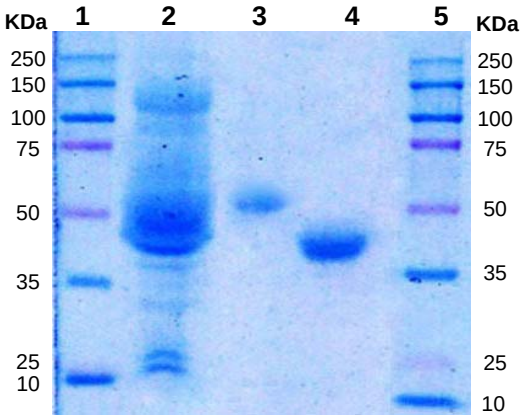
Fig. S1

A

PaDa-I mutant	Total activity (U/mL)	[Protein] (mg/mL)	Specific activity (U/mg)	Purification factor
Culture broth	3.4	0.14	25	1
55-85% $(NH_4)_2SO_4$	320	1.92	167	6.7
Cation exchange SP Sepharose	133	0.35	381	15.2
Anion exchange MonoQ	174	0.21	828	33.1

B



C

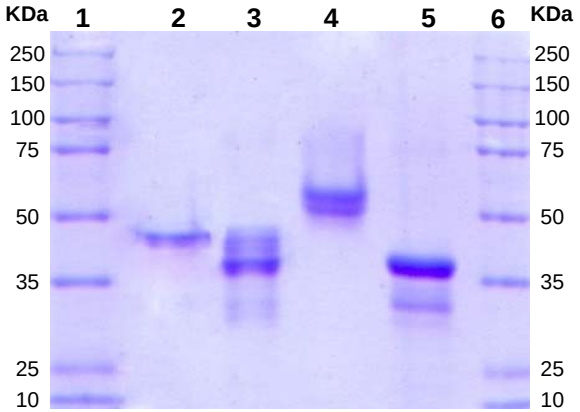


Fig. S2

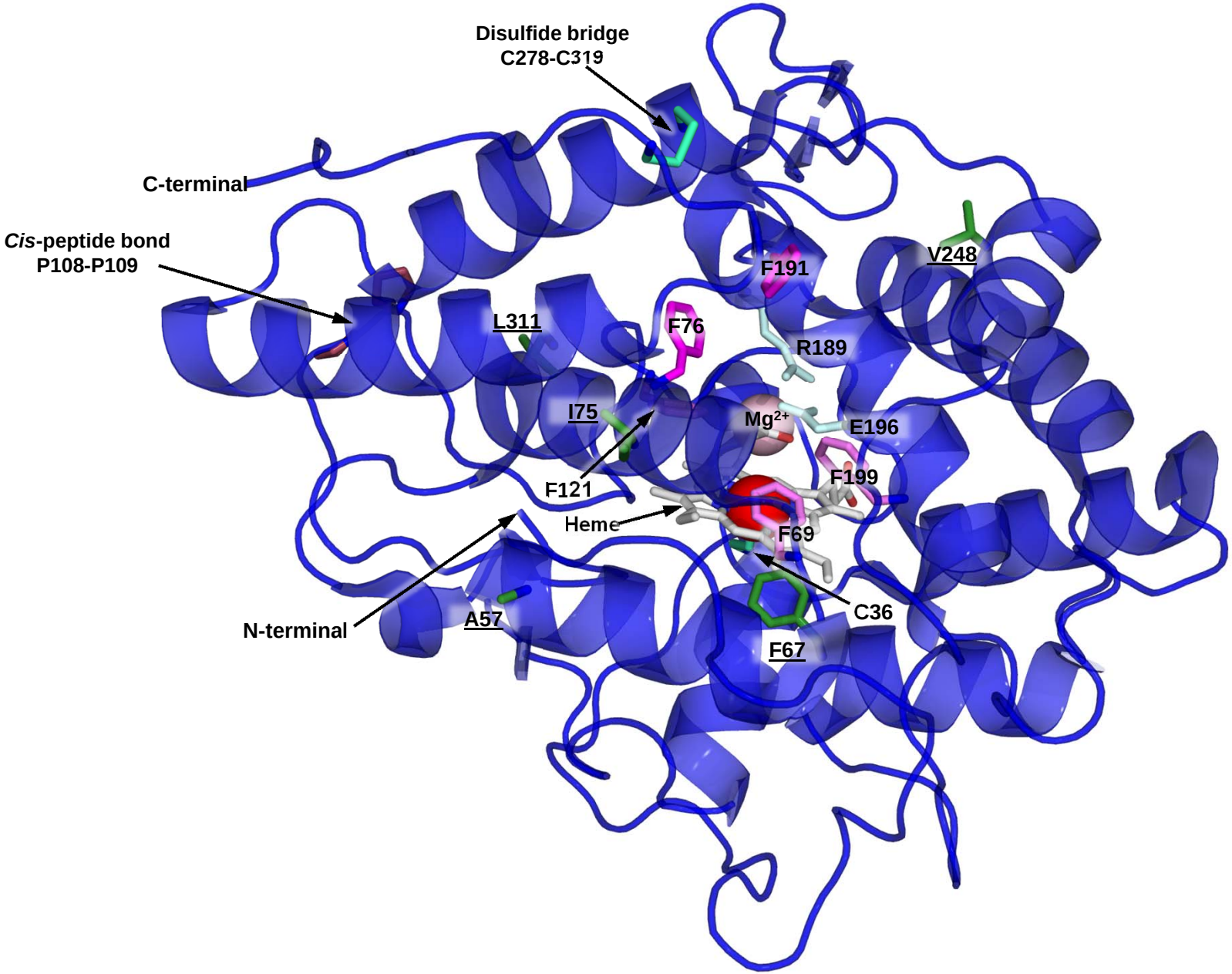

Disulfide bridge
C278-C319
C-terminal
Cis-peptide bond
P108-P109
V248
F191
L311
F76
R189
I75
Mg2+
E196
F199
F121
Heme
F69
C36
N-terminal
A57
F67

Table S1. Strategies and mutations obtained along the directed UPO1 evolution.

	Parental type	Generation of diversity	Mutational rate (mutations/1000bp)	Library size (nº clones)	Total clones	Selected mutants	Improvements referred to n-UPO1 parent (ABTS screening)*	New mutations	Suggested Recombination events	Codon usage (%) parental/mutant
1stG	n-UPO1	Taq/$MnCl_2$	Low (1-3)	522	2088	1A11 (n-UPO1, Mutaz)	13 (pH 3.5; 1mM H_2O_2)	L67F ($_{199}$**C**TC/**T**TC$_{201}$); H251H ($_{751}$CA**T**/CA**C**$_{753}$)		4/47 60/40
		Mutazyme II	Medium (4-9)	522		3C2 (n-UPO1, Mutaz)	9 (pH 3.5; 1mM H_2O_2)	I248V ($_{742}$**A**TA/**G**TA$_{744}$); F311L ($_{931}$TT**C**/TT**A**$_{933}$)		20/16 47/27
	α-UPO1	Taq/$MnCl_2$	Low (1-3)	522						
		Mutazyme II	Medium (4-9)	522						
2ndG	1A11, 3C2	Taq/$MnCl_2$+ *in vivo* DNA shuffling	Low (1-3) (Taq)	1044	2088					
		Mutazyme II + *in vivo* DNA shuffling	Medium (4-9) (Mutazyme II)	1044		12C12 (Mutaz)	57 (pH 3.5; 1mM H_2O_2)	A[21]D ($_{61}$G**C**C/G**A**C$_{63}$)	L67F(1A11) with I248V and F311L (3C2)	28/38
3rdG	12C12	IvAM	Low (1-3) and medium (4-9)	2088	2610	I13D3	70 (pH 3.5; 1mM H_2O_2)	V75I ($_{223}$**G**TC/**A**TC$_{225}$)		24/30
		Morphing at the leader	Low (1-3 per segment, 129bp)	522		M6D4	84 (pH 3.5; 1mM H_2O_2)	R[15]G ($_{43}$**A**GG/**G**GG$_{45}$)		17/9
						M2B5	65 (pH 3.5; 1mM H_2O_2)	A[14]V ($_{40}$G**C**G/G**T**G$_{42}$)		8/15
						M5D2	87 (pH 3.5; 1mM H_2O_2)	F[12]Y ($_{34}$T**T**C/T**A**C$_{36}$)		47/50
						M4D8	63 (pH 3.5; 1mM H_2O_2)	L4S ($_{10}$T**T**A/T**C**A$_{12}$)		27/19
4thG	I13D3, M5D2 and M4D8	Taq/$MnCl_2$ + *in vivo* DNA shuffling	Low (1-3)	2088	2262	22A10	150 (pH 4.4; 2mM H_2O_2)	V57A ($_{169}$G**T**G/G**C**G$_{171}$)	F[12]Y and A[21]D (M5D2) with L67F, V75I, I248V, F311L (I13D3)	15/8
						3F10	31 (pH 4.4; 2mM H_2O_2)	T120P ($_{358}$**A**CC/**C**CC$_{360}$)		25/13
	M5D2, M2B5, M6D4 and I13D3	Site-directed mutagenesis (iProof)	0	174		2A12	132 (pH 4.4; 2mM H_2O_2)	F[12]Y ; A[14]V ; R[15]G		
5thG	2A12 and 22A10	Site-directed mutagenesis (iProof)	0	87	87	**PaDa-I**	162 (pH 4.4; 2mM H_2O_2)	V57A		

*From 1st to 3rd generation, screening was carried out in the presence of hemoglobin as external heme supply that produced a strong background due to interactions with ABTS assay. To reduce the unwanted signal, reaction mixture was set at lower [H_2O_2] (1 mM) and pH (3.5) than that of the optimum for UPO1. In generations 4 and 5, UPO1 mutants were produced without hemoglobin allowing the use of optimum reaction conditions (2mM H_2O_2, pH 4.4). See also **Figure 1**.

Table S2. Mutations in mature PaDa-I variant. See also **Figures 5, S2**.

Mutation	Secondary structure motif	Relative position	Distance to heme-Fe (Å)	H bonding surrounding residues	
				Before mutation	After mutation
V57A	Alpha helix	Surface (in front of N-terminal)	15.7	N61 (x2)	N61 (x2)
L67F	Alpha helix	Near heme domain (close to F69)	8.1	V63	V63
V75I	Alpha helix	Surface (close to F76)	12.1	N71, Y79	N71, Y79
I248V	Alpha helix	Surface (next to F191, access to the heme channel)	17.2	V244	V244
F311L	Alpha helix	Near C-terminal	14.0	N307, G314	N307, G314

Table S3. Primers used in the directed UPO evolution study.

Primer name	Sequence (5' → 3')
RMLN	CCTCTATACTTTAACGTCAAGG
RMLC	GCTTACATTCACGCCCTCCC
psn-apo1 N	*CGGGATCCATGAAATATTTTCCCCTGTTCCCAACCTTG
apo1 C (2)	*AAGGAAAAAAGCGGCCGCTCAATCTCGCCCGTATGGGAAG
apo-dir	GAGCCAGGATTACCTCCTG
apo-rev	**AACTAATTACATGATGCGGCCCTCTAGATGCATGCTCGATCAATCTCGCCCGTATGG
alpha-aporev	**GCAGAGCTATTCTCGAGAGGACCAGGAGGTAATCCTGGCTCGCTTCAGCCTCTCTTTCTC
Morph psn apo1 rev	CAGAGCTATTCTCGAGAGGA
Morph psn apo1 dir	GAGCCAGGATTACCTCCTGG
apo1secdir	GAAGGCGACGCCAGTATGACC
apo1secrev	GGTCATACTGGCGTCGCCTTC
PSN*R	GTCAGGAAAAGCAACGACCCCCACTGCGTAGACCAAGGTTGGGAACAGG
PSN*F	***CCTGTTCCCAACCTTGGTCTACGCAGTGGGGGTCGTTGCTTTTCCTGAC
2A12*REV	GAACCGCGTTTATTATTTGCGCCGGGGTTGCAACGCCATTTC
2A12*DIR	***GAAATGGCGTTGCAACCCCGGCGCAAATAATAAACGCGGTTC

*The target sequences for BamHI (psn-apo1 N) and NotI (apo1 C (2)) are underlined.
** Overhangs for *in vivo* cloning are underlined.
*** The changed nucleotides are underlined.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Molecular mass and degrees of glycosylation of $_{wt}$UPO1 and the PaDa-I mutant. (**A**) Purification table for PaDa-I. (**B**) SDS-PAGE (PVDF membrane). Lanes: 1 and 5, protein markers; 2, PaDa-I culture broth; 3, purified PaDa-I; 4, purified $_{wt}$UPO1. (**C**) Deglycosylation/SDS-PAGE with PNGase F. Lanes: 1 and 6, protein markers; 2, $_{wt}$UPO1; 3, deglycosylated $_{wt}$UPO1; 4, PaDa-I mutant; 5, deglycosylated PaDa-I mutant. See also **Table 1**.

Figure S2. Structural model of PaDa-I. Mutations V57A, L67F, V75I, I248V and F311L are shown in green and underlined. The heme group is shown in CPK coloring, Fe^{3+} in red and the structural Mg^{2+} in salmon. The model shows the Cys36 axial heme ligand, the disulfide bridge formed between Cys278 and Cys319, and the *cis*-peptide bond between P108 and P109. The catalytic pocket contains 5 Phe residues that mediate the adjustment of the substrates (Phe 69, Phe 76, Phe 121, Phe 191 and Phe 199: in pink) and two catalytic residues (R189 and E196: light cyan). See also **Figure 5**, **Table S2**.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reagents and Enzymes

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DMP (2,6-dimethoxyphenol), veratryl alcohol, benzyl alcohol, hemoglobin from bovine blood, Taq DNA polymerase for random mutagenesis and the *Saccharomyces cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain). NBD (5-nitro-1,3-benzodioxole) was acquired from TCI America (USA). The cDNA of *upo*1 (clone C1A-2) from *Agrocybe aegerita* was provided by Dr. Martin Hofrichter (Pecyna et al. 2009). The *Escherichia coli* XL2-Blue competent cells and the Genemorph II Random Mutagenesis kit (Mutazyme II) were obtained from Stratagene (La Jolla, CA, USA). The uracil independent and ampicillin resistance shuttle vector pJRoC30 was from the California Institute of Technology (CALTECH, USA). The protease deficient *S. cerevisiae* strain BJ5465 was obtained from LGCPromochem (Barcelona, Spain) and the pGAPZαA vector containing α-factor prepro-leader was from Invitrogen. The Zymoprep Yeast Plasmid Miniprep kit and Zymoclean Gel DNA Recovery kit were from Zymo Research (Orange, CA, USA). The NucleoSpin Plasmid kit was purchased from Macherey-Nagel (Germany) and the restriction enzymes BamHI, XhoI, EcoRI and NotI were from New England Biolabs (Hertfordshire, UK). The high fidelity polymerase iProof was acquired from Bio-Rad (USA). The oligonucleotides were synthesized by Isogen Life Science (Barcelona, Spain). All chemicals were reagent-grade purity.

Culture Media

Sterile minimal medium contained 100 mL 6.7% filtered yeast nitrogen base, 100 mL 19.2 g/L filtered yeast synthetic drop-out medium supplement

without uracil, 100 mL filtered 20% raffinose, 700 mL *dd*H_2O and 1 mL 25 g/L filtered chloramphenicol. SC drop-out plates contained 100 mL 6.7% filtered yeast nitrogen base, 100 mL 19.2 g/L filtered yeast synthetic drop-out medium supplement without uracil, 20 g autoclaved bacto agar, 100 mL 20% filtered glucose, 1 mL 25 g/L filtered chloramphenicol and *dd*H_2O to 1,000 mL. Sterile expression medium contained 720 mL autoclaved YP, 67 mL 1 M filtered KH_2PO_4 pH 6.0 buffer, 111 mL 20% filtered galactose, 22 mL filtered $MgSO_4$ 0.1 M, 31.6 mL absolute ethanol, 1 mL 25 g/L filtered chloramphenicol and *dd*H_2O to 1,000 mL. From 1st to 3rd generation, 2.75 mL of filtered hemoglobin 20g/L were added. For large scale cultures, the expression medium was supplemented with 300mg/L of filtered hemoglobin. YP medium contained 10 g yeast extract, 20 g peptone and *dd*H_2O to 650 mL. YPD solution contained 10 g yeast extract, 20 g peptone, 100 mL 20% sterile glucose, 1 mL 25 g/L chloramphenicol and *dd*H_2O to 1,000 mL. Luria-Bertani (LB) medium was prepared with 5 g yeast extract, 10 g peptone, 10 g NaCl, 100 mg ampicillin and *dd*H_2O to 1,000 mL.

Constructions of Fusion Genes

The gene used as starting point was the cDNA of *upo*1 (clone C1A-2) from *A. aegerita,* cloned in the pSTBlue-1 plasmid. Four UPO1 constructs (α-UPO1, α*-UPO1, n*-UPO1 and n*-3F10) were engineered to switch signal peptides in the shuttle vector pJRoC30. The pSTBlue1-upo1 vector was used as a template to amplify the original *upo1* gene (containing native signal peptide-upo1, n-upo1). PCR reactions were performed in a final volume of 50 µL containing 3 % DMSO, 0.5 µM psn-apo1 N, 0.5 µM apo1 C (2), 1 mM dNTPs (0.25 mM each), 0.02 U/µL of iProof DNA polymerase, and 0.2 ng/µL pSTBlue1-upo1. The thermal cycling programme was: 98ºC for 30 s (1 cycle), 98ºC 10 s, 52ºC for 25 s, 72ºC for 1 min (28 cycles) and 72ºC for 10 min (1

cycle). The n-upo1 product was digested with BamHI and NotI, and ligated by the T4 DNA ligase to the pJRoC30 plasmid linearized with the same restriction enzymes, giving rise to pJR-n-upo1.

pJR-α-upo1 and pJR-α*-upo1: Both fusions were obtained by *in vivo* overlap extension (IVOE, Alcalde, 2010). Firstly, the sequence corresponding to the mature protein of the *upo*1 gene was amplified from pSTBlue1-upo1 with the primers apo-dir and aporev-RMLC. The α-factor pre-proleader and the evolved (α*) factor pre-proleader were amplified from templates pJR-α-PM1 and pJR-α*-OB1, respectively (Maté et al., 2010) using primers RMLN and alpha-aporev. PCR reactions were performed in a final volume of 50 µL containing 3 % DMSO, 0.5 µM primer N, 0.5 µM primer C, 1 mM dNTPs (0.25 mM each), 0.02 U/µL of iProof DNA polymerase, and 0.2 ng/µL of template. The thermal cycling programme was: 98ºC for 30 s (1 cycle), 98ºC 10 s, 49ºC for 25 s (α-factors) / 48ºC for 25 s (*upo1*), 72ºC for 30 s (α-factors) /1 min (*upo1*) (28 cycles) and 72ºC for 10 min (1 cycle). The amplified PCR fragments were purified as detailed in Experimental Procedures and transformed in *S. cerevisiae* with the pJRoC30 linearized plasmid (BamHI and XhOI) for *in vivo* cloning.

n*-UPO1 and n*3F10: The evolved signal peptide (n*) was amplified from 2A12 mutant using primers RMLN and Morph psn apo1 rev. The UPO1 native mature sequence was amplified from n-upo1 whereas the mature 3F10 mutant was amplified from its own template. For the mature sequence amplifications the primers used were Morphpsnapo1dir and RMLC. PCR experiments were performed in a final volume of 50 µL containing 3 % DMSO, 0.5 µM primer N, 0.5 µM primer C, 1 mM dNTPs (0.25 mM each), 0.02 U/µL of iProof DNA polymerase, and 0.2 ng/µL of template. Two different thermal cycling programmes were employed: i) 98ºC for 30 s (1 cycle), 98ºC 10 s, 47ºC

for 25 s, 72ºC for 10 s (28 cycles), 72ºC for 10 min (1 cycle) to amplify the n*; ii) 98ºC for 30 s (1 cycle), 98ºC 10 s, 52ºC for 25 s, 72ºC for 40 s (28 cycles), 72ºC for 10 min (1 cycle) to amplify the mature protein sequences. The amplified PCR fragments were purified as detailed in Experimental Procedures. Cloning was performed *in vivo* by transforming the corresponding PCR fragments along with the linearized gene using a ratio signal sequence: mature protein: linearized plasmid of 2: 2: 1.

High-Throughput Dual-Screening Assay

Individual clones were picked and inoculated in sterile 96-well plates (Greiner Bio-One GmbH, Germany) containing 50 µL of minimal medium per well. In each plate, column number 6 was inoculated with the corresponding parent type, and one well (H1-control) was inoculated with untransformed *S. cerevisiae* cells. Plates were sealed to prevent evaporation and incubated at 30°C, 220 RPM and 80% relative humidity in a humidity shaker (Minitron-INFORS, Biogen, Spain). After 48 h, 160 µL of expression medium were added to each well, and the plates were incubated for 48 h. The plates (master plates) were centrifuged (Eppendorf 5810R centrifuge, Germany) for 10 min at 3,500 RPM at 4°C. 20 µL of supernatant were transferred from the master plate to two replica plates by a robot (Liquid Handler Quadra 96-320, Tomtec, Hamden, CT, USA). 180 µL of reaction mixture with ABTS or NBD were added to each replica plate. ABTS reaction mixture contained 100 mM sodium phosphate/citrate buffer at pH 3.5 (from 1st to 3rd generation) or at pH 4.4 (for generations 4 and 5), 0.3 mM ABTS and 1 mM H_2O_2 (from 1st to 3rd generation) or 2 mM H_2O_2 for generations 4 and 5. Reaction mixture with NBD contained 100 mM potassium phosphate buffer pH 7.0, 1 mM NBD, 15% acetonitrile (ACN) and 1 mM H_2O_2. Plates were stirred briefly and the initial absorptions at 418 nm ($\varepsilon_{ABTS^{\bullet+}}$ = 36,000 M^{-1} cm^{-1}) and 425 nm (ε_{NBD} = 9,700 M^{-1} cm^{-1}) were

recorded in the plate reader (SPECTRAMax Plus 384, Molecular Devices, Sunnyvale, CA). The plates were incubated at room temperature until a green (ABTS) or yellow (NBD) color developed, and the absorption was measured again. The values were normalized against the parental type in the corresponding plate. To rule out false positives, two re-screenings were carried out. Finally a third re-screening was performed in order to assess kinetic stability.

First Re-screening

Aliquots of 5 µL of the best ~50 clones of the screening were transferred to new sterile 96-well plates with 50 µL of minimal medium per well. Columns 1 and 12 plus rows A and H were not used to prevent the appearance of false positives. After 24 h of incubation at 30°C and 220 RPM, 5 µL were transferred to the adjacent wells and further incubated for 24 h. Finally, 160 µL of expression medium were added and plates were incubated for 48 h. Accordingly, every single mutant was grown in 4 wells. Parental type was subjected to the same procedure (lane D, wells 7-11). Plates were assessed using the same HTS protocol of the screening described above.

Second Re-screening

An aliquot from the wells with the best ~10 clones of first re-screening was inoculated in 3 mL of YPD and incubated at 30°C and 220 RPM for 24 h. Plasmids from these cultures were extracted with Zymoprep Yeast Plasmid Miniprep kit. Due to the impurity of the zymoprep product and the low concentration of extracted DNA, the shuttle vectors were transformed into super-competent *E. coli* cells XL2-Blue and plated onto LB-amp plates. Single colonies were picked and used to inoculate 5 mL LB-amp media and were grown overnight at 37°C and 250 RPM. Plasmids were then extracted by

NucleoSpin Plasmid kit and competent *S.cerevisiae* cells were transformed with these plasmids and with the parental type. Five colonies of every single mutant were picked and re-screened as described above.

Third Re-screening: Thermostability Assay

A single colony from the *S. cerevisiae* clone containing the corresponding mutant gene was selected from an SC drop-out plate, inoculated in 3 mL of minimal medium and incubated for 48 h at 30ºC and 220 RPM (Minitron-INFORS, Biogen, Spain). An aliquot of the cells was removed and inoculated in a final volume of 5 mL of minimal medium (optical density, OD_{600}=0.25). The cells were incubated for two complete growth phases (6 to 8 h). Thereafter, 9 ml of expression medium was inoculated with the 1 mL preculture in a 100 mL flask (OD_{600}=0.1). After incubating for 72 h at 25ºC and 220 RPM (UPO activity was maximal; OD_{600}=25-30), the cells were separated by centrifugation for 10 min at 4,500 RPM (4ºC) and the supernatant was double-filtered (using both glass membrane and a nitrocellulose membrane of 0.45 µm pore size). Appropriate dilutions of supernatants were prepared in such a way that aliquots of 20 µL gave rise to a linear response in kinetic mode. 50 µL supernatant were used for each point in a gradient scale ranging from 30 to 80°C. This gradient profile was achieved using a thermocycler (Mycycler, Bio-Rad, USA). After 10 min of incubation, samples were removed and chilled out on ice for 10 min. After that, samples of 20 µL were removed and incubated at room temperature for 5 min. Finally, samples were subjected to the same ABTS colorimetric assay described above for the screening (100 mM sodium phosphate/citrate buffer pH 4.4, 0.3 mM ABTS and 2 mM H_2O_2). Thermostability values were calculated from the ratio between the residual activities incubated at different temperature points and the initial activity at room temperature. The T_{50} value was determined by the transition midpoint of

the inactivation curve of the protein as a function of temperature, which in our case was defined as the temperature at which the enzyme lost 50% of its activity following an incubation of 10 minutes.

Production and Purification

$_{wt}$UPO1 was produced and purified as described elsewhere (1).

Production of recombinant UPO variants in *S. cerevisiae:* A single colony from the *S. cerevisiae* clone containing the parental or mutant *upo*1 gene was picked from a SC drop-out plate, inoculated in minimal medium (20 mL) and incubated for 48 h at 30°C and 220 RPM. An aliquot of cells was removed and used to inoculate minimal medium (100 mL) in a 500 mL (at a OD_{600} of 0.25). The cells completed two growth phases (6–8 h) and then expression medium (900 mL) was inoculated with the pre-culture (100 mL) (OD_{600} of 0.1). After incubating for 72 h at 25°C and 220 RPM (maximal UPO activity; OD_{600} = 25–30), the cells were recovered by centrifugation at 4,500 RPM (4°C) and the supernatant was double-filtered (using both glass membrane and a nitrocellulose membrane of 0.45 μm pore size).

Purification of recombinant UPO variants: Recombinant UPO purification was achieved by cationic exchange chromatography and anion exchange chromatography (ÄKTA purifier, GE Healthcare). The crude extract was first submitted to a fractional precipitation with ammonium sulfate (55%, first cut) and after removing the pellet, the supernatant was again precipitated with ammonium sulfate (85%, second cut). The final pellet was re-suspended in buffer sodium phosphate/citrate 10 mM at pH 4.3 (buffer A), and the sample was filtered and loaded on to a strong cation-exchange column (HiTrap SP FF GE Healthcare) pre-equilibrated with buffer A. The proteins were eluted with a linear gradient from 0 to 25% of buffer A within 55 mL of NaCl and from 25 to

100% within 5mL at a flow rate of 1 mL/min. Fractions with UPO activity *vs* ABTS were harvested, concentrated, dialyzed against buffer Bis Tris 10 mM at pH 6.5 (buffer B) and loaded onto a 10 μm high resolution anion-exchange Biosuite Q column (Waters) pre-equilibrated with buffer B. The proteins were eluted with a linear gradient from 0 to 15% within 40 mL of NaCl and from 15 to 100% within 5mL at a flow rate of 1 mL/min. The fractions with UPO activity *vs* ABTS were pooled, dialyzed against buffer potassium phosphate 50mM at pH 7.0 and stored at 4ºC. The Reinheitszahl values (*Rz* A_{418}/A_{280}) achieved were around 2. Throughout the purification protocol, the fractions were analysed by SDS/PAGE on 12% gels and the proteins were stained with colloidal Coomassie Blue (Bio-Rad). The concentrations of all crude protein extracts were determined using the Bio-Rad protein reagent and BSA as standard. One unit of ABTS-activity was defined as the amount of enzyme that oxidizes 1 μmol of ABTS per min in 100 mM sodium phosphate/citrate buffer pH 4.4 containing 2 mM H_2O_2.

MALDI–TOF-MS Analysis and pI Determination

The MALDI–TOF-MS experiments were performed on an Autoflex III MALDI-TOF-TOF instrument with a smartbeam laser (Bruker Daltonics). The spectra were acquired using a laser power just above the ionization threshold, and the samples were analysed in the positive-ion detection and delayed extraction linear mode. Typically, 1,000 laser shots were summed into a single mass spectrum. External calibration was performed, using the BSA from Bruker, covering the range 15,000–70,000 Da. Purified UPOs (8 μg each) were subjected to two-dimensional electrophoresis gel in order to determine the pI. These determinations were carried out at the Proteomic and Genomic Services from CIB (CSIC, Spain).

Steady-state Kinetic Constants

ABTS kinetic constants for UPO1 were estimated in 100 mM sodium phosphate/citrate pH 4.4 containing 2 mM H_2O_2; for the rest of the substrates in 100 mM potassium phosphate pH 7.0 containing 2 mM H_2O_2 (benzyl and veratryl alcohols) or 1 mM H_2O_2 (NBD). H_2O_2 kinetic constants were estimated using benzyl alcohol as reducing substrate at the corresponding saturated conditions. Reactions were performed by triplicate and substrates oxidations were followed by measuring the absorption at 418 nm for ABTS, ε_{418} =36,000 M^{-1} cm^{-1}; DMP, ε_{469}=27,500 M^{-1} cm^{-1}; NBD, ε_{425}=9,700 M^{-1} cm^{-1}; veratryl alcohol, ε_{310}=9,300 M^{-1} cm^{-1} and benzyl alcohol, ε_{280}=1,400 M^{-1} cm^{-1}. To calculate the values of K_m and $k_{cat,}$ the average V_{max} was represented versus substrate concentration and fitted to a single rectangular hyperbola function in SigmaPlot 10.0, where parameter a was equaled to k_{cat} and parameter b was equaled to K_m.

pH Activity Profiles

Appropriate dilutions of enzyme samples were prepared in such a way that aliquots of 20 µL gave rise to a linear response in kinetic mode. The optimum pH activity was determined using 100 mM sodium phosphate/citrate buffer at different pH values for ABTS (0.3 mM ABTS and 2 mM H_2O_2), NBD (1 mM NBD, 15% acetonitrile and 1 mM H_2O_2) and DMP (1 mM DMP and 2 mM H_2O_2). The activities were measured in triplicate mode, and the relative activity (in percent) is based on the maximum activity for each variant in the assay

DNA sequencing

Plasmid-containing variant *upo*1 genes were sequenced by using an ABI 3730 DNA Analyzer/Applied Biosystems Automatic Sequencer from Secugen (Spain). The primers used were: RMLN; apo1secdir; apo1secrev and RMLC.

Protein modelling

The structure of wild-type UPO1 (purified from *A. aegerita* culture) at a resolution of 2.1 Å (protein Data Bank Europe [PDB] accession number 2YOR) was used as a template to generate a molecular model where the new found mutations were mapped. The resulting model was analyzed with PyMOL Molecular Visualization System (http:// pymol.org)

Supplemental References:

1. **Ullrich R, Liers C, Schimpke S, Hofrichter, M.** 2009. Purification of homogeneous forms of fungal peroxygenase. Biotechnol. J. **4**: 1619-1626.