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Supplementary Information for

Genetically Encoded Fluorophenylalanines Enable Insights into the Recognition of Lysine Trimethylation by an Epigenetic Reader

Yan-Jiun Lee, M. J. Schmidt, Jeffery M. Tharp, Annemarie Weber, Amber L. Koenig, Hong Zheng, Jianmin Gao, Marcey L. Waters, Daniel Summerer,* and Wenshe R. Liu*

Email: Daniel.summerer@tu-dortmund.de and wliu@chem.tamu.edu

Supplementary Methods

Chemicals

F_5F, F_3F, and F_2F' were purchased from ChemImpex Inc (Wood Dale, IL, USA). F_4F was synthesized according to a reported procedure.[1] F_2F was purchased from CombiBlocks Inc (San Diego, CA, USA).

Cloning and Expression of the C-terminal catalytic domain of PylRS-AA and PylRS_AAAF

For construction of expression constructs, a PylRS_wt gene and a PylRS_AF gene were amplified with primers oDaS105 (TTT TTC TGC AGT CAA TGA TGA TGA TGA TGA TGC AGG TTC GTA GAG ATC) and oDaS106 (TTT TTA GAT CTA TGC CAG CAC TGA CAA AAT CCC AAA CC) from pDaS69 (pEVOL_PylRS_wt) and pDaS70 (pEVOL_PylRS_AF) and cloned into the BglII and PstI sites of pDaS69 (pEVOL_PylRS_wt) to shorten the araBAD-controlled PylRS gene and remove the glnS´-controlled PylRS gene. The N346A and C348A mutations were introduced by Quickchange mutagenesis on the resulting plasmids pMoS221 (pEVOL_PylRS_single_wildtype_short_His6) and pMoS222 (pEVOL_PylRS_single_ AF_short_His6) with primers oMoS_402 (N346A_C348A_fwd, GGA GTT TAC CAT GCT GGC ATT TGC ACA AAT GGG TTC AGG TTG TAC TCG) and oMoS_403 (N436A_C348A rev, CAA CCT GAA CCC ATT TGT GCA AAT GCC AGC ATG GTA AAC TCC TCC AGA TG), resulting in plasmids pMoS230 (pEVOL_PylRS_single_wildtype_N346A_C348A_short_His6) and pMos231 (pEVOL_PylRS_single_AF_N346A_C348A_short_His6).

Expressions were essentially performed as described previously.[2] Briefly, an overnight culture of *E. coli* Top10 transformed with pMos230 or pMos231 was grown in LB media supplemented with 34 µg/ml chloramphenicole and diluted 50-fold into 1 L of the same medium. The culture was grown at 37 °C and 160 rpm shaking and induced at an OD_{600} nm of 0.4 - 0.6 with 0.02% (w/v) L-arabinose. After 4 hours growth under the same incubation conditions, culture was harvested by centrifugation. Cell lysis was achieved by four passages through a precooled French pressure cell. Both PylRS mutants were purified by Ni^{2+} affinity chromatography (HisTrap, GE Healthcare) followed by size exclusion chromatography (Superdex75, GE Healthcare) in 10 mM potassium phosphate pH 7.4, 300 mM NaCl, 5 mM $MgCl_2$, and 5 mM β-mercaptoethanol. The solutions were concentrated to a final protein concentration of 15 mg/ml.

Amino Acid Sequences of the C-terminal catalytic domains (amino acids 188-454) of PylRS_wt as well as PylRS_AA and PylRS_AAAF used for crystallization

>PylRS_wt
MPALTKSQTDRLEVLLNPKDEISLNSGKPFRELESELLSRRKKDLQQIYAEERENYLGKLEREITRF
FVDRGFLEIKSPILIPLEYIERMGIDNDTELSKQIFRVDKNFCLRPMLAPNL**Y**NYLRKLDRALPDPIK
IFEIGPCYRKESDGKEHLEEFTML**N**F**C**QMGSGCTRENLESIITDFLNHLGIDFKIVGDSCMV**Y**GDTL

DVMHGDLELSSAVVGPIPLDREWGIDKPWIGAGFGLERLLKVKHDFKNIKRAARSESYYNGISTNL
HHHHHH*

>PylRS_AA
MPALTKSQTDRLEVLLNPKDEISLNSGKPFRELESELLSRRKKDLQQIYAEERENYLGKLEREITRF
FVDRGFLEIKSPILIPLEYIERMGIDNDTELSKQIFRVDKNFCLRPMLAPNLANYLRKLDRALPDPIK
IFEIGPCYRKESDGKEHLEEFTML**A**F**A**QMGSGCTRENLESIITDFLNHLGIDFKIVGDSCMVFGDTL
DVMHGDLELSSAVVGPIPLDREWGIDKPWIGAGFGLERLLKVKHDFKNIKRAARSESYYNGISTNL
HHHHHH*

>PylRS_AAAF
MPALTKSQTDRLEVLLNPKDEISLNSGKPFRELESELLSRRKKDLQQIYAEERENYLGKLEREITRF
FVDRGFLEIKSPILIPLEYIERMGIDNDTELSKQIFRVDKNFCLRPMLAPNL**A**NYLRKLDRALPDPIK
IFEIGPCYRKESDGKEHLEEFTML**A**F**A**QMGSGCTRENLESIITDFLNHLGIDFKIVGDSCMV**F**GDTL
DVMHGDLELSSAVVGPIPLDREWGIDKPWIGAGFGLERLLKVKHDFKNIKRAARSESYYNGISTNL
HHHHHH*

Positions 306, 346, 348 and 384 targeted for Mutation are bold and underlined.

Crystallization and X-Ray Crystallographic Data Acquisition and Processing

Prior to crystallization, the proteins were supplemented with 10 mM AMPPNP (Jena Biosciences). PylRS_AA was mixed in a 1:1 ratio with a reservoir solution containing 100 mM TrisHCl pH 9.5, 200 mM $MgCl_2$, and 20% (w/v) PEG4000; PylRS_AAAF was mixed in a 1:2 ratio with a reservoir solution containing 100 mM TrisHCl pH 8.5, 200 mM $MgCl_2$, and 18% (w/v) PEG4000. Crystals were grown by the vapor-diffusion hanging drop method at 18 °C. Cryostabilization of the crystals was achieved by soaking in reservoir solution supplemented with 30 % (v/v) ethylene glycol. Crystals were flash-frozen in liquid nitrogen and diffraction data was collected at beamline X06DA at the Swiss Light Source of the Paul Scherrer Institute, Villigen, Switzerland. The XDS package was used for data reduction and processing. [3]
The proteins crystallized in space group C2 with similar cell dimensions. The structures were solved by rigid-body refinement against pdb entry 4CS2.[2] Model building and refinement was done with COOT[4] and PHENIX,[5] respectively. MolProbity[6] was used for structure validation during refinement.

Table of Data Collection and Refinement Statistics

	PylRS_AA in complex with AMPPNP	PylRS_AAAF in complex with AMPPNP
PDB ID	5K1P	5K1X
Data collection		
Beamline	X06DA	X06DA
Wavelength (Å)	1.0000	1.0000
Space group	C2	C2
Cell dimensions		
a, b, c (Å)	102.49, 44.36, 64.31	102.02, 44.14, 64.31
α, β, γ (°)	90, 99.92, 90	90, 100.01, 90
Resolution range (Å)	40.61 – 1.499 (1.553 – 1.499)	43.18 – 1.95 (2.02 – 1.95)
Total reflections	146162 (14070)	59393 (1968)
Unique reflections	44122 (4370)	19540 (1293)
Completeness (%)	96 (97)	94 (63)
Mean *I/σ(I)*	11.64 (1.03)	7.45 (0.67)
R_{meas} (%) [a]	6.4 (119.4)	13.18 (122.6)
$CC_{1/2}$ (%) [b]	99.9 (43.6)	99.5 (38)
Refinement		
R_{work} / R_{free}	16.5 / 19.6	19.7 / 22.7
No. of non-hydrogen atoms	2536	2362
Protein	2230	2180
Ligands	33	45
Water	273	137
Protein residues	270	268
R. m. s. Deviations		
Bond lengths (Å)	0.010	0.005
Bond angles (°)	1.10	0.77
Ramachandran (%)		
Favored	98	98
Allowed	1.8	2.2
Outlier	0	0
B-factors		
Average B-factor	29.8	37.4
Protein	28.8	37.1
Ligands	23.8	33.5
Water	39.2	43.5

Values in parentheses correspond to highest resolution shell. [a] for definition of $R_{meas,}$ see [7]; [b] for definition of $CC_{1/2,}$ see [8].

Recognition F_5F by PylRS-AA

Two previously constructed plasmids pEVOL-pylT-PylRS-AA (pEVOL-pylT-N346A/C348A) and pET-pylT-sfGFP2TAG were used to transform *E. coli* BL21 cells.[9] The transformed cells were grown in the GMML medium(M9 minimal medium supplemented with 1% glycerol, 300 μM leucine, 2 M $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% NaCl, 25 μg/mL kanamycin and 100 μg/mL ampiccilin) with or without the supplement of 5 mM F_5F. Cells were grown at 37 °C in an incubating shaker (225 r.p.m.) and protein expression was induced by adding 0.2% arabinose and 1 mM IPTG when OD_{600} reached 0.6. After 20 h incubation, cells were harvested, resuspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) and sonicated. The cell lysate was clarified by centrifugation (30 min, 11000 g, 4 °C). The supernatant was subjected to Ni-NTA affinity chromatography according to manufacturer's protocol. The supernatant was incubated with Ni-NTA resin (Qiagen) (2 h, 4 °C). The slurry was then loaded to a column and the protein-bound resin was washed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 45 mM imidazole, pH 8.0) followed by eluting the bound protein with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was buffer-exchanged to Tris-HCl buffer (50 mM, pH 7.4) and concentrated using an Amicon Ultra-15 Centrifugal Filter (10 kD MWCO, Millipore). Purified sfGFP proteins expressed in conditions with and without the supplement of F_5F were analyzed by SDS-PAGE for comparison (**Supplementary Figure 1**). The sfGFP concentration was quantified by UV absorbance at 485 nm using literature reported extinction coefficient 8.33×10^4 M^{-1} cm^{-1}.

Library construction and selection of PylRS-AS

Library Construction

The plasmid pBK-PylRS-AA was derived from pBK-MmPylRS plasmid reported previously.[9, 10] This plasmid was used a template to construct a library with randomization at A348. This construction of this library was based on Phusion DNA polymerase (NEB, Ipswich, MA, USA)-catalyzed site-directed mutagenesis. Two synthesized degenerated primers (IDT, Coralville, IA, USA): YJL055 (5'- NNKCAGATGGGATCGGGATGTACAC -3') and YJL056 (5'-GAAGTTCAGCATGGTAAACTCTTCGAGGTGTTC -3') where N=A/C/T/G; T, K=G/T; M=C/A were used to PCR amplify the template DNA. The afforded PCR product was phosphorylated with T4 PNK, ligated with T4 DNA ligase, and then used to transform Top10 competent cells.

Library Screening

This single site library in pBK plasmid together with another selection plasmid pY+ was used to transform the Top10 *E. coli* strain. pY+ plasmid contains genes encoding pylT, type I chloramphenicol acetyltransferase with an amber mutation at D112 and green fluorescent protein (GFPuv) with an amber mutation at Q204.[10] The library-containing cells were grown on a GMML plate with 50 μg/mL kanamycin, 25 μg/mL tetracycline, and 1% LB. Single colonies were randomly selected and inoculated to LB medium with 50 μg/mL kanamycin and 25 μg/mL tetracycline on 96-well plates. After incubating the plates at 37

°C for 3 h, the cell in each well was dotted onto the screening plates containing 68 μg/mL chloramphenicol, 50 μg/mL kanamycin, 25 μg/mL tetracycline and with 1 mM F_5F. The plates without F_5F served as a control. Plates were incubated at 37 °C. Both cell viability and fluorescence intensity were evaluated during the screening process. The clones with strong fluorescence on the F_5F-containing plate and low viability and fluorescence on the control plate were selected as the active clones and were amplified from the corresponding wells in the 96-well plate. The pBK plasmid of the active clones was isolated and sequenced. Most of the active clones were converged to contain N346A/C348A mutations (**Supplementary Figure 2**).

Expression of fluorinated sfGFP proteins using PylRS-SA

pEVOL-pylT-PylRS-AS Construction

The pEVOL-PylT-PylRS-AS plasmid was derived from the pEVOL-PylT plasmid reported previously.[9] The PylRS-AS gene were PCR amplified from the identified pBK-PylRS-AS plasmid using primers pEVOL-PylRS-SpeI-F (5'-gaggaaactagtatggataaaaaaccactaaacactctg-3') and pEVOL-PylRS-SalI-R (5'-tgatggtcgactcacaggttggtagaaatcccgtt-3'). The PCR product was digested by SpeI and SalI restriction enzymes, and ligated to a similarly digested pEVOL-PylT plasmid.

Protein Sequnce of sfGFP

MA**S**KGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFI**C**TTGKLPVPWPTLVTTL
TYGVQ**C**FSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGID
FKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVL
LPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYKGSHHHHHH
*The fluorophenylalanine incorporation site is highlighted in **red**.

Incorporation of fluorophenylalanines into sfGFP

E. coli BL21(DE3) cells were cotransformed with pET-PylT-sfGFPS2TAG and pEVOL-PylT-PylRS-AS. The transformed cells were used to inoculate LB medium and was grown at 37 °C with agitation until OD_{600} reached 1.0-1.2. Then the cells were pelleted and washed with isotonic saline or PBS buffer (x3). After medium shift to the GMML medium supplemented with 1 mM IPTG, 0.2% arabinose, and one of desired fluorophenylalanine (3 mM), the cells were incubated at 37 °C with agitation for 20 h. All fluorophenylalanines except F_4F were purchased from commercial sources . F_4F was synthesized according to a previous reported protocol. After centrifugation, the cell pellet was resuspended in ice cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication. The lysate was centrifuged (10000 g, 4 °C, 1 h) and the supernatant was subjected to Ni-NTA affinity chromatography according to the manufacturer's protocol. The supernatant was incubated with Ni-NTA resin (Qiagen) (2 h, 4 °C). The slurry was then loaded to a column and the protein-bound resin was washed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 45 mM imidazole, pH 8.0) followed by eluting the bound protein with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was

buffer-exchanged to Tris-HCl buffer (50 mM, pH 7.4) and concentrated using an Amicon Ultra-15 Centrifugal Filter (10 kD MWCO, Millipore). The quality of the purified protein was analyzed by SDS-PAGE and ESI-MS. The sfGFP concentration was quantified by UV absorbance at 485 nm using literature reported extinction coefficient 8.33×10^4 M^{-1} cm^{-1}.

Expression and purification of Mpp8C-SUMO-6xHis proteins

DNA Sequence of Mpp8C

5'-atgggcgaagacgtgttcgaagtagagaagatcctggacatgaagaccgaaggcggtaaagttctgtacaaagtgcgctggaa aggctatacttctgacgatgacacctgggaaccagagatccatctggaagattgtaaagaagttctgctggaatttcgcaagaagat tgccgagaacaaagctaaa- 3'

Mpp8C-SUMO Expression Vector Construction

The codon optimized Mpp8C gene was synthesized by Epoch (Epoch Life Science, Sugar Land, TX, USA). It was PCR amplified from the synthetic construct using primers YJL057 (5'-CCACCATG GGCGAAGACGTGTTCGAAGTAG-3') and YJL058 (5'-AACCATGGCACCT TGGAAATACAGGTTCTCTTTAGC-3'). A pET28-SUMO-6xHis plasmid (a gift from Prof. C. Hilty, Department of Chemistry, Texas A&M) was digested with the NcoI restrict enzyme. The digested site was ligated with the aforementioned NcoI-digested PCR product using T4-DNA ligase to afford pET28b-Mpp8C-SUMO-6xHis. The Mpp8C-SUMO-6xHis gene was then PCR amplified from the template plasmid pET28b-Mpp8C-SUMO-6xHis using primers YJL057 (5'- CCACCATG GGCGAAGACGTGTTCGAAGTAG-3') and YJL094 (5'-GATCCTGCAGTTAGTGGTGGTGGTGGTGG ACCACCAATCTGTTCTCTGTGAGCC-3'). The PCR-Amplified product was inserted between NcoI and PstI sites of a pBAD plasmid downstream of an *araBAD* promoter to generate pBAD-Mpp8C-SUMO-6xHis. The amber mutation at the F59 site was then introduced using Phusion DNA polymerase-based site-directed mutagenesis.

Protein Sequence of MPP8CD-SUMO-6xHis

MGEDV**F**EVEKILDMKTEGGKVLYKVR**W**KG**Y**TSDDDTWEPEIHLEDCKEVLLEFRKKIAENKAKE LSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFL YDGIRIQADQTPEDLDMEDNDIIEAHREQIGGHHHHHH

*The underline highlights the Mpp8C sequence and the fluorophenylalanine incorporation site is marked in **red**.

Incorporation of fluorophenylalanines into sfGFP

The purification protocol was modified from the fluorinated sfGFP expression and purification. *E. coli* strain Top10 were cotransformed with plasmids pBAD-Mpp8C-F59TAG-SUMO-6xHis and pEVOL-pylT-PylRS-AS. The transformed cells were used to inoculate LB media and grown at 37 °C with agitation until the OD_{600} reached 1.0-1.2. Then the cells were pelleted and washed with isotonic saline or PBS buffer (x3). After medium shift to GMML medium supplemented with 1 mM IPTG, 0.2% arabinose, and 3 mM fluorophenylalanine, the cells were incubated at 37 °C with agitation for 20 h. After

centrifugation to collect cells, the cell pellet was resuspended in ice cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication. The lysate was centrifuged (10000 g, 4 °C, 1 h) and the supernatant was subjected to Ni-NTA affinity chromatography according to the manufacturer's protocol. The supernatant was incubated with Ni-NTA resin (Qiagen) (2 h, 4 °C). The slurry was then loaded to a column and the protein-bound resin was washed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 45 mM imidazole, pH 8.0) followed by eluting the bound protein with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was buffer-exchanged to Tris-HCl buffer (50 mM, pH 7.4) and concentrated using an Amicon Ultra-15 Centrifugal Filter (10 kD MWCO, Millipore). The quality of purified protein was analyzed by SDS-PAGE.

To further improve the quality of protein, the buffer exchanged Mpp8C was subjected to FPLC mounted with a QSepharose HP column where the Mpp8C was eluted at roughly 37% NaCl concentration of buffer B (20 mM TrisHCl pH 8, 1 M NaCl). The original running buffer A contains the same composition as buffer B without NaCl. The collected fractions were dialyzed to a fluorescence polarization assay buffer containing 50 mM potassium phosphate pH 8.0 and 25 mM NaCl and then concentrated. Protein concentration was determined by absorbance spectroscopy using predicted extinction coefficient (ε_{280} = 15470 M^{-1} cm^{-1}, MW = 19640.12).

Fluorescence polarization binding assays

Fluorescence polarization was performed on a BioTek Synergy H1 plate reader by setting it on automatic gain and 100 flashes. FP binding assays were performed under conditions of 50 mM potassium phosphate pH 8.0 and 25 mM NaCl and in the presence of 100 nM fluorescein-labeled peptide. A series of diluted Mpp8C proteins were prepared in a range of nM to sub-mM concentrations on a black solid-bottom 96-well plate. Binding curves were analyzed by non-linear least-squares fitting of the raw fluorescence polarization data to the Hill equation to obtain K_d values.[11]

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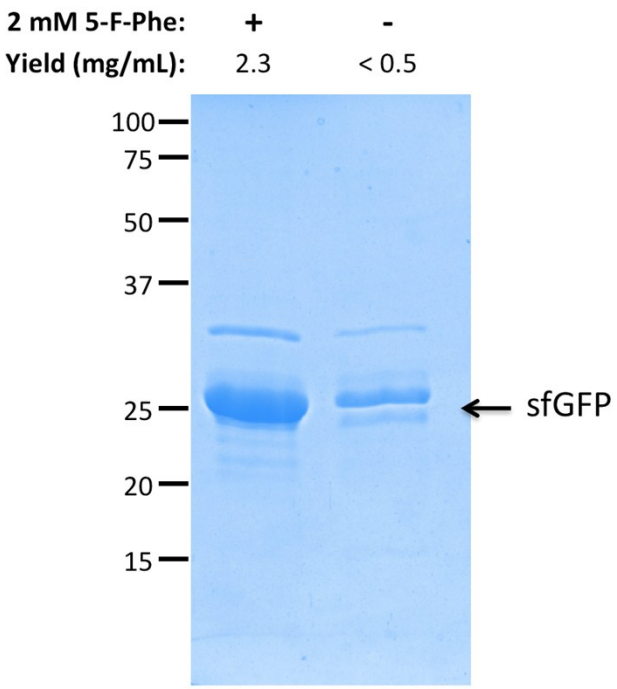
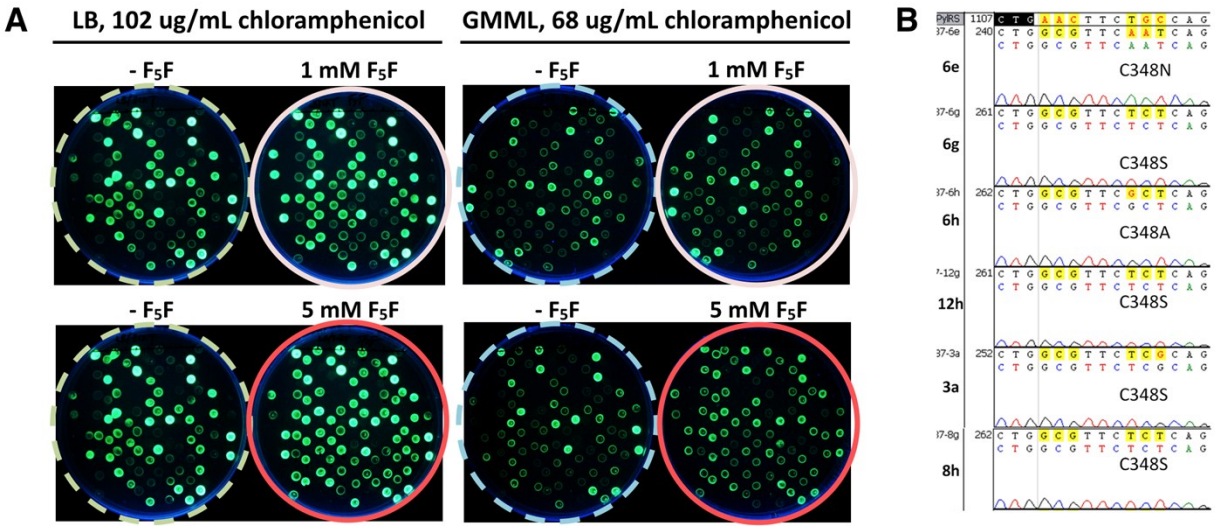
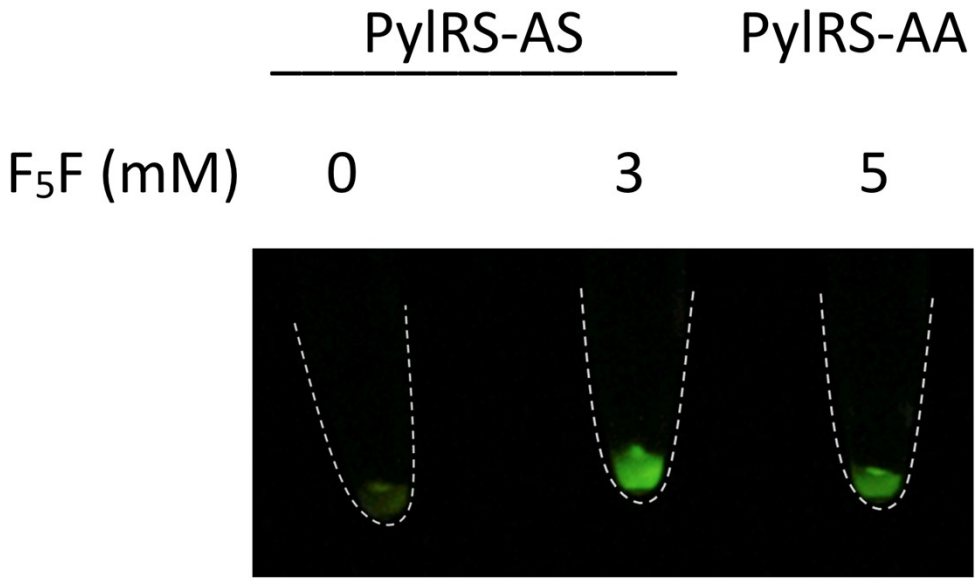


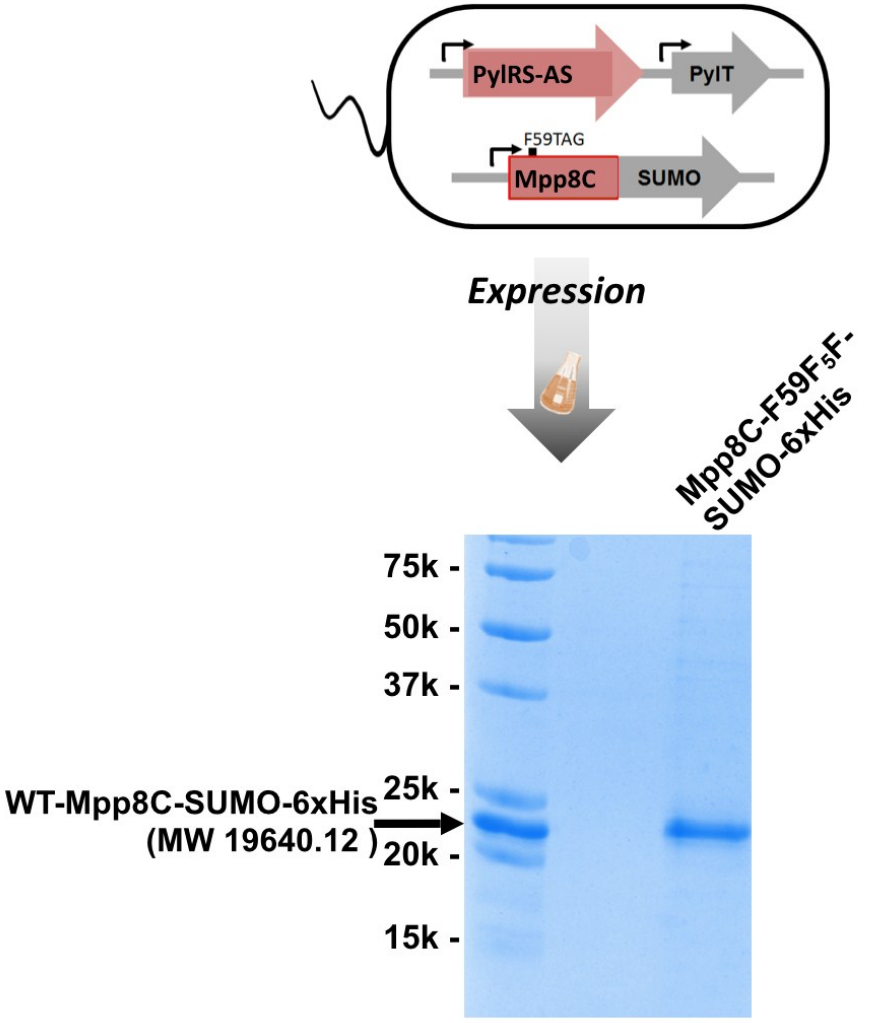
Supplementary Figure 1: PylRS-AA recognizes F_5F for its selective incorporation into the S2 position of sfGFP.



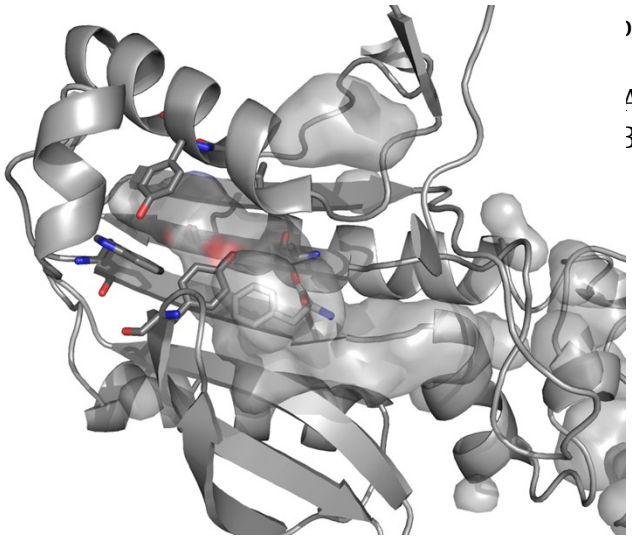
Supplementary Figure 2: Selections of clones that recognize F_5F. 3CKT means 3 times of regular used chloramphenicol concentration (34 μg/mL), 50 μg/mL kanamycin, and 25 μg/mL tetracycline.



Supplementary Figure 3: A small scale test to show that PylRS-AS performs better than PylRS-AA. BL21(DE3) cells were cotransformed with pEVOL-PylT-PylRS-AA or pEVOL-pylT-PylRS-AS and pET-PylT-sfGFP2TAG. Cells were grown in GMML supplemented with 3 mM F_5F for the PylRS-AS mutant and 5 mM F_5F for the PylRS-AA mutant.



Supplementary Figure 4: A diagram to illustrate the expression of fluorinated Mpp8C and the SDS-PAGE analysis of purified wild type and the F59F_5F mutant of Mpp8C.



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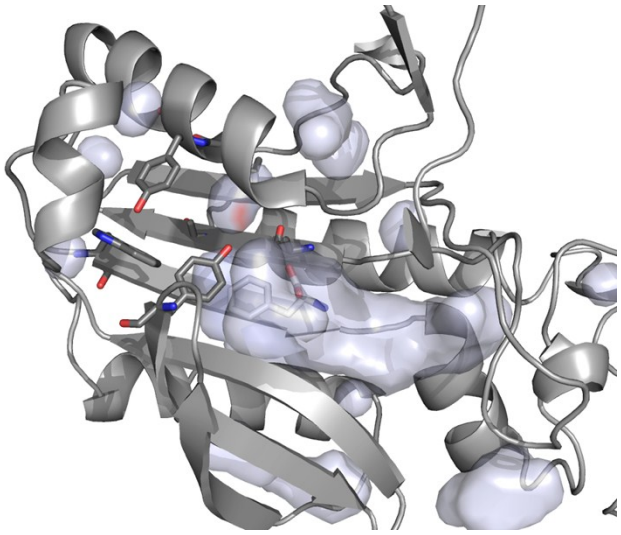

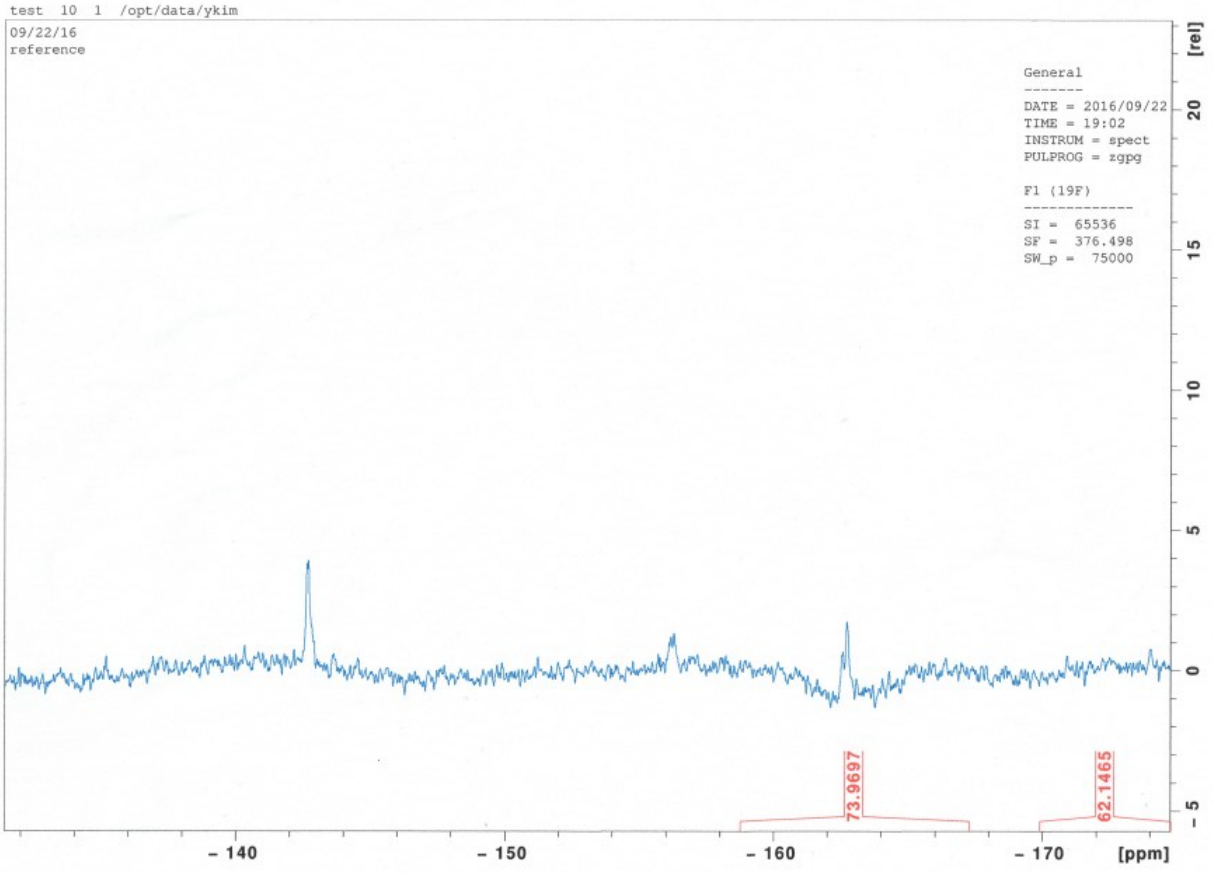

Supplementary Figure 6: ^{19}F NMR signals of Mpp8C-F_5F.