IDENTIFICATION OF THE ROLES OF THE SMALL UBIQUITIN-RELATED MODIFIER (SUMO) IN CELLULAR STRESS RESPONSE

by
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Abstract

Proper protein folding is essential for cell function, as misfolding leads to cell stress and cytotoxicity. Misfolded proteins are associated with several serious diseases in humans such as Parkinson's and Alzheimer's diseases. The posttranslational modifier SUMO has been implicated in several of these diseases, including cystic fibrosis, amyotrophic lateral sclerosis, and other neurodegenerative diseases. However, the mechanism by which SUMO affects protein folding and stress response is not well understood. To address this question, we investigated a SUMO mutant that is sensitive to canavanine, an arginine analog that induces protein misfolding. We characterized this mutant as deficient in binding to proteins containing SUMO-interacting motifs (SIMs) and hypothesized that a high-copy suppressor screen would identify proteins that function downstream of SUMO in the protein stress response. We found that San1, a ubiquitin E3 ligase involved in nuclear protein quality control, can suppress the sensitivity of our mutant to canavanine. Interestingly, the RING domain required for E3 ligase activity is not necessary for suppression, but the unstructured N-terminus of San1 is both necessary and sufficient for suppression. The N-terminus has a chaperone-like function, binding to unstructured, misfolded proteins. Our findings suggest that sumoylation may either play a similar role and promote proper protein folding, or function to prevent the accumulation of toxic misfolded proteins in the cell.

Mentor/Principal Investigator: Michael J. Matunis, Ph.D.

Advisor: Robert D. Horner, Ph.D.

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1. Introduction

Posttranslational protein modifications, including phosphorylation, acetylation, and ubiquitination function to regulate nearly all cellular processes. These modifications affect protein function by controlling stability, localization, and interactions with other proteins. Posttranslational modifications often undergo shifts and changes between sets of proteins when cells encounter a new environment. Changes in the overall landscape of these modifications are associated with cellular stress response pathways, including oxidative stress, genotoxic stress, and protein misfolding stress. By studying posttranslational modifications during cell stress, we can learn more about how the cell reacts to stress and what goes wrong in diseases associated with cell stress such as diabetes (1), cancer (2), and neurodegenerative diseases (3).

1.1 The SUMO Pathway

The Small Ubiquitin-related MOdifier, or SUMO, is a prominent posttranslational modification in the cell. SUMO is a small, 100 kD peptide with one isoform in invertebrates and four isoforms in vertebrates. In all organisms, SUMO is conjugated to other proteins through an E1 activating, E2 conjugating, and E3 ligating enzyme cascade (4, 5, 6). In this cascade, there is only one E1 enzyme, called Aos1/ Uba2 in yeast, and a single E2 enzyme, called Ubc9 in yeast, however there are numerous E3 ligases which are thought to have varying specificity to the many SUMO substrates. The consensus site for SUMO conjugation is ψKXE, where ψ is a large hydrophobic residue, K is the lysine of SUMO that is conjugated to substrates, X is any amino acid, and E is glutamic acid (7). On the substrate side, the SUMO-interaction

motif (SIM) consists of an essential hydrophobic core that is often juxtaposed with a cluster of acidic residues (8). SUMO conjugation is reversible, with sentrin-specific proteases (SENPs) deconjugating SUMO from its substrates when it is no longer required (9). SUMO conjugation and deconjugation regulate many cellular processes, including mitosis, nuclear transport, and DNA repair (10).

1.2 SUMO in Cellular Stress Response

SUMO has been implicated in the cellular response to many types of stresses, including hypoxic stress (11), genotoxic stress (12), and protein misfolding stress (3). Each of these types of stress is a causative factor in one or more human diseases. Hibernation and hypothermia in ground squirrels has been used by Lee et al. as a model for ischemia, a lack of oxygen evident in cells during heart attacks and kidney failure. While these animals are in hibernation, there is a strong increase in sumoylation in

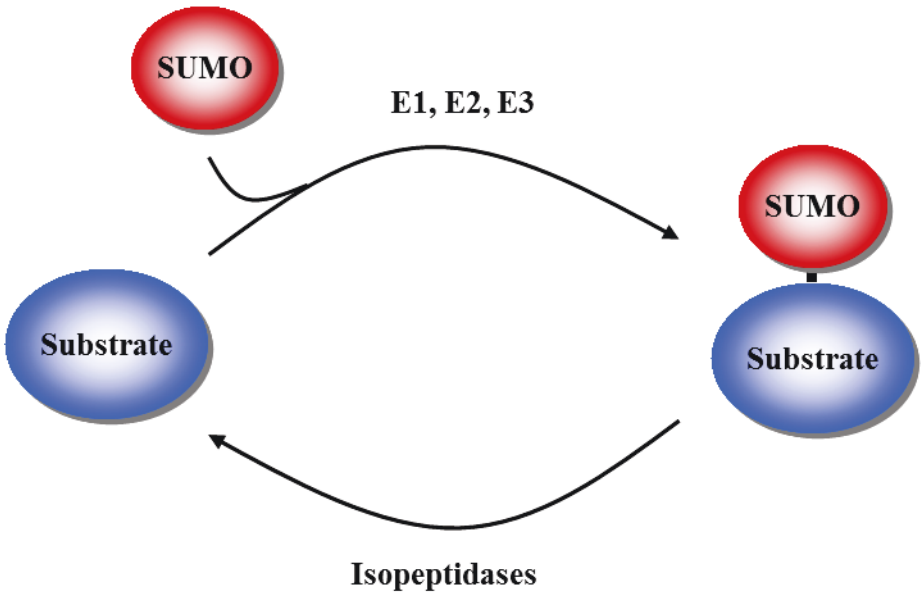


Figure 1: The SUMO Conjugation and Deconjugation Pathway. SUMO is conjugated by an E1, E2, E3 enzyme cascade to its substrates, then deconjugated by isopeptidases known as sentrin-specific proteases, or SENPs.

brain, liver, and kidney cells (13). This increase is thought to be protective, as siRNAs which inhibit global sumoylation are associated with increased apoptosis during hypoxic stress (14). Additionally, during renal ischemia, there is a strong increase in high-molecular-weight conjugates of SUMO which correlates with increased cell survival (15).

Genotoxic stress is also very dangerous to cells; DNA damage and misregulation of the repair pathways are often involved in the formation of cancer. BRCA1, the gene commonly found mutated in breast cancer, is a ubiquitin ligase that localizes to DNA damage sites. BRCA1 modification by SUMO is required for its proper function at DNA damage sites in response to genotoxic stress (12). SUMO and the E2 enzyme Ubc9 are also generally required for nuclear integrity. Lack of Ubc9 in mice causes embryonic lethality due to chromosomal defects and alterations to nuclear organization such as disassembled nucleoli, misshapen nuclei, and mislocalized RanGAP1 and Ran (16).

1.3 SUMO in Protein Misfolding Stress

Many human diseases are caused by misfolded proteins, including cystic fibrosis (17, 18), amyotrophic lateral sclerosis (ALS) (19), Parkinson's disease (20), and other neurodegenerative disorders (3). SUMO has been found to associate with many of the causative misfolded proteins. The most common mutation causing cystic fibrosis is a phenylalanine deletion at amino acid 508 in the cystic fibrosis transmembrane conductance receptor (CFTR) (17, 18). This mutant CFTR associates with heat shock protein 27, which interacts with Ubc9 to selectively sumoylate the misfolded protein leading to proteasomal degradation via the SUMO targeted ubiquitin ligase RNF4 (17,

18).

ALS is characterized by the progressive degeneration of motor neurons in both the spinal cord and the brain, and one feature of this disease is the aggregation of proteins that form inclusion bodies containing both ubiquitin and SUMO. Several proteins that are mutated in familial forms of ALS are targets of SUMO, and SUMO appears to be an important regulator for several proteins directly involved in the mechanisms underlying ALS (19). The hypoxic response is partially mediated by hypoxia inducible factor (HIF1), the sumoylation of which promotes its translocation to the nucleus, while the oxidative stress response involves desumoylation of another protein, p300, which then binds with HIF1 (19).

Misfolded proteins are also the cause of spinocerebellar ataxia, a polyQ disease, which involves a mutated and misfolded form of ataxin1 (3). The promyelocytic leukemia protein (PML) interacts with misfolded proteins in the nucleus and conjugates them with SUMO, which then attracts RNF4 and targets the proteins to proteasomal degradation (11). Deficiency in PML prevents sumoylation of ataxin1 and exacerbates pathological phenotypes in a mouse model of spinocerebellar ataxia (11). Overall, many human pathogenic diseases are caused by cellular stress, particularly protein misfolding stress, making the study of these cellular processes an important pursuit.

1.4 Rationale for Study

Much research has been done on the role of SUMO in protein misfolding stress, but there is a lack of information on the molecular mechanisms by which SUMO protects the cell from stress-induced damage. A large scale mutagenesis project has

been undertaken to investigate which residues on SUMO are required for its role in various cell stress responses (Newman H, unpublished) (Figure 2). These studies led to the identification of a mutant form of SUMO, containing lysine to alanine substitutions at residues 38 and 40, which sensitizes yeast to various stresses including protein misfolding. We investigated how mutations in these two residues affect the role of SUMO in the protein misfolding stress response using a high-copy suppressor screen. We hypothesized that the resulting suppressor proteins would reveal insights into the molecular mechanisms by which sumoylation protects cells from toxic misfolded proteins.

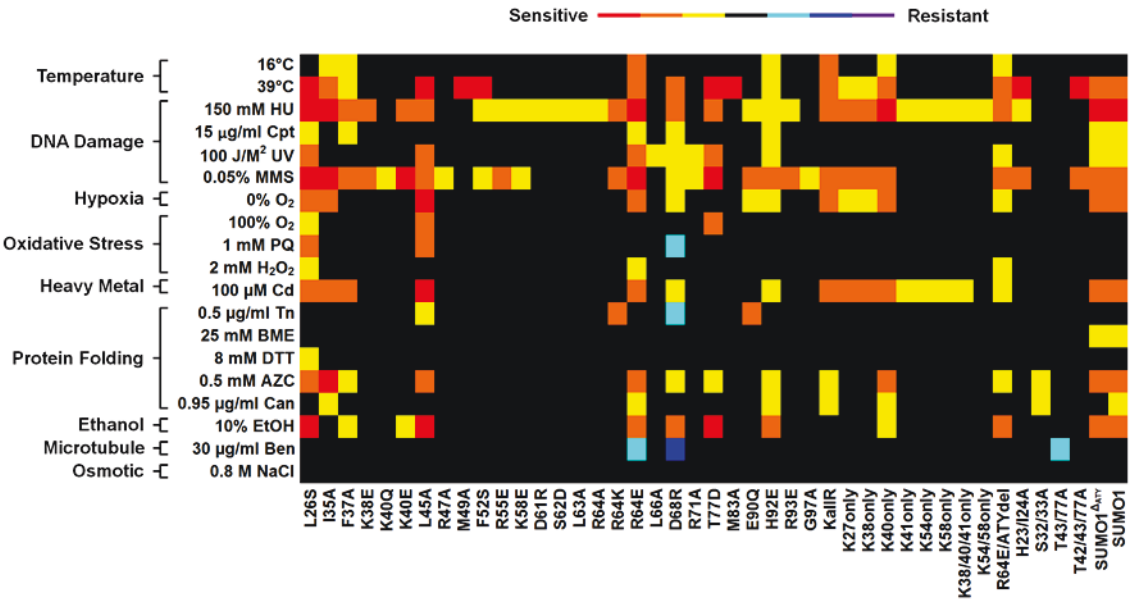


Figure 2: Mutagenesis Project. Heat map displaying sensitivity of various strains from mutagenesis project.

2. Materials and Methods

2.1 Methods

ATP Depletion and Recovery

Cells were grown overnight to saturation in the appropriate synthetic media at 30°C. The cells were diluted to OD_{600} 0.25 in synthetic media the following day and were allowed to grow to OD_{600} 0.8. One milliliter of cells was added to each tube and spun at 5,000 rpm for 2 minutes. The cells were then washed in 1 mL of fresh media, pelleted, then resuspended in 1 mL of fresh media. One sample was harvested and the pellet was frozen in liquid nitrogen as the starting sample. The remaining tubes of cells were washed with 1X PBS, harvested, then resuspended in 1 mL of ATP depletion solution (10 mM sodium azide, 10 mM 2-deoxyglucose in PBS). Cells were incubated in the ATP depletion solution at 30°C for 2, 4, or 8 minutes. Cells were then pelleted and frozen in liquid nitrogen. The remaining cells were washed once with 1 mL of 1X PBS, harvested, and resuspended in 1 mL of synthetic medium. Cells were allowed to recover at 30°C for 2 or 4 minutes. Following recovery, the cells were pelleted and frozen in liquid nitrogen.

Yeast Protein Extract

Cell pellets were resuspended in 200 µL of 20% TCA and 100 µL of acid-washed glass beads then vortexed for 8 minutes at 4°C. One milliliter of 5% TCA was added to each sample followed by incubation on ice for 10 minutes. One milliliter of each sample was transferred to a preweighed tube and centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and the tubes were reweighed to get the sample weight.

Pellets were resuspended in sample buffer (125 mM Tris-Cl ph 6.8, 4% SDS, 20% Glycerol, 10% beta-mercaptoethanol, 0.05% bromophenol blue, and 0.02 µL 2M Tris Base/µL) to a standardized concentration, then boiled for 5 minutes and centrifuged for 5 minutes at 13,000 rpm.

Western Blotting

Five microliters of each whole cell lysate was loaded onto a 12.5% SDS-PAGE gel. The gel was run at 200V for 40 minutes, and the protein was transferred onto a nitrocellulose membrane at 100V for 2 hours. The membrane was blocked with 5% milk with Tween overnight, then treated with primary antibody (αSUMO or αtubulin in 2% BSA in 1X PBS) for one hour. After 30 minutes of washing with Tween+ST (0.05% Tween in 50mM Tris-Cl pH7.6 and 140 mM NaCl), the membrane was treated with secondary antibody (αrabbit in 2% BSA in 1X PBS) for one hour. After washing, the membrane was treated with ECL from Amersham according to the manufacturer's protocol. Signal was detected through film exposure and development

Yeast Transformation

Yeast culture(s) of OD_{600} 0.4-0.8 were spun down in 1-2 mL aliquots at 5000rpm for 2 minutes. Cells were washed twice with 1 mL of 0.1 M LiOAc, then resuspended in 250 µL of 0.1 M LiOAc. A mixture of 620 µL 50% PEG, 90 µL 1M LiOAc and 10 µL ssDNA (40 µL for high efficiency transformations) was added to the cells along with the transformation DNA. Tubes were inverted gently to mix, then incubated at 30°C for 40 minutes, mixing by inversion every 10 minutes. 100 µL DMSO was added and cells were incubated at 42°C for 20 minutes, mixing every 10 minutes. Tubes were spun

down at 5000 rpm for 2 minutes and washed once with 5mM $CaCl_2$. Cells were resuspended in 150 μL $CaCl_2$ and plated on SC-Ura-Arg plates (SC-Ura-Arg+Can 1ug/ mL for initial screening).

Yeast Plasmid Miniprep

Plasmids were isolated using the Qiagen miniprep kit (catalog #27106). 2-5mL of cells were harvested by centrifugation at 2000 rpm and resuspended in 250 μL Buffer P1 containing 0.1 mg/mL RNase A. 50-100uL of acid washed glass beads were added to the cells and the sample was vortexed for 5 minutes in 4°C room. The supernatant was transferred to a fresh microcentrifuge tube. 250 μL lysis buffer was added and the tube was inverted gently 4-6 times to mix, then incubated at room temperature for 5 minutes. 350 μL of neutralization buffer was added and the tube was inverted immediately but gently 4-6 times. The tube was then centrifuged 10 minutes at 14,000 rpm. Lysate was transferred to a spin column, then centrifuged 1 minute at 14,000 rpm, discarding flow through. 750 mL wash buffer was added and centrifuged for 1 minute. Flow through was discarded and the tube was centrifuged for one additional minute. The spin column was placed in a microcentrifuge tube and 25 μL elution buffer was added. The tube was left to stand for 1 minute, then centrifuged 1 minute.

5FOA Assay

Putative suppressor colonies were patched onto plates containing 0.1% 5-fluoroorocetic acid and grown for two days. Remaining colonies no longer contained the library plasmids with the URA3 marker. Once the colonies no longer contained plasmids, they were again tested for resistance to canavanine. Colonies resistant to canavanine after

this test were discarded as their resistance did not come from overexpression of a yeast gene but rather a mutation in the genome.

E. coli Transformation

Competent DH5α cells were thawed in hand and placed on ice for 10 minutes. DNA was added to each tube and the tubes incubated on ice for 30 minutes. Tubes were heat shocked at 42°C for 1 minute, then placed back on ice for 2 minutes. 800 μL of 1X SOC (Super Optimal Broth with catabolite repression) was added and the tubes were shaken at 37°C for 1 hour. Cells were plated on LB plates containing 100 μg/mL of carbenicillin and grown at 37°C overnight.

E. coli Plasmid Miniprep

ThermoScientific GeneJET plasmid miniprep kit (Life Technologies, catalog #K0503) was used for all minipreps out of E. coli.

2.2 Materials

Dropout Mix: 0.5g Adenine, 2.0g Alanine, 2.0g Asparagine, 2.0g Aspartic acid, 2.0g Cysteine, 2.0g Glutamine, 2.0g Glutamic acid, 2.0g Glycine, 2.0g Histidine, 2.0g Inositol, 2.0g Isoleucine, 10.0g Leucine, 2.0g Lysine, 2.0g Methionine, 0.2g para-Aminobenzoic acid, 2.0g Phenylalanine, 2.0g Proline, 2.0g Serine, 2.0g Threonine, 2.0g Tryptophan, 2.0g Valine

Synthetic Complete Media: 2.0g Dropout Mix, 6.67g Yeast Nitrogen Base without Amino acids, 2% Glucose, 1L H2O; for plate media include 20g Agar per liter.

ATP Depletion Solution: 10 mM sodium azide, 10 mM 2-deoxyglucose in 1X PBS.

Plasmids: All transformation were done using the PJEF2626 vector, a high-copy yeast expression vector containing an *E. coli* replication site, carbenicillin resistance gene, and URA3 marker.

For the 2-hybrid assay, genes tested for interaction were cloned into plasmids containing a Gal4 promotor and either an activating domain (AD) or a binding domain (BD).

The yeast genomic library was obtained from Dr. Pamela Meluh.

Cloning work was carried out in the PGEMT-EZ vector according to manufacturer's protocol, followed by restriction enzyme digest and insertion into PJEF2626.

San1 Cloning Primer Pairs:

San1 Region	**Primer Pairs**
Promotor (P)	Forward: ATGCGGTACCAGGACACAATCTGAATCCTCCA Reverse: ATGCGTCGACAATGAACAAAAGCTGAGGTTACA
Promotor + N-terminus (PN)	Forward: ATGCGGTACCAGGACACAATCTGAATCCTCCA Reverse: ATGCGTCGACTTATAAGGGCCCTTCGGCCTT
Promotor + N-terminus +RING (PNR)	Forward: ATGCGGTACCAGGACACAATCTGAATCCTCCA Reverse: ATGCGTCGACTTAACAAAGGGGACAAGAATTTTCT
RING + C-terminus + Terminator (RCT)	Forward: ATGCGTCGACATGTGTAGTATATGTTATGACGAA-TATGA Reverse: ATGCGAGCTCTTACTTGCTGGAGACATGCA
C-terminus + Terminator (CT)	Forward: ATGCGTCGACATGAGACAAAA-GATCAGCGAATCTG Reverse: ATGCGAGCTCTTACTTGCTGGAGACATGCA
Terminator (T)	Forward: ATGCGTCGACACAATACCGCTTCTGAAGTATG Reverse: ATGCGAGCTCTTACTTGCTGGAGACATGCA

3. Results

3.1 SUMO K38/40A double mutant is sensitive to canavanine

To study the role of SUMO in stress response, a library of SUMO mutants was generated in which each mutant either had one residue mutated to alanine or multiple residue changes. The library was integrated into yeast and resulting strains were screened for sensitivity to various stress conditions (Newman H, unpublished). As this study is focused on protein folding stress, we screened the collection for strains sensitive to canavanine, an arginine analog that induces misfolding when incorporated into proteins. From this screening, we identified one mutant in which the lysines at positions 38 and 40 were mutated to alanine (K38/40A) which was particularly sensitive to growth in the presence of canavanine. The locations of K38 and K40 in SUMO are shown in Figure 3A. To demonstrate that the canavanine sensitivity of the K38/40A mutant was due to the mutations in SUMO, I transformed wildtype and mutant cells with a vector containing the yeast isoform of SUMO, *SMT3*, and performed a spotting assay on plates containing 1 μg/mL of canavanine. Cells were also transformed with an empty vector for a negative control (Figure 3B). The mutant strain transformed with the *SMT3* grew equally well as wildtype transformants, while the mutant transformed with an empty vector did not grow as well. This confirms that the deficiency in the K38/40A SUMO mutant strain is caused by defective SUMO and not some other factors.

3.2 Characterization of SUMO levels and conjugation in K38/40A mutant

Since previous studies have shown canavanine treatment to induce sumoylation (21), we next looked at the profile of sumoylation in both mutant and wildtype strains.

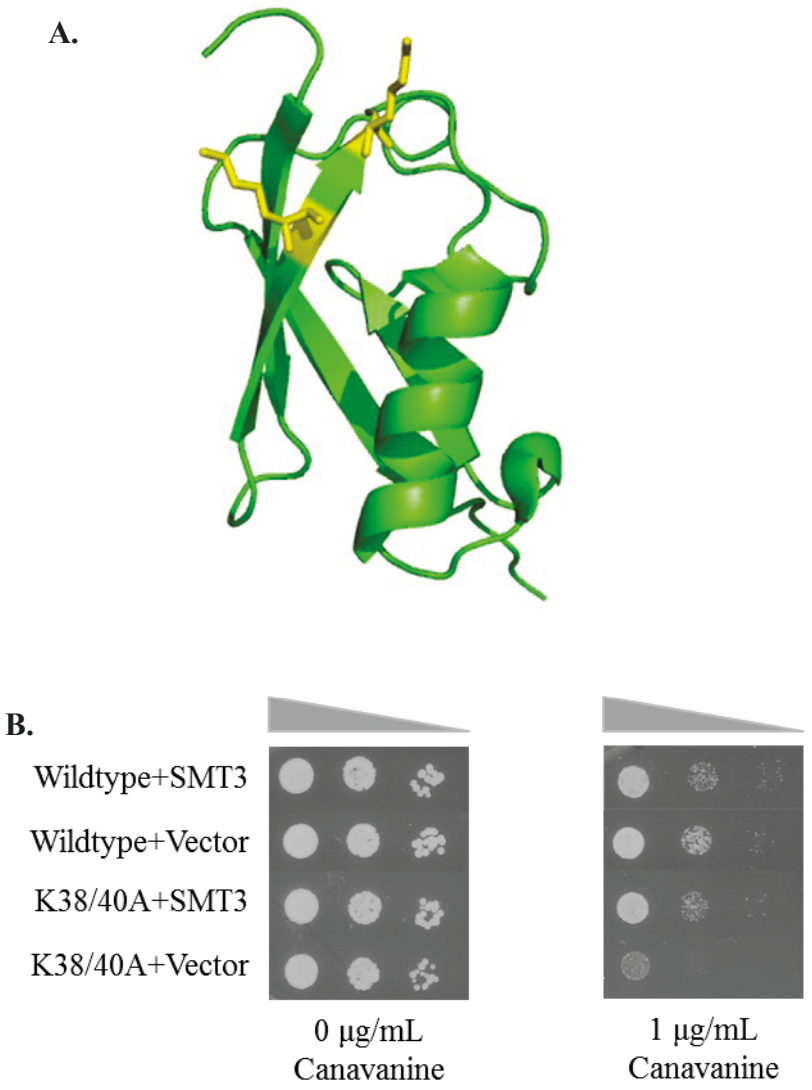


Figure 3. SUMO K38/40A Mutant Sensitivity to Canavanine. A. Structure of SUMO with mutated residues indicated in yellow. **B.** Wildtype and K38/40A strains were transformed with high-copy *SMT3* or empty vector and spotted onto canavanine plates.

Whole cell lysates of mutant and wildtype strains with and without canavanine treatment were probed for SUMO (Figure 4A). Overall, both strains showed an increase in high molecular weight conjugates under canavanine treatment. The mutant strain seemed to show a greater increase in high molecular weight conjugates, however there was not a corresponding decrease in free SUMO levels.

Since the sumoylation levels were not different between mutant and wildtype strains, we also wanted to characterize the conjugation and deconjugation efficiency of SUMO in mutant and wildtype strains. In the very dynamic sumoylation cycle, SUMO conjugation is ATP dependent but deconjugation is not, therefore we performed an ATP depletion and recovery assay. Mutant and wildtype cells grown to log phase in synthetic complete media were resuspended in ATP depletion media for 0, 2, 4, or 8 minutes. After an 8 minute depletion, cells were allowed to recover in synthetic complete media for 2 or 4 minutes. Cells were pelleted immediately after treatment and whole cell lysates were probed for SUMO protein levels (Figure 4B). SUMO conjugation levels decreased with similar kinetics in both mutant and wildtype strains. However, nearly complete recovery of sumoylation was observed in the wildtype strain within 2 minutes of ATP restoration, while the mutant had not fully recovered even after 4 minutes.

3.3 Interaction between K38/40A mutant and downstream SIM-containing proteins

The lower than normal levels of free SUMO observed in the K38/40A mutant strain, along with the apparently reduced conjugation efficiency could potentially explain the observed canavanine sensitivity of this strain. To test this hypothesis, we overexpressed the K38/40A on a high-copy expression vector in the mutant strain and

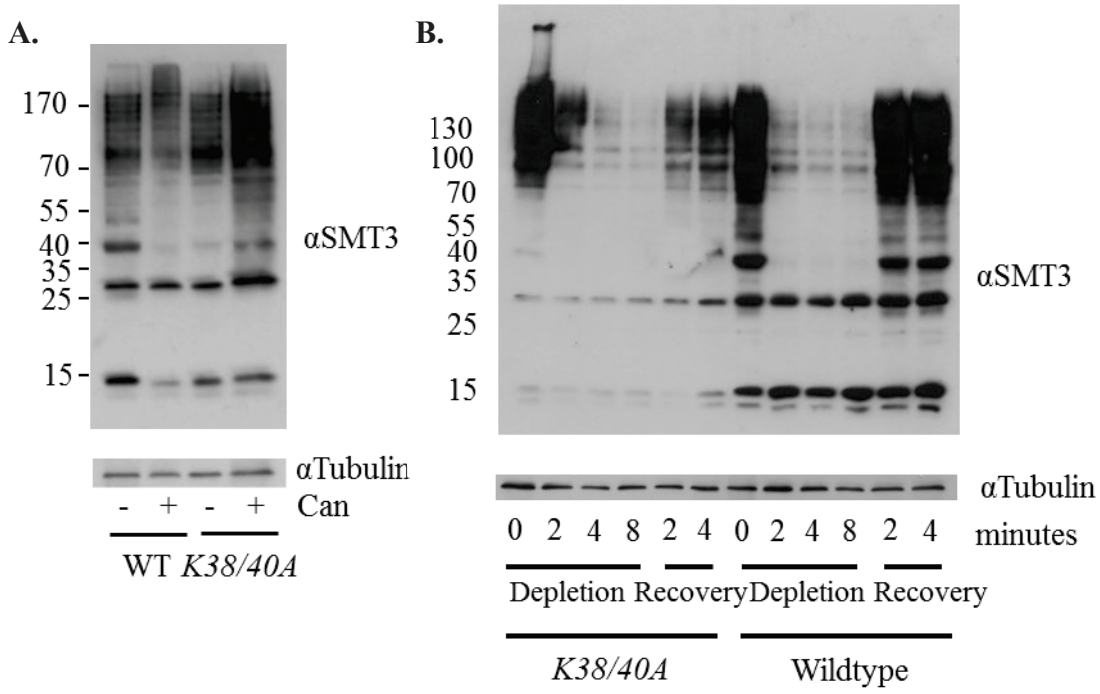


Figure 4: Sumoylation levels and ATP Depletion Assay. A. Wildtype and mutant strains were treated with 0 or 60 μg/mL of canavanine for 2 hours. Whole cell lysates were probed for SUMO, with tubulin as a loading control. **B.** Mutant and wildtype strains were depleted of ATP for 0, 2, 4, or 8 minutes. After 8 minutes of depletion, 2 or 4 minutes of recovery were allowed. Whole cell lysates were probed for SUMO, with tubulin as a loading control.

performed a spotting assay. The overexpression restored sumoylation levels to near wildtype, but did not suppress canavanine sensitivity (Lu J, unpublished), thus indicating that reduced SUMO expression and conjugation could not alone explain the phenotype.

K38 and K40 are positioned on a surface of SUMO involved in interactions with SIM-containing proteins (Figure 3A). We therefore hypothesized that the K38/40A

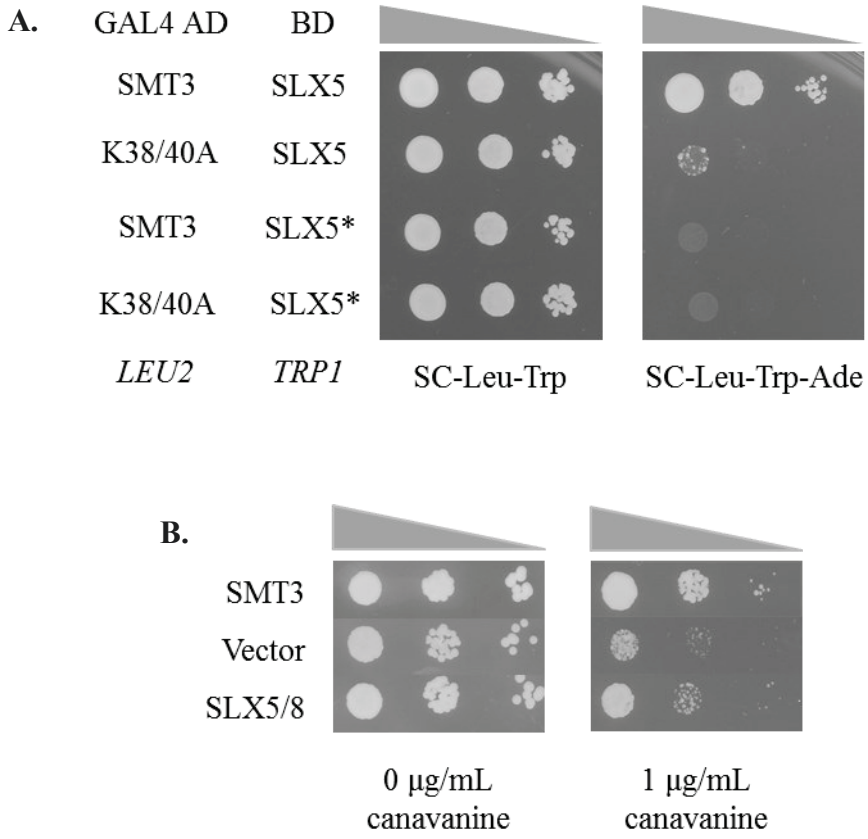


Figure 5: SLX5/8 Suppression and Binding Assay. A. A two-hybrid assay was performed with *SMT3* or K38/40A and *SLX5* or *SLX5* with a SIM mutation (*SLX5**). Strains were plated on SC-Leu-Trp as a control and SC-Leu-Trp-Ade to determine interaction. **B.** A high-copy plasmid containing *SLX5/8* was transformed into the K38/40A strain and tested on canavanine with *SMT3* as a positive control and an empty vector as a negative control.

mutant may be deficient in interactions with SIM-containing effector proteins functioning downstream of SUMO conjugation. To examine interactions of the K38/40A mutant with such proteins, we investigated the interactions of the mutant with Slx5/8, a SUMO-targeted ubiquitin E3 ligase (22, 23). Slx5/8 is a heterodimer in which Slx5 contains a tandem SIMs that bind tightly to polymeric SUMO and Slx8 contains a catalytic RING domain. A two-hybrid assay was performed with wildtype and K38/40A mutant SUMO and wildtype Slx5 or a SIM-defective Slx5 mutant (Slx5*). As expected, strains expressing Slx5* showed no interaction with either wildtype or mutant SUMO, highlighting the importance of the SIM for SUMO binding. When examining interactions with wildtype Slx5, we observed much weaker interactions with the K38/40A mutant compared to wildtype SUMO, indicating that the mutations do affect SIM binding as hypothesized (Figure 5A).

Because the K38/40A mutant SUMO has reduced affinity for Slx5, we hypothesized that overexpression of Slx5 may suppress the canavanine sensitivity of the K38/40A mutant strain. To test this, I expressed the *SLX5* and *SLX8* genes together on a high-copy vector in the mutant strain and performed a spotting assay on plates containing 1 μg/mL of canavanine. As hypothesized, the Slx5/8 complex suppressed sensitivity to canavanine (Figure 5B). Taken together, our findings demonstrate that the K38/40A SUMO mutant is deficient in binding to Slx5 and likely to other SIM-containing proteins. Moreover, our findings also indicate that the Slx5/8 SUMO-targeted E3 ligase is an important downstream effector of sumoylation and its role in protein misfolding. The *SLX5/8* overexpression assay also establishes the feasibility of

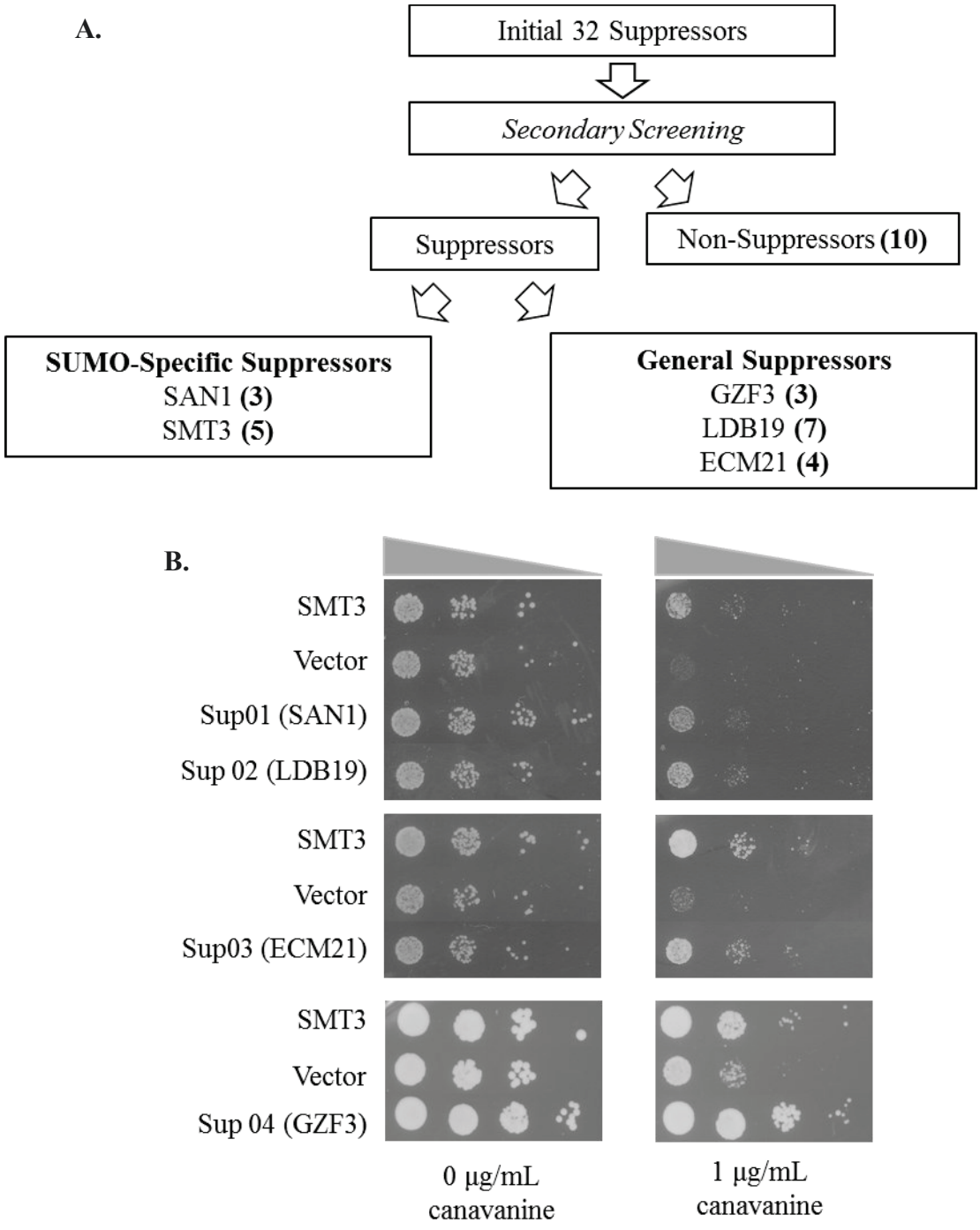
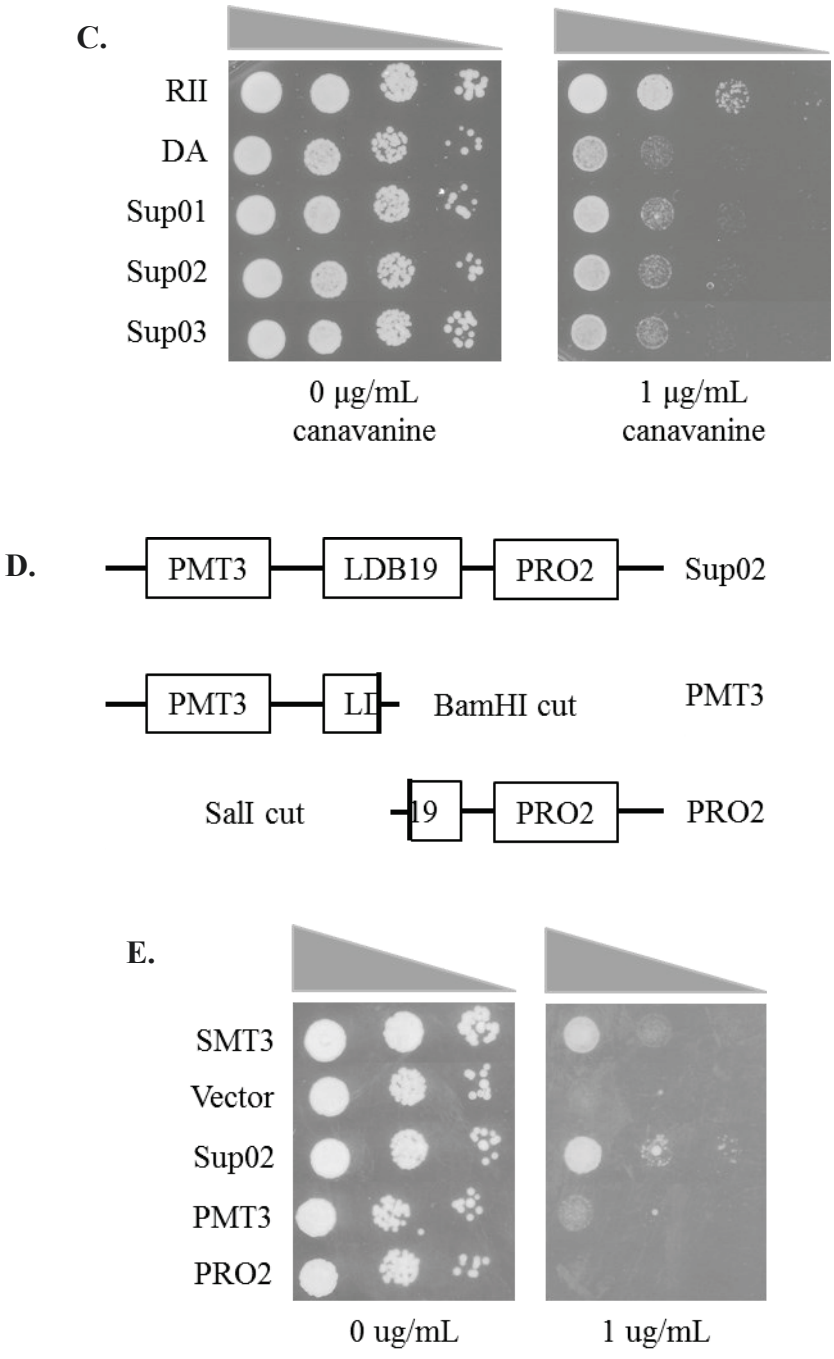


Figure 6: Experimental Design and Screening Experiments. A. After initial identification of suppressors, a secondary screening was performed. Suppressors falling into each category indicated with parentheses. Plasmids were retransformed to confirm suppression **(B)** and then treated with 5FOA and retested for canavanine resistance **(C).** Plasmids were sequenced and cutbacks were performed with restriction enzymes to determine what gene on the plasmid was suppressing **(D,E).**



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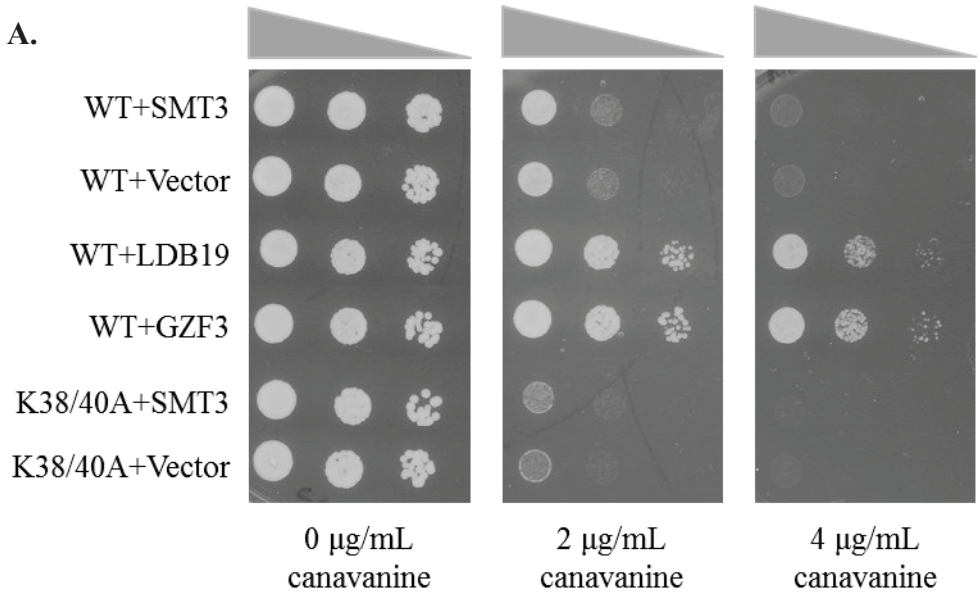
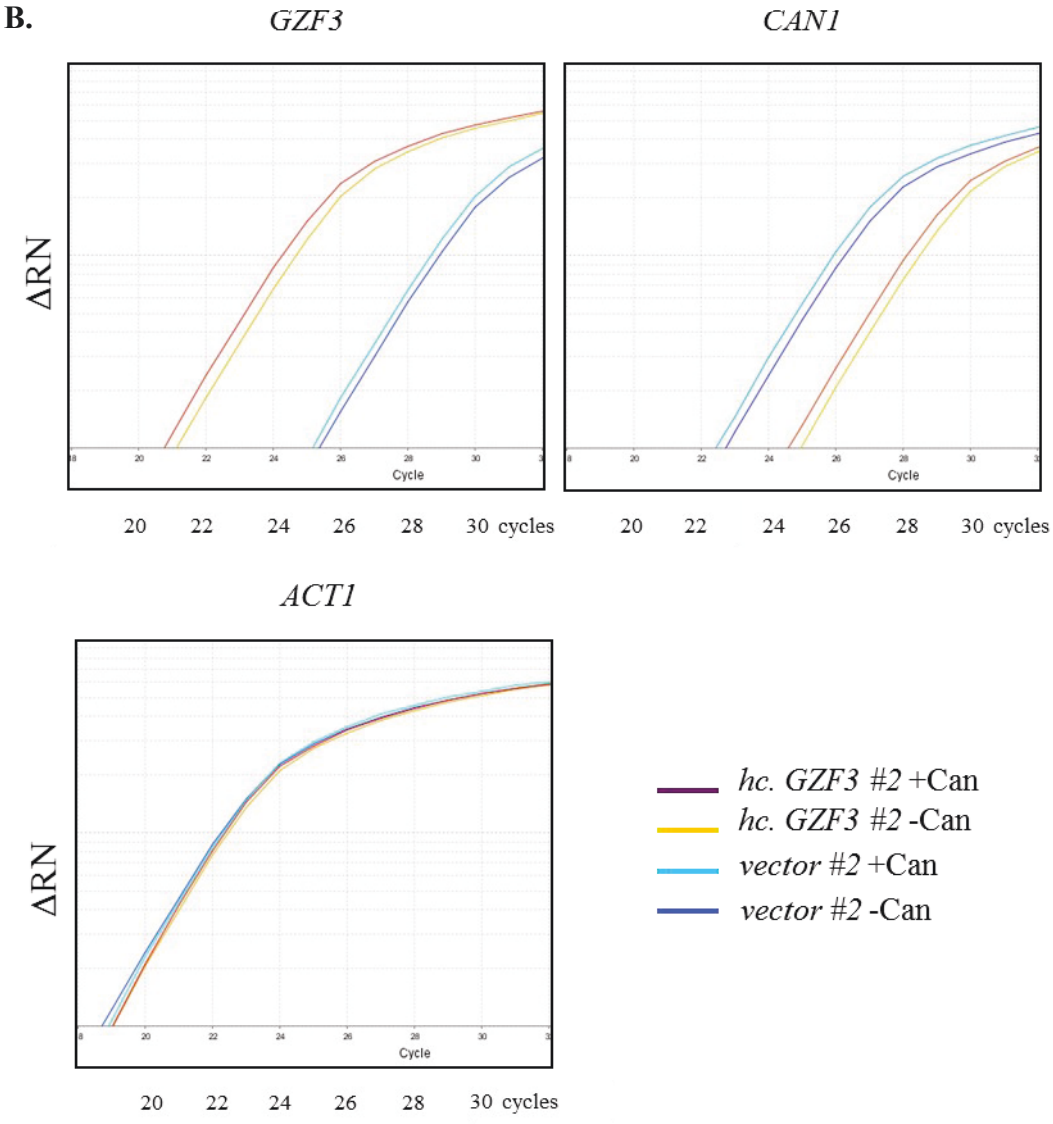
a high-copy suppressor screen to identify downstream targets of SUMO that are affected by the K38/40A mutation.

3.4 High copy suppression screen in K38/40A mutant

The ability to suppress the canavanine sensitivity of the K38/40A mutant strain with Slx5/8 overexpression suggested that an unbiased high copy suppressor screen could be used to find other downstream effectors of SUMO. To examine this, I transformed a library of yeast genomic DNA on a high-copy URA3 vector into the K38/40A mutant and grew the cells onto SC-Ura-Arg plates containing 1 μg/mL of canavanine. One control plate (SC-Ura-Arg without canavanine) was used to determine genome coverage for the transformation. I calculated that the genome was covered approximately 5 times based on the number of transformants on the control plate. From the canavanine plates I initially identified 32 colonies as suppressors based on colony size after two days of growth. Plasmids obtained from these suppressors were then subjected to secondary screening (Figure 6A), which included retransformation and growth on canavanine and a 5FOA sensitivity assay (Figure 6B and C). This screening removed 10 plasmids that did not show plasmid-specific suppression after being transformed back into the mutant strain and tested for suppression after 5FOA-induced loss of plasmid. The remaining 22 plasmids were sequenced and subjected to cutback experiments in order to determine the segments of the yeast genome responsible for

Figure 7 (Next Page): Suppression of Wildtype Sensitivity. (A). Two suppressors, *LDB19* and *GZF3*, were transformed into the wildtype strain to test general suppression of canavanine sensitivity. **(B).** *GZF3* was transformed into the WT strain and RT-PCR was used to determine mRNA levels of *CAN1* in the presence and absence of canavanine.


A.
WT+SMT3
WT+Vector
WT+LDB19
WT+GZF3
K38/40A+SMT3
K38/40A+Vector
0 µg/mL canavanine
2 µg/mL canavanine
4 µg/mL canavanine


B.
GZF3
CAN1
ΔRN
Cycle
20 22 24 26 28 30 cycles
ACT1
hc. GZF3 #2 +Can
hc. GZF3 #2 -Can
vector #2 +Can
vector #2 -Can

conferring suppression to the K38/40A mutant. As an example, Figure 6D shows one suppressor (Sup02) that contained three genes: *PMT3*, *LDB19*, and *PRO2*. I transformed cutback plasmids as well as the original suppressor into the mutant strain and performed a spotting assay on plates containing 1 μg/mL of canavanine (Figure 6E). Neither cutback suppressed K38/40A sensitivity, indicating that *LDB19* represents the suppressor gene. This approach was repeated and of the 22 suppressor plasmids, seven were found to suppress through *LDB19*, three through *GZF3*, four through *ECM21*, three through *SAN1* and five through *SMT3*.

3.5 SUMO-independent suppression of canavanine sensitivity

Ldb19 and Ecm21 are regulators of endocytosis (24) while *GZF3* is a transcriptional regulator (25), and all three of these genes are involved in the regulation or turnover of the canavanine transporter Can1. We therefore hypothesized that high copy expression of these genes could confer canavanine resistance by reducing levels of Can1 transporter at the plasma membrane. If true, these genes would be predicted to suppress canavanine sensitivity in wildtype strain as well as the K38/40A SUMO mutant strain. To test this prediction, I transformed one of the endocytosis regulators, *LDB19*, and the transcriptional regulator, *GZF3*, into wildtype cells and performed a spotting assay on plates containing 2 and 4 μg/mL of canavanine (Figure 7A). Strains carrying high-copy *LDB19* or *GZF3* grew better than those carrying *SMT3* or empty vector on canavanine plates, which is consistent with the prediction that these genes are affecting Can1 independently of SUMO.

GZF3 is a transcription factor that negatively regulates expression of the *CAN1* gene (26). We performed an RT-PCR experiment in order to examine the effect of

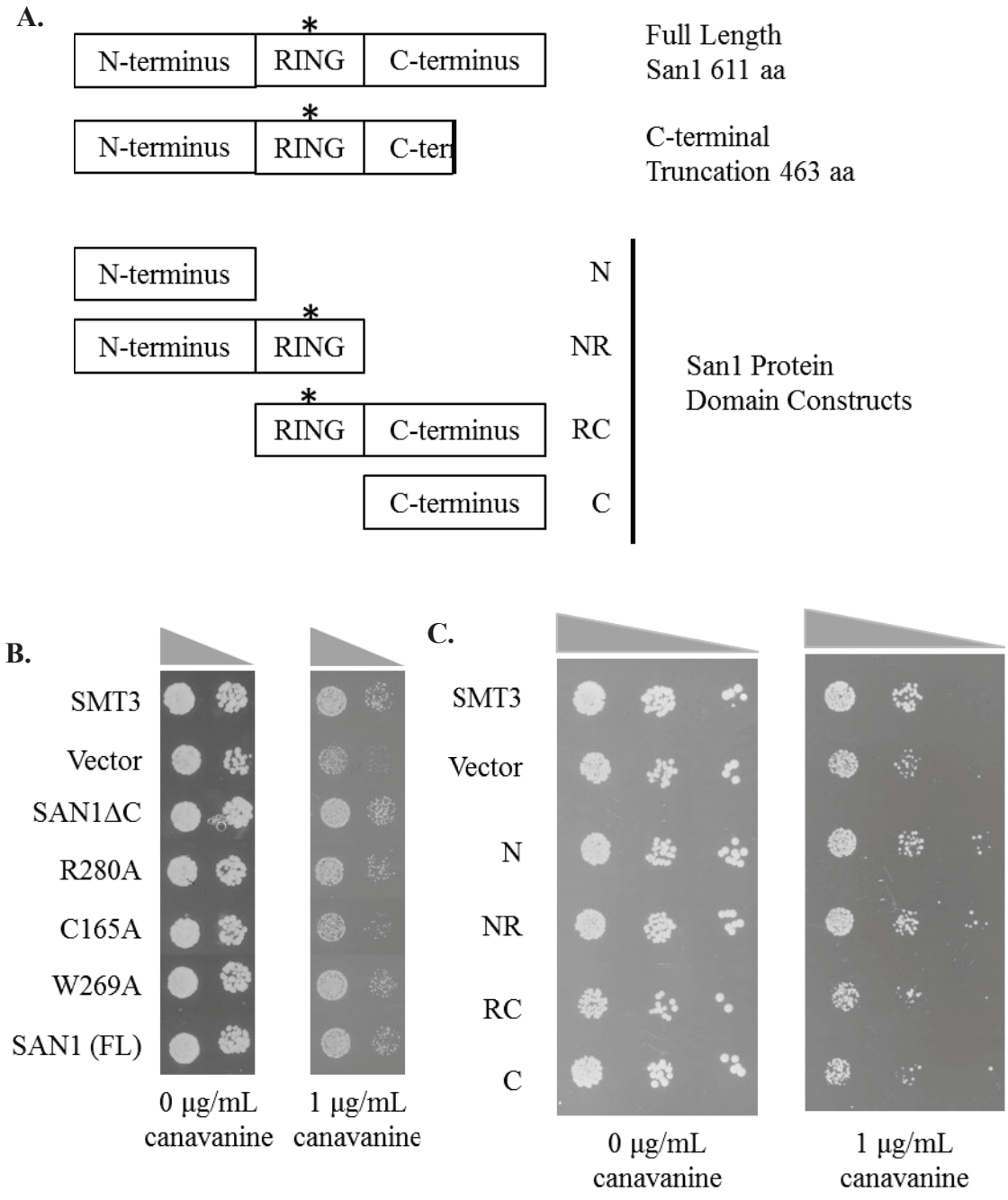


Figure 8: San1 Suppression. A. Diagram of *SAN1* protein domains, including unstructured N- and C-termini and a catalytic RING domain. * Denotes NLS. **B.** *SAN1* mutants were made in the catalytic RING domain and tested for their ability to suppress canavanine sensitivity. **C.** *SAN1* constructs were transformed into K38/40A strain and tested for suppression of canavanine sensitivity.

GZF3 overexpression on the expression levels of the *CAN1* in the presence or absence of canavanine (Figure 7B). As expected, strains transformed with a high-copy *GZF3* vector showed increased *GZF3* expression levels and decreased *CAN1* expression levels. Treatment with canavanine had no noticeable effect on the expression levels of either gene, and *ACT1* expression levels were constant across all strains. These results demonstrate that *GZF3* suppresses canavanine sensitivity in a SUMO-independent manner by affecting *CAN1* expression and canavanine import.

3.6 San1 N-terminus suppresses K38/40A sensitivity to canavanine

Having excluded *LDB19*, *ECM21*, and *GZF3* as general suppressors, we next turned our focus to *SAN1*, a gene that encodes a ubiquitin E3-ligase and whose suppressing activity was specific to our K38/40A mutant (data not shown). San1 is of particular interest because it has been implicated in protein quality control in the nucleus (27). San1 has three distinct domains, including a catalytic RING domain containing a nuclear localization signal (NLS) and unstructured N- and C-termini (27) (Figure 8A). While the catalytic domain facilitates ubiquitylation of protein substrates, the unstructured termini function to recognize and bind unfolded and unstructured regions of proteins. Of the three original *SAN1*-containing plasmids found in our suppressor screen, two contained the full-length *SAN1* gene while one contained a partial gene encoding for the N-terminal region, the RING domain, and only part of the C-terminal region (Figure 8A). Each of the three plasmids conferred approximately equivalent resistance to canavanine, which indicates that the C-terminus is not required for suppressor function.

To explore whether or not the RING E3 ligase activity was required for suppression, I transformed three catalytic mutants of *SAN1* (R280A, C165A, and W269A) into the mutant strain along with a C-terminal truncation mutant and a full-length version and performed a spotting assay on plates containing 1 μg/mL of canavanine (Figure 8B). All of the catalytic mutants and the C-terminal truncation mutant San1 grew equally well on the canavanine plates, showing that the ubiquitin E3 ligase activity of San1 is not required for suppressing canavanine sensitivity.

To further define the domains of *SAN1* that are responsible for suppressing the mutant sensitivity to canavanine, I generated four constructs with different combinations of the RING domain and the N- and C-termini (Figure 8A). I then transformed these four constructs into the K38/40A strain and performed a spotting assay on plates containing 1 μg/mL of canavanine (Figure 8C). While constructs lacking the N-terminus failed to suppress the mutant sensitivity, each of the constructs with the N-terminus conferred resistance to canavanine. This demonstrates that the N-terminus of San1 is both necessary and sufficient for the suppression of the K38/40A mutant sensitivity to canavanine.

4. Discussion

Protein quality control is a vital cellular process: misfolded proteins are the cause of many human diseases including cystic fibrosis (17, 18), Parkinson's disease (20), Alzheimer's disease, and other neurodegenerative diseases (3). The posttranslational modifier SUMO is known to be involved in protein quality control in the nucleus (28). It is thought that PML recognizes and attaches SUMO to misfolded proteins, which allows the proteins to be tagged with ubiquitin by RNF4 and marked for degradation (10, 29). In this capacity, RNF4 operates as a SUMO-targeted ubiquitin ligase (STUbL), a class of ubiquitin E3 ligases that recognize SUMO-tagged proteins that need to be degraded by the proteasome (30, 31).

Here we investigate a mutant form of SUMO with lysines at residues 38 and 40 changed to alanines. These mutations occur in the SIM-binding domain of SUMO and cause a particular sensitivity to drugs that induce protein misfolding (Figure 3). This sensitivity is influenced by reduced affinity of the mutant to the SUMO-interacting motif of substrates (Figure 4, 5). In a successful high-copy suppressor screen, we were able to identify four suppressors of canavanine sensitivity along with wildtype *SMT3*. As all four suppressor genes and *SMT3* were found a minimum of three times (Figure 7A), we appear to have saturated the suppressors that can be found from this genomic library.

The K38/40A mutant-specific sensitivity to canavanine can be suppressed by the overexpression of San1 (Figure 8), a ubiquitin E3 ligase that localizes to the nucleus and specifically targets aberrant proteins for degradation (27). While previous studies have shown that San1 requires its catalytic domain and NLS for proper function (27), our

results show that the N-terminus is necessary and sufficient for suppression of the SUMO K38/40A mutant sensitivity to canavanine (Figure 8C). Preliminary data from a two-hybrid assay suggests that full-length San1 does not interact with wildtype or mutant SUMO, which would rule out the idea that San1 sequesters SUMO or SUMO-modified proteins. Instead, we hypothesize that San1 is compensating for reduced levels of sumoylation on misfolded proteins and therefore decreased activity of STUbLs. Given that the N-terminus of San1 has no known NLS, this seems to indicate that it is operating in the cytoplasm when suppressing canavanine sensitivity. Neither SUMO nor San1 is known to affect protein quality control outside of the nucleus. If the N-terminus of San1 can be verified as localizing to the cytoplasm, this would open interesting research questions into the function of San1 and SUMO in protein quality control in the cytoplasm.

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6. Curriculum Vitae

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EDUCATION

Johns Hopkins University — Baltimore, MD

Master of Science in Molecular and Cellular Biology, *candidate* — May 2015

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Undergraduate GPA: 3.42

HONORS/AWARDS

Dean's List — Fall 2013

Provost Undergraduate Research Award ($2400) — Summer 2013

Beta Beta Beta Honor Society, Associate Member and Secretary — April 2013

RESEARCH EXPERIENCE

Research Assistant — August 2012-present

Johns Hopkins Bloomberg School of Public Health — Baltimore, MD
Matunis Lab, Department of Biochemistry and Molecular Biology

- Characterize defects of SUMO mutant strain of *S. cerevisiae* using Western blotting and immunofluorescence
- Perform high-copy suppressor screen to study effects of canavanine on SUMO mutant using transformation and plasmid preparation
- Design and perform own experiments, such as cloning and primer design and 2-hybrid assays, with approval from primary investigator

POSTER PRESENTATIONS

Carson CN, Lu J, Matunis MJ. Protecting Cells from Cytotoxic Stress: Characterizing the Roles of Sumoylation. Poster presented at Provost Undergraduate Research Award Poster Session, Johns Hopkins University, April 2014; Baltimore, MD.

TEACHING EXPERIENCE

Teaching Assistant, General Biology Course and Lab — August 2014-present

Johns Hopkins University — Baltimore, MD
Department of Biology

- Lead section of 20 undergraduate students
- Explain and supervise lab methods including PCR, electrophoresis, and dissections
- Grade weekly lab assignments and exams for the lecture course

SKILLS

Lab Techniques: plasmid transformations into *S. cerevisiae* and *E. coli*; plasmid preparation, cloning and primer design, immunofluorescence microscopy, western blotting, yeast 2-hybrid assay, yeast culture

Lab Equipment: immunofluorescent microscope, spectrophotometer, centrifuge, autoclave

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