FEMS Yeast Research, 19, 2019, foz078

doi: 10.1093/femsyr/foz078
Advance Access Publication Date: 11 November 2019
Research Article

RESEARCH ARTICLE

Overexpression of the peroxin Pex34p suppresses impaired acetate utilization in yeast lacking the mitochondrial aspartate/glutamate carrier Agc1p

Chalongchai Chalermwat[1], Thitipa Thosapornvichai[2], Parith Wongkittichote[3,4], John D. Phillips[5], James E. Cox[6,7], Amornrat N. Jensen[8], Duangrurdee Wattanasirichaigoon[3] and Laran T. Jensen[2,*,†]

[1]Graduate Program in Molecular Medicine, Faculty of Science, Mahidol University, 272 Rama 6 Road, Ratchathewi, Bangkok 10400 Thailand, [2]Department of Biochemistry, Faculty of Science, Mahidol University, 272 Rama 6 Road, Ratchathewi, Bangkok 10400 Thailand, [3]Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, 270 Rama 6 Road, Ratchathewi, Bangkok 10400, Thailand, [4]Department of Pediatrics, St. Louis Children's Hospital, Washington University School of Medicine, 1 Brookings Drive, St. Louis, MO 63130, USA, [5]Department of Internal Medicine, Division of Hematology, University of Utah, 30 N 1900 E, Salt Lake City, UT 84132, USA, [6]Metabolomics Core Research Facility, University of Utah, 15 N Medical Drive East, Salt Lake City, UT 84112, USA, [7]Department of Biochemistry, University of Utah, 15 N Medical Drive East, Salt Lake City, UT 84112, USA and [8]Department of Pathobiology, Faculty of Science, Mahidol University, 272 Rama 6 Road, Ratchathewi, Bangkok 10400, Thailand

*Corresponding author: Department of Biochemistry, Faculty of Science, Mahidol University, 272 Rama 6 Road, Bangkok 10400, Thailand. Tel: +66-2-201-5460; Fax: +66-2-354-7174; E-mail: laran.jen@mahidol.ac.th

One sentence summary: Bypass of aspartate-glutamate carrier deficiency through overexpression of the peroxin *PEX34* is mediated by redirection of acetyl-CoA toward energy production together with utilization of an alternative mitochondrial redox shuttle.

Editor: Cristina Mazzoni

†Laran T. Jensen, http://orcid.org/0000-0003-3199-1743

ABSTRACT

PEX34, encoding a peroxisomal protein implicated in regulating peroxisome numbers, was identified as a high copy suppressor, capable of bypassing impaired acetate utilization of *agc1*Δ yeast. However, improved growth of *agc1*Δ yeast on acetate is not mediated through peroxisome proliferation. Instead, stress to the endoplasmic reticulum and mitochondria from *PEX34* overexpression appears to contribute to enhanced acetate utilization of *agc1*Δ yeast. The citrate/2-oxoglutarate carrier Yhm2p is required for *PEX34* stimulated growth of *agc1*Δ yeast on acetate medium, suggesting that the suppressor effect is mediated through increased activity of a redox shuttle involving mitochondrial citrate export. Metabolomic analysis also revealed redirection of acetyl-coenzyme A (CoA) from synthetic reactions for amino acids in *PEX34* overexpressing yeast. We propose a model in which increased formation of products from the glyoxylate shunt, together

Received: 31 May 2019; **Accepted:** 10 November 2019

with enhanced utilization of acetyl-CoA, promotes the activity of an alternative mitochondrial redox shuttle, partially substituting for loss of yeast *AGC1*.

Keywords: aspartate-glutamate carrier; peroxin; *PEX34*; suppressor; acetate utilization; AGC1

INTRODUCTION

Mitochondrial carrier proteins (MCPs) link biochemical pathways in the mitochondrial matrix and cytosol through the movement of metabolites across the inner mitochondrial membrane and are involved in the proper function of both compartments (Kunji 2004; Palmieri 2004). In many cases, MCPs can mediate secondary active transport by coupling the transport of one molecule with the movement of another substrate against its relative concentration gradient (Palmieri and Pierri 2010). The MCP family consists of 35 members in yeast, 58 members in *Arabidopsis thaliana* and 53 members in humans (Palmieri and Pierri 2010). MCPs transport a wide range of solutes including protons, nucleotides, amino acids, carboxylic acids, inorganic ions and cofactors. Members of this transporter family are involved in metabolic pathways such as metabolism of fatty acids, amino acids, nucleic acids, as well as urea production, heme synthesis and metal ion homeostasis (Palmieri 2004, 2014).

Aspartate/glutamate carrier (AGC) isoforms, AGC1 (aralar, *SLC25A12*) and AGC2 (citrin, *SLC25A13*), function to export aspartate from mitochondria in exchange with cytosolic glutamate (Palmieri *et al.* 2001b). In addition, two glutamate carriers, GC1 (*SLC25A22*) and GC2 (*SLC25A18*), have overlapping functions with the AGCs, although they are glutamate/H^+ symporters (Fiermonte *et al.* 2002). Thus, it is possible for cells to have four distinct transporters to facilitate the import of glutamate into mitochondrial. While AGC and GC isoforms are involved in a similar biochemical process, they do not appear to be functionally redundant as they are expressed in different types of tissue (Kobayashi *et al.* 1999; Iijima *et al.* 2001; Begum *et al.* 2002; Fiermonte *et al.* 2002). AGC1 is highly expressed in brain, spinal cord, skeletal muscle, kidney and heart (Ramos *et al.* 2003). In contrast, AGC2 is expressed ubiquitously but appears to have a primary role in the liver, where AGC1 is absent (del Arco, Agudo and Satrustegui 2000; Iijima *et al.* 2001). The GC isoforms also show distinct tissue distribution with GC1 expressed at much higher levels in liver, pancreas, spleen and kidney although both GC1 and GC2 exhibit similar levels of expression in the brain (Fiermonte *et al.* 2002).

Mutations in AGC2, resulting in lowered or absent carrier activity, are associated with two age-dependent disorders: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM#605814) and type II citrullinemia (CTLN2, OMIM#603471) (Kobayashi *et al.* 1993; Saheki *et al.* 2002). NICCD patients display intrahepatic jaundice and multiple metabolic abnormalities, with these symptoms typically disappearing by the age of one year (Kobayashi *et al.*1993). CTLN2 develops in adults and if untreated can lead to death due to hyperammonemic encephalopathy and complications of brain edema (Kobayashi *et al.* 1993). The carrier frequency for AGC2 mutations is very high in Asia (1 in 70 individuals) (Yamaguchi *et al.* 2002; Lu *et al.* 2005; Song *et al.* 2007; Kobayashi *et al.* 2008; Tabata *et al.* 2008; Wongkittichote *et al.* 2013a), and can also be found in populations from the Middle East, Europe and North America, although at a lower frequency (Dimmock *et al.* 2009).

The exchange of cytosolic aspartate for glutamate in mitochondria by AGC2 is involved in several metabolic processes including the malate-aspartate shuttle, that functions in the movement of reducing equivalents between the cytosol and mitochondria, regulating the redox balance between these compartments (Bakker *et al.* 2001; Begum *et al.* 2002; Palmieri 2004). The malate-aspartate shuttle requires two carriers to complete the cycle, an AGC isoform and a malate/2-oxoglutarate antiporter. Malate is imported into mitochondria where it is converted to oxaloacetate, reducing NAD^+ to NADH. Oxaloacetate is then converted to aspartate and exported from mitochondria using the AGC. Cytosolic aspartate can then be converted back to oxaloacetate and then malate consuming a molecule of NADH, regenerating NAD^+. 2-Oxoglutarate exported into the cytosol is required for the formation of glutamate, which is imported into mitochondria by AGC and utilized as an amino donor for aspartate synthesis. The result of the malate-aspartate shuttle is the movement of NADH inside the mitochondrial matrix (Palmieri and Pierri 2010). However, loss of AGC2 activity in CTLN2 patients is thought to prevent the transfer of reducing equivalents from cytosolic NADH into mitochondria, leading to lowered ATP production as well as inadequate supplies of cytosolic NAD^+ for pathways such as ureogenesis and glycolysis (Saheki *et al.* 2005).

Movement of reducing equivalents into mitochondria can be facilitated through substrate exchange cycles beyond the malate/aspartate shuttle. Mitochondrial carrier proteins have been characterized as that involved in the exchange of reducing equivalents through shuttle systems involving pyruvate, citrate and malate (Bakker *et al.* 2001). An example of this is the 2-oxoglutarate/citrate shuttle involved in citrate cycling that can facilitate the movement of cytosolic NADPH equivalents into mitochondria. This shuttle utilizes 2-oxoglutarate for the production of isocitrate in the cytosol with the consumption of NADPH. After isomerization to citrate and import into mitochondria, 2-oxoglutarate is regenerated with the production of mitochondrial NADPH (Jiang *et al.* 2016). It is possible that activation of the 2-oxoglutarate/citrate or other redox shuttles could reduce the effects of AGC deficiency.

Current therapies for CTLN2 are limited with liver transplantation, the most effective treatment. However, the low number of donors, as well as the cost and complications of liver transplantation, restricts its use in the treatment of CTLN2 (Saheki *et al.* 2002; Saheki and Kobayashi 2002). Administration of arginine (Imamura *et al.* 2003; Dimmock *et al.* 2007) or mixtures of amino acids have been shown to have some efficacy in the treatment of CTLN2 through limiting ammonia levels in the blood following carbohydrate ingestion (Saheki and Kobayashi 2002). Intravenous treatment with sodium pyruvate has also shown promise in reducing CTLN2 symptoms (Mutoh *et al.* 2008), presumably promoting the conversion of cytosolic NADH to NAD^+ through the activity of lactate dehydrogenase (Moriyama *et al.* 2006).

Positive effects in CTLN2 patients from these supplements may be mediated at least in part through MCPs that function in the transport of readily interconvertible molecules. An example of this is the functional redundancy between the mitochondrial pyruvate carrier (MPC) and an alanine carrier. The presence of the alanine carrier allows the bypass of MPC deficiency by alanine supplementation through pyruvate-alanine cycling (McCommis *et al.* 2015). In addition, changes in metabolism in response to the loss of function in an MCP can also promote

bypass of the impaired activity. The adaptation following the disruption of MCP activity appears to require changes in several other pathways to compensate for a metabolic defect (Jiang *et al.* 2017). Thus, MCPs provide cells with the potential for maintaining essential homeostatic processes through redundant or partially redundant activities.

The baker's yeast *Saccharomyces cerevisiae* has been shown to be a suitable model system for the study of several human diseases (Steinmetz *et al.* 2002; Perocchi, Mancera and Steinmetz 2008) including AGC2 deficiency (Cavero *et al.* 2003; Wongkittichote *et al.* 2013b). One of the powerful tools available using *S. cerevisiae* is the ability to search for genetic suppressors that can help to identify functional relationships between genes that may not have been uncovered using other techniques (Forsburg 2001). In this study, we utilized a high copy suppressor screen to identify genes that were capable of bypassing the need for *AGC1* (the yeast homologue of human AGC1 and AGC2) when overexpressed using growth on medium containing acetate as a selection. Overexpression of *PEX34*, encoding an integral membrane protein localized to peroxisomes, was capable of partially bypassing impaired acetate utilization of *agc1*Δ yeast. We report that the ability of *PEX34* to function as a bypass suppressor of *agc1*Δ yeast appears to be mediated through activation of endoplasmic reticulum and mitochondrial stress responses and altered utilization of acetyl-CoA.

MATERIALS AND METHODS

Yeast strains

Yeast strains used in this study were derived from BY4741 (*Mat a, leu2*Δ*0, met15*Δ*0, ura3*Δ*0, his3*Δ*1*) or BY4742 (*Mat α, leu2*Δ*0, lys2*Δ*0, ura3*Δ*0, his3*Δ*1*) (Brachmann *et al.* 1998). Single deletion strains were obtained from Open Biosystems, Inc. (Huntsville, AL, USA) Disruption of *AGC1* was generated with plasmid pCC001 resulting in strains CC001 (BY4742 *agc1*Δ), CC002 (*pex11*Δ, *agc1*Δ), CC003 (*pex25*Δ, *agc1*Δ), CC004 (*pex27*Δ, *agc1*Δ), CC005 (*cit2*Δ, *agc1*Δ), CC006 (*mdh3*Δ, *agc1*Δ), CC007 (*odc1*Δ, *agc1*Δ), CC008 (*ctp1*Δ, *agc1*Δ), CC009 (*yhm2*Δ, *agc1*Δ), CC010 (*rtg2*Δ, *agc1*Δ) and LJ464 (*ymc2*Δ, *agc1*Δ). Gene deletions were verified by *in vivo* PCR with a BioRad MJ Mini thermocycler (Hercules, CA) using flanking primers (Longtine *et al.* 1998). Strain PW001 (BY4741 *agc1*Δ) has been described previously (Wongkittichote *et al.* 2013b). Yeast transformations were performed using the lithium acetate procedure (Gietz and Schiestl 1991). Cells were propagated at 30°C either in enriched yeast extract, peptone-based medium supplemented with 2% glucose (YPD), synthetic complete (SC) (Sherman, Fink and Lawrence 1978) or synthetic acetate (SA) medium containing 100 mM sodium acetate, pH 5 (Cavero *et al.* 2003).

Screen for *agc1*Δ suppressors

The *S. cerevisiae* AB320 genomic library in YEp13 (American Type Culture Collection, Manassas, VA, USA) has been described previously (Nasmyth and Reed 1980). The library was transformed into the *agc1*Δ strain and colonies were isolated after 10 days that allowed for growth on SA medium. Plasmids were rescued and transformed into fresh *agc1*Δ cells to verify the ability of plasmids to promote the utilization of acetate.

Plasmids

The *AGC1* disruption plasmid pCC001 was generated by PCR amplifying upstream (−931 to −114) and downstream sequences (+2499 to +3478) of *ACG1* introducing BamHI and SalI (upstream) or EcoRI and BamHI (downstream) restriction sites. Following digestion, the *AGC1* DNA fragments were inserted into pRS403 (*HIS3*) (Sikorski and Hieter 1989). Transformation of yeast strains with pCC001 digested with *Bam*HI resulted in the deletion of *AGC1* sequences −113 to +2498. Yeast expression plasmids for *PEX34* (pST001), *KAR4* (pLJ517), *PEX11* (pCC002), *MDH3* (pCC030) and *CIT2* (pCC003) utilized YCplac33 (*CEN URA3*) containing the *TPI1* promoter and *PGK1* terminator (Jensen *et al.* 2019). A GFP-CIT2 fusion was derived by replacing *SMF1* sequences in plasmid GFP-SMF1 (Sullivan *et al.* 2007) with the *CIT2* ORF resulting in plasmid pCC014. *PEX34* and *PEX11* expression plasmids containing *LEU2* selection were generated by first introducing an XhoI site downstream of the *PGK1* terminator sequence in pST001 and pCC002 using the QuickChange mutagenesis procedure (Stratagene, Santa Clara, CA, USA). The *TPI1* promoter-ORF-*PGK1* terminator fragments were excised with SpeI and XhoI and ligated into pRS315 (*CEN, LEU2*) (Sikorski and Hieter 1989) resulting in plasmids pCC008 (*PEX34*) and pCC011 (*PEX11*). For *lacZ* reporter plasmids, promoter sequences from *ACT1* (−761 to +9), *CIT2* (−638 to +7) and *FMS1* (−859 to +10) were PCR amplified, digested with SalI and BamHI and ligated into pLGΔ178 (Guarente 1983) resulting in plasmids pLJ519 (*ACT1-lacZ*), pCC005 (*CIT2-lacZ*) and pLJ503 (*FMS1-lacZ*). The DNA sequence integrity of all plasmids was verified by DNA sequencing (Macrogen, Seoul, Rep. of Korea). The following plasmids have been described previously: pADHHARFP-PTS1 (PTS1-RFP) (Zipor *et al.* 2009), pSM1960 (Sec63-RFP) (Metzger *et al.* 2008) and pPW433 (unfolded protein response element, UPRE-*lacZ*) (Cox, Shamu and Walter 1993).

Fluorescence imaging

Signals from green fluorescent protein (GFP) and red fluorescent protein (RFP) fusions PTS1-RFP (peroxisome marker) (Zipor *et al.* 2009), Sec63p-RFP (ER marker) (Metzger *et al.* 2008) and GFP-Cit2p were visualized in live cells (Sundin *et al.* 2004; Jensen *et al.* 2009) and viewed directly at a magnification of 60× with an FV10i-DOC confocal laser scanning microscope (Olympus, Tokyo, Japan), equipped with universal plan super apochromat phase-contrast oil-immersion objective (Olympus Bioimaging Center, Mahidol University). The number of peroxisomes in each strain examined was evaluated by manually counting peroxisomes from 50 to 100 cells showing PTS1-RFP fluorescence.

Growth tests

The ability of yeast strains to utilize acetate as a carbon source using both agar plates and liquid SA medium was performed according to established procedures (Cavero *et al.* 2003). Growth of BY4741 (WT) transformed with the control plasmid (pRS425) (Sikorski and Hieter 1989) and PW002 (*agc1*Δ::*URA3*) containing pRS425 or high copy suppressor clones was monitored with SA agar plates and liquid medium. Growth of strains BY4742 (WT), CC001 (*agc1*Δ::*HIS3*) or double deletion strains transformed as indicated with vector control (pRS316) (Sikorski and Hieter 1989), and the overexpression plasmids pST001 (*PEX34*), pLJ517 (*KAR4*), pCC002 (*PEX11*), pCC030 (*MDH3*) or pCC003 (*CIT2*), was examined with SA liquid medium. Growth was monitored by measuring the OD_{600} at 48, 72 and 96 hours. Results for growth are presented as the % of wild-type control.

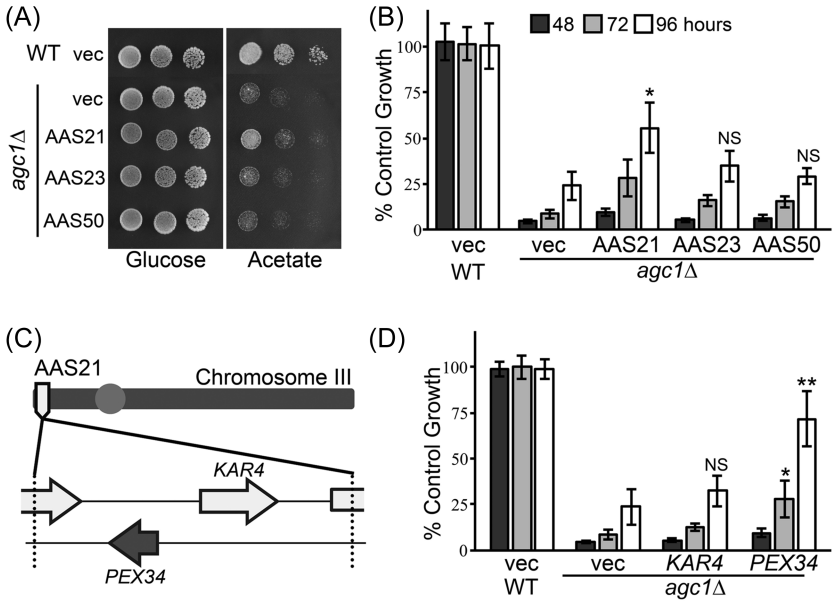

Figure 1. *PEX34* is a suppressor of impaired acetate utilization in *agc1*Δ yeast. **(A)** Growth of WT (BY4741) and *agc1*Δ (PW001) transformed with pRS315 (vector) or three potential high copy suppressors was monitored by spotting 10^5, 10^4 or 10^3 cells on solid SD and SA medium followed by incubation at 30°C for 3 days (SD) or 10 days (SA). **(B)** Transformants from panel (A) were grown in liquid SA medium lacking leucine and OD_{600} nm was recorded at 48, 72 and 96 hours. Results are normalized to WT cells containing the vector control = 100%. **(C)** The region of chromosome III present on plasmid AAS21 showing the position of *PEX34* and *KAR4*. **(D)** Growth of WT (BY4742) and *agc1*Δ (CC001) transformed with pRS316 (vector) or *PEX34* (pST001) and *KAR4* (pLJ517) overexpression plasmids utilizing the strong *TPI1* promoter in liquid SA medium lacking uracil. OD_{600} nm measurements were performed as described in (B). Values are mean ± SD ($n = 3$) and $^*P < 0.05$ and $^{**}P < 0.01$ were determined using Student's *t*-test.

Metabolomics analysis

Yeast were grown in synthetic medium containing 1% sodium pyruvate lacking uracil to late log phase. Cells were collected by centrifugation, washed with deionized water and resuspended in SA medium lacking uracil. After incubation at 30°C for 12 hours, cells were collected, washed, and metabolites were extracted with 80% ethanol. Samples were completely dried using a lyophilizer (FTS Systems Inc., Stone Ridge, NY, USA) prior to analysis. GC-MS analysis was performed with a Waters GCT Premier mass spectrometer fitted with an Agilent 6890 gas chromatograph and a Gerstel MPS2 autosampler. Dried samples were derivatized with O-methoxylamine hydrochloride (MOX) in pyridine for 1 hour at 30°C followed by further derivatization using N-methyl-N-(trimethylsilyl)trifluoroacetamide for 1 hour at 37°C prior to injection. Data was collected using MassLynx 4.1 software (Waters, Milford, MA, USA). Metabolites were identified and their m/z peak area was recorded using QuanLynx (Waters). Metabolite identity was established using a combination of an in-house metabolite library developed using pure purchase standards and the commercially available NIST library.

β-Galactosidase assays

Transformants containing the *UPRE-lacZ* (pPW344) (Cox, Shamu and Walter 1993), *CIT2-lacZ* (pCC005), *FMS1-lacZ* (pLJ503) or *ACT1-lacZ* (pLJ519) reporters were grown using the same procedure as for metabolomics analysis except both uracil and leucine were absent in the growth medium. β-Galactosidase activities were assayed using ONPG as a substrate, measuring absorbance at 412 nm (Giacomini *et al.* 1992), and results from two independent transformants, assayed at least in duplicate. Results for *UPRE*, *CIT2* and *FMS1* reporters were normalized using *ACT1-lacZ* levels. Values are mean ± standard deviation (SD) presented normalized to WT with vector control = 1.

Statistical analysis

Experimental data are reported as the mean ± the standard deviation. Significant differences among groups are indicated with, $^*P < 0.05$ and $^{**}P < 0.01$ or between groups with, # $P < 0.05$ and ## $P < 0.01$. Data were analyzed with one-way ANOVA with post-hoc Tukey test or Student's t-test as appropriate.

RESULTS

Overexpression of *PEX34* can partially bypass the growth defect of *agc1*Δ cells on acetate

Cells lacking *AGC1* exhibited impaired growth with acetate as the sole carbon source (Cavero *et al.* 2003). To identify extragenic suppressors, the *agc1*Δ strain was transformed with a high copy yeast genomic library (Nasmyth and Reed 1980) and colonies that displayed the ability to utilize acetate were isolated. Approximately 10 000 colonies were screened and the plasmid isolated from AAS21 allowed for partial complementation of the *agc1*Δ growth defect on acetate (Fig. 1). Sequencing of plasmid AAS21 revealed the presence of two complete genes from chromosome III, *PEX34* and *KAR4*. The coding sequences for these genes were PCR amplified and cloned into an expression plasmid utilizing the *TPI1* promoter (Jensen *et al.* 2019). Expression of *KAR4* did not promote the growth of the *agc1*Δ strain on acetate medium, while *PEX34* expression was sufficient to bypass the *agc1*Δ acetate growth defect, confirming the identity of *PEX34* as the suppressor.

Pex11p protein family members are not required for *PEX34*-mediated enhanced utilization of acetate in *agc1*Δ yeast

Pex34p, Pex11p, Pex25p and Pex27p are thought to function together to regulate peroxisome numbers and can induce

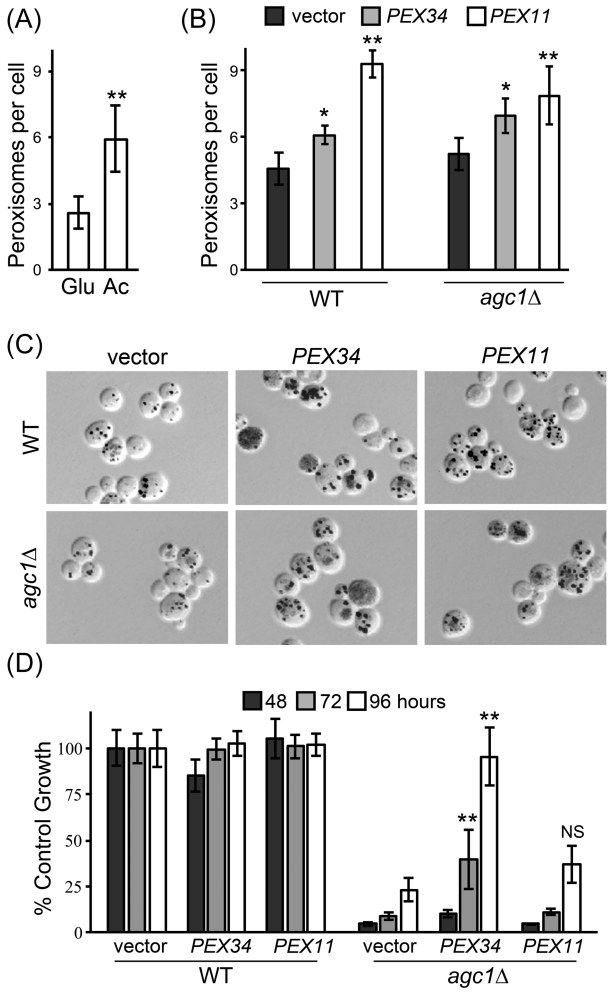

Figure 2. Peroxisome proliferation is not sufficient to promote acetate utilization in *agc1*Δ cells. **(A)** Peroxisome number in WT (BY4742) transformed with pADHHARFP-PTS1 (PTS1-RFP) grown in SD or SA medium. Peroxisomes were visualized using confocal microscopy at a magnification of ×60 in strains WT (BY4742) and *agc1*Δ (CC001) strains co-transformed with pADHHARFP-PTS1 (PTS1-RFP) and pRS316 (vector), pST001 (*PEX34*) or pCC002 (*PEX11*) with **(B)** quantitation of peroxisome numbers and **(C)** representative images from fluorescence microscopy. **(D)** Growth of WT (BY4742) and *agc1*Δ (CC001) strains transformed with pRS316 (vector), pST001 (*PEX34*) or pCC002 (*PEX11*) in SA medium lacking uracil was monitored as described in Fig. 1D at 48, 72 and 96 hours. Values are mean ± SD and $^{**}P < 0.01$ was determined using Student's *t*-test.

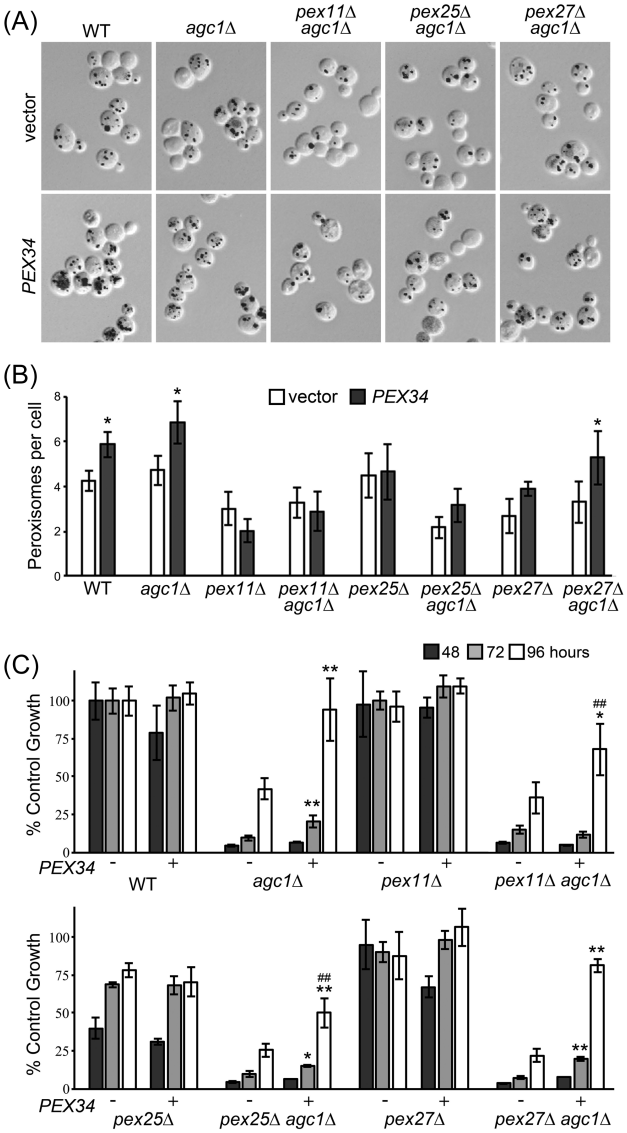

Figure 3. Peroxins involved in regulating peroxisome number are not required for *PEX34* bypass of impaired acetate utilization in *agc1*Δ cells. **(A)** Peroxisomes were visualized using confocal microscopy at a magnification of ×60 in WT (BY4742), *agc1*Δ (CC001), *pex11*Δ *agc1*Δ (CC002), *pex25*Δ *agc1*Δ (CC003), and *pex27*Δ *agc1*Δ (CC004) strains co-transformed with pADHHARFP-PTS1 (PTS1-RFP) and pRS316 (vector) or pST001 (*PEX34*). **(B)** Peroxisome numbers in strains *pex11*Δ, *pex25*Δ, *pex27*Δ, and those indicated in panel (A). **(C)** Growth of WT (BY4742), *agc1*Δ (CC001), *pex11*Δ (17129), *pex11*Δ *agc1*Δ (CC002), *pex25*Δ (12140), *pex25*Δ *agc1*Δ (CC003), *pex27*Δ (12449) and *pex27*Δ *agc1*Δ (CC004) strains transformed with pRS316 (–) or pST001 (*PEX34*) (+) in SA medium lacking uracil was monitored as described in Fig. 1B at 48, 72 and 96 hours. Values are mean ± SD ($n = 4$) and $^{*}P < 0.05$ and $^{**}P < 0.01$ were determined with one-way ANOVA with post-hoc Tukey test or Student's *t*-test as appropriate.

proliferation when overexpressed (Rottensteiner *et al.* 2003; Tam *et al.* 2003; Tower *et al.* 2011). This suggested the possibility that increased peroxisome numbers may be involved in suppression of the *agc1*Δ defect. Growth in acetate increased peroxisome numbers in WT yeast, monitored by observing the PTS1-RFP marker (Fig. 2A). However, *PEX34* and *PEX11* when expressed from the *TPI1* promoter could further increase peroxisome numbers both in WT and *agc1*Δ strains grown in acetate medium (Fig. 2B and C). A significant fraction of WT and *agc1*Δ cells overexpressing *PEX34* exhibited what appear to be vacuolar PTS1-RFP fluorescence (Figure S1, Supporting Information), although the identity of these signals has not been confirmed. In contrast to *PEX34*, overexpression of *PEX11*, while capable of increasing peroxisome numbers, did not promote a significant increase in the utilization of acetate by the *agc1*Δ strain (Fig. 2D).

PEX11, *PEX25* and *PEX27* have been reported to be required for peroxisome proliferation induced by *PEX34* overexpression in oleic acid-containing medium (Tower *et al.* 2011). These genes were also found to be important for peroxisome proliferation from *PEX34* overexpression in acetate medium (Fig. 3A and B). However, peroxisome numbers in the *pex27*Δ *agc1*Δ strain were responsive to *PEX34* overexpression. Deletion of either *PEX11* or *PEX25* in the *agc1*Δ background significantly reduced, but did not eliminate, and improved acetate utilization from overexpression of *PEX34* (Fig. 3C). In contrast, acetate utilization in the *pex27*Δ *agc1*Δ strain overexpressing *PEX34* was similar to *agc1*Δ cells. Together, these observations indicate that *PEX34*-mediated

peroxisome proliferation may be involved in the bypass of impaired growth of the *agc1*Δ strain on acetate medium.

PEX34 overexpression induces ER stress

Peroxisomes are derived through both the division of pre-existing peroxisomes and *de novo* formation from the endoplasmic reticulum (ER) (Lazarow 2003; van der Zand, Braakman and Tabak 2010; van der Zand *et al.* 2012). We examined whether *PEX34* overexpression was causing stress to the ER. Monitoring activation of the ER unfolded protein response (UPR) using a UPRE-*lacZ* reporter revealed a significant increase in reporter activity in both WT and *agc1*Δ cells from *PEX34* overexpression. WT yeast overexpressing *PEX34* exhibited roughly a 2-fold increase in UPRE-*lacZ* activity, compared to control cells. In contrast, *PEX34* overexpression in *agc1*Δ cells induced an almost 10-fold increase in UPRE-*lacZ* activity (Fig. 4A). No significant change in UPRE-*lacZ* activity was detected in yeast overexpressing *PEX11*.

Examining ER structure using Sec63p-RFP, a component of the translocon embedded in the ER membrane (Young *et al.* 2001; Metzger *et al.* 2008), also revealed significant changes in cells overexpressing *PEX34*. Cells containing the control plasmid or overexpressing *PEX11* exhibited perinuclear fluorescence, typical for ER. However, in yeast overexpressing *PEX34* the signal for Sec63p-RFP was observed in punctate structures at a high frequency, although the effect was more pronounced in the *agc1*Δ strain (Fig. 4B). ER stress and UPR signaling pathways participate in the regulation of lipid metabolism (Travers *et al.* 2000; Volmer and Ron 2015) and the punctate structures may be ER-derived lipid droplets. However, the identity of these structures has not been established.

Mitochondrial retrograde signaling is activated by PEX34 overexpression in *agc1*Δ yeast

Activation of the mitochondrial retrograde signaling pathway is another mechanism in yeast for increasing peroxisome numbers (Epstein *et al.* 2001). The expression of *CIT2*, encoding a peroxisome citrate synthase, is activated in response to mitochondrial stress (Liao *et al.* 1991). Using a *CIT2* promoter-*lacZ* reporter we observed only a small increase in *CIT2* expression in WT cells overexpressing *PEX34*. Surprisingly, *agc1*Δ yeast containing the control plasmid displayed a 3-fold decrease in *CIT2* expression compared to the WT strain. *PEX34* overexpression restored *CIT2* levels in the *agc1*Δ strain to near WT levels, while overexpression of *PEX11* did not enhance activity from the *CIT2-lacZ* reporter (Fig. 5A).

Cit2p is present in both the cytoplasm and peroxisomes (Nakatsukasa *et al.* 2015) and we evaluated if *PEX34* overexpression would alter the localization of this protein. As seen in Fig. 5B, GFP-Cit2p was primarily observed in the cytoplasm in WT and *agc1*Δ yeast containing the control plasmid or overexpressing *PEX11*. In contrast, GFP-Cit2p was seen as punctate structures, most likely peroxisomes, in cells overexpressing *PEX34*. The shift in Cit2p localization may enhance the activity of the glyoxylate cycle, promoting acetate utilization.

The contribution of retrograde regulation on promoting growth on acetate of the *agc1*Δ strain overexpressing *PEX34* was evaluated using both deletion and overexpression of peroxisomal citrate synthase (*CIT2*) and malate dehydrogenase (*MDH3*). The deletion of *CIT2* in the WT strain resulted in the inability to grow on acetate, consistent with a previous report (Nakatsukasa *et al.* 2015); similarly, the *agc1*Δ *cit2*Δ strain

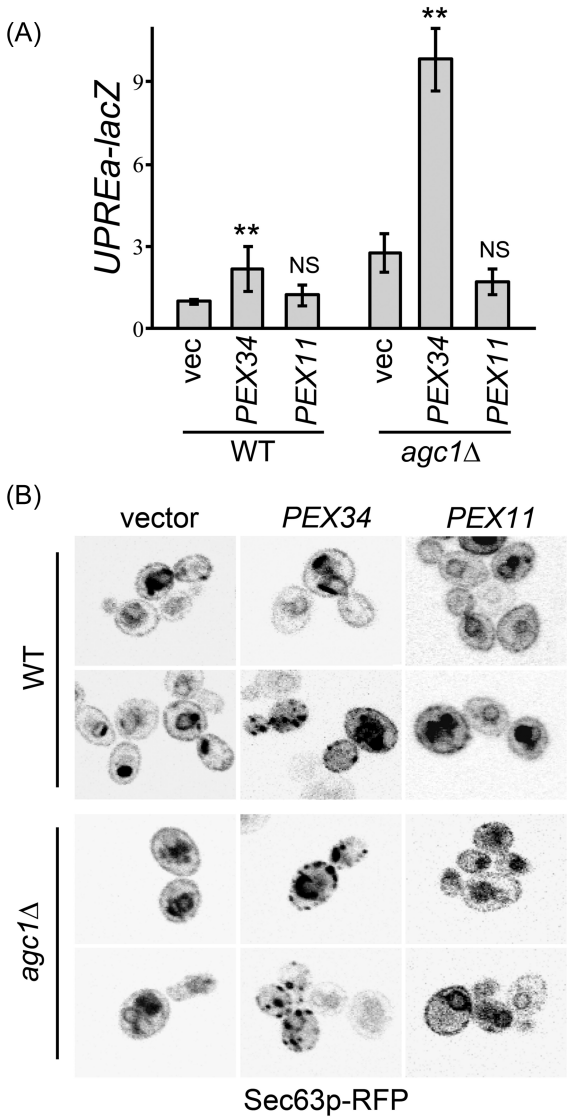

Figure 4. ER stress is induced by the overexpression of *PEX34*. **(A)** Activation of the ER unfolded protein response (UPR) was monitored in WT (BY4742) and *agc1*Δ (CC001) strains co-transformed with either or pPW433 (UPRE-*lacZ*) or pLJ519 (*ACT1-lacZ*) and pRS316 (vector), pST001 (*PEX34*) or pCC002 (*PEX11*). UPRE-*lacZ* results, relative to *ACT1-lacZ* in matched samples, are mean ± SD ($n = 4$). Values are normalized to WT with control vector = 1. Values are mean ± SD and $^{**}P < 0.01$ was determined using Student's *t*-test. **(B)** ER morphology was examined in WT (BY4742) and *agc1*Δ (CC001) strains co-transformed with pSM1960 (Sec63-RFP) and pRS315 (vector), pCC008 (*PEX34*) or pCC011 (*PEX11*) using confocal microscopy at a magnification of ×60. The punctate fluorescence observed in *PEX34* overexpressing cells is thought to be from lipid droplets.

was unable to grow on acetate. In addition, overexpression of *PEX34* was not sufficient to promote growth on acetate of *agc1*Δ *cit2*Δ cells. This indicates that *CIT2* is either required for enhanced acetate utilization mediated by *PEX34* or alternatively that acetate usage is impaired by deletion of *CIT2* beyond the ability of *PEX34* to bypass the *agc1*Δ growth defect. In contrast to the results with *CIT2*, deletion of *MDH3* did not significantly limit growth on acetate medium and *PEX34* was capable of promoting acetate utilization in the *agc1*Δ *mdh3*Δ strain. Deletion of *RTG2*, encoding a key regulator of the retrograde response (Liao and Butow 1993), also prevented *PEX34*-mediated acetate utilization (Fig. 5C), likely due to decreased expression of *CIT2*

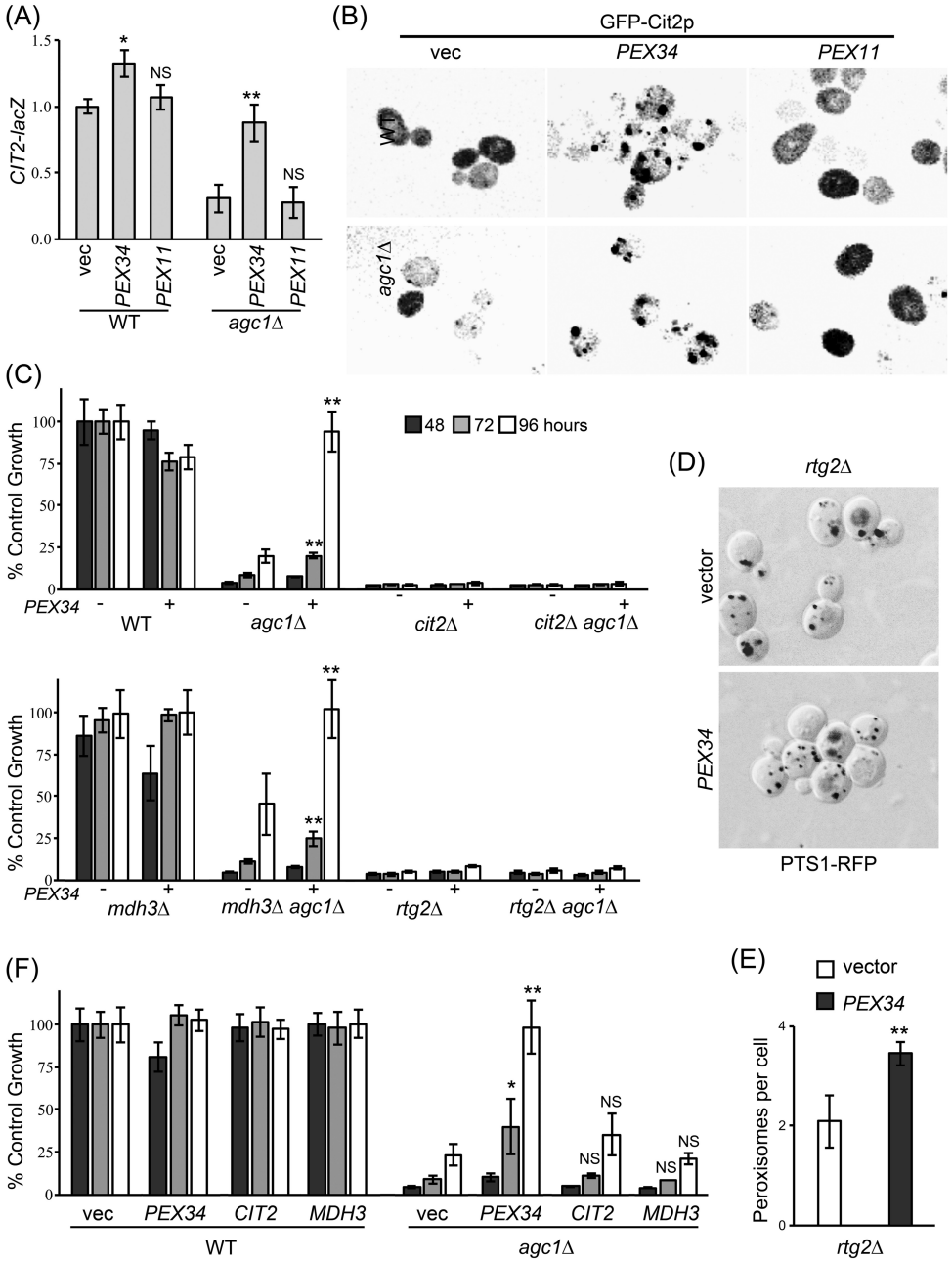

Figure 5. *PEX34* overexpression enhances mitochondrial retrograde signaling in *agc1*Δ yeast. **(A)** Activation mitochondrial retrograde signaling was monitored in WT (BY4742) and *agc1*Δ (CC001) strains co-transformed with either or pPW433 (*CIT2-lacZ*) or pLJ519 (*ACT1-lacZ*) and pRS315 (vector), pCC008 (*PEX34*) or pCC011 (*PEX11*). *CIT2-lacZ* results, relative to *ACT1-lacZ* in matched samples, are mean ± SD ($n = 4$). Values are normalized to WT with control vector = 1. **(B)** Cit2p localization was examined in WT (BY4742) and *agc1*Δ (CC001) strains co-transformed with pCC014 (GFP-Cit2p) and pRS315 (vector), pCC008 (*PEX34*) or pCC011 (*PEX11*) using confocal microscopy at a magnification of ×60. **(C)** Growth of WT (BY4742), *agc1*Δ (CC001), *cit2*Δ (13 485), *cit2*Δ *agc1*Δ (CC005), *mdh3*Δ (13 775), *mdh3*Δ *agc1*Δ (CC006), *rtg2*Δ (14619) and *rtg2*Δ *agc1*Δ (CC010) strains transformed with pRS316 (–) or pST001 (*PEX34*) (+) in SA medium lacking uracil was monitored as described in Fig. 1B. **(D)** Peroxisomes were visualized using confocal microscopy at a magnification of ×60 in *rtg2*Δ (14619) yeast co-transformed with pADHHARFP-PTS1 (PTS1-RFP) and pRS316 (vector) or pST001 (*PEX34*). **(E)** Peroxisome numbers from strains shown in panel (D). **(F)** Growth of WT (BY4742) and *agc1*Δ (CC001) transformed with pRS316 (vector), *PEX34* (pST001), *CIT2* (pCC003) or *MDH3* (pCC030) overexpression plasmids utilizing the strong *TPI1* promoter in SA medium lacking uracil was monitored as described in Fig. 1B at 48, 72 and 96 hours. Values are mean ± SD ($n = 3$) and $^{*}P < 0.05$ and $^{**}P < 0.01$ were determined using Student's *t*-test.

and other genes in the RTG pathway. In addition to its role in regulating retrograde signaling, *RTG2* also influences peroxisome numbers with yeast grown in oleate (Chelstowska and Butow 1995; Kos *et al.* 1995). Similarly, peroxisome numbers were reduced in the *rtg2*Δ strain in acetate medium (Fig. 5D and E).

Overexpression of either *CIT2* or *MDH3* in *agc1*Δ cells was not sufficient to promote growth on acetate to levels seen with *PEX34* overexpression (Fig. 5F). While the retrograde response appears to be induced from *PEX34* overexpression, enhanced expression of *CIT2* alone does not appear sufficient to bypass impaired acetate utilization due to deletion of *AGC1*.

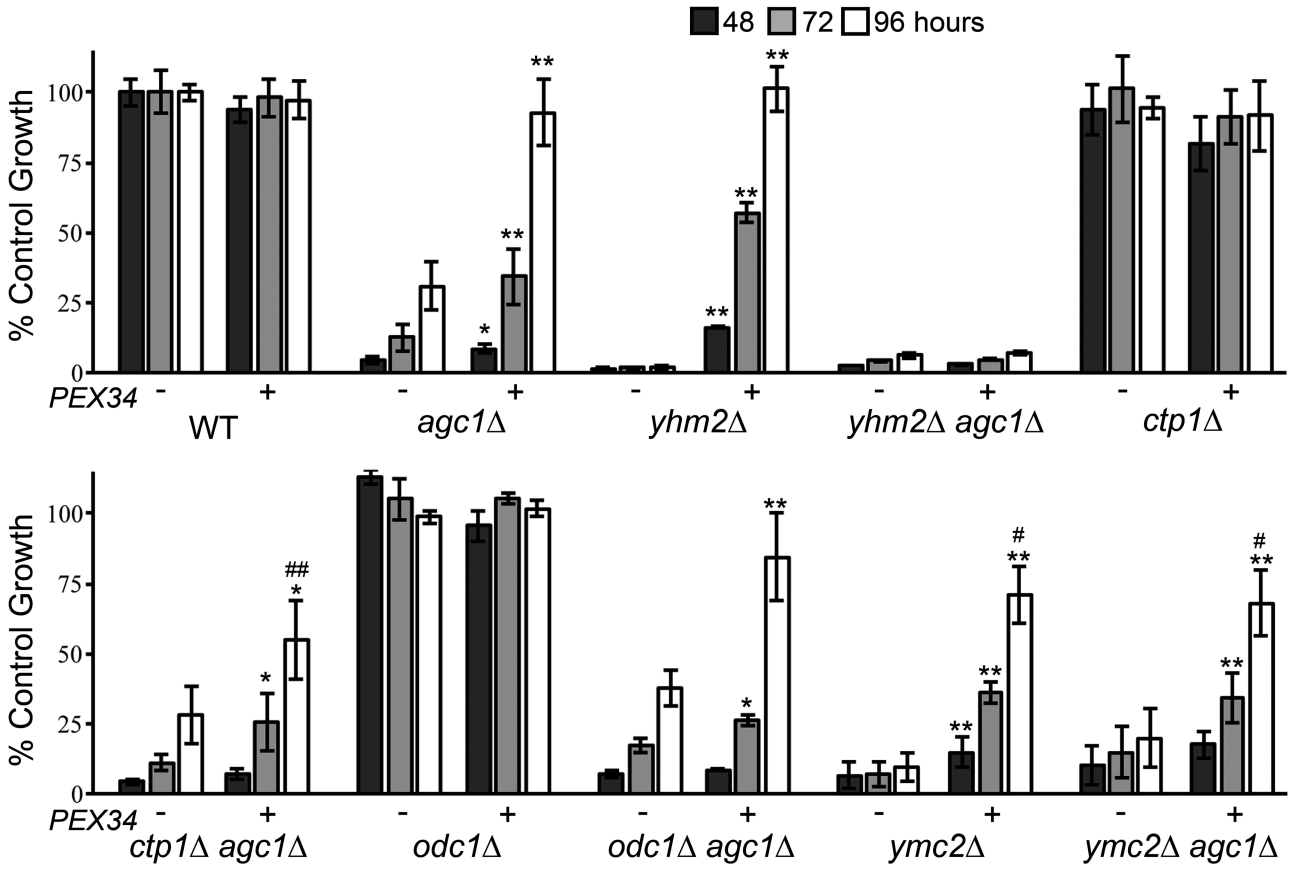

Figure 6. The citrate/2-oxoglutarate exchanger Yhm2p is required for *PEX34* bypass of impaired acetate utilization in *agc1*Δ yeast. Growth of WT (BY4742), *agc1*Δ (CC001), *yhm2*Δ (10827), *yhm2*Δ *agc1*Δ (CC009), *ctp1*Δ (15739), *ctp1*Δ *agc1*Δ (CC008) and *odc1*Δ (16480), and *odc1*Δ *agc1*Δ (CC007) strains transformed with pRS316 (–) or pST001 (*PEX34*) (+) in SA medium lacking uracil was monitored as described in Fig. 1B at 48, 72 and 96 hours. Values are mean ± SD ($n = 3$) and $^{*}P < 0.05$ and $^{**}P < 0.01$ for within each group and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ for comparison with *agc1*Δ were determined using Student's *t*-test.

YHM2 is required for PEX34-mediated bypass of impaired acetate utilization in *agc1*Δ cells

Yeast deleted for *AGC1* are impaired for mitochondrial import of glutamate and export of aspartate (Palmieri *et al.* 2001b; Cavero *et al.* 2003). However, other mitochondrial carriers (MCPs) may have functions that partially overlap with Agc1p and could participate in the *PEX34*-mediated bypass of impaired acetate utilization of *agc1*Δ cells. We examined whether the MCPs Yhm2p, Odc1p or Ctp1p were required for the *PEX34*-mediated stimulation of *agc1*Δ acetate utilization. Both Odc1p and Yhm2p are capable of importing 2-oxoglutarate into mitochondria (Palmieri *et al.* 2001a; Castegna *et al.* 2010) and we speculated that increasing 2-oxoglutarate levels in the mitochondria may partially alleviate the loss of glutamate import from Agc1p. Deletion of *ODC1* did not have a significant effect on either growth on acetate or the ability of *PEX34* to stimulate acetate utilization in cells lacking *AGC1*. In contrast, loss of *YHM2* led to impaired growth on acetate medium; surprisingly, *PEX34* overexpression was capable of bypassing the growth defect of the *yhm2*Δ strain. However, *PEX34* overexpression did not provide any measurable enhancement to acetate utilization in yeast lacking both *AGC1* and *YHM2* (Fig. 6). As Yhm2p can function in the export of citrate from mitochondria (Castegna *et al.* 2010), we examined whether loss of another citrate exporter, Ctp1p (Kaplan *et al.* 1995), would similarly impede the ability of *PEX34* to promote growth on acetate of cells lacking *AGC1*. Deletion of *CTP1* alone did not alter growth on acetate. However, the ability of *PEX34* to promote acetate utilization in the *agc1*Δ *ctp1*Δ strain was reduced compared to *agc1*Δ cells (Fig. 6). Together these results suggest an important role of citrate export in the *PEX34*-mediated bypass of *AGC1* deficiency.

We also examined whether Ymc2p, an MCP involved in mitochondrial import of glutamate, was important for *PEX34*-mediated bypass of *AGC1*. Enhanced mitochondrial glutamate influx from elevated Ymc2p levels can compensate for the loss of *AGC1* (Porcelli *et al.* 2018). Impaired acetate utilization was observed in both *ymc2*Δ and *agc1*Δ *ymc2*Δ cells under our culture conditions. While overexpression of *PEX34* could promote acetate utilization in *ymc2*Δ and *agc1*Δ *ymc2*Δ cells, growth was reduced compared to the *agc1*Δ strain (Fig. 6). This suggests that Ymc2p may have a minor role in *PEX34*-mediated enhanced acetate utilization.

Based on these findings, it appears that deletion of either *YHM2* or *AGC1* substantially impairs growth on acetate medium, which can be bypassed with overexpression of *PEX34*. However, one of these genes is required for the *PEX34* suppressor effect, as no growth enhancement was observed from *PEX34* in the *agc1*Δ *yhm2*Δ strain.

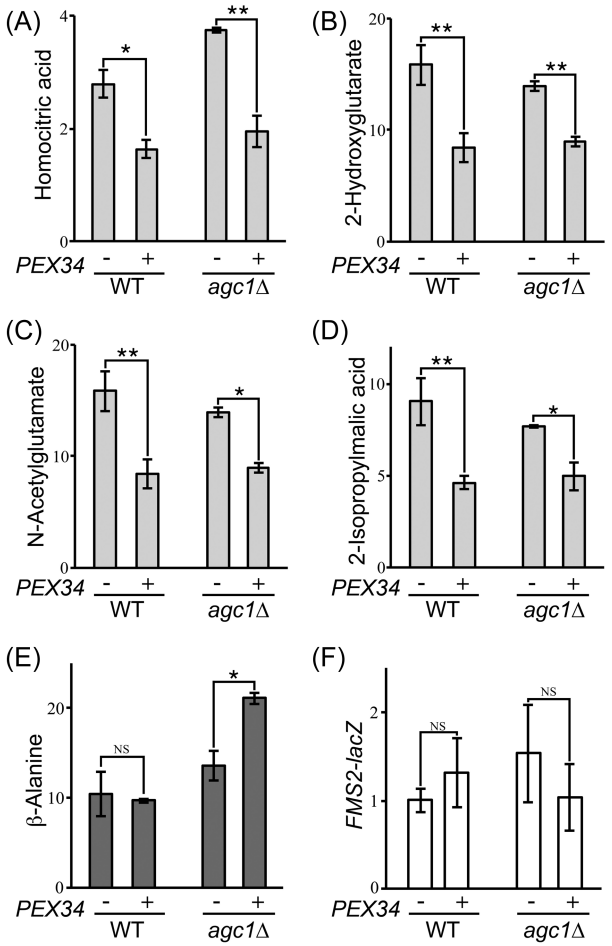

Figure 7. Metabolomic profiling indicates altered acetyl-CoA utilization in *PEX34* overexpressing yeast. Metabolite abundance was measured in WT (BY4742) and *agc1*Δ (CC001) yeast transformed with the control plasmid, pRS316 (–) or *PEX34* plasmid pST001 (+). Results for **(A)** homocitrate, **(B)** 2-hydroxyglutarate, **(C)** *N*-acetylglutamate, **(D)** 2-isopropylmalate and **(E)** β-alanine are reported as intensity counts. Values are mean ± SD ($n = 3$) and $^{*}P < 0.05$ and $^{**}P < 0.01$ were determined using Student's *t*-test. **(F)** *FMS1-lacZ* analysis was performed as described in Fig. 4,; values are mean ± SD ($n = 3$).

PEX34 overexpression reduces the abundance of compounds requiring acetyl-CoA for synthesis

A comparison of metabolite profiles from WT and *agc1*Δ yeast containing the control plasmid or overexpressing *PEX34* revealed several significant differences. One major change due to *PEX34* expression in *agc1*Δ cells was a 2-fold increase in the accumulation of β-alanine (Fig. 7A). The formation of β-alanine is required for the synthesis of pantothenate and subsequently coenzyme A (CoA), with the rate-limiting step in β-alanine synthesis mediated by a polyamine oxidase, encoded by *FMS1* (White, Gunyuzlu and Toyn 2001). However, using an *FMS1-lacZ* reporter did not reveal enhanced *FMS1* transcription (Fig. 7B), suggesting another mechanism is involved in increasing β-alanine levels in *agc1*Δ cells. Interestingly, the abundance of many metabolites that require acetyl-CoA for their synthesis is reduced in both WT and *agc1*Δ cells overexpressing *PEX34*. These include homocitric acid, 2-hydroxyglutarate, *N*-acetylglutamate, and 2-isopropylmalate (Fig. 7C–F). For each of these metabolites, a statistically significant decrease was noted in WT and *agc1*Δ cells overexpressing *PEX34* relative to the control strains. The abundance of *N*-acetylglycine in cells overexpressing *PEX34* also displayed a downward trend; however, the differences did not reach statistical significance. The reduced abundance of metabolites requiring acetyl-CoA as a substrate may indicate that *PEX34* overexpression is redirecting acetyl-CoA utilization, perhaps in favor of energy production at the expense of synthesis reactions.

DISCUSSION

Our search for potential therapeutic targets using a yeast genetics suppressor screen has identified *PEX34*, encoding a peroxisomal integral membrane protein (Tower *et al.* 2011), as a bypass suppressor of *AGC1* deficiency. Pex34p participates with other peroxins in the regulation of peroxisome populations (Tower *et al.* 2011) and was recently found to be involved in tethering of peroxisome-mitochondria contact sites (Shai *et al.* 2018). Mitochondria and peroxisomes have overlapping functions in several pathways including metabolite production, fatty acid metabolism, and both contain redox shuttles to move reducing equivalents from the cytosol to the interior of these organelles (Bakker *et al.* 2001; Rottensteiner and Theodoulou 2006; Antonenkov and Hiltunen 2012). Initially, we speculated that increased peroxisome numbers may be enhancing the activity of a peroxisomal redox shuttle, partially offsetting loss of the malate-aspartate shuttle in *agc1*Δ yeast. However, overexpression of *PEX11*, encoding another peroxin protein involved in regulating peroxisome numbers, increased peroxisome numbers but was not capable of enhancing the growth of *agc1*Δ cells on acetate medium. This indicates that increasing peroxisome numbers alone is not sufficient to bypass impaired acetate utilization in *agc1*Δ cells.

Similar to the findings from Tower *et al.* (2011), we observed that *PEX11*, *PEX25* and to a lesser extent *PEX27* were required for increased peroxisome numbers from *PEX34* overexpression. In addition, the deletion of *PEX11* or *PEX25* in the *agc1*Δ strain reduced the ability of *PEX34* to promote growth on acetate. In the *pex27*Δ *agc1*Δ strain *PEX34* was capable of increasing peroxisome numbers as well as promoting growth on acetate medium to similar levels as seen in *agc1*Δ cells. ER stress resulting from overexpression of *PEX34* may also promote increased peroxisome numbers. Thus, a connection between *PEX34*-mediated peroxisome proliferation and bypass of *AGC1* deficiency may exist.

If a peroxisome redox shuttle is not mediating *PEX34* bypass of *agc1*Δ deficiency, then cells may be forced to rely on alternative mitochondrial redox shuttles for the movement of reducing equivalents from the cytosol to mitochondria. A candidate for an alternative redox shuttle appears to involve the export of mitochondrial citrate. We observed that loss of the citrate/2-oxoglutarate exchanger Yhm2p prevented *PEX34*-mediated bypass of *AGC1* deficiency on acetate medium. The relevance of Yhm2p to CTLN2 is not clear as a citrate/2-oxoglutarate carrier has not been observed in mammalian cells. If a similar bypass for AGC deficiency through *PEX34* overexpression can occur in mammalian cells it would be mediated through another citrate exporting carrier protein.

Our analysis did not identify a mitochondrial carrier that functions with Yhm2p to facilitate the movement of reducing equivalents into mitochondria. However, Yhm2p-mediated citrate export from mitochondria (Castegna *et al.* 2010) could be utilized by the glyoxylate pathway to generate malate and succinate. Succinate, malate or other molecules derived from these metabolites may facilitate the transfer of reducing equivalents

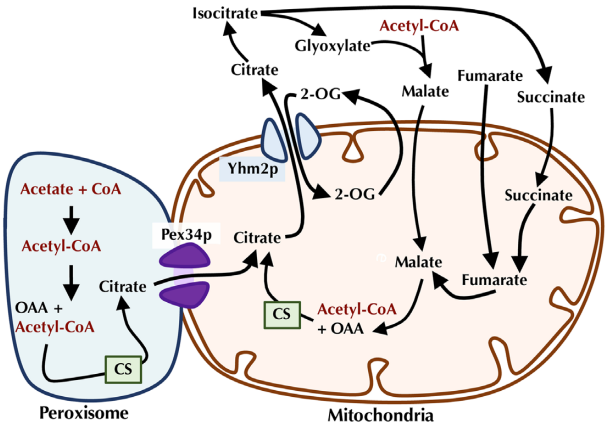

Figure 8. Model for *PEX34*-mediated bypass of *AGC1* deficiency. Cells overexpressing *PEX34* alter their metabolism allowing for the use of an alternative redox shuttle to provide reducing equivalents to mitochondria. Citrate produced either in mitochondria or peroxisomes by citrate synthases (CS) through combining oxaloacetate (OAA) with acetyl-CoA can be utilized by the glyoxylate pathway to generate malate, fumarate and succinate, which is then imported into mitochondria to resupply reducing equivalents. Formation of peroxisome-mitochondria contact sites from *PEX34* overexpression may also facilitate function of the alternative redox shuttle through direct movement of citrate from peroxisomes to mitochondria. The identity of the MCP(s) that functions with Yhm2p in the proposed redox shuttle is not known.

from the cytosol to mitochondria in the absence of *AGC1*. A similar citrate/dicarboxylate shuttle has been proposed in *Yarrowia lipolytica* involving YlYhm2p and an unidentified second MCP (Yuzbasheva *et al*. 2019). In *S. cerevisiae* candidate carriers that may function with Yhm2p include Dic1p, a dicarboxylate carrier capable of exchanging malate or succinate for phosphate (Palmieri *et al*. 1999), Sfc1p, a succinate/fumarate exchanger (Palmieri *et al*. 1997), Ctp1p, a proposed malate/citrate exchanger (Kaplan *et al*. 1995), the oxodicarboxylate carriers Odc1p and Odc2p implicated in the export of oxoglutarate in exchange with malate (Palmieri *et al*. 2001a), as well as fumarate/malate and fumarate/aspartate exchange reactions (Atlante, Gagliardi and Passarella 1998; Pallotta, Fratianni and Passarella 1999). However, we could not examine the role of *DIC1* or *SFC1* on *PEX34*-mediated bypass of impaired growth of *agc1*Δ, as loss of these genes prevents the utilization of acetate as a sole carbon source (Palmieri *et al*. 1997, 1999). Fumarate/malate and fumarate/aspartate exchange could also not be evaluated as the identity of the carrier proteins that possess these activities have not been established. Loss of Odc1p, the major isoform of the oxodicarboxylate carrier (Palmieri *et al*. 2001a), did not prevent *PEX34* bypass of *AGC1* deficiency suggesting that this activity is not involved or that the Odc2p isoform alone is sufficient. Deletion of *CTP1* reduced but did not abolish *PEX34*-mediated bypass of impaired growth on acetate medium in *AGC1* deleted cells. This suggests that the Ctp1p malate/citrate carrier may participate, but is not essential, for the movement of reducing equivalents in cells overexpressing *PEX34*. Regardless of the carrier(s) coupled with Yhm2p, the result of these reactions is expected to be increased movement of reducing equivalents into mitochondria. A model for *PEX34* mediated bypass of impaired acetate utilization in *agc1*Δ cells is presented in Fig. 8.

In addition to promoting peroxisome proliferation Pex34p is involved in the formation or stabilization of peroxisome-mitochondria contact sites (Shai *et al*. 2018). Organelle contact sites have multiple functions including the exchange of lipids, ions and metabolites (Elbaz and Schuldiner 2011). Citrate was found to be transferred from peroxisomes to mitochondria through expanded contact sites following *PEX34* overexpression. A similar transfer of citrate between peroxisomes and mitochondria was not observed by overexpression of *FZO1*, encoding another tethering protein, suggesting that Pex34p functions in a specific transfer of metabolites between compartments (Shai *et al*. 2018). Interestingly, Pex11p is also involved in peroxisome-mitochondrial contacts through a separate complex, the ER-mitochondria encounter structure (ERMES) (Mattiazzi Usaj *et al*. 2015). Pex11p forms a large pore in the peroxisomal membrane and is predicted to be involved in the transfer of free fatty acids between peroxisomes and mitochondria, although the substrate has not been determined (Mindthoff *et al*. 2016). In any case, the substrate transferred between peroxisomes and mitochondria through Pex34p and Pex11p appear distinct. This may explain why bypass of AGC deficiency is mediated only by overexpression of *PEX34*, even though overexpression of *PEX34* and *PEX11* both promote peroxisome proliferation.

Metabolomic analysis also revealed altered acetyl-CoA utilization from *PEX34* overexpression in both WT and *agc1*Δ cells. Overexpression of *PEX34* significantly lowers the abundance of precursors for lysine (homocitrate), leucine (α-isopropylmalate) and arginine (*N*-acetylglutamate) that require acetyl-CoA for their synthesis. The level of β-alanine, a precursor required for CoA synthesis, was also significantly higher in *agc1*Δ yeast overexpressing *PEX34*, which could promote the enhanced formation of CoA. Our findings are consistent with *PEX34* overexpression causing an increase in the CoA/acetyl-CoA ratio, resulting in CoA inactivation of isopropylmalate synthase, homocitate synthase (Tracy and Kohlhaw 1975; Tan-Wilson and Kohlhaw 1978), and N-acetylglutamate synthase (Wipe and Leisinger 1979), limiting acetyl-CoA consumption by these biosynthetic pathways. More importantly, conditions producing a high CoA/acetyl-CoA ratio can redirect acetyl-CoA for energy production (Hampsey and Kohlhaw 1981) and this could promote acetate utilization in *agc1*Δ yeast overexpressing *PEX34*.

Overall, we suggest that altered acetyl-CoA utilization and increased activity of the glyoxylate pathway from mitochondrial stress, mediated by overexpression of *PEX34*, combined facilitate bypass of *AGC1* deficiency in yeast cells. Consistent with this model, dietary supplement of medium-chain triglycerides (MCTs) to CTLN2 patients is thought to partially alleviate symptoms through increasing acetyl-CoA supply for use as an energy source in hepatic cells (Hayasaka *et al*. 2014). Interventions directed at enhancing the supply of metabolic intermediates capable of delivering reducing equivalents to mitochondria, such as succinate, and promoting utilization of acetyl-CoA for energy production together with dietary MCTs therapy may have a greater effect on relieving symptoms of CTLN2 patients.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

FUNDING

This work was supported by a joint grant from Faculty of Science, Mahidol Universityand Ramathibodi Hospital Faculty of Medicine (LJ and DW), Mahidol University (DW), the Medical Scholars Program of Mahidol University (PW) and a grant from the Thailand Research Fund IRG5980008. DW is a recipient of Research Career Development Award, Faculty of Medicine Ramathibodi Hospital.

ACKNOWLEDGMENTS

We thank the Olympus Bioimaging Center, Mahidol University, for providing equipment for imaging studies and S. Michaelis, J. Gerst, S. Tongpradit and W. Chindaudomsate for providing plasmids.

Conflict of interest. None declared.

REFERENCES

Antonenkov VD, Hiltunen JK. Transfer of metabolites across the peroxisomal membrane. *Biochim Biophys Acta* 2012;**1822**:1374–86.

Atlante A, Gagliardi S, Passarella S. Fumarate permeation in normal and acidotic rat kidney mitochondria: fumarate/malate and fumarate/aspartate translocators. *Biochem Biophys Res Commun* 1998;**243**:711–8.

Bakker BM, Overkamp KM, van Maris AJ *et al.* Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 2001;**25**:15–37.

Begum L, Jalil MA, Kobayashi K *et al.* Expression of three mitochondrial solute carriers, citrin, aralar1 and ornithine transporter, in relation to urea cycle in mice. *Biochim Biophys Acta* 2002;**1574**:283–92.

Brachmann CB, Davies A, Cost GJ *et al.* Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 1998;**14**:115–32.

Castegna A, Scarcia P, Agrimi G *et al.* Identification and functional characterization of a novel mitochondrial carrier for citrate and oxoglutarate in *Saccharomyces cerevisiae*. *J Biol Chem* 2010;**285**:17359–70.

Cavero S, Vozza A, del Arco A *et al.* Identification and metabolic role of the mitochondrial aspartate-glutamate transporter in *Saccharomyces cerevisiae*. *Mol Microbiol* 2003;**50**:1257–69.

Chelstowska A, Butow RA. RTG genes in yeast that function in communication between mitochondria and the nucleus are also required for expression of genes encoding peroxisomal proteins. *J Biol Chem* 1995;**270**:18141–6.

Cox JS, Shamu CE, Walter P. Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* 1993;**73**:1197–206.

del Arco A, Agudo M, Satrustegui J. Characterization of a second member of the subfamily of calcium-binding mitochondrial carriers expressed in human non-excitable tissues. *Biochem J* 2000;**345**:725–32.

Dimmock D, Kobayashi K, Iijima M *et al.* Citrin deficiency: a novel cause of failure to thrive that responds to a high-protein, low-carbohydrate diet. *Pediatrics* 2007;**119**:e773–7.

Dimmock D, Maranda B, Dionisi-Vici C *et al.* Citrin deficiency, a perplexing global disorder. *Mol Genet Metab* 2009;**96**:44–9.

Elbaz Y, Schuldiner M. Staying in touch: the molecular era of organelle contact sites. *Trends Biochem Sci* 2011;**36**:616–23.

Epstein CB, Waddle JA, Hale WT *et al.* Genome-wide responses to mitochondrial dysfunction. *Mol Biol Cell* 2001;**12**:297–308.

Fiermonte G, Palmieri L, Todisco S *et al.* Identification of the mitochondrial glutamate transporter. Bacterial expression, reconstitution, functional characterization, and tissue distribution of two human isoforms. *J Biol Chem* 2002;**277**:19289–94.

Forsburg SL. The art and design of genetic screens: yeast. *Nat Rev Genet* 2001;**2**:659–68.

Giacomini A, Corich V, Ollero FJ *et al.* Experimental conditions may affect reproducibility of the beta-galactosidase assay. *FEMS Microbiol Lett* 1992;**79**:87–90.

Gietz RD, Schiestl RH. Applications of high-efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast* 1991;**7**:253–63.

Guarente L. Yeast promoters and lacZ fusions designed to study expression of cloned genes in yeast. *Methods Enzymol* 1983;**101**:181–91.

Hampsey DM, Kohlhaw GB. Inactivation of yeast alpha-isopropylmalate synthase by CoA. Antagonism between CoA and adenylates and the mechanism of CoA inactivation. *J Biol Chem* 1981;**256**:3791–6.

Hayasaka K, Numakura C, Toyota K *et al.* Medium-chain triglyceride supplementation under a low-carbohydrate formula is a promising therapy for adult-onset type II citrullinemia. *Mol Genet Metab Rep* 2014;**1**:42–50.

Iijima M, Jalil A, Begum L *et al.* Pathogenesis of adult-onset type II citrullinemia caused by deficiency of citrin, a mitochondrial solute carrier protein: tissue and subcellular localization of citrin. *Adv Enzyme Regul* 2001;**41**:325–42.

Imamura Y, Kobayashi K, Shibatou T *et al.* Effectiveness of carbohydrate-restricted diet and arginine granules therapy for adult-onset type II citrullinemia: a case report of siblings showing homozygous SLC25A13 mutation with and without the disease. *Hepatol Res* 2003;**26**:68–72.

Jensen LT, Carroll MC, Hall MD *et al.* Down-regulation of a manganese transporter in the face of metal toxicity. *Mol Biol Cell* 2009;**20**:2810–9.

Jensen LT, Phyu T, Jain A *et al.* Decreased accumulation of superoxide dismutase 2 within mitochondria in the yeast model of Shwachman–Diamond syndrome. *J Cell Biochem* 2019;**120**:13867–80.

Jiang L, Boufersaoui A, Yang C *et al.* Quantitative metabolic flux analysis reveals an unconventional pathway of fatty acid synthesis in cancer cells deficient for the mitochondrial citrate transport protein. *Metab Eng* 2017;**43**:198–207.

Jiang L, Shestov AA, Swain P *et al.* Reductive carboxylation supports redox homeostasis during anchorage-independent growth. *Nature* 2016;**532**:255–8.

Kaplan RS, Mayor JA, Gremse DA *et al.* High-level expression and characterization of the mitochondrial citrate transport protein from the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 1995;**270**:4108–14.

Kobayashi K, Saheki T, Song YZ *et al.* Citrin deficiency. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, Ameniya A (eds). *Gene Reviews*. Seattle, WA: 1993.

Kobayashi K, Sinasac DS, Iijima M *et al.* The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. *Nat Genet* 1999;**22**:159–63.

Kobayashi K, Ushikai M, Song Y-Z *et al.* Overview of citrin deficiency: SLC25A13 mutations and the frequency. *J Appl Clin Pediatr* 2008;**23**:1553–7.

Kos W, Kal AJ, van Wilpe S *et al.* Expression of genes encoding peroxisomal proteins in *Saccharomyces cerevisiae* is regulated by different circuits of transcriptional control. *Biochim Biophys Acta* 1995;**1264**:79–86.

Kunji ER. The role and structure of mitochondrial carriers. *FEBS Lett* 2004;**564**:239–44.

Lazarow PB. Peroxisome biogenesis: advances and conundrums. *Curr Opin Cell Biol* 2003;**15**:489–97.

Liao X, Butow RA. RTG1 and RTG2: two yeast genes required for a novel path of communication from mitochondria to the nucleus. *Cell* 1993;**72**:61–71.

Liao XS, Small WC, Srere PA *et al*. Intramitochondrial functions regulate nonmitochondrial citrate synthase (CIT2) expression in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1991;**11**:38–46.

Longtine MS, McKenzie A, III, Demarini DJ *et al*. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 1998;**14**:953–61.

Lu YB, Kobayashi K, Ushikai M *et al*. Frequency and distribution in East Asia of 12 mutations identified in the SLC25A13 gene of Japanese patients with citrin deficiency. *J Hum Genet* 2005;**50**:338–46.

Mattiazzi Usaj M, Brloznik M, Kaferle P *et al*. Genome-wide localization study of yeast Pex11 identifies peroxisome–mitochondria interactions through the ERMES complex. *J Mol Biol* 2015;**427**:2072–87.

McCommis KS, Chen Z, Fu X *et al*. Loss of mitochondrial pyruvate carrier 2 in the liver leads to defects in gluconeogenesis and compensation via pyruvate-alanine cycling. *Cell Metab* 2015;**22**:682–94.

Metzger MB, Maurer MJ, Dancy BM *et al*. Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. *J Biol Chem* 2008;**283**:32302–16.

Mindthoff S, Grunau S, Steinfort LL *et al*. Peroxisomal Pex11 is a pore-forming protein homologous to TRPM channels. *Biochim Biophys Acta* 2016;**1863**:271–83.

Moriyama M, Li MX, Kobayashi K *et al*. Pyruvate ameliorates the defect in ureogenesis from ammonia in citrin-deficient mice. *J Hepatol* 2006;**44**:930–8.

Mutoh K, Kurokawa K, Kobayashi K *et al*. Treatment of a citrin-deficient patient at the early stage of adult-onset type II citrullinaemia with arginine and sodium pyruvate. *J Inherit Metab Dis* 2008;**31**:S343–7.

Nakatsukasa K, Nishimura T, Byrne SD *et al*. The ubiquitin ligase SCF(Ucc1) acts as a metabolic switch for the glyoxylate cycle. *Mol Cell* 2015;**59**:22–34.

Nasmyth KA, Reed SI. Isolation of genes by complementation in yeast: molecular cloning of a cell-cycle gene. *Proc Natl Acad Sci USA* 1980;**77**:2119–23.

Pallotta ML, Fratianni A, Passarella S. Metabolite transport in isolated yeast mitochondria: fumarate/malate and succinate/malate antiports. *FEBS Lett* 1999;**462**:313–6.

Palmieri F. The mitochondrial transporter family (SLC25): physiological and pathological implications. *Pflugers Arch* 2004;**447**:689–709.

Palmieri F. Mitochondrial transporters of the SLC25 family and associated diseases: a review. *J Inherit Metab Dis* 2014;**37**:565–75.

Palmieri F, Pierri CL. Mitochondrial metabolite transport. *Essays Biochem* 2010;**47**:37–52.

Palmieri L, Agrimi G, Runswick MJ *et al*. Identification in *Saccharomyces cerevisiae* of two isoforms of a novel mitochondrial transporter for 2-oxoadipate and 2-oxoglutarate. *J Biol Chem* 2001a;**276**:1916–22.

Palmieri L, Lasorsa FM, De Palma A *et al*. Identification of the yeast ACR1 gene product as a succinate–fumarate transporter essential for growth on ethanol or acetate. *FEBS Lett* 1997;**417**:114–8.

Palmieri L, Pardo B, Lasorsa FM *et al*. Citrin and aralar1 are Ca(2+)-stimulated aspartate/glutamate transporters in mitochondria. *EMBO J* 2001b;**20**:5060–9.

Palmieri L, Vozza A, Honlinger A *et al*. The mitochondrial dicarboxylate carrier is essential for the growth of *Saccharomyces cerevisiae* on ethanol or acetate as the sole carbon source. *Mol Microbiol* 1999;**31**:569–77.

Perocchi F, Mancera E, Steinmetz LM. Systematic screens for human disease genes, from yeast to human and back. *Mol Biosyst* 2008;**4**:18–29.

Porcelli V, Vozza A, Calcagnile V *et al*. Molecular identification and functional characterization of a novel glutamate transporter in yeast and plant mitochondria. *BBA-Bioenergetics* 2018;**1859**:1249–58.

Ramos M, del Arco A, Pardo B *et al*. Developmental changes in the Ca2+-regulated mitochondrial aspartate-glutamate carrier aralar1 in brain and prominent expression in the spinal cord. *Brain Res Dev Brain Res* 2003;**143**:33–46.

Rottensteiner H, Stein K, Sonnenhol E *et al*. Conserved function of pex11p and the novel pex25p and pex27p in peroxisome biogenesis. *Mol Biol Cell* 2003;**14**:4316–28.

Rottensteiner H, Theodoulou FL. The ins and outs of peroxisomes: co-ordination of membrane transport and peroxisomal metabolism. *Biochim Biophys Acta* 2006;**1763**:1527–40.

Saheki T, Kobayashi K. Mitochondrial aspartate glutamate carrier (citrin) deficiency as the cause of adult-onset type II citrullinemia (CTLN2) and idiopathic neonatal hepatitis (NICCD). *J Hum Genet* 2002;**47**:333–41.

Saheki T, Kobayashi K, Iijima M *et al*. Metabolic derangements in deficiency of citrin, a liver-type mitochondrial aspartate-glutamate carrier. *Hepatol Res* 2005;**33**:181–4.

Saheki T, Kobayashi K, Iijima M *et al*. Pathogenesis and pathophysiology of citrin (a mitochondrial aspartate glutamate carrier) deficiency. *Metab Brain Dis* 2002;**17**:335–46.

Shai N, Yifrach E, van Roermund CWT *et al*. Systematic mapping of contact sites reveals tethers and a function for the peroxisome-mitochondria contact. *Nat Commun* 2018;**9**:1761.

Sherman F, Fink GR, Lawrence CW. *Methods in Yeast Genetics*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 1978.

Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces ceresiviae*. *Genetics* 1989;**122**:19–27.

Song YZ, Ushikai M, Sheng JS *et al*. SLC25A13 gene mutation analysis in a pedigree of neonatal intrahepatic cholestasis caused by citrin deficiency. *Zhonghua Er Ke Za Zhi* 2007;**45**:408–12.

Steinmetz LM, Scharfe C, Deutschbauer AM *et al*. Systematic screen for human disease genes in yeast. *Nat Genet* 2002;**31**:400–4.

Sullivan JA, Lewis MJ, Nikko E *et al*. Multiple interactions drive adaptor-mediated recruitment of the ubiquitin ligase rsp5 to membrane proteins *in vivo* and *in vitro*. *Mol Biol Cell* 2007;**18**:2429–40.

Sundin BA, Chiu CH, Riffle M *et al*. Localization of proteins that are coordinately expressed with Cln2 during the cell cycle. *Yeast* 2004;**21**:793–800.

Tabata A, Sheng JS, Ushikai M *et al*. Identification of 13 novel mutations including a retrotransposal insertion in SLC25A13 gene and frequency of 30 mutations found in patients with citrin deficiency. *J Hum Genet* 2008;**53**:534–45.

Tam YY, Torres-Guzman JC, Vizeacoumar FJ *et al*. Pex11-related proteins in peroxisome dynamics: a role for the novel peroxin Pex27p in controlling peroxisome size and number in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2003;**14**:4089–102.

Tan-Wilson A, Kohlhaw GB. Specific, reversible inactivation of yeast beta-hydroxy-beta-methylglutraryl-CoA reductase by CoA. *Biochem Biophys Res Commun* 1978;**85**:70–6.

Tower RJ, Fagarasanu A, Aitchison JD *et al*. The peroxin Pex34p functions with the Pex11 family of peroxisomal divisional

proteins to regulate the peroxisome population in yeast. *Mol Biol Cell* 2011;**22**:1727–38.

Tracy JW, Kohlhaw GB. Reversible, coenzyme-A-mediated inactivation of biosynthetic condensing enzymes in yeast: a possible regulatory mechanism. *Proc Natl Acad Sci USA* 1975;**72**:1802–6.

Travers KJ, Patil CK, Wodicka L *et al*. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 2000;**101**:249–58.

van der Zand A, Braakman I, Tabak HF. Peroxisomal membrane proteins insert into the endoplasmic reticulum. *Mol Biol Cell* 2010;**21**:2057–65.

van der Zand A, Gent J, Braakman I *et al*. Biochemically distinct vesicles from the endoplasmic reticulum fuse to form peroxisomes. *Cell* 2012;**149**:397–409.

Volmer R, Ron D. Lipid-dependent regulation of the unfolded protein response. *Curr Opin Cell Biol* 2015;**33**:67–73.

White WH, Gunyuzlu PL, Toyn JH. *Saccharomyces cerevisiae* is capable of *de novo* pantothenic acid biosynthesis involving a novel pathway of beta-alanine production from spermine. *J Biol Chem* 2001;**276**:10794–800.

Wipe B, Leisinger T. Regulation of activity and synthesis of N-acetylglutamate synthase from *Saccharomyces cerevisiae*. *J Bacteriol* 1979;**140**:874–80.

Wongkittichote P, Sukasem C, Kikuchi A *et al*. Screening of SLC25A13 mutation in the Thai population. *World J Gastroenterol* 2013a;**19**:7735–42.

Wongkittichote P, Tungpradabkul S, Wattanasirichaigoon D *et al*. Prediction of the functional effect of novel SLC25A13 variants using a *S. cerevisiae* model of AGC2 deficiency. *J Inherit Metab Dis* 2013b;**36**:821–30.

Yamaguchi N, Kobayashi K, Yasuda T *et al*. Screening of SLC25A13 mutations in early and late onset patients with citrin deficiency and in the Japanese population: identification of two novel mutations and establishment of multiple DNA diagnosis methods for nine mutations. *Hum Mutat* 2002;**19**:122–30.

Young BP, Craven RA, Reid PJ *et al*. Sec63p and Kar2p are required for the translocation of SRP-dependent precursors into the yeast endoplasmic reticulum *in vivo*. *EMBO J* 2001;**20**:262–71.

Yuzbasheva EY, Agrimi G, Yuzbashev TV *et al*. The mitochondrial citrate carrier in *Yarrowia lipolytica*: its identification, characterization and functional significance for the production of citric acid. *Metab Eng* 2019;**54**:264–74.

Zipor G, Haim-Vilmovsky L, Gelin-Licht R *et al*. Localization of mRNAs coding for peroxisomal proteins in the yeast, *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 2009;**106**:19848–53.