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Sumoylation in *Aspergillus nidulans*: *sumO* inactivation, overexpression and live-cell imaging

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Abstract

Sumoylation, the reversible covalent attachment of small ubiquitin-like modifier (SUMO) peptides has emerged as an important regulator of target protein function. In *Saccharomyces cerevisiae*, but not in *Schizosaccharomyces pombe*, deletion of the gene encoding SUMO peptides is lethal. We have characterized the SUMO-encoding gene, *sumO*, in the filamentous fungus *Aspergillus nidulans*. The *sumO* gene was deleted in a diploid and *sumO*⁺ haploids were recovered. The mutant was viable but exhibited impaired growth, reduced conidiation and self-sterility. Overexpression of epitope-tagged SumO peptides revealed multiple sumoylation targets in *A. nidulans* and SumO overexpression resulted in greatly increased levels of protein sumoylation without obvious phenotypic consequences. Using five-piece fusion PCR, we generated a *gfp-sumO* fusion gene expressed from the *sumO* promoter for live cell imaging of GFP-SumO and GFP-SumO-conjugated proteins. Localisation of GFP-SumO is dynamic, accumulating in punctate spots within the nucleus during interphase, lost at the onset of mitosis and re-accumulating during telophase.

Keywords

SUMO; conidiation; sexual development; fusion PCR; cell cycle

Introduction

Sumoylation, the covalent addition of a small ubiquitin-like modifier (SUMO) peptide, is a conserved post-translational modification that can influence the activity, interaction or sub-cellular location of a diverse array of proteins (Melchior, 2000; Verger *et al.*, 2003). The addition of SUMO peptides to target proteins is catalysed by a series of enzymatic steps in which the SUMO peptide precursor is first cleaved to produce a mature peptide with a C-

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terminal di-glycine motif (Johnson *et al.*, 1997; Kamitani *et al.*, 1997). SUMO peptides are activated by the E1-activating enzyme and transferred to the E2-conjugating enzyme Ubc9 for covalent attachment, via the diglycine motif, to a lysine residue on the target protein by a member of the E3-SUMO ligase family (Johnson and Blobel, 1997; Schwarz *et al.*, 1998; Takahashi *et al.*, 2001). The covalent attachment of SUMO peptides to proteins is reversible by a family of SUMO-specific proteases (Gong *et al.*, 2000; Gong and Yeh, 2006; Li and Hochstrasser, 1999; Yeh *et al.*, 2000).

In higher eukaryotes, sumoylation is involved in cell cycle progression, genome stability, DNA repair, transcriptional regulation and signal transduction (see Gill, 2004; Johnson, 2004; Verger *et al.*, 2003 for reviews). Proteomic analyses in *Saccharomyces cerevisiae* has revealed that sumoylation is involved in a similarly broad range of functions including transcription, translation, DNA replication and stress responses (Denison *et al.*, 2005; Hannich *et al.*, 2005; Panse *et al.*, 2004; Wohlschlegel *et al.*, 2004; Zhou *et al.*, 2004). SUMO peptides are a highly conserved family of proteins found in all eukaryotes. In vertebrates, the SUMO family comprises four genes while eight *SUMO* genes have been identified in *Arabidopsis thaliana* (Bohren *et al.*, 2004; Kurepa *et al.*, 2003; Su and Li, 2002). In contrast, the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* each contain a single gene (*SMT3* and *pmt3*, respectively) (Giaever *et al.*, 2002; Johnson *et al.*, 1997; Tanaka *et al.*, 1999). Deletion of *SMT3* in *S. cerevisiae* is lethal whereas the *pmt3*[•] mutant in *S. pombe* is viable but shows severely reduced growth and aberrant cellular and nuclear morphologies (Giaever *et al.*, 2002; Johnson *et al.*, 1997; Tanaka *et al.*, 1999).

We have identified the *sumO* gene encoding the SUMO peptide in *Aspergillus nidulans* and show that inactivation of this gene is not lethal in this filamentous fungus. The role of sumoylation has been investigated by determining the *in vivo* effects of gene inactivation and of *sumO* overexpression. The tagging of the SUMO peptide with GFP has revealed a dynamic pattern to the sub-cellular localization of sumoylated proteins during the cell-cycle.

Materials and Methods

Fungal strains, media and molecular methods

Complete and ANM minimal media were as described by Cove, (1966) and YAG medium contained 5g/L of yeast extract and 20g/L D-glucose. Minimal medium contained 1% glucose or xylose where indicated to induce expression from the *xylP* promoter. Nitrogen sources were added at a concentration of 10 mM unless otherwise indicated. For testing sensitivity to compounds, 0.0025% methyl methanesulfonate (MMS) or 5 mM hydroxyurea (HU) was added to glucose minimal medium containing ammonium tartrate as the nitrogen source. Glufosinate solution was prepared as described (Nayak *et al.*, 2006) and used at a final concentration of 25µl/ml. Benlate® (1µg/ml) was added to complete medium to induce haploidisation. Standard genetic manipulations and gene symbols were as described (Clutterbuck, 1974; Todd *et al.*, 2007a, b). For sexual development in homokaryons, MH1 (*biA1*) and MH10992 (*biA1*; *wA3*; *sumO*[•]) conidia were inoculated onto solid biotin-supplemented minimal medium with 10 mM sodium nitrate as the nitrogen source, incubated at 37°C for 2 days before the plate was sealed and further incubated at 37°C for 7 days. For analysis of sexual development in heterokaryons wild type (A234: *yA2 pabaA1*)

and *sumO*[•] (MH10992: *biA1*; *wA3*; *sumO*[•]) were inoculated to solid complete medium and grown for 2 days. A mixture of hyphae from each parent was transferred to unsupplemented solid 10 mM sodium nitrate-minimal medium, incubated at 37°C for 2 days, sealed and incubated for a further 7 days. The preparation of protoplasts and transformation was as described (Andrianopoulos and Hynes, 1988). Colony growth (hyphal extension) of MH1 (*biA1*) and MH10992 (*biA1*; *sumO*[•]) was measured as the radius of the colony from the point of conidial inoculation to the outermost edge on solid complete medium incubated at 37°C. Spore count was as described (Todd *et al.*, 2006). TNO2A7 (*nkuA*:*argB* *pyroA4* *pyrG89* *riboB2*) was the recipient for the *gfp-sumO* construct. R153 (*wA3*; *pyroA4*) was used as a control strain to verify that *gfp-sumO* fully substitutes for the *sumO* gene. Time lapse GFP-SumO images were made with LO1655 (*pyrG89* *Aspergillus fumigatus* *pyrG*⁺; *pabaA1*; *gfp-sumO* *mipAR243*) or with progeny of a cross between LO1761 (mCherry- γ -tubulin, *pyrG89* *Aspergillus fumigatus* *pyrG*⁺; *pabaA1*; *pyroA4*; *riboB2*) and LO1655. Standard molecular methods for DNA manipulations, nucleic acid blotting and hybridization were as previously described (Sambrook *et al.*, 1989). Genomic DNA was isolated as described (Lee and Taylor, 1990). Oligonucleotide sequences are listed in Table 1. DNA sequencing was performed by the Australian Genome Research Facility (Brisbane, Australia).

Isolation and deletion of the *A. nidulans* *sumO* gene

The single *A. nidulans* *sumO* gene AN1191.3 was identified by tblastx and blastp searches (Altschul *et al.*, 1990) using *S. cerevisiae* Smt3 (Accession no. AAB01675) against the *A. nidulans* genome database (Aspergillus Sequencing Project. Broad Institute of MIT and Harvard (<http://www.broad.mit.edu>)). The open reading frame was annotated using the GeneScan algorithm in Biomanager of ANGIS (<http://biomanager.angis.org.au>) and confirmed by alignment with the full-length EST sequence (Genbank Accession no. AA965561). A 3962 bp PCR product containing the *sumO* gene and flanking sequences was amplified from wild-type (MH1: *biA1*) genomic DNA using *sumo-F* (-2153 to -2135) and *sumo-R* (+1809 to +1788) primers. The fragment was cloned into pGEMT-easy (Promega) (pCW6726) and sequenced. The *HindIII*/*SmaI* fragment of pCW6726, which includes the entire *sumO* coding region, was replaced by the *HindIII*/*EcoRV* fragment of pMT1612 containing the dominant selectable marker *Bar* conferring glufosinate resistance (Monahan *et al.*, 2006), to generate pCW6042. A linear PCR product generated using *sumo-F* and *sumo-R* primers from pCW6042 was transformed into a diploid strain (MH6590: *suA1adE20* *yA2*; *AcrA1*; *galA1*; *pyroA4*; *facA303*; *sB3*; *nicB8*; *riboB2* / *biA1*; *wA3*; *amdI9*; *niiA4* *riboB2* *facB101*). *sumO*[•] heterozygotes were identified by Southern blot analysis using a PCR product amplified with *sumo-F3* (-26 to -5) and *sumo-R* (+1809 to +1788) as a probe.

Generation of a *xyIP(p)sumO^{FLAG}* over-expression strain

A PCR product, amplified from wild-type genomic DNA with *sumo-F3* (-26 to -5) and *sumo-R* primers (+1809 to +1788), was cloned into the *EcoRV* site of pBluescriptSK⁺ (pCW6572). The *SmaI*/*HindIII* fragment of pCW6572 was sub-cloned into the *SmaI*/*HindIII* sites of pSM6130, which carries the xylose inducible promoter (*xyIP(p)*) (Zadra *et al.*, 2000), to give rise to pCW6574. The *Bar* gene was then cloned as an *EcoRV* fragment from

pSM5962 into the *Sma*I site of pCW6574 to create pCW6575. Flag-sumo-InvF and Flag-sumo-InvR primers, each containing the sequences for half of the Flag epitope (underlined in Table 1) were used for inverse PCR on pCW6575 and the product was digested using the *Eco*RV restriction site (in bold in Table 1) incorporated into the Flag-sumo-InvR primer and self-ligated to introduce the full Flag epitope coding sequence in-frame between the third and fourth codons of the *sumO* gene (pCW6747). Cotransformation of this construct used the *pyroA* selectable marker plasmid pI4 (Osmani *et al.*, 1999) and a *sumO*⁺ recipient (MH10996: *biA1*; *wA3*; *pyroA4*; *sumO*⁺). To target the *xylP(p)sumO^{FLAG}* construct to *sumO*, pCW6747 was transformed into MH11036 (*pyroA4*; *nkuA::argB*⁺; *riboB2*), glufosinate resistant transformants were isolated and homologous integration at *sumO* was confirmed by Southern blot analysis.

Detection of SumO^{FLAG} and SumO^{FLAG}-modified proteins

50 micrograms of crude protein extracts from the specified growth conditions were resolved on denaturing 15% SDS-PAGE, blotted onto PVDF membrane (Millipore) and probed with Anti-FLAG (M2, Sigma) antibody at 1/10,000 dilution followed by anti-mouse IgG HRP (Promega) at 1/4000 dilution and chemiluminescence with the ECL Plus Western blotting detection system (Amersham).

GFP tagging of SumO

Five separate fragments were made by PCR. Three fragments of the *sumO* gene were amplified from *A. nidulans* genomic DNA: the 5' untranslated region from −1589 to −521 relative to the start codon of *sumO* was amplified with primers P1 and P3, a fragment (−521 to −1) containing the *sumO* promoter was amplified with primers P4 and P5 and a fragment (+1 to +1009) carrying the *sumO* coding sequence and a region of the 3' untranslated region was amplified using primers P6 and P8. Primers P3, P4, P5 and P6 were synthesized with extensions that overlap *A. fumigatus pyrG* and *gfp* amplified from plasmid pFN03 (Yang *et al.*, 2004). A fragment carrying the *A. fumigatus pyrG* gene (Weidner *et al.*, 1998) was amplified with primers pyrGF2 and pyrGR and a fragment carrying the GFP coding sequence was amplified with primers LIZP186 and LIZP184. The 3' primer (LIZP184) was designed to encode five glycine-alanine repeats at the C-terminus of GFP to provide a flexible linker. The five PCR fragments were mixed as template for the fusion PCR and amplified with nested primers P2 and P7 (see Fig. 4).

Live imaging of fluorescent proteins

Conidia were inoculated into minimal medium with appropriate supplements in LAB-TEK, 8-well chambered coverglasses (Nunc 155411) and grown for approximately 16 hours at 30°C before observations. TNO2A7 transformants for GFP-SumO observations were grown in minimal medium supplemented with pyridoxine and riboflavin. As riboflavin is fluorescent, it was washed out immediately before imaging. Several *gfp-sumO* transformants were imaged and gave the same GFP-SumO localization patterns. Time lapse images were made with one transformant, LO1655. Progeny of a cross between LO1761 and LO1655 were used for dual mCherry-γ-tubulin and GFP-SumO imaging. Growth and imaging was

the same as for the *gfp-sumO* transformants except that the growth medium was supplemented additionally with *p*-amino benzoic acid.

Image sets were collected with two Olympus IX 71 microscopes. Both were equipped with 1.42 N.A. planapochromatic objectives. One was equipped with a Hamamatsu ORCA ER ccd camera, a Prior shutter and excitation filter wheel and a Semrock GFP/DsRed-2X-A “Pinkel” filter set [459-481 nm bandpass excitation filter for GFP and a separate 546-566 nm bandpass excitation filter for mCherry, a dual reflection band dichroic (457-480 nm and 542-565 nm reflection bands, 500-529 and 584-679 nm transmission bands) and dual wavelength bandpass emission filter (500-529 nm for GFP and 584-679 nm for mCherry)]. The other microscope was equipped with a Hamamatsu ORCA ERAG camera, Prior shutter, Prior excitation and emission filter wheels, and a Semrock GFP/DsRed2X2M-B dual band “Sedat” filter set [459-481 nm bandpass excitation filter for GFP and a 546-566 nm bandpass excitation filter for mCherry), dual reflection band dichroic (457-480 nm and 542-565 nm reflection bands, 500-529 and 584-679 nm transmission bands) and two separate emission filters (499-529 nm for GFP and 580-654 nm for mCherry)]. Because of the greater selectivity between the two wavelengths, the dual filter wheel setup was used for most dual wavelength imaging. Both systems were driven with Slidebook software (MacIntosh version). Z-series stacks were deconvolved using the constrained iterative method with Slidebook software.

Results

A single SUMO peptide-encoding gene is present in *A. nidulans*

A single gene (AN1191.3), designated *sumO*, was identified in the *A. nidulans* genome sequence using the Blastp algorithm for sequences predicted to encode a SUMO-1-like peptide. The *sumO* gene was PCR-amplified from genomic DNA, cloned and sequenced (Materials and Methods). Comparison of the *sumO* genomic sequence and a full-length *sumO* EST (Genbank Accession No. AAB01675) revealed a single 98 bp intron (+223 to +320). The predicted *sumO* gene product of 94 amino acids shows considerable similarity to *S. cerevisiae* Smt3 (63%) and *S. pombe* Pmt3 (68%) and the di-glycine residues required for target attachment are conserved (Fig. 1A).

Considering the pleiotropic roles of the *SUMO* orthologs and the lethal phenotype of the *smt3* mutant in *S. cerevisiae*, we replaced the entire *sumO* open reading frame with the dominant selectable marker *Bar* (Straubinger *et al.*, 1992) in the diploid recipient MH6590 (Fig. 1B). Glufosinate resistant transformants were isolated and a transformant (MH10968) with the expected genomic digestion pattern for a heterozygous *sumO* diploid was chosen and haploidised. Glufosinate resistant *sumO* haploids were recovered, indicating that deletion of *sumO* is not lethal in *A. nidulans*. The resistance marker segregated in the haploidization progeny with the *facB101* mutation on linkage group VIII, consistent with the physical location of the *sumO* gene (AN1191.3).

Deletion of the *sumO* gene has pleiotropic effects

The *sumO* mutant has a severely reduced growth phenotype on complete medium. This phenotype is less extreme on ANM minimal or on YAG medium. After 5 days incubation on complete medium at 37°C, the wild-type strain produced large smooth-edged colonies, whereas the *sumO* mutant formed smaller colonies with ragged edges suggesting non-uniform growth arrest at the periphery of the colony (Fig. 2A). Measurement of colony growth showed that wild-type reproducibly maintained a linear growth rate whereas the growth of the *sumO* mutant gradually slowed and ultimately ceased approximately three days after inoculation (Fig. 2B). The timing of growth cessation in the *sumO* mutant showed considerable variation between replicates consistent with a stochastic basis for the growth arrest (Fig. 2B). Microscopic examination of the *sumO* mutant after 5 days growth on complete medium did not reveal an aberrant morphology or branching at the hyphal tip (data not shown). Re-introduction of the *sumO* gene by transformation fully restored wild-type growth (data not shown) confirming that the growth defect was specific to the loss of *sumO* function. In *S. pombe*, the *pmt3* mutant displays sensitivity to heat, DNA-damaging agents and DNA synthesis inhibitors (Tanaka *et al.*, 1999). The *sumO* mutant exhibited slightly increased sensitivity to the DNA damaging agent methylmethanesulphonate (MMS) and to the DNA synthesis inhibitor hydroxyurea (HU) compared with the wild-type (Fig. 2D). However, there was no evidence of increased heat sensitivity, as the relative growth of the *sumO* mutant was not markedly affected at 42°C compared with 37°C on either minimal medium (Fig. 2D) or complete medium (data not shown).

A. nidulans undergoes two developmental programs: asexual development, leading to the formation of asexual reproductive structures (conidiophores) and asexual spores (conidia), and sexual development, which generates meiotic products (ascospores) contained within fruiting bodies (cleistothecia) surrounded by Hülle cells. The production of conidia was substantially reduced in the *sumO* mutant compared with wild type (Fig. 2C), although the morphology of individual *sumO* conidiophores appeared normal (data not shown). As *A. nidulans* is homothallic, the production of viable ascospores does not normally require a mating partner. While *sumO* mutant homokaryons (MH10992: *biA1 sumO*) produced normal Hülle cells the cleistothecia were very small compared with those formed by wild-type homokaryons (*biA1*) (Fig. 2E-G) and failed to produce progeny. Microscopic examination revealed that the cleistothecia did not contain ascospores (data not shown). Therefore, the *sumO* mutant is self-sterile indicating a requirement for sumoylation of key proteins involved in development of viable meiotic progeny. Hybrid cleistothecia formed by a [wild type + *sumO*] heterokaryon contained viable progeny and the *sumO* mutation segregated as a single gene in the progeny indicating that the *sumO* mutation is recessive in the heterokaryon and ascospores carrying this mutation were able to germinate.

Over-expression of SumO is not detrimental

Overexpression of the SumO peptide was achieved by fusing *sumO*-coding sequences to the highly inducible *xyIP* promoter (Zadra *et al.*, 2000). As commercially available anti-SUMO antibodies raised against the *S. cerevisiae* and Smt3 *Arabidopsis* SUMO-1 proteins did not cross-react with *A. nidulans* SumO-conjugated proteins in Western blot analysis (data not shown), the SumO peptide was tagged with the FLAG epitope for detection (Materials and

Methods). Introduction of the FLAG tag between residues three and four of SumO did not affect its function as the modified *sumO*^{FLAG} gene fully complemented the growth phenotype of the *sumO*[•] mutant in co-transformation experiments using the *pyroA* gene on pI4 (Osmani *et al.*, 1999) as the selectable marker (data not shown).

The *xylP(p)::sumO*^{FLAG} construct (pCW6747) was integrated by a single homologous recombination event at the *sumO* locus (Fig. 3A). Western blot analysis using Anti-FLAG antibodies detected low levels of a variety of sumoylated proteins in protein extracts prepared from mycelia grown in glucose medium indicating that SumO^{FLAG} is expressed at low levels from the *xylP* promoter even in the absence of inducer (Fig. 3B). Unconjugated SumO^{FLAG} was detected at approximately 10.4 kDa. Extracts prepared from mycelia grown on xylose medium to induce SumO^{FLAG} expression contained increased amounts of sumoylated proteins and elevated levels of free, unconjugated SumO peptide (Fig 3B). Remarkably, while overexpression of SumO led to a dramatic increase in the abundance of sumoylated proteins, growth tests revealed no obvious phenotypic difference between overexpression and wild-type strains on glucose or xylose media containing ammonium, alanine, proline or nitrate as sole nitrogen sources, on xylose medium containing MMS or HU or at 42°C (data not shown). Therefore, SumO peptide overexpression did not detectably interfere with the normal function of cellular proteins involved in growth or metabolism.

In vivo localization of SumO

To examine the distribution of SumO over time in living cells, we created a Green Fluorescent Protein (GFP)-SumO fusion expressed under the control of the endogenous *sumO* promoter. As SUMO peptides are ligated to target proteins by their C-terminus, GFP was fused to the N-terminus of SumO. To construct a transforming DNA fragment that carried a *gfp-sumO* fusion gene under the control of the *sumO* promoter, we developed a fusion PCR procedure in which five fragments were fused in a single fusion PCR reaction (Fig. 4). The linear product was transformed into an *nkuA*[•] strain (TN02A7) to facilitate gene targeting (Nayak *et al.*, 2006). Transformants with the desired integration at the *sumO* locus were identified by diagnostic PCR and confirmed by Southern blot analysis. Growth rates of the GFP-SumO transformants were the same as the wild-type control strain R153 indicating that fusion of GFP to SumO did not affect SumO conjugation to target proteins or SumO function. Several GFP-SumO transformants examined by fluorescence microscopy gave identical localization patterns for GFP-SumO and thus for sumoylated proteins.

In interphase cells, sumoylated proteins were highly concentrated in the nucleus (Fig. 5). Within the nucleus, there were a number of spots with higher concentrations of sumoylated proteins and the concentration of sumoylated proteins was lower in the nucleolus than in the surrounding nucleoplasm (Fig. 5A). Through-focus series revealed that the spots were not confined to the nuclear periphery but were located throughout the nucleoplasm. In *S. pombe*, GFP-Pmt3 is predominately nuclear and forms intense spots, which are colocalised with the spindle pole body in interphase cells (Tanaka *et al.*, 1999). To investigate whether any of the GFP-SumO spots corresponded to the spindle pole body, we crossed the GFP-tagged *sumO* gene into a strain (LO1761) carrying an mCherry γ -tubulin fusion (constructed by Yi Xiong and Dr Edyta Szewczyk), a diagnostic spindle pole body marker. Examination of Z-series

stacks revealed that there was no preferential association of GFP-SumO with the spindle pole body (Fig. 5A).

To monitor the localization of sumoylated proteins through the cell cycle, we collected four-dimensional image sets (Z-series collected over time) (Fig. 5B-E). Sumoylated proteins were concentrated in the nucleus throughout interphase. As the cells entered mitosis, sumoylated proteins disappeared rapidly from the nucleoplasm, apparently exiting the nucleus. The nuclear envelope is known to become permeable at the onset of mitosis (De Souza *et al.*, 2004). Sumoylated proteins re-accumulated in the nucleoplasm in telophase.

Discussion

An incredibly diverse range of cellular proteins serve as substrates for the covalent attachment of SUMO peptides in vertebrates and in yeast (e.g. Hannich *et al.*, 2005; Wohlschlegel *et al.*, 2004). It is not clear how sumoylation affects the various cellular processes in which it is involved and the consequences of SUMO attachment vary between substrates. The covalent linkage of SUMO peptides can directly affect the activity of target proteins by altering their protein or DNA interactions, subcellular location or by acting as an antagonist to ubiquitination (Johnson, 2004). In addition, SUMO peptides can participate in non-covalent interactions and SUMO modification of certain target proteins may facilitate their interaction with other proteins bearing a SUMO-binding motif (SBM). The SBM of human RanBP2/Nup358 important for interaction with sumoylated RanGAP1 has been identified and a similar SBM sequence has been defined in *S. cerevisiae* by two-hybrid analysis (Song *et al.*, 2004; Hannich *et al.*, 2005). However, as *S. cerevisiae* and *S. pombe* mutants lacking the SUMO conjugating enzyme (encoded by the *UBC9* and *hus5* genes, respectively) show similar defects to mutants lacking SUMO peptides (Johnson and Blobel, 1997; Al-Khodairy *et al.*, 1995), it is clear that the most critical functions of SUMO require covalent attachment.

In *A. nidulans*, as in *S. cerevisiae* and *S. pombe*, SUMO peptides are encoded by a single gene. The phenotypes of the *S. cerevisiae* *SMT3* and *S. pombe* *pmt3* deletion mutants are not equivalent as the *S. pombe* mutant is viable whereas the *S. cerevisiae* mutant is lethal (Giaever *et al.*, 2002; Johnson *et al.*, 1997; Tanaka *et al.*, 1999). As the situation in the filamentous fungi was unknown, we created the *A. nidulans* *sumO*[•] mutant in a diploid context and then uncovered the mutation by haploidisation. This mutant was found to be viable although it exhibited pleiotropic defects including a reduced capacity for continued hyphal growth, an increased sensitivity to DNA damaging agents and affects on both asexual and sexual reproduction. Our data demonstrate that SumO is not essential for mitosis in *A. nidulans* unlike *S. cerevisiae* where sumoylation is required for mitotic cell cycle progression and chromosome segregation (Biggins *et al.*, 2001; Dieckhoff *et al.*, 2004; Meluh and Koshland, 1995; Motegi *et al.*, 2006). However, the stochastic nature of hyphal growth cessation, the reduced levels of conidiation and increased sensitivity to DNA damaging agents suggest *sumO* may have an ancillary role in mitosis. SUMO plays a role in maintaining proper chromosome segregation, telomere length and the DNA damage response in *S. pombe* (Ho and Watts, 2003). We established that SumO is required for sexual development as reduced fruiting body size and self-sterility were observed in *sumO*[•].

homokaryons. In *S. cerevisiae*, Smt3 is associated with the synaptonemal complex (Cheng *et al.*, 2006; Hooker and Roeder, 2006) and sumoylation of the transcription factor Ste12 promotes mating (Wang and Dohlman, 2006).

Using GFP-SumO we were able to follow changes in the sub-cellular location of SUMO peptides through the cell cycle. These studies revealed that sumoylated proteins and/or SUMO peptides were localized throughout the nucleus concentrated as punctate spots in chromatin-free sub-nuclear regions. These spots are not associated with spindle pole bodies whereas in *S. pombe* Pmt3 fused to GFP is localized in intense spots in the nucleus corresponding to spindle pole bodies (Tanaka *et al.*, 1999). Human SUMO-1/2/3 peptides are localized on the nuclear membrane, nuclear bodies and cytoplasm, respectively (Su and Li, 2002). Many of the cellular processes influenced by sumoylation, including transcription factor modification, DNA repair and chromosomal maintenance and stability, occur within the nucleus. Imaging in live cells using GFP-tagged SumO revealed a fascinating link between SumO subcellular distribution and nuclear division. In interphase cells, GFP-SumO accumulated within the nucleus but GFP-SumO fluorescence was undetectable from entry to mitosis until telophase. It is not known whether this cyclic concentration and loss of the GFP signal is due to exit of sumoylated nuclear proteins as the nuclear envelope becomes permeable or changes in the sumoylation state of target proteins during the cell cycle. The co-localisation of SUMO-conjugating and SUMO-deconjugating enzymes with the nuclear pore suggests that sumoylation of nuclear proteins may be a dynamic and rapid process associated with nucleocytoplasmic transport (Hang and Dasso, 2002; Smith *et al.*, 2004; Zhang *et al.*, 2002).

Many processes in which sumoylation has been implicated are central to cell growth and genome integrity and are likely to be conserved across the eukaryota. We have demonstrated using denaturing SDS-PAGE that epitope-tagged SumO peptides can be covalently attached to a large number of *A. nidulans* proteins. The unique features of the filamentous fungi and the amenability of genetic analysis in certain of these species offers significant opportunities to further explore these processes. Furthermore, it is remarkable, given the range of processes in which sumoylation has been implicated, that SumO overexpression produced no obvious growth or developmental phenotype. This may provide an additional tool for further study of this important post-translational modification providing valuable insights into the influence of sumoylation on key cellular processes in filamentous fungi.

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Fig. 1. Deletion of *sumO* in *A. nidulans*

(A) Alignment of the *A. nidulans* SumO predicted peptide sequence (An_SumO) with the SUMO homologues from *Saccharomyces cerevisiae* (Sc_Smt3: Accession no. AAB01675), *Schizosaccharomyces pombe* (Sp_Pmt3: Accession no. BAA32595), *Homo sapiens* (Hs_SUMO1: Accession no. AAH06462), *Xenopus laevis* (Xl_SUMO: Accession no. CAB09807) and *Drosophila melanogaster* (Dm_Smt3: Accession no. AAD19219). Identical residues present in at least half of the sequences are indicated by the black boxes, whereas gray shading represents similar residues. Sequences were aligned using Clustal W (Thompson *et al.*, 1994) in Biomanager (<http://biomanager.angis.org.au>) with manual manipulation where necessary and shaded by MacBoxshade v2.15E. The conserved di-glycine motif required for SUMO conjugation to target proteins is boxed. (B) Gene replacement strategy for *sumO*. The predicted *sumO* open reading frame (AN1191.3) was gene replaced with a dominant selectable marker, *Bar*, in one homologue in a diploid strain (MH6590).

Fig. 2. The *sumO* mutant shows growth, asexual development and sexual development phenotypes

(A) The growth of the wildtype strain (MH1) and the *sumO* mutant (MH10992) on complete media after 5 days incubation at 37°C. (B) The radius of the colony at various time intervals after inoculation on complete medium and incubation at 37°C was determined as a measure of hyphal extension. Four replicates are shown. (C) Asexual spore (conidia) numbers were determined for wildtype (MH1) and *sumO* (MH10992) strains after 48 hours growth on complete media or minimal media with 10 mM ammonium tartrate as nitrogen source at 37°C. Average number of spores x10⁶ per cm² is shown with standard error in parentheses. (D) Growth of wildtype (MH1) and *sumO* (MH10992) strains on complete media, minimal media with 10 mM ammonium tartrate and minimal media containing 0.0025% methyl methanesulfonate (MMS) or 5 mM hydroxyurea (HU) at 37°C and minimal media at 37°C or 42°C for 2 days. (E) and (F) Sexual development of wildtype and *sumO* homokaryons. MH1 and MH10992 strains were grown on solid minimal media with 1% glucose and 10 mM sodium nitrate at 37°C for 2 days, the plates were sealed to induce sexual development and further incubated at 37°C for 7 days. Cleistothecia (S) and conidiophores (A) are indicated by arrows and the scale bar represents 100 μm. (G) Sizes of the cleistothecia from wildtype and *sumO* were compared. The scale bar represents 50 μm.

Fig. 3. Over-expression of *sumO*^{FLAG} in *A. nidulans*

(A) The *xyIP(p)sumO^{FLAG}* construct was targeted to the genomic *sumO* locus by single crossover. The solid black box within the *sumO* coding region indicates the relative position of the introduced FLAG epitope. (B) Western blotting of 50 μ g of crude protein extracts from the wildtype and *xyIP(p)sumO* strains grown in minimal media containing either 1% glucose (G) or 1% xylose (X) with 10 mM ammonium tartrate at 37°C for 16 hours. Sumoylated proteins were detected using α -FLAG antibodies. Molecular weight markers are shown. * indicates unconjugated SumO^{FLAG} peptide.

Fig. 4. Creation of GFP-*sumO* by five-piece fusion PCR

(A) Amplification of three fragments from genomic DNA, the 5' untranslated region (5' UTR) (shown in black), the *sumO* promoter (yellow), and the *sumO* coding sequence (red) and 3' untranslated region (3' UTR) (black). Primers P5 and P6 carry extensions that hybridize to the GFP coding sequence and primers P3 and P4 carry extensions that hybridize to a fragment carrying the *Aspergillus fumigatus pyrG* gene (*AfpyrG*) (Weidner *et al.*, 1998). (B) After primer and nucleotide removal, these fragments were mixed with *AfpyrG* and GFP fragments and amplified with primers P2 and P7, which are nested primers. The five pieces hybridize during fusion PCR and amplification results in a single fragment as shown. Primers were designed such that GFP is fused in frame with the *sumO* coding sequence and the fusion is under control of the endogenous *sumO* promoter. Upon transformation this fragment integrates at the *sumO* locus by homologous recombination, replacing the endogenous *sumO* gene with the construct shown.

Fig. 5. SumO distribution in living cells

(A) A maximum intensity projection of a Z-series stack of GFP-SumO and mCherry-γ-tubulin. There are multiple intense spots of SumO in each nucleus. The mCherry-γ-tubulin localizes to spindle pole bodies (arrows) and there is no increased concentration of SumO at the spindle pole bodies. The insert shows the second nucleus from the right prior to deconvolution for comparison. (B-E) GFP-SumO in mitosis. Each panel is a maximum intensity projection of a deconvolved Z-series stack from a time-lapse image set. The time (in minutes) from the beginning of image collection is at the lower left of each panel. The images were collected with minimal exposure to minimize bleaching, and at wider Z-series intervals than panel A so the GFP-SumO spots are less clear. SumO is present in G₂ nuclei (B), is nearly undetectable in mitotic nuclei (C), reaccumulates in daughter nuclei as they exit mitosis (D) and is present in G₁ nuclei (E). B-E are the same magnification (scale in E).

Table 1

Oligonucleotides used in this study

sumo-F: 5'-TCGTGCCGTTGCTGTTAC-3'
sumo-R: 5'-CACCCCAGACTGACGCCATAC-3'
sumo-F3: 5'-TCGATAACTCCCAAATAGTTTC
Flag-sumo-InvF: <u>GATGACGATAAA</u> CCATCTGCTCCCACTCCTGAGGC
Flag-sumo-InvR: <u>TACGATATCTTTGTA</u> ATCAGACATTGTTGAAACTATTTGG
P1: GAGCGCTGAGTCGGCGAGGTA
P2: TACTTACATTCAATGACGCG
P3: CGAAGAGGGTGAAGAGCATTGAGTATCCAAGCGAATTACATCATG
pyrGF2: CAATGCTCTTCACCCTCTTCG
PyrGR: CTGTCTGAGAGGAGGCACTGATGC
P4: CATCAGTGCCTCCTCTCAGACAGCAGGAGCAAATTGTACTTG
P5: AGTGAAAAGTTCTTCTCCTTTACTCATTGTTGAAACTATTTGGGAG
LIZP186: ATGAGTAAAGGAGAAGAAGCTTTTCACT
LIZP184: GGCACCGGCTCCAGCGCCTGCACCAGCTCCTTTGTATAGTTCATCCATGCC
P6: GGAGCTGGTGCAGGCGCTGGAGCCGGTGCCATGTCTGATCCATCTGCTCCAC
P7: AGGAACAGCGAGCTTACGA
P8: ATTCGAGCGCCCAAGAGGACGG