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The K. Marxianus Small Protein Modifier Sumo Enhances Stress Tolerance In The Mesophilic Yeast S. Cerevisiae

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The *K. marxianus* small protein modifier SUMO enhances stress tolerance in the mesophilic yeast *S. cerevisiae*

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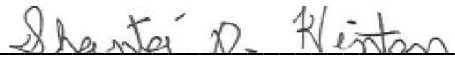

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COMPLIANCE PAGE

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ABSTRACT

SUMO, a small ubiquitin-like modifier protein, becomes attached to specific eukaryotic proteins to modulate their function and activity. The importance of SUMO modification in cell cycle progression, transcriptional regulation, and DNA damage-related processes has been firmly established. In contrast, a SUMO-dependent Stress Response (SSR) exists, but this process remains ill-defined. When cells are exposed to proteotoxic and genotoxic stressors, the SSR involves a rapid and dramatic increase in SUMO-modified proteins. The SSR is believed to play a cytoprotective role for normal cells, but it may also enhance the robustness of cancerous cells and eukaryotic pathogens. To test our hypothesis that SUMO and SUMO pathway components play a role in stress tolerance, we utilize the stress tolerant yeast *Kluyveromyces marxianus* (Km). Unlike *Saccharomyces cerevisiae* (Sc) cells, Km cells thrive at temperatures of up to 49°C and are highly resistant to oxidative stress and UV irradiation. By utilizing Km, we aimed to **I)** establish whether sumoylation and the SSR play a role in stress tolerance of Km and **II)** identify specific SUMO pathway components involved in stress tolerance of Km. Our results reveal that Km displays a distinct SSR. Additionally, by cloning nine Km SUMO pathway genes and using CRISPR/Cas9 technology, we were able to replace Sc SUMO with its Km ortholog and found that this replacement enhances the resistance of Sc cells to oxidative stress.

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This Thesis is dedicated to my family who have always given me their support.

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CHAPTER 1: INTRODUCTION

1.1 The cell & stress

Environmental stressors, including temperature extremes, oxidizing agents, radiation, shear and osmotic stress are extrinsic stressors that damage cells and tissues (Díaz-Villanueva et al., 2015). Intrinsic stress, in contrast, can stem from reactive oxygen species (ROS) produced due to normal (or abnormal) metabolic processes, telomere shortening, DNA damage, or the expression of aggregation prone proteins or oncogenes (reviewed in Fields & Johnson 2010; Tomasetti et al., 2017). For example, elevated temperatures interfere with the proper folding of proteins, and protein misfolding may lead to the accumulation of non-functional proteins and protein aggregates, which interfere with normal metabolic processes and overwhelm the cell's ability to clear them (Vidair et al., 1996). UV and ionizing radiations in contrast can produce ROS, which damage proteins or DNA (reviewed in Sinha & Häder 2002). In summary, a cell's exposure to extrinsic cellular stress can majorly disrupt a cell's proteome and genome, potentially leading to premature aging, diseases including cancer and neurodegenerative disorders, or cell death. (reviewed in Tomasetti et al., 2017; Fields & Johnson 2010).

1.2 The cellular response to stress

Cells have evolved several mechanisms to alleviate the harmful effects of proteotoxic and genotoxic stressors (reviewed in Fulda et al., 2010). Generally, a cell's response to stress depends on the severity of the stressor. In case that cellular components are damaged beyond the point of recovery, i.e. excessive levels of insoluble protein aggregates and/or irreparable DNA damage, cell will initiate programmed cell

death via apoptosis or necrosis (reviewed in Fink & Cookson 2005; Fulda et. al 2010). Apoptosis is a tightly caspase-controlled dismantling of cellular components, resulting in cell shrinkage, disruption of the cell membrane, and blebbing. Necrosis is a rapid swelling and bursting of the cell, which causes an influx of calcium ions to be released in the extracellular matrix, resulting in activation of intracellular nucleases that destroy cell debris (Fink & Cookson 2005).

While increased levels of insoluble protein aggregates challenge the fitness of the cell and are considered a hallmark for development of neurodegenerative diseases (Lamark & Johansen 2012), they initially form as a cytoprotective response to proteotoxic stressors (Kopito, 2000). One form of protein aggregates, called inclusion bodies, form to effectively compartmentalize misfolded proteins and prevent their unspecific interaction with other stable proteins (Taylor et al., 2003; Kopito, 2000). During the cellular post-stress recovery period, these aggregates can then be degraded via proteasomes or chaperone-mediated autophagy. Proteasomes are barrel-like protein complexes that function to break down unfolded/damaged proteins tagged via polyubiquitination while chaperone-mediated autophagy involves the selective delivery of these protein aggregates to the lysosome via the Hsp70/Hsc70 chaperone protein (Lamark & Johansen 2012; Kaushik & Cuervo 2018).

To prevent cell death and minimize irreversible damage, cells have evolved several mechanisms to mitigate damage to their constituent biomolecules, collectively known as the cellular stress response (Fulda et al., 2010). Focusing on proteotoxic stress, exposure to elevated temperatures, oxidative stress or other proteotoxic insults trigger a protective transcriptional reprogramming, during which general transcription and

translation is paused while expression of a set of stress response proteins is increased (Fulda et al., 2010). One example is the increased expression of heat shock proteins (Hsps) due to activation of heat shock transcription factors (HSFs). Specifically, upon stress exposure monomeric HSF1 is re-localized to the nucleus, where it forms transcriptionally active trimers that trigger transcription of Hsps, especially Hsp90 and Hsp70. Generally, Hsp90 facilitates re-folding and re-solubilization of proteins while the Hsp70 chaperones help mediate degradation of unfolded proteins that are past the point of recovery or prevents them from being misfolded during synthesis in the first place. Other proteins, like Hsp104 in yeast, rescue denatured proteins from their previous aggregate forms (Glover & Lindquist, 1998). Concomitantly, the pause in general transcription during stress exposure redirects cellular resources as cells refold or dispose of damaged proteins (Shi et al., 1998; Zou et al., 1998).

1.3 PTMs & the stress response

Post translational modification pathways (PTMs), such as ubiquitination, phosphorylation, and sumoylation play an important role in stress signaling and response, as well (reviewed in Zhang et al., 2015). For example, the degradation of protein aggregates via proteasomes is dependent on the ubiquitin-proteasome system (UPS). Unfolded proteins are marked for degradation with chains of ubiquitin, a small modifier protein: Ubiquitin receptors on the proteasome interact with polyubiquitinated proteins and begin the process of their unfolding and subsequent hydrolyzation into short peptides (Cundiff et al., 2019). Other key PTM pathways, namely phosphorylation, plays critical roles in stress signaling. For example, the eIF2α initiating factor prevents its binding to

eIF2B, which results in a pause to the costly protein translation and redirects the cell's resources to the stress response (Dunand-Sauthier et al., 2005).

Little is known about the role of the SUMO pathway in the cellular stress response. In response to most stressors, eukaryotic cells display a dramatic and rapid global increase in conjugation of proteins by the small post-translational protein modifier, SUMO, in a process known as the SUMO stress response (SSR) (Zhou et al., 2004; Lewicki et al., 2015). SUMO is a small ubiquitin-like modifier protein involved in many key cellular processes, including transcription regulation, cell cycle control, DNA repair, and protein turn-over (reviewed in Kerscher et al., 2006; Müller et al., 2001; Gill 2004; Andreou & Tavernarakis 2009; Eifler & Vertegaal 2015) and only recently new details of the role that SUMO plays in stress tolerance have shifted into focus (reviewed in Enserink et al., 2015).

SUMO has been conserved in eukaryotic organisms from yeast to plants to mammals. In budding yeast (*Saccharomyces cerevisiae* or Sc), SUMO is encoded by a single gene, SMT3 (suppressor of MIF2). Mammalian cells express at least four isoforms: SUMO1, SUMO2, SUMO3, and SUMO4 while plants, for example *Arabidopsis thaliana*, express as many as eight different SUMO isoforms (Kurepa et al., 2003). SUMO becomes conjugated to specific cellular proteins, requiring a dedicated enzymatic cascade, consisting of SUMO E1 activating heterodimers Aos1 and Uba2, E2 conjugating Ubc9 conjugating, and E3 ligating Siz/PIAS family of enzymes. SUMO target proteins often contain a SUMO consensus motif (ψKXE) that is recognized by the SUMO E2 Ubc9 prior to sumoylation (**Fig. 1**) (reviewed in Kerscher et al., 2006; Xie et al., 2007). SUMO2/3 can also form SUMO chains via their internal lysine residues, and, in some cases, substrate proteins with SUMO chains become modified with ubiquitin and may be degraded via

SUMO-targeted ubiquitin ligases (STUbLs), such as the enzymes making up the Slx5/Slx8 complex (reviewed in Sriramachandran & Dohmen 2014).

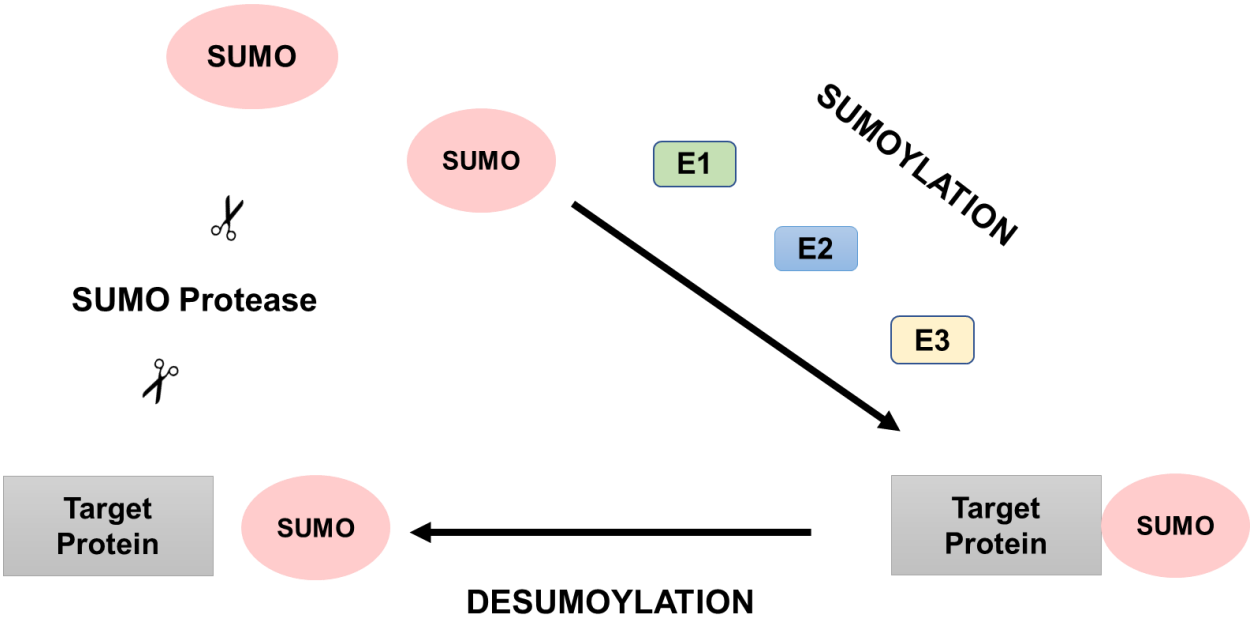


Figure 1. The sumoylation pathway. Schematic of the SUMOylation pathway. The small modifier protein, SUMO, is initially encoded as an inactive precursor. Then, SUMO gets processed into its active form via the SUMO protease Ulp1. Through an enzymatic cascade involving E1, E2, and E3 enzymes, SUMO gets conjugated to specific target proteins. SUMO can get deconjugated from the target protein via the same SUMO protease (Ulp1) (reviewed in Kerscher et al. 2006).

1.4 The SUMO stress response (SSR)

The SSR is the process of a dramatic and rapid global increase in conjugation of proteins with SUMO, which has been consistently observed in eukaryotic organisms from yeast to mammals when they are exposed to most types of extrinsic stressors, including elevated temperatures, hyperosmotic shock, and presence of reagents that generate ROS (Lewicki et al., 2015). Increasing stress levels cause a rise in sumo conjugate levels,

but interestingly, de novo SUMO synthesis is *not* required for this the SSR; this phenomenon utilizes the existing pool of SUMO for conjugation of target proteins. In yeast, the SSR relies on SUMO E3 ligase Siz1 for conjugation and the SUMO protease Ulp2 for deconjugation post-stress (Lewicki et al., 2015).

How does the SSR mediate stress tolerance? Recently, several pathways have emerged that show a key influence of sumoylation levels on the cellular stress response although many details remain elusive. In the nucleus, it has been suggested that the SSR enables a wave of sumoylation as part of the protective transcriptional reprogramming (Seifert et al., 2015; Lewicki et al., 2015; Mollapour et al., 2014); the majority of the sumoylation during SSR takes place within the nucleus, and SUMO conjugation appears essential for stability of nuclear protein complexes that are associated with active genes during stress (Zhang et al., 2015; Seifert et al., 2015). During heat stress, SUMO2 has been reported to accumulate at nucleosome-depleted and active DNA regulatory binding sites of chromatin (Seifert et al., 2015). SUMO modulation as part of the SSR is also reported to aid in disassembly of defective RNA polymerase complexes, namely RNA polymerase III (RNAPIII) and RNAPII, in a process involving the recruitment of the STUbL Slx5/8 to one of the complex's subunits and the complex's subsequent ubiquitination although, surprisingly, the Slx5/8 is not required for this degradation to proceed so details still remain unclear (Heckmann et al., 2019; Wang et al., 2018).

A stress induced increased sumoylation is also observed in the cytoplasm, and several studies suggest that as part of the SSR, sumoylation helps maintain target protein solubility to prevent or minimize accumulation of harmful protein aggregates (Liebelt et al., 2019; Ma et al., 2020; Datwyler et al., 2011; Guo & Henley, 2014). A study shows that

in chaperone-depleted cells, sumoylated proteins retained their conjugated state longer than WT cells. Specifically, increase in SUMO2/3 in response to heat stress is reported to work hand in hand with the ubiquitin-proteasome system (UPS) to prevent it from becoming overloaded with accumulated protein aggregates (Liebelt et al., 2019). Liebelt et al. (2019) proposes that, regardless of the functional state of proteins, their sumoylation keeps them soluble, allowing the UPS more time to sort through terminally damaged versus recoverable proteins.

Analogous to the role of SUMO in heat shock, SUMO modification of mitochondrially-targeted proteins appears to play an important role in mitochondrial protein quality control, especially for the ones that are misfolded or incorrectly targeted. For example, in yeast, truncated mitochondrial proteins Ilv6 and Adh3 that lack their matrix-targeting sequence (MTS) fail to import into the mitochondria and show increased sumoylation. These mutant proteins are subsequently degraded. Supporting this, retention of both sumoylated Ilv6 and Adh3 is also observed when Hsp70 or proteasomes are depleted (Paasch et al., 2018).

Additionally, studies show that SUMO levels and crosstalk of sumoylation signals may help in different phases of the cellular response from being instantly cytoprotective, to recovery from early stages of apoptosis, or to proceeding to irreversible necrosis. For example, during hypoxia and/or ischemic injury, Drp1, a dynamin related GTPase that is required for mitochondrial fission occurring in the cytosol (Bleazard et al., 1999; Smirnova et al., 2001; Fonseca et al., 2016) is modified by SUMO1 and/or SUMO2/3 (Harder et al., 2004; Guo et al., 2013). It has been reported that overexpression of either SUMO1 or the SUMO E3 ligase, MAPL, (the SUMO ligase for Drp1) results in increased stabilization of

Drp1, which in turn leads to the subsequent cytochrome *c* release and mitochondrial hyperfragmentation, which are prerequisites for apoptosis to occur (Wasiak et al., 2007; Prudent et al., 2015). Importantly, cells with reduced levels of SUMO1-sumoylated-Drp1 show a reduction of cytochrome *c* in the cytosol, and consequently a delayed apoptosis (Prudent et al., 2015). This contrasts with the role which has been proposed for modification of Drp1 with SUMO2/3 (Guo et al., 2013). SUMO2/3 conjugation of Drp1 has been reported to prevent binding of Drp1 to the mitochondrial outer membrane (Guo et al., 2013). During hypoxia, decreased levels of the SUMO protease, SENP3, leads to increased Drp1 sumoylation with SUMO2/3, and Drp1 binding activity to the mitochondrial outer membrane is reduced, effecting a delay in apoptosis (Guo et al., 2017). Hence, persistent Drp1 modification with SUMO2/3 may prevent mitochondrial fission/fragmentation and subsequent apoptosis (Guo & Henley 2014). It is not currently clear whether SUMO1 and SUMO2/3 modification of Drp1 represent competing or redundant processes that control stress-induced mitochondrial fission and apoptosis.

Interestingly, while SUMO2/3 sumoylation of Drp1 prevents a pro-apoptotic process during hypoxia (Guo et al., 2013), SUMO2/3 sumoylation of the protein, FADD promotes hypoxia-induced necrosis (Choi et al., 2017). High levels of calcium, which occur during hypoxia or ischemic injury, initiate the translocation of sumoylated FADD (the fas associated death domain protein) to the mitochondrial outer membrane (Kawahara et al., 1998; Matsumura et al., 2000; Choi et al., 2017). FADD sumoylation is required for this translocation and the subsequent mitochondrial fragmentation. HeLa cells expressing a FADD 3KR mutant that cannot be modified with SUMO2/3 show a 30% decrease in mitochondrial fragmentation in comparison to the cells expressing WT FADD

(Choi et al., 2017). Knocking out the SUMO E3 ligase PIAS3, which facilitates FADD sumoylation, also significantly suppresses calcium-induced necrosis (Choi et al., 2017).

1.5 Yeast as a genetic model system to study SUMO

Budding yeast, *S. cerevisiae*, is a popular mesophilic genetic model system, with ~60% of its genome conserved in mammals, including the SUMO pathway (Botstein & Fink 2011; Yang et al., 2017). *S. cerevisiae* is genetically tractable, simple to use, and displays the SSR (Zhou et al., 2004). Intriguingly, a stress tolerant yeast, *Kluyveromyces marxianus* (*K. marxianus* or Km), which is closely related to *S. cerevisiae* can withstand oxidative stress, UV irradiation, and temperatures of up to 49°C (Yamamoto et al., 2015; Pinheiro et al., 2002) (**Fig. 2**). These properties of *K. marxianus* have already proven useful for industrial applications using high-temperature bioreactors as well as research applications involving unstable proteins (Limtong et al. 2007; Löser et al., 2015). Km and Sc are part of the same subclade (*Saccharomycetes*) and share ~78% genetic similarity, which extends to their respective SUMO pathways (**Table 1**) (Lertwattanasakul et al., 2015; Peek et al., 2018),

The stress resistance of Km is attributed to enhanced protein stability due to more compact protein structures (Peek et al., 2018; Yamamoto et al., 2015). Intriguingly, work from our lab suggests that post-translational protein modification with SUMO may play a pivotal role in Km's stress tolerance. For example, we found that Km Ulp1 (a SUMO protease), unlike its budding yeast ortholog Sc Ulp1, shows a remarkable ability to withstand both temperature and oxidative stressors (Peek et al., 2018), leading us to hypothesize that the SUMO pathway plays a part in the stress tolerance of Km.

Table 1. List of *K. marxianus* sumoylation proteins, their function, and amino acid identity with *S. cerevisiae* sumoylation proteins. Amino acid identity data was obtained from Peek et al. (2018).

Km SUMO Pathway Protein	Description and Function	Amino Acid (aa) Identity with Sc
Smt3	Small Ubiquitin-like modifier protein (SUMO), which gets conjugated to specific target proteins involved in cell cycle control, DNA repair, signal transduction, and stress response	85%
Ulp1	Ubiquitin-like protease 1; processes SUMO from its inactive form to its active form and is also responsible for desumoylation of target proteins.	51%
Ulp2	Ubiquitin-like protease 2; involved in SUMO processing and localization of SUMOylated proteins.	44%
Aos1	SUMO E1 dimer, which activates SUMO before its conjugation to target proteins.	55%
Uba2	SUMO E1 dimer, which activates SUMO before its conjugation to target proteins.	62%
Ubc9	SUMO E2 enzyme, which enables SUMO conjugation to target proteins.	83%
Siz1	SUMO E3 ligase, which aids SUMO conjugation to target proteins.	43%
Mms21	SUMO E3 ligase, which aids SUMO conjugation to target proteins.	29%
Slx5/8	SUMO targeted ubiquitin ligase, which may lead to the conjugated protein’s degradation	39%/48%

1.6 The role of SUMO in the stress tolerance of *K. marxianus*

As detailed above, the specific mechanisms via which the SSR helps cellular stress tolerance remain unclear. We know from previous studies that sumoylation is essential in the stress response of single cell eukaryotes such as the yeast *C. albicans* and the green algae *C. reinhardtii.* In *C. albicans*, *smt3/smt3* null mutants can grow as wildtype (WT) cells, but are hypersensitive to a range of stressors, including elevated temperatures, increased NaCl, and H_2O_2 exposure amongst other proteotoxic insults (Leach et al., 2011). Similarly, in *C. reinhardtii*, comparable results have been observed when knocking out the SUMO E2 activating enzyme, Ubc9, which made these algae cells hypersensitive to the set of stressors (Knobbe et al., 2014). Hence, given the necessity of SUMO for the stress tolerance of these single celled eukaryotes, and given that *K. marxianus* displays an enhanced stress tolerance, we wanted to know whether this yeast's stress tolerance properties are related to its SUMO pathway. In support of this, in a previous study (Peek et al., 2018), we demonstrated that the Km SUMO protease Ulp1 – in comparison to its Sc ortholog (Sc Ulp1) – shows enhanced capabilities to withstand elevated temperatures and chemical denaturants. The SENP/Ulp1 family bind and cleave a variety of SUMO isoforms and SUMO conjugates. Peek et al. (2018) used a SUMO-trapping mutant of KmUlp1, KmUTAG, to investigate SUMO binding in the presence of proteotoxic stress. Analysis indicated that SUMOtrapping by KmUTAG is considerably more stress-tolerant than the SUMO-trapping by ScUTAG (Peek et al., 2018). Specifically, SUMO-trapping by KmUTAG is much less affected by multiple stressors, including the presence of H_2O_2, the denaturant UREA, and exposure to the elevated temperature of 42°C (Peek et al., 2018).

Considering these results, we hypothesized the resilience observed in Km Ulp1 is present in the rest of the Km SUMO pathway proteins, and the resilient SUMO pathway in Km is what enables the enhanced stress tolerance of *K. marxianus*. The goal of this research was to first show the presence of the SSR for the stress tolerant yeast strain, *K. marxianus*, and then to identify and characterize the SUMO components involved in *K. marxianus*' tolerance to stress. Here, we show **I)** the distinct SSR pattern observed in *K. marxianus*, and whether **II)** differing expression of single or multiple Km SUMO genes, or **III)** replacement of the Sc SUMO gene with its Km ortholog, Km SUMO enhances *S. cerevisiae* cells' stress tolerance when they are exposed to elevated temperatures, H_2O_2, and UV radiation treatment.

CHAPTER 2: MATERIALS & METHODS

2.1 Yeast and bacterial strains, and media

The yeast and bacterial strains used in this study are listed under Appendix: Supplementary Tables 2 and 3, respectively. W303, MHY500, and MHY501 were the wildtype (WT) Sc strains used. Pigmented WT Sc strains were a gift from Maitreya Dunham. WT Km (BY28353) was purchased from Yeast Genetics Resource Center, Osaka University, Japan. All bacterial strains used were DH5α strain unless otherwise noted. Yeast and bacterial media were prepared at 2x concentration as previously described (Guthrie & Fink, 1991). Lab grade dextrose, sucrose, raffinose, and galactose were purchased from either Genesee Scientific or Thermo Fisher Scientific. The antibiotic Carbenicillin was used at 60 ug/mL concentration for selection of bacterial colonies, and the antibiotic G418 was used at 250 ug/mL concentration for selection of yeast colonies as instructed by Sambrook and Russell (2001, p. 1.9, 16.48). All yeast strains were grown at 30°C for up to 72 hours unless otherwise noted (*smt3-331* was grown at 25°C). All bacterial strains were grown at 37°C for up to 16 hours. Mating crosses of Sc strains containing pYES2.1/URA3 or pAG425/LEU2 Km SUMO pathway genes were performed following the Clontech Yeast Protocols Handbook (2009, p. 44). Empty vectors pRS426 (BOK 344) and pAG425GAL-ccdB were transformed into Sc yeast strains to be used as controls.

2.2 Yeast overexpression vectors and sequencing primers

Km SUMO pathway genes were PCR amplified from the WT Km genomic DNA, using the appropriate primers (listed under Appendix: Supplementary Table 4) and were

cloned into the overexpression vectors pYES2.1/V5-His-TOPO (Life Technologies) and pAG425GAL-ccdB (Addgene #14153) by following the Life Technologies pYES2.1 TOPO® TA Expression Kit manual (#K4150-01). Any Sc SUMO pathway genes cloned into overexpression vectors were PCR amplified from the W303 strain using the appropriate primers listed under Supplementary Table 4. All constructs were sequence confirmed using the appropriate primers listed under Supplementary Table 4.

2.3 Yeast CRISPR vector, gRNA oligos, DNA repair templates, and gDNA extracts

The CRISPR/Cas9 plasmid pML104 was purchased from Addgene (#67638) and mini-prepped from dam-/dcm- competent bacterial cells (NEB, #C2925I) prior to linearization by SwaI (NEB, #R0604S) and BclI (NEB, #R0160S) double digestion (BOK 1594). The dam-/dcm- competent cells and the enzymes were purchased from New England Biolabs, MA. Guide RNA oligonucleotides were designed using the website, http://wyrickbioinfo2.smb.wsu.edu/crispr.html and are listed under Appendix: Supplementary Table 4. Guide RNA oligonucleotide hybridization and ligation into linearized pML104 CRISPR/Cas9 plasmid were performed by adapting methods from Laughery et al. (2015) (see Appendix for details). All ligated Sc gRNA/pML104 plasmids were sequence confirmed using the T3 forward primer (OOK 1075). Repair template constructs were generated from PCR amplified Km SUMO pathway genes with primers containing 40 nucleotides of flanking regions. The PCR products were cleaned using the IBI Scientific PCR cleanup kit (#IB47010). Co-transformations of repair templates and pML104 were performed by adapting methods from the Clontech Yeast Protocols Handbook (2009, p. 18-19). Km replacements were confirmed by PCR and sequencing.

PCR products that matched the expected size of Km replacements were sent for sequence confirmation.

2.4 Yeast stress tolerance assays

For conducting chronic elevated temperature stress, plates containing yeast spotting or streaks were placed at a range of different temperatures from 37°C – 4°C for 24 to 72 hours. For conducting acute temperature stress, liquid yeast cultures were places at temperatures of 42°C – 55°C for 30 seconds to 5 minutes. Chronic oxidative stress was induced by addition of 2.5 mM to 5.0 mM of H_2O_2 in yeast media plates as described in Spencer et al. (2014). Acute oxidative stress was induced via treating liquid yeast cultures with 25 mM – 50 mM H_2O_2 for 15 to 30 minutes as indicated. Genotoxic stress was induced via UV treatment of plates containing spotted yeast or streaks at 90 mJ – 125 mJ. Other chronic stress reagents tested were 5%-8% EtOH, 0.5 M NaCl, 100 ug/mL L-canavanine, and 0.1 M hydroxy urea, which were added to media plates as described in Tekarslan-Sahin et al. (2018).

2.5 Yeast two-hybrid assays

The Km SMT3 gene was PCR amplified using the pOAD/BD forward and reverse primers (OOKs 1126 & 1227) and cloned into the Gal4-binding-domain prey (pOBD) plasmid (BOK 312) (Yeast Resource Center, WA). The construct was sequence confirmed and transformed into an AH109 strain that already contained an ScSlx5-AD bait plasmid from a previous study (YOK 1224) (Westerbeck et al., 2014). KmSmt3-BD and ScSlx5-AD fusion constructs interaction was confirmed on selective media plates

lacking leucine, tryptophan, histidine, and adenine (the reporter genes), and interaction strength was measured by conducting an ONPG assay for colonies with confirmed KmSmt3-BD and ScSlx5-AD interaction as described in the Clontech Yeast Protocols Handbook (2009, p. 44). The ONPG assay was performed for overnight grown colonies before and after treatment with 25 mM of H_2O_2 for 30 minutes.

2.6 Competition Assay with Pigmented Yeast Strains

The SUMO gene was replaced with Km SUMO in WT Sc blue strains (YOK 3734) as described in **2.3**. Cultures of the Sc blue strain with Km SMT3 (YOK 3745) and WT Sc pink strain (YOK 3733) were each grown overnight in 5 mL of YPD plus 250 ug/mL G418 rotating at 30°C. After 16-19 hours, 20 uL of the grown YOK 3745 was added to 20 uL of YOK 3733 in a new Eppendorf tube and vortexed. 5 uL of the vortexed mix was added to 5 mL of fresh YPD plus 250 ug/mL G418 and again incubated overnight, rotating at 30°C. After 16-18 hours, the 5 mL of mixed cell culture was split into two 2.5 mL volumes and 25 mM of H_2O_2 was added to one of the 2.5 mL volumes (25 mM H_2O_2 was made from a 1.0 M H_2O_2 stock solution (3.0%, CVS commercial grade). Both untreated and H_2O_2 treated cells were incubated for an additional 30 minutes rotating at 30°C. Cells were then spun down and washed of tubes. Then, the untreated cell culture was diluted 1:10,000 and the H_2O_2 treated cells were diluted 1:5. 150 uL of each dilution was plated onto a set of six YPD + 250 ug/mL media plates (n=3). Plates were incubated at 30°C for up to five days. This pigmented yeast competitive oxidative stress tolerance assay was adapted from the yeast competition protocol (Maitreya Dunham Lab, WA).

2.7 Protein extracts and anti-bodies

Proteins were extracted from WT Km and WT Sc cells before and after treatment with H_2O_2 (2.5 – 100 mM for 20 minutes) as detailed in Szymanski & Kerscher (2013). Proteins were run on NuPAGE 4-12% Bis-Tris gels (Life Technologies) for 50 minutes at 200 V in 1x MOPS buffer. The proteins were transferred to a polyvinylidene diflourine membrane (PVDF) (Millipore) by semi-dry transfer in semi-dry transfer buffer (192 mM glycine, 250 mM Tris Acetate at pH 8.8, 20% methanol) for 30 minutes at 19 V. The blot was blocked in OneBlock™ Western-CL blocking buffer (Genesee Scientific, #20313) for an hour and then in OneBlock containing primary Sc anti-SUMO antibody (a gift from Michael Matunis, JHMI) (1:10,000 dilution) overnight rocking at 4°C. After three, five-minutes washes in TBS plus TWEEN20 (1 mL TWEEN20/ 1L of 150 mM NaCl and 50 mM Tris-HCL at pH 7.4), the blot was incubated in OneBlock containing secondary goat anti-rabbit antibody (LI-COR, #925-68071) (1:5,000 dilution) for 1-3 hours and then washed again in TBS plus TWEEN20 for three, 15-minute periods. Blot were images using the Odyssey imaging system (LI-COR Biosciences).

CHAPTER 3: RESULTS

3.1 *K. marxianus* shows a distinct SSR in response to stress

Despite *K. marxianus* showing enhanced stress tolerance to a range of different stressors, we couldn't find a figure that compares these properties next to *S. cerevisiae*, so we decided to first illustrate the stress tolerance of Km in comparison to the mesophilic Sc by conducting a spotting stress assay: Overnight cultures of the wildtype (WT) Sc strain (W303) and WT Km strain (B28353) were serially diluted and spotted onto four plates with different conditions. One plate was left unmodified and incubated at 30°C for control while the rest either contained 2.5 mM H_2O_2, were incubated at 45°C, or treated with 125 mJ of UV. As expected, our assay shows that when exposed to any type of stressor, Km cells show superior stress tolerance in comparison to Sc (**Fig. 2**).

Why Km displays these stress tolerant properties is not well-understood or well-studied, and there is nothing known about the SSR in Km. Therefore, we wanted to establish whether K. marxianus cells display a similar pattern of increased SUMO levels as observed in Sc when exposed to stress. We visualized SUMO levels of wildtype (WT) Km versus WT Sc cells, using western blotting and a cross-reactive anti-SUMO antibody to detect the SSR in both Km and Sc cells before and after treatment with zero to 100 mM H_2O_2 for 20 minutes. Our results suggest that, like *S. cerevisiae* cells, *K. marxianus* cells display a stress-dependent increase of sumoylated protein conjugates. **Figure 3** shows both Km and Sc protein sumoylation levels under untreated conditions, 2.5 mM H_2O_2, and 25 mM H_2O_2. Both Sc and Km cells show increased sumoylation when exposed to H_2O_2 with a particular increase in sumoylation of proteins with high molecular weights. The amount of SUMO, too, appears to increase on the same proteins with

increasing H_2O_2 concentration. This may be an indication of SUMO chain formations or presence of multiple SUMO moieties on one target protein. Our observations indicate that the SSR appears less pronounced in Km cells, however, there is still a clear and distinct increase in SUMO levels when these cells are exposed to acute oxidative stress.

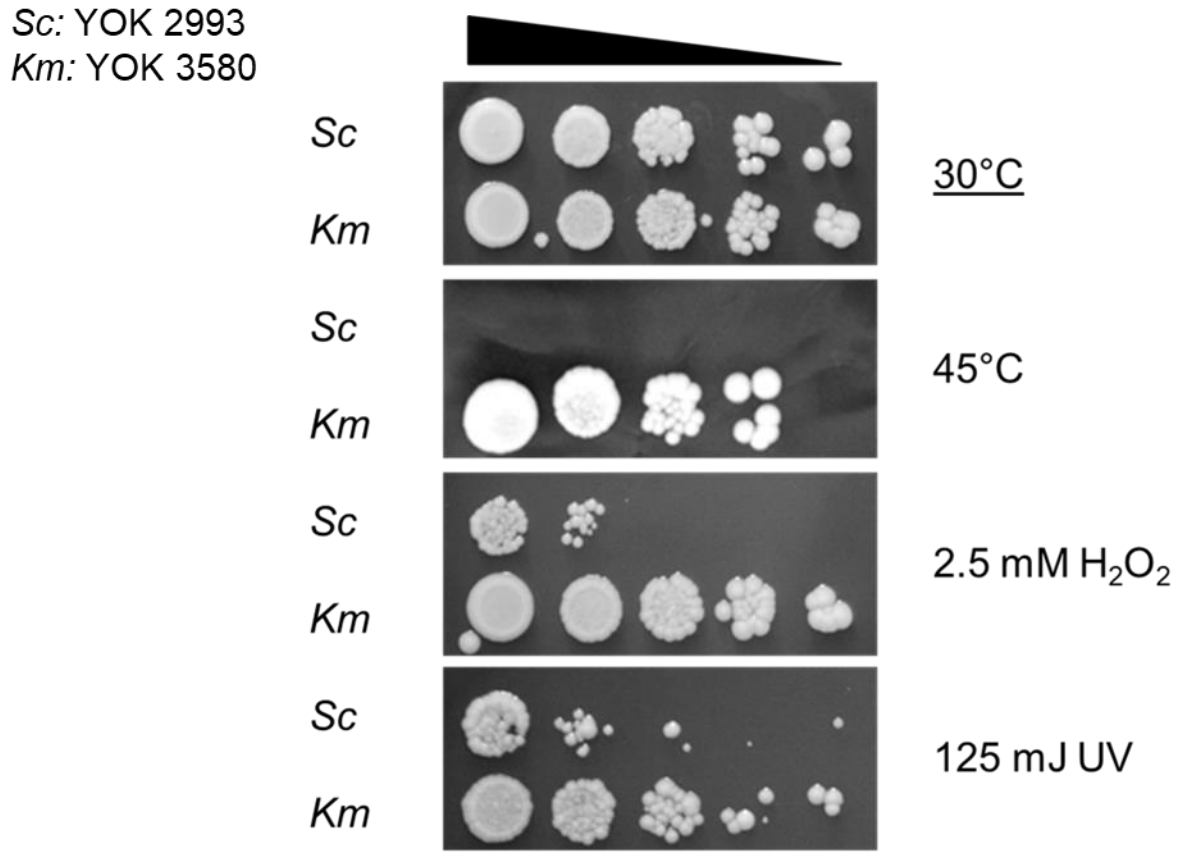


Figure 2. Superior stress tolerance of *K. marxianus*. Comparison of *K. marxianus*' growth to *S. cerevisiae*'s under temperature (45°C) and oxidative (2.5 mM H_2O_2) stress, and after UV radiation (125.0 mJ UV) after incubation period of 72 hours. 1 OD_{600} of overnight colony cultures were harnessed, and they were serially 10-fold diluted and spotted, from left to right, on YPD media. The control with untreated, optimal conditions is underlined.

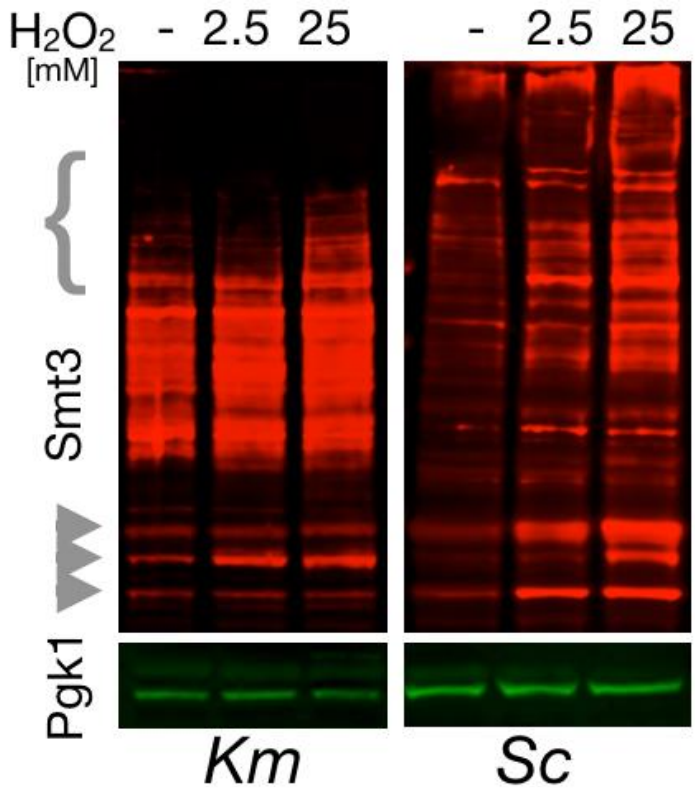


Figure 3. The SSR is present in *K. marxianus*. The SSR observed in *K. marxianus* and *S. cerevisiae* yeast. Km and Sc cells (YOKs 2993 and 3580, respectively) were treated with the indicated concentration of H_2O_2 for a period of 20 minutes. Grey arrows and bracket point to proteins with a notable increase in sumoylation. Proteins were extracted and western blotted with cross-reactive antibodies to PGK1 and SMT3 (SUMO).

3.2 Overexpression of Km SUMO pathway genes causes lethal phenotypes

Having observed the SSR in *K. marxianus* we expected that overexpression of specific Km SUMO pathway genes may enhance stress tolerance of WT Sc cells. It has been shown in other contexts that ectopic expression of SUMO E2 conjugating and E3 ligating enzymes, which help speed the sumoylation process, can improve stress tolerance: for example, 1) overexpression of SUMO E2 Ubc9 protects neuroblastoma cells when exposed to ischemia (Lee et al. 2011), and 2) overexpression of SUMO E3 Siz1 increases *Solanum lycopersicum*'s (tomato's) tolerance to heat (Zhang et al. 2017).

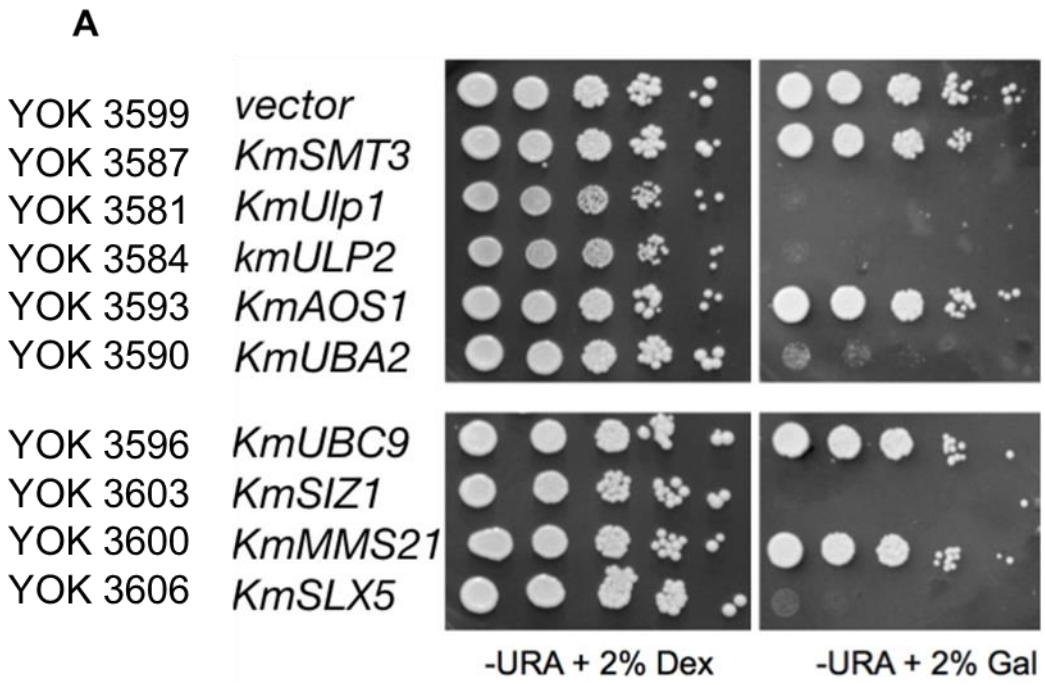
Therefore, we wanted to investigate whether *Sc* cells overexpressing SUMO pathway genes from the stress tolerant Km show any enhanced ability against a series of stressors. For this purpose, nine genes of the Km sumoylation pathway were PCR-amplified from WT Km strain (BY28353) and cloned into a high copy (multi-copy 2μm) galactose-inducible overexpression vector (pYES2.1/V5-His-TOPO). These included genes encoding for the Km SUMO protein, Smt3, two SUMO proteases KmUlp1 and KmUlp2, the two E1 enzyme heterodimers, KmAos1 and KmUba2, the E2 enzyme KmUbc9, the E3 enzymes KmSiz1 and KmMms21, and the STUbL KmSlx5. All constructs were sequence confirmed and complementation tested. Next, WT Sc cells from the W303 strain were transformed with these genes, and viability of the transformants were tested before and after overexpression of inserted Km genes, using the strong inducible Gal promoter. Briefly, on dextrose, the expression from the GAL1 promoter is repressed by repressors and inhibitory proteins and there is little to no expression. On other sugars, such as sucrose and raffinose, inhibitory proteins are removed from the promoter region, allowing for moderate levels of expression. On galactose, repressors are completely removed and there are high levels of expression (Bro et al., 2005). This allowed us to "tune" levels of expression of inserted Km genes depending on the type of carbon source provided for the yeast cells with dextrose inducing little to no expression and galactose inducing the most amount of overexpression. Interestingly, Gal overexpression of five Km SUMO pathway genes resulted in a lethal phenotype for Sc (**Fig. 4A**): They included the Km proteases Ulp1 and Ulp2, the E1 heterodimer Uba2, the E3 enzyme Siz1, and the STUbL Slx5. Intriguingly, only overexpression of the Sc orthologs Ulp1 and Slx5 cause similar lethality in Sc while overexpression of Sc orthologs Uba2 and Ulp2 result in

decreased growth rates (Yoshikawa et al., 2011). There is no previously recorded data on Sc Siz1 overexpression effects (see yeastgenome.org).

3.3 Paired overexpression of Km SUMO pathway genes suppresses lethal phenotypes

Next, we wanted to investigate whether any combination of two Km genes will rescue the phenotypes of the lethal Km genes overexpression. We cloned our PCR-amplified Km genes in a new high copy (multi-copy, 2μm) Gal inducible overexpression vector (pAG425GAL-ccdB) with a different selectable marker (LEU2). Therefore, we were able to mate haploid WT Sc transformants from the MHY500 strain that contained single plasmid-borne Km genes (with the LEU2 selectable marker) to ones containing the URA3 selectable marker, resulting in diploid WT Sc transformants containing two Km genes. Results show overexpression of certain pairs of Km genes were observed to be viable despite some having previously been lethal if overexpressed on their own (**Fig. 4B**). For example, overexpression of the *KmSMT3* and *KmSLX5* genes together was not lethal for the Sc cells despite *KmSLX5* overexpression causing a lethal phenotype when expressed singly in Sc cells. Similarly, overexpression of *KmSIZ1* and *KmUBC9* (but not *KmSIZ1* and *KmSLX5*) was viable despite overexpression of *KmSIZ1* being lethal on its own. The combination of these two overexpression pairs are interesting, as they do not necessarily re-balance SUMO homeostasis that may have been perturbed by the single overexpression levels: both SIZ1 and UBC9 overexpression, for example, help sumoylation proceed faster. This suggests the lethality due to overexpression of Km SUMO pathway proteins may be due to titration of essential sumoylated proteins. This

dominant phenotype can be suppressed by co-overexpression of a second Km protein, possibly because it binds the first one. This possibility and its implications are further discussed below (see **Discussion 4.2**).



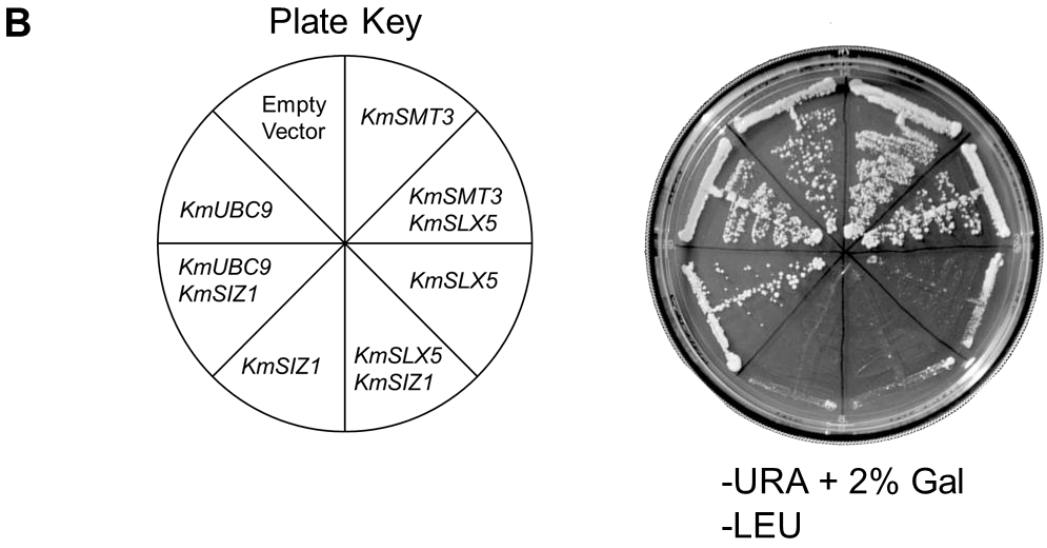


Figure 4: Km SUMO pathway gene overexpression causes lethal phenotypes, which can be suppressed by other Km SUMO pathway genes. (A) Overexpression of five Km SUMO pathway genes from the strong inducible GAL promoter (*KmULP1*, *KmULP2*, *KmUBA2*, *KmSIZ1*, and *KmSLX5*) is lethal in Sc. Dex (Dextrose – induces little to no expression). Gal (Galactose – induces strong overexpression). Cells were spotted onto -URA selective media and incubated at 30°C for a period of 72 hours. **(B)** Effects of single versus double Km gene(s) overexpression on viability of WT Sc. Combinations of the *KmUBC9* and the *KmSIZ1* genes, and *KmSMT3* and *KmSLX5* genes appear to rescue the lethal phenotypes displayed by the *KmSIZ1* and the *KmSLX5* genes when overexpressed on their own. Transformed cells were streaked on –LEU -URA + 2% galactose selective media and incubated at 30°C for a period of 72 hours.

3.4 Overexpression of Km SUMO pathway genes does not enhance stress tolerance in WT Sc

To test whether the single or paired overexpression of Km SUMO pathway genes enhance the stress tolerance properties of Sc cells, we performed stress assays for our diploid MHY500 and haploid W303 Sc transformants, using a set of different acute and chronic stressors, including acute heat shock (40-55°C) and chronic heat exposure (37-45°C), chronic oxidative stress (1-5 mM H_2O_2), the use of the genotoxic hydroxy urea (0.1 M), and different levels of UV treatments (90 mJ – 125 mJ) on both serial dilutions of overnight grown colonies and streaking batches of colonies. We also “tuned” expression levels for each stress assay that we ran with 2% dextrose as control, 2% sucrose and 1.8%+0.2% raffinose as mild and moderate expression levels, and 2% gal as high expression levels. There were no significant advantages observed in growth of the Sc cells overexpressing either single or paired Km SUMO pathway genes (see Appendix for some examples).

3.5 *KmSMT3* complements *smt3-331* growth defect at elevated temperatures

Based on the results we obtained from the overexpression stress assays, we reasoned that overexpression of Km SUMO pathway genes, in addition to the Sc cell's endogenous SUMO pathway genes, could mask the effects of Km gene expressions. Therefore, we decided to next transform a stress sensitive Sc mutant, *smt3-331*, with the *KmSMT3* (SUMO) gene. The *smt3-331* strain is a conditional SUMO mutant, which grows best at room temperature (25°C) and cannot grow at temperatures of 35°C and beyond (Biggins et al. 2001). To test whether *KmSMT3* is capable of either suppressing or complementing the lethal phenotype of *smt3-331* at elevated temperatures, we transformed the *KmSMT3* overexpression plasmid (BOK 1546) in *smt3-331*. As expected, the untransformed *smt3-331* strain fails to grow above 35°C. **Figure 5** shows that both *KmSMT3 and ScSMT3* complement *the smt3-331* growth defect at temperatures up to 37°C, with *KmSMT3* overexpression enhancing *smt3-331*'s heat tolerant to degrees of up to 40°C (although the this part of the experiment could not always be repeated), raising the possibility that *KmSMT3* imparts temperature-tolerant properties to *S. cerevisiae* cells.

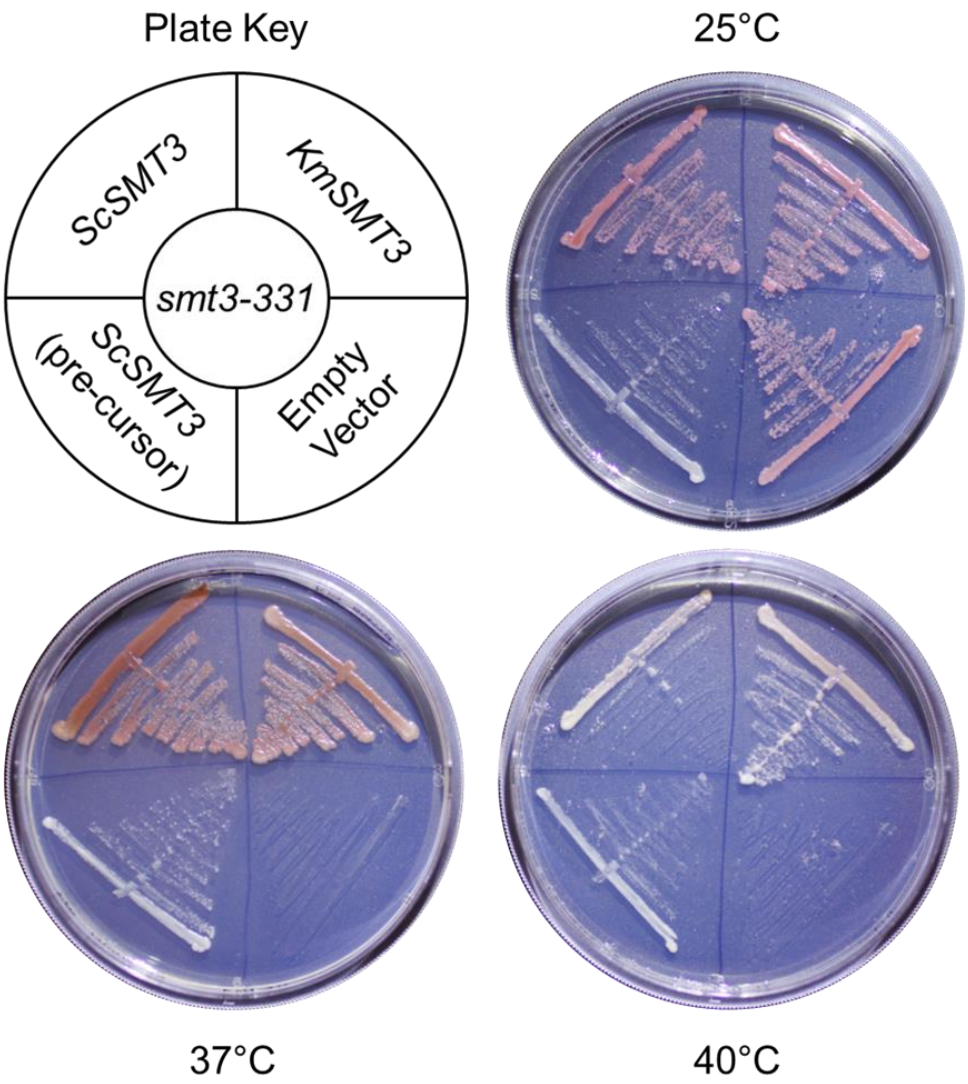


Figure 5: Both *Km* and *Sc* SMT3 support *smt3-331* growth at elevated temperatures. Expression of the both the Km and Sc SUMO gene, *SMT3*, complements a *smt3-331* temperature sensitive SUMO mutant at 37°C, which is a lethal temperature for this strain. Additionally, *KmSMT3* expression enabled enhanced stress tolerance at 40°C. Transformed cells of the *smt3-331* mutant strain were streaked on -URA + 1.8% raffinose & 0.2% galactose selective media, and incubated at temperatures of 25 – 40°C for a period of 72 hours.

3.6 *ScSMT3* replacement with *KmSMT3* increases resistance to oxidative stress

Encouraged by our finding that KmSmt3 successfully suppressed the temperature sensitive growth defect of *smt3-331* at 37°C and beyond, we tried a different approach to answer the question of whether Km SUMO pathway genes enable enhanced stress tolerance for Sc: Instead of using yeast expression vectors, we integrated the *KmSMT3* gene into the chromosomal ScSMT3 locus to replace the endogenous SUMO gene. Using this approach, the Km SUMO gene is integrated into the Sc genome, and variation in expression levels and the heterologous expression of two SUMO orthologs are no longer a concern. For the replacement, we used a CRISPR/Cas9 approach. Adapting methods described in Laughery et al. (2015), we cloned *ScSMT3* specific target sequences into the guide RNA expression cassette of Cas9 plasmid pML104 (Addgene plasmid #67638 – see Appendix for oligonucleotides' targeting sequences). This construct and a *KmSMT3* PCR-amplified fragment fitted with *ScSMT3* specific overhangs were co-transformed into WT W303 *Sc* cells, where the Cas9 protein is guided to the cut region on the SMT3 locus specified by the gRNA sequence that we ligated into the pML104 plasmid. The co-transformed *KmSMT3* repair template is subsequently recombined to the Sc genome at the cut site of Cas9. Replacement with *KmSMT3* was sequence confirmed (**Fig. 6A**) for several colonies, which were used for stress tolerance assays that included control strains that did not contain *KmSMT3*. Briefly, colonies expressing *KmSMT3* or *ScSMT3* were overnight grown in YPD liquid media, serially diluted, and spotted on YPD media or media that was either placed in a stressful environment (i.e. elevated temperatures) or contained a stressor (i.e. hydrogen peroxide, NaCl, and treated with UV). Remarkably, when compared to the *ScSMT3* expressing controls, cells carrying the *KmSMT3* replacement

showed improved growth on hydrogen peroxide media (**Fig. 6B**). However, Sc cells with *KmSMT3* did not display similar enhanced stress tolerance to any of the other stressors tested (data not shown).

To investigate why *KmSMT3* enables Sc cells with enhanced stress tolerance against oxidative stress, we compared the amino acid sequences of *KmSMT3* and ScSMT3 (**Fig. 6C**) and were able to detect differences in the position and the number of lysine residues residing on SUMO consensus motifs, which are regions on the SUMO protein that enable the binding of other SUMO for SUMO chain formation. Additionally, we noted shift differences in the stretch of hydrophobic residues. The implications of these findings are discussed further below (See **Discussion 4.3**).

3.7 Replacements of remaining Km SUMO pathways genes in Sc were unsuccessful.

We also attempted to replace the remaining Km SUMO pathway genes in Sc cells. We constructed the pML104 plasmids and the Km repair templates required for the replacement for each of the remaining Km SUMO pathway genes (see Appendix), but our transformations failed to yield any successful replacements. We believe this is due to the gRNA target sequence low efficiency rate, high levels of homologous regions between our repair templates and the cut site regions, and the general larger sizes of the remaining Km SUMO pathway genes that were to be replaced. For future studies, using different sets of gRNA target sequences will most likely provide us with more successful attempts of Km SUMO pathway gene replacements.

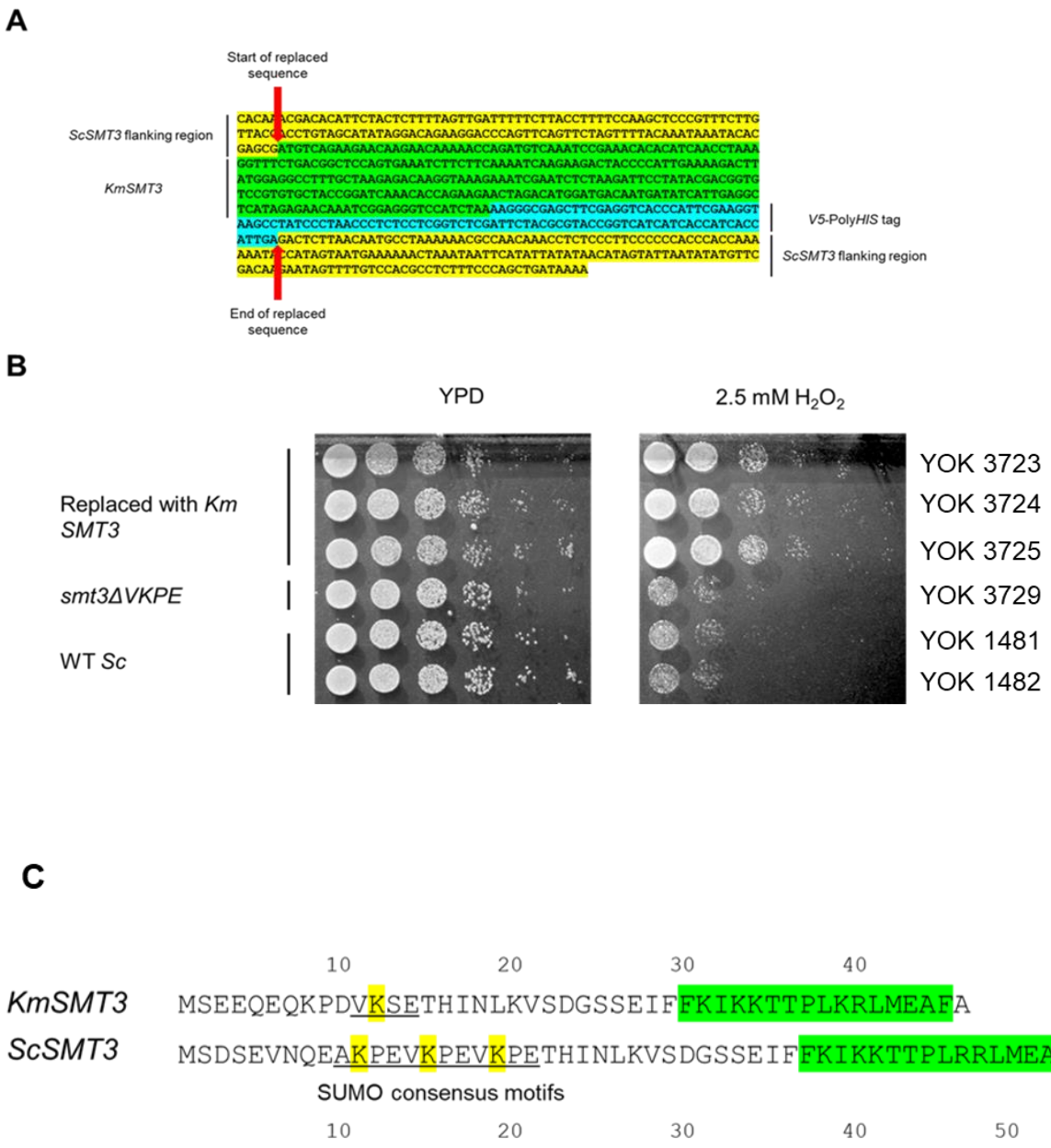


Figure 6: *KmSMT3* replacement enhances stress tolerance of *S. cerevisiae* to H_2O_2. **(A)** Sequence confirmation from a successful *KmSMT3* replacement (YOK 3723). The yellow shows the *ScSMT3* locus flanking regions, the green shows the entire *KmSMT3* sequence (in place of *ScSMT3*), and the blue shows the accompanying V5-PolyHIS tag. The red arrows point at where the *KmSMT3* sequence starts and ends. **(B)** Serial dilutions of three isolates with *KmSMT3* (YOKs 3723, 3724, and 3725) were spotted onto YPD and H_2O_2 plates. smt3ΔVKPE is a mutant (YOK 3729). Two *ScSMT3* WT controls are spotted at the bottom (YOKs 1481 and 1482). Plates were incubated at 30°C for 72 hours. **(C)** Amino acid sequence comparison between *KmSMT3* and *ScSMT3*. The yellow highlights lysine residues residing on SUMO consensus motifs, the SUMO consensus motifs are

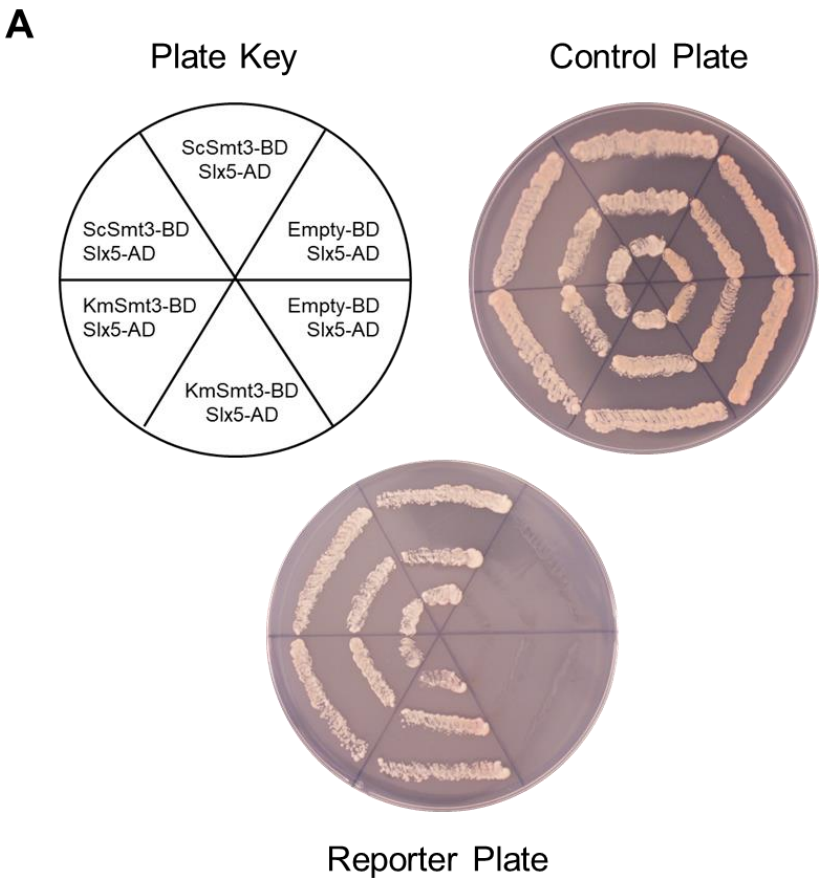
underlined, and the green highlights the stretch of hydrophobic amino acid residues that make up the core for SUMO interacting motifs (SIMs).

3.8 KmSmt3 interacts with ScSlx5

The enhanced stress tolerance enabled by *KmSMT3* may be due to this protein interacting differently with key Sc SUMO pathway proteins. We chose to assess the interaction of *Km*Smt3 with the SUMO-targeted ubiquitin ligase (STUbL) Slx5. Slx5 is part of the Slx5/Slx8 complex and is a heterodimer that depends on four SUMO interacting motifs (SIMs) to bind to sumoylated proteins. The binding of Slx5/8 to these proteins mediates their ubiquitination (reviewed in Heideker et al. 2009; Wang & Prelich 2009).

To compare the interaction between KmSmt3 and ScSmt3 with ScSlx5, PCR-amplified *KmSMT3* was cloned into a yeast two-hybrid bait construct (Gal4-BD fusion) and transformed into a two-hybrid reporter Sc strain (AH109) that already contained a Gal4-AD fusion of ScSlx5 (**Fig. 7B**). An empty Gal4-BD and an ScSmt3 fusion with Gal4-BD were also transformed in the strain as negative and positive controls respectively. The interaction between the proteins were assessed based on expression of Adenine and Histidine reporter genes. As expected, KmSmt3 and ScSmt3 interact with ScSlx5 (**Fig. 7A**). To assess the strength of the interaction between KmSmt3 and ScSmt3 with ScSlx5, we performed an *in-vitro* ONPG assay. We observed that the KmSmt3-ScSlx5 has a x0.4-fold increase binding activity in comparison to ScSmt3-ScSlx5. **Figure 7C** shows the error bars not overlapping, but due to small sample size (n=3), the increase is not significant ($P < 0.192433$). More interesting is the observation that KmSmt3-ScSlx5 retained its

increased binding activity when the ONPG assay was performed with cells that had been previously treated with acute oxidative stress (25 mM H_2O_2 for 30 minutes) ($P < 0.089663$). Further repeats of this assay will clarify the differences in interaction strength between ScSmt3 and KmSmt3 with ScSlx5, but it appears that KmSmt3 at least as strong a binding interaction to ScSlx5 as ScSmt3 does.



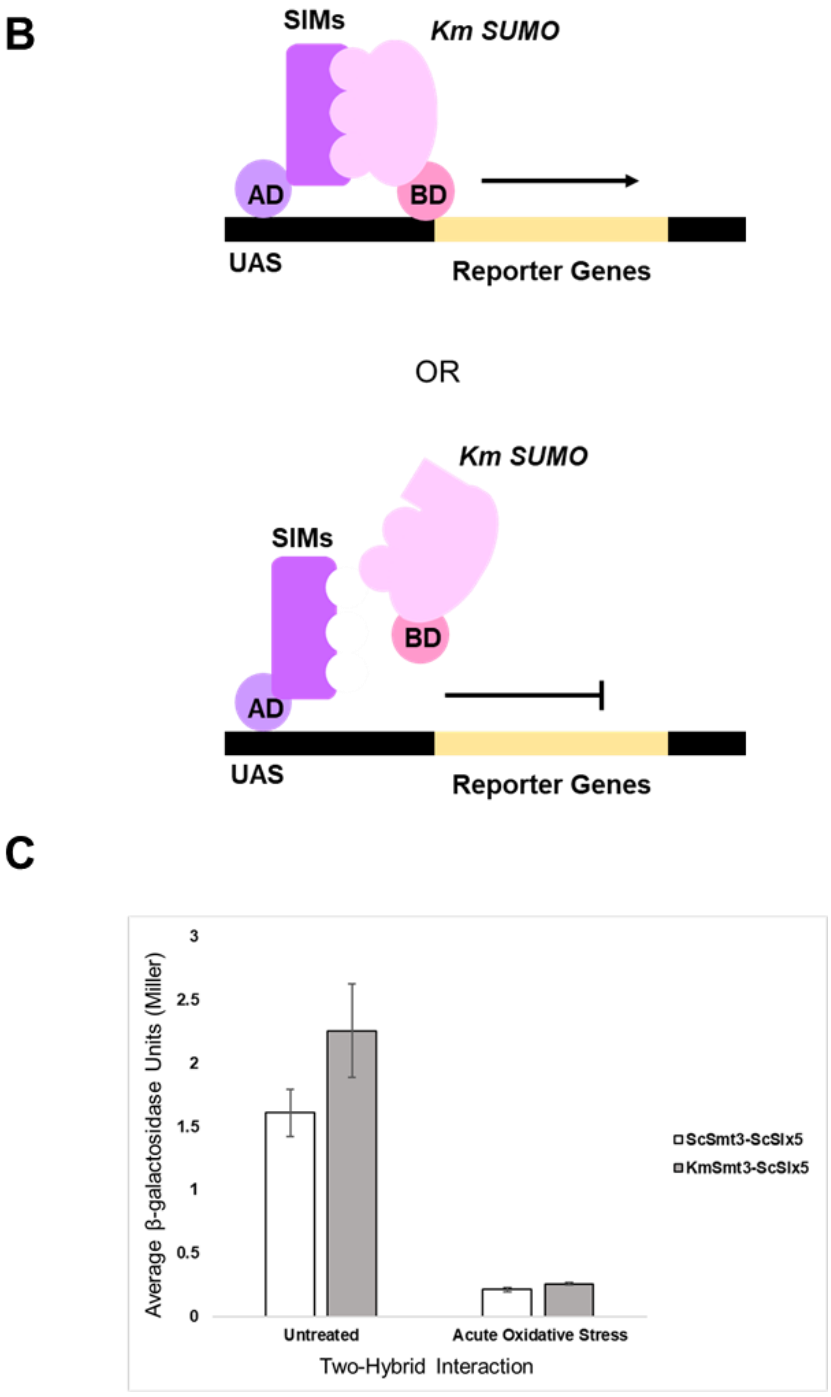


Figure 7: KmSmt3 interacts with ScSlx5. (A) Six isolate groups of AH109 strains containing KmSMT3-BD (BOK 1621) and ScSLX5-AD (BOK 289), ScSMT3-BD (BOK 295) and ScSLX5-AD (BOK 289) (positive control), and empty vector-BD (BOK 313) and ScSLX5-AD (BOK 312) (negative control) were batched on selective -LEU and -TRP media to confirm transformation, and on selective -LEU -TRP -ADE -HIS media to test for interactions. **(B)** Schematic showing the *KmSMT3-BD* and *ScSLX5-AD* fusions and how

the *SMT3* interaction with SUMO interacting motifs (SIMs) is tested. **(C)** An *in-vitro* ONPG assay was conducted with overnight cultures of AH109 strains expressing KmSmt3-BD + ScSlx5-AD, ScSmt3-BD + ScSlx5-AD, and empty vector-BD + ScSlx5-AD that were either untreated or exposed to 30 minutes of H_2O_2. OD_{425} of each culture was measured, and β-galactosidase activity was calculated. For both untreated and treated cultures, KmSmt3-ScSlx5 shows a higher binding activity in comparison to ScSmt3-ScSlx5 (n=3; untreated $P < 0.192433$; acute oxidative stress $P < 0.089663$).

3.9 KmSmt3 significantly enhances resistance to oxidative stress in a quantifiable yeast colony color competition assay

We sought to further establish the enhanced oxidative stress tolerance properties of KmSmt3 replacement in Sc with a quantifiable yeast colony color competition assay. We utilized WT Sc strains that have been engineered to express pink and blue pigments. Using the same CRISPR/Cas9 methods described in **3.6**, we replaced Sc SUMO with Km SUMO in a WT Sc blue strain. Replacement with *KmSMT3* was sequence confirmed for two blue Sc colonies (YOKs 3745 and 3746), one which was then grown with WT pink Sc (YOK 3733) in YPD media as a 1:1 mix. The 1:1 mixed culture was then plated onto media plates before and after acute H_2O_2 treatment (25 mM for 30 minutes). After an incubation period of 3-5 days, oxidative stress tolerance was scored based on number of colonies for each color (blue or pink). Intriguingly, the number of blue Sc colonies carrying the *KmSMT3* replacement significantly outnumber the WT pink Sc colonies that express *ScSMT3* ($P > 6.95744E-5$) (**Fig. 8B**).

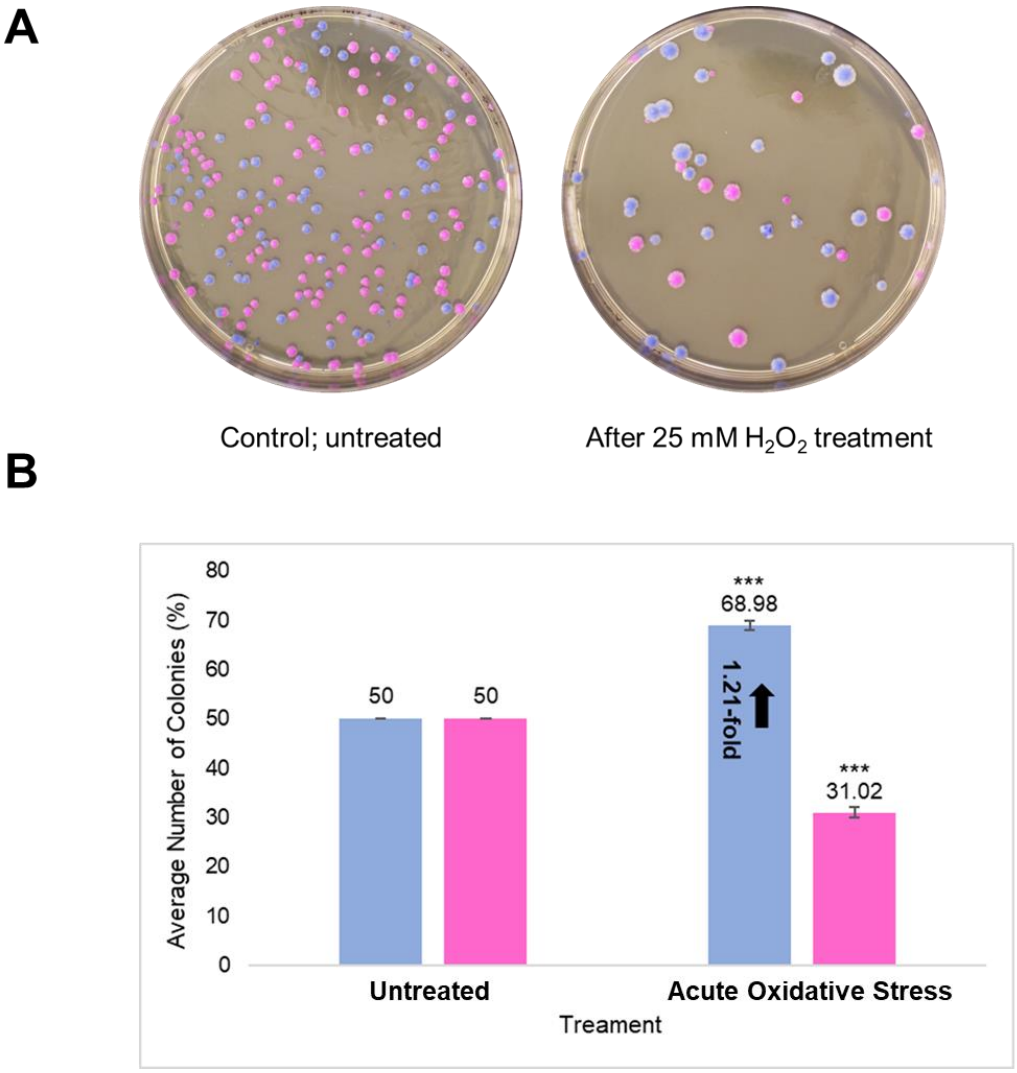


Figure 8: Blue Sc colonies with *KmSMT3* significantly outnumber Pink WT Sc colonies after H_2O_2 treatment. Panel **(A)** shows 1:1 mixed blue Sc with KmSmt3 (YOK 3745) and WT pink Sc colonies (YOK 3733) growing before and after acute oxidative stress treatment. 5 OD_{600} of overnight 1:1 mixed cultures of blue and pink cells were left either untreated or treated with 25 mM H_2O_2 for 30 minutes. Untreated cells were diluted 1:10,000 (to avoid confluency) and H_2O_2 treated cells were diluted 1:5 and plated onto YPD media containing G418 (for pigment selection). Plates were incubated at 30°C for up to five days. **(B)** Number of blue and pink colonies were counted for untreated and H_2O_2 treated cells. The final count for each sample was normalized for WT pink Sc colonies base growth advantage (15.63%) and dilution factor (x10,000 / 5). Average percentage of each sample was graphed. The graph illustrates a significant x1.21-fold increase in colony number of Sc blue cells carrying *KmSMT3* over colony number of WT Sc pink cells (n=3; P > 6.95744E-5).

CHAPTER 4: DISCUSSION

4.1 Is there a SUMO stress response (SSR) in *K. marxianus*?

The presence of the SSR in H_2O_2 treated *K. marxianus* cells, as visualized by the anti-SUMO antibody, supports our hypothesis that *K. marxianus* cells involve the SUMO pathway in their remarkable stress tolerance properties. Regarding differences in levels of sumoylation between Km and Sc cells, it's important to note that the polyclonal anti-SUMO antibody we used was raised against Sc. Therefore, little interpretation could be made based on differences in sumoylation levels. However, differences (and similarities) in patterns of sumoylation between Km and Sc cells can be interpreted. In both Km and Sc cells, specific proteins show increased sumoylation in response to increased H_2O_2 concentration (indicated by the gray arrows in **Fig. 3**), which is consistent with an increase of sumoylation due to stress (See **Introduction 1.4**).

Additionally, in Km cells, we observed an increase of sumoylated conjugates with high molecular weights (from 125 kDa up to 200 kDa, indicated by the gray bracket). High molecular weight SUMO conjugates can be indicators of an increase in SUMO chain formations and/or poly-SUMO attachments to target proteins. However, it is only in Sc cells that we detect an increase of sumoylated conjugates with molecular weights as high as 250 kDa. This could be due to Km proteins having shorter sequences in general (Yamamoto et al., 2015), or it may indicate that Km cells produce fewer SUMO chains and/or poly-SUMO modified proteins in comparison to Sc proteins during H_2O_2 exposure. There are no previous studies that investigate how the length of SUMO chains may affect the cellular stress response, but increased and constant levels of poly-SUMO are linked to slow growth and replication stress sensitivity (Békés et al., 2011). Furthermore, during

the recovery period post stress, only cells with depleted levels of chaperone proteins and proteasomes retain sumoylated conjugates for a longer period, indicating that desumoylation is an essential step for recovery (Schwartz et al., 2007; Liebelt et al., 2019). Hence, it can be speculated that having fewer SUMO chains and poly-SUMO moieties on proteins is due to faster desumoylation of conjugates during the post-stress recovery phase of Km cells. In other words, Km cells may recover faster from stress exposure due to quicker turn-over of stress induced SUMO chains.

It is also possible that the increase of high molecular weight sumoylated conjugates is not due to increased poly-sumoylation of the same target proteins, but rather an increase in sumoylation of high molecular weight target proteins. This means that the difference in pattern of sumoylation between Km and Sc cells observed, especially at high molecular weights, is due to the sumoylation of *different* target proteins between the two strains, and hence activation of alternative stress response mechanisms. To determine the reason for increase of high molecular weight SUMO conjugates, a SUMO pulldown assay on select high molecular weight SUMO targets may be conducted after stress exposure, and the sumoylated protein can be identified using mass spec approaches.

4.2 Does the overexpression of Km SUMO pathway proteins enhance stress tolerance in Sc?

Out of our Sc transformants, each overexpressing one of the nine Km SUMO pathway proteins, five resulted in lethality after galactose induction (refer to **Fig. 4**), and out of the four-remaining viable transformants, none enhanced stress tolerance when

expressed in WT Sc. We interpret these lethality phenotypes mainly as a consequence of the effects of overexpressing proteins, which are: stoichiometric imbalance, promiscuous interactions, and pathway modulations (reviewed in Moriya 2015). All the cloned Km genes encode proteins that interact with SUMO and may **1)** deplete SUMO modified proteins **2)** cause hyper-sumoylation, and/or **3)** induce hypo-sumoylation in case of Ulp1 or Ulp2 overexpression.

Additionally, we observed that co-overexpression of certain Km SUMO pathway genes can suppress the lethal effects of KmSlx5 and KmSiz1; KmUbc9 can suppress KmSiz1 lethality while KmSmt3 can suppress KmSlx5 lethality. It can be reasoned that the increase in the E2 conjugating enzyme Ubc9 causes a rise in SUMO that is ready to bind to the E3 Siz1, and therefore prevents Siz1's lethal interactions with other proteins. This suppression may also be due to Siz1 and Ubc9 binding to each other as previous studies have shown these two proteins directly interactive with each other (Johnson & Gupta 2001). Similarly, Smt3 is able to suppress Slx5 lethality likely because the excess Smt3 can bind to all the four SIMs on Slx5, rendering this STUbL inactive. However, it's important to note that the results of this experiment remain unexplained and must be confirmed as there are no previous studies establishing the effects of co-overexpressing these pairs of SUMO pathway proteins.

Results from overexpression of *KmSMT3* in the *smt3-331* strain (Refer to **Fig. 5**) indicates that *KmSMT3* can reliably complement *smt3-331* lethal effects at 37°C, but a robust increase in stress tolerance at 40°C was not repeatedly observed. One possible explanation is that excess *levels* of Km SUMO pathway proteins produced in addition to the endogenously expressed SUMO pathway proteins may interfere with and mask the

true effects of the Km SUMO pathway proteins on stress tolerance. This possibility was addressed by complete replacement of Sc SUMO with Km SUMO.

4.3 Does replacing *ScSMT3* with *KmSMT3* in *S. cerevisiae* cells enhance stress tolerance?

KmSmt3 ability to suppress H_2O_2 sensitivity in both pigmented and non-pigmented WT Sc cells after complete replacement of ScSmt3 (Refer to **Figs 6B & 8**) suggests that a specific set of proteins may rely on sumoylation to enable stress tolerance of the cell. For example, proteins that are involved in the yeast response to oxidative stress, specifically to H_2O_2, are the superoxide dismutases MnSod (encoded by *SOD2*) and Cu/ZnSod (encoded by *SOD1*), which function to remove superoxide anions from the cytoplasm (reviewed in Jamieson 1998) and are known substrates of SUMO (Zhong & Xu 2008). So, for example, it may be possible that KmSmt3 increases SOD stability and activity during and after stress exposure. Interestingly, KmSmt3 replacement did not enhance the stress tolerance of Sc cells against other tested stressors, like heat exposure/shock and UV treatment. This further points to the possibility that the enhanced stress tolerance of Sc cells with KmSmt3 may be mainly due to the ability of KmSmt3 conjugating (or not conjugating) to certain target proteins that may be specifically involved in the oxidative stress response of the cell (such as MnSod and Cu/ZnSod), but not necessarily in the heat shock and/or the DNA damage response. In future studies, this hypothesis can be tested by purifying Km or Sc modified MnSod and Cu/ZnSod and assessing their stability and activity in in vitro assays (Weydert & Cullen, 2010).

Whether Km SUMO may be capable of different target binding activities in comparison to Sc SUMO needs to be further studied. The comparative analysis of Km and Sc SUMO protein sequences reveals that while three (out of nine) lysine residues that enable SUMO chain formation in Sc are located on a repeating SUMO consensus motif sequence at positions 11, 15, and 19 in the N-terminal region of SUMO (Bylebyl et al., 2003), the *K. marxianus* lysine residues are positioned differently, with only one lysine residue residing in a SUMO consensus motif (at position 12) (Refer to **Figure 6C**). This may implicate that the SUMO chain formation in *K. marxianus* may be limited and occurs at a different position of the SUMO protein. The latter may also be the reason why there is reduced sumoylation observed on the western blot in **Fig. 3**. We also noted a shift in the position of the stretch of hydrophobic residues (V/I/L) that are part of the of *KmSMT3* that accepts the SIM of SUMO interacting proteins, such as the STUbLs, Slx5 (Xu et al., 2014). However, the length and the identity of the amino acids that occupy this SIM region are identical in both Km and Sc SUMO. The potential for limited SUMO chain formation may render KmSmt3 more organized and compact in comparison to ScSmt3. However, besides our two-hybrid interaction assays with Slx5 that suggest an increase in interaction between KmSmt3 and SIMs, we currently have no experimental data to support these hypotheses. Further analysis of *KmSMT3* modification is required.

Comparative analysis of the protein hydrophobicity of KmSmt3 and ScSmt3 show that KmSmt3 has an average hydrophobicity score of -0.82584 (Hydro. / Kyte & Doolittle) and ScSmt3 an average score of -0.88275 (Hydro. / Kyte & Doolittle), making KmSmt3 x0.07 less hydrophobic than ScSmt3 (*ExPASy* Protscale analysis tool). Higher hydrophobicity is a contributing factor to protein aggregation (Calamai et al., 2003).

Although the difference between Km and Sc SUMO hydrophobicity score is minimal, if we consider that several SUMO moieties and/or SUMO chains can bind to the same target protein, this slight difference in hydrophobicity adds up, and may be a contributing factor to explain Sc with *KmSMT3*'s enhanced tolerance to oxidative stress due to reduced potential for protein aggregation.

Finally, results of our 2-hybrid KmSmt3-ScSlx5 interaction assay support the possibility that KmSmt3 enhances Sc stress tolerance against oxidative stress because of higher efficiency in binding of Km SUMO to the SIMs. An increased binding activity of Smt3 to Slx5 may cause a more efficient turnover of SUMO modified proteins by STUbLs. In other words, increased binding activity of STUbLs could mark sumoylated proteins for more efficient ubiquitination, which during stress is essential for clearing the cells of unfolded proteins (Cundiff et al., 2019). For future studies, it would be interesting to design a competitive binding assay for *KmSMT3* and *ScSMT3* with *ScSLX5* to establish whether *KmSMT3* more efficiently binds SIMs in the presence of *ScSMT3* (Pollard, 2017).

4.4 Significance

Understanding how SUMO contributes to stress tolerance and what specific properties make a protein more stress resilient than its ortholog is significant because it may help us understand the conserved stress tolerance mechanisms in normal and diseased cells. For example, we may understand the role that SUMO plays in stress tolerance of eukaryotic pathogens: Several pathogenic yeasts have evolved to be highly stress resistant and render antifungal drug treatments ineffective. Classes of the yeast *Candida*, *Aspergillus*, and *Cryptococcus* are the predominant cause of nosocomial

infections, which lead to mortalities nearly 40% of the time (reviewed in Revie et al., 2018), but studying SUMO and its role in stress tolerance has already shown that the *Candida albicans* yeast can become stress sensitive (Leach et al., 2011). Additionally, cancer cells are known to survive despite hypoxia, protein misfolding, high mutational load, and chemotherapy treatments, and it has been proposed that cancer cells rely on SUMO dysregulation for their survival in such hostile environments (reviewed in Seeler & Dejean 2017). Specifically, the SUMO E2 and E3 conjugating and ligating enzymes show elevated levels in tumors, suggesting cancer cells rely on increased sumoylation for survival (Moschos et al., 2010; Seeler & Dejean 2017). In contrast, upregulation of some SUMO proteases has also been linked to the development of some cancers, such as breast cancer (Li et al., 2014). Finally, sumoylation has been observed to co-localize with neural inclusions and aggregating proteins in several neurodegenerative diseases, such as multiple system atrophy (MSA), Huntington's, and Parkinson's (reviewed in Dorval & Fraser 2007; Eckermann 2013), and a previous study shows that STUbLs are essential for survival of yeast cells expressing an aggregation-prone protein (Ohkuni et. al, 2018).

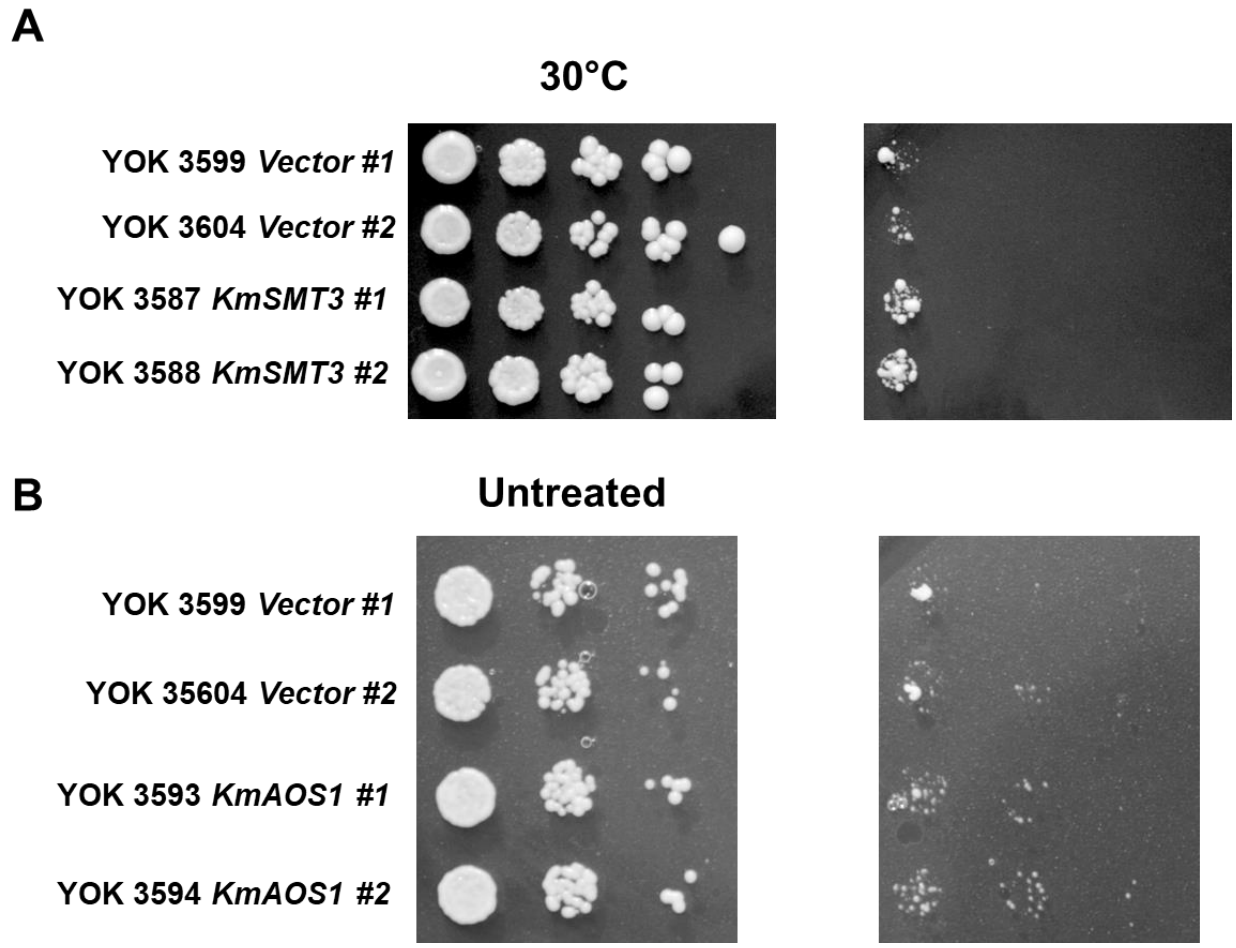
Given what we will learn from SUMO and sumoylation in stress tolerance, it's important to investigate how or whether we can modulate its role to reduce stress tolerance in eukaryotic pathogens and cancerous cells, and to prevent and/or minimize aggregating proteins. In summary, we foresee that this research has the potential to open new avenues for pharmacological intervention to enhance stress resistance pathways in normal cells (i.e. prevent protein aggregation) or reduce stress in diseased cells and eukaryotic pathogens.

4.5 Future directions

One critical experiment to conduct in the future is to replace KmSmt3 with ScSmt3 in *K. marxianus* to see whether *K. marxianus*' stress tolerance will diminish when exposed to H_2O_2. To do so, the same CRISPR/Cas9 approach can be used, but a Km specific CRISPR plasmid is needed. The Kerscher lab is currently working on customizing the pML104 CRISPR plasmid for use in *K. marxianus*. Another important future experiment is comparing sumoylation levels between WT *S. cerevisiae* cells and *S. cerevisiae* cells with KmSmt3 replacement in the presence of absence of H_2O_2. Detecting differences in pattern and/or levels of sumoylation in combination with mass spec can help us identify the pivotal stress-resistance proteins.

In depth analysis of previously conducted experiments is also important. First, the co-overexpression of Km SUMO pathway genes and their suppression effects needs to be confirmed via experimental repeats in multiple strain backgrounds. Second, results of the pigmented yeast competition assay can be strengthened by repeating the assay with additional isolates of the WT Sc pink and blue strains, and with switching the pigmented Sc strain with a *KmSMT3* integrant. The enhanced stress tolerance against oxidative stress enabled by Km Smt3 can also be further characterized by measuring other aspects of this experiment, such as the time it takes for the first colonies to grow after H_2O_2 treatment, and by assessing cell morphologies that may be indicators of arrested or dying cells, such as enlarged buds (reviewed in Lippuner et al. 2014). Ultimately, we hope that these studies will help us identify the specific mechanism and protein targets involved in SUMO-dependent stress tolerance.

Appendix



Supplementary Figure 1. Examples of additional stress tolerance assays performed with Km SUMO pathway genes overexpression. (A) Comparison of WT Sc plus empty vector (BOK 344) (YOKs 3599 and 35604) and Sc plus Km *SMT3* isolates' (YOKs 3587 and 3588) growth at elevated temperatures (40°C) and **(B)** comparison of WT Sc plus empty vector and Sc plus Km *AOS1* isolates (YOKs 3593 and 3594) growth after treatment with 90.0 mJ UV show no apparent growth advantages. 1 OD_{600} of overnight colony cultures were harnessed, and they were serially 10-fold diluted and spotted, from left to right, on YPD media. Plates were incubated for 72 hours.

Supplementary Table 1. Growth scores for Km SUMO pathway gene co-overexpression in MHY500/501. (YOKs 1481/1482).

Km SUMO Pathway Genes Paired	Growth on 2% Galactose
ULP1 + UBA2 (BOK 1548 + BOK 1547)	+*
ULP1 + ULP2 (BOK 1548 + BOK 1569)	+*
ULP1 + SLX5 (BOK 1548 + BOK 1570)	+*
ULP1 + SIZ1 (BOK 1548 + BOK 1567)	-
ULP1 + SMT3 (BOK 1548 + BOK 1568)	+*
ULP1 + UBC9 (BOK 1548 + BOK 1566)	+*
ULP1 + AOS1 (BOK 1548 + BOK 1542)	+*
ULP1 + MMS21 (BOK 1548 + BOK 1540)	+*
ULP2 + UBA2 (BOK 1569 + BOK 1547)	-
ULP2 + SLX5 (BOK 1569 + BOK 1570)	-
ULP2 + SIZ1 (BOK 1569 + BOK 1567)	-
ULP2 + SMT3 (BOK 1569 + BOK 1568)	+*
ULP2 + UBC9 (BOK 1569 + BOK 1566)	+*
ULP2 + AOS1 (BOK 1569 + BOK 1542)	+*
ULP2 + MMS21 (BOK 1569 + BOK 1540)	+*
UBA2 + SLX5 (BOK 1547 + BOK 1570)	-
UBA2 + SIZ1 (BOK 1547 + BOK 1567)	-
UBA2 + SMT3 (BOK 1547 + BOK 1568)	-/+
UBA2 + UBC9 (BOK 1547 + BOK 1566)	-/+
UBA2 + AOS1 (BOK 1547 + BOK 1542)	-/+
UBA2 + MMS21 (BOK 1547 + BOK 1540)	-/+
SLX5 + SIZ1 (BOK 1552 + BOK 1567)	-
SLX5 + SMT3 (BOK 1552 + BOK 1568)	+

SLX5 + AOS1 (BOK 1552 + BOK 1542)	-/+
SLX5 + UBC9 (BOK 1552 + BOK 1566)	-/+
SLX5 + MMS21 (BOK 1552 + BOK 1540)	-
SIZ1 + SMT3 (BOK 1544 + BOK 1568)	-/+
SIZ1 + AOS1 (BOK 1544 + BOK 1542)	-/+
SIZ1 + UBC9 (BOK 1544 + BOK 1566)	-
SIZ1 + MMS21 (BOK 1544 + BOK 1540)	-/+
SMT3 + AOS1 (BOK 1546 + BOK 1542)	+
SMT3 + UBC9 (BOK 1546 + BOK 1566)	+
SMT3 + MMS21 (BOK 1546 + BOK 1540)	+
UBC9 + AOS1 (BOK 1541 + BOK 1542)	+
UBC9 + MMS21 (BOK 1541 + BOK 1540)	+
MMS21 + AOS1 (BOK 1540 + BOK 1542)	+

Supplementary Table 2. Yeast strains used in this study.

Name:	**Pertinent Genotypes or Parent Strains:**	**Plasmids:**	**Reference:**
YOK2993	W303; WT Sc mat α *leu2-3,112 trp1-1, ura3-1, ade2-1, his3-11,15*	-	Rothstein, 1983
YOK1481	MHY500; WT Sc mat a *his3-200 leu2-3,112 ura3-52 lys2-801 trp1-1*	-	Rothstein, 1983
YOK1482	MHY501; WT Sc mat α *his3-200 leu2-3,112 ura3-52 lys2-801 trp1-1*	-	Hochstrasser et al., 1993
YOK3733	M. Dunham 3443 Pink Sc mat a *leu2Δ ura3Δ his3Δ1 met15Δ lys'2*	pMS008/G418	Jeff Boeke Laboratory, NYU
YOK3734	M. Dunham 3449 Blue Sc mat a *leu2Δ ura3Δ his3Δ1 met15Δ lys'2*	pMS003/G418	Jeff Boeke Laboratory, NYU
YOK1220	AH109 mat a Sc Reporter strain *his3, ade2, trp1, leu2*	-	Clontech Laboratories

YOK3621	*smt3-331* mat a *ura3-1 leu2,3-112 his3-11*	-	Biggins et al., 2001
YOK3580	BY28353 *ura3-1, ade2-2, leu2-2*	-	Yeast Genetics Resource Center, Osaka University
YOK3581	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km ULP1/URA3 (BOK 1554) #1	This study
YOK3582	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km ULP1/URA3 (BOK 1554) #2	This study
YOK3583	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km ULP1/URA3 (BOK 1554) #3	This study
YOK3584	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km ULP2/URA3 (BOK 1549) #1	This study
YOK3585	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km ULP2/URA3 (BOK 1549) #2	This study
YOK3586	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km ULP2/URA3 (BOK 1549) #3	This study
YOK3587	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SMT3/URA3 (BOK 1546) #1	This study
YOK3588	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SMT3/URA3 (BOK 1546) #2	This study
YOK3589	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SMT3/URA3 (BOK 1546) #3	This study
YOK3590	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km UBA2/URA3 (BOK 1547) #1	This study
YOK3591	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km UBA2/URA3 (BOK 1547) #2	This study
YOK3592	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km UBA2/URA3 (BOK 1547) #3	This study
YOK3593	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km AOS1/URA3 (BOK 1543) #1	This study
YOK3594	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km AOS1/URA3 (BOK 1543) #2	This study
YOK3595	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km AOS1/URA3 (BOK 1543) #3	This study
YOK3596	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km UBC9/URA3 (BOK 1553) #1	This study

YOK3597	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km UBC9/URA3 (BOK 1553) #2	This study
YOK3598	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km UBC9/URA3 (BOK 1553) #3	This study
YOK3600	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km MMS21/URA3 (BOK 1540) #1	This study
YOK3601	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km MMS21/URA3 (BOK 1540) #2	This study
YOK3602	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km MMS21/URA3 (BOK 1540) #3	This study
YOK3603	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SIZ1/URA3 (BOK 1545) #1	This study
YOK3604	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SIZ1/URA3 (BOK 1545) #2	This study
YOK3605	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SIZ1/URA3 (BOK 1545) #3	This study
YOK3606	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SLX5/URA3 (BOK 1552) #1	This study
YOK3607	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SLX5/URA3 (BOK 1552) #2	This study
YOK3608	W303 WT Sc mat α	pYES2.1 V5-His-TOPO Km SLX5/URA3 (BOK 1552) #3	This study
YOK3599	W303 WT Sc mat α	pRS426/URA3 (BOK 344) #1	This study
YOK3609	W303 WT Sc mat α	pRS426/URA3 (BOK 344) #2	This study
YOK3610	W303 WT Sc mat α	pRS426/URA3 (BOK 344) #3	This study
YOK3611	*smt3-331*	pYES2.1 V5-His-TOPO Km SMT3/URA3 (BOK 1546) #1	This study
YOK3612	*smt3-331*	pYES2.1 V5-His-TOPO Km SMT3/URA3 (BOK 1546) #2	This study
YOK3624	*smt3-331*	pYES2.1 V5-His-TOPO Sc SMT3/URA3 (BOK 1562)	This study

YOK3617	*smt3-331*	pRS426/URA3 (BOK 344) #1	This study
YOK3618	*smt3-331*	pRS426/URA3 (BOK 344) #2	This study
YOK3828	MHY500/MHY501 Diploid	pRS426/URA3 (BOK 344)	This study
YOK3631	MHY500 mat a	pYES2.1 V5-His-TOPO Km SMT3/URA3 (BOK 1546)	This study
YOK3632	MHY501 mat α	pYES2.1 V5-His-TOPO Km SMT3/URA3 (BOK 1546)	This study
YOK3633	MHY500 mat a	pYES2.1 V5-His-TOPO Km ULP1/URA3 (BOK 1554)	This study
YOK3634	MHY501 mat α	pYES2.1 V5-His-TOPO Km ULP1/URA3 (BOK 1554)	This study
YOK3635	MHY500 mat a	pYES2.1 V5-His-TOPO Km ULP2/URA3 (BOK 1549)	This study
YOK3636	MHY501 mat α	pYES2.1 V5-His-TOPO Km ULP2/URA3 (BOK 1549)	This study
YOK3637	MHY500 mat a	pYES2.1 V5-His-TOPO Km UBC9/URA3 (BOK 1553)	This study
YOK3638	MHY501 mat α	pYES2.1 V5-His-TOPO Km UBC9/URA3 (BOK 1553)	This study
YOK3639	MHY500 mat a	pYES2.1 V5-His-TOPO Km SIZ1/URA3 (BOK 1544)	This study
YOK3640	MHY501 mat α	pYES2.1 V5-His-TOPO Km SIZ1/URA3 (BOK 1544)	This study
YOK3641	MHY500 mat a	pYES2.1 V5-His-TOPO Km SLX5/URA3 (BOK 1552)	This study

YOK3642	MHY501 mat α	pYES2.1 V5-His-TOPO Km SLX5/URA3 (BOK 1552)	This study
YOK3723	MHY500 mat a	with Km SMT3 replacement #1	This study
YOK3725	MHY500 mat a	with Km SMT3 replacement #2	This study
YOK3724	MHY501 mat α	with Km SMT3 replacement #1	This study
YOK3726	MHY501 mat α	with Km SMT3 replacement #2	This study
YOK3727	MHY501 mat α	with Km SMT3 replacement #3	This study
YOK3728	MHY501 mat α	with Km SMT3 replacement #4	This study
YOK3737	MHY501 mat α	with Km SMT3 replacement #5	This study
YOK3738	MHY501 mat α	with Km SMT3 replacement #6	This study
YOK3745	M. Dunham 3449 Blue Sc	with Km SMT3 replacement #1/G418	This study
YOK3746	M. Dunham 3449 Blue Sc	with Km SMT3 replacement #2/G418	This study
YOK3747	AH109	ScSlX5-AD/LEU2 (BOK289) + KmSMT3-BD/TRP1 #1	This study
YOK3748	AH109	ScSlX5-AD/LEU2 (BOK289) + KmSMT3-BD/TRP1 #2	This study
YOK3749	AH109	ScSlX5-AD/LEU2 (BOK289) + KmSMT3-BD/TRP1 #3	This study
YOK3729	MHY500/1 *smt3ΔVKPE*	Sequence listed under “Other Sequences”	This study

Supplementary Table 3. Plasmids used in this study.

Name:	Gene(s):	Based on:	Description:	Reference:
BOK1540	Km MMS21 #4	pYES2.1 V5-His-TOPO	SUMO E3	This study
BOK1542	Km AOS1 #7	pYES2.1 V5-His-TOPO	SUMO E1	This study
BOK1543	Km AOS1 #8	pYES2.1 V5-His-TOPO	SUMO E1	This study
BOK1544	Km SIZ1 #6	pYES2.1 V5-His-TOPO	SUMO E3	This study
BOK1545	Km SIZ1 #7	pYES2.1 V5-His-TOPO	SUMO E3	This study
BOK1546	Km SMT3 #7	pYES2.1 V5-His-TOPO	SUMO	This study
BOK1562	Sc SMT3 #4	pYES2.1 V5-His-TOPO	**Sc** SUMO	This study
BOK1547	Km UBA2 #7	pYES2.1 V5-His-TOPO	SUMO E1	This study
BOK1548	Km ULP1 #6	pYES2.1 V5-His-TOPO	SUMO Protease	This study
BOK1554	Km ULP1 #1	pYES2.1 V5-His-TOPO	SUMO Protease	This study
BOK1549	Km ULP2 #6	pYES2.1 V5-His-TOPO	SUMO Protease	This study
BOK1552	Km SLX5 #6	pYES2.1 V5-His-TOPO	STUbL	This study
BOK1553	Km UBC9 #1	pYES2.1 V5-His-TOPO	SUMO E2	This study
BOK1566	Km UBC9	pAG425Gal-ccdB-HA	SUMO E2	This study
BOK1567	Km SIZ1	pAG425Gal-ccdB-HA	SUMO E3	This study
BOK1568	Km SMT3 #1	pAG425Gal-ccdB-HA	SUMO	This study

BOK1569	Km SMT3 #2	pAG425Gal-ccdB-HA	SUMO	This study
BOK1570	Km SLX5	pAG425Gal-ccdB-HA	STUbL	This study
BOK1593	-	pML104 CRISPR/Cas9	**Not** in dam- strain	Addgene/67638/ Laughery et al., 2015
BOK1594	-	pML104 CRISPR/Cas9	In dam-/dcm- strain (NEB# C2925I)	This study
BOK1606	ScSMT3 gRNA #1.1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc SMT3	This study
BOK1607	ScSMT3 gRNA #1.2	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc SMT3	This study
BOK1612	ScAOS1 gRNA #1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc AOS1	This study
BOK1613	ScSLX5 gRNA #1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc SLX5	This study
BOK1614	ScUBC9 gRNA #1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc UBC9	This study
BOK1615	ScUBA2 gRNA #1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc UBA2	This study
BOK1616	ScSIZ1 gRNA #1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc SIZ1	This study
BOK1617	ScMMS21 gRNA #1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc MMS21	This study
BOK1618	ScULP1 gRNA #1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc ULP1	This study
BOK1619	ScULP2 gRNA #1	pML104 CRISPR/Cas9	For CRISPR/Cas9 targeting cut in Sc ULP2	This study
BOK1621	KmSMT3-BD	pOBD-2	Bait; For two-hybrid assay	This study

Supplementary Table 4. Oligonucleotides used in this study, including sequencing primers and guide RNAs.

Name:	Construct:	Description:	Reference:
OOK1081	Km SMT3 Forward Primer	For cloning into vectors	This study
OOK1082	Km SMT3 Reverse Primer	For cloning into vectors; contains stop codon	This study
OOK1089	Km AOS1 Forward Primer	For cloning into vectors	This study
OOK1090	Km AOS1 Reverse Primer	For cloning into vectors; contains stop codon	This study
OOK1091	Km UBA2 Forward Primer	For cloning into vectors	This study
OOK1092	Km UBA2 Reverse Primer	For cloning into vectors; contains stop codon	This study
OOK1093	Km SIZ1 Forward Primer	For cloning into vectors	This study
OOK1094	Km SIZ1 Reverse Primer	For cloning into vectors; contains stop codon	This study
OOK1095	Km MMS21 Forward Primer	For cloning into vectors	This study
OOK1096	Km MMS21 Reverse Primer	For cloning into vectors; contains stop codon	This study
OOK1097	Km SLX5 Forward Primer	For cloning into vectors	This study

OOK1098	Km SLX5 Reverse Primer	For cloning into vectors; contains stop codon	This study

OOK1105	Km ULP1 Forward Primer	For cloning into vectors	This study
OOK1106	Km ULP1 Reverse Primer	For cloning into vectors	This study
OOK1107	Km ULP2 Forward Primer	For cloning into vectors	This study
OOK1117	Km ULP2 Reverse Primer	For cloning into vectors	This study
OOK1109	Km UBC9 Forward Primer	For cloning into vectors	This study
OOK1110	Km UBC9 Reverse Primer	For cloning into vectors	This study
OOK1128	pYES2.1 V5-His-TOPO Site Directed Mutagenesis (SDM) Forward Primer	For removing stop codons; usable for all Km genes from the pYES vector	This study
OOK1129	pYES2.1 V5-His-TOPO SDM Reverse Primer for Km AOS1	For removing Km AOS1 stop codon; used to make BOK and YOK	This study
OOK1130	pYES2.1 V5-His-TOPO SDM Reverse Primer for Km UBA2	For removing Km UBA2 stop codon; used to make BOK 1590	This study
OOK1131	pYES2.1 V5-His-TOPO SDM Reverse Primer for Km SLX5	For removing Km SLX5 stop codon; used to make BOKs 1579 and 1580	This study
OOK1132	pYES2.1 V5-His-TOPO SDM Reverse Primer for Km MMS21	For removing Km MMS21 stop codon; used to make BOK 1591	This study
OOK1133	pYES2.1 V5-His-TOPO SDM Reverse Primer for Km SIZ1	For removing Km SIZ1 stop codon; used to make BOK 1608	This study

OOK1135	pYES2.1 V5-His-TOPO SDM Reverse Primer for Km SMT3	For removing Km SMT3 stop codon	This study
OOK1146	pYES2.1 Reverse Sequencing Primer	For reverse sequencing pYES2.1 plasmids	This study
OOK1163	Sc SMT3 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1164	This study
OOK1164	Sc SMT3 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1163	This study
OOK1169	Sc ADE2 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1169	This study
OOK1170	Sc ADE2 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1170	This study
OOK1171	Sc ULP1 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1172	This study
OOK1172	Sc ULP1 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1171	This study
OOK1173	Sc ULP2 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1174	This study
OOK1174	Sc ULP2 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1173	This study
OOK1175	Sc AOS1 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1176	This study
OOK1176	Sc AOS1 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1175	This study
OOK1177	Sc UBA2 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1178	This study

OOK1178	Sc UBA2 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1177	This study
OOK1179	Sc UBC9 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1180	This study
OOK1180	Sc UBC9 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1179	This study
OOK1181	Sc SIZ1 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1182	This study
OOK1182	Sc SIZ1 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1181	This study
OOK1183	Sc MMS21 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1183	This study
OOK1184	Sc MMS21 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1182	This study
OOK1185	Sc SLX5 gRNA Oligo 1	For gRNA hybridization to ligate with pML104; use with OOK1186	This study
OOK1186	Sc SLX5 gRNA Oligo 2	For gRNA hybridization to ligate with pML104; use with OOK1185	This study
OOK1150	Km SMT3 Forward Primer with Sc SMT3 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1168	Km SMT3 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1195	Km ULP1 Forward Primer with Sc ULP1 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study

OOK1196	Km ULP1 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1197	Km ULP2 Forward Primer with Sc ULP2 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1198	Km ULP2 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1152	Km AOS1 Forward Primer with Sc AOS1 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1189	Km AOS1 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1199	Km UBA2 Forward Primer with Sc UBA2 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1200	Km UBA2 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1192	Km UBC9 Forward Primer with Sc UBC9 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1191	Km UBC9 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1201	Km SIZ1 Forward Primer with Sc SIZ1 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1202	Km SIZ1 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1203	Km MMS21 Forward Primer with Sc MMS21 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study

OOK1204	Km MMS21 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1154	Km SLX5 Forward Primer with Sc SLX5 overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1190	Km SLX5 Reverse Primer with V5-PolyHIS overhang	For constructing CRISPR/Cas9 repair template from pYES2.1 vector	This study
OOK1158	Sc SMT3 Forward Flanking Primer	For confirming Km SMT3 replacement in Sc strain	This study
OOK1157	Sc SMT3 Reverse Flanking Primer	For confirming Km SMT3 replacement in Sc strain	This study
OOK1207	Sc ULP1 Forward Flanking Primer	For confirming Km ULP1 replacement in Sc strain	This study
OOK1220	Km ULP1 Reverse Mid Primer	For confirming Km ULP1 replacement in Sc strain	This study
OOK1209	Sc ULP2 Forward Flanking Primer	For confirming Km ULP2 replacement in Sc strain	This study
OOK1221	Km ULP2 Reverse Mid Primer	For confirming Km ULP2 replacement in Sc strain	This study
OOK1217	Sc AOS1 Forward Flanking Primer	For confirming Km AOS1 replacement in Sc strain	This study
OOK1225	Km AOS1 Reverse Mid Primer	For confirming Km AOS1 replacement in Sc strain	This study
OOK1211	Sc UBA2 Forward Flanking Primer	For confirming Km UBA2 replacement in Sc strain	This study

OOK1222	Km UBA2 Reverse Mid Primer	For confirming Km UBA2 replacement in Sc strain	This study
OOK1205	Sc UBC9 Forward Flanking Primer	For confirming Km UBC9 replacement in Sc strain	This study
OOK1206	Sc UBC9 Reverse Flanking Primer	For confirming Km UBC9 replacement in Sc strain	This study
OOK1213	Sc SIZ1 Forward Flanking Primer	For confirming Km SIZ1 replacement in Sc strain	This study
OOK1223	Km SIZ1 Reverse Mid Primer	For confirming Km SIZ1 replacement in Sc strain	This study
OOK1215	Sc MMS21 Forward Flanking Primer	For confirming Km MMS21 replacement in Sc strain	This study
OOK1224	Km MMS21 Reverse Mid Primer	For confirming Km MMS21 replacement in Sc strain	This study
OOK265	Sc SLX5 Forward Flanking Primer	For confirming Km SLX5 replacement in Sc strain	This study
OOK266	Km SLX5 Reverse Mid Primer	For confirming Km SLX5 replacement in Sc strain	This study
OOK1228	Sc UBC9 gRNA Oligo 2.1	For new gRNA hybridization to ligate with pML104; use with OOK1229	This study
OOK1229	Sc UBC9 gRNA Oligo 2.2	For new gRNA hybridization to ligate with pML104; use with OOK1228	This study
OOK 1122	GAL1 Forward Primer	For pYES2.1 construct sequence confirmation	This study
OOK 1123	V5 C-terminus Reverse Primer	For pYES2.1 construct sequence confirmation	This study

Supplementary Table 5. CRISPR/Cas9 Targeting Cut Site Sequence.

Oligo Pair Names:	CRISPR/Cas9 Targeting Sequence:
OOKs 1163 and 1163 for Sc SMT3	5’ CCAGAGGTCAAGCCAGAAGTCAA 3’
OOKs 1169 and 1170 for Sc ADE2	5’ GCACAAAAGTTAGAAACTGTCGG 3’
OOKs 1171 and 1172 for Sc ULP1	5’ TTGATTATAAAGATGCGATTAGG 3’
OOKs 1173 & 1174 for Sc ULP2	5’ CCGCTAGACACCTTGAACAGCTC 3’
OOKs 1175 & 1176 for Sc AOS1	5’ ATGATAGACAGATTCGTCTATGG 3’
OOKs 1177 & 1178 for Sc UBA2	5’ CCAGTAACAAAAGGACAAAGTTA 3’
OOKs 1179 & 1180 for Sc UBC9	5’ CCTCCTTTTATGCTTACATTCTG 3’
OOKs 1228 & 1229 for Sc UBC9	5’ GAAGGTACAAACTGGGCGGGTGG 3’
OOKs 1181 & 1182 for Sc SIZ1	5’ ATGAAACGCCTGGGCCTGATAGG 3’
OOKs 1183 & 1184 for Sc MMS21	5’ GATGAAAGAATCTCAGGAACAGG 3’
OOKs 1185 & 1186 for Sc SLX5	5’ CCCATCAGACAATAATCCAAATG 3’

Other Sequences:

YOK3729(smt3ΔVKPE)>

CACAAACGTACACATTCTACTCTTTTAGTTGATTTTTCTTACCTTTTCCAAGCTCCCGTTTCTTGTTACCACCTGTA
GCATATAGGACAGAAGGACCCAGTTCAGTTCTAGTTTTACAAATAAATACACGAGCGATGTCGGACTCAGAAGTCAA
TCAAGAAGCTAAGCCAGAAGTCAAGCCTGAGACTCACATCAATTTAAAGGTGTCCGATGGATCTTCAGAGATCTTCT
TCAAGATCAAAAAGACCACTCCTTTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGACAGGGTAAGGAAATGGACTCC
TTAAGATTCTTGTACGACGGTATTAGAATTCAAGCTGATCAGACCCCTGAAGATTTGGACATGGAGGATAACGATAT
TATTGAGGCTCACAGAGAACAGATTGGTGGTGCTACGTATTAGGACTCTTAACAATGCCTAAAAAACGCCAACAAAC
CTCTCCCTTCCCCCCACCCACCAAAAAATACCATAGTAATGAAAAAACTAAATAATTCATATTATATAACATAGTAT
TAATATATGTTCGACAAGAATAGTTTTGTCCACGCCTCTTTCCCCAGCTGATAA

BOK1621(KmSMT3-BD)>

GAL4-DBD Km SMT3 start
ᵛAGATCGAATTCCAGCTGACCᵛATGTCAGAAGAACAAGAACAAAAACCAGATGTCAAATCCGAAACACACATCAACCT
AAAGGTTTCTGACGGCTCCAGTGAAATCTTCTTCAAAATCAAGAAGACTACCCCATTGAAAAGACTTATGGAGGCCT
TTGCTAAGAGACAAGGTAAAGAAATCGAATCTCTAAGATTCCTATACGACGGTGTCCGTGTGCTACCGGATCAAACA

CCAGAAGAACTAGACATGGATGACAATGATATCATTGAGGCTCATAGAGAACAAATCGGAGGGACCATGGCAATTCC
GGGGATCCGTCGACCTGCAGAGATCTATGAATCGTAGATACTGAAAAACCCCGCAAGTTCACTTCAACTGTGCATCG
TGCACCATCTCAATTTCTTTCATTTATACATCGTTTTGCCTTCTTTTATGTAACTATACTCCTCTAAGTTTCAATCT
TGGCCATGTAACCTCTGATCTATAGAATTTTTTAAATGACTAGAATTAATGCCCATCTTTTTTTTGGACCTAAATTC
TTCATGAAAATATATTACGAGGGCTTATTCAGAAGCTTTGGACTTCTTCGCCAGAGGTTTGGTCAAGTCTCCAATCA
AGGTTGTCGGCTTGTCTACCTTGAAAATTTACGAAAAGATGGAAAAGGGTCAAATCGTTGGTAGATACGTTGTTGAC
ACTTCTAAATAAGCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAATAAGTGTATACAA
ATTTTAAAGTGACTCTTAGTTTTAAAACGAAAATTCTTATTCTTGAGTAACTCTTTCCTGTAGGTCAGTTGCTTTCT
CAGGTATAGCATGAGGTCGCTCTTATTGACCACACCTCTACCGGCATGCCGAGCAAATGCCTGCAAATCGCTCCCCA
TTTCACCCAATTGTAGATATGCTAACTCCAGCAATGAGTTGATGAATCTCGGTGTGTATTTTATGTCCTCAGAACAC
CTGTGAATCGTTCTCACACGGTCCTTAATACGAAAGGCCCACCGATCGCCCTCCCATACCGAAGGCGACCGAGCATT
TTTCACATG

CRISPR/Cas9 Replacement Protocol:

gRNA hybridization:

1. Guide RNAs were designed using the below website and oligos were ordered from Integrated DNA Technologies (IDT):
   http://wyrickbioinfo2.smb.wsu.edu/crispr.html
2. gRNA oligo pairs were hybridized at a concentration of 3 uM in a solution of 1x T4 DNA ligase buffer:
   a. 1 uL of gRNA oligo 1 (30 uM concentration) + 1 uL of gRNA oligo 2 (30 uM concentration) + 1 uL of 10x T4 DNA ligase buffer were added to 7 uL of water
   b. The hybridization reaction was held at 95°C for 6 minutes, and then at 70 cycles of decreasing 1°C per minute to reach a final temperature of 25°C.
3. Hybridized reaction was run on agarose gel to confirm DNA presence.
4. Hybridized gRNA was stored at 4°C for use in the cloning steps that followed.

Cloning:

1. 1 uL of the purchased pML104 plasmid was transformed into dam-/dcm- competent cells (NEB# C2925I).
2. Selected colony of pML104 in dam-/dcm- strain was mini-prepped.
3. Resulting mini-prepped plasmid was double digested with the enzymes SwaI (NEB# R0406S) and BclI (NEB# R0160S):
    a. 7 uL of pML104 plasmid + 1 uL of SwaI + 4 uL of 3.1 buffer were added to 27 uL of water.
    b. The digestion reaction was incubated at overnight at room temperature (25°C).
    c. SwaI activity was then stopped by incubating the reaction at 65°C for 20 minutes.
    d. 1 uL of BclI was added to the reaction.
    e. The digestion reaction was then incubated at 50°C for a minimum of 2 hours (maximum 6 hours).
4. The entire double digested reaction was run on an agarose gel.
5. A band of ~11 kb was cut from the gel and cleaned using the IBI Scientific PCR/Gel fragment cleanup kit.
6. The hybridized guide DNA was ligated to the clean, linearized pML104 plasmid:
    a. 1 uL of the hybridized guide DNA + ~100 ng of linearized pML104 plasmid + 2.5 uL of T4 ligase buffer + 1 uL T4 ligase was added to water to total a 25 uL ligation reaction.
7. The ligation reaction was incubated overnight at 16°C.

8. 1-2 uL of the overnight ligated reaction was then transformed into competent cells and plated onto LB + carbenicillin plates.
9. Selected colonies were mini-prepped, and sequence confirmed using a T3 forward primer.

Repair-template construction:

1. Forward and reverse primers with overhangs were designed with the following formula in mind:
    a. For forward primers: 40 nucleotides before start of targeted Sc gene + 20 nucleotides of “replacement” Km gene starting from ATG
    b. For reverse primers: 40 nucleotides of reverse V5-PolyHIS region of the pYES2.1 vector + 20 nucleotides from the end of “replacement” Km gene.
2. pYES2.1 Km SUMO pathway genes plasmids were used as DNA templates for the PCR reaction.
3. Repair templates were PCR amplified using the Q5® High-Fidelity 2x master mix (NEB# M0492S) with the following program:
    a. 98°C for 30s
    b. 98°C for 10s
    c. 50 – 72°C for 30s (depends on primer G-C%; use the NEB T_m Calculator)
    d. 70°C for 30s/kb
    e. Repeat b-d for 35 cycles
    f. Hold at 4°C
4. 0.4 uL of the KLD enzyme mix (NEB# M0554S) was added to 20 uL of the PCR reaction and incubated overnight at room temperature (25°C).

5. The PCR + KLD enzyme mix was cleaned using the IBI Scientific PCR cleanup kit.

Co-transformation:

1. WT Sc (YOKs 1481 or 1482) was grown overnight in YPD media, rotating at 30°C.
2. Overnight grown cells were centrifuged and spent media was removed.
3. Pellet was washed with water 1x.
4. Pellet was resuspended in 1-5 mL of 0.05 M Lithium Acetate/0.5x TE buffer and incubated rotating at 30°C for 3-6 hours. (volume of LiAc/TE mix added depends on size of the pellet).
5. After 3-6 hours, cells were centrifuged and resuspended in residual LiAc/TE supernatant.
6. 10 uL of clean repair template (~1,000 ng) + 1-2 uL of ligated pML104 plasmid (~500 ng) + 2.5 uL of ssDNA were added to 50 uL of competent cells.
7. 400 uL of 40% PEG was added to the competent cells' mix.
8. Cells were heat shocked at 42°C for 30 minutes.
9. After heat shock, 100 uL of cells were plated onto -URA selective media.
10. Plates were incubated at 30°C for 2-3 days.
11. Resulting colonies were either plated onto YPD or 5-FOA media to lose pML104 plasmid.

Replacement confirmation:

1. Genomic DNA of selected co-transformed colonies was extracted.

2. 1 uL of the genomic DNA was used as DNA template for PCR amplification of the targeted region with flanking forward and reverse primers.
3. PCR products were run on agarose gel for:
    a. Confirming DNA presence.
    b. Checking for size shifts if one was expected for a replacement.
4. PCR samples with putative replacements were cleaned using the IBI Scientific PCR cleanup kit and sent for sequencing (usually with the forward flanking primer).

Competition Assay with Pigmented Yeast Strain Protocol:

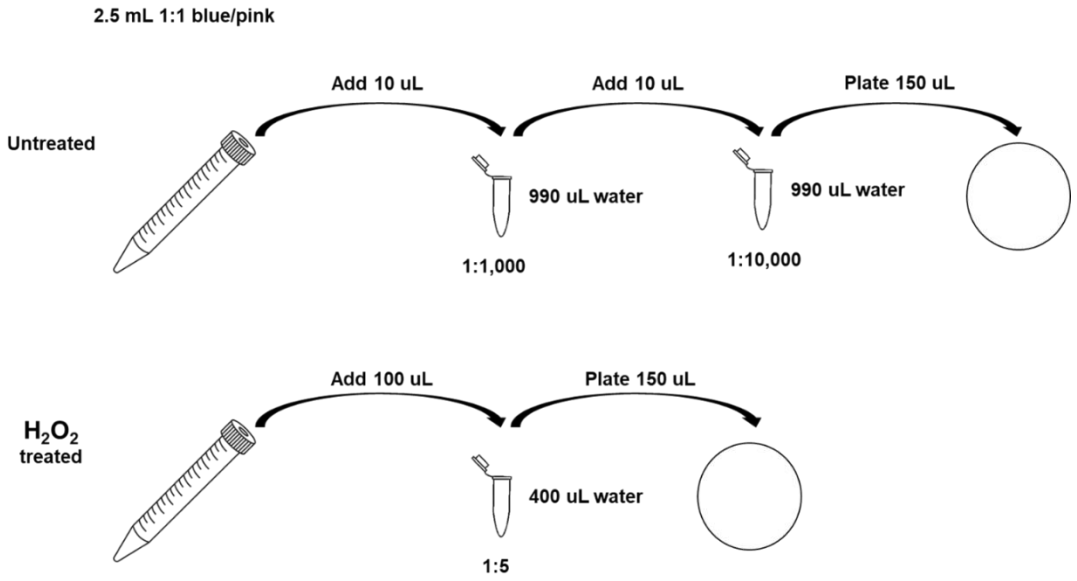
Co-transformation:

1. *ScSMT3* in WT blue Sc (YOK 3734) was replaced with *KmSMT3* following the CRISPR/Cas9 replacement protocol. YOKs 3745 and 3746 were confirmed with *KmSMT3* replacements.

Competition Assay:

1. Single colonies of YOK 3733 (WT pink Sc) and YOK 3745 (Sc blue with *KmSMT3*) were each grown overnight in 5 mL of YPD media plus 250 ug/mL G418, rotating at 30°C for 16-19 hours.
2. 20 uL of each overnight grown culture was taken and mixed in an Eppendorf tube for a total 40 uL 1:1 mix of YOK 3733 and YOK 3746.
3. The 1:1 mix was thoroughly vortexed.

4. 5 uL of the 1:1 mix was then added to 5 mL of fresh YPD media plus 250 ug/mL G418 and grown overnight, rotating at 30°C for 16-18 hours.
5. The overnight grown 1:1 mixed culture was then split into two separate, 15 mL conical tubes (2.5 mL of 1:1 mixed culture in each tube).
6. 25 mM of H_2O_2 was added to one of the conical tubes. No additions were made to the other tube.
    a. For a final concentration of 25 mM of H_2O_2 in 2.5 mL, 62.5 uL of 3% H_2O_2 (~1.0 M) was added to the conical tube.
7. Both tubes (H_2O_2 treated and untreated) were incubated for an additional 30 minutes rotating at 30°C.
8. Immediately after 30 minutes, both tubes were centrifuged at top speed for 2 minutes.
9. Spent media was removed.
10. Tubes were washed 1x with water.
11. Washed pellets from each tube were resuspended in 2.5 mL of water.
12. The untreated culture was diluted 1:10,000.
13. The H_2O_2 treated culture was diluted 1:5.
14. 150 uL of each culture was plated onto YPD + 250 ug/mL G418 plates.
15. Plating was repeated with two more pairs of plates for a total sample number of 3 (n=3).
16. Plates were incubated at 30°C for up to 5 days.



Supplementary Figure 2. Schematic of the competition assay culture dilution steps. As part of the competition assay protocol, 2.5 mL of untreated mix of blue/pink cells were diluted 1:10,000 before plating and 2.5 mL of H_2O_2 treated blue/pink cells were diluted 1:5 before plating. Dilution of untreated cells is much larger to avoid confluency.

Counting colonies:

1. Once colonies appeared on the plates (after 3 days), number of pink versus blue colonies on each of the untreated versus H_2O_2 treated plates were counted.
2. Base growth advantage of WT Sc pink cells was noted based on the higher number of pink colonies growing on untreated plates in comparison to Sc blue cells with KmSmt3.
3. Average percent difference between number of WT Sc pink and Sc blue cells with KmSmt3 on untreated plates was calculated.
    a. Example: If 82 blue colonies and 116 pink colonies were counted on the untreated plate, blue colonies made up 41.4% of the colony population while pink colonies made up 58.6% of the colony population and pink colonies

had a (58.6 – 41.4 = 17.2) 17.2% base growth advantage over blue colonies.

   b. This calculation was made for all three untreated plates and the percent growth advantage was averaged to be 15.63% for pink colonies.

4. To normalize the data for pink colonies base growth advantage, 15.63% was added to number of blue colonies counted on both the untreated and H_2O_2 treated plates. This resulted in a 1:1 ratio of pink versus blue colonies on the untreated plates.

   a. Example: If there were 371 pink colonies and 720 blue colonies on a H_2O_2 treated plate, 170.52 was added to the number of blue colonies to account for the pink colonies 15.63% base growth advantage:

      i. 371 + 720 = 1,091

      ii. 15.63% of 1,091 is 170.52

5. To normalize the data for dilution factor, numbers for blue and pink colonies of untreated plates were multiplied by 10,000 and then divided by 5 (to match the 1:5 dilution made for H_2O_2 treated colonies). This step is only necessary if planning to represent the **number** of colonies on the graph. However, graphing **percentage** number of colonies is recommended because the number of untreated colonies are usually too high to fit on the same graph as H_2O_2 treated colonies.

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