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SUMOlock reveals a more complete *Aspergillus nidulans* SUMOylome

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Abstract

SUMOylation, covalent attachment of the small ubiquitin-like modifier protein SUMO to proteins, regulates protein interactions and activity and plays a crucial role in the regulation of many key cellular processes. Understanding the roles of SUMO in these processes ultimately requires identification of the proteins that are SUMOylated in the organism under study. The filamentous fungus *Aspergillus nidulans* serves as an excellent model for many aspects of fungal biology, and it would be of great value to determine the proteins that are SUMOylated in this organism (i.e. its SUMOylome). We have developed a new and effective approach for identifying SUMOylated proteins in this organism in which we lock proteins in their SUMOylated state, affinity purify SUMOylated proteins using the high affinity S-tag, and identify them using sensitive Orbitrap mass spectroscopy. This approach allows us to distinguish proteins that are SUMOylated from proteins that are binding partners of SUMOylated proteins or are bound non-covalently to SUMO. This approach has allowed us to identify 149 proteins that are SUMOylated in *A. nidulans*. Of these, 67 are predicted to be involved in transcription and particularly in the regulation of transcription, 21 are predicted to be involved in RNA processing and 16 are predicted to function in DNA replication or repair.

Graphical Abstract

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Keywords

SUMO; SUMOylation pathway; SUMOylated proteins; *Aspergillus nidulans*

Introduction

SUMO is a small ubiquitin-like molecule that is covalently linked to a number of proteins in cells, modifying their activities, and it plays an important role in regulation of many cellular functions [reviewed in (Melchior et al., 2003; Schwartz and Hochstrasser, 2003; Verger et al., 2003; Dohmen, 2004; Johnson, 2004; Muller et al., 2004; Hay, 2005; Mukhopadhyay and Dasso, 2007; Dasso, 2008; Meulmeester and Melchior, 2008; Mukhopadhyay and Dasso, 2009)]. Four SUMO family proteins, SUMO-1, SUMO-2, SUMO-3 and SUMO-4, are found in vertebrates, but the yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and the filamentous fungus *Aspergillus nidulans* each have a single SUMO gene (Meluh and Koshland, 1995; Johnson and Blobel, 1997; Tanaka et al., 1999; Wong et al., 2008). In *S. cerevisiae*, deletion of the SUMO gene, *SMT3*, is lethal whereas deletion of the *S. pombe* SUMO gene, *pmt3⁺*, is not lethal but greatly reduces growth (Tanaka et al., 1999).

SUMO, as translated, cannot be conjugated to proteins. A small C-terminal peptide must first be cleaved by SUMO isopeptidases, leaving two glycines at the C-terminus. SUMOylation then occurs by consecutive actions of a heterodimeric E1 activating enzyme and an E2 conjugating enzyme. The E2 enzyme, SUMO and one of several SUMO E3 ligases form a complex with the target protein resulting in SUMOylation. The E3 ligase is thought to determine substrate specificity. The isopeptidases involved in the cleavage of SUMO prior to conjugation can also remove SUMO from proteins and SUMOylation is, thus, reversible [reviewed by (Mukhopadhyay and Dasso, 2007)] and the released SUMO can be reused by the SUMOylation machinery (Schwienhorst et al., 2000).

A wide variety of proteins have been reported to be SUMOylated *in vivo* indicating that SUMOylation is involved in a large number of cellular processes. One potentially versatile approach to understanding the functions of SUMO is to determine which proteins in the cell are SUMOylated. Isolation and characterization of SUMOylated proteins purified from living organisms have been attempted using several different organisms. Among fungi,

SUMOylation has been extensively studied in the yeasts *S. cerevisiae* (Panse et al., 2004; Wohlschlegel et al., 2004; Zhou et al., 2004; Denison et al., 2005; Hannich et al., 2005; Albuquerque et al., 2013) and *S. pombe* (Nie et al., 2015). Up to three hundred proteins were reported to be SUMOylated in these studies. A significant number of proteins were detected in multiple studies, and these are likely to be genuinely SUMOylated. The number and identities of SUMOylated proteins varied widely from one study to another, however, for reasons that are not completely clear. This variation could reflect organismal differences, methodological differences or differences in growth or culture conditions.

Filamentous fungi in general, and the genus *Aspergillus* in particular, are extremely important medically, agriculturally and economically (Meyer et al., 2016). Invasive pulmonary aspergillosis is a leading cause of death for patients with acute leukemia and for hematopoietic stem cell transplantation patients, and it is a significant problem for other immune compromised patients including those with HIV/AIDS (Pagano et al., 2006). About 40% of patients with invasive *Aspergillus* infections die from the infection (Pagano et al., 2006). *Aspergillus spp.* produce aflatoxins (among the most carcinogenic substances known) that contaminate the food chain causing cancer, food wastage and sometimes killing humans or animals outright (Cullen and Newberne, 1993). On the positive side, *Aspergillus spp.* are the source of many medically important compounds (e.g. the anti-cholesterol agent lovastatin) and industrially important chemicals (e.g. citric acid) and enzymes (Meyer et al., 2016).

Aspergillus nidulans is the model genetic and experimental organism for the genus *Aspergillus* and it has one of the best genetic and molecular genetic systems developed in fungi. As mentioned, *A. nidulans* has a single SUMO gene, *sumO*. Results of its deletion, overexpression and localization have been reported (Wong et al., 2008). It is not essential for viability but deleting it does cause some growth reduction as well as self-sterility and reduced conidiation. Imaging GFP-SUMO revealed that SUMOylated proteins are concentrated in the nucleoplasm in interphase with particularly high concentrations at punctae in nuclei. GFP-SUMO leaves the nucleus at mitotic onset (Wong et al., 2008), when nuclear pore complexes become partially disassembled allowing nucleo-cytoplasmic mixing (De Souza et al., 2004). SUMO re-accumulates in the nucleus at mitotic exit after nuclear pore complexes reassemble and reinstate nuclear cytoplasmic transport. Deletion of *sumO* alters secondary metabolite production, dramatically decreasing production of austinol and dehydroaustinol as well as the aflatoxin-related compound sterigmatocystin, while increasing production of asperthecin (Szewczyk et al., 2008).

Genes of the SUMOylation pathway in *A. nidulans* have been identified and deleted and the effects of the deletions on development have been analyzed (Harting et al., 2013), revealing that SUMOylation plays an important role in asexual and sexual development. Harting et al. (2013) created a tap-tagged version of SUMO and used it to purify candidate SUMOylated or SUMO-interacting proteins. A total of 56 such proteins were identified, and they were particularly enriched for proteins involved in transcription and histone modifications.

We have developed a new approach that has allowed us to develop a more complete catalog of SUMOylated proteins. By deleting the SUMO isopeptidase genes and modifying the

sumO gene we are able to lock proteins in their SUMOylated state *in vivo*. We are then able to purify them using a high-affinity tag and identify them using sensitive Orbitrap mass spectroscopy. This approach, that we call SUMOlock for brevity, has allowed us to identify, with excellent confidence, 149 proteins that are SUMOylated in growing hyphae of *A. nidulans* as well as additional proteins that form complexes with SUMOylated proteins.

The great majority of SUMOylated proteins fell into a small number of functional groups. Notably, 67 are predicted to function in transcription and, particularly, the regulation of transcription, 21 are predicted to function in RNA processing, and 16 in DNA replication and repair. These results indicate a major role for SUMO in the regulation of these processes.

Results

Identification of SUMOylation pathway genes of *A. nidulans*

Through a series of BLAST searches, we have identified the *A. nidulans* SUMOylation pathway genes (Table 1), and our results largely confirm those of Harting *et al.* (2013). [Note: the gene numbers we use (e.g. AN10240) are from the current annotation available at the Aspergillus Genome Database (AspGD) and the Fungal and Oomycete Genomics Resources database (FungiDB)]. Harting *et al.* (2013) have tentatively identified AN10240 as a homolog of *S. cerevisiae mms21*, an E3 ligase, but they did not give it a gene name.] Our results confirm this identification, and we designate it *mmsU*. In addition, we were able to identify AN4497 as an additional SUMO E3 ligase not found by Harting *et al.* AN4497 is a homolog of the *S. cerevisiae* Siz1 and Siz2 E3 ligases. Harting *et al.* have already identified AN10822 as a homolog of Siz1 and Siz2 and designated it SizA (gene = *sizA*). We give AN4497 the designation *sizB*. The *A. nidulans* genome did not contain an ortholog of the *S. cerevisiae* Cst9 E3 ligase.

Phenotypes of deletions of SUMOylation pathway genes

We have previously deleted the *A. nidulans sumO* gene (Wong *et al.*, 2008), and Harting *et al.* (2013) have deleted several SUMO pathway genes. The phenotypes of deletions of the E3 ligases have not been reported. We deleted each of the SUMO pathway genes including the E3 ligases by replacing them with selectable markers. For initial deletions of all genes except *mmsU*, we used the *Aspergillus fumigatus pyrG* gene (*AfpyrG*) (Weidner *et al.*, 1998) as the selectable marker. We subsequently repeated the *ulpB* deletion using the *Aspergillus terreus pyrG* gene (*AtpyrG*) (Dohn *et al.*, 2018). For *mmsU* we used the *A. fumigatus riboB* gene (*AfriboB*) (Nayak *et al.*, 2006). To create multiple deletions in the same strain we deleted additional genes by replacing them with *AfriboB* or the *A. fumigatus pyroA* gene (*AfpyroA*) (Nayak *et al.*, 2006). Genotypes of all strains including deletion strains are given in Table 2. Growth phenotypes of the deletions are shown in Fig. 1 and 2 and Fig. S1. Deletion of *sumO* (*sumO*⁻) causes only a small reduction in radial colony growth but causes a great reduction in conidiation. In Fig. 1 all strains produce green conidia and the reduction of conidiation is illustrated by the fact that poorly conidiating colonies are buff colored rather than green. Deletion of the E1 or E2 genes is predicted to prevent SUMOylation and, as anticipated, deletion of either of the two E1 component genes *ubaB*

(Fig. 1) or *aosA* (Fig. S1) gave a phenotype essentially identical to *sumO* as did deletion of the E2 gene, *ubcN* (Fig. 1). Of the E3 ligase deletions, *sizA* and *sizB* caused no detectable inhibition of growth and the *sizA*, *sizB* double deletion grew almost as well as non-deletion controls (Fig. 1). Deletion of *mmsU*, however, resulted in much stronger growth inhibition and the triple E3 deletion caused very strong inhibition of growth (Fig. 1).

Deletion of the isopeptidase *ulpA* caused a smaller inhibition of growth and conidiation (Fig. 2) than *sumO*. With respect to the deletion of *ulpB*, our results were not the same as those of Harting et al., and they require discussion. We initially replaced the coding sequence of *ulpB*, as annotated in the database available at AspGD (<http://www.aspgd.org>) and FungiDB (<http://fungidb.org/fungidb>), with the *A. fumigatus pyroA* gene. Subsequently we obtained RNA-seq and CAGE-RNA-seq data (Fig. S2) that indicated that the initiation codon for *ulpB* is 429 bp upstream of the annotated start site and that there is an intron in the region between the actual and annotated start sites. The predicted coding sequence of the reannotated protein is 94 amino acids longer than the predicted coding sequence from AspGD/FungiDB. We have included our proposed reannotation of *ulpB* in Fig. S3 and the predicted amino acid sequence of UlpB in Fig. S4. Following our reannotation, we deleted the coding sequence of *ulpB* based on the new annotation, and we repeated our deletion of the coding sequence from the previous annotation. For these deletions we replaced the target sequences with the *A. terreus pyrG* gene (Dohn et al., 2018). Phenotypes were identical for deletions based on both the old and new annotations. We anticipated that both deletions would give the same phenotype because even the shorter deletion would remove most of the coding sequence.

Transformations resulted in two types of colonies. Some transformants were thin and very slow growing. Others were more robust but had uneven edges as is typical of heterokaryon transformants. Diagnostic PCR on spores from the robust colonies revealed bands diagnostic for both the deletion and the wild-type (wt) copy of *ulpB*, revealing that these transformants were heterokaryons or diploids. Heterokaryons containing both transformed (parental) and untransformed nuclei often form during transformation in *A. nidulans*. When deletion of the target gene is lethal or results in poor growth, there is a selection for maintenance of the heterokaryon, with transformed nuclei carrying the selectable marker and parental nuclei carrying a functional copy of the target gene (a process termed heterokaryon rescue) (Osmani et al., 1988; Oakley et al., 1990; Osmani et al., 2006). Since *A. nidulans* conidia are uninucleate, conidia carry parental or transformed nuclei but not both. If conidia from a heterokaryon are spread on selective media, conidia carrying parental nuclei lack the selective marker and will not grow. Conidia carrying transformed nuclei will display the phenotype of the target deletion. In this case spreading conidia from the heterokaryons on selective medium resulted in poorly growing colonies very similar to the small, slow growing colonies seen on transformation plates. Although these colonies grew poorly and did not conidiate well, we were able to cut them from the selective plates and subculture them on to YAG medium plus riboflavin and 1.0 M sucrose. This medium is selective for the *pyrG* marker, preventing growth of conidia carrying parental nuclei, and we have found that 1.0 M sucrose often facilitates conidiation. As we had hoped, we were able to obtain enough conidia to carry out diagnostic PCR (Fig. S5), and we found that these colonies carried deletions of *ulpB* and did not carry the wt copy. LO11584, a PCR-verified deletion of *ulpB*

created by replacement of *ulpB* with *AtpyrG* is shown in Fig. 2. These data indicated very strongly that deletion of *ulpB*, whether based on the old or new annotation, causes a strong restriction of growth and conidiation. We have also obtained corroborating evidence from crosses that the *ulpB* deletion is very sick (discussed below).

We anticipated that if we deleted both *ulpA* and *ulpB*, SUMO would not be processed, and the phenotype would be the same as the SUMO deletion. We, therefore, deleted *ulpB* (older annotation) in a *ulpA* deletion strain and found, indeed, that the phenotype of the double deletion strain was essentially the same as that of the *sumO* deletion and much better than the *ulpB* deletion alone (Fig. 2).

We were able to clarify the phenotype of the *ulpB* deletion by crossing LO3886 to LO1501. LO3886 carries an S-tag preprocessed *sumO* gene (inserted using *AfriboB* as a selectable marker, discussed below) as well as a deletion of *ulpA* by replacement with *AfpyrG* and a deletion of *ulpB* by replacement with *AfpyrA*. LO1501 carries wild-type alleles of all SUMOylation pathway genes and the selectable markers *pyrG89*, *riboB2*, *pyrA4*, and *pabaA1*. In such a cross, segregants that carry *ulpB* and *ulpA*⁺ will be pyr⁻ and pyr⁺. No pyr⁻, pyr⁺ colonies were obtained in 100 segregants obtained by spreading ascospores on fully supplemented medium, whereas other expected classes were obtained in reasonable numbers. This result indicates that *ulpA*⁺, *ulpB*⁻ segregants are dead or grow so slowly that they are obscured by other, more rapidly growing, segregants. Our results in creating *ulpB* deletions by transformation suggested that the latter possibility was likely, so we spread ascospores at very low density on fully supplemented media containing 1.0 M sucrose which facilitates conidiation of *ulpB* transformants. Of 43 progeny tested, three were pyr⁻, pyr⁺ indicating that they are *ulpA*⁺ and *ulpB*⁻, and each of these grew slowly. Interestingly, each of these three progeny was also ribo⁺ indicating that they carried the preprocessed *sumO* construct. In spite of the poor growth, we were able to obtain enough DNA from two of these progeny to carry out diagnostic PCR, and we found that they both carried *ulpB* (Fig. S5). We compared the growth of *ulpB* strains with and without the preprocessed SUMO. The *ulpB* strain with preprocessed SUMO grew poorly, but clearly better than the *ulpB* strains with wt SUMO. This result reveals that part of the growth restriction caused by *ulpB* is due to its role in SUMO processing. Since growth is still strongly restricted even when SUMO is preprocessed, however, *ulpB* must have important functions in addition to processing SUMO. Overall, these data confirm our conclusion from transformation data that deletion of *ulpB* in a *ulpA*⁺ background results in strains that grow very slowly and conidiate poorly.

Identification of SUMOylated proteins in exponentially growing *A. nidulans* hyphae

Construction of strains that allow affinity purification of proteins locked in their SUMOylated state—We have used our ability to manipulate the *A. nidulans* SUMOylation pathway to develop a sensitive method for identifying proteins that are SUMOylated *in vivo* in *A. nidulans*. Our basic approach was to affinity tag SUMO, use the tag to purify SUMOylated proteins, and then analyze them by mass spectroscopy, although to do this effectively required several steps as we will discuss. As an affinity tag, we chose the S-tag because it is small (16 amino acids in the version we used, see Fig. S6) and it binds

to the S-protein with great specificity and high affinity (Kim and Raines, 1993) and has previously been found to work well in *A. nidulans* (Ahn and Morris, 2001; Zhuang et al., 2007; Liu et al., 2010). It also binds to the S-protein under semi-denaturing conditions, which should allow us to distinguish proteins that are SUMOylated from those that are bound non-covalently to SUMO or complexed tightly with a SUMOylated protein.

Since the C-terminus of SUMO must be intact for SUMO to be processed, we fused the S-tag peptide sequence to the 5' end of the *sumO* coding sequence using the construction strategy detailed in the Materials and Methods and Fig. S7 and S8. We also included a 5 glycine-alanine (GA) linker between the S-tag and the *sumO* coding sequence. This linker is flexible and extends the S-tag some distance from SUMO. It should allow easy access of the S-protein to the S-tag during affinity purification. We transformed this construct into *A. nidulans* strain LO1385 replacing the wt *sumO* gene. We designated the resulting strain LO2715. In LO2715, the S-tagged *sumO* construct was the only copy of the *sumO* gene in the genome, its expression was driven by its normal promoter, and it supported normal growth and conidiation, indicating that it is functional (Fig. 2).

We tested the efficacy of this approach by using the S-protein to affinity purify SUMOylated proteins from this strain and an untagged control strain. We used the procedure developed by Liu et al. (2010). Briefly, we harvested hyphae, froze them in liquid nitrogen, freeze dried them, disrupted them by grinding with a mortar and pestle, and suspended the hyphal powder in extraction buffer (see Materials and Methods). We precipitated S-tagged proteins with S-protein beads and, after multiple washes in extraction buffer, the S-protein beads were boiled in SDS sample buffer and the released proteins were run on an SDS gel (Fig. 3). The extraction buffer contained 300 mM NaCl and 0.2% NP40 and, thus, proteins that are weakly bound to SUMOylated proteins should be washed off. As expected, the S-protein beads pulled down few proteins in the untagged strain. In the tagged strain, the S-tagged SUMO was pulled down by the S-protein beads (Fig. 3), demonstrating that the affinity purification worked. Only a few more protein bands were visible in the tagged strain, however, than in the untagged strain.

SUMOylation is reversible, with SUMO being removed by the same isopeptidases that process SUMO so that it can be conjugated to target proteins. If proteins are transiently SUMOylated (for example at specific points in the cell cycle), only a small fraction of the protein molecules may be SUMOylated at any given time and they may not be easily detected. We therefore employed an approach, based on the work of Hannich et al. (2005), that would lock proteins in their SUMOylated state to allow easier detection.

We transformed an S-tag SUMO construct into a strain in which *ulpA* was deleted (LO2592), creating strain LO3608. We also transformed the S-tag SUMO construct into strain LO2870, in which both of the isopeptidases were deleted, creating strain LO3612. Since *pyrG89* is complemented in LO2592 and LO2870, we created a transforming fragment in which *AfriboB* was the selectable marker (Fig. S8). In both cases the chromosomal copy of *sumO* was replaced, leaving the S-tag-*sumO* as the only copy of the *sumO* gene in the genome. Because the isopeptidases are required, however, for removal of

the C-terminus to allow conjugation of SUMO to target proteins, deletion of the isopeptidase genes will prevent SUMOylation.

To circumvent this problem, we created an N-terminally S-tagged *sumO* construct in which the codons encoding the two C-terminal amino acids were removed from the *sumO* gene. This created an S-tag “preprocessed” SUMO with a C-terminal GG sequence (Fig. S9) that, in principle, should allow the molecule to be conjugated normally to proteins *in vivo* without having to be processed by the isopeptidases (Smith et al., 2004). This construct was transformed into a strain with a wt SUMO pathway (creating strain LO3860). Through a series of crosses, we created strain LO3888 that carries S-tag preprocessed *sumO* and *ulpA*, as well as strain LO3886 that carries S-tag preprocessed *sumO* and deletions of both *ulpA* and *ulpB*. The *ulpA*, *ulpB* gene replacements and the S-tag preprocessed *sumO* in strain LO3886 were verified by diagnostic PCR and Southern hybridizations (Fig. S10). In each of these strains, the N-terminally tagged preprocessed *sumO* gene was the only *sumO* gene in the genome and it was under control of the normal *sumO* promoter. The tagged, preprocessed *sumO* gene was not toxic in a strain wt for all other SUMO pathway genes, nor in a *ulpA* deletion strain (Fig. 2). Importantly, it restored near normal conidiation to the *ulpA*, *ulpB* double deletion strain, indicating that it restores SUMO function (Fig. 2). This result also suggests, perhaps surprisingly, that locking proteins *en masse* in their SUMOylated state is not particularly harmful to hyphal growth and conidiation. Finally, we were able to isolate and verify segregants of a cross between LO3886 (S-tag-preprocessed-*sumO*, *ulpA*, *ulpB*) and LO1501 that carried the preprocessed S-tag *sumO* as well as *ulpB*. Although growth was strongly inhibited in these strains, they grew significantly better than *ulpB* strains with wt *sumO* (LO11581 in Fig. 2), revealing that the preprocessed S-tag *sumO* partially corrected the growth defect caused by *ulpB*.

As anticipated, S-tag affinity purification with preprocessed S-tag SUMO in *ulpA* single deletion or *ulpA* and *ulpB* double deletion strains resulted in the recovery of SUMO and many additional SUMOylated proteins (Fig. 3). These data confirmed the efficacy of our approach. It is notable that nearly as many protein bands were seen in the *ulpA* deletion as in the *ulpA*, *ulpB* double deletion. This result indicates that UlpA is the main deSUMOylating enzyme, consistent with the results of Harting *et al.* (2013). However, there are clearly protein bands in the double deletion that are not recovered with the single deletion. This result indicates that UlpB specifically deSUMOylates a relatively small set of proteins.

Identification of SUMOylated proteins in exponentially growing *A. nidulans* hyphae

We analyzed the proteins pulled down by S-protein beads using mass spectroscopy. We used the preparation scheme outlined in Fig. S11, which is based on the approach of De Souza *et al.*, (2014). After affinity purification and washing, the S-protein beads with bound proteins were placed in SDS-PAGE sample buffer and boiled, which denatures and dissociates bound proteins. They were then loaded onto an SDS-PAGE gel and subjected to brief electrophoresis. In this procedure the proteins stack and are thereby greatly concentrated. They are run a very short distance into the separating gel, cut out, digested with trypsin and subjected to LC-MS/MS. This separates proteins, fragments them into peptides, and sequences the peptides. We used a sensitive Orbitrap mass analyzer as a detector. Peptide

sequences were used to search the Broad Institute *A. nidulans* genome predicted protein database [no longer accessible but the annotated genome is available at FungiDB (fungidb.org)], allowing the identification of the proteins binding to the S-protein beads. Since Orbitrap mass spectroscopy is very sensitive, it might detect proteins that bind non-specifically, in small amounts, to the S-protein beads. We, therefore used a wt strain, LO1, in which SUMO was not S-tagged as a control. In the wt strain we detected 129 proteins in one run and 132 in a second run. Only 57 of these proteins were found in both of the runs, however, and most of these proteins are predicted to be very abundant, consistent with the possibility that trace amounts of abundant proteins are not completely eliminated from the S-protein beads. We next carried out the same procedure with strain LO3886 that carries an S-tag-preprocessed SUMO as well as *ulpA* and *ulpB*. In three runs we detected 295, 319 and 336 proteins. Substantially more proteins were, thus, detected in LO3886 than in the control strain.

Although the conditions of our non-denaturing wash were fairly vigorous, proteins that are not SUMOylated, but are complexed tightly with SUMOylated proteins, may remain associated with the S-protein beads and would be detected. In addition, some proteins bind tightly, but non-covalently, to the SUMO molecule itself and might also be detected. To differentiate between SUMOylated proteins and those that are simply complexed tightly with SUMO or with SUMOylated proteins, we carried out two runs in which we washed the S-tag beads under non-denaturing conditions followed by 3.0 M urea washes. The urea washes should dissociate protein complexes, releasing proteins that are complexed with SUMOylated proteins. They should also release proteins that are bound non-covalently to SUMO, but they will not release the SUMOylated proteins themselves (for the difference in protein content between normal wash and urea wash samples, see Fig. S12). Orbitrap mass spectroscopy identified 284 proteins in one run and 261 in the second. As a control, extracts from the wt control were subjected to the same treatment and 82 proteins were identified in one run and 77 in a second.

These data allowed us to determine which proteins were genuinely SUMOylated using the following criteria. Proteins that 1) were detected in the S-tag-preprocessed SUMO strain in at least four of five experiments (three without urea and two with urea), 2) were detected after urea treatment (see criteria below) and 3) were not detected in the wt control strain LO1, were considered to be SUMOylated. Proteins that were detected in both LO1 and LO3886 (s-tag-preprocessed-*sumO*, *ulpA*, *ulpB*) required a more complex analysis. Traces of abundant proteins bound to S-protein beads may be detected by Orbitrap mass spectroscopy even in strains in which SUMO is not S-tagged (e.g. LO1). While the majority of these proteins are probably not SUMOylated, some of them certainly may be. To distinguish between those that are SUMOylated and those that are not, we looked at the MASCOT protein scores in the mass spectroscopy data. The MASCOT score is complex, but for a given protein it is dependent on the abundance of the protein in the sample. We arbitrarily, but reasonably, set a threshold of 20% for exclusion of proteins. Thus, if the mean MASCOT protein score for a particular protein in the five wt control datasets was 20% or more of the mean score in the five LO3886 datasets, the data were not deemed to provide adequate evidence that the protein was SUMOylated. In practice, few proteins were near this

cutoff (Spreadsheet S1, SUMOylated proteins tab, background/signal column). Most proteins were clearly SUMOylated or not.

We also used the datasets to identify proteins that are not SUMOylated but are tightly complexed with SUMOylated proteins or are bound non-covalently to SUMO itself. For proteins that were present in all three datasets from non-urea-washed material, we applied the 20% protein score cutoff relative to the wt control datasets as described. For the proteins that met this criterion, if the mean protein score for proteins in the two data sets obtained with 3.0 M urea washed material was less than 10% of the score in the three datasets without the urea wash, we considered the proteins to be tightly complexed with SUMOylated proteins, or non-covalently bound to SUMO rather than being SUMOylated themselves (Spreadsheet S1, Binding Partners tab, urea wash/reg wash column). In practice only one protein in this category was near the 10% cutoff (AN1456). Five proteins were well below the cutoff and 36 in this category were not detected at all in the urea washed sample. Similarly, the scores of most proteins that we have categorized as SUMOylated were reduced very little by the urea wash, and many increased, firmly establishing them as SUMOylated. There were, however, sixteen proteins the scores of which after the urea wash were between 11 and 35% of their pre-wash scores. We have listed them as SUMOylated in Spreadsheet A1, but acknowledge that these proteins could be in complexes that somehow partially survive the urea wash. It is notable that 10 of these have been reported to be SUMOylated in other organisms, but it is not clear that the methods employed in these studies would unambiguously distinguish SUMOylated proteins from proteins complexed with SUMOylated proteins or bound non-covalently to SUMO.

Using these criteria, we identified 149 proteins as being definitely SUMOylated and 42 proteins as being either tightly complexed with SUMOylated proteins or non-covalently bound to SUMO. The abundances of proteins vary, however, and we reasoned that detection of low abundance SUMOylated proteins may be marginal. We have therefore defined a class of proteins as “possibly SUMOylated.” These proteins were detected in three of five datasets and the urea wash data indicated that they were SUMOylated rather than non-covalently bound to SUMO or complexed with SUMOylated proteins (Spreadsheet S1, Possible SUMOylated tab). Similarly, we have also defined a class of proteins as possible binding partners of SUMOylated proteins. These proteins were detected twice in non-denatured samples, but their MASCOT scores were reduced 90% or more by the 3.0 M urea wash. A full list of these proteins is in Spreadsheet S1, Possible binding partners tab.

Automated BLAST searches have been carried out for the genes of the *A. nidulans* genome and are available at AspGD (www.aspgd.org/) along with additional functional annotation data. We examined each of the SUMOylated proteins for function based on the AspGD data, NCBI BLAST data and literature searches when appropriate. We classified SUMOylated proteins into the categories shown in Fig. 4 and Spreadsheet S1, SUMOylated proteins tab. For simplicity we have listed each protein in a single category, but we note that it is becoming increasingly clear that many proteins are multifunctional and that there may be some overlapping of categories. Topoisomerases, for example, play critical roles in DNA replication, DNA repair, and transcription (Wang, 2002). We have included them in the DNA replication category but note that they could reasonably be added to other categories.

The most striking result was the very large number of proteins that are involved in transcription and, in particular, the regulation of transcription. Fully 67 of 149 SUMOylated proteins are predicted to play a role in the regulation of transcription. Of these, 39 proteins are predicted to be transcription factors or part of the transcription apparatus. We grouped these proteins together because SUMOylation is usually regulatory and SUMOylation of RNA polymerase III, for example, is likely to regulate RNA polymerase III-driven transcription. That being said, the great majority of these proteins were predicted to be transcription factors. In addition, 28 proteins were predicted to regulate transcription at the chromatin level. As discussed below, these included members of a number of chromatin level regulatory complexes. Another major category was RNA processing, with 21 proteins predicted to function in various aspects of this process. The final major category was DNA replication and repair, with 16 proteins in this group. Replication and repair were grouped together because some proteins (e.g. DNA ligase, Proliferating Cell Nuclear Antigen) are involved in both replication and repair, while others (e.g. DNA glycosylases) appear to be involved exclusively in DNA repair, and others (e.g. primases) primarily in DNA replication.

Five SUMOylation pathway proteins were SUMOylated. SUMOylation of SUMO pathway proteins has been reported in *S. cerevisiae* (Panse et al., 2004; Wohlschlegel et al., 2004; Zhou et al., 2004; Denison et al., 2005; Hannich et al., 2005; Albuquerque et al., 2013) and in *A. nidulans* (Harting et al., 2013). Harting et al. found that AosaA, UbcN, and SizA were pulled down by tap-tagged SUMO (Harting et al., 2013). We found that UbcN and SizA are SUMOylated, but we did not detect AosaA as being SUMOylated. Rather we detected it as a possible binding partner of a SUMOylated protein. This is as expected because it is a binding partner of UbaB, which we found to be SUMOylated. In addition, we found all three of the E3 enzymes to be SUMOylated.

In addition, six genes were predicted to be involved in cell cycle control. Note, however, that although they were not grouped with cell cycle control proteins, proteins required for DNA replication are obviously required for cell cycle progression as are the two members of the condensin complex that plays a role in chromosomal condensation in mitosis. Alteration of activities of any of these proteins would likely have significant cell cycle effects. Perhaps surprisingly, four ubiquitin pathway proteins were SUMOylated. Although cytoskeletal proteins were strikingly absent, two members of the TCP1 complex, which is a chaperone for tubulins and actin, are SUMOylated. Finally, 30 SUMOylated proteins were outside of these categories or their functions could not be deduced from BLAST searches, in some cases because there were no known homologs.

The proteins that we have designated as possibly SUMOylated and binding partners of SUMOylated proteins are also enriched for these categories (Spreadsheet S1, Binding Partners and Possible Binding Partners tabs). Comfortingly, the great majority of binding partners of SUMOylated proteins are predicted to be in complexes with proteins that we have determined to be SUMOylated (compare Spreadsheet S1, SUMOylated proteins and Binding Partners tabs).

Discussion

A new method for identifying SUMOylated proteins

The facts that *sumO* and SUMOylation pathway genes are not essential in *A. nidulans*, and that good methods have been developed for manipulation of genes in this organism, have allowed us to develop an efficient and direct method for identifying proteins that are SUMOylated *in vivo*. While affinity purification tags have been used to purify SUMOylated proteins in several studies, the S-tag is particularly effective because of its high binding affinity for the S-protein (Connelly et al., 1990). By combining the S-tag, deletions of the SUMO isopeptidases and deletion of two C-terminal amino acids of SUMO to form a preprocessed SUMO, we have been able to lock proteins in their SUMOylated state and to purify them efficiently. The fact that the S-tag remains bound to the S-protein under semi-denaturing conditions has additionally allowed us to identify proteins that are not SUMOylated themselves, but are tightly complexed with SUMOylated proteins or bind tightly, but non-covalently, to SUMO.

The *A. nidulans* SUMOylome

Our SUMOylome data indicate that most SUMOylated proteins are involved in a relatively small number of important cellular processes. Keeping in mind the fact that SUMOylation is generally involved in regulation of activity or of protein/protein interactions, our data point to a central role for SUMOylation in the regulation of these processes in *A. nidulans*.

The most striking feature of the *A. nidulans* SUMOylome is the number of proteins that are involved in transcription (fully 67 of 149 SUMOylated proteins). These data are consistent with the findings of Harting et al. in *A. nidulans* (Harting et al., 2013) as well as with findings in other organisms (Rosonina et al., 2017). Also striking is the number of classes of proteins included within this group. There are general (basal) transcription factors such as members of the TFIIA, TFIID and TFIIE complexes, there are general transcription repressors and co-repressors, there are bZip transcription factors, zinc finger transcription factors, a forkhead transcription factor, and transcription factors that regulate specific processes such as nitrogen metabolism (MeaB and AreB), heat shock protein expression (Hsf1), penicillin biosynthesis (AnbH1), etc. The SUMOylated proteins predicted to be involved in chromatin-level regulation of transcription include members of important chromatin modifying complexes such as the RSC complex, the SAGA complex, the INO80 complex, the ISW2 complex and the COMPASS complex. Note that while each of these complexes regulates transcription, some have additional activities as well.

As might be anticipated, components of the aforementioned chromatin modifying complexes figure prominently among the binding partners of SUMOylated proteins. It is worth noting, moreover, that many transcription factors, such as those required for development, expression of secondary metabolites and growth on minimal media would likely not be expressed under our growth conditions. The total number of SUMOylated proteins involved in the regulation of transcription may therefore be considerably higher than the ones we have detected. These results point to a particularly important role for SUMO in the regulation of transcription in *A. nidulans*.

Five SUMO pathway proteins were found to be SUMOylated. Two were SUMO E1 and E2 molecules (product of AN2450 and AN4399) which are covalently bound to SUMO as SUMO is processed (Gareau and Lima, 2010). More interestingly, each of the three SUMO E3 ligases was also SUMOylated. Since these proteins are not supposed to be covalently bound to SUMO as part of the SUMOylation process, SUMO might play a role in their regulation. Four proteins were related to ubiquitination, a putative E1 ubiquitin activating enzyme (AN10266), a putative E3 ubiquitin ligase (AN6574), a strange, but evolutionarily conserved, protein in which ubiquitin is fused to the ribosomal C-terminal extension protein (AN4872) and a similarly strange but evolutionarily conserved protein in which ubiquitin is fused to ribosomal protein L40 (AN4016).

Another major category of SUMOylated proteins were DNA replication and repair proteins (16 proteins). These are overlapping categories to some extent, because, for example, DNA ligase is required for both normal DNA replication and repair. However, some proteins are exclusive to one process or the other. Eight proteins were predominantly involved in DNA replication. They include the genes that encode topoisomerase I (AN0253), topoisomerase II (AN5406), three components of the DNA polymerase/primase α complex (AN10755, AN10855, AN3033), a protein involved in the initiation of replication (AN1221), a sliding clamp protein for DNA polymerase δ (AN10087), and a component of the clamp loading replication factor C complex (AN6303). Five SUMOylated proteins are involved predominantly in DNA repair: AN3629 and AN10978 which are DNA glycosylases, AN10434 which is involved in mms22-dependent DNA repair, AN3947 which is a DNA lyase, and AN3372 which is a nibrin homolog involved in double strand break repair. DNA ligase (AN6069) is essential for DNA replication and many forms of DNA repair, and PCNA is also important for both.

Twenty-one SUMOylated proteins are involved in RNA processing. They are predicted to play roles in a variety of aspects of RNA processing, (3' end cleavage, polyadenylation, tRNA maturation, rRNA maturation, splicing, etc.). Two members of the condensin complex are SUMOylated. Condensin is involved in chromosomal condensation and a number of other activities related to chromosome maintenance (Hirano, 2012).

In all, 117/149 proteins are predicted to interact with nucleic acids or chromatin. This is consistent with the observation that SUMO is highly concentrated in nuclei in *A. nidulans* (Wong et al., 2008). Finally, three chaperone proteins were SUMOylated, the *A. nidulans* HSP90 homolog and two proteins that are predicted to be components of the TCP1 chaperone complex (the products of AN1851 and AN5713).

The *A. nidulans* SUMOylation pathway and phenotypes of SUMO pathway deletions

The phenotypes of most of the SUMOylation pathway genes were consistent with the phenotypes noted by Harting et al. (2013). However, we were able to extend the work of Harting et al. by deleting additional genes and by creating double and triple deletions. In addition, we did note one significant discrepancy between our results and those of Harting et al.

With respect to the E3 deletions, our data reveal that MmsU is the most important E3 ligase with respect to hyphal growth and conidiation. In its absence, growth is severely restricted, while elimination of *sizA* and *sizB*, singly or together, results in little inhibition of growth. Since Mms21/Nse2 is an integral component of the Smc5/6 complex, and its physical presence, but not SUMO ligase activity, is essential for growth in yeasts (McDonald et al., 2003; Andrews et al., 2005; Zhao and Blobel, 2005), the strong growth defect of *mmsU* deletion strains may be caused by a loss of function of the Smc5/6 complex rather than defects in SUMOylation. Deletion of *sizA* and *sizB*, however, does exacerbate the growth defect of *mmsU*. This result suggests that there is some functional redundancy between SizA/SizB and MmsU. It is noteworthy that the growth reduction caused by the deletion of all three E-3 ligases is much greater than that caused by deletion of *sumO*, which eliminates all SUMOylation. We speculate that this is due principally to the loss of function of the Smc5/6 complex caused by deletion of *mmsU*, coupled with an additive growth reduction due to reduction of SUMOylation caused by the absence of E-3 ligase activity.

SUMO isopeptidases not only cleave preprocessed SUMO such that it can be ligated to proteins, but they also remove SUMO from proteins. Deletion of the *A. nidulans* SUMO isopeptidase genes, *ulpA* and *ulpB*, in combination, was anticipated to eliminate SUMO processing and, thus, eliminate SUMOylation. The phenotype of our double deletion was indistinguishable from the *sumO* deletion as anticipated. We note that *ulpA*, *ulpB* double deletion strains carrying pre-processed S-tag SUMO grow near normally. In these strains, proteins are predicted to be locked in the SUMOylated state and our data indicate that this is the case. General inhibition of deSUMOylation, thus, does not cause strong growth inhibition.

When *ulpA* alone was deleted, there was, at most, a small reduction in growth and conidiation, indicating that UlpB is able to process SUMO such that SUMOylation still occurs. The small reduction in growth and conidiation in *ulpA* deletants may be due to a reduction in the rate of SUMO processing or the failure of *ulpA* to remove SUMO from specific target proteins as Fig. 3 indicates. If this is the case, this removal failure does not cause problems that result in major inhibition of growth.

One significant difference in our results from the results of Harting et al. is the phenotype of the deletion of *ulpB*. We found that *ulpB* caused a very strong inhibition of colonial growth (Fig. 2). While Harting et al. did not show a colonial growth phenotype for the *ulpB* deletion, it is difficult to imagine that the strong growth inhibition we found would not have been noted. Our results were verified by diagnostic PCR (Fig. S5), Southern hybridizations (Fig. S10) and crosses. In addition, the deletion of *ulpB* based on the AspGD/FungiDB annotation gave the same phenotype as the deletion based on our later annotation. Deletions based on either annotation would eliminate most of the gene and certainly render it dysfunctional.

Preprocessed S-tag *sumO* restores some growth to the *ulpB* deletion (Fig. 2), indicating that a portion of the growth reduction is related to reduction of SUMO processing. However, growth was still greatly reduced relative to the *ulpA*, *ulpB* double deletion in which SUMO processing is predicted to be eliminated completely. This result indicates that the bulk of

growth inhibition is due to something other than failure of SUMO processing. Fig. 3 suggests that UlpB may be specifically required for deSUMOylation of a set of proteins (i.e. some proteins are recovered in the double deletant that are not pulled down in the *ulpA* single deletant). Bearing in mind that the major functions of SUMOylation are to regulate protein activities and interactions, we hypothesize that failure of deSUMOylation of these proteins in a background in which other proteins are SUMOylated and deSUMOylated normally creates an imbalance of activities that results in strong growth inhibition, much stronger growth inhibition than that caused by complete inhibition of SUMOylation or deSUMOylation. If this hypothesis is correct, it indicates the importance of deSUMOylation and the specificity of UlpB in targeting a subset of proteins for deSUMOylation.

Related to this, we noted that the *ulpB* deletion strains exhibited a high frequency of spontaneous reversion. In our model, a high spontaneous reversion frequency would be expected because, in a *ulpB* strain, any mutation that rendered *sumO* or *ulpA*, or *ubaB*, or *aosA* or *ubcN* dysfunctional would eliminate SUMOylation resulting in a *sumO* phenotype, which grows much better than the *ulpB* deletant.

Materials and Methods

Strains and media

The strains of *A. nidulans* used in this study are listed in Table 2. Minimal medium (MM) [10 g D-glucose, 6 g NaNO₃, 0.52 g KCl, 0.52 g MgSO₄·7H₂O, 1.52 g KH₂PO₄, 400 µl of a trace element solution adapted from (Vishniac and Santer, 1957) per liter] and complete medium (YG) [20 g D-glucose, 5 g yeast extract, 400 µl of a trace element solution adapted from (Vishniac and Santer, 1957) per liter] with appropriate supplements [uridine (2.442 mg/ml), uracil (1 mg/ml), riboflavin (2.5 µg/ml), pyridoxine (0.5 µg/ml)] when needed were used. 1.5% agar was added for solid medium.

Genome database search

To identify genes of SUMOylation pathway genes, BLASTP searches were carried out for the predicted products of the *A. nidulans* genome at AspGD (www.aspgd.org/) using SUMOylation pathway protein sequences of *S. cerevisiae* SUMOylation pathway genes as queries.

Gene manipulations

For transformation of *A. nidulans* strains and for preparation of linear DNA fragments to be used for transformation, we followed the method described earlier (Nayak et al., 2006; Szweczyk et al., 2006). We disrupted SUMO pathway genes by using fusion PCR to create linear molecules in which DNA flanking the target gene was amplified and these flanks were fused to a selectable marker. The result was a linear molecule in which the entire coding sequence of the target gene was replaced by the selectable marker. Transformation and selection on appropriate media resulted in the replacement of the coding sequence of the target gene with the marker gene.

To construct S-tag-SUMO we used the fusion PCR approach shown in Fig. S7 resulting in a linear DNA fragment that consisted of 1) a 1011 bp region upstream of *sumO*, 2) a selectable marker, the *A. fumigatus pyrG* gene (*Afp_{pyrG}*, 1885 bp), 3) a 512 bp fragment containing the *sumO* promoter sequence, 4) the start codon followed by 48 bp encoding the S-tag which was followed by a 30 bp sequence encoding a 5 glycine/alanine linker, and 5) a 971 bp fragment consisting of the *sumO* sequence and 3' flanking DNA. This fragment was cloned into pCR-Blunt II-TOPO (Invitrogen) creating plasmid pLO105 and verified by sequencing. To create an initial S-tag SUMO strain, the insert in pLO105 was amplified by PCR to create a linear transforming molecule. This molecule was used to transform strain LO1385 creating strain LO2715 in which the chromosomal copy of *sumO* was replaced with the S-tag version.

To construct strains expressing S-tag SUMO in the SUMO isopeptidase deletion strains, we needed to create a transforming fragment carrying *AfriboB* as a selectable marker rather than *Afp_{pyrG}* because the isopeptidases had been deleted by replacement with *Afp_{pyrG}* and the *pyrG* selection was, thus, not available. We did this by creating a transforming fragment like the one in pLO105 except that *AfriboB* replaced *Afp_{pyrG}* (Fig. S8). We transformed this construct into *A. nidulans* strains LO2592 (*ulpA*⁻) and LO2870 (*ulpA*⁻, *ulpB*⁻) creating strains LO3608 and LO3612 respectively.

We constructed a preprocessed version of S-tag-SUMO by deleting the two C-terminal amino acids, leaving the two glycines required for conjugation as shown in Fig. S9). We transformed the resulting fusion PCR product into LO1723 (*ulpA*⁺, *ulpB*⁺), creating strain LO3860. LO3860 was crossed to LO2870 (*ulpA*⁻, *ulpB*⁻). LO4654 (S-tag preprocessed SUMO, *ulpA*⁺, *ulpB*⁺) was a green segregant from this cross. LO3881, a white (*wA2*), S-tag-preprocessed-*sumO*, *ulpA*⁺, *ulpB*⁺ segregant from this cross was crossed to LO2870 (*ulpA*⁻, *ulpB*⁻) and the progeny included segregants LO3886 (S-tag-preprocessed-*sumO*, *ulpA*⁺, *ulpB*⁺) and LO3888 (S-tag-preprocessed-*sumO*, *ulpA*⁺, *ulpB*⁺). LO3860, LO3886 and LO3888 were confirmed by Southern hybridization of genomic DNA (Figure S10).

S-tag affinity purification and MASS spectrometry analysis

SUMOylated proteins were affinity purified using an S-tag affinity purification system. Conidia of each strain were cultured in liquid complete medium (YG) at 37°C in a gyrotory shaker for 14–16 hours, at which time the cultures were in exponential growth phase. Mycelia were harvested by filtration through Miracloth (Calbiochem), frozen in liquid nitrogen, freeze dried and processed for affinity purification using S-protein beads (Novagen), essentially following a method described previously (Liu et al., 2010). Briefly, freeze-dried mycelia were pulverized in mortar and pestle and resuspended in an extraction buffer (EX-buffer) [25 mM Tris-HCl, pH7.5, 300 mM NaCl, 5.0 mM EDTA, 15 mM EGTA, 10 mM NaF, 1 mM DTT, 0.2% NP-40, 1.0 mM iodoacetamide, 1 tablet/ 7 ml complete MINI EDTA-free protease inhibitor cocktail (Roche)]. S-protein beads were washed 6X with EX-buffer and an aliquot was reserved as a non-denatured sample. The remaining beads were suspended in EX-buffer with 3 M urea, incubated on ice for 30 minutes, then washed with EX buffer + 3.0 M urea three times. All proteins bound to the S-protein beads were released from the S-protein beads by boiling in SDS-containing buffer, collected by running

in SDS-PAGE gels until proteins had entered into the separating gel a short distance (Liu et al., 2010; De Souza et al., 2014) and processed for Orbitrap LC-MS/MS (Thermo Fisher) analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SUMO	small ubiquitin-like modifier
tap-tag	tandem affinity protein-tag

References

- Ahn C, Morris NR, 2001 Nudf, a fungal homolog of the human LIS1 protein, functions as a dimer in vivo. *J Biol Chem* 276, 9903–9909. [PubMed: 11134054]
- Albuquerque CP, Wang G, Lee NS, Kolodner RD, Putnam CD, Zhou H, 2013 Distinct SUMO ligases cooperate with Esc2 and Slx5 to suppress duplication-mediated genome rearrangements. *PLoS Genet* 9, e1003670. [PubMed: 23935535]
- Andrews EA, Palecek J, Sergeant J, Taylor E, Lehmann AR, Watts FZ, 2005 Nse2, a component of the Smc5–6 complex, is a SUMO ligase required for the response to DNA damage. *Mol Cell Biol* 25, 185–196. [PubMed: 15601841]
- Connelly PR, Varadarajan R, Sturtevant JM, Richards FM, 1990 Thermodynamics of protein-peptide interactions in the ribonuclease S system studied by titration calorimetry. *Biochemistry* 29, 6108–6114. [PubMed: 2383573]
- Cullen JM, Newberne PM, 1993 Acute hepatotoxicity of aflatoxins. In: DL, E., JD, G., (Eds.s) *The toxicology of aflatoxins: human health, veterinary, and agricultural significance*, Academic Press, London, pp 1–26.
- Dasso M, 2008 Emerging roles of the SUMO pathway in mitosis. *Cell Div* 3, 5. [PubMed: 18218095]
- De Souza CP, Hashmi SB, Osmani AH, Osmani SA, 2014 Application of a new dual localization-affinity purification tag reveals novel aspects of protein kinase biology in *Aspergillus nidulans*. *PLoS One* 9, e90911. [PubMed: 24599037]
- De Souza CP, Osmani AH, Hashmi SB, Osmani SA, 2004 Partial nuclear pore complex disassembly during closed mitosis in *Aspergillus nidulans*. *Curr. Biol* 14, 1973–1984. [PubMed: 15556859]
- Denison C, Rudner AD, Gerber SA, Bakalarski CE, Moazed D, Gygi SP, 2005 A proteomic strategy for gaining insights into protein sumoylation in yeast. *Mol Cell Proteomics* 4, 246–254. [PubMed: 15542864]
- Dohmen RJ, 2004 SUMO protein modification. *Biochim Biophys Acta* 1695, 113–131. [PubMed: 15571812]
- Dohn JW, Grubbs AW, Elizabeth Oakley C, Oakley BR, 2018 New Multi-Marker Strains and Complementing Genes for *Aspergillus nidulans* Molecular Biology. *Fungal Genet Biol* 111, 1–6. [PubMed: 29309843]

- Gareau JR, Lima CD, 2010 The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* 11, 861–871. [PubMed: 21102611]
- Hannich JT, Lewis A, Kroetz MB, Li SJ, Heide H, Emili A, Hochstrasser M, 2005 Defining the SUMO-modified proteome by multiple approaches in *Saccharomyces cerevisiae*. *J Biol Chem* 280, 4102–4110. [PubMed: 15590687]
- Harting R, Bayram O, Laubinger K, Valerius O, Braus GH, 2013 Interplay of the fungal sumoylation network for control of multicellular development. *Mol Microbiol* 90, 1125–1145. [PubMed: 24279728]
- Hay RT, 2005 SUMO: a history of modification. *Mol Cell* 18, 1–12. [PubMed: 15808504]
- Hirano T, 2012 Condensins: universal organizers of chromosomes with diverse functions. *Genes Dev* 26, 1659–1678. [PubMed: 22855829]
- Johnson ES, Blobel G, 1997 Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. *J. Biol. Chem* 272, 26799–26802. [PubMed: 9341106]
- Johnson ES, 2004 Protein modification by SUMO. *Annu. Rev. Biochem* 73, 355–382. [PubMed: 15189146]
- Kim JS, Raines RT, 1993 Ribonuclease S-peptide as a carrier in fusion proteins. *Protein Sci* 2, 348–356. [PubMed: 8453373]
- Liu HL, Osmani AH, Ukil L, Son S, Markossian S, Shen KF, Govindaraghavan M, Varadaraj A, Hashmi SB, De Souza CP, Osmani SA, 2010 Single-step affinity purification for fungal proteomics. *Eukaryot Cell* 9, 831–833. [PubMed: 20363899]
- McDonald WH, Pavlova Y, Yates JR, Boddy MN, 2003 Novel essential DNA repair proteins Nse1 and Nse2 are subunits of the fission yeast Smc5-Smc6 complex. *J Biol Chem* 278, 45460–45467. [PubMed: 12966087]
- Melchior F, Schergaut M, Pichler A, 2003 SUMO: ligases, isopeptidases and nuclear pores. *Trends Biochem Sci* 28, 612–618. [PubMed: 14607092]
- Meluh Pamela B., Koshland D, 1995 Evidence that the MIF2 gene of *Saccharomyces cerevisiae* encodes a centromere protein with homology to the mammalian centromere protein CENP-C. *Mol. Biol. Cell* 6, 793–807. [PubMed: 7579695]
- Meulmeester E, Melchior F, 2008 Cell biology: SUMO. *Nature* 452, 709–711. [PubMed: 18401402]
- Meyer V, Andersen MR, Brakhage AA, Braus GH, Caddick MX, Cairns TC, de Vries RP, Haarmann T, Hansen K, Hertz-Fowler C, Krappmann S, Mortensen UH, Peñalva MA, Ram AFJ, Head RM, 2016 Current challenges of research on filamentous fungi in relation to human welfare and a sustainable bio-economy: a white paper. *Fungal Biol Biotechnol* 3, 6. [PubMed: 28955465]
- Mukhopadhyay D, Dasso M, 2007 Modification in reverse: the SUMO proteases. *Trends Biochem Sci* 32, 286–295. [PubMed: 17499995]
- Mukhopadhyay D, Dasso M, 2009 The SUMO pathway in mitosis. In: Wilson VG, (Eds.) *SUMO Regulation of Cellular Processes*, Springer Science+Business Media B. V., pp 153–169.
- Muller S, Ledl A, Schmidt D, 2004 SUMO: a regulator of gene expression and genome integrity. *Oncogene* 23, 1998–2008. [PubMed: 15021887]
- Nayak T, Szewczyk E, Oakley CE, Osmani A, Ukil L, Murray SL, Hynes MJ, Osmani SA, Oakley BR, 2006 A versatile and efficient gene-targeting system for *Aspergillus nidulans*. *Genetics* 172, 1557–1566. [PubMed: 16387870]
- Nie M, Vashisht AA, Wohlschlegel JA, Boddy MN, 2015 High Confidence Fission Yeast SUMO Conjugates Identified by Tandem Denaturing Affinity Purification. *Sci Rep* 5, 14389. [PubMed: 26404184]
- Oakley BR, Oakley CE, Yoon Y, Jung MK, 1990 γ -Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell* 61, 1289–1301. [PubMed: 2194669]
- Osmani AH, Oakley BR, Osmani SA, 2006 Identification and analysis of essential *Aspergillus nidulans* genes using the heterokaryon rescue technique. *Nat. Protoc* 1, 2517–2526. [PubMed: 17406500]
- Osmani SA, Engle DB, Doonan JH, Morris NR, 1988 Spindle formation and chromatin condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. *Cell* 52, 241–251. [PubMed: 3277718]

- Pagano L, Caira M, Candoni A, Offidani M, Fianchi L, Martino B, Pastore D, Picardi M, Bonini A, Chierichini A, Fanci R, Caramatti C, Invernizzi R, Mattei D, Mitra ME, Melillo L, Aversa F, Van Lint MT, Falcucci P, Valentini CG, Girmenia C, Nosari A, 2006 The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. *Haematologica* 91, 1068–1075. [PubMed: 16885047]
- Panse VG, Hardeland U, Werner T, Kuster B, Hurt E, 2004 A proteome-wide approach identifies sumoylated substrate proteins in yeast. *J Biol Chem* 279, 41346–41351. [PubMed: 15292183]
- Rosonina E, Akhter A, Dou Y, Babu J, Sri Theivakadacham VS, 2017 Regulation of transcription factors by sumoylation. *Transcription* 8, 220–231. [PubMed: 28379052]
- Schwartz DC, Hochstrasser M, 2003 A superfamily of protein tags: ubiquitin, SUMO and related modifiers. *Trends Biochem Sci* 28, 321–328. [PubMed: 12826404]
- Schwiehorst I, Johnson ES, Dohmen RJ, 2000 SUMO conjugation and deconjugation. *Mol Gen Genet* 263, 771–786. [PubMed: 10905345]
- Smith M, Bhaskar V, Fernandez J, Courey AJ, 2004 *Drosophila* Ulp1, a nuclear pore-associated SUMO protease, prevents accumulation of cytoplasmic SUMO conjugates. *J Biol Chem* 279, 43805–43814. [PubMed: 15294908]
- Szewczyk E, Chiang Y-M, Oakley CE, Davidson AD, Wang CCC, Oakley BR, 2008 Identification and characterization of the asperthecin gene cluster of *Aspergillus nidulans*. *Appl. Environ. Microbiol* 74, 7607–7612. [PubMed: 18978088]
- Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA, Oakley BR, 2006 Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat. Protoc* 1, 3111–3120. [PubMed: 17406574]
- Tanaka K, Nishide J, Okazaki K, Kato H, Niwa O, Nakagawa T, Matsuda H, Kawamukai M, Murakami Y, 1999 Characterization of a fission yeast SUMO-1 homologue, pmt3p, required for multiple nuclear events, including the control of telomere length and chromosome segregation. *Mol Cell Biol* 19, 8660–8672. [PubMed: 10567589]
- Verger A, Perdomo J, Crossley M, 2003 Modification with SUMO. A role in transcriptional regulation. *EMBO Rep* 4, 137–142. [PubMed: 12612601]
- Vishniac W, Santer M, 1957 The thiobacilli. *Bacteriol. Rev* 21, 195–213. [PubMed: 13471458]
- Wang JC, 2002 Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* 3, 430–440. [PubMed: 12042765]
- Weidner G, d'Enfert C, Koch A, Mol PC, Brakhage AA, 1998 Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5'-monophosphate decarboxylase. *Curr. Genet* 33, 378–385. [PubMed: 9618589]
- Wohlschlegel JA, Johnson ES, Reed SI, Yates JR, 2004 Global analysis of protein sumoylation in *Saccharomyces cerevisiae*. *J Biol Chem* 279, 45662–45668. [PubMed: 15326169]
- Wong KH, Todd RB, Oakley BR, Oakley CE, Hynes MJ, Davis MA, 2008 Sumoylation in *Aspergillus nidulans*: *sumO* inactivation, overexpression and live-cell imaging. *Fungal Genet. Biol* 45, 728–737. [PubMed: 18262811]
- Zhao X, Blobel G, 2005 A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. *Proc Natl Acad Sci U S A* 102, 4777–4782. [PubMed: 15738391]
- Zhou W, Ryan JJ, Zhou H, 2004 Global analyses of sumoylated proteins in *Saccharomyces cerevisiae*. Induction of protein sumoylation by cellular stresses. *J Biol Chem* 279, 32262–32268. [PubMed: 15166219]
- Zhuang L, Zhang J, Xiang X, 2007 Point mutations in the stem region and the fourth AAA domain of cytoplasmic dynein heavy chain partially suppress the phenotype of NUDE/LIS1 loss in *Aspergillus nidulans*. *Genetics* 175, 1185–1196. [PubMed: 17237507]

Figure 1.

Phenotypes of SUMO pathway deletants. The genes deleted are listed under the figure. All strains used in this figure produce green conidia, and strains that produce fewer conidia appear pale. The strains pictured are, from top left, LO2059 (*sumO*), LO2606 (*ubaB*), LO2609 (*ubcN*), LO2797 (*sizB*), LO2799 (*sizA*), LO5338 (*mmsU*), LO1 (wt), LO2874 (*sizA* , *sizB*), LO5336 (*sizA* , *sizB* , *mmsU*) (see Table 2 for full genotypes).

Figure 2.

Phenotypes of isopeptidase deletions and effects of the expression of S-tag-preprocessed SUMO on the growth of *A. nidulans*. The isopeptidase deletions are indicated at the top of the figure and the versions of SUMO carried by the strains are indicated at the left. Top row: strains carrying wt *sumO*. The *ulpA* deletion shows a slight reduction in conidiation. The *ulpB* deletion shows almost no growth and the *ulpA*, *ulpB* double deletion is essentially identical to the *sumO* deletion (see Fig. 1). Second row: Arrows indicate suppression of isopeptidase deletion defects by S-tag-preprocessed SUMO (S-tag PP'd SUMO). The S-tag-preprocessed SUMO improves the growth of the *ulpB* deletion relative to the strain carrying wt *sumO*. The S-tag preprocessed SUMO also improves the conidiation of the *ulpA*, *ulpB* double deletion relative to the strain carrying wt *sumO* or S-tag *sumO*. Incubation was at 37°C. The strains used are, from left to right, row 1: LO1 (wt), LO2592 (*ulpA*), LO11584 (*ulpB*), LO2870 (*ulpA*, *ulpB*); row 2: LO4654 (S-tag-preprocessed-*sumO*), LO3888 (S-tag-preprocessed-*sumO*, *ulpA*), LO11581 (S-tag-preprocessed-*sumO*, *ulpB*), LO3886 (S-tag-preprocessed-*sumO*, *ulpA*, *ulpB*); row 3: LO2715 (S-tag-preprocessed-*sumO*), LO3608 (S-tag-*sumO*, *ulpA*), NT (not tested) and LO3612 (S-tag-preprocessed-*sumO*, *ulpA*, *ulpB*). See Table 2 for full genotypes.

Figure 3.

Gel electrophoresis analysis of proteins pulled down with S-protein beads. Strain names are indicated at the top of each lane. Below the strain names are the versions of SUMO carried by the strain and below those are the versions of SUMO isopeptidases carried by the strain. Dashes indicate that the strain carries the wt allele and circles indicate that the strain carries the mutant form indicated at the left. An arrow near the bottom of the gel indicates the migration position of S-tagged SUMO. The migration positions of molecular weight markers are indicated to the left of the gel. As expected, since SUMO in the wt strain carries no S-tag, few proteins are pulled down and SUMO is not pulled down. Slightly more proteins are pulled down in a strain carrying S-tag SUMO and wt alleles of *ulpA* and *ulpB*. Many proteins are pulled down in the strain carrying a deletion of *ulpA* and the S-tag-preprocessed SUMO (S-tag PP'd), and several additional proteins are pulled down in the strain carrying S-tag-preprocessed SUMO as well as deletions of both *ulpA* and *ulpB*.

Figure 4.
Functional classifications of SUMOylated proteins in *A. nidulans*.

Table 1.

SUMO and SUMO pathway genes in *A. nidulans*. E-values are from BLAST searches with the *S. cerevisiae* homolog. Note Siz1 and Siz2 each have strong homology to AN4497 and AN10822. AN10822 (*sizA*) has stronger homology to both Siz1 and Siz2 than does An4497 (*sizB*).

Pathway component	<i>S. cerevisiae</i> homolog (a.a.'s)	<i>A. nidulans</i> gene AspGD number (a.a.'s)	E-value
SUMO	Smt3 (101)	<i>sumO</i> AN1191 (94)	1.6E-23
E1 activating enzyme components	Uba2 (636)	<i>ubaB</i> AN2450 (610)	1.6E-92
	Aos1 (347)	<i>aosA</i> AN2298 (396)	2.1E-51
E2 conjugating enzyme	Ubc9 (157)	<i>ubcN</i> AN4399 (157)	5.6E-51
E3 ligases	Cst9 (482)	no homologue	> 0.57
	Siz1 (904)	<i>sizB</i> AN4497* (1,117)	4.6E-07
		<i>sizA</i> AN10822 (504)	1.6E-50
	Siz2 (726)	<i>sizB</i> AN4497* (1,117)	5.8E-07
		<i>sizA</i> AN10822 (504)	1.1E-36
	Mms21 (267)	<i>mmsU</i> AN10240 (502)	3.3E-06
Proteases (Isopeptidases)	Ulp1 (621)	<i>ulpA</i> AN2689 (965)	1.5E-13
	Ulp2 (1,034)	<i>ulpB</i> AN8192 (1,051)	2.1E-17

Table 2.

Genotypes of strains used in this study. Note: LO1 is the genome sequenced strain. Although the genome sequenced strain was listed as FGSC A4, it is actually a separate strain. LO1 carries *veA1* whereas FGSC A4 is *veA+*. All other strains used in this study also carry *veA1*.

LO1	wt
LO1385	<i>nkuA::argB, pyrG89, pyroA4</i>
LO1501	<i>nkuA::argB, fwA, pyrG89, pabaA1, pyroA4, riboB2,</i>
LO1723	<i>nkuA::argB, wA2, pyroA4, riboB2</i>
LO2059	<i>nkuA::argB, pyrG89, pyroA4, riboB2, argB2, sumO (AN1191)::AfpyrG</i>
LO2592	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ulpA (AN2689)::AfpyrG</i>
LO2606	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ubaB (AN2450)::AfpyrG</i>
LO2609	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ubcN (AN4399)::AfpyrG</i>
LO2715	<i>nkuA::argB, pyrG89, pyroA4, riboB2, S-tag-sumO (An1191)-AfpyrG</i>
LO2797	<i>nkuA::argB, pyrG89, pyroA4, riboB2, sizB (AN4497)::AfpyrG</i>
LO2799	<i>nkuA::argB, pyrG89, pyroA4, riboB2, sizA (AN10822)::AfpyrG</i>
LO2870	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ulpA (AN2689)::AfpyrG, ulpB (AN8192)::AfpyroA</i>
LO2874	<i>nkuA::argB, pyrG89, pyroA4, riboB2, sizB (AN4497)::pyroA, sizA (AN10822)::pyrG</i>
LO3608	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ulpA (AN2689)::AfpyrG, S-tag-sumO (AN1191)-AfriboB</i>
LO3612	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ulpA (AN2689)::AfpyrG, ulpB (AN8192)::AfpyroA, S-tag-sumO (AN1191)-AfriboB</i>
LO3860	<i>nkuA::argB, argB2, pyroA4, riboB2, S-tag-preprocessed-sumO (AN1191)-AfriboB, wA2</i>
LO3881	<i>nkuA::argB, pyrG89, riboB2, pyroA4, S-tag-preprocessed-sumO (AN1191)-AfriboB, wA2, (argB2?)</i>
LO3886	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ulpA (AN2689)::AfpyrG, ulpB (AN8192)::AfpyroA, S-tag-preprocessed-sumO (AN1191)-AfriboB, (argB2?)</i>
LO3888	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ulpA(AN2689)::AfpyrG, S-tag-preprocessed-sumO (AN1191)-AfriboB, (argB2?)</i>
LO4654	<i>nkuA::argB, pyrG89, pyroA4, riboB2, S-tag-preprocessed-sumO (AN1191)-AfriboB, (argB2?)</i>
LO5336	<i>nkuA::argB, argB2, pyrG89, pyroA4, riboB2, sizB (AN4497)::AfpyroA, sizA (AN10822)::AfpyrG, mmsU (AN10240)::AfriboB</i>
LO5338	<i>nkuA ::argB, pyroA4, riboB2, mmsU (AN10240)::AfriboB</i>
LO6789	<i>nkuA ::argB, pyrG89:pPL6 (pyrG⁺), pyroA4, riboB2, aosA (AN2298)::AfriboB</i>
LO11581	<i>nkuA::argB, pyrG89, pyroA4, riboB2, ulpB (AN8192)::AfpyroA, S-tag-preprocessed-sumO (AN1191)-AfriboB, (argB2?)</i>
LO11584	<i>nkuA ::argB, pyrG89, pyroA4, riboB2, ulpB (AN8192)::AtpyrG</i>