

## Supplemental Information

### Ideal bioorthogonal Reactions Using A Site-Specifically Encoded Tetrazine Amino Acid

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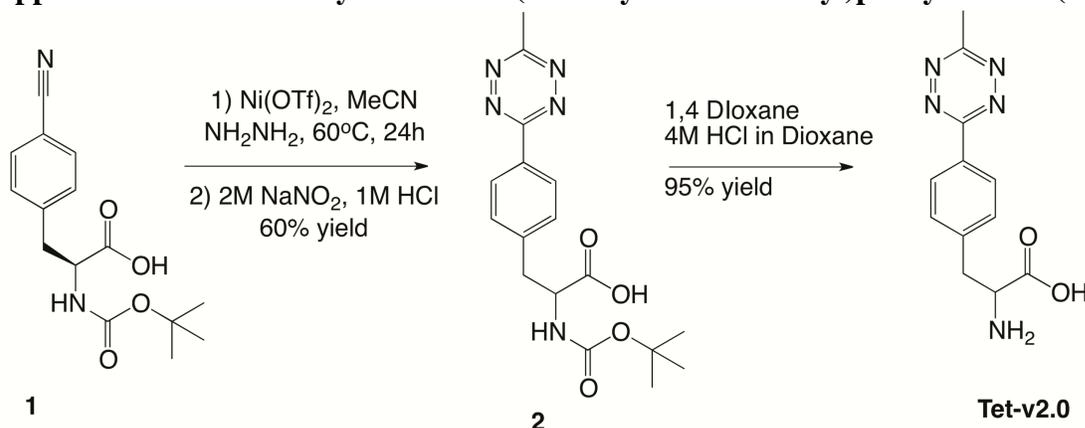
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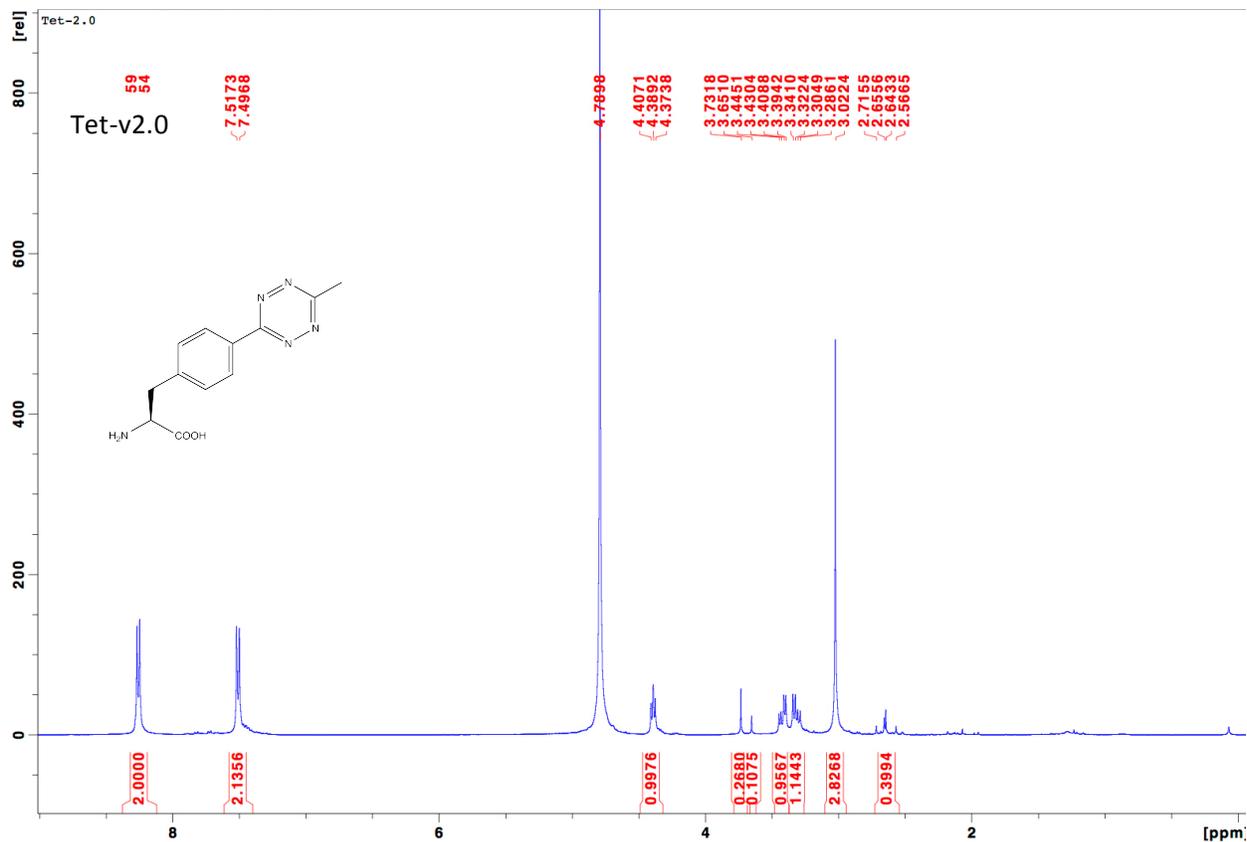
### Synthesis of 4-(6-methyl-s-tetrazin-3-yl)phenylalanine (Tet-v2.0)

#### Supplemental Scheme 1. Synthesis of 4-(6-methyl-s-tetrazin-3-yl)phenylalanine (Tet-v2.0)

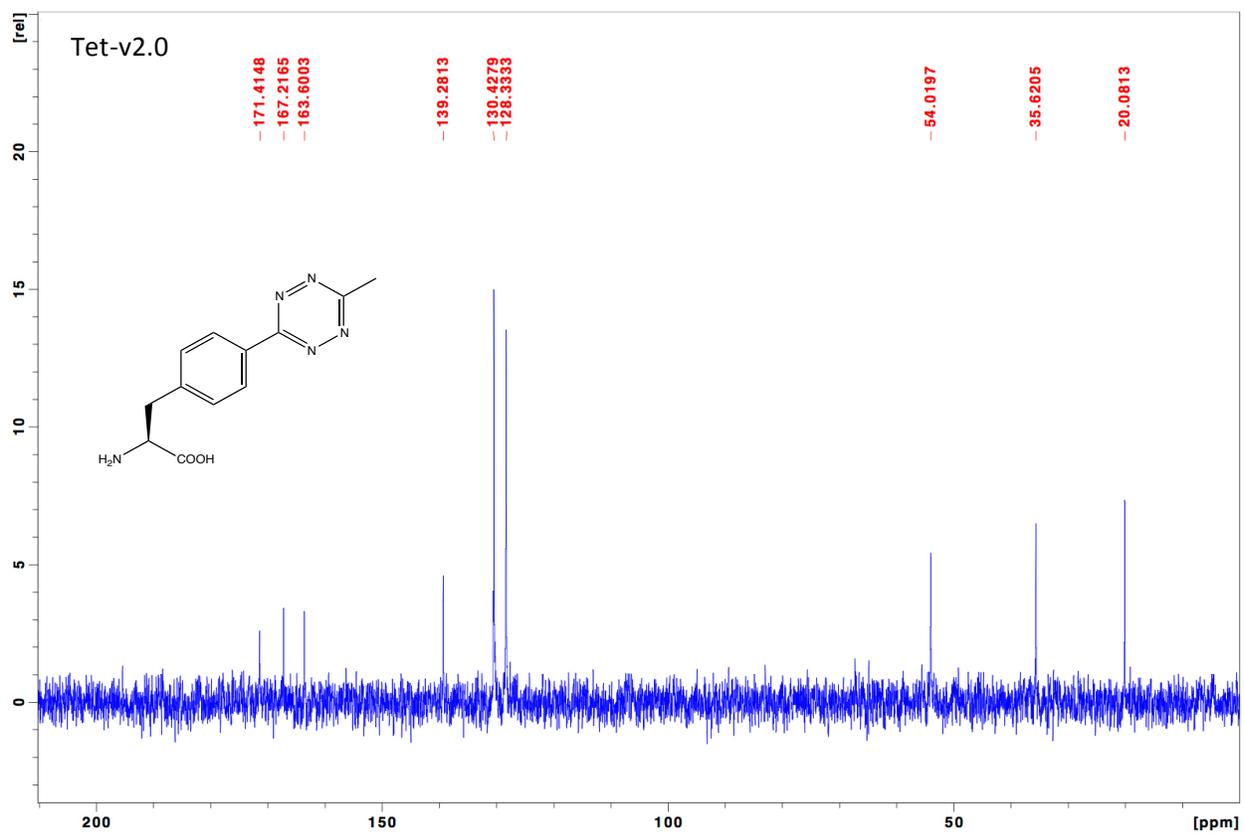


*N*-(*tert*-Butoxycarbonyl)-4-(6-methyl-1,2,4,5-tetrazin-3-yl)-*L*-phenylalanine (**2**)<sup>1</sup> A dry, 75 mL heavy walled reaction tube was equipped with a stir bar and was charged with **1** (500 mg, 1.72 mmol), Ni(OTf)<sub>2</sub> (307.3 mg, 0.86 mmol), and acetonitrile (1.8 mL, 34.4 mmol). The flask was purged with argon for 20 min. Anhydrous hydrazine was added to the mixture (2.7 mL, 86 mmol), the tube was sealed, and the reaction mixture was heated to 60°C for 24 hr. The reaction was allowed to cool to room temperature and slowly opened to air. Sodium nitrite (2 M, 8 mL) was added to the flask and the contents were cooled to 0°C. 1 N HCl was added slowly until gas evolution ceased and the pH of the mixture was acidic (~3). The mixture was then diluted with EtOAc and the layers separated. The aqueous layer was extracted with EtOAc (2x). The combined organic layers were washed with brine, dried with Na<sub>2</sub>SO<sub>4</sub>, and concentrated under reduced pressure. Silica gel flash column chromatography (50% ethyl acetate in hexanes with 1% acetic acid) yielded 370 mg of **2** (1.03 mmol, 60%) in the form of a red oil. R<sub>f</sub>=0.33 in 50% ethyl acetate in hexanes with 1% acetic acid; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.53 (d, 2H), 7.39 (d, 2H), 5.0 (d, 1H), 4.66 (m, 1H), 3.37-3.15 (dd, 2H), 3.09 (s, 3H), 1.39 (s, 9H).

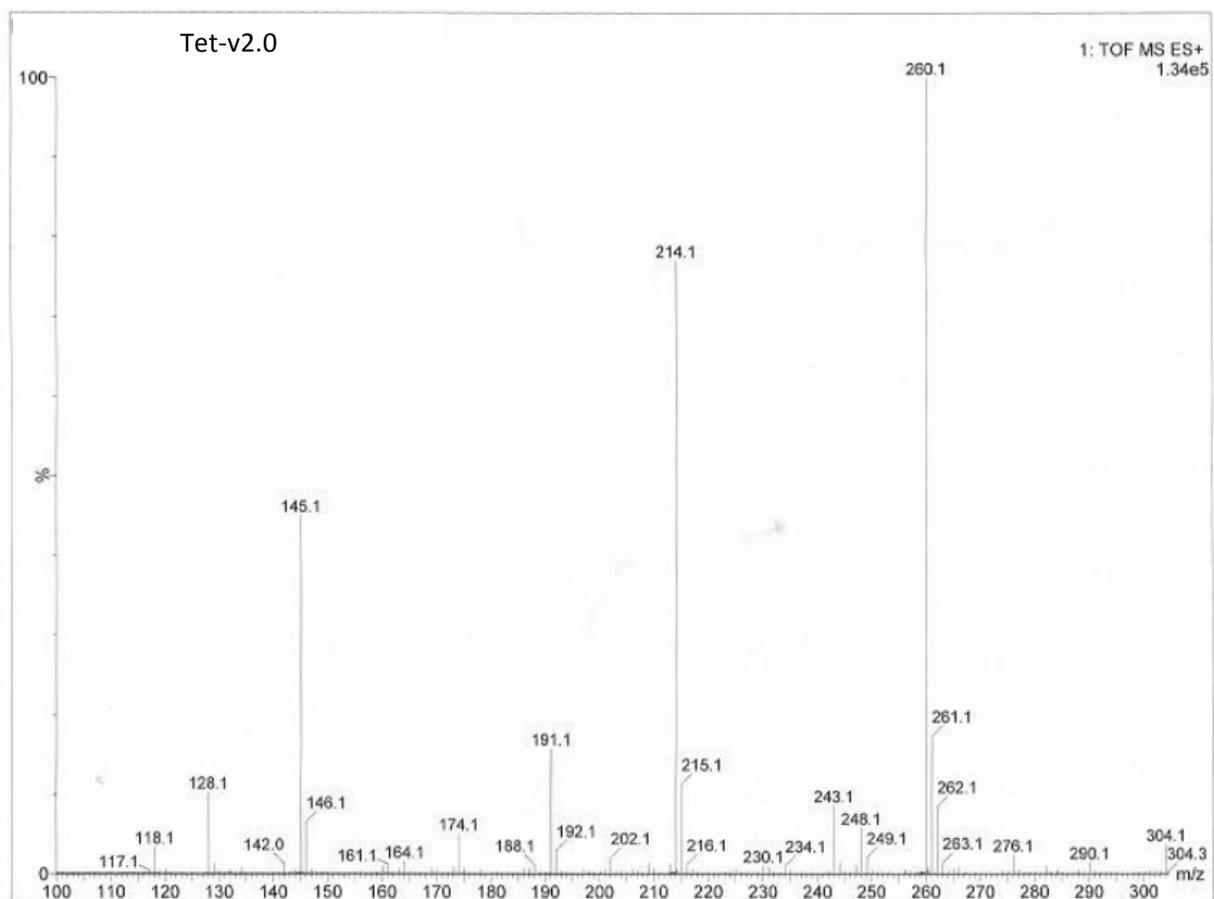
4-(6-methyl-1,2,4,5-tetrazin-3-yl)-L-phenylalanine hydrochloride salt (**3**) A dry 100 mL flask containing **2** (480 mg, 1.34 mmol) was charged with and then purged with argon for 10 min. This oil was dissolved in 4 mL of 1,4 dioxane then 4 M HCl in 1,4 dioxane (6 mL) was added over 20 sec. The reaction was allowed to stir for 12 hours at room temperature. The reaction was concentrated under reduced pressure, then dissolved in EtOAc, and concentrated down to a red powder. The red solid was washed with pentanes and collected via filtration to afford 377 mg of **Tet-v2.0** (1.18 mmol, 95%). <sup>1</sup>H NMR (400MHz, CD<sub>3</sub>OD) δ 8.48 (d, 2H), 7.48 (d, 2H), 4.29 (t, 1H), 3.41-3.20 (m, 2H), 2.97 (s, 3H) (Sup. Fig. 1).



Supplemental Figure 1. <sup>1</sup>H NMR of 4-(6-methyl-s-tetrazin-3-yl)phenylalanine (Tet-v2.0)



Supplemental Figure 2:  $^{13}\text{C}$  NMR of 4-(6-methyl-*s*-tetrazin-3-yl)phenylalanine (Tet-v2.0)



**Supplemental Figure 3. MS of 4-(6-methyl-s-tetrazin-3-yl)phenylalanine (Tet-v2.0)**

**Supplemental Table 1. Components for autoinducing and non-inducing mediums, for final volume of 500 mL.**

	A) Autoinduction medium	B) Non-inducing medium	C) Autoinducing plates
5% aspartate, pH 7.5	25 mL	25 mL	25 mL
10% glycerol	25 mL	-	25 mL
25× 18 amino acid mix	20 mL	20 mL	20 mL
50× M	10 mL	10 mL	10 mL
leucine (4 mg/mL), pH 7.5	5 mL	5 mL	5 mL
20% arabinose	1.25 mL	-	1.25 mL
1 M MgSO <sub>4</sub>	1 mL	1 mL	1 mL
40% glucose	625 μL	6.25 mL	125 μL
Trace metals	100 μL	100 μL	100 μL

### **Selection of aminoacyl-tRNA synthetases specific for Tet-v2.0**

The library of aminoacyl-tRNA synthetases was encoded on a kanamycin (Kn) resistant plasmid (pBK, 3000 bp) under control of the constitutive *Escherichia coli* GlnRS promoter and terminator. The aminoacyl synthetase library (3D-Lib) was randomized as follows: Leu65, His70, Gln155, and Ile159 were randomized to all 20 natural amino acids; Tyr32 was randomized to 15 natural amino acids (less Trp, Phe, Tyr, Cys, and Ile); Asp158 was restricted to Gly, Ser, or Val; Leu162 was restricted to Lys, Ser, Leu, His, and Glu; and Phe108 and Gln109 were restricted to the pairs Trp-Met, Ala-Asp, Ser-Lys, Arg-Glu, Arg-Pro, Ser-His, or Phe-Gln. The library plasmid, pBK-3D-Lib, was moved between cells containing a positive selection plasmid (pCG) and cells containing a negative selection plasmid (pNEG).

The positive selection plasmid, pCG (10000 bp), encodes a mutant *Methanococcus jannaschii* (Mj) tyrosyl-tRNA<sub>CUA</sub>, an amber codon-disrupted chloramphenicol acetyltransferase, an amber codon-disrupted T7 RNA polymerase, a T7 promoter controlled green fluorescent protein gene, and the tetracycline (Tet) resistance marker. The negative selection plasmid, pNEG (7000 bp), encodes the mutant tyrosyl-tRNA<sub>CUA</sub>, an amber codon-disrupted barnase gene under control of an arabinose promoter and rrnC terminator, and the ampicillin (Amp) resistance marker. pCG electrocompetent cells and pNEG electrocompetent cells were made from DH10B cells carrying the respective plasmids and stored in 100 μL aliquots at -80 °C for future rounds of selection.

The synthetase library in pBK-3D-Lib was transformed by electroporation into DH10B cells containing the positive selection plasmid, pCG. The resulting pCG/pBK-3D-Lib-containing cells were amplified in 1 L of 2×YT with 50 μg/mL Kn and 25 μg/mL Tet with shaking at 37 °C. The cells were grown to saturation, then pelleted at 5525 rcf, resuspended in 30 mL of 2×YT and 7.5 mL of 80% glycerol, and stored at -80 °C in 1 mL aliquots for use in the first round of selections.

For the positive selection, 2 mL of pCG/pBK-3D-Lib cells were thawed on ice before addition to 1.2 L of room temperature 2×YT media containing 50 μg/mL Kn and 25 μg/mL Tet. After incubation (11 h, 250 rpm, 37 °C), a 200 μL aliquot of these cells was plated on eleven 15 cm GMM-L-agar plates containing 50 μg/mL Kn, 25 μg/mL Tet, and 60 μg/mL chloramphenicol

(Cm). The positive selection agar medium also contained 1 mM **Tet-v2.0**. After spreading, the surface of the plates was allowed to dry completely before incubation (37 °C, 15 h). To harvest the surviving library members from the plates, 10 mL of 2×YT (50 µg/mL Kn, 25 µg/mL Tet) was added to each plate. Colonies were scraped from the plate using a glass spreader. The resulting solution was incubated with shaking (60 min, 37 °C) to wash cells free of agar. The cells were then pelleted, and plasmid DNA was extracted. For the first positive selection a Qiagen midiprep kit was used to purify the plasmid DNA. For all other plasmid purification steps a Thermo Scientific miniprep kit was used to purify the plasmid DNA. The smaller pBK-3D-Lib plasmid was separated from the larger pCG plasmid by agarose gel electrophoresis and extracted from the gel using the Thermo Scientific gel extraction kit.

The purified pBK-3D-Lib was then transformed into pNEG-containing DH10B cells. A 100 µL sample of pNEG electrocompetent cells was transformed with 50 ng of purified pBK-3D-Lib DNA. Cells were rescued in 1 mL of SOC for 1 h (37 °C, 250 rpm) and the entire 1 mL of rescue solution was plated on three 15 cm LB plates containing 100 µg/mL Amp, 50 µg/mL Kn, and 0.2% L-arabinose. Cells were collected from plates and pBK-3D-Lib plasmid DNA was isolated in the same manner as described above for positive selections.

In order to evaluate the success of the positive and negative selection based on variation in synthetase efficacy (as opposed to traditional survival/death results), the synthetases resulting from the selection rounds were tested with the pALS plasmid. This plasmid contains the sfGFP reporter with a TAG codon at residue 150 as well as tyrosyl-tRNA<sub>CUA</sub>. When a pBK plasmid with a functional synthetase is transformed with the pALS plasmid and the cells are grown in the presence of the appropriate amino acid on autoinduction agar, sfGFP is expressed and the colonies are visibly green.

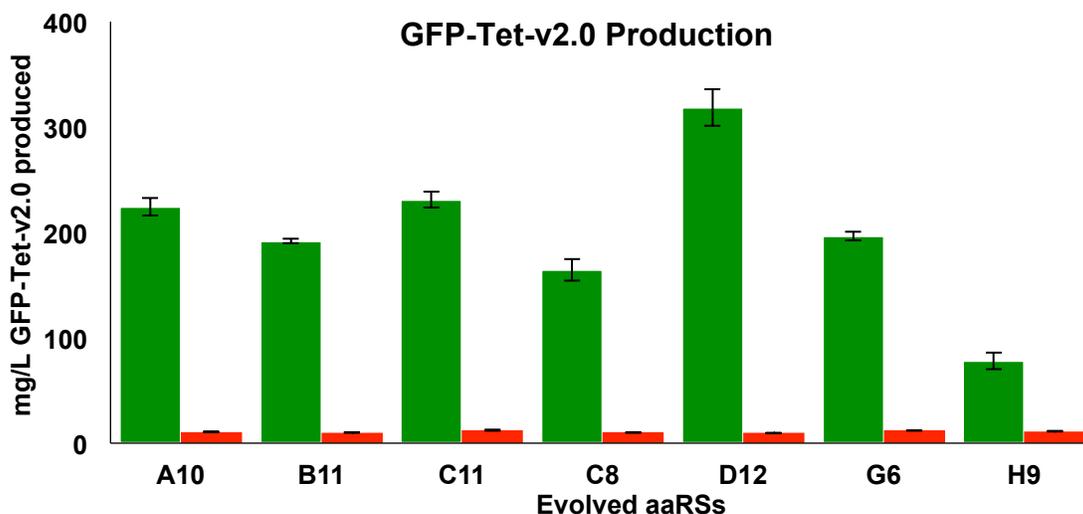
One microliter of each library resulting from the second positive and the second negative rounds of selection was transformed with 60 µL of pALS-containing DH10B cells. The cells were rescued for 1 hr in 1 mL of SOC (37 °C, 250 rpm). A 250 µL and 50 µL volume of cells from each library were plated on autoinducing minimal media with 25 µg/mL Kn, 50 µg/mL Tet, and 1 mM **Tet-v2.0**. Plates were grown at 37 °C for 24 hours and then grown on the bench top, at room temperature, for an additional 24 hours. Autoinducing agar plates were prepared by combining the reagents in Sup. Table 1C with an autoclaved solution of 40 g of agarose in 400 mL water. Sterile water was added to a final volume of 500 mL. Antibiotics were added to a final concentration of 25 µg/mL Tet and 50 µg/mL Kan.

A total of 96 visually green colonies were selected from the two 1 mM **Tet-v2.0** plates and used to inoculate a 96-well plate containing 0.5 mL per well non-inducing minimal media (Sup. Table 1B, with sterile water added to a final volume of 500 mL) with 25 µg/mL Kn, 25 µg/mL Tet. After 24 hours of growth (37 °C, 250 rpm), 5 µL of these non-inducing samples were used to inoculate 96-well plates with 0.5 mL autoinduction media (Sup. Table 1C, with sterile water added to a final volume of 500 mL) containing 25 µg/mL Kn, 25 µg/mL Tet with and without 1 mM **Tet-v2.0**.

Fluorescence measurements of the cultures were collected 36 hours after inoculation using a BIOTEK® Synergy 2 Microplate Reader. The emission from 528 nm (20 nm bandwidth) was summed with excitation at 485 nm (20 nm bandwidth). Samples were prepared by diluting suspended cells directly from culture 2-fold with phosphate buffer saline (PBS). Seven expressions showed high efficiency with **Tet-v2.0** and good fidelity without ncAA.

### Fluorescence analysis of highest-fluorescing clones

Non-inducing cultures (3 mL) with 25 µg/mL Kn and 25 µg/mL Tet were grown to saturation (37 °C with shaking at 250 rpm) from the top seven expressions in the 96 well plate analysis. Autoinduction cultures (5 mL) with 25 µg/mL Kn and 25 µg/mL Tet were inoculated with 30 µL of non-inducing cultures and grown with and without 1 mM **Tet-v2.0** at 37 °C with shaking at 250 rpm. After approximately 40 hours, fluorescence was assessed as described (Sup. Fig. 4). The top seven performing clones were sequenced revealing seven unique clones (Supplemental Table 2).



**Supplemental Figure 4.** Fluorescence measurements of seven synthetases with GFP ncAA-reporter. Green represents colonies induced in media containing 1 mM **Tet-v2.0**, while red represents colonies induced in the absence of UAA. Expressions of 500 µL were grown for 36 hours before dilution of suspended cells directly from culture 2-fold with phosphate buffer saline (PBS). Fluorescence measurements were collected using a BIOTEK® Synergy 2 Microplate Reader.

Mj Tyr parent	Tyr32	Leu65	Phe108	Gln109	Asp158	Leu162
A10	Ala	Ala	Glu	His	Gly	Ser
B11	Ala	Ser	Gln	Glu	Gly	Ala
C8	Ala	Ala	His	Ser	Ser	Gly
C11	Ala	Val	Asp	His	Gly	Ser
<b>D12</b>	<b>Gly</b>	<b>Gln</b>	<b>Ser</b>	<b>Asp</b>	<b>Ser</b>	<b>Asn</b>
E12	Ala	Ala	Leu	Pro	Gly	Gly
G6	Ala	Ser	Ala	Glu	Asn	Ala
H9	Ala	Ser	Gln	Asp	Ser	Ala

**Supplemental Table 2. Sequence of top performing Tet-RSs.** The D12 synthetase (bold) was moved into the pDule plasmid for protein expression.

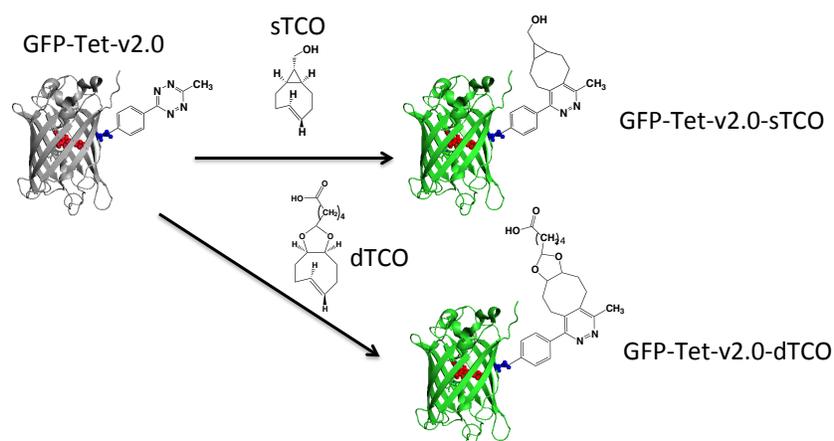
## Generation of pDule-tet-v2.0

The top performing Tet-RS was moved from the *pBK-D12* plasmid to the pDule plasmid (*pDule-tet2.0*). The *pDule* plasmid was generated by amplifying the *MjYRS* gene from the *pBK* plasmid isolated from the library using primers RSmovf (5'-CGCGCGCCATGGACGAATTTGAAATG-3') and RSmover (5'-GACTCAGTCTAGGTACCCGTTTGAAACTGCAGTTATA-3'). The amplified DNA fragments were cloned into the respective sites on the *pDule* plasmids using the incorporated NcoI and KpnI sites.

## Expression and Purification of GFP-Tet-v2.0

DH10B *E. coli* cells co-transformed with *pDule-tet2.0* and *pBad GFP-TAG150* vector were used to inoculate 5 mL of non-inducing medium containing 100 µg/mL Amp and 25 µg/mL Tet. The non-inducing medium culture was grown to saturation with shaking at 37 °C, and 1 mL was used to inoculate 0.1 L autoinduction medium with 100 µg/mL Amp and 25 µg/mL Tet, and 1 mM Tet-v2.0 (0.1 L of media grown in 500 mL plastic baffled flasks). After 40 hours of shaking at 37 °C, cells were collected by centrifugation. Standard expression aeration is important to maintain appropriate tetrazine proteins oxidation state labeled in vivo and in vitro because of redox cycling detected NMR study.

The protein was purified using BD-TALON cobalt ion-exchange chromatography. The cell pellet was resuspended in wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7) and lysed using a microfluidizer (final volume 35 mL). The lysate was clarified by centrifugation, applied to 0.5 mL bed-volume resin, and bound for 20 min. Bound resin was washed with >50 volumes wash buffer. Protein was eluted from the bound resin with 2.0 mL of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole pH 7) until the resin turned pink and the color of the eluent the column was no longer green. The elution concentrations were checked with a Bradford protein assay. The protein was desalted into PBS pH 7.4 using PD10 columns.



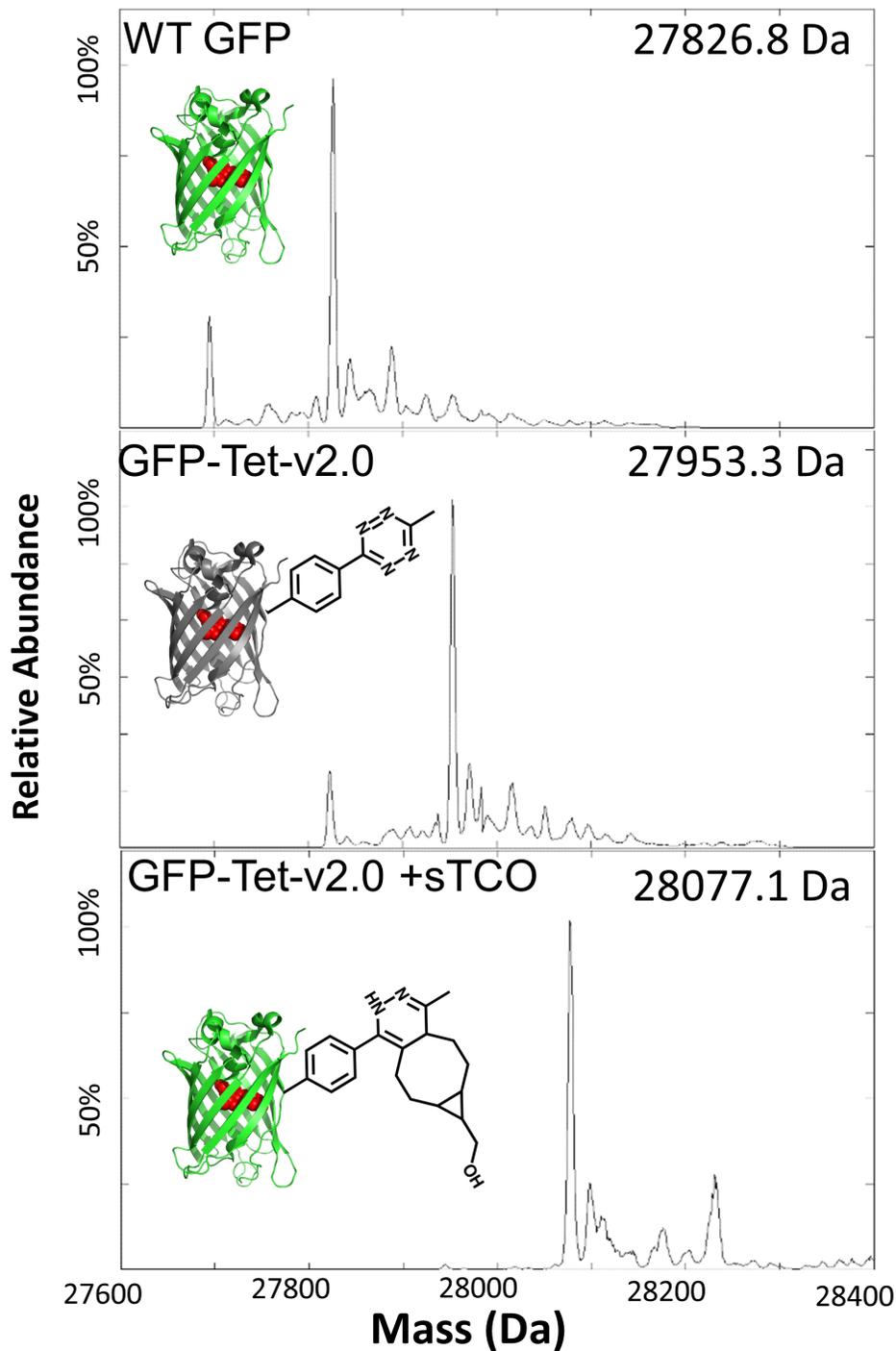
## Supplemental Scheme 2. Reaction of GFP-Tet-v2.0 with sTCO and dTCO.

## Rapid in vitro labeling of Tet-v2.0 containing protein with sTCO and dTCO

Pure GFP-Tet-v2.0 at 3  $\mu\text{M}$  in 20 mM ammonium acetate pH 7 was incubated with dioxilane fused trans cyclooctene (dTTCO) at a final concentration of 39  $\mu\text{M}$ . The reaction was run for 5 min at room temperature before desalting with PD10 columns into 20 mM ammonium acetate pH 7. These samples were frozen and vacuum dried. WT GFP and GFP-Tet-v2.0 with no addition of **dTTCO** were run in parallel as controls.

#### **Rapid *in vivo* labeling of Tet-v2.0 containing protein with sTCO**

DH10B cells in 50 mL autoinduction media expressing cytosolic GFP-Tet-v2.0 were pelleted at 2000 rcf for 5 min. The cells were washed 3 times with 5 mL PBS. Aliquots of 1 mL cells were centrifuged (2000 rcf, 5 min) and stored at  $-80\text{ }^{\circ}\text{C}$ . Cells were thawed on ice and resuspended in 5 mL PBS. The cells were incubated at  $37\text{ }^{\circ}\text{C}$  for 3 hours with heavy aeration. A stock solution of **sTCO** was prepared in MeOH with a concentration of 1.0 mM. Cells were diluted in a cuvette (100  $\mu\text{L}$  cells to 2.9 mL PBS). The fluorescence of the reaction was monitored (488 nm excitation, 509 nm emission, 5 mm slit width). Excess addition of sTCO was performed as a positive control (50  $\mu\text{L}$  1.0 mM). The first three sub-stoichiometric additions (3  $\mu\text{L}$  1.0 mM) were allowed 3 min to equilibrate before 250  $\mu\text{L}$  of buffer was removed to assess the sTCO concentration. After 5 min the subsequent addition was performed. The fourth addition of sTCO consisted of an excess of sTCO (41  $\mu\text{L}$  1.0 mM). Control additions of sTCO and subsequent removal were performed for GFP TAG150 cells grown in the absence of Tet-v2.0 and for PBS buffer alone.



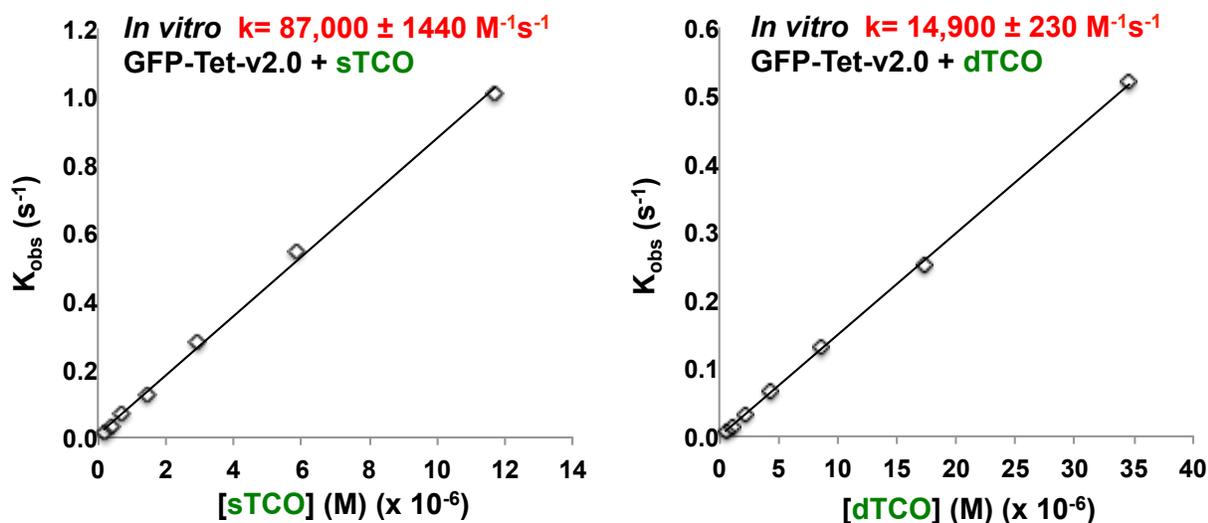
**Supplemental Figure 5. *In vitro* incubation of GFP-Tet-v2.0 with sTCO.** ESI-MS of proteins; GFP-Tet-v2.0, GFP-Tet-v2.0-sTCO, and GFP-Tet-v2.0-dTCO, demonstrates specific and quantitative labeling of GFP-Tet-v2.0 and no background labeling of WT GFP. A: ESI-MS TOF analysis of WT GFP shows a single major peak at 27826.8 Da  $\pm$  1 Da (expected 27827.3) B: ESI-MS-TOF analysis of GFP-Tet-v2.0 shows a single major peak at 27953.3 Da  $\pm$  1 Da which is in

agreement with the expected mass (27954.5 Da). C: ESI-MS-TOF analysis of GFP-Tet-v2.0-sTCO shows a single major peak at 28077.1 Da  $\pm$  1 Da (expected 28078.7 Da). This shows the expected molecular weight difference of 124.2 Da from GFP-Tet-v2.0 demonstrating specific and quantitative conversion to GFP-Tet-v2.0-sTCO. C: ESI-MS-TOF analysis of GFP-Tet-v2.0 incubated with dTCO shows a single major peak at 28181.9 Da  $\pm$  1 Da (expected 28180.7 Da). This shows the expected molecular weight difference of 226.2 Da from GFP-Tet-v2.0 demonstrating specific and quantitative conversion to GFP-Tet-v2.0-dTCO. Each sample did show a small peak at -131  $\pm$  1 Da indicating minor amounts of peptidase-based removal of N-terminal methionines and +22 sodium adducts.

### MS Analysis of GFP-Tet-v2.0, GFP-Tet-v2.0-sTCO, GFP-Tet-v2.0-dTCO.

Purified WT GFP and GFP-Tet-v2.0 were diluted to a concentration of 1 mg/mL and reacted with  $\sim$ 5 equivalents of sTCO overnight. Protein was desalted on Millipore C<sub>4</sub> zip tips and analyzed using an FT LTQ mass spectrometer at the Oregon State University mass spectrometry facility (Sup. Fig. 5).

Cells that expressed GFP Tet-v2.0 were reacted with  $\sim$ 5 equivalents of sTCO overnight. Protein was purified as described above. Protein in elution buffer was desalted on Millipore C<sub>4</sub> zip tips and analyzed using an FT LTQ mass spectrometer at the Oregon State University mass spectrometry facility (Sup. Fig. 5).



### Supplemental Figure 6. *In vitro* kinetic analysis of GFP-Tet-v2.0 with sTCO and dTCO.

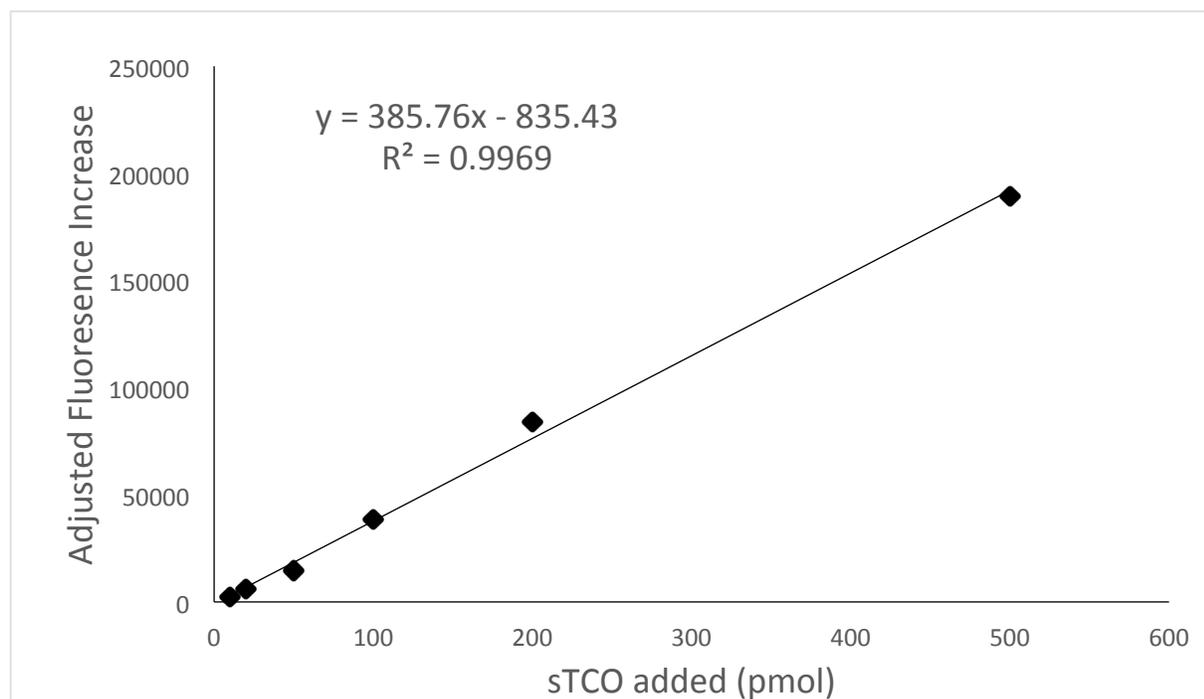
Determined first order rate constants were plotted against sTCO or dTCO concentrations. Standard curves were fit to the data and 2<sup>nd</sup> order rate constants were calculated.

Seven stock solutions of sTCO in methanol were prepared (0.585 mM, 0.293 mM, 0.146 mM, 0.0731 mM, 0.0366 mM, 0.0183 mM, and 0.00914 mM). Solid dTCO was dissolved in methanol

to generate seven stock dilutions (1.73 mM, 0.864 mM, 0.432 mM, 0.216 mM, 0.108 mM, 0.054 mM, 0.027 mM). Kinetic trials containing 30  $\mu\text{L}$  of GFP-Tet-v2.0 (1  $\mu\text{M}$  in PBS) in 2.5 mL of 1x PBS buffer at 21°C were initiated by adding 50  $\mu\text{L}$  of an sTCO or dTCO stock solution. Reactions were monitored by observing the fluorescence increase from product formation (Excitation 488 nm with a 1.25 nm slit, Emission 510 nm with a 4 nm slit). Fluorescence measurements for each trial were run until a constant emission intensity was reached indicating a completed reaction. A unimolecular rate constant was obtained for each concentration and all seven unimolecular rate constants were used to obtain a bimolecular rate constant (Sup. Fig. 6).

#### ***In vivo* kinetic analysis of GFP-Tet-v2.0 with sTCO.**

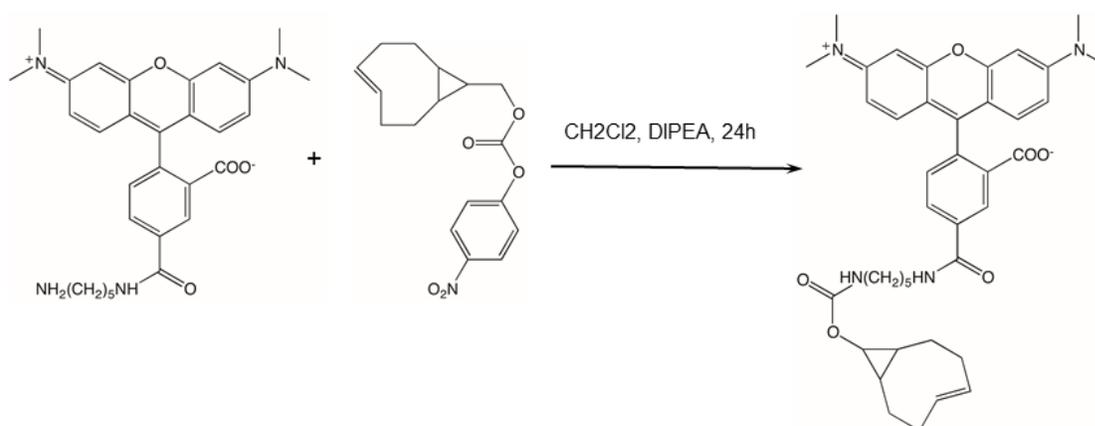
A 50 mL GFP-150-Tet-v2.0 cell pellet was resuspended and washed in 50 mL PBS buffer three times. Six kinetic trials containing 100  $\mu\text{L}$  of the cell solution added to 2.85 mL of 1x PBS buffer were initiated by adding 50  $\mu\text{L}$  of one of six stock solutions of sTCO (613, 306, 153, 76.6, 38.3, 19.1  $\mu\text{M}$ ). Kinetics trials were stirred continuously and monitored by observing the fluorescence increase from product formation (Excitation 488 nm with 2 nm slit, Emission 506 nm with 5 nm slit, 0.1 sec integration time, and 2 sec increments). Fluorescence measurements for each trial were run until a constant emission intensity indicative of a complete reaction was obtained. A unimolecular rate constant was obtained for each concentration and all six unimolecular rate constants were used to obtain a bimolecular rate constant.



**Supplemental Figure 7. Calibration Curve for Determining sTCO Concentration.** The adjusted fluorescence increase of additions of sTCO to GFP-Tet-v2.0 were plotted against the amount of sTCO. Unknown concentrations of sTCO were then added and the resulting adjusted fluorescence increase was used to determine the concentration of the unknown values.

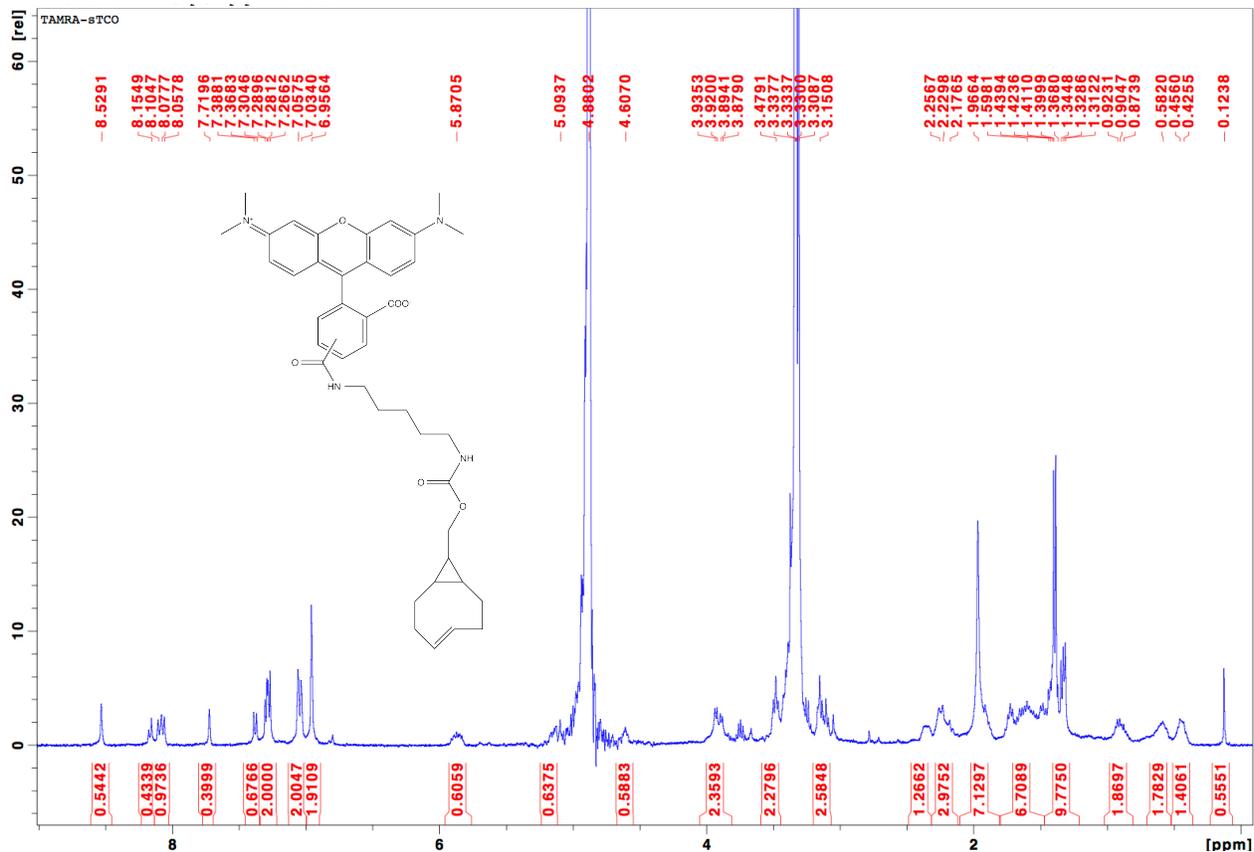
### Assay of sTCO concentration in supernatant of sub-stoichiometric additions

The buffer samples from the sub-stoichiometric reactions described above were centrifuged (2000 rcf, 5 min) and 200  $\mu\text{L}$  of supernatant was removed for analysis. To assay the concentration the supernatant was reacted with excess purified GFP-Tet-v2.0 and the fluorescence increase of the GFP-Tet-v2.0 was compared to a standard curve. To generate the standard curve 10  $\mu\text{L}$  GFP-Tet-v2.0 was added to 2990  $\mu\text{L}$  PBS. Known amounts of pure sTCO (10-1000 pmol) were added in volumes of 10-50  $\mu\text{L}$ . The increase in fluorescence of the GFP-Tet-v2.0 was measured and the dilution factor was accounted for to result in an adjusted fluorescence increase. The quantity of sTCO was plotted against the adjusted increase and the data were fit with a least squares regression line ( $R^2 = 0.9969$ ) (Sup. Fig. 7). The adjusted fluorescence increases were measured for 20-50  $\mu\text{L}$  additions of supernatant from the PBS, GFP-Tet-v2.0 cells, and the minus amino acid cells. GFP-Tet-v2.0 cells had significant background due to trace amounts of GFP in the supernatant. The background was accounted for by subtracting the adjusted fluorescence increase from addition of the supernatant to 3000  $\mu\text{L}$  PBS buffer alone. The standard curve was used to determine quantities and concentrations of sTCO present in the supernatants and error was determined as the error in the standard curve.



### Supplemental Scheme 3: Synthesis of TAMRA linked sTCO

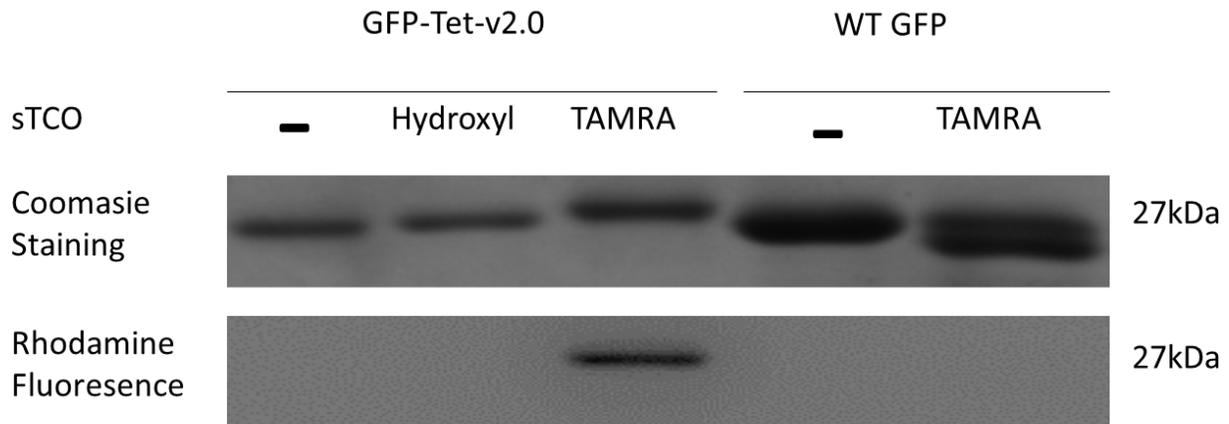
A dry 25 mL pear shaped flask was charged with (19.7 mg, 31.3  $\mu\text{mol}$ ) tetramethylrhodamine 5-(and 6)-carboxamide cadaverine TFA salt that was partially dissolved in anhydrous dichloromethane (1 mL, 15 mmol). Diisopropylethylamine (20.6  $\mu\text{L}$ , 0.118 mmol) was added and resulted in the complete dissolution of tetramethylrhodamine 5-(and 6)-carboxamide cadaverine. A solution was prepared of (activated sTCO) (18.8 mg, 59.2  $\mu\text{mol}$ ) in dichloromethane (1 mL, 15 mmol) and was added to the mixture. The reaction was allowed to proceed at room temperature under argon for 16 hours. The mixture was dried under reduced pressure and purified using silica gel flash column chromatography (9:1 dichloromethane:methanol) to yield TAMRA-sTCO (13.2 mg, 61%), a red solid.  $^1\text{H}$  NMR (400MHz,  $\text{C}_2\text{D}_6\text{OS}$ )  $^1\text{H}$  NMR(400MHz,  $\text{DMSO D}_6$ )  $\delta$ - 8.82 (t, 1H), 8.69 (t, 1H), 8.44 (s, 1H), 8.22 (d, 1H), 8.17 (d, 1H), 8.06 (d, 1H), 7.63 (s, 1H), 7.32 (d, 1H), 7.08 (m, 2H), 5.776 (m, 2H), 5.067 (m, 2H) (Sup. Fig. 8). MS (ESI)  $[\text{M}+\text{H}]$  calcd. for  $\text{C}_{41}\text{H}_{49}\text{N}_4\text{O}_6+$ ,  $[\text{M}+\text{H}]$ : 693.36 ; found: 693.40.



**Supplemental Figure 8. <sup>1</sup>H NMR of TAMRA-sTCO**

### SDS-PAGE analysis of GFP-Tet-v2.0 incorporation and reactivity

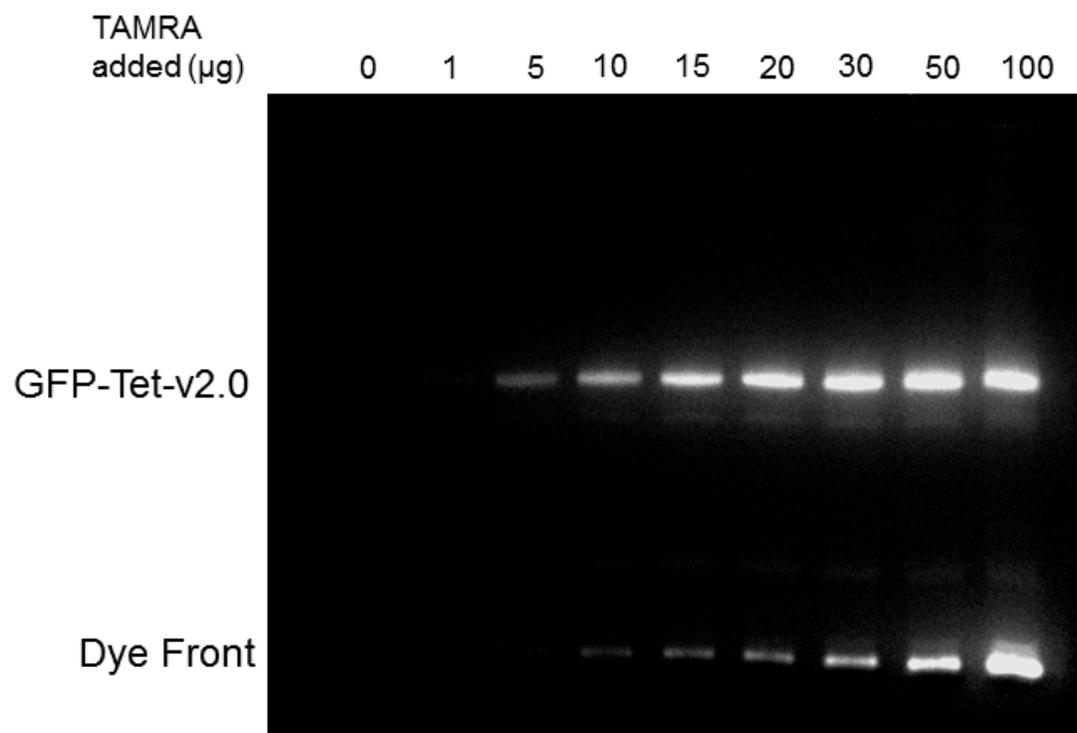
DH10B cells expressing GFP-Tet-v2.0 from 50 mL autoinducing media with 1 mM Tet-v2.0 were washed 3 times with 5 mL PBS and resuspended in 5 mL PBS. The cells were incubated at 37°C for 3 hours. Cells were aliquotted into 1 mL samples and centrifuged (2000 rcf, 5 min) and stored at -80°C. Similarly, WT-GFP expressed cells and cells expressed in the absence of Tet-v2.0 were also aliquotted and centrifuged. TAMRA-sTCO (173  $\mu$ L, 1 mM) was added to a WT-GFP and GFP-Tet-v2.0 aliquot and allowed to react overnight. Additionally, sTCO was added to an aliquot of GFP-Tet-v2.0 and allowed to react overnight. The aliquots were subsequently lysed and purified using BD-TALON cobalt ion exchange chromatography eluting into 0.5 mL. Equal volumes of purified protein were mixed with 2x Laemmli Buffer and heated at 95°C for 20 min. Samples were analyzed using SDS-PAGE (7  $\mu$ L sample, 15% Acrylamide gel, 180 V, 70 min) (Sup. Fig. 9). The gel was fluorescently imaged prior to staining with Coomassie. After staining and destaining, the gel was imaged again and the fluorescent image was aligned with the Coomassie-stained image using the ladders visible in the unstained image. Fluorescent signal from the TAMRA label was only present when the dye was reacted with GFP-Tet-v2.0.



**Supplemental Figure 9. SDS-PAGE analysis of GFP-Tet-v2.0 reactivity.** Reaction of TAMRA-sTCO with GFP-Tet-v2.0 resulted in a slight upward gel shift in comparison to the unreacted and sTCO reacted forms. In addition a fluorescent band was visible for the TAMRA-sTCO reacted GFP-Tet-v2.0. No shift was present or fluorescence was observed for WT GFP incubated with TAMRA-sTCO.

#### TAMRA labeling of GFP-Tet-v2.0 in cell lysate

GFP-Tet-v2.0 expressing *E. coli* cells were resuspended in PBS and lysed. The lysate was clarified via centrifugation (21036 rcf, 45 min) and the supernatant was decanted and stored at 4°C. A mixture of protease inhibitors (400  $\mu$ L) were added to 20 mL of supernatant. The clarified lysate was divided into 8 x 1 mL aliquots. TAMRA-sTCO (1 mg/mL in methanol, 0-100  $\mu$ L) was added to each aliquot. Each sample was allowed to react for 1 hr at room temperature. The samples were mixed with 2x Laemmli Buffer and heated at 95°C for 20 min. The samples were analyzed via SDS-PAGE (10  $\mu$ L sample, 15% Acrylamide gel, 200V, 50 min)(Sup. Fig. 10). The dye front was not allowed to run off the gel. Samples were fluorescently imaged prior to staining with Coomassie.

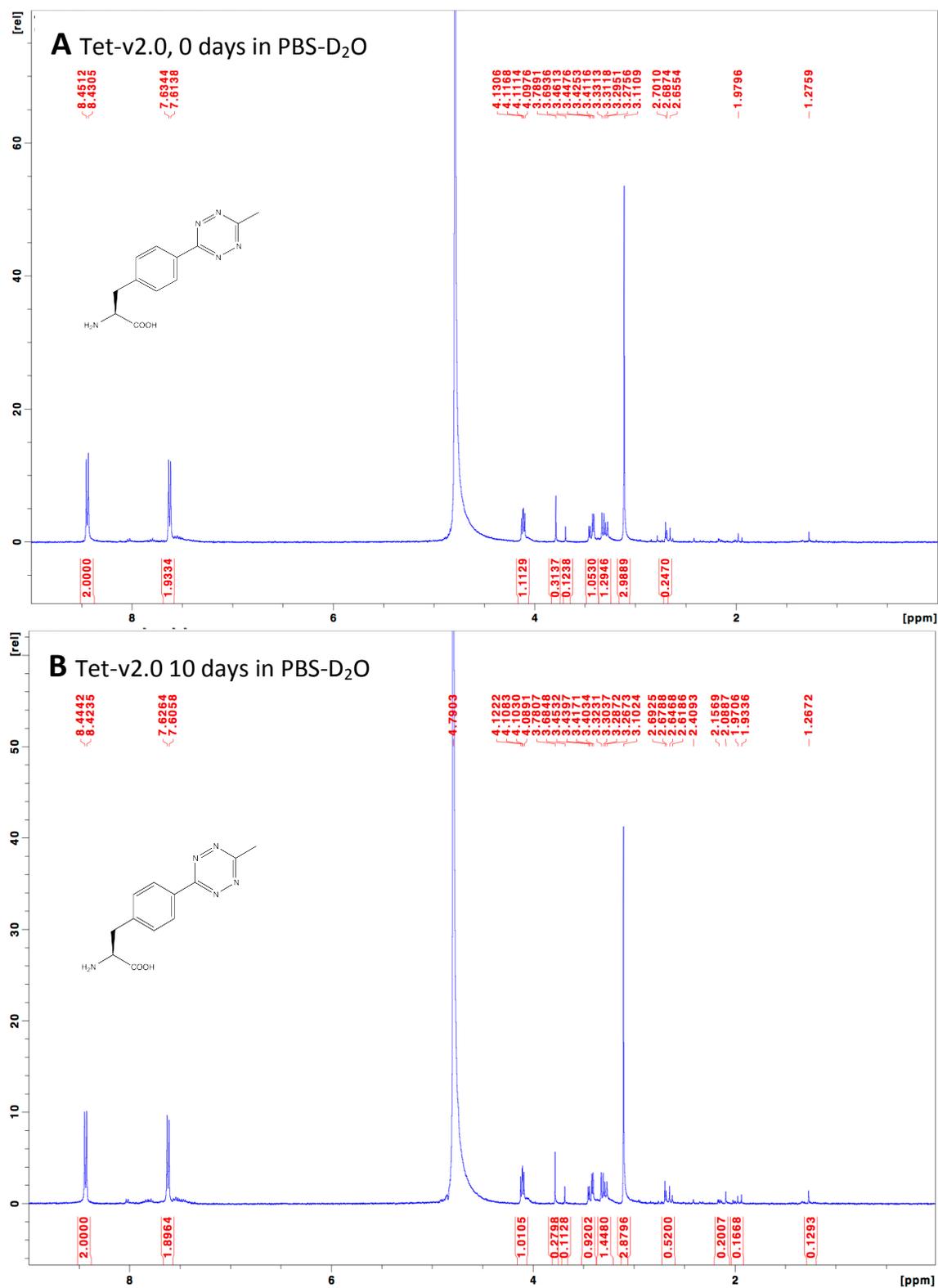


**Supplemental Figure 10. Fluorescent SDS-PAGE analysis of GFP-Tet-v2.0 reaction.**

Reaction of TAMRA-sTCO with GFP-Tet-v2.0 resulted in two prominent fluorescent bands. The bands correspond to migration of TAMRA-sTCO reacted with GFP-Tet2.0 and unreacted TAMRA-sTCO. The low background signal detected at the dye front in the sub-stoichiometric reactions likely resulted from incomplete purification of TAMRA-sTCO from isomerized cyclopropane-fused cis-cyclooctene-linked-TAMRA and unreacted TAMRA starting materials.

**Stability assessment of Tet-v2.0 in aqueous solutions.**

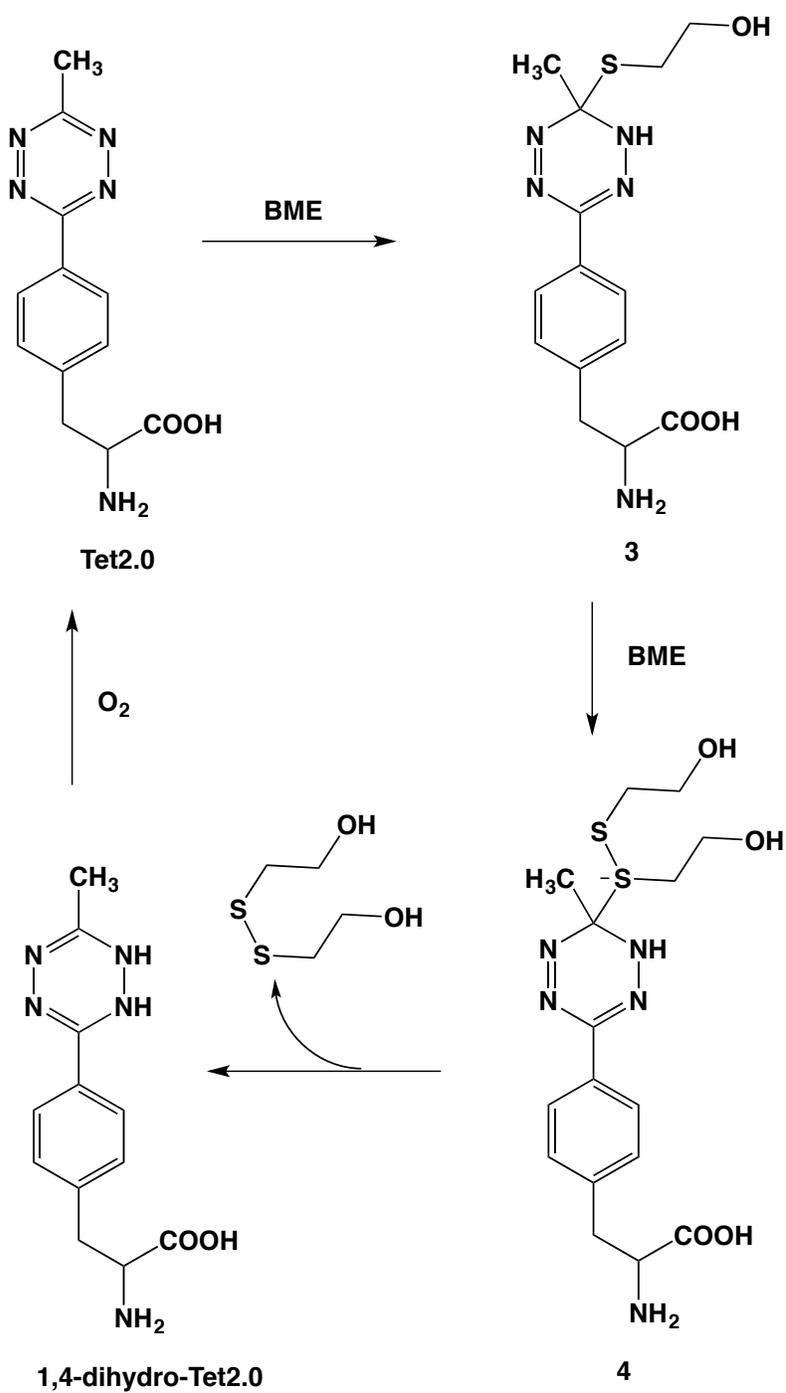
A solution of Tet-v2.0 (5 mM) in deuterated PBS was created.  $^1\text{H}$  NMRs were obtained after 0 and 10 days. The sample showed no significant degradation over time (Sup. Fig. 11).



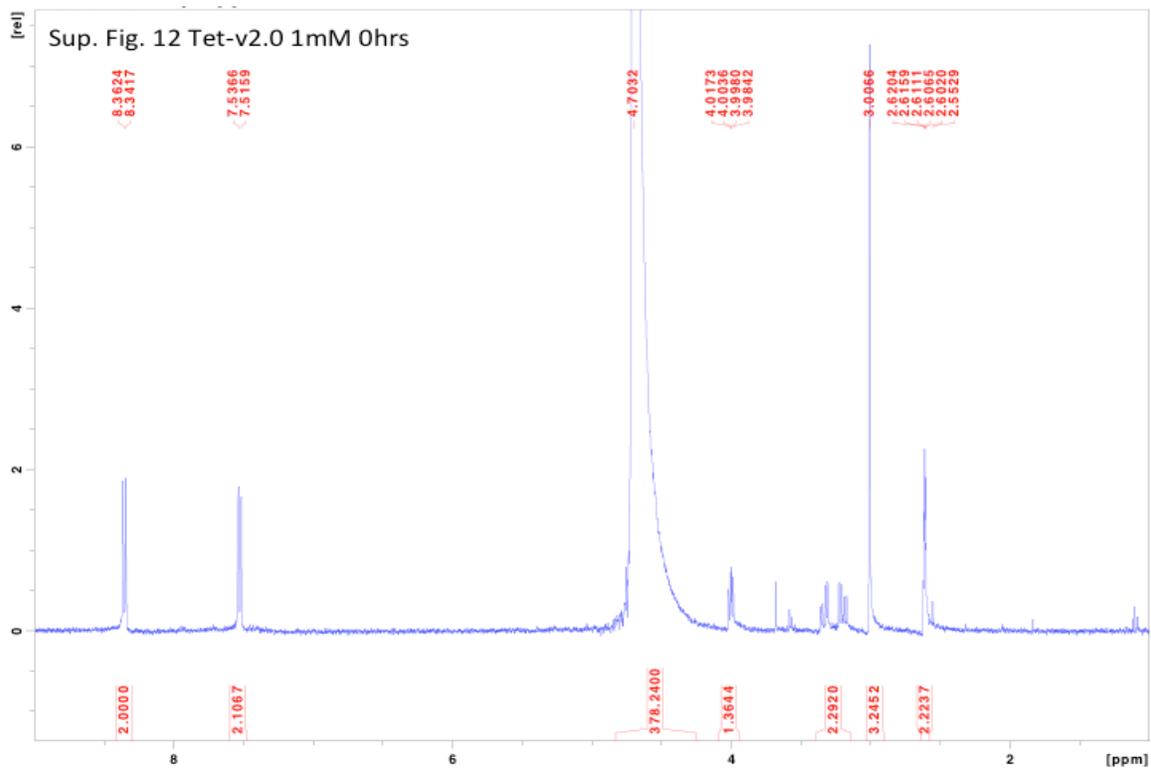
**Supplemental Figure 11. <sup>1</sup>H NMR of Tet-v2.0 in dPBS (A) <sup>1</sup>H NMR of Tet-v2.0 upon dissolution in deuterated PBS. (B) <sup>1</sup>H NMR of Tet-v2.0 in deuterated PBS after 10 days. Tet-v2.0 shows no significant degradation after 10 days.**

### **H<sup>1</sup> NMR analysis of tetrazine reactivity with thiols.**

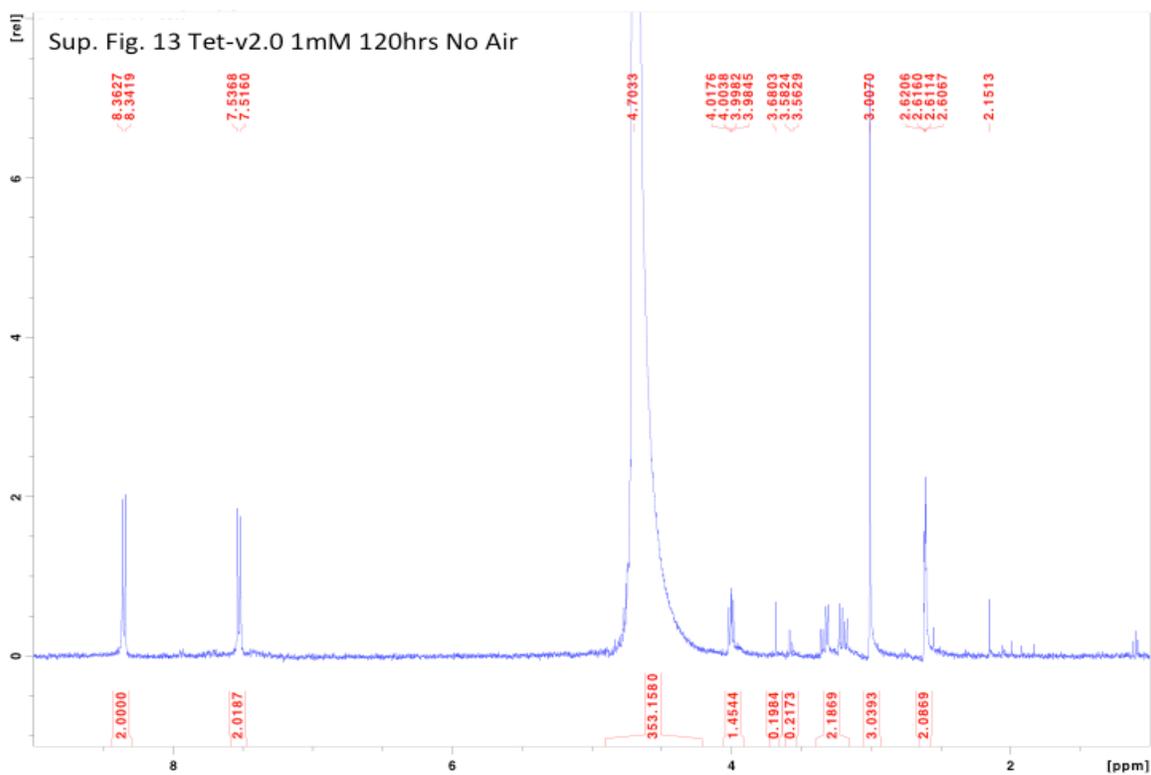
In addition to measuring Tet-v2.0 stability in aqueous solution (Sup. Fig. 11, 12-14), we reacted Tet-v1.0 and Tet-v2.0 with thiols and assessed potential side reactions. Initially, we dissolved Tet-v1.0 and Tet-v2.0 in a 1 mM solution of phosphate buffered D<sub>2</sub>O (40 mM, pH 7.4) (Sup. Fig. 12, 15) and reacted them with 1 mM 2-mercaptoethanol (Sup. Fig. 18, 21). The reaction was set up under both oxygen rich (3 mL of solution in a 25 mL test tube shaking at 120 rpm) and oxygen deficient (inside a sealed NMR tube) conditions. The concentrations used were selected as they match the concentration of tetrazines used in media during protein expression. The reactions were monitored over the course of 5 days (120 hours) and no side products were identified (Sup. Fig. 19, 20, 22, 23). However, in both solution with Tet-v1.0 and Tet-v2.0 the 2-mercaptoethanol was found to oxidize to 2-hydroxyethyl disulfide more rapidly than observed in absence of tetrazine (Sup. Fig. 24-26). This suggests that Tet-v1.0 and Tet-v2.0 could be catalyzing the oxidation of 2-mercaptoethanol. To investigate further we increased the concentration of Tet-v2.0 and 2-mercaptoethanol (Sup. Fig. 27). The concentration of Tet-v2.0 was fixed at 5 mM while the concentration of 2-mercaptoethanol was varied in the range 3-20 mM. The NMRs corresponding to Tet-v2.0 reaction with 20 mM 2-mercaptoethanol are shown as representative of all concentrations (Sup. Fig. 27-30). Tet-v1.0 could not be obtained at higher concentrations due to insufficient solubility in water. Under oxygen deficient conditions at higher concentrations, Tet-v2.0 was found to react with 2-mercaptoethanol to form dihydro-Tet-v2.0 (Sup. Fig. 28). In contrast under oxygen rich conditions, no dihydro-Tet-v2.0 was isolated, but all 2-mercaptoethanol (4 equivalents) was oxidized to 2-hydroxyethyl disulfide (Sup. Fig. 29). This suggests that Tet-v2.0 is reduced by 2-mercaptoethanol to form dihydro-Tet-v2.0 and subsequently oxidized by oxygen to reform Tet-v2.0. Under oxygen deficient conditions the dihydro-Tet-v2.0 product cannot be oxidized leading to its buildup in solution and detection in the NMR analysis. When the dihydro-Tet-v2.0 is moved to oxygen rich conditions the tetrazine is reoxidized and Tet-v2.0 is reformed. After 24 hours all dihydro-Tet-v2.0 was converted to Tet-v2.0 confirming the reaction is catalytic (Sup. Fig. 30). Under conditions used in protein expression (50 mL media in 250 mL baffled flasks, 1 mM Tet-v2.0) this reaction did not change the Tet-v2.0 concentration or impact the reactivity of Tet-v2.0 with sTCO. This reaction has potential to act as a switch and enable tetrazine reactivity with sTCO to be turned on or off dependent the addition of excess thiols and removal of oxygen.



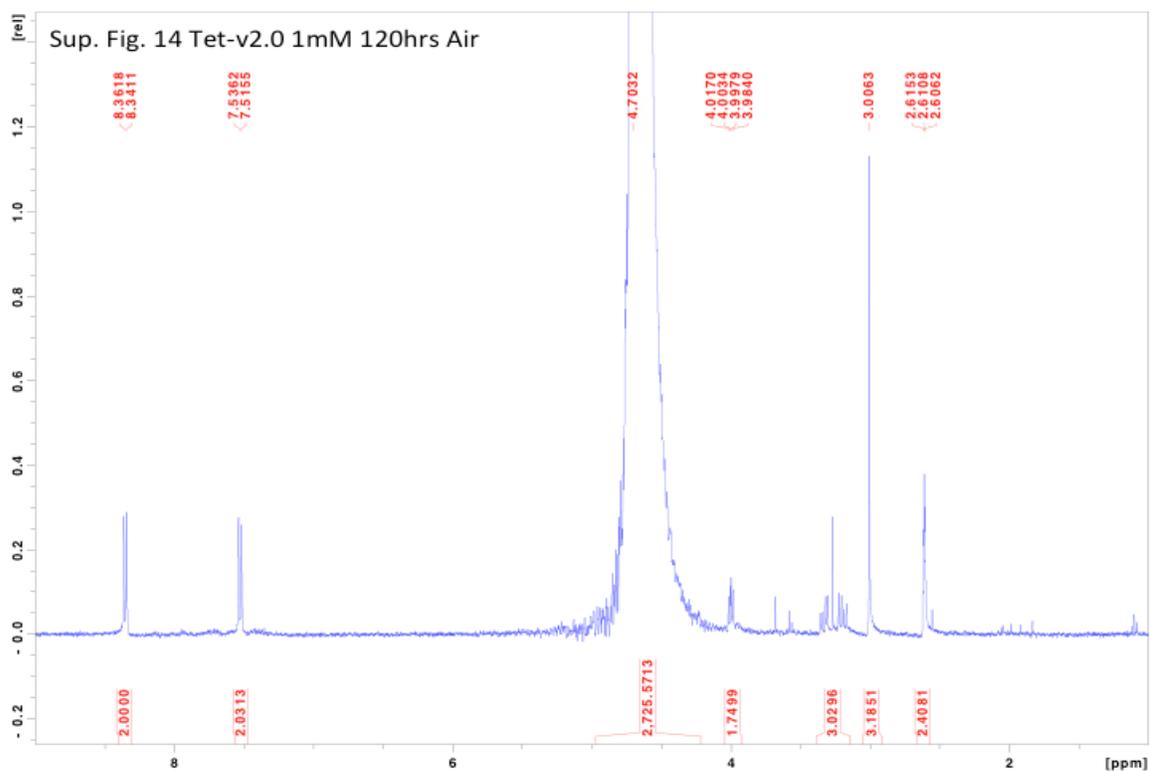
**Supplemental Scheme 3.** Proposed reaction for catalytic cycle of Tet-v2.0 with thiols and oxygen.



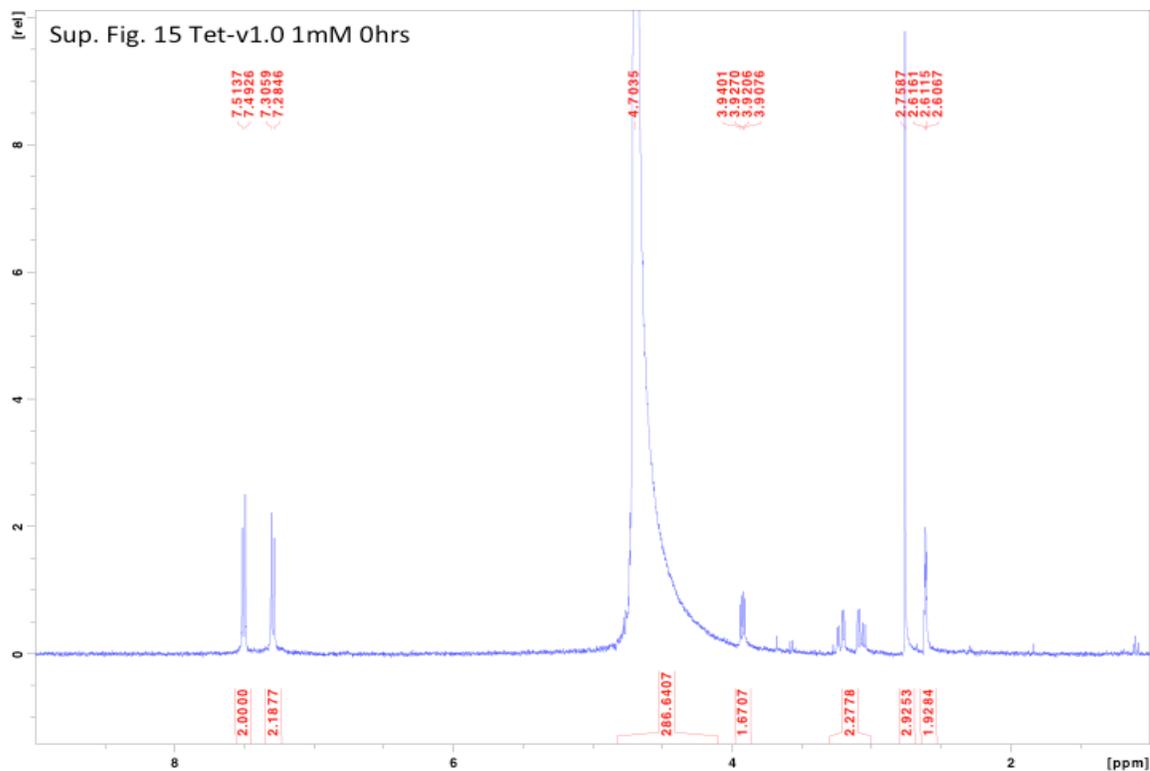
**Supplemental Figure 12.**  $^1\text{H}$  NMR of 1 mM Tet-v2.0 in deuterated phosphate buffer (40 mM, pH 7.4) after 0 hours.



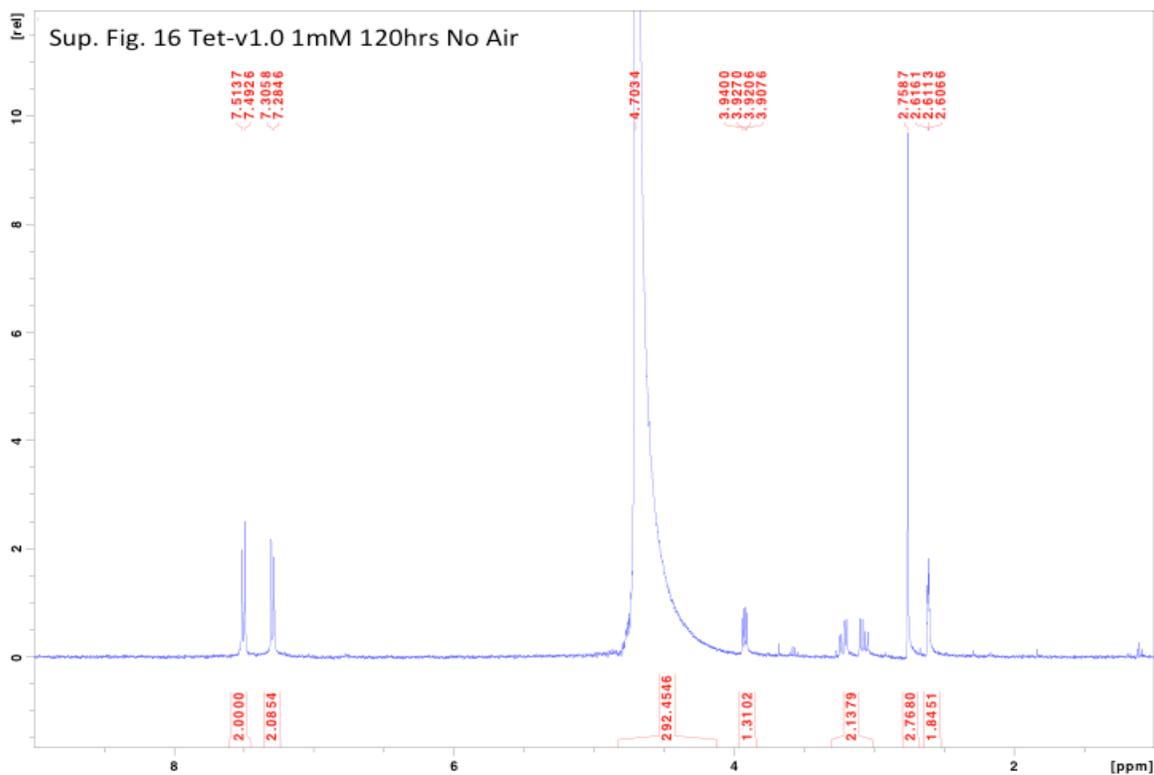
**Supplemental Figure 13.**  $^1\text{H}$  NMR of 1 mM Tet-v2.0 in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen deficient conditions. No significant change is observed.



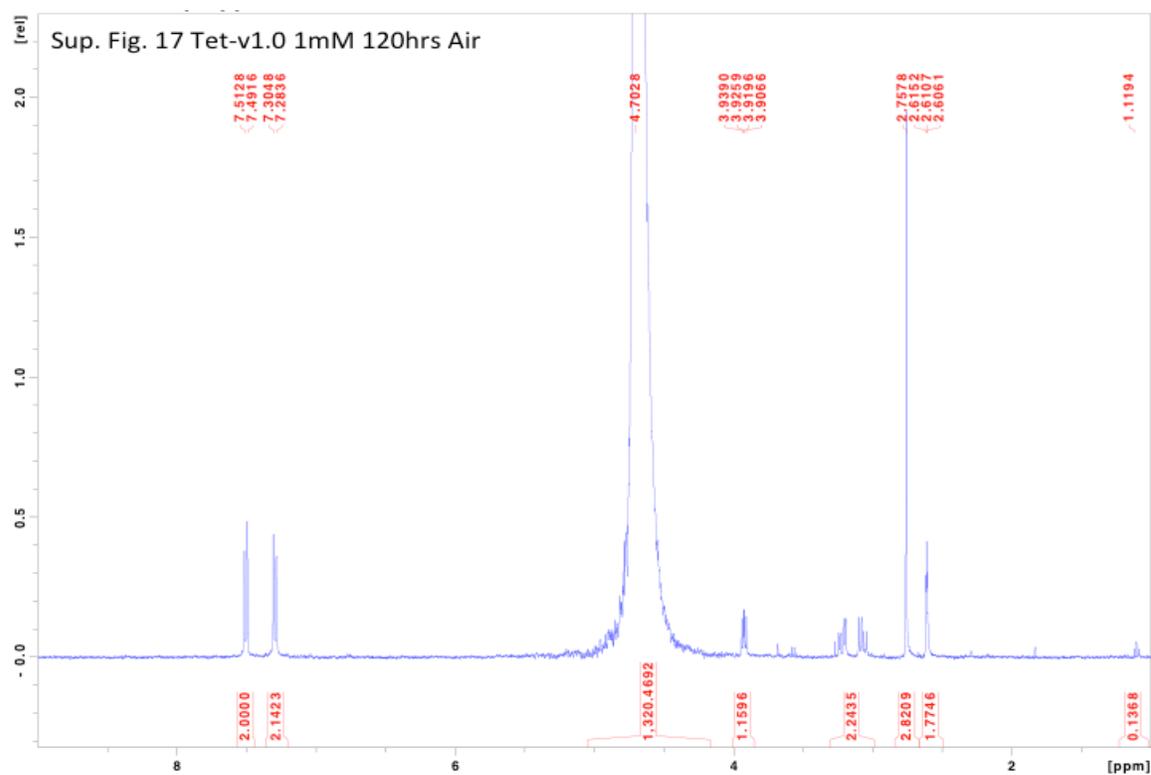
**Supplemental Figure 14.**  $^1\text{H}$  NMR of 1 mM Tet-v2.0 in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen rich conditions. No significant change is observed.



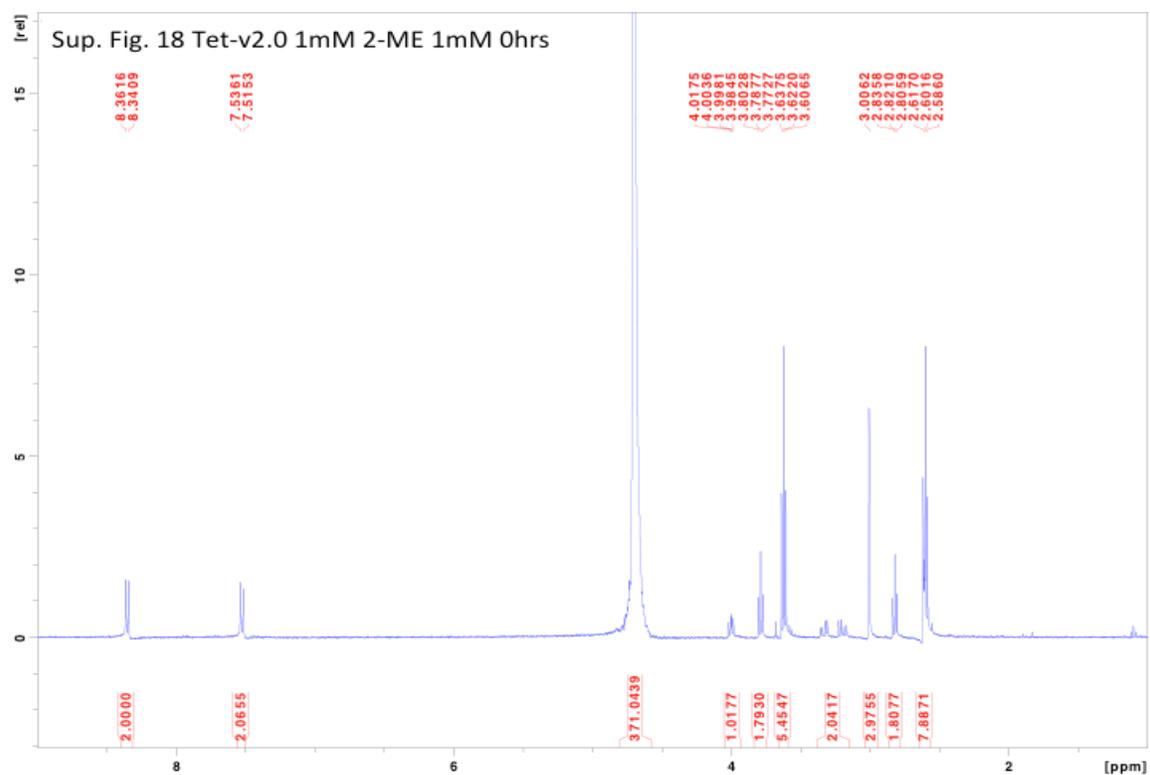
**Supplemental Figure 15.**  $^1\text{H}$  NMR of 1 mM Tet-v1.0 in deuterated phosphate buffer (40 mM, pH 7.4) after 0 hours.



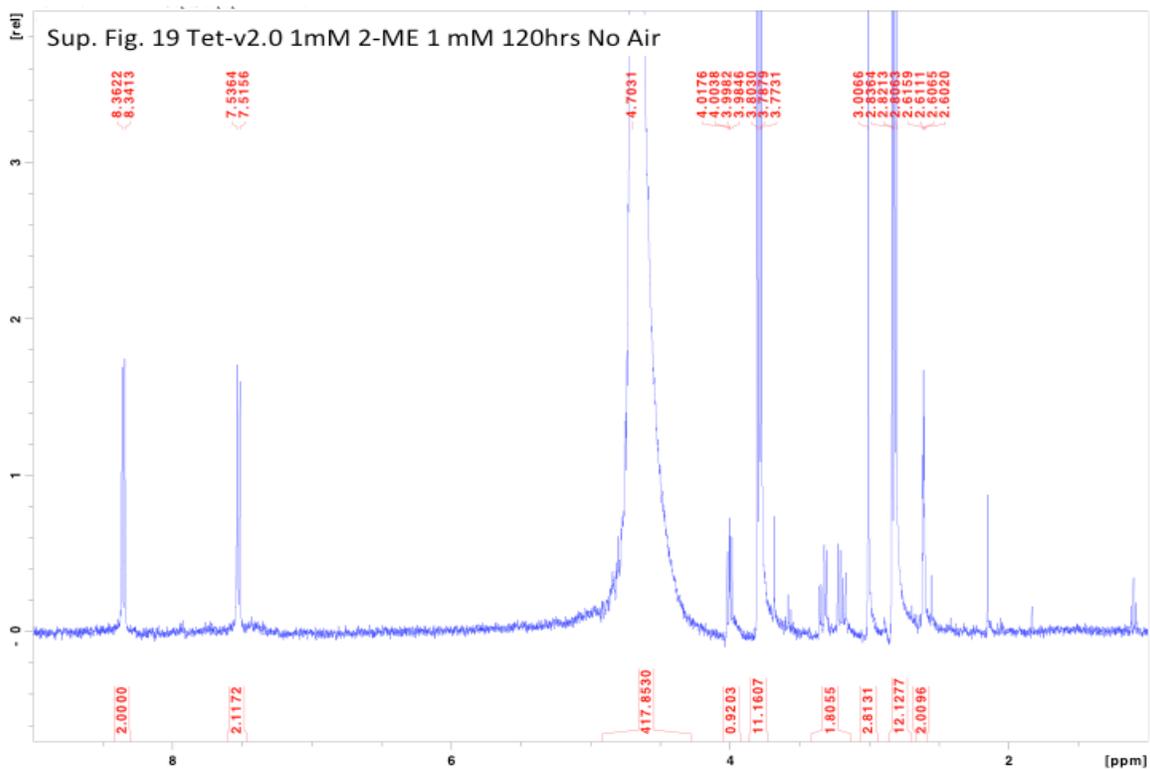
**Supplemental Figure 16.**  $^1\text{H}$  NMR of 1 mM Tet-v1.0 in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen deficient conditions. No significant change is observed.



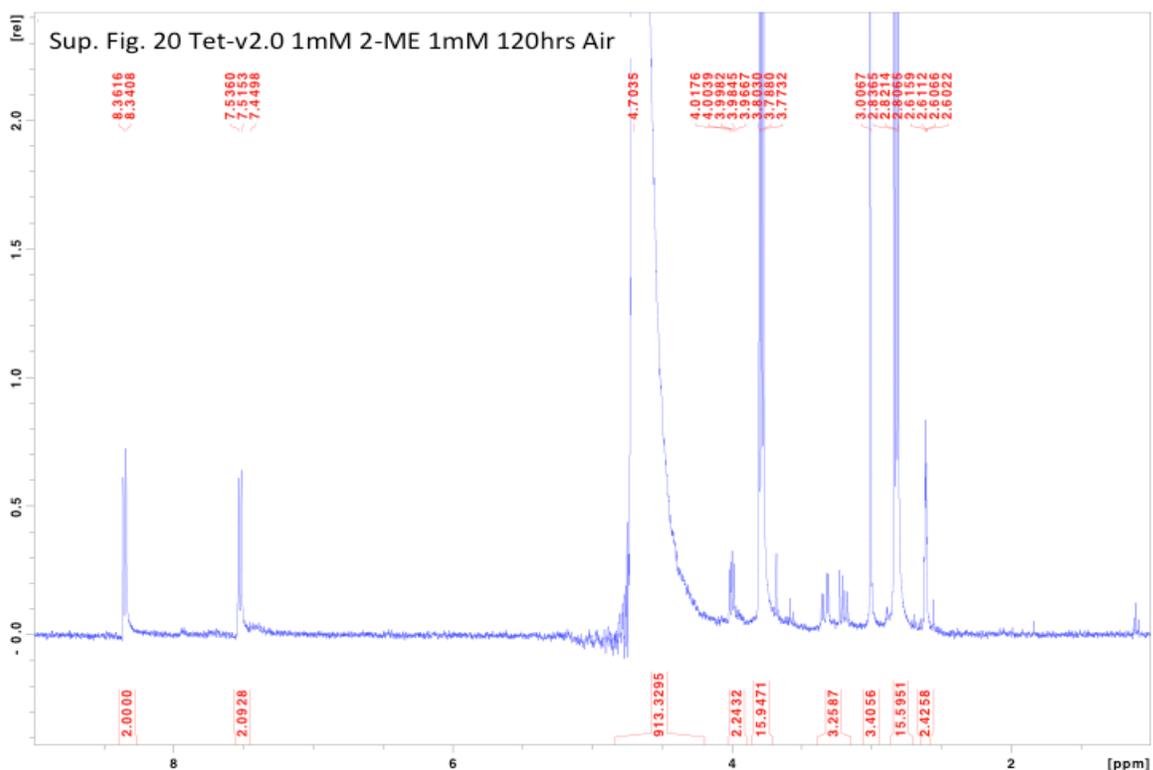
**Supplemental Figure 17.**  $^1\text{H}$  NMR of 1 mM Tet-v1.0 in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen rich conditions. No significant change is observed.



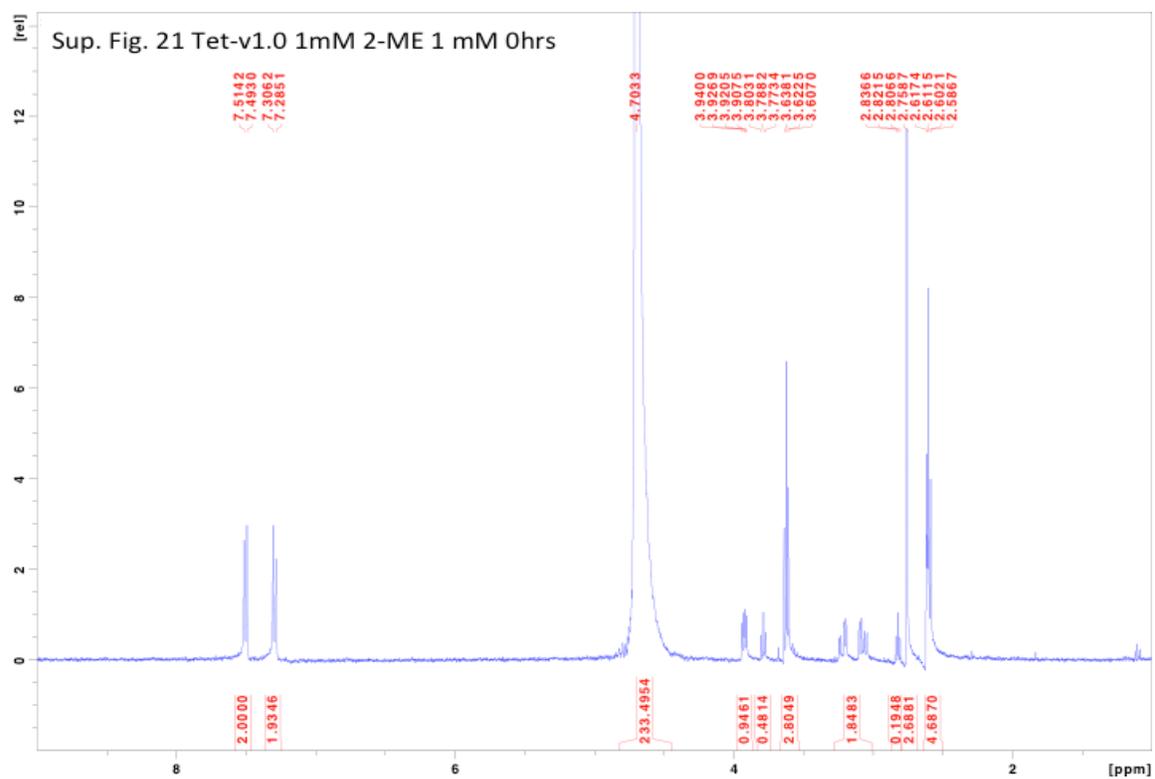
**Supplemental Figure 18.**  $^1\text{H}$  NMR of 1 mM Tet-v2.0 with 1 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 0 hours.



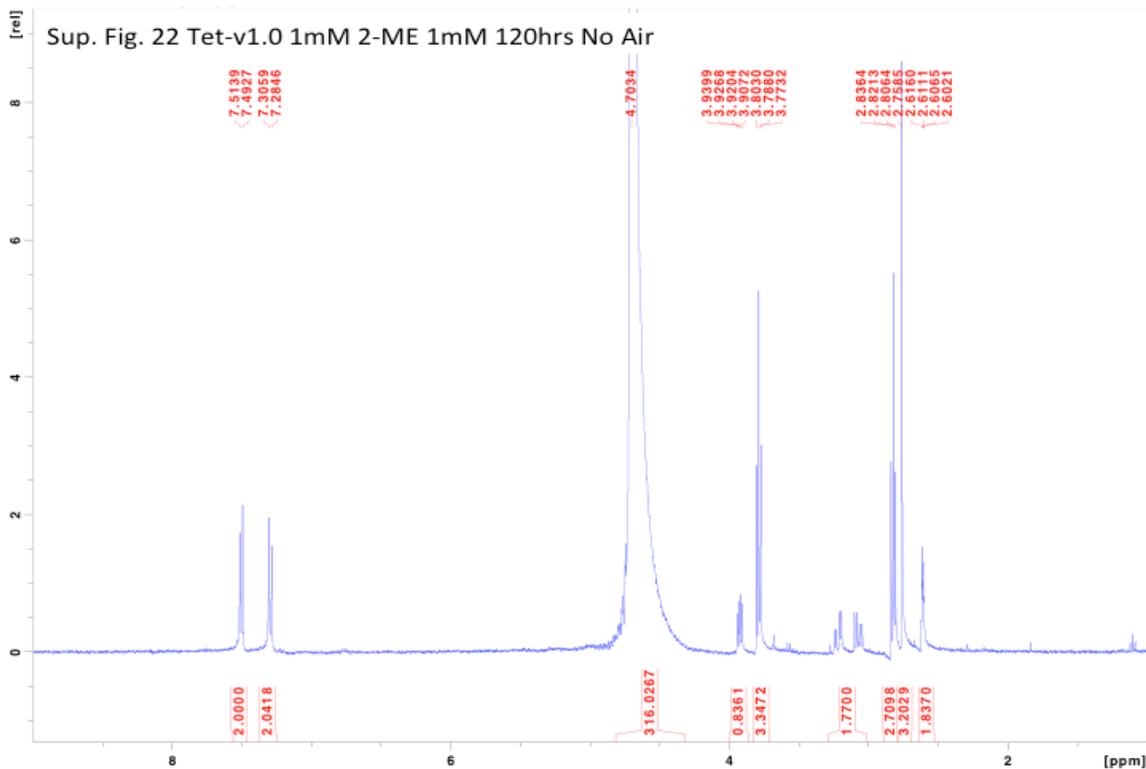
**Supplemental Figure 19.**  $^1\text{H}$  NMR of 1 mM Tet-v2.0 with 1 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen deficient conditions. 2-mercaptoethano is converted to 2-hydroxyethyl disulfide.



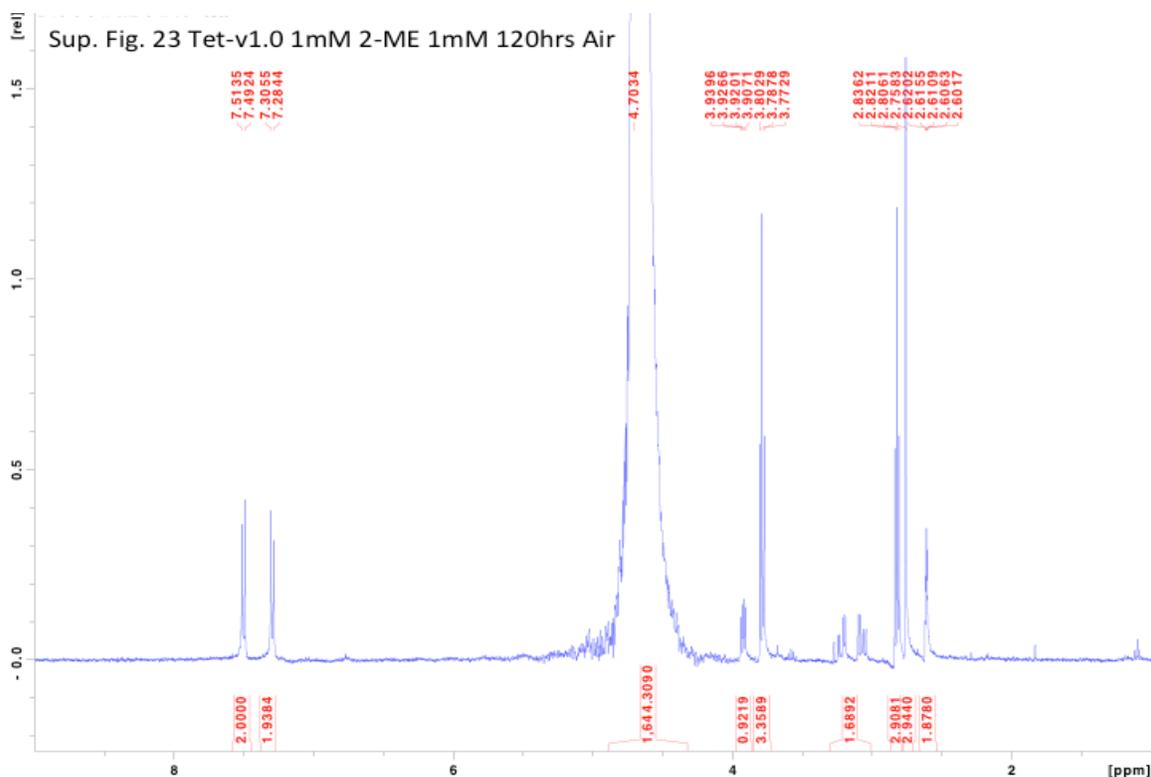
**Supplemental Figure 20.**  $H^1$  NMR of 1 mM Tet-v2.0 with 1 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen rich conditions. 2-mercaptoethanol is converted to 2-hydroxyethyl disulfide.



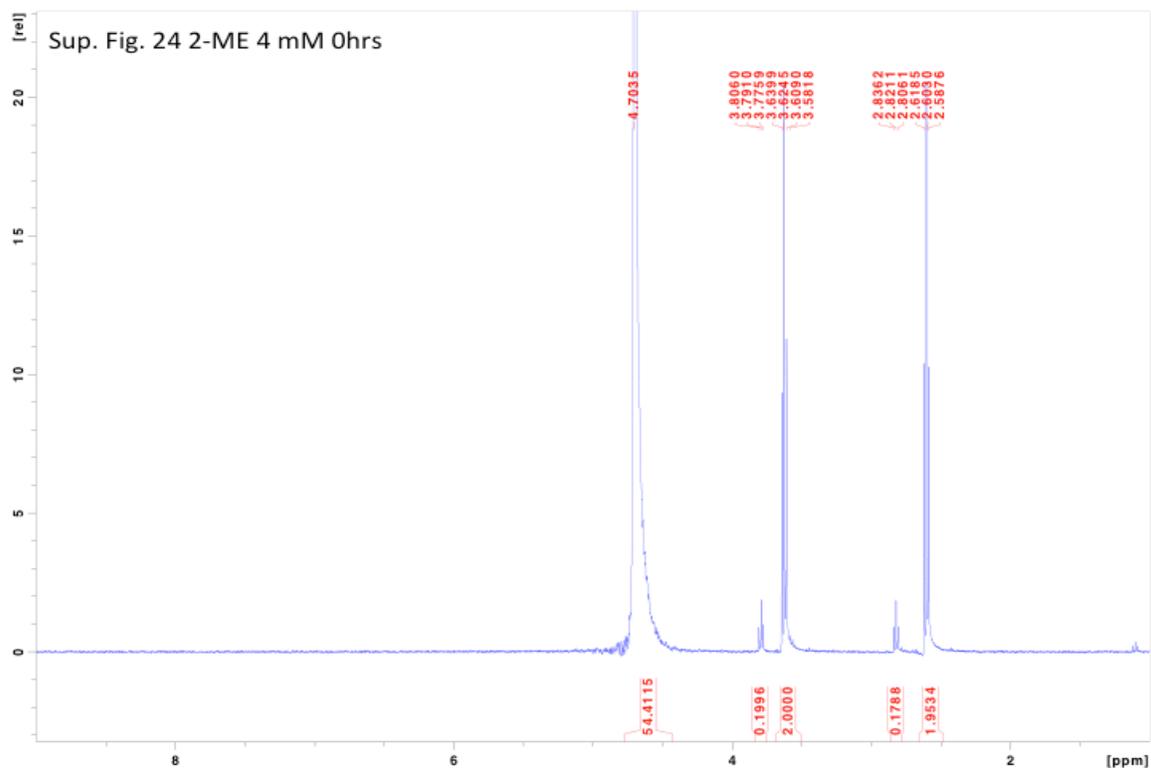
**Supplemental Figure 21.**  $H^1$  NMR of 1 mM Tet-v1.0 with 1 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 0 hours.



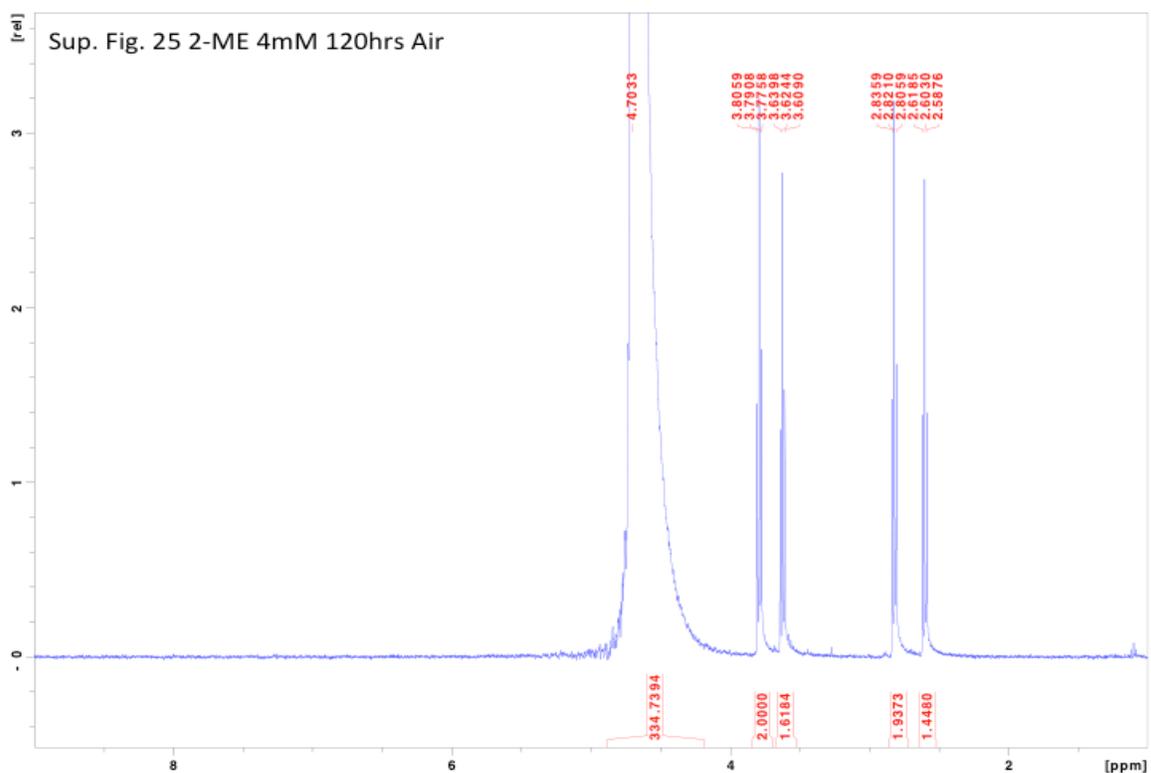
**Supplemental Figure 22.**  $^1\text{H}$  NMR of 1 mM Tet-v1.0 with 1 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen deficient conditions. 2-mercaptoethanol is converted to 2-hydroxyethyl disulfide.



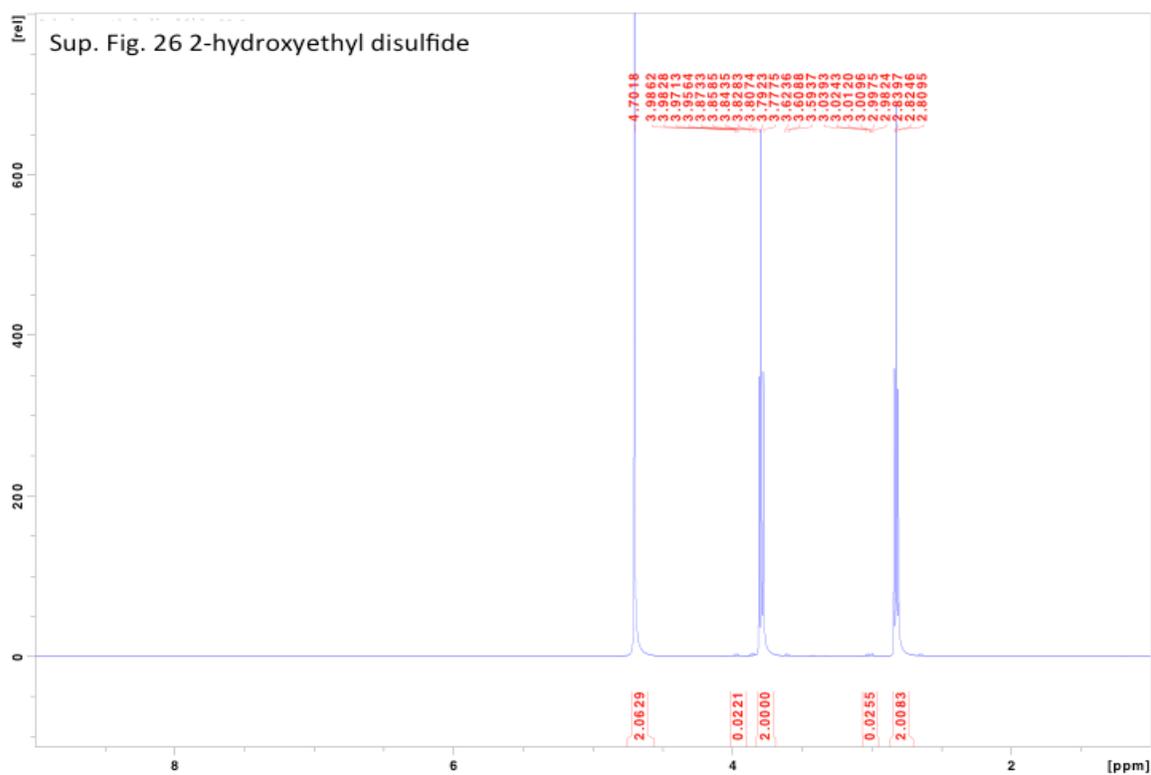
**Supplemental Figure 23.**  $^1\text{H}$  NMR of 1 mM Tet-v1.0 with 1 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen rich conditions. 2-mercaptoethanol is converted to 2-hydroxyethyl disulfide.



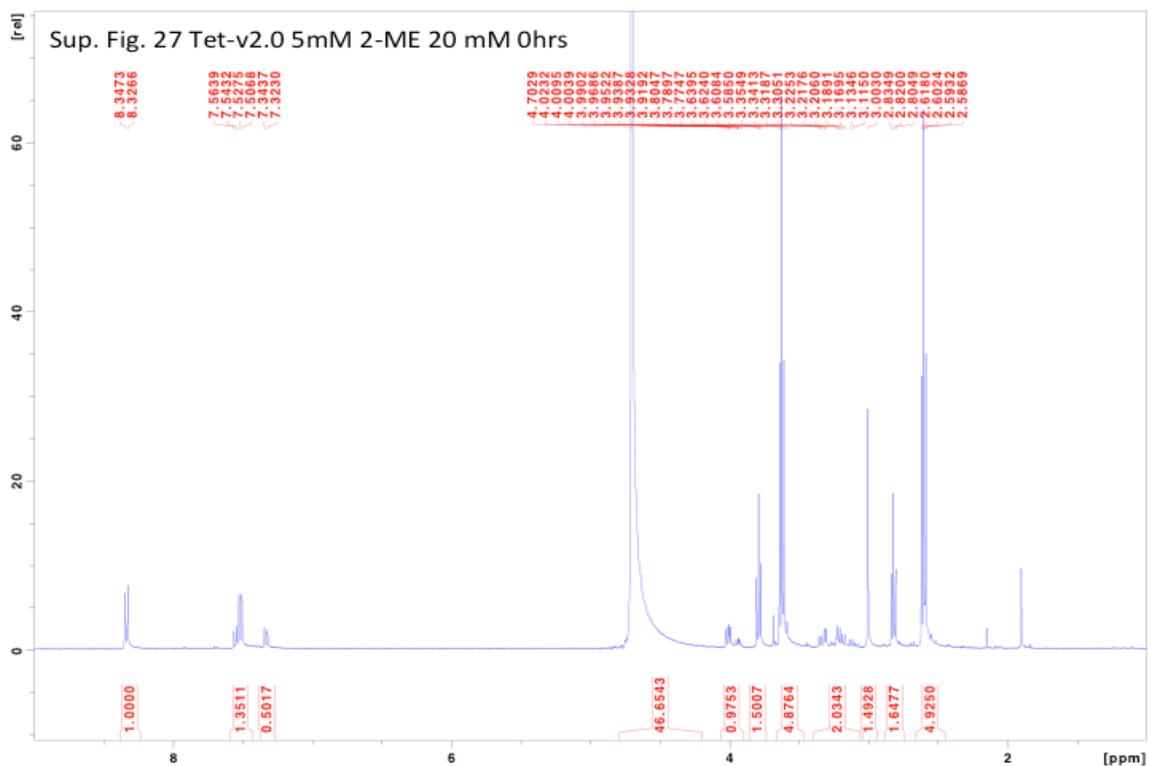
**Supplemental Figure 24.**  $H^1$  NMR of 4 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 0 hours. A mixture of the 2-mercaptoethanol and 2-hydroxyethyl disulfide is observed.

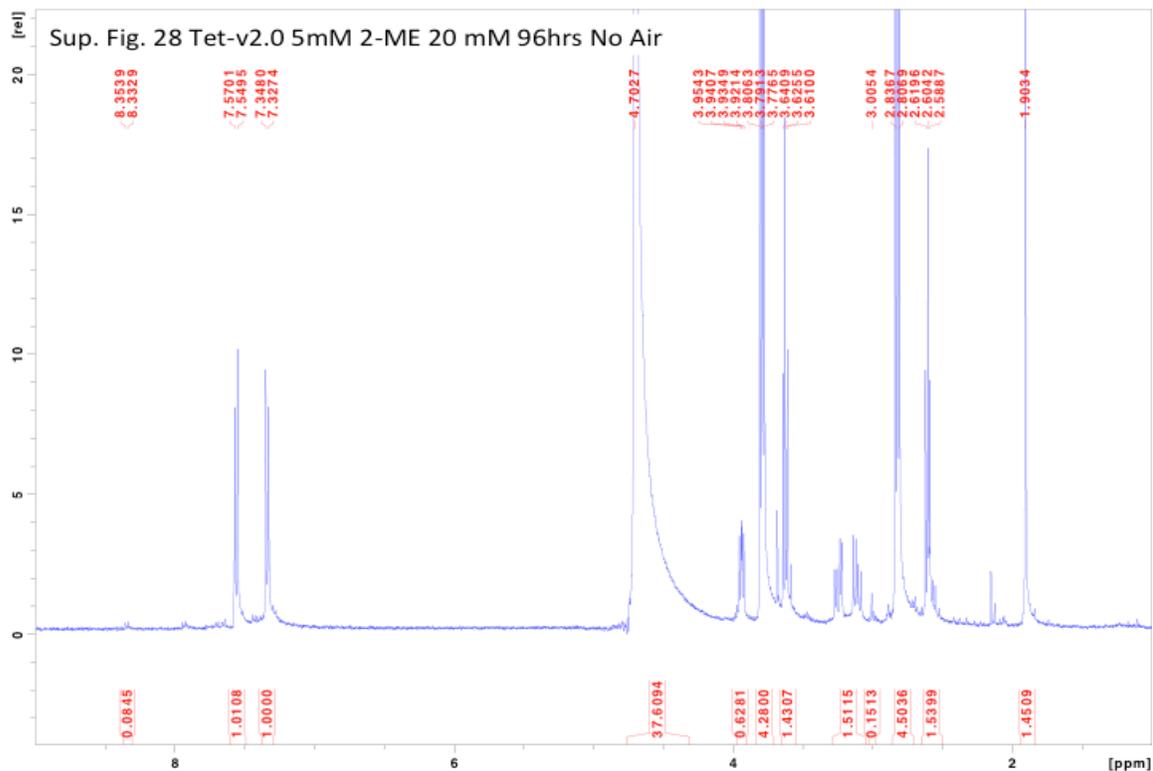


**Supplemental Figure 25.**  $H^1$  NMR of 4 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 120 hours under oxygen rich conditions. An increase in the quantity of 2-hydroxyethyl disulfide is observed.

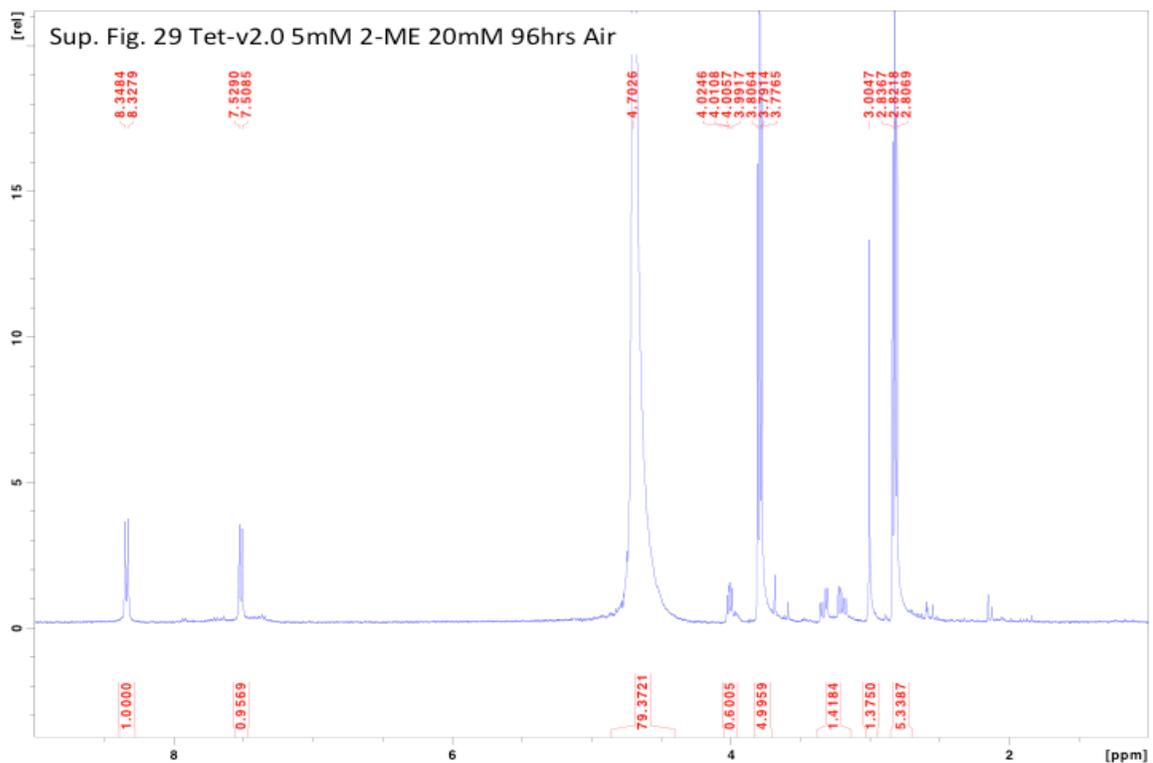


Supplemental Figure 26.  $^1\text{H}$  NMR of buffered 2-hydroxyethyl disulfide.

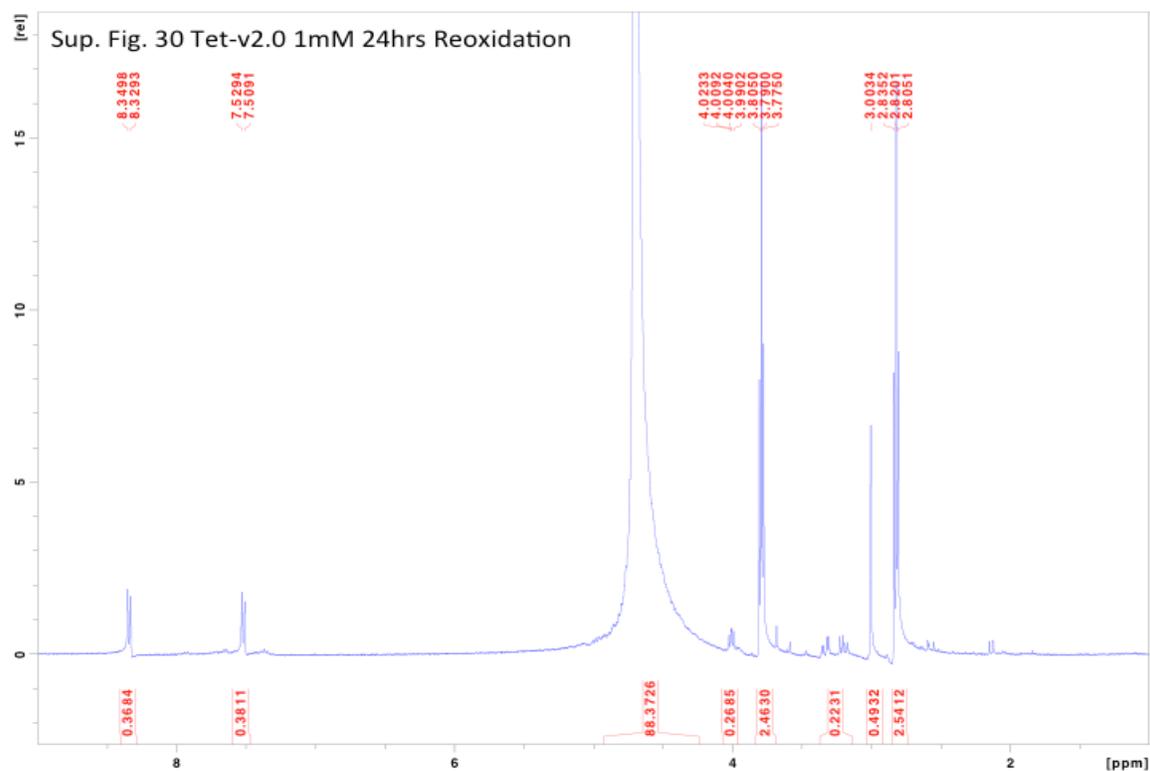




**Supplemental Figure 28.**  $^1\text{H}$  NMR of 5 mM Tet-v2.0 with 20 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 96 hours under oxygen deficient conditions. Dihydro-Tet-v2.0 is the dominant form of Tet-v2.0.



**Supplemental Figure 29.**  $^1\text{H}$  NMR of 5 mM Tet-v2.0 with 20 mM 2-mercaptoethanol in deuterated phosphate buffer (40 mM, pH 7.4) after 96 hours under oxygen rich conditions. Dihydro-Tet-v2.0 is not present in significant quantities.

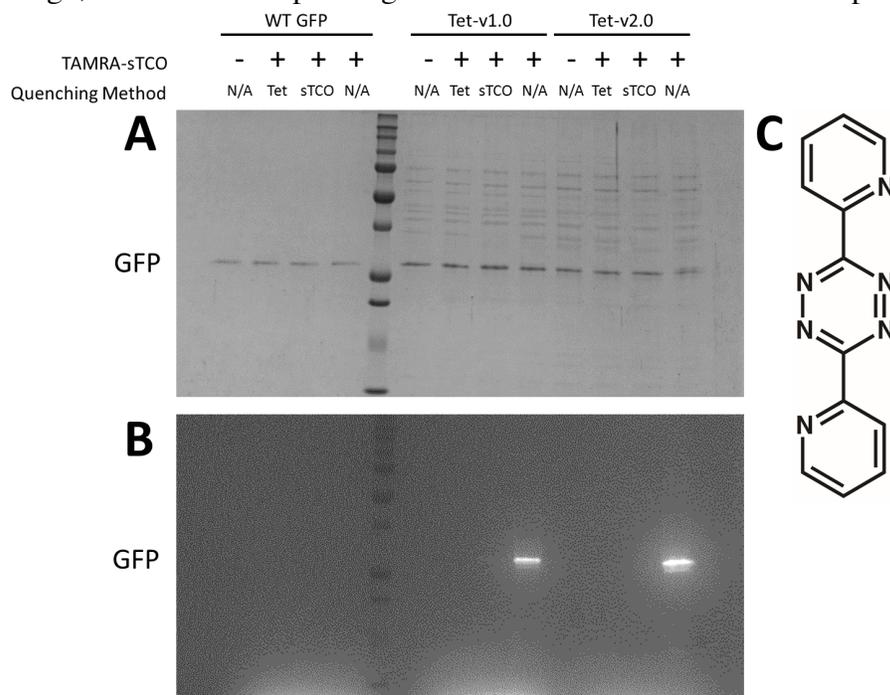


**Supplemental Figure 30.**  $^1\text{H}$  NMR of sample from Sup. Fig. 28 after exposure to an oxygen rich environment after 24 hrs. Chemical shifts indicate oxidation of dihydro-Tet-v2.0 to Tet-v2.0.

### Labeling of GFP-Tet protein with fluorescent dye

We compared the ability of the reaction between sTCO and tetrazines to label GFP-Tet-v2.0 and GFP-Tet-v1.0 with a fluorescent dye in cell lysate. In order to analyze the reactions quantitatively, we evaluated two different methods of quenching the reaction. The first method involved addition of excess 3,6-Di-2-pyridal-1,2,4,5-tetrazine to react with and remove unreacted sTCO from solution. The second method of quenching involves the addition of an excess amount of sTCO to remove unreacted tetrazine functional groups from solution. The effectiveness of these quenching methods was tested by reacting GFP-Tet-v1.0 and GFP-Tet-v2.0 in cell lysate with TAMRA-sTCO and quantifying the relative fluorescent intensities of their corresponding bands using SDS-PAGE. Cell lysate containing GFP-Tet-v1.0 and GFP-Tet-v2.0 ( $20\ \mu\text{L}$ ,  $\sim 1.25\ \mu\text{M}$  GFP), as well as WT GFP ( $20\ \mu\text{L}$ ,  $\sim 1.25\ \mu\text{M}$  GFP) was quenched via the addition of either sTCO (1 mM in  $5\ \mu\text{L}$  methanol) or 3,6-Di-2-pyridal-1,2,4,5-tetrazine (1 mM in  $5\ \mu\text{L}$  dimethylformamide) and after 1 min reacted with  $\sim 0.33$  equivalents of TAMRA-sTCO in  $5\ \mu\text{L}$  methanol. As a negative control, samples were run without the addition of TAMRA-sTCO, and as a positive control, samples were reacted with TAMRA-sTCO 1 min prior to addition of the 3,6-Di-2-pyridal-1,2,4,5-tetrazine quenching agent (1 mM in  $5\ \mu\text{L}$  dimethylformamide). Samples were allowed to react 1 min after the addition of the last reagent before mixing with  $30\ \mu\text{L}$  2x Laemmli Buffer and heating for 10 min at  $99.5\ ^\circ\text{C}$ . The samples were analyzed via SDS-PAGE ( $10\ \mu\text{L}$  sample, 15% Acrylamide gel,

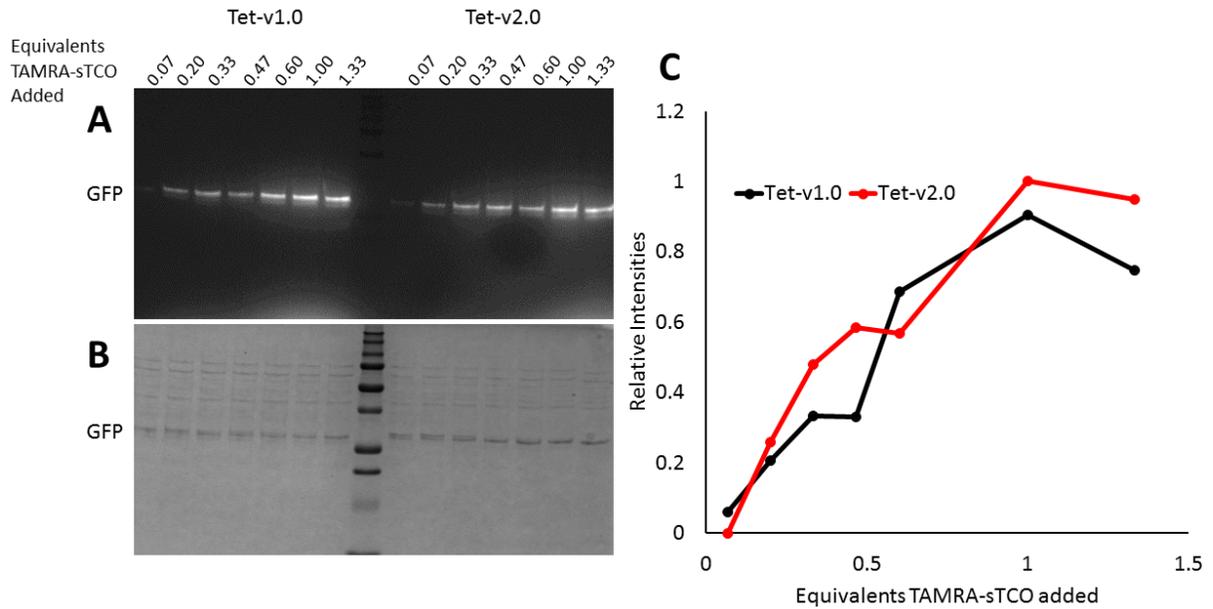
200V, 40 min) (Sup. Fig. 31). Samples were fluorescently imaged prior to staining with Coomassie. Both the absence of fluorescent bands at the expected molecular weight outside of the positive control indicates that the addition of excess sTCO and excess tetrazine are sufficient to quench this reaction. In addition, the absence of fluorescently labeled bands outside of GFP and the dye front indicate that this reaction is selective towards Tet-v2.0. To further demonstrate this we have increased the brightness on the fluorescently imaged gel to the point where there is a clear halo effect around the two observed bands and from the dye front at the bottom of the image. Even with this bright image, no bands corresponding to side reactions other cellular components are observed.



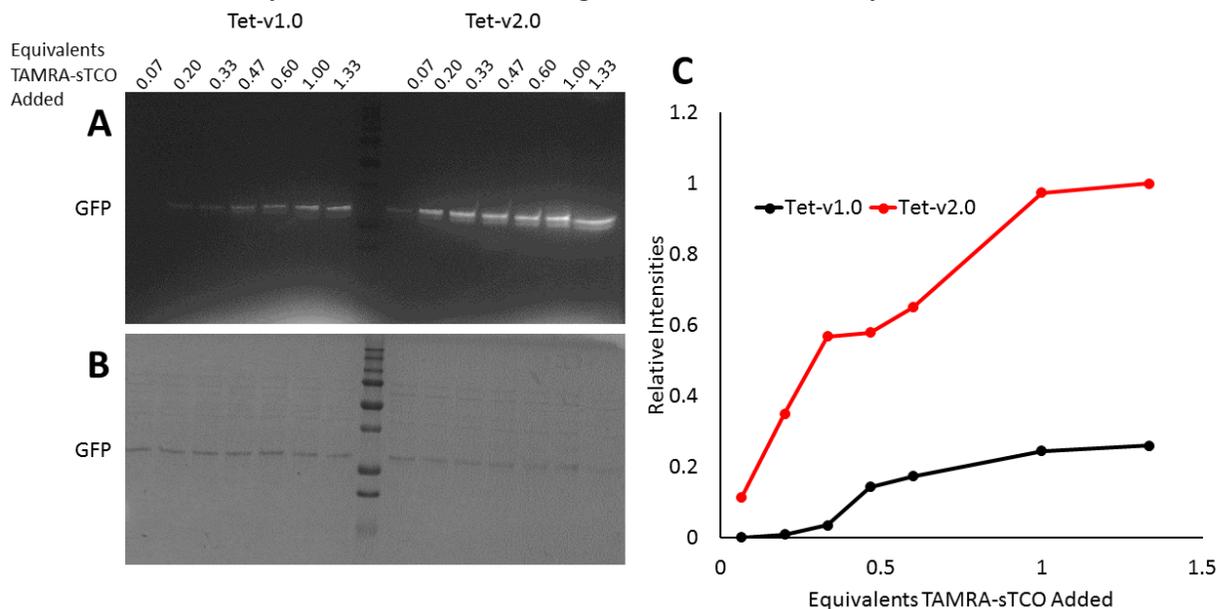
**Supplemental Figure 31. Quenching of the reaction between tetrazine and sTCO.**

(A) SDS-PAGE of WT GFP, GFP-Tet-v1.0, and GFP-Tet-v2.0 reacted with TAMRA-sTCO in cell lysate. (B) Rhodamine fluorescence of the above gel. Bands are present at the expected molecular weight of GFP only when TAMRA-sTCO is added prior to the addition of the quenching agent. (C) Structure of 3,6-Di-2-pyridal-1,2,4,5-tetrazine.

GFP-Tet-v1.0 and GFP-Tet-v2.0 (20  $\mu$ L,  $\sim$ 1.25  $\mu$ M GFP in cell lysate) were then reacted with various concentrations of TAMRA-sTCO spanning from 0.07 to 1.33 equivalents. The reactions were quenched through the addition of 3,6-Di-2-pyridal-1,2,4,5-tetrazine (1 mM, 5  $\mu$ L in dimethylformamide) after 15 sec and 15 min, and analyzed using SDS-PAGE(Sup. Fig. 32, 33). The fluorescent intensity of bands corresponding to reaction of tetrazine-GFP and sTCO-TAMRA was quantitated. After 15 min of reaction, GFP-Tet-v1.0 and GFP-Tet-v2.0 had similar intensities when labeled with the same amount of TAMRA-sTCO. Both Tet-v1.0 and Tet-v2.0 underwent sub-stoichiometric labeling. However, after shorter 15 s reactions, Tet-v2.0 reacted to completion at sub-stoichiometric quantities of label whereas Tet-v1.0 did not completely react.



**Supplemental Figure 32. 15 min reaction between sTCO-TAMRA and GFP-tetrazine.** (A) Fluorescent SDS-PAGE analysis of GFP-Tet-v1.0, and GFP-Tet-v2.0 reacted with TAMRA-sTCO in cell lysate. Bands are only observed at the expected molecular weight of GFP and at the dye front. (B) Coomassie staining of the above gel. (C) Quantitation of the fluorescent intensity of the gel in A at the position of GFP. As the concentration of TAMRA-sTCO increases, both Tet-v1.0 and Tet-v2.0 react completely. Fluorescence intensity is normalized to the highest observed intensity.



**Supplemental Figure 33. 5 min reaction between sTCO-TAMRA and GFP-tetrazine.** (A) Fluorescent SDS-PAGE analysis of GFP-Tet-v1.0, and GFP-Tet-v2.0 reacted with TAMRA-sTCO in cell lysate. Bands are only observed at the expected molecular weight of GFP and at the dye front. (B) Coomassie staining of the above gel.

(C) Quantitation of the fluorescent intensity of the gel in A at the position of GFP. As the concentration of TAMRA-sTCO increases, only Tet-v2.0 is observed to react completely. Partial reaction is seen for Tet-v1.0. Fluorescence intensity is normalized to the highest observed intensity.