A LYSINE DESERT PROTECTS SUMO-TARGETED Ub LIGASES FROM AUTO-UBIQUITINATION AND PROTEOLYSIS TO MAINTAIN GENOME STABILITY IN YEAST

by

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ABSTRACT OF THE DISSERTATION

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Post-translational modification by SUMO (Small Ubiquitin-like MOdifier) regulates the enzymatic activity, subcellular localization, and protein-protein interactions of modified target proteins. SUMO has also been implicated in proteasome-dependent protein degradation with the identification of a special class of enzymes termed, SUMO-Targeted Ubiquitin Ligases (STUbLs). These conserved enzymes typically possess multiple, tandem SUMO-Interaction Motifs and a RING domain that together facilitate the conjugation of ubiquitin to SUMO-modified target proteins. Originally discovered as essential gene pair for the viability of RecQ helicase-deficient yeast, *SLX5* and *SLX8* code for a heterodimeric STUbL. Two novel alleles of *SLX5* were isolated in a forward genetic screen for suppressors of genome instability. A combination of genetic and biochemical experiments indicate that these alleles disrupt a conserved lysine desert, unique to STUbLs, leading to Slx5's auto-ubiquitination and proteasome-mediated degradation. The results are consistent with the idea that STUbLs employ a lysine desert as a defense against self-destruction.

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DEDICATION

To my daughter Roshni, the reason of my happiness everyday.

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CHAPTER I

INTRODUCTION

DNA repair and SUMO

The cellular genome is constantly impacted both intrinsic and extrinsic damaging agents. To maintain and protect their genomic integrity, eukaryotic cells have evolved a number of sophisticated mechanisms –collectively called DNA Damage Response. This involves several repair pathways, often are regulated by a post-translational modification (PTM) of the pathway components. PTMs range from addition of small functional groups to conjugation of an entire polypeptide to the substrate undergoing modification. Of the latter, ubiquitination and SUMOylation are the most famous examples. Tasks for PTMs include recognition of the DNA damage to assemble repair factors and checkpoint regulation. To orchestrate a quick, effective reaction to damaging stimuli, a wide variety of PTMs such as phosphorylation (or other chemical modifications), ubiquitination and SUMOylation are generally engaged. However, unlike phosphorylation and ubiquitination where mechanistic roles in DNA repair are well documented, the role of SUMO-conjugation is only beginning to be understood(Cremona, Sarangi et al. 2012) (Hoege, Pfander et al. 2002, Pfander, Moldovan et al. 2005, Richly, Rape et al. 2005, Sacher, Pfander et al. 2006). Here, I will discuss basic principles of SUMOylation, the enzymes involved and the relationship between the SUMO and Ub pathways.

SUMOylation

The budding yeast *SMT3* is an essential gene (Meluh and Koshland 1995, Matunis, Wu et al. 1998, Giaever, Chu et al. 2002), and belongs to a family of ubiquitin-like (UBL) proteins consisting of SUMO-1 (Matunis, Wu et al. 1998), PIC1(Boddy, Howe et al. 1996) and sentrin (Okura, Gong et al. 1996). For the sake of uniformity, I will refer to yeast protein Smt3 as SUMO (Small Ubiquitin-like MOdifier). Like Ub, SUMO is synthesized as a 101 amino acid long precursor (Johnson and Blobel 1997), which must be processed (by cleaving off 3 residues) to reveal a C-terminal di-glycine motif required for attachment to a Lys residue in the substrate. The maturation of the precursor is accomplished by a specific SUMO-protease, Ulp1(Li and Hochstrasser 1999). The process of SUMO conjugation bears striking resemblance to ubiquitination, both involving three main steps: activation of the mature form, transfer of activated form to the conjugating enzyme and its final ligation to the substrate by the SUMO E3 ligases. Finally, SUMOylation is also a dynamic process (Fig. 1) that can be quickly reversed by the SUMO isopeptidases (Li and Hochstrasser 1999, Li and Hochstrasser 2000).

1. Activation: Aos1/Uba2

The first step in the SUMO cycle following generation of the mature form is ATP-dependent activation by a single heterodimeric E1 Aos1/Uba2 (Johnson, Schwienhorst et al. 1997). This involves formation of a thio-ester bond between the active site Cys177 of Uba2 and the C-terminal carboxyl group revealed in the mature

SUMO. Because deletions of *SMT3, AOS1* and *UBA2* are lethal, SUMO conjugation is vital for cell viability.

2. Conjugation: Ubc9

Out of the 13 E2 enzymes identified in yeast, only two are essential: *UBC3* and *UBC9*. The latter, first mistaken as a Ub conjugating enzyme (Seufert, Futcher et al. 1995), is in fact the only SUMO conjugating enzyme in yeast (Johnson and Blobel 1997). This is in sharp contrast with the Ub pathway that has multiple UBCs (Ubiquitin Conjugating enzymes). Although the final conjugation of SUMO to the substrates is normally mediated by another set of enzymes, E3 ligases, Ubc9 is sometimes sufficient to achieve the ligation in yeast as well as humans (Desterro, Thomson et al. 1997, Sampson, Wang et al. 2001).

3. Ligation: Siz1, Siz2 and Mms21

Saccharomyces cerevisiae possess three known SUMO E3 ligases, namely Siz1, Siz2 and Mms21. Out of these, Siz1 is responsible for most of the SUMO-conjugation in the cell (Johnson and Gupta 2001). These enzymes belong to Siz/PIAS (Protein Inhibitor of Activates STAT) family of proteins that contain a central SP-RING domain– a putative Zinc-binding RING-like domain, which is critical for ligase activity *in vivo* and *in vitro*. The N-terminal conserved region comprises of a PINIT domain needed for substrate binding in certain cases and a SAP (Scaffold attachment factor, Acinus, PIAS) domain that binds DNA. The individual roles of each of the structural features have not been fully characterized (Takahashi and

Kikuchi 2005). There seems to be an overlap between the function of Siz1 and Siz2, for example regulation of the endogenous 2-micron circle in budding yeast or SUMOylation of Flp1 (Chen, Reindle et al. 2005) and Top2 (Reindle, Belichenko et al. 2006). In a *siz1Δ siz2Δ* mutant, an unregulated, high copy number of the 2-micron circle confers a nibbled colony morphology. It still remains unclear as to how just a handful of E3-ligases can faithfully and selectively ligate SUMO to their substrates.

4. De-SUMOylation: Ulp1, Ulp2 and Wss1

As mentioned before, SUMOylation is a reversible modification. The two major and functionally unique SUMO proteases in yeast are encoded by *ULP1*, an essential gene (Li and Hochstrasser 1999) and *ULP2* (Li and Hochstrasser 2000). There exist six mammalian homologs namely: SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7 (Mukhopadhyay and Dasso 2007). Originally identified as a protease required for cell-cycle progression, Ulp1 processes the SUMO precursor to reveal its di-glycine at the C-terminus. As *ulp1Δ* mutants were rescued by over-expression of only the mature form of SUMO, processing the SUMO precursor appears to be the essential aspect of Ulp1 activity. The Ulps share a conserved 200-residue fragment called the Ulp domain (UD). Cells expressing the Ulp1-UD fragment alone have no growth defect and an isolated Ulp domain has robust protease activity *in vitro*. However, the *in vivo* efficiency of this fragment is weaker than the full-length enzyme as evidenced by the accumulation of SUMO conjugates. This indicates that although the enzymatic activity resides exclusively in the Ulp domain, the N-terminal region is critical for its regulation.

The amino-terminus of Ulp1 features two domains that localize the enzyme at the nuclear envelope proteins Kap60/Kap95 and Kap121 (Li and Hochstrasser 2003, Panse, Kuster et al. 2003). Here, Ulp1 is tethered to the nuclear pore complex (NPC) through proteins such as Nup60, Mlp1/2 and the Nup84 nucleoporin complex (Zhao, Wu et al. 2004, Palancade, Liu et al. 2007, Goeres, Chan et al. 2011). Intriguingly, a Ulp1 variant lacking the N-terminal region can compensate for the loss of Ulp2 isopeptidase (Li and Hochstrasser 2003). Thus, Ulp1 is excluded from Ulp2 substrates through restricted subcellular localization by its N-terminal domain. The nuclear pore association is not limited to the yeast enzyme as two of the mammalian homologs (SENP1 and SENP2) are also known to localize to the NPCs (Hang and Dasso 2002). Additionally, *ulp1* mutants share the nibbled colony phenotype (Dobson, Pickett et al. 2005) of the conjugation mutants (Chen, Reindle et al. 2005), which indicates that *ulp1* phenotypes originate from an inadequate SUMO processing, not reduced de-SUMOylation.

Ulp2 was first identified as a SUMO specific protease owing to its weak sequence similarity to Ulp1(Li and Hochstrasser 2000). Ulp2 also contains a Ulp domain but does not participate in processing of the SUMO precursor. Rather than localizing to nuclear envelope, this protease is found spread in the nucleoplasm. Cells lacking *ULP2* are viable but grow slowly and show a severe sensitivity to high temperatures, hydroxyurea (HU) and MMS induced DNA damage. Additionally, *ulp2Δ* cells are also defective in sporulation, recovery from checkpoint arrest (Strunnikov, Aravind et al. 2001) and display abnormally high rates of chromosomes and plasmid loss (Li and

Hochstrasser 2000). One of the most striking phenotypes of the *ulp2Δ* mutation is the excessive accumulation of highly SUMO-modified species in the cell. These high molecular SUMO conjugates appear to contribute to the *ulp2Δ* sickness as limiting poly-SUMO chain formation relieves the strain's sensitivity to high temperature, HU, caffeine and dimethyl formamide (DMF) (Bylebyl, Belichenko et al. 2003). Apparently, the sickness of the *ulp2Δ* cells results from accumulation toxic uncleaved chains and not due to a depletion of free SUMO (Schwienhorst, Johnson et al. 2000). Together, these observations suggest that poly-SUMO conjugates are the primary substrates for Ulp2.

Poly-SUMO chains

The yeast SUMO and two (SUMO2, SUMO3) of the four mammalian homologs have been shown to form poly-SUMO chains, both *in vitro* and *in vivo* (Johnson and Gupta 2001, Tatham, Jaffray et al. 2001, Bencsath, Podgorski et al. 2002). A thorough study determined that out of a total of nine lysines, the yeast SUMO preferentially forms chains using one or more of the three N-terminal Lys residues, i.e. Lys11, Lys15 and Lys19 (Bylebyl, Belichenko et al. 2003). Each of these Lys residues is a part of short consensus sequences (ΨKxE, where Ψ is a hydrophobic residue and x is any residue) that are preferred sites for SUMO conjugation (Rui, Fan et al. 2002). This feature of SUMO pathway is distinct from Ub conjugation for which no such motifs have been identified thus far. However, in a wild type situation, poly-SUMO chain formation is dispensable and an *smt3-3KR* (K11, K15, K19 each substituted by an Arg) bearing yeast mutant has no discernable phenotype. An *smt3-allR* mutant, where all Lys residues have been mutated, exhibits slow growth (Bylebyl, Belichenko et al. 2003). Although functional significance of poly-Ub chains in proteasomal targeting is well characterized (Deveraux, Ustrell et al. 1994, Johnson 2002), the biological role of poly-SUMO chains has remained an enigma.

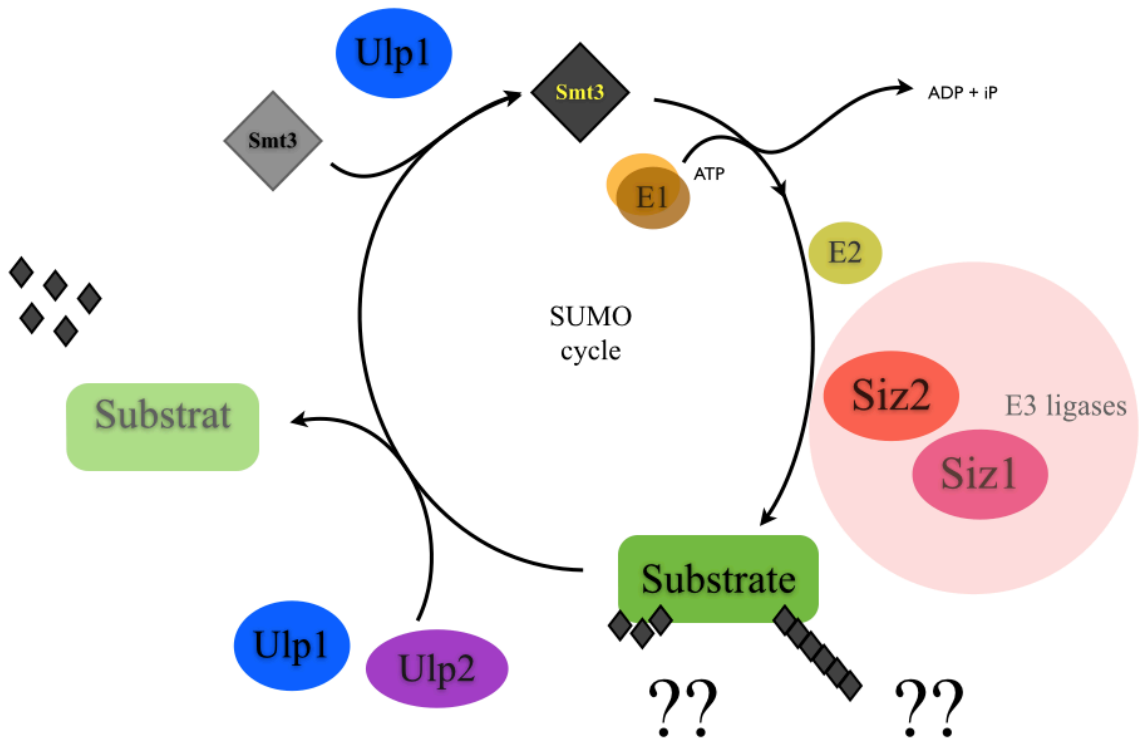


Fig. 1: Schematic representation of events in SUMO conjugation.

The mature form of Smt3, processed by Ulp1 from a precursor peptide is conjugated to the substrates via a cascade involving unique activating (E1) and conjugating enzymes (E2) and a set of ligases (E3) in an ATP-dependent manner. Modification by SUMO can be monomeric, multimeric or polymeric and is readily reversed by SUMO proteases, Ulp1 and Ulp2.

SUMO-Targeted Ub Ligases

Despite the resemblance between SUMO and Ub in terms of their protein sequences (18% identical), conjugation-deconjugation cascades and a propensity for chain formation, the functional consequences of each modification are unique. The targeting of substrates for proteasomal degradation via K-48 linked chains is known to be a Ub-modification phenomenon (Arnason and Ellison 1994). However the discovery of a novel class of Ub ligases elucidates the crosstalk between the SUMO and Ub pathways. SUMO-Targeted Ubiquitin Ligases (STUbLs) are specialized enzymes that conjugate Ub to a previously SUMO-modified substrate (Uzunova, Gottsche et al. 2007). Two distinct features characterize these enzymes: 1) multiple, tandem SUMO Interaction Motifs (SIMs) and 2) a RING domain that bears the Ub-conjugating activity. The SIMs are capable of binding more than one SUMO moieties concomitantly giving the enzymes their name (Prudden, Pebernard et al. 2007). In certain cases, these enzymes can also recognize proteins that contain SUMO-like domains (SLDs) but no SIMs (Xie, Rubenstein et al. 2010). The significance of these enzymes is apparent as they are functionally conserved from budding (Slx5-Slx8) and fission (Rfp-Slx8) yeast to humans (RNF4). One obvious difference among species is that the mammalian homologs form homodimers instead of heterodimers. Multiple reports have implicated STUbLs in maintenance of genomic integrity of the cell (Mullen, Kaliraman et al. 2001, Ii, Fung et al. 2007, Prudden, Pebernard et al. 2007, Galanty, Belotserkovskaya et al. 2012, Guzzo, Berndsen et al. 2012, Groocock, Nie et al. 2014). However, the understanding of their precise role and the

mechanism of action through which they preserve the genomic stability are poorly understood.

Saccharomyces cerevisiae Slx5-Slx8 complex

Slx5-Slx8 heterodimer was first identified as one of the three protein complexes required for viability in cells lacking the yeast RecQ helicase, Sgs1 (Mullen, Kaliraman et al. 2001). Slx5-Slx8 was directly implicated in suppression of gross chromosomal re-arrangements and unprompted DNA lesions as *slx5Δ* and *slx8Δ* strains showed high mutational rates, a mitotic delay, an activated checkpoint and sensitivity to only prolonged HU exposure (Zhang, Roberts et al. 2006). Slx5-Slx8 has been found to negatively regulate recombination highlighting its importance for stability of DNA repeats and extra-chromosomal DNA (Burgess, Rahman et al. 2007). Functionally, the heterodimer was first linked to the SUMO pathway as *slx5* and *slx8* mutants suppressed a *mot1* temperature sensitive allele, a transcriptional regulation mutant. Both *slx5Δ* and *slx8Δ* have genetic interactions with E1, E2 and two (Siz1, Siz2) E3 enzymes of the SUMO cycle (Wang, Jones et al. 2006). However, Slx5-Slx8 possesses Ub-ligase activity contingent on poly-SUMOylation of the substrate resulting in the latter's proteasome-dependent degradation (Uzunova, Gottsche et al. 2007, Xie, Kerscher et al. 2007, Mullen and Brill 2008). The partiality for substrates attached to poly-SUMO chains is easy to comprehend because of the several SIMs in the Slx5 subunit. Interestingly, *slx5Δ* and *slx8Δ* (Mullen unpublished data) mutations show synthetic lethality in combination with the *smt3-3KR* allele that has limited poly-SUMO chain formation capability

(Uzunova, Gottsche et al. 2007). This observation is counterintuitive because if the goal of Slx5-Slx8 is to lower SUMO conjugates, inhibiting poly-SUMOylation would be expected to have a positive effect on *slx5Δ* or *slx8Δ*. However, the synthetic lethality of *slx5Δ smt3-3KR* (or *slx8Δ smt3-3KR*) suggests that the Slx5-Slx8 activity is targeted toward the modified substrate and not the control of chain length. Consistent with the genetics, Slx5-Slx8 can only ligate Ub to one of the three N-terminal lysines in yeast SUMO (K11, K15 or K19). The Ub is added to the terminal SUMO in a polymeric chain that modifies the substrate (Mullen and Brill 2008)

Extensive biochemical studies (Yang, Mullen et al. 2006) have revealed that Slx5 and Slx8 co-purify when expressed in *E.coli*. The resulting complex is highly stable even under high salt conditions or in the presence of a detergent. Additionally, each subunit is capable of binding dsDNA, which is cooperatively enhanced in the complex. Both subunits possess one C-terminal RING domain (Ub ligase) that is each essential for *in vivo* function. The heterodimer achieves auto-ubiquitination with Slx5 serving as a better substrate *in vitro*. In the same study, the interaction between Slx5 and Slx8 was shown to be mediated by their RING domains and both are essential for the activity of the heterodimer (Ii, Fung et al. 2007). Since no authentic substrates of this complex have been identified, their essential function in an *sgs1Δ* strain can only be speculated to target one or more poly-SUMOylated proteins (especially because *sgs1Δ* cells do accumulate such high molecular weight conjugates). In addition to the RING domain, SUMO binding is also a vital aspect of the Slx5-Slx8 function in the *sgs1Δ* mutant (Mullen and Brill 2008). Collectively, the

current literature supports the model represented in Fig. 2. The present study is focused on Slx5 subunit of the complex discussed above.

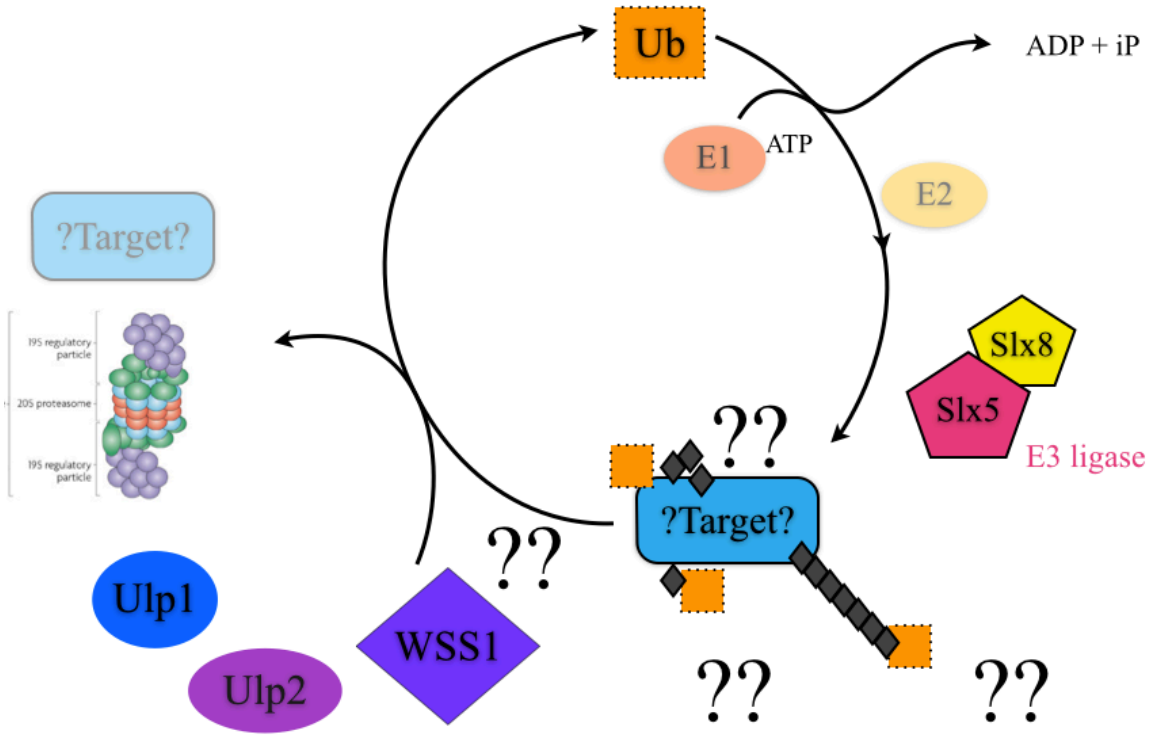


Fig. 2: Schematic representation of Ub-conjugation to SUMOylated substrates. STUbLs conjugate ubiquitin to previously SUMO-modified substrate via the Ub E1-E2 cascade and target the substrate to 26S proteasome.

Wss1: A Metalloprotease linked to SUMO pathway

Over-expression of *WSS1* is one of the few known ways to suppress *sgs1Δ slx5Δ* synthetic lethality (Mullen, Chen et al. 2010). *WSS1* was originally discovered as a high-copy- weak suppressor of *smt3-331* (a temperature sensitive allele of yeast SUMO or *SMT3*) (Biggins, Bhalla et al. 2001). Independently, *wss1Δ* mutants have no observable phenotype except sensitivity to continued exposure to UV light (O'Neill, Hanway et al. 2004), however such cells show a severe synthetic growth defect in combination with mutations, like *sgs1Δ* among others (Tong, Evangelista et al. 2001, Pan, Ye et al. 2006). Based on computational analysis, Wss1 was identified as the founding member of the WLM family of metalloproteases (Wss1-like Metalloproteases) (Iyer, Koonin et al. 2004). The characteristic WLM domain is typically identified by HEXsHX6H motif where s is a small amino acid. In addition to the SIMs at its C-terminus, Wss1 possesses two distinct features: the VIM (VCP-interaction motif) and SHP box-like motif for binding Cdc48 segregase (Stapf, Cartwright et al. 2011, Bergink, Ammon et al. 2013, Stingele, Schwarz et al. 2014). In addition to the structural elements of the encoded protein, *WSS1*'s genetic interactions with *SLX5, SLX8, ULP1* and *ULP2* also implicate it in both the UPS (ubiquitin proteasome system) and the SUMO metabolism. It was also proposed that Wss1 may indirectly facilitate target degradation owing to its physical association with the proteasome (Mullen, Chen et al. 2010). A recent study advanced this idea and demonstrated indiscriminate proteolysis of all (tested) DNA bound proteins by Wss1 (Stingele, Schwarz et al. 2014). This study also deemed the SIMs of Wss1

beneficial but not absolutely necessary for its cellular function. The most important finding of this study was that Wss1 is involved in processing of Top1-DNA lesions, identical to a result presented in a later section here. There are several indications that Wss1 and Slx5-Slx8 functions have some overlap, for instance deletion of each results in high rates of gross chromosomal rearrangements (Burgess, Rahman et al. 2007, Stingele, Schwarz et al. 2014), over-expression of either *SLX5* or *WSS1* rescues *ulp1-ts* slow growth (Mullen, Chen et al. 2010, Mullen, Das et al. 2011) as well as suppression of gross chromosomal rearrangements. The current study presents yet another link between *WSS1* and *SLX5* reiterating their compensatory roles with respect to each other.

Tyrosyl-DNA Phosphodiesterase 1

The cellular DNA must be compact for obvious reasons, however at the same time it should be accessible to carry out transcription and replication (Gartenberg and Wang 1992, Lowary and Widom 1997). Top1 alters the topology of supercoiled DNA as needed for either replicative or transcriptional activity (Brill, DiNardo et al. 1987). The mechanism by which Top1 achieves this task is relatively well understood, briefly: Top1 makes a transient break in the DNA backbone, permitting relaxation and resealing of the strand (Wang 1985). The cleavage is accompanied by the formation of a covalent bond between the active site Tyr in Top1 and the 3'-end of the sugar-phosphate backbone. In cases where the re-ligation step fails due to any reason, the persistent Top1-DNA covalent complexes can prove to be deadly because of stalled replication forks.

TDP1 was first discovered in *Saccharomyces cerevisiae* as the gene responsible for the Tyr-DNA phosphodiesterase activity present in the cell lysates (Pouliot, Yao et al. 1999). It was expected that Tdp1 should be involved in the repair of Top1-DNA adducts. Lately, the repertoire of Tdp1 substrates has expanded to include Top2-DNA adducts, both in yeast (Nitiss, Malik et al. 2006) as well as humans (Murai, Huang et al. 2012). However, due to the existence of redundant pathways (for example Rad1-10) to repair Top1-DNA covalent complexes, *tdp1Δ* mutants are largely aphenotypic (Pouliot, Robertson et al. 2001, Liu, Pouliot et al. 2002, Vance and Wilson 2002). In this report, I studied in detail the recently reported genetic interaction between *TDP1* and *WSS1* (Dixon, Fedyshyn et al. 2008).

CHAPTER II

Materials and Methods

Yeast strains:

Table 1 lists the strains used in this study. All strains are W303 derivatives, maintained on 1% yeast extract, 2% peptone and 2% dextrose medium, unless stated otherwise. The yeast gene deletion library used in this study was obtained from Open BioSystems, Inc. Strain construction, propagation and yeast transformation were carried out as per standard protocols described in (Adams 1997). Gene disruptions carried out using PCR were designed to replace entire Open Reading Frames (ORFs) and marked with antibiotic resistance.

In hydroxyurea sensitivity experiments, the drug was added to a final concentration of 0.1M in minimal medium lacking leucine. Plates were allowed to solidify and dry overnight before pinning the cell suspensions.

Yeast spot-dilution growth experiments were performed as follows: Each transformant was resuspended in sterile water, homogenized by vortexing to determine OD_{600}, resuspended at an OD=3, and diluted in 10-fold increments in a 96-well plate. Approximately 5 µl of cells were transferred by pinning onto the appropriate plates.

Plasmid constructions:

Phusion DNA polymerase was used to amplify genes to be sub-cloned into pRS400 series vectors (Sikorski and Hieter 1989). For the generation of point mutants, site-directed mutagenesis was performed using primer sets specific to the desired constructs and two-rounds of PCR.

Suppressor screen:

A 50 mL culture of yeast strain NJY3292 in YPD broth was used to spread 10^7 cells on synthetic complete plates supplemented with 5-FOA (5-Floroorotic Acid). Plates were incubated at 30˚C for 7 days before selecting the fastest growing colonies- potential suppressor mutants. Each colony was first tested for presence of p*WSS1/ADE3/URA3* by patching on YPD plates. Since the parent strain is *ade2 ade3,* cells growing as red colonies indicate the presence of the balancer plasmid. True suppressors were obtained after confirming the absence of *WSS1* by PCR using *WSS1* specific primers.

NJY3288 was mutagenized using ethyl MethaneSulfonate (EMS; Sigma, St, Louis) as follows: A confluent culture of the yeast strain grown in rich medium was harvested and resuspended in sodium phosphate (0.1M, pH=7.0) to get 1 mL suspension with 2 x 10^9 cells. Following addition of 50 mL of EMS, cells were incubated at 30˚C while shaking gently. 100 mL suspensions was collected from the bulk after every 15 minutes-interval for an hour. Each aliquot was neutralized with 4 mL of 5% sodium thiosulfate solution (freshly prepared) and stored at 4˚C. A small portion from each of these samples was used to determine cell viability by plating on YPD medium. The 30 minute treatment of cells with EMS caused ~72% lethality and was used to select for sectoring colonies and/or fast growing colonies on 5-FOA containing synthetic complete solid medium (Boeke, Trueheart et al. 1987).

Cloning by plasmid complementation:

To clone suppressors containing recessive mutations, suppressed strains (*tdp1Δ wss1Δ supx*) were first transformed with *pWSS1/ADE3/URA3,* followed by transformation with a tiling library of *LEU2*-marked multi-copy plasmids. Transformants were spread on minimal medium plates containing 2 μg/mL adenine, but lacking leucine. When the suppressor mutation was complemented by one/more of the plasmids, transformants retained p*WSS1/ADE3/URA3* and formed red colonies (that were inviable on medium lacking leucine and containing 5-FOA. Such colonies were used to either recover the plasmid to be sequenced and to extract genomic DNA in order to identify the suppressor allele by PCR amplification and sequencing of candidate genes contained on the rescuing tiling plasmid.

Yeast cell extracts for anti-Smt3 and anti-Slx5 immunoblotting:

Cellular extracts from yeast cells were prepared under denaturing conditions. Cells equivalent to OD_{600}= 15.0 were harvested from exponentially growing cultures (in minimal media for plasmid retention) by centrifugation ay 6000g. Pellet was washed with cold distilled water, centrifuged and transferred to a microfuge tube. Using a 4 N freshly prepared, pre-chilled NaOH stock, lysis solution was prepared to have 1.85 N NaOH, 7.5% BME (b-mercaptoethanol), 5 mg/mL Leupeptin, 10 mg/mL PepstatinA and 20 mM Iodoacetamide. A volume of lysis solution twice the size of cell pellet (approximate) was used to uniformly lyse the cell pellet on ice for 10 minutes, followed by addition of an equal amount of 50% TCA (Tri-Chloroacetic Acid) solution fro another 10 minutes. Precipitated protein

was collected by high-speed centrifugation at 4˚C, followed by a large volume (2x lysis volume) wash with 90% acetone stored at -20˚C. Finally, precipitates were dried and resuspended in 200 mL of Solution A (0.5M Tris Base, 6% SDS) by gentle sonication. Finally, 200 mL of Solution B (25% Glycerol and 1.1M BME) was added to each sample. Before subjecting the samples to SDS-PAGE, they were heated at 95˚C for 5 minutes and centrifuged again to separate the insoluble fraction.
For immuno-blotting against Smt3, proteins were always transferred on the nitrocellulose in a wet chamber.

SUMOylation and Auto-ubiquitination Assay:

For *in vitro* SUMOylation assay the following reaction conditions were used: 30 nM Aos1/Uba2, 200nM Ubc9, 1μM SMT3 and1mM creatine phosphate, 0.1 mg/ml creatine phosphate kinase (ATP regeneration system) in the presence of 2mM ATP. Each assay was performed with 500ng Slx5/8 dimer for 20 minutes incubation at 30°C.

Ubiquitination assays in Figure 27 E used 20nM Ube1, 200nM Ubc4, 1μM Ub, 1mM creatine phosphate, 0.1mg/ml creatine phosphate kinase with 2mM ATP and 500ng Slx5/8 dimer. The reactions were incubated for 35 minutes at 30°C. For the assays in Fig 19, Slx5-Slx8 protein complexes (50 nM each) were used to carry out auto-ubiquitination in the presence of 5 nM hUbe1 (Boston Biochem.), 140 nM Ubc5, 2 mM ATP and 100 nM Ub (wt). Reactions were assembled on ice and transferred to 30˚C after the addition of Ube1 for 20 or 40 minutes. 3X Laemmli's buffer was added to stop the reactions, samples were heated to 95˚C for 5 minutes prior to loading on

protein gel. 10% polyacrylamide gels were used for most immuno-blots against Slx5, unless otherwise stated.

Ni-NTA affinity pull down

Denaturing extracts were prepared from the cultures (containing His-tagged proteins) as detailed above. However, the precipitates obtained after TCA-treatment were re-solubilized in Buffer G: a 25 mM sodium phosphate buffer (pH = 8.0) containing 6 M Gn-HCl (Guanidine Hydrochloride), 300 mM NaCl, 5% Glycerol, 0.01% NP-40 (Nonidet-P 40) and 1 mM DTT (freshly added). For instance, precipitates from 10 OD_{600}s worth of cells were re-solubilized in 2 mL of the Buffer G. This was done at room temperature for an hour on a 360˚ nutator. Solubilized proteins were separated away from the insoluble fraction by high-speed centrifugation. Samples of lysates were collected for analysis later. The pH of this soluble fraction was tested using the pH strip and adjusted to 8.0 by using 1 M Tris base, if required. Finally, this fraction was incubated with 40 mL of pre-equilibrated Ni-NTA agarose beads (Qiagen) as 50% slurry in Buffer G for 4-12 hours in the presence of 5 mM imidazole at 4˚C. Unbound fraction was removed by short (1 min), low-speed centrifugation. Beads were washed twice with re-solubilization buffer and finally with buffer containing 25mM Tris (pH=8.0), 8.0 M Urea, 150 mM NaCl, 5% glycerol, 1 mM DTT and 0.05% Tween.

Table 1: List of strains

JMY447	MATα *ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 mus81-10::KAN top1-8::LEU2*	
JMY3105	MAT**a** *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 can1-100 RAD5*	
JMY3107	MATα *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 can1-100 RAD5*	
JSY3238	MATa *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 tdp1Δ::HGR can1-100 RAD5*	This Study
MSY3249	MATa *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 wss1::NAT can1-100 RAD5*	This Study
NJY3250	MATa *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 can1-100 wss1-10::KAN::loxP tdp1::HGR ulp2-1::HIS3 RAD5*	This Study
NJY3288	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 can1-00 wss1Δ::NAT tdp1Δ::HGR RAD5* plus pNJ7478 (*WSS1/ADE3/URA3*)	This study
NJY3292	MATα *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-00 wss1Δ::NAT tdp1Δ::HGR RAD5* plus pNJ7478 (*WSS1/ADE3/URA3*)	This Study
PSY3296	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 can1-00 supz wss1Δ::NAT tdp1Δ::HGR RAD5* plus pNJ7478 (*WSS1/ADE3/URA3*)	This Study
PSY3297	MATα *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-00 supy wss1Δ::NAT tdp1Δ::HGR RAD5* plus pNJ7478 (*WSS1/ADE3/URA3*)	This Study
PSY3401	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 lys2 can1-00 CEN14::cit1Δ::KAN wss1Δ::NAT tdp1Δ::HGR RAD5* plus pNJ7478 (*WSS1/ADE3/URA3*)	This Study
PSY3402	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 lys2 can1-00 CEN13::KAN wss1Δ::NAT tdp1Δ::HGR RAD5* plus pNJ7478 (*WSS1/ADE3/URA3*)	This Study
PSY3434	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 can1-100 ulp1Δ-10::KAN wss1Δ::NAT* plus p7352 (*ULP1/ADE3/URA3*)	This Study
PSY3435	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 can1-100 ulp1Δ-10::KAN wss1Δ::NAT* plus pRL7636 (*ULP1/TRP1/CEN*) RAD5	This Study
PSY3457	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 can1-100 ulp1Δ-10::KAN wss1Δ::NAT* plus pRL7637 (Ulp1prom-*ulp1-ΔN346/TRP1/CEN*) RAD5	This Study
PSY3458	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 can1-100 ulp1Δ-10::KAN* plus pRL7637 (Ulp1prom-*ulp1-ΔN346/TRP1/CEN*) RAD5	This Study
PSY3473	MAT a *ade2-1 ade3 ura3-1or -52 his3-11,15 or -Δ200 trp1-1 leu2-3,112 lys2-801 siz1Δ::LEU2 siz2Δ::TRP1 wss1Δ::NAT tdp1Δ::HGR* plus pNJ7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3477	MAT a *ade2-1 ade3 ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-100 nth2Δ::KAN tdp1Δ::NAT wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study

PSY3478	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-100 irc22Δ::KAN wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3479	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-100 leu1Δ::KAN wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This study
PSY3480	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-100 irc22Δ::KAN wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3481	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-100 inp51Δ::KAN wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3482	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-100 sys1Δ::KAN wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3483	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-100 cit3Δ::KAN wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3484	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 lys2 can1-100 top1-8::LEU2 wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3485	MATa *ade2-1 ade3 ura3-1* or *-52 his3-11, 15 trp1-1 leu2-3, 112 lys2-801 siz1Δ::LEU2 siz2Δ::TRP1 wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3486	MATa *ade2-1 ura3-1* or *-52 his3-11, 15* or *-Δ200 trp1-1 leu2-3, 112 lys2-801 siz1Δ::LEU2 wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3487	MATa *ade2-1 ade3 ura3-1* or *-52 his3-11, 15* or *-Δ200 trp1-1 leu2-3, 112 lys2-801 siz2Δ::TRP1 wss1Δ::NAT*	This Study
PSY3488	MATa *ade2-1 ade3 ura3-1* or *-52 his3-11, 15 trp1-1 leu2-3, 112 LYS2 siz2Δ::TRP1 wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3489	MATa *ade2-1 ade3::hisG ura3-1* or *-52 his3-11, 15 trp1-1 leu2-3, 112 LYS2 can1-100 sup88-2 wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3491	MATa *ade2-1 ade3::hisG ura3-1* or *-52 his3-11, 15 TRP1 leu2-3, 112 LYS2 can1-100 sup92-h tdp1Δ::HGR wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This study
PSY3492	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 LEU2::pRS405 LYS2 can1-100 tdp1Δ::HGR wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3493	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 TRP1 leu2-3, 112 can1-100 sup88-3 tdp1Δ::HGR wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3494	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 can1-100 slx5Δ::TRP1 tdp1Δ::HGR wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3498	MAT a *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 LYS2 leu2-3, 112 can1-100 sup88-9*	This Study
PSY3499	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 can1-100 slx8Δ::KAN tdp1Δ::HGR wss1Δ::NAT*	This Study

PSY3500	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 LYS2 leu2-3, 112 can1-100 slx8Δ::KAN tdp1Δ::HGR wss1Δ::NAT* plus pRL7478 (*WSS1/ADE3/URA3*) RAD5	This Study
PSY3554	MATa *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 tdp1Δ::HGR can1-100* RAD5	This Study
PSY3620	MAT a *ade2-1 ADE3 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 tdp1Δ::HGR can1-100 TOP1-K65,91,92,600R*-His6-HA::*HIS3* RAD5	This Study
PSY3621	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 tdp1Δ::HGR wss1Δ::NAT can1-100 smt3-3KR(G98)* RAD5	This Study
PSY3623	MAT a *ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 LYS2 tdp1Δ::HGR wss1Δ::NAT smt3Δ::KAN can1-100* plus p7232 (GPD1prom-*SMT3/TRP1/CEN*) RAD5	This Study
PSY3684	MAT a *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3, 112 lys2 can1-100 smt3Δ::KAN tdp1Δ::HGR wss1Δ::NAT plus* p7232 (*SMT3/TRP1/CEN*) and p7478 (*WSS1/ADE3/URA3*)	This Study
PSY3704	MATa *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 LYS2 leu2-3,112 can1-100 siz1Δ::NAT::loxP RAD5*	This Study
PSY3710	MATa *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 can1-100 slx5Δ::TRP1 wss1Δ::NAT tdp1Δ::HGR* plus pNJ7478 (*WSS1/ADE3/URA3*) *RAD5*	This Study
PSY3723	MATα *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 LYS2 tdp1Δ::HGR wss1Δ::NAT uls1Δ:: KAN +pNJ7478 (WSS1/ADE3/URA3)*	This Study
PSY3730	MATa *ade2 ADE3 ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 tdp1Δ::HGR wss1Δ:: NAT can1-100 TOP1-K65,91,92,600R-His6-HA::HIS3 + pNJ7478 (WSS1/ADE3/URA3) RAD5*	This Study
PSY3731	MATα *ade2 ADE3 ura3-1 his3-11,15 lys2 leu2-3,112 TRP1 tdp1Δ::HGR can1-100 wss1Δ:: NAT TOP1-K65,91,92,600R-His6-HA::HIS3 + pNJ7478 (WSS1/ADE3/URA3)*	This Study
PSY3732	MATα ade2 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 lys2 tdp1Δ::HGR wss1Δ:: NAT can1-100 TOP1-K65,91,92,600R-His6-HA::HIS3 + pNJ7478 (WSS1/ADE3/URA3)	This Study
PSY3775	Mat α *ade2-1 ade3:: hisG ura3-1 his3-11,15 leu2-3,112 TRP1 LYS2 slx5-Q362K*	This Study
PSY3780	Mat *α ade2-1 ade3:: hisG ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 slx5-E350K*	This Study
PSY3867	MATa *ade2-1 ade3::hisG ura3-1* or *-52 his3-11, 15 leu2-3, 112 LYS2 can1-100 siz1Δ::NAT siz2Δ::KAN slx5Δ::HGR* plus p7494(*SLX5/ADE3/URA3*) RAD5	This Study
PSY3868	MATa *ade2-1 ade3::hisG ura3-1 or -52 his3-**Δ**200 or -11,15 leu2-3,112 trp1-1 LYS2 ulp2Δ::NAT slx5Δ::HGR RAD5*	This Study
PSY3884	MAT *α ade2-1? ade3::hisG? ura3-1 or -52 his3-**Δ**200 or -11,15 leu2-3,112 trp1-1 LYS2 slx5Δ::HGR RAD5*	This Study

CHAPTER III

The inviability of *tdp1Δ wss1Δ* mutants

and its rescue by SUMO mutants

Cells lacking *TDP1* and WSS1 have reduced viability

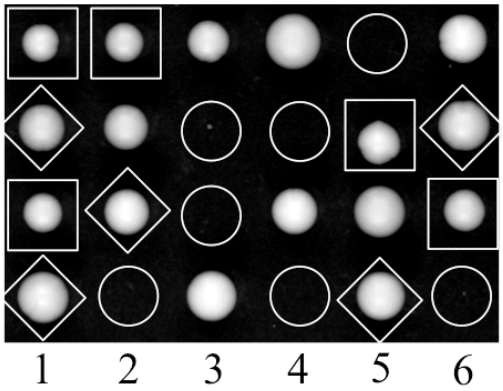
The role of SUMO modification in the maintenance of genomic integrity has emerged only recently. A high throughput study reported recently that cells carrying *tdp1Δ wss1Δ* mutation had a slow growth phenotype (Dixon, Fedyshyn et al. 2008). While Tdp1 function in repairing DNA-Top1 adducts is well studied (Liu, Pouliot et al. 2004), the biological role of Wss1 is under active investigation (Biggins, Bhalla et al. 2001, Liu, Pouliot et al. 2004, Stingele, Schwarz et al. 2014). In order to learn more about Wss1 and its role in the SUMO pathway, a cross between haploids carrying *tdp1Δ* and *wss1Δ* was dissected to obtain *tdp1Δ wss1Δ* cells. In our hands, the *tdp1Δ wss1Δ* mutant showed a synthetic lethal phenotype, where double mutant spores either failed to germinate (4 out of 10) or didn't proliferate past a few cell divisions (Fig. 3A).

To rule out germination defects, the diploid was dissected (Fig. 3B) in the presence of a single copy "balancer" plasmid expressing *WSS1* (p*WSS1/ADE3/URA3*). The balancer plasmid confers a red color, as both parental strains are *ade2 ade3*. Tetrad 4 lacked the plasmid and the genotype of 4d was inferred from its three siblings. All *tdp1Δ wss1Δ* mutant progeny contained the balancer plasmid, suggesting that the double mutant was synthetically lethal. One double mutant was transformed with single copy plasmids expressing *TDP1*, *WSS1* or no insert. The transformants were streaked on synthetic complete plates containing 5-FOA to select against the *URA3* marked balancer (Fig. 3C). None of the *tdp1Δ wss1Δ* cells were able to grow without either the *WSS1* or *TDP1* plasmids,

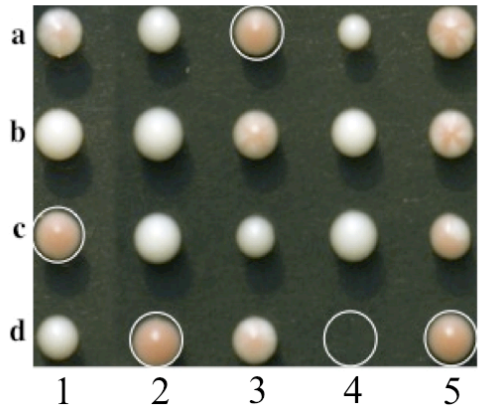
clearly showing that the double mutants are inviable, independently of any germination defects. Since synthetic lethality is more severe than the reported synthetic sickness (Dixon, Fedyshyn et al. 2008), the double mutants were constructed and tested in another genetic background, BY4247 (data not shown). The results were identical to those in the W303 background, allowing the conclusion that the *tdp1Δ wss1Δ* mutations are synthetically lethal.

A recent study identified Wss1 as a DNA-dependent protease (Stingele, Schwarz et al. 2014) containing a central DNA binding region, two Cdc48 binding motifs (VIM/SHP), a metalloprotease domain and a couple of SIMs at the C-terminal tail. *wss1* alleles lacking either the protease activity or Cdc48 binding motifs were found unable to complement the *tdp1Δ wss1Δ* synthetic growth defect. To observe the phenomenon first hand, I used same *wss1* variants that were subcloned into either single or multi-copy vectors. In my experiments, the *wss1-VIM/SHP* mutant (Cdc48 binding defective) showed same results when expressed from a CEN-based plasmid. However, when constitutively expressed from a multi-copy plasmid, this allele restored WT-like growth in the *tdp1Δ wss1Δ* strain (Fig. 3D). Hence, I concur that the inadequate complementation of *tdp1Δ wss1Δ* by *wss1-VIM/SHP* allele is indeed due to the lack of Cdc48 binding.

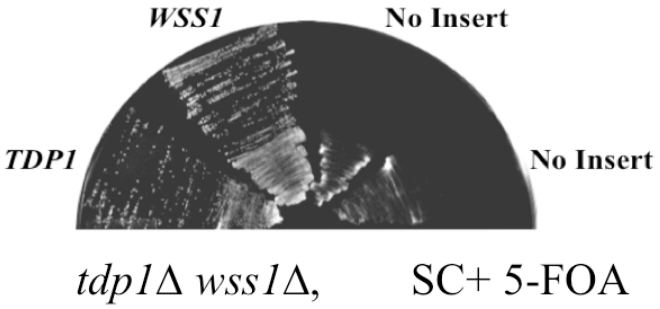
A. ***tdp1Δ* x *wss1Δ***



B. ***tdp1Δ* x *wss1Δ***



C.



D.

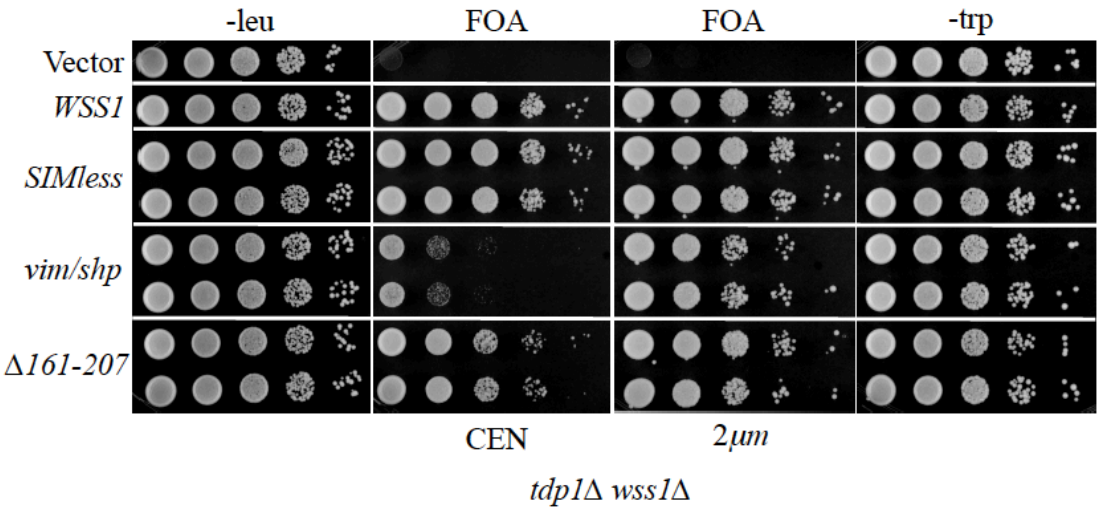


Fig. 3: *TDP1* and *WSS1* display a synthetic lethal interaction A. A cross between NJY3238 (*tdp1Δ*) and NJY3249 (*wss1Δ*) was sporulated and micro-dissected. Shown above are representative tetrads, numbered 1 through 6, photographed following 7 days of incubation at 30˚C on YPD. Squares depict *wss1Δ*, diamonds are *tdp1Δ*, and circles are *tdp1Δ wss1Δ*. Genotype of the double mutants was inferred from the siblings. **B.** Spores from the cross NJY3238 (*tdp1Δ*) x NJY3249 (*wss1Δ*) containing pNJ7478 (*WSS1/URA3/ADE3*) were micro-dissected and photographed after 3 days of growth on YPD at 30˚C. Cells bearing *tdp1Δ wss1Δ* mutation are circled. **C.** PSY3288 (*tdp1Δ wss1Δ* + p*WSS1/ADE3/URA3*) was transformed with single copy plasmids carrying *WSS1, TDP1* or no insert. Transformants were streaked on synthetic medium containing 5-FOA lacking leucine and photographed after 3 days of incubation at 30˚C. **D.** PSY3288 (*tdp1Δ wss1Δ* + p*WSS1*) was transformed with single and multi-copy vectors expressing either no insert or indicated *wss1* constructs. Transformants were pinned on selective plates as shown. Growth was recorded following 3 days of incubation at 30˚C.

Synthetic lethality of *tdp1Δ wss1Δ* is suppressed by perturbation of the SUMO pathway

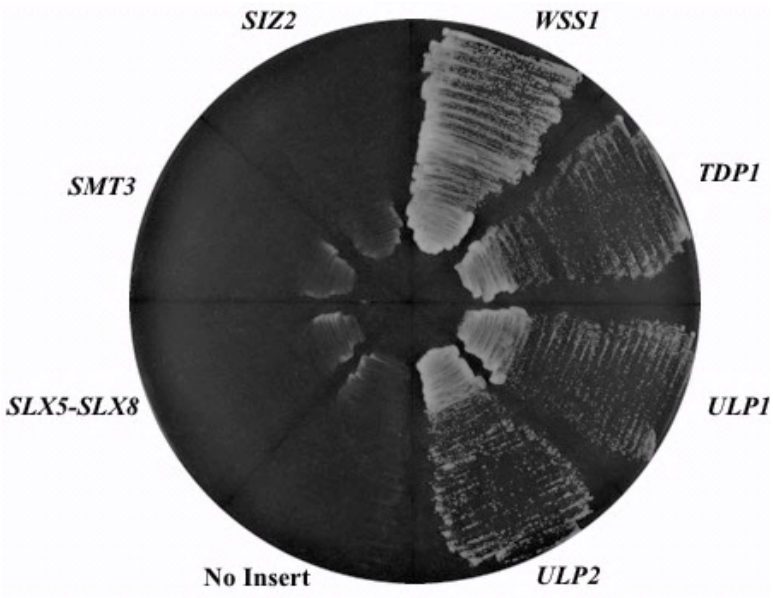
Genetic screening is a powerful tool that can help identify gene function(s) as well as the molecular details of cellular processes. I employed both reverse and forward genetic screens to obtain suppressors of the synthetic lethal phenotype of *tdp1Δ wss1Δ* cells.

High dosage complementation screen

WSS1 has been shown to genetically interact with many of the SUMO-pathway components. For instance, over-expression of *WSS1* suppresses the *smt3-331* ts allele (Biggins, Bhalla et al. 2001). High copy *WSS1* also suppresses the simultaneous loss of both the SUMO proteases, Ulp1 and Ulp2, and the synthetic lethality of the *sgs1Δ slx5Δ* (or *slx8Δ*) mutants (Mullen, Chen et al. 2010) . This suggests that these genes act in pathway(s) parallel to that involving *WSS1*. Therefore, I chose to over-express genes encoding SUMO-conjugation enzymes in a strain carrying *tdp1Δ wss1Δ.* For this purpose, I selected a double mutant that had always contained the balancer plasmid pNJ7478 (*WSS1/ADE3/URA3*) and transformed it with multi-copy plasmids carrying no insert or *ULP1, ULP2, SLX5*, *SLX8, SMT3,* and *SIZ2* (Results are summarized in Table 2). As positive controls, single copy plasmids carrying *WSS1* or *TDP1* were also transformed. In addition to testing their individual over-expression, *SLX5* and *SLX8* were concomitantly over-expressed in *tdp1Δ wss1Δ* using the same multi-copy vector. As seen in Fig. 4, only

ULP1 and *ULP2* over-expression rescued the *tdp1Δ wss1Δ* mutants. One of several ideas consistent with this result is that *WSS1* is normally involved in de-SUMOylation (directly or indirectly) of one or more substrates and that loss of WSS1 activity can be suppressed by elevating Ulp1 or Ulp2 levels in the cell.

SC + 5-FOA



All strains are *tdp1Δ wss1Δ*

Fig. 4: *ULP1* and *ULP2* over-expression suppresses *tdp1Δ wss1Δ* lethality. NJY3288 (*tdp1Δ wss1Δ +* p*WSS1/URA3*) was transformed with multi-copy plasmids constitutively expressing the indicated genes. The *WSS1* and *TDP1* plasmids are single-copy with endogenous promoters. Transformants were streaked on synthetic complete 5-FOA to select against p*WSS1/URA3* and photographed following 4 days of growth at 30˚C.

Table 2: Candidate genes over-expressed in *tdp1Δ wss1Δ* in search for high copy suppressors.

S.No.	Over-expressed gene	Growth on SC + 5-FOA
1	*ULP2*	+
2	*ULP1*	+
3	*SIZ2*	-
4	*SLX5*	-
5	*SLX8*	-
6	*SLX5-SLX8*	-
7	*SMT3*	-

Gene deletions that suppress *tdp1Δ wss1Δ* synthetic lethality

(a) *top1Δ*

Although very little is known about the biological significance of Wss1, Tdp1 has been shown to alleviate Top1-related DNA damage (Liu, Pouliot et al. 2004). Thus, the first candidate gene deletion was *top1Δ*. To obtain a triple mutant, a *top1Δ* strain was mated to a *tdp1Δ wss1Δ* strain carrying the plasmid *WSS1/ADE3/URA3* (pNJ7478). Diploids were selected and sporulated, followed by micro-dissection. As expected, triple mutants carrying the *tdp1Δ wss1Δ top1Δ* were viable (Fig. 5). In fact, elimination of *TOP1* restored wild type growth to the *tdp1Δ wss1Δ* cells. This result directly implicates Wss1 in the repair of the Top1-DNA covalent complexes. More importantly, this perhaps the first evidence of crosstalk between the Tdp1 and SUMO pathways.

(b) *siz2Δ*

SIZ2 and *SIZ1* are the two major yeast SUMO-E3 ligases. Together, they account for about 90% of the total SUMO conjugation in yeast. This suggests that excessive and/or inaccurate SUMOylation of one or more substrates could be causing the synthetic lethality of *tdp1Δ wss1Δ* mutants. I crossed *siz1Δ siz2Δ wss1Δ* triple mutant cells to *tdp1Δ wss1Δ* mutants and obtained *tdp1Δ wss1Δ siz1Δ* and *tdp1Δ wss1Δ siz2Δ* deletion strains. I found that only the *siz2Δ* triple mutant could rescue *tdp1Δ wss1Δ* lethality (Fig. 5). This suppression suggests that at least one substrate of Siz2 is toxic when SUMOylated in the absence of both Tdp1 and Wss1.

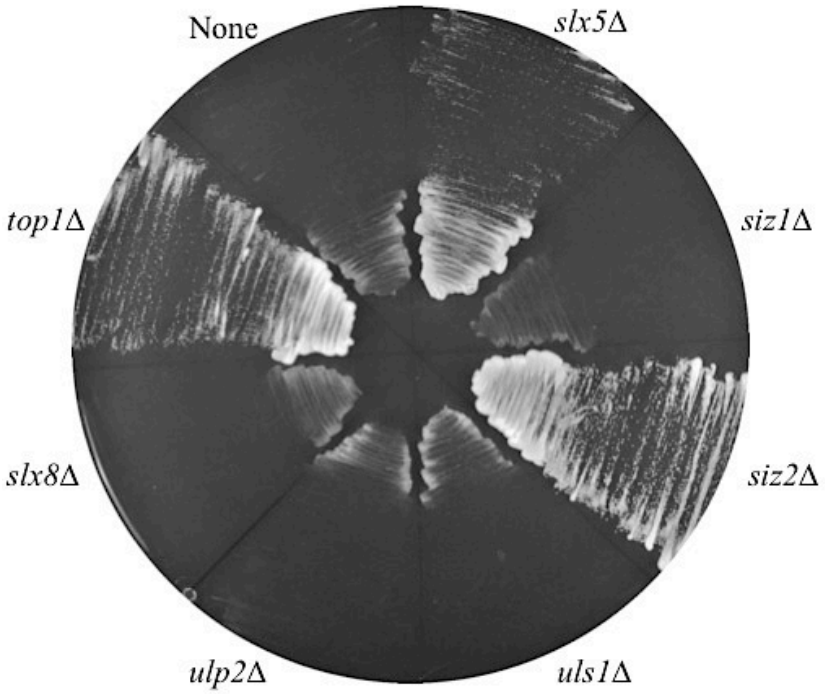
Interestingly, Siz2 responsible for a "wave" of SUMOylation of HR pathway proteins after DNA damage (Psakhye and Jentsch 2012). It seems likely that the toxic substrate(s) SUMOylated by Siz2 in a *tdp1Δ wss1Δ* mutant is involved in DNA repair.

(c) *slx5Δ*

While the over-expression of *WSS1* compensates for the absence of Slx5 in *sgs1Δ* cells (Mullen, Chen et al. 2010), on the contrary increasing Slx5 levels did not restore viability to the *tdp1Δ wss1Δ* mutant (Fig. 4). Owing to the established genetic interaction between *WSS1* and *SLX5*, I decided to test the effect of *slx5Δ* on the growth of *tdp1Δ wss1Δ* cells. Consequently, a strain carrying *slx5Δ* was crossed to cells bearing the *tdp1Δ wss1Δ* mutation in the presence of the balancer plasmid (p*WSS1/ADE3/URA3*). Surprisingly, deletion of *SLX5* allowed the *tdp1Δ wss1Δ* cells to survive in the absence of the balancer, though the growth of the triple mutant was very slow (Fig. 5). Knowing that the *siz2Δ tdp1Δ wss1Δ* strain is viable, the suppression of the double mutant by deleting *SLX5* appears counterintuitive. Since the *slx5Δ* and *slx8Δ* cells are known to accumulate mutations at a high rate (Burgess, Rahman et al. 2007), the poor suppression in *tdp1Δ wss1Δ slx5Δ* cells could be attributed to spontaneously arising weak suppressor mutations. However, a *tdp1Δ wss1Δ slx8Δ* mutant, similarly generated using a genetic cross between an *slx8* mutant and *tdp1Δ wss1Δ* kept alive by the balancer, was not suppressed. Until now, *slx5Δ* and *slx8Δ* have been believed to be phenotypically identical (Mullen, Kaliraman et al. 2001), the disparity of these mutants in a *tdp1Δ wss1Δ* genetic background is the first one observed to my knowledge. Since, most apparent

distinction between Slx5 and Slx8 is the longer N-terminal SIM containing region in the former, I proposed that the *tdp1Δwss1Δ slx5Δ* mutant is alive due to the absence of the SUMO-Slx5 interaction.

SC + 5-FOA



All strains are *tdp1Δ wss1Δ*

Fig. 5: *slx5Δ*, but not *slx8Δ*, can rescue *tdp1Δ wss1Δ*. Triple mutant progeny born with *pWSS1/ADE3/URA3* (7478) in crosses between NJY3287 (*tdp1Δ wss1Δ* +p7478) and NJY3253 (*tdp1Δ top1-8*)*, NJY3288 (*tdp1Δ wss1Δ* + p7478) and PSY3472 (*siz1Δ siz2Δ*), JMY2559 (*slx5Δ slx8Δ*) or JMY3024 (*uls1Δ*) were streaked on synthetic complete medium containing 5-FOA and photographed following 3 days of incubation at 30˚C. (* *top1-8* is a null allele)

Table 3: List of candidate genes tested in deletion screen. Triple mutants with *tdp1Δ wss1Δ* were streaked on 5-FOA synthetic complete medium. Triple mutants that germinated with the balancer plasmid (p*WSS1/ADE3/URA3*) were chosen to eliminate extraneous suppressor mutations that might arise during germination.

S.No.	Gene knocked out	Growth on SC + 5-FOA
1	*ulp2Δ*	-
2	*top1-8*	++
3	*siz1Δ*	-
4	*siz2Δ*	+
5	*slx5Δ*	+/-
6	*slx8Δ*	-
7	*uls1Δ*	-
8	*nup60Δ*	++

Structure –Function analyses of *SLX5*

To examine the mechanism of suppression by *slx5Δ*, I made systematic truncations of *SLX5* (Fig. 6) expressed from the endogenous promoter on single copy plasmids. A *tdp1Δ wss1Δ slx5Δ* strain with the *WSS1* balancer plasmid was transformed with the truncated plasmids. For each transformant, ten fold serial dilutions were pinned on minimal medium lacking leucine and on leucine deficient medium supplemented with 5-FOA to select against the balancer. Plates were incubated at 30˚C and photographed after 3 days (Fig. 7). Interestingly, deletion of the first 100 amino acids of Slx5 improved growth in the triple mutant and this suppression was strengthened by an additional 100 amino acid deletion. As indicated in Fig. 6, the N-terminal region of Slx5 contains four known SUMO-interaction motifs (SIMs), all residing within the first 200 residues (Uzunova, Gottsche et al. 2007, Mullen and Brill 2008, Perry, Tainer et al. 2008) The rescue of *tdp1Δ wss1Δ* by deletion of this region implies that the interaction of Slx5 with SUMO, presumably conjugated to one or more substrates, is intolerable in this genetic background. Disruption of this interaction by eliminating the SIMs relieves the synthetic lethality of *tdp1Δ wss1Δ* mutants. Consistent with the observation that *slx5Δ* and not *slx8Δ* suppresses the synthetic lethality, an enzymatically dead slx5-*ring*$^{-}$ does not suppress the *tdp1Δ wss1Δ* phenotype any more than the null. This indicates that eliminating ubiquitin ligase activity alone is not sufficient to improve the suppression of the triple mutant. Deleting the first 300 or 400 amino acids did not improve the suppression of synthetic lethality, which is likely because these constructs are essentially null mutations (Mullen and Brill 2008). I conclude that

although loss of Slx5 allows *tdp1Δ wss1Δ* double mutants to survive, eliminating the SIM's while retaining the ubiquitin ligase activity allows even better growth.

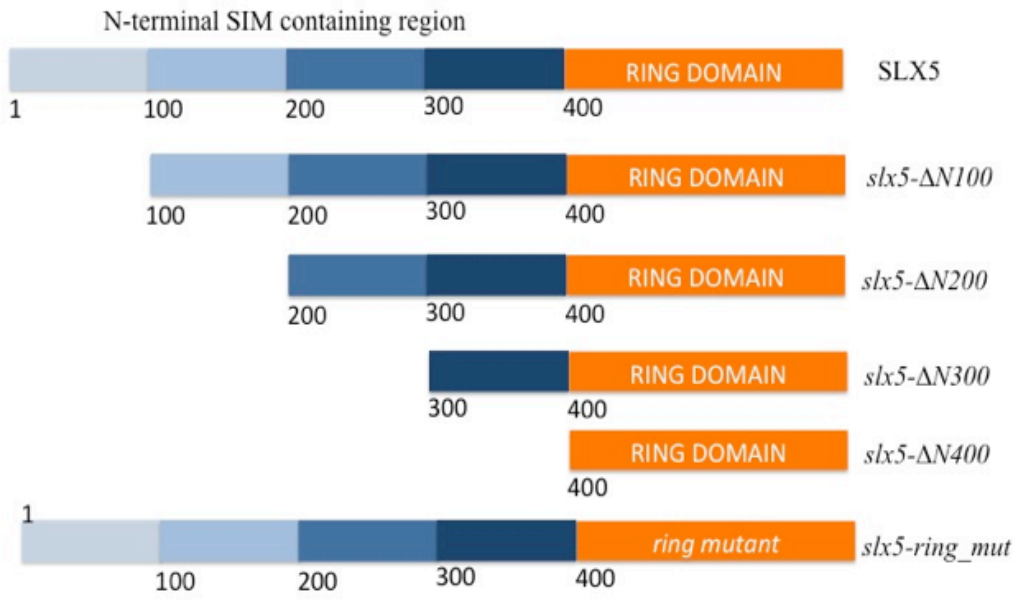


Fig. 6: Schematic representation of *SLX5* truncations. Diagrams of the *slx5* constructs used in the structure-function study are shown, highlighting the relative lengths of individual fragments tested.

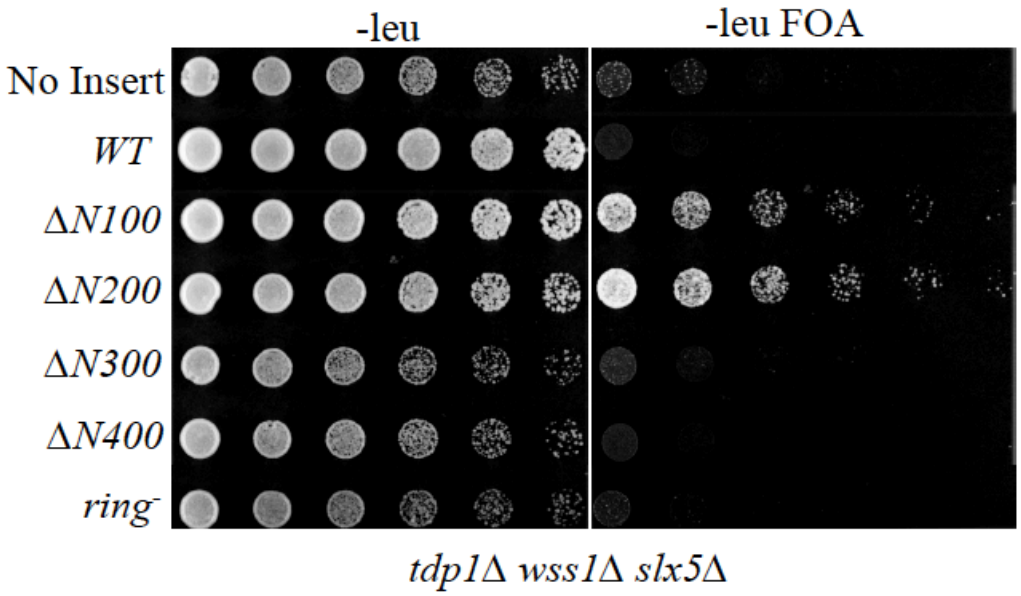


Fig. 7: Structure-Function analyses of *SLX5* in *tdp1Δ wss1Δ*. Transformants of PSY3494 (*tdp1Δ wss1Δ slx5Δ* + p*WSS1*) expressing the indicated genes were pinned on the indicated plates. Plates were photographed following 3 days of incubation at 30˚C (–leu for 2 days).

Over-expression of Slx5 is detrimental to *wss1Δ*

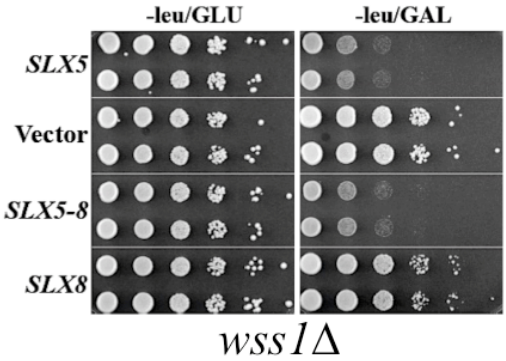
The data from the reverse genetic screen are consistent with the idea that the *tdp1Δ wss1Δ* cells suffer from accumulation of poly-SUMO conjugates or some other form of hyper-SUMOylation. This is because genetic mutation (*siz2Δ*, Fig. 5) expected to lower the abundance of such species reverse the synthetic lethal phenotype of the double mutant. As discussed before, Slx5-Slx8 enzyme prefers to ubiquitinate poly-SUMO modified substrates, signaling their proteasomal degradation (Uzunova, Gottsche et al. 2007, Mullen and Brill 2008). Thus, the failure of Slx5-Slx8 over-expression to restore viability to the *tdp1Δ wss1Δ* mutant was unexpected (Fig. 4). One explanation for this could be that the substrates causing lethality are not normal Slx5-Slx8 targets and are bound by SIMs of Slx5 erroneously. Evidence for such an event(s) was first reported in a study demonstrating the phenomenon of SUMO chain accumulation following over-expression of SIM-containing proteins in WT cells (Uzunova, Gottsche et al. 2007). The ability of N-terminal truncations to suppress *tdp1Δ wss1Δ* synthetic lethality suggests that the N-terminus in general, or the SIMs in particular, are toxic in either *tdp1Δ*, *wss1Δ*, or *tdp1Δ wss1Δ* cells.

One way to test the hypothesis that the N-terminus of Slx5 is toxic in these various backgrounds is to overexpress them in the relevant mutant backgrounds. Thus, to determine the effect of their over-expression in a *wss1Δ* mutant, multi-copy plasmids expressing *SLX5*, *SLX8* or *SLX5-SLX8* under the *GAL1-10* promoter were transformed into cells lacking the genomic copy of *WSS1*, but containing the

balancer plasmid p*WSS1/ADE3/URA3*. As shown in Fig. 8A, over-expression of *SLX5*, but not *SLX8*, is toxic to *wss1Δ* cells. The *GAL1-10* promoter is known to be induced in the presence of galactose. Thus, excess Slx5 protein appears to drive the sickness of the transformants bearing *SLX5* on galactose plates. In fact, the Slx5 SIMs appears to be specifically responsible for the sickness in *wss1Δ* cells because I could overexpress an Slx5-SIMless construct without a significant reduction in growth (Fig. 8B). The *slx5-SIMless* allele is a full-length allele in which all four SIM's are mutated by changing 3 or 4 amino acids in each SIM.

Our lab has also tested whether the sickness (or toxic phenotype) due to the overexpression of Slx5 SIMs is associated with poly-SUMO chain accumulation. To do this, whole cell extracts of *wss1Δ* over-expressing *SLX5*, *slx5-SIMless, slx5-ΔC219, slx5-ΔC126* and *slx5-ΔN400* were prepared. As shown in Fig. 8C, high molecular weight SUMO-conjugates were seen in all cultures in which the *slx5* alleles possessed normal SUMO-binding capability. Furthermore, it was established that the accumulation of such conjugates correlated perfectly with the various *slx5* alleles that reduced the growth of wss1Δ cells when overexpressed (Fig. 8C). These results are consistent with the idea that the N-terminal SIMs are likely to be toxic in various SUMO mutants, including *tdp1Δ wss1Δ*.

A.



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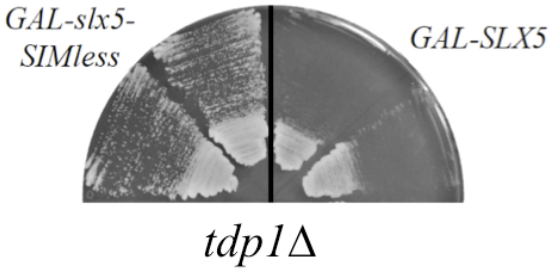
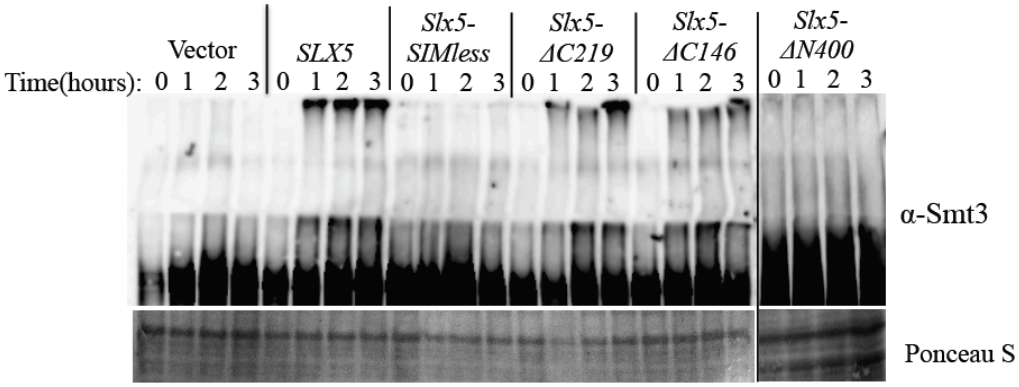
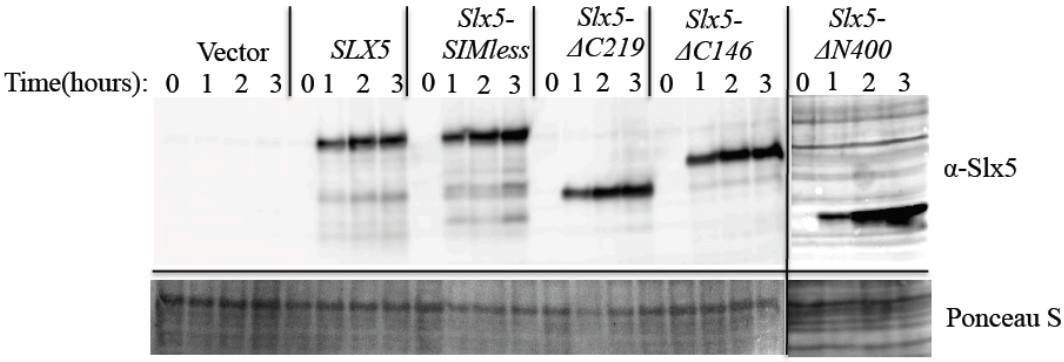


C.



D.



A, C, and D courtesy: Letzibeth Mendez-Rivera

Fig. 8 SUMO binding by Slx5 is deleterious to *wss1Δ* A. A *wss1Δ* (LMY3274) strain carrying pNJ7478 (*WSS1/ADE3/URA3*) was transformed with multi-copy plasmids containing indicated inserts under the control of *GAL1-10* inducible promoter. Transformants were pinned on selective plates as shown. Growth was recorded after 3 days of incubation at 30˚C. **B.** A *tdp1Δ* mutant (NJY3238) was transformed with multi-copy plasmids expressing either *SLX5* or *slx5-SIMless* under *GAL10* promoter. Transformants were streaked on galactose containing minimal medium lacking leucine. The plate was photographed after 2 days of growth at 30˚C **C.** Cultures of *wss1Δ* (LMY3274) transformed with indicated multi-copy plasmids were grown in minimal medium containing raffinose (non-repressing sugar) to log phase, followed by supplementation with galactose to induce gene expression. Denatured extracts were prepared at indicated time points. Lysates were subjected to SDS-PAGE and immunoblotted against Smt3. **D.** Samples from extracts in B were also immunoblotted against Slx5 at each time point. Membranes stained with Ponceau S are shown as loading controls.

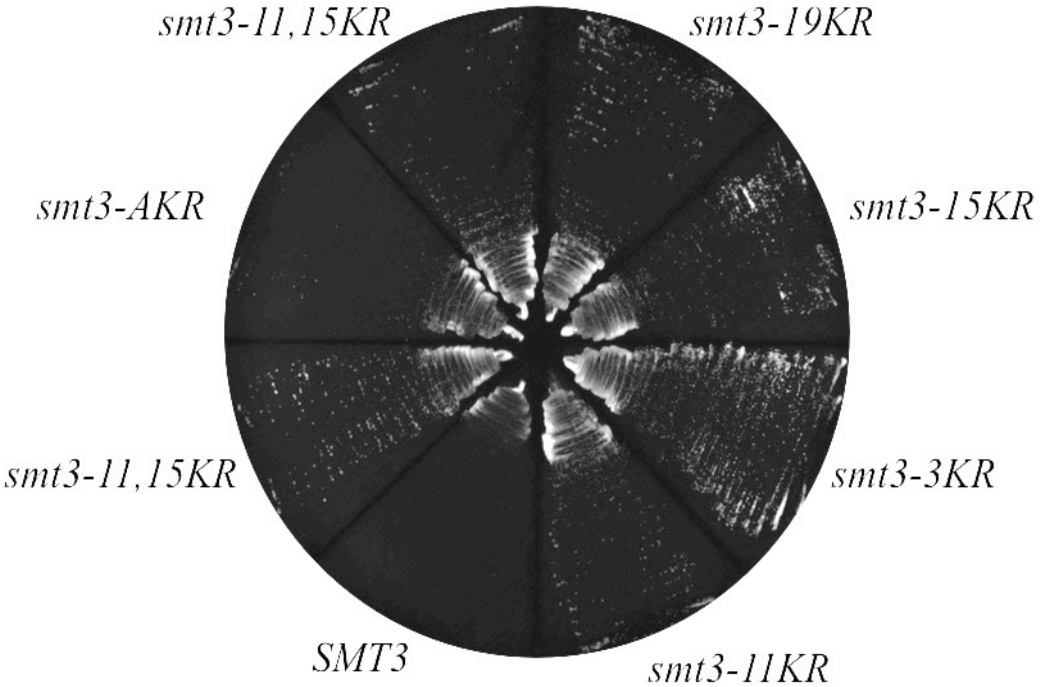
Poly-SUMO chain formation is deleterious to cells lacking both *TDP1* and *WSS1*

Knowing that Siz2-driven SUMOylation of certain substrate(s) is a potential cause of lethality in *tdp1Δ wss1Δ*, I wanted to know if the toxicity was due to poly-SUMOylation of this substrate(s). Smt3, the yeast SUMO can form polymeric chains *in vivo*, usually through one of its first three lysines, i.e. K11, K15 and K19 (Bylebyl, Belichenko et al. 2003). For this analysis, I used the *smt3-3KR* allele that encodes arginine residues instead of lysines at these three positions. Although the resulting protein can be normally conjugated to substrates, it forms aberrant poly-smt3-3KR chains with markedly reduced efficiency (Bylebyl, Belichenko et al. 2003, Mullen, Das et al. 2011). To determine the effect of disrupting natural poly-SUMO chain formation in the *tdp1Δ wss1Δ* mutant, I crossed an *smt3-3KR* strain to a *tdp1Δ wss1Δ* strain complemented by the p*WSS1* balancer. All triple mutants from the cross were viable and sectored, indicating that the balancer plasmid was dispensable (data not shown). This indicates that poly-SUMOylation presumably on one or more substrates is toxic for cells lacking Tdp1 and Wss1. Additionally, this modification could perhaps be achieved by Siz2.

Next, I needed to determine if these toxic poly-SUMO chains in the *tdp1Δ wss1Δ* strain derive from one of the three lysines missing in the *smt3-3KR* mutant. *SMT3* is an essential gene, so constructing a *tdp1Δ wss1Δ smt3Δ* strain required two balancer plasmids: p*SMT3/TRP1* and p*WSS1/ADE3/URA3*. An *smt3Δ* + p*SMT3/TRP1* strain was crossed to a *tdp1Δ wss1Δ* + p*WSS1/ADE3/URA3* strain, sporulated, dissected, and the triple mutant carrying both plasmids was found. The triple mutant was transformed with plasmids containing *smt3-11KR*, *smt3-15KR* and *smt3-19KR*

variants and combinations of lysine mutations (Fig. 9). All transformants were streaked on synthetic complete medium containing FAA to select against the p*SMT3*/*TRP1*, followed by streaking on plates containing 5-FOA. The results indicate that in *tdp1Δ wss1Δ* cells, lowering poly-SUMO chain formation by mutating at least one of the three lysines suppresses the synthetic lethality.

-leu + 5-FOA

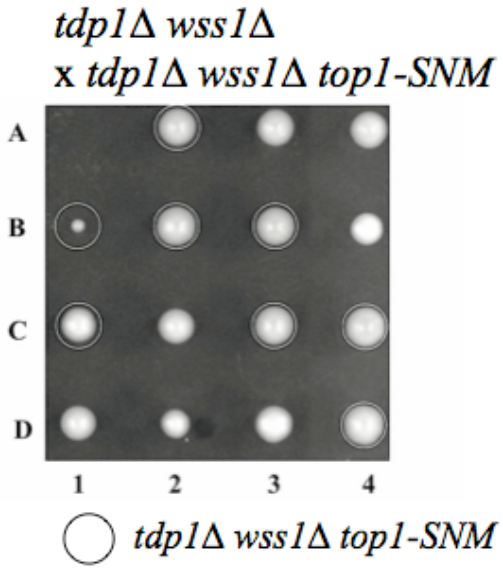


tdp1Δ wss1Δ smt3Δ

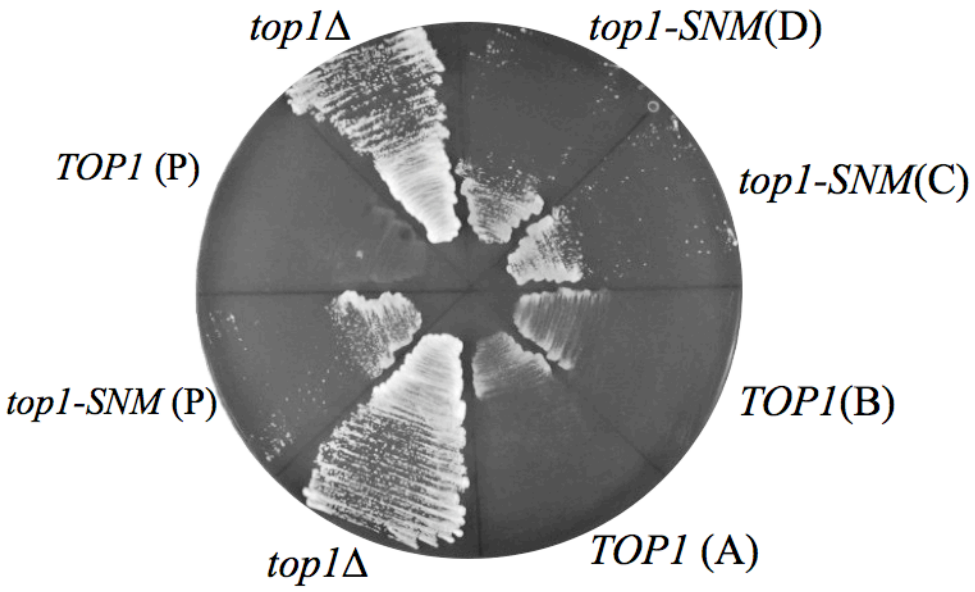
Fig. 9: Limiting poly-SUMOylation rescues *tdp1Δ wss1Δ*. PSY3684 (*tdp1Δ wss1Δ smt3Δ* + pNJ7478 + p*GPD1-SMT3/TRP1*) was transformed with the indicated alleles of SMT3. All transformants were streaked on FAA containing synthetic complete medium to shuffle out the *pGPD1-SMT3/TRP1*. Single colonies were then picked and streaked on synthetic complete medium supplemented with 5-FOA to select against the pNJ7478 (*WSS1*) and plate was photographed after 4 days of incubation at 30˚C.

The above data strongly suggest that formation of poly-SUMO chains is at least one of the causes of lethality in *tdp1Δ wss1Δ* cells. Next, I wanted to determine if there is a specific substrate that, when poly-SUMOylated, causes death in *tdp1Δ wss1Δ* cells. Top1 is the best characterized substrate of Tdp1 activity and, as Top1 is modified by SUMO *in vivo* (Chen, Silver et al. 2007) it stood out as a potential candidate. To test the hypothesis that SUMOylated Top1 could be the toxic substrate leading to inviability of *tdp1Δ wss1Δ* cells, I crossed *top1-SNM* (non SUMOylatable allele: *top1-K65, 91, 92, 600R*) (Chen, Silver et al. 2007) into a *tdp1Δ wss1Δ* genetic background. Triple mutants thus obtained were viable. The triple mutant progeny that germinated without the balancer started out as slow growing colonies (e.g. Fig. 10A, spore clone 1B), whereas triple mutants born with the balancer plasmid (pNJ7478) grew at the same rate as *tdp1Δ wss1Δ* double mutants with the balancer. Additionally, all triple mutants were able to grow on 5-FOA synthetic complete medium (Fig. 10B), indicating that a non-SUMOylatable top1 allows *tdp1Δ wss1Δ* cells to live, though very slowly.

A.



B.



All strains are *tdp1Δ wss1Δ* SC + 5-FOA

Fig. 10: Top1 SUMOylation is toxic to *tdp1Δ wss1Δ*. A NJY3288 (*tdp1Δ wss1Δ* +pNJ7478 (*WSS1*)) was crossed to PSY3732 (*tdp1Δ wss1Δ top1-SNM*) complemented by pNJ7478. Diploids were sporulated, micro-dissected and allowed to grow on rich medium for 4 days at 30˚C. Circles indicate triple mutant containing *top1-SNM* **B.** Progeny from tetrad 4 were streaked onto a synthetic complete plate containing 5-FOA, along with parents (P) and NJY3347 (*tdp1Δ wss1Δ top1Δ*) as controls. All strains were born with the balancer p*WSS1*.

Forward genetic screen to obtain suppressors

To initiate a forward screen, I selected a *tdp1Δ wss1Δ* strain (PSY3292) that had always contained the balancer plasmid (*WSS1/ADE3/URA3)*. About 10^7 cells from an overnight culture were spread on a synthetic complete plate containing 5-FOA (Fig. 11). Following several days of incubation at 30˚C, only a few colonies appeared on the plate. Additionally, from another balanced *tdp1Δ wss1Δ* strain (PSY3288), a log phase culture was treated with EMS (ethyl methane sulfonate) before plating cells on 5-FOA containing medium.

To eliminate false positives due to mutations in *URA3*, colonies from screening plates were streaked onto YPD, and those that formed red colonies (due to the *ADE3* balancer gene) were discarded. Genomic DNA was prepared from all remaining candidates to confirm by PCR that *WSS1* from the balancer had not integrated into the genome. Confirmed suppressors were then backcrossed to the parent strain of the opposite mating type. Diploids plus balancer were selected, sporulated and micro-dissected. Only suppressors that segregated 2:2 in the progeny (indicating a single mutation) were selected for further analysis. In order to determine if the suppressors were dominant or recessive, the backcrossed diploids were streaked onto FOA plates next to their respective suppressed parent . The backcrossed diploids of recessive mutations were unable to survive without the balancer due to complementation of the suppressor by WT copy, while dominant mutants formed FOA-resistant diploids. An example for each type is shown in Fig. 13. Table 4 lists the suppressors isolated and the genetic information obtained.

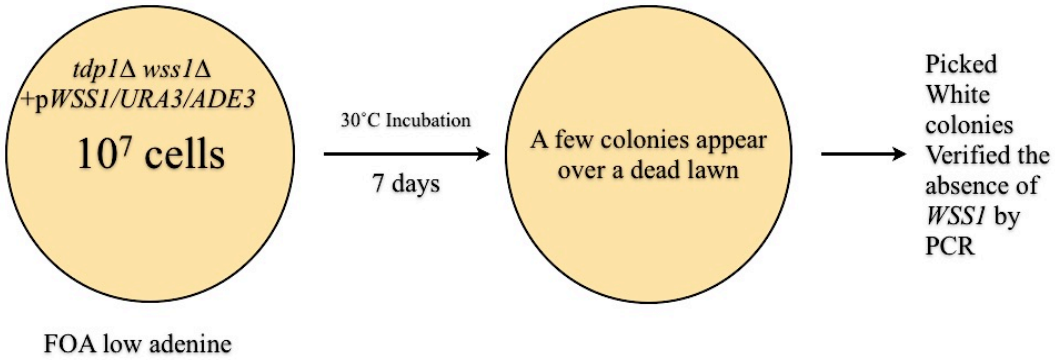


Fig. 11: Schematic representation of method of suppressor isolation.

Table 4: Strains containing spontaneous suppressor mutations.

A list of strains that acquired at least one mutation allowing faster growth than the original *tdp1Δ wss1Δ* parents.

S. No.	Recessive/ Dominant	Centromere-linked?	Gene involved	Mutation
PSY3296 (*supz*)	Partially dominant	No	*SIZ2*	V301STOP
PSY3297 (*supy*)	Recessive	Yes/ChrI	*NUP60*	V30STOP
PSY3489 (sup88-2)	Partially dominant	Yes/ChrIV	*SLX5*	E350K
PSY3493 (sup88-3)	Partially dominant	Yes/ChrXV	*TOP1*	Q668STOP
PSY3498 (sup88-9)	Recessive	Yes/ChrXVI	*ULP1*	W163STOP
PSY3491 (sup92-h)	Recessive	Yes/ChrIV	*SLX5*	Q362K

2. Premature STOP codon in *TOP1*: *top1-Q668STOP*

In the forward screen, one of the suppressors was found to be centromere-linked (Table 4), by comparing segregation patterns with the centromere-linked *TRP1*. As *TOP1* is centromere-linked, *TOP1* from the suppressor was sequenced and the suppressor mutation was identified as *top1-Q668STOP*.

Since the active site of Top1 is Y727, I suspected that the mutant allele must be null. To confirm this, I transformed a *mu81Δ top1Δ* strain with plasmids expressing either *TOP1, top1-Q668STOP* or no gene. Cells with the *mus81Δ* mutation are sensitive to camptothecin (Liu, Pouliot et al. 2002) but become resistant when *TOP1* is deleted. As shown in Fig. 12C, the *top1-Q668STOP* mutant didn't allow the *mus81Δ top1Δ* cells to grow in the presence of CPT, indicating that it is a null allele. Furthermore, the *top1-Q668STOP* allele suppresses the synthetic lethality of *tdp1Δ wss1Δ* just as well as empty vector (Fig. 12C).

Hence, it seems that the pathways involving *TDP1* and *WSS1* intersect at the Top1-linked DNA lesions. These findings add to the growing body of data linking SUMO pathway to modulation of DNA repair. Interestingly, loss of *TOP1* suppresses synthetic lethality observed in a variety of combinations of SUMO and DNA repair mutations (e.g., *siz1 siz2 rad52; siz1 siz2 rad27; ulp1-Δ1-160 rad52*) (Chen, Silver et al. 2007)

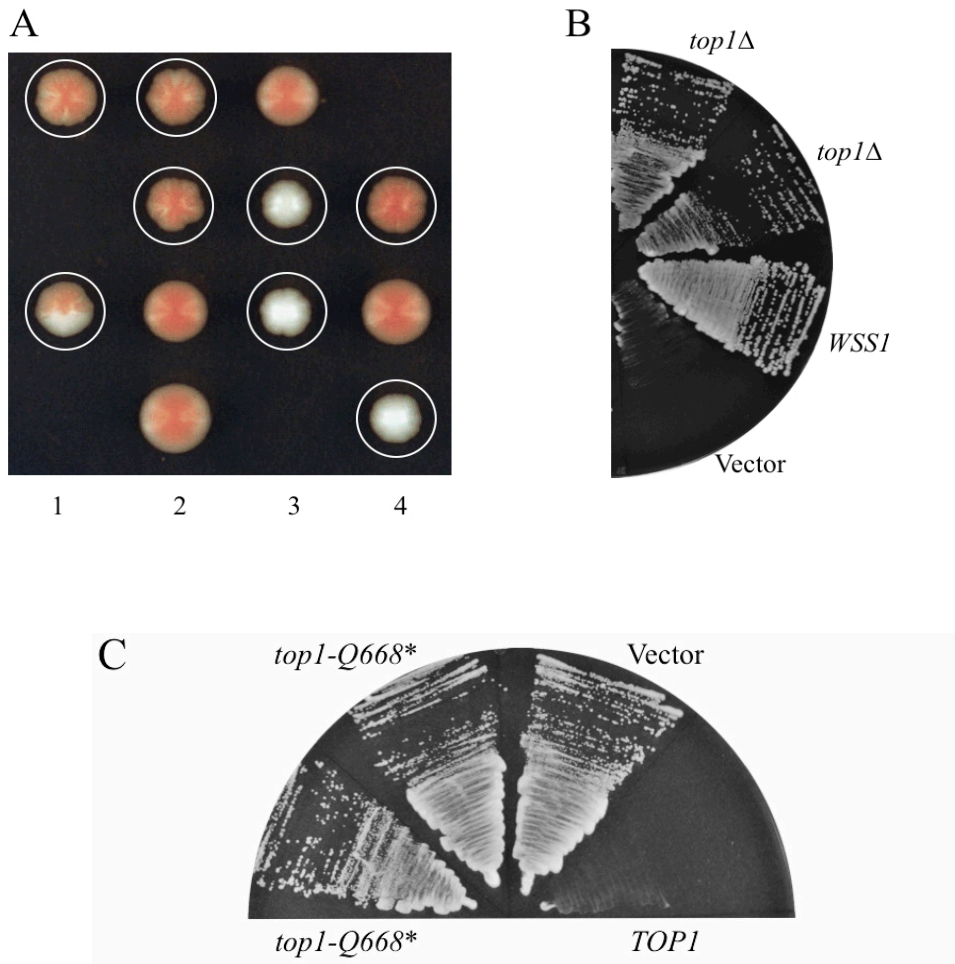


Fig. 12: *top1Δ* relieves the synthetic lethality of *tdp1Δ wss1Δ*. A. PSY3498 (*tdp1Δ wss1Δ sup88-3*) was crossed to NJY3288 (*tdp1Δ wss1Δ* + p*WSS1/ADE3/URA3*), sporulated, micro-dissected and allowed to grow on YPD for 4 days at 30˚C. Circles identify *tdp1Δ wss1Δ sup88-3*, with red color indicating the presence of p*WSS1*. **B.** PSY3548 (*tdp1Δ wss1Δ top1-8*) plus p*WSS1/URA3* was streaked on 5-FOA plates lacking leucine, along with NJY3288 transformed with either p*WSS1/LEU2* or empty vector. Plate was photographed after 4 days at 30˚C. **C.** *mus81Δ top1Δ* transformed with indicated plasmids on YPD containing 50mg/mL of camptothecin, after 4 days at 30˚C.

2. Premature STOP codon in *SIZ2* : *siz2-V301

As listed in Table 4, the suppression in PSY3296 (aka, sup '*z*') was determined through a backcross to be a single gene mutation. All of the progeny with the mutation showed a nibbled colony morphology. We analyzed FOA^R and *TRP1* segregation patterns and found that this suppressor was not centromere-linked. Since the diploid analysis showed that the suppressor was dominant (Fig. 13), complementation could not be used to identify the gene, so I tried a speculative approach instead. There are only a few mutations, not centromere-linked, that cause a nibbled colony phenotype, e.g. *slx8, siz1, siz2, ulp1* and *ulp2*, etc. Therefore, I sequenced these ORF's and their promoter regions from the suppressor strain. Sequence analysis of *SIZ2* revealed a frame shift mutation causing a premature stop codon at amino acid 301. *SIZ2* belongs to a conserved family of SUMO E3 ligases that stimulate conjugation of SUMO to substrates. As the RING domain of Siz2 resides between residues 300 and 400 approximately, this allele is most likely a null (Johnson and Gupta 2001).

3. Premature STOP codon in NUP60: nup60-*V30**

The strain PSY3297 was shown to have a single gene suppressor that was recessive (Fig. 13) and strongly centromere-linked (Table 4). To clone this mutant by plasmid complementation, I transformed the balancer plasmid back into PSY3297. Then, a tiling library of multi-copy plasmids containing sequential sets of WT yeast ORFs was transformed into this balanced strain. Transformants were streaked onto FOA to identify the tiling plasmid containing the WT copy of the suppressor, which

should die on FOA. Most of the plasmids comprising the tiling library bear clusters of genes. Furthermore, I had combined multiple plasmids to form pools of these plasmids to accelerate results. Following identification of the pool that contained plasmid bearing WT copy of the suppressor, I repeated the transformation with individual plasmids. The plasmid that complemented the suppressed strain contained *NUP60*, among other genes (Fig. 14). As *NUP60* has known ties to the SUMO specific protease, Ulp1 (Panse, Kuster et al. 2003, Palancade, Liu et al. 2007), I directly amplified the corresponding ORF from the test strain and identified a premature STOP in the gene (Table 4).

As expected, a triple mutant obtained by dissecting a cross between *tdp1Δ wss1Δ* and *nup60Δ* (from a commercial knockout library) also suppressed the synthetic lethality of *tdp1Δ wss1Δ* (data not shown).

Nup60 is a nucleoporin and was identified as one of the components of the nuclear pore complex (NPC) that is required to dock and stabilize Ulp1 at the pore (Panse, Kuster et al. 2003, Palancade, Liu et al. 2007). Thus, the *nup60Δ* strain has delocalized Ulp1. This is thought to result in either unregulated or a lowering of global SUMOylation due to insufficient Ulp1 activity to mature SUMO. Since the growth defect of *tdp1Δ wss1Δ* is rescued by overexpression of both *ULP1* and *ULP2* (latter not involved in SUMO processing) it is more likely that the suppression seen in *tdp1Δ wss1Δ nup60Δ* is due to de-SUMOylation of certain substrate(s) that are not the usual target(s) of this enzyme.

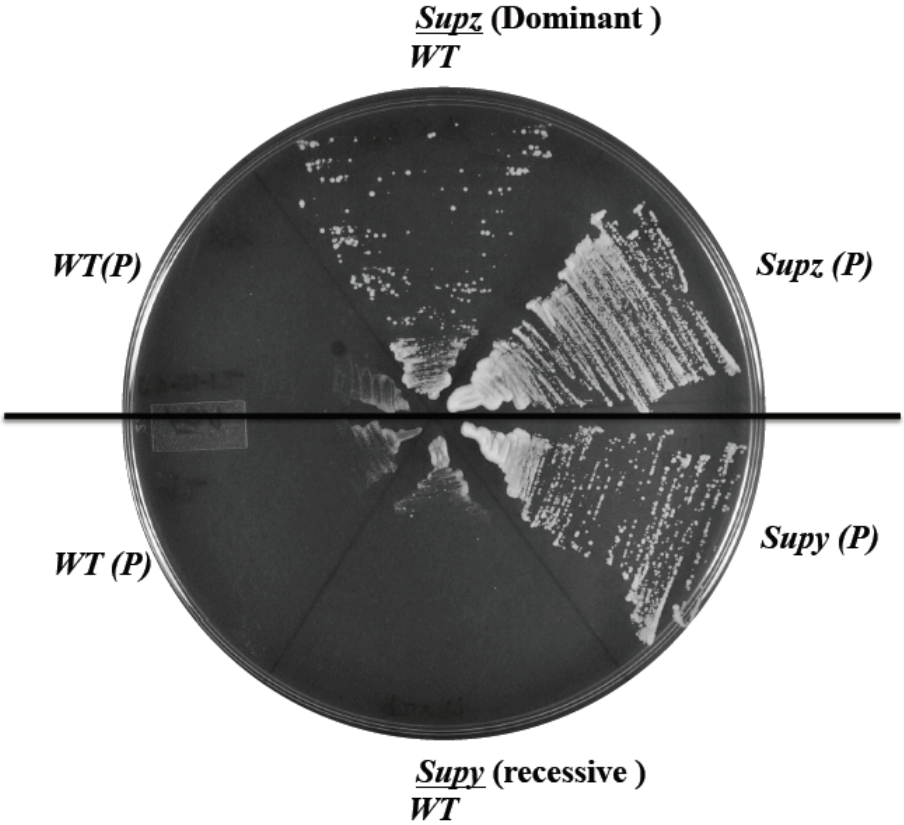


Fig. 13. Determination of recessive or dominant phenotype of suppressor mutants. Strains carrying suppressor mutations were backcrossed: PSY3296 (*tdp1Δ wss1Δ supz*) X NJY3288 (*tdp1Δ wss1Δ* + pNJ7478) and PSY3297 (*tdp1Δ wss1Δ supy*) X NJY3292 (*tdp1Δ wss1Δ* + pNJ7478). Haploid parents (P) and diploid *tdp1Δ wss1Δ* strains, with suppressor mutations as indicated, were streaked on synthetic complete medium containing 5-FOA. Plate was photographed after 4 days of incubation at 30˚C.

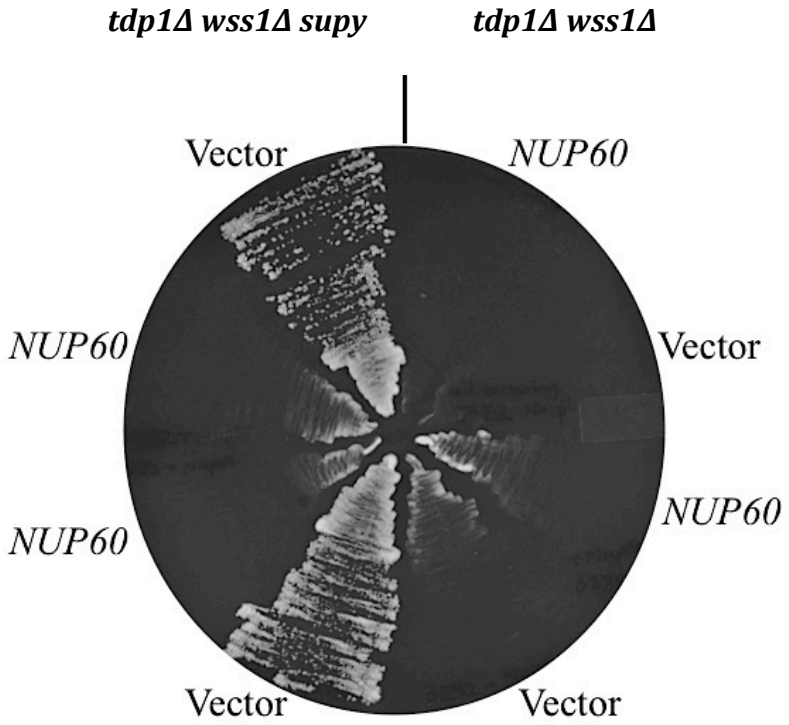


Fig. 14: Suppressor mutation "*supy*" lies in *NUP60.* PSY3297 (*tdp1Δ wss1Δ supy*) was first transformed with balancer plasmid, pNJ7478 (*WSS1*) and then with either *NUP60* containing multi-copy plasmid or an empty vector. Transformants were streaked on synthetic medium supplemented with 5-FOA, but lacking leucine. NJY3288 (*tdp1Δ wss1Δ* +pNJ7478) was also transformed with same plasmids to serve as control (right half of the plate) for the experiment. Plate was photographed after 3 days of incubation at 30˚C.

4. Premature STOP codon in *ULP1*: *ulp1-W163

Some of the suppressors of *tdp1Δ wss1Δ* were obtained through EMS mutagenesis. Genetic analyses of one such suppressor (PSY3549 or sup88-9) revealed that suppressor mutation was (i) monogenic (FOA^R: FOA^S :: 2:2) , (ii) recessive and (iii) weakly CEN-linked (P:NP:T :: 6:6:6). I constructed a set of strains in the *tdp1Δ wss1Δ* background in which each chromosome was marked with KAN^R (or LEU2) in close proximity to its centromere. If a suppressor mutant was linked to a centromere, crossing this mutant to a strain bearing that marked centromere strain would yield higher than expected parental di-type progeny). This particular suppressor mutation was acquired in an EMS-treated strain, several sequential backcrosses were performed to avoid detecting extraneous mutations during genome wide sequence analysis. Table 4 lists the information gained through the backcrosses. Genomic DNA from a seventh generation complete tetrad (two mutants and two wild type siblings) was sent for commercial Genome-Wide Association Studies (GWAS). I then compared the sequencing results of wild-type siblings to suppressed mutant siblings and looked for single nucleotide polymorphisms (SNPs) unique to the mutant strains. A premature stop codon (W163STOP) in *ULP1* was found in one of the mutants but not in the wild-type siblings. We then amplified and sequenced the *ULP1* ORF from genomic DNA of all four siblings. Indeed, both mutant strains contained the same SNP in *ULP1,* whereas their WT siblings had WT *ULP1*.

As *ULP1* is an essential gene, *ulp1-W163** cannot be a null allele. I therefore suspect that this allele allows initiation of protein synthesis at a methionine downstream from the natural start site. This would result in an N-terminally

truncated protein, presumably unable to interact with the nuclear pore. Unbound functional Ulp1 could suppress *tdp1Δ wss1Δ* lethality in a manner similar to the *nup60Δ* mutation. Consistent with this theory, I found that the *ulp1-ΔN346* could also suppress *tdp1Δ wss1Δ* phenotype (data not shown).

5. Novel *slx5* alleles: *slx5-Q362K* and *slx5-E350K*

The forward genetic screen delivered two novel alleles of *SLX5* as suppressors of synthetic lethality of cells lacking *TDP1* and *WSS1.* These alleles are characterized in greater detail later.

Discussion

In this study, the interaction between *TDP1* and *WSS1* was demonstrated to be synthetically lethal. This observation is different from the literature (Dixon, Fedyshyn et al. 2008, Baryshnikova, Costanzo et al. 2010, Stingele, Schwarz et al. 2014) that showed the phenotype of *tdp1Δ wss1Δ* as a slow growth. I speculate that this difference is because of the ability of *tdp1Δ wss1Δ* cells to easily acquire weak suppressor mutations. In order to avoid the selection of such suppressors, my screen was designed to use *tdp1Δ wss1Δ* cells that were complemented by a balancer plasmid (p*WSS1/ADE3/URA3*) until applying the FOA selection. In addition, only suppressors conferring robust growth were chosen for further examination. Thus, I conclude that *tdp1Δ wss1Δ* mutants display a synthetic lethal interaction but weak suppressor mutations arise frequently giving the false impression of a slow growth defect. I suggest that the published studies of viable tdp1 wss1 cells contain such suppressors.

The fact that *TDP1* and *WSS1* interact genetically is a preliminary evidence for the role of SUMO pathway in the repair of DNA damage . The results described above revealed a definite connection between the SUMO pathway and cellular processes involving *TDP1*. Because the lethality could suppressed by over-expression of de-SUMOylases Ulp1 and Ulp2, the *tdp1Δ wss1Δ* phenotype possibly arises, at least in part, from an abnormal build-up of highly SUMOylated substrate(s). Whether such an accumulation is directly causing sickness was tested in this study by deletion of *SIZ1* and SIZ2, the two main SUMO E3 ligases

(Hochstrasser 2001, Johnson and Gupta 2001). Although Siz1 is responsible for the bulk of SUMOylation in the cell, a *siz1Δ* didn't rescue the *tdp1Δ wss1Δ* synthetic lethality. Interestingly, deletion of *SIZ2* in a *tdp1Δ* strain restored growth rate comparable to wt cells. Reportedly, there is an overlap between the substrates SUMOylated by Siz1 and Siz2, but it is mostly Siz2 that promotes ligation of SUMO to the proteins involved in the homologous recombination repair (HRR) (Psakhye and Jentsch 2012, Jentsch and Psakhye 2013). Therefore, a Siz2-dependent SUMO-modification of possibly of the HRR pathway components appears to be harmful for cells missing both Tdp1 and Wss1. My results also support the idea that in the absence of Tdp1, Wss1 either prevents SUMOylation or promotes de-SUMOylation of certain substrate(s). Additionally, it seems that attachment of Smt3-3KR (a mutant SUMO-defective in chain formation) to such substrate(s) is tolerable to the double mutants. This suggests that poly-SUMOylation of one or more substrates is deleterious in the when Tdp1 and Wss1 are unavailable. While speculating what such substrates could be, I found out that Top1, the best-characterized Tdp1 substrate, is SUMOylated in vivo (Chen, Silver et al. 2007). As described before, *top1Δ* suppresses the synthetic lethality of the *tdp1Δ wss1Δ* mutation allowing for a robust growth comparable to that of a wt strain. When tested, a *top1-SNM* ("SUMO No More") allele also suppressed the lethality of *tdp1Δ wss1Δ* mutant, however the suppression was much weaker than that seen in *top1Δ tsp1Δ wss1Δ* mutant case. Top1 has several hundred Lys residues but the *top1-SNM* allele has only four Lys to Arg substitutions (K65, 91, 92, 600R), which do not eliminate SUMO modification of the resulting protein entirely (Chen, Silver et al. 2007). Thus, the weak suppression

that it provides could possibly be due to the SUMOylation on one or more of the several Lys still present in the mutant protein. It is clear, however, that the parallel pathways involving Tdp1 and Wss1 intersect at Top1. If the role of the Wss1 pathway is to avoid SUMO modification of Top1 can only be speculated from the available data. A recent report that Top1 is a direct substrate of Wss1 protease corroborates this notion (Stingele, Schwarz et al. 2014), however the activity of Wss1 in that case was independent of SUMO modification.

Many of the suppressor mutants obtained in the forward screen confirmed my choice of candidate genes tested. From the latter screen, I already knew that *top1Δ, siz2Δ* and *slx5Δ* individually suppressed the *tdp1Δ wss1Δ* phenotype. So, it was reassuring to identify spontaneous suppressor mutations in these genes. Although *top1* and *siz2* alleles were null, *slx5* suppressor mutants didn't phenocopy *slx5Δ*. Since the identification of Slx5-Slx8 complex more than a decade ago (Mullen, Kaliraman et al. 2001), its role in promoting genomic stability still remains to be fully elucidated. My results have revealed additional information about the Slx5 and Slx8 mutants. Previously understood to be functionally indistinguishable, this study demonstrated a clear distinction between mutations in *SLX5* and *SLX8*. Although cells carrying *tdp1Δ wss1Δ slx5Δ slx8Δ* were viable, a triple mutant carrying *slx8Δ* was no better than *tdp1Δ wss1Δ* combination. Thus, I suppose that only Slx5 subunit's activity is detrimental to *tdp1Δ wss1Δ* cells. This hypothesis is further supported by the observation that out of *SLX5* and *SLX8* pair, only the over-expression of *SLX5* is toxic to cells carrying single *tdp1Δ* or *wss1Δ* mutation. Further,

that this effect of *SLX5* over-expression is independent of the extent of *SLX8* expression. As the most obvious difference between Slx5 and Slx8 protein is the presence of a much larger, SIM-containing N-terminal sequence in Slx5, our lab tested the role of SIMs of Slx5 in promoting the toxicity associated with *SLX5*-overexpression. Therefore, an *slx5-SIMless* allele was over-expressed in single *tdp1Δ* or *wss1Δ* mutants. As predicted, the over-expression of *slx5-SIMless* didn't show any toxic effect on either *wss1Δ* (Méndez-Rivéra 2014, unpublished data) or *tdp1Δ* mutant. The SIMs of Slx5 bind multiple SUMOs in tandem effectively, so much so that Slx5-Slx8 complex was initially shown to stimulate SUMOylation *in vitro* (Ii, Mullen et al. 2007). Therefore, we proposed and tested the idea that the overexpression toxicity of *SLX5* stems from aberrant poly-SUMO chain formation promoted by SIMs of Slx5. As shown in Fig. 8 C and D, SIMs do mediate the toxic phenotype associated with *SLX5* overexpression (Méndez-Rivéra 2014, unpublished data). Finally, the *slx5-Q362K* and *slx5-E350K* suppressor mutations identified in the forward screen delivered an important opportunity to study Slx5 more closely. The findings related to these alleles are described in the following chapter.

CHAPTER IV

A Lysine Desert in Slx5

Slx5-Slx8 enzyme belongs to a special class of ubiquitin ligases known as SUMO-Targeted Ubiquitin Ligases. These conserved enzymes are characterized by the presence of several consecutive SUMO Interaction Motifs (SIMs) and a RING domain (Ub ligase domain) that work together to attach ubiquitin to SUMO-modified substrates (Uzunova, Gottsche et al. 2007). Cells lacking *SLX5*, *SLX8* or both show accumulation of high molecular weight SUMO conjugates indicating that the heterodimer down-regulates SUMO-modified proteins (Mullen and Brill 2008). Both Slx5-Slx8 and the human homolog (RNF4) have been shown to preferentially catalyze the ubiquitination of poly-SUMO conjugates (Mullen and Brill 2008, Tatham, Geoffroy et al. 2008, Rojas-Fernandez, Plechanovova et al. 2014). The biological significance of the poly-SUMO conjugates is still unknown and whether these poly-SUMO chains are tethered to any substrate(s) is unknown and remains a subject for further studies.

As described in the previous chapter (III), I isolated two novel alleles of *SLX5* in a forward genetic screen for suppressors of *tdp1Δ wss1Δ* synthetic lethality: *slx5-Q362K* and *slx5-E350K*. Here, I characterize these alleles through a series of genetic and biochemical assays. The results show that Slx5-Q362K and Slx5-E350K are prone to self-degradation in a proteasome dependent manner, manifesting a partially defective phenotype of the suppressor mutants. As a result of this screen, I discovered uniquely conserved lysine deserts in SUMO-Targeted Ubiquitin Ligases across species as a mechanism against auto-ubiquitination.

Novel *slx5* alleles suppress *tdp1Δ wss1Δ*

The forward screen yielded two novel and distinct *SLX5* alleles: *slx5-Q362K* and *slx5-E350K* (Table 2) as suppressors of *tdp1Δ wss1Δ* synthetic lethal phenotype.

1. Identification of *slx5-Q362K* as a suppressor of *tdp1Δ wss1Δ* lethality by complementation

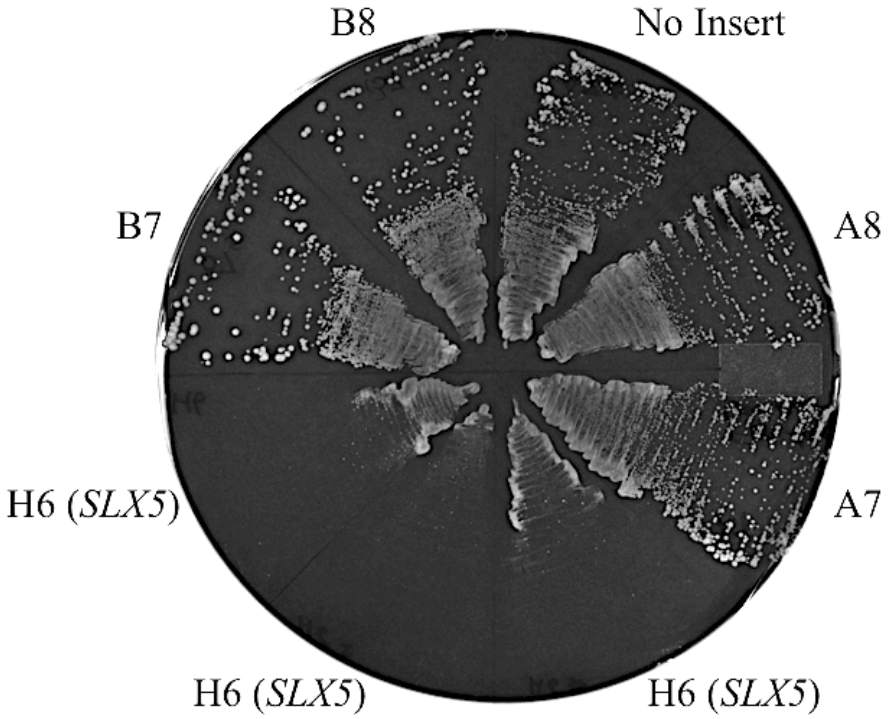
The *slx5-Q362K* allele was cloned by means of genetic analyses of a backcross (to the parent of opposite mating type) followed by DNA sequencing. *SLX5* is located on chromosome IV about10 Kb away from the centromere. Another gene on chromosome IV that lies in close proximity to the centromere is *TRP1*. When FOA^R segregation patterns were analyzed in the backcross with respect to *TRP1*, I found that the suppressor mutation was linked to *TRP1*, (8 Parental: 0 Non-Parental: 0 Tetratype). That is, not only was the mutant gene located close to a centromere; it was on the same chromosome as *TRP1*. Because the mutation was recessive (Table 4), I used plasmid complementation to identify the mutation. Plasmids from a tiling library expressing gene clusters around CEN IV were transformed into the suppressed strain. I observed that the plasmid containing *SLX5* complemented the triple mutant, which now died on synthetic complete medium containing 5-FOA (Fig. 15). The specific mutation was pinpointed by sequencing the *SLX5* ORF PCR-amplified from the suppressed strain's genomic DNA.

2. Identification of *slx5-E350K* as a suppressor of *tdp1Δ wss1Δ* lethality by CEN-linkage

One suppressor isolate acquired through treatment with EMS also showed *TRP1*-linkage in the backcross. This mutant was partially dominant and could not be cloned by the method of complementation. Since I had already discovered an *slx5* mutant as a suppressor, I sequenced *SLX5*'s promoter and ORF amplified from the genomic DNA and found a point mutation at amino acid 350 from glutamate (E) to lysine (K).

To confirm that *slx5-E350K* was the mutation providing the suppression, I first used a *tdp1Δ wss1Δ slx5Δ* strain covered by p*WSS1.* Indeed, *slx5-E350K* conferred robust suppression, unlike the wild-type transformant that died upon loss of p*WSS1* (Fig. 16). As previously discovered, I again observed that *slx5Δ* relieved the synthetic lethality of *tdp1Δ wss1Δ*, but it grew slower than *tdp1Δ wss1Δ slx5-E350K.*

-leu + 5-FOA



tdp1Δ wss1Δ supx

Fig. 15: Complementation of *slx5-Q362K* mutation by *SLX5*. PSY3490 (*tdp1*Δ *wss1*Δ *supx*) was first transformed with p*WSS1/ADE3/URA3* and then with a series of individual tiling library plasmids (pRS425 based) around CEN IV (10 individual plasmids on either side). Shown above are representative transformants (containing tiling library's unique multi-copy plasmids labeled A7, B7, A8, B8 and H6) streaked on 5-FOA containing minimal medium lacking leucine. Only the transformants possessing *SLX5* expressing plasmids (H6) lost suppression.

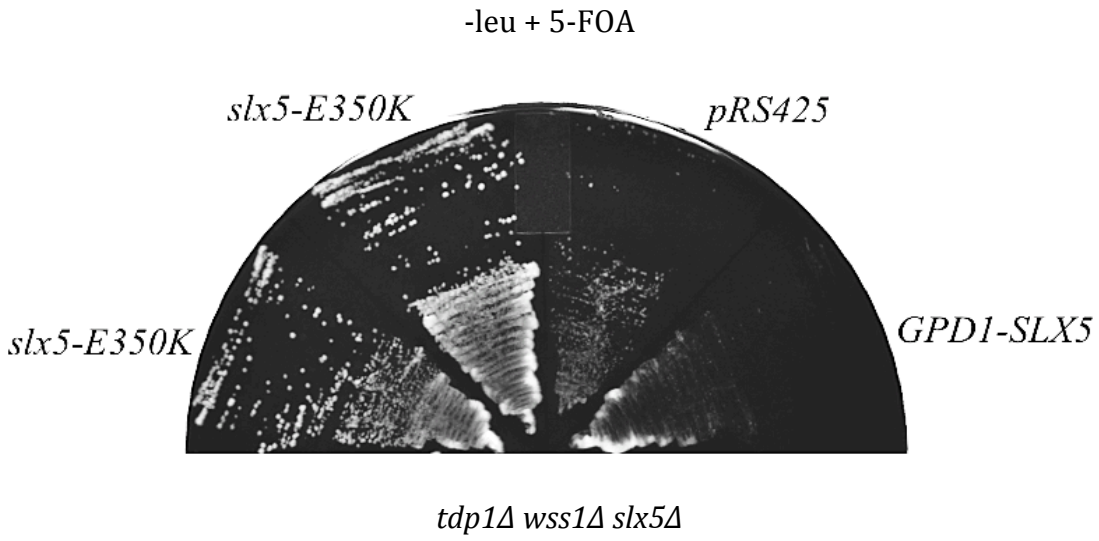


Fig. 16: *slx5-E350K* suppresses *tdp1Δ wss1Δ* lethal phenotype. PSY3494 (*tdp1Δ wss1Δ slx5Δ* + p*WSS1/ADE3/URA3*) was transformed with plasmids expressing *slx5-E350K*, *SLX5* or empty vector (pRS425). Transformants were then streaked onto minimal medium lacking leucine, but containing 5-FOA to select against p*WSS1*. The plate was photographed following 4 days of incubation at 30˚C. NOTE: To ensure constant expression of *SLX5* from a CEN-based vector, a constitutive promoter was used here. (*SLX5* driven by the endogenous promoter has the same phenotype as shown Fig 17.)

Is the change to a lysine residue crucial?

I was intrigued to observe that both *slx5* suppressors had mutations resulting in substitutions to lysines. Such a mutation can have the following two implications. One, the lysine side chain causes a structural change by introducing a positive charge. The second possibility is that the substitution permits post-translational modification of the protein either by Ub, SUMO, or alkylation, each of which occurs on lysine side chains (Wilkinson 1987, Takahashi, Iwase et al. 1999).

To test our predictions, I generated "arginine alleles" (*slx5-Q362R* and *slx5-E350R*) through site-directed mutagenesis and subcloned these alleles under its natural promoter in a single copy vector (Fig. 17, upper panel). Surprisingly, unlike *slx5-K* mutants, *slx5-R* alleles did not suppress the synthetic lethality displayed by *tdp1Δ wss1Δ* cells. The simplest explanation of this result is that the Slx5-K mutants undergo post-translational modification at the lysine since arginine has the same relative size and charge.

SUMO modification is known to occur preferentially at a consensus sequence YKxE, where Y is a large hydrophobic residue and x can be any residue. Again using site-directed mutagenesis, I engineered an *slx5* variant such that Leu354 and His355 were changed to Ile and Lys, respectively. These two changes created a consensus SUMOylation sequence between amino acids 354-357. I refer to this allele as *slx5-YKxE*. To serve as a control, I made the analogous mutant except that Lys was replaced with Arg to yield Slx5-Y<u>R</u>xE (L354, H355 to I354, R355). Both constructs were put in single copy vector under the natural *SLX5* promoter and transformed into cells of the genotype *tdp1Δ wss1Δ slx5Δ*, but constantly complemented by the

balancer p*WSS1/ADE3/URA3*. Only *slx5-YKxE,* and not *slx5-YRxE,* conferred rapid growth to the *tdp1Δ wss1Δ* strain (Fig. 17, lower panel). This favored the idea that SUMO modification of Slx5-Q362K and Slx5-E350K could be key to their distinct phenotype. However, since no conserved motif for ubiquitin conjugation exists, I am unable to rule out the possibility that this lysine is modified by Ub and not SUMO.

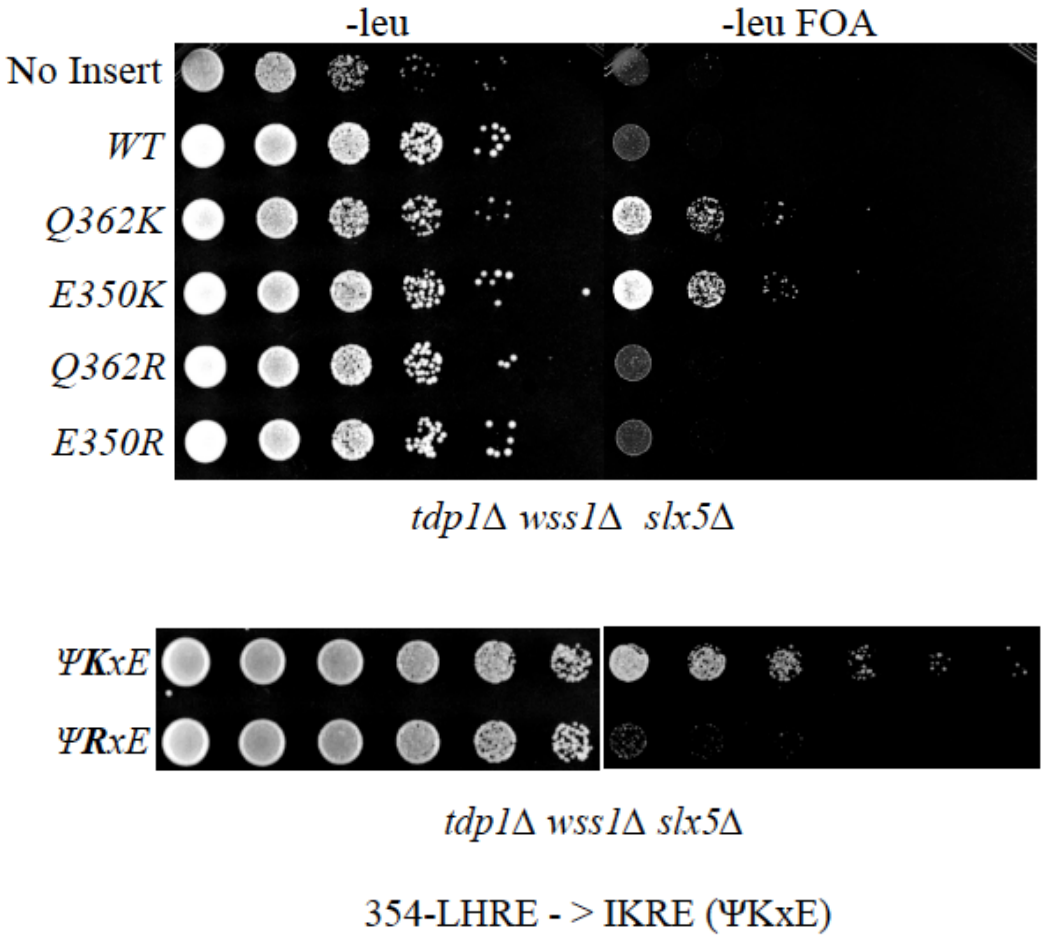


Fig. 17 Importance of lysine residues for the suppressor phenotype. Single-copy plasmids expressing the indicated *SLX5* alleles under its natural promoter were transformed into a *tdp1Δ wss1Δ slx5Δ* strain (PSY3710) that had always contained the balancer plasmid (p*WSS1*). Cells of each transformant were then diluted in 10-fold (upper panel) or 5-fold (lower panel) serial dilutions starting at an OD_{600} of 3. Cells (5 µl) were then pinned onto synthetic medium lacking leucine either with or without 5-FOA. Plates were photographed following 3 days of growth at 30˚C.

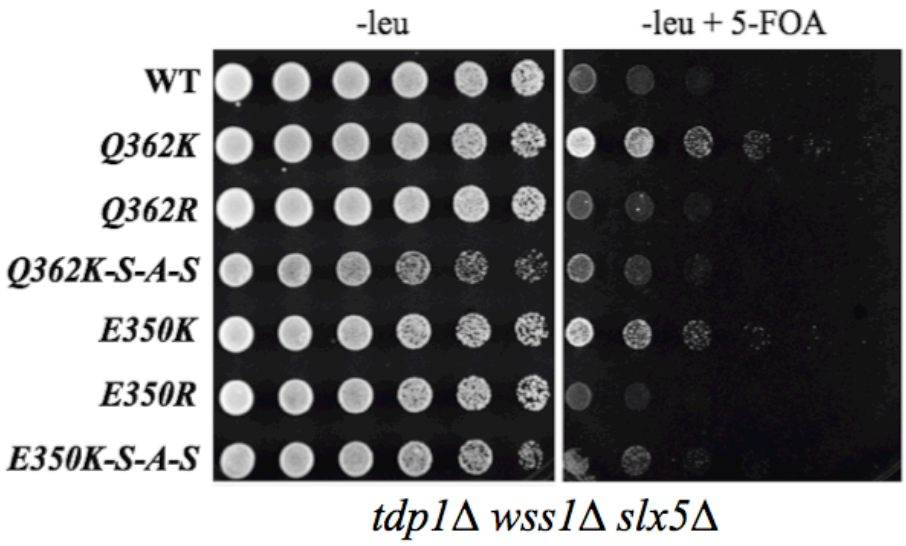
Suppression of the *tdp1Δ wss1Δ* phenotype by *slx5-K* alleles requires an active Ub-ligase.

Based on their rapid growth, I concluded that suppression by any of the (three) *slx5-K* alleles was better than that of the *slx5Δ* null mutation (Fig. 5, 16 and 17). This suggests that the mutant protein is providing a growth advantage to the suppressed strain. From the structure-function analyses of *SLX5* (described in Chapter III, Fig. 6), I concluded that the Ub-ligase activity of Slx5-Slx8 was not detrimental to *tdp1Δ wss1Δ* mutants. This notion was reinforced by the fact that only *slx5Δ,* and not *slx8Δ*, suppressed the *tdp1Δ wss1Δ* synthetic-lethal phenotype (Fig. 5). The Ub ligase activity of Slx5-Slx8 requires the RING domains of both subunits to be active (Ii, Fung et al. 2007). Therefore, I considered the possibility that the proteins encoded by the *slx5-k* alleles are likely to have an intact Ub ligase and that the enhanced suppression of the *tdp1Δ wss1Δ* synthetic-lethal phenotype relied on it. To test this idea, I created compound alleles (i.e. *slx5-Q362K-SAS* and *slx5-E350K-SAS)* such that the resulting proteins have a non-functional RING domain in addition to the lysine substitution. As shown in Fig. 18A, suppression of the synthetic lethal phenotype of *tdp1Δ wss1Δ* by the *slx5-K* alleles is dependent on an intact Ub-ligase.

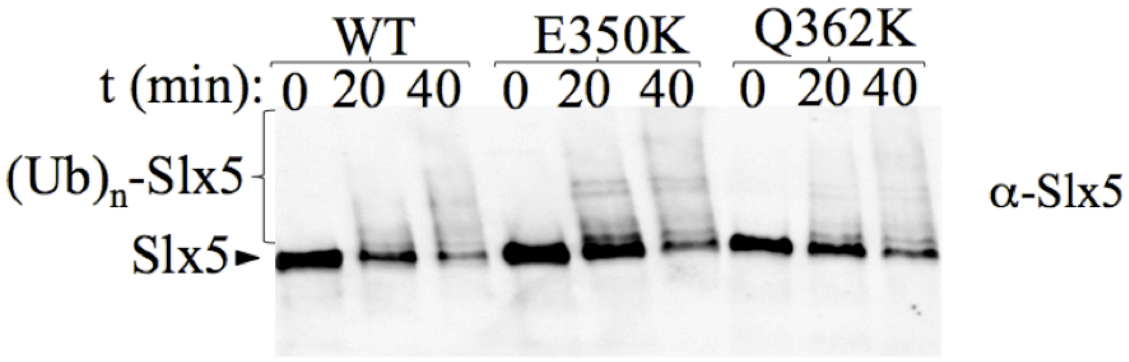
In addition to the genetic assay, I thought of comparing the activity of the Slx5-K mutant protein to the WT in heterodimeric complexes with Slx8, *in vitro*. This would eliminate the possibility that the K-mutations were suppressing via a change in Ub ligase activity. Therefore, the *slx5-K* alleles were subcloned into a bacterial expression vector for the purification of Slx5-Slx8 heterodimers from *E. coli*. Purified complexes were then allowed to undergo auto-ubiquitination in the presence of

ATP, Ube1(activating enzyme, E1), Ubc5 (conjugating enzyme, E2) and free Ub (Fig. 18). The reaction products were immuno-blotted with antibody against Slx5 to detect high-molecular weight Slx5-Ub conjugates, which appear as smears extending up from the unmodified Slx5 band. Supporting our inference from the genetic results, the data in Fig. 18B demonstrates that both mutant heterodimers displayed the same level of auto-ubiquitination as that of the WT protein complex

A.



B.



Auto-ubiquitination assays

Fig. 18: Ubiquitin ligase activity is critical for optimal suppression A. Transformants of PSY3710 (*tdp1Δ wss1Δ slx5Δ* + p*WSS1*) expressing the indicated genes were subjected to 10 fold serial dilutions (starting at OD_{600}=3.0) and pinned (5 µl) onto medium lacking leucine and on medium lacking leucine but containing 5-FOA. Plates were photographed following 3 days of incubation at 30˚C (–leu for 2 days). **B.** Purified heterodimers (50 nM each) of Slx5-Slx8, Slx5-E350K-Slx8 and Slx5-Q362K-Slx8 were subjected to auto-ubiquitination using 5 nM Ube1, 140 nM Ubc5 and 100 nm Ub, in the presence of 2 mM rATP at 30˚C. Reactions were terminated at the indicated times and analyzed using SDS-PAGE (10% polyacrylamide gel) and western blotting against Slx5.

The *slx5-K* alleles are partially defective in-vivo.

Since the Slx5-K-Slx8 proteins displayed WT levels of Ub ligase activity *in vitro*, I took a different approach to better appreciate their mechanism of suppression. I subjected the *slx5-K* alleles to a series of tests comparing their phenotypes to *slx5Δ*. Interestingly, the *slx5-k* mutants exhibited wt or hypomorphic phenotypes, depending on the assay, whereas *slx5-R* alleles were always WT. The following results support the hypothesis that the lysine-containing mutants are hypomorphs.

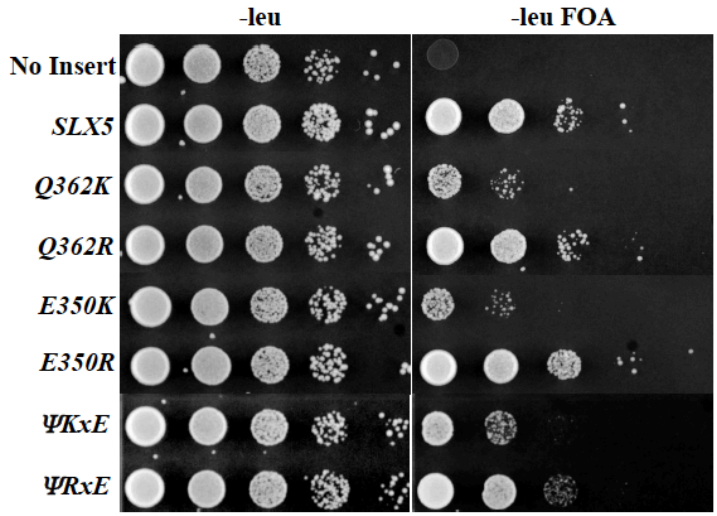
1. *slx5-K* alleles weakly complement *sgs1Δ slx5Δ* synthetic lethality.

Slx5 was discovered as a part of heterodimeric ubiquitin ligase Slx5-Slx8, which is essential for viability in *sgs1Δ* cells (Mullen 2001). Extensive structure-function studies have been performed (Mullen and Brill 2008) on both subunits of this complex leading to the identification of the minimal regions of each protein needed for survival as *sgs1Δ*. Also reported in the above study is the fact that the ubiquitin ligase activity of Slx5-Slx8 is essential in *sgs1Δ* cells. As a test of the *in vivo* activity of the suppressor alleles, an *sgs1Δ slx5Δ* kept alive by p*SGS1/ADE3/URA3* was individually transformed with single-copy plasmids expressing *slx5-K*, *slx5-R, slx5-ΨKxE, slx5-ΨRxE* and *SLX5*. In contrast to the *in vitro* assay, the *slx5-K* mutations could not provide perfect complementation of *slx5Δ* in an *sgs1Δ* background (Fig. 19A). I hypothesized that the difference between any *in vitro* and *in vivo* efficacy is due to a post-translational modification of the lysine side chain by either SUMO or ubiquitin. To unify my results in Chapter III with this conclusion, I proposed that, in

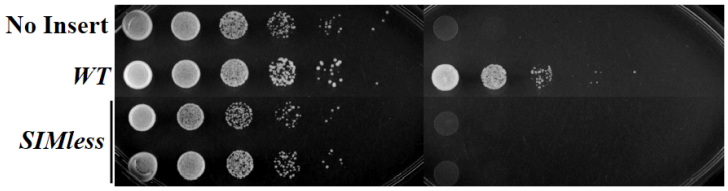
case of SUMOylation of Slx5-K, the SUMO Interaction Motifs (SIMs) of the protein might be occluded from a target(s) of this SUMO-Targeted Ub-Ligase hence limiting the range of substrates it might act on. To test this theory, I constructed a new *slx5-SIMless* mutant such that the protein would be incapable of interacting with SUMO (Uzunova, Gottsche et al. 2007), and I transformed it into two strains: *tdp1Δ wss1Δ slx5Δ* plus p*WSS1/ADE3/URA3* and *sgs1Δ slx5Δ* plus p*SGS1/ADE3/URA3*. As expected, the *slx5-SIMless* allele provided a level of suppression that was similar to that of the *slx5-K* alleles in *tdp1Δ wss1Δ* cells, but exhibited a null phenotype in *sgs1Δ slx5Δ* cells (Fig. 19B). This difference between the *slx5-K* and *slx5-SIMless* might be attributed to the existence of small population of unmodified Slx5-K protein still capable of binding and ubiquitinating SUMOylated targets, whereas Slx5-SIMless's SUMO-binding is abolished a 100% at all times.

Additionally, modification by SUMO could also result in auto-ubiquitination followed by proteasomal degradation, which would exhibit an *slx5Δ slx8Δ* phenotype if the efficiency of auto-ubiquitination is high. If the Slx5-K proteins were undergoing Ub-modification, I would likely see an overall null effect again. To summarize, as shown in Figures 16 and 17, *slx5- ΨKxE* phenocopied the original suppressor mutants in *tdp1Δ wss1Δ* strain and the Slx5-K proteins have no obvious defect in the Ub ligase activity. Therefore, the inhibitory effect of SUMOylating Slx5-K could be the cause of their weak complementation of *sgs1Δ slx5Δ* lethality. However, it is still possible that auto-ubiquitination and proteasomal degradation is partial and provides the precise level of active STUbL to suppress *tdp1Δ wss1Δ* and partially complement *slx5Δ slx8Δ*

A.

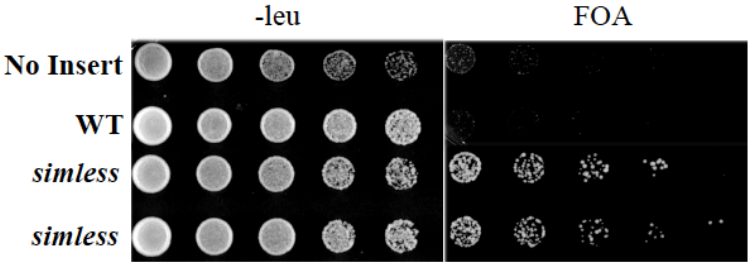


sgs1Δ slx5Δ



sgs1Δ slx5Δ

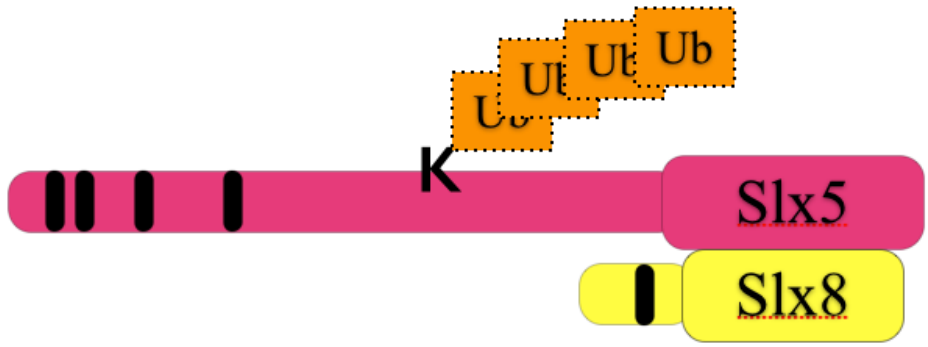
B.



tdp1Δ wss1Δ slx5Δ

Fig. 19: Mutant alleles partially complement *sgs1Δ slx5Δ* A. Single copy plasmids expressing the indicated genes with endogenous promoter were transformed into *sgs1Δ slx5Δ* (JMY2924) balanced by p*SGS1/ADE3/URA3*. Individual transformants were then streaked onto a synthetic complete plate containing 5-FOA to select against the balancer. The plate was photographed following 3 days of incubation at 30˚C.

A.



B.

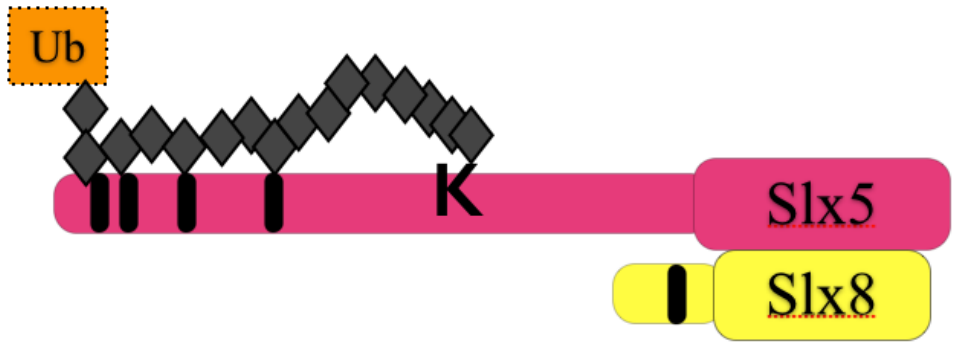


Fig. 20 Hypothetical models for the modification and mis-regulation of Slx5-K mutant proteins.

A. Auto-ubiquitination leading to proteasomal degradation.

B. SUMOylation, poly-SUMOylation and SUMO-dependent auto-ubiquitination. This is expected to result in occlusion of the Slx5 SIMs and proteasomal degradation.

2. *slx5-K* mutants show a SUMO-defective phenotype

In a *tdp1Δ wss1Δ* background, the *slx5-K* alleles displayed a nibbled-colony morphology that is characteristic of *slx5Δ* and a number of other SUMO mutations, e.g, *slx8Δ*, *ubc9-1*, *ulp2Δ*, *ulp1-ΔN346*, *siz1Δ siz2Δ* etc. (Zhao, Wu et al. 2004, Ii, Fung et al. 2007, Mullen, Chen et al. 2010). To determine the colony morphology of the mutant alleles in an otherwise wild-type genetic background, I isolated them away from *tdp1Δ* or *wss1Δ* by crossing to WT strains. Unexpectedly, the *slx5-K* alleles were nibbled like *slx5Δ*, independent of the *tdp1Δ* or *wss1Δ* status of the cells. Additionally, *slx5-R* mutants still phenocopied WT and formed round colonies. This indicates that the *slx5-K* mutants suffer from a perturbation of the SUMO pathway. It should be noted that the nibbled-colony phenotype can occur due to mutation of either Slx5's SIMs or its RING-domain (Fig. 21A).

3. *slx5-K* mutants are resistant to hydroxyurea

The *slx5Δ* phenotypes include sensitivity to hydroxyurea (HU) (Ii et. al. 2007, Mullen 2011). Therefore, I tested the *slx5-K* alleles for HU sensitivity as well (Fig. 22). Surprisingly, the mutant showed no response to 0.1M HU, a phenotype resembling that of wild type *SLX5*. By assaying other *slx5* strains, I observed that the sensitivity of *slx5* mutants to HU most likely derives from the loss of Ub ligase activity and not a lack of SUMO binding. Taken together, the HU resistance indicates that the Slx5-K mutant proteins must retain at least some Ub ligase activity *in-vivo*. Such a result is consistent with the protein's Ub ligase activity *in-vitro*.

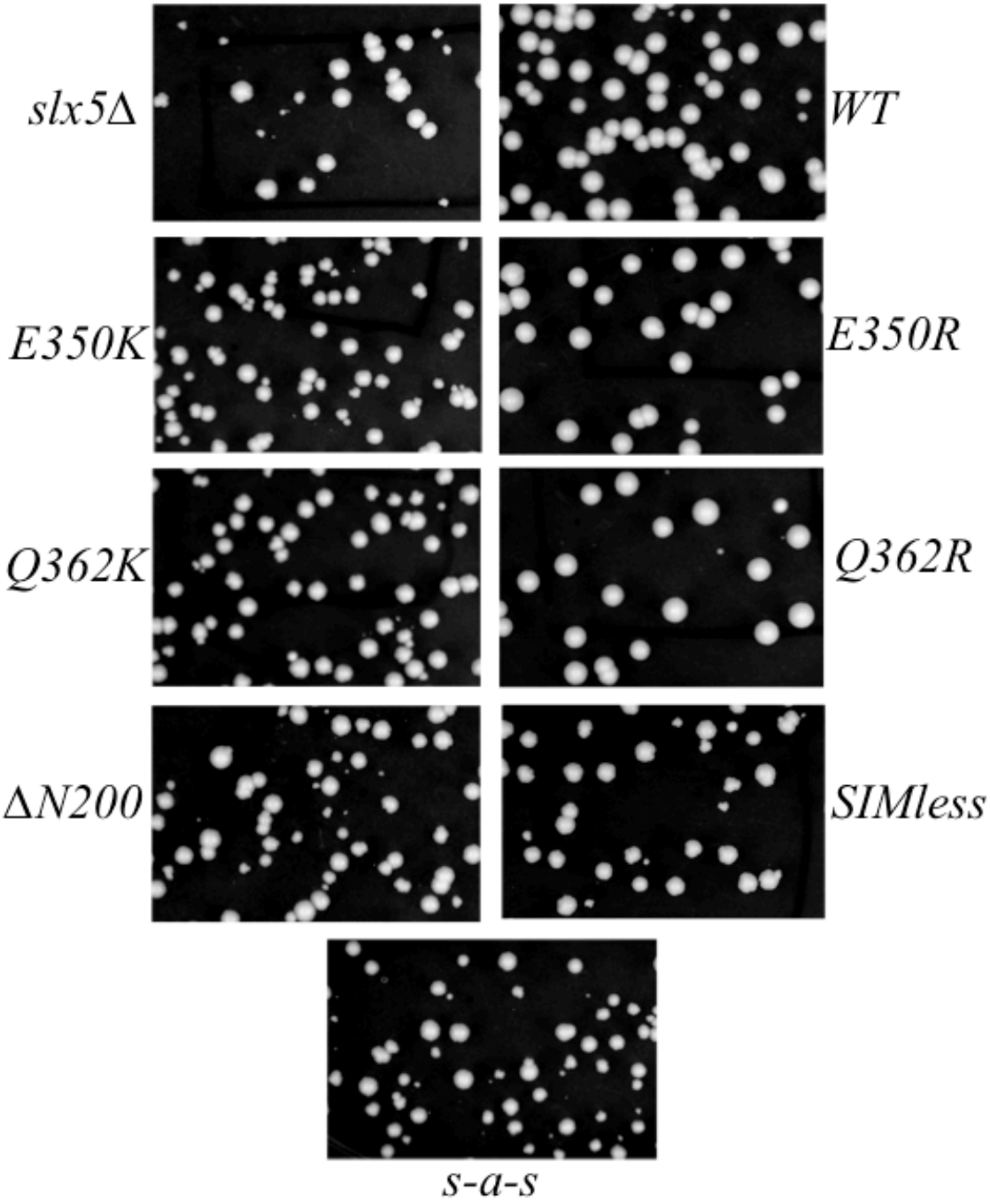


Fig. 21 A An *slx5Δ* strain transformed with single copy plasmids expressing indicated alleles or an empty vector (*slx5Δ*)

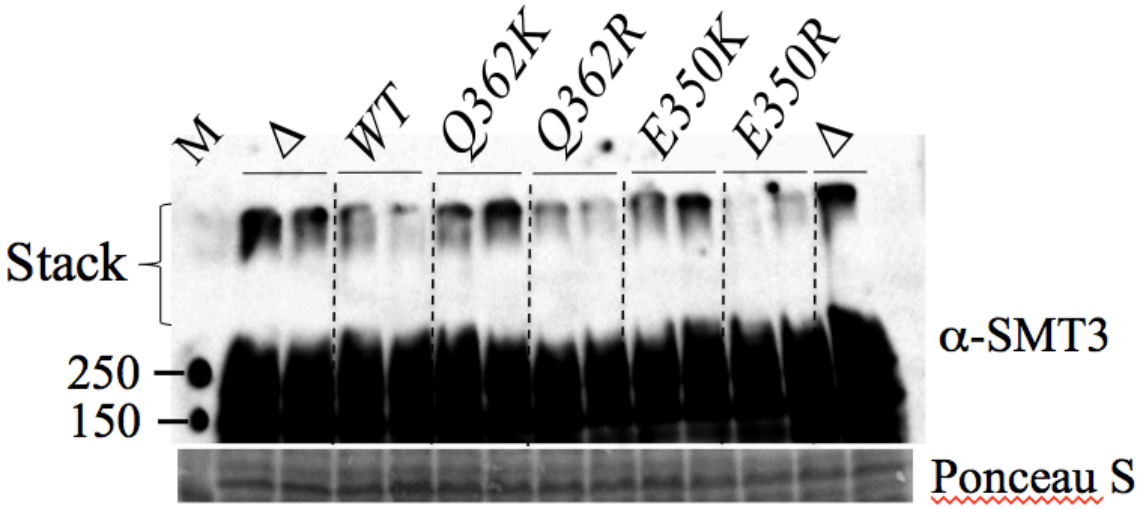


Fig. 21B Whole cell extracts of *slx5Δ* strain carrying single copy plasmids expressing indicated genes or an empty vector (Δ)

Fig. 21: *slx5-Q362K* and *slx5-E350K* emulate *slx5Δ* with respect to its nibbled colony morphology. A. Strains PSY3884 (*slx5Δ*) was transformed with single copy plasmids containing either no insert or indicated *slx5* alleles under the control of endogenous Slx5 promoter. Transformants were allowed to grow on selective medium for 3 days at 30˚C before photographing the plates **B.** Exponential cultures of *slx5Δ* cells expressing indicated genes from single copy plasmids were harvested to obtain 10 ODs worth of cells. Denaturing extracts were prepared from each culture, separated using SDS-PAGE (10% polyacrylamide gel) and immuno-blotted using Smt3 anti-serum. Before immuno-blotting the membrane was also stained using Ponceau S to serve as loading control.

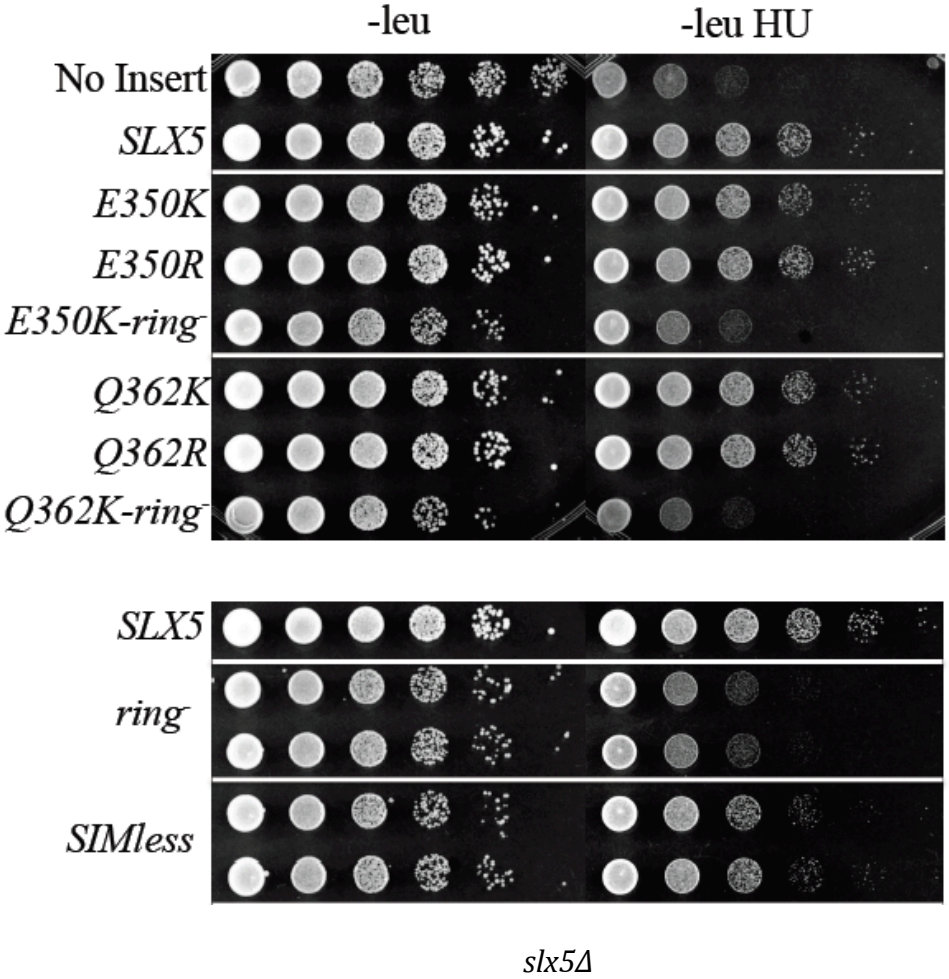


Fig. 22: *slx5-k* mutants are resistant to hydroxyurea. PSY3884 (*slx5Δ*) was transformed with a single copy plasmid expressing indicated gene or an empty vector. Transformants were pinned on minimal plates lacking leucine, with or without 0.1M HU and photographed after 2 days of incubation at 30˚C.

4. Genetic interaction between *ULP2* and *slx5* alleles

ULP2 encodes for a SUMO isopeptidase (Li and Hochstrasser 2000)and is known to preferential act on poly-SUMO chains (Bylebyl, Belichenko et al. 2003).It was recently reported that *slx5Δ* suppresses certain phenotypes associated with *ulp2Δ*. For example, *ulp2Δ slx5Δ* cells show reduced temperature sensitivity, HU sensitivity and an improvement in overall growth rate when compared to *ulp2Δ* alone (Mullen, Das et al. 2011). I thought it would be interesting to observe the behavior of *slx5* alleles in context of a *ulp2Δ* as it might shed light on the functions of Slx5 that are responsible for generating these phenotypes in the absence of Ulp2. For this purpose, a *ulp2Δ slx5Δ* double mutant was transformed with a single copy plasmid expressing wt or mutant *SLX5*. Figure 23 shows a remarkable contrast in the behavior of the suppressor mutants (*slx5-Q362K* and *slx5-E350K*) and *SLX5*. As expected, introduction of wt *SLX5* restored the slow growth typical of *ulp2Δ* cells. In contrast, the *slx5-K* alleles enhanced their growth rate! The control alleles, *slx5-Q362R* and *slx5-E350R* that cannot suppress *tdp1Δ wss1Δ*, mimicked the wild type *SLX5* behavior in reducing the growth rate of *ulp2Δ slx5Δ* cells. One interpretation of this result is that the *slx5-K* alleles retain an activity beneficial to *ulp2Δ* cells but lack an antagonizing activity. An alternative interpretation, discussed at the end of this chapter, is that the *slx5-K* alleles reduce but do not eliminate Slx5 activity.

One way of looking at this data, vis-à-vis *SLX5*, is that *ulp2Δ* cells recapitulate the synthetic lethal phenotype of *tdp1Δ wss1Δ* cells. First, although *ulp2Δ* strains are slow growing and *tdp1Δ wss1Δ* cells are lethal, complete deletion of *SLX5* relieves both defects. Second, the *slx5-K* alleles suppress both phenotypes better than the

slx5Δ null. And third, both phenotypes can be suppressed by limiting poly-SUMO chain formation as is evident from *smt3-3KR* phenotypes (Fig. 9) and as reported for *ulp2Δ smt3-3KR* (Bylebyl, Belichenko et al. 2003). Taken together, I conclude that wt Slx5 is detrimental to the growth of these cells, however Slx5-K activity is significantly better for them than having no Slx5 activity at all. Finally, based on these observations, along with the suppression of *tdp1Δ wss1Δ* cells by over-expression of *ULP2* (Fig. 4), it would be reasonable to postulate that Wss1 is involved in de-SUMOylation of one or more substrates in *tdp1Δ* cells.

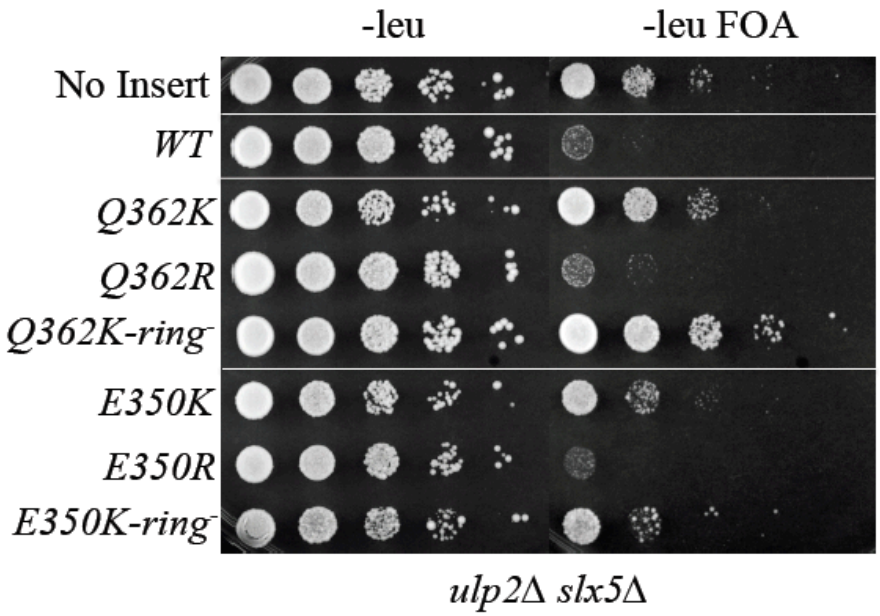


Fig. 23: The *slx5-K* suppressor mutants suppress the *ulp2Δ* slow growth phenotype. PSY3868 (*ulp2Δ slx5Δ*) was transformed first transformed with p*ULP2/ADE3/URA3* (pMS7481), followed by either empty vector or vector containing the indicated *slx5* alleles. Following 10-fold serial dilutions were pinned onto minimal medium lacking leucine, with or without 5-FOA. The plates were photographed following 3 days of growth at 30˚C.

5. The *slx5-K* alleles phenocopy the *SLX5* null mutant in *siz1Δ siz2Δ* cells

In contrast to the observation in *ulp2Δ* cells, *SLX5* is essential for the growth of cells lacking the two major SUMO E3 ligases, namely Siz1 and Siz2 (Wang, Jones et al. 2006, Mullen and Brill 2008). This genetic interaction provided an additional opportunity to study the behavior of *slx5-K* mutants. Consequently, I generated a *siz1Δ siz2Δ slx5Δ* strain in the presence of a balancer plasmid, p*SLX5/ADE3/URA3* through a genetic cross between *siz1Δ siz2Δ* and *slx5Δ* mutants. This strain was transformed with single copy plasmids expressing different *slx5* alleles under the control of its endogenous promoter, and tested for growth in the absence of the balancer plasmid. As shown in Fig. 24 A, the *slx5-K* alleles were unable to complement the loss of *SLX5* in this triple mutant. Once again, the *slx5-R* variants were indistinguishable from WT. Interestingly I also observed that, similar to the observations in the *sgs1Δ slx5Δ* mutant, SUMO-binding as well as the Ub-ligase activity of Slx5 are vital for robust growth of *siz1Δ siz2Δ* cells (Fig. 24 B). These results suggest that the Slx5-K proteins lack a specific Slx5 activity or a sufficient level of Slx5 activity.

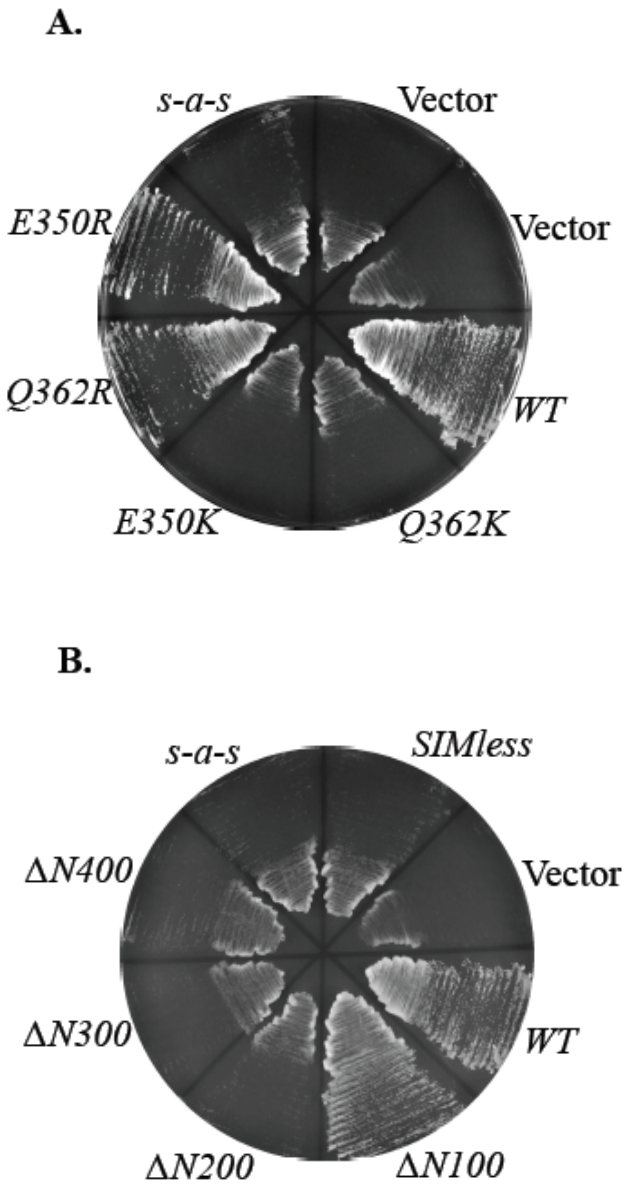


Fig. 24: *siz1Δ siz2Δ* cells need SUMO-targeted ubiquitination activity of Slx5 A. PSY3867 (*siz1Δ siz2Δ slx5Δ*) mutant complemented by a p*SLX5/ADE3/URA3* balancer plasmid was transformed with single copy plasmids expressing the indicated *slx5* constructs. Transformants were streaked on minimal medium plates lacking leucine, but supplemented with 5-FOA to force the loss of the balancer. Growth was recorded following two days of incubation at 30˚C.

A lysine desert is conserved in SUMO-Targeted Ubiquitin Ligases

Upon first discovering the *slx5-Q362K* and *slx5-E350K*, I noted that both alleles had mutations introducing lysines and that they lay outside of the RING domain and the four SUMO-Interaction Motifs (SIMs). I was intrigued by this observation because SIM-mediated SUMO targeting and the Ub-ligase activity are the only known functions of Slx5. To gather more information, I took a closer look at the amino acid sequence to better understand these mutations. Fig. 25 shows a schematic representation of primary structural features of Slx5. Analysis of the protein sequence revealed that there were only two lysines in the first 430 amino acids (~70%) of Slx5, and a stretch of 398 aa within the N-terminus with no lysines. This 398 aa lysine desert is not lacking in basic residues as it contains 60 arginines. It should be noted that lysine residues represent 5.8% of all amino acids in the Expasy database. I then noted that our suppressor mutations mapped to this 398 aa lysine desert. A similar but proportionally smaller region was also identified in Slx5's partner subunit, Slx8.

In order to determine whether the lysine desert was significant or just a random occurrence, I tested whether it was conserved in STUbLs (SUMO Targeted Ubiquitin Ligases) from other fungal species (Fig. 26). Amino acid sequence analysis confirmed the presence of lysine deserts comprising ~50% to 80% of each protein that was analyzed. The conservation of the lysine desert in homodimeric, mammalian homologs (RNF4) highlights the significance of this novel structural element in STUbLs.

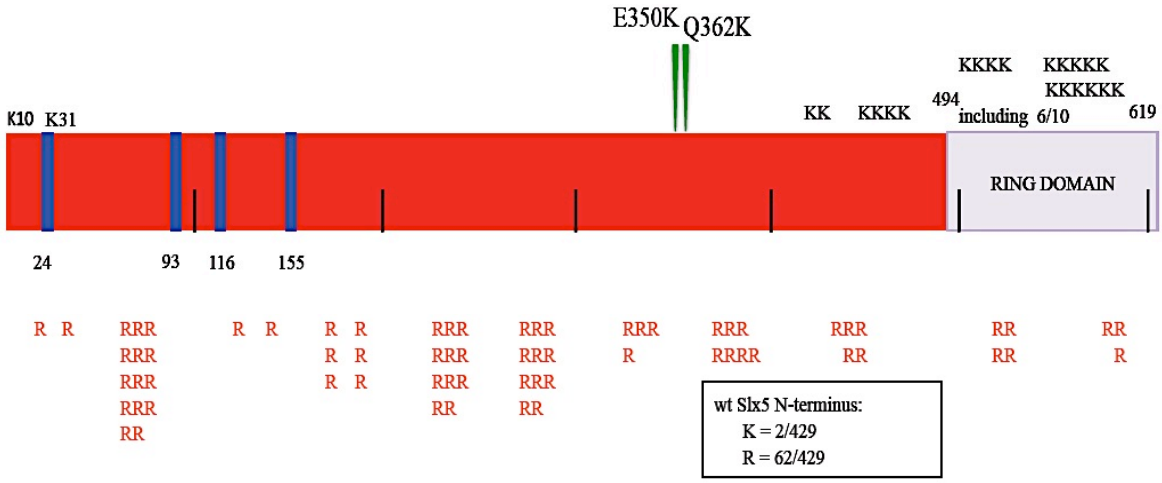


Fig. 25: A lysine desert in yeast Slx5 protein: Shown is a schematic drawing of Slx5 with the red region representing the residues N-terminal of the RING domain. Also shown are the positions of all lysine and arginine residues above and below the schematic, respectively. The relative locations of the suppressor mutations are also shown. Blue bars depict SUMO-Interaction Motifs (SIMs) that are responsible for SUMO-binding. Small vertical back bars designate 100 aa increments.

Fig. 26: Lysine deserts in SUMO-targeted Ubiquitin Ligases. (Upper) Amino acid sequences of Slx5 (I), Slx8 (II) and RNF4 (III) homologs from a variety of fungal and higher-cell organisms were identified and annotated. (Lower) Examples of Ub ligases and SIM-containing proteins from budding yeast were analyzed for comparison. lysine residues are represented by a blue **K** with yellow highlighting, and arginines are indicated by a red R. In each case, the longest aa sequence uninterrupted by a lysine is shaded in gray. The length of the shaded region is presented at the top of each sequence and as a percentage of the total protein. C-terminal RING domains are underlined and, if relevant, SIMs are highlighted in green.

I. Fungal Slx5 homologs.

```
>SceSLX5.619 (S. cerevisiae) (398=64%)
MHSDTNGRTKSNNSPSDNNPNETVILIDSDKEEDASIREANLPVRLYPDRRVGRRRDALNRFVRSDSRSRNSQRTHITASSERPDFQ
ANNDDITIIREVGRFFGDDGPIDPSAHYVDLDQEPGSETLETPRTIQVDNTNGYLNDNGNNNESDDGLTIVEERTTRPRVTLNLPGG
ERLEVTATTTDIPIRRSFEFQEDLGASRRQLLRRSATRARNLFVDRSDENDEDWTDDTHNLPEAIQRARRESRMRMSRRIAERQRRV
QQQRVSSDENISTSIRLQSIRERIQSYTPDIRSAFHRAESLHEFRSILQNVAPITLQECEEELMALFTEFRNQLLQNWAIDRVRNTQ
EEALRLHREALERQERTAGRVFHRGTLRESITNYLNFNGEDGFLSRLWSGPALSDADEERHTQNIIDMIQEREERERDVVMKNLMNK
TRAQQEEFEARAASLPEGYSASFDTTPKMKLDITKNGKEETIIVTDDDLAKTLEDIPVCCLCGAELGVGIPDDFTGISQKDRGVSFE
GLVSKYKFHCPYQTLARPSMLDRDLSKRTFIASCGHAFCGRCFARIDNAKKKSKMPKKKLAQLKGSAHPDNYGPKLCPADSCKKLIR
SRGRLKEVYF

>SpaSlx5.615 (S. paradoxus) (394=64%)
MHGDTNGRTKSNDNPAGNSPNETVILIDSDKEEDASLREANLPVRLYPDRRVGRQRDALNRFVRSDSRNRNSQRTHTTASSESFDPQ
ASNDDLTIIREVGRFFGDDGPIDPSAHYVDLDQEPGSELLETPRTIQVDNTNGYLNDNDNDDGLTIVEERTTRPRVTLNLPGGERLE
VTATTTDIPIRRSFEFQEDLAASRRQLLRRSATRARNLFVDRSDENDEDWTEDTHNLPEAIQSARRESRMRMSRRIAERQRRVQQQR
MSSDENMSTSISLQSIRERIQSYTPDIRSAFHRAESLHEFRSILQNVAPITLQECEEELMALFTEFRNQLLQNWAIDRVRNTQEEAL
RLHREALERQERTTGRVFHRGAFRESITNYLNFNGEDGFLSRLWSGSVLSDADEERHTQNIIDMIQEREERERDVVMKNLMNKTRAQ
QEEFEAKAASLPENYSASFDTTPKMKLDIAKNGKEETIIVTDDDLAKTLEDIPVCCLCGAELGVGIPDDFTGISQKDRGISFEGLVS
KYKFHCPYQTLARPSVLDRDLSKRTFIASCGHAYCGRCFARIDNAKKKSKMPKKKLAQLKGSAHPDNYGPKLCPANSCKKLIRSRGR
LKEVYF

>NcaSlx5.613 (Naumovozyma castellii) (Saccharomycetes) (423=69%)
MSTTTDHHQQDPIITIESDPEDDARIRNDQQQIIRLYPDARTDRPRDSMNRFVDAQGSNRAIRSTPDLSHLNDDQDQDQDDDDDITI
VREIVNPNVARDDAPINPDAEFIDLDQEVPYANATIPRPRTIQIITPSPPQQQQDDDLVITQERTMRPTVTLNLPGGQTLQVDATPT
DQLARSSFEWQENIPLARRQLLRRSTNRANSLFFHDDHESDNDNGSVPLPPNVIRLRQRQQQQNREEPPPPPPPQDDANPIMTRLRT
RINTYSPDIQSAFNHAHSLHEFTSILQNVAPLTLQECGDELLSLFTQYRSQLMVNWATRRVRSSQQNTSSSNHRQRNLRNLRRNGAL
RVTPNANGFNVNLARLFLMNQLGINNGNDEDQYFTGAYNSEDDADHNGDDEDDTQTQNIINMIQRREEQEHDTRTKSFMARTKPQQD
AFKKRANELPENYSANFNTEPTMKLDIKKNGKEETIIVEDNDAAKNYIEVPACTLCGVELGVGIPKDFQGINDTDRGGSFESLVSKY
KFHCPYQSLAKPSQLDKDLSQRVFMASCGHTFCGRCYARIDNAKSKSKLPKKTLKQLKGSSHPDNYGPRVCPADSCRSLLRVKTKMR
EVFF

>YliSlx5.332 (Yarrowia lipolytica) (Saccharomycetes) (179=54%)
MTPQRECDPITVLDDETEVVGSTDCIPEPASEDPFIVISSDEEDNTNTDTGNYRSRTPEIIDISDTERPDEPEEEEEDPRLNMERIF
MNSFQQDQHEYLNNGFGFFRQPHYPVRAERNPGQPSFSPRQFAHYHHFISRLQGIPGLGFGGPNMREDPAFEEDLQRAMRASMEDSL
SNFGPKKVSHPEPPTTTTEGYTRSVRKRQVVCCALCRSDLGVGIPPETKKPSKSSSPKKRKRFSRFSDLTDVERTLSKRMFFTKCGH
VYCGLCVQFIKRRRTSRQKKEDKSNINVDCITDFKGRYVDTPFSLIDHCVVPGCKEKLPRSRGKFFKEMFT
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>CalSlx5.602 (*Candida albicans*) (Saccharomycetes) (430=71%)
MNSDNNPTREPGFKRRKIEPEIRNNNSPQQATSNPGSNDQVIEILSDEETAPPLQSTNTVVHLDTESEDSSLSDIDYRDDDNNGDAN
NDVQIVSVNTANPTPLSTPSSNLNHSGVPTNTNTNNNNNNNNRNGEDRDEDDDDLQILGVRNIYDPNNGRLSTPVGDIPFVREDNDY
TIVEDAPRRNLNPAIARAIRRILDMERRYPLPSQEQIRQILMENRPWMQVSQSEFRDRRVTFDNSAGGGGEEGGGGGGESQPRPQNY
HEALQESARRALAELAAMREAAERAERENSSRQGENSFGFGTPRRSYVELTENETSQPVAVPLPRQRSVHFTEEALRRGNGGPTFSE
SRDGNIIRRERRQPVRYTDSPQFLHNLPFSGAVVHRVPHELRFLGNTFHDVGFALDNLMDDSDTPRGFNSAIERSIMQRIEDDTNRM
IDRRIAQETNYNKKTKNEIEKKIANQDERHTSNIKSNENLVCELCNIVLGEGAPDDFKGDIRYNEKFSKYCETYNCQAPWFCVYPFT
EVDIELSKRIFVAKCGHLFCGRCVKNIGNRPKSRKSKTSNANSSTAEISILNPKFYAPSTCPELQCQKKFSSKSFTEV
YF

>DhaSlx5.412 (Debaryomyces hansenii) (Saccharomycetes) (262=64%)
MPKDPIDMSRNGEYLANMENEGENGALDSQQEVIEILTDDEEDNRESSFGSNNITNSEEPLFVREHNDDDDIQITGANTMNANERAP
RIIRRRSNAAHMDQSRNVRRRHNDDEDDIQIIDERPAERQLIPPVDMLPSYASDSLRRRVQGIETPIGVFQSNENSSTSFSGSESER
GLRYTEPPRRNPLRSRRARRMRPPRNDFVALDHFVLSQQLYHNLYGNWNGHEDLPNADAVEGSVMARIERDNENAVDQRLQNENIFN
RAALKNKKEIAEKELPGYTNDIKPDSNICCDLCGIILGEGIPDDFKPDVYYNDEENFKKFCEDYKVQAPWFCIRQCLPVDVDLSKRV
FVAKCGHIYCGRCVKNIGNRPSGRRKQNQKLTIDNPLVYAPRKCTNSECNLSFTGKKSFTELYF

>KlaSlx5.607 (*Kluvermyces lactis*) (Saccharomycetes) (417=69%)
MTEQGCVIEIDSDPELDAQIREEYAPRRLIPDRRRNRPRDHLSRFVSNAEDRNVAPIAPATDLPVSTDGEDLEITAEVSLRPDGPIN
DDAEYINLDNYRASNNGTVVVDHREGDDDLMLVEERIQESGRPVLLQLPAGQTLQVDASWNELPVGRSFQNQVYRNPNQLRRLRRHI
DHATAMFMGGYNQSGTGDSDFSDNDADYIPPDIVNERNQYTMQTRMREQRERQNQMQRLQRAADETIDSLDPDLRSLFFESQSLSQF
REGLARFNFPPARLEQLLGQYRDFRARAMQSWAEGRRTRSMSYQNRNSLRPQRTRSGTQQNHSRGSNGDSRDNALYQQMLFGMPDDD
ENMDGNNFTGTTQSLQRIISNTMNRRFGNLPFGVGPEFGFYEDGDDAANTELIIRMIQEREERDASTRTKKLNEVTKSQQQKYIDKA
NSLPEGYSASFNTAPLMSMTMENEGREETVMVTDDVAAKTYIDVPVCVLCGVELGVGIPDDWKGVSEEDKGVSFEALQSHYKFHCPY
QTLARPTLVDRDLSRRTYVSSCGHTFCGRCQVRIKNARDISAKEKNKLKNSQGPSHPDNYGPKKCPAEGCNSNLRKKGIMREVYF

>AgoSlx5.557 (*Ashbya gossypii*) (Saccharomycetes) (366=66%)
MTDPNAARNAVIEIESDPEEDQRIINQYRQTRLVPDRRLNRPRDAANRFVSVSGRVAAAAGEGDDEVEIVNEVYLDDEGPIDSTADF
VDLDEQQGPSSSMVINHDGGGDDDVAIIEERTTHPTFLLNLPNGQTLRISGSVHDRPLRRSFETQRTARANMLRRAARSAQRLFMNE
NDGEPSEREASDREYLPQSVLRQRQQALMQERLQRQREHARQHEHIADPQVDALAPELRSIFYHAETLHEVRTMLNASGLGTSAAAM
QDLLQLYMQFRSRQINNWARQRAQEFRHGSAREADHAQDDSASRARLRNPALNAQRRNSFTSYVLARSLGGFGVWPNDFFGDDDEVT
QNIIDIIQAREERDLDSRKRKYMEDTKSQQQAFVARAQRLPEGYYASFDPTPKMKMTLEKNGKTEEVVVADDLAAKSYVEVPVCCLC
GVELGLGIPDDFAGISAVDRGVSFESLVSKYDFHCPYQALAHPSIADRDLSRKTFVAHCGHTFCGRCFARINNAKRFSKISKKRLAE
LHGPSHPDNYGPRVCPAEGCRGQLRTRGKMREVFF

>TdeSlx5.556 (*Torulaspora delbrueckii*) (Saccharomycetes) (364=66%)
MVGGSANYVGPSDPVSNSDDVILIESDDEQHSSRDSGLRSSTTRDTMSPNSNGSNQSEVVSRESSRNADSVSPMGSTAEYIDLEMEP
AGNEEQDDMIVYDERLSTPRDAEDDELIFVQEQTRPSTIQLNLPGRNPMRITATEYDRPARRSFEQQASNRQLRHQTRVARRQPMRR
AARRANHSMNGYESDDGGLTSNRNQINYDPEFAAIQRRLDSYPLDIRSAFLHAQNMNEFSSIIENVDPLTWQDCRYDLERLFMDYRR
MMARRAAQIGQNMPGYRSPPNRTQGNADLLRRLGPLGVRIGIGTGMGESLTHYILEPGIFGYGSFRMIGNPGGFDDGMEEEARTQSI
LNAIQEREEQERDAQTKSNMEKTRSQEENFIEKCKNLPEGYSASFDTTPTTVRLEPLNDGNKQAIVVEDESAIAEWEEIAVCCLCGV
ELGVGLPDDFTGISADDRHLSFDELVTKYCSHCPYQSLTSANGTERALSMKTFVASCGHTFCGRCVVRIENARTHSKSSKKELSKFK
GPLHPDNYGPRLCPATGCKQNIRLGRTKMREAFF

>MguSlx5.402 (*Meyerozyma guilliermondii*) (Saccharomycetes) (223=56%)
MSVNIHRRRSGRALDRESHEGKQDTNLEPSEVNQNENNNRNSILRNSSEPVLESVTDQEVVEVLSDEGDSIEPLSMLSDHEDIEITG
VNMVPQSRIFPSRIRHRRIRPHTRNVRLRRDQDTSSDDIQIVDERPVLPLAQFDDTEEFRRALMGRFGTLESPIGTFEGDFGRVVSV
ITGMNRWRRLADFGSTPRRHLRRRRPVRYTSEPQYELEFPFLEGPSSDEASAMERSIMERIDRENDRDLDGKLRQENKYNTKQLHAK
QAIAANEHHGYTNDINSQTAFTCELCAVPLGTGIPEEFTPNPKYDANLERYSRLYRVQAPWFCSRQISPVDIDLSKRVFVAKCGHVY
CGRCIKNIGNRPRQKRASKQLTIENPGLSCPRRCPAPECNLEFRGKRTFTELYF

>CfaSlx5.379 (*Cyberlindnera fabianii*) (Saccharomycetes) (215=57%)
MPPPEVIEVCSGSEGEDDIQILEARPITDSRRQRSPTRDTRHSRRLVRSSDDVEIVEERQVEPALHEEIYLPDGPHALPNTRRPNRE
NASFDRRRSTRRRQQMRDRRERERQELEREAQELLLDEEIHRGHQPFWVGIRQPLWRSSFDGAQQALNRVWVGRQPNRHQAERQGLT
FEMLNDHFNHLLYGQQNADAGDAIPPDVMAAIQASEERVINKKIEVNYEATKKVQSDIQDRINNIKEPYTCAISEDEEYACVLCGVT
LGGGIPSDFAPPKLTKPLAALQEKNDVQAPYEAMKLVTDVDRDLSKRIFSARCGHTYCGRCVKNISNVKGSMKEVRKKGIKRTDNVI
DNPFVYAPLKCVGGDCQQRLGGRTAFFELFL

>SpRFP1.254 (*S. pombe*) (Schizosaccharomycetes) (163=80%)
MQFNGSNGIDESSVIDLTRSPSPPVETSISSTNIIDLDAIPDDSFPSSPVLSPRRRRMNRRRNERSRNFPSNHLSYLEDMIYLGPQV
STRRSSSRRDLMGMIARTFPEFSSVNSLSPSLFQLIVNRMRFDAIHPEWTNGSDDEYFSNHFEESYDDFTSSLENIKQSYKPPGPPK
SGFTRSFNNDTLMVCPRCQEPLGTSKSKEKSALWATKCGHVYCGSCAKVLKTSKRSQSKCLVNDCGRYLNTKNAMWELFY

>SpRFP2.205 (*S. pombe*) (Schizosaccharomycetes) (115=56%)
MNLHGLELPGRDQRLSPEVIDLTEDIEDDGADVSEVTLLDLTRIPEFQPRRRIRTSRNHLDANLSNVPTINSIPSPVTRPPVAVGGG
IFYGARRTRNRSQTQRRTLLENGFRNSRKKAQDSSNSIAERVSPPPGFCYDVHPHNNIACAKCGNELVSDEKKSIFAAKCGHLFCST
CAKELRKKTVPCPVQHCRKRITKKFIFPLYL

II. Fungal Slx8 homologs.

```
>SceSlx8.274 (134=49%)
MARRPDNQNPEGENLRIKRVRLESVRQNDEEEENEVSRTQNIVTDNRHDSPEAVVEIIGERALENTSEEDGDDDLSLFRALEEDPGS
DHNTSNNDSGNHDRETMHTEEPEASSGNNITLTNNVEELHTMDVLSQTANTPSASPMLDAAPPTTKPGTNSKEQTVDLTADAIDLDA
EEQQVLQISDDDFQEETKEAPKEYGAAKDYRCPICFEPPETALMTLCGHVFCCPCLFQMVNSSRTCRQFGHCALCRSKVYLKDVRLI
ILRKKQVKKKVKS

>AgoSlx8.241 (117=49%)
MAGERGGAALGAPLGGDEADGAQAEEWNTDGGTQESGASSESEAPARKRRRVGRNRQMYDSDDSDYDGSPPQVAVGDSAAGRAAAAT
ARADESGDGSQSSVELADEGSAGGGEEPDIVYLEARGPVSGSVVDVEAEARLQQVVEIPDEEVGEEQTRLGGDRPVECKKAMDYKCP
ICFDPPEAALMTPCGHIYCTVCLFQMVNSSRGYRRNGQCALCRKDVKLKEVGLVVLRKKRVRKQSAE

>KlaSlx8.279  (96=34%)
MVNEDDSLRVVSSSDTTDQLQLVPGLSMGQSTVRTGEDEDERIVTGRRRHELQESETDIEEEEEELRRKSRRISRTSIVSIDDADIL
SETQHVTEIGDTTEQSNLATAPPQEVSDNEEPAIISTADYGVAYQNVQDQSAEAQDQEEEEEEEELHEAVGEGDDDDIKVLTEEEQI
KAQQVIEIDDEDETEGHKSGKDMSETPLETKKAADYVCPICMEPPEAALVTKCGHVFCTTCLYGMVNSSKGNGRRNGLCALCRENVK
LQDLRLIVMRKNRIRKPN

>YliSlx8.254  (89=35%)
MSSTILFSSRFTRRLSLLTISHTPNQVSPVLKPKQSPFFSPTKHHTKLQTVHRQQVQRQAMSGTPLSVHSSASTPESRMLTINDTDT
DNSSQPLYIDSGDEETHNVIDLDASDASDYDSNDDDGLDLSAPPPDQHSKLREVKCPICLEPPDRLCVTECGHLYCGDCVFKALSSG
VRASDSVGECSICRKSVVYKNVVFLETRMGPLLKDDDDEYETDDSIEDDRNVKVQKMHESLTYDLDEVDTLDLTPSLPDA

>CalSlx8.129  (57=44%)
MSTPNVTNISSDDEDDEIEVLEFRKLTQDLIDNPTQPEERRITKKLSDVECPICFDEVRRAITTSCGHVFCLECIERSIS
SSHARGQVRSSQRGRGLCPLCRKQVVFKETIPLKMKKAEKIDKPDLPPK

>NcaSlx8.263  (160=61%)
MGKLRSDGPSKDEPEPREKESVKRRRIDNDHHNEATTGVNDEEESIDVLDNDDDSVEIVHENAAPASPEDLLIRALEEGLAETPSME
DNLHETNATATPALNTDNTQGQTAENIDESVVINSQPVTSNTRTPTGVSAASSVVTPRVLDTSGFDPLIEAIDLEAAEQQVIQIPDD
EIEDEIQRGKKQETYRPVTEYRCPICMDPPETALIAPCGHVFCCDCLFQMVNSSRTYRKDGHCALCRKEVRLRDVKLIILRKKRIKK
VA

>SpoSlx8.269  (161=60%)
MPPAHKRDTNVRNLSAPYNIPSQSARVAAGNAAINRRRSSPVENSPGNGFPVSEDATDYPSGTTSENESLPLNRAPRSLREVASELA
QEETLPVETSDLNIDVESEVFDLEDINFQNDADDINQRFTYNNHPASVENSLTNVNSIHAQPTTISDMIDLTDETSYDPRKQKFEQG
KNPSTTNAEIEKEEPSKKQVVPSSQRLADYKCVICLDSPENLSCTPCGHIFCNFCILSALGTTAATQKCPVCRRKVHPNKVICLEMM
LGSQKKKS

>NcrSlx8.428 (281=66%)
MDDDFPLSDAAYDDIFADLDLDALPPPLSSVAHPNPGVPVPQSLPSLAPLLQQYPAPNPNSNSDNSQIHGQRQQHPHNIPGQTTNIL
HSLSIQPQAPSPANAYITPRTSLPCPHPQARAEPGRHLDNRQSQAAYLPPASRALPPPRPLPPALSFLLSGSSSTPDSRGTSFDNNA
DPALNTNLATGSRAPGLFVTPHRSDREPNSDDFLNELASRDFSSPSLPPQTPRHLGDLLSPSRNSCNNTMPSRRDESAAEGSRRRSR
GTSHNIVSLPTLPPQSSGAPKRKREDGLEIRAHKRKAHVLLSSDDEKDPFGDDNFMDVVDLADTEEVPESMRAKPRPKNEIKLSAFQ
CVICMDNVTGLTVTHCGHLFCSECLHSALTIDPTKRTCPVCRQKIDKAPIGGKWTAKAKGYYPLELKLVTKKSLGKRAAQ
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III. RNF4 homologs.

```
>DmeDegringoladeA.319 (D. melanogaster) (248=78%)
MSDSTIFRFNSSILDQSADSNVSSSSSNSSVSDSSVDSHSSIESNSGHNTSVESQSSAESDSSADSHSSLERPSPVERDLSVESDSS
SATNTSESSVGEASNQLQSPQSNNPLNMANLSVSTEDAHSQIRSLTQDVIQMEAELDRMNRYCEEVVAQIDTFATRTSTPTTRRIRR
RTSPVEVIDLSHLDRAPPVRSARNRDPDAFIDLCTPEGPRSRTVNLHSNDSLTILPRRSAENDPVVDLDVASPPKRVNRDIDESQKE
ELYKCPICMDSVSKREPVSTKCGHVFCRECIETAIRATHKCPICNKKLTARQFFRIYL

>GgaRNF4.194   (G. gallus) (92=47%)
MSTTPRKRRGGSANSRQAQKRSRLIASTTEMASEGEPIELEESAGEEVVDLTCESSDPVVVDLTHNDSIVIVEENQQRRRNLRLRGQ
RQSDSCVLSSDDEDETRDNDVYVTDKVSRELGPLEDETASSKPSGTVSCPICMDGYSEIVQSGRLIVSTKCGHVFCSQCLRGSLRNA
NSCPTCRKKLTHRQYHPIYI

>XlaRNF4.161 (X. laevis)(83=52%)
MTAVTEAIELESGEEVVDLTCESAEPVVVDLTNNDLSINDSVVIVEDTPRQRRAVSRTSQQTSSCVLSSDDEDSRDTEQVGTNKDIS
STGDGSSRSSSGKVSCPICMDCYSEIVQSGRLIVSTKCGHIFCSQCLRDALKNAPSCPTCRKKLNHKQYHPIYI

>DreRNF4.184 (D. rerio)  (123=67%)
MSTTVTQRKRRTSTTCSRRGNSKRNRAQMSQTVMETIDVLENDRTNSEDVVDLTCEGSEPAVVDLTNNDSIVVVEDGVQRRVGPCTE
SYVLSSDEEEESSLRLSPGLLSSLRDSSRARSTPGAISCPVCMDVYSEIMDSGRLMVSTKCGHLFCSQCIRDSLSRAHSCPTCRKKL
THKQYHPIYI
```

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>BtaRNF4.186    (B. taurus)(85=46%)
MSTRKRRGGTVNSRQAQKRTRESTSPPEMALEAEPVELVESAGDEIVDLTCESLEPVVVDLTHNDSVVIVEERRRPRRNGRRLRQDH
ADSCVVSSDEEELARDKDVYVTTHSPRSAATGFRPSGTVSCPICMDGYSEIVQNGRLIVSTECGHIFCSQCLRDSLKNANTCPTCRK
KMSHKRYHPIYI

>RnoRNF4.194 (R. norvegicus)(83=43%)
MSTRNPQRKRRGGAVNSRQTQKRTRETTSTPEISLEAEPIELVETVGDEIVDLTCESLEPVVVDLTHNDSVVIVEERRRPRRNGRRL
RQDHADSCVVSSDDEELSKDKDVYVTTHTPRSTKDEGTTGLRPSGTVSCPICMDGYSEIVQNGRLIVSTECGHVFCSQCLRDSLKNA
NTCPTCRKKINHKRYHPIYI

>MmuRNF4.194    (M. musculus)(85=44%)
MSTRNPQRKRRGGTVNSRQTQKRTRETTSTPEVSLETEPIELVETVGDEIVDLTCESLEPVVVDLTHNDSVVIVEERRRPRRNGRRL
RQDHADSCVVSSDDEELSRDKDVYVTTHTPRSTKDDGATGPRPSGTVSCPICMDGYSEIVQNGRLIVSTECGHVFCSQCLRDSLKNA
NTCPTCRKKINHKRYHPIYI

>HsaRNF4.190 (H. sapiens)(149=78%)
MSTRKRRGGAINSRQAQKRTREATSTPEISLEAEPIELVETAGDEIVDLTCESLEPVVVDLTHNDSVVIVDERRRPRRNARRLPQDH
ADSCVVSSDDEELSRDRDVYVTTHTPRNARDEGATGLRPSGTVSCPICMDGYSEIVQNGRLIVSTECGHVFCSQCLRDSLKNANTCP
TCRKKINHKRYHPIYI
```

IV. Budding yeast SIM-containing proteins.

```
>SceNis1.407 (67=17%)
METYETSIGTQSYPPTLFPPPLGTGGFTTSGYIHALVDSTSNSNSNSNTNSNTNSNTNSNSDTKIPIVQISDDSHITHDSFKPYMEY
HDASHLRNRNISKADQVESTEVMEQFTQWSNYKMRSRSPTINAKPIRHTSQRRTDFTSKNELSKFSKNHNFIFHKGFLKRQHSIRRE
DRQAKVRSRFRSKKELTSVLNYIELEQMDIANVLASQPVNLHAIRNLTSRDPAVTPIPFLRSQMYATSSRPPYLRNRSISRKLPKSQ
PGSLPTTMPATATKTIKQNSTTPTTRSVYNKNVGRSNTSPSVLYHPKRRGKLNTKSHARKEQLLLELWREYLMLVITQRTQLRLTLL
CSPGSASNESSVCSSNASDLDMSLLSTPSSLFQMAGETKSNPIIIPDSQDDSILSSDPF

>SceFir1.876  (58=7%)
MSLPVTPVKSKVCSTLSIQHEMDHDQCRDVLCPRENLLAINRTNNIESVAIPRQRSSKNKKPHEHTQSQVRFSIPDPNEISQDSPLK
IVFPKSGNETERRMSTSSLLMNSHGHLVDMHSKILVDVPKEVWKFHHNRRKKCESRHRKTRSDVRSNTSSSGKEPNHSRSKSLQSII
VDTMNTYRATEVETSINENTSNISQVSPLNLSFDRPPPLTPEKNLYLTPESPLNRYHLPVPLEISLPPYLSPQNKDKKRSSLVYDGD
GYSQFQEGNTSSSTESSLEQPSSSYSDEDDSIPYAHHDVSFELNNADADKLLGIDENANVNLKIQRRNLKNPQHIKSKTDRECEEKN
TEKNVSLKILSTPNKLIDIPDLEHMKSPPSTGLNGTLKFFQQFEPSEEPTSPTRQVNPESLDKLDMSFKFPSSTTNNNVDKVHENRN
SGDTNNEDFLKVDTSPVNQSFESRRQMLIDLQKSPTNNNPRIHKHRRSRSVHNIDDISLNFEATSTPPAPTSAPSIPVEHSNPCTSI
EIPKRSPLRFTSSPKTSDIPPEAQSPKNGSFLQEISVPSIQIIPDESISHTREPSPSLIECPEDENEAFSTEVADHSIAIISETKSV
PSIEPFKPLSSFNSFGQEIQNKEPTPLNQTPTDLIGKQRNCVNPHSIPFSVLSSNSQSSQSGSSKSSYNSEFSSNTAITDTTSQPSV
TINRSTLEHNFIEEKRSIKNLGHGPPSQKNNYSFPRNKNTPSNRHDLDFNTIYEKRDGKMVEVILLDEDEDVGLKNNDISRTRVCHA
QKAKNEQQKKRLSHCNEILGMCDKTADDAKRIIYQLVNEKHKFSEKGQQTKPKKSRVLPPLPFPLYDEKGNSLIPNKYQSSIHNDIP
SHRKLK
```

IV. Budding yeast SIM-containing proteins.

```
>SceNis1.407 (67=17%)
METYETSIGTQSYPPTLFPPPLGTGGFTTSGYIHALVDSTSNSNSNSNTNSNTNSNTNSNSDTKIPIVQISDDSHITHDSFKPYMEY
HDASHLRNRNISKADQVESTEVMEQFTQWSNYKMRSRSPTINAKPIRHTSQRRTDFTSKNELSKFSKNHNFIFHKGFLKRQHSIRRE
DRQAKVRSRFRSKKELTSVLNYIELEQMDIANVLASQPVNLHAIRNLTSRDPAVTPIPFLRSQMYATSSRPPYLRNRSISRKLPKSQ
PGSLPTTMPATATKTIKQNSTTPTTRSVYNKNVGRSNTSPSVLYHPKRRGKLNTKSHARKEQLLLELWREYLMLVITQRTQLRLTLL
CSPGSASNESSVCSSNASDLDMSLLSTPSSLFQMAGETKSNPIIIPDSQDDSILSSDPF

>SceFir1.876  (58=7%)
MSLPVTPVKSKVCSTLSIQHEMDHDQCRDVLCPRENLLAINRTNNIESVAIPRQRSSKNKKPHEHTQSQVRFSIPDPNEISQDSPLK
IVFPKSGNETERRMSTSSLLMNSHGHLVDMHSKILVDVPKEVWKFHHNRRKKCESRHRKTRSDVRSNTSSSGKEPNHSRSKSLQSII
VDTMNTYRATEVETSINENTSNISQVSPLNLSFDRPPPLTPEKNLYLTPESPLNRYHLPVPLEISLPPYLSPQNKDKKRSSLVYDGD
GYSQFQEGNTSSSTESSLEQPSSSYSDEDDSIPYAHHDVSFELNNADADKLLGIDENANVNLKIQRRNLKNPQHIKSKTDRECEEKN
TEKNVSLKILSTPNKLIDIPDLEHMKSPPSTGLNGTLKFFQQFEPSEEPTSPTRQVNPESLDKLDMSFKFPSSTTNNNVDKVHENRN
SGDTNNEDFLKVDTSPVNQSFESRRQMLIDLQKSPTNNNPRIHKHRRSRSVHNIDDISLNFEATSTPPAPTSAPSIPVEHSNPCTSI
EIPKRSPLRFTSSPKTSDIPPEAQSPKNGSFLQEISVPSIQIIPDESISHTREPSPSLIECPEDENEAFSTEVADHSIAIISETKSV
PSIEPFKPLSSFNSFGQEIQNKEPTPLNQTPTDLIGKQRNCVNPHSIPFSVLSSNSQSSQSGSSKSSYNSEFSSNTAITDTTSQPSV
TINRSTLEHNFIEEKRSIKNLGHGPPSQKNNYSFPRNKNTPSNRHDLDFNTIYEKRDGKMVEVILLDEDEDVGLKNNDISRTRVCHA
QKAKNEQQKKRLSHCNEILGMCDKTADDAKRIIYQLVNEKHKFSEKGQQTKPKKSRVLPPLPFPLYDEKGNSLIPNKYQSSIHNDIP
SHRKLK
```

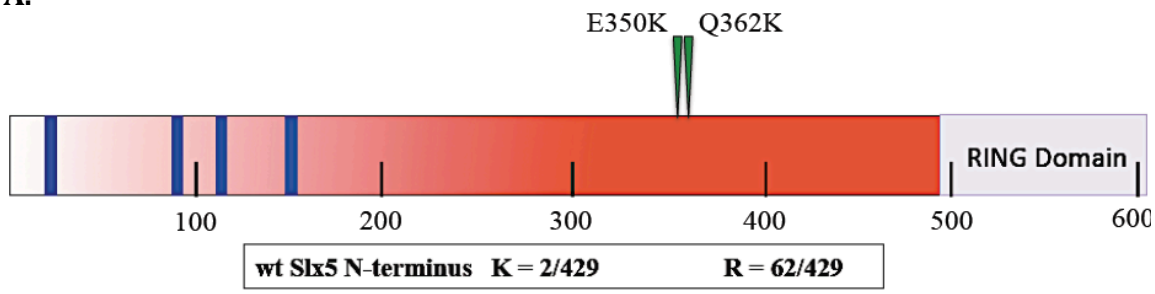
```
>SceRad5.1169   (59=5%)
MSHIEQEERKRFFNDDLDTSETSLNFKSENKESFLFANSHNDDDDDVVVSVSDTTEGEGDRSIVPVRREIEEEGQNQFITELLRIIP
EMPKDLVMELNEKFGSQEEGLSLALSHYFDHNSGTSISKIPSSPNQLNTLSDTSNSTLSPSSFHPKRRRIYGFRNQTRLEDKVTWKR
FIGALQVTGMATRPTVRPLKYGSQMKLKRSSEEISATKVYDSRGRKKASMASLVRIFDIQYDREIGRVSEDIAQILYPLLSSHEISF
EVTLIFCDNKRLSIGDSFILQLDCFLTSLIFEERNDGESLMKRRRTEGGNKREKDNGNFGRTLTETDEELESRSKRLALLKLFDKLR
LKPILDEQKALEKHKIELNSDPEIIDLDNDEICSNQVTEVHNNLRDTQHEEETMNLNQLKTFYKAAQSSESLKSLPETEPSRDVFKL
ELRNYQKQGLTWMLRREQEFAKAASDGEASETGANMINPLWKQFKWPNDMSWAAQNLQQDHVNVEDGIFFYANLHSGEFSLAKPILK
TMIKGGILSDEMGLGKTVAAYSLVLSCPHDSDVVDKKLFDIENTAVSDNLPSTWQDNKKPYASKTTLIVVPMSLLTQWSNEFTKANN
SPDMYHEVYYGGNVSSLKTLLTKTKTPPTVVLTTYGIVQNEWTKHSKGRMTDEDVNISSGLFSVNFYRIIIDEGHNIRNRTTVTSKA
VMALQGKCKWVLTGTPIINRLDDLYSLVKFLELDPWRQINYWKTFVSTPFESKNYKQAFDVVNAILEPVLLRRTKQMKDKDGKPLVE
LPPKEVVIKRLPFSKSQDLLYKFLLDKAEVSVKSGIARGDLLKKYSTILVHILRLRQVCCHPGLIGSQDENDEDLSKNNKLVTEQTV
ELDSLMRVVSERFDNSFSKEELDAMIQRLKVKYPDNKSFQSLECSICTTEPMDLDKALFTECGHSFCEKCLFEYIEFONSKNLGLKC
PNCRNQIDACRLLALVQTNSNSKNLEFKPYSPASKSSKITALLKELQLLQDSSAGEQVVIFSQFSTYLDILEKELTHTFSKDVAKIY
KFDGRLSLKERTSVLADFAVKDYSRQKILLLSLKAGGVGLNLTCASHAYMMDPWWSPSMEDQAIDRLHRIGQTNSVKVMRFIIQDSI
EEKMLRIQEKKRTIGEAMDTDEDERRKRRIEEIQMLFE
```

Overcoming *tdp1Δ wss1Δ* lethality by disruption of Slx5's lysine desert.

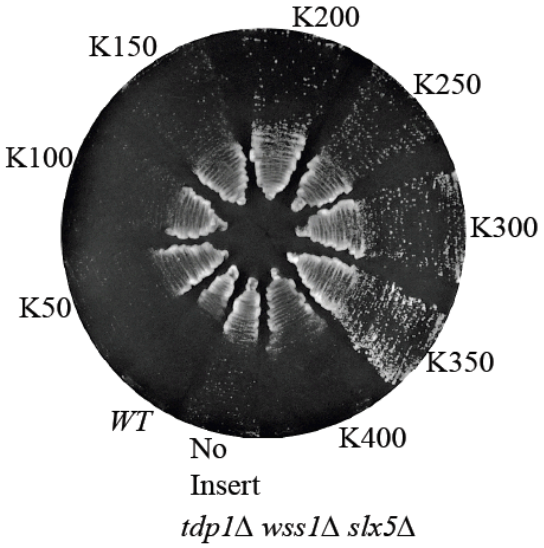
As described above, I learned that *slx5-K* mutations suppressed the synthetic-lethal phenotype of *tdp1Δ wss1Δ* mutants and that the suppression was specifically dependent on the lysine and a functional RING domain. Additionally, the mutations in these alleles disrupt a region of Slx5 that is largely devoid of lysine residues. To comprehend the need for a lysine desert, so well conserved across species, I decided to make systematic disruptions in this region again utilizing the lethality of *tdp1Δ wss1Δ* cells.

First, I constructed individual *slx5* alleles, each carrying a unique lysine mutation, to systematically disrupt the lysine desert at 50 amino acid intervals. Each mutant gene was subcloned into a single copy plasmid, each of which was then used to individually transform a *tdp1Δ wss1Δ slx5Δ* strain complemented by the p*WSS1/ADE3/URA3* balancer. Single colonies from each transformation were subsequently streaked on minimal medium supplemented with 5-FOA to select against the balancer. Introduction of lysine in Slx5's at the very N-terminal region did not suppress the synthetic lethality of *tdp1Δ wss1Δ*. However, the lysines introduced at amino acid positions 150, 200, 250 and 300 restored viability to these cells with the effect being strongest with the *slx5-K300* allele based on growth rate (Fig. 27 B). All the variants were also tested for the ability to complement *sgs1Δ slx5Δ* cells (Fig. 27 C). With the exception of *slx5-K400,* all variants complemented this mutant, irrespective of their differences in *tdp1Δ wss1Δ* cells. .

A.


E350K
Q362K
RING Domain
100
200
300
400
500
600
wt Slx5 N-terminus K = 2/429 R = 62/429

B.


K200
K150
K250
K100
K300
K50
K350
WT
No Insert
K400
tdp1Δ wss1Δ slx5Δ

C.

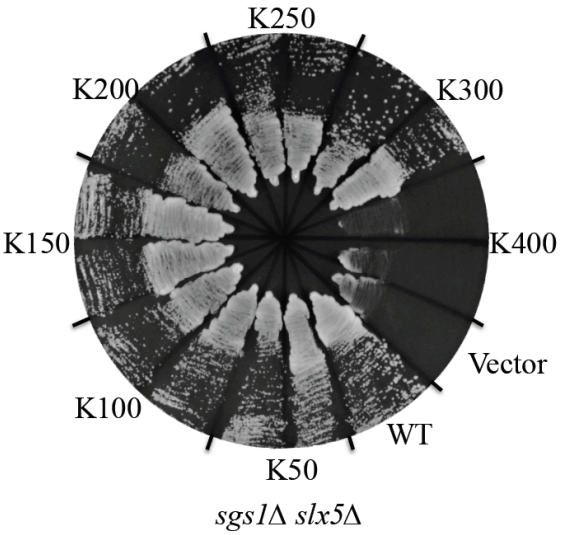

K250
K200
K300
K150
K400
Vector
K100
WT
K50
sgs1Δ slx5Δ

Fig. 27 Systematic disruption of the Slx5 lysine desert A. A diagram of Slx5 protein showing SIMs (blue bars), the RING domain, and the relative locations of original suppressor lysine mutations. **B.** The strain *tdp1Δ wss1Δ slx5Δ* (PSY3710) carrying the pWSS1/ADE3/URA3 plasmid, was transformed with single-copy plasmids expressing indicated *SLX5* mutant genes and streaked on minimal medium plates containing 5-FOA but lacking leucine. Plates were photographed following 2 days of incubation at 30˚C. **C.** An *sgs1Δ slx5Δ* (NJY2462) mutant kept alive by *pSGS1/ADE3/URA3* was transformed with the same plasmids as in (B). Transformants were then streaked onto synthetic complete medium supplemented with 5-FOA and allowed to grow for 3 days at 30˚C before photographing.

The lysine desert: A self-preservation mechanism

Based on the data accumulated so far, I hypothesized that the lysine desert in STUbLs was a means against self-degradation via auto-ubiquitination. The availability of lysine residues (structure-permitting) in these enzymes could render them highly susceptible to auto-ubiquitination, followed by proteasome-mediated degradation. I tested this hypothesis by first determining steady state levels of Slx5-K variants and the wild type protein. Single copy plasmids expressing $(His)_6$- tagged versions of *SLX5, slx5-Q362K, slx5-Q362R, slx5-E350K* and *slx5-E350R* were transformed into cells carrying *slx5Δ* alone. Exponentially growing cultures were harvested for each transformant to obtain cells equivalent to OD_{600}= 20. Cells were lysed under denaturing conditions for maximum lysis efficiency and to minimize proteolysis. These extracts were then used to perform His-tag assisted pull downs using Ni-NTA agarose beads. In accordance with our predictions, the amount of Slx5-K protein was lower in each mutant's case (Fig. 28 A). This supports at least part of the hypothesis that disruption of the lysine desert in Slx5 leads to a reduction in protein level. Since the levels of Slx5-R proteins were no different than the natural protein, it can be safely assumed that introducing a positively charged residue at amino acid 350 or 362 does not alter the protein structure and hence the Slx5-K mutants have relatively normal structural stability, especially since arginine side chains have a stronger positive charge than the lysine side chains.

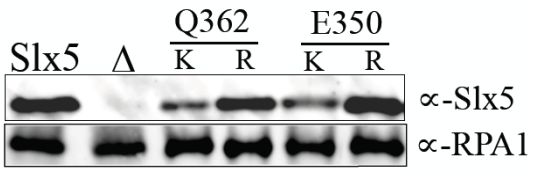
As a further test of our hypothesis, I wanted to know if the lower expression is due to auto-ubiquitination or mediated through another Ub-ligase, and if so,

whether it required proteasomal degradation. If the turnover was self-inflicted, then inactivating the Ub-ligase (using our well-studied ring$^-$ allele containing the "SAS" triple mutation in the RING domain, ref) in a compound allele should restore the levels of the Slx5-K proteins. For this purpose, I again used cultures from *slx5Δ* transformed (as above) with plasmids expressing $(His)_6$-tagged Slx5-Q362K-ring$^-$ and Slx5-E350K-ring$^-$, in addition to those used before. Denaturing extracts were prepared and treated as before. Indeed, the inactivation of ligase brought about a significant enhancement in the abundance of Slx5-K proteins. This suggests that it is not abnormally low synthesis of Slx5-K proteins but an increase in degradation that leads to their lower abundance. Additionally, the degradation appears to be mediated via auto-ubiquitination as the levels of the compound Slx5-K-ring$^-$ mutant were greater than the Slx5-K mutants. Since the steady state levels of the compound mutants were greater than that of the Slx5-R variants and wild type proteins (Fig. 28 B), then it is likely that wt Slx5 is normally subject to a low level of auto-ubiquitination and degradation. As shown in Figure 28 D, this idea was confirmed by immunoblotting wt and *slx5-ring$^-$* cells.

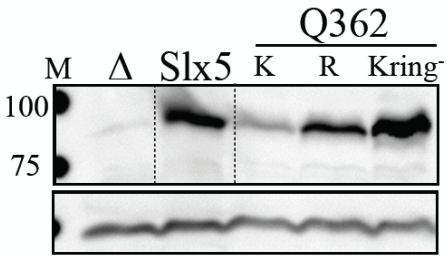
Finally, to ultimately prove the above proteolytic model, I attempted to show that the decrease in Slx5-K abundance was due to proteasomal degradation as opposed to another pathway. To prove this, I treated *slx5-E350K* cells with the proteasomal inhibitor MG132. As shown in Figure 28 C, the levels of Slx5-E350K were increased following MG132 treatment. Slx5-K proteins were also better

substrates for auto-ubiquitination, which unlike the touted SUMO-targeting activity didn't care for SUMO modification (Fig. 28 D)

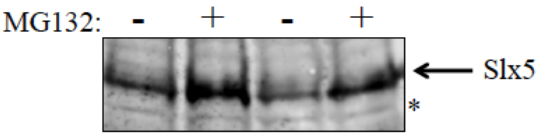
In summary, the *tdp1Δ wss1Δ* suppressor screen selected for mutations in *SLX5* that resulted in a single lysine residue being inserted into a conserved lysine desert and rendering the proteins subject to auto-ubiquitination and proteasomal turnover. Under these conditions, the level of mutant Slx5-K protein was low enough to eliminate the detrimental effects caused by the Slx5 SIMs, and high enough to provide optimal growth to the *tdp1Δ wss1Δ* strain and the *ulp2Δ* mutant. However, the levels of Slx5-K protein were insufficient to provide growth to *siz1Δ siz2Δ* cells or optimal growth to *sgs1Δ* cells.



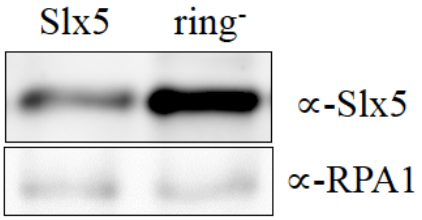
A.



B. α-Slx5 (lower panel is a non-specific signal used as control)

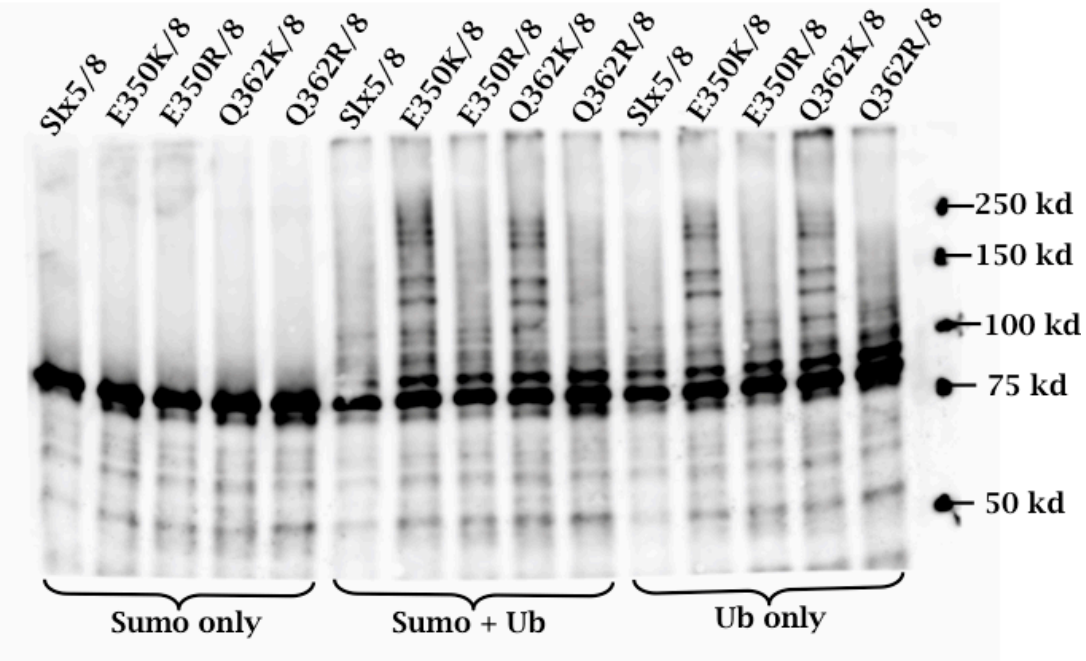


C.



D.

E.


Slx5/8
E350K/8
E350R/8
Q362K/8
Q362R/8
Slx5/8
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Q362K/8
Q362R/8
Slx5/8
E350K/8
E350R/8
Q362K/8
Q362R/8
250 kd
150 kd
100 kd
75 kd
50 kd
Sumo only
Sumo + Ub
Ub only

Fig. 28: Slx5-K mutants undergo auto-ubiquitination and proteasome-mediated degradation A. An *slx5Δ* (PSY3884) strain was transformed with single copy plasmids expressing either no insert or indicated $(His)_6$ tagged *slx5* alleles. Cells equivalent to OD_{600}=20 were harvested from log-phase cultures and lysed under denaturing conditions. Extracts were incubated with Ni-NTA agarose beads to pull down the proteins. Samples were subjected to SDS-PAGE and immunoblotted using a-Slx5 rabbit anti-serum. **B.** Exponentially growing cultures of *slx5Δ* (PSY3884) transformants were harvested to obtain cells equivalent to OD_{600}= 10.0. Denaturing extracts were directly subjected to SDS-PAGE and immuno-blotted with antibody against Slx5. The lower panel displays a non-specific band in each lane and shows equal loading of samples. **C.** The *slx5-E350K* mutant was grown to an OD=0.5 and was treated for 2 hours with either 75 μM MG132 in DMSO (+) or DMSO alone (-). Following the treatment, NaOH/TCA extracts were made from 10 OD of cells, and a portion corresponding to 0.25 OD was subjected to immuno-blotting as above. **D.** Either wt or *slx5-ring*$^-$ cells were immuno-blotted as above while control extracts were immunoblotted with antibody against the large subunit of RPA as an internal loading control. **E.** SUMOylation assays were carried out using 30 nM Aos1/Uba2, 200nM Ubc9, 1μM SMT3,1mM creatine phosphate, 0.1 mg/ml creatine phosphate kinase, in the presence of 2mM ATP, 500ng Slx5/8 dimer, 20 min at 30°C.
The Ubiquitination conditions were 20nM Ube1, 200nM Ubc4, 1μM Ub, 1mM creatine phosphate, 0.1mg/ml creatine phosphate kinase, 2mM ATP and 500ng Slx5/8 dimer, 35 min at 30°C. Reactions were stopped by addition for 3x Laemmli's buffer, subjected to SDS-PAGE and immuno-blotted against Slx5.

Discussion

In this chapter, I have described the novel *slx5-Q362K* and *slx5-E350K* alleles obtained in a forward genetic screen. These two alleles were found to have an interesting mix of *slx5Δ* and *SLX5* phenotypes. The superior suppression conferred by the *slx5-k* alleles suggests that only part of the Slx5-Slx8 activity is harmful to the cells lacking *TDP1* and *WSS1.* This is consistent with the observation that neither *slx8Δ* (Fig 5) nor the enzymatically inactive *slx5-ring*$^{-}$ (Fig. 7) suppressed the lethal phenotype of *tdp1Δ wss1Δ* mutation. In the previous chapter, I also discussed that over-expression of *SLX5*, derived from the SIMs in the N-terminal region, is toxic to *tdp1Δ* and *wss1Δ* single mutants. Thus, a strain carrying *tdp1Δ wss1Δ* mutation appears to be sensitive to the SIMs even at the endogenous level of expression. This is confirmed by the observation that an allele lacking the SIMs, *slx5-SIMless* mimicks the phenotype of *slx5-K* mutants in the *tdp1Δ wss1Δ* genetic background (Fig. 19). Due to this result and the observation that another allele inserting an artificial SUMOylation consensus site in Slx5 could restore viability to *tdp1Δ wss1Δ* cells, I was tempted to think that the Slx5-K mutant proteins undergo SUMO-modification at the Lys substitution. Further, I hypothesized that this could result in an intra-molecular, physical interaction between the SIMs and the modifying SUMO moieties occluding the SIMs from other SUMOylated substrates. Additional supporting evidence for this hypothesis is the fact that the suppression of *tdp1Δ wss1Δ* by the *slx5-K* alleles can be reversed by expressing just the N-terminal SIM containing fragment of Slx5 (Appendix 1). As alluded to previously, *tdp1Δ wss1Δ* mutants are more sensitive to the region of Slx5 than either single mutant, accordingly the above

reversal of suppression did not require overexpression of the N-terminal fragment. As fascinating as this theory is, it has several weaknesses. First, neither Lys 350 nor Lys 362 was predicted to undergo SUMOylation by a web-based software, SUMOPlot™ (see Appendix 2). Second, although the artificial site exactly matches the SUMOylation consensus motif, the possibility of a Ub conjugation at the same site cannot be excluded. Further, Slx5-K proteins pulled down from denatured cell extracts (supplemented with protease inhibitors) showed no obvious SUMO-modification (anti-SUMO blots were not shown). The absence of SUMOylated Slx5 in these extracts, in fact, favors the idea of Ub-attachment, in which case modified forms would be undetectable owing to immediate degradation of such species (at least in cells carrying wt Ub and no proteasome inhibition). Furthermore, *slx5-K200, -K250, -K300* alleles phenocopied the original suppressor alleles (and the artificial SUMO-site allele) despite the fact that these Lys substitutions are not a part of any SUMOylation consensus motif. Therefore, the mechanism of suppression achieved by the *slx5-K* alleles is probably mediated by a Ub-modification at the lysine substitutions.

The partial complementation *of sgs1Δ slx5Δ* lethality by the *slx5-k* mutants and their weak SUMO-defective phenotypes suggest that the steady state levels of Slx5-K proteins are lower than wt, regardless of the genetic background. Thus, the unique phenotypes of *slx5-K* alleles are independent of the cellular *TDP1* and *WSS1* status. These Lys residues perhaps cause some change in the overall structure of the protein, however such variation is not likely to be the source of *slx5-K* phenotypes.

This is evident from the sharp contrast between phenotypes of *slx5-K* and *slx5-R* alleles. The collective data suggests that the Lys substitutions are sites for protein modification, most likely ubiquitination.

Ub-conjugation is well known to target the modified proteins to the 26S proteasome (Deveraux, Ustrell et al. 1994). I examined the steady state levels of Slx5-K variants with respect to WT protein and found that the Slx5-K proteins are indeed less abundant (Fig. 28 A). More interestingly, the lower abundance is almost certainly due to auto-ubiquination (Fig. 28 B). In addition, I showed that the auto-ubiquitination leads to proteasomal degradation because the levels of Slx5-E350K protein increased in cells treated with the proteasome inhibitor, MG132. These observations, together with the conservation of the lysine deserts in STUbLs suggested that wt Slx5 could undergo the same auto-ubiquitination-dependent turnover using its natural lysine residues. I confirmed this by showing that the levels of Slx5-ring$^-$ protein are indeed elevated compared to wt Slx5. The simplest explanation of this result is that the Slx5-Slx8 Ub-ligase is needed to reduce Slx5 protein levels. If this is true, then one would predict that the levels of Slx5 would be elevated in an *slx8Δ* or slx8-ring$^-$ strain. Indeed, a search of the literature provided evidence in support of this idea (Ii, Fung et al. 2007).

Epilogue

This study advances the current understanding of the role played by the SUMO pathway in orchestrating DNA repair. A previously reported link between *TDP1*and the SUMO pathway was explored further revealing greater involvement of SUMO in genome maintenance than was known.

The role of *WSS1* in SUMO de-conjugation was also confirmed. Further studies are expected to reveal whether this aspect of Wss1 function in the cell is direct or indirect.

This work identifies a novel structural feature in STUbLs that is evolutionarily conserved and was termed lysine desert due to the region being devoid of Lys residues. Additionally, a probable auto-regulation mechanism for the yeast STUbL Slx5-Slx8 was also discussed.

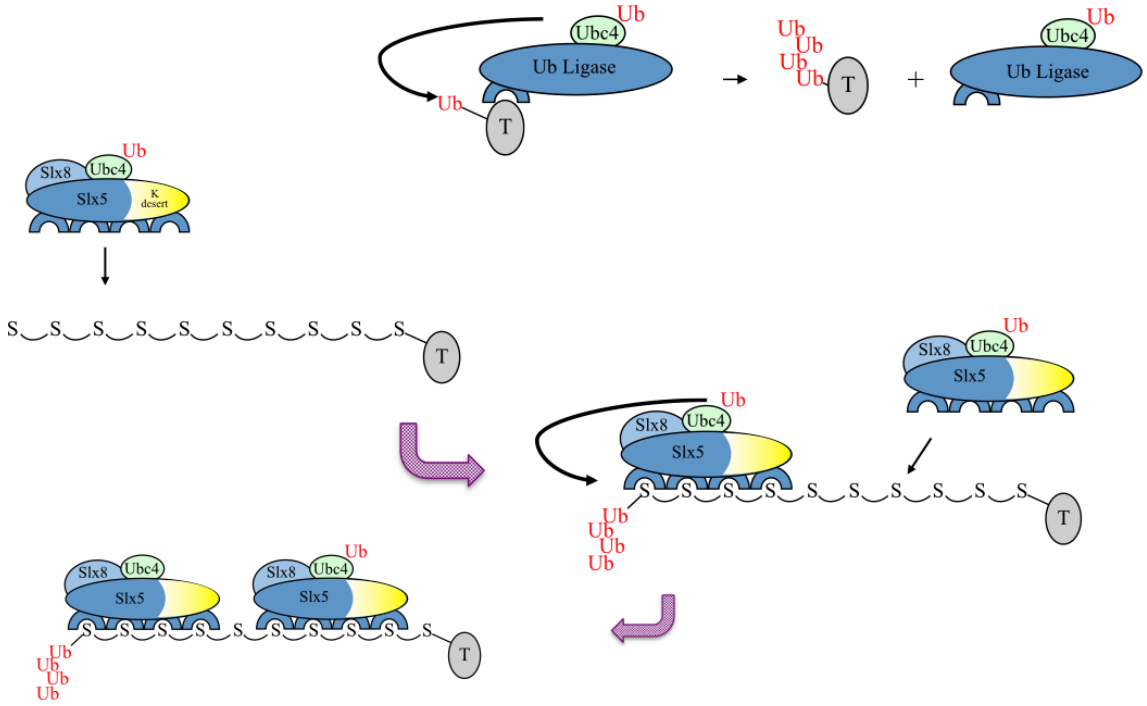

Fig 29: The lysine desert in STUbLs prevents self-destruction via auto-ubiquitination

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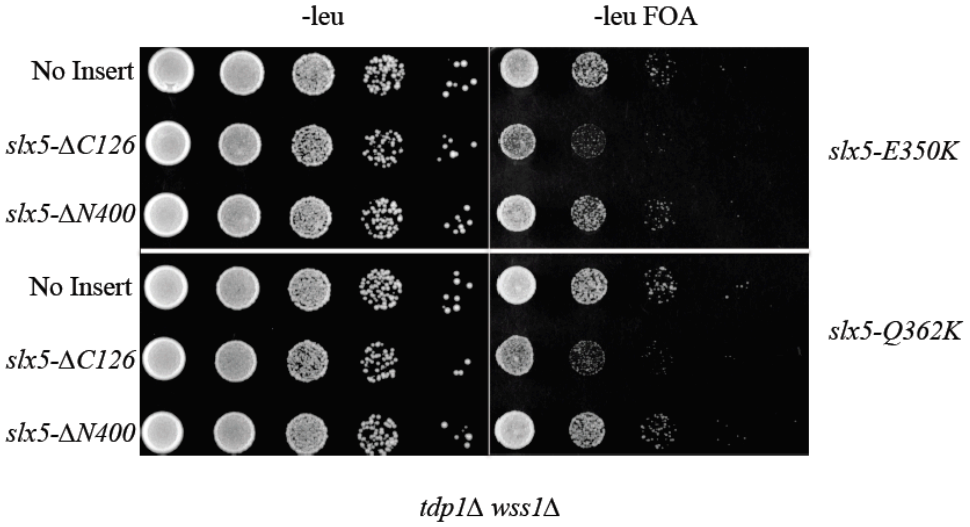
Appendix 1: The N-terminal region of Slx5 is deleterious to *tdp1Δ wss1Δ* mutants. The original suppressed strains, *tdp1Δ wss1Δ slx5-E350K* and *tdp1Δ wss1Δ slx5-Q362K* were first transformed with pNJ7478 (*WSS1/ADE3/URA3*) followed by transformations with single copy plasmids expressing indicated Slx5 truncations. Transformants were serially diluted and pinned on selective plates with or without 5-FOA. Plates were photographed following 3 days of incubation at 30˚C



Appendix 2: Predictions of SUMOylation probability using SUMOplot™ software. The web-based program predicts probability of a protein sequence of being modified by SUMO. Motifs highlighted in red are predicted to be the most favored SUMOylation site and motifs with low probability are indicated as blue.

2-1: A snapshot of SUMOylation site prediction for Slx5.

Protein ID:	gi|398364839|ref|NP_010271.3|
Defintion:	SUMO-targeted ubiquitin ligase complex subunit SLX5 [Saccharomyces cerevisiae S288c]
Length:	619 aa

```
  1 MHSDTNGRTK SNNSPSDNNP NETVILIDSD KEEDASIREA NLPVRLYPDR
 51 RVGRRRDALN RFVRSDSRSR NSQRTHITAS SERPDFQANN DDITIIREVG
101 RFFGDDGPID PSAHYVDLDQ EPGSETLETP RTIQVDNTNG YLNDNGNNNE
151 SDDGLTIVEE RTTRPRVTLN LPGGERLEVT ATTTDIPIRR SFEFQEDLGA
201 SRRQLLRRSA TRARNLFVDR SDENDEDWTD DTHNLPEAIQ RARRESRMRM
251 SRRIAERQRR VQQQRVSSDE NISTSIRLQS IRERIQSYTP DIRSAFHRAE
301 SLHEFRSILQ NVAPITLQEC EEELMALFTE FRNQLLQNWA IDRVRNTQEE
351 ALRLHREALE RQERTAGRVF HRGTLRESIT NYLNFNGEDG FLSRLWSGPA
401 LSDADEERHT QNIIDMIQER EERERDVVMK NLMNKTRAQQ EEFEARAASL
451 PEGYSASFDT TPKMKLDITK NGKEETIIVT DDDLAKTLED IPVCCLCGAE
501 LGVGIPDDFT GISQKDRGVS FEGLVSKYKF HCPYQTLARP SMLDRDLSKR
551 TFIASCGHAF CGRCFARIDN AKKKSKMPKK KLAQLKGSAH PDNYGPKLCP
601 ADSCKKLIRS RGRLKEVYF
```

Motifs with high probability
Motifs with low probability
Overlapping Motifs

2-2: A snapshot of SUMOylation site prediction for Slx5-Q362K (Arrow points to the location of substitution)

Protein ID:	N/A
Defintion:	N/A
Length:	619 aa

```
  1 MHSDTNGRTK SNNSPSDNNP NETVILIDSD KEEDASIREA NLPVRLYPDR
 51 RVGRRRDALN RFVRSDSRSR NSQRTHITAS SERPDFQANN DDITIIREVG
101 RFFGDDGPID PSAHYVDLDQ EPGSETLETP RTIQVDNTNG YLNDNGNNNE
151 SDDGLTIVEE RTTRPRVTLN LPGGERLEVT ATTTDIPIRR SFEFQEDLGA
201 SRRQLLRRSA TRARNLFVDR SDENDEDWTD DTHNLPEAIQ RARRESRMRM
251 SRRIAERQRR VQQQRVSSDE NISTSIRLQS IRERIQSYTP DIRSAFHRAE
301 SLHEFRSILQ NVAPITLQEC EEELMALFTE FRNQLLQNWA IDRVRNTQEE
351 ALRLHREALE RKERTAGRVF HRGTLRESIT NYLNFNGEDG FLSRLWSGPA
401 LSDADEERHT QNIIDMIQER EERERDVVMK NLMNKTRAQQ EEFEARAASL
451 PEGYSASFDT TPKMKLDITK NGKEETIIVT DDDLAKTLED IPVCCLCGAE
501 LGVGIPDDFT GISQKDRGVS FEGLVSKYKF HCPYQTLARP SMLDRDLSKR
551 TFIASCGHAF CGRCFARIDN AKKKSKMPKK KLAQLKGSAH PDNYGPKLCP
601 ADSCKKLIRS RGRLKEVYF
```

Motifs with high probability
Motifs with low probability
Overlapping Motifs

2-3: A snapshot of SUMOylation site prediction for Slx5-E350K (Arrow points to the location of substitution)

Protein ID:	N/A
Defintion:	N/A
Length:	619 aa

```
  1 MHSDTNGRTK SNNSPSDNNP NETVILIDSD KEEDASIREA NLPVRLYPDR
 51 RVGRRRDALN RFVRSDSRSR NSQRTHITAS SERPDFQANN DDITIIREVG
101 RFFGDDGPID PSAHYVDLDQ EPGSETLETP RTIQVDNTNG YLNDNGNNNE
151 SDDGLTIVEE RTTRPRVTLN LPGGERLEVT ATTTDIPIRR SFEFQEDLGA
201 SRRQLLRRSA TRARNLFVDR SDENDEDWTD DTHNLPEAIQ RARRESRMRM
251 SRRIAERQRR VQQQRVSSDE NISTSIRLQS IRERIQSYTP DIRSAFNAE
301 SLHEFRSILQ NVAPITLQEC EEELMALFTE FRNQLLQNWA IDRVRNTQEK
351 ALRLHREALE RQERTAGRVF HRGTLRESIT NYLNFNGEDG FLSRLWSGPA
401 LSDADEERHT QNIIDMIQER EERERDVVMK NLMNKTRAQQ EEFEARAASL
451 PEGYSASFDT TPKMKLDITK NGKEETIIVT DDDLAKTLED IPVCCLCGAE
501 LGVGIPDDFT GISQKDRGVS FEGLVSKYKF HCPYQTLARP SMLDRDLSKR
551 TFIASCGHAF CGRCFARIDN AKKKSKMPKK KLAQLKGSAH PDNYGPKLCP
601 ADSCKKLIRS RGRLKEVYF
```

Motifs with high probability

Motifs with low probability

Overlapping Motifs