

Cell-cycle-dependent localisation of Ulp1, a *Schizosaccharomyces pombe* Pmt3 (SUMO)-specific protease

Deborah L. Taylor, Jenny C. Y. Ho, Alejandro Oliver and Felicity Z. Watts*

Genome Damage and Stability Centre, School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK

*Author for correspondence (e-mail: f.z.watts@sussex.ac.uk)

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Summary

We report here on the characterisation of Ulp1, a component of the SUMO modification process in *S. pombe*. Recombinant *S. pombe* Ulp1 has de-sumoylating activity; it is involved in the processing of Pmt3 (*S. pombe* SUMO) and can, to a limited extent, remove Pmt3 from modified targets in *S. pombe* cell extracts. *ulp1* is not essential for cell viability, but cells lacking the gene display severe cell and nuclear abnormalities. *ulp1*-null (*ulp1.d*) cells are sensitive to ultraviolet radiation in a manner similar to *rad31.d* and *hus5.62*, which have mutations in one subunit of the activator and the conjugator for the ubiquitin-like protein SUMO respectively. However *ulp1.d* cells are less sensitive to ionising radiation and hydroxyurea (HU) than are *rad31.d* and *hus5.62*. *ulp1*-null cells are defective in

processing precursor Pmt3 and display reduced levels of Pmt3 conjugates compared with wild-type cells. The slow growth phenotype of *ulp1* null cells is not substantially rescued by over-expression of the mature form of Pmt3 (Pmt3-GG), suggesting that the de-conjugating activity of Ulp1 is required for normal cell cycle progression. During the S and G2 phases of the cell cycle the Ulp1 protein is localised to the nuclear periphery. However, during mitosis the pattern of staining alters, and during anaphase, Ulp1 is observed within the nucleus. Ulp1 localisation at the nuclear periphery is generally re-established by the time of septation (S phase).

Key words: SUMO, Pmt3, Ulp1, Cell cycle

Introduction

In *S. pombe*, processes required for the maintenance of genetic integrity have been defined through the analysis of radiation-sensitive mutants and the subsequent identification of genes involved in a range of different DNA damage responses, for example, nucleotide excision repair, recombination repair and DNA integrity checkpoints (Carr et al., 1994; Caspari and Carr, 1999; Murray et al., 1992; Tavassoli et al., 1995). During our characterisation of these processes we identified the *rad31.1* mutant (Shayeghi et al., 1997), which we demonstrated to be epistatic with *hus5.62* (al-Khodairy et al., 1995). These two mutants exhibit a moderate sensitivity to DNA damaging agents such as UV and ionising radiation and to the DNA synthesis inhibitor, hydroxyurea (HU) (al-Khodairy et al., 1995; Shayeghi et al., 1997). The mutants also display similar defects in cell and nuclear morphology, displaying a frequent 'cut' phenotype in the absence of DNA damage and the occurrence of chromosome fragmentation. Cells display a slow growth phenotype and increased mini-chromosome loss compared with wild-type cells. Null alleles of *rad31* and *hus5* have similar, but somewhat more severe, phenotypes.

The *S. pombe rad31* and *hus5* genes, which were initially proposed to be involved in SUMO modification as a result of their high level of sequence identity to the *S. cerevisiae AOS1* and *UBC9* genes (al-Khodairy et al., 1995; Johnson et al., 1997; Shayeghi et al., 1997; Tanaka et al., 1999), have recently been shown to be required for SUMO modification by their activity in an in vitro SUMO (Pmt3) modification system (Ho

et al., 2001). Specifically, the *rad31* and *hus5* genes encode one subunit of the heterodimeric activator and the conjugator, respectively, for the *S. pombe* SUMO protein, Pmt3. Pmt3 is a member of a Ubl (ubiquitin-like) family, which has been identified independently in many organisms, and its members are known variously as SUMO, PIC1, sentrin and UBL1 in mammalian cells, as Smt3 in *S. cerevisiae* and as Pmt3 in *S. pombe* (Yeh et al., 2000).

The Ubls are expressed as precursor molecules that, like ubiquitin, require C-terminal processing to reveal the double glycine (GG) motif needed for conjugation to the target lysine residue (Johnson et al., 1997). The conjugation of SUMO and other Ubls such as Rub1/NEDD8 to substrate proteins is similar to that of ubiquitination and requires activation and conjugation of the Ubl by E1 and E2 enzymes (Desterro et al., 1997; Johnson and Blobel, 1997). Conjugation is initiated by an activation enzyme (E1), which, in an ATP-dependent reaction, forms a thiolester linkage with the Ubl. The Ubl is then transferred to a conjugating enzyme (E2), forming a similar thiolester linkage, before transfer to a target lysine residue of the substrate protein. Unlike the E1 required for ubiquitin activation, which is a monomer, the E1 (or SAE) required for SUMO activation is a heterodimer in which the two subunits are related to the N- and C-terminal domains of the ubiquitin E1 enzymes (Johnson et al., 1997). In *S. pombe*, the Rad31 protein acts in conjunction with Fub2 (Tanaka et al., 1999) to form the E1 heterodimer (Tanaka et al., 1999; Ho et al., 2001).

The mechanism of deconjugation of Ubls from targets is similar to deconjugation of ubiquitin from its targets, which occurs through the action of the Dub (de-ubiquitinating) enzymes. It appears that cells contain multiple SUMO proteases, suggesting that there may be some specificity in their location and/or action. Recently identified cysteine proteases with specificity for SUMO/Smt3 hydrolysis in vitro include mammalian SMT3IP1, SUSP1 and SENP1 (Gong et al., 2000; Kim et al., 2000; Nishida et al., 2000) and *S. cerevisiae* ScUlp1 and ScUlp2 (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000).

Several targets of SUMO modification have been identified in mammalian systems including RanGAP1, PML, p53 and IκBα (e.g. Boddy et al., 1996; Desterro et al., 1998; Gostissa et al., 1999; Mahajan et al., 1997; Matunis et al., 1998; Rodriguez et al., 1999; Sternsdorf et al., 1997), and although a definitive role for modification has yet to be recognised, most modified targets are localised in the nucleus or at the nuclear envelope. Exceptions to this are the *S. cerevisiae* septins Cdc3, Cdc11 and Shs1, which are modified by the *S. cerevisiae* SUMO homologue Smt3 and are located at the mother/bud neck in a cell-cycle-dependent manner (Johnson and Blobel, 1999).

To further our understanding of Pmt3 modification in fission yeast and to facilitate the identification of modified targets, we have initiated a characterisation of the Pmt3-specific proteases in *S. pombe*. We report here on *S. pombe* Ulp1 and show that it is a 65 kDa protein that is capable of processing the Pmt3 precursor to produce a protein corresponding in size to that expected of the mature form and is able to function to a limited extent in the removal of Pmt3 from target proteins in cell extracts. The *ulp1* null allele is viable and its phenotype is described here. The Ulp1 protein is localised at the nuclear envelope throughout S phase and G2 but is observed within the nucleus during mitosis.

Materials and Methods

Strains, plasmids and growth media

The *S. pombe* strains used are listed in Table 1. *E. coli* strains used were NM522 and BL21. pRSETB (Clontech) and pET15b (Novagen) were used to produce in-frame 6×His-tag fusion proteins in *Escherichia coli*. pREP41 (Basi et al., 1993) and pREP41HA (Craven et al., 1998), both containing a modified version of the thiamine repressible *nmt1* promoter, were used for expression in *S. pombe*. pREP41HA allows the in-frame cloning of the Pmt3 or Pmt3-GG ORFs using *NdeI* and *BamHI* cloning sites to produce a haemagglutinin-tagged protein. Procedures and growth media used for the routine culture and maintenance of *E. coli* and *S. pombe* strains were as described previously (Murray et al., 1992).

Table 1. *S. pombe* strains used in this study

Strain	Genotype
sp.011	<i>ade6.704, leu1.32, ura4.D18, h⁻</i>
sp.117	<i>ade6.704/ade6.704, leu1.32/leu1.32, ura4.D18/ura4.D18, h⁺/h⁺</i>
sp.301	<i>rad17::ura4, ade6.704, leu1.32, ura4.D18, h⁻</i>
sp.611	<i>ulp1-13MYC, ade6.704, leu1.32, ura4.D18, G418^R, h⁻</i>
sp.651	<i>ulp1::ura4, ade6.704, leu1.32, ura4.D18, h⁻</i>
sp.666	<i>ulp1::ura4, rad17::ura4, ade6.704, leu1.32, ura4.D18, h⁺</i>

Construction of myc epitope tagged *ulp1* strain

A C-terminal myc-epitope-tagged *ulp1* strain was generated using the G418 selectable marker module and methodology described in Bahler et al. (Bahler et al., 1998). Oligonucleotides Ulp1-MH 5' TGTTCTGTTTCAGTTTAGTCAAAAATGATATGCCTGAACTACGAATCAAAATGGCAGCTAGTATTATTGATGCACAAAATCTATCCGGATCCCGGGGTTAATTA 3' and Ulp1-rev 5' ATGCGGGTATTTTTTTTTCAAAAGCTATATTTGCTGAGATTCCTGGATTCAGATTATTAATTAGAGTATTCCCTGCTAGAATTCGAGCTCGTTTAAA 3' were generated with 80 bases of homology to the target sequence followed by 20 bases of 3' homology to the plasmid sequence. Integration was confirmed by PCR using the primers KanMXR 5' CGGATGTGATGTGAGAAGTGTATCCTAGC 3' and Ulp1-6 5' TATACTTGTAAGCTTCTCACG 3'. The tagged strain did not display any obvious growth or morphological abnormalities and thus resembles wild-type strains.

Cloning and expression of full-length and C-terminally truncated *pmt3*

The *S. pombe pmt3* sequence was amplified by PCR using the following primers SEN4: 5' CTGAGAACATATGTCTGAATC 3' and SUMO2: 5' TTGAGAGGATCCACGTATGG 3' to produce a full-length clone. The C-terminally truncated Pmt3 ORF (Pmt3-GG) was amplified using primers SEN4 and pGG: 5' GGATCCTAAC-CACCTAACTGTTCT 3'. Both ORFs were subsequently cloned as *NdeI*-*BamHI* fragments in the *E. coli* expression vector pET15b (Novagen). Full-length and truncated versions of Pmt3 (Pmt3-GG) were expressed with N-terminal 6×His tags and affinity purified using Ni²⁺ agarose according to the manufacturers instructions (Novagen).

Cloning and expression of Ulp1

The oligonucleotide primers Ulp1X 5' CTCGAGAATGATAGGA-AAACGCAATGC 3' and Ulp1S 5' CCCGGGTTGCTGTTGTGCG-CATCAGC 3' were used for PCR to amplify the *S. pombe ulp1* coding sequence. A 1.9 kb fragment was generated and cloned into pGEM5Zf⁺ for sequencing. The ORF was then subcloned as an *XhoI*-*SmaI* fragment into pRSETB. 6×His-tagged Ulp1 protein was expressed from the T7 promoter of pRSETB in *E. coli* BL21 cells. Cell lysis was performed by sonication, and Ulp1 was purified on Talon metal affinity resin according to the manufacturer's instructions (Clontech). Protein-containing fractions were pooled and dialysed overnight into 50 mM sodium phosphate pH 7.5, 150 mM NaCl, 1 mM DTT before being snap frozen and stored at -80°C.

Creation of the *ulp1* null allele

The *ulp1* null allele was created by replacing a 1.1 kb *BamHI*-*SalI* fragment within the *ulp1* coding sequence with the *S. pombe ura4* gene. A 2.5 kb *XhoI*-*SmaI* fragment was excised and used to transform a diploid strain. Replacement of the wild-type allele was confirmed by Southern blot analysis.

Ulp1 protease assays

S. pombe native cell extracts were prepared in cleavage buffer (10 mM sodium phosphate pH 7.0, 150 mM NaCl, 1 mM DTT (Sigma)) from cells grown to a density of 10⁶ cells/ml in rich medium. Precursor and truncated Pmt3 (Pmt3-GG, used as a control) were synthesised either in a coupled in vitro transcription and translation system using the TnT system according to the manufacturer's instructions (Promega) with [³⁵S]methionine or purified as 6×His tag fusion proteins from *E. coli* BL21 transformed with either pET15b-Pmt3 or pET15b-Pmt3-GG using Ni²⁺ agarose. Processing and deconjugation assays were carried out in cleavage buffer in a total volume of 20 µl at 30°C for 30 minutes unless otherwise stated. Samples were analysed by SDS-

PAGE. ^{35}S -labelled proteins were detected using a phosphorimager. Recombinant Pmt3 produced in *E. coli* and Pmt3 species in *S. pombe* cell extracts were detected by immunoblotting using polyclonal anti-Pmt3 antisera.

Generation of antibodies and immunological methods

A fragment encoding an N-terminally truncated version of Pmt3 (N Δ Pmt3) was amplified from a cDNA library (Fikes et al., 1990) using primers pmt3-1 5' CTGAGAACATATGTCTGAATCACC 3' and pmt3-4 5' TGGAGAGGATCCACGTATTGG 3' and cloned into the *E. coli* expression vector pET15b (Novagen) and expressed as a 6 \times His fusion in *E. coli* BL21 cells. Pmt3 protein was purified using Ni $^{2+}$ agarose affinity chromatography and used for polyclonal antisera production in rabbits (as described in (Ho et al., 2001)). Anti-myc antibodies were purified from cell supernatant (cell line CRL1729, from ATCC) using protein G-sepharose. Anti-HA antisera were from Babco, anti-tubulin antisera were from Sigma and anti-His antisera were from Pharmacia.

Western blotting was carried out as described (Harlow and Lane, 1988) on whole cell yeast lysates prepared by lysis in 150 mM NaCl, 10 mM NaPO $_4$ pH 7.0, 1 mM DTT or on TCA extracts of whole cells prepared as described in Caspari et al. (Caspari et al., 2000). Immunofluorescence was carried out as described in Moreno et al. (Moreno et al., 1991) using formaldehyde fixation.

Analysis of DNA damage responses

UV irradiation was carried out on freshly plated cells using a Stratagene 'Stratalinker'. Gamma irradiation was carried out using a [^{137}Cs] gamma source with a dose rate of 12 Gy/min. Sensitivity to hydroxyurea (HU) was tested in liquid cultures at HU concentrations of 20 mM.

Results

Cloning the Ulp1 ORF from genomic DNA and expression of the recombinant protein

The predicted *S. pombe* *ulp1* gene (accession number AL021839, protein ID CAA17063.1) encodes a protein of 64.9 kDa with significant homology to other recently identified Ubl proteases (Li and Hochstrasser, 1999). The full-length Ulp1

open reading frame was amplified by PCR from *S. pombe* genomic DNA using primers Ulp1X and Ulp1S as described in the Materials and Methods. The generated fragment was subcloned into the *E. coli* expression vector pRSETB and expressed as an N-terminal 6 \times His-tagged fusion protein in *E. coli* BL21 cells. Recombinant Ulp1, purified by immobilisation on Talon affinity resin, was observed to migrate close to the predicted size of 64.9 kDa (Fig. 1A, lane 2).

Ulp1 processes the C terminus of Pmt3 precursor

Like ubiquitin and other Ubls, SUMO is produced as a precursor molecule, which requires C-terminal processing involving the removal of a few amino acids at the C terminus to reveal the double glycine (GG) motif required for conjugation to an internal lysine residue of the substrate. In the case of Pmt3, the equivalent processing reaction would be expected to remove six amino acids (CTHLCL) to produce the mature form. To determine whether Ulp1 functions in the processing of Pmt3 in *S. pombe*, we analysed the ability of Ulp1 to process the Pmt3 precursor protein to its mature form. We expressed the full-length [^{35}S]methionine-labelled precursor form of Pmt3 in vitro and incubated it with recombinant Ulp1 produced in *E. coli* or with an equal volume of an equivalent fraction of an extract of *E. coli* BL21 cells transformed with empty vector. Products were analysed by SDS PAGE and compared with a truncated version of Pmt3 (Pmt3-GG) synthesised in vitro, which lacks the last six amino acids. The results of the cleavage assay verify that Ulp1 is proficient in the hydrolysis of Pmt3 in vitro (Fig. 1B, lanes 1-3). The processed product produced by Ulp1 (lane 3) migrates with a mobility identical to that of the synthetic, truncated Pmt3-GG (lane 2). No processing is observed in the negative control (lane 10). To ensure that the processing observed in lane 3 was not due to an activity in the reticulocyte lysate used to prepare the Pmt3 substrate, we assayed Ulp1 on full-length Pmt3 purified from *E. coli* (lane 13). Comparison with the full-length Pmt3 and Pmt3-GG controls (lanes 11 and 12 respectively) indicates that Pmt3 purified from *E. coli* is also processed. The processed

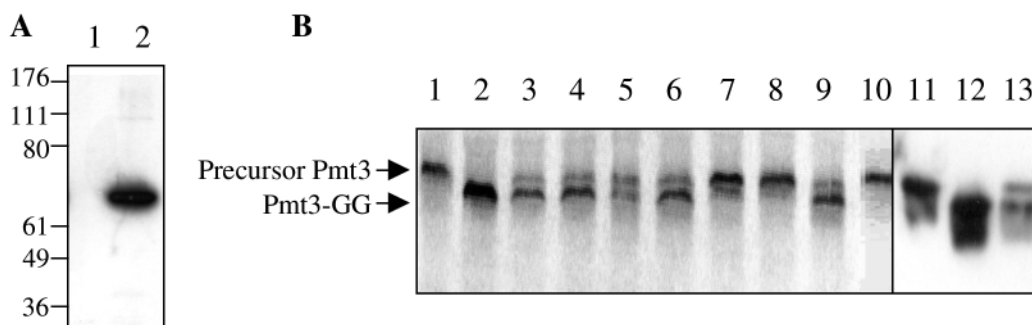


Fig. 1. Ulp1 processes the Pmt3 precursor. (A) Recombinant 6 \times His-tagged Ulp1 expressed in *E. coli*. Extracts were made from cells transformed with empty pRSETB (lane 1) or pRSETB-Ulp1 (lane 2), separated by SDS PAGE (10% polyacrylamide) and western blotted using anti-His antisera. (B) Recombinant Ulp1 was tested for Pmt3-processing activity as described in Materials and Methods. Lanes 1-10, ^{35}S -labelled Pmt3 or Pmt3-GG produced in the TnT system. Lane 1, full-length Pmt3 with no additions; lane 2, Pmt3-GG with no additions. Lanes 3-9 full-length Pmt3 incubated with 0.72 μg of recombinant Ulp1. Lane 3, no inhibitors; lane 4, 1:1000 mammalian protease inhibitor cocktail; lane 5, 1:1000 yeast protease inhibitor cocktail; lane 6, 5 mM benzamide; lane 7, 10 mM iodoacetamide; lane 8, 10 mM NEM; lane 9, 1 mM PMSF; lane 10, 2 μl elution fraction from *E. coli* BL21 + pRSETB. Lanes 11-13, full-length Pmt3 (600 ng) or Pmt3-GG (600 ng) purified from *E. coli* BL21 + pET15b-Pmt3 or *E. coli* BL21 + pET15b-Pmt3-GG respectively. Lane 11, full-length Pmt3, no additions; lane 12, Pmt3-GG no additions; lane 13 full-length Pmt3 incubated with 0.72 μg Ulp1. All samples were incubated at 30°C for 30 minutes.

product is identical in size to that observed following processing of Pmt3 produced in reticulocyte lysate (lane 3). These data implicate Ulp1 as a Pmt3 protease in fission yeast.

Sequence analysis indicates that *S. pombe* Ulp1 is a member of a family of cysteine proteases. Other members of this family, for example, *S. cerevisiae* Ulp1 (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000), have been shown to be inhibited by N-ethyl maleimide (NEM) and iodoacetamide. We wished to determine whether the Pmt3-processing activity of Ulp1 was also affected by these inhibitors or by any other protease inhibitors used in the preparation of *S. pombe* cell extracts. Of the inhibitors we tested, only iodoacetamide (10 mM) and NEM (10 mM) inhibited Ulp1 activity (Fig. 1B, lanes 7 and 8 respectively). The mammalian and yeast protease inhibitor cocktails, benzamidine and PMSF, had no effect on processing (Fig. 1B, lanes 4-6 and 9). Similar results were obtained using Pmt3 purified following over-expression in *E. coli* (data not shown).

Ulp1 can release Pmt3 from conjugates in *S. pombe* extracts

We next asked if Ulp1 is also involved in deconjugation of Pmt3 from modified substrates. In freshly prepared *S. pombe* cell extracts of cells grown in rich medium to around 10^6 cells/ml (early-mid exponential phase), the majority of Pmt3 cross-reacting material is of M_r greater than 100 kDa, and unconjugated Pmt3 monomer is not detected (Fig. 2A, lane 1). We therefore used such extracts to assay Ulp1 protein for its ability to cleave Pmt3 conjugates as this would allow us to observe the release of Pmt3 from high molecular weight species. Incubation of cell extracts with Ulp1 results in the appearance of free, unconjugated Pmt3 (Fig. 2A, lanes 3-6), and this is dependent on the presence of added Ulp1 as incubation of the extracts in the absence of Ulp1 does not release the Pmt3 monomer (Fig. 2A, lane 2). Deconjugation of Pmt3 from high M_r species was not observed following incubation of extracts with 2 μ l of the Talon column fraction equivalent to that containing the Ulp1 protein but prepared from *E. coli* cells transformed with empty vector (data not shown). These data indicate that Ulp1 is capable of releasing Pmt3 from high M_r conjugates. Interestingly, some high molecular weight species that cross-react with anti-Pmt3 antisera remain even when the period of incubation is increased

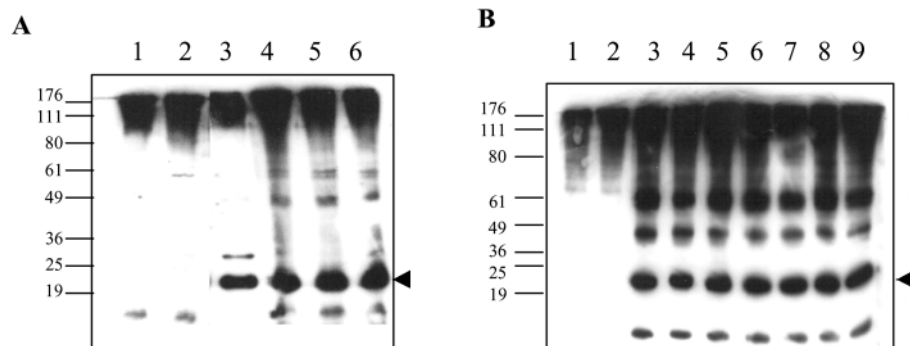
to 40 minutes (Fig. 2A lanes 4-6). The nature of these high M_r species is unknown, but may represent Pmt3-modified targets that are not affected by Ulp1 protease activity. If this is the case, Ulp1 may not be the only protease involved in the Pmt3 deconjugation pathway in fission yeast.

The two cysteine protease inhibitors, iodoacetamide and NEM, which inhibit the Pmt3-processing activity of Ulp1, and the serine protease inhibitor PMSF, have no effect on Pmt3 deconjugating activity (Fig. 2B, lanes 4-6) even using concentrations of NEM as high as 100 mM or a combination of 10 mM or 20 mM each of NEM and iodoacetamide (data not shown). To ensure that the lack of inhibition was not due to quenching of the inhibitors in the yeast cell extract, Ulp1 protein was pre-incubated in the presence of 100 mM iodoacetamide, 100 mM NEM or 5 mM PMSF before being added to the cell extract. Pre-incubation of Ulp1 had no effect on the deconjugating activity of Ulp1 (Fig. 2B, lanes 7-9).

Ulp1-null cells display aberrant cell and nuclear morphologies

We next disrupted the genomic copy of *ulp1* to determine whether the *ulp1* gene is essential for cell viability (Fig. 3A). Tetrad analysis of the heterozygous *ulp1*⁺/*ulp1*⁻ diploid initially suggested that *ulp1* was essential as no *ura*⁺ colonies were obtained from 20 dissected tetrads. In parallel we also undertook random spore analysis in case germination of *ulp1*⁻ spores was a rare event. Ten days after plating spores from the *ulp1*⁺/*ulp1*⁻ heterozygote, very small colonies were obtained on YES medium that were subsequently shown to be *ura*⁺, whereas the more abundant, larger colonies, which appeared three days after plating on YES, were all found to be *ura*⁻ when tested on selective medium. Microscopic analysis of the *ura*⁺ colonies indicates a range of cell and nuclear abnormalities (Fig. 3B). *ulp1.d* (*ulp1*-null) cells are generally elongated compared with wild-type cells, and, in some cases the nucleus is displaced from the expected position within the cell. Many *ulp1*-null cells have irregular shapes, whereas others are multiply septated. The doubling time of *ulp1.d* cells is around 5 hours compared with 2.5 hours for wild-type cells. The *ulp1.d* phenotype is thus similar to the phenotypes of *rad31.d* and *hus5.62*, which are defective in one subunit of the Pmt3 activator and the Pmt3 conjugator, respectively (al-Khodairy et al., 1995; Shayeghi et al., 1997).

Fig. 2. Ulp1 can remove Pmt3 from conjugates in *S. pombe* cell extracts. Recombinant Ulp1 was tested for deconjugating activity using wild-type (sp.011) *S. pombe* cell extract corresponding to 0.3 OD₆₀₀ units of cells per assay. Assays were analysed by SDS PAGE (12.5% polyacrylamide in (A)) and 10% polyacrylamide in (B)) and western blotted with anti-Pmt3 antisera. (A) Lanes 1 and 2, no additions; lanes 3-6, 3.7 μ g Ulp1. Lane 1, 0 minutes; lanes 2,6, 40 minutes at 30°C; lane 3, 5 minutes at 30°C; lane 4, 10 minutes at 30°C, lane 5, 20 minutes at 30°C. (B) Lanes 1,2, no additions; lanes 3-6, 3.7 μ g Ulp1; lane 4, 10 mM iodoacetamide; lane 5, 10 mM NEM; lane 6, 0.5 mM PMSF. Lanes 7-9, 3.7 μ g Ulp1 preincubated for 10 minutes on ice in the presence of 100 mM iodoacetamide (lane 7), 100 mM NEM (lane 8) or 5 mM PMSF (lane 9) before addition of cell extract. Lanes 2-9, incubated at 30°C for 30 minutes.



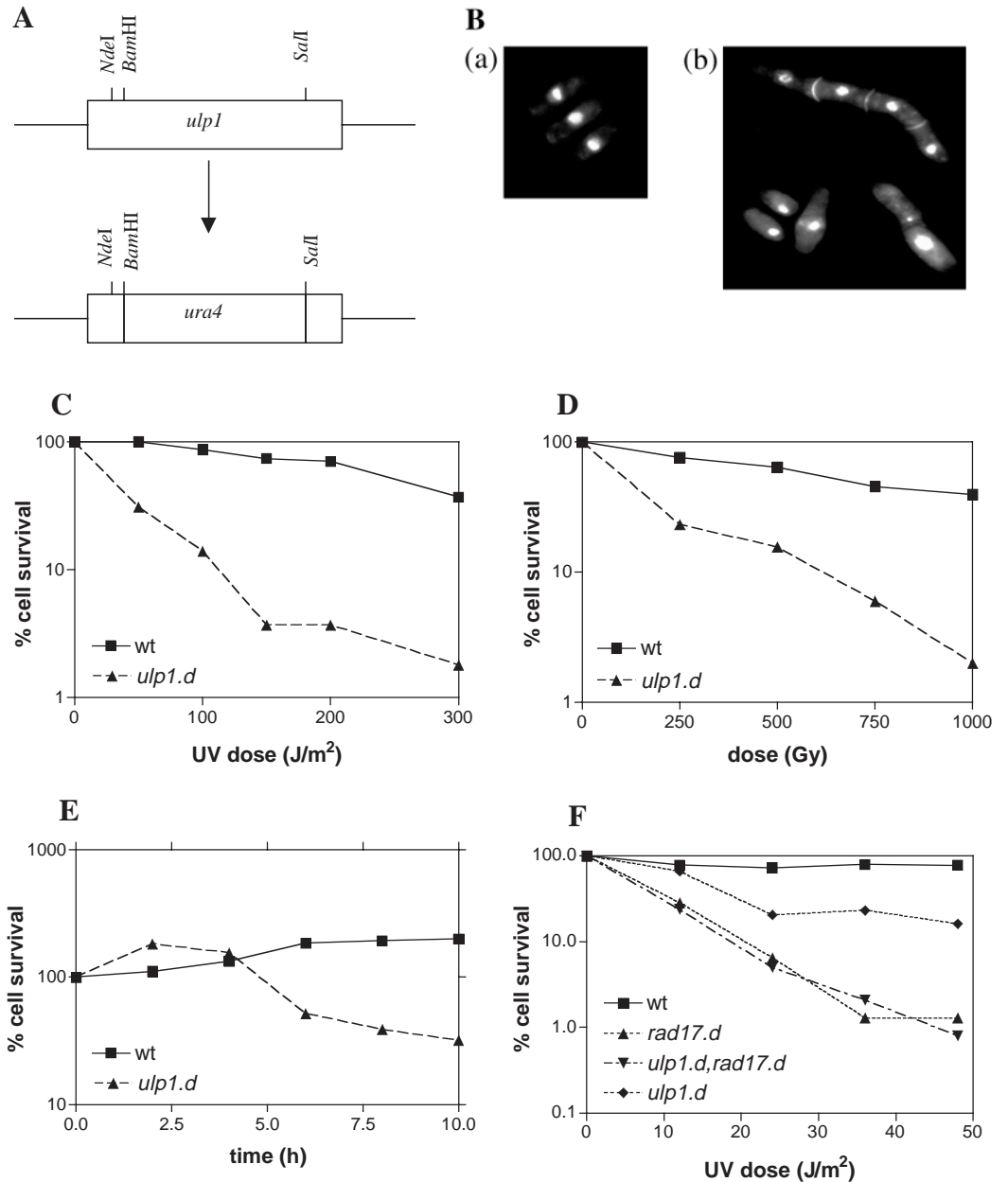


Fig. 3. Disruption of the *ulp1* gene. (A) *ulp1* disruption strategy. A 1.1 kb *Bam*HI-*Sal*I fragment within the *ulp1* coding sequence was replaced with the *ura4* gene. (B) Microscopic analysis of *ulp1*-null cells. Wild-type, sp.011, (a) and *ulp1.d*, sp.651, (b) cells were fixed in 70% ethanol, stained with DAPI and analysed using a Zeiss Axioskop 2 microscope. (C-E) Response of *ulp1*-null cells to UV and ionising radiation and the DNA synthesis inhibitor HU. Wild-type (sp.011) and *ulp1.d* (sp.651) were exposed to a range of doses of UV (C), ionising radiation (D) or 20 mM HU and samples taken over 10 hours (E). (F) Epistasis analysis. Wild-type (sp.011), *ulp1.d* (sp.651), *rad17.d* (sp.310) and *ulp1.d,rad17.d* (sp.666) cells were subject to UV radiation.

The *rad31* and *hus5* genes are required for the DNA damage response, as the *rad31.d* and *hus5.62* strains are sensitive to UV and ionising radiation and the DNA synthesis inhibitor HU (al-Khodairy et al., 1995; Shayeghi et al., 1997). Fig. 3C-E show that *ulp1*-null cells are also sensitive to DNA damage and HU. The level of sensitivity to UV radiation is similar to that of *rad31.d* and *hus5.62* cells, but *ulp1.d* cells are less sensitive than *rad31.d* and *hus5.62* to ionising radiation and HU (for example, *rad31.d* has an approximately 0.1% survival rate at 1000 Gy compared with 3% for *ulp1.d* and a 10% survival rate in response to 8 hours incubation in the presence of HU as compared to 40% for *ulp1.d*) (al-Khodairy et al., 1995; Shayeghi et al., 1997). The *ulp1*-null cells similar to *rad31*-null cells showed hypersensitivity to 10 mM caffeine (data not shown), and the slow growth of *ulp1.d* cells is partially rescued by growth on plates containing 1 M sorbitol (data not shown).

The *rad31.d* and *hus5.62* mutants are defective in a pathway

that also involves the DNA integrity checkpoint genes (al-Khodairy et al., 1995; Shayeghi et al., 1997), and more specifically, *hus5.62* is defective in the recovery from checkpoint arrest. To determine whether *ulp1* also functions in a checkpoint-dependent process, a range of double mutants was made and analysed for sensitivity to UV radiation. As shown in Fig. 3F, the *ulp1.d,rad17.d* double mutant displays no increase in sensitivity over the *rad17.d* single mutant, indicating that *ulp1* and *rad17* function in a common pathway. Similar results were obtained with other checkpoint *rad* mutants (data not shown).

ulp1-null cells have reduced levels of Pmt3 conjugates that are restored by over-expression of mature Pmt3

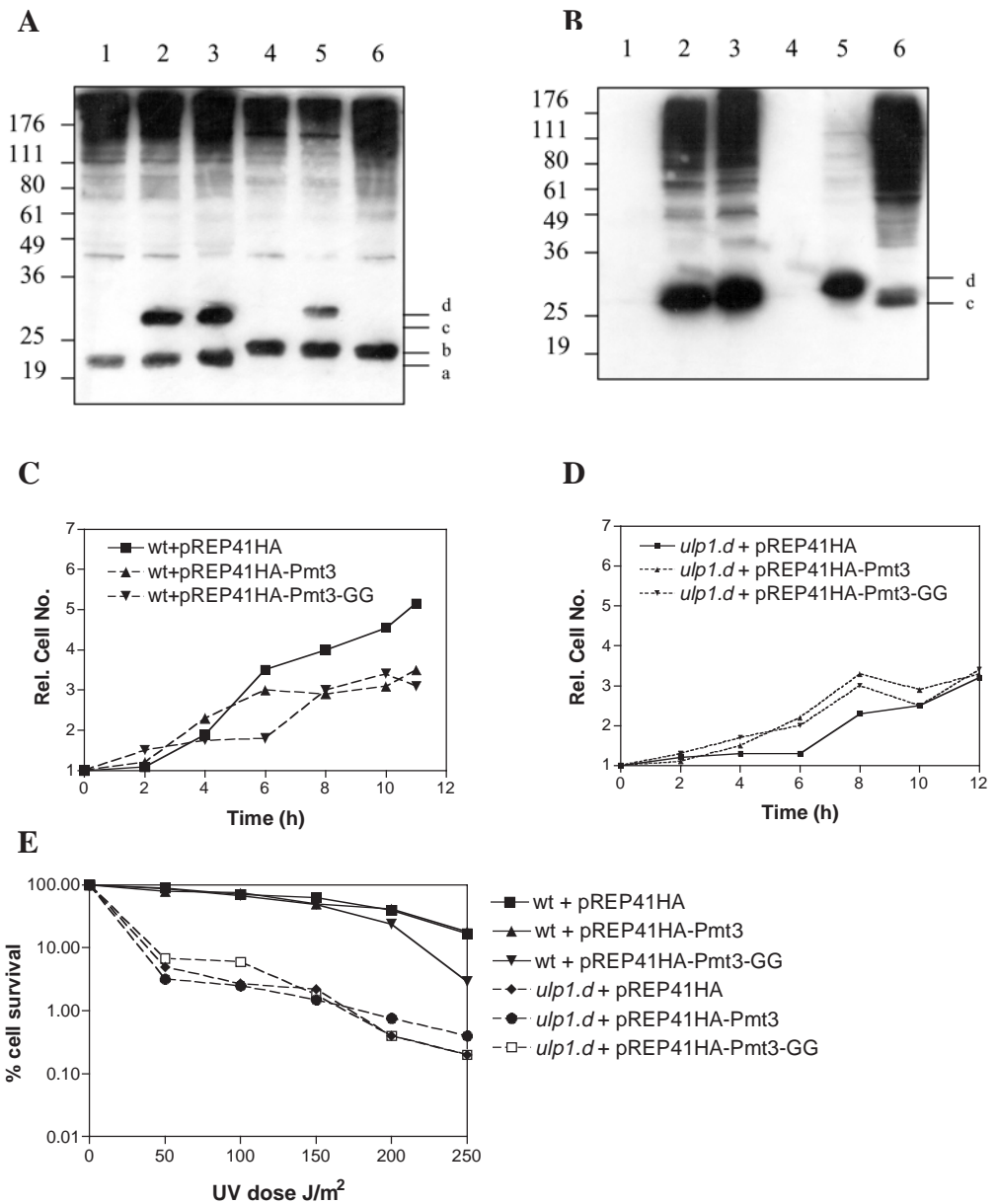
As we have shown that Ulp1 can remove Pmt3 from high molecular weight conjugates in cell extracts (Fig. 2A), we

wished to determine whether the *ulp1*-null allele showed any difference in the pattern of Pmt3 conjugates when compared with wild-type cells and if so, whether expression of mature Pmt3 had any effect on the pattern of conjugates. Wild-type and *ulp1*-null cells were transformed with pREP41HA-Pmt3, pREP41HA-Pmt3GG and the empty vector pREP41HA. Transformed cells were grown in selective medium to mid-late exponential phase, conditions where some unconjugated Pmt3 is observed in cells (unlike the situation in cells grown to early exponential phase in rich medium when free Pmt3 is not detected, (Fig. 2A, lane 1)). Western analysis using anti-Pmt3 antisera on extracts from cells transformed with empty vector (Fig. 4A) shows that the *ulp1*-null strain has a reduced level of Pmt3 conjugates (lane 4) compared with the level of conjugates observed in wild-type cells (lane 1). (Similar results are obtained with untransformed cells, data not shown.) These data suggest that the loss of Pmt3 processing activity in *ulp1* null

cells is limiting the extent of Pmt3 conjugation to substrate proteins.

We next investigated whether it was possible to bypass the loss of Pmt3-processing activity in the *ulp1*-null cells by over-expressing mature Pmt3 (Pmt3-GG). Wild-type cells expressing either HA-Pmt3 or HA-Pmt3-GG show no major changes in the pattern of Pmt3 conjugates detected using anti-Pmt3 antisera when compared with extracts from cells transformed with the empty vector (Fig. 4A, lanes 1-3). Probing with anti-HA antisera indicates that HA-tagged species are equally abundant in wild-type cells transformed with either HA-Pmt3 or HA-Pmt3-GG, implying that both the precursor and mature forms of Pmt3 can be used for incorporation into high molecular weight conjugates (Fig. 4B, lanes 2 and 3). In contrast *ulp1*-null cells are less able than wild-type cells to incorporate HA-Pmt3 species when provided with HA-tagged precursor Pmt3 (Fig. 4A and B, lane 5).

Fig. 4. Effect of over-expressing Pmt3 and Pmt3-GG in wild-type and *ulp1.d* cells. (A,B) Total cell extracts from wild-type (sp.011) (lanes 1-3) and *ulp1.d* (sp.651) cells (lanes 4-6) transformed with pREP41HA (lanes 1,4), pREP41HA-Pmt3 (lanes 2,5) or pREP41HA-Pmt3-GG (lanes 3,6) and grown for 18 hours in selective medium lacking thiamine were analysed by western blotting with anti-Pmt3 antisera (A) or anti-HA antisera (B). Equal amounts of protein were loaded in each lane as determined by staining with Coomassie Brilliant Blue (data not shown). (a) Pmt3-GG, (b) full-length Pmt3, (c) HA-Pmt3-GG, (d) HA-Pmt3. (C,D) Wild-type (sp.011) and *ulp1.d* (sp.651) cells transformed with pREP41HA, pREP41HA-Pmt3 or pREP41HA-Pmt3-GG were grown for 12 hours to mid-log phase in selective medium lacking thiamine. Cells were then diluted ($t=0$) to 10^6 cells/ml in fresh thiamine-free medium and counted every 2 hours. (E) Transformed cells were grown for 18 hours to 4×10^6 cells/ml in selective media lacking thiamine and then subjected to UV radiation as indicated.



However *ulp1*-null cells use HA-Pmt3-GG efficiently, with the result that HA-tagged species are as abundant in *ulp1.d* + HA-Pmt3-GG (Fig. 4B, lane 6) as they are in the equivalent wild-type transformant (lane 3).

Fig. 4 confirms that *ulp1*-null cells are defective in the processing of precursor Pmt3 to the mature form. In this experiment, wild-type cells (Fig. 4A, lanes 1-3) accumulate an anti-Pmt3 cross-reacting species of approximately 22 kDa, representing Pmt3-GG (the mature form of Pmt3). Wild-type cells transformed with either HA-Pmt3 or HA-Pmt3-GG also accumulate another species of around 26 kDa, representing HA-Pmt3-GG (Fig. 4A and B, lanes 2 and 3). In contrast, the smallest anti-Pmt3 cross-reacting species observed in *ulp1*-null cells (lanes 4-6) is around 23 kDa, that is, about 1 kDa larger than the smallest species observed in wild-type cells (lanes 1-3) and consistent with the size expected for full-length Pmt3. Additionally, *ulp1*-null cells transformed with HA-Pmt3 (Fig. 4A and B, lane 5) accumulate an anti-HA cross-reacting species slightly larger than the 26 kDa species observed in HA-Pmt3 transformed wild-type cells (Fig. 4A and B, lane 2), consistent with it being the HA-tagged form of full-length Pmt3. Fig. 4B, lane 6 indicates that very little HA-tagged Pmt3-GG monomer is present in *ulp1.d* cells transformed with HA-Pmt3GG, this is likely to be due to the fact that much of it is conjugated to targets in *ulp1*-null cells.

We were interested in determining whether expression of Pmt3-GG could rescue the slow growth phenotype or the UV radiation sensitivity of *ulp1.d* cells. Cells transformed with the appropriate plasmids were grown for 12 hours in selective liquid medium in the absence of thiamine to mid-log phase and then diluted to 10^6 cells/ml in fresh thiamine-free medium (time=0). The cell number was monitored every 2 hours for up to 12 hours. Over-expression of full-length or mature Pmt3 in wild-type cells has a deleterious effect on cell growth rate after 18-20 hours under de-repressing conditions (Fig. 4C). In

contrast, over-expression of full-length or mature Pmt3 in *ulp1.d* cells is not deleterious and may provide a very slight increase in the rate of growth 18-20 hours after release into thiamine-free medium (Fig. 4D). However, any increase in the rate of cell division is short lived, and after 20-22 hours the rate resembles that of cells transformed with empty vector. Over-expression of full-length or mature Pmt3 has no effect on UV radiation survival rates for either wild-type (sp.01) or *ulp1.d* cells (Fig. 4E).

The level of Ulp1 protein is affected by increased temperatures but not by exposure to HU or ionising radiation

To investigate Ulp1 protein expression in cells, we used a strain (sp.611) containing a myc-epitope-tagged genomic copy of *ulp1*. Fig. 5A (lane 1) shows that the myc-tagged Ulp1 protein migrates as a doublet of Mr between 90-100 kDa. The increased size observed here compared with the size of Ulp1 purified from *E. coli* (Fig. 1A) is accounted for by the presence of 13 myc tags (approximately 17.2 kDa). The nature of the doublet is unknown but may reflect proteolysis of Ulp1, an internal translation initiation site, post-translational processing or alternative splicing.

The level of the Ulp1 protein does not change in cells exposed to 20 mM HU for 3 hours (Fig. 5A, lane 2) or in cells 1.5 hours after exposure to 500 Gy ionising radiation (lane 3). In contrast, the level of Ulp1 and the proportion of the upper and lower bands change in response to different temperatures. In cells grown at 25°C and then shifted to 30°C Fig. 5B (lane 2), the level of Ulp1 protein is similar to the level observed in cells maintained at 25°C (lane 1). However, when cells are incubated at 35.5°C for 3 hours (lane 3) the amount of the upper species decreases with a concomitant increase in the lower species and an overall reduction in the total amount of the protein.

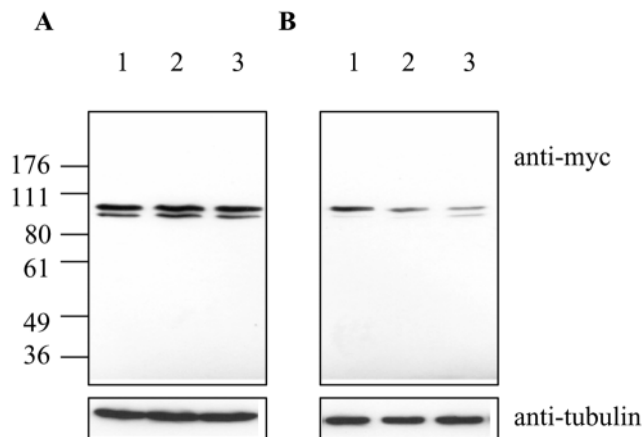


Fig. 5. Ulp1 protein levels vary in response to increased temperature. Strain sp.611 (*ulp1-13myc*) was grown overnight to mid-log phase at 30°C (A) or at 25°C (B). TCA extracts were analysed by SDS PAGE and western blotting using anti-myc antisera (upper panels) or anti-tubulin antisera (lower panels). (A) Effect of ionising radiation and HU. Lane 1, no treatment; lane 2, 20 mM HU for 3 hours; lane 3 cells exposed to 500 Gy ionising radiation and incubated for a further 1.5 hours at 30°C. (B) Effect of temperature. Lane 1, no treatment; lane 2, 3 hours at 30°C; lane 3, 3 hours at 35.5°C.

Localisation of Ulp1 changes through the cell cycle

The two *S. cerevisiae* Smt3 proteases are differentially localised within the cell (Li and Hochstrasser, 2000; Schwienhorst et al., 2000). To identify the intracellular localisation of the *S. pombe* Ulp1 protein, immunofluorescence analysis with anti-myc antisera was undertaken using strain sp.611, which contains a myc-epitope-tagged genomic copy of *ulp1*. Many cells in an exponentially growing culture display strong staining at the nuclear periphery (Fig. 6A) in a pattern similar to that observed with *S. cerevisiae* Ulp1. However, we noticed that in a significant proportion of cells this peripheral nuclear staining was not evident. We therefore investigated whether the localisation of Ulp1 was cell cycle dependent. The majority of cells (approximately 80%) in an exponentially growing culture of wild-type *S. pombe* are in G2. In these cells, Ulp1 localises predominantly to the nuclear periphery (Fig. 6Ba,b). As cells enter mitosis (c,d), the staining remains strong, but becomes more diffuse in the region of the nucleus. During anaphase (e-h) the intensity of the Ulp1 staining decreases as Ulp1 staining appears within the nucleus. The staining within the nucleus persists as the spindle elongates (i-l) and is weak during G1 (m-o). The Ulp1 staining at the nuclear periphery reappears at around the time of septation (p,q), which is

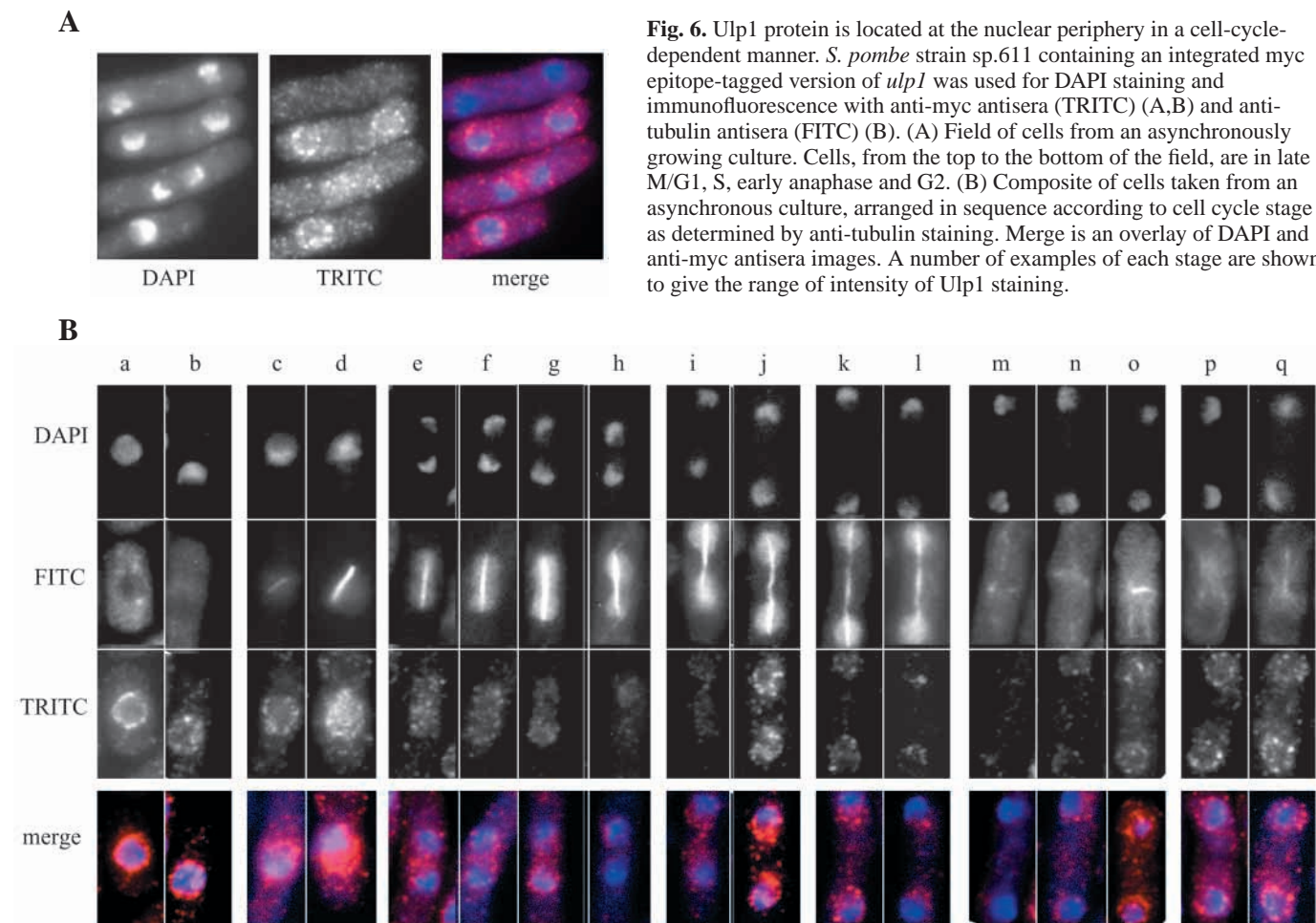


Fig. 6. Ulp1 protein is located at the nuclear periphery in a cell-cycle-dependent manner. *S. pombe* strain sp.611 containing an integrated myc epitope-tagged version of *ulp1* was used for DAPI staining and immunofluorescence with anti-myc antisera (TRITC) (A,B) and anti-tubulin antisera (FITC) (B). (A) Field of cells from an asynchronously growing culture. Cells, from the top to the bottom of the field, are in late M/G1, S, early anaphase and G2. (B) Composite of cells taken from an asynchronous culture, arranged in sequence according to cell cycle stage as determined by anti-tubulin staining. Merge is an overlay of DAPI and anti-myc antisera images. A number of examples of each stage are shown to give the range of intensity of Ulp1 staining.

equivalent to S phase in *S. pombe*. Throughout most stages of the cell cycle, weak punctate staining of Ulp1 is also observed in the cytoplasm, for example, Fig. 6A.

Discussion

In order to further our studies of proteins required for SUMO modification in *S. pombe*, we have begun a characterisation of *S. pombe* Ulp1. Recombinant Ulp1 protein purified from *E. coli* is capable of processing precursor Pmt3 to the size expected of mature Pmt3 and can, to a limited extent, remove Pmt3 from high molecular weight conjugates in *S. pombe* cell extracts. *ulp1*-null cells accumulate unprocessed Pmt3 and have a reduced level of Pmt3 conjugates. These data suggest that deconjugation of Pmt3 from conjugates by Ulp1 may be a minor role compared to its processing activity. The main Pmt3 deconjugating activity may, as is the case in *S. cerevisiae*, be due to the action of a second Pmt3 protease. A second potential Pmt3-specific protease, identified in *S. pombe* through its homology with the *S. cerevisiae* Ulp2 enzyme (Li and Hochstrasser, 1999), is a candidate for the main deconjugating activity.

In *S. pombe*, the Ulp1 protein migrates as a doublet. The doublet is observed in TCA extracts of cells and in native cell extracts in the presence of protease inhibitors, suggesting that it is not due to proteolysis. It is also unlikely to be due to

phosphorylation, as incubation with λ protein phosphatase has no effect on the doublet (data not shown). We are currently investigating whether the *ulp1* mRNA is differentially spliced and, if so, whether the different protein products have different activities.

Deletion analysis indicates that *ulp1* is not essential for viability, in contrast to the situation in *S. cerevisiae* (Li and Hochstrasser, 1999). The reason for this difference between the two yeasts is not known, but *SMT3*, the *S. cerevisiae* SUMO gene, is essential for cell viability whereas *S. pombe* *pmt3* is not (Tanaka et al., 1999). The low frequency of recovery of *ulp1*-null cells (approximately 1-2% of the number of wild type cells obtained) might be due to poor spore germination in *ulp1*-null cells. *S. pombe* *ulp1*-null cells isolated here display severe cell and nuclear abnormalities. This is reminiscent of the defects observed in *rad31.d* and *hus5.62* cells, which have mutations in other components of the sumoylation pathway. Crosses of *ulp1.d* with *rad31.d* produced very few asci, with the result that we have so far been unable to obtain a *ulp1.d,rad31.d* double mutant to confirm genetically that the two genes function in the same process(es). The range of phenotypes such as the sensitivity to UV radiation and caffeine (a molecule likely to have a number of effects on the cell including the ability to over-ride the S-M checkpoint (Moser et al., 2000)) and the rescue by the osmotic stabiliser, sorbitol, displayed by *ulp1.d*, *rad31.d* and *hus5.62* implies that Pmt3

modification is likely to have a number of functions within cells.

Over-expression of full-length or mature Pmt3 was unable to rescue the slow growth phenotype of *ulp1.d* cells. The reason for this is unknown, but since over-expression of Pmt3 or Pmt3-GG in wild-type cells is deleterious, the amount of Pmt3/Pmt3-GG in cells appears to be critical. The inability of increased levels of Pmt3-GG to rescue substantially the slow growth or UV-sensitive phenotypes of *ulp1.d* cells could be due to the requirement for the deconjugating activity of Ulp1, which is presumed to be still defective in these transformed cells. This would imply that there is/are Pmt3-modified target(s) with key roles in cell cycle progression and/or the DNA damage response that require Ulp1 for deconjugation.

In *S. pombe*, GFP-Pmt3 has been shown to be localised predominantly within the nucleus and, more specifically, to associate with the SPB during interphase (Tanaka et al., 1999). During prometaphase and metaphase, the Pmt3 staining at the SPB disappears and cells in anaphase display weak Pmt3 staining between the SPBs. After anaphase Pmt3 reappears at the SPBs. During S and G2, we detect myc-tagged Ulp1 at the nuclear periphery consistent with data from *S. cerevisiae* (Schwienhorst et al., 2000) where Ulp1 colocalises with the nuclear pore complex. Unlike the case in *S. cerevisiae*, we observe the pattern of Ulp1 staining to change as cells enter mitosis. The changes that we observe at the early stages of mitosis coincide with the changes observed in Pmt3 localisation (Tanaka et al., 1999). The pattern of Ulp1 staining in Fig. 6B(d) suggests that Ulp1 might be transiently located at the ends of the spindle. As is observed with GFP-Pmt3 (Tanaka et al., 1999), myc-tagged Ulp1 appears to be localised in the DAPI-negative parts of the nucleus during anaphase. (Fig. 6Be-1).

Data from work in *Drosophila* and mammalian cells have implicated SUMO modification as having a role in the nuclear import of proteins (e.g. Mahajan et al., 1998; Saitoh et al., 1998). Thus the function of Ulp1 at the nuclear periphery during S and G2 may be to provide mature Pmt3, either as a result of processing or deconjugating Pmt3 from target proteins and recycling it for subsequent conjugation to target proteins destined for nuclear import. The relocation of Ulp1 during mitosis may be responsible for the alteration of the Pmt3 modification state of proteins required specifically for mitosis, the identity of which remain to be determined. A critical role for Ulp1 at G2/M has been shown in *S. cerevisiae*, where a temperature-sensitive mutant of *ulp1* arrests at G2/M at the restrictive temperature (Li and Hochstrasser, 1999).

An important group of proteins that undergo cell-cycle-dependent sumoylation are the *S. cerevisiae* septins (Johnson and Blobel, 1999). The septins, which are located at the mother/bud neck (where they are required for cytokinesis) are maximally sumoylated during mitosis. At this time of the cell cycle, SUMO (Smt3) is clearly visible in rings at the mother/bud neck. These proteins might thus be candidate proteins affected by the activity of Ulp1. However, a similar staining pattern for Pmt3 during cytokinesis in *S. pombe* has not been reported (e.g. Tanaka et al., 1999), suggesting that the septins may not be major SUMO-modified targets in *S. pombe*.

Another possible target is the centromere protein CENP-C, as temperature-sensitive mutations in CENP-C have been

shown to be suppressed by over-expression of SUMO (Fukagawa et al., 2001; Meluh and Koshland, 1995). The *S. cerevisiae* CENP-C homologue *MIF2* has been shown to be required for maintaining the integrity of the mitotic spindle during anaphase (Brown et al., 1993), and recently, a temperature-sensitive CENP-C mutant in vertebrate cells has been shown to display metaphase delay and chromosome mis-segregation (Fukagawa et al., 2001).

One of our aims is to identify *S. pombe* proteins modified by Pmt3. As SUMO conjugates are unstable and/or present at low levels in cells (e.g. Gostissa et al., 1999) we wished to determine whether it was possible to stabilise them by inhibiting the deconjugating activity of Pmt3-specific proteases, such as Ulp1. However, despite using high concentrations of known cysteine protease inhibitors we were unable to inhibit deconjugation of Pmt3 from high molecular weight species using either NEM or iodoacetamide. Another possible method for stabilising Pmt3-modified species in order to facilitate their identification might be to deplete the deconjugating enzymes. We are currently analysing Pmt3-modified species present in *ulp1.d* cells where the processing defect is rescued by the over-expression of HA-Pmt3-GG.

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