Supporting information for

Eukaryotic TYW1 Is A Radical SAM Flavoenzyme

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1. Methods and Materials

1.1 Cloning of *Sc*TYW1. The gene, codon optimized for expression in *E. coli*, corresponding to TYW1 (YPL207W) from *S. cerevisiae* was obtained from GenScript in a pUC57 vector. The gene sequence is shown in **Fig S.1**. The portion of the sequence coding for amino acids 46-810 was PCR amplified using the forward primer 5′-AAAAAAAA*CATATG*GGTTCACAGGACTCGC-3′ and the reverse primer 5′-AAAAAA*AAGCTT*ATCAGGCCGGAATCGGCG-3′ with the italicized sequences encoding *Nde*I (forward primer) and *Hind*III (reverse primer) restriction sites (italic). The PCR product and pET28JT vector (1) were digested with *Nde*I, *Hind*III, and CIP, and then ligated using T4 DNA ligase to form plasmid pAY788. This plasmid contains an N terminal Histag followed by a Tobacco Etch Virus nuclear-inclusion-a endopeptidase (TEV) cleavage site and then *Sc*TYW1. The resulting DNA sequence of the gene and tag are shown in **Fig S.2**. The protein sequence is shown in **Fig S.3**. With this construct, upon cleavage by TEV we obtained a protein with residues QGHM at the N-terminus, which starts at amino acid 46 of YPL207W.

1.2 Production of *Sc*TYW1. *E. coli* BL21-DE3 phage T1 resistant cells (NEB C2527) were co-transformed with pAY788 and pPH151, which encodes the *E. coli sufABCDSE* operon (2), and plated on a Lysogeny broth (LB), Lennox formulation, agar plate containing 34 μg/mL chloramphenicol and kanamycin. A single colony was picked and used to inoculate a 125 mL culture of LB, containing 34 μg/mL each of chloramphenicol and kanamycin, and grown overnight at 37 °C.

Ten mL of the overnight culture was used to inoculate twelve 2.8 L Fernbach flasks containing 1 L of Terrific broth (TB), 34 μg/mL each of kanamycin and chloramphenicol, and 1x of a trace metal solution (3). The cells were grown at 37 °C with shaking at 175 rpm to an OD_{600} ~0.6, at which point the temperature was reduced to 18 °C. One hour later solid riboflavin (~200 mg), isopropyl ß-D-1-thiogalactopyranoside (500 μM final concentration), and iron(III)chloride ($FeCl_3$) (50 μM final concentration) were added. The shaking speed was reduced further to 125 rpm and the growth continued for ~16 h. The cells were harvested by centrifugation at 5,000 xg and flash frozen. The typical yield is ~7 g/L of culture.

1.3 Purification of *Sc*TYW1 (UniProtKB Q08960).The purification was carried out in a Coy chamber (97% N_2, 3% H_2). The cell paste from 12 L of growth (~80 g) was resuspended in 50 mM potassium phosphate (KPi) (pH 7.4) buffer containing 0.5 M potassium chloride (KCl), 50 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 1 mM FMN, and 100 μg/mL lysozyme. Cells were lysed using a Branson sonifier at 50% amplitude for 25 min total with 15 s bursts of sonication, followed by 60 s breaks while stirring on ice to avoid excess heating of the solution. The cell lysate was clarified by centrifugation at 35,000 xg for 1 h. at 4 °C.

Using an AKTA prime plus, the clarified lysate was loaded onto four 5 mL HisTrap FF columns connected in series, charged with Ni^{2+} and equilibrated in 50 mM KPi (pH 7.4) buffer containing 0.5 KCl and 50 mM imidazole. The columns were washed with the same buffer and bound protein was eluted via a step gradient to equilibration buffer containing 0.5 M imidazole. Brown/black fractions were pooled, solid dithiothreitol (DTT) was added (2 mM final concentration) along with 10 mg of TEV protease and stirred at room temperature for 4 h.

Precipitated protein was removed by centrifugation at 35,000 xg at 4 °C for 10 min and the clarified protein solution was desalted into 50 mM KPi (pH 7.4) buffer containing 0.5 KCl and 50 mM imidazole using a Sephadex G25 column (18 X 2.5 cm). The protein was passed back over the four serially connected HisTrap columns equilibrated in 50 mM KPi (pH 7.4) buffer containing 0.5 KCl and 50 mM imidazole. The flowthrough was collected, flash-frozen in liquid nitrogen, and stored at -80 °C.

*Sc*TYW1 was further purified by anion exchange chromatography. For this step, the protein solution from above was thawed, solid FMN was added to a final concentration of 0.5 mM, followed by a 10-fold dilution by the addition of 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid)-sodium hydroxide (pH 7.4) (PIPES-NaOH) buffer to decrease the KCl concentration to ~ 50 mM prior to loading onto a Q-Sepharose FF column (10 X 1.7 cm) equilibrated in 20 mM PIPES-NaOH (pH 7.4). The column was washed with the loading buffer and the bound protein was eluted with a 0.25 L gradient to 20 mM PIPES-NaOH (pH 7.4) buffer containing 0.5 M KCl. Fractions containing protein were identified via SDS-PAGE and were pooled and concentrated to approximately 20 mL using an Amicon stirred cell under nitrogen gas pressure. The protein was flash-frozen in liquid nitrogen and stored at -80 °C. Prior to reconstitution, the protein was thawed and desalted into 50 mM PIPES-NaOH (pH 7.4) buffer containing 0.5 M NaCl and 2 mM DTT using the Sephadex G25 column. A Bradford protein assay with BSA as standard (ThermoScientific) was used to determine the protein concentration. Tenfold molar excess of $FeCl_3$, sodium sulfide, and a fivefold molar excess of FMN, dissolved in water, were added to the solution while stirring and the solution was incubated at room temperature for 4 h. Precipitated protein was removed by centrifugation and the solution was desalted into 50 mM PIPES-NaOH (pH 7.4) buffer containing 0.15 M KCl and 2 mM DTT using the Sephadex G25 column.

The final gel filtration step in the purification removes high molecular weight components, which are often observed after reconstitution. In this step, the protein was concentrated to ~3 mL and loaded onto a Sephacryl S-300 HR column (60 X 2.5 cm) equilibrated in 50 mM PIPES-NaOH (pH 7.4) buffer containing 0.15 M KCl and 2 mM DTT. The fractions containing protein were identified via color and SDS-PAGE and concentrated to ~3 mL. Aliquots were flash-frozen in liquid nitrogen and stored at -80 ºC. The concentration of this protein was typically ~150 μM as determined by Bradford assay with BSA as standard.

1.4 Production and purification of TEV protease. TEV protease was produced and purified as described previously (1,4). The stock concentration was determined to be 93 μM.

1.5 Production and purification of *M. jannaschii* TYW1 (*Mj*TYW1) (UniProtKB Q57705). *Mj*TYW1 was produced and purified as described previously (4).

1.6 Preparation of SAM. SAM was synthesized as described previously (4,5).

1.7 Purification of soluble RNA from *S. cerevisiae* YPL207w deletion strain. RNA was purified from *S. cerevisiae* YPL207w deletion strain (YSC1-21-547084 Thermo Scientific) as previously described (4), with a method adapted from (6).

1.8 Amino acid analysis of *Sc*TYW1. Amino acid analysis of the protein was carried out at the UC Davis Genomics Center revealing a correction factor of 0.77 to relate the Bradford assays to amino acid content.

1.9 Flavin cofactor analysis. Protein was precipitated by the addition of 30% (w/v) trichloroacetic acid (TCA) in water to a final concentration of 10% (v/v). Precipitated protein was removed by centrifugation and an aliquot of the supernatant was analyzed by a Thermo Vanquish UHPLC and the analytes were separated using a Thermo Hypersil gold C18 column (150 x 2.1 mm) 1.9 µ particle size.

The UHPLC was run with a flow rate of 0.2 mL/min with buffer A consisting of 0.1% (v/v) TFA (v/v) water and buffer B consisting of 0.1% TFA (v/v) in acetonitrile. The separation program was as follows: 0-6 min, 0% B; 6-30 min, 0-100% B; 30.1-35 min, 100% B; 35.1-39.8 min, 0% B. UV-vis absorbance were measured at 375 and 450 nm.

The identity of the flavin cofactor was verified by comparison of retention times with FMN and FAD standards. The concentration of the FMN cofactor was determined by comparing the area of the peak at 375 and 450 nm to a standard curve of FMN. The FMN standard was dissolved in water and acidified by the addition of 30% (w/v) TCA to a final concentration of 10% (v/v) and the concentration determined by UV-visible spectroscopy on an Agilent 8454 UV-visible spectrophotometer at 445 nm with ε_{445} 12,500 $M^{-1}cm^{-1}$.

This was performed on three different preparations of *Sc*TYW1.

1.10 Iron analysis of ScTYW1. *Sc*TYW1 was diluted to a concentration of 1 µM by addition of 1 % (v/v) nitric acid. Iron concentration was determined by inductively coupled plasma mass spectrometry at the ICP-MS metals and strontium isotope facility within the Department of Geology and Geophysics at the University of Utah.

This was performed on three different preparations of *Sc*TYW1.

1.11 UV-vis spectroscopy of *Sc*TYW1. All measurements were recorded on an Agilent 8453 UV-visible spectrophotometer inside a Coy anaerobic chamber (97% N_2, 3% H_2). All samples were diluted with 50 mM PIPES-NaOH (pH 7.4) buffer containing 150 mM KCl and 2 mM DTT. The protein concentration in the oxidized and reduced spectra is 10 µM. The protein was reduced by the addition of dithionite (final concentration of 0.1 mM) and the spectrum recorded. The spectrum of the flavin cofactor was obtained by addition of 12 µL of 30% TCA to 120 µL of 25 µM *Sc*TYW1. Protein was removed by centrifugation and an absorbance spectrum of the supernatant was recorded.

1.12 *Sc*TYW1 activity assays. All assays were setup in a Coy chamber (97% N_2, 3% H_2) using anaerobic solutions. Assays were carried out in 50 mM PIPES-NaOH (pH 7.4) buffer containing 2 mM SAM, 10 mM dithionite, 2 mM 1,2,3-$^{13}C_3$-pyruvate, 144 µg RNA (from *S. cerevisiae* TYW1 deletion strain), and 15 µM ScTYW1 in a final volume of 100 µL. Samples were incubated at 30 ºC for 4 h. and were quenched by flash freezing in liquid nitrogen. RNA was then extracted and analyzed as described below. This assay was performed in duplicate.

1.13 *Sc*TYW1 assays with varying FMN concentrations. All assays were setup in a Coy chamber (97% N_2, 3% H_2) using anaerobic solutions. Assays were carried out in 50 mM PIPES-NaOH (pH 7.4) buffer containing 2 mM SAM, 2 mM 1,2,3-$^{13}C_3$-pyruvate, 144 µg RNA (from *S. cerevisiae* TYW1 deletion strain), 2 mM NADPH and 15 µM ScTYW1. The following concentrations of FMN were added: 0, 0.5, 1, 2.5, 5, 7.5, 10, 20, 40, and 60 µM. This was in a final volume of 100 µL and samples were incubated at 30 ºC for 4 h. The samples were quenched and analyzed as described below. This assay was performed in triplicate.

1.14 *Sc*TYW1 assays with different reductants. All assays were setup in a Coy chamber (97% N_2, 3% H_2) using anaerobic solutions. Assays were carried out in 50 mM PIPES-NaOH (pH 7.4) buffer containing 2 mM SAM, 2 mM 1,2,3-$^{13}C_3$-pyruvate, 144 µg RNA (from *S. cerevisiae* TYW1 deletion strain), and 15 µM ScTYW1 in a final volume of 400 µL. The reductants were at the following concentrations when present: dithionite – 10 mM, NADPH – 2 mM, NADH – 2 mM, FAD – 20 µM, and FMN – 20 µM. The mixtures were incubated at 30 ºC, and aliquots (100 µL) were removed at 1, 2, and 4 h. The aliquots were quenched by flash freezing in liquid nitrogen and storing at -20 ºC until the RNA was extracted as described below. This assay was performed a minimum of three times

1.15 Comparison of *Mj*TYW1 and *Sc*TYW1 activity in the presence of different reductants. All assays were setup in a Coy chamber (97% N_2, 3% H_2) using anaerobic solutions. These samples contained 50 mM PIPES-NaOH (pH 7.4) buffer containing 2 mM SAM, 1,2,3-$^{13}C_3$-pyruvate, 144 µg RNA (from *S. cerevisiae* TYW1 deletion strain) and 15 µM of either ScTYW1 or MjTYW1. The reductants were at the following concentrations when present: dithionite – 10 mM, NADPH – 2 mM, NADH – 2 mM, FAD – 20 µM, and FMN – 20 µM. The assays containing ScTYW1 were incubated at 30 ºC and those containing MjTYW1 at 50 ºC. These were 100 µL reactions and were quenched as described below after 4 h. This assay was performed in triplicate.

1.16 Reduction of FMN with NADPH in solution. All measurements were recorded on an Agilent 8453 UV-visible spectrophotometer inside a Coy anaerobic chamber (97% N_2, 3% H_2) in the dark. One chamber of a mixing cuvette contained 4 mM NADPH in 50 mM PIPES-KOH (pH 7.4) and the other chamber an equal volume of 40 µM FMN in water. The spectrophotometer was blanked with the NADPH solution. The reaction was initiated by inversion of the cuvette and the absorbance spectrum recorded at 0, 10, 20, and 30 min.

1.17 RNA digestion and analysis. RNA was extracted from assays and digested to the nucleoside level as described previously (4) with one exception. The P1 digestion took place at 45 °C for 14 h. Prior to analysis via LC-MS, 60 µL of digested nucleosides was combined with 5 µL of 1 µM 2′-deoxy-4-demethylwyosine (dimG-14), and 63 µL was injected onto a Thermo Vanquish UHPLC connected in series to a Q-Exactive mass spectrometer.

Analytes were separated using a Thermo Hypersil gold C18 column (150 x 2.1 mm) 1.9 µ particle size. The separation program was described previously(4). The Q Exactive mass spectrometer was operated in positive ion mode, with a resolution of 70,000 and a scan range of 300 to 400 *m/z*.

2. Figure S.1.

CATATGGACGGTTTTCGTGTGGCGGGTGCCCTGGTCGTTGGTGCTCTGACGGCGGCGTATCTGTACTTC
GGCGGTCGCTTCTCGATTGCCCTGGTCATTATCGTGGGCTATGGTATCTACTGCAACGAAGCATCCGGC
GGTTCACAGGACTCGCAAGAAAAACTGGATCTGAATAAGCAGCAGAAAAAACCGTGCTGTAGTGACAAA
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CTGGTTGGCTATACCGTCCTGGGCCTGGGTGACTCGGAAAGCTGGCCGGAAAAATTTTGCTACCAGGCA
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GTGAAAGGCGCAACCTTCTCCGGTTCTAGTGATGGCAACGGTAATCCGCTGACGATGCAGAACATTCCG
TTCTACGAAGAATGCGTGAAGTTCGTTAAAGCCTTCACCACGGAACTGCAACGTCGCGGTCTGCATTAC
GACCTGGCGGCCGAACATGCACACAGCAACTGTCTGCTGATCGCTGATACCAAGTTTAAAATTAATGGC
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ATGGATTACCTGGAAAAAACGCCGGAATGGGCGCTGTTTGGCAACGGCGGTTTCGCCCCGGGTAATACC
CGTGTTTATCGCAAAGATAAAAAGAAACAGAATAAAGAAAACCAAGAAACGACCACCCGTGAAACCCCG
CTGCCGCCGATTCCGGCCTGATAAGCTT

Figure S.1. DNA sequence of codon optimized *Sc*TYW1. The *Nde*I and *Hind*III sites are underlined.

3. Figure S.2.

ATGGGCAGCAGC*CATCATCATCATCATCAC*AGCAGC**GAGAACCTGTACTTCCAGGGC**CATATGGGTTCA
CCGGAGGACTCGCAAGAAAAACTGGATCTGAATAAGCAGCAGAAAAAACCGTGCTGTAGTGACAAAAAG
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GATGAACTGACCAACGAACCGAAACTGCTGAATCTGGATGACCTGAGCGATTTTGATGACTATTTCATC
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CGTGCTGATCATTGGATCAGCCGTCTGGGCGGTCGTCGCATTTTCCCGCTGGGCAAGGTTTGTATGAAA
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ATTATCTATGAATACGATGAAAACGCGGACAGCGAAGAAGATGAAGAAGAAGGCAATGGTTCTGATGAA
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GTTTATCGCAAAGATAAAAAGAAACAGAATAAAGAAAACCAAGAAACGACCACCCGTGAAACCCCGCTG
CCGCCGATTCCGGCCTGAT<u>AAGCTT</u>

Figure S.3. The DNA sequence of the gene expressed in this study. The restriction sites used to clone the gene into the vector are underlined. The His_6-tag is shown in italics. The TEV protease site is shown in bold.

4. Figure S.3.

MGSSHHHHHHSSENLYFQGHMGSPEDSQEKLDLNKQQKKPCCSDKKIADGGKKTGGCCSDKKNGGGKGG
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VFSKNSSSNSRVGKKLSVSKKIGPDGLIKSALTISNETLLSSQIYVLYSSLQGAASKAAKSVYDKLKEL
DELTNEPKLLNLDDLSDFDDYFINVPVENALYVLVLPSYDIDCPLDYFLQTLEENANDFRVDSFPLRKL
VGYTVLGLGDSESWPEKFCYQAKRADHWISRLGGRRIFPLGKVCMKTGGSAKIDEWTSLLAETLKDDEP
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HSGVKICRWTKNELRGKGSCYKKSLFNIASSRCMELTPSLACSSKCVFCWRHGTNPVSKNWRWEVDEPE
YILENALKGHYSMIKQMRGVPGVIAERFAKAFEVRHCALSLVGEPILYPHINKFIQLLHQKGITSFLVC
NAQHPEALRNIVKVTQLYVSIDAPTKTELKKVDRPLYKDFWERMVECLEILKTVQNHQRTVFRLTLVKG
FNMGDVSAYADLVQRGLPGFIEVKGATFSGSSDGNGNPLTMQNIPFYEECVKFVKAFTTELQRRGLHYD
LAAEHAHSNCLLIADTKFKINGEWHTHIDFDKFFVLLNSGKDFTYMDYLEKTPEWALFGNGGFAPGNTR
VYRKDKKKQNKENQETTTRETPLPPIPA

Figure S.3. The protein sequence of *Sc*TYW1 used in this study. The portion removed following TEV cleavage is shown underlined.

5. Figure S.4.

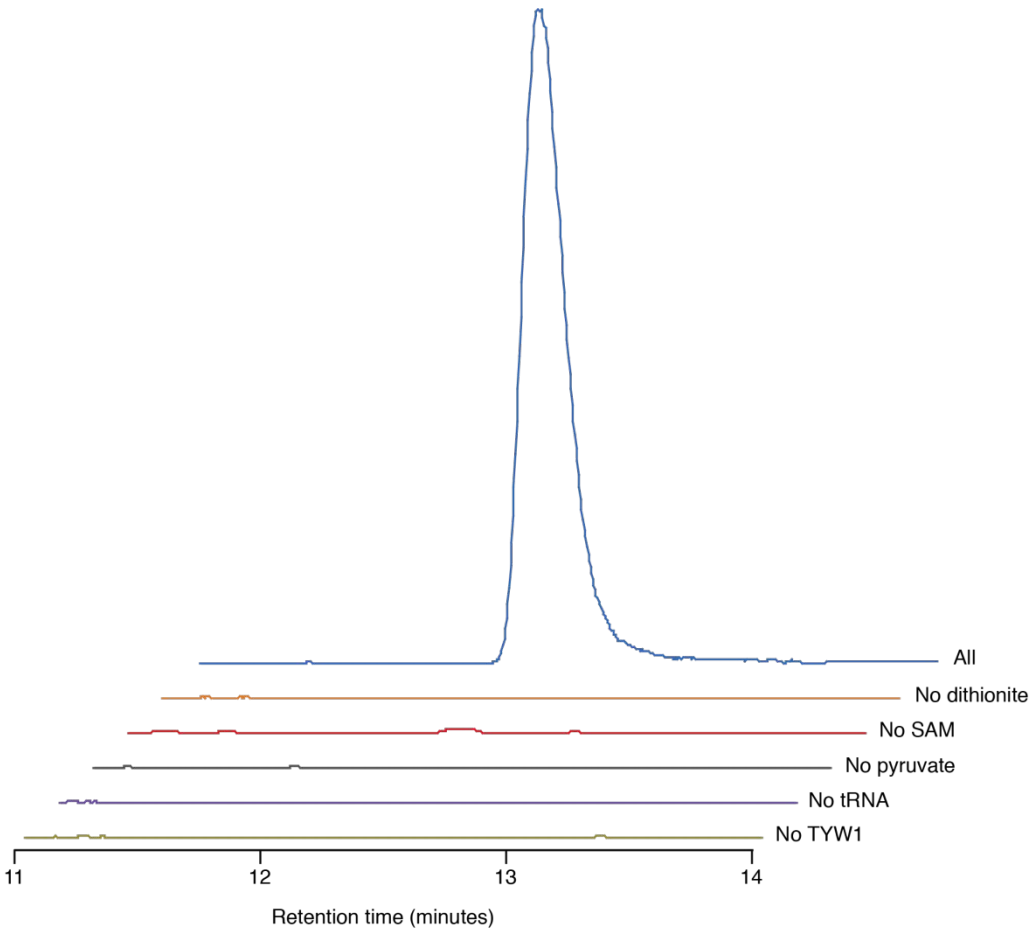

Figure S.4. *Sc*TYW1 catalyzes the conversion of m^1G to imG-14. Extracted ion chromatogram at *m/z* 324.1-324.2 of the digested RNA obtained from the reaction mixtures shows that formation of the modified base requires the presence of pyruvate, SAM, and a reductant.

6. Figure S.5.

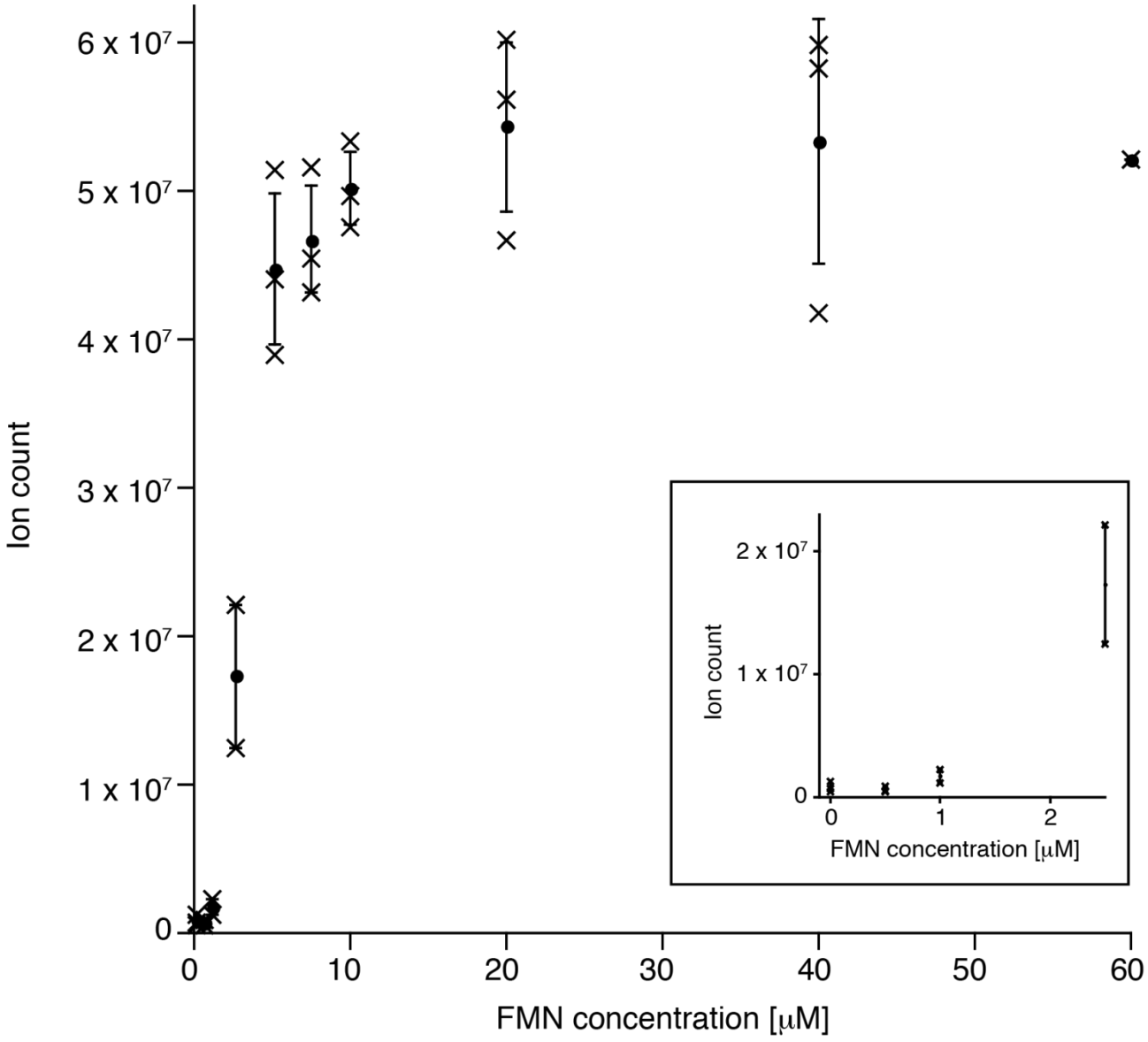

Figure S.5. The product formed by *Sc*TYW1 after 4 hours when incubated with the following concentrations of FMN: 0, 0.5, 1, 2.5, 5, 7.5, 10, 20, 40, and 60 μM. The inset panel has a smaller scale, so the data at 0, 0.5, 1, and 2.5 μM is visible. X represents the experimental data points, the dots represent the mean, and the error bars represent one standard deviation from the mean.

7. Figure S.6.

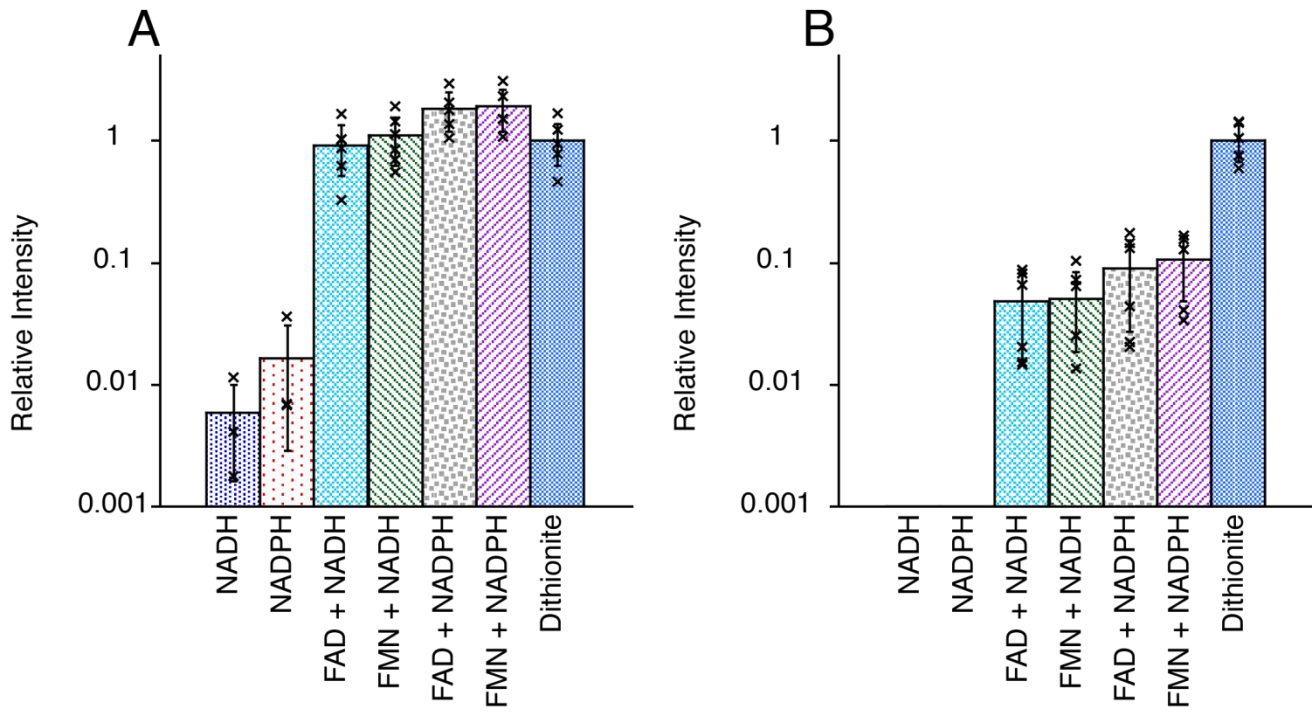

Figure S.6. A comparison of the level of product made by *Sc*TYW1 (panel A) and *Mj*TYW1 (panel B) with different reductants. The data were normalized to dithionite in both the *Sc* and *Mj*TYW1 cases. The error bars represent one standard deviation from the mean and x represents the data points.

7. **Figure S. 7**.

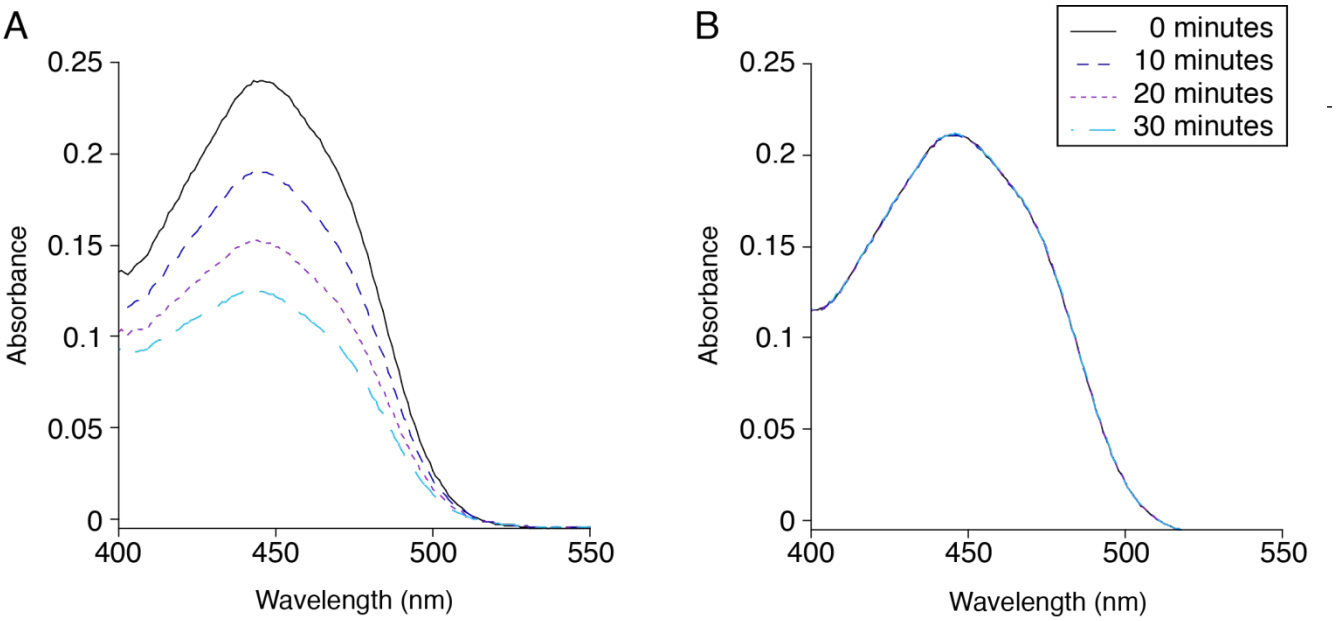

Figure S. 7. Panel A shows the absorbance spectrum between 400 and 550 nm of 20 µM FMN when incubated with 2 mM NADPH. Panel B shows the absorbance of 20 µM FMN in the absence of pyridine nucleotide. The spectrum is shown at 0, 10, 20, and 30 min in both cases.

8. References

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