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expression of a novel member of the mitochondrial carrier f
ues defects in both DNA and RNA metabolism
east mitochondria

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t The *PIF1* and *MRS2* gene products have pre- been shown to be essential for mitochondrial naintenance at elevated temperatures and mito- ial group II intron splicing, respectively, in the *accharomyces cerevisiae*. A multicopy suppressor e of rescuing the respiratory deficient phenotype ted with null alleles of either gene has been iso- This suppressor is a nuclear gene that was called *MRS12*. The *RIM2/MRS12* gene encodes a pre- protein of 377 amino acids that is essential for ondrial DNA metabolism and proper cell . Inactivation of this gene causes the total loss of ondrial DNA and, compared to wild-type rho° s, a slow-growth phenotype on media containing . Analysis of the RIM2/MRS12 protein se- suggests that *RIM2/MRS12* encodes a novel r of the mitochondrial carrier family. In particu- pical triplicate structure, where each repeat con- two putative transmembrane segments separat- hydrophilic loop, can be deduced from amino quence comparisons and the hydropathy profile M2/MRS12. Antibodies directed against the erminus of RIM2/MRS12 detect this protein in ondria. The function of the RIM2/MRS12 and the substrates it might transport are dis-

rds Mitochondrial carrier · Nucleic acids
opy suppressor

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Introduction

Mitochondria have evolved a crucial dependen clear gene products for the replication and exp their genomes. Selection of recombinant clone suppress defects by overexpression has been u yeast *Saccharomyces cerevisiae* to analyze rat steps in biological pathways and to understan molecular interactions and cell structure (rev Rine 1991). Suppressor genes that rescue defec mitochondrial (mt) DNA or mtRNA metab involved in mitochondrial replication, tran splicing and translation (reviewed by Griv Costanzo and Fox 1990 ; Pon and Schatz 1991 al. 1993).

Among the nuclear genes whose products a to take part in mtDNA metabolism or mito gene expression in yeast are *PIF1* and *MRS2*. gene encodes a DNA helicase involved in mt combination, repair and stability (Lahaye e and references therein). Although its function pensable under normal growth condition (Foury and Lahaye 1987), the *PIF1* gene pr pears essential for mtDNA metabolism at 36° perature at which *pif1* null mutants lose all (rho^0) and thus become unable to utilize mentable carbon sources like glycerol (Van D 1992; and references therein).

The *MRS2* gene product is required for gr tron splicing and is involved in the assembly o ponents of the respiratory chain. Even in a stra of mitochondrial introns, *MRS2* gene disruptio ciated with the absence of cytochrome *aa3* a crease in cytochrome *b* spectral bands. When p a multicopy plasmid it acts as a suppressor of NA splicing defect caused by a single base pai in domain 3 of the mitochondrial group II in the first intron of the cytochrome *b* gene (K 1987).

We have taken advantage of the condition type exhibited by the *pif1* null mutants to is

nes which, when overexpressed, can rescue ure-sensitive defect of these mutants, and ntify new factors involved in mtDNA In a previous report, we have characterized component of the mtDNA replication ap- ded by one of these suppressors - the mito- gle-stranded DNA binding protein (SSB) he *RIM1* gene (Van Dyck et al. 1992).

ar approach, we have isolated multicopy of the respiratory defect of a *mrs2* null mu- them are the *MRS3* and *MRS4* genes en- embers of the mitochondrial carrier family enberger et al. 1991; Waldherr et al. 1993). ppressors of the *mrs2* null phenotype have lated (Waldherr et al. 1993).

eport the cloning and characterization of a apable of rescuing both the *pif1* and *mrs2* efect, called *RIM2/MRS12*. *RIM2/MRS12* 7 amino acid polypeptide that is essential metabolism and proper cell growth; inacti- nuclear gene results in the complete loss of , relative to wild-type rho^0 controls, in a phenotype on media containing glucose. he RIM2/MRS12 protein sequence sug- *M2/MRS12* encodes a new member of the ticular, a typical triplicate structure, where consists of two putative transmembrane arated by a hydrophilic loop, can be de- equence comparisons and the hydropathy M2/MRS12. Antibodies directed against erminus of RIM2/MRS12 detect this itochondria. The functions of the RIM2/ ein and the substrates it might transport ssed.

methods

nd growth media

analysis of the suppression of the *mrs2* respiratory t strains used for *PIF1* suppression studies are he standard wild-type strains W303-1B (*MATα 15 leu2-3,112 ura3-1 trp1-1 can1-100* [rho$^+$]) and Ta *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-* Rothstein).

ll mutants used for the cloning experiments are Ta *pif1::LEU2 ade2-1 his3-11,15 leu2-3,112 ura3-1* [rho$^+$]) and αEVI-1a (*MATα pif1::LEU2 ade2-1 3,112 ura3-1 trp1-1 can1-100* [rho$^+$]). These two genic and issued from a cross between αW303-1B/ Lahaye 1987) and W303-1A. They are unable to ol at 36° C, due to the disruption of the *PIF1* gene. strain EVW used for *RIM2* disruption experi- om crosses between W303-1B and W303-1A. Dis- ried out by the one-step gene replacement method 983). The resulting diploid EVRT (*2 η RIM2/* vas used for tetrad dissection and analysis. The tic segregants were used in some experiments: *ATa ade2-1 trp1-1 his3-11,15 leu2-3,112* [rho$^+$]), *ATα ade2-1 trp1-1 his3-11,15 leu2-3,112*[rho$^+$]), *ATa ade2-1 trp1-1 his3-11,15 ura3-1 rim2::URA3*

The genotypes and origins of the yeast strains used for analysis of the suppression of the *mrs2* respiratory de DBY747, DBY747/M1301, GW/gd2-21.2 and GW7/gd2-21.2 have all been described before (Wiesenberger et al. 1992). Stai from the strain DBY747 (*MATa leu2-112 his3-1 ura3-52 trp1* [rho$^+$ mit$^+$], ATCC 44774) strains DBY747/M1301 (isogeni the nuclear markers but carrying a single-bp deletion in stem the mt group II intron bI1) and GW7:gd2-21.2 (with a nu *mrs2::HIS3* disruption, [rho$^+$ mit$^+$]) have been constructec this work. In a similar way, the strain GW7/gd2-21.2 (*mrs2::HIS3* [rho$^+$ mit$^+$ ΔΣaI ΔΣbI Δω]) was derived f DBY747/wo (lacking all known mitochondrial introns, i.e. Δ ΔΣaI, Δω; a gift from I. Bousquet, Centre de Génét Moléculaire, CNRS, Gif-sur-Yvette).

The following media were used to grow the strains: gluc rich medium [2% glucose, with 2% yeast extract (Kat) or yeast extract (Difco) and 2% bactopeptone (Difco)]; glycerol- medium [2% glycerol, with 2% yeast extract (Kat) or 1% y extract (Difco), 2% bactopeptone (Difco), 2% glycerol); min medium (2% glucose, 0.7% yeast nitrogen base (Difco)] sup mented with the auxotrophic requirements (40 mg/l); galac medium [2% galactose, 0.7% yeast nitrogen base (Difco) sup mented with the auxotrophic requirements (40 mg/l)]. Solid m were prepared with 2% Difco agar (minimal media) or 2% agar (rich media)

DAPI staining and visualization of DNA

Total cell DNA was stained with the DNA-specific dye 4′,6 amidino-2-phenylindole (DAPI) according to Williamson Fennel (1979), using ethanol as fixative. A 100 µl portion of culture were mixed with 200 µl of ethanol and incubated at r temperature for 20 min. Cells were washed once with 400 µ H_20 and resuspended in 100 µl of H_20. Then 10 µl of D [0.5 µg/ml) were added to 3 µl of this suspension and the cells examined by fluorescent microscopy. Module U1 of a Pol microscope (Reichert-Jung) equipped with a 200 W mercury l was used for this purpose.

DNA and RNA manipulations

Standard protocols were used for *Escherichia coli* and yeast tr formations, plasmid DNA preparations, yeast total DNA is tion, gel electrophoresis, nick translations, Southern and No ern blotting hybridizations using nitrocellulose membranes (niatis et al., 1982). Dideoxy sequencing was achieved on dou stranded (ds) DNA or single-stranded (ss) DNA by the metho Sanger et al. (1977) using T7 DNA polymerase (Pharmacia). D fragments to be sequenced were obtained by unidirectional d tions using exonuclease III and mung bean nuclease.

Cloning and subcloning of the *RIM2/MRS12* gene

Yeast genomic libraries constructed in the shuttle vectors YC YEp24 and pHCG3 were used to transform the *pif1* null mu aEVII-4b or αEVI-1a to uracil prototrophy. Ura3$^+$ transform were gridded on minimal medium supplemented with the requ amino acids before being replicated onto glycerol medium incubated at 36° C for 1 day. They were then replicated f second time onto the same medium and incubated at 36° C f days. This second replica-plating, which eliminates all non- pressed *pif1* null clones, allowed the selection of two clone which growth on glycerol medium at 36° C was partially rest upon transformation. Approximately 8000 Ura$^+$ transform were screened. *RIM2* was uncovered on the rescuing plasmid from a DNA library constructed in vector pHCG3 (a cos vector that contains the yeast selectable marker *URA3*, and

ion and the selectable marker *TRP1*; it was recently
FL45S (Bonneaud et al. 1991). Since DNA fragments
the *E.coli* origin of replication and the *amp*^{r} marker
r pHCG3 would readily religate and transform bacte-
ragments were expected to prevail over pFL45 recombi-
compose most of this library if transformants were
ly for Amp^r. Therefore, in order to ensure the efficient,
cloning of DNA sequences from the insert of cos8, we
ntage of the fact that the yeast selectable marker *TRP1*
pFL45 is able to complement the corresponding mu-
.coli. An appropriate bacterial recipient strain, MC1066
74 *hsd*R *gal*U *gal*K *rps*L *trp*C *leu*B *pyr*F::Tn5 (*Kn*^{r}); a
f François Hilger, Gembloux], was thus used for the
ation, and plasmid DNA was recovered from Trp$^+$ Am-
rmants selected on M9 minimal medium supplemented
(Maniatis et al. 1982). This mini-library was then used
m the yeast strain aEVII-4b to growth on glycerol at

tives of plasmid pFL45-8-1 were constructed as follows.
FL45-H, pFL45-E, and pFL45-S were constructed by
respectively a 1.1 kb *Hin*dIII fragment, a 4.5 kb *Eco*RI
r a 2.35 kb *Sal*I fragment from pFL45-8-1 and selfligat-
sulting plasmid. To construct plasmids pFL45-S1 and
a 2.35 kb *Sal*I fragment from pFL45-8–1 was inserted
ientations into pFL45 cut by *Sal*I.
nstruction of the yeast genomic library used for selec-
recombinant plasmid YEpMW12 has been described
(Waldherr et al. 1993). The multicopy yeast/*E. coli*
tor YEp351 (Hill et al. 1986), capable of complementing
rophs in yeast and allowing identification of recombi-
nids by screening for α-complementation in *E. coli*, was
onstructing the genomic library and for the subcloning
ts. The *E. coli* strains DH5α [*recA1 endA1 gyrA96 thi-1*
upE44 recA1 lac(F′*proAB lacI*^{q}*ZΔ*M15, Tn*10 tet*^{R})]
thersburg, Md.) and XL1-blue (*recA1 endA1 gyrA thi-1*
K-,mK+) supE44 relA1 Δ(lacZYA-argF)U169, F-
M15; Stratagene] were used as host for plasmid con-

onstruction and *RIM2/MRS12* disruption

K-*RIM2::URA3*, which contains the *rim2::URA3* al-
onstructed in two steps. A 1.65 kb *Eco*RI-*Sal*I fragment
the *RIM2* gene from plasmid pFL45-8–1 was inserted
escript SK(-) cut with *Eco*RI and *Sal*I to create SK-
418 bp *Hin*dIII-*Eco*RV fragment internal to the *RIM2*
lasmid SK-*RIM2* was then replaced by a 1.1 kb *Hin*dI-
agment containing the *URA3* marker. The resulting
SK-*RIM2::URA3* was digested with *Eco*RI and *Xho*I
site lies in the polylinker of pBluescriptSK(-)], and the
NA fragment containing the *rim2::URA3* allele was
to replace one of the corresponding wild-type copies in
strain EVW. The disruption was verified by Southern
sing a 1.1 kb *Hin*dII fragment overlapping *RIM2* as a

napping of the *RIM2/MRS12* gene

full-length separated yeast chromosomes on a Biodyne
ane (a kind gift of Jean-Claude Jauniaux, Bruxelles) was
th a 1.1 kb *Hin*dII fragment overlapping *RIM2/MRS12*.
filters containing a set of ordered DNA clones covering
genome were a kind gift of Linda Riles and Maynard
shington University, St. Louis). The 1.1 kb *Hin*dII frag-
rlapping *RIM2/MRS12* used as a probe in this experi-
cted clones 3201, 3680 and 5150.

Group 1991). We used the program BESTFIT to alig
MRS12 with members of the MCF. The multiple sequer
ment was refined using the program MACAW (Schu
1991). Dot plots were performed with the programs CO
and DOTPLOTS using a window size of 30 and a stringe

Detection of RIM2/MRS12 in mitochondria

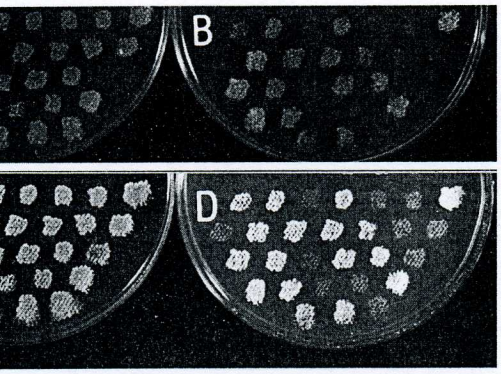
The strains aEVRT-7a (*RIM2*) and EVRT-8a (*RIM2*,
the plasmid RIM2/PFL45-8–1 were grown in glycerol
while the strain aEVRT-7b (*rim2::URA3*) was grown in
medium. Cells were harvested in the exponential phase
and mitochondria were isolated by a combination of the
reported by Daum et al. (1982) and Yaffe (1991). Mitopl
prepared by incubation of the mitochondria at 0° C for
the presence of 0.3 mg digitonin per mg protein in a
containing 0.6 M mannitol, 10 mM TRIS-HCl, 2 mM
0.1% BSA, pH 6.8. The reaction was stopped by a tenfol
and centrifugation. The mitoplasts were frozen, thawed
ed in 50 mM sodium carbonate, pH 9.6. Inner membra
isolated by centrifugation at 178000 × g for 10 min in a
Airfuge microcentrifuge. Inner membrane proteins isol
the same volume of mitoplasts (volume accessible to trit
ter) were subjected to SDS-polyacrylamide gel electr
For western blotting, the proteins were transferred onto
bilon-P (Millipore) membrane. Immunodetection was c
using an antibody raised against a synthetic peptide
1:50 dilution) that includes residues 2–12 of the NH_2
part of RIM2/MRS12. The preparation of this antibo
described elsewhere (Hellin and Sluse, unpublished res
reaction was visualized by ^{35}S-labeled protein A (Amers
autoradiography.

Results

Independent approaches have led to the conclus
the same gene, named *RIM2/MRS12*, can act a
ticopy suppressor of two types of defect in mito
al nucleic acid metabolism. The *RIM2* gene wa
ered as a suppressor of a DNA helicase defect
chondria, and is allelic to *MRS12*, which was i
as a suppressor of a mitochondrial RNA splicin

RIM2, a partial suppressor of a DNA helicase in yeast mitochondria

RIM2 was originally isolated from a wild-type
library constructed in the cosmid vector pHC
suppressor rescuing the temperature sensi
growth defect of the *pif1* null strain aEVII-4b
erol (Fig. 1). A restriction enzyme analysis of t
ing plasmid cos8 indicated that neither *PIF1*
the previously uncovered suppressor *RIM*
present on this plasmid (data not shown). It w
fore concluded that the rescuing activity is a
with a novel suppressor gene. This gene wa
RIM2.



mid cos8 rescues the temperature sensitivity of the aEVII-4b on glycerol. The *pif1* null strain aEVII- med with a yeast genomic library based on the (*URA3*) and Ura$^+$ transformants displaying a otype at the restrictive temperature were selected. of the *URA3* marker and the growth phenotype 6° C in mitotic segregants issued from transfor-/cos8 is illustrated. Transformants aEVII-4b/cos8 ernight in glucose-rich medium at 28° C before single colonies on glucose-rich medium. Individ- e then gridded on glucose plates and replica-plat- cerol-rich medium, (**C**) glucose-rich medium and dium lacking uracil. Plates **A**, **C** and **D** were incu- Plate B was incubated at 36° C for 1 day before ated for a second time on the same medium. The ows the growth at 28° C after 2 days and the 2 days after the second replica-plating. As illus- 3 marker and the Glycerol$^+$ phenotype at 36° C nitotic segregants

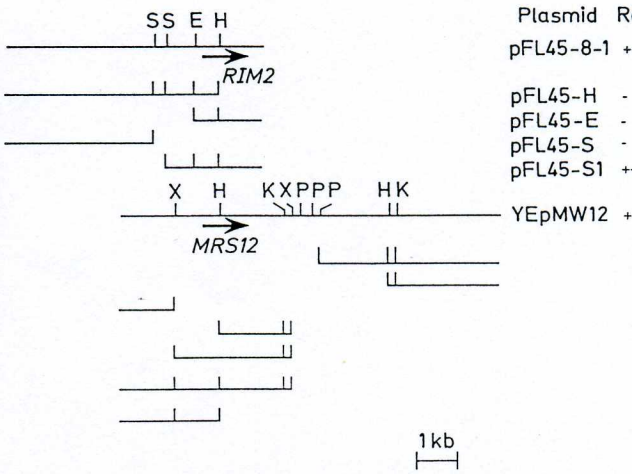


Fig. 2 Physical map of RIM2/MRS12 and flanking sequen The *top line* represents a partial restriction map of the *RI MRS12* gene and surrounding sequences. The *arrow* repres the *RIM2/MRS12* ORF and indicates the direction of transc tion. To identify the gene of interest, deleted subclones were c structed from pFL45-8–1 and YEpMW12 and tested for t suppressor activity. PFL45-8–1 derivatives were tested for t ability to rescue the mitochondrial defect displayed by the null strain aEVII-4b on glycerol at 36° C. YEpMW12 derivati were tested for their ability to restore glycerol growth in the str DBY747Δmrs2. (*E Eco*RI, *H Hin*dIII, *S Sal*I, *K Kpn*I, *P Pst*I *Xba*I. Note that not all of these restriction enzymes were use independent analyses of pFL45-8–1 and YEpMW12)

ppressor of a mitochondrial RNA splicing

selected from a wild-type genomic library the multi-copy YEp351 vector as a phe- ssor of the *mrs2* null strain GW/gd21.2 (a DBY747 that contains mitochondrial in- xpression of *MRS12* restores the glycerol e *mrs2* mutant both in intron-containing ss strains but surprisingly Northern analy- chondrial transcripts have revealed only a ation of the group II intron splicing defect his strain (data not shown). In contrast to nd *MRS4* genes, ovexpression of *MRS12* ess the defect caused by the mit$^-$ mutation ron bI1.

nd chromosomal localization
S2

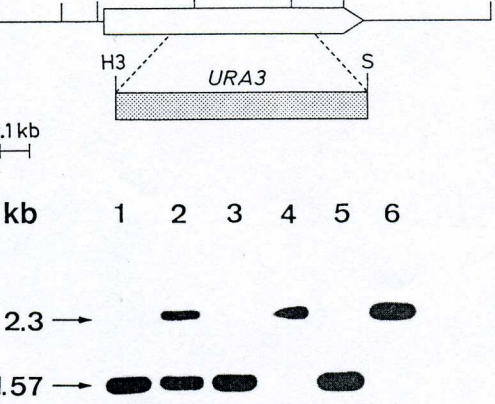
y of cos8 DNA sequences was constructed *u*3A partial digests of this plasmid into the ctor pFL45, and this mini-library was used the yeast strain aEVII-4b to growth on ° C. Plasmid pFL45-8-1, which contains a nsert (Fig. 2), was found to rescue the tem- itivity of the *pif1* null strain. To identify tives of this plasmid were then constructed and tes for their ability to rescue the ts growth defect of str aEVII-4b on glycerol (Fig. 2). From these experime it was concluded that the *RIM2/MRS12* gene resides a 2.35 kb *Sal*I fragment on pFL45-8–1 (one of the *S* sites lies in the polylinker of pFL45).

In a similar way, the *RIM2/MRS12* gene was loc ized on the 9 kb insert of plasmid YepMW12 by c structing differentially deleted clones that were tes for *mrs2* suppressor activity (Fig. 2). The gene was fou to reside on a 2.9 kb *Xba*I fragment.

Sequencing of the rescuing regions of pFL45-8-1 a YEpMW12 revealed that the inserts of these plasm overlapped, with their non-overlapping segments c taining sequences upstream and downstream, resp tively, of an open reading frame (ORF) correspond to the *RIM2/MRS12* gene (Fig. 2). This fragment of *S. cerevisiae* genome has already been characterized detail during the systematic sequencing of chromoso II (Demolis et al. 1993) and its localization confirmed the physical mapping of *RIM2/MRS12* between *su* and *CDC28* on the right arm of this chromosome (d not shown, L. Riles, personal communication).

Sequence and expression of the *RIM2/MRS12* gene

The *RIM2/MRS12* gene is identical to ORF YBR1



ruption of the *RIM2/MRS12* gene. **A** The construction lete one copy of the *RIM2/MRS12* gene in the diploid N (*RIM2/RIM2*). The *URA3* marker is represented by ox. (*E Eco*RI, *V Eco*RV, *H2 Hin*dII, *H3 Hin*dIII, *S Sma*I, *Spe*I. Only the relevant sites are indicated) **B** Southern nalysis of *RIM2/MRS12* disruption. Genomic DNA diploid strains EVW (lane 1) and EVRT (*RIM2/ 43*) (lane 2) and meiotic segregants (lanes 3, 4, 5 and 6) ed with *Spe*I plus *Eco*RI and probed with the 1.1 kb gment described in **A**. The 1.57 kb band corresponds to pe *RIM2/MRS12* gene. Disruption of this gene results of 2.3 kb

The nucleotide sequence is available in EMBL, k and DDBJ nucleotide sequence databases un- accession number Z21487 (partial sequence of EMBL X69839). The *RIM2/MRS12* gene en- 77 amino acids protein with a predicted molec- ght of 42.1 kDa and a calculated isoelectric 10.15. The nucleotide distribution around the rt codon (5′-aaagATGcct-3′) represents a good the yeast consensus sequence (5′-a/yaa/taAT- (reviewed by Cigan and Donahue 1987).

RIM2/MRS12 gene is transcribed into an mR-

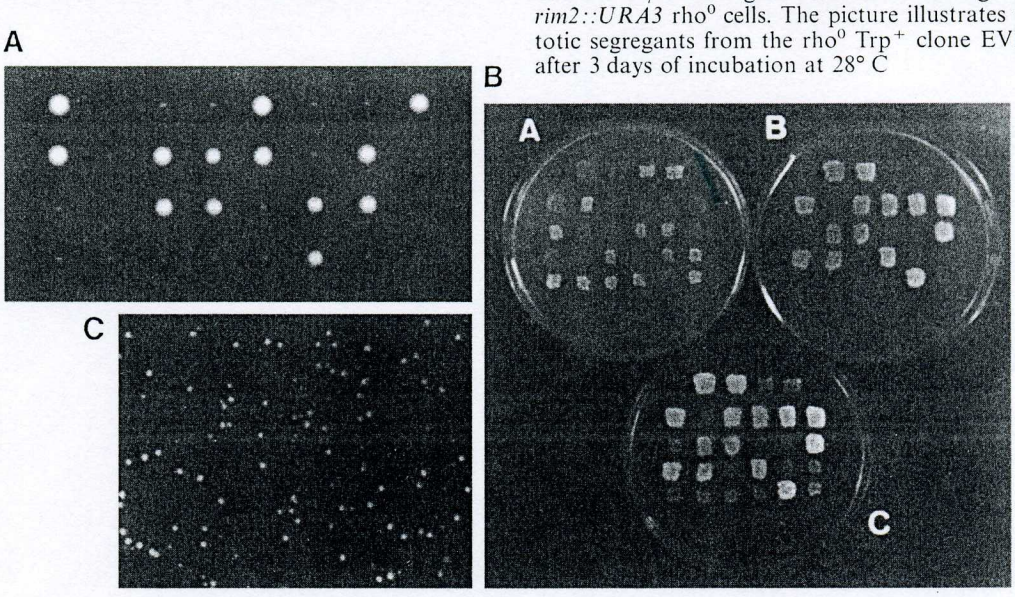
ATG start codon.

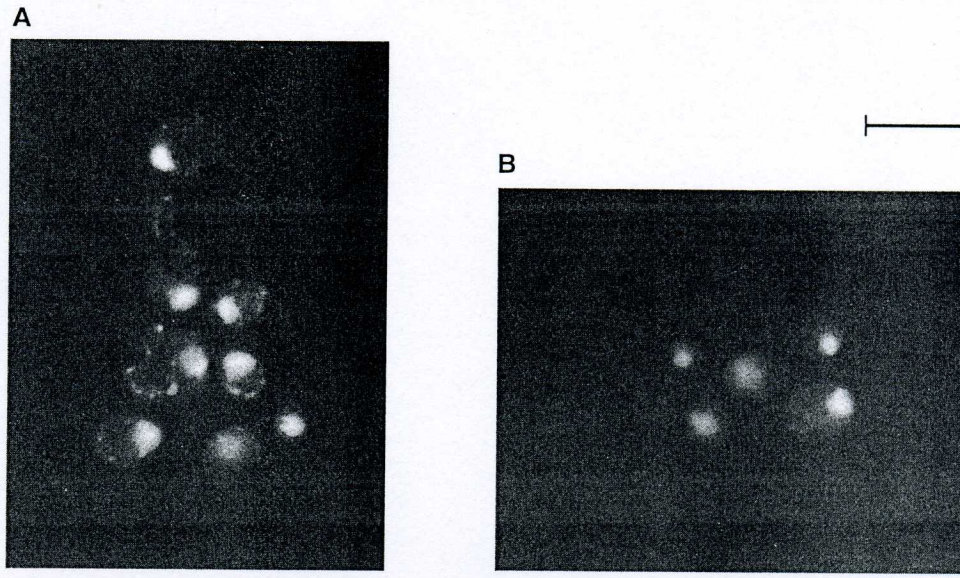
The *RIM2/MRS12* gene is essential for mtDNA maintenance and proper cell grow

In order to determine whether the *RIM2/MRS* product performs an essential function, a constr made in which a 418 bp *Eco*RV-*Hin*dIII DNA fr internal to the *RIM2* ORF was replaced by lectable marker *URA3*, as illustrated in Fig. 3 deletion allele was then used to replace one of th sponding wild-type *RIM2* copies in a diploid str placement was verified by Southern blot hybri (Fig. 3B). The resulting diploid, called EVR sporulated and the products of meiosis were d onto complete medium containing glucose befo analyzed.

All four spores were found to be viable in mo tetrads, indicating that *RIM2* is not essential viability. However, *rim2* null colonies displayed growth phenotype (Fig. 4A, see below). In additi were unable to grow on medium containing the mentable substrate glycerol as the sole carbon

Fig. 4A–C Phenotypes associated with *RIM2/MRS12* di **A** illustrates the slow-growth phenotype associated wit ruption of the *RIM2/MRS12* gene. Tetrads issued diploid EVRT (*RIM2/rim2::URA3*) were dissected on rich medium. The plate was photographed after 48 h o tion at 28° C. In most of the tetrads, all four spores a However, these spores segregate 2:2 as large and small The small colonies are Ura$^+$ and therefore carry a disru of *RIM2/MRS12*. **B** Some of these spores were analyzed ca-plating on (A) minimal medium lacking uracil, (B) gly medium, and (C) glucose-rich medium. Plates were phot after 1 day of incubation at 28° C. A 2:2 cosegregati *rim2::URA3* allele and the Glycerol$^-$ phenotype is ob The *RIM2/MRS12* gene rescues the slow-growth phe *rim2::URA3* rho^0 cells. The picture illustrates the grow totic segregants from the rho^0 Trp$^+$ clone EVRT-8b/p after 3 days of incubation at 28° C





n2::URA3 cells mitochondrial e presence of *RIM2* and **B** ells was tested A-specific dye -2-phenylindole najor source of (A) and (B) DAPI-stained Small spots of cence corre- tDNA are ob- *RIM2* cells. ents 10 μm

efective mitochondrial functions (Fig. 4B). efect was confirmed by the observation that pres failed to grow on glycerol even when diploid EVRT were dissected directly on (data not shown). Since the defects of the ains could be rescued if diploid EVRT was with plasmid pFL45-8–1 prior to sporula- t shown, see also below), it was concluded phenotype was due to the disruption of

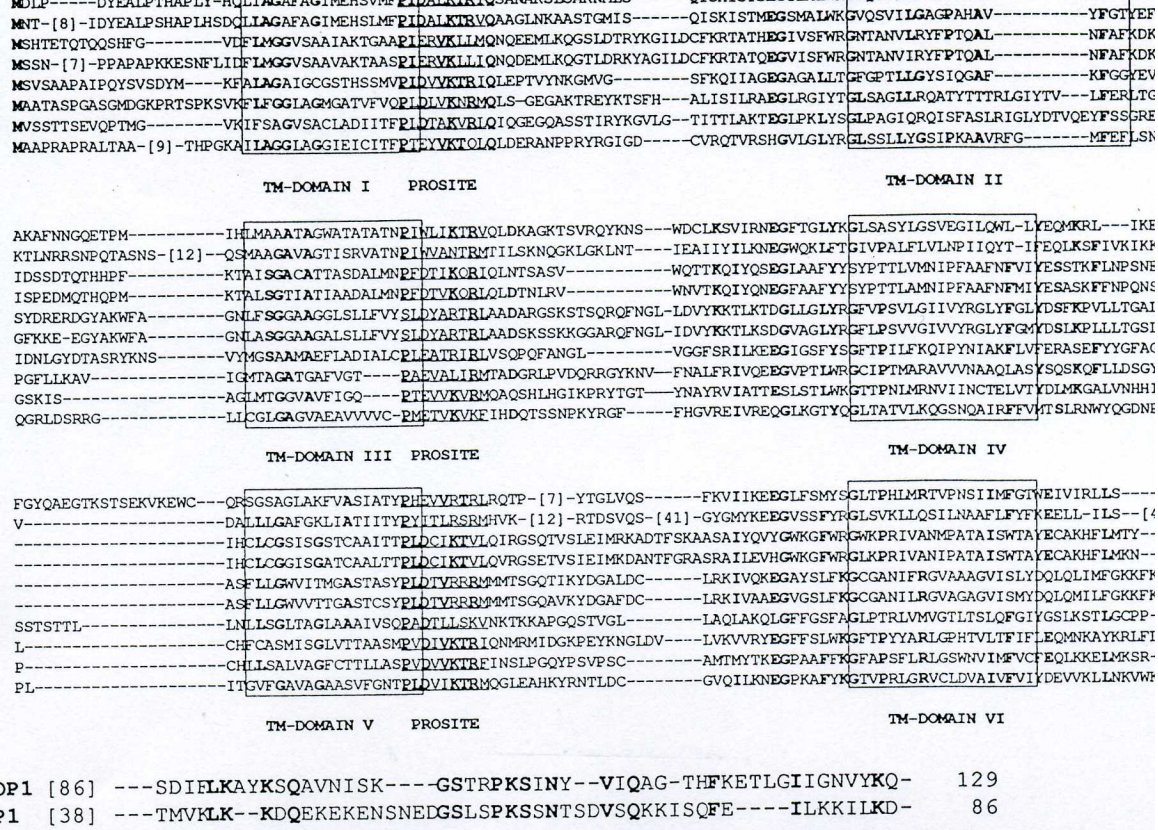
e the integrity of the mtDNA in *rim2* null ated mitotic segregants from strain EVRT- -1 (a *rim2::URA3* [rho$^+$] spore issued from VRT previously transformed with pFL45- grown in glucose-rich medium. These e tested for presence of the plasmid and ycerol. As expected, the loss of the plasmid- gene always resulted in a Glycerol$^-$ pheno- n tested the Glycerol$^-$ colonies for the pres- NA. No signal was detected when total ed from these cells was probed with a mtD- t from the *oli1* region (data not shown). tion suggested that cells lacking a function- e may be devoid of mtDNA (rho^0). That the s indeed essential for mtDNA maintenance ed by the observation that no mtDNA cted by staining these cells with the DNA- DAPI (Fig. 5).

re *rim2* null strains incapable of maintain- ondrial genome, but they also exhibit a t. This was first indicated by the slow- otype of the *rim2* null colonies following Fig. 4A). To learn more about this pheno- pared the growth of rho^0 mutants from nd *rim2::URA3* genetic backgrounds. For , EVRT-8b/pFL45-8–1, a *rim2::URA3* containing a plasmid-borne *RIM2* gene, rho^0 by ethidium bromide treatment. A one was selected and grown for 32 h in glucose-rich medium before being spread for si colonies on this medium. Two types of colonies (l and small) were clearly visible after 3 days (Fig. 4C). large colonies, which represented about 80% of the p ulation, were Trp$^+$ and therefore exhibited a phenot consistent with the presence of *RIM2* on plas pFL45-8–1. In contrast, all small colonies consiste Trp$^-$ cells. Taken together, these data indicate *RIM2* is required for proper cell growth. Fina *rim2::URA3* cells did not display a temperature-se tive growth phenotype on glucose at 36° C (data shown).

RIM2 encodes a new member of the MCF

The predicted primary structure of the RIM2/MR protein was compared with sequence databases us the FASTA (Pearson and Lipman 1988) and BLA (Altschul et al. 1990) search programs. Signific matches were identified with members of a protein f ily that span the inner mitochondrial membrane, MCF (reviewed by Aquila et al. 1987; Klingenb 1989; Kuan and Saier 1993). While the best stuc member of the MCF is the ADP/ATP carrier AA from *S. cerevisiae* (Nelson et al. 1993), numerous quences of proteins belonging to this family have b identified in various organisms. In addition, homol of the MCF have been identified in amyloplasts (St van et al. 1991) and peroxisomes (Jank et al. 1993) multiple sequence alignment of RIM2/MRS12 v some of these carriers is presented in Fig. 6 (top).

Members of the MCF consist of three repeating quences, each containing two putative membrane-sp ning helices separated by a hydrophilic region. In ac tion, the threefold presence of the energy tran protein signature P × (DE) × (LIVAT)(RK) × (I (LIVMFY) (PROSITE PDOC00189 ; Bairoch 1993) hallmark of this protein family. This signature is



B Alignment of the deduced amino acid sequence of the RS12 protein with members of the mitochondrial carrier Multiple sequence alignment of RIM2/MRS12 (*RIM2_* 21487) with the peroxisomal membrane protein PMP47 *CANBO*, McCammon et al. 1990) and mitochondrial of the carrier protein family including the two isoforms nd MRS4 (MRS3/4_YEAST, Wiesenberger et al. 1991) been shown previously to be involved in mtRNA splic- bers of the mitochondrial carrier family (MCF) that have een assigned a transported substrate are the ADP/ATP *AC1/2_YEAST*, Adrian et al. 1986; Lawson and Douglas e phosphate carrier (*MPCP_YEAST*, Phelps et al. 1991), utarate/malate shuttle (*M2OM_BOVIN*, Runswick et al. e uncoupler protein (*UCP_RAT*, Bouillaud et al. 1986), ricarboxylate transporter (*RATCTP2X_1*, Kaplan et al. quences of the N-terminal, central and C-terminal re- the proteins are arranged in three blocks to show the of sequences of the same repeat as well as between the eats. Transmembrane (*TM*-) domains are *boxed* accord- ose of the yeast ADP/ATP carrier (Nelson et al. 1993). d amino acid residues are represented in *bold letters*. The E energy transfer protein signature is *underlined*. The t shows the conserved transmembrane only, neglecting ophilic loop of the carriers. **B** Alignment of the first hy- loop of RIM2/MRS12 and PMP47. The alignment was sing the computer program MACAW (Schuler et al. ote that the homologous stretch GSxxPKSxN does not ny known PROSITE motif

3 times in RIM2/MRS12 (Fig. 6, top). In order tain internal homologies in RIM2/MRS12, a d analysis of the 377 amino acids long RIM2/M quence was performed against itself and memb MCF (Fig. 7). Stretches of homologous amino displaced approximately 100 and 200 residue tively, from the main diagonal. This pattern the existence of three internal repeats withi MRS12, each consisting of about 100 residues. more, six hydrophobic transmembrane domai proposed from the sequence comparisons (Fi and fit well the hydropathy profile of RIM2 (data not shown; Demolis et al., 1993). Each RIM2/MRS12 (repeat I-III) comprises two h bic – putative transmembrane (TM) – domain ed by a hydrophilic region. These observation RIM2/MRS12 as a new member of the MCF

With a predicted molecular weight of 42.1 RIM2/MRS12 protein constitutes one of th members of its family. This large size is attrib part to the length of the N-terminal extension the first repeat (about 50 residues for RIM2 between 10 and 35 for the other carriers). In the internal hydrophilic loop of repeat I in th MRS12 protein is much longer (66 residues) t of repeats II (44 residues) and III (42 residues), all repeats of most other carriers (about 40 Finally, with a size of about 35 residues, the

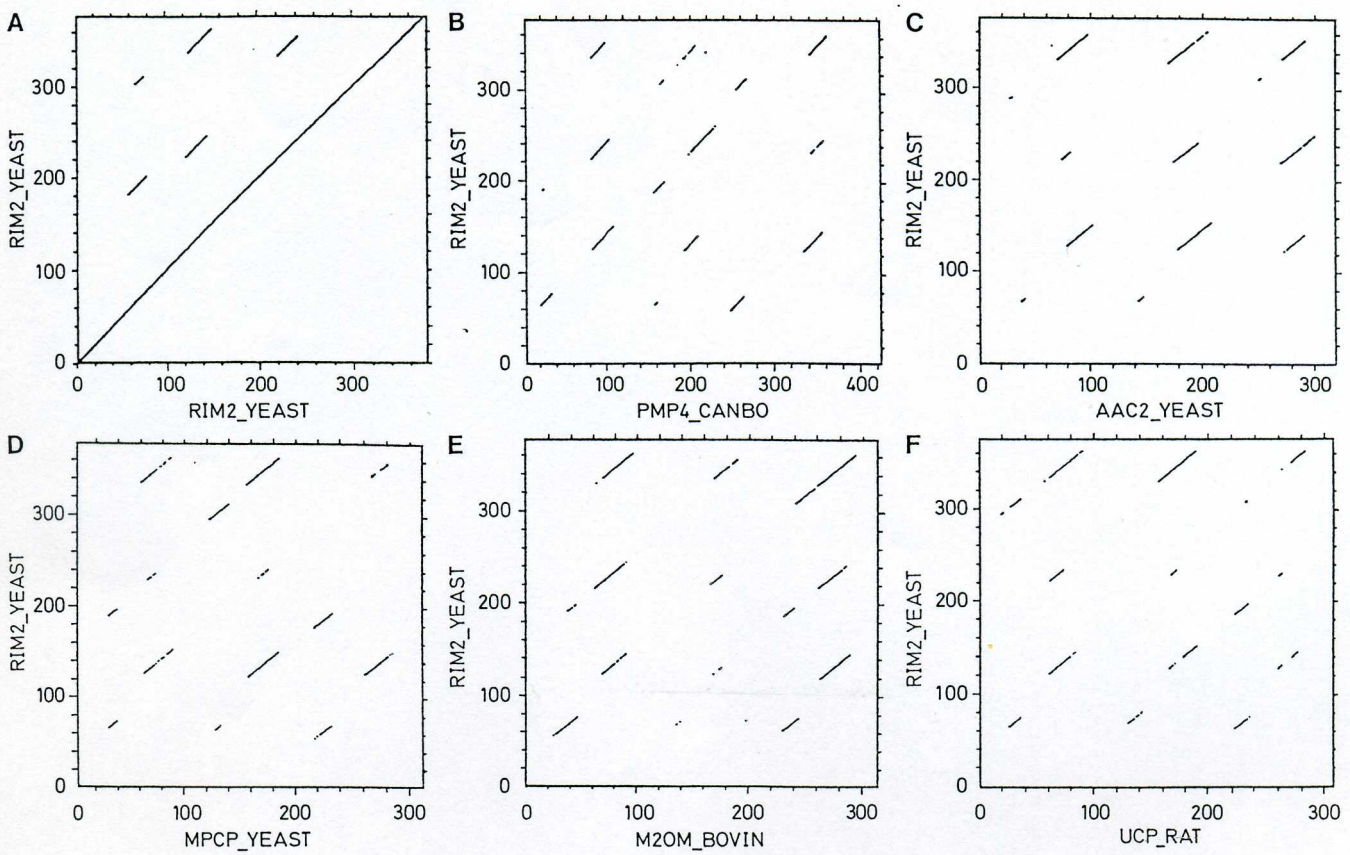


Fig. 7A–F Dot plot comparisons of the amino acid sequence of RIM2/MRS12 against itself and against sequences of other members of the carrier family. The RIM2/MRS12 sequence was compared with itself, PMP47 from *Candida boidinii*, the yeast ADP/ATP carrier AAC2, the yeast phosphate carrier MPCP, the bovine oxoglutarate malate shuttle M2OM and the rat uncoupler protein UCP. The sequences for the carriers were taken from the references listed in Fig. 6

connecting repeats II and III in RIM2/MRS12 is larger than in any other member of this family. It is tempting to speculate that these observations reflect the functional specialization of RIM2/MRS12 during evolution. Comparison of RIM2/MRS12 with PMP47, a peroxisomal homolog of the MCF from the methylotrophic yeast *Candida boidinii* (McCammon et al. 1990; Jank et al. 1993), might be interesting in this regard. The internal hydrophilic loops in repeat I of RIM2/MRS12 and PMP47 are indeed of similar length and share a homologous stretch of nine residues that reads GSxxP-KSxN (Fig. 7B). The significance of this observation is still unknown. This conserved region does not represent any known PROSITE motif. Besides, PMP47 also contains an extended loop between TM domains V and VI, whereas RIM2/MRS12 does not. Finally, there is no N-terminal extension preceding the first repeat of PMP47 (Fig. 7A).

Mitochondrial localization of the RIM2/MRS12 protein

All the data presented above suggest that the RIM2/MRS12 protein functions in the mitochondria. As a first step to determine the cellular location of the *RIM2/MRS12* gene product, a polyclonal antiserum was raised against a synthetic peptide (pep1) corresponding to amino acids 2–12 of the deduced RIM2/MRS12 protein, and affinity-purified antibodies were used in western blotting experiments to probe mitochondrial proteins from the strains EVRT-7a (*RIM2*), EVRT-7b (*rim2::URA3*) and EVRT-8a/pFL45-8–1 (a *RIM2* strain carrying the plasmid pFL45-8–1). As illustrated in Fig. 8 (lanes 3, 4), antibodies to pep1 antiserum show the presence of a major protein of about 42 kDa, consistent with the predicted molecular weight of the RIM2/MRS12 protein. That this protein indeed corresponds to RIM2/MRS12 is further confirmed by the observation that it is not detected in the *rim2* null strain (lanes 5, 6) while it is present at an increased level in the strain carrying the *RIM2* gene on a multicopy plasmid (lanes 1, 2). Finally, our observation that antibodies directed against the N-terminus of the RIM2/MRS12 protein are able to detect RIM2/MRS12 in mitochondria suggests that this protein is imported into the organelle without any cleavable amino-terminal presequence. The lack of a N-terminal presequence in the RIM2/MRS12 protein is further supported by the acidic character of its N-terminus (with 11 acidic residues and only 5 basic

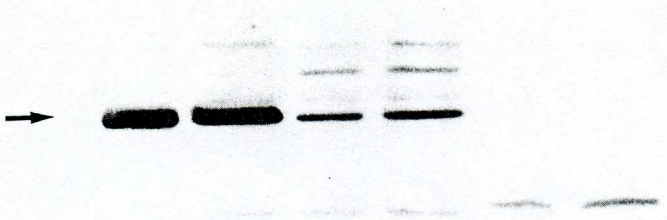


Fig. 8 The RIM2/MRS12 protein is present in mitochondria. Western blot of mitochondrial preparations obtained from various yeast strains shows the presence of the RIM2/MRS12 protein in the inner mitochondrial membrane from a wild-type *RIM2* strain (EVRT-7a, lanes 3, 4) and a strain carrying *RIM2* on a multicopy plasmid (EVRT-8a/pFL45-8–1, lanes 1, 2). RIM2/MRS12 is not detected in a *rim2* deletion mutant (EVRT-7b, lanes 5, 6). Inner membrane proteins from 0.18 µl (lanes 1, 3, 5) and 0.36 µl (lanes 2, 4, 6) mitoplasts (mitoplast volume that is accessible to ^3H_2O) were subjected to SDS-polyacrylamide gel electrophoresis. The RIM2/MRS12 protein is indicated by an *arrow*

residues in the first 45 amino acids of the protein). The absence of N-terminal targeting presequence is a characteristic feature of the MCF (reviewed by Pfanner and Neupert 1990).

Discussion

Overexpression of a novel gene, *RIM2/MRS12*, rescues both a DNA helicase and a RNA splicing defect in yeast mitochondria. The notion that RIM2/MRS12 is involved in mitochondrial metabolism is further supported by the two following observations. First, inactivation of the *RIM2/MRS12* gene not only impairs mitochondrial functions but also causes the total loss of mtDNA. Second, antibodies directed against an N-terminal peptide of the protein detect RIM2/MRS12 in mitochondria. Yet, far from encoding a component of the machinery of mtDNA replication or mtRNA splicing, the *RIM2/MRS12* gene identifies a new member of the MCF, suggesting that the control of mitochondrial nucleic acid metabolism by RIM2/MRS12 is mediated at the level of mitochondrial transport.

Mitochondrial carriers constitute important transport systems that ensure the traffic of solutes through the inner mitochondrial membrane, therefore maintaining an essential connection between mitochondrial and cytosolic functions (Aquila et al. 1987; Klingenberg 1989; 1990). A large variety of substrates (including adenine nucleotides, inorganic anions and cations, mono-, di-, and tricarboxylic acids, amino acids, fatty acids; Kramer and Palmieri 1989) are known to be specifically transported across the inner mitochondrial membrane by these proteins. The mitochondrial solute carriers sequenced to date form an homologous protein

Fig. 7).

While the ligand(s) transported by RIM2/MR are at present not known, RIM2/MRS12 is so far only member of the MCF whose elimination causes loss of mtDNA. Although we cannot rule out the pc bility that such a severe phenotype is due to a pertu tion of the integrity of the inner mitochondrial m brane, our data suggest that this phenotype reflec direct and important involvement of the RIM2/MR protein in mitochondrial nucleic acid metabolism particular, it is tempting to speculate that RI MRS12 transports mtDNA precursors or compou necessary for the mtDNA synthesis machinery. It been shown that isolated yeast mitochondria inco rate externally added deoxyribonucleotides to syn size mtDNA de novo (Mattick and Hall 1977). T RIM2/MRS12 could encode a translocator for deox bonucleotides. Assuming a limiting pool of deox bonucleotides in yeast mitochondria, such an hypo sis would provide an attractive explanation to our servation that, when present on a multicopy plas the *RIM2/MRS12* gene rescues the temperature-se tive loss of mtDNA exhibited by cells lacking the D helicase PIF1.

On the other hand, overexpression of *RIM2/MR* also rescues defects in mtRNA group II intron spli and mitochondrial assembly/expression caused *MRS2* gene disruption. This additional role for RI MRS12 in mitochondrial biogenesis suggests that cofactors controlling both cytochrome oxidase ass bly (or expression), mitochondrial group II intron s ing and mtDNA synthesis make better candidate our search for a substrate transported by the RI MRS12 protein. In this regard, an attractive (altho not exclusive) candidate is Mg^{2+}, for which mitoc dria are important stores. Although the adenine nu otide carrier has been proposed to participate in M transport in rat liver mitochondria under certain co tions (Romani et al. 1991), the major translocator of cation remains to be characterized. Finally, the suppression phenotype displayed by RIM2/MRS shared by two previously uncovered carrier pro with unknown functions, MRS3 and MRS4 (Wald et al. 1993 and references therein). Unlike *RI MRS12*, however, disruption of the *MRS3* and *M* genes has no apparent effect on mitochondrial funct or cell viability (Wiesenberger et al. 1991). With re to a possible role in mtDNA metabolism, it will t fore be interesting to learn whether overexpressio *MRS3* and *MRS4* is able to rescue the mitochon thermosensitivity of a *pif1* null mutant.

Several hypotheses could explain the growth d of the *rim2* null strains. One could consider tha substrate transported by RIM2/MRS12 from the sol to mitochondria or vice versa is important for p er cellular function. This defect could also result

the accumulation of untransported substrates in the cytosol. Finally, in addition to its presumed transport function, RIM2/MRS12 might also be involved in the mitochondrial structure, the maintenance of which appears necessary to eukaryotic life.

This work has shown that overexpression of the same gene product can suppress defects in several mitochondrial pathways. Another puzzling observation is the large number of genes whose overexpression can rescue *mrs2* mutants, since a dozen complementation groups have thus far been discovered. Some of these suppressors may be auxiliary factors that interact with the mtRNA splicing machinery. Many, however, are certainly modulators of the intramitochondrial environment, which facilitate group II intron splicing, in agreement with the observation that these introns are capable of efficient self-splicing in vitro. That this view also holds for suppressors of mtDNA metabolism defects is illustrated by the *RIM1* and *RIM2* genes. Thus, while it is not surprising that the *RIM1* gene is a partial suppressor of a DNA helicase defect since it encodes a ssDNA binding protein that is an essential component of the mtDNA replication apparatus, and plays a role in the maintenance of unwound DNA, it is unlikely that RIM2, a mitochondrial carrier, interacts directly with the mtDNA. The transport function of RIM2, MRS3 and MRS4 suppressors suggests that several solutes are rate-limiting in mitochondrial metabolism even though the loss of mtDNA in *rim2/mrs12* null mutants argues in favor of a more direct involvement of this gene in nucleic acid metabolism. Thus, search for suppressors of defects in mitochondrial nucleic acid metabolism has revealed the existence of gene products and metabolites that are rate-limiting in mitochondrial metabolism and suggests the existence of alternative pathways that can compensate for nucleic acid metabolism defects.

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