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Minireview

MINIREVIEW

¡Viva la mitochondria!: harnessing yeast mitochondria for chemical production

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One sentence summary: Minireview on the state of the art of mitochondrial engineering for chemical production in yeast.

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ABSTRACT

The mitochondria, often referred to as the powerhouse of the cell, offer a unique physicochemical environment enriched with a distinct set of enzymes, metabolites and cofactors ready to be exploited for metabolic engineering. In this review, we discuss how the mitochondrion has been engineered in the traditional sense of metabolic engineering or completely bypassed for chemical production. We then describe the more recent approach of harnessing the mitochondria to compartmentalize engineered metabolic pathways, including for the production of alcohols, terpenoids, sterols, organic acids and other valuable products. We explain the different mechanisms by which mitochondrial compartmentalization benefits engineered metabolic pathways to boost chemical production. Finally, we discuss the key challenges that need to be overcome to expand the applicability of mitochondrial engineering and reach the full potential of this emerging field.

Keywords: mitochondria; metabolic engineering; mitochondrial engineering; metabolism; chemical production; biotechnology

ABBREVIATIONS

3β-HSD:	*H. sapiens* 3β-hydroxy steroid dehydrogenase
Ack:	*A. nidulans* acetate kinase
ACL:	ATP-dependent citrate lyase
ACS:	acetyl-coA synthetase
ACTGlu:	N-acetyl-L-glutamate
ACTOrn:	N-acetylornithine
ADH1:	alcohol dehydrogenase
ADH2:	glucose-repressible alcohol dehydrogenase
Adhe2:	*C. beijerinckii* alcohol dehydrogenase
ADHs:	alcohol dehydrogenases
ADS:	*A. annua* amorpha-4,11-diene synthase
ADX:	*H. sapiens* adrenodoxin
AGC1:	mitochondrial amino acid transporter
AKG:	α-ketoglutarate
ALD6:	cytosolic aldehyde dehydrogenase
ALDC:	*B. amyloliquefaciens* acetolactate decarboxylase
ALS:	*B. subtilis* acetolactate synthase
ARG Syn:	argening biosynthesis genes ARG6/5/8
ARG2:	acetylglutamate synthase
ARG7:	mitochondrial ornithine acetyltransferase
BAT1:	mitochondrial branched-chain amino acid (BCAA) aminotransferase
BAT2:	cytosolic branched-chain amino acid (BCAA) aminotransferase

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Bcd:	clostridia crotonyl-CoA reductase
BDH1:	NAD-dependent (R,R)-butanediol dehydrogenase
BDH2:	putative medium-chain alcohol dehydrogenase
CimA:	*L. interrogans* citramalate synthase
CIT2:	citrate synthase
cMAE1:	cytosolic malic enzyme
Crt:	*C. beijerinckii* crotonase
CYP11B1:	*H. sapiens* 11β-steroid hydroxylase
CYP17A1:	*H. sapiens* 17α-steroid hydroxylase
CYP21A1:	*H. sapiens* 21-steroid hydroxylase
DMAPP:	isomer dimethylallyl pyrophosphate
ECM1:	pre-ribosomal factor
ERG10:	acetyl-CoA C-acetyltransferase
ERG12:	mevalonate kinase
ERG13:	3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase
ERG19/MDVD1:	mevalonate pyrophosphate decarboxylase
ERG20:	farnesyl pyrophosphate synthetase
ERG8/PMK:	phosphomevalonate kinase
FAEE:	fatty acid ethyl esters
FDP:	farnesyl diphosphate (FDP)
FDPS:	*A. thaliana* farnesyl diphosphate synthase
FPS:	*G. gallus* farnesyl diphosphate synthase
FRA2:	transcriptional repressor of iron regulon
GES:	*O. basilicum* and *C. roseus* geraniol synthase
Glu:	glutamate
GPP:	geranyl disphosphate
HaAOX1:	*H. anomala* NADH alternative oxidase
Hbd:	*C. beijerinckii* 3-hydroxybutyryl-CoA dehydrogenase
HOM2:	aspartic beta semi-aldehyde dehydrogenase
HOM3:	aspartate kinase
HOM6:	homoserine dehydrogenase
IDI1:	IPP isomerase
ILV1:	threonine deaminase
ILV2:	acetolactate synthase
ILV3:	dihydroxyacid dehydratase
ILV5:	acetohydroxyacid reductoisomerase
ILV6:	regulatory subunit of acetolactate synthase
IPP:	isopentenyl pyrophosphate
KDCs:	α-Keto-acid decarboxylases
KGD2:	dihydrolipoyl transsuccinylase
KIC:	α-ketoisocaproic acid
LAT1:	dihydrolipoamide acetyltransferase component (E2) of the PDC
LEU1:	isopropylmalate isomerase
LEU2:	β-isopropylmalate dehydrogenase
LEU3:	zinc-knuckle transcription factor
LEU9/LEU4:	α-isopropylmalate synthase
LPD1:	lipoamide dehydrogenase component (E3) of the pyruvate dehydrogenase
LplA/LplA2:	*E. faecalis* Lipoate–protein ligase A
MAE1:	mitochondrial malic enzyme
MDH2:	cytoplasmic malate dehydrogenase
MLS1:	malate synthase
MPC:	mitochondrial pyruvate carriers
NFS1:	a mitochondrial cysteine desulfurase
OAC1:	mitochondrial inner membrane transporter of α-isopropylmalate
ODC1:	mitochondrial inner membrane transporter of α-ketoglutarate
Orn:	ornithine
PDA1:	E1 alpha subunit of the pyruvate dehydrogenase (PDH) complex
PDB1:	E1 beta subunit of the pyruvate dehydrogenase (PDH) complex
PDC1/PDC5/PDC6:	pyruvate decarboxylase isozymes
PDH:	pyruvate dehydrogenase
Pta:	*B. subtilis* phosphotransacetylase
PYC2:	pyruvate carboxylase isoform
SDH1:	succinate dehydrogenase flavoprotein subunit
SFC1:	succinate–fumarate transporter
SUCLG2:	succinyl-CoA synthetase
Ter:	*T. denticola* trans-enoyl-CoA reductase
tHMG1:	truncated HMG-CoA reductase
THR1:	homoserine kinase
THR4:	threonine synthase
TIM:	translocase of the inner membrane
TOM:	translocase of the outer membrane
TPS1:	*C. sinensis* valencene synthase
XpkA:	*A. nidulans* xylulose-5-phosphate phosphoketolase
α-KIV:	α-ketoisovalarate
α-KMV:	alpha-keto-beta-methylvalerate
α-KV:	α-Ketovalerate

INTRODUCTION

The yeast *Saccharomyces cerevisiae*, a preferred workhorse organism in biotechnology, has been engineered to produce a broad range of valuable products, such as biofuels, commodity and specialty chemicals, and pharmaceuticals (Nielsen and Keasling 2016). Metabolic engineering aims to direct metabolic flux toward desired products by overcoming different challenges, such as achieving appropriate levels and specificity of enzyme activity, obtaining adequate substrate availability, prevailing over competing endogenous pathways, keeping metabolic cofactors balanced, relieving or bypassing metabolic bottlenecks, and reducing the toxicity of intermediates and products (Keasling 2010; Angeles-Martinez and Theodoropoulos 2015; Yadav *et al.* 2017; Kuroda *et al.* 2019). Despite these challenges, researchers have been very successful at engineering and optimizing metabolic pathways traditionally in the yeast cytosol, effectively treating cells as 'bags of enzymes'. However, like all eukaryotic cells, yeast have a complex subcellular architecture comprised of many different compartments, which creates not only additional challenges but also potential opportunities for yeast metabolic engineering.

Surrounded by a cell wall and membrane, the yeast cytosol is the site where many biochemical reactions in the cell occur. Within the cytosol, there are various membrane-enclosed organelles that have evolved functionally specialized compartments (Alberts *et al.* 2002). Due to this high complexity in cellular organization, proteins and metabolites must be transported across multiple membranes for proper organellar and cellular function. This cellular architecture can present obstacles to yeast metabolic engineering when endogenous resources needed for product biosynthesis, such as enzymes, metabolites or cofactors, are isolated in different subcellular compartments. This contrasts with bacterial systems, in which spatial segregation of metabolic resources is not usually considered due to the lack of membrane-bound organelles.

Motivated by the success of engineered bacterial strains and perceiving that subcellular compartmentalization is largely a disadvantage, yeast metabolic engineers typically avoid organelles and favor localization of engineered pathways in the cytosol when developing yeast strains for chemical production (Krivoruchko and Nielsen 2015; Lian, Mishra and Zhao 2018). This is true for heterologous pathways, in which enzymes from other organisms are almost always by default expressed in the yeast cytosol, as well as for native pathways, in which organelle localization signals are often removed from enzymes to favor their cytosolic localization (Shiba et al. 2007; Xu, Liu and Chen 2012; Wess, Brinek and Boles 2019). In many cases, this strategy can be successful at rewiring metabolic pathways to be, not only completely functional in the cytosol but also capable of being optimized to achieve high fluxes toward desired products. However, challenges may arise when stable or active enzymes cannot be expressed in the cytosol, when there is limited availability of substrates or cofactors, when cytosolic side reactions lead to lower metabolic flux or toxic by-products, or when the cell secretes the intermediate metabolites (Borodina and Nielsen 2014; Hammer and Avalos 2017a). In many cases, these obstacles may be mitigated or even resolved by harnessing yeast organelles for metabolic pathway reconstruction.

The yeast cell contains a variety of organelles, each with a unique environment optimal for a particular set of biochemical reactions. This includes the peroxisome, with its high protein concentration and very active fatty acid metabolism; the endoplasmic reticulum, which, in addition to its oxidative environment, offers an extensive intracellular membrane network for protein anchoring and, along with the Golgi apparatus, serves as the conduit for protein secretion and glycosylation; the vacuole, which has a relatively low pH and the ability to store ions, metabolites, proteins and, especially under starvation conditions, carries out the degradation of proteins and even entire organelles; and the mitochondria, which has a relatively high pH, a reducing environment, and is known as the powerhouse of the cell due to its role in ATP generation (Hammer and Avalos 2017a). Metabolic pathways for chemical production have been engineered in each of these organelles, demonstrating important advantages over their cytosolic counterparts by exploiting the increased availability and/or activity of enzymes, precursors or cofactors found in these compartments (Hammer and Avalos 2017a). Compartmentalization can also reduce by-product formation and loss of secreted intermediates, by physically separating metabolites from competing pathways or cellular export mechanisms (Farhi et al. 2011; Lv et al. 2016; Yee et al. 2019). Furthermore, assembling complete pathways in a single organelle can improve pathway efficiency by eliminating the need to transport intermediates across multiple membranes (Avalos, Fink and Stephanopoulos 2013; Hammer and Avalos 2017b). The realization of these advantages in recent years launched a new field of yeast subcellular metabolic engineering, which we recently reviewed (Hammer and Avalos 2017a).

The mitochondrion is one of the most extensively studied organelles in cellular physiology, as well as one of the most heavily used for compartmentalization of engineered biosynthetic pathways. The yeast mitochondria have been harnessed to produce a variety of chemicals, including alcohols, terpenoids, sterols, organic acids and other valuable products. This review focuses on the specific advantages of targeting metabolic pathways to the yeast mitochondria. We describe the strategies that have been employed to take advantage of mitochondrial metabolism and physiology for metabolic engineering. We analyze the different mechanisms by which mitochondrial compartmentalization has helped to improve the biosynthesis of desired products. Finally, we highlight the current challenges of mitochondrial compartmentalization and discuss the potential for future innovations.

THE MITOCHONDRIAL ENVIRONMENT

The evolution of cellular compartmentalization has allowed the cell to develop functionally specialized aqueous compartments separate from the cytosol (Alberts et al. 2002). Enclosed by two lipid bilayers, the mitochondrion offers two different environments, each with particular physiological properties. The outer membrane contains large porins that allow the free exchange of small molecules (<5 kDa) with the cytosol, which makes the intermembrane space (IMS) chemically similar, but not equivalent, to the cytosol (Alberts et al. 2002; Malina, Larsson and Nielsen 2018) (Fig. 1). For example, the IMS has been reported to offer an environment that is similar in pH, but more oxidizing than that of the cytosol (Fig. 1) (Orij et al. 2009). In contrast, the inner membrane is more selective to small molecule permeation, which makes the chemical composition of the mitochondrial matrix distinct from all other cellular compartments. The matrix environment is more basic and has a higher reduction potential than both the IMS and the cytosol (Hu, Dong and Outten 2008; Orij et al. 2009). Thus, the unique physicochemical environment of mitochondria could be advantageous for the activity of some enzymes or metabolic reactions, in which case, targeting biosynthetic pathways to this organelle would be beneficial.

Mitochondria also offer a unique repertoire of enzymes and metabolites that can be exploited for metabolic engineering. They contain a rich proteome of ~1000 different proteins (Vögtle et al. 2017), which are differentially expressed at different growth stages and distributed across their sub-compartments (Vögtle et al. 2017; Di Bartolomeo et al. 2020). The matrix is a more enzymatically diverse sub-compartment than the IMS, containing up to 400% more proteins (Vögtle et al. 2017). In addition, compared with the cytosol, the yeast mitochondrion has a higher concentration of key precursor metabolites and cofactors that are essential to many biosynthetic pathways including acetyl-CoA (Galdieri et al. 2014), pyruvate (while respiring) (Park, Kim and Hahn 2016), iron-sulfur clusters (Schilke et al. 1999), NADH, NAD^+, FAD and α-KIV (Fig. 1) (Li, Liu and Chen 2015). A recent study in yeast reported that out of 72 measurable metabolites, 8 were exclusively detected in mitochondria, some of which are interesting for metabolic engineering applications (Pan et al. 2018), such as squalene and sterols. Furthermore, there are important metabolites and cofactors that are constantly being exchanged between the mitochondria and the cytosol (Fig. 1 and Table 1). Thus, the natural pool of key cofactors and metabolites in the mitochondria can inform scientists of which biosynthetic pathways may benefit from mitochondrial localization.

Mitochondrial acetyl-CoA

Acetyl-CoA is a key precursor in many metabolic pathways, including some utilized to produce pharmaceuticals, dietary supplements, vitamins, bioplastics and biofuels (Luengo et al. 2003; Kozak et al. 2014; Krivoruchko et al. 2015). While acetyl-CoA is present in multiple compartments at different levels, it is estimated that acetyl-CoA concentrations are as much as 20 to 30 times higher in the mitochondria than in the nucleocytosolic space in metabolically arrested fermenting cells (Weinert et al. 2014). In the mitochondria, acetyl-CoA is synthesized from

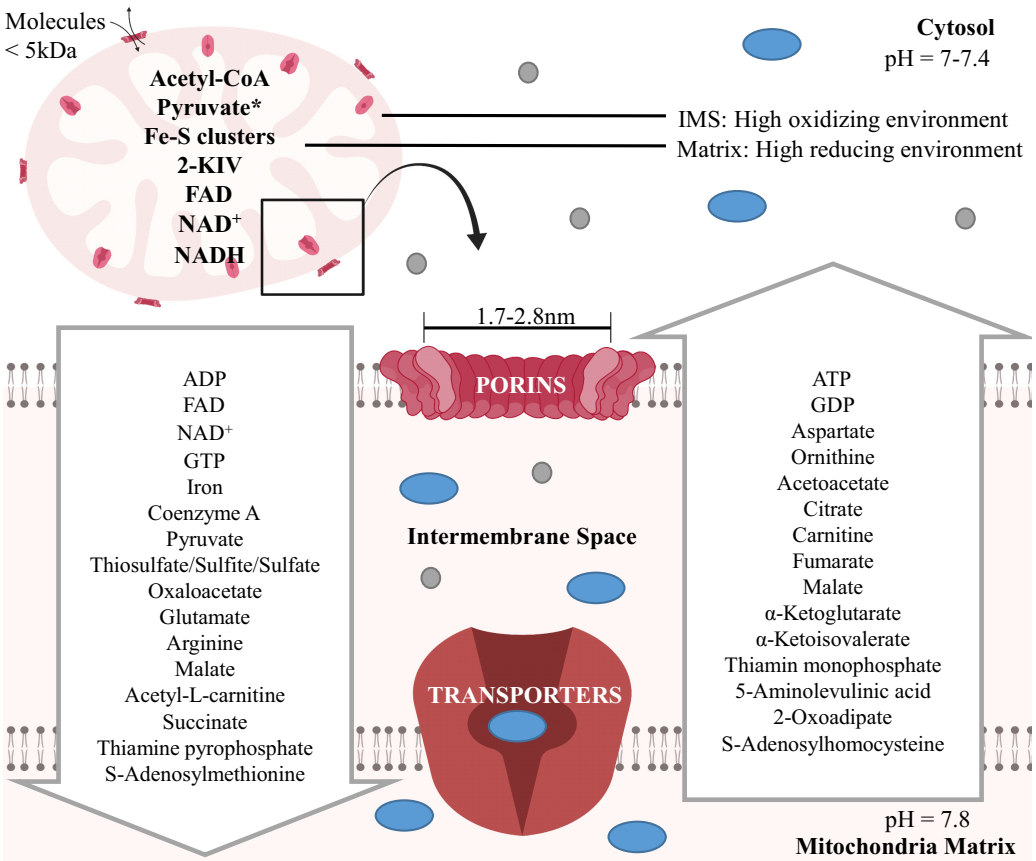

Figure 1. Mitochondrial environment. The mitochondria have been reported to have higher levels of acetyl-CoA, pyruvate, 2-KIV, FAD, NAD^+ and NADH than the cytosol. However, these observations may change depending on the conditions. For example, pyruvate is only found in higher concentration in the mitochondria during respiring conditions. In addition, various important metabolites and cofactors are exchanged between the mitochondria and cytosol, shown inside the large arrows (see Table 1 for references). Partially adapted from Herrmann and Riemer (2010). Partially made using BioRender.

Table 1. Studies reporting directional transport of key metabolites in yeast mitochondria (see Fig. 1).

Metabolite imported		Metabolite exported	
FAD/ADP/NAD^+	(Tzagoloff *et al.* 1996; Bamber *et al.* 2006)	*ATP/GDP*	(Bamber *et al.* 2007)
GTP	(Vozza *et al.* 2004)	*Aspartate*	(Cavero *et al.* 2003)
Coenzyme A	(Prohl *et al.* 2001)	*Ornithine*	(Palmieri *et al.* 1997b)
Pyruvate	(Hildyard and Halestrap 2003)	*Acetoacetate*	(Nałęcz *et al.* 1991)
Iron	(Lange, Kispal and Lill 1999)	*Citrate*	(Kaplan *et al.* 1995)
Thiosulfate/Sulfite/sulfate	(Palmieri *et al.* 1999b)	*Carnitine*	(Palmieri *et al.* 1999a)
Oxaloacetate	(Palmieri *et al.* 1999b)	*Fumarate*	(Palmieri *et al.* 1997a)
Glutamate	(Cavero *et al.* 2003)	*Malate*	(Palmieri *et al.* 2006)
Arginine	(Soetens *et al.* 1998)	*α-Ketoglutarate*	(Palmieri *et al.* 2001)
Malate	(Palmieri *et al.* 2006)	*α-Ketoisovalerate*	(Yuan *et al.* 2017)
Acetyl-L-carnitine	(Palmieri *et al.* 1999a)	*Thiamin monophosphate*	(Marobbio *et al.* 2002)
Succinate	(Palmieri *et al.* 1997a)	*5-Aminolevulinic acid*	(Hoffman, Góra and Rytka 2003)
Thiamine pyrophosphate	(Marobbio *et al.* 2002)	*2-Oxoadipate*	(Palmieri *et al.* 2001)
S-adenosylmethionine	(Marobbio *et al.* 2003)	*S-adenosylhomocysteine*	(Marobbio *et al.* 2003)
		Iron-sulfur precursors	(Kispal *et al.* 1999)

pyruvate by the pyruvate dehydrogenase (PDH) complex, while in the nucleus and cytosol it is produced from acetate by acetyl-CoA synthetases (ACSs) (Shiba *et al*. 2007; Krivoruchko *et al*. 2015; van Rossum *et al*. 2016). However, cytosolic acetyl-CoA production is limited by the activity and regulation of the ACS enzymes (Shiba *et al*. 2007), and competition with ethanol fermentation. Therefore, the higher enzyme activity of the PDH complex, in conjunction with the smaller volume of the mitochondria, leads to the greater availability of acetyl-CoA within the mitochondria (Shiba *et al*. 2007; Weinert *et al*. 2014). Furthermore, in oleaginous yeasts, such as *Yarrowia lipolytica*, mitochondria contribute additional acetyl-CoA to the cytosolic pool through the production of citrate, which gets exported from mitochondria and converted to acetyl-CoA by ATP-dependent citrate lyase (ACL) (Krivoruchko *et al*. 2015). Acetyl-CoA metabolism and synthesis have been extensively described in the context of yeast metabolic engineering in two excellent reviews (Krivoruchko *et al*. 2015; van Rossum *et al*. 2016).

Iron-sulfur clusters

Biogenesis of iron-sulfur (Fe-S) clusters occurs predominantly within the mitochondria in yeast (Schilke *et al*. 1999) and at lower capacity in the cytosol (Sharma *et al*. 2010). These clusters are cofactors that are required for proper *in vivo* activity and assembly of some enzymes. This is true for native enzymes that have evolved for optimized efficiency in the mitochondria and for heterologous enzymes for which the mitochondria provide the necessary ingredients for activity (Milne *et al*. 2016). Furthermore, the mitochondria are also essential for the proper assembly of both cytosolic and nuclear Fe-S cluster dependent proteins (Malina, Larsson and Nielsen 2018). Mitochondrial Fe-S cluster biogenesis has been demonstrated to be intimately tied to iron regulation, with yeast responding to defects in mitochondrial Fe-S biogenesis by upregulation of the iron regulon and mitochondrial iron uptake (Sharma *et al*. 2010). The modification of iron homeostasis for increased activity of Fe-S cluster dependent enzymes is discussed below. There are >200 different classes of enzymes that depend on Fe-S clusters (Bandyopadhyay, Chandramouli and Johnson 2008) and have potentially much to gain by being targeted to mitochondria for improved activity.

TRADITIONAL MITOCHONDRIAL PATHWAY ENGINEERING

Biosynthetic pathways that natively involve mitochondrial metabolism are of immense importance to the field of metabolic engineering. Valuable products have been derived from mitochondrial pathways, such as for amino acid, organic acid and alcohol biosynthesis. In this review, we use the term 'traditional mitochondrial engineering' to refer to approaches that follow two major traditional strategies to engineer native mitochondrial pathways: (i) upregulation of mitochondrial metabolic pathways, and (ii) mitochondrial pathway decompartmentalization. Thus, our definition of traditional mitochondrial engineering excludes more recent efforts to compartmentalize non-mitochondrial pathways in this organelle. In this section, we review these two approaches commonly used to increase end-product titers from native mitochondrial pathways and discuss the advantages and drawbacks of these techniques. Because extensive work in this space has been devoted for the production of higher alcohols, our discussion will concentrate on these products, while highlighting how these strategies have been also applied for the biosynthesis of other chemicals.

Upregulation of mitochondrial metabolic pathways

Important biosynthetic pathways that natively localize (partially or completely) to the yeast mitochondria have been engineered for chemical production while keeping the metabolic enzymes in their native localization. This approach may involve the manipulation of central carbon metabolism, overexpression or deregulation of metabolic enzymes involved in the biosynthetic pathways, protein engineering, deletion or suppression of competing pathways and elimination of cofactor imbalance. A main advantage of this strategy is having all enzymes expressed in their native location, which increases the chance that they will have optimal activity.

Upregulation of biosynthetic pathways for chemical production is a hallmark of metabolic engineering, including for native mitochondrial pathways. This is typically achieved by overexpressing genes encoding key enzymes in the biosynthetic pathways of interest, using constitutive or inducible promoters, and increasing the copy number of such genes by means of single or multiple genomic integrations, or using low (CEN-ARS) or high copy (2μ) episomal plasmids. Because this approach keeps each enzyme in the pathway in their native compartment, there is no procedural difference with conventional metabolic engineering, as mitochondrial enzymes are simply cloned with their natural mitochondrial localization signals. Following this strategy, mitochondrial enzymes have been overexpressed for the production of several chemicals, including those involved in branched-chain alcohols (isobutanol, 3-methyl-1-butanol and 2-methyl-1-butanol) (Avalos, Fink and Stephanopoulos 2013; Matsuda *et al*. 2013; Park, Kim and Hahn 2014; Hammer and Avalos 2017b; Yuan *et al*. 2017), *n*-butanol (Si *et al*. 2014; Shi *et al*. 2016), fumarate (Chen *et al*. 2015; Chen, Zhu and Liu 2016) and L-ornithine (Qin *et al*. 2015; Fig. 2 and Table 3). In the traditional approach, mitochondrial reactions are often preceded and/or followed by metabolic steps catalyzed by enzymes in the cytosol (whether overexpressed or not) because that is the compartment where they naturally occur.

Central carbon metabolism can pose a major challenge for yeast mitochondrial engineering (as for any type of metabolic engineering), as it often directs metabolic flux away from the product of interest and into competing pathways. This is particularly problematic when trying to make products from glucose or other fermentable carbon sources, as yeast has evolved to repress respiration in the presence of glucose concentrations as low as 0.1 g/L in favor of adopting a fermentative metabolism (Yin *et al*. 2003). This phenomenon, known as the Crabtree effect, results in the diversion of most of the metabolic flux toward ethanol production. Thus, any biosynthetic pathway starting from glucose, whether cytosolic or compartmentalized in an organelle, is challenged by the significant production of ethanol as a by-product. Pathways involving mitochondrial metabolism are particularly susceptible as the Crabtree effect also suppresses general mitochondrial activity (Heyland, Fu and Blank 2009).

Nevertheless, chemical production from metabolic pathways involving mitochondria can be improved by suppressing ethanol production. Central carbon metabolism diverts flux to ethanol biosynthesis through decarboxylation of pyruvate to acetaldehyde by pyruvate decarboxylases (PDCs), encoded by *PDC1*, *PDC5* and *PDC6*, but mostly by Pdc1p (Hohmann and Cederberg 1990;

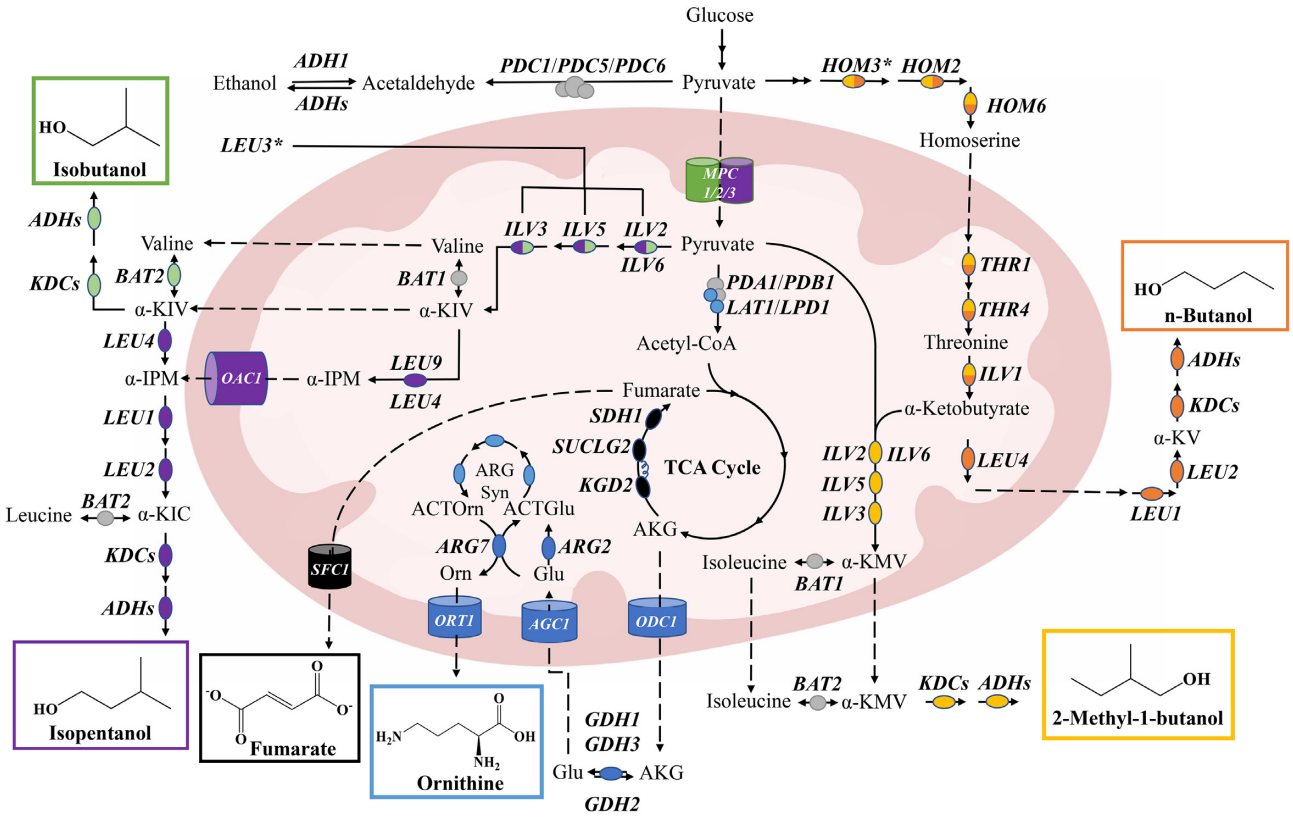

Figure 2. Traditional engineering of biosynthethic pathways that partially localize to the mitochondria. Metabolic pathways involving mitochondria that have been engineered through traditional metabolic engineering: isobutanol (green), isopentanol (purple), fumarate (black), ornithine (blue), 2-methyl-1-butanol (yellow) and *n*-butanol (orange). The L-ornithine pathway described above also includes modifications to the urea cycle that are not shown for clarity. Transporters that have been overexpressed to increase product formation are identified. Gray indicates genes whose expression was not modified. Dashed arrows represent transport across the mitochondrial membranes. Mutants are shown with asterisk (*). Curly line connecting *KGD2* and *SUCLG2* represents a protein fusion.

Kondo *et al.* 2012). Acetaldehyde is then reduced to ethanol by several alcohol dehydrogenases (ADHs), but mainly by the one encoded by *ADH1* (Flikweert *et al.* 1999). Pathways that compete with ethanol biosynthesis for pyruvate or acetaldehyde, have been enhanced by deleting one or more PDCs or ADHs, respectively. Strains with the *PDC1* and *PDC5* double deletion are unable to grow in glucose concentrations that trigger the Crabtree effect, or without a C2 carbon source (usually ethanol or acetate) because the loss of acetaldehyde production by these mutations prevents them from making acetyl-CoA in the cytosol and nucleus (Flikweert *et al.* 1999). Therefore, it is a significant challenge to work with strains lacking pyruvate decarboxylase activity.

While reducing ethanol by-product formation in glucose fermentations can result in major increases in yields of engineered mitochondrial pathways, deleting other relatively minor competing pathways can also lead to significant improvements. For example, reducing formation of the following by-products by deleting specific genes (shown in parentheses) increases isobutanol production: isobutyrate (*ALD6*), leucine (*LEU1/LEU4/LEU9*), isoleucine (*ILV1*), valine (*BAT1*), glycerol (*GPD1/2*) and acetyl-CoA (*PDA1/PDB1/LAT1/LPD1*) (Park, Kim and Hahn 2014, 2016; Ida *et al.* 2015; Lee *et al.* 2018; Wess, Brinek and Boles 2019). Reduced isoleucine production by deleting *ILV6/ILV2* also enhances *n*-butanol synthesis (Si *et al.* 2014). Similarly, by-products derived from the tricarboxylic acid (TCA) cycle have been reduced by deleting *KGD2*, enhancing L-ornithine production (Qin *et al.* 2015). Deleting genes that consume the product of interest is also effective; for example, deletion of *ADH1* decreases *n*-butanol consumption, improving titers (Si *et al.* 2014). The mitochondrion itself provides an extra layer of protection by separating valuable metabolites from competing pathways in the cytosol.

Traditionally, engineered mitochondrial pathways can also be enhanced by manipulating their regulatory mechanisms. Metabolic flux through the traditionally engineered isobutanol pathway can be increased by eliminating the regulation of branched-chain amino acid (BCAA) biosynthesis by valine, which occurs in mitochondria. This can be best observed in *bat1*Δ strains fermenting in media without valine or by expressing a valine-insensitive *ILV6* mutant instead of the wild type (Hammer and Avalos 2017b). Similarly, a threonine-insensitive *HOM3* mutant has been used to enhance the production of *n*-butanol (Shi *et al.* 2016). A different strategy to deregulate biosynthetic pathways is to express variants of their transcriptional regulators containing mutations that cause them to lose sensitivity to feedback inhibition. Such is the case of the α-isopropylmalate-insensitive *LEU3*, which induces expression of genes involved in BCAA biosynthesis in response to low levels of this leucine precursor (Park, Kim and Hahn 2014; Shi *et al.* 2016). Thus, as in traditional metabolic engineering, understanding the natural regulatory mechanisms of mitochondrial biosynthetic pathways offers effective strategies to enhance chemical production.

The efficiency of metabolic reactions can often be limited by the availability of required cofactors. In yeast, isobutanol synthesis may cause an NADPH shortage that limits production (Matsuda *et al.* 2013). *Saccharomyces cerevisiae* does not encode a nucleotide transhydrogenase enzyme that catalyzes NADPH formation from NADH and $NADP^+$, and successful heterologous expression of bacterial enzymes has not replenished the NADPH pool (Matsuda *et al.* 2013). Therefore, redox cofactors have been balanced for isobutanol production by overexpressing pyruvate carboxylase (*PYC2*), malate dehydrogenase (*MDH2*) and malic enzyme (*MAE1*) in the cytosol (in a decompartmentalized pathway) or in its native mitochondrial location to produce NADPH (Fig. 3) (Matsuda *et al.* 2013; Ida *et al.* 2015). Regeneration of NADPH in the mitochondria or cytosol in an *lpd1*Δ background, with the mitochondrial and cytosolic BCAA biosynthetic pathway upregulated, results in a strain that produces 221 ± 27 mg/L of isobutanol, a near 5-fold increase in titers in comparison to sole upregulation of BCAA biosynthesis (Matsuda *et al.* 2013). In a separate study, upregulation of *H. anomala* NADH alternative oxidase (*HaAOX1*) and endogenous NADH dehydrogenase (*NDI1*) in yeast mitochondria was shown to alleviate the cofactor imbalance in L-ornithine production, leading to a 37.7% increase in titers (Qin *et al.* 2015). Heterologous expression of *HaAOX1* also upregulates all steps of the TCA cycle, which offers a possible target for valuable products that branch from the TCA cycle (Fig. 3). Thus, improving the redox cofactor balance of engineered metabolic pathways, whether they are mitochondrial or decompartmentalized, benefits production.

Increased flux through mitochondrial pathways can result in bottlenecks in the transport of metabolites in and out of the organelle. A strategy to overcome this challenge is to overexpress mitochondrial carriers. For example, the mitochondrial pathway for arginine biosynthesis has been engineered to produce L-ornithine. Overexpression of *ORT1*, a mitochondrial L-ornithine exporter, resulted in a 44% increased production of L-ornithine in comparison to only overexpression of the arginine biosynthetic genes (*ARG2/5/6/7/8*) (Fig. 2). L-ornithine titers were further improved by 30% through additional overexpression of *AGC1*, a mitochondrial importer of glutamate required for arginine biosynthesis (Fig. 2) (Qin *et al.* 2015). Overexpression of *ODC1*, an α-ketoglutarate mitochondrial exporter, did not lead to any further improvements in titers (Qin *et al.* 2015). In the biosynthesis of branched-chain higher alcohols (BCHAs), overexpressing the α-isopropylmalate (α-IPM) transporter *OAC1* increases isopentanol production and reduces isobutanol by-product formation (Yuan *et al.* 2017). Similarly, the pyruvate mitochondrial carriers (*MPC1*, *MPC2* and *MPC3*) have been overexpressed to increase the import of pyruvate, the starting metabolite of BCHA biosynthesis, to increase isobutanol and isopentanol production (Fig. 2) (Park, Kim and Hahn 2016). While this strategy can be very effective, it is challenged by the incomplete picture we currently have of mitochondrial carriers, in terms of their existence, number and molecular identity.

Mitochondrial decompartmentalization

While traditional metabolic engineering is effective at developing strains to produce chemicals derived from mitochondrial metabolic pathways, this approach often results in bottlenecks created by the need to transport metabolites in and out of mitochondria. An alternative strategy to prevent these bottlenecks is to re-localize enzymatic steps from mitochondria to the cytosol (Krivoruchko *et al.* 2013; Blazeck *et al.* 2015). This process of 'mitochondrial decompartmentalization' can be achieved by overexpressing the endogenous genes encoding mitochondrial enzymes with their mitochondrial localization signal (MLS) removed, or by introducing heterologous genes that naturally lack MLS, such that these enzymes are targeted to the cytosol.

Acetyl-CoA is a precursor to many products of interest. Because the levels of this metabolite in the cytosol are low compared with mitochondria, there have been many efforts to increase cytosolic pools of acetyl-CoA, which are thoroughly examined in an excellent review (van Rossum *et al.* 2016). A common strategy to achieve this is to overexpress ACS in the cytosol. This approach is sometimes called the 'PDH bypass' for its ability to circumvent the activity of mitochondrial PDH, which normally produces most of the acetyl-CoA in the cell. The PDH bypass was first employed for the synthesis of amorphadiene (Fig. 4), and entails overexpressing the endogenous *ALD6* gene encoding an aldehyde dehydrogenase that oxidizes acetaldehyde to acetate, and an acetyl-CoA synthetase variant from *Salmonella enterica* (*SeACS*) that is insensitive to feedback inhibition (Shiba *et al.* 2007). A similar approach has been utilized to produce α-santalene and PHB (Fig. 4) (Kocharin *et al.* 2012; Chen *et al.* 2013). Production of α-santalene was further improved by the deletion of *MLS1* and *CIT2*, encoding peroxisomal enzymes that utilize acetyl-CoA in the glyoxylate cycle (Chen *et al.* 2013). Flux into the PDH bypass can also be improved by heterologous overexpression of the phosphoketolase (PK) pathway (Fig. 4), which utilizes xylulose-5-phosphate phosphoketolase (*xpk*A) and acetate kinase (*ack*) from *A. nidulans* to convert the carbon source to acetate. Simultaneous overexpression of the PK pathway and *SeACS* gene increases production of PHB and FAEE (Kocharin *et al.* 2012; Kocharin, Siewers and Nielsen 2013; de Jong *et al.* 2014). Alternatively, expression of phosphotransacetylase (*pta*) from *B. subtilis* to bypass ACS and synthesize acetyl-CoA directly from PK intermediate acetyl-phosphate (acetyl-P) has been used for FAEE synthesis (Fig. 4) (de Jong *et al.* 2014). However, flux through these PK engineered pathways during growth in glucose appears to be low (Kocharin, Siewers and Nielsen 2013; de Jong *et al.* 2014; van Rossum *et al.* 2016).

Another strategy to increase cytosolic acetyl-CoA is to introduce heterologous PDH complexes from bacterial species, which lack MLS and are thus retained in the cytosol. This has been done by simultaneously overexpressing the E1α, E1β, E2 and E3 subunits that make the PDH complex from *E. faecalis* (*Ef*PDH), which was shown to be active by rescuing the growth defect phenotype caused by deleting both endogenous ACS genes (*ACS1* and *ACS2*) (Fig. 4) (Kozak *et al.* 2014). However, the activity of *Ef*PDH required the heterologous expression of genes involved in protein lipoylation (*lpla*, *lpla2*) or external supply of lipoic acid, which is an important cofactor for PDH. Reconstruction of an *Escherichia coli* PDH complex in the yeast cytosol has also been reported for the production of *n*-butanol (Lian *et al.* 2014; Lian and Zhao 2016).

A different strategy to increase cytosolic acetyl-CoA still relies on mitochondrial metabolism. Oleaginous yeasts accumulate high levels of cytosolic acetyl-CoA-derived triacylglycerides through the activity of a native ACL, which produces acetyl-CoA in the cytosol from citrate exported from mitochondria (Boulton and Ratledge 1981). Because non-oleaginous yeasts lack this key enzyme, several ACLs from different species have been tested in *S. cerevisiae*, revealing that those from *A. nidulans (An)* and *Y. lipolytica (Yl)* can increase cytosolic acetyl-CoA to boost the production of mevalonate and *n*-butanol, respectively (Fig. 4) (Lian *et al.* 2014; Rodriguez *et al.* 2016). Although *YlACL* was shown to increase *n*-butanol synthesis by 2-fold in one study (Lian *et al.*

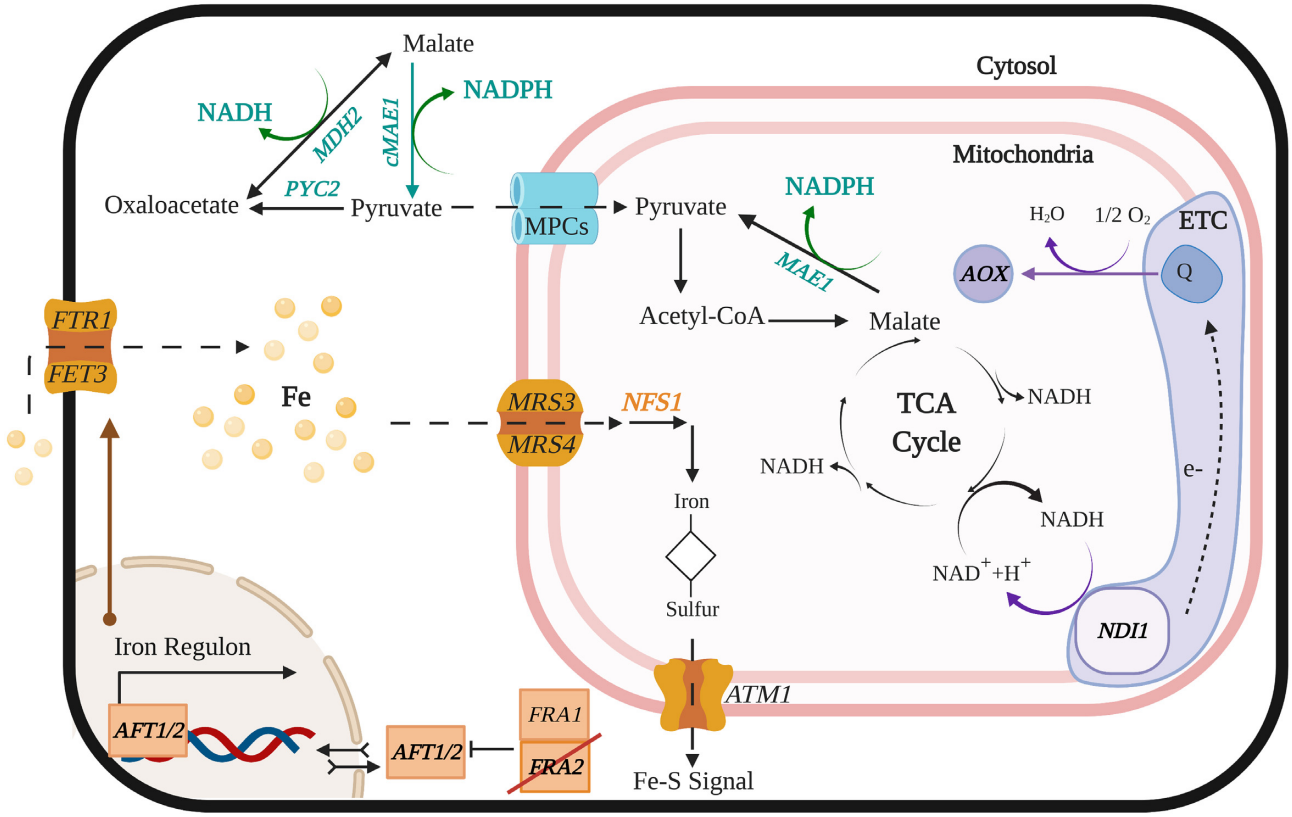

Figure 3. Engineering the mitochondrial environment. Deletion of *FRA2* and overexpression of *NFS1* or *AFT1/2* increase iron-sulfur cluster biogenesis. The Fe-S signal is triggered by iron-sulfur cluster precursors that are exported from the mitochondria. Overexpression of mitochondrial *MAE1* and cytosolic *cMAE1*, *PYC* and *MDH2* mitigates NADPH cofactor imbalance. Mitochondrial pyruvate carriers (*MPC1*, *MPC2* and *MPC3*) are shown in cyan (MPCs) and the electron transport chain (ETC) in purple. Overexpression of *NDI1* and *AOX*, which are components of the ETC, results in the upregulation of the TCA cycle and increased NADH formation. Made using BioRender.

2014), it did not help improve medium-chain fatty alcohol production in another (Sheng, Stevens and Feng 2016). The activity of *AnACL* has been reported to be approximately an order of magnitude higher than that of *YlACL* and other ACLs (Rodriguez *et al.* 2016). Furthermore, the efficiency of *AnACL* expressed in yeast is highlighted by its ability to decrease cellular citrate levels by 46% in a strain that accumulates citrate due to deletion of mitochondria isocitrate dehydrogenase (*IDH1*) (Rodriguez *et al.* 2016). This finding demonstrates that even in decompartmentalization approaches, some level of mitochondrial engineering can yield beneficial results.

Biosynthetic pathways for the production of higher alcohols, including *n*-butanol and isobutanol, have been reconstructed in the yeast cytosol (Fig. 4) (Generoso *et al.* 2015). The key to obtaining a cytosolic pathway for *n*-butanol production was to overexpress the PDH bypass (*ALD6* and the feedback-insensitive *SeACS*) to increase cytosolic pools of acetyl-CoA, as well as the heterologous genes *hbd*, *crt*, *ter/bcd* and *adhe2* from the ABE (acetone-butanol-ethanol) pathway of *C. beijerinckii and T. denticola* (Fig. 4) to convert acetyl-CoA to *n*-butanol (Krivoruchko *et al.* 2013; Swidah *et al.* 2018) reaching as much as 16.3 mg/L (Krivoruchko *et al.* 2013). In a separate study, cytosolic production of *n*-butanol was further improved to 120 mg/L by combining the overexpression of *SeACS*, *YlACL* and cytosolic PDH from *E. coli* in a strain with gene deletions in *ADH1*, *ADH4*, *GPD1* and *GPD2* to reduce/eliminate ethanol and glycerol by-product formation (Fig. 4) (Lian *et al.* 2014).

Isobutanol biosynthesis has also been reconstructed in the yeast cytosol (Brat *et al.* 2012; Wess, Brinek and Boles 2019). To achieve this, the MLS peptides of *ILV2*, *ILV5* and *ILV3* were removed or shortened to decompartmentalize the BCAA biosynthetic pathway to the cytosol. Additional overexpression of the α-ketoacid decarboxylase (*ARO10*) and alcohol dehydrogenase (*ADH2*) results in a strain that could produce 630 mg/L of isobutanol (Brat *et al.* 2012). Paradoxically, achieving these titers required the deletion of the endogenous copy of *ILV2* and exclusion of valine from the medium, which exerts its inhibitory effect through Ilv6p in mitochondria (Brat *et al.* 2012). However, these requirements were not reported in some subsequent similar strains (Matsuda *et al.* 2013). Isobutanol titers were further improved to 2.08 g/L by deleting several genes from competing pathways, including enzymes for the biosynthesis of 2,3-butanediol (*BDH1/BDH2*), leucine (*LUE4/LEU9*), pantothenate (*ECM31*) and isoleucine (*ILV1*), in a strain overexpressing the cytosolic isobutanol pathway, which represents a 200-fold increase in titers compared with wild type (Wess, Brinek and Boles 2019). However, given the paradoxical results mentioned above, and by the authors' own conclusions, an important mitochondrial contribution to isobutanol biosynthesis in these and other similar strains cannot be ruled out (Wess, Brinek and Boles 2019). Nevertheless, this approach offers important advantages, including the elimination of bottlenecks caused by intracellular metabolite transport across the mitochondrial membrane, and the utilization of high metabolic fluxes in the cytosol. It can also

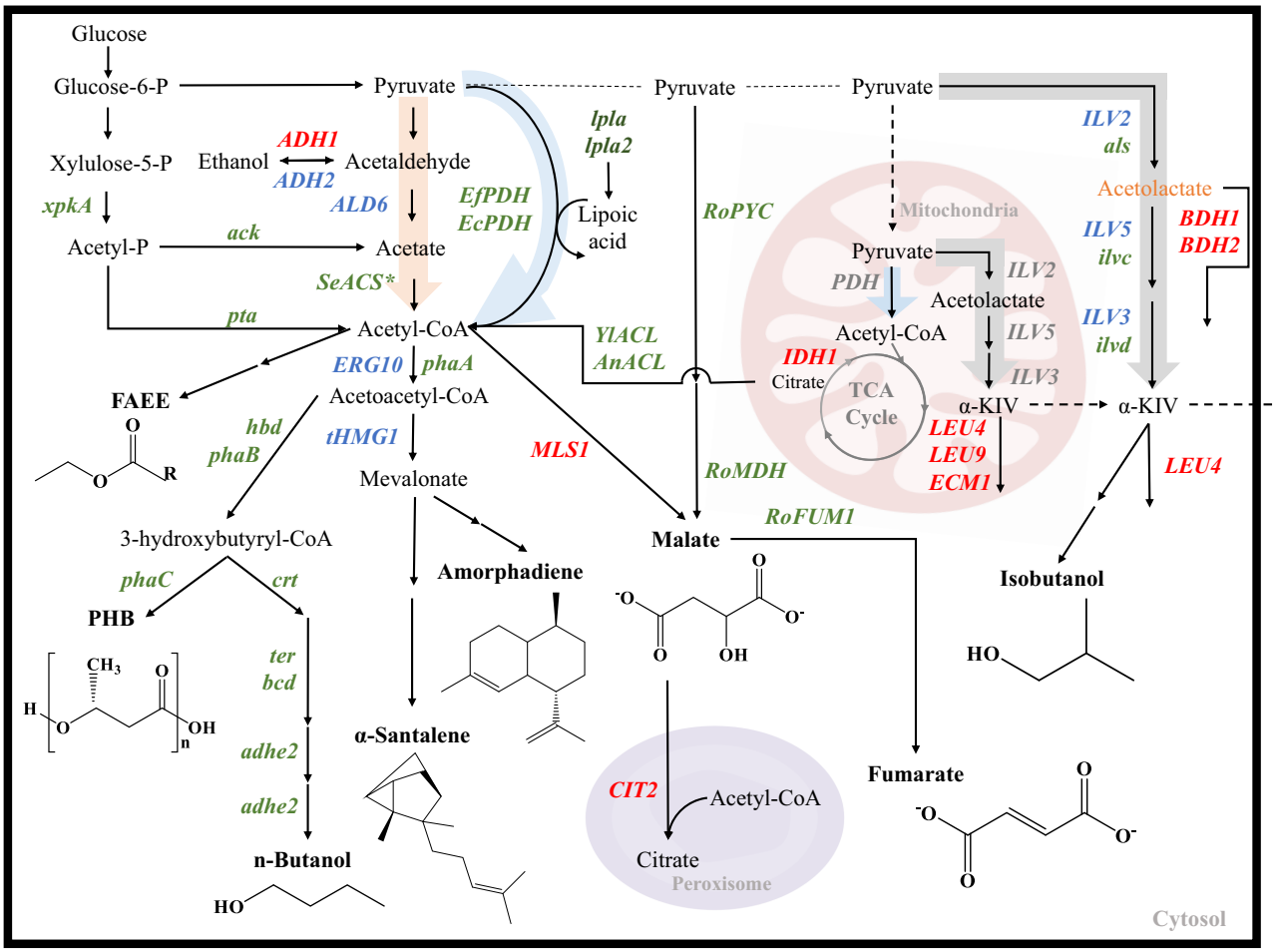

Figure 4. Decompartmentalization of mitochondrial pathways to the cytosol. Metabolic pathways that involved re-localizing one or more mitochondrial enzymatic steps to the yeast cytosol, including for the production of fatty acid ethyl esters (FAEE), *n*-butanol, isobutanol, α-santalene, polyhydroxybutyrate (PHB) and amorphadiene. Thick colored arrows denote pathways with equivalent enzymatic steps in mitochondria and cytosol: conversion of pyruvate to acetyl-CoA (blue), upstream BCAA biosynthetic pathway (gray) and PDH bypass (orange). Overexpressed endogenous genes are shown in blue, heterologous genes are shown in green, genes whose mitochondrial enzymatic activities have been decompartmentalized to the cytosol are shown in gray and genes deleted to enhance product formation are shown in red. Repetitive arrows represent multiple catalytic steps. Partially made using BioRender.

present new challenges, such as the production of toxic metabolites in the cytosol by the decompartmentalized acetolactate synthase (*ILV2*) (Park and Hahn 2019), and loss of the ketol-acid intermediate to the extracellular environment (Milne *et al.* 2016).

MITOCHONDRIAL COMPARTMENTALIZATION OF METABOLIC PATHWAYS

The opposite approach to pathway decompartmentalization is targeting the enzymes of a pathway to the mitochondrial matrix. This is achieved by fusing to enzymes N-terminal MLSs, which typically get cleaved off upon entry into the mitochondrial matrix, thus minimizing the possibility of interference with enzymatic activity (Table 2) (Hurt *et al.* 1985; Avalos, Fink and Stephanopoulos 2013; Burkhart *et al.* 2015). There are two general mechanisms by which targeting metabolic pathways to mitochondria may be advantageous: the biochemical environment and the physical separation from the cytosol. The highly selective permeability of the inner mitochondrial membrane maintains a unique biochemical environment that provides increased access to key enzymes, cofactors and metabolites that may benefit some metabolic pathways, as well as specific physicochemical properties (e.g. pH, dissolved oxygen, ionic composition) that may be advantageous for some heterologous enzymes. In addition, the inner membrane provides a physical barrier that isolates the mitochondria from the rest of the cellular environment, and limits its matrix to a much smaller volume, relative to the cytosolic volume. This isolation can prevent intermediate metabolites of targeted pathways from being consumed by competing pathways in the cytosol, secreted across the plasma membrane to the extracellular environment, or toxic elsewhere in the cell. Also, the smaller volume of mitochondria can increase the local concentrations of metabolites, enzymes or cofactors, which may increase the rates of metabolic reactions (Avalos, Fink and Stephanopoulos 2013; Li, Liu and Chen 2015). Thus, compartmentalizing engineered metabolic pathways in mitochondria may offer several advantages, including increased access to key metabolites, cofactors and enzymes, enhanced enzymatic activity, improved pathway specificity with decreased by-product formation and intermediate metabolite toxicity, and elimination of metabolite transport bottlenecks. Below we summarize how different pathways have benefitted from each of these advantages of mitochondrial compartmentalization (Fig. 5).

Table 2. Mitochondrial localization signals. List of genes whose mitochondrial localization signals have been used to synthetically target proteins to mitochondria.

Mitochondria compartment	Signal gene origin	Amino acid fragment	MLS cleavage[2]	MLS confirmed to be sufficient to cause mitochondrial localization[1]	References and notes
Intermembrane space	*CYB2*	1-88	Confirmed	Yes	(Hu, Dong and Outten 2008)[1], (Beasley, Müller and Schatz 1993)[2]
Matrix	*COXIV**	1-26	Confirmed	Yes	(Sesaki and Jensen 1999; Hu, Dong and Outten 2008)[1], (Hurt *et al.* 1985)[2], Quantification of mitochondrial targeting (Avalos, Fink and Stephanopoulos 2013), Most often used signal for metabolic engineering
	*COXVI**	1-41	Confirmed	Yes (unpublished)	(Hammer, Zhang and Avalos 2020)[1], (Burkhart *et al.* 2015)[2], Targeting efficiency comparable to COXIV (unpublished)
	*IDH1**	1-11	Predicted		(Salusjärvi *et al.* 2017)[#], (Vögtle *et al.* 2009)[2]
	*CYB2**	1-39	Confirmed	Yes	(Shi *et al.* 2016)[1], (Beasley, Müller and Schatz 1993)[2]
	*CAT2**	1-136	Confirmed[+]	Yes	(Shi *et al.* 2016)[1], (Burkhart *et al.* 2015)[2]
	*CDC9**	1-47	Predicted	Yes	(Willer *et al.* 1999)[1,2]
	ACO1	1-19	Confirmed	Yes	(Regev-Rudzki, Yogev and Pines 2008)[1,2]
	FUM1	1-30	Confirmed	Yes	(Regev-Rudzki, Yogev and Pines 2008)[1,2]
	SOD2	1-27	Confirmed[+]	Yes	(Jensen and Culotta 2000)[1], (Burkhart *et al.* 2015)[2]
	PSU9	1-69		Yes	(Gallas *et al.* 2006)[1]

*Denotes that the signal has been utilized to target enzymes to mitochondria for metabolic engineering.
[+]Confirmed in a high-throughput study.
[#]The signal in this study was used to target a protein to the mitochondria but no data confirming localization was provided.

Products derived from BCAA metabolism

The biosynthesis of BCAAs initiates in mitochondria by the activity of five enzymes (encoded by *ILV1, ILV2, ILV3, ILV5* and *LEU4*) (Kohlhaw 2003). Therefore, several metabolic pathways to produce chemicals derived from BCAAs have been targeted to the mitochondria to benefit from the availability of these enzymes, their substrates and cofactors, as well as the environment in which their activities have evolved to be optimal. Targeting the enzymes involved in the Ehrlich amino acid degradation pathway (α-KDCs and ADHs) to mitochondria significantly improves the production of isobutanol, isopentanol and 2-methyl-1-butanol, by as much as 260%, 370% and 500%, respectively, relative to overexpressing these enzymes in their native cytosolic compartment (Avalos, Fink and Stephanopoulos 2013). This was first demonstrated using a 2μ plasmid to introduce the metabolic pathway, achieving as much as 635 mg/L of isobutanol, 95 mg/L of isopentanol and 118 mg/L of 2-methyl-1-butanol from glucose (Avalos, Fink and Stephanopoulos 2013); and later by integrating the pathway in chromosomal delta-integration sites of retrotransposon (Ty) elements, achieving ~600 mg/L of isobutanol and trace amounts of C5 alcohols from galactose (Yuan and Bun Ching 2014). This pathway benefits from mitochondrial compartmentalization largely due to improved access to the α-KIV intermediate, which is more abundant in mitochondria than in the cytosol (Avalos, Fink and Stephanopoulos 2013), as well as higher availability of Fe-S clusters, required for the activity of Ilv3p. In addition, quantitative immunoblotting measurements showed that enzymes targeted to mitochondria can reach higher local concentrations than in the cytosol, due to the smaller volume of this organelle (Avalos, Fink and Stephanopoulos 2013). In a more recent study, it was shown that dynamically controlling this pathway and ethanol biosynthesis (an essential pathway that competes for pyruvate) with optogenetics significantly increases isobutanol (8.49 g/L) and 2-methyl-1-butanol (2.38 g/L) production (Zhao *et al.* 2018). Thus, it is possible to not only target non-native enzymes to mitochondria to assemble complete metabolic pathways in this organelle but also significantly boost the flux through these pathways to increase chemical production even under fermentation conditions.

A key mechanism by which targeting downstream enzymes to mitochondria improves the biosynthesis of BCAA-derived products is that it alleviates metabolic bottlenecks in the endogenous BCAA biosynthetic pathway caused by the need to transport intermediate metabolites across the mitochondrial membrane. For BCHA production, the native localization of upstream pathway enzymes (Ilv1p, Ilv2p/Ilv6p, Ilv3p and Ilv5p) in mitochondria, and downstream enzymes (KDC and ADH) in the cytosol introduce bottlenecks due to the need to export α-KIV, α-KMV and α-IPM from mitochondria to produce isobutanol, 2-methyl-1-butanol and isopentanol, respectively (Fig. 1) (Hammer and Avalos 2017b; Yuan *et al.* 2017). Because these intermediates are polar, they most likely need carriers to cross the mitochondrial membrane, which becomes a limiting step when the upstream enzymes are overexpressed. Overexpressing the mitochondrial carrier for α-IPM (*OAC1*) can mitigate, though not completely eliminate, the bottleneck created by the need to export this intermediate in isopentanol production (Fig. 3) (Yuan *et al.* 2017). Similar experiments for isobutanol and

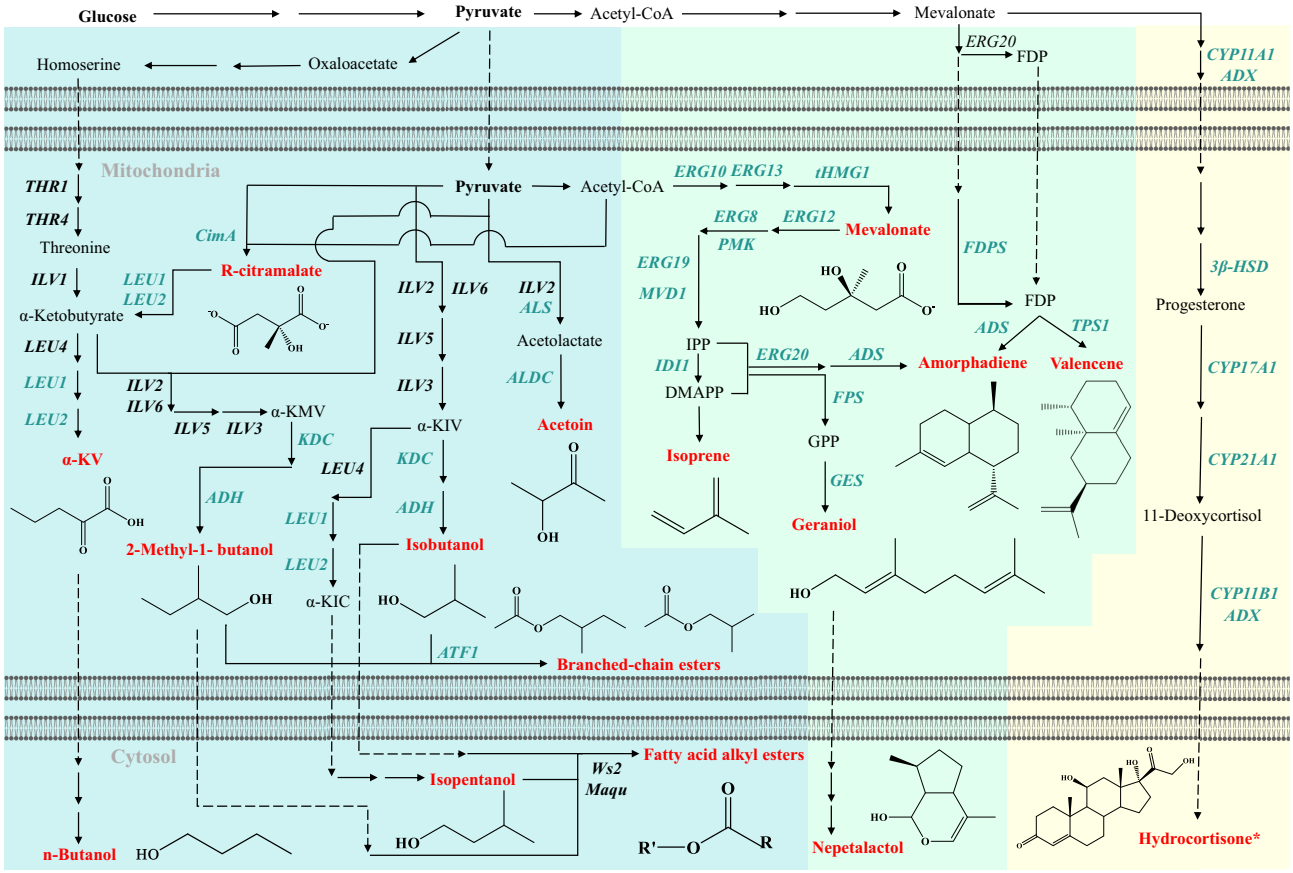

Figure 5. Metabolic pathways that benefit from mitochondrial compartmentalization. Products derived from three major metabolic pathways that have been targeted to mitochondria, including from branched-chain amino acid metabolism (blue), the terpenoid pathway (green) and sterol biosynthesis (yellow). Targeting key non-mitochondrial enzymes (teal) in these pathways to mitochondria increases production of chemicals of interest (red). Multiple consecutive arrows represent multiple steps in a pathway. Dashed arrows indicate product/metabolite transport across the mitochondrial membranes. *Most enzymes in hydrocortisone biosynthesis are membrane bound.

2-methyl-1-butanol production have not been possible because the mitochondrial carriers for α-KIV, α-KMV have not been identified. However, targeting KDCs and ADHs to mitochondria eliminates bottlenecks of α-KIV and α-KMV transport, increasing the production of isobutanol and 2-methyl-1-butanol, respectively (Avalos, Fink and Stephanopoulos 2013; Hammer and Avalos 2017b). Because BCHAs are less polar than their metabolic precursors, they are able to diffuse across membranes without transporters, which effectively eliminates bottlenecks caused by carrier-mediated metabolite transport (Avalos, Fink and Stephanopoulos 2013; Hammer and Avalos 2017b).

Targeting enzymes to mitochondria can also be used to increase the specificity of recursive α-ketoacids biosynthetic pathways by introducing desirable metabolite transport bottlenecks (Hammer, Zhang and Avalos 2020). Extensive carbon chain elongation for the production of higher α-ketoacid derived alcohols is hampered by early termination of the pathway at shorter α-ketoacid intermediates due to the promiscuous activity of KDCs. This can be mitigated to some extent by using engineered KDC variants that show some preference for larger α-ketoacids (Zhang *et al.* 2008; Hammer, Zhang and Avalos 2020). However, early termination of backbone elongation still limits the production of larger BCHAs. In yeast, this challenge can be addressed by targeting the elongation enzymes (Leu4p, Leu2p, Leu1p) to mitochondria and separating them from the termination enzymes (KDCs and ADHs), which are kept in the cytosol (Hammer, Zhang and Avalos 2020). This introduces a kinetic bottleneck in the termination pathway by requiring the 2-ketoacid intermediates to be exported from mitochondria before they can be processed by termination enzymes in the cytosol, giving elongation enzymes in the mitochondria a competitive advantage to divert the α-ketoacid intermediate through the iterative cycle. With this strategy, it is possible to achieve a ratio of isopentanol to isobutanol titers of 14:1, the highest specificity for isopentanol production (93%) reported thus far, and boost isopentanol production to as much as 1.26 ± 0.06 g/L (Hammer, Zhang and Avalos 2020). This study illustrates how the segregation of elongation enzymes in mitochondria can increase the cycling efficiency of recursive pathways by preventing early termination.

These natural BCHA biosynthetic pathways can be used in combination with alternative enzymes targeted to mitochondria or the cytosol to produce other products of interest, such as *n*-butanol and branched-chain esters (BCEs). Separation of threonine biosynthesis in the mitochondria from the Ehrlich degradation pathway in the cytosol prevents the diversion of α-ketobutyrate to *n*-propanol to improve *n*-butanol production (Si *et al.* 2014). Re-localizing *LEU1*, *LEU2* and *LEU4* in mitochondria, while overexpressing mitochondrial *ILV1*, *LEU5* (involved in importing CoA into mitochondria), and a feedback-insensitive *HOM3** mutant in the cytosol, in an *adh1*Δ strain increased production to 243 mg/L (Shi *et al.* 2016). Complementing this pathway with citramalate synthase (*CimA*) from

L. interrogans, which shortens the number of steps from pyruvate to α-ketobutyrate (Fig. 5), overexpressing the *LEU4* homolog *LEU9* and cysteine desulfurase *NFS1*, involved in Fe-S biogenesis (Fig. 2), in mitochondria, as well as the Ehrlich degradation enzymes (*ARO10* and *ADH7*) in the cytosol, resulted in a strain that produces 1.05 g/L of *n*-butanol in a bioreactor (Shi *et al.* 2016). In addition, mitochondrial production of BCHAs has been extended to produce BCEs by re-localizing the alcohol acetyl-transferase Atf1p to mitochondria (Yuan, Mishra and Ching 2016). This approach takes advantage of both the increased BCHA production obtained by targeting the complete isobutanol pathway to mitochondria, and the higher pools of Acetyl-CoA in this organelle to produce 155.4 mg/L of isobutyl acetate and 119.6 mg/L of 2-methyl-1-butyl acetate. Unexpectedly, 138.8 mg/L of isopentyl acetate (3-MBA) was also observed, raising the possibility that some cytosolically produced isopentanol was entering the mitochondria to be acetylated by Atf1p (Yuan, Mishra and Ching 2016), or that some Atf1p remains in the cytosol. Localization of Atf1p in the cytosol increased the total BCA production, but shifted the product selectivity to favor 3-MBA (Yuan, Mishra and Ching 2016), most likely because isopentanol synthesis was not compartmentalized. Similarly, the isobutanol pathway compartmentalized in mitochondria has been utilized in conjunction with cytosolic expression of wax ester synthases, *ws2* and *Maqu_0168,* from *Marinobacter sp.* to produce up to 230 mg/L fatty acid short- and branched-chain alkyl esters (Teo *et al.* 2015). These studies demonstrate how mitochondrial pathways can be built upon with additional enzymes, targeted to either mitochondria or the cytosol, to produce different chemicals.

Heterologous pathways can also be localized to the mitochondria to utilize branched-chain amino acid biosynthesis intermediates to make other products of interest. Cytosolic expression of acetolactate decarboxylase from *B. amyloliquefaciens* (*BaALDC*) for acetoin production is inefficient due to the need to transport acetolactate from the mitochondria to the cytosol (Li *et al.* 2014). However, targeting *BaALDC* to the mitochondria led to a 181% increase in acetoin yields (Li, Liu and Chen 2015). To further improve pathway efficiency, the authors expressed in the mitochondria the acetolactate synthase (an *ILV2* ortholog) from *B. subtilis* (*BsALS*), which preferentially produces acetolactate over α-ketobutyrate. Co-expression of *BsALS* and *BaALDC* in mitochondria led to not only 20% higher acetoin production but also 22% higher accumulation of acetolactate, compared with co-expressing the same two enzymes in the cytosol (Li, Liu and Chen 2015). The accumulation of acetolactate also indicated that ALDC conversion of acetolactate to acetoin could be further optimized, suggesting this pathway might benefit from a more efficient acetolactate decarboxylase. Additionally, overexpression of *MPC1* and *MPC2* to increase mitochondrial pyruvate importation boosts acetoin titers to 3.26 g/L (Fig. 5) (Table 3). This demonstrates that, as in traditional metabolic engineering approaches to manipulate endogenous mitochondrial pathways (Fig. 1), overexpressing metabolite transporters, specifically *MPC1* and *MPC2*, can increase flux through engineered mitochondrial pathways to boost product formation.

Terpenoids

Terpenoids encompass a large class of natural products that are of high relevance to the biosynthesis of advanced biofuels, commodity chemicals and specialty chemicals. Many important drugs, such as Taxol or Artemisinin, utilized in anti-cancer and anti-malarial treatments, respectively, are derived from terpenoids (Wang, Tang and Bidigare 2005). Extracting many of these terpenoids from their native hosts, however, can be limited by low yields, raising the cost of production (Farhi *et al.* 2011). Therefore, engineering biosynthetic pathways for terpenoid products and precursors in yeast is an attractive alternative to produce these chemicals, for which mitochondrial engineering can provide some advantages.

Early attempts to utilize yeast mitochondria to produce plant sesquiterpenes uncovered a previously unknown mitochondrial pool of farnesyl diphosphate (FPP), whose origins are still not fully understood (Farhi *et al.* 2011). Localizing the synthases for amorphadiene (*AaADS*), valencene (*CsTPS1*) and farnesyl diphosphate (*AtFDPS*) in mitochondria leads to an 8- and 20-fold improvement in valencene (300 μg/L) and amorphadiene (20 mg/L) production, respectively, when compared with concentrations achieved from cytosolic expression of the same synthases (Table 3) (Farhi *et al.* 2011). However, since FPP is produced in the cytosol, further improvement of terpenoid production is limited by the need to import FPP across the mitochondrial membrane, especially given that the FPP mitochondrial carrier is unknown. This bottleneck was evident in the observation that overexpressing the cytosolic mevalonate pathway decreases amorphadiene production only when *AaADS* is targeted to the mitochondria but not when expressed in the cytosol (Yuan and Ching 2016). These findings suggest that transport of FPP across the mitochondria membrane is inefficient and highly regulated (Yuan and Ching 2016), which creates a bottleneck.

The mevalonate pathway was later fully reconstructed inside mitochondria, taking advantage of the substantial mitochondrial pool of acetyl-CoA, from which the mevalonate pathway originates, and eliminating the bottleneck caused by FPP importation (Fig. 5). This approach has been utilized to produce amorphadiene (Yuan and Ching 2016), geraniol (Yee *et al.* 2019) and isoprene (Lv *et al.* 2016) in mitochondria, leading to 1.3-, 6- and 1.7-fold increases in production, respectively, when compared with cytosolic overexpression of the mevalonate pathway and terpenoid synthases (Fig. 5) (Table 3) (Lv *et al.* 2016; Yuan and Ching 2016; Yee *et al.* 2019). For amorphadiene production, this corresponds to a titer of 427 mg/L, reflecting a 3.5-fold increase when compared with a previously constructed strain with an engineered PDH bypass for higher acetyl-CoA levels (120 mg/L) (Shiba *et al.* 2007). While geraniol is already a valuable fragrance and drug precursor, the geraniol produced in mitochondria was further processed in the endoplasmic reticulum and cytosol of the same strain to produce nepetalactol, a valuable precursor of monoterpene indole alkaloid drugs (Yee *et al.* 2019). Furthermore, simultaneous overexpression of the cytoplasmic and mitochondrial isoprene pathways improves production by 2.1-fold compared with only overexpressing the mitochondrial pathway (see Mixed Approach below). These studies combine the benefit of using an abundant mitochondrial metabolite, acetyl-CoA, with the ability to eliminate the need to import a rate-limiting metabolite, FPP, across the mitochondrial membrane.

Compartmentalizing the mevalonate pathway in mitochondria also helps decrease the formation of unwanted by-products that would more readily occur in the cytosol. Using the mitochondria to protect important intermediates that would otherwise be consumed by competing reactions improves the production of amorphadiene (Farhi *et al.* 2011; Yuan and Ching 2016), valencene (Farhi *et al.* 2011), isoprene (Lv *et al.* 2016) and geraniol (Yee *et al.* 2019) (Fig. 5 and Table 3). When producing isoprene, monoterpenes or sesquiterpenes, it is necessary to reduce the metabolic flux being diverted toward farnesol, squalene and

Table 3. Summary of metabolic pathways harnessing mitochondria. Studies in which mitochondrial engineering improved product titers. Bold titers represent highest titers obtained by targeting one or more enzymes to mitochondria. Bold superscripts indicate that the specific intervention was involved in the highest titer reported.

Metabolic product	*Yeast species*	*Native non-mitochondrial localization*		*Native mitochondrial localization*		*Synthetically localized to mitochondria*	*Highest titer reported*	*References*
		Overexpressed	Deleted	Overexpressed	Deleted			
n-Butanol	*S. cerevisiae*	*HOM3*[1]* *HOM3*[2] *HOM2*[1,2] *HOM6*[1,2] *LEU4*[1,2] *LEU1*[1,2] *LEU2*[1] *LlKivD*[1,2] *ARO10*[1,2]* *ADH7*[1]	*ADH1*[1,2]	*THR1*[1,2] *THR4*[1,2] *ILV1*[1,2] *LEU4*[1,2] *LEU5*[1] *NFS1*[1] *CHA1*[2]	*ILV2*[2] *ILV6*[2] *ILV3*[2]	*LEU1*[1] *LEU2*[1] short*LEU4*[1] *LiCimA*[1] *MjCimA*[1] *GsCimA*[1]	**1050 mg/L**[1] 242.8 mg/L[2]	(Shi *et al.* 2016)[1] (Si *et al.* 2014)[2]
Isobutanol	*S. cerevisiae*	OptoPDC1[7] *PYC2*[2] *MDH2*[2] *cMAE1*[2] *LEU3*[3] *LEU3*[3,4]* *BAT2*[6] *ARO10*[3,4,5] *LlKivD*[2,5] *ADH2*[3,4] *ADH6*[2] *ADH7*[5] *LlAdhA*[5]	*PDC1*[7] *PDC5*[7] *PDC6*[7] *ALD6*[2,3,4] *BAT2*[6] *LEU2*∼[3]* *LEU4*[4]	*MPC1*[4] *MPC2*[4] *MPC3*[4] *MAE1*[4] OptoILV2[7] *ILV2*[1,2,3,4,5,6,7] *ILV5*[1,3,4,5,6,7] *ILV3*[1,3,4,5,6,7]	*LPD1*[4] *ILV1*[2] *ILV6*[6] *ILV6*[6]* *LEU4*[4] *BAT1*[3,4,6,7] *ECM31*[2]	*ARO10*[1,4,5,6,7] *LlKivD*[5] *ADH2*[4] *ADH7*[1,5] *LlAdhA*[5,6,7]*	**600 mg/L**[1] 224 mg/L[2] 376.9 mg/L[3] **330.9 mg/L**[4] **635 mg/L**[5] **1245 mg/L**[6] **8490 mg/L**[7]	(Yuan *et al.* 2014)[1] (Ida *et al.* 2015)[2] (Park, Kim and Hahn 2014)[3] (Park, Kim and Hahn 2016)[4] (Avalos *et al.* 2013)[5] (Hammer *et al.* 2017b)[6] (Zhao *et al.* 2018)[7]
2-Methyl-1-butanol	*S. cerevisiaeS. cerevisiae*	OptoPDC1[3] *BAT2*[2] *ARO10*[1] *LlKivD*[1] *LlAdhA*[1]	*PDC1*[3] *PDC5*[3] *PDC6*[3] *BAT2*[2]	OptoILV2[3] *ILV2*[1,2,3] *ILV5*[1,2,3] *ILV3*[1,2,3]	*ILV6*[2] *ILV6*[2]* *BAT1*[2,3]	*ARO10*[1,2,3] *LlKivD*[1] *ADH7*[1] *LlAdhA*[1,2,3]*	**118 mg/L**[1] **203 mg/L**[2] **2380 mg/L**[3]	(Avalos *et al.* 2013)[1] (Hammer *et al.* 2017b)[2] (Zhao *et al.* 2018)[3]
Isopentanol	*S. cerevisiae*	*LEU3*[1,4]* *LEU4*[1] *LEU4*[1,4]* *LEU1*[2,4] *LEU2*[1,2,4] *ARO10*[1,2,3] *LlKivD*[3] *ADH2*[1] *ADH7*[2,3] *LlAdhA*[3]	*ALD6*[1] *LEU4*[4]	*ILV2*[1,2,3,4] *ILV5*[1,2,3,4] *ILV3*[1,2,3,4] *OAC1*[2] *LEU4*[1] *LEU4*[1,4]* *LEU9*[2]	*BAT1*[1,4] *OAC1*[4] *LEU4*[4] *LEU9*[4]	*LEU4*[4]* *LEU1*[4] *LEU2*[4] *ARO10*[3] *LlKivD*[3] *ADH7*[3] *LlAdhA*[3]*	765.7 mg/L[1] 561.21 mg/L[2] **95 mg/L**[3] **1240 mg/L**[4]	(Park, Kim and Hahn 2014)[1] (Yuan *et al.* 2017)[2] (Avalos *et al.* 2013)[3] (Hammer *et al.* 2020)[4]
L-Ornithine	*S. cerevisiae*	*PYC2*[1] *MTH1-ΔT* *CAR1*[1] *EcArgA*[1] *EcArgB*[1] *CgArgC*[1] *CgArgD*[1] *CgArgJ*[1] *GLT1* *GLN1* *GDH1*[1] *GDH3*	*ARG3∼[1]* *CAR2*[1]	*IDP1* *ACO2* *CIT1* *PDA1* *PDA1** *ODC1* *AGC1*[1] *ORT1*[1] *ARG2* *ARG7* *ARG5* *ARG6* *ARG8* *HaAOX1* *NDI1*	*KGD2*		5,100 mg/L	(Qin *et al.* 2015)
Fumarate	*C. glabrata*	*ASL*[1] *SpMAE1*[1,2] *RoPYC*[2] *RoMDH*[2] *RoFUM1*[2]	*PDC6∼[2]* *RHR2∼[2]*	*SFC1*[1,2] *KGD2*[1] *SUCLG2*[1] *SDH1*[1,2] *KGD2-SUCLG2*[1,2]	*ARG8*[1] *THI2*[2] *FUM1*[2]		15.76 g/L[1] 33.13 g/L[2]	(Chen *et al.* 2015)[1] (Chen *et al.* 2016)[2]

Table 3. Continued

Metabolic product	Yeast species	Native non-mitochondrial localization		Native mitochondrial localization		Synthetically localized to mitochondria	Highest titer reported	References
		Overexpressed	Deleted	Overexpressed	Deleted			
Glycolic acid	*S. cerevisiae*	*CsXylB*[1] *CsXylD*[1] *EcYagE*[1] *EcYjhH* *EcAldA*[1] *LhLdhL*[1]	*FRA2*[1]			*XylD*	1 g/L	(Salusjärvi *et al.* 2017)
Hydrocortisone	*S. cerevisiae*	*CYP11A1*[1] *ADX*[1]	*ATF2*[1] *GCY1*[1] *YPR1*[1]	*ARH1*[1]		*ADX*[1] *ADR* *3β-HSD*[1] *CYP11B1*[1] *CYP17A1*[1] *CYP21A1*[1]	**11.5 μg/mL**	(Szczebara *et al.* 2003)
Amorphadiene	*S. cerevisiae*	*AsADS*[1,2] *tHMG**[1] *FDPS*[1] *ERG10*[2] *ERG13*[2] *tHMG1*[2] *ERG12*[2] *ERG8*[2] *ERG19*[2] *IDI1*[2] *ERG20*[2]			*BTS1*[1]	*AsADS*[1,2] *FDPS*[1] *ERG10*[2] *ERG13*[2] *tHMG1*[2] *ERG12*[2] *ERG8*[2] *ERG19*[2] *IDI1*[2] *ERG20*[2]	**20 mg/L**[1] **427 mg/L**[2]	(Farhi *et al.* 2011)[1] (Yuan *et al.* 2016)[2]
Valencene	*S. cerevisiae*	*CsTPS1*[1] *tHMG**[1] *FDPS*[1]			*BTS1*[1]	*CsTPS1*[1] *FDPS*[1]	**300 μg/L**	(Farhi *et al.* 2011)
Geraniol *Hydroxygeraniol* *Nepetalactol*	*S. cerevisiae*	*GgFPS* *ObGES* *CsGOR*[1] *CsG8H*[1] *CsISY*	*OYE2* *OYE3*[1]		*BTS1*	*ERG10*[1] *ERG13*[1] *tHMG1*[1] *ERG12*[1] *ERG8*[1] *ERG19*[1] *IDI1*[1] *GgFPS*[1] *ObGES*[1]	**7 mg/L227 mg/ L5.9 mg/L**	(Yee *et al.* 2019)
Isoprene	*S. cerevisiae*	*ERG10* *HMGS* *HMG1* *ERG12* *MPK* *MVD1* *IDI1* *KvISPS*[1]	*ERG20*~			*ERG10*[1] *HMGS*[1] *HMG1*[1] *ERG12*[1] *MPK*[1] *MVD1*[1] *IDI1*[1] *KvISPS*[1]	**2527 mg/L**	(Lv *et al.* 2016)
Acetoin	*C. glabrata*	*BaALDC*		*MPC1*[1] *MPC2*[1]		*BsALS*[1] *BaALDC*[1]	**3.26 g/L**	(Li *et al.* 2015)
Branched-chain esters: *isobutyl acetate* *3-Methyl-1-butyl acetate* *2-Methyl-1-butyl acetate*	*S. cerevisiae*	*PYC2* *MDH2*[1] *ARO10* *ADH7* *ATF1*[1]		*ILV2*[1] *ILV3*[1] *ILV5*[1] *MAE1*[1]		*ARO10*[1] *ADH7*[1] *ATF1*[1]	**260.2 mg/L** **296.1 mg/L** **289.6 mg/L**	(Yuan *et al.* 2016)
Fatty acid alkyl ester	*S. cerevisiae*	*Ws2* *Maqu**[1]	*OPI1*[1] *RPD3*	*ILV2*[1] *ILV3*[1] *ILV3*[1]		*ARO10*[1] *ADH7*[1]	**230 mg/L**	(Teo *et al.* 2015)

*Denotes a mutant protein. ~Denotes downregulation instead of deletion.

ergosterol, ideally without introducing auxotrophies. This is particularly challenging for monoterpenes, as the same farnesyl pyrophosphate synthase (encoded by *ERG20*) catalyzes the formation of both GPP (the precursor of monoterpenes) and FPP (a by-product of monoterpenes and precursor of sesquiterpenes, squalene and ergosterol). However, segregating terpenoid synthesis in the mitochondria has resulted in as much as 50% and 80% decrease in farnesol and squalene by-product formation, respectively (Lv *et al.* 2016; Yee *et al.* 2019). In conjunction, these findings show that pathway segregation in organelles is an efficient method for decreasing by-product formation by isolating the pathway of interest.

Sterols

Sterols are lipids, also derived from the mevalonate pathway, with many biological and pharmacological roles. Hydrocortisone is a valuable anti-inflammatory and precursor for the synthesis of other drugs with anti-inflammatory and antiproliferative properties. Yeast mitochondria were first harnessed for sterol biosynthesis by the heterologous reconstruction of the mammalian hydrocortisone pathway (Szczebara *et al.* 2003). Researchers demonstrated that yeast mitochondria contain enzymes capable of transferring electrons to mammalian adrenodoxin (ADX), allowing for *in vivo* recapitulation of mammalian cytochrome P450 (CYP) enzymatic activity (Dumas *et al.* 1996). These findings spurred the reconstruction of the entire hydrocortisone pathway in yeast, which involved the heterologous expression of *ADX*, *CYP11β1*, *3β-HSD*, *CYP17A1* and *CYP21A1* within mitochondria, as well as other heterologous proteins, *CYP11A1* and *ADX*, expressed in the cytosol to produce 11.5 μg/mL of hydrocortisone (Fig. 5 and Table 3). Although this titer was relatively low, this case shows the potential to functionally express completely heterologous complex pathways involving multiple cytochrome P450 enzymes in yeast mitochondria.

Mixed approach

Some strains are engineered following a mixed approach in which the same (or equivalent) biosynthetic pathways are simultaneously targeted to both the cytosol and mitochondria. Even when metabolic pathways are targeted to mitochondria, leakage of their intermediate metabolites from the mitochondria to the cytosol may occur, diverting some metabolic flux away from the pathway of interest toward competing pathways in the cytosol. For most metabolites, it is difficult to genetically address this challenge because the identity of mitochondrial carriers responsible for their leakage is unknown. This occurs in terpenoid production, in which overexpression of mevalonate in the mitochondria with a cytosolic synthase still results in increased production of terpenoids when compared with the wild type (Yee *et al.* 2019). This suggests that mevalonate intermediates are leaking from the mitochondria into the cytosol, through an unknown carrier, and entering the cytosolic terpenoid pathway (Yee *et al.* 2019). Even when a known carrier is deleted to prevent unwanted leakage of a precursor, it does not necessarily result in a substantial increase in the product of interest, probably due to the existence of multiple redundant carriers. This was observed when deletion of the α-IMP carrier *OAC1* failed to substantially increase isopentanol production in a strain with Leu4p, Leu1p and Leu2p compartmentalized in mitochondria (Hammer, Zhang and Avalos 2020). An effective strategy to boost production when facing this challenge is to supplement the mitochondrial pathway with a cytosolic counterpart to help funnel the leaked intermediates back into the biosynthetic pathway of interest. This strategy has been demonstrated in the production of isoprene (Lv *et al.* 2016), isopentanol (Hammer, Zhang and Avalos 2020) and *n*-butanol (Shi *et al.* 2016). The mixed approach is likely to improve production in strains with other engineered mitochondrial pathways, as long as it does not interfere with the initial motivation for compartmentalizing the pathway, such as to avoid toxicity of the pathway in the cytosol.

Exploiting and engineering iron-sulfur cluster biogenesis

Mitochondria offer a more favorable environment than the cytosol for the expression of some bacterial enzymes that require Fe-S clusters (Salusjärvi *et al.* 2017). Targeting XylD, a *C. crescentus* D-xylonate dehydratase, to the mitochondria improves enzyme activity by 14-fold compared with expression in the cytosol (Salusjärvi *et al.* 2017). The activity of mitochondrially targeted XylD further improves to 24-fold when *FRA2*, a negative regulator of the iron regulon, is deleted (Fig. 3) (Salusjärvi *et al.* 2017). A separate study showed that overexpression of *NFS1*, a mitochondrial cysteine desulfurase important in Fe-S biogenesis, increases *n*-butanol titers, presumably by improving the Fe-S-dependent activity of Ilv3p in the pathway (Fig. 3) (Shi *et al.* 2016). Furthermore, overexpression of transcription factors *AFT1/2*, which regulate iron utilization and homeostasis, has also been reported in the patent literature to increase the activity of dihydroxyacid dehydratases (of which Ilv3p is a member) localized in the cytosol or mitochondria, improving alcohol production (Dundon *et al.* 2011). However, a similar strategy to increase the activity of cytosolic Fe-S-dependent 6-phosphogluconate dehydratase (PGDH) from *E. coli* by overexpressing *AFT1*, or by localizing it to the mitochondria was not effective (Benisch and Boles 2014). Unfortunately, it is unclear whether the lack of PGDH activity was due to a missing chaperone needed for proper protein folding, inactivation of the enzyme by reactive oxygen species and insufficient repair mechanisms, or improper recognition of the Fe-S site by the yeast machinery. These findings not only highlight the complexity of expressing Fe-S-dependent enzymes in yeast but also demonstrate the potential of utilizing the mitochondria to enhance the activity of these enzymes.

CHALLENGES AND FUTURE OPPORTUNITIES OF MITOCHONDRIAL COMPARTMENTALIZATION

Mitochondrial compartmentalization of metabolic pathways still faces important challenges that not only hinder the broader applicability of this approach but also offer areas of opportunity for innovation and discovery. A better understanding of the mitochondrial environment, its dependence on carbon source and media conditions, and how it changes during the course of fermentation will improve our understanding of how it might impact (positively or negatively) compartmentalized pathways. Metabolite transport across the mitochondrial membrane, protein translocation into the mitochondrial matrix, changes in the activity of targeted enzymes, and mitochondrial activity, dynamics and morphology pose other important questions and opportunities, some of which we discuss in this section.

An important limitation in mitochondrial engineering is our incomplete picture of metabolite transport across the mitochondrial membrane. Recognizing the metabolites that are imported

or exported from mitochondria and identifying the mitochondrial carriers responsible for this transport will greatly impact our decisions to compartmentalize pathways and our capabilities to enhance their performance. Overexpressing mitochondrial pyruvate carriers (*MPC1* and *MPC2*) has improved some compartmentalized pathways that start from pyruvate (Fig. 5) (Li, Liu and Chen 2015; Park, Kim and Hahn 2016). Similarly, the α-IPM carrier *OAC1* has been overexpressed (Yuan *et al.* 2017) or deleted (Hammer, Zhang and Avalos 2020) to improve mitochondrial pathways with some success. However, this approach remains largely untapped because most relevant mitochondrial carriers remain unknown. Identifying and overexpressing carriers for other metabolites of interest could make it possible to compartmentalize pathways that initiate from different metabolites, or enhance mitochondrial export of desirable products or precursors. Alternatively, knowing what carriers are responsible for the unwanted secretion of intermediate metabolites would make it possible to delete them to improve metabolic flux and product formation. One can also envision the possibility of engineering mitochondrial carriers to alter their substrate specificity and enable the transport of new classes of metabolites and products in and out of mitochondria for metabolic engineering applications. Thus, improving our understanding of mitochondrial carriers will likely expand dramatically the application and capabilities of mitochondrial engineering.

Recapitulating the activity of targeted enzymes is sometimes challenging, even for enzymes expected to benefit from mitochondrial compartmentalization. This includes enzymes that should benefit from the availability of mitochondrial cofactors as well as enzymes previously expressed successfully in mitochondria of other microbial species. For example, mitochondrial compartmentalization of glutamate dehydrogenases (encoded by *GDH1* and *GDH2*) to improve L-ornithine production had the opposite effect (Qin *et al.* 2015). This was unexpected, as L-ornithine biosynthesis naturally occurs in mitochondria from glutamate, whose pools should have increased by targeting Gdh1p or Gdh2p to this organelle. Similarly, expressing the *E. coli* 6-phosphogluconate dehydratase (PGDH) in mitochondria or cytosol did not result in active enzyme (Benisch and Boles 2014). Because PGDH is an Fe-S cluster-dependent enzyme, the expectation was that targeting this enzyme to mitochondria would improve its activity. In separate studies, reconstructing a heterologous biosynthetic pathway for itaconic acid in mitochondria increases production in *A. niger* (Blumhoff *et al.* 2013), but not when targeted to the mitochondria or cytosol of *S. cerevisiae* (Blazeck *et al.* 2014), indicating that pathways that work well in the mitochondria of one species are not necessarily effective in the mitochondria of another species. Such negative results are not unique to mitochondrial engineering, of course, and are often found in traditional metabolic engineering as well; but it is worth noting that some negative results may arise from challenges particular to mitochondrial engineering. For example, the mitochondrial environment may be unfavorable for some of these enzymes; the availability of ammonia in mitochondria may be insufficient for Gdh1p or Gdh2p activity; or the Fe-S assembly machinery and chaperones available in yeast mitochondria may be incompatible with bacterial PGDH. Furthermore, targeted enzymes may not always be stable in the mitochondrial environment or survive the translocation process, which involves post-translational transport of proteins through outer and inner membrane transporters (TOMs and TIMs) (Becker and Wagner 2018), often requiring the assistance of cytosolic and mitochondrial chaperones. Therefore, improving our understanding of the mitochondrial environment, the compatibility of Fe-S accessory proteins across species, and the molecular mechanism of protein mitochondrial import will help improve our ability to compartmentalize stable and active enzymes in mitochondria.

Another significant challenge is increasing mitochondrial activity, especially under fermentation conditions. Yeast mitochondria are highly dynamic and undergo significant changes in number, size, surface area to volume ratio, localization, volume and activity depending on growth conditions, carbon source, metabolic state, mutation and other factors (Jensen *et al.* 2000; Okamoto and Shaw 2005). When respiring, the cell contains smaller, more numerous and active mitochondria, localized closer to the periphery of the cell, than when fermenting. Crabtree-positive yeasts, including *S. cerevisiae,* prefer to ferment in the presence of glucose, repressing respiration and mitochondrial activity, which constrains mitochondrial pathways engineered to produce chemicals from glucose. This phenomenon was evident in recent studies in which mitochondrial production of isobutanol from xylose was 2-fold to almost 6-fold higher than from glucose; the key difference between these substrates being that xylose does not induce a Crabtree effect (Kwak and Jin 2017), and thus maintains approximately a 6-fold higher mitochondrial activity than glucose (Zhang *et al.* 2019; Lane *et al.* 2020). These results suggest that mitochondrial engineering should be more effective in Crabtree-negative yeasts than in the two Crabtree-positive species in which it has been implemented thus far (*S. cerevisiae* and *C. glabrata*). It also suggests that engineering mitochondrial physiology to increase their number, morphology, localization and activity could have at least as much of an impact in improving mitochondrial biosynthetic pathways than following traditional approaches in metabolic engineering such as metabolic enzyme overexpression, gene deletion or pathway deregulation. The prospect of engineering mitochondria to be as active and numerous in fermenting cells as they naturally are in respiring ones represents an exciting area of opportunity to advance the field of mitochondrial compartmentalization of engineered pathways.

The lack of high-throughput screening of mitochondrially compartmentalized pathways is another obstacle to accelerating the development of strains with engineered mitochondria. A variety of biosensors to measure different aspects of mitochondrial physiology have been developed, such as redox potential, pyruvate transport and pH (Zhang and Avalos 2017). However, they have not been applied for high-throughput screening of mitochondrial metabolic pathways. A recently developed biosensor for BCAA-derived products is the first biosensor capable of monitoring the metabolic flux through a mitochondrial biosynthetic pathway (Zhang *et al.* 2020). This biosensor was used to develop high-throughput screens for isobutanol or isopentanol production. It will be instrumental in improving strains for the production of these chemicals and potentially other BCAA-derived products, and may be useful to study non-respiratory mitochondrial metabolic activity.

CONCLUSIONS

The subcellular architecture of yeast can present formidable obstacles for metabolic engineering when the endogenous resources for pathways of interest are distributed across different compartments separated by membranes of highly selective permeability. In this regard, the simpler architecture of bacterial cells can be advantageous, as most of their metabolism takes place in one contiguous compartment. However, in recent years

metabolic engineers have started to take advantage of the different subcellular compartments that yeast has to offer to improve the activity, specificity and innocuousness of engineered pathways (Hammer and Avalos 2017a).

Of the different yeast organelles, the mitochondrion is one of the most attractive for metabolic engineering and, as such, one of the most extensively utilized for the production of valuable chemicals thus far. The richness of metabolic pathways, enzymes, metabolites and cofactors contained in mitochondria is unrivaled by other organelles in yeast. Furthermore, our ability to target exogenous enzymes into the mitochondrial matrix with good efficiency has enabled the assembly of a variety of different pathways in this organelle. As detailed in this review, the extent to which the mitochondria are involved in engineered pathways is also very flexible. The applications range from simply utilizing metabolic pathways naturally housed in mitochondria at their basal or upregulated levels, to overexpressing partial or complete biosynthetic pathways not natively found in yeast.

Several obstacles need to be overcome in order to expand the applicability of mitochondrial engineering and realize its full potential. Important advances in our fundamental understanding of mitochondrial physiology and metabolism are still needed to solve the remaining challenges. However, the prospective capabilities of a fully developed framework for mitochondrial engineering make this ambitious goal worth pursuing. If researchers in the field succeed, then having mitochondria will be one of the biggest advantages of using yeast for metabolic engineering.

AUTHOR CONTRIBUTIONS

LD, JML and JLA wrote the manuscript.

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