Supporting Information

Supplemental Figures

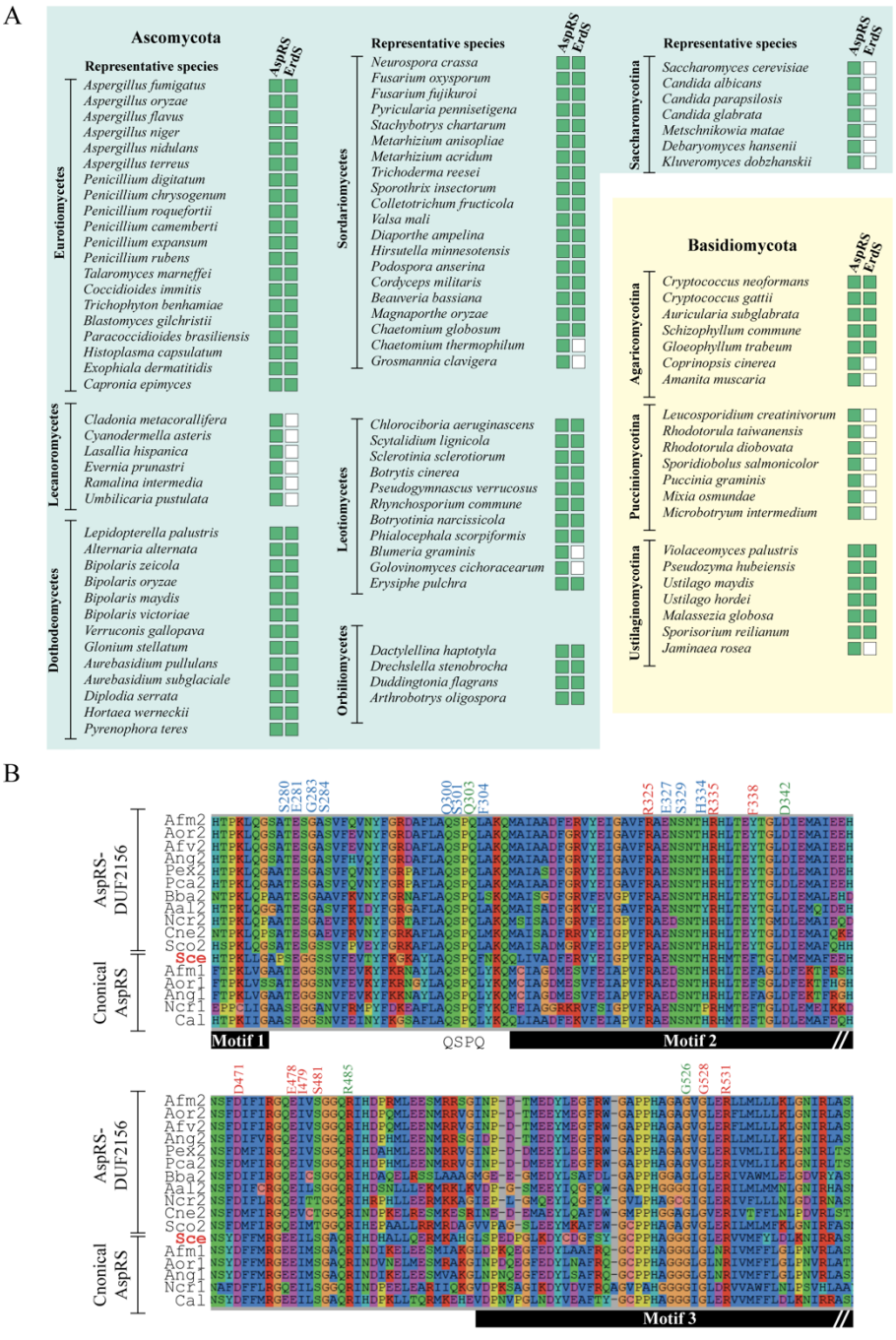


Figure S1: (**A**) Distribution in representative species of each class of Dikarya of the presence (green box) or absence (white box) of a canonical AspRS and ErdS. (**B**) Conservation of the consensus class II aaRS motifs 1, 2 and 3 in the alignment of 11 ErdS (AspRS-DUF2156) and 6 canonical AspRSs. *Afm: Aspergillus fumigatus, Aor: Aspergillus oryzae, Afv: Aspergillus flavus, Ang: Aspergillus niger, Pex: Penicillium expansum, Pca: Penicillium camemberti, Bba:*

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

Beauveria bassiana, Aal: Alternaria alternata, Ncr: Neurospora crassa, Cne: Cryptococcus neoformans, Sco: Schizophyllum commune, Sce: Saccharomyces cerevisiae, Cal: Candida albicans. Numberings are indicated relative to the *Sce* AspRS sequence. Residues involved in the recognition of ATP, and aspartate are indicated in red and green respectively, whereas residues responsible for the $tRNA^{Asp}$acceptor arm recognition through its 3'-CCA are indicated in blue.

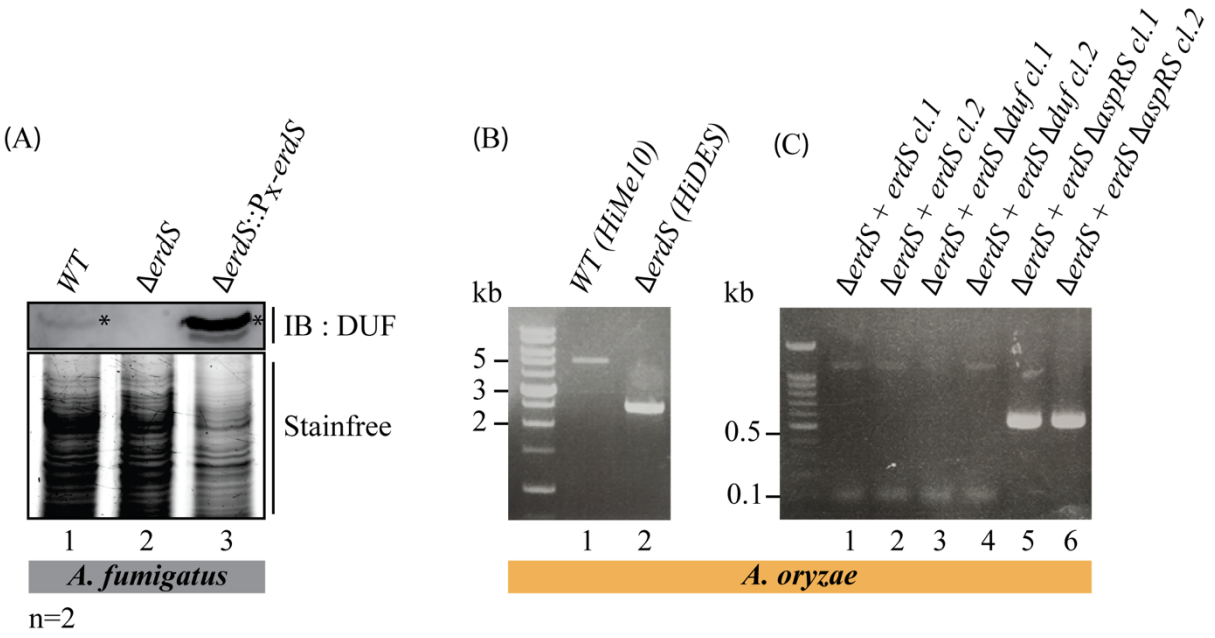


Figure S2: (**A**) Confirmation of *erdS* deletion in Δ*erdS* (lane 2) and complementation in Δ*erdS::*P*x-erdS* (lane 3) extracts using immunodetection of ErdS with anti-ErdS polyclonal antibodies (WT as a control) (n=2). Stainfree: loading control. The uncropped Western Blot is shown in **Figure S8**. (**B**) Confirmation of the presence of*erdS* in *Aor* HiMe10 (lane 1) and *erdS* deletion in *Aor*HiDES (lane 2) by PCR amplification. (**C**) PCR confirmation of Δ*erdS* complemented with *erdS* (lanes 1 and 2), *erdS*Δ*duf* (lanes 3 and 4) or *erdS*Δ*aspRS* (lanes 5 and 6) in *Aor* HiDES. Molecular weight markers are indicated in kb. Primers used for construction verification are listed in **Table S1 and S2**.

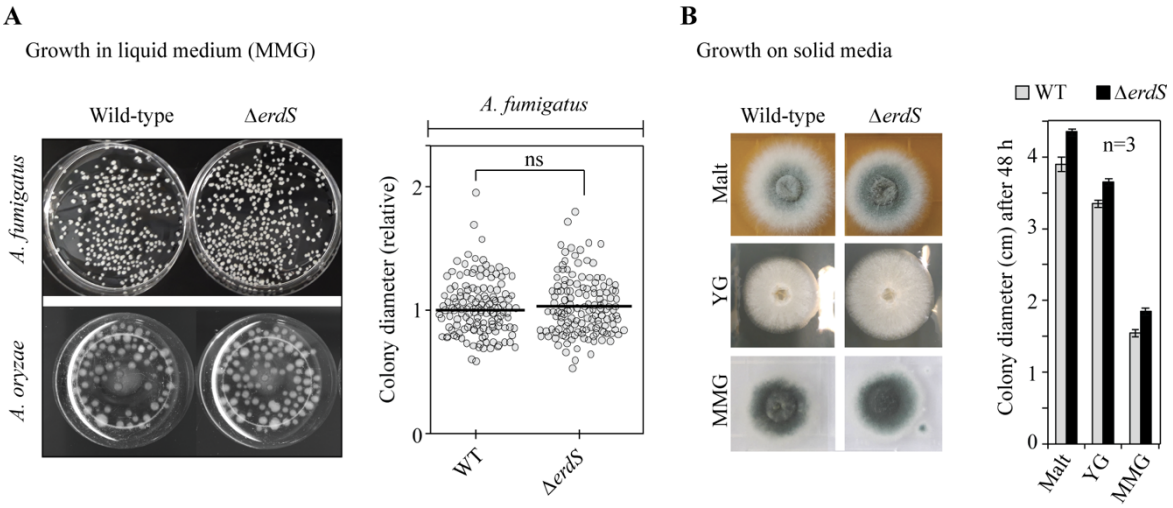


Figure S3: The *erdS* gene is not essential and its deletion does not affect growth or colony morphology. **(A)***A. fumigatus* and *A. oryzae* WT and Δ*erdS* strains were grown in liquid media under agitation (200 rpm) at 37°C and the resulting spherical colonies (pellets) obtained after 24 hrs were photographed (left panel). In the case of *Afm*, 140 colonies (pellets) of the WT and Δ*erdS* strains were measured (pixel area of individual pellets) and each represented in a dot plot. The geometric mean of pellets' sizes of the WT strain was normalized to 1. No significant (ns, t-test) differences were detected between both strains, indicating the absence of growth defects in the Δ*erdS* strain. **(B)** Growth was also analyzed on solid agar plates containing 3 different media: Malt extract, YG (Yeast extract 0.5 % (w/v), Glucose 2 %) and MMG (*Aspergillus* minimal medium with 1 % glucose). Six µL of a freshly prepared 10^6 conidia/mL suspension were spotted on plates and growth monitored (37 °C) up to 4 days. Results after 48 hours of growth are represented. Colony diameters of mycelia were monitored. No significant difference between the WT and Δ*erdS* strains was detected, indicating that mycelia expansion is not significantly altered upon deletion of the *erdS* gene in *Afm*.

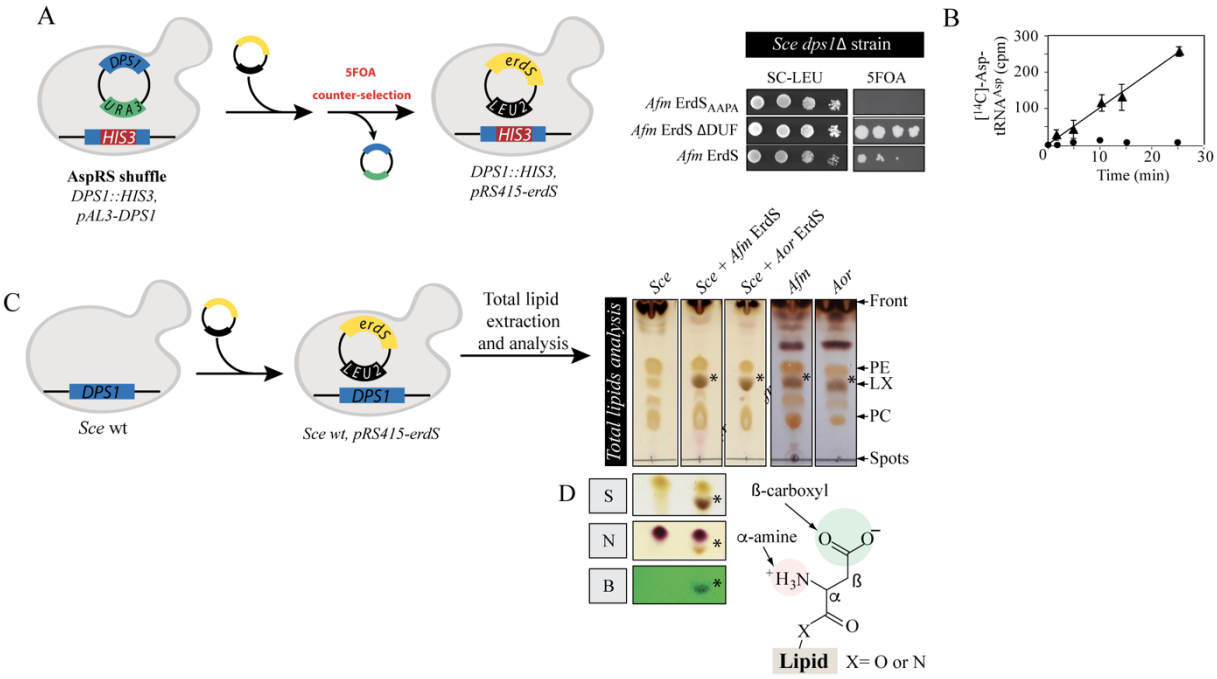


Figure S4:(**A**) Plasmid shuffling experiments in the Δ*dps1 Sce* strain. Drop tests were performed on SC-LEU and 5FOA media with Δdps1 strains carrying plasmids expressing the indicated *Afm* ErdS isoform: $ErdS_{AAPA}$ corresponds to ErdS mutated in the QSPQ motif of the AspRS domain, ErdS ΔDUF corresponds to ErdS with the DUF2156 domain deleted and ErdS corresponds to the full-length protein version. (**B**) *In vitro*$tRNA^{Asp}$ aminoacylation assay performed with purified recombinant *Afm* ErdS (triangles) in the presence of pure *Sce* $tRNA^{Asp}$, ATP, and [^{14}C]-Asp. Circles: control without enzyme. (**C**) Total lipids were extracted from the 3 indicated *Sce* strains and from *Afm* and *Aor*, separated by TLC in the $CHCl_3$: MeOH: H_2O (130:50:8 v/v/v) solvent and stained with a $MnCl_2$/sulfuric acid treatment. The novel ErdS-dependent lipid (lipid X (LX), marked with *), also present naturally in *Afm* and *Aor* was detected. PE: phosphatidylethanolamine, PC: phosphatidylcholine. (**D**) An Asp residue is likely linked to any lipid via an ester (α-COO^-) or amide (N-containing) bond and have free α-NH_3^+ and β-COO^- groups. TLCs of total lipids extracted from *Sce* strain expressing *Afm* ErdS were stained with $MnCl_2$/sulfuric acid (S), ninhydrin (N) or bromocresol green (B) dyes and supported that lipid X contained an amine and carboxyl groups.

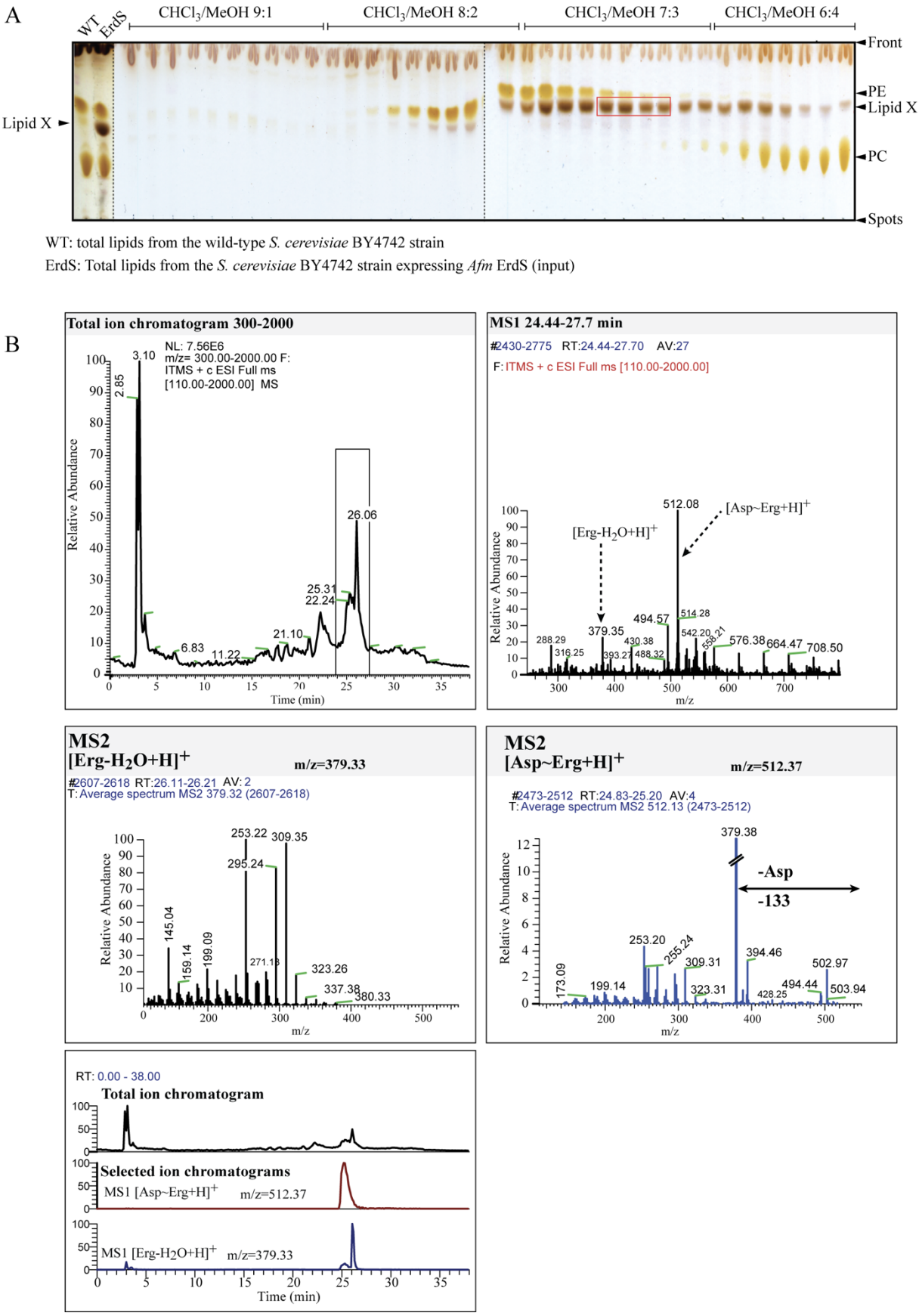
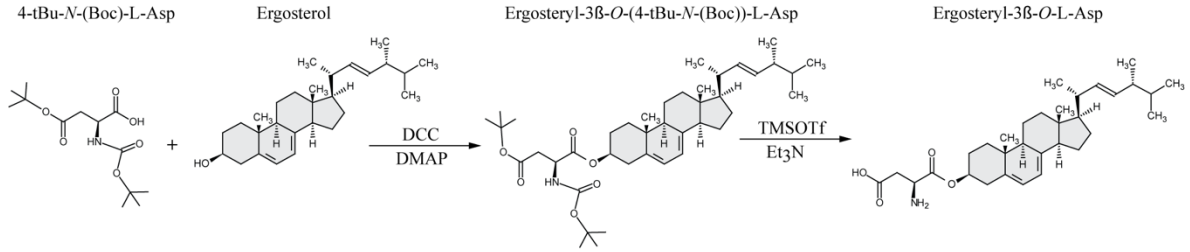


Figure S5: Purification of Erg-Asp from *Sce* and detection of Erg-Asp in total lipids from *Afm*. (**A**) Erg-Asp was purified from *Sce* expressing the *Afm* ErdS protein. Total lipids were loaded

on a silica gel 60 glass column, washed with $CHCl_3$, followed by acetone, and polar lipids were eluted using $CHCl_3$/MeOH mixture varying from 9/1 to 6/4 ratio. Fractions enriched in Erg-Asp were collected, pooled, and analyzed by MS/MS. Total lipids from a WT *Sce* strain that does not produce Erg-Asp were treated equally, and fractions corresponding to the elution peak of Erg-Asp were collected, pooled and used as a negative control for MS/MS (see **Fig. 4**). (**B**) In parallel, total lipids from *Afm* were extracted and directly analyzed. The figure shows the LC-ESI-MS/MS analysis of total lipid extract from *Afm*. 10 µL of total lipids from *Afm* were analyzed with a liquid chromatograph Surveyor Plus coupled with a LTQ-XL ion trap analyzer (Thermo Finnigan). The column was a Cogent Diamond Hydride (250×4.6 mm, 4 µm, Microsolv, Eatontown, NJ, USA) ran in hydrophilic interaction chromatography (HILIC) conditions. Mass spectrometer was run in positive mode, and MS/MS spectra were acquired for the 15 most intense peaks. Erg-Asp and Erg were identified with their MSMS spectra. Selected ion chromatograms show that these lipids had a retention time of 24.1 and 26.1 min, respectively.

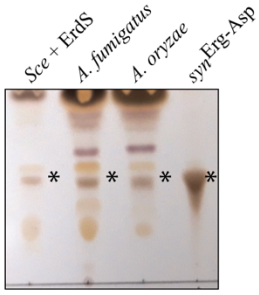
A



B

position	^{1}H(ppm)	^{13}C(ppm)	position	^{1}H(ppm)	^{13}C(ppm)
1		38,240	**17**		57,047
2		29,436	**18**	0.668, s	12,511
3		76,965	**19**	0.981, s	16,506
4		41,807	**20**		40,287
5		139,166	**21**	1.057, d	21,652
6	5.573, m	119,660	**22**	5.228, m	133,256
7	5.396, m	117,531	**23**	5.228, m	136,980
8		142,791	**24**		44,347
9		47,397	**25**		30,735
10		37,253	**26**	0.868, d	20,082
11		22,071	**27**	0.847, d	20,468
12		38,922	**28**	0.944, d	18,207
13		43,936	**1'**		172,663
14		55,691	**2'**	4.307, m	50,603
15		24,019	**3'**		34,368
16		28,844	**4'**		168,89

D



* Erg-Asp

Solvent system:
$CHCl_3$:MeOH:H_2O (130/50/8)

C

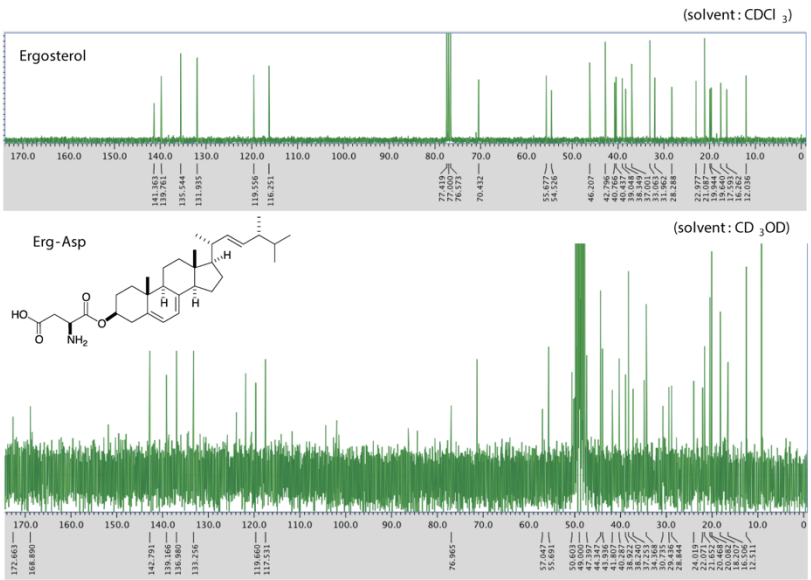


Figure S6: Chemical synthesis of Erg-Asp. (**A**) Two-step synthesis scheme of Erg-Asp (Ergosteryl-3β-*O*-L-aspartate) from Erg and 4-tBu-*N*-(Boc)-L-Asp. (**B**, **C**) NMR chemical shift assignments of synthetic Erg-Asp ($_{syn}$Erg-Asp) and [^{13}C] NMR spectrum of synthetic ergosteryl-3β-*O*-L-aspartate. (**D**) Comparison of $_{syn}$Erg-Asp (lane 4) with Erg-Asp extracted from a *Sce* strain expressing *Afm*ErdS (lane 1), from *Afm* and *Aor* (lanes 2, 3).

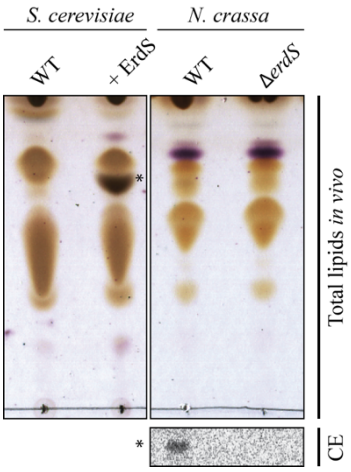


Figure S7: Total lipids from WT and Δ*erdS* strains of *Ncr* were extracted and separated by TLC with the $CHCl_3$(n=2): MeOH: H_2O (130:50:8 v/v/v) solvent and stained with a $MnCl_2$/sulfuric acid treatment. Control Sce strains that express (+) or not (WT)ErdS were used as controls. Erg-Asp is marked with *. The expression of ErdS was tested in crude protein extracts (CE) from the WT and Δ*erdS* strains of *Ncr* using the LA assay with [^{14}C]-Asp to monitor Erg-[^{14}C]Asp synthesis (n=2). To obtain a clear visualization of the Erg-[^{14}C]Asp band, after reaction and separation in TLC, at least 8 hrs of exposure (phosphorimaging) were required, which suggested that ErdS was weakly expressed or that a putative Erg-Asp hydrolase was expressed and masked ErdS activity by cleaving the Erg-[^{14}C]Asp product.

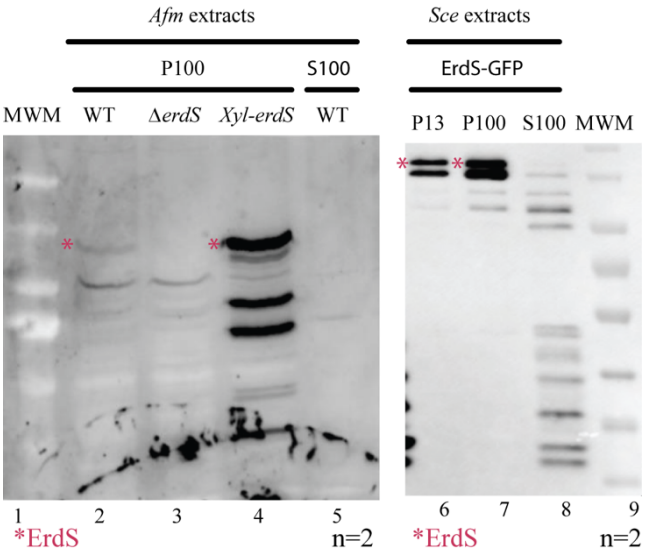


Figure S8: *Afm* ErdS colocalizes with membrane fractions. Western blot using anti-ErdS antibodies (1/1000 dilution) after 12 % SDS-PAGE (n=2). Lanes 1 &9: molecular weight marker; lanes 2 and 5: P100 andS100 fractions of the *Afm* WT strain extract; lane 3: P100 fraction of the *Afm* Δ*erdS* strain extract; lane 4: P100 fraction of an extract of the *Afm* Δ*erdS* strain complemented with the *erdS* gene under the dependence of a xylose promoter (overexpresses ErdS in the presence of xylose); lanes 6-8: P13, P100 andS100 fractions of an extract of the *Sce* strain overexpressing *Afm* ErdS. Note that lanes 2-4 represent the uncropped Western blot shown in Figure S2.

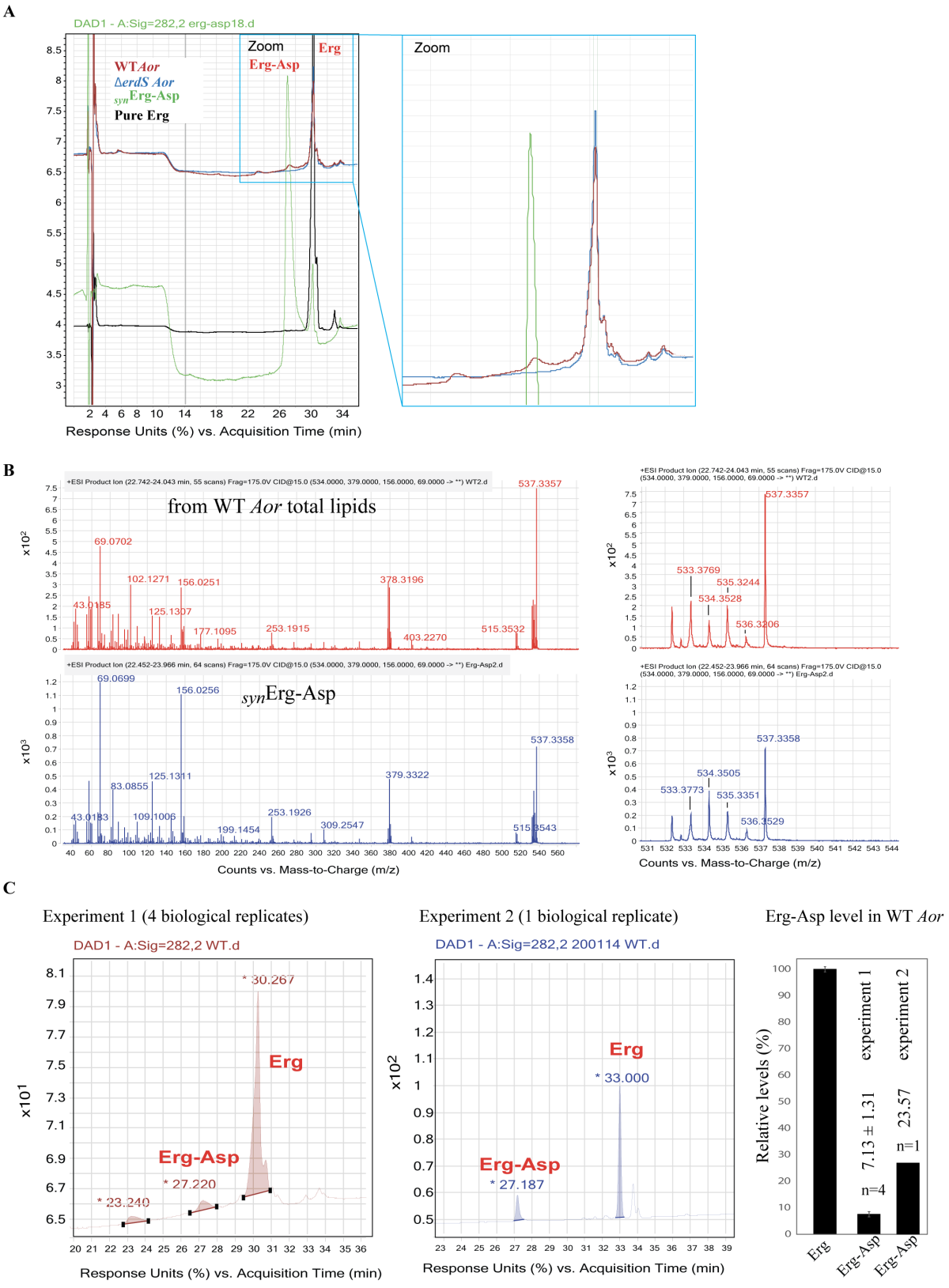


Figure S9:Detection and quantification of Erg-Asp in *Aor*.(A) Lipids extracted from *Aor* were fractionated on a TSKgel ODS80$_{TM}$ QA (C18) (2.0×150 mm, 5mm) column using a continuous gradient from 100 % MeOH:H_2O (4:1, v:v) (solvent A) to 100 % MeOH:CH_2Cl_2 (3:1, v:v)(solvent B) between 5 and 34 min at a flow rate of 0.2 mL/min at 25°C. Eluted compounds were monitored at 282 nm and

peaks of interest were submitted to an Accurate-Mass Q-TOF LC/MS (Agilent Technologies 6520) to confirm their identity **(B)** (upper spectrum: total lipids from WT *Aor* and bottom spectrum: $_{syn}$Erg-Asp). Lipids from a WT *Aor* strain (Erg and Erg-Asp present) and from a Δ*erdS* strain (no Erg-Asp) were analyzed and compared to the elution profile of pure Erg or of our synthetic (chemically synthesized) $_{syn}$Erg-Asp, as a reference (**A** and **B**).**(C)** Lipids from a WT *Aor* strain, having both Erg and Erg-Asp, were analyzed on a TSKgel ODS80$_{TM}$ QA (C18) (2.0×150 mm, 5mm) column with a continuous gradient from 100 % solvent A to 100 % B between 5 and 34 min and analyzed with an Accurate-Mass Q-TOF LC/MS (Agilent Technologies 6520) (experiment 1, 4 independent biological replicates). Quantification of peaks area showed that Erg-Asp represents 7.13±1.31 % of total Erg. Total lipids were also analyzed on a TSKgel ODS80$_{TM}$ QA (C18) (2.0×150 mm, 5mm) column in an independent biological replicate (experiment 2). In this case, quantification showed that Erg-Asp represented 27.2 % of total Erg. Discrepancies likely come from the solubility properties of Erg-Asp in extraction solvents, the amount found in experiment 2 representing an upper limit. Therefore, Erg-Asp is seemingly produced under standard growth conditions at levels between 7 and 20 %, but most likely ~10 % of total free Erg in *Aor*, which is in good correlation with TLC profiles obtained with *Aor* total lipids, but also with its close relative *Afm*.

Supplemental Tables

Table S1: Bacterial and fungi strains used in this study

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Escherichia coli XL-1 Blue:endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(r_K- m_K+)	Agilent (Stratagene)	Catalog#200249
Escherichia coli Rosetta-2: strain B F– *ompT gal dcmlonhsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λ^S))*, carrying the pRARE2 plasmid	Merck Millipore	71400-3
Escherichia coli DH5α: F– endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoRnupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ^-	Invitrogen	C404003
Organisms/Strains		
Saccharomyces cerevisiae strains		
Saccharomyces cerevisiae BY4742*: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf	
Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3*	Dr. Gilbert Eriani	Ador *et al*, 1999
Saccharomyces cerevisiae BY4742*: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* + pRS415-*erdSAfm*	This paper	N/A
Saccharomyces cerevisiae BY4742*: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* + pRS415-*erdSAor*	This paper	N/A
Saccharomyces cerevisiae BY4742*: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* + pRS415-*erdS ΔDUF2156Afm*	This paper	N/A
Saccharomyces cerevisiae BY4742*: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* + pRS415-*erdSΔaspRSAfm*	This paper	N/A
Saccharomyces cerevisiae BY4742*: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* + pRS415-*erdS$_{AAPA}$Afm*	This paper	N/A
Saccharomyces cerevisiae BY4742*: MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* + pRS414-*erdS ΔDUF2156Afm* (AspRSonly)+ pRS415-*erdS ΔaspRSAfm* (DUF2156 only)	This paper	N/A
Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*erdSAfm*	This paper	N/A

Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*erdSAor*	This paper	N/A
Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*erdS ΔDUF2156Afm*	This paper	N/A
Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*erdSΔaspRSAfm*	This paper	N/A
Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*erdS$_{AAPA}$Afm*	This paper	N/A
Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*erdS* W785A *Afm*	This paper	N/A
Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*erdS* W785A *Afm*	This paper	N/A
Saccharomyces cerevisiae YAL3 *Δdps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*erdS* W785H *Afm*	This paper	N/A
Saccharomyces cerevisiae YAL3 Δ*dps1: MATa ura3-52 lys2-801am trp1-63 his3-200 leu2-1 ade2-450 ade3-1483 dps1::HIS3* + pRS415-*er**d**S* R789A *Afm*	This paper	N/A
*Aspergillus fumigatus*strains		
Aspergillus fumigatus CEA17^{KU80}*: pyrG akuB::pyrG (ΔakuB^{KU80})*	Prof. J.-P. Latgé. Pasteur Institute, Paris, France	Da Silva Ferreira ME et al., 2006
Aspergillus fumigatus CEA17^{KU80}*Δer**d**S (hph): pyrG akuB::pyrG (ΔakuB^{KU80}) **erd**S::hph*	This paper	N/A
Aspergillus fumigatus CEA17^{KU80}*Δ**erd**S: pyrG akuB::pyrG (ΔakuB^{KU80}) Δ**erd**S* (hph cassette excised)	This paper	N/A
Aspergillus fumigatus CEA17^{KU80}*Δ**erd**S::P$_{xyl}$-**erd**S: pyrG akuB::pyrG (ΔakuB^{KU80}) Δ**erd**S::[5'-utr- P$_{xyl}$-**erd**S-trpCterm-hph-3'-utr]*	This paper	N/A
Aspergillus oryzae strains		
Aspergillus oryzae RIB40*: wild-type*	H. Nakajima. Meiji University, Tokyo, Japan	Kuroki Y *et al.*, 2002

Aspergillus oryzae HiMe10: *ΔligD::AnpyrG-300bp ΔpyrG*	H. Nakajima Meiji University, Tokyo, Japan	Kuroki Y *et al.*, 2002
Aspergillus oryzae HiDES*Δerd*S: Δ*ligD::AnpyrG-300bp,* Δ***pyrG,*** Δ***AorErd****S::AnpyrG-300bp*	This paper	N/A
Aspergillus oryzae HiDEC *Δ**erd**S+aspRS:* Δ*ligD::AnpyrG-300bp,* Δ*pyrG,* Δ*Ao-e**rd**S::AnpyrG-300bp, niaD::(AoaspRSniaDAnpyrG)*	This paper	N/A
Aspergillus oryzae HiDEC *Δ**erd**S+duf2156:* Δ*ligD::AnpyrG-300bp,* Δ*pyrG,* Δ*Ao-e**rd**S::AnpyrG-300bp, niaD::(Aoduf2156 niaDAnpyrG)*	This paper	N/A
Aspergillus oryzae HiDEC ***ΔerdS+erd****S:* Δ***ligD::AnpyrG-300bp,*** Δ***pyrG,*** Δ***AoErdS::AnpyrG-300bp,*** ***niaD::(Aoerd****SniaDAnpyrG)*	This paper	N/A
Neurospora crassa strains		
Neurospora crassa 74-OR23-1VA : wild-type*, matA*	FGSC	FGSC #2489
*Neurospora crassa Δ**erd**S:erd**S::hph, matA*	FGSC	FGSC #20236
*Neurospora crassa ΔeraH:**erd**H::hph, matA*	FGSC	FGSC #20235
Other fungal strains		
*Aspergillus flavus*CA14: Δ*pyrG* Δ*ku80*	FGSC	FGSC #A1421
Beauveria bassiana NRRL 20698*:* wild-type	ATCC	ATCC 90517
Schizophyllum commune H4-8*:* wild-type	FGSC	FGSC #9210
*Aspergillus niger:*wild-type	University of Strasbourg	N/A
*Candida albicans:*wild-type	University of Strasbourg	N/A
Candida parapsilosis:wild-type	University of Strasbourg	N/A
*Geotrichum candidum:*wild-type	University of Strasbourg	N/A
*Penicillium expansum:*wild-type	University of Strasbourg	N/A
*Penicillium camemberti:*wild-type	University of Strasbourg	N/A
*Alternaria alternata:*wild-type	University of Strasbourg	N/A
*Cryptococcus neoformans:*wild-type	University of Strasbourg	N/A

Final plasmid	DNA template	Primers (5' --> 3')
Construction of donor plasmids containing erdS variants - Gateway BP reactions		
pDONR221-erdS-afm_Sce_opt	pUC57-erdS-afm_Sce_opt	GW_s : GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATG

(GW BP reaction)		
		GW_as : GGGGACTTTGTACAAGAAAGCTGGGTC
pDONR221-erdS-Δduf2156 (GW BP reaction)	pUC57-erdS-afm_Sce_opt	FF001 : GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAATCAAGAGAGCATTATCC
		FF003 : GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAGGTGGAAAAGATTTTGGATC
pDONR221-erdS-ΔaspRS (GW BP reaction)	pUC57-erdS-afm_Sce_opt	dufssDRS_s : GGGGACAAGTTTGTACAAAAAAGCAGGCTTCatgAGACACCCTGAAAGTTCTACAATAGAACC
		GW_as : GGGGACTTTGTACAAGAAAGCTGGGTC
pDONR221-erdS-aor (GW BP reaction)	A. oryzaegDNA	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCatgtccatcaaacgggccc
		GGGGACCACTTTGTACAAGAAAGCTGGGTCttagtcttcgaaaaagtgaagg
pDONR221-erdS-afm_Sce_opt (GW BP reaction)	pUC57-erdS-afm_Sce_opt	GW_s : GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATG
		GW_as : GGGGACTTTGTACAAGAAAGCTGGGTC
pDONR221-erdS-Δduf2156 (GW BP reaction)	pUC57-erdS-afm_Sce_opt	FF001 : GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAATCAAGAGAGCATTATCC
		FF003 : GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAGGTGGAAAAGATTTTGGATC
pDONR221-erdS-ΔaspRS (GW BP reaction)	pUC57-erdS-afm_Sce_opt	dufssDRS_s : GGGGACAAGTTTGTACAAAAAAGCAGGCTTCatgAGACACCCTGAAAGTTCTACAATAGAACC
		GW_as : GGGGACTTTGTACAAGAAAGCTGGGTC
pDONR221-erdS-aor (GW BP reaction)	A. oryzaegDNA	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCatgtccatcaaacgggccc
		GGGGACCACTTTGTACAAGAAAGCTGGGTCttagtcttcgaaaaagtgaagg
pDONR221-erdS-afm_Sce_opt (GW BP reaction)	pUC57-erdS-afm_Sce_opt	GW_s : GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATG
		GW_as : GGGGACTTTGTACAAGAAAGCTGGGTC
Construction of *S. cerevisiae* expression plasmids - isoThermal assembly		
pRS415-GPD-erdS-aor	pDONR221-erdS-aor	#101 : gaactagtggatcccccatcacaagtttGTACAAAAAAGCAGGCTTC
		#102 : cttgatatcgaattcctgcagcccatcaCCACTTTGTACAAGAAAGCTGGGTC
	pRS415-GPD-X (LR recombined plasmid)	#104 : tgatgggctgcaggaattcgatatcaag
		#103 : aaacttgtgatgggggatccactagttc
pRS415-GPD-erdS-afm	pDONR221-erdS-afm	#101 : gaactagtggatcccccatcacaagtttGTACAAAAAAGCAGGCTTC
		#102 : cttgatatcgaattcctgcagcccatcaCCACTTTGTACAAGAAAGCTGGGTC
	pRS415-GPD-X	#104 : tgatgggctgcaggaattcgatatcaag
		#103 : aaacttgtgatgggggatccactagttc
pRS415-GPD-erdS-Δduf2156	pDONR221-erdS-Δduf2156	#101 : gaactagtggatcccccatcacaagtttGTACAAAAAAGCAGGCTTC
		#102 : cttgatatcgaattcctgcagcccatcaCCACTTTGTACAAGAAAGCTGGGTC
	pRS415-GPD-X	#104 : tgatgggctgcaggaattcgatatcaag
		#103 : aaacttgtgatgggggatccactagttc
pRS415-GPD-erdS-ΔaspRS	pDONR221-erdS-ΔaspRS	#101 : gaactagtggatcccccatcacaagtttGTACAAAAAAGCAGGCTTC
		#102 : cttgatatcgaattcctgcagcccatcaCCACTTTGTACAAGAAAGCTGGGTC
	pRS415-GPD-X	#104 : tgatgggctgcaggaattcgatatcaag
		#103 : aaacttgtgatgggggatccactagttc

pRS415-AspRS$_{AAPA}$-DUF2156	pRS415-GPD-erdS-afm	FF048 : TTTTAGCCGCTGCTCCTGCTTTGGCAAAGCAAATGGCCATC
		FF049 : CTTTGCCAAAGCAGGAGCAGCGGCTAAAAATGCATCTCTACCG
pRS415-GPD-erdS-aor	pDONR221-erdS-aor	#101 : gaactagtggatcccccatcacaagtttGTACAAAAAAGCAGGCTTC
Construction of *E. coli* overexpression plasmids - isoThermal assembly		
pMtevGWA-erdS-afm	pDONR221-erdS-afm	#142 : GGTACCGGATCTTACATCACAAGTTTGTACAAAAAAGCAGGCTTC
		#134 : GTGGTGGTGGTGCTCGAGGTACATCAACTTTGTACAAGAAAGCTGGGTC
	pMtevGWA-X	#114 : TGATGTACCTCGAGCACCACCACCAC
		#135 : AAACTTGTGATGTAAGATCCGGTACC
pMtevGWA-erdS-∆duf2156	pDONR221-erdS-∆duf2156	#142 : GGTACCGGATCTTACATCACAAGTTTGTACAAAAAAGCAGGCTTC
		#134 : GTGGTGGTGGTGCTCGAGGTACATCAACTTTGTACAAGAAAGCTGGGTC
	pMtevGWA-X	#114 : TGATGTACCTCGAGCACCACCACCAC
		#135 : AAACTTGTGATGTAAGATCCGGTACC
pMtevGWA-erdS-∆aspRS	pDONR221-erdS-∆aspRS	#142 : GGTACCGGATCTTACATCACAAGTTTGTACAAAAAAGCAGGCTTC
		#134 : GTGGTGGTGGTGCTCGAGGTACATCAACTTTGTACAAGAAAGCTGGGTC
	pMtevGWA-X	#114 : TGATGTACCTCGAGCACCACCACCAC
		#135 : AAACTTGTGATGTAAGATCCGGTACC
pMtevGWA-AspRS$_{AAPA}$-DUF2156	pMtevGWA-erdS-afm	FF048 : TTTTAGCCGCTGCTCCTGCTTTGGCAAAGCAAATGGCCATC
		FF049 : CTTTGCCAAAGCAGGAGCAGCGGCTAAAAATGCATCTCTACCG
pMtevGWA-erdH	*Afm*Genomic DNA	NY119 : AAGCAGGCTTCATGGCCTCTCATGCCCCTC
		NY120 : gAAAGCTGGGTCCTATCTGTCAAAAATCGC
	pMtevGWA-X	NY121 : GACAGATAGGACCCAGCTTTcttgtacaaagtgg
		NY122 : ATGAGAGGCCATGAAGCCTGCTTTTTTGTACaaac
pMtevGWA-erdH-S153A	pMtevGWA-erdH	NY145 : TGAGTGGTTTCGCTGCGGGCGGCAACCTCGCTG
		NY146 : TTGCCGCCCGCAGCGAAACCACTCAGAGCAATTC
pMtevGWA-erdH-D277A	pMtevGWA-erdH	NY147 : TCTGCGAATGGGCTATGCTGATGAACGAGGGC
		NY148 : TTCATCAGCATAGCCCATTCGCAGATGTACAG
Deletion and complementation cassettes for *Afm* strains - isoThermal assembly		
pJET1.2-erdS délétion cassette *Afm*	pSK529 (Hartmann et al., 2010)	FF166 : TATAGGTCAATAGAGTATACTTATTTG
		FF167 : TATTATGCTCAACTTAAATGACCTAC
	*Afm*CEA17ΔakuB^{KU80}gDNA	FF168 : cccgggTACGTTGGTACGTGAAAGAAATGG
		FF169 : TAAGTATACTCTATTGACCTATAGGTGACGGATGAGAGACCACC
		FF170 : GGTCATTTAAGTTGAGCATAATAAGTGGCAGATGTACCAAGCCC
		FF171 : cccgggTACGCCGCCTCCCTTCCCATTGC
pJET1.2-Pxyl-erdS	pJET1.2-erdS deletion cassette *Afm*	FF271 : CAAAGCACGTTTGATCGACATCTGCAgttggttcttcgagtcgatgaatg
		FF272 : TGCACTTCTTTGAAGACTAAGGATCCCCGACGCCGACCAACACCGCC
	Afm CEA17ΔakuB^{KU80}gDNA	FF273 : ATGTCGATCAAACGTGCTTTGTCTAAG
		FF274 : TTAGTCTTCAAAGAAGTGCAGAACCG
pJET1.2-erdS délétion cassette *Afm*	pSK529 (Hartmann et al., 2010)	FF166 : TATAGGTCAATAGAGTATACTTATTTG
		FF167 : TATTATGCTCAACTTAAATGACCTAC
	*Afm*CEA17ΔakuB^{KU80}gDNA	FF168 : cccgggTACGTTGGTACGTGAAAGAAATGG
		FF169 : TAAGTATACTCTATTGACCTATAGGTGACGGATGAGAGACCACC
		FF170 : GGTCATTTAAGTTGAGCATAATAAGTGGCAGATGTACCAAGCCC
***Afm* ∆erdS strains verification**		
	*Afm*CEA17ΔakuB^{KU80}gDNA	FF684 : GGAGATTGGATATGGATGAAGTGAAC
		FF685 : GTTGCCAACGTCGAGAAAACC
		NY15 : GAGCTGATGCTTTGGGCCGAGGACTGC
		NY16 : GCAGTCCTCGGCCCAAAGCATCAGCTC
	*Afm*CEA17ΔakuB^{KU80}gDNA	FF684 : GGAGATTGGATATGGATGAAGTGAAC
		FF685 : GTTGCCAACGTCGAGAAAACC
		NY15 : GAGCTGATGCTTTGGGCCGAGGACTGC

		NY16 : GCAGTCCTCGGCCCAAAGCATCAGCTC
Deletion and complementation cassettes for *Aor* strains - restriction and ligation		
pTAnpyrG	*A. nidulans* A26	AnpyrG-F : CCCCCCCGGGCTAGGCGCAATCCCTG
		AnpyrG-R : GGGGACTAGTGCCGGCTTAACCACAG
pAnpyrG-MR	pTAnpyrG	AnpyrG-SpeI-FF : CCCCACTAGTCTCGTCGGCTCTTTTCGCAA
		AnpyrG-SphI-FR : GGGGGCATGCGTAGAGGGTGCGGAGAACA
pUCdn	*Aor*RIB40*genomic DNA*	AoErdS-3'-SmaI-F : GACTAACCCGGGGCCTTCTTATGTTAGGCGTTTG
		AoErdS-3'-EcoRI-R : CTAGTCGAATTCTTCGAACCAGAATTAACGCTAC
pUCflk	*Aor*RIB40*genomic DNA*	AoErDS-5'-HindIII-F : ACCTGCAAGCTTCTTTAATATCCCGAGAATACTCG
		AoErDS-5'-PstI-R : TGATGACTGCAGTTGTGTAGCGGACGATAG
pDAor-ErDS	pAnpyrG-MR	AnpyrG-MR-SmaI-F primer : CTTCATCCCGGGCTAGGCGCAATCCCTGTC
		AnpyrG-MR-PstI-R primer : CTTCATCCCGGGCTAGGCGCAATCCCTGTC
pTAnpyrG	*A. nidulans* A26	AnpyrG-F : CCCCCCCGGGCTAGGCGCAATCCCTG
		AnpyrG-R : GGGGACTAGTGCCGGCTTAACCACAG
pAnpyrG-MR	pTAnpyrG	AnpyrG-SpeI-FF : CCCCACTAGTCTCGTCGGCTCTTTTCGCAA
		AnpyrG-SphI-FR : GGGGGCATGCGTAGAGGGTGCGGAGAACA
pUCdn	*Aor*RIB40*genomic DNA*	AoErdS-3'-SmaI-F : GACTAACCCGGGGCCTTCTTATGTTAGGCGTTTG
		AoErdS-3'-EcoRI-R : CTAGTCGAATTCTTCGAACCAGAATTAACGCTAC
pUCflk	*Aor*RIB40*genomic DNA*	AoErDS-5'-HindIII-F : ACCTGCAAGCTTCTTTAATATCCCGAGAATACTCG
		AoErDS-5'-PstI-R : TGATGACTGCAGTTGTGTAGCGGACGATAG
Deletant and complemented *Aor* strains verification		
	HiMe10, HiDES (gDNA)	P1 : AoErdS-5'-HindIII-F : ACCTGCAAGCTTCTTTAATATCCCGAGAATACTCG
		P4 : AoErdS-3'-EcoRI-R : CTAGTCGAATTCTTCGAACCAGAATTAACGCTAC
	HiDES + erdS, HiDES + erdSΔduf (gDNA)	P15 : AoErdS-486-F : TGCCAAGCTGGTTTTCCTTG
		P16 : AoErdS-575-R : ATGGCGATGGAATTCTTGCC
	HiDES + erdSΔaspRS (gDNA)	P10 :AoErdS-PmaCI-R : AGACACGTGTTAGTCTTCGAAAAAGTGAAGGACAG
		P17 : AoErdS-2274F : GGCGATCCTTTGTGCGATTC

Supplemental Materials and Methods

Media and growth conditions

For routine growth and maintenance of *Aspergillus fumigatus (Afm)* and *Aspergillus oryzae (Aor)*, fresh conidia were spread on Malt extract agar (ThermoScientific) plates or slants and incubated at 37 °C (*Afm*) or 30 °C (*Aor*). Mycelia were then incubated 7 days for *Afm* or 10 days for *Aor* until they produced enough conidia. *N. crassa* (*Ncr*) was grown in Vogel's agar medium. Nutrient-rich (NR) liquid or agar medium was composed of glucose 4 % (w/v), peptone 1 % (w/v) and yeast extract (YG) 1 % (w/v) (with 1.5 % w/v agar for plates or slants). Standard *Aspergillus* minimal medium Glucose (MMG) was composed as follow: For 1 L, it contained glucose (1 % w/v), ammonium tartrate dibasic(0.92 g, 5 mM, *i.e.*, 10 mM ammonium), salts (10 mL of a 50 X solution containing KCl 26 g/L, $MgSO_4$ $7H_2O$ 26 g/L, KH_2PO_4 76 g/L) and trace elements (0.5 mL of a 1000 X solution containing: $FeSO_4$ $7H_2O$ 1 g, Na_2EDTA 10 g, $ZnSO_4$ $7H_2O$ 4.4 g, H_3BO_3 2.2 g, $MnCl_2$ $4H_2O$ 1 g, $CoCl_2$ $6H_2O$ 0.32 g, $CuSO_4$ $5H_2O$ 0.32 g and Na_2MoO_4 0.8 g for 200 mL adjusted at pH 6.5). For MMX media, it contained 1 % (w/v) xylose as the carbon source. Solid media contained 1.5 % (w/v) agar. Agar plates or slants were incubated at 37°C (*Afm*) or 30 °C (*Aor*, *Ncr*) in the dark for indicated periods of time. Liquid cultures were incubated in glass flasks at 37 °C for *Afm* or 30 °C for *Aor*, *Ncr* and

all other filamentous fungi tested under shaking (220 rpm) for 24 (*Afm*) to 48 h (other fungi), until enough cells or mycelia were produced. *Sce*, *Candida* spp. and *C. neoformans* (*Cne*) were grown in NR medium for 24 h at 30 °C under agitation (220 rpm).

Spores/conidia preparation

Spores from 7 days for *Afm* or 10 days-old for *Aor* Malt agar slants were resuspended by addition of 5 mL sterile Tween 20-H_2O (0.05 % v/v) and vortexing, then the conidia were filtered with Cell Strainer filters (EASY strainer^{TM} Greiner Bio-One), and the concentration was determined with a hemacytometer. Conidia were stored in Tween 20-H_2O (0.05 % v/v) at 4°C in the dark up to 1 week. For *Ncr*, conidia were harvested and treated similarly using 1 M sterile sorbitol.

Mycelia harvesting from liquid cultures

Liquid cultures to produce mycelia were inoculated with 10^6 to 10^7 conidia/mL in 50 mL liquid MMG for *Afm* or NR for *Aor*, incubated for 24 h at 37 °C (*Afm*) or 30° C (*Aor*) in the dark under agitation (220 rpm). Mycelia were then filtrated through two layers of gauze, rinsed twice with 50 mL sterile H_2O, and squeezed to eliminate excess water. Mycelia were directly used to extract total lipids.

Construction of cassettes for *A. fumigatus*

For the construction of Δ*erdS*mutants of *Afm*, 1000 bp of the 5'-upstream and of the downstream regions were amplified by PCR (using primer pairs FF#168 + FF#169 andFF#170 + FF#171, respectively) from *Afm*CEA17Δ*akuB*^{KU80}genomic DNA. Primers contained a 25 bp sequence at the 3' and 5' extremities, respectively, to obtain sequence tags corresponding to the 5' and 3' extremities of the *six*-P_{xyl}-β*rec-trpC-hygB-six* resistance cassette (1) flanked by *Sma*I restriction sites. The *six*-P_{xyl}-β*rec-trpC-hygB-six* resistance cassette was amplified by PCR (FF#166 and FF#167), and the 3 resulting fragments were fused using the Gibson method as described (2) to obtain the 5'-UTR-*six*-P_{xyl}-β*rec-trpC-hygB-six*-3'-UTR cassette flanked by the *Sma*I restriction sites. This cassette was ligated into a pJET1.2 plasmid using the CloneJET PCR Cloning Kit (ThermoScientific) following the manufacturer's instructions, to obtain the pJET1.2-Δ*erdS*deletion plasmid. The integrity of the deletion cassette was checked by PCR and sequencing. Thexylose-inducible *erdS* complementation cassette was constructed by replacing the β- *rec* gene in the 5'-UTR-*six*-P_{xyl}-β*rec-trpC-hygB-six*-3'-UTR cassette by the

wild-type *erdS* gene, so that it is under the control of the xylose promoter (P_{xyl}) (3) and the *trpC* terminator. The pJET1.2-Δ*erdS* plasmid was reverse-amplified with primers FF#271 and FF#272 to obtain the pJET1.2-Δ*erdS* open plasmid (pJET1.2-*eraS*-β*rec*$^{0,\ open}$) deprived of the β-*rec* gene. This PCR fragment was submitted to *Dpn*I digestion at 37 °C, 1 h, to remove the pJET1.2-Δ*erdS* matrix and purified using the Nucleospin Gel and PCR clean-Up Kit (Marcherey-Nagel). The *erdS* gene was amplified from *Afm*CEA17 24 ΔakuB^{KU80}enomic DNA using primers FF#273 and FF#274 with 20 pb 5'- and 3'-tags corresponding to the flanking regions of the β-rec gene. The pJET1.2-*eraS*-β*rec*$^{0,\ open}$ and *erdS* fragments were fused using the Gibson procedure (2) to obtain the pJET1.2-P_{xyl}-*erdS* (precisely, the pJET1.2 plasmid containing the 5'-UTR-*six*-P_{xyl}-*erdS-trpC-hygB-six*-3'-UTR cassette) integration plasmid. This cassette was designed to be integrated by homologous recombination at the Δ*erdS* locus. Because the β-*rec* gene was removed, once integrated at the Δ*erdS* locus, the 5'-UTR-*six*-P_{xyl}-*eraS-trpC-hygB-six*-3'-UTR cassette could not be excised.

Construction of mutants and complemented strains in *A. fumigatus*

Deletion of the *erdS* gene was obtained through homologous recombination with the deletion cassettes described above. Transformations of *Afm* were performed by electroporation of swollen conidia essentially as described (4) and we used 10 µg of linearized (*Sma*I-digested) deletion plasmid.

From the primary hygromycin-resistant colonies, conidia were isolated on individual Malt agar plates containing 150 µg/mL hygromycin B. After 3 days, conidia from single isolated colonies were scrapped and transferred on Malt agar slants containing 150 µg/mL hygromycin B and incubated 5 days at 37 °C. Genomic DNA was extracted (5)from mycelium cultures of Δ*erdS* mutants and the disruption of the *Afm-erdS* gene was confirmed by PCR (Primers FF#684 + NY#015 and NY#016 + FF#685) and by Western-blot. The hygromycin resistance cassette was then self-excised as described (1) on xylose-containing MM, and clones verified by PCR with primers FF#684 and FF#685.

To complement this Δ*erdS* strain, P_{xyl}-*erdS* cassette was inserted at the Δ*erdS* locus with the same protocol but the cassette could not be excised since, for this construct, the β-recombinase gene (1) was replaced by the *erdS* open reading frame.

Construction of cassettes for *A. oryzae*

For the disruption of *Aor-erdS* in *Aor*, *pyrG* marker recycling method was used (6). The first 300 bp of the coding sequence (starting from the ATG codon) of *Aspergillus nidulanspyrG* was amplified (AnpyrG-300 bp) and ligated to the 3' end of *pyrG* gene, immediately after the stop codon. This will bring the first 300 bp sequence directly attached to the 3' end of the *pyrG* gene in tandem repeats. The *pyrG* gene was amplified by PCR from *A. nidulans* A26 genomic DNA with the primers AnpyrG-F and AnpyrG-R. The PCR product was then ligated into pT7Blue using TA-cloning kit to yield pTAnpyrG. AnpyrG-300bp was amplified from pTAnpyrG with the primers AnpyrG-SpeI-FF and AnpyrG-SphI-FR. The resulting fragment was ligated into pTAnpyrG digested with *Spe*I and *Sph*I to yield the pAnpyrG-MR.

To construct the *Aor-erdS* gene disruption cassette, the upstream (1.0 kb) and the downstream (1.0 kb) flanking region of *Aor-erdS* ORF were amplified by PCR from *Aor*RIB40 genomic DNA using primer pairs AoErDS-5'-HindIII-F and AoErDS-5'-PstI-R for the upstream, and AoErDS-3'-SmaI-F and AoErDS-3'-EcoRI-R for the downstream regions. The downstream fragment was ligated into pUC118 vector digested with *Sma*I and *EcoR*I to yield the pUCdn, and then the upstream fragment was ligated into pUCdn digested with *Hind*III and *Pst*I to yield the pUCflk. The AnpyrG-MR was amplified from pAnpyrG-MR with the primers AnpyrG-MR-SmaI-F and AnpyrG-MR-PstI-R, and ligated into pUCflk digested with *Sma*I and *Pst*I to yield the pDAor-ErDS.

Construction of mutants and complemented strains in *A. oryzae*

The conidial suspension of *Aor*HiMe10was inoculated in 50 mL GPYU broth (2 % glucose, 1 % polypeptone, 0.5 % yeast extract, 0.1 % uridine, 0.1 % uracil) and incubated for 18 h at 30 ˚C and 160 rpm. Then mycelia were harvested by filtration with sterile Myracloth, washed with sterile distilled water and the cell walls digested with 1 % yatalase (Takara), 0.6 M $(NH_4)_2SO_4$, 50 mM maleate buffer (pH 5.5) at 30 ˚C for 3 h. Protoplast conversion was monitored by microscopic observation. After removing hyphal debris by filtration, the protoplast suspension was diluted 1:1 with Solution I (1.2 M sorbitol, 50 mM $CaCl_2$, 35 mM NaCl, 10 mM Tris-HCl, pH 7.5). Protoplasts were collected by centrifugation (2000 rpm, 8 min, 4 ˚C) and washed twice with Solution I. Finally, protoplasts were resuspended in Solution I at $2x10^7$ cells mL^{-1}. Of the protoplast suspension, 200 µL was mixed with 15 µg of linearized disruption cassette and incubated on ice for 30 min, then to the mixture was added 1.35 mL Solution II (60 % PEG4000, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5). After 20 min incubation at room temperature (RT), the mixture was diluted with 5 mL Solution I and centrifuged (2000 rpm, 8 min, 4 ˚C). The precipitates were suspended in 250 µL Solution I and added in 5 mL Top agar (selective media

including 1.2 M sorbitol and 0.8 % agar) and then the transformation mix overlaid on selective agar media (3 % sucrose, 0.3 % $NaNO_2$, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % KCl, 0.001 % $FeSO_4 \cdot 7H_2O$ and 1.2 M sorbitol). The plates were incubated at 30 ˚C for seven days. From the primary transformation plates, conidia were isolated from individual colonies using a flamed loop and transferred on individual Czapek-Dox agar plates to allow isolated sporulating colonies to form. After isolating three times to a single colony, conidia were inoculated into 5 mL GPY broth and incubated for 18 h at 30 ˚C and 160 rpm. Mycelia were harvested by filtration through Miracloth, washed with water, subjected to genomic DNA extraction with the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. After isolation, the disruption of the *Aor-eraS* gene was confirmed by PCR with QuickTaq HS DyeMix (TOYOBO)using primers AoEraS-F2 and AnpyrG-F2 that were designed based on homologous region and the AnpyrG-MR, respectively. The AnpyrG-MR was self-excised on PDU agar media containing 5-FOA. The resulting disruption mutant was named HiDES.

To complement the *Aor* Δ*eraS* disruption mutant, the plasmid pUAES was constructed, which harbors the *Aor-eraS* gene, the promoter region of *Aor-eraS* and the terminator of *agdA*. The *Aor-eraS* gene was amplified by PCR using the primers PEraS-Sse8387I-F and AoEraS-PmaCI-R digested with *Sse8387*I and *PmaC*I, and then ligated into pUenoP digested with *Pst*I and *Sma*I. The resulting pUAES plasmid containing the *niaD* gene was introduced into HiDES to obtain HiDEC. Clones were isolated and identified as described above.

Induction of *erdS* expression with xylose

Liquid cultures were performed as described in the previous section, and mycelia harvested similarly. Then washed mycelia were resuspended in fresh minimal media containing 1 % glucose (w/v) or 1 % xylose (w/v) as the sole carbon source, to induce or not ErdS and Erg-Asp overproduction in the Δ*erdS*::P_{xyl}-*erdS* strain of *Afm*. Mycelia were then harvested as described and lipids extracted as specified below.

Construction of *Sce* and *E. coli* expression plasmids

The *Afm erdS* open reading frame (*AFUA_1g02570*) was synthesized (Genscript®) with codon optimization for expression in *Sce*, amplified by PCR (Primers GW_s and GW_as) and cloned in a pDONR221-*ccdB* vector (kanamycin resistance marker) (Gateway technology) using a BP reaction as described (7). To construct the *erdS*Δ*duf2156* and *erdS*Δ*aspRS* variants, the *erdS* sequence was amplified with primers pairs FF#001/FF#003 and

Dufssdrs_sens/GW_as, respectively, that contained *attB1* and *attB2*5'-tags, and introduced in the pDONR221-*ccdB* with a BP reaction, as described (7). To transfer the *erdS*, *erdS*Δ*duf2156* and *erdS*Δ*aspRS* constructs in destination vectors (pDEST was pRS415-Gpd or pRS414-Gpd), we first amplified the corresponding ORFs from the pDONR vectors with primers #101 and #102. We reverse-amplified a pDEST vector containing another unrelated ORF (already recombined) with primers #104 and #103 to obtain the open form of the plasmid without this unrelated ORF. Fragments (*erdS*, *erdS*Δ*duf2156* and *erdS*Δ*aspRS*) were introduced in the open pDEST form separately using the Gibson assembly procedure as described (2) and recombinant plasmids verified by PCR, restriction analysis and sequencing. The same procedure was used to clone the *erdS* gene from *Aor* (primers FF#099 and FF#100 to insert the ORF in the pDONR and primers #101 and #102 to transfer in the pDEST vectors).

To construct vectors enabling the expression of Maltose-binding protein fusions in *E. coli*, the codon-optimized *erdS* ORFs and variants were PCR-amplified using primers #142 and #134, and the pDEST (pMTevGWA) reverse-amplified with primers #114 and #135 before performing the Gibson assembly. The same was applied for the *erdH* gene (*AFUA_1g02580*, no introns) that was PCR-amplified from *Afm*KU80 with primers NY#119 and NY#120 and inserted directly in the pMTevGWA open vector generated by reverse-amplification with primers NY#121 and NY#122.

Site directed mutagenesis was performed with primer pairs containing selected mutations (indicated and described in supplementary table), as previously described (8).

Plasmid-shuffling complementation assays in *S. cerevisiae*

Plasmid shuffling experiments were conducted in the Δ*dps1 Sce* strain (9) rescued with a wild-type *DPS1* gene copy cloned in an *URA3*-bearing plasmid. All *Afm* or *Aor erdS* constructs to be tested were cloned in p415 (LEU) plasmids, transformed in the Δ*dps1* strain, and shuffled essentially as described (10, 11).

Proteins extraction and Western blots

For yeast crude extract preparation, 1 OD_{600nm} of cells were resuspended and incubated 10 min in 500 µL of pre-cooled NaOH 0.185 N, then precipitated by adding 50 µL of Trichloroacetic acid (TCA) 100 % and incubated 10 min on ice. Finally, the samples were centrifuged at 13, 000 x *g* for 15 min and the resulting pellets were resuspended in 100 µL of

Laemmli Sample Buffer. Then, 8 µL of each sample was then resolved on 10 % SDS-PAGE gels. Samples were separated by using a BioRad Mini-PROTEAN electrophoresis apparatus. For western blotting, proteins were transferred onto PVDF membranes that were blocked in 5 % (w/v) skimmed milk in TBS-Tween (TBS 1X, Tween-20 0.3 % (v/v)) for 1 h at RT. Primary antibodies (polyclonal anti-DUF2156, Covalab, France, anti-PGK) were incubated overnight at 4 °C and then washed several times with TBS-Tween. Membranes were then incubated for 1 h with HRP-conjugated secondary antibodies (Goat anti-rabbit for anti-DUF2156 and Goat anti-mouse for anti-PGK) at RT. Revelation was performed with the BioRad clarity western ECL Kit and monitored in a BioRad ChemiDoc Touch® apparatus.

Recombinant protein expression and purification

For recombinant protein purification, we modified the pMGWA vector (described in (7)) by introducing the Maltose Binding Protein (MBP) tag coding sequence 5' to a TEV cleavage site (TevCS). The gene encoding the protein of interest (*erdS*, *erdH* and mutants) was then cloned into this pMtevGWA using the above described cloning method. The resulting MBP-TevCS-X expression plasmids were then transformed in *E. coli* Rosetta-2 strains. Transformed bacteria were grown in LB medium containing ampicillin (150 µg/ml) and shaken at 37 °C, until an OD_{600nm} ~ 0.5-0.6 was reached. Cultures were then chilled on ice for 30 min and protein overexpression was induced with 0.1 mM IPTG for 12 h at 18 °C. Finally, cells were harvested by centrifugation (5000 x *g*, 15 min, 4 °C). The resulting cell pellet (~9 g) was resuspended in 30 mL of Tris-HCl pH 7 250 mM lysis buffer, containing NaCl 300 mM, KCl 30 mM, Glycerol 5 % (v/v), Tween 20 0.25 % (v/ v), TritonX-100 0.1 % (v/v), 2-mercaptoethanol 5 mM, Na_2EDTA 0.5 mM and a protease inhibitor cocktail (Roche Complete, EDTA-free). Cell lysis was performed by sonication (6 x 1 min, amplitude 28 %) (Vibracell, 72408) with intermediate cooling on ice. The lysate was centrifuged at 13, 000 x *g*, 20 min and the supernatant directly incubated with 1 mL of equilibrated amylose resin (NEB Amylose Resin, E8021S) at 4 °C on a rotating wheel. After transfer on an column (BioRad), the flow through was collected, beads where washed with 5 column volumes (CV) of wash buffer (Tris-HCl pH 7 50 mM, NaCl 300 mM, KCl 30 mM, Glycerol 5 % (v/v), Tween-20 0.25 % (v/v), 2-mercaptoethanol 10 mM) and the recombinant protein was eluted with 5 x 1 mL of elution buffer (Wash buffer supplemented with 2 % (p/v) of maltose monohydrate (Sigma-Aldrich). The collected fractions were then analyzed on 10 % SDS-PAGE gels. Fractions containing MBP-TevCS-X (X: ErdS, ErdH or mutants) protein were pooled. When required, the MBP-tag was cleaved off using the TEV

protease, as described. To separate the protein of interest from the MBP-tag and some other contaminants, fractions were injected on a HiLoad 16/600 Superdex 200 pg set up on a ÄKTA pure chromatography system (GE Healthcare). Finally, the fractions containing the protein of interest where pooled, concentrated to a final concentration of 1 mg/mL and stored at -20 °C with 30 % glycerol until use.

***S. cerevisiae* tRNA^{Asp} purification**

Extraction and purification of tRNA^{Asp} from *Sce* was performed as described elsewhere (12).

***In vitro*tRNA^{Asp}aspartylation assay**

Aspartylation of tRNA^{Asp} was performed as and adapted from (13, 14). Briefly, pure MBP-ErdS (or mutants) or ErdS (or mutants) without the N-terminal MBP tag were used. Aminoacylation reactions were performed in a Na-HEPES 100 mM pH 7.2 buffer containing KCl 30 mM, $MgCl_2$, 12 mM, ATP 10 mM, BSA 0.1 mg/mL, pure yeast tRNA^{Asp} (10 µM), [U-^{14}C]-Asp (280 cpm/pmol, Perkin Elmer) in a final volume of 100 µL. Reactions were initiated by adding 1 µg of enzyme pre-incubated at 30°C, and incubated at 30 °C. At each time points, 10 µL of reaction mix were removed, spotted onto Whatman paper filters (2 x 2 cm) and plunged into a 5 % TCA solution in a glass beaker to precipitate [^{14}C]-Asp-tRNA^{Asp}. Papers were washed 3 times for 15 min in 5 % TCA to remove residual free [^{14}C]-Asp, and 3 times 5 min in 100 % ethanol and air-dried. Radioactivity of precipitated [^{14}C]-Asp-tRNA^{Asp} was counted in a scintillation counter. Control experiments were performed with a reaction mix deprived of enzyme to obtain the background counts.

Chemical synthesis of ergosteryl-3β-*O*-L-aspartate and NMR analyses

4-*tert*-Butyl-1-ergosteryl *N*-(*tert*-butoxycarbonyl)-*L*-aspartate

Ergosterol (400 mg) and 4-*tert*-butyl-*N*-(*tert*-butoxycarbonyl)-*L*-aspartate (300 mg in Et_2O (30 ml)) at RT was added *N*,*N*′-dicyclohexylcarbodiimide (300 mg) and *N*,*N*-dimethyl-4-aminopyridine (amount of catalyst) and stirred for 3 h at RT. The mixture was extracted with EtOAc and washed with 1N HCl, sat. $NaHCO_3$ and sat. brine. The organic layer was dried over Na_2SO_4. Removal of the organic solvent *in vacuo* gave the crude product. Purification by silica gel column chromatography (hexane/EtOAc = 15:1) gave the product (480 mg) in 70 % yield. NMR was measured using JEOL ECP-500 (^{1}H at 500 MHz, ^{13}C at 125 MHz) with $CDCl_3$ (99.8% atom ^{2}H, Kanto Chemical, Japan) as a solvent with a solvent signal of δ 7.26 ppm for ^{1}H and δ 77.0 ppm for ^{13}C as references for chemical shifts.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.617 (s, 3H), 0.814 (d, *J* = 6.60 Hz, 3H), 0.830 (d, *J* = 6.60 Hz, 3H), 0.908 (d, *J* = 6.60 Hz, 3H), 0.940 (s, 3H), 1.028 (d, *J* = 6.60 Hz, 3H), 1.438 (s, 9H), 1.446 (s, 9H), 2.704 (dd, *J* = 4.40, 16.86 Hz, 1H), 2.887 (dd, *J* = 4.77, 16.86 Hz, 1H), 4.475 (m, 1H), 4.762 (m, 1H), 5.190 (m, 2H), 5.368 (m, 1H), 5.545 (m, 1H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 12.022, 16.140, 17.571, 19.618, 19.930, 20.972, 21.073, 22.947, 28.020, 28.258, 28.291, 33.051, 36.405, 37.022, 37.786, 38.962, 40.417, 42.767, 42.768, 45.941, 50.192, 54.475, 55.642, 74.123, 79.861, 81.554, 116.214, 120.291, 131.941, 135.525, 138.197, 141.625, 155.518, 170.135, 170.653.

Ergosteryl-3β-*O*-L-aspartate

To 4-*tert*-Butyl-1-ergosteryl *N*-(*tert*-butoxycarbonyl)-*L*-aspartate (290 mg) in CH_2Cl_2 was added Et_3N (200 μL). After cooling with an ice bath, the mixture was added TMSOTf (300 μL) and stirred for 24 hrs at 0 °C. The mixture was extracted with $CHCl_3$ and washed with sat. $NaHCO_3$. The organic layer was dried over Na_2SO_4. Removal of the organic solvent *in vacuo* gave the crude product. Purification by silica gel column chromatography ($CHCl_3$ ~ $CHCl_3$/MeOH = 5:1) gave the product. NMR was measured with CD_3OD (99.8 % atom ^{2}H, Merck, Switzerland) as a solvent with a tetramethylsilane signal of δ 0.00 ppm for ^{1}H and a solvent signal of δ 49.0 ppm for ^{13}C as references for chemical shifts.

In silico analyses and determination of the phylogenomic distribution of ErdS

BLAST analyses

The protein sequence of the *Afm* AspRS-DUF2156 (ErdS) (encoded by the *AFUA_1G02570* gene in the Af293 strain) was used to perform PSI-BLAST searches (15) using non-redundant protein sequences databases and limited to fungi with 20,000 target sequences. Convergence was reached after 2 iterations. Sequences were then filtered using a home-made Python algorithm to recover only sequences of 250 to 2000 residues in length (7584 sequences) to exclude short and truncated versions of protein domains (AspRS and/or DUF2156). These filtered sequences were used to construct a protein size distribution plot in order to determine the average length of AspRS-DUF2156 proteins. Filtered sequences were then used to build a local BLAST database and a local BLAST search was performed using the sequence of the DUF2156 domain of *Afm* AspRS-DUF2156 as a query to detect DUF2156-containing proteins in the distribution plot (1006 sequences).

Phylogenomics analyses: To determine the phylogenomic distribution of AspRS-DUF2156 (ErdS), we used the *Afm* AspRS-DUF2156 sequence to retrieve by BLAST (15) all orthologs in each fungal class that belongs to the Dikarya sub-kingdom (Eurotio-, Lecanoro-, Xylono-, Dothideo-, Sordario-, Leotio-, Pezizo-, Orbioliomycetes, Saccharo- and Taphrinomycotina for ascomycetes, and in Agarico-, Puccinio- and Ustilagomycotina for basidiomycetes) and counted proteins containing a DUF2156 domain with length >750 residues, to take into account only complete and full-length AspRS-DUF2156 fusions, and not truncated forms that could account for sequencing or protein sequence prediction errors. No AspRS-DUF2156 fusions were detected outside Dikarya. We mapped the presence/absence of ErdS in each class onto the phylogenetic tree of fungi available at the JGI MycroCosm website (16). Distribution of ErdS was also monitored in the indicated selected species. In each case, we also indicated the presence of a canonical AspRS, involved in protein synthesis, for comparison.

Sequence alignments and analyses: Eleven ErdS (AspRS-DUF2156) protein sequences from *Aspergillus fumigatus*, *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus niger*, *Penicilliumexpansum*, *Penicillium camemberti*, *Beauveria bassiana*, *Alternaria alternata*, *Neurosporacrassa*, *Cryptococcus neoformans* and *Schizophyllum commune* were used, together with 6 canonical AspRS sequences from *Saccharomyces cerevisiae*, *Candida albicans*, *A. fumigatus*, *A. oryzae*, *A. niger* and *N. crassa* to obtain a multiple alignment of all proteins with the Muscle program (17, 18) included in the Seaview package. Class II aaRS motifs 1, 2 and 3 (19) and the AspRS-specific QSPQ (20, 21) sequences were localized using the well characterized *S. cerevisiae*AspRS(20, 22). Residues known to be crucial for $tRNA^{Asp}$ acceptor arm, ATP, L-Asp or the Asp~AMP intermediate binding were identified according to the residues that had been experimentally identified in the yeast AspRS(9, 20, 23). The N-terminal extensions, AspRS and DUF2156 domains boundaries were determined according to the alignment.

Protein structure modelling and analysis: The amino acid sequence of the *Afm* AspRS-DUF2156 (ErdS) protein was used on the Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (24) to obtain structurespredictions of either the AspRS (residues 1-593) or DUF2156 (residues 594-947) domains independently. Because sequence similarity with bacterial DUF2156 was very low (~20 %), the GNAT I and II subdomains – as well as the intercalated $\alpha^{(+)}$ helix – of the *Afm*DUF2156 (residues 594-947) were localized in the Phyre2 model by comparison with the

crystal structures of the DUF2156 domains of bacterial aaPGSs (25). The $\alpha^{(+)}$ helix of the DUF2156 domain was identified based on the model of the interaction inferred between $tRNA^{Ala}$ and the $\alpha^{(+)}$ helix of *P. aeruginosa* AlaPGS (25) where it was suggested to contact and recruit the Ala-$tRNA^{Ala}$ acceptor arm.

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