Novel Components of an Active Mitochondrial K⁺/H⁺ Exchange

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Defects of the mitochondrial K⁺/H⁺ exchanger (KHE) result in increased matrix K⁺ content, swelling, and autophagic decay of the organelle. We have previously identified the yeast Mdm38 and its human homologue LETM1, the candidate gene for seizures in Wolf-Hirschhorn syndrome, as essential components of the KHE. In a genome-wide screen for multicopy suppressors of the pet− (reduced growth on nonfermentable substrate) phenotype of mdm38Δ mutants, we now characterized the mitochondrial carriers PIC2 and MRS3 as moderate suppressors and MRS7 and YDL183c as strong suppressors. Like Mdm38p, MRS7p and YDL183cp are mitochondrial inner membrane proteins and constituents of ~500-kDa protein complexes. Triple mutant strains (mdm38Δ mrs7Δ ydl183cΔ) exhibit a remarkably stronger pet− phenotype than mdm38Δ and a general growth reduction. They totally lack KHE activity, show a dramatic drop of mitochondrial membrane potential, and heavy fragmentation of mitochondria and vacuoles. Nigericin, an ionophore with KHE activity, fully restores growth of the triple mutant, indicating that loss of KHE activity is the underlying cause of its phenotype. Mdm38p or overexpression of MRS7p, YDL183cp, or LETM1 in the triple mutant rescues growth and KHE activity. A LETM1 human homologue, HCCR-1/LETMD1, described as an oncogene, partially suppresses the yeast triple mutant phenotype. Based on these results, we propose that YDL183p and the Mdm38p homologues MRS7p, LETM1, and HCCR-1 are involved in the formation of an active KHE system.

The high, inside negative membrane potential (Δψ) of mitochondria favors uptake of cations through the inner mitochondrial membrane. Potassium is an osmotically active ion and the most abundant cation in the cytosol and in the mitochondrial matrix. The uncontrolled influx of K⁺ into mitochondria causes an increase of osmotic pressure of the organelles and their swelling. The presence of K⁺/H⁺ exchangers in mitochondria which, driven by the inside-directed pH gradient, extrude excess K⁺ from mitochondria was already postulated in the 1960s by Mitchell (1). Although the KHE as been studied extensively by physiological methods, its molecular identity remained obscure. Recently, our studies identified Mdm38/LETM1 as major players of this extrusion system (2–4).

Phenotypic analyses of mdm38Δ are consistent with the loss of KHE activity (4). These included increased matrix K⁺ content, swelling, and fragmentation of mitochondria, reduced mitochondrial Δψ, as well as reduced growth of cells on nonfermentable substrate. Further tests involving submitochondrial inner membrane particles (SMPs) confirmed the near total lack of KHE activity (2). Addition of the synthetic KHE nigericin to mdm38Δ cells restored all mitochondrial functions, including growth on nonfermentable substrates, Δψ, morphology, and KHE activity (4, 5). This result strongly supported the conclusion that Mdm38 acts as an essential regulator or subunit of the mitochondrial KHE, because it is unlikely that a protein with only one transmembrane domain like Mdm38 forms the KHE itself. Mdm38p is conserved in all eukaryotic organisms. The human homologue, LETM1, has been implicated in the Wolf-Hirschhorn syndrome (6). The yeast Saccharomyces cerevisiae encodes a homologue, YPR125w, YPR125w had initially been identified as a multicopy suppressor of mutants lacking the mitochondrial Mg²⁺ transporter MR52 and was named MRS7 (7). YPR125w/MRS7, also named YLH47 for yeast LETM1 homologue of 47 kDa (6), encodes a protein located in mitochondria (4, 8). Although disruption of MRS7 has a weak phenotype, its overexpression restores growth of mdm38Δ strains, showing a functional homology to Mdm38p (4). The human genome also encodes a second member of the Mdm38/LETM1 family, named HCCR-1 or LETMD1, which was found to be overexpressed in various human cancer cells (9). Here, we characterize the role of four yeast multicopy suppressors of mdm38Δ as well as of LETM1 and HCCR-1 with

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4 and an additional reference.
10 This paper is dedicated to the memory of Rudolf Schweyen, who tragically passed away during the writing of this manuscript.
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The abbreviations used are: KHE, K⁺/H⁺ exchanger; SMP, submitochondrial particle; FMN-44, N-(3-triethylammoniumpropyl)-4-[4-(3-diethylamino)phenyl]hexatrienyl]pyridinium dibromide; GFP, green fluorescent protein; HA, hemagglutinin; CoIP, coimmunoprecipitation; ORF, open reading frame; Ni-NTA, nickel-nitrilotriacetic acid; GFP, green fluorescent protein; BCFCEF, 2′,2′-bis(carboxyethyl)-5,6-carboxyfluorescein; BN, Blue Native; YFP, yellow fluorescent protein; PBF, potassium-binding benzoferan isophthalate.
respect to their potential to restore K⁺/H⁺ exchange activity. We find that Mdm38p, Mrs7p and Ydl183cp are part of a large mitochondrial KHE protein complex. We discuss its putative composition and assemble the additive effects resulting from the triple deletion of MDM38, MRS7, and YDL183c.

**EXPERIMENTAL PROCEDURES**

*Yeast Strains and Growth Media*—The *S. cerevisiae* strains W303 (ATCC accession number 2012239) and BY747 (ATCC accession number 204659) were used as wild type. W303 *mdm38::HIS3* termed *mdm38Δ* was described previously (4). W303 cells were grown in YPD (yeast extract, bacto peptone, 2% dextrose), YPG (2% glycerol) or YPGal (yeast extract, bacto peptone, 2% galactose) media as indicated. YPG plates were supplemented with 2 μM nigericin when indicated. Synthetic minimal media (S-Gal, synthetic medium containing 2% galactose, or SD, synthetic medium with 2% dextrose, 2% glucose) were supplemented with amino acids and bases when appropriate.

Genomically tagged versions of *MDM38* and *MRS7* were constructed by homologous recombination. The TAG and the selection marker *TRPI-1* were amplified by PCR from the vector pBS1479 (10). The following primers were used to create a C-terminally tagged version of *MDM38* with His₆, and the TAG tag consisting of two immunoglobulin binding domains of protein A and the calmodulin-binding peptide: *MDM38*HisTAGp, 5'-TACCTCCCCGCGATCAAGCTCGGAAGACTTTTTGCTTACTTAAAGAAGATCATCACCATCACCATCACTCCATGGAAAAGAGAAG-3'; *MDM38*HisTAGp, 5'-CCTGATGTAACCTGTGTCATGGAAAAGAGAAG-3. For tagging *MRS7* with His₆ and TAG, the following primers were used: *MRS7*HisTAGp, 5'-AAGGCGAGGAGAGACCATCACCATCACCATCACCATCATCATCGAGAAAAGAGAAGAAG-3'; *MRS7*HisTAGp, 5'-AGAGCATTAGCTTACCTGTGTGAGAAGACACCATCGCTGCAGGTCGAC-3. To create chromosomal, C-terminally His₆-tagged versions of *MDM38* and *MRS7*, the following forward primers were used: *MDM38*Hisfw, 5'-TACCTCCCCGCGATCAAGCTCGGAAGACTTTTTGCTTACTTAAAGAAGATCATCACCATCACCATCACTCCATGGAAAAGAGAAG-3'. The following primers were used for creating chromosomal, C-terminal One-StrEP (11)-tagged versions of *MDM38* and *MRS7*, the One-StrEP sequence (based on the plasmid pEXP-IBA103, IBA BioTAGnology) was synthesized (Eurofins MWG GmbH) and cloned into the BamHI-linearized pBS1479 plasmid. Chromosomal integration was performed using the forward primers: *MDM38*OneStrEPfw, 5'-CATCCTCCCCGCGATCAAGCTCGGAAGACTTTTTGCTTACCTTAAGAAAGATGAGAATTGGTATTTCAGG-3'; and *MRS7*OneStrEPfw, 5'-GATCCGACACCAAGCCTATCGAGAAGGAGGAGGAGAAGGAGAAGGAGATTTGTATTTTAGATTAAAGCGTACGCTGCAGGTCGAC-3. Following verification of the correct gene replacement using analytical PCR (12), the selection marker was removed with the CRE recombinase containing vector pHS63 (13). *YDL183c* deletion in *BY747* was performed by replacement with the *LEU2* disruption cassette, using the primers 5'-GTCGACGATTATTGCAAATTAATTGAAACTACCTGTCGAGGTCGAGGATTTTATCCATGATACGTTCAATATTTATCCGCAG-3' and 5'-ACCTGTATATTTATCCATGATACGTTCAATATTTATCCGCCAGGTCGAGGATTTTATCCATGATACGTTCAATATTTATCCAGG-3'.

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**Plasmid Constructs**—To provide *MRS7*, *MDM38*, and *YDL183c* with a C-terminal GFP tag, the entire respective ORFs were cloned into the centromeric vector pUG35 (14). *MRS7* coding sequence was amplified by PCR from *YEpl51-MRS7* plasmid (7) with the 5' primer 5'-ACAAAGAATTCATGCTGGAATACCGTCGCTGAC-3' and the 3' primer 5'-ACGTCGAC-CTTCTCCTTTGATGGC-3' (EcoRI and Sall sites are underlined). The putative candidates were cloned into the EcoRI/Sall sites of the plasmid pUG35 carrying the methionine promoter. To clone *YDL183c* into the pUG35 plasmid, the entire ORF sequence was amplified by PCR from W303 genomic DNA by use of 5' primer 5'-GCCGATCCATGATACGTTCAATATTTTACGCCGAC-3' and 3' primer 5'-GCCGATCCATGATACGTTCAATATTTTACGCCGAC-3'.
GAGATTTCG-3’ introducing the underlined BamHI and Sall restriction sites. The amplified fragment was cloned into the BamHI- and Sall-linearized pUG35 plasmid. A C-terminally GFP-tagged version of the entire ORF MDM38 was obtained by cloning MDM38 in pUG35 by use of the forward primer 5’-TAATATGGATCCATGGATCAATG-3’ and the reverse primer 5’-AATATCTATCGATCT-3’. To express YDL183c from its own promoter and in fusion with the triple HA epitope at the C-terminal end, the entire ORF was amplified from MGC IRAT human (Invitrogen 6009854) with the triple HA epitope at the C-terminal end, the entire ORF was amplified from W303 genomic DNA with the primers 5’-CCAGGGTGTG-3’ and 5’-GCGTCGACCAATTTGTTTTTCTTTGAGATTCTTCCC‘-3’ and inserted in the SacI/Sall sites of the YCP33-HA vector. The vectors YCP33-MDM38-HA and Yep351-MDM38-HA expressing Mdm38p under the control of its native promoter and pVTU103-LETM1-HA expressing the human LETM1 from the native promoter and pVTU103 vector.

Isolation and Subfractionation of Mitochondria—Yeast mitochondria used for ion-flux measurements were isolated from cells growing overnight to stationary phase. For all other experiments, cells were grown to $A_{600}$ = 1. Mitochondrial isolation and mitoplast preparation were done as described previously (15). Protein extraction with sodium carbonate was performed according to Ref. 16 followed by protein precipitation with trichloroacetic acid and Western blotting analysis. Proteinase K protection experiments were performed as described previously (17). Resuspended mitoplasts were incubated in the presence or absence of proteinase K as indicated for 20 min, and the proteinase K reaction was inactivated with 1 $\mu$m phenylmethylsulfonyl fluoride, and the proteins were trichloroacetic acid-precipitated. 50 $\mu$g of protein were loaded in each lane of a 12.5% SDS–PAGE, transferred onto polyvinylidene difluoride membrane, and immunoblotted in Tris-buffered saline/Tween plus 2.5% dry milk with the antibodies against the following: HA (laboratory stock; hexokinase-1 (Biotrend); F1 plus 2.5% dry milk with the antibodies against the following: HA and the reverse primer 5’-GACTTGACGGC-3’ of the YCp33-HA vector. The vectors YCp33-MDM38-HA and Yep351-MDM38-HA expressing Mdm38p under the control of its native promoter and pVTU103-LETM1-HA expressing the human LETM1 from the native promoter and in fusion

Coimmunoprecipitation (CoIP)—UltraLink immobilized protein A, covalently bound to HA aspergillus with the cross-linker, was kindly provided by A. Pichler. Isolated mitochondria (2 mg of protein) expressing Yep-MDM38-HA (70 kDa) and either pUG-MDM38-GFP (92 kDa), YDL183-GFP (64 kDa), or AIF-GFP (68 kDa) were solubilized for 30 min in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS containing 1.2% n-dodecyl-$d$-malto side (Sigma)) and protease inhibitor mixture (complete Mini, Roche Applied Science) plus 1 mM phenylmethylsulfonyl fluoride and, after a clarifying spin, incubated under rotation for 1 h with 10 $\mu$l of the HA-coupled beads, washed four times in RIPA buffer, and eluted in Laemmli buffer. Proteins were separated on 12.5% SDS–PAGE, transferred, and analyzed by immunoblotting with GFP (Roche Applied Science) and HA (laboratory stock) antibodies.

Measurements of the Mitochondrial Membrane Potential—The membrane potential of isolated mitochondria was recorded in an LS 55 fluorescence spectrometer (PerkinElmer Life Sciences) by monitoring the fluorescence of 5,5’,6,6’-tetra chloro-1,1,3,3’-tetraethylbenzimidazolylcarbocyanine iodide
K⁺/H⁺ Exchange Measurements in SMPs—Preparations of SMPs and loading with the K⁺- and H⁺-sensitive fluorescent dyes potassium-binding benzofuran isophthalate (PBFI) and BCECF (both Invitrogen) were made as described previously (2). SMPs were treated with 1 μM antimycin A and 1 μM oligomycin prior to measurements. To determine the kinetics of K⁺ and H⁺ transport across the membrane, 150 mM KCl was added to the SMPs. When indicated, incubation of SMPs with 10 μM nigericin (Sigma) was done at room temperature for 5 min before the measurements. All measurements were repeated at least three times with different preparations of SMPs.

Confocal Microscopy—The plasmids pHS72 (TOM72-YFP) (21) and pYX232-ntGFP (22) were gifts from H. Sesaki and B. Westermann, respectively, and served to label mitochondria. Alternatively, mitochondria were labeled with Mitotracker Red (100 nM). Vacuoles were stained with FM4-64 in a final concentration of 10 μM (Molecular Probes). Microscopy settings were used as described previously (5).

Electron Microscopy—Cells were harvested at logarithmic growth phase (A₆₀₀ = 1). Cryofixation, freeze substitution, thin sectioning, and image acquisition were performed as described previously (5).

RESULTS

PIC2, MRS3, MRS7, and a Novel Gene YDL183c Act as Multicopy Suppressors of mdm38Δ—Absence of Mdm38p in yeast cells (mdm38Δ mutants) results in reduced growth on nonfermentable substrate (pet− phenotype) (4). According to our previous data, the antibiotic nigericin, a KHE ionophore, acts as a multivalent suppressor of the mdm38Δ deletion phenotype. It restores cell growth on nonfermentable substrate, KHE activity, and Δψ and reverts matrix swelling and fragmentation of mitochondria (5).

To identify proteins substituting similarly for the function of MDM38, we have screened a yeast genomic library and selected suppressor genes that, being overexpressed, restored growth of mdm38Δ on nonfermentable substrate (Fig. 1). Among the suppressors, we found three previously described genes, PIC2, MRS3, and MRS7, encoding mitochondrial proteins. PIC2 and MRS3 encode mitochondrial carrier proteins involved in P₇ transport and Fe³⁺ accumulation, respectively (23, 24). MRS7 encodes a functional homologue of Mdm38p, located in the inner membrane of mitochondria, with a weak deletion phenotype depending on the strain (7, 8). Additionally, we found one not yet characterized gene, YDL183c.

Up-regulation of Pic2, Mrs3, Mrs7, Ydl183c, or Human LETM1 Increases the Mitochondrial Membrane Potential of mdm38Δ—Δψ was found to be moderately reduced in ydl183cΔ or mrs7Δ and substantially impaired in mdm38Δ mitochondria. Having determined that overexpression of Pic2p, Mrs3p, Mrs7p, and Ydl183cp rescued the nonfermentative growth of mdm38Δ, we asked whether this positive growth effect also correlated with a rise of the mitochondrial Δψ of the mdm38Δ mutant. Although Δψ was slightly increased upon overexpression of Pic2p and more significantly upon overexpression of Mrs3p in mdm38Δ, overexpression of Mrs7p and Ydl183cp in the mutant restored Δψ close to the wild-type levels (Table 1).

Mitochondrial Morphology Is Restored upon Overexpression of the Suppressor Genes in mdm38Δ—Next, we investigated whether high copy expression of the suppressor genes reversed the fragmentation of mdm38Δ mitochondria. For this purpose, mdm38Δ cells expressing a GFP targeted to the mitochondrial matrix and a vector with or without the suppressor genes were observed under the confocal microscope. Mitochondria from mdm38Δ cells transformed with the empty vector appeared fragmented into large unconnected spheres (Fig. 2a). Compared with wild-type cells, mdm38Δ cells displayed wild-type-like elongated tubular mitochondria in only about 3% of the population. Mutant mdm38Δ cells overexpressing PIC2 exhibited a heterogeneous mixture of spherical and tubular mitochondria, indicating a partial reversion of the phenotype (Fig. 2c). Overexpression of MRS3, YDL183c, or MRS7 resulted in a tubular mitochondrial network (Fig. 2, d–f, respectively, and Table 2) similar to that displayed by wild-type cells (Fig. 2b). The percentage of elongated tubular mitochondria was shifted to almost 80% upon overexpression of Pic2 and to about 95% when Mrs3, Mrs7, or Ydl183c was overexpressed (Table 2).

YDL183c Is a Strong Suppressor for Mitochondrial KHE Activity in mdm38Δ—Because swelling, depolarization, and fragmentation of mdm38Δ mitochondria result from loss of mitochondrial KHE activity and mitochondrial K⁺ overload (5), we next asked whether overexpression of the suppressor genes restored the mitochondrial defects by modulating the KHE activity.
TABLE 1
Relative Δψ of mdm38Δ (Δ) and mdm38Δ mrs7Δ ydl183cΔ (ΔΔΔ) mutants in function of the overexpressed suppressor genes
The relative Δψ of mitochondria are expressed in % relatively to hyperpolarization of the probe with nigericin. ND, no data.

| Strains          | Vectors        | Empty | PIC2 | MRS3 | MRS7 | YDL183 | MDM38 | LETM1 |
|------------------|----------------|-------|------|------|------|--------|-------|-------|
| Δ                |                | 48    | 62 ± 3.4 | 85 ± 2.7 | 98 ± 3 | 92 ± 6 | 95 ± 6 | ND    |
| ΔΔΔ              |                | 17 ± 2 | ND   | ND   | 89 ± 3.3 | 78.6 ± 3.3 | 62.3 ± 3.4 | 82.3 ± 5.2 |

FIGURE 2. Mitochondrial morphology in function of overexpression of the proteins Pic2, Mrs3, Mrs7, or Ydl183c in W303 mdm38Δ mutant cells.
Mitochondrial morphology of cells cotransformed with a mitochondrial matrix targeted GFP (pYX232-mtGFP) and the vector without (a) or with the following suppressor genes: PIC2 (c), MRS3 (d), YDL183c (f), and MRS7 (e) were compared with wild-type (WT) cells (b). Cells were grown in galactose-containing medium and analyzed by differential interference contrast (Nomarski) and confocal fluorescence microscopy.

TABLE 2
Mitochondrial morphology of W303 mdm38Δ cells in function of the overexpressed suppressor gene
Strains were grown overnight, and mitochondrial morphology was visualized by detection of the expression of the mitochondrial targeted matrix GFP under fluorescence microscopy. Cells were counted with hidden identity.

| Strain            | Total cells | % cells with fragmented mitochondria |
|-------------------|-------------|--------------------------------------|
| Wild type         | 602         | 2.6 ± 1.6                            |
| mdm38Δ            | 680         | 89.0 ± 5.8                           |
| mdm38Δ + (MRS7)n  | 1660        | 7.3 ± 2.6                            |
| mdm38Δ + (PIC2)n  | 870         | 20.3 ± 4.3                           |
| mdm38Δ + (MRS3)n  | 1126        | 10.6 ± 6.3                           |
| mdm38Δ + (YDL183)n| 1040        | 7.7 ± 3.8                            |

We have developed a method to measure the KHE activity across the mitochondrial inner membrane using SMPs with entrapped K⁺⁻ and H⁺⁻ sensitive fluorescent dyes PBFI and BCECF (2). This approach allows controlling internal and external ion milieus at will and recording of both proton and potassium fluxes. As shown previously and here in Fig. 3, SMPs prepared from wild-type mitochondria exhibited rapid, reciprocal translocation of K⁺⁻ and H⁺⁻ driven by concentration gradients of either. In contrast, SMPs from mdm38Δ failed to exhibit changes in [H⁺⁻], and those in [K⁺⁻] were drastically reduced. Nigericin restored K⁺⁻ and H⁺⁻ translocation in mutant SMPs to the wild-type level (Fig. 3B) (3).

SMPs were then prepared from mdm38Δ mitochondria overexpressing the respective suppressors (Fig. 3A). Overexpression of the phosphate carrier Pic2p showed a mild increase in K⁺⁻ fluxes and a stronger increase in H⁺⁻ fluxes (Fig. 3A, round dotted line). Interestingly, when K₂HPO₄ was used as K⁺⁻ salt instead of KCl, K⁺⁻ fluxes were not significantly re-established, whereas H⁺⁻ fluxes reached wild-type levels (data not shown), which are consistent with the role of Pic2 as PO₄⁻⁻/H⁺⁻ transporter (23). Overexpression of Msr3p poorly restored the K⁺⁻ and H⁺⁻ fluxes (Fig. 3A, black broken line). However, overexpression of Ydl183cp restored the K⁺⁻ and H⁺⁻ exchange activity to a wild-type level, as did overexpression of Msr7p, the yeast Mdm38p homologue (Fig. 3A, black solid line and gray square dotted line, respectively) or addition of nigericin. These results confirmed that, in contrast to Pic2p or Msr3p, Ydl183cp, like Msr7p, can fully substitute for Mdm38p in providing mitochondria with KHE activity.

Ydl183cp Is an Integral Mitochondrial Protein—YDL183c encodes a protein of 320 amino acids with a molecular mass of about 37 kDa. The computer programs DAS and TMPRED (available on line) predict one transmembrane domain (196–212 amino acids) and a potential N-terminal mitochondrial targeting sequence (Nomarski) and confocal fluorescence microscopy.

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To determine the cellular localization of Ydl183c, cells expressing the fusion protein Ydl183c-GFP from the MET promoter encoded on the centromeric plasmid pUG35 were stained with Mito Tracker Red. Fluorescence confocal microscopy revealed the colocalization of GFP and red fluorescence, indicating the mitochondrial localization of Ydl183c-GFP (Fig. 4B). To confirm these data, biochemical studies were performed with cells expressing the low copy vector encoding Ydl183c from its own promoter and C-terminally tagged with the triple hemagglutinin (HA) epitope. Cell fractionation and immunoblotting showed Ydl183c-HA protein to cofractionate with a mitochondrial protein (Porin1, Por1p), whereas the cytosolic protein hexokinase 1 (Hxk1p) was detected in the post-mitochondrial fraction, excluding the possibility of cross-
contamination of cytoplasmic and mitochondrial fractions (Fig. 4C, panel a). Fractionation of mitochondria into pellet and supernatant upon alkaline sodium carbonate treatment released the membrane-associated β subunit of the F1-ATPase (F1β) almost entirely into the supernatant, whereas the membrane protein Por1p was retained in the pellet fraction containing integral proteins. Ydl183c-HA was found in the membrane pellet (Fig. 4C, panel b, lane P). However, in contrast to Por1p, Ydl183c-HA was also partially found in the soluble fraction (Fig. 4C, panel b, lane SN). These results indicated that Ydl183cp is inserted into one of the mitochondrial membranes where it can be partly released by alkaline treatment.

For further determination of the topology of Ydl183cp, intact mitochondria were first treated with or without proteinase K. Ydl183cp was not degraded upon addition of proteinase K (data not shown). Mitoplasts were prepared by osmotic swelling and rupture of the mitochondria. Mitoplasts containing the inner membrane were treated with proteinase K (Fig. 4C, panel c). To control the intactness of mitoplasts, the topology of known proteins was also tested. Tim44p, a matrix-sided protein of the inner membrane, remained protected from proteinase K, indicating that the mitoplasts were intact. In contrast, Yme1p, an inner mitochondrial membrane protein partially exposed to the outside of mitoplasts, was accessible to proteinase K indicating that the outer membrane was disrupted. The C-terminally tagged Ydl183cp was resistant to 40 g/ml proteinase K, whereas it became accessible to higher proteinase K concentrations. Proteinase K at 120 µg/ml degraded most of Ydl183-HA without generating proteolytic C-terminal fragments. When mitoplast were lysed with Triton X-100 and then treated with proteinase K, the protein was entirely degraded. Altogether, although alkaline extraction released some of the protein, these results qualify Ydl183p as an integral protein of the inner mitochondrial membrane, with a C_out (facing the intermembrane space) topology. Degradation of Ydl183c-HA occurred only in presence of high concentrations of proteinase K as compared with Yme1, either because it is shielded by other proteins or Ydl183cp is intrinsically more resistant to proteinase K.

Synthetic Growth Effect of Triple Disruptions of MDM38, MRS7, and YDL183c—The W303 and DBY ydl183c disruption strain showed reduced growth on nonfermentable carbon sources (YPG) at high temperature (37 °C) (data not shown). Reduced growth on nonfermentable substrate was also reported by Volckaert et al. (25) for a FY ydl183c mutant at 30 °C and 37 °C. The double disruptants ydl183c mrs7Δ exhibited a mild growth reduction on nonfermentable sub-

FIGURE 3. KHE activity of mdm38Δ SMPs in the function of the suppressors Pic2, Mrs3, Mrs7, and Ydl183c. Submitochondrial inner membrane particles were prepared from wild-type and mdm38Δ mutant cells with entrapped K⁺-sensitive PBF1 or H⁺-sensitive BCECF. Ratios of K⁺-bound or H⁺-bound to -unbound dyes were recorded at 25 °C at resting conditions and upon the addition of 150 mM KCl. A shown are the effects on K⁺ and H⁺ fluxes in SMPs upon overexpression of the suppressor genes in W303 mdm38Δ. SMPs were prepared from mitochondria of wild type (WT) (black thin dashed line) or mutant mdm38Δ cells carrying the empty plasmid (gray solid) or the suppressor plasmid containing the genes PIC2 (black dotted line), Mrs3 (black bold dashed line), Mrs7 (gray square dotted line), or YDL183c (black solid line). B, increase of [K⁺]i and [H⁺]i observed in SMPs from DBY wild-type (black dashed line), single mutant mdm38Δ (gray dashed line), or triple mutant mdm38Δ mr7Δ ydl183cΔ (black thin solid line) in the absence of nigericin or mdm38 (gray bold solid line) and mdm38Δ mr7Δ ydl183cΔ (black bold solid line) in the presence of nigericin.
Novel Players in Mitochondrial KHE

Figure 4. Ydl183cp is a component of the mitochondrial inner membrane. A, Ydl183cp is a member of a novel protein family. Homologous proteins were identified by a BLAST search. A sequence alignment (ClustalW) of Ydl183cp and its homologues in A. thaliana (A.t.) and Neurospora crassa (N.c.) is shown here. Identical amino acids are highlighted in black and similar amino acids in gray. The putative potential N-terminal mitochondrial targeting sequence is marked with a dotted bar and the putative transmembrane domain with a solid bar. B, localization of the Ydl183c-GFP fusion protein analyzed under confocal microscopy. W303 cells expressing the Ydl183c-HA fusion protein (YCp-MCM3) are shown here. Identical subcellular and submitochondrial localization of Ydl183cp. Panel a, W303 cells expressing the Ydl183c-HA fusion protein (Ycp-YDL183c-HA, 42 kDa) were grown to log phase in galactose-containing medium at 28 °C. Mitochondria are labeled with MitoTracker red chloromethyl-X-rosamine. C, subcellular and submitochondrial localization of Ydl183cp. Panel a, W303 cells expressing the Ydl183c-HA fusion protein (Ycp-YDL183c-HA, 42 kDa) were grown to log phase in galactose-containing medium at 28 °C. Mitochondria are labeled with MitoTracker red chloromethyl-X-rosamine.
Novel Players in Mitochondrial KHE

A

28

16

35.5

YPD

YPG

mdm38

mdm38Δ mrs7Δ

mdm38Δ ydl183cΔ

mhd1Δ mrs7Δ ydl183cΔ

mdm38Δ

mdm38Δ mrs7Δ

mdm38Δ ydl183cΔ

mdm38Δ mrs7Δ ydl183cΔ

mdm38Δ

mdm38Δ mrs7Δ

mdm38Δ ydl183cΔ

mdm38Δ mrs7Δ ydl183cΔ

mdm38Δ

B

YPD 16°C YPG 16°C YPD 20°C YPG 20°C

WT

mdm38Δ ydl183cΔ mrs7Δ

mdm38Δ ydl183cΔ mrs7Δ + (MRS7/H)

mdm38Δ ydl183cΔ mrs7Δ + MDM38

mdm38Δ ydl183cΔ mrs7Δ + (YDL183c/H)

mdm38Δ ydl183cΔ mrs7Δ + (LETM1/H)

C

16 16 28 28

YPD YPG YPD YPG

-YPG YPG YPG negicerin

WT

mdm38Δ

mdm38Δ mrs7Δ

mdm38Δ ydl183cΔ

mdm38Δ mrs7Δ ydl183cΔ

FIGURE 5. Deletion growth phenotypes. A, serial dilutions of DBY. mdm38Δ, mdm38Δ mrs7Δ, mdm38Δ ydl183cΔ, and mdm38Δ mrs7Δ ydl183cΔ mutants were spotted onto YDP and YPG and incubated at the indicated temperatures. Growth on 28, 35.5, and 16 °C was observed after 3, 5, and 8 days, respectively. B, DBY wild-type (WT) and mdm38Δ mrs7Δ ydl183cΔ triple mutant cells expressing an empty control vector (pUG35) or YCp33-MDM38-HA, pUG35-MRS7-GFP, pUG35-YDL183c-GFP, or pVT-U-LETM1-HA. Serial dilutions were spotted onto YPD and YPG plates and incubated for 10 days at 16 °C or 3 or 5 days at 28 and 37 °C on YPD or YPG, respectively. C, effect of negicerin on the nonfermentative growth of DBY747 mdm38Δ single, mdm38Δ mrs7Δ, mdm38Δ ydl183cΔ double, and mdm38Δ mrs7Δ ydl183cΔ triple mutant cells. Serial dilutions of the wild-type and mutant cells were spotted onto YPD and YPG plates containing (+) or not (−) 2 mM negicerin and incubated 10 days at 16 °C and 5 days at 28 °C.

terized as a mitochondrial protein (26). We verified its subcellular localization when heterologously expressed in yeast. Cell fractionation of wild-type (data not shown) and mdm38Δ mrs7Δ ydl183cΔ triple mutant cells expressing HCCR-1-His and Western blotting analysis revealed that HCCR-1 was detected as a protein of 35 kDa in the total and mitochondrial fractions (Fig. 6C). Por1p was also recovered in total and mitochondrial fractions and Hxk1p in total and cytoplasmic fractions. Accordingly, in yeast HCCR-1-His vesicles were visible in each section, all looking almost alike in size and electron density. The recognition of single or double vesicle-surrounding membranes was the only morphological criterion to discriminate between mitochondrial and vacuolar vesicles. Yet a distinction of the organelles was not always possible (Fig. 7B, panels a and b, right panels). Surprisingly, a large number of cells showed vesicular mitochondria containing undefined material suggesting either internalized membranes or paracrystalline structures (Fig. 7B, panel b, right panel). Most was exclusively found in mitochondria, although in significantly less abundant amounts than Por1p (Fig. 6C).

Severe Loss of the Mitochondrial Membrane Potential in the Absence of Mrs7p, Ydl183cp, and Mdm38p—Most importantly, the mitochondrial Δψ was dramatically reduced in the triple mutant mdm38Δ mrs7Δ ydl183cΔ (Table 1). We tested if overexpression of the individual suppressors also restored the mitochondrial Δψ in the triple mutant mdm38Δ mrs7Δ ydl183cΔ. We found that expression of Mdm38p and overexpression of Mrs7p or Ydl183cp restored the reduced Δψ of the triple mutant to a reasonable level (Table 1), a result comparable with that observed after overexpression of human LETM1. These findings suggest that cellular growth and increase of mitochondrial Δψ are mechanistically linked.

Dramatic Changes of Organelle Morphology in mdm38Δ mrs7Δ ydl183cΔ Cells—In addition to growth impairment and profound depolarization, the triple deletion mutant mdm38Δ mrs7Δ ydl183cΔ differed most strikingly from the mdm38Δ single deletion mutant in its organellar morphology. Confocal microscopic analysis of triple mutant cells expressing the mitochondrial matrix-targeted GFP showed that mitochondria appeared fragmented in spherical units, were less numerous than in the single mdm38Δ mutant, and were somewhat clumped together. Furthermore, staining of cells with the specific vacuole dye FM4-64 consistently showed a multiple lobed morphology of the vacuoles (Fig. 7A). To look into the structure of the organelles at higher resolution, electron microscopy was performed. Remarkably, numerous
in the \textit{mdm38} \textit{Δ} single mutant SMPs were fully eliminated in the triple mutant (Fig. 3B). However, KHE was fully active in \textit{yd1183c} \textit{Δ} and moderately reduced in \textit{mrs7} \textit{Δ} (data not shown). Consistent with data reported above on cell growth and \( \Delta \psi \), disruption of all three genes had additive effects on the KHE activity. However, preincubation of the \textit{mdm38} \textit{Δ} \textit{mrs7} \textit{Δ} \textit{yd1183c} \textit{Δ} SMPs with nigericin led to the activation of the \( K^+ \) and \( H^+ \) transport across the SMPs membrane, although not to full wild-type levels (Fig. 3B).

\( K^+ \) and \( H^+ \) flux measurements carried out in triple mutant SMPs revealed efficient restoration of activities by Mdm38p expressed from a single copy vector, whereas its homologue Mrs7p required expression from a multicopy vector (Fig. 8). Black solid line, and \( A \), black square dotted line, respectively. Overexpression of Ydl1183cp also restored some of the \( K^+ / H^+ \) fluxes in the triple mutant but not fully (Fig. 8A, black broken line). Finally, overexpression of the human homologue of Mdm38p, LETM1 in the triple mutant strains, restored most of the KHE activity (Fig. 8B, gray solid line).

\textit{Ydl1183c} \textit{p}, \textit{Mrs7} \textit{p}, and \textit{Mdm38} \textit{p} Form High Molecular Weight Complexes—The genetic data presented here support the notion that importantly, wild-type-like morphology of the cells was restored upon addition of nigericin (Fig. 7B, panel \textit{c}) with reversion of mitochondria from swollen, fragmented, and electron-dense organelles. This key finding links the morphological phenotype of the triple mutant to a defect of \( K^+ \) homeostasis, which can be compensated by nigericin.

For better discrimination of the origin of the visualized organelles, we used a mitochondrial YFP targeted to the outer membrane (pHS72) and the vacuolar stain FM4-64. Confocal microscopy showed stained wild-type mitochondria and vacuoles as clearly distinct organelles (Fig. 7C, panels \textit{a}–\textit{d}). In contrast, triple mutant cells exhibited widely overlapping fluorescence of FM4-64 (vacuoles) and YFP directed to the outer mitochondrial membrane (Fig. 7C, panels \textit{e}–\textit{h}), indicative of the colocalization of both organelle markers that occurs in mitophagy (27). Mitochondrial KHE Is Totally Absent in the Triple Deletion Strain \textit{mdm38} \textit{Δ} \textit{mrs7} \textit{Δ} \textit{yd1183c} \textit{Δ}—Remarkably, SMPs from the triple mutant \textit{mdm38} \textit{Δ} \textit{mrs7} \textit{Δ} \textit{yd1183c} \textit{Δ} failed to exhibit \( H^+ \) fluxes, and most importantly, residual \( K^+ \) fluxes observed

\textbf{FIGURE 6.} Suppression effect of human HCCR-1. \textit{A}, sequence alignments of Mdm38, Mrs7, Let1m1, and HCCR-1. ClustalW alignments of the amino acid sequences over the homologous regions are shown. Identities are highlighted in \textit{black} and similarities in \textit{gray}. Amino acid residues identical over all four sequences are in \textit{boldface} and \textit{boxed}. \textit{Bar} is over the transmembrane domain. \textit{B}, growth effect of HCCR-1 expression in yeast triple \textit{mdm38} \textit{Δ} \textit{mrs7} \textit{Δ} \textit{yd1183c} \textit{Δ} mutants (\( \Delta \Delta \Delta \)). Wild-type (WT) and triple mutant cells expressing pVTU103 with or without HCCR-1 were spotted onto SD-ura, YPD, and YPG plates and grown at the indicated temperatures for 6, 3, and 6 days, respectively. \textit{C}, subcellular localization of HCCR-1 in yeast. Yeast triple \textit{mdm38} \textit{Δ} \textit{mrs7} \textit{Δ} \textit{yd1183c} \textit{Δ} expressing HCCR-1 were fractionated into total (\( T \)), mitochondrial (\( M \)), and post-mitochondrial (\( C \)) fractions, and Western blotting was performed.

\begin{table}
\begin{tabular}{|c|c|c|c|}
\hline
 & SD-ura & YPD & YPG \\
\hline
\textit{WT} & 28 & 37 & 28 \\
\hline
\textit{Δ} \textit{Δ} \textit{Δ} & 28 & 37 & 28 \\
\hline
\textit{Δ} \textit{Δ} \textit{Δ} + HCCR-1 & 28 & 37 & 28 \\
\hline
\end{tabular}
\end{table}

\textbf{FIGURE 7.} \textit{A}, mitochondrial membrane (Fig. 7 \textit{C}, panels \textit{a}–\textit{d}). In contrast, triple mutant cells exhibited widely overlapping fluorescence of FM4-64 (vacuoles) and YFP directed to the outer mitochondrial membrane (Fig. 7 \textit{C}, panels \textit{e}–\textit{h}), indicative of the colocalization of both organelle markers that occurs in mitophagy (27).

Mitochondrial KHE Is Totally Absent in the Triple Deletion Strain \textit{mdm38} \textit{Δ} \textit{mrs7} \textit{Δ} \textit{yd1183c} \textit{Δ}—Remarkably, SMPs from the triple mutant \textit{mdm38} \textit{Δ} \textit{mrs7} \textit{Δ} \textit{yd1183c} \textit{Δ} failed to exhibit \( H^+ \) fluxes, and most importantly, residual \( K^+ \) fluxes observed
Novel Players in Mitochondrial KHE

A

Nomarski

GFP

FM4-64

Overlay

B

C

a

b

c

a

b

c

d

e

f

g

h
the anti-His antibody revealed that Mdm38-His (∼67 kDa) migrated at ∼500, <232, and <140 kDa (Fig. 9A, left panel). Of note, Mdm38-His was detected in protein complexes of the same molecular weights irrespective of the presence of Mrs7p or Ydl183cp (data not shown). Furthermore, Mrs7-His (53 kDa) appeared in three bands around ∼500 kDa and an additional band of >232 kDa (Fig. 9B, lane 1). BN-PAGE analysis of solubilized mitochondria expressing Ydl183-GFP yielded a product of an apparent molecular mass of about 67 kDa, which corresponds to its molecular weight as GFP-tagged monomer and to additional bands of <232 kDa, representing YDL183GFP-containing complexes. However, in the background of a mutant mdm38Δ strain, Ydl183-GFP partly shifted to a major band of ∼500 kDa (Fig. 9C).

Next, we affinity-purified the chromosomally His-tagged Mdm38p and Mrs7p. The proteins were bound to the resin and eluted from the column prior to analysis on BN-PAGE. Surprisingly, despite changing the experimental conditions such as the incubation times with the Ni-NTA beads or using different detergents or NaCl concentrations, the eluted Mdm38-His exclusively appeared as a single band of a molecular mass slightly smaller than 232 kDa (Fig. 9A, middle panel). This was an unexpected result. To find out if the protein complex of ∼500 kDa containing the His-tagged Mdm38p as detected on BN prior affinity purification had become inaccessible to the column possibly because it was hidden by additional proteins of the larger complex, we decided to affinity-purify Mdm38 fused to the One-STrEP tag. This tag containing a linker region makes the tagged component of a protein complex more accessible to the column. In fact, using the chromosomal One-STrEP-tagged version of MDM38, the purified Mdm38p was recovered within high molecular complexes ranging between ∼500 and < 600 kDa in addition to the complex of <232 kDa (Fig. 9A, right panel).

Affinity chromatography of solubilized mitochondria chromosomally expressing Mrs7-His followed by BN-PAGE recovered Mrs7-His within three complexes of <140, >232, and between 440 and 669 kDa as seen in Fig. 9B, left panel, lane 2. Similar results were obtained using Mrs7OneStrep instead of Mrs7His (Fig. 9, right panel). In the next step, we solubilized mitochondria from chromosomally Mrs7-His-tagged cells coexpressing either YCp-Mdm38-ΔHA or YCp-Ydl183-HA. Mitochondrial expression of Mdm38-ΔHA and Ydl183c-HA was confirmed by Western blotting (data not shown). Affinity purification followed by BN-PAGE and Western blotting analyses, including immunodetection with anti-His and HA antibodies, was performed. Although the anti-His antibody recognized Mrs7-His, neither Mdm38-ΔHA nor Ydl183c-HA was detectable when the eluted fractions were probed with the anti-HA antibody, excluding a direct interaction of Mrs7-His and Mdm38-ΔHA or Mrs7-His and Ydl183c-HA (Fig. 10, A and C, respectively). Second dimension SDS-PAGE confirmed that Mdm38-ΔHA was not part of the Mrs7-His complex (Fig. 10B, right panel). Taken together, our experiments did not suggest any direct interaction between Mdm38-ΔHA and Mrs7-His.

These results are in contrast to data reported previously by Frazier et al. (8), indicating a direct interaction of a protein A-tagged Mdm38 with numerous other mitochondrial proteins, including Mrs7p. In fact, when we used a strain expressing the Mrs7 protein C-terminally tagged with a His fused to protein A (Mrs7-His-TAP), we found that Mdm38 coeluted with Mrs7-His-TAP in the ∼232-kDa complex (Fig. 11, B and C). However, a direct interaction between Mrs7-His-TAP and Ydl183c was not detectable (Fig. 10C). We asked whether the tagged suppression of the mutant phenotype. Mrs7-His or Mrs7-His-TAP was introduced into the mdm38Δ mutant, and nonfermentative growth was tested. We found that mdm38Δ cells expressing Mrs7-His-TAP did not grow as well as Mrs7-His or wild-type cells (Fig. 11D). Thus, these data altogether suggest that Mrs7p and Mdm38p are not interacting directly.

We used CoIP to ask whether Mdm38 homo- or hetero-oligomerizes with Ydl183c. Mdm38 self-oligomerization was confirmed by CoIP experiments performed on mdm38Δ cells coexpressing Mdm38-ΔHA (72 kDa) and Mdm38-GFP (92 kDa). Although Mdm38-ΔHA was successfully bound to HA-coated protein A beads, only Mdm38-GFP was pulled down, and Ydl183c-GFP did not copurify in the protein A-bound fractions like Aif-GFP serving as negative control (Fig. 12).

**DISCUSSION**

We previously characterized Mdm38p as a mitochondrial protein essential for KHE activity (2, 4). Because this protein has only one transmembrane domain, it appears unlikely to be solely responsible for the KHE process. To explore the possible existence of additional proteins involved in KHE, we carried out a genome-wide suppressor screen.

We identified the mitochondrial carriers Pic2p and Mrs3p as weak suppressors. Their overexpression rescued the growth defect of mdm38Δ. We showed that mitochondrial morphology of mdm38Δ was restored to wild-type upon overexpression of PIC2 and MRS3. However, in mdm38Δ SMPs, KHE was not seen after overexpression of Pic2p and was only marginally restored by Mrs3p. Because overexpression of Pic2p, a P1 carrier, had no effect on mitochondrial K+ fluxes in mdm38Δ mitochondria and resulted in a marginal increase of the mitochondrial Δψ, we hypothesize that a contribution to Δψ above a threshold is sufficient to heal the growth and morphologyphe-
notype. Alternatively, Pic2p might act indirectly by modulating proton fluxes or mitochondrial pH. Overexpression of Mrs3p, a Fe\(^{2+}\)/H\(^{100}\) carrier, increased the mitochondrial Δ\(ψ\) and moderately the KHE. Deletion of \(MRS3\) had no effect on KHE, and \(mrs3\Delta\) \(mdm38\Delta\) mutants remained without synthetic phenotype,\(^5\) excluding a role of Mrs3p as the KHE. These findings suggest an indirect role of Mrs3p on the KHE activity. In fact, several ions have been stated to play a direct or indirect role in mitochondrial K\(^+\) homeostasis (28–31).

The suppressor screen also identified two additional genes, \(MRS7\) and the novel gene \(YDL183c\) encoding an unknown protein, which were found to restore both growth and KHE activity of \(mdm38\Delta\) mutant cells. Although Mdm38p and Mrs7p are phylogenetically related, Ydl183cp is likely not related to them. Each of these proteins contains a single transmembrane domain and appears to be part of a high molecular weight protein complex. They are functionally redundant in establishing a functional KHE in mitochondria.

In contrast to Mdm38p, absence of either Mrs7p or Ydl183cp alone or in combination did not seriously affect the growth of yeast cells. Yet the triple mutant \(mdm38\Delta \ mrs7\Delta \ ydl183c\Delta\) had a dramatically stronger negative growth phenotype than the single \(mdm38\Delta\) mutant or double mutants. This mutant completely failed to grow on respiratory substrates and exhibited a strain- and temperature-dependent reduced growth on fermentable substrates. This synthetic phenotype of the triple mutant indicates the following: (i) all three proteins are functionally expressed in yeast; (ii) loss of all three proteins dramatically impairs mitochondrial volume homeostasis through a disturbance that can be rescued by nigericin; and (iii) impaired volume homeostasis causes mitochondrial dysfunction affecting cell vitality as indicated by the reduction in growth on fermentable substrates.

Overexpression of Mrs7p or Ydl183cp fully compensated the growth defects of the \(mdm38\Delta\) single and the \(mdm38\Delta \ mrs7\Delta \ ydl183c\Delta\) triple disruptant. Accordingly, either protein could fully substitute for Mdm38p when expressed at high abundance. Addition of the exogenous KHE nigericin equally compensated for the growth defect of \(mdm38\Delta\) single as well as for the even stronger growth defects of the triple mutant. This finding supports the conclusion that the triple mutant growth phenotype is essentially due to a lack of KHE activity.

This important point was proved by a direct test for KHE activity on inner membrane SMPs, a system that entirely avoids any interference of osmotically swollen mutant mitochondria.

\(^5\) G. Wiesenberger and K. Nowikovsky, unpublished data.

**FIGURE 8.** KHE activity of \(mdm38\Delta \ mrs7\Delta \ ydl183c\Delta\) SMPs. \([K^+]\)-driven changes of \([K^+]\) and \([H^+]\) in submitochondrial inner-membrane particles prepared from wild-type and \(mdm38\Delta \ mrs7\Delta \ ydl183c\Delta\) mutant cells with entrapped \(K^+\)-sensitive PBFI or \(H^+\)-sensitive BCECF were recorded as described in Fig. 3. A, effect of overexpression of Mrs7p (black square dotted line) or Ydl183cp (black thin dashed line) on \([K^+]\)-driven changes of \([K^+]\) and \([H^+]\) in DBY triple mutant \(mdm38\Delta \ mrs7\Delta \ ydl183c\Delta\) SMPs (black solid line) in comparison with wild-type SMPs (black dotted line). B, effect of Mdm38p (expressed from YCP33, bold black solid line) or LETM1 (expressed from pVTU- (bold gray solid line) on \([K^+]\)-driven changes of \([K^+]\) and \([H^+]\) in DBY \(mdm38\Delta \ mrs7\Delta \ ydl183c\Delta\) SMPs (black thin solid line) in comparison with wild-type SMPs (bold square dotted line).
Novel Players in Mitochondrial KHE

A genome-wide screen in Drosophila S2 cells recently identified LETM1 as strongly affecting mitochondrial Ca\(^{2+}\) and H\(^+\) homeostasis. Absence of LETM1 resulted in reduced mitochondrial Ca\(^{2+}\) uptake \textit{in situ}, a finding that led the authors to conclude that Letm1 is the mitochondrial Ca\(^{2+}\)/H\(^+\) antiporter (35). This conclusion is puzzling, because down-regulation of the mitochondrial Ca\(^{2+}\)/H\(^+\) exchanger would rather have been expected to result in decreased Ca\(^{2+}\) efflux and therefore in increased mito-

A comparison between single and triple mutants clearly revealed that the single \textit{mdm38}\(\Delta\) mutant retained a minor KHE activity, whereas the triple mutant totally lacked this activity. Thus, single and triple mutants most likely differ only in the degree to which they have lost KHE activity. These data correlate well with the reduction in growth of the single and triple mutants. However, mild reduction in KHE activity detected in single mutants \textit{mrs7}\(\Delta\) and \textit{ydl183c}\(\Delta\) indicates that expression of \textit{M}rs7p and/or \textit{Y}dl183p is necessary for full KHE activity in wild-type cells. Yet the physiological effects of \textit{mrs7}\(\Delta\) or \textit{ydl183c}\(\Delta\) mutations are too weak to result in reduced growth of mutant cells. In assays performed on the single \textit{mdm38}\(\Delta\) mutant, overexpression of \textit{Y}dl183cp or Mrs7 could equally restore transmembranal K\(^{+}\)/H\(^+\) fluxes, like \textit{M}dm38p or LETM1. However, in the triple mutant \textit{mdm38}\(\Delta\) \textit{mrs7}\(\Delta\) \textit{ydl183c}, \textit{Y}dl183cp restored the mitochondrial KHE to a lesser extent than \textit{M}dm38p \textit{Mrs7p}, or LETM1, most likely resulting from a less abundant expression of \textit{Y}dl183c as compared with \textit{M}dm38 or \textit{Mrs7} (shown in supplemental Fig. S4). Taken together, these results indicate that \textit{M}dm38p, \textit{Mrs7p}, and \textit{Y}dl183cp are functionally redundant, but only \textit{M}dm38p is essential.

Mitochondrial depolarization in the mutants and its rescue by expression of either \textit{M}dm38p or \textit{Mrs7p} or \textit{Y}dl183cp correlated with loss and recovery of KHE activity, respectively, and with cell growth that was mildly affected on YPG when \(\Delta\psi\) fell below 60% of wild-type values and increasingly more with lower values. The lowest \(\Delta\psi\) values observed in the triple mutant also affected growth on fermentable substrate, indicating that essential functions of mitochondria, possibly protein import, were affected. The loss of \(\Delta\psi\) in mitochondria of the disruptants may be a direct consequence of the absence of H\(^+\) fluxes into the mitochondria in exchange for the efflux of K\(^{+}\) rather than an additional effect resulting from the K\(^{+}\) accumulation and swelling of mitochondria.

Total loss of KHE activity of the triple mutant was accompanied by more dramatic changes in organelle morphology than in the single \textit{mdm38}\(\Delta\) mutant. Both mitochondria and vacuoles appeared to be heavily fragmented and were shown to frequently colocalize, suggesting intense mitophagy. Notably, hyperosmotic stress has been reported to result in significant changes of the vacuole morphology of wild-type cells. In fact, the one to three large vacuoles usually present in wild-type cells underwent fragmentation to numerous smaller multilobe vacuoles (32). Interestingly, treatment of triple mutant cells with the K\(^{+}\)/H\(^+\) ionophore nigericin efficiently reversed swelling and restored a near normal mitochondrial network. As this involves the mitochondrial fusion (33, 34), we assume that proteins regulating the fusion activity are not affected by the absence of \textit{M}dm38, \textit{Mrs7}, and \textit{Y}dl183c. Vacular fragmentation was efficiently reverted together with re-establishing mitochondrial KHE activity by nigericin (data not shown). This raises the question of how the loss of KHE and swelling of mitochondria cause fragmentation of the vacuole. In sum, this study provides strong evidence for a role of all three proteins in contributing to an active mitochondrial KHE.
Figure 10. Interaction of Mrs7-His with Mdm38-HA and YDL183c-HA. A, affinity chromatography and preparative BN-PAGE of solubilized mitochondria coexpressing chromosomally His-tagged Mrs7 and extra-chromosomal YCp-Mdm38-HA in different backgrounds as follows: wild-type (WT) (lanes 1 and 2) and mdm38Δ (lanes 3–5). 120 μl (lanes 1, 3, and 5) and 60 μl (lanes 2 and 4) of the eluted fractions were applied to the same gel. Lanes 1–4 were probed with an antibody against His. Lane 5 served for the additional immunodetection with an antibody against HA. M, marker. B, second dimension SDS-PAGE of lane 3. Left panel, the antibody against His recognizes a product of ~55 kDa corresponding to Mrs7-His. The signal is in perfect agreement with the signals of the first dimension (BN-PAGE). Right panel, immunodetection with anti-HA antibody of the same blot after mild stripping. C, affinity chromatography and BN-PAGE of solubilized mitochondria coexpressing chromosomally His-tagged Mrs7 (lanes 1 and 3) or His-TAP-tagged Mrs7 (lanes 2 and 4) and extra-chromosomal Ydl183-HA (lanes 1–4). Lanes 1 and 2 and lanes 3 and 4 were probed with antibodies against HA and His, respectively.

Mitochondrial Ca\(^{2+}\) accumulation, because mitochondrial cation/H\(^+\) antiporters protect cells from mitochondrial cation overload by mediating cation efflux from energized mitochondria (1). Jiang et al. (35) also found that reconstitution of LETM1 in liposomes catalyzed Ruthenium red-sensitive Ca\(^{2+}\)/H\(^+\) exchange, which raises further questions since decades of work on mitochondria indicate that Ca\(^{2+}\)/H\(^+\) exchange is insensitive to Ruthenium red (36). Previous evidence that LETM1 is essential for mitochondrial K\(^+/\)H\(^+\) exchange is compelling (4, 5, 37), and the present study demonstrated that LETM1 fully restores mitochondrial KHE activity of the yeast triple mutant mdm38Δ mrs7Δ ydl183c like the exogenous bona fide KHE nigericin. We believe that further studies will
be needed to clarify whether the highly conserved LETM1 proteins exert different cation-specific functions in different eukaryotic organisms or rather disturbances of mitochondrial 
K⁺ homeostasis can secondarily affect mitochondrial cation transport. Relevant to this discussion, we have shown that yeast mitochondria depleted of Mdm38p display a considerably reduced influx of Mg²⁺ and Ca²⁺ resulting from decreased mitochondrial (4).

Human LETM1 has previously been shown to be part of a complex of about 550 kDa by CoIP of GFP- and HA-tagged isomers (38). Rehling and co-workers (8) reported an interaction of Mdm38-protein A with various proteins, including numerous mitochondrial ribosomal proteins and Mrs7p. In our hands, hetero-oligomerization of Mrs7p with Mdm38p was not

**FIGURE 11. Interaction of Mdm38p with Mrs7-His and Mrs7-His-TAP.** A, affinity chromatography and BN-PAGE of solubilized DBY mitochondria expressing either chromosomally His-tagged or His-TAP-tagged Mrs7. Eluted fractions 1–2 containing Mrs7-His were applied on lanes 1 and 2 and 5 and 6 and eluted fractions 1–2 containing Mrs7-His-TAP on lanes 3 and 4 and 7 and 8. BN-PAGE was performed and followed by immunostaining with an antibody against His (lanes 1–4) and Mdm38p (lanes 5–8). M, marker. B, preparative affinity chromatography and BN-PAGE of DBY mitochondria expressing chromosomally Mrs7-His-TAP prior to second dimension SDS-PAGE. The membrane was first incubated with an anti-His primary antibody (lane 1). Thereafter, the blot was mildly stripped and reincubated with an antibody against Mdm38p (lane 2). C, second dimension SDS-PAGE. Left panel, the blot was probed with the anti His antibody. Right panel, same blot probed with the anti Mdm38p antibody after mild stripping of the membrane. D, suppression effect of Mrs7-His and Mrs7-His-TAP in *mdm38Δ*. DBY wild type (WT) with YEp112 empty and *mdm38Δ* with YEp112 empty, MRS7-His, or MRS7-His-TAP were grown overnight. Serial dilutions were spotted onto YPD and YPG plates and incubated at the indicated temperatures.

**FIGURE 12. CoIP of isolated *mdm38Δ* mitochondria coexpressing YEp-MDM38-HA (72 kDa) and pUG-MDM38-GFP (92 kDa) (A), YDL183-GFP (65 kDa) (B), or AIF-GFP (68 kDa) (C). F, flow-through fraction; B, HA-coated protein A-bound fraction.**
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