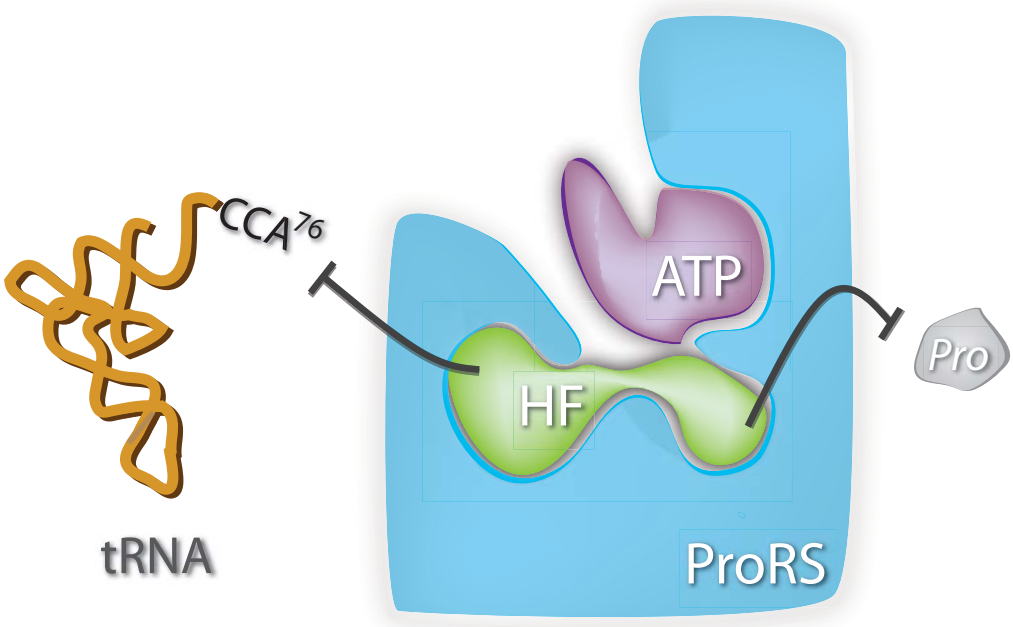
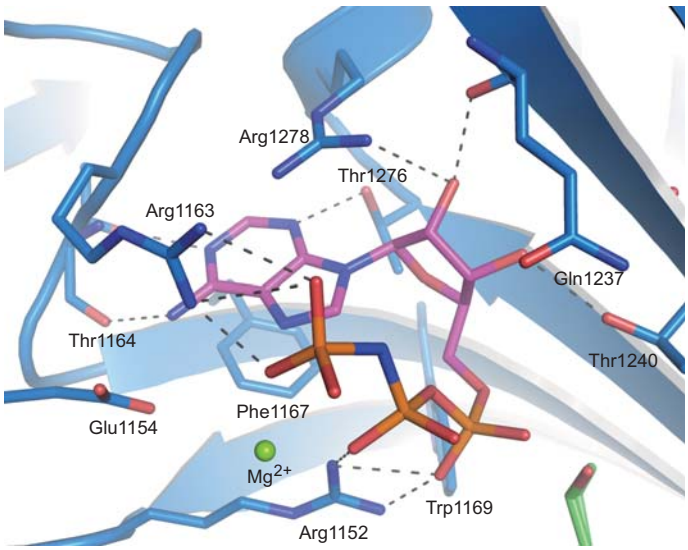
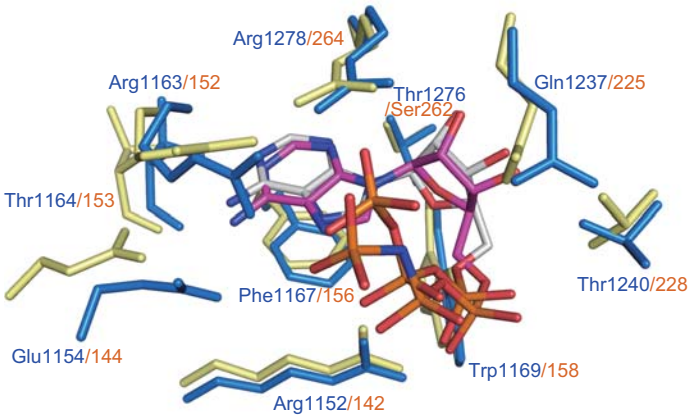
SUPPLEMENTARY INFORMATION



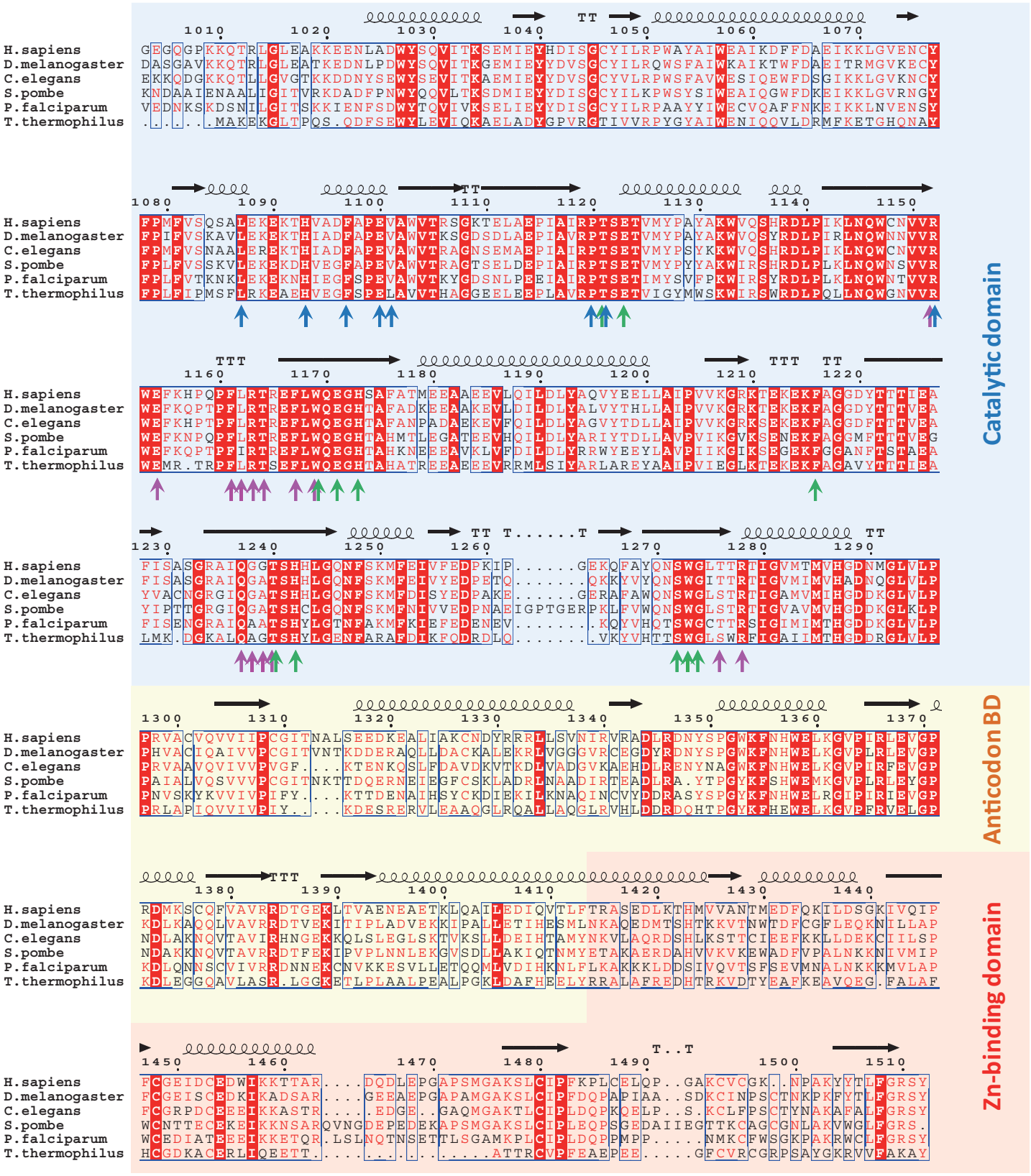
Supplementary Figure 1 | HF is an ATP-dependent dual-site inhibitor of ProRS. HF occupies both the proline binding pocket and the pocket for the 3'-end of $tRNA^{Pro}$, and blocks the binding of these two substrates to ProRS.



Supplementary Figure 2 | ATP[a] binds to ProRS. Stick model cartoons present the interactions between ATP[a] and ProRS. The hydrogen bonds are indicated with dashes.



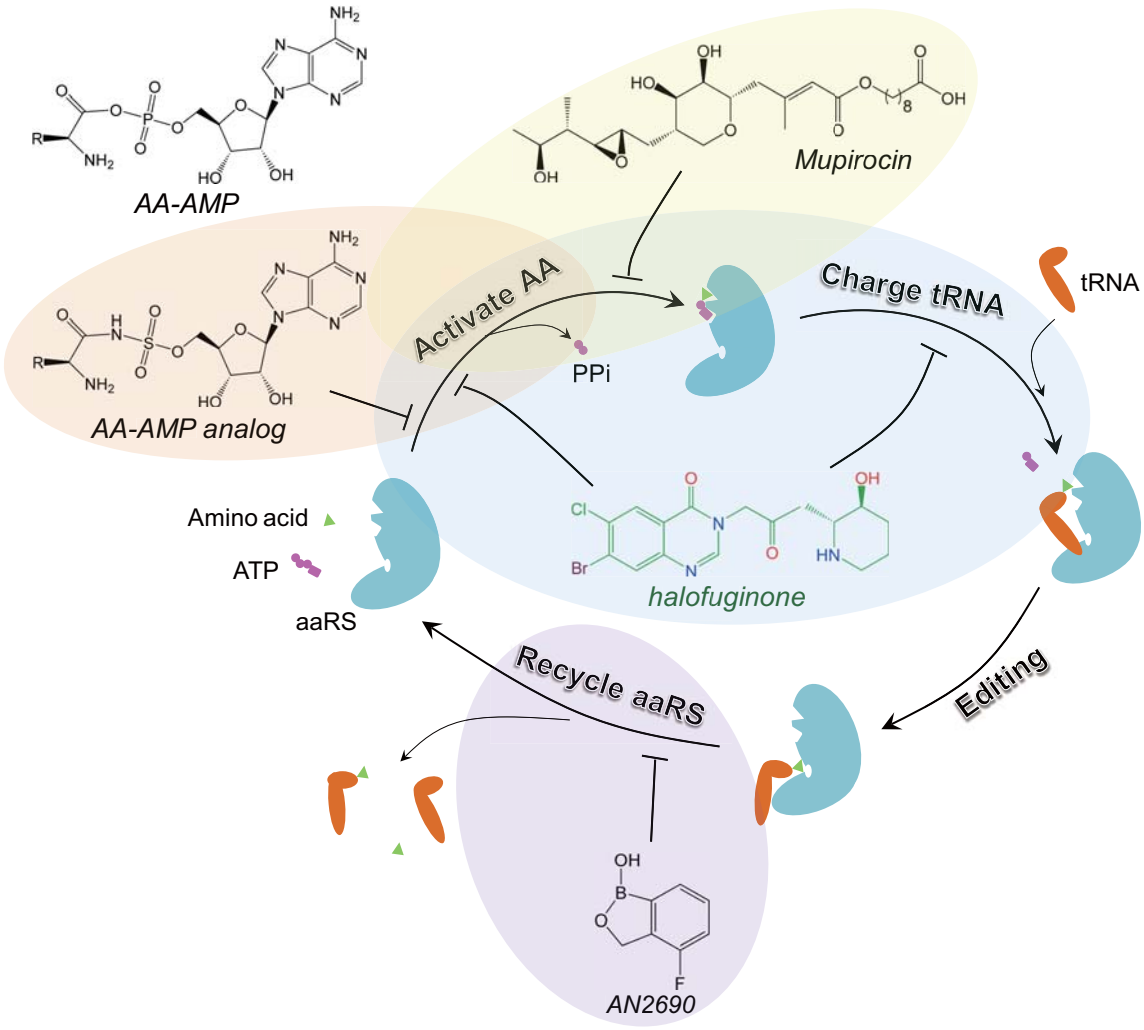
Supplementary Figure 3 | Conformation comparison of ATP[a] and ATP in different protein-ligand complexes. Superposition of the structure of eukaryote-like *T. thermophilus* ProRS (yellow) with bound ATP (gray-white, PDB 1H4Q), and of human ProRS (blue) with bound ATP[a] (magenta) (and HF (omitted)), shows that the bound nucleotides are superimposable. Thus, the ATP-dependent binding of HF to human ProRS does not change the orientation of ATP in the catalytic site.



X Conserved
X Partial conserved
X Not conserved

Residues within 4 Å of ATP[a]
Residues within 4 Å of halofuginone piperidine ring
Residues within 4 Å of halofuginone 4-quinazolinone group

Supplementary Figure 4 | Sequence alignment of eukaryotic or eukaryotic-like ProRSs. Sequence alignments reveal the conserved halofuginone and ATP binding sites amongst eukaryotic ProRSs. Symbolic notations for the colored residues and arrows are given at the bottom of the figure. Along the top of each segment of the alignment, the locations of helices (spirals), β-strands (arrows) and turns (T…T) are annotated.



Supplementary Figure 5 | Three different kinds of inhibitors block different steps of aminoacyl-tRNA synthesis. The AA-AMP mimics, including the "operational" mimic Mupirocin, an antibiotic targeting prokaryotic isoleucyl-tRNA synthetase, block the activation of amino acids. AN2690, an antifungal agent targeting leucyl-tRNA synthetase, traps tRNA at the editing site and blocks the synthetase recycle. Halofuginone is a dual site inhibitor blocking both amino acid activation and tRNA charging.

Supplementary Table 1 | Data collection and refinement statistics.

	ProRS:HF:ATP[a] ternary complex
Data collection	
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	72.54, 93.10, 87.00
α, *β*, *γ* (°)	90.00, 107.95, 90.00
Resolution (Å)	50.00-2.00 (2.03-2.00)*
R_{merge}	6.0 (58.2)
$I/\sigma I$	26.2 (3.5)
Completeness (%)	98.6 (98.5)
Redundancy	4.8 (4.5)
Refinement	
Resolution (Å)	46.43-2.00
No. reflections	69440
$R_{work/}$ R_{free}	20.4/22.7
No. atoms	
Protein	7671
Ligand/ion	114
Water	204
B-factors	
Protein	50.4
Ligand/ion	43.2
Water	46.6
R.m.s deviations	
Bond lengths (Å)	1.065
Bond angles (°)	0.007

*Highest resolution shell is shown in parenthesis.