Title: Optogenetic-mediated cellular arrest and characterization of stress due to blue light and cryptochromes CRY2 and CIB1 in *S. cerevisiae*.

Goal: To create a blue light inducible construct to promote cell arrest in *S. cervisiae*, quantitatively assess the response of yeast to blue light and the cryptochromes CRY2 and CIB1, and create a transcriptional switch to minimize baseline noise from the light-inducible transcription factor.

Background and significance: *S. cerevisiae* cell cycle is well characterized and Far1, an inhibtor which interferes Cdc28, a cyclin which coordinates the cell cycle, has been shown to be an essential gene for cell cycle arrest (1). However, the current set of tools to induce cell cycle arrest and synchronize cells for study are limited, expensive, and non-gratuitous in the best case inducing the full mating pathway and leading to cell deformity. Far1 mutant Far1-22, which lacks a phosphorylation site, has been shown to cause cell arrest in not only **a** and α cells but also in **a**/α diploids (2). I propose the use of an optogenetic transcription factor to quickly and efficiently induce cell cycle arrest by promoting Far1-22 in order to synchronize cells and study the cell cycle.

Arabidopsis cryptochromes CRY2 and CIB1 (3), blue light inducible proteins, have been modified in *S. cerevisiae* to create a light inducible transcription factor consisting of two subunits, with CRY2 paired with a zinc finger binding domain and CIB1 to an activating domain. This approach permits sensitive, fast, and nearly gratuitous transcriptional regulation of single genes in *S. cerevisiae* by replacing their corresponding DNA binding domain with the zinc finger domain. In order to provide a more robust basis for this tool, I also propose to analyze the effects of blue light and the toxicity of the cryptochromes to *S. cerevisiae* and address the issue of noisy baseline transcription of the construct.

Aim 1: *Construction of a system to optogenetically promote cell arrest in yeast.* I will construct a plasmid with a method to efficiently arrest *S. cerevisiae* cells in the G1 phase by induction with blue light. A DNA binding domain corresponding to the binding motif of the CRY2/CIB1 complex will be placed in front of Far1-22 in a plasmid additionally containing the CRY2 subunit of the complex by homologous recombination in yeast (construct from Megan McClean). A plasmid with wild-type Far1p will be constructed to control for plasmid toxicity. After confirmation of plasmid sequence, transformed yeast will be exposed to a range of fluence rates of blue light (from 42 to 120 20 mmol m^2 s^1) and cells will be visualized by microscopy to confirm cellular arrest. The sensitivity of growth to the level of Far1-22p will be assessed quantitatively by measuring OD_{600} of cells grown at various light intensities and qualitatively by the rate of colony formation on plates with minimal media (in glycerol). As the blue light induced transcription factor may exhibit leakage, the same set of assessments will be performed on cells transformed with Far1-22 under the GAL promoter in minimal media with 2% glucose and a range of glucose from 0% to 2% at 0.5% intervals (plasmid from D. Botstein). Far1-22p has a longer lifetime than Far1p ($\sim$ 120 rather than $\sim$ 30 minutes). Due to the stability of the protein and depending on the growth of the construct, I may have to reduce the baseline level of expression of the gene from the CRY2 promoter (as addressed in Aim 2). If due to stability of Far1-22p it is necessary to reduce the level of Far1-22p, it has been shown to be possible to degrade Far1-22p and not impact the cellular arrest phenotype by expressing non-localized cdc4, which degrades Far1p in the wild-type cell (4).

Aim 2: *Creation of an optogenetic switch.* I hypothesize that background light will induce a low level of transcription of any gene of interest from the CRY2 mediated promoter.

A system characterized by low baseline transcription and a rapid increase in promoter mediated transcription to quickly raise the expression of a gene of interest, the creation of an optogenetic switch, would provide a useful method for constitutively turning on genes in the ce In particular, in the case of induction of genes which may interfere with cell growth, such as Far1-22, low baseline levels of transcription may interfere with normal function. To decrease the sensitivity of light induced transcription, I will place the DNA binding domain corresponding to the transcription factor construct in front of the CIB1 subunit to form a positive feedback mechanism to regulate the level of the transcription factor. Instead of constitutively expressing the T positive feedback coupled with transcriptional noise will create a bistable system where transcription will jump from low to high levels upon induction by light and will return to baseline after removing light stimulus. I will assess expression levels with both a GFP reporter and by measuring cell arrest relative to controls with ACT1 promoters. a suitable baseline level of transcription is not achieved, I will attempt to reduce the signal by expressing reporters from weaker DNA binding domains or prevent the accumulation of TF by rapidly degrading the CIB1 subunit by tagging it with a N-degron-tag and inducing the TEV protease (5).

Aim 3: *Characterization of yeast response to CRY2 and CIB1 induction under blue light.* To assess the impacts of using blue light to express genes with CRY2/CIB1 I will investigate the effect of blue light on yeast. Growth rate and gene expression will be measured at a range of fluence rates of blue light (from 42 to 120 20 mmol m^2 s^1) in *S. cerevisiae* cells transformed with the plasmids containing the constructs under the ACT1 promoter. I will measure growth using OD_{600} readings and perform microarrays for the fluence rates with measured growth rate defects of more than 2%, using uninduced samples as control. To analyze microarray da I will cluster genes by expression and search for affected pathways and potential binding sites to the CRY2/CIB1 construct. If growth defects are not correlated with gene expression, I will measure growth with CRY2 and CIB1 expressed under weaker constitutive promoters to test for toxicity.

roader Impacts: Completion of my aims will not only lead to the creation of a simple and efficient method for cell synchronization, but my analysis of the optogenetic transcription factor system will inform use of this tool in future single gene studies, in particular for network analysis. Furthermore, the creation of a bistable switch-like system for using the transcription factor will also add to the possibilities for network study; both in quickly activating and deactivating single gene.

References:

(1) Chang, F., Herskowitz, I. (1992). Phosphorylation of FAR1 in response to alpha-factor: a possible requirement for cell-cycle arrest. Molecular biology of the cell, 3(4), 445.

(2) Henchoz, S., ... Peter, M. (1997). Phosphorylation-and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. Genes and development, 11(22), 3046-3060.

(3) Liu, H.,... Lin, C. (2008). Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in Arabidopsis. Science, 322(5907), 1535-1539.

(4) Blondel, M.,... Peter, M. (2000). Nuclear-specific degradation of Far1 is controlled by the localization of the F-box protein Cdc4. The EMBO journal, 19(22), 6085-6097.

(5) McIsaac,R.S.,... Botstein,D. (2011) Fast-acting and nearly gratuitous induction of gene expression and protein depletion in Saccharomyces cerevisiae. Mol. Biol. Cell., 22, 4447−4459

Rough Timeline for QCB 301

Week of 10/14

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	Plasmids Obtain GAL + Far1-22 from Botstein Lab **Plasmids** Obtain construct from McClean Lab. **Prep** Plan out and order primers. **Prep** If needed, order oligos for weaker binding DBDs.	**Rap1** RNA purification and send to sequencing.	**Controls** If I have them already, transform yeast with GAL + Far1-22 plasmid. Also with only the blue light construct plasmids. **Shift Schedule** If this is done today, do the GAL experiments next Tuesday.	**Today or next week.** PCR amplify fragments for *Far1-22* and *Far-WT* from Botstein plasmid. **Today or next week.** PCR amplify fragments for *DBDs* from McClean plasmid.	**By today** Have obtained plasmids Planned recombination Ordered primers/oligos.	

Week of 10/21

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	PCR If not done yet, do PCR. Also amplify Far1 WT **mutPCR** If I want to do this, do it today (recombination later or tomorrow). **Recombination** Put together Far1-22/WT + DBD strains.	**GAL gradient** Transform yeast with GAL plasmid (Botstein). **Light Control** Transform yeast with two blue light plasmids. **GAL media** Set up dosages of galactose and glucose for gradient work.	**Construction** Store some cells from recombination. Extract plasmid and transform *E. coli*. **Positive Feedback** Plan primers to recombine zinc finger promoter with ACT1 promoter in plasmid.	**Mini-prep** Extract plasmid and purify Send to sequencing. **GALp gradient** Store cultures for next week. **Light Control** Store cultures for next week.	**Mini-prep** Do today if a day behind. **By today** Recombined plasmid strains. Grow up yeast; transfer plasmid to bacteria. Send to sequencing?	

Week of 10/28

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Tests this week If we have weak DBDs for Far1-22, also include in tests.	**GAL gradient** Taking transformed cells, set up several tubes of liquid culture to measure OD_{600} over time. Come in earlier today ($\sim 12-1:00$). **GAL gradient** Meanwhile add portioned gal/glu to cells and measure speed of cell arrest by microscopy. **Protein Stability** Measure when	**GAL** Yesterday's experiments should spill over to here. **Otherwise** Go over sequencing results and pick out cultures. **Tomorrow** Plan out the strains and the timing for tomorrow based on GAL results.	**Test Construct Arrest** Testing Far1-22, Far1WT, and (maybe) Far1-22 + weak DBDs. Grow at several light intensities and measure OD_{600} over time intervals. Plate in parallel and leave over several hours to assess rate of colony formation at different lights. Visualize cells from liquid culture by microscopy at time points given by GAL work.	**Test Construct Recovery** Using results from yesterday, choose light intensities that cause cell arrest, stimulate arrest, and then cover in foil for ~ 120 min to degrade Far1-22. Plate some cells and also visualize by microscopy.	**Construct Recovery** Check plates; visualize cells on slide to check for synchronization. Check for residual effects by measuring OD_{600} against a control and an uninduced sample. **By today** Have done at least GAL experiments and have construct arrest. Construct recovery would be optimal.	

Week of 11/04

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Light Control This week and next. **Alternatives** Start this week. Take any day to finish other work.	**RNA-seq** RNA-seq at 0, 60, 120 minutes of light intensity (IF a growth defect has been observed).	**Positive Feedback** Create fragment with zinc finger promoter (PCR) OR *weak DBD*. Linearize plasmid extracted from *E. coli* (sequencing step). **Prep** Blue light control cultures for tomorrow.	**Positive Feedback** Recombine to construct positive feedback CRY2 or CIB2 with DBD fragment and plasmid in yeast. Do so in the plasmid that does NOT contain Far1-22. **Blue Light** Taking control cells, measure growth relative to no light and no CRY2/CIB1 controls using OD_{600}.	**Blue Light** Microarrays for controls and intensities (Not sure of procedure. Consult.) Should also overlap into next week?	**Positive Feedback** Extract plasmid and transform *E.coli* (or transform next week). Also transform yeast with the other half of the plasmid, containing Far1-22 (so that I can do experiments on toxicity of the cryptochromes). **By today** Prepared to test alternates. Half done with the blue light controls. Have finished the construct tests.	

Week of 11/11

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Light Control Finish this week. **Alternatives** Positive feedback AND weak DBDs. Also this week. Take any day to finish other work.	**Light Microarrays** Do today if not last Thursday. **Positive Feedback** Extract plasmid from bacteria, miniprep, and send to sequence.	**Positive Feedback** Not waiting on sequencing, test growth by measuring OD_{600}, as with earlier constructs. Plate as well to see colony formation at different intensities.	**Alternatives and Recovery** Test recovery in similar way to construct in the alternative strategies. Plate cells and visualize by microscopy.	**Alternatives and Recovery** Check plates. Check cultures. Visualize cells to check for synchronization. Check residual effects as well by measuring growth.	**By today** Finished blue light testing. Tested alternate constructs.	

All weeks after this: analysis and buffer time.

If able to, continue experiments with western blots and/or further characterization of blue light effect on yeast with different promoters to test for toxicity (such as the ones in alternative experiments).