Supplementary Information

YidC assists the stepwise and stochastic folding of membrane proteins

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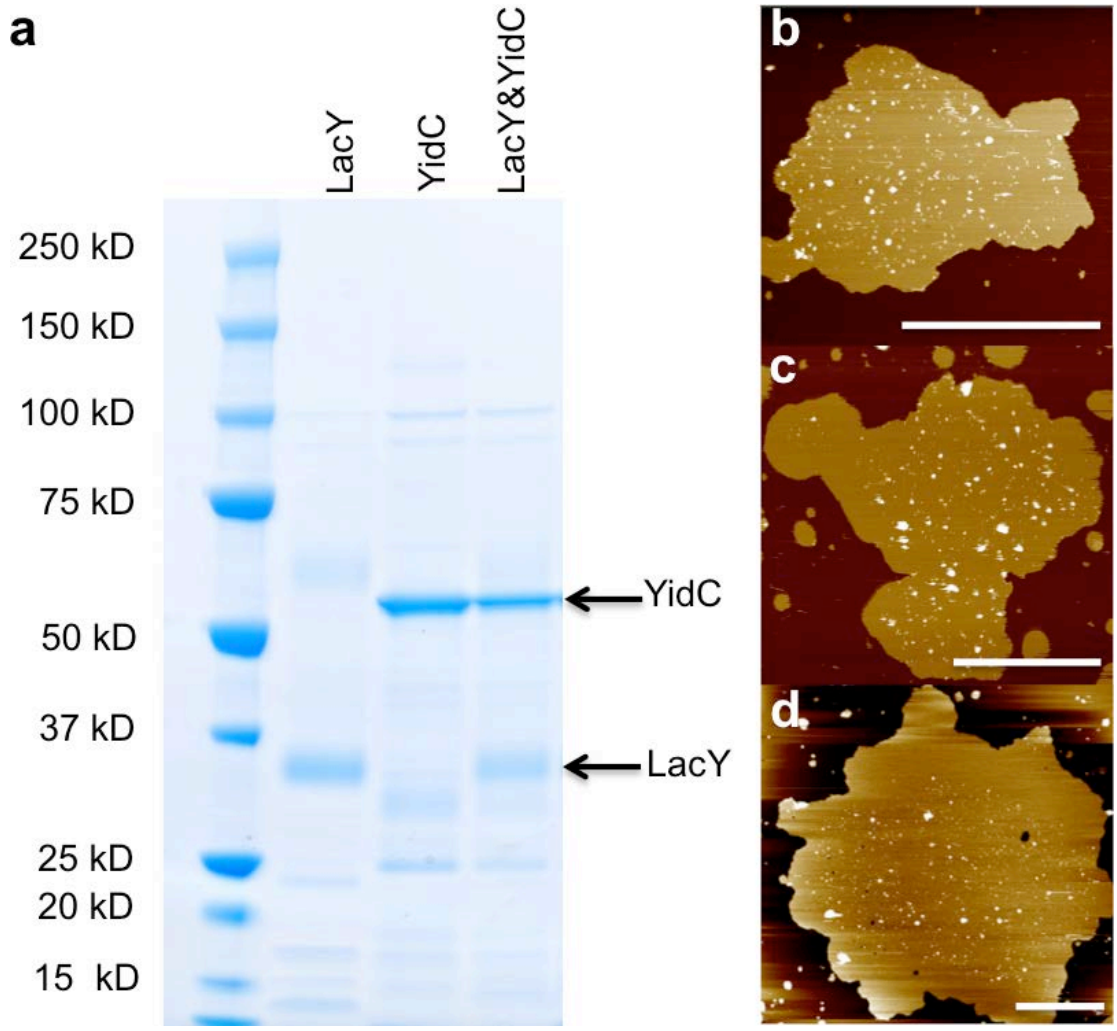
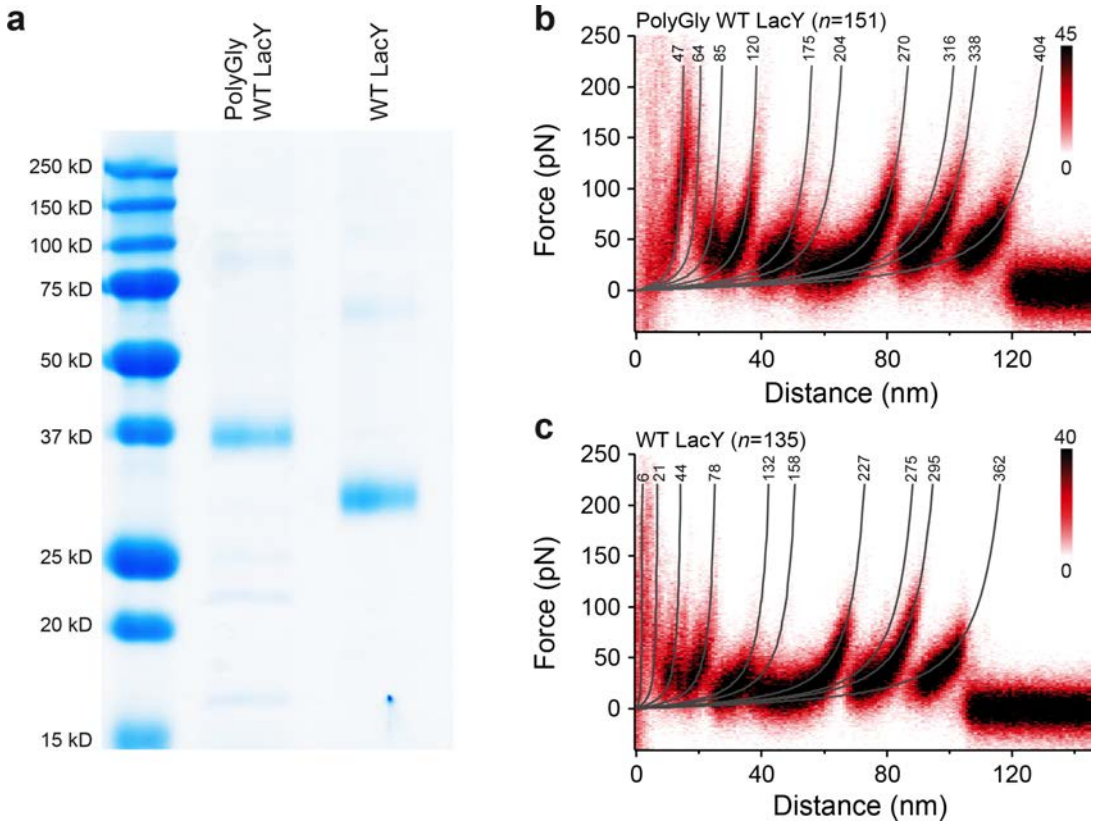
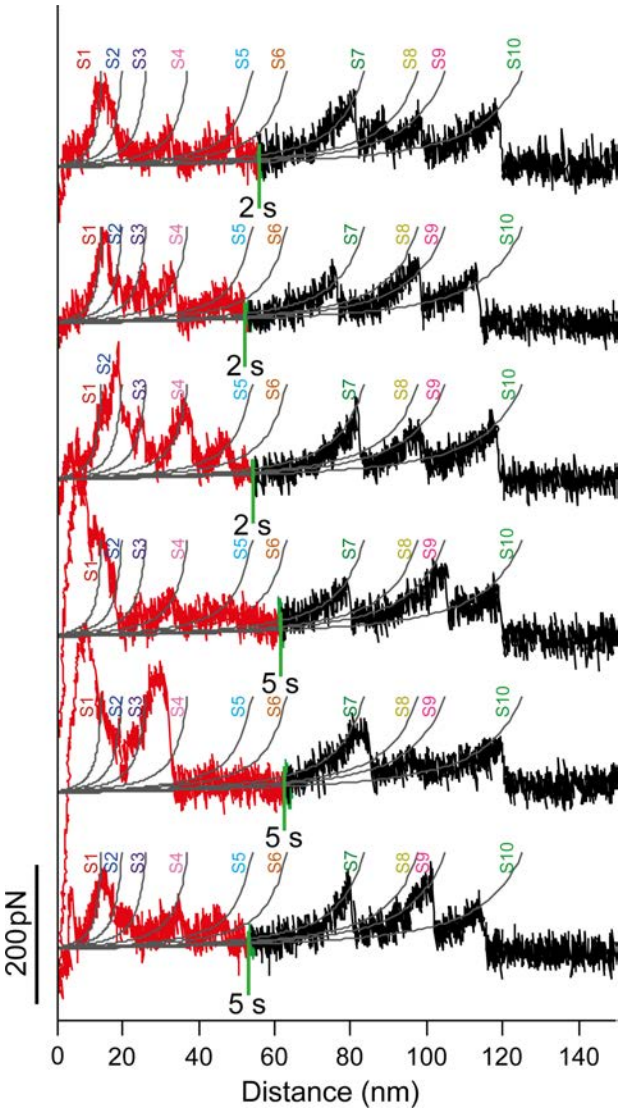
Supplementary Results



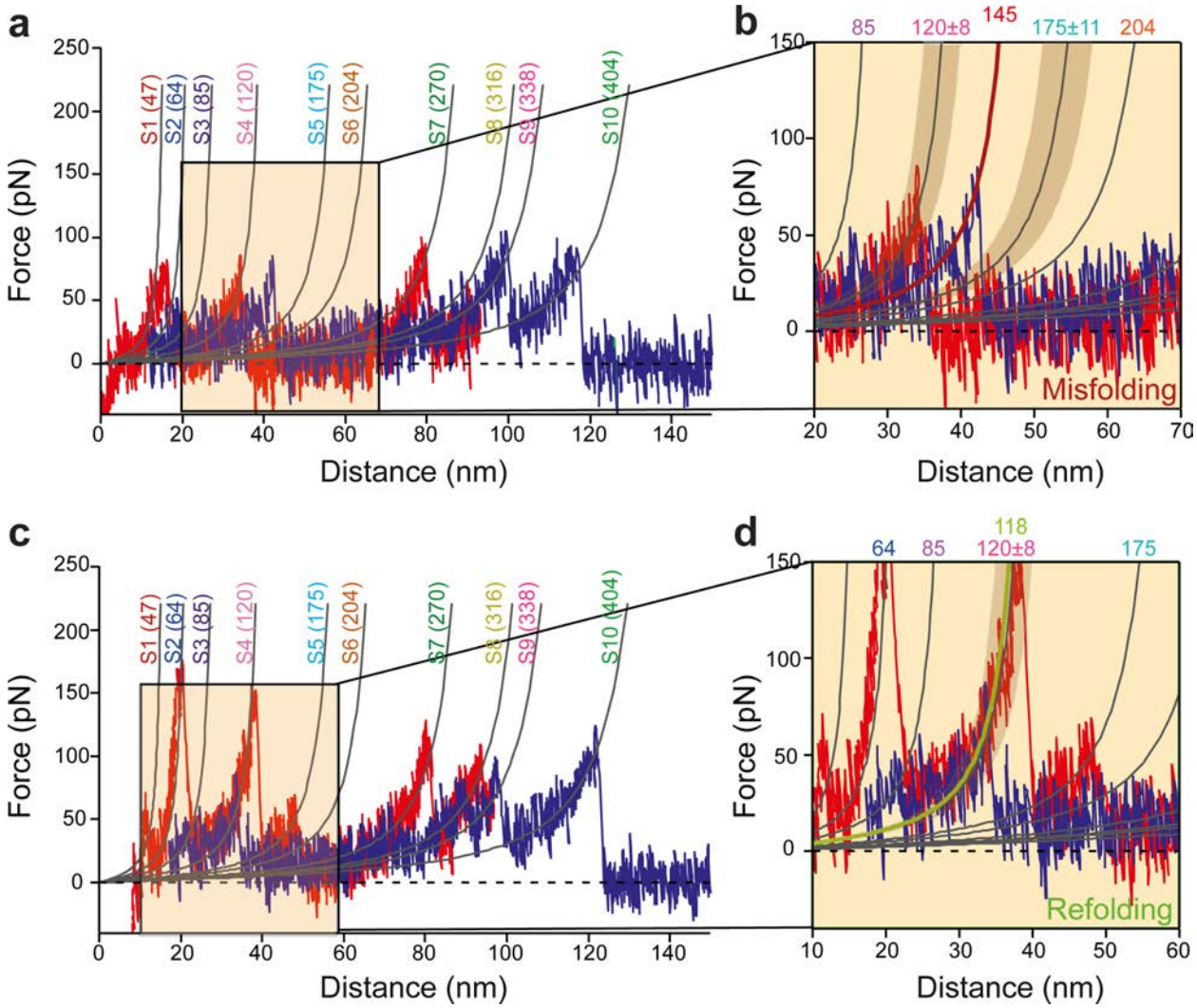
Supplementary Figure 1. Purified and co-reconstituted LacY and YidC. (**a**) SDS gel (acrylamide concentration 12%) of polyGly wild-type (WT) LacY (second line), YidC (third line), and polyGly WT LacY co-reconstituted with YidC (fourth line). Although YidC and LacY have been co-reconstituted at similar concentrations the fourth line suggests an excess of YidC. This apparent difference may be due to the compactness of the YidC and LacY bands and their different efficiency of staining. The C-terminal end of WT LacY has been elongated by a 36 amino acid (aa) long polyglycine (polyGly) tail [GSM(G_{11})EAVEEAVEEA(G_{11})S] followed by 8 aa long His_8-tag. (**b-d**) AFM topographs of (**b**) polyGly WT LacY, (**c**) YidC, and (**d**) polyGly WT LacY and YidC co-reconstituted into PE:PG (ratio 3:1) liposomes. The lipid membranes protrude 4.0 ± 0.3 nm (average ± s.d.; n = 93) from the supporting mica, thus showing that upon adsorption to mica the proteoliposomes open and adsorb as single layered membrane patches. Protrusions assigning membrane proteins homogeneously distributed in the lipid membranes and projected (**b**) 1.7 ± 0.4 nm (n = 36), (**c**) 1.9 ± 0.6 nm (n = 27), and (**d**) 1.8 ± 0.5 nm (n = 34) from the lipid membrane. Further, the protrusions showed widths ranging from ≈ 4–20 nm, indicating that they represent individual or assemblies of membrane proteins. The results thus show that all three reconstitutions successfully embedded LacY, YidC, or LacY and YidC into phospholipid membranes. AFM topographs were taken in buffer solution (200 mM KCl, 20 mM Hepes, pH 7.4) at room temperature as described[1]. Scale bars, 1 µm.



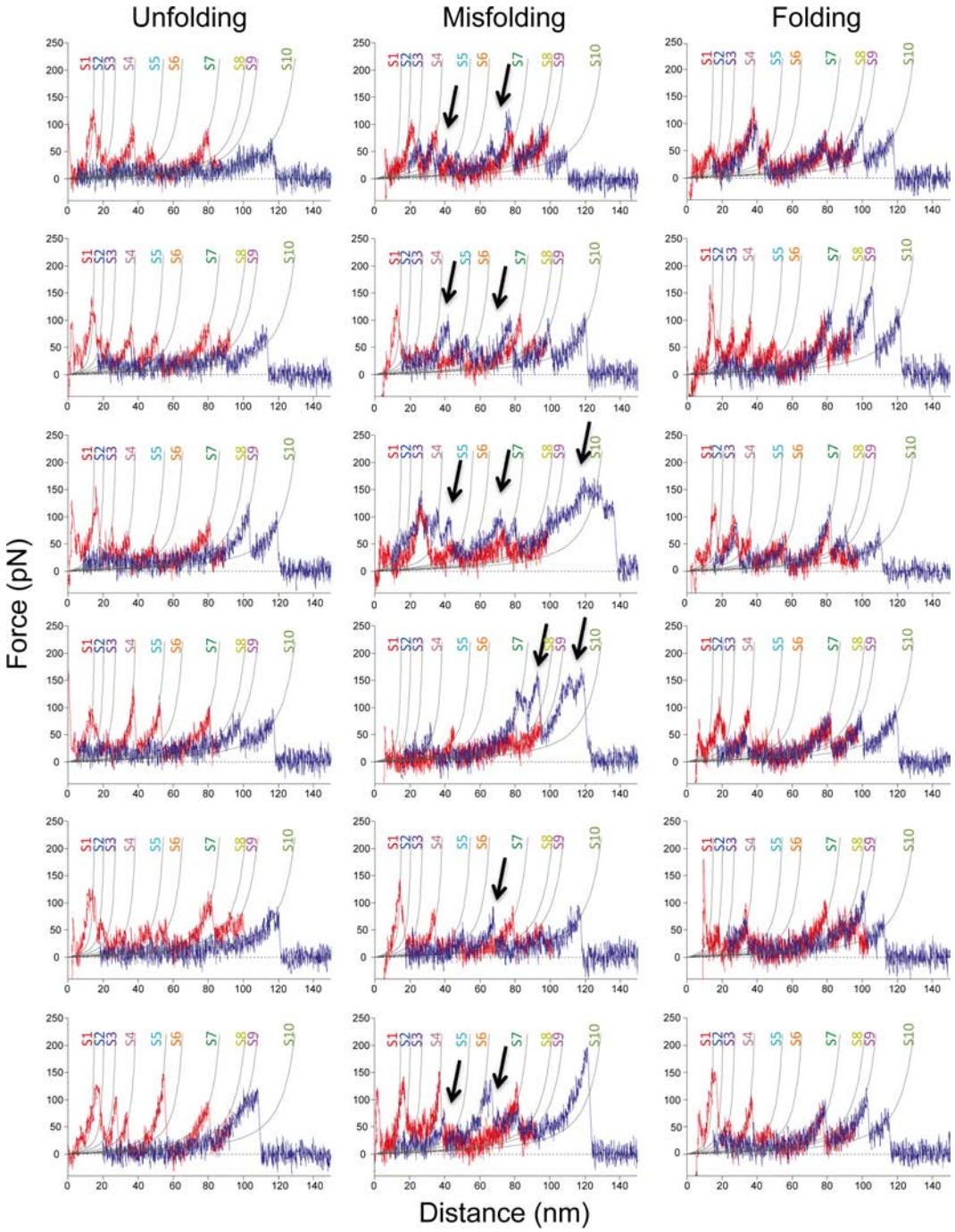
Supplementary Figure 2. Single-molecule force spectroscopy (SMFS) of elongated wild-type (polyGly WT) LacY and wild-type (WT) LacY reconstituted into PE:PG (3:1) liposomes reveals the same unfolding pattern. (**a**) SDS gel (acrylamide concentration is 12%) of purified LacY in proteoliposomes. The C-terminal end of WT LacY is either functionalized by a 6 amino acid (aa) long His-tag (WT LacY) or elongated by a 36 aa long polyglycine (polyGly) tail [GSM(G11)EAVEEAVEEA(G11)S] followed by 8 aa long His-tag (polyGly WT LacY). (**b**,**c**) Density plot of superimposed force-distance curves recorded either upon mechanically unfolding of polyGly WT LacY (**b**) or of WT LacY (**c**). *n* gives the number of force-distance curves superimposed. Fitting of each force peak of the force-distance curves with the worm-like-chain (WLC) model (black thin lines)[1,2] provides the contour length of the unfolded and stretched polypeptide (given in amino acids (aa) at the top of each WLC curve). Force-distance curves recorded for polyGly WT LacY show the same unfolding force peak pattern as recorded for WT LacY except that the characteristic force peak pattern observed for polyGly WT LacY is shifted to ≈ 40 aa longer contour lengths. This shift corresponds to the ≈ 36 aa long polyGly tail and His_8-tag elongating the C-terminal end. Accordingly, polyGly WT LacY and WT LacY describe the same mechanical unfolding from the C-terminal end. Note that unfolding polyGly WT LacY detects the first unfolding force peak at a distance of ≈ 10–15 nm corresponding to a contour length of 47 aa (fitted by the first WLC curve). In contrast, unfolding WT LacY without an elongated C-terminal hardly reveals this unfolding force peak, which is masked by unspecific interactions occurring when the AFM stylus and the protein membrane surface are in close proximity (< 15 nm).



Supplementary Figure 3. **Mechanically unfolding single LacY molecules in two timely separated phases.** In the first unfolding phase the AFM stylus was attached to the C-terminal end of LacY and retracted by ≈ 55 nm to partially unfold the LacY molecule (red colored section of the force-distance curve). After this, the AFM stylus was held at the position of ≈ 55 nm for a given waiting time of 2 s or 5 s (green line). Then AFM stylus was completely withdrawn (black colored section of the force-distance curve). The WLC curves, which were taken from the native fingerprint spectrum of WT LacY (**Fig. 1c**), allow estimating whether the unfolding force peaks change after leaving the partially unfolded LacY molecule for several seconds in the membrane. Colored labels at each WLC curve name the stable structural segments (S1–S10) unfolded by a particular force peak. This comparison shows that the unfolding force peaks recorded upon the second phase of unfolding occurred at exactly the same position as detected upon initial unfolding of WT LacY (**Fig. 1c**). The experiment demonstrates that partially unfolded LacY molecules left for several seconds in the membrane do not collapse. Furthermore, because the unfolding force peaks are at exactly the same position as detected upon unfolding of native WT LacY we can conclude that the partially unfolded LacY did not undergo significant structural changes during the time course of seconds. Unfolding experiments were performed in buffer solution (50 mM KPi, pH 7.2) at room temperature.

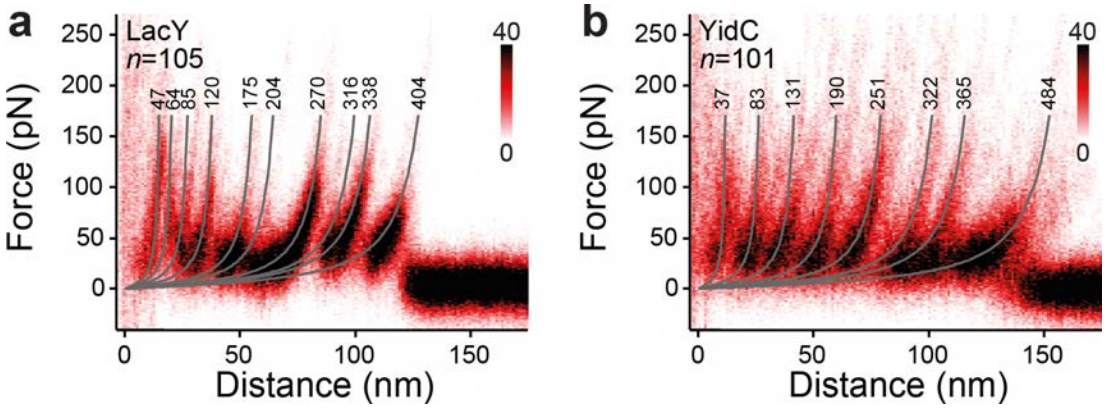


Supplementary Figure 4. Criteria of classifying unfolding, misfolding and refolding events of LacY. (**a,b**) Detecting a misfolding event of LacY. The red force-distance curve shows the initial partial unfolding of LacY. Colored labels at the top of each WLC curve give the mean contour length of the force peak in aa (in parenthesis) and the stable structural segment (S1-S10) unfolded by the force peak. After initial unfolding the unfolded polypeptide is brought back to the membrane surface for relaxation. After a time of 2 s left for the unfolded polypeptide to refold the LacY molecule is completely unfolded (blue force-distance curve). The additional force peak detected in the second unfolding curve (blue) locates at a position located between the force peaks detecting the unfolding of structural segments S4 and S5. (**b**) This additional force peak, which locates at a contour length of 145 aa, does not correspond to the native unfolding force peak pattern of WT LacY as indicated by the WLC fits, their mean contour lengths and their s.d. (**Fig. 1c,d**). (**c,d**) Detecting a folding event of LacY. The red force-distance curve shows the initial partial unfolding of LacY. After this the unfolded polypeptide is brought back to the membrane surface for relaxation. After a time of 2 s left for the unfolded polypeptide to refold the LacY molecule is completely unfolded (blue force-distance curve). (**d**) This second unfolding force-distance curve (blue) shows force peaks only at positions observed for the native unfolding force peak pattern of WT LacY, which has been indicated by the WLC fits, their mean contour lengths and their s.d. (**Fig. 1c,d**). SMFS experiments were performed in buffer solution (50 mM KPi, pH 7.2) at room temperature.

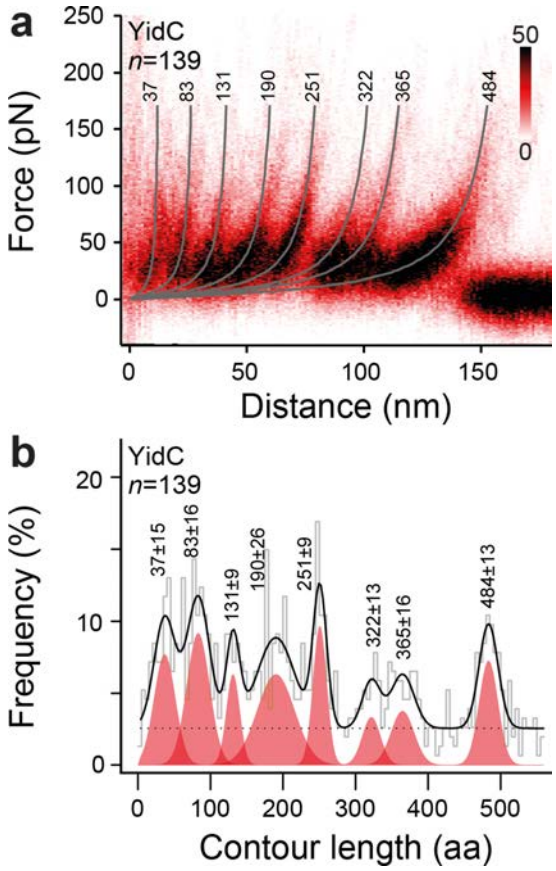


Supplementary Figure 5. Unfolding, misfolding and refolding events of single WT LacY in the absence of YidC. After partial unfolding of a single LacY (red force-distance curve) the AFM stylus holding the C-terminal end of the unfolded polypeptide was brought in close proximity (≈ 10 nm) to the membrane. Then, the unfolded polypeptide was allowed refolding for 2 s, LacY was completely unfolded (blue force-distance curve) to see whether the unfolded polypeptide remained unfolded, misfolded or refolded. Typical force-distance curves of refolded LacY (blue curves) were classified in three classes: (i) unfolding is indicated when the force-distance curve of the before ahead unfolded portion of LacY showed no force peak and thus no stable segments refolded. (ii) misfolding is indicated when the force-distance curve of the before ahead unfolded portion of LacY showed at least one force peak at a position not corresponding to the native unfolding force peak pattern of WT LacY in terms of mean contour

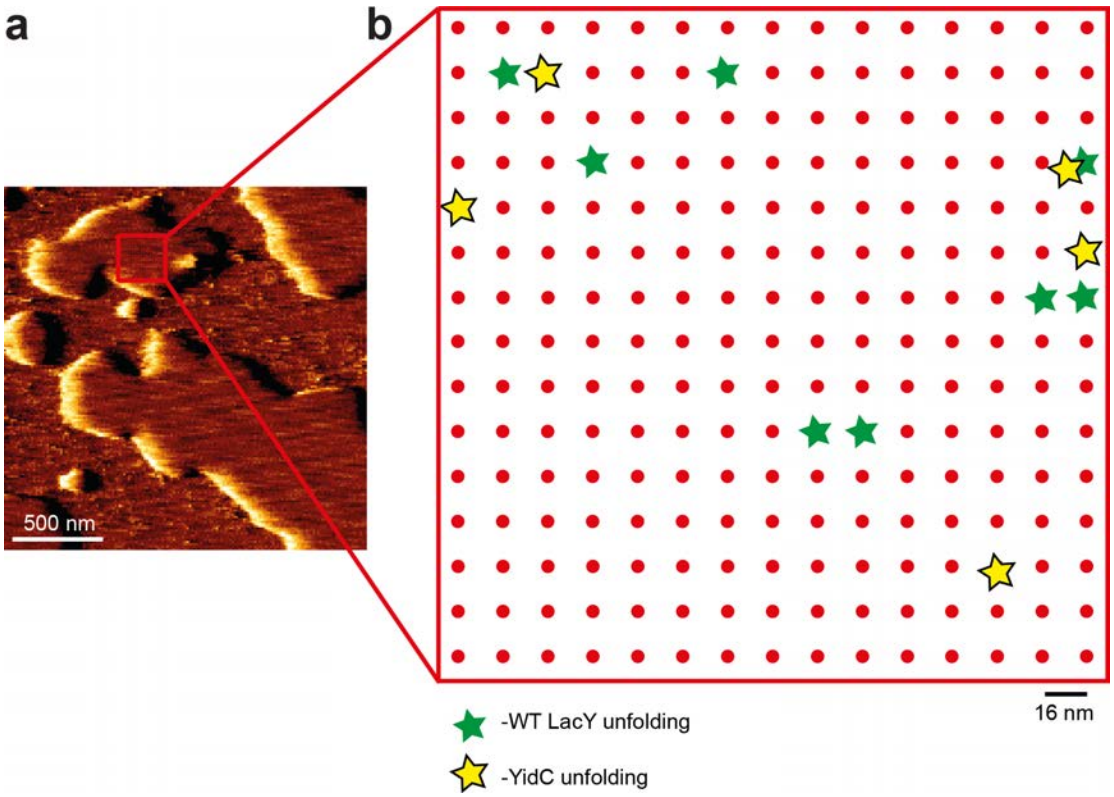
lengths ± s.d. (**Fig. 1c,d** and **Supplementary Fig. 4**). Black arrows point examples of non-native force peaks indicating misfolding events of LacY. (iii) folding is indicated when the force-distance curve of the before ahead unfolded portion of LacY showed only force peaks corresponding to the native unfolding force peak pattern of WT LacY in terms of mean ± s.d.. The folding examples show force peaks, which indicate correctly folded structural segments but differ in their position and occurrence. This variation between the folding experiments indicates that the structural segments insert in an apparently random order. WLC fits corresponding to the mean contour lengths of force peaks detected in the native unfolding force spectrum are represented by grey curves. Colored labels at each WLC curve name the stable structural segments (S1–S10), which unfolding is described by the force peak fitted. SMFS experiments were performed in buffer solution (50 mM KPi, pH 7.2) at room temperature.



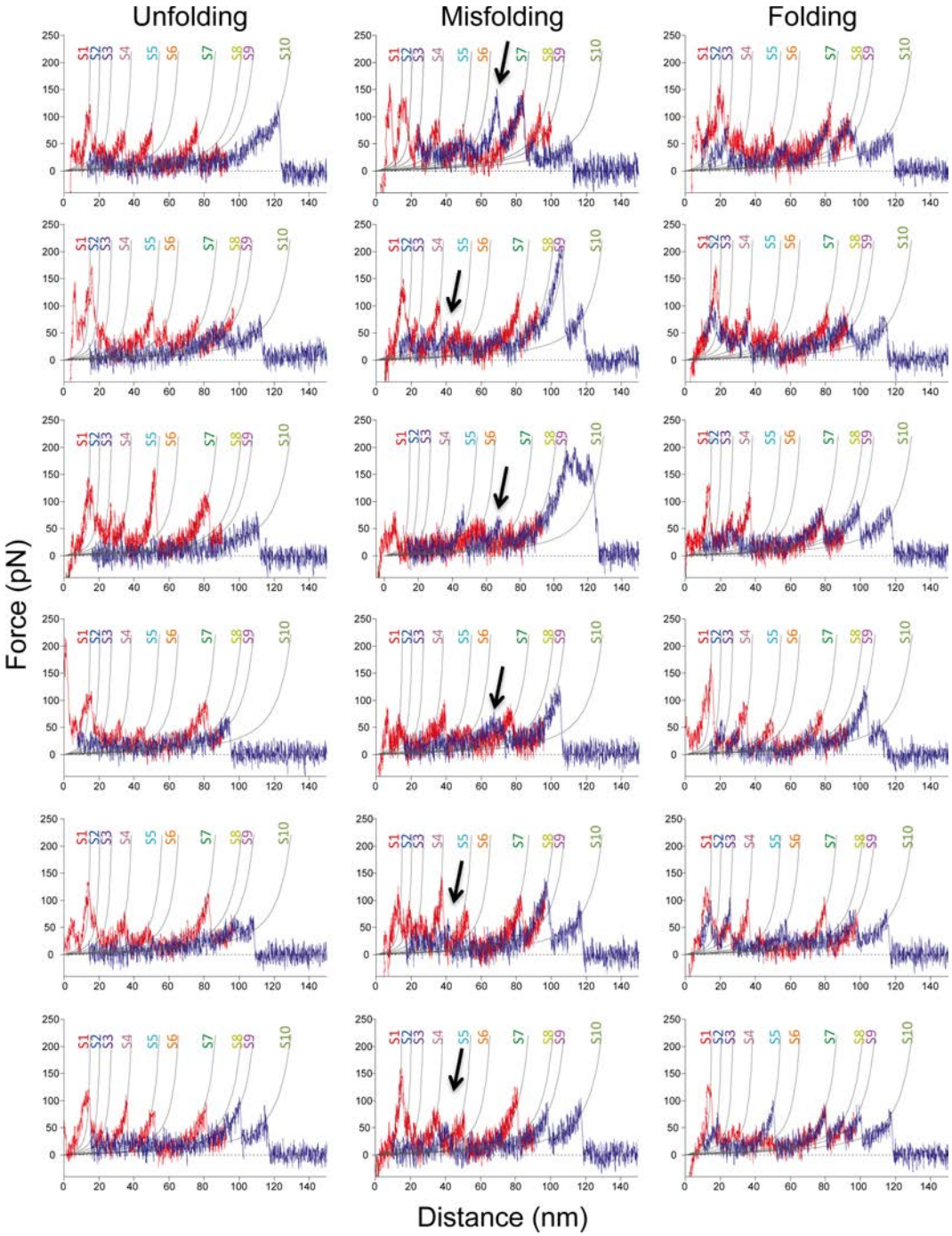
Supplementary Figure 6. The two distinct unfolding patterns of WT LacY and YidC show that both membrane proteins have been successfully co-reconstituted in PE:PG (ratio 3:1) liposomes. (**a,b**) Density plots of superimposed force-distance curves reveal two distinct force peak patterns matching either (**a**) force-distance curves recorded from LacY (**Fig. 1c**) or (**b**) force-distance curves recorded from YidC (**Supplementary Fig. 7**). WLC fits corresponding to the mean contour lengths of each force peak are displayed by grey curved lines. Numbers at the end of each WLC curve give the mean contour length (in aa). *n* gives the number of force-distance curves superimposed and analyzed. SMFS experiments were performed in buffer solution (50 mM KPi, pH 7.2) at room temperature.



Supplementary Figure 7. Unfolding force peak pattern of YidC. (**a**) Density plot of superimposed force-distance curves observed upon mechanical unfolding of YidC reconstituted into PE:PG (ratio 3:1) liposomes. WLC fits corresponding to the mean contour lengths of each force peak are represented by grey curves. Numbers at the end of each WLC curve give the mean contour length (in aa). (**b**) Contour length histogram of force peaks. Histograms were fitted with a Gaussian mixture model [3]. Each Gaussian distribution gives the mean contour length of a force peak class. Eight force peak classes were found for YidC. The black solid line shows the sum of weighted contour lengths for all force peak classes. The dashed line represents the uniform baseline noise. Numbers at the top of each WLC curve and histogram peak represent the mean contour length in aa ± s.d.. *n* gives the number of force-distance curves superimposed and analyzed. SMFS experiments were performed in buffer solution (50 mM KPi, pH 7.2) at room temperature.

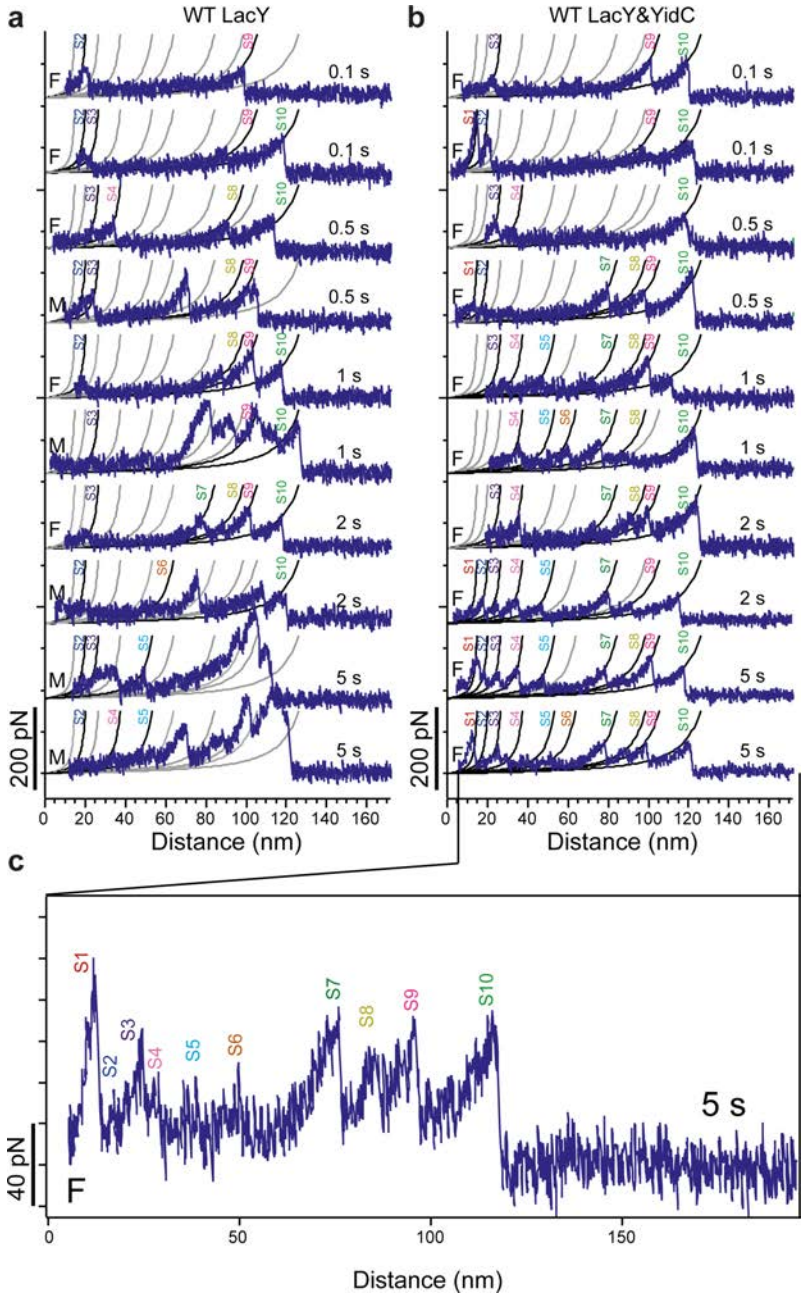


Supplementary Figure 8. Distribution of LacY and YidC reconstituted in phospholipid membranes. (**a**) AFM deflection image of polyGly WT LacY and YidC co-reconstituted into PE:PG (ratio 3:1) membranes. After contact mode AFM imaging (**a**), a grid (red frame) was defined from which force-distance curves were recorded from evenly distributed pulling positions (red dots). This grid is shown at higher magnification in (**b**). Every red dot indicates the position where AFM stylus was approached to attach and withdrawn to mechanically unfold single membrane proteins. To compensate for the low attachment rate of the membrane proteins to the AFM stylus, every dot was approached 100 times with the AFM stylus. The force-distance curves recorded during this process showed either no attached protein or corresponded either to the native unfolding pattern of WT LacY or YidC. Green stars indicate the position at which the force-distance curve detected the native unfolding pattern of WT LacY, and yellow stars indicate the native unfolding pattern of YidC (**Supplementary Fig. 6**). Experiments were performed in 50 mM KP_i at pH 7.2 and 25°C. The Figure shows that LacY and YidC are in sufficiency close proximity that YidC could directly promote refolding of LacY. For our refolding experiments, it does not matter whether contact between the two proteins is static or transient. It has been shown previously that membrane proteins in lipid membranes adsorbed to a mica support exhibit reduced mobility, but are not immobilized[4]. Thus, both LacY and YidC can likely diffuse in the adsorbed phospholipid membrane under these conditions.



Supplementary Figure 9. Unfolding, misfolding and refolding events of single WT LacY in the presence of YidC. After partial unfolding of a single LacY (red force-distance curve) the AFM stylus holding the C-terminal end of the unfolded polypeptide was brought in close proximity (≈ 10 nm) to the membrane. Then, the unfolded polypeptide was allowed refolding for 2 s, LacY was completely unfolded (blue force-distance curve) to see whether the unfolded polypeptide remained unfolded, misfolded or refolded. Typical force-distance curves of refolded LacY (blue curves) were classified in three classes: (i) unfolding is indicated when the force-distance curve of the before ahead unfolded portion of LacY showed no force peak and thus no stable segments refolded. (ii) misfolding is indicated when the force-distance curve of the before ahead unfolded portion of LacY showed at least one force peak at a position not corresponding to the native unfolding force peak pattern of WT LacY in terms of mean contour

lengths ± s.d. (**Fig. 1c,d** and **Supplementary Fig. 4**). Black arrows point examples of non-native force peaks indicating misfolding events of LacY. (iii) folding is indicated when the force-distance curve of the before ahead unfolded portion of LacY showed only force peaks corresponding to the native unfolding force peak pattern of WT LacY in terms of mean ± s.d.. The folding examples show force peaks, which indicate correctly folded structural segments but differ in their position and occurrence. This variation between the folding experiments indicates that the structural segments insert in an apparently random order. WLC fits corresponding to the mean contour lengths of force peaks detected in the native unfolding force spectrum are represented by grey curves. Colored labels at each WLC curve name the stable structural segments (S1–S10), which unfolding is described by the force peak fitted. SMFS experiments were performed in buffer solution (50 mM KPi, pH 7.2) at room temperature.



Supplementary Figure 10. Additional SMFS data showing that in the presence of YidC the unfolded LacY polypeptide takes variable folding intermediates towards the full refolding. Force-distance curves recorded of before ahead unfolded LacY molecules that were allowed refolding in the (**a**) absence and in the (**b**) presence of YidC. Experiments are conducted as described in **Fig. 2,** but the refolding time is varied from 0.1 s (top) to 5 s (bottom). WLC curves with the structural segment indicated at their ends are taken from the native fingerprint spectrum of LacY (**Fig. 1c**). WLC curves matching the refolding force peaks in terms of mean ± s.d. (**Supplementary Fig. 4**) are colored black, those not matching any force peak are colored grey. Force-distance curves marked M indicate misfolding, those marked F indicate folding. (**c**) The last force-distance curve taken from (**b**) shown at higher magnification to display the force peaks in greater detail. The raw data complements the single-molecule refolding experiments shown in **Fig. 4**.

Supplementary References

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2. Bustamante, C., Marko, J.F., Siggia, E.D. & Smith, S. Entropic elasticity of lambda-phage DNA. *Science* **265**, 1599-600 (1994).
3. Kawamura, S. et al. Kinetic, energetic, and mechanical differences between dark-state rhodopsin and opsin. *Structure* **21**, 426-37 (2013).
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Statistical Significance Tests

Refolding time	*P*-value from Z-test for "Folding"	*P*-value from Z-test for "Misfolding"	*P*-value from Z-test for "Unfolded"
0.1 s	0.0285	0.0002	0.04741
0.5 s	0.0009	< 0.0001	0.2609
1 s	< 0.0001	< 0.0001	0.7908
2 s	0.0007	< 0.0001	0.1382
5 s	< 0.0001	< 0.0001	N/A

Supplementary Table 1. Statistical significance of the differences between the refolding experiments of LacY conducted in the presence of YidC (WT LacY & YidC) and the control group (WT LacY) obtained for refolding times 0.1–5 s. Values are obtained from two-tailed Z-tests. The experimental data analyzed is taken from **Fig. 4b,c**.

Refolding time	*P*-value from Fisher's exact test	*P*-value from Chi-square test
0.1 s	0.0009	0.0010
0.5 s	< 0.0001	< 0.0001
1 s	< 0.0001	< 0.0001
2 s	< 0.0001	< 0.0001
5 s	< 0.0001	< 0.0001

Supplementary Table 2. Statistical significance of the differences between the groups of refolding experiments of LacY conducted in the presence of YidC (WT LacY & YidC) and the control group (WT LacY) obtained from Chi-square tests and two-tailed Fisher's exact tests for refolding times 0.1–5 s. The experimental data analyzed is taken from **Fig. 4b,c**.

Group	*P*-value from Z-test for "Folding"	*P*-value from Z-test for "Misfolding"	*P*-value from Z-test for "Unfolded"
WT LacY & BSA	0.6701	0.6643	0.9882
WT LacY & lysozyme	0.7771	0.9496	0.5794

Supplementary Table 3. Statistical insignificance of the differences between the groups of refolding experiments of LacY conducted in the presence of BSA (WT LacY & BSA) or lysozyme (WT LacY & lysozyme) *vs* the control group (WT LacY). Values are obtained from two-tailed Z-tests. The experimental data analyzed is taken from **Fig. 3c,d**.

Group	*P*-value from Fisher's exact test	*P*-value from Chi-square test
WT LacY & BSA	0.9460	0.9064
WT LacY & lysozyme	0.9038	0.8504

Supplementary Table 4. Statistical insignificance of the differences between the groups of refolding experiments of LacY conducted in the presence of BSA (WT LacY & BSA) or lysozyme (WT LacY & lysozyme) *vs* the control group (WT LacY). Values are obtained from Chi-square and two-tailed Fisher's exact tests. The experimental data analyzed is taken from **Fig. 3c,d**.