

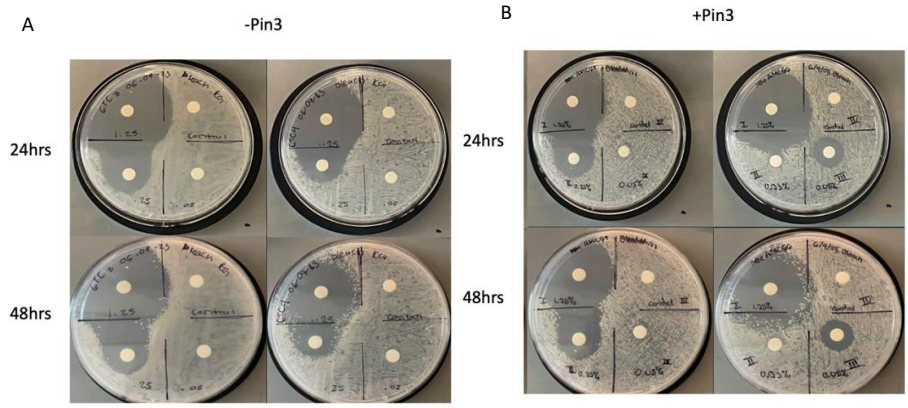
Candida albicans are a single celled, commensal, and pathogenic fungus, which can be found within humans. Although usually harmless, a change in its growth pattern can pose serious health risks for immuno-compromised individuals. Their virulence can be attributed to changes in their growth morphologies. *C.albicans* can change between a budding yeast form and a hyphae form. When in its hyphal morphology, the yeast forms elongated, filamentous structures. It is in the hyphal form that the yeast can cause damage to host cells, through the secretion of proteins that penetrate host cells.

In the presence of host cells, C. *albicans* can encounter numerous forms of stress including oxidative and thermal stress*. C.albican*s can respond to stress with heat shock proteins and stress response signaling pathways. The stress responses of a similar organism, *Saccharomyces cerevisiae,* have been heavily studied, thus becoming the model organism of this study. From research on *Saccharomyces cerevisiae*, it has been found that Pin3 is a protein which regulates protein aggregation and stabilizes heritable protein aggregates called, "prions" in the presence of stress (Chernova et al., 2011). When *Saccharomyces cerevisiae* is under heat stress conditions, Pin3 levels are high and protein aggregation increases. However, in the absence of Pin3, protein aggregation is sensitive to heat stress (Gibney et al., 2013). Although Pin3 is also found in *Candida albicans,* much less is known about it than in *Saccharomyces cerevisiae.* In a study focused on proteotoxic stress in *C.albicans*, it was found that *C.albicans* is resistant to protein aggregation during stress conditions (Leach et al., 2017*)*. However, it remains unknown if Pin3 is involved in *C.albicans* resistance to protein aggregation. The objective of this study was to analyze Pin3 levels after exposing *C.albicans* to various stress conditions. We aimed to determine if Pin3 levels increased in the presence of stress, thus potentially responsible for *C. albicans* resistance to protein aggregation.

When inside of a host, *C. albicans* may encounter oxidative stress in the presence of a macrophage. Thus, oxidative stress may affect Pin3 levels when *C.albicans* signal for a stress response. Bleach and hydrogen peroxide were used as the oxidative agents against *C. albicans* strains with and without Pin3. To measure the effects of these agents, a halo disk assay was used. Overnight cultures of strains with and without Pin3 were grown in YPD+ Uridine (Uri) media. The following day, the strains were spread onto Synthetic Complete plates. Serial dilutions of each agent were made from the stock solutions, resulting and water was utilized as a control for both agents. The disks were soaked in each

concentration and placed onto a quadrant of plates inoculated with either Wild Type (WT) or Pin3 deletion strains. The diameters of the zones of inhibition were measured after 24 and 48 hours. The zones of inhibition were expected to indicate if Pin3 affected the strains' resistance or sensitivity to oxidative stress.

Bleach Halo Assay



Hydrogen peroxide Halo Assay

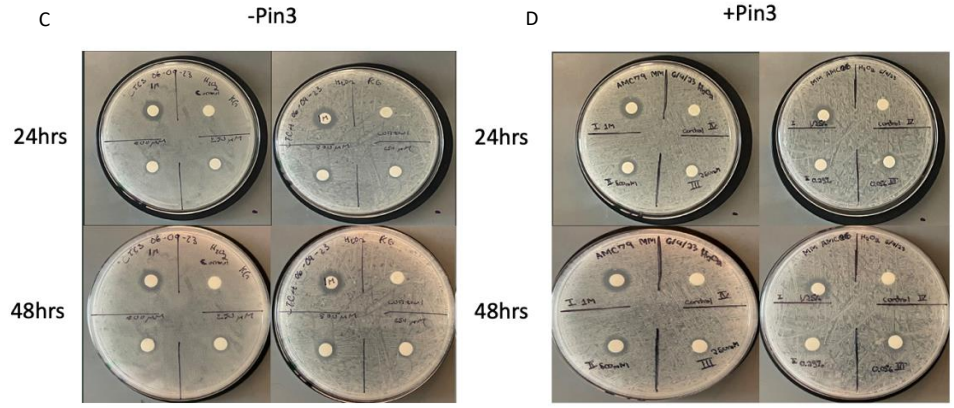


figure 1. **Halo Disk assay to measure sensitivity to Oxidative stress on *C.albicans* strains with and without Pin3**

(A) Disks with bleach were placed on quadrants of *C.albicans* without Pin3. Zones of inhibition are observable at a 25% and 125% concentration. **(B)** Disks with bleach were placed on quadrants of *C.albicans* with Pin3. Zones of inhibition are observable at a 25% and 125% concentration. **(C)** Disks with hydrogen peroxide were placed on quadrants of *C.albicans* without Pin3. A zone of inhibition was visible on all plates at 1000mM. **(D)** Disks with hydrogen peroxide were placed on quadrants of plates with Pin3. A zone of inhibition was visible on all plates at 1000mM.

As the concentrations of bleach and hydrogen peroxide increased, the radii for the zones of inhibition increased (fig 1A-1D). Overall *C.albicans* strains with and without Pin3, had the largest zones of inhibition at the highest concentrations. Although measurements were taken at 24 hours and 48 hours, there were little to no differences in the zones of inhibition for neither types of strains (fig. 1A &1B). For *C.albicans* strains without Pin3, the diameters for the zones of inhibition, when exposed to the highest concentration of bleach, ranged between 40-48mm (fig. 1A). Similarly, for *C.albicans* strains with Pin3 and exposed to bleach, the diameters ranged between 36-100mm (fig. 1B). For both types of strains, a zone of inhibition was only visible for the 25% and 125% concentrations of bleach. A zone of inhibition was not present for the lowest concentration of bleach, 5%. In the presence of hydrogen

peroxide, the results did not seem to differ between *C.albicans* strains with and without Pin3 (fig. 1C & 1D). A zone of inhibition was visible at the highest concentration, 1000mM. At the highest concentration of hydrogen peroxide, the diameters for strains without Pin3 ranged from 11.5-12mm(fig. 1C). Similarly, at the highest concentration of hydrogen peroxide for strains with Pin3, the diameters ranged from 11-13mm (fig. 1D). It can be stated that both types of *C.albicans* strains were sensitive to both oxidative stressors at high concentrations. However, there was an increased sensitivity to bleach as opposed to hydrogen peroxide. The zones of inhibition in the presence of bleach were almost twice as large than those of hydrogen peroxide (fig 1A & 1B). It does not appear that the presence of Pin3 affects sensitivity to a given oxidative stressor. The results between strains with and without Pin3 were similar across oxidative stressors. Thus these findings do not support that Pin3 in *C.albicans* affects resistance to oxidative stress.

To determine if heat shock affected levels of Pin3 in *C.albicans,* strains with and without Pin3 were placed in a 42ºC water bath. To prevent the yeast from acquiring thermotolerance, the cells were initially resuspended in pre-heated medium before being directly placed into the water bath. The strains with Pin3 were heat shocked for intervals of 0, 5, 10,20, and 30 minutes. The strains without Pin3 was utilized as a control group and only heat shocked for 0 and 15 minutes.

After exposing *C.albicans* to a stress condition such as heat shock or hyphae induction, the cells were lysed to visualize levels of Pin3 through a western blot. To release the proteins within the cells, they were lysed by bead beating. The non-soluble proteins were conserved by removing the supernatant. The non-soluble proteins were normalized for protein concentration and samples were loaded onto a protein gel. The proteins from the gel were transferred onto a membrane via a gel Trans Blot Turbo Transfer system. Both primary antibodies, such as Pin3, and secondary antibodies, anti-rabbit, would be added onto the membrane. Levels of Pin3 were the visualized utilizing a western blot imaging system, G:Box.

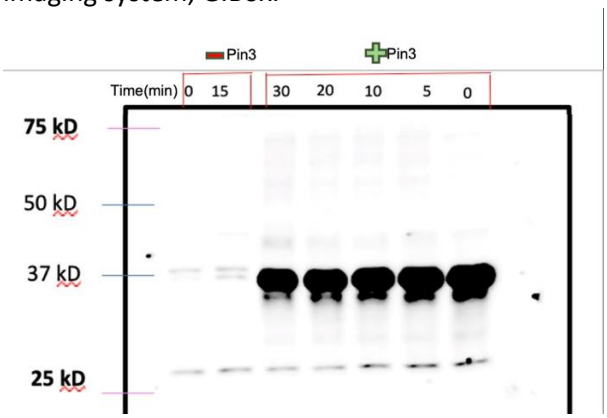


Figure 2. **Pin3 levels during intervals of Heat Shock**
Intervals of heat shock did not affect Pin3 levels, as levels remained the same in *C.albicans* strains with Pin3.

The immunoblot results demonstrated that Pin3 was not present in the *C.albicans* strain with a Pin3 deletion (fig. 1). At neither interval of 0 or 15 minutes of 42° heat shock exposure, was Pin3 present. These results were expected, as the strain without Pin3 was included as a negative control. In the *C.albicans* strains with Pin3, Pin3 was present at all intervals. However, the relative thickness of the bands remained the same across, thus suggesting that Pin3 levels were unchanged. From the lack of correlation between heat shock exposure and Pin3 levels, it can be interpreted that heat shock did not affect *C.albicans* expression of Pin3. It was originally hypothesized that in the presence of heat shock, Pin3 in *C.albicans* may respond similarly to Pin3 in S.ce*revisiae*. During a study that analyzed Pin3 levels in S.*cerevisiae* after heat shock, it was found that Pin3 levels seemed to increase during heat shock intervals ranging from 5 minutes to 30 minutes (Chernova, et al 2011). Since Pin3 levels remained the

same throughout C.albicans exposure to heat shock, the results of Pin3 in S.cerevisiae could not be applied to this study. It does not appear that an increase in Pin3 is involved in *C.albicans* response to thermal stress.

To measure the effects of a hyphae morphology on Pin3 levels, C.*albicans* were placed in two forms of hyphae inducing media. Overnight cultures of wildtype *C.albicans* were transferred to prewarmed hyphae inducing media. The media included, YPD+ Fetal Calf Serum, RPMI. YPD+Uri was utilized as a control. The cultures were incubated between 3 to 4 hours.

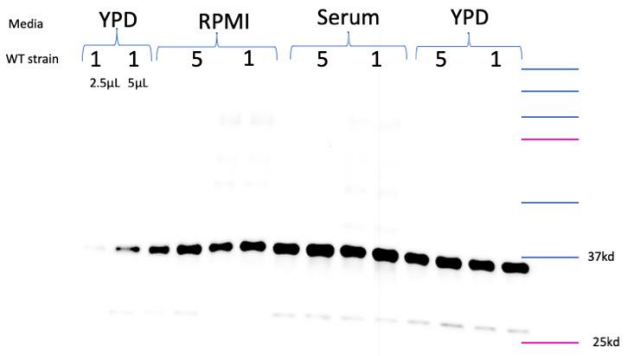


Figure 3. **Pin3 levels after inducing hyphal growth**
Pin3 levels appear the highest after being placed in fetal calf serum media. In comparison to growth in YOPD, Pin3 levels appeared to be less in the after growth in RPMI media.

Wild type (WT) strains 1 and 5 were grown in hyphae inducing media, including calf serum and RPMI (fig.2). An immunoblot with anti-Pin3, showed that across all media, there was not a visible difference between the Pin3 levels of WT strains. Both WT1 and WT5 had similar Pin3 levels for each respective media. The WT strains grown in YPD served as a control group, in which hyphal growth was not expected. In calf serum samples there seemed to be an increase in Pin3 levels as opposed to YPD samples. However, when comparing YPD and RPMI, there seemed to be a decrease in Pin3 levels. The diluted 5µl sample of YPD is visibly similar in size to the RPMI samples. Thus, the dilutions of YPD served to show that in RPMI, Pin3 was twofold down expressed in comparison to YPD. Between the two hyphae inducing media, Pin3 levels were greater with calf serum than with RPMI. Since Pin3 levels increased with calf serum but decreased with RPMI, it can be interpreted that upwards or downwards expression of Pin3 is dependent on a given growth media rather than on hyphae induction. When yeast cells are in the presence of calf serum or RPMI, specific signaling pathways are activated to induce the switch from yeast to hyphal growth. The different signaling pathways may be responsible for the differences in Pin3 levels found in the study (Feng et al., 1999) (Sato et al.,2020). Pin3 levels are baseline in YPD, which is when they are in a yeast-budding morphology. However, Pin3 levels increase during hyphae formation in calf serum. The original hypothesis that hyphae induction may be linked to an increase in Pin3 levels and its association with *C.albicans*, is not entirely supported by the findings of this study. Pin3 levels can vary depending on the media and signaling pathway that is induced.

Acknowledgments

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