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The *LETM1/YOL027* Gene Family Encodes a Factor of the Mitochondrial K^+ Homeostasis with a Potential Role in the Wolf-Hirschhorn Syndrome*

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The yeast open reading frames *YOL027* and *YPR125* and their orthologs in various eukaryotes encode proteins with a single predicted trans-membrane domain ranging in molecular mass from 45 to 85 kDa. Hemizygous deletion of their human homolog *LETM1* is likely to contribute to the Wolf-Hirschhorn syndrome phenotype. We show here that in yeast and human cells, these genes encode integral proteins of the inner mitochondrial membrane. Deletion of the yeast *YOL027* gene (*yol027*Δ mutation) results in mitochondrial dysfunction. This mutant phenotype is complemented by the expression of the human *LETM1* gene in yeast, indicating a functional conservation of LetM1/Yol027 proteins from yeast to man. Mutant *yol027*Δ mitochondria have increased cation contents, particularly K^+ and low-membrane-potential ΔΨ. They are massively swollen *in situ* and refractory to potassium acetate-induced swelling *in vitro*, which is indicative of a defect in K^+/H^+ exchange activity. Thus, *YOL027/LETM1* are the first genes shown to encode factors involved in both K^+ homeostasis and organelle volume control.

Respiring mitochondria maintain a membrane potential ($\Delta\Psi$)[1] of −150 to −180 mV (ΔΨ, inside negative). This high ΔΨ constitutes a large driving force for the electrophoretic influx of cations, either through specific channels or by diffusion through the membrane. Several cation channels have been characterized physiologically (reviewed in Refs. 1 and 2), and recently, a single one has been identified molecularly (3). These transport systems seem to have intrinsic control mechanisms which ensure that the matrix cation concentrations stay within physiological ranges, far below chemical equilibrium.

Diffusive permeability of the inner mitochondrial membrane to ions is generally low but physiologically significant, as it lowers the pH gradient and membrane potential. Moreover, if not counteracted by extrusion, steadily increasing concentrations of matrix cations (and of compensating anions) will lead to an imbalance of osmotic pressure across the inner mitochondrial membrane. As a consequence, water will pass through the membrane, causing excessive swelling and eventual rupture of the organelle (1, 2, 4).

As first proposed by P. Mitchell (5), mitochondria have carrier systems allowing the electroneutral exchange of cations against H^+ (and anions against OH^-). These exchangers counteract the ΔΨ-driven cation leakage of the membrane and also cation imbalances due to changes in mitochondrial physiology. Mitochondrial cation distribution is, therefore, a steady state, in which the accumulation ratio is modulated by the relative rates of cation influx and efflux by means of separate pathways.

Many physiological studies have been devoted to cation/H^+ exchange systems (reviewed in Ref. 1). With respect to the most abundant cations in cells and mitochondria, K^+ (150 mM) and Na^+ (5 mM), researchers agree on the existence of two separate antiporters in mammalian cells, a selective Na^+/H^+ exchanger, and an unselective K^+/H^+ exchanger transporting virtually all alkali ions. Given the particularly high concentration of K^+ in cells and mitochondria, the unselective exchanger is referred to most commonly as the K^+/H^+ exchanger (reviewed in Ref. 1). This exchanger has pronounced sensitivity to matrix [Mg^{2+}] (K_i of 0.3–0.4 mM in mammalian mitochondria), timolol, and quinine. Proteins of apparent molecular masses of 82 and 59 kDa constitute the unselective mitochondrial K^+/H^+ exchanger and the selective Na^+/H^+ exchanger, respectively (6, 7). Attempts to identify the gene encoding the K^+/H^+ have not been successful yet (2), and a report on the identification of the yeast *NHE2* and its mammalian homolog *NHE6* as encoding the mitochondrial Na^+/H^+ exchanger (8) have been questioned recently (9).

In the course of characterizing a set of yeast genes potentially encoding mitochondrial cation transport proteins (10) we focused on the yeast genes *MRS7* and *YOL027*, as well as their human homolog *LETM1* (leucine zipper/EF-hand-containing trans-membrane domain; Ref. 11), which are representatives of a novel eukaryotic gene family with hitherto unknown function. Hemizygous deletion of a region on human chromosome 4 (4p16.3), including *LETM1* and several other genes, causes the Wolf-Hirschhorn syndrome. Recent data reveal that the full Wolf-Hirschhorn syndrome phenotype, including neuromuscular features, such as seizures correlates with the deletion of the *LETM1* gene (12).

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[1] The abbreviations used are: ΔΨ, membrane potential; KOAc, potassiumacetate; HA, hemagglutin; GFP, green fluorescent protein; YPGal, yeast extract, bacto peptone, 2% galactose; YPD, yeast extract, bacto peptone, 2% dextrose; SMP, submitochondrial particles; DCCD, dicyclohexylcarbodiimide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; A23187, 4-bromo-calcium ionophore A23187; TM, transmembrane.

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We report here on the mitochondrial localization of the human LetM1 protein and of its yeast homologs, Yol027p and Ypr125p (Mrs7p), on their functional homology, and on the effects resulting from the disruption of the yeast genes on mitochondrial functions. The results indicate a role of Yol027p in mitochondrial K^+ homeostasis. As compared with wild-type mitochondria, mutant *yol027*Δ mitochondria exhibit severely reduced potassium acetate (KOAc)-induced swelling, which is indicative of a lack or reduction in K^+/H^+-exchange activity. We discuss the possibility that *YOL027* encodes either the K^+/H^+ exchanger itself or an essential cofactor thereof.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Media—The following strains of *Saccharomyces cerevisiae* were used: GA74–1A (13), DBY747 (ATCC no. 204659), and W303 (ATCC no. 201239), all of which served as wild-type strains, and DBY747 *mrs2*Δ (14). Yeast growth media were as described previously (14).

Hemagglutin (HA)-tagged and Green Fluorescent Protein (GFP)-tagged Genes—A transposon-tagged *YOL027c*-containing DNA fragment (15) was used to replace the chromosomal copy of *YOL027c* in strain GA74–1A. Upon Cre-mediated recombination, a variant of the *YOL027c* gene was obtained which had a triple HA-tag inserted in-frame after codon 469. This HA-tagged version of the *YOL027c* gene had no apparent phenotypic effect on the growth of the mutant strain compared with the isogenic wild-type strain.

The *YOL027c* gene sequence (nucleotides −426 (relative to the start codon) to +1721) was PCR-amplified from W303 genomic DNA by use of an oligonucleotide 5′ primer carrying a natural SacI site and a 3′ primer that introduced a PstI site upstream of the stop codon. The SacI-PstI fragment of this product was inserted into YCp33-HA vector, resulting in plasmid YCp-*YOL027*-HA.

YPR125 coding sequence (1356 nucleotides from 1 to +1356) was PCR-amplified from the same yeast strain, introducing recognition sites for EcoRI and SalI, followed by insertion of this fragment into the EcoRI and SalI sites of the centromeric vector pUG35. The resulting plasmid pUG35-*YPR125*-GFP expressed the *YPR125* gene under the *MET25* promoter and in-frame with the GFP coding sequence following at its 3′ end. The YEp-*YPR125* construct (YEp-MW7) had been cloned by Waldherr *et al*. (10).

To clone the human *LETM1* cDNA, SuperScript II reverse transcriptase (Invitrogen) and an oligo(dT) primer were used for first-strand synthesis on poly(A)-enriched template RNA isolated from a PA-1 human teratocarcinoma cell line. PCR fragments covering the entire coding sequences (from nucleotide 1 to +2590) were amplified, and the purified PCR product was cloned to the pGEM-T vector (Promega), and a C-terminal HA-tag was added by PCR. The PCR product was digested with XhoI and HindIII and cloned into the corresponding sites of the yeast expression vector, pVT103-U. An XhoI-EcoRI PCR fragment was cloned into the pEGFP-N1 mammalian expression vector, thus creating an in-frame fusion with the enhanced GFP (EGFP) sequence carried on the vector.

Gene Deletion—Complete disruptions of *YOL027*, *MRS2*, and *MRS7* were performed according to the one-step replacement protocol described in Ref. 16 in the diploid yeast strain W303 and the haploid DBY747. Disruption of *YOL027* resulted in a deletion of 1702 nucleotides (from the start codon to nucleotide −19, relative to the stop codon) of the *YOL027* open reading frame (named *yol027*Δ mutant). Spores derived from this diploid strain were found to be viable. A disruption of the same size then was obtained in DBY747 (haploid). Disruption of the open reading frames *MRS2 (mrs2*Δ) and *MRS7* (*mrs7*Δ) resulted in deletions of 1218 nucleotides (from nucleotide −49 relative to the start codon to nucleotide −243 relative to the stop codon) and of 1356 nucleotides (from the start to the stop codon).[2]

W303 *yol027*Δ*mrs2*Δ and *yol027*Δ*mrs7*Δ double-mutant strains were then obtained by crossing the *yol027*Δ strain with the *mrs2*Δ or *ypr125*Δ strain, respectively. Diploids were sporulated, and the haploid double mutants were identified among the meiotic progeny by screening for the appropriate combination of disruption markers.

Isolation and Subfractionation of Mitochondria—GA74–1A were grown in lactate medium (17), W303 cells in complete YPGal medium or synthetic S-galactopyranoside (S-Gal) medium containing 2% galactose. Strain DBY747 was cultivated in YPD medium. For ion-influx measurements, cells were grown to stationary phase; for all other applications, cells were harvested at A_{600} of 1. Mitochondria were prepared according to Ref. 18 and suspended in breaking buffer (0.6 M sorbitol HEPES-KOH, pH 7.4) prior to further use.

Na_2CO_3 extraction of proteins from membranes and mitoplast preparation was as described in Refs. 20 and 18, respectively.

Yeast mitochondria obtained by differential centrifugation were diluted to a protein concentration of 10 mg/ml with 10 mM HEPES-Tris-Cl, pH 7.4, and a final osmolarity of 0.1 M. After a 20-min incubation on ice, the resulting mitoplasts were collected by centrifugation (40,000 × *g* for 10 min at 4 °C). To obtain submitochondrial particles (SMP), mitoplasts were resuspended in sucrose buffer (250 mM sucrose, 10 mM Tris-Cl, pH 7.4). The mitoplast suspension was sonified for 3 min with maximum intensity in a Bandelin sonicator UW70/GM70. After removing unbroken mitochondria (10-min centrifugation at 10,000 × *g*), SMPs were collected by centrifugation at 100,000 × *g* for 1 h and resuspended in 1 ml of sucrose buffer.

Antibodies used for immunodetection were as described in Ref. 19 or kindly provided by Hans van der Spek (Tom44p), Jan Brix (Tom70p), Doron Rapaport (Aac2p), and Thomas D. Fox (Yme1p). Protein-antibody complexes were visualized on Western blots using the SuperSignal™ West Pico system (Pierce). For fluorimetric determination of cation concentrations, measurements of matrix concentrations of free Mg^{2+} and Ca^{2+} ($[Mg^{2+}]_m$ and $[Ca^{2+}]_m$) were performed in mitochondria from the DBY747 background as described in Ref. 3.

KOAc-induced Swelling of Isolated Mitochondria—Mitochondria were prepared from DBY747 or W303 wild-type cells and from isogenic *yol027*Δ mutant cells, resuspended in 0.6 M sorbitol buffer Tris-Cl, pH 7.4, to a final concentration of 10 mg of protein per ml. Aliquots of 100 μl of this suspension were incubated for 5 min at 25 °C with antimycin A (final concentration of 5 μM) and then transferred into cuvettes containing 1 ml of swelling buffer (55 mM KOAc, 5 mM TES, 0.1 mM EGTA, and 0.1 mM EDTA). Recording of A_{540} (Hitachi U-2000 spectrophotometer) was started immediately thereafter.

To deplete mitochondria of endogenous Mg^{2+}, the 4-bromo-calcium ionophore A23187 (0.5 μM) and EDTA (10 mM) were added prior to the KOAc treatment. To observe inhibition of swelling carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), quinine, or dicyclohexylcarbodiimide (DCCD) (final concentrations of 0.5 μM, 200 μM, and 1 mM, respectively) were added to A23187/EDTA-treated mitochondria prior to the KOAc treatment.

Confocal Fluorescence Microscopy—Cells transformed with plasmids expressing GFP fusion proteins were stained with 10 μM rhodamine B hexyl ester (Molecular Probes) or 25 nM Mito-Tracker red chloromethyl-X-rosamine and examined by laser confocal microscopy using a Leica TCS4D laser confocal microscope. GFP and rhodamine B were excited by 488 and 543 nm laser lines, respectively, and detected simultaneously at their emission maxima. Mitochondrial polarization was observed by laser confocal microscopy as described in Ref. 3.

Electron Microscopy—W303 cells were grown at 28 °C in YPGal to an A_{600} of 1.2, fixed for 30 min in 3.7% formaldehyde, spheroblasted with zymolyase at 0.5 mg/g of cells, and washed in phosphate-buffered saline. Spheroblasts were pelleted and resuspended in 2% glutaraldehyde in 0.15 M Sorensen′s buffer (pH 7.4) for postfixation overnight at 4 °C. Subsequently, the cell suspensions were filled into cellulose tubes (200 μm in diameter), infiltrated with 1% OsO_4 for 1 h, dehydrated in ethanol, and embedded in epoxy resin Agar 100 (Agar Scientific Ltd, UK). Thin sections were cut on a Reichert Ultracut S microtome, mounted on copper grids, and contrasted by uranyl acetate and lead citrate. Grids were examined at 60 kV using a JEM-1210 electron microscope (Jeol Ltd., Japan).

RESULTS

LetM1p, YOL027p, and Ypr125p Are Members of a Novel Eukaryotic Protein Family—LETM1, a human open reading frame of unknown function, is part of most deletions in chromosome 4 causing Wolf-Hirschhorn syndrome. It encodes a protein of 83.4 kDa (11). Homologs of this protein have been detected in all heavily sequenced eukaryotes. The genome of the yeast *S. cerevisiae* contains two open reading frames (*YOL027* and *YPR125*) encoding *LETM1* homologs of 573 and 454 amino acids, respectively, with about 40% sequence identity.

Although the size variation among these homologs is high, lower eukaryotes, animals, and plants have at least one predicted transmembrane domain (Fig. 1*A*). In addition, most proteins in animals and plants have one or two predicted EF-

[2] L. Zotova, unpublished data.

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hand calcium-binding domains in their C-terminal extensions, and the mammalian ones contain a leucine zipper region (Fig. 1*A*), as first noted in Ref. 11.

Full-sequence alignments of homologs from plant, human, and yeast (Fig. 1*B*) reveal that members of this new protein family are highly conserved in their middle parts (about 40% amino acid identity). The region predicted to contain a transmembrane domain is particularly well conserved (*TM*, *boxed* in Fig. 1*B*). Three prolines within the putative α helical transmembrane sequence (Fig. 1*C*) are remarkable. Prolines, forming molecular hinges, have been observed repeatedly in transmembrane α helices of proteins, notably in ion channels and G protein-coupled receptors (21).

LetM1p and Ypr125p Localize to Mitochondria—The human *LETM1* gene and the yeast *YPR125* gene were C-terminally tagged with the GFP-epitope. The LetM1-GFP fusion protein was transiently expressed from the vector pEGFP-N1 in the mouse NIH/3T3 embryonic fibroblast cell line. Fluorescence confocal microscopy revealed the co-localization of the GFP fluorescence with Mito-Tracker fluorescence of mitochondria (Fig. 2, *a–c*). When expressed under control of the methionine promoter from a yeast low-copy plasmid (pUG35), the Ypr125-GFP fusion protein co-localized with Mito-Tracker fluorescence, visualizing a distinct tubular network typical of yeast mitochondria (Fig. 2, *d–f*).

Yol027p Is an Integral Protein of Mitochondrial Inner Membrane—A *YOL027*-HA allele (triple HA tag C-terminally fused to the *YOL027* open reading frame and inserted at the chromosomal locus; see "Experimental Procedures") was used to determine the subcellular localization of Yol027p by cell fractionation and immunoblotting. Total cell content (*T*), post-mitochondrial supernatant (*C*), and mitochondrial (*M*) fractions were separated by SDS-PAGE and analyzed by immunoblotting (Fig. 3*A*, *lanes T*, *C*, *M*). The cytosolic fraction was characterized by the presence of hexokinase Hxk1p, a soluble protein. Yol027-HAp was found exclusively in the total cell content and mitochondrial fractions, as were the ADP/ATP carrier Aac2p, an integral protein of the inner membrane, and the β subunit of the F1 ATPase, F1β, a protein associated with the matrix side of the inner membrane.

Treatment of mitochondria by alkaline sodium carbonate (20) solubilized the membrane-associated ATPase subunit F1β (Fig. 3*A*, *lane SN*), but not the integral membrane protein Aac2p (Fig. 3*A*, *lane P*). Yol027-HA protein also stayed in the pellet fraction, thus qualifying it as an integral membrane protein. Cell fractionation, sodium carbonate extraction, and immunoblotting also revealed that Ypr125-GFP and LetM1-GFP behaved as integral proteins of a mitochondrial membrane (data not shown).

To further determine to which of the two mitochondrial membranes Yol027-HA localizes, whole mitochondria, mitoplasts, and SMPs of a *yol027*Δ mutant strain expressing a C-terminally HA-tagged Yol027 protein from a single-copy plasmid were obtained, and the accessibility of their proteins by proteinase K was studied (Fig. 3*B*). In whole mitochondria, all tested proteins were protease resistant, except Tom70p, an outer membrane protein protruding to the surface. Upon disintegration of the membranes by Triton X-100, all proteins were digested by proteinase K, showing that none of them was intrinsically protease-resistant.

Mitoplasts were characterized by (*i*) the absence of Tom70p, pointing to an efficient removal of the outer membrane, (*ii*) by protection of the matrix-sided integral membrane protein Tim44 from degradation by proteinase K, (*iii*) by the degradation of Yme1p, an inner membrane protein with domains exposed to the intermembrane space and the matrix, and (*iv*) shortening of Aac2p, the ADP/ATP carrier, an inner mitochondrial membrane protein partially exposed to the outside of mitoplasts. Mitoplasts contained Yol027p, but in a proteinase K-resistant form, which implies that no part of this protein protrudes to the intermembrane space to such an extent that it is rendered protease-sensitive.

Sonication of mitoplasts is known to result in the formation of SMPs with a majority of inside-out vesicles (22). Consistently, we found that Tim44p became protease-sensitive, whereas Aac2p lost its protease-sensitivity. The presence of Yol027-HA in these SMPs confirmed its nature as a membrane protein, and its protease-sensitivity indicated that it was exposed to the surface of the SMPs. This change in protease sensitivity of Yol027-HA and Aac2p indicates that sonication of mitoplasts under the conditions used here led to a very large fraction of inside-out particles, allowing the conclusion that the C terminus of Yol027p is located in the mitochondrial matrix.

Disruption of the YOL027 Gene—To investigate the function of Yol027p, the *YOL027* coding sequence was replaced by the *HIS3MX6* cassette in the diploid yeast strain W303 (see "Experimental Procedures"). After sporulation of the resulting heterozygous strain and tetrad dissection, *yol027*Δ spores were found to exhibit reduced growth on non-fermentable carbon sources (YPEG) at 28 °C and nearly no growth at 37 °C (Fig. 4) and at 18 °C (data not shown). Fermentative growth of the mutant (on YPD) was also reduced, as compared with that of the isogenic wild-type (Fig. 4). When grown on glucose containing media, *yol027*Δ strains were mitotically unstable, throwing off rho$^-$ cells (having macro-deletions in mitochondrial DNA) at a moderate rate (data not shown).

Disruption of *YPR125* had no apparent phenotype. Disruption of both *YOL027* and *YPR125* (*yol027*Δ *ypr125*Δ mutant) led to a phenotype indistinguishable from the one exhibited by the *yol027*Δ mutant (data not shown). Because *YOL027* and *YPR125* are multicopy suppressors of the *mrs2*Δ *petite* phenotype, defective in mitochondrial Mg^{2+} influx (3, 10), we also investigated the phenotypes of a *yol027*Δ *mrs2*Δ double mutant. This mutant was unable to grow on non-fermentable substrate at any temperature and proved to be rho^o (devoid of mitochondrial DNA; data not shown). Simultaneous deletion of *YOL027* and *MRS2* thus results in a more pronounced (synthetic) growth defect than single deletions of each of these two genes.

Functional Homology of Yeast and Human LetM1p—To find out if the human LetM1p is a functional homolog of Yol027p, we transformed a *yol027*Δ strain with a plasmid expressing the *LETM1* gene from the strong, constitutive *ADH1* promoter on a multicopy plasmid ((*LETM1*)*n*). As a control, the strain was also transformed with the empty plasmid and a plasmid containing the *YOL027* coding region. As shown in Fig. 4, expression of (*LETM1*)*n* restored growth of the *yol027*Δ mutant, although not as well as expression of (*YOL027*)*n*. Apparently, LetM1p is targeted to the yeast mitochondria and can functionally replace its Yol027 homolog. The yeast homolog *YPR125*, expressed from a multicopy plasmid, also restored growth of the *yol027*Δ mutant strain (Fig. 4).

*Mg^{2+} and Ca^{2+} Influx into Wild-type and Mutant yol027*Δ *Mitochondria*—Partial suppression of the *mrs2*Δ phenotype by (*YOL027*)*n* or by (*YPR125*)*n* suggested to us that these two proteins might be involved in mitochondrial cation homeostasis. Comparing influx of Mg^{2+} and Ca^{2+} into isolated mitochondria (3), we observed a considerably reduced influx of both Mg^{2+} and Ca^{2+} into mutant *yol027*Δ mitochondria as compared with wild-type mitochondria (Fig. 5, *A* and *B*). Although an increase in external Mg^{2+} or Ca^{2+} elicited an initial rapid response, influx quickly ceased, leading to steady-state plateau

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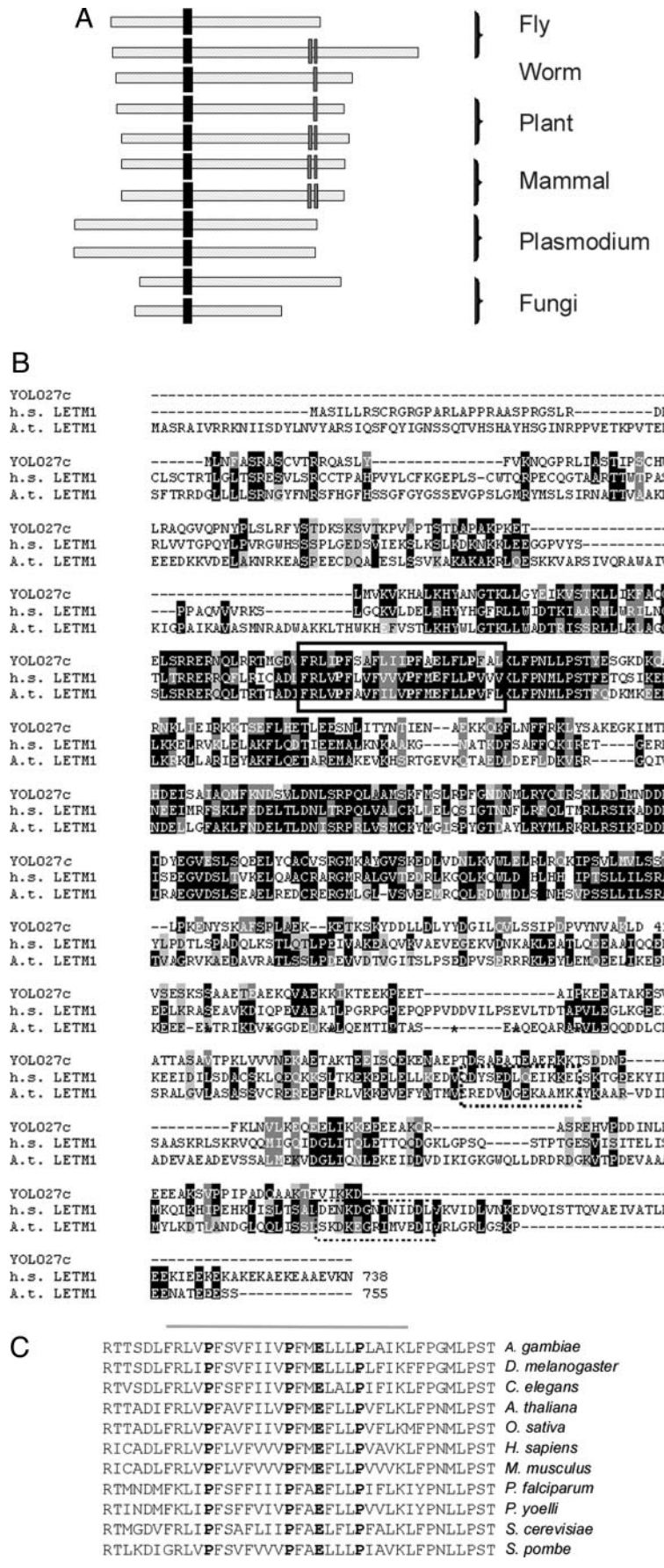

FIG. 1. **Alignments of Yol027p with its homologs.** *A*, schematic alignment. Homologs are represented by *horizontal bars* of lengths proportional to their molecular masses, and their predicted transmembrane domains (*black box*) are aligned. The locations of putative EF-hand domains are indicated by *gray boxes*. *Drosophila melanogaster*, gi 19922902 ref NP_611922.1; *Anopheles gambia* gi 31236990 ref XP_319522.1; *C. elegans* gi 17561656 ref NP_506381.1; *Arabidopsis thaliana* gi 15232180 ref NP_191541.1; *Oryza sativa* gi 38345383 emb CAD41252.2; *Homo sapiens* gi 6912482 ref NP_036450.1; *Mus musculus* gi 33416528 gb AAH55865.1; *Plasmodium falciparum* gi 23510156 ref NP_702822.1; *Plasmodium yoelli* gi 23479373 gb EAA16221.1; *S. cerevisiae* gi 6324546 ref NP_014615.1; and *Schizosaccharomyces pombe* gi 19114560 ref NP_593648.1. *B*, full-sequence alignment. Proteins encoded by the yeast *YOL027* gene, the human *LETM1* gene, and their plant homolog (*A. thaliana*) are aligned. Identical and conserved amino acids are highlighted in *black* and *gray*, respectively. The single predicted transmembrane domains of the proteins are *boxed* (*continuous lines*). The C-terminal part of the sequences contains putative EF-hand Ca^{2+}-binding sites (*box*, *dotted lines*) and a putative leucine zipper motif (*asterisks*). Both have originally been noted for LetM1p (11), and they are not conserved throughout all of the organisms. *C*, multiple alignment of the exceptionally well conserved transmembrane domain of *YOL027* with representatives of its orthologs from other eukaryotic organisms (*A*). The α-helical region is indicated by a *bar*. The conserved proline residues and a glutamic acid residue within this domain are *highlighted*.

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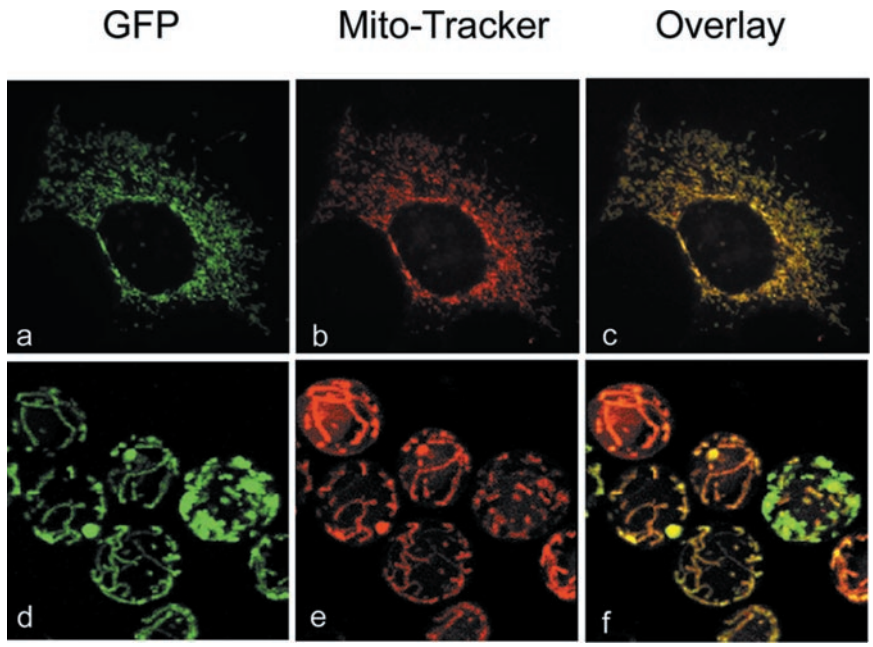

FIG. 2. **Subcellular localization of LetM1 and Mrs7p by fluorescence microscopy.** *In vivo* co-localization of GFP-tagged *LETM1* and Mito-Tracker red chloromethyl-X-rosamine in mitochondria of mouse NIH/3T3 cells (*a–c*) and of GFP-tagged *YPR125* and rhodamine B hexyl ester in mitochondria of yeast W303 cells (*d–f*). The NIH/3T3 mouse fibroblast (*a–c*) cells were transiently transfected with the pEGFP vector carrying the human *LETM1* gene tagged with EGFP at its C terminus. W303 cells expressing C-terminally GFP-tagged *YPR125* gene from the centromeric plasmid pUG35-*YPR125*-GFP (*d–f*) were grown in S-Gal medium at 28 °C to log phase and examined by confocal microscopy.

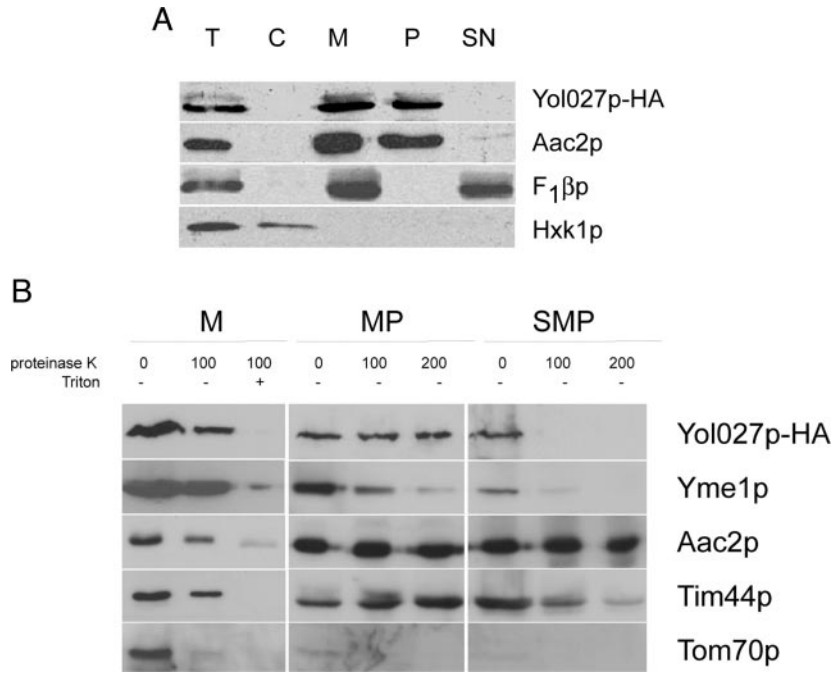

FIG. 3. **Subcellular and submitochondrial localization of Yol027p.** *A*, transformants of the yeast strain GA74–1A expressing an internally tagged *YOL027-HA* gene (see "Experimental Procedures") were grown in synthetic media containing 2% lactate. Protoplasts were homogenized (*T*) and separated into a mitochondrial (*M*) and a postmitochondrial fraction (*C*). Purified mitochondria were treated with 0.1 M Na_2CO_3 and fractionated by centrifugation at 100,000 × *g* into pellet (*P*) and supernatant (*SN*). Proteins were separated by SDS-PAGE and analyzed by immunoblotting using antibodies to the HA epitope, the mitochondrial proteins Aac2 and $F_1\beta$, and the cytosolic protein Hxk1. *B*, W303 *yol027*Δ mutant cells expressing a C-terminally HA-tagged Yol07p (from plasmid YCplac33) were grown in S-Gal-urea medium. Intact mitochondria, (*M*, *left panel*), mitoplasts (*MP*, *middle panel*), and submitochondrial particles (*SMP*, *right panel*) were incubated without and with proteinase K (in the indicated concentrations) or proteinase K plus Triton X-100. Proteins were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to HA, to the inner membrane proteins Tim44, Aac2, and Yme1, and to the outer membrane protein Tom70.

levels considerably lower in *yol027*Δ than in wild-type mitochondria (Fig. 5, *A* and *B*). This result indicates to us that the Mg^{2+} and Ca^{2+} transport systems are active, but saturation of influx is reached at comparatively low intramitochondrial cation concentrations.

As shown by Ref. 3, the driving force for Mg^{2+} uptake by Mrs2p is the internally negative membrane potential $\Delta\Psi$ of about −150 mV in mitochondria. We speculated that the absence of Yol027p might result in a reduced $\Delta\Psi$ and, hence, reduced Mg^{2+} and Ca^{2+} influx, whereas overexpression of Yol027p might have increased $\Delta\Psi$ and thus improve Mg^{2+} influx in *mrs2*Δ (by so far unknown pathways). In fact, addition of the exogenous cation/H^+ exchanger nigericin, which is known to enhance $\Delta\Psi$ in respiring mitochondria, was found to stimulate Mg^{2+} influx into *yol027*Δ cells to a considerable extent (Fig. 5*A*).

*Effects of yol027*Δ *Mutation on Mitochondrial* $\Delta\Psi$ *and* K^+ *Concentrations*—To determine the $\Delta\Psi$ of mitochondria isolated from wild-type and mutant *yol027*Δ cells, we used JC-1, a fluorescent imidazole cyanine dye that stays monomeric at low

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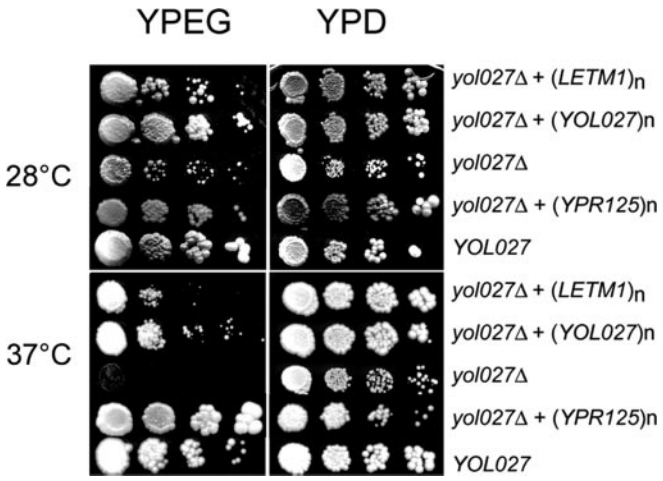

FIG. 4. **Growth defects of the W303 *yol027*Δ mutant and complementation by *YOL027* and its homologs *YPR125* and *LETM1*.** A *yol027*Δ disruptant of strain W303 was transformed either with the plasmid pVT-U103 without inserts (marked *yol027*Δ), with inserts *YOL027* or *LETM1*, or with plasmid YEp351 with insert *YPR125* (marked (*YOL027*)n, (*LETM1*)n, and (*YPR125*)n, respectively). Serial dilutions of transformants and the wild-type strain W303 (marked *YOL027*) were spotted onto YPD and YPEG plates and grown at 28 °C or for 3 and 5 days, respectively, or at 37 °C for 3 and 6 days, respectively.

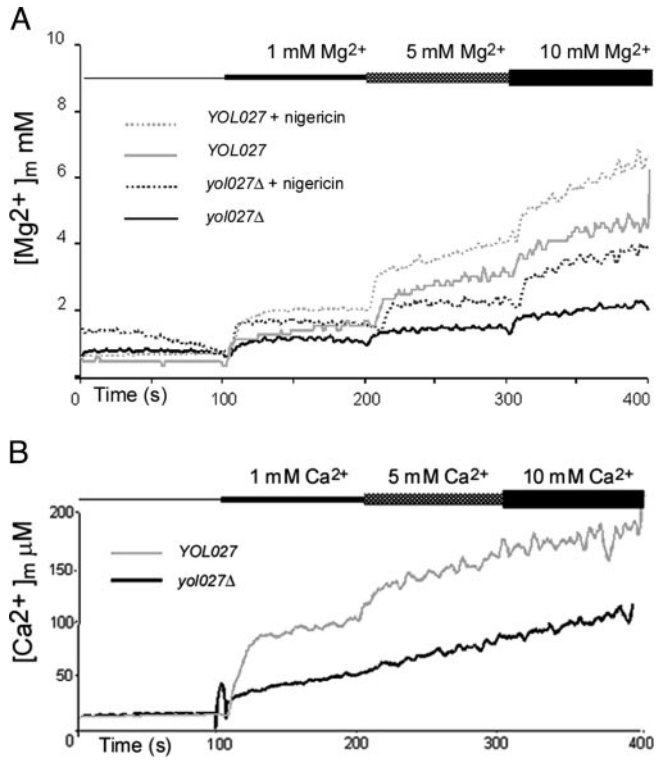

FIG. 5. **Mg^{2+} and Ca^{2+} influx into isolated mitochondria of DBY747 wild-type and *yol027*Δ disruptant cells.** Mitochondria were loaded with Mg^{2+}-sensitive mag-fura-2 (*A*) and Ca^{2+}-sensitive fura-2 dyes (*B*). Fluorescence was determined as described under "Experimental Procedures." Resting concentrations of free Mg^{2+} ($[Mg^{2+}]_m$) and Ca^{2+} ($[Ca^{2+}]$)were determined in buffers nominally free of divalent cations and changes of Mg^{2+} and Ca^{2+} dependent upon external ion concentrations were recorded over time. Addition of external ion concentrations are marked by *bars*. Mg^{2+} measurements were also carried out after treatment of mitochondria with nigericin. *Continuous lines*, wild-type traces; *dotted lines*, mutant *yol027*Δ traces. *Continuous* and *dotted lines* represent measurements without or with nigericin, respectively.

ΔΨ, has a yellowish-green fluorescence, and aggregates with increasing ΔΨ, thereby shifting from green to orange-red fluorescence (23).

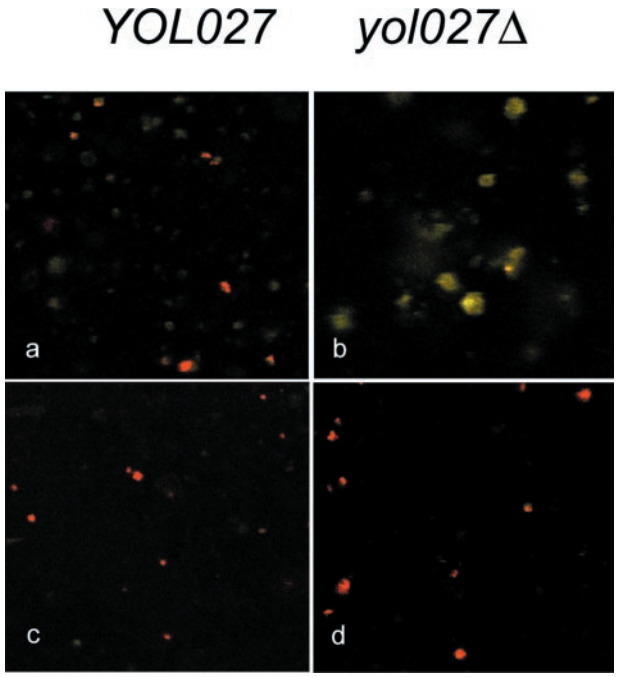

FIG. 6. **Membrane potential in isolated mitochondria of W303 wild-type and *yol027*Δ cells.** Fluorescence confocal microscopy of mitochondria isolated from wild-type strain W303 and its isogenic mutant *yol027*Δ incubated in 0.6 M sorbitol buffer in the presence of the ΔΨ-sensitive fluorescent dye JC-1 without (*a*, *b*) or with (*c*, *d*) the addition of the K^+/H^+ exchanger nigericin.

As shown in Fig. 6*a*, mitochondrial preparations of wild-type yeast cells exhibit red fluorescence, which is consistent with a high ΔΨ. Yellow and green spots point to heterogeneity of the membrane potential among mitochondrial particles and even within particles, a phenomenon that has previously been described for mammalian mitochondria (23). In contrast to the wild-type mitochondria, mutant *yol027*Δ mitochondria exhibited a green fluorescence (Fig. 6*b*), indicative of low ΔΨ. Valinomycin (a K^+ ionophore) and high external KCl concentrations dissipated the ΔΨ to the same extent in mutant and wild-type mitochondria, resulting in green to yellow fluorescence (data not shown). Addition of nigericin, an electroneutral K^+/H^+ exchanger, to respiring mitochondria resulted in an equal fluorescence in wild-type and *yol027*Δ particles with compact red spots, indicative of the restoration of a high ΔΨ (Fig. 6, *c* and *d*). Nigericin thus fully compensated for the absence of the Yol027 protein, which points to a possible defect in K^+ homeostasis in the *yol027*Δ mutant.

By the use of atomic absorption spectrometry of mitochondrial matrix extracts (24), we observed a drastic increase of the potassium content in *yol027*Δ mitochondria (230 nmol/mg of protein) as compared with wild-type mitochondria (125 nmol/mg of protein). Mitochondrial contents of other metals/elements in mutant mitochondria such as magnesium and sodium were modestly increased by about 40 and 10%, respectively (data not shown).

*Increased Volume of yol027*Δ *Mitochondria*—Increased osmolality, resulting from the net uptake of cations, is expected to be compensated for by an influx of water and swelling of the organelle. In fact, *yol027*Δ mutant mitochondria proved to be heavily swollen as compared with wild-type mitochondria, both *in situ* and *in vitro*. Transmission electron micrographs (Fig. 7*A*) revealed enlarged *yol027*Δ organelles, lacking tubular shaped cristae and other electron-dense material. Laser confocal microscopy (Fig. 7*B*) of isolated mitochondria also showed that *yol027*Δ organelles *in vitro* are much larger than their

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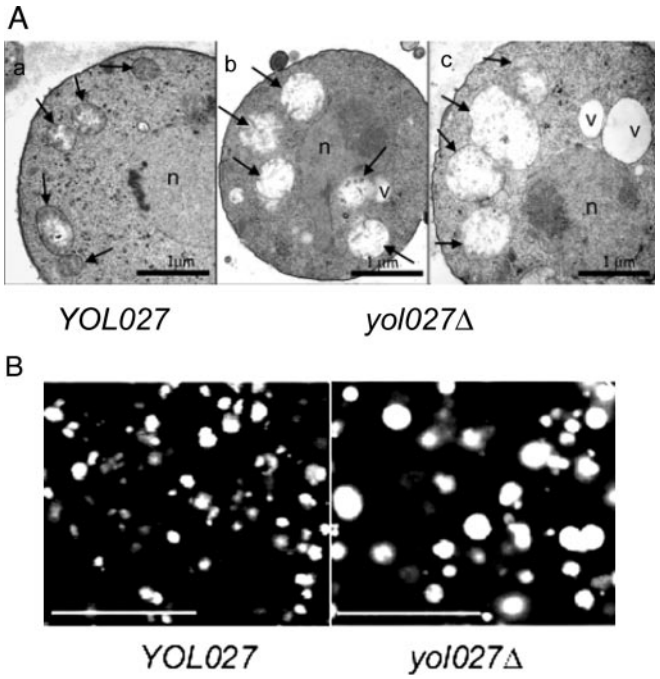

FIG. 7. **Increased mitochondrial volume in *yol027*Δ mutant cells.** *A*, transmission electron microscopy of wild-type and *yol027*Δ cells. *n*, nucleus; *v*, vacuole; *black arrows* point to the mitochondria. *Bars*, 1 μm. W303 wild-type cells (*A*) and isogenic mutant *yol027*Δ cells (*B*, *C*) were grown in YPGal to an A_{600} of 1.2 and then spheroblasted and fixed for electron microscopy. *B*, confocal microscopy of isolated mitochondria. Mitochondria isolated from wild-type W303 cells and from isogenic mutant *yol027*Δ cells expressing a matrix-targeted GFP construct were examined under confocal microscopy. *Bars*, 10 μm.

wild-type counterparts prepared and kept in the same buffers. Combined with the data presented above, these findings suggested to us that *yol027*Δ mutant mitochondria might be defective in K^+ homeostasis and possibly in K^+/H^+ exchange, which is of prime importance for the control of matrix K^+ concentration and volume of the organelle.

KOAc-induced Mitochondrial Swelling—Swelling of isolated mitochondria, determined by light scattering, is a widely accepted optical technique to determine monovalent cation transport across the inner mitochondrial membrane (Refs. 25–27, and reviewed in Refs. 1 and 28). When incubated in KOAc, isolated mitochondria rapidly take up the protonated form of acetic acid. Its ionization in the mitochondrial matrix leads to acidification of the matrix and activation of the K^+/H^+ exchange system. In non-respiring mitochondria, this results in the net accumulation of potassium acetate, uptake of water, and swelling of the organelle, which can be measured as a decrease in light scattering or absorbance (2, 25).

Mitochondria of the wild-type strain DBY747 used here showed little change in absorption at 540 nm upon addition of KOAc (Fig. 8*A*). Rapid swelling was observed, however, upon addition of the ionophore A23187 and EDTA, depleting the system of divalent cations. This effect was abolished in the presence of the protonophore CCCP, consistent with the notion that KOAc-mediated swelling is dependent upon a pH gradient activating the K^+/H^+ exchange. The addition of Mg^{2+} at molar concentrations exceeding those of EDTA resulted in partial inhibition (data not shown). Quinine, a known inhibitor of the K^+/H^+ exchange reaction, as well as DCCD strongly inhibited swelling (Fig. 8*A*).

This data parallels previous findings on KOAc-induced swelling of mammalian and yeast mitochondria, except that the need for Mg^{2+} depletion from the matrix of yeast mitochondria was not observed previously (25–27). The use of mitochondria from different yeast strains may explain this minor discrepancy of results. In fact, when using mitochondria of wild-type strain W303, we also observed spontaneous swelling in KOAc, which was poorly enhanced by Mg^{2+} depletion (data not shown).

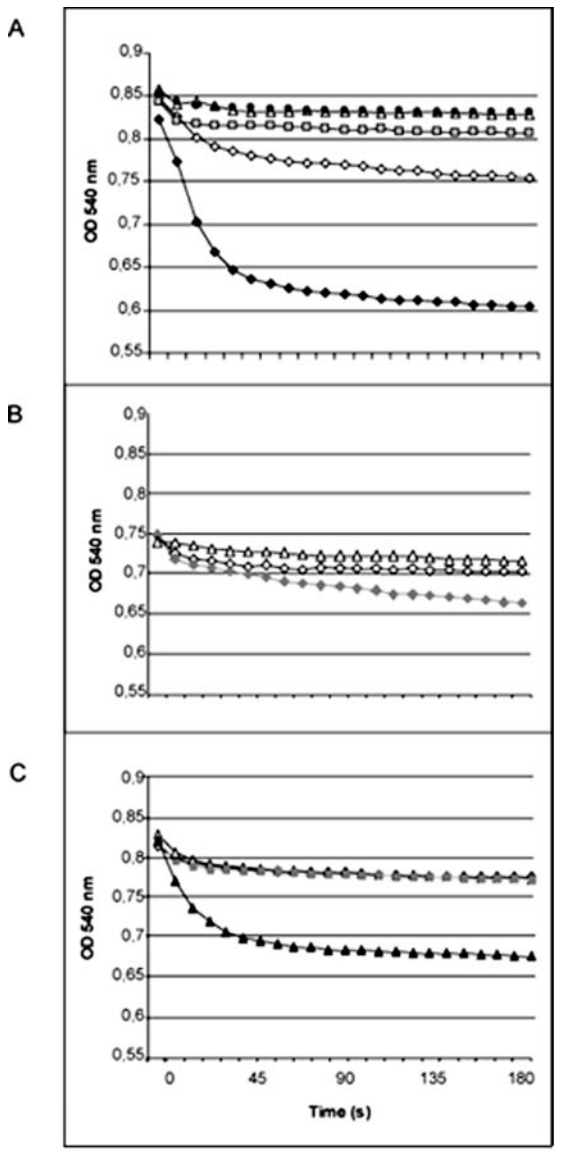

FIG. 8. **KOAc-induced swelling of wild-type and *yol027*Δ mutant mitochondria.** Mitochondria were prepared from DBY747 wild-type cells and isogenic *yol027*Δ mutant cells, resuspended in 0.6 M sorbitol buffer, pH 7.4, to a final concentration of 10 mg of protein per ml. 100 μl of this suspension was incubated 5 min with antimycin A at a prior time and then added into cuvettes containing 1 ml of swelling buffer (55 mM KOAc, 5 mM TES, 0.1 mM EGTA, and 0.1 mM EDTA). Recording of A_{540} was started immediately thereafter. *A*, wild-type mitochondria without A23187/EDTA (◇), with A23187/EDTA (◆), or with A23187/EDTA and CCCP(●), or quinine (△), or DCCD (▨). *B*, *yol027*Δ mitochondria without A23187/EDTA (◇), with A23187/EDTA (◆), or with A23187/EDTA and quinine (△). *C*, *yol027*Δ + (LETM1)n with A23187/EDTA + DCCD (■), with A23187/EDTA (▲), or with A23187/EDTA and quinine (△).

Preparations of mutant *yol027*Δ mitochondria from either strain (DBY747 or W303), at concentrations similar to those of wild-type cells, exhibited reduced absorbance at resting conditions (Fig. 8*B* and data not shown). This indicates that the organelles were swollen prior to the addition of KOAc, which is consistent with the microscopic data presented above. Furthermore the *yol027*Δ mitochondria failed to exhibit rapid swelling in KOAc (plus A23187 and EDTA) (Fig. 8*B*). These results are

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fully consistent with the notion of a severe reduction in K^+/H^+ exchange activity in *yol027*Δ mutant mitochondria.

Expression of LetM1, the human homolog of Yol027, in the DBY747 *yol027*Δ mutant strain resulted in a partial restoration of KOAc-dependent swelling of mitochondria (Fig. 8*C*). Rapid swelling of those mitochondria was similar to that of wild-type mitochondria in that it was sensitive to quinine and to DCCD. This partial restoration of swelling parallels our finding that *LETM1* is able to complement the respiratory growth defect of the *yol027*Δ mutant (compare Fig. 4) and indicates that the human LetM1 protein is the functional homolog of the yeast Yol027 protein. Their effects upon KOAc-induced swelling of mitochondria suggests a role for both proteins in K^+/H^+ exchange.

DISCUSSION

Homologs of the human WHSCR2 (Wolf-Hirschhorn Syndrome Critical Region 2) candidate disease gene *LETM1* are ubiquitous in eukaryotes (11, 12). The genome of the yeast *S. cerevisiae* harbors two homologs, *MRS7* (*YPR125*) and *YOL027*. *MRS7* was isolated as a multi-copy suppressor of a mutant defective in mitochondrial Mg^{2+} influx (3, 10). Deletion of *YOL027* has been shown to cause changes in mitochondrial morphology and suggested a mitochondrial location of the *YOL027* gene product (29). A recent report involving GFP-tagged versions of LetM1p (30), as well as this study on human LetM1p, yeast Yol027p, and yeast Ypr125p, confirm a mitochondrial location of LetM1p and its yeast homologs.

Disruption of *YOL027* (*yol027*Δ mutation) is shown here to result in a defect in respiratory growth, which is consistent with its mitochondrial location. This phenotype can be suppressed by over-expression of the human *LETM1* gene in yeast, which is indicative of homologous functions of the *LETM1* and *YOL027* gene products. *YPR125* can also suppress the *yol027*Δ mutant phenotype, but a *ypr125*Δ mutant did not reveal any obvious growth phenotype.

LetM1, Yol027p and Ypr125p are shown here to be integral membrane proteins. This is consistent with the presence of a predicted, highly conserved transmembrane domain in a central part of all members of this family. By fractionation of mitochondria and immunoblotting, we show that Yol027p is a protein of the inner mitochondrial membrane, exposing the C-terminal part of its sequence toward the mitochondrial matrix. Our finding that no part of Yol027p (or Ypr125p) protrudes to the surface of mitoplasts to an extent that it would be protease-sensitive is at variance with the computer prediction of a single transmembrane domain. Only further studies will reveal if Yol027p has a second, so far unrecognized TM domain, or if its amino-terminal sequence is present on the outside of the inner membrane but embedded into this membrane or otherwise protected from protease degradation.

Detailed phenotypic analysis of the *yol027*Δ mutant in comparison to its isogenic wild-type disclosed pleiotropic mitochondrial defects, namely an elevated intramitochondrial potassium level, a swollen appearance of the organelle, and a drastically reduced $\Delta\Psi$. All of these phenotypic features of *yol027*Δ mutant mitochondria are consistent with an essential role of the *YOL027* gene product in mitochondrial K^+ homeostasis, possibly in the K^+/H^+ exchange system. Full restoration of $\Delta\Psi$ by the addition of the exogenous K^+/H^+ ionophore nigericin to isolated organelles supports the assumption that a lack in K^+/H^+ exchange activity accounts for the mutant phenotypes of the *yol027*Δ mutant.

The activity of the K^+/H^+ exchange system can be unmasked by incubation of isolated mitochondria in KOAc. In non-respiring mitochondria, this treatment results in the uptake of acetic acid, acidification of the matrix, and activation of K^+/H^+ antiport, with a net increase of $[K^+]_i$ and swelling of the organelle (25). Although mammalian mitochondria exhibit rapid swelling only upon prior depletion of divalent cations, yeast mitochondria have been reported to swell spontaneously (26, 27).

Mitochondrial preparations of the wild-type yeast strain W303 exhibited spontaneous rapid swelling in KOAc (observed as a decrease in absorbance at 540 nm; data not shown), whereas mitochondria of the wild-type strain DBY747 responded to KOAc addition with a minor change in absorbance (compare Fig. 8) and showed rapid swelling only upon Mg^{2+} depletion. Thus, concerning their Mg^{2+} sensitivity of swelling, mitochondria of the latter yeast strain behave similarly to those of mammalian cells. As observed here, rapid KOAc-induced swelling was abolished by quinine or DCCD. This parallels previous studies and is likely to result from K^+/H^+ exchange activity.

Mutant *yol027*Δ mitochondria failed to show rapid KOAc-induced swelling. However, swelling was restored to a considerable extent by expression of human *LETM1* in *yol027*Δ mutant cells. These data support the hypothesis that a lack of Yol027 proteins results in a defect in mitochondrial K^+/H^+ exchange activity. A lack of this activity is sufficient to explain the phenotypic effects observed with *yol027*Δ mitochondria: they have highly increased matrix K^+ content (nearly 2-fold in *yol027*Δ mitochondria as compared with wild-type mitochondria), which we interpret as resulting from uncompensated K^+ leakage in *yol027*Δ. Increased osmolarity is accompanied by an increase in mitochondrial volume (reviewed in Ref. 28). By using electron microscopy and confocal fluorescence microscopy of whole cells and isolated mitochondria, we observed drastically altered mitochondrial volume and shape in the *yol027*Δ mutant, which is consistent with the predicted increased mitochondrial volume as a consequence of elevated K^+ content. We also found that mutant mitochondria have lower membrane potential than those of isogenic wild-type cells. This may result in part from the observed increase in matrix K^+ content in *yol027*Δ cells. Additionally, the disturbed cation homeostasis may have affected the activity of the respiratory chain or other proton extrusion systems of the organelle. Finally, the exogenous cation/H^+ ionophore nigericin was found to restore $\Delta\Psi$ in *yol027*Δ mutant mitochondria *in vitro*, presumably by replacing the endogenous K^+/H^+ exchanger.

The data presented here are consistent with an essential role of the Yol027/LetM1 proteins in mitochondrial K^+/H^+ exchange. We cannot discriminate whether Yol027/LetM1 constitutes the exchanger itself or a regulatory factor associated with it. The exceptionally high sequence conservation in the single transmembrane domain might be in favor of a role in transport. Yet, an exchanger with just one transmembrane domain would be unprecedented.

This defect in K^+ homeostasis of *yol027*Δ mutant yeast cells causes a pronounced defect in growth on non-fermentable substrate. A weaker effect upon fermentable substrate, indicating that the absence of the *YOL027*-encoded protein did not only affect mitochondrial energy conservation systems, but also mitochondrial functions relevant for other processes, such as protein import into the organelles (31), which is dependent upon $\Delta\Psi$. In addition, this single deletion causes instability of mitochondrial DNA. These findings clearly reveal an important role of Yol027/LetM1 in mitochondrial physiology, consistent with their proposed prominent role in K^+ extrusion from the organelles. Our finding of mitochondrially defective, but viable, *yol027*Δ (as well as *yol027*Δ*mrs7*Δ) mutant yeast cells indicates that a total breakdown of the mitochondrial K^+ homeostasis is being prevented in the absence of Yol027 (and its homolog Mrs7/Ypr125) by the activity of other factors.

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Knock-down of LetM1p activity seems to severely hamper the development of *Caenorhabditis elegans* embryos and larvae.[3] Reduction in LetM1 thus seems to have a more serious effect upon the life or development of a multicellular organism than upon that of a unicellular yeast. Provided that the correlation of the hemizygous deletion of *LETM1* correlates with the classical Wolf-Hirschhorn syndrome phenotype in humans (12), a change in the *LETM1* gene dose seems sufficient to provoke a neuromuscular defect. This result follows the pattern of many reports showing that (*i*) haploinsufficiency can have dramatic effects on human health, and (*ii*) neuromuscular disease phenotypes result from mitochondrial dysfunction (32).

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