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Published in:
Nature Communications

Link to article, DOI:
10.1038/ncomms15587

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Citation (APA):
Yu, T., Zhou, Y. J., Wenning, L., Liu, Q., Krivoruchko, A., Siewers, V., Nielsen, J., & David, F. (2017). Metabolic engineering of Saccharomyces cerevisiae for production of very long chain fatty acid-derived chemicals. Nature Communications, 8, Article 15587. https://doi.org/10.1038/ncomms15587

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Metabolic engineering of *Saccharomyces cerevisiae* for production of very long chain fatty acid-derived chemicals

Tao Yu¹², Yongjin J. Zhou¹²†, Leonie Wenning¹², Quanli Liu¹², Anastasia Krivoruchko¹²³, Verena Siewers¹², Jens Nielsen¹²⁴⁵ & Florian David¹²³

Production of chemicals and biofuels through microbial fermentation is an economical and sustainable alternative for traditional chemical synthesis. Here we present the construction of a *Saccharomyces cerevisiae* platform strain for high-level production of very-long-chain fatty acid (VLCFA)-derived chemicals. Through rewiring the native fatty acid elongation system and implementing a heterologous *Mycobacteria* FAS I system, we establish an increased biosynthesis of VLCFAs in *S. cerevisiae*. VLCFAs can be selectively modified towards the fatty alcohol docosanol (C22H₄₆O) by expressing a specific fatty acid reductase. Expression of this enzyme is shown to impair cell growth due to consumption of VLCFA-CoAs. We therefore implement a dynamic control strategy for separating cell growth from docosanol production. We successfully establish high-level and selective docosanol production of 83.5 mg l⁻¹ in yeast. This approach will provide a universal strategy towards the production of similar high value chemicals in a more scalable, stable and sustainable manner.

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Very-long-chain fatty acids (VLCFAs; 22–26 carbons) are essential biological components, which are incorporated in triacylglycerol molecules, sphingolipids, cuticle or waxes. VLCFAs such as erucic acid, as well as VLCFA-derived products such as very-long-chain fatty alcohols (VLCFAlc) (for example, docosanol) and very-long-chain fatty waxes (for example, Jojoba oil) represent important classes of valuable chemicals, which are widely used as lubricants, detergents, polymers, photographic film-processing agents, coatings, cosmetics and pharmaceuticals.

VLCFA chemicals are mainly extracted from natural sources or synthesized from petrochemical feedstocks. For natural sources, such as vegetable oils, the content of VLCFA derivatives is usually very low and only limited sources are available. When produced from petrochemical feedstocks, VLCFAs are synthesized chemically through oligomerization of ethylene followed by other modifications, which causes production cost increase with chain length. Thus, a more scalable, stable and sustainable production route is needed. One promising solution is microbial production of VLCFAs and their derivatives from renewable feedstocks.

Here we engineered the FA metabolism for production of these VLCFA chemicals by the yeast Saccharomyces cerevisiae, a robust and industrially established microorganism for sustainable production of several products on the market. As an initial target product we focused on docosanol, a saturated VLCFA with a chain length of 22 carbons, which is used as an emollient, emulsifier, thickener in cosmetics and nutritional supplements with a market volume of 40,000 ton per year. Furthermore, docosanol has been approved by the Food and Drug Administration as a pharmaceutical antiviral agent for reducing the duration of cold sores caused by the herpes simplex virus.

Administration as a pharmaceutical antiviral agent for reducing docosanol has been approved by the Food and Drug Administration and industrially established microorganism for sustainable production of VLCFAs and derived products (Fig. 1).

Results

VLCFA chain length control by FA elongation system.

Normally, yeast generates very low amounts of VLCFAs (≥ C20). They are derived from the major FA components of C16/18 LCFAs through several catalytic steps carried out by the intrinsic elongation system. β-Ketoacyl-CoA synthases, also known as elongases, catalyse the first and rate-limiting step of the elongation process by condensing acyl-CoA with malonyl-CoA building blocks. These elongases determine the substrate specificity and final product profile of the whole elongation system. In S. cerevisiae, the elongases Elo1, Elo2 and Elo3 are required for VLCFA synthesis. These elongases have different substrate and product specificities: Elo1 has specificity for the elongation of C12–16 to C16–18 FAs, whereas Elo2 elongates C16–18 up to C22 and Elo3 elongates C18 up to C26 (refs 18,24). We thus modulated these elongase genes for enhancing VLCFAs pools with chain length ≥ C22.

We first engineered the VLCFAs biosynthesis in the LCFCA-overproducing strain JV03, in which the LCFA-CoA-consuming pathways including β-oxidation, triacylglycerol (TAG) and sterol ester synthesis were deleted (CEN.PK 113-5D are1Δ dga1Δ are2Δ bpa1Δ pox1Δ, Fig. 2a). This strain JV03 produced a small fraction of C26, whereas C22 and C24 VLCFAs were not detectable (Supplementary Fig. 1a). To drive the FA pool towards C22, we deleted the ELO3 gene to block C26 biosynthesis, which led to production of C22 VLCFAs (strain TY001, Supplementary Figs 1b and 2b). Subsequently, the ELO2 gene was overexpressed in JV03 elo3Δ strain (TY002, Supplementary Table 5), which further increased the C22 VLCFA level by almost 1.5-fold (Fig. 2b). On the other hand, when overexpressing the ELO3 gene in the JV03 background strain TY003, the C26 VLCFA level increased almost twofold (Supplementary Fig. 1d). These
results clearly showed that the chain length of VLCFAs could be selectively modulated by engineering the intrinsic yeast elongation system. Despite using an engineered strain, the overall production of C22 FAs was lower than 1 mg g⁻¹ dry cell weight (DCW).

VLCFAs chain length control by Mycobacterium FAS I system. To overcome the limitation of the intrinsic yeast elongation system, we focused on implementing the mycobacterial FAS system into yeast. In mycobacteria, this multimeric enzyme produces C₁₆/C₁₈ FAs for the intrinsic cellular demand, as well as system FAS I into yeast. In mycobacteria, this multimeric enzyme system, we focused on implementing the mycobacterial FAS to overcome the limitation of the intrinsic yeast elongation system. Despite using an engineered strain, the overall production of C22 FAs was lower than 1 mg g⁻¹ dry cell weight (DCW).

VLCFAs are specifically elongated to a VLCFA of choice via the intrinsic elongation system or Mycobacteria FAS I (MbFAS I) and further modified to the target product of interest. Product selectivity is determined by choice of enzymes and background strain.
Statistical analysis was performed using a Student's t-test (one-tailed; *P < 0.05; **P < 0.01 and ***P < 0.001; two-sample unequal variance). At least two independent measurements were performed for each experiment and the mean ± s.d. of three biological replicates of a representative measurement is shown. All cells were grown as described in experimental procedures.

FAS and (2) using the exogenous MvFAS circumvents the intrinsic regulation mechanisms. Thus, the mycobacterial FAS I system allows for efficient production of specific VLCFAs. In the future, other VLCFAs could be produced via selectively choosing the particular mycobacterial FAS system that corresponds to the VLCFA chain length desired, as summarized in Supplementary Table 1.

Selection of FA reductase for docosanol production. After establishing the VLCFAs biosynthesis platform, we aimed to engineer it towards docosanol production, a high-value VLCFA-derived chemical (Fig. 3a). For this purpose, two main challenges have to be faced: first, the low level of VLCFAs asks for efficient ‘terminal enzyme(s)’ to convert VLCFAs towards relevant chemicals. Second, with much higher levels of cellular C16 and C18 fatty acyl-CoAs, the terminal enzyme(s) should have high catalytic specificity. For docosanol production, we tested five fatty

Figure 2 | Engineering production of C_{22} VLCFAs by FA chain-length control in yeast. (a) Schematic overview of two strategies for chain length control towards VLCFAs. The dotted lines indicate multiple steps and solid lines a single step. Overexpressed genes are shown in blue (endogenous) or green (heterologous). Competitive pathways were eliminated by deleting corresponding genes (marked with X). Elo1, Elo2 and Elo3, yeast FA elongases 1, 2 and 3; MbFAS, FAS I system from Mycobacterium; ScFAS, S. cerevisiae FAS. (b) GC–MS analysis of docosanoic acid (C_{22}O) generated by yeast elongation system in strains TY001 (JV03 elo3Δ) and TY002 (JV03 elo3Δ pELO2). Statistic analysis was performed using a Student’s t-test (one-tailed; *P < 0.05; **P < 0.01 and ***P < 0.001; two-sample unequal variance). At least two independent measurements were performed for each experiment and the mean ± s.d. of three biological replicates of a representative measurement is shown. (c) GC–MS analysis of docosanoic acid generated in strain TDY7005 (MATa lys2 ura3–52 trp1Δ elo2Δ elo2Δ::kanMX elo3Δ::TRP1/pELO3) and strain TY004 (MATa lys2 ura3–52 trp1Δ elo2Δ elo2Δ::kanMX elo3Δ::TRP1/pGPD415-MvFAS-Acp5)). Statistical analysis was performed using a Student’s t-test (one-tailed; *P < 0.05. **P < 0.01 and ***P < 0.001; two-sample unequal variance). At least two independent measurements were performed for each experiment and the mean ± s.d. of three biological replicates of a representative measurement is shown. All cells were grown as described in experimental procedures.

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Figure 3 | Engineering production of docosanol (C_{22}H_{46}O) in yeast. (a) Schematic biosynthetic pathway for docosanol. AtFAR, fatty acyl-CoA reductase from A. thaliana (AT5g22500). (b) GC–MS analysis of docosanol production in TY002 (JV03 elo3Δ pELO2) with or without expression of atfar. Statistical analysis was performed using a Student’s t-test (one-tailed; *P < 0.05, **P < 0.01 and ***P < 0.001; two-sample unequal variance). At least two independent measurements were performed for each experiment and the mean ± s.d. of three biological replicates of a representative measurement is shown. (c) Final OD_{600} of strains TY002 and TY012, with or without expression of the fatty acyl-CoA reductase gene atfar. Statistical analysis was performed using a Student’s t-test (one-tailed; *P < 0.05, **P < 0.01 and ***P < 0.001; two-sample unequal variance). At least two independent measurements were performed for each experiment and the mean ± s.d. of three biological replicates of a representative measurement is shown. (d) VLCFA chain-length profiles of strain TY002 (JV03 elo3Δ pELO2). IS (internal standard). (e) VLCFAic profiles of docosanol producing strain TY012 (JV03 elo3Δ pVLCFAic07). Typical GC–MS total ion chromatograms (TIC) of total FAs or fatty alcohols extracted from strain TY002 (retention time from 16 to 21 min) and strain TY012 (retention time from 16 to 21 min). FAME and fatty alcohols were identified by retention time and comparison with the mass spectral library. All cells were grown as described under experimental procedures.
acyl-CoA reductases that are capable of specifically converting VLCFA-CoAs towards VLCFAcals (AmFAR from Apis mellifera39, AtFAR from Arabidopsis thaliana36, CIFAR from Calanus finmarchicus39, ScFAR from Simmondsia chinesis30 and TaFAR from Triticum aestivum31, detailed description in Supplementary Table 8). We compared their activity in the strains with increased C22-CoA pools. TY001 (JV03 elo3Δ) and TY002 (JV03 elo3Δ pELO2) were transformed with the respective plasmids resulting in strains TY006–TY015 (Supplementary Table 2). The fatty alcohol profile analysis revealed that most of the reductases tested did not show any detectable docosanol production, probably due to low conversion efficiency and specificity (Supplementary Table 8). Only atfar expression in strain TY002 led to the production of detectable amounts of 1.1 mg l⁻¹ docosanol (Fig. 3b and Supplementary Table 2). Interestingly, no viable transformants were obtained when attempting to introduce atfar expressing plasmid to the strain TY001 (JV03 elo3Δ) (without ELO2 overexpression) (Supplementary Table 2). This might be attributed to the fact that the AtFAR-deprived essential C22-CoA towards docosanol biosynthesis. ELO2 overexpression in strain TY012 (JV03 elo3Δ pVLCFAlc07) restored the cell growth possibly by providing higher levels of VLCFAs (Fig. 2b) for both docosanol biosynthesis and cell growth. Still, the biomass yield was much lower (final OD₆₀₀ = 0.3) than for the control strain without expressing atfar (final OD₆₀₀ = 6.0) (Fig. 3c). These results suggested that the docosanol biosynthesis deprived the cell of VLCFAs for cell growth. The metabolic flux therefore should be carefully balanced, to enable better cell proliferation and higher docosanol production. Furthermore, atfar expression in yeast resulted in specific production of docosanol (Fig. 3d,e), proving the high selectivity of this enzyme. This specificity was also shown resulting in specific production of docosanol (Fig. 3d,e), proving the concept of docosanol production in yeast (Figs 3b and 4a). However, the titres remained very low due to the impaired cell growth caused by deprivation of essential precursors C22-CoA. Thus, we needed to fine tune the expression of these genes to balance cell growth and product formation. To reach this goal, we designed a dynamic control system where cell growth and docosanol production were separated into two different phases by using carbon source-dependent promoters (Fig. 4b and Supplementary Fig. 8). To make the system as stringent and robust as possible, we tested the strategies in JV03 elo3Δ strain. As a first attempt to increase cell growth with atfar expression, we expressed ELO3 under the control of HXT1 promoter, which is activated by high level of glucose and becomes repressed as the level of glucose decreases33. For atfar expression, we selected HXT7, ADH2, ICL1 and GAL1 promoters33, which are repressed by high level of glucose and activated when the cells are in the ethanol phase or exposed to galactose as inducer, respectively (Fig. 4b). In addition, to evaluate constitutive expression of the atfar gene, we also tested the TDH3 and TEF1 promoters. The results of this screening are summarized in Supplementary Table 3 and highlighted in Fig. 4c (strains TY016-TY027). The strain TY018, having a deletion of ELO3 and atfar expressed under GAL1 promoter control, showed the highest docosanol production of 4.2 mg l⁻¹, which is fourfold higher compared with the previous proof-of-concept strain TY012 (Figs 3b and 4c). The biomass yield was almost 80% of the control strain TY001 (without atfar expression). 

**Dynamic control system to increase docosanol production.** We successfully demonstrated the concept of docosanol production in yeast (Figs 3b and 4a). However, the titres remained very low due to the impaired cell growth caused by deprivation of essential precursors C22-CoA. Thus, we needed to fine tune the expression of these genes to balance cell growth and product formation. To reach this goal, we designed a dynamic control system where cell growth and docosanol production were separated into two different phases by using carbon source-dependent promoters (Fig. 4b and Supplementary Fig. 8). To make the system as stringent and robust as possible, we tested the strategies in JV03 elo3Δ strain. As a first attempt to increase cell growth with atfar expression, we expressed ELO3 under the control of HXT1 promoter, which is activated by high level of glucose and becomes repressed as the level of glucose decreases33. For atfar expression, we selected HXT7, ADH2, ICL1 and GAL1 promoters33, which are repressed by high level of glucose and activated when the cells are in the ethanol phase or exposed to galactose as inducer, respectively (Fig. 4b). In addition, to evaluate constitutive expression of the atfar gene, we also tested the TDH3 and TEF1 promoters. The results of this screening are summarized in Supplementary Table 3 and highlighted in Fig. 4c (strains TY016-TY027). The strain TY018, having a deletion of ELO3 and atfar expressed under GAL1 promoter control, showed the highest docosanol production of 4.2 mg l⁻¹, which is fourfold higher compared with the previous proof-of-concept strain TY012 (Figs 3b and 4c). The biomass yield was almost 80% of the control strain TY001 (without atfar expression). Further expression of ELO3 under HXT1 promoter control completely restored the cell growth, but docosanol biosynthesis was totally abolished. In the strains carrying HXT1p-ELO3, tuning the atfar expression with promoters HXT7p or ADH2p resulted in docosanol production of 0.8 and 0.2 mg l⁻¹, respectively, which was much lower than the TY018 strain. These results indicated that ELO3 disruption is essential for supplying C22-CoA for docosanol production and a strict growth phase-dependent control of atfar is necessary for fine tuning cell growth and increasing final docosanol titres. In conclusion, the dynamic control of gene expression successfully solved the growth defect caused by precursor deprivation which laid the foundation for high-level production of docosanol.

**Efficient pathway reconstruction for production of docosanol.** Although FA metabolism was successfully engineered for docosanol production, the titre needs to be further improved. Owing to the non-functional FA storage and degradation, the growth rate and final cell biomass of the JV03 background strain are lower than that of wild-type strain35. We thus re-engineered FA elongation and reduction towards docosanol production in the more robust background strain CEN.PK113-5D. With the recently established CRISPR/Cas9 technology34,35 and modular pathway engineering36 for yeast genetic engineering, we could easily transfer our metabolic engineering strategy to different genetic background strains. Here we mainly focused on integrating the genes into chromosome instead of using episomal expression to get more stable genotypes37. We reconstructed the entire pathway for docosanol production introducing modifications regarding chain-length control, terminal FAR enzyme expression and further optimization using the dynamic control system (Fig. 5a). First, we deleted ELO3 gene and integrated the atfar gene under GAL1 promoter control at the site of ELO3 simultaneously; the resulting strain TY028 produced 0.65 mg l⁻¹ docosanol. Further overexpressing ELO2 gene under the control of GAL10 promoter (strain TY029) improved the docosanol production by 4-fold (2.4 mg l⁻¹) compared with the previous strain TY028. To provide more acyl-CoA precursors for the endoplasmic reticulum-localized yeast elongation system, we overexpressed ELO1 gene under GAL7 promoter (strain TY030, Supplementary Fig. 9). However, this modification had only a marginal effect on docosanol production (Fig. 5b), suggesting that precursor supply for the elongation system in terms of acyl-CoAs seemed not to be the rate limiting step at this point. In parallel, we aimed to further increase FA production by integrating and overexpressing a constitutive active version of the ACC1** enzyme (Accr5659A.S1157A) in strain TY028, thereby leading to an increased precursor supply of malonyl-CoA38. At the same time, we implemented the deletion of the GAL1 gene to avoid potential inducer consumption and ensure stable GAL1 promoter performance39. The resulting strain TY033 could generate 1.4 mg l⁻¹ docosanol, more than a twofold increase compared with the previous strain TY028. If further combined with the overexpression of ELO2 and ELO1, the docosanol production was remarkably increased to 5.7 mg l⁻¹ (strain TY034) and 51 mg l⁻¹ (strain TY035), respectively (Fig. 5b). This highlights that upregulating the elongation system, especially ELO1, only becomes beneficial when sufficient malonyl-CoA precursors for FA production are provided. By implementing the MvFAS system, the production level of docosanol was further increased. TY036, which provided increased malonyl-CoA precursor supply through ACC1** overexpression, generated docosanol up to 40 mg l⁻¹. When combining both the yeast elongation system and the MvFAS strategy in strain TY037, the level of docosanol...
was increased to 83.5 mg l⁻¹, an almost 80-fold increase compared with the proof-of-concept strain TY012 (JV03 elo3Δ pVLCFAH07) (Figs 3b and 5b). Even after 80-fold improvement in production, docosanol is still the major fatty alcohol peak (Supplementary Fig. 5), demonstrating the high potential and selectivity of the implemented docosanol production pathway.

**Discussion**

VLCFAs and their derivatives are important precursors for valuable chemicals used in the food, drug, chemical and cosmetic industry. Here we established a yeast-based production platform for the production of high-value chemical docosanol. This was accomplished by overexpressing a highly active and specific FAR
enzyme to generate VLCFAcs and by controlling FA chain length towards VLCFAs.

During the last years there have been several reports on engineering FA metabolism for long-chain FA-derived chemical and biofuel production\textsuperscript{10,11,40–42} with the main focus on introducing heterologous pathways to transform cellular abundant FAs (mainly C\textsubscript{16}/\textsubscript{18}). Here we succeeded to engineer FA metabolism towards VLCFA-derived chemical production. Unlike abundant C\textsubscript{16}/\textsubscript{18} FAs, the VLCFAs biosynthesis is strictly regulated, which makes it challenging to redirect the cellular metabolism towards the production of VLCFA-derived chemicals with specific chain length, such as docosanol. In our study, single modification of VLCFA biosynthesis using the mycobacterial MvFAS resulted in an accumulation of only C\textsubscript{26} FA, but no C\textsubscript{22} and C\textsubscript{24} FAs (Supplementary Fig. 3). This suggests that the cellular elongation system is highly interconnected with the heterologous FAS. Thus, for the production of specific VLCFA chemicals, the intrinsic elongation system should be carefully tuned, depending on the target product of interest. Here we found that disruption of ELO3 and overexpression of ELO1 and ELO2 are the best combination for increased docosanol production. However, engineering the elongation system only produced small amounts of docosanol (1.1 mg l\textsuperscript{-1}, Fig. 3b). By additionally introducing the MvFAS system, as well as enhancing the precursor supply, we significantly improved docosanol production by 80-fold to 83.5 mg l\textsuperscript{-1} (Fig. 5b). A heterologous mycobacterial FAS I system was successfully constructed by fusing the phosphopantetheinyl transferase domain with the main FAS enzyme and was proven to be functional and efficient for VLCFA biosynthesis, and to enable high-level docosanol production in yeast (Fig. 5b).

Another challenge for producing VLCFA-derived chemicals is the need for a ‘terminal enzyme’ to selectively transform the precursor VLCFA-CoA into a relevant product, as VLCFA-CoA levels are much lower compared with C\textsubscript{16}/\textsubscript{18}-CoAs. Thus, an efficient and specific FAR was selected, enabling the high level and targeted production of docosanol (Fig. 3e and Supplementary Fig. 5b). However, an efficient FAR might drain VLCFA-CoA precursors towards the end product, resulting in insufficient VLCFAs supply for cell growth. Correspondingly, we observed that constitutive expression of atfar decreased the biomass yield significantly (Fig. 3c). This is counterproductive as a certain amount of biomass is necessary to enable high-level production of the product of interest. The problem was solved by fine-tuning target gene expression using galactose-inducible promoters, thereby dynamically controlling the product pathway and separating the cell growth from the docosanol production phase. Targeted pathway overexpression and control via GAL promoters were previously used to optimize artemisinin production. The strategy was applied to avoid build-up of any potentially toxic intermediates in the mevalonate pathway, which may have resulted in strain instability\textsuperscript{39}. Furthermore, in our study the strains benefited from this effect. When implementing the constitutively active version of the Acc1 enzyme, high levels of precursor are generated but at the same time this strain shows high instability\textsuperscript{39}, possibly due to significant deprivation of the acetyl-CoA pool. To further improve docosanol production in the future, different strategies for increasing cytosolic acetyl-CoA supply could be evaluated\textsuperscript{43}.

Recently, several studies showed the reconstruction of fatty acyl-CoA\textsuperscript{16,44} or FA\textsuperscript{10} reduction pathways in S. cerevisiae, which enabled the production of about 100–300 mg l\textsuperscript{-1} fatty alcohols with a chain-length mixture of C\textsubscript{10}–C\textsubscript{18}. Although facing additional challenges when producing VLCFAs and derived products, we reached a similar range in our studies with a final docosanol titre of 83.5 mg l\textsuperscript{-1} with high purity.

These results clearly demonstrate that we were successful in establishing and combining three main platforms facilitating targeted VLCFAs and derived chemicals production in yeast (Fig. 1): (1) providing increased precursor supply for FA production, (2) tuning of yeast elongation system and choice of mycobacterial FAS I system for targeted chain length control of VLCFAs, and (3) choice of terminal enzyme and dynamic pathway control to generate the final product of interest. Our findings suggests that other chain length VLCFAs could be produced in a similar manner by tuning endogenous elongases, along with selection of the appropriate mycobacterial FAS I system. Depending on the background strains used and the terminal enzyme of choice, this technology would enable the production of various VLCFAs and derived products. These can be used for both bulk and specialty chemicals. In biobased production, the rate and yield on glucose are the main determinants for production costs, and hereby determining the cost competitiveness on the market. For low-priced bulk chemicals such as, for example, erucic acid, one has to operate near the theoretical yield, whereas for high-priced products, for example, waxes for cosmetics, lower yields are acceptable. At the same time, for products with higher chemical complexity more engineering has to be done to facilitate production. We show in this study that we were able to establish a production platform where we targeted several modifications, such as FA chain length and specific conversion to a C22 fatty alcohol, and even though we reach reasonable titres, further improvements are required to reach a commercial process.

However, our proof-of-principle demonstration that yeast can be used for production of long-chain fatty alcohols will facilitate the construction of microbial cell factories for the production of various new products in this area, in particular considering that industry favours the use of yeast as a robust and industrially established production host.

**Methods**

**Strains and reagents.** Primers, plasmids and S. cerevisiae strains used are listed in Supplementary Tables 4, 5 and 7. PrimeStar DNA polymerase was purchased from Takara Bio. Taq DNA polymerase, restriction enzymes, DNA gel purification and plasmid extraction kits were purchased from Thermo Scientific. The Gibson Assembly Cloning Kit was purchased from NEB. Yeast plasmid Miniprep 1 kits were purchased from Zymo Research. All oligonucleotides (Supplementary Table 4) were synthesized at Sigma-Aldrich. All chemicals including analytical standards were purchased from Sigma-Aldrich, unless stated otherwise. All codon optimized heterologous genes were synthesized (Genscript) and listed in Supplementary Table 6.

**Strain cultivation.** Yeast strains for preparation of competent cells were cultivated in YPD consisting of 10 g l\textsuperscript{-1} extract (Merck Millipore), 20 g l\textsuperscript{-1} peptone, (Difco) and 20 g l\textsuperscript{-1} sugar (Merk Millipore). Strains containing URA3 marker-based plasmids or cassettes were selected on synthetic complete media without uracil, which consisted of 6.7 g l\textsuperscript{-1} yeast nitrogen base without amino acids (Formedium), 0.77 g l\textsuperscript{-1} CSM without uracil (Formedium), 20 g l\textsuperscript{-1} glucose (Merk Millipore) and 20 g l\textsuperscript{-1} agar (Merck Millipore). The URA3 maker was removed and selected against on synthetic complete media + 5-FOA plates, which contained 6.7 g l\textsuperscript{-1} yeast nitrogen base, 0.77 g l\textsuperscript{-1} CSM and 0.8 g l\textsuperscript{-1} -5-FOA. Strains containing the kanMX cassette were selected on YPD plates containing 200 mg l\textsuperscript{-1} G418 (Formedium). Shake flask batch fermentations for the production of fatty alcohols were carried out in minimal medium\textsuperscript{10,45} containing 30 g l\textsuperscript{-1} glucose with or without 0.5% galactose supplemented with 60 mg l\textsuperscript{-1} uracil if needed. Cultures were inoculated, from 24 h precultures, at initial OD\textsubscript{600} of 0.05 in 15 ml minimal medium and cultivated at 200 r.p.m., 30°C for 72 h. For Afla/Asfa2 strain, C\textsubscript{16}p (5 mM palmitic acid) and C\textsubscript{18}s (5 mM stearic acid) free FAs were dissolved in ethanol/Tween 20 (1:1) to get 100 ml 100 × FA stock medium, then sterilized by membrane filtration and were added as described before\textsuperscript{27}.

**Synthetic genes.** S. cerevisiae codon-optimized synthetic genes of atfar (GenBank: AE089345.1), atfar (NCBI reference sequence: NP_197642.1), atfar (NCBI accession number ADJ56408), scfar (NCBI accession number AF149917), tafar (TAaA; NCBI accession number CAD62962), acp (NCBI reference sequence:
Subsequently, 3–4 clones with correct module integration were cultivated overnight. MvFAS4 was amplified by primer pair pTY017/pTY018. The

Supplementary Table 6. synthesized by GenScript, Inc. (Supplementary Table 4. To delete fragment was amplified by primer pair pTY001/pTY002 using the plasmid

MVFS4 via Gibson cloning. These plasmids were used to transform TY001

digested by BamHI. The ELO2 gene was ligated into the BamHI site of pSPGM2 to generate pELO2. The plasmid pSPGM2 was amplified by primer pair pTY011/pTY012. The whole expression cassette MvFAS-(GGGGS)3-AcpS was assembled in three parts: GPD-MvFAS2, Dp415GPD and amfar promoter and MvFAS2 were fused together by overlapping PCR to generate strain TY031. Correct colonies were confirmed by primer pairs pTY012/pTY013. The double-strand break of ELO3 and integrate the accI1** (Acc6089311071)** gene, accI1** was amplified by primer pair pTY071/pTY072, which comprised 100 bp overhang sequences homologous to up- and downstream regions of ELO3. The gRNA expression vector and repair fragment or primers were used to transform the IMX81 strain to generate strain TY031. Correct strains were confirmed by primer pairs pTY070/pTY072 and primer pTY073/pTY074. For deletion of ELO3, the 2 μm fragment of pROS10 was amplified by primer pair pTY086/pTY087 and the gRNA expression vector pROS10-grnaELO3. The double-strand break of ELO3 was repaired by primer pair pTY065/pTY076, which comprised 60 bp sequences homologous to both up- and downstream regions of ELO3. The gRNA expression vector and repair primers were used to transform strain TDY7005 to obtain strain TDY032. Correct colonies were confirmed by primer pair pTY067/pTY077. To repair the double-strand break of ELO3 and integrate the atfar gene under control of the GAL1 promoter, the upstream fragment of ELO3 was amplified from CEN.PK113-11C genomic DNA with primer pair pTY094/pTY095. The GAL1 promoter was amplified from CEN.PK113-11C genomic DNA with primer pair pTY100/pTY101. The 2 μm fragment and the backbone of pROS13 were fused using Gibson Assembly to generate the gRNA expression vector pROS13-grnaELO3. The double-strand break of ELO3 was repaired by primer pair pTY096/pTY097, which comprised 60 bp sequences homologous to up- and downstream regions of ELO3. The gRNA expression vector and repair fragment or primers were used to transform the IMX81 strain to generate strains TDY033 and TDY028, respectively. To repair the double-strand break of ELO3 and integrate the atfar and ELO2 gene, the upstream fragment of ELO3 was amplified from CEN.PK113-11C genomic DNA with primer pair pTY091/pTY092. The G4L cassette was amplified from primer pair pTY091/pTY092. The 2 μm fragment and the backbone of pROS13 were fused using Gibson Assembly to generate the gRNA expression vector pROS13-grnaELO3. The double-strand break of ELO3 was repaired by primer pair pTY096/pTY097, which comprised 60 bp sequences homologous to up- and downstream regions of ELO3. The gRNA expression vector and repair fragment or primers were used to transform the IMX81 strain to generate strains TDY034 and TDY028, respectively. To repair the double-strand break of ELO3 and integrate the atfar gene and ELO2 gene, the upstream fragment of ELO3 was amplified from CEN.PK113-11C genomic DNA with primer pair pTY091/pTY092. The G4L cassette was amplified from primer pair pTY091/pTY092. The 2 μm fragment and the backbone of pROS13 were fused using Gibson Assembly to generate the gRNA expression vector pROS13-grnaELO3. The double-strand break of ELO3 was repaired by primer pair pTY096/pTY097, which comprised 60 bp sequences homologous to up- and downstream regions of ELO3. The gRNA expression vector and repair fragment or primers were used to transform the IMX81 strain to generate strains TDY034 and TDY028.
pair pT082/pTY081. The afar was amplified by primer pair pT082/pTY081. The FBA1 terminator was amplified by primer pair pT082/pTY081. The downstream region of ELO2 was amplified from CEN.PK113-1C genomic DNA with primer pair pTY091/pTY092. Then, these fragments were fused together by overlapping PCR to obtain the two integration cassettes ELO3up-3DHH2-ELO1-GAL7p-CYC1-ELO2 and ELO2-GAL10-GAL1p-FAIR-FBA1-ELO3sw. The gRNA expression vector and repair fragments were used to transform TY031 on IMX58 to generate strains TY035 and TY030, respectively. The strains TY035 and TY033 were transfected with plasmid pSGPM2-MVFA-ACp5 to generate the strains TY036 and TY037 (Supplementary Figs 9 and 10).

Total FA identification and quantification using GC-MS. Briefly, cell pellets were collected from 20 ml cell culture and then freeze dried for 48 h. Ten milligrams or 40 OD500 of cell culture was prepared in an extraction tube. As extraction solvent, a solution of 1 ml hexane/2 ml of 14% BF3 in MeOH was used, which contained heneicosanol as internal fatty alcohol standard. The extracted fraction was dried by rotary evaporation and dissolved in ethyl acetate10.

Fatty alcohol identification and quantification. For fatty alcohol identification and quantification, cell pellets were collected from 5 ml cell cultures and then freeze dried for 72 h. As extraction solvent, a 2:1 chloroform:methanol solution was used, which contained heneicosanol as internal fatty alcohol standard. The extracted fraction was dried by rotary evaporation and dissolved in ethyl acetate10.

Quantification of fatty alcohols was performed on the same gas chromatography–mass spectrometry (GC-MS) system as used for FAME analysis or a GC–FID system (Thermo Fisher Scientific). The GC programme was as follows: initial temperature of 45 °C hold for 5 min; ramp to 140 °C at a rate of 10 °C min⁻¹ and hold for 10 min; ramp to 310 °C at a rate of 15 °C min⁻¹ and hold for 5 min. The alcohol quantification was as follows: initial temperature of 45 °C hold for 2.5 min; ramp to 220 °C at a rate of 20 °C min⁻¹ and hold for 2 min; ramp to 300 °C at a rate of 20 °C min⁻¹ and hold for 5 min. The temperature of inlet, mass transfer line and ion source were kept at 250 °C, 300 °C and 230 °C, respectively. The flow rate of the carrier gas (helium) was set at 1.0 ml min⁻¹ and data were acquired at full scan mode (50–650 m/z). Final quantification was performed with Xcalibur software.

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information file or available from the corresponding author upon reasonable request.

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Acknowledgements

This work was funded by the Novo Nordisk Foundation and Knut and Alice Wallenberg’s foundation, which are gratefully acknowledged. We thank Professor Teresa M. Dunn (Uniformed Services University, Bethesda, Maryland) for kindly sharing the elo2Δelo3Δ strain, Professor Hans-Joachim Schüller (University of Greifswald, Greifswald, Germany) for kindly sharing the fas1Δfas2Δ strain, Alexandra Bergman for help with constructing the ELO3 deletion cassette, Raphael Ferreira for help with constructing the CRISPR/Cas9 plasmid, Tyler Doughty for help with final polishing of the manuscript, and Sakda Khoomrung and Julia Karlsson for their help with GC analysis. We also appreciate the helpful discussion with Yun Chen, Zhizhui Zhu, Mingtao Huang, Jiufu Qin, David Bengולהohn, Boyang Ji, Yongqiang Wei and Yi Liu.

Author contributions

T.Y. and F.D. conceived the study. T.Y. designed and performed all the experiments and analysed the data. F.D. and Y.J.Z. assisted with experimental design and data analysis. Q.L.L. assisted with experimental performance. L.W. assisted with constructing the ELO3 deletion strains. T.Y., F.D., Y.J.Z., L.W., Q.L.L., A.K., V.S. and J.N. wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing interests: T.Y., F.D., Y.J.Z., L.W., A.K., V.S. and J.N. have filed a patent (‘Fungal cells and methods for production of very long chain fatty acid derived products’, number PCT/US/62/142,236) for protection of part of the work described herein. F.D., J.N., A.K. and V.S. are shareholders in Biopetrolia AB. All other authors declare no competing financial interests.

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How to cite this article: Yu, T. et al. Metabolic engineering of Saccharomyces cerevisiae for production of very long chain fatty acid derived chemicals. Nat. Commun. 8, 15587 doi: 10.1038/ncomms15587 (2017).

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