The Role of Pds1 in Cell Size Control in Budding Yeast Cells

Quincy Okobi, Ricardo M. Leitao, and Douglas R. Kellogg

Department of Molecular, Cell and Developmental Biology, University of California,

1156 High Street, Santa Cruz, CA 95064, USA

Abstract

Cell size is controlled by cell size checkpoints, which ensure that key cell cycle transitions only occur when sufficient growth has been achieved. Previous studies have suggested that control of cell growth and size occurs primarily in G1 phase; however, our recent studies have shown that growth and size are controlled primarily during mitosis. We have also shown that a phosphatase called $PP2A^{Rts1}$ controls the amount of growth that occurs during mitosis, although the targets of $PP2A^{Rts1}$ are poorly understood. A key player in mitotic control is Pds1, which controls metaphase duration by inhibiting cleavage of proteins that hold chromosomes together. In response to DNA damage, phosphorylation of Pds1 results in a longer metaphase. We hypothesize that phosphorylation of Pds1 in response to nutrient changes controls the duration of growth during mitosis, and that $PP2A^{Rts1}$ controls Pds1 phosphorylation. To test this hypothesis, we will use Phos-tag™ gels to analyze phosphorylation of Pds1 cells growing in rich or poor nutrients, which show different durations of growth in mitosis. We will also generate a mutant version of Pds1 that cannot be phosphorylated and determine whether it causes defects in cell size control. Our hypothesis predicts that the pds1 phosphorylation site mutant will have a shortened metaphase and a reduced cell size.

Introduction

The significance of cell size

The coordination of cell proliferation and cell growth is essential for life. For example, the increase in the size of pancreatic beta cells during pregnancy is key for an appropriate response to the high insulin demand [1]. Normal cells control their size through various size checkpoints that coordinate growth with cell cycle progression. The specific mechanism behind cell growth and cell cycle progression are, however, not well understood. Misregulation of cell size checkpoints may have a detrimental effect on cell size which is known to be a universal feature of cancer cells. [2]. With a new-found insight in cell growth and its association with cancer and other aspects in human health, certain vulnerabilities may be discovered. Recent work in our lab connects cell growth with progression through mitosis in *Saccharomyces cerevisiae.*

Cell growth is regulated for cell cycle progression

If cells proliferated without growing, they would get progressively smaller and there would be no net increase in total cell mass. Therefore, in most proliferating cell populations cell growth must accompany cell division. Previous studies suggest that in order to achieve cell cycle progression, an adequate amount of cell growth is required. In Prescott's 1956 study, *Ameoba proteus* cells underwent a process known as amputation where one quarter to one half of cytoplasmic material was periodically extracted for several months during development. The limitation of cell size by amputation caused the cells to halt cell division and to undergo further growth [6]. Cells may have undergone stress for two months but did not develop a mechanism to counter the removal

cytoplasmic compartments. The removal of cytoplasmic compartments may have induced cellular repair machinery, ultimately arresting its cell life cycle. Another study has shown that cell size checkpoints delay key cell cycle transitions until a certain size threshold has been achieved [16]. In this study, the length of G1 phase was shown to be inversely proportional to the initial cell size. In addition, all cells reached approximately the same size at completion of G1, independently of their initial cell size. These studies have shown that completion of G1 and entry into the next phase of the cell cycle requires cells to reach a critical cell size.

Rich and poor nutrients alter cell growth via checkpoints

In single-celled organisms such as the budding yeast *Saccharomyces cerevisiae*, efficient cell growth and cell division are nutrient dependent. In a previous study on *S. cerevisiae,* reduced cell size was observed after growing in poor nutrients [15]. The production of small unbudded cells under poor nutrient conditions suggested that cell division in yeast could be completed without the normal amount of growth [15]. Cells altered their cell cycle checkpoints to compensate for the poor media environment. Nutrient value plays a significant role in the expression of cell size checkpoints and their very own role in cell maturation. Daughter cells are born at a significantly smaller size in poor nutrients [6]. Evidence suggests that the reduced size of daughter cells in poor nutrients is a simple consequence of reduced growth rate. The cell alters the duration of the cell cycle to compensate for the reduced growth rate seen in poor nutrients. These altered growth rates ultimately lead to variability in cell size and shape [7]. Cells may be placed in poor nutrients but will compensate by adjusting its cell cycle time course, ultimately leading to a smaller cell. Therefore, rich nutrients consequently lead to faster growth rates and larger cells.

A protein named $PP2A^{Rts1}$ is a phosphatase required to maintain the normal growth that cells undergo. rts1Δ cells do not adapt to nutrients, resulting in a large cell size independent of the. Nutrient conditions control cell size and entry into the cell cycle via $PP2A^{Rts1}$ [7]. $PP2A^{Rts1}$ is regulated for nutrient modulation of cell size and is directly affected by nutrient conditions. This nutrient modulation mechanism is necessary so the cell can evaluate and adjust the duration of cell cycle accordingly.

Swe1 partially controls the duration of mitosis

Swe1, the budding yeast homolog of human Wee1 kinase, regulates mitotic progression via inactivation of Cdk1[8,10]. Swe1 restrains Cdk1 activity by placing an inhibitory phosphate on its Tyr^{19} within its catalytic subunit [13]. De-phosphorylation of Cdk1 is required for the metaphase-anaphase transition [10]. Swe1 activity is controlled by the phosphatase $PP2A^{Rts1}$. $PP2A^{Rts1}$ leads to the inactivation of Swe1 which in turn allows CDK1 activity (Figure 2) [6]. Switching cells to poor carbon is likely to induce inactivation of $PP2A^{Rts1}$. In these circumstances, Swe1 remains active for longer periods of time inducing a mitotic delay. The persistence of Swe1 activity is what permits cells to compensate for the lower growth rate observed in poor nutrients [10]. During this mitotic delay, the cell has enough time to grow before it moves onto anaphase and ultimately cytokinesis [9].

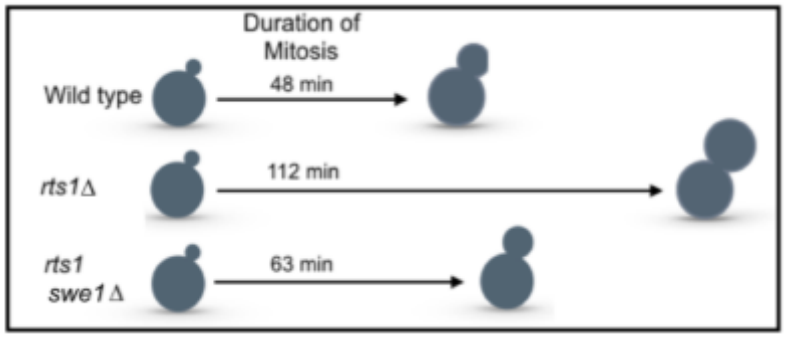

Figure 1: The duration of mitosis was measured in wildtype, rts1Δ, and rts1Δ/ swe1Δ cells. Wildtype cells had the shortest period of mitosis with 48 minutes. Rts1Δ cells had a significantly longer mitotic stage with 112 minutes. Lastly rts1Δ/ swe1Δ double mutant cells mitotic stage lasted for 63 minutes.

Despite the observation that *swe1*Δ cells have a shorter duration of mitosis, mitosis still lasts for a longer period of time than that of a wild type cells in rich nutrients [10]. This suggests that another mechanism is responsible for the prolonged duration of mitosis observed in poor nutrients (Figure 1).

Pds1 is a key candidate that could control the duration of mitosis in response to nutrients

The observation that swe1 mutant cells in poor media still have a mitosis longer than wild type cells in rich media suggests that an alternative pathway has influence on the duration of mitosis. Proteome-wide mass spectrometry data results show that in *rts1*Δ cells, a protein named Pds1 (securin) is hyper-phosphorylated [8]. Pds1 binds to and inhibits a protease named separase, which, when released, cleaves the cohesions that hold sister chromatids together during metaphase [11]. Entry into anaphase requires Pds1 degradation via the Anaphase Promoting Complex (APC). The APC is a large, multi-subunit ubiquitination machine essential for the proteolysis of several proteins at the onset of and during anaphase [14]. Data suggests that in the presence of DNA damage, Pds1 is hyper-phosphorylated, no longer making it a target for the

APC, halting the cell cycle to allow for DNA repair [10]. In addition to the described role of Swe1, we further hypothesize that nutrient dependent phosphorylation of Pds1 is responsible for the delay seen in mitosis when cells are grown in poor media. Progression through mitosis requires Pds1 de-phosphorylation via $PP2A^{Rts1}$. We will test how phosphorylation of Pds1 affects mitotic progression and cell size. Because a pds1Δ would fail to function both as a co-chaperone translocating separase into the nucleus and as an inhibitor of the protease separase, deleting Pds1 is not a viable approach [15]. Mutating the phosphorylation sites of Pds1 rather than creating a deletion would be a more viable option considering that a deletion may have unintended effects that may be detrimental to the cell.

Working model

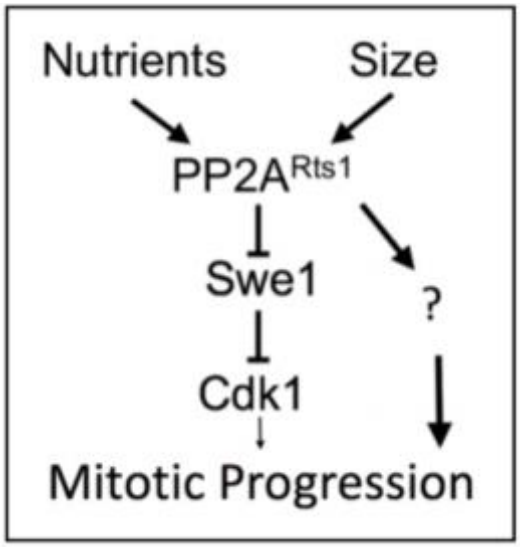

Figure 2: Current model of nutrient condition and its downstream effects leading to mitotic progression. We currently believe that the pathway above is responsible for the regulation of mitosis. $PP2A^{Rts1}$ is dependent on nutrient conditions and indirectly effects the phosphorylation status of Swe1 allowing CDK1 to promote mitotic progression. It is also believed that $PP2A^{Rts1}$ is responsible for the dephosphorylation of Pds1 allowing for the onset of anaphase.

Materials and Methods

Yeast Strains

Table 1

Strain	MAT	Genotype
DK186	a	bar1
DK1778	a	PDS1-HA
DK2678	a	PDS1-HA + rts1Δ
Clone 2	a	pds1-4A

Primers

Table 2

Primer	Sequence
Pds1-12	5’ -TTCCGGCATTAACTTTCCAC- 3’
Pds1-13	5’ -ACATACGGTAACGGCTCCTG- 3’

Measurement of WT Pds1 hyper-phosphorylation in rich & poor nutrients

Western Blotting

DK186, DK1778, DK2678 were inoculated in YPD rich media and YEP-G poor media overnight. Cells were lysed in a Multibeater-16 (BioSpec Products, Inc.) at 14000rpm for 2 min in the presence of 165 µl of sample buffer (65 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 50 mM NaF, 100 mM glycerolphosphate, 5% 2-mercaptoethanol, and bromophenol blue). PMSF was added to the sample buffer to a final concentration of 2 mM immediately before cell lysis.

After lysis, samples were centrifuged for 2 min at 13,000 rpm and placed in a boiling water bath for 7 min. After boiling, the samples were centrifuged for 2 min at 13,000 rpm and loaded on an SDS polyacrylamide gel.

SDS-PAGE was performed on a Phos-tag™ gel at a constant current of 20 mA & 165V for 3hr & 30mins. For PDS1 blots, electrophoresis was performed on 10% polyacrylamide gels until a 43-kD pre-stained marker ran to the bottom of the gel. Proteins were transferred onto nitrocellulose membranes for 7 min using Trans-turbo™ transfer buffer.

Blots were probed overnight at 4°C with affinity-purified mouse monoclonal antibodies raised against Pds1. All blots were probed with HRP-conjugated donkey anti-mouse secondary antibody (GE Healthcare, cat# NA934) for 75 min at room temperature. Secondary antibodies were detected via chemiluminescence using WesternBright Quantum substrate (Advansta). Blots were exposed to film or imaged using a ChemiDoc™ MP System (Bio-Rad).

After mutated strains were attained. They too were tested on a Phos-tag™ with the previous strains mentioned above. This was conducted to determine the measurement of mutant pds1-4A hyper-phosphorylation in rich & poor nutrients.

Measurement of PDS1 phosphorylation following transition to poor nutrients

Nutrients shift and Western Blot

Cell lines Dk1777 and DK2678 were grown in a 50ml conical tube overnight in YPD and adjusted to an OD of 0.5 to 1. Cells were initially collected in a 1.6ml micro centrifuge tube for

an initial time point of 0 minutes. 30mls of cells were added onto the filter gizmo. Cells were filtered onto nitrocellulose filter paper previously blocked with YPD. Cells were then washed 2 times using 20ml of YPge via fast pippet. A timer was set as soon as cells were primarily rinsed with YPge. Cells were then resuspended in 30ml of YPge. Cells were then placed in a rotating water bath set at 30ºC. 1.6 ml of cells were collected every 2.5 minutes following YPge washes. Wild type cells were collected at time points 0, 2.5, 5, 7.5, 10, 15, and 30 minutes. For the rts1Δ, a time point of 5 mins was not collected due to time constraints. However, time points 7.5, 10 and 15 were collected. Once cells were collected, they were centrifuged at 14,000 RPM for 15 seconds. The supernatant was discarded as 1 cap of acid washed beads were added to the micro centrifuge tube. All samples were frozen down in -80ºC

SDS-PAGE was performed on a Phos-tag™ gel at a constant current of 20 mA & 165V for 3hr & 30mins. For PDS1 blots, electrophoresis was performed on 10% polyacrylamide gels until a 43-kD pre-stained marker ran to the bottom of the gel. Proteins were transferred onto nitrocellulose membranes for 7 min using Trans-turbo™ transfer buffer.

Mutation of Pds1 Phospho-sites

PCR

The master mix consisted of 1X PCR Buffer, dNTP's, Pds1-12, Pds1-13, Phusion Enzyme and dH_2O. The positive control included WT Pds1-HA locus DK1778. The negative control consisted of the Pds1-URA- Pds1 DNA fragment. The PCR tubes were placed in a Thermocycler. The process of the Thermocycler went as follows: incubate in 98°C for 30

seconds, 29 cycles of 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds, finally 72°C for 10 minutes. Products were finally stored in 4°C. To evaluate the success of the PCR samples, each PCR product and a DNA ladder was run through gel electrophoresis at 110V for approximately an hour until the dye front was at the bottom of the gel. These samples were Pds1-12 & Pds1-13 products. The next two samples served as a positive (+) and negative control (-).

DNA precipitation

600µl of PCR product was pooled into a 1.6ml micro centrifuge tube. 600µl of phenol chloroform was added to the tube. After vortexing, the tube was centrifuged at 14,000 RPM for 5min. 500µl of the supernatant was collected. 500µl of chloroform was added. After vortexing, the tube was centrifuged at max speed for 5 min. 400µl of supernatant was collected. 800µl of EtoH was added to the micro centrifuge. The sample was cooled down at -20˚C for two hours. After centrifugation, the supernatant was discarded. The micro centrifuge tubes were placed in the 37˚C incubator to dry the pellet. The dry DNA pellet was re-suspended in 50µl of H2O.

Transformations

DK1778 cells were grown in 15ml conical tube overnight in YPD to an OD of 0.5 to 1. Cells were then pelleted (~1 minutes at 4400 rpm). Cells were then centrifuged for 1min at 4.4RPM in Eppendorf 5702. They were then washed 2 times in 15mls of LiOAc. Cells were re-suspended and pelleted in 100µl LiOAc Mix. 50µl of PCR product,15µl of 10 mg/ml Salmon Sperm DNA, 100µl cells in LiOAc mix and 0.7ml PEG Mix were all placed in a 1.6ml micro centrifuge tube and incubated at 30ºC for 60 minutes. Cells were heat shocked at 42°C in a water bath for 20

minutes. Cells were pelleted and the supernatant was discarded. then re-suspended in 125 µl of YPD. Cells were then plated on a selection media (-LEU) plate and left overnight for colony growth. Replica plates were grown onto FOA.

DNA extraction

Once cell colonies grew from the media plates, colonies were tested via gel electrophoresis. Once the samples ran down the gel, a fragment from each well was excised and placed into a 1.6ml micro centrifuge tube. Per each mg of agarose excised, 3x of Buffer QG was added to the tube. Tubes were then incubated in a 50ºC water bath for 10 minutes. Tubes were then vortexed for complete solubilization. The solution color was noted for contamination. Being any other color assured that the experiment was being conducted correctly. Shortly after, 10µl of 3M sodium acetate pH5 was added to the tube. 1µl of isopropanol per each mg of agarose was added to the tube. A mini-elute spin column was prepared and the samples to each column were added. These columns were left to sit for 1 minute then centrifuged for 1 minute at 14,000 rpm. The flow through was discarded and 500µl of buffer QG was added. The tube was centrifuged and the flow through was discarded once again. 750µl of Buffer PE was added and left to sit for 5 minutes. After centrifugation and discarding the flow through again, discard flow trough. The column was placed in a new tube and 30-50µl EB buffer was added onto the membrane and left to sit for 4 minutes. Samples were diluted in 30µl of EB buffer and were left in -80°C. Samples were then sent for sequencing at University of California Berkeley DNA Sequencing Facility for further results.

Results and Discussion

Clb2 presence marks mitotic duration

To determine the duration of mitosis in rich nutrients, we synchronized cells using alpha factor and grew them in a rich carbon source. We were able to determine the duration of mitosis by measuring the levels of the mitotic cyclin Clb2. Clb2 is involved in the regulation of the G2/M phase transition of the mitotic cell cycle. Clb2 also regulates mitotic spindle elongation and spindle pole body separation. Peak levels of Clb2 were found to be at time points 90 and 105 (Figure 3).

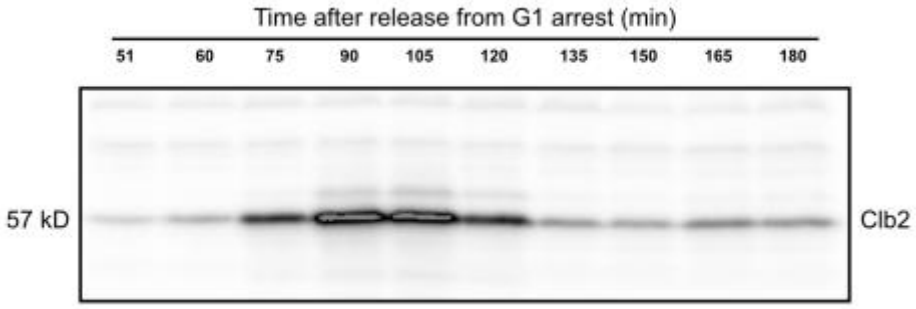

Figure 3: The Clb2 blot consisted of time points after a cell cycle arrest. Time points were collected approximately every 15mins after 51 mins.

Measurement of WT Pds1 hyper-phosphorylation in rich & poor nutrients

Because Pds1 was suspected of partially contributing to the mitotic delay observed in poor nutrients, Phos-tag™ gels were used to determine if Pds1 phosphorylation is affected in response to different nutrient sources. We cultured WT and rts1Δ cells in a rich carbon source (2% dextrose) and in a poor carbon source (2% glycerol + 2% ethanol) and analyzed the Pds1

phosphorylation levels. While considering that $PP2A^{Rts1}$ is responsible for the inactivation of Pds1, we expected to see a higher form of Pds1 phosphorylation in WT when grown in poor nutrients as when compared to cells in rich nutrients. This would corroborate with previous findings considering that $PP2A^{Rts1}$ activity is likely nutrient dependent. $PP2A^{Rts1}$ in poor nutrients would result in its inactivation and the prolonged activation of pds1. In rts1Δ cells we would expect to see the same trend of prolonged Pds1 hyperphosphorylation. $PP2A^{rts1}$ behaves similarly when its inhibited by nutrients or deleted (Figure 4).

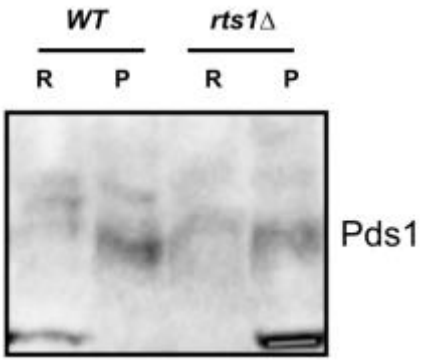

Figure 4: Samples were loaded in order from WT(Rich), WT(Poor), rts1Δ(Rich) and rts1Δ(Poor). R- Rich. P-Poor.

Pds1-HA hyperphosphorylation shifts in response to nutrient value

Phos-tag™ gels were used to determine if Pds1 changes in phosphorylation upon nutrient shift. Pds1 in rich media may exist in multiple phosphoforms given that it is in a log phase culture. Shifting *wild type* cells from a rich carbon source (YPD) to poor carbon source (YPge) caused further phosphorylation of Pds1 and the loss of some lower phosphorylated forms. Time points 0 minutes and 2.5 minutes consist of lower phosphoforms of pds1, while there is a shift in phosphorylation status at time points 5 minutes and on. Deletion of rts1 induced a more drastic

phosphorylation of Pds1 upon shift to poor nutrients, and further loss of the lower phosphoforms. The 4.5-minute mark of the rts1Δ shows an abrupt shift from the 0-minute mark.

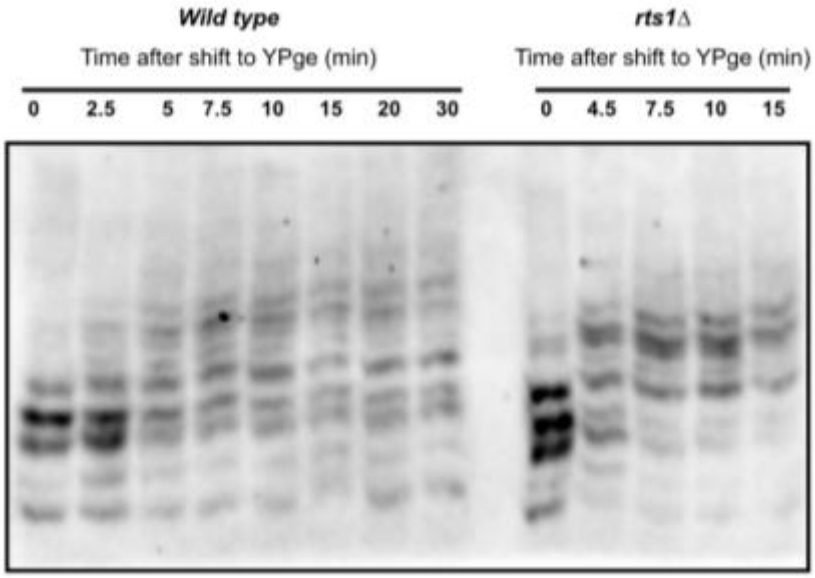

Figure 5: Time points for wildtype cells were collected approximately every 2.5 -10 mins after being shifted to poor nutrients. Time points for rts1Δ cells were collected every 4-5 mins after the nutrient shift.

Pds1-4A mutants fail to undergo phosphorylation in response to poor nutrients

As previously stated in our hypothesis, nutrient dependent phosphorylation of Pds1 is responsible for the delay seen in mitosis when cells are grown in poor media therefore progression through mitosis requires Pds1 de-phosphorylation via $PP2A^{Rts1}$. A mutant version of Pds1 that could not be phosphorylated was generated to test this hypothesis. The DNA samples sent for sequencing all tested positive for alanine incorporation. "TCCTCC" & "AGTTCC" where the phosphorylatable serine regions that were mutated into "GCAGCA" (Alanine) sequences. A PCR was performed and the product was sent into sequencing for confirmation. (Figure 6A).

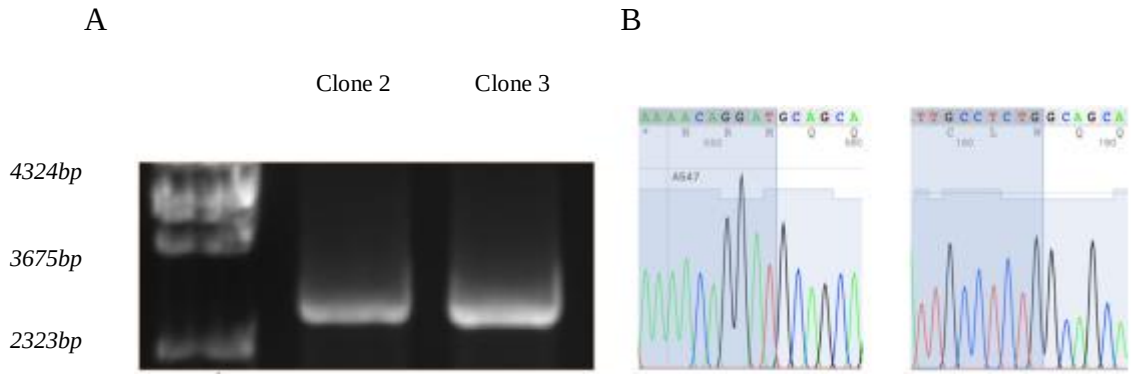

Figure 6A: The expected product was 2996bp. The figure shows banding patterns between 3675bp and 2323bp. Figure 6B shows sequencing results received. The GCAGCA codons were correctly incorporated in the right location.

Now that mutation of the Pds1 was finally achieved, we tested whether this mutant results in changed phosphorylation of Pds1. To do so we compared the phosphorylation of Pds1 in *wild type* cells against Pds1-4A mutants in rich and poor carbon. In addition, we also compared the phosphorylation of Pds1-4A to that of an rts1Δ mutants which we have shown to result in Pds1 hyperphosphorylation in both nutrient conditions. We observed slightly higher levels of phospohorylation in rts1mut cells compared to *wild type*. We believe that the increase in phosphorylation should have been higher, but this can be explained bearing in mind many of the cells were in log phase. Non-synchronous log phase grown yeast cells pass continuously through the cell cycle, and a log phase culture will have cells in all phases of the cell cycle. Because Rts1 is a key player controlling the duration of G1 and rts1mut cells have a prolonged G1, it is possible that in a log phase culture a greater percentage of rts1mut cells are in G1, a time when Pds1 is not active. Lower phosphorylation forms in the pds1-4A mutant compared to wild type strain support the hypothesis that pds1-4A is an unphosphorylatable protein. (Figure 7).

In contrast when grown in poor nutrients, wildtype and rts1Δ cells have a very high phosphorylation form. In poor nutrients, $PP2A^{Rts1}$ is rendered inactive allowing higher forms of

Pds1 phosphorylation to persist. The pds1-4A mutant in poor nutrients behaved the same as in rich nutrients, the two failed to become phosphorylated and reverted the trend of the previous two samples (Figure 7).

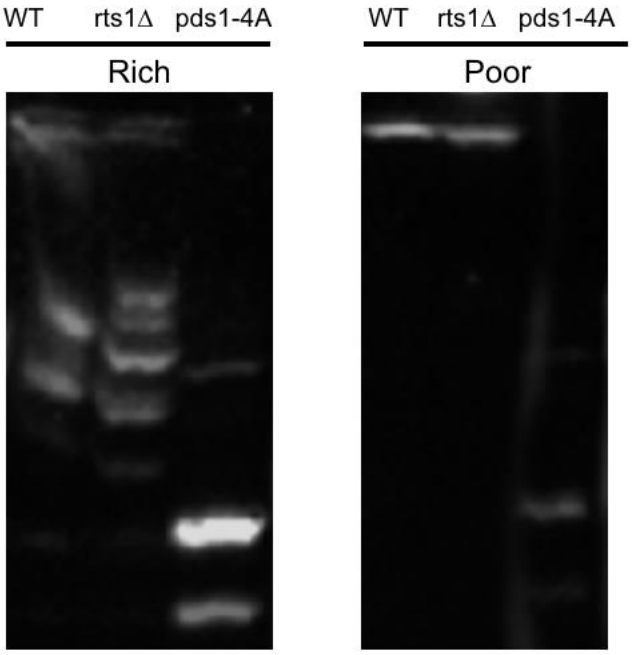

Figure 6: Pds1-4A mutants fail to undergo nutrient dependent phosphorylation changes. 15µl of each sample was loaded. The loading order went as follows; Dk186, WT, rts1Δ and pds1-4A mutant.

After observing pds1-4A mutant phosphorylation trends, it would be ideal to compare wildtype cells with pds1-4A/swe1Δ or pds1-4A/rts1Δ double mutants. Observing the phosphorylation status of these mutants will give more insight into the regulation of this pathway. To get a more direct answer on how cell size is being altered, future experiments involving coulter counter and fluorescence microscopy may be of interest.

Acknowledgments

This study was supported by the national Institutes of Health (NIH) grants to Douglass Kellogg. Ricardo Leitao R.L. is a scholar supported by a fellowship from the “Fundação para a Ciência e a Tecnologia” (FCT), with funds from “Programa Operacional Potencial Humano/ Fundo Social Europeu” (POPH/FSE), under the fellowship SFRH/BD/75004/2010. We thank the Kellogg Lab members for their helpful suggestions.

Bibliography

1. Alberts, Bruce, et al. "Molecular Biology of the Cell (Garland Science, New York, 2014)."
2. Artiles, Karen, et al. "The Rts1 regulatory subunit of protein phosphatase 2A is required for control of G1 cyclin transcription and nutrient modulation of cell size." *PLoS Genet* 5.11 (2009): e1000727.
3. Cameron, I. L., and D. M. Prescott. "Relations between cell growth and cell division: V. Cell and macronuclear volumes of Tetrahymena pyriformis HSM during the cell life cycle." *Experimental cell research* 23.2 (1961): 354-360.
4. Dhawan, Sangeeta, Senta Georgia, and Anil Bhushan. "Formation and regeneration of the endocrine pancreas." *Current opinion in cell biology* 19.6 (2007): 634-645.
5. Johnston, G. C., J. R. Pringle, and L. H. Hartwell. "Coordination of growth with cell division in the yeast Saccharomyces cerevisiae." *Experimental cell research* 105.1 (1977): 79-98.
6. Leitao, Ricardo Mendes, Annie Pham, and Douglas Kellogg. "The duration of mitosis and daughter cell size are modulated by nutrients in budding yeast." bioRxiv (2016): 080648.
7. Lianga, Noel, et al. "A Wee1 checkpoint inhibits anaphase onset." *J Cell Biol*201.6 (2013): 843-862.
8. Moinfar, Farid. *Essentials of diagnostic breast pathology: a practical approach*. Springer Science & Business Media, 2007.

9. Peters, Jan-Michael. "The anaphase promoting complex/cyclosome: a machine designed to destroy." *Nature reviews Molecular cell biology* 7.9 (2006): 644-656.
10. Raspelli, Erica, et al. "Budding yeast Swe1 is involved in the control of mitotic spindle elongation and is regulated by Cdc14 phosphatase during mitosis." *Journal of Biological Chemistry* 290.1 (2015): 1-12.
11. Sudakin, Valery, et al. "The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis." *Molecular biology of the cell* 6.2 (1995): 185-197.
12. Wang, Hong, et al. "Pds1 phosphorylation in response to DNA damage is essential for its DNA damage checkpoint function." *Genes & development*15.11 (2001): 1361-1372.
13. Zapata, Jessica, et al. "PP2ARts1 is a master regulator of pathways that control cell size." *J Cell Biol* 204.3 (2014): 359-376.