Mechanisms Underlying Mitochondrial Quality Control and Cytokinesis in Budding Yeast

Part I: Mitochondrial Remodeling Through the Proteasome is Critical for Mitochondrial Quality Control in Budding Yeast

Part II: Aim44p Regulates Phosphorylation of Hof1p to Promote Contractile Ring Closure During Cytokinesis in Budding Yeast

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ABSTRACT

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This work discusses both mechanisms underlying mitochondrial quality control and cytokinesis in the budding yeast *Saccharomyces cerevisiae*. As these topics are quite different, their presentation has been divided into two parts, “Part I: Mitochondrial Remodeling Through the Proteasome is Critical for Mitochondrial Quality Control in Budding Yeast” and “Part II: Aim44p Regulates Phosphorylation of Hof1p to Promote Contractile Ring Closure During Cytokinesis in Budding Yeast.” In Part I, we show that the proteasome is critical for cellular fitness in response to chronic, low levels of mitochondrial reactive oxygen species (ROS) in budding yeast. Deleting *DOA1*, which is required for ubiquitin-mediated degradation, *UFD5*, which promotes proteasome gene expression, or *NAS2*, which promotes proteasome regulatory particle assembly, increases the sensitivity of yeast to chronic, low levels of mitochondrial ROS. In contrast, deleting *ATG32*, a gene required for mitophagy, other autophagy genes, non-essential chaperones including prohibitins, or mitochondrial proteins including the Lon protease (Pim1p) or *YME1*, does not affect cellular fitness under these conditions. Doa1p binds with Cdc48p and Vms1p, which associates with mitochondria and promotes extraction of ubiquitinated proteins from the organelle for proteasomal degradation in a pathway called mitochondria-associated degradation (MAD). Elevated mitochondrial ROS increases protein ubiquitination, ubiquitination of the mitochondrial

protein aconitase and expression of key MAD proteins. Interestingly, down-regulating ER-associated degradation (ERAD), which shares some common proteins with MAD, can promote cell growth under conditions of elevated mitochondrial ROS. Finally, deletion of *DOA1* results in increased sensitivity of yeast and yeast mitochondria to oxidative stress. Mitochondria in *doa1*Δ cells are more oxidized than mitochondria in wild-type or *atg32*Δ cells under conditions of elevated mitochondrial ROS. Moreover, deletion of *DOA1* results in a decrease in chronological lifespan. These findings support a critical role for the proteasome and MAD in mitochondrial quality control, which in turn affects cellular fitness, in response to chronic, low levels of mitochondrial ROS.

In Part II, we show that the protein product of *YPL158C,* Aim44p, undergoes septin-dependent recruitment to the site of cell division. Aim44p co-localizes with Myo1p, the type II myosin of the contractile ring, throughout most of the cell cycle. The Aim44p ring does not contract when the actomyosin ring closes. Instead, it forms a double ring that associates with septin rings on mother and daughter cells after cell separation. Deletion of *AIM44* results in defects in contractile ring closure. Aim44p co-immunoprecipitates with Hof1p, a conserved F-BAR protein that binds both septins and type II myosins and promotes contractile ring closure. Deletion of *AIM44* results in a delay in Hof1p phosphorylation, and altered Hof1p localization. Finally, overexpression of Dbf2p, a kinase that phosphorylates Hof1p and is required for re-localization of Hof1p from septin rings to the contractile ring and for Hof1p-triggered contractile ring closure, rescues the cytokinesis defect observed in *aim44*Δ cells. Our studies reveal a novel role for Aim44p in regulating contractile ring closure through effects on Hof1p.

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CHAPTER 1 - INTRODUCTION TO PART I: Mitochondrial Remodeling Through the Proteasome is Critical for Mitochondrial Quality Control in Budding Yeast

INTRODUCTION

Mitochondria and Age-related Disease

Mitochondria are essential organelles for aerobic energy mobilization, biosynthesis of amino acids, fatty acids, heme, steroid hormones, pyrimidine, and iron-sulfur complexes, calcium (Ca^{2+}) storage and buffering, and the regulation of apoptosis. Increasing evidence also links mitochondria to aging and age-related diseases. Some of the age-related diseases associated with changes in mitochondria or mutations in mitochondrial proteins are Parkinson's disease (PD), Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis (ALS), and Huntington's disease (HD) (Gibson *et al.*, 2010). The studies linking these diseases to alterations in mitochondrial morphology, mitochondrial content, and function include a wide range of observations in human patients through autopsy analysis and in many model systems, including tissue culture and rodents. In many cases, it is not clear whether the mitochondrial changes associated with disease are causal or a consequence of the primary cause of each disease. Nonetheless, the link between mitochondria and age-related disease is well-founded.

PD is the second most common neurodegenerative disorder in the world and is thought to affect about 1% of the population over the age of 60 in industrialized countries (de Lau and Breteler, 2006). PD is clinically characterized by rigid movements, bradykinesia, and resting tremor (Beal, 2002; Lin and Beal, 2006). Pathologically, the disease is associated with degeneration of neurons in the substantia nigra and the presence of Lewy bodies, which are cytoplasmic aggregates containing α-synuclein and

ubiquitin (Lin and Beal, 2006). PD was first linked to mitochondria in the early 1980s when drug users developed a type of PD as a result of using a "synthetic heroin" containing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces a metabolite, 1-methyl-4-phenylpyridinium (MPP^+), that inhibits complex I of the mitochondrial electron transport chain (ETC) (Langston *et al.*, 1983; Canet-Avilés *et al.*, 2004; Lin and Beal, 2006). Complex I is also the target for inhibition by rotenone in an animal model for PD (Betarbet *et al.*, 2000; Beal, 2005). Other studies revealed that PD is associated with mutations in mitochondrial DNA (mtDNA) and nine nuclear genes, including α-synuclein, Parkin, ubiquitin carboxy-terminal hydrolase L1, phosphatase and tensin homologue (PTEN)-induced kinase 1 (PINK1), leucine-rich-repeat kinase 2 (LRRK2), DJ-1/Park7, nuclear receptor NURR1, high-temperature requirement A 2 (HTRA2), and tau. Parkin, PINK1, LRRK2, HTRA2, and DJ-1 are linked to mitochondrial function, providing additional support for a role for mitochondria in PD (Lin and Beal, 2006; Westerlund *et al.*, 2011).

AD is the most common neurodegenerative disorder in the world and, according to the World Health Organization, may contribute to 60-70% of all cases of dementia. AD is clinically characterized by a decline in cognitive function and memory, resulting in dementia (Beal, 2002; Lin and Beal, 2006). Pathologically, AD is associated with protein aggregates, called plaques, composed largely of amyloid-β (Aβ) peptide and neurofibrillary tangles composed primarily of hyperphosphorylated tau, localized mainly in the cerebral cortex and hippocampus (Beal, 2002; Lin and Beal, 2006; Gibson *et al.*, 2010). The amyloid precursor protein (APP) has targeting sequences for endoplasmic

reticulum (ER) and mitochondria, and blocks import of proteins into mitochondria in tissue culture and mouse models (Lin and Beal, 2006). Increased production of the Aβ peptide, a hallmark of AD, decreases mitochondrial function by inhibiting cytochrome oxidase activity and increasing free-radical production (Crouch *et al.*, 2005; Manczak *et al.*, 2006). There is also a decrease in glucose metabolism in AD brain (Gibson *et al.*, 2010). The observed decreases in mitochondrial metabolic function may be explained by the binding of the Aβ peptide to mitochondrial enzymes, including α-ketoglutarate dehydrogenase complex (KGDHC), thereby inhibiting their enzymatic functions (Gibson *et al.*, 2010).

ALS is a fairly rare but very debilitating and terminal disease. It is estimated that one to two people in 100,000 are diagnosed with ALS within the United States per year (Miller *et al.*, 2009). ALS is clinically characterized by muscle weakness, muscular atrophy, and muscle spasms (Beal, 2002; Lin and Beal, 2006). Pathologically, the disease is associated with death of upper and lower motor neurons in the cortex, brainstem, and spinal cord (Beal, 2002; Lin and Beal, 2006). In patients with ALS, there are defects in mitochondrial morphology, amount, and/or localization in the spinal cord, nerves, and muscle tissue (Lin and Beal, 2006). In a mouse model of ALS, mitochondria have defects in Ca^{2+} uptake, a phenotype that occurs before symptoms present (Damiano *et al.*, 2006; Lin and Beal, 2006).

HD occurs in five to ten individuals per 100,000 people worldwide (Driver-Dunckley E, 2007). HD is characterized clinically by involuntary movements, psychotic episodes, and

dementia (Beal, 2002; Lin and Beal, 2006). Pathologically, the disease is associated with the death of long projection neurons in the cortex and striatum (Beal, 2002; Lin and Beal, 2006). HD is associated with an expansion of a CAG trinucleotide repeat in the huntingtin (*HTT*) gene, resulting in an expanded polyglutamine region in the translated protein (Lin and Beal, 2006). Complexes II and III of the ETC in brain from HD patients have decreased activity (Gu *et al.*, 1996; Lin and Beal, 2006). Additionally, lymphoblast mitochondria from HD patients have decreased membrane potential and are less capable of storing Ca^{2+} than mitochondria from lymphoblasts derived from control patients (Panov *et al.*, 2002). This observation was reproduced in brain mitochondria from a mouse model of HD (Panov *et al.*, 2002). These mitochondrial defects are thought to be a direct consequence of the mutant huntingtin protein interacting with the outer mitochondrial membrane (OMM) through its N terminus, an interaction that has been observed both with immunoelectron microscopy and via subfractionation experiments (Panov *et al.*, 2002; Choo *et al.*, 2004). Mitochondrial respiration and ATP production are decreased in striatal cells in HD mouse models (Milakovic and Johnson, 2005; Lin and Beal, 2006). Another explanation for the mitochondrial dysfunctions associated with HD is the interaction of the mutant huntingtin protein with transcription factors, including p53, which is a tumor suppressor known to control the expression of genes involved in mitochondrial functions and oxidative stress (Luthi-Carter and Cha, 2003). Not surprisingly, the mitochondrial poisons malonate and 3-nitroproprionic acid result in an HD-like disorder in mice (Gibson *et al.*, 2010).

While some individuals live their entire lives with mutations in mitochondrial proteins, including PINK1 and Parkin, associated with age-related neurodegenerative diseases, symptoms of disease occur only later in life. This indicates that changes occur in mitochondria with age that allow disease pathology to emerge. Indeed, aging is the greatest risk factor for disease with clear mitochondrial defects and dysfunction, such as AD, PD, ALS, and HD.

Mitochondria and Aging

According to the World Health Organization, increased life expectancy and decreased fertility rates are contributing to a worldwide increase in the population of individuals over the age of 60 years, making this cohort the fastest growing age group. Aging is an intuitive concept for humans and is associated with recognizable phenotypic traits. However, for the sake of studying this process, a concrete definition of aging is required. Biological aging is defined as a progressive decrease in the capacity of an organism to combat stress, damage, and disease, resulting in an increased frequency of mortality with age (Jazwinski, 2002).

Defects in mitochondrial function have been linked to aging. For example, mutations in mtDNA, especially point mutations and deletions, can produce aging phenotypes. Mutations in the mtDNA polymerase (Polγ) of mice that affect its proofreading function but leave its DNA replication function unaltered, resulted in premature aging, characterized by an inability to gain weight, balding, decreased bone mineral density, and decreased lifespan (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). In these models,

the aging phenotypes are due to decreased activity of respiratory enzymes, which results in decreased ATP production within mitochondria (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). For example, an increase in the amount of point mutations in cytochrome oxidase in neurons is associated with a decrease in its enzymatic activity and an increase in aging phenotypes (Lin *et al.*, 2002).

In addition to the specific changes in mitochondria and mitochondrial function that occur with the age-related diseases discussed above, increased oxidative stress and oxidative damage also link mitochondria and age-related diseases. In fact, oxidative damage is associated not only with the pathologies of age-related disease, but also with normal aging. Oxidative damage as a result of reactive oxygen species (ROS) increases in aged cells in many organisms including bacteria (Dukan and Nyström, 1998); yeast (Stadtman, 1992; Aguilaniu *et al.*, 2003; Reverter-Branchat *et al.*, 2004); and mammals, including rodents and humans (Hamilton *et al.*, 2001; Gibson *et al.*, 2010).

ROS are highly reactive oxygen-containing molecules with an unpaired electron in their valence shell. ROS include oxygen ions, free radicals, and peroxides (Fig. 1.1). ROS react readily with proteins, lipids, and nucleic acids and can irreversibly alter them through protein carbonylation, lipid peroxidation, or by modifying guanine to 8-oxoguanine in nucleic acids (Beal, 2002). Enzymes containing iron-sulfur (Fe-S) clusters, such as aconitase, readily react with $O_2^{\bullet-}$, which damages them and renders them non-functional (Winterbourn, 2008; Merksamer *et al.*, 2013). Protein carbonylation occurs most commonly by the oxidation of the side chains of arginine, lysine, proline,

and threonine residues (Berlett *et al.*, 1998). ROS are produced as a part of the normal catalytic functions of several enzymes, such as NADPH oxidases in the cytosol and mitochondria, xanthine oxidase, cyclooxygenases, cytochrome p450 enzymes, and lipoxygenases (Finkel, 2011). ROS are also a by-product of mitochondrial respiration, making mitochondria the largest endogenous source of ROS (Guarente, 2008). During oxidative phosphorylation, protons are pumped across the IMM from the matrix to the IMS and the ETC facilitates the transfer of electrons from a series of complexes and finally to oxygen, culminating in the synthesis of ATP (Gruber *et al.*, 2013). During the transfer of electrons through the ETC, there are several opportunities for electrons to be transferred to oxygen, resulting in ROS production. Among the ETC components, Complexes I and III have the highest incidence of electron transfer to oxygen (Brand *et al.*, 2004; Gruber *et al.*, 2013). Approximately 1-5% of the oxygen consumed during oxidative phosphorylation is converted into ROS as a result of electron leakage from the ETC (Herrero *et al.*, 2008; Westermann, 2008).

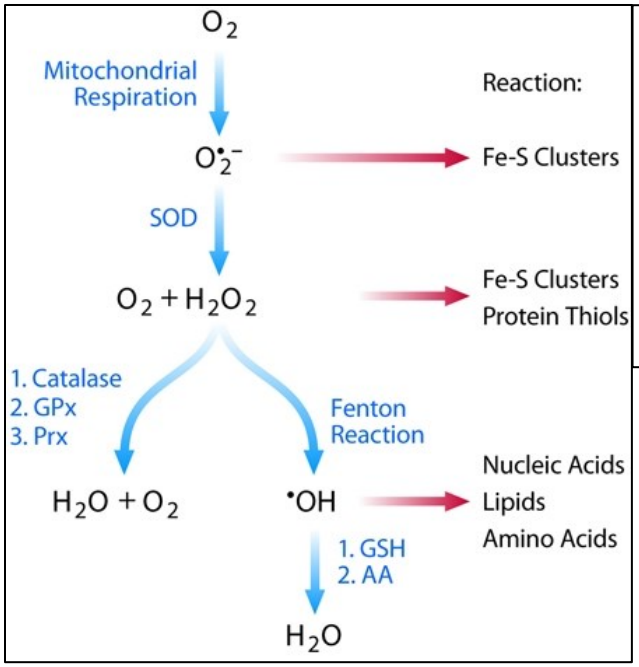

Figure 1.1. Mitochondria are the greatest endogenous source of ROS, which damage cellular components, such as proteins, nucleic acids, and lipids. Cells have developed several antioxidant mechanisms to detoxify ROS and prevent their permanent damage to the cell. This figure is from Merksamer *et al.*, 2013.

Under conditions of normal physiological levels of ROS production, ROS play important signaling and regulatory roles. A benefit of H_2O_2 serving as a signaling molecule is its small size and high diffusion rate (Sundaresan *et al.*, 1995). For example, in metazoans, ROS play a role in cell signaling in response to growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and angiotensin II (Finkel, 2011). For example, when platelet-derived growth factor PDGF stimulates rat vascular smooth muscle cells, H_2O_2 concentration increases dramatically 50 to 100-fold for a period of several minutes (Sundaresan *et al.*, 1995). H_2O_2 then acts as a second messenger, the downstream response to which includes tyrosine kinase signaling and activation of gene expression (Sundaresan *et al.*, 1995; Bae *et al.*, 1997). The exact mechanism by which H_2O_2 induces these changes is not well understood, but it is proposed to be through the reversible modification of protein tyrosine phosphatases (Hecht and Zick, 1992).

Transiently inhibiting tyrosine phosphatase activity results in a temporary shift in the normal balance between kinase and phosphatase activity. At least one cysteine residue is conserved and essential for catalytic activity of tyrosine phosphatases, and formation of a cysteine-phosphate intermediate in which a covalent thiol phosphate bond occurs at one of these cysteine residues is an essential part of their catalytic mechanism (Shibanuma *et al.*, 1990; Rao and Berk, 1992; Ohba *et al.*, 1994). H_2O_2 is proposed to directly oxidize cysteine sulfhydryl groups within tyrosine phosphatase, inhibiting activity (Hecht and Zick, 1992; Hockenbery *et al.*, 1993; Sundaresan *et al.*, 1995). Consistent with this, the addition of a reducing reagent, dithiothreitol (DTT), can reverse the inhibitory effects of H_2O_2 on tyrosine phosphatase activity in a dose-dependent manner (Hecht and Zick, 1992).

As detailed above in the example of growth factor response and ROS production, the regulatory role of ROS is likely mediated through the ability of ROS to introduce reversible conformational changes in proteins through the oxidation of cysteine residues (Finkel, 2011). There are many more examples that describe the normal signaling of ROS within the cell. ROS also play a role in cell signaling in the inflammatory response (Finkel, 2011). In mammalian cells, the accumulation of mitochondria-produced ROS activates the NLRP3 inflammasome, which in turn activates innate immune defenses through a cascade of signaling (Zhou *et al.*, 2011). Many transcription factors are also regulated by ROS, including Nrf2 and FoxO transcription factors, which control the expression of genes that encode for antioxidants (Singh *et al.*, 2010; de Keizer *et al.*, 2011). These processes are reviewed in greater detail elsewhere (Finkel, 2011). Taking

these examples, into account, it is clear that while ROS do contribute to cellular damage, some level of ROS within the cell is essential for normal cellular functions.

ROS-induced damage to DNA, lipids, and proteins may result in aging phenotypes and increased probability of death (Yan *et al.*, 1997; Przybyla-Zawislak *et al.*, 1999; Zhou *et al.*, 2008). As a result of the wide range of damage that ROS can elicit, the ROS generated by mitochondria not only have the potential to produce damage locally, but also throughout the cell. The Free Radical Theory of Aging was first proposed by D. Harman in the 1950s (Harman, 1956). In this theory, Harman postulates that the free radicals produced endogenously by the cells of an organism can contribute to the demise of the organism in the same manner as the irradiation of organisms, which also produces free radicals, induces mutations, cancer, and aging phenotypes (Hempelmann and Hoffman, 1953; Harman, 1956). He suggests that the propagation of free radicals damages cellular components, including nucleic acids, which could result in deleterious mutations, even causing cancer, and the resulting damage accumulates with age (Harman, 1956).

Several lines of evidence support the Free Radical Theory of Aging. Indeed, oxidative damage to proteins increases with age in human muscles, the eye lens, and brain tissue (Beal, 2002) and oxidative damage is a common feature of age-associated neurodegenerative pathology (Gibson *et al.*, 2010). Interestingly, there is more damage from oxidative stress than plaques and tangles of protein aggregates in brain of AD patients (Calingasan *et al.*, 1999; Gibson *et al.*, 2010) and an increase in superoxides

occurs prior to plaque formation in mouse models of AD (Gibson *et al.*, 2010). Importantly, the decrease in glucose metabolism and KGDHC activity in AD brain (discussed above) may be a result of oxidative stress because KGDHC is particularly sensitive to oxidative damage (Gibson *et al.*, 2010).

There is also a large body of evidence linking ROS to aging. In a 2006 publication, Bonawitz, *et al.* find that a mutant yeast strain with reduced lifespan generates increased mitochondrial ROS. Overexpression of mitochondria-targeted enzymes, such as superoxide dismutase or catalase, that break down ROS, expression of enzymes such as methionine sulphoxide reductase that repair oxidative damage to proteins, or treatment with antioxidants like vitamin E, reduces ROS levels and extends lifespan in yeast (Bonawitz *et al.*, 2006), the nematodes *Turbatrix aceti* (Vinegar eels), *Caenorbabditis elegans*, and *Caenorhabditis briggsae* (Epstein and Gershon, 1972; Kahn-Thomas and Enesco, 1982; Zuckerman and Geist, 1983; Harrington and Harley, 1988), *Drosophila melanogaster* (Sun and Tower, 1999) and mice (Schriner *et al.*, 2005). Moreover, deletion of the gene *MRG19*, which encodes for a nuclear ubiquitin protein ligase binding protein that may regulate the utilization of non-fermentable carbon sources, results in elevated ETC protein expression, decreased mitochondrial ROS, and lifespan extension in budding yeast (Kharade *et al.*, 2005).

Caloric restriction (CR) has also been shown to increase lifespan and healthspan in yeast, worms, flies, rodents, and even, though to a lesser extent, monkeys (Fontana *et al.*, 2010). The mechanism by which CR extends lifespan and decelerates aging

phenotypes is complex, but it is thought to be partially through increasing resistance to oxidative stress. Rats exposed to CR had elevated transcription of genes involved in combating ROS (Sreekumar *et al.*, 2002). Other mechanisms by which CR affects lifespan are reviewed elsewhere (Fontana *et al.*, 2010; Amigo and Kowaltowski, 2014).

The finding that antioxidant treatment can extend lifespan in model organisms raised the possibility that antioxidants may serve as therapeutic agents to prevent aging and age-related diseases. However, antioxidants have been studied in several clinical trials with varying outcomes (Gruber *et al.*, 2013). MitoQ is a mitochondria-targeted antioxidant (Gruber *et al.*, 2013). Its antioxidant component is ubiquinone, which protects against lipid peroxidation (Gruber *et al.*, 2013). The ubiquinone portion of MitoQ is conjugated to a lipophilic cation, triphenylphosphonium (TPP), which is taken up by mitochondria due to the relative negative charge of the mitochondrial matrix compared to the cytoplasm and the hydrophobic phenyl groups surrounding the positively charged phosphorus atom (Gruber *et al.*, 2013). Administration of MitoQ to PD patients for one year had no obvious effect on the clinical progression of the disease (Snow *et al.*, 2010). However, it is possible that the PD symptoms in these patients had progressed beyond a point from which they could be reversed or halted (Smith and Murphy, 2011; Gruber *et al.*, 2013). In contrast, treatment of patients with hepatitis C-induced chronic inflammation with MitoQ results in a decrease in plasma levels of alanine aminotransferase (ALT), which is a marker of liver damage (Gane *et al.*, 2010). It remains to be shown whether antioxidants will prove as successful therapeutics for age-

related diseases, and only further investigation through clinical trials can test this possibility.

Mitochondrial Quality Control Mechanisms

The total mitochondrial proteome includes over 1,000 different proteins, and considering the role of mitochondria in carrying out a wide range of essential functions, it is fitting that mitochondrial protein quality control mechanisms have evolved to maintain mitochondrial health, and consequently overall cell function and fitness (Prokisch *et al.*, 2004). With age-associated increases in ROS, quality control mechanisms may become impaired or overburdened, leading to increased damage to proteins, nucleic acids, and lipids, and consequently, reduced lifespan (Laun *et al.*, 2001; Deas *et al.*, 2009; Ugidos *et al.*, 2010).

Several mechanisms to prevent the damaging consequences of ROS exist within mitochondria. There are four major mitochondrial quality control pathways: (1) Antioxidants that degrade ROS or repair oxidatively damaged proteins. (2) Mitochondrial chaperones and proteases that promote re-folding or degradation, respectively, of misfolded proteins. (3) Mitophagy, the specific degradation of mitochondria by the vacuole (the lysosome in mammalian cells). (4) A newly identified pathway, mitochondria-associated degradation (MAD), in which mitochondrial proteins are ubiquitinated, extracted from the organelle, and degraded by the proteasome.

The relative contributions of these quality control mechanisms to mitochondrial maintenance are still not well understood, nor is it known whether they are differentially activated temporally in response to stress, or whether different types of stress preferentially activate specific pathways. Each mechanism will be discussed in greater detail below.

Mitochondrial Antioxidants

Protein sulfhydryl/thiol groups are extremely sensitive to oxidative stress (Herrero *et al.*, 2008). This is because they can become progressively oxidized (forming sulphenic, sulphinic, and sulphonic acid groups), eventually reaching a point at which the oxidation is irreversible, rendering the protein permanently damaged (Herrero *et al.*, 2008). Antioxidant systems exist throughout the cell to regulate ROS levels, thereby preventing irreversible oxidative damage to proteins, lipids, and nucleic acids. Antioxidants can be divided into two groups: (1) enzymes that act directly on ROS to convert them to less toxic forms and (2) enzymes that control the redox states of protein thiol/sulfhydrl groups, preventing irreversible oxidative damage. Both types of antioxidants will be discussed below, with a focus on mitochondrial antioxidant systems.

Superoxide dismutases (SODs) are enzymes with antioxidant activities that catalyze the conversion of $O_2^{\bullet-}$ to H_2O_2 (Herrero *et al.*, 2008) (Fig. 1.1). SOD activity depends upon redox active metal ions (Fridovich, 1995; Culotta *et al.*, 2006). There is a cytosolic SOD (Sod1p), which depends upon copper and zinc metal ions for activity, and a mitochondrial matrix SOD (Sod2p), which depends upon a manganese metal ion for

activity (Culotta *et al.*, 2006). Interestingly, a small portion of Sod1p localizes to the mitochondrial IMS (Sturtz *et al.*, 2001).

While H_2O_2 is less reactive than $O_2^{\bullet-}$, it can still cause oxidative damage and it can diffuse freely throughout the cell. The antioxidant enzyme catalase, which exists in budding yeast as a cytosolic enzyme (Ctt1p), and as a peroxisome enzyme (Cta1p), catalyzes the conversion of H_2O_2 to H_2O and O_2 (Herrero *et al.*, 2008) (Fig. 1.1). If H_2O_2 is not fully reduced to H_2O and O_2 by catalase, it can be partially reduced to the very reactive oxidant, the hydroxyl radical ($^{\bullet}OH$). The most well-known of these partial reduction reactions is the Fenton reaction, in which iron is the electron donor (Herrero *et al.*, 2008) (Fig. 1.1).

Peroxidases have antioxidant enzymatic activities that can reduce inorganic and organic peroxides into their detoxified alcohols through the use of cysteine thiols in their active sites (Herrero *et al.*, 2008). Two electron donors function as cofactors in these reactions, depending on the type of peroxidase. Glutathione peroxidases (GPXs) use glutathione as the electron donor. Glutathione is the most abundant non-protein thiol within cells (Hwang *et al.*, 1992). It exists in both its reduced state (GSH), which can function as an antioxidant, and its oxidized state, glutathione disulfide (GSSG) (Hwang *et al.*, 1992) (Fig. 1.1). Glutathione reductase catalyzes the reduction of GSSG to GSH, keeping the intracellular ratio of GSH to GSSG high, generally with more than 99% in the reduced state (Beer *et al.*, 2004). *S*-glutathionylation is a reaction in which protein thiol/sulfhydryl groups react with GSH to form mixed disulphide groups (Herrero *et al.*,

2008). The reversibility of this reaction makes it protective, preventing progressive oxidation of sulphydryl/thiol groups that irreversibly damage proteins under conditions of oxidative stress (Herrero *et al.*, 2008). There exits both a cytosolic and mitochondrial GSSG/GSH pool, the redox states of which are independently regulated (Beer *et al.*, 2004). Interestingly, treating cells with the reduced form of glutathione increases the lifespan of yeast under conditions of oxidative stress (Nestelbacher *et al.*, 2000). Thioredoxin peroxidases, also called peroxiredoxins, which will not be discussed in detail here, function similarly. For a more extensive review of this topic, reference Herrero *et al*., 2008.

Mitochondrial Chaperone Proteins

Protein misfolding exposes hydrophobic residues that are normally not exposed and that can seed the misfolding of other proteins and produce protein aggregates. Protein aggregates can produce toxic gain-of-function phenotypes and are associated with disease states, such as AD (aggregation of Aβ and tau), PD (aggregation of α-synuclein), HD (aggregation of huntingtin), and prion diseases (aggregation of the prion protein) (Morimoto, 2006, 2008). Chaperone proteins are diverse families of large proteins that promote proper folding of newly synthesized proteins, re-folding of misfolded proteins, stabilization of unfolded proteins, and unfolding of proteins for degradation by proteolytic machinery (Mayer, 2010). Unlike enzymes that have evolved to have catalytic sites designed to work on specific substrate proteins, chaperone proteins have evolved to have more general associations with a wide range of proteins based on hydrophobic interactions (Saibil, 2013).

One major class of molecular chaperones is the ATP-dependent chaperone family called the heat shock proteins (HSPs). While there are several orthologues of these proteins, including Hsp60p and Hsp70p, localized to various cellular compartments, only their mitochondrial variants will be discussed in depth here. Hsp60ps, also called chaperonins, are divided into two groups (Mayer, 2010). Group I is common to all prokaryotes, mitochondria, and plastids (Gutsche *et al.*, 1999; Spiess *et al.*, 2004). Group II is common to archaea and the cytosol of eukaryotes (Gutsche *et al.*, 1999; Spiess *et al.*, 2004). Group I Hsp60p chaperonins are ATPases that are composed of two stacked rings, each containing seven subunits, which surround a central hydrophobic substrate binding pocket (Mayer, 2010). Group I chaperonins also require a co-chaperone to enclose the substrate within the chamber (Mayer, 2010). In the ATP-free state, the hydrophobic region of a chaperonin is exposed, and can bind to exposed hydrophobic regions on an unfolded protein (Mayer, 2010). ATP binding to the first ring induces conformational changes in the chaperonin which decrease the hydrophobicity of the protein interior (Mayer, 2010). Refolding of the bound protein occurs upon ATP hydrolysis, which occurs slowly, allowing time for proper re-folding of the substrate. Binding of ATP to the second ring results in release of the folded protein. At this stage, ADP bound to the first ring is also released (Mayer, 2010). If the substrate is not properly folded, the chaperonin undergoes another ATP cycle (Mayer, 2010).

Hsp70p plays an important role in chaperoning proteins as they translocate across membranes (Koll *et al.*, 1992). Since the majority of mitochondrial proteins are encoded

by nuclear genes and imported across one or more mitochondrial membranes, Hsp70p chaperones are important for mitochondrial protein maintenance. When a protein is delivered to the mitochondrial matrix, the protein substrate is transferred from Hsp70p to Hsp60p, which promotes proper folding (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). However, if the protein is ultimately to be delivered to the intermembrane space (IMS), it remains bound to Hsp60p until it again traverses the inner mitochondrial membrane (IMM) (Koll *et al.*, 1992). The deletion of either *HSP70* or *HSP60* is lethal (Bender *et al.*, 2011).

The prohibitin (PHB) complex is a conserved protein chaperone composed of the homologous scaffold proteins Phb1p and Phb2p (Van Aken *et al.*, 2010). While there are many prohibitin homologues, individual cells contain multiple prohibitin proteins. Therefore, these proteins may not be fully functionally redundant (Van Aken *et al.*, 2010). Phb1 was originally identified in 1989 in a screen for possible tumor suppressors that are normally highly expressed in rat liver cells (McClung *et al.*, 1989; Thuaud *et al.*, 2013). The prohibitins are known for their diverse roles in many different signaling pathways involved in the immune response, responses to growth factors and steroid hormones, and in the regulation of metabolism, mitochondrial biogenesis, cell migration, and even cell division and survival (Thuaud *et al.*, 2013). However, for the purposes of this work, the discussion of Phb1p and Phb2p focuses on their roles in mitochondrial quality control.

Phb1p and Phb2p bind with each other to form heterodimers that assemble into a circular supercomplex of 12 to 16 heterodimers. The complex localizes to the IMM through the hydrophobic N-terminal domains of both Phb1p and Phb2p, and protrudes into the IMS (Van Aken *et al.*, 2010; Thuaud *et al.*, 2013). Plants in which the prohibitins are silenced or mutated exhibit stunted growth and abnormal root meristem morphology (Jiang *et al.*, 2006; Van Aken *et al.*, 2007). Furthermore, mitochondrial morphology may be regulated by the prohibitins. Tobacco plants and *Arabidopsis* lacking functional prohibitins have abnormal mitochondrial cristae and swollen mitochondria, respectively (Ahn *et al.*, 2006; Van Aken *et al.*, 2007). Silencing PHBs in petunia plants results in increased respiration rates (Chen *et al.*, 2005). In *C. elegans* and mice, the prohibitins are required for embryonic development and embryonic fibroblast proliferation (Artal-Sanz *et al.*, 2003; Park *et al.*, 2005; 2008; He *et al.*, 2008).

The PHB complex promotes folding of newly synthesized proteins, including ETC complex I proteins (Nijtmans *et al.*, 2000). The PHB complex may carry out this function by binding to newly synthesized proteins and preventing their degradation by the mitochondrial *m*-AAA protease (Steglich *et al.*, 1999). Phb1p promotes the stability of mtDNA encoded subunits of the respiratory chain by regulating mtDNA copy number (Kasashima *et al.*, 2008). Prohibitins may contribute to mtDNA stability through their role in maintaining normal organization of the IMM, which binds to mtDNA nucleoids (Artal-Sanz and Tavernarakis, 2009).

PHB1 and *PHB2* undergo stress-activated expression and have been implicated in mitochondrial quality control and aging (Artal-Sanz and Tavernarakis, 2009; Van Aken *et al.*, 2010). Interestingly, silencing of prohibitins in tobacco plants results in aging phenotypes including premature leaf senescence, cell death, increased ROS production and decreased mitochondrial membrane potential, suggesting a role for the prohibitins in aging (Van Aken *et al.*, 2010). Consistent with this, inhibition of PHB complex formation results in decreased replicative lifespan and other aging phenotypes in yeast (Coates *et al.*, 1997; Berger and Yaffe, 1998). There is also evidence that prohibitins themselves become damaged with age. In rats, PHB1 becomes increasingly phosphorylated with age and during the initiation of apoptosis (Thompson *et al.*, 1999). It is speculated that this modification might decrease PHB complex formation by preventing binding of PHB1 and PHB2 (Van Aken *et al.*, 2010).

Mitochondrial Proteases

Mitochondrial proteases degrade mitochondrial proteins that fail to be properly folded. Two important classes of age-related proteases found in mitochondria are the ATPases associated with diverse cellular activities (AAA) proteases, exemplified by Yme1p and the Lon proteases.

AAA proteases are ATP-dependent metallopeptidases that contain an N-terminal transmembrane domain, an ATPase domain, and a C-terminal metallopeptidase domain (Janska *et al.*, 2013). The *i*- and *m*-AAA proteases span the IMM and expose their catalytic domains to the IMS and the mitochondrial matrix, respectively (Janska *et al.*,

2013). Both the *i*- and *m*-AAA proteases contain a pocket that binds to unfolded, but not folded proteins (Janska *et al.*, 2013). This structural feature suggests that the AAA proteases do not recognize specific protein sequences, but rather, they degrade unstructured and flexible substrates (Janska *et al.*, 2013).

Yme1p is the catalytic subunit of a homooligomeric *i*-AAA protease complex that acts on membrane-bound or membrane-associated mitochondrial proteins. Yme1p protease complex substrates include respiratory chain subunits, unassembled small Tim proteins (which mediate protein import across the IMM), and Ups1p and Ups2p (mitochondrial IMS proteins that play a role in phospholipid metabolism) (Potting *et al.*, 2010; Baker and Haynes, 2011; Böttinger and Becker, 2012; Rugarli and Langer, 2012; Voos, 2013). Furthermore, yeast cells lacking *YME1* accumulate protein aggregates in the IMS, indicating that Yme1p may play both proteolytic and chaperone roles (Schreiner *et al.*, 2012). For unknown reasons, cristae morphology is also abnormal in *yme1Δ* cells (Schreiner *et al.*, 2012). Recent studies indicate that Yme1p cleaves the mitophagy protein, Atg32p, at its C-terminus, allowing Atg32p to efficiently bind Atg11p, which is necessary for efficient mitochondria-specific autophagy (Wang *et al.*, 2013), a process that will be discussed below.

The ATP-dependent protease known as the Lon protease was originally shown to degrade misfolded proteins in *Escherichia coli* (Ngo *et al.*, 2013). The Lon protease has three domains, one that binds ATP, another that binds the protein substrate, and a third that comprises the proteolytic site (Ugarte *et al.*, 2010). While ATP hydrolysis is required

to unfold proteins for degradation by the Lon protease, ATP hydrolysis is not required to degrade unfolded proteins, but it does serve to increase the rate of substrate degradation (Van Melderen *et al.*, 1996; Ondrovicová *et al.*, 2005). The Lon protease homologue in yeast, Pim1p, localizes to the mitochondrial matrix (Bender *et al.*, 2011).

Phenotypic analysis of Lon protease deletion mutants supports a role for this protein in mitochondrial quality control. Deletion of *PIM1* in yeast results in an increase in 14 carbonylated proteins (most of which are mitochondrial metabolic enzymes or subunits of the respiratory chain) (Bayot *et al.*, 2010), an increase in ubiquitinated cytosolic proteins, a decrease in 20S proteasome capacity (Erjavec *et al.*, 2013), and defects in maintenance of mtDNA and mitochondrial respiratory activity (Van Dyck *et al.*, 1994; van Dyck *et al.*, 1998). During oxidative stress, Pim1p degrades dihydroxyacid dehydratase and aconitase (Bender *et al.*, 2011). Under heat stress, Pim1p degrades a subunit of the F_1F_O ATPase and the β subunit of MPPβ, a matrix processing peptidase (Suzuki *et al.*, 1994). In human tissue culture cells, knockdown of the Lon protease results in mitochondrial dysfunction, mitochondrial morphology defects, accumulation of electron-dense bodies within mitochondria, and cell death (Levine, 2005; Ngo and Davies, 2009). Indeed, in human tissue culture models, stress including heath shock, serum starvation, and oxidative stress, result in an increase in the expression of the Lon protease (Ngo and Davies, 2009).

Other studies further support a role for the Lon protease in aging. Lon protease mRNAs, steady-state protein levels, and protease activity decrease in muscle tissue of old mice

compared to young mice (Lee *et al.*, 1999), in liver of old rats (Bakala *et al.*, 2003), and in the heart tissue of old rats (Delaval *et al.*, 2004), respectively. Moreover, aged yeast have a decrease in Pim1p proteolytic activity, and increases in electron-dense inclusion bodies, oxidative protein damage, and ubiquitinated cytosolic proteins (Erjavec *et al.*, 2013). Consistent with this link between the Lon protease and aging, yeast lacking *PIM1* exhibit a decrease in replicative lifespan (Erjavec *et al.*, 2013).

Peptide products of the AAA proteases are between 10 and 20 amino acids, are usually about three kDa, and are either exported from the mitochondrion or degraded further by various oligopeptidases (Janska *et al.*, 2013). A membrane channel called the half-type ABC-transporter, Mdl1p, in the IMM mediates export of these peptides from the matrix to the IMS (Young *et al.*, 2001; Augustin *et al.*, 2005; Kambacheld *et al.*, 2005). From the IMS, peptides less than two kDa diffuse through the semi-permeable outer mitochondrial membrane (OMM) to the cytosol (Neutzner et al., 2007).

Mitophagy, the Specific Degradation of Mitochondria by the Lysosome/Vacuole

Autophagy is a catabolic process by which lysosomes/vacuoles degrade proteins and organelles in response to starvation, developmental cues, or defects in protein or organelle function. The three major types of autophagy are macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Martinez-Vicente and Cuervo, 2007). In macroautophagy, a portion of the cytosol or an organelle is engulfed by a double-membrane compartment called an autophagosome. The contents of the autophagosome are degraded upon fusion with the lysosome (mammals) or the vacuole

(yeast). Although up-regulated under stress conditions, macroautophagy is normally active at a basal level and is essential for the viability of certain cell types, notably neurons (Hara *et al.*, 2006). Microautophagy involves direct engulfment of cellular components by the lysosome/vacuole (Martinez-Vicente and Cuervo, 2007). CMA does not require an autophagosome and is a selective process in which the cargo contains a specific motif that is recognized by a cytosolic chaperone and allows for autophagy of the cargo (Martinez-Vicente and Cuervo, 2007).

Several specific types of macroautophagy have been identified. For example, pexophagy is the selective autophagy of peroxisomes (Dunn *et al.*, 2005) and mitophagy is the selective autophagy of mitochondria (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009). In yeast, the autophagy-related (ATG) gene *ATG32* is essential for mitophagy, but is not required for general autophagy or other types of selective autophagy (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009). Atg32p is an integral OMM protein and plays a role in mitophagy under starvation conditions (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009) (Fig. 1.2). Phosphorylation of Atg32p at S114 results in binding of Atg32p to Atg11p and recruitment of Atg11p to the mitochondrial surface (Aoki *et al.*, 2011). At the OMM, Atg11p serves as an adaptor protein that recruits other autophagy-related proteins to the OMM, leading to engulfment of mitochondria by an autophagosome, and their subsequent transport to and degradation by the vacuole (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009).

Another yeast OMM protein, Uth1p, is also necessary for mitophagy (but not general autophagy) under conditions of nitrogen starvation and rapamycin treatment (Kissová *et al.*, 2004). The yeast mitochondrial protein Aup1p is a phosphatase localized to the IMS that is also necessary for efficient mitophagy during stationary phase (Tal *et al.*, 2007; Abeliovich, 2011). Aup1p plays a role in the retrograde signaling (RTG) pathway, whereby mitochondria experiencing stress conditions send signals to the nucleus that prompt downstream stress response signals (Journo *et al.*, 2009). Aup1p regulates the phosphorylation of Rtg3p, a transcription factor that controls the transcription of genes involved in the RTG pathway response (Journo *et al.*, 2009). Interestingly, during stationary phase mitophagy, different mitochondrial matrix proteins undergo vacuolar degradation at different rates, indicating that there may be distinct compartmentalization of different proteins that coincides with stationary phase mitophagy (Abeliovich *et al.*, 2013). Unfortunately, the authors of this work did not investigate whether the distinctly segregated matrix proteins that undergo vacuolar degradation at varying rates were distinguishable based on quality, so it remains undetermined whether this mechanism is related to mitochondrial quality control.

In many cultured mammalian cell models, including primary neurons, cancer cell lines, such as HeLa cells, and mouse embryonic fibroblasts, mitophagy has a direct role in eliminating severely damaged mitochondria (Narendra and Youle, 2011). Treatment of these cells with proton ionophores results in loss of mitochondrial membrane potential ($\Delta\psi$), and with prolonged treatment, apoptosis. Mitochondria in carbonyl cyanide m-

chlorophenylhydrazone (CCCP)-treated cells are targeted for degradation by the lysosome (Jin *et al.*, 2010; Vives-Bauza *et al.*, 2010; Narendra and Youle, 2011).

Other studies support a role for PINK1 and Parkin, targets for mutation in familial PD, in marking damaged mitochondria for degradation by mitophagy. PINK1 accumulates on the surface of mitochondria with low Δψ, which leads to recruitment of Parkin to that site (Fig. 1.2). The ubiquitination of mitochondrial proteins by Parkin leads to recruitment of proteins necessary for autophagosome formation, including LC3 II, to the organelle. LC3 II-labeled mitochondria accumulate in the perinuclear region, a subcellular location associated with autophagy. Parkin has the capacity to ubiquitinate OMM proteins, including the mitofusins (Mfn1 and 2), mitochondrial Rho GTPases RhoT1 and 2 (Miro in *D. melanogaster*), and the voltage dependent transporters VDAC1, 2, and 3 (Wang *et al.*, 2011b; Sarraf *et al.*, 2013).

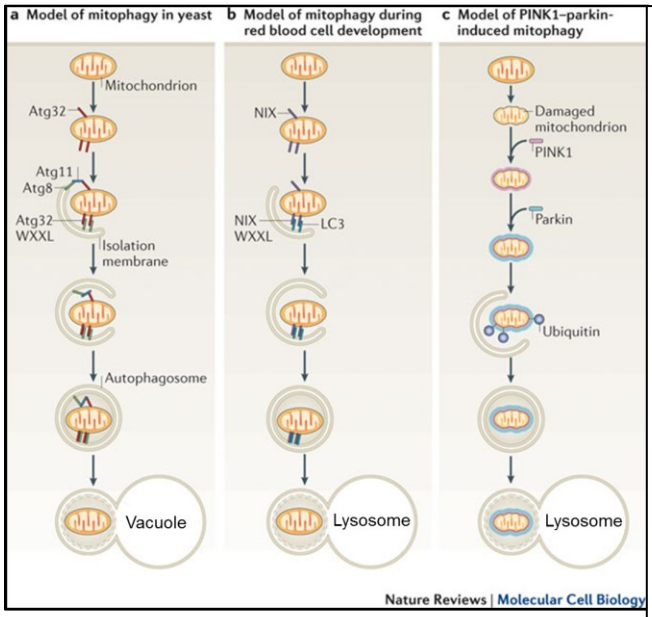

Figure 1.2. (a) In yeast, mitophagy is activated under starvation conditions. Mitophagy in yeast requires the integral mitochondrial protein, Atg32p, which binds to the adaptor protein Atg11p. (b) In mammalian cells, mitophagy is required for red blood cell maturation to produce a mature red blood cell, which is devoid of mitochondria. In this scenario, mitophagy is mediated through NIX. NIX and Atg32p may be orthologues, as they share the WXXL binding motif necessary for the binding of NIX to LC3, and of Atg32p to the LC3 yeast homologue, Atg8p. (c) Mitophagy plays a role in mitochondrial quality control in mammalian cells, targeting severely damaged mitochondria with low Δψ for lysosomal degradation. This figure is modified from Youle and Narendra, 2011.

Interestingly, PINK1 and Parkin may act together to modulate mitochondrial homeostasis through mitochondrial motility. Miro is a protein that anchors kinesin to the OMM, and is necessary for mitochondrial movement. PINK1 phosphorylates Miro, which results in its ubiquitination by Parkin and degradation by the proteasome, defects in recruitment of kinesin to the organelle, and defects in mitochondrial movement (Wang *et al.*, 2011b). Together, these findings support the model that PINK1 and Parkin target less fit mitochondria for mitophagy and play a role in mitochondrial quality control in cultured neurons and other metazoan tissue culture models when mitochondria are damaged beyond repair.

In other cell types, including yeast and reticulocytes, mitophagy has been implicated in mitochondrial *quantity* control, not mitochondrial *quality* control. In yeast, Atg32p-mediated mitophagy is activated by nitrogen starvation (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009). The mitochondria marked for degradation in this pathway are not degraded because they are damaged or less fit; instead, they are degraded to provide essential macromolecules for survival during starvation. Elimination of mitochondria when they are no longer beneficial to the cell also occurs during red blood cell development and is mediated by the OMM protein Nix. In reticulocytes, Nix mediates the degradation of all mitochondria to generate the mature organelle-free red blood cell (Kanki, 2010) (Fig. 1.2). Interestingly, Atg32p and Nix share the WXXL motif, which binds to the autophagic machinery protein LC3A in mammals, and its homologue in yeast, Atg8p. Thus, Atg32p and Nix may be orthologues that control mitochondrial quantity (Kanki, 2010).

The Ubiquitin-Proteasome System and Mitochondrial Protein Degradation

Components of the ubiquitin-proteasome system (UPS) include ubiquitin, enzymes that catalyze the tagging of substrates with ubiquitin, and the proteasome (Fig. 1.2). Ubiquitin is a 76-amino acid residue polypeptide, containing seven lysine residues, that covalently binds with proteins in a post-translational modification event (Walczak *et al.*, 2012; Clague *et al.*, 2013). The proteasome is a protein complex that forms a hollow cylindrical structure (Alberts *et al.*, 2002). ATPases located near the entrance of the cylinder unfold the ubiquitinated protein substrate. Proteases that face the interior of the cylindrical chamber catalyze degradation of unfolded, ubiquitinated proteins (Alberts *et al.*, 2002). During protein ubiquitination, ubiquitin is first bound to a ubiquitin-activating enzyme (E1) containing an active-site cysteine that binds to the carboxy-terminal glycine of ubiquitin via a reactive thioester bond (Walczak *et al.*, 2012). The ubiquitin is then transferred to a ubiquitin conjugating enzyme (E2) through a similar mechanism (Lorick *et al.*, 2006; Walczak *et al.*, 2012). E2s mediate the transfer of ubiquitin to E3 ligases, which bind to substrate proteins and catalyze the transfer of ubiquitin to a lysine residue of the substrate protein or to a chain of ubiquitin monomers bound to a substrate (Lorick *et al.*, 2006). Polyubiquitination can occur via Lys48 or Lys63 chain extension. Lys48 is associated with marking substrates for proteasomal degradation, while Lys63 polyubiquitin chains play a role in signaling for DNA repair, autophagy, and endocytosis (Sims *et al.*, 2012). It is worthy to note that ubiquitination is reversible. Deubiquitylating enzymes (DUBs) can remove ubiquitin from a protein substrate. This process contributes to maintaining free ubiquitin levels in cells, reversing the targeting of a protein for degradation by the proteasome, and regulating the dynamics of other

processes for which ubiquitination is a signal, such as in endocytosis, which is reviewed elsewhere (Walczak *et al.*, 2012; Clague *et al.*, 2013).

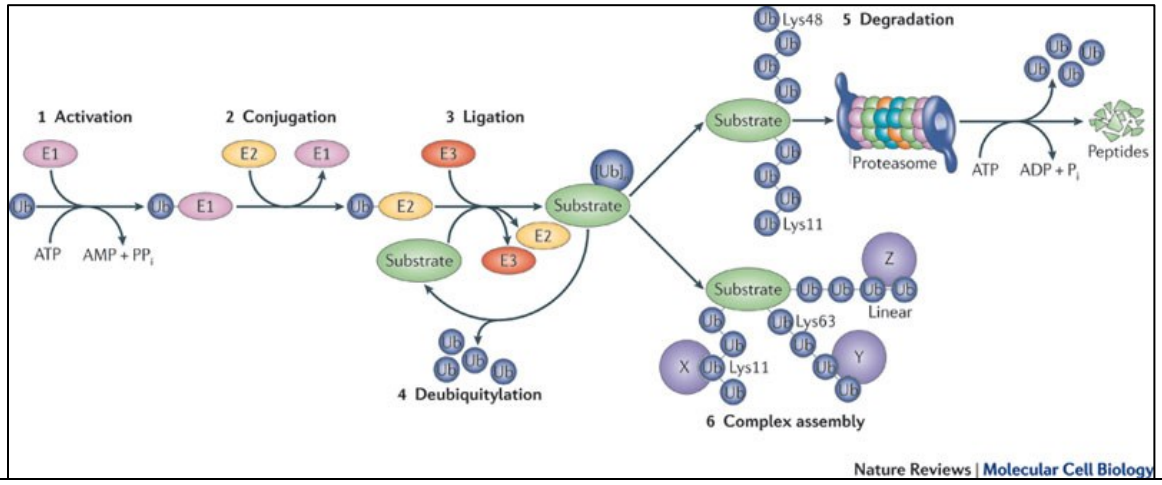

Figure 1.3. Major players in the UPS are ubiquitin, enzymes that catalyze the tagging of substrates with ubiquitin, and the proteasome. The proteasome is a large hollow cylindrical protein complex. ATPases located near the entrance of the cylinder unfold the ubiquitinated protein substrate, after which the substrate is degraded by proteases facing the interior of the chamber. Ubiquitin is first bound to a ubiquitin-activating enzyme (E1), and then transferred to a ubiquitin conjugating enzyme (E2). E2s mediate the transfer of ubiquitin to E3 ligases, which bind to substrate proteins and catalyze the transfer of ubiquitin to the substrate protein or to a chain of ubiquitin monomers bound to a substrate. Deubiquitylating enzymes (DUBs) can remove ubiquitin from a protein substrate. This figure is from Vucic *et al.*, 2011.

The UPS was originally thought to degrade only cytosolic proteins. However, degradation of Fzo1p, a yeast integral OMM GTPase that promotes mitochondrial fusion, depends upon ubiquitin, the activity of the 20S proteasomal core particle, and the ATPase subunit of the 19S regulatory complex of the proteasome (Escobar-Henriques *et al.*, 2006; Neutzner *et al.*, 2007). The mammalian mitofusins, Mfn1 and Mfn2, are also degraded by the UPS (Tanaka *et al.*, 2010). Interestingly, the protein FK506 (Fpr3p in yeast) anchors the 26S proteasome to membranes of organelles including mitochondria (Nakagawa *et al.*, 2007). Thus, it is now clear that the UPS also targets mitochondrial proteins for degradation. Moreover, evidence suggests that

intramitochondrial proteins, such as the integral mitochondrial IMM uncoupling protein, Ucp2p, are also degraded by this process (Azzu and Brand, 2010).

Degradation of mitochondrial proteins by the UPS occurs via the mitochondria-associated degradation pathway (MAD) (Neutzner *et al.*, 2007). MAD is similar to the pathway by which misfolded proteins in the ER are eliminated, ER-associated degradation (ERAD) (Werner *et al.*, 1996). Both the ERAD and MAD pathways are conserved from yeast to mammals. In the ERAD pathway, misfolded proteins in the ER lumen bind chaperone proteins, are retro-translocated across the ER membrane through the protein translocating translocon complex (composed of Sec61p, Sbh1p, and Sss1p) where they are ubiquitinated, and are subsequently pulled from the membrane and degraded by the proteasome (Neutzner *et al.*, 2007). In MAD, mitochondrial proteins are also ubiquitinated, either by mitochondrial E3 ligases, such as Mdm30p, or by cytosolic E3 ligases that are recruited to mitochondria, such as Rsp5p (Neutzner *et al.*, 2007). The energy necessary to remove the ubiquitinated protein from the membrane is provided by an AAA ATPase called Cdc48p in yeast or VCP/p97 in mammals. Currently, the mechanism whereby proteins are retro-translocated across mitochondrial membranes is not well understood.

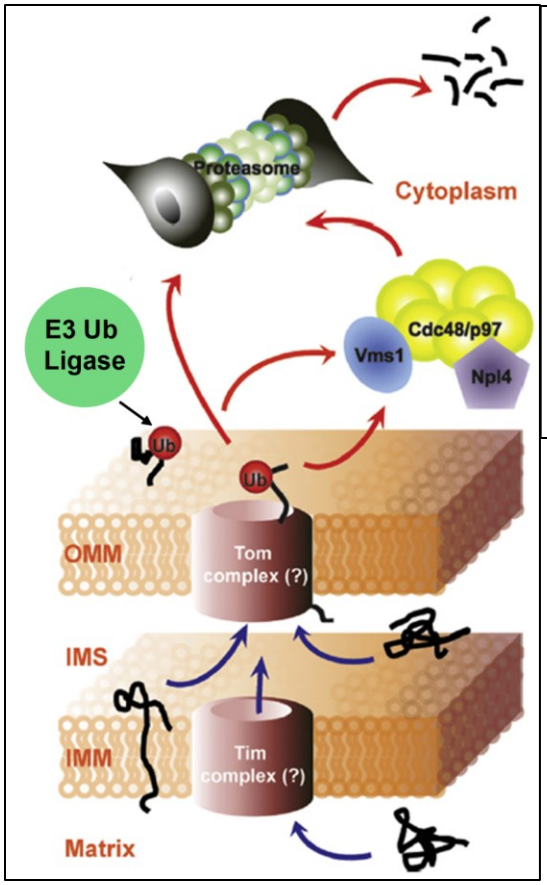

Figure 1.4. In MAD, mitochondrial proteins are ubiquitinated, either by mitochondrial E3 ligases or cytosolic E3 ligases that are recruited to mitochondria. The energy necessary to remove the ubiquitinated protein from the membrane is provided by an AAA ATPase called Cdc48p in yeast or VCP/p97 in mammals. Once in the cytosol, ubiquitinated protein substrates are degraded by the proteasome. This figure is modified from Heo and Rutter, 2011.

Cdc48p is one subunit of a polyubiquitin-selective segregase complex involved in ERAD and MAD. It is recruited to ER or mitochondria, extracts ubiquitinated proteins from these organelles, and presents ubiquitinated proteins to the proteasome for degradation (Heo *et al.*, 2010). Cdc48p is an essential protein, and therefore, much of its characterization in yeast has depended on use of a temperature-sensitive mutant (Moir *et al.*, 1982). In ERAD, Cdc48p binds with Npl4p and Ufd1p, the last of which confers specificity for ER targeting. In MAD, Cdc48p is thought to bind with Npl4p and Vms1p. Npl4p is a cofactor of the Cdc48p segregase complex that assists in the degradation of polyubiquitinated proteins by the proteasome (Hitchcock *et al.*, 2001). One group

postulates that Vms1p confers specificity for mitochondrial targeting of Cdc48p under certain conditions (Heo *et al.*, 2010; Heo and Rutter, 2011). Heo et al. (2010) find that a functional Vms1p-GFP fusion protein is cytosolic in glucose-based synthetic medium, glycerol-based rich and synthetic medium, and raffinose-based rich and synthetic medium (Heo *et al.*, 2010). However, they found that Vms1p-GFP localizes to mitochondria in rho^0 yeast cells that lack mtDNA, in cells lacking the mitochondrial superoxide dismutase (*SOD2*), in late log phase yeast, and in yeast treated with antimycin A, oligomycin, CCCP/FCCP or H_2O_2 and rapamycin (Heo *et al.*, 2010). Based on these results, they concluded that low or dissipated $\Delta\psi$ and/or oxidative stress induces Vms1p-GFP to localize to mitochondria (Heo *et al.*, 2010). The Cdc48p-Npl4p-Vms1p segregase complex colocalizes with mitochondria mainly during stress, but also to some extent under normal conditions, raising the possibility that MAD plays a role in mitochondrial protein quality control under all conditions (Heo *et al.*, 2010; Shanbhag *et al.*, 2012). Finally, Cdc48p can localize to some extent to mitochondria even in cells lacking *VMS1* (Heo *et al.*, 2010). Therefore, Vms1p contributes to but is not essential for targeting of the Cdc48p segregase complex to the organelle.

Doa1p, a protein containing a ubiquitin binding domain, is necessary for ubiquitin-mediated degradation and interacts physically with Cdc48p (Mullally *et al.*, 2006). Doa1p, degradation of alpha 1, was originally characterized for its involvement in the degradation of a yeast transcriptional regulator called the alpha two repressor, that controls the mating type of budding yeast (Hochstrasser and Varshavsky, 1990). Doa1p facilitates the formation of the Doa1p-Cdc48p-ubiquitin complex, bringing Cdc48p into

contact with ubiquitinated substrates (Mullally *et al.*, 2006). The human homologue of Doa1p, PLAA, plays an analogous role, serving as an ubiquitin cofactor that recruits the Cdc48p homologue, p97/VCP, to ubiquitinated substrates (Mullally *et al.*, 2006). Doa1p also plays a role in ubiquitin homeostasis, maintaining total cellular ubiquitin levels (Lis and Romesberg, 2006).

The human homologue of Cdc48p, VCP or p97, has been linked to diseases of the nervous system, muscle, and bone, including ALS, Parkinsonisms, frontotemporal dementia, Paget's disease, and Charcot-Marie-Tooth Disease (Watts *et al.*, 2004; Weihl *et al.*, 2007; Badadani *et al.*, 2010; Yamanaka *et al.*, 2012; Feely *et al.*, 2014). Moreover, recent findings revealed a link between mitochondrial oxidative stress and ubiquitin-dependent proteolysis in both *C. elegans* and human cell lines from patients with defects in mitochondrial metabolism (Segref *et al.*, 2014). *C. elegans* harboring mutations that increase levels of mitochondrial ROS are defective in the degradation of a GFP-based cytosolic protein substrate of the UPS, presumably because the proteasome is actively engaged in degrading damaged mitochondrial proteins (Segref *et al.*, 2014). Degradation of the cytosolic proteins by the proteasome under conditions of mitochondrial oxidative stress can be restored both by antioxidants and overexpression of proteins that increase the assembly and activity of the 26S proteasome (Vilchez *et al.*, 2012; Segref *et al.*, 2014).

The Effect of Different Methods of Mitochondrial Stress Induction in Laboratory Experiments on Our Understanding of Mitochondrial Quality Control Mechanisms

Available evidence indicates that mitophagy is activated only during starvation, or when mitochondria are no longer needed for cellular function, as in the case of erythrocyte maturation or during the degradation of paternal mitochondria in fertilization, or when mitochondria are beyond the point of repair in a stressed cell (Schweers *et al.*, 2007; Sandoval *et al.*, 2008; Kanki *et al.*, 2009b; Okamoto *et al.*, 2009; Al Rawi *et al.*, 2011; Sato and Sato, 2011; Bhatia-Kiššová and Camougrand, 2013; Deffieu *et al.*, 2013). As described below, there is growing skepticism that mitophagy is used for mitochondrial quality control during normal cellular homeostasis.

First, many studies use long-term (e.g., 24 hr) treatment with proton ionophores to induce mitochondrial stress. However, proton ionophores disrupt all membranes in the cell and induce apoptosis. Thus, it is possible that the autophagy observed under these conditions is a consequence of cellular pathology and not of perturbation of mitochondria. Second, recent evidence indicates that bulk autophagy, but not specific degradation of mitochondria, is increased during conditions of mitochondrial stress in patients with mitochondrial diseases. This group used skin fibroblast cultures from 11 patients with known, distinct OXPHOS defects (Morán *et al.*, 2014). Interestingly, from these patients, only two of the 11 cell lines showed decreased mitochondrial Δψ (Morán *et al.*, 2014). Furthermore, these findings lend credence to the already existing skepticism regarding the finding that mitophagy is a major contributor to mitochondrial quality control during normal cellular homeostasis. Patient cells with defects in complex I did have elevated recruitment of Parkin to mitochondria and elevated autophagosome accumulation (Morán *et al.*, 2014), but this group did not find any increase in mitophagy

in these cells. Thus, they conclude that mitochondrial defects can induce an increase in autophagosome formation, suggesting that bulk autophagy may be activated without activating mitophagy (Morán *et al.*, 2014).

Taking into account the controversy regarding the role of mitophagy in maintaining mitochondrial quality control, it is evident that greater care should be taken when studying quality control of mitochondria, particularly regarding the manner and extent to which mitochondria are stressed in laboratory experiments. Moreover, although multiple mechanisms for mitochondrial quality control have been identified, their relative contribution to mitochondrial maintenance under conditions of low-level mitochondrial stress has not been assessed. Here, we studied the relative contributions of mitophagy, autophagy, select mitochondrial chaperonins and proteases, and MAD and the proteasome in mitochondrial quality control under conditions that were designed to mimic one aspect of aging: chronic low levels of mitochondrial ROS. The agent that we used for these studies to induce mitochondrial oxidative stress is paraquat (1,1'-dimethyl-4,4'-bipyridinium) (PQ).

PQ is a small molecule that is taken up across the IMM in a Δψ-dependent manner, making it more mitochondria-specific than proton ionophores (Bus *et al.*, 1976; Cochemé and Murphy, 2008). PQ is an herbicide that has been etiologically linked as a factor in PD in humans (Dinis-Oliveira *et al.*, 2006). Mitochondria take up PQ only as the dication (PQ^{2+}), not the radical monocation ($PQ^{\bullet+}$) (Fig. 1.5) (Cochemé and Murphy, 2008). One of the first effects observed upon PQ treatment is mitochondrial swelling

(Hirai *et al.*, 1992; Wang *et al.*, 1992). Due to the very negative reduction potential of PQ, only a very limited number of molecules within the cell are capable of reducing it (Bus and Gibson, 1984). Inside the mitochondrial matrix, PQ undergoes single-electron reduction (primarily by NADPH dehydrogenase in yeast and by complex I in mammals) to form a radical that reacts rapidly with oxygen to form superoxides (Bus *et al.*, 1976; Cochemé and Murphy, 2008) (Fig. 1.5).

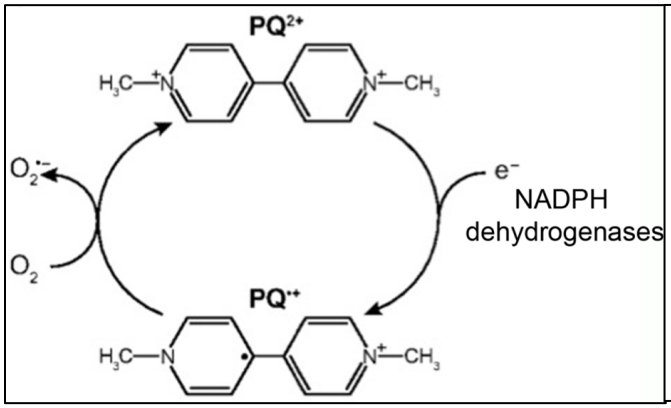

Figure 1.5. PQ is a small molecule that is taken up across the IMM in a Δψ-dependent manner and undergoes single-electron reduction (by NADPH dehydrogenase in yeast) to form a radical that reacts with oxygen to form superoxides in the mitochondrial matrix. This figure is modified from Cocheme and Murphy, 2008.

The mitochondrial specificity of PQ is well established. Down-regulation of the mitochondrial superoxide dismutase in *D. melanogaster* decreased lifespan and increased signs of oxidative damage with PQ treatment (Kirby *et al.*, 2002). Mice deficient in both the mitochondrial superoxide dismutase and glutathione peroxidase 1, both enzymes with antioxidant properties, were more sensitive to PQ, having decreased lifespans and also showing signs of oxidative stress (Van Remmen *et al.*, 2004). Furthermore, overexpressing the mitochondrial catalase in *D. melanogaster* protected flies from PQ toxicity more effectively than overexpression of the cytosolic catalase (Mockett *et al.*, 2003). Similarly, yeast are more efficiently protected from PQ-induced

toxicity by mitochondrial targeting of the human peroxiredoxin 5 (an antioxidant enzyme that mitigates hydrogen peroxide and alkyl hydroperoxides) than cytosolic targeting (Tiên Nguyên-nhu and Knoops, 2003). Yeast cells in which the mitochondrial superoxide dismutase is deleted are also more sensitive to PQ treatment than wild-type cells (Cochemé and Murphy, 2008). Further supporting the detrimental effect of PQ on mitochondrial fitness, yeast cells are more sensitive to PQ when grown on non-fermentable media than when grown on fermentable media (Cochemé and Murphy, 2008).

In these studies, we use mild PQ treatment to induce mild, chronic mitochondria-specific stress to model stress associated with aging. To identify genes and pathways that are critical for cellular fitness under conditions of low levels of mitochondrial oxidative stress, we performed a candidate-based screen to identify genes that, when deleted, increase the sensitivity of candidate *Saccharomyces cerevisiae* (budding yeast) to the growth inhibiting effect of low levels of PQ. We identified the proteasome, and not mitophagy or select proteases or chaperones, as critical for promoting mitochondrial health under these conditions.

CHAPTER 2 - Mitochondrial Remodeling Through the Proteasome is Critical for Mitochondrial Quality Control in Budding Yeast

ABSTRACT

Many pathways can repair or eliminate damaged mitochondria, yet their relative contributions in response to specific stress conditions are not well understood. We show that the proteasome is critical for cellular fitness in response to chronic, low levels of mitochondrial reactive oxygen species (ROS) in the budding yeast *Saccharomyces cerevisiae*. Deleting *DOA1*, which is required for ubiquitin-mediated degradation, *UFD5*, which promotes proteasome gene expression, or *NAS2*, which promotes proteasome regulatory particle assembly, increases the sensitivity of yeast to chronic, low levels of mitochondrial ROS. In contrast, deleting *ATG32*, a gene required for mitophagy, other autophagy genes, non-essential chaperones including prohibitins, or mitochondrial proteins including the Lon protease (Pim1p) or *YME1*, does not affect cellular fitness under these conditions. Doa1p binds with Cdc48p and Vms1p, which associates with mitochondria and promotes extraction of ubiquitinated proteins from the organelle for proteasomal degradation in a pathway called mitochondria-associated degradation (MAD). Elevated mitochondrial ROS increases protein ubiquitination, ubiquitination of the mitochondrial protein aconitase and expression of key MAD proteins. Interestingly, down-regulating ER-associated degradation (ERAD), which shares some common proteins with MAD, can promote cell growth under conditions of elevated mitochondrial ROS. Finally, deletion of *DOA1* results in increased sensitivity of yeast and yeast mitochondria to oxidative stress. Mitochondria in *doa1*Δ cells are more oxidized than mitochondria in wild-type or *atg32*Δ cells under conditions of elevated mitochondrial ROS. Moreover, deletion of *DOA1* results in a decrease in chronological lifespan. These findings support a critical role for the proteasome and MAD in mitochondrial quality

control, which in turn affects cellular fitness, in response to chronic, low levels of mitochondrial ROS.

INTRODUCTION

Mitochondria are essential organelles for oxidative phosphorylation, the Krebs cycle, fatty acid biosynthesis, Ca^{2+} storage and buffering, and the regulation of apoptosis. Increasing evidence also links mitochondria to aging and age-related diseases. Some of the age-related diseases associated with changes in mitochondria or mutations in mitochondrial proteins are Parkinson's disease, Alzheimer's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis. While some individuals live their entire lives with mutations in mitochondrial proteins, including Pink1 and Parkin, that are associated with age-related neurodegenerative diseases, disease symptoms occur only later in life. This indicates that changes occur in mitochondria with age that allow disease pathology to emerge. Reactive oxygen species (ROS) are a candidate for linking mitochondria, the normal progression of aging, and age-related diseases. ROS-induced oxidative damage increases with age in many cell types (e.g. bacteria: (Dukan and Nyström, 1998); yeast: (Stadtman, 1992; Aguilaniu *et al.*, 2003; Reverter-Branchat *et al.*, 2004); mammals, such as in rodents and humans: (Hamilton *et al.*, 2001; Gibson *et al.*, 2010)). ROS, such as oxygen ions, free radicals, and peroxides, are a by-product of mitochondrial respiration, making mitochondria the largest endogenous source of ROS (Guarente, 2008).

Three major quality control pathways for mitochondria are mitochondrial chaperones and proteases that promote re-folding and degradation, respectively, of misfolded proteins; mitophagy, the specific autophagic degradation of mitochondria by the vacuole (the lysosome in metazoans); and the mitochondria-associated degradation pathway

(MAD) in which mitochondrial proteins are ubiquitinated, extracted from the organelle, and degraded by the proteasome (Artal-Sanz and Tavernarakis, 2009; Ngo and Davies, 2009; Heo and Rutter, 2011; Narendra and Youle, 2011). However, the relative contributions of these quality control mechanisms in mitochondrial maintenance are still not well understood, nor is it known whether they are differentially activated temporally in response to stress, or whether different types of stress preferentially activate a specific pathway. Many studies use proton ionophores to induce stress, damaging mitochondria beyond repair and activating mitophagy in mammalian cells (Narendra and Youle, 2011). However, recent studies have questioned the primary role of mitophagy as a response to low-level, repairable mitochondrial damage. These studies suggest that mitophagy may be activated only when mitochondria are beyond the point of repair in a stressed cell, during starvation, or when mitochondria are no longer needed for cellular function, as in the case of erythrocyte maturation or during the degradation of paternal mitochondria in fertilization (Schweers *et al.*, 2007; Sandoval *et al.*, 2008; Kanki *et al.*, 2009b; Okamoto *et al.*, 2009; Al Rawi *et al.*, 2011; Sato and Sato, 2011; Bhatia-Kiššová and Camougrand, 2013; Deffieu *et al.*, 2013).

MAD is similar to the pathway by which misfolded proteins in the endoplasmic reticulum (ER) are eliminated, called the ER-associated degradation pathway (ERAD) (Werner et al., 1996; Neutzner et al., 2007)). The ERAD and MAD pathways are homologous in yeast and mammals. In the ERAD pathway, misfolded proteins in the ER lumen bind chaperone proteins, are retro-translocated across the ER membrane through the protein translocating translocon complex where they are ubiquitinated, pulled from the

membrane, and degraded by the proteasome (Neutzner *et al.*, 2007). In MAD, mitochondrial proteins are also ubiquitinated, either by mitochondrial E3 ligases, such as Mdm30p, or by cytosolic E3 ligases that are recruited to mitochondria, such as Rsp5p (Neutzner *et al.*, 2007).

Cdc48p in yeast (VCP/p97 in mammals) is a subunit of a polyubiquitin-selective segregase complex involved in ERAD and MAD. It is a AAA ATPase that is recruited to ER or mitochondria, where it generates the energy to extract ubiquitinated proteins from these organelles and presents ubiquitinated proteins to the proteasome for degradation (Heo *et al.*, 2010). It is an essential protein and much of its characterization in yeast has used a temperature-sensitive mutant (Moir *et al.*, 1982). In ERAD, Cdc48p binds with Npl4p and Ufd1p, the latter of which confers specificity for ER-targeting. In MAD, Cdc48p is thought to bind with Npl4p and Vms1p. Vms1p facilities but is not essential for targeting of the Cdc48p-Npl4p-Vms1p segregase complex to mitochondria (Heo *et al.*, 2010). The Cdc48p-Npl4p-Vms1p segregase complex colocalizes with mitochondria mainly during elevated mitochondrial stress. It also associates with mitochondria under normal conditions, raising the possibility that MAD plays a role in mitochondrial protein quality control under all conditions (Heo *et al.*, 2010; Shanbhag *et al.*, 2012). Importantly, the human homologue of Cdc48p, VCP/p97, has been linked to diseases of the nervous system, muscle, and bone, including ALS, Parkinsonisms, frontotemporal dementia, Paget's disease, and Charcot-Marie-Tooth Disease (Watts *et al.*, 2004; Weihl *et al.*, 2007; Badadani *et al.*, 2010; Yamanaka *et al.*, 2012; Feely *et al.*, 2014).

Doa1p, a protein containing a ubiquitin binding domain, is necessary for ubiquitin-mediated degradation and directly interacts with Cdc48p (Mullally *et al.*, 2006). Doa1p facilitates the formation of the Doa1p-Cdc48p-ubiquitin complex, bringing Cdc48p into contact with ubiquitinated substrates (Mullally *et al.*, 2006). The human homologue of Doa1p, phospholipase A2 activating protein (PLAA), plays an analogous role, serving as a ubiquitin cofactor that recruits p97/VCP to ubiquitinated substrates (Mullally *et al.*, 2006). Doa1p also plays a role in ubiquitin homeostasis, recycling ubiquitin from the proteasome degradation pathway into other pathways, including that of the DNA damage response (Lis and Romesberg, 2006).

Several recent studies have linked mitochondrial protein quality control to the cytosolic ubiquitin-proteasome system (UPS). For example, a recent study found that aged yeast have a decrease in Pim1p proteolytic activity, an increase in electron-dense inclusion bodies, and increased oxidative protein damage (Erjavec *et al.*, 2013). They also find that cells lacking *PIM1* experience a decrease in replicative lifespan and an increase in oxidized and ubiquitinated cytosolic proteins and a decrease in 20S proteasome capacity (Erjavec *et al.*, 2013). Based on these results, the authors propose that the loss of Pim1p activity may cause a release of damaged mitochondrial proteins to the cytoplasm by an unknown mechanism, where they are degraded by the 20S proteasome, which may become overburdened by the increase in ubiquitinated protein substrates (Erjavec *et al.*, 2013). Another recent study revealed a link between mitochondrial oxidative stress and ubiquitin-dependent proteolysis in both *Caenorbabditis elegans* and human cell tissue culture lines from patients with defects in

mitochondrial metabolism (Segref *et al.*, 2014). *C. elegans* harboring mutations that increase levels of mitochondrial ROS are defective in the degradation of a GFP-based cytosolic protein substrate of the UPS, presumably because the proteasome is actively engaged in degrading damaged mitochondrial proteins (Segref *et al.*, 2014). The decrease in the degradation of the cytosolic GFP-based proteasome substrate under conditions of elevated ROS can be relieved by antioxidants or by overexpression of components of the UPS known to increase the assembly and activity of the 26S proteasome (Vilchez *et al.*, 2012; Segref *et al.*, 2014).

Paraquat (PQ) is a small molecule that is taken up across the mitochondrial inner membrane (IMM) in a membrane potential (Δψ)-dependent manner and undergoes single-electron reduction (by NADPH dehydrogenases in yeast) to form a radical that reacts with oxygen to form superoxides (Bus *et al.*, 1976; Cochemé and Murphy, 2008). In these studies, we use mild PQ treatment to induce mild, chronic mitochondria-specific stress, to model stress associated with aging. To identify genes and pathways that are critical for cellular fitness under conditions of low levels of mitochondrial stress, we performed a candidate screen to identify genes that, when deleted, increase the sensitivity of yeast cells to the growth-inhibiting effect of low levels of PQ. We find that the proteasome, and not mitophagy or select proteases or chaperones, is critical for promoting mitochondrial health under these conditions.

RESULTS

Paraquat toxicity severely inhibits cell growth rate in cells compromised in the ubiquitin-proteasome system

Treatment of cells with PQ induces mitochondrial oxidative stress by creating elevated ROS within the mitochondrial matrix (Bus *et al.*, 1976; Cochemé and Murphy, 2008). We confirmed that PQ treatment results in a dose-dependent decrease in growth rates of wild-type (WT) yeast cells (Fig. 2.1 A). Growth is blocked by treatment with 5 mM PQ and the maximum growth rate is reduced by 40% with 2.5 mM PQ treatment. Therefore, treatment with 2.5 mM PQ was selected as the condition to induce chronic, yet mild mitochondrial oxidative stress to model the type of stress associated with aging (Fig. 2.1 B).

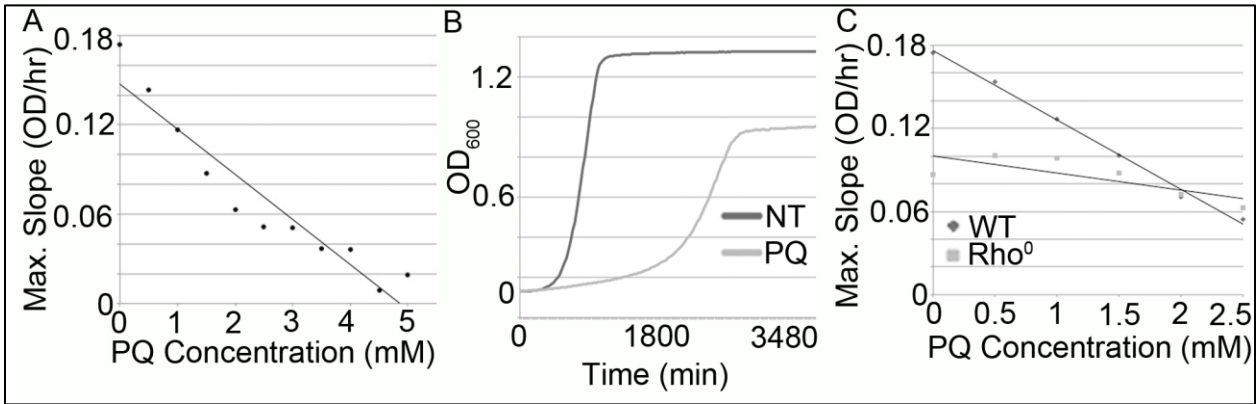

Figure 2.1. Cell growth rate is impaired by PQ treatment, which induces mitochondria-specific toxicity. Growth rates in nutrient-rich glucose-based medium were monitored by measuring optical density at 600 nm (OD_{600}) every 20 min for 3 days. **(A)** Dose-dependent growth inhibition of WT yeast cells by PQ. **(B)** Growth curves of non-treated WT cells (NT, dark grey) and cells grown with 2.5 mM PQ (PQ, light grey). **(C)** Rho^0 cells, which lack mtDNA, were generated by the deletion of *MGM101*, a gene required for mtDNA maintenance. Mitochondrial function is required for the effects of low levels of PQ on cell growth.

To assess the specificity of PQ for mitochondria, we tested the effect of PQ treatment on the growth of rho^0 cells, yeast that have no mitochondrial DNA (mtDNA) and no mitochondrial respiratory or translational activity. Rho^0 cells were generated by the deletion of *MGM101*, a gene required for mtDNA maintenance, and without which, mtDNA is lost. PQ at levels ≤ 2.5 mM has no effect on the growth rate of rho^0 cells (Fig. 2.1 C). Thus, PQ effects on cell growth are dependent upon and therefore specific to mitochondria.

To investigate the relative contributions of mitophagy, select mitochondrial proteases and chaperone proteins, and the UPS to mitochondrial quality control under conditions of chronic, mild mitochondrial oxidative stress, we tested the effect of 2.5 mM PQ treatment on the growth rate of yeast lacking non-essential genes in these pathways. We reasoned that loss of factors that maintain and/or repair mitochondria undergoing oxidative stress would increase the sensitivity of cells to the growth-inhibiting effects of PQ.

We investigated deletions of eight autophagy genes: *atg1Δ*, *atg5Δ*, *atg7Δ*, *atg8Δ*, *atg9Δ*, *atg11Δ*, *atg12Δ*, and *atg32Δ*. Atg1p, Atg5p, Atg7p, Atg8p, Atg9p, Atg11p, and Atg12p are involved in important steps in autophagy, including autophagosome formation and the cytoplasm-to-vacuole targeting (cvt) pathway, which is itself required for vacuolar function by contributing to the delivery of at least two hydrolases, aminopeptidase I and α-mannosidase, to the vacuole, where they are later processed to their enzymatically active forms (Suzuki and Ohsumi, 2007; May *et al.*, 2012). In

addition, Atg11p is an adaptor protein for two specific types of macroautophagy: pexophagy, peroxisome-specific autophagy, and mitophagy, mitochondria-specific autophagy (Farré *et al.*, 2008; Kanki *et al.*, 2009b; Okamoto *et al.*, 2009). Atg32p is involved in mitophagy, but not in general autophagy (Kanki *et al.*, 2009b; Okamoto *et al.*, 2009).

We also screened four mutants involved in the UPS and MAD pathways: *vms1Δ*, *doa1Δ*, *nas2Δ*, and *ufd5Δ*. As described above, Vms1p contributes to but is not essential for targeting of the Cdc48p-Npl4p-Vms1p segregase complex to mitochondria. Doa1p is a WD repeat protein that is associated with the segregase complex, required for proteasomal degradation of ubiquitinated proteins, and controls cellular ubiquitin levels. Ufd5p stimulates the expression of proteasome genes (Mannhaupt *et al.*, 1999; Ng, 2000; Xie and Varshavsky, 2001; Owsianik *et al.*, 2002). Nas2p promotes the assembly of the proteasome regulatory particle (Watanabe *et al.*, 1998; Funakoshi *et al.*, 2009; Saeki *et al.*, 2009).

To examine the role of mitochondrial proteases and chaperone proteins, we studied seven additional strains: *pim1Δ*, *yme1Δ, yta10Δ, yta12Δ, phb1Δ*, *phb2Δ*, and *phb1Δphb2Δ*. Lon proteases (Pim1p in yeast) are evolutionally conserved AAA serine proteases that usually localize to the mitochondrial matrix. Pim1p is implicated in the degradation of aggregated mitochondrial polypeptides that accumulate with elevated ROS (Bender *et al.*, 2011). Yme1p is the catalytic subunit of the *i*-AAA protease complex localized to the IMM and is responsible for the degradation of unfolded or

misfolded proteins (Schnall *et al.*, 1994; Schreiner *et al.*, 2012). Yta10p and Yta12p are components of the *m*-AAA protease that are responsible for the degradation of unfolded or misfolded proteins, and are required for the proper assembly of various mitochondrial enzyme complexes (Arlt *et al.*, 1996, 1998). Prohibitins are conserved, ubiquitous membrane proteins that are essential for cell proliferation, development in many eukaryotes, and lifespan control (Van Aken et al., 2010; Artal-Sanz and Tavernarakis, 2009; Osman et al., J Cell Sci 2009). Deletion of the prohibitins in yeast, *PHB1* and *PHB2*, results in elevated ROS, defects in mitochondrial morphology, defects in the organization of mitochondrial nucleoids and cristae, and defects in the phospholipid composition of mitochondrial membranes. Emerging studies support a role for the prohibitins as protein and lipid scaffolds for quality control of the IMM (Osman *et al.*, 2009).

The growth rates of these strains in nutrient-rich, glucose-based medium are very similar (Fig. 2.2 A). When treated with 2.5 mM PQ, the growth rate of each strain decreases, as expected (Fig. 2.2 B). Surprisingly, yeast bearing deletions in the autophagy, mitophagy, mitochondrial proteases, or prohibitin genes do not exhibit a significant increase in sensitivity to the growth-inhibiting effects of 2.5 mM PQ treatment. In contrast, deletion of genes associated with the proteasome, *DOA1*, *UFD5*, and *NAS2,* results in increased sensitivity to chronic low levels of mitochondrial ROS (Fig. 2.2 B). PQ treatment inhibits WT cell growth 2.8 fold. In contrast, PQ-treated *doa1*Δ, *nas2*Δ, or *ufd5*Δ cells exhibited 51, 24, and 14 fold decreases in growth rates, respectively. For our work, we continue to study *doa1Δ* and *atg32Δ* strains as model

knockout mutants that are very affected and not significantly affected, respectively, by 2.5 mM PQ. Consistent with our previous finding that 2.5 mM PQ induces mitochondrial specific stress in WT yeast cells, we find that rho^0 *doa1Δ* and *atg32Δ* strains are also more resistant to PQ than their non-rho^0 controls (Fig. 2.2 C).

To further test whether chronic low levels of mitochondrial oxidative stress induce mitophagy, we directly probed for the degradation of mitochondria by the vacuole using a well-established method. In this assay, a mitochondrial protein, Cit1p, was tagged at its C-terminus with GFP. Whole cell extract from cells expressing Cit1p-GFP under either nitrogen-starvation conditions or either treated or non-treated with PQ was collected and analyzed by Western blotting. Due to the protease-resistant nature of GFP, the appearance of a free-GFP band indicates the occurrence of mitophagy (Kanki *et al.*, 2009a). As expected, we find that nitrogen starvation at 4, 8, and 12 hr induces mitophagy (Fig. 2.2 D). Consistent with our finding that cells lacking genes involved in general autophagy and mitophagy are not more sensitive to PQ treatment than WT cells, we find that mild PQ treatment does not induce mitophagy (Fig. 2.2 D).

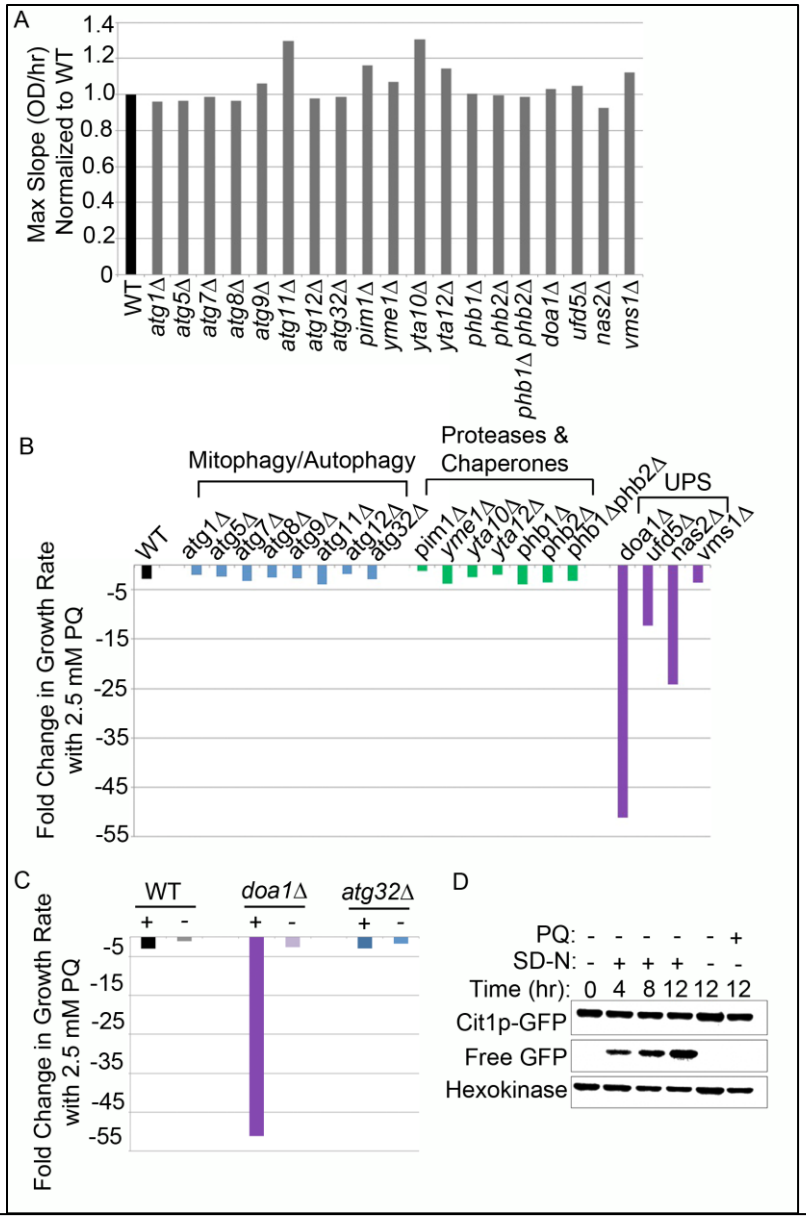

Figure 2.2. (A) WT and mutant strains grow at very similar rates in nutrient-rich glucose-based media in the absence of PQ. Growth rate was monitored as described in Fig. 2.1. **(B)** Genes of the UPS, but not other quality control pathways, are involved in the cellular response to PQ stress. Fold change is the ratio of maximum growth rate in 2.5 mM PQ versus that in the absence of PQ. Growth rates were monitored as described in Fig. 2.1. **(C)** Consistent with our findings in Fig. 2.1 C, rho^0 (indicated by -) *doa1Δ* and *atg32Δ* cells are more resistant to 2.5 mM PQ than their non-rho^0 controls (indicated by +). Rho^0 cells construction and cell growth rate measurements were performed as described in Fig. 2.1. **(D)** Cells expressing Cit1p-GFP were grown in glucose media before being either exposed to nitrogen starvation (SD-N) or 2.5 mM PQ. Whole cell extract was collected and analyzed by Western blotting and probed with anti-GFP and anti-hexokinase antibodies. A band representing free GFP, which is protease resistant, indicates the occurrence of mitophagy.

Proteins including mitochondrial aconitase are ubiquitinated and degraded by the UPS in response to elevated mitochondrial oxidative stress

If the UPS contributes to mitochondrial quality control during elevated oxidative stress in the organelle, then there should be elevated levels of ubiquitinated proteins under these conditions and those ubiquitinated proteins should be degraded by the proteasome. To test this hypothesis, we treated WT cells with the proteasome inhibitor MG132 in the presence and absence of PQ (Fig. 2.3). We find that PQ treatment results in a 79% increase in total cellular levels of ubiquitinated proteins. Thus, protein ubiquitination is a normal response to chronic, mild mitochondrial oxidative stress. Equally important, treatment with PQ and MG132 results in a 627% increase in ubiquitinated protein levels (Fig. 2.3). Thus, protein ubiquitination is a response to elevated mitochondrial ROS and the vast majority of these ubiquitinated proteins are degraded by the proteasome. Consistent with this, we find that protein ubiquitination in PQ- and MG132-treated *doa1*Δ cells is significantly lower than that observed in WT cells under the same conditions (Fig. 2.3). These studies provide additional support for a major role for the UPS in mitochondrial quality control when the organelle is challenged with low levels of chronic oxidative stress.

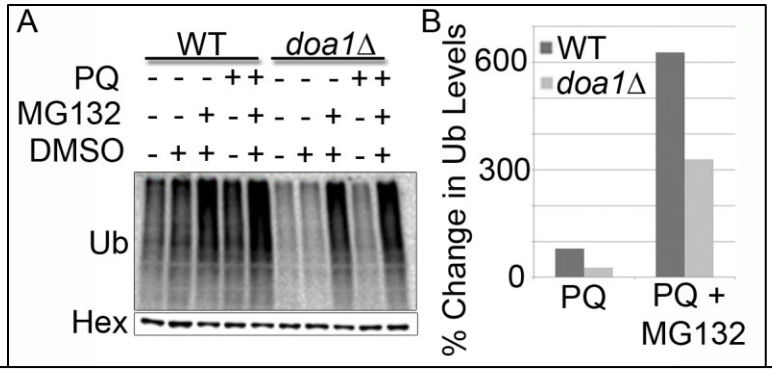

Figure 2.3. Doa1p-dependent ubiquitination of proteins occurs as a response to PQ treatment. **(A)** Western blot of whole-cell extracts from WT and *doa1Δ* cells with the indicated treatments. DMSO is the vehicle for MG132. Cells were incubated with 2.5 mM PQ for 8 hrs followed by incubation with PQ and either MG132 or DMSO for an additional 4 hrs. The extract was probed with antibodies to ubiquitin (Ub, upper panel) and hexokinase (Hex, lower panel), which serves as a loading control. Results are representative of 3 independent experiments. **(B)** Densitometric quantitation of Ub levels normalized to hexokinase.

Next, we determined whether a specific mitochondrial protein is ubiquitinated and degraded by the UPS when yeast cells are challenged with elevated mitochondrial ROS. Aconitase, Aco2p, is a component of the mitochondrial TCA cycle (Przybyla-Zawislak *et al.*, 1999) that reacts readily with superoxides (Yan *et al.*, 1997). Oxidative damage decreases Aco2p enzymatic activity, which is associated with aging and decreased lifespan (Yan *et al.*, 1997). We tested whether Aco2p undergoes increased ubiquitination and UPS-mediated degradation with PQ treatment. To do this, we immunoprecipitated Aco2p tagged with 13Myc from whole cell extracts of WT cells treated with PQ, MG132, or PQ with MG132 and tested whether the protein was ubiquitinated by Western blot analysis using antibodies raised against ubiquitin. In cells treated with PQ, there is a 16% increase in the ubiquitination of Aco2p-13Myc compared to untreated cells (Fig. 2.4). Treatment with PQ and MG132 results in a 115% increase in Aco2p ubiquitination. Thus, a mitochondrial protein, Aco2p, is ubiquitinated

in response to elevated mitochondrial oxidative stress and the proteasome is required for its degradation. These results further support that mitochondrial proteins that are damaged by elevated mitochondrial ROS are ubiquitinated and eliminated by UPS-mediated degradation.

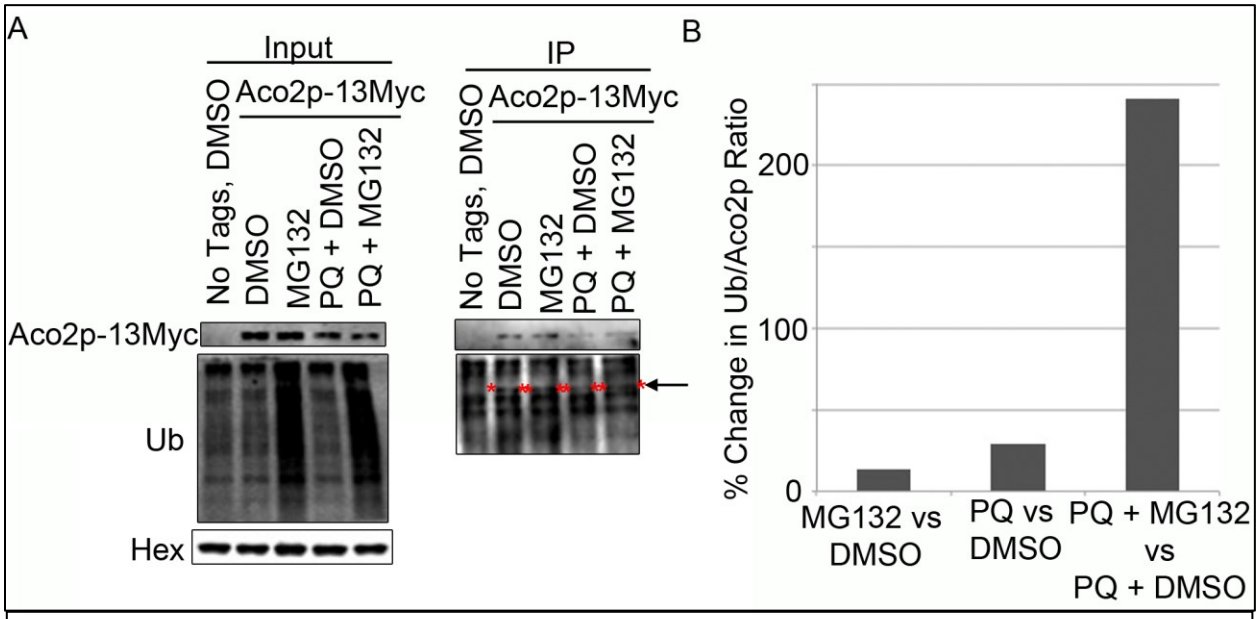

Figure 2.4. Aco2p, a mitochondrial protein, becomes ubiquitinated with PQ treatment and is degraded by the proteasome. **(A)** Western blot of input and immunoprecipitated protein from whole-cell extracts of WT cells expressing Aco2p-13Myc. Cells were treated with PQ and MG132 as described in Fig. 2.3. Proteins were immunoprecipitated with an anti-Myc antibody. Blots were probed with antibodies against Myc and ubiquitin (Ub). Input was also probed with anti-hexokinase (Hex) as a loading control. The arrow and red asterisks indicate Ub signal that co-migrates with the Aco2p-13Myc band in the immunoprecipitate. Cells with no fusion proteins (No Tags) were included as a negative control to demonstrate antibody specificity. Blot is representative of 3 independent experiments. **(B)** Quantitation by scanning densitometry of ubiquitinated aconitase levels in the blot shown in (A).

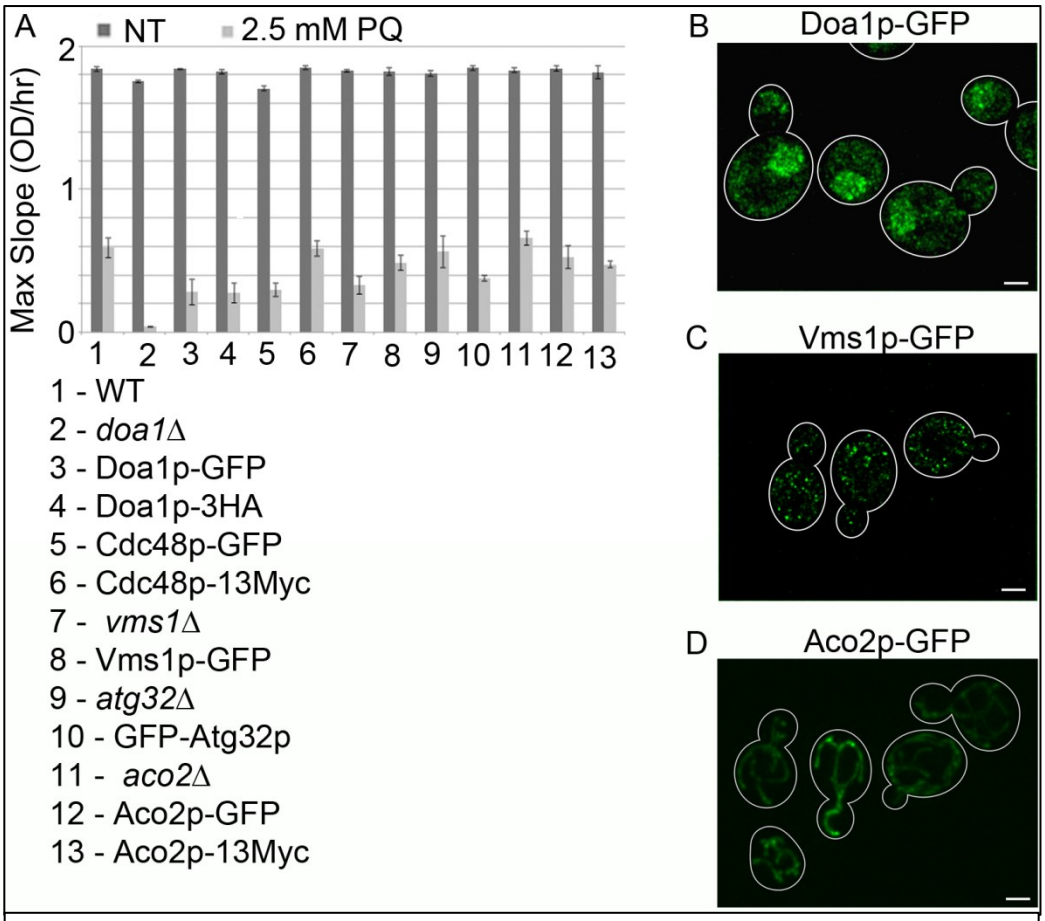

Figure 2.5. Growth rates and localizations of fusion proteins used in this study. **(A)** Growth rates were monitored as described in Fig. 2.1. A fusion protein is considered functional if the strain maintains a growth rate comparable to that of WT cells. Error bars represent standard error of the mean. **(B)** Localization of Doa1p-GFP. **(C)** Localization of Vms1p-GFP. **(D)** Localization of Aco2p-GFP. All cells in (B-D) were grown in synthetic complete glucose-based media and imaged by fluorescence microscopy. Images were rendered as described in the Methods. Scale bars = 1 μm.

Elevated mitochondrial ROS results in upregulation of MAD proteins

The studies described above focused on the proteasome and ubiquitin in mitochondrial quality control during chronic, mild oxidative stress. Next, we studied the role of MAD proteins in that process. We tested whether Cdc48p, the essential component of the MAD complex, responds to chronic, elevated mitochondrial oxidative stress. To do this,

we generated strains in which Cdc48p was tagged with either GFP or 13Myc at its C-terminus (Fig. 2.5). To determine whether the tags compromise function, we assessed growth rates of yeast expressing tagged proteins in the presence and absence of PQ. Previous studies revealed that tagging Cdc48p at its C-terminus does not compromise function (Heo *et al.*, 2010). We find that the growth rate of cells expressing Cdc48p tagged with 13Myc or GFP in the absence of PQ was indistinguishable from that of untreated WT cells. However, under more stringent conditions that require defenses against oxidative stress, we see that tagging of Cdc48p with GFP but not 13Myc may compromise function: yeast expressing Cdc48p-GFP exhibit increased sensitivity to the growth-inhibiting effects of PQ. We observe similar results with Doa1p; tagging Doa1p has no effect on growth in the absence of PQ, but does inhibit growth upon PQ treatment. This provides evidence for a role for MAD and Doa1p in the cellular response to elevated mitochondrial ROS.

Since tagging of Cdc48p with 13Myc does not compromise function but tagging of Cdc48p with GFP does compromise function, we used Cdc48p tagged with 13Myc and GFP to study the effect of PQ on Cdc48p levels and localization. Cdc48p-13Myc and Cdc48p-GFP localize as diffuse and punctate structures in both the cytosol and the nuclear ER (Fig. 2.6 A). Interestingly, on average, the number of punctate structures formed by Cdc48p-GFP doubles from 4 to 8 upon treatment with PQ (Fig. 2.6 A and B). Moreover, using Cdc48p-GFP together with mCherry-tagged mitochondrial citrate synthase (Cit1p) to visualize mitochondria, we find that (1) there is a general increase in cytosolic as opposed to nuclear ER-associated Cdc48p-GFP; and (2) some of the

punctate structures formed by Cdc48p-GFP after PQ treatment localize near mitochondria (Fig. 2.6 C). However, it is difficult to draw any conclusions regarding Cdc48p localization with respect to mitochondria because Cdc48p is abundant and localizes throughout the entire cell.

We do, however, observe that the steady-state levels of Cdc48p, Vms1p, and Doa1p, components or associates of the components of the MAD segregase complex, increase with PQ treatment (Fig. 2.6 D and E). We analyzed whole-cell extracts by Western blotting in cells expressing fusion proteins (Fig. 2.4) Cdc48p-13Myc, Doa1p-3HA, and Vms1p-GFP and find that these proteins increase in expression with PQ treatment by, on average, 34, 46, and 45%, respectively (Fig. 4 E). These data further support a role for MAD in mitochondrial quality control under conditions of chronic, mild elevated mitochondrial oxidative stress.

Since the ERAD and MAD pathways share Cdc48p, the major component of the segregase complex, we reasoned that down-regulation of ERAD may promote MAD by allowing more of the complex to localize with mitochondria instead of ER. To test this hypothesis, we used a strain in which *UBX2*, a component of ERAD that does not overlap with MAD, was deleted. Ubx2p is an integral ER-membrane protein that binds to the cytosolic Cdc48p complex, physically linking it to the ER (Schuberth *et al.*, 2004; Neuber *et al.*, 2005; Schuberth and Buchberger, 2005). While the WT and *ubx2Δ* strains grow at very similar rates under non-treated conditions, growth of the *ubx2Δ* cells is less

compromised by PQ than the WT strain (Fig. 2.6 F). These data suggest that promoting MAD increases cellular fitness in response to elevated mitochondrial ROS.

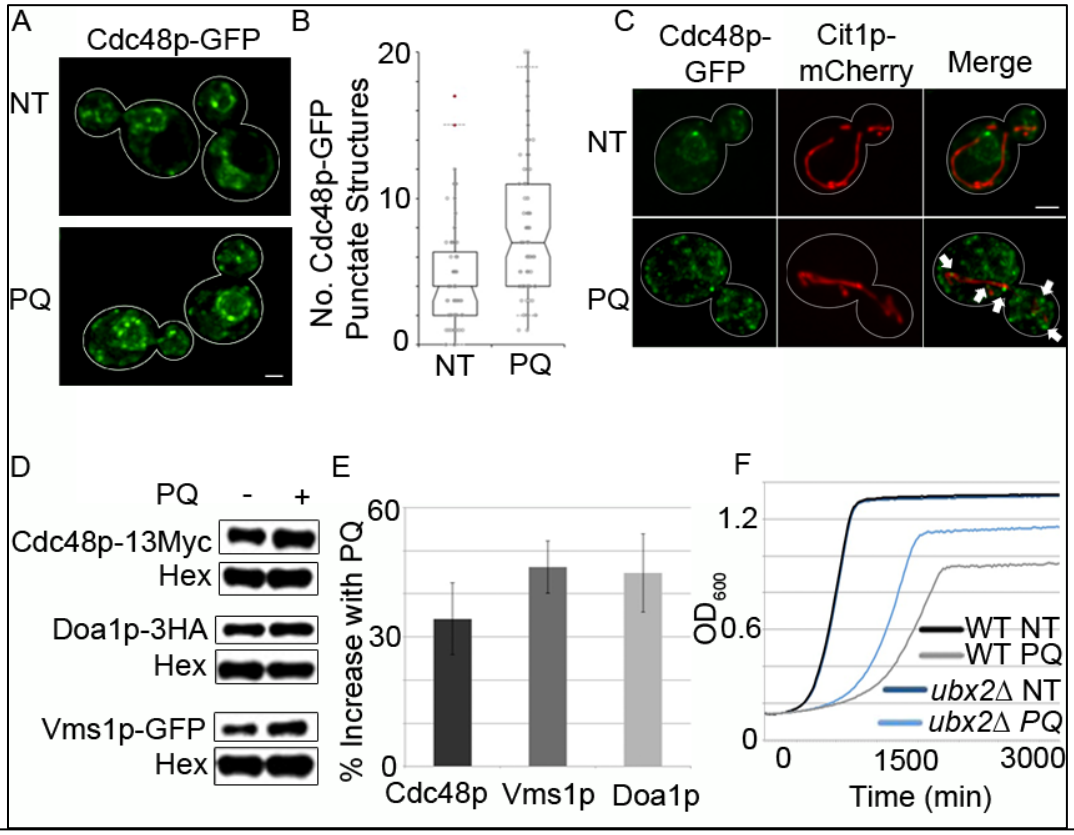

Figure 2.6. Proteins of the MAD segregase complex and associated components respond to elevated mitochondrial ROS. **(A)** Cdc48p-GFP puncta are more numerous after PQ treatment. WT cells expressing Cdc48p-GFP are shown in the absence (NT) or presence (PQ) of paraquat. **(B)** Quantitation of the number of Cdc48p puncta in cells propagated in the absence (NT) or presence (PQ) of PQ (average of 3 independent experiments, n ≥ 105 cells). The difference in number of puncta is statistically significant by the Kruskal-Wallis test ($p < 0.0001$). Error bars represent standard error of the mean. **(C)** Cdc48-GFP puncta partially colocalize with mitochondria after PQ treatment. WT cells expressing Cdc48p-GFP and the mitochondrial protein Cit1p-mCherry are shown. Cells in (A-C) were grown to mid-log phase in synthetic complete glucose-based media and imaged by fluorescence microscopy. Deconvolved maximum intensity projections are shown. **(D)** Levels of the MAD segregase complex and associated components increase upon PQ treatment. Cells were grown in glucose-based medium with or without 2.5 mM PQ for 12 hr. Western blots of whole-cell extracts were probed with the indicated antibodies. Hexokinase (Hex) is a loading control. Blot is representative of 5 independent experiments. **(E)** Quantification of Western blots from 5 independent experiments carried out as described in (D), by densitometry. Increases in all three protein levels upon PQ treatment are statistically significant by Student's t-test. ($p < 0.005$) Error bars represent standard error of the mean. **(F)** Growth rates of WT and *ubx2*Δ strains in the absence (NT) and presence (PQ) of PQ were determined as for Fig. 2.1. Curves shown are representative of >3 independent experiments.

The UPS contributes to mitochondrial quality control in response to elevated ROS in the organelle and this response is linked to aging

Finally, we tested mitochondrial fitness upon PQ treatment and whether the UPS or mitophagy promote mitochondrial health when there is an increase in mitochondrial oxidative stress. To address these questions, we used a ratiometric redox biosensor targeted to the mitochondrial matrix, mito-roGFP1 (Dooley *et al.*, 2004; Hanson *et al.*, 2004; Vevea *et al.*, 2013). When targeted to the mitochondrial matrix, mito-roGFP1 is capable of sensing the redox state of mitochondria, which is an indicator of mitochondrial fitness, since a more oxidizing environment within the mitochondria is less healthy and indicative of oxidative stress. Using microscopy and ratiometric image analysis, we assessed the redox state of mitochondria in WT, *doa1Δ*, and *atg32Δ* cells under non-treated and PQ-treated conditions. As expected, PQ treatment produced a more oxidizing environment in mitochondria in each of these strains (Fig. 2.7 A and B). Interestingly, however, *doa1Δ* cells are more sensitive in terms of redox state to 2.5 mM PQ treatment than either WT or *atg32Δ* cells, indicating that they have less healthy mitochondria under the same stress conditions (Fig. 2.7 A and B). These results further support a role for the UPS, and not mitophagy, under conditions of chronic, mild elevated mitochondrial oxidative stress.

Given the known link between elevated oxidative stress and aging, we next sought to determine whether Doa1p may play a role in aging. Specifically, we tested whether *doa1Δ* cells might have a shorter chronological lifespan (CLS) than WT cells. CLS in

yeast is a measure of longevity in a population of cells that are no longer replicating, having reached either the post-diauxic phase or stationary phase, and is thus a measure of the ability of cells to survive under stress (Fabrizio and Longo, 2003; Longo *et al.*, 2012). We also included *atg32Δ* cells in our CLS studies. Previous studies have shown that autophagy indeed promotes normal CLS in yeast (Alvers *et al.*, 2009; Longo *et al.*, 2010; Orozco *et al.*, 2012; Aris *et al.*, 2013). Autophagy has also been show to extend lifespan in worms (Meléndez *et al.*, 2003). Consistent with this, we find that *atg32Δ* cells have a shorter CLS than WT cells (Fig. 2.7 C). Importantly, we find that the CLS of *doa1Δ* cells is shorter than that of either WT or *atg32*Δ cells (Fig. 2.7 C). These data indicate a role for Doa1p in normal lifespan under stress conditions.

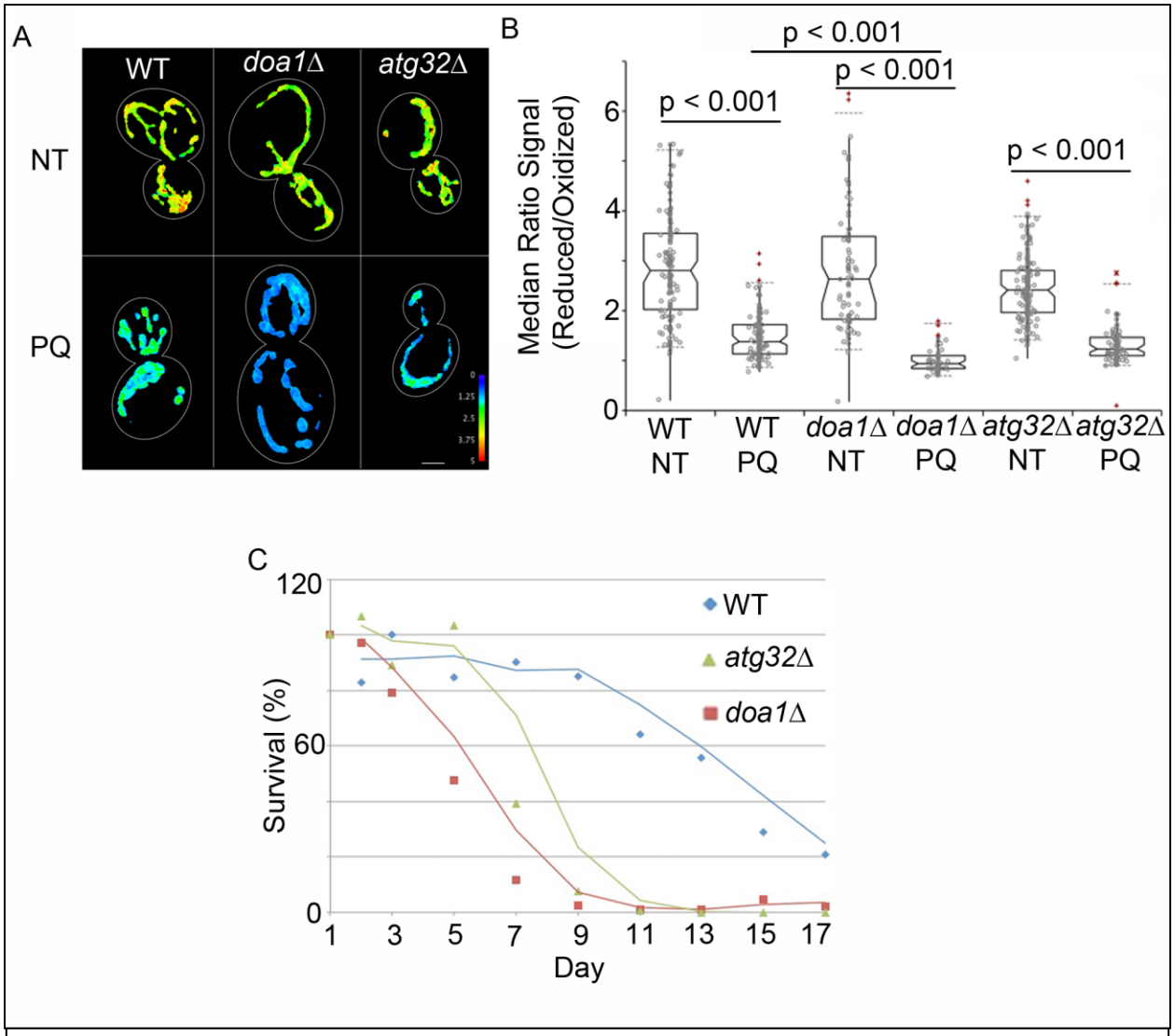

Figure 2.7. Doa1p plays a role in mitochondrial quality control in response to oxidative stress. **(A)** WT, *doa1Δ*, and *atg32Δ* cells expressing a plasmid-borne redox-sensitive GFP targeted to the mitochondrial matrix (mito-roGFP1) were grown in glucose-based media either with (PQ) or without (NT) 2.5 mM PQ for 12 hr. Cells were imaged during mid-log phase. The ratio of the reduced to oxidized roGFP signal was calculated and displayed so that blue represents a more oxidizing environment and red represents a more reducing environment. Maximum-intensity projections of wide-field z series are shown. Images are representative of the median values shown in (B). Scale bars = 1 μm. **(B)** Quantitation of the ratio of the reduced to oxidized roGFP signal. Horizontal bars indicate statistically significant differences ($p<0.0001$, Kruskal-Wallis significance test). $n > 100$ cells per condition for each strain. **(C)** CLS of WT, *doa1Δ*, and *atg32Δ* strains. Cells were grown in synthetic complete glucose-based media. Survival was measured each day as described in Materials and Methods. The scatter plot is representative of 3 independent trials. Moving average trend lines are shown.

DISCUSSION OF RESULTS

While there are many mechanisms to promote repair of mitochondria or elimination of the organelle when it is damaged beyond repair, the relative contributions of these pathways to mitochondrial quality control under conditions that mimic aging are not well understood. Here, we mimicked one aspect of aging by exposure of yeast cells to chronic, low levels of elevated mitochondrial ROS and we tested the relative contributions of autophagy, mitophagy, mitochondrial proteases, mitochondrial chaperone proteins, and the UPS to the maintenance of the fitness of mitochondria and of the cells as a whole. Our studies support a role for the UPS and for MAD, a recently identified system for ubiquitination and extraction of damaged proteins from mitochondria and targeting them for degradation by the UPS. Indeed, this is the first study to demonstrate a role for the MAD pathway in repair of specific defects in mitochondrial proteins. Thus, MAD is an important pathway allowing the repair of mitochondria by removing damaged components, rather than eliminating the still repairable organelle.

Second, we find that mitophagy and autophagy do not contribute significantly to mitochondrial quality control in response to elevated mitochondrial ROS. Previous studies support a role for autophagy and mitophagy in degradation of mitochondria under conditions of nitrogen starvation in yeast, elimination of mitochondria to produce the organelle-free red blood cell, and elimination of mitochondria that are damaged beyond repair in neurons and other mammalian cells (Schweers *et al.*, 2007; Sandoval *et al.*, 2008; Kanki *et al.*, 2009b; Okamoto *et al.*, 2009; Al Rawi *et al.*, 2011; Sato and

Sato, 2011; Bhatia-Kiššová and Camougrand, 2013; Deffieu *et al.*, 2013). We confirmed a role for Atg32p in chronological lifespan, a condition where cells are exposed to severe nutrient limitations and high levels of stressors including oxidative stress. Here, we provide evidence that mitophagy and autophagy are not critical for the fitness of mitochondria and overall cell health in response to chronic, low-level increases in mitochondrial ROS.

We also find that the Lon protease, other mitochondrial proteases, and prohibitins do not contribute significantly to maintenance of mitochondria with low oxidative stress. This is surprising in light of the fact that Lon proteases and prohibitins are conserved and ubiquitous and that the prohibitins are essential for development and implicated in lifespan control. This raises the possibility that there are other challenges to mitochondria where these pathways are critical for mitochondrial repair and maintenance, or perhaps suggests that they too become damaged under conditions of chronic elevated ROS. Since defects in mitochondrial ATP production and not mitochondrial ROS have been implicated in aging in a mouse model that exhibits elevated mutation rates in mtDNA, it is possible that these pathways are critical for maintenance of IMM proteins including components of the respiratory chain and ATP synthase. Further studies may reveal if this model is relevant and whether other challenges require repair by the Lon protease and prohibitins. It also remains to be determined how damaged proteins are identified in mitochondria and translocated to the surface of the organelle for ubiquitination and degradation by MAD and the UPS.

FUTURE DIRECTIONS

The human genome encodes 345 really interesting new gene (RING) finger domain proteins, which are domains associated with E3 ubiquitin ligases. Of these, 54 have predicted membrane-spanning domains and nine are localized to mitochondria (Neutzner *et al.*, 2007). It is predicted that these mitochondrial E3 ubiquitin ligases are localized such that their catalytic domains face the cytosol, and thus can ubiquitinate proteins within the mitochondrial membrane, but not within the internal compartments of the mitochondria (Neutzner *et al.*, 2007). Interestingly, proteins localized within the mitochondria, such as Ucp2p, an integral IMM protein, and Aco2p, a mitochondrial matrix enzyme, can nevertheless become ubiquitinated (Azzu and Brand, 2010; Ziv *et al.*, 2011; Kolawa *et al.*, 2013). Based on the predicted topology of the mitochondrial E3 ubiquitin ligases, it is unclear how internal mitochondrial proteins such as these can become ubiquitinated. Future work can investigate this phenomenon.

One possibility is that damaged proteins from within the mitochondria may become mislocalized, and may gain access to other mitochondrial compartments, such as contact sites between the IMM and the OMM. Proteins that reach contact sites might translocate to the OMM, bringing them within reach of E3 ubiquitin ligases. Alternatively or additionally, there may be a mechanism in MAD that is analogous to that which has been described in ERAD allowing the translocation of a misfolded protein to the OMM for subsequent ubiquitination and proteasomal degradation. Most membrane proteins, secretory proteins, and proteins destined for the ER membrane must be translocated from the cytosol across the ER membrane (Kopito, 1997; Matlack *et al.*, 1998).

Translocation occurs through a protein translocation channel called the Sec61p complex (Matlack *et al.*, 1998). Interestingly, the Sec61p complex also functions in the translocation of misfolded proteins from within the ER lumen to the ER membrane where they can be ubiquitinated and degraded in a proteasome-dependent manner in the protein quality control pathway ERAD (discussed in Chapter 1) (Wiertz *et al.*, 1996; Kopito, 1997; Pilon *et al.*, 1997; Plemper *et al.*, 1997). Mitochondrial proteins are imported into the mitochondria across the OMM through the translocase of the outer membrane (TOM) complex and proteins destined for the matrix or IMM are translocated to their final destinations through the translocase of the inner membrane (TIM) complex (Hewitt *et al.*, 2014). It is reasonable to speculate that misfolded or loosely folded mitochondrial proteins from within the mitochondria may be retro-translocated through the existing TIM and TOM import machineries. It is possible that damaged proteins bind the import machinery through regions that are not normally exposed when they are properly folded, such as hydrophobic stretches. Once they reach the OMM, they could be ubiquitinated by E3 ligases and removed for proteasome-dependent degradation in a manner similar to the retro-translocation that occurs through the Sec61p complex in ER.

It would additionally be interesting to determine whether some E3 ubiquitin ligases are responsible for ubiquitinating mitochondrial proteins that are normally (under non-stress conditions) degraded by the proteasome, while others are responsible for ubiquitinating mitochondrial proteins that become damaged under stress conditions. To investigate this question, it would be possible to use strains from the yeast deletion collection in which mitochondrial E3 ubiquitin ligases are deleted (those that are not lethal upon

deletion) and to expose these cells to both normal and stress conditions, and to probe for the levels of ubiquitination of mitochondrial proteins (or a selection of candidate proteins). Upon deleting select mitochondrial E3 ligases, large scale mass spectrometry studies could be used to investigate the ubiquitination profile of mitochondrial proteins under stress and non-stress conditions to gain an understanding of the differences in the mitochondrial protein ubiquitination profile under the two conditions. An E3 ubiquitin ligase that plays a critical role in the ubiquitination of damaged mitochondrial proteins would show a lower level of ubiquitination of these proteins under stress conditions than the WT strain and would be more sensitive to mitochondrial oxidative stress. For these studies, it would also be useful to inactivate the machinery that removes ubiquitinated proteins from the OMM. Identification of an E3 ubiquitin ligase that plays an important role in ubiquitinating damaged mitochondrial proteins would provide a new target for therapeutic intervention.

Nas4p (also known as Rpn6p) is a non-ATPase regulatory subunit of the lid portion of the 26S proteasome that is essential for the assembly and function of the 26S proteasome (Saito *et al.*, 1997; Finley *et al.*, 1998; Santamaria *et al.*, 2003). Given the important role of this protein in the UPS, another future experiment would be to overexpress Nas4p in WT cells and deletion strains that show extreme sensitivity to PQ (such as *doa1Δ*, *nas2Δ*, and *ufd5Δ* cells). *NAS4* has been shown to have a positive genetic interaction with *DOA1* and the protein product of *NAS4* has been shown to interact physically with Nas2p (Collins *et al.*, 2007; Saeki *et al.*, 2009). Thus, overexpression of Nas4p may provide particularly interesting results, as we find that

strains in which *DOA1* and *NAS2*, respectively, are deleted are the two most PQ-sensitive strains in our panel (Fig. 2.2 B). It is likely that overexpression of Nas4p may result in reduced sensitivity of WT, *doa1Δ*, *nas2Δ*, and *ufd5Δ* cells to PQ. If overexpression of these genes that promote the UPS are found to increase resistance of yeast cells to PQ, our model would predict that this overexpression would also extend chronological lifespan. Indeed, previous work shows that increasing levels of Ufd5p (also known as Rpn4p or Son1p), a transcription factor that stimulates the expression of proteasome genes, increases UPS activity and results in increased resistance of yeast cells to toxins and extends replicative lifespan (Kruegel *et al.*, 2011). Yeast cells overexpressing Ump1p, a chaperone that contributes to proper assembly of the 20S proteasome, are less sensitive to oxidative stress and have extended chronological lifespan, further supporting a critical role for the proteasome in quality control under stress conditions (Chen *et al.*, 2006).

While overexpression studies that are designed to increase the capacity of the UPS, and thus increase resistance to PQ toxicity and perhaps extend chronological lifespan in yeast would be informative, overexpression studies are often not physiologically relevant, as they can result in aberrant protein localizations and functions. Therefore, other strategies should be employed to increase proteasome capacity in a manner less likely to be disruptive of normal cellular functions. One possibility is through mutation of Ubr2p and Mub1p, which are both required for the turnover of Rpn4p (discussed above). Ubr2p is a cytosolic ubiquitin ligase that ubiquitinates Rpn4p and Mub1p is a Ubr2p-associated protein that aids in this function (Kruegel *et al.*, 2011). Deletion of

these genes elevates Rpn4p levels, thereby increasing proteasome capacity without the introduction of an overexpressed plasmid-borne protein (Kruegel *et al.*, 2011). It would be interesting to test whether deletion of *UBR2* and *MUB1* could increase resistance of yeast cells to PQ and extend chronological lifespan. In performing this experiment, one must keep in mind that the deletion of *UBR2* and *MUB1* may have unknown consequences if they are involved in the degradation of other yet unidentified protein substrates. Additionally, the proteasome components that accumulate upon the deletion of *UBR2* and *MUB1* may over time become less efficient if they are not turned over at their normal rates. Regardless of these possible detrimental consequences, it is worth testing this hypothesis.

Although data from this study clearly supports a critical role for the proteasome, and not mitophagy, in mitochondrial quality control under conditions of chronic, low levels of mitochondrial oxidative stress and identifies some major players in this quality control pathway, other specific genes that also play important roles in mitochondrial quality control under these conditions can be identified. The screening protocol designed in this study could be applied to genome-wide or other unbiased screens to aid the discovery of other factors in mitochondrial quality control.

MATERIALS AND METHODS

Strains and plasmids

S. cerevisiae strains used in this study are summarized in Supplemental Table 2.1. All yeast strains were created in the BY4741 wild-type background. Deletion strains were purchased from Open Biosystems (Huntsville, AL). *CDC48, DOA1*, and *VMS1* were tagged at the C-terminus with GFP by transformation with a PCR-amplified GFP tagging cassette from the pFA6a-GFP system (Addgene, Cambridge, MA) containing *HIS3* and 40 bp homology to the endogenous locus. *ACO2* was tagged at the C-terminus with GFP by transformation with a PCR-amplified GFP tagging cassette from the pFA6a-GFP system (Addgene, Cambridge, MA) containing *KanMX6* and 40 bp homology to the endogenous locus. To tag Cit1p at its C-terminus with mCherry in cells expressing Cdc48p-GFP, cells were transformed with a PCR-amplified pcY3090-02 plasmid containing a *hphMX4* gene for selection with hygromycin B and 40 bp homology to *CIT1* (Young *et al.*, 2012). *CDC48* and *ACO2* were tagged at the C-terminus with 13Myc by transformation with a PCR-amplified pFA6a-13Myc plasmid containing a *His3Mx6* or *KanMX6* gene for selection, respectively, and 40 bp homology to the *CDC48* or *ACO2* endogenous locus. *DOA1* was tagged at the C-terminus with 3HA by transformation with a PCR-amplified pFA6a-3HA plasmid containing a *KanMX6* gene for selection, respectively, and 40 bp homology to the endogenous locus. The *PHB2* genomic locus was disrupted in *phb1Δ* cells by transformation with a PCR-amplified sequence containing HIS3 from the pFA6a-HIS3MX6 and 40 bp homology to the endogenous locus. The rho^0 strains were generated by deleting *MGM101* by disrupting its genomic locus by transformation with a PCR-amplified sequence containing LEU2 from the

plasmid derived from the pOM cassette system (Tamm, 2009) and 40 bp homology to the endogenous locus. The mitochondrial-targeted redox sensitive green fluorescent protein (mito-roGFP1) (Vevea *et al.*, 2013) was transformed into WT, *atg32Δ*, and *doa1Δ* cells.

Following transformation using the lithium acetate method, cells were selected on solid media with the appropriate selective solid media (Gietz *et al.*, 1995). In-frame tagging or disruption of a gene was verified using PCR amplification. *E. coli* DH5-α competent cells (Stratagene, USA) were used for amplification of all plasmids used in this study. Plasmid clones were recovered from bacteria with a MiniPrep kit (Qiagen, Valencia, CA).

Media, culture conditions, and drug treatments

E. coli was cultivated in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1% sodium chloride, pH 7.0) at 37 °C with ampicillin (100 g/L) to select for plasmid-carrying cells. *S. cerevisiae* strains were grown in synthetic complete (SC) medium at 30 °C on a rotary shaker at 225 rpm, as previously described (Fehrenbacher *et al.*, 2004). Synthetic complete (SC) medium, media for selection of auxotrophs, and solid media were prepared according to Sherman (2002). For selection of antibiotic-resistant cells, geneticin (G418) sulfate (Invitrogen, Carlsbad, CA) was added at 200 µg/mL for cells expressing the *kanMX6* gene, and at 300 µg/mL for cells expressing *hphMX4*. Starvation experiments were performed in synthetic minimal medium lacking nitrogen

(SD-N), made as previously described (Kanki *et al.*, 2009b). This medium contains 0.17% yeast nitrogen base without amino acids and 2% glucose.

Cells were treated with 2.5 mM paraquat (Santa Cruz Biotechnology, Dallas, TX) for 12 hr, in glucose-based media, unless otherwise stated. Cells that were treated with the proteasome inhibitor Z-Leu-Leu-Leu-al (MG132) (Sigma, St. Louis, MO) were grown in synthetic medium (0.17% yeast nitrogenous base without ammonium sulfate) supplemented with 0.1% proline, amino acids, and 2% glucose. SDS (0.003%) was added 2 hrs prior to MG132 treatment. Cultures were treated with a final concentration of 75 µM MG132 in dimethyl sulfoxide (DMSO) (Molecular biology grade, Sigma, St. Louis, MO), or the vehicle alone, and incubated under normal growth conditions for 4 hr (Liu *et al.*, 2007).

Growth rate analysis

Cells were grown at 30°C in glucose-based media in a sterile Corning 96-well clear flat bottom polystyrene TC-treated microplate (Life Sciences, Manassas VA, product number 3596) with the cover on. Growth rate was measured by optical density (OD) measurements at 600 nm every 20 min for 3 days using a Tecan Nanoquant plate reader (Tecan US, Morrisville, NC). The plate reader was operated using Magellan software. To prepare initial cultures for inoculation, stationary-phase precultures were diluted and grown to mid-log phase before being diluted to an OD_{600} of 0.07 in a total of 500 µL. These cultures were further diluted to an OD_{600} of 0.0035 by adding 10 µL culture 200 µL of the appropriate media within a single well of the 96-well plate. Cells

were grown in either standard medium or medium containing 2.5 mM paraquat. Each sample within the 96-well plate was prepared in quadruplicates, and growth rate analyses were replicated at least three times.

Chronological lifespan assay

Cultures were inoculated from a single colony into synthetic dextrose complete medium with 4-fold excess of the supplements for which the BY4741 strain is auxotrophic, and grown overnight with shaking at 30 °C (Fabrizio and Longo, 2003). The next morning, cultures were diluted 1:200 into 10 mL of fresh synthetic complete medium with a flask-to-culture volume ratio of 5:1. This incubation time-point was considered day 0**.** An aluminum cap covered each flask to reduce the level of oxygen in the flask. Flasks were left to shake at 30 °C at 200 rpm to allow cells to grow logarithmically until they reached the mostly non-dividing high-metabolism postdiauxic phase within 24 hr (Longo *et al.*, 2012). While traditionally, every two days starting on day 3, an aliquot is removed from the culture and diluted according to the estimated survival and plated onto yeast peptone dextrose (YPD) plates, we began our dilutions on day 1 to incorporate more data points. The plates were incubated at 30°C for 2-3 days. Viability of the cells in the flask at the time that the yeast cells were plated was assessed by counting colony forming units (CFU). Traditionally, viability at day 3, when the great majority of the cells stop dividing, is defined as the initial survival (100%), but for our assay, we defined the initial survival as survival at day 1.

Microscopy

Unless otherwise stated, all imaging was performed in live cells. Single slides were not imaged for more than 15 min. For the localization studies, we used an AxioObserver.Z1 microscope equipped with a Colibri LED excitation source (Carl Zeiss Inc., Thornwood, NY), a wide-field camera (Orca ER, Hamamatsu Photonics, Bridgewater, NJ), and Axiovision or ZEN acquisition software (Zeiss). We used a 100x/1.3 NA EC PlanNeofluar objective (Zeiss) and set the camera to 1x1 binning to optimize spatial resolution. Three-dimensional images over time were acquired by obtaining z-series with 0.5-µm spacing. To visualize mCherry, we used an excitation of 570 nm and a standard rhodamine/RFP filter set. To visualize GFP, we used excitation at 470 nm (100% LED power) and a standard GFP filter set.

Imaging for the mito-roGFP1 studies was performed as previously described (Vevea *et al.*, 2013). Briefly, the oxidized and reduced forms were excited with LEDs at 365nm an 470 nm, respectively, and emission was collected through a standard GFP filter set with the excitation filter removed. For the Cdc48p-GFP studies, imaging was performed on an Axioskop 2 microscope (Zeiss) with a 100x/1.4 NA Plan-Apochromat objective and an Orca 1 cooled charge-coupled device (CCD) camera (Hamamatsu) or an E600 microscope (Nikon) with a Plan-Apo 100x/1.4 NA objective and an Orca-ER cooled CCD camera with FITC filter sets. Hardware was controlled by Openlab software (Perkin-Elmer, Waltham, MA). Three-dimensional images over time were acquired by obtaining z series with 0.3-µm spacing using a piezoelectric focus motor mounted on the objective lens (Polytech PI, Auburn, MA). Images were deconvolved and rendered using Volocity software (Perkin-Elmer).

Image processing and analysis

Images were deconvolved using a constrained iterative restoration algorithm in Volocity software. Maximum projections were reconstructed using Volocity or ImageJ (Schneider *et al.*, 2012). For statistical analysis, p values were calculated with the Student's t-test (two-tailed distribution) or the Kruskal-Wallis significance test.

Protein methods

Protein was isolated from yeast cells as previously described (Boldogh *et al.*, 1998). Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Hexokinase was detected using a rabbit polyclonal anti-hexokinase antibody (Cat. No. LS-C59302) purchased from LifeSpan Biosciences, Inc. (Seattle, WA). HA-tagged proteins were detected using a monoclonal mouse anti-HA antibody (clone 16B12, Cat. No. MMS-101P, Covance, Princeton, NJ). GFP was detected using a rabbit polyclonal anti-GFP antibody (Cat. No. ab290) purchased from Abcam (Cambridge, MA). Ubiquitin was detected using a mouse monoclonal anti-ubiquitin antibody (clone Ubi-1/042691GS, Cat. No. MAB1510) purchased from EMD Millipore (Billerica, MA). Horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) or DyLight 650 fluorescent secondary antibodies (Rockland, Gilbertsville, PA) were used for detection of protein bands for Western blotting. Signal was detected using the Luminata Forte Western Blot HRP substrate (Millipore). Luminescence was recorded using a ChemiDoc MP imaging system and analyzed using Image Lab software (Bio-Rad, Hercules, CA).

Table 2.1. Yeast strains used in this study were constructed as described in the Methods section. All yeast strains were created in the *BY4741* wild-type background.

Strain	Genotype	Source
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems (Huntsville, AL)
5063	*MAT**a** doa1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
3985	*MAT**a** vms1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
3048	*MAT**a** pim1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
7144	*MAT**a** yme1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
148	*MAT**a** yta10Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
6224	*MAT**a** yta12Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
4762	*MAT**a** phb1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
5884	*MAT**a** phb2Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
4547	*MAT**a** atg1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
2103	*MAT**a** atg5Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
7090	*MAT**a** atg7Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
3104	*MAT**a** atg8Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
3847	*MAT**a** atg9Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems

Strain	Genotype	Source
5468	*MAT**a** atg11Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
3357	*MAT**a** atg12Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
2305	*MAT**a** atg32Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
3716	*MAT**a** ufd5Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
1399	*MAT**a** nas2Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
6486	*MAT**a** fpr3Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
7022	*MAT**a** aco2Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
DAY019	*MAT**a** phb1Δ::kanMX6 phb2Δ::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY020	*MAT**a** cit1-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY021	*MAT**a** cdc48-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY022	*cit1-mcherry::hphMX4 cdc48-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY023	*MAT**a** doa1-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY024	*MAT**a** vms1-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY025	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [p416GPD mito-roGFP::URA3]*	This study
DAY026	*MAT**a** doa1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [p416GPD mito-roGFP::URA3]*	This study
DAY027	*MAT**a** atg32Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [p416GPD mito-roGFP::URA3]*	This study
DAY028	*MAT**a** cdc48-13Myc::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study

Strain	Genotype	Source
DAY029	*MAT**a** doa1-3HA::KANMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY030	*MAT**a** doa1-3HA::KanMX6 Cdc48-13Myc::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY031	*MAT**a** aco2-13Myc::KanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY032	*MAT**a** aco2-GFP::KanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY033	*MAT**a** mgm101Δ::LEU2 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY034	*MAT**a** doa1Δ::kanMX6 mgm101Δ::LEU2 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY035	*MAT**a** atg32Δ::kanMX6 mgm101Δ::LEU2 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study

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CHAPTER 3 - INTRODUCTION TO PART II: Aim44p Regulates Phosphorylation of Hof1p to Promote Contractile Ring Closure During Cytokinesis in Budding Yeast

INTRODUCTION

A Brief History of the Study of Cytokinesis

Cytokinesis is the division of cytoplasm between dividing cells and thus is a process that naturally lends itself to be studied closely with karyokinesis, the division of the nucleus (Beams and Kessel, 1976). The study of cell division is essential to our understanding of the transmission of genetic material, cell growth, cell differentiation, and malignancy. As scientists have been interested in cell division for over a century, hundreds of earlier studies have made use of the light microscope (Beams and Kessel, 1976). However, new techniques and improvements to older techniques that arose starting in the 1950s, such as electron microscopy, autoradiography, phase contrast, microdissection, and chemicals to promote and inhibit cell growth, to name a few, allowed for rapid progression of the field (Beams and Kessel, 1976).

The fertilized egg and early blastomere of zebrafish and amphibians are some of the early classic model systems for studying cell division (Beams and Kessel, 1976). Currently, echinoderm embryos are among the most popular model system for studying this process. Studies using these models and scanning electron micrographs allowed for the observation and characterization of the cleavage furrow, the contractile ring, and spindle fibers (Beams and Kessel, 1976). Other early studies were performed in tissue culture models, such as HeLa cells (Beams and Kessel, 1976). Despite the fact that the study of cytokinesis dates back to the 19th century, the field is expanding considerably as a major topic of research for researchers not only interested in understanding cell

division as an essential process for life, but also for investigators interested in studying its role in human disease, especially cancer (Burgess, 2005).

Among all of the events that occur throughout the cell cycle, it is interesting to note that cytokinesis may be the most divergent among different cell types. In the most extreme case exemplifying these differences, some cells do not even undergo cell division following nuclear division, as in the case of the slime mold *Physarum* (Hyams, 2005). In the case of plants and fungi, cell division includes the formation of a cell wall between the dividing cells (discussed in detail below). Yet even in the case of cell wall formation during cell division, differences exist in the site of origin and direction in which the wall is deposited in different cell types (Hyams, 2005). Metazoan cell division does not include cell wall deposition. Instead, animal cells generally divide through the constriction of an invagination in the cell membrane called a cleavage furrow (discussed in detail below) (Hyams, 2005). These unique differences, among others, have presented a challenge in studying cytokinesis compared to other events throughout the cell cycle, but also contribute to making this field a broad and intriguing realm of study.

Overview of Key Events in Animal Cell Cytokinesis

Selection of the Site of Cell Division

In metazoan cells, several models have been proposed to explain the role of microtubules and the mitotic apparatus in selecting the site of the division plane. In 1940, experiments employing microtubule inhibitors and cold temperatures to depolymerize microtubules revealed a role for microtubules in establishing the position

of the cleavage furrow (Beams W. H. and Evans T.C., 1940). To discuss current models of site selection for the cleavage furrow in cytokinesis, it is necessary to understand different microtubule orientations. Kinetochore microtubules span the chromosomes and the spindle poles (Fig. 3.1) (Burgess and Chang, 2005). Spindle midzone microtubules are microtubule bundles that stretch from the centrosomes towards the midzone of the mitotic apparatus and do not make contact with chromosomes (Fig. 3.1) (Burgess and Chang, 2005). Equatorial microtubules and polar microtubules are types of astral microtubules (Fig. 3.1) (Burgess and Chang, 2005). Equatorial astral microtubules stem from the centrosomes towards the cleavage furrow (Fig. 3.1) (Burgess and Chang, 2005). Polar astral microtubules extend from the centrosomes towards the polar cell surface, away from the midzone (Fig. 3.1) (Burgess and Chang, 2005).

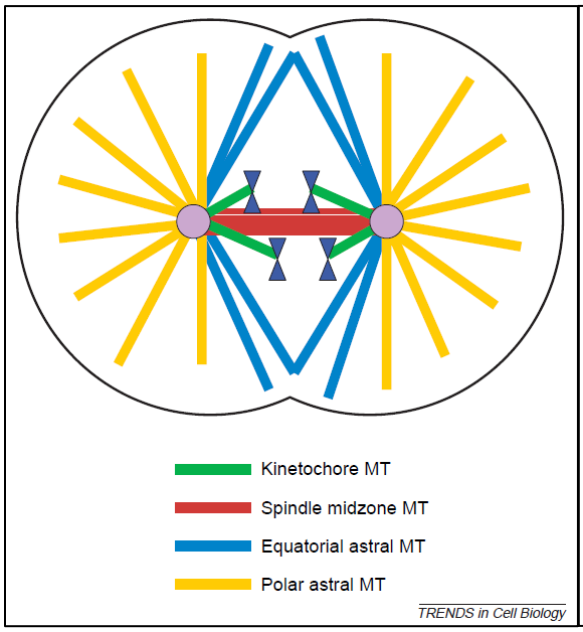

Figure 3.1. Understanding the current models of site selection for the cleavage furrow in cytokinesis requires understanding different types of microtubules, which are defined for this purpose in terms of their geometrical relationships. Kinetochore microtubules span the chromosomes and the spindle poles (green). Spindle midzone microtubules are microtubule bundles that stretch from the centrosomes towards the midzone of the mitotic apparatus and do not make contact with the chromosomes (red). Equatorial astral microtubules stem from the centrosomes towards the cleavage furrow (blue). Polar astral microtubules extend from the centrosomes towards the polar cell surface, away from the midzone (yellow). This figure is from Burgess and Chang, 2005.

In the **equatorial stimulation model** of cytokinesis, equatorial astral microtubules send a positive signal to the cortex to induce cleavage furrow formation (Burgess and Chang, 2005). This model is supported by the observation of Ray Rappaport and colleagues in which they performed micromanipulation experiments on echinoderm eggs and used brightfield and phase-contrast microscopy to visualize the mitotic apparatus (Burgess and Chang, 2005). For example, modulating echinoderm eggs into unnatural cylindrical shapes such that astral rays are unlikely to reach the polar cortex revealed that the eggs divided at the plane between the spindle poles (Rappaport, 1960). Additionally, physically blocking the asters from contacting the equatorial region prevents normal cleavage, further supporting an active role in the microtubule apparatus on cleavage furrow formation (Rappaport, 1968; Rappaport and Rappaport, 1983).

The **polar relaxation model** proposes that astral microtubules signal to the polar cortex to inhibit cortical tension, preventing furrow formation at the poles, yet allowing formation at the metaphase plate. This model is supported by the observation that equatorial astral microtubules in egg models are less dense at the cortex of the cleavage furrow than in the polar areas (Asnes and Schroeder, 1979; White and Borisy, 1983; Dechant and Glotzer, 2003; Yoshigaki, 2003). However, manipulation of the astral microtubule density did not change the localization of cleavage furrow formation, arguing against this model (Rappaport, 1960; Rappaport and Rappaport, 1984).

The **spindle midzone model** proposes that proteins in the midzone, along with antiparallel, non-kinetochore associated microtubules communicate with the cortex to

stimulate cleavage furrow formation (Burgess and Chang, 2005). It should be noted that these models may not be mutually exclusive and future work in this area may build upon one or more of these models.

Assembly, Contraction, and Disassembly of the Contractile Components

While obvious differences were observed between the early model systems used to study cell division, a great many commonalities between their cell division processes were noted, especially regarding the cleavage furrow and the existence of microfilaments in the region of the cleavage furrow that were likely providing the driving force for cell-cell separation (Schroeder, 1990). For example, in all systems studied, the cleavage furrow was identified as a marker of the division site, appearing as an indentation or ingression on the cell's surface that becomes progressively deeper until the point of cell-cell separation.

The concept that a force ultimately drives cell division was originally proposed by Douglas Marsland in the early 1950s and later modified by Lewis Wolpert (Marsland and Landau, 1954; Wolpert, 1960; Schroeder, 1990). By 1960, scientists speculated that cleavage furrow closure might occur by a mechanism much like that by which muscle contraction occurs, though there were no experiments that specifically provided evidence for this idea (Schroeder, 1990). It was not until after this hypothesis was proposed that actin was eventually identified in non-muscle cells (Schroeder, 1990). Later, experiments revealed "actin-sized" microfilaments localized in the cleavage furrow, supporting the hypotheses of Marsland and Wolpert, and within several years,

the microfilaments were identified biochemically as F-actin (Schroeder, 1990). The remainder of this section will highlight the great progress made in the field since the early observations of the cleavage furrow and will discuss the current state of the field in understanding the molecular composition of the contractile components, as well as their regulation.

The contractile ring mediates cell division in many cell types. The contractile ring is composed of F-actin, myosin II, and about 50 accessory proteins (Wolfe and Gould, 2005). Contractile ring assembly is mediated by a GTP-bound, active pool of RhoA (Rho1p in budding yeast) molecules (Piekny *et al.*, 2005). The Rho GTPase family of proteins is made up of small proteins composed of a GTPase domain and an additional approximately 30 amino acid residues. Some of these additional residues undergo posttranslational modifications that alter the localization of the protein and promote interaction with lipid membranes including the plasma membrane, endosomes, and the Golgi apparatus (Piekny *et al.*, 2005). Rho GTPases play an important role in the regulation of the actin cytoskeleton and myosin II dynamics, implicating them in a wide range of cellular functions, including cytokinesis (Piekny *et al.*, 2005).

In metazoan cells, which will be the focus of this section, RhoA is the main Rho GTPase that regulates assembly of the contractile ring, the forces that promote its contraction, and the stability of the contractile ring in late stages of cytokinesis (Piekny *et al.*, 2005). In fact, inhibiting RhoA through knockdown by RNA interference in *Caenorbabditis elegan* embryos and by chemical inhibition in *Xenopus* embryos resulted in defects in

cortical ring contraction and furrow formation in most cases (Kishi *et al.*, 1993; Drechsel *et al.*, 1997; Jantsch-Plunger *et al.*, 2000; Yüce *et al.*, 2005).

The active, GTP-bound form of RhoA promotes cytokinesis by binding to and regulating effector proteins such as the formin family, the Rho-dependent kinase (ROK) and the Citron kinase (Piekny *et al.*, 2005). As expected, formins promote the polymerization of actin essential for the assembly of the contractile ring (Piekny *et al.*, 2005). Specifically, the formins that regulate cytokinesis are normally auto-inhibited by the binding of their C- and N-terminal domains (Alberts, 2001). RhoA binds to a region of the formins that partially overlaps with the C-terminal region normally involved in the autoinhibited-binding state, thus allowing the formins to promote actin polymerization (Otomo *et al.*, 2005; Rose *et al.*, 2005).

RhoA activation indirectly regulates contractile ring constriction by directly activating the kinase activity of ROK, which in turn regulates myosin (Piekny *et al.*, 2005). The phosphorylation of a conserved serine residue (S19) within one of the regulatory light chains of myosin II promotes myosin filament assembly (Piekny *et al.*, 2005). This activates the actin-dependent ATPase activity of the motor domain and ultimately provides the driving force for actomyosin ring constriction. While the S19 residue of myosin can be phosphorylated by other kinases, such as MLCK and Citron kinase, suggesting some functional redundancy, inhibiting ROK activity results in either a delay or complete failure of cleavage furrow ingression in human tissue culture cells, *Drosophila melanogaster*, and *C. elegans* (Kosako *et al.*, 2000; Piekny and Mains,

2002; Royou *et al.*, 2002). Interestingly, ROK not only phosphorylates myosin, but also prevents its dephosphorylation by inhibiting the phosphatase responsible for dephosphorylation at the key S19 residue (Kimura *et al.*, 1996; Piekny and Mains, 2002).

The activation of Citron kinase by GTP-bound RhoA plays a role in late stages of ring closure to varying extents, depending on cell type (Piekny *et al.*, 2005). The localization of Citron kinase to the cleavage furrow in both human and *D. melanogaster* depends upon active RhoA (Eda *et al.*, 2001; Shandala *et al.*, 2004). In mice, deleting Citron kinase produces cytokinesis defects in only a few tissues, having the greatest detrimental effect on the brain and central nervous system tissues compared to other tissues (Di Cunto *et al.*, 2000). In contrast, in *D. melanogaster*, Citron kinase is essential for cytokinesis in all cell types (Shandala *et al.*, 2004). In flies lacking Citron kinase, the furrow fully forms and constricts, but later regresses upon becoming unstable, accompanied by aberrant furrow formation (D'Avino *et al.*, 2004; Echard *et al.*, 2004; Naim *et al.*, 2004). Although evidence supports a role for activated Citron kinase in the stabilization of the actomyosin ring in late stages of contraction, an exact mechanism for this regulatory role is unknown. Interestingly, *in vitro* studies reveal that Citron kinase can phosphorylate myosin II at two residues, Thr18 and Ser19 (Yamashiro *et al.*, 2003). Moreover, in normal rat kidney epithelial (NRK) cell lines, the di-phosphorylated form of myosin has a more constrained localization to the cleavage furrow, especially late in cytokinesis, than the mono-phosphorylated form (Yamashiro *et al.*, 2003).

In a 1972 publication, Thomas E. Schroeder noted that during the first cleavage in the eggs of *Arbacia punctulata* (sea urchin), the contractile ring exists from only about 20 seconds after anaphase to the end of furrow ingression, for a total duration of only approximately six to seven minutes (Schroeder, 1972). He also noted that during contraction, the volume of the contractile ring components decreases, indicating that the contraction of the ring occurs concomitantly with ring disassembly (Schroeder, 1972). In 1995, Gunsalus *et al*. suggest that the contractile ring disassembles as a result of an actin-binding protein, such as the cofilin family of proteins that depolymerize actin filaments and this view remained widely-accepted for another decade (Gunsalus *et al.*, 1995). In 2005, two publications, one by Murthy and Wadsworth and the other by Wang and colleagues, find evidence that the myosin ATPase activity is required for actin disassembly in the contractile ring using cultured mammalian cells (Guha *et al.*, 2005; Murthy and Wadsworth, 2005). Using fluorescence recovery after photobleaching (FRAP) experiments, both groups used drug treatments such as blebbistatin to inhibit the ATPase activity of myosin and observed that this decreased the dynamics of the fluorescently-labeled actin within the contractile ring (Guha *et al.*, 2005; Murthy and Wadsworth, 2005). This work provides compelling evidence for the ATPase activity of myosin II in the disassembly of the actomyosin ring during ring constriction, an observation that was made over 40 years ago but which is still not mechanistically well understood.

The work of Jian-Qiu Wu and Thomas D. Pollard provided the field of cytokinesis with great insight into not only the composition of the contractile ring, but also its temporal

regulation. In their work using the fission yeast *Schizosaccharomyces pombe*, they used fluorescence microscopy and flow cytometry to measure the amount of nearly 30 cytoskeletal and signaling YFP fusion proteins and to monitor their localizations during the cell cycle in *S. pombe* (Wu and Pollard, 2005). Importantly, they verified the functionality of each fusion protein and used the native promoters of each to ensure physiological activity and proper stoichiometry during their studies (Wu and Pollard, 2005). The data provided by this study demonstrate just how intricate and coordinated the role of each protein is within the entire process of cell division and emphasize the importance of this process in the propagation of genetic inheritance in a population of dividing cells.

While this section focused on the assembly and constriction of the contractile ring during cytokinesis in animal cells, it should be noted that this is covered with more specific relation to the budding yeast *Saccharomyces cerevisiae* (the model system used in this work) in greater detail in the Introduction to Chapter 4. Additionally, the roles of the septins, which also form rings at the start of cell division and play critical roles in cytokinesis are discussed below, with specific attention given to their functions in yeast, the organism in which they were discovered.

New Membrane Insertion at the Division Site

Contractile ring constriction is accompanied by the addition of new membrane material to the division site, allowing for the necessary expansion of this region to accommodate cell separation (Wolfe and Gould, 2005). In this process, the secretory apparatus is re-

directed towards the site of cell division (Wolfe and Gould, 2005). The secretory apparatus, including the exocyst complex, plays a key role in the delivery of secretory vesicles, generally derived from the Golgi, to the plasma membrane for fusion (Ting *et al.*, 1995; TerBush *et al.*, 1996; Grindstaff *et al.*, 1998). Treating cells with brefeldin A (BFA), an Arf GTPase inhibitor that blocks the anterograde transport of vesicles from the ER, thereby causing Golgi disruption, blocking cytokinesis in its late stages, causing failure of cell division in the first zygotic division of *C. elegan* embryos (Donaldson and Jackson, 2000; Skop *et al.*, 2001). This observation indicates that the membrane trafficked to the site of cell division, and thus contributing to membrane expansion, is indeed Golgi-derived. In yeast, cell membrane deposition is also accompanied by the synthesis and deposition of new cellular wall components in a process called septation, which will be discussed below.

Cytokinesis in Budding Yeast

In the budding yeast *S. cerevisiae*, cytokinesis is highly homologous to cytokinesis in animal cells, sharing both structures and mechanisms (Wloka and Bi, 2012). For example, actomyosin contractile ring formation and constriction is a homologous mechanism in yeast and animal cells (Bi et al., 1998). While septation, or cell wall deposition (discussed in more detail below), occurs in yeast but not in animal cells, this mechanism is considered analogous to the deposition of the extracellular matrix and its remodeling that occurs during cell division in mammals (Bi and Park, 2012). Taking these similarities and differences into account, yeast are an exceptional model organism for studying cell division because actomyosin contractile ring closure and septation can

be perturbed in yeast without causing lethality, giving this model organism a major advantage over animal cells (Rodriguez and Paterson, 1990; Lippincott and Li, 1998b; Schmidt *et al.*, 2002). Additionally, many conditional lethal mutants have already been developed for this organism, facilitating studies that would be difficult or impossible in other organisms. Yeast cells are also readily genetically manipulatable and can be easily used to generate genetic knockouts and genetically encoded fusion proteins regulated by their endogenous promoters.

Overview of the Septins in Yeast Cell Division

Septins were first discovered in the budding yeast *S. cerevisiae* in the early 1970s during a mutagenesis screen performed by Lee Hartwell to identify genes involved in cell division (Hartwell, 1971). The protein products of the four septins discovered in this screen (Cdc3p, Cdc10p, Cdc11p, and Cdc12p) were later determined by fluorescence microscopy to localize to the mother-bud neck in budding yeast (Haarer and Pringle, 1987; Kim *et al.*, 1991). Septins are highly conserved proteins, existing in all known eukaryotes except for land plants (Saarikangas and Barral, 2011). However, the number of septin genes between organisms differs greatly and alternative splicing in some organisms contributes to further diversity (Cao *et al.*, 2007; Pan *et al.*, 2007; Saarikangas and Barral, 2011). While they are conserved, septins do not share high sequence homology (Mostowy and Cossart, 2012).

The septins are a subfamily of GTP-binding proteins that form hetero-oligomeric filament-forming complexes (Pan *et al.*, 2007; Bertin *et al.*, 2008, 2012; Garcia *et al.*,

2011). Septins are known for their formation of hetero-complexes which polymerize into higher-order structures, including filaments, rings, and cage-resembling structures (Saarikangas and Barral, 2011; Mostowy and Cossart, 2012). It is their ability to form filamentous structures that has gained them recognition as a noncanonical fourth component of the cytoskeleton (Mostowy and Cossart, 2012).

The filamentous structures formed by septins are non-polar, similar to the intermediate filaments of the cytoskeleton, but distinct from actin and microtubule filaments (Mostowy and Cossart, 2012). During septin polymer formation, septins interact via their GTP-binding domains and through their N- and C-terminal domains (Sirajuddin *et al.*, 2007, 2009; Bertin *et al.*, 2008; Zent *et al.*, 2011). GTP hydrolysis may regulate septin-septin binding by stimulating conformational changes in these domains (Sirajuddin *et al.*, 2007, 2009). Interestingly, septins can interact physically with actin (Kinoshita *et al.*, 2002; Joo *et al.*, 2007), microtubules (Surka *et al.*, 2002; Nagata *et al.*, 2003; Kremer *et al.*, 2005; Spiliotis *et al.*, 2008; Bowen *et al.*, 2011; Sellin *et al.*, 2011), and phospholipid membranes (Tanaka-Takiguchi *et al.*, 2009; Bertin *et al.*, 2010), and these interactions between septins and actin, microtubules, and phospholipids may regulate, promote, and stabilize septin filament formation (Mostowy and Cossart, 2012). Interaction of septins with phospholipid membranes may also contribute to their function in modulating membrane morphology (Tanaka-Takiguchi *et al.*, 2009). Finally, septins are post-translationally modified by phosphorylation (Barral *et al.*, 1999; Mortensen *et al.*, 2002; Dobbelaere *et al.*, 2003; Versele and Thorner, 2004; Garcia *et al.*, 2011), sumoylation (Johnson and Blobel, 1999; Johnson and Gupta, 2001), and ubiquitylation (Zhang *et al.*,

2000), and these modifications may also regulate septin filament assembly and/or disassembly.

The septins provide structural support for assembly and maintenance of the actomyosin ring at the bud neck, and play a role in primary septum formation in yeast (which will be discussed in more detail in the following section) (Longtine *et al.*, 1996). Immediately before bud emergence, the septins localize to the presumptive bud site as unorganized septin patches. These patches then form an organized, single cortical ring (Fig. 3.2) (Iwase *et al.*, 2006; Merlini and Piatti, 2011). During bud growth, the septin ring forms an hourglass structure (the septin collar) at the mother-bud neck, and at the onset of cytokinesis, the mitotic exit network (MEN) signals the septins to form a double ring that surrounds the actomyosin ring (Cid *et al.*, 2001; Lippincott *et al.*, 2001). The double septin ring structure is maintained throughout cytokinesis. Thereafter, the mother and daughter cell each retain a single septin ring at the cell division site (Cid *et al.*, 2001).

Interestingly, fluorescence recovery after photobleaching experiments revealed that the septin single ring and double ring structures are dynamic, while the septin hourglass structure is not (Caviston *et al.*, 2003; Dobbelaere *et al.*, 2003). Formation of the septin hourglass structure is regulated by kinases that are recruited to the bud neck in a septin-dependent manner (Merlini and Piatti, 2011). Formation of the hourglass structure requires phosphorylation of several septins by the kinases Cla4p and Gin4p, which are themselves phosphorylated by Elm1p (Sreenivasan and Kellogg, 1999; Dobbelaere *et al.*, 2003; Versele and Thorner, 2004). Later, dephosphorylation of the

septin Shs1p by Rts1p, the catalytic subunit of the protein phosphatase 2A (PP2A) is necessary for septin reorganization from the hourglass structure to double rings (Dobbelaere *et al.*, 2003). The septins are organized parallel to the mother-bud axis within the hourglass structure, and then reorient 90 degrees such that they are perpendicular to the mother-bud axis in the septin double rings (Vrabioiu and Mitchison, 2006; DeMay *et al.*, 2011).

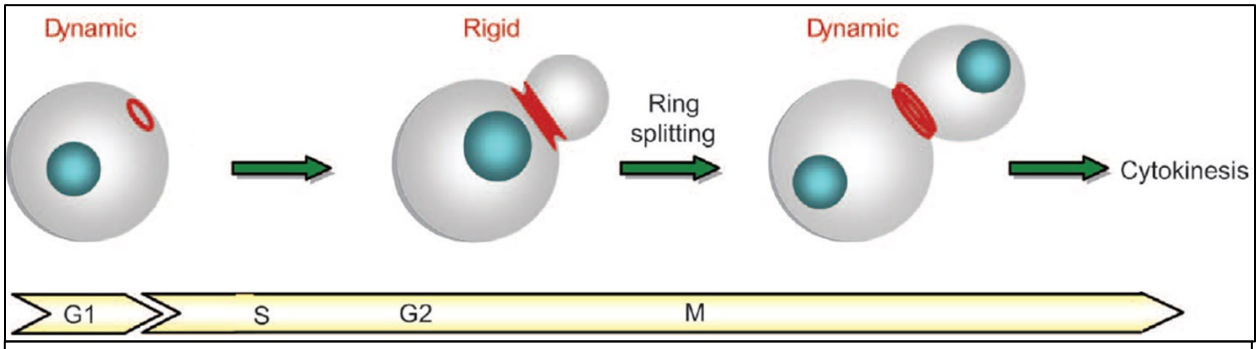

Figure 3.2. In budding yeast, immediately before bud emergence, the septins localize to the presumptive bud site as unorganized septin patches, which rapidly form an organized, single cortical ring. During bud growth, the septin ring forms an hourglass structure at the mother-bud neck. At the onset of cytokinesis, the MEN signals the septins to form a double ring that surrounds the actomyosin ring. The double septin rings are maintained throughout cytokinesis, after which the mother and daughter cell each retain a single septin ring at the cell division site. Figure modified from Merlini and Piatti, 2011.

Septins also function as a lateral diffusion barrier during cell division. Specifically, septins decrease the diffusion rate of membrane-associated molecules, thereby promoting the asymmetric distribution of proteins critical for cytokinesis to the mother-bud neck by (Longtine and Bi, 2003; Dobbelaere and Barral, 2004; Saarikangas and Barral, 2011).

Finally, septins play a role in ensuring proper chromosome segregation during cell division. They regulate spindle positioning, in part, through effects on microtubule capture and shrinkage (Kusch *et al.*, 2002). During this process, cytoplasmic microtubules emerging from the spindle pole bodies (SPBs) extend, contract, survey the cell cortex and ultimately make contact with dynein in the bud tip. This in turn leads to dynein-dependent pulling forces on cytoplasmic microtubules, which is critical for positioning the nucleus within the bud neck (Palmer *et al.*, 1992; DeZwaan *et al.*, 1997). Septins are required for the interaction of the microtubules with the bud neck (Kusch *et al.*, 2002). They are also required for recruitment of kinases including Elm1p, Gin4p, and Hsl1p to the bud neck, which in turn is necessary for the asymmetric distribution of dynein at the spindle poles (Merlini and Piatti, 2011).

Recently, septins have also been implicated in regulating the spindle position/orientation checkpoint (SPOC). This checkpoint is responsible for delaying the exit from mitosis and cytokinesis if the mitotic spindle is not properly oriented along the axis of cell division, allowing time for correction of the misalignment (Merlini and Piatti, 2011). Activation of this checkpoint requires septin ring formation and several kinases that are recruited to the bud neck in a septin-dependent manner (Fraschini *et al.*, 2006).

CHAPTER 4 - Aim44p Regulates Phosphorylation of Hof1p to Promote Contractile Ring Closure During Cytokinesis in Budding Yeast

ABSTRACT

While actomyosin and septin ring organization and function in cytokinesis are thoroughly described, little is known regarding the mechanisms whereby the actomyosin ring interacts with septins and associated proteins to coordinate cell division. Here, we show that the protein product of *YPL158C,* Aim44p, undergoes septin-dependent recruitment to the site of cell division. Aim44p co-localizes with Myo1p, the type II myosin of the contractile ring, throughout most of the cell cycle. The Aim44p ring does not contract when the actomyosin ring closes. Instead, it forms a double ring that associates with septin rings on mother and daughter cells after cell separation. Deletion of *AIM44* results in defects in contractile ring closure. Aim44p co-immunoprecipitates with Hof1p, a conserved F-BAR protein that binds both septins and type II myosins and promotes contractile ring closure. Deletion of *AIM44* results in a delay in Hof1p phosphorylation, and altered Hof1p localization. Finally, overexpression of Dbf2p, a kinase that phosphorylates Hof1p and is required for re-localization of Hof1p from septin rings to the contractile ring and for Hof1p-triggered contractile ring closure, rescues the cytokinesis defect observed in *aim44*Δ cells. Our studies reveal a novel role for Aim44p in regulating contractile ring closure through effects on Hof1p.

INTRODUCTION

Key Events during Cytokinesis in Budding Yeast

In the budding yeast *S. cerevisiae*, cytokinesis involves actomyosin contractile ring closure and septum formation at the mother-bud neck (Bi *et al.*, 1998). In the budding yeast, septin proteins play an instrumental role in establishing the cell division plane and recruit other components of the cytokinetic machinery (Wolfe and Gould, 2005). In haploid budding yeast, sites of cell division occur adjacent to each prior division site (Chant, 1999). In diploid budding yeast, bud emergence on the opposite pole of the prior site of division (Chant, 1999). The actomyosin ring contains F-actin formed by formin-dependent processes (Fang *et al.*, 2010; Wloka and Bi, 2012). During actomyosin ring assembly Myo1p, the only myosin-II heavy chain in budding yeast, is recruited to the site of cell division in late G1 by septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p, and Shs1p/Sep7p), the septin-interacting protein Bni5p, the essential myosin light chain (Mlc1p), and an IQGAP family protein (Iqg1p/Cyk1p) in a highly regulated fashion (Epp and Chant, 1997; Bi *et al.*, 1998; Lippincott and Li, 1998b; Lee *et al.*, 2002). Constriction of the actomyosin ring provides a force for ingression of the plasma membrane. During its constriction, Myo1p disassembles from the ring (Tully *et al.*, 2009) and vesicles containing materials essential for the formation of new plasma membrane are deposited at the cell division site (Fang *et al.*, 2010).

In yeast cells, cytokinesis also involves septation, the deposition of cell wall between mother and daughter cells. After actomyosin ring constriction begins, a specialized extension of the cell wall, the primary septum, begins to form at the bud neck through

reactions catalyzed by chitin synthase II (Chs1p) (Sburlati and Cabib, 1986). The primary septum is completed after contractile ring closure and separation of the plasma membranes of mother and daughter cells. Thereafter, a secondary septum is laid down on each side of the primary septum. Finally, hydrolytic enzymes are released from the daughter cell to degrade the primary septum and part of the secondary septum, allowing for cell separation (Kuranda and Robbins, 1991; Kovacech *et al.*, 1996; Baladrón *et al.*, 2002).

Coordination of the Septins and Actomyosin Contractile Ring Closure by Hof1p

Several proteins have been implicated in interactions between the septins and the actomyosin ring, including Hof1p, an F-BAR protein that regulates contractile ring closure. F-BAR proteins are membrane-associated proteins that sense membrane curvature and bind preferentially to curved membranes. F-BAR proteins also can link the plasma membrane to the actin cytoskeleton in processes such as cytokinesis and endocytosis (Oh *et al.*, 2013). The localization of Hof1p during cell division is highly dynamic and tightly regulated. Hof1p binds to septins and type II myosins (Myo1p in yeast) and is recruited to septin rings at the mother-bud neck. The N-terminus of Hof1p interacts with the septin Cdc10p, while the C-terminus of Hof1p interacts with Myo1p (Meitinger *et al.*, 2013a; Oh *et al.*, 2013). The ring structure formed by Hof1p at the mother-bud neck first forms on the mother side of the bud neck, after which a second Hof1p ring forms on the daughter side (Vallen *et al.*, 2000).

The localization of Hof1p is also regulated by the Mitotic Exit Network (MEN). The MEN is a GTPase signaling cascade, which includes several key kinase activity events, originating at the spindle pole body (Wolfe and Gould, 2005). High levels of Cdk1p-Cyclin-B activity throughout the cell division process prevents premature cytokinesis, ensuring that this process occurs only after the inheritance of genetic material and organelles (Wolfe and Gould, 2005). One role of the high levels of Cdc1p-Cyclin-B is to phosphorylate the MEN protein kinase Cdc15p, preventing its localization to the daughter poles, which is essential for cytokinesis (Jaspersen and Morgan, 2000; Menssen *et al.*, 2001). Once Cdc1p-Cyclin-B is inactivated, the MEN becomes activated. Upon activation of the MEN pathway, Cdc14p is released from its prior nucleolar sequestration (Jaspersen *et al.*, 1998; Stegmeier *et al.*, 2002). Cdc14p is a conserved protein phosphatase that inactivates Cdc1p-Cyclin-B, further stimulating the MEN pathway (Visintin *et al.*, 1998; Shou *et al.*, 1999).

One of the most downstream events of MEN activation, which is of particular relevance to this work, is the translocation of the Dbf2p-Mob1p protein kinase complex (Hwa Lim *et al.*, 2003; Meitinger *et al.*, 2011). Phosphorylation of Hof1p by the MEN kinase complex Mob1p-Dbf2p at the S313 residue results in release of Hof1p from septin rings and association of the protein with the actomyosin ring (Meitinger *et al.*, 2011, 2013a). Actomyosin ring closure is also regulated by the MEN (Vallen *et al.*, 2000; Lippincott *et al.*, 2001; Luca *et al.*, 2001). Recent studies indicate that the SH3 domain of Hof1p is critical for maintaining the symmetry of Myo1p ring constriction during cytokinesis (Korinek *et al.*, 2000; Vallen *et al.*, 2000; Oh *et al.*, 2013) and that phosphorylation of

Hof1p by Mob1p/Dbf2p at S533 and S563 promotes its function in contractile ring closure (Meitinger *et al.*, 2013a). Hof1p function in contractile ring closure also requires interaction of Hof1p with Inn1p, an essential protein that localizes to the contractile ring and is required for coordinating contractile ring closure with plasma membrane ingression (Sanchez-Diaz *et al.*, 2008).

Evidence also supports a role for Hof1p in septation, deposition of a cell wall at the site of mother-bud separation. First, there is a temporal link. The single ring formed by Hof1p does not fully contract, but only partially constricts, during contractile ring closure. Then, the Hof1p single ring divides to form two rings at the onset of septation (Vallen *et al.*, 2000). Second, there is a physical link. Hof1p also interacts with the chitin synthase, Chs2p, an enzyme that is required for primary septum formation. Thus, Hof1p also may coordinate actomyosin contractile ring closure with primary septum formation (Oh *et al.*, 2013).

At the end of the cell division cycle, Hof1p is degraded. Hof1p degradation begins in late mitosis and is mediated by the Grr1p E3 ligase (Blondel *et al.*, 2005; Stockstill *et al.*, 2013). It is possible that Hof1p degradation is related to the regulation of actomyosin ring closure. However, this link between Hof1p and ring closure is controversial (Blondel *et al.*, 2005; Stockstill *et al.*, 2013).

A Potential Role for Aim44p in Cytokinesis

At the onset of these studies, Aim44p, the protein product of *YPL158C*, was characterized as a protein of unknown function. However, genome-wide screens revealed a role for Aim44p in mitochondrial DNA (mtDNA) inheritance (Hess *et al.*, 2009) and in the cell cycle (Doolin *et al.*, 2001; Wang *et al.*, 2011a). A screen for mutations leading to altered inheritance of mitochondria (AIM) revealed that yeast cells lacking *YPL158C* have an increased loss of mtDNA compared to wild-type cells (Hess *et al.*, 2009). However, this study revealed no other defects in mitochondrial biogenesis or inheritance in cells lacking *YPL158C*.

Other studies revealed a potential role for Aim44p in the cell cycle. A global protein localization study revealed that the Aim44p protein fused to GFP at the C-terminus localizes to the mother-bud neck (Huh *et al.*, 2003). Consistent with this, Aim44p has physical interactions with several cell division proteins, such as Hof1p, Boi1p and Boi2p, Cyk3p, and Kip3p (Wong *et al.*, 2007; Tonikian *et al.*, 2009; Hoppins *et al.*, 2011). Finally, *AIM44* expression is regulated by the yeast transcription factor Swi5p, which targets genes involved in mating type switching, exit from mitosis, and cell wall function (Doolin *et al.*, 2001; Cocklin *et al.*, 2011).

While this work was in preparation, another group reported a role for Aim44p in cell polarity and septation during cell division and renamed the protein GTPase-mediated polarity switch 1 (Gps1p) (Meitinger *et al.*, 2013b). This group obtained evidence that Aim44p contributes to maintaining cell polarity in a Rho1p-dependent manner (Meitinger *et al.*, 2013b). Rho1p is a Rho-GTPase family protein involved in regulating cell polarity

and that functions in secondary septum formation and cell-cell separation (Meitinger *et al.*, 2013b). Meitinger et al. also find a role for Aim44p in preventing premature activation of Cdc42p, thus preventing a phenotype characterized by “re-budding” inside the old bud site that they observe in *aim44Δ* cells (Meitinger *et al.*, 2013b).

In our work, we show novel functions for Aim44p during the cell division process. Namely, we find that Aim44p is recruited to the bud neck by the septins, where it regulates Hof1p phosphorylation and localization, and promotes contractile ring closure, a process common to cell division in all cell eukaryotes (Wolken *et al.*, 2014). We also provide evidence that Aim44p interacts physically with Hof1p (Wolken *et al.*, 2014). Thus, Aim44p is a novel regulator of contractile ring closure in budding yeast.

RESULTS

*aim44*Δ cells exhibit defects in contractile ring closure during cytokinesis

We find that deletion of *AIM44* results in a multibudded phenotype, which is a hallmark of cytokinesis failure. In the wild-type cells used for these studies, 18.8% ± 1.0% cells examined are multibudded (García-Rodríguez *et al.*, 2009). Deletion of *MYO1*, a known mediator of cytokinesis, results in a 69.2% increase in multibudded cells compared to wild-type cells (61.2% ± 13.5%, p = 0.006). Similarly, in *aim44*Δ cells, we detect a 69% increase in multibudded cells compared to wild-type cells (60.7% ± 3.6%, $p = 4x10^{-5}$) (Fig. 4.1 A). From these data, we conclude that Aim44p contributes to mother-daughter separation in *S. cerevisiae*.

Failure of yeast cells to separate can indicate a defect in contractile ring closure or septation. To determine whether *aim44*Δ cells have septation defects, cells were treated with zymolyase, an enzyme isolated from *Arthrobacter luteus* that digests cell wall polymers (Lippincott and Li, 1998a). Zymolyase treatment results in cell separation in strains with septation defects, such as *cbk1*Δ cells, but not in yeast with defects in contractile ring closure (Hartwell, 1971). The proportion of *cbk1*Δ cells in multibudded clusters significantly decreases after digestion with zymolyase (before digestion: 34% ± 6%; after digestion: 7% ± 2%, p = 0.002) (Fig. 4.1 B). In contrast, zymolyase treatment has no significant effect on the multibudded phenotype of *aim44*Δ cells (before digestion: 44% ± 8%; after digestion: 48% ± 7%, p = 0.5) (Fig. 4.1 B). Thus, the multibudded phenotype of *aim44*Δ cells is not due to defects in septation that are sensitive to zymolyase treatment.

In light of this, we studied the effect of deletion of *AIM44* on contractile ring closure. Myo1p, the type II myosin of the actomyosin ring, was tagged at its chromosomal locus using GFP in wild-type and *aim44*Δ cells. Previous studies indicate that this tag has no effect on Myo1p function (Lippincott and Li, 1998b). Deletion of *AIM44* does not affect assembly of Myo1p into a ring at the bud neck (Fig. 4.1 C). However, deletion of *AIM44* results in defects in contractile ring closure. In synchronized wild-type cells, contractile ring closure, visualized as a decrease in the diameter of the Myo1p-GFP-labeled contractile ring, occurs in 91% of the cells analyzed (n = 66) and is completed within 10 min from the onset of contraction (Fig. 4.1 C). In contrast, the contractile ring fails to close in 50% of the *aim44*Δ cells examined (n = 52) (Fig. 4.1). Notably, in the *aim44*Δ cells that do undergo contractile ring constriction, the rate of ring closure is not statistically different from that in wild-type cells (p = 0.96). However, in *aim44Δ* cells with contractile ring closure defects, Myo1p-GFP persists as a ring that does not contract and ultimately dissipates (Fig. 4.1 C). These *aim44*Δ cells eventually develop another bud and become multibudded. These findings are consistent with a role for Aim44p in regulating contractile ring closure.

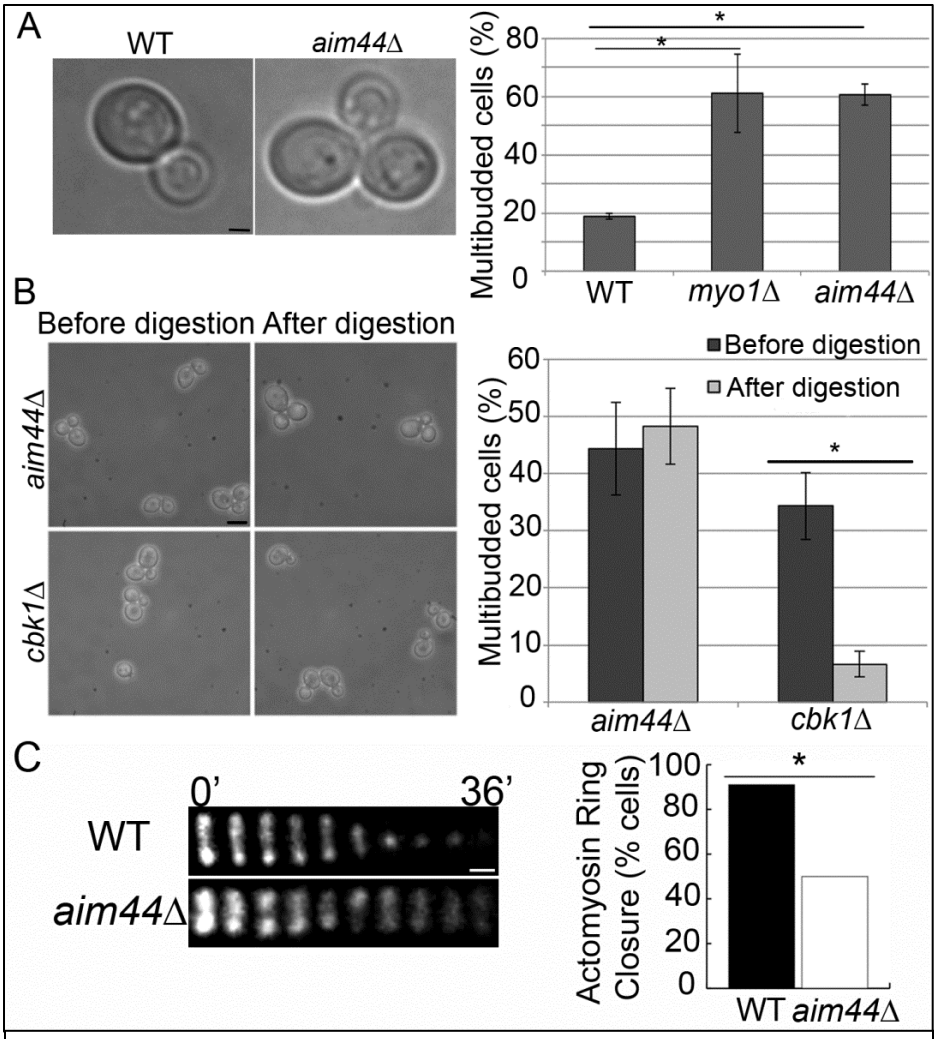

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Figure 4.1. *aim44*Δ cells have a defect in contractile ring closure during cytokinesis. (A) Wild-type and *aim44*Δ cells were grown to late-log phase (OD_{600} = 1.5) in synthetic complete (SC) glucose-based medium at 30°C and the percentage of cells in multibudded clusters was determined. **Left**, transmitted-light image of wild-type cell with a single bud and an *aim44*Δ cell with multiple buds. **Right**, quantitation of the multibudded phenotype in wild-type, *myo1*Δ, and *aim44*Δ cells. *myo1Δ* cells, which have defects in contractile ring constriction, exhibit higher levels of multibudded cells compared to wild-type cells (p = 0.006). The *aim44Δ* cells show a statistically significant increase in the level of multibudded cells over wild-type ($p = 4.0 \times 10^{-5}$). Error bars show standard deviations from 3 independent experiments. n ≥ 100 cells per strain per experiment. Scale bar = 1 μm. (B) The percentage of multibudded cells in *aim44*Δ and *cbk1*Δ cells was determined before and after treatment with zymolyase 20T (0.1 mg/ml for 10 min at RT). **Left**, phase-contrast images of *aim44*Δ and *cbk1*Δ cells before and after zymolyase treatment. **Right**, quantitation of multibudded phenotype before and after treatment. Zymolyase treatment results in cell separation in the *cbk1*Δ septation mutant (p = 0.002), but not in *aim44*Δ cells (p = 0.5). Errors bars show standard deviations from n > 800 cells per strain. Scale bar = 5 μm. (C-D) Wild-type and *aim44*Δ cells expressing *MYO1* C-terminally tagged at its chromosomal locus with GFP were grown to mid-log phase and synchronized in G_1 phase by incubation with pheromone (10 μM α-factor) for 2 hrs at 30°C. Cells were then washed and placed in fresh media. The contractile ring was visualized beginning 60 min after release from G_1 arrest by time-lapse imaging at 4-min intervals over a 40 min period. (C) Montage of the contractile ring in single cells over time. In wild-type cells (top panel), contractile ring closure is complete within 10 min. In contrast, in the *aim44*Δ cell shown, the contractile ring does not close during the 40-min imaging period (bottom panel). Scale bar = 0.3 μm. (D) Quantitation of the number of wild-type and *aim44*Δ cells that exhibit contractile ring closure (n = 52 and 66 for wild-type and *aim44*Δ cells, respectively; $p = 7 \times 10^{-7}$, chi-squared test). Results shown are pooled from 3 independent time-lapse imaging experiments.

Aim44p-GFP assembles into a ring structure at the bud neck that transiently co-localizes with the septins and the contractile ring

In yeast and other cell types, proteins that mediate or regulate cytokinesis localize to the site of cell division and often associate with either the actomyosin contractile ring or septin rings. High-throughput subcellular localization experiments revealed that Aim44p localizes to the bud neck (Huh *et al.*, 2003). We tagged endogenous *AIM44* at its C-terminus with GFP and find that Aim44p-GFP is fully functional (Fig. 4.2). Aim44p-GFP localizes to a ring at the incipient bud site (Fig. 4.3). During early stages of bud development, Aim44p-GFP forms a single ring extending around the bud neck,

consistent with the identification of Aim44p as a bud-neck protein (Huh *et al.*, 2003). After contractile ring closure, the Aim44p-GFP ring thickens and separates into two rings; each ring remains at the site of cell division on the mother and daughter cells after cell separation (Fig. 4.3).

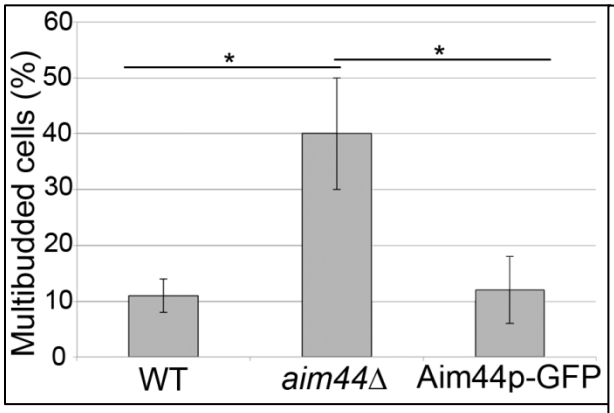

Figure 4.2. Aim44p-GFP is a functional fusion protein. The fusion of a C-terminal GFP tag to Aim44p does not produce a multibudded phenotype in cells in late-log phase liquid culture (SC), indicating that the tag does not interrupt its normal function in promoting cell division. The percentage of multibudded cells in the Aim44p-GFP expressing cells is not statistically different from wild-type cells ($p = 0.9$, Student's t-test). *aim44Δ* cells are multibudded compared to wild-type and Aim44p-GFP expressing cells ($p = .0085$ and $p = 0.01$, respectively, Student's t-test). Error bars represent standard deviation of 3 independent experiments. $n \geq 100$ cells for each strain in each independent experiment.

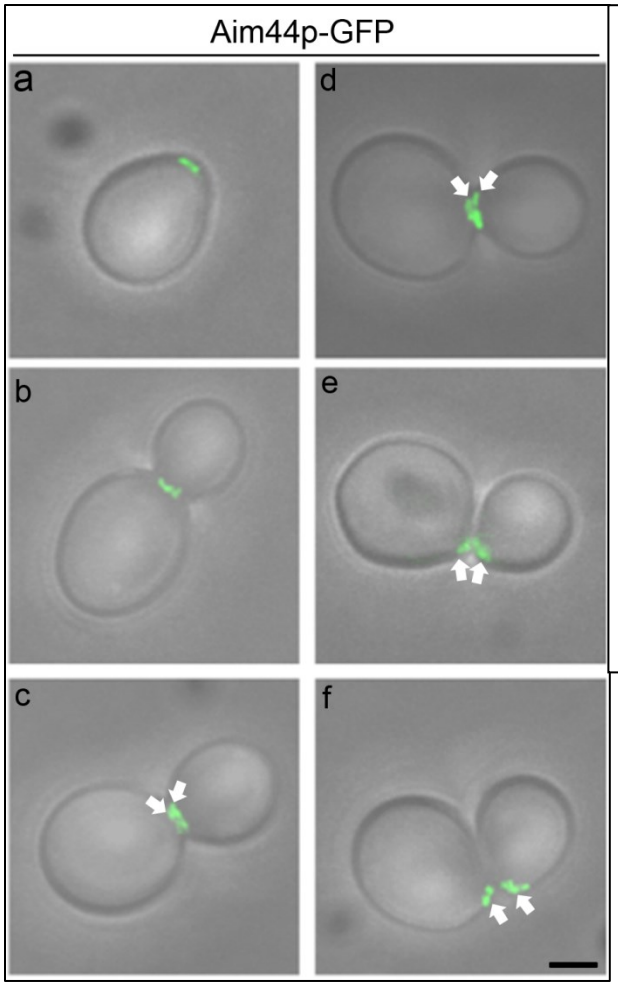

Figure 4.3. Aim44p-GFP localization throughout the cell cycle. Cells expressing *AIM44* tagged at its chromosomal locus with GFP (green) were grown as for Fig. 4.1 and imaged by fluorescence and phase contrast microscopy. Micrographs of the fluorescence images superimposed on transmitted-light images depict representative cells at different stages in the cell division cycle. Aim44p-GFP is recruited to the selected bud site where it forms a ring structure **(a)**. As the bud emerges and grows, Aim44p-GFP localizes to a single ring **(b)** and later to a double ring (**c** and **d**, highlighted with white arrows) at the bud neck. Finally, Aim44p persists as a ring on newly separated mother and daughter cells (**e** and **f**, highlighted with white arrows). Scale bar (bottom right) = 1 μm.

Since the localization pattern of Aim44p-GFP during mitosis resembles that of the septins, we studied the localization of Aim44p-GFP relative to the septin Cdc3p, tagged at its C-terminus with mCherry, in wild-type yeast cells (Fig. 4.4). At the beginning of the cell division cycle, Aim44p-GFP and Cdc3p-mCherry are recruited to concentric rings at the selected bud site. During early bud development, the single Aim44p-GFP and septin rings partially co-localize. Later in the cell cycle, when septins localize to double rings, Aim44p-GFP localizes to a single ring between the septin double rings. Later in the cell cycle, when septin rings decrease in size, Aim44p-GFP localizes to two rings that are

within and partially overlap with the double septin rings. Finally, after cell separation, the double septin and Aim44p rings separate such that the mother and daughter cells each have an Aim44p ring within a septin ring.

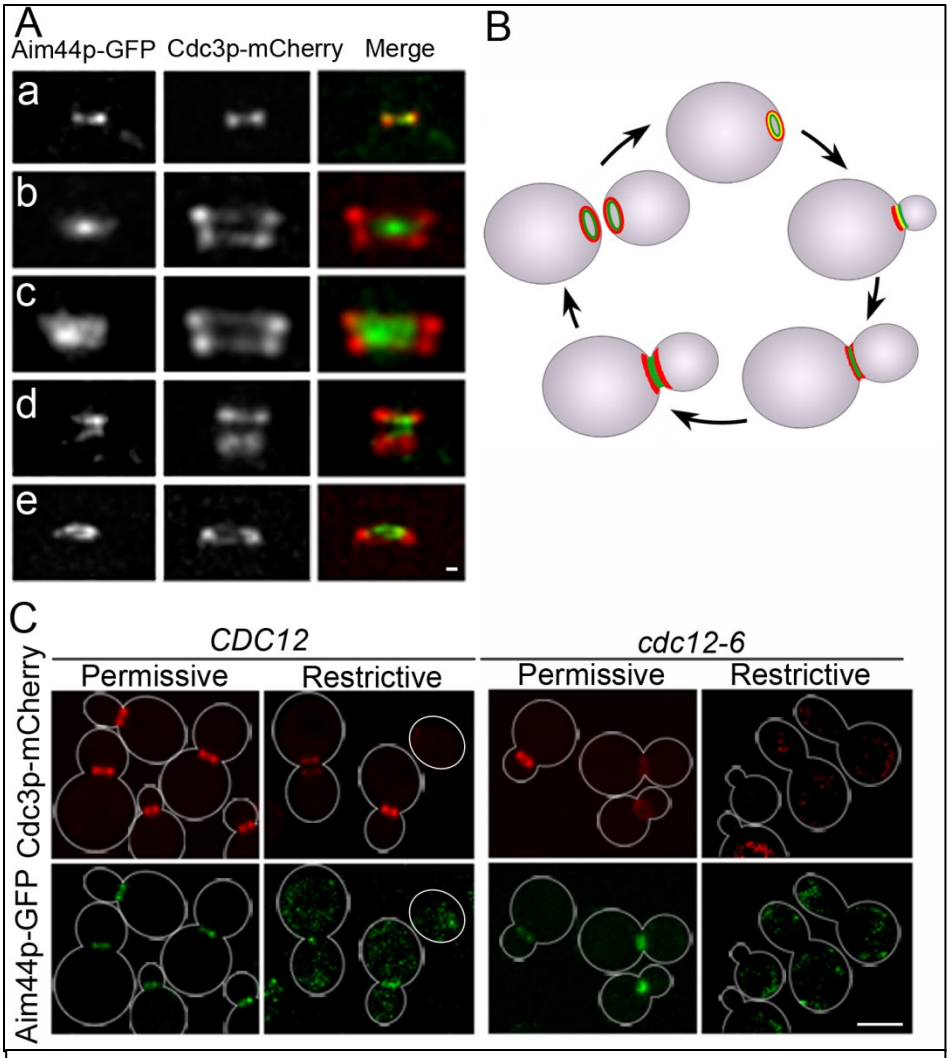

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Figure 4.4. Aim44p undergoes septin dependent assembly into rings that encircled by septin rings. Wild-type yeast expressing *AIM44* tagged at its chromosomal locus with GFP (green) and plasmid-borne Cdc3p-mCherry (red) were grown to mid-log phase (OD_{600} = 0.1-0.5) in SC and imaged by fluorescence microscopy. Representative images show the localization of Aim44p-GFP and the septin ring marked by Cdc3p-mCherry at the bud neck at various points throughout the cell division cycle. (A) Projections of deconvolved z-series showing side views of Aim44p-GFP (green) and Cdc3p-mCherry (red) at the mother-bud neck. Concentric single rings of Aim44p-GFP and septins during early bud development **(a)**. A single Aim44p ring between two double septin rings in a cell with a medium to large bud **(b)**. After contractile ring closure, Aim44p-GFP forms a thick single ring that is concentric and partially overlapping with the septin rings **(c)**. After cell division, both Aim44p-GFP and Cdc3p-mCherry are present as rings on both the mother and daughter cells, marking the former site of cell division **(d)**. Concentric rings of Cdc3p-mCherry and Aim44p-GFP in a cell after cell separation **(e)**. Scale bar = 0.3 μm. (B) Schematic of the localizations of Aim44p-GFP (green) and the septin rings (red) throughout the cell division cycle. (C) A temperature-sensitive septin strain, *cdc12-6,* and the wild-type parent *CDC12* strain expressing Aim44p-GFP (green) and a plasmid-borne septin Cdc3p-mCherry (red) were grown to mid-log phase at permissive temperature (RT). An aliquot of each strain was shifted to restrictive temperature (34°C) for 2 hr and all cells were imaged by fluorescence microscopy. Shift of *cdc12-6* mutant cells to restrictive temperature results in localization of Aim44p-GFP and Cdc3p-mCherry to abnormal diffuse and punctate cytosolic structures. Cell outlines based on transmitted-light images are shown in white. Scale bars = 5 μm.

Since Aim44p colocalizes with the single septin ring early in the cell division cycle and septins serve as a scaffold for assembly of the contractile ring, we tested whether Aim44p ring assembly is septin-dependent. We detect both the single and double septin rings in *aim44Δ* cells (Fig. 4.5 A). Thus, septin ring assembly does not require Aim44p. In contrast, Aim44p-GFP ring assembly is dependent upon septin ring formation. Specifically, we find that shift of the temperature-sensitive septin mutant *cdc12-6* to restrictive temperatures disrupts Aim44p localization (Fig. 4.4 C). Incubation of wild-type *CDC12* cells at 34°C results in a modest (10.6%) decrease in Aim44p localization to rings. In contrast, shift of the *cdc12-6* mutant from room temperature (RT) to 34°C results in complete loss of septin rings, as described previously (Cid *et al.*, 2001), and complete loss of detectable Aim44p rings (rings present in 98.5% of cells at permissive

temperature and 0% at restrictive temperature; n ≥ 200 for each sample). In *cdc12-6* cells at 34°C, Aim44p-GFP and Cdc3p-mCherry localize to punctate cytosolic structures. From these data, we conclude that septin ring formation is required for Aim44p ring formation.

We also examined the localization of Aim44p-GFP relative to the contractile ring component Myo1p, which was tagged at its C-terminus with mCherry. Deletion of *MYO1* does not affect Aim44p-GFP ring formation (Fig. 4.5 B). However, Aim44p-GFP co-localizes with the Myo1p-mCherry-labeled contractile ring when both proteins form rings at the presumptive bud site and through most of the cell division cycle (Fig. 4.6 A and B). Interestingly, the ring formed by Aim44p-GFP does not contract when the contractile ring contracts. Instead, the Aim44p ring thickens and forms a double ring, which separates concomitant with cell separation such that mother and daughter cells each retain one Aim44p ring (Fig. 4.6). The relative localization of Cdc3p-mCherry and Myo1p-GFP during the cell cycle is shown in Fig. 4.7.

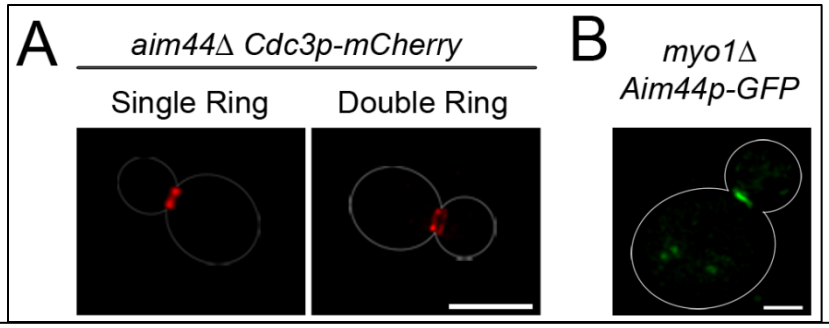

Figure 4.5. Localization of Cdc3p-mCherry and Myo1p-GFP during cell division in wild-type cells. Cells expressing *MYO1* tagged with GFP at its chromosomal locus and plasmid-borne Cdc3p-mCherry were grown to mid-log phase and imaged by fluorescence microscopy. Deconvolved projections of representative images of Cdc3p-mCherry (red) and Myo1p-GFP (green) at the bud neck of cells with small, medium, and large buds (**a-c**, respectively) and separated mother and daughter cells **(d)**. **(a)** Myo1p co-localizes with a single septin ring in a small-budded cell. **(b-c)** In cells bearing medium to large buds, the septin ring thickens and forms 2 rings that surround the single Myo1p ring. **(d)** After contractile ring closure and cell separation, mother and daughter cell each have a single septin ring. Scale bar = 0.3 μm.

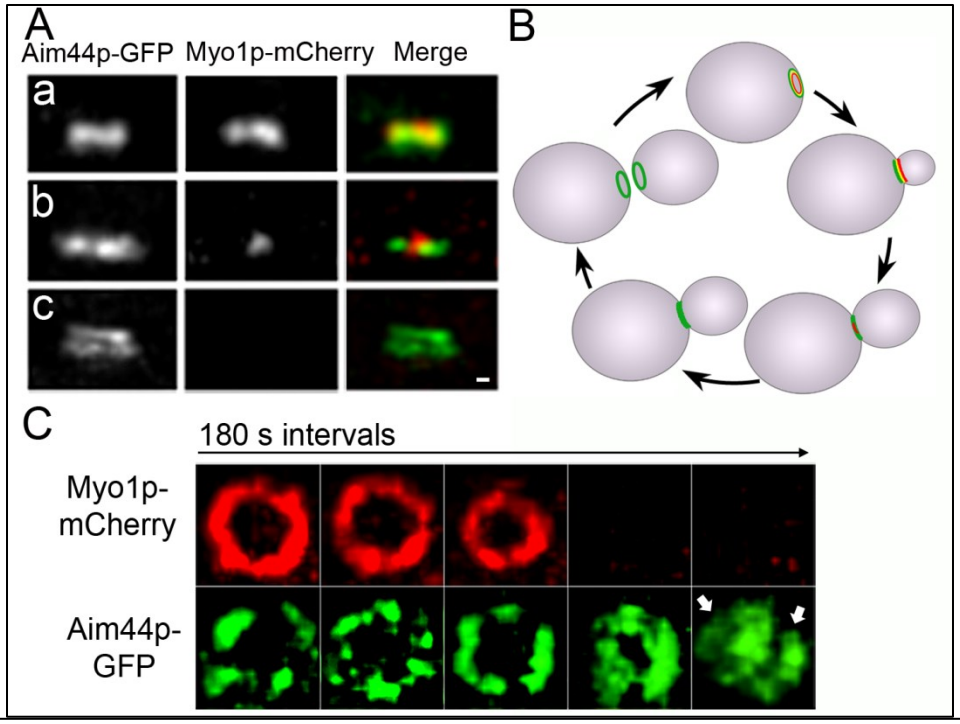

Figure 4.6. Localization of Aim44p-GFP and Myo1p-mCherry during cell division in wild-type cells. *AIM44* and *MYO1* were tagged with GFP and mCherry, respectively, at their chromosomal loci. Representative images show the bud neck of mid-log phase cells imaged by fluorescence microscopy at different points throughout the cell division cycle. (A) Projections of deconvolved z-series showing side views of Aim44p-GFP (green) and Myo1p-mCherry (red). Single rings formed by Aim44p-GFP and Myo1p-mCherry characteristic of early bud growth **(a)**. Aim44p-GFP remains a single ring that co-localizes with the actomyosin ring through most of the cell division cycle. During contractile ring closure, the diameter of the actomyosin ring decreases **(b)**. However, the Aim44p ring does not contract. Aim44p forms a double ring after contractile ring closure **(c)**. Scale bar = 0.3 µm. (B) Schematic of the localizations of Aim44p-GFP (green) and the actomyosin contractile ring (red) throughout the cell division cycle. (C) Cells expressing Myo1p-mCherry and Aim44p-GFP were treated with pheromone as for Fig. 1, immobilized in a microfluidic chamber such that the mitotic rings were localized parallel to the microscope stage and imaged 60 – 90 min after release from pheromone-induced G_1 arrest. Z-series were obtained every 3 min to observe ring dynamics before, during, and after contractile ring closure. The Myo1p-mCherry ring contracts, but the Aim44p-GFP ring does not. After completion of contractile ring closure, Aim44p-GFP forms two rings (marked by 2 white arrows).

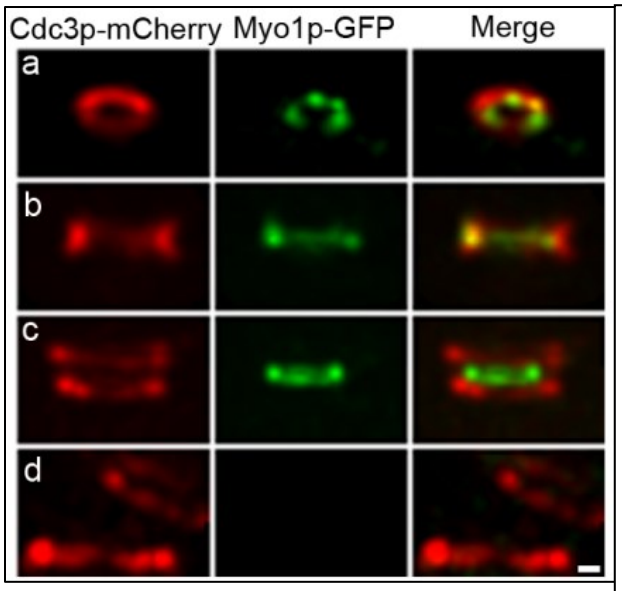

Figure 4.7. Localization of Cdc3p-mCherry and Myo1p-GFP during cell division in wild-type cells. Cells expressing *MYO1* tagged with GFP at its chromosomal locus and plasmid-borne Cdc3p-mCherry were grown to mid-log phase and imaged by fluorescence microscopy. Deconvolved projections of representative images of Cdc3p-mCherry (red) and Myo1p-GFP (green) at the bud neck of cells with small, medium, and large buds (**a-c**, respectively) and separated mother and daughter cells **(d)**. **(a)** Myo1p co-localizes with a single septin ring in a small-budded cell. **(b-c)** In cells bearing medium to large buds, the septin ring thickens and forms 2 rings that surround the single Myo1p ring. **(d)** After contractile ring closure and cell separation, mother and daughter cell each have a single septin ring. Scale bar = 0.3 μm.

Aim44p co-immunoprecipitates with Hof1p and regulates Hof1p phosphorylation and localization

Here, we investigated another regulator of contractile ring closure, Hof1p, and obtained evidence for a role for Aim44p in regulating Hof1p phosphorylation and localization. First, we confirmed that Hof1p undergoes cell cycle-dependent changes in abundance and phosphorylation. Specifically, we detect changes in the electrophoretic mobility of Hof1p over time after release from G_1 arrest. The mobility shifts are sensitive to treatment with calf intestinal alkaline phosphatase, and are therefore due to Hof1p phosphorylation (Fig. 4.8).

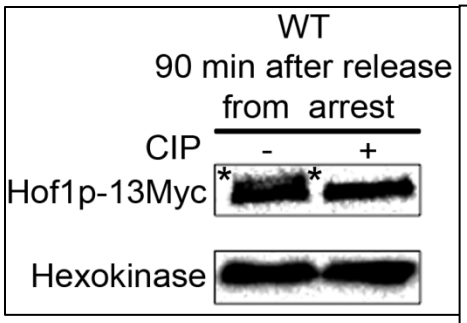

Figure 4.8. The lower-mobility band containing Hof1p-13Myc is due to phosphorylation of the protein. Wild-type and *aim44Δ* cells expressing Hof1p tagged with 13Myc at its chromosomal locus were synchronized as for Figure 1. An aliquot was removed 90 min after release from G_1 arrest for extraction of protein. Half of the protein sample was left untreated and the other half was treated with 10 units of calf intestinal alkaline phosphatase (CIP) for 1 hr at 37°C. Western blots are shown in which Hof1p was detected using anti-Myc antibody and the load control, hexokinase, was detected using a polyclonal anti-hexokinase antibody. *, lower mobility band(s) of Hof1p-13Myc. The lower mobility Hof1p-13Myc band(s) is phosphorylated, as measured by sensitivity to CIP treatment.

Next, we assessed the effect of deletion of *AIM44* on the level and phosphorylation state of Hof1p. Hof1p-13Myc is phosphorylated 75 min after release from G_1 arrest in wild-type cells and 90 min after release from G_1 arrest in *aim44*Δ cells (Fig. 4.9 A). Moreover, in wild-type cells, Hof1p undergoes dephosphorylation 105 min after release from G_1 arrest, but this does not occur until 120 min after release from G_1 arrest in *aim44*Δ cells. Thus, deletion of *AIM44* results in a delay in cell cycle-regulated phosphorylation and dephosphorylation of Hof1p.

Total levels of Hof1p are also affected by deletion of *AIM44* (Fig. 4.9 B). In wild-type cells, we detect an increase in the level of Hof1p from 30-75 min after release from pheromone-induced G_1 arrest and a decline in Hof1p levels from 75-120 min after release from G_1 arrest. Deletion of *AIM44* alters cell cycle-linked changes in Hof1p levels. Specifically, we observe a decrease in the rate of cell cycle-linked increases in Hof1p levels, a delay in the time when maximum Hof1p levels occurs (90-105 min for

*aim44*Δ cells versus 75 min for wild-type cells), and a 22% decrease in the maximum level of Hof1p. Using spindle length as an indicator of mitotic progression, we find that deletion of *AIM44* has no effect on cell cycle progression (Fig. 4.10). Thus, deletion of *AIM44* affects the abundance and phosphorylation of Hof1p, without affecting overall rates of cell cycle progression prior to the point of cell division.

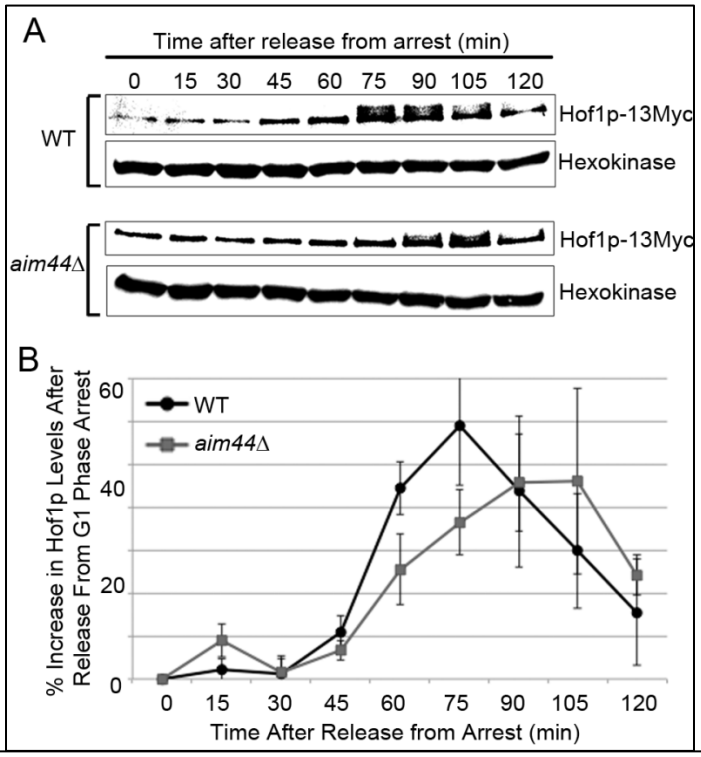

Figure 4.9. Deletion of *AIM44* results in defect in cell cycle-linked changes in Hof1p abundance and phosphorylation. Wild-type and *aim44Δ* cells expressing Hof1p tagged with 13 copies of Myc at its chromosomal locus were synchronized as for Figure 6. Aliquots were removed from cell cultures for protein extraction every 15 min for 120 min after release from pheromone-induced G_1 arrest. (A) Western blot decorated with antibodies against the Myc epitope on Hof1p and the loading control, hexokinase. The shift in electrophoretic mobility of Hof1p detected at 75-105 min in wild-type cells and 90-105 min in *aim44*Δ cells represents phosphorylation, as determined by sensitivity to treatment with calf intestinal alkaline phosphatase (Figure 4.8). (B) Relative levels of Hof1p-13Myc were quantified by scanning densitometry. Quantifications are averages from Western blots from 3 independent experiments. Error bars represent standard error of the mean.

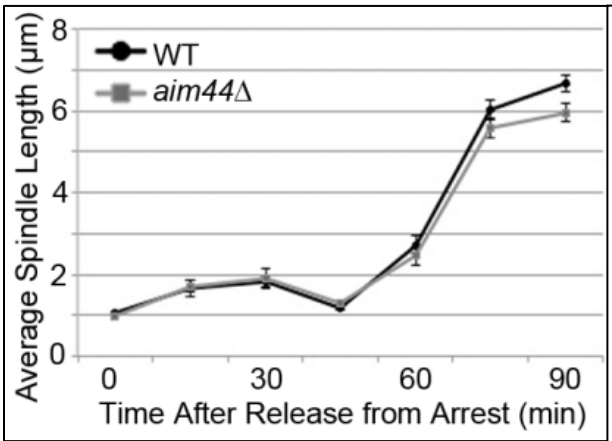

Figure 4.10. Deletion of *AIM44* inhibits cytokinesis but does not affect rates of cell cycle progression. Cell cycle progression was analyzed by quantifying spindle length every 15 min for 90 min after release from pheromone-induced arrest in G_1 phase in wild-type and *aim44Δ* cells expressing tubulin tagged at its C-terminus with GFP. Cells were fixed using paraformaldehyde and stained with the DNA-binding dye DAPI and imaged. These data are pooled from two independent experiments and measurements average n ≥ 100 cells per strain. Error bars represent standard error of the mean.

Since deletion of *AIM44* results in defects in phosphorylation of Hof1p and since Dbf2p catalyzes phosphorylation of Hof1p, we assessed the effect of overexpression of Dbf2p on cytokinesis in *aim44*Δ cells. We confirmed that incubation in galactose-based media results in overexpression of Dbf2p in wild-type cells and *aim44*Δ cells. We also found that the level of overexpression of Dbf2p is similar in both cell types (Fig. 4.11 A). Next, we monitored cytokinesis, by quantitation of the number of multibudded cells, in cells with wild-type or elevated levels of Dbf2p. Overexpression of Dbf2p has no effect on the level of multibudded cells observed in wild-type cells ($p = 0.52$). In contrast, overexpression of Dbf2p results in a decrease in the number of multibudded cells from 55% in *aim44*Δ cells to 24% in *aim44*Δ cells that overexpress Dbf2p ($p = 0.002$) (Fig. 4.11 B). Thus, the defect in cytokinesis observed upon deletion of *AIM44* can be rescued by overexpression of a kinase that catalyzes phosphorylation of Hof1p.

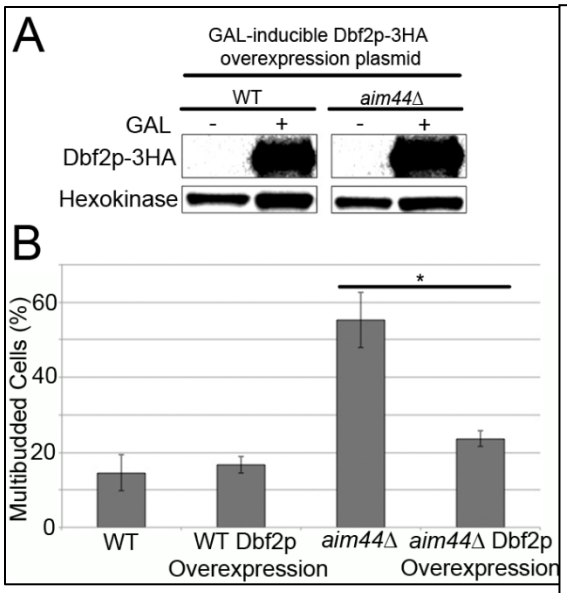

Figure 4.11. The cell division defect of *aim44Δ* cells is alleviated with overexpression of the kinase, Dbf2p. Wild-type and *aim44Δ* cells were transformed with a plasmid bearing the *DBF2* gene that was tagged with HA and expressed under control of the GAL-inducible promoter. The cells were grown to late-log phase (OD_{600} = 1.5) in synthetic complete (SC) glucose-based medium of galactose-based medium at 30 °C. (A) Western blots showing the level of Dbf2p-HA detected using anti-HA antibodies and of the load control, hexokinase detected with anti-Hxk1p antibody, in whole cell extracts of glucose or galactose grown cells. (B) The percentage of multibudded in wild-type and *aim44*Δ cells that express either endogenous Dbf2p or overexpress Dbf2p was determined as for Figure 1. Error bars show standard deviations from 3 independent experiments. n ≥ 100 cells per strain per experiment.

As phosphorylation of Hof1p affects its localization, we carried out time-lapse imaging to study the effect of deletion of *AIM44* on Hof1p dynamics. We confirmed that Hof1p-GFP localizes to a double ring at the bud neck in a wild-type strain expressing Hof1p C-terminally tagged at its chromosomal locus with GFP (Fig. 4.12). Later in the cell cycle, Hof1p localizes to a single, medial ring that undergoes partial constriction as the actomyosin ring contracts. It then separates into two rings that localize to both mother and daughter cells after cell division. 85% of wild-type cells examined display this pattern of Hof1p-GFP dynamics (Fig. 4.12 B). In contrast, 40% of *aim44Δ* cells exhibit defects in Hof1p dynamics ($p = 0.0003$). In these cells, Hof1p localizes to a double ring at the bud neck that does not undergo partial contraction during the 3-hr imaging period (Fig. 4.12 B). *aim44*Δ cells that exhibit defects in Hof1p dynamics also do not undergo

cell separation. Thus, deletion of *AIM44* results in defects in Hof1p abundance, phosphorylation, dynamics and function in promoting contractile ring closure.

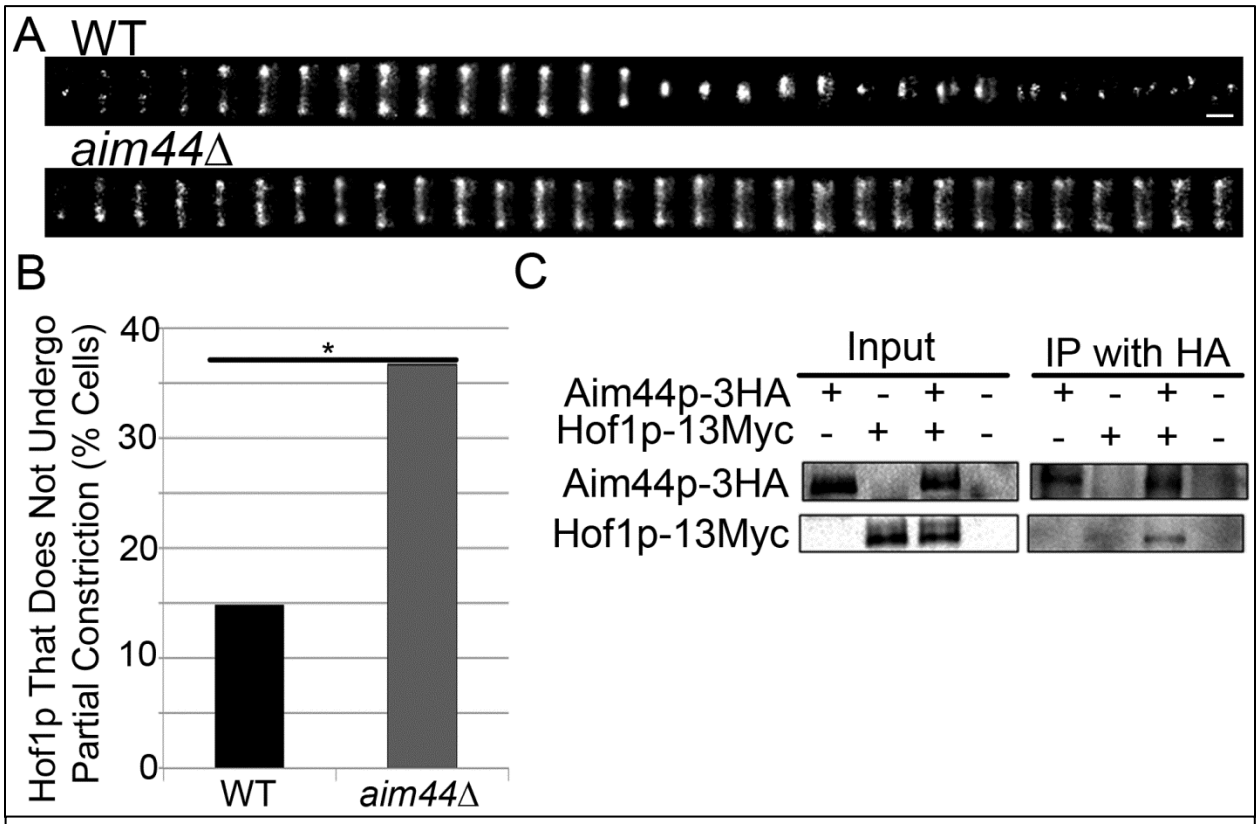

Figure 4.12. Aim44p regulates Hof1p localization and dynamics at the bud neck and co-immunoprecipitates with Hof1p. (A) Wild-type and *aim44Δ* cells expressing Hof1p-GFP were synchronized as for Figure 6. Hof1p-GFP were imaged every 5 min beginning 60 min after release from G_1 arrest for a total time of 3 hrs. Montages showing changes in Hof1p localization and dynamics at the bud neck as a function of time after release from pheromone-induced G_1 arrest in wild-type cells (top panel) and *aim44*Δ mutants (bottom panel). Scale bar = 0.3 µm. (B) Quantitation of wild-type and *aim44*Δ cells that exhibit Hof1p-GFP ring contraction ($p = 0.0003$, chi-squared test). n = 101 and 120 for wild-type and *aim44*Δ cells, respectively. Data are pooled from 2 independent experiments. (C) Yeast cells expressing no tagged proteins, Aim44p-3HA, Hof1p-13Myc, or both Aim44p-3HA and Hof1-13Myc were grown to mid-log phase and treated with pheromone as for Fig. 1. Whole cell extracts were prepared from each sample 75 min after release from pheromone-induced G_1 arrest, and samples were immunoprecipitated using anti-HA antibodies. Western blots show whole cell extracts and immunoprecipitated proteins detected using anti-HA and anti-Myc antibodies. Hof1p-Myc co-immunoprecipitates with Aim44p-HA.

To further characterize the mechanism of Aim44p function in regulation of Hof1p, we tested whether the two proteins interact. Previous genome-wide two-hybrid screens revealed that Hof1p and Aim44p have the capacity to bind to each other (Tonikian *et al.*, 2009). We tested whether this interaction is physiologically significant. To do so, we tagged Aim44p and Hof1p at their chromosomal loci in wild-type cells with HA and Myc epitopes, respectively. Cells were then synchronized and immunoprecipitation was carried out with whole cell extracts prepared 75 min after release from pheromone-induced G_1 arrest. We find that Aim44p co-immunoprecipitates with Hof1p. Interestingly, Aim44p associates primarily with the unphosphorylated form of Hof1p (Fig. 4.12 C).

DISCUSSION OF RESULTS

Our studies support a role for Aim44p in actomyosin contractile ring closure during cytokinesis. First, the localization of Aim44p is consistent with a role in this process. Specifically, we find that Aim44p forms a single ring within the single septin ring at the bud neck early in the cell cycle. The Aim44p ring colocalizes with the actomyosin ring throughout the cell cycle until contractile ring closure. During contractile ring closure, the Aim44p ring does not contract. After contractile ring closure, the Aim44p ring appears to thicken and forms two rings that are within and closely opposed to the double septin rings. Finally, during cell separation, the double rings of septins and Aim44p also separate such that a single Aim44p ring remains within a single septin ring on both mother and daughter cells, marking the site of division. The localization of Aim44p to the actomyosin ring at the bud neck is dependent upon the septins but not upon Myo1p, the type II myosin of the contractile ring in yeast.

Aim44p is not required for assembly of septin or actomyosin rings at the bud neck. However, it does regulate actomyosin ring closure. Deletion of *AIM44* results in a statistically significant increase in multibudded cells and a decrease in the frequency of contractile ring closure. In *aim44*Δ cells that do undergo contractile ring constriction, the rate of ring closure is not significantly different from that observed in wild-type cells. Thus, Aim44p is not required for formation of structures underlying the contractile ring, but it regulates the efficiency of contractile ring closure.

Consistent with this, we find that deletion of *AIM44* affects the phosphorylation and localization of Hof1p, a protein that localizes to the bud neck, binds to septins and Myo1p, regulates contractile ring closure, and is detected as an Aim44p binding partner in a genome-wide protein interaction screen (Tonikian *et al.*, 2009). We find that cell cycle-regulated changes in Hof1p abundance and phosphorylation are altered in *aim44*Δ cells. We also detect defects in Hof1p localization in *aim44*Δ cells. In wild-type cells, Hof1p assembles as two rings on septin rings at the bud neck early in the cell division cycle. In response to a series of phosphorylations, Hof1p then moves from septin rings to the actomyosin ring. The Hof1p ring undergoes partial contraction with the actomyosin ring, ultimately associating with septin rings on mother and daughter cells upon cell separation.

Recent studies indicate that phosphorylation of Hof1p affects its binding to the septins, which in turn affects septin stability, in addition to its function in regulating actomyosin ring closure (Meitinger *et al.*, 2013a). Although we find that assembly of the septin ring at the bud neck is not dependent upon Aim44p, it is possible that the maintenance of a stable septin scaffold may also be affected by Aim44p regulation of Hof1p phosphorylation.

However, we find that deletion of *AIM44* results in defects in Hof1p localization. In *aim44*Δ cells, Hof1p localizes to two large rings. Therefore, it is likely that Hof1p can associate with septin rings in *aim44*Δ cells. However, in *aim44*Δ cells with contractile ring closure defects, Hof1p does not undergo cell cycle-linked movement from double

rings to the single actomyosin ring. We also find that Aim44p co-immunoprecipitates with Hof1p, and that it is recovered primarily with the unphosphorylated form of Hof1p. Finally, we find that overexpression of Dbf2p, the kinase that catalyzes phosphorylation of Hof1p which leads to movement of Hof1p from the septin rings to the actomyosin ring, rescues the contractile ring closure defect in *aim44*Δ cells.

These findings support a model for Aim44p regulation of contractile ring closure. According to this model, Aim44p assembles into a ring at the interface between septin and actomyosin rings early in the cell division cycle. Later in the cell cycle, as Hof1p levels increase and it assembles into a ring within the septin ring, Aim44p interacts with the unphosphorylated form of Hof1p. This interaction then promotes cell cycle-regulated phosphorylation of Hof1p by Mob1p/Dbf2p, which leads to movement of Hof1p from the septin rings to the contractile ring and to Hof1p-triggerred contractile ring closure. Aim44p remains associated with septin rings during contractile ring closure, septation, and cell-cell separation, as rings that are encircled by septin rings.

During the preparation of this manuscript, Meitinger and coworkers (2013b) found that wild-type Aim44p (referred to in that work as Gps1p) localizes to structures at the bud neck that are similar to those that we report and can bind directly to two signal transduction GTPase proteins, Cdc42p and Rho1p, which also localize to the bud neck during cytokinesis. They identified a role for Aim44p in regulating secondary septum formation through effects on Rho1p. They also observe a role for Aim44p in preventing the activation of Cdc42p at the bud neck after cytokinesis, and find that this mechanism

insures that new daughter cells are not produced at the site of cytokinesis from the previous round of cell division.

Meitinger et al. (2013b) argue that Aim44p does not function in contractile ring closure based on findings that contractile ring closure occurs at wild-type rates in *aim44*Δ cells. We also find that actomyosin ring closure occurs at the wild-type rate in *aim44Δ* cells that do undergo this process. Importantly, however, we find that the actomyosin ring fails to close in 50% of *aim44Δ* cells. Thus, we find that Aim44p regulates the efficiency of contractile ring closure, rather than the rate of closure.

Moreover, Meitinger et al., (2013b) find that there is a defect in secondary septum formation in *aim44*Δ cells. We find that zymolyase-dependent cell wall degradation under conditions that allow for cell separation in a known septation mutant, *cbk1*Δ, does not result in separation of multibudded *aim44*Δ cells. These findings indicate that *aim44*Δ cells either have a cytokinesis defect that appears prior to the septation defect, or they have septation defects that are not similar to those of *cbk1*Δ cells.

Finally, recent studies indicate that Rho1p and Cdc42p are activated during actomyosin ring assembly, inactivated during actomyosin ring contraction, and activated again during septum formation (Atkins *et al.*, 2013; Onishi *et al.*, 2013). Therefore, it is possible that defects in Rho1p and/or Cdc42p regulation may contribute to the defects in contractile ring closure in *aim44*Δ cells.

Taken together, this and other recent work indicates that Aim44p has multiple functions late in the yeast budding process. It functions in contractile ring closure through effects on Hof1p phosphorylation and localization in addition to its role in septation through effects on Cdc42p. Indeed, Meitinger et al. (2013b) find that an *aim44*Δ *hof1*Δ mutant exhibits more severe growth defects than either *aim44*Δ or *hof1*Δ single mutant yeast. This is genetic evidence that Aim44p has Hof1p-independent functions in addition to the Aim44p- and Hof1p-dependent function in contractile ring closure described here. In summary, our studies reveal a novel role for Aim44p in contractile ring closure through effects on Hof1p phosphorylation and localization to the actomyosin ring.

FUTURE DIRECTIONS

Since Hof1p, septins, and contractile ring components are conserved, it is possible that there is an unidentified orthologue of Aim44p in other cell types. While cell division can readily be studied in yeast because either septation or actomyosin contractile ring closure can be compromised without causing lethality, gene deletion screens to identify a mutant with phenotypes similar to those of *aim44Δ* cells would be unlikely to succeed as an experimental approach for identifying the potential mammalian orthologue of Aim44p. However, mutagenesis resulting in partial loss of function in a mammalian system may allow identification of an orthologue of *AIM44*. Mutations found by this approach would be expected to affect the dynamics and localization of the Hof1p mammalian homolog, and result in the misregulation of its phosphorylation patterns during cell division.

Another line of future investigation would seek to understand how Aim44p contributes to the coordinated regulation of the septins and actomyosin ring closure. One important set of experiments towards this end would be structure-function studies on truncations of the Aim44p protein, which is about 84.8 kDa in molecular weight and composed of 758 amino acids. Visualization of truncated Aim44p-GFP fusion proteins by fluorescence microscopy could identify which domain(s) are necessary for bud-neck localization. Expression of a truncated Aim44p fused to a small tag (such as 3HA, as was used in this study) in a strain also expressing Hof1p tagged with a small tag (such as 13Myc, as was used in this study) could elucidate which domain of Aim44p is necessary for binding of the protein to Hof1p (either directly or indirectly). A truncation of

Aim44p that prevents binding of the protein to Hof1p would result in the inability to pull down both proteins via co-immunoprecipitation.

MATERIALS AND METHODS

Strains, plasmids, and genetic manipulation

S. cerevisiae strains used in this study are summarized in Table 4.1 (see below). All yeast strains were created in the BY4741 wild-type background except for the temperature-sensitive septin strain (*YEF743 cdc12-6*) and the wild-type control (*YEF6866 CDC12*). The two *YEF* strains were kindly provided by Dr. Erfei Bi.

AIM44 was tagged at its C-terminus with GFP by transformation with a PCR-amplified GFP tagging cassette from the pFA6a-GFP system (Addgene, Cambridge, MA) containing *HIS3* and 40 bp homology to the endogenous locus. The protein Hof1p was fused with 13 copies of Myc at its C-terminus tag by transformation with a PCR-amplified pFA6a-13Myc plasmid containing a *His3Mx6* gene for selection and 40 bp homology to *HOF1*. The *AIM44* genomic locus was disrupted in cells expressing Myo1p-GFP and Hof1p-GFP by transformation with a PCR-amplified cassette from pFA6a-KanMX6 containing the kanMX selectable marker and 40 bp homology to the endogenous locus. The *AIM44* genomic locus was disrupted in *Hof1-13Myc:His3MX6* cells by transformation with a PCR-amplified sequence containing LEU2 derived from the pOM cassette system (Tamm, 2009) and 40 bp homology to the endogenous locus. Similarly, the *MYO1* genomic locus was disrupted by transformation with a PCR-amplified sequence containing LEU2 derived from the pOM cassette system and 40 bp homology to the endogenous locus. To tag Myo1p with mCherry, cells were transformed with a PCR-amplified pcY3090-02 plasmid containing a *hphMX4* gene for selection with hygromycin B and 40 bp homology to *MYO1* (Young *et al.*, 2012). *TUB1* was tagged at

its C-terminus with GFP by transformation with DNA from the plasmid pTS988 digested with the restriction enzyme XbaI (New England Biolabs, Ispwich, MA) in wild-type and *aim44Δ* cells. *AIM44* was tagged with 3HA at its C-terminus in wild-type and Hof1p-13Myc expressing cells by transformation with a PCR-amplified cassette from the pFA6a-3HA system containing *kanMX6* and 40 bp homology to the endogenous locus. Construction of the *cbk1Δ* strain (García-Rodríguez *et al.*, 2009), Myo1p-GFP expression strain (Huckaba *et al.*, 2006), and the *myo1Δ* strain (Higuchi *et al*., 2013) are described in previous work. In the *YEF6866 CDC12* and *YEF743 cdc12-6* strains, a C-terminus GFP tag was fused to *AIM44* by transformation with a PCR-amplified kanMX-encoding cassette from pFA6a-GFP(S65T)-kanMX6 containing 40 bp homology to the endogenous locus. These strains were then transformed with the YIP128-CDC3-mCherry plasmid, kindly provided by Dr. John Pringle (Stanford University, Palo Alto, CA, USA) *aim44Δ* cells and cells expressing Aim44p-GFP and Myo1p-GFP were also transformed with the YIP128-CDC3-mCherry plasmid. Following transformation using the lithium acetate method, cells were selected on solid media with the appropriate selective solid media (Gietz *et al.*, 1995). For the Dbf2p overexpression studies, wild-type and *aim44Δ* cells were transformed with an ORF expression vector plasmid, pGB1805, with a GAL inducible promoter controlling the overexpression of Dbf2p tagged with an HA epitope (Thermo Scientific, Pittsburgh, PA). In-frame tagging or disruption of a gene was verified using PCR amplification. *E. coli* DH5-α competent cells (Stratagene, USA) were used for amplification of all plasmids used in this study. Plasmid clones were recovered from bacteria with a MiniPrep kit (Qiagen, Valencia, CA).

Media and culture conditions

E.coli was cultivated in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1% sodium chloride, pH 7.0) at 37 °C with ampicillin (100 g/L) to select for plasmid-carrying cells. *S. cerevisiae* strains were grown in synthetic complete (SC) medium at 30 °C on a rotary shaker at 225 rpm, as previously described (Fehrenbacher *et al.*, 2004). Solid medium was prepared as above with the addition of agar (2% w/v). For selection of genetically manipulated cells, SC medium was prepared without histidine for selection of cells auxotrophic for *HIS3*, without leucine for selection of cells auxotrophic for *LEU2*, or with the addition of geneticin (G418) sulfate (200 µg/mL, Invitrogen, Carlsbad, CA) for cells expressing the *kanMX6* gene. Media was prepared with galactose instead of glucose as the carbon source for experiments requiring media containing galactose. For experiments with temperature-sensitive *YEF6866* and *YEF743* strains expressing Aim44p-GFP and Cdc3p-mCherry, cells were grown as described above, but using 20 °C and 34 °C as permissive and restrictive temperatures, respectively. Other yeast methods were performed as previously described (Sherman, 2002).

Quantification of multibudded cells

To quantify the frequency of multibudded cells, cultures were grown to late-log phase in SC medium to an optical density (OD_{600}) of at least 1.5. Cells were sonicated and visualized using a light microscope. Data shown was pooled from three independent experiments. To check for a cytokinesis defect, cells were incubated with zymolyase 20T (0.1 mg/mL) at room temperature for 10 min.

Synchronization of cells

To induce cell-cycle arrest in G_1 phase, mid-log phase cultures were treated with 10 μM α-factor (Genemed Synthesis Inc.) in fresh SC for 2 h at 30 °C. Arrest was verified by light microscopy, indicated by at least 70% of cells exhibiting a schmoo morphology. Cells were released from arrest by washing 3 times in cold (4 °C) water before resuspending in fresh SC medium.

Preparation of cells for microscopy, microscopy, and image analysis

For the localization studies in fixed cells, we used an AxioObserver.Z1 microscope equipped with a Colibri LED excitation source, a wide-field camera (Orca ER, Hamamatsu Photonics, Bridgewater, NJ), and Axiovision acquisition software. We used a 100x/1.3NA EC PlanNeofluar objective (Zeiss) and set the camera to 1x1 binning to optimize spatial resolution. To visualize mCherry, we used an excitation of 570 nm and a standard rhodamine/RGP filter set with an exposure time of 300 ms for Cdc3p-mCherry and 100 ms for Myo1p-mCherry. To visualize GFP, we used excitation at 470 nm (100% LED power) and a standard GFP filter set with an exposure time of 500 ms for Aim44p-GFP and 300 ms for Myo1p-GFP. We acquired z stacks consisting of 13 slices with 0.5 μm spacing. The time-lapse imaging of Hof1p-GFP expressing cells was also performed using this microscope and the 470 nm LED for excitation with an exposure time of 125 ms. For this experiment, cells were imaged using the CellASIC (EMD Millipore, Billerica, MA) microfluidic flow chamber (Y04C plate) controlled by the

ONIX Control System and software. Cells were imaged every 5 min for 3 hrs beginning 1 h after release from arrest in G_1 phase.

For time-lapse imaging wild-type cells expressing Myo1-mCherry and Aim44p-GFP, cells were held upright in microfluidic chambers composed of polydimethylsiloxane (PDMS) with a depth of 20 μm and a diameter of 6 μm, such that actomyosin and Aim44p rings were positioned parallel to the microscope state. Imaging was carried out using a spinning disk confocal microscope consisting of the CSU-X1 spinning disk attachment (Yokogawa Electronic Corporation, Tokyo, Japan) on a Nikon Ti Eclipse inverted microscope (Nikon Inc., Melville, NY) equipped with and EMCCD cameras (DU-897, Andor Technologies, plc, Belfast, UK; Evolve, Photometrics, Tucson, AZ), 488 and 561 nm, 50 mW lasers and a CFI Plan Apo 100X □□1.45 NA oil objective (Nikon Inc., Melville, NY). Aim44p-GFP was imaged using 488 nm excitation illumination with 500 ms exposure times and 20% laser power. Myo1p-mCherry was imaged using 561 nm excitation illumination with 300 ms exposure times and 30% laser power.

Experiments with temperature-sensitive strains were performed using a Nikon Eclipse Ti microscope with a 60x/1.4NA oil PlanApo Nikon objective and Perfect Focus System, equipped with a Hamamatsu 10-600 Orca R^2 camera, an ASI Nano-Drive piezoelectric focus drive (Applied Scientific Instruments, Eugene, OR), a Lambda smart shutter controller and emission filter wheel (Sutter Instruments, Novato, CA), spectral LMM5 100 mW lasers, and a Yokogawa (Sugar Land, TX) CSU 10 spinning-disk confocal attachment upgraded with a Borealis system (Spectral Applied Research, Natick, MA).

The 491 and 561 nm lasers were used for excitation at 100% with exposure times of 800 and 225 ms for green and red, respectively.

Live-cell imaging for the actomyosin contractile ring studies was performed using agarose pads as previously described (Fehrenbacher *et al.*, 2004). Imaging was performed on an Axioskop 2 microscope (Zeiss) with a 100x/1.4 NA Plan-Apochromat objective and an Orca 1 cooled charge-coupled device (CCD) camera (Hamamatsu) or an E600 microscope (Nikon) with a Plan-Apo 100x/1.4 NA objective and an Orca-ER cooled CCD camera with FITC filter sets. Hardware was controlled by Openlab software. Three-dimensional images over time were acquired by obtaining optical sections in 0.5 µm spacing using a piezoelectric focus motor mounted on the objective lens (Polytech PI, Auburn, MA). An exposure time of 250 ms was used to visualize Myo1p-GFP. Cells were imaged every 4 min for about 48 min beginning 60 min after release from arrest in G1 phase.

For the cell cycle progression analysis in wild-type and *aim44Δ* cells expressing Tub1-GFP, cells were fixed using 3.6% fixed using paraformaldehyde and stained with the DNA-binding dye DAPI (4',6-diamidino-2-phenylindole; Life Technologies, Grand Island, NY) mixed with Mounting Solution, as described previously (Swayne *et al.*, 2010). Fixed cells were then imaged on an Axioskop 2 microscope (Zeiss) with a 100x/1.4 NA Plan-Apochromat objective and an Orca 1 cooled charge-coupled device (CCD) camera (Hamamatsu). Z-sections with 0.5 µm spacing were acquired.

Image processing and analysis

Images were deconvolved using a constrained iterative restoration algorithm in Volocity software (Perkin-Elmer, Waltham, MA). Maximum projections were reconstructed using Volocity software or ImageJ (Schneider *et al.*, 2012). Volocity was also used for measurement of spindle length. For statistical analysis, p values were calculated with the Student's t-test (two-tailed distribution), the chi-squared test, or Kruskal-Wallis analysis.

Protein methods

Protein was isolated from yeast cells as previously described (Boldogh *et al.*, 1998). Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Calf intestinal alkaline phosphatase (New England Biolabs, Ipswich, MA) treatment was carried out using 10 units of enzyme for 1 h at 37 °C. 13Myc-tagged proteins were probed on nitrocellulose membrane using a monoclonal anti-Myc (9E10 1-1) primary antibody (Evan *et al.*, 1985) and a horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI). HA-tagged proteins were detected using a monoclonal mouse anti-HA antibody (12CA5, Roche, Nutley, NJ). Hexokinase was detected using a rabbit polyclonal anti-hexokinase antibody (Cat. No. LS-C59302) purchased from LifeSpan Biosciences, Inc. (Seattle, WA). Signal was detected using the SuperSignal West Pico chemiluminescent substrate (Pierce). Luminescence was recorded using a ChemiDoc MP imaging system and analyzed using Image Lab software (Bio-Rad, Hercules, CA). For the co-immunoprecipitation experiments, protein extracts were added to Dynabeads® Protein G (Life Technologies, Grand Island, NY)

according to the manufacturer's instructions and incubated with a monoclonal mouse anti-HA antibody (clone 12CA5, Cat. No. 11 583 816 001, Roche, Nutley, NJ) to mediate the pull-down of Aim44p-3HA. The antibodies used for probing the nitrocellulose membrane were a monoclonal rabbit anti-Myc antibody (71D10, Cat. No. 2278, Cell Signaling, Danvers, MA) and a monoclonal mouse anti-HA antibody (clone 16B12, Cat. No. MMS-101P, Covance, Princeton, NJ).

Table 4.1. Yeast strains used in this study were constructed as described in the Methods section. All yeast strains were created in the *BY4741* wild-type background except for the temperature-sensitive septin strain (*YEF743 cdc12-6*) and the wild-type control (*YEF6866 CDC12*). The two *YEF* strains were kindly provided by Dr. Erfei Bi (The University of Pennsylvania, Philadelphia, PA, USA).

Strain	Genotype	Source
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems (Huntsville, AL)
7394	*MAT**a** aim44Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
ISY007	*MAT**a** cbk1Δ:HIS3 leu2Δ0 met15Δ0 ura3Δ0*	(García-Rodríguez *et al.*, 2009)
THY158	*MAT**a** MYO1-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	(Huckaba *et al.*, 2006)
RHY045	*MAT**a** his3Δ1 myo1Δ::LEU2 met15Δ0 ura3Δ0*	(Higuchi *et al.*, 2013)
DAY001	*MAT**a** aim44Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* [pYIP128-CDC3-mCherry::LEU2]	This study
DAY002	*MAT**a** MYO1-GFP:HIS3 aim44Δ::kanMX6 leu2Δ0 met15Δ0 ura3Δ0*	This study

Strain	Genotype	Source
DAY003	*MAT**a** AIM44-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY004	*MAT**a** AIM44-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0* [pYIP128-CDC3-mCherry::LEU2]	This study
DAY005	*MAT**a** AIM44-GFP::HIS3 myo1Δ::LEU2 met15Δ0 ura3Δ0*	This study
DAY006	*MAT**a** AIM44-GFP::HIS3 MYO1-mCherry::hphMX4 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY007	*MAT**a** MYO1-GFP::HIS3 CDC3-mCherry::hphMX4 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY008	*MAT**a** HOF1-13Myc::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY009	*MAT**a** HOF1-13Myc::HIS3 aim44Δ::LEU2 met15Δ0 ura3Δ0*	This study
LPY057-1	*MAT**a** HOF1-GFP::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study
DAY010	*MAT**a** aim44Δ::KANMX6 HOF1-GFP::HIS3 met15Δ0 ura3Δ0*	This study
DAY011	*CDC12+ AIM44-GFP::KanMX6 ura3 leu2Δ0 HIS3+ TRP1+* [pYIP128-CDC3-mCherry::LEU2]	This study
DAY012	*cdc12-6 AIM44-GFP::KanMX6 ura3 leu2Δ0 HIS3+ TRP1+* [pYIP128-CDC3-mCherry::LEU2]	This study
DAY013	*MAT**a** his3Δ1 TUB1-GFP::LEU2 met15Δ0 ura3Δ0*	This study
DAY014	*MAT**a** aim44Δ::kanMX6 his3Δ1 TUB1-GFP::LEU2 met15Δ0 ura3Δ0*	This study
DAY015	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [*pGB1805-*DBF2::URA3]*	This study
DAY016	*MAT**a** aim44Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [*pGB1805-*DBF2::URA3]*	This study
DAY017	*MAT**a** AIM44-3HA::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	This study

Strain	Genotype	Source
DAY018	*MAT**a** AIM44-3HA::kanMX6 HOF1-13Myc::HIS3 leu2Δ0 met15Δ0 ura3Δ0*	This study

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