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

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Running Title: Effects of modulated sumoylation on cell fitness

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ABSTRACT

Sumoylation is an essential, conserved protein modification with hundreds of targets. Compared to the related modification ubiquitination, few 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 on cell growth, we examined yeast strains that artificially display elevated or reduced sumoylation. Remarkably, reducing sumoylation levels by >70% has no effect on cell fitness, indicating that most SUMO conjugation events are not required for normal growth. Surprisingly, among a panel of stress conditions, only elevated temperatures severely impacted growth of cells harbouring constitutively low sumoylation levels. 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 basal level of sumoylation for growth, but that normal levels are required pre-emptively to facilitate survival at elevated temperatures.

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 ~12 kDa 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)). 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 (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. In addition to stress, we found that cellular SUMO conjugation levels change with ploidy, culture density, and nutrient availability. By examining mutant yeast strains that harbour altered sumoylation levels, either constitutively or conditionally, we determined that a moderate increase or a 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 the rapid loss of cellular sumoylation, 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 a yeast SUMO antibody. 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, implying 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, Smt3, 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") 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, whereas SUMO proteases recognize and deconjugate isopeptide bonds. This analysis indicates 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) or ethanol (10% for 60 min) stress resulted in a two- to four-fold increase in SUMO conjugation levels (Fig. 1B) (Zhou *et al.* 2004; Lewicki *et al.* 2015). However, neither heat shock (15 min at 37°C) or exposure to oxidative conditions (100 mM H_2O_2 for 5 min)

influenced cellular sumoylation levels in our analysis, whereas others have seen significantly increased sumoylation under oxidative stress (Zhou *et al.* 2004; Lewicki *et al.* 2015). 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 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, genetic background.

Modestly elevated sumoylation levels have no effect on yeast growth

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 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.

As a different approach to artificially and conditionally elevate SUMO conjugation levels, we targeted the major SUMO protease, Ulp1. A yeast strain was generated that expresses Ulp1 as a fusion with a C-terminal HA tag and an auxin-inducible degron (AID; Ulp1-AID*-HA). The presence of the AID causes fused proteins to be targeted for ubiquitination and proteasome-mediated degradation when the plant hormone auxin is included in growth media (Nishimura and Kanemaki 2014). When the Ulp1-AID*-HA strain was exposed to auxin over a time course, levels of Ulp1 gradually decreased to ~40% at 60 min, and levels of cellular sumoylation were noticeably increased after 90 min to ~1.4-fold (Fig. 2D). Using a spot assay, we tested whether this level of elevated sumoylation affects yeast growth. However, we detected no difference in growth between the Ulp1-AID-HA strain and its corresponding parent strain, Parent-AID, which has an unmodified *ULP1* gene, in the presence or absence of auxin (Figs. 2E). This indicates that a moderate increase in cellular sumoylation levels has no apparent effect on yeast growth.

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, this is expected to cause a significant reduction in overall cellular sumoylation levels (Cubenas-Potts *et al.* 2013). 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 70% 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 ~70% 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 (Fig. 3C, D). 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 a 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 shown below, that small differences in our growth curves, specifically at the level of cell saturation, 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 constitutively display 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; *ubc9-1*, which harbours a point mutation that partially reduces Ubc9 activity (Betting and Seufert 1996); and their wild-type parent strains (*SIZ1*, Parent-AA, and *UBC9*, respectively). 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, and

ethanol stress (Figs. 4B, C). Surprisingly, this indicates that normal sumoylation levels are not required for survival during these types of stress.

In contrast to the other stress conditions tested, strains Ubc9-AA and *ubc9-1*, 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). 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 surviving 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). To determine what effect such low levels of sumoylation has on yeast growth, we carried out spot assays and generated growth curves. In both assays, the Ubc9-TO strain grew as well as its parental strain (Parent-TO in Fig. 5) in the absence of doxycycline. When both strains were grown in culture in the presence of the drug, then spotted onto medium supplemented with doxycycline, a marked growth defect was observed in the Ubc9-TO strain (Fig. 5B). However, the growth curves for the two strains were virtually identical and unaffected by doxycycline (Fig. 5C). Using the Ubc9-TO system, these experiments confirm that a dramatic reduction in cellular sumoylation levels is well-tolerated by yeast, particularly during growth in liquid culture.

To determine why the growth defect in rapamycin-treated Ubc9-AA is more severe than the defect seen in the doxycycline-treated Ubc9-TO strain (compare Figs. 3C and 5B), we compared their sumoylation levels when the strains were grown and analyzed side-by-side. In the same experiment, we included the *siz1Δ* strain and its parent. Among the strains and conditions tested, *siz1Δ* showed low sumoylation levels, followed by less sumoylation in the untreated Ubc9-AA strain, then the Ubc9-TO strain treated with doxycycline, and, finally, the rapamycin-treated Ubc9-AA strain showed the lowest level of sumoylation (Fig. 5D). Quantification of sumoylation levels indicated that rapamycin-treated Ubc9-AA strain harbours 20% less sumoylation than the doxycycline-treated Ubc9-TO strain, which is consistent with the more significant growth defect seen in the former. In summary, the doxycycline treated Ubc9-TO strain shows intermediate levels of sumoylation compared to untreated and rapamycin-treated Ubc9-AA, and, accordingly, it shows intermediate growth effects, with normal liquid growth but defective growth on solid medium.

Next, we examined whether the reduced sumoylation level in doxycycline treated Ubc9-TO confers temperature-sensitivity to the strain, as in the Ubc9-AA and *ubc9-1* strains. Cultures of Ubc9-TO and its parent were grown for several hours at 30°C, untreated or in the presence of doxycycline, then growth was assessed by spot assays and liquid growth curves with incubation at 37°C. Compared to the normal temperature, incubation at the elevated temperature conferred no apparent additional growth defect on doxycycline treated Ubc9-TO, as determined by spot assay (compare Figs. 5B and E). However, a small but significant reduction in culture density was detected specifically for the Ubc9-TO strain grown at 37°C in the presence of doxycycline (Fig. 5F). Although the effects in the Ubc9-TO strain are not as dramatic as in the Ubc9-AA strain, potentially because they derive from genetically different background strains, these results support that reduced sumoylation has an impact on heat tolerance in yeast.

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 primarily intended as a method of reducing cellular sumoylation levels, we realized that the system could also be used to examine the stability of SUMO modifications. Specifically, by preventing 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. 6A). The residual level of SUMO conjugation did not further decrease with longer exposure to the drug, and might derive from cytoplasmic 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. 6A 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. 6A, B). 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 carrier, 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. 6C). However, treatment with rapamycin in MG132-treated cells, for either 30 or 8 min, still resulted in the rapid disappearance of SUMO conjugates (Fig. 6C, D). 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 M G132 (Collins *et al.* 2010). Nonetheless, deletion of *PDR5* had no effect on the loss of SUMO conjugates in MG132- and rapamycin-treated cells (Fig. 6E). 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 SUMO conjugation. This was apparent by comparing sumoylation levels in yeast grown in rich vs 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. Yeast cultures are typically treated with stressors when they are growing exponentially, as in Fig. 1B, indicating that stress elevates 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 other that cells elevate sumoylation globally by inactivating or deactivating SUMO proteases (Sydorskyy *et al.* 2010; Pinto *et al.* 2012; Lewicki *et al.* 2015). Accordingly, we generated a yeast strain in which one of the two SUMO proteases, Ulp1, is conditionally depleted upon treatment with auxin. Indeed, Ulp1 depletion by this method resulted in a modest increase in global sumoylation, which had no apparent affect on growth.

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 conjugation events 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.

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 the 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 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 30% of the normal levels of SUMO conjugation, this observation suggests that the accumulation of misfolded proteins destined for proteasomal degradation does not trigger a sumoylation response in budding yeast, as it does is higher eukaryotes.

By examining growth in the Ubc9-AA strain and others that express partially defective or diminished Ubc9, we determined that yeast can tolerate dramatic reductions in sumoylation levels under normal growth. Whereas a reduction to ~30% 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 (W303a and BY4741, respectively), and that genetic variations between the strains might have an impact on specific

tolerance levels. Nonetheless, these results suggest that somewhere between 70% and 95% of SUMO conjugation events are not required for normal growth, and might therefore serve a protective, pre-emptive role in anticipation of stress-inducing changes in the environment. However, reduced sumoylation 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)). These observations align with the proposal that the SSR is a consequence of altered transcription genome-wide, which involves a wave of transcription-mediated SUMO conjugation, and suggests that elevated sumoylation plays no protective role for these stresses (Lewicki *et al.* 2015). Reduced cellular sumoylation levels did elicit sensitivity to elevated temperatures, however, 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, 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 for 5 min; 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; 0.5 mM auxin (indole-3-acetic acid), for times indicated; 300 µg/mL hygromycin B for solid media. 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 to 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 in the morning 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, pellets were then 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 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 – 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 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 or H3 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 and growth rates were 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 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.

ACKNOWLEDGEMENTS

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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, unconjugated 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. Unconjugated SUMO is shown in a separate immunoblot than the SUMO conjugates. **(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. Relative sumoylation levels, shown in the graph at bottom, were determined as indicated in the Materials and Methods.

Figure 2. Moderately elevated sumoylation levels do not affect growth. (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*. **(D)** A yeast strain was generated that expresses the major SUMO protease Ulp1 as a fusion with the minimal auxin-induced degron (AID*) and 6xHA tag (Ulp1-AID*-HA). Cultures of the strain were grown in YPD and treated with auxin for the indicated times, then protein extracts were prepared and analyzed by SUMO, HA, and GAPDH immunoblots. **(E)** The Ulp1-AID*-HA strain, and its otherwise isogenic parent strain, Parent-AID, were grown in YPD medium, or YPD supplemented with auxin, for several hours, then spotted onto YPD plates, untreated or supplemented as indicated, with serial five-fold dilutions spotted in each successive column. The AID*-HA fusion is marked with a gene that confers resistance to hygromycin, and the spot assay at the bottom confirms that the Ulp1-AID*-HA strain, but not the parent, is marked as such. Plates were imaged after two days of growth.

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 lysates were prepared and analyzed by SUMO and GAPDH immunoblots. **(B)** Lysates were prepared from cultures of W303a, Parent-AA, Ubc9-AA, or of the 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, as in Fig. 2E, 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 measurements were made every 15 min. 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.

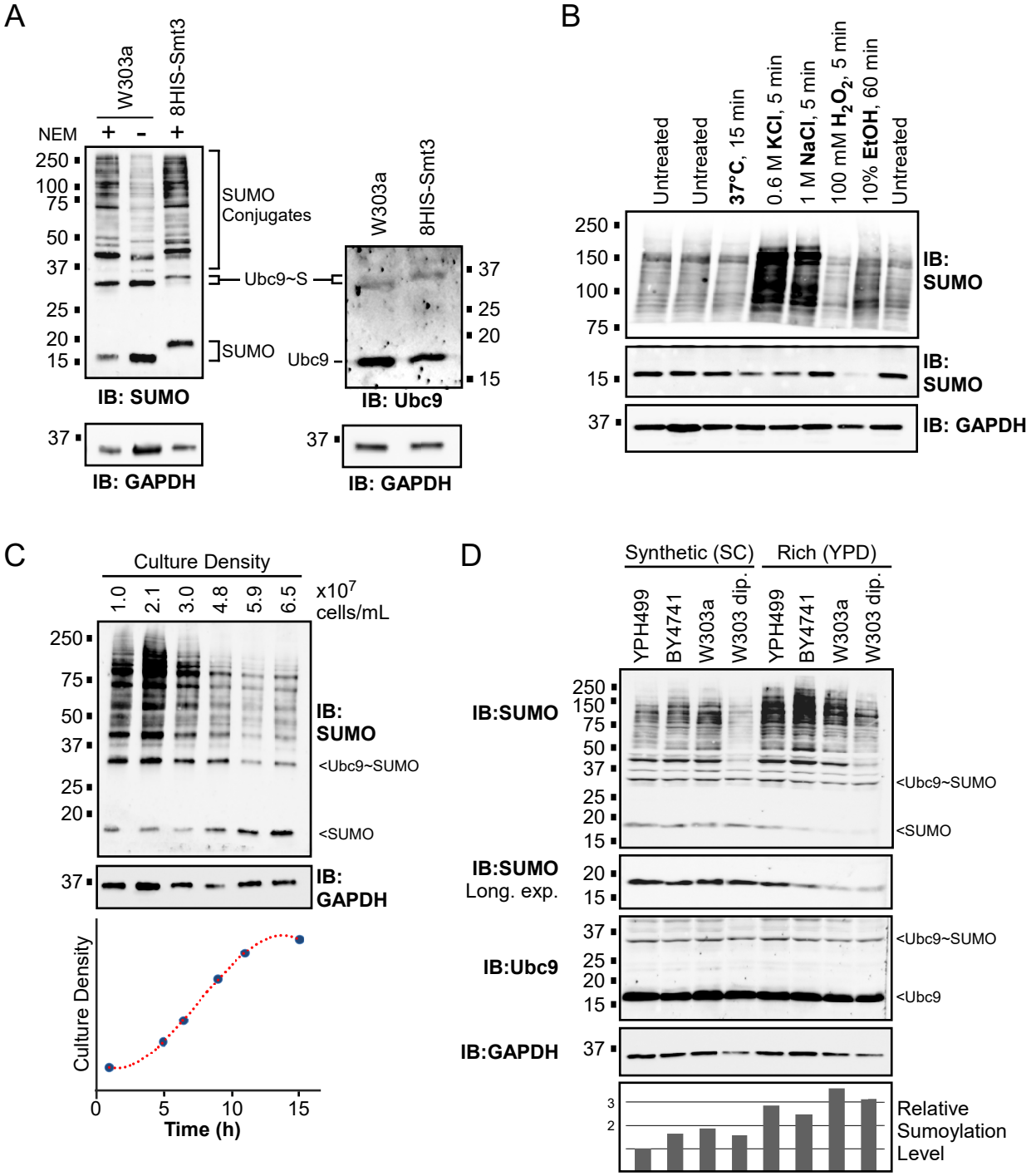
Figure 4. Yeast strains with constitutively low levels of sumoylation cannot grow at elevated temperatures. (A) Protein extracts were prepared from cultures of the indicated strains and analyzed by SUMO and GAPDH immunoblots. **(B)** Spot assays were performed, as in Fig. 2E, with the indicated strains, 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." For the Parent-AA and Ubc9-AA set, rapamycin treatment was included for the 37°C condition only. **(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 immunoblot. **(B)** A spot assay was performed, as in Fig. 2E, with the Ubc9-TO and Parent-TO strains, onto either YPD medium, or YPD supplemented with

doxycycline. Plates incubated at 30°C and were imaged after one day of growth (~30 h). **(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 30°C. The four curves essentially overlap each other. **(D)** Cultures of the *SIZ1*, s*iz1Δ*, 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. **(E)** A spot assay was performed as in *B*, except that incubation was at 37°C, and plates were imaged after one day. **(F)** Growth curves were generated as in *C*, except that samples were incubated at 37°C.

Figure 6. Many SUMO conjugation events are highly transient. (A) 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. **(B)** 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). **(C)** 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. **(D)** As in *C*, except samples were treated with rapamycin for 8 min. **(E)** 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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15
SUMO Conjugates
Ubc9~S
SUMO
IB: SUMO
37
IB: GAPDH
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8HIS-Smt3
37
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20
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Ubc9
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37
IB: GAPDH
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150
100
75
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15
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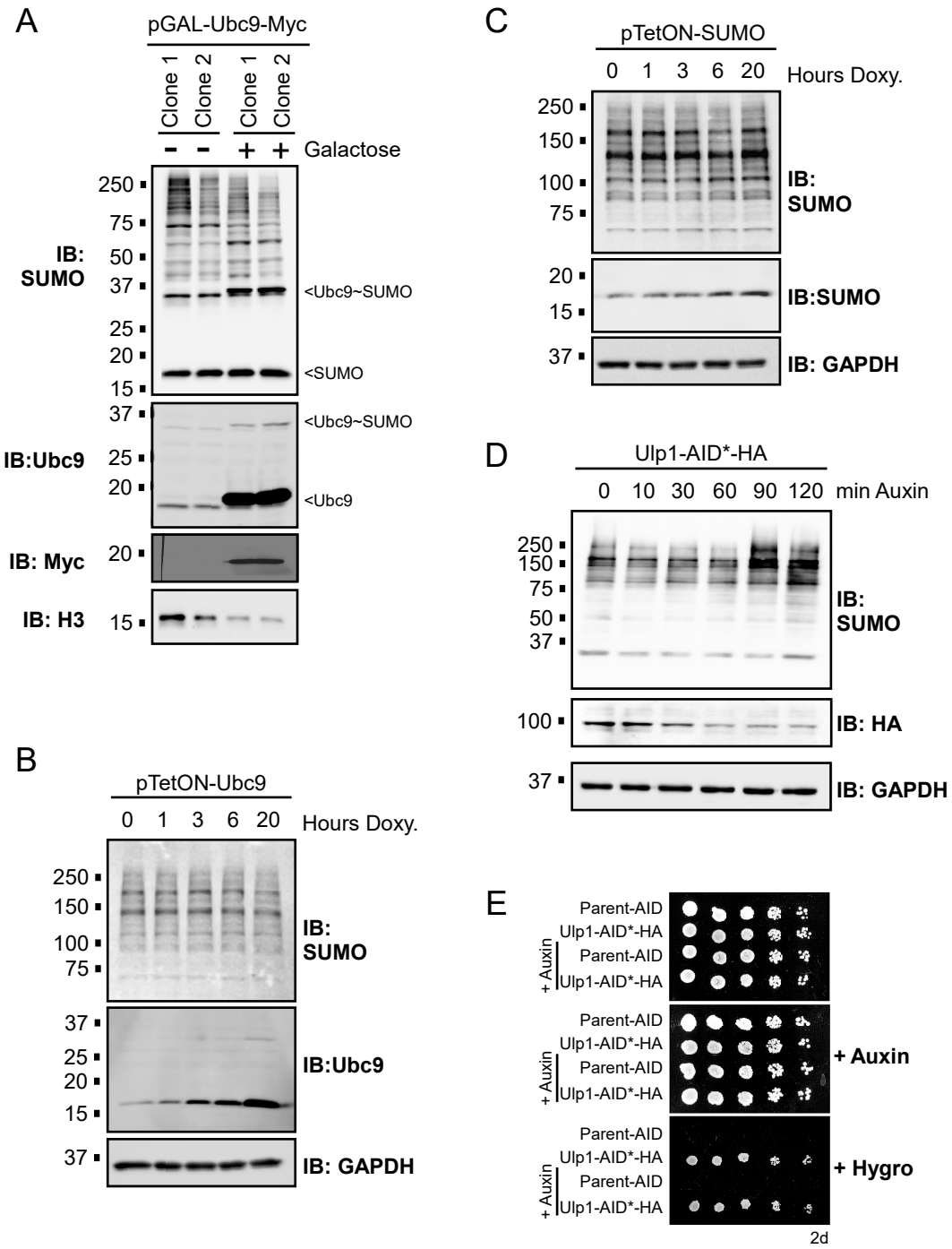
Relative Sumoylation Level

Figure 2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Clone 2
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IB: SUMO
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IB: HA
37
IB: GAPDH
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+ Auxin
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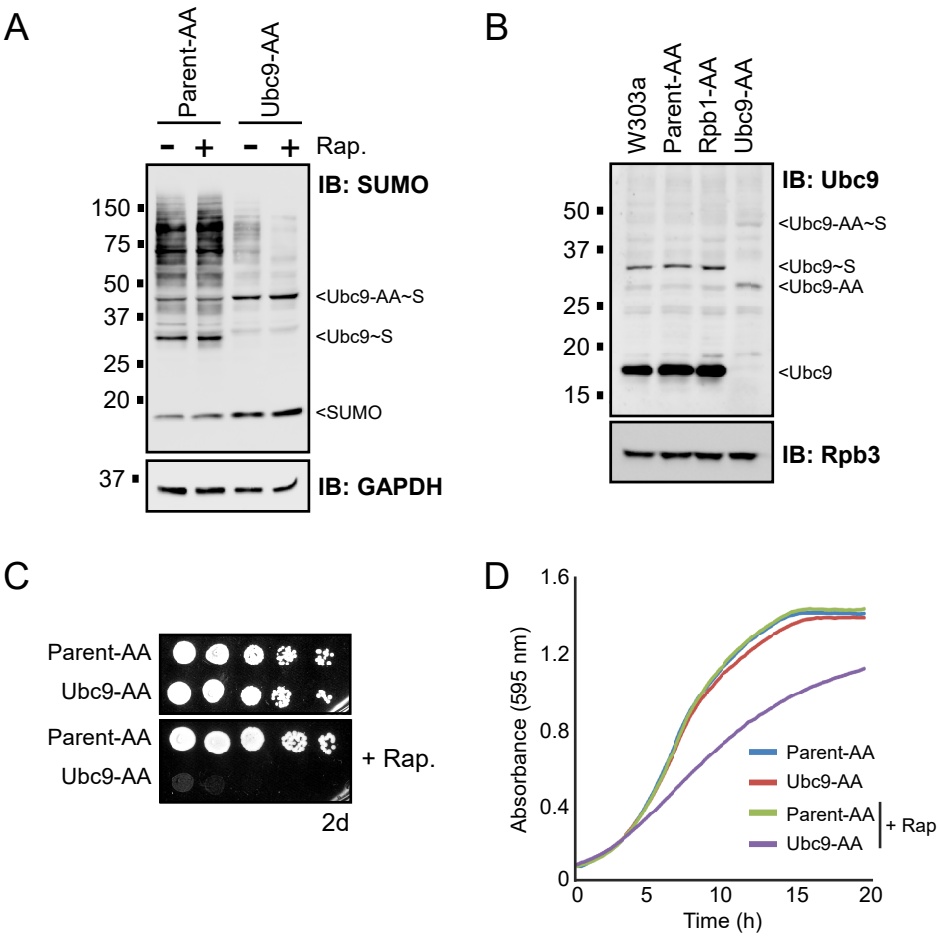
+ Hygro
2d

Figure 3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Ubc9-AA
Parent-AA
Ubc9-AA
+ Rap.
2d
D
Absorbance (595 nm)
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1.2
0.8
0.4
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5
10
15
20
Time (h)
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Ubc9-AA
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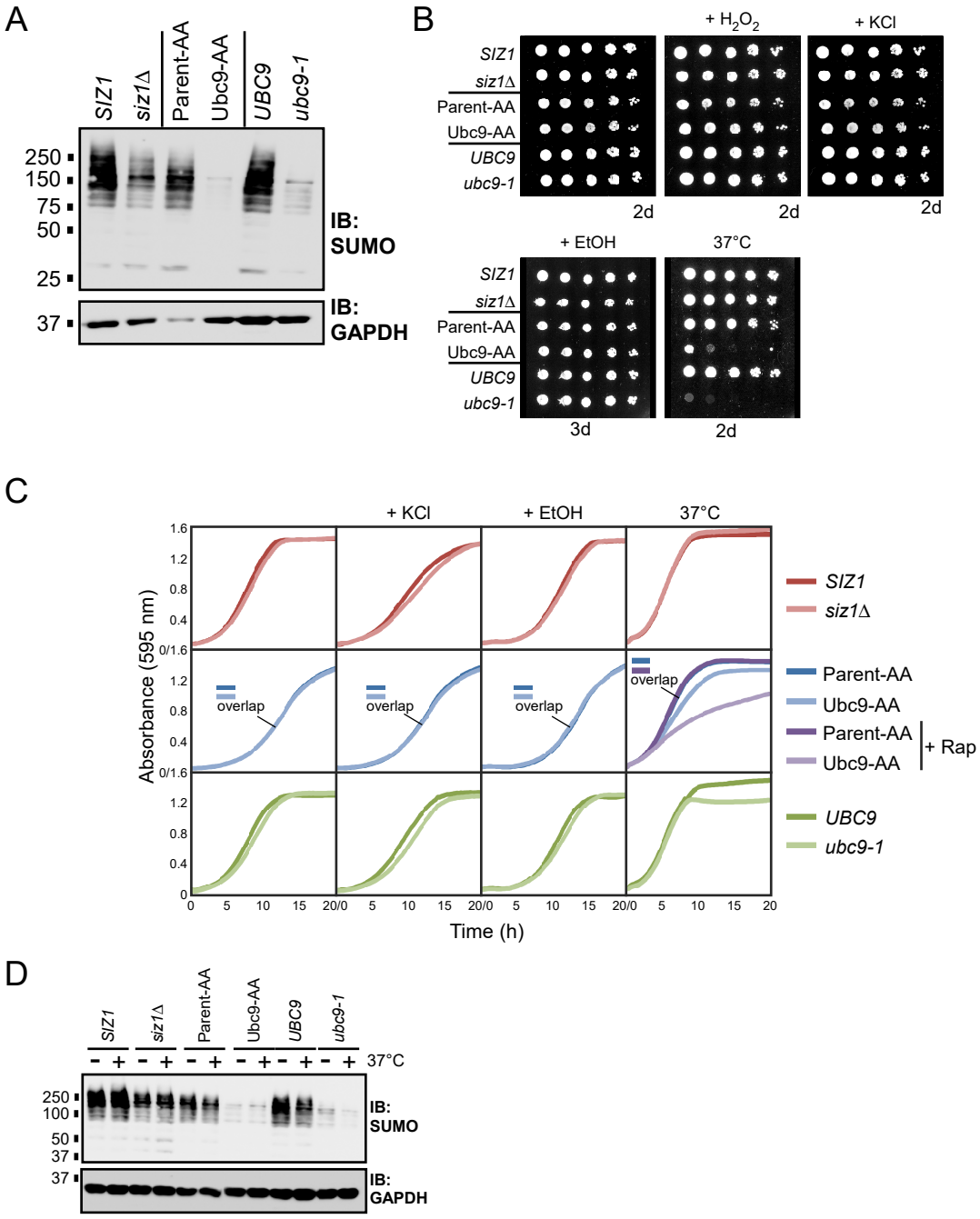
Ubc9-AA
+ Rap

Figure 4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+ KCl
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UBC9
ubc9-1
2d
2d
2d
+ EtOH
37°C
3d
2d
C
+ KCl
+ EtOH
37°C
Absorbance (595 nm)
overlap
SIZ1
siz1Δ
Parent-AA
Ubc9-AA
Parent-AA
Ubc9-AA
+ Rap
UBC9
ubc9-1
Time (h)
D
SIZ1
siz1Δ
Parent-AA
Ubc9-AA
UBC9
ubc9-1
37°C
250
100
50
37
37
IB: SUMO
IB: GAPDH

Figure 5

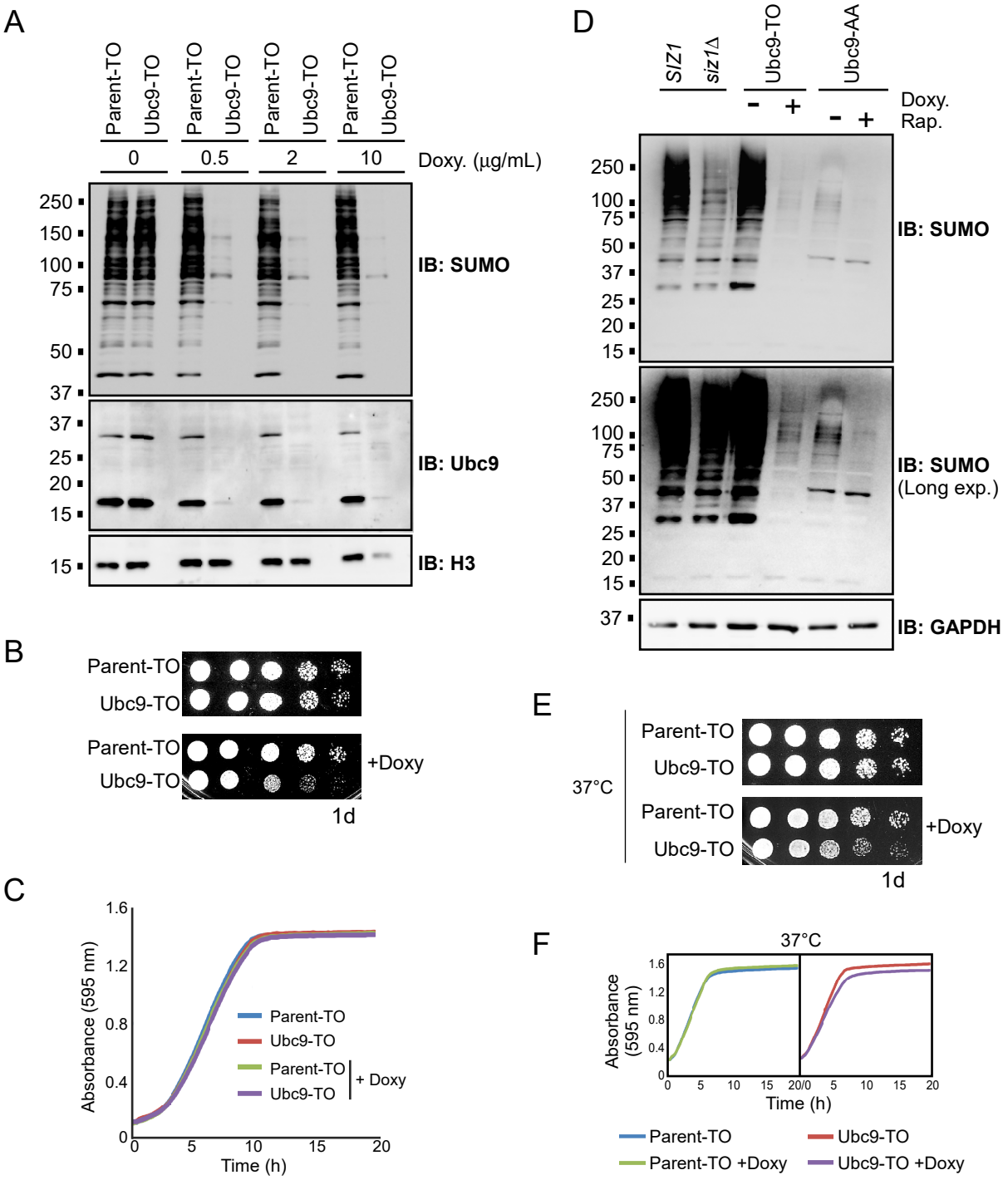
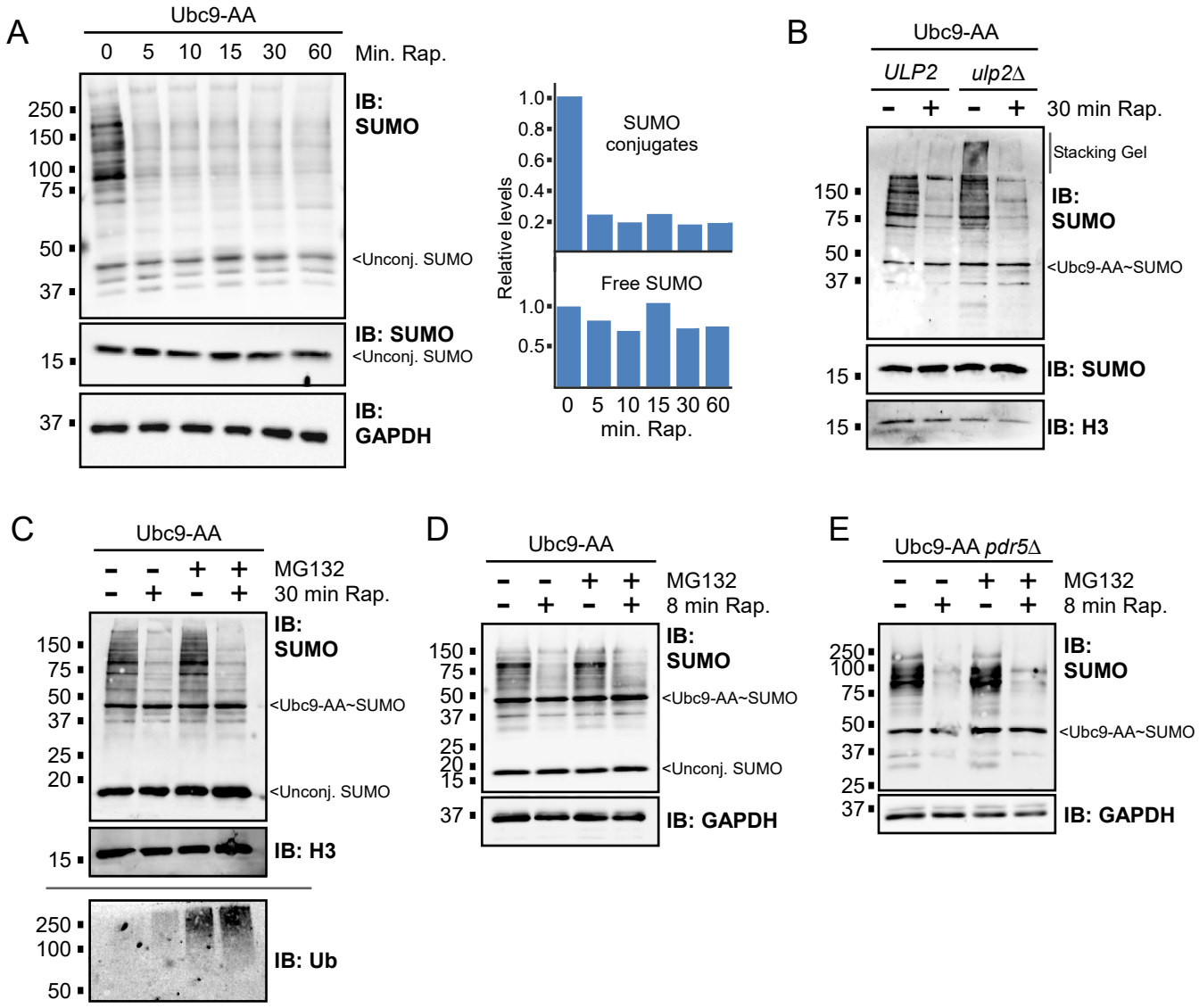

A
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
0
0.5
2
10
Doxy. (µg/mL)
250
150
100
75
50
37
IB: SUMO
37
25
20
15
IB: Ubc9
15
IB: H3
B
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
+Doxy
1d
C
Absorbance (595 nm)
1.6
1.2
0.8
0.4
0
0
5
10
15
20
Time (h)
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
+ Doxy
D
SIZ1
siz1Δ
Ubc9-TO
Ubc9-AA
-
+
-
+
Doxy.
Rap.
250
100
75
50
37
25
20
15
IB: SUMO
250
100
75
50
37
25
20
15
IB: SUMO
(Long exp.)
37
IB: GAPDH
E
37°C
Parent-TO
Ubc9-TO
Parent-TO
Ubc9-TO
+Doxy
1d
F
37°C
Absorbance
(595 nm)
1.6
1.2
0.8
0.4
0
0
5
10
15
20/0
5
10
15
20
Time (h)
Parent-TO
Ubc9-TO
Parent-TO +Doxy
Ubc9-TO +Doxy

Figure 6


A
Ubc9-AA
0 5 10 15 30 60 Min. Rap.
250
150
100
75
50
37
15
37
IB: SUMO
<Unconj. SUMO
IB: SUMO
<Unconj. SUMO
IB: GAPDH
SUMO conjugates
Free SUMO
Relative levels
1.0
0.8
0.6
0.4
0.2
1.0
0.5
0 5 10 15 30 60
min. Rap.
B
Ubc9-AA
ULP2
ulp2Δ
- + - + 30 min Rap.
Stacking Gel
150
75
50
37
15
15
IB: SUMO
<Ubc9-AA~SUMO
IB: SUMO
IB: H3
C
Ubc9-AA
- - + + MG132
- + - + 30 min Rap.
150
75
50
37
25
20
15
250
100
50
IB: SUMO
<Ubc9-AA~SUMO
<Unconj. SUMO
IB: H3
IB: Ub
D
Ubc9-AA
- - + + MG132
- + - + 8 min Rap.
150
75
50
37
25
20
15
37
IB: SUMO
<Ubc9-AA~SUMO
<Unconj. SUMO
IB: GAPDH
E
Ubc9-AA pdr5Δ
- - + + MG132
- + - + 8 min Rap.
250
100
75
50
37
25
37
IB: SUMO
<Ubc9-AA~SUMO
IB: GAPDH