Characterization of a yeast strain that is deficient in protein sumoylation

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science

GRADUATE PROGRAM IN BIOLOGY

YORK UNIVERSITY

TORONTO, ONTARIO

September 2017

ABSTRACT

Sumoylation, the covalent attachment of the SUMO peptide (*s*mall *u*biquitin-related modifier) to cellular proteins, is a post-translational modification affecting many cellular processes, including transcription. Sumoylated proteins are found specifically on the promoters of transcriptionally active genes. Since the role of SUMO on the promoters for constitutive genes remains largely unknown, we applied the "anchor-away" technology to generate a yeast strain in which the SUMO conjugating enzyme, Ubc9, could be depleted from nuclei, allowing us to study the effects on transcription.

The strain had significantly lower SUMO levels upon rapamycin treatment. However, it also had low levels of basal sumoylation, which reduced further with rapamycin. This rapid disappearance of sumoylated proteins shows that sumoylation is very labile, probably because of the SUMO protease Ulp1's activity. The lower sumoylation in the strain was associated with decreased transcription, reduced RNA Pol II recruitment and lower levels of active histone marks for some genes.

ACKNOWLEDGMENTS

Ever since my first meeting with Dr Emanuel Rosonina at the LSB elevator before he introduced me to his lab, I have learnt a tremendous amount thanks to his unparalleled expertise in the field. The knowledge that I obtained from the lab during my time through his mentorship went much further than Biological techniques or theoretical concepts; he has also taught me to look at failed western blots and inconsistent qPCR data differently, and in a much more positive light. His patience, calmness & compassion for his students, and his remarkable efficiency in running the lab (except for the time when he asked us to call him the polysyllabic 'Emanuel' instead of the monosyllabic 'Sir' which we had been using), have certainly been hugely inspiring.

Although this thesis stars sumoylation and yeast, it could not have been completed without a star-studded crew from the Rosonina lab that kept me motivated for the past 3 years. I would like to thank all of the lab members, past and present: Akhi (for answering all the questions that I bombarded her with and for treating me as a younger brother), Veroni (for being a friend who first helped me ease into the lab), Yimo, Marjan, Jessica (for always spreading happiness in the air), Farhaan (for redefining humor every day), Areeb (for many enlightening conversations), Justin, Giovanni, Russell, Arman, Ben, Eva and Tammi. I would also like to thank Sang, Jessica, Myat, Mohammed and Shailee from the 3rd floor for making LSB my second home.

Last, but by no means the least, I would like to thank Dr Peter Cheung, Dr Mark Bayfield & Dr Anthony Scime (for agreeing to be in my committee despite their hectic schedules) and Dr Arthur Hilliker & Dr Pat Lakin-Thomas (for being my first mentors in research).

Table of Contents

List of tables

List of Figures

Chapter 1: Introduction

1.1 Post-translational Modifications:

Post-translational modifications (PTMs) are processing events which can change the properties of proteins by proteolytic cleavage or by the covalent addition of a modifying group to one or more amino acid residues (15). These modifications can be extremely diverse and can determine a protein's activity state, localization, turnover, interactions with other proteins and more (15). Some of the common PTMs that are studied around the world include acetylation, methylation, glycosylation, phosphorylation, ubiquitination and sumoylation. Many PTMs can also work in combination with others as part of extensive 'crosstalk' to enhance the level of complexity in the cell.

1.2 Sumoylation:

Sumoylation is a post-translational modification that affects proteins in a vast range of cellular processes (1-3). It involves the covalent reversible attachment of the SUMO peptide (*s*mall *u*biquitin-related <u>mo</u>difier) to specific lysine residues of cellular proteins. The modified residues usually fall within a consensus motif, ΨKxE (Ψ is a large hydrophobic residue, K the residue modified and x any amino acid) (7) (Fig 1A). Other post-translational modifications, such as phosphorylation, can also stimulate sumoylation (18). Although the small ~12 kDa SUMO peptide shares a similar three-dimensional structure as ubiquitin (Fig 1B), they share less than 20% amino-acid sequence identity and are different in their overall surface-charge distribution. All SUMO proteins have an unstructured stretch of 10-25 amino acids at their N termini which is not seen in any other ubiquitin-related proteins (Fig 1A). The budding yeast *Saccharomyces cerevisiae* expresses a single form of SUMO, encoded by the essential *SMT3* gene, while three active isoforms, SUMO-1, SUMO-2 and SUMO-3, exist in vertebrates. The SUMO-2 and SUMO-3 proteins share ~97% sequence identity and are sometimes collectively termed as SUMO-2/3 (19). SUMO-1 is approximately 48% identical to SUMO-2 and 46% identical to SUMO-3 (20), while also having the highest sequence identity to the yeast Smt3 protein

(19) (Fig 1A). However, it does not form polysumoylated chains whereas the mammalian SUMO-2/3 and the yeast Smt3 do (21).
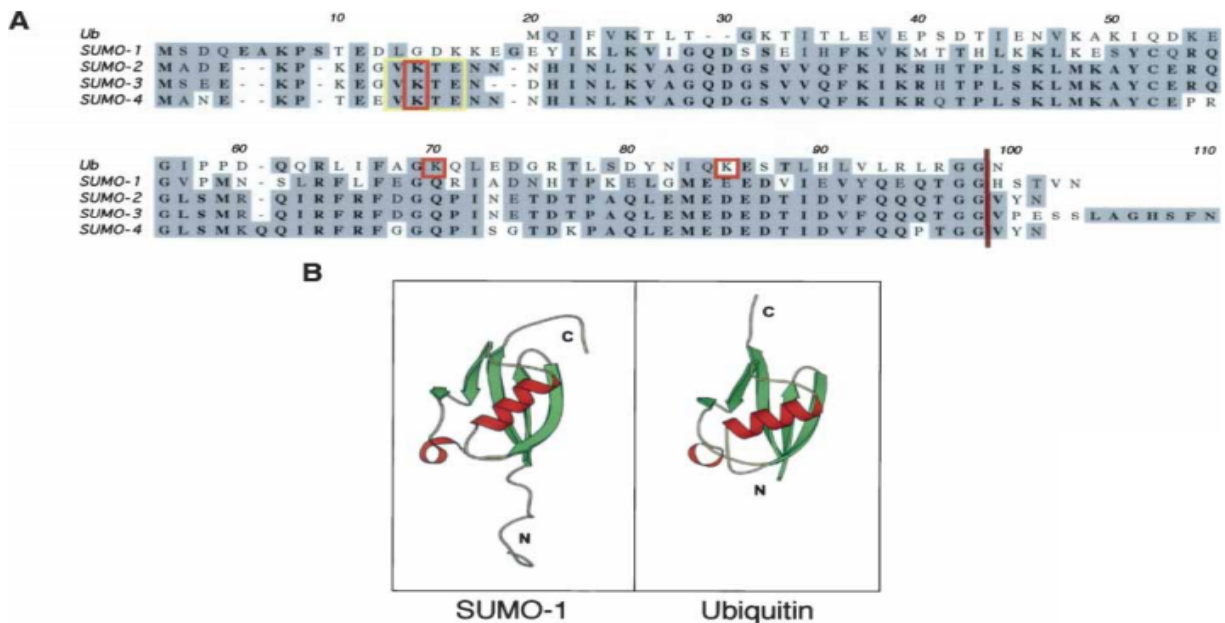
Figure 1: SUMO vs Ubiquitin

A) Amino acid sequence alignments of ubiquitin and the four human SUMO homologs. Identities indicated in bold and similarities are shaded. The SUMO consensus motif for SUMO chain formation on SUMO-2, SUMO-3 and SUMO-4 is boxed in yellow, with the SUMO acceptor lysine (K) in the motif boxed in red. Ubiquitin Ls48 and Lys63, the common sites for ubiquitin polymerization, are boxed in red. The cleavage site, used to produce mature SUMO peptides with C-terminal glycine-glycine residues, is also indicated. B) Ribbon diagrams show the three-dimensional structural similarity between SUMO-1 and ubiquitin. Secondary structures indicated: β sheets are green and α helices are red. Note that SUMO has an N-terminal extension not seen in ubiquitin. Figure adapted from Gill (40).

1.3 The Mechanism of Sumoylation:

SUMO, like other members of the ubiquitin-like protein (UBL) family, covalently attaches to its target proteins in a process that is very similar to ubiquitin-protein conjugation, involving a cascade of enzymatic activities involving E1-activting enzymes, a sole E2-conjugating enzyme, and in some cases, an E3 ligating enzyme (22). SUMO is initially synthesized as an inactive precursor with an extension on the peptide's C-terminus (19). A SUMO protease (Ubiquitin-like specific protease (Ulp1)

in yeast or SENtrin-specific protease (SENP) in humans) then cleaves downstream of a Gly-Gly motif

at the C-terminal proximal end to form mature SUMO ready to conjugate (Fig 2).
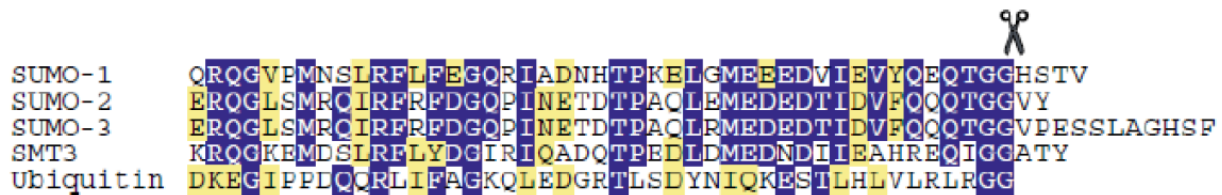
Figure 2: Di-glycine motif of the Ubl protein family

The SUMO peptide is part of the Ubl superfamily of proteins. These proteins require processing to reveal a di-glycine motif. This motif is used during its conjugation to target proteins (41).

The carboxy-terminus of this mature SUMO is activated by the heteromeric SUMO-activating enzyme

E1 (Aos1/Uba2 in yeast and Sae1/Sae2 in mammals) in an ATP-dependent manner, forming a high-

energy thioester bond with the E1. SUMO is then transferred to the active-site cysteine of the E2

SUMO-conjugating enzyme (Ubc9) before being transferred to the lysine side chain on the target

proteins (19). Protein sumoylation can be facilitated by one of a small number of SUMO E3 ligases,

such as Siz1, Siz2, Zip3 and Mms21, that have been discovered in yeast (24-25), usually accentuating

conjugation specificity (23) (Fig 3). Target proteins can be sumoylated on a single lysine

(monosumoylation), on multiple lysines (multisumoylation), or SUMO chains can be formed on one or

more lysine residues (polysumoylation) (19).
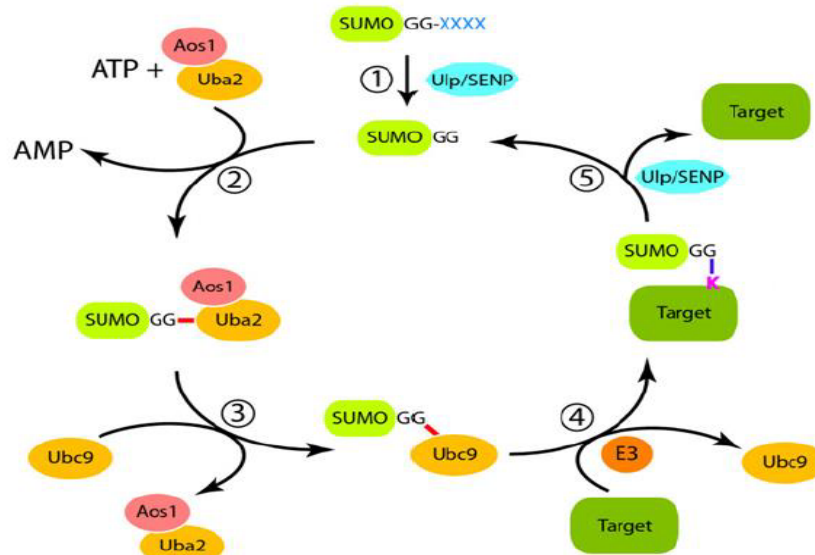
Figure 3: The Sumoylation pathway

Pre-mature SUMO undergoes maturation through SUMO-specific protease cleavage to display its C-terminal Gly-Gly motif (Step 1). This mature SUMO is then activated by the E1 heterodimer enzyme in an ATP-dependent reaction (Step 2). The activated SUMO is then transferred to Ubc9, the sole E2 enzyme (Step 3). E3 ligases can sometimes help Ubc9 transfer the SUMO peptide to the specific lysine acceptor site of the target proteins (Step 4). Sumoylation can be removed from the conjugated species by the action of SUMO proteases (Step 5). Adapted from Dasso (42).

1.4 De-Sumoylation:

Being a reversible PTM, SUMO modifications can be removed from its substrates by SUMO –specific proteases, which have isopeptidase activity. These proteases specifically cleave the bond between the SUMO and its substrate. Three known classes of SUMO proteases, with the SENP family being the largest, have been identified in vertebrates, with seven well characterized members in humans. In yeast, where the SUMO proteases were initially identified, Ulp1 and Ulp2 have been found performing this role (Table 1). With multiple enzymes at play, sumoylation is a highly dynamic process in which the steady-state levels of SUMO are tightly regulated by the complex interplay of the SUMO E1, E2, E3 and various proteolytic enzymes and plays crucial roles in many cellular pathways (26-27).

Table 1: Known enzymes involved in the sumoylation pathway in yeast and mammals

Proteins/Enzymes	Yeast (*S. cerevisiae)*	Mammals (*H. sapiens*)
SUMO	Smt3	SUMO-1, SUMO-2 and SUMO-3
E1 (activating enzyme)	Aos1, Uba2	Sae1 and Sae2
E2 (conjugating enzyme)	Ubc9	Ubc9
E3 (ligating enzyme)	Siz1, Siz2, Mms21 and Zip3	PIAS1, PIAS3, PIASxα, PIASxβ, PIASy, RanBP2, TOPORS, PC2, HDAC4 and Mms21
SUMO proteases	Ulp1 and Ulp2	SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7

Not surprisingly, dysregulation of sumoylation is associated with numerous diseases, including Alzheimer's, Huntington's, Parkinson's and many cancers (28-29). For instance, in metastatic breast cancer cells, sumoylation levels are significantly elevated, while both Ubc9 and SUMO protease expression levels were found to be altered in a large number of tumors (30-32).

1.5 Connection to the Ubiquitin Proteolysis Pathway:

Although sumoylation has not been directly found to signal protein degradation, it can act as a secondary signal for proteolysis (33). Poly-sumoylated proteins can be recognized by the SUMO-interacting motifs (SIMs) of a class of E3 ubiquitin ligating enzymes, also known as SUMO-targeted ubiquitin E3 ligases (STUbLs). A RING finger domain of the STUbL, along with an E2 ubiquitin conjugating enzyme, then results in the ubiquitination of the target protein (Fig 4). The 26S proteasome can then be recruited eventually degrading the protein (34). The sumoylation of acute promyelocytic

leukemia (PML) protein is recognized by the RNF4 STUbL in this fashion, facilitating its

polyubiquitination and proteasome-dependent degradation (35) (Fig 4). However, in some cases,

sumoylation can also compete with ubiquitin for specific Lys acceptor sites, thus preventing its

ubiquitination and degradation by the proteasome (36).
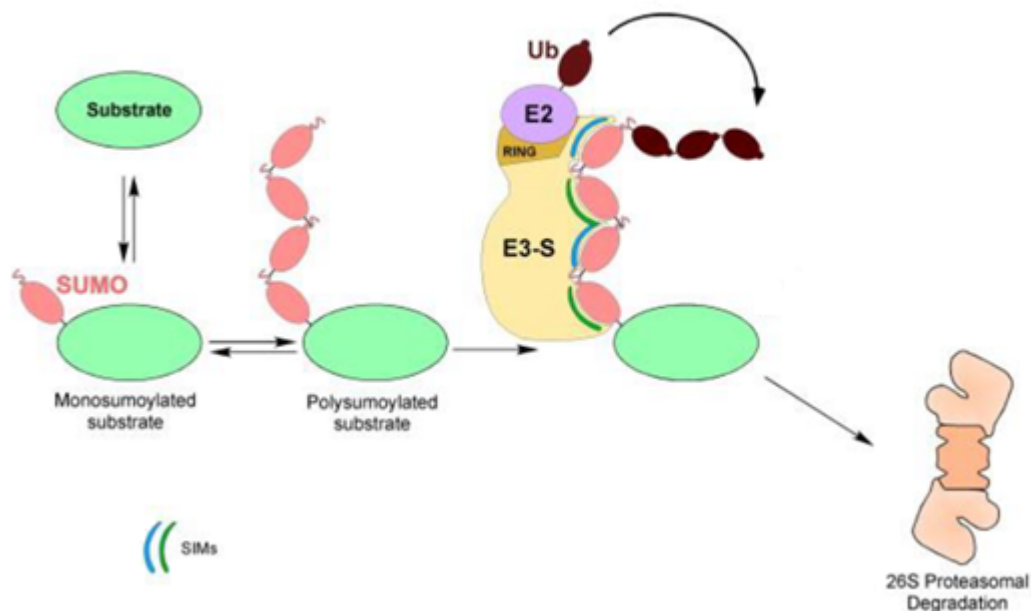
Figure 4: Schematic Diagram of the connection between sumoylation and ubiquitination

In some cases, poly-sumoylation can lead to interaction with the SIMs on the E3 ubiquitin ligases for sumoylated proteins
(E3-S). These enzymes bind to the E2 ubiquitin conjugating enzyme through a RING domain conjugated to it, beginning
poly-ubiquitination. This protein can then be degraded by the 26S proteasome (34).

1.6 Sumoylation of Transcription Factors:

More than 6000 proteins have been recognized as potential targets for sumoylation (4)

with the process being implicated in controlling many facets of cell physiology, including cell-

cycle regulation, transport, DNA replication and repair, and transcription (5-8). Although

sumoylated proteins are found throughout the cell, those involved in transcription are one of the

largest groups of sumoylation targets (2, 3). This included many transcriptional activators, repressors, co-regulators and general transcription factors in both yeast and mammalian cells. Sumoylation's effect on different transcription factors is diverse. However, in a vast majority of cases, this modification is associated with transcriptional repression, accomplished by different SUMO-mediated mechanisms. For instance, the sumoylation of transcription factor Elk1 stimulates the recruitment of the histone deacetylase HDAC-2, reducing the histone acetylation levels and thereby inhibiting transcription (37). However, when this pathway is activated by ERK, Elk-1 is phosphorylated together with a loss of SUMO and HDAC leading to a full activation of target genes. Several studies have also found evidence of sumoylation being involved in activating transcription. For example, the SUMO modification of p53 enhances its ability to activate reporter genes possibly by competing against Mdm2-mediated ubiquitination, which would otherwise target it for degradation (38-39).

Chromatin immunoprecipitation (ChIP) experiments in budding yeast showed that Ubc9 is recruited to promoters of inducible genes when they are activated, which coincides with the striking appearance of sumoylated proteins in this region (9). Further, impairing sumoylation (with a strain expressing partially defective Ubc9) does not affect induction of these genes, but rather prolongs their expression, suggesting that sumoylation of proteins at promoters of activated genes is important for shutting genes off when appropriate. Analysis of the inducible *ARG1* gene showed that sumoylation promotes the clearance of its activator, Gcn4, from its promoter, which can explain how sumoylation facilitates shutting genes off (9)

ChIP analyses, including genome-wide studies, showed that sumoylated proteins are also found at promoters of constitutively active genes, but not repressed or silent genes, in both yeast and human cells, pointing to a correlation between sumoylation and active transcription (9-10).

However, Ubc9 was not detected at promoters of constitutive yeast genes suggesting that promoter-associated proteins are sumoylated before they associate with these genes. This also suggests that sumoylation plays a different role at constitutive gene promoters than at inducible promoters. One of the main goals of this study was to determine how constitutive expression is affected by sumoylation.

Most literature on the function of sumoylation on gene promoters in the context of transcription is focused on transcription factors that are gene specific. These experiments resort to mutating sumoylation sites by mutation of the target lysine residue(s) to non-sumoylatable arginine(s), thereby blocking sumoylation of the specific protein. Since both the *SMT3* and *UBC9* are essential genes, examining the effects of sumoylation using *SMT3* and/or *UBC9* null strains is not possible. As such, the function of global sumoylation on constitutive gene expression remains largely unknown.

1.7 The Anchor Away Technique:

The anchor away (AA) technique was developed by Haruki et el. (11) as a method of conditionally depleting nuclei of specific proteins in living yeast cells to study the consequences of their absence in nuclear processes. By this technique, a nuclear protein of interest (the target) is relocalized to the cytoplasm by conditionally tethering it to an abundant cytoplasmic protein (the anchor) by rapamycin-induced heterodimerization. Specifically, the system takes advantage of the massive flow of ribosomal proteins through the nucleus during maturation prior to their cytoplasmic localization as a method of anchoring the nuclear protein of interest. In the anchor away strain, the ribosomal protein L13a (RPL13A) is fused to the human 12 kDa FK506 binding protein 12 (FKBP12) at its C-terminus. In the same yeast strain, the target nuclear protein of interest is expressed as a fusion to the 11 kDa FRB domain (FKBP12-rapamycin-binding

domain) of human mTOR (mammalian target of rapamycin), also at the C-terminus. When rapamycin is added to the cells, it binds to the FKBP12 domain, establishing an interaction surface for the FRB domain to form a tight ternary complex of nanomolar dissociation constant (11) (Fig 5). Although rapamycin is toxic to wild-type yeast, the strains used for this technique are derived from a rapamycin-resistant strain containing a mutated *TOR1*. They are also deleted for the *FPR1* gene, the yeast homolog of human FKBP12, which is the most abundant FK506 and rapamycin binding protein in *S. cerevisiae*. Deletion of *FPR1* confers rapamycin resistance, while also reducing competition between FPR1 and the anchor-FKBP12 construct for binding to the FRB domain (11).

The Anchor Away technique has been used a number of times to test the effects of depleting selected proteins from the nucleus. For instance, the technique was used to conditionally deplete Kin28, the TFIIH subunit that phosphorylates the CTD of RNA polymerase II, from the nucleus. It was shown that this depletion resulted in a dramatic increase in Mediator occupancy at the core promoter, which suggested that Kin28 was stimulating rapid promoter escape through dissociation of the Mediator from the preinitiation complex (PIC) (45). Similarly, Subramanian et al. (46) used the anchor away system to test whether the synaptonemal complex (SC) protein Zip1 was responsible for the loss of meiotic kinase, Mek1during meiosis. The conditional nature of the system offered an advantage over using Zip1 null strains as it helped circumvent potential pleiotropic effects of earlier roles of Zip1 in centromere pairing and double stranded break (DSB) repairs (46).

1.8 Overall Goal and Hypothesis of the Study:

This study was aimed at examining the role of nuclear sumoylation, particularly with respect to gene expression and constitutive transcription. To answer this question, the anchor

away system described above was used to conditionally deplete Ubc9 from yeast nuclei. The engineered anchor away strains in the experiments were derived from a rapamycin-resistant W303a strain of yeast, and express Ubc9-FRB fusion protein. The addition of rapamycin stimulates the dimerization of Ubc9-FRB and RPL13A-FKBP12, depleting the Ubc9 from the nucleus as the ternary complex is migrated to the cytoplasm (Fig 5). This is tantamount to preventing *de novo* sumoylation as Ubc9 is the sole conjugating E2 enzyme found in yeast cells.

Considering that sumoylated proteins are detected on active gene promoters (9), it was hypothesized that SUMO plays a critical role in this process, potentially impairing the transcription of many constitutive genes.

Once the specialized anchor away strains were engineered, the effects of Ubc9 nuclear depletion on cell viability, growth rates, constitutive gene expression and the efficiency of RNA Pol II recruitment were examined. Qualitative assays were also used to elucidate the localization of sumoylated proteins and to examine the levels of different histone marks (H3K4me3 and H3K9ac) as a function of reduced sumoylation. Our results showed that the anchor away strain had significantly lower SUMO levels upon rapamycin treatment, which also corresponded with lower growth rates (short-term) and cell viability (long-term).The strain, however, had lower basal sumoylation compared to the parental strain (i.e. even in the absence of rapamycin), and some constitutive gene promoters that started off with lower SUMO levels, did not show a further drop with rapamycin treatment. Sumoylation, however, was mostly associated with increased transcription and RNA Pol II recruitment.
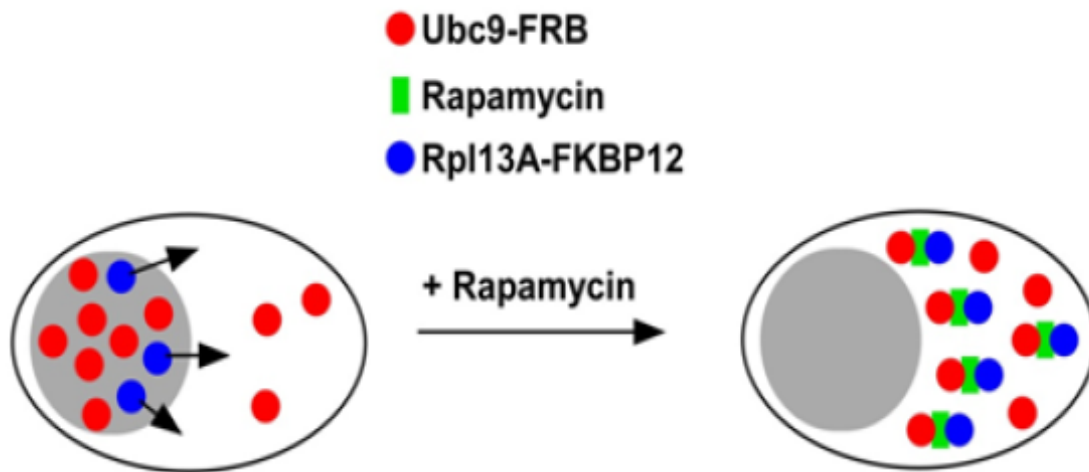
Figure 5: The Anchor Away System Schematic

In the specially engineered anchor away strain, the target protein (Ubc9) is fused to the FRB domain of mTOR, while the anchor protein RPL13A is fused to FKBP12. Upon adding rapamycin, FKBP12 and FRB dimerize. Since RPL13A leaves the nucleus upon maturation, the ternary complex (consisting of the target, anchor and rapamycin) is moved to the cytoplasm (11). This results in Ubc9 being sequestered from the nucleus to the cytoplasm.

Chapter 2: Materials and Methods

2.1 Yeast Strains and Plasmids:

Several yeast (*Saccharomyces cerevisiae*) strains were used in this study. Strains were derived from the W303 background strain. Strains expressing fusion proteins were marked on the genome with the KanMX marker gene cassette, and were generated by homologous recombination as previously outlined in literature (9). The presence of the fusion tag sequence in the strains was then confirmed by PCR and growth in the appropriate media.

2.2 Yeast Media and Growth Conditions:

Yeast cultures (10 – 50 mL) were grown at 30°C in Synthetic Complete (SC; 0.17% YNB, 0.5% ammonium sulfate, and 2% glucose) media to an optical density A_{595} (O.D.) of 0.5-0.7, before treating them with rapamycin (stored in dimethyl sulfoxide, DMSO) at a final concentration of 1 μg/mL. Some cultures were treated solely with DMSO as a control.

For the RNA extraction and ChIP assays, the cultures were subjected to an additional treatment with the Alpha-factor mating pheromone (Zymo Research, 0.42 mg in 50 ml cultures) for 3 hours before the rapamycin treatment. This pheromone was used to arrest and synchronize yeast cells at the G1 phase before blocking d*e novo* sumoylation by rapamycin treatment. The synchronization resulted in a homogenous cell culture which was important to isolate the effects of the experiment from the effects of normal cell-cycle progression (43).

2.3 Transformation:

The appropriate strain was inoculated overnight and grown to saturation. It was then diluted and incubated in 10 mL of SC-media for 3 hours at 30° C the next day. The cells were then harvested by centrifugation at 3000 *g* for 5 min., and subsequently washed with 10 mL of sterile water. The pellet

was resuspended in 0.1 M LiAc, transferred to a new microfuge tube and centrifuged twice at top speed for 15 seconds. The cells were then resuspended in Transformation Mix (240 µL of PEG 3500 50% w/v, 36 µL of 1.0 M LiAc, 25 µL of boiled SS- carrier DNA, 40 µL of water & 10 µL of a cleaned PCR product with the required DNA), vortexed for 1 minute and then incubated in a 42° C water bath for 40 min. Following this incubation, the samples were centrifuged at top speed for 30 seconds and the transformation mix was removed. The yeast cells were then plated on to the appropriate plate at 30° C for 3 days.

2.4 Spot Assay:

Cells were grown in appropriate liquid growth medium overnight, and their optical densities determined the following morning. Approximately 10,000 cells of each strain were spotted next to each other in the first position, and serial five-fold dilutions were spotted in adjacent positions, on appropriate solid medium plates. These plates were then incubated at 30˚C and were imaged for the growth of yeast. Images were taken daily for up to three days.

2.5 Liquid Growth Assay:

The anchor away strain and the control parental strain were inoculated overnight in SC media and grown to saturation. Each strain was then diluted to an OD of 0.05 in 100 mL of SC media. Each of the culture was then split into two treatment groups (50 mL each) with half of them receiving 1:1000 addition of dimethyl sulfoxide (DMSO) or 1 mg/mL rapamycin dissolved in DMSO. Over a 34 hour period, 12 OD readings were taken and recorded. The time points included were 0, 2, 4, 10, 12, 16, 24, 26, 28, 30, 32 and 34 hours after the initial treatment. The experiment was repeated three times in total and the results were averaged and graphed using Microsoft Excel with the standard deviation shown as error bars on the graph.

2.6 Chromatin Fractionation:

The strains were incubated and grown to saturation overnight. The next day, the cultures were diluted to 25 mL at an O. D of 0.2-0.25. They were then grown to an O. D of 0.5-0.6 before being treated with rapamycin (or control DMSO) for 30 min. The cells were pelleted at 2000 g or a minute at room temperature. They were then resuspended in 6.25 mL of a buffer consisting of 100 mM PIPES/KOH pH 9.4 &10µMDTT with rapamycin (or control DMSO), followed by incubation for 10 min. at 30° C with agitation. The cells were spun down (2000 g at room temperature for 3 min.) and resuspended again in 2.5 mL of a buffer consisting of 0.6 M sorbitol & 25 mMTris-HCl pH 7.5in YPD medium with rapamycin (or control DMSO). 10-15 mg/mL of the lysing enzyme zymolyase was also added to each sample at this point, followed by incubation for 20 min. at 30° C with agitation. The cells were again spun down (2000 g at room temperature for 3 min.) and resuspended in 2.5 mL of a buffer containing 0.7 M sorbitol, 25 mM Tris-HCl pH 7.5 in YPD medium with rapamycin (or control DMSO), followed once again by incubation for 20 min. at 30° C with agitation. The cells were pelleted by spinning them down at 2000 g in room temperature for 3 min. and then washed 3 times, each with 1 mL of lysis buffer (0.4 M sorbitol, 150 mM potassium acetate, 2 mM magnesium acetate, 20 mM PIPES/KOH pH 6.8, 0.1% NP40, 1 mM PMSF, yeast protease inhibitor cocktail & 0.25 g NEM/100 mL buffer). The cells were then resuspended in 400 µL lysis buffer, followed by the addition of Triton-X 100 to a final concentration of 1%, mixing gently. Some of this lysed sample was removed and an equal volume of 2X SDS-PAGE sample buffer (4% SDS, 20% glycerol, bromophenol blue, 10% beta-mercaptoethanol and 140 mMTris-HCl pH 8) was added to it. This served as the 'whole cell extract' control. The remaining lysed material was transferred to a microfuge tube and spun down at 20,817 g for 15 min. at 4° C. The supernatant obtained from this spin was labeled as the 'soluble' fraction and was mixed with an equal volume of 2X SDS-PAGE sample buffer (4% SDS, 20% glycerol,

bromophenol blue, 10% beta-mercaptoethanol and 140 mM Tris-HCl pH 8). The pellet obtained from the spin was washed once with 500μL lysis buffer and resuspended in 100μL of 2X SDS-PAGE sample buffer (4% SDS, 20% glycerol, bromophenol blue, 10% beta-mercaptoethanol and 140 mM Tris-HCl pH 8). This was labeled as the 'chromatin' fraction. All the three fractions (whole cell extract, chromatin and soluble) were then boiled for 5 min., cooled and finally frozen at -20º C or loaded directly to the SDS gels.

2.7 Non-Denatured Yeast Extraction

The strains were incubated, grown to saturation overnight and diluted to an O. D of 0.2-0.25 the next day. After reaching an O.D between 0.5 – 1.0, the cells were appropriately treated with rapamycin (or DMSO as a control) for 30 min., and then washed in IP buffer (50 mM Tris-HCl, pH 8 & 150 mM NaCl, 0.1% NP40, 1 mM PMSF, yeast protease inhibitor cocktail & 0.25 g NEM/100 mL buffer) and subsequently resuspended in 0.5 mL of IP buffer with 0.1 mM DTT and transferred to new microfuge tubes. 0.25 grams of acid washed glass beads were added to the tube and the cells were vortexed at 4º C for 30 min. (vortexed for 15 min., 5 min. on ice followed by vortexing again for 15 min.). The lysate/supermatant was transferred to a new microfuge tube and was centrifuged at 4º C at top speed for 5 min. The supernatant obtained from this spin was transferred to a new tube and this step was repeated. An equal volume of 2X SDS-PAGE sample buffer (4% SDS, 20% glycerol, bromophenol blue, 10% beta-mercaptoethanol and 140 mM Tris-HCl pH 8) was added to the lysate and was boiled for 3 min.

2.8 Western Blot & Antibodies:

Equal amounts of protein samples were resolved on an appropriate-percentage SDS-PAGE gel (7.5%, 10% or 12.5%) and transferred to nitrocellulose membranes (Biorad) followed by incubation with

blocking buffer [5% milk in 1X PBST {0.05% Tween-20 in 1% PBS (Fischer Scientific)}] for 30 min. at room temperature. The membranes were then probed with appropriate antibodies at the appropriate concentrations [GAPDH (1:5000), H3 (1:1000), H3K4me3 (1:1000), H3K9ac (1:1000), Smt3 (1:3000; for detection of sumoylation) & Ubc9-CTD (1:1000; for detecting the C terminal domain of the conjugation enzyme Ubc9)], overnight at 4˚C. Following incubation with the primary antibodies, these membranes were washed 3 times in 1X PBST for 5 min. each at room temperature. The blots were then incubated with the appropriate HRP conjugated secondary antibody (1:2000 for anti-goat IgG & 1:5000 for anti-rabbit IgG; Thermo Fisher Scientific) for 30 min. at room temperature. The blots were then washed 3 times again and were later detected by chemiluminescence (ECL, Bio-Rad) using autoradiography or a MicroChemi chemiluminescence imager (DNR).

2.9 RNA Extraction and Reverse Transcription-PCR (RT-PCR):

To extract RNA, 10 mL yeast cultures were grown under appropriate conditions with the necessary treatments as described above. Samples were pelleted by centrifugation at 3000 g for 3 min. at 4°C and washed twice with ice-cold AE buffer (50 mM sodium acetate, pH 5.2 and 10 mM EDTA, pH 8.0). The pellet was then re-suspended in 400 µL ice-cold AE buffer and 40 µL of 10 % SDS was added, followed by the addition of 440 µL of phenol (pH 5.2). Samples were then chilled in a dry ice/ethanol bath (made by crushing dry ice to powder and then mixing it with 95% ethanol to form a thick slurry mixture) for 5 min. and then transferred to a 65°C water bath for 5 min. followed by vigorous vortex-mixing for 30 s. After repeating this freeze/thaw/vortex cycle, samples were centrifuged at 16,000 g for 7 min. and the aqueous layers were transferred to micro-centrifuge tubes for standard phenol-chloroform extraction and ethanol precipitation of RNA.

For reverse-transcription, 12µg RNA samples were treated with DNAse I (New England Biolabs) and approximately 1µg of this DNA-free-RNA was used for cDNA synthesis using the Super Script II

reverse transcriptase reaction (Biorad) according to the manufacturer's instructions. For quantification,

qPCRs were performed using SYBR green mix (Froggabio) according to the manufacturer's

instructions. Transcripts were normalized to 25S rRNA and the levels were calculated by the $2^{-\Delta\Delta CT}$

method (44). All experiments were performed at least three times and the average values are shown

with standard deviations shown as error bars. Primer sequences are listed in Appendix G.

2.10 Chromatin Immunoprecipitation (ChIP):

Yeast strains were inoculated in 50 mL of SC medium at 30°C and grown to an optical density A_{595}

(O.D.) of 0.5-0.6.The cultures were then treated with rapamycin (or DMO as a control) as mentioned

above for 20 min., followed by cross-linking with 1.1% formaldehyde for 20 min., before quenching

with 450 mM of glycine for 5 min. The samples were then pelleted by centrifugation and washed with

ice-cold TBS (20mM Tris-HCl, pH 7.5 and 150mM NaCl), followed by washing with ChIP buffer

(50mM HEPES-KOH, pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium

deoxycholate and 0.1% SDS). The samples were then resuspended once again in ChIP buffer and then

subjected to beating with glass beads followed by sonication to shear chromatin to fragments of ~500

bp length. Followed by centrifuging the samples for 5 min., NaCl was added to the isolated

supernatants to a final concentration of 425 mM. A small amount of the supernatant (40μL) was stored

at -20°C as input, and the rest of the salt-adjusted supernatant was incubated overnight at 4°C with

washed Protein G agarose beads in addition to 1 μg of the appropriate antibody for IP. The following

day, beads were washed first in ChIP buffer with 275mM NaCl, followed by the ChIP buffer with

500mM NaCl and then with an additional washing buffer (10mM Tris-HCl, pH 8, 0.25M LiCl, 1mM

EDTA, 0.5% NP-40 and 0.5% sodium deoxycholate) and finally with Tris-EDTA buffer (10mM Tris-

HCl, pH 8 and 1mM EDTA). Between each buffer wash, the samples were incubated at room

temperature for 4 min. The beads were then incubated with ChIP elution buffer (50mM Tris-HCl, pH

7.5, 10mM EDTA and 1% SDS) for 10 min. at 65°C. The samples were then centrifuged at 8000 g and the supernatants were treated with proteinase K (BioShop) at 42°C for 1 hour, and then transferred to 65°C for 4 hours to overnight to reverse cross-links. Each ChIP experiment was performed at least three times and the average of quantitative PCR (qPCR) analyses are presented relative to levels at an untranscribed region of chromosome V (Chr V), with standard deviations shown as error bars. The value for each sample is calculated as follows:

Percent Chr V levels = 100*2^(-ΔCt[normalized ChIP to Chr V levels]). Primer sequences used for qPCR are listed in Appendix E.

2.11 Genomic DNA Extraction:

10 mL of yeast culture was incubated in the appropriate media overnight to saturation. The cells were centrifuged at 3000 g for 5 min. the next day, followed by the pellet being resuspended in 0.5 mL of sterile water and washed. 0.2 mL of extraction buffer (40 μL PEG 3500 50% w/v, 36 μL LiAc 1.0 M, 25 μL boiled SS-carrier DNA, 10 μL cleaned PCR product & 40 μL ddH2O), 0.3 g of acid-washed baked glass beads and 0.2 mL of phenol-chloroform (1:1) were then added to the pellet, followed by vortexing it for 3 min. After adding 0.2 mL of TE, pH 7.4 to the samples, the cells were then centrifuged at top speed for 5 min., followed by the aqueous layer being transferred to a new microfuge tube. 1 mL of absolute ethanol was then added, and mixed by inversion followed by centrifugation at top speed for 1 min. The supernatant was subsequently removed by decanting, and the pellet resuspended in 0.4 mL of 1X TE (10XTE buffer has 440 mL ddH2O, 50 mL 1 M Tris-HCl pH 7.5 and 10 mL 500 mM EDTA pH 8.0). 3 μL of 10 mg/mL RNAse A was added to the cells and incubated at 37°C for 5 min. 10 μL of 4 M ammonium acetate and 1 mL of absolute ethanol were added to re-precipitate the DNA. Samples were then inverted and centrifuged at top speed for 2 min. Finally, the supernatant was removed and the pellet was resuspended in 50 μL of TE.

2.12 Microscopy:

The strains (parental and Ubc9-FRB) were incubated at 30° C in SC media overnight, and the cultures were treated with 1 mg/mL rapamycin for 0, 15 or 30 minutes. A drop of the strain(s) was placed on a glass slide and observed under a light microscope at 100 X magnification. Images were recorded using the microscopy software Motic Images 2.0.

Chapter 3: Results

3.1 Preparing the Anchor away strains

To examine the effects of the absence of nuclear sumoylation on cell functions, particularly transcription, we applied the anchor away system to the sole conjugating enzyme, Ubc9. A homologous recombination-based transformation procedure was used to generate the anchor-away strain which expresses Ubc9 fused to the FRB domain of mTOR.

The pFA6a-FRB-KanMX plasmid (which had an FRB domain fused to the KanMX marker), used as a PCR template to generate a cassette consisting of the FRB domain fused to the KanMX marker, was flanked by regions of homology with sequences surrounding the stop codon of *UBC9*. After confirming the size of the expected product using gel electrophoresis (Fig 6A), a phenol chloroform extraction was done on the PCR products to clean it from other impurities. This was then transformed into a strain where the human FKBP12 protein had been fused to the yeast ribosomal protein RPL13A. The homologous recombination-based transformation resulted in the generation of the anchor-away strain which expressed Ubc9 fused to the FRB domain of mTOR. The parental strain also contains a mutated *TOR1* making it resistant to rapamycin. The transformation was done on a YPD plate with the antibiotic G418 (0.3mM) in it in order to select for the colonies which had incorporated the KanMX marker in them, providing resistance against the antibiotic. Successful transformants were selected and were further grown in YPD media with G418 in order to fully confirm whether the strains had the KanMX insert successfully present in them. Genomic DNA from the strains that grew successfully was extracted, their concentrations measured and tested by PCR for their presence of the inserted fusion protein (Fig 6, B & C). Successful transformants were then confirmed by sequencing and Ubc9-FRB fusion strain 1 was selected to proceed to further experiments. A control western blot assay was also performed on the Ubc9-FRB fusion strain lysates to confirm the presence of the FRB domain (Fig 6 D).
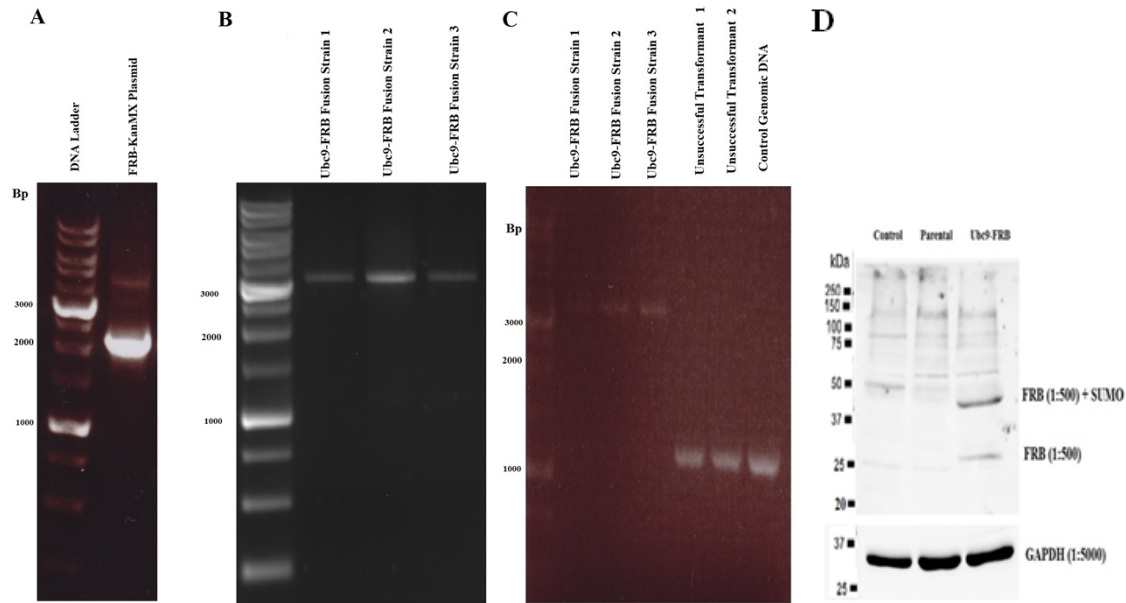
Figure 6: Preparing the Ubc9-FRB Anchor Away Strain

A: The pFA6a-FRB-KanMX plasmid (which had an FRB domain fused to the KanMX marker) was amplified by PCR using primers to tag the forward and reverse base-pairs of Ubc9. The size of the expected product (~ 2000 bps) was confirmed by gel electrophoresis. After cleaning the PCR products with a phenol chloroform extraction, this was later transformed into a strain where the human FKBP12 protein was fused to the yeast ribosomal protein RPL13A. The homologous recombination-based transformation resulted in the generation of the anchor-away strain which expressed Ubc9 fused to the FRB domain of mTOR. **B**: Genomic DNA from the strains that grew successfully was extracted and tested by PCR for their presence of the inserted fusion protein. A & D primers for Ubc9 were used to amplify the region of interest. Bands around ~3000 bps indicated that the Ubc9 gene locus, 1118 bps long, was successful in incorporating the FRB-KanMX plasmid, ~ 2000 bps long. **C**: Further PCR was performed using an unrelated genomic DNA without the fusion segment as a control. The results confirmed that the Ubc9-FRB fusion DNA (~3000 bps) was longer than the control DNA (1118bps) by roughly the same size as the plasmid it incorporated (~ 2000 bps). **D**: A control western blot assay performed on the Ubc9-FRB fusion strain lysate probing for FRB revealed its presence in the strain, while being absent in control strains as expected.

3.2 Testing the Ubc9 anchor away strain

To confirm that the Ubc9 anchor away fusion strain ("Ubc9-FRB") was indeed deficient in nuclear sumoylation when exposed to rapamycin, cell lysate from the fusion strain (as well as from the parental

strain) was extracted after treating the cells with rapamycin for various times, and analyzed by western blot with a SUMO antibody. As expected, while the parental strain was unaffected by rapamycin treatment, the Ubc9-FRBstrain showed a marked depletion of sumoylated proteins, from as quickly as five min. after the treatment (Fig 7). This quick activation of the anchor away system was expected, considering that rapidity was one of the advantages of using this system (11). Levels of the constitutive GAPDH protein, used as a control in the western blot, remained fairly consistent, even with the depletion of sumoylated proteins in this blot (Fig 7). The dramatic disappearance of sumoylation from the lysate in the rapamycin-treated Ubc9-FRB strain suggests that most sumoylated proteins are nuclear.

This assay also showed that the system was successful in conditionally depleting sumoylation with rapamycin treatment.

To determine the localization of sumoylated proteins in the cell, a chromatin fractionation was then performed, followed by a SUMO western blot. The results show that the great majority of sumoylated proteins are associated with chromatin (Fig 8). Upon rapamycin treatment, these levels, once again, showed a marked depletion indicating that the anchor away system is effective in blocking nuclear sumoylation events (Fig 8).The association of most SUMO targets with chromatin indicates that SUMO likely participates in many aspects of chromatin biology. This result was also consistent with other studies which detected a high level of sumoylation associated with promoters of active genes. There is a conspicuous band seen on the SUMO blot in this assay, weighing around 42 kDa (Fig 8). We hypothesize that this band corresponds to the Ubc9-FRB fusion protein itself (~ 30 kDa) conjugated to SUMO (~ 12 kDa). If so, the presence of this band solely in the chromatin fraction, even with rapamycin treatment, would indicate that the enzyme was present in the nucleus, contradictory to the theory behind the anchor-away technique. Nonetheless, the conditional depletion of sumoylated proteins with the treatment suggests that the enzyme activity was impaired simply by association with

rapamycin. It was also notable that this band is more intense when treated with rapamycin, implying that this treatment resulted in Ubc9 being unable to conjugate SUMO to its target proteins and essentially remaining stuck with the peptide (Fig 8).
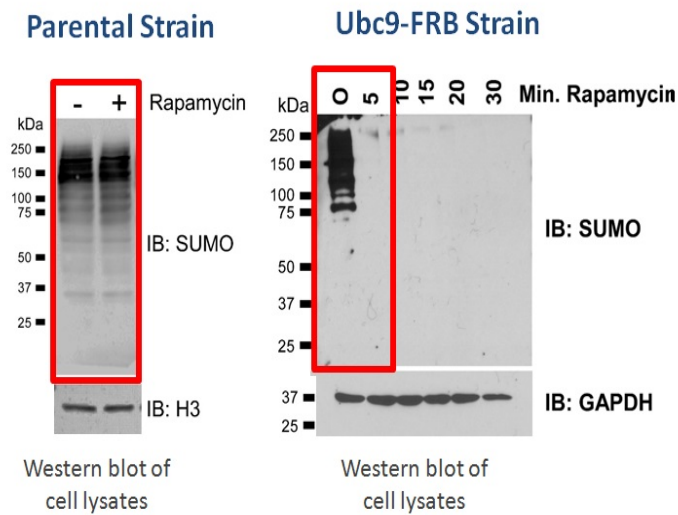
Figure 7: Depletion of sumoylated proteins is rapid upon rapamycin addition

Cell lysates were extracted from the parental and the Ubc9-FRB fusion strains and a western blot assay was performed using a SUMO antibody. While the parental strain was unaffected with rapamycin treatment, the Ubc9-FRB fusion strain showed a rapid decrease in sumoylated proteins in as little as 5 min. indicating that the anchor away system blocked *de novo* sumoylation rapidly. GAPDH is shown as a control.
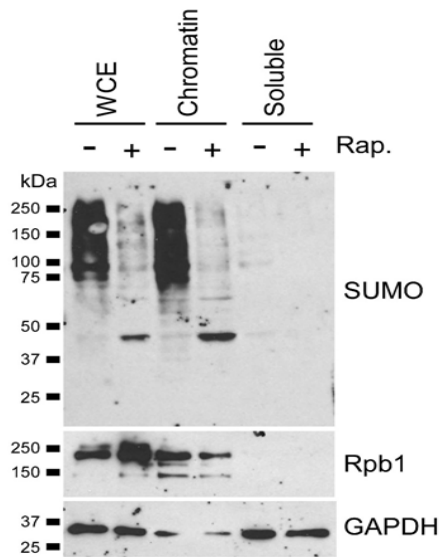
Figure 8: Most sumoylated proteins are present on the chromatin

Chromatin fractionation followed by a western blot analysis of the Ubc9-FRB fusion strain revealed that most sumoylated proteins are found associated with chromatin. There is a significant decrease in chromatin-associated sumoylation upon treatment with rapamycin (30 min.) indicating that the anchor away system is effective. Rpb1 (largest subunit of RNA Polymerase II) and GAPDH are shown as controls for the chromatin and soluble fractions, respectively.

In order to confirm the transit (or lack of transit) of the Ubc9 enzyme from the nucleus to the cytoplasm upon rapamycin treatment, a chromatin fractionation was once again performed followed by a western blot assay probing for Ubc9. The Ubc9 band (~ 30 kDa) did not disappear from the chromatin fraction with rapamycin treatment, as would be expected if Ubc9-FRB was re-localized to the cytoplasm (9A). There were two additional sets of bands seen in the Ubc9 blot (around 37 kDa and 42 kDa). The 42 kDa band is most likely the Ubc9-FRB fusion protein conjugated to the SUMO peptide (Fig 9A). This hypothesis is strengthened as these bands are also seen on the corresponding SUMO blot. The increased intensity of the 42 kDa band in the chromatin fraction, as seen in Fig 8, is seen here as well with rapamycin treatment, showing that Ubc9 was unable to conjugate SUMO to its target proteins, thus leaving the enzyme associated with the peptide (Fig 9B). However, rapamycin treatment did deplete sumoylated proteins as expected in the strain, strengthening our hypothesis that

binding of rapamycin to Ubc9-FRB itself impairs its SUMO conjugation activity, but does not cause the evacuation of nuclear Ubc9 to the cytoplasm. Regardless of the mechanism, the Ubc9-FRB strain that we generated is effective in conditionally and rapidly blocking sumoylation, and further analysis was carried out with this strain (Fig 9B).
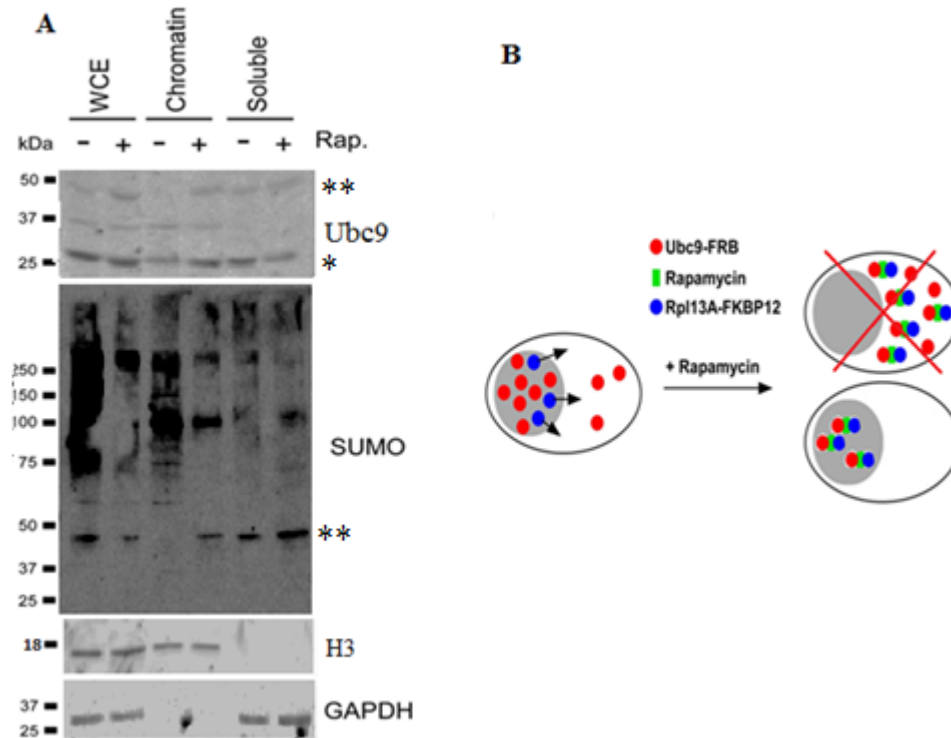
Figure 9: Ubc9 may not be leaving the nucleus upon rapamycin treatment in the Ubc9-FRB strain

A: Chromatin fractionation followed by a western blot analysis of the Ubc9-FRB fusion strain revealed that the conjugating enzyme Ubc9 does not dissociate from chromatin upon rapamycin treatment. * indicates the Ubc9 band (~ 30 kDa) while the ** in the Ubc9 and SUMO blots indicate the Ubc9 fused with SUMO (~ 42 kDa). The extra bands seen ~ 37 kDa are most likely non specific bands. B: Although the evacuation of the Ubc9 enzyme is theoretically required for the anchor-away system to work, the depletion of sumoylated proteins upon rapamycin treatment indicates that the system is effective regardless of the enzyme's presence in the nucleus. As indicated in figure 7, most sumoylated proteins are found associated with the chromatin fraction. H3 and GAPDH are shown as controls for the chromatin and soluble fractions, respectively.

3.3 Comparing the sumoylation levels of the anchor away strain with the parental strain

We wished to determine whether the FRB fusion to Ubc9 adversely affects its function by comparing protein sumoylation levels in the Ubc9-FRB strain (in the absence of rapamycin) with sumoylation levels in the parental strain. Lysate from the Ubc9-FRB fusion strain was extracted and analyzed by western blot with a SUMO antibody to compare the sumoylation levels in the parental strain and an unrelated control strain. The analysis revealed that the fusion strain has lower levels of SUMO-conjugation, even in the absence of rapamycin, compared to the parental strain and a control strain (Fig 10). As we previously observed (Fig 7), upon rapamycin treatment, the sumoylation levels in the fusion strain dropped further while the parental and control strains remained unaffected (Fig 10).This indicates that a caveat of using the strain is that it displays reduced basal sumoylation levels, even when grown in the absence of rapamycin. Nonetheless, we reasoned that the strain is suited for two types of analysis: (1) It can be used to study the consequences of constitutively reduced levels of sumoylation, by comparing the parental strain and the Ubc9-FRB strain in the absence of rapamycin, and (2) it can be used to study the effects of a rapid, inducible loss of sumoylation, by treating the Ubc9-FRB strain with rapamycin.
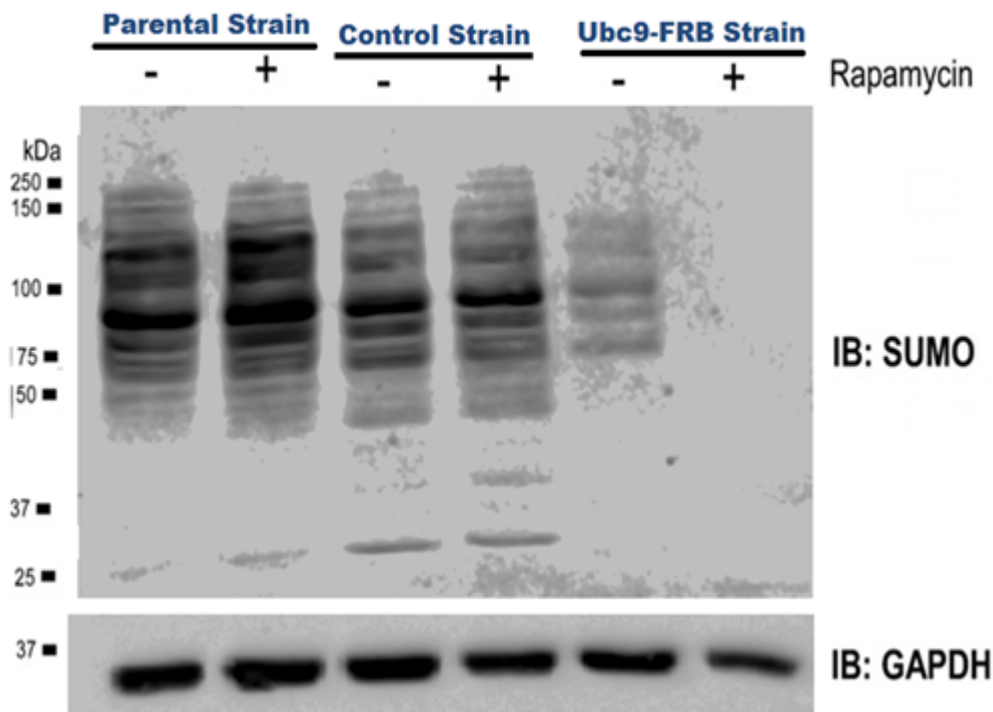
Figure 10: The Ubc9-FRB fusion strain starts off with a lower level of sumoylation compared to other strains

Lysate extraction followed by western blot analysis of the Ubc9-FRB fusion strain showed that it had significantly lower basal levels of sumoylation to begin with, compared to the parental and control strain, even before rapamycin treatment. This level reduced further upon treating with rapamycin (30min.) in these strains, although the control strains remained unaffected with the same treatment. GAPDH was used as a control (See Appendix A for the western blot assay using lysate volumes normalized to the band intensities).

3.4 Characterizing the growth patterns of the anchor away strain

In order to determine whether reduced sumoylation affects cell growth, we compared growth patterns of the Ubc9-FRB fusion strain with the parental strain, using a "spot assay" on standard rich medium (YPD) and on YPD medium supplemented with rapamycin (1 μg/mL). The strains had comparable growths on the standard YPD plates over 2 - 3 days (Fig 11). The parental strain (used as the control) grew similarly in the YPD plates supplemented with rapamycin. However, the Ubc9-FRB fusion strains did not grow on the rapamycin plates indicating that rapamycin was uniquely toxic for these

strains (Fig 11). Since the Ubc9-FRB strain displays dramatically reduced sumoylation upon exposure

to rapamycin, it could be surmised that some level of sumoylation is essential for yeast cell survival.
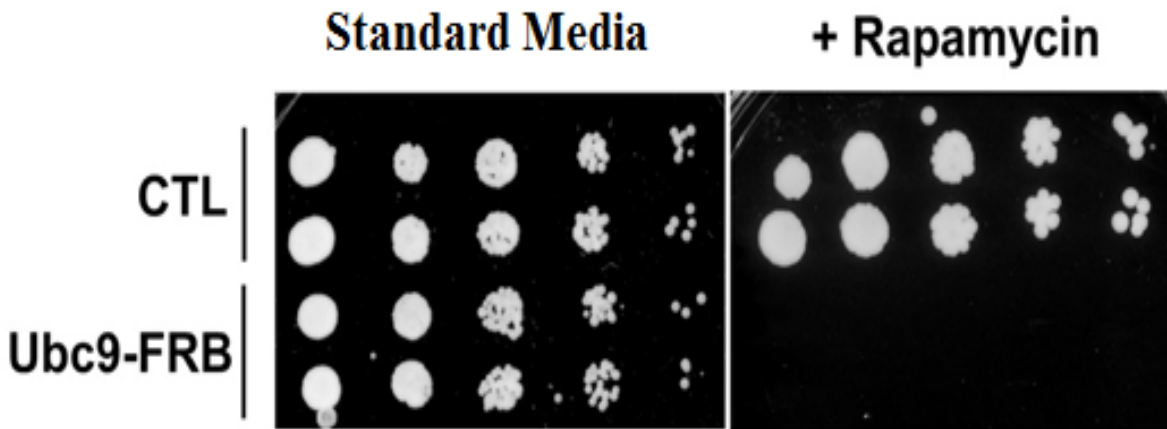
Figure 11: A functional Ubc9 is essential for yeast survival

A spot assay showing the growth of yeast strains on standard (YPD) plates with/without rapamycin ($1\ \mu g/mL$).It is to be noted that while the control and fusion strains grew similarly on the standard plates, the Ubc9-FRB fusion strains did not grow on rapamycin plates indicating that the system is effective and that a functional Ubc9 is essential for yeast survival.

A liquid growth assay was then conducted to further explore the growth impairment in the Ubc9-FRB

fusion cells and to narrow down the timeframe that the cells start to die off. The fusion strain as well

as the parental strain, which was used as the control in this assay, were treated with rapamycin (or

with DMSO as a control), and the optical densities (ODs) of cultures were measured across 34 hours.

The untreated parental (parental + DMSO), untreated anchor away strain (anchor-away +DMSO) and

the treated parental strain (parental + rapamycin) had similar growth patterns throughout the assay,

showing a normal exponential growth curve that enters a flat, stationary phase (peaking at an average

OD of 3.93, 3.87 & 3.64 respectively) (Fig 12). The treated Ubc9-FRB strain (anchor-away +

rapamycin) began to plateau from the 6 – 10 hour mark and showed no further increase in cell density

for the duration of the assay, ending with an average OD of 0.970. It is of interest that the treated

anchor away curve showed a similar trend to the other three curves until around the five hour mark,

from which it began to diverge (Fig 12). Since most of the experiments described below were performed within an hour of rapamycin treatment, the results obtained are most likely due to the drop in sumoylation levels, and are not a side-effect of the cells dying in general.

Light microscopy analysis revealed that there was a greater number of dead yeast cells, characterized as sessile, dark and clumped structures, observed in the Ubc9-FRB strain compared to the parental strain after rapamycin treatment (Fig 13). The assay was performed at two time intervals (15 & 30 min.) after the initial rapamycin treatment.
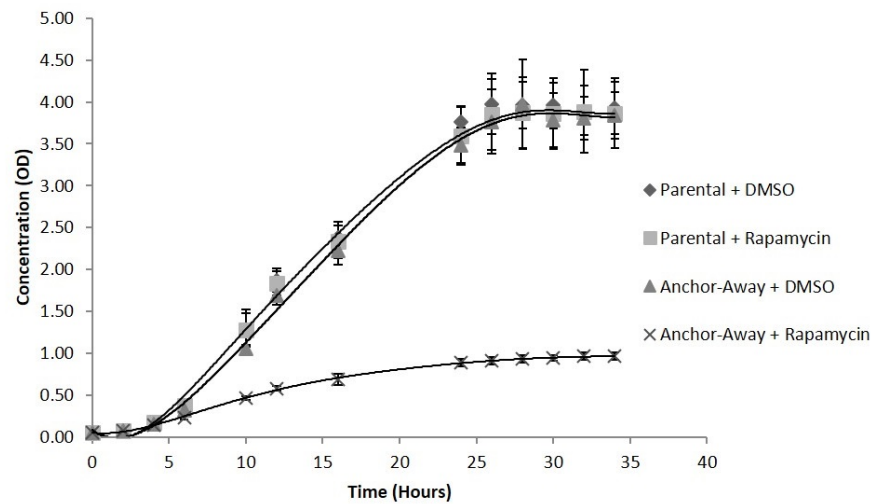
Figure 12: The Ubc9-FRB fusion strain grew similarly to the parental strain until rapamycin treatment
A liquid growth assay showing the growth of the parental and Ubc9-FRB fusion strains measured after rapamycin (or DMSO control) treatment across 35 hours. The fusion strain and the parental strain had similar growth rates. However, although the parental strain was unaffected with rapamycin treatment, the fusion strain started to die off 4-5 hours after treatment. The measurements were obtained using a spectrophotometer after the initial treatments (1 μg/mL) and were averaged across three replicates. A polynomial trend line was chosen to illustrate the trend. (See Appendices B, C & D for the raw data).

(Result & Fig. 12 obtained from: Farhaan Khan)

Rapamycin (minutes) – 15 30

Parental Strain

Anchor-Away Strain

25μm
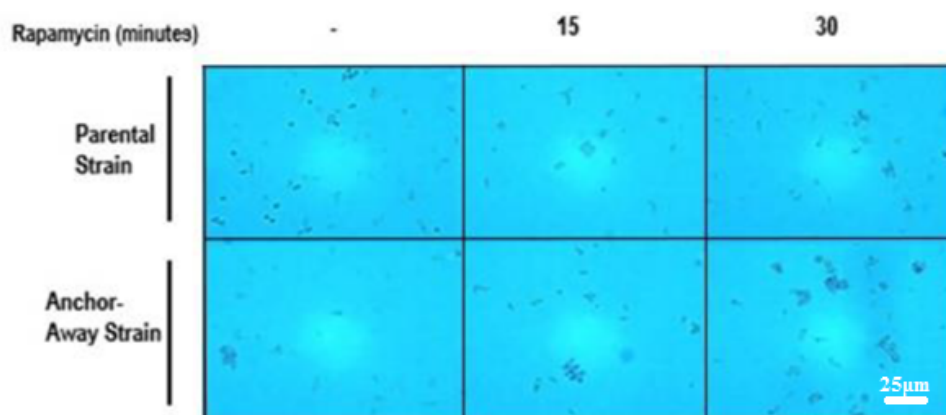
Figure 13: Rapamycin treatment resulted in the accumulation of dead Ubc9-FRB fusion cells
Microscopy revealed that rapamycin treatment resulted in the increased accumulation of dead cells for the Ubc9-FRB fusion strain compared with the parental strain. The parental and Ubc9-FRB fusion strains were incubated in rapamycin for 0 min (lane 1), 15 min. (lane 2) and 30 min. (lane 3). Dead yeast cells were defined as sessile, dark and possibly in clusters. Samples were placed on a slide and viewed through a light microscope at 100 X magnification. Images were acquired from a microscopy program which took pictures from the microscope's field of view.

(Result & Fig. 13 obtained from: Farhaan Khan)

3.5 Effects of reduced sumoylation on transcription of constitutive genes

Once the Ubc9-FRB strain was characterized, sumoylation was blocked by adding rapamycin to the cells in order to determine the effects of reduced sumoylation on transcription of constitutive genes. We performed RT-PCR and ChIP analyses, followed by quantitative PCR assays for both these experiments. The *DBP2* gene was chosen as a model gene to examine due to its high expression levels, and because *DBP2* transcripts have a very short half-life (3 min.), which means that steady-state RNA levels better reflect transcription rates of the gene. Although rapamycin was not expected to affect the parental strains, the *DBP2* transcript levels decreased following 30 min. of rapamycin treatment, suggesting that rapamycin itself has a negative effect on *DBP2* expression (Fig 14). However, levels of *DBP2* were dramatically lower in the Ubc9-FRB strain compared to the parental strain, even in the

absence of rapamycin (Fig 14). Addition of rapamycin did not change *DBP2* levels in the

Ubc9-FRB strain, implying that further blocking sumoylation did not have a noticeable effect

on its expression. To verify that our procedure was effective in detecting changing levels of

DBP2 transcription, we examined DBP2 mRNA levels in an Rpb1-FRB strain. In the control

strain, rapamycin treatment triggers the anchor-away system to sequester Rpb1, the largest

subunit of RNA Polymerase II, from the nucleus, effectively blocking transcription by RNA

Polymerase II transcribed genes in these strains. Indeed, treatment of the Rpb1-FRB strain

resulted in an expected decrease in the mRNA levels of the *DBP2*gene (Fig 14). Our analysis

of the *DBP2* suggests that constitutively reduced levels of sumoylation result in impaired

expression of the gene, but that further reduction of sumoylation, by treatment of Ubc9-FRB

with rapamycin, has no effect.
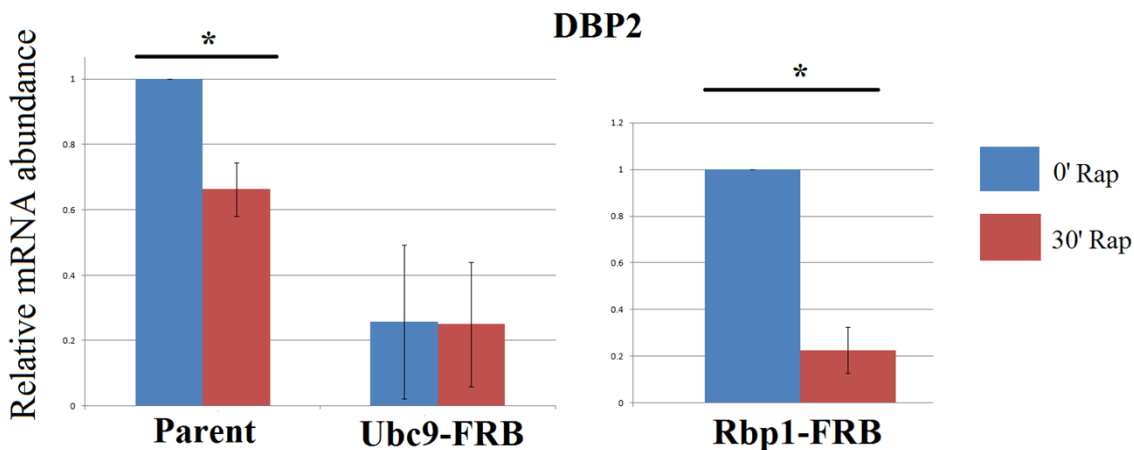
Figure 14: The effects of blocking sumoylation on the mRNA levels of DBP2, a constitutive gene, in the Ubc9-FRB fusion strain

The mRNA levels of the *DBP2* gene in the Ubc9 fusion strain were compared with the parental control strain after alpha-factor and rapamycin treatment. The results showed that rapamycin had an effect on the parental strain for the gene. The mRNA levels of the Ubc9-FRB fusion strain, however, did not change, even with rapamycin treatment. Notably, the Ubc9-FRB fusion strain had lower levels of the *DBP2* transcript compared to the parental strain, to begin with. The control Rpb1-FRB fusion strain, where the addition of rapamycin triggers Rpb1 evacuation from the nucleus subsequently resulting in decreased transcription, expectedly showed a drop in *DBP2* mRNA levels. Assays were normalized to 25S rRNA and the results were averaged across 4 replicates, with * $p < 0.05$.

To support our RT-PCR analyses, we next examined the *DBP2* gene by Rpb1 and SUMO ChIP. The Rpb1-FRB strain was again used as a control. ChIP analysis shows that there is less SUMO and Rpb1 associated with the promoter of *DBP2* in the untreated Ubc9-FRB strain than in the control strain (Fig 15). Interestingly, no further reduction in SUMO levels was observed in the Ubc9-FRB strain on the *DBP2* promoter with rapamycin treatment (Fig 15). This suggests that the minimal level of promoter-associated sumoylation was already achieved on DBP2, even before rapamycin treatment. As expected, the Rpb1-FRB strain showed a significant reduction in the Rpb1 levels with rapamycin treatment. For the Ubc9-FRB strain, there was also no change in the Rpb1 occupancy levels on the *DBP2* promoter with rapamycin treatment (Fig 15). Although rapamycin did not further reduce promoter-associated SUMO levels on *DBP2*, this ChIP analysis correlates promoter-associated sumoylation levels with transcription levels, suggesting that SUMO has a positive effect on *DBP2* expression. To determine whether this is a general phenomenon, three other transcripts were analyzed, including a highly-expressed, but more stable mRNA, *PMA1*, a ribosomal protein, *RPP2B*, and an RNA transcribed by RNA polymerase III, sNR52. As shown in Appendix E & F, variable effects were observed.
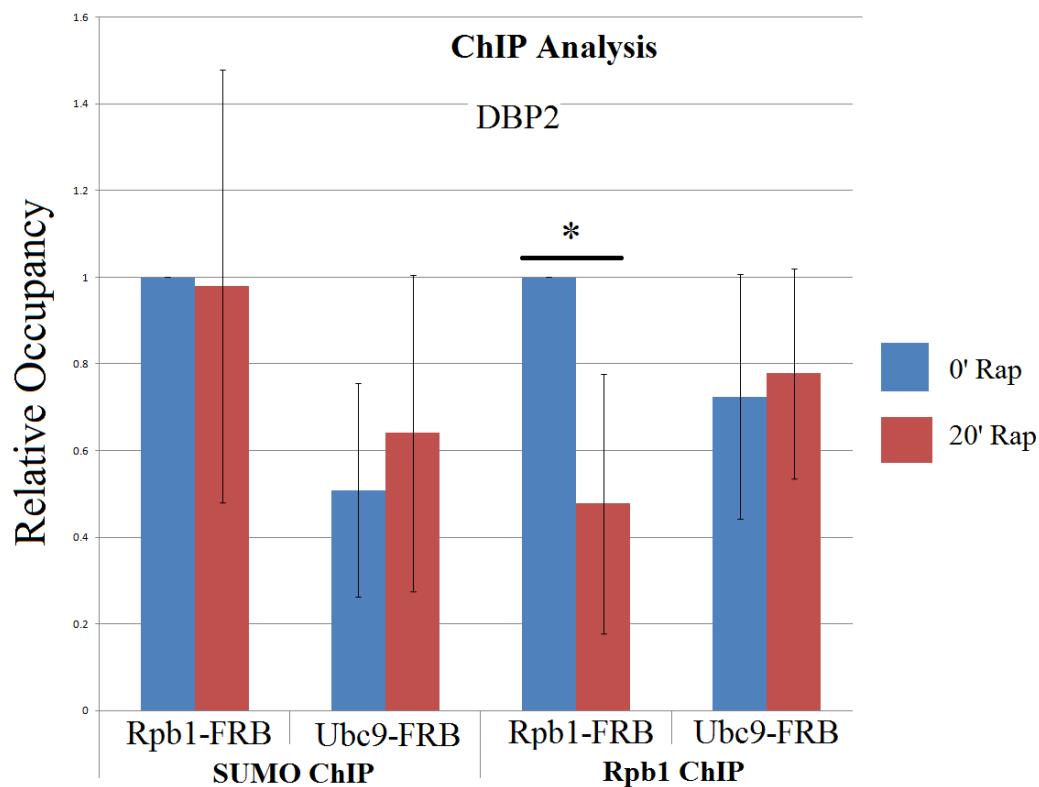
Figure 15: Blocking sumoylation does not alter recruitment of Rpb1 to the DBP2 constitutive gene promoter in the Ubc9-FRB fusion strain.

Several ChIP assays were performed to look at Rpb1 recruitment to the *DBP2* constitutive gene promoter. The results showed that there wasn't a marked difference in the polymerase recruitment for *DBP2* in the Ubc9 fusion strain with rapamycin treatment. SUMO ChIP results, looking at the SUMO levels on these promoters, also showed that even with rapamycin addition, there wasn't a further reduction in SUMO levels for the *DBP2* gene promoter, perhaps because they start off with lower SUMO levels even before rapamycin treatment. The control Rpb1-FRB fusion strain, where the addition of rapamycin triggers Rpb1 evacuation from the nucleus, expectedly showed a drop in Rpb1 levels at the *DBP2* promoter, upon rapamycin treatment. Assays were performed after alpha-factor treatment, normalized to Chr V (an untranscribed region of the chromosome) and the results were averaged across 3 replicates, with * $p < 0.05$.

3.6 Effects of reduced sumoylation on histone marks

Trimethylation of histone H3 at Lys 4 (H3K4me3) and acetylation of histone H3 at Lys 9 (H3K9ac) are histone modifications that are considered indicators of genes that are transcriptionally active (17). To determine whether reduced sumoylation results in changes to transcription-associated histone marks, we performed Western Blot assays of the lysates

extracted from the Ubc9-FRB fusion strain (and the control parental strain) with or without the treatment of rapamycin. The strains were probed for the active histone marks H3K4me3 & H3K9ac. Although there was significantly less global sumoylation in the Ubc9-FRB strain, which was further depleted after rapamycin treatment, cellular levels of H3K4me3 or H3 K9ac marks in these strains were not affected (Fig 16).
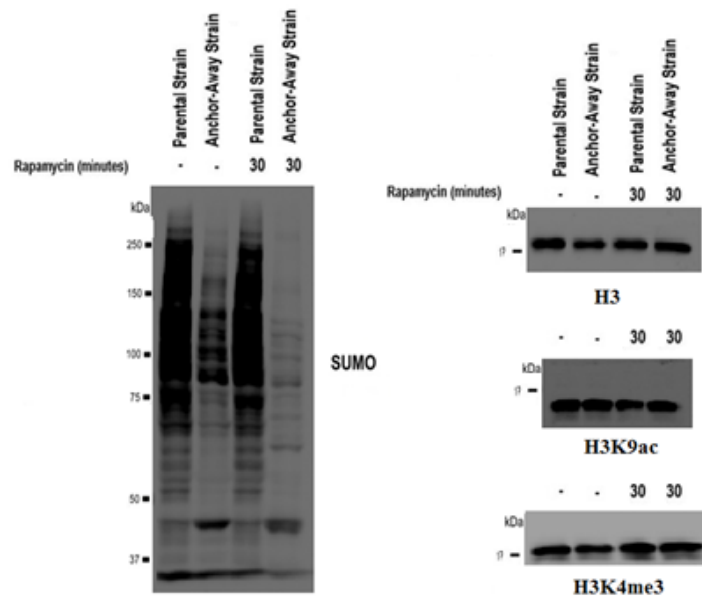
Figure 16: Rapamycin treatment resulted in no difference in various histone marks in the Ubc9-FRB strains

Western Blots probing the parental and Ubc9-FRB fusion strains for histone marks H3K4me3 & H3K9ac after a rapamycin treatment condition revealed no difference. As expected, the Ubc9-FRB fusion strain showed a marked depletion in sumoylation upon rapamycin treatment indicating that sumoylation levels do not influence the H3K4me3 or H3K9ac marks globally in the fusion strain.

(Fig. 16 obtained from: Akhi Akhter)

ChIP assays were then conducted to determine whether levels of the H3K9ac histone mark on various constitutive gene promoters was affected by impaired sumoylation in the Ubc9-FRB fusion strain. The Rpb1-AA strain, which was used as a control, showed a significant reduction in the H3K9ac mark with rapamycin treatment, indicating that reduced transcription of *DBP2*

and *RPP2B* triggered by the anchor-away system was associated with lower levels of these activating marks on their promoters (Fig 17). In the Ubc9-FRB strain, the H3K9ac modification levels on the selected gene promoters were unaffected by rapamycin treatment (Fig 17). However, an examination of levels of the histone modification in untreated Ubc9-FRB cells shows that both the *DBP2* and *RPP2B* gene promoters have significantly lower levels of H3K9ac compared with the control (Fig 17). This correlates reduced sumoylation levels on promoters of these genes (Figs 15 and Appendix F) with reduced levels of a histone mark that is associated with active transcription.
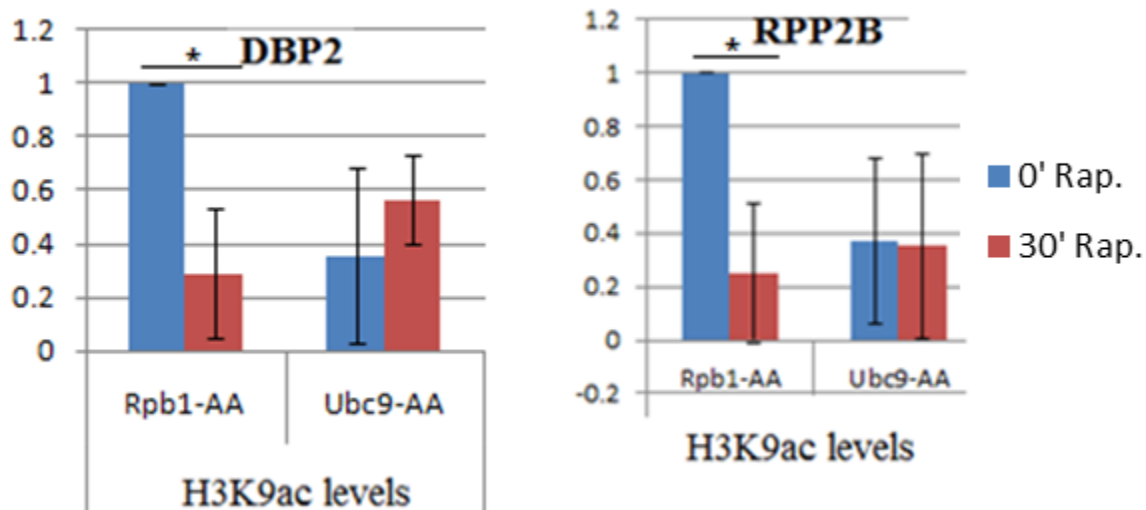
Figure 17: Rapamycin treatment resulted in no difference in the H3K9ac mark in the selected Ubc9-FRB strain promoters

The levels of histone mark H3K9ac on various constitutive promoters of the Ubc9-FRB fusion strain were examined. Although the control Rpb1-AA strain showed a reduction in the mark with rapamycin treatment, the Ubc9 strain did not show the same trend. H3K9ac is mostly associated with active transcription and this coincided with the reduced transcription seen in the Rpb1-AA strain upon rapamycin treatment. Experiments were performed and the results were averaged across 3 replicates and normalized to ChrV (an untranscribed region of the chromosome) levels, followed by normalizing it to total H3 levels. Assays were conducted after 3 hours of alpha factor treatment with * $p < 0.05$.

3.7 Enzymes responsible for the rapid turnover of SUMO

Although rapamycin-induced inhibition of Ubc9 activity is expected to conditionally and rapidly block only *de novo* sumoylation, the total sumoylated proteins in the Ubc9-FRB strain diminish rapidly, in as little as in 5 min. (Fig 7). This indicates that most SUMO modifications are highly transient involving rapidly sumoylating new proteins in conjunction with a quick turnover of already sumoylated proteins. This observation poses an interesting question: What enzyme(s) is responsible for maintaining this quick SUMO turnover? There were three candidate possibilities which we considered to be responsible for the rapid disappearance of sumoylated proteins: degradation of sumoylated proteins through the 26S proteasome, or removal of SUMO modifications by either the Ulp1 or Ulp2 SUMO-specific proteases (i.e. desumoylation enzymes). While the 26S proteasome degrades polyubiquitinated proteins (and thus, indirectly, sumoylated proteins as discussed in section 1.5 in the Introduction), the SUMO proteases cleave the bond between SUMO and its substrate (discussed in section 1.4 in the Introduction). Several experiments were performed to narrow down the enzyme(s) involved.

To determine whether the 26S proteasome was implicated, Ubc9-FRB cells were treated with the proteasome inhibitor MG132 prior to treatment with rapamycin. With controls, four conditions were tested: strains without MG132 or rapamycin, strains without MG132 but treated with rapamycin for 30 min., strains with MG132 but no rapamycin and strains with MG132 and treated with rapamycin for 30 min. (Fig 18). A western blot assay probing for SUMO showed that despite the lack of a functioning 26S proteasome, there was still a depletion of sumoylated proteins with rapamycin treatment (Fig 18). This indicated that the degradation of sumoylated proteins by the proteasome was probably not the reason for the quick SUMO turnover. To ensure that the MG132 treatment was effective at blocking the 26S proteasome, lysates from the samples were analyzed with the FK1 antibody, which recognizes polyubiquitinated proteins of high molecular weight. Indeed, MG132 treatment stabilized ubiquitinated proteins, indicating that the proteasome had been inactivated (Fig 18).

To test if Ulp2 was responsible for the rapid turnover of SUMO signal, *ULP2* was deleted from the

Ubc9-FRB anchor-away strain using a URA3-MX3 deletion cassette and homologous recombination.

A western blot assay of the lysates extracted (with or without rapamycin treatment) from the *ULP2*

deleted Ubc9-FRB fusion strain probing for SUMO showed the same pattern as the experiments done

using the Ubc9-FRB fusion strain with *ULP2* intact (Fig 19). It is to be noted that the *ulp2Δ* strain had

more proteins that had higher molecular weights than the parental anchor-away strain, a characteristic

expected of a strain lacking the SUMO protease Ulp2 (Fig 19). However, depletion of sumoylated

proteins with rapamycin treatment in the *ulp2Δ* was comparable to the parental Ubc9-FRB fusion strain

(Fig 19). This indicates that desumoylation by Ulp2 is also not responsible for the rapid loss of

sumoylation during rapamycin-induced inactivation of Ubc9. Future analysis will determine whether

the essential SUMO protease Ulp1 is responsible for the phenomenon.
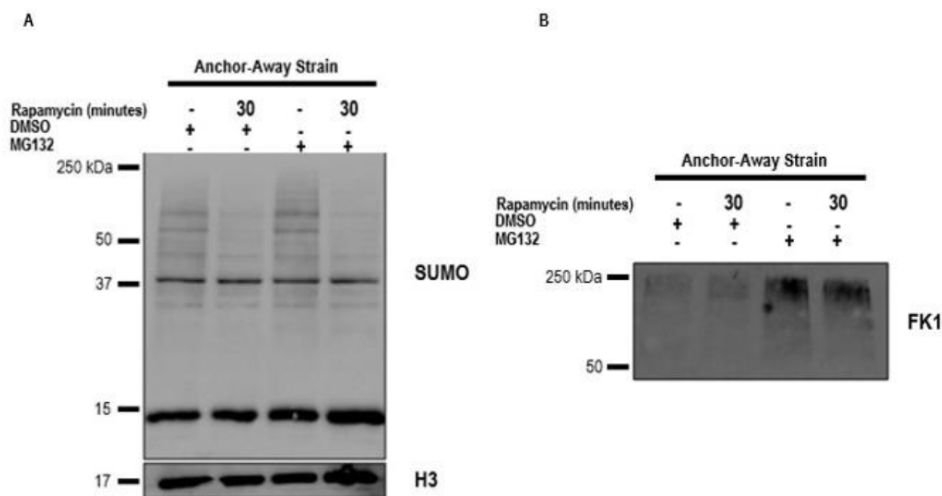
Figure 18: 26S proteasome is most likely not responsible for the depletion of sumoylated proteins upon rapamycin treatment in the Ubc9-FRB fusion strain

A Western Blot assay of the Ubc9-FRB fusion strain lysates after MG132 and rapamycin treatment was performed probing for SUMO and FK1. **(A & B)** From left to right Lanes 1-2: contain lysates incubated for 30 min. in DMSO, Lanes 3-4: lysates incubated in 75µM MG132. Lanes 2 & 4 were treated with rapamycin. Lane 4 of **A** showed that there was depletion of sumoylated proteins with rapamycin treatment, even when the 26S proteasome was not functioning due to MG132 treatment. Lanes 3 &4 from **B** showed the presence of poly-ubiquitinated proteins, recognized by the FK1 antibody, when treated with MG132, implying that it was successful in blocking the 26S proteasome. H3 is used in **A** as a control.

(Result & Fig. 18 obtained from: Farhaan Khan)
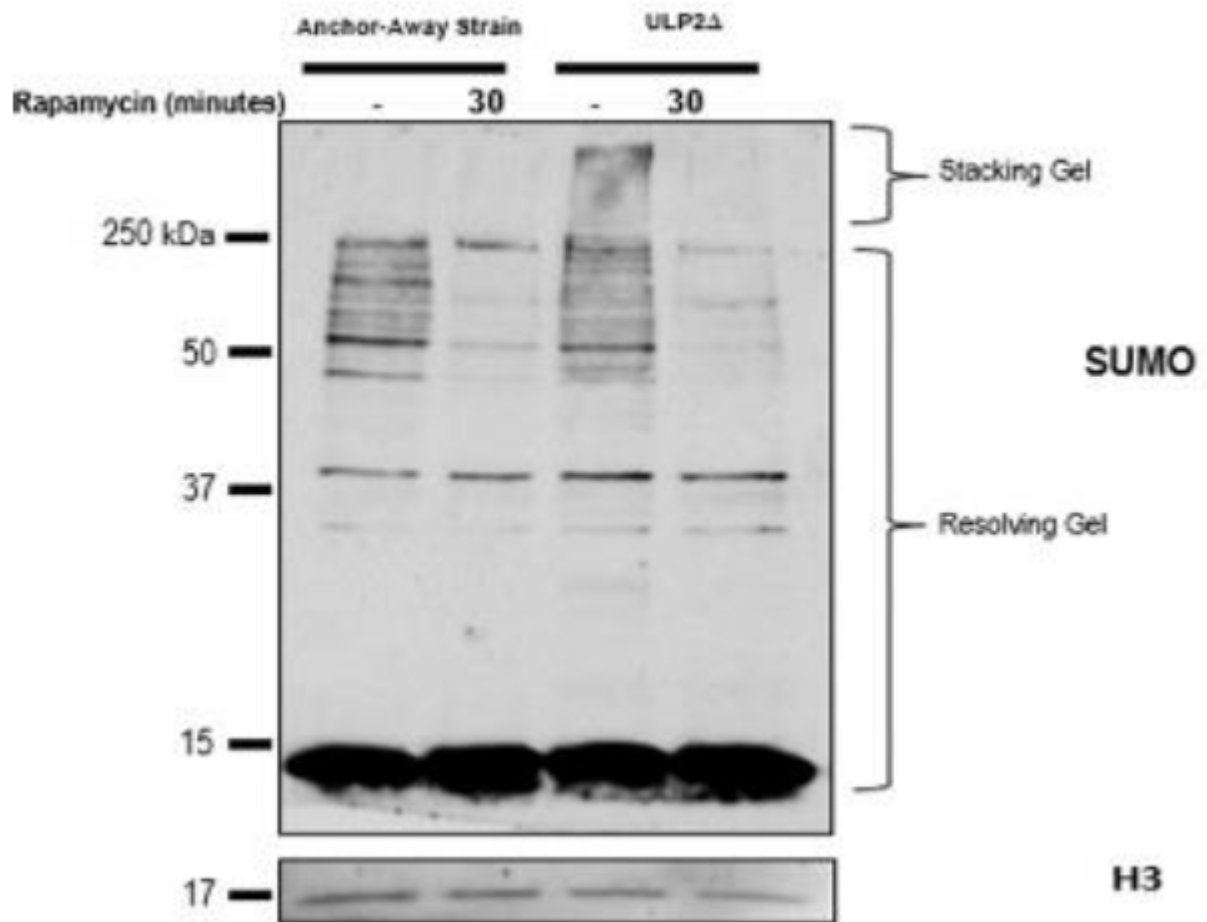
Figure 19: Ulp2 is most likely not responsible for the depletion of sumoylated proteins upon rapamycin treatment in the Ubc9-FRB fusion strain

A Western Blot assay of the Ubc9-FRB fusion strain and *Ulp2Δ* strain, after rapamycin treatment, was performed probing for SUMO. From left to right, Lanes 1-2: contain the anchor away strain lysates incubated without (lane 1) or with (lane 2) rapamycin, Lanes 3-4: *Ulp2Δ* strain lysates incubated without (lane 3) or with (lane 4) rapamycin. The depletion of sumoylated proteins with rapamycin treatment persisted even for the Ubc9-FRB anchor-away strains with deleted *ULP2*.

(Result & Fig. 19 obtained from: Farhaan Khan)

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Chapter 4: Discussion

Sumoylation, the covalent reversible attachment of the SUMO peptide to lysine residues of cellular proteins, affects proteins in a vast range of cellular processes. Due to the finding that proteins involved in transcription comprise one of the largest groups of sumoylation targets, and that sumoylated proteins are associated with transcriptionally active genes, particularly at their promoters, it is likely that sumoylation functions in regulating transcription and other levels of gene expression. The sole SUMO conjugating enzyme Ubc9's recruitment to a number of different inducible gene promoters when they were activated also implies a general role for this PTM during the gene induction process (9). Since the exact function of protein sumoylation on gene promoters still remains largely unknown, a system to study global sumoylation by blocking the process can be extremely powerful. The anchor-away system that was developed and characterized in this project was used for this purpose and helped examine the effects of impaired sumoylation under essentially normal growth conditions. The distinct advantage of using this system was that it allowed for the conditional and rapid inactivation of Ubc9, tantamount to preventing *de novo* sumoylation. Both *SMT3*, the only SUMO isoform in yeast, and *UBC9*, the only known SUMO conjugating enzyme, are essential genes making the usage of null mutants impossible (22). Although there have been previous studies using a temperature sensitive *ubc9-1* strain for examining the effects of reduced cellular sumoylation (9), the experiments using the strain had to be performed during heat shock due to the strain expressing an unstable mutant form of the enzyme, only inactive at elevated temperatures. Further, the overall sumoylation was abnormal with the strain displaying growth defects, even at normal temperatures. Using the novel Ubc9-FRB strain no longer relied on the previously utilized stress inducing techniques which could increase the likelihood of introducing experimental artifacts and was thus, theoretically superior (11). Characterizing this strain and testing out some of the effects of blocking sumoylation in regulating transcription were the major objectives of this project.

Sumoylation levels in the Ubc9-FRB fusion strain compared to the parental strain and an additional control strain showed that there were much lower levels of sumoylated proteins in this strain, which could be even further reduced with rapamycin. A growth assay on solid medium ("drop test"), liquid growth assay and microscopy experiments revealed that while the parental strain and the Ubc9-FRB strain grew similarly on standard YPD plates, with similar cell densities and were visually similar under the microscope, rapamycin treatment resulted in the latter strain not being able to survive and were visually seen as sessile and in clusters. The ODs of these strains diverged from around 4-5 hours after rapamycin treatment from the parental strains, which were unaffected. This showed that sumoylation was essential for cell viability and conditionally blocking *de novo* nuclear sumoylation events by rapamycin treatment was lethal for the cells. However, it is also to be noted that, in the absence of rapamycin, the Ubc9-FRB strain grew fairly similarly to the parental strain despite having constitutively lower levels of sumoylation, indicating that this characteristic of the strain was not lethal.

Consistent with the western blot analysis, ChIP analysis of some gene promoters showed lower sumoylation levels compared to the control strain. Combined with the mRNA analysis, for the constitutive gene *DBP2*, there were much lower levels of the transcript in the Ubc9-FRB fusion strain coinciding with the lower SUMO levels on this promoter. These results suggest that sumoylation is mostly associated with increased transcription and RNA Pol II recruitment in constitutive genes. This effect, however, was not seen on all the genes examined indicating that sumoylation may have different effects on different constitutive genes, perhaps depending on which transcription factors are involved. It is also to be noted that the lower half-life of the *DBP2* transcript means that steady-state RNA levels better reflect transcription rates of the gene compared to the others examined. .A further drop in global sumoylation, induced by rapamycin in the Ubc9-FRB fusion strain, did not further reduce promoter-associated sumoylation, and also did not further drop transcription levels suggesting that the minimal

level of promoter-associated sumoylation was already achieved on DBP2, even before rapamycin treatment.

Blocking sumoylation in the Ubc9-FRB fusion strain did not alter the levels of various activating histone marks globally. The levels of the activating mark H3K9ac on different constitutive gene promoters also showed a similar trend in the fusion strain as blocked sumoylation did not alter these levels. However, it was once again seen that for *DBP2* (and for *RPP2B*), the Ubc9-FRB fusion strain started off with much lower levels of the activating mark H3K9ac. Combined with the mRNA analysis and previous ChIP assays, the lower sumoylation levels on the *DBP2* promoter in the Ubc9-FRB fusion strain was associated with lower expression levels of the transcript and with lower levels of the activating H3K9ac mark. This indicated that reduced sumoylation is most likely associated with decreased transcription levels, at least for the *DBP2* gene in these strains. The significant reduction of the H3K9ac mark in the Rpb1-FRB fusion strain upon rapamycin treatment indicates that active histone marks may be affected by reduced transcription. Thus, it is likely that although lesser sumoylation in the Ubc9-FRB fusion strain correlated with lesser H3K9ac marks on the *DBP2* promoter, this could be a consequence of lesser transcription of the gene in these strains to begin with.

By inhibiting Ubc9 function, the anchor-away system used in this study, triggered by rapamycin, theoretically blocks only *de novo* sumoylation, and all previously sumoylated proteins should have been detected if the modification were stable. However, the rapid decrease in sumoylated proteins in the Ubc9-FRB fusion strain upon rapamycin treatment indicates that the PTM is surprisingly very labile. MG132 treatment, which inhibits the 26S proteasome, did not reverse the rapamycin-triggered disappearance of SUMO conjugates, indicating that SUMO conjugates are not necessarily prone to rapid degradation. A western blot of the *ULP2Δ* strain generated from the Ubc9-FRB fusion strain revealed that de-conjugation of SUMO by the SUMO protease Ulp2 was also not responsible for the depletion of sumoylated proteins. This was slightly expected, as *ULP2* is not an essential protease in

yeast (47). *ULP1*, on the other hand, encodes an essential SUMO protease, with *ulp1* null mutants being generally lethal (48 & 49). Thus, a temperature sensitive *ulp1* strain will be used to confirm whether de-conjugation by the enzyme could be the cause for the SUMO depletion observed in previous experiments. Considering that the impairment of the Ulp1 protease causes an overall elevation in sumoylation levels in yeast cells (50), it is likely that inhibiting the Ulp1 enzyme would rescue the drops in SUMO levels seen in the Ubc9-FRB fusion strain with rapamycin treatment, and that the enzyme is the primary contributor to the SUMO turnover making it a transient PTM.

Chapter 5: Future Directions

Future experiments can utilize immunofluorescence microscopy to demonstrate the exact location of Ubc9 and confirm its presence in the nucleus after rapamycin treatment. Although not described in this thesis, there was a second anchor-away strain designed with an extra green fluorescent protein (GFP) tagged next to FRB in the fusion protein. This strain permits the use of GFP microscopy to observe Ubc9 in the strain. New strains can be designed for the anchor-away technique to serve as 'replicates' for the same experiments conducted using the present strain to confirm the consistency of the obtained results. Instead of looking at individual transcript levels or gene promoters, a global analysis of different mRNA levels and promoters using ChIP seq and RNA-seq can be performed to shed more light on the patterns seen on different genes during blocked sumoylation. Although only active histone marks H3K4me3 and H3K9ac were looked at in this project, future experiments can look at other epigenetically relevant marks and modifications to see if sumoylation is associated with them. Lastly, the *ULP1* mutant genotype described in this thesis can be made from transforming the Ubc9-FRB fusion strain to see if the lack of functioning Ulp1 in these strains could rescue the depletion of sumoylated proteins seen with rapamycin treatment. This could confirm Ulp1's role in the rapid SUMO turnover, thus making it essential in maintaining steady-state SUMO levels. Although there are caveats in using the strain designed in this project, it still is an extremely useful tool to study global sumoylation due to its conditional and rapid ability to block *de novo* sumoylation in the cells.

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Appendix
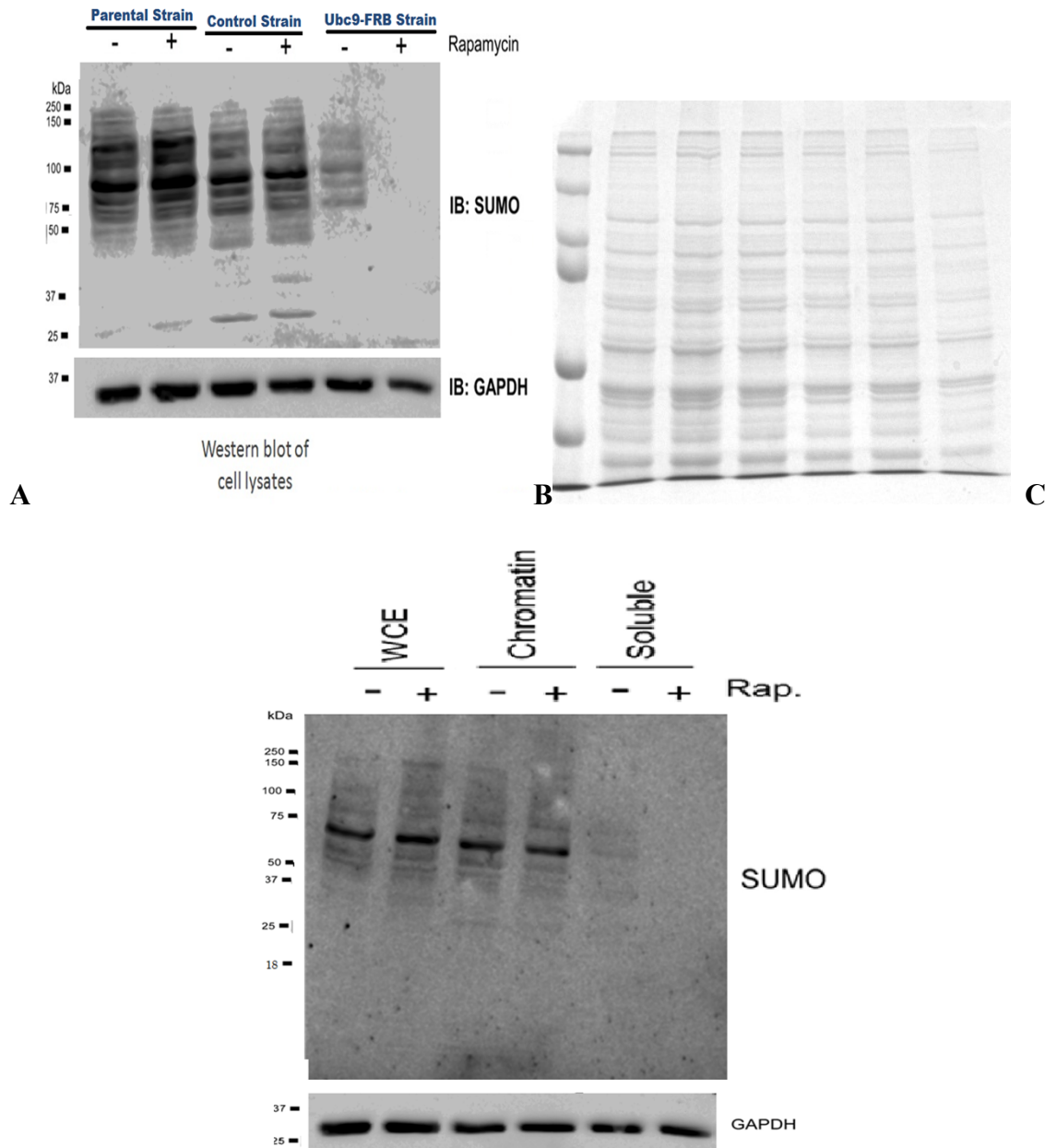
APPENDIX A: Comparing the sumoylation levels of the Ubc9-FRB fusion strain with the control strains

A: The initial western blot analysis of the Ubc9-FRB fusion strain with the parental and control strain showed that the samples were slightly uneven (Fig 10). **B**: A coomassie strain of the gel revealed the same with lane 6 having the least amount of the lysate. **C**: The samples were reloaded, after adjusting the volumes for the band intensities measured using the ImageJ software. The pattern seen was the exact same as before although since the samples were re-run after a few weeks, the amount of sumoylated proteins had decreased.

APPENDIX B: Liquid growth assay trial data

ODs collected during liquid growth assay. YER013 refers to the parental strain, YJBa001 is the anchor away strain.

TRIAL1

			OD			
	Time	Hour	YER013+ DMSO	YER013+ Rapamycin	YJBa001+ DMSO	YJB00a001 + Rapamycin
	7:00AM	0	0.050	0.050	0.050	0.050
	9:00AM	2	0.085	0.087	0.074	0.072
	11:00AM	4	0.182	0.190	0.140	0.132
	1:00PM	6	0.380	0.386	0.268	0.212
	5:00PM	10	1.550	1.510	1.098	0.442
	7:00PM	12	1.922	1.896	1.660	0.556
	11:00PM	16	2.470	2.440	2.240	0.648
	7:00AM	24	3.810	3.750	3.580	0.861
	9:00AM	26	4.170	4.160	3.980	0.884
	11:00AM	28	4.100	4.110	4.340	0.903
	1:00PM	30	4.110	3.960	3.910	0.915
	3:00PM	32	4.060	3.950	3.970	0.926
	5:00PM	34	4.120	4.000	4.020	0.942

TRIAL2

	Time	Hour	YER013+ DMSO	YER013+ Rapamycin	YJBa001+ DMSO	YJB00a001 + Rapamycin
	7:00AM	0	0.050	0.050	0.050	0.050
	9:00AM	2	0.069	0.071	0.069	0.071
	11:00AM	4	0.166	0.170	0.170	0.142
	1:00PM	6	0.381	0.385	0.350	0.251
	5:00PM	10	1.074	1.126	1.014	0.489
	7:00PM	12	1.718	1.672	1.596	0.602
	11:00PM	16	2.176	2.108	2.058	0.772
	7:00AM	24	3.570	3.205	3.235	0.940
	9:00AM	26	3.560	3.370	3.320	0.964
	11:00AM	28	3.640	3.380	3.360	0.980
	1:00PM	30	3.645	3.400	3.420	0.987
	3:00PM	32	3.560	3.365	3.510	1.010
	5:00PM	34	3.570	3.400	3.520	1.023

TRIAL3

	Time	Hour	YER013+ DMSO	YER013+ Rapamycin	YJBa001+ DMSO	YJB00a001 + Rapamycin
	7:00AM	0	0.050	0.050	0.050	0.050
	9:00AM	2	0.085	0.084	0.085	0.085
	11:00AM	4	0.168	0.165	0.160	0.150
	1:00PM	6	0.380	0.365	0.336	0.254
	5:00PM	10	1.190	1.204	1.060	0.476
	7:00PM	12	1.978	1.934	1.806	0.590
	11:00PM	16	2.510	2.450	2.387	0.660
	7:00AM	24	3.920	3.840	3.631	0.870
	9:00AM	26	4.202	4.020	3.995	0.894
	11:00AM	28	4.140	4.120	4.210	0.910
	1:00PM	30	4.120	4.220	4.020	0.934
	3:00PM	32	4.080	4.345	3.940	0.959
	5:00PM	34	4.100	4.200	3.980	0.946

APPENDIX C: Averaged liquid growth assay data

ODs collected during liquid growth assay. YER013 refers to the parental strain, YJBa001 is the anchor away strain.

AVERAGE

TIME	Hour	YER013+ DMSO	YER013+ Rapamycin	YJBa001+ DMSO	YJBa001+ Rapamycin
7:00AM	0	0.050	0.050	0.050	0.050
9:00AM	2	0.080	0.081	0.076	0.076
11:00AM	4	0.172	0.175	0.157	0.141
1:00PM	6	0.380	0.379	0.318	0.23
5:00PM	10	1.271	1.280	1.057	0.469
7:00PM	12	1.873	1.834	1.687	0.583
11:00PM	16	2.385	2.333	2.228	0.693
7:00AM	24	3.767	3.598	3.482	0.890
9:00AM	26	3.977	3.850	3.765	0.914
11:00AM	28	3.960	3.870	3.970	0.931
1:00PM	30	3.958	3.860	3.783	0.945
3:00PM	32	3.900	3.887	3.807	0.965
5:00PM	34	3.930	3.867	3.840	0.970

## APPENDIX D: Standard deviation of liquid growth assay trial data

YER013 refers to the parental strain. YJBa001 is the anchor away strain. STDEV was calculated using the excel function "=STDEV'

STDEV

| TIME	Hour	YER013+ DMSO	YER013+ Rapamycin	YJBa001+ DMSO	YJBa001+ Rapamycin
7:00AM	0	0.000	0.000	0.000	0.000
9:00AM	2	0.009	0.009	0.008	0.008
11:00AM	4	0.009	0.013	0.015	0.009
1:00PM	6	0.001	0.012	0.044	0.023
5:00PM	10	0.248	0.203	0.042	0.024
7:00PM	12	0.137	0.142	0.108	0.024
11:00PM	16	0.182	0.195	0.165	0.068
7:00AM	24	0.179	0.344	0.215	0.043
9:00AM	26	0.362	0.422	0.385	0.044
11:00AM	28	0.278	0.424	0.532	0.043
1:00PM	30	0.271	0.419	0.319	0.037
3:00PM	32	0.295	0.493	0.257	0.042
5:00PM	34	0.312	0.416	0.278	0.046

(Result & Appendices B, C & D obtained from: Farhaan Khan)

mRNA Analysis
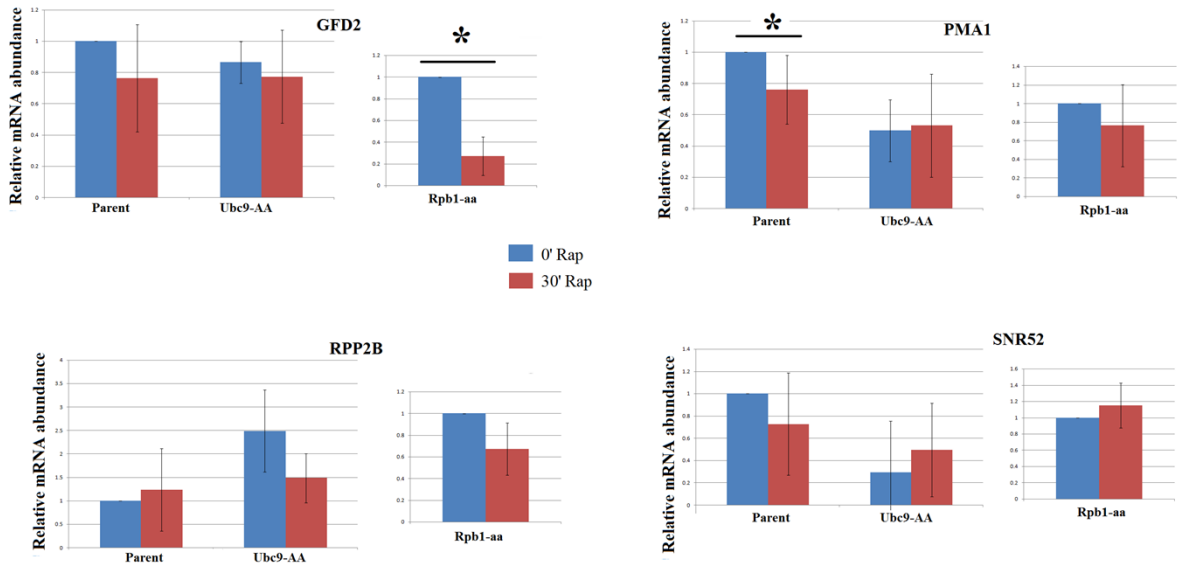
APPENDIX E: The effects of blocking sumoylation on the mRNA levels of various constitutive genes in the Ubc9-FRB fusion strain.

The mRNA levels of various constitutive genes in the Ubc9 fusion strain were compared with the parental control strain after rapamycin treatment. The results showed that rapamycin had an effect on the parental strain for the *PMA1* gene. The mRNA levels of the Ubc9-FRB fusion strain, however, did not change, even with rapamycin treatment. Notably, the Ubc9-FRB fusion strain had lower levels of some transcripts (such as PMA1) compared to the parental strain, to begin with. The control Rpb1-AA strain, where the addition of rapamycin triggers Rpb1 evacuation from the nucleus subsequently resulting in decreased transcription, expectedly showed a drop in mRNA levels in all the genes looked at. SNR52, a gene transcribed by RNA Pol III, is also used as an additional control. Assays were conducted after 3 hours of alpha factor treatment, normalized to 25S rRNA and the results were averaged across 4 replicates, with * $p < 0.05$.

ChIP Analysis

PMA1

RPP2B

Legend: 0' Rap (blue), 20' Rap (red)

SNR52
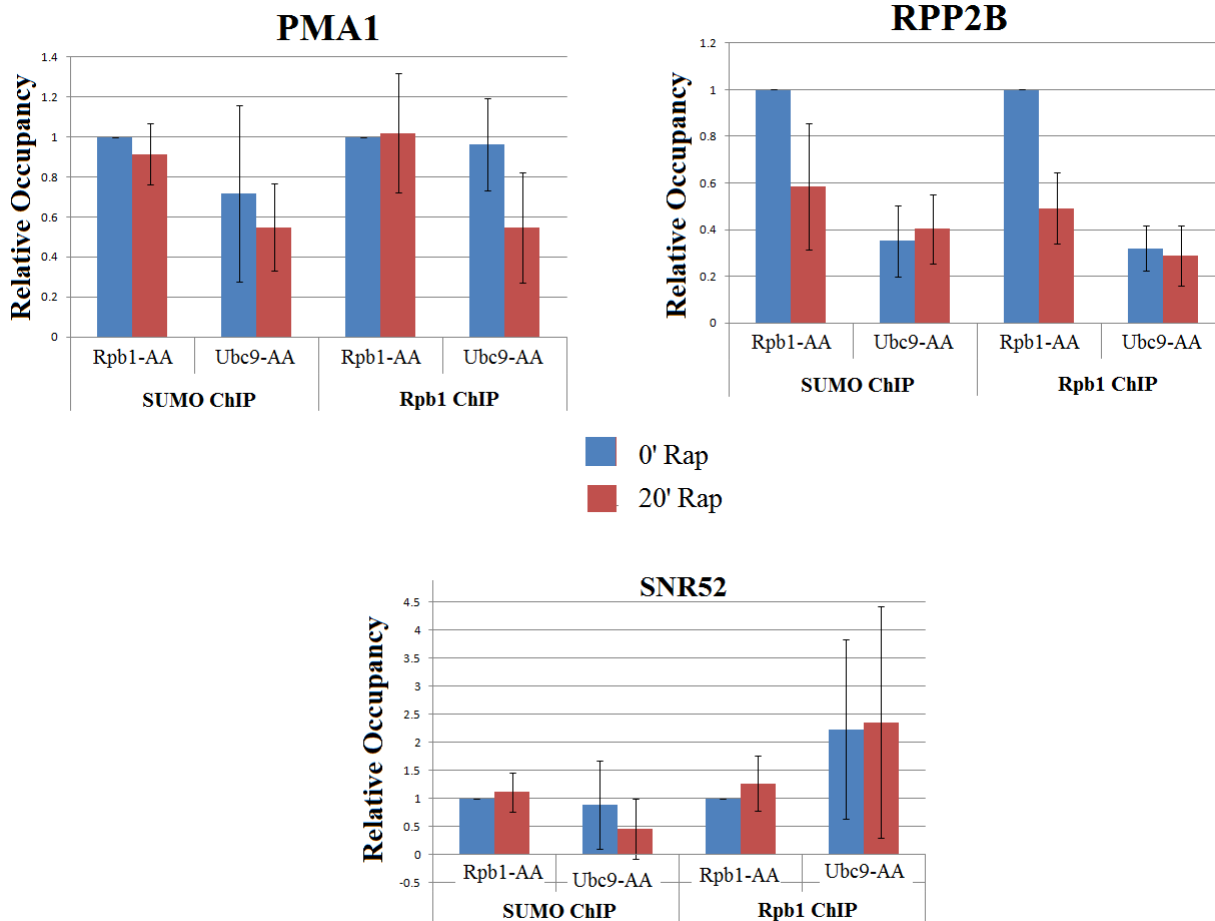
APPENDIX F: Blocking sumoylation does not alter recruitment of Rpb1 in many constitutive promoters in the Ubc9-FRB fusion strain

Several ChIP assays were performed to look at Rpb1 recruitment to the promoters of various constitutive genes. The results showed that there wasn't a marked difference in the polymerase recruitment for the fusion strains. SUMO ChIP results, looking at the SUMO levels on these promoters, also showed that even with rapamycin addition, there wasn't a further reduction in SUMO levels for most promoters, perhaps because they start off with lower SUMO levels to begin with. Notably, *SNR52*, which starts off with a much higher level of sumoylation compared to other genes, did indeed show a drop in SUMO levels with rapamycin treatment. Assays were conducted after 3 hours of alpha factor treatment, normalized to Chr V (an untranscribed region of the chromosome) and the results were averaged across 3 replicates, with * p <0.05.

APPENDIX G: Primers used for qPCR (ChIP and RT-PCR)

Gene	Region	Primer Sequence (5' to 3')	Direction
DBP2	Promoter	GATAAAAATACACCTTCGTA	Forward
		GAATGAGATACAGAAAAGTA	Reverse
	ORF	AAGAGAAGATGGATGGCCCG	Forward
		TGACATCGATACCTCTGGCG	Reverse
RPP2B	Promoter	GATATTTGGACTTACTCTAA	Forward
		GTTTAAGCGGTAATAAATTC	Reverse
	ORF	GTCGGTGCTGAAGTCGATGA	Forward
		TCCAAAGAGCCCTTACCTTCC	Reverse
PMA1	Promoter	CAATTATGACCGGTGACGAAAC	Forward
		AATCGAAACTAATGGAGGGGAG	Reverse
	ORF	CTGGTCCATTCTGGTCTTCTATC	Forward
		TCAGACCACCAACCGAATAAG	Reverse
SNR52	Promoter & ORF	TGACATTAGCGTGAACAATCTCTG	Forward
		TTTCAGAAGGAAGGCAACATAAG	Reverse
GFD2	ORF	TAAGCGGCATGGACGATGTT	Forward
		ATGGGCACCACTTTAGGCTC	Reverse