De-sumoylation of RNA polymerase III lies at the core of the Sumo stress response in yeast

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Abstract

Posttranslational modification by small ubiquitin‐like modifier (Sumo) regulates many cellular processes, including the adaptive response to various types of stress, referred to as the Sumo stress response (SSR). However, it remains unclear whether the SSR involves a common set of core proteins regardless of the type of stress or whether each particular type of stress induces a stress-specific SSR that targets a unique, largely non-overlapping set of Sumo substrates. In this study, we used mass spectrometry and a gene ontology approach to identify differentially sumoylated proteins during heat stress, hyperosmotic stress, oxidative stress, nitrogen starvation, and DNA alkylation in Saccharomyces cerevisiae cells. Our results indicate that each stress triggers a specific SSR signature centered on proteins involved in transcription, translation, and chromatin regulation. Strikingly, while the various stress-specific SSRs were largely non-overlapping, all types of stress tested here resulted in de-sumoylation of subunits of RNA polymerase III, which correlated with a decrease in tRNA synthesis. We conclude that de-sumoylation and subsequent inhibition of RNA polymerase III constitutes the core of all stress-specific SSRs in yeast.

Rapidly sensing and relaying stimuli is a key aspect of normal cellular physiology, and is of great importance for maintaining cellular homeostasis during environmental stress. To survive stress, cells rewire their transcriptome to promote the transcription of key genes whose products will help to adapt to the stressful environment. Rewiring of transcriptional programs typically involves rapid posttranslational modification (PTM) of signaling proteins and transcription factors.

Sumoylation is a conserved PTM involving the covalent attachment of the Small
Ubiquitin-like Modifier (Sumo) to target proteins, and plays a critical role in cellular responses to stress. Plants express eight versions of Sumo while mammals express four Sumo isoforms (Sumo-1, -2, -3 and -4) (1). In contrast, the budding yeast *Saccharomyces cerevisiae* expresses one Sumo isoform encoded by the essential gene *SMT3* (2). Protein sumoylation is a reversible process that follows an ordered series of events. Sumo is initially synthesized as an inactive precursor, which is proteolytically processed by the yeast Sumo proteases Ulp1 and Ulp2 (SSEN1 to SSEN6 in mammals) at the C-terminal tail to yield an active conjugatable form (3,4). Subsequently, an E1 activating enzyme consisting of the Aos1/Uba2 heterodimer (Sae1/Sae2 in mammals) (5,6), transfers Sumo to the sole E2 conjugating enzyme Ubc9, which is conserved from yeast to humans (5,6). Ubc9 then transfers Sumo to one of its substrates, either with or without the help of E3 enzymes (7,8). Finally, the Sumo proteases Ulp1 and Ulp2 can remove Sumo to complete the Sumoylation cycle (3).

Sumoylation of a protein may influence its fate and activity. For instance, Sumo conjugation can induce conformational changes (9,10); hide or reveal interacting motifs, thereby altering protein-protein or protein-DNA interactions (11-14); affect cellular localization (15); compete with other PTMs (16,17); alter enzymatic activity (18); or affect protein stability (19). Sumo is often simultaneously conjugated to a group of proteins that belong to the same complex or act within the same pathway, a phenomenon referred to as waves of sumoylation or Sumo spray, which is assumed to reinforce the activation of a given biological process, such as DNA damage checkpoint activation and DNA repair (11,20).

Under homeostatic conditions, a large portion of Sumo substrates is involved in the process of transcription. It was long thought that sumoylation generally had an inhibitory effect on transcription, an assumption that has since been challenged (reviewed in (21)). Indeed, we have shown that chromatin-bound Sumo promotes the expression of pro-growth genes (22,23). More specifically, we found that sumoylation of the transcription factor Rap1 promotes the interaction between Rap1 and the RNAPIII transcriptional machinery, thereby stimulating transcription of ribosomal protein genes (RPG) (22). We have also recently shown that sumoylation of RNA polymerase III (RNAPIII) promotes tRNA synthesis (23).

Interestingly, the Ubc9-Sumo pathway also has important functions in the response to environmental stress, a phenomenon often referred to as the “Sumo Stress Response” (SSR) (24). For example, it has been observed that proteins involved in transcriptional regulation, chromatin remodeling or DNA repair become highly sumoylated upon environmental stress, including hyperosmotic and heat stress (25). Conversely, the SSR can also involve de-sumoylation of certain substrates. For instance, we have shown that nitrogen starvation, as well as treatment of cells with the TORC1 inhibitor rapamycin, results in de-sumoylation of components of RNAPIII, leading to a strong reduction in RNAPIII activity (23).

Although several studies have analyzed stress-induced changes in cellular sumoylation patterns, it remains unclear whether the SSR involves a common set of core Sumo substrates that are altered regardless of the type of stress, or whether the SSR rather targets a unique set of proteins depending on each particular type of stress. In this study, we used a proteomic approach to gain more insight into the SSR. We found only limited overlap between SSRs induced by different types of stress, suggesting that each form of stress triggers a unique SSR. However, we did find that de-sumoylation and inactivation of the RNAPIII machinery were a common response to all forms of stress. We conclude that de-sumoylation and inactivation of the RNAPIII machinery constitute a common core component of the SSR.

**Results**

**A comparative analysis of Sumo Stress Targets in various stress conditions**

To better define the *S. cerevisiae* SSR, we performed affinity purification combined with mass spectrometry to identify Sumo stress targets (SSTs), i.e. sets of differentially sumoylated proteins in various stress conditions. Cells expressing HIS6-FLAG-tagged Sumo were exposed to heat, hyperosmotic and oxidative stresses, DNA alkylation and nitrogen starvation, followed by purification of Sumo under denaturing conditions (Fig. 1A). As assessed by western blotting, all five stresses correlated with an overall increase in Sumo conjugation, which is usually referred to as the SSR (24)(Suppl. Fig. S1). A comparative analysis of differentially sumoylated proteins was performed in these five stress conditions (i.e. increased or decreased sumoylation compared to normal growth...
Differentially sumoylated proteins were then reproducibly identified by mass spectrometry in two biological repeats, although fold changes varied to some degree (Fig. 1B and C; Suppl. Fig. S2 and Suppl. Table S1). This revealed little overlap between the various treatments (Fig. 1B-E, Suppl. Table S1 and S2), suggesting that each stress triggers a stress-specific SSR. However, a small set of proteins was globally de-sumoylated independently of the type of cellular injury (Fig. 1B-D, Suppl. Fig. S2 and Suppl. Table S1 and S2). This cluster included subunits of the three RNA polymerases, as well as subunits of their general transcription machineries (Fig. 1C and Suppl. Table S2). Strikingly, only three proteins were de-sumoylated upon all five stresses: Rpe37, Ret1 and Rpc82 (Fig. 1C, Suppl. Fig. S2 and Suppl. Table S1), which are three subunits of RnapIII. Furthermore, a fourth RnapIII subunit, Rpc53, was de-sumoylated in all stresses with the exception of one nitrogen starvation biological replicate (Fig. 1C and Suppl. Fig. S2) where it fell just below the cut-off. These data indicate that different stresses trigger stress-specific SSRs, and that RnapIII subunits are de-sumoylated upon each stress tested here (also see below).

Additionally, three proteins showed increased sumoylation upon four stresses but not oxidative stress: the Ribosomal RNA processing element (RRPE)-binding protein Sib3 and two TCA cycle enzymes Sdh1 and Lpd1 (Suppl. Table S1 and S2). Similarly, the Structural Maintenance of Chromosome (Smc) 5 and Smc6 subunits of the Smc5/6 complex, which is a known Sumo target involved in the DNA damage response, exhibited increased sumoylation in all stresses with the exception of Osmotic stress, where, conversely, both Smc5 and Smc6 showed a significant decrease in sumoylation (Suppl. Table S1 and S2).

To gain further insight into the cellular processes and biological pathways that are targeted by the different SSRs, we performed Gene Ontology (GO) analysis of the proteomic datasets. Comparative Gene Ontology (GO; Biological Process) analysis of differentially sumoylated substrates revealed that proteins that were differentially sumoylated upon heat shock, hyperosmotic stress and nitrogen starvation were mainly involved in chromatin organization, whereas DNA alkylation and oxidative stress resulted in differential sumoylation of the translation machinery (Fig. 1F). Surprisingly, there was substantial overlap between several stresses, notably nitrogen starvation, heat stress and hyperosmotic stress (Fig. 1F). Thus, although the differentially sumoylated substrates varied between different stresses, they broadly fell into the same biological process with “Transcription” being the sole biological process that appeared in all five stresses. These data indicate that the SSR affects similar cellular processes yet targets specific sets of proteins according to the type of cellular injury.

We also performed comparative Gene Ontology (GO; Cellular Component) analysis to identify protein complexes that are differentially targeted by the various stresses. As expected, RnapIII was the only common complex to all five stresses (Fig. 1G). Hyperosmotic stress, nitrogen starvation and heat stress exhibited a certain degree of overlap in chromatin-associated complexes, whereas DNA alkylation and oxidative stress overlapped under the “cytosolic ribosome” term.

Taken together, our data suggest that each of the cellular stresses we have tested triggers its own unique SSR that involves a particular set of proteins (discussed in further detail below), whereas RNA polymerase complexes and their respective transcription machineries, and the RnapIII complex in particular, are the only SSTs common to each SSR.

**Heat stress-regulated sumoylome**

Next, we wished to gain further insight into the various stress-specific SSRs. We identified 211 proteins that were reproducibly differentially sumoylated upon exposure to elevated temperature (Suppl. Table S1). We identified several regulators of the septin cytoskeleton (Fig. 2A), including the septins Cdc3, Cdc11 and Shs1, which have previously been shown to be Sumo-modified proteins (26) and which all became hyposumoylated following heat stress (Suppl. Table S2). We also identified thirteen components of the SWI/SNF superfamily-type complex, which became hypersumoylated upon heat stress and four that became hyposumoylated. These chromatin remodeling complexes have previously been shown to be Sumo modified upon exposure to elevated temperatures in the plant *Arabidopsis thaliana* (27), suggesting evolutionary conservation of this stress response. Other proteins that became hypersumoylated are the Smc2 and Smc4 subunits of the condensin complex and four components of the aforementioned eight-subunits Smc5/6 complex.
Similarly, three subunits of the four-subunit HIR (Histonet Regulation) complex, Hir1, Hir2 and Hpc2 also appeared more sumoylated. As mentioned above, exposure to heat resulted in de-sumoylation of all three transcription machineries (Fig. 2A): The RNAPIII subunits Ret1, Rpc37, Rpc82 and Rpc53, the TFIIIC subunits Brf1 and Bdp1, the TFIIIA Pzf1, and the RNAPII subunit Rpb4. Finally, Rpo26 (common to all three polymerases) was also de-sumoylated after heat stress (Suppl. Fig. S2 and Suppl. Table S1).

Hyperosmotic stress-regulated sumoylome

We identified 128 differentially sumoylated substrates after hyperosmotic stress. We found that 87 out of 128 substrates were de-sumoylated (Suppl. Table S1). These de-sumoylated proteins participate in various cellular processes and the vast majority of differentially sumoylated proteins comprised nuclear proteins (Suppl. Table S2 and Fig. 2B). A prominent group of differentially sumoylated proteins included transcription factors previously shown to participate in general stress responses, including Crz1, Asg1 and Yhb1 (28-30), as well as the osmotic stress-regulated transcription factor Sko1 (31). In addition to these DNA sequence-specific transcription factors, we found that several subunits of chromatin silencing and remodeling complexes are Sumo targets, such as the RSC (Remodeling the Structure of Chromatin)-type complex, and two SWI/SNF superfamily-type complexes Ino80 and Swr1 (Fig. 2B, Suppl. Table S1 and Suppl. Table S2). Furthermore, multiple subunits of RNAPI, RNAPII and RNAPIII were de-sumoylated upon hyperosmotic stress, as well as the basal transcription factors TFIIA and TFIIF, and subunits of the Mediator complex, whereas subunits of the TFIID (Taf4, Taf5, Taf7, Taf8 and Taf12) showed increased sumoylation. In agreement with our findings, a previous study also found that hyperosmotic stress affects sumoylation of multiple components of the chromatin remodeling SWI/SNF complex, as well as subunits of TFIID (25). Finally, we found that a major target of the hyperosmotic SSR was the RNAPIII machinery, including subunits of the RNAPIII basal transcription factors TFIIIB and TFIIIC, all of which were de-sumoylated (Fig. 2B, Suppl. Fig. S2 and Suppl. Table S2).

Oxidative stress-regulated sumoylome

Upon exposure to H$_2$O$_2$, 40 proteins were found differentially sumoylated, which is the smallest number of all the stresses we tested (Suppl. Table S1). Ribosome components were most frequently observed to be differentially sumoylated, including 12 ribosomal proteins and one ribosome-associated protein (Sro9), which underwent increased sumoylation (Fig. 3A and Suppl. Table S2). The few proteins that became less sumoylated after oxidative stress were the RNAPIII subunits Rpc37, Ret1, Rpc53 and Rpc82 as well as two subunits of the Tec1-Ste12-Dig1 translational repressor complex involved in filamentous growth (Fig 1C, Fig. 3A and Suppl. Table S1).

DNA alkylation stress-regulated sumoylome

DNA alkylation stress resulted in differential sumoylation of 60 Sumo substrates, including proteins involved in diverse DNA repair pathways that were previously reported to be Sumo substrates upon exposure to DNA damage (Suppl. Table S1). These include Mag1, which is a 3-methyl-adenine DNA glycosylase involved in the Base Excision Repair pathway, and Saw1, which is involved in DNA repair by recruiting the Rad1-Rad10 complex. We also identified the aforementioned Smc5/6 complex (Suppl. Table S2 and Fig. 3B). In addition, multiple components of the translational machinery were differentially sumoylated after treatment with alkylating agents, including eight ribosomal proteins that were more sumoylated and one component of the 66S pre-ribosomal particle, Ebp2, which was de-sumoylated (Suppl. Table S1 and S2). Similar to the other stresses, RNAPIII was among the most represented complex, with five subunits being de-sumoylated (Ret1, Rpc82, Rpc37, Rpc53 and the TFIIIB subunit Bdp1). In addition, one subunit of RNAPI (Rpa43) and one subunit shared by all three polymerases (Rpo26) were de-sumoylated in response to treatment with alkylating agents (Fig. 3B, Suppl. Fig. S2 and Suppl. Table S1).

Nitrogen starvation stress-regulated sumoylome

We identified 105 proteins that were differentially sumoylated after nitrogen stress, 65 of which showed increased sumoylation (Suppl. Table S1). The septin ring organization complex became de-sumoylated, which was also observed after heat and hyperosmotic stresses (Fig. 3C). Interestingly, our data suggest that some Sumo targets display a sumoylation behavior during nitrogen starvation that is opposite to that of hyperosmotic stress. For instance, hyperosmotic stress resulted in de-sumoylation of the Sir2-containing RENT complex subunits, the Smc5/6 complex and the chromosomal passenger proteins
(CPC) Brl1 and Sli15, whereas nitrogen starvation stress resulted in a significant increase in their sumoylation levels (Fig. 2B, Fig. 3C and Suppl. Table S1). Proteins that became de-sumoylated mainly include RNAPI subunits Rpa43 and Rpa34, RNAPIII subunits Ret1, Rpc82 and Rpe37 and the RNAPII-associated transcription factor Pafl (Suppl. Fig. S2). Two proteins that are particularly relevant for the response to nitrogen starvation are the transcription factors Gcn4 and Stp1, which are involved in the transcription of amino acid biosynthetic genes and amino acid permease genes, respectively (32). Gcn4 is a known Sumo target (33) and showed increased Sumo modification after nitrogen starvation, while Stp1 became de-sumoylated (Fig. 3C and Suppl. Table S1). Interestingly, a unique feature of the nitrogen starvation stress response was an apparent increase in sumoylation of multiple subunits of the mitochondrial respiratory chain, with the notable exception of Gcv2 (glycine decarboxylase), which was de-sumoylated (Suppl. Table S1 and S2).

De-sumoylation of RNAPIII constitutes the core of the SSR

Among the most consistently regulated SSTs were subunits of RNAPIII and its general transcription factors (Fig. 1B-D and Suppl. Table S1 and S2). We validated these MS data by immunoblotting. Sumo conjugated proteins were affinity purified from exponentially growing or stressed yeast cultures under denaturing conditions and SDS-PAGE immunoblotting was performed with antibodies recognizing HA-tagged Rpc82 (Fig. 4A) or Myc-tagged Ret1 (Fig. 4B). Sumoylation of Rpc82 was significantly reduced following exposure to heat stress, nitrogen starvation, hyperosmotic stress and DNA damage, whereas de-sumoylation of Rpc82 in response to oxidative stress appeared to be prone to experimental variation for reasons we do not currently understand (Fig. 4A). Sumoylation of Ret1 was also strongly reduced after exposure to all stressors (Fig. 4B).

Physiological relevance of stress-induced de-sumoylation of Rpc82 and Ret1

We previously reported that TORC1 inhibition and nitrogen starvation result in de-sumoylation of Ret1 and Rpc82, which decreases their presence at class III genes (RNAPIII-transcribed genes) (23). We then asked whether oxidative stress, hyperosmotic stress, heat stress and DNA damage provoke a similar response as nitrogen starvation. We analyzed the association of Rpc82 and Ret1 to class III genes by ChiP-qPCR following exposure to stress. We found that Rpc82 occupancy was significantly reduced at tDNALeu in response to heat stress, hyperosmotic stress, oxidative stress, alkylation stress and nitrogen starvation stress (Fig. 4C and F). These forms of stress also reduced Rpc82 levels at SCR1, which is another non-coding RNA transcribed by RNAPIII, although the reduction in Rpc82 levels at this gene did not appear to be significant under oxidative stress (Fig. 4C and F). Ret1 occupancy also appeared significantly decreased at tDNALeu after exposure to stress, with the exception of oxidative stress, which only caused a slight, non-significant reduction in Ret1 levels (Fig. 4D and F). Furthermore, Ret1 levels at SCR1 were only significantly reduced after hyperosmotic stress and nitrogen starvation (Fig. 4E and F).

We previously reported that Rpc82 sumoylation, but not Ret1 SUMOylation, was necessary to promote RNAPIII assembly and transcriptional activity at class III genes (23). tRNA molecules are transcribed as precursors (pre-tRNAs) that undergo sequential post-transcriptional modifications to generate mature tRNAs. These steps include removal of 3’ and 5’ ends, addition of 3’ and 5’ tails, intron removal for intron-containing tRNAs and a number of nucleoside modifications. Some of these modifications may impair analysis of RNA levels by RT-qPCR, because they block efficient conversion into cDNA (34,35). Given that mature tRNAs are very stable, it is important to study the immature form to determine the effect of a given stimulus on tDNA transcription. Therefore, to assess whether tDNA transcription was affected by stress, we performed Northern blotting using probes specific for the precursors and mature forms of tRNALeu and tRNATrp. Both tRNALeu and tRNATrp are synthesized from intron-containing genes (Fig. 4G), and mature tRNAs migrate faster than the unprocessed intron-containing forms. We observed that mature tRNALeu and mature tRNATrp levels were unaffected in response to cell stress (Fig. 4H and I). In contrast, the relative
abundance of precursor tRNA$^{\text{Leu}}$ and tRNA$^{\text{Trp}}$ were significantly decreased following exposure to stress (Fig. 4H and I).

Taken together, these results suggest that stress generally triggers de-sumoylation of RNAPIII subunits Ret1 and Rpc82, which affects their occupancy at tDNA genes, resulting in reduced tRNA synthesis.

Discussion

Sumo has previously been implicated in adaptation to cellular stress and cell stress can induce increased global sumoylation, which is referred to as the Sumo Stress Response (reviewed in (24)). Despite the preexisting literature on stress-induced changes in the sumoylation levels of several Sumo substrates (36-38), it remains unclear whether the SSR constitutes a broad pathway consisting of a common set of Sumo targets (regardless of the type of stress), or whether each type of stress triggers its own specific SSR involving a unique core set of proteins.

In this study, we addressed this question by exploring the SSR induced by five different forms of stress, i.e. heat stress, hyperosmotic stress, oxidative stress, DNA alkylation stress and nitrogen starvation stress. We isolated sumoylated proteins and performed a comparative analysis of targets obtained for each stress using mass spectrometry. Interestingly, each form of stress resulted in differential sumoylation of largely non-overlapping sets of Sumo substrates. The only Sumo targets shared by each SSRs were the RNAPIII subunits Rpc37, Ret1 and Rpc82, which invariably became de-sumoylated upon cellular injury. We conclude that each stress triggers its own unique SSR, in some cases partially overlapping with SSRs of other forms of stress. A notable exception is de-sumoylation of RNAPIII components, which appears to be a key effect shared by all SSRs.

Different stresses trigger stress-specific SSRs

We observed limited overlap between the SSR patterns induced by the five different stressors that we have tested. However, we found that SSTs belong to a relatively small number of 26 mainly nuclear protein complexes involved in transcription, chromatin organization, centromeric function, translation and energy metabolism. Of these 26 complexes, 19, 15, 13, 8 and 5 complexes were differentially sumoylated upon osmotic stress, nitrogen starvation, heat stress, DNA alkylation and oxidative stress, respectively. These data suggest that each SSR mobilizes the sumoylation pathway in a stress-specific manner (Suppl. Table S2).

The nature of these 26 complexes is remarkably consistent with Sumo targets identified in previous studies (33). It will be interesting to determine the physiological effect of Sumo on these complexes under normal growth conditions versus stress conditions. Given that Sumo is thought to act as a molecular glue that stabilizes protein-protein interactions (21), it is tempting to hypothesize that the SSR regulates the activity of these protein complexes by controlling their stability/assembly. Indeed, differential sumoylation triggered by DNA damage and nitrogen starvation affects the stability of DNA repair complexes and transcription factor complexes (20,22,23).

We have identified SSTs that belong to the general transcription machinery, including the three RNA polymerases and their GTFs (Suppl. Table S1 and S2); for instance, Sumoylation of TFIIIA, TFIIIB, TFIIIC, RNAPIII and RNAPI, all of which are involved in synthesis of factors involved in mRNA translation, is particularly affected in response to multiple forms of stress, emphasizing the importance for the cell to regulate its translational capacity under conditions that may compromise cellular integrity. A large number of SSTs belong to complexes involved in chromatin organization and regulation (Suppl. Table S2). The Nua4, Ino80, SWR1 and RSC-type complexes were all differentially sumoylated in several, yet non-overlapping stresses. This suggests that in response to each stress Sumo may regulate a specific pathway leading to chromatin remodeling and potential stress-specific differential gene regulation.

In response to all stresses other than oxidative stress, Sumo is differentially linked to cytoplasmic protein complexes that belong to the mitochondrial respiratory chain (Suppl. Table S2). This is consistent with the rewiring of cellular energy metabolism in response to stress. Nevertheless, the effect of Sumo on energy production by mitochondria during SSR remains to be clarified.

A large part of the cell’s energy is devoted to the translation process and decreasing translational capacity helps cells survive stressful conditions (39). Several ribosomal proteins (RPs) and ribosome-associated factors were differentially sumoylated (mainly hyper-sumoylated) in all stresses but oxidative stress.
Whether sumoylation regulates ribosomal assembly or activity during various SSRs remains to be determined.

Sumoylation is a dynamic process and different stressors induce different kinetics of SSR activation and inactivation (25). It is possible that stress-specific SSRs share a greater number of common SSTs than the three that we identified in the present study (i.e. Rpc82,Rpc37 and Ret1, see below), depending on the time the cell is exposed to a given stressor. One limitation of our study is that we analyzed single time points, and it will be interesting to perform time-course analyses with multiple stresses to determine the degree of overlap between stress-dependent SSRs at high temporal resolution.

**De-sumoylation of RNAPIII constitutes the core of the SSR**

PTMs of the RNAPIII and subsequent alteration of tRNA synthesis is still an understudied field (40). In normally growing cells, sumoylated RNAPIII and TORC1 maintain high translational capacity by sustaining strong levels of tRNA synthesis and RPG transcription (22,23). Under these conditions the active conformation of the RNAPIII holoenzyme is supported by the sumoylation of its subunit Rpc82, which promotes tDNA transcription (23). However, nitrogen starvation or treatment with the TORC1 inhibitor rapamycin triggers the de-sumoylation of several RNAPIII subunits, including Rpc82, Ret1 and Rpc53. This results in reduced Rpc82 levels at RNAPIII-transcribed genes, such as tDNAs and SCR1, and subsequent inhibition of tRNA synthesis.

In the present study we reiterated these findings, and more strikingly, we found thatRpc82, Ret1 andRpc37 sumoylation was reduced upon all stresses that we have tested. We also found thatRpc53 was de-sumoylated upon all stresses except for one nitrogen starvation biological replicate. In this replicate, althoughRpc53 sumoylation was reduced, it did not reach the significance cut-off (Fig. 1 and Suppl. Fig. 2). However, we previously detected Rpc53 de-sumoylation upon nitrogen starvation or rapamycin treatment using label free MS and SILAC, and these findings were confirmed by western blotting (23). We therefore believe that despite minor experimental variation, Rpc53 is a fourth subunit of RNAPIII belonging to the core of the SSR. Now, the question is: why are these four subunits invariably de-sumoylated upon stress?

Interestingly Ret1-Rpc82 on one side andRpc37-Rpc53 on the other side physically interact and form two sub-complexes of the RNAPIII holoenzyme, which interact with TFIIIB and TFIIIC, respectively (41,42). Given the preeminent role of Sumo in regulating protein-protein interactions, it is plausible that the stability of these two subcomplexes within the holoenzyme, or their interaction with TFIII-B or -C, is altered by de-sumoylation during the SSR. This could result in the destabilization of the RNAPIII transcripational complex and decreased tRNA synthesis. This hypothesis is supported by the lower levels ofRpc82 and Ret1 detected at tDNA genes in all stresses, which correlated with a decrease in tRNA synthesis (Fig. 4). These data indicate that cells carefully regulateRpc82, Ret1, Rpc37 and Rpc53 sumoylation to control tRNA synthesis under optimal growth conditions and during cell stress.

Contrary to Rpc82, we observed that recruitment of Ret1 and Brf1 (a subunit of TFIIIB) was impaired mainly at tRNA genes, whereas it was only moderately affected at SCR1 in three of the five stresses that were tested (Fig. 4). This suggests that stress predominantly regulates RNAPIII assembly at tRNA genes rather than at other genes transcribed by the RNAPIII such as SCR1 (Fig. 4). This could be due to the different promoter and gene structure of SCR1 compared with tRNA genes, and may involve additional regulatory pathways during SSR.

Another interesting question is the relevance of our findings to mammalian cells. Indeed, previous high-throughput MS studies found that the human RNAPIII subunits RPC3, RPC4 and RPC5 (yeast Rpc82, Rpc53 and Rpc37, respectively) are Sumo substrates in normally growing Hela cells (43). Interestingly, RPC3 and RPC4 are differentially sumoylated in response to heat shock, and RPC4 and RPC5 are de-sumoylated upon MMS treatment (43,44). Of note, in these studies the human RNAPI subunit RPA34 appeared de-sumoylated in response to heat stress and MMS, which we have also found upon starvation and osmotic stress in yeast. Strong sumo signals were also found at tRNA genes in K562 cells using ChIP-seq (37,45,46). These Sumo signals decreased at tRNA genes upon heat shock, suggesting that, as we previously found in yeast (23), de-sumoylation of the RNAPIII complex also occurs during the stress response in mammals. These data indicate that differential Sumo modification of basal
transcription factors may constitute an evolutionarily conserved regulatory hub altering tRNA (and rRNA) synthesis in response to stress. As mentioned above, previous reports showed that the TORC1 pathway and the Sumoylation pathway cooperate to trigger a decrease in translational capacity that allows cells to survive during nutrient stress (22,23,39,47,48). In the present study we extend the role of the sumoylation pathway beyond cellular response to starvation, by showing that inhibition of tRNA synthesis via de-sumoylation of Rfc82, Ret1, Rpc37 (and possibly Rpc53) is triggered by several other stressors. We propose that while each stress triggers a stress-specific SSR, de-sumoylation of RNAPIII and subsequent inhibition of tRNA synthesis is a general mechanism of adaptive response to stress that constitutes the core of each SSR.

**Experimental procedures**

**Yeast strains and growth conditions**

*Saccharomyces cerevisiae* strains used in this study are derivatives of either the S288c strains RDKY3615 (49) or BY4741 and are listed in Suppl. Table S3. Yeast genetic manipulations were performed using standard gene-replacement methods or intercrossing. Yeast strains were grown at 30 °C to mid-logarithmic phase in Complete Synthetic Medium (CSM) (2 % glucose, 0.67 % yeast nitrogen base w/o amino acids, 0.08 % complete dropout mixture). Heat stress was performed at 42 °C in pre-heated CSM. Hyperosmotic stress was performed in CSM in which glucose was substituted with 1.5 M Sorbitol. Oxidative stress and DNA Damage were induced with addition of respectively 0.75 mM hydrogen peroxide or 0.1 % Methyl Methanesulfonate. Strains were grown in minimal medium (2 % glucose, 0.17 % yeast nitrogen base w/o amino acids w/o ammonium sulphate) to induce nitrogen starvation. For hyperosmotic and oxidative stresses and DNA damage samples, cells were harvested after 30 minutes of treatment. Heat stress and nitrogen starvation samples were harvested after 10 minutes or 3 hours of treatment respectively.

**Whole cell lysates for western blotting**

Cells (10 mL) were collected by centrifugation, washed with cold water and flash frozen in liquid nitrogen. Pellets were resuspended in 250 μL lysis solution [1.85 M NaOH, 7.4 % β-mercaptoethanol (vol/vol)]. After 10 minutes on ice, 250 μL of 55 % trichloroacetic acid (TCA) were added and samples were incubated 10 min on ice. Proteins were pelleted by centrifugation (10 min, 4 °C, 10 000 rpm). The remaining pellets were washed 30 min at -20 °C with 90 % acetone. Pellets were then resuspended in SDS-PAGE sample buffer and incubated at 95 °C for 10 min prior to SDS-PAGE (Suppl. Fig S1).

**Sumo pull-down under denaturing conditions for MS analysis**

Sumo pull-downs were performed as previously described (22) in two independent biological repeats. Briefly, for each growth condition, 1L of cells expressing HIS6-FLAG-Smt3 were harvested, washed in cold water and flash frozen in liquid nitrogen. Lysates were prepared by alkaline lysis (1.85 M NaOH) in presence of β-mercaptoethanol [7.5 % (vol/vol)] and TCA protein precipitation. Proteins were pelleted by centrifugation, washed with cold water and resuspended in buffer A (6 M guanidine-HCl, 100mM NaH2PO4, 10 mM Tris-HCl, 0.05 % Tween; adjusted to pH 8.0). The suspension was homogenized at room temperature for 1 h and insoluble material was removed by centrifugation. The supernatants were supplemented with 10 mM imidazole and incubated overnight at 4 °C in presence of Ni-NTA agarose beads (Qiagen). The beads were washed in buffer A and buffer C (8 M urea, 100 mM, 10 mM Tris-HCl, 0.05 % Tween; adjusted to pH 6.3) and proteins bound to the Ni-NTA agarose were eluted in buffer C supplemented with 250 mM imidazol. Proteins were precipitated with 55 % TCA and the pellets washed with -20 °C acetone before proceeding to proteolytic digestion.

**Sumo pull-down under denaturing conditions for western blotting**

Cells (20 mL) were collected by centrifugation, washed in ice-cold water and flash frozen. Pellets were resuspended in lysis buffer (8 M urea, 50 mM Tris pH 8.0, 0.05 % SDS) supplemented with 10 mM NEM and PIC and cells were lysis by mechanical disruption with glass beads. Cells debris were discarded by centrifugation and 5 % of the supernatant was saved as input material. The remaining supernatant was incubated with 50 μL Ni-NTA agarose beads and incubated overnight at 4 °C. The beads were washed three times for 5 min with wash buffer (8 M urea, 50 mM Tris pH 8.0, 200
mM NaCl, 0.05 % SDS) supplemented with 5 mM imidazol. Proteins bound to the resin were eluted by addition of loading buffer (8 M urea, 10 mM MOPS, 10 mM EDTA, 1 % SDS, 0.01 % bromophenol blue, pH 6.8). Samples were incubated 10 min at 65 °C prior to SDS-PAGE.

**Mass spectrometry and data analyses**

Protein pellets were solubilized in 50 mM NH₄HCO₃ containing 2% rapigest surfactant (Waters), reduced using DTT (5 mM, 45 min, 56°C) and digested overnight at 37°C with trypsin (Promega). Rapigest was removed by acidification (0.5-1% trifluoroacetic acid), and peptide samples were de-salted using c18 StageTips prior to MS analysis.

Tandem mass spectrometry (LC-MS/MS) analyses were performed using an Easy nLC1000 LC system (Thermo Electron) connected to a quadrupole Orbitrap (QExactive Plus) mass spectrometer (Thermo Electron) and involved a nanoelectrospray ion source (EasySpray; Thermo Electron). An EasySpray analytical column (C18, 2 µm beads, 100 Å, 75 µm inner diameter; Thermo) was used for peptide separation, with a flow rate of 0.3 µL/min, and solvent gradient of 2% to 30% (v/v) ACN in 0.1% (v/v) formic acid for 120 minutes, after which columns were washed using 90% (v/v) ACN in 0.1% (v/v) FA for 20 minutes. All LC-MS/MS analyses involved data-dependent acquisition (DDA) where selected peptides were fragmented using high-energy collision dissociation. Ions selected for MS/MS were dynamically excluded for 30 seconds.

Protein identification and label-free quantification was performed using the MaxQuant software (50). Statistical analyses were performed in the Perseus software (51) using log2-transformed LFQ intensities. For analysis of de-sumoylation events, proteins that were not significantly enriched in the SUMO pull down in mid-logarithmic phase compared to non-tagged SUMO controls were removed. For analysis of increased sumoylation events, proteins that were not significantly enriched in SUMO pull downs under a stress condition compared to non-tagged SUMO controls were removed. Comparisons were then performed between each SUMO pull down under a stress condition and the SUMO pull down in mid-logarithmic phase. Samples were compared using a Student’s T-test method (permutation-based FDR correction (250 randomisations); FDR cut-off=0.05; S0=0.1). Proteins were considered significantly reduced or increased under a specific stress condition if they passed the Student’s T-test significance threshold across two biological repeat experiments. For display purposes, samples were also compared using Volcano plots as performed in Perseus.

Network enrichment analysis of each list of significant proteins was performed using Metascape (52). Processed data for all proteins enriched in at least one stress condition versus the mid-logarithmic control are displayed in Suppl. Table S1 All raw MS data and MaxQuant search parameter details are available via the PRIDE database (see details below). Experiment labels for PRIDE data are as follows: control/un-tagged (S1 or 398); no treatment/mid-logarithmic phase (S2 or 484); nitrogen starved (S3 or SD); oxidative stress (S4 or H₂O₂); osmotic stress (S5 or Sorbitol); DNA damage (S6 or MMS); heat shock (S7 and S8 in biological repeat 2). Biological repeat 1 includes an extra experiment (S7 or 46c) that was not used for final analyses.

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed as previously described (53). Briefly, 50 mL of mid-log phase cells were fixed with 1% (vol/vol) formaldehyde for 30 min and formaldehyde was quenched by adding glycine to a final concentration of 125 mM. Cells were centrifuged and pelleted were washed with cold Tris-buffered saline then lysed in lysis buffer [50 mM Hepes-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% (vol/vol) Trition X-100, 0.1% (wt/vol) Na-deoxycholate, and protease inhibitor mixture]. Samples were sonicated twice using a diagenode Bioruptor Twin, for 15 min with alternating cycles of 30s pulses. Resulting samples were centrifuged and the supernatant was collected. Chromatin immunoprecipitation was performed over-night at 4°C on 100 μg of chromatin. Beads were washed with the lysis buffer then washed with the lysis buffer supplemented with 500 mM NaCl. Beads were further washed with wash buffer [10 mM Tris (pH 8.0), 250 mM LiCl, 0.5% (vol/vol) Nonidet P-40, 0.5% (wt/vol) Na-deoxycholate and 1 mM EDTA] and with a buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0 prior elution in 50 mM Tris (pH 8.0), 10 mM EDTA, 1 % (vol/vol) SDS at 65 °C. The cross-link was reverse through at 65°C for 6 hours. RNA and proteins were degraded with RNase and proteinase K treatments, respectively and DNA was purified using QIAquick PCR purification kit (Qiagen). DNA fragments were analysed by qPCR with an AB StepOnePlus machine (Applied Biosystem) using primers amplifying tDNALeu...
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(forward: GTCTAAGGCGCCTGATTCAAGA, reverse: GTTCACTGCGGTCAAGAT) or SCR1 (forward: GAATTCTGGCCGAGGAACAAATCC, reverse: CAGCTCTGCCCAGGACAAATTAC).

Northern blotting

Cells (10 mL) were harvested by centrifugation, washed in ice-cold water and frozen in liquid nitrogen. Total RNA was extracted using mirVANA miRNA isolation kit (Thermo Fisher Scientific) following the manufacturer instructions. After heat denaturation at 55 °C for 5 min, samples were separated by 12% PAGE in 1x taurine at 200 V for 80 min. tRNAs were transferred to a nylon membrane (Hybond XL, GE Healthcare) by electroblotting in 1x taurine at RT and 200 mA. RNAs were UV-crosslinked to membranes (120 mJ cm⁻² in a CL-1000 UV-Crosslinker, UVP) and prehybridization, hybridization and washing steps were performed. tRNAs were detected by hybridization of ³²P 5'-end-labelled oligonucleotides probes complementary to specific sequences of their non-intron regions. Immature forms and other posttranscriptional modifications migrate slower than the mature forms. Hybridized probes were removed from the filters by boiling in 0.1% SDS. The sequences of the probes are CAGGAATTGAACCTGCAACCCTTC for tRNA¹⁰¹(CCA) and GGTTGCTAAGAGATTCGAACTC for tRNA¹⁰¹(CAA). Hybridization signals were detected using a phosphorimager and analyzed with the ImageQuant TL software.

Data Deposition

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (54) partner repository with the dataset identifier PXD013884.
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FOOTNOTES
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The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013884.

The abbreviations used are: Sumo, small ubiquitin-like modifier; SSR, sumo stress response; SST, sumo stress target; PTM, posttranslational modification; tRNA, transfer RNA; RNAPI, II and III, RNA polymerase I, II and III; GO, gene ontology; GTF, general transcription factor
**Figure 1. Characterization of Sumo proteomes in five stress conditions by mass spectrometry.**

A, experimental workflow for Ni-NTA enrichment of sumoylated proteins in diverse stress conditions. Exponentially growing cells expressing HIS6-FLAG-SMT3 were exposed to different cellular stressors as indicated on the scheme. Cells were lysed under denaturing conditions and sumoylated proteins were enriched with Ni-NTA affinity matrix. Sumo substrates in each condition were identified by mass spectrometry. B-C, heat maps showing specific signatures of differentially sumoylated proteins under different stress conditions. All identified sumo targets are shown in (B), whilst (C) shows only transcription machineries subunits. Relative changes in the sumoylation status of identified targets after each stress are displayed, where green denotes an increase in sumoylation and red denotes a decrease. Values from two biological replicates per stress are depicted. The star to the right of each heat map highlights proteins displaying a SSR that is consistent across all five stresses. D-E, circos plot representation of overlaps between the different stresses for proteins enriched in Sumo pull-downs. The outer arcs (multiple colors) on each plot represent a given stress, whilst the inner arcs, (blue) represent proteins depleted (D) or enriched (E) from Sumo pull-downs in the given stress. Lines connect the same proteins shared by multiple stresses. F-G, heatmap of differentially enriched Biological Processes (GOBP) (F) or Cellular Components (GOC) (G) based on Gene Ontology IDs generated using Metascape (52). The GO terms were assigned from the list of proteins identified in Sumo pull-downs in stressed vs. unstressed samples. The respective \(-\log_{10}(p)\) values are visualized with a color scale ranging from 0 for no representation to +20 for overrepresentation. NT: No Treatment, HS: Heat Stress, Os: Osmotic stress, Ox: Oxidative stress, DA: DNA Alkylation, -N: Nitrogen starvation.

**Figure 2. Identification of sumo-modified complexes: Heat stress and osmotic stress**

Networks generated for differentially sumoylated substrates following Heat stress (A) and Osmotic stress (B). Nodes represent proteins and edges represent protein-protein associations. De-sumoylated proteins are circled in red whereas hypersumoylated proteins are circled in blue. Enriched complexes are depicted with different colors (see legend). Black nodes represent proteins not associated with enriched complexes. Networks were generated using the STRING database (55).

**Figure 3. Identification of sumo-modified complexes: Oxidative stress, DNA alkylation and nitrogen starvation**

Networks generated for differentially sumoylated substrates following Oxidative stress (A), DNA alkylation (B) and Nitrogen starvation (C). Nodes represent proteins and edges represent protein-protein associations. De-sumoylated proteins are circled in red whereas hypersumoylated proteins are circled in blue. Enriched complexes are depicted with different colors (see legend). Black nodes represent proteins not associated with enriched complexes. Networks were generated using the STRING database (55).

**Figure 4. De-sumoylation of Ret1 and Rpc82 upon stress rewire their binding to class III genes and affect RNAIII transcriptional activity**

A-B, validation of MS data. Sumo pull-downs were performed under denaturing conditions using cell lysates of 6HF-SMT3 cells grown in the indicated conditions. The levels of copurifying Rpc82-HA and Ret1-MYC were analyzed by western blotting using anti-HA (A) and anti-MYC (B) antibodies, respectively. Lower graphs display the quantification of Rpc82-HA (A) and Ret1-MYC (B) signals. Error bars indicate the deviation from the average of three different experiments. * p<0.05, ** p<0.01, *** p<0.001, n.s. non-significant (Student’s T test). UT: untagged, NT: No Treatment, HS: Heat Stress, Os: Osmotic stress, Ox: Oxidative stress, DA: DNA Alkylation, -N: Nitrogen starvation. C-E, stress reduces the recruitment of RNAIII holoenzyme and general transcription factor TFIIIB at class III genes. The occupancy of RNAIII and TFIIIB subunits were analyzed by ChIP-qPCR. Yeasts were stressed as indicated in Fig. 1 and the relative levels of Rpc82-HA (C), Ret1-MYC (D) and Brf1-GFP (E) at IDN4LEU and SCR1 were determined by ChIP-qPCR using anti-HA, -MYC and –GFP antibodies, respectively. F, schematic representation of the regions where primers used for ChIP anneal. G, schematic representation of Northern blotting probes. H, precursor tRNAs (pre-tRNA) do not accumulate under stress. Pre-tRNA levels were analyzed by Northern blotting. Northern blots are representative of three independent biological replicates of experiments. Wild-type cells were grown at 30°C to exponential phase (No treatment, NT) then stressed as specified in Fig. 1. Total RNA was isolated and
analyzed by Northern hybridization with oligonucleotide probes specific to precursor and mature tRNA_{Leu(CAA)} (top) and tRNA_{Trp(CCA)} (middle). Loading control is provided by ethidium bromide-stained 5.8S rRNA (bottom). \( I \), ratios of precursor over mature tRNAs. Errors bars, SEM of three different experiments. * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \), n.s. non-significant (Student’s T test).
Increased sumoylation upon stress
Decreased sumoylation upon stress
Condensin complex
Not affiliated to a complex
Transcriptional repressor complex
Nuclear nucleosome
HIR complex
SWI/SNF superfamily-type complex
Septin cytoskeleton
Oxidoreductase complex
General transcription machineries
Ammonium sulfate complex
Smc5-Smc6 complex
NelS complex
Condensin complex
Hairpin complex
Increased sumoylation upon stress
Decreased sumoylation upon stress
Increased sumoylation upon stress
Decreased sumoylation upon stress
Not affiliated to a complex

General transcription machineries
Oxidoreductase complex
Smc5-Smc6 complex
Cytosolic ribosomes
Increased sumoylation upon stress
Decreased sumoylation upon stress
Not affiliated to a complex

Spindle midzone
DNA packaging
SWI/SNF complex
Septin cytoskeleton
Smc5-Smc6 complex
RENT complex
Increased sumoylation upon stress
Decreased sumoylation upon stress
Not affiliated to a complex

Cytosolic ribosomes
Tec1-Ste12-Dig1 complex
General transcription machineries
Oxidoreductase complex
Smc5-Smc6 complex
Cytosolic ribosomes
Increased sumoylation upon stress
Decreased sumoylation upon stress
Not affiliated to a complex
Nguéa P et al., Figure 4

A) 

B) 

C) 

D) 

E) 

F) 

G) 

H) 

I) 

J) 

K) 

L) 

M) 

N) 

O) 

P) 

Q) 

R) 

S) 

T) 

U) 

V) 

W) 

X) 

Y) 

Z)
De-sumoylation of RNA polymerase III lies at the core of the Sumo stress response in yeast
Aurélie Nguéa P, Joseph Robertson, Maria Carmen Herrera, Pierre Chymkowitch and Jorrit M. Enserink

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