Characterization of SUMO-conjugating enzyme mutants in *Schizosaccharomyces pombe* identifies a dominant-negative allele that severely reduces SUMO conjugation

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The phenotypes of mutants defective in the *Schizosaccharomyces pombe* SUMO (small, ubiquitin-like modifier)-conjugating enzyme Hus5 (the homologue of Ubc9) show that it is required for recovery from S-phase arrest. Unlike the case with ubiquitination, where ligases are required, SUMO-conjugating enzymes are sufficient for substrate recognition and conjugation of SUMO on to target proteins, at least *in vitro*. Thus SUMO-conjugating enzymes are likely to be important regulators of sumoylation. Here, we report on the characterization of two *hus5* alleles. Although *hus5.17* and *hus5.62* respond in a similar manner to UV and ionizing radiation, they have different responses to the DNA-synthesis inhibitor, hydroxyurea. In addition, SUMO (Pmt3) is mislocalized in *hus5.17* cells, but not in *hus5.62* mutant cells. The mutations in *hus5.62* and *hus5.17* map to Ala[129] and the 5′ splice site of intron 2 respectively. We have characterized the Hus5.62 protein and shown, *in vitro*, that it still interacts with SUMO and at least one protein, Rad22, which is a SUMO-modified target. The Hus5.62 protein is also capable of forming a thioester link with SUMO, but it does not function in sumoylation assays, either in the modification of Rad22 or in SUMO chain formation. When overexpressed in wild-type *S. pombe* cells, the Hus5.62 protein has a dominant-negative effect on sumoylation.

Key words: DNA damage response, *hus5*, hydroxyurea, *S. pombe*, sumoylation, *UBC9*.

INTRODUCTION

SUMO (small, ubiquitin-like modifier) is one of a number of ubiquitin-like proteins that are covalently attached to target proteins. In the case of SUMO, most of these proteins are nuclear [1], and the role of SUMO modification appears to be target-specific. For example, SUMO modification of PML is required for the assembly of PODs (PML oncogenic domains), and the subsequent recruitment of Daax and Sp100 proteins [2–4], whereas in the case of Ran-GAP (GTPase-activating protein) it is required to localize Ran-GAP to the nuclear-pore complex through its association with RanBP2 [1,5]. Additionally, SUMO modification of inhibitory $\kappa B\alpha$ ($I\kappa B\alpha$) has been shown to prevent $I\kappa B\alpha$ from being ubiquitinated, and hence degraded, by ubiquitin-dependent proteolysis [6]. Sumoylation has also been shown to affect the activity of several transcription factors, including p53 [7,8], and the activity of a range of different enzymes, including the histone deacetylase HDAC4 [9] and human thymine DNA glycosylase [10].

SUMO conjugation to target proteins requires a series of enzymic steps (reviewed in [11]). These include the processing of precursor SUMO to the mature form, followed by the ATP-dependent formation of a thioester bond between SUMO and a cysteine residue within a heterodimeric activator [12]. SUMO is then transferred to a conjugating enzyme, where it also forms a thioester, before being attached via an ϵ-isopeptide linkage to a lysine residue on target proteins [13–15]. *In vitro*, the conjugating enzyme is sufficient for attachment of SUMO to target proteins. However, the identification of SUMO ligases has been described recently. These proteins facilitate the production of SUMO chains, and appear to have a role in attaching SUMO, either as chains or monomers, to target proteins [16,17].

The SUMO-modification system is well conserved in eukaryotes. In *Schizosaccharomyces pombe*, the SUMO protein is encoded by the *pmt3* gene [18]. Precursor Pmt3 is processed by at least one specific protease, Ulp1 [19]. The mature form is then activated by a heterodimer of Rad31 and Fub2 [18,20] and is conjugated to target proteins by a conjugating enzyme, Hus5, the homologue of Ubc9 [21,22]. Several laboratories have demonstrated that mutations in the Pmt3/SUMO-modification process result in cells that are sensitive to DNA-damaging agents and hydroxyurea (HU), an inhibitor of DNA synthesis (for references, see e.g. [18,20,21]). Such mutants also display a defect in cell-cycle progression (see, e.g. [18,21]). Interestingly, mutants defective in the SUMO-processing and modification enzymes display subtly different phenotypes. For example, a null mutant in one of the SUMO-processing enzymes, Ulp1, responds differently to DNA-damaging agents compared with certain alleles of *hus5* [19,22].

Since Ubc9 (Hus5) is likely to be an important regulator of sumoylation, we have undertaken an analysis of the phenotypes of two *hus5* mutants, and compared them with the phenotype of the *rad31* null mutant. With the aim of determining why the two *hus5* mutants result in different phenotypes, we have identified the nature of the mutations in the two alleles. *hus5.17* contains a point mutation in the 5′ splice site of intron 2, whereas the mutation in *hus5.62* results in an Ala[129]→Val substitution. Since Ala[129] maps close to the SUMO and target protein-binding sites, we have investigated whether the Hus5.62 protein is able to interact with both Pmt3 (SUMO) and the target protein Rad22. We have also

Abbreviations used: GAP, GTPase-activating protein; GST, glutathione S-transferase; HU, hydroxyurea; RT, reverse transcription; SUMO, small, ubiquitin-like modifier.

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analysed the Hus5.62 protein for its ability to sumoylate Rad22 *in vitro*, and for its ability to form Pmt3 chains. We show in the present study that the Hus5.62 protein is unable to sumoylate Rad22 or form Pmt3 chains, although it can still interact with Pmt3 and the Pmt3-modified target Rad22, and can form a thioester bond with Pmt3. Overexpression of *hus5.62* in wild-type cells has a dominant-negative effect on sumoylation *in vivo*.

EXPERIMENTAL

Strains and plasmids

The *S. pombe* strains used in the present study were sp.011 *ade6.704, leu1.32, ura4.*Δ*18, h*$^-$, sp.333 *rad31 : :ura4, ade6.704, leu1.32 h*$^-$ [20], sp.715 *hus5.17, ade6.704, leu1.32, ura4.*Δ*18, h*$^-$ [21] and sp.654 *hus5.62, ade6.704, leu1.32, ura4.*Δ*18, h*$^+$ [21]. Mutant *hus5* alleles were amplified by PCR using primers Hus5-*Nde* (5′-CATATGTCATCTCTTTGTAAAACGC-3′) and Hus5-*Bam* (5′-GGATCCAGTCAGTTTATGGAGC-3′). The Hus5.62-containing plasmid was created by using the Quikchange Mutagenesis Kit (Stratagene) with mutagenic primers Hus5-Ala^{129}$\rightarrow$Val F(forward) (5′-GTATATGCTTCTG-TTTGAACGGGAGAGGCAATATTAG-3′) and Hus5-Ala^{129}$\rightarrow$ Val R(reverse) 5′-CTAATATTGCCTCTCCCGTTCAAACAG-AAGCATATAC-3′), according to the manufacturer's instructions. The cloning of the *rad31*, *fub2*, *pmt3* and *pmt3-GG* open reading frames has been described elsewhere [22].

For expression in *Escherichia coli*, Hus5 wild-type protein and variants were expressed as glutathione S-transferase (GST) fusion proteins using a modified form of pGEXGH (a gift from H. Lindsay at the University of Sussex). For expression in *S. pombe*, the *hus5* sequences were cloned into pREP41 under the control of the *nmt1* promoter [23].

Molecular-biology techniques

Reverse transcription (RT)-PCR was performed on mRNA as follows: 50 ml of exponential-growth-phase culture was harvested by centrifugation for 5 min at 5000 rev./min (1000 ***g***) at 4 °C. The cell pellet was resuspended in 200 μl of water, and transferred to a tube containing 500 μl of acid phenol, 100 μl of chloroform, 500 μl of detergent [9.6% (v/v) Decon/20 mM sodium acetate, pH 4.0] and glass beads. Cells were lysed in a Ribolyser with 3 $\times$ 20 s bursts, followed by a 10 min incubation on ice. The homogenate was centrifuged at 13 000 rev./min (5000 ***g***) for 10 min at 4 °C. Supernatant (500 μl) was then transferred to 1 ml of 100 % ethanol and incubated at −70 °C for 1 h. RNA was harvested by centrifugation at 13 000 rev./min (5000 ***g***) for 30 min at 4 °C, and the pellet was resuspended in 50 μl of 1 mM sodium citrate. DNA was removed using a DNA-free DNase kit (Abgene) following the manufacturer's instructions. cDNA was prepared using a first-strand synthesis kit (Abgene), again following the manufacturer's instructions.

In vitro Pmt3 modification system

The *S. pombe in vitro* Pmt3 modification system has been described elsewhere [22]. The *hus5.62* sequence was cloned into modified pGEXGH as an *Nde*I–*Bam*HI fragment, and Hus5.62 protein was expressed in *E. coli* BL21. Modification of Rad22 was carried out using ^{35}S-labelled Rad22 prepared in the TnT system (Promega). Pmt3 chain formation was assessed using His-Pmt3-GG (the mature form of Pmt3 [22]) produced in *E. coli* in the *in vitro* system, followed by Western blotting with anti-Pmt3 sera.

Thioester bond formation was assayed under conditions similar to those employed for assaying Pmt3 modification, except that the incubation time was reduced to 15 min. Samples were split into two, with one sample being treated with standard SDS sample buffer containing 2-mercaptoethanol, and the other treated with SDS sample buffer without 2-mercaptoethanol.

Protein extraction and immunological methods

Total protein extracts were prepared from exponentially growing *S. pombe* cells using trichloroacetic acid, as described in [24]. GSH-affinity purification of GST–Hus5 fusion protein was undertaken as described previously [22]. Immunolocalization of proteins was performed as described in [25]. The production and characterization of the anti-Pmt3 sera was as described previously [22]. The anti-tubulin monoclonal antibody was obtained from Sigma (T5168) and the anti-His$_6$ sera were from Pharmacia.

Sensitivity to DNA-damaging agents

Sensitivities to UV and ionizing radiation were assessed as described in [26]. HU sensitivity was measured in rich medium in the presence of 6 mM HU.

RESULTS

Comparison of *hus5.17* and *hus5.62* phenotypes

Null mutants of *pmt3* and *rad31* (which encodes one subunit of the Pmt3 activator) display severe morphological abnormalities [18,20], whereas the *hus5* null allele is essentially non-viable [21]. In contrast with the *hus5* null mutant, the *hus5.62* and *hus5.17* alleles are viable [21], but the mutations result in severe growth and morphological abnormalities. This suggests that the mutant proteins may have some residual or partial activity. We decided to undertake a further characterization of the nature of the defects in *hus5.17* and *hus5.62* to gain more insights into Hus5 (Ubc9) function. We therefore compared the phenotypes of the *hus5.17* and *hus5.62* mutants. Figures 1(A) and 1(B) show that the *hus5.17* and *hus5.62* alleles display sensitivities to UV and ionizing radiation that are generally similar to each other and to those observed for *rad31.d*. However, the two *hus5* alleles differ in their response to the DNA-synthesis inhibitor, HU (Figure 1C). *hus5.17* cells display loss of cell viability after 2 h in HU, while the *hus5.62* allele resembles wild-type cells during the first 8 h exposure to HU, after which cell viability decreases. The data for *hus5.62* are consistent with those in a previous study [21], which demonstrate that *hus5.62* is defective in recovery from S-phase arrest. The *hus5.17* response to HU suggests that the mutation in this strain is more deleterious than that in the *hus5.62* strain.

We have previously demonstrated that the *hus5.62* allele is defective in Pmt3 modification [22]. Therefore we compared the pattern of Pmt3- (SUMO)-conjugates in wild-type, *hus5.17* and *hus5.62* mutant cells. In wild-type cells, we observed substantial cross-reaction, with the occurrence of high-molecular-mass species of size 50 kDa upwards (Figure 1D, lane 1). The levels and nature of the cross-reacting species in *hus5.62* (lane 2) and *hus5.17* (lane 3) are similar to each other, but are substantially reduced compared with the level observed in wild-type cells (lane 1). In both *hus5.62* and *hus5.17*, there is a strong signal at approx. 20 kDa corresponding to free Pmt3.

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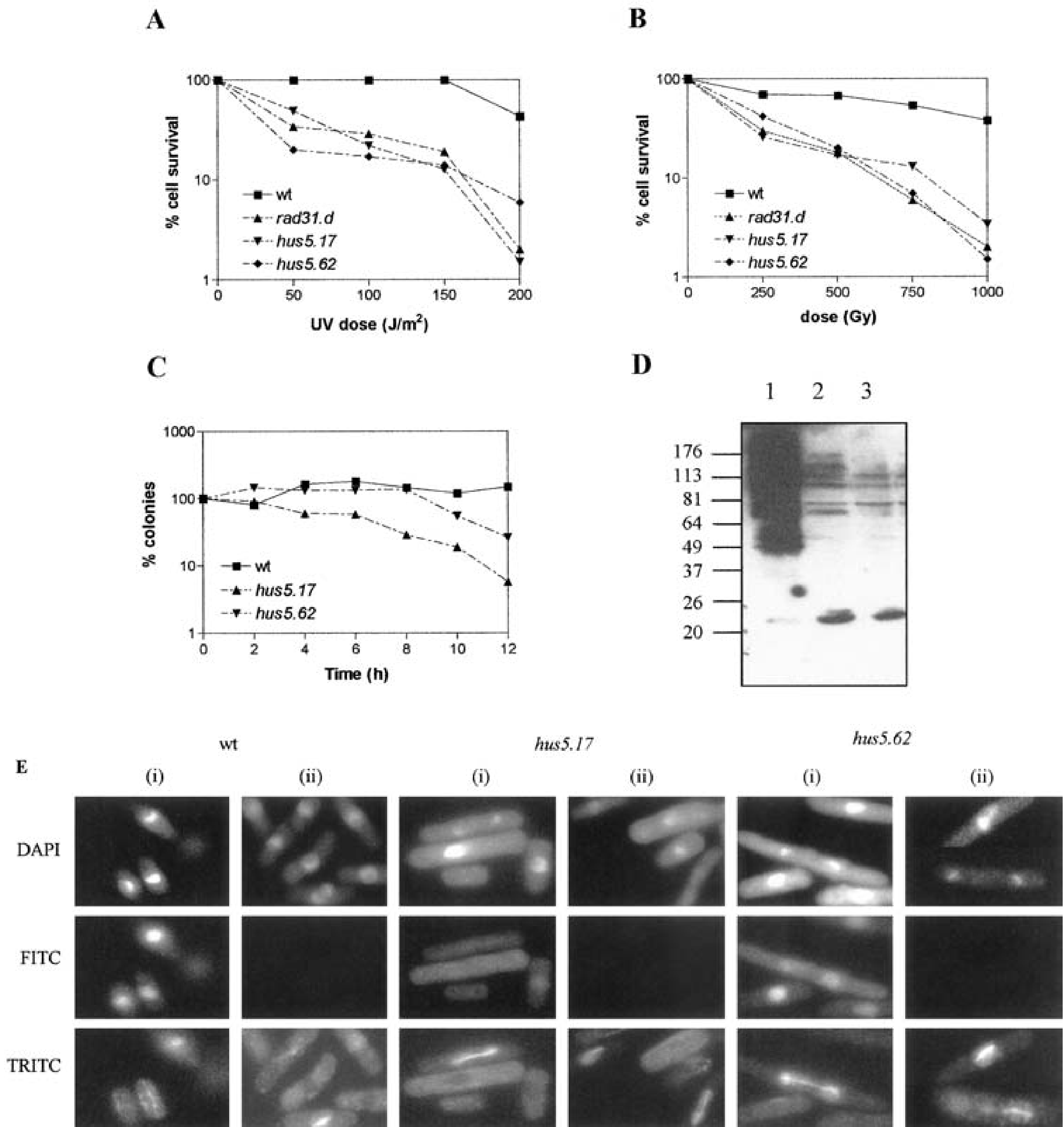

Figure 1 Phenotypic analysis of *rad31.d*, *hus5.17* and *hus5.62*

Shown is the response of wild-type (wt), *rad31.d*, *hus5.17* and *hus5.62* strains to UV light (**A**), ionizing radiation (**B**) and 6 mM HU (**C**). (**D**) Pmt3 conjugates. Total-cell extracts of exponentially growing cells were analysed by Western blot analysis with anti-Pmt3 sera. Lane 1, wt (sp.011) ; lane 2, *hus5.62* (sp.654) ; lane 3, *hus5.17* (sp.715). (**E**) Intracellular localization of Pmt3, showing : cells incubated with anti-Pmt3 (rabbit) and anti-tubulin monoclonal (mouse) antibodies (columns i) or cells incubated with pre-immune serum (rabbit) and anti-tubulin monoclonal antibodies (columns ii). In both cases, cells were incubated with FITC-conjugated anti-rabbit Ig and tetramethylrhodamine β-isothiocyanate (TRITC)-conjugated anti-mouse Ig antibodies. Strains used were as described for (**D**). DAPI, 4,6-diamidino-2-phenylindole.

We next investigated whether the localization of Pmt3 was affected in the *hus5.17* and *hus5.62* mutants (Figure 1E). Pmt3 is localized predominantly within the nucleus in wild-type cells, as demonstrated previously [18]. In the majority of *hus5.17* cells, Pmt3 staining is weak, but detectable, and in these cells, Pmt3 is dispersed throughout them. This is also the case with *rad31.d* (results not shown). In contrast, in *hus5.62* cells, Pmt3 localization resembles that observed in wild-type cells. These data suggest further that the mutations in the *hus5.17* and *hus5.62* alleles have different effects on Hus5 activity.

Sequence analysis of the *hus5.17* and *hus5.62* alleles

In order to identify the nature of the mutation in *hus5.62* and *hus5.17* alleles, the mutant sequences were amplified by PCR, as described in the Experimental section. Analysis of the *hus5.62* sequence indicates that it contains a mutation in the *hus5*-coding sequence, resulting in an $Ala^{129}\rightarrow Val$ substitution (Figure 2A). *S. pombe* Hus5 and Ubc9 proteins from *Saccharomyces cerevisiae* and humans are highly conserved, particularly within the region containing Ala^{129}. Comparison of the Hus5 protein's primary

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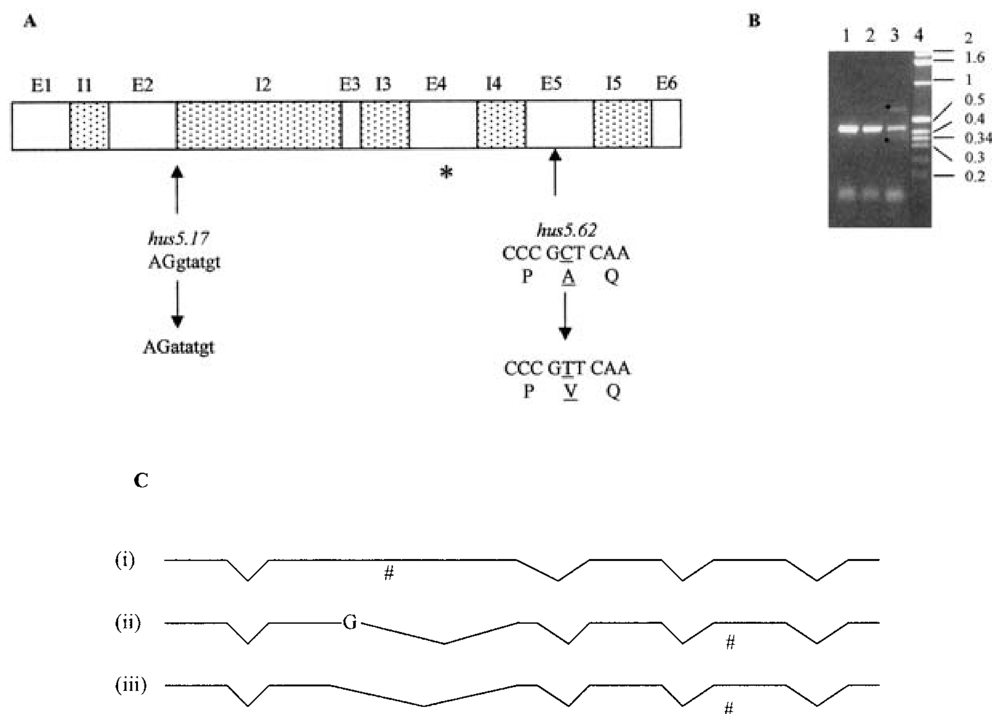

Figure 2 Analysis of the *hus5.17* and *hus5.62* mutations

(**A**) Sites of the *hus5.17* and *hus5.62* mutations. Open boxes represent exons; dot-shaded boxes represent introns. The asterisk (*) indicates the position of the active-site Cys[93]. Uppercase letters for the DNA sequence show the coding sequence, whereas the lowercase letters show intronic sequence. (**B**) Transcripts from the *hus5* locus identified by RT-PCR. Lane 1, wild-type (sp.011); lane 2, *hus5.62*; lane 3, *hus5.17*; lane 4, positions of size markers (in kb). The asterisks (*) indicate the minor products produced from *hus5.17* that are shown in (**C**). (**C**) Sequence analysis of RT-PCR products indicates the potential for three aberrant proteins of 71 (i), 120 (ii) and 95 amino acids (iii). G, extra G within splice product (ii); #, premature stop codon.

structure with the primary structure and the crystal structure of Ubc9 [27,28] indicates that Ala[129] is located within the region of Hus5 that is located between the active-site cysteine residue (Cys[93]) and the region that is predicted to interact with the Lys-Xaa-Glu motif (where 'Xaa' denotes 'any amino acid'), which is the SUMO-modification consensus sequence in Pmt3-modified proteins.

In contrast with the *hus5.62* mutation, which results in a single amino acid substitution, the *hus5.17* allele contains a mutation in the 5′ splice site of intron 2, which would prevent correct splicing and, if no in-frame splice occurred, result in a truncated protein of 71 amino acids. To determine whether the *hus5.17* RNA undergoes aberrant splicing at intron 2, we used RT-PCR to analyse the mRNA specie(s) in *hus5.17*. Figure 2(B) indicates that in both wild-type (lane 1) and *hus5.62* cells (lane 2), one mRNA species of approx. 490 nt is produced. In contrast, in *hus5.17* cells (lane 3) three transcripts are produced from the *hus5* locus. Sequence analysis of the *hus5.17* transcripts indicates that they all encode aberrant proteins (Figure 2C). One transcript [labelled in Figure 2C as (i)] of 711 nt still contains intron 2, and would result in a protein of 71 amino acids, encoded by the first two exons and terminating at a stop codon within intron 2. Another transcript [Figure 2C, (ii)] of 493 nt contains an extra G at the mutant 5′ splice site, which would produce a frameshift and would result in a protein of 120 amino acids, and in the third transcript of 416 nt [Figure 2C, (iii)], an incorrect 5′ splice site within exon 2 has been used, which results in deletion of the remainder of exon 2 and produces a frameshift which would give rise to a protein of 95 amino acids. None of the three predicted proteins from these *hus5.17* mRNAs would contain the active-site Cys[93] or the proposed target binding region if the correct ATG were used as the initiating codon. In none of the three cases is there an ATG upstream and in-frame with the sequence yielding Cys[93]. This suggests that any protein products produced from these transcripts are very unlikely to encode the catalytic site of Hus5.

Hus5.62 acts as a dominant-negative suppressor of Pmt3 modification

We next investigated whether overexpression of the mutant Hus5.62 protein had any effect on wild-type cells. Figure 3(A) indicates that the levels of high-molecular-mass, Pmt3-containing species in cells overexpressing wild-type Rad31 (lane 1), Fub2 (lane 2) or wild-type Hus5 (lane 3) are similar to those observed in cells transformed with empty vector (lane 6). Additionally, there is no change in Pmt3-containing species in cells transformed with *hus5.17* (lane 4). However, overexpression of Hus5.62 protein results in a substantial decrease in the level of high-molecular-mass, Pmt3-containing species (lane 5). These results indicate that, when overexpressed in wild-type cells, Hus5.62 has a dominant-negative effect on the Pmt3 modification process.

Since Pmt3 modification is required for the response to DNA damage, we sought to determine whether overexpression of Hus5.62 in wild-type cells affected the response of cells to DNA-damaging agents. Figure 3(B) indicates that wild-type cells overexpressing wild-type Hus5, Hus5.17 or Hus5.62 behave as wild-type cells transformed with empty vector when exposed to UV light. Furthermore, overexpression of wild-type Hus5, Hus5.17 or Hus5.62 proteins does not affect the response of wild-type cells to either HU or the microtubule-destabilizing drug thiabendazole (results not shown). Therefore, despite the

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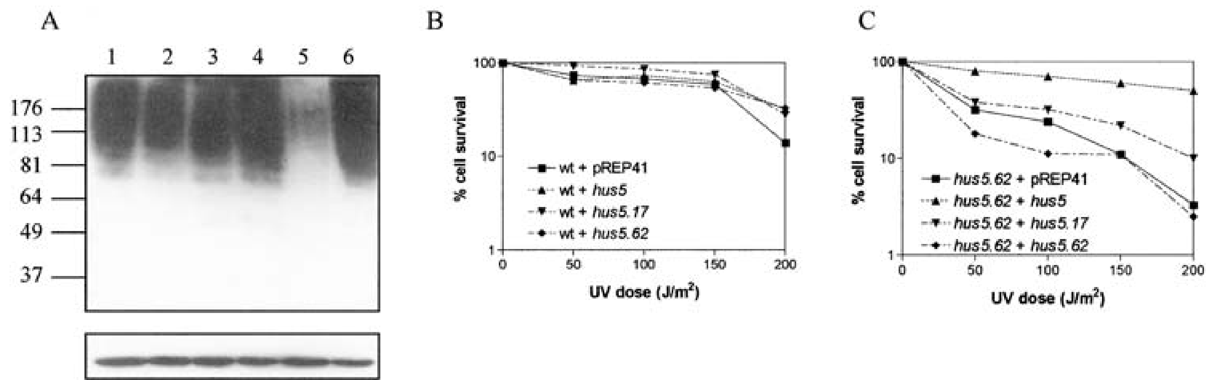

Figure 3 Hus5.62 acts as a dominant-negative suppressor of Pmt3 modification

(**A**) Total-cell extracts of wild-type cells transformed with pREP41-*rad31* (lane 1), pREP41-*fub2* (lane 2), pREP41-*hus5* (lane 3), pREP41-*hus5.17* (lane 4), pREP41-*hus5.62* (lane 5) and pREP41 (lane 6) and grown under de-repressing conditions for 18 h in the absence of thiamine were subjected to Western blot analysis using anti-Pmt3 sera (upper panel) or anti-tubulin sera (lower panel, i.e. the loading control). (**B** and **C**) Response to UV light of wild-type (**B**) and *hus5.62* (**C**) cells overexpressing wild-type and mutant forms of Hus5. Transformed cells were grown under de-repressing conditions, as described for (**A**).

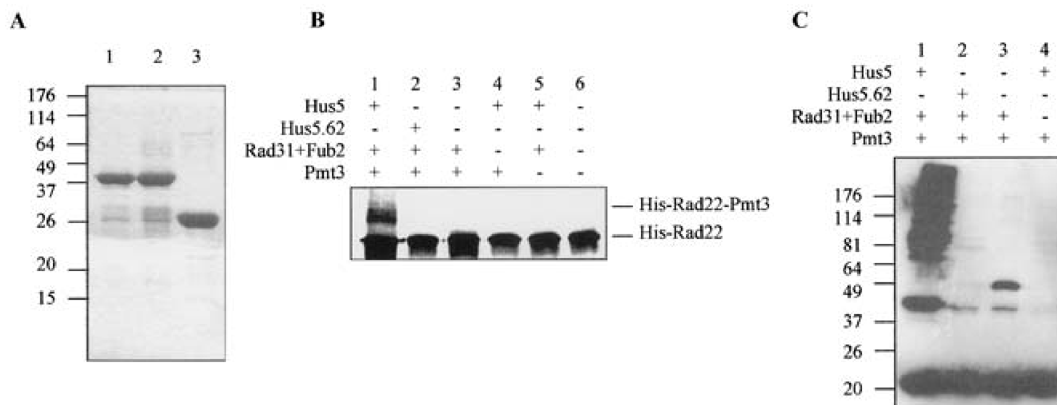

Figure 4 Assay of Hus5 activity in an *in vitro* Pmt3-modification system

(**A**) Purification of wild-type and Hus5.62 proteins. Lane 1, GST–Hus5 ; lane 2, GST–Hus5.62 ; lane 3, GST. (**B**) Assay for sumoylation of Rad22 *in vitro*. *In vitro* sumoylation assays were carried out using ^{35}S-labelled Rad22, as indicated. (**C**) Assay for SUMO chain formation. All proteins in this assay were purified from *E. coli*.

fact that the cells have decreased levels of high-molecular-mass Pmt3-containing species, there is no effect on response to DNA damage.

Overexpression of protein from the *hus5.17* allele partially complements the UV-sensitive phenotype of *hus5.62*

The fact that the *hus5.17* and *hus5.62* mutations are not lethal, unlike the *hus5* null mutation, suggests that these alleles produce proteins that have some residual or partial activity. We therefore sought to determine whether the *hus5.17* allele produced a protein that was able to fully or partially complement the *hus5.62* mutation. Figure 3(C) shows that, indeed, *hus5.62* cells transformed with the genomic copy of the *hus5.17* sequence are less sensitive to UV radiation than are cells transformed with empty vector.

Testing Hus5.62 protein for activity in an *in vitro* Pmt3-modification system

The results of overexpression of Hus5.62 protein in wild-type cells suggests that Hus5.62 protein is likely to be defective in Pmt3 modification. We therefore tested whether this was the case using an *in vitro* Pmt3 modification system. GST–Hus5 and GST–Hus5.62 proteins were purified from *E. coli* (Figure 4A) and were observed to migrate with similar mobilities. We compared the ability of the purified wild-type Hus5 and Hus5.62 proteins to modify Rad22, a previously characterized Pmt3-modified target in *S. pombe* [22]. Figure 4(B) indicates that, as previously demonstrated, in the presence of wild-type Hus5, Rad22 is modified by Pmt3 (lane 1) in a manner dependent on Hus5 (lane 3), Rad31+Fub2 (lane 4) and Pmt3 (lane 5). In contrast, Hus5.62 is unable to support the modification of Rad22 (lane 2) *in vitro*.

Smt3 (the *S. cerevisiae* SUMO protein) has been shown to form chains, both *in vivo* and *in vitro* [16]. We therefore investigated whether Pmt3 was capable of forming chains *in vitro*, as this could be used to assay the sumoylating activity of *hus5.62*. Figure 4(C) shows that Pmt3 is able to form chains *in vitro* using wild-type Hus5 in the presence of Rad31+Fub2 (lane 1), but not in the absence of Hus5 (lane 3) or in the absence of Rad31+Fub2 (lane 4). When Hus5.62 is used instead of wild-type Hus5 (lane 2), no Pmt3 chains are formed, confirming that Hus5.62 does not have sumoylating activity.

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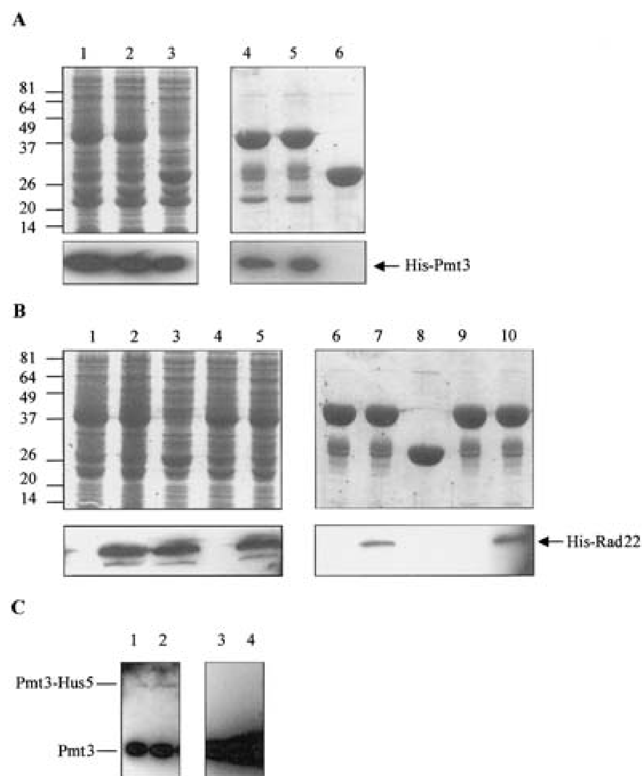

Figure 5 Interaction of Hus5 and Hus5.62 with Pmt3 and Rad22

(**A**) Interaction of Hus5 and Hus5.62 with Pmt3. Lanes 1–3, total protein extract of cells expressing GST–Hus5, GST–Hus5.62 or GST with His-Pmt3 respectively; lanes 4–6, GST–pull-down assay. Upper panel, Coomassie Brilliant Blue stain; lower panel, Western blot with anti-His_6 sera. Lanes 1 and 4 show GST–Hus5+His_6-Pmt3; lanes 2 and 5 show GST–Hus5.62+His_6-Pmt3, lanes 3 and 6 show GST+His_6-Pmt3. (**B**) Interaction of Hus5 and Hus5.62 with Rad22. Lanes 1–5, total protein extract of cells expressing GST–Hus5, GST–Hus5.62 or GST with His_6-Rad22; lanes 6–10, GST pull-down assay. Upper panel, Coomassie Brilliant Blue stain; lower panel, Western blot with anti-His_6 sera. Lanes 1 and 6, GST–Hus5+empty pET15b vector; lanes 2 and 7, GST–Hus5+His_6-Rad22; lanes 3 and 8, GST+His_6-Rad22; lanes 4 and 9, GST–Hus5.62+empty pET15b vector; lanes 5 and 10, GST–Hus5.62+His_6-Rad22. (**C**) Thioester formation. Pmt3, Rad31+Fub2 were incubated at 30 °C for 15 min with wild-type Hus5 (lanes 1 and 3) or Hus5.62 (lanes 2 and 4). Lanes 1 and 2, samples without 2-mercaptoethanol; lanes 3 and 4, samples with 2-mercaptoethanol.

Hus5.62 is capable of interacting with Pmt3 and the target protein Rad22

We next sought to identify the nature of the defect in the Hus5.62 protein. Therefore we investigated whether the mutant Hus5.62 protein was still able to interact with Pmt3. This was tested using a GSH-affinity purification assay from *E. coli* cell extracts. Figure 5(A) indicates that both wild-type Hus5 and Hus5.62 proteins interact with Pmt3 (lanes 4 and 5 respectively). Pmt3 is not pulled down in the absence of the GST-tagged Hus5 proteins (lane 6).

Since the Hus5.62 protein is able to interact with Pmt3, it is possible that the loss of function due to the $Ala^{129} \rightarrow Val$ mutation in Hus5.62 affects the binding of target proteins to Hus5. We therefore investigated whether Hus5.62 is still capable of interacting with Rad22 protein (Figure 5B). Lanes 7 and 10 show that both wild-type Hus5 and Hus5.62 proteins are capable of binding Rad22 *in vitro*. Lane 8 indicates that Rad22 is not pulled down in the absence of either of the Hus5 proteins.

Figure 5(A) shows that Hus5.62 is capable of interacting with Pmt3. However, in order for sumoylation to occur, a thioester bond is required between Cys^{93} of Hus5 and the C-terminal glycine residue of the mature form of Pmt3. We next tested whether Hus5.62 was able to form a thioester bond with Pmt3. Wild-type Hus5 and Hus5.62 proteins were incubated for 15 min with Pmt3-GG in the presence of Rad31+Fub2 under conditions used to test for sumoylation. The samples were split into two and treated either with or without 2-mercaptoethanol (4 %), which has been shown previously to destroy thioester bonds [15]. Figure 5(C) indicates that, as expected, in the presence of wild-type Hus5 and in the absence of 2-mercaptoethanol an anti-Pmt3 cross-reacting species of 60–65 kDa is observed (lane 1). This is the size expected for Hus5–Pmt3. This species is not detected when samples are incubated with 2-mercaptoethanol (lane 3), indicating that the linkage is sensitive to the presence of thiol groups, and hence the species represents the Hus5–Pmt3 thioester. The same size product is also detected using Hus5.62 protein (lane 2), and this is also sensitive to incubation with 2-mercaptoethanol (lane 4). This indicates that, like the wild-type Hus5 protein, the mutant protein is able to form a thioester bond with Pmt3.

DISCUSSION

Deletion of *UBC9* from *S. cerevisiae* or *hus5* from *S. pombe* indicates that these genes are essential for cell viability [21,29]. More specifically, Ubc9 has been shown to be required for cell-cycle progression during G_2- or early M-phase, and Hus5 has been shown to required for the recovery from S-phase arrest [21]. In order to further our understanding of the function of the SUMO-conjugating enzyme, Hus5, we have compared the phenotypes of two *hus5* mutations and identified the nature of the defects in the respective alleles. The phenotypes of the two *hus5* alleles described here, *hus5.17* and *hus5.62*, and the fact that deletion of the *hus5* gene is essentially lethal, are consistent with the notion of Hus5 participating in a number of different cellular functions. The fact that *hus5.17* and *hus5.62* cells are viable compared with *hus5* null cells, which are essentially non-viable, implies that *hus5.17* and *hus5.62* are partially functional, i.e.: (1) the essential function(s) of *hus5* is provided by the *hus5.17* and *hus5.62* alleles; (2) *hus5.17* is lacking a function(s) that is required for the early response to HU (likely to be the arresting of S-phase); and (3) *hus5.62* is deficient in a process(es) that is required for recovery from S-phase arrest.

The *hus5.62* mutation is an Ala $\rightarrow$ Val substitution at amino acid position 129, which results in loss of sumoylation activity in an *in vitro* assay. The $Ala^{129} \rightarrow Val$ mutation maps within the region of the protein which, by analogy with Ubc9, has been shown to be required to interact with proteins that undergo sumoylation [28,30]. The importance of this residue for sumoylation has been shown by Bernier-Villamor et al. [28], who undertook detailed mutational analysis of residues in this region. In this case, mutation of Ala^{129} to Cys resulted in reduced ability to sumoylate a fragment of Ran-GAP1 and loss of ability to sumoylate a fragment of p53 and IκBα.

Analysis of recombinant proteins expressed together in *E. coli* shows that the $Ala^{129} \rightarrow Val$ mutation does not affect binding of the sumoylation target, Rad22 or Pmt3. Additionally, Hus5.62 is also capable of forming a thioester bond with Pmt3. This might be expected, since the Cys^{93} residue is retained in this protein. The fact that Hus5.62 forms a thioester link with Pmt3 and can bind target proteins implies that the $Ala^{129} \rightarrow Val$ mutation inhibits the transfer of Pmt3 to target proteins. Ala^{129} has been shown by NMR spectroscopy of human Ubc9 to undergo substantial chemical shift on binding peptides corresponding to sequences surrounding SUMO-1 conjugation sites [30]. On binding peptides, the side chain of Ala^{129} approaches Cys^{93} so that the two side

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chains are less than 5 Å apart (1 Å = 0.1 nm). Since valine has a bulkier side chain than alanine, mutation of Ala[129] to valine may result in steric hindrance of Cys[93], and thus eliminate sumoylating activity. Another formal possibility is that the binding affinity of Hus5 for Rad22 is reduced in the Hus5.62 protein, and that this reduced affinity is not observed in the binding studies due to the relatively large amounts of Hus5.62 protein present. However, the sumoylation assays involved similar amounts of Hus5 protein (3 μg) suggesting that the major defect is not due to reduced substrate binding of Hus5.62.

The *hus5.17* mutation is in the 5′ splice site of intron 2. Analysis of three major transcripts produced by this allele (i–iii) indicates that none of them encodes the catalytic Cys[93] in-frame with an initiating methionine residue. The reason for the viability of the *hus5.17* strain is therefore unknown, but possible explanations include: (1) the strain has acquired a suppressor mutation; (2) the N-terminus has a function that is independent of the catalytic and target binding sites; and/or (3) despite the mutation, the splicing machinery is capable of splicing correctly at very low levels, which we were unable to detect by RT-PCR from mRNA. The first possibility is less likely than the other two, since we obtain viable *hus5.17* cells after back-crossing to wild-type, suggesting that viability is not due to a suppressor, unless this suppressor is closely linked to the *hus5* gene. The second possibility is that the N-terminus has a function that is independent of sumoylation, the nature of which is not obvious. Analysis of the structure of Ubc9 indicates that the N-terminus of the protein forms a discrete domain and a loop which is not present in Ubc proteins, which are involved in conjugating ubiquitin to target proteins [28], suggesting a SUMO-specific role for the N-terminus of Ubc9/Hus5. Ubc9 has been shown to be present at nuclear-pore complexes and to interact with the nucleoporin RanBP2/Nup358 [31], which has been shown recently to have SUMO ligase activity [32]. Thus a function of the N-terminus of Hus5 might be to interact with other factors, such as a putative *S. pombe* SUMO ligase, or other components of the sumoylation machinery, e.g. to affect their activity or stability. The third possibility (that a wild-type transcript is capable of being produced) has not been ruled out at this stage, but such a transcript was not detected in this study.

In both the *hus5.62* and *hus5.17* mutants, Pmt3 conjugation is substantially reduced. The two mutants differ in the effect on Pmt3 localization: in the *hus5.62* mutant, Pmt3 is correctly localized within the nucleus, whereas in *hus5.17* cells Pmt3 is mislocalized, as is the case in the *rad31* null mutant. Correct localization of Ubc9 has recently been shown to be important for the localization of SUMO within the nucleus [31]. The data described here indicate that active Hus5 (Ubc9) is not required for entry of Pmt3 into the nucleus, and that the Hus5.62 protein will allow correct localization of Pmt3.

Overexpression of Hus5.62, although resulting in a decrease in overall levels of sumoylation, does not affect cell growth or viability. The ability of cells to survive with substantially reduced levels of SUMO conjugates has recently been observed with the *S. cerevisiae* SUMO ligase null mutants [16]. The reason why cells are viable with such low levels of sumoylation is not clear. It may be due to the fact that sufficient sumoylation is still occurring, in both cases, to allow normal cell function, or that sumoylation of many target proteins is not necessary for cell survival. Studies are currently under way to identify the nature of any proteins that may still be modified in *hus5.62*, but not in *hus5.17*, to allow us to investigate further the role of SUMO modification in the recovery of cells from S-phase arrest.

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