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Methanol biotransformation toward high-level production of fatty acid derivatives by engineering the industrial yeast *Pichia pastoris*

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Methanol-based biorefinery is a promising strategy to achieve carbon neutrality goals by linking CO_2 capture and solar energy storage. As a typical methylotroph, *Pichia pastoris* shows great potential in methanol biotransformation. However, challenges still remain in engineering methanol metabolism for chemical overproduction. Here, we present the global rewiring of the central metabolism for efficient production of free fatty acids (FFAs; 23.4 g/L) from methanol, with an enhanced supply of precursors and cofactors, as well as decreased accumulation of formaldehyde. Finally, metabolic transforming of the fatty acid cell factory enabled overproduction of fatty alcohols (2.0 g/L) from methanol. This study demonstrated that global metabolic rewiring released the great potential of *P. pastoris* for methanol biotransformation toward chemical overproduction.

metabolic engineering | methylotrophic yeast | synthetic biotechnology | oleochemicals | biofuels

Significance

Methanol-based biomanufacturing is considered a promising way to achieve carbon neutrality. However, efficient methanol biotransformation toward chemical production is challenging. To address this challenge, we enhanced the precursor supply and coenzyme regeneration in *Pichia pastoris*, combined with reinforcing methanol assimilation, which resulted in significant improvements in fatty acid production. Furthermore, we offered an effective approach for rapidly constructing versatile cell factories that share common precursors. This study represents an important step forward in the rewiring of *P. pastoris* as industrial microbial cell factories for chemical production.

The rapid growth of the global energy demand has exacerbated environmental and climate issues and enhanced the need for sustainable feedstocks. Methanol is considered as an ideal one-carbon (C1) resource for the chemical industry and biomanufacturing (1) since it can be produced from CO_2 and hydrogen (electrolysis of water) by photocatalysis (2) or electrocatalysis (3). This strategy is considered one of the most promising ways to achieve carbon neutrality, and cheap sources and flexible production processes have lowered the cost of methanol and increased the competitiveness of methanol biomanufacturing (Fig. 1). Furthermore, methanol is easy to store and transport and is thus considered an excellent energy carrier and a promising building block for synthesizing complex chemicals, and its high reduction potential is beneficial for achieving higher product yields (4, 5).

The core consideration in methanol-based biomanufacturing is the construction of efficient microbial cell factories for synthesis of target products from methanol. However, the complex metabolism of methanol and the fatal toxicity of formaldehyde, the intermediate of methanol metabolism, bring challenges in driving metabolic flux toward target chemicals (6). Recently, great efforts have been made in engineering synthetic methylotrophs such as *Escherichia coli* (7–9) and *Corynebacterium glutamicum* (10) for methanol utilization by constructing heterologous and artificial utilization pathways (11, 12). However, the methanol utilization efficiency is extremely low for the synthesis of target chemicals (13–16). Alternatively, native methylotrophs might be promising hosts for methanol-based biomanufacturing given their capacity for methanol metabolism. Among them, methylotrophic yeasts show great potential in methanol biotransformation because of their natural efficiency in methanol assimilation and available methanol induction system (17). In particular, *Pichia pastoris* (syn. *Komagataella phaffii*) is an important industrial yeast that has been recognized as a GRAS (generally regarded as safe) microbe and is widely used for the production of recombinant proteins (18), such as human serum albumin (19) and plectasin (20). In addition, the Crabtree-negative phenotype facilitates the use of *P. pastoris* for high-cell-density fermentation in industrial processes (6). Although some chemicals have been synthesized from methanol in *P. pastoris* (21–26), the efficiency needs to be largely improved. Previous studies have elucidated the basic features of the methanol metabolism in *P. pastoris* (27–31), but more efforts are still needed to accurately regulate the methanol metabolism to promote chemicals production.

Fatty acids and their derivatives are promising raw materials for manufacturing advanced biofuels, detergents, lubricants, surfactants, etc. (32). Microbes have been extensively engineered for overproduction of fatty acid derivatives from glucose (33–36). For example, we previously extensively reprogrammed the metabolism of *Saccharomyces cerevisiae* from alcoholic fermentation to lipogenesis, which enabled the production of 33.4 g/L free fatty acids (FFAs). However, there has been no report of the production of fatty acid derivatives from methanol thus far, which might be attributed to the complex regulation of methanol metabolism. Recently, we and others developed efficient genetic tools for *P. pastoris* with

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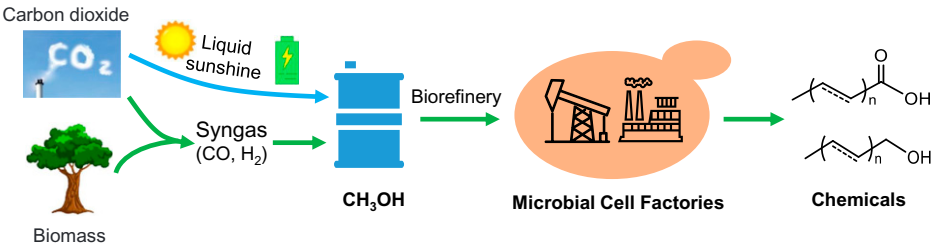

Fig. 1. The concept of methanol-based biomanufacturing from renewable resources. Methanol can be produced economically and in a large scale from renewable resources and by using green energy, representing an ideal feedstock for future biomanufacturing, and will contribute to the carbon neutrality goal by capturing CO_2.

high homologous recombination, various promoters, and genome integration sites (37–43), which should facilitate precise and global metabolic rewiring of *P. pastoris* for methanol biotransformation.

Here, we comprehensively engineered the central metabolism of *P. pastoris* for overproduction of FFAs (23.4 g/L) and fatty alcohols (2.0 g/L) from methanol by enhancing the supply of precursors and cofactors. A metabolic transforming strategy was developed for the rapid transformation of an FFA cell factory toward fatty alcohols overproduction. The engineering endeavors described here provide insight into methanol metabolism, which should be beneficial for the production of other chemicals from methanol.

Results

Engineering a *P. pastoris* Chassis for FFA Production. We first tried to establish a platform *P. pastoris* strain for FFA overproduction. In yeast, fatty acids are de novo synthesized by cytosolic type I fatty acid synthase (Fas) as fatty acyl-CoAs by using acetyl-CoA as a primer and malonyl-CoA as an extension unit. Trace amounts of FFAs can be synthesized from fatty acyl-CoA through a nonspecific thioesterase (Tes), and excess amounts of FFAs can be rapidly converted back to fatty acyl-CoA under the catalysis of fatty acyl-CoA synthetases (36) (Fig. 2*A*). Consistent with this, the wild-type strain GS115 accumulated only ∼50 mg/L fatty acids using either glucose or methanol as the carbon source (Fig. 2*B*). A search of the genome of *P. pastoris* identified two fatty acyl-CoA synthetase genes, namely, *FAA1* and *FAA2*. Deletion of *FAA1* (strain PC101) significantly improved FFA accumulation and further deletion of *FAA2* (strain PC103) slightly improved FFA production (Fig. 2*B*), which suggested that *FAA1* played the dominant role in FFA activation. Knocking out the fatty acyl-CoA oxidase gene *POX1* to reduce the β-oxidation of fatty acyl-CoA did not increase FFA production. We thus retained *POX1* in subsequent experiments since it may play an important role in peroxisome function. The engineered strain PC103 produced 860 and 415 mg/L FFAs from 20 g/L glucose and 20 g/L methanol with cell densities (OD_{600}) of 15.2 and 8.9, respectively (Fig. 2*B*). This result demonstrated that methanol metabolism was much more rigid for FFA synthesis and needed to be carefully rewired. The percentage of C18:2 fatty acids from cells cultured with methanol was slightly higher than that of cells cultured with glucose, although the unsaturated fatty acids content was more than 65% (Fig. 2*C*).

Rewiring Carbon Metabolism to Improve FFA Production from Methanol. The lower production of FFAs from methanol than from glucose suggested that the metabolic flux needed to be enhanced. We thus used a bottom-up rational strategy to improve the biosynthesis of fatty acids from methanol in *P. pastoris* (Fig. 3*A*). Knocking out *FAA1* and *FAA2* in PC111B, a homologous recombination-enhanced strain PC110 with a genome-integrated *CAS9* gene at *PNSI-3* site (37), exhibited similar FFA production as strain PC103 (Figs. 2*B* and 3*B*).

Acetyl-CoA and malonyl-CoA are the direct precursors of fatty acid synthesis, and one molecule of acetyl-CoA is required for each two-carbon unit extension of the fatty acid carbon chain. Therefore, sufficient metabolic flux of acetyl-CoA and malonyl-CoA is crucial for fatty acid synthesis. We first tried to enhance the supply of acetyl-CoA. Overexpressing ATP:citrate lyase gene (*MmACL*) from *Mus musculus*, catalyzing the decomposition of citric acid to form acetyl-CoA and oxaloacetate

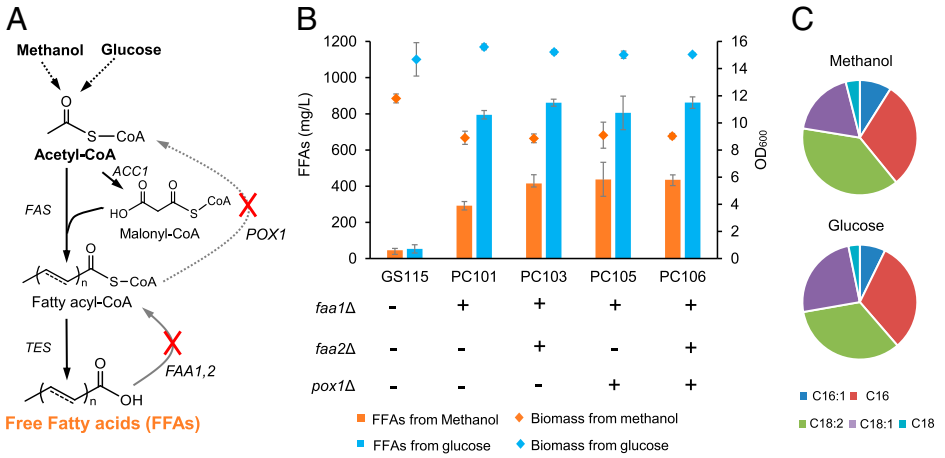

Fig. 2. Blocking FFA activation and consumption in *P. pastoris*. (*A*) Schematic view of the interruption of fatty acids consumption. (*B*) FFA titers of engineered strains in shake flasks after 96 h of cultivation in 20 g/L glucose medium or 120 h of cultivation in 20 g/L methanol medium at 220 rpm and 30 °C. The histogram shows the FFA titers, and the rhombus scatter points show the biomass of engineered strains. (*C*) FFA profiles of the strain PC103 in methanol- and glucose medium. Error bars correspond to the SD of the mean ± SD ($n = 3$, corresponding to three biological replicates).

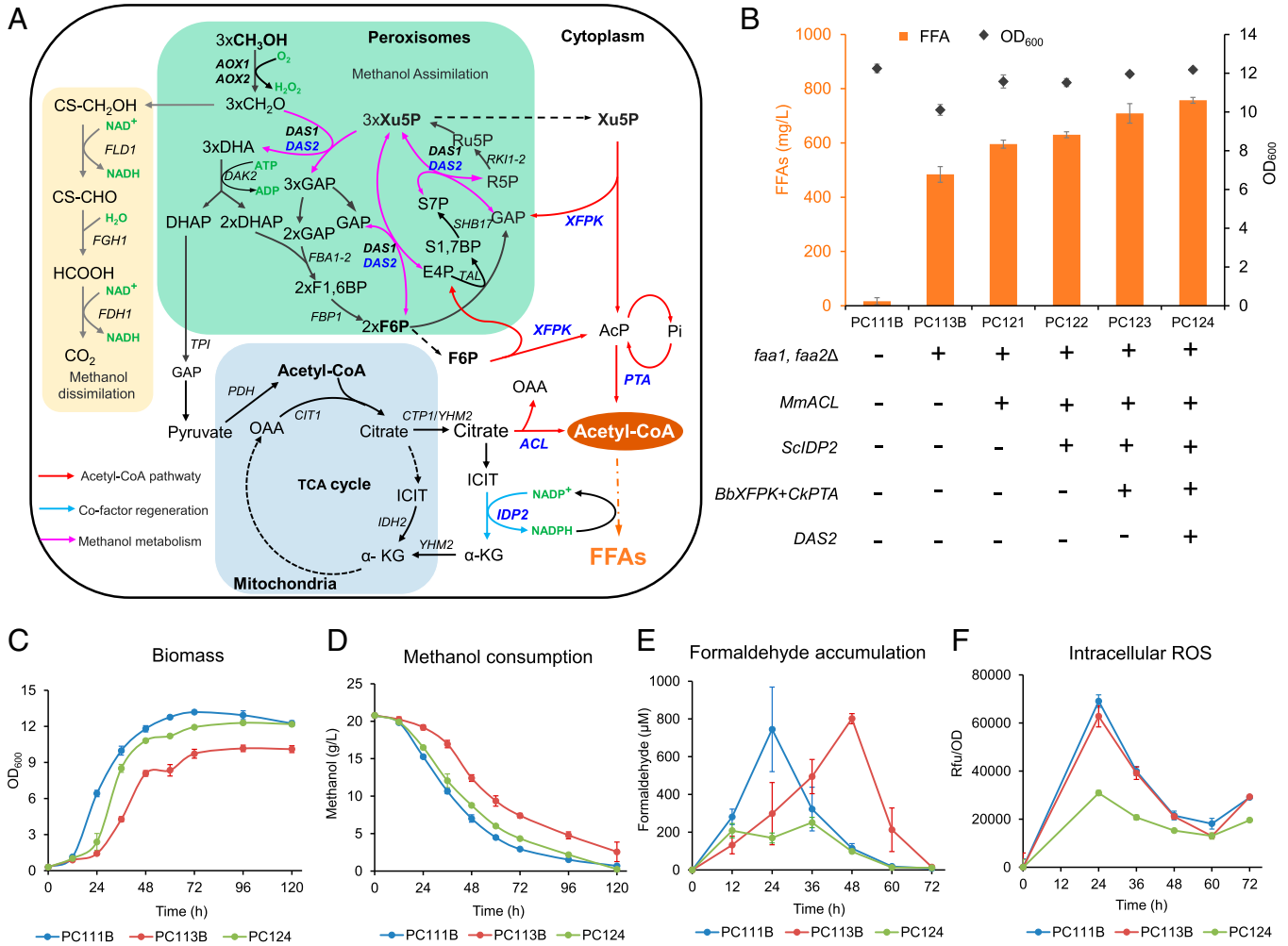

Fig. 3. Metabolic rewiring for FFA production in *P. pastoris*. (*A*) Schematic illustration of rewired central metabolism for improved FFA production from methanol. Overexpressed genes are shown in bright blue. The *Mus musculus* ATP:citrate lyase gene *MmACL* was integrated into the genomic *PNSI-2* site; the *Bifidobacterium breve* phosphoketolase gene *BbXFPK* and *Clostridium kluyveri* phosphotransacetylase gene *CkPTA* were integrated into the genomic *PNSIII-5* site. For NADPH regeneration, the *S. cerevisiae* $NADP^+$-dependent isocitrate dehydrogenase gene *ScIDP2* was integrated at the *PNSI-4* site. Another copy of endogenous *DAS2* was overexpressed at the *PNSII-4* site to improve formaldehyde assimilation. (*B*) FFA production and cell density of strains in shake flasks after 120 h of cultivation at 220 rpm and 30 °C with 20 g/L methanol minimal medium. (*C–F*) Growth curves, methanol consumption, formaldehyde accumulation and intracellular ROS levels. Error bars represent SD of triplicate samples.

(36), improved FFA production by 23% (596 mg/L in strain PC121) compared to PC113B (Fig. 3*B*). The phosphoketolase-phosphotransacetylase (PK-PTA) pathway was hypothesized to be beneficial for enhancing acetyl-CoA supply by diverting carbon flux from glycolysis and pentose phosphate pathway via acetyl phosphate (AcP) (44–46). The heterologous PK-PTA pathway was constructed by expressing *BbPK* from *Bifidobacterium breve* and *CkPTA* from *Clostridium kluyveri*, which significantly increased FFA production to 709 mg/L (strain PC123). The intracellular malonyl-CoA content is usually very low (47). Here, the promoter replacement method was used for malonyl-CoA engineering. Unfortunately, no transformants were obtained either by P_{GAP} or P_{TEF1}, which was speculated to be caused by the cytotoxicity of malonyl-CoA.

Since FFA biosynthesis requires a large amount of NADPH, we also tried several strategies to increase the NADPH supply. The $NADP^+$-dependent isocitrate dehydrogenase (Idp2) is responsible for converting isocitrate to α-ketoglutaric acid and producing NADPH in the cytoplasm (33). Overexpressing *ScIDP2* from *S. cerevisiae* in strain PC122 slightly increased FFA production (Fig. 3*B*). We also constructed an artificial malate cycle in the cytoplasm (36) to improve NADPH supply (*SI Appendix*, Fig. S1*A*), which resulted in a lower FFA production regardless of whether the strong or moderate promoters were used (*SI Appendix*, Fig. S1*B*). Cofactor quantification showed that the *P. pastoris* wild-type strain PC111B had much higher NADPH/$NADP^+$ levels (0.62 at 18 h and 0.29 at 42 h) than *S. cerevisiae* (0.39 at 18 h and 0.04 at 42 h) when cultured in glucose media (*SI Appendix*, Fig. S2). More importantly, methanol-cultured *P. pastoris* PC111B had significantly higher NADPH/$NADP^+$ levels (1.00 at 24 h, and 1.07 at 48 h) than *S. cerevisiae*. Although FFA biosynthesis consumed a large amount of NADPH in strains PC113B and PC124, their NADPH/$NADP^+$ levels were still much higher than that of *S. cerevisiae*. These results suggested that methanol-cultured *P. pastoris* cells had sufficient NADPH supply for FFA biosynthesis, which might explain why the NADPH engineering strategies were successful in *S. cerevisiae* (33) but failed in *P. pastoris*.

We then overexpressed the mitochondrial acid transporter Yhm2 from *S. cerevisiae* (33) to drive metabolic flux through cytoplasmic ATP:citrate lyation and isocitrate dehydrogenation, which, however, dramatically lowered FFA production and biomass yield (*SI Appendix*, Fig. S1*B*). This observation suggested that the metabolic flux of tricarboxylic acid (TCA) cycle in *P. pastoris* was not strong enough when using methanol as carbon source compared

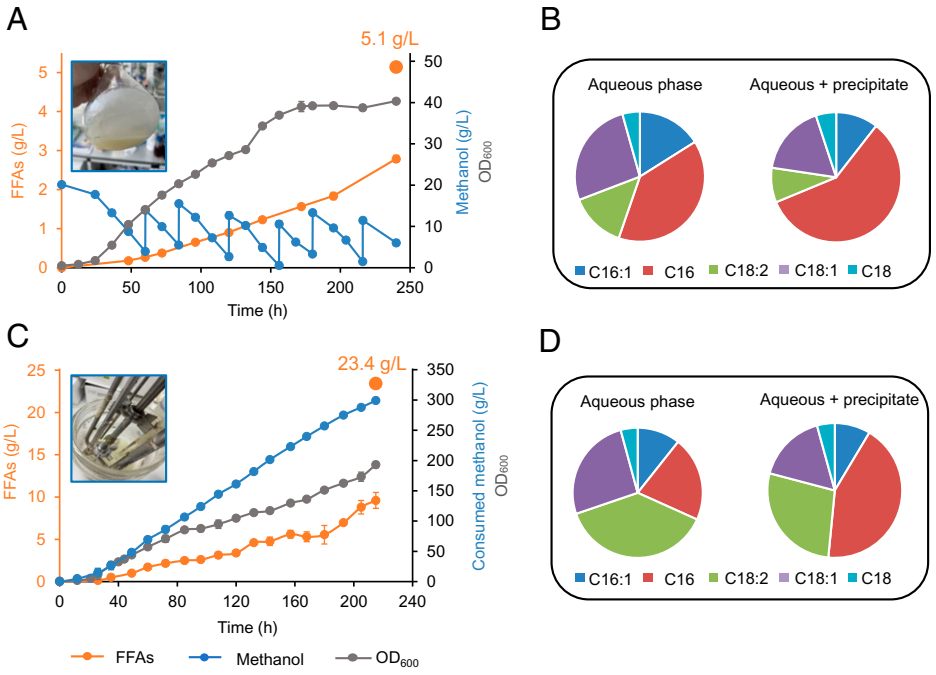

Fig. 4. Fed-batch fermentation for FFA production. Fed-batch fermentation of PC124H in shake flasks (*A*) or 1 L bioreactor (*C*). PC124H is a prototrophic strain with complementation of the *HIS4* marker in PC124. Time courses of fatty acid titers (in orange), growth curves (in gray) and methanol consumption (in blue) are shown. Fatty acids production at the end of fermentation are also shown as filled orange circles. FFA profile of the strain PC124H in shake flasks (*B*) and bioreactors (*D*). Due to the precipitation, ethyl acetate was used for the dissolution of all fatty acids. Error bars correspond to the SD of the mean ($n = 3$ for shake flask fed-batch, corresponding to three biological replicates; $n = 2$ for bioreactor fed-batch, corresponding to two independent biological samples).

to that of the glucose-based TCA cycle in *S. cerevisiae* (33). As xylulose-5-phosphate (Xu5P) is a critical precursor for methanol assimilation in methylotrophic yeast (48), we constructed a compensation pathway for Xu5P supply by expressing the fructose-1,6-bisphosphatase gene (*FBP1*) and D-ribulose-5-phosphate 3-epimerase gene (*RPE1*), as well as the glucose-6-phosphate dehydrogenase gene (*ZWF1*) and 6-phosphogluconate dehydrogenase gene (*GND2*) for NADPH generation (*SI Appendix*, Fig. S3*A*). However, these engineering endeavors resulted in reduced FFA production (*SI Appendix*, Fig. S3*B*).

Formaldehyde is the first intermediate of methanol utilization and confers the main toxicity that impairs cellular robustness (16). Indeed, we found that the FFA-overproducing strain PC113B exhibited decreased cell density (Fig. 3*C*) and methanol utilization (Fig. 3*D*) with higher formaldehyde accumulation (Fig. 3*E*) than the control strain PC111B. This observation suggested that FFA biosynthesis caused cellular stress and drove enhanced methanol oxidation toward a high level of formaldehyde accumulation. Interestingly, engineering the central metabolism to improve the supply of acetyl-CoA and NADPH (strain PC123) resulted in reduced formaldehyde accumulation (*SI Appendix*, Fig. S4), which suggested that a smoother metabolic flux of methanol utilization was driven toward FFA production. To further drive formaldehyde assimilation, we expressed an additional copy of the endogenous dihydroxyacetone synthase gene *DAS2* (strain PC124), which further decreased formaldehyde accumulation with higher biomass yield and methanol utilization (Fig. 3*C*–*E*). In particular, the accumulation of formaldehyde in strain PC124 was much lower than that in the non-FFA producing strain PC111B (Fig. 3*E*). Furthermore, *DAS2* overexpression together with central metabolism rewiring (strain PC124) significantly reduced the reactive oxygen species (ROS) stress (Fig. 3*F*) and improved FFA production (Fig. 3*B*) compared with the parent strain PC113B, which suggested that enhancing formaldehyde assimilation was beneficial for detoxification and improving cellular robustness. These results showed that global rewiring of the central metabolism was essential for driving the metabolic flux toward product biosynthesis with relieving the toxicity caused by methanol oxidation.

Fed-Batch Fermentation for FFA Production. To test the potential of our engineered strain for FFA production from methanol, we carried out fed-batch fermentation in shake flasks and bioreactors by using the prototrophic strain PC124H (in situ restoration of the *HIS4* gene in PC124). In shake-flask fed-batch fermentation, the engineered strain produced 5.1 g/L FFAs with 2.8 g/L in the aqueous phase (Fig. 4*A* and *B*). In the bioreactor process, the FFA titer reached 23.4 g/L (9.6 g/L in the aqueous phase) with a methanol consumption rate of 1.4 g/L/h (Fig. 4*C* and *D*). The FFA yield (0.078 g/g methanol, 24.4% of the theoretical yield) was comparable with the current maximal FFA yield from glucose in *S. cerevisiae* (33), indicating the outstanding potential of our engineered strain for industrial application. Interestingly, the percentage of unsaturated fatty acids (C18:1, C18:2, and C16:1) from bioreactor-cultured cells (54%) was much higher than that of shake flask-cultured cells (Fig. 4*B* and *D*), which might be attributed to the more abundant supply of oxygen in the bioreactor, since oxygen acts as an electron acceptor in the desaturation procedure (49).

Metabolic Transforming for the Production of Fatty Alcohols. Fatty alcohols are widely used as detergents, cosmetic ingredients, pharmaceutical ingredients, and potential substitutes for fossil fuels. We thus explored the de novo synthesis of fatty alcohols from methanol in *P. pastoris*. Two main fatty alcohol biosynthetic pathways have been constructed in *S. cerevisiae* (36, 50): the FFA reduction pathway and the fatty acyl-CoA reduction pathway (Fig. 5*A*). Construction of the FFA reduction pathway in the FFA-overproducing background by expressing the codon-optimized carboxylic acid reductase gene *MmCAR* from *Mycobacterium marinum*, the cofactor 4′-phosphopantetheinyl transferase

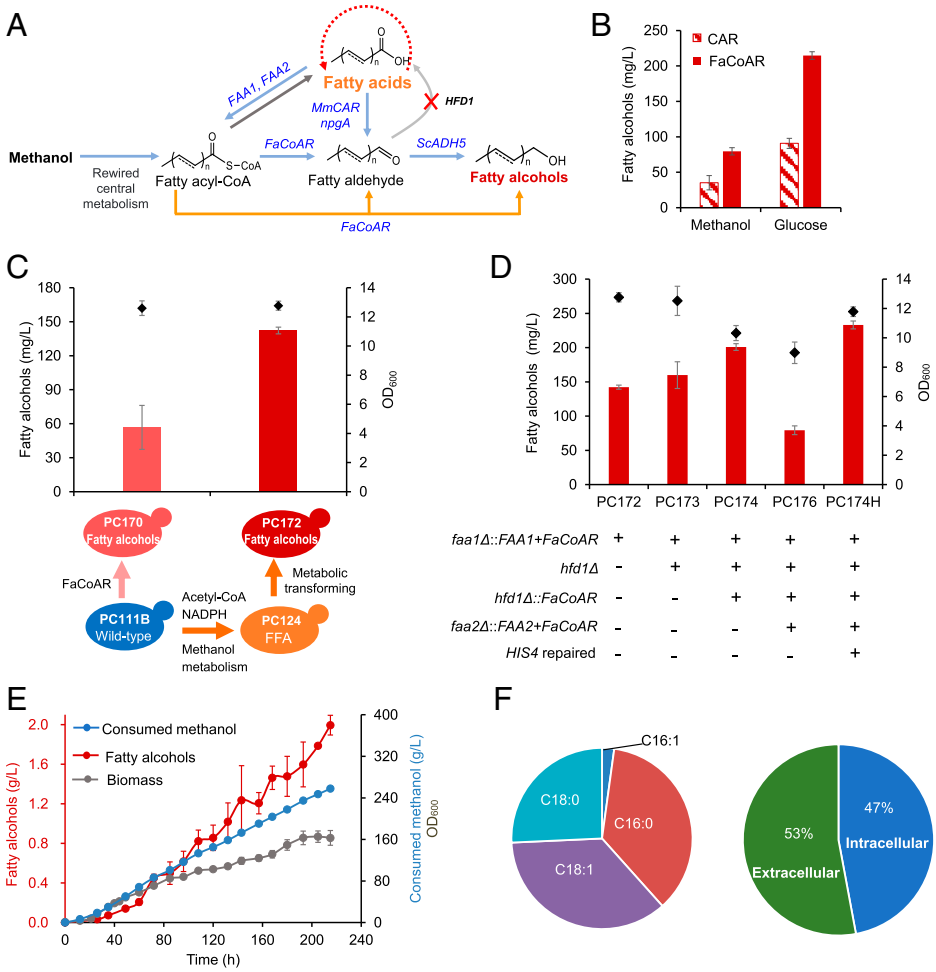

Fig. 5. Metabolic transforming for fatty alcohol production. (*A*) Schematic illustration of metabolic transforming of an FFAs-overproducing strain for overproduction of fatty alcohols. (*B*) Comparison of FFA reduction (*CAR*) and fatty acyl-CoA reduction (*FaCoAR*) pathways for fatty alcohol production from methanol. (*C*) Comparing fatty alcohol production between the wild-type and metabolic rewired strain. One copy of the *FaCoAR* gene was expressed in PC111B (generating strain PC170) and PC124 (generating strain PC172 by simultaneously reintroducing *FAA1*). (*D*) Optimization of fatty acyl-CoA reduction pathway. The engineered strains were cultivated in shake flasks containing 20 g/L methanol for 120 h at 220 rpm and 30 °C. (*E*) Fed-batch fermentation of the prototrophic strain PC174H. Time courses of fatty alcohol titers (deep red), growth curve (gray-purple) and consumption of methanol (blue) are shown. (*F*) The composition (*Left*) and distribution (*Right*) of fatty alcohols from fed-batch fermentation. Error bars correspond to the SD of the mean (n = 3, corresponding to three biological replicates).

gene *NpgA* from *Aspergillus nidulans* and alcohol dehydrogenase *ScADH5* from *S. cerevisiae*, produced 35.2 and 90.8 mg/L fatty alcohols from methanol and glucose, respectively. Expressing the fatty acyl-CoA reductase gene *FaCoAR* resulted in much higher fatty alcohol production than that obtained from FFA reduction pathway (Fig. 5*B*), which was consistent with our previous observation (37). The low titer of fatty alcohols from FFA reduction pathway might be attributed to FFAs secretion, which makes it difficult in access and utilization of the substrate FFAs.

We next tried to enhance the supply of fatty acyl-CoA to improve fatty alcohol biosynthesis by using the fatty acyl-CoA reduction pathway. It is easy to get the idea to use metabolic engineering strategies as described for FFA production such as enhancing the supply of acetyl-CoA, NADPH, and methanol assimilation (Fig. 3*B*), which, however, is time consuming when conducting multiple rounds of genetic manipulation. We thus transformed the FFA-overproducing strain toward fatty alcohol production by restoring *FAA1* and *FAA2* together with genomic integration of *FaCoAR* to their original genomic locus. Consistent with this, integration of one copy of *FaCoAR* with restoration of *FAA1* in the FFA-overproducing strain PC124 resulted in strain PC172 and enabled the production of 142 mg/L fatty alcohols, which was 2.5 times higher than the titer of the wild-type–derived strain PC170 without engineered central metabolism (Fig. 5*C*). Reintroducing *FAA1* diminished FFA accumulation (*SI Appendix*, Fig. S5), again suggesting that *FAA1* plays a major role in activating FFAs in *P. pastoris*. We speculated that *FaCoAR* expression might be a bottleneck in the background of enhanced acetyl-CoA and NADPH levels, and our previous studies showed that deletion of the aldehyde dehydrogenase gene *HFD1* was beneficial for fatty alcohol production by blocking the consumption of fatty aldehydes in *S. cerevisiae* (36, 50). We therefore knocked out *HFD1* by integrating another copy of the *FaCoAR* gene at the *HFD1* site (strain PC174), which improved fatty alcohol production by 41% (201 mg/L), while *HFD1* deletion alone (strain PC173) resulted in only 10% improvement of fatty alcohol production (Fig. 5*D*). Further integration of another copy of the *FaCoAR* gene at the genomic site *FAA2* (strain PC176) resulted in a significant decrease in fatty alcohol production and cell growth. This result suggested that two copies of *FaCoAR* were optimal for transforming cellular fatty acyl-CoA toward fatty alcohol production and that excessive *FaCoAR* expression placed a metabolic burden by draining cellular fatty acyl-CoA.

For fed-batch fermentation, a prototrophic strain PC174H was constructed by in situ restoring the auxotrophic marker gene *HIS4* in PC174, which produced 233 mg/L fatty alcohols in shake-flask

Table 1. Chemical production from methanol in *P. pastoris*

Chemicals	Titer (g/L)	Yield (g/g)	Cultivation condition	Ref.
D-lactic acid	3.48	N.C.*	YPM†, Test tube	(21)
Dammarenediol-II	4.8×10^{-3}	N.C.*	YPM, squalene	(67)
Citrinin	6×10^{-4}	1.2×10^{-4}	Methanol (MM‡)	(22)
6-Methylsalicylic acid	2.2	9×10^{-3}	Glycerol, methanol (MM) Fed-batch	(23)
Chondroitin sulfate	2.1	N.C.*	Glycerol, methanol (MM) Fed-batch	(24)
Lovastatin	0.25	1×10^{-3}	Glycerol, methanol (MM) Fed-batch	(25)
Monacolin J	0.59	2×10^{-3}		
Malic acid	2.8	N.C.*	Methanol, Yeast extract Fed-batch	(26)
(+)-Nootkatone	0.21	5×10^{-4}	Glucose, methanol (MM) Fed-batch	(68)
Fatty alcohols	2.0	8×10^{-3}	Methanol (MM), Fed-batch	This study
Fatty acids	23.4	7.8×10^{-2}	Methanol (MM), Fed-batch	This study

*N.C., not calculated due to the lack of methanol consumption data or containing complex media component such as yeast extract.
†YPM, a nutrient-rich medium containing yeast extract, peptone, and methanol.
‡MM, minimal medium.

cultivation (Fig. 5*D*). Fed-batch cultivation of PC174H in bioreactor resulted in high-level production of fatty alcohols of 2.0 g/L with a methanol consumption rate of 1.2 g/L/h and cell density (OD_{600}) of 163 (Fig. 5*E*). The main components of fatty alcohols (Fig. 5*F*) were hexadecanol (C16:0, 36%), octadecanal (C18:0, 26%), and 9-octadecenal (C18:1, 36%). It is worth mentioning that 53% of the fatty alcohols were secreted (Fig. 5*F*), while previous studies showed that fatty alcohols mainly accumulated in the cells when glucose was used as the carbon source (51–55).

Discussion

Methanol is a promising feedstock for biomanufacturing by linking clean energy storage and CO_2 capture (1). Engineering efficient microbial cell factories is one of the key steps for economical methanol biotransformation. Here, we harnessed the industrially important yeast *P. pastoris* for high-level production of fatty acids and fatty alcohols from sole methanol as the carbon source in minimal media (Table 1) by rewiring the central metabolism and metabolic transforming. We rationally designed and engineered the cellular metabolism using a bottom-up approach: 1) engineering FFA accumulation by blocking FFA consumption; 2) enhancing the supply of precursor acetyl-CoA and cofactor NADPH; and 3) enhancing methanol assimilation. This engineering endeavor resulted in the comparable production of FFAs and fatty alcohols with those from glucose in *S. cerevisiae* (33) and much higher than several other chemicals biosynthesized from methanol (Table 1). Furthermore, the secretion of FFAs and fatty alcohols should be convenient for subsequent separation and purification.

P. pastoris is considered an ideal host for methanol-based biomanufacturing due to its natural ability of methanol utilization. Long-term evolution results in efficient methanol utilization with high-level expression of alcohol oxidase 1 (Aox1) (30). Thus, the promoter of *AOX1* (P_{AOX1}) can drive high-level production of proteins at the gram-per-liter level with methanol induction (56). However, economical production of small molecules requires much higher titers with relatively low costs, which can be realized by global rewiring of the central metabolism and balancing of the biosynthetic pathways. The established efficient genetic engineering tools (18, 56–58) can facilitate comprehensive metabolic engineering of *P. pastoris* as described in this study. For example, we used a combination of methanol-induced and constitutive promoters for pathway construction and optimization (*SI Appendix*, Table S2), which showed good performance in enhancing the production of FFAs and fatty alcohols with marginal effects on cellular fitness.

Currently, it is still challenging in engineering methanol biotransformation toward efficient chemical biosynthesis and the milligram-per-liter level of production is far from the requirement for industrial application (59). As the precursor of fatty acid biosynthesis, acetyl-CoA is a key central intermediate involving several cellular processes with tight regulation (60). Acetyl-CoA is mainly formed from pyruvate by mitochondrial pyruvate dehydrogenase (Pdh) and then enters the TCA cycle, or PDH bypass in the cytoplasm. During glucose metabolism, the TCA cycle is the main process for providing cellular energy, which indicates the relative abundance of acetyl-CoA flux. In methanol metabolism, the main energy source is methanol dissimilation rather than the TCA cycle (48), which suggests the relatively limited availability of acetyl-CoA and more challenges in enhancing the acetyl-CoA supply by using methanol as the carbon source. Indeed, most acetyl-CoA engineering strategies that were successful in improving FFA production from glucose in *S. cerevisiae* (33) failed to improve FFA production from methanol in *P. pastoris*. Only expressing *MmACL* and a PK-PTA pathway significantly improved FFAs biosynthesis, which suggested that the central metabolism was relatively rigid and required exploration of alternative engineering targets.

The cofactor NADPH is also essential for fatty acid biosynthesis, driving two reduction steps (reduction of β-ketoacyl-ACP and enoyl-ACP) during one round of extension. The $NADP^+$-dependent isocitrate dehydrogenase (Idp2) slightly promoted FFA production by enhancing NADPH regeneration. This reaction might compete with citrate that is also used for acetyl-CoA supply, and this carbon-energy trade-off might offset the positive effect on FFA biosynthesis. Here, we observed that *P. pastoris* had much higher cellular NADPH/$NADP^+$ levels than *S. cerevisiae*, which suggested *P. pastoris* is an ideal host for NADPH-dependent chemical biosynthesis.

As described previously, high level of *AOX1* expression is essential for efficient methanol utilization by oxidizing methanol toward the active intermediate formaldehyde (30). However, the accumulation of formaldehyde causes toxicity stress (48), and we found that FFA biosynthesis further exacerbated formaldehyde accumulation. Overexpression of the dihydroxyacetone synthase

gene *DAS2*, for accelerating formaldehyde assimilation, significantly improved FFA production and decreased formaldehyde accumulation to a much lower level than that of the wild-type strain. This observation showed that accelerating formaldehyde utilization is a feasible approach to drive methanol biotransformation.

The construction of microbial cell factories is time-consuming and laborious since it requires multiple rounds of genetic manipulation. Here, we developed a metabolic transforming strategy for rapidly transforming an FFA-overproducing strain toward fatty alcohol overproduction by taking advantage of the enhanced supply of acetyl-CoA and NADPH. Two rounds of genetic manipulation realized the construction of a fatty alcohol overproducer, while building from scratch required six rounds of genetic engineering. Thus, this concept can be easily applied to construct versatile cell factories for production of a variety of chemicals, particularly those with common precursors such as acetyl-CoA, as described here.

In summary, we successfully reprogrammed the metabolism of *P. pastoris* for high-level production of FFAs and fatty alcohols by using methanol as the sole carbon source. The strategies can be used for other methylotrophic yeasts and even other nonconventional microbes, with the rapid expansion of the genetic engineering toolbox (61, 62).

Materials and Methods

Plasmids and Strains. *E. coli* DH5α was used for plasmid propagation, and *P. pastoris* GS115 was used as the host for yeast strains construction. All the plasmids and strains used in this study are shown in *SI Appendix*, Tables S1 and S2, respectively. PC111B derived from PC110 with a Cas9 expression cassette at the neutral site *PNSI-3* (37). The engineering strategy and the flowcharts of yeast strain construction are shown in *SI Appendix*, Fig. S6.

Genetic Manipulation. For construction of gRNA expression plasmids, 20 bp target sequences of gRNAs for genome targeting were designed by using the web toolbox (http://chopchop.cbu.uib.no). pPICZ-Cas9-gPOX1 for targeting *POX1* was constructed as follows. The promoter part and gRNA part were obtained by PCR amplification with primers AOX1t-Kpn-ARS-R/gPOX1-F and HTX1-Cas9-F/gPOX1-R, respectively. The two parts were fused by overlap-extension PCR, and then ligated to the linearized pPICZ-Cas9-gRNA backbone (digested by Kpn I and Spe I) by Gibson cloning. Transformations were screened on LB (with 5 g/L NaCl) agar plate with 25 μg/L Zeocin. pCAI-gFAA1t and pCAI-gFAA2t were constructed as follows. Primers gFAA1t-F/gFAA1t-R and gFAA2t-F/gFAA2t-R were used to amplify the vector skeleton of pCAI-gRNA, and 20 bp target sequences of T_{FAA1} and T_{FAA2} were introduced. PCR products after digested by DpnI were directly transformed into DH5α competent cells. Transformations were screened on LB agar plate with 100 μg/L kanamycin. Other plasmids used in this study have been described in our previous studies (37).

For seamless deletion of *POX1*, 1 kb homologous arms flanking the ORF were amplified from genomic DNA by using the primers POX1-HRUP-F/POX1-HRUP-R1 and POX1-HRDN-F1/POX1-HRDN-R, which were used for constructing the seamless-knockout donor through overlap-extension PCR. The expression cassettes were also constructed by overlap-extension PCR. All the endogenous genes were amplified from the genomic DNA of *P. pastoris* GS115. The gene fragments of *ScPYC1* and *Sc'MDH3* (without mitochondrial localization signal) were amplified from *S. cerevisiae* S288C genomic DNA. *OpRPE1* was amplified from *Ogataea polymorpha* NCYC495 genomic DNA. All the other heterologous genes were codon optimized and chemical synthesized at Genewiz. Custom DNA oligonucleotide primers were synthesized by Sangon Biotech, and the sequences are listed in *SI Appendix*, Table S3. Information of integration sites has been given in our previous study (37).

Strain Cultivation. Shake-flask batch fermentations for production of fatty acids and fatty alcohols were carried out in 100 mL shaking flasks with a working volume of 20 mL minimal medium, which contained 2.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 20 g/L glucose or 20 g/L methanol, trace metal and vitamin solutions supplemented with 40 mg/L histidine if needed. The yeast cells were cultivated at 30 °C, 220 rpm (Zhichu Shaker ZQZY-CS8) for 5 d with initial OD_{600} of 0.3 when using methanol as the carbon source, or 4 d with initial OD_{600} of 0.1 when using glucose as the carbon source. The shake-flask fed-batch fermentations in 250 mL shake conical flasks with an initial working volume of 50 mL and an initial OD_{600} of 0.5. The temperature was set as 30 °C, and agitation was set as 220 rpm. The pH was adjusted to 5.6 by adding 4 M KOH every 24 h. During the fed-batch process, 632 μL of methanol was supplemented each time when the methanol concentration in the medium was lower than 10 g/L. The cells were fed with 0.5 mL 10× supplementary components containing 25 g/L $(NH_4)_2SO_4$, 144 g/L KH_2PO_4, 5 g/L $MgSO_4{\cdot}7H_2O$, 10× trace metal, and 10× vitamin solutions at the same time.

The fed-batch fermentation was conducted in 1.0 L DasGip Parallel Bioreactors System (DasGip) bioreactors with an initial working volume of 300 mL The initial batch stage was conducted with an inoculation OD_{600} of 1.0 in minimal medium containing 2.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 10 g/L methanol, trace metal, and vitamin solutions. The temperature, agitation, aeration, and pH were monitored and controlled using a DasGip Control 4.0 System. The temperature was kept at 30 °C, the initial agitation was set to 400 rpm and increased to maximally 1,200 rpm depending on the dissolved oxygen (DO) level. Aeration was initially set as 36 sL/h and increased to maximally 48 sL/h depending on the DO level. The dissolved oxygen level was maintained above 30%, the pH was kept at pH 5.6 by automatic addition of 4 M KOH and 2 M HCl. During the fed-batch cultivation, the cells were fed with 600 g/L methanol and 10× supplementary components [25 g/L $(NH_4)_2SO_4$, 144 g/L KH_2PO_4, 5 g/L $MgSO_4{\cdot}7H_2O$, 10× trace metal, and 10× vitamin solutions]. The methanol replenishment rate was 1.0 to 1.5 mL/h to keep the methanol concentration below 20 g/L. The feeding speed of supplementary component solution was set as half of feeding speed of methanol solution.

Extraction and Quantification of Products and Metabolites. The extraction and quantification of FFAs were performed as our previous studies (33, 36) with slight modification. For aqueous FFA analysis, 100 μL cell cultures from shake flask were diluted 2-fold with water and those from bioreactor were diluted 10-fold. To analyze total fatty acids including those in precipitation, 5 mL and 100 mL ethyl acetate were added into the shake flask and bioreactor, respectively, and stirred for 30 min to dissolve fatty acid precipitation. The solution was stratified after standing for 10 min. The ethyl acetate phase in the supernatant samples was removed by rotary evaporation after 50-fold dilution. The followed extracted step was manipulated according to the reported methods (36). The extracted methyl esters were resuspended in hexane and analyzed by gas chromatography (Focus GC, Thermo Fisher Scientific) equipped with a Zebron ZB-5MS GUARDIAN capillary column (30 m * 0.25 mm * 0.25 μm; Phenomenex). The GC program was as follows: initial temperature of 40 °C, held for 2 min; ramp to 130 °C at a rate of 30 °C per minute, then raised to 280 °C at a rate of 10 °C per min and held for 3 min. Fatty alcohols were extracted and quantified as a previous report (36, 50) by using 2 mL cell culture from shake flasks and 0.2 mL cell culture from fed-batch fermentation.

The methanol concentration was determined by high-performance liquid chromatography analysis. One milliliter broth sample was centrifuged at 12,000 × g for 10 min and then filtered through a 0.2 μm syringe filter and analyzed with an Aminex HPX-87G column (Bio-Rad) on a 1260 infinity II HPLC (Agilent) equipped with a differential refractive index detector. The samples were eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min at 50 °C for 26 min. Formaldehyde was quantified as described in reference (63) with slight modification. Briefly, 0.2 mL samples were mixed with 0.2 mL 10% trichloroacetic acid, 0.1 mL 2,4-dinitrophenylhydrazine (DNPH, 0.1 g/L) and 0.5 mL acetonitrile via vigorous vortexing at 1,600 rpm for 1 min, then incubated in a 60 °C water bath for 30 min. After incubation, samples were centrifuged at 12,000 × g for 10 min and filtered through a 0.2 μm syringe filter. Formaldehyde-DNPH was then quantified by using an HPLC (LC-2030, Shimadzu) equipped with an UV detector and a C18 reversed-phase column, with 65% acetonitrile as the mobile phase.

Quantification of Cellular NADP(H). The quantification method was modified from the previous references (64, 65). About 4×10^8 cells of each sample were collected and washed twice with cold PBS. Cells were centrifuged (4,000 × g at 4 °C for 5 min) and immediately resuspended in 0.3 mL of 0.2 M HCl (for

$NADP^+$) or 0.2 M NaOH (for NADPH). The cells were vigorously shaken with appropriate amount of glass beads to crush the cells at low temperature, and then incubated at 60 °C for 30 min. Next, 0.3 mL of 0.2 M NaOH (for $NADP^+$) and 0.2 M HCl (for NADPH) were added for the neutralization reaction. Samples were centrifuged at 12,000 × g for 5 min.

The cycling assay (*SI Appendix*, Fig. S2*A*) was performed by using a reagent mixture consisting of equal volumes of 1.0 M Bicine buffer (pH 8.0), 30 mM glucose-6-phosphate, 40 mM EDTA (pH 8.0), 4.2 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), twice the volume of 16.6 mM phenazine ethosulfate, and three volumes of water, previously incubated at 25 °C. The following volumes were added to 96-well plates: 10 μL neutralized extract, 180 μL reagent mixture. At last, the reaction was started by adding 10 μL of yeast glucose-6-phosphate dehydrogenase (200 U/mL, A003981, Sangon Biotech). The absorbance at 570 nm was recorded for 2 min at 25 °C by microplate reader (Tecan Spark 3). All fluorescence measurements were normalized to the protein concentration which was calculated by the BCA assay kit (Sangon Biotech).

ROS Measurement. The cellular ROS level was estimated by using the oxidant-sensitive probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as described previously (66). Samples were collected every 12 h during the first 3 d of cultivation and washed twice with distilled water. The cells were then resuspended in 0.5 mL 10 mM PBS (pH 7.0) containing 10 μM DCFH-DA, and incubated at 37 °C for 30 min. Fluorescence was measured with λEX 485 nm and λEM 525 nm using a SPARK multifunctional microplate platform (Tecan) with a gain value of 50.

Data Availability. All study data are included in the article and/or supporting information.

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