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** 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 **yol027Δ** 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 (Δψ\(^+\)) of −150 to −180 mV (Δψ, inside negative). This high Δψ constitutes a large driving force for the electrochemical 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 m\(\text{M}\)) and Na\(^+\) (5 m\(\text{M}\)), 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\(^2+\)] \((K_m \approx 0.3–0.4 \text{ mM in mammalian mitochondria})\), 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).
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 yol027Δ mitochondria exhibit severely reduced potassium acetate (KOAc)-induced swelling, which is indicative of a lack or reduction in K⁺ 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 DBY77 mrs2Δ (14). Yeast growth media were as described previously (14).

Hemagglutinin (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 YOLO27c 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 to that of the isogenic wild-type strain.

The YOL027c gene sequence (nucleotides 1 to 1262, relative to the start codon) to +1721 was PCR-amplified from W303 genomic DNA by use of an oligonucleotide 5′-primer carrying a yeast 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 YEp353 HA- vector, resulting in plasmid YCp-YOP1027-HA.

YPR125 coding sequence (1356 nucleotides from 1 to +1356) was PCR-amplified from the same yeast strain, introducing recognition sites for EcoRI and Sall, followed by insertion of this fragment into the EcoRI and SalI sites of the centromeric vector pUG35. The resulting plasmid for EcoRI and SalI, followed by insertion of this fragment into the EcoRI end. The YEp-YOL027-HA tagging Genes—

**Gene Deletion**—Complete disruptions of YOL027, MR2Δ, and MR7Δ 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 yol027Δ 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 MR2Δ (mrs2Δ) and MR7Δ (mrs7Δ) resulted in deletions of 1218 nucleotides (from nucleotide 49 relative to the start codon to nucleotide 2343 relative to the stop codon) and of 1356 nucleotides (from the start to the stop codon).

W303 diploids were grown at 25 °C in YPGal to an A₆₀₀ of 1.2, fixed for 30 min in 3.7% formaldehyde, spheroplasted with zymolyase at 0.5 mg/ml of cells, and washed in phosphate-buffered saline. Spheroplasts were pelleted and resuspended in 5% Sörensen’s buffer (pH 7.4) for postfixation overnight at 4 °C. Subsequently, the cell suspensions were filtered into cellulosic tubes (200 μm in diameter), infiltrated with 1% OsO₄ 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 microscope, 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. 1A). In addition, most proteins in animals and plants have one or two predicted EF-

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

Full-sequence alignments of homologs from plant, human, and yeast (Fig. 1B) 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. 1B). Three prolines within the putative α helical transmembrane sequence (Fig. 1C) 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 YOL275p 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. 3A, 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. 3A, lane SN), but not the integral membrane protein Aac2p (Fig. 3A, 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 yol027Δ 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. 3B). 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, yol027Δ 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, yol027Δ strains were mitotically unstable, throwing off rho0 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 (yol027Δ ypr125Δ mutant) led to a phenotype indistinguishable from the one exhibited by the yol027Δ mutant (data not shown). Because YOL027 and YPR125 are multisecoporators of the mrs2Δ petite phenotype, defective in mitochondrial Mg2+ influx (3, 10), we also investigated the phenotypes of a yol027Δ mrs2Δ double mutant. This mutant was unable to grow on non-fermentable substrate at any temperature and proved to be rho0 (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 yol027Δ strain with a plasmid expressing the *LETM1* gene from the strong, constitutive ADH1 promoter on a multicopy plasmid (∆*LETM1*). As a control, the strain was also transformed with the empty plasmid and a plasmid containing the TOL027 coding region. As shown in Fig. 4, expression of ∆*LETM1* restored growth of the yol027Δ 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 yol027Δ mutant strain (Fig. 4).

Mg2+ and Ca2+ Influx into Wild-type and Mutant yol027Δ Mitochondria—Partial suppression of the mrs2Δ phenotype by (YOL027) or by (YPR125) suggested to us that these two proteins might be involved in mitochondrial cation homeostasis. Comparing influx of Mg2+ and Ca2+ into isolated mitochondria (3), we observed a considerably reduced influx of both Mg2+ and Ca2+ into mutant yol027Δ mitochondria as compared with wild-type mitochondria (Fig. 5, A and B). Although an increase in external Mg2+ or Ca2+ elicited an initial rapid response, influx quickly ceased, leading to steady-state plateau
FIG. 1. Alignments of Yol027p with its homologs. A, schematic alignment. Homologs are represented by horizontal bars of length 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 31236890 ref XP.319522.1; C. elegans gi 17561656 ref NP.506381.1; Arabidopsis thaliana gi 15232180 ref NP.191541.1; Oriza 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 6245456 ref NP.014615.1; and Schizosaccharomyces pombe gi 19114590 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 domain of the proteins are boxed (continuous lines). The C-terminal part of the sequences contains putative EF-hand Ca2+-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.
levels considerably lower in yol027Δ 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 mrs2Δ (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 yol027Δ cells to a considerable extent (Fig. 5A).

Effects of yol027Δ Mutation on Mitochondrial \(\Delta \Psi\) and K\(^{+}\) Concentrations—To determine the \(\Delta \Psi\) of mitochondria isolated from wild-type and mutant yol027Δ cells, we used JC-1, a fluorescent imidazole cyanine dye that stays monomeric at low...
Mitochondrial $K^+$ Homeostasis

Increased Volume of yol027Δ 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, yol027Δ mutant mitochondria proved to be heavily swollen as compared with wild-type mitochondria, both in situ and in vitro. Transmission electron micrographs (Fig. 7A) revealed enlarged yol027Δ organelles, lacking tubular shaped cristae and other electron-dense material. Laser confocal microscopy (Fig. 7B) of isolated mitochondria also showed that yol027Δ organelles in vitro are much larger than their control.
Mitochondrial $K^+$ Homeostasis

Fig. 7. Increased mitochondrial volume in yol027Δ mutant cells. A, transmission electron microscopy of wild-type and yol027Δ cells. n, nucleus; v, vacuole; black arrows point to the mitochondria. Bars, 1 μm. W303 wild-type cells (A) and isogenic mutant yol027Δ cells (B, C) were grown in YPGal to an $A_{500}$ 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 yol027Δ cells expressing a matrix-targeted GFP construct were examined under confocal microscopy. Bars, 10 μm.

Mitochondria of the wild-type strain DBY747 used here showed little change in absorption at 540 nm upon addition of KOAc (Fig. 8A). 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 pH gradient activation of the K'/H+ exchange system. 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. 8A).

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).

Preparations of mutant yol027Δ mitochondria from either strain (DBY747 or W303), at concentrations similar to those of wild-type cells, exhibited reduced absorbance at resting conditions (Fig. 8B 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 yol027Δ mitochondria failed to exhibit rapid swelling in KOAc (plus A23187 and EDTA) (Fig. 8B). These results are
fully consistent with the notion of a severe reduction in K\(^{+}\)/H\(^{+}\) exchange activity in yol027Δ mutant mitochondria.

Expression of LetM1, the human homolog of Yol027, in the DBY747 yol027Δ mutant strain resulted in a partial restoration of KOAc-dependent swelling of mitochondria (Fig. 8C). Rapid swelling of those mitochondria was similar to that of wild-type mitochondria in that it was sensitive to quinine and DCCD. This partial restoration of swelling parallels our finding that LETM1 is able to complement the respiratory growth defect of the yol027Δ 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, confirms a mitochondrial location of LetM1p and its yeast homologs.

Disruption of *YOL027* (yol027Δ 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 yol027Δ mutant phenotype, but a ypr125Δ 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 yol027Δ 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 ΔΨ. All of these phenotypic features of yol027Δ 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 ΔΨ 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 yol027Δ 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\(^{+}\)] 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 yol027Δ mitochondria failed to show rapid KOAc-induced swelling. However, swelling was restored to a considerable extent by expression of human LETM1 in yol027Δ 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 yol027Δ mitochondria: they have highly increased matrix K\(^{+}\) content (nearly 2-fold in yol027Δ mitochondria as compared with wild-type mitochondria), which we interpret as resulting from uncompensated K\(^{+}\) leakage in yol027Δ. 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 yol027Δ 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 yol027Δ 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 ΔΨ in yol027Δ 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 yol027Δ 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 ΔΨ. 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, yol027Δ (as well as yol027Δ mrs7Δ) 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.
Knock-down of LetM1p activity seems to severely hamper the development of Caenorhabditis elegans embryos and larvae. 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).

Acknowledgments—We thank I. Lichtscheidl, Vienna, for facilitating the use of the laser confocal microscopy, M. Snyder for providing a transposon-tagged Yol057 DNA fragment, H. Van der Spek (Amsterdam), J. Brix (Freiburg), D. Rapoport (Munich), and T. D. Fox (Ithaca) for providing antisera, and L. Zotova, M. Piskacek, and J. Weghuber (all Vienna) for communicating unpublished results. We thank F. Eisenhuber (Vienna) for supporting us with protein sequence analysis, A. Gruschopf and A. Ragnini (Vienna) for helpful suggestions, and M. Iliiev for excellent technical assistance.

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K. Nowikovsky, unpublished data.
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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J. Biol. Chem. 2004, 279:30307-30315.
doi: 10.1074/jbc.M403607200 originally published online May 11, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403607200

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