Mechanistic insight into light-dependent recognition of Timeless by *Drosophila* Cryptochrome

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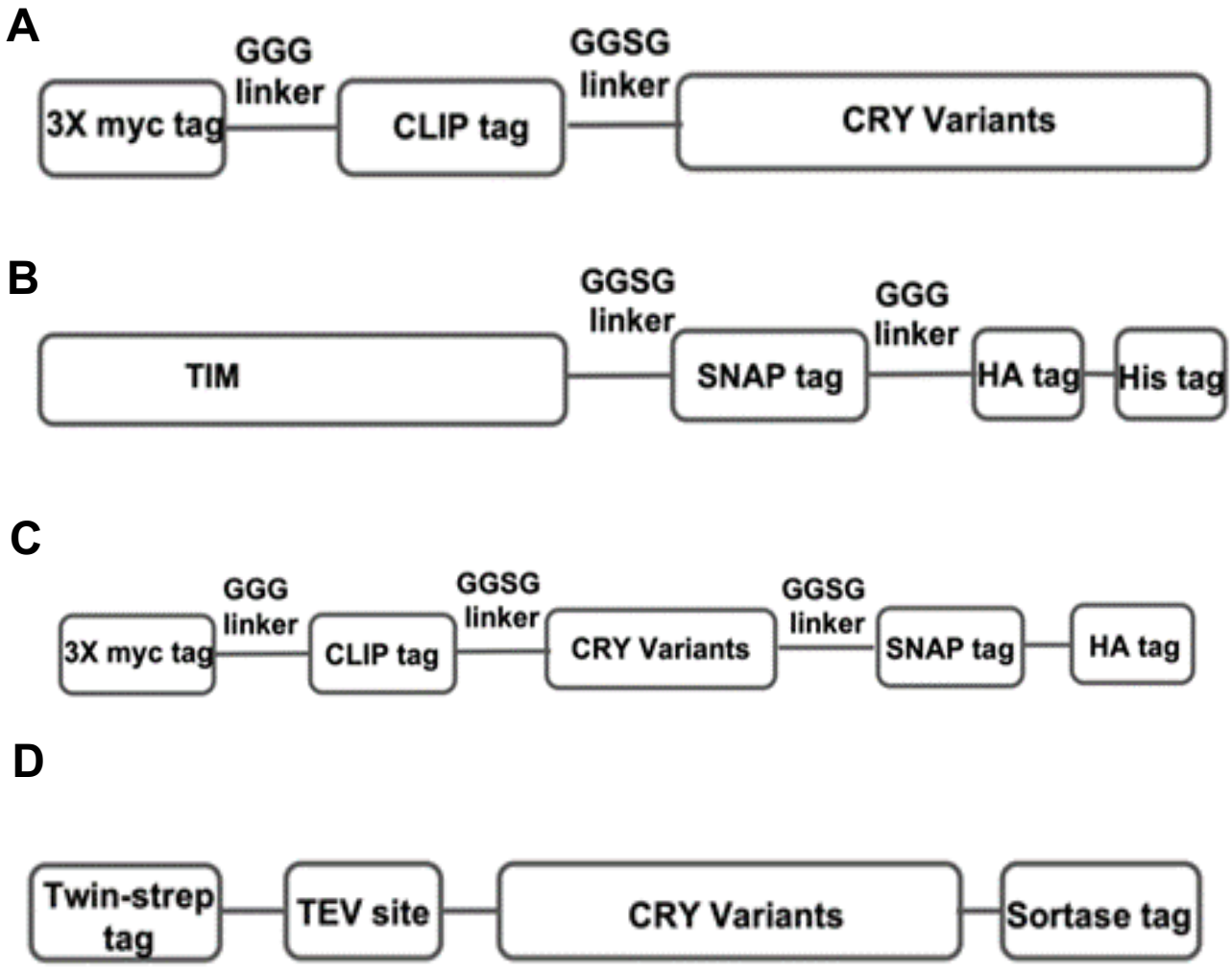

Figure S1: Domain maps of fusions proteins used in this study, Related to Figures 1-4. (A) CLIP-CRY (in pAC5.1), (B) TIM-SNAP-HA (in pAC5.1), CLIP-CRY-SNAP (in pAC5.1) and (D) CRY with sortase tag (in pET28a(+)) fusion proteins made for this study.

Table S1 Primer sequences for Q5 polymerase-based mutagenesis and DNA construct sequences, Related to Figure 1.

Primer name	Sequences
H378A-Forward	ATGGCTCCACgccACGCTGCGCAAC
H378K-Forward	ATGGCTCCACaagACGCTGCGCAAC
H378R-Forward	ATGGCTCCACcgcACGCTGCGCAAC
H378N-Reverse	ATGGCTCCACaacACGCTGCGCAAC
H378Q-Forward	ATGGCTCCACcagACGCTGCGCAAC
H378L-Reverse	ATGGCTCCACctgACGCTGCGCAAC
H378F-Forward	ATGGCTCCACttcACGCTGCGCAAC
H378-Reverse	CCCTCGGCCAGGAGTTGT
H377A/H378A-Forward	GGGATGGCTCgctgccACGCTGCGCAACACCG
H377A/H378A-Reverse	TCGGCCAGGAGTTGTCGC
H377A-Forward	GGGATGGCTCgctCATACGCTGCGCAAC
H377L-Forward	GGGATGGCTCctgCATACGCTGCG
H377-Reverse	TCGGCCAGGAGTTGTCGC
Kozak sequence	AAAAATGG
3x myc- CLIP-CRY in pAC5.1 vector	ATGGAGCAGAAGCTGATCTCAGAGGAGGACCTGGGAGGAGGAGAACAAAA ATTAATAAGTGAAGAAGACCTGGGCGGCGGCGAGCAGAAGCTGATCTCAG AGGAGGACCTGGGAGGAGGAATGGACAAAGACTGCGAAATGAAGCGCACC ACCCTGGATAGCCCTCTGGGCAAGCTGGAACTGTCTGGGTGCGAACAGGG CCTGCACCGTATCATCTTCCTGGGCAAAGGAACATCTGCCGCCGACGCCG TGGAAGTGCCTGCCCCAGCCGCCGTGCTGGGCGGACCAGAGCCACTGATC CAGGCCACCGCCTGGCTCAACGCCTACTTTCACCAGCCTGAGGCCATCGA GGAGTTCCCTGTGCCAGCCCTGCACCACCCAGTGTTCCAGCAGGAGAGCT TTACCCGCCAGGTGCTGTGGAAACTGCTGAAAGTGGTGAAGTTCGGAGAG GTCATCAGCGAGAGCCACCTGGCCGCCCTGGTGGGCAATCCCGCCGCCAC CGCCGCCGTGAACACCGCCCTGGACGGAAATCCCGTGCCCATTCTGATCC CCTGCCACCGGGTGGTGCAGGGCGACAGCGACGTGGGGCCCTACCTGGGC GGGCTCGCCGTGAAAGAGTGGCTGCTGGCCCACGAGGGCCACAGACTGGG

	CAAGCCTGGGCTGGGTGGTGGCTCAGGAATGGCCACGCGAGGGGCGAATG TGATTTGGTTTCGCCATGGATTGCGCCTCCATGATAATCCCGCTCTATTG GCCGCCCTCGCCGATAAGGATCAGGGTATAGCCCTAATTCCCGTTTTCAT ATTCGATGGAGAGAGTGCAGGTACCAAGAATGTGGGTTACAATCGGATGC GTTTCCTCCTGGACTCGTTGCAGGACATCGATGATCAGCTACAGGCGGCA ACTGATGGACGTGGACGCCTCCTGGTCTTCGAGGGCGAACCGGCTTATAT CTTCCGCCGGCTACATGAGCAAGTGCGTCTGCACAGGATTTGCATAGAGC AGGACTGCGAGCCAATTTGGAATGAGCGCGATGAAAGCATCCGTTCTCTA TGTCGGGAGCTGAATATCGACTTTGTCGAGAAGGTATCACACACGCTTTG GGATCCGCAATTGGTGATTGAGACCAATGGTGGCATTCCACCGCTGACCT ACCAAATGTTCCTGCACACGGTGCAAATTATTGGGCTTCCACCGCGTCCC ACCGCCGATGCTCGACTAGAAGACGCCACCTTTGTCGAGCTGGACCCCGA GTTCTGCCGAAGTCTTAAGTTGTTCGAGCAGCTGCCCACGCCGGAGCACT TCAATGTGTATGGAGACAACATGGGCTTCCTGGCCAAGATTAACTGGCGC GGCGGAGAAACACAGGCCTTACTTCTGTTGGATGAGCGTCTTAAAGTGGA GCAGCATGCGTTTGAGCGTGGATTTTATCTGCCCAACCAGGCACTGCCCA ATATCCACGACTCGCCAAAATCGATGAGCGCCCATCTGCGCTTTGGTTGC CTTTCGGTACGTCGCTTCTACTGGAGCGTCCACGATCTCTTCAAGAATGT CCAGTTGCGCGCCTGTGTGCGGGGCGTTCAGATGACTGGCGGCGCGCACA TCACGGGACAGTTGATCTGGCGAGAGTACTTCTACACCATGTCGGTGAAC AATCCAAACTACGATCGCATGGAGGGCAATGACATCTGCCTGAGCATCCC GTGGGCTAAGCCGAACGAAAATCTCCTGCAGAGCTGGCGTTTAGGCCAAA CGGGATTCCCGCTCATCGACGGCGCCATGCGACAACTCCTGGCCGAGGGA TGGCTCCACCATACGCTGCGCAACACCGTGGCCACCTTTCTCACGCGCGG CGGTTTGTGGCAGAGCTGGGAGCATGGACTGCAGCACTTTCTGAAGTATC TGCTGGATGCGGATTGGTCGGTCTGCGCTGGCAACTGGATGTGGGTATCC AGCTCGGCGTTTGAAAGGCTGCTGGACTCCTCCCTGGTCACCTGCCCGGT GGCATTGGCCAAGCGACTTGATCCGGATGGCACCTACATCAAGCAGTACG TCCCGGAGTTGATGAATGTGCCCAAGGAATTTGTTCACGAGCCCTGGCGA ATGTCTGCCGAGCAGCAGGAGCAGTACGAGTGCCTGATCGGAGTCCATTA TCCGGAGCGGATCATTGATTTGTCCATGGCCGTAAAGCGAAACATGCTGG CCATGAAGTCTCTCCGAAATTCGCTGATCACCCCGCCGCCGCATTGCCGA CCATCCAACGAGGAGGAAGTGCGTCAGTTCTTCTGGCTGGCCGACTAA
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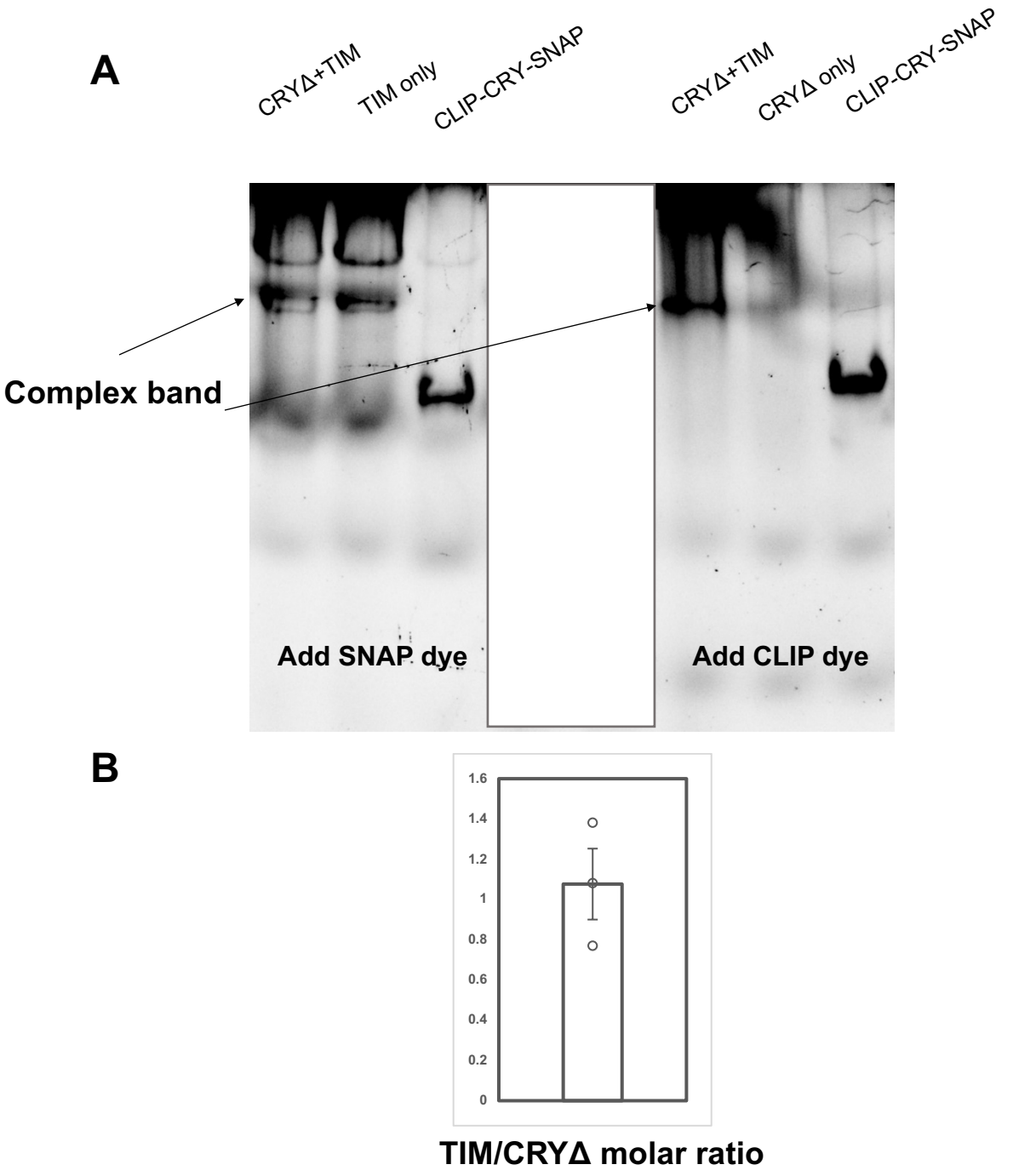


Figure S2: Stoichiometry of CRY binding to TIM, Related to Figure 2. (A) Representative Clear-Native (CN)-PAGE gel of purified CLIP-CRY:TIM-SNAP complexes after dye addition and labeling. Fluorescence detected and imaged by a ChemiDoc (BioRad). Samples on the left were only labeled with SNAP dye and samples on the right were labeled with CLIP dye. Known amount of a CLIP-CRY-SNAP fusion provides the standard for normalization. (B) Quantification of the normalized TIM and CRYΔ fluorescent signal. Error bars represent the SEM for n = 3.

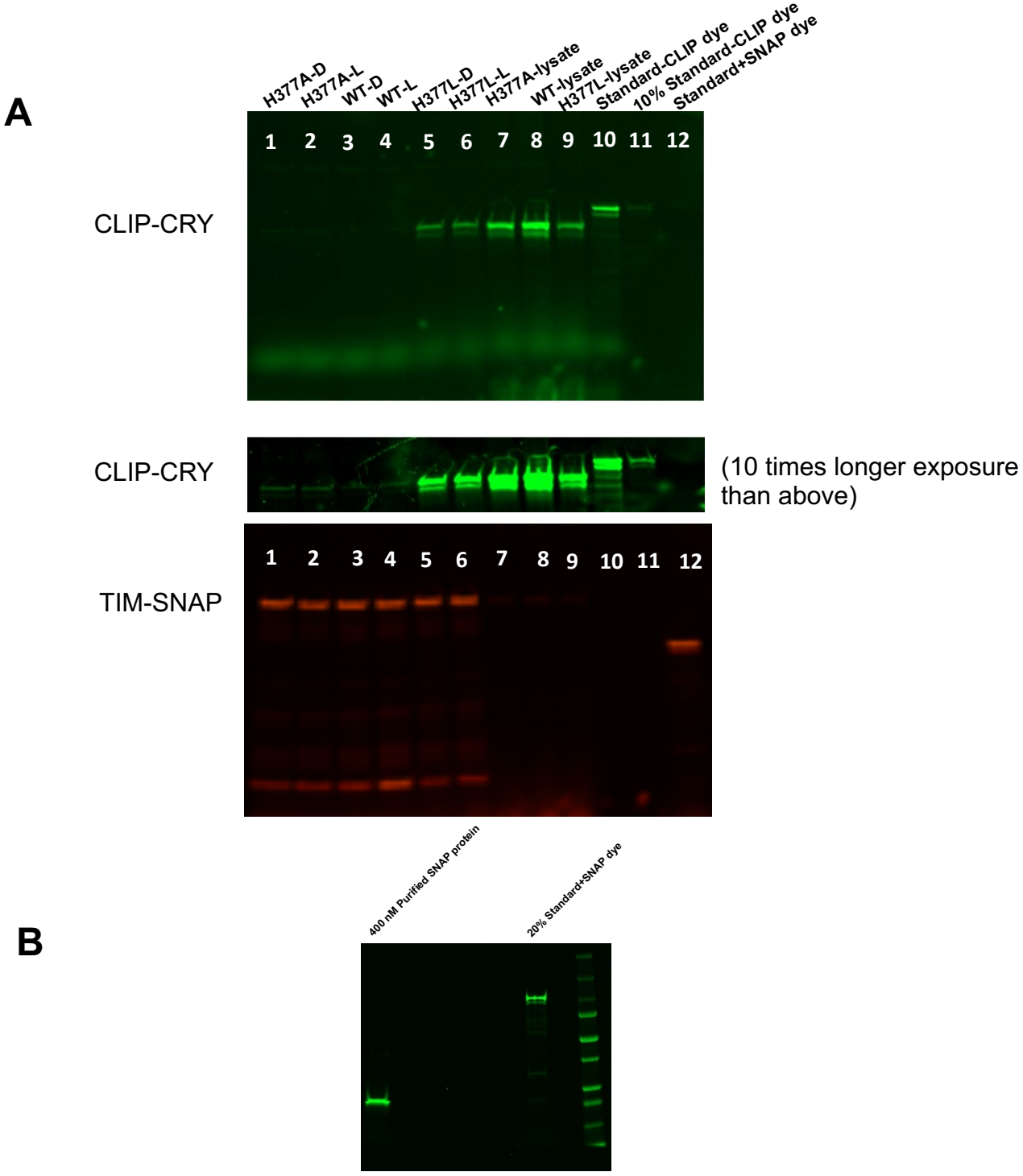


Figure S3: Multiplex imaging of TIM and CRY bands, Related to Figures 2 and 3. (A) Lane 1-6 show TIM and CRY SWFTI signals on HA resin, whereas lane 7-9 show the signals in lysate samples. Lane 10-12 exhibit fluorescent signals from the internal standard CLIP-CRY-SNAP. To prevent fluorescence crosstalk, Lane 10 and 11 have the standard only mixed with CLIP dye, whereas lane 12 contains the standard with only SNAP dye. In lane 11 the standard was diluted to 10%. (B) The quantification of standard by purified SNAP proteins. The standard was diluted to 20% and mixed with SNAP dye.

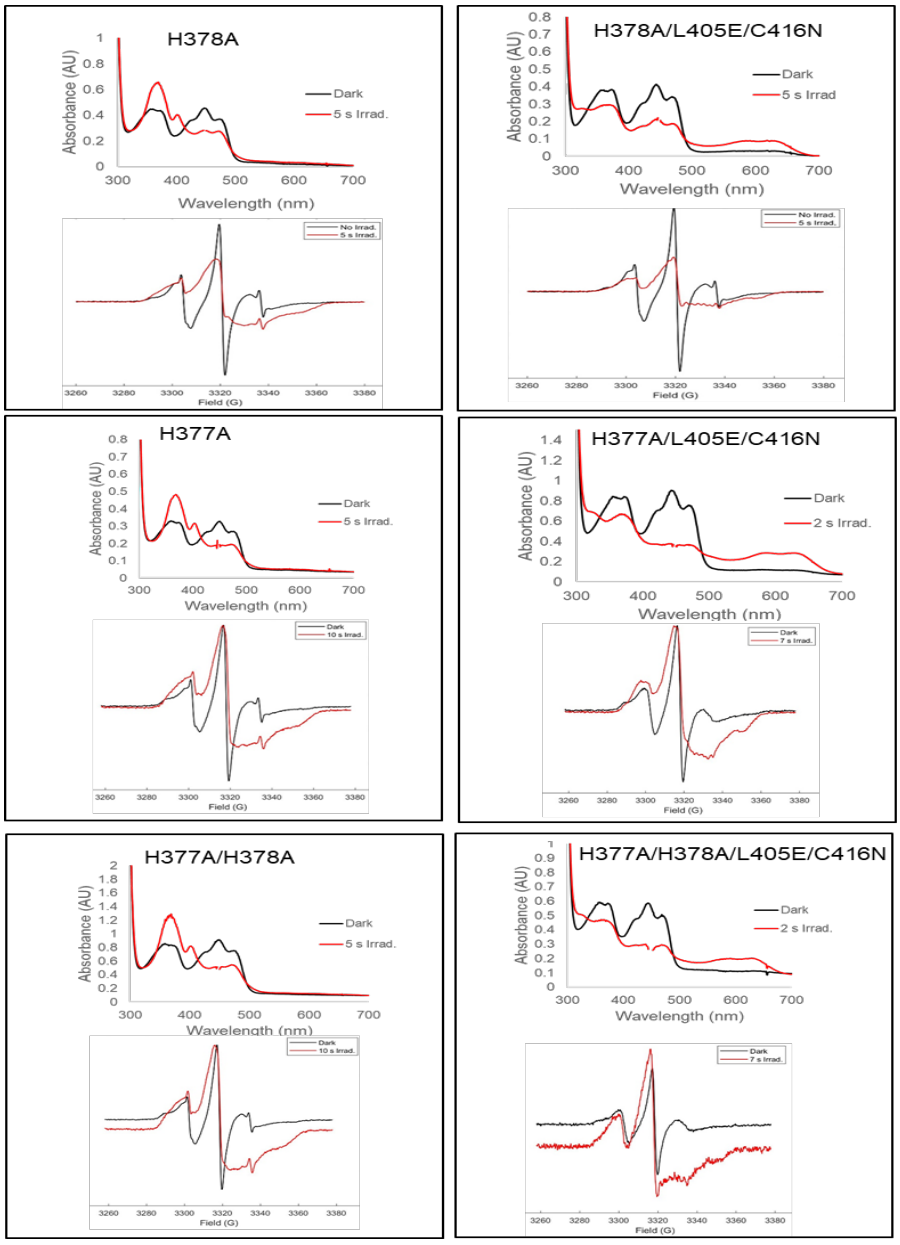


Figure S4: Spectroscopy of CRY variants, Related to Figure 4. UV-Vis and cw-ESR spectra of His377 and His378 alanine variants in the dark and after 2-5 sec of blue light exposure at 440 nm. The left column are all variants in the WT background, while the right column is those in the L405E/C416N background. In UV-Vis (top half of each panel), all WT background variants produce the ASQ with a characteristic absorbance at 364 and 403 nm, while the L405E/C416N background variants produce the NSQ (broadband feature ~550-650 nm). Gaps in spectra are at the excitation laser wavelength; Time of irradiation for full reduction varies between 2-5 second depending on laser alignment with respect to the cuvette. cwESR (bottom half of each panel) spectra of all dCRY variants labeled at the C-terminus with a nitroxide label. All spectra were recorded at X-band in deuterated buffer. Broadened features in light-state spectra reflect overlapping flavin and nitroxide features.

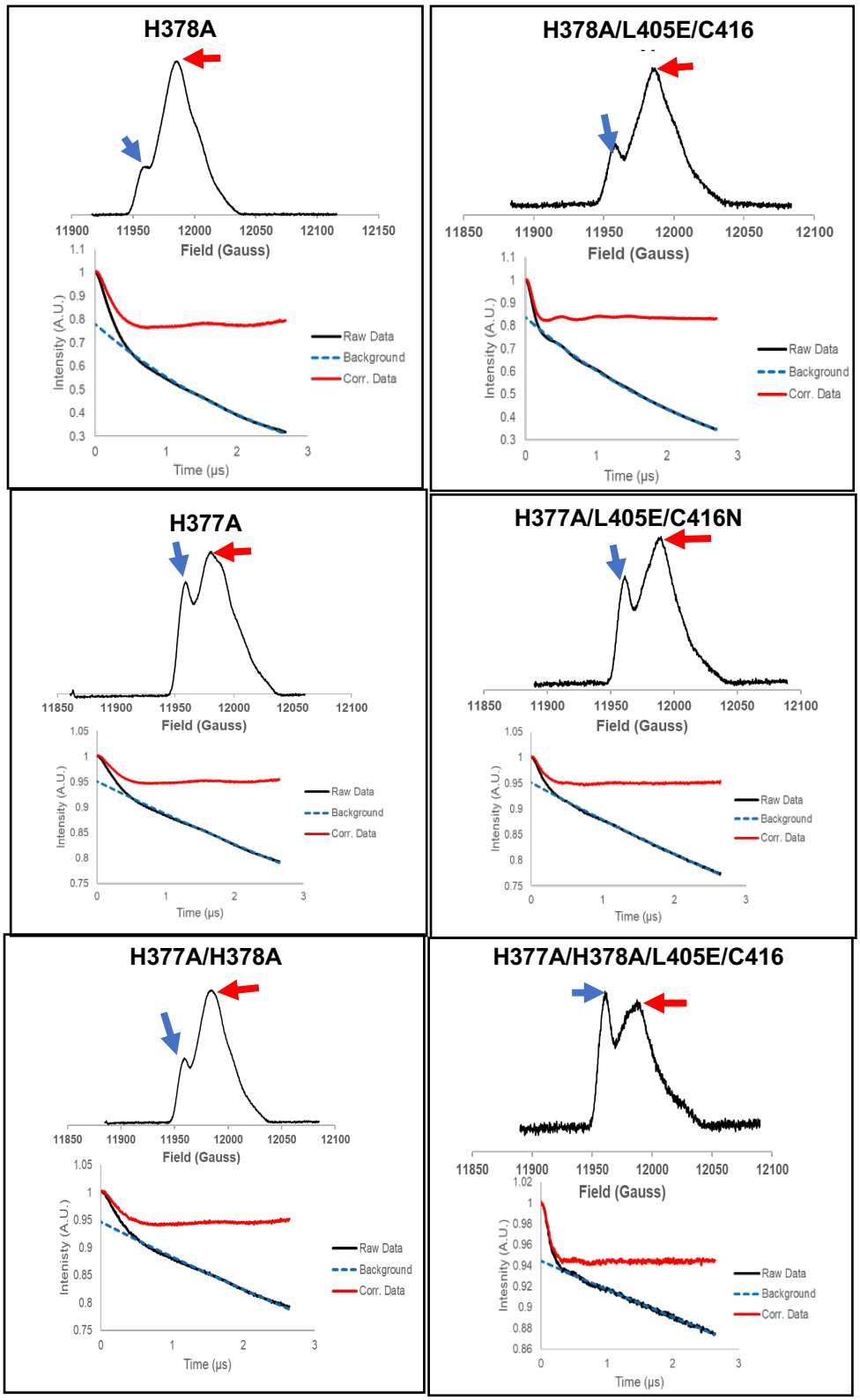


Figure S5: Representative field swept echoes (FSE), primary DEER traces, background subtractions, and corrected signals of H377/H378 variants after light irradiation at Q-band, Related to Figure 4. Probe (nitroxide) and pump (flavin) pulses are marked by arrows blue and red arrows, respectively, and are separated by 84 MHz (~30G).

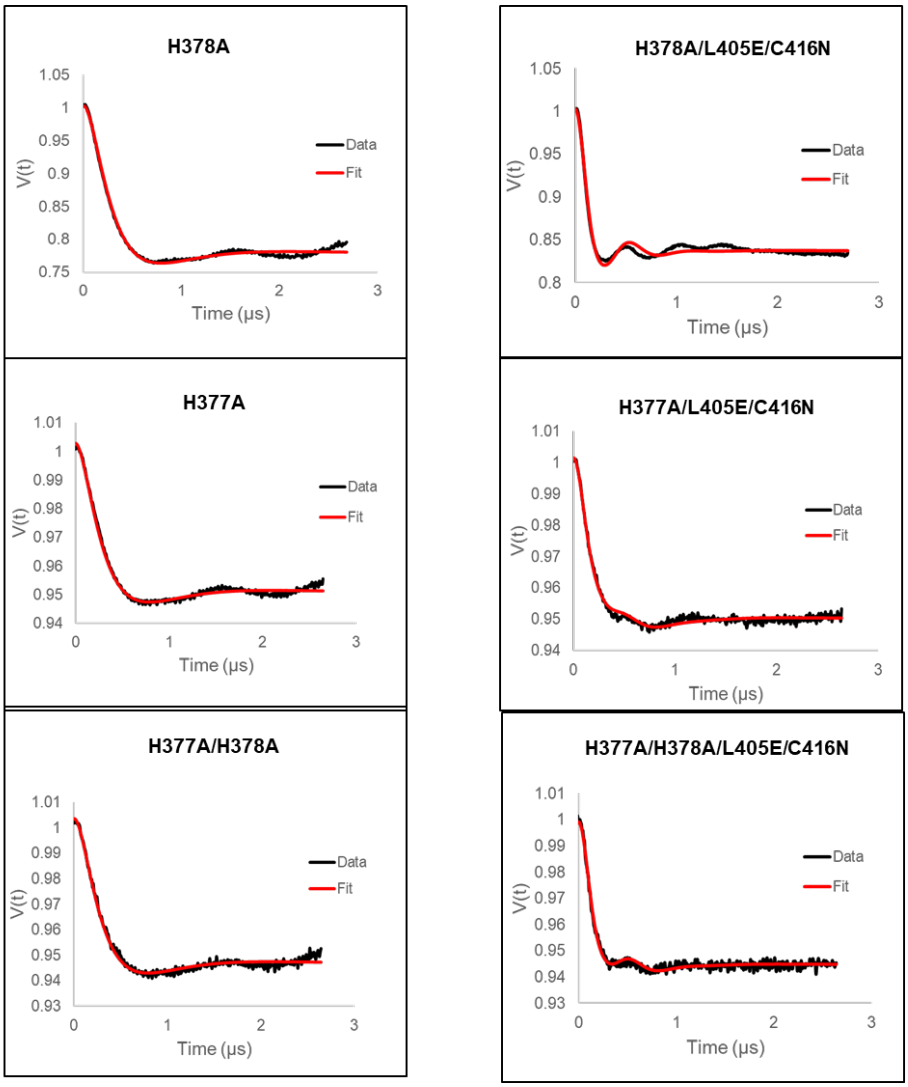


Figure S6: Time domain traces of all alanine variants and their respective fits carried out based on a restrained two-state model previously established with DD, Related to Figure 4. All ASQ forming variants (left) were fit with one component, while all NSQ forming variants (right) were fit using two components. Fittings were carried out as detailed in the **Methods**.

Table S2: Details of restrained DD fittings, Related to Table 1 and Figure 4.

Variant	% Undocked	χ_v^2 (error in fit)
H377A	100*	3.1**
H378A	100*	7.1**
H377A/H378A	100*	1.4
H377A/L405E/C416N	63 ± 1 49 ± 13 58 ± 5***	1.4 1.1 1.0
H378A/L405E/C416N	17 ± 0.3 32 ± 6 29 ± 4***	24.** 1.0 0.9
H377A/H378A/L405E/C416N	42 ± 2 54 ± 30 39 ± 17***	1.0 1.4 1.0

Table S2: Distance distributions obtained from one and two gaussian fittings of time domains by DD. Residual error χ_v^2 values are determined by DD and indicate the robustness of the fit to the data. The $\langle R \rangle$,σ with the short component listed first, and long component listed second. H377A, H378A, and H377A/H378A are all WT-like and were fit best using only the undocked component (*). The relatively high error (**) reflects the small amplitude and broad features of this component. Samples measured at pH 7 are indicated by (***).

Table S3: Percent undocked state for Ala variants using linear combination fit, Related to Figure 4.

Variant	% Undocked
H377A	100
H378A	100
H377A/H378A	100
H377A/L405E/C416N	50 ± 5*
H378A/L405E/C416N	16 ± 1.3*
H377A/H378A/L405E/C416N	36 ± 6*

Table S3: Values determined by fitting the time domain traces of the H377A/H378A variants with a linear combination of the time domain traces of the WT & EN variant (described in the methods section). Uncertainty values obtained for n = 3 samples (*).

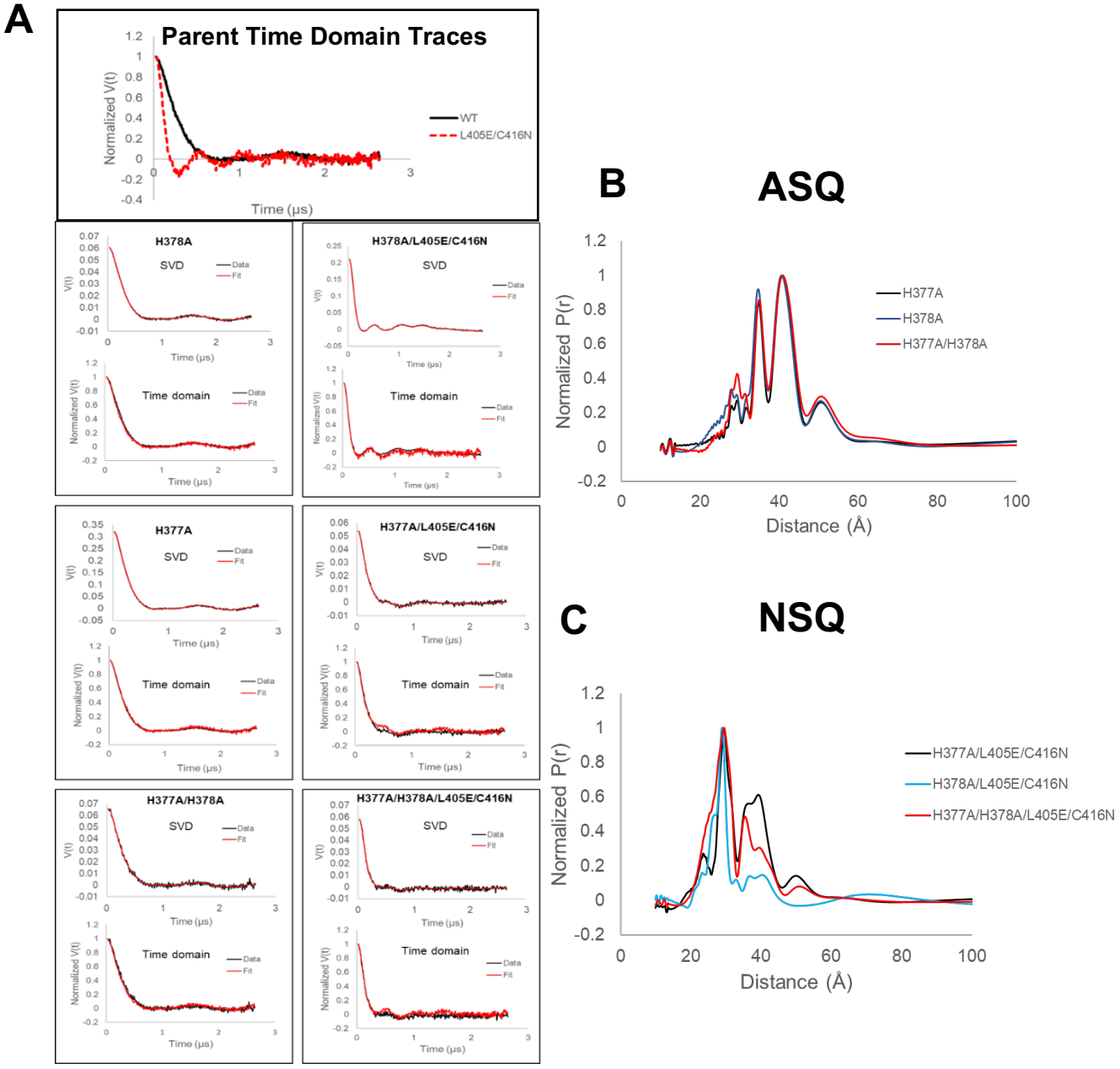
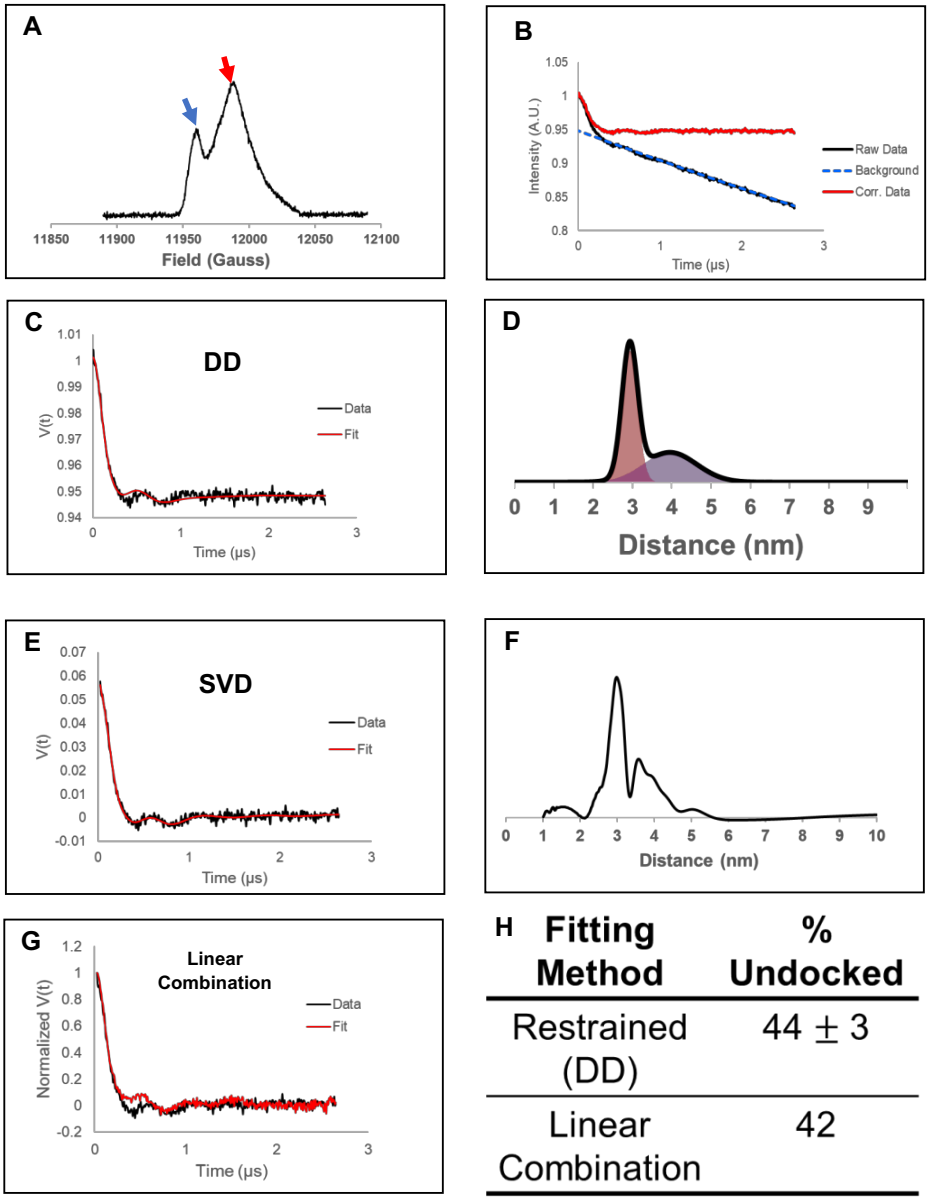


Figure S7: Analysis of alanine variant DEER data with the SVD and time-domain methods, Related to Figure 4. (**A**) Parent time trace components shown at top, followed by fits to experimental data to provide the component weights of Table S2. (**B and C**) Distance distributions obtained using the SVD method for all the alanine variants. The SVD fits qualitatively agrees with the DD and linear combination fits. The ASQ (WT background) forming variants (**B**) have larger distance peaks at ~35-45 Å corresponding to the undocked state. whereas the NSQ (EN background) forming variants (**C**) have peaks at both ~25-30 Å and 35-45 Å, thereby indicating a mixture of the docked and undocked states.



H

Fitting Method	% Undocked
Restrained (DD)	44 ± 3
Linear Combination	42

Figure S8: H377L/L405E/C416N DEER data, Related to Figure 4. (**A**) Field swept echo with probe and pump pulses marked with blue and red arrows, respectively. (**B**) Primary DEER trace along with background subtracted data, and corrected signals obtained from DD. (**C**) Time domain fitting using restrained two component fit ($\langle R \rangle$,**σ** the docked and undocked states are (29.3, 2.0 Å) and (39.5, 6.8 Å), respectively; χ_v^2=0.4). (**D**) Distance distribution calculated by DD, corresponding to 44 ± 3% undocked. (**E**) Time domain fitting using the SVD method. (**F**) Distance distribution calculated by SVD. (**G**) Linear combination of time domain fit, corresponding to 42% undocking. (**H**) Table summarizing results of undocking from quantifiable fitting methods.

Table S4: Gibson Assembly master mix, Related to Figures 2, 3 and 4.

	Stock concentration (U/μL)	Final Concentration (U/μL)	Volume (μL)
Q5 reaction buffer	5 X	1.3 X	234
Q5 DNA polymerase	2	0.033	14.8
Taq DNA ligase	40	5.3	119.2
T5 Exonuclease (dilute with 1X NEbuffer 4)	0.5	0.005	9
PEG-8000	50%	6.7%	120.6
NAD^+	50 mM (33.2 mg/ml)	1.3 mM	23.4
dNTPs	2.5 mM	0.27 mM	97.2
Water			282
		Total	900

Reagents all purchased from New England Biolabs, except PEG-8000 (Cat# HR21-535, Hampton Research) and NAD^+ (Cat# 16077, Cayman Chemical).