RNA-dependent sterol aspartylation in fungi
Nathaniel Yakobov, Frédéric Fischer, Nassira Mahmoudi, Yusuke Saga, Christopher Grube, Hervé Roy, Bruno Senger, Guillaume Grob, Shunsuke Tatematsu, Daisuke Yokokawa, et al.

To cite this version:

HAL Id: pasteur-02869570
https://hal-pasteur.archives-ouvertes.fr/pasteur-02869570
Submitted on 16 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives| 4.0 International License
RNA-dependent sterol aspartylation in fungi

Nathaniel Yakobov, Frédéric Fischer, Nassira Mahmoudi, Yusuke Saga, Christopher D. Grube, Hervé Roy, Bruno Senger, Guillaume Grob, Shunshuke Tatematsu, Daisuke Yokokawa, Isabelle Mouyna, Jean-Paul Latgé, Harushi Nakajima, Tetsuo Kuroishi, and Hubert D. Becker

Université de Strasbourg, CNRS, Génétique Moléculaire, Génomique, Microbiologie, UMR 7156, 67084 Strasbourg Cedex, France; School of Agriculture, Meiji University, Kawasaki 214-8571, Japan; Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32826; and Unité des Aspergillus, Département de Mycologie, Institut Pasteur, 75724 Paris Cedex 15, France

Edited by Dieter Soll, Yale University, New Haven, CT, and approved May 11, 2020 (received for review February 20, 2020)

Diverting aminoacyl-transfer RNAs (tRNAs) from protein synthesis is a well-known process used by a wide range of bacteria to aminoacylate membrane constituents. By tRNA-dependently adding amino acids to glycerolipids, bacteria change their cell surface properties, which intensifies antimicrobial drug resistance, pathogenicity, and virulence. No equivalent aminoacylated lipids have been uncovered in any eukaryotic species thus far, suggesting that tRNA-dependent lipid remodeling is a process restricted to prokaryotes. We report here the discovery of ergosteryl-3β-O-L-aspartate (Erg-Asp), a conjugated sterol that is produced by the tRNA-dependent addition of aspartate to the 3β-OH group of ergosterol, the major sterol found in fungal membranes. In fact, Erg-Asp exists in the majority of fungi, including species of biotechnological interest, and, more importantly, higher fungi. We uncovered that removal of the Asp modifier from Erg-Asp is catalyzed by a second enzyme, ErdH, that is a genuine Erg-Asp hydrolase participating in the turnover of the conjugated sterol in vivo. Phylogenomics highlights that the entire Erg-Asp synthesis/degradation pathway is conserved across “higher” fungi. Given the central roles of sterols and conjugated sterols in fungi, we propose that this tRNA-dependent ergosterol modification and homeostasis system might have broader implications in membrane remodeling, trafficking, antimicrobial resistance, or pathogenicity.

Significance

Bacteria are known to add amino acids (aa) to membrane lipids to resist antimicrobials and escape immune responses. This surface lipid aminoacylation process requires diverting aminoacyl-tRNAs from protein synthesis. While widespread in bacteria, no analogous lipid remodeling system had thus far been evidenced in eukaryotes. We uncovered that most fungi tRNA-dependently add aspartate onto ergosterol (ergosteryl-3β-O-L-aspartate [Erg-Asp]), the major sterol found in fungal membranes. Asp addition is catalyzed by an ergostereryl-3β-O-L-aspartate synthase (ErdS) and its removal by a dedicated hydrolase (ErdH). This pathway is conserved across “higher” fungi, including pathogens. Given the central roles of sterols and derivatives in fungi, we propose that the Erg-Asp homeostasis system might impact membrane remodeling, trafficking, antimicrobial resistance, or pathogenicity.

RNA-dependent aminoacylation

Remodeling of membranes and lipid modifications are processes used by living cells to interact with and adapt to their environment. They are notably critical for host/pathogens interactions, antimicrobial resistance, and virulence in both bacteria (1, 2) and fungi (3). MprFs are bacterial virulence factors that transfer amino acids (aa) onto membrane glycerolipids in a so-called aminoacylation reaction (4). This process requires aminoacyl-transfer RNAs (aa-tRNA) that are first synthesized by aminoacyl-tRNA synthetases (aaRS) (5), prior transfer of the aa moiety onto an aminoacylated lipid acceptor substrate, that is, phosphatidylethanolamine, cardiolipin, or diacylglycerol (4). The aminoacyl-tRNA transferase (AAT) module, that catalyzes the transfer, belongs to the DUF2156 family and recognizes both the aa-tRNA and the lipid substrates (6). Glycerolipid aminoacylation modifies the overall charge, fluidity, and permeability of bacterial membranes, which enhances antimicrobial resistance, explaining why these enzymes have been termed multiple peptides resistance factors (MprF) (4). Glycerolipid aminoacylations also affect host/pathogen interactions and have been shown to potentiate immune escape and to increase virulence of pathogens (7). In Pseudomonas aeruginosa (8), Enterococcus faecium (9), and Agrobacterium tumefaciens (10), extracytoplasmic esterases of the VirD or α/β-hydrolase family participate in the homeostasis of aminoacylated lipids and hydrolyze the modifying aa from lipids.

So far, only glycerolipids have been shown to be aminoacylated and only by MprFs in prokaryotes. In the present study, we show that eukaryotes aminoacylate sterols in a tRNA-dependent modification pathway that mimics those described in bacteria. In higher fungi, we functionally characterized a novel enzyme, the ergosteryl-3β-O-L-aspartate synthase (ErdS), composed of a DUF2156/AAT domain fused to an aspartyl-tRNA synthetase (AspRS) paralog. We show that ErdS synthesizes ergosteryl-3β-O-L-aspartate (Erg-Asp), a previously undetected conjugated sterol. Moreover, most of higher fungi (Dikarya), including opportunistic human pathogens such as Aspergillus fumigatus (Afm), encode this ErdS and produce Erg-Asp, suggesting that the system is widespread in “higher” fungi. We also evidenced that these species express a dedicated Erg-Asp hydrolase (ErdH) that deacylates Erg-Asp and whose gene is almost always located in a two-gene cluster next to the erdS gene. In conclusion, our data unravel an evolutionary conserved sterol–aa conjugation pathway. We discuss the potential implications of this pathway in the context of lipid homeostasis and antimicrobial resistance.


The authors declare no competing interest.

This article is a PNAS Direct Submission.

This open access article is distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).  

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2003266117/-/DCSupplemental.  

www.pnas.org/cgi/doi/10.1073/pnas.2003266117

PNAS Latest Articles | 1 of 10

Downloaded by guest on June 18, 2020
Results

DUF2156 Proteins Are Present in Fungi. Although DUF2156 domains were thought to be absent or rare outside prokaryotes (11), we identified, in most higher fungi, DUF2156-containing proteins fused to the C terminus of an aaRS-like domain (Fig. L4 and SI Appendix, Fig. S1A), that we named ErDS for reasons that will be presented below. This protein architecture, previously detected in two fungi (12), is actually widespread across fungi, including the well-characterized Afm, Aspergillus oryzae (Aor), or Neurospora crassa (Ncr) (Fig. L4). Firstly, sequence analyses and structure predictions of the detected DUF2156 domains suggested that they all adopt the characteristic double Gcn5-like N-acyltransferase (GNAT) fold observed in bacterial MprFs (6). In fungal DUF2156 domains, the GNAT I (recognition of the acceptor substrate) and II (transferase activity) subdomains of the double GNAT fold are separated, like in bacteria, by a positively charged N-terminal domain (Fig. 1C), that we named ErDS for reasons that will be presented below. This protein architecture, previously detected in two fungi (12), is actually widespread across fungi, including the well-characterized Afm, Aspergillus oryzae (Aor), or Neurospora crassa (Ncr) (Fig. L4). Firstly, sequence analyses and structure predictions of the detected DUF2156 domains suggested that they all adopt the characteristic double Gcn5-like N-acyltransferase (GNAT) fold observed in bacterial MprFs (6). In fungal DUF2156 domains, the GNAT I (recognition of the acceptor substrate) and II (transferase activity) subdomains of the double GNAT fold are separated, like in bacteria, by a positively charged α(II) helix involved (Fig. 1A and B) in the binding of aa-tRNAs (6), suggesting that 1) they could indeed be aa-tRNA-utilizing modules and 2) they might transfer the aa moiety of aa-tRNAs onto lipids. Second, the aaRS domains contain bona fide AspRS signature sequences including the known residues (13) involved in tRNAAsp, Asp, and adenosine 5′-triphosphate (ATP) binding ubiquitously found in functional AspRSs (SI Appendix, Fig. S1B). These considerations prompted us to propose the following working hypothesis: The AspRS domain of ErDS would generate Asp-tRNAAsp from ATP, l-Asp, and tRNAAsp, which would then be transferred to the DUF2156 module, thought to carry the AAT activity, to transfer the Asp residue onto a yet-to-be-identified lipid (Fig. 1C).

ErdS Is Essential for the Synthesis of a Lipid in Filamentous Fungi. In order to test our hypothesis on the function of ErDSs, we deleted the corresponding erds genes in two fungi (Fig. 2A and SI Appendix, Fig. S2), namely, the human opportunistic pathogen Afm and the species of biotechnological and industrial interest Aor. Both Δerds mutants grew normally on solid media (Fig. S3), demonstrating that the genes to encode proteins composed of an AspRS domain N-terminally fused to a DUF2156 domain. Protein domains were delimited by confronting data obtained from Protein Families database (PFAM) and from multiple alignments as described in SI Appendix, Supplementary Materials and Methods. Critical positively charged residues in the alpha helix (α(II)) separating both GNAT folds are indicated (orange). (B) Comparison of the DUF2156 structure of AlapGS (alanyl-phosphatidylglycerol synthase, MprF) from P. aeruginosa to the Phyre2 prediction of Afm DUF2156. GNAT I and II subdomains are highlighted in gray and green, respectively, with the positively charged α(II) helix in blue. (C) Schematic representation of the hypothetical ErDS reaction mechanism during which tRNAAsp shift from the AspRS catalytic site (position 1: tRNAAsp asparylation) to the DUF2156 active site (position 2: Asp transfer from Asp-tRNAAsp onto a lipid substrate). The α(II) helix is indicated in blue, and the active site is indicated in green. Aspartate is represented in orange.

To dissect the mechanism of LX synthesis, we transfected the synthetic LX, hypoththesized to rely on the sole ErDS enzyme, to the budding yeast Saccharomyces cerevisiae (Sce), in which we over-expressed Afm ErDS. Switching to a heterologous yeast system was dictated for three main reasons: 1) Genetic engineering is far more time consuming in Afm than in Sce; 2) no DUF2156-containing proteins were detected in Sce (SI Appendix, Fig. S1A); and 3) the lipid synthesis pathways are conserved across ascomycetes (amigo.geneontology.org/amigo/term/GO:0006629), and thus the lipid contents of these species belonging to the same phylum are likely to be very similar (14). To start with and test whether the AspRS moeity was functional, Afm ErDS or ErDS-ΔDUF, that is an ErDS form that comprises only the AspRS domain, was expressed in the Sce Δdps1 strain (15) (Δdps1 encodes the essential AspRS), and complementation of the Δdps1 mutation lethality was tested by plasmid shuffling (Fig. 3A and SI Appendix, Fig. S4). Results show that both ErDS and ErDS-ΔDUF functionally replaced Sce AspRS in vivo. This was not observed with a catalytic null mutant (ErDSΔAAPA) of the AspRS domain (Fig. 3A and SI Appendix, Fig. S4A), in which the OSPQ signature motif (16) was mutated to AAPA. In addition, in vitro aminoacylation assays with purified recombinant Afm ErDS and Sce tRNAAsp confirmed ErDS’s capacity to generate Asp-tRNAAsp (SI Appendix, Fig. S4B). Interestingly, full-length ErDS displays a lower complementation efficiency of the Sce Δdps1 strain compared to ErDS-ΔDUF (SI Appendix, Fig. S4A). We interpreted that, in the full-length ErDS, the DUF2156 domain likely transfers...
Asp from Asp\text{-}tRNA^{\text{Asp}} onto an accepting lipid, thereby decreasing the amount of Asp\text{-}tRNA^{\text{Asp}} available for protein synthesis and impacting growth.

To determine whether the DUF2156 module of ErdS transfers Asp from Asp\text{-}tRNA^{\text{Asp}} onto lipids in vivo, as suggested from the results obtained in Aspergillus spp. (Fig. 2 B and C), we analyzed the total lipid composition of a WT Scex strain bearing plasmids that express Afm or Aor full-length or truncated ErdSs (Fig. 3A). Expression of proteins was confirmed by Western blot with anti-ErdS antibodies that recognize both Afm and Aor ErdS (Fig. 3B), and analyses of total lipids by TLC revealed the presence of an additional brownish lipid, absent in WT Scex strains, when Afm and Aor ErdS were expressed. This lipid presents the same migration profile as the LX detected in Afm and Aor (SI Appendix, Fig. S4C), strongly suggesting that heterologous expression of Afm or Aor ErdS in Scex allows complete and correct synthesis of LX. Positive staining of LX with ninhydrin (detection of primary amine) and bromocresol green (detection of carboxyl) further suggested that it contained an aa moiety, likely L\text{-}Asp ester- or amide-linked through its \(\alpha\text{-}NH_3^+\) and \(\beta\text{-}COO^-\) groups (SI Appendix, Fig. S4D).

To investigate whether ErdS transfers Asp from Asp\text{-}tRNA^{\text{Asp}} onto a lipid in a DUF2156-dependent manner, we analyzed the total lipids produced by ErdS\text{-}\text{ΔDUF}, ErdS\text{-}\text{ΔAspRS}, and ErdS\text{AAPA} expressed in Scex (Fig. 3B). Expression of the different constructs was confirmed by anti-ErdS Western blot (Fig. 3B). The ErdS\text{ΔDUF} could not be detected, because our antibodies were raised against the DUF2156 domain; however, complementation of the lethal \(\Delta\text{dps1}\) mutation in Scex by ErdS\text{ΔDUF} confirmed that the AspRS moiety was expressed and functional (SI Appendix, Fig. S4A). LX spots were quantified and normalized to that of PE (phosphatidylethanolamine) (LX/PE ratio). All TLCs are representative of at least two independent experiments \((n = 2)\).

**Fig. 2.** Identification of an Aspergillus lipid species, whose synthesis requires ErdS. (A) Deletions of \(\text{erdS}\) from \(\text{Afm}\) and from \(\text{Aor}\) were performed by homologous recombination (see SI Appendix for details). The genotypes of the strains are indicated. For \(\text{Afm}\), the \(\Delta\text{erdS}\) strain shown corresponds to the strain after excision of the deletion cassette, whereas, for the complemented \(\Delta\text{erdS}\) strain, the selection marker is still present. For \(\text{Aor}\) the ORF of \(\text{erdS}\) was replaced by a \(\text{pyrG}\)-containing module and subsequently excised by selection on 5-FOA medium. Complementation was operated by ectopic expression of \(\text{erdS}\text{ΔDUF}, \text{erdS}\text{ΔAspRS}\), or \(\text{erdS}\) (B and C) Total lipids extracted from the different strains described in A were analyzed by TLC and stained with a sulfuric acid/MnCl\(_2\) solution. TLC plates were observed either under white light or under UV light. Cultures were done in glucose or xylose containing media; * indicates the LX. (D) Quantification of the TLCs shown in B and C. LX signal (number of pixels) was normalized to that of PE (phosphatidylethanolamine) (LX/PE ratio). All TLCs are representative of at least two independent experiments \((n = 2)\).
Sce strain that expresses ErdS with a catalytically null AspRS moiety (Sce+ErdSAPA), LX levels mirrored those of the Sce+ErdS-ΔAspRS and Sce+ΔA+ΔD strains (Fig. 3B). These experiments show, in addition, that the DUF2156 domain of ErdS can efficiently utilize Sce Asp-tRNAAsp, regardless of whether it was generated by the Afm AspRS moiety of ErdS or by the endogenous Sce AspRS.

To better characterize the ErdS catalytic mechanism, we developed an in vitro lipid aminoacylation assay (LA assay), using purified Afm or Aor recombinant ErdS incubated with purified Sce tRNAAsp, ATP, and [14C]-Asp in the presence of Sce total lipids (Fig. 3C). Reaction products from the LA assay were analyzed by TLC (Fig. 3D and E). Results confirmed that Afm or Aor recombinant ErdSs catalyzed the formation of a [14C]-labeled lipid (Fig. 3D). Adding RNase or removing lipids from the LA assay abolished synthesis of [14C]-lipid, thereby proving the tRNA and lipid dependency of LX synthesis by Afm ErdS (Fig. 3E). When the recombinant Afm ErdS-ΔDUF protein was used, synthesis of the [14C]-lipid was abolished, proving that the reaction is, indeed, DUF2156 dependent (Fig. 3D). Likewise, when ErdS-ΔAspRS or ErdSAPA were used in the LA assay, synthesis of the [14C]-lipid was abolished (Fig. 3D), but fully restored upon addition of the AspRS domain of ErdS in trans. These data confirm results obtained in Sce but also that the DUF2156 is capable of capturing Asp-tRNAAsp formed by an AspRS expressed in trans (Fig. 3D).

Finally, crude extracts from WT Afm or Aor mycelia also catalyzed a [14C]-Asp-tRNAAsp-dependent [14C]-lipid synthesis in vitro, whereas crude extracts from ΔerdS mutant strains did not (Fig. 3F). However, for reasons that will be discussed below, the ErdS activity was hardly detectable in crude extracts, especially for Aor, when compared to recombinant ErdS.

Alternatively, these results show that ErdS produces Asp-tRNAAsp from ATP, L-Asp, and tRNAAsp in its AspRS domain,
and that the Asp-tRNA\textsuperscript{Asp} product likely shifts from the AspRS active site to the DUF2156 module, that transfers the Asp acylating tRNA\textsuperscript{Asp} onto a lipid, which mirrors the activity of bacterial DUF2156 proteins (4).

**Liquid chromatography-MS/MS Reveals that ErdS Aspartylates Ergosterol.** To identify the substrate lipid of ErdS, we fractionated total lipids from an ErdS overexpressing \textit{Sce} strain by column chromatography and isolated LX at \textasciitilde80\% purity (SI Appendix, Fig. S5A). Fractions were submitted to mass spectrometry (MS) analyses and compared to equivalent fractions obtained from total lipids of a WT yeast strain. Strikingly, MS electrospray ionization quadrupole time-of-flight (MS-ESI-QTOF) spectra revealed two peaks, absent in WT fractions, with \textit{m/z} of 379.3309 and 534.3565 that were submitted to a second round of MS/MS collision-induced dissociation QTOF (MS/MS-CID-QTOF) (Fig. 4A). Contrary to bacterial MprF-aminoacylated lipids, results were not compatible with LX being a glycerolipid derivative. Fragmentation pattern of the first peak was rather consistent with a dehydrated ergosterol (Erg) (\textit{m/z} 379.3355), which was supported by the presence of characteristic additional Erg fragmentation products (Fig. 4A) (17). The second peak gave similar fragmentation products together with that of Asp (\textit{m/z} of 156.0265, \([\text{C}_6\text{H}_7\text{NO}_4\text{Na}]+\)) and of an aspar-tylated form of Erg (534.3547, \([\text{M} \sim \text{aspartyl+Na}]+\)). Results were consistent with an Erg moiety esterified with Asp on the \(\beta\)-OH group in position 3 of the A ring, making this species an Erg-Asp (Fig. 4B). Finally, liquid chromatography (LC)-ESI-MS/MS analyses of total lipids extracted directly from \textit{AfM} confirmed that Erg-Asp was indeed present (SI Appendix, Fig. S5). In parallel, we chemically synthesized Erg-Asp that we compared, on TLC, to Erg-Asp present in total lipids of \textit{AfM}, \textit{Aor}, or \textit{Sce} expressing \textit{AfM} ErdS and confirmed that, as expected, it shared the exact same migration properties (SI Appendix, Fig. S6).

To confirm that Erg is the substrate of ErdS, we performed LA assays using either \([\text{H}]+\)-Erg and cold Asp or cold Erg and \([\text{14C}]+\)-Asp. Analyses of reaction products by TLC revealed that ErdS indeed produced \([\text{H}]+\)-Erg-Asp (Fig. 4C) or \([\text{14C}]+\)-Erg-Asp (Fig. 4D) in a tRNA-dependent manner, and that it requires L-Asp, ATP, and tRNA\textsuperscript{Asp} for activity. Interestingly, in the presence of cholesterol (Cho), the animal equivalent of Erg and \([\text{14C}]+\)-Asp, ErdS also produced cholesteryl-aspartate (Cho-Asp) (Fig. 4D), indicating that it likely has a relaxed specificity toward...
its sterol substrate. Overall, our results demonstrate that ErdSs represent a novel type of bifunctional AspRS/ergosteryl-aspartate synthases that we named ergosterol-3β-O-t-aspartate synthases or ErdS, with “Er” standing for “ergosteryl,” “d” for Asp, and “S” for synthase.

**Fungi Having ErdS also Encode a Dedicated Erg-Asp Hydrolase Involved in Erg-Asp Turnover.** Bacteria that possess MprFs are also equipped with dedicated hydrolases that remove aa modifiers from lipids, and whose gene usually lays next to mprF genes (8, 9). In *Af* (*ermS*), we observed that the *ermS* gene (4FA U1g02570 in *Af* 239, AO090005000838 in *Aor* RIB40, and NCU007082 in *Ncr* OR7434) is found close to a gene encoding a protein belonging to the α/β-hydrolase family (PFAM: PF07875) (4FA U1g02580, AO090005000837, and NCU007081, respectively) (Fig. 5A). These α/β-hydrolases all contain three conserved residues, Ser153, Asp277, and His307 (numbering of *Af*), that are typical of the catalytic triad of esterases/lipases, a finding supported by structure predictions (Fig. 5A).

To determine whether these esterases were involved in the Erg-Asp metabolic pathway, we used *Ncr* because, in this fungus, despite the presence of an *ermS* gene (Fig. 5A and SI Appendix, Fig. S14), we could not observe Erg-Asp in total lipids, although an ErdS activity could be detected using the LA assay in crude protein extracts (SI Appendix, Fig. S7). The WT, Δ*ermS*, and Δ*esterase* deletion mutants of *Ncr* were obtained from the Fungal Genetic Stock Center (FGSC) (18, 19). We compared total lipids profiles of *Ncr* WT, Δ*ermS*, and Δ*esterase* using TLC (Fig. S5B). As stated, Erg-Asp was undetectable in the WT or Δ*ermS* strains of *Ncr* but accumulated at high levels in the Δ*esterase* strain (Fig. 5B), suggesting that the enzyme was most likely responsible for the absence of detectable Erg-Asp in WT *Ncr*. This esterase, seemingly involved in Erg-Asp degradation, was therefore named ErdH, for Erg-Asp hydrolase, and the Δ*esterase* strain was renamed Δ*ermH* (Fig. 5A and B). Additionally, we monitored the Erg-[14C]-Asp synthesis activity by LA assay in protein extracts from WT *Ncr*, Δ*ermS*, and Δ*ermH* strains. Our data show that, while almost undetectable under the conditions tested in the WT and Δ*ermS* strains, Erg-[14C]-Asp levels were much higher (>23-fold) in the Δ*ermH* strain (Fig. 5C). This is again in agreement with ErdH being involved in Erg-Asp deacylation, which most likely masked the Erg-Asp synthesis activity of ErdS in the WT *Ncr* strain. Similarly, Erd-Asp synthesis was hardly monitored in vitro using total protein extracts of *Af* and *Aor* (Fig. 3F), suggesting either low expression levels of ErdS or that ErdH hydrolyzed the Erg-Asp product.

In order to confirm that ErdH was indeed an Erg-Asp hydrolase, we used an in vitro Erg-Asp deacylation assay. Erg-[14C]-Asp incubated for 30 min with purified recombinant *Af* ErdH was almost entirely hydrolyzed (88 ± 2%), while, in the same reaction performed without ErdH, Erg-[14C]-Asp levels remained stable (Fig. 5D). In addition, purified ErdH mutants of the catalytic triad (S153A, D277A, and H307A) did not hydrolyze Erg-[14C]-Asp in vitro (Fig. 5D). All these results demonstrate the presence of two enzymes in fungi that regulate the synthesis and degradation of Erg-Asp, namely ErdS and ErdH.

**An Evolutionary Conserved Synteny between *erdS* and *erdH*. ErdS and ErdH are present in *Af*, *Aor*, and *Ncr*, with their respective genes found at the same locus and encoded as divergent expression units. In order to analyze whether ErdS and ErdH are more generally found in fungi and/or other eukaryotes, we performed bioinformatics searches (SI Appendix, Supplementary Materials and Methods). We first searched ErdS sequences among eukaryotes using the basic local alignment search tool (BLAST) with the *Af* ErdS sequence as a probe and found 7,584 protein sequences (only those with lengths 200 to 2,000 residues), that corresponded to homologous aaRSs such as asparaginyl- and lysyl-tRNA synthetases. Among them, 1,006 (13.3%) were bona fide DUF2156-containing

---

**Fig. 5.** Detection and characterization of ErdH in fungi carrying ErdS. (A) Schematic representation of the genomic context of the *erdS* (yellow) and *erdH* (for Erg-Asp hydrolase, in green) genes locus from *Af*, *Aor*, and *Ncr* that highlights that *erdS* and *erdH* are found in a divergent orientation. Phylog2-based self-hydrolase–like predicted structure of *Af* ErdH and active site alignments of *Af*, *Aor*, and *Ncr* ErdHs. Ser-Asp-His catalytic triads of self-hydrolases/lipases (S153, D277, and H307 in *Af* ErdH) are displayed on the structure prediction and the alignment. (B) Total lipids from WT, Δ*ermS*, and Δ*ermH* were separated by TLC and stained with sulfuric acid/MnCl2 and observed under UV or visible light (n = 3); * indicates Erg-Asp. (C) In vitro measurements of Erg-[14C]-Asp synthesis by LA assay using protein extracts from the WT, Δ*ermS*, and Δ*ermH* strain protein extracts, using pure ScetrNAAso as a substrate (n = 2). (D) In vitro measurement of the Erg-[14C]-Asp hydrolase activities of purified recombinant WT (n = 3) or catalytic mutant Ncr ErdHs (n = 1). (C and D) The Student’s t test was used to determine significance of the means of the data; ***P < 0.005.
proteins, with 780 (77%) being ErdS with a minimal length of \( \sim 800 \) aa (no N- or C-terminal extensions) and a maximal length of 1,417 aa (with long N- and/or C-terminal appendages). “Standalone” DUF2156 domains (23%) were also detected and often accounted for ErdS forms with either the AspRS or the DUF2156 domain truncated. A closer look at the phylogenomic distribution of complete ErdS (AspRS-DUF2156 fusions) highlighted that they are present only in “higher” fungi (Dikarya, both Ascomycota and Basidiomycota), and specifically among 9 out of the 13 fungal lineages analyzed (Fig. 6A), with the notable exception of Saccharomyces (including Sce), Lecanoromycetes, and Pezizomycetes. ErdS is present in prominent human pathogens such as 

\text{Afv} \ (20), Histoplasma capsulatum, or Cryptococcus neoformans (Cne), plant pathogens such as Fusarium oxysporum, Magnaporthe oryzae, or Ustilago maydis, insect pathogens like Beauveria bassiana or Metarhizium spp., and also fungi of biotechnological interest like Aor or Ncr. We then visualized the synteny of the erdS gene in various fungi and observed that, in most of the cases, the erdH gene was almost always present next to erdS with the same divergent orientation in numerous species, showing that the linkage between the two genes is conserved. In fungi lacking erdS, the erdH genes were also absent, as judged from the absence of proteins with significant sequence homology to ErdH (Fig. 6A). Overall, the whole ErdS/ErdH enzymatic pathway seemed conserved across “higher” fungi.

Finally, in order to confirm that Erg-Asp is synthesized in several representative ErdS/ErdH-containing fungi, we extracted and analyzed, by TLC, total lipids from a collection of 15 fungal species (13 Ascomycota and 2 Basidiomycota). As expected from the absence of an erdS gene, Erg-Asp was absent in the four tested Ascomycota and one Basidiomycota species tested (Fig. 6B). The only exceptions were Ncr (Ascomycota), as already explained above, and Cne (Basidiomycota), for which the erdS gene regulation and/or the activity of ErdH could also account for the absence of Erg-Asp. Taken together, our results confirm that Erg-Asp is likely widely distributed and conserved across fungi.

Discussion

A wide range of bacteria possess membrane proteins with an AAT activity, namely MprFs, that reroute aa-tRNAs from protein synthesis to cell surface remodeling, thereby intensifying antimicrobial resistance, pathogenicity, and/or virulence (4, 7, 10).
Ever, suggest that ErdH might be mitochondrial in the subcellular localization of either ErdS or ErdH. Bioinformatics predictions (Wolf PSORT, MitoProt II software), however, suggest that ErdH might be mitochondrial in Aspergillus spp., while ErdS is predicted to be cytoplasmic and/or nucleocytoplasmic, a fact that is not unusual for aASs (23). Also, no plausible membrane-spanning or membrane-anchoring helices could be predicted in ErdS or ErdH, which differs from bacterial homologs: MprFs have N-terminal membrane-spanning domains, and aminocoy-glycerolipid hydrolases have N-terminal secretion signals to ensure correct periplasmic or extracellular localization (4). Very preliminary subcellular fractionation experiments suggest that, in A. fumigatus or in the heterologous model Sce, ErdS is not soluble but is associated with membranes, but the nature of those membranes remains unknown. Of note, in any case, is that membrane association of ErdS—to recognize Erd—requires that the enzyme be exposed to the cytoplasmic space, where its tRNA^{Asp}, Asp, and ATP substrates are present (SI Appendix, Fig. S8).

Erd is the major sterol found in fungal plasma membranes (24). Its function is analogous to that of Cho in mammals, which includes modulating permeability and fluidity of the lipid bilayer (25, 26) and regulating the activity of membrane proteins, such as G protein-coupled receptors (27) or V-ATPases (28). Erd, together with sphingolipids, regulates membrane trafficking, which is illustrated in filamentous species, such as Aspergillus spp., by a constant supply of Erd at the tip of mycelia to form lipid microdomains that are crucial to polarized cell growth (29). Erd also plays multiple roles in pathogenicity and in antifungal resistance (24, 30, 31). Incidentally, because Erd biosynthesis pathway differs from that found in humans for Cho, it is one of the main targets of current antifungal drugs (32).

Although we deciphered the function of ErdS and ErdH, the role of Erd-Asp in the biology of fungi now remains to be discovered, and this will be the focus of our future work. The viability of the ΔerdS strains of A. fumigatus, A. oryzae, and N. crassa shows that neither ErdS nor ErdH is essential under standard growth conditions. However, as discussed below, this fact does not exclude functions that could become essential or contribute to the overall fitness of fungi under challenging conditions, such as in the presence of membrane-targeting antifungals or antimicrobial peptides, which may explain the evolutionary conservation of the enzymatic system across Dikarya. As a first hypothesis, asparafylation of ErdS by ErdH might play a role similar to aminocoacylated glycerolipids in bacteria, regarding antimicrobial resistance (4, 7, 11, 32). Indeed, addition of t-Asp on the neutral and highly hydrophobic Erd leads to a novel zwiterionic sterol, which may significantly change Erd physicochemical properties within membranes, by influencing its phase behavior, its lateral diffusion, and/or its interaction with other lipidic. Local Erd-Asp synthesis might, in turn, influence membranes’ overall properties such as interface hydration, membrane proteins composition, permeability, and/or fluidity, or the activity and/or localization of membrane proteins. Therefore, Erd-Asp may participate in membrane remodeling processes that might have an impact on antifungal resistance, notably, in the case of polyenes, known to directy interact with Erd to form pores, since asparafylation of the 3β-OH could change their interactions with Erd (33), a hypothesis that we will explore. Erd-Asp levels (7 to 20% of free Erd in A. oryzae; SI Appendix, Fig. S9) upon growth in rich medium seem incompatible with an overall surface remodeling; however, nothing is currently known on the regulation of Erd-Asp synthesis and the amount of modification that can occur under challenging conditions. As an illustration, in Sce, an Erd acetylation/deacylation cycle, that functionally resembles our Erd/S-ErdH enzymatic system, involves the Atf2 acetyltransferase and the Sayl esterase. This nonessential enzymatic system protects cells from the accumulation of toxic Erd intermediates within membranes, and from the effect of membrane-disrupting agents such as the antifungal agent eugenol (34), suggesting that the ErdS/ErdH system and Erd asparafylation might be of importance in resistance when Erd biosynthesis inhibitors, such asazole derivatives, that trigger accumulation of toxic Erd intermediates, are used against fungi. This could be tested by determining the susceptibility of WT (Erd-Asp-containing), ΔerdS (Erd-Asp-deficient), ΔerdH (Erd-Asp-accumulating),
or overexpressing (Erg-Asp-overproducing) strains in the presence ofazole derivatives.

While lipid modifications are usually involved in cells’ surface remodeling and antimicrobial resistance in bacteria (7, 35), they also often participate in membrane trafficking and lipid-derived signaling in eukaryotes (3), like, for example, in the case of phosphoinositides or sphingolipids (36). Erg-Asp is produced at levels (7 to 20% of free Erg in Aor; SI Appendix, Fig. S9) that could be compatible with such cellular functions. Ergosteryl-3β-O-glucoside (Erg-Glc), another sterol conjugate, produced by Erg glycosylation, is nonessential, but known to actively participate in the regulation of autophagy in several yeasts and in Aor (37, 38), likely through the recruitment of protein partners on membrane structures (39). It is therefore possible that Erg aspartylation could intervene in the recruitment of protein factors on membranes (Erg-containing microdomains, etc.) and influence regulatory or trafficking pathways. Finally, bacterial esterases that remove aa modifications from glycolipids also influence virulence in A. tumefaciens (10). Those observations in various bacterial lipid aminoaoylation systems or fungal sterol modification pathways raise the question of the contribution of Erg aspartylation and deacetylation to those processes in fungi. Given the conservation of the ErdS/ErdH enzymatic system across Dikarya, these aspects will now have to be addressed experimentally to decipher the function of Erg-Asp.

Materials and Methods

Materials, Strains, and Growth Condition. The erds codon-optimized synthetic gene was purchased from the Genscript Corporation. The anti-DUF2156 polyclonal antibody production was performed by Covalab (R&D in Bio-technology). Some of the fungal strains were ordered from the Fungal Genetics Stock Center. The CEA17 ΔakubNΔerdS strain of Aor (40) and the R84Δ lo strain of Aor (41) have been used as parental strain for this study. The routine growth and maintenance of Afm and Aor are described in SI Appendix, Supplementary Materials and Methods. The strains (bacterial and fungi) used in this study are listed in SI Appendix, Table S1.

Total Lipid Extraction from Sce and from Filamentous Fungi. Lipid extraction protocols for Sce and filamentous fungi were adapted from the Bligh and Dyer procedure (42). Briefly, Sce cells were grown in synthetic complete (SC) medium without leucine (SC-LEU) (MP Biomedicals) until optical density 600 nm (OD600 nm) reached 1.0. An equivalent of 150 OD600 nm of Sce cells were centrifuged at 4,000 × g for 15 min at 4 °C. The cell pellet was resuspended in 1 mL of Na-acetate 120 mM pH 4.5. Mechanical cell disruption was performed in a FastPrep-24 apparatus (MP Biomedicals, serial no. 10020698) in the presence of 400 μL of glass beads (0.25 mm to 0.5 mm, Roth) at 6 m/s, for 1 min repeated 6 times, with cooling on ice between each step; 3.75 vol % of MeOH:CHCl3 2:1 (v:v) were added, and the mixture was incubated on a rotating wheel at 4 °C for 3 h. Then, 1.25 vol % of CHCl3 and Na-acetate 120 mM pH 4.5 were successively added and mixed by vortexing. The mixture was then centrifuged at 4,000 × g for 10 min to obtain two phases. The lower organic phase, containing the lipids, was transferred into a new tube, dried under vacuum, and stored at −20 °C until use or resuspended in 50 μL to 200 μL of MeOH:CHCl3 1:1 (v:v) for analysis by TLC.

For filamentous fungi, the same protocol was applied on 2 g of mycelia that were ground in a mortar with a pestle in liquid nitrogen to obtain a fine powder. Furthermore, the incubation on the rotating wheel was performed overnight at 4 °C.

Lipid X Purification. LX was purified from adaptation of the flash chromatography procedure used for bacterial aminoaoylated lipids (11). Total lipids extracted from 500 OD600 nm of Sce cells were solubilized in 200 μL of chloroform and loaded on a 1.5-ml silica gel (Sigma-Aldrich, pore size 60) glass column preequilibrated with CHCl3. After absorption of lipids on the column, nonpolar lipids and glycolipids were eluted with 10 mL of chloroform followed by 10 mL of acetone, respectively. To elute polar lipids, various ratios of CHCl3:MeOH from 9:1 to 6:4 (vol/vol) (20 mL each) were used, and elution fractions were collected in 2-ml samples in glass tubes. Fractions were dried under vacuum, resolubilized in 35 μL of MeOH:CHCl3 (1:1, v:v), and visualized by TLC.

TLC Analyses. Dried lipid samples were resuspended in MeOH:CHCl3 (1:1, vol/vol) and spotted onto 10 × 20 cm silica gel on TLC A1 foils (Sigma-Aldrich). TLC was developed with a CHCl3:MeOH:H2O (130:50:8, v:v:v) mobile phase (10 min at room temperature [RT]), and plates were stained with either MnCl2/sulphuric acid or ninhydrin or bromocresol green. MnCl2/sulphuric acid, a universal staining (43), was prepared with 0.8 g of MnCl2 tetrahydrate dissolved in 240 mL of 50% (vol/vol) MeOH supplemented with 9 mL of concentrated sulphuric acid. To detect primary amines (pink color), ninhydrin 0.4% (vol/vol) (Sigma-Aldrich) was used. To reveal carboxylate-containing compounds (blue color) with a pH below 5.0 (44), bromocresol green 0.06 g (Sigma-Aldrich) dissolved in absolute ethanol and NaOH 0.1 M was used. In the case of MnCl2/sulphuric acid and ninhydrin stains, plates were heated at 100 °C until coloration developed, while direct ultraviolet (UV) (254 or 315 nm) visualization was performed with bromocresol green. Quantification of LX (Erg-Asp), PE, or PG spots was performed using the ImageJ software. Lipid spots signals (number of pixels, N) were normalized to that of PE (NPE/NLPE).

In Vitro LA Assay. Prior to lipid aminoaoylation, the in vitro tRNAAsp aspar- tylation was performed as described in SI Appendix, Supplementary Materials and Methods. Lipid or steroid aspar- tylation assays were adapted from lipid aminoaoylation protocols used for bacterial MprFs (42). Briefly, reactions were performed in the following aminoaoylation mixture: Na-Hepes 100 mM pH 7.2 buffer containing KCl 30 mM, MgCl2 12 mM, ATP 10 mM, bovine serum albumin 0.1 mg/mL, pure yeast tRNAAsp (10 μM) (46), L-[1-14C]-Asp (280 cpm/pmol, Perkin-Elmer, NEC268E050UC) in a final volume of 50 μL. To test the transfer of [14C]-Asp onto lipids, total lipids were added to a final concentration of 2 mg/mL, and commercial pure sterols (Erg and Cho) were added to a final concentration of 0.5 mg/mL. When [3H]-Erg (10 Ci/mmol, Hartmann Analytic) was used, 20,000 cpm were added to the reaction mix. The resulting suspension was sonicated for 30 s in a sonicator bath at RT, and hydrophilic interaction liquid chromatography (HILIC) conditions with a binary gradient running from 99.7:0.3 (A:B) to 75:25 in 40 min, where A was water, and temperature of the ESI was 380 °C. Full-scan spectra were collected in the 110 to 2,000 m/z range, and data-dependent Zoom and MS2 spectra were acquired on the 15 most intense peaks. Data were analyzed with XcaliburQual Browser.

In Vitro Lipid Deacylation Assay. To generate Erg-[14C]-Asp, we used the protocol described above for LA assay. RNase A was added and incubated for 20 min at 30 °C to hydrolyze RNA** and stop the RNase-dependent deacylation catalyzed by ErdS. Then, 0.1 μM of purified recombinant ErgS or ErdH variants were added, and the reaction mixture was incubated for 30 min at 30 °C. Lipids were extracted with the Bligh and Dyer procedure as described above, dried, resuspended in CHCl3:MeOH (1:1, vol/vol), and analyzed on TLC with the CHCl3:MeOH:H2O (130:50:8, v:v:v) solvent, and results were analyzed by phosphorimaging as described above.

Yakovov et al.
Statistical Analyses. The Student’s t test was used to determine significance of the means of the data.

Materials and Data Availability. All available data are already included in the manuscript. All materials and strains are freely available (contact: h.becker@unistra.fr; frfischer@unistra.fr).

ACKNOWLEDGMENTS. This work was supported by the Fondation pour la Recherche Médicale (FRM), to H.D.B. (Grant DBF20160635713), by “Mito-Cross” Laboratory of Excellence (Grant ANR-10-IDEX-0002-02 to H.D.B.), by the University of Strasbourg (to H.D.B.), by an IDEX from the University of Strasbourg (W17RAT81 to F.F.), the National Center for Scientific Research (H.D.B., B.S., F.F., and N.Y.), and the Meiji University (T.K., Y.S.T., D.Y., and H.N.). N.Y. was supported by a fellowship from the French Ministère de l’Enseignement Supérieur et de la Recherche, and N.M. was supported by a postdoctoral fellowship from the FRM (Grant DBF20160635713). H.R. and C.D.G. were supported by NIH Grant 1R21AI144481-01. We thank J.-P.L., Dr. I. Mouyna, Dr. A. Beauvais, and Dr. T. Fontaine (Institut Pasteur, Paris, France) for providing A. fumigatus strains and deletion cassette templates, for having trained F.F. on the manipulation of the strains, and for thoughtful discussions. We thank H.N. for A. oryzae strains and related plasmids and Maryline Brock (University of Strasbourg) for other fungal strains. In addition, we thank Dr. Sylvie Friant for critical review, experimental suggestions, and careful reading of the manuscript.