Supporting Information

Supporting Information materials and methods

Reduction-oxidation cycle of GkR2loxI for ESI-LCMS

*Gk*R2loxI was prepared for redox experiments as follows. Samples at 7 mg/ml protein were reconstituted in an anaerobic glove box with O_2 level lower than 10 ppm at first with 1.8 equivalents of $MnCl_2$ for 30 minutes and subsequently with 1.8 equivalents of $(NH_4)_2Fe(SO_4)$ for 30 minutes. The protein was collected in a glass HPLC-vial with sealing caps and transferred out of the glove box, where it was incubated with pure O_2 gas for 30 minutes. The sample was then transferred back into the glove box and chemically reduced by addition of 0.01 mM methyl viologen and 16 mM sodium dithionite (final concentrations). Then, the sample was let stand with open lid for at least 30 minutes to make sure no oxygen was left in solution. Samples of 30 μl were collected before and after metal reconstitution, and after each oxidation and reduction steps. The reducing and oxidizing state of the solution was monitored visually thanks to the shift in color of methyl viologen. The samples were immediately mixed with 60 μl GC-grade chloroform (Sigma-Aldrich) and vortexed for 30 seconds. A volume of 30 μl of LC-grade methanol (Sigma-Aldrich) was subsequently added to each sample, which was vortexed for other 30 seconds and centrifugated at 1000 x g for 10 minutes. The chloroform phase was finally extracted and dried under N_2 gas in an HPLC vial, before being stored at -80 °C. Two negative controls were prepared: a first one in which the samples underwent the redox process in metal-free state and a second negative control was prepared reconstituting the protein with exclusively 2 equivalents of $(NH_4)_2Fe(SO_4)$ for 30 minutes.

Supporting Information figures and table

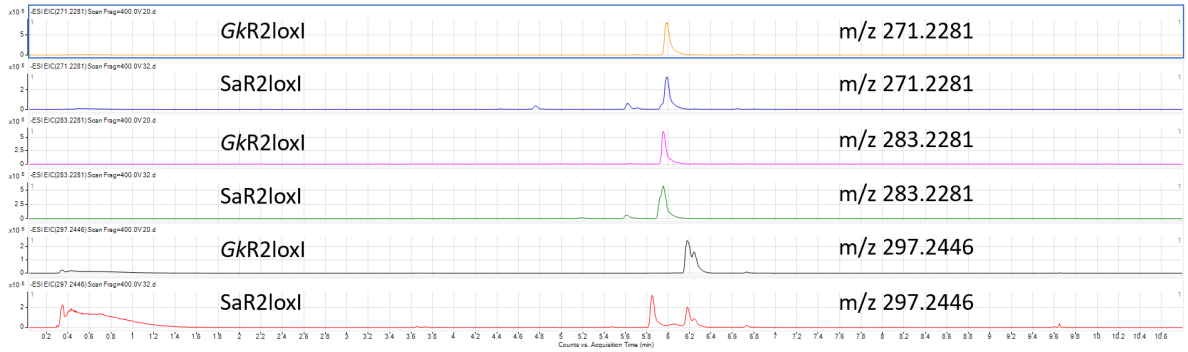

Fig. S1. Extracted Ion Chromatograms (EICs) for *Gk*R2loxI and *Sa*R2loxI showing the presence of the same ligands in both proteins. The chromatogram shows the extracted m/z for 271, 283 and 297 m/z. From the top, EIC signal of m/z 271 in *Gk*R2loxI and *Sa*R2loxI, respectively, followed by m/z 283 in *Gk*R2loxI and *Sa*R2loxI, respectively, and finally the EIC signal of m/z 297 in *Gk*R2loxI and *Sa*R2loxI, respectively. The signals are identifiable with the same retention time in both samples, indicating a similar ligand specificity in both proteins. We therefore continued our ligand identification on *Gk*R2loxI instead of analyzing both samples.

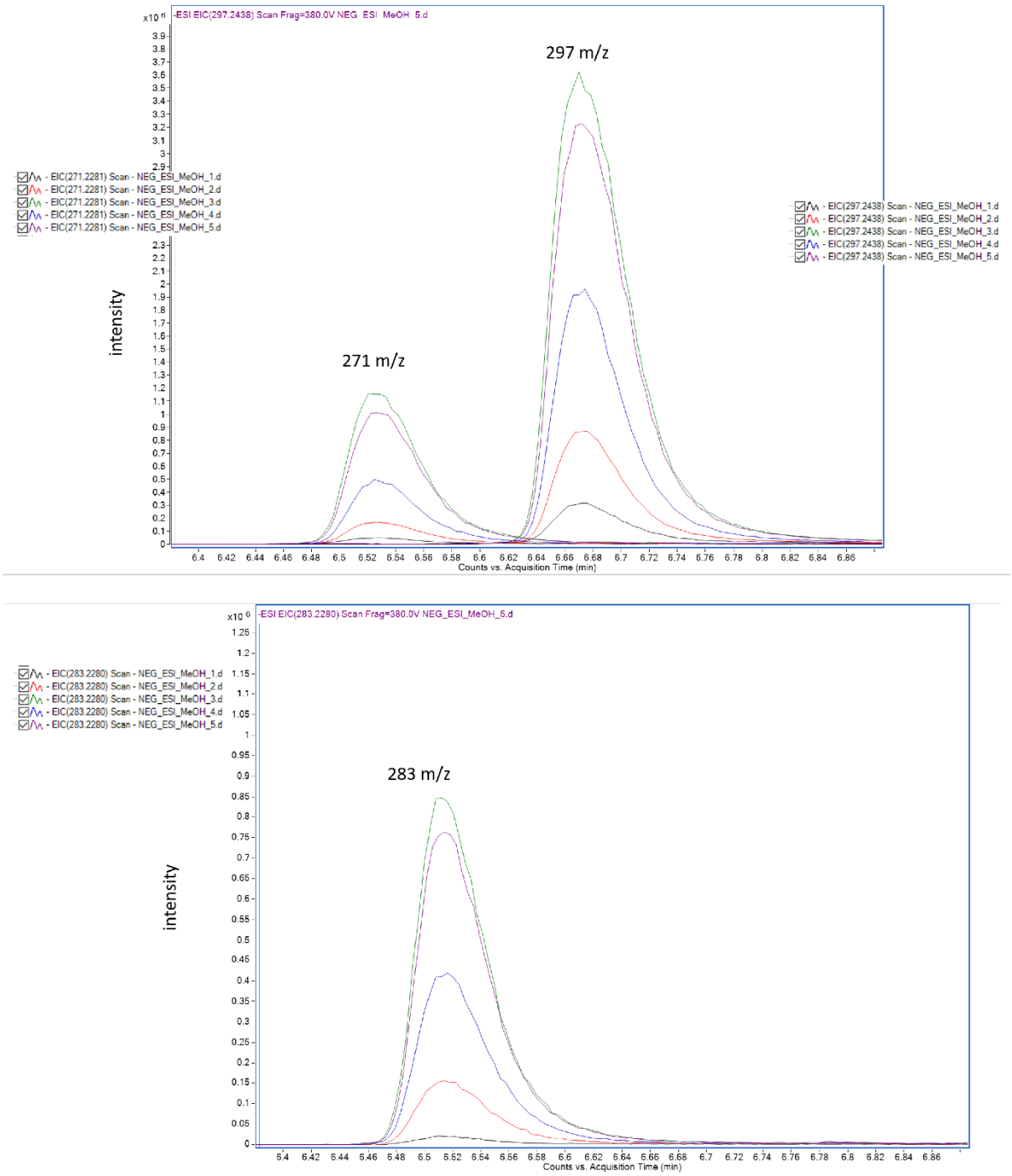

Fig. S2. Extracted Ion Chromatograms (EICs) of the ligands identified in *Gk*R2loxI after reduction-oxidation cycle. The chromatograms show the presence of the peaks 271 m/z, 283 m/z and 297 m/z in *Gk*R2loxI during redox cycle. Sample 1 (black) is the metal-free protein before metal reconstitution and in anaerobic conditions. Sample 2 (red), is the protein after Mn/Fe reconstitution in anaerobic conditions. Sample 3 (green) is sample 2 after incubation with pure O_2 gas for 30 minutes. Sample 4 (blue) is sample 3 after reduction with dithionite for 30 minutes in anaerobic environment. Sample 5 (purple) is sample 4 after further incubation with pure O_2 gas for 30 minutes. The intensities are not normalized but methyl viologen was used as internal standard to determine the relative concentration of each sample against each other. The chromatograms indicate no changes in the ligand content of *Gk*R2loxI after reduction-oxidation cycle. Furthermore, no newly formed compounds were identifiable in the chromatograms.

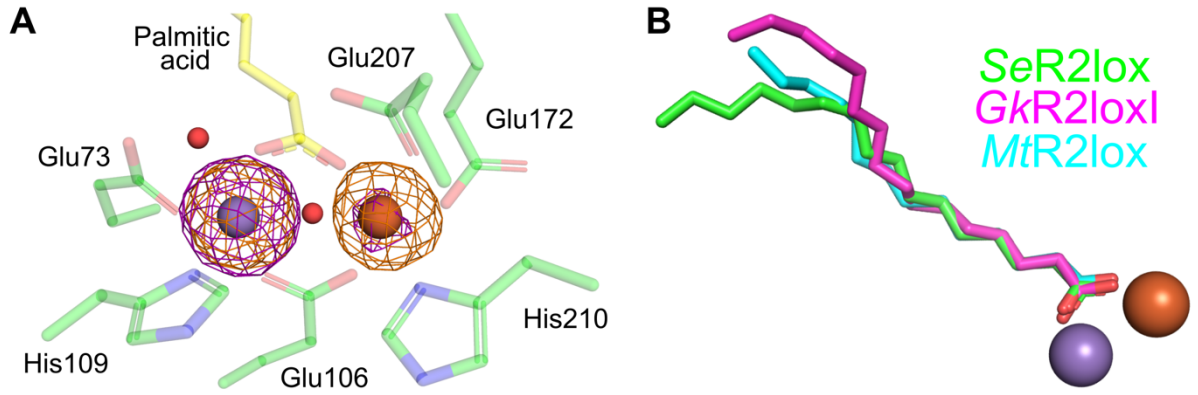

Fig. S3. Metal center in *Se*R2lox and ligands modeled in R2lox proteins. A. Metal ions in *Se*R2lox were identified by X-ray anomalous diffraction data collected at the Fe- and Mn-edge (wavelengths of 1.73 Å and 1.88 Å, respectively). Resulting anomalous maps are contoured at 6 σ and shown in orange and purple for Fe- and Mn-edge, respectively. **B.** Superimposed ligands modeled in R2lox proteins, *i.e.*, myristic acid (C_{14}) in *Mt*R2lox, and palmitic acid (C_{16}) in *Gk*R2loxI and *Se*R2lox (PDB ID: 3ee4, 4hr0 and 7qbp, respectively).

Table S1. Data collection and refinement statistics.
Statistics for the highest-resolution shell are shown in parentheses.

	***Se*R2lox**	***Sa*R2loxII**
PDB ID	7qbp	7qbk
Wavelength (Å)	0.9184	0.984
Resolution range (Å)	46.14 - 1.38 (1.43 - 1.38)	41.91 - 2.26 (2.34 - 2.26)
Space group	$P4_32_12$	$P6_4$
Unit cell a, b, c (Å) α, β, γ (°)	 103.173, 103.173, 61.574 90, 90, 90	 128.029, 128.029, 41.894 90, 90, 120
Total reflections	1789725 (175009)	61888 (6396)
Unique reflections	68604 (6772)	17196 (1739)
Multiplicity	26.1 (25.8)	3.6 (3.7)
Completeness (%)	99.99 (100.00)	91.80 (93.53)
Mean I/σ(I)	29.88 (1.79)	6.67 (0.91)
Wilson *B* factor (Å)	19.05	41.35
R_{merge}	0.06269 (1.782)	0.1587 (1.442)
R_{meas}	0.06397 (1.818)	0.1858 (1.683)
R_{pim}	0.01261 (0.3558)	0.0948 (0.8499)
$CC_{1/2}$	1 (0.755)	0.991 (0.398)
CC*	1 (0.927)	0.998 (0.755)
Reflections used in refinement	68604 (6772)	17183 (1736)
Reflections used for R_{free}	3431 (339)	859 (87)
R_{work}	0.1503 (0.2362)	0.2016 (0.2814)
R_{free}	0.1647 (0.2733)	0.2392 (0.3334)
Number of non-hydrogen atoms	2565	2342
Macromolecules	2363	2287
Ligands	56	2
Solvent	177	53
Protein residues	291	284
RMSD, bonds (Å)	0.018	0.003
RMSD, angles (°)	1.38	0.55
Ramachandran favored (%)	97.58	96.07
Ramachandran allowed (%)	2.42	3.57
Ramachandran outliers (%)	0.00	0.36
Rotamer outliers (%)	0.41	0.41
Clashscore	2.36	3.91
Average *B* factor (Å^2)	25.33	46.27
Macromolecules (Å^2)	24.44	46.22
Ligands (Å^2)	30.16	53.41
Solvent (Å^2)	36.60	47.81