Mitochondrial iron uptake is of key importance both for organellar function and cellular iron homoeostasis. The mitochondrial carrier family members Msr3 and Mrp4 (homologues of vertebrate mitoferrin) function in organellar iron supply, yet other lower efficiency transporters may exist. In Saccharomyces cerevisiae, overexpression of Rim2 (MRS12) encoding a mitochondrial pyrimidine nucleotide transporter can overcome the iron-related phenotypes of strains lacking both Msr3 and Mrp4. In the present study we show by in vitro transport studies that Rim2 mediates the transport of iron and other divalent metal ions across the mitochondrial inner membrane in a pyrimidine nucleotide-dependent fashion. Mutations in the proposed substrate-binding site of Rim2 prevent both pyrimidine nucleotide and divalent ion transport. These results document that Rim2 catalyses the co-import of pyrimidine nucleotides and divalent metal ions including ferrous iron. The deletion of Rim2 alone has no significant effect on mitochondrial iron supply, Fe–S protein maturation and haem synthesis. However, Rim2 deletion in mrs3Δ/mrs4Δ cells aggravates their Fe–S protein maturation defect. We conclude that under normal physiological conditions Rim2 does not play a significant role in mitochondrial iron acquisition, yet, in the absence of the main iron transporters Msr3 and Mrp4, this carrier can supply the mitochondrial matrix with iron in a pyrimidine-nucleotide-dependent fashion.

Key words: iron metabolism, mitochondrial carrier family, metal transport, nucleotide transport, sub-mitochondrial particle.

INTRODUCTION

Iron is an essential element for virtually all living cells. It is required for a variety of cellular processes including respiration, metabolite biosynthesis, DNA synthesis and repair, ribosomal biogenesis and oxygen transport in vertebrates. Homoeostasis of iron has to be tightly regulated, as intracellular free iron may potentially produce reactive oxygen species that are highly toxic at elevated levels. On the other hand, the availability of iron is limited due to the low solubility of ferric iron (Fe3+), the predominant form in aerobic environments [1,2]. Therefore all organisms have evolved sophisticated mechanisms that guarantee a tight balance between cellular iron acquisition, its intracellular distribution and storage, and the demands of cellular iron-utilizing systems (reviewed in [3–10]).

In eukaryotes, the major site of iron utilization is the mitochondrion, where haem and ISCs (Fe–S clusters) are synthesized, e.g. for the respiratory chain [11–13]. Haem is an essential protein cofactor, and yeast cells with defects in haem synthesis are not viable unless supplied with an external source of haem [14]. In addition, several cytosolic and nuclear Fe–S proteins perform indispensable tasks in ribosome assembly and function, DNA synthesis and repair, and the regulation of telomere length [15–18]. The assembly of cellular Fe–S proteins requires the mitochondrial ISC assembly machinery, which includes many components essential for cell viability [13,19,20].

The mitochondrial ISC assembly system also plays an important role in the regulation of cellular iron homoeostasis in eukaryotes [7,9,10,12,13,19]. The requirement of the mitochondrion in the maturation of essential Fe–S and haem proteins suggests that the transport of iron into the mitochondrial matrix is a crucial process, and its functional impairment is expected to be lethal.

In yeast and vertebrates, the role of the mitochondrial carriers Msr3 and Mrp4 (mouse and zebrafish mitoferrin 1 and 2) in mitochondrial iron uptake is well established [21–27]. Yeast Msr3 and Mrp4 are required for mitochondrial iron acquisition to support haem and ISC synthesis, especially under iron-limiting conditions [21–23]. In human and zebrafish, mitoferrin 1 is restricted to the erythroid system and is essential for haem biosynthesis, but has no detectable function in other tissues [25,28]. Ablation of the mouse and zebrafish mitoferrin 1 gene is lethal [25,29]. Mitoferrin 2 is expressed mostly in non-erythroid cell types and its deletion results in lower haem levels and reduced Fe–S protein biogenesis [26]. Additional functional relatives of Msr3, Mrp4 and mitoferrin have been characterized in Drosophila melanogaster and Caenorhabditis elegans [30,31]. Homozygous deletion of the corresponding gene in rice results in a lethal phenotype [32].

Despite the importance of mitochondrial iron, deletion of both Msr3 and Mrp4 in yeast (strain mrs3Δ/mrs4Δ) did not affect cell viability, suggesting the existence of additional mitochondrial iron transporters. Recently, the MCF (mitochondrial carrier family)
protein Rim2 [33,34] was found to play a role in mitochondrial iron homoeostasis by influencing mitochondrial haem and Fe–S protein biosynthesis [35,36]. Originally, RIM2/MRS12 had been identified as a multi-copy suppressor of mutations in PIF1 encoding a mitochondrial DNA helicase, and in MRS2 encoding the mitochondrial Mg$_2^+$ channel [37,38]. A role of Rim2 in mitochondrial iron supply was suggested from a further reduction in the growth rate of mrs3/4Δ cells. However, mrs3/4Δrim2Δ triple mutants are still viable, and low amounts of iron in the growth media obliterated this defect [35,36]. Functional reconstitution of Rim2 convincingly demonstrated its function as a pyrimidine nucleotide transporter [33,39], raising the interesting question of how Rim2 might affect mitochondrial iron metabolism. Deletion of RIM2 causes a rho0 phenotype (loss of mtDNA), which is fully consistent with a physiological role of Rim2 in mitochondrial pyrimidine nucleotide uptake. However, in some strain backgrounds, deletion of RIM2 is lethal, suggesting that the protein may perform additional functions [35].

In the present study we addressed the question of how the pyrimidine nucleotide transporter Rim2 might influence mitochondrial iron acquisition in the absence of Mrs3 and Mrs4. We tested the possibility that Rim2 might co-import iron (and other divalent metal ions) and pyrimidine nucleotides. A co-import scenario seems plausible, since nucleotides are known to tightly bind and chelate iron. The present biochemical study aims to clarify the relative roles of this carrier in the transport of pyrimidine nucleotides and (potentially) iron. We addressed further the functional relevance of Rim2 for mitochondrial iron supply under normal physiological conditions, i.e. when cells can rely on Mrs3 and Mrs4 as the default iron transporters.

EXPERIMENTAL

Strains and growth conditions and plasmids

Yeast strains used in the present study are listed in Supplementary Table S1 (at http://www.biochemj.org/bj/455/bj4550057add.htm). If not stated otherwise, the strain background was W303-1A. rho0 derivatives were obtained by ethidium bromide treatment [40]. Cells were cultivated in rich medium (YP) or minimal medium containing all recommended supplements (SC) containing either 2% (w/v) glucose (D) or 3% (w/v) glycerol (G) as carbon source [41]. Iron-depleted minimal medium was prepared using yeast nitrogen base lacking FeCl$_3$, (ForMedium) [42]. Medium for cultivation under iron-depriving conditions was supplemented with 50–100 μM BPS (bathophenanthroline sulfonate). For information on plasmids see Supplementary Table S2 (at http://www.biochemj.org/bj/455/bj4550057add.htm). The rim2-1 allele (G245Y S246A) was created by PCR-mediated site-directed mutagenesis. High-copy suppressors were identified by screening strain GW403 (W303-1A; mrs3/4Δ) transformed with a yeast genomic library (provided by the Nasmyth laboratory, Department of Biochemistry, University of Oxford, Oxford, U.K.). Transformants were replica-plated on to iron-depriving (YPD + 80 μM BPS or SD (synthetic dropout) – leucine + 80 μM BPS + 5 μM FeCl$_3$) and cadmium-containing (YPD + 40 μM CdCl$_2$) media.

Pyrimidine uptake of isolated mitochondria

The method described by Mattick and Hall [43] was modified in the following way: 100 μg of isolated mitochondria from DBY747-derived strains [at 10 mg/ml, in buffer A (0.6 M sorbitol and 20 mM Tris/HCl, pH 7.4)] were incubated with 12.5 μCi of [3H]dTTP (15 Ci/mmol, PerkinElmer) at 28°C for 10 min. The solution was pipetted on to a glass fibre filter (Whatman, 55 mm diameter, GFC) presoaked with buffer A while vacuum was applied. The filter was washed with 25 ml of buffer A and the bound radioactivity was quantified by scintillation counting. The inhibitor BPS was added to mitochondria prior to incubation with [3H]dTTP.

Iron uptake into SMPs (submitochondrial particles)

Mitochondria (110 mg at 10 mg/ml) from strain DBY747 or GW403 transformed with the required plasmids were diluted 5-fold with 10 mM Tris/HCl, pH 7.4, incubated on ice for 20 min and centrifuged at 27000 rev./min for 10 min at 4°C in a TLA 100.4 rotor (Beckman). The pellet was resuspended in 1 ml of 250 mM sucrose and 10 mM Tris/HCl, pH 7.4. The solution was sonicated three times for 1 min at maximum intensity in an ice bath and then centrifuged for 10 min at 10000 rev./min at 4°C. The supernatant was transferred into a new tube and centrifuged at 60000 rev./min for 1 h at 4°C. The pellet was resuspended in 1 ml of sucrose buffer (250 mM sucrose and 10 mM Tris/HCl, pH 7.4). For loading, 5 μl (50 μM) of PGSK (PheGreen™ SK) were added to the SMPs and sonicated three times for 1 min and centrifuged for 10 min at 60000 rev./min at 4°C. The pellet was washed with 1 ml of sucrose buffer, the centrifugation was repeated and the pellet was resuspended in 1 ml sucrose buffer. The fluorescence was measured using the PerkinElmer LS55 spectrofluorometer. Excitation and emission for PGSK were 506 and 520 nm respectively. For measurement of uptake, 100 μl of loaded SMPs were diluted in 1.9 ml of sucrose buffer, 5 μM FeSO$_4$ and equal amounts of nucleotides were added. Calibration of metal uptake and controls of SMPs were performed as described in [27].

Miscellaneous methods

The following previously published methods were used: manipulation of DNA and PCR [44]; transformation of yeast cells [45]; isolation of yeast mitochondria and post-mitochondrial supernatant [46]; immunostaining [47]; in vivo labelling of yeast cells with $^{59}$FeCl$_3$ (ICN) and immunoprecipitation of Fe–S proteins or haem [42]; determination of enzyme activities of catalase, MDH (malate dehydrogenase) [42] and aconitate [48]; the promoter strength of the FET3 and FIT3 genes [42]. The error bars represent either the S.D. or the S.E.M., as indicated (n>4). Data were subjected to repeated measures one-way ANOVA with Newman–Keuls statistical tests using Prism software. Unless indicated otherwise, differences were considered statistically significant (P < 0.05).

RESULTS

RIM2 is a specific suppressor of MRS3 and MRS4

We initially tried to identify genes encoding mitochondrial iron transporters in addition to the mitochondrial carriers Mrs3 and Mrs4 by performing a screen for multi-copy suppressors of yeast cells deleted for both MRS3 and MRS4 (mrs3/4Δ). mrs3/4Δ mutants display a severe growth defect under iron-limiting conditions (Figure 1A and [49]) and are hypersensitive to cadmium [21]. We screened a Saccharomyces cerevisiae genomic DNA library in the 2 μ plasmid YEp181 for genes that restored these two phenotypes of mrs3/4Δ cells, and found RIM2/MRS12 as a suppressor gene. Expression of RIM2 under the control of the strong TetO$_2$ promoter fully restored the growth defect of mrs3/4Δ cells under iron-depriving conditions and in the presence of elevated levels of CdCl$_2$ (Figure 1A, and Supplementary
Rim2 promotes a pyrimidine nucleotide-specific import of iron into mitochondria

The above results as well as data provided by others [35,36] are consistent with the idea that the pyrimidine nucleotide carrier Rim2 is directly or indirectly involved in iron import into mitochondria. In order to investigate the molecular mechanism of how Rim2 supports this process, we analysed the transport of iron across the mitochondrial inner membrane in vitro using SMPs loaded with the divalent metal-chelator PGSK. This method had been developed previously to study mitochondrial iron uptake by Mrs3 and Mrs4 carriers [27]. For the present study, we isolated mitochondria from mrs3/4Δ cells either overproducing Rim2 from vector Yepl351-RIM2 or harbouring the empty vector Yepl351. SMPs were prepared and loaded with PGSK which displays a specific fluorescence quenching at 506 nm upon binding of Fe2+. When SMPs from mrs3/4Δ cells with or without RIM2 overexpression were incubated with iron alone, no change in fluorescence was observed (Figure 1B). However, when iron was provided together with dCTP, PGSK fluorescence was quenched in SMPs from cells overproducing Rim2. This was not the case with SMPs from control cells transformed with the empty vector. Iron uptake into SMPs from Rim2 overexpressing mrs3/4Δ cells was approximately 65% of that seen in wild-type SMPs (see Figure 1B, right-hand panel). Furthermore, quenching occurred only when dCTP was present on the outside of the SMPs, but not when dCTP was enclosed solely within the SMPs (Figure 1C). These results indicate that iron is transported across the mitochondrial inner membrane of SMPs containing high levels of Rim2 in a dCTP-dependent fashion, suggesting a co-import of iron and nucleotide.

Since Rim2 is a pyrimidine-specific nucleotide transporter, we tested which nucleotides support iron transport into SMPs. We incubated PGSK-loaded SMPs from mrs3/4Δ cells overexpressing RIM2 with various nucleotides followed by the addition of iron. PGSK quenching was observed only when SMPs were pre-incubated with pyrimidine nucleotides (CTP, dCTP, TTP and dTTP), but not with the purines dGTP and dATP (Figure 2A). When SMPs were incubated with increasing amounts of TTP and CTP we found that PGSK quenching correlated with the concentration of TTP/CTP applied outside of the SMPs (Figure 2B and results not shown). From these results we conclude that iron transport by Rim2 is pyrimidine-specific and concentration-dependent.

Mutations in the putative substrate-binding site impair transport of both pyrimidine nucleotides and iron

Mitochondrial carriers from S. cerevisiae with known substrate specificities have been classified into three major groups, i.e. adenine nucleotide, keto acid and amino acid transporters [34,50]. Amino acid sequence analyses within these groups allowed the identification of residues potentially interacting with the substrate molecules transported by mitochondrial carriers. Based on these results, Kunji and Robinson [34,50] proposed a substrate-binding site composed of three so-called contact sites which are conserved among the members of each functional group. In their structural model these contact sites are located inside a channel/pore formed by the carriers, and they are required for specific binding and transport of the substrate. We intended to investigate: (i) whether these contact sites are required for transport of pyrimidine nucleotides; and (ii) whether impaired pyrimidine nucleotide uptake would affect iron transport. To this end, we mutated residues Gly245 and Ser246 in the contact site II of Rim2 to Tyr245 and Ala246 respectively (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550057add.htm). Furthermore, Rim2 rescued the defects of mrs3/4Δ cells in the biosynthesis of haem and the maturation of the mitochondrial aconitase, but only when Rim2 was overproduced at high levels (Supplementary Figure S1). In contrast, overexpression of other members of the MCF, such as the phosphate transporters Mir1 and Pic2, and the S-adenosylmethionine transporter Pet8/Sam5, did not suppress the growth defect of mrs3/4Δ mutant cells (Figure 1A). This indicates that the high-copy suppressor phenotype of RIM2 is specific and most likely related to the function of Mrs3 and Mrs4 in cellular iron metabolism.
with mitochondria from wild-type cells or mitochondria from rim2Δ cells containing Rim2-1 mutant protein did not show any uptake of dTTP that could be inhibited by BPS (Figure 3C). These results indicate that an intact contact site II is essential for efficient pyrimidine nucleotide transport into mitochondria.

Finally, we tested whether mutated Rim2-1 is able to transport iron. To this end, we prepared SMPs from mitochondria isolated from mrs3/4Δ cells transformed with either the wild-type or the contact site II mutated version of RIM2. As expected, neither protein was able to mediate iron transport across the mitochondrial inner membrane in the absence of pyrimidine nucleotides (Figure 4A). However, in presence of dCTP, CTP, dTTP or TTP, wild-type Rim2, but not Rim2-1, was capable of iron import. In contrast, dGTP did not support this reaction. Collectively, the findings demonstrate that a functional substrate-binding site is essential for Rim2-mediated pyrimidine nucleotide and iron trafficking.

**Figure 3** Mutation of the ‘contact site II’ motif inactivates pyrimidine nucleotide transport by Rim2

(A) RIM2/rim2-1::kanMX4 diploid cells harbouring either the empty plasmid YCplac33, pMH3 (RIM2.YCplac33) or pMH5 (rim2-1.YCplac33) were sporulated and tetrads were dissected. Tetrads were replica-plated on to SD–ura, YPD + G418, YPD or YP + glycerol (YPD) agar plates. (B) Serial dilutions (1:10) of DBY747 wild-type (WT) cells and the isogenic mrs3/4Δ mutant harbouring the empty plasmid YCplac33 vector, pAS12 (RIM2.pCM189) or pMH2 (rim2-1.pCM189) were spotted on agar plates containing YPD, YPD supplemented with 100 μM BPS and 2 μM FeCl₃ and YPD and 20 μM CdCl₂, as indicated. Plates were incubated either at 37°C (YPD) or 30°C (BPS plus Fe and Cd) for 2 days. (C) Mitochondria isolated from yeast strain DBY747 rho0 transformed with YCplac33 or from the isogenic rim2Δ carrying the empty plasmid (YCplac33) or plasmids with RIM2 (pMH3) or rim2-1 (pMH5) were incubated with 12.5 μCi [3H]dTTP or with [3H]dTTP and 20 mM BPS for 10 min. Samples were filtered through a glass fibre filter and radioactivity bound to the filters was quantified by scintillation counting. Results are means ± S.D. (n=3). The significance of the differences between mean values was determined by one-way ANOVA (P<0.05) followed by pairwise comparisons with the control group using unpaired t tests. Differences were considered statistically significant, except between wild-type and rim2Δ pMH3.

**Figure 2** The Rim2-dependent iron transport into SMPs is specific for pyrimidine nucleotides

SMPs from mrs3/4Δ cells overexpressing Rim2 were loaded with PGSK (see Figure 1). SMPs were pre-incubated with (A) the indicated nucleotides (5 μM each) or (B) TTP at the indicated concentrations. Iron was added as indicated and the iron-dependent fluorescence quenching of PGSK was measured.

To test the functionality of the resulting protein, termed Rim2-1, the following experiments were performed. First, a diploid heterozygous (RIM2/rim2Δ) strain was transformed with either a CEN plasmid carrying the wild-type RIM2 or the mutated version, or with the empty plasmid as a control. These transformants were pre-incubated with (G418R) carrying the wild-type RIM2 or the mutated version, and the empty plasmid (YCplac33) or plasmids with RIM2 (pMH3) or rim2-1 (pMH5) were sporulated and tetrads were dissected. Tetrads were replica-plated on to SD–uracil, YPD + G418, YPD or YP + glycerol (YPD) agar plates. (B) Serial dilutions (1:10) of DBY747 wild-type (WT) cells and the isogenic mrs3/4Δ mutant harbouring the empty YCplac33 vector, pAS12 (RIM2.pCM189) or pMH2 (rim2-1.pCM189) were spotted on agar plates containing YPD, YPD supplemented with 100 μM BPS and 2 μM FeCl₃ and YPD and 20 μM CdCl₂, as indicated. Plates were incubated either at 37°C (YPD) or at 30°C (BPS plus Fe and Cd) for 2 days. (C) Mitochondria isolated from yeast strain DBY747 rho0 transformed with YCplac33 or from the isogenic rim2Δ carrying the empty plasmid (YCplac33) or plasmids with RIM2 (pMH3) or rim2-1 (pMH5) were incubated with 12.5 μCi [3H]dTTP or with [3H]dTTP and 20 mM BPS for 10 min. Samples were filtered through a glass fibre filter and radioactivity bound to the filters was quantified by scintillation counting. Results are means ± S.D. (n=3). The significance of the differences between mean values was determined by one-way ANOVA (P<0.05) followed by pairwise comparisons with the control group using unpaired t tests. Differences were considered statistically significant, except between wild-type and rim2Δ pMH3.

Thirdly, pyrimidine nucleotide uptake assays with isolated mitochondria were carried out (Figure 3C). For this experiment we used rho0 strains, since the RIM2 deletion results in the loss of mtDNA. Nucleotide transport by Rim2 is strongly inhibited by BPS [33]. We therefore performed the uptake assays in parallel in the presence and absence of BPS, in order to distinguish Rim2-dependent pyrimidine nucleotide uptake from transport by other systems and/or unspecific binding. In contrast with mitochondria from wild-type rho0 cells or mitochondria from rim2Δ cells complemented with RIM2, organelles from rim2Δ cells containing Rim2-1 mutant protein did not show any uptake of dTTP that could be inhibited by BPS (Figure 3C). These results indicate that an intact contact site II is essential for efficient pyrimidine nucleotide transport into mitochondria.

Finally, we tested whether mutated Rim2-1 is able to transport iron. To this end, we prepared SMPs from mitochondria isolated from mrs3/4Δ cells transformed with either the wild-type or the contact site II mutated version of RIM2. As expected, neither protein was able to mediate iron transport across the mitochondrial inner membrane in the absence of pyrimidine nucleotides (Figure 4A). However, in presence of dCTP, CTP, dTTP or TTP, wild-type Rim2, but not Rim2-1, was capable of iron import. In contrast, dGTP did not support this reaction. Collectively, the findings demonstrate that a functional substrate-binding site is essential for Rim2-mediated pyrimidine nucleotide and iron trafficking.

**Rim2 can also transport copper and zinc**

We also tested whether Rim2 supports the transport of other divalent metal ions across the mitochondrial inner membrane. To follow the uptake of these metals into the mitochondrial matrix space, we used the ability of metal ions such as Cu²⁺, Zn²⁺ and...
Rim2 was compromised. The mitochondrial inner membrane via Rim2 in the presence of divalent iron, copper and zinc ions were transported across treated with the ionophore pyrithione (Figure 4B). Taken together, and the iron-dependent fluorescence quenching was determined. Fe^{2+} concentrations within SMPs (Fe^{2+}) were calculated from five independent measurements as described previously [27]. SMPs from the indicated cells were prepared, loaded with PGSK and pre-incubated with 5 μM CTP before addition of the indicated divalent cations. Fluorescence quenching was measured and the ion concentrations within SMPs ([Me^{2+}]) were calculated as above. SMPs pre-treated with the ionophore pyrithione were used as a control for ion uptake. Data were analysed by ANOVA and differences were considered statistically significant (P < 0.05). Error bars indicate the S.D. (n≥3).

Cd^{2+} to quench the fluorescence of PGSK. This approach had shown that Msr3 and Mr4 can transport Cu^{2+}, but not Zn^{2+}, ions, whereas Cd^{2+} is transported independently of Msr3 and Mr4 [27]. Using SMPs prepared from wild-type and various mutant mitochondria, we found that Cu^{2+} ions were transported across the mitochondrial inner membrane by either Msr3 and Mr4 or by wild-type Rim2 in the presence of CTP, but not by mutated Rim2-1 (Figure 4B). Transport of Zn^{2+} ions was also detectable, but occurred only in SMPs prepared from cells overexpressing wild-type Rim2, but not in SMPs from wild-type or Msr3/Mr4-deficient cells. In contrast, Cd^{2+} ions crossed the mitochondrial inner membrane independently of both Msr3 and Mr4 [27] and Rim2, as the quenching pattern of PGSK upon addition of Cd^{2+} ions was similar to that of Fe^{2+} ions in SMPs pre-treated with the ionophore pyrithione (Figure 4B). Taken together, divalent iron, copper and zinc ions were transported across the mitochondrial inner membrane via Rim2 in the presence of pyrimidine nucleotides, but not when the transport activity of Rim2 was compromised.

**RIM2 deletion does not affect haem synthesis**

Our results are in obvious conflict with those of a recent analysis of the role of Rim2 in mitochondrial iron uptake [35]. That study concluded that iron transport by Rim2 is not linked to its role in pyrimidine nucleotide transport. The central conclusion was mainly based on the observation that RIM2 depletion (in a galactose-regulated yeast strain) affected *in vivo* haem synthesis, but not pyrimidine import into isolated mitochondria. In order to address these differences, we analysed haem synthesis by radiolabelling of yeast cells with 55Fe *in vitro* [42,51]. A series of isogenic RIM2 deletion mutants was created in the W303-1A strain background, in which RIM2 was deleted either alone or in combination with MRS3 and MRS4. Since rim2Δ cells lose mtDNA [37], the resulting deletion strains were compared with corresponding isogenic rhoΔ strains. Remarkably, *in vivo* haem synthesis was consistently 1.5-fold higher in rim2Δ than in rhoΔ wild-type cells, and virtually unchanged in mrs3Δ/mrs4Δ/rim2Δ triple mutants compared with mrs3Δ/mrs4Δ/rhoΔ cells (Figure 5A). These data argue against a specific requirement of Rim2 for haem formation.

In order to exclude that these discrepancies on Rim2 function are due to differences in strain backgrounds, we repeated the analysis of Figure 5(A) using the strain background BY4742. These rim2Δ cells did not elicit any significant growth defect and in *vivo* haem synthesis was only marginally reduced in comparison with BY4742 rhoΔ wild-type cells (Figures 5B and 5C). Strikingly, haem synthesis significantly increased upon deletion of RIM2 in BY4742 mrs3Δ/mrs4Δ cells (Figure 5B). Taken together, these data strongly argue that Rim2 is dispensable for mitochondrial iron supply for *in vivo* haem synthesis, even though our data support the previously made observation that overproduction of Rim2 cures the haem synthesis defect of mrs3Δ/mrs4Δ cells [35,36] (see Supplementary Figure S1).

**Rim2 supports iron import for ISC biogenesis in mrs3Δ/mrs4Δ cells**

In order to explore whether the Rim2-mediated co-import of iron that is seen under Rim2-overproducing conditions is physiologically relevant *in vivo*, we investigated mitochondrial Fe–S protein biogenesis. For this we followed the maturation
of mitochondrial aconitase Aco1, mitochondrial biotin synthase Bio2 and cytosolic isopropylmalate isomerase Leu1 in W303-1A strains deleted for RIM2 by $^{55}$Fe radiolabelling of cells and immunoprecipitation with specific antibodies. $^{55}$Fe bound to the immunobeads reflects the amount of de novo ISC assembly on these Fe–S proteins, and was quantified by scintillation counting (Figures 6A–6C). All three Fe–S proteins were radiolabelled to a similar extent in both rho$^0$ wild-type and rim2Δ cells. These data exclude a major role of Rim2 in mitochondrial iron supply for mitochondria that contain Mrs3 and Mrs4. Furthermore, deletion of RIM2 did not induce the iron-responsive promoters FIT3 or FET3 which are highly expressed upon impaired mitochondrial Fe–S protein biogenesis [7] (Figures 6E and Supplementary Figure S3 at http://www.biochemj.org/bj/455/bj4550057add.htm). The triple-deleted mrs3Δ/4Δ/rim2Δ cells and mrs3Δ/4Δ rho$^0$ showed similar low levels of iron insertion into aconitase (Figure 6A). Iron binding to Bio2 and Leu1 were 2.5-fold lower in mrs3Δ/4Δ/rim2Δ cells than in mrs3Δ/4Δ rho$^0$, yet residual iron binding was close to background levels in both cells (Figures 6B and 6C). Since apoprotein levels were similar in all cells (Supplementary Figures S3 and S4 at http://www.biochemj.org/bj/455/bj4550057add.htm), these differences in iron insertion likely reflect a further, yet small decline in mitochondrial iron supply for Fe–S protein biogenesis upon deletion of RIM2 in mrs3Δ/4Δ cells. Steady-state enzyme activities of aconitase were also 1.7-fold reduced in mrs3Δ/4Δ/rim2Δ cells compared with mrs3Δ/4Δ cells (Figure 6D) and the activity of the iron-responsive FIT3 promoter was 3.7-fold higher in mrs3Δ/4Δ/rim2Δ cells compared with mrs3Δ/4Δ cells (Figure 6E). Taken together, deletion of RIM2 alone does not affect Fe–S protein biogenesis efficiency. However, RIM2 deficiency in mrs3Δ/4Δ cells results in a further small decline in Fe–S protein maturation. This indicates that, in the absence of Mrs3 and Mrs4, the mitochondrial pyrimidine nucleotide carrier Rim2 can weakly supply iron to mitochondria leading to slightly better Fe–S protein maturation than in triple-deleted mrs3Δ/4Δ/rim2Δ cells. This conclusion is consistent with the observation that high-copy expression of RIM2 suppresses the phenotype of mrs3Δ/4Δ strains. However, the fact that the deletion of RIM2 alone has no obvious consequences on mitochondrial iron supply indicates that Rim2 does not play a major physiological role in this process independently of Mrs3 and Mrs4.

In order to further verify the latter conclusion, we compared the relative effects of deleting either MRS3 or RIM2 in a rho$^0$mrs4Δ strain. Although deletion of MRS3 led to a severe growth defect on SD minimal medium as expected, the deletion of RIM2 did not affect growth, neither alone nor together with a deletion of MRS4, and even not on iron-limited growth medium (Figure 7A). Consistent with this, deletion of RIM2 in rho$^0$mrs4Δ cells did not cause any defects in $^{55}$Fe incorporation into haem or aconitase (Figures 7B and 7C). Rather, a slight increase relative to rho$^0$mrs4Δ cells was observed. The double deletion of MRS3 and MRS4, however, caused a significant impairment of both processes, and resulted in an induction of the FET3 promoter (Supplementary Figure S3). This characteristic feature of cells with impaired mitochondrial Fe–S protein maturation was not seen in the mrs4Δ/rim2Δ strain. In summary, these results confirm that Mrs3 and Mrs4 function as major mitochondrial iron importers. In contrast, yeast cells do not utilize Rim2 for mitochondrial iron acquisition in the presence of Mrs3 and Mrs4 under normal conditions. Deletion of RIM2 in mrs3Δ/4Δ cells, however, induces a small, but significant, further decline in the maturation of mitochondrial Fe–S proteins, indicating that Rim2 may function as a backup iron importer. This view is consistent with our observation that Rim2 can transport ferrous iron and other


pyrimidine nucleoside triphosphates requires the efflux of a
exchange mechanism by which the carrier-mediated uptake of
component of mitochondrial pyrimidine nucleotide supply [37].

fully consistent with a physiological role of Rim2 as a major
analysed by ANOVA and differences were considered statistically significant (P

to aconitase was determined by immunoprecipitation and scintillation counting. Data were
B
was extracted with butyl acetate and quantified by scintillation counting (μ

0.05). Results
C
for certain metal ions, as Fe2
counter ions for nucleotide transport, it showed some preference
in Rim2. Even though Rim2 can use various divalent ions as
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Sal1 that are assumed to bind the Mg2
ion are not conserved
in Rim2. Even though Rim2 can use various divalent ions as
as it is present at much higher concentrations in the cell than
Fe2
or Zn2
. Under certain conditions of cytosolic iron overload, as found in cells lacking the mitochondrial
carriers Mrs3 and Mrs4, or the vacuolar divalent metal transporter
Ccc1 [36], Fe2
might be co-imported by Rim2 with nucleotides

divalent metal ions into mitochondria in a pyrimidine nucleotide-
dependent fashion.

DISCUSSION
The conserved mitochondrial solute carriers Mrs3 and Mrs4 play
a central role in mitochondrial iron uptake in cells from yeast
to man [21,22,25,26]. In addition, the mitochondrial pyrimidine
nucleotide transporter Rim2 was isolated in a synthetic lethal
 genetic screen for cells with defects in mitochondrial iron
metabolism in S. cerevisiae and therefore represents a possible
candidate for a low-affinity mitochondrial iron uptake system
in parallel with Mrs3 and Mrs4 [35,36]. Deletion of RIM2 exhibited
concordant defects in mitochondrial iron-dependent pathways
and in mitochondrial pyrimidine transport, but the mechanism
connecting pyrimidine transport to mitochondrial iron availability
was not clarified. In fact, one study concluded that the basis for the
defects in iron utilization in Rim2-depleted mitochondria cannot
directly be attributed to pyrimidine transport, suggesting that the
latter is only a secondary function of this solute carrier [35].

Previous analysis of purified reconstituted Rim2 clearly
demonstrated its function as a mitochondrial transporter for
pyrimidine nucleotides. Pyrimidine (deoxy)-di- and (deoxy)-tri-
phosphates were shown to be the major substrates of Rim2
[33,39]. Deletion of RIM2 causes a loss of mtDNA which is
fully consistent with a physiological role of Rim2 as a major
component of mitochondrial pyrimidine nucleotide supply [37].
Nucleotides are transported almost exclusively by a substrate-
exchange mechanism by which the carrier-mediated uptake of
pyrimidine nucleoside triphosphates requires the efflux of a
counter-substrate, mainly pyrimidine nucleoside monophosphates
[33,39]. A potential metal requirement of the transport reaction
was not addressed in the reconstituted transport system.

In the present study, we show that Rim2 directly mediates
the transport of divalent metals across the mitochondrial inner
membrane. Metal transport was strictly dependent on the presence
of pyrimidine nucleotides in a concentration-dependent manner.
Pyrimidine nucleotides were required on the same side of the
membrane as the metal, strongly suggesting that Rim2 co-imports
nucleotides and a divalent cation. Consistent with this conclusion,
mutations in contact site II of the common substrate-binding
pocket of mitochondrial nucleotide carriers [34,50] abolished both
pyrimidine nucleotide and divalent metal import by Rim2. Co-
import of substrates with bound cations is not uncommon for
mitochondrial carriers, as several of these carry out a proton−
substrate symport such as the oxodicarboxylate carrier and the
GTP/GDP carrier [52]. A co-import of divalent metals in complex
with pyrimidine nucleotides can fully explain the suppressor
function of high levels of Rim2 for defects in the mitochondrial
transporters Mrs2 (for Mg2
), and Mrs3 and Mrs4 (for Fe2
) [22,57]. In this respect, Rim2 is similar to the calcium-dependent
mitochondrial ADP/ATP carrier Sal1, that transports nucleotides
in complex with Mg2
[34,53]. The transport mechanism of both
carriers, however, must differ considerably, as the two charged
amino acids in contact site I of the substrate binding site of
Sal1 that are assumed to bind the Mg2
ion are not conserved
in Rim2. Even though Rim2 can use various divalent ions as
counter ions for nucleotide transport, it showed some preference
for certain metal ions, as Fe2
, Cu2
 or Zn2
 were transported
in vitro with different efficiencies. Under normal physiological
conditions, the co-imported cation most likely may be Mg2
, as
it is present at much higher concentrations in the cell than
the trace elements Fe2
, Cu2
 or Zn2
. Under certain conditions of
cytosolic iron overload, as found in cells lacking the mitochondrial
carriers Mrs3 and Mrs4, or the vacuolar divalent metal transporter
Ccc1 [36], Fe2
 might be co-imported by Rim2 with nucleotides
to a significant extent.

The suggestion of a strict nucleotide/metal co-import by Rim2
contradicts previous work which concluded that the functions
of Rim2 in pyrimidine nucleotide and iron import are probably
separate [35]. This central conclusion was mainly based on the
observation that RIM2 depletion (in a galactose-regulated
yeast strain) affected haem synthesis, but not pyrimidine import
into isolated mitochondria under low-salt conditions in vitro. In
contrast with our study that employed rim2Δ deletion strains,
the depletion of Rim2 was reported to induce strong effects on
mitochondrial iron-dependent processes [35]. However, low levels
of Rim2 cause the loss of mtDNA [37], which is associated
with drastic changes in mitochondrial metabolism and gene
expression [54]. In particular, conditions of acute loss of mtDNA
induce massive transient changes in all aspects of cellular iron
homoeostasis [55] that likely contribute significantly to the
strong mitochondria-related effects that were observed upon
Rim2 depletion in rho
 strains. Thus the use of rho
 strains
makes it difficult to distinguish the potential role of Rim2 in
mitochondrial iron transport from its established function in
mitochondrial DNA maintenance [37]. In order to avoid this
problem, our analysis was carried out exclusively with rho
 strains in two different yeast strain backgrounds. In the present study,
msr3Δ rim2Δ cells consistently showed slightly higher rather
than impaired haem synthesis activities in vivo compared with
msr3Δ rho
 cells, excluding a critical role of Rim2 in iron
supply for haem. Nevertheless, this effect differed from the effect
on Fe–S protein maturation. The msr3Δ rim2Δ cells showed
slightly lower efficiencies in Fe–S protein maturation compared
with the isogenic msr3Δ cells, a finding consistent with a
role of Rim2 in mitochondrial iron supply. The relatively weak effect on Fe–S protein maturation and the absence of an effect on haem synthesis suggests that Rim2 only plays a minor role in mitochondrial iron supply and under special conditions, e.g. in the absence of Mrs3 and Mrs4. As shown by our in vitro iron uptake experiments, Rim2 may contribute to mitochondrial iron influx by co-importing pyrimidine nucleotides together with ferrous iron. From these results, there is no evidence justifying the assumption that Rim2 transports a second solute beside pyrimidine nucleotides that specifically promotes mitochondrial iron utilization as previously suggested [35]. RIM2 was reported to be essential in the YPH499/500 strain background [35] allowing the conclusion that BY4742 and W303 strains possess an as-yet uncharacterized backup system for iron supply to haem that is missing in the YPH background which may explain the essential character of RIM2 in this latter background. However, we consider this unlikely, as we were able to delete RIM2 in YPH499/500 cells by PCR-mediated gene replacement. Similar to the case of a significant role in mitochondrial iron influx under normal physiological conditions, RIM2 deletion had hardly any influence on mitochondrial Fe–S proteins or haem synthesis in wild-type rho0 cells. This is consistent with a previous study that reported only mild effects compared with the deletion of Mrs3 and Mrs4 [36]. Taken together, these observations suggest that Rim2 is not a major iron transporter in parallel with Mrs3 and Mrs4 under normal physiological conditions. Beneficial effects of Rim2 on mitochondrial iron utilization are consistently seen upon overexpression of the gene in cells deleted for MRS3 and MRS4 [35,36] (the present study). These findings suggest that Rim2 can indeed substitute Mrs3 and Mrs4 function and may act as a physiological low-affinity iron import system under certain conditions, such as cytosolic iron overload. Notably, according to systematic transcription analyses, RIM2 is not strongly regulated in S. cerevisiae, with less than 2-fold changes in transcription under all conditions tested (see http://www.yeastgenome.org).

It is evident from the present study that Rim2 does not play a significant role in mitochondrial iron influx under normal physiological conditions. RIM2 deletion had hardly any influence on mitochondrial Fe–S proteins or haem synthesis in wild-type rho0 cells. This is consistent with a previous study that reported only mild effects compared with the deletion of Mrs3 and Mrs4 [36]. Taken together, these observations suggest that Rim2 is not a major iron transporter in parallel with Mrs3 and Mrs4 under normal physiological conditions. Beneficial effects of Rim2 on mitochondrial iron utilization are consistently seen upon overexpression of the gene in cells deleted for MRS3 and MRS4 [35,36] (the present study). These findings suggest that Rim2 can indeed substitute Mrs3 and Mrs4 function and may act as a physiological low-affinity iron import system under certain conditions, such as cytosolic iron overload. Notably, according to systematic transcription analyses, RIM2 is not strongly regulated in S. cerevisiae, with less than 2-fold changes in transcription under all conditions tested (see http://www.yeastgenome.org).

An iron-responsive expression of RIM2 reminiscent of that of MRS4 was not reported [56]. Thus there is no evidence that yeast actually utilizes elevated Rim2 levels in order to enhance mitochondrial iron influx. In vertebrates, the mitochondrial pyrimidine nucleotide carrier PNC1 is inducible by insulin and the insulin-like growth factor IGF-I (insulin-like growth factor 1), and PNC1 expression was significantly increased in several cancer cell lines [57,58]. The IGF-I signalling pathway is important in promoting cell growth and proliferation and can directly enhance mitochondrial activity. However, the significance of PNC1 overexpression for enhanced mitochondrial activity is unclear.

Taken together, our work shows that the mitochondrial carrier Rim2 supports the co-import of pyrimidine nucleotides and divalent metals including ferrous iron. This import mechanism easily explains how this carrier promotes mitochondrial iron influx in the absence of the major mitochondrial iron importers Mrs3 and Mrs4 and/or under conditions of cytosolic iron overload. Although Rim2 can mediate low-affinity iron uptake into mitochondria, our results of the present study provide little evidence that Rim2 contributes significantly to mitochondrial iron supply under normal physiological conditions. Even a triple deletion of RIM2, MRS3 and MRS4 is not lethal in several S. cerevisiae strain backgrounds. This suggests the existence of other low-affinity mitochondrial iron transporters which may fulfill this task in the absence of the major iron transporters Mrs3 and Mrs4.

AUTHOR CONTRIBUTION

Elisabeth Froschauer, Nicole Rietzschel, Melanie Hassler, Markus Binder, Ulrich Mühlenhoff and Gerlinde Wiesenberger conducted the experiments. Statistical analyses were performed by Ulrich Mühlenhoff and Melanie Hassler. Rudolf Schwheyen, Roland Lill, Ulrich Mühlenhoff and Gerlinde Wiesenberger conceived and designed the experiments. Roland Lill, Ulrich Mühlenhoff and Gerlinde Wiesenberger wrote the paper.

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Import of iron into mitochondria
SUPPLEMENTARY ONLINE DATA

The mitochondrial carrier Rim2 co-imports pyrimidine nucleotides and iron

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Figure S1 Rim2 overexpression rescues the iron-related phenotype of mrs3/4Δ

(A) W303-1A wild-type (WT) transformed with pCM189 and the isogenic mrs3/4Δ mutant strain transformed with either the empty pCM189 or pCM189 harbouring Rim2 were grown in liquid SD — uracil medium. Cells were diluted to D600 = 1 and serial 1:10 dilutions were spotted on to SD — uracil, YPD plus 100 μM BPS or SD — uracil medium supplemented with 10 μM CdCl2 and cultivated for 2 days at 30°C. (B and C) rho0 derivatives of W303-1A wild-type and mrs3/4Δ cells harbouring either YCplac33-RIM2 (↑), pCM189-RIM2 (↑↑) or the empty vector were radiolabelled with 55Fe, and 55Fe binding to haem (B) and aconitase (C) was determined. Results are means±S.E.M. (n=4). Data were analysed by ANOVA and differences were considered statistically significant (P < 0.05) or not (n.s.). 3/4Δ, mrs3/4Δ/mrs4Δ; strain background W303-1A. Note that the rescue of the biochemical parameters of mrs3/4Δ cells in (B) and (C) is only observed upon strong overexpression of Rim2.

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4 Deceased.
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Figure S2  The Rim2 G245Y S246A mutant is expressed in S.cerevisiae

(A) DNA sequences and deduced amino acid sequences of wild-type RIM2 (upper rows) and the mutated version rim2-1 (lower rows). (B) Mitochondria were isolated from mrs3/4Δ transformed with the empty pCM189 or with pCM189 expressing either the wild-type (WT) RIM2 or the mutated version (rim2-1; G245Y S246A) as indicated. A total of 40 mg of each protein extract were separated on SDS/PAGE (12% gel), transferred on to a nitrocellulose membrane and immuno-decorated with an anti-Rim2 antibody (left-hand panel). The Coomassie Blue stain of a duplicate gel served as a loading control (right-hand panel).

Figure S3  Rim2 overexpression rescues the iron-related phenotype of mrs3/4Δ

(A) The indicated strains in an W303 background strain were transformed with reporter plasmid FET3-GFP and pCM189-RIM2 (↑↑) as indicated. The activity of the FET3 promoter was determined from the GFP-specific fluorescence of whole cells cultivated in SD medium supplemented with 50 μM ferric ammonium citrate. Results are means±S.E.M. (n=4). (B) Levels of the indicated mitochondrial proteins in cell extracts of the indicated strains were detected by immunostaining. Cells were cultivated in SD medium prior to analysis. WT, wild-type.

Figure S4  RIM2 deletion affects Fe–S protein maturation only in the absence of Mrs3 and Mrs4

W303-1A rim2Δ and rho0Δ derivatives of isogenic wild-type (WT) and mrs3/4Δ were cultivated in iron-free SD minimal medium. Cells were lysed and aconitase, biotin synthase Bio2 and cytosolic Leu1 were immunoprecipitated with specific antibodies. Protein levels in cell extracts (~30 mg protein (15 mg in case of Bio2)) and immunoprecipitates (one third of an immunoprecipitation from 200 mg cell extract (one sixth in the case of Bio2)) were determined by immunostaining of Western blots. Bio2 was overproduced from vector p426TDH3. Note that due to the restoration of the LEU2 marker, Leu1 levels are lower in mrs4::LEU2 backgrounds than in W303 (leu2-3-112). In leu2 strains, the Leu3 transcription factor is constitutively active, resulting in high Leu1 expression levels [15].

Figure S5  RIM2 is not essential in YPH backgrounds

RIM2 was deleted by PCR-mediated gene replacement with a HIS3 marker cassette in YPH499 and YPH500. (A) Histidine prototrophic transformants were streaked together with the corresponding wild-type (as indicated) on SC minimal media supplemented with glucose or glycerol and incubated for 3 days. Five out of seven prototrophs each displayed the petite phenotype of rim2Δ cells. (B) The histidine prototrophic clone rim2Δ 1 in YPH499 background was analysed for the insertion of the HIS3 cassette in the RIM2 locus by PCR.
## Table S1  Yeast strains used in the present study

Gene disruptions and promoter exchanges were generated by PCR-based gene replacement and verified by PCR as described previously [3,5]. Yeast cells were transformed by the lithium acetate method [6]. Rho 0 derivatives of the strains were created by ethidium bromide treatment [7]. LoxP-disrupted strains were created by PCR-based gene replacement using pUG72 and pUG73 as templates and subsequent loss of markers mediated by CRE recombinase [3].

<table>
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## Table S2  Plasmid constructs used in the present study

For expression of RIM2 under the control of the strong inducible TetO 7 promoter, the coding sequence of RIM2 was amplified from genomic DNA from DBY747 using the primers MRS12-3 (5′-CCAGGATCCTCAAGGAGATAAAGATGC-3′) and MRS12-4 (5′-AACGCTGCAGCTAGCGGCCGCCCGATAGTAGCCTTATAAC-3′). The PCR product was cloned into the BamHI and PstI restriction site of pCM189. The rim2-1 allele (G245Y S246A) of vector pMH2 was created by PCR-mediated site-directed mutagenesis of pAS12 using the primers mrs12mGSfw (5′-TTGAGTGCCTCTTATTTGTATGCTGTTGAAGGAATTC-3′), mrs12mGSrv (5′-ATATAATAGCCATTGAAGAATTCCTTCAACAGCATAAATAAGAGGCACTCAAC-3′), pCM189fw (5′-AAATTACCGGATCCATTCG-3′) and pCM189rv (5′-CATAACTAATTACATGATGC-3′). The mutations (underlined in the primer sequences above) were introduced into the single-copy plasmid pOLU2 by replacing a HincII fragment containing part of the open reading frame and 3′ untranslated sequences by a HincII fragment from pMH2 containing the mutated DNA and the CYC1 terminator from the pCM189 backbone, creating pMH5. The wild-type control (pMH3) was constructed in the same way from pAS12.

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