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Changes in Cell Morphology and the Cellular Localization of Protein Kinase Dsk1 in *Schizosaccharomyces pombe* in Response to Butylated Hydroxyanisole

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**Changes in Cell Morphology and the Cellular Localization of Protein Kinase Dsk1
in *Schizosaccharomyces pombe* in Response to Butylated Hydroxyanisole**

A Thesis Presented

By

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To the Keck Science Department

Of the Claremont Colleges

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Abstract

Dsk1 is the *Schizosaccharomyces pombe* functional homolog of human SRPK1, an SR protein kinase that regulates localization and function of SR protein splicing factors involved in transcription, alternative splicing, and mRNA export. It has been shown that a Dsk1 deletion strain of *S. pombe* is sensitive to exposure to butylated hydroxyanisole (BHA), a phenol derivative commonly used as a food preservative. Little is known about how BHA interacts with cells on a functional level, although it has been shown to be cytotoxic and tumorigenic. The aims of this thesis are to study the effect of BHA on eukaryotic cells and the possible involvement of Dsk1 protein kinase in the cellular response network to BHA through the use of fluorescence microscopy. The results showed that in BHA-treated cells, Dsk1 exhibits reduced nuclear localization and increased incidence of cytoplasmic clusters as well as a series of changes in cellular morphology. These observations imply that the function of Dsk1 is altered in response to BHA, consistent with genomic data collected by the Tang Lab. Thus, this study provides a basis for a series of future studies that will reveal in more detail how BHA affects fission yeast cells, and potentially gene or protein functional homologs in human cells.

Introduction

Schizosaccharomyces pombe as a model organism

Schizosaccharomyces pombe, also known as fission yeast, is a useful model organism for the study of essential cellular processes, despite the fact that humans and yeast diverged from each other approximately one billion years ago. The fission yeast genome, sequenced in 2002, consists of three linear chromosomes, with approximately 5,100 protein-coding genes (Wood, et al. 2012). Although impressive, these numbers are dwarfed by the 23 chromosomes and 25,000 protein-coding genes found in human nuclei (Lander et al., 2001). At a glance, it may seem that fission yeast and humans do not have much in common at all. In truth, however, when it comes to core cellular processes, fission yeast has proved an indispensable tool for the study of cell biology.

Fission yeast is an ideal organism for study of the cell cycle and essential cellular mechanisms because it is well characterized, relatively simple, easy to grow, and maintains functional homology of many genes with higher eukaryotes. It also has introns and exons, which can be argued to make it more similar to higher eukaryotes than budding yeast (Tang et al., 1998). In the modern era of fission yeast research, databases like PomBase allow researchers to access up-to-date information about fission yeast genes, proteins, and current publications (Wood, et al, 2012). The cell cycle of fission yeast is very well understood, and many mutants exist that we can use to manipulate the progression of the cell throughout the cell cycle. In contrast with human tissue cultures, fission yeast are much easier and cheaper to grow and maintain. In addition to all of these things—and the crux of what makes fission yeast so valuable as a model organism—essential cellular processes are extremely well conserved. The likelihood that a mutation

in the most crucial genes would allow survival is so low that these genes have evolved very little over time. Thus, even though so many other genes have evolved as humans have, those genes that control crucial cellular processes stay profoundly similar. So highly conserved are these major genes among eukaryotes, that if one is deleted in fission yeast, it can often be functionally replaced by the human homolog of that gene (Lee and Nurse, 1987).

Paul Nurse demonstrated the power of this concept when he applied a cross-species complementation assay to fission yeast. Using the *cdc2^{ts}* mutant strain, he systematically transfected an entire human cDNA library into fission yeast. The strain he used had a temperature sensitive mutation in *cdc2*, a protein he had shown was required for the fission yeast to progress through the cell cycle. What he found was that despite all the differences between fission yeast and humans, there was indeed a human gene that could replace the function of mutant *cdc2* at the restrictive temperature (Lee and Nurse, 1987). At the time, this gene was called *CDC2Hs*, but now it is known as Cdk1. Paul Nurse and his colleagues had not only presented the answer to one of the major questions still at hand during the time, but also demonstrated that fission yeast maintain a compelling level of genetic homology. The fact that this quality can be found in a relatively simple, well-characterized organism allows researchers to study cellular mechanisms at a basic level before moving on to more complex eukaryotes.

Genome-wide sensitivity assays

In a field like genomics, studying human cells offers vast pools of information to wade through, and often it is hard to tell where to start. However, because many genes

are well conserved between fission yeast and humans, preliminary studies in fission yeast can offer a catalog of genes of interest for more targeted research. For example, genomic comparison has produced a catalog of 289 disease-related genes that humans share with fission yeast. Genes associated with cancer make up the largest subset of those 289 genes (Wood et al., 2002). This makes sense, considering that cell cycle genes are among those most profoundly conserved between humans and fission yeast, and cancer is a cell cycle disorder.

Another strategy that is often employed using fission yeast is genome-wide sensitivity assays. These assays use deletion libraries to determine how different genes respond to a certain compound. If a given deletion is found to be sensitive to a compound, it can be inferred that the gene may be required for the proper response to damage or stress induced by the compound. In contrast, if a deletion is found to be resistant, possible explanations include gene involvement in an arrest pathway response, or inhibition of arrest factors triggered by the presence of the compound. These assays, while not meant to do much else other than identify possible targets for further study, are ideal applications of fission yeast as a model organism.

Butylated hydroxyanisole (BHA) and its effect on cells

Many synthetic antioxidants are used in foods as preservatives. One of the most common of these is butylated hydroxyanisole, or BHA. This chemical, classified as a phenol-derivative, helps preserve foods by inhibiting lipid peroxidation (Kahl, 1984). In addition to this effect, BHA has been shown to help cells defend against acute radiation and chemical toxicity (Yu et al., 2000). Despite its documented benefits, BHA has also

been shown in multiple studies to promote cancer formation in the forestomachs of multiple mammalian species (Ito et al., 1983; Nera et al., 1988). Analysis of the carcinogenic qualities of BHA show that it has documented cytotoxicity and is implicated in triggering apoptosis through mitochondria-directed pathways. This cytotoxicity may trigger genotoxic effects that could contribute to carcinogenesis. Mitochondria can be driven to induce apoptosis in a myriad of ways. Loss of metabolic capacity or a sudden increase in membrane permeability causes the release of cytochrome *c*, signaling the cleavage of pro-caspases into active apoptotic caspase-3 (Budihardjo et al., 1999). BHA was shown to promote changes in mitochondrial membrane permeability and release of cytochrome *c* (Yu et al., 2000). The specific mechanism behind this effect, as well as other elements of BHA-induced cytotoxicity remain to be elucidated.

In order to identify the genes involved in the response to BHA, a genome-wide sensitivity assay has been performed in the Tang Lab. Among the more than 2,000 deletion strains screened, this assay revealed that a deletion strain missing the gene encoding Dsk1 protein kinase shows a sensitive phenotype (data not published, Tang Lab). After a preliminary library of sensitive and resistant strains was established, a sorbitol-rescue assay was done to rule out potential osmotic stress. The scope of this assay was extremely wide, with over 5,000 strains screened (data not published, Tang Lab).

SR protein-specific kinase Dsk1 and SR proteins

Dsk1 was discovered as a novel protein in fission yeast in 1993. Researchers studying the cold-sensitive gene *dis1* found that multiple copies of a then-unknown gene

could suppress the *dis1* phenotype. They named this novel protein *dis1*-suppressing protein kinase, or *dsk1*. Upon further characterization, it has been found that Dsk1 phosphorylates serine/arginine residues, and can autophosphorylate both tyrosine and serine-arginine residues. Phosphorylation states and expression of Dsk1 change during the cell cycle (Takeuchi and Yanagida, 1993, Tang, et al., 2012). When Dsk1 is immunoprecipitated from mitotically blocked cell extracts, the phosphorylated form prevails. In contrast, in G1, S, or G2 phases, Dsk1 is not phosphorylated. Overexpression of Dsk1 using an *nmt+* promoter causes cell elongation and delay of the G2/M checkpoint (Takeuchi and Yanagida, 1993).

Further investigation of Dsk1 established it as a serine/arginine protein kinase (SRPK), and later as the yeast functional homolog of human SRPK1 (Tang et al., 1998, 2000). The SRPK class of kinases specifically phosphorylates SR proteins, which are splicing factors essential for transcription-coupled splicing of pre-mRNA (Misteli and Spector, 1999). Among the characteristics shared by SR proteins are domains of alternating serine and arginine amino acid residues, known as RS domains. It has been established that both SRPKs and the similarly functioning LAMMER kinase family (human homologs are the Clk/Sty family) hyperphosphorylate the RS domains of SR proteins (Misteli et al., 1998). Phosphorylation of SR proteins by SRPKs and LAMMER kinases modulates their activity and subcellular localization (Colwill et al., 1996; Ghosh and Adams, 2011; Misteli, et al., 1998; Huang, et al., 2004). It is important to note that there is a significant amount of variation in function of SR proteins. Some act as exon enhancers, others as shuttling transcription factors. The shared RS domain of SR proteins allows SRPKs and LAMMER kinases to participate in regulation of each of these

functions, emphasizing their importance within the cell. Indeed, the viability of Δ sk1 Δ kic1 double deletion mutants is severely impaired (Tang et al., 2002).

A significant theme in SR protein regulation is cellular localization. Changes in phosphorylation levels at the RS domain of SR proteins affect their subcellular localization, thus altering their distribution within the cell. In humans, hyperphosphorylated SR proteins bind to exon enhancing sequences along pre-mRNA, contributing to the decision making process of alternative splicing (Zahler et al., 1992; Kohtz et al., 1994; Montes et al., 2012). After splicing, dephosphorylation of SR proteins is associated with nuclear export of properly spliced mRNA sequences. Once exported from the nucleus, SR proteins are hyperphosphorylated once more and transported back to the nucleus (Figure 1). When in the nucleus, SR proteins localize to nuclear speckles, putative storage sites present during interphase (Spector et al., 1993)

<http://www.pnas.org/content/101/26/9666.full.pdf>

Figure 1. Schematic model of general SR protein function, showing phosphorylation dependent localization of SR proteins (Huang et al., 2004).

Visualization of splicing factors including SR proteins has revealed that they aggregate into bright spots in the nucleus (Figure 2). These spots have since been termed “nuclear speckles.” Analysis of nuclear speckles using electron microscopy has shown that nuclear speckles consist of two types of structures: interchromatin granule clusters (IGCs) and perichromatin fibrils (PFs). Interchromatin granule clusters are approximately 20-25 nm in diameter and are found in the interchromatin space within the nucleus.

Perichromatin fibrils often radiate from IGCs and can be found throughout the nucleoplasm (Sacco-Bubulya and Spector, 2002). Further investigation of these structures has revealed that PFs are the site of most active transcription in the nucleus, and that relatively little transcription occurs at IGCs (Misteli et al., 1998). SR proteins and other splicing factors are thought to aggregate in IGCs, forming the nuclear speckles seen using fluorescence microscopy. However, SR proteins must be shuttled from these sites to transcription sites in order to properly operate in pre-mRNA splicing (Caceres, et al., 1997, Misteli et al., 1998).

As has been stated above, it is clear that in both yeast and humans, localization of SR proteins to the nucleus requires Dsk1 and Kic1 (a LAMMER kinase), respectively (Figure 2; Misteli et al., 1998). It is now thought that once in the nucleus, SR proteins undergo another phosphorylation event that modulates their localization to nuclear speckles (Gui et al., 1994; Colwill et al., 1996; Misteli and Spector, 1997, 1999; Cazalla et al., 2002). This event is thought to alter the protein-protein interactions that allow SR proteins to aggregate into IGCs. More specifically, when SR proteins are hyperphosphorylated, they dissociate from IGCs, and when they are hypophosphorylated, they localize to IGCs (Sacco-Bubulya and Spector, 2002). Several lines of evidence support this conclusion. In humans, deletions in the RS domain of SR proteins prevent redistribution from nuclear speckles to sites of pre-mRNA (Misteli, et al., 1998). Additionally, overexpression of either SRPK or Clk/Sty causes loss of SR protein localization to speckles (Colwill, et al., 1996, Gui, et al., 1994, Sacco-Bubulya and Spector, 2002). Thus, SRPKs, LAMMER kinases are not only implicated in the regulation of SR protein translocation to the nucleus, but also in the shuttling of SR

proteins from IGCs to sites of active transcription. Taken together, these findings suggest that SRPKs and LAMMER kinases and their yeast homologs are involved in wide scale and fine-tuning of SR protein localization and function throughout the cell cycle.

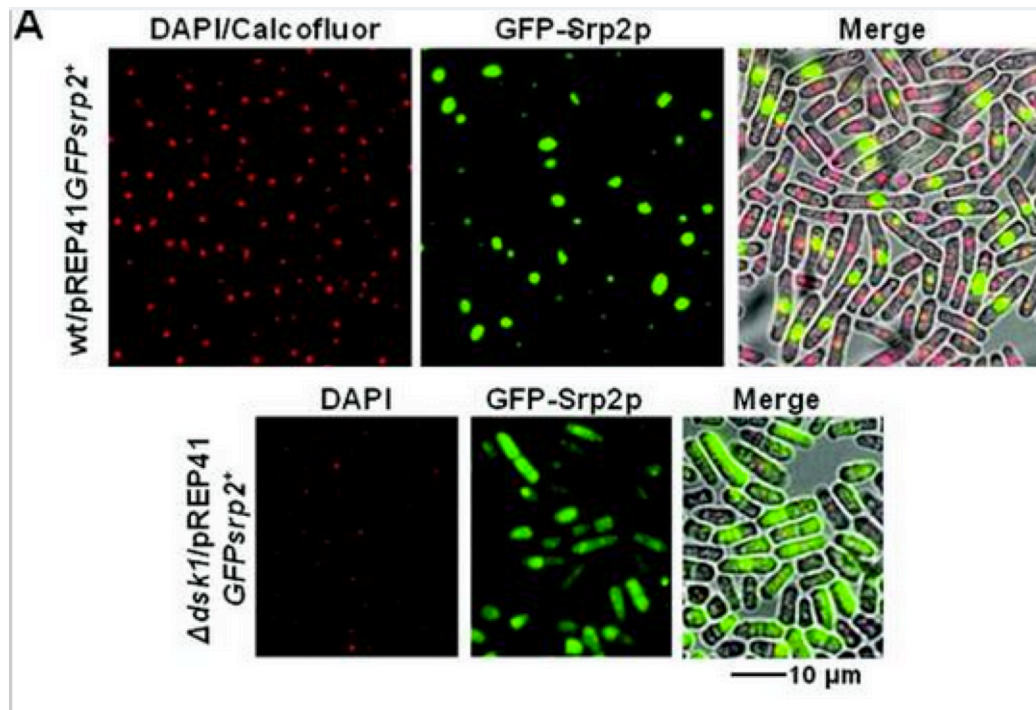


Figure 2. Localization of GFP tagged Srp2, a yeast SR protein, in either a wild type or $\Delta dsk1$ background. While localization to the nucleus is clear in the wild type strain (top), deletion of Dsk1 causes diffuse presence of Srp2 throughout the cell (Tang et al., 2007).

SRPKs and LAMMER kinases are constitutively active, which suggests that their regulation depends on cellular localization patterns (Ghosh and Adams, 2011). In human models, SRPK1 is sequestered in the cytoplasm where it hyperphosphorylates newly synthesized SR proteins, allowing their entry into the nucleus. Upon recognition of certain signaling events, dependent on events such as stress, DNA damage, or progression of the cell cycle, SRPK1 translocates to the nucleus, where it can facilitate

dissociation of SR proteins from speckles (Giannakouros et al., 2011). This seemingly binary subcellular regulation model is less clear in yeast, where *in vivo* studies using fluorescence microscopy show that Dsk1 tagged with GFP is present in both the nucleus and the cytoplasm, but shows stronger signals in the nucleus in all phases of the cell cycle (Tang, et al., 2012).

Understanding Dsk1 deletion sensitivity to BHA

Data collected in the genome wide kinase deletion sensitivity assay has revealed that the Dsk1 deletion strain of yeast is sensitive to BHA exposure (data not published, Tang Lab). It is possible that fission yeast cells tolerate environmental exposure to BHA by altering gene expression patterns. Based on the close functional relationship between Dsk1 and mRNA processing, Dsk1 may be required for this reprogramming. Therefore, its absence may lead to increased sensitivity in the Dsk1 deletion strain. Additionally, because SRPKs are constitutively active and are subject to regulation through subcellular localization, changes in localization may be indicative of regulation of gene expression levels through Dsk1 sequestration. If this is the case, I hypothesize that treatment with BHA will lead to changes in cellular localization of Dsk1 in fission yeast cells.

In order to test this hypothesis, a number of techniques were used. Fluorescence microscopy of a strain with a Dsk1-GFP fusion protein produced endogenously from its native promoter will show potential changes in localization within the nucleus. This method can start to address possible causes of Dsk1 deletion sensitivity to BHA. Because of conserved homology between Dsk1 and Srp1 and human SRPK1 and SRSF1, the results from these experiments extend beyond yeast. Although the findings stated here

cannot be directly applied to human biology, they offer a strong basis from which further research can expand.

Materials and Methods

Table 1. *S. pombe* strains used

Strain	Genotype	Reference/source
501	<i>h⁻ ade6-704 leu1-32 ura4-D18</i>	Carr, University of Sussex
ZT501Dsk1GFP	<i>h⁻ ade6-704 leu1-32ura4-D18 dsk1-GFP::kanMX6</i>	Tang, et al., 2012

Yeast Cultures

Precultures of wild type 501 and ZT501Dsk1GFP were made by isolating a colony of each strain from a master plate and introducing it to a sterile culture tube containing 5 mL of yeast extract plus supplements (YES) (Table 1). All cultures were grown at 30 °C unless otherwise indicated. Precultures were left to grow overnight. The next day, the OD₅₉₅ of the precultures was measured to determine cell density. Enough preculture was transferred to 15 mL of minimal media (MM) containing 225 mg/L each of adenine, histidine, uridine, and leucine to ensure a final cell concentration not higher than 2×10^7 cells/mL after 24 hours. This density is within log phase. At zero hours, each of these cultures was either treated with 0.4 mM BHA dissolved in 1% DMSO or with only 1% DMSO (control). The concentration of BHA used in these cultures corresponds to the IC₇₀ of BHA. The generation times of the BHA treated and untreated cells were 5 and 3.5 hours, respectively.

Fluorescence Microscopy

At 5, 10, and 24 hours, approximately 1×10^7 cells from these cultures were harvested using a microcentrifuge. These cells were rinsed three times with sterile water by adding 1 mL water, vortexing, then spinning down the cells using a microcentrifuge, and removing the supernatant. After washing, the cells were resuspended in cold 70% ethanol to fix the cells. These samples were kept in the refrigerator until slides could be made. To make slides, fixed cells were rehydrated in 1 mL of sterile H₂O and their concentration was checked using a light microscope to ensure that the cells were dense enough to be easily found under the microscope, but not so dense as to create multiple layers of cells on the slide. Five μ L of this cell solution was pipetted onto a clean slide and allowed to evaporate over a hot plate on very low heat. Once evaporated, 5 μ L of a 9:1 solution of DAPI:calcofluor was added. This DAPI/calcofluor solution was made using the same protocol as in Tang, et al., 2012. A coverslip was placed over the solution and the slide was sealed using clear nail polish and kept refrigerated in the dark until observation. The cells were observed using an Olympus IX81 motorized inverted fluorescence microscope with magnification of 1000x and ImagePro Plus version 6.3 (MediaCybernetics, Inc., Bethesda, MD, USA.)

Results

Cells treated with BHA show a series of morphological changes

Figure 3 shows changes in cell shape, size, and septation as a result of BHA treatment. Among the changes seen in BHA treated cells are elongation (Figure 3B, panel

iv), loss of rod shape (Figure 3B, panels ii and iii), and extremely thick, abnormal septa (Figure 3B, panel i). The very thick septa could be stained with calcofluor, but this did not happen all the time (data not shown). This may be because calcofluor stains new cell wall growth most efficiently and some of the wide septa were relatively old. Many treated cells also lost the rod shape that is characteristic of fission yeast cells, instead becoming circular, club-shaped, or globular.

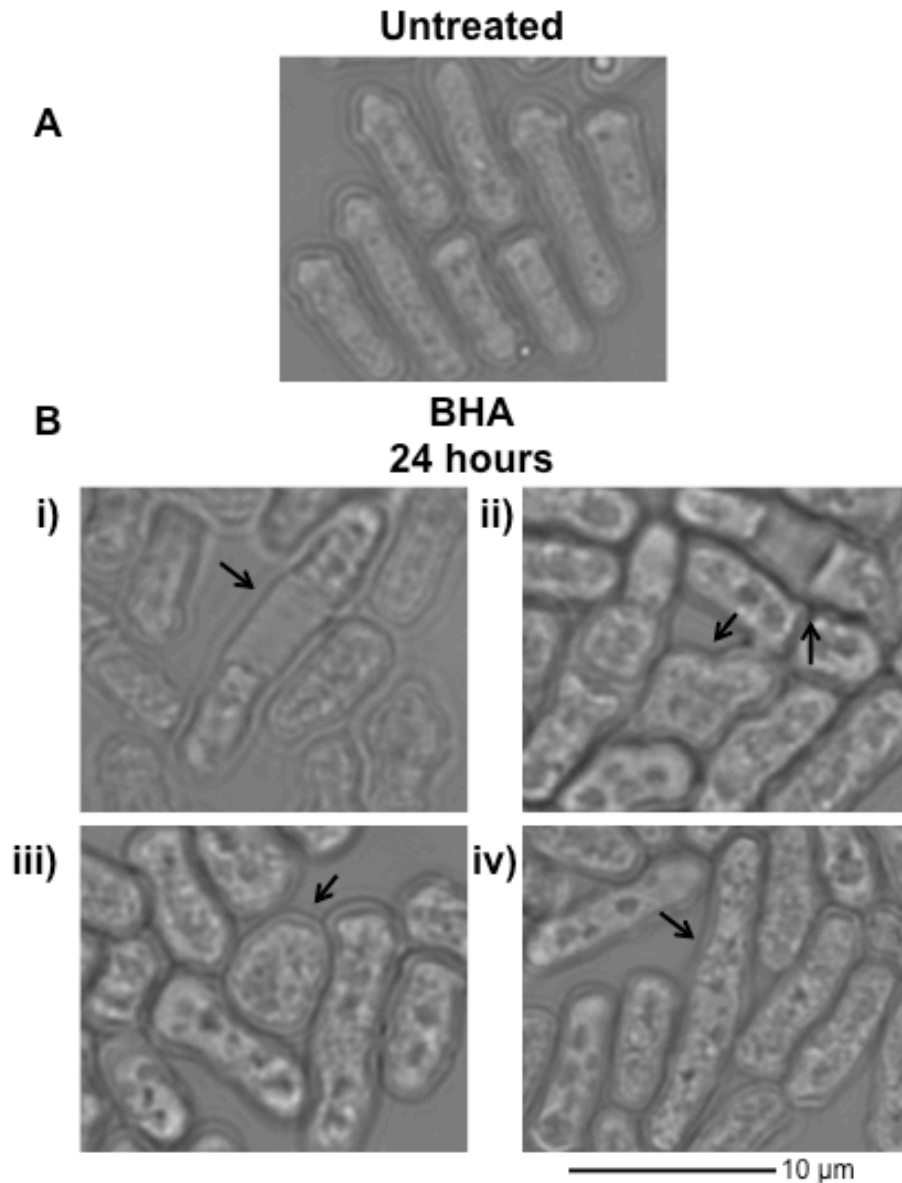


Figure 3. Phase contrast microscopy shows a series of morphological changes in response to BHA. Cell size and shape of (A) *Dsk1GFP* cells treated with 1% DMSO (control) for 24 hours and (B) *Dsk1GFP* cells treated with 0.4 mM BHA for 24 hours. Original magnification 1000x, scale bar represents 10 μ m. Black arrows represent cells showing altered features.

Untreated Dsk1 has a diffuse cytoplasmic signal and a stronger nuclear localization signal

Fluorescence microscopy of *dsk1GFP* cells confirmed the previously established localization patterns of wild type Dsk1 in an untreated environment (Tang et al., 2012). Dsk1 is present throughout the cytoplasm but excluded from vacuoles (Figure 5). Furthermore, a markedly stronger GFP signal colocalizes with DAPI staining of DNA, showing that Dsk1 is present in higher quantities in the nucleus (Figure 4). With this localization pattern confirmed, changes in localization when *dsk1GFP* cells are treated with BHA can be compared to this control.

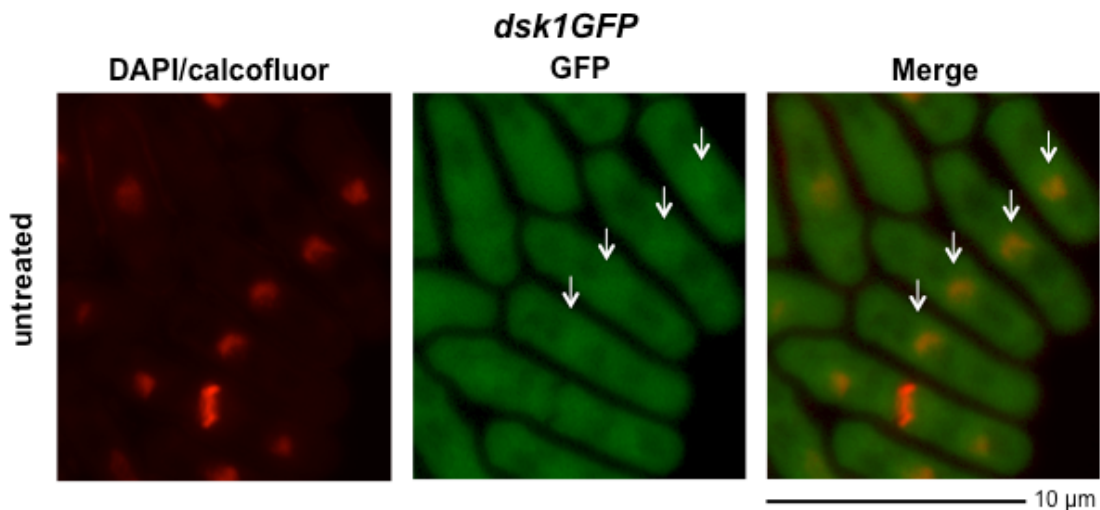


Figure 4. Untreated *dsk1GFP* cells show a diffuse cytoplasmic localization pattern as well as distinct nuclear localization. *Dsk1GFP* cells were grown in MM + 1% DMSO (control) then fixed and observed using fluorescence microscopy (left panel) and DAPI/calcofluor (right panel). Merged images were generated by overlapping GFP and DAPI images. Original magnification 1000x, scale bar represents 10 μm. White arrows mark sites of nuclear localization.

BHA treated dsk1GFP shows reduced nuclear localization and the appearance of cytoplasmic clusters

Figure 5 shows the localization of Dsk1 when *S. pombe* cells were treated with BHA compared to cells treated with 1% DMSO as a control. When exposed to BHA, two major observable changes in Dsk1 localization appear. The first is reduced nuclear localization. While in untreated cells, the *dsk1GFP* fusion protein has a clearly stronger GFP signal at nuclei, in BHA treated cells, the GFP signal is no stronger than its cytoplasmic background. Quantification of this phenotype reveals that the number of cells displaying reduced nuclear localization increases with time exposed to BHA. By 24 hours, approximately 24.5% of cells showed poor Dsk1 localization to nuclei, which is more than 13 times that of untreated cultures (Figure 6A).

The second major phenotypic change observed in BHA treated cells was that Dsk1 appears to form cytoplasmic clusters that are often nowhere near the nucleus. Cells often showed multiple clusters per cell, as is seen in the 24-hour panels of BHA treated cells (Figure 5). Quantification of this phenotype showed a 2.5 fold increase in the percentage of cells displaying cytoplasmic Dsk1 clusters relative to control populations (Figure 6B).

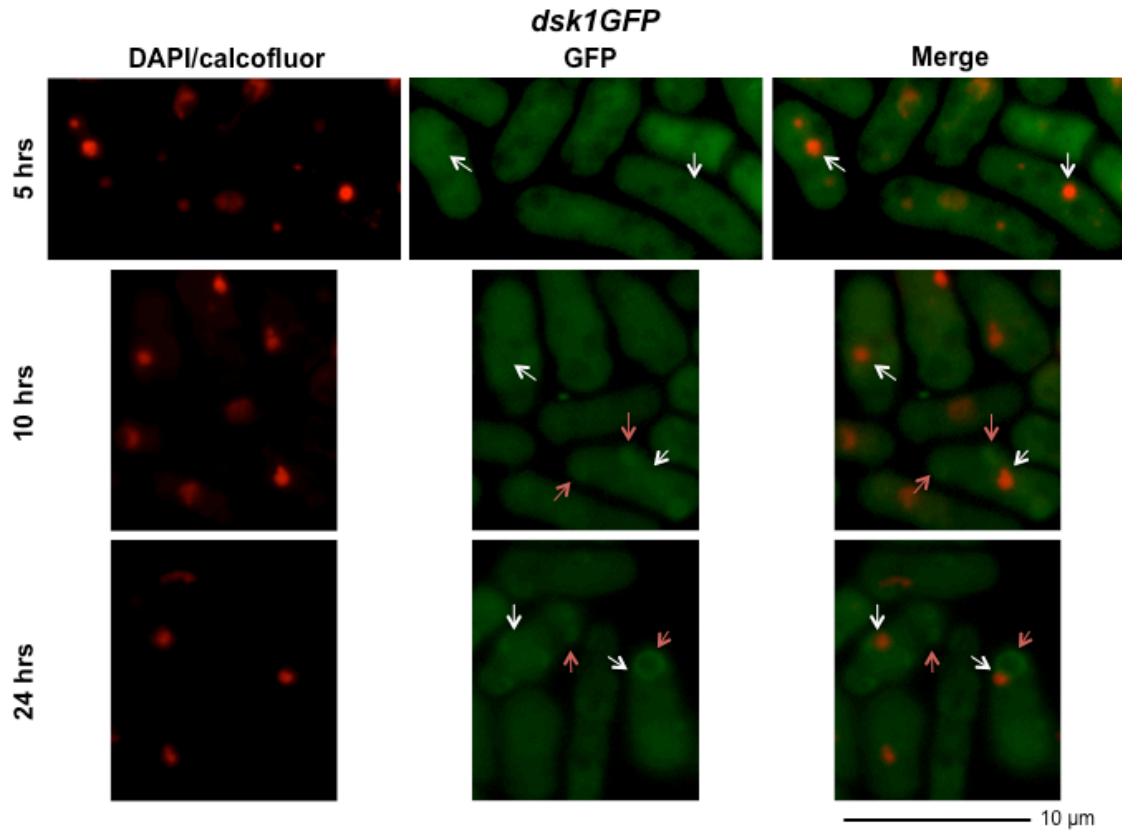


Figure 5. Changes in the cellular localization of Dsk1 in BHA treated *dsk1GFP* cells. *Dsk1GFP* cells were grown for 5, 10, or 24 hours in MM + 0.4 mM BHA then fixed and observed using fluorescence microscopy for GFP (left panels) and DAPI/calcofluor (middle panels). Merged images were generated by overlapping GFP and DAPI images. Original magnification 1000x, scale bar represents 10 μ m. White arrows mark sites of reduced nuclear localization in BHA treated *dsk1GFP* cells, red arrows mark Dsk1 cytoplasmic clusters.

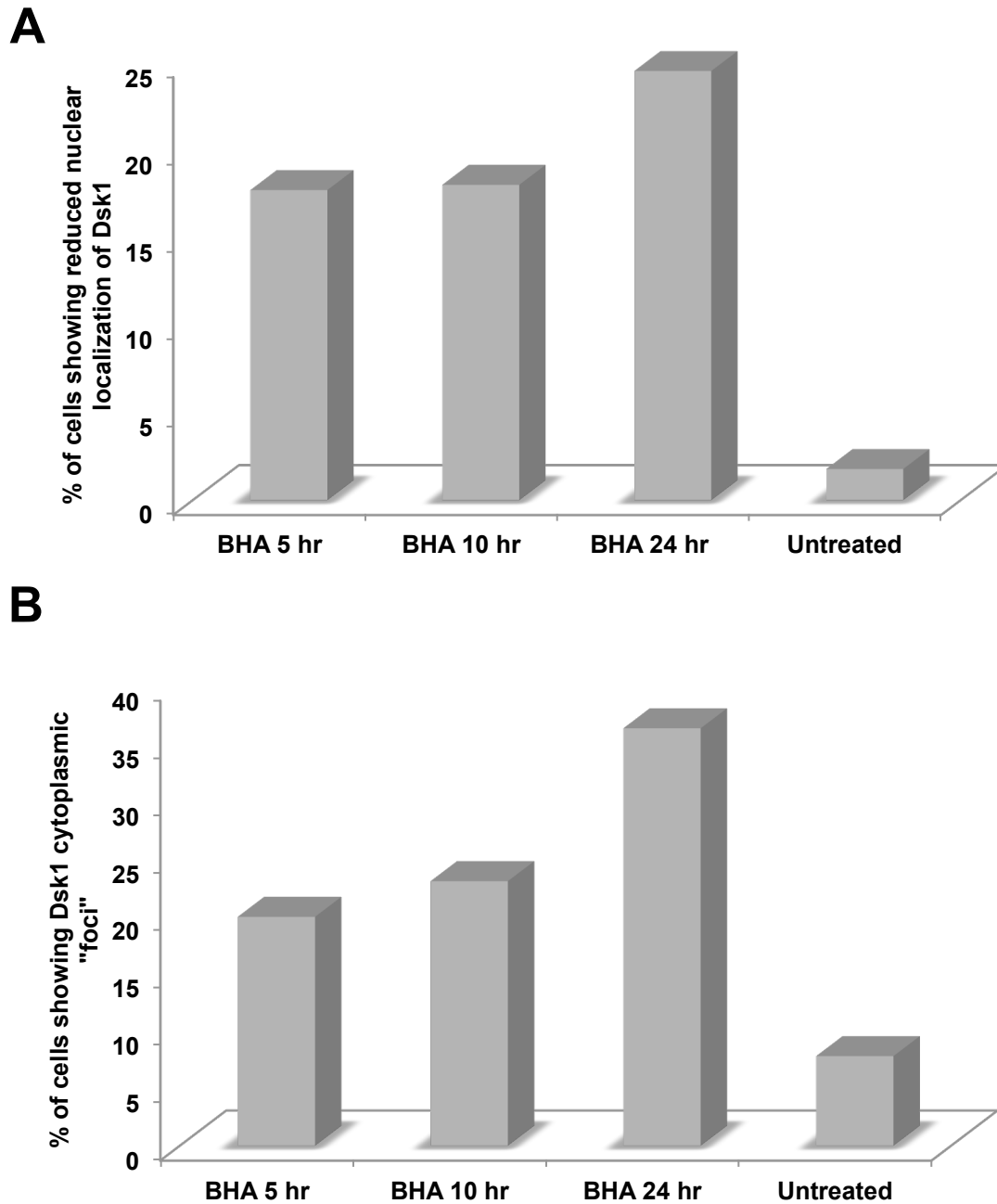


Figure 6. Quantitative analysis of changes in Dsk1GFP cellular localization induced by BHA. Phenotypes were quantified by calculating the percent of cells that displayed either reduced localization (A) or cytoplasmic clusters (B) in *dsk1GFP* cells treated with BHA after 5, 10, and 24 hours, as well as untreated control cells. At least 100 cells were counted for each treatment, data is combined from three separate trials.

Overall, it is clear that treatment with BHA causes morphological changes as well as changes in the localization of Dsk1 in *S. pombe*. Taken together, these results offer some novel observations of the effect of BHA on a fission yeast homolog of a major class of splicing factor regulators. That this is the case implies some functional relationship between Dsk1 and the cell's overall response to BHA.

Discussion

Treatment of fission yeast cells with BHA leads to changes in overall morphology and increased vacuole abundance

One of the most easily observed changes in morphology in treated yeast was an increase in the abundance of vacuoles in each cell. This was especially obvious upon visualization with GFP, where the vacuoles could be seen as dark circular shapes with no GFP signal (Figure 5). In both fission yeast and budding yeast, vacuoles are used as a method of detoxification and their presence could indicate that the cell is attempting to sequester BHA from the rest of the cytoplasm to reduce its cytotoxic effects (Ortiz et al., 1992). Additional morphology changes seen in fission yeast treated with BHA included both elongated and shortened phenotypes, loss of the characteristic rod shape, and very thick septa. These types of morphology changes indicate that BHA may affect more than one mechanism. Figure 7 shows how various cell cycle mutants lead to specific morphological changes in combination with BHA treatment. Each mutant represents what occurs when the functional protein is disrupted, demonstrating its role in maintaining proper morphological features. The simultaneous presence of many of these

phenotypes in populations of treated yeast emphasizes that BHA has strong, pleiotropic effects on cell populations.

<http://genesdev.cshlp.org/content/11/22/2939/F2.expansion.html>

Figure 7. Yeast mutations and resulting morphological changes. Yeast deletions and temperature sensitive mutations lead to the above morphological changes (Gould and Simanis, 1997).

Reduced Dsk1 nuclear localization

In control populations of yeast, Dsk1 shows a strong cytoplasmic signal and a slightly stronger but clearly visible presence at nuclei (Figure 4). Such a localization pattern is well explained by data that shows that Dsk1 has functions in both the nucleus and the cytoplasm. Dsk1 present in the cytoplasm hyperphosphorylates SR proteins, causing them to be transported into the nucleus. Once in the nucleus, subsequent hyperphosphorylation events precipitate their dissociation from nuclear speckles so they can localize to sites of transcription and splicing (Sacco-Bubulya and Spector, 2002). However in BHA treated yeast, approximately 25% of the time Dsk1 nuclear accumulation like what is seen in untreated cells fails to occur. There are a number of potential consequences of reduced nuclear localization in fission yeast.

As stated above, we know that hyperphosphorylation by SRPKs is necessary for SR proteins to delocalize from nuclear speckles and subsequently act in constitutive and alternative splicing (Sacco-Bubulya and Spector, 2002). If Dsk1 in BHA treated cells does not localize to nuclei with the same efficiency as untreated cells, it is possible that SR proteins are not hyperphosphorylated and released from speckles at normal rates. This

may cause changes in mRNA transcription, splicing, or export. The process that dictates exon inclusion is a finely tuned competition of both enhancing and silencing elements, and even a slight change in SR protein concentration or binding capacity due to changes in phosphorylation could strongly alter how pre-mRNA is spliced (Xiao and Manley, 1997). Research on SRPKs in humans has illustrated that increased translocation of SRPKs to the nucleus alters splice site selection, thus the opposite, reduced nuclear localization, could do the same (Zhong et al., 2009).

The result of this may ostensibly be the production of alternative gene products that act in a response pathway to BHA. Without Dsk1, a deletion strain would not be able to respond to BHA by altering Dsk1 subcellular localization. This may impair the ability of the cell to prompt SR proteins to alter gene expression patterns, preventing the creation of products that aid in the response to BHA, ultimately leading to the sensitive phenotype observed from the genomic screening assay. Such changes in expression patterns may also be responsible for generating the pleiotropic morphological effects seen in BHA treated yeast (Figure 3).

Alternative splicing has essential functions in differentiation, development, and various stress responses, and is tissue specific (Stamm, 2002). If alternative splicing is significantly affected by BHA exposure, it may also explain why it causes the development of tumors in very specific tissue types even though it has been shown not to be directly mutagenic. Perhaps through activating specific alternative splicing events, BHA can trigger oncogenic mechanisms. To further investigate this possibility it is important to know whether or not the reduced nuclear localization of Dsk1 is significantly affecting hyperphosphorylation levels of SR proteins or their localization to

nuclear speckles. This knowledge would link the findings of this paper to the functionality of SR proteins and to mRNA processing and splicing. Additionally, an assay that compared levels of mRNA isoforms between BHA treated and control cells would shed further light onto the possibility that BHA acts through this mechanism.

BHA-treated yeast grow approximately 40% slower than control yeast treated with 1% DMSO (see methods). It is possible that if Dsk1 cannot localize efficiently to the nucleus to effect SR protein nuclear speckle dissociation, that mRNA production in general would be less efficient. Such inefficient mRNA processing has been shown to slow yeast growth (Pandit et al., 2009). Inhibition of pre-mRNA processing itself would interfere with the production of maintenance proteins necessary for cell growth. Furthermore, it has been shown that improperly spliced RNA is retained in the nucleus, offering another mechanism through which BHA could negatively affect the availability of necessary mRNA templates for protein synthesis (Fasken and Corbett, 2009).

BHA results in Dsk1 cytoplasmic clusters

Another major observation in treated populations was that Dsk1 appears to aggregate in cytoplasmic clusters upon treatment with BHA. In light of observations that suggest that SRPKs are often regulated by altering their presence within the cell, This phenomenon may be connected to reduced nuclear localization. Recent research in humans has shown that SRPK1 is anchored to specific heat shock chaperone proteins while in the cytoplasm, preventing them from translocating to the nucleus without specific types of signals (Zhong et al., 2009; Zhou et al., 2012). It is possible that the clusters seen in BHA treated cells are aggregations of Dsk1/chaperone complexes in the cytoplasm and that BHA is interrupting the signaling pathway necessary for Dsk1 to be

released from these complexes. The increased presence of such clusters over time in the treatment environment suggests support for this hypothesis; as such complexes seem to build up over time.

In order to further address this possibility, we could observe the coimmunoprecipitants of Dsk1 from BHA-treated or control lysates. This would suggest that a similar mechanism occurs in yeast as does in humans, as well as provide support for the reasoning that chaperone interactions are causing the development of Dsk1 clusters in BHA treated cultures.

Concluding remarks

The results shown in this experiment demonstrate the strong, pleiotropic effects of BHA on wild type fission yeast expressing a Dsk1GFP fusion protein. Morphological changes such as increased vacuole formation are correlated with the presence of cytotoxic elements (Ortiz et al., 1992). More data is necessary to determine whether or not the increased incidence of vacuoles is a response to BHA cytotoxicity. Additionally, the observations collected throughout this research imply that BHA changes localization patterns of Dsk1. The reduction of Dsk1 presence within the nucleus, as well as the development of clusters of Dsk1 in the cytoplasm are evidence of this conclusion. The result of these changes may affect nuclear events in which Dsk1 is involved, namely, the regulation of mRNA splicing and processing factors. This may lead to a reprogramming of gene expression patterns, which would allow the cell to cope with environmental exposure to BHA. If alternative gene expression patterns must be activated in order to respond to BHA exposure, then regulation of Dsk1 activity through subcellular

sequestration to the cytoplasm rather than to the nucleus may reflect such an attempt to alter expression patterns. This model also highlights the importance of Dsk1 in responding to BHA, supporting unpublished data from the Tang Lab that a Dsk1 deletion strain is sensitive to BHA.

Further investigation of the observed changes in cellular localization and their effect on the phosphorylation states and localization patterns of SR proteins is necessary to provide more evidence that Dsk1 acts in the response to BHA by altering gene expression through regulation of mRNA processing. Future experiments should attempt to observe whether or not BHA impedes the ability of Dsk1 to hyperphosphorylate SR proteins, as well as look at downstream events such as the effect of BHA on alternative splicing isoforms. Additionally, because of the close functional relationship between Dsk1 and Kic1, it would be interesting to make observations of the effect of BHA on Kic1 cellular localization using a Kic1GFP strain.

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