Normal levels of cellular sumoylation are largely dispensable for growth but facilitate heat tolerance in yeast

Marjan Moallem*, Akhi Akhter*, John Babu*, Giovanni L. Burke,

Farhaan Khan, Benjamin G. Bergey, Mohammad S. Baig,

J. Bryan McNeil, and Emanuel Rosonina[#]

Running Title: Effects of reduced sumoylation on cell fitness

Department of Biology, York University, Toronto, Ontario, Canada

*Equal contributions

#Address correspondence to Emanuel Rosonina, rosonina@yorku.ca

ABSTRACT

Sumoylation is an essential, conserved protein modification with hundreds of targets. Compared to the related modification ubiquitination, a small number of enzymes are involved in SUMO conjugation and deconjugation, including a single conjugase, Ubc9, in yeast and mammals. This suggests that cells can simultaneously control the sumoylation level of numerous proteins by regulating just one enzyme of the SUMO pathway. Such modulated levels of cellular sumoylation are observed in response to a number of stress conditions, which typically cause a rapid and dramatic increase in overall sumoylation. Here, we demonstrate that ploidy, culture density, and nutrient availability also affect global sumoylation levels in yeast. To determine the effects of modulated cellular sumoylation levels on cell growth, we examined engineered yeast strains that harbour reduced global sumoylation. Remarkably, reducing SUMO conjugation levels by >75% has no effect on cell fitness, indicating that most sumoylation events are not required for normal growth. Surprisingly, strains with constitutively low sumoylation levels show no growth defects when exposed to a number of stress conditions except that they are highly sensitive to elevated temperatures. Consistent with the fact that Ubc9 and SUMO are essential, however, cells displaying less than ~5% of normal sumoylation levels show significantly impaired growth, even at normal temperatures. Finally, we demonstrate that many sumoylation events are highly transient, requiring constant de novo sumoylation to maintain steady state levels. Together, our results suggest that cells need only a low level of sumoylation for growth, but that normal levels are required pre-emptively to facilitate survival when temperatures rise.

INTRODUCTION

Nearly one tenth of all human and budding yeast proteins have been identified as putative targets of SUMO post-translational modification in normal growth conditions, many of which are involved in gene expression and chromatin maintenance or regulation (Makhnevych *et al.* 2009; Albuquerque *et al.* 2015; Hendriks *et al.* 2017; Esteras *et al.* 2017; Zhao 2018). Sumoylation involves the covalent attachment of a SUMO peptide to the side chain of specific Lys residues, through isopeptide bonds, that often lie within a SUMO consensus motif (Flotho and Melchior 2013; Hendriks *et al.* 2017). The effects of protein sumoylation are largely target-specific, but include altered localization, activity, stability, and association with chromatin (Flotho and Melchior 2013; Chymkowitch *et al.* 2015; Rosonina *et al.* 2017; Zhao 2018). At the molecular level, these effects are usually mediated by altered protein-protein interactions, with SUMO occluding the interaction of the sumoylated target with binding partners in some cases, or facilitating interactions with binding partners that harbour SUMO interacting motifs (SIMs) in other cases (Gareau and Lima 2010; Flotho and Melchior 2013). Although many proteins are sumoylated, typically, only a small fraction (<5%) of polypeptides of each target protein is modified at any one time, which is partly the result of the constitutive activity of SUMO proteases, including the SENP family of isopeptidases in human cells (Hickey *et al.* 2012). There are two SUMO proteases in yeast, Ulp1, which is essential for viability, and Ulp2, which prevents accumulation of polysumoylate chains that form when conjugated SUMO peptides themselves become sumoylated (Li and Hochstrasser 2000; Bylebyl *et al.* 2003).

The name SUMO, an acronym for small ubiquitin-like modifier, derives from its similarity to the eukaryotic protein modifier, ubiquitin (Mahajan *et al.* 1997). In addition to structural similarity between SUMO and ubiquitin peptides, SUMO conjugation involves a

cascade of enzymatic activities that is analogous to ubiquitination: activation by an E1 class enzyme and target conjugation by E2 enzymes, which can be facilitated by E3 SUMO ligases that enhance sumoylation and target Lys specificity (Bayer *et al.* 1998; Sheng and Liao 2002; Flotho and Melchior 2013). In sharp contrast with ubiquitination, however, the number of SUMO conjugation and deconjugation enzymes is small, with both yeast and mammals harboring a single conjugase, Ubc9. As such, eukaryotic cells can simultaneously control the sumoylation level of hundreds of substrate proteins by regulating the activity of just one SUMO conjugating or deconjugating enzyme at a time. For example, exposure of budding yeast to ethanol triggers the nucleolar sequestration of the SUMO protease Ulp1, which results in a global increase in protein sumoylation within minutes (Sydorskyy *et al.* 2010).

Such rapid increases in global levels of SUMO conjugation occur in response to a variety of stress conditions, and are collectively referred to as the SUMO stress response (SSR; (Lewicki *et al.* 2015; Enserink 2015; Niskanen and Palvimo 2017; Nguéa P *et al.* 2019)). Exposure of mammalian cells to heat, oxidative, osmotic, or ethanol stress results in a spike in sumoylation levels caused by increased conjugation of numerous proteins with SUMO isoforms SUMO2 and/or SUMO3, specifically (Saitoh and Hinchey 2000; Golebiowski *et al.* 2009; Hendriks *et al.* 2014). As with sequestration of Ulp1 in yeast upon exposure to ethanol, the SSR that occurs with heat shock in human cells is at least partly caused by inactivation of multiple SENPs, suggesting that constitutive desumoylation is required for maintaining steady state levels of sumoylation (Pinto *et al.* 2012). In addition to ethanol exposure, yeast also respond with dramatically elevated SUMO conjugation levels in response to oxidative or osmotic conditions (Zhou *et al.* 2004; Lewicki *et al.* 2015). Inhibitors of transcription virtually eliminate the SSR in yeast, implying that elevated sumoylation is largely a consequence of stress-induced transcriptional

reprogramming and is not necessarily linked to the stress itself (Lewicki *et al.* 2015). However, SUMO2/3 isoforms are required for human cells to survive heat shock, and elevated levels of sumoylation appear to have a protective effect on neuronal tissue during exposure to reduced oxygen and glucose (Lee *et al.* 2009, 2011; Golebiowski *et al.* 2009; Flotho and Melchior 2013; Guo and Henley 2014). This suggests that elevated sumoylation itself serves a role in the adaptation of cells to unfavourable conditions, but the physiological effects of modulated cellular sumoylation levels are largely unknown.

To explore this, here we examined how altered sumoylation levels affect cell fitness in yeast. Besides stress, we found that cellular SUMO conjugation levels change with ploidy, culture density, and nutrient availability. By examining mutant yeast strains that harbour reduced sumoylation levels, either constitutively or conditionally, we determined that dramatic reduction in global sumoylation is well-tolerated in yeast grown in normal, non-stress conditions. However, reduced sumoylation results in increased sensitivity to high temperature, but, surprisingly, it had no effect on growth in response to other types of stress, including oxidative, osmotic and ethanol stress. Finally, we demonstrate that blocking de novo sumoylation results in a rapid and dramatic reduction of SUMO conjugation levels, suggesting that the modification is highly transient for many conjugates. Our results support a model in which sumoylation plays a largely pre-emptive role, specifically in preparing cells for potential exposure to high temperatures.

RESULTS

Cellular sumoylation levels are modulated under different conditions

To explore how cellular sumoylation levels change in response to different environments, yeast strains were grown in various conditions, then cell lysates or protein extracts

were prepared and examined by immunoblot with an antibody that recognizes the sole SUMO isoform in yeast, Smt3. Immunoblots of lysates prepared from yeast grown in synthetic complete (SC) medium at the standard temperature of 30°C were probed with this antibody and showed numerous bands, indicating that many proteins are sumoylated in normally growing yeast (Fig. 1A). The intensity of bands ranging from ~40 kDa to over 250 kDa dropped significantly if *N*-ethylmaleimide (NEM), which inhibits cysteine proteases, including SUMO proteases, was excluded during lysate preparation, supporting that these bands correspond to SUMO-conjugated proteins (Fig. 1A). The antibody also detected unconjugated SUMO, which migrates at ~16 kDa in the sample prepared from the common lab strain W303a, or ~19 kDa in the sample from a strain that expresses the yeast SUMO peptide as a fusion with an 8xHIS tag (8HIS-Smt3; Fig. 1A). The intense band seen at ~32 kDa in the immunoblot most likely corresponds to Ubc9 charged with a SUMO peptide ("Ubc9~SUMO" or "Ubc9~S" in diagrams) since (i) it co-migrates with Ubc9~SUMO as detected in a Ubc9 immunoblot (Fig. 1A, right), (ii) its migration in the 8HIS-Smt3 strain is retarded to a similar degree in the SUMO and Ubc9 blots, and (iii) the absence of NEM did not decrease the intensity of the band, which is expected for Ubc9~SUMO since SUMO attaches to Ubc9 through a thioester bond, not an isopeptide bond which is recognized by SUMO proteases. This analysis confirms that, with this antibody, we can detect high levels of protein sumoylation in normally growing yeast in addition to unconjugated SUMO and SUMO-charged Ubc9.

Next, we compared cellular sumoylation levels when the W303a strain was exposed to a number of stress conditions. Consistent with previous studies, osmotic (0.6 M KCl or 1M NaCl for 5 min), oxidative (100 mM H_2O_2 for 5 min), or ethanol (10% for 60 min) stress resulted in an increase in SUMO conjugation levels (Fig. 1B) (Zhou *et al.* 2004; Lewicki *et al.*

2015). In our hands, a brief heat shock (5 min at 42°C) or exposure to reduced temperatures (14°C for 60 min) only modestly increased cellular sumoylation levels compared to untreated cultures (Fig. 1B). We then tested whether factors other than stress can affect SUMO conjugation levels. The W303a strain was grown in liquid culture and protein extracts were generated from aliquots taken from the culture at various time points and examined by SUMO immunoblot. Remarkably, the level of sumoylation dropped dramatically as the culture density increased, suggesting that depletion of nutrients, or physiological changes that accompany the approach to culture saturation (e.g. diauxic shift), results in reduced SUMO conjugation levels (Fig. 1C). Finally, we compared sumoylation levels in different common lab strains of yeast grown in either SC or nutrient-rich (YPD) medium. Minor differences were seen between the YPH499, BY4741, and W303a haploid yeast strains, but significantly less sumoylation was detected in a diploid yeast strain (W303 diploid), and approximately two times more sumoylation was detected when strains were grown in YPD versus SC medium (Fig. 1D). Together, our results indicate that yeast cells modulate cellular sumoylation levels in response to a number of factors including certain types of stress, culture density, nutrient availability, ploidy, and to a lesser extent, strain genetic background.

Upregulation of Ubc9 or SUMO protein levels does not increase SUMO conjugation

To explore the physiological effects of modulated sumoylation, we attempted to artificially elevate cellular sumoylation levels through different strategies. First, we generated a plasmid from which Myc-epitope tagged Ubc9 overexpression can be induced by growth in the presence of galactose (pGAL-Ubc9-Myc). The plasmid was introduced into the W303a strain, and growth in galactose strongly induced overexpression of Ubc9, including Ubc9~SUMO (Fig. 2A). However, we found that the addition of galactose in the medium itself has a reducing effect

on sumoylation levels, and no increase was detected. We then transformed yeast with a plasmid that expresses the *UBC9* gene, with no epitope tag fusions, under control of a tetracycline inducible promoter (pTetON-Ubc9). Exposing this strain to the tetracycline analog doxycycline for increasing durations resulted in significantly elevated Ubc9 expression, but this had no effect on cellular sumoylation levels (Fig. 2B). In a similar fashion, increasing expression of the SUMO peptide, using a plasmid with a tetracycline inducible *SMT3* gene, did not result in increased SUMO conjugation levels (Fig. 2C). Together, these results indicate that cellular sumoylation levels are not restricted by a limited abundance of Ubc9 or unconjugated ("free") SUMO. Indeed, whereas SUMO conjugation levels varied significantly among the strains examined in Fig. 1D, Ubc9 protein levels were essentially constant, implying that regulating the abundance of Ubc9 is not a strategy by which cells modulate sumoylation levels.

Yeast cells can tolerate dramatically reduced sumoylation levels

To explore the physiological consequences of reduced global sumoylation, we applied the Anchor Away methodology to Ubc9 (Haruki *et al.* 2008). By this system, Ubc9 is fused with the FRB domain of human mTOR ("Ubc9-AA") in a yeast strain that also expresses the ribosomal protein Rpl13A fused to human FKBP12, which binds FRB in the presence of rapamycin. Exposing the Ubc9-AA strain to rapamycin causes nuclear Ubc9-AA to bind Rpl13A-FKB12, which is then rapidly translocated to the cytoplasm, effectively depleting the nucleus of Ubc9 and de novo sumoylation. As most sumoylated proteins are nuclear and associated with chromatin (Cubenas-Potts *et al.* 2013), this is expected to cause a significant reduction in overall cellular sumoylation levels. The Anchor Away parent strain, Parent-AA, harbours a mutation in the *TOR1* gene such that exposure to rapamycin has no effect (see Figs. 3A, C, D). We compared sumoylation levels in the Parent-AA and Ubc9-AA strains, which are isogenic except for the C-

terminal FRB fusion on Ubc9 in the latter. Strikingly, even in the absence of rapamycin, about 75% less sumoylation was detected in the Ubc9-AA strain, indicating that the FRB fusion itself hampers Ubc9 activity (Fig. 3A). This might reflect lower abundance of Ubc9-AA compared to normal Ubc9, as detected in a Ubc9 immunoblot (Fig. 3B), but we cannot rule out that the fusion inhibits binding of the Ubc9 antibody, which recognizes the C-terminus of Ubc9. Addition of rapamycin for 30 min during growth caused a further dramatic reduction of overall sumoylation levels (to ~3% of parental levels) in the Ubc9-AA strain, which is consistent with the exclusion of Ubc9-AA from the nucleus in these conditions (Fig. 3A). The Ubc9-AA system, therefore, allows us to examine the effects of constitutively low SUMO conjugation levels which can be further reduced by exposure to rapamycin.

We then compared growth rates of the Parent-AA and Ubc9-AA strains in the presence or absence of rapamycin. Despite the ~75% reduction in sumoylation levels in Ubc9-AA, the strains grew equally well in untreated SC medium, indicating that normal levels of SUMO conjugation are not needed for growth in standard conditions on solid medium (Fig. 3C) or during exponential growth in liquid culture (Fig. 3D). Further reducing sumoylation levels by the addition of rapamycin, however, was lethal to the Ubc9-AA strain, as indicated by spot assay (Fig. 3C). This striking effect is supported by the liquid growth assay that showed significantly slowed escape from lag phase and attenuated exponential growth for Ubc9-AA in the presence of rapamycin, which had no effect on the Parent-AA strain (Fig. 3D). We note, as also shown below, that small differences in our growth curves, specifically at the level of cell saturation, generally relate to much more dramatic growth defects in spot assays. In any case, our data indicates that, strikingly, yeast growth is unaffected by a dramatic reduction in cellular sumoylation levels, although levels of ~3% are lethal.

A threshold level of cellular sumoylation is required for survival at high temperatures

Why do cells maintain levels of sumoylation that are significantly higher than needed for normal growth? Considering that there are links between sumoylation levels and stress (e.g. Fig. 1B) (Lewicki *et al.* 2015; Enserink 2015), we wished to determine whether normal sumoylation levels might play a pre-emptive role in preparing cells for possible exposure to unfavourable conditions. We compared growth rates during exposure to stress of yeast strains that display constitutively reduced sumoylation, without the need for drug treatment. Namely, these are: *siz1Δ*, which lacks the *SIZ1* gene that encodes a major SUMO E3 ligase (Flotho and Melchior 2013); the Ubc9-AA strain described above; and *ubc9-1*, which harbours a point mutation that reduces Ubc9 activity (Betting and Seufert 1996). The corresponding wild-type parent strains (*SIZ1*, Parent-AA, and *UBC9*, respectively) where included in the analysis. As shown in Fig. 4A, the mutant strains show different levels of reduced sumoylation relative to each other and to their parent strains. Nonetheless, spot assays and liquid growth assays showed that all the sumoylation-deficient strains grew essentially as well as their parent strains when exposed to oxidative, osmotic, or ethanol stress (Figs. 4B, C). Surprisingly, this indicates that normal sumoylation levels are not required for survival after exposure to these types of stress.

In contrast to these stress conditions, strains Ubc9-AA and *ubc9-1*, the latter of which was first described as a temperature-sensitive mutant (Betting and Seufert 1996), both showed severe growth defects when incubated at the elevated temperature of 37°C (Fig. 4B). Consistent with this, in a liquid growth assay, these mutant strains showed impaired growth compared to their parent strains during the exponential phase or during entry into the stationary phase (diauxic shift) at 37°C, leading to reduced culture density at saturation (Fig. 4C). In the liquid growth assay, the addition of rapamycin exacerbated the growth defect for the Ubc9-AA strain at the

elevated temperature, but had no effect on the parent strain, which supports the idea that significantly reduced sumoylation levels preclude growth during temperature stress (Fig. 4C). The *siz1Δ* strain did not show a growth defect at 37°C, likely because sumoylation levels in that strain are not sufficiently reduced when compared with the Ubc9-AA and *ubc9-1* strains. Although heat shock results in a rapid elevation of sumoylation levels in human cells (Golebiowski *et al.* 2009), we did not observe a significant change in cellular sumoylation levels in yeast after heat shock in any of the strains tested (Fig. 4D, and see Fig. 1B and (Lewicki *et al.* 2015)). This suggests that a threshold level of cellular sumoylation is needed *prior* to exposure for yeast to survive elevated temperatures, and it supports the idea that normal sumoylation levels serve a pre-emptive role in protecting yeast from changing temperatures.

Effects of reduced sumoylation in an inducible system

To examine the effects of reduced cellular sumoylation levels using an additional approach, we employed the Ubc9-TO ("Tet-Off") strain, in which the *UBC9* gene is under the control of a tetracycline repressible promoter (Mnaimneh *et al.* 2004). The strain was grown for several hours in medium containing varying concentrations of doxycycline, then lysates were prepared and analyzed by SUMO and Ubc9 immunoblots. Even at a low concentration of doxycycline (0.5 µg/mL), both Ubc9 and overall SUMO conjugation showed a dramatic reduction to less than 5% of their normal levels (Fig. 5A). When compared side-by-side with the Ubc9-AA strain, the doxycycline-treated Ubc9-TO strain displayed less overall SUMO conjugation than in the untreated Ubc9-AA strain, but approximately 20% more sumoylation than in rapamycin-treated Ubc9-AA cells (Fig. 5B). We then examined how well the Ubc9-TO strain tolerates low sumoylation levels, and whether reduced sumoylation also confers temperature sensitivity in this strain. Despite the dramatic reduction in sumoylation levels,

treatment with doxycycline had no effect on exponential growth of the Ubc9-TO strain at 30°C, which is consistent with a high tolerance of yeast cells for low levels of sumoylation at normal temperatures (Fig. 5C). When the analysis was performed at elevated temperatures, however, Ubc9-TO cells treated with doxycycline displayed a growth defect that was more pronounced at 39.5°C than at 37°C, whereas the drug had no effect on the isogenic Parent-TO strain at any temperature (Fig. 5C). Using this different system, these observations support our finding that many SUMO conjugation events are dispensable under optimal growth conditions but are needed to prepare cells for elevated temperatures.

We then examined how reduced sumoylation affects growth of the Ubc9-TO strain on solid medium using spot assays. Parent-TO and Ubc9-TO strains were grown in culture for several hours, either untreated or in the presence of doxycycline, then spotted onto the same medium in solid form. In contrast to the liquid growth assays, compared to the Parent-TO strain, a modest growth defect was observed in the Ubc9-TO strain when grown in the presence of doxycycline (Fig. 5D). This is consistent with the observation that doxycycline-exposed Ubc9-TO harbours an intermediate level of sumoylation compared to untreated Ubc9-AA cells, which show no growth defect, and rapamycin-exposed Ubc9-AA cells, which are inviable on solid medium (see Fig. 5B and compare Fig. 3C with 5D). Whereas yeast can tolerate a significant reduction in sumoylation levels under normal conditions, these observations further support the idea that there is a specific threshold level of sumoylation that is required for normal cell function.

Unexpectedly, the modest growth defect of doxycycline-treated Ubc9-TO was not exacerbated when spot assays were performed at elevated temperatures (Fig. 5D). As the Ubc9-TO and Parent-TO strains derive from a different background yeast strain (BY4741) than the

Ubc9-AA and Parent-AA strains (W303a), we explored whether yeast strains of different origins show differential sensitivity to high temperatures. Indeed, as shown in Fig. 5E, BY4741, and its derived strain Parent-TO, show strikingly higher thermotolerance than W303a-derived strains, including the Parent-AA strain and the parental strain for *ubc9-1*, *UBC9*. Although this inherent high tolerance for elevated temperatures may obscure temperature sensitivity to reduced sumoylation levels in the Ubc9-TO strain when spot assays are performed, the liquid growth assays shown in Fig. 5C support our finding that yeast cells with reduced SUMO conjugation levels show growth defects at higher temperatures.

Evidence that sumoylation is a highly transient modification

As explained above, adding rapamycin to cultures of the Ubc9-AA strain causes the Ubc9-AA fusion protein to translocate to the cytoplasm, thereby preventing de novo sumoylation events in the nucleus. Although SUMO conjugation levels in the Ubc9-AA strain are constitutively low (see Fig. 3A), the strain responds normally to a variety of stress conditions by elevating sumoylation globally (Fig. 6A), implying that mechanisms of regulating protein sumoylation are intact in this strain. Whereas it was primarily intended as a method of reducing cellular sumoylation levels, we realized that the Ubc9-AA strain could also be used for examining the stability of SUMO modifications. Specifically, by blocking de novo sumoylation in the nucleus, where the bulk of sumoylated proteins are located, we could examine how long SUMO modifications last. To explore this, we grew a culture of the Ubc9-AA strain and collected samples at various times after the addition of rapamycin for analysis by SUMO immunoblot. Remarkably, about 80% of the SUMO signal that corresponds to SUMO conjugated proteins disappeared just five minutes after rapamycin treatment (Fig. 6B). The residual level of SUMO conjugation did not further decrease with longer exposure to the drug,

and might derive from non-nuclear SUMO conjugates, or from particularly stable nuclear SUMO modifications. The rapid disappearance of the bulk of SUMO conjugates suggests that SUMO modifications are highly transient and require constant de novo sumoylation to maintain a steady state.

The rapid loss of the SUMO signal in the immunoblot in Fig. 6B could be explained by a high level of constitutive deconjugation of SUMO peptides from target proteins, or from rapid degradation of SUMO-modified proteins. Arguing against the idea that desumoylation is predominantly responsible, we did not detect a corresponding increase in the abundance of free SUMO peptides when the level of sumoylation dropped upon addition of rapamycin, and deletion of the non-essential SUMO protease Ulp2 did not prevent the disappearance of SUMO conjugates (Figs. 6B, C). To determine whether the bulk of SUMO conjugates in the Ubc9-AA strain are prone to degradation through the 26S proteasome, we treated cultures of the strain with the inhibitor, MG132. Because they contain a cell wall, budding yeast are generally impermeable to MG132, but can be treated with low concentrations of sodium dodecyl sulfate (SDS) to increase sensitivity to the drug (Liu *et al.* 2007). Cultures of the Ubc9-AA strain were treated with SDS for three hours prior to exposing them to MG132 or its solvent, DMSO, followed by treatment with rapamycin or DMSO. Cultures treated with MG132 showed significantly increased levels of high molecular weight ubiquitin conjugates, which indicates that inhibition of the 26S proteasome was successful (Fig. 6D). However, treatment with rapamycin in MG132-treated cells, for either 30 or 8 min, still resulted in the rapid disappearance of SUMO conjugates (Fig. 6D, E). As an alternative to treatment with SDS, deletion of the *PDR5* gene, which encodes a plasma membrane multi-drug transporter protein, also increases sensitivity to MG132 (Collins *et al.* 2010). Nonetheless, deletion of *PDR5* had no effect on the loss of SUMO conjugates in

MG132- and rapamycin-treated cells (Fig. 6F). Together, our results indicate that SUMO conjugation is a highly transient modification for many proteins, but our data suggests that the loss of bulk sumoylation when de novo sumoylation is blocked is not due to desumoylation or degradation of SUMO conjugates through the 26S proteasome.

DISCUSSION

Over 550 proteins have been identified as SUMO conjugates in normally growing yeast through proteomics analyses ((Makhnevych *et al.* 2009; Albuquerque *et al.* 2015; Esteras *et al.* 2017), and addition studies cited therein). Whereas, in some published studies, immunoblots show few yeast SUMO conjugates except in stress conditions, our SUMO immunoblots readily detect numerous sumoylated species in non-stressed yeast, indicating that they are a good reflection of the sumoylation status of the cells. As such, we were able to identify factors and conditions, in addition to stress, that affect global sumoylation in yeast. Specifically, we found that diploid cells harbour less sumoylation than their haploid counterparts, and that nutrient availability has a positive effect on levels of SUMO conjugation. This was apparent by comparing sumoylation levels in yeast grown in rich versus synthetic medium, and by monitoring sumoylation in a growing liquid culture. Sumoylation levels were greatest during exponential growth, but dropped dramatically with the diauxic shift, which occurs as glucose becomes depleted. Typically, to elicit a stress response, yeast cultures are treated with stressors when they are growing exponentially, as in Fig. 1B. This indicates that stress can elevate sumoylation levels beyond the maximal level seen in cultures during normal growth. These results demonstrate that sumoylation levels can be shifted dramatically both upward, as during stress, and downward, when cultures reach saturation.

Considering that many SUMO targets are involved in gene expression, metabolism, and cell cycle, it might be expected that dramatic shifts in overall SUMO conjugation affect cell growth (Makhnevych *et al.* 2009; Albuquerque *et al.* 2015; Hendriks *et al.* 2017; Esteras *et al.* 2017). Our initial strategies to explore this involved upregulating the abundance of the SUMO conjugase, Ubc9, or the SUMO peptide itself, as similar approaches have been used previously to elevate sumoylation levels in mammalian systems (Lee *et al.* 2009, 2011). However, in our analysis, global sumoylation levels were unaffected, indicating that Ubc9 and SUMO peptides are not limiting, and that it is unlikely that yeast cells modulate sumoylation levels by regulating the abundance of these proteins. This is consistent with the finding of others that cells can elevate sumoylation globally by inactivating or deactivating SUMO proteases (Sydorskyy *et al.* 2010; Pinto *et al.* 2012; Lewicki *et al.* 2015).

To examine the effects of reduced global sumoylation on cell fitness, we first used the Anchor Away methodology with Ubc9 to conditionally block de novo sumoylation events in the nucleus (Haruki *et al.* 2008). Nuclear depletion of Ubc9 by this system had a surprisingly rapid effect, in which most SUMO conjugations disappeared within a few minutes. Although this might reflect that SUMO modifications are highly labile, there is no evidence that the dramatic reduction in SUMO conjugation levels was due to desumoylation, as there was no concomitant increase in the abundance of free SUMO. This suggests that the bulk of sumoylated proteins detected in the Ubc9-AA strain are rapidly degraded. Consistent with this, many sumoylated proteins, particularly those that are polysumoylated, can be targeted for degradation through the action of SUMO-targeted ubiquitin ligases (STUbLs), that bind and ubiquitinate SUMO chains, which directs the conjugated proteins to the 26S proteasome for proteolysis (Sriramachandran and Dohmen 2014). However, inhibition of the 26S proteasome using MG132 had no effect on

the disappearance of SUMO conjugates when Ubc9 was depleted, suggesting that many sumoylated proteins in yeast are destined for rapid degradation through a pathway that does not involve typical ubiquitin-mediated proteolysis. Alternatively, the disappearance of the SUMO signal might be attributed to frequent desumoylation of targets by the essential SUMO protease Ulp1, followed by the rapid degradation of the SUMO peptide itself. However, we were unable to generate viable yeast strains harbouring defective Ulp1 mutants in the Ubc9-AA background to test this.

Treatment of mammalian cells with proteasome inhibitors, such as MG132, leads to a significant increase in SUMO conjugates, primarily with the SUMO2/3 isoforms, which includes accumulation of proteins that are modified with both SUMO and ubiquitin (Schimmel *et al.* 2008; Tatham *et al.* 2011; Lamoliatte *et al.* 2017). As this MG132-dependent increase in sumoylation levels was shown to be dependent on protein synthesis, it is believed that misfolded newly synthesized polypeptides are normally targeted by sumoylation as part of their degradation pathway, and that these become detectable upon proteasomal inhibition with MG132 treatment (Tatham *et al.* 2011; Častorálová *et al.* 2012). In our analysis, proteasomal inhibition with MG132 did cause an accumulation of polyubiquitinated proteins, as expected, but no significant change to overall sumoylation levels was observed. Although these experiments were performed in the Ubc9-AA strain, which harbours ~25% of the normal levels of SUMO conjugation, this observation suggests that any accumulation of misfolded proteins destined for proteasomal degradation that might occur in budding yeast does not trigger a sumoylation response as it does in higher eukaryotes.

By examining growth of the Ubc9-AA strain and others that express partially defective or diminished levels of Ubc9, we determined that yeast can tolerate dramatic reductions in

sumoylation levels under normal growth. Whereas a reduction to ~25% of normal sumoylation levels in the Ubc9-AA strain results in no growth defect, the tetracycline-treated Ubc9-TO strain, which harbours ~5% of normal sumoylation, shows a modest growth defect on solid medium. We note that the Ubc9-TO and Ubc9-AA strains derive from different backgrounds (BY4741 and W303a, respectively), and that genetic variations between the strains might have an impact on specific tolerance levels. Nonetheless, these results suggest that somewhere between 75% and 95% of SUMO conjugation events are not required for normal growth, and might rather serve a protective, pre-emptive role in anticipation of stress-inducing changes in the environment. However, reduced sumoylation levels did not impact growth when cells were challenged with osmotic, oxidative, and ethanol stress, all of which can trigger elevated sumoylation in the SUMO stress response (SSR; (Zhou *et al.* 2004; Lewicki *et al.* 2015)). Our observations align with the proposal that the SSR is primarily a consequence of altered transcription genome-wide, which involves a wave of transcription-mediated SUMO conjugation, and suggest that high levels of overall sumoylation do not play a protective role during these stresses (Lewicki *et al.* 2015). However, further analysis is needed to determine whether elevated sumoylation of specific substrates functions in cellular stress responses. In contrast to these stresses, reduced cellular sumoylation levels did elicit sensitivity to elevated temperatures, particularly in the Ubc9-AA and *ubc9-1* strains for which higher temperatures are lethal, which is consistent with the finding that sumoylation facilitates heat tolerance in human cells and plants (Yoo *et al.* 2006; Golebiowski *et al.* 2009; Zhang *et al.* 2018). To understand why, among stressors, resistance to high temperatures specifically shows a dependence on sumoylation levels prior to exposure, future studies will be key to determine, at the molecular level, how the modification functions as a general thermoprotectant. Taken together, our study demonstrates that the bulk of sumoylation

events are dispensable for normal growth in yeast but suggests that SUMO modifications serve proactively to protect cells from the effects of elevated temperatures.

MATERIALS AND METHODS

Yeast media and growth

Yeast strains and plasmids used in this study are listed and described in Table S1. Previously unreported strains were generated by transformation with plasmids or with PCR-generated linear fragments that were incorporated at specific genomic loci through homologous recombination (Knop *et al.* 1999). Notes on plasmid construction are included in Table S1.

Unless otherwise noted, yeast were grown at 30°C in synthetic complete (SC) medium (per litre: 1.74 g yeast nitrogen base without ammonium sulfate or amino acids; 5 g ammonium sulfate; complete amino acid mix or drop-out mix, as appropriate; 2% glucose; and for solid medium, 20 g of agar, and the pH was adjusted to 5.8 – 6.0 before autoclaving). YPD medium consists of, per litre, 10 g yeast extract, 20 g peptone, and 2% glucose, with 20 g of agar for solid medium. Where appropriate, the following were added to media (final concentrations indicated): 2% galactose for 90 min; 0.6 M KCl for 5 min for liquid cultures, 1 M KCl on solid media; 1M NaCl; 100 mM H_2O_2 for 5 min for liquid cultures, 1 mM H_2O_2 for solid media; 10% ethanol for 60 min for liquid cultures, 7% ethanol for solid media; 1 µg/mL rapamycin for 30 min, or as indicated; doxycycline, 10 µg/mL, or as indicated, for duration of culture growth. Where rapamycin was added, the control or "untreated" sample was supplemented with the same volume of its carrier, DMSO. For inhibition of the 26S proteasome, 75 µM of MG132 was added to cultures for 1 hour prior to further treatment or harvesting, or the same volume of DMSO was added for "untreated" samples. To increase permeability of the drug for the Ubc9-AA strain, the method of Liu et al. was used (Liu *et al.* 2007).

Protein lysates and extracts

Protein samples were generated under non-denaturing conditions ("lysates") or through precipitation with trichloroacetic acid (TCA; "protein extracts"). In all cases, overnight cultures were diluted to an absorbance (595 nm) of ~0.2 in a volume of 10 to 50 mL, then grown to exponential phase (absorbance < 1.0), unless otherwise noted. Samples were treated as indicated above, if appropriate, then harvested by centrifugation at 3000 *g* for 5 min. For lysate preparation, cells pellets were washed with ice-chilled IP buffer (50 mM tris-HCl, pH8; 150 mM NaCl; 0.1% Nonidet P-40 (NP40); supplemented with a protease inhibitor cocktail, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 2.5 mg/mL *N*-ethylmaleimide (NEM)) at a volume equal to the culture volume, then resuspended in 500 µL of IP buffer. Acid-washed glass beads (0.25 g) were then added, and samples were vortexed at 4°C for 30 min, with a 5-min ice incubation after the first 15 min. Lysates were transferred to fresh microfuge tubes and clarified by two rounds of 5-min centrifugation at maximum microfuge speed at 4°C. Prior to analysis by immunoblot, an equal volume of 2X sample buffer (140 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 0.02% bromophenol blue; supplemented with 10% 2-mercaptoethanol prior to use) was added, and samples were boiled for 4 min.

For protein extract preparation through TCA precipitation, volumes equal to 2.5 absorbance units (595 nm) of the exponentially growing yeast cultures, treated if necessary, were centrifuged at 3000 *g*, then resuspended in 1 mL of ice-cold water. 150 µL of freshly prepared extraction buffer (1.85 NaOH; 7.5% 2-mercaptoethanol) was added, and the mixed samples were incubated on ice for 10 min. Then, 150 µL of cold 50% TCA was added, samples were mixed, then incubated again on ice for 10 min before they were centrifuged at 4°C at maximum microfuge speed. Supernatants were removed and the protein pellets were resuspended in 100 µL

of 2X sample buffer, with 5 to 10 µL of Tris-HCl, pH 8 added if the sample was yellow in colour, to raise the pH and restore the blue colour. Samples were boiled for 4 min, centrifuged for 3 min at maximum microfuge speed to separate debris, then used for immunoblot analysis.

Immunoblots and quantifications

Protein samples were analyzed by standard polyacrylamide gel electrophoresis (PAGE) techniques, followed by "wet" transfer, or by rapid semi-dry electrotransfer using a PowerBlotter (Invitrogen), to nitrocellulose membranes which were then blocked with a 5% milk solution in phosphate-buffered saline (PBS) supplemented with 0.05% tween (PBST), and incubated in a solution of the appropriate primary antibody in 5% milk. Three ten-minute washes in PBST were carried out before and after incubation with secondary antibody (in a 1% milk solution). Chemiluminescence-based imaging was performed using the MicroChemi imager (DNR). For quantification of signals, TIFF-format images were analyzed using ImageJ software (NIH). Specifically for determining levels of sumoylation levels, all SUMO signals situated above the 37 kDa marker were considered SUMO conjugates, and normalization was made to the corresponding signal from GAPDH immunoblots. For sumoylation level quantification of immunoblots that included samples from the Ubc9-AA strain, since Ubc9-AA~SUMO migrates at ~45 kDa, only bands above this mark were considered for all samples. Antibodies used for immunoblot analyses were: 1:500 SUMO/Smt3 (y-84; Santa Cruz, sc-28649); 1:3000 GAPDH (Sigma,G9545); 1:500 Ubc9 (Santa Cruz, sc-6721); 1:3000 histone H3 (Abcam, ab1791); 1:1000 Myc epitope tag (Sigma 05-724); 1:500 Ubiquitin (FK1; Cayman, 14219); 1:1000 Rpb3 (Abcam; ab202893).

Spot assays and liquid growth assays

Yeast strain fitness was examined by spot assays and liquid growth analysis. For spot assays, growing cultures were diluted to an absorbance (595 nm) of 0.2, then four five-fold serial dilutions were prepared. Three microlitres of each dilution were then spotted in an array on the appropriate solid medium plate. By this method, approximately 10,000 cells are spotted in the first spot. Plates were left to dry, then incubated inverted at the appropriate temperature (usually 30°C) for several days, with images taken daily. To generate growth curves, growing cultures were diluted to an absorbance of 0.2 – 0.5 in appropriate medium that was supplemented as indicated above, if necessary. Each sample was transferred, in triplicate, to wells in a 96-well microtiter dish, and inserted into an accuSkan absorbance microplate reader (Fisherbrand), which shook and incubated samples at the appropriate temperature while taking absorbance measurements (595 nm) at 15-min intervals over a course of 20 hours. Background absorbance readings were made from wells containing uninoculated medium, and the background-subtracted average absorbance readings, and standard deviations, are listed in Table S2. Curves were generated with the average values and shown in the figures without standard deviations, since they were minimal and to simplify the diagrams. Because the light path distance in the accuSkan is less than 1 cm, absorbance readings are not directly comparable to values obtained through a standard spectrophotometer.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR; grant number MOP-142282) to E.R.

REFERENCES

Albuquerque C. P., Yeung E., Ma S., Fu T., Corbett K. D., Zhou H., 2015 A Chemical and Enzymatic Approach to Study Site-Specific Sumoylation (MJ Edelmann, Ed.). PLoS One **10**: e0143810.

Bayer P., Arndt A., Metzger S., Mahajan R., Melchior F., Jaenicke R., Becker J., 1998 Structure determination of the small ubiquitin-related modifier SUMO-1. J. Mol. Biol. **280**: 275–286.

Betting J., Seufert W., 1996 A yeast Ubc9 mutant protein with temperature-sensitive in vivo function is subject to conditional proteolysis by a ubiquitin- and proteasome-dependent pathway. J. Biol. Chem. **271**: 25790–25796.

Bylebyl G. R., Belichenko I., Johnson E. S., 2003 The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast. J Biol Chem **278**: 44113–44120.

Častorálová M., Březinová D., Švéda M., Lipov J., Ruml T., Knejzlík Z., 2012 SUMO-2/3 conjugates accumulating under heat shock or MG132 treatment result largely from new protein synthesis. Biochim. Biophys. Acta - Mol. Cell Res. **1823**: 911–919.

Chymkowitch P., Nguéa P A., Enserink J. M., 2015 SUMO-regulated transcription: Challenging the dogma. BioEssays **37**: 1095–1105.

Collins G. A., Gomez T. A., Deshaies R. J., Tansey W. P., 2010 Combined chemical and genetic approach to inhibit proteolysis by the proteasome. Yeast **27**: 965–74.

Cubenas-Potts C., Matunis M. J., Cubeñas-Potts C., Matunis M. J., 2013 SUMO: a multifaceted modifier of chromatin structure and function. Dev. Cell **24**: 1–12.

Enserink J. M., 2015 Sumo and the cellular stress response. Cell Div. **10**: 4.

Esteras M., Liu I.-C., Snijders A. P., Jarmuz A., Aragon L., 2017 Identification of SUMO conjugation sites in the budding yeast proteome. Microb. Cell **4**: 331–341.

Flotho A., Melchior F., 2013 Sumoylation: A Regulatory Protein Modification in Health and Disease. Annu. Rev. Biochem. **82**: 357–385.

Gareau J. R., Lima C. D., 2010 The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. Nat. Rev. Mol. Cell Biol. **11**: 861–871.

Golebiowski F., Matic I., Tatham M. H., Cole C., Yin Y., Nakamura A., Cox J., Barton G. J., Mann M., Hay R. T., 2009 System-wide changes to SUMO modifications in response to heat shock. Sci. Signal. **2**: ra24.

Guo C., Henley J. M., 2014 Wrestling with stress: roles of protein SUMOylation and deSUMOylation in cell stress response. IUBMB Life **66**: 71–77.

Haruki H., Nishikawa J., Laemmli U. K., 2008 The anchor-away technique: rapid, conditional establishment of yeast mutant phenotypes. Mol. Cell **31**: 925–932.

Hendriks I. A., D'Souza R. C. J., Yang B., Verlaan-De Vries M., Mann M., Vertegaal A. C. O., 2014 Uncovering global SUMOylation signaling networks in a site-specific manner. Nat. Struct. Mol. Biol. **21**: 927–936.

Hendriks I. A., Lyon D., Young C., Jensen L. J., Vertegaal A. C. O., Nielsen M. L., 2017 Site-specific mapping of the human SUMO proteome reveals co-modification with phosphorylation. Nat. Struct. Mol. Biol.

Hickey C. M., Wilson N. R., Hochstrasser M., 2012 Function and regulation of SUMO proteases. Nat. Rev. Mol. Cell Biol. **13**: 755–766.

Knop M., Siegers K., Pereira G., Zachariae W., Winsor B., Nasmyth K., Schiebel E., 1999 Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast **15**: 963–972.

Lamoliatte F., McManus F. P., Maarifi G., Chelbi-Alix M. K., Thibault P., 2017 Uncovering the

SUMOylation and ubiquitylation crosstalk in human cells using sequential peptide immunopurification. Nat. Commun. **8**: 14109.

Lee Y. J., Castri P., Bembry J., Maric D., Auh S., Hallenbeck J. M., 2009 SUMOylation participates in induction of ischemic tolerance. J. Neurochem. **109**: 257–67.

Lee Y., Mou Y., Maric D., Klimanis D., Auh S., Hallenbeck J. M., 2011 Elevated Global SUMOylation in Ubc9 Transgenic Mice Protects Their Brains against Focal Cerebral Ischemic Damage (A Meisel, Ed.). PLoS One **6**: e25852.

Lewicki M. C., Srikumar T., Johnson E., Raught B., 2015 The S. cerevisiae SUMO stress response is a conjugation-deconjugation cycle that targets the transcription machinery. J. Proteomics **118**: 39–48.

Li S. J., Hochstrasser M., 2000 The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. Mol Cell Biol **20**: 2367–2377.

Liu C., Apodaca J., Davis L. E., Rao H., 2007 Proteasome inhibition in wild-type yeast Saccharomyces cerevisiae cells. Biotechniques **42**: 158,160,162.

Mahajan R., Delphin C., Guan T., Gerace L., Melchior F., 1997 A Small Ubiquitin-Related Polypeptide Involved in Targeting RanGAP1 to Nuclear Pore Complex Protein RanBP2. Cell **88**: 97–107.

Makhnevych T., Sydorskyy Y., Xin X., Srikumar T., Vizeacoumar F. J., Jeram S. M., Li Z., Bahr S., Andrews B. J., Boone C., Raught B., 2009 Global map of SUMO function revealed by protein-protein interaction and genetic networks. Mol. Cell **33**: 124–135.

Mnaimneh S., Davierwala A. P., Haynes J., Moffat J., Peng W. T., Zhang W., Yang X., Pootoolal J., Chua G., Lopez A., Trochesset M., Morse D., Krogan N. J., Hiley S. L., Li Z., Morris Q., Grigull J., Mitsakakis N., Roberts C. J., Greenblatt J. F., Boone C., Kaiser C. A.,

Andrews B. J., Hughes T. R., 2004 Exploration of essential gene functions via titratable promoter alleles. Cell **118**: 31–44.

Nguéa P A., Robertson J., Herrera M. C., Chymkowitch P., Enserink J. M., 2019 De-sumoylation of RNA polymerase III lies at the core of the Sumo stress response in yeast. J. Biol. Chem.: jbc.RA119.009721.

Niskanen E. A., Palvimo J. J., 2017 Chromatin SUMOylation in heat stress: To protect, pause and organise?: SUMO stress response on chromatin. BioEssays **39**: 1–10.

Pinto M. P., Carvalho A. F., Grou C. P., Rodríguez-Borges J. E., Sá-Miranda C., Azevedo J. E., 2012 Heat shock induces a massive but differential inactivation of SUMO-specific proteases. Biochim. Biophys. Acta - Mol. Cell Res. **1823**: 1958–1966.

Rosonina E., Akhter A., Dou Y., Babu J., Sri Theivakadadcham V. S., 2017 Regulation of transcription factors by sumoylation. Transcription **8**: 220–231.

Saitoh H., Hinchey J., 2000 Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J. Biol. Chem. **275**: 6252–6258.

Schimmel J., Larsen K. M., Matic I., Hagen M. van, Cox J., Mann M., Andersen J. S., Vertegaal A. C. O., 2008 The ubiquitin-proteasome system is a key component of the SUMO-2/3 cycle. Mol. Cell. Proteomics **7**: 2107–22.

Sheng W., Liao X., 2002 Solution structure of a yeast ubiquitin-like protein Smt3: The role of structurally less defined sequences in protein-protein recognitions. Protein Sci. **11**: 1482–1491.

Sriramachandran A. M., Dohmen R. J., 2014 *SUMO-targeted ubiquitin ligases*.

Sydorskyy Y., Srikumar T., Jeram S. M., Wheaton S., Vizeacoumar F. J., Makhnevych T., Chong Y. T., Gingras A.-C., Raught B., 2010 A Novel Mechanism for SUMO System

Control: Regulated Ulp1 Nucleolar Sequestration. Mol. Cell. Biol. **30**: 4452–4462.

Tatham M. H., Matic I., Mann M., Hay R. T., 2011 Comparative proteomic analysis identifies a role for SUMO in protein quality control. Sci. Signal. **4**: rs4.

Yoo C. Y., Miura K., Jin J. B., Lee J., Park H. C., Salt D. E., Yun D.-J., Bressan R. A., Hasegawa P. M., 2006 SIZ1 Small Ubiquitin-Like Modifier E3 Ligase Facilitates Basal Thermotolerance in Arabidopsis Independent of Salicylic Acid. Plant Physiol. **142**: 1548–1558.

Zhang S., Wang S., Lv J., Liu Z., Wang Y., Ma N., Meng Q., 2018 SUMO E3 Ligase SlSIZ1 Facilitates Heat Tolerance in Tomato. Plant Cell Physiol. **59**: 58–71.

Zhao X., 2018 SUMO-Mediated Regulation of Nuclear Functions and Signaling Processes. Mol. Cell **71**: 409–418.

Zhou W., Ryan J. J., Zhou H., 2004 Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stresses. J. Biol. Chem. **279**: 32262–32268.

FIGURE LEGENDS

Figure 1. Different factors and growth conditions affect cellular sumoylation levels. (A) W303a and 8HIS-Smt3 yeast strains were grown in SC medium at the standard temperature (30°C) and whole cell lysates were prepared under non-denaturing conditions, in the presence of NEM, except where indicated. Lysates were analyzed by SUMO, GAPDH, and Ubc9 immunoblots. The positions of SUMO conjugates, Ubc9~SUMO ("Ubc9~S"), unconjugated SUMO ("Free SUMO"), and uncharged Ubc9 are indicated. **(B)** W303a cultures were grown in SC medium at 30°C to exponential phase, then left untreated or exposed to the indicated stress conditions. Protein extracts were prepared and analyzed by SUMO and GAPDH immunoblots. Different exposures are shown for each of the four immunoblots. **(C)** Aliquots of a W303a culture grown in SC medium at 30°C were collected at six intervals over 15 hours and protein extracts were prepared and analyzed by SUMO and GAPDH immunoblots. The culture density (cells/mL) at each time point is indicated at top and depicted in the growth curve below. **(D)** The indicated common lab yeast strains, including haploid and diploid W303 ("W303a" and "W303 dip.," respectively), were grown in either SC or YPD medium to exponential phase, then protein extracts were generated and analyzed by SUMO, Ubc9, and GAPDH immunoblots.

Figure 2. Increasing abundance of Ubc9 or free SUMO does not increase SUMO conjugation levels. (A) Two clones of the W303a strain harbouring a plasmid that expresses Myc-tagged Ubc9 from a galactose-inducible promoter (pGAL-Ubc9-Myc) were grown in the absence or presence of galactose, then lysates were prepared and analyzed by SUMO, Ubc9, Myc, and histone H3 immunoblots. **(B)** A W303a strain transformed with a plasmid that expresses Ubc9 from a tetracycline-inducible promoter (pTetON-Ubc9) was treated with the tetracycline analog doxycycline ("Doxy.") for the indicated times, then lysates were prepared

and analyzed by SUMO, Ubc9, and GAPDH immunoblots. **(C)** A W303a strain ectopically expressing the SUMO peptide, without its C-terminal proteolytic fragment, from a tetracycline-inducible promoter (pTet-ON-SUMO), was analyzed as in *B*.

Figure 3. Yeast cells can tolerate a dramatic reduction in cellular sumoylation levels. (A) Cultures of the Ubc9 Anchor Away strain, Ubc9-AA, and its parent, Parent-AA, were left untreated or treated with rapamycin ("+Rap.") for 30 min, then extracts were prepared and analyzed by SUMO and GAPDH immunoblots. SUMO conjugation levels were quantified by densitometry and the average and standard deviations (error bars) from three experiments are plotted at right. **(B)** Lysates were prepared from cultures of W303a, Parent-AA, Ubc9-AA, or from an unrelated control Anchor Away strain, Rpb1-AA, and analyzed by immunoblots with antibodies that recognize Ubc9 or a subunit of RNA Polymerase II, Rpb3, which serves as a loading control. **(C)** A spot assay was performed with the Ubc9-AA and Parent-AA strains, onto either SC medium, or SC supplemented with rapamycin. Plates were imaged after two days of growth. **(D)** Cultures of the Ubc9-AA and Parent-AA strains were prepared in SC medium, with or without rapamycin, at an absorbance (595 nm) of ~0.2, then grown for 20 hours while absorbance (culture density) measurements were made every 15 min. The two Parent-AA curves are virtually indistinguishable and are therefore marked with "overlap." Triplicate cultures were grown for each sample, and the average values were plotted to create the growth curves shown. Values for average absorbance measurements and standard deviations for each triplicate set are listed in Table S2.

Figure 4. Yeast strains with constitutively low levels of sumoylation cannot survive elevated temperatures. (A) Protein extracts were prepared from cultures of the indicated strains grown in untreated SC medium and analyzed by SUMO and GAPDH immunoblots. **(B)** Spot assays were

performed with the indicated strains, onto either SC medium, or SC supplemented with the indicated stressors (see Materials and Methods for details) and incubated at the standard growth temperature of 30°C, or at the elevated temperature of 37°C. Plates were imaged after the indicated number of days. **(C)** Growth curves were generated, as in Fig. 3D, for the indicated strains and conditions (see Materials and Methods for details). Curves that overlap significantly and are indistinguishable are marked with "overlap." Individual graphs include two curves except for the Parent-AA and Ubc9-AA set at 37°C, which includes untreated and rapamycin-treated samples for both strains. **(D)** Cultures of the indicated strains were grown at 30°C, then left untreated or incubated at 37°C for 15 min before protein extracts were prepared and analyzed by SUMO and GAPDH immunoblots.

Figure 5. Effects of induced reduction of sumoylation levels on cell growth. (A) Cultures of a strain expressing the *UBC9* gene from a tetracycline-repressible promoter, Ubc9-TO, and its otherwise isogenic parent strain, Parent-TO, were exposed to the indicated concentrations of the tetracycline analog doxycycline for several hours, then lysates were prepared and examined by SUMO, Ubc9 and H3 immunoblots. **(B)** Cultures of the Ubc9-TO, and Ubc9-AA strains were grown, side-by-side, and treated with either doxycycline or rapamycin, where indicated, or left untreated. Lysates were prepared and analyzed by SUMO and GAPDH immunoblots. **(C)** Growth curves were generated, as in Fig. 3D, for the Ubc9-TO and Parent-TO strains grown in the absence or presence of doxycycline at the indicated temperatures. **(D)** Spot assays were performed with the Ubc9-TO and Parent-TO strains, onto either SC medium, or SC supplemented with doxycycline. Plates were incubated at the indicated temperatures and were imaged after one day (~30 h) or two days of growth, as indicated. As none of the strains could grow in the presence of doxycycline at 39.5°C on solid medium, a temperature of 38.5°C was

used as the highest temperature for this experiment. **(E)** A spot assay was performed comparing growth of the indicated background and parental yeast strains at 30°C, 37°C, or 39.5°C, for the indicated number of days.

Figure 6. Many SUMO conjugation events are highly transient. (A) The Ubc9-AA strain was grown to exponential phase in liquid culture then treated with the indicated stressors. Extracts were prepared and analyzed by SUMO and GAPDH immunoblots. No rapamycin was used in this experiment. **(B)** The Ubc9-AA strain was grown in culture, treated with rapamycin, and aliquots were taken at the indicated times after treatment. Protein extracts were prepared and analyzed by SUMO and GAPDH immunoblots. The levels of SUMO conjugation and unconjugated SUMO were quantified (see Materials and Methods) relative to GAPDH and graphed at right as the average and standard deviations of three experiments. **(C)** Versions of the Ubc9-AA strain with the *ULP2* gene deleted (*ulp2Δ*) or intact (*ULP2*) were grown in culture and either left untreated or treated with rapamycin for 30 min. Lysates were then prepared and analyzed by SUMO and H3 immunoblots. Polysumoylated conjugates accumulate in *ulp2Δ* yeast strains and can be seen in the stacking gel (Bylebyl *et al.* 2003). **(D)** SDS-treated Ubc9-AA cultures were exposed to DMSO (-) or MG132 (+) for 30 min, then treated with rapamycin or left untreated for an additional 30 min. Lysates were then prepared and analyzed by SUMO, H3, and ubiquitin (Ub) immunoblots. **(E)** As in *D*, except samples were treated with rapamycin for 8 min. **(F)** A version of the Ubc9-AA strain lacking the *PDR5* gene was treated with MG132 for 30 min and/or rapamycin for 8 min, or left untreated, and lysates were analyzed by SUMO and GAPDH immunoblots.

Figure 1

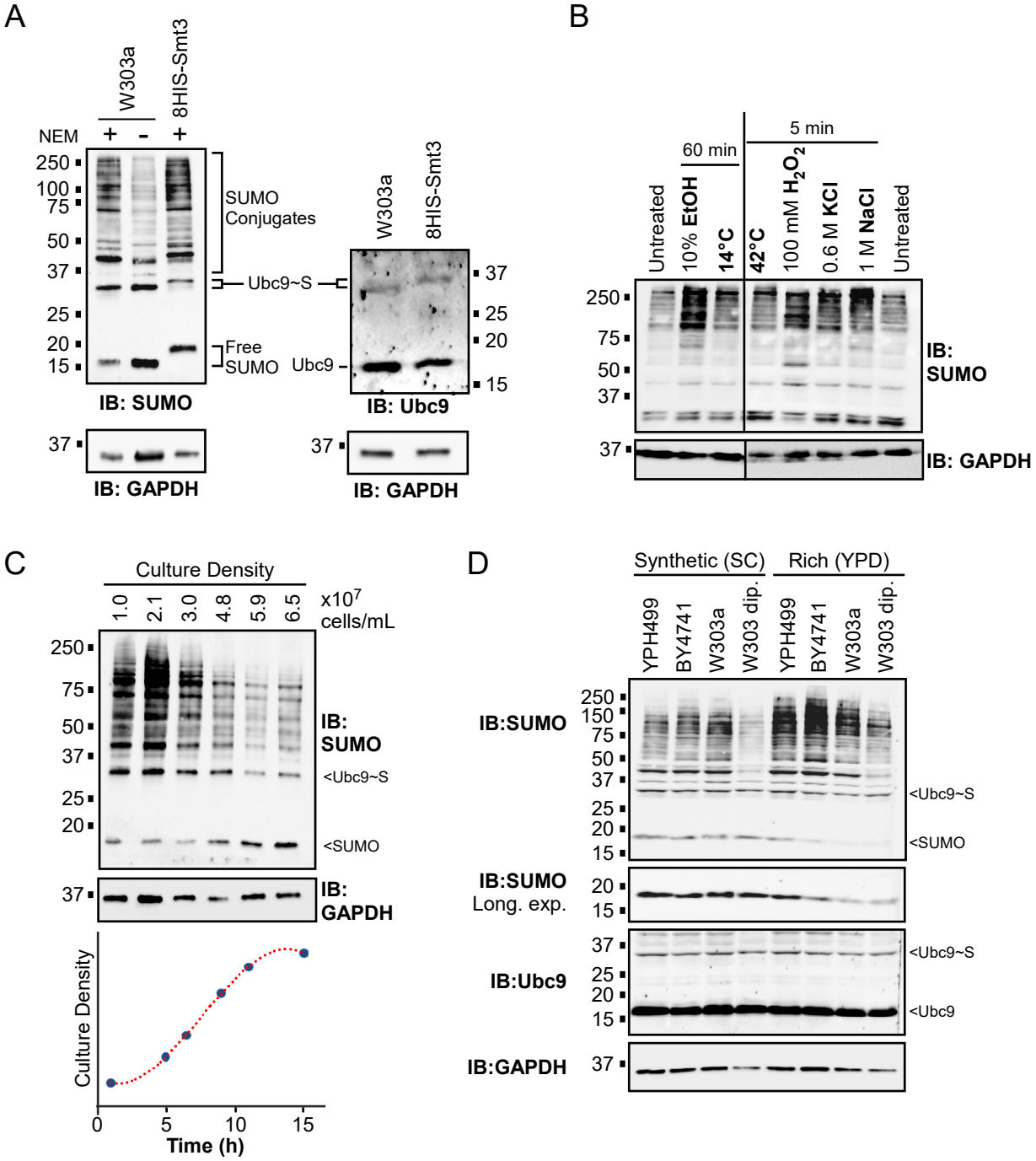

A
W303a
8HIS-Smt3
NEM
+
-
+
250
100
75
50
37
25
20
15
SUMO Conjugates
Ubc9~S
Free SUMO
Ubc9
IB: SUMO
IB: Ubc9
IB: GAPDH
B
Untreated
10% EtOH
14°C
42°C
100 mM H2O2
0.6 M KCl
1 M NaCl
Untreated
60 min
5 min
IB: SUMO
IB: GAPDH
C
Culture Density
1.0
2.1
3.0
4.8
5.9
6.5
x10^7 cells/mL
IB: SUMO
<Ubc9~S
<SUMO
IB: GAPDH
Culture Density
Time (h)
D
Synthetic (SC)
Rich (YPD)
YPH499
BY4741
W303a
W303 dip.
IB:SUMO
IB:SUMO Long. exp.
IB:Ubc9
IB:GAPDH
<Ubc9~S
<SUMO
<Ubc9

Figure 2

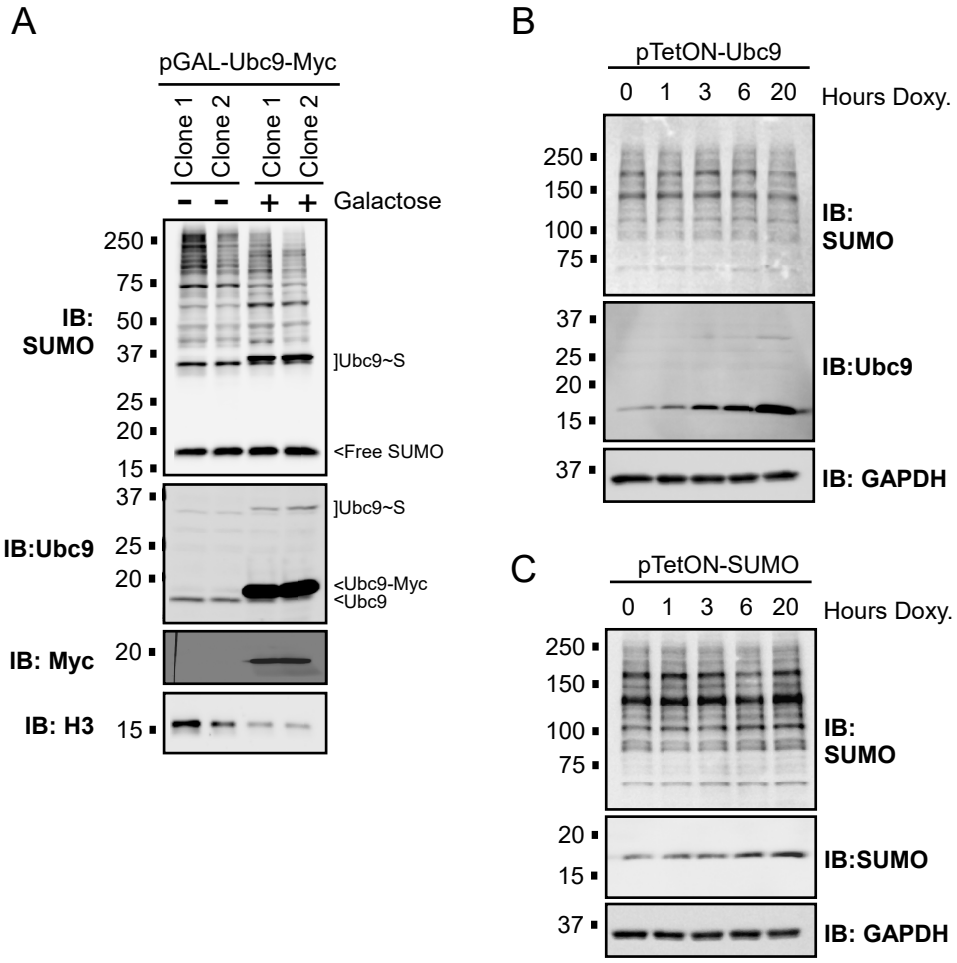

A
pGAL-Ubc9-Myc
Clone 1
Clone 2
Clone 1
Clone 2
- - + + Galactose
250
75
50
37
25
20
15
IB: SUMO
]Ubc9~S
<Free SUMO
37
25
20
IB:Ubc9
]Ubc9~S
<Ubc9-Myc
<Ubc9
20
IB: Myc
15
IB: H3
B
pTetON-Ubc9
0 1 3 6 20 Hours Doxy.
250
150
100
75
IB: SUMO
37
25
20
15
IB:Ubc9
37
IB: GAPDH
C
pTetON-SUMO
0 1 3 6 20 Hours Doxy.
250
150
100
75
IB: SUMO
20
15
IB:SUMO
37
IB: GAPDH

Figure 3

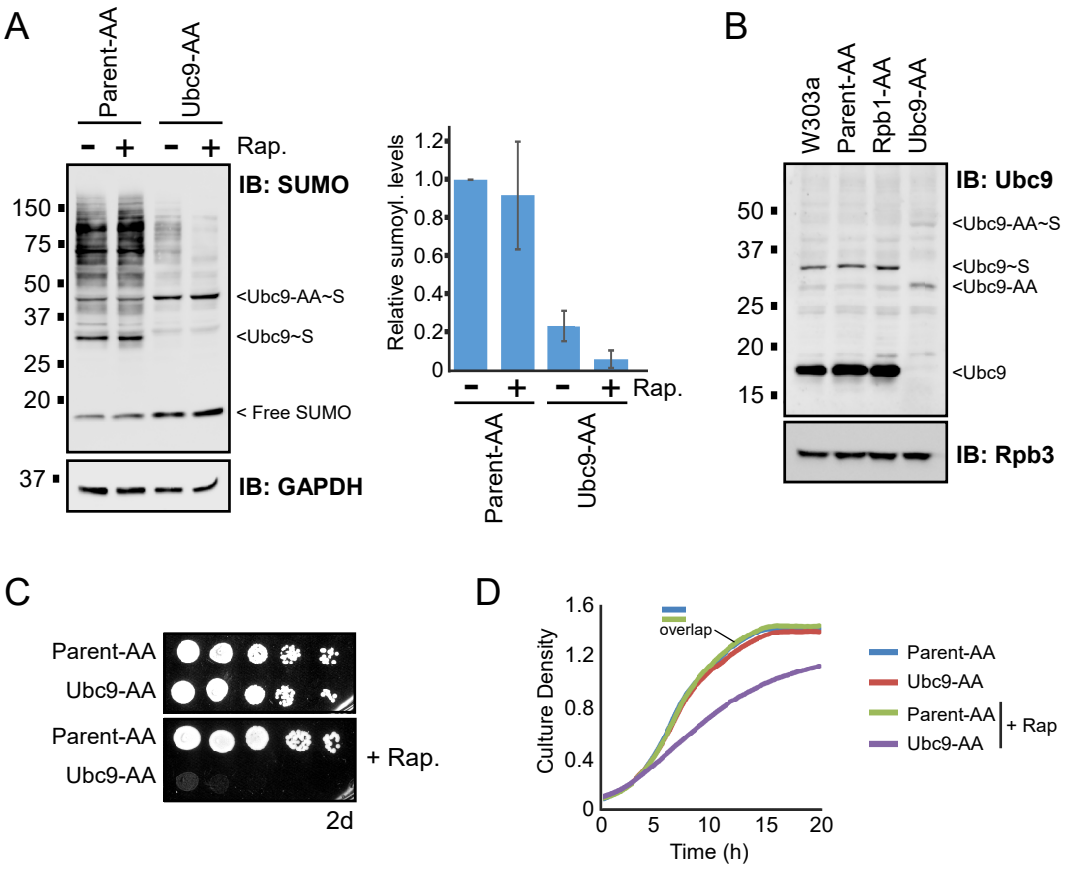

A
Parent-AA
Ubc9-AA
- + - + Rap.
IB: SUMO
150
75
50
37
25
20
<Ubc9-AA~S
<Ubc9~S
< Free SUMO
37
IB: GAPDH
Relative sumoyl. levels
1.2
1.0
0.8
0.6
0.4
0.2
0
- + - + Rap.
Parent-AA
Ubc9-AA
B
W303a
Parent-AA
Rpb1-AA
Ubc9-AA
IB: Ubc9
50
37
25
20
15
<Ubc9-AA~S
<Ubc9~S
<Ubc9-AA
<Ubc9
IB: Rpb3
C
Parent-AA
Ubc9-AA
Parent-AA
Ubc9-AA
+ Rap.
2d
D
Culture Density
1.6
1.2
0.8
0.4
0
overlap
0
5
10
15
20
Time (h)
Parent-AA
Ubc9-AA
Parent-AA
Ubc9-AA
+ Rap

Figure 4

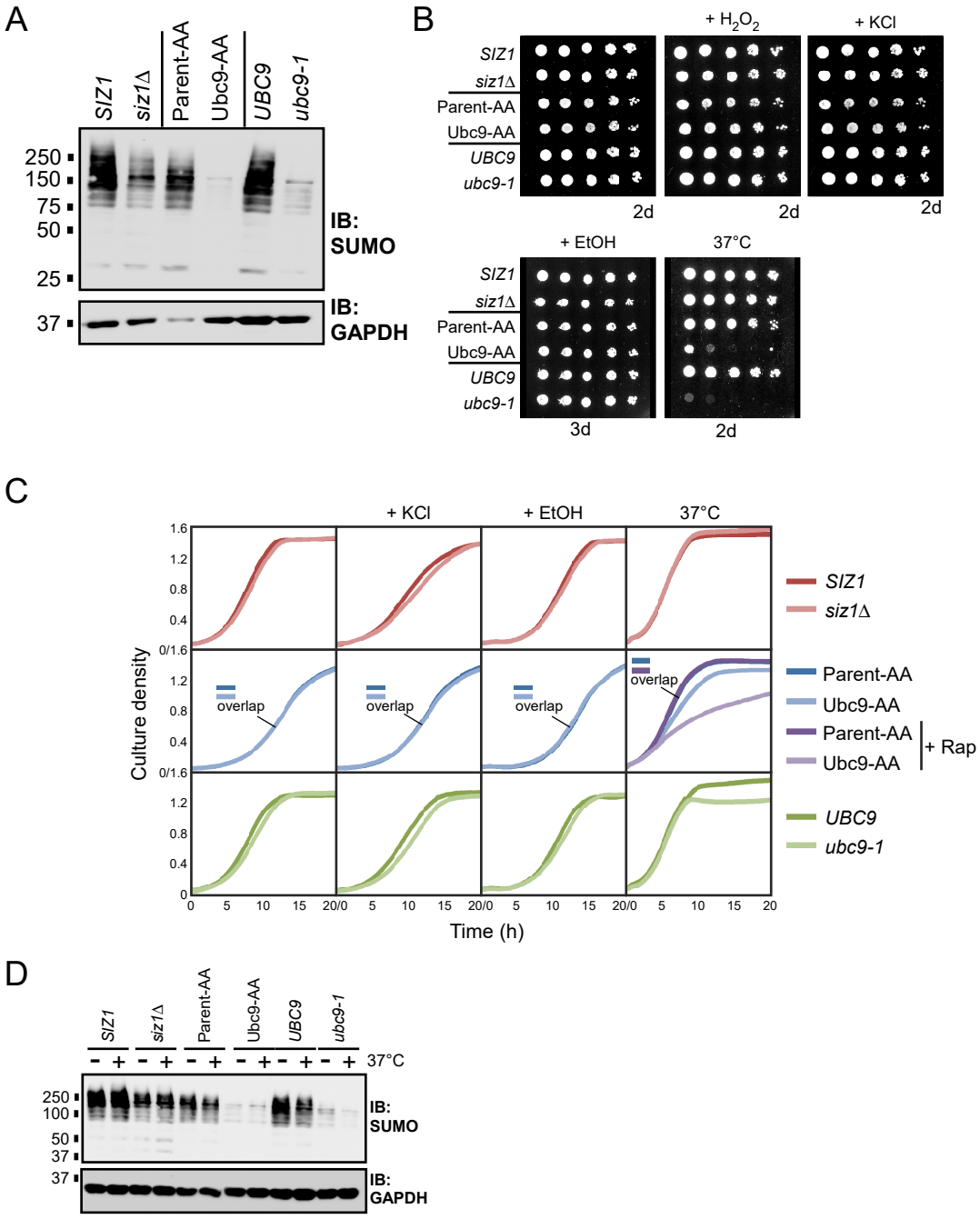

A
SIZ1
siz1Δ
Parent-AA
Ubc9-AA
UBC9
ubc9-1
250
150
75
50
25
37
IB: SUMO
IB: GAPDH
B
+ H2O2
+ KCl
SIZ1
siz1Δ
Parent-AA
Ubc9-AA
UBC9
ubc9-1
2d
2d
2d
+ EtOH
37°C
3d
2d
C
+ KCl
+ EtOH
37°C
Culture density
overlap
Time (h)
SIZ1
siz1Δ
Parent-AA
Ubc9-AA
Parent-AA
Ubc9-AA
+ Rap
UBC9
ubc9-1
D
37°C
250
100
50
37
37
IB: SUMO
IB: GAPDH

Figure 5

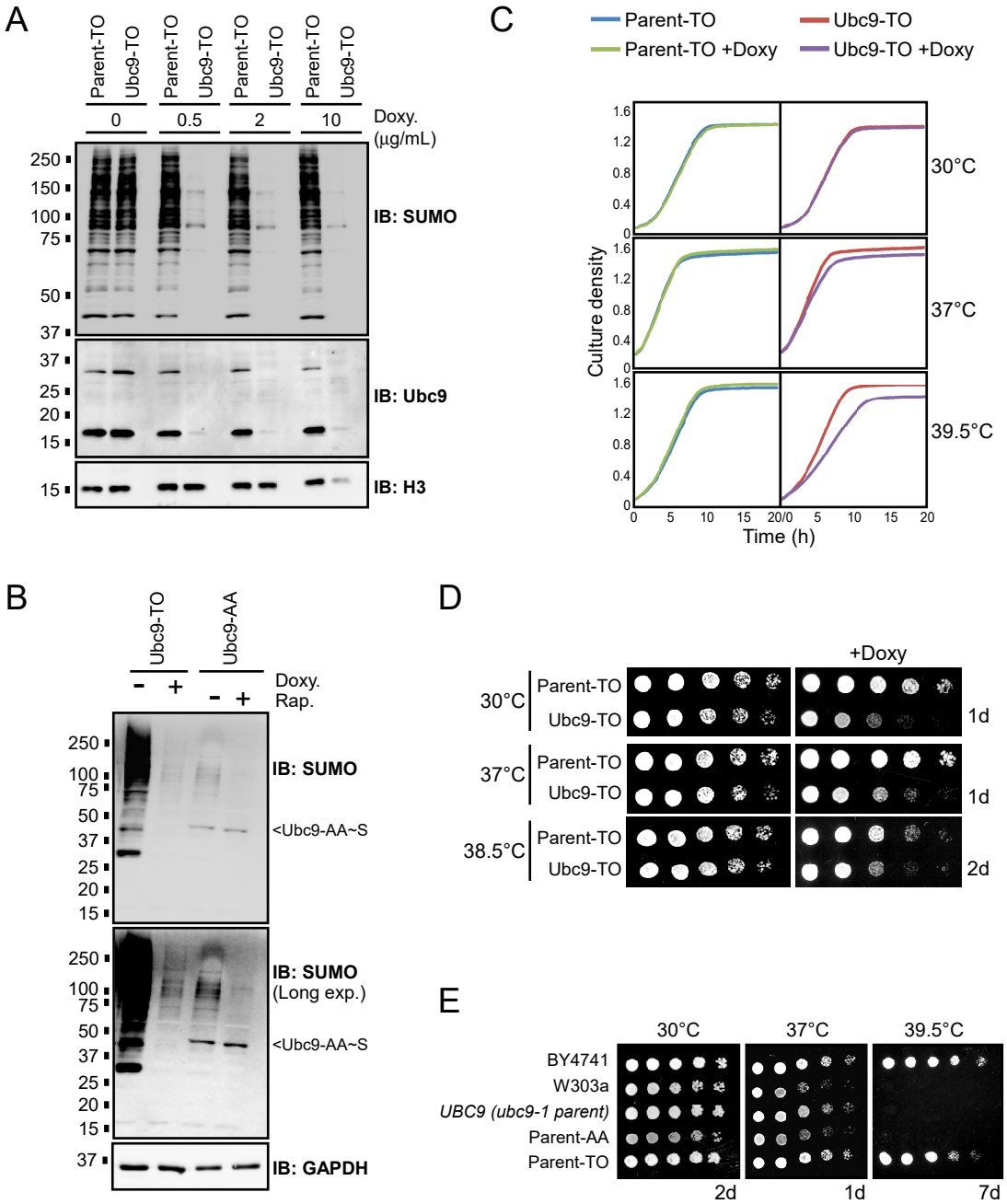
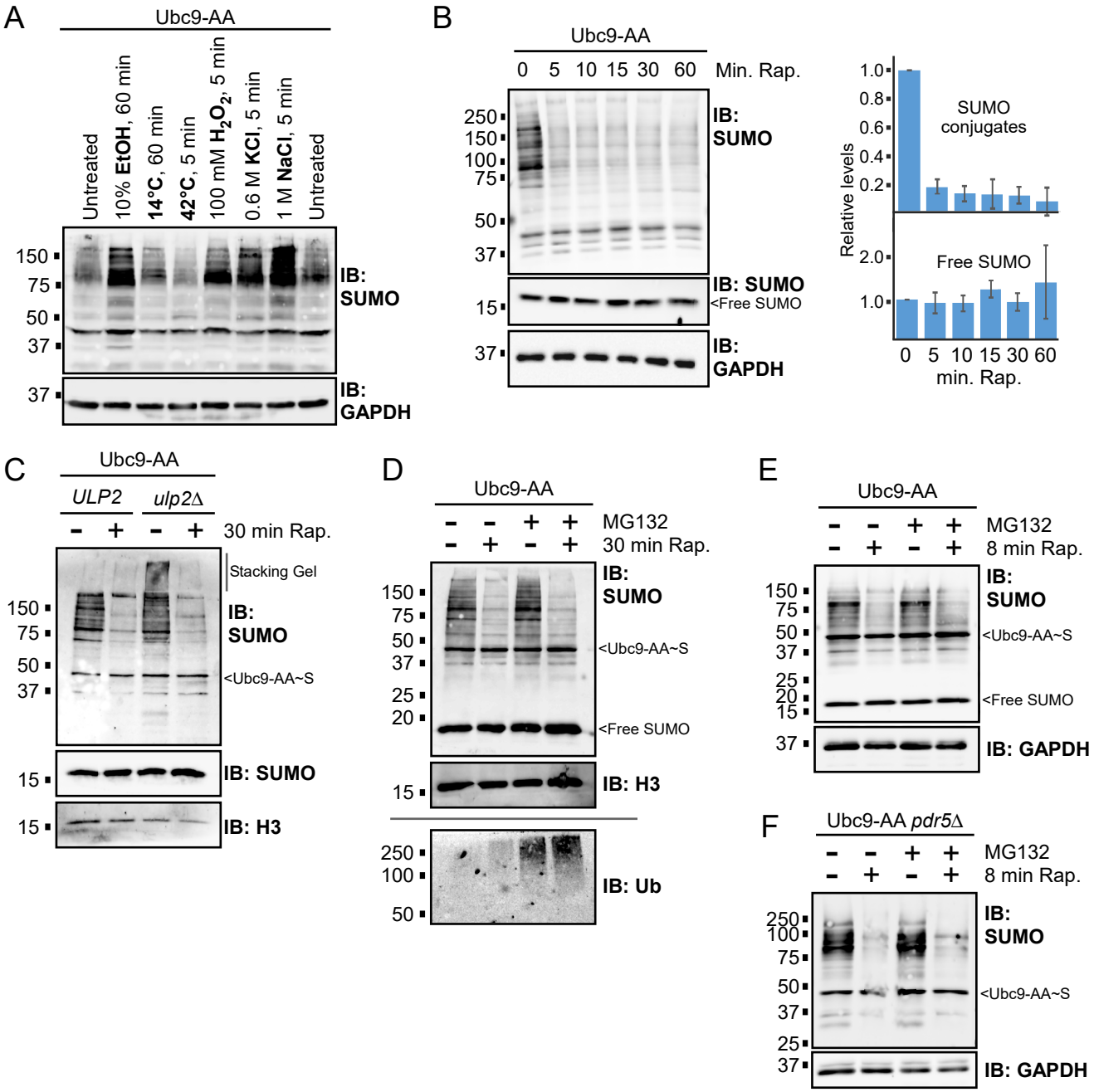

A
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
0
0.5
2
10
Doxy. (µg/mL)
250
150
100
75
50
37
IB: SUMO
37
25
20
15
IB: Ubc9
15
IB: H3
B
Ubc9-TO
Ubc9-AA
- +
- +
Doxy.
Rap.
250
100
75
50
37
25
20
15
IB: SUMO
<Ubc9-AA~S
250
100
75
50
37
25
20
15
IB: SUMO (Long exp.)
<Ubc9-AA~S
37
IB: GAPDH
C
Parent-TO
Ubc9-TO
Parent-TO +Doxy
Ubc9-TO +Doxy
Culture density
1.6
1.2
0.8
0.4
0
30°C
37°C
39.5°C
0
5
10
15
20/0
5
10
15
20
Time (h)
D
+Doxy
30°C
Parent-TO
Ubc9-TO
1d
37°C
Parent-TO
Ubc9-TO
1d
38.5°C
Parent-TO
Ubc9-TO
2d
E
30°C
37°C
39.5°C
BY4741
W303a
UBC9 (ubc9-1 parent)
Parent-AA
Parent-TO
2d
1d
7d

Figure 6


A
Ubc9-AA
Untreated
10% EtOH, 60 min
14°C, 60 min
42°C, 5 min
100 mM H2O2, 5 min
0.6 M KCl, 5 min
1 M NaCl, 5 min
Untreated
150
75
50
37
IB: SUMO
37
IB: GAPDH
B
Ubc9-AA
0 5 10 15 30 60 Min. Rap.
250
150
100
75
50
37
IB: SUMO
15
IB: SUMO
<Free SUMO
37
IB: GAPDH
Relative levels
1.0
0.8
0.6
0.4
0.2
SUMO conjugates
2.0
1.0
Free SUMO
0 5 10 15 30 60
min. Rap.
C
Ubc9-AA
ULP2
ulp2Δ
- + - + 30 min Rap.
Stacking Gel
150
75
50
37
IB: SUMO
<Ubc9-AA~S
15
IB: SUMO
15
IB: H3
D
Ubc9-AA
- - + + MG132
- + - + 30 min Rap.
150
75
50
37
25
20
IB: SUMO
<Ubc9-AA~S
<Free SUMO
15
IB: H3
250
100
50
IB: Ub
E
Ubc9-AA
- - + + MG132
- + - + 8 min Rap.
150
75
50
37
25
20
15
IB: SUMO
<Ubc9-AA~S
<Free SUMO
37
IB: GAPDH
F
Ubc9-AA pdr5Δ
- - + + MG132
- + - + 8 min Rap.
250
100
75
50
37
25
IB: SUMO
<Ubc9-AA~S
37
IB: GAPDH