A conserved evolutionary mechanism permits Δ9 desaturation of very-long-chain fatty acyl lipids

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Δ9 fatty acyl desaturases introduce a cis–double bond between C9 and C10 of saturated fatty acyl chains. From the crystal structure of the mouse stearoyl-CoA desaturase (mSCD1) it was proposed that Tyr-104, a surface residue located at the distal end of the fatty acyl binding pocket plays a key role in specifying 18C selectivity. We created mSCD1-Y104G to test the hypothesis that eliminating this bulky side chain would create an opening and permit the substrate’s methyl end to protrude through the enzyme into the lipid bilayer, facilitating the desaturation of very-long-chain (VLC) substrates. Consistent with this hypothesis, Y104G acquired the ability to desaturate 24C and 26C acyl-CoAs while maintaining its Δ9-regioselectivity. We also investigated two distantly related very-long-chain fatty acyl (VLCA) desaturases from Arabidopsis, ADS1.2 and ADS1.4, which have Ala and Gly, respectively, in place of the gatekeeping Tyr found in mSCD1. Substitution of Tyr for Ala and Gly in ADS1.2 and ADS1.4, respectively, blocked their ability to desaturate VLCAFs. Further, we identified a pair of fungal desaturase homologs which contained either an Ile or a Gly at this location and showed that only the Gly-containing desaturase was capable of very-long-chain desaturation. The conserved desaturase architecture wherein a surface residue with a single bulky side chain forms the end of the substrate binding cavity predisposes them to single amino acid substitutions that enable a switch between long- and very-long-chain selectivity. The data presented here show that such changes have independently occurred multiple times during evolution.

Fatty acyl desaturases are a class of enzymes that are capable of inserting a double bond into fatty acyl chains. There are two classes of evolutionarily unrelated fatty acyl desaturases, i.e. soluble and integral membrane-bound desaturases (1). Both classes contain di-iron center active sites that bind and activate molecular oxygen to abstract two hydrogens from the acyl chain to introduce a double bond. Soluble desaturases are mainly found in the plastids of higher plants, whereas the integral membrane desaturases are distributed more widely in the endomembrane systems of most eukaryotes and some prokaryotes (1–4). Desaturases typically act on acyl chains that are esterified to a carrier moiety. For example, soluble desaturases act on acyl chains esterified to acyl carrier protein (ACP), whereas integral membrane desaturases act on acyl chains esterified to either CoA or lipid head groups. Mammalian stearoyl-CoA desaturases are located within the endoplasmic reticulum (ER), where they convert stearoyl-CoA to oleoyl-CoA. Mutations associated with stearoyl-CoA desaturases (SCDs) in humans have been associated with cancer, obesity, diabetes, and other cellular disorders (5, 6).

The early availability of crystal structures of several soluble desaturases facilitated detailed studies of their substrate chain-length specificity and regioselectivity (7, 8). For example, mutation of two amino acids within the substrate-binding channel of the castor Δ9-18:0-ACP desaturase was sufficient to alter the chain-length specificity from C18 to C16 (9). In follow-up work comparing structural models of acyl-ACP desaturases from cat claw and castor bean, a single amino acid located at the end of the substrate-binding channel was identified as the prime determinant of chain-length specificity by Cahoon et al. (10).

The lack of crystal structures of integral membrane desaturases has long hampered progress toward understanding the factors governing their specificity. Indeed, the structures of members of the integral membrane class lagged behind those of soluble desaturases by almost two decades, when two mammalian SCD1 structures were published almost simultaneously (11, 12). In addition to providing clues as to their own substrate selectivity, homology between desaturases allows them to be used to model others for which structures are not currently available. Their architecture consists of four transmembrane helices with a cytosolic “cap” domain containing the catalytic di-metal center. Although the iron ions shown to be biochemically essential for function (13) were absent from these structures, a di-zinc site was identified adjacent to the substrate-binding cavity that was coordinated by a set of conserved histidine residues that coordinate the di-iron center (1). The crystallized di-zinc form was catalytically inactive but is useful for framing testable hypotheses regarding specificity determinants (14). The substrate-binding cavities of mammalian SCD1s are boomerang-shaped blind-ending cavities like those previously reported for soluble acyl-ACP desaturases. Bai et al. (11) proposed that the acyl carbonyl of the substrate forms a hydrogen bond with the Trp-258 at the opening of the cavity of the mouse SCD1, precisely positioning the C9 and C10 of the acyl chain in the vicinity of the catalytic di-metal center, thereby predisposing Δ9-regioselective desaturation. Moreover, the di-metal center is located at the apex of the bend in the channel, forcing the substrate to adopt an eclipsed conformation, orienting the two pro-R hydrogens toward the di-metal site facilitating the introduction of a cis–double bond.

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Very-long-chain fatty acyl desaturation

Acyl-CoA desaturases are commonly responsible for producing monounsaturated fatty acids in animal and fungal species, whereas the bulk of monounsaturated fatty acids produced in plants is formed by acyl-ACP desaturases in the plastid (15). However, Arabidopsis has a family of integral membrane desaturases, i.e. acyl-CoA desaturases—like enzymes annotated as Arabidopsis desaturases (ADS) that are also capable of converting saturated fatty acyl lipids to their monounsaturated equivalents (16–18). They are principally involved in the biosynthesis of very-long-chain monounsaturated fatty acids in the cytoplasm (16, 17) that can be converted to very-long-chain unsaturated fatty alcohols for wax or cutin production (19). Members in this family have a broad spectrum of substrate specificity. Heterologous expression of ADS1 in Brassica juncea reduced the levels of total saturated fatty acids (20). The specificity of ADSs were experimentally evaluated by their heterologous expression in baker’s yeast (16). It has also been shown that the head group to which the saturated fatty acid is esterified can affect desaturase regioselectivity and that targeting ADS enzymes to either the ER or plastid can affect their specificity because each compartment contains its own characteristic head groups (17). Bioinformatic analyses of membrane desaturases have also correlated the presence or absence of charged residues close to the opening of substrate-binding channels to various head group specificities (18).

Although these enzymes are biochemically or physiologically characterized, the detailed structural factors for determining the specificity are still unclear. Particularly, whether the structural determinant proposed from mammalian stearoyl-CoA desaturases can be experimentally validated and if so whether it is a general mechanism shared by distantly related desaturase homologs that use lipid head groups in place of CoA. In this work, we used a mammalian SCD crystal structure to empirically test the proposed chain-length specificity hypothesis. We then used homology modeling to test the chain-length specificity determinants of ADS and fungal acyl-CoA desaturases and show that mechanisms governing specificity appear to be conserved across these architecturally conserved, but evolutionarily diverged enzymes.

Results

Testing the role of mSCD1 Tyr-104 in determining the C18 chain-length specificity

Mammalian SCD1 belongs to a class of desaturases that insert a double bond at defined number of carbons from the carboxyl moiety. The availability of mammalian SCD1 structures allows us to test predictions of structure–function relationships by constructing and expressing mutants in bakers’ yeast and investigating their biochemical consequences. The mSCD1 has a mushroom-shaped architecture (Fig. 1A) consisting of four transmembrane helices capped by a soluble substrate-binding domain containing a di-iron active site which activates molecular oxygen to effect catalysis (11). The substrate-binding pocket consists of a blind ending, boomerang-shaped channel that can accommodate a linear fatty acyl chain. The distal end of the channel is sealed by a highly conserved large hydrophobic residue, Tyr-104 (Fig. 1B) that has been proposed to specify C18/C16 chain-length specificity. An exception is an SCD from Calanus hyperboreus with a Thr at the corresponding location, which can desaturate very-long-chain fatty acyl CoA (21). Tyr-104 is a surface residue located within the membrane-spanning domain of the desaturase on the opposite face of the desaturase relative to the opening of the substrate-binding channel. We therefore set out to test the hypothesis that SCD Tyr-104 determines its C18 specificity.

We substituted Tyr-104 in mSCD1 for Gly to create an opening at the end of the channel, to test the hypothesis that it could acquire the ability to desaturate chain lengths greater than C18 by allowing the methyl end of the fatty acyl chain to protrude through the newly formed opening. Yeast deletion strain fat1Δ was used as a host for heterologous expression because it accumulates substantial amounts of VLCFAs, of C24 and C26 chain length (Fig. 2C) that could serve as potential substrates if the mutant were to acquire the ability to desaturate chains longer than C18 (22, 23). The portion of chromatograms corresponding to the elution times for VLCFA methyl esters from cells expressing mSCD1-Y104G reveal the accumulation of some 24:1 and 26:1 fatty acids (Fig. 2A) in addition to 16:1 and 18:1, indicating that mSCD1-Y104G can desaturate 16:0 and 18:0 in addition to C24 and C26 substrates. In control experiments, cells expressing native mSCD1 (Fig. 2B) showed no detectable desaturation with the VLCFA substrates, nor did cells transformed with an empty vector (Fig. 2C). In all cases, the major unsaturated fatty acid species are 16:1 and 18:1, which likely arise from the native yeast Δ9 desaturase. These experiments support the hypothesis that Tyr-104 can be considered to be a gatekeeping site with respect to chain-length specificity.

Mutation of Tyr-104 does not change SCD regioselectivity

SCDs are responsible for introducing the first double bond into fatty acyl chain. The double bond is exclusively introduced at Δ9 position. It is proposed that the regioselectivity of mSCD1 is determined by the hydrogen bond formed between the carbonyl group of the substrate fatty acyl chain and the

Figure 1. Structure of mSCD1. A, the mSCD1 (PDB ID: 4YMK) structure is shown in pink. The two metal ions are shown as red spheres. An 18C fatty acyl chain shown in blue is modeled in the substrate binding channel contoured in gray. B, a cross-section of mSCD1 shows the substrate-binding channel with bound 18C substrate. Tyr-104, which seals the end of the substrate-binding channel is shown in green. Trp-258, which was proposed to interact with the substrate acyl chain is shown in magenta.
secondary amine group $N^e_1$ of Trp-258 (11). Therefore, the above mutations should not affect the regiospecificity of the native and mutant enzymes. To test this, we performed double bond positional analysis by derivatization with dimethyl disulfide (DMDS) and evaluated the fragmentation pattern by GC-MS. DMDS analysis shows that the mutant enzyme (mSCD1-Y104G) desaturates its substrates at $\Delta 9$ position (Fig. 2, D and E), i.e. the same as its parental enzyme, indicating the mutagenesis of the chain-length specificity determining residue only influenced the enzyme’s chain-length specificity without changing regioselectivity.

**Fatty acyl lipid desaturases use the same mechanism for determining their substrate chain-length selectivity**

Unlike mammals, plants do not have SCDs to desaturate fatty acyl–CoA. Instead, Arabidopsis has an eight-member family of membrane-bound acyl lipid desaturases. Two members, i.e. AtADS1.2 (At1g06090) and AtADS1.4 (At1g06120), can perform $\Delta 9$ desaturation. They share limited homology with mSCD1 (i.e. 23% identity, 39% similarity and 23% identity, 38% similarity, respectively). However, multiple sequence alignment shows that the nine histidine residues that are proposed to coordinate the catalytic iron ions are well conserved (Fig. S1).

We used the crystal structure of the mouse stearoyl-CoA desaturase to construct structural models for AtADS1.2 and AtADS1.4. Like mSCD1, the models for AtADS1.2 and AtADS1.4 are mushroom shaped and contain a boomerang-shaped substrate-binding channel (Fig. 3, A and B, respectively). In contrast to the blind-ending substrate-binding cavity of mSCD1, the substrate-binding channels of AtADS1.2 and AtADS1.4 are channels with openings on both sides of the proteins (Fig. 3, C and D, respectively), consistent with their reported ability to use VLC fatty acyl lipid substrates (16). To test this experimentally, we expressed AtADS1.2 and AtADS1.4 in the fat1Δ yeast strain. In both cases, substantial quantities of 24:1 and 26:1 accumulated (Fig. 4, A, C and E). These monounsaturated fatty acids have $\Delta 9$ double bonds as confirmed by DMDS analysis (Fig. 4, F and G). Multiple sequence alignment shows that at the position corresponding to mSCD1–Tyr-104, AtADS1.2 and AtADS1.4 contain Ala and Gly, respectively (Fig. S1). Therefore, these two enzymes have an open conformation at the end of their substrate-binding channels, which enables them to accept fatty acyl chains longer than 18 carbons. We tested the effects of mutating these residues to Tyr and challenged them with VLC substrates. As expected, this

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**Figure 2. Fatty acid methyl ester analysis of S. cerevisiae fat1Δ expressing different mSCD1 variants.** A–C, mSCD1-Y104G (A); mSCD1 (B); empty vector control (C). D and E, mass spectral profile for the dimethyl sulfide adducts corresponding to 24:1$\Delta 9$ and 26:1$\Delta 9$ species, respectively, from A. Relative abundance of fatty acids are shown in Table S2.

**Figure 3. Homology model of AtADS1.2 (A and C) and AtADS1.4 (B and D).** The catalytic metal ions are shown as red spheres. The substrate-binding channel is contoured in gray. A 26C fatty acyl chain in the substrate-binding pocket is shown in blue. The cross-sections of the AtADS1.2 and AtADS1.4 surfaces with the opening of the channel that accommodates 26C fatty acyl chain are shown in C and D respectively.
mutation occludes the methyl end of the substrate-binding channel and prevents the enzymes from desaturating fatty acyl chains longer than C18 (Fig. 4, B and D). In addition, closing of the gatekeeping site in plant fatty acyl desaturase did not inactivate the enzyme. For instance, AtADS1.2-A65Y expressing oleD yeast can accumulate 18:1D9 (Fig. S2 and Table S4), whereas oleD with empty vector cannot produce D9 mono-unsaturated fatty acid.

Similar chain-length specificity determination mechanisms also exist in fungal membrane desaturases

The above data showed that both animal and plant membrane front-end fatty acyl desaturases exploit the same mechanisms to determine their substrate chain length by using a gatekeeping residue at the end of their substrate-binding pocket. We next tested if this mechanism is common to fungal fatty acyl desaturases. Using AtADS1.4 amino acid sequence as a query, we searched into the nonredundant protein database of fungal fatty acyl desaturases and identified several candidate enzymes with a Gly at their gatekeeping sites. We selected the enzyme from Paxillus involutus ATCC 200175 with accession number KIJ06932.1 (hereafter 6932) for further test (Fig. 5A and B). We synthesized the encoding gene, expressed it in yeast and tested its activity. As shown in Fig. 5C, 6932 clearly showed the activity to desaturate VLCFAs to produce its cognate mono-unsaturated products up to 26C (Fig. 5, D–F), suggesting the gatekeeping chain-length specificity determination mechanism is conserved among front-end desaturases. Moreover, the mutations did not affect the D6 regiospecificity of the enzyme (Fig. S3, E and F). Collectively, these data demonstrated that the mechanism for determining substrate chain-length specificity is widely conserved for front-end desaturases from different species and across different kingdoms.

mSCD1 Trp-258 is not essential for specifying D9 regiospecific desaturation

In the structural model of mSCD1, Trp-258 forms a hydrogen bond with the carbonyl group of oleoyl-CoA, which was hypothesized to position the fatty acyl–CoA for D9 regioselective desaturation (11). Interestingly, AtADS1.2 and AtADS1.4 also have a Trp at the corresponding position, suggesting that AtADS1.2, AtADS1.4 and/or other D9 fatty acyl–CoA desaturases may use a similar mechanism for D9-specific desaturation. Although from a structural view it appeared that mSCD1 Trp-258 plays an important role as a reference point for positioning the acyl chain, there was no empirical evidence to support this view. Because VLCFA desaturation products do not naturally occur in yeast cells, we took advantage of the ability of mSCD1-Y104G to desaturate very-long-chain fatty acyl chains. Into this mutant we combined a series of mutations at position 258 to investigate the influence on regioselectivity of Trp at this position. Mutations of Trp-258 to most other residues, such as Arg, Gln, His, Ile, Leu, Lys, Met and Glu, resulted in a total loss of mSCD1-Y104G activity (data not shown), validating the

Figure 4. Fatty acid methyl ester and dimethyl sulfide derivative analyses of S. cerevisiae fat1Δ expressing ADS1.2 and ADS1.4 and variants thereof. A–E, fatty acid methyl ester profiles: AtADS1.2 (A); AtADS1.2-A65Y (B); AtADS1.4 (C); AtADS1.4-G65Y (D); empty vector (E). F and G, show the mass ion profile for the dimethyl sulfide adducts corresponding to 24:1Δ9 and 26:1Δ9 peaks, respectively. In A, Relative abundance of fatty acids are shown in Table S3.
importance of this residue. Interestingly, mutants in which Trp-258 was substituted by Phe or Tyr (Fig. 6, A–E) partially retained desaturation activity and maintained Δ9 regioselectivity (Fig. 6, F and G).

**Discussion**

In this work we have tested hypotheses that were proposed from inspection of the crystal structure of the mSCD1. Specifically, we provide experimental evidence to support the hypo-
thesis that Tyr-104 is key to specifying C18 substrate chain-length specificity to mSCD1. Substituting a small residue in place of the Tyr allows VLCFA to bind to the desaturase and insert a Δ9 double bond. That this is possible is related to two features that are observable from the crystal structure. First, Tyr-104 forms not only the end of the substrate-binding cavity, but also a surface residue located within a hydrophobic domain which is predicted to be positioned within the lipid bilayer. Thus, in the Y104G mutant, there is an opening through which the hydrophobic methyl end of the substrate can protrude, which will then intercalate with the acyl chains of the lipid bilayer, obviating the energetically unfavorable solvation of the methyl end of the acyl chain that would be necessary if the opening was in a hydrophobic domain.

Another consequence of the conserved architecture of the integral membrane desaturases in which residue 104 is solely responsible for ending the substrate-binding cavity is that mutations that convert this large hydrophobic residue to a smaller one can potentially switch C18 chain-length specificity to accept VLCFA of C22 or more. That minimal changes to the DNA sequence at the residue 104 codon are sufficient to make this transformation is reflected by the parallel independent evolution of VLCFA desaturation capability in related desaturases from different evolutionary lineages including animals, plants, and fungi investigated here.

We also investigated the hypothesized involvement of mSCD1 residue Trp-258 for the Δ counting mechanism of mSCD1, i.e. acting as a reference point by forming a hydrogen bond with the carbonyl group of oleate. Whereas most mutations resulted in loss of catalytic activity, mutations in which Trp-258 was substituted to phenyl ring-containing residues, i.e. Phe and Tyr, partially retained desaturation activity and maintained Δ9 regioselectivity. This implies that π electron interactions of phenyl ring-containing residues play an important role in substrate binding and/or orientation.

Membrane-bound desaturases occur widely across the kingdoms of life, being present in some prokaryotes and most eukaryotes. They play important roles in controlling the desaturation status of fatty acids and thereby their physical properties like melting temperatures which directly affect the fluidity of cellular membranes. Unsaturated fatty acids can also serve as important signaling molecules, such as insect pheromone mimics, and some VLCFA are metabolized to alkanes and alkenes that are important extracellular components with roles in water conservation. It is therefore of great interest to understand the structural and functional relationships of desaturases and related enzymes. Until recently, researchers wanting to investigate substrate specificities or catalysis mechanisms were limited to domain swap or site-specific mutagenesis approaches in conjunction with heterologous expression and lipid analysis. For example, the comparison of an atypical SCD from a copepod species C. hyperboreus that naturally accumulates wax esters with Δ11 fatty alcohols with the SCD from the same species revealed the residues that likely determine the substrate chain length of the SCDs (21). However, these studies were limited by the lack of structural context with which to interpret their findings. The recent determination of two mammalian SCD structures revealed a blind-ending substrate-binding channel that accommodates up to 18C substrates, which allowed us to test hypotheses with respect to substrate chain length and regioselectivity for SCDs, and to create testable models for other SCD-like enzymes from plants that potentially share similar structures and/or substrate determination mechanisms with SCDs.

The substrate chain-length specificity of the evolutionary and structurally distinct, soluble acyl-ACP desaturases has been extensively studied, where the preference for long-chain fatty acyl ACPs can be modulated by amino acid substitutions of cavity-lining residues. In contrast, VLCFAs are usually esterified to a CoA or lipid head groups that are not recognized by acyl-ACP desaturases (16, 24, 25). Modifications of such lipid molecules are mediated by membrane desaturases located in the ER of higher organisms. Nonetheless, both soluble desaturases and integral membrane desaturases employ similar mechanisms for recognizing substrate acyl chain lengths, where the nature of side chains of substrate-binding channel-forming residues and volume of the substrate-binding pocket define the substrate acyl chain length. The substrate-binding pocket of soluble desaturases is fully enclosed within the enzymes, therefore the acyl chain-length specificity can be finely tuned between 18C and 16C or even 14C by varying the side chain size and/or properties of the channel-forming residues, whereas the membrane desaturases use a single gatekeeping residue to discriminate between long fatty acyl chain (16C and 18C) and very-long fatty acyl chain (22C-26C) substrates. The inability of integral membrane desaturase to finely distinguish fatty acyl chains beyond 20C suggests that the opening of the gatekeeping residue allows fatty acyl chains longer than 20C to penetrate the channel with no constraining residues to further recognize different chain lengths. That the gatekeeping residue is located on the surface of membrane desaturases from homology modeling analysis also corroborated this hypothesis, where substitution of the gatekeeping site for a smaller side chain residue (e.g Gly, Ala) makes the substrate-binding channel an internal tunnel that traverses between two sides of the enzyme so that the very-long fatty acyl chain can enter the tunnel from one end and protrude the channel at the other end. The methyl portion of fatty acyl chain that protrudes from the substrate-binding channel likely intercalates with the acyl chains that form the lipid bilayer into which the desaturase is embedded, thereby permitting the intercalation of varying acyl chain lengths. Changes from a bulky to a small gatekeeping residue can thus be likened to opening a window, allowing the desaturation of fatty acyl chains of various lengths beyond that constrained by the presence of a bulky residue at the gatekeeper position.

This mechanism of acyl chain-length specificity determination involves changes at only one residue. That mutations at this single location can occur easily is evidenced by their occurrence across different kingdoms. Phylogenetic analysis shows that fatty acyl desaturases from the same organism typically cluster together (Fig. 5B), suggesting that mutations occur after the evolutionary divergence of different organisms, following a gene duplication event. That nature has selected the enzymes with different substrate chain-length specificity to exist in one organism and the same mutation has been selected several
times further implies a widespread biological importance of monounsaturated VLCFA across different kingdoms. Indeed, the marine copepode accumulates high-level monounsaturated VLCFA to serve as energy storage molecules (26); plants accumulate monounsaturated VLCFA in response to cold environments (16, 27). Some fungi, e.g. Phycymices blakesleeanus, incorporate VLC saturated and unsaturated fatty acids in their sporangiophore waxy cuticles to prevent dehydration (28). These VLCFA-recognizing enzymes have independently evolved in response to evolutionary pressures within different lineages via natural selection.

In summary, front-end integral membrane desaturases from organisms across different kingdoms share a common chain-length specifying mechanism whereby the channel is either closed, forming a pocket that accommodates chain lengths of up to C18, or open, forming a tunnel, in which case varying lengths of substrates longer than C20 can be accommodated while the Δ-counting desaturation position remains unaffected. This is because regioselectivity is based on the distance between Trp-258 (or its equivalent) and the di-iron site where the active oxygen that initiates desaturation is formed. While the Trp-258 and sequence alignments from PROMALS3D were used as inputs.

Bioinformatic analysis

The sequences of mouse stearoyl-CoA desaturase 1 (mSCD1), human stearoyl-CoA desaturase 1 (hSCD1), human stearoyl-CoA desaturase 5 (hSCD5), AtADS1.2, and AtADS1.4 were extracted from NCBI with the following accession numbers: AAA40103, NP_005054, NP_001032671, NP_172099, and NP_172102, respectively. Multiple sequence alignments were performed using the PROMALS3D (29) online server. The fungal fattyacyl desaturases are obtained by blast search in NCBI nonredundant fungi protein database using AtADS1.4 sequence as a query. The resulting sequences were parsed and manually selected according to their consensus at the sites that form the end of their substrate-binding pockets. The selected fattyacyl desaturase is from Paxillus involutus ATCC 200175 with NCBI accession number KIJ06932.1. The phylogenetic tree was constructed using MEGA7 with neighbor-joining method (30, 31).

Homology modeling of ADS1.2 and ADS1.4 3D structure

Homology modeling was performed using MODELLER (32) (version 9.15) based on the mSCD1 structure (PDB ID 4YMK) and sequence alignments from PROMALS3D were used as inputs.

Expression vector construction

The nucleotide sequence encoding yeast Ole1 leader sequence (the first 27 amino acids) was N-terminally fused to all the desaturases by overlap extension PCR. The resulting PCR products were then inserted into pCR8/GW-TOPO to generate donor vectors. The promoter sequence of Saccharomyces cerevisiae translational elongation factor 1 (TEF1) (425 bp upstream of the start codon) was used to replace the GAL1 promoter in pYES-DEST52 to generate the expression vector pTEF-DEST. The genes encoding different desaturases were then introduced to pTEF-DEST by LR reactions.

Site-directed mutagenesis

The oligonucleotides listed in Table S1 were used to introduce point mutations into desaturases. Site-directed mutagenesis was performed by QuikChange method (Agilent Technologies). All mutants were confirmed by DNA sequencing prior to functional analysis.

Yeast transformation and culture

The fat1Δ yeast strain (BY4741; MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YBR041w::kanMX4) was obtained from Invitrogen (Cat. no. 95400, clone ID 3178). Ole1Δ (L8-14C) yeast knockout mutant (MATa ole1::LEU2 leu2-3 leu2-112 trp1-1, ura3-52 his4) was from the laboratory stock. The desaturase expression vectors were transformed into S. cerevisiae fat1Δ or ole1Δ using Yeastmaker™ Yeast Transformation System 2 (Clontech, Mountain View, CA, USA) following the manufacturer’s protocols. Transformants were selected by prototrophy using S.D.-Ura plates. Positive colonies were inoculated into fresh S.D.-Ura media for propagation at 30°C. For culturing ole1Δ, 0.2 mM cis-10-heptadecenoic acid was added into the medium. For expression, yeast cultures were inoculated into fresh S.D.-Ura at a final A600 of ~0.05 and incubated in an orbital shaker oscillating at 250 rpm and 20°C for 60 h.

Fatty acid compositional analysis

To analyze the fatty acid composition of desaturase-expressing fat1Δ strains, yeast cells were isolated by centrifugation at 3000 × g for 10 min and washed twice with 5 ml ddH2O. An internal standard, 5 μg heptadecanoic acid was added to each sample. Cells were dried under a stream of nitrogen. Total fatty acid methyl esters (FAME) were prepared by incubation with 1 ml BCl3-methanol (Sigma-Aldrich) at 100°C for 90 min. FAMEs were extracted by adding 1 ml ddH2O and 3 ml hexane. After vigorously mixing, the hexane phase was transferred to a fresh tube and dried under a stream of nitrogen. The FAME products were then dissolved in 200 μl of hexane and subjected to analysis by an Agilent GC (Model no. 7890A) equipped with a 5975C inert Mass Selective Triple-Axis Detector. The samples were separated on an Agilent J&W DB-23 capillary column (30 m × 0.25 μM × 0.25 μM). The oven temperature was set to ramp from 100°C to 225°C at a rate of 10°C/min, then to 250°C at a rate of 15°C/min and hold for 10 min. The flow rate of helium carrier gas was set at 1 ml/min. For double bond position analysis, we used dimethyl disulfide (Sigma-Aldrich)
Very-long-chain fatty acyl desaturation
derivatization and followed the protocol in Christie and Han (33).

Data availability
All data are contained within the manuscript.

Author contributions—Y. C., C.-J. L., and J. S. conceptualization; Y. C., J. C., C.-J. L., and J. S. formal analysis; Y. C., X.-H. Y., C.-J. L., and J. S. investigation; Y. C., X.-H. Y., and J. C. methodology; Y. C., C.-J. L., and J. S. writing-original draft; Y. C., X.-H. Y., C.-J. L., and J. S. writing-review and editing; C.-J. L. and J. S. funding acquisition.

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Abbreviations—The abbreviations used are: ACP, acyl carrier protein; ER, endoplasmic reticulum; SCD, stearoyl-CoA desaturase; ADS, Arabidopsis desaturases; VLC, very-long-chain; VLCFA, very-long-chain fatty acyl; DMDS, dimethyl disulfide.

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