A Possible Conserved Role for SR Protein Kinases in the Regulation of Cell Death

Thesis by

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Abstract:

Apoptosis, programmed cell death, is a process that mammalian cells frequently undergo to suppress tumors and regulate cell growth. It is unclear whether or not apoptosis is conserved across both multicellular and unicellular eukaryotes. In baker's yeast, *Saccharomyces cerevisiae*, specific stress conditions can cause nuclear DNA fragmentation and cytoplasmic release of reactive oxygen species (ROS), the molecular markers of apoptosis in mammalian cells [1,2]. However, it is unclear whether these markers are part of a fungal specific stress response pathway or indicate that the machinery controlling cell death is conserved. One complication to resolving this question has been that the probes used to quantify DNA fragmentation and ROS levels are usually used to stain fixed cells. This means cells are only observed at a fixed point in time and the fate of a cell following appearance of apoptotic markers cannot be tracked. We are unable to confirm yeast cell death without observing the cells after the markers of cell death appear because we do not know if the presence of ROS is indicative of a stress response or cell death pathway. To address this we developed a novel technique using live-cell confocal microscopy to quantify the levels of ROS in yeast grown in different stress conditions. Preliminary data show that once ROS appear in a cell it never buds again over a 10-hour time course. This correlation between expression of the mammalian apoptotic markers and loss of cell viability in yeast suggest that this is a cell death pathway. Here, we focus on a potentially conserved role for SR protein kinases in the regulation of apoptosis in yeast and mammals using this system. Previous work has demonstrated a role for the mammalian SRPK2 in apoptosis. Specifically, SRPK2 appears to be cleaved in a caspase dependent system;

the N-terminus then translocates to the nucleus where it may be involved in a nuclear apoptotic pathway [3]. Yeast encode for a single SR protein, Sky1 [4]. In response to cation toxicity, such as LiCl, we see induction of apoptosis markers in yeast. Interestingly, the deletion of SKY1 reduces the presence of the molecular markers of apoptosis compared to wild-type (WT) in both standard (1% and 2% respectively) and stress conditions (3% and 14% respectively) [5]. Consistent with this, cells that carry a sky1Δ grow better than WT under stress conditions; moreover, cells that have constructs that overexpress SKY1 grow much worse under stress conditions. Using our live-cell microscopy assay, we quantify and compare release of ROS in WT to sky1Δ constructs and WT to sky1-overexpression constructs. Furthermore, we are investigating whether the cleavage of Sky1 is conserved by using yeast with N- and C-terminal fluorescent tags on Sky1. Determining that these results are representative of an apoptotic cell death pathway in yeast opens the door to using this genetically facile model organism to further probe the molecular mechanics of apoptosis.

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Introduction: A Conserved Role for SR Protein Kinases in the Induction of Apoptosis

Cell death

Apoptosis is the process of regulated or induced cell death. It plays a key role in mammalian tumor suppression in addition to fetal development. Apoptosis is morphologically different from other forms of cell death such as necrosis [6]. Indicators include DNA condensation and fragmentation, release of cytochrome c from the mitochondria, externalization of phosphatidylserine, and membrane blebbing [2,7].

Cellular factors involved in apoptosis

Because apoptosis is a vital component of various processes such as cell turn over and development and functioning of the immune system, the process and markers have been extensively studied. Over induction of apoptosis may be a factor in neurodegenerative diseases and autoimmune disorders [8]. On the other hand when cells are unresponsive to apoptotic signaling they may grow out of control and develop into a tumor. Many proteins and enzymes, namely Apoptosis inducing factor (AIF), and various caspases have been identified; however, the molecular mechanisms of the apoptotic pathway have yet to be elucidated [8]. A proposed mechanism is shown in figure 1. Induction of apoptosis in this case is caused by extracellular signaling from surrounding tissue cells.

Some proteins with known regulatory roles in cell proliferation have also been shown to play a role in apoptosis; SRPK2, a protein in a family of Serine/ Arginine protein-specific kinases is a notable example. SRPKs phosphorylate SR domain-containing proteins involved in pre-mRNA splicing and mRNA transport (Figure 2) [3,9]. In an *in vitro* study

using mammalian neuronal cells Hong et al. 2011 show SPRK2 is cleaved by caspases in apoptotic cells. Caspases are a class of protease enzymes that specifically recognize and Asp residue at the substrate P1 site [10]. The Asp-139 and Asp-403 residues in SPRK2 are the putative cleavage sites with the prior as the principal position. The study also proposed that after cleavage the N-terminus of SRPK2 translocates to the nucleus and induces apoptotic cell death (Figure 3). Apoptosis was triggered most often in cells treated with fluorescently tagged N-terminal SRPK2 fragments. Other fragments showed no significant apoptosis levels. Conversely, Akt phosphorylation of SRPK2 promotes binding of a 14-3-3 protein which was demonstrated to inhibit apoptotic proteolytic cleavage of the SRPK2. 14-3-3 proteins are regulatory molecules conserved across all eukaryotes [3]. This *in vitro* analysis brings us another step closer to elucidating the mechanism of apoptotic cell death; however, it does not provide a timeline for the sequence of events proposed.

Conservation of a mechanism across all eukaryotes?

To fully investigate the molecular mechanism, a model organism should be chosen such that steps may be observed chronologically *in vivo*. *Saccharomyces cerevisiae* (baker's yeast) may provide a solution. The yeast form fungi are genetically facile single-celled eukaryotes that reproduce quickly and have many homologs to mammalian proteins. In addition they provide the unique advantage of genetic tractability via simultaneous gene manipulation. Furthermore, yeast side-step a key technical drawback of mammalian studies because we can rely on more than apoptotic markers and the assays that quantify them – we can directly observe the number of dead versus living cells rather

than solely relying on the apoptotic markers and the assays that detect them [7]. Though it is unclear whether a single-celled organism can undergo apoptosis, previous research has shown the presence of cell death markers in yeast (Figure 4) [1].

When treated with a cell growth inhibitor, yeast exhibit chromatin condensation and fragmentation and phosphatidylserine is exposed to the outer membrane [1]. These are two classic markers of apoptotic-like cell death. In addition, oxidative stress leads to the accumulation of reactive oxygen species (ROS) which is caused by the release of cytochrome c from the mitochondria, another key sign of apoptosis [11,12]. The conservation of these cell death phenotypes suggests apoptosis is conserved across all eukaryotes; however, it is unclear if the markers are a part of a fungal-specific stress response pathway. Because budding yeast grow in colonies it is plausible that stress conditions induce apoptotic-like death in older or less fit cells to promote colony fitness. With less competition for resources the colony carrying near-identical genetic lines has a better chance of survival [13].

Homologs

Two key mammalian apoptosis homologs have been identified in yeast: apoptosis inducing factor 1 (AIF1) and yeast caspases 1 (YCA1) [14,15]. AIF1 is a non-essential gene that effects mitochondrial cell death. It also translocates to the nucleus in response to apoptotic stimuli and is a putative reductase [16]. YCA1 is a Ca^{2+} dependent cysteine protease. The monomeric metacaspase may regulate stress induced apoptosis by cleaving substrates in response to stress. It also plays a role in

clearing insoluble protein aggregates during normal cell growth [14]. Low external concentrations of hydrogen peroxide can induce apoptosis-like death via the production and accumulation of ROS. AIF1 activity, mitochondrial Cytochrome c release, and ROS activate YCA1 leading to caspase-dependent cell death [17].

Yeast SR Proteins, their kinases, and a misconception

Because SR proteins were identified as regulators of alternative splicing, it was thought to be of little possibility that they were conserved in yeast which have less sophisticated splicing. Being particularly powerful genetic tools, a yeast SR protein would provide an excellent model for *in vivo* study of physical and genetic interaction [5]. Three candidate yeast SR proteins have been identified as Npl3, Gbp2, and Hrb1 which are involved in mRNA export rather than alternative splicing like their mammalian homologs. However, Npl3 plays a role in pre-mRNA splicing in yeast by promoting splicing recruitment of splicing factors to chromatin [18].

Npl3 shares several features with mammalian SR proteins. In addition to similar structures including two N-terminal RNA recognition motifs and a C-terminal RS domain, the RS domain is subject to phosphorylation. *In vitro* Npl3 can be phosphorylated by the mammalian SRPK1 and the yeast SR protein kinase, Sky1 [9,18]. The mammalian SRPKs and Sky1 have similar structures and are primarily located in the cytoplasm.

A Strange Result

Prior to studying apoptosis, the Whitworth research group focused on mRNA processing. An assay probing for genes involved the regulation of gene expression in response to environmental conditions provided a strange result. The yeast strain carrying a deletion of sky1 (Δsky1) (Table 1) has a healthier growth phenotype than wild-type (WT) cells when both are grown in the presence of stress agents. On plates lacking the stress agent, the Δsky1 yeast is aphenotypic. It seemed strange that the deletion of a regulatory gene could give such a result. This led the Whitworth Group to investigate a potential conserved role of Sky1 in the regulation of apoptosis [5].

Using DAPI to visualize nuclear DNA fragmentation in fixed cells, the percentage of cells in a colony with the presence of DNA fragmentation was quantified. The specific strains studied were WT, Δsky1, and Δnpl3. All strains were grown both in the presence of and in the absence of stress. Unsurprisingly, colonies of WT cells with the oxidative stress agent $CuSO_4$ had significantly higher percentages of cells staining positive for nuclear DNA fragmentation than WT colonies without the $CuSO_4$ (Figure 5) [5]. On the other hand, colonies of cells carrying the Sky1 deletion showed about a 2% increase in DNA fragmentation from non-stress to stress conditions. Colonies of Δnpl3 gave similar results to that of Δsky1 [5]. These data lead us to hypothesize that Sky1 plays a larger role than regulating a response to stress, rather it may be necessary for the induction of cell death via DNA fragmentation. In addition to quantifying nuclear DNA fragmentation, the release of ROS over time was studied. Consistent with the other data, the ROS

assay demonstrated that colonies of ∆sky1 yeast have smaller percentages of cells inducing the apoptotic marker than WT cells [5].

Interestingly, an inducible nuclear localization signal (NLS) tag on Sky1 is constitutively toxic to yeast. Even in the presence of endogenous cytoplasmic Sky1, growth is inhibited when the NLS-Sky1 is induced [19]. One explanation to this result is that nuclear Sky1 plays a role in the induction of apoptosis. This is consistent with the *in vitro* data that suggests the N-terminus of SRPK2, the mammalian homolog of Sky1, is translocated to the nucleus following apoptotic cleavage of the protein; rather than inhibiting cell growth the presence of an SR protein kinase in the nucleus could be kickstarting cell death machinery. It is possible that both the full-length protein and the N-terminus are sufficient for such regulation.

Conservation of Cell Death Machinery in Yeast?

Though the primary function of Sky1 and Npl3 is mRNA export (Figure 2), some data suggest the proteins may also play a role in programmed cell death. Given the proposed function of the mammalian homolog SRPK2, Sky1 is an attractive candidate for exploring the regulatory network between mRNA processing and apoptosis. Determining that the apoptosis-like markers in yeast indeed do proceed apoptotic-like death will open the door to uncovering the molecular mechanics of the induction of these markers. The power of yeast genetics will also allow us to characterize the specific machinery of Sky1 as well as demonstrate the conservation of apoptosis and the role of SR protein kinases across eukaryotes.

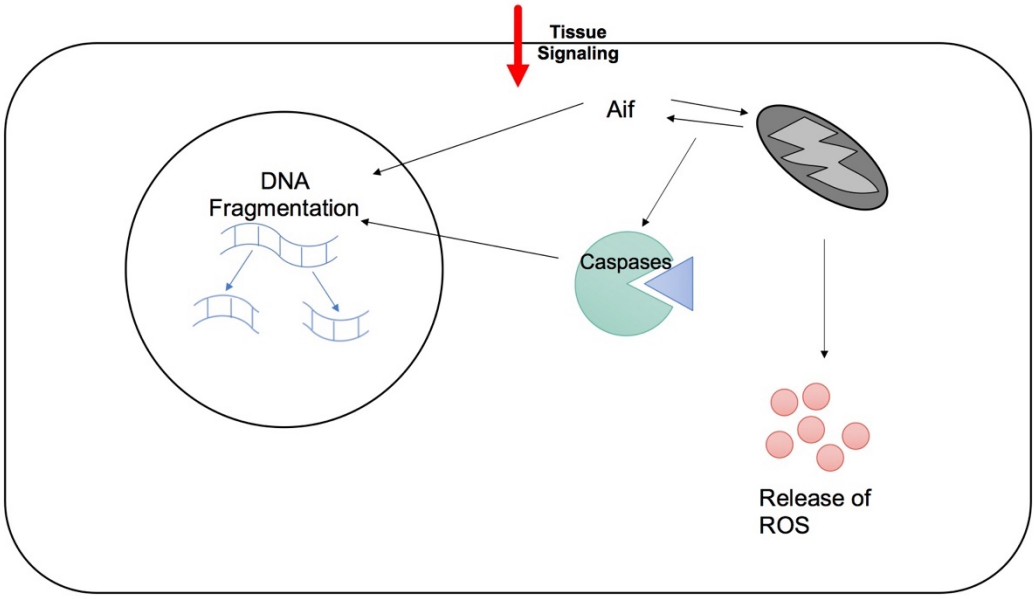


Figure 1: Phenotypic Markers of Mammalian Apoptosis

When surrounding tissue signals a cell for apoptosis, the cell prepares for death by releasing reactive oxygen species (ROS) and fragmenting its DNA to prevent recovery. In addition, membrane blebbing, the pinching off of the cellular membrane, encapsulates organelles and nuclear fragments [8].

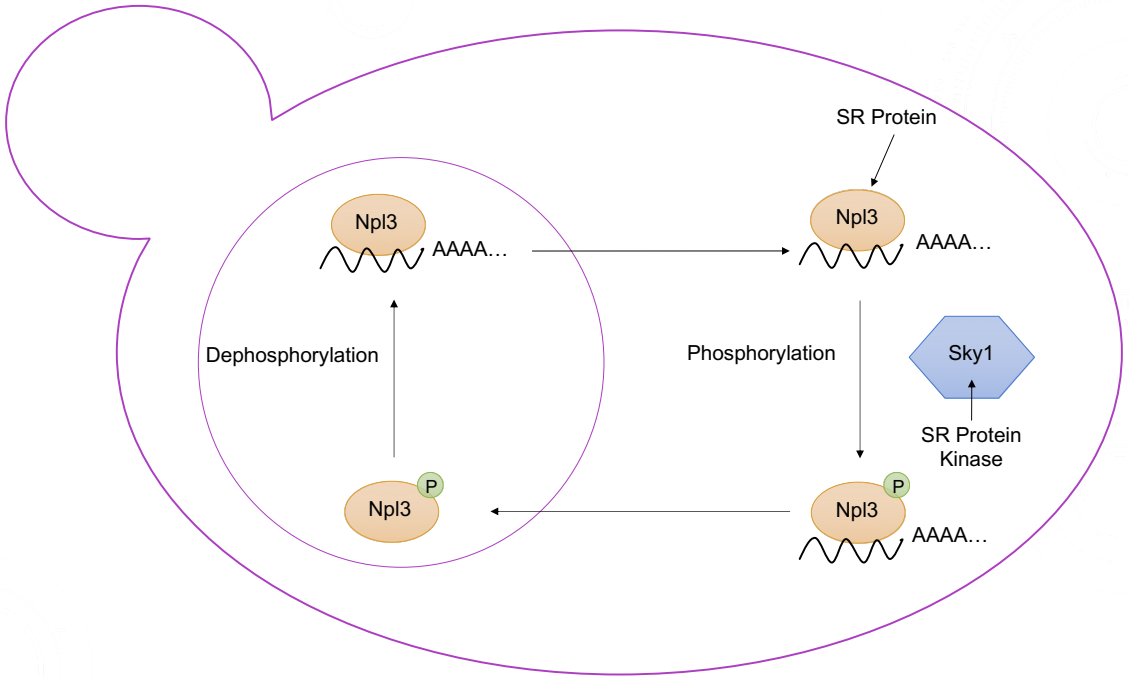


Figure 2: Known Role of Sky1 in Yeast

Sky1 is an SR protein kinase known to phosphorylate the mRNA export protein Npl3. Phosphorylation causes Npl3 to release mRNA and allows it to return to the nucleus.

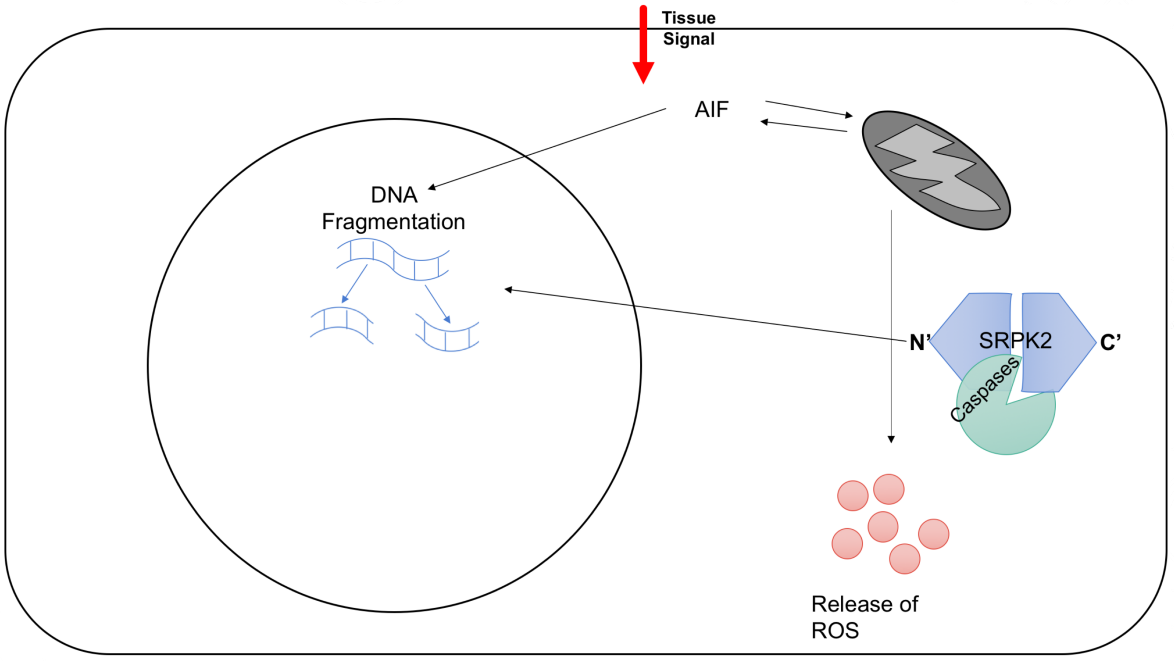


Figure 3: N-terminal Cleavage of SRPK2 in an Apoptotic Cell

The schematic shows caspases cleaving SRPK2 only in an apoptotic cell. We predict that Sky1, the mammalian homolog, will be cleaved by a metacaspase under stress conditions and the N-terminus translocates to the nucleus.

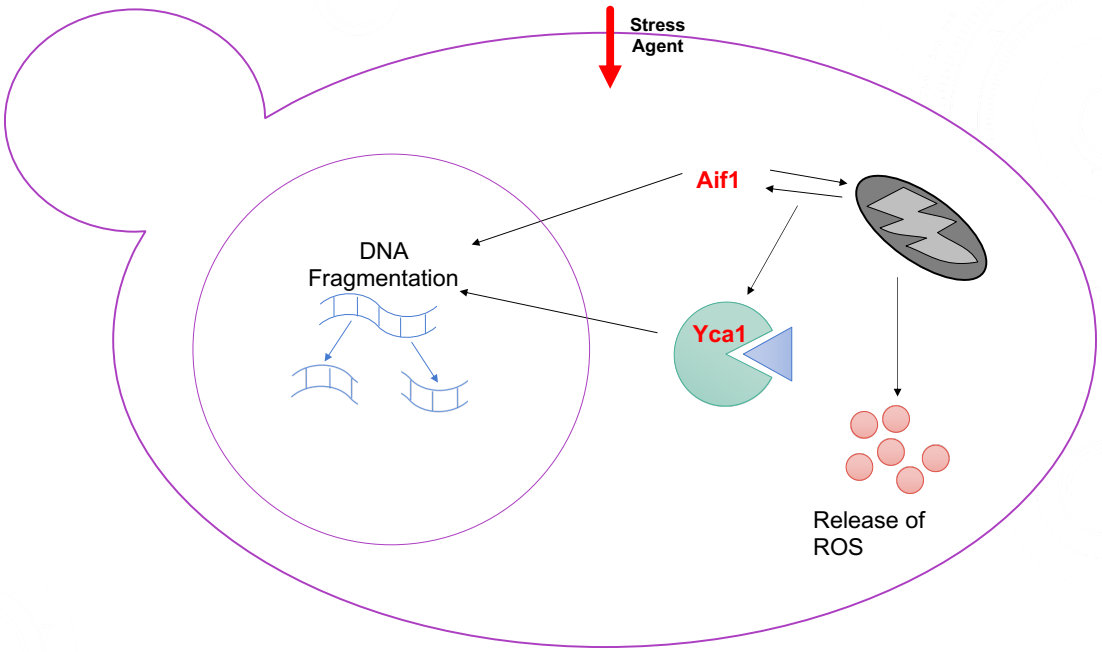


Figure 4: Proposed Apoptotic Pathway in Yeast

Because yeast are single-celled organisms, environmental stress may act as the cell death signal. In stress conditions yeast exhibit 2 phenotypic markers of apoptosis: release of ROS and Nuclear DNA fragmentation [1]. Yeast have a cell wall which may prevent membrane blebbing. Shown in red are the yeast proteins believed to be involved as they are homologs of the mammalian apoptotic proteins.

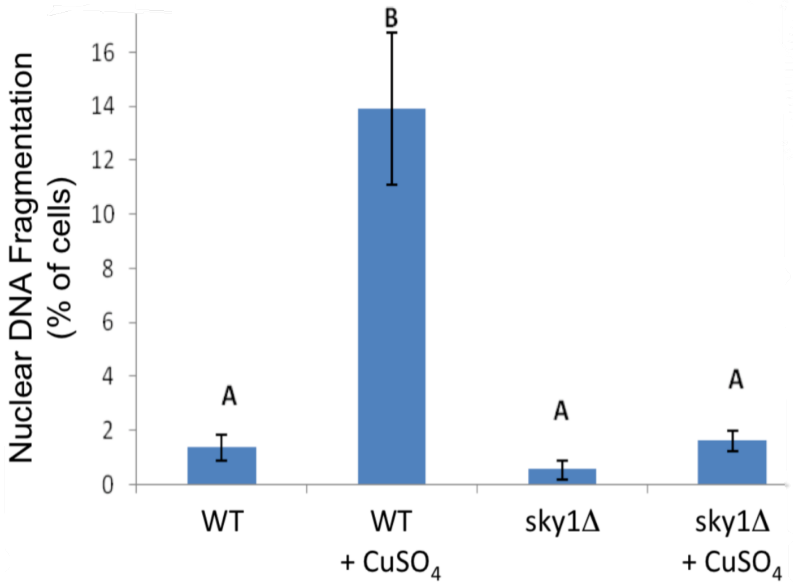


Figure 5: Previous Nuclear DNA Fragmentation Results

Nuclear DNA fragmentation of Δsky1 and wild-type cells in apoptotic stress conditions quantified by the percentage of cells in a colony with the presence of DNA fragmentation. These data were obtained by a Erica Schwotzer and published in her honors thesis [5].

Yeast Nomenclature	Description
SKY1	Wild-type version of gene
∆sky1	A mutant cell line in which the entire SKY1 gene has been deleted
Sky1	The Sky1 protein

Table 1: A Guide to Yeast Nomenclature

This table demonstrates how yeast genes, mutations, and proteins are differentiated from one another. If all letters are capitalized, the gene is WT. If all lower case with a ∆ in front then the gene has been deleted. The first letter capitalized indicates a protein.

Chapter 1: A Novel Technique for Quantifying Cell Death in Yeast

Scientific context:

While our lab's previous data indicate the deletion of SKY1 decreases the percent of cells that stain positive for nuclear DNA fragmentation and reactive oxygen species, these data are insufficient to indicate direct involvement in apoptosis. It is possible that Sky1 is involved in a stress response pathway and the markers appear as a result. All these data were obtained using fixed cell microscopy meaning cells were terminated before quantifying apparent makers [5]. Previous mammalian apoptosis studies have used fixed-cell microscopy to probe for apoptotic markers and provide no indication of how a cell responds after the appearance of the markers; therefore, it is not possible to say if ROS and nuclear DNA fragmentation precede cell death when using similar techniques with yeast. Recovery would be indicative of a stress response mechanism while cell death in conjunction with the cell death markers supports our hypothesis of a role for Sky1 in apoptosis. Therefore, identifying apoptosis using nuclear DNA fragmentation and release of ROS in single-celled organisms is controversial among yeast biochemists due to aberrations in fixed-cell microscopy.

Experimental Design:

To address the previous technical limitations of the fixed cell assays, we developed a novel live-cell microscopy technique using a scanning laser confocal microscope and low concentrations of Dihydroethidium (DHE) dye that stains red for ROS. I have optimized the assay conditions to achieve relatively high penetrance of ROS release while maintaining sub lethal doses. This assay allows us to track the cell cycle progression for individual cells across dozens of population doublings.

Finding the optimal concentration of the oxidative stress agents proved to be more difficult than expected. Growth assays before had been performed on enriched agar plates or stirring in growth media; however, in the live-cell assay the yeast cells were stuck to the bottom of the wells with the growth medium and stress agents above them (Figure 6). This paired with a small well size meant that stress agent concentrations were far lower than our expected values.

Using a 384-well plate with the confocal, we are able to run 9 strains and conditions combinations simultaneously (Figure 7). Due to small aberrations in the plates we are unable to prepare more than 9 wells at time; the plates are not perfectly level so the farther we travel from the starting point, the worse the focus on the confocal becomes. Limiting the number of simultaneous wells also gives each strain/ growth condition more data points during the 10-hr time course of the overall run. The typical run contains 3 strains of yeast, 1 of which is always WT, while the other two carry deletions for apoptotic regulating genes such as YCA1, AIF1, and SKY1.

Results and Discussion:

Before beginning work with the confocal microscopy assay, I performed a procedure commonly referred to as “frogging” to investigate the effects of SKY1 expression on growth. The method is used to observed growth phenotypes of many yeast strains through a series of dilutions; the yeast are grown on enriched agar plates with or without stress agents. We compared WT, ∆sky1, and strains that overexpress SKY1 to carrying

degrees using increasingly strong promotors on both standard and stress plates. On the standard growth plates all strains had a healthy growth phenotype while in the presence of stress only the Δsky1 yeast grew comparatively well. WT and sky1-overexpression were less fit in the presence of stress and the sky1-overexpression with the strongest promotor grew the least (Figure 8). This indicates that Sky1 plays a role in a cell death or stress response process. To determine if Sky1 played a role in apoptosis per-say we began the live-cell microscopy assay using the Δsky1 strain and strains carrying deletions for other apoptotic genes while probing for apoptotic markers.

With the optimized conditions for the live-cell microscopy assay the incubation chamber on the confocal microscope allowed us to grow cells for an extended period of time. This provides ROS and growth data across various time points during, on average, a 10-hour time course. Unsurprisingly, all strains in YNB, a standard growth media grew well and showed low evidence of apoptosis which is consistent with the growth assays on enriched agar plates. When oxidative stress such as hydrogen peroxide is added to the media WT cells show an increase in the number of cells undergoing apoptosis while strains carrying the aforementioned deletions show little to no spikes in apoptotic cells (Figure 9). The strains that we have yet to test with this assay, but have with growth phenotypes, are those that carry an overexpression of SKY1. We expect that with each degree of overexpression we will have increasing populations of apoptotic cells.

In addition to demonstrating that SKY1 follows similar trends to other cell death genes with regards to the presence of apoptotic markers, we are able to track cell cycle

progression. We observe the appearance of ROS when a yeast cell turns red and watch non-red, ie non-apoptotic, cells continue to grow around them. The cells that turn red do not divide again over a 10-hour time course and are therefore dead and have undergone apoptosis (Figure 10). In future experiments we will expand the assay to include yeast strains carrying an overexpression of SKY1.

Methods and Materials:

Frogging

Overnight incubations of yeast cultures were back diluted and grown until the culture reached an OD of 0.1. Each culture was then transferred to a sterile 96 well plate and diluted by half 5 times. The yeast were then spotted onto agar growth plates such that an equal volume of each dilution was placed in a decreasing order. The plates were grown at 30C and imaged every day for several days to compare the growth phenotypes of various strains.

Yeast Strains

All *Saccharomyces cerevisiae* used were in a BY4741 (S288Ca) background. The genotype is MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [14]. The deletion strains came from the non-essential deletion collection [20]. The sky1 overexpression strains were constructed in the Whitworth lab as describe in Erica Schwotzer's honors thesis [5].

Live-cell Microscopy assay

As before, overnight incubations of yeast cultures were back diluted and grown until the culture reached an OD of 0.1. A 384-well plate is prepared by washing wells with 95% ethanol and aspirating the liquid off. The wells are rinsed with sterile ddH_2O to remove any excess ethanol which would inhibit growth. A final wash with polylysine is performed to help stick the yeast to the bottom of the wells and prevent the cells from floating away during imaging. The overnight cultures are diluted 1:100 before loading into wells. The DHE is diluted in growth media to a final concentration of 2.8 μg/mL. Stress agents that may be added are hydrogen peroxide – final concentrations of 0.14mM or 0.16mM – or Copper (II) sulfate (2mM).

The incubation chamber on the scanning-laser confocal microscope is set to 30C and the humidity control to 75%. The microscope is set that each well is scanned approximately every 14 minutes; several depths are measured at each well to optimize to range of focus. All data are processed in Fiji (imageJ) and cells are counted by hand.

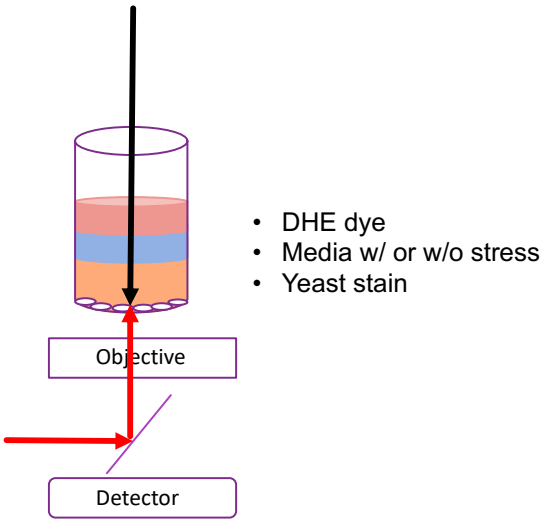
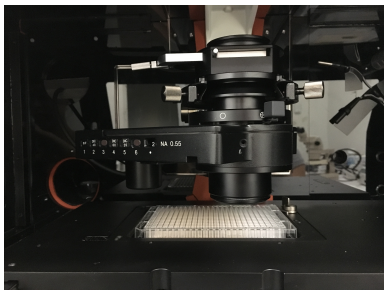
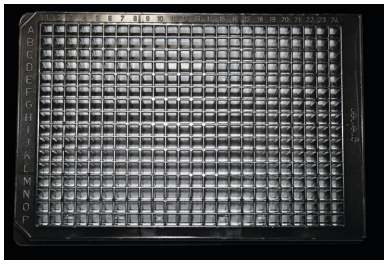


Figure 6: Schematic of Individual Well Set-Up for Confocal Microscopy

The confocal takes images via white light for stereo images and lasers for fluorescent dyes. In both cases light passes through the sample and objective and is reflected back to the detector. Each well is first prepared with polylysine to help the yeast stick to the bottom of the well. On top of the yeast sample we add growth media with or without a stress agent. The DHE Dye is added just before the confocal run begins to reduce photobleaching during well preparation.





- 30º C
- Humidity control
- 8-10 hours

Figure 7: Live-Cell Microscopy Set-Up

Using the Laser Scanning Confocal Microscope in the Washington and Lee University IQ Center we developed an assay that allows us to image growing cells over a 10-hour time course. We can monitor ROS presence in cells using low concentrations of DHE which stain red for the marker. The set up in the IQ center allows us to maintain a plate at 30 °C, the optimal growth temperature for yeast, as well as control humidity to prevent the wells containing yeast from drying out during the confocal runs.

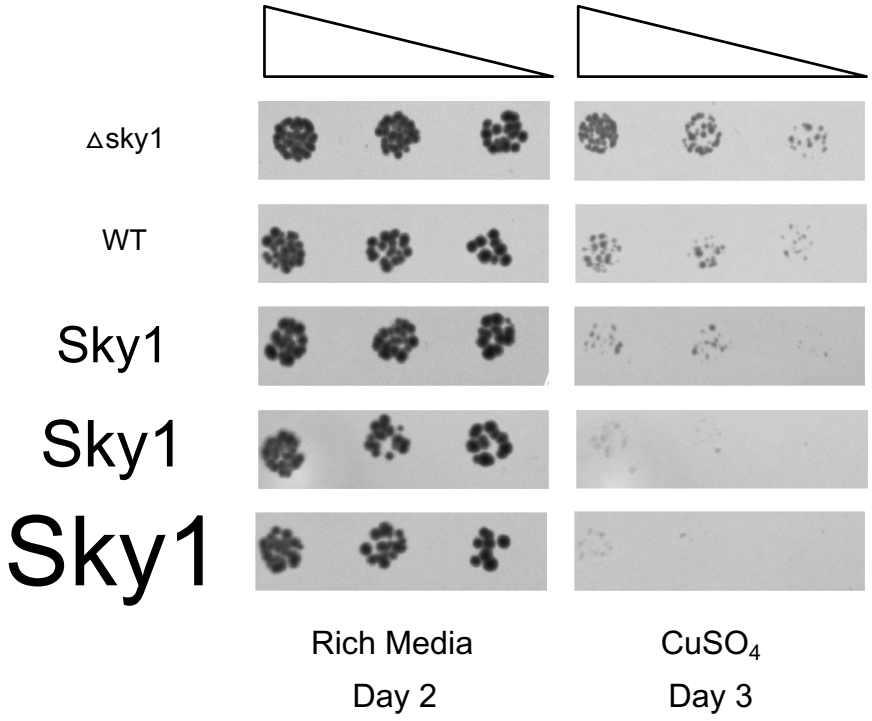


Figure 8: Growth Phenotypes of Sky1 Mutants Compared to WT

Cells carrying a deletion of Sky1 (Δsky1) have increased fitness compared to WT cells when grown in stress. The overexpression strains of Sky1 have decrease fitness compared to WT; the higher the overexpression the sicker the cells become. Text size is used to represent degree of overexpression.

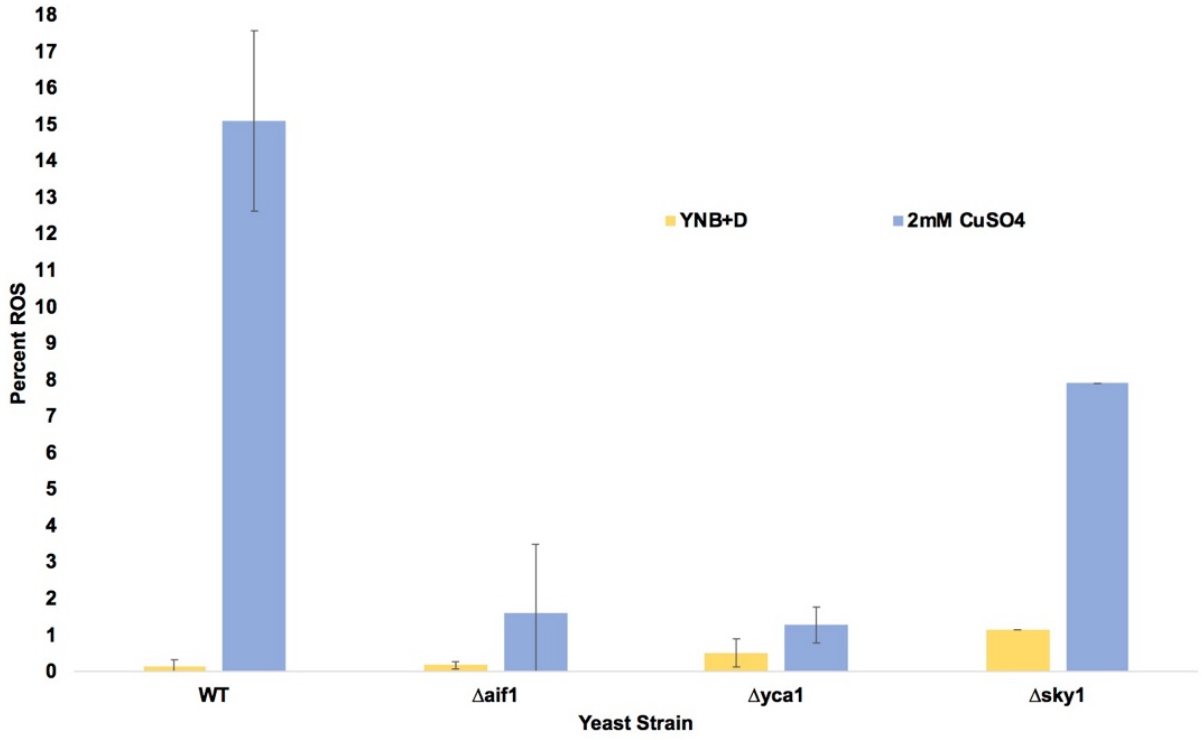


Figure 9: %ROS in Cells in Live-cell Microscopy Assay

The graph indicates the %ROS in a colony at the ending timepoint. These data are consistent with fixed cell microscopy results. Deletion strains of apoptotic related genes have increased fitness compared to WT in stress. Percentages are (number of cells that show ROS (i.e. red cells)/ total number of cells) *100. Cells that grew into view or floated out of view were excluded from final counts.

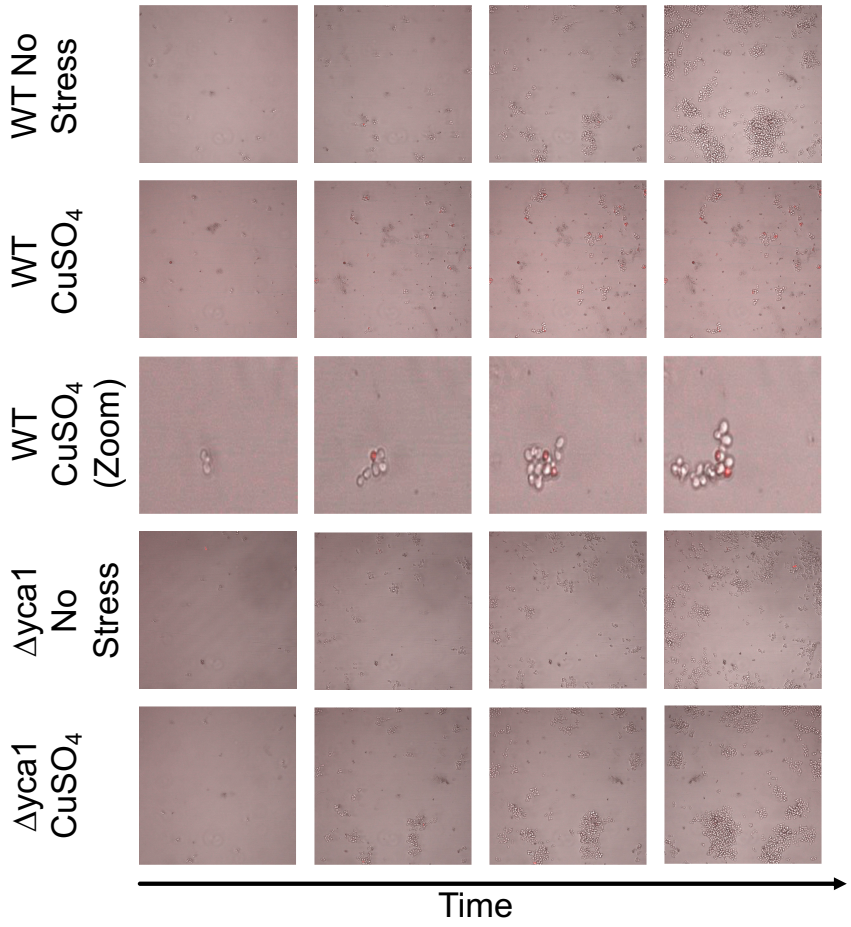


Figure 10:Live-Cell Confocal Microscopy Images

Images taken over a 10-hr. time course. Red indicates (+) DHE staining for ROS. A cell (+) for DHE does not divide again.

Chapter 2: Investigating the Role of Sky1 in Apoptosis

Scientific Context:

SRPK2, the mammalian homolog of the yeast SR protein kinase Sky1, has been shown *in vitro* to play a role in apoptosis via caspase mediated cleavage and nuclear translocation [3]. In addition, an NLS tag on Sky1 was shown to be constitutively toxic in yeast [19]. Both of these suggest Sky1 plays a nuclear role in the induction of apoptosis; however, the cleavage of the SR protein kinase is not something that has been specifically studied *in vivo*. Evidence of such an event in yeast may provide supporting evidence for the mammalian study and be sufficient to implicate Sky1 as a regulator of apoptosis in yeast. Controlling the induction of apoptosis also opens many avenues for investigating downstream factors allowing us to elucidate the mechanism of programmed cell death.

Experimental Design:

Because we have established a method for tracking cell cycle progression and apoptosis in real time through our live-cell microscopy assays, we already have a viable method for studying the cleavage and translocation of Sky1 in apoptotic yeast cells. The scanning laser confocal microscope allows us to simultaneously track several dyes and fluorescent proteins. To determine if the N-terminus of Sky1 translocates to the nucleus we can tag both the N' and C' termini with different fluorescent proteins that do not share an absorption spectrum with DHE to track the fluorophores' location in conjunction with the appearance of ROS.

There are several methods to add fluorescent labels to proteins. One is using the Gateway Cloning technology with Addgene cloning vectors that encode for a fluorescent protein on either the N' or C' terminus of an insertable gene [21,22]. The resulting plasmid was then transformed into yeast (Figure 11). To tag both termini, I inserted Sky1 into two plasmids one of which contained an N-terminal tag and the other a C-terminal tag and transformed both plasmids into yeast.

As new gene editing technology became available, I began investigating CRISPR-Cas9 as a tool to insert the fluorescent tags (Figure 12) [23]. The Wyrick group at Washington State University developed a vector to insert a CRISPR-Cas 9 system into Saccharomyces cerevisiae (Figure13) [24]. This method provided us a way to fluorescently label Sky1 and maintain a better control of its expression within cells.

Results and Discussion:

Using the Gateway Cloning system, I created a library of plasmids in *Escherichia coli* that are ampicillin resistant and contain Sky1 with either a C-terminal eGFP, eYFP, or HA tag or an N-terminal Cerulean tag (Table 2). The plasmids also encode for either Uracil or Leucine, two components our lab strain of yeast gets from its surrounding environment, so that the plasmids are positively selected for when Uracil or Leucine is withheld from the growth media. Because yeast grew on the selective plates, the plasmid transformations were successful and the yeast should express a fluorescently labeled Sky1 which was confirmed using a fluorescent microscope. The yeast were then stored in a long-term freezer stock at -80 °C for future use.

Upon retrieving the yeast from prolonged storage we observed very few cells were exhibiting fluorescence. All cells were propagated using selective pressure, so the plasmid should have still been intact. Because the plasmids had been inserted into WT yeast, the new strains carried an overexpression of SKY1 which leads to decreased fitness and may have been mildly toxic. To address this, the same protocols were repeated with the Δsky1 strain as opposed to WT. Using the Δsky1 strain helped the issue some, but not to the extent that most cells showed some fluorescence; we believe the strong promotor used in the plasmid exceeds WT SKY1 expression levels. One explanation for the cells surviving the selective pressure but somehow failing to express the fluorescently labeled protein is the portion of the plasmid that encodes for Uracil and/or Leucine is used to repair the deletion in the parent strain via homologous recombination. While unlikely, the event is more probable than a blank plasmid successfully transforming because the Gateway vectors contain a gene that is constitutively toxic, so only cells with plasmids that have the proper gene inserted in them can survive [22].

This set-back and its potential cause inspired me to investigate using a CRISPR-Cas9 system in yeast. By designing a gene construct that encodes for a fluorescent protein directly before SKY1 or directly after and dropping the stop codon with several dozen base pairs of similarity to WT SKY1 on either end, it is possible to use CRISPR to force the homologous recombination to insert the fluorescent protein without effecting endogenous expression of the gene [24]. Codon optimization for yeast significantly increases the relative intensity of an inserted fluorophore, so the gene construct was

optimized for the organism during the design process [25]. Amplifying and purifying the designed gene construct has proved to be more of a challenge than expected and this part of the project has not been completed to date. We believe a PCR inhibitor may have been included in the original construct which contributed to the difficulty. In addition, the primers for the construct either amplify a portion of the gene or dimerize, so gel excision is necessary. The plasmid containing the CRISPR elements has been prepared via restriction enzyme digests. The short guide RNA that will be a part of the plasmid vector has also been designed and prepared. Interestingly, we were unable to synthesize a gene construct for the C terminal end of SKY1 because of a 17 Adenine repeat in the C-terminal open reading frame following the stop codon. The repeating region is close enough to the C-terminus that it cannot be excluded without shortening the homologous region for CRISPR to the point it will be far less effective.

The next step is to redesign the gene fragments such that they are easier to amplify. Once this occurs the insertion to the plasmid should be relatively straightforward. With fluorescently labeled Sky1, we will be able to track its movements in apoptotic cells in conjunction with our current live-cell microscopy assay. If the N-terminus of Sky1 translocates to the nucleus in apoptotic cells which can be visually determined then we can confirm its role in the cell death pathway.

Methods and Materials:

Plasmids

The *S. cerevisiae* Advanced Gateway Destination Vectors were a gift from Susan Lindquist (Addgene kit #1000000011) [21]. Sky1 was inserted onto the N-terminal Cerulean, C-terminal eGFP, eYFP, and HA vectors with GPD and GAL promoters. The vector for the N-terminal tag encodes Leucine, and the C-terminally tagged vectors encode Uracil and should be propagated with selective pressure where Leucine or Uracil is withheld from growth media. Protocols from the LR and BP Clonase II kits were followed to construct the plasmids [22]. Zymo mix-and- go competent *E. coli* were used in relevant transformation steps and the recommended protocol was followed [26]. Once transformed into *E. coli*, plasmids were extracted using a mini or maxi prep kit from Zymo research. The provided protocol was followed using maximum centrifuge time and rcf recommendations [27].

PCR amplification of SKY1 and primers

Yeast genomic DNA was added to the PCR reaction with 1μmol of forward and reverse primer. QuestTaq Premix was used in most of the PCR reactions. In creating the CRISPR gene fragment, Phusion HF with 10mM DNTPs and Phusion DNA polymerase was used in PCR reactions.

Yeast transformations

Several yeast colonies from a YPD agar plate or selective YNP agar plate were added to a mixture of 5mM Lithium Acetate, 50μM EDTA, and 0.5mM Tris-HCl (pH 8.0) and

centrifuged for 4 sec to form a pellet. 0.1mg of boiled salmon or herring sperm ssDNA and 1μg of transforming DNA were added to the pellet and suspended in a 40% PEG3350/ Lithium acetate mix. This was left to incubate at room temperature overnight then plated on the appropriate selective YNB agar plate (either -Leu or -Ura) and incubated a 30C for several days.

Gene fragment and gRNA design

The gene fragment ordered from Eurofins was designed using the sequence of SKY1 and fluorescent proteins from the yeast genome database and SnapGene respectively [14,28]. The sequences were codon optimized using GeneArt by ThermoFischer Scientific. We used the Atum CRISPR gRNA design and ordered it from Eurofins.

CRISPR Plasmid preparation

The plasmid was extracted from *E. coli* and cut at the BclI-HF and SwaI sites during two separate restriction enzyme digests. The New England BioLabs recommended protocol was followed for each digest.

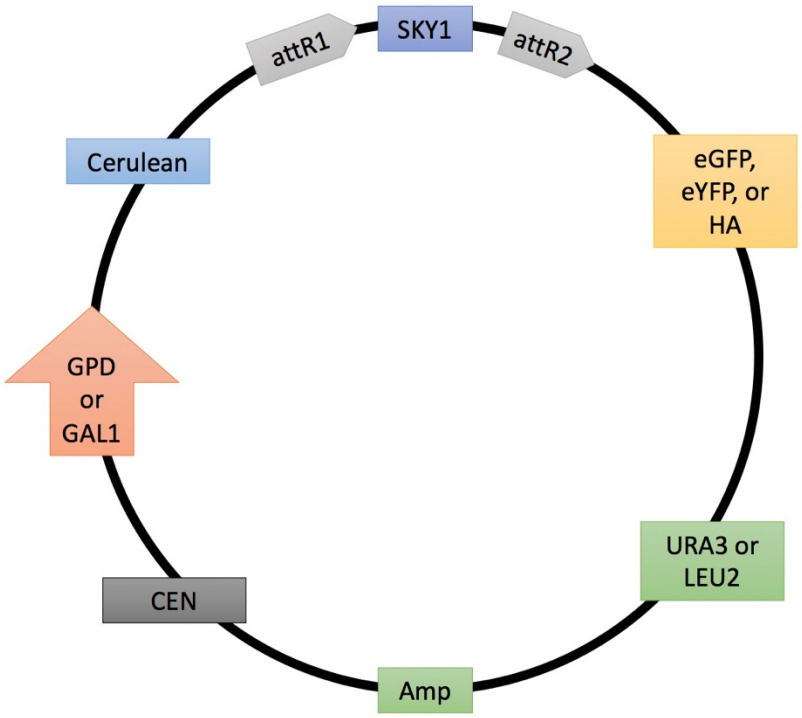


Figure 11: Gateway Destination Vectors

The destination vectors with inserted SKY1 contain either a GPD or GAL1 promoter. The N-terminal cerulean plasmid has the URA3 marker and the C-terminal tags have a LUE2 marker. All plasmids encode for ampicillin resistance and contain a low-copy CEN replicator [21].

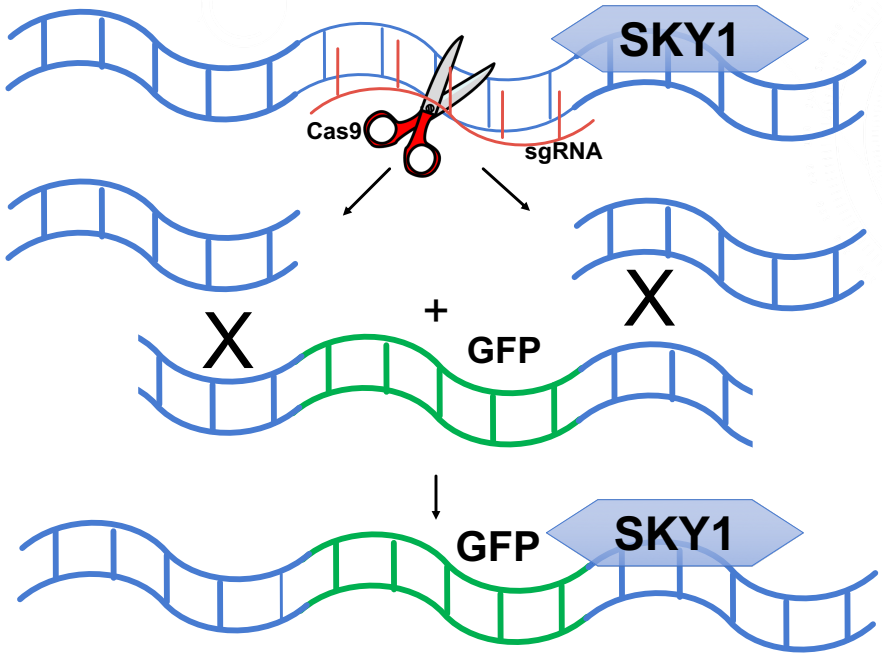


Figure 12: CRISPR Machinery

Cas9 uses a small gRNA to make a double stranded cut in DNA which uses homologous recombination to make a repair. By inserting a gene with homologous flanks we can force the repair machinery to insert genetic code such as a fluorescent protein. With the CRISPR plasmid containing Cas9 and a short guide RNA for the 5'UTR we will be able to insert a fluorescent protein upstream of Sky1. The same method will be used for a C-terminal tag.

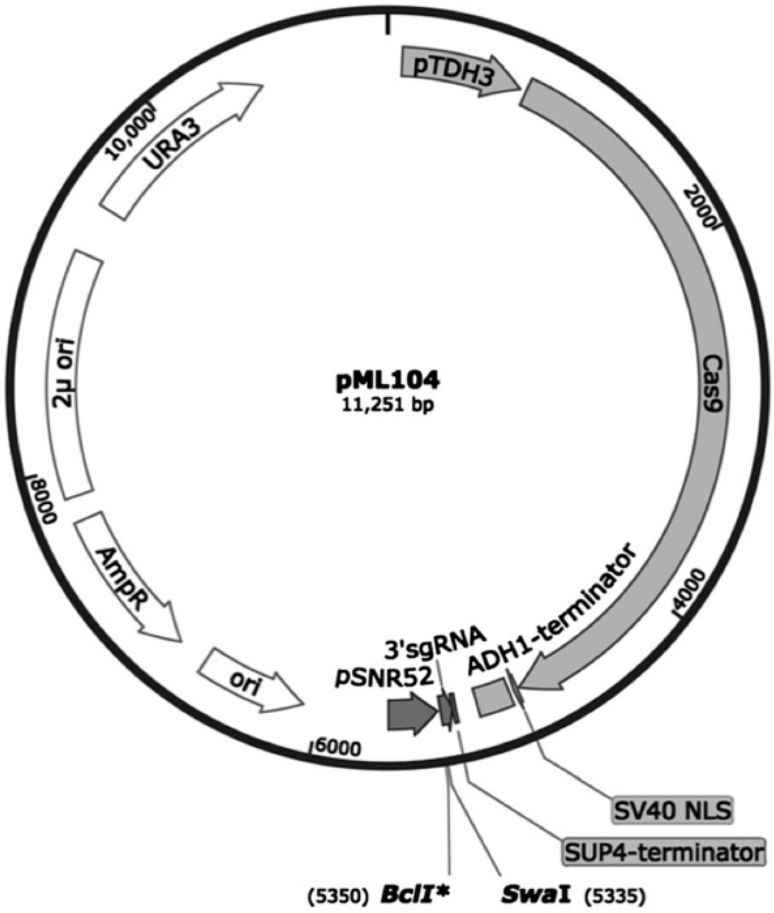


Figure 13: Map of CRISPR Cas-9 Plasmid Provided by the Wyrick Group

The vector uses a 2-micron high copy replicator and encodes for ampicillin resistance. A gene can be inserted between the two restriction sites. This plasmid map was published by Laughery et al. 2015 [24].

Tag	Terminus	Selectivity	Promoter	Replicon
Cerulean	N	URA3	GPD	CEN (low-copy)
eYFP	C	LEU2	GPD	CEN (low-copy)
eGFP	C	LEU2	GPD	CEN (low-copy)
HA	C	LEU2	GPD	CEN (low-copy)
N/A	N/A	URA2	GPD	CEN (low-copy)
Cerulean	N	URA3	GAL1	CEN (low-copy)
eYFP	C	LEU2	GAL1	CEN (low-copy)
eGFP	C	LEU2	GAL1	CEN (low-copy)
HA	C	LEU2	GAL1	CEN (low-copy)
N/A	N/A	URA2	GAL1	CEN (low-copy)

Table 2: Constructed Gateway Plasmids

10 unique SKY1 plasmids were created each with a different tag or promoter. The table outlines the various combinations.

Future Direction:

Once Sky1 is successfully labeled with fluorescent tags, we will have the ability to track its location in the live-cell microscopy assay. If the N-terminus of Sky1 is translocated to the nucleus only in apoptotic cells then we will investigate if the protein fragment is sufficient to induce apoptosis. By placing an inducible NLS tag on the N-terminus we will be able to control its localization within a cell. If nuclear localization of N-terminal Sky1 results in cell death as determined by the presence of ROS as we predict, then we will have a more efficient route for inducing apoptosis than oxidative stress.

With the ability to induce apoptosis in an entire population of cells we can use the power of yeast genetics to probe for other apoptotic regulating genes. With the inducible NLS tag on Sky1, nuclear expression of the N-terminal fragment can be turned on by shifting the carbon source to galactose while probing for apoptotic markers with the live-cell ROS assay. This method also allows us to screen for other nuclear genes in yeast knockout mutants since any cell that survives under these conditions is failing to induce cell death.

Conclusion:

SR proteins and their kinases play many roles in eukaryotes and may play an important one in the induction of apoptosis. In an ongoing project we are examining the mechanism by which Sky1 is required for apoptosis through the development of a novel live-cell microcopy assay which probes for ROS in apoptotic conditions. In addition we have studied the effects of overexpressing Sky1 on the induction of cell death. This work may lead to the elucidation of a Sky1 mediated apoptotic mechanism. Future work will investigate whether this mechanism and other potential apoptotic genes are conserved in higher eukaryotes.

More broadly, understanding the mechanisms of apoptosis is essential to investigating its various clinical benefits. If there is conservation in cell death control mechanics in the model organism yeast, we have a potentially powerful platform to do drug screening in. It could also lead to advancements in cancer treatment whereby drugs induce apoptosis in tumor cells.

Resources:

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