Ubiquitin-Dependent Proteolysis of the Yeast Mating-Type Regulator Matα1

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Abstract

Cell type in budding yeast is controlled by the master regulatory transcription factors encoded at the mating-type (*MAT*) locus Matα1, Matα2, and Mat$\underline{a}$1. In homothallic strains, the $\underline{a}$ and α haploid cell types are unstable states, and cell type switching can occur with high frequency. To facilitate such efficient switching, the Mat transcription factors are unstable proteins. At least one of these factors, Matα2, is degraded by the ubiquitin-proteasome system (UPS). It stands to reason that the UPS would also degrade the other α-specific regulator, Matα1. Understanding how Matα1 is degraded and if it is degraded coordinately with Matα2 will provide a model to study other phenotypic switching events. Here, I demonstrate that Matα1 is a substrate for the UPS and is targeted for proteolysis by multiple E2 and E3 enzymes, including one common pathway with Matα2: Ubc4/Ubc5/Slx5-Slx8. Interestingly, Matα1 turnover requires an intact sumoylation pathway as well as poly-SUMO chains, whereas Matα2 does not. Using a series of Matα1 deletion mutants fused to Ura3 (the normally stable uracil biosynthetic enzyme), I have also isolated several regions within Matα1 that are necessary for rapid turnover potentially corresponding to the multiple ubiquitin ligases. Interestingly, a region coinciding with the alpha1 domain (residues 75-150), while not sufficient to confer wild-type levels of instability to Ura3, is dependent upon Slx5-Slx8. Taken together, these data show that while the degradation pathways of Matα1 and Matα2 share commonalities, the details of the pathways are unique for each factor.

Chapter I

Introduction: Phenotypic Switching, the Ubiquitin-Proteasome System, and Yeast Mating-type Biology

Phenotypic Switching

Differential gene expression is fundamental to many biological processes, such as the patterning of a body plan during embryogenesis or the specification of tissues during organogenesis. Each differentiated cell in a multi-cellular organism contains the same genetic information, but only utilizes a portion of it. When producing a cell lineage, one founder cell can give rise to two differently specified cells based on different expression patterns activated in those progeny. The ability to expressing a subset of this genome is critical, for example, in establishing a neuron that is distinct from an erythrocyte. Without differential gene expression, cellular specialization could not take place leading to a developmental halt. Studying the mechanisms that orchestrate changes in gene expression programs is an active and important area of scientific investigation. Understanding these mechanisms will not only further our knowledge regarding basic cell and organism development, but also regarding diseases arising from aberrant of differential gene expression such as cancer.

Mechanisms for regulating phenotypic switches

Regulating a phenotypic switch poses two distinct problems for a cell. The first problem is how to decide which genes to turn on and at what time. Most research focuses on this aspect of cellular differentiation. The second problem arises once the gene expression programs have changed. Even if protein synthesis is turned off, the cell is still left with already-made factors

that determined its previous phenotype. It must inactivate these factors in order to complete a faithful switch to the new phenotype (Mohn and Schubeler, 2009; Reik, 2007).

There are at least three ways that inactivation of the prior regulators could occur, which are not necessarily mutually exclusive. First, the protein could undergo a post-translational modification, such as phosphorylation much like the antiapoptotic factor BCL-2. BCL-2 is phosphorylated by stress response kinases to inactivate at the G2/M transition allowing apoptosis to occur (Yamamoto et al., 1999). A second mechanism to inactivate factors responsible for the previous phenotype is to relocate the protein to a different cellular compartment. The myocyte enhancer factor-2 (MEF2) transcription factors are responsible for activating skeletal myogenesis, but are repressed by histone deacetylases (Lu et al., 2000a; Miska et al., 1999; Molkentin et al., 1995; Sparrow et al., 1999). To initiate differentiation of muscle cells the histone deacetylase HDAC5 is shuttled out of the nucleus relieving repression of the MEF2 transcription factors (Lu et al., 2000b; McKinsey et al., 2000). While these two mechanisms described above would serve to inactivate factors determining the previous phenotype, protein modifications and cellular relocations can be reversed. A permanent method of removing the factors responsible for the previous state is protein degradation. Once a genetic switch in cell fate has occurred, rapid proteolysis of these factors would efficiently remove them allowing for faithful phenotypic switching.

Phenotypic switches regulated by proteolysis

There are several examples of phenotypic switches regulated by proteolysis, specifically by the ubiquitin-proteasome system (UPS), in the literature. In *C. elegans*, Mei-1 and Mei-2 make up the subunits for katanin, a microtubule severing complex. Following meiosis, Mei-1 and Mei-2 are rapidly degraded in order for the transition from a small meiotic spindle to a large mitotic spindle to occur (Clark-Maguire and Mains, 1994; Srayko et al., 2000). Also, the asymmetric division of germ line and somatic cells in *C. elegans* is regulated by ubiquitin-dependent proteolysis. This asymmetry is accomplished by the soma-specific degradation of the zinc-finger proteins, Pie-1, Mex-1 and Pos-1. This degradation is blocked in germ line cells by a protein called Par-1 (DeRenzo et al., 2003). In the yeast, *Sacchromyces cerevisiae*, phenotypic switching between haploid mating-types appears to be regulated by protein turnover since all three of the mating-type transcription factors are short-lived (Hochstrasser and Varshavsky, 1990; Johnson, 1997). The degradation of the transcription factor Matα2 is the best characterized in this switching event and has been shown to be required for a switch from the α mating-type to the a mating-type to occur (Laney and Hochstrasser, 2003). It stands to reason that the other yeast mating-type transcription factors are also regulated by proteolysis.

The Ubiquitin-Proteasome System

Clearly, the ubiquitin-proteasome system is an important component of phenotypic switching. The UPS is a complex system that is utilized in the regulation of many cellular functions. This system is compromised of several components: the 26S proteasome, and a 76 amino acid protein called ubiquitin, and an enzyme cascade responsible for conjugating ubiquitin to proteins destined for degradation. In fact, the UPS is responsible for a majority of protein degradation (Goldberg, 2005).

The 26S Proteasome

The ubiquitin-proteasome system operates by targeting proteins for degradation to the large, multi-subunit, ATP-dependent protease known as the 26S proteasome. The proteasome is compromised of two sub-complexes (Figure I-1). The first sub-complex is the 19S regulatory particle that can be further broken down into the base and the lid. The base, which is made up of six enzymes in the AAA family of ATPases and three non-ATPase proteins, sits over the channel in the 20S particle created by the α subunits. The lid is made of entirely non-ATPase proteins. These two components function together in recognition of and subsequent unfolding of target substrates (Fu et al., 2001; Glickman et al., 1998a).

To enter the proteasome target proteins must be deubiquitylated and at least partially unfolded (Liu et al., 2006). Deubiquitylation and unfolding of polyubiquitylated substrates is mediated by the 19S subunit and

deubiquitylating enzymes associated with it (Glickman and Ciechanover, 2002; Glickman and Maytal, 2002; Schmidt et al., 2005). The unfolded substrate can then enter the narrow channel of the 20S subunit, which is gated by the N-terminal tails of the subunits in the α rings (Groll et al., 2000; Groll et al., 1997). Recognition and unfolding of target proteins requires ATP, but translocation through the active site of the 20S subunit, the second component of the 26S proteasome, does not require hydrolysis of ATP (Liu et al., 2006; Smith et al., 2005). The 26S proteasome can efficiently degraded unfolded targets, but not folded proteins, in the presence of non-hydrolyzable ATP (Smith et al., 2005).

The 20S proteolytic subunit is responsible for dismantling target proteins into small peptides, usually only 7-9 amino acids long (Voges et al., 1999). The 20S particle is a cylinder made up of a stack of four heptameric rings with the two outer rings consisting of the seven α subunits and the two inner rings containing the β subunits. The protease active sites are confined to the large central chamber within the 20S particle (Groll et al., 1997; Unno et al., 2002). The two outer α rings have a small opening which only permits unfolded proteins to enter (Groll et al., 2000).

The actual destruction of proteins occurs within the β rings of the 20S particle. These rings contain the three catalytic centers of the proteasome, which each have a conserved lysine required for proteolysis. The catalytic core of the proteasome has chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing activities (Heinemeyer et al., 1997; Voges et al., 1999).

Ubiquitylation

Ubiquitin, with only 76, highly conserved amino acids, serves as the targeting flag for a wide range of cellular processes including degradation by the 26S proteasome. This attachment most often occurs through the formation of a covalent bond between an ε-amino group of a lysine within the target and the C-terminal glycine residue (G76) of ubiquitin. An enzyme cascade is utilized to form this isopeptide linkage between the substrate and ubiquitin, and between ubiquitin molecules.

A ubiquitin-activating enzyme, E1, initiates this cascade. Binding of ATP and ubiquitin to the ubiquitin-activating enzyme creates an adenylated ubiquitin that can be transferred to its active site cysteine (Haas et al., 1982). A thiol ester bond is formed between a cysteine in the active site of the E1 and the C-terminus of the adenylated ubiquitin (Haas et al., 1982; Hershko et al., 1983). The activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme, E2, which interacts with a ubiquitin ligase, or E3. The ubiquitin ligase serves to deliver the ubiquitin moiety to the target substrate either by bringing the substrate and the E2 together or through transfer first to the E3 and then to the intended protein (Haas et al., 1982; Hershko et al., 1983; Pickart, 2001) (Figure I-2).

This enzyme cascade is hierarchal in that substrate specificity increases as you progress through the three groups of enzymes. In most organisms, there is only one E1, which serves to activate ubiquitin for all subsequent target substrates. At the next level is only a handful of E2s. Eleven enzymes are

present in *Saccharomyces cerevisiae* that conjugate ubiquitin, for example. Each one of these ubiquitin-conjugating enzymes will function with a set of E3s, which there are a myriad (Pickart, 2001). This enzymatic organization is the basis for target specificity in the UPS with ubiquitin ligases being the final determinant of target choice (Figure I-2).

There are 3 families of ubiquitin ligases which are based on the domains responsible for ubiquitylating their targets: HECT domains, RING-finger domains, and U-box domains (Patterson, 2002). A HECT domain, Homologous to the E6-AP Carboxy Terminus, is a highly conserved domain that forms a thiol-ester bond with ubiquitin via an active-site cysteine residue before transferring it to its target substrate (Huibregtse et al., 1995; Jackson et al., 2000; Scheffner et al., 1995). The namesake ubiquitin ligase for this family, E6-AP, recognizes and targets p53 for ubiquitylation (Scheffner et al., 1993).

RING (Really Interesting New Gene) domains, which are cysteine-rich zinc finger domains: three β sheets, an α helical domain, and two loops structured around cysteine residues charged with zinc (Freemont et al., 1991). These domains function in protein-protein interactions in a wide variety of cellular processes by acting as a molecular scaffold (Borden, 2000). The first RING domain-containing protein to be linked with ubiquitylation was Rad18. Rad18 promotes the ubiquitylation of histones (Bailly et al., 1997; Deshaies and Joazeiro, 2009). RING domains do not appear to from a bond with ubiquitin, but mediate its transfer from an activated ubiquitin-conjugating enzyme to the target substrate (Jackson et al., 2000; Patterson, 2002). This novel mechanism

for ubiquitin transfer was elucidated by the discovery of Rbx1/Roc1 as part of the SCF ubiquitin ligase complex, and demonstrated that the ubiquitin ligase activity of SCF requires the RING domain of Rbx1/Roc1 (Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999).

The third ubiquitin ligase domain, the U-box domain, is a poorly conserved 75 amino acid motif similar to RING domains, but lacks the cysteine residues necessary for chelating zinc (Aravind and Koonin, 2000). In the place of cysteine residues, U-box domains have charged and polar amino acids that are necessary to maintain the domain's structure and activity (Aravind and Koonin, 2000; Vander Kooi et al., 2006). This domain is named for its presence in the ubiquitin chain elongator, Ufd2 (Koegl et al., 1999). *In vitro* ubiquitylation experiments have demonstrated that this domain is required for the ubiquitylation of target substrates (Hatakeyama et al., 2001; Jiang et al., 2001).

Types of ubiquitin attachments

The enzyme cascade described above is universal to all ubiquitin-mediated processes. The number of ubiquitin molecules attached to a substrate and the overall architecture of the ubiquitin signal are somewhat unique to the various ubiquitin-mediated processes. Since ubiquitin is used in so many cellular processes being able to identify differences in the signal placed on the target protein are necessary to ensure it reaches its destination.

The unique features of these ubiquitin signals are likely the key to proper recognition and sorting within the cell.

Ubiquitin is generally conjugated to a lysine residue within the target protein (Chau et al., 1989). The lysine chosen for ubiquitylation can be critical. For example, the lysine residues within Sic1, an important regulator for the G1 to S phase transition of the cell cycle, are required for its rapid turnover (Nugroho and Mendenhall, 1994; Petroski and Deshaies, 2003a; Schwob et al., 1994). While one multiubiquitin chain is sufficient to degrade Sic1, placement of the chain influences the rate of Sic1 degradation (Petroski and Deshaies, 2003a). Interestingly, only chains conjugated to one of six N-terminal lysines near the phosphorylation-dependent degradation signals are capable of efficiently targeting Sic to the proteasome (Petroski and Deshaies, 2003a, b). Furthermore, the transcriptional activator Met4 must be inactivated by an SCF ubiquitin ligase Met30 in order for faithful cell cycle progression (Patton et al., 1998; Rouillon et al., 2000; Skowyra et al., 1997). Met30 ubiquitylates Met4 specifically at K163. Mutating K163 to an arginine constitutively activates Met4 inhibiting cell cycle progression (Flick et al., 2004).

While lysine residues are the most common site of ubiquitin attachment (Chau et al., 1989), other residues have been identified as acceptors of ubiquitin. For example, a transcriptional activator involved in muscle development, MyoD, the N-terminal residue is the site of ubiquitin conjugation. In fact, a free N-terminus is both necessary and sufficient for ubiquitin

conjugation and subsequent degradation of MyoD (Breitschopf et al., 1998). Additionally, ubiquitylation can occur on non-lysine residues such as cysteine. A viral E3, Mir1, from Kaposi's sarcoma-associated herpes virus, can conjugate ubiquitin to a cysteine residue in MHC I (major histocompatibility complex class I). These attachments are sensitive to reducing agents, such as β-mercaptoethanol, whereas ubiquitin attachments to lysine residues are not (Cadwell and Coscoy, 2005).

In addition to alterations in ubiquitin placement within a target protein, the number of ubiquitins attached at one site as well as how they are linked together can be diverse. A tetraubiquitin K48-linked chain has long been thought to be the minimum required length for recognition as a proteolytic signal (Thrower et al., 2000), but other types of ubiquitin chains have recently been established as proteasome targeting as well. *In vitro* studies have suggested that K63-linked chains can signal the degradation of their target (Hofmann and Pickart, 2001). Mixed chains of K11, K48, and K63 linkages can target cyclin B for proteolysis (Kirkpatrick et al., 2006). The Anaphase-Promoting Complex is a RING domain-containing ubiquitin ligase, which preferentially creates chains linked via K11 for targeting substrates to the proteasome (Jin et al., 2008). Additionally, ubiquitin can be linked together via isopeptide bonds between the N-terminus of one ubiquitin and the C-terminus of another forming what has been termed a linear ubiquitin chain, which are structurally similar to K63-linked chains (Kirisako et al., 2006;

Komander et al., 2009). These linear chains are sufficient to direct a synthetic substrate, Ub-GFP, for degradation (Kirisako et al., 2006).

Interestingly, multiubiquitin K48-linked chains do not always target substrates for degradation. The transcriptional activator Met4 is conjugated with a K48-linked chain. This chain, however, does not signal its degradation. Blocking ubiquitylation of Met4, while causing it to become constitutively active, does not stabilize the protein (Flick et al., 2004). This results indicates a proteasome-independent role for a K48-linked multiubiquitin chain.

Ubiquitin is not obligated to form chains. Monoubiquitylation has been shown to participate in such processes as viral budding (Patnaik et al., 2000), and histone modification (Robzyk et al., 2000). It is also sufficient to direct proteasomal processing of the NF-κB precursor, p105 (Kravtsova-Ivantsiv et al., 2009). A methylated version of ubiquitin, which is incapable of polymerizing chains of any type, has been shown to direct the in vitro degradation of the protein lysozyme, though at a reduced rate (Hershko and Heller, 1985). In addition, the myogenesis regulator Pax3 is targeted to the 26S proteasome by a single ubiquitin moiety (Boutet et al., 2007).

Examples also exist in the literature of proteins being targeted for degradation in a ubiquitin-independent manner (Jariel-Encontre et al., 2008). A ubiquitin-dependent and a ubiquitin-independent pathway is responsible for the degradation of Rpn4, a yeast transcriptional activator of proteasomal genes (Ju and Xie, 2004; Mannhaupt et al., 1999; Xie and Varshavsky, 2001). Both pathways must be disabled in order to stabilize Rpn4 (Ju and Xie, 2004).

However, a ubiquitin-independent pathway as the sole method of proteasomal targeting has only been solidly established for ornithine decarboxylase (ODC). ODC is degraded by the proteasome, but is directed there by a protein called antizyme (Murakami et al., 1992a; Murakami et al., 1992b; Murakami et al., 1992c). Both ODC and antizyme contain domains that are required for the turnover of ODC. Neither domain is sufficient on its own suggesting that the two domains must cooperate in some manner to target ODC to the proteasome (Verma and Deshaies, 2000). The cyclin-dependent kinase inhibitor $p21^{CIP1}$ also appears to be degraded in a ubiquitin-independent manner. $p21^{CIP1}$ is stabilized in the presence of proteasomal inhibitors, but its degradation is not impaired by removal its lysines or by expression of a ubiquitin mutant that can not make lysine-linked chains (Sheaff et al., 2000).

Types of Degradation Signals

The enzyme cascade responsible for ubiquitylating target proteins may be the reason for substrate specificity, but what directs a ubiquitin ligase to its target (Pickart, 2001)? Some type of signal is necessary to mark a protein for degradation to maintain specificity and allow proteolysis to function as a regulatory mechanism. This substrate recognition signal, or degron, is often the interaction surface between the target protein and the specific ubiquitin ligase. Several types of degrons have been identified. These signals have been characterized by their amino acid sequence, specific post-translational modifications, and structural motifs (Ravid and Hochstrasser, 2008).

Degradation signals characterized by amino acid sequence

The PEST motif was discovered when Rogers et al. (1986) performed a survey of the literature and noted many rapidly degraded proteins contain one or more regions rich in proline, glutamic acid, serine, and threonine. These so-called PEST regions are often flanked by positively charge residues (Rogers et al., 1986). In fact, removal of a 37 base pair PEST sequence from the C-terminus of Cln2, a yeast G1 cyclin increases steady state levels of this protein and enhances its stability. This sequence is not sufficient, however, to confer instability and leads to the conclusion that additional signals may lie within Cln2 (Salama et al., 1994).

Another degron characterized by amino acid sequence is the cyclin destruction box. Experiments in *Xenopus* extracts revealed that the N-terminal region of cyclin B is both necessary and sufficient for its turnover during mitosis. The degradation of cyclin B signals the cell's exit from mitosis and entry into the next cell cycle. Further mutational analysis identified the sequence RTVLGVIGD as important for the mitotic destruction of cyclin B. This nine amino acid sequence termed the "destruction box" is conserved in many other cyclins with particularly strong homology seen in the first (R) and fourth (L) residues. By mutating the highly conserved arginine in the first position degradation as well as ubiquitylation of cyclin B is inhibited suggesting that recognition of this substrate is mediated through the "destruction box" (Glotzer et al., 1991).

Consistent with the sequence identified in cyclin B being a substrate recognition motif for degradation machinery, point mutations in the cyclin B destruction box homolog (RAALGNISN) at conserved residues R and L inhibit degradation of cyclin A at the crucial switch from mitosis to interphase (Kobayashi et al., 1992). Though both sequences serve as degradation signals, there are differences in how cyclins A and B are recognized by the ubiquitylation machinery. The cyclin A destruction box can not functionally substitute for its counterpart found in cyclin B (King et al., 1996). Furthermore, destruction boxes alone are not sufficient to direct proteolysis. In order to confer instability on protein A from *Staphylococcus aureus*, residues surrounding the cyclin B destruction box totalling 27 base pairs are required (Glotzer et al., 1991).

An additional method for marking a protein for degradation is through its N-terminal residue and is referred to as the N-end rule. Having a stabilizing residue (Met, Gly, Val, Pro, Cys, Ala, Ser, or Thr) versus having a de-stabilizing residue (Glu, Gln, Asp, Asn, Ile, Leu, Phe, Trp, Tyr, His, Lys, Arg) plays a large role in determining the half-life of β-galactosidase and mouse dihydrofolate reductase (Bachmair et al., 1986; Bachmair and Varshavsky, 1989). Additionally, a second amino acid is required for this N-terminal residue to efficiently function as a determinant of stability. A spatially accessible lysine is required for the efficient turnover of an N-end rule substrate (Bachmair and Varshavsky, 1989).

Ubiquitin as a signal for E3 recognition

The ubiquitin fusion degradation is a pathway that has been characterized in yeast for synthetic substrates consisting of a non-removable ubiquitin molecule fused to the N-terminus of a substrate. This N-terminal ubiqutin moiety acts as a degron signaling recognition of the substrate by a ubiquitin ligase. The substrate used to identified this pathway was Ub-Pro-βGal (Johnson et al., 1992). To-date no physiological substrate has been identified. This pathway utilizes the activities of the ubiquitin-conjugating enzymes Ubc4 and Ubc5. A series of proteins known as Ufd1, Ufd2, Ufd3, Ufd4, and Ufd5 have been identified in association with this pathway (Johnson et al., 1992; Johnson et al., 1995) and are conserved in mammalian cells (Dantuma et al., 2000).

The functions of the UFD proteins have all been determined. Ufd1 functions post-ubiquitylation through recognition of already modified substrates. In a complex with the AAA-ATPase Cdc48 and Npl4, Ufd1 presents ubiquitylated substrates to the proteasome (Braun et al., 2002; Ye et al., 2001). Ufd2 is a U box-containing ubiquitin ligase, which serves to elongate ubiquitin chains already initiated by other ubiquitin ligases and interacts with the Cdc48 (Koegl et al., 1999). Ufd3, also known as Doa1, is a WD repeat protein that also interacts with Cdc48 (Ghislain et al., 1996; Hochstrasser and Varshavsky, 1990). Ufd4, an E3 whose ligase activity is derived from a HECT-domain, is known to ubiquitylate substrates with Ubc4 or Ubc5 (Ju et al., 2007). Interestingly, Ufd4 interacts with the N-terminal ubiquitin molecule of UFD substrates via a series

of Armadillo Repeat Motifs (ARM) (Ju et al., 2007). Each repeat consists of 3 α–helices, and appears structurally similar to other ubiquitin-binding domains such as UBA and CUE domains (Dieckmann et al., 1998; Kang et al., 2003; Mueller and Feigon, 2002; Ohno et al., 2005). Ufd5, more commonly called Rpn4, is responsible for the transcriptional activation of proteasome genes (Mannhaupt et al., 1999; Xie and Varshavsky, 2001).

Phosphodegrons

Phosphorylation can also be used to mark a protein for degradation. Sic1, an inhibitor of the B-type cyclin dependent kinase (S-CDK), is needed to maintain G1 and prevent premature DNA synthesis in yeast (Mendenhall, 1993; Nugroho and Mendenhall, 1994; Schwob et al., 1994). Phosphorylation of Sic1 by Cln-CDK triggers binding to a constitutively active E3, SCF^{Cdc4} (Skowyra et al., 1997) allowing activation of S-CDK and subsequent DNA synthesis (Verma et al., 1997). Interestingly, six different phosphorylation events are necessary for recognition by SCF^{Cdc4}. Processive phosphorylation of Sic1 is key in timing the G1 to S phase transition (Nash et al., 2001). The requirement for multiple phosphorylation events allows time for Sic1 to function in its specified manner before being turned over (Deshaies and Ferrell, 2001). Like Sic1, many of the known examples of phosphodegrons have been found to involve SCF-type E3 complexes such as $p27^{Kip1}$ and cyclin E, which are recognized by SCF^{Skp2} (Tsvetkov et al. 1999, Yeh et al. 2001), and Emi1, IκBα, and β-catenin

recognized by $SCF^{\beta TrCP}$ (Margotinn-Goguet et al. 2003, Yaron et al. 1998, Hart et al. 1999).

SUMO-dependent ubiquitylation

SUMO stands for Small Ubiquitin-like MOdifier. Just as its name implies, SUMO is a part of the ubiquitin-like family of proteins and has been implicated in an array of cellular functions (Johnson, 2004; Kerscher et al., 2006). SUMO, like ubiquitin, is covalently attached to target proteins by a three-step enzyme cascade. The process of sumoylation, however, requires SUMO-specific enzymes (Johnson and Blobel, 1997; Johnson and Gupta, 2001; Johnson et al., 1997; Zhao and Blobel, 2005). Interestingly, SUMO and ubiquitin can function together as they do in the degradation of the yeast Flp recombinase (Chen et al., 2005; Xiong et al., 2009). Sumoylation may also be functioning as a type of degron. As research continues, more evidence is being found to support the idea that SUMO is a key player in substrate recognition for the ubiquitin-proteasome system (Chen et al., 2005; Uzunova et al., 2007; Wang et al., 2006; Wang and Prelich, 2009; Xiong et al., 2009). Sumoylation, like phosphorylation, could be used to create a functional degradation signal allowing for precise recognition and timing of degradation.

This apparent pathway overlap converges on a family of ubiquitin ligase called SUMO-Targeted Ubiquitin Ligases (STUbLs) (Mullen and Brill, 2008; Prudden et al., 2007; Sun et al., 2007; Xie et al., 2007). This family is evolutionarily conserved, with members found in slime mold, yeast, and man

(Prudden et al., 2007). STUbLs not only contain a RING domain giving them ubiquitin ligase activity, but they also have conserved motifs that interact with SUMO called SIMs for SUMO-Interacting Motifs (Hannich et al., 2005; Minty et al., 2000; Reverter and Lima, 2005; Song et al., 2004). SIMs, like those found in Slx5 (Xie et al., 2007), might be specially designed to recognize Sumo-dependent degradation signal.

The heterodimer Slx5-Slx8 in *Saccharomyces cerevisiae* is a STUbL. Two *in vivo* substrates have been identified for this STUbL: Flp recombinase, which is involved in the propagation of the yeast 2μm plasmid, and a mutant version of Mot1, which binds to TATA-binding protein (Chen et al., 2005; Wang et al., 2006; Wang and Prelich, 2009; Xiong et al., 2009). Slx5-Slx8 is also known to function with the ubiquitin-conjugating enzyme Ubc4 (Uzunova et al., 2007; Yang et al., 2006). Both Slx5 and Slx8 contain RING domains (Ii et al., 2007; Xie et al., 2007). Slx5 is proposed to be the substrate recognition portion of the heterodimer since it contains SIM sequences shown to interact with SUMO (Cook et al., 2009; Xie et al., 2007). Slx8 modulates DNA binding through its N-terminus forming a complex with double stranded DNA suggesting that this heterodimer can target substrates on DNA (Yang et al., 2006).

Masking degrons protects substrates from degradation

While post-translational modifications offer a method for timing degradation of particular target proteins, another mechanism would be to physically occlude the degron from the ubiquitylation machinery. Physical

occlusion can occur through transcriptional activation domains, or other domains such as protein-protein interaction regions overlapping a degradation signal. This overlap would cause competition between factors that bind these domains thereby slowing down the rate of degradation. This mechanism provides a functionally engaged protein protection from proteolysis (Salghetti et al., 2000). Several cases of transcriptional activation domains overlapping with degrons have been described including: Myc, Gcn4, and p53 (Fields and Jang, 1990; Hope et al., 1988; Salghetti et al., 1999; Salghetti et al., 2000). Another example of a degron overlapping with a functional domain is found in the yeast mating-type transcription factor, Matα2. A degradation signal, Deg1, located at the N-terminus, overlaps with the protein-protein interaction surface required to form a heterodimer with the a-specific protein Mata1 (Johnson et al. 1998).

Yeast mating-type biology

In discussing the ubiquitin-proteasome system many examples can be found involving proteins from the yeast, *Saccharomyces cerevisiae*. In addition, the mating-type switching system in yeast functions to specify different cell fates in this unicellular organism and affords an in-depth investigation of cellular differentiation controlled by differential gene expression. This system is ideal for exploring how the UPS can aid in regulating switches in cellular phenotypes.

Haploid cell-types and their genetic regulation

Saccharomyces cerevisiae exists as two different haploid cell types (a and α), which are characterized by the expression of type-specific genes. These different expression patterns are governed by a group of regulatory transcription factors encoded at the mating-type (*MAT*) locus (Klar et al., 1981; Nasmyth et al., 1981; Tatchell et al., 1981). For example, α cells carry the *MATα* allele that encodes two different regulatory activities needed to define the α state: the Matα1 activator turns on the expression of the α-specific genes, while the Matα2 repressor blocks the transcription of genes specific to the opposite a cell type. In a cells, Mata1 is expressed but has no known function and genes specific to a cells are expressed because there is no Matα2 to repress them (Strathern et al., 1981) (Figure I-3).

Mating-type switching

Interestingly, these mating phenotypes are unstable states and switching between a and α can occur in the span of a single cell cycle (Hicks and Herskowitz, 1976; Strathern et al., 1979a; Strathern et al., 1979b) (Figure I-3). A genetic switch of the information contained at the *MAT* locus set offs this phenotypic change (Hicks and Herskowitz, 1977; Strathern et al., 1979a; Takano et al., 1973). The genetic switch is initiated by the HO (HOmothallic) endonuclease, but only in mother cells. Daughter cells do not alter their mating-type until they have completed one cell cycle and, thus, become a mother cell (Nasmyth, 1983). In daughter cells, expression of HO endonuclease is repressed due to the asymmetric localization of Ash1 mRNA at the distal tip

of the daughter bud (Long et al., 1997). In mother cells, the HO endonuclease creates a site-specific, double strand break at the *MAT* locus (Kostriken and Heffron, 1984; Kostriken et al., 1983; Strathern et al., 1982). This break is repaired by homologous recombination using information contained at one of two silent loci, HMR$\underline{a}$ or HMLα. An α cell will almost always use HMR$\underline{a}$ and an $\underline{a}$ cell will consistently use HMLα to repair the break though the mechanisms that these cells use in donor loci preference are different (Klar et al., 1982; Wu and Haber, 1995; Wu et al., 1996).

Following the genetic rearrangement, the cell type-specific gene expression programs, including the transcription factors that establish these states, must be labile to facilitate such a rapid switch in cellular phenotype. Indeed, the Mat regulators are short-lived proteins, with the ubiquitin-proteasome pathway promoting the instability of these transcription factors (Hochstrasser et al., 1991; Hochstrasser and Varshavsky, 1990; Johnson, 1997).

Haploid cells of opposite types mate to form a diploid

Switching between mating-type is not the only phenotypic switch observed in yeast. A second switch occurs when $\underline{a}$ and α cells mate to form a diploid cell (Figure I-3). Mating is initiated when cells of opposite type sense mating-type specific pheromones via G protein-coupled receptors (Bender and Sprague, 1989; Jackson et al., 1991). Binding of these pheromones to the receptors sets off a signal transduction pathway that is common to both mating-types (Hartwell, 1980; Nakayama et al., 1987). Haploid cells respond

only to the pheromone from the opposite mating-type; diploid cells respond to neither pheromone (Jackson and Hartwell, 1990a, b). Response to pheromone results in a cell cycle arrest at G1 and directional cell growth towards the cell of opposite mating-type to eventually form a diploid cell (Bucking-Throm et al., 1973; Hartwell, 1973; Wilkinson and Pringle, 1974).

Diploid cells are distinct from haploid cells in chromosome number as well as their inability to respond to mating pheromones and ultimately mate (Jackson and Hartwell, 1990a, b). Diploid cells can also undergo meiotic divisions to form four haploid spores, two of each mating type (Roman et al., 1955). Furthermore, differences between haploid cells and diploid cells are observed with respect to the proteolysis of the Matα transcription factors (Johnson, 1997; Johnson et al., 1998).

Matα2

Matα2 is the transcriptional repressor of a-specific genes and is expressed in α cells (Strathern et al., 1981). Matα2 accomplishes robust repression of its target genes by recruiting the general repressors Tup1 and Ssn6 to a-specific gene promoters (Keleher et al., 1988; Keleher et al., 1992). In diploid cells, it forms a heterodimer with Mata1 to repress haploid specific genes as well as α-specific genes by repressing the expression of Matα1 (Dranginis, 1990; Goutte and Johnson, 1988; Herskowitz, 1989)(Figure I-3). As mentioned previously, the regulation of Matα2 during mating-type switching is well characterized. Its degradation is required for faithful mating-type

switching to occur. If Matα2 is stabilized, the switch from the α to the a cell-type is impaired (Laney and Hochstrasser, 2003).

Proteolysis of Matα2

Matα2 turnover proceeds through two different ubiquitin-dependent pathways. One pathway utilizes a degradation signal in the N-terminus of Matα2, called Deg1, which is recognized by the ubiquitin-conjugating enzymes Ubc6 and Ubc7 and the ubiquitin ligase Doa10 (Chen et al., 1993; Swanson et al., 2001). Deg1 is defined by a hydrophobic amphipathic helix (Johnson et al., 1998; Ravid and Hochstrasser, 2008). The region containing Deg1 interacts with one of Matα2's co-repressors, Tup1 (Chen et al., 1993; Komachi et al., 1994). This interaction inhibits degradation protecting the active fraction of Matα2 from proteolysis (Laney et al., 2006). Additionally, Deg1 is masked in diploid cells by Matα2's heterodimerization with Mata1. The formation of this heterodimer stabilizes each protein in diploid cells by a steric mechanism that occludes the degradation signals in both Mata1 and Matα2 (Johnson et al., 1998). Both interactions serve to stabilize Matα2 so that its short half-life does not interfere with the stable function it is required to perform (Johnson et al., 1998; Laney et al., 2006). In contrast, Matα1 has been observed to be more rapidly degraded in diploid cells than in haploid cells (Johnson, 1997). This difference between the Matα transcription factors makes sense biologically. Matα2 is functionally active in diploid cells, whereas the activating function of Matα1 is hypothesized to be detrimental to the diploid cell phenotype.

The second pathway that degrades Matα2 requires the ubiquitin-conjugating enzymes Ubc4 and Ubc5 (Chen et al., 1993). The STUbL Slx5-Slx8 functions with Ubc4 and Ubc5 through an unknown degradation signal located in the C-terminus of Matα2. Though Matα2 proteolysis is not SUMO-dependent, there is speculation that this degron may structurally mimic poly-SUMO chains allowing it to interact with Slx5 and its SUMO interacting motifs (Y. Xie and M. Hochstrasser, personal communication).

Matα1

Matα1 is the transcriptional activator of a-specific genes and is expressed in α cells (Strathern et al., 1981). Upon switching mating-types from an α cell to an a cell, presumably Matα1 must be inactivated just as Matα2 must for a phenotypic switch to occur (Laney and Hochstrasser, 2003). Not much is known about the inactivation of Matα1 other than its mRNA is short lived, and it is a rapidly degraded protein (Herrick et al., 1990; Johnson, 1997).

Matα1 mRNA is rapidly degraded

The messenger RNA for Matα1 is rapidly removed from the cell with a half-life of 3.5 minutes. This instability is governed by a 65 nucleotide stretch (nt 201-266) found in the coding region of Matα1 called the Matα1 instability element (MIE) (Caponigro et al., 1993). Translation through this region is required for mRNA decay (Hennigan and Jacobson, 1996; Parker and Jacobson, 1990). Rapid decay of Matα1 mRNA would aid in eliminating it following a

genetic switch from an α cell to an a cell, but already translated Matα1 protein would remain to activate α-specific genes unless it is cleared from the cell.

Matα1 is a short-lived protein

Matα1 is a rapidly degraded protein with a half-life of approximately 5-7 minutes. This observation was made using a highly over-expressed version of Matα1 driven by the GAL promoter. Using this over-expression, a monoubiquitylated form of Matα1 has also been detected (Johnson, 1997). It remains to be determined whether or not the data collected from this over-expression is relevant to physiological levels of Matα1.

Matα1 interacts cooperatively with Mcm1 to activate transcription

To understand how Matα1 is removed from a rapidly switching cell, it is important to consider interactions this protein makes with cofactors, which could potentially modulate its stability. The first 57 amino acids of Matα1 are necessary for transcriptional activation of α-specific genes (Sengupta and Cochran, 1991). Matα1 interacts with the transcription factor Mcm1 to activate transcription of α-specific genes (Figure I-4) (Bender and Sprague, 1987; Sengupta and Cochran, 1991). The Mcm1-Matα1 complex binds to α-specific genes at a sequence termed the P'Q box with Matα1 binding to the Q portion and Mcm1 binding to the P' portion, which is an imperfect palindrome (Hagen et al., 1993; Jarvis et al., 1988). Mcm1 can bind α-specific genes in the absence of Matα1 (Tan et al., 1988), but requires the mating-type regulator to

achieve the correct conformation of the protein-DNA complex in order to activate transcription (Carr et al., 2004). Together, they bind a single strand of DNA at the P'Q box (Grayhack, 1992).

Matα1 interacts with the middle third of Mcm1 (Mead et al., 2002; Tan and Richmond, 1990). The portion of Matα1 that interacts with Mcm1, however, is unknown. It is hypothesized to be a region of high sequence homology among Matα1 homologs termed the α1 domain (Coppin et al., 1997; Yuan et al., 1993). The α1 domain lies within residues 88-144 of Matα1 (Figure I-4). The possibility also exists that this region is the DNA binding domain since no such domain has been identified for Matα1. The function of the α1 domain remains to be determined.

Matα1 interacts with Ste12 in response to pheromone

In addition to interacting with Mcm1, Matα1 also interacts with the transcription factor Ste12 (Yuan et al., 1993) to activate α–specific genes in response to pheromone (Sengupta and Cochran, 1991). Residues 45-57 of Matα1 are required for the induction of gene expression in response to pheromone, the so-called pheromone response element (Figure I-4) (Sengupta and Cochran, 1991). Although Ste12 can bind to a consensus seven base pair sequence, such elements are not present in α–specific genes (Sengupta and Cochran, 1990). Instead, Ste12 is targeted to these genes through a direct protein-protein interaction with Matα1 (Sengupta and Cochran, 1991; Yuan et al., 1993).

Goals of this study

The ability to adopt a new, differentiation phenotype is fundamental to cellular development, and it is critical that the cell is able to clear its palette of any factors determining the previous state. Without this ability, a complete phenotypic switch could not take place. The yeast mating-type switching system offers an ideal model system to study the regulation of factors from the eclipsed state. Understanding how factors responsible for engendering mating-type are inactivated during switching to the opposite cell type will offer insight into the regulation of cellular differentiation in many systems. The goal of the project described in these pages has been to characterize the turnover of Matα1, the activator of α-specific gene transcription. Is the short-lived Matα1 targeted for degradation by the UPS? What factors are involved in targeting Matα1 to the proteasome? What about Matα1 targets it for proteolysis? Is the degradation of Matα1 and Matα2 coordinately regulated?

In the coming chapters, I present data showing that Matα1 is a substrate for the UPS. I observe stabilization of Matα1 when the function of the proteasome is impaired by either genetic or chemical means. Furthermore, proteolysis of this transcription factor is dependent upon multiple ubiquitin-associated enzymes. The yeast ubiquitin-conjugating enzymes Ubc4, Ubc5, and Ubc13 and the yeast ubiquitin ligases Ufd4, Dma1, Dma2, and Slx5-Slx8 are all required for rapid degradation of Matα1. Since the STUbL Slx5-Slx8 is involved, I investigated the role SUMO in Matα1 turnover. I found that rapid proteolysis of Matα1 requires an intact Sumoylation pathway and poly-SUMO chains. While

genetic analysis of these enzymes indicate one common pathway, I propose a model in which multiple pathways are functioning to ubiquitylate Matα1 above a set threshold for targeting it to the proteasome.

In order to recognize and ubiquitylate Matα1, these enzymes require a degradation signal. I found through a series of Matα1 deletion mutants that several regions within Mata1 are required for its rapid turnover including a region in the N-terminal half of the protein and at the extreme C-terminus. Furthermore, I present data to show that a region overlapping with the alpha1 domain (residues 75-150) is dependent upon the STUbL Slx5-Slx8.

These studies begin to answer whether or not the master regulators Matα1 and Matα2 are coordinately degraded during a switching event. There are distinct similarities and differences between the degradation pathways of these master regulators. Matα1 and Matα2 share a common pathway, Ubc4/Ubc5/Sl5-Slx8, but the nuances of this pathway are unique for each transcription factor. Both Ubc4 and Ubc5 must be removed to observe a defect in Matα1 degradation. However, A single deletion of Ubc4 is sufficient to alter the stability of Matα2 (Chen et al., 1993). Moreover, SUMO does not play a role in Matα2 turnover (Y. Xie and M. Hochstrasser, personal communication), but is required for rapid removal of Matα1. Whether or not there is a coordinated signal for the destruction of these regulators remains to be determined.

The proteolytic regulation of Matα1 is more complex than one would have initially imagined. The results presented here not only characterize the degradation pathway of Matα1, they also shed light on how mating-type

switching is regulated in yeast and how phenotypic changes are regulated in other systems. I have found a common link between how the two α-specific transcription factors are regulated, but have also shown that distinct mechanisms function to ensure their removal. This data adds to the on-going debate about proteolytic signals and raises new questions for the ubiquitin-proteasome and sumoylation fields.

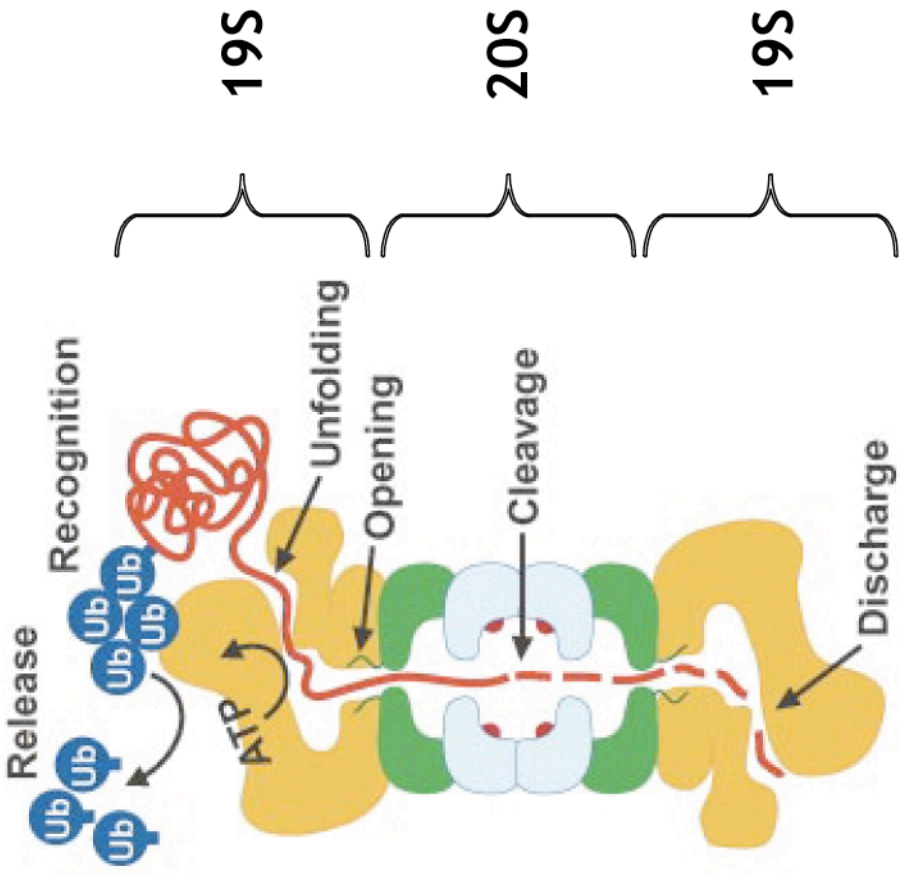

19S
20S
19S
Recognition
Unfolding
Opening
Cleavage
Discharge
Release
ATP
Ub

Figure I-1. Schematic of the 26S Proteasome. A schematic representation of the 26S proteasome shows the core 20S particle in green and gray bound at either end by the 19S regulatory subunit in gold. The 19S can cap one or both ends serving as the recognition module for the 26S proteasome. The 20S particle cleaves polypeptides as they transverse through its central pore. Adapted from: (Vierstra, 2003).

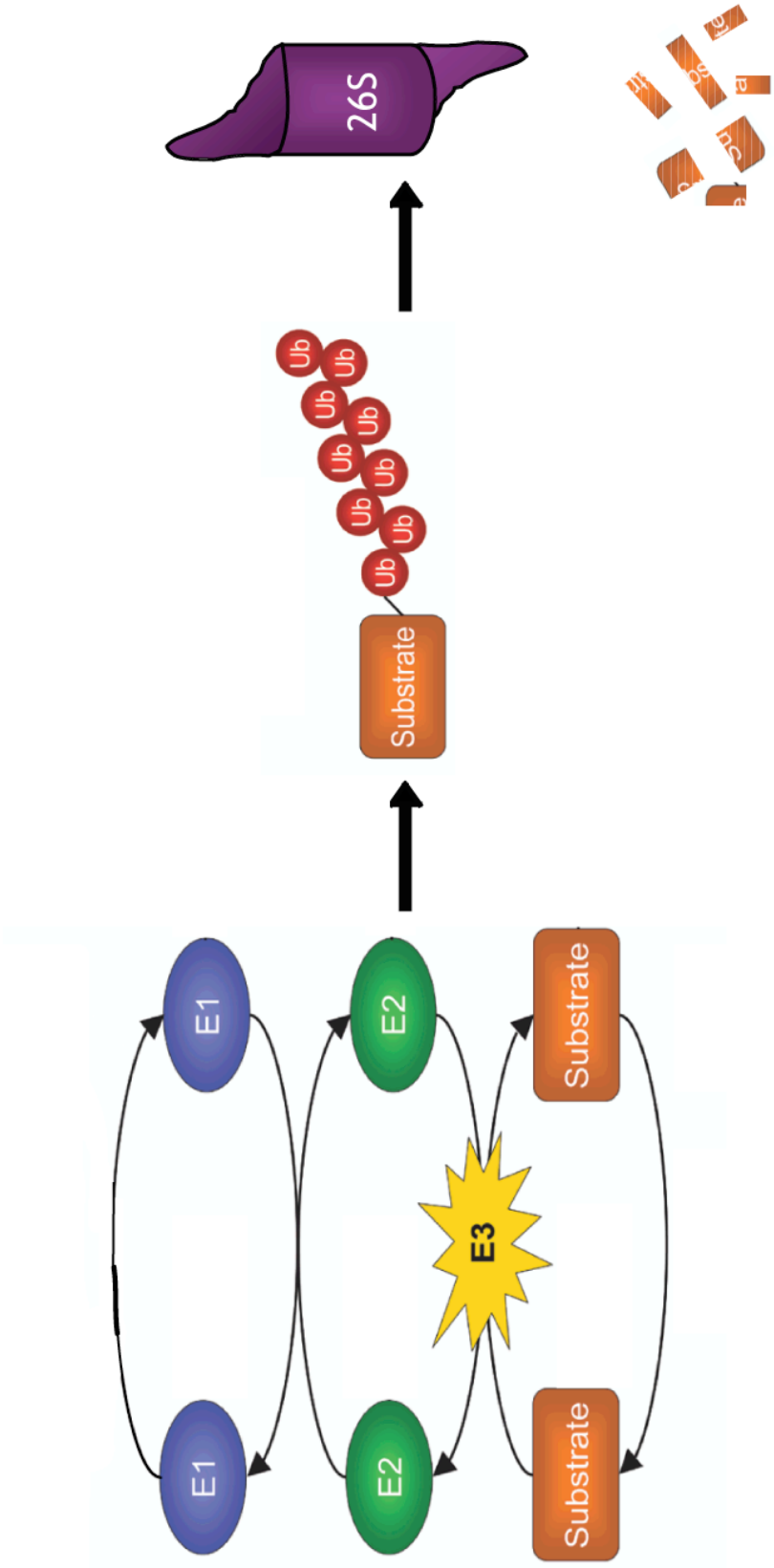

26S
Ub
Substrate
E1
E2
E3
Substrate
E1
E2
Substrate

Figure I-2. Schematic of the Ubiquitin-Proteasome Pathway. Target proteins are ubiquitylated through an enzyme cascade consisting of three separate enzymatic steps. To target a substrate to the proteasome for degradation it is thought that a minimum of four ubiquitin molecules linked via lysine 48 are necessary. Substrates appendend with a ubiquitin tag sufficient for directing it to the proteasome are recognized and degraded. Adapted from: (Passmore and Barford, 2004).

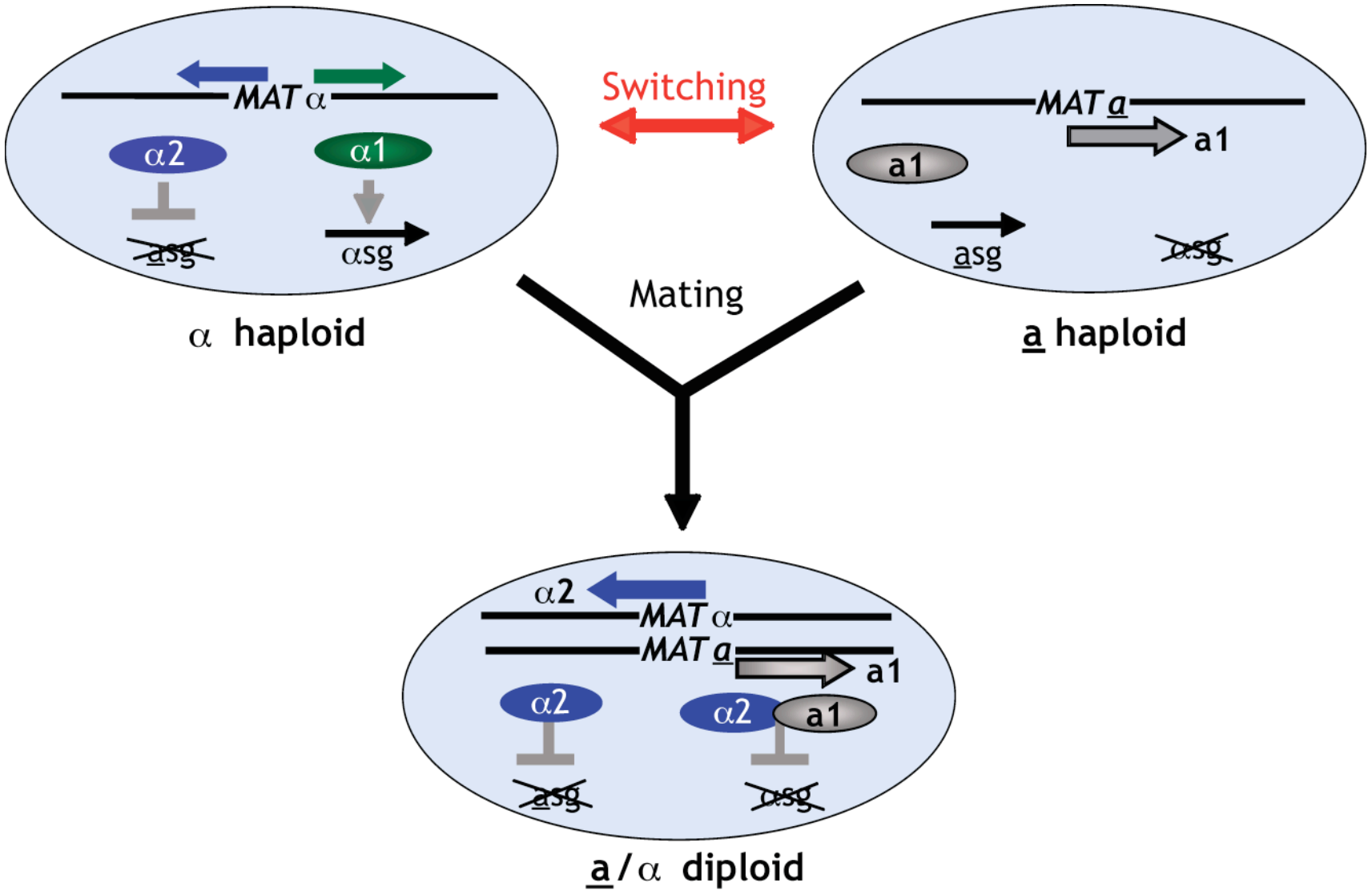

MAT α
α2
α1
asg
αsg
α haploid
Switching
MAT a
a1
a1
asg
asg
a haploid
Mating
α2
MAT α
MAT a
a1
α2
α2
a1
asg
asg
a / α diploid

Figure I-3. Cell-type Specific Regulation in Yeast. Yeast cells exist in one of two haploid cell-types: a or α. These states are determined by the transcription factors encoded at the *MAT* (mating-type) loci. Haploid cells can switch their mating-types and cells of opposite mating-types can mate to form a diploid.

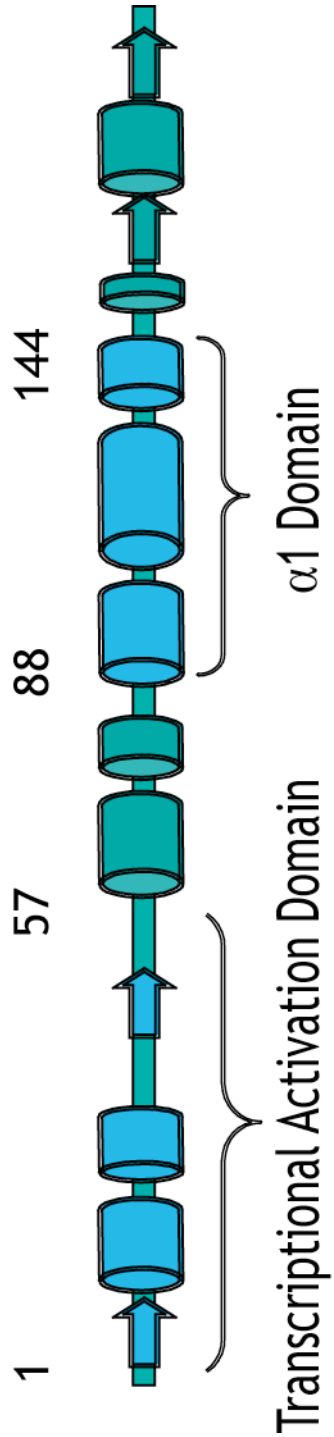

1
57
88
144
Transcriptional Activation Domain
α1 Domain

Figure I-4. Schematic of Matα1. Drawing representing the predicted secondary structure for Matα1. Arrows represent β-sheets and cylinders represent α-helices. The transcriptional activation domain has been mapped to residues 1-57 with 45-57 being important for pheromone response. A region of high homology among Matα1 proteins in fungi called the α1 domain lies within residues 88-144.

Chapter II

Characterization of the Proximal Degradation Pathway for Matα1

Abstract

Yeast cells can undergo phenotypic switching between two distinct haploid mating-types (a and α), which are determined by information encoded at the MAT locus. This locus encodes a set of master regulatory transcription factors that determine cell phenotype by regulating cell type-specific genes. One such regulator called Matα1 functions as a transcriptional activator of genes specifically expressed in α cells and appears to be a short-lived protein. Here, I show that rapid removal of Matα1 is governed by the ubiquitin-proteasome system. Rapid turnover of Matα1 requires the ubiquitin-conjugating enzymes Ubc4 and Ubc5 and the ubiquitin ligase Ufd4, a member of the ubiquitin fusion degradation pathway, as well as the SUMO-targeted ubiquitin ligase Slx5-Slx8. The degradation of Matα1 is also dependent upon an intact SUMO pathway and the ability of the cell to make SUMO chains. I have also found that Ubc13, but not the E2 variant, Mms2, it normally forms a heterodimer with, is required for turnover of Matα1. Dma1 and Dma2, yeast orthologs to the Ubc13-associated, human ubiquitin ligase Rnf8, are also necessary for rapid removal of this transcription. These data suggest that Matα1 is removed by a complex proximal degradation pathway involving not only ubiquitylation, also sumoylation of Matα1 and identify the first endogenous, physiological substrate for Slx5-Slx8.

Introduction

The ubiquitin-proteasome system functions to target particular proteins for rapid degradation. This system affords the cell a mechanism with which to finely regulate cellular processes that ultimately govern phenotype (Hochstrasser, 1996). A series of *trans*-acting factors regulate degradation of substrates. The overall pathway of factors that direct a protein for proteolysis can be divided into two distinct pathways: proximal and distal. The proximal pathway consists of enzymes responsible for tagging a protein with ubiquitin (Hershko et al., 1983). The distal pathway is made up of factors that either function to elongate ubiquitin chains (so-called E4s) as well as chaperone molecules that aid in delivering the target substrate to the proteasome (Hohfeld et al., 2001; Hoppe, 2005).

The ubiqutin-proteasome system has been shown to mediate the turnover of Matα2, one of the transcription factors involved in the mating-type switching system of the yeast *Sacchromyces cerevisiae*. Yeast mating-type (a or α) is determined by a set of transcription factors encoded at the *MAT* locus (Matα1, Matα2, or Mata1) (Hicks and Herskowitz, 1977; Strathern et al., 1979a; Takano et al., 1973). The Mat transcription factors have all been shown to have short half-lives (Hochstrasser and Varshavsky, 1990; Johnson, 1997). The rapid removal of these master regulators is hypothesized to permit efficient phenotypic changes to occur once the genotypic switch that initiates mating-type switching has taken place. This hypothesis has only been proven for Matα2 (Laney and Hochstrasser, 2003).

Matα2 is a transcriptional repressor which functions in the α cell type to repress a-specific genes. Interestingly, two distinct, proximal degradation pathways regulate Matα2. One pathway operates through the ubiquitin-conjugating enzymes Ubc6 and Ubc7 via the ubiquitin ligase Doa10 (Chen et al., 1993; Swanson et al., 2001), and the second pathway utilizes Ubc4 and Ubc5 (Chen et al., 1993) through the heterodimer Slx5-Slx8 (Y. Xie and M. Hochstrasser, personal communication). In an α cell, there is another Mat transcription factor present: Matα1. Matα1 is the transcriptional activator of α-specific genes (Strathern et al., 1981). Since Matα2 and Matα1 are both α-specific transcription factors and both need to be degraded upon the phenotypic switch to an a-cell, one could hypothesize that their degradation is coordinated.

To investigate this hypothesis, I first determined that Matα1 is a substrate for the ubiquitin-proteasome system. I will also present evidence that rapid turnover of Matα1 requires multiple ubiquitin-conjugating enzymes and at least four ubiquitin ligases. Epsistasis experiments indicate that these enzymes are all functioning in one genetic pathway. Though genetically indistinguishable, these enzymes could be functioning in separate pathways to ubiquitylate Matα1 above a set threshold required for targeting to the proteasome. Additionally, my investigations suggest that degradation of Matα1 requires an intact SUMO pathway. The characterization of Matα1's proximal degradation pathway highlights not only a common pathway for removing

Matα2 and Matα1, but also indicates that there are distinct mechanism in place to ensure the removal of both of these factors.

Results

Proteasome-dependence

To assess whether or not Matα1 is a substrate for degradation by the proteasome, I asked if Matα1 is stabilized when the proteasome is inactivated. I chemically inactivated the proteasome with the drug MG-132 and then performed a cycloheximide chase assay. In the presence of MG-132, Matα1 is strongly stabilized (Figure II-1) demonstrating that turnover of Matα1 is proteasome-dependent.

Although deletion of Rpn13, a non-ATPase proteasome subunit within the 19S regulatory particle and one of several ubiquitin receptors (Husnjak et al., 2008; Verma et al., 2000), shows little to no effect on the turnover rate of Matα1, I did observe stabilization of Matα1 in the presence of two other proteasome mutants: *cim5-1* and *cim3-1*. At the restrictive temperature of 37°C, I saw increased stabilization in the presence of both temperature-sensitive alleles *cim5-1* and *cim3-1* (Figure II-2). The *cim* mutations lie within the ATPases Rpt1 and Rpt 6, respectively, in the 19S regulatory subunit of the proteasome (Glickman et al., 1998b; Rubin et al., 1998). While *cim5-1* does appear to have a larger effect on Matα1's stability, both mutations increased Matα1's apparent half-life to greater than 90 minutes. It is not surprising that the *rpn13Δ* altered Matα1 turnover very little as compared to the *cim*

mutations, because Rpn13, unlike the *cim* mutations, is not required for cell viability suggesting that the proteasome can maintain some level of function without this subunit.

Next, I tested whether or not the other major degradation pathway in yeast, the vacuole, is required for Matα1 turnover. To do so, I analyzed degradation of Matα1 in the presence of mutations that impaired vacuolar proteases. I observed a modest, two-fold effect on Matα1's turnover when the function of vacuolar proteases, Pep4 and Prb1, were impaired (Figure II-2). This data indicates that the proteasome is the major player in ridding the cell of Matα1.

Ubiquitin-dependence

Most proteins targeted for destruction by the proteasome are directed by a ubiquitin signal. A ubiquitin-activating enzyme is necessary to attach ubiquitin to a substrate. In yeast there is only one such enzyme, Uba1, which is required for cell viability (McGrath et al., 1991). In strains containing a temperature-sensitive allele *uba1-204*, the ubiquitin-activating enzyme can be inactivated at the restrictive temperature (37°C) and ubiquitylation of substrates is impaired (Ghaboosi and Deshaies, 2007). I used this strain to determine if turnover of Matα1 is mediated by a ubiquitin-dependent conjugation pathway.

At the restrictive temperature (37°C), I observed mild stabilization of Matα1 suggesting that its rapid turnover is dependent upon a functional Uba1

(Figure II-3A). I was surprised by how mild the effect was on Matα1. The uba1-204 is known to revert if returned to the permissive temperature or if left on ice for an extended amount of time (Ghaboosi and Deshaies, 2007). Even though I attempted to maintain conditions in which Uba1 would remain inactive, the modest stabilization could be due to some level of reactivation of the allele. Additionally, residual activity of the mutant allele even at the restrictive temperature could be sufficient for turnover of Matα1.

To further test the hypothesis that degradation of Matα1 is ubiquitin-dependent, I tested its stability in a *doa4Δ*. Doa4 is a de-ubiquitylating enzyme that when deleted decreases the cellular levels of available ubiquitin (Swaminathan et al., 1999). My evaluation of the turnover of Matα1 in this strain showed, again, only a mild alteration. Two interpretations of this result can be made. One, turnover of Matα1 is not largely dependent upon ubiquitin, or two, cellular levels of ubiquitin in a *doa4Δ* are still sufficient for turnover of Matα1 (Figure II-3B).

While both of the experiments described above ask whether turnover of Matα1 is dependent upon ubiquitylation, both are indirect tests of this question. To directly assay for ubiquitylation of Matα1, I attempted to immunoprecipitate Matα1 and then immunoblot for ubiquitin. My attempts using tagged forms of Matα1 driven by the ADH promoter were unsuccessful. A monoubiquitylated form of Matα1 has been detected previously using a GAL driven Matα1 (Johnson, 1997). While there are concerns that over-expressing a protein can drive non-physiological ubiquitylation, it is equally possible that

the levels of Matα1 expressed from the ADH promoter in my experiments are below the limit of detection for these types of assays. I have evidence identifying multiple ubiquitin conjugating enzymes and ubiquitin ligases that play a role in the turnover of Matα1, which will be discussed later in this chapter. This data strongly suggests that Matα1 is ubiquitylated. Other methods need to be attempted to directly show ubiquitylation of Matα1.

Ubiquitin-conjugating enzymes

There are 11 enzymes that conjugate ubiquitin to substrates in *Sacchromyces cerevisiae*. I analyzed the levels of either steady-state or newly-synthesized Matα1 over time to determine if any of the 11 ubiquitin-conjugating enzymes are involved in Matα1 degradation (data not shown). I found that Matα1 is stabilized 3-fold in a *ubc13Δ* by cycloheximide chase (Figure II-4A). Ubc13's best-characterized function is to make polyubiquitin chains linked through lysine 63. To make these alternative chains, Ubc13 forms a heterodimer with a ubiquitin-conjugating enzyme variant called Mms2 (Brusky et al., 2000; Hofmann and Pickart, 1999; Ulrich and Jentsch, 2000). This heterodimer is important in the error-free DNA postreplication repair pathway (Brusky et al., 2000). Interestingly, I found that the Ubc13's effect on Matα1 is independent of Mms2 as well as Rad5, a ubiquitin ligase involved in postreplication repair (Lemontt, 1971; Torres-Ramos et al., 2002), ruling out K63 linkages and the DNA damage repair pathway (Figure II-4A).

In addition to Ubc13, I found that Matα1 is also stabilized in an *ubc4Δubc5Δ* double mutant approximately six-fold over wild type. Unlike Matα2, a ubc4Δ or a ubc5Δ single deletion does not stabilize Matα1 to any degree, even though these ubiquitin-conjugating enzymes are nearly identical (Figure II-4B) (Chen et al., 1993; Seufert and Jentsch, 1990). The effect of the double mutant on Matα1 is much greater than the effect I observed for *ubc13Δ* indicating an additional role for Ubc4 and Ubc5.

Ubc4/Ubc5-associated ubiquitin ligases

There are numerous ubiquitin ligases in yeast. In an attempt to identify ubiquitin ligases responsible for Matα1 degradation, I tested candidate proteins reported in the literature to function with the ubiquitin-conjugating enzymes I identified. One such candidate is a HECT-domain containing ligase, Ufd4, which is a member of the ubiquitin-fusion degradation pathway and is known to ubiquitylate substrates with Ubc4 or Ubc5 (Johnson et al., 1995; Ju et al., 2007). In a *ufd4Δ* strain, Matα1 is stabilized three-fold, suggesting that Ufd4 is required for its rapid turnover (Figure II-5A).

Slx5 and Slx8 form a heterodimer and are also known to function with Ubc4/5 (Uzunova et al., 2007; Yang et al., 2006). This heterodimer is a SUMO (small ubiquitin-like modifier)-Targeted Ubiquitin Ligase (STUbL) (Wang et al., 2006). It has also been observed to function in the degradation pathway of Matα2 turnover, but appears to do so in a SUMO-independent manner (Y. Xie and M. Hochstrasser, personal communication). Matα1 is stabilized three-fold

in either *slx5Δ* or *slx8Δ* by cycloheximide chase (Figure II-5A). Interestingly, I do not observe any stabilization of Matα1 in either single deletion when I analyze them by pulse-chase experiment (data not shown). This difference is most likely due to the nature of the two assays. Cycloheximide chase is an analysis of the steady-state pool of protein, while pulse-chase assays only provide information regarding newly synthesized proteins. The distinction between the two assays for Slx5 and Slx8 may suggest a difference in how newly synthesized and "old" Matα1 are degraded.

A double mutant of *slx5Δ* and *slx8Δ* exhibits the same change in kinetics, indicating that Slx5 and Slx8 are indeed in the same pathway with regards to Matα1 turnover (Figure II-6). Since this heterodimer has been observed to play a role in regulating Matα2, I also asked if the other ubiquitin ligase in the Matα2 degradation pathway, Doa10 (Swanson et al., 2001), was participating in the regulation of Matα2. While turnover of Matα1 is dependent upon Slx5-Slx8, it is not dependent upon Doa10 (Figure II-6).

Ris1 and Rsp5 are two more ubiquitin ligases known to cooperate with Ubc4/5 (Hoppe et al., 2000; Uzunova et al., 2007). A *ris1Δ* and a temperature-sensitive allele of *RSP5*, *rsp5-2*, do not alter Matα1's stability (Figure II-5B). This data indicates that Matα1's stability is dependent specifically upon Ufd4, and Slx5-Slx8 and not any Ubc4/5-dependent ubiquitin ligase.

Degradation of Matα1 is SUMO-dependent

Since Slx5-Slx8 has been shown to be associated with the SUMO pathway (Wang et al., 2006), I wanted to determine if Matα1 proteolysis is dependent upon this pathway. The SUMO-activating enzyme, Uba2, the SUMO-conjugating enzyme, Ubc9, and de-sumolyating enzyme, Ulp1, are all essential in yeast (Dohmen et al., 1995; Johnson and Blobel, 1997; Johnson et al., 1997; Li and Hochstrasser, 1999). I used temperature-sensitive alleles of these enzymes to determine if Matα1 turnover requires an intact sumolyation pathway. Matα1 is stabilized by a mutation in Uba2, albeit mildly, with only two-fold stabilization (Figure II-7A).

The mild effect observed with the temperature-sensitive alleles could be due to the strength of the alleles, or because of cell-cycle defects caused by these mutations (Seufert et al., 1995). To rule out that the stabilization of Matα1 in these strains is not due to secondary cell-cycle defects caused by these mutations, I tested Matα1 stability in cells mutant for known SUMO ligases. A double deletion of Siz1 and Siz2 and a deletion of the RING domain of Mms21 all stabilize Matα1 with the same kinetics as *slx5Δ* or *slx8Δ* (Figure II-7B) (Johnson and Gupta, 2001; Takahashi et al., 2001; Zhao and Blobel, 2005). Therefore, an intact sumoylation pathway is necessary for rapid turnover of Matα1. These data are consistent with a model in which sumolyation of Matα1 makes it a substrate for ubiquitylation by the STUbL Slx5-Slx8.

Given that SUMO is conjugated to substrates just as ubiquitin is and can form poly-SUMO chains on a target protein, I hypothesized that SUMO chains

might be required for Matα1 proteolysis (Kim et al., 2002; Schwartz and Hochstrasser, 2003). To investigate this hypothesis, I used a mutant in which all lysines within the *SMT3* gene that encodes SUMO are mutated to arginines (*smt3allR)*. *smt3allR* is incapable of forming poly-SUMO chains. I also tested a mutant in which only the first three N-terminal lysines were changed to arginines (*smt3-3NR*) (Bylebyl et al., 2003) since most poly-SUMO chains appear to assemble with the first three lysines of *SMT3* (E. Johnson, personal communication). The *smt3-3NR* mutant should also abrogate poly-SUMO chain formation via those residues (Bylebyl et al., 2003). Interestingly, Matα1 is stabilized in the presence of *smt3allR* (Figure II-7B), but not in the presence of *smt3-3NR* mutant (Figure II-7C). This observation indicates that while degradation of Matα1 requires lysine residues within SUMO, it does not require the first three lysines within *SMT3* for chain formation.

Ubc13-associated ubiquitin ligases

Previously, I ruled out the error-free DNA postreplication repair pathway normally associated with Ubc13 when characterizing *mms2Δ* and *rad5Δ* (Figure II-4A). Interestingly, it has recently been shown that Ubc13 can form non-K63 linked chains in the absence of an E2 variant (Petroski et al., 2007). Furthermore, the mammalian ubiquitin ligase Rnf8 has also been shown to function with Ubc13 independently of its E2 variant partner (Plans et al., 2006). There are two orthologous proteins to Rnf8 in yeast: Dma1 and Dma2, which have been shown to play a role in the cell cycle via Ubc4/5 and Ubc13

(Loring et al., 2008). Deletions of either Dma1 or Dma2 stabilize steady-state levels of Matα1 by three-fold (Figure II-8) indicating that both are involved in the proteolysis of Matα1. Additionally, the double mutant stabilizes Matα1 to the same extent as the single mutations (Figure II-8) suggesting that Dma1 and Dma2 function in the same genetic pathway.

Genetic characterization of Matα1's degradation pathway

Deleting one of the ubiquitin ligases I identified in a strain already deficient for Ubc13 does not further increase the stability of Matα1 indicating, by standard interpretations, that they all fall within one genetic pathway (Figure II-9). I was unable to verify genetically that Ufd4 or Slx5-Slx8 are in the same pathway as Ubc4 and Ubc5 as suggested by the literature (Ju et al., 2007; Uzunova et al., 2007; Yang et al., 2006) since these *ubc4Δubc5Δ* strains grow extremely poorly. In fact, these mutations are not viable in some genetic backgrounds. Adding further deletions to the *ubc4Δubc5Δ* strain was possible, but the manipulations necessary to perform the cycloheximide chase assays were not tolerated by the strains.

Surprisingly, all double mutant combinations of the ubiquitin ligases demonstrated identical kinetics to those observed in the single deletions (Figure II-10). The only exception is *ufd4Δdma1Δ*, which is stabilized more than six-fold (Figure II-11). This data suggest that while Dma1 and Ufd4 are in parallel pathways, all of the other ubiquitin ligases are in genetically indistinguishable pathways.

Interestingly, the deletion of Dma2 suppresses the stabilization observed for *ufd4Δdma1Δ* as indicated by the degradation rate of Matα1 in *ufd4Δdma1Δ*dma2*Δ* (Figure II-11). I would hypothesize that the target of this negative regulation by Dma2 is Slx5 and/or Slx8 (or possibly an unidentified factor), because they are the only factors remaining in the *ufd4Δdma1Δ*dma2*Δ* strain. In the absence of Dma2, one or both of these factors are up regulated allowing for efficient turnover of Matα1.

Additionally, Slx5 and Slx8 seem to be behaving independently of one another in some instances. These differences are noted in the fold change in degradation kinetics for *slx5Δdma1Δ*dma2*Δ* (six-fold) and *slx8Δdma1Δ*dma2*Δ* (three-fold) as well as *ufd4Δslx5Δdma1Δ*dma2*Δ* (six-fold) and *ufd4Δslx8Δdma1Δ*dma2*Δ* (three-fold) (Figure II-10, 12). In theses mutants, the deletion of Slx8 doesn't increase the stabilization of Matα1 observed in the other single mutants while the deletion of Slx5 increases it to six-fold. This difference is surprising given that *slx5Δ* and *slx8Δ* behave similarly with respect to Matα1 turnover, and a *slx5Δslx8Δ* indicates that these two enzymes function in the same pathway (Figure II-6) consistent with their described role as a heterodimer (Yang et al., 2006).

Discussion

Mating-type switching in yeast predicts the rapid inactivation of the transcription factors that determine the previous mating-type. This inactivation has already been shown to rely on protein degradation for the

transcriptional repressor Matα2 (Hochstrasser and Varshavsky, 1990). The finding that its counterpart, Matα1, is also a short-lived protein prompted these studies. My findings demonstrate that Matα1 is subject to protein degradation by the ubiquitin-proteasome system (Figure II-1, II-2, II-3). Moreover, its degradation requires multiple enzymes involved in ubiquitin conjugation suggesting that Matα1 is directly ubiquitylated, though I cannot rule out an indirect effect.

Degradation of Matα1 requires multiple ubiquitylation enzymes

My experiments indicate that degradation of Matα1 requires both Ubc4 and Ubc5. Additionally, the turnover of this transcription factor is dependent upon Ubc13, but not Mms2 (Figure II-4). I have also identified four ubiquitin ligases that are necessary for rapid proteolysis of Matα1: Ufd4, Slx5-Slx8, Dma1, and Dma2 (Figure II-5, 8). While Dma1 and Dma2 could be functioning with either Ubc4/5 or Ubc13 (Loring et al., 2008), Ufd4 and Slx5-Slx8 are known only to function with Ubc4/5 (Ju et al., 2007; Uzunova et al., 2007; Yang et al., 2006).

The finding that turnover of Matα1 is dependent Slx5-Slx8 led to further investigations into the role of SUMO in this degradation pathway. Alterations in the sumoylation pathway led to stabilization of Matα1 suggesting that an operational sumoylation pathway is required for proteolysis of Matα1 (Figure II-7A). Moverover, a mutant form of Smt3 that cannot form poly-SUMO chains does not support turnover of Matα1, further implicating SUMO in this

degradation pathway (Figure II-7B). Whether or not Matα1 is directly sumoylated remains to be determined. This finding, however, points to Matα1 being the first physiologically relevant substrate for the SUMO-targeted ubiquitin ligase Slx5-Slx8.

Model for the proximal degradation pathway of Matα1

Although the epistasis experiments indicate that all ubiquitin enzymes required for Matα1 turnover fall into one genetic pathway with only separable functions for Dma1 and Ufd4 (Figure II-10), I would offer an alternative explanation given the number of enzymes involved in this pathway. Perhaps Ufd4, Dma1, Dma2, and Slx5-Slx8 all function in parallel pathways, some of which are stronger than the others (Figure II-13). Ufd4 and Dma1 are the strongest of the 4 pathways. Dma2 and Slx5-Slx8 are minor pathways, but still important enough to alter Matα1's degradation kinetics by three-fold when inactivated. These separate pathways work together to ubiquitylate Matα1 to a certain threshold. To alter the stability Matα1 more than three-fold as in the single ubiquitin ligase mutants, several pathways must be inactivated. The number of pathways that need to be shut down depends on the strength of the ubiquitin ligase in directing turnover of Matα1. Ufd4 and Dma1 are strong enough to alter Matα1's degradation rate together without further mutations, but the other pathways require at least three pathways to be inactivated (Figure II-11, 12).

Additionally, if Dma2 has a negative role as well as a positive role in the turnover of Matα1 (Figure II-13), it is possible that its negative activity is complicating the epistatic analysis I performed. Those experiments indicate that Dma2 is in the same genetic pathway as the other ubiquitin ligases I identified (Figure II-10, 11), but in the absence of Dma2 other factors are potentially being upregulated and masking any additional stabilization caused by the absence of Dma2's positive activity.

Coordinate degradation of Matα1 and Matα2

The data I have presented indicates that while there are similarities between the degradation of Matα1 and Matα2, there are also distinct differences. Like Matα1, degradation of Matα2 requires Ubc4 and Ubc5 (Chen et al., 1993). However, unlike Matα2, no effect is observed with regards to Matα1 turnover unless both of these nearly identical ubiquitin-conjugating enzymes are removed (Figure II-4A). An additional commonality can be found in that degradation of both transcription factors requires the heterodimer Slx5-Slx8, though degradation of Matα2 does not require any part of the sumoylation pathway (Y. Xie and M. Hochstrasser, personal communication).

Degradation of Matα2 requires a second pathway: Ubc6 and Ubc7 via Doa 10 (Chen et al., 1993; Swanson et al., 2001). This pathway is not required for proteolysis of Matα1 (Figure II-6). Degradation of Matα1 utilizes several distinct factors from Matα2. The ubiquitin ligase for the ubiquitin fusion degradation pathway known to function with Ubc4 (Johnson et al., 1995; Ju et

al., 2007), Ufd4, is required for turnover of Matα1 (Figure II-5A), but has not been identified for Matα2. Additionally, the ubiquitin conjugating enzyme Ubc13 and the Rnf8 orthologs Dma1 and Dma2 (Loring et al., 2008; Plans et al., 2006) are not necessary for turnover of Matα2, but are required for rapid removal of Matα1 (Figure II-4B, 8).

Further investigation into how the similar Ubc4 and Ubc5 pathway via Slx5-Slx8 are recognizing and targeting Matα1 and Matα2 could shed light onto the possible coordinated degradation of these master regulators. Are they being recognized at a similar location (i.e. on DNA)? Are they being recognized by a similar degradation signal?

Methods

Plasmids

Information regarding plasmid construction can be found in the appendix (Table 1).

Yeast Strains

Information regarding strain construction can be found in the appendix (Table 2).

Pulse-chase and TCA lysis

Cells were grown to mid log phase (OD_{600} 0.5 - 1.0) in a minimal media lacking the appropriate amino acid for plasmid selection. Approximately 10 cell

ODs were harvested by centrifugation (3000rpm for 5 minutes at room temperature). Cells were washed twice with 1ml of wash buffer (1X SD, 2% glucose, no amino acids) and transferred to 1.5mL Eppendorf tubes. Cells were resuspended in 200μL of minimal media lacking methionine and the amino acid necessary for plasmid selection. The tubes were put into a dry bath at 30°C. Subsequently, 100-200μCi of [35S] Trans-Label (MP Biomedicals and PerkinElmer) was added to each tube. Cells were allowed to label for 10 minutes keeping them suspended by periodic vortexing. Cells were spun for 10 seconds in a microfuge to pellet them. The supernatant was removed and 200μL of chase buffer (1X SD, 2% glucose, 10mM methionine, 0.5mg/ml cycloheximide, and the required amino acids). Immediately following addition of the chase buffer, 50μL of cells were transferred to an Eppendorf tube containing 50μL of stop buffer (40mM methionine, and 20mM NaN_3) on ice. This step was repeated for each subsequent time point. After the time course was finished each sample was washed one time with ice-cold dH_2O.

Protein was extracted using a TCA lysis as previously described (Loayza et al., 1998). Samples were normalized within a time course by determining the TCA precipitable counts and transferring the normalized volumes to fresh 1.5mL Eppendorf tubes on ice. To immunoprecipitate 3HA tagged Matα1, 4μL of 25 mg/mL HA antibody (12CA5, Roche) was added to each sample and mixed by inversion at 4°C overnight. Samples were then spun down (13Krpm for 15 minutes at 4°C) to remove any non-specific aggregates, and supernatants were transferred to fresh tubes containing 20μL of a 50% slurry of Protein A-agarose

(Repligen). Samples were rotated end-over-end at 4°C to keep beads in suspension for 1-2 hours. Samples were washed 3-4 times with TCA dilution buffer plus 0.1% SDS. Following the last wash, 50μL of 2X Laemmli loading buffer was added and samples were boiled for 3 minutes.

To visualize, the entire sample was loaded on a large format 0.75mm SDS-PAGE (10-12%). After running overnight, the gel was fixed in 10% MeOH/ 10% acetic acid, placed on Whatman paper, and dried in a gel dryer. The gel was then exposed to a phosphoimager screen overnight and analyzed using a Typhoon.

Cycloheximide chase and NaOH/SDS lysis

Cells were grown to mid log phase (OD_{600} 0.5 -1.0) in minimal media lacking the appropriate amino acid for plasmid selection. Cycloheximide was added to a final concentration of 0.2 mg/mL from a stock of 10 mg/mL in dH_20 to each culture. Immediately after cycloheximide addition, 2.5 ODs of cells were harvested and put in a 50mL conical tube containing 10mL of ice-cold dH_20 and 30mM NaN_3 and placed on ice. Cells were harvested in this way for each subsequent time point.

Cells were collected by centrifugation (3000rpm for 5 minutes at 4°C). The supernatants were poured off, and cell pellets were resuspended in 1mL of ice-cold dH_20 and transferred to 1.5mL Eppendorf tubes. Cells were re-pelleted by centrifugation (13K for 1 minute at room temperature). The supernatant was aspirated off, and the pellet was resuspended in 200μL of 0.1M NaOH and

allowed to incubate at room temperature for 5 minutes. Cells were again collected by centrifugation (13K for 1 minute at room temperature). The supernatant was removed and the pellet was resuspended in 50μL of 1X Laemmli Loading Buffer and boiled for 3 minutes (Kushnirov, 2000).

Samples were then spun down (13K for 3 minutes at room temperature) and 1 OD was loaded of each sample (unless otherwise noted) onto a large format 1.5 mm SDS-Page (10-12%). After running overnight, gels were transferred and visualized by Western blotting. Where indicated a cycloheximide chase was performed followed by an immunoprecipitation of the HA tag which was conducted as described for pulse-chase experiments. These samples were also visualized by Western blotting.

Western blotting

To visualize an SDS-PAGE by Western blotting, gels were transferred to a nitrocellulose membrane (Immobilin PVDF) in 1X Transfer Buffer diluted from a 10X stock (250mM Tris-OAc, pH 8.8, 2M glycine, and 0.1% SDS) and 20% methanol at 20 volts for 1.5 hours. The membrane was incubated on a rocker in 2% milk in TBST (20mM Tris-HCL, pH 7.6, 150mM NaCl, 0.1% Tween) for at least 1 hour at room temperature. Primary was added at a dilution of 1:1000 (25 mg/mL α-HA, 12CA5, Roche) to 1% milk in TBST and incubated on a rocker overnight at 4°C. The membrane was washed 3 times for 5 minutes each with TBST and then incubated on a rocker at room temperature for 30 minutes to 2 hours in Seablock (Pierce Chemical) with Qdot 605 α-mouse diluted 1:1000

(Invitrogen). The membrane was then washed 3 times for 5 minutes each with 3X TBST (as described above, except 0.3% Tween instead of 0.1%) and 2 times for 5 minutes each with TBST. The membrane was then visualized and subsequently analyzed using a Typhoon and ImageQuant software. Data was normalized to the zero time point within individual experiments to determine percents remaining. Replicates were then averaged.

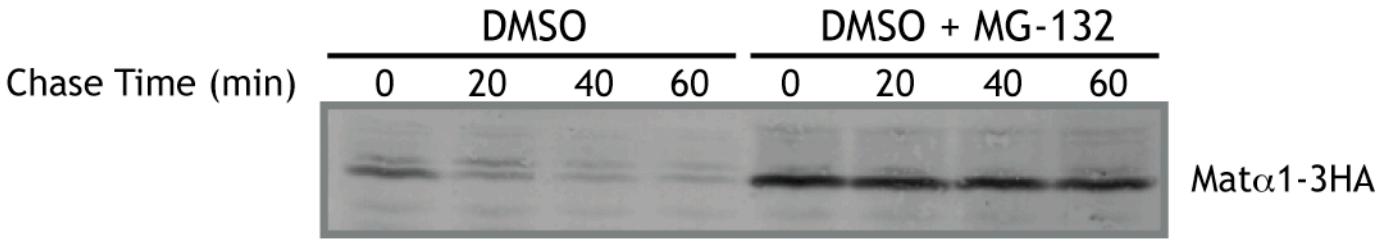
Chemical inhibition of the proteasome

To inhibit the proteasome, I treated growing cultures of JY470 (*pdr5Δ*) expressing p414ADH-α1-3HA with 25μg/mL of MG-132 (Biomol International) dissolved in DMSO for 30 minutes at 30°C. Control cultures were treated with DMSO alone (Gardner et al., 2005; Lipford and Deshaies, 2003). Following treatment, I continued with a cycloheximide chase as described above.

Treatment of strains containing temperature-sensitive alleles

All temperature-sensitive strains were grown at 30°C initially and then shifted to 37°C for one hour. Following the temperature shift, I proceeded with either a cycloheximide chase or a pulse-chase experiment as described above, except for strains containing the *uba1-204* allele. These strains were kept at 37°C throughout the lysis protocol to prevent reactivation of the allele as previously described (Ghaboosi and Deshaies, 2007). Wild type control strains were subjected to the same conditions as the temperature sensitive strains.

A.


DMSO
DMSO + MG-132
Chase Time (min)
0
20
40
60
0
20
40
60
Matα1-3HA

B.

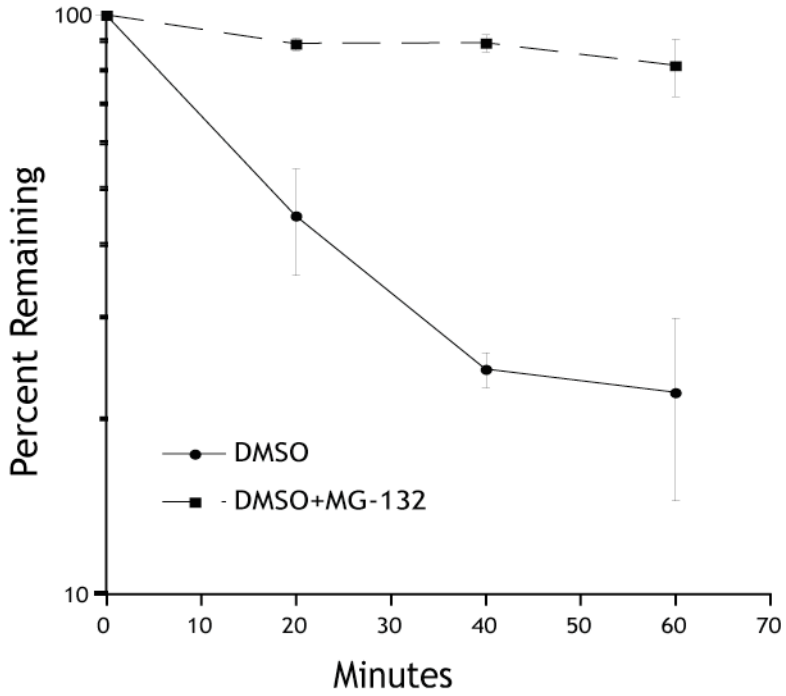

100
10
Percent Remaining
DMSO
DMSO+MG-132
0
10
20
30
40
50
60
70
Minutes

Figure II-1. Matα1 is stabilized in the presence of the proteasome inhibitor MG-132. A. Representative cycloheximide chase experiment in which cells (*pdr5Δ* - JY470) expressing p414ADH-α1-3HA were treated with DMSO alone or with DMSO containing 25μg/mL MG-132. In cells treated with MG-132 the signal for Matα1 persists throughout the time course. B. Quantification of multiple cycloheximide chase experiments showing the percent of Matα1 remaining at each time point.

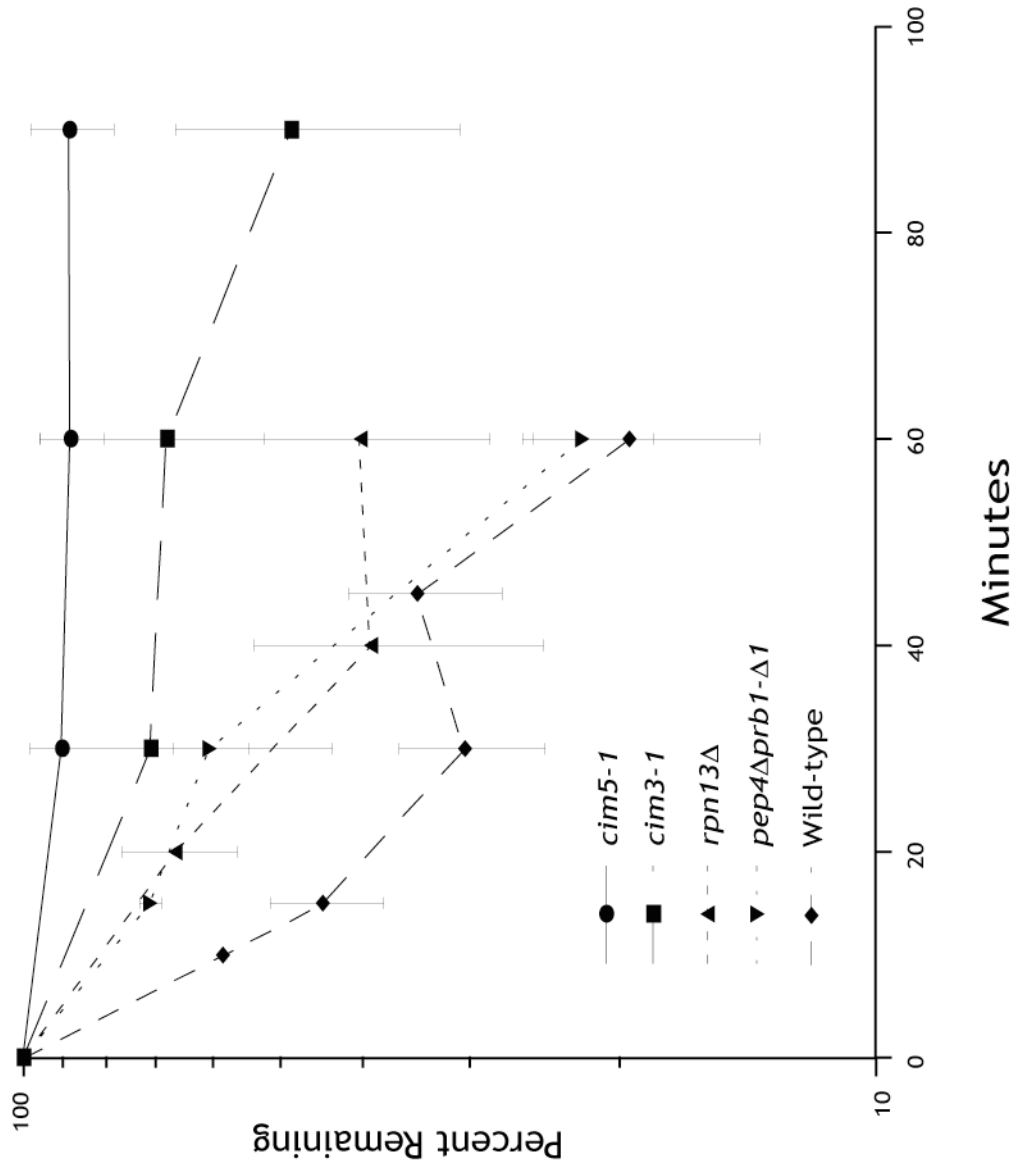

Percent Remaining
100
10
0
20
40
60
80
100
Minutes
cim5-1
cim3-1
rpn13Δ
pep4Δprb1-Δ1
Wild-type

Figure II-2. Proteasome mutants stabilize Matα1. Quantification of multiple cycloheximide chase experiments showing the percent of Matα1 (p413ADH-α1-3HA) remaining at each time point. *cim5-1*(JY142) and *cim3-1* (JY138)are temperature sensitive mutants of Rpt1 and Rpt6, respectively. *pep4Δprb1-Δ1* (MHY1061) is deficient in protein turnover mediated by vacuolar proteases.

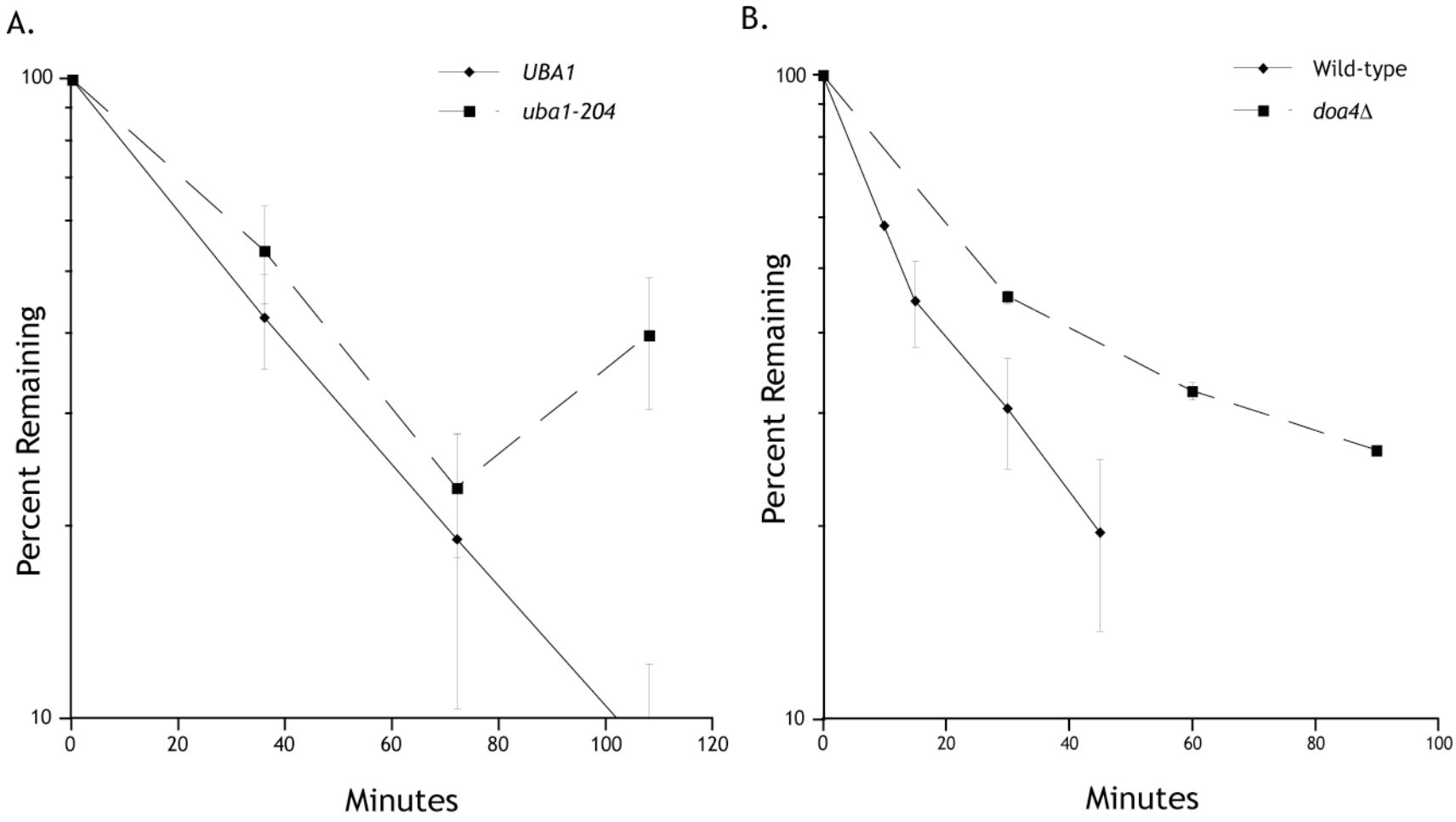

A.
100
10
Percent Remaining
UBA1
uba1-204
0
20
40
60
80
100
120
Minutes
B.
100
10
Percent Remaining
Wild-type
doa4Δ
0
20
40
60
80
100
Minutes

Figure II-3. Matα1 turnover is ubiquitin-dependent. A. Quantification of cycloheximide chase experiments in strains containing *UBA1* (RJD3268) and *uba1-204* (RJD3269) showing the percent of Matα1 (p414ADH-α1-3HA) remaining at each time point. Time-courses for both strains were performed at 37°C following a one-hour incubation at this temperature. B. Quantification of cycloheximide chase experiments in *doa4Δ* (MHY623) showing the percent of Matα1 (p414ADH-α1-3HA) remaining at each time point. This strain is slow growing due to the mutation and overall protein expression levels are decreased. For cycloheximide chase experiments using the *doa4Δ* strains, three cellular ODs per time point were visualized and quantified.

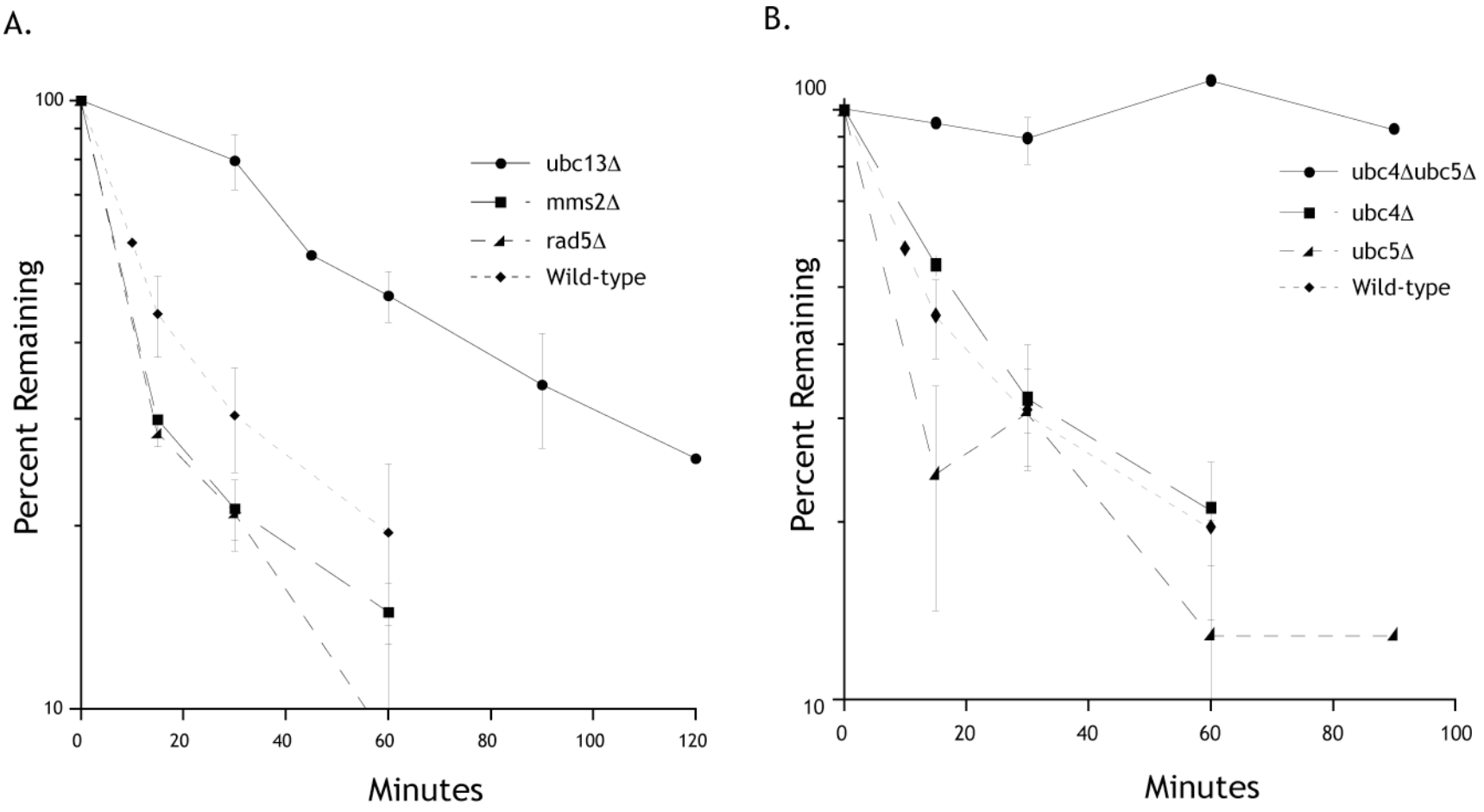
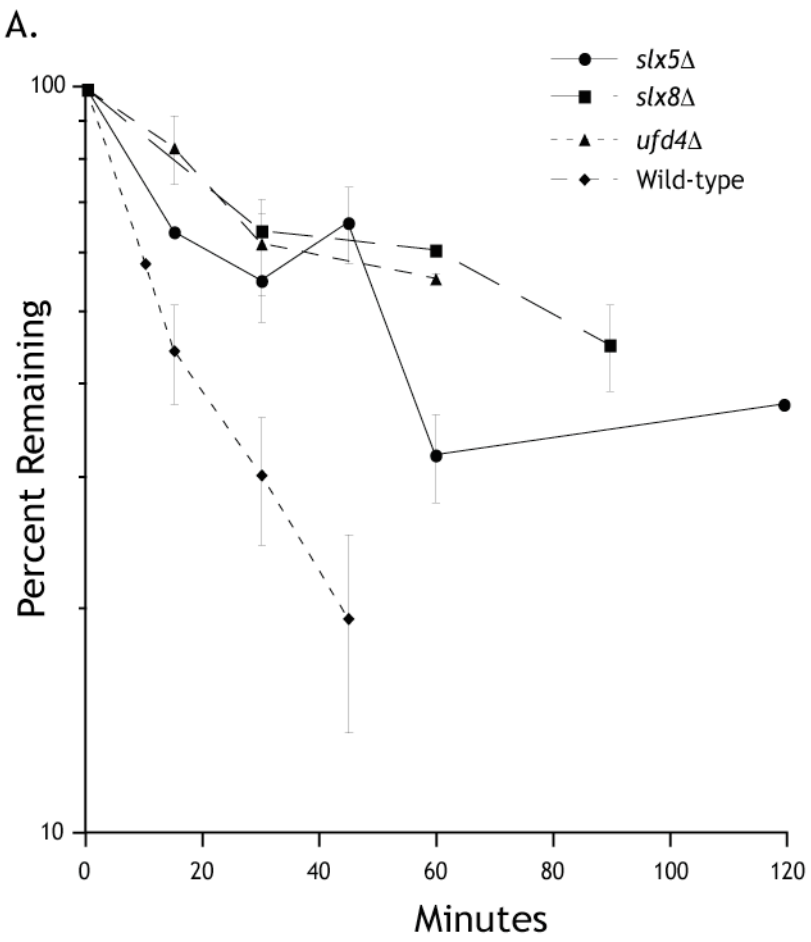

A.
100
10
Percent Remaining
0
20
40
60
80
100
120
Minutes
ubc13Δ
mms2Δ
rad5Δ
Wild-type
B.
100
10
Percent Remaining
0
20
40
60
80
100
Minutes
ubc4Δubc5Δ
ubc4Δ
ubc5Δ
Wild-type

Figure II-4. Multiple ubiquitin-conjugating enzymes are involved in Matα1 proteolysis. A. Quantification of cycloheximide chase experiments in *ubc13Δ* (JY389), *mms2Δ* (JY507), and *rad5Δ* (JY527) strains showing the percent of Matα1 (p414ADH-α1-3HA) remaining at each time point. B. Quantification of cycloheximide chase experiments in *ubc4Δubc5Δ* (JY597), *ubc4Δ* (MH498), and *ubc5Δ* (MHY499) showing the percent of Matα1 (p414ADH-α1-3HA) remaining at each time point.


A.
slx5Δ
slx8Δ
ufd4Δ
Wild-type
100
10
Percent Remaining
0
20
40
60
80
100
120
Minutes

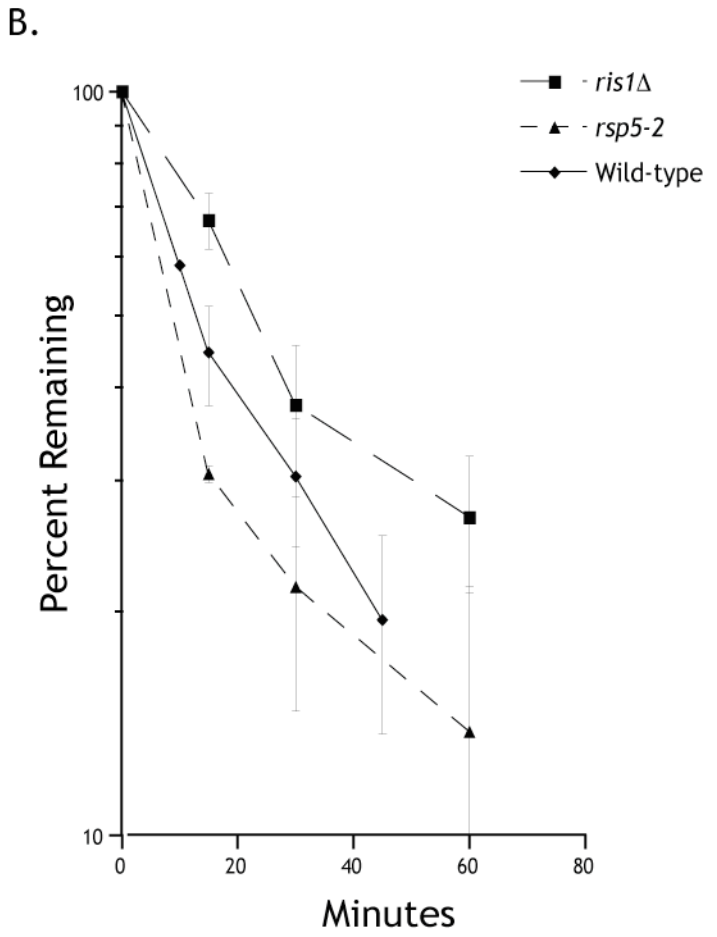

B.
ris1Δ
rsp5-2
Wild-type
100
10
Percent Remaining
0
20
40
60
80
Minutes

Figure II-5. Several Ubc4-dependent ubiquitin ligases mitigate degradation of Matα1. A. Quantification of cycloheximide chase experiments in *slx5Δ* (MHY3712), *slx8Δ* (MHY3716), and *ufd4Δ* (JY612) showing the percent remaining of Matα1 (p414ADH-α1-3HA) at each time point. B. Quantification of cycloheximide chase experiments in *ris1Δ* (JY680), and *rsp5-2* (MHY2480) showing the percent remaining of Matα1 (p414ADH-α1-3HA) at each time point.

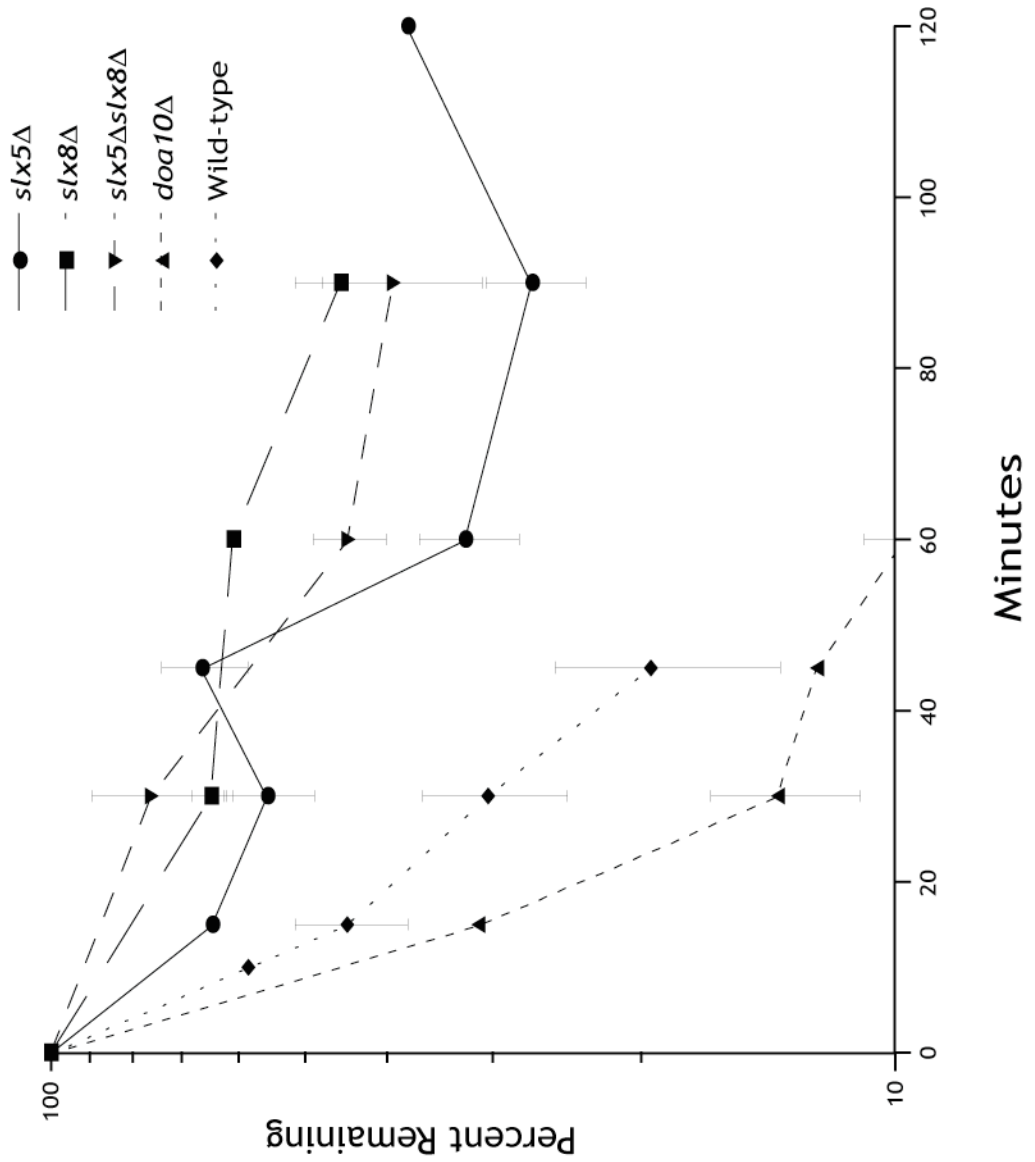

slx5Δ
slx8Δ
slx5Δslx8Δ
doa10Δ
Wild-type
Percent Remaining
100
10
0
20
40
60
80
100
120
Minutes

Figure II-6. The heterodimer Slx5-Slx8 is required for Matα1 turnover.

Quantification of cycloheximide chase experiments in *slx5Δ* (MHY3712) *slx8Δ* (MHY3716), *slx5Δ slx8Δ* (MHY3714) and *doa10Δ* (MHY1631) showing the percent remaining of Matα1 (p414ADH-α1-3HA) at each time point.

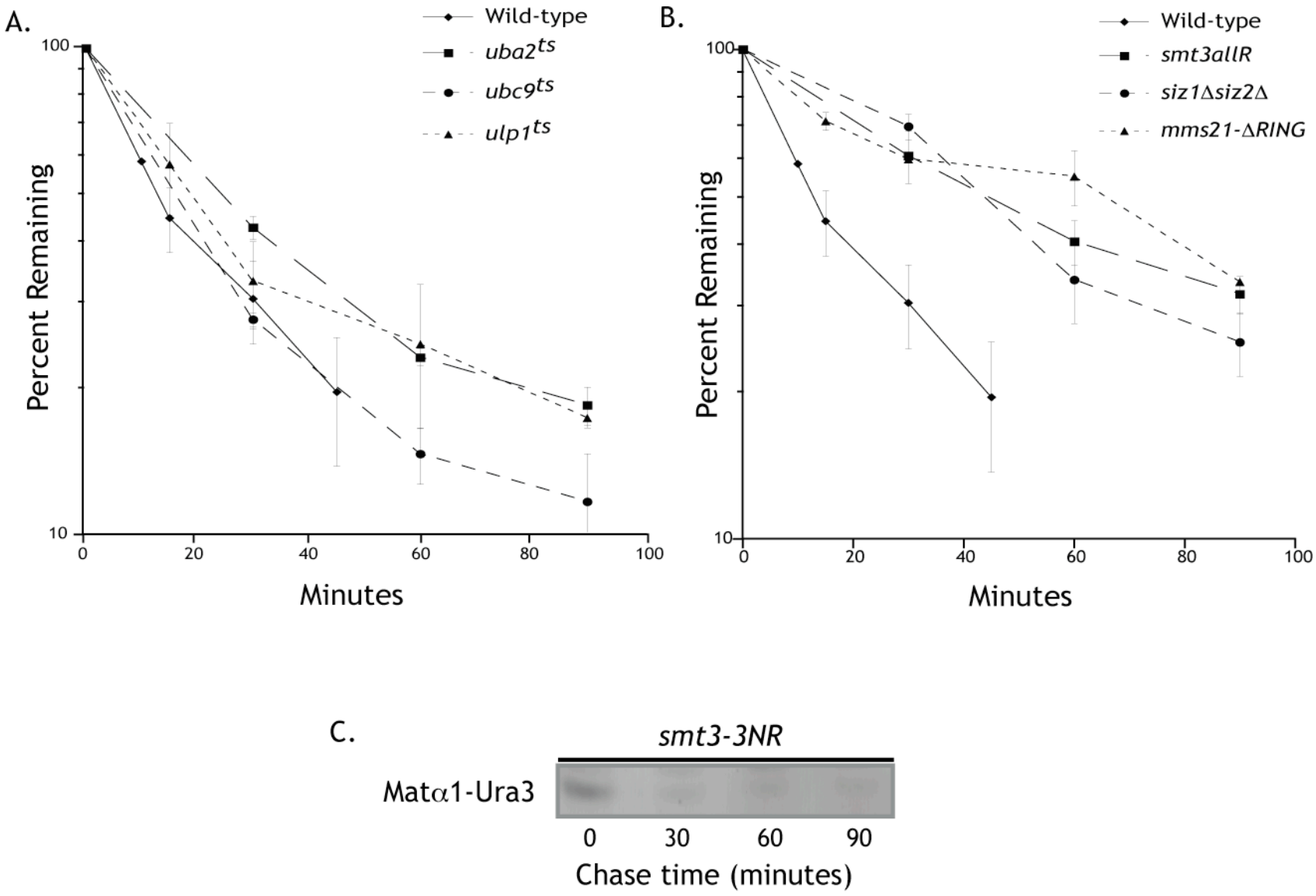

A.
Wild-type
uba2ts
ubc9ts
ulp1ts
Percent Remaining
100
10
0
20
40
60
80
100
Minutes
B.
Wild-type
smt3allR
siz1Δsiz2Δ
mms21-ΔRING
Percent Remaining
100
10
0
20
40
60
80
100
Minutes
C.
smt3-3NR
Matα1-Ura3
0
30
60
90
Chase time (minutes)

Figure II-7. Proteolysis of Matα1 requires an intact Sumoylation pathway. A. Quantification of cycloheximide chase experiments in *uba2*^{ts} (MHY4664), *ubc9*^{ts} (MHY3997) and *ulp1*^{ts} (MHY2423) showing the percent remaining of Matα1 (p413ADH-α1-3HA) at each time point. B. Quantification of cycloheximide chase experiments in *smt3allR* (JY817), *mms21-ringΔ* (JY764), *siz1Δ*siz2Δ (JY802) showing the percent remaining of Matα1 (p413ADH-α1-3HA) at each time point. C. Cycloheximide chase experiment showing the rapid turnover of p413ADH-α1-3HA in *smt3-3NR* (JY818).

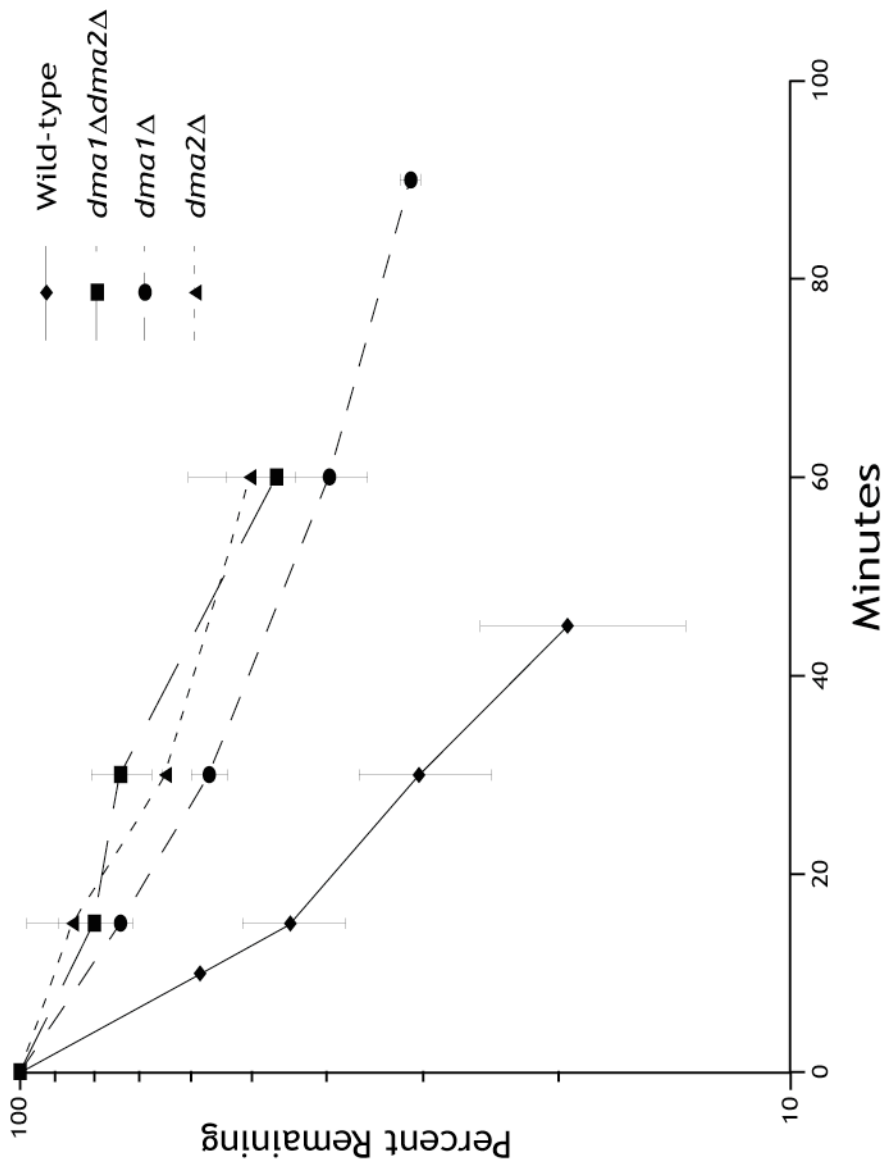

Wild-type
dma1Δdma2Δ
dma1Δ
dma2Δ
Percent Remaining
100
10
0
20
40
60
80
100
Minutes

Figure II-8. Matα1 turnover is dependent upon Rnf8 orthologs, Dma1 and Dma2. Quantification of cycloheximide chase experiments in *dma1Δ* (JY710), *dma2Δ* (JY714), *dma1Δ*dma2Δ (JY706) showing the percent of Matα1 (p414ADH-α1-3HA) remaining at each time point.

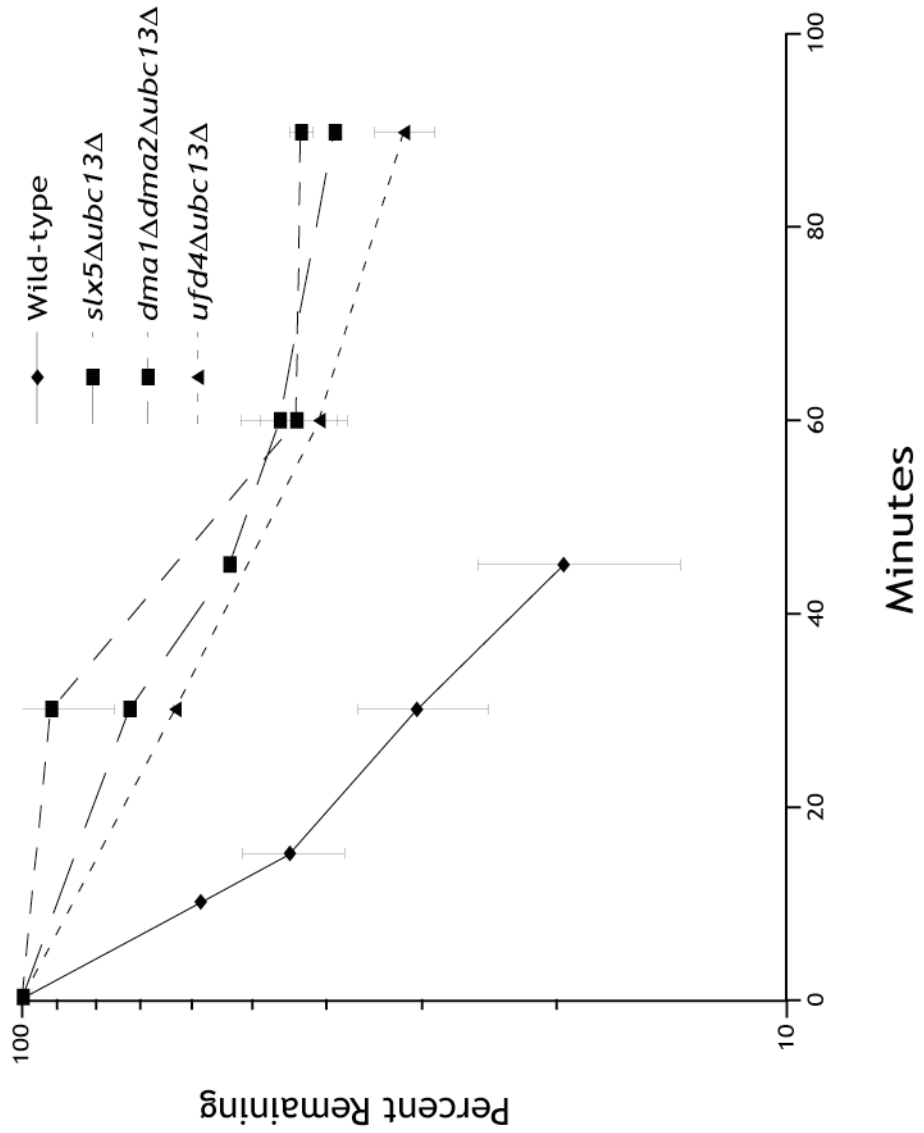

Wild-type
slx5Δubc13Δ
dma1Δdma2Δubc13Δ
ufd4Δubc13Δ
Percent Remaining
100
10
0
20
40
60
80
100
Minutes

Figure II-9. Ubc13 and the ubiquitin ligases responsible for Matα1 turnover are in the same genetic pathway. Quantification of cycloheximide chase experiments in *slx5Δubc13Δ* (JY642), *ufd4Δubc13Δ* (JY734), *dma1Δ*dma2*Δubc13Δ* (JY753) showing the percent remaining of Matα1 (p414ADH-α1-3HA) at each time point.

Strain name	Half Life	Genotype
MHY501	1	Wild-type
MHY3712	3	*slx5Δ*
MHY3716	3	*slx8Δ*
JY612	3	*ufd4Δ*
JY710	3	*dma1Δ*
JY714	3	*dma2Δ*
MHY3861	3	*slx5Δslx8Δ*
JY751	3	*slx5Δufd4Δ*
JY743	3	*slx8Δufd4Δ*
JY706	3	*dma1Δdma2Δ*
JY798	#	*dma1Δslx8Δ*
JY788	3	*dma1Δslx5Δ*
JY794	6	*dma1Δufd4Δ*
JY800	3	*dma2Δslx8Δ*
JY790	3	*dma2Δslx5Δ*
JY792	3	*dma2Δufd4Δ*
JY745	2	*ufd4Δdma1Δdma2Δ*
JY739	6	*slx5Δdma1Δdma2Δ*
JY768	6	*ufd4Δslx5Δdma1Δ*
JY776	6	*ufd4Δslx8Δdma2Δ*
JY774	*6	*ufd4Δslx8Δdma1Δ*
JY796	3	*slx8Δdma1Δdma2Δ*
JY770	3	*ufd4Δslx5Δdma2Δ*
JY772	6	*ufd4Δslx5Δdma1Δdma2Δ*
JY778	*3	*ufd4Δslx8Δdma1Δdma2Δ*

Figure II-10. Summary of the fold changes in Matα1 stability observed in strains deficient for one or more ubiquitin ligases. Half-life: 1= Wild type (approximately 15 minutes), 2= two-fold (approximately 30 minutes), 3= three-four-fold (approximately 45-60 minutes), and 6= six-fold (approximately 90 minutes). # - no data due to weak signal, * - large error between individual experiments.

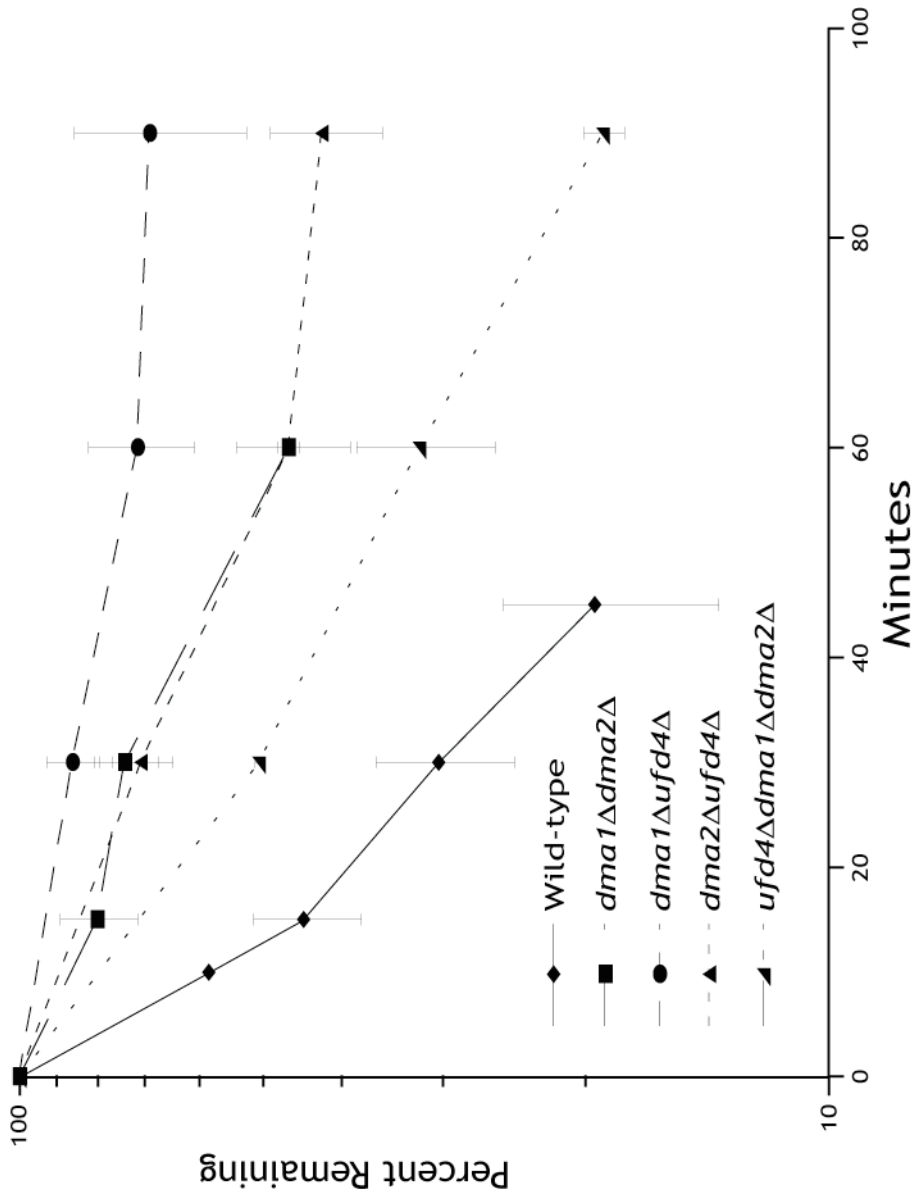

Percent Remaining
100
10
0
20
40
60
80
100
Minutes
Wild-type
dma1Δdma2Δ
dma1Δufd4Δ
dma2Δufd4Δ
ufd4Δdma1Δdma2Δ

Figure II-11. Ufd4 and Dma1 function in parallel pathways. Quantification of cycloheximide chase experiments in *dma1Δufd4Δ* (JY794), *dma2Δufd4Δ* (JY792), *dma1Δ*dma2*Δ* (JY706), and *ufd4Δdma1Δ*dma2*Δ* (JY745) of Matα1 (p414ADH-α1-3HA) showing the percent remaining at each time point.

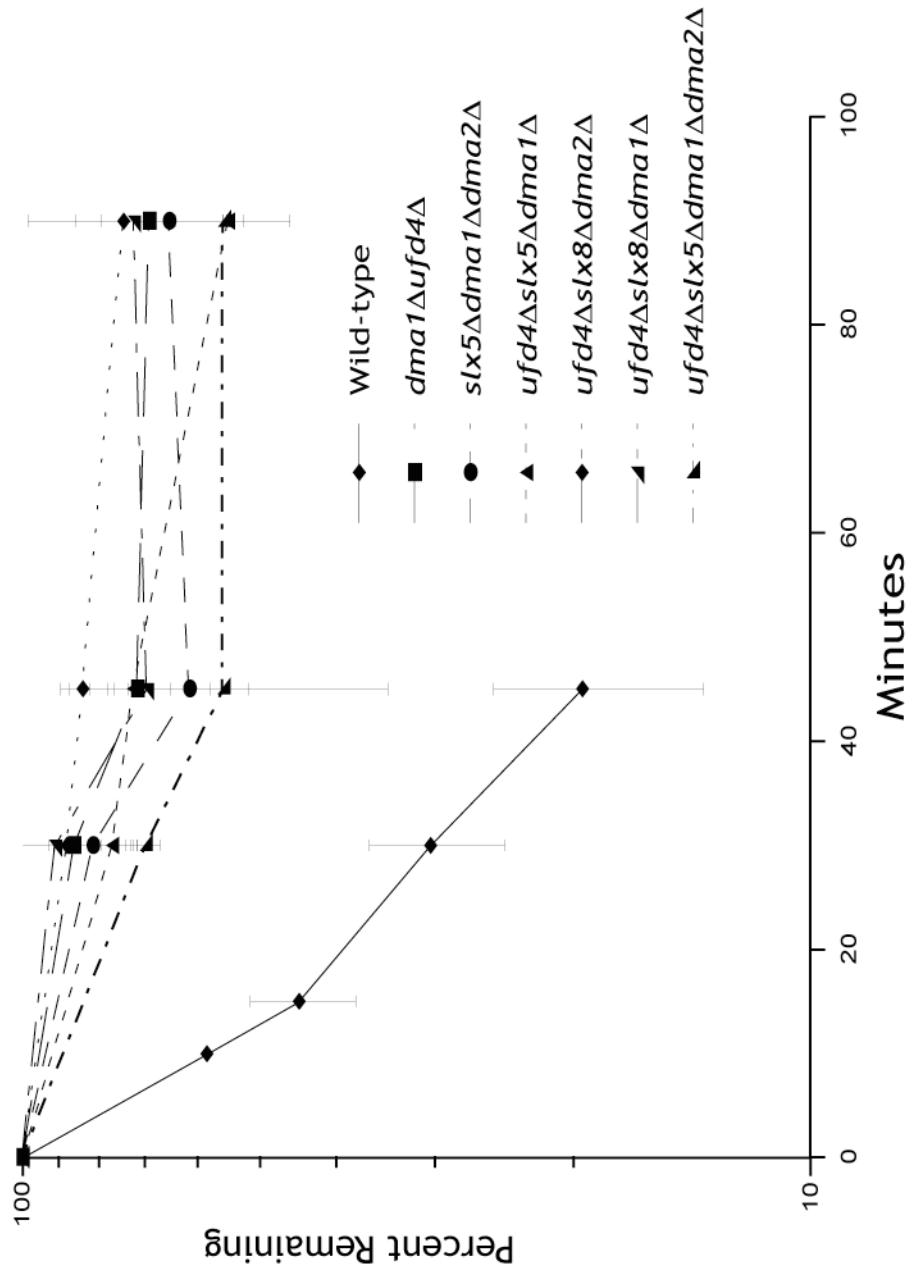

Percent Remaining
100
10
0
20
40
60
80
100
Minutes
Wild-type
dma1Δufd4Δ
slx5Δdma1Δdma2Δ
ufd4Δslx5Δdma1Δ
ufd4Δslx8Δdma2Δ
ufd4Δslx8Δdma1Δ
ufd4Δslx5Δdma1Δdma2Δ

Figure II-12. Matα1 is stabilized six-fold in mutants lacking two or more ubiquitin ligases. Quantification of cycloheximide chase experiments in *dma1Δufd4Δ* (JY794), *slx5Δdma1Δdma2Δ* (JY739), *ufd4Δslx5Δdma1Δ* (JY768), *ufd4Δslx8Δdma2Δ* (JY776), *ufd4Δslx8Δdma1Δ* (JY774), and *ufd4Δslx5Δdma1Δdma2Δ* (JY772) showing the percent remaining of Matα1 (p414ADH-α1-3HA) at each time point.

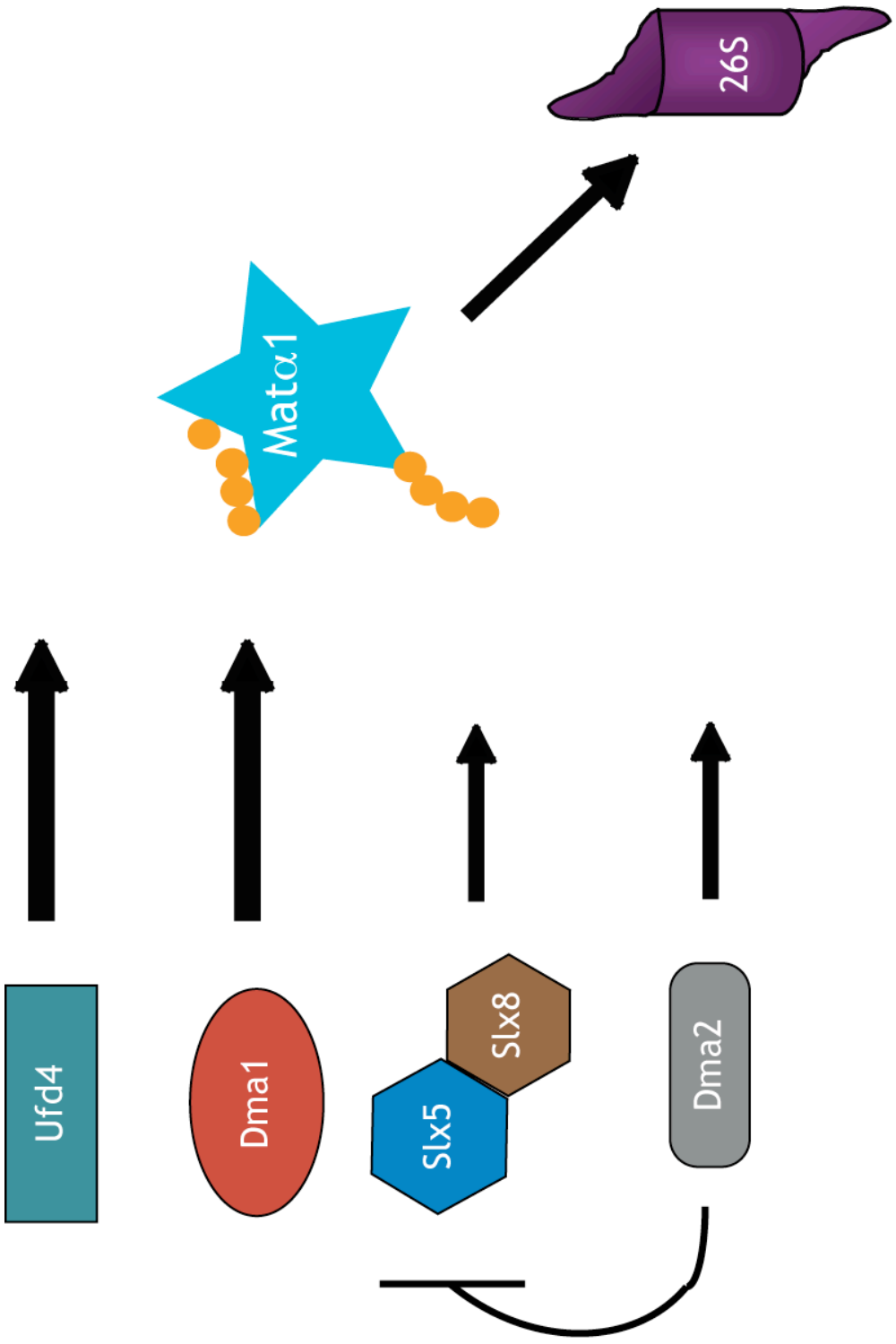

26S
Matα1
Ufd4
Dma1
Slx8
Slx5
Dma2

Figure II-13. Proposed model for the proximal degradation pathway of Matα1. In this model, all four ubiquitin ligases identified in this study are acting in parallel pathways to ubiquitylate Matα1. This ubiquitylation will continue until a certain threshold has been reached in order to target it to the proteasome for destruction. In addition to its positive role in Matα1 proteolysis, Dma2 is also hypothesized to be negatively regulating Slx5-Slx8.

Chapter III

Characterization of Degradation Signals within Matα1

Abstract

The a and α cell types present in yeast are determined by transcription factors encoded at the MAT locus: Mata1, Matα1, and Matα2. Yeast cells can switch between these two phenotypes suggesting that the mating type regulators must be rapidly inactivated. In fact, all three are unstable and both Matα1 and Matα2 are known to be substrates for the ubiquitin-proteasome system (UPS). Matα1, in particular, is targeted to the proteasome by multiple ubiquitin-conjugating enzymes and ubiquitin ligases. It is not known what characteristics of Matα1 are recognized by these enzymes to make it a target for proteolysis as there are no sequence motifs within the transcription factor corresponding to known protein degradation signals. Here, I identify multiple regions that are necessary for rapid turnover of Matα1. Furthermore, residues 75-150 are required for Slx5-Slx8-dependent degradation of Matα1.

Introduction

Haploid yeast cells are capable of switching their mating-type at each cell cycle (Hicks and Herskowitz, 1977; Strathern et al., 1979a; Takano et al., 1973). Inactivation of the transcription factors (Matα1, Matα2, and Mata1) that engender each cell type is necessary for this phenotypic switch to occur. All three of these master regulators have been shown to be short-lived proteins (Hochstrasser and Varshavsky, 1990; Johnson, 1997). For example, Matα2 is degraded by the ubiquitin-proteasome pathway and its degradation is necessary for efficient mating-type switching to occur (Hochstrasser and Varshavsky,

1990; Laney and Hochstrasser, 2003). Additionally, I found that the UPS is responsible for the rapid removal of Matα1, and that it is targeted for degradation by multiple ubiquitin-congjugating enzymes and ubiquitin ligases including the SUMO-dependent ligase Slx5-Slx8 (Chapter II).

Much research has focused on describing features within a protein that signal its turnover. Specific regions or signals that confer instability have been identified in many proteins. These regions, often called degrons, can be defined either by amino acid sequence or structure. Additionally, degrons are not necessarily sites of ubiquitylation. They are the site or region of a protein that is recognized by the ubiquitylation machinery.

For example, the G1 cyclin Cln2 has a 37 base pair consensus sequence known as a PEST sequence, which is necessary for its rapid turnover (Salama et al., 1994). Another consensus sequence called the cyclin destruction box is conserved in many cyclins (Glotzer et al., 1991; King et al., 1996). Degradation can also be signaled by the presence of a particular amino acid at the extreme N-terminus of a protein. Amino acids which qualify for this N-end Rule are Glu, Gln, Asp, Asn, Ile, Leu, Phe, Trp, Tyr, His, Lys, Arg (Bachmair et al., 1986; Bachmair and Varshavsky, 1989). Several other types of degradation signals have been described such as the phosphorylation dependent signal found in Sic1, an inhibitor of the S phase B-type cyclin (Clb)-cyclin dependent kinase (S-CDK), which requires precisely six phosporylation events before recognition by the ubiquitin ligase SCF^{Cdc4} can occur. Matα2 contains a degron defined by structure. This degron, called Deg1, is recognized by the ubiquitin ligase

Doa10 via the hydrophobic face of an amphipathic helix (Johnson et al., 1998; Swanson et al., 2001).

No degradation signal, defined by either structure or sequence, has been characterized for the yeast transcription factor Matα1. Also, there is no sequence within Matα1 to suggest a conserved degradation signal like the PEST sequence or cyclin destruction box. Since degradation signals can be defined by a variety of characteristics, such as sequence or structure, I took two different approaches. I used PCR-mediated mutagenesis (Muhlrad et al., 1992) and analysis of a series of nested truncations to determine the nature of the degradation signal that targets Matα1 for rapid proteolysis. I found that more than one region of Matα1 is necessary for its turnover. In particular, 75 amino acids within the C-terminal half of Matα1 are targeted for degradation by Slx5-Slx8.

Results

PCR-mediated mutagenesis

Matα1 fused to Ura3 is sufficient to target this normally stable uracil biosynthetic enzyme for rapid proteolysis (Figure III-1A). Matα1-Ura3 is unable to support growth in the absence of uracil consistent with it being a short-lived protein (Figure III-1B). In a strain (*doa4Δ*) with decreased cellular levels of ubiquitin and therefore an impaired UPS, the fusion protein can support growth on media lacking uracil suggesting that it is stabilized (Figure III-1B). This result is surprising given that biochemical analysis of unfused Matα1 in a doa4Δ

mutant strain are only mildly stabilized (Figure II-3). Perhaps the strength of the growth phenotype observed in Figure III-B is due not only to the poor growth phenotype of the strain, but also to the strength of Matα1 stabilization. Moreover, Matα1-Ura3 is a stabilized in an slx8Δ mutant strain demonstrating that the pathway targeting wild-type Matα1 is also targeting the Matα1-Ura3 fusion protein (Figure II-5A, III-1C). These results confirmed that Matα1 fused to Ura3 could serve as a reagent for characterizing the degradation signals within Matα1.

I utilized PCR-mediated mutagenesis (Muhlrad et al., 1992) to ask whether specific amino acids within Matα1 are responsible for its rapid turnover. While wild-type Matα1-Ura3 fusions are not sufficient to support growth on media lacking uracil (because the fusion is rapidly degraded), mutations that stabilized Matα1 should grow on this media (Figure III-1). Using this selection procedure, I isolated several candidate amino acid changes that were potentially altering the half-life of Matα1 based on their ability to grow on media lacking uracil using this method (Figure III-2A). However, I observed no deviation from the wild-type half-life for any of the mutants by pulse-chase (data not shown) suggesting that the stability of these fusions was not altered. Therefore, the growth observed on plates could be due to a change in the mutant Matα1's expression levels allowing the cell to overcome the rapid degradation of the fusion and produce enough uracil to survive. The lack of true positives may also indicate that a single amino acid change is not sufficient to alter the stability of Matα1.

Nested truncations

Since mutagenesis did not yield positive results, I also created and analyzed deletion mutants of Matα1. A series of truncations were made based on secondary structure predictions in order to preserve the native fold of Matα1. Each mutant, lacking a stop codon, was fused to Ura3. Depending on where the deletion was made, the truncation would offer information on whether a particular region is necessary or if it is sufficient for turnover of Matα1.

The deletion mutants were first tested for growth on media lacking uracil. Deleting the C-terminal half of the protein (Δ93-175) yielded a derivative that was degraded like the full-length protein, as indicated by its lack of growth on media lacking uracil. However, removing an additional 35 amino acids (Δ58-175) stabilized the fusion protein, since robust growth on this media was observed. Further deletions (Δ28-175) also resulted in a presumably stable Matα1-Ura3 fusion protein (Figure III-2C). These results imply that those 35 residues removed from Δ93-175 (58-92) are required for turnover of Matα1.

Progressive truncations from the N-terminus of Matα1 (Δ2-106 and Δ2-139) continued to be sufficient for degradation of the Matα1-Ura3 fusion protein as indicated by the lack of growth in the absence of uracil. In contrast, a small, 32 amino acid fragment containing residues 107-139 (Δ2-106; Δ140-175) can support growth under these conditions, indicating that these amino acids are not sufficient for targeting substrates for degradation. Taken together, these results suggest that the C-terminal 35 residues of Matα1 (140-

175) contain a degron both necessary and sufficient for directing turnover of Ura3 (Figure III-2B).

The truncations were then analyzed by pulse-chase experiments in wild-type cells, which measures the degradation of newly-synthesized protein, to verify that the growth phenotypes observed on media lacking uracil are due to changes in protein degradation. Consistent with the plating assays described above, the data obtained showed that removing half of Matα1 from the C-terminus (Δ93-175) did not stabilize the protein. Removing an additional 35 amino acids (Δ58-175) stabilized the fusion to Ura3 approximately three-fold (Figure III-3, 4). This biochemical analysis supports the conclusion from the plating assays that amino acids 58-92 are necessary for rapid degradation of Matα1.

Additionally, half of Matα1 could be removed from the N-terminus (Δ2-107) without significantly altering the degradation kinetics of Ura3 as indicated by pulse-chase analysis (Figure III-3, 5). Deletion of another 32 residues yielded the smallest N-terminal truncation analyzed containing only the last 35 amino acids of Matα1 (Δ2-139). This fragment was still sufficient to direct turnover of Ura3 as suggested by the growth phenotypes on media lacking uracil (Figure III-5, 2B). The 32 amino acids removed to create Δ2-139 (Δ2-106; Δ140-175), however, are not sufficient to target Ura3 for proteolysis (Figure III-5). These results demonstrate that residues the C-terminus of Matα1 (residues 140-175) are both necessary and sufficient for to confer instability on the normally stable protein Ura3 (Figure III-3).

I also analyzed an additional 75 amino acid fragment from the middle of Matα1. This fragment contains residues 75-150 (Δ2-74; Δ151-175) of Matα1 and is not sufficient for directing turnover of Ura3 (Figure III-5). This truncation further narrows the residues necessary for proteolysis of Matα1. Together with the data from the progressive N-terminal and C-terminal truncations, this data suggests that regions amino acids 58-74 and 150-175 are required for turnover of newly synthesized Matα1 (Figure III-3).

Slx5-Slx8-dependent Degradation Signal

The truncations were also analyzed by cycloheximide chase, which reports on the steady-state pool of protein as opposed to monitoring newly-synthesized protein with pulse-chase experiments. Analysis by cycloheximide chase also allows the truncations to be characterized in strains mutant for ubiquitin ligases required for turnover of the steady-state pool and not the turnover of newly-synthesized Matα1 (Chapter II). While I have identified regions necessary and sufficient for targeting the normally stable Ura3 for degradation by pulse-chase analysis, I do not know if these truncations are subject to degradation pathways that are physiologically relevant to Matα1. They are potentially mis-folded proteins that are recognized for destruction by quality control pathways. Testing the deletion mutants by cycloheximide chase will determine if the truncation is biologically relevant to wild-type Matα1, and it will potentially connect a degradation signal with a ubiquitin ligase pathway.

First, I used steady-state analysis (cycloheximide chase) to characterize the degradation of the Matα1 deletion mutants. Then, I analyzed unstable truncations in cells mutant for either Slx5 or Slx8, which form a heterodimer that is categorized as a SUMO-targeted ubiquitin ligase (Wang et al., 2006; Yang et al., 2006). A truncation removing approximately half of Matα1 (Δ93-175), which was sufficient for targeting Ura3 for degradation by pulse-chase, was undetectable by this steady-state evaluation, suggesting that this fragment is so rapidly degraded that it does not accumulate to observable levels. Furthermore, removing the next 35 residues (Δ58-175) did not stabilize the steady-state pool of the Ura3 fusion protein as it did for the newly-made pool. I did not observe stabilization until I removed another 20 residues (Δ28-175), indicating that the extreme N-terminus of Matα1 is not capable of conferring instability to Ura3 (Figure III-6, 7). This difference observed between evaluation by cycloheximide chase and pulse-chase suggests that residues 28-57 are necessary for turnover of the steady-state pool of Matα1.

The unstable, C-terminal deletion mutants were then tested in either slx5Δ or slx8Δ mutant cells. A truncation containing only the N-terminal half of Matα1 (Δ93-175) was still not detectable in these mutants nor was the truncation containing only the first 57 residues (Figure III-7). These results indicate that turnover of these truncations is not dependent on Slx5-Slx8 and may not be physiologically relevant to the turnover of Matα1 (Figure III-6).

Consistent with the pulse-chase assays I preformed (Figure III-5), I was able to remove half of the protein from the N-terminus (Δ2-71) and still

maintain turnover of Ura3 (Figure III-8). However, the degradation of this deletion mutant is not altered in *slx8Δ* cells, indicating that residues 72-175 are either recognized by a different Matα1-required ubiquitin ligase or as a mis-folded protein by a physiologically irrelevant pathway (Figure III-6). Next, I analyzed a truncation from the middle region of Matα1 lacking the extreme N- and C-termini (Δ2-24; Δ151-175) and two smaller fragments that divided the larger deletion mutant in two (Δ2-24; Δ85-175 and Δ2-74; Δ151-175). The larger fragment (Δ2-24; Δ151-175) and the N-terminal half of this fragment (Δ2-24; Δ85-175) did not produce a reliable signal sufficient for quantification (data not shown). The C-terminal half of the larger truncation (Δ2-74; Δ151-175), which contained 75 amino acids, was stabilized three-fold over wild-type. Interestingly, this truncation was further stabilized in an *slx8Δ* strain (Figure III-8). While amino acids 75-150 of Matα1 are not sufficient to direct Ura3 for proteolysis with the same kinetics as full-length Matα1, these data suggest that the level of turnover that they do imbue is dependent upon Slx5-Slx8. Given that there are multiple ubiquitin ligases required for Matα1 turnover, it is not surprising that a single degradation signal is not sufficient for degradation with the same kinetics as full-length Matα1.

Combining the cycloheximide chase data for the N-terminal and C-terminal truncations suggests that residues 28-74 of Matα1 are necessary for turnover of Matα1 as indicated by stability of the first 27 amino acids of Matα1 fused to Ura3 (Δ28-175) and the 75 amino acids from the C-terminal half of Matα1 (Δ2-74 ; Δ151-175). However, a degradation pathway, not

physiologically relevant to the proteolysis of Matα1, could be recognizing this region (residues 28-74) since neither unstable C-terminal truncation (Δ93-175 nor Δ58-175) is dependent upon Slx5-Slx8. Alternatively, amino acids 28-74 could be recognized by one of the other ubiquitin ligases required for Matα1 degradation. I was unable to test this hypothesis directly as a deletion mutant containing these residues (Δ2-24; Δ85-175) was inexplicably unreliable and inconsistent. Taken together, these results demonstrate that there is more than one region of Matα1 required for targeting it to the proteasome.

Discussion

Matα1, like the other mating-type transcription factors in yeast, is known to be a short-lived protein (Johnson, 1997). I have shown that the UPS degrades Matα1 and that this degradation requires multiple ubiquitin-conjugating enzymes and ubiquitin ligases (Chapter II). Here, I have presented data suggesting that this degradation is directed by more than one *cis*-acting degradation signal within Matα1. Having multiple degradation signals is not unheard of. Matα2 also appears to contain two degrons. The first, Deg1, is defined by its structure, and is recognized by Doa10 (Johnson et al., 1998; Swanson et al., 2001). A second degradation signal is hypothesized to reside within Matα2, but has yet to be fully characterized. The second pathway, Ubc4/Ubc5/Slx5-Slx8, required for Matα2 turnover recognizes this signal (Y. Xie and M. Hochstrasser, personal communication).

For Matα1, analysis of newly-synthesized protein demonstrates that residues 58-92 are necessary to direct the turnover of the normally stable Ura3 protein. In addition, amino acids 140-175 are both necessary and sufficient for targeting Ura3 for destruction (Figure III-3, 4, and 5). Evaluation of the steady-state pool of Matα1 by cycloheximide chase indicates a slightly different region (Figure III-6). Though not verified directly by a truncation containing these amino acids, analysis of the N- and C-terminal truncations indicates that amino acids 28-74 are necessary for turnover of Matα1.

An Slx5-Slx8-dependent degron overlaps with the alpha1 domain

A deletion mutant containing 75 amino acids from the C-terminal half of Matα1 (Δ2-74; Δ151-175) shows stabilization of the Ura3 fusion protein by pulse-chase and cycloheximide chase (Figure III-5, 8). By cycloheximide chase, it is stabilized three-fold over wild-type. Interestingly, this truncation is stabilized to an even greater extent in *slx8Δ* cells, indicating that it contains a degron responsive to the STUbL, Slx5-Slx8 (Figure III-8). Matα1 is targeted to the proteasome by several ubiquitin ligases. To stabilize Matα1 more than three-fold, a combination of these ligases must be removed (Chapter II). It stands to reason that a single degradation signal would not be sufficient to confer the levels of instability observed for wild-type Matα1, which requires multiple pathways, to a normally stable protein.

Residues 75-150 coincide with a region of Matα1 termed the alpha1 domain, because is highly conserved among fungi. The function of this region

is unknown, but is hypothesized to either be the interaction surface between Matα1 and its transcriptional activation partner Mcm1 or Matα1's DNA binding domain (Coppin et al., 1997; Yuan et al., 1993). Could this overlap with a functional domain imply that even while Matα1 is actively engaged, Slx5-Slx8 is able to recognize and target Matα1 for ubiquitylation? Slx8 modulates DNA binding for the Slx5-Slx8 heterodimer conceivably placing it at or near α-specific gene promoters and able to target functionally-engaged Matα1 (Yang et al., 2006). The SUMO-interacting motifs in Slx5 would able to interact with sumoylated Matα1 and further modify it with ubiquitin (Xie et al., 2007). This model would serve to remove active Matα1 from DNA once its function is complete. Using chromatin immunoprecipitation experiments, one could determine if Slx5-Slx8 is present at or near α-specific gene promoters, and if Matα1 occupancy at these promoters is altered in *slx5Δ* or *slx8Δ* cells. Mutational analysis of this region could also yield information about the basis for recognition of Matα1 by Slx5-Slx8. Is recognition mediated by amino acid sequence or secondary structure of residues 75-150? Furthermore, is this region actually sumoylated and, therefore, a target for the sumo interacting motifs within Slx5 (Minty et al., 2000; Xie et al., 2007)?

Additional Degrons

I have identified three segments of Matα1 that are required to confer instability to Ura3. Evaluation of the newly-synthesized pool of Matα1 by pulse-chase indicates that amino acids 140-175 are necessary and sufficient for

turnover of the Ura3 fusion protein. Furthermore, residues 58-92 are necessary to direct turnover of Ura3 (Figure III-3, 4, and 5). Analysis of steady-state protein levels revealed an overlapping region, residues 28-74 are necessary for turnover of the Ura3 fusion protein (Figure III-6, 7, and 8). These deletion mutants could be identified as cellular garbage and destroyed by another pathway, which is not relevant to the turnover of wild-type Matα1. Alternatively, the regions may represent additional degrons that are recognized by one of the other ubiquitin ligases I have identified: Ufd4, Dma1, and Dma2 (Chapter II). The only way to distinguish between these two alternative hypotheses is to analyze the Matα1 truncations in the context of deletions of Ufd4, Dma1, and Dma2.

Further analysis of the truncations would also help to determine if all of the ligases involved in turnover of Matα1 are recognizing the same degron or if they are recognizing distinct degradation signals. Given that the Slx5-Slx8-dependent signal is a region (amino acids 75-150) that already shows some stabilization in wild-type cells, I would argue that each ubiquitin ligase is recognizing its own signal. Moreover, I posit that each signal alone may not be sufficient to direct turnover of a normally stable protein to the levels observed for wild-type Matα1. With this hypothesis in mind, it might be useful to evaluate more closely the deletion mutants that showed varying degrees of stabilization over wild-type by pulse-chase such as the extreme C-terminus of Matα1 (Δ2-139), or the N-terminal fragment containing the transcriptional activation domain (Δ58-175). Expressing theses deletion mutants in cells

mutant for Ufd4, Dma1, or Dma2 could potentially stabilize them further identifiying another ubiquitin ligase-dependent degron.

Important Residues within Matα1

In addition to examining structural regions and domains within Mata1, I also utilized a PCR-mediated mutagenic approach to ask if specific residues were required for Matα1 turnover. Using this plating assay, I identified specific amino acids that may be important (Figure III-2A), but could not be verified by biochemical analysis of newly synthesized protein (data not shown). This data suggests that single amino acid changes are not sufficient to alter Matα1 degradation kinetics. However, an alternative explanation may exist based on the differences I observed between pulse-chase and cycloheximide experiments for Slx5 and Slx8. It is possible that the mutants I have isolated are not sufficient to alter the degradation rate of newly synthesized Matα1, but do alter the turnover kinetics of the steady-state pool of Matα1 allowing the fusion protein to persist long enough to support growth on media lacking uracil. To test this hypothesis, the mutants I identified by PCR-mediated mutagenesis should be analyzed by cycloheximide chase experiments.

Methods

Plasmids

Information regarding plasmid construction can be found in Appendix 7 (Table 1).

Yeast Strains

Information regarding strain construction can be found in Appendix 7 (Table 2).

PCR-mediated mutagenesis

PCR-mediated mutagenesis was performed as previously described (Muhlrad et al., 1992). The plasmid p414ADH-α1-Ura3-3HA(ΔPstI) was used as template for PCR reactions. Primers used for amplification were located in the ADH promoter 5' to Matα1 and 3' to Matα1 in the open reading frame of Ura3. PCR products were transformed alongside a gapped plasmid made from p414ADH-α1-Ura3-3HA(ΔPstI), which was digested with SpeI and PstI removing the wild-type copy of Matα1, using standard yeast protocols. Upon transformation into yeast the gapped plasmids were repaired using the PCR product via homologous recombination (Parker and Mulhrad). Transformants were plated directly on SD-trp-ura, which is a minimal media lacking tryptophan (to select for the plasmid) and uracil (to select for stabilizing mutants).

On days 3-5, colonies were picked and streaked to SD-trp-ura to verify their phenotype. Strains that reproduced the growth phenotype on SD-trp-ura were grown overnight in 2mL of SD-trp (to maintain the plasmid). Mutant plasmids were extracted as previously described (Hoffman and Winston, 1987). These extractions were taken to completion even though some protocols suggest that one can electroporate into *E. coli* immediately following

chloroform extraction. I found that my samples contained too much salt at that step for electroporation. Once DNA was extracted, plasmids were rescued into *E. coli* by electroporation. Cells were plated on LB + ampicillin to select for the plasmid. Rescued plasmids were transformed into the wild-type strain MHY501 and checked for growth on SD-trp-ura. The plasmids capable of supporting growth were sent to Cornell University DNA Sequencing Facility for sequencing.

Construction of nested truncations

Nested truncations were designed based on secondary structure predictions from "JPred", website available courtesy of the University of Dundee, Scotland (www.compbio.dundee.ac.uk/www-jpred). Primers for N-terminal truncations were designed to include a start codon (ATG). Each truncation was first cloned into a TA cloning kit (Invitrogen), sequenced, and then subcloned via restriction digest with SpeI and PstI into p414ADH-α1-Ura3-3HA(ΔPstI) replacing the full length Matα1 open reading frame.

Pulse-chase and TCA lysis

Cells were grown to mid log phase (OD_{600} 0.5 - 1.0) in a minimal media lacking the appropriate amino acid for plasmid selection. Approximately 10 cell ODs were harvested by centrifugation (3000rpm for 5 minutes at room temperature). Cells were washed twice with 1ml of wash buffer (1X SD, 2% glucose, no amino acids) and transferred to 1.5mL Eppendorf tubes. Cells were

resuspended in 200μL of minimal media lacking methionine and the amino acid necessary for plasmid selection. The tubes were put into a dry bath at 30°C. Subsequently, 100-200μCi of [35S] Trans-Label (MP Biomedicals and PerkinElmer) was added to each tube. Cells were allowed to label for 10 minutes keeping them suspended by periodic vortexing. Cells were spun for 10 seconds in a microfuge to pellet them. The supernatant was removed and 200μL of chase buffer (1X SD, 2% glucose, 10mM methionine, 0.5mg/ml cycloheximide, and the required amino acids). Immediately following addition of the chase buffer, 50μL of cells were transferred to an Eppendorf tube containing 50μL of stop buffer (40mM methionine, and 20mM NaN_3) on ice. This step was repeated for each subsequent time point. After the time course was finished each sample was washed one time with ice-cold dH_2O.

Protein was extracted using a TCA lysis as previously described (Loayza et al., 1998). Samples were normalized within a time course by determining the TCA precipitable counts and transferring the normalized volumes to fresh 1.5mL Eppendorf tubes on ice. To immunoprecipitate 3HA tagged Matα1, 4μL of 25 mg/mL HA antibody (12CA5, Roche) was added to each sample and mixed by inversion at 4°C overnight. Samples were then spun down (13Krpm for 15 minutes at 4°C) to remove any non-specific aggregates, and supernatants were transferred to fresh tubes containing 20μL of a 50% slurry of Protein A-agarose (Repligen). Samples were rotated end-over-end at 4°C to keep beads in suspension for 1-2 hours. Samples were washed 3-4 times with TCA dilution

buffer plus 0.1% SDS. Following the last wash, 50μL of 2X Laemmli loading buffer was added and samples were boiled for 3 minutes.

To visualize, the entire sample was loaded on a large format 0.75mm SDS-PAGE (10-12%). After running overnight, the gel was fixed in 10% MeOH/ 10% acetic acid, placed on Whatman paper, and dried in a gel dryer. The gel was then exposed to a phosphoimager screen overnight and analyzed using a Typhoon.

Cycloheximide chase and NaOH/SDS lysis

Cells were grown to mid log phase (OD_{600} 0.5 -1.0) in minimal media lacking the appropriate amino acid for plasmid selection. Cycloheximide was added to a final concentration of 0.2 mg/mL from a stock of 10 mg/mL in dH_20 to each culture. Immediately after cycloheximide addition, 2.5 ODs of cells were harvested and put in a 50mL conical tube containing 10mL of ice-cold dH_20 and 30mM NaN_3 and placed on ice. Cells were harvested in this way for each subsequent time point.

Cells were collected by centrifugation (3000rpm for 5 minutes at 4°C). The supernatants were poured off, and cell pellets were resuspended in 1mL of ice-cold dH_20 and transferred to 1.5mL Eppendorf tubes. Cells were re-pelleted by centrifugation (13K for 1 minute at room temperature). The supernatant was aspirated off, and the pellet was resuspended in 200μL of 0.1M NaOH and allowed to incubate at room temperature for 5 minutes. Cells were again collected by centrifugation (13K for 1 minute at room temperature). The

supernatant was removed and the pellet was resuspended in 50μL of 1X Laemmli Loading Buffer and boiled for 3 minutes (Kushnirov, 2000).

Samples were then spun down (13K for 3 minutes at room temperature) and 1 OD was loaded of each sample (unless otherwise noted) onto a large format 1.5 mm SDS-Page (10-12%). After running overnight, gels were transferred and visualized by Western blotting. Where indicated a cycloheximide chase was performed followed by an immunoprecipitation of the HA tag which was conducted as described for pulse-chase experiments. These samples were also visualized by Western blotting.

Western blotting

To visualize an SDS-PAGE by Western blotting, gels were transferred to a nitrocellulose membrane (Immobilin PVDF) in 1X Transfer Buffer diluted from a 10X stock (250mM Tris-OAc, pH 8.8, 2M glycine, and 0.1% SDS) and 20% methanol at 20 volts for 1.5 hours. The membrane was incubated on a rocker in 2% milk in TBST (20mM Tris-HCL, pH 7.6, 150mM NaCl, 0.1% Tween) for at least 1 hour at room temperature. Primary was added at a dilution of 1:1000 (25 mg/mL α-HA, 12CA5, Roche) to 1% milk in TBST and incubated on a rocker overnight at 4°C. The membrane was washed 3 times for 5 minutes each with TBST and then incubated on a rocker at room temperature for 30 minutes to 2 hours in Seablock (Pierce Chemical) with Qdot 605 α-mouse diluted 1:1000 (Invitrogen). The membrane was then washed 3 times for 5 minutes each with 3X TBST (as described above, except 0.3% Tween instead of

0.1%) and 2 times for 5 minutes each with TBST. The membrane was then visualized and subsequently analyzed using a Typhoon and ImageQuant software. Data were normalized to the zero time point within individual experiments to determine percents remaining. Replicates were then averaged.

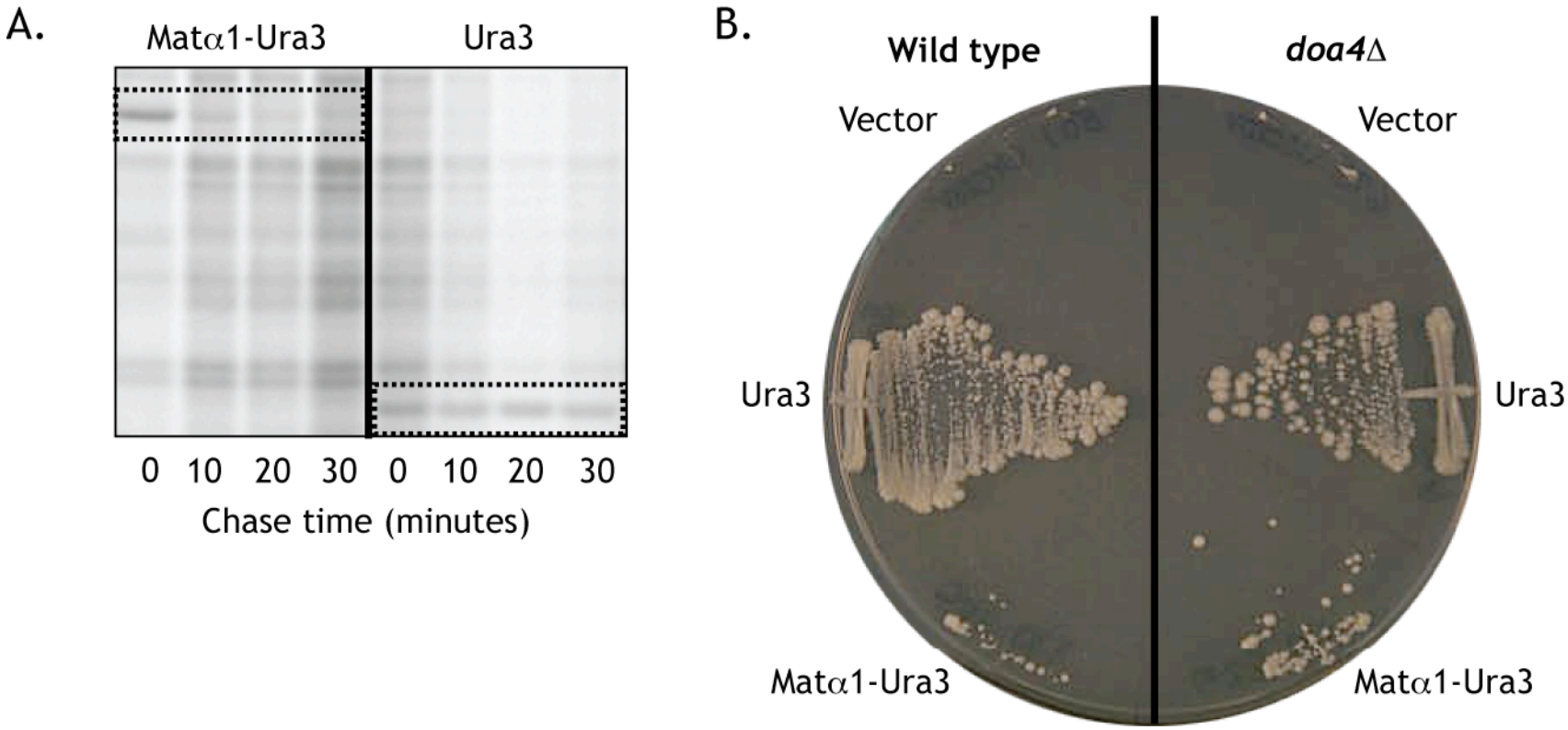

A.
Matα1-Ura3
Ura3
0 10 20 30 0 10 20 30
Chase time (minutes)
B.
Wild type
doa4Δ
Vector
Vector
Ura3
Ura3
Matα1-Ura3
Matα1-Ura3
C.
Wild-type
slx8Δ
Matα1-Ura3
0 15 30 60 0 15 30 60
Chase time (minutes)

Figure III-1. Matα1-Ura3 is a rapidly degraded protein. A. Pulse-chase assay of p414ADH-α1-URA3-3HA (Matα1-Ura3), or p414TEF-URA-3HA (Ura3) in wild-type cells (MHY501) showing that while Ura3 is normally a stable protein fusion of Matα1 to Ura3 targets it for rapid degradation. B. p414ADH (Vector), p414ADH-α1-URA3-3HA (Matα1-Ura3), or p414TEF-URA-3HA (Ura3) in MHY501 (wild-type) or MHY623 (*doa4Δ*) grown on SD-trp-ura for 3 days at 30°C. p414ADH-α1-URA3-3HA is unable to support growth on this media in wild-type cells (MHY501), but in cells with decreased cellular levels of ubiquitin, *doa4Δ* (MHY623), growth is observed. C. Cycloheximide chase of p414ADH-a1-Ura3-3HA (Matα1-Ura3) in either wild-type cells (MHY501) and in an *slx8Δ* (MHY3716).

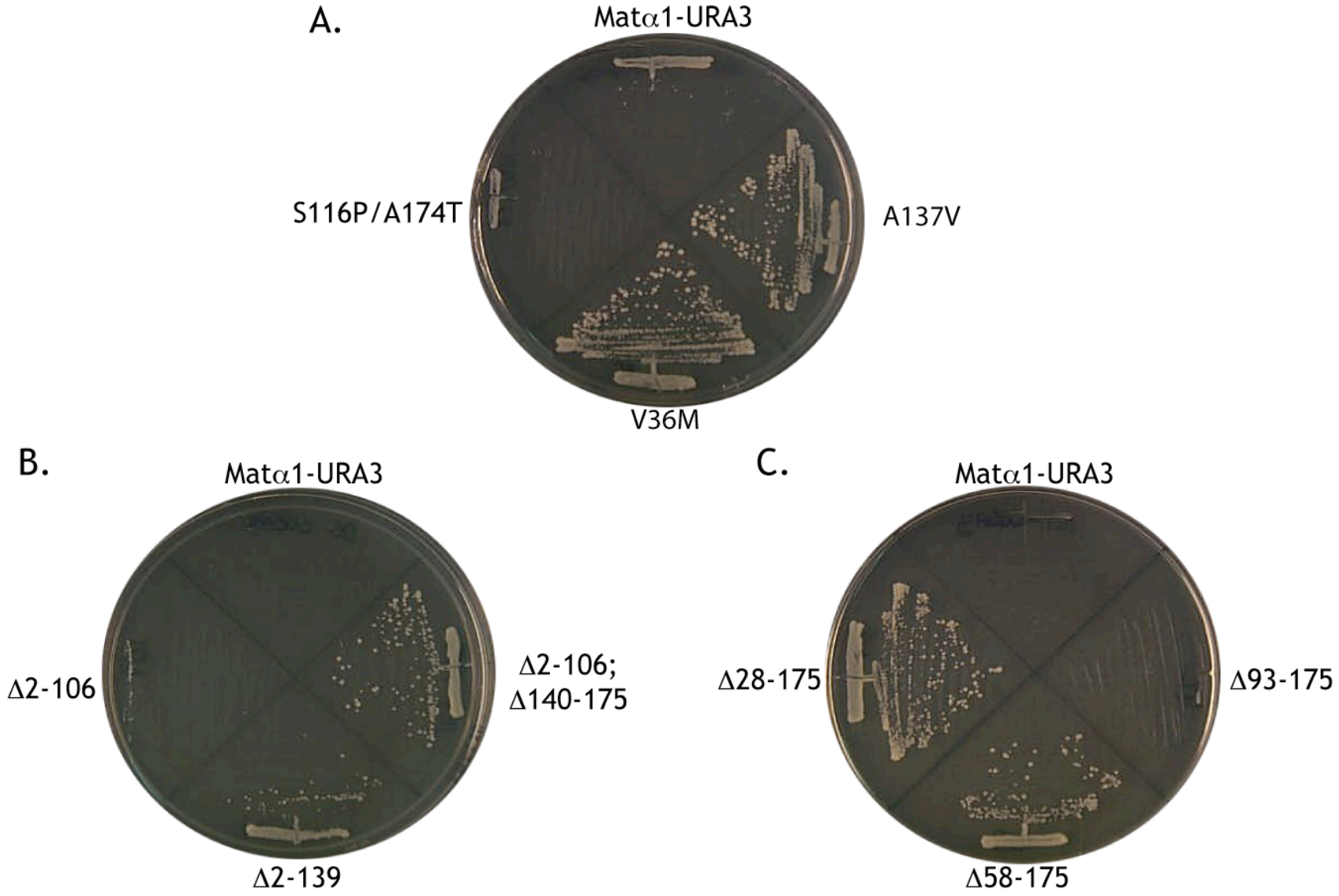

A.
Matα1-URA3
S116P/A174T
A137V
V36M
B.
Matα1-URA3
Δ2-106
Δ2-106;
Δ140-175
Δ2-139
C.
Matα1-URA3
Δ28-175
Δ93-175
Δ58-175

Figure III-2. Mutants of Matα1 fused to Ura3 permit growth on media lacking uracil. A. Point mutants isolated from a PCR-mediated mutagenic screen grown on SD-trp-ura in wild-type cells (MHY501) grown for 3 days at 30°C. B. Nested truncations grown on SD-trp-ura in wild-type cells (MHY501) for 3 days at 30°C.

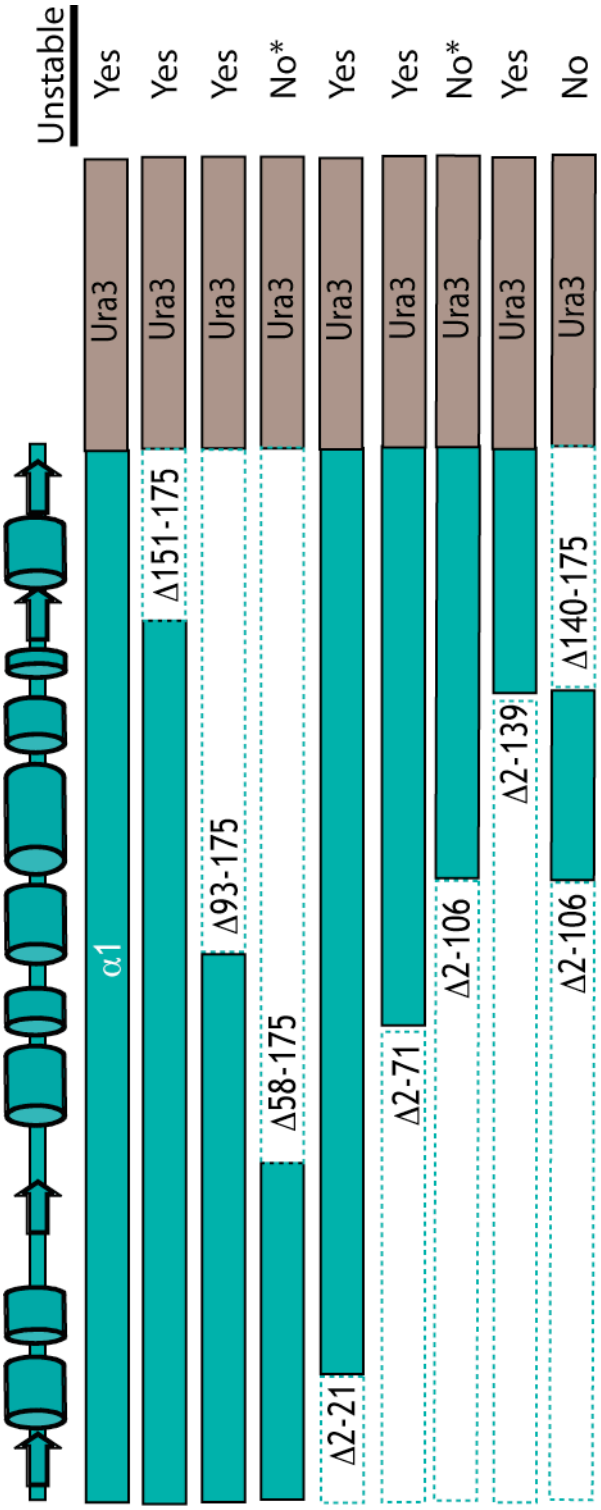

Unstable
Yes
Yes
Yes
No*
Yes
Yes
No*
Yes
No
Ura3
Ura3
Ura3
Ura3
Ura3
Ura3
Ura3
Ura3
Ura3
α1
Δ151-175
Δ93-175
Δ58-175
Δ2-21
Δ2-71
Δ2-106
Δ2-139
Δ140-175
Δ2-106

Figure III-3. Summary of pulse-chase data for Matα1 truncations. Cylinders represent β-barrels and arrows represent α-helices in Matα1's predicted secondary structure. Wild-type half-life of the Matα1-Ura3 fusion is approximately 5 minutes. "Yes" indicates half-life within two-fold of wild-type (5-10 minutes). "No*" indicates a half-life that is two- to three-fold more stable than wild-type. These truncations have a half-life between 10-15 minutes (See Figure III-4, and 5). "No" indicates a half-life of three-fold or greater over wild-type.

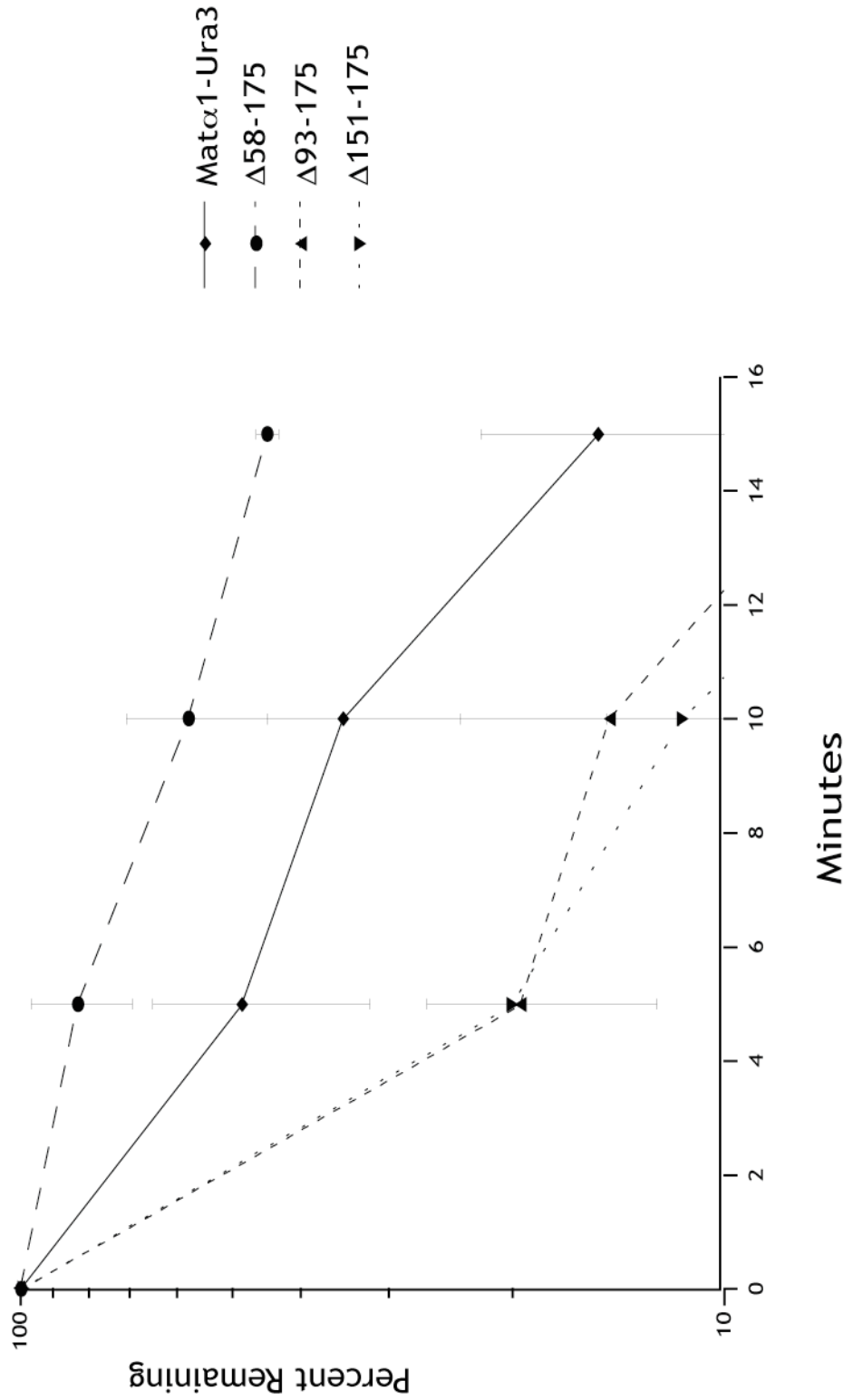

Matα1-Ura3
Δ58-175
Δ93-175
Δ151-175
Percent Remaining
100
10
0
2
4
6
8
10
12
14
16
Minutes

Figure III-4. Residues 58-92 are required for Matα1 turnover.

Quantification of pulse-chase experiments for C-terminal truncations summarized in Figure III-3. Graph shows percent remaining of Matα1 fragments fused to Ura3 at each timepoint. All fusion proteins were expressed in wild-type cells.

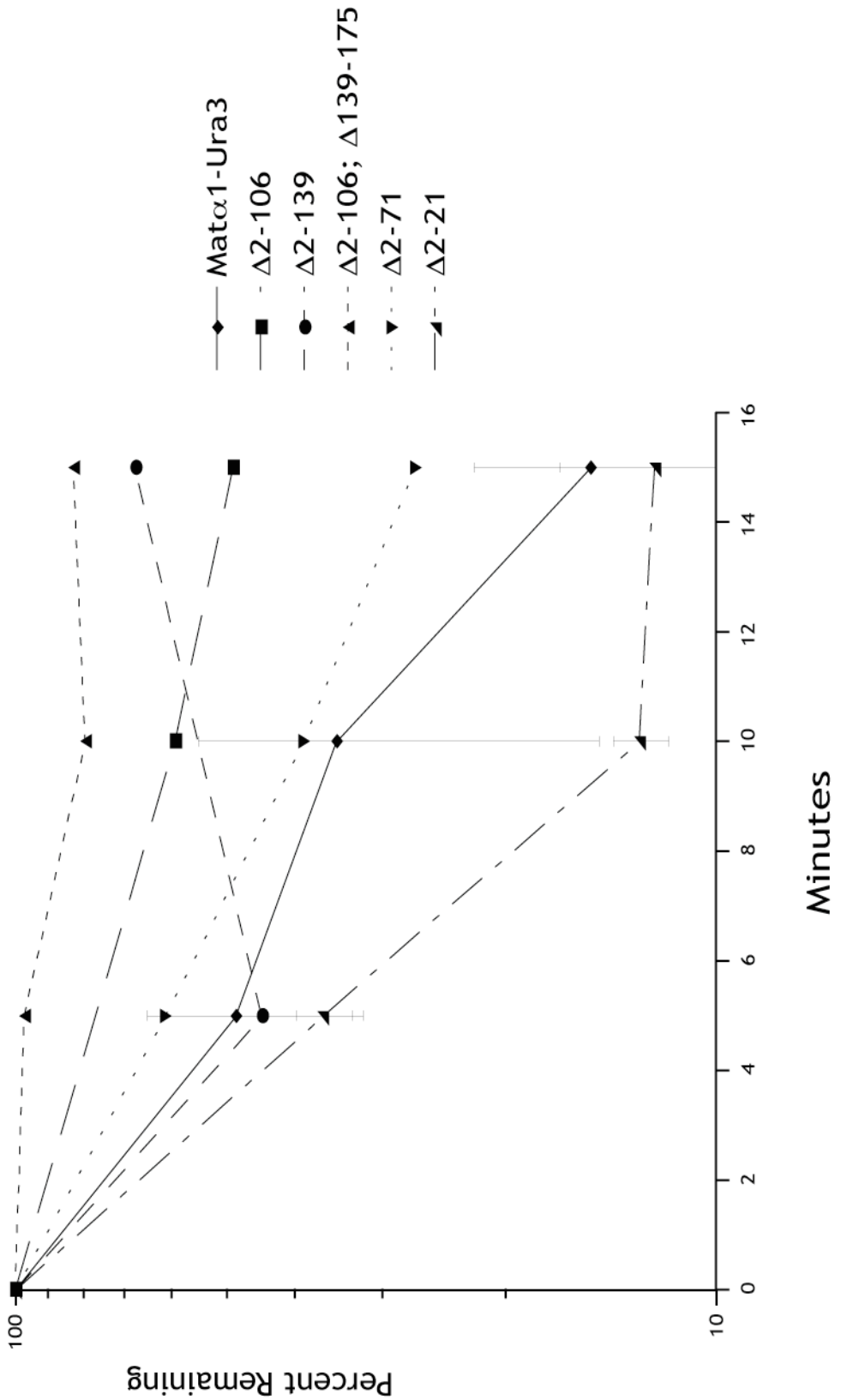

Matα1-Ura3
Δ2-106
Δ2-139
Δ2-106; Δ139-175
Δ2-71
Δ2-21
Percent Remaining
100
10
0
2
4
6
8
10
12
14
16
Minutes

Figure III-5. Amino acids 140-175 of Matα1 are necessary and sufficient to direct turnover of Ura3. Quantification of pulse-chase experiments for N-terminal truncations summarized in Figure III-3. Graph shows percent remaining of Matα1 fragments fused to Ura3 at each timepoint. All fusion proteins were expressed in wild-type cells.

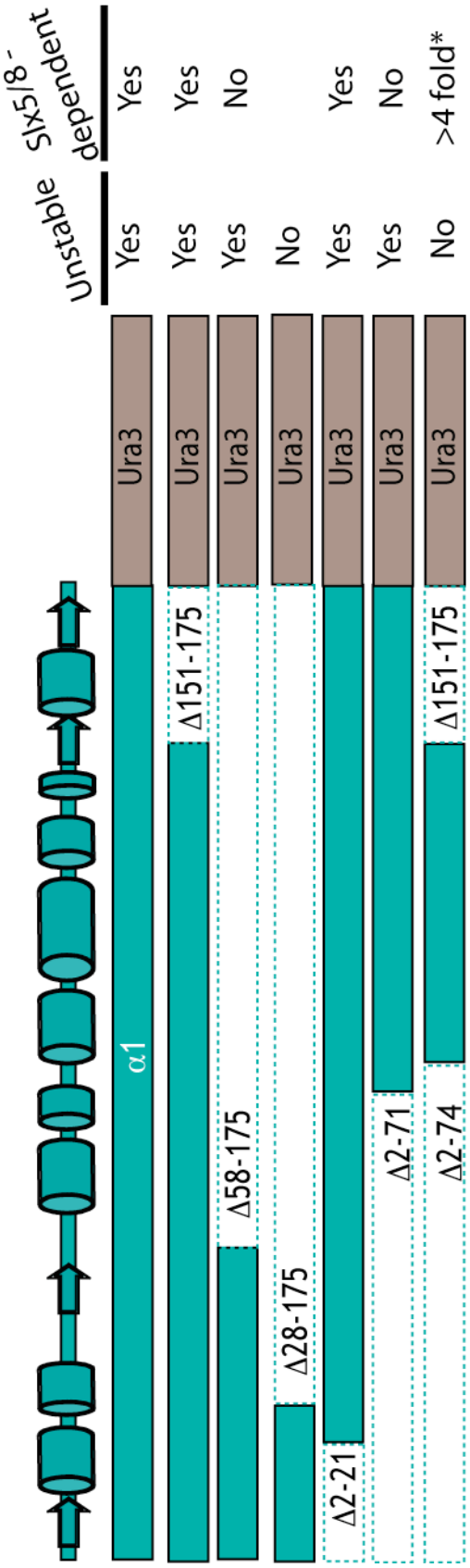

Unstable
Slx5/8- dependent
Yes
Yes
Yes
Yes
No
No
Yes
Yes
No
Yes
No
>4 fold*
Ura3
Ura3
Ura3
Ura3
Ura3
Ura3
Ura3
α1
Δ151-175
Δ58-175
Δ28-175
Δ2-21
Δ2-71
Δ2-74
Δ151-175

Figure III-6. Summary of cycloheximide chase data for Matα1 truncations.

Summary of cycloheximide chase analysis of nested truncations. Cylinders represent β-barrels and arrows represent α-helices in Matα1's predicted secondary structure. Wild-type half-life of the Matα1-Ura3 fusion is approximately 5 minutes. "Yes" indicates half-life within two-fold of wild-type (5-10 minutes). "No" indicates a half-life of three-fold or greater over wild-type. * - The half-life of Δ2-74; Δ151-175 in wild-type cells is three-fold over full-length Matα1. However, this truncation is stabilized to an even greater extent in *slx8Δ* cells. See Figure III-7.

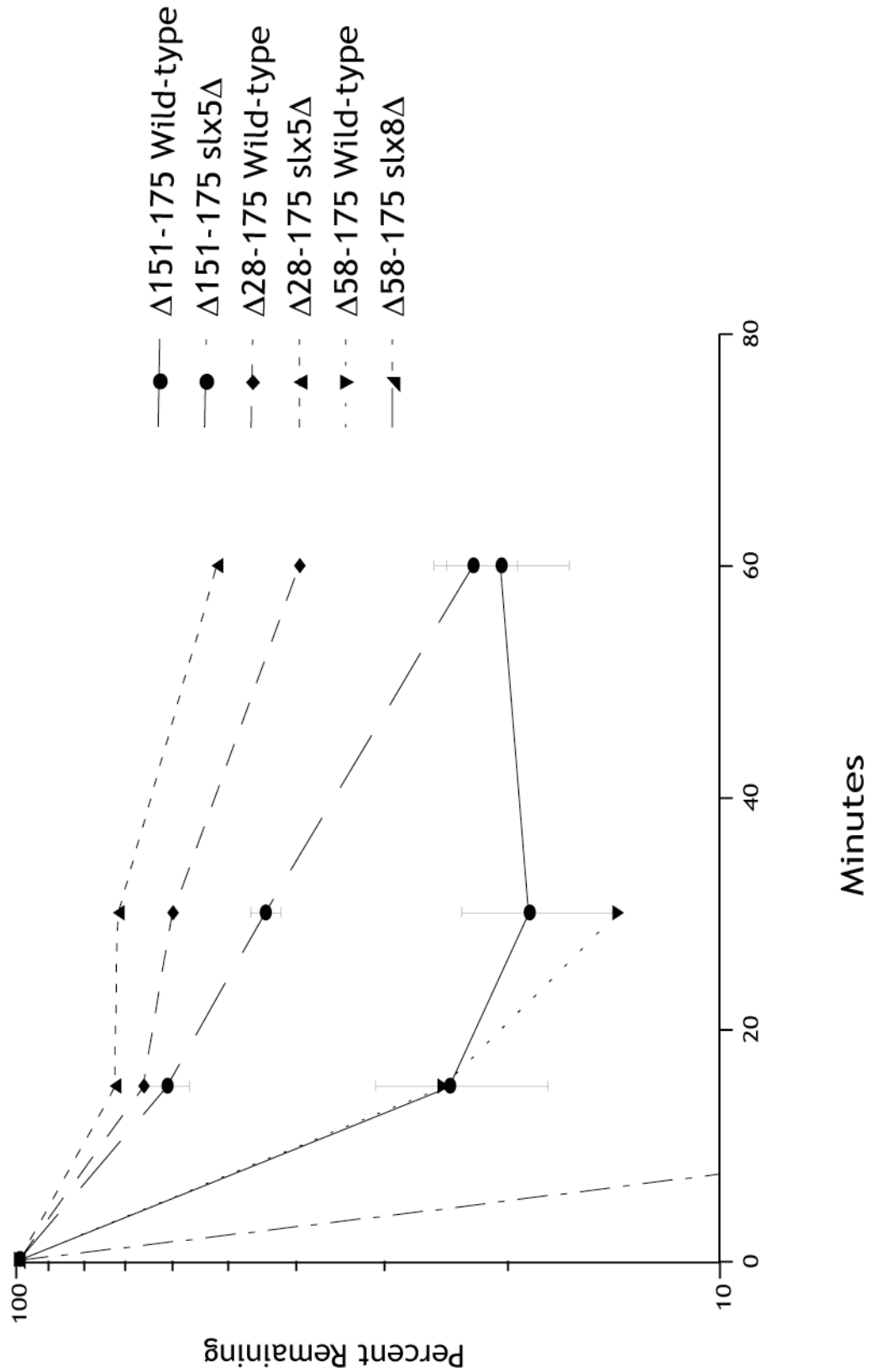

Δ151-175 Wild-type
Δ151-175 slx5Δ
Δ28-175 Wild-type
Δ28-175 slx5Δ
Δ58-175 Wild-type
Δ58-175 slx8Δ
Percent Remaining
100
10
0
20
40
60
80
Minutes

Figure III-7. The extreme N-terminus of Matα1 is not a degron.

Quantification of cycloheximide chase experiments for C-terminal truncations summarized in Figure III-6. Graph show percent of Matα1 fragment fused to Ura3 remaining at each time point. Truncations were expressed in either wild-type, *slx5Δ*, or *slx8Δ* cells as indicated.

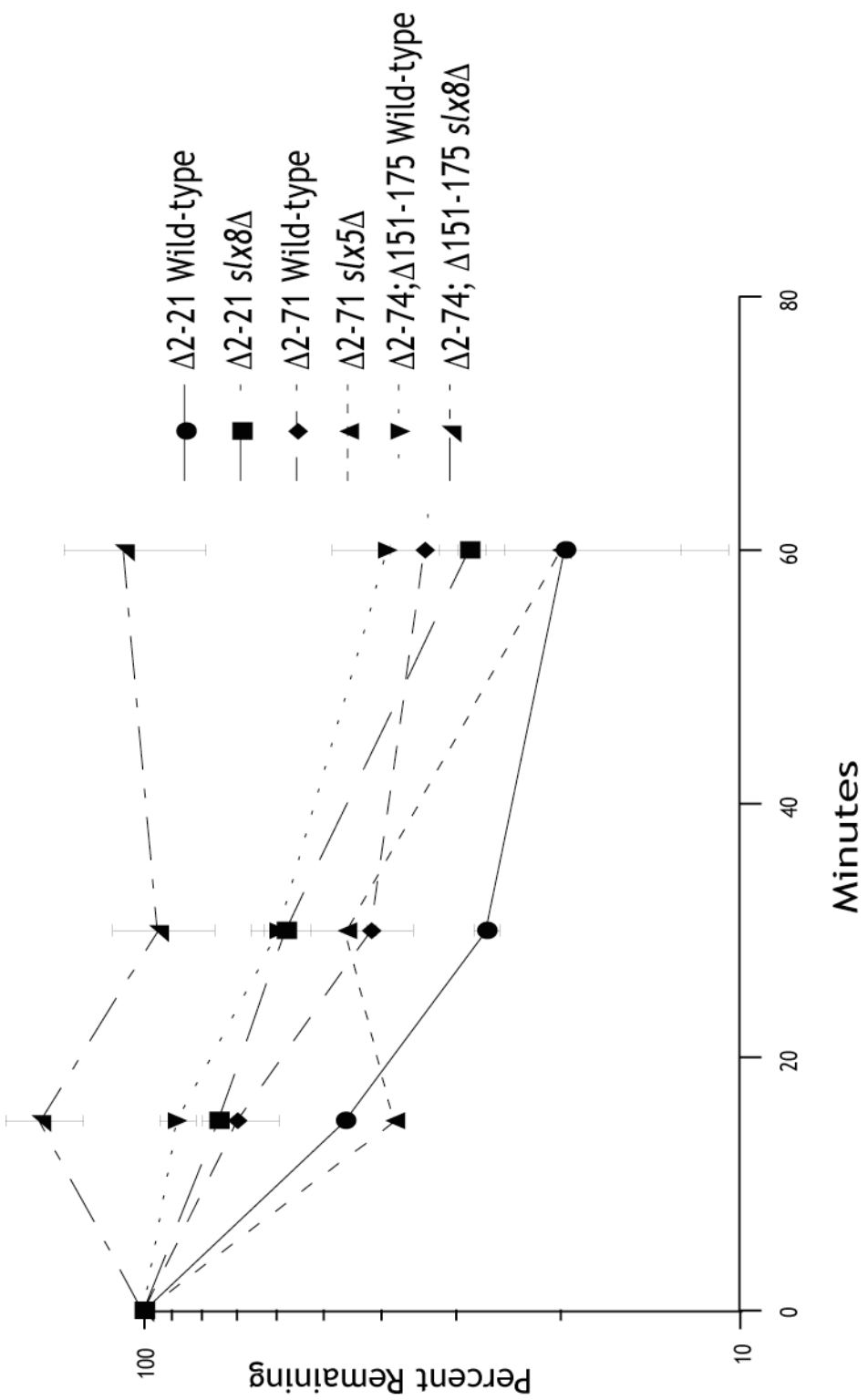

Δ2-21 Wild-type
Δ2-21 slx8Δ
Δ2-71 Wild-type
Δ2-71 slx5Δ
Δ2-74;Δ151-175 Wild-type
Δ2-74; Δ151-175 slx8Δ
Percent Remaining
100
10
0
20
40
60
80
Minutes

Figure III-8. Residues 75-150 of Matα1 are an Slx5-Slx8-dependent degron.

Quantification of cycloheximide chase experiments for N-terminal truncations summarized in Figure III-6. Graph shows percent remaining of Matα1 fragment fused to Ura3 at each time point. Truncations were expressed in either wild-type, *slx5Δ*, or *slx8Δ* cells as indicated.

Chapter IV

Discussion

Major Conclusions

A number of conclusions can be made from the experiments I have presented in these pages. The first major conclusion is that Matα1 is rapidly degraded by the ubiquitin-proteasome system. Proteasome-dependence is evidenced by stabilization of Matα1 in the presence of the proteasomal inhibitor MG-132 and in strains expressing proteasomal mutants (Figure II-1, 2). This work also suggests that degradation of Matα1 is ubiquitin-dependent (Figure II-3, 4, 5, and 8).

Second, I have shown that multiple ubiquitin-conjugating enzymes and ubiquitin ligases mediate the turnover of Matα1 (Figure II-4, 5, and 8). Epistasis experiments indicate that these enzymes all function in the same genetic pathway, with the exception of Ufd4 and Dma1 (Figure II-11). I would argue for an alternative interpretation; A certain level of ubiquitin activity must be removed in order to observe stabilization of Matα1 greater than three-fold, which is the level observed for single mutants (Figure II-12). I propose that these pathways are functioning independently of one another to ubiquitylate Matα1 to the level necessary for targeting it to the proteasome.

In addition, I have shown that degradation of Matα1 is dependent upon an intact sumoylation pathway as well as poly-SUMO chains (Figure II-7). Interestingly, Matα1 turnover does not require the first three lysines in SUMO, which are the sites most often chosen for SUMO conjugation of other proteins (Bylebyl et al., 2003). The dependence on SUMO chains and the requirement

for the SUMO-targeted ubiquitin ligase (STUbL), Slx5-Slx8 strongly suggest that Matα1 is sumoylated prior to recognition for ubiquitylation.

Finally, I have made observations regarding the signals that target Matα1 for proteolysis. There are multiple regions within Matα1 that are necessary to direct the normally stable uracil biosynthetic enzyme, Ura3, for rapid proteolysis. These regions fall in the N-terminal half of Matα1 (residues 58-92 by analysis of newly synthesized protein levels and residues 28-74 by evaluation of steady state protein levels) and in the extreme C-terminus (amino acids 140-175 when analyzing newly-made protein) (Figure III-3, 6). Additionally, I found that a region in the C-terminal half of Matα1 that overlaps with the alpha1 domain (residues 75-150) is not sufficient to direct turnover of Ura3 to the same extent as full-length Matα1. Fusion of this Matα1 fragment with Ura3 results in a three-fold stabilization over wild-type. However, this deletion mutant is dependent upon Slx5-Slx8 as it is stabilized to an even greater extent in *slx8Δ* cells (Figure III-8). These results suggest that amino acids 75-150 contain a degron that is recognized by Slx5-Slx8.

Taken together, the data presented in this study identify the factors responsible for targeting Matα1 to the proteasome. These factors may be recognizing separate and distinct degradation signals within Matα1. Moreover, the results of this study highlight the cross-talk that occurs between protein modification pathways. In this case, there is cross-talk between sumoylation and ubiquitylation.

Relevance to the Field

Yeast mating biology: similarities and differences with Matα2 degradation

Matα1 and Matα2 are both transcription factors that are expressed in α cells. When cells undergo a switch from α to a, both regulators must be removed. One objective of this study was to look at whether or not the degradation of Matα1 and Matα2 is coordinately regulated. Indeed, these factors do have one pathway in common. Both are targeted for degradation by the ubiquitin conjugating enzymes Ubc4 and Ubc5 most likely through the SUMO-Targeted Ubiquitin Ligase (STUbL), Slx5-Slx8. However, the specifics of how this pathway regulates each transcription factor differ.

A defect in turnover kinetics is observed for Matα2 with either a ubc4Δ or a ubc5Δ (Chen et al., 1993), while a deletion of both is required to see any effect on Matα1's stability (Figure II-4B). Since Ubc4 and Ubc5 are nearly identical and thought to be redundant, one must wonder what is the basis for this difference. Perhaps the level of one is sufficient to degrade Matα1, but full levels of both enzymes are required to achieve wild-type turnover kinetics in the case of Matα2.

There are also differences with respect to the STUbL, Slx5-Slx8. Although the heterodimer is required for the degradation of either regulator, SUMO-dependence is only observed for the degradation of Matα1. Turnover of Matα2 does not appear to require SUMO (Y. Xie and M. Hochstrasser). The reason why one factor requires SUMO and the other does not remains to be determined. Perhaps, sumoylation of Matα1 is required to increase its binding

affinity with Slx5-Slx8 and is not necessary for recognition of Matα2. Alternatively, something inherent about the Slx5-Slx8-dependent degron in Matα2 could function to interact with the heterodimer in place of a poly-SUMO chain.

SUMO-targeted ubiquitin ligases

There are, at present, only two known targets for Slx5-Slx8. One is a mutant version of the yeast protein Mot1, which interacts with the TATA-binding protein (Wang et al., 2006; Wang and Prelich, 2009). The second is Flp recombinase (Chen et al., 2005; Xiong et al., 2009), which is involved in propagation of the yeast 2μm plasmid. The 2μm plasmid's only known function is to self-propagate (Jayaram et al., 2004). Matα1 is the first physiologically relevant substrate to be identified for Slx5-Slx8. The identification of Matα1 offers an experimental tool for further investigations into how ubiquitin and SUMO are working together to direct protein degradation. Moreover, studies with Matα1 can be compared and contrasted with the data obtained from Flp recombinase and from the mutant version of Mot1.

Additionally, the findings in this study indicate a role for Slx5-Slx8 in the turnover of Matα1. It is noteworthy that Slx5 and Slx8 may be making individual contributions in Matα1 degradation as indicated by epistasis experiments (Figure II-10) and discussed in Chapter II. It is possible that Slx5 and Slx8 may not be an obligate heterodimer and in fact, may have individual

roles in targeting Matα1 for ubiquitylation. Further investigation into these individual roles is warranted.

Ubiquitin-dependent proteolysis

I have found that Matα1's proximal degradation pathway is not a standard enzyme cascade. Turnover of most proteins is regulated by one ubiquitin-conjugating enzyme and one ubiquitin ligase. There are examples, such as Matα2, where there are two pathways regulating a protein's cellular levels. In this case, however, multiple ubiquitin-conjugating enzymes and multiple ubiquitin ligases are working in concert to clear Matα1 from the cell. There are, perhaps, four separate ubiquitylation cascades regulating this transcriptional activator. The degradation pathway for Matα1 offers a unique model to consider when investigating the turnover of other proteins. Whether or not all of these pathways are, in fact, separable and working towards a threshold of ubiquitylation needs to be further tested.

Future Directions

Ubiquitylation of Matα1

Directly observing ubiquitylation of a protein is technically challenging. These proteins are short-lived and, therefore, not generally abundant cellular factors. There is also an army of enzymes that remove ubiquitin moieties from target substrates. Additionally, ubiquitylated forms of the protein are a small fraction of the overall pool. While there are chemical inhibitors of de-

ubiquitylating enzymes and one can artificially alter protein expression levels, these modifications sometimes are not enough to visualize substrate ubiquitylation. These factors contributed to my unsuccessful attempts to detect ubiquitylation of Matα1.

Ubiquitylation of Matα1 has been observed previously (Johnson, 1997). However, this observation was made under conditions in which Matα1 was highly over-expressed using the GAL promoter. One concern is that highly over-expressing Matα1 could drive non-physiologic ubiquitylation. I tried to detect ubiquitylated forms of an over-expressed Matα1, but from a much weaker promoter, ADH. This line of experimentation should not be abandoned as I have not exhausted all possible methods of detection. It is possible that trying to immunoprecipitate larger amounts of Matα1 would permit detection of ubiquitylated forms. Moverover, attempting to detect ubiquitylated Matα1 by mass spectroscopy, which is a much more sensitive technique than immunoblotting, may be a fruitful alternative.

Proximal degradation pathway for Matα1

I have shown that three ubiquitin-conjugating enzymes and four ubiquitin ligases are required for turnover of Matα1. These findings raise some very interesting questions. First, are these factors functioning in separate pathways? Epistasis experiments indicate that all factors are in the same genetic pathway except for Ufd4 and Dma1. However, Ufd4 is in the same genetic pathway as Slx5, Slx8, and Dma2, and Slx5, Slx8, and Dma2 are

genetically indistinguishable from Dma1. I have proposed that all four factors are functioning in separate pathways, but appear to be genetically linked because Matα1 degradation is dependent upon a threshold of ubiquitylation. I am suggesting that you must remove multiple factors to decrease the level of ubiquitylation, thereby, increasing Matα1 stability. To test this hypothesis, I would propose two sets of experiments: directly assaying levels of ubiquitylation, and *in vitro* ubiquitylation studies.

Once a direct test of Matα1 ubiquitylation has been established, the assay can be used to ask whether or not a decrease in ubiquitylation can be observed when members of the degradation pathway are removed. Does this decrease continue as more players are deleted? One possible complication is that the level of decrease may be below the limit of detection.

The second set of experiments is *in vitro* ubiquitylation experiments. If the pathways are indeed separable, then reconstituting only one set of enzymes would be required to ubiquitylate Matα1. For example, if Ubc4 is in one pathway with Dma2, then only these two enzymes are required to ubiquitylate Matα1 *in vitro*. *In vitro* ubiquitylation experiments would also serve to confirm which ubiquitin-conjugating enzymes function with each of the ubiquitin ligases to modify Matα1. The same caveat would apply to these studies as applies to directly assaying levels of ubiquitylation. One pathway may not be sufficient to ubiquitylate Matα1 to a detectable level and may require experimental conditions to be adjusted in order to visualize ubiquitylation of Matα1.

Another prediction of the model that each of the four individual ubiquitin ligases are working toward a particular level of ubiquitylation is that each pathway is somehow limiting on its own. In other words, I would predict based on this model that over-expressing factors in one pathway in the absence of another pathway would rescue the single deletion phenotype I observed (Figure II-9). For example, does over-expression of Ubc13 and/or Dma1 rescue the phenotype observed in an *slx5Δ*?

The next set of questions regarding the proximal degradation pathway concerns the individual ubiquitin ligases. First, is Matα1 being recognized by Ufd4 in the same manner it recognizes ubiquitin fusion degradation (UFD) substrates? The armadillo (ARM) repeats in Ufd4 have been shown to interact with the N-terminal ubiquitin in UFD substrates (Ju et al., 2007). Mutating the ARM repeats, or removing them altogether could address the question of Ufd4 recognition. Does Matα1 degradation in the presence of a mutant Ufd4 lacking the ARM repeats mimic a ufd4Δ strain?

Second, I have demonstrated that the yeast Rnf8 homologs, Dma1 and Dma2, are required for turnover of Matα1 (Figure II-8). Initial epistasis experiments put them in the same pathway with Ubc13 (Figure II-9). I was unable to complete the epistasis experiment between Dma1, Dma2, and Ubc4 and Ubc5 due to the severe cell growth phenotype of *ubc4Δubc5Δ*. The *in vitro* ubiquitylation assays mentioned above would be one way to determine which ubiquitin-conjugating enzymes Dma1 and Dma2 are cooperating with.

In addition, my epistasis experiments (Figure II-10) open up questions regarding the role of Dma2. Matα1 in a *dma1Δdma2Δ* strain and a *dma2Δufd4Δ* strain is stabilized three-fold. In a *dma1Δufd4Δ* strain, Matα1 is stabilized six-fold. Removing Dma2 from a *dma1Δufd4Δ* strain suppresses the stabilization of Matα1 (Figure II-10). What is the nature of the suppression phenotype observed in a *ufd4Δdma1Δdma2Δ* strain? I have hypothesized that the suppressor phenotype is due to the lack of Dma2 and that it actually functions to repress Slx5 and/or Slx8. To test this hypothesis, a simple examination of Slx5 and Slx8 levels in cells lacking Dma2 by Western blotting should yield the answer. My hypothesis predicts that levels of Slx5 and/or Slx8 would be higher in a *dma2Δ* strain, because suppression of this heterodimer would be relieved.

Third, the STUbL Slx5-Slx8 has also been identified in this study as a modulator of Matα1 proteolysis (Figure II-5, 6). Several questions have arisen from this result, including whether or not Slx5 and Slx8 have separable functions with regards to turnover of Matα1 as mentioned previously. By further characterizing their role in Matα1 proteolysis, I believe these questions will begin to be answered. This characterization should begin by dissecting the functional elements of within Slx5 and Slx8. Does a mutant Slx5 lacking its SUMO-interacting motifs alter the stability of Matα1 (Xie et al., 2007)? Is Mata1 stabilized in the presence of an Slx8 mutant that can no longer bind DNA (Yang et al., 2006)?

Sumoylation of Matα1

I have also shown that an intact sumoylation pathway as well as lysines within Smt3 are required for degradation of Matα1 (Figure II-7). Whether or not Matα1 is directly sumoylated remains to be determined. Experiments to determine the sumoylation status of Matα1 are just as described for directly demonstrating ubiquitylation of Matα1: immunoprecipitation of Matα1 followed by immunoblotting for SUMO. Directly assaying sumoylation comes with the same issues and concerns mentioned above for directly assaying ubiquitylation. I have attempted this experiment at the most basic level. It should be attempted again on a larger scale. Additionally, pursuing mass spectroscopy of Matα1 may prove useful in detecting this potential modification as well. Mass spectroscopy might also shed light on the nature of the sumoylation. Is sumoylation of Matα1 occuring at independent sites from ubiquitylation? Or is sumolyation making the base of a chain and then either capped by a single ubiquitin molecule or extended by a polyubiquitin chain?

Regulation of "new" vs. "old" Matα1

While characterizing the degradation of Matα1, I made an interesting observation. I observed differences in protein turnover rates for certain mutants depending on whether I was using pulse-chase experiments or cycloheximide chase assays. These differences most likely arose from the inherent differences between the assays. Pulse-chase experiments report only on newly synthesized protein, whereas cycloheximide chase analysis gives

information on the steady-state pool of protein. Interestingly, I observed that Matα1 is stabilized only two-fold in a *ubc13Δ* strain by pulse-chase, but three-fold in the same mutant background by cycloheximide chase (Figure II-4A). Also, I noted that Slx5-Slx8 modulate only the steady-state pool of Matα1 and do not affect turnover of newly-made Matα1 (Figure II-5A). While the Ubc13 result might only be a slight nuance in the differences between the assays, the distinction between "new" and "old" Matα1 made by Slx5-Slx8 may point to an interesting regulatory mechanism. Perhaps, Slx5-Slx8 can only recognize Matα1 that has been matured or activated in someway. Given the fact that Slx5-Slx8 is a STUbL, it is possible that this mark of maturation is sumoylation. One way to test this hypothesis would be to construct a Matα1-SUMO fusion and ask if the turnover of newly-synthesized Matα1-SUMO is dependent upon Slx5-Slx8 by pulse-chase.

Matα1's degradation signals

In this study, I found that more than one region of Matα1 is required for its turnover. Interestingly, a 75 amino acid stretch from the C-terminal half of Matα1 (Δ2-74; Δ151-175), while not sufficient to direct rapid turnover of the normally stable Ura3 protein, is dependent on Slx5-Slx8. The level of turnover residues 75-150 are capable of directing (three-fold greater than full-length Matα1) is abrogated in *slx8Δ* cells (Figure III-8). The fact that this degron is incapable of directing the same turnover kinetics observed for full-length Matα1 is not surprising given that it is not the transcription factor's only

degradation signal. Since there are multiple pathways and multiple degrons in Matα1, it is logical that these signals may not be sufficient on their own for rapid degradation. A deletion of Matα1 lacking only this region (Δ75-150) would aid in further establishing it is role in Matα1 turnover. I would predict that the turnover of Matα1 lacking amino acids 75-150 would mimic an *slx5Δ* or an *slx8Δ* strain.

Furthermore, amino acids 75-150 coincide with a highly conserved region within Mata1 called the alpha1 domain (residues 88-144). The function of this domain is unknown, but it is thought to be either the DNA binding domain or possibly the surface that interacts with the transcription factor Mcm1 (Coppin et al., 1997; Yuan et al., 1993). It is intriguing that this functional domain overlaps with the Slx5-Slx8-dependent signal I have identified. Is a functionally engaged Matα1 protected from degradation? Does a change, such as sumoylation of Matα1, allow for recognition by Slx5-Slx8?

Other regions of Matα1 are also important for its turnover (Figure III-3, 6). The regions of Matα1 that are sufficient to target Ura3 for degradation, but do not show Slx5-Slx8-dependence, should be analyzed in *ufd4Δ*, *dma1Δ*, and *dma2Δ* strains as they are also required for Matα1 degradation (Figure II-5, 8). These analyses would determine if the same pathways that recognize the full-length protein are recognizing these truncations or if a physiologically irrelevant pathway is targeting them for proteolysis. They would also determine if all of the ubiquitin ligases involved in proteolysis of Matα1 are recognizing the same degradation signal or different signals. I would

hypothesize based on the evidence presented for the Slx5-Slx8-dependent degron that they are each recognizing a distinct signal. Analyzing Matα1 deletion mutants that show mild stabilization (two- to three-fold) in wild-type cells in the context of these ubiquitin ligase mutants should also be considered. Since there are multiple pathways and the evidence suggests multiple degrons, a single degradation signal is not likely capable of conferring wild-type Matα1 levels of turnover to a normally stable protein.

Big Picture Model for Matα1 Degradation

Based on the data presented in this work, I hypothesize that Matα1 could be targeted for degradation by Slx5-Slx8 while bound to DNA. It is possible that a transcriptionally active Matα1 engaged on DNA, possibly through the alpha1 domain, is sumoylated. This sumoylation causes Matα1 to become accessible to the STUbL, Slx5-Slx8. The DNA binding domain found in Slx8 potentially puts the heterodimer within close proximity to a functionally engaged Matα1 permitting interaction of Slx5's SUMO-interacting motifs with a sumoylated Matα1 (Xie et al., 2007; Yang et al., 2006). Matα1 is, subsequently, ubiquitylated. Further ubiquitylation of Matα1 by Slx5-Slx8, Ufd4, Dma1, and Dma2 continues until the threshold necessary for targeting to the proteasome is met and Matα1 is degraded. The scenario I have suggested makes several assumptions, but it provides a model that can begin to be tested and reworked as new data is uncovered.

Appendix

Additional Observations and Yeast Strains and Plasmid Tables

Site of Matα1 Ubiquitylation

A K48-linked poly-ubiquitin chain targets most proteins degraded by the proteasome. This chain is generally attached covalently to a lysine within the target substrate (Chau et al., 1989). Ubiquitylation of a target can be directed to a specific lysine as is the case with yeast iso-2-cytochrome c (Sokolik and Cohen, 1991). In contrast, ubiquitylation can be promiscuous targeting a number of available lysines. For example, there are multiple lysines within cyclin B, p53, and c-jun that can be targeted for ubiquitylation (King et al., 1996; Rodriguez et al., 2000; Treier et al., 1994). Examples also exist in which lysines surrounding a degradation signal are the preferred site of ubiquitylation, as is the case with GCN4 and lysines surrounding its PEST signal(Kornitzer et al., 1994). In addition to investigating the *cis*-acting degradation signals within Matα1, I also was interested in determining the site of ubiquitylation within Matα1. In addition to determining whether or not there is a single specific site of ubiquitylation for Matα1, I was hoping to create a reagent for further studies into the physiological relevance of Matα1 proteolysis.

To begin to investigate the site of ubiquitylation within Matα1, I first analyzed a mutant version of Matα1 lacking all of its lysines (p414ADH-$\alpha1^{KR}$-3HA). I started with a mutant devoid all lysines, because of the promiscuity of ubiquitylation. Surprisingly, this mutant was only stabilized two- to three-fold over wild-type in pulse-chase experiments (Figure A-1). Next, I asked whether the turnover of Mat$\alpha1^{KR}$ is dependent upon one of the known *trans*-acting

factors that regulate degradation of Matα1: Ubc13. Matα1^{KR} turnover in *ubc13Δ* cells mimicked that of Matα1 in the same strain, but it was also very similar to the turnover I observed for Matα1^{KR} in wild type cells (Figure A-1). There are two interpretations of this data: Matα1^{KR} is a substrate for Ubc13 that utilizes the internal lysines of Matα1 putting Ubc13 and ubiquitylation of an internal lysine in the same genetic pathway, or Matα1^{KR} is not being recognized by Ubc13. The other *trans*-acting factors I have identified should be tested to distinguish between these two interpretations and to determine which pathway, if any, is recognizing Matα1^{KR}.

Taken together, these data suggest that while internal lysines are required for rapid degradation of Matα1, other non-canonical sites of ubiquitylation, or a ubiquitin-independent pathway could be targeting it for destruction. A ubiquitin-independent pathway is unlikely given the number of ubiquitin-conjugating and ubiquitin ligases I have found to be required for Matα1 turnover. Alternatively, the data could indicate that when internal lysines are not available, alternative sites of ubiquitylation, such as the extreme N-terminus or alternative amino acids, can and will be utilized even if they are not the usually the preferred targets.

To test whether the N-terminus of Matα1 is a site of ubiquitylation, I assayed the turnover of an N-terminally tagged Matα1, p414ADH-6HA-α1. This tagged version of Matα1 is greatly stabilized in either pulse-chase experiments or cycloheximide chase assays (Figure A-2A, B). The tag is potentially having

one of two effects that I will address separately: blocking the site of ubiquitylation or sterically occluding access to a degradation signal.

The tag could be blocking the N-terminus from being ubiquitylated. Matα1 is not predicted to be a substrate for acetylation, but the sequence at the N-terminus of the 6HA tag is predicted to be acetylated (Kiemer et al., 2005; Polevoda and Sherman, 2003). This predicted acetylation could be preventing the ubiquitylation machinery from placing a ubiquitin moiety at the amino terminus of the tag. To test this hypothesis, I took two approaches. First, I asked whether the deletion of Ard1, part of the N-terminal acetylation complex NatA (Mullen et al., 1989; Park and Szostak, 1992), would relieve the stabilization I observed with 6HA tagged Matα1. However, in *ard1Δ* mutant cells, N-terminally tagged Matα1 remains stabilized (Figure A-3A). No definitive conclusion can be made from this data, as I do not know whether the deletion of Ard1 did, in fact, relieve the hypothetical acetylation of N-terminally tagged Matα1. Second, I looked at Matα1 mutants in which the N-terminal sequence was replaced with that of known acetylation targets Pre1, Pup2, and Pre10 (Kimura et al., 2000). These mutants were all tagged at the C-terminus with a 3HA epitope. None of the mutants showed any change over wild-type degradation kinetics (Figure A-3B). Again, I can draw no conclusion regarding these assays, as I do not know whether or not adding the sequences from Pre1, Pup2, or Pre10 was successful in making Matα1 a substrate for acetylation. Further testing, such as analysis by mass spectroscopy, to determine if the N-terminus of Matα1 is a site of ubiquitylation is necessary.

The second possible reason that N-terminally tagging Matα1 results in stabilization is that the large tag is sterically occluding access to the degradation signal. It is difficult to make an educated guess about what region of Matα1 could potentially be occluded, since I have no information about how the fusion protein, or the native protein may be folding. It is conceivable that it could be blocking access to amino acids 52-75 of Matα1 that I have shown to be required for degradation of the protein in an Slx5-Slx8 dependent manner, but given the degree of stabilization an additional role such as blocking the site of ubiquitylation is likely.

Regardless of how an N-terminal 6HA tag is stabilizing Matα1, this construct provides a valuable reagent. This stabilized version of Matα1 could be used to replace wild-type Matα1 in a homothallic, or mating-type switching competent, strain since it can complement a *matα1Δ* (Figure A-2C). A switching competent strain expressing this stabilized version of Matα1 could be used in quantitative mating experiments to analyze whether or not rapidly degrading Matα1 is biologically relevant.

Discussion and Future Directions

There are many examples in the field showing that ubiquitylation is not often confined to a specific lysine (King et al., 1996; Rodriguez et al., 2000; Treier et al., 1994), but there are far fewer examples of ubiquitylation on alternative sites such as the extreme N-terminus (Breitschopf et al., 1998) or alternative amino acids (Cadwell and Coscoy, 2005). Given this information, I

was surprised to find that a lysine-less Matα1 is stabilized only two- to three-fold more than wild-type Matα1 (Figure A-1). This data suggests that alternative sites within Matα1 can be used for attachment of ubiquitin. This construct could be analyzed in the context of deletions of the other ubiquitin ligases I have identified to determine if one or more of them are mediating degradation of this mutant Matα1.

Furthermore, N-terminally tagging Matα1 prevents rapid turnover of the protein suggesting that the N-terminus should be examined more extensively as a possible site of ubiquitylation. Shortening the N-terminal tag, by using only one HA or a 3HA tag, or adding a small number of arbitrary amino acids to the N-terminus could yield more information as to how this tag is altering Matα1's degradation rate. Analysis of Matα1 by mass spectroscopy might also offer insight into the site of ubiquitylation and whether or not it is occurring at the extreme N-terminus of the protein.

The secondary reason for pursuing this line of experimentation was to construct a reagent for further study of the biological relevance of Matα1 proteolysis. The assumption in the field is that degradation of Matα1 is vital to faithful mating-type switching. This assumption is based largely on the current understanding of yeast mating-type biology and the fact that degradation of Matα2 is required for efficient mating-type switching (Laney and Hochstrasser, 2003). This assumption bears experimental verification. The N-terminally tagged Matα1 construct, 6HA-Matα1, is functional making it an ideal reagent for investigating the biological relevance of Matα1. By replacing the

endogenous copy of Matα1 with the N-terminally tagged version in a homothallic strain, which is capable of mating-type switching, quantitative mating assays can be preformed to determine if switching is impaired when Matα1 persists in a cell.

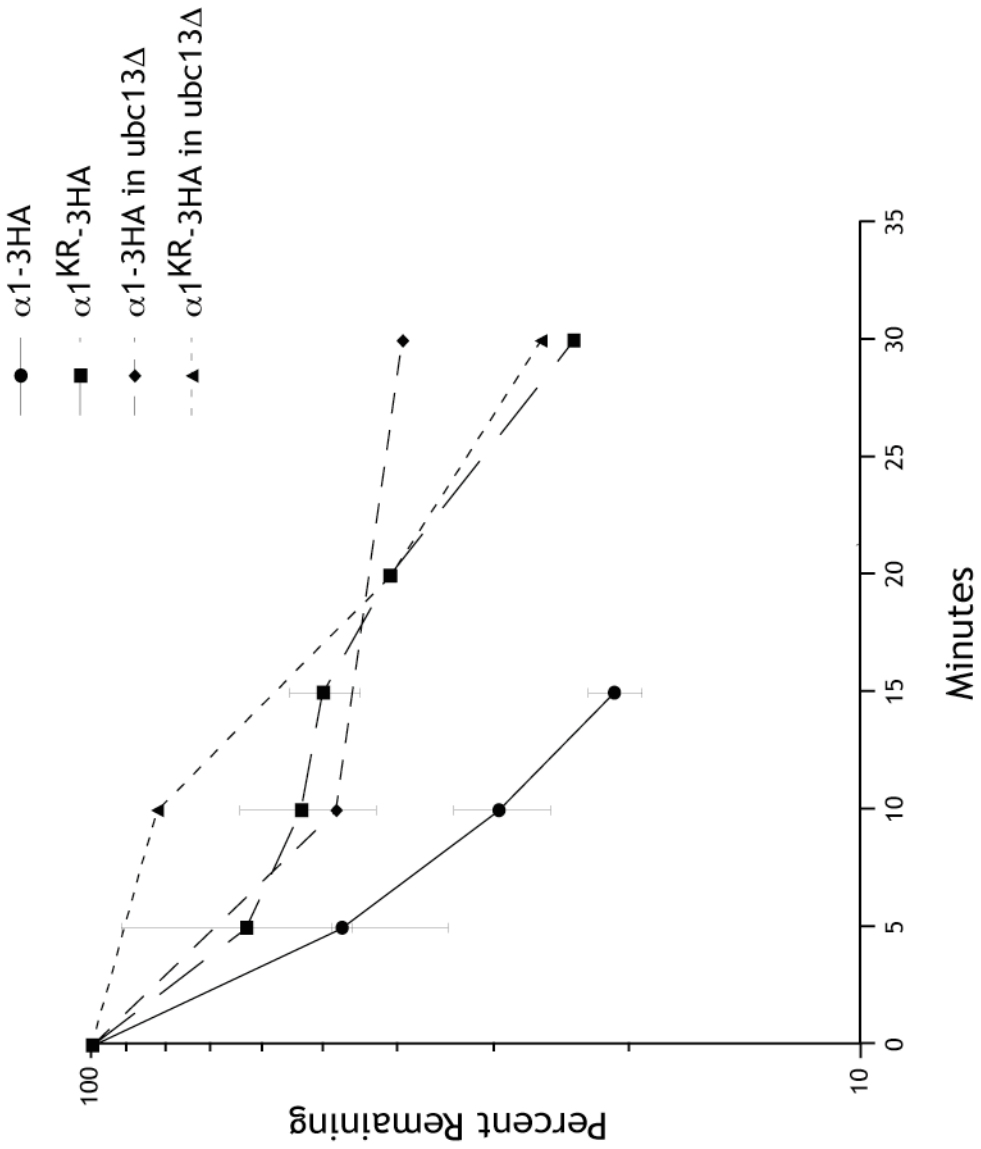
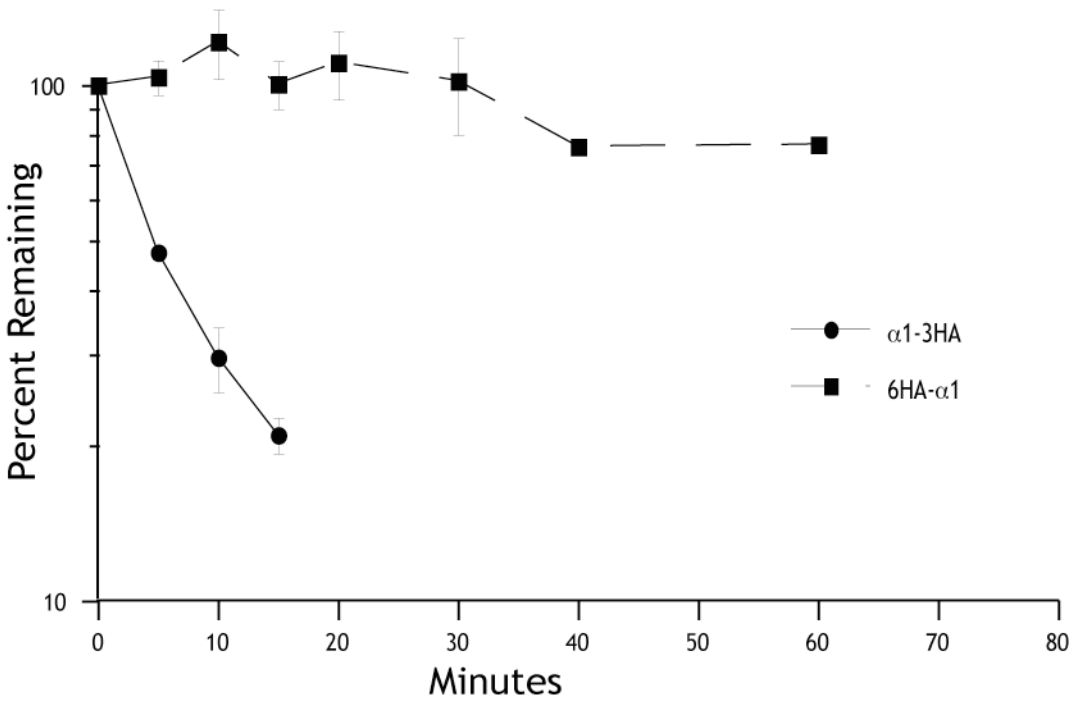

α1-3HA
α1KR-3HA
α1-3HA in ubc13Δ
α1KR-3HA in ubc13Δ
Percent Remaining
100
10
0
5
10
15
20
25
30
35
Minutes

Figure A-1. Lysine-less Matα1 is stabilized only 2-3 fold. Quantification of pulse-chase experiments analyzing p414ADH-α1-3Ha and p414ADH-$\alpha1^{KR}$-3HA in either wild type cells (MHY501) or *ubc13Δ* (JY389). While Matα1 has a half-life of 5-7 minutes by pulse-chase assay, Mat$\alpha1^{KR}$ has a half-life of approximately 14-15 minutes. Additionally, deletion of Ubc13 does not further exacerbate the half-life alteration.

A.


100
10
Percent Remaining
0
10
20
30
40
50
60
70
80
Minutes
α1-3HA
6HA-α1

B.

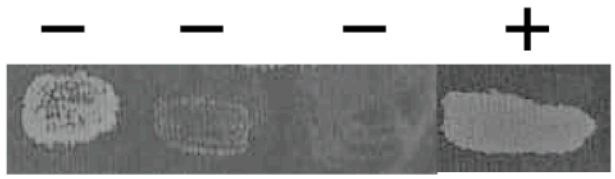

−
−
−
+
6HA-α1
α
a
α1Δ
α1Δ

Figure A-2. N-terminally tagged Matα1 is strongly stabilized.

A. Quantification of pulse-chase experiments showing that p414ADH-6HA-α1 is strongly stabilized as compared to a C-terminally tagged Matα1 (p414ADH-α1-3HA) in wild type cells (MHY501). B. Strains are patched onto plate lacking all amino acids and inoculated with the mating-type tester D585a. N-terminally tagged Matα1 is able to complement a *Matα1Δ* (JY357) as indicated by growth. a refers to D585a and α refers to M84α and are used here as controls.

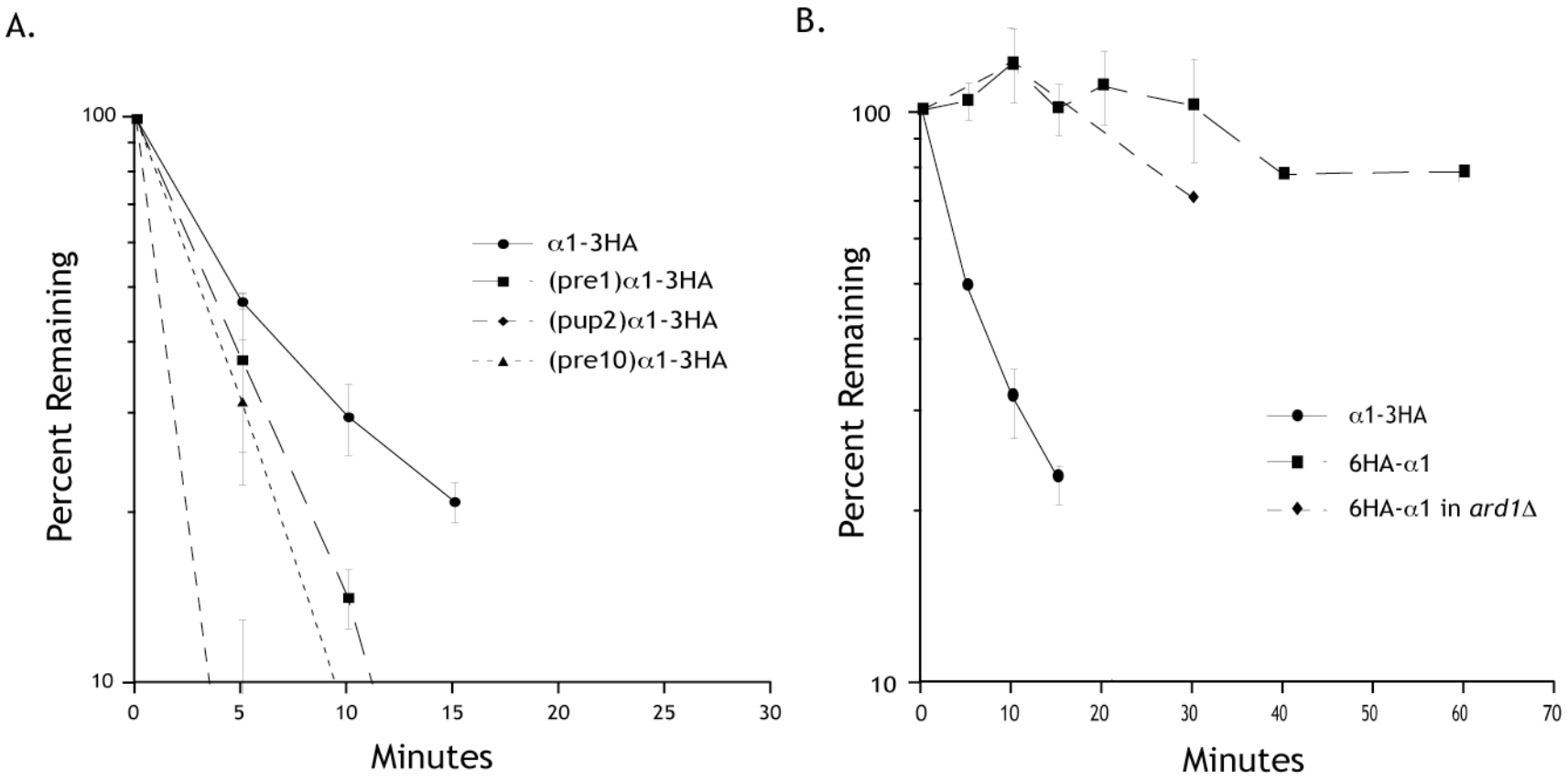

A.
100
10
Percent Remaining
0
5
10
15
20
25
30
Minutes
α1-3HA
(pre1)α1-3HA
(pup2)α1-3HA
(pre10)α1-3HA
B.
100
10
Percent Remaining
0
10
20
30
40
50
60
70
Minutes
α1-3HA
6HA-α1
6HA-α1 in ard1Δ

Figure A-3. Acetylation mutants are unable to alter the phenotype of either C-terminally or N-terminally tagged Matα1. A. Quantification of pulse-chase experiments in wild type cells (MHY501) showing that replacing the N-terminus of Matα1 with that of known acetylation targets, Pre1, Pup2, or Pre10, does not cause the protein to be stabilized. B. Quantification of pulse-chase experiments of wild type cells (MHY501) or an *ard1Δ* (JY608) expressing p414ADH-6HA-α1 shows that removing Ard1 does not relieve the stabilization observed for N-terminally tagged Matα1.

Nature of Matα1 Ubiquitylation

The prevailing thought in the ubiquitin field has been that substrates are directed to the proteasome by lysine 48-linked poly-ubiquitin chains (Chau et al., 1989; Thrower et al., 2000). In recent years, examples of alternative chains targeting substrates to the proteasome (such as mixed linkage chains in the case of cyclinB1 or K63-linked chains) have been uncovered (Hofmann and Pickart, 2001; Kirkpatrick et al., 2006). Even though the shortest signal thought to be recognized by the proteasome has always been a tetraubiquitin chain (Thrower et al., 2000), there is also evidence that monoubiquitylation can direct substrates to the proteasome (Boutet et al., 2007).

To ask what type of ubiquitylation is required for Matα1 turnover, I over-expressed copper-driven ubiquitin mutants in wild-type cells expressing p414ADH-α1-3HA. In the presence of ubiquitin mutants incapable of making K48-linkages (Ub^{K48R} or $Ub^{K48R/G76A}$) or a mutant that cannot form chains by any lysine linkage (Ub^{KO} or $Ub^{KO/G76A}$), two substrates known to be targeted to the proteasome by poly-ubiquitin chains, Matα2 and $Rpn4_{1-229}$-3HA, are stabilized (Hochstrasser et al., 1991; Johnson, 1997; Ju and Xie, 2004) (Figure A-4B,C). However, under the same conditions, Matα1 turnover is unaltered (Figure A-4A).

This data suggests that lysine-linked ubiquitin chains are not important for degradation of Matα1, and points to monoubiquitylation being sufficient for turnover. These experiments cannot rule out short, lysine-linked chains that utilize endogenous, wild-type ubiquitin present in the cells. Additionally,

linear ubiquitin chains, in which ubiquitin moieties are covalently attached via the C-terminal glycine and the N-terminal methionine (Kirisako et al., 2006), cannot be excluded by this data either. Interestingly, LUBAC, the ligase complex that forms linear ubiquitin chains is required for the turnover of a synthetic ubiquitin fusion degradation pathway substrate, Ub-GFP (Kirisako et al., 2006). Given the involvement of a ubiquitin ligase from the ubiquitin fusion degradation pathway, Ufd4, in the destruction of Matα1, linear ubiquitylation is an interesting lead to follow-up on.

Discussion and Future Directions

Many questions remain regarding the nature of Matα1 ubiquitylation. A monoubiquitylated form of Matα1 has been detected by greatly overexpressing the protein (Johnson, 1997). I have shown that lysine-linked ubiquitin chains are not required for Matα1 turnover (Figure A-4), but I cannot rule out the formation of short chains in these assays. To rule out short ubiquitin chains made utilizing endogenous ubiquitin, a method has been described in which the mutant copy of ubiquitin can be expressed in yeast cells as the only copy of ubiquitin. The cells express a wild type copy of ubiquitin driven by the GAL promoter. The expression of the mutant copy is induced at the same time the wild type copy is repressed by adding glucose to the media (Kravtsova-Ivantsiv et al., 2009). Analyzing the turnover of Matα1 under these conditions would rule out any contribution that endogenous ubiquitin might be making and strengthen the case for either linear ubiquitin chains or monoubiquitylation.

This observation leaves open the possibility that either linear ubiquitin chains or monoubiquitylation (a single moiety or multiple monoubiquitylation events) are required. To determine if the linear ubiquitin chains are involved in Matα1 degradation, one could use a methylated form of ubiquitin, which has no acceptors available for conjugation (lysines or a free amino group at the N-terminus of ubiquitin) in an *in vitro* ubiquitylation assay. If methylated ubiquitin is still sufficient for Matα1 degradation this strongly suggests that monoubiquitylation is involved. However, if Matα1 degradation is abrogated in the presence of methylated ubiquitin, then linear ubiquitin chains are implicated.

Directly assaying Matα1 ubiquitylation in the presence of a lysine-less ubiquitin mutant could offer answers to this question. If a monoubiquitylated species of Matα1 is detected, then monoubiquitylation is sufficient for its turnover. Detection of poly-ubiquitylated Matα1 under these conditions would suggest linear ubiquitylation. Furthermore, analysis by mass spectroscopy could potentially address the site as well as the nature of Matα1 ubiquitylation.

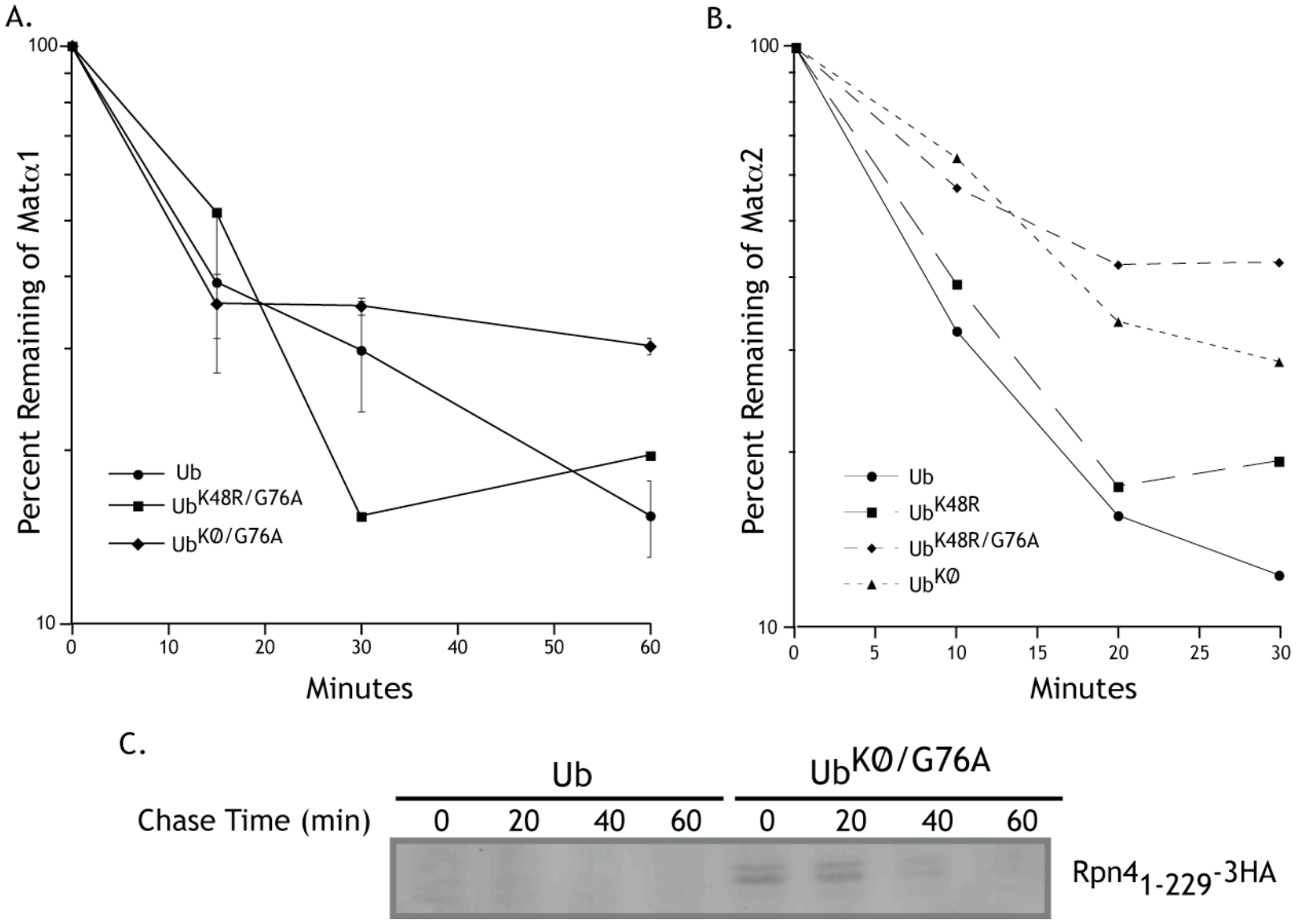

A.
Percent Remaining of Matα1
100
10
0
10
20
30
40
50
60
Minutes
Ub
Ub^K48R/G76A
Ub^KØ/G76A
B.
Percent Remaining of Matα2
100
10
0
5
10
15
20
25
30
Minutes
Ub
Ub^K48R
Ub^K48R/G76A
Ub^KØ
C.
Ub
Ub^KØ/G76A
Chase Time (min)
0
20
40
60
0
20
40
60
Rpn4 1-229 -3HA

Figure A-4. Rapid degradation of Matα1 is not dependent upon canonical ubiquitin chains. A. Matα1's stability is not altered in the presence of over-expressed wild type ubiquitin (p425CUP1-Ub), $Ub^{K48R/G76A}$ (p425CUP1- $Ub^{K48R/G76A}$), Ub^{K0} (p425CUP1- Ub^{K0}), or $Ub^{KO/G76A}$ (p425CUP1- $Ub^{KO/G76A}$) as assayed by cycloheximide chase. B. Pulse-chase analysis of Matα2 over-expressed from YEp13-Matα in the presence of over-expressed wild type ubiquitin (YEp96), Ub^{K48R} (YEp110), $Ub^{K48R/G76A}$ (pUb204), or Ub^{K0} (p424CUP1- Ub^{K0}). Matα2 is stabilized 2-fold in both $Ub^{K48R/G76A}$ and Ub^{K0}. C. Cycloheximide chase of an N-terminally tagged fragment of Rpn4, p314CUP1/HA-$Rpn4_{1-229}$, is stabilized in the presence of $Ub^{KO/G76A}$ (p425CUP1- $Ub^{KO/G76A}$).

The Distal Degradation Pathway for Matα1

While investigating the *trans*-factors involved in Matα1 degradation, I found that Matα1 turnover requires the ubiquitin ligase Ufd4, which is a member of the ubiquitin fusion degradation pathway. Another member of the ubiquitin fusion degradation pathway is Ufd2 (Johnson et al., 1995). Ufd2 is a U-box containing ubiquitin ligase that functions as a chain assembly factor to elongate chains started by other ubiquitin ligases, making them more appealing targets for destruction by the proteasome. In addition, Ufd2 has been shown to interact with the AAA-ATPase Cdc48 (Koegl et al., 1999), which another member of the Laney lab has shown to be required for Matα1 proteolysis (R. Brese, unpublished).

Intriguingly, deletion of Ufd2 stabilizes Matα1 approximately three- to four-fold over wild-type (Figure A-5). Epistasis experiments put Ufd2 in the same genetic pathway as Ufd4, Slx5, and Slx8, since double deletion combinations between Ufd2 and any of these ubiquitin ligases results in the same change in Matα1 turnover observed for single deletions of these factors (Figure A-5). This data suggests that Ufd2 is functioning in a distal degradation pathway to target Matα1 to the proteasome. It is potentially elongating shorter chains or extending monoubiquitylation events started by the members of the proximal pathway. The involvement of this chain elongator and the data I have suggesting that Matα1 turnover is independent of lysine-linked chains (Figure A-4) points to linear ubiquitin chains having a role in degradation of Matα1.

Discussion and Future Directions

In addition to characterizing the proximal degradation pathway for Matα1, I have found a factor involved in Matα1 degradation post-ubiquitylation. Ufd2 is required for Matα1 turnover (Figure IV-5), and my initial epistasis experiments suggest that it is functioning in the same genetic pathway as Slx5, Slx8, and Ufd4. Is Ufd2 in the same pathway as Dma1 and/or Dma2?

Additionally, the AAA-ATPase Cdc48 also modulates Matα1 degradation (R. Brese, unpublished). Cdc48 and Ufd2 are known to interact (Koegl et al., 1999). Are they interacting in this instance? What role does Cdc48 have in Matα1 turnover? Cdc48 has been shown to pull proteins out of the endoplasmic reticulum and into the cytosol for degradation (Ye et al., 2003). Cdc48 could be functioning to physically remove Matα1 from its transcriptionally active state on DNA. Chromatin immunoprecipitation experiments looking at the occupancy of Matα1 in temperature-sensitive mutants of Cdc48 could begin to address these questions. These types of experiments have already been used to show that Cdc48 is functioning to remove Matα2 from DNA (Wilcox and Laney, 2009, submitted). Cdc48 could aid in the quick removal of both Matα1 and Matα2 from their active state on DNA allowing for a quick transition between α and <u>a</u> cell types upon mating-type switching.

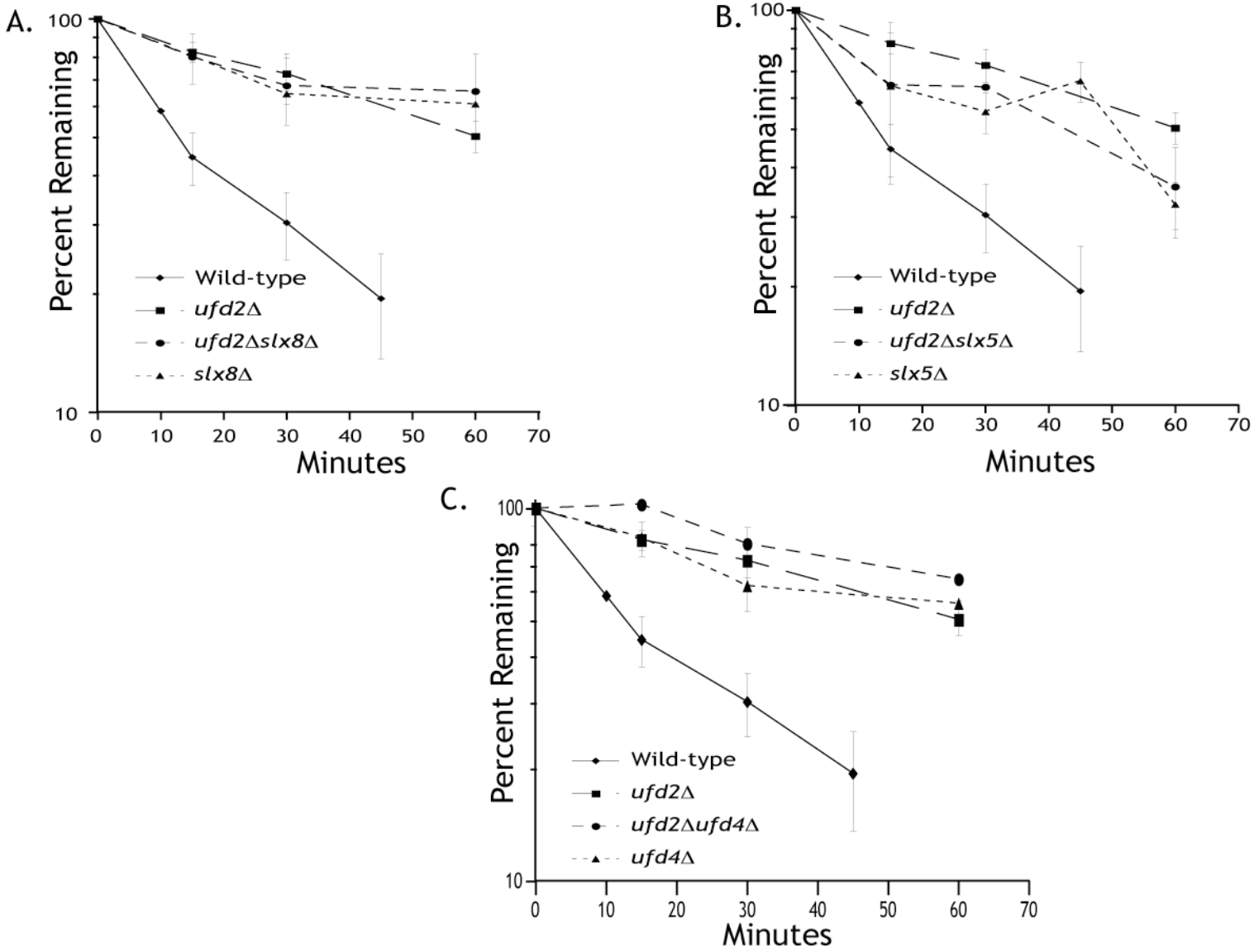

A.
Percent Remaining
100
10
0
10
20
30
40
50
60
70
Minutes
Wild-type
ufd2Δ
ufd2Δslx8Δ
slx8Δ
B.
Percent Remaining
100
10
0
10
20
30
40
50
60
70
Minutes
Wild-type
ufd2Δ
ufd2Δslx5Δ
slx5Δ
C.
Percent Remaining
100
10
0
10
20
30
40
50
60
70
Minutes
Wild-type
ufd2Δ
ufd2Δufd4Δ
ufd4Δ

Figure A-5. Matα1 is stabilized in a *ufd2Δ*. A. Quantitation of cycloheximide Chase experiments showing that Ufd2 (JY604) and Slx8 (MHY3716) are in the same genetic pathway. B. Quantitation of cycloheximide chase experiments showing that Ufd2 (JY604) and Slx5 (MHY3712) are in the same genetic pathway. C. Quantitation of cycloheximide chase experiments showing that Ufd2 (JY604) and Ufd4 (JY612) are in the same genetic pathway.

Ploidy-specific Degradation Rates for Matα1

It has been previously observed that Matα1 is degraded more rapidly in diploid cells than it is in haploid cells (Johnson, 1997). There are two distinct differences between haploid and diploid cells. First, haploid cells have one genetic complement while diploid cells contain two. Second, diploid cells have both MAT$\underline{a}$ and MATα alleles giving them a unique gene expression program from haploid cells (Figure I-3). Either of the two differences could be responsible for the diploid-specific degradation rate observed for Matα1.

I used a plating assay to begin to distinguish between the two possibilities. For these assays, I used a Matα1 fused to the uracil biosynthetic enzyme Ura3. Previously, I had shown that this fusion driven by the ADH promoter (p414ADH-α1-URA3-3HA) does not support growth on minimal media lacking uracil (Figure III-1). However, if expression of this fusion is driven by a stronger promoter, TEF (Mumberg et al., 1995), I observe that the fusion (p414TEF-α1-URA3-3HA) is able to support growth on media lacking uracil in haploid strains, but not in diploid strains (Figure A-6A). This difference in growth phenotype is seen, even though degradation rates for either a fusion driven by the ADH promoter or the TEF promoter in haploids cells are indistinguishable (Figure A-6B).

The unique gene expression program present in diploids cells stems from the presence of both $\underline{a}$ and α mating-type alleles allowing for the formation of an $\underline{a}$1/α2 heterodimer. I used diploid strains mutant for either $\underline{a}$1 ($\underline{a}1^{-}/\alpha$ diploid: MHY892) or α2 ($\underline{a}/\alpha 2^{-}$ diploid: MHY940) to ask whether altering this

heterodimer would result in a haploid-like growth phenotype on media lacking uracil. These mutations do not alter the growth phenotype observed for wild type diploid cells (Figure A-6A). Additionally, I assayed the growth phenotype of a diploid strain containing two copies of MATα (α/α diploid: JY367) constructed by over-expressing the HO endonuclease to permit genetic switching and then selecting strains that were able to mate. This alternative diploid was also unable to support growth in the absence of uracil just like a wild type diploid strain (Figure A-6C). In a counter-experiment, I added either a plasmid expressing the *MAT*$\underline{a}$ allele (YEp13-MAT$\underline{a}$) or the parent vector (YEp13) to an α haploid strain to ask if the haploid strain could be converted to a diploid-like growth phenotype. However, the haploid cells expressing the TEF driven fusion were still able to grow on media lacking uracil regardless of the addition of the *MAT*$\underline{a}$ allele (Figure A-6D).

Having genetically ruled out the unique mating-type gene expression program in diploids, I then used the same plating assay to ask about chromosome number. Using the alternative diploids I had constructed previously, I mated an $\underline{a}/\underline{a}$ diploid (JY368) and an α/α diploid (JY367) to obtain a tetraploid strain ($\underline{a}/\underline{a}/\alpha/\alpha$: JY376). I then plated this polyploid strain onto media lacking uracil. I let the strains grow at 30°C for five to seven days (two to three days longer than the other plating assays). The additional incubation time allowed the diploid strain to begin to form colonies in the absence of uracil, but the tetraploid strain had not started to grow suggesting that the degradation rate of the fusion is even faster with increased chromosomal ploidy

(Figure A-7A). A pilot pulse-chase experiment comparing the degradation of p414TEF-α1-URA3-3HA in either haploid or diploid cells confirms that turnover of the fusion is somewhat faster in diploid cells (Figure A-7B).

The genetic evidence presented by these plating assays suggests that chromosomal ploidy is the cause of Matα1's increased rate of turnover in diploid cells, and the initial biochemical data agrees with this conclusion. The factors I have identified as part of Matα1's degradation pathway could be used to further probe this question. Rendering a diploid strain deficient of one copy of a *trans*-acting factor could potentially result in a haploid-like degradation rate.

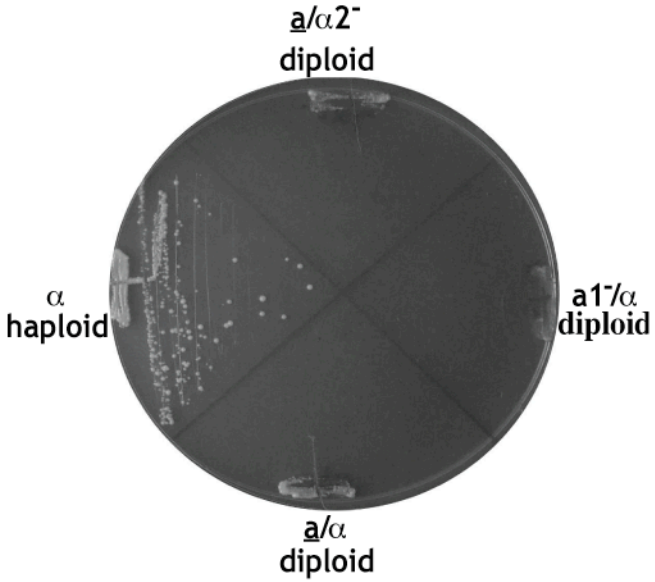
Discussion and Future Directions

Diploid cells degrade Matα1 at a slightly faster rate than haploids cells (Johnson, 1997). Additionally, plating assays and initial biochemical data suggest that cells of increasing ploidy can degrade Matα1 at an even higher rate. My experiments also implicate ploidy as the basis for the cell-type specific degradation of Matα1 and not the cell type-specific expression of the mating-type program.

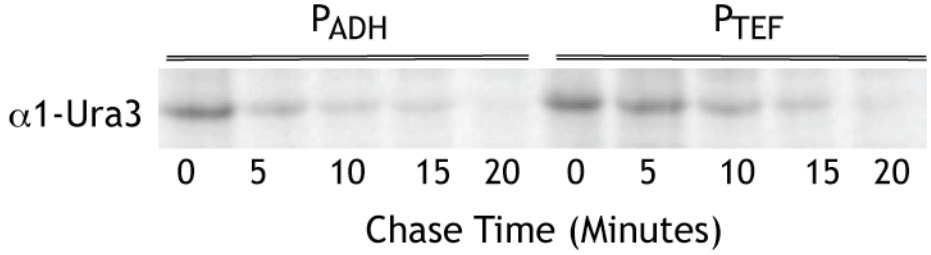
To further dissect this basis, one could turn to the factors I have identified as regulators of Matα1. For example, does removing one copy of Ubc13 from a diploid cell alter the degradation rate of Matα1 to what I observed in haploid cells? If the answer is yes, it suggests that a dosage effect of Ubc13 is causing the difference in turnover rates, and one would predict

that over-expressing Ubc13 in a haploid cell would increase degradation of Matα1. This line of experimentation could be repeated for the other factors I have identified.

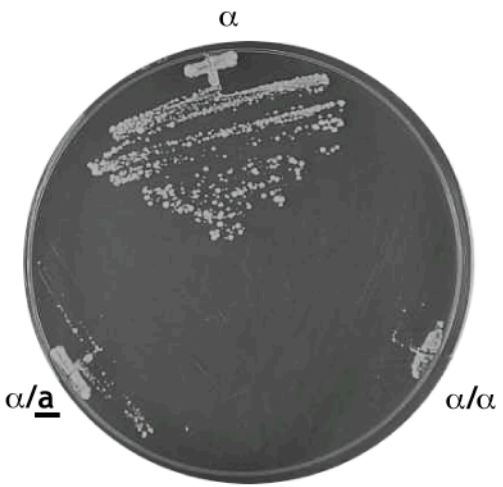
A.


a/α2⁻
diploid
α
haploid
a1⁻/α
diploid
a/α
diploid

B.


PADH
PTEF
α1-Ura3
0 5 10 15 20 0 5 10 15 20
Chase Time (Minutes)

C.


α
α/a
α/α

D.

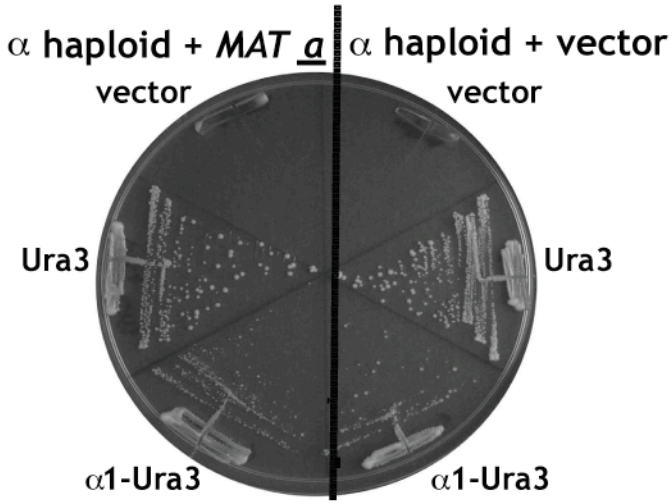
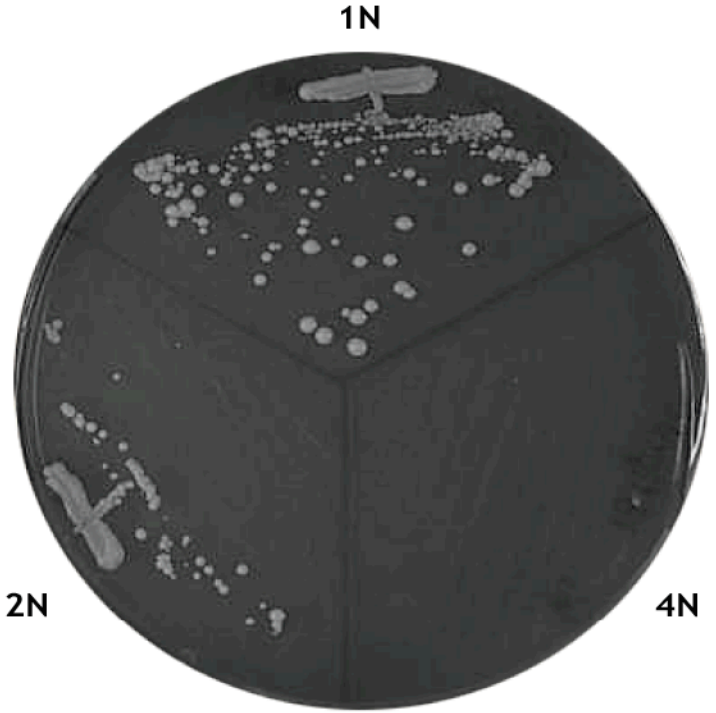

α haploid + MAT a
vector
α haploid + vector
vector
Ura3
Ura3
α1-Ura3
α1-Ura3

Figure A-6. Altering the a/α gene expression program does not alter the degradation kinetics observed for haploid and diploid cells. All strains are transformed with p414TEF-α1-URA3-3HA unless otherwise noted. A. SD-trp-ura plate showing growth phenotypes of an α haploid (MHY501), an a/α diploid (MHY606), an $a1^{-}/\alpha$ diploid (MHY892), and an a/α 2-diploid (MY940). B. Pulse-chase assay showing that both the p414ADH-α1-URA3-3HA (P_{ADH}) and p414TEF-α1-URA3-3HA (P_{TEF}) fusions are rapidly degraded. C. SD-trp-ura plate showing growth phenotype of an α haploid (MHY501), an a/α diploid (MHY606), and an α/α diploid (JY367). D. SD-trp-ura plate showing growth phenotype for an α haploid (MHY501) transformed with either a vector (YEp13) or a plasmid expressing the *MATa* allele (YEp13-MATa) and one of the following, vector (p414TEF), Ura3 (p414TEF-URA3-3HA), or α1-URA3 (p414TEF-α1-URA3-3HA).

A.


1N
2N
4N

B.

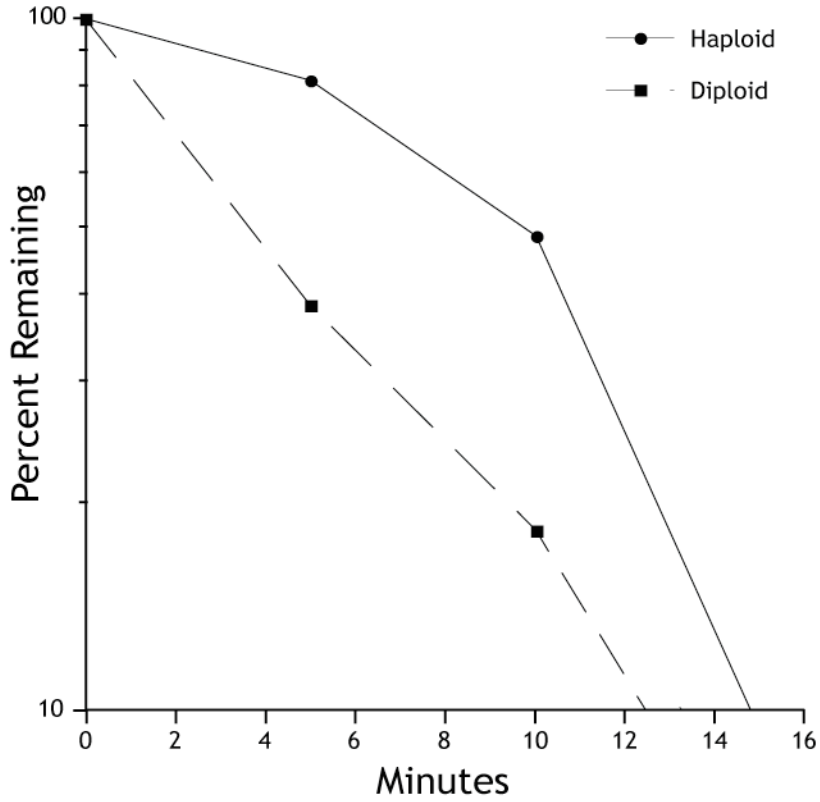

100
10
Percent Remaining
0
2
4
6
8
10
12
14
16
Minutes
Haploid
Diploid

Figure A-7. An increase in chromosomal ploidy results in an increase in the rate of Matα1 degradation. A. SD-trp-ura plate showing growth phenotype for an α haploid (1N: MHY501), an a/α diploid (2N: MHY606), and an a/a/α/α tetraploid (4N: JY376) all of which are expressing p414TEF-α1-URA3-3HA. B. Quantificatiomn of pulse-chase experiments showing that turnover of p414TEF-α1-URA3-3HA is faster in diploid cells (MHY606) than in haploid cells (MHY501).

Methods for Appendices

Plasmids

Information regarding plasmid construction can be found in Appendix 7 (Table 1).

Yeast Strains

Information regarding strain construction can be found in Appendix 7 (Table 2).

Plating assays

Cells containing the plasmid p414TEF-α1-URA3-3HA were struck onto SD-trp-ura, a minimal media lacking tryptophan (to select for the plasmid) and uracil (to select for stabilized fusions). Plates were placed at 30°C for 3-5 days.

Copper induction

Plasmids driven by the *CUP1* promoter were induced by adding 100μM $CuSO_4$ to cells 6-7 hours prior to the start of either a pulse-chase or cycloheximide chase experiement.

Pulse-chase and TCA lysis

Cells were grown to mid log phase (OD_{600} 0.5 - 1.0) in a minimal media lacking the appropriate amino acid for plasmid selection. Approximately 10 cell

ODs were harvested by centrifugation (3000rpm for 5 minutes at room temperature). Cells were washed twice with 1ml of wash buffer (1X SD, 2% glucose, no amino acids) and transferred to 1.5mL Eppendorf tubes. Cells were resuspended in 200μL of minimal media lacking methionine and the amino acid necessary for plasmid selection. The tubes were put into a dry bath at 30°C. Subsequently, 100-200μCi of [35S] Trans-Label (MP Biomedicals and PerkinElmer) was added to each tube. Cells were allowed to label for 10 minutes keeping them suspended by periodic vortexing. Cells were spun for 10 seconds in a microfuge to pellet them. The supernatant was removed and 200μL of chase buffer (1X SD, 2% glucose, 10mM methionine, 0.5mg/ml cycloheximide, and the required amino acids). Immediately following addition of the chase buffer, 50μL of cells were transferred to an Eppendorf tube containing 50μL of stop buffer (40mM methionine, and 20mM NaN_3) on ice. This step was repeated for each subsequent time point. After the time course was finished each sample was washed one time with ice-cold dH_2O.

Protein was extracted using a TCA lysis as previously described (Loayza et al., 1998) except for experiments looking at Matα2. Samples were normalized within a time course by determining the TCA precipitable counts and transferring the normalized volumes to fresh 1.5mL Eppendorf tubes on ice. To immunoprecipitate 3HA tagged Matα1, 4μL of 25 mg/mL HA antibody (12CA5, Roche) was added to each sample and mixed by inversion at 4°C overnight. Samples were then spun down (13Krpm for 15 minutes at 4°C) to remove any non-specific aggregates, and supernatants were transferred to

fresh tubes containing 20μL of a 50% slurry of Protein A-agarose (Repligen). Samples were rotated end-over-end at 4°C to keep beads in suspension for 1-2 hours. Samples were washed 3-4 times with TCA dilution buffer plus 0.1% SDS. Following the last wash, 50μL of 2X Laemmli loading buffer was added and samples were boiled for 3 minutes.

For experiments analyzing Matα2, proteins were extracted from cells over-expressing Matα2 from the plasmid Yep13-Matα using an SDS lysis. Following the chase, 50μl aliquots were taken at indicated time-points and transferred to Eppendorf tubes containing 50μl of 2X SDS lysis buffer (2% SDS, 90mM HEPES, pH 7.5, and 30mM DTT). Tubes were then boiled for 5-8 minutes and placed on ice. The protocol described by (Loayza et al., 1998) was then followed. Matα2 was immunoprecipitated with 5μl of α-Mat α2 sera (antibody a gift from M. Hochstrasser).

To visualize, the entire sample was loaded on a large format 0.75mm SDS-PAGE (10-12%). After running overnight, the gel was fixed in 10% MeOH/ 10% acetic acid, placed on Whatman paper, and dried in a gel dryer. The gel was then exposed to a phosphoimager screen overnight and analyzed using a Typhoon.

Cycloheximide chase and NaOH/SDS lysis

Cells were grown to mid log phase (OD_{600} 0.5 -1.0) in minimal media lacking the appropriate amino acid for plasmid selection. Cycloheximide was added to a final concentration of 0.2 mg/mL from a stock of 10 mg/mL in dH_20

to each culture. Immediately after cycloheximide addition, 2.5 ODs of cells were harvested and put in a 50mL conical tube containing 10mL of ice-cold dH_20 and 30mM NaN_3 and placed on ice. Cells were harvested in this way for each subsequent time point.

Cells were collected by centrifugation (3000rpm for 5 minutes at 4°C). The supernatants were poured off, and cell pellets were resuspended in 1mL of ice-cold dH_20 and transferred to 1.5mL Eppendorf tubes. Cells were re-pelleted by centrifugation (13K for 1 minute at room temperature). The supernatant was aspirated off, and the pellet was resuspended in 200μL of 0.1M NaOH and allowed to incubate at room temperature for 5 minutes. Cells were again collected by centrifugation (13K for 1 minute at room temperature). The supernatant was removed and the pellet was resuspended in 50μL of 1X Laemmli Loading Buffer and boiled for 3 minutes (Kushnirov, 2000).

Samples were then spun down (13K for 3 minutes at room temperature) and 1 OD was loaded of each sample (unless otherwise noted) onto a large format 1.5 mm SDS-Page (10-12%). After running overnight, gels were transferred and visualized by Western blotting. Where indicated a cycloheximide chase was performed followed by an immunoprecipitation of the HA tag which was conducted as described for pulse-chase experiments. These samples were also visualized by Western blotting.

Western blotting

To visualize an SDS-PAGE by Western blotting, gels were transferred to a nitrocellulose membrane (Immobilin PVDF) in 1X Transfer Buffer diluted from

10X stock (250 mM Tris-OAc, pH 8.8, 2M glycine, and 0.1% SDS) and 20% Methanol at 20V for 1.5 hours. The membrane was incubated on a rocker in 2% milk in TBST (20 mM Tris-HCL, pH 7.6, 150 mM NaCl, 0.1% Tween) for at least 1 hour at room temperature. Primary was added 1:1000 (25 mg/mL α-HA, 12CA5, Roche or 1:1000 α-Matα2) to 1% milk in TBST and incubated on a rocker overnight at 4°C. The membrane was washed 3 times for 5 minutes each with TBST and then incubated on a rocker at room temperature for 30 minutes to 2 hours in Seablock (Pierce Chemical) with Qdot 605 α-mouse diluted 1:1000 (Invitrogen). The membrane was then was 3 times for 5 minutes each with 3X TBST (0.3% Tween instead of 0.1%) and 2 times for 5 minutes each with TBST. The membrane was then visualized and subsequently analyzed using a Typhoon and ImageQuant software. Data was normalized to the zero time-point within individual experiments to determine percents remaining. Replicates were then averaged.

Table 1 Plasmids

Plasmid Name	Description	Source
pFA6a-3HA-kanMX6		(Longtine et al., 1998)
pfA6a-URA3-3HA-KanMX6	*URA3* amplified with PacI ends cloned into pfA6a-3Ha-KanMX6	This Study
p414ADH		(Mumberg et al., 1995)
p414ADH(ΔPstI)	p414ADH digested with PstI, overhangs removed with T4 Pol Reaction, and religated.	This Study
p414ADH-α1-3HA(ΔPstI)	PstI/AscI fragment containing 3HA and ADH terminator from pFA6a-3HA-kanMX6 replaced URA3-3HA in p414ADH-α1-URA3-3HA(ΔPstI)	This Study
p413ADH-α1-3HA	SacI-P_{ADH}-α1-3HA-AscI fragment was cloned into pRS413	This Study
pBluescript/α1-noLys	*MATα1* with SpeI/PstI ends with all lysine residues changed to arginines was constructed by PCR in a series of steps with primers containing K to R mutations and put in pBluescript	This Study
p414ADH/α1KR-3HA	$MAT\alpha1^{KR}$ with SpeI/PstI ends from pBluescript/α1-noLys was cloned into a similarly digested p414ADH-α1-3HA(ΔPstI)	This Study
p414ADH/6XHA	6XHA tag was amplified from pYM3 with NheI/SpeI ends, sequenced and sub-cloned into SpeI-digested p414ADH.	This Study
p414ADH/6XHA-α1	*MATα1* ORF with SpeI/PstI ends, cloned into p414ADH/6XHA	This Study
p414ADH-α1-URA3-3HA(ΔPstI)	SpeI/BglII fragment removed from p414ADH-α1-URA3-3HA and ligated into p414ADH(ΔPstI) using SpeI/BamHI.	This Study
p414ADH-72-α1-URA3-3HA(ΔPstI)	Amino acids 72-175 of Matα1; includes a start codon	This Study
p414ADH-140-α1-URA3-3HA(ΔPstI)	Amino acids 140-175 of Matα1; includes a start codon	This Study
p414ADH-22-α1-URA3-3HA(ΔPstI)	Amino acids 22-175 of Matα1; includes a start codon	This Study
p414ADH-107-139-α1-URA3-3HA(ΔPstI)	Amino acids 107-139 of Matα1; includes a start codon	This Study
p414ADH-92-α1-URA3-3HA(ΔPstI)	Amino acids 1-92 of Matα1	This Study
p414ADH-57-α1-URA3-3HA(ΔPstI)	Amino acids 1-57 of Matα1	This Study
p414ADH-27-α1-URA3-3HA(ΔPstI)	Amino acids 1-27 of Matα1	This Study
p414ADH-107-α1-URA3-3HA(ΔPstI)	Amino acids 107-175 of Matα1; includes a start codon	This Study
p414ADH-25-150α1-URA3-3HA(ΔPstI)	Amino acids 25-150 of Matα1; includes a start codon	This Study

Plasmid Name	Description	Source
p414ADH-75-150α1-URA3-3HA(ΔPstI)	Amino acids 75-150 of Matα1; includes a start codon	This Study
p414ADH-150α1-URA3-3HA	Amino acids 1-150 of Matα1	This Study
p414ADH-116&174-α1-URA3-3HA(ΔPstI)	S116P and A174T mutations in the ORF of *MATα1*	This Study
p414ADH-A137V-α1-URA3-3HA(ΔPstI)	A137V mutation in the ORF of *MATα1*	This Study
p414ADH-M36V-α1-URA3-3HA(ΔPstI)	M36V mutation in the ORF of *MATα1*	This Study
p414ADH-pre1alpha1-3HA	N-terminal sequence of Pre1 replaces that of Matα1	This Study
p414ADH-pup2alpha1-3HA	N-terminal sequence of Pup2 replaces that of Matα1	This Study
p414ADH-pre10alpha1-3HA	N-terminal sequence of Pre10 replaces that of Matα1	This Study
p414TEF		(Mumberg et al., 1995)
p414TEF-α1	*MATα1* ORF amplified with SpeI and PstI ends was cloned into p414TEF	This Study
p414TEF-α1-URA3-3HA	PstI fragment containing URA3-3HA from pfA6a-URA3-3HA-kanMX6 was cloned into p414TEF-α1	This Study
p414TEF-URA3-3HA	PstI fragment containing URA3-3HA from pfA6a-URA3-3HA-kanMX6 was cloned into p414TEF	This Study
YEp96	P_{CUP1}/Yeast Ub on 2μ/TRP1-marked plasmid	(Ecker et al., 1987)
YEp110	P_{CUP1}/ Ub^{K48R} on 2μ/TRP1-marked plasmid	(Hochstrasser et al., 1991)
pUB204	P_{CUP1}/$Ub^{K48R/G76A}$ on 2μ/TRP1-marked plasmid	(Willems et al., 1996)
pUb197	Ub^{K63R} in Yep96 backbone	(Spence et al., 1995)
p424Cup1-Ub^{KO}	P_{CUP1}/Human ubiquitin with no lysines on 2μ/TRP1 plasmid	
p425CUP1-Ub	BamHI/HindIII fragment from YEp96 cloned into similarly digested pRS425	This Study
p425CUP1-Ub^{K48R}	BamHI/HindIII fragment from YEp110 cloned into similarly digested pRS425	This Study
p425/P_{CUP1}-$Ub^{K48R/G76A}$	BamHI/HindIII fragment from pUB204	This Study
p425Cup1-Ub^{K63R}	BamHI-HindIII fragment containing Ub^{K63R} from pUb197 was cloned into a similarly digested pRS425.	This Study
p425Cup1-$Ub^{K63R/G76A}$	SalI fragment from p425Cup1-$Ub^{K48R/G76A}$ cloned into similarly digested p425Cup1-Ub^{K63R}.	This Study
p425Cup1-Ub^{KO}	BamHI/KpnI fragment with Cup1 promoter and Ub^{K0} from p424Cup1-Ub^{K0} subcloned into pRS425	This Study

Plasmid Name	Description	Source
P425Cup1-$Ub^{KO/G76A}$	Replaced XhoI fragment from p425Cup1-Ub^{KO} with XhoI fragment from p425Cup1-$Ub^{K63R/G76A}$	This Study
YEp13		(Broach et al., 1979)
YEp13-Mat<u>a</u>	HindIII fragment of *MAT<u>a</u>* in YEp13	(Hall and Johnson, 1987)
YEp13-Matα	HindIII fragment of *MAT*α in YEp13	(Hall and Johnson, 1987)
p314Cup1/HA-$RPN4_{(1-229)}$	Cup1 driven N-terminal fragment (amino acids 1-229) of Rpn4	(Ju and Xie, 2004)

Table 2 Yeast Strains

Strain	Abbreviation	Genotype	Source
D585a	a mating tester	*MATa, lys1*	
M84α	α mating tester	*MATα, leu1*	
JY357	*matα1Δ*	matα1Δ::HIS3MX6*, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1*	This study
MHY501	Wild type 1N	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1*	(Chen et al., 1993)
MHY606	Wild type 2N	*MATa/MATα, his3-Δ200/his3-Δ200, leu2-3,112/leu2-3,112, ura3-52/ura3-52, lys2-801/lys2-801, trp1-1/trp1-1*	(Chen et al., 1993)
JY138	*cim3-1*	*MATα, ade 2-101, his3-Δ200, leu2-Δ1, lys2-801, ura3-52, adh4::URA3-TEL-VIIL, DIA5-1, cim3-1*	Laney lab
JY142	*cim5-1*	*MATα, ade 2-101, his3-Δ200, leu2-Δ1, lys2-801, ura3-52, adh4::URA3-TEL-VIIL, DIA5-1, cim5-1*	Laney lab
JY560	*rpn13Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, rpn::HIS3*	This study
JY470	*pdr5Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, pdr5::HIS3*	This study
MHY1061	*pep4Δprb1-Δ1*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, pep4::HIS3, prb1-Δ1::KAN*	(Swaminathan et al., 1999)
RJD3268	*UBA1*	MATa, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, uba1Δ::KanMX, pRS313-UBA1	(Ghaboosi and Deshaies, 2007)
RJD3269	*uba1-204*	MATa, ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100, uba1Δ::KanMX, pRS313-uba1-204	(Ghaboosi and Deshaies, 2007)
MHY623	*doa4Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, doa4::LEU2*	(Papa and Hochstrasser, 1993)
MHY498	*ubc4Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1,* ubc4-Δ1::HIS3	(Chen et al., 1993)
MHY499	*ubc5Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ubc5-Δ1::LEU2*	(Chen et al., 1993)
JY597	*ubc4Δubc5Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ubc4::HIS3, ubc5-Δ1::LEU2*	This study
JY389	*ubc13Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ubc13::KAN*	This study
JY507	*mms2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, mms2::HIS3*	This study
JY527	*rad5Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, rad5::HIS3*	This study
MHY1631	doa10*Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, doa10-Δ1::HIS3*	(Swanson et al., 2001)
JY612	*ufd4Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd4::hphMX*	This study
MHY3712	*slx5Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, slx5::KAN*	Y. Xie/ M. Hochstrasser

Strain	Abbreviation	Genotype	Source
MHY3716	*slx8Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, slx8::KAN*	Y. Xie/ M. Hochstrasser
JY680	*ris1Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ris1::hphMX4*	This study
JY710	*dma1Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::hphMX4*	This study
JY714	*dma2 Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma2::KAN*	This study
MHY2480	*rsp5-2*	*MATα*, lys2-801, his3Δ200, trp1-1, ura3-52::rsp5-2::URA3, leu2-3,112, rsp5Δ::HIS3	(Hoppe et al., 2000)
JY642	*slx5Δubc13Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, slx5::KAN, ubc13::KAN*	This study
JY734	*ufd4Δubc13Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd4::hphMX4, ubc13::KAN*	This study
JY762	*slx8Δubc13Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, slx8::KAN, ubc13::KAN*	This study
JY753	*dma1Δdma2Δubc13Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::hphMX4, dma2::KAN, ubc13::KAN*	This study
JY706	*dma1Δdma2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::hphMX4, dma2::KAN*	This study
MHY3861	*slx5Δslx8Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, slx5::KAN, slx8::KAN*	Y. Xie/ M. Hochstrasser
JY751	*slx5Δufd4Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd4::hphMX4, slx5::KAN*	This study
JY743	*slx8Δufd4Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd4::hphMX4, slx8::KAN*	This study
JY798	*dma1Δslx8Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::HIS3, slx8::KAN*	This study
JY788	*dma1Δslx5Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::HIS3, slx5::KAN*	This study
JY794	*dma1Δufd4Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::HIS3, ufd4::hphMX4*	This study
JY800	*dma2Δslx8Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma2::CaURA3, slx8::KAN*	This study
JY790	*dma2Δslx5Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma2::CaURA3, slx5::KAN*	This study
JY792	*dma2Δufd4Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma2::CaURA3, ufd4::hphMX4*	This study

Strain	Abbreviation	Genotype	Source
JY745	*ufd4Δdma1Δdma2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::hphMX4, dma2::KAN, ufd4::hphMX4*	This study
JY739	*slx5Δdma1Δdma2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::hphMX4, dma2::KAN, slx5::KAN*	This study
JY768	*ufd4Δslx5Δdma1Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::hphMX4, ufd4::hphMX4, slx5::KAN*	This study
JY776	*ufd4Δslx8Δdma2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd4::hphMX4, dma2::CaURA3, slx8::KAN*	This study
JY774	*ufd4Δslx8Δdma1Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd4::hphMX4, slx8::KAN, dma1::HIS3*	This study
JY796	*slx8Δdma1Δdma2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::HIS3, dma2::CaURA3, slx8::KAN*	This study
JY770	*ufd4Δslx5Δdma2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd4::hphMX4, dma2::CaURA3, slx5::KAN*	This study
JY772	*ufd4Δslx5Δdma1Δdma2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd4::hphMX4, slx5::KAN, dma1::HIS3, dma2::CaURA3*	This study
JY778	*ufd4Δslx8Δdma1Δdma2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, dma1::HIS3, dma2::CaURA3, slx8::KAN, ufd4::hphMX4*	This study
JY604	*ufd2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd2::hphMX4*	This study
JY741	*ufd2Δslx8Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, slx8::KAN, ufd2::hphMX4*	This study
JY749	*ufd2Δslx5Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd2::hphMX4, slx5::KAN*	This study
JY732	*ufd4Δufd2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ufd2::hphMX4, ufd4::hphMX4*	This study
JY608	*ard1Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, ard1::KAN*	This study
MHY3997	*ubc9^{ts}*	*MATα, his3-Δ200, leu2-3,112::LEU2::ubc9-1, ura3-52, lys2-801, trp1-1, gal2, ubc9::TRP1*	Y. Xie/ M. Hochstrasser
MHY4664	*uba2^{ts}*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, gal2, uba2::KAN, pIS1-uba2^{ts} #9*	Y. Xie/ M.Hochstrasser
MHY2423	*ulp1^{ts}*	*MATα, his3-Δ200, leu2-3,112::LEU2::ulp1^{ts}(3-33), ura3-52, lys2-801, trp1-1, ulp1::his3::URA3*	D. Schwartz/ M. Hochstrasser
JY764	*mms21-ringΔ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, mms21-Δring::KAN*	This study

Strain	Abbreviation	Genotype	Source
JY802	*siz1Δsiz2Δ*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, siz2::hphMX4, siz1::KAN*	This study
JY817	*smt3allR*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, smt3allR::TRP1*	This study/ E. Johnson
JY818	*smt3-3NR*	*MATα, his3-Δ200, leu2-3,112, ura3-52, lys2-801, trp1-1, smt3-R11, 15, 19::TRP1*	This study/E. Johnson
JY367	α/α	*MATα/MATα, his3-Δ200/his3-Δ200, leu2-3,112/leu2-3,112, ura3-52/ura3-52, lys2-801/lys2-801, trp1-1/trp1-1*	This study
JY368	a/a	*MATa/MATa, his3-Δ200/his3-Δ200, leu2-3,112/leu2-3,112, ura3-52/ura3-52, lys2-801/lys2-801, trp1-1/trp1-1*	This study
JY376	4N	*MATa/MATa/MATα/MATα, his3-Δ200/his3-Δ200/his3-Δ200/his3-Δ200, leu2-3,112/leu2-3,112/leu2-3,112/leu2-3,112, ura3-52/ura3-52/ura3-52/ura3-52, lys2-801/lys2-801/lys2-801/lys2-801, trp1-1/trp1-1/trp1-1/trp1-1*	This study
MHY892	a1$^-$/α	*mata1-aX20/MATα, trp1/trp1, leu2/leu2, ura3/ura3, his4/his4*	(Johnson et al., 1998)
MHY940	a/α2$^-$	*MATa/matα2-αX182, trp1/trp1, leu2/leu2, ura3/ura3, his4/his4*	(Johnson et al., 1998)

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