

## **Supplementary Information**

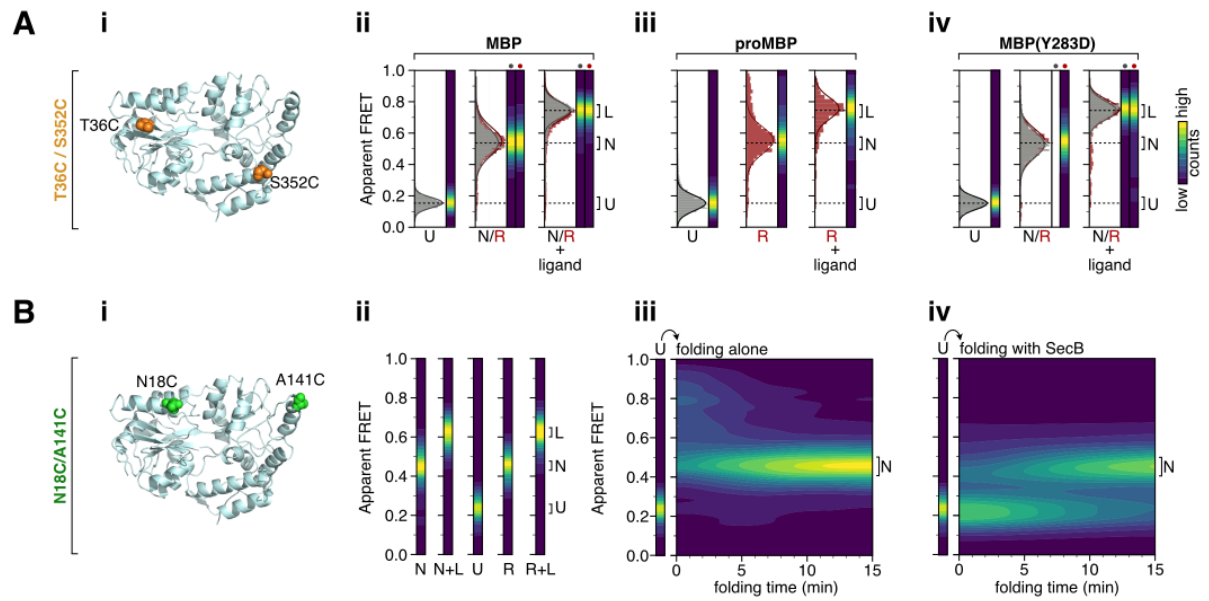
### **How SecB maintains clients in a translocation competent state**

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**Supplementary Figure 1 | Refolding of MBP derivatives, monitored by smFRET.**

**A.** The 36C/352C cysteine pair.

**i.** Position of the cysteine pair (shown as spheres) used for fluorophore labeling shown on the MBP structure (PDB code: 1ANF)

**ii to iv.** Apparent FRET histograms of the indicated steady states of MBP, proMBP and MBP(Y283D). Native MBP (N, in grey) can bind maltotriose (L, grey). Urea-unfolded MBP (U, 8 M urea) can refold to its native state (R, 200-fold dilution in native buffer, red) and bind maltotriose (R red + ligand). To facilitate visualization, the histograms were represented by kernel-density estimation and visualized as color maps ranging from purple to yellow / low to high event numbers (right slices).

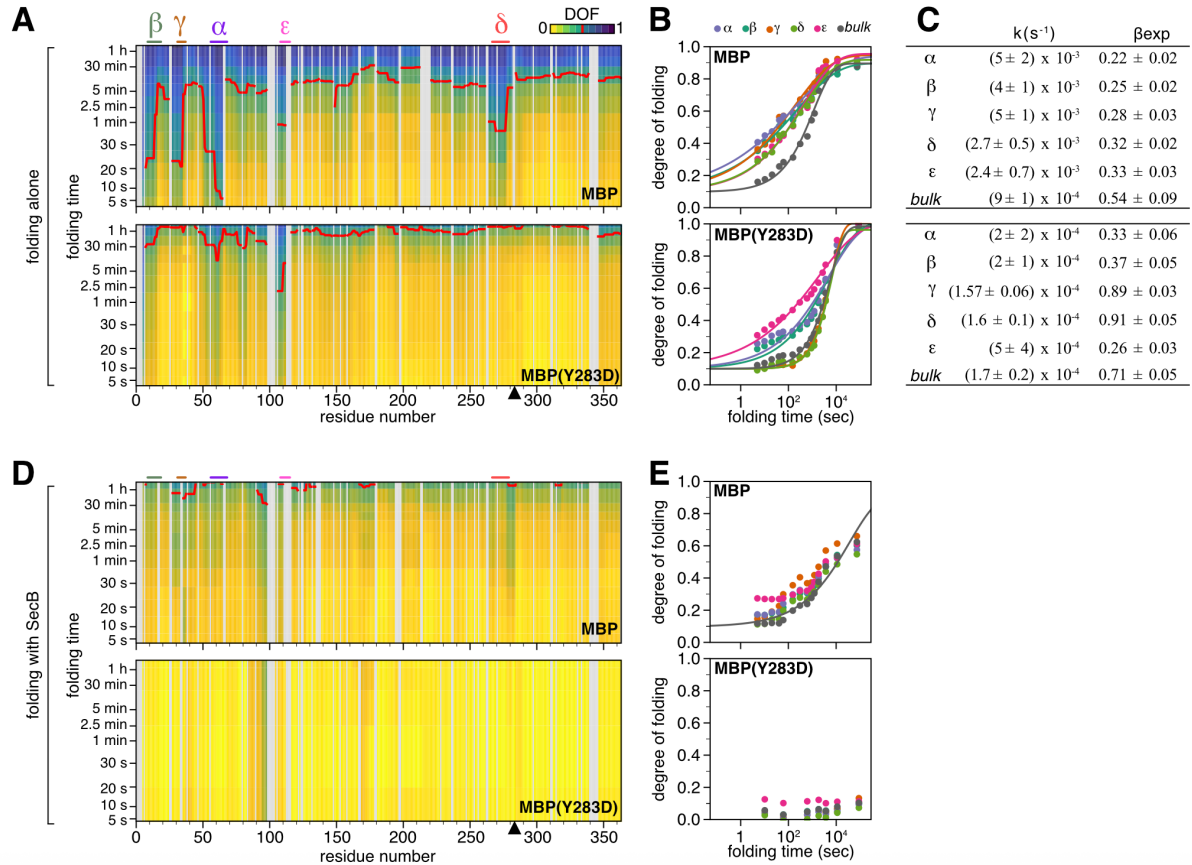
**B.** The 18C/141C cysteine pair:

**i.** Position of the cysteine pair (shown as spheres) used for fluorophore labeling shown on the MBP structure (PDB code: 1ANF)

**ii.** Apparent FRET values for the indicated steady states of MBP carrying fluorophores on the 18C/141C cysteine pair, as in A.ii-iv.

**iii.** Refolding of MBP<sub>18C/141C</sub> was monitored by smFRET, as in Fig 1A.

**iv.** Refolding of MBP<sub>18C/141C</sub> in the presence of SecB<sub>4</sub>, by smFRET, as in Fig 2A



### Supplementary Figure 2 | Refolding of MBP in the absence/ presence of SecB, monitored by local HDX-MS.

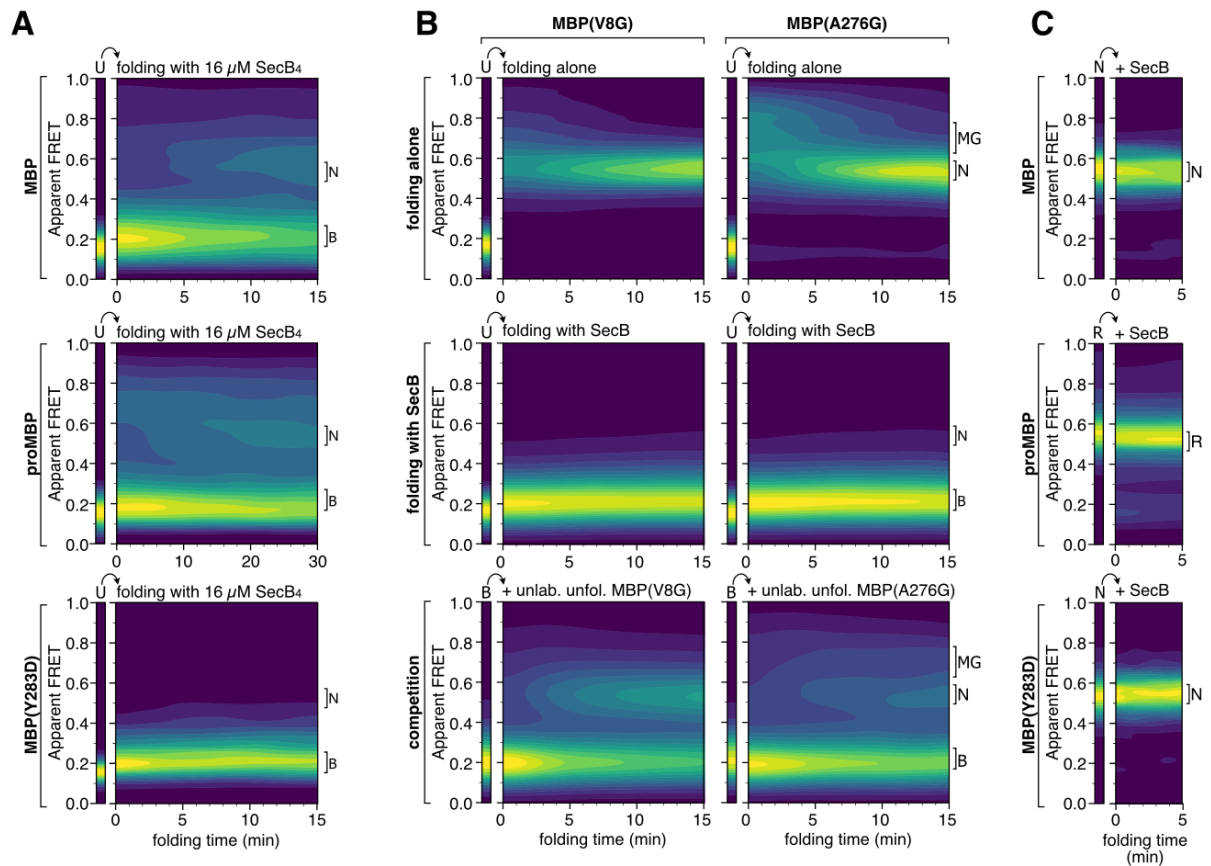
**A.** Refolding of unfolded MBP, or MBP(Y283D), in native buffer (30-fold dilution, to 0.2 M urea) for the indicated time, before samples were pulse-labelled in D<sub>2</sub>O (10 sec), quenched (ice), and MS analyzed (see M&M). The determined D-uptake per peptide (Supplementary Data 1) was converted to degree of folding per residue following normalization using PyHDX (DOF; Supplementary Data 2) and is shown as a color code ranging from yellow=0 DOF (i.e. unfolded protein in 6 M urea) to purple=1 DOF (i.e. native protein). Grey: prolines / non-identified regions. Greek letters: foldons in order of appearance. Black arrow: Y283D mutation on the linear sequence. Red line: 0.5 DOF.

**B.** DOF as function of time for individual foldons and bulk protein for MBP (top) and MBP(Y283D) (bottom). Lines are stretched exponential fits ( $\sim (1 - \exp(-(kt)^\beta))$ ). Initial DOF at  $t=0$  is set to 0.1 while asymptote values are fit parameters (limited to 1) to obtain good fits.

**C.** Fit parameters of stretched exponential fits. For MBP (top), fitted foldon rates are clustered into two groups, where foldons α, β and γ have similar rates while the remaining foldons δ and ε fold slower. All foldons have stretched stretching exponent  $\beta_{exp}$  well below one, ranging from  $\beta_{exp} = 0.22$  (α) to  $\beta_{exp} = 0.33$  (ε), indicative of heterogeneous folding pathways with many kinetic traps or folding steps. The final folding step in the bulk protein with  $\beta_{exp}$  closer to one is more characteristic of a single folding step. For MBP(Y283D), foldons α, β and ε showed similar heterogeneity compared to WT, albeit with a reduced folding rate. Foldons γ and δ, other the other hand, are absent as they fold identical to bulk protein as a single folding step.

**D.** As in a. but SecB<sub>4</sub> (1:1 client:SecB<sub>4</sub> molar ratio) is present in the native buffer.

**E.** as in b. but in the presence of SecB. Since no folding plateau was reached the asymptote value for the fit was set to the mean asymptote value of MBP (0.9 DOF), yielding a bulk folding rate of  $2.8 (\pm 0.8) \times 10^{-5} s^{-1}$  with  $\beta_{exp} = 0.36$ .

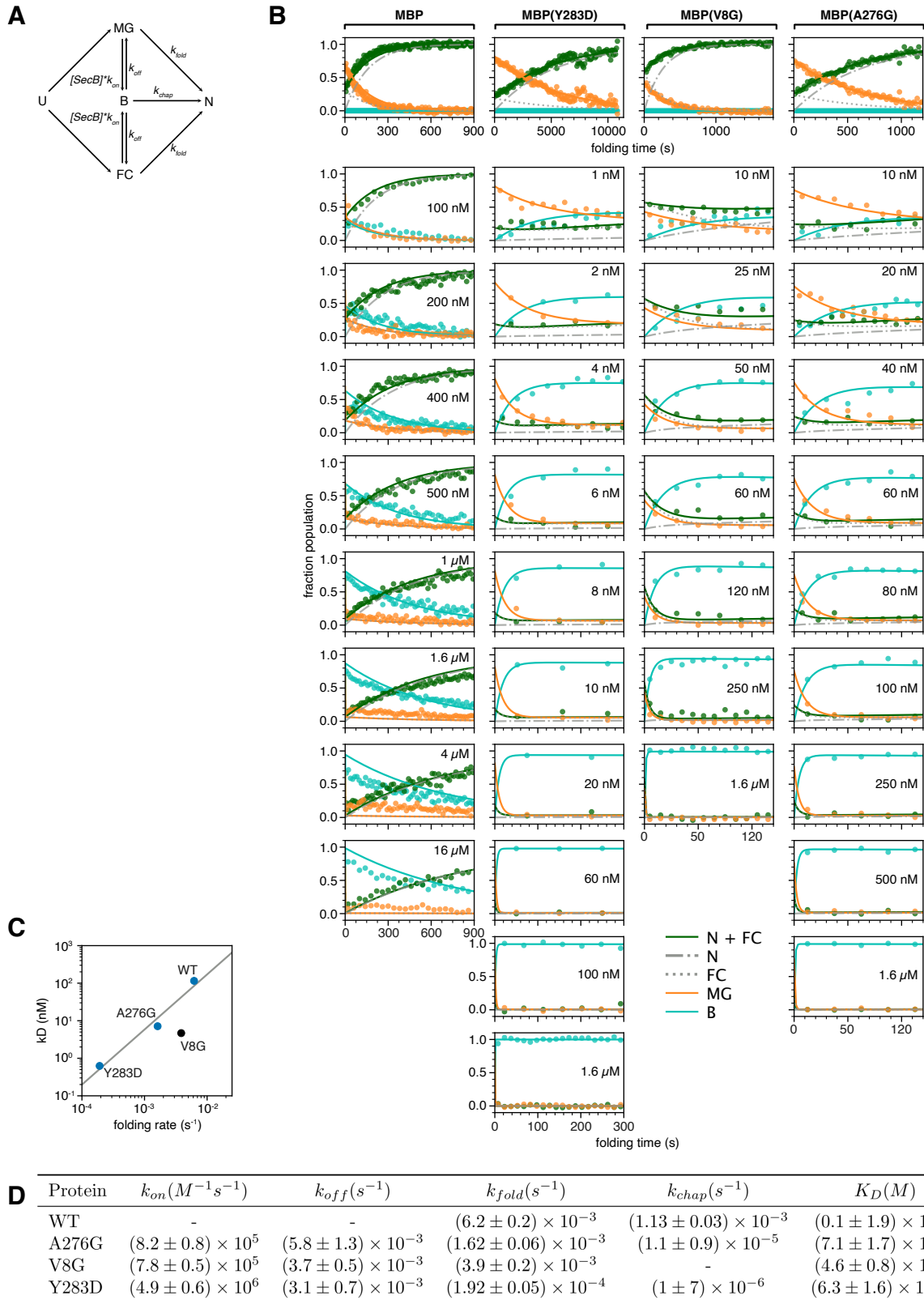


**Supplementary Figure 3 | Effect of SecB on the folding of MBP, monitored by smFRET.**

**A.** Unfolded proteins (U, left slices; as indicated) were refolded in the presence of SecB<sub>4</sub> excess (16  $\mu$ M). Apparent FRET values over course of time (right panels) are visualized as in Fig 1A.

**B.** Effect of the indicated single-point mutations on the refolding of MBP alone (top), in the presence of SecB (1.6  $\mu$ M, middle); or unfolded proteins were allowed to fold in the presence of SecB for 5 seconds before adding excess of unlabeled/unfolded client (2.5  $\mu$ M, competition, bottom).

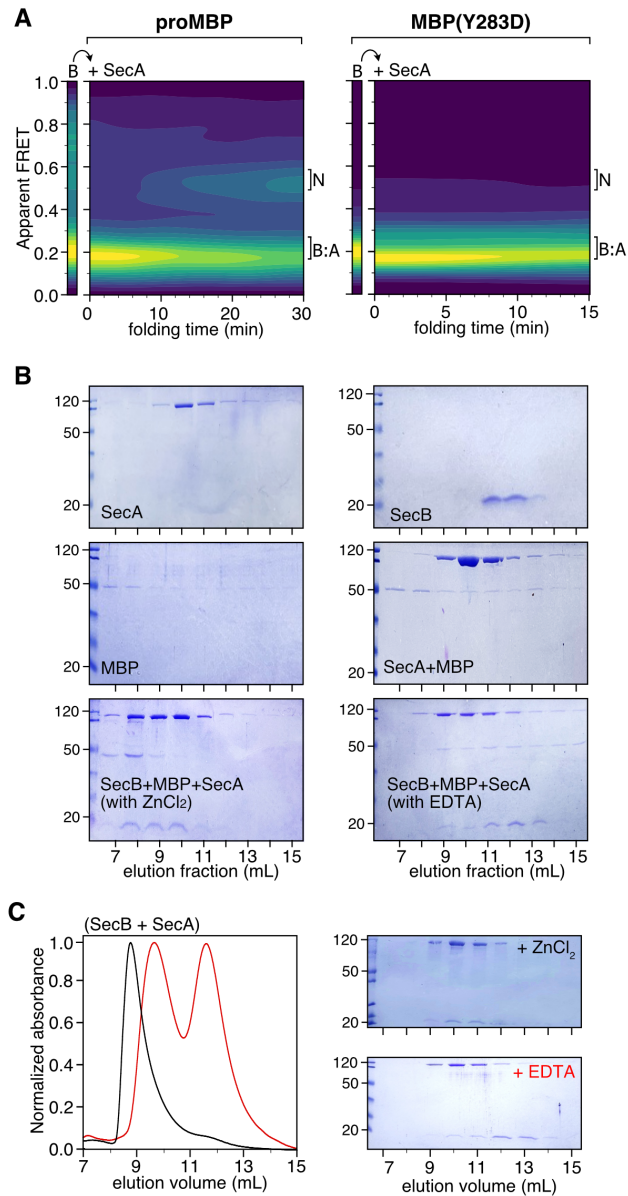
**C.** Native MBP and MBP(Y283D), or refolded proMBP (2 hours at room temperature) (left slices), were mixed with SecB<sub>4</sub> (1.6  $\mu$ M). Apparent FRET as a function of time (right panels) are visualized as in A.



(legend on the next page)

**Supplementary Figure 4 | Global fit of folding kinetics of MBP WT, Y283D, V8G and A276G in the presence of various concentrations of SecB.**

- A.** Kinetic model used in global fits. The model assumes that both the MG and FC states are recognized by SecB with identical  $k_{on}/k_{off}$  rates and fold with equal rates  $k_{fold}$  when free from SecB. An on-chaperone pathway is included with rate  $k_{chap}$ . The forward rate to formation of the SecB complex is  $[SecB] \cdot k_{on}$ . V8G was fitted with a model without on-chaperone folding since including it resulted in the fit hitting a constraint boundary ( $k_{chap} > 0$ ).
- B.** Measured population kinetics from fitting gaussian mixture modules to smFRET data of MG, FC/ $N_{burst}$  (burst-phase native) and B states of MBP derivatives (points) and global fits (lines). Measured  $E_{app}(FC/N_{burst})$  is modelled as the sum of folding core (FC, dotted grey) and native (as modelled, dotted dashed grey) populations.
- C.** Obtained dissociation constants from fitting for MBP and folding mutants versus their folding rate. The gray line is power-law fit (linear relation in log-log space, excluding V8G), relating the energy barriers of folding and chaperone release.
- D.** Fit parameters ( $k_{on}$ ,  $k_{off}$ ,  $k_{fold}$ ,  $k_{chap}$ ) and resulting dissociation constant ( $k_{off}/k_{on}$ ). For WT, on and off rates are not resolved since the protein folds fast and SecB affinity is low. Reported errors are standard errors (see methods). The high standard error of WT  $K_D$  is attributed to high covariance/standard error on rates  $k_{on}/k_{off}$ .



### Supplementary Figure 5 | SecA stabilizes the MBP:SecB<sub>4</sub> complex.

**A.** Urea-unfolded proMBP or MBP(Y283D), as indicated, were allowed to refold in the presence of SecB<sub>4</sub> (1.6  $\mu$ M) for 5 seconds and form the SecB-bound state (B; left slices) before adding SecA<sub>2</sub> (2.5  $\mu$ M; right panels), as in Fig 4B, by smFRET.

**B.** SDS-PAGE analysis of the fractions from SEC-MALS. Uncropped gels presented in Fig 4D.

**C.** SEC-MALS analysis of the SecA<sub>2</sub>:SecB<sub>4</sub> complex (15  $\mu$ M, 1:1 molar ratio, left panel) in a buffer containing ZnCl<sub>2</sub> (30  $\mu$ M, black line) or EDTA excess (2 mM, red line). SDS-PAGE analysis of the indicated chromatographic fractions (right panels).

**Supplementary Table 1 | List of cloned genes and primers.**

| <b>Gene</b>                  | <b>Reference</b> | <b>Description</b>                                                                                                                                                                                                                                                                                                                 |
|------------------------------|------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>mbp-his</i>               | <b>pIMBB1506</b> | Maltose binding protein with C-terminal his-tag                                                                                                                                                                                                                                                                                    |
| <i>proMBP(T62C/S378C)</i>    | <b>pLMB1916</b>  | As in de Boer <i>et al.</i> (2021) <sup>46</sup>                                                                                                                                                                                                                                                                                   |
| <i>mbp(T36C/S352C)</i>       | <b>pLMB1917</b>  | As in de Boer <i>et al.</i> (2021) <sup>46</sup>                                                                                                                                                                                                                                                                                   |
| <i>mbp(V8G/T36C/S352C)</i>   | <b>pLMB2173</b>  | Template: pLMB1917<br>V8G primers: X2206 (5' GAT AAT GTC AGG GCC <b>ATT</b> GCC AGT TGC CGC AAC 3') and X2207 (5' GCC GTT AAT CCA GAT <b>GCC</b> CAG TTT ACC TTC TTC 3')                                                                                                                                                           |
| <i>mbp(T36C/A276G/S352C)</i> | <b>pLMB2050</b>  | Template pLMB1917<br>A276G primers: X1575 (5' CCG AAC AAA GAG CTG <b>GGG</b> AAA GAG TTC CTC GAA 3') and X1577 (5' TTC GAG GAA CTC TTT <b>CCC</b> CAG CTC TTT GTT CGG 3')                                                                                                                                                          |
| <i>mbp(T36C/S352C/Y283D)</i> | <b>pLMB2049</b>  | Template: pLMB1917<br>Y283D primers: X1573 (5' GAG TTC CTC GAA AAC <b>GAT</b> CTG CTG ACT GAT GAA 3') and X1574 (5' TTC ATC AGT CAG CAG <b>ATC</b> GTT TTC GAG GAA CTC 3')                                                                                                                                                         |
| <i>mbp(N18C/A141C)</i>       | <b>pLMB2206</b>  | Template: pIMBB1506<br>N18C primers: X2438 (5' GGC GAT AAA GGC TAT <b>TGC</b> GGT CTC GCT GAA GTC 3') and X2439 (5' GAC TTC AGC GAG ACC <b>GCA</b> ATA GCC TTT ATC GCC 3')<br>A141C primers: X2436 (5' GAT AAA GAA CTG AAA <b>TGC</b> AAA GGT AAG AGC GCG 3') and X2437 (5' CGC GCT CTT ACC TTT <b>GCA</b> TTT CAG TTC TTT ATC 3') |

**Supplementary Table 2 | List of buffers**

|          |                                                                                  |
|----------|----------------------------------------------------------------------------------|
| Buffer A | 50 mM Tris-HCl pH 8.0, 400 mM NaCl, 5 mM imidazole, and 5% glycerol              |
| Buffer B | 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM imidazole, and 5% glycerol               |
| Buffer C | 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 200 mM imidazole, and 5% glycerol             |
| Buffer D | 50 mM Tris-HCl pH 8.0, 50 mM NaCl, and 5% glycerol                               |
| Buffer E | 50 mM Tris-HCl pH 8.0, 50 mM NaCl, and 50% glycerol                              |
| Buffer F | 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 1 M urea and 1% (v/v) Triton X-100 |
| Buffer G | 50 mM Tris-HCl pH 8.0, 400 mM NaCl, 1 mM DTT, 5 mM imidazole and 6 M urea        |
| Buffer H | 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 200 mM imidazole, and 6 M urea                |
| Buffer I | 50 mM Tris-HCl pH 8.0, 50 mM NaCl, and 6M urea                                   |
| Buffer J | 50 mM Tris-HCl pH 8.0, 400 mM NaCl and 5% glycerol                               |
| Buffer K | 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5% glycerol                                  |
| Buffer L | 50 mM Tris-HCl pH 7.4, 200 mM KCl, 1 mM MgCl <sub>2</sub> and 10% glycerol       |
| Buffer M | 50 mM Tris-HCl pH 7.4, 1 M KCl, 1 mM MgCl <sub>2</sub> and 10% glycerol          |
| Buffer N | 50 mM Tris-HCl pH 7.4, 50 mM KCl and 10% glycerol                                |
| Buffer O | 50 mM Tris-HCl pH 8.0, 50 mM NaCl, and 1 mM Trollox                              |
| Buffer P | 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM Trollox and 8 M urea                     |
| Buffer Q | 25 mM Tris-HCl pH 8.0, 25 mM KCl and 8 M urea                                    |
| Buffer R | 25 mM Tris-HCl pH 8.0 and 25 mM KCl                                              |

**Supplementary Table 3 | Gaussian mixture model: fitting parameters**

| Client     | N-state |          | MG-state |          | B-state |          |
|------------|---------|----------|----------|----------|---------|----------|
|            | $\mu$   | $\delta$ | $\mu$    | $\delta$ | $\mu$   | $\delta$ |
| WT         | 0.55    | 0.07     | 0.77     | 0.11     | 0.24    | 0.08     |
| proMBP     | 0.55    | 0.08     | 0.79     | 0.09     | 0.24    | 0.10     |
| MBP(Y283D) | 0.53    | 0.08     | 0.77     | 0.11     | 0.21    | 0.08     |
| MBP(V8G)   | 0.55    | 0.07     | 0.62     | 0.17     | 0.23    | 0.08     |
| MBP(A276G) | 0.53    | 0.07     | 0.76     | 0.11     | 0.23    | 0.08     |

**Supplementary Table 4 | Gaussian mixture model: replicability**

| Client     | MG-state   |                    |   | B-state    |                    |   |
|------------|------------|--------------------|---|------------|--------------------|---|
|            | Mean value | Standard deviation | n | Mean value | Standard deviation | n |
| WT         | 0.77       | 0.01               | 5 | 0.24       | 0.01               | 7 |
| proMBP     | 0.79       | 0.00               | 8 | 0.23       | 0.01               | 5 |
| MBP(Y283D) | 0.77       | 0.01               | 3 | 0.21       | 0.01               | 2 |
| MBP(V8G)   | 0.66       | 0.07               | 3 | 0.24       | 0.01               | 3 |
| MBP(A276G) | 0.76       | 0.01               | 3 | 0.23       | 0.01               | 2 |

**Supplementary Table 5 | Number of bursts accumulated in smFRET**

| <b>Conditions</b>               |                                                                                              | <b>Bursts</b> | <b>Figure</b> |
|---------------------------------|----------------------------------------------------------------------------------------------|---------------|---------------|
| <b>MBP</b><br>T36C/S352C        | Native state                                                                                 | 10 431        | S1A.ii        |
|                                 | Native state with ligand                                                                     | 7 256         | S1A.ii        |
|                                 | Unfolded state                                                                               | 5 970         | S1A.ii        |
|                                 | Refolded state                                                                               | 7 452         | S1A.ii        |
|                                 | Refolded state with ligand                                                                   | 4 285         | S1A.ii        |
|                                 | Folding alone                                                                                | 52 214        | 1A            |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub>                                                   | 60 028        | 2A.i          |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub> then competition (2.5 $\mu$ M unlabeled)          | 11 453        | 2A.iv         |
|                                 | Folding alone 5 seconds then with 1.6 $\mu$ M SecB <sub>4</sub>                              | 7 395         | 3A            |
|                                 | Folding with 2.5 $\mu$ M SecA <sub>2</sub>                                                   | 4 509         | 4A            |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub> 5 seconds then with 2.5 $\mu$ M SecA <sub>2</sub> | 392           | 4B            |
|                                 | Folding with 16 $\mu$ M SecB <sub>4</sub>                                                    | 3 700         | S3A           |
|                                 | Native with 1.6 $\mu$ M SecB <sub>4</sub>                                                    | 5 550         | S3C           |
| <b>proMBP</b><br>T36C/S352C     | Unfolded state                                                                               | 141           | S1A.iii       |
|                                 | Refolded state                                                                               | 2 776         | S1A.iii       |
|                                 | Refolded state with ligand                                                                   | 238           | S1A.iii       |
|                                 | Folding alone                                                                                | 80 860        | 1B            |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub>                                                   | 60 735        | 2A.ii         |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub> then competition (2.5 $\mu$ M unlabeled)          | 13 026        | 2A.v          |
|                                 | Folding alone 10 seconds then with 1.6 $\mu$ M SecB <sub>4</sub>                             | 8 904         | 3B            |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub> 5 seconds then with 2.5 $\mu$ M SecA <sub>2</sub> | 7 336         | S5A           |
|                                 | Folding with 16 $\mu$ M SecB <sub>4</sub>                                                    | 73 972        | S3A           |
|                                 | Refolded with 1.6 $\mu$ M SecB <sub>4</sub>                                                  | 7 787         | S3C           |
| <b>MBP(Y283D)</b><br>T36C/S352C | Native state                                                                                 | 10 414        | S1A.iv        |
|                                 | Native state with ligand                                                                     | 1 271         | S1A.iv        |
|                                 | Unfolded state                                                                               | 2 563         | S1A.iv        |
|                                 | Refolded state                                                                               | 339           | S1A.iv        |
|                                 | Refolded state with ligand                                                                   | 281           | S1A.iv        |
|                                 | Folding alone                                                                                | 73 194        | 1C            |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub>                                                   | 32 036        | 2A.iii        |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub> then competition (2.5 $\mu$ M unlabeled)          | 31 917        | 2A.iv         |
|                                 | Folding alone 5 seconds then with 1.6 $\mu$ M SecB <sub>4</sub>                              | 3 214         | 3B            |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub> 5 seconds then with 2.5 $\mu$ M SecA <sub>2</sub> | 755           | S5A           |
|                                 | Folding with 16 $\mu$ M SecB <sub>4</sub>                                                    | 6 554         | S3A           |
|                                 | Native with 1.6 $\mu$ M SecB <sub>4</sub>                                                    | 8 929         | S3C           |
|                                 | Folding with 1 nM SecB <sub>4</sub>                                                          | 2 703         | 3D            |
| <b>MBP(V8G)</b><br>T36C/S352C   | Unfolded state                                                                               | 1 354         | S3B           |
|                                 | Folding alone                                                                                | 39 150        | S3B           |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub>                                                   | 54 501        | S3B           |
|                                 | Folding with 1.6 $\mu$ M SecB <sub>4</sub> then competition (2.5 $\mu$ M unlabeled)          | 86 735        | 2A.iv         |
|                                 | Folding alone 5 seconds then with 1.6 $\mu$ M SecB <sub>4</sub>                              | 3 214         | 3B            |

|                   |                   |                                                                                     |        |         |
|-------------------|-------------------|-------------------------------------------------------------------------------------|--------|---------|
| <b>MBP(A276G)</b> | <b>T36C/S352C</b> | Unfolded state                                                                      | 2 583  | S3B     |
|                   |                   | Folding alone                                                                       | 36 091 | S3B     |
|                   |                   | Folding with 1.6 $\mu$ M SecB <sub>4</sub>                                          | 27 229 | S3B     |
|                   |                   | Folding with 1.6 $\mu$ M SecB <sub>4</sub> then competition (2.5 $\mu$ M unlabeled) | 33 694 | 2A.iv   |
|                   |                   | Folding alone 5 seconds then with 1.6 $\mu$ M SecB <sub>4</sub>                     | 8 777  | 3B      |
| <b>MBP</b>        | <b>N18C/A141C</b> | Native state                                                                        | 1 939  | S1B.ii  |
|                   |                   | Native state with ligand                                                            | 1 220  | S1B.ii  |
|                   |                   | Unfolded state                                                                      | 942    | S1B.ii  |
|                   |                   | Refolded state                                                                      | 1 699  | S1B.ii  |
|                   |                   | Refolded state with ligand                                                          | 1 509  | S1B.ii  |
|                   |                   | Folding alone                                                                       | 8 613  | S1B.iii |
|                   |                   | Folding with 1.6 $\mu$ M SecB <sub>4</sub>                                          | 14 934 | S1B.iv  |