A Role for Ubiquitination in Mitochondrial Inheritance in *Saccharomyces cerevisiae*

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Abstract. The smm1 mutation suppresses defects in mitochondrial distribution and morphology caused by the mdm1-252 mutation in the yeast *Saccharomyces cerevisiae*. Cells harboring only the smm1 mutation themselves display temperature-sensitive growth and aberrant mitochondrial inheritance and morphology at the nonpermissive temperature. smm1 maps to RSP5, a gene encoding an essential ubiquitin-protein ligase. The smm1 defects are suppressed by overexpression of wild-type ubiquitin but not by overexpression of mutant ubiquitin in which lysine-63 is replaced by arginine. Furthermore, overexpression of this mutant ubiquitin perturbs mitochondrial distribution and morphology in wild-type cells. Site-directed mutagenesis revealed that the ubiquitin ligase activity of Rsp5p is essential for its function in mitochondrial inheritance. A second mutation, smm2, which also suppressed mdm1-252 defects, but did not cause aberrant mitochondrial distribution and morphology, mapped to BUL1, encoding a protein interacting with Rsp5p. These results indicate that protein ubiquitination mediated by Rsp5p plays an essential role in mitochondrial inheritance, and reveal a novel function for protein ubiquitination.

Key words: mitochondria • ubiquitin • yeast • organelle inheritance • cytoskeleton

Mitochondria propagate by growth and division of preexisting mitochondria (Palade, 1983; Attardi and Schatz, 1988). Therefore, an essential component of cell proliferation is the distribution of mitochondria to daughter cells before cytokinesis. In the yeast *Saccharomyces cerevisiae*, mitochondrial inheritance involves the vectorial transfer of mitochondria from the mother portion of the cell into the developing daughter bud (Stevens, 1981). Cellular components required for this process have been identified through the analysis of mutant yeast strains that display defects in mitochondrial distribution and morphology, the mdm mutants (M CCConnell et al., 1990; Berger and Yaffe, 1996). These studies have revealed the importance of three integral proteins of the mitochondrial outer membrane (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997), as well as key functions for several cytoplasmic proteins (M CCConnell and Yaffe, 1992; Hermann et al., 1997), but additional components and basic underlying mechanisms remain to be identified.

Mitochondrial inheritance in budding yeast requires Mdm1p, an intermediate filament-like protein localized to a series of punctate structures distributed throughout the cytoplasm (M CCConnell and Yaffe, 1992). The distribution, stability, and apparent composition of these structures suggest that they function as part of a cytoskeleton-like system that mediates mitochondrial and nuclear positioning (M CCConnell and Yaffe, 1992, 1993). In mdm1-1 mutant cells at the nonpermissive temperature, these punctate structures disassemble and mitochondrial transmission to buds is defective (M CCConnell and Yaffe, 1992). Microscopic analysis of mdm1-1 mutant cells has also revealed a role for Mdm1p in the transmission of nuclei to daughter buds, and mdm1-1 cells display defects in orientation of the mitotic spindle (M CCConnell and Yaffe, 1992; Fisk and Yaffe, 1997). These functions of M dm1p in mitochondrial and nuclear inheritance have been uncoupled in a series of additional mutant mdm1 alleles that cause defects exclusively in mitochondrial or nuclear inheritance (Fisk and Yaffe, 1997).

The mdm1-252 mutation causes mitochondrial inheritance defects but has no effect on nuclear segregation (Fisk and Yaffe, 1997). To identify proteins that interact with Mdm1p as part of its function in mitochondrial inheritance, second-site suppressors of mdm1-252 were isolated. A nalysis of two of these suppressors has revealed a role for the ubiquitin-protein ligase, Rsp5p, in mitochondrial inheritance.
### Materials and Methods

#### Yeast Strains and Genetic Methods

*S. cerevisiae* strains used in this study are listed in Table I. Strains MY Y 290, MY Y 291, and MY Y 298 (Smith and Yaffe, 1991, MY Y 403 [McConnell and Yaffe, 1992], MY Y 535 [Nicholas and Yaffe, 1996], and MY Y 700-704 [Fisk and Yaffe, 1997]) have been described previously. Strains MY Y 298 and MY Y 299 were isolated from a backcross of strain MY Y 803 to strain MY Y 291. Strains MY Y 802 and MY Y 803 were isolated from a backcross of strain MY Y 803 to strain MY Y 291. Strains MY Y 818 and MY Y 819 were isolated from a backcross of strain MY Y 803 to strain MY Y 291. Strain MY Y 820, a M A T a strain marked with LEU 2 at the R S P 5 locus, was created as described below. Strain MY Y 823 was created by crossing strain MY Y 809 to strain X 21803A (Yeast Genetics Stock Center), sporulating the resulting diploid, and isolating a temperature-sensitive, U R A - , U R A 3 - strain. Strain MY Y 825 was created by disrupting one copy of R S P 5 with HIS 3 in MY Y 298 as described below. Strains MY Y 826 and MY Y 827 in which B U L 1 is replaced by LEU 2 were generated as described below. Strains MY Y 826, MY Y 829, MY Y 832, MY Y 833, and MY Y 834 were generated by transformation of MY Y 825 with plasmids R S P 316-R S P 5, R S P 516-s mm 1 , R S P 516-mdp 1-13, R S P 516-mdp 1-14, respectively, sporulation of transformed strains, and recovery of H I S - , U R A - , haploid spores. Strains L H Y 1 (R H 448), L H Y 180 (R H 316), L H Y 192 (R H 312), L H Y 201 (R H 3096), L H Y 183 (R H 3147), and L H Y 21 (R H 3097) were obtained from Linda H icke (Northwestern University) and have been described previously (H icke and R iezman, 1996). Media and genetic analyses were as described previously (R ose et al., 1996).

#### Isolation of Second Site Suppressors of mdm1-252

Second-site suppressors were identified by pseudoreversion analysis. Approximately 5 × 10^6 M Y Y 721 (mdm1-252) cells were plated at a density of 0.5–10^8 cells per plate onto yeast extract/peptone/glucose (Y PD) agar medium at 37°C. Cells were replica-plated every day for 5 d onto prewarmed Y PD agar plates at 37°C. Clones able to grow as serial reversion colonies at 37°C were tested for the ability to grow as single isolated colonies at 37°C. Mitochondrial distribution and morphology of apparent revertant colonies were analyzed by staining with the mitochondria-specific vital dye 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI) as described previously (McConnell and Yaffe, 1992). To determine if pseudorevertants harbored second site suppression mutations, candidate strains were backcrossed to strain MY Y 291 and meiotic progeny were analyzed for temperature-sensitive growth defects in the presence of wild-type M D M 1.

#### Phenotypic Analysis

Mitochondrial inheritance in living cells was analyzed by D A S P M 1 stain-1.

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1. Abbreviations used in this paper: C I P , calf intestinal phosphatase; D A P 1 , 4,6-diamidino-2-phenylindole; D A S P M 1 , 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide; Y PD , yeast extract/peptone/glucose.
ing of cells grown in liquid cultures as described previously (McConnell and Yaffe, 1992). Cellular distribution of Mdm1p structures, mitochondrial outer membranes, and microtubules was examined by indirect immu-
nofluorescence microscopy as described previously (Fisk and Yaffe, 1997). Nuclear and mitochondrial DNA was visualized by fluorescence microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI) (Mc-
Connell and Yaffe, 1992; Williamson and Fennell, 1975).

Cloning of smm1 and smm2

Strain MYY823 was transformed with two different genomic libraries in the centromere-based vectors Y Cps0 (Rose et al., 1987) and pSB32 (Rose and Broach, 1991). Four plasmids capable of completely complementing the temperature-sensitive growth of smm1 were isolated: YCps0-3.1 and Y Cps0-3.10 from the Rose library and pSB8-b and pSB-16 from the pSB32-
based library. DNA sequence was determined for each of the gen-
omic DNA inserts in these plasmids using the primer 322 BamHI cd and BamHI cccw sequencing primers (Promega). DNA sequence analysis of plasmid inserts was compared to the Saccharomyces Genome Database using the BLAST program (Altschul et al., 1990).

Strain MY821 was transformed with a genomic DNA library in plas-
mid Y Ep13 (Broach et al., 1979). Three plasmids (Y Ep13.1, Y Ep13.2-2, and Y Ep13.4-1) containing identical yeast DNA inserts were isolated and analyzed as described above.

Integrative Mapping

The smm1 mutation was mapped to the RSP5 locus by integrative trans-
formation and genetic analysis. The integrating vector pRS305-RSP5 was linearized by digestion with MscI and transformed into strain MY829. The resulting strain was sporulated, and a haploid spore with the LEU2 gene integrated at the RSP5 locus was identified (strain MY820). This strain was crossed to smm1 strain MY808, the diploid was sporulated, and haploid progeny were scored for Leu+ growth at 37°C. No recombi-
nants were identified from 28 tetrads, indicating that smm1 was linked to within 1.7 cm of RSP5.

The smm2 mutation was mapped to the BUL1 locus by analyzing mei-
otic progeny resulting from a cross of strain MY81313 (smm2) to strain MYY826, which contained a disrupted copy of BUL1 (bul1:LEU2). A mong 20 tetrads analyzed, all spores were temperature-sensitive, indicating no recombination between smm2 and bul1 and revealing genetic linkage of these two loci within 2.5 cm.

Plasmid Construction

Plasmids pRS316-RSP5 and pRS305-RSP5 were created by cloning the 4,031-bp XbaI-XhoI fragment containing the RSP5 gene from plasmid Y Cps0-3.1 into the XbaI and Xhol sites of pRS316 (Sikorski and Hieter, 1989) and into the Sall and Xbal sites of pRS305 (Sikorski and Hieter, 1989), respectively.

Plasmid pBS-BUL1 was created by cloning the 5,456-bp NheI-SalI fragment from Yepl3-2 between the Sall and Sall sites of pbT1005 (Stragene). Plasmid pBS-ΔbUL1 was created by replacing the region of pBS-BUL1 between the outermost EcoRV sites of LEU2 with LEU2 as described above. A fter digestion of pBS-BUL1 with EcoRV and treatment of calf intesti-
nal phosphatase (CIP), the 5,834-bp vector backbone was isolated by gel purification. A 2,237-bp fragment containing the LEU2 gene was isolated from plasmid Yepl3 after digestion with Sall and Xhol and treatment with the Klenow fragment of DNA polymerase I and ligated into the pBS-
BUL1 EcoRV-deleted backbone.

Plasmid pTER21, pTER22, pTER3, encoding mutant ubiquitin (K29R, K48R, and K63R mutations), respectively (under control of the CUP1 promoter, were described previously (Ellison and Hochstrasser, 1991; A rason and Ellison, 1994). Plasmid plu b, which contains wild-type ubiquitin driven by the CUP1 promoter, was created by replacing the BglII-Sall fragment from pTER22 with the BglII-Sall fragment from plasmid Yepl5 (Ellison and Hochstrasser, 1991).

Plasmid pBS-smm1 was isolated by plasmid-mediated gap repair (Orr-
Weaver et al., 1983), as described below. Plasmids pRS316-Ca, pRS316-
mpd1-1, pRS316-mpd1-13, and pRS316-mpd1-14 were generated by PCR-
mediated site-directed mutagenesis and fragment-mediated gap repair of pRS316-RSP5 as described below. Plasmid pS426-smm1 was created by replacing the 3,897-bp region between the BspaI and Xhol sites of pRS426-RSP5 with the corresponding fragment from pSB-smm1. Plasmid pS316-smm1 was created by replacing the 3,897-bp BspaI-XhoI frag-

ment of pRS316-RSP5 with the corresponding fragment from pRS316-

smm1.

Mutational Analysis and Mutagenesis

The smm1 mutation was shown to lie within RSP5 by plasmid-mediated gap repair (Orr-Weaver et al., 1983). Plasmid pSB-H8 was digested with PvuI and A pal. The resulting vector backbone (17,006 bp) was gel puri-

fied and cotransformed with the same enzyme combination, dephosphorylated with CIP, and transformed into MY808. 24 independent Leu+ isolates were tested for the ability to grow at 37°C. A plasmid, designated pSS-
smm1, was isolated from one of the resulting Leu+ temperature-sensitive clones. The DNA region of this plasmid between PvuI and A pal was se-
quenced, and the only mutation found was a change of G to T at nucleo-
 tide 2358 of RSP5. The sequence of this region of the RSP5 locus was also determined from genomic DNA isolated from MY808 and MYB823 by asymmetric PCR (McCabe, 1990) as described previously (Fisk and Yaffe, 1997). For both strains, the only mutation found was G to T at nucleotide 2258 of RSP5, resulting in the substitution of valine for glycine at position 753 of RSP5 (both numbers are relative to +1 of the RSP5 open reading frame).

A diploid strain deleted for one copy of RSP5 (strain MY 825) was de-

vided from strain MY 829 by PCR-mediated gene disruption using the H153 gene as a selectable marker as previously described (Berger and Yaffe, 1998). A strain deleted for BUL1 was created by transforming the wild-type diploid strain MY829 with plasmid pBS-ΔBUL1 which had been digested with BglII and EcoRV. A Leu+ transformant was then sporulated, and Leu+ haploid spores of MATa (MY 816) and MAt (MY 817) genotypes were isolated. Gene disruptions were confirmed by PCR analysis.

The site-directed mutation C777A was created in RSP5 by PCR in a manner similar to that described by Imhof and M.Cordon (1996), and it was cloned into pRS316-RSP5 by fragment-mediated gap repair as follows. Two separate PCR reactions were performed using pRS316-

RSP5 as a template. The first reaction used the primer pair 5’-CCTCA-

CAACTTAAAGCTG-3’ and 5’-GCGAAGGGGATG-3’ (binds in the multicloning site of pRS316-RSP5, on the 3‘ side of RSP5), and the second used the primer pair 5’-CAGTCTTCATCACTG-3’ and 5’-CTCTTGGATACG-3’ (the lower case letters in these primer sequences indicate mutagenic nucleotides). The products of these two reactions, which overlap by 20 nucleotides, were combined and amplified in the absence of primers to create a final PCR product con-
taining the C777A mutation and spanning from nucleotide 2134 of RSP5 across the pRS316 multicloning site on the 3‘ side of RSP5. Plasmid pRS316-RSP5 was digested with MfeI and SacII and dephosphorylated by CIP treatment. The 7,576-bp vector backbone was gel purified and trans-
fected into MY823 together with the final PCR product. SacII cuts pRS316-RSP5 outside of the yeast insert such that one end of the digested vector only has homology with the cotransformed PCR product. Therefor-
ne the only event that can repair the gapped plasmid is recombination with the PCR product to generate pRS316-CA. Transformation of MY823 with digested vector alone yielded nine Ura+ clones, which were cotransformed with digested vector and the C777A-containing PCR prod-
uct yielded 323 Ura- clones. A Ura- clones testes failed to grow at 37°C. Plasmids were isolated from these clones and digested with NiaI to verify the presence of C777A, and with BmaI to verify the absence of smm1. One of these clones was designated pRS316-Ca, and sequenced to verify that C777A was the only nucleotide change.

The RSP5-mdp1 mutations (Zoladek et al., 1997) were created in pRS316-RSP5 by the method described above, using the following muta-
tagenic primers: mdp1-1, 5’-AGTGGATTGTTGACACAAACTG-3’; 5’-ACGTATTGGTCAATACTG-3’; mdp1-13, 5’-GATATGCGT-
GaTTACCAGAG-3’; 5’-GCCGGTGTTAGCTAAATG-3’; mdp1-14, 5’-GCTGGTACAAGCAGTAAAATG-3’; and 5’-ACTCTTTAAACGCCTTAGGG-3’. Plasmids were isolated from yeast and se-

quenced to verify that the mdp1 mutations were the only changes present.

Results

Isolation of Second-Site Suppressors of mdm1-252

To identify proteins interacting with Mdm1p to mediate mitochondrial distribution and morphology, second site suppressor analysis was performed using the mdm1-252
mutation. This mutation causes temperature-sensitive growth and defects in mitochondrial distribution and morphology, but does not cause abnormal nuclear inheritance (Fisk and Yaffe, 1997). Eight clones capable of growth at 37°C were identified from ~5 x 10^8 cells. Genetic and microscopic analysis revealed that two of these strains possessed single, distinct mutations which suppressed both temperature-sensitive growth (Fig. 1 A) and mitochondrial distribution and morphology defects (Fig. 1 B) caused by mdm1-252. These suppressor mutations also conferred temperature-sensitive growth on cells when separated genetically from the original mdm1-252 lesion (Fig. 2). These new mutations, smm1 and smm2 (suppressor of Mdm1p-dependent mitochondrial inheritance defects), were found to be unlinked to MDM1.

The specificity of suppression by the smm1 and smm2 mutations was determined by crossing cells harboring these lesions to a collection of 10 otherwise isogenic strains containing different mdm alleles (Fisk and Yaffe, 1997) and analyzing the phenotypes of haploid progeny harboring both the mdm allele and the suppressor mutation. Suppression of mdm1 by smm1 was highly allele-specific: smm1 failed to suppress the mutant phenotypes of any allele other than mdm1-252. In contrast, smm2 partially suppressed the mutant phenotypes of several mdm alleles including mdm1-202, mdm1-204, mdm1-251, and mdm1-252. Each of these smm2-suppressed mdm1 mutations is a dominant allele (Fisk and Yaffe, 1997). Additionally, smm2 displayed nonallelic noncomplementation with the recessive mdm1 alleles: heterozygous mdm1/MDM1 smm2/SMM2 diploids displayed temperature-sensitive growth.

**smm1 Affects Mitochondrial Distribution and Morphology**

Cells harboring the smm1 or smm2 mutation were analyzed microscopically to assess the possible effect of these lesions on mitochondrial distribution and morphology. At a permissive temperature (23°C), mitochondrial distribution and morphology in smm1 cells was indistinguishable from wild-type cells (Fig. 3 A). In contrast, cells harboring the smm1 mutation displayed dramatic aberrations in mitochondrial distribution and morphology, similar to those caused by mdm1-252, after incubation at 37°C (Fig. 3 A). Indirect immunofluorescence microscopy further revealed that mitochondria in smm1 cells at 37°C formed small round structures of uniform size which failed to enter buds in a large proportion of cells (Fig. 3 B). DAPI staining of smm1 cells indicated that the smm1 mutation was specific for mitochondrial inheritance, as there was no detectable defect in nuclear segregation (Fig. 3 B). Finally, there appeared to be no effect of smm1 on the distribution or stability of Mdm1p cytoplasmic structures (Fig. 3 B).

**smm1 Is a Mutation in RSP5**

To understand the mechanism of suppression of mdm1-252, smm1 was cloned by complementation. Four plasmids which completely restored growth at 37°C to smm1 cells were isolated. These plasmids also corrected defects in mitochondrial distribution and morphology. Restriction enzyme and nucleotide sequence analysis revealed that these plasmids contained overlapping inserts of yeast genomic DNA corresponding to a 5.6-kb region of chromosome V. Transformation of smm1 cells with different DNA fragments derived from the genomic inserts localized complementing activity to RSP5, an essential gene encoding a ubiquitin-protein ligase containing a HECT domain (Huibregtse et al., 1995). Integrative mapping (as described in Materials and Methods) confirmed that the smm1 mutation mapped to RSP5.

The smm1 mutation was mapped within RSP5 by plasmid-mediated gap repair, and the molecular identity of the mutation was determined by nucleotide sequencing of the
appropriate region of the mutant gene. This analysis revealed a single change in the mutant gene, a transversion of G to T at nucleotide 2258. This mutation in the region of RSP5 corresponding to the conserved HECT domain (Huibregtse et al., 1995) leads to a change of glycine-753 to valine.

smm2 Is a Mutation in BUL1
To investigate suppression by the smm2 mutation, genomic plasmids that complemented the temperature-sensitive phenotype of smm2 mutant cells were isolated. Three complementing plasmids contained the same genomic DNA insert encoding two genes, RCE1 and BUL1, located on chromosome XIII. The complementing activity was mapped to BUL1 by subcloning and retesting complementation in smm2 cells. The isolated BUL1 gene was shown to correspond to sequences from the smm2 locus by integrative transformation and mapping. BUL1 was previously shown to encode a 109-kD protein which binds to the ubiquitin-ligase Rsp5p (Yashiroda et al., 1996).

The smm2 mutation did not cause defects in mitochondrial distribution or morphology (see above). To examine further a possible role for BUL1 in mitochondrial inheritance, a bul1-null allele was created. Like the smm2 mutant, cells deleted for BUL1 (Δbul1) displayed temperature-sensitive growth but no alteration in mitochondrial distribution or morphology at either permissive or nonpermissive temperature (data not shown). Aditionally, the bul1-null mutation failed to suppress mdm1-252.

Ubiquitin Overexpression Influences Mitochondrial Inheritance
Previously, several mutations in the HECT domain of Rsp5p were shown to be suppressed by ubiquitin overexpression (Zoladek et al., 1997). To test whether the smm1 mutation could be similarly suppressed, smm1 mutant cells were transformed with plasmids encoding either wild-type or mutant ubiquitin expressed from the copper-inducible CUP1 promoter. These cells were incubated at 37°C in the presence of 100 μM CuSO₄ (to induce high levels of ubiquitin) and examined by microscopy to assess effects on mitochondrial distribution and morphology. Overexpression of wild-type ubiquitin was found to suppress the mitochondrial morphology and distribution defects of smm1 cells (Fig. 4 A). The specificity of this suppression was investigated by overexpression of versions of ubiquitin mutated in one of three critical lysine residues, K29R (UbK29R), K48R (UbK48R), or K63R (UbK63R). Overexpression of UbK29R or UbK48R suppressed the mitochondrial morphology and distribution defects, similar to suppression by wild-type ubiquitin (data not shown). However, the UbK63R mutant failed to suppress smm1 (Fig. 4 A). No effect of ubiquitin overexpression was observed in mdm1-252 cells at either permissive or nonpermissive temperatures (data not shown).

In control experiments, the effect of ubiquitin overexpression was examined in wild-type cells. Overexpression of wild-type ubiquitin (Fig. 4 A), UbK29R, or UbK48R had no apparent effect on mitochondrial distribution or morphology. However, expression of UbK63R perturbed mitochondrial inheritance in wild-type cells (Fig. 4, A and B). A quantitative analysis of this effect revealed that ~30% of MYY290 cells expressing UbK63R displayed buds devoid of mitochondria, a frequency similar to that seen in MYY823 (smm1) cells (Fig. 4 A). In addition, these cells possessed empty buds of medium to large size and displayed pronounced mitochondrial aggregations (Fig. 4 B). The effect of ubiquitin overexpression was identical whether copper induction was simultaneous with temperature shift or if ubiquitin overexpression was induced for 2 h before temperature shift (data not shown). These results suggest that the formation of polyubiquitin

Figure 2. smm1 and smm2 confer temperature-sensitive growth. Strains MYY803 and MYY804 were backcrossed to MYY291 to separate mdm1-252 from smm1 and smm2, respectively. Spores of the indicated genotypes were plated on YPD medium and incubated 48 h at 37°C. (A) Growth at 37°C of spores from a cross of MYY803 and MYY291; (B) growth at 37°C of spores from a cross of MYY804 and MYY291.
chains linked via lysine-63 is essential for mitochondrial inheritance.

**smm1 Is Distinct from Previously Identified RSP5 Mutations**

Several other temperature-sensitive mutations in RSP5, the mdp1 mutations, were shown to map to the HECT domain (Zoladek et al., 1997). To determine if these mutations behave like smm1 with respect to mitochondrial inheritance, the effect of three previously described lesions, mdp1-1, mdp1-13, and mdp1-14, on suppression of mdm1-252 and on mitochondrial distribution in the MYY 290 genetic background was examined. As expected, each of the mutations conferred a temperature-sensitive growth phenotype on otherwise wild-type cells, but none of the three mdp1 mutations suppressed the mdm1-252 phenotypes (data not shown).

To determine whether the mdp1 mutations affected mitochondrial distribution and morphology, cells were incubated at 37°C and examined microscopically after staining with DASPMI. mdp1-1 caused a modest defect in mitochondrial distribution and morphology, although considerably less than that caused by smm1 (Fig. 5). Neither mdp1-13 nor mdp1-14 caused any defect in mitochondrial inheritance or morphology relative to wild-type RSP5 (Fig. 5). These results indicate that the smm1 mutation confers properties unique from those caused by previously described mutations in RSP5. These findings are largely consistent with a previous study (Zoladek et al., 1995) which found that the mdp1 mutation in a different strain background caused no defects in mitochondrial distribution or morphology.

**Ubiquitination Is Required for Mitochondrial Inheritance**

The smm1 mutation lies in the HECT domain of Rsp5p, suggesting that the mutation may affect ubiquitin ligase activity or substrate specificity. To test whether the ubiquitin ligase activity of Rsp5p is required for mitochondrial inheritance, a mutation in a key residue of the active site...
of Rsp5p was created. The conversion of cysteine-777 to alanine (C777A) was shown previously to destroy the ability of Rsp5p to form a covalent intermediate with ubiquitin, thereby destroying its ubiquitin ligase activity (Huibregtse et al., 1995). The C777A mutation was generated in a plasmid-borne copy of RSP5, and the plasmid was transformed into smm1 cells. The C777A mutant rsp5 failed to complement either the smm1 temperature-sensitive growth phenotype (Fig. 6 A) or the smm1 defects in mitochondrial distribution and morphology (Fig. 6 B). These results demonstrate that the ubiquitin ligase activity of Rsp5p is essential for its function in mitochondrial inheritance.

To further evaluate the requirement of ubiquitination for mitochondrial inheritance, mitochondrial distribution and morphology were examined in cells defective for ubiquitin-conjugating enzymes (E2-type enzymes). S. cerevisiae possesses genes encoding more than a dozen different E2 proteins, but much of the cytoplasmic ubiquitination activity depends on two proteins with redundant specificity, Ubc4p and Ubc5p (Seufert and Jentsch, 1990; Seufert et al., 1990). Strains deleted for UBC4, UBC5, or UBC1 (or combinations of two of these genes) were examined by fluorescence microscopy after mitochondrial staining. Cells with null mutations in both UBC4 and UBC5 displayed aggregated mitochondria and daughter buds devoid of mitochondria (Fig. 7). Both mutant traits were more prevalent after incubation of cells at 37°C, and a quantitation of this effect after 4 h at 37°C revealed 86% of cells with aggregated mitochondria and 52% of budded cells with empty daughter buds. These mutant traits were not apparent in cells with only ubc4 or ubc5 mutations, nor were they found in ubc1 or ubc1 ubc4 mutants (data not shown). In addition, the inheritance and distribution of nuclei were normal in the ubc4 ubc5 mutant cells (data not shown). These results suggest that the ubiquitin-conjugat-
ing enzymes Ubc4p and Ubc5p mediate ubiquitination reactions essential for mitochondrial inheritance.

**Discussion**

We have uncovered a novel role for protein ubiquitination in mitochondrial inheritance. This role was revealed through the characterization of smm1, a mutation that suppresses the mitochondrial distribution and morphology defects caused by mdm1-252. Six key findings support this new function for ubiquitination. First, smm1 mapped to RSP5, an essential gene encoding a ubiquitin-protein ligase. Second, the smm1 mutation alone conferred conditional defects in mitochondrial distribution and morphology. Third, the defects caused by smm1 were complemented by wild-type Rsp5p but not by mutant Rsp5p lacking ubiquitin ligase activity. Fourth, smm2, a second mutation suppressing mdm1-252, mapped to BUL1, a gene encoding a protein that binds to Rsp5p and facilitates its activity (Yashiroda et al., 1996). Fifth, overexpression of a mutant form of ubiquitin which blocks elongation of certain polyubiquitin chains also caused aberrant mitochondrial distribution and morphology in wild-type cells. Finally, depletion of two ubiquitin-conjugating enzymes, Ubc4p and Ubc5p, caused defective mitochondrial morphology and inheritance.

Protein ubiquitination has been found to play a key role in a variety of cellular functions. Prominent among these roles is the ubiquitin-mediated targeting of cytosolic proteins for degradation by the proteosome (Hochstrasser, 1996; Pickart, 1997). Similarly, the proteosome-dependent turnover of several membrane proteins of the endoplasmic reticulum is initiated by their ubiquitination (Brody and McCracken, 1997; Hampton and Bhakta, 1997). Ubiquitination of plasma membrane proteins including the yeast Fur4p (Galan et al., 1996), Ste2p (Hicke and Riezman, 1997), and...
ubiquitination in mitochondrial inheritance represents a novel cellular function for this consequential covalent modification.

The smm1 and mdm1-252 mutations display highly specific reciprocal suppression. smm1 did not suppress any other mdm1 allele, nor was mdm1-252 or any other mdm1 allele suppressed by other rpsp5 mutations. This specificity and reciprocity suggests that Rsp5p and Mdm1p interact directly to effect normal mitochondrial inheritance. The results suggest further that the smm1 and mdm1-252 mutations interfere individually with this interaction but that the combination of the two lesions restores functionality. Similar reciprocal suppression has been demonstrated for specific mutations in actin and actin-binding protein Sac6p in S. cerevisiae (A dams and B otstein, 1989; A dams et al., 1989). Future studies will evaluate the possible direct binding or transient interactions of Rsp5p and M dm 1p.

The mapping of the smm2 mutation to BUL1 further supports a role for ubiquitination in mitochondrial inheritance. The BUL1 product, Bul1p, was previously identified as a protein that binds to Rsp5p (Y ashiroda et al., 1996), and it has been proposed to function as a cofactor, modulating the activity or specificity of the ubiquitin ligase. A similar role is played by the E 6 protein of human papilloma virus in the ubiquitination of p53 (H ubregtse et al., 1991). Although smm2 suppressed mdm1-252, it also displayed genetic interactions with several other mdm1 alleles, indicating that Bul1p may not interact directly with M dm 1p. Furthermore, neither smm2 nor the bul1-null mutation caused any apparent defect in mitochondrial inheritance, indicating that Bul1p is not normally involved in this process. One hypothesis consistent with these observations is that one of three Bul1p homologues in S. cerevisiae, Y ml 111p, Y nr068p, or Y nr069p, may be required for mitochondrial inheritance, and the smm2 mutation might allow Bul1p to supplement or interfere with the activity of its homologue.

The target of Rsp5p-mediated ubiquitination associated with mitochondrial inheritance is unknown. Rsp5p was previously shown to ubiquitinate a variety of cellular substrates including the large subunit of RNA polymerase II (H ubregtse et al., 1997) as well as the plasma membrane proteins Fur4p (G alan et al., 1996) and Gap1p (Springael and A ndre, 1998). M dm 1p appeared to be a reasonable candidate for ubiquitination by Rsp5p, but no evidence of such ubiquitination was obtained. In particular, immunoblot analysis of cellular proteins separated by two-dimensional polyacrylamide gel electrophoresis failed to reveal either ubiquitin associated with M dm 1p or higher molecular weight forms of M dm 1p that might represent ubiquitinated species (data not shown). Other candidates for ubiquitination include proteins integral or bound to the mitochondrial outer membrane. The identification of the relevant Rsp5p substrates may emerge from analysis of proteins ubiquitinated in wild-type cells but not in smm1 or mdm1-252 mutant cells at 37°C.

What might be the role of ubiquitination in mitochondrial inheritance? One possibility is that the ubiquitination of one or more key proteins initiates changes in the interaction of mitochondria with M dm 1p structures. In this model, Rsp5p might first bind to M dm 1p and then ubiquitinate a nearby target protein. The consequence of this activity could be either to promote or diminish an interaction between the target protein and M dm 1p. For example, ubiquitination might promote an association of mitochondria with M dm 1p structures as a critical step in the mitochondrial distribution process. Alternatively, ubiquitination of a mitochondrial surface protein might facilitate its dissociation from M dm 1p structures to mobilize mitochondria. The direct effect of ubiquitination could be to target the ubiquitinated substrate for degradation or, alternatively, ubiquitination might function like other types of covalent modifications to alter the activity or structure of the target protein. The dependence of mitochondrial inheritance on lysine-63 (K 63) of ubiquitin may provide a clue to the fate of the ubiquitinated target protein. Fur4p modification by Rsp5p-dependent addition of K 63-linked polyubiquitin chains leads to the internalization of this protein from the plasma membrane in a proteosome-independent event (G alan and H aguenauer-T sapis, 1997). In contrast, the addition of polyubiquitin chains linked through lysine-48 leads to the proteosome-dependent degradation of the M A T a2 transcriptional regulator (H ochstrasser et al., 1991). Because Rsp5p-dependent formation of K 63-linked chains appears to be required for mitochondrial inheritance, ubiquitination may play a proteosome-independent role in mitochondrial inheritance. The identification of the relevant substrates of ubiquitination and a characterization of these proteins' molecular interactions should uncover biochemical details of the function of ubiquitination in mitochondrial inheritance.

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