Supporting Information for

Incorporation of fluorotyrosines into ribonucleotide reductase using an evolved, polyspecific aminoacyl-tRNA synthetase

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Materials and Methods

I. Synthesis of fluorotyrosines

Fluorotyrosines (F_nYs, n=2-4) were synthesized enzymatically from pyruvate, ammonia, and the appropriate fluorinated phenol, with tyrosine phenol lyase (TPL) as the catalyst.[1,2] The desired products were isolated in 60-85% yield and characterized by ^{1}H NMR, ^{19}F NMR, and absorption spectroscopy. TPL was expressed, purified, and assayed following the previously reported protocol.[1]

II. Aminoacyl-tRNA synthetase selection

Two cycles of positive and negative selection followed by a final positive selection round were performed on *Mj*TyrRS libraries containing random mutations at positions 32Y, 65L, 70H, 108F, 109Q, 158D, 159I, and 162L. Positive selection rounds were performed on glycerol minimal media with leucine (GMML)/agar containing 2,3,5-trifluorotyrosine (2,3,5-F_3Y, 1 mM), kanamycin (Km, 50 μg/mL), tetracycline (Tet, 12.5 μg/mL), and chloramphenicol (Cm, 40 μg/mL). Negative selections were performed on LB agar supplemented with Km (50 μg/mL), ampicillin (Amp, 100 μg/mL), and 0.2% arabinose (ara). Following the final positive selection round, individual colonies were picked and grown in a 96 well format in 2XYT media (with Km and Tet) overnight to saturation. The cultures were diluted to OD_{600} of 0.01 and stamped on positive selection plates with varied Cm concentrations (0-200 μg/mL) in the presence and absence of 2,3,5-F_3Y (1 mM). Colonies able to sustain growth above 100 μg/mL Cm only in the presence of unnatural amino acid (UAA) were then selected and sequenced (**Table S1, Figure S1**). The two best hits (E3 and E11, Table S1) were cloned into the pEVOL[3] expression plasmid for further assays.

III. Polyspecificity Assay

The pEVOL-2,3,5-F_3Y-RS constructs for hits E3 and E11 were co-transformed with pET-GFP_{Y151X}[3] into BL21(DE3) cells and grown in the presence of Amp (100 μg/mL) and Cm (50 μg/mL). Colonies were selected and grown to saturation overnight at 37 ^{o}C, then used to inoculate 2XYT media (10 mL) at OD_{600} 0.2. The culture was grown to OD_{600} 0.8 and induced with 0.02% w/v ara and isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM), then aliquoted (100 μL/well) into a 96 well plate containing 80 different UAAs (1 mM).[4] An initial fluorescence measurement was taken using a Spectramax Gemini EM (Ex/Em= 395/509 nm; Molecular Probes) to normalize for background fluorescence. The plate was incubated at 30 ^{o}C for 16 h followed by a second fluorescence measurement. Appropriate controls were utilized including three wells containing 2,3,5-F_3Y, three wells containing no UAA, three wells of uninduced culture, and three wells containing a bacterial culture with no GFP plasmid. The relative fluorescence of each well was then plotted to determine potential polyspecificity (**Figure S4**). Significant increases in GFP expression was observed for wells containing 3-FY (well H4, Figure S4), 3-chlorotyrosine (E3), and 2,3,5-F_3Y (H11).

IV. Verification of Polyspecificity

The pEVOL-2,3,5-F_3Y-RS constructs for hits E3 and E11 were co-transformed with pET-GFP_{Y151X} into BL21(DE3) cells and grown in the presence of Amp (100 μg/mL) and Cm (50 μg/mL). Colonies were selected and grown to saturation overnight at 37 °C, and then used to inoculate 2XYT media (10 mL) at OD_{600} 0.2. The culture was grown to OD_{600} 0.8, at which point 0.02% ara, IPTG (1 mM), and the appropriate F_nY (1 mM) were added, followed by incubation at 30 °C for 16 h. Cells were harvested by centrifugation and lysed using Bug Buster (Novagen) and the protein was purified on Ni-NTA spin columns (Qiagen) according to the manufacturer's protocol. Purified GFP was then analyzed by LC/MS on an Agilent 1100 Series LC/MSD. The chromatographic peak corresponding to GFP (between 6.3 min and 7.1 min) was charge deconvoluted using Agilent LC/MSD ChemStation software (revision B.03.02). Deconvolution parameters were set to higher than M_r = 20,000 and lower than M_r = 30,000, maximum charge = 50, and minimum peaks in set = 3–8. Error in measurements was ± 0.02%, as determined from external control samples (**Figure S5**).

V. UAA Uptake

Uptake studies were performed for 2,3-F_2Y, 2,3,5-F_3Y, and 2,3,5,6-F_4Y in BL21(DE3) cells as previously described.[3] The soluble fraction of cell lysate was then analyzed by LC/MS analysis. While 2,3-F_2Y and 2,3,5-F_3Y were easily detected, 2,3,5,6-F_4Y was present at much lower levels, suggesting limited cellular uptake. This finding corroborates protein expression experiments which indicated low incorporation of F_4Y into GFP and RNR (see main text and **Table S2**).

VI. Expression of His-$Y_{730}F_n$Y-α2s and His-$Y_{731}F_n$Y-α2s

A generalized protocol was established for the expression of all F_nY-containing α2s. *E. coli* BL21-DE3 cells (Invitrogen) were co-transformed with pET-28a-$nrdA_{Y730TAG}$ (or pET-28a-$nrdA_{Y731TAG}$), encoding for α with an N-terminal His_6 tag and a stop codon at the site of one of the two conserved Ys,[5] and pEVOL-F_3YRS-E3. Cells were plated on LB-agar plates supplemented with 50 μg/ml Km and 35 μg/ml Cm at 37 °C. After 14 h, single colonies were harvested and inoculated into 8 mL of 2XYT with antibiotics and grown at 37 °C, 225 rpm. After ~12 h, the small culture was inoculated into fresh 2XYT (100 mL, pH 7.0) with the same antibiotics at a 1:100 dilution. The culture was grown at 37 °C, 225 rpm until reaching an OD_{600} ~0.3, at which point F_nY (500 mM stock solution in water, NaOH solubilized) was added to the culture to a final concentrations of 0.7 - 2 mM. After 5 min, the F_3Y-RS was induced with ara (0.05% w/v). At OD_{600} ~0.6, *nrdA* expression was induced with 1 mM IPTG. The cells were allowed to grow for an additional 4 h (final OD_{600} >3), then harvested by centrifugation (3,000 x *g*, 10 min, 4 °C). No toxicity from F_nYs (up to 2 mM) was observed as determined by comparison to the OD_{600} of a flask without F_nY. Yields of 0.5-0.6 g cell paste/100 mL culture were obtained. Success of expression was assessed by 8% SDS-PAGE (**Figure S6a**).

VII. Purification His-$Y_{730}F_nY$-α2s and His-$Y_{731}F_nY$-α2s

The cell pellet (~1.0 g) was resuspended in lysis buffer (50 mM NaH_2PO_4, 5% glycerol, pH 7.2) with 1.0 mM PMSF and 10 mM β-ME at 5 mL buffer/g of cell paste, homogenized, and lysed via two passes though a French pressure cell at 14,000 psi. Cell debris was cleared by centrifugation (30,000*g*, 25 min, 4 °C), and the resulting pellet discarded. To the supernatant was added DNase (NEB) at 10 U/mL. The resulting solution was incubated with rocking for 30 min at 4 °C. To the supernatant was added Ni-NTA agarose (Qiagen, 1.0 mL), and the buffer adjusted to contain 300 mM NaCl. The resulting slurry was incubated with rocking for 1 h at 4 °C. The slurry was loaded into a column and allowed to gravity pack, and the column was washed with 30 column volumes (CVs) of wash buffer (lysis buffer + 300 mM NaCl + 10 mM imidazole) with 1.0 mM PMSF and 10 mM β-ME. The protein was eluted with a linear gradient (12 mL x 12 mL) of 10 to 250 mM imidazole in wash buffer. Protein-containing fractions were pooled, diluted 5-fold with storage buffer (50 mM Tris, 5% glycerol, 10 mM DTT, pH 7.6), and concentrated on an Amicon YM 30 membrane to give ~10 mg protein/g of cell paste. The purified protein (**Figure S6b**) contained a mixture of full-length and 729- (or 730-)truncated α, with the ratio of the two species varying according to the suppression efficiency achieved with the respective F_nY. This efficiency varied as follows: 2,3,5-F_3Y ≈ 3,5-F_2Y > 2,3,6-F_3Y ≈ 2,3-F_2Y > 2,3,5,6-F_4Y.

Separation of the truncated protein from the desired F_nY-α2 was achieved by FPLC. The concentrated protein sample after Ni-NTA chromatography was injected onto a POROS HQ/20 column (16 mm x 100 mm, 20 mL). The column was washed with 50 mM NaCl in storage buffer for 1 CV at a rate of 2 mL/min. The protein was then eluted with a gradient of 75 mM to 500 mM NaCl over 3 CVs at a rate of 2 mL/min. Protein-containing fractions were assessed by 8% SDS-PAGE, and full-length fractions were diluted 5-fold into storage buffer without salt and concentrated on an Amicon YM-30 membrane. Final yield of purified proteins are reported in **Table S2**.

VIII. Expression and purification of His-Y_{356}(2,3,5)F_3Y-β2

His-Y_{356}(2,3,5)F_3Y-β2 was expressed following the general protocol for F_nY-containing α2s, substituting pET-28a-*nrdA* with pET-9d-*nrdB*(TAG_{356}), which encodes for β with an N-terminal His_6 tag and no linker[6] and an amber stop codon at the position of interest. Yields of 0.4-0.5 g cell paste/100 mL culture were obtained. Expression was assessed by 10% SDS-PAGE.

His-Y_{356}(2,3,5)F_3Y-β2 was purified by Ni-NTA chromatography following the protocol described for F_nY-α2s, except that reductant was omitted from the buffer (no β-ME or DTT). Yield after affinity purification was 13 mg/g of cell paste, with the isolated fraction containing a statistical mixture of 355-truncated homodimer (β′β′), heterodimer (ββ′), and full-length homodimer (ββ). The mixed-fraction was purified further by FPLC. The entire protein fraction was injected onto a POROS HQ/20 column (16 mm x 100 mm, 20 mL), and the column was washed with 150 mM NaCl in 50 mM Tris, 5% glycerol, pH 7.6 for 1 CV at a rate of 1.5 mL/min. The protein was then eluted with a gradient of 150 mM to 450 mM NaCl over 6 CVs at the same rate (120 mL linear gradient). Protein-containing fractions were assessed by 10% SDS-PAGE and the desired fractions were pooled and concentrated. The protocol allowed for baseline separation between the truncated homodimer, the heterodimer, and the full-length homodimer.

ESI-MS analysis was conducted on a sample of the purified His-Y_{356}(2,3,5)F_3Y-β2 at the Proteomics Core Facility in the Koch Center for Integrative Cancer Research (MIT). The protein was bound to a protein microtrap (Michrom BioResources) and desalted by HPLC (50% water – 50% acetonitrile – 0.1% formic acid) immediately prior to MS analysis. Molecular weight measurements were made by LC-MS on a QSTAR Elite quadrupole-TOF mass spectrometer, which had been externally calibrated to a resolution of ~50 ppm. The spectrum indicated a major peak with MW of 44,454 Da (calculated: 44,451 Da).

IX. Expression and purification of Y_{122}(2,3,5)F_3Y-β2 or Y_{122}(3,5)F_2Y-β2

E. coli TOP10 cells were co-transformed with pBAD-*nrdB*(TAG_{122})[7] and pEVOL-F_3YRS-E3 and plated on LB-agar plates supplemented with 100 μg/ml Amp and 35 μg/ml Cm at 37 °C. A small culture was grown as described above and was used to inoculate 5 x 500 mL of 2XYT at a 100-fold dilution. The cells were grown until reaching an OD_{600} of 0.3, at which point F_nY was added to the media to a final concentration of 0.7 mM. At an OD_{600} of 0.5, the F_nY-RS and *nrdB* gene were induced with ara (0.05% w/v). The cells were grown for an additional 4-5 h to a final OD_{600} of ~1.5, then harvested by centrifugation (3,000 x *g*, 10 min, 4 °C). Yields of 2 g/L were obtained. Success of expression was assessed by 10% SDS-PAGE.

Y_{122}(2,3,5)F_3Y-β2 (or Y_{122}(3,5)F_2Y-β2) was purified by anion-exchange chromatography following a previously reported protocol.[7] 30-35 mg of protein per g of cell paste was obtained. The protein Y_{122}(2,3,5)F_3Y/Y_{356}F-β2 was expressed from pBAD-*nrdB*($TAG_{122}TTT_{356}$) and pEVOL-F_3YRS-E3 and purified in an analogous fashion.

X. Activity assays of Y_{122}(2,3,5)F_3Y-β2 and Y_{122}(3,5)F_2Y-β2

The activities of Y_{122}(2,3,5)F_3Y-β2 and Y_{122}(3,5)F_2Y-β2 were examined by the spectrophotometric assay for nucleotide reduction. Briefly, ribonucleotide reductase activity was monitored through a continuous coupled-enzyme assay in which the consumption of NADPH was determined by monitoring the decrease in absorbance at 340 nm. α2 (2.5 μM), ATP (3 mM), CDP (1 mM), TR (30 μM), and TRR (0.5 μM) were mixed in assay buffer (50 mM Hepes, 15 mM $MgSO_4$, 1 mM EDTA, pH 7.6) in a final volume of 300 μL. The assay was initiated by addition of β2 (0.5 μM) and NADPH (0.2 mM) and the change in A_{340} was monitored for 1 min.

XI. Characterization of 2,3,5-F_3Y_{122}• and 3,5-F_2Y_{122}• by 9 GHz EPR spectroscopy

EPR spectra were recorded at 77 K on a Bruker EMX X-band spectrometer equipped with a quartz finger dewar containing liquid N_2 in the Department of Chemistry Instrumentation Facility. EPR parameters were as follows: microwave frequency = 9.34 GHz, power = 30 μW, modulation amplitude = 1.5 G, modulation frequency = 100 kHz, time constant = 5.12 ms, scan time = 41.9 s. EPR spin quantitation was carried out by calculating the corrected double integral intensity of the spectra and comparing it to the spectra of a Cu(II) standard[8] and the Y_{122}• of a wt-β2 sample, in which the radical concentration had been established using the dropline correction method.[9] EPR spectra were simulated with the EasyFit function of EasySpin[10] (version 3.1.7) using the simulation parameters of Y_{122}• and 3-FY_{122}• as starting points (**Table S3**).

XII. In vitro reconstitution of 2,3,5-F_3Y_{122}•

The iron contained in as-isolated $Y_{122}(2,3,5)F_3Y$-β2 (or $Y_{122}(2,3,5)F_3Y/Y_{356}F$-β2) was reduced and removed under anaerobic conditions using dithionite, methyl viologen, and ferrozine following a previously-reported protocol.[5] The differic-F_3Y• cofactor of the resulting apo protein was reconstituted with Fe^{2+} and O_2, as previously reported.[9]

XIII. EPR reaction of of $Y_{122}(2,3,5)F_3Y$-β2 with wt-α2, CDP, and ATP

Pre-reduced wt-α2 and ATP were mixed rapidly on ice with $Y_{122}(2,3,5)F_3Y$-β2 (0.4 Y•/β2) and CDP in assay buffer to give final concentrations of 40 μM, 3 mM, 40 μM, and 1 mM. The reaction was hand-quenched at 20 s in acetone/dry ice and its EPR spectrum recorded using the parameters described above. An analogous experiment was conducted between $Y_{122}(2,3,5)F_3Y$-β2 and $Y_{731}F$-α2, and between $Y_{122}(2,3,5)F_3Y/Y_{356}F$-β2 (0.9 Y•/β2) and wt-α2. Spin quantitation was conducted as described above, and revealed no total spin loss during any of the three reactions. Deconvolution of the composite reaction spectra was conducted with an in-house, Excel-based program using the known spectrum of 2,3,5-F_3Y• from the $Y_{122}(2,3,5)F_3Y$-β2 mutant. The subtraction was optimized using the fluorine hyperfine coupling features observed at the low field and high field region of the spectrum. The resulting spectrum is designated the "new radical."

Supplementary Tables and Figures

Table S1. Sequences of aaRS library members surviving the final positive selection round. Yellow highlighting indicates sequence convergence among clones. Red boxes indicate positional preferences that emerged in the selection process. Clones E3 and E11 were deemed the best candidates for further study on the basis of their performance on the final selection round (Figure S1).

Clone	32	65	70	108	109	114	158	159	162
WT	Y	L	H	F	Q	Y	D	I	L
E11	H	Y	G	Y	A	Y	N	I	R
D11	H	Y	G	Y	A	Y	N	I	R
E4	H	Y	G	Y	A	Y	N	I	R
G6	H	N	G	Y	S	Y	S	I	R
C10	H	N	P	S	S	Y	H	I	T
E3	L	G	N	F	Q	Y	S	Y	H

Figure S1. Selective growth of individual clones at Cm concentrations of 100 μg/mL in the absence (left) and presence (right) of 2,3,5-F_3Y (1 mM). The two most robust clones in the right panel are hits E11 (second row, position 1) and E3 (second row, position 2). Clones A5 and B6 showed background growth in the absence of 2,3,5-F_3Y and thus were not pursued for study.

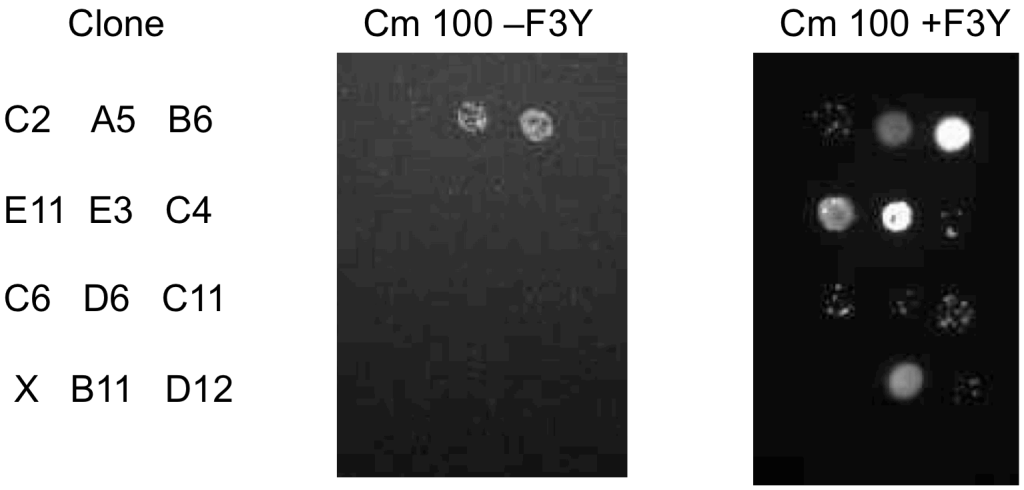
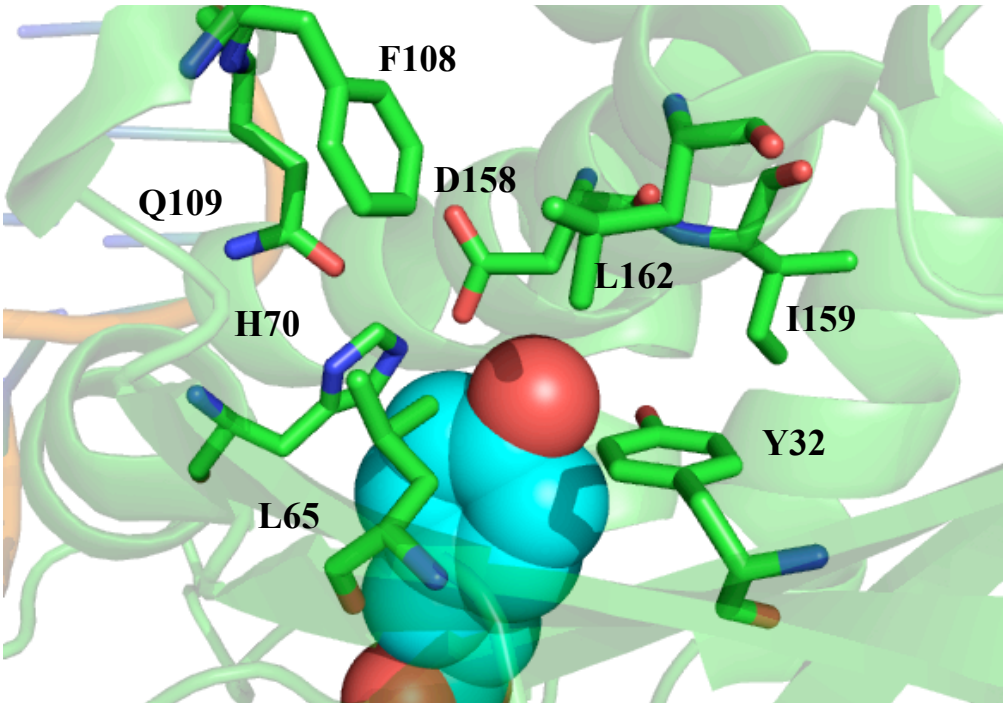


Figure S2. A) Structure of substrate-bound wt *Mj*TyrRS and locations of mutated residues. The active site of the wt *Mj*TyrRS/tRNA/Tyr ternary complex (PDB 1J1U) is shown.[11] Residues that are mutated in the TyrRS libraries are drawn in sticks, and the Tyr substrate is shown in space-filling model; B) Mutations of E3 and E11 RS relative to the native *Mj*TyrRS.

(A)



(B)

Clone	32	65	70	108	109	158	159	162
WT	Y	L	H	F	Q	D	I	L
E3	L	G	N	F	Q	S	Y	H
E11	H	Y	G	Y	A	N	I	R

Figure S3. Incorporation of 2,3,5-F_3Y (**1**) into GFP. A) SDS PAGE analysis of the expression of a GFP possessing a TAG stop codon at position 151 using aaRSs E3 and E11 in the presence and absence of **1**. Red arrow indicates the position of full-length GFP. B) LC/MS confirmation of incorporation of **1** into GFP. Observed mass: 27759 Da (expected: 27758 Da).

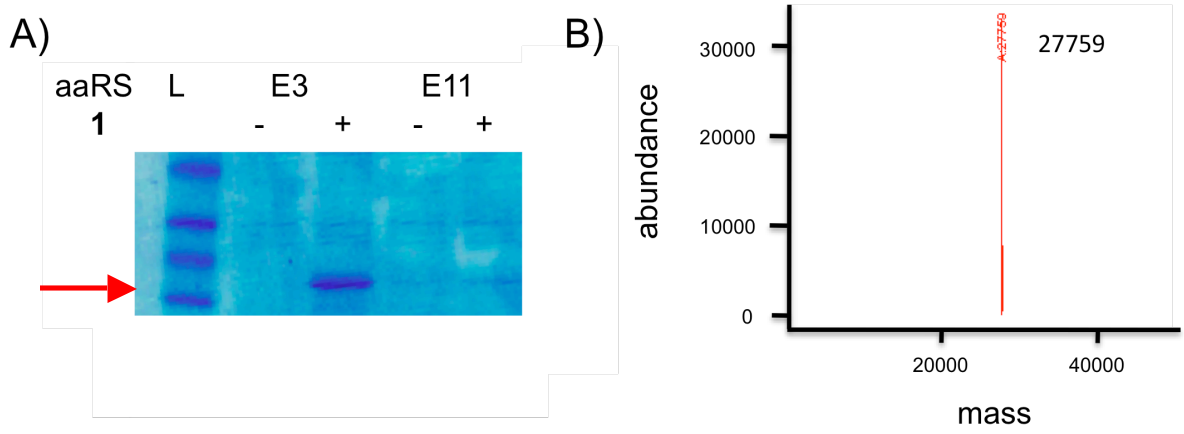


Figure S4. Polyspecificity fluorescence assay for 2,3,5-F_3Y-RS-dependent incorporation of UAAs into GFP_{Y151X}. A 96-well plate contains different UAAs (1 mM) incubated with BL-21(DE3) cells harboring pET-GFP_{Y151X} and the pEVOL-2,3,5-F_3Y-E3 RS (similar results were observed for pEVOL-2,3,5-F_3Y-E11; data not shown) in 2XYT media. Fluorescence was measured 16 h after induction of GFP expression at 30 ^{o}C and corrected for background by a non-induced culture. Wells containing 3-FY (H4), 3-chlorotyrosine (E3), and 2,3,5-F_3Y (H11) showed the greatest response.

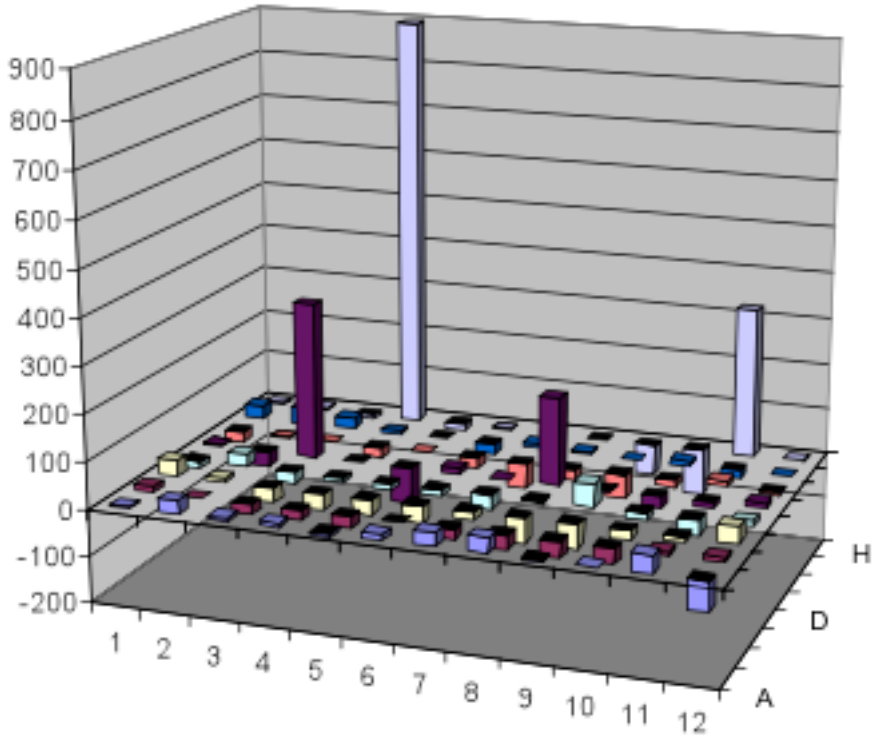


Figure S5. LC/MS analysis of purified GFP incorporating different fluorotyrosine analogs. Calculated MWs are 27758 (2,3,5-F_3Y and 2,3,6-F_3Y), 27776 (2,3,5,6-F_4Y) and 27740 (2,3-F_2Y and 3,5-F_2Y). The large error in the F_4Y measurement is likely related to difficulty in detecting the very low levels of isolated protein. The green peak in the spectrum with 3,5-F_2Y is a small amount of protein that co-purifies with GFP during Ni-NTA purification (GFP purity is ≥90%).

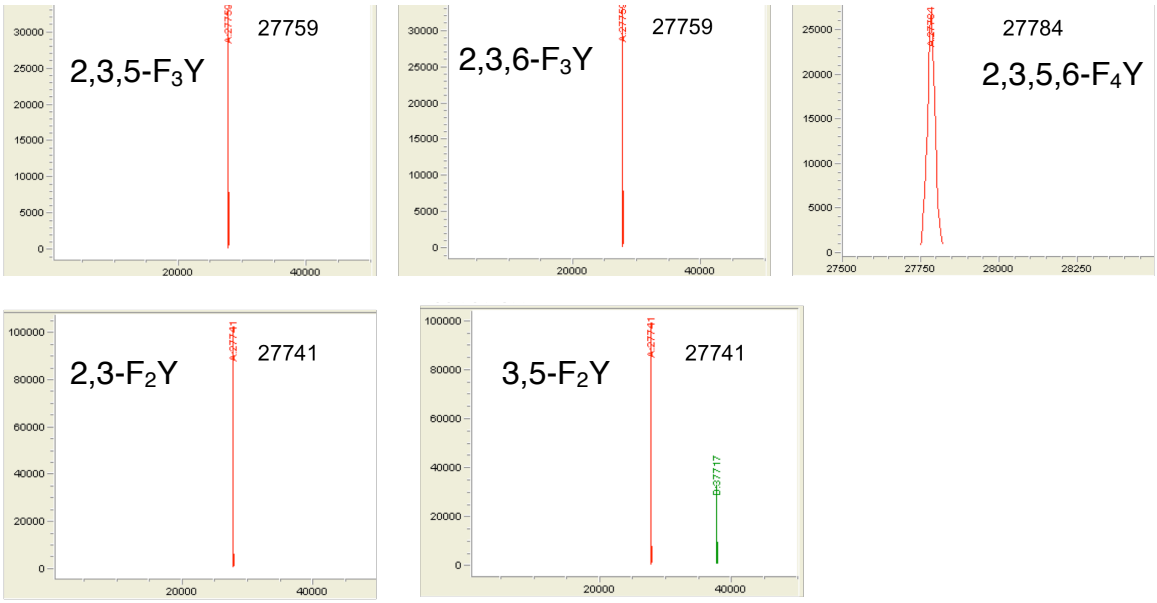


Figure S6a. Expression of His-Y_{730}(2,3,5)F_3Y-α2 using the E3 (panel A) and E11 (panel B) F_nY-RSs analyzed by SDS-PAGE (8%) of whole cells four hours after IPTG-induction of the *nrdA* gene. Lanes are as follows: (1) His-α standard, (2) no F_3Y in media, (3) 1 mM F_3Y in media, (4) 2 mM F_3Y in media, and (5) His-α standard.

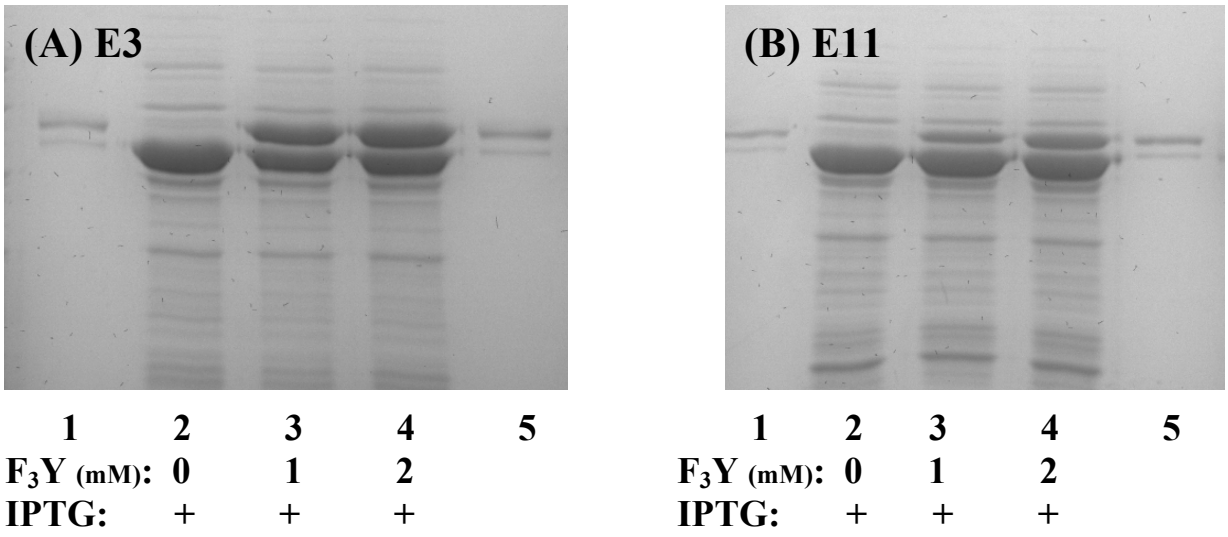


Figure S6b. Purification of His-Y_{730}(2,3,5)F_3Y-α2. Lanes (from left to right) contain the following: (1) His-α(wt) standard; (2) soluble fraction after lysis; (3) insoluble fraction after lysis; (4) flow-through upon column loading; (5) elution fractions from Ni column with increasing [imidazole]; (6) His-α(wt) standard

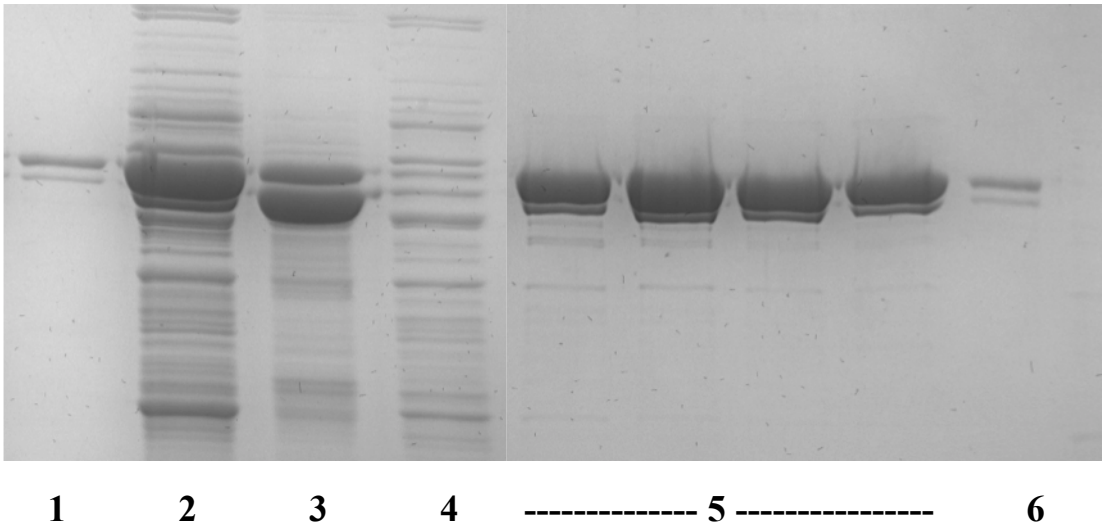


Table S2. Properties of F_nYs and their incorporation into positions in *E. coli* RNR α2 and β2

F_nY	**1**	**2**	**3**	**4**	**5**	**6**
pK_a[a]	6.4	8.4	7.8	7.2	7.0	5.6
E_p (mV)[a]	853	705	810	755	911	968
Yield at 730-α2[b]	10	-[c]	3	3	2.5	<0.3
Yield at 731-α2	4	-	-	3	-	-
Yield at 356-β2	2	-	-	-	-	-
Yield at 122-β2	35	-	-	30	-	-

a. Originally reported elsewhere.[2,12] *b*. Yield of purified F_nY-RNRs reported in mg of protein/g of cell paste. [UAA] in the growth media varied from 0.7 – 2 mM depending on the F_nY. *c*. Not determined.

Table S3:EPR simulation parameters for 3,5-F_2Y_{122}• and 2,3,5-F_3Y_{122}•. Simulations were conducted with the EasyFit function in EasySpin[10] using the parameters for 3-FY_{122}• as a starting point.[2]

(A) 3,5-F_2Y_{122}•

Nucleus	A_x (MHz)	A_y (MHz)	A_z (MHz)
^{19}F	-21	-10	160
^{19}F	1	13	-154
$^{1}H^{a}$	49	52	46

a. Second β-methylene proton has coupling constants <10 MHz.

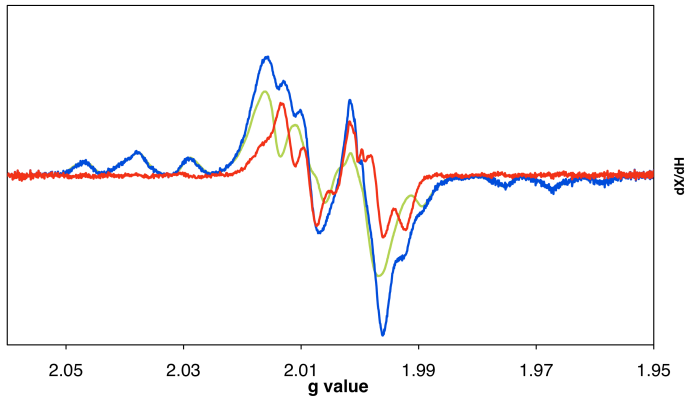
(B) 2,3,5-F_3Y_{122}•

Nucleus	A_x (MHz)	A_y (MHz)	A_z (MHz)
^{19}F	15	3	53
^{19}F	-15	-3	183
^{19}F	18	3	-152
$^{1}H^{a}$	52	46	36

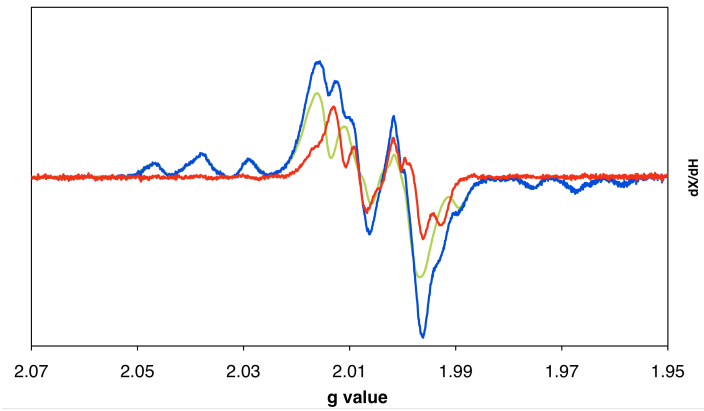
a. Second β-methylene proton has coupling constants <10 MHz.

Figure S7. EPR spectra of the reactions of $Y_{122}(2,3,5)F_3Y$-β2, CDP, and ATP with (A) wt-α2 or (B) $Y_{731}F$-α2, and (C) $Y_{122}(2,3,5)F_3Y/Y_{356}F$-β2 with wt-α2, CDP, and ATP. Reaction spectrum is in blue, residual $F_nY\bullet$ is in green, and net spectrum (i.e., new radical) is in red.

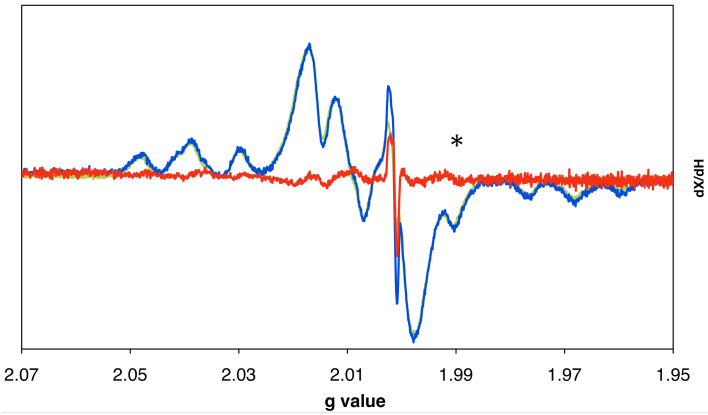
(A) $Y_{122}(2,3,5)F_3Y$-β2 with wt-α2:



(B) $Y_{122}(2,3,5)F_3Y$-β2 with $Y_{731}F$-α2:



(C) $Y_{122}(2,3,5)F_3Y/Y_{356}F$-β2 with wt-α2:



*The sharp g ~ 2 feature is associated with an impurity in the N_2 finger dewar used to collect data at 77 K.

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