The mitochondrial ABC transporter Atm1p functions as a homodimer

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Abstract The ATP-binding cassette (ABC) transporters constitute one of the largest families of proteins in evolution. The *ATM1* gene of the yeast *Saccharomyces cerevisiae* encodes an ABC protein, which is localized to the mitochondrial inner membrane. A deletion of *ATM1* results in the accumulation of up to a 30-fold excess of mitochondrial iron, loss of mitochondrial cytochromes and abnormalities of cytosolic iron metabolism. In this study, we have evaluated the role of conserved sequence elements in Atm1p in its function and dimerization in vivo. We report that conserved residues in the Walker A and B motifs of the nucleotide binding domain, which are required for ATP binding and hydrolysis, are essential for Atm1p function. In addition, we provide evidence that ATP binding is important for Atm1p dimerization.

Keywords: ABC transporter; Mitochondrion; Iron metabolism; Fe-S protein

1. Introduction

The ATP-binding cassette (ABC) transporters constitute one of the largest families of proteins in evolution with numerous examples present in archea, eubacteria, and eukaryotes. The majority of these proteins are involved in the energy-dependent transport of substrates across cellular membranes, and their impaired function is related to a wide variety of inherited diseases. Well-known examples of ABC transporters include the cystic fibrosis transmembrane conductance regulator (CFTR) and the multidrug resistance proteins. The canonical structure of a functional transporter includes two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). While TMDs determine substrate specificity, the NBDs serve as the motors, coupling the energy of ATP binding and hydrolysis to transmembrane transport.

The *ATM1* gene of the yeast *Saccharomyces cerevisiae* encodes an ABC transporter whose disruption results in the accumulation of up to a 30-fold excess of mitochondrial iron, loss of mitochondrial cytochromes, oxidative damage to the mitochondrial DNA, and decreased levels of cytosolic heme proteins [1,2]. Deletion of *ATM1* also results in cytosolic iron starvation and loss of cytosolic Fe-S proteins [3,4]. Atm1p is localized to the mitochondrial inner membrane with its carboxy terminal NBD in the matrix, which predicts that it functions to export substrate from the matrix to the intermembrane space [2]. The specific function of Atm1p has not been identified, but based on the phenotype of Δ*atm1* cells, roles in mitochondrial iron export and/or Fe-S cluster assembly have been proposed [3,4].

The structure and function of ABC transporters has been intensively investigated. As a result, the specific functions of conserved sequence elements are being defined. Previous studies of Atm1p have been based primarily on the analysis of yeast in which the *ATM1* gene has been deleted, but a systematic analysis of specific sequence elements has not been undertaken. In this paper, we describe studies to assess the role of conserved elements in NBD in the function of Atm1p. We report that mutation of residues that affect either ATP binding or hydrolysis results in a loss of Atm1p function in vivo. Additionally, we have determined that Atm1p functions as a homodimer and that mutations in the NBD affect dimer stability.

2. Materials and methods

2.1. Yeast strains and culture conditions

With the exception of the ATP-binding analysis, all studies were performed in the W303 strain (*MATa, ade2, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*). Disruption of the chromosomal *ATM1* gene was done using a targeting allele made by inserting the *HIS3* gene into the unique Tth1 1 1 I and *Xba*I sites in the *ATM1* gene. The ATP-binding experiments were done in strain YKB5 (*MATa, Δura3, his4-519, leu2-3,112, atm1::LEU2*) [2]. Growth and manipulations of yeast were performed using standard procedures [5]. Yeast were grown in Hartwell's Complete (HC) medium (0.145% yeast nitrogen base with amino acids, 0.38 M $(NH_4)_2SO_4$, and either 2% glucose or lactate). *Escherichia coli* manipulations were performed using the strains DH5α and TOP1 OF' (Invitrogen) according to standard procedures [6].

2.2. Construction of plasmids

Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The template for mutagenesis consisted of the entire Atm1p open reading frame, along with approximately 500 bp of 5'-flanking sequence and a C-terminal epitope tag, in YEplac112 (*TRP1*, HA tag) or YEplac195 (*URA3*, V5 tag). The following primers were used for mutagenesis:

K475M – forward (5′-GGTCTTCGGGCAGTGGGATGTCCAC-CATTTTAAAATTAGTC), reverse (5′-GACTAATTTTAAAATG-GTGGACATCCCAAGACC); E598A – forward (5′-CATGTTTTT-TGACGCGGCCACAAGCGCC), reverse (5′-GGCGGCTTGTGG-CCGCGTCAAAAAACATG). For the complementations assays, wild type and mutant alleles of *ATM1* were expressed in a centromeric

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Abbreviations: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; HC, Hartwell's complete medium; IP, immunoprecipitation; NBD, nucleotide binding domain; TMD, transmembrane domain

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plasmid with a *LEU2* marker that was generated from YCplac33 via marker swap [7]. To test for dominant-negative effects, the K475M were expressed in wild type cells under the control of the galactose inducible *GAL1* promoter in pYES 2.0.

2.3. Immunoprecipitation

Mitochondria (2 mg for proteins expressed in a centromeric plasmid, or 500 μg for high copy plasmids) were solubilized in immunoprecipitation buffer (IP; 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.2% Triton X-100) and incubated overnight at 4 °C with 1 μg of either anti-HA or anti-V5 antibodies (Invitrogen). Antigen-antibody complexes were precipitated with protein-A Sepharose beads for 90 min at 4 °C, collected by centrifugation, washed three times with IP buffer, and resuspended in Laemmli sample buffer [8]. Proteins were separated by SDS–PAGE and transferred to PVDF membranes by electroblotting. Western-blot analysis was performed with either anti-HA (1:1000) or anti-V5 (1:5000) antibodies, and a horseradish peroxidase conjugated secondary antibody, and visualized by ECL [9].

2.4. ATP binding

These studies were done in a Δ*atm1* strain generated in the YKB5 background, which has a relatively mild phenotype [2]. This allowed for the growth and isolation of mitochondria from cells expressing the mutant *ATM1* alleles in the absence of any wild type protein. Mitochondria were isolated from Δ*atml* cells expressing V5 epitope-tagged versions of either the wild type or mutant Atm1p from high copy plasmids. Binding of [^{32}P]-labeled ATP was assayed using a method based on a published procedure [10]. Mitochondrial samples were subjected to immunoprecipitation with anti-V5 antibody, and after collection on protein-A Sepharose, the samples were divided into two fractions. Unlabeled ATP was added to a final concentration of 5mM to one of the aliquots, followed by the addition of 2 μCi of 8-azido-[^{32}P]-ATP (ALT) to both samples. Photo-crosslinking was performed for 3 min at 245 nm. Samples were then washed several times with IP buffer, separated by SDS–PAGE, and visualized by autoradiography of the dried gel.

2.5. Miscellaneous techniques

Protein concentration was determined using the BCA kit (Pierce) with bovine serum albumin as a standard. Yeast and bacterial transformations were done using standard techniques [5,6]. Mitochondria were isolated from cells grown to mid log phase [$A_{600} = 0.6$] in HC-2% lactate by differential centrifugation [11].

3. Results and discussion

3.1. Role of conserved residues in the nucleotide binding domain

ABC transporters function in the transmembrane transport of a diverse array of substrates, ranging from metal ions to peptides. As a result, the sequences of their TMDs, which determine substrate specificity, have undergone significant evolutionary divergence. In contrast, the NBDs, whose ATPase activity energizes transport, show significant sequence conservation [12] and have a common three-dimensional structure [13–15]. Within the NBD, the highest conservation is seen in the Walker A and B motifs, which are common to many ATPases, and the signature sequence, which is characteristic of ABC transporters [16–18]. These conserved elements are required for ATP binding and hydrolysis [19].

The sequences of the Walker A and B motifs of Atm1p and several closely related yeast ABC transporters are shown in Fig. 1. The Walker A lysine, corresponding to lysine 475 in Atm1p, is found in all ABC transporters [20]. We found that substitution of this lysine with methionine (K475M) resulted in a complete loss of Atm1p function (Fig. 2A, B). Western-blot analysis demonstrated the presence of similar levels of the wild type and K475M mutant proteins (Fig. 2C), indicating that the inability of this allele to complement Δ*atml* cells was not due to decreased protein expression. Furthermore, the K475M mutant could be co-immunoprecipitated with the wild type protein (see below, Fig. 4C), indicating that it was properly localized to the mitochondrial inner membrane. Residues in the Walker A motif have been shown by X-ray crystallography to interact with the β and γ phosphate groups of ATP [13], suggesting that the inactivation of Atm1p by the K475M mutation may be due to decreased ATP binding. Therefore, we assessed ATP binding by the K475M mutant using the photo cross-linkable ATP analog 8-azido [^{32}P]ATP. ATP binding was significantly decreased by the K475M mutation (Fig. 3A). A Western-blot of the same mitochondrial samples used for ATP binding showed a high level of expression of the K475M protein (Fig. 3B), confirming that the decreased signal in Fig. 3A was due to an effect of the mutation on ATP binding and not a lack of protein.

The ATP binding experiments were carried out in cells of the YKB5 background, which can be cultured in the absence of any functional Atm1p [2,21]. The use of this strain was necessary, since a Δ*atm1* mutation is lethal in the W303 strain that was used for the complementation analysis in Fig. 2 (see Section 2). A comparison of Figs. 2 and 3 CB shows variability between the level of expression of the plasmid borne alleles of *ATM1* between these strains and amongst the YKB5 cells expressing the different *ATM1* genes. However, both the ATP binding and Western blotting shown in Fig. 3 were done using the same sample of isolated mitochondria, and thus the levels of protein seen in Fig. 3B are an accurate reflection of the level of each protein present in Fig. 3A.

Studies of both prokaryotic and eukaryotic ABC transporters have shown that the glutamate immediately adjacent to the Walker B motif (Fig. 1) is essential for ATP hydrolysis [13,19]. We found that substitution of the corresponding glutamate of Atm1p with alanine (E598A) resulted in a loss of function (Fig. 2A, B) but did not affect the level of expressed protein (Fig. 2C), or its ability to associate with the wild type protein (see below, Fig. 4B). In contrast with the K475M

	Walker A	Signature	Walker B
ATM1	GSSGSGKST	.....ISGGEKQRLAIARVLLKNARIMFFDEATSALD	
MDL1	GPSGSGKST	.....LSGGQKQRIALARAFLLDPAVLILDEATSALD	
MDL2	GPSGRGKST	.....LSGGQKQRIAIARALIKKPTILILDEATSALD	
STE6N	GKSGSGKST	.....LSGGQQQRVAIARAFIRDTPILFLDEAVSALD	
STE6C	GESGTGKST	.....LSGGQAQRLCIARALLRKSKILILDECTSALD	
	*		*

Fig. 1. Sequence comparison of conserved regions of yeast ABC transporters. The aminoacid sequence of the region of Atm1p containing the Walker A, B and the signature sequences is compared to other members of the MDR sub-group of yeast ABC proteins. STE6N and STE6C correspond to the amino and carboxy terminal nucleotide binding domains of Ste6p, respectively. The consensus residues of each motif are underlined. The conserved Walker A lysine and the glutamate adjacent to the Walker B sequence are indicated with an asterisk.

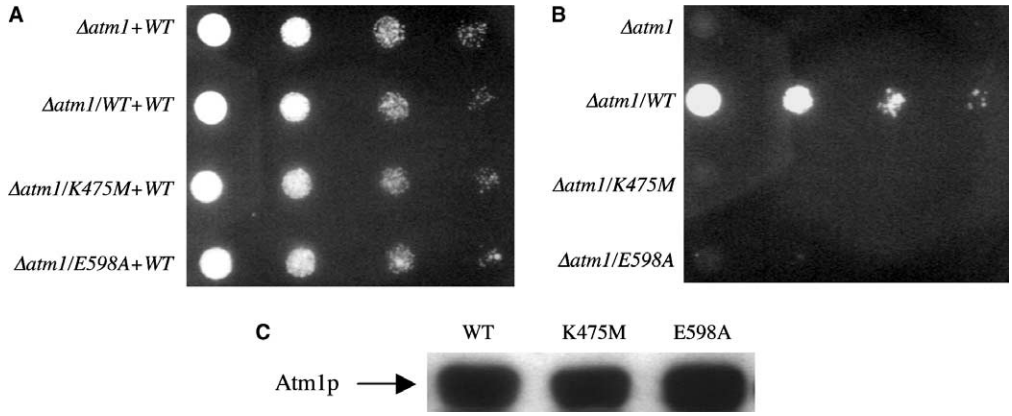

Fig. 2. Complementation assays for the Walker A (K475M) and Walker B (E598A) mutants. A, Δ*atm1* cells containing a wild type allele of *ATM1* on a plasmid with a *URA3* selectable marker (Δ*atm1*+*WT*) were transformed with a second plasmid bearing a *LEU2* selectable marker and expressing the wild type *ATM1* gene (Δ*atm1*/*WT*+*WT*), an *ATM1* allele with the K475M mutation (Δ*atm1*/*K475M*+*WT*), or an *ATM1* allele with the E598A mutation (Δ*atm1*/*E598A*+*WT*). Equal numbers of cells from each strain were serially diluted and dotted onto HC/2% glucose medium lacking uracil and leucine to select for cells containing both plasmids. B, Cells from each of the strains were plated on HC/2% glucose medium lacking leucine and containing 5′-fluoroorotic acid, which is toxic to cells that express the *URA3* gene, to select for cells which have lost the wild type allele *of ATM1*. C, Western-blot analysis of immunoprecipitations of Atm1p from mitochondria isolated from cells expressing the wild type, K475M, or E598A alleles. Immunoprecipitations and Western blotting were done with a mouse monoclonal antibody against the V5 epitope tag, located at the carboxyl terminus of each expressed protein.

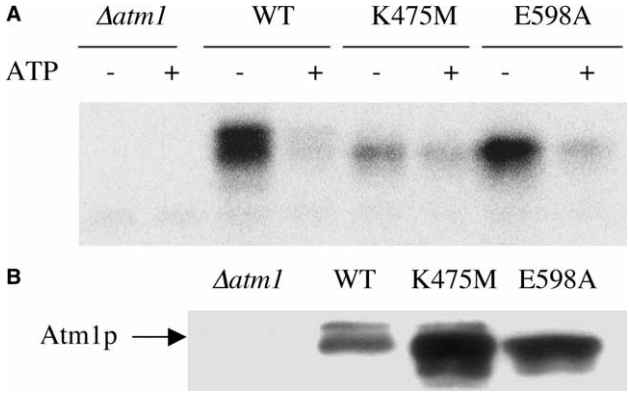

Fig. 3. Analysis of ATP binding. A, Mitochondria from Δ*atm1* cells or Δ*atm1* cells expressing a plasmid borne copy of the WT, K475M, or E598A alleles were purified and subjected to immunoprecipitation with an anti-V5 antibody. Immunoprecipitated proteins were incubated with 8-azido-[^{32}P]ATP in the presence or absence of an excess of unlabeled ATP. Analysis of the amount of bound [^{32}P]ATP was visualized by autoradiography on dried gels following SDS–PAGE. B, Aliquots of the isolated mitochondria used for ATP-binding experiments were subjected to immunoprecipitation and Western blotting using anti-V5 antibodies.

mutant, the E598A mutation resulted in only a small decrease in ATP binding (Fig. 3). This is similar to results reported from studies of the NBD of the closely related yeast Md11p, which demonstrated a 3000-fold decrease in ATPase activity but no decrease in ATP binding following mutation of this conserved glutamate [22].

We have observed that both the wild type and mutant forms of Atm1p often migrate as a doublet on SDS–PAGE (Fig. 3). The presence of the double band is inconsistent and is seen in blots from lysates of whole mitochondria as well as following immunoprecipitation. With the wild type protein, both of these bands were capable of binding ATP (Fig. 3A). The ratio between the two bands was identical under reducing and non-reducing conditions and was unaffected by alkaline phosphatase treatment (not shown). These data indicate that differences in intramolecular disulfide bonds and phosphorylation are not likely responsible for the presence of the two species of Atm1p. The faster migrating species may result from partial proteolysis, but further characterization will be required to address this question.

Most ABC proteins function as ATP-dependent membrane transporters and share the common requirement for an active ATPase. The isolated NBD of the closely related *Schizosaccharomyces pombe* Atm1 protein has been demonstrated to have ATPase activity, though its functional role has not been investigated [23]. We have shown that the wild type Atm1p binds ATP and that mutation of the conserved Walker A lysine resulted in significantly decreased ATP binding and a loss of function. We also observed that a mutation of glutamate 598, which is predicted to be the catalytic residue and essential for ATPase activity [13,19], inactivated Atm1p. Collectively, these results demonstrate that Atm1p requires an active ATPase for its function.

3.2. Atm1p forms a homodimer

The four-domain structure of a functional ABC transporter can be formed from a single polypeptide or by the association of two to four separate subunits [24]. In eukaryotes, most ABC proteins are encoded as single polypeptides, containing two TMDs and two NBDs [25]. A small number are formed by the dimerization of half molecules composed of single transmembrane and nucleotide binding domains [26].

Atm1p is a half ABC transporter with an amino terminal TMD and a carboxy terminal NBD. Thus, it is predicted to function as a dimer. The yeast genome encodes four additional half ABC transporters with this structural organization that could potentially form a heterodimer with Atm1p [27]. Two of these proteins (Pxa1p and Pxa2p) are localized to the peroxisome [28] and the other two (Md11p and Mdl2p) are mitochondrial [29]. We recently identified Mdl1p as a high copy suppressor of an Δ*atml* mutation, but we found no evidence that it forms a heterodimer with Atm1p or has a physiologic

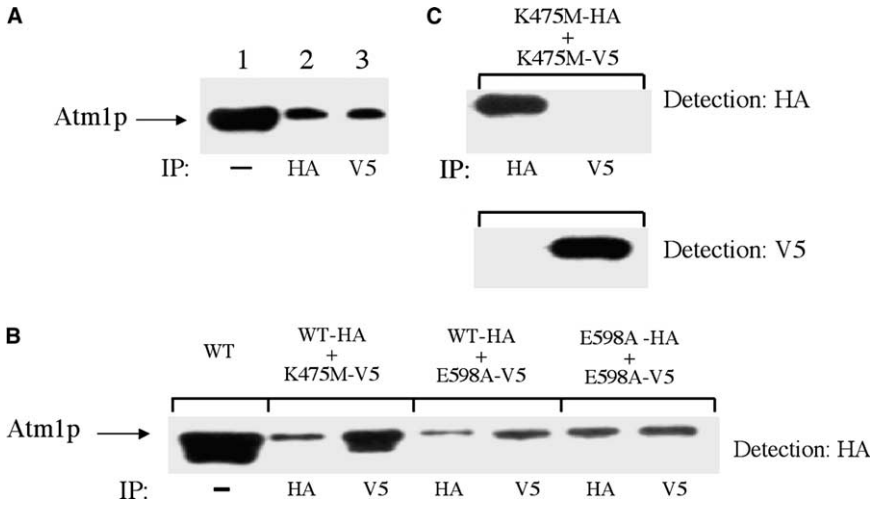

Fig. 4. Co-immunoprecipitation analysis of Atm1p. A, Mitochondria were isolated from a yeast strain expressing both HA and V5 epitope tagged alleles of *ATM1* and analyzed by immunoprecipitation. Western-blot analysis was performed with an anti-HA antibody. Lane 1: mitochondrial lysate, Lane 2: HA immunoprecipitation, Lane 3: V5 immunoprecipitation. B, Mitochondria were isolated from yeast strains expressing the indicated alleles of Atm1p and subjected to immunoprecipitation with anti-HA and anti-V5 antibodies. Western-blot analysis was done with an anti-HA antibody. C, Western-blot analysis using either an anti-HA antibody (upper panel) or an anti-V5 antibody (lower panel). WT-HA, wild type protein with HA epitope tag; K475M-V5, K475M mutant protein with V5 epitope tag; K475M-HA, K475M mutant protein with HA epitope tag; E598A-V5, E598A mutant protein with V5 epitope tag; E598A-HA, E598A mutant protein with HA epitope tag.

role in iron metabolism [21]. Deletion of *MDL2* has no effect on growth in minimal glucose media [29], whereas a deletion of *ATM1* results in a severe growth defect (Fig. 2B), making it unlikely that Atm1p functions as a heterodimer with Mdl2p.

To determine whether Atm1p forms a homodimer, we constructed alleles with either an HA or V5 epitope tag and co-expressed them in yeast. The epitope tags were located at the carboxyl terminus of the protein and did not affect the ability of the tagged proteins to complement a Δ*atm1* strain (not shown), confirming that both tagged proteins were functional and properly localized. Immunoprecipitation using monoclonal antibodies against the HA and V5 tags demonstrated that Atm1p forms a homodimer (Fig. 4A). However, we do not know whether Atm1p functions as a dimer, or as part of a larger oligomeric complex. The human ABCG2, which like Atm1p is a half ABC transporter, forms a homotetramer [30]. Using gel filtration chromatography, we have observed monomer, dimer, and higher molecular weight forms of Atm1p (not shown). We are currently characterizing the oligomeric form of Atm1p.

3.3. ATP binding is required for dimerization of Atm1p

The dimerization of ABC transporters involves interactions between the TMDs and the NBDs. Structural studies of bacterial ABC transporters have shown that dimerization of the NBDs results in the sandwiching of an ATP molecule between the Walker A motif of one subunit and the signature sequence of a second subunit [13,31]. The DNA repair protein Rad50, which contains an ABC-type NBD, also requires ATP for dimerization [32]. These and other studies have led to the hypothesis that the energy associated with ATP binding drives the dimerization of the two NBDs [13,14,32]. Having observed that the K475M mutation significantly decreased ATP binding, we looked at whether this mutation also affected the ability of the protein to form a stable homodimer [K475M:K475M], or a heterodimer with the wild type protein [K475M:WT]. We observed that the K475M and WT proteins could be co-immunoprecipitated (Fig. 4B), whereas despite multiple attempts, we were unable to co-immunoprecipitate a K475M homodimer (Fig. 4C). Similar experiments with the E598A mutant demonstrated both homodimers [E598A:E598A] and heterodimers [E598A:WT] in yeast mitochondria (Fig. 4B). These results suggest that ATP-dependent interactions between the NBDs are crucial for the formation of a stable Atm1p dimer.

Current models for the mechanism of transport by most ABC proteins include a requirement for two functional NBD ATPases [33]. Thus, a heterodimer of active and inactive monomers is predicted to be non-functional. Studies with ABCD1 (ALDP), a homodimeric human half ABC transporter of the peroxisome, have shown that co-expression of wild type and mutant proteins leads to a loss of function of the wild type protein via a dominant-negative effect [34]. The ability of the inactive K475M and E598A mutant proteins to dimerize with wild type Atm1p predicts that their overexpression in wild type cells could also have a dominant-negative effect. To test this hypothesis, wild type cells were transformed with a plasmid containing the wild type, K475M, or E598A mutant allele of *ATM1* under the control of the galactose inducible *GAL1* promoter. Surprisingly, we found no difference between the growth of cells overexpressing the wild type and mutant forms of Atm1p in either glucose- or galactose-containing medium (Fig. 5). We also found no differences in mitochondrial iron content between the strains overexpressing the wild type, K475M and E598A mutant proteins (not shown).

The lack of a dominant-negative effect of the K475M and E598A alleles of *ATM1* could result from preferential association between wild type proteins, such that even in the presence of an excess of mutant monomers, sufficient numbers of functional wild type dimers are formed to allow for cellular survival. Alternatively, our observations may indicate that the two NBDs of the Atm1p homodimer are actually functionally asymmetric and that only one active ATPase is required for function. ABC transporters in which the two NBDs are known

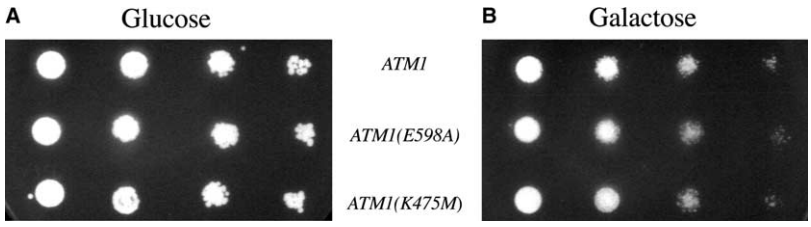

Fig. 5. Test for dominant-negative effects of the Walker A (K475M) and Walker B (E598A) mutants on wild type yeast. The wild type, K475M, or E598A alleles of *ATM1* were expressed in WT cells under the control of the galactose inducible *GAL1* promoter in pYES 2.0. Equal numbers of cells from each strain were serially diluted and dotted onto a -URA plate containing either glucose or galactose.

to be functionally asymmetric have been identified, including several that perform non-classical ABC functions (i.e., they are proteins that do not simply translocate substrates across membranes in an ATP-dependent manner). For example, the sulfonylurea receptors (SUR1, 2A, 2B) are ABC proteins that function as regulators of ATP sensitive potassium channels [35], and the CFTR functions as both a chloride channel and a regulator of other plasma membrane ion channels [12,36]. The potential asymmetry of the Atm1p ATPase suggests that it may also perform a function other than that of an ATP-dependent transporter. However, understanding the reason for the lack of a dominant-negative effect of the mutant proteins, and determining the true role of Atm1p in Fe-S cluster biosynthesis will require further in vitro and in vivo studies.

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