

1 Two Disease-Causing SNAP-25B Mutations Selectively Impair SNARE
2 C-terminal Assembly

3
4 **Aleksander A. Rebane^{a,b,c}, Bigeng Wang^{a,1}, Lu Ma^a, Sarah M. Auclair^a, Hong Qu^a, Jeff**
5 **Coleman^a, Shyam Krishnakumar^a, James E. Rothman^{a,*}, and Yongli Zhang^{a,*}**

6
7 ^aDepartment of Cell Biology, Yale School of Medicine, New Haven, CT 06511, USA

8 ^bIntegrated Graduate Program in Physical and Engineering Biology

9 ^cDepartment of Physics, Yale University, New Haven, CT 06511, USA

10 ¹Current address: Department of Physics, Columbia University, New York, NY 10027, USA

11 *Correspondence: james.rothman@yale.edu, yongli.zhang@yale.edu

12

13 **KEYWORDS**

14 Optical tweezers, SNARE assembly, membrane fusion, protein folding, neuropathy

15 **ABSTRACT**

16 Synaptic exocytosis relies on assembly of three soluble N-ethylmaleimide-sensitive factor
17 attachment protein receptor (SNARE) proteins into a parallel four-helix bundle to drive
18 membrane fusion. SNARE assembly occurs by step-wise zippering of the vesicle-associated
19 SNARE (v-SNARE) onto a binary SNARE complex on the target plasma membrane (t-SNARE).
20 Zippering begins with slow N-terminal association followed by rapid C-terminal zippering,
21 which serves as a power stroke to drive membrane fusion. SNARE mutations have been
22 associated with numerous diseases, including neurological disorders. It remains unclear how
23 these mutations affect SNARE zippering, partly due to difficulties to quantify the energetics and
24 kinetics of SNARE assembly. Here, we used single-molecule optical tweezers to measure the
25 assembly energy and kinetics of SNARE complexes containing single mutations I67T/N in
26 neuronal SNARE synaptosomal-associated protein of 25 kDa (SNAP-25B), which disrupt
27 neurotransmitter release and have been implicated in neurological disorders. We found that both
28 mutations significantly reduced the energy of C-terminal zippering by ~ 10 $k_B T$, but did not affect
29 N-terminal assembly. In addition, we observed that both mutations lead to unfolding of the C-
30 terminal region in the t-SNARE complex. Our findings suggest that both SNAP-25B mutations
31 impair synaptic exocytosis by destabilizing SNARE assembly, rather than stabilizing SNARE
32 assembly as previously proposed. Therefore, our measurements provide insights into the
33 molecular mechanism of the disease caused by SNARE mutations.

34

35 **HIGHLIGHTS**

- 36
- The mechanism by which two SNAP-25B mutations cause disease is unclear.
- 37
- The mutations greatly weaken SNARE C-terminal zippering.

- 38 • The mutations do not affect SNARE N-terminal assembly.
- 39 • The mutations impair t-SNARE folding.
- 40 • The mutations impair SNARE assembly and thus lead to impaired neurotransmission.

41

42 **Abbreviations**

43 SNARE – soluble N-ethylmaleimide-sensitive factor attachment protein receptors

44 VAMP2 – vesicle-associated membrane protein 2

45 SNAP-25 – synaptosomal-associated protein of molecular weight 25 kDa

46 v-SNARE – vesicle-associated SNARE

47 t-SNARE – target membrane-associated SNARE

48 NTD – N-terminal domain of the SNARE complex

49 CTD – C-terminal domain of the SNARE complex

50 LD – linker domain of the SNARE complex

51 FEC – Force-extension curve

52 HMM – Hidden Markov modeling

53

54 **Glossary**

55 **Ternary complex** – SNARE complex comprising VAMP2, SNAP-25, and syntaxin that exhibits
56 a four-helix coiled-coil structure.

57 **t-SNARE complex** – Partially structured SNARE complex comprising SNAP-25 and syntaxin
58 located on the target membrane.

59 **trans-SNARE** – Partially assembled ternary SNARE complex where complementary v- and t-
60 SNAREs bridge two membranes in *trans*. This intermediate is formed by vesicle priming and

61 acts as a precursor to the final fusion step.

62 **Layers** – Buried, inward-facing amino acid residues between helices in the four-helix bundle

63 structure of the SNARE complex numbered from -7 to +8 from the N-terminus to C-terminus.

64 The residues in the 0 layer are either glutamine or arginine, whereas residues in other layers are

65 hydrophobic.

66 **Equilibrium force** – Force at which a two-state transition exhibits 50% unfolding probability.

67 **Equilibrium transition rate** – Transition rate at equilibrium force, where folding and unfolding

68 rates are equal.

69

70 **INTRODUCTION**

71 Intracellular trafficking and secretion relies on soluble *N*-ethylmaleimide-sensitive factor

72 attachment protein receptors (SNAREs) to fuse cargo-containing vesicles to target membranes

73 [1, 2]. Complementary SNAREs are C-terminally anchored to the vesicles (*v*-SNARE) or the

74 target membranes (*t*-SNARE) [3]. In the case of synaptic vesicle exocytosis, the *v*-SNARE

75 consists of the vesicle-associated membrane protein 2 (VAMP2) and the *t*-SNARE comprises a

76 partially structured binary complex of 25 kDa synaptosomal-associated protein B (SNAP-25B)

77 and syntaxin 1A [4-8]. When in proximity, *v*- and *t*-SNAREs zipper from their N-terminal to C-

78 terminal ends to form a stable four-helix bundle, contributing one and three helices, respectively

79 (Fig. 1) [9-12]. Energy that is released during SNARE assembly lowers the energy barrier posed

80 by membrane-membrane repulsion and thereby accelerates the fusion process. The tight

81 association of the four-helix bundle is mediated by 15 layers of hydrophobic amino acids

82 (numbered from -7 to +8) and a central ionic layer (“0” layer) in the core of the bundle [13].

83 Point mutations that disrupt these hydrophobic layers in the N-terminal domain (Figure 1, NTD)

84 or C-terminal domain (CTD) impair vesicle docking at the plasma membrane and Ca^{2+} -triggered
85 membrane fusion, respectively [10, 14, 15]. Therefore, assembly of each SNARE domain
86 corresponds to a distinct stage in synaptic exocytosis with unique function.

87 SNARE mutations have been implicated in various diseases or disorders, including
88 neurological disorders, cancer, immunodeficiency, and diabetes [16-20]. Particularly, SNARE
89 mutations have been identified in patients with congenital myasthenic syndrome, a group of
90 inherited diseases of the neuromuscular junction that are characterized by fatigable muscle
91 weakness [21-23]. In two cases of interest, the dominant disease-causing mutation affects codon
92 67 of SNAP-25B, which lies in the +4 hydrophobic layer of the SNARE CTD (Fig. 1). In the
93 first case, a human patient carrying the SNAP-25B mutation I67N suffers from myasthenia,
94 cerebellar ataxia, cortical hyperexcitability, and intellectual disability [21]. Transfected into
95 bovine chromaffin cells, the mutant SNAP-25B impairs evoked exocytosis. In the second case,
96 SNAP-25B I67T was identified in the blind-drunk mouse [16]. The mouse exhibits ataxic gait at
97 around 4 weeks of age, as well as impaired sensorimotor gating, an important component of the
98 schizophrenia phenotype related to altered sensory processing. Transfected into murine cortical
99 brain cells and pancreatic beta-cells, the I67T mutant impaired both constitutive and evoked
100 exocytosis, with markedly reduced replenishment of the readily releasable pool of vesicles.
101 Surprisingly, *in silico* modeling and melting temperature measurements of the mutant SNARE
102 complex suggest that the mutation I67T stabilized the SNARE four-helix bundle. Consequently,
103 the mutation was expected to facilitate, not impair membrane fusion, since more energy is
104 released during SNARE assembly to drive exocytosis. Thus, it remains controversial how the
105 two SNAP-25B mutations impair synaptic transmission.

106 SNARE assembly is difficult to study using traditional bulk assays. The experimental
107 challenge is to resolve the multiple intermediates of SNARE assembly under conditions that
108 mimic membrane fusion in the presence of force, and to minimize misfolding of the SNARE
109 complexes [11, 12, 24, 25]. We have developed a high-resolution optical tweezers approach to
110 apply precisely known pulling forces on a single cytosolic SNARE complex molecule to mimic
111 membrane repulsion during membrane fusion, while observing its folding/unfolding in real-time,
112 on sub-millisecond timescale and at sub-nanometer resolution [15, 26-29]. These time-resolved
113 force-extension measurements have yielded the assembly energetics and kinetics of SNARE
114 cytosolic domain, along with the structures of key folding intermediates [12, 15, 25]. We have
115 identified at least three stages of synaptic ternary SNARE assembly - NTD, CTD, and the linker
116 domain (LD) - and found that CTD stability is particularly sensitive to mutations in its
117 hydrophobic layers +4 to +6 [12, 15]. Thus, the energy released during CTD assembly can serve
118 as the power stroke that drives membrane fusion [30]. In this work, we hypothesized that the
119 SNAP-25B mutations I67N and I67T cause the synaptic malfunction by impairing SNARE
120 assembly. To test the hypothesis, we used optical tweezers to measure the assembly energetics
121 and kinetics of both complexes with mutant SNAP-25B. We find that the mutants greatly
122 destabilized the ternary complex CTD without affecting the NTD, and disrupted the partially
123 structured C-terminal portion of the t-SNARE binary complex.

124

125 **RESULTS**

126 **SNAP-25B Mutations Destabilize SNARE CTD**

127 To study SNARE assembly, we tethered single cytosolic SNARE complexes between
128 two polystyrene beads trapped in two tightly focused laser beams and pulled the complexes by

129 separating the two optical traps (Fig. 1) [12, 29]. On one side of the complex, the VAMP2 C-
130 terminus was attached to an anti-digoxigenin-coated bead via a digoxigenin-functionalized 2,260
131 bp DNA handle [31]. On the other side, the syntaxin C-terminus was biotinylated using an Avi-
132 tag and directly attached to a streptavidin-coated bead. To facilitate SNARE refolding, we cross-
133 linked VAMP2 and syntaxin with a disulfide bridge at their N-termini (-6 layer) [15]. We applied
134 force on the tethered molecule by controlling the distance between the two optical traps and
135 simultaneously measured the tether extension by monitoring bead displacements from the trap
136 centers [27, 28]. The SNAREs were fully assembled when the tether was initially formed. We
137 then pulled and subsequently relaxed the SNARE complex by gradually increasing and
138 decreasing the trap separation, respectively. Figure 2a shows the resulting force-extension curves
139 (FECs) for WT and SNAP-25B mutants I67T/N, with black and cyan curves corresponding to
140 the pulling and relaxation phases, respectively. FECs comprise continuous stretches (fit by red
141 curves), regions of extension flickering, and discrete extension jumps (gray arrow). Continuous
142 signals stem from elastic stretching of both the DNA handle and any unfolded polypeptides [32],
143 while the protein remains in a single folding state (indicated by the corresponding state number).
144 Flickering represents reversible protein unfolding/refolding transitions between two or more
145 discrete states [33]. Lastly, jumps in the signal indicate irreversible unfolding/refolding
146 transitions between states that are separated by a high energy barrier and cannot reach
147 thermodynamic equilibrium during pulling or relaxation.

148 The WT SNARE complex (in state 1) disassembled in three reversible and one
149 irreversible steps (Figs. 2a & b). The first transition between states 1 and 2 occurred at an
150 equilibrium force of 11.6 (± 0.6 , standard deviation, N=29) pN and represents reversible
151 unfolding/refolding of the LD. The subsequent transition between states 2 and 3 at 16.5 (± 0.8 ,

152 N=90) pN stemmed from the folding and unfolding of the CTD. The last transition between
153 states 3 and 4 at 17.2 (± 0.8 , N=73) pN was associated with the NTD. Pulled to even higher force,
154 the SNARE underwent an irreversible transition from state 4 to 5 as SNAP-25B dissociated from
155 the t-SNARE complex. The remaining unfolded SNAREs could not refold even after relaxing to
156 low force. Thus, the LD, CTD, and NTD in the WT complex exhibited distinct stabilities, with
157 the CTD unfolding at significantly greater force than the LD. All these measurements on the WT
158 SNARE complex are consistent with previous reports [12, 15, 30].

159 In contrast, both SNAP-25B mutants unfolded in only two reversible steps. In both cases,
160 the intermediate state 2 (LD unfolded four-helix bundle state) disappeared and the LD and the
161 CTD folded and unfolded as a single unit at considerably lower force than the WT CTD, but
162 close to the WT LD. We measured equilibrium force 12.0 (± 0.5 , N=15) pN for I67T and 10.5
163 (± 0.8 , N=37) pN for I67N (Fig. 2a). These measurements suggest that both SNAP-25B
164 mutations significantly destabilized the CTD. Consequently, the CTD now exhibited similar (for
165 I67T) or even lower (for I67N) mechanical stability than the LD, leading to simultaneous folding
166 and unfolding transitions of both domains. However, the NTDs in both mutants unfolded at
167 forces equal to WT within experimental error, with equilibrium force of 17.0 (± 0.7 , N=14) pN
168 for I67T and 16.8 (± 0.8 , N=30) pN for I67N. In summary, the FECs show that both mutations
169 specifically destabilized the CTD of the four-helix bundle while leaving the NTD unaffected.

170

171 **Quantification of SNARE Zippering Energetics, Kinetics, and Intermediates**

172 To quantify the energetics and kinetics of the mutant SNARE complexes, we measured a
173 series of extension trajectories at distinct trap separations or mean forces. The forces were
174 chosen so as to sample the entire force region where the transition occurred. Figure 3 shows

175 excerpts from typical extension trajectories of the LD/CTD transition in I67T and I67N mutants,
176 as well as the CTD transition in WT SNARE complexes (black traces). To verify the two-state
177 nature of the transitions, we plotted the probability density distributions of the extensions and
178 found that double-Gaussian functions fit the bimodal distributions well (green curves). The
179 extension fluctuation around each peak was mainly caused by Brownian motion of the trapped
180 beads [34]. The area below each Gaussian function represents the probability of the
181 corresponding state. An increase in force led to an increase in the unfolding probability, as is
182 expected for typical force-induced two-state transitions under equilibrium conditions [26, 35].
183 Besides a reduction in equilibrium force, both mutations slowed down the folding and unfolding
184 processes. Thus, we conclude that the SNAP-25B mutations not only destabilize the CTD, but
185 also slow down CTD zippering.

186 We used hidden Markov modeling (HMM) to derive the state transitions underlying each
187 extension trajectory obscured by noise (Materials and Methods) [34]. HMM yielded noise-free
188 idealized transitions (Fig. 3, red traces), which closely match the corresponding extension
189 trajectories. Furthermore, HMM revealed the average state extensions and forces, as well as the
190 unfolding probabilities and folding/unfolding rates. The force-dependent unfolding probabilities
191 follow a sigmoidal curve (Fig. 4a, upper panel), similar to that seen in denaturant-based protein
192 folding experiments, with force acting a similar role as the denaturant [36, 37]. Similarly,
193 logarithms of the force-dependent unfolding rates (lower panel, solid symbols) and folding rates
194 (hollow symbols) increase and decrease approximately exponentially in the force region tested,
195 respectively. We were able to accurately determine SNARE zippering kinetics from extensive
196 measurements on single SNARE complexes (Fig. 4a,b). In addition, results from different
197 molecules were highly consistent (Fig. 4c).

198 To determine the free energies and conformations of the folded, unfolded, and transition
199 states at zero force, we simultaneously fit the measured unfolding probabilities, transition rates,
200 and extension changes by a non-linear model (Fig. 4a, curves) [35]. The model describes the
201 observed two-state transition in terms of a force-dependent folding energy landscape comprising
202 folded, unfolded, and transition states. This model allows us to calculate the unfolding
203 probability based on the Boltzmann distribution and the folding/unfolding rates according to the
204 Kramers' theory at each force. For each state, we calculated the total energy of the system
205 including the potential energies of two beads in optical traps, entropic energies of the stretched
206 DNA handle and polypeptides, and the intrinsic free energy of the protein at zero force. We
207 described the DNA and unfolded polypeptides using the worm-like chain model (Eq. 3), which
208 relates the polymer's force-dependent extension and entropic energy to the its contour length and
209 flexibility [32]. The DNA contour length is a known experimental parameter (2,260 bp or 768.4
210 nm), but the contour length of the unfolded polypeptide needs to be determined, since it depends
211 on the folding state of the protein. Thus, our model features two fitting parameters for each state:
212 its free energy at zero force and the contour length of the unfolded, stretched polypeptide. We
213 therefore obtained the energies and polypeptide contour lengths of all states at zero force by
214 fitting the HMM results with model predictions (see Materials and Methods for details). Notably,
215 the model fitting (Fig. 4a, curves) accurately reproduces the experimentally determined HMM
216 results (symbols).

217

218 **Disease-causing Mutations Differentially Affect NTD and CTD Assembly**

219 Model fitting confirmed that the two-state transitions in I67T and I67N correspond to
220 coupled folding of the CTD (+3 layer to +8 layer) and the LD (+8 layer to cytosolic C-terminus)

221 [15, 35]. We derived coupled LD/CTD folding energies of 23 (± 3) $k_B T$ for I67T and 19 (± 3) $k_B T$
222 for I67N, where k_B is the Boltzmann constant and T the absolute temperature (Fig. 4c, red bars).
223 For WT, we added the folding energies of 25 (± 2) $k_B T$ for the CTD and of 8 $k_B T$ (± 2) $k_B T$ for the
224 LD, yielding a combined LD/CTD energy of 33 (± 3) $k_B T$. Therefore, the I67T and I67N
225 mutations destabilize the LD/CTD by 10 $k_B T$ and 14 $k_B T$, respectively. The equilibrium
226 LD/CTD transition rates of I67T (30 s^{-2}) and I67N (10 s^{-2}) were reduced by three-fold and ten-
227 fold, respectively, compared to that of the WT CTD (100 s^{-2}) (Fig. 4a, lower panel). The
228 reconstructed energy landscape at zero force (Fig. 4d) supports this observation. In particular, the
229 mutations give rise to a small energy barrier (0.5 $k_B T$ for I67T and 2 $k_B T$ for I67N) for the
230 LD/CTD transition. These findings demonstrate that the two disease mutations greatly
231 destabilized the LD/CTD.

232 In contrast to LD and CTD assembly, the SNAP-25B mutations have negligible effect on
233 the NTD. Using the methods introduced above, we determined the force-dependent unfolding
234 probabilities and transition rates for the NTD (Fig. 4b). The mutants have the same equilibrium
235 forces and rates as the WT within experimental error. Model fitting yielded NTD folding
236 energies of 37 (± 4) $k_B T$ for I67T, 36 (± 3) $k_B T$ for I67N, and 38 (± 2) $k_B T$ for WT (Fig. 4c, gray
237 bars). In all cases, NTD folding involved association of VAMP2 with the t-SNARE complex
238 from -6 to +3 layers and faced no energy barrier at zero-force (Fig 4d). In summary, both SNAP-
239 25B mutations only destabilize C-terminal assembly and are therefore expected to selectively
240 impair the fusion step of synaptic exocytosis.

241

242 **SNAP-25B Mutations Impair t-SNARE Folding**

243 Next, we investigated how the mutations affect the cytosolic t-SNARE complex. In this
244 case, we pulled the t-SNARE complex from the C-terminus of syntaxin and the C-terminus of
245 SN1 domain in SNAP-25B (Fig. 5a), as previously described [38]. The two SNARE proteins
246 were crosslinked at the N-termini of both SNARE domains. To prevent t-SNARE misfolding, we
247 first formed the ternary SNARE complex and then removed the VAMP2 molecule by
248 disassembling the ternary complex *in situ*, generating the unfolded t-SNARE complex (Figs. 5a
249 & b, state ii). Interestingly, even in this new pulling direction, the CTD of the mutant ternary
250 SNARE complex reversibly unfolded at significantly lower force than the WT complex (green
251 arrows), consistent with a weak CTD in the mutants. As the syntaxin-SNAP-25B conjugate was
252 relaxed to around 5 pN, both WT and mutant t-SNAREs reversibly folded into the t-SNARE
253 complex (state 3). Figure 5c shows typical extension trajectories of the mutant and WT t-SNARE
254 folding transitions near equilibrium force (black traces). The mutant t-SNARE complexes exhibit
255 lower equilibrium forces than WT t-SNARE complex, suggesting that the mutations weaken the
256 t-SNARE complex. In addition, the extension change accompanying the folding transition is
257 reduced in the mutants with respect to WT, indicating that the mutant t-SNAREs are less
258 structured than the WT. We then quantified the force-dependent unfolding probabilities and
259 unfolding/refolding rates for this transition using HMM (Fig. 6a, symbols). Model fitting (Fig.
260 6a, curves) revealed greatly reduced mutant t-SNARE folding energies of 6 (± 2) $k_B T$ and 7 (± 2)
261 $k_B T$ for I67T and I67N, respectively, compared to 12 (± 3) $k_B T$ for WT (Fig 6b). The derived
262 zero-force energy landscape (Fig. 6c) shows that the mutations result in a ~ 9 $k_B T$ energy barrier
263 near the -3 layer, compared to the ~ 6 $k_B T$ energy barrier near the +1 layer in WT. The folded
264 states of the mutants are less structured than in WT, with I67T and I67N structured to +1 and +2
265 layers, respectively, compared to WT, which is structured to +5 layer. Together, these data show

266 that the mutations disrupt the C-terminal portion of the t-SNARE complex and thereby reduce t-
267 SNARE folding energies by at least 5 k_BT.

268

269 **DISCUSSION**

270 We used optical tweezers to determine the effect of disease-causing SNAP-25B
271 mutations I67T and I67N on the energetics, kinetics, and intermediates of SNARE complex
272 assembly. To our knowledge, these are the first single-molecule measurements to elucidate the
273 molecular mechanism of disease-causing SNARE mutations. We show that the mutations, which
274 lie in the +4 hydrophobic layer in the CTD, selectively destabilize LD/CTD assembly by at least
275 10 k_BT (Fig. 4c). Previous studies have demonstrated that mutations that destabilize the C-
276 terminal assembly severely impair Ca²⁺-triggered membrane fusion [10, 14, 15]. In particular,
277 the +4 layer mutation VAMP2 L70A, which was shown to destabilize LD/CTD assembly by 10
278 k_BT [15], dramatically reduces Ca²⁺-triggered neurotransmitter release in chromaffin cells [14].
279 The equally great destabilization of LD/CTD assembly in the SNAP-25B mutants is therefore
280 expected to strongly inhibit membrane fusion, consistent with the reduced spontaneous and
281 evoked neurotransmitter release observed *in vivo* [16, 21].

282 NTD assembly mediates vesicle docking and forms the partially assembled *trans*-SNARE
283 intermediate that acts as a precursor to vesicle priming and Ca²⁺-triggered fusion [2, 12, 14, 15].
284 We found that the SNAP-25B mutations have no effect on NTD assembly, which suggests that
285 mutant SNAREs can participate in vesicle docking likely as well as their WT counterpart.
286 Furthermore, vesicle docking is mediated by multiple copies of *trans*-SNARE complexes [39,
287 40]. Therefore, in cells that express both WT and mutant SNAREs, a docked vesicle should
288 contain equal numbers of WT and mutant *trans*-SNARE complexes on average. It is likely that

289 Ca^{2+} -triggered vesicle fusion is abolished by a single copy of defective trans-SNARE complex in
290 a docked vesicle [15]. Thus, our findings may account for the dominant disease phenotype of
291 both SNAP-25 mutations.

292 The total energy released by assembly of a v-SNARE and preformed t-SNARE into a
293 single ternary SNARE is 60 $k_B T$ for I67T, 55 $k_B T$ for I67N, and 71 $k_B T$ for the WT.
294 Additionally, the energy of t-SNARE formation is 6 $k_B T$ and 7 $k_B T$ for I67T and I67N mutants,
295 respectively, and 12 $k_B T$ for the WT. Thus, the I67T and I67N mutations reduce the total
296 SNARE complex formation energy by 17 $k_B T$ and 20 $k_B T$, respectively, compared to the WT.
297 Our results contrast with the report by Jeans et al. [16]. Based on an increase in melting
298 temperature for the I67T ternary SNARE, these authors suggest that the mutation increased the
299 thermodynamic stability of the ternary SNARE complex. Consequently, they reasoned that the
300 reduced *in vivo* exocytosis stems from the impaired vesicle recycling, as increased SNARE
301 stability might hinder SNARE disassembly and recycling for subsequent rounds of fusion. We
302 note that SNARE complexes melt far from thermodynamic equilibrium and thus the melting
303 temperature of the SNARE complex mainly represents the energy barrier of SNARE unfolding,
304 instead of thermodynamic stability of the SNARE complex. In contrast, our single-molecule
305 measurement is conducted under thermodynamic equilibrium and yields the free energy of
306 SNARE folding and assembly [25]. We therefore suggest that in addition to impairing the
307 replenishment of the readily releasable pool by a yet unknown mechanism, the SNAP-25B
308 mutations compromise the ternary SNARE's ability to drive membrane fusion. In summary, our
309 findings provide hitherto missing molecular detail on how single SNARE mutations can impair
310 synaptic transmission to a degree that leads to neurological disorders such as congenital
311 myasthenic syndrome.

312

313 **MATERIALS AND METHODS**

314 **SNARE Proteins**

315 We employed the cytosolic domain of mouse VAMP2 (residues 1-96) with a C-terminal
316 linker sequence (GGSGNGSGGLSTPSRGG), followed by a FLAG tag (DYKDDDDK) [12].
317 For the ternary SNARE complex pulling experiment, we engineered a cysteine via Q36C site-
318 directed mutagenesis (Agilent Technologies) to facilitate crosslinking to syntaxin at the -6 layer
319 [15]. Additionally, to allow covalent attachment to the DNA handle, we mutated a serine in the
320 linker (underlined in the sequence) to a cysteine. The syntaxin construct comprised the cytosolic
321 domain of rat syntaxin 1A (residues 1-265, mutation C145S) with a C-terminal linker sequence
322 (GGSGNGGSGS), followed by an Avi-tag (GLNDIFEAQKIEWHE) [12]. The -6 layer cysteine
323 in syntaxin was added by site-directed mutagenesis L205C [15]. For t-SNARE complex pulling,
324 we instead added a cysteine at the -8 layer by mutating H199C [38]. The VAMP2 and syntaxin
325 genes were cloned into the pET-SUMO vector (Thermo Fisher). For the full-length mouse
326 SNAP-25B, we replaced all intrinsic cysteines with serines (mutations C85S, C88S, C90S,
327 C92S) and inserted it into the pET-28a vector. For the t-SNARE complex pulling experiment, we
328 additionally mutated S25C to facilitate crosslinking to syntaxin at the -8 layer and N93C to allow
329 for covalent attachment of the DNA handle.

330 We expressed all proteins in BL21 Gold (DE3) cells (Agilent Technologies) and purified
331 the proteins using nickel nitriloacetic acid beads (GE Healthcare Lifesciences) and the buffer
332 containing 25 mM HEPES, 400 mM KCl, 1 mM TCEP, 10 mM imidazole, and 10% glycerol.
333 After purification, we enzymatically biotinylated syntaxin using the biotin ligase BirA (Avidity),
334 leading to biotin conjugation to the underlined lysine in the Avi-tag sequence [29]. For VAMP2

335 and syntaxin, the N-terminal SUMO protein was cleaved along with the His-tag using SUMO
336 protease. To form the SNARE complex, we mixed syntaxin, SNAP-25B, and VAMP2 at a molar
337 ratio of 1:1:2, followed by an overnight incubation at 4 °C, in the presence of 3 mM Tris(2-
338 carboxyethyl) phosphine (TCEP). Then the SNARE complex was purified using the N-terminal
339 His-tag on SNAP-25B, followed by overnight incubation in the absence of TCEP at 4 °C to
340 allow disulfide bond formation between VAMP2 and syntaxin (for ternary SNARE pulling
341 experiment) or SNAP-25B and syntaxin (for t-SNARE pulling experiment).

342

343 **High-Resolution Optical Tweezers**

344 We used home-built dual-trap optical tweezers with interferometric detection, as previously
345 described [27, 28]. Briefly, we used a 1064 nm laser beam to form the optical traps. To this end,
346 we expanded, collimated, and then split the beam into two orthogonally polarized beams, each
347 corresponding to one trap. We reflected one beam by a mirror that could be tipped and tilted
348 along two axes with high precision by virtue of a nano-positioning stage (Mad City Labs), thus
349 controlling the beam's path relative to the other. The two beams were subsequently combined
350 and expanded once more, and finally focused by a water-immersion 60X objective with
351 numerical aperture of 1.2 (Olympus) to form two optical traps. The outgoing laser beams were
352 collimated by an identical objective and split again by polarization. The separated beams were
353 each projected onto a position-sensitive detector (Pacific Silicon Sensor) to detect bead
354 displacements from the trap center using back-focal-plane interferometry [41]. The force
355 constants and the constants to convert detector signal to bead displacement were calibrated using
356 the Brownian motion of the trapped beads. The force, bead displacement, trap separation, and
357 other experimental parameters were acquired at 20 kHz, filtered online to 10 kHz and stored on

358 hard-disc. Importantly, the tether extension was directly calculated by subtracting the bead radii
359 and bead displacements from the trap separation.

360

361 **Single-Molecule Experiments**

362 We covalently attached a 2,260 bp DNA handle to the C-terminal cysteine on VAMP2 (for
363 ternary complex pulling) or on SNAP-25B (t-SNARE pulling). This was done by mixing the
364 purified SNARE complex with DNA handle at 100:1 molar ratio, as is described in detail
365 elsewhere [29]. The final DNA handle concentration was approximately 150 nM. A 2 μ L aliquot
366 of the protein-DNA mixture was incubated with 20 μ L anti-digoxigenin antibody-coated
367 polystyrene beads of 2.17 μ m diameter (Spherotech) for 15 minutes. Then the mixture was
368 diluted with 1 mL PBS and injected into the top channel of a microfluidic chamber (for further
369 details on the microfluidics, please see [29]). Streptavidin-coated beads of 1.86 μ m diameter
370 were injected into the bottom channel of the chamber. Both bottom and top channels were
371 connected to a central channel by capillary tubes. The beads were trapped in the central channel
372 by sequentially approaching the top and bottom capillary tubes, out of which flowed a steady
373 stream of anti-digoxigenin and streptavidin beads, respectively. Once one of each bead was
374 trapped, a single SNARE complex was tethered between them by bringing the two beads close.
375 The tethered molecule was pulled and relaxed by increasing or decreasing the trap separation at
376 10 nm/s, respectively, or held at a constant average force by keeping the trap separation constant.
377 The optical tweezers experiment was conducted in PBS at 23 (\pm 1) $^{\circ}$ C. To prevent oxidative
378 photodamage by the strong trapping beams, we supplemented the PBS buffer with an oxygen
379 scavenging, as described elsewhere [29].

380

381 **Data Analysis**

382 The data analysis to derive the intermediate structures and energies was performed as
383 described in detail elsewhere [35]. Briefly, we obtained extension trajectories of
384 folding/unfolding transitions at stepwise constant average forces by holding the protein at
385 constant trap separations. The trajectories were mean-filtered to a bandwidth of 200 Hz or 1 kHz.
386 We calculated the histogram distribution of the extension trajectories and determined the number
387 of states by fitting the distribution with multiple-Gaussian functions. We then determined the
388 state populations and transition rates, along with the state extensions and forces, using hidden
389 Markov modeling (HMM) [34]. The idealized, noise-free trajectories were calculated using the
390 Viterbi algorithm [42].

391 We calculated the state structures and energies at zero force by fitting the HMM-derived
392 observables with a non-linear model. In this model, we chose the contour length of the unfolded,
393 stretched portion of the protein L as the reaction coordinate to describe unfolding of the
394 SNAREs along a pathway inferred from the crystal structure of the fully assembled SNARE
395 complex [9]. Unfolding along the inferred pathway occurs by peeling off of the protein from the
396 coiled-coil structure, starting from the C-terminus, while leaving the remaining, folded structure
397 unperturbed (for more details, see [35]). To derive the conformations and free energies of folded,
398 unfolded, and transition states, we defined a simplified energy landscape $\{(L_i, V_i)\}$, where L_i is the
399 contour length of the unfolded peptide in the i -th state and V_i the associated free energy at zero
400 force. The $\{(L_i, V_i)\}$ were determined by fitting the HMM-derived observables with a model that
401 relates the experimental observables to the simplified energy landscape. The model expresses
402 the mean extension of the i -th state, X_i , as

403
$$X_i = x^{(m)}(F_i, L) + H(F_i, L_i) + x^{(DNA)}(F_i), \quad (1)$$

404 where $x^{(m)}$ is the extension of the unfolded, stretched polypeptide, H is the extension of the
 405 folded, structured portion of the protein, $x^{(DNA)}$ is the extension of the DNA handle, and F_i is the
 406 mean state force. The extensions $x^{(m)}$ and $x^{(DNA)}$ are implicitly defined in terms of state force F_i ,
 407 using the Marko-Siggia formula for the worm-like chain:

408
$$F_i = \frac{k_B T}{P} \left[\frac{1}{4 \left(1 - \frac{x}{L}\right)^2} + \frac{x}{L} - \frac{1}{4} \right], \quad (2)$$

409 where P and L are the persistence length and contour length of the polymer, respectively. For
 410 DNA, we adopt $P_{DNA} = 40nm$ and $L_{DNA} = 0.34 \frac{nm}{bp} \cdot 2260bp = 768.4nm$ for a 2,260 bp DNA
 411 handle. For polypeptide, we use $P_m = 0.6nm$ and $L = L_i$. We calculated the extension of the
 412 folded protein portion H using the freely jointed chain model

413
$$H_i = -\frac{k_B T}{F_i} + h(L_i) \coth\left(\frac{F_i h(L_i)}{k_B T}\right), \quad (3)$$

414 where $h(L_i)$ is the size of the structured portion of the protein along the pulling direction. The
 415 functional dependence of this core size h on the contour length L was directly determined from
 416 the protein crystal structure. A further constraint on the model is given by the relation of the trap
 417 separation D to the tether extension X_i , i.e.

418
$$D = X_i + \frac{F_i}{k_{traps}} + r_{strep} + r_{adig}, \quad (4)$$

419 where F_i/k_{traps} is the total displacement of the two beads from the traps, $k_{traps} = k_1 k_2 / (k_1 + k_2)$ is
420 the effective stiffness of the two traps, and $r_{strep} + r_{adig}$ the sum of the bead radii. We get the state
421 force at trap separation D by substituting Eqs. (1) to (3) into Eq. (4) and solving for F_i .
422 Consequently, we also get the state extension X_i for a given state contour length L_i by plugging
423 the calculated state force into Eq. (1).

424 The state populations and transition rates are determined from the free energy differences
425 between the states. The free energies G_i are calculated as the sum

$$426 \quad G_i = G^{(DNA)}(F_i) + G^{(m)}(F_i, L_i) + \frac{F_i^2}{2k_{traps}} + G^{(h)}(F_i, L_i) + V_i, \quad (5)$$

427 where $G^{(DNA)}$ and $G^{(m)}$ are the elastic energies of the DNA handle and unfolded polypeptide,
428 $F_i^2 / 2k_{traps}$ is the potential energy of the trapped beads, $G^{(h)}$ is the entropic energy of the
429 structured protein that arises from rotational degrees of freedom, and V_i is the intrinsic, force-
430 independent free energy of the protein, which is unknown and thus set as a fitting parameter. The
431 elastic energies $G^{(DNA)}$ and $G^{(m)}$ are given by the worm-like chain model as

$$432 \quad G^{(m/DNA)} = \frac{k_B T}{P} \frac{L}{4 \left(1 - \frac{x}{L}\right)} \left[3 \left(\frac{x}{L}\right)^2 - 2 \left(\frac{x}{L}\right)^3 \right]. \quad (6)$$

433 Similarly, the entropic, rotational energy of the structured core is given as

$$434 \quad G_i^{(h)} = k_B T \left\{ -1 + \frac{Fh(L_i)}{k_B T} \coth \left(\frac{Fh(L_i)}{k_B T} \right) + \ln \left[\frac{\frac{Fh(L_i)}{k_B T}}{\sinh \left(\frac{Fh(L_i)}{k_B T} \right)} \right] \right\}. \quad (7)$$

435 With the state energies G_i defined, we can calculate the state populations P_i using the Boltzmann
436 distribution, i.e.

$$437 \quad P_i = \frac{e^{-\frac{G_i}{k_B T}}}{e^{-\frac{G_f}{k_B T}} + e^{-\frac{G_u}{k_B T}}}, \quad (8)$$

438 where G_f and G_u are the system energies for the folded and unfolded states, respectively.

439 Additionally, we can calculate the folding and unfolding rates k_f and k_u , respectively, using

440 Kramers' equation

$$441 \quad \frac{k}{k_m} = k_m e^{-\frac{G^\dagger - G_u}{k_B T}} \quad (9)$$

442 and

$$443 \quad \frac{k}{k_m} = k_m e^{-\frac{G^\dagger - G_f}{k_B T}}, \quad (10)$$

444 where G^\dagger is the system energy of the transition state, and the pre-factor k_m is the diffusion-
445 limited rate constant in the absence of an energy barrier. We adopted $k_m = 10^6 \text{ s}^{-1}$, consistent
446 with the fastest folding speeds observed for short helical proteins.

447 Last, we used non-linear least-squares method to fit the HMM-derived mean state
448 extensions and forces, as well as the state populations and transition rates at all experimental trap
449 separations with the model-based calculations, while using the state contour lengths and protein
450 free energies at zero force as fitting parameters. The resulting best-fit parameters yield the
451 simplified energy landscape at zero force that defines the energetics, kinetics, and state structures
452 of the two-state transition. For the ternary SNARE complex, we evaluated the NTD, CTD, and

453 LD separately, where applicable. The full assembly energy landscape was then compiled from
454 the individual transitions.

455

456 **AUTHOR CONTRIBUTIONS**

457 A.A.R, L.M, S.K, J.E.R, and Y.Z. designed the experiments. A.A.R, B.W, L.M, Q.H., S.M.A.,
458 and J.C. performed the experiments. A.A.R, B.W, S.K., J.E.R., and Y.Z. analyzed and
459 interpreted the data. A.A.R, S.K., J.E.R, and Y.Z., wrote the article.

460

461 **ACKNOWLEDGEMENTS**

462 This work was supported by the NIH grants F31GM119312 to A. R., R01GM093341 and
463 R01GM120193 to Y. Z., and R01DK027044 to J. E. R. We acknowledge support from the
464 Raymond and Beverly Sackler Institute for Biological, Physical, and Engineering Sciences at
465 Yale.

466 **FIGURE LEGENDS**

467

468 **FIGURE 1** SNARE complex and experimental setup. The ternary SNARE complex forms a
469 parallel four-helix bundle that is stabilized by inward-facing residues in layers -8 to +8.
470 Engineered cysteines at the -6 layer create a disulfide bridge between syntaxin and VAMP2 to
471 facilitate SNARE re-assembly. SNARE assembly occurs by sequential folding of the N-terminal
472 domain (NTD), the C-terminal domain (CTD), and the linker domain (LD). The N-terminal Habc
473 domain in syntaxin recruits other proteins to regulate SNARE assembly [43, 44], but minimally
474 affects ternary SNARE assembly in the absence of these regulatory proteins in our assay [15].
475 Disease-causing mutations SNAP-25B I67T and I67N disrupt the hydrophobic contacts in the +4
476 layer.

477

478 **FIGURE 2** SNAP-25B mutations destabilize SNARE CTD. (a) Force-extension curves (FECs)
479 obtained by pulling (black) or relaxing (cyan) single SNARE complexes. SNARE complexes.
480 Different SNARE folding states are marked by red numbers of states depicted in b. These states
481 are derived from continuous regions in the FECs (red solid curves) or regions with discrete but
482 distinct extensions (red dashed lines) based on the worm-like chain model [32]. (b) Diagrams of
483 different SNARE folding states. The folding states of the WT SNARE complex include the fully
484 assembled SNARE state (state 1), the LD-unfolded four-helix bundle state (2), the partially
485 zippered state (3), the unzipped state (4), and the fully unfolded state (5). Folding of both
486 SNARE complexes containing SNAP-25B mutations bypasses the state 2.

487

488 **FIGURE 3** Representative extension-time trajectories containing the LD/CTD transition for
489 I67T and I67N or the CTD transition for WT. The mean force F was kept constant for each
490 trajectory by fixing the distance between two optical traps. Red traces represent idealized state
491 trajectories as determined by hidden Markov Modeling (HMM). Double-Gaussian fits (green) of
492 the extension probability density distributions reveal transitions between the two discrete states
493 indicated by their corresponding state numbers (Fig. 2b). All extension traces share the same
494 length and time scale bars, except for the trace at the bottom, which has a different time scale bar
495 for a close-up view.

496

497 **FIGURE 4** Zippering energy and kinetics of WT and mutant SNARE complexes. (a, b) Force-
498 dependent unfolding probabilities (top panel) and transition rates (bottom panel) for CTD and
499 LD/CTD transitions (a) or NTD transitions (b). Symbols denote measurements from time-
500 extension trajectories for CTD transition in WT (black circles) and LD/CTD transition in I67T
501 (red diamonds) or I67N (blue squares). Folding and unfolding rates are shown as hollow and
502 solid symbols, respectively. Curves represent fitting results with a non-linear two-state model.
503 (c) Comparison of NTD (gray) and LD/CTD (red) zippering energies between WT and mutant
504 SNARE complexes. (d) Simplified energy landscape of SNARE zippering at zero force. The
505 abscissa denotes the VAMP2 residue to which the SNARE complex is structured starting from
506 the crosslinking site at -6 layer (residue 36). The regions corresponding to NTD, CTD, and LD
507 are marked at the top of the graph. The derived stable and transition states are denoted by solid
508 and hollow symbols, respectively. Solid lines denote an arbitrary interpolation between the
509 calculated states to guide the eye.

510

511 **FIGURE 5** Structures and dynamics of WT and mutant t-SNARE complexes. (a) The correctly
512 folded t-SNARE complex (state iii) is prepared by completely unfolding a ternary SNARE
513 complex (state i) *in situ* at high force and subsequent refolding the remaining t-SNAREs (state
514 ii). Note that SNAP-25B contains an N-terminal SNARE domain (SN1) and a C-terminal
515 SNARE domain (SN2) connected by a disordered linker. The t-SNARE complex is pulled from
516 the C-termini of syntaxin and SN1. (b) FECs obtained by pulling t-SNARE complexes in ternary
517 SNARE complexes (black) and then relaxing the t-SNARE complexes alone (cyan). Green
518 arrows indicate LD/CTD transitions in ternary SNARE complexes. (c) Representative extension-
519 time trajectories for the t-SNARE folding/unfolding transition near equilibrium force. Double-
520 Gaussian fits (green) of the extension histogram distributions confirm the two-state nature of the
521 transition. Red traces represent idealized state trajectories as determined by HMM.

522

523 **FIGURE 6** Folding energies, kinetics, and conformations of t-SNARE complexes. (a) Force-
524 dependent unfolding probabilities (top panel) and transition rates (bottom panel) of the t-SNARE
525 complex. Symbols denote experimental measurements for WT (black circles), I67T (red
526 diamonds), and I67N (blue squares). Folding and unfolding rates are shown as hollow and solid
527 symbols, respectively. Best-fits with a two-state model are shown as curves. (b) Comparison of
528 t-SNARE folding energies between WT and mutant complexes. (c) Simplified folding energy
529 landscapes for t-SNARE complexes. The abscissa denotes the syntaxin residue to which the t-
530 SNARE complex is structured starting from the crosslinking site at -8 layer (residue 199).
531 Locations of corresponding hydrophobic and ionic layers are marked on top of the graph. The
532 derived stable and transition states are shown as solid and hollow symbols, respectively, for WT
533 (black), I67T (red), and I67N (blue).

534 **REFERENCES**

- 535 [1] Sudhof TC, Rothman JE. Membrane fusion: grappling with SNARE and SM proteins.
536 *Science*. 2009;323:474-7.
- 537 [2] Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, et al.
538 SNAREpins: Minimal machinery for membrane fusion. *Cell*. 1998;92:759-72.
- 539 [3] Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, et
540 al. SNAP receptors implicated in vesicle targeting and fusion. *Nature*. 1993;362:318-24.
- 541 [4] Bennett MK, Calakos N, Scheller RH. Syntaxin - a synaptic protein implicated in docking of
542 synaptic vesicles at presynaptic active zones. *Science*. 1992;257:255-9.
- 543 [5] Trimble WS, Cowan DM, Scheller RH. VAMP-1 - a synaptic vesicle-associated integral
544 membrane-protein. *Proc Natl Acad Sci USA*. 1988;85:4538-42.
- 545 [6] Baumert M, Maycox PR, Navone F, Decamilli P, Jahn R. Synaptobrevin - an integral
546 membrane-protein of 18000 daltons present in small synaptic vesicles of rat-brain. *EMBO J*.
547 1989;8:379-84.
- 548 [7] Sudhof TC, Baumert M, Perin MS, Jahn R. A synaptic vesicle membrane-protein is
549 conserved from mammals to *Drosophila*. *Neuron*. 1989;2:1475-81.
- 550 [8] Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE, et al. The
551 identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by
552 neuronal subpopulations. *J Cell Biol*. 1989;109:3039-52.
- 553 [9] Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex
554 involved in synaptic exocytosis at 2.4 angstrom resolution. *Nature*. 1998;395:347-53.
- 555 [10] Sorensen JB, Wiederhold K, Muller EM, Milosevic I, Nagy G, de Groot BL, et al.
556 Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory
557 vesicles. *EMBO J*. 2006;25:955-66.
- 558 [11] Pobbati AV, Stein A, Fasshauer D. N- to C-terminal SNARE complex assembly promotes
559 rapid membrane fusion. *Science*. 2006;313:673-6.
- 560 [12] Gao Y, Zorman S, Gundersen G, Xi ZQ, Ma L, Sirinakis G, et al. Single reconstituted
561 neuronal SNARE complexes zipper in three distinct stages. *Science*. 2012;337:1340-3.
- 562 [13] Fasshauer D, Sutton RB, Brunger AT, Jahn R. Conserved structural features of the synaptic
563 fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci USA*.
564 1998;95:15781-6.
- 565 [14] Walter AM, Wiederhold K, Bruns D, Fasshauer D, Sorensen JB. Synaptobrevin N-
566 terminally bound to syntaxin-SNAP-25 defines the primed vesicle state in regulated exocytosis. *J*
567 *Cell Biol*. 2010;188:401-13.
- 568 [15] Ma L, Rebane AA, Yang G, Xi Z, Kang Y, Gao Y, et al. Munc18-1-regulated stage-wise
569 SNARE assembly underlying synaptic exocytosis. *eLIFE*. 2015;4:e09580.
- 570 [16] Jeans AF, Oliver PL, Johnson R, Capogna M, Vikman J, Molnar Z, et al. A dominant
571 mutation in SNAP25 causes impaired vesicle trafficking, sensorimotor gating, and ataxia in the
572 blind-drunk mouse. *Proc Natl Acad Sci USA*. 2007;104:2431-6.
- 573 [17] Garcia-Reitböck P, Anichtchik O, Bellucci A, Iovino M, Ballini C, Fineberg E, et al.
574 SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's
575 disease. *Brain*. 2010;133:2032-44.
- 576 [18] Johnson RD, Oliver PL, Davies KE. SNARE proteins and schizophrenia: linking synaptic
577 and neurodevelopmental hypotheses. *Acta Biochim Pol*. 2008;55:619-28.

578 [19] Spessott WA, Sanmillan ML, McCormick ME, Kulkarni VV, Giraudo CG. SM protein
579 Munc18-2 facilitates transition of Syntaxin 11-mediated lipid mixing to complete fusion for T-
580 lymphocyte cytotoxicity. *Proc Natl Acad Sci USA*. 2017;114:E2176-E85.
581 [20] Stockli J, Fazakerley DJ, James DE. GLUT4 exocytosis. *J Cell Sci*. 2011;124:4147-58.
582 [21] Shen XM, Selcen D, Brengman J, Engel AG. Mutant SNAP25B causes myasthenia, cortical
583 hyperexcitability, ataxia, and intellectual disability. *Neurology*. 2014;83:2247-55.
584 [22] Engel AG, Shen XM, Selcen D, Sine SM. Congenital myasthenic syndromes: pathogenesis,
585 diagnosis, and treatment. *Lancet Neurol*. 2015;14:420-34.
586 [23] Avdoshenko SM, Makarov DE. Reaction Coordinates and Pathways of Mechanochemical
587 Transformations. *J Phys Chem B*. 2016;120:1537-45.
588 [24] Weninger K, Bowen ME, Chu S, Brunger AT. Single-molecule studies of SNARE complex
589 assembly reveal parallel and antiparallel configurations. *Proc Natl Acad Sci USA*.
590 2003;100:14800-5.
591 [25] Zhang YL. Energetics, kinetics, and pathway of SNARE folding and assembly revealed by
592 optical tweezers. *Protein Sci*. 2017;26:1252-65.
593 [26] Bustamante C, Chemla YR, Forde NR, Izhaky D. Mechanical processes in biochemistry.
594 *Annu Rev Biochem*. 2004;73:705-48.
595 [27] Sirinakis G, Ren YX, Gao Y, Xi ZQ, Zhang YL. Combined and versatile high-resolution
596 optical tweezers and single-molecule fluorescence microscopy. *Rev Sci Instrum*.
597 2012;83:093708.
598 [28] Moffitt JR, Chemla YR, Izhaky D, Bustamante C. Differential detection of dual traps
599 improves the spatial resolution of optical tweezers. *Proc Natl Acad Sci USA*. 2006;103:9006-11.
600 [29] Jiao JY, Rebane AA, Ma L, Zhang YL. Single-molecule protein folding experiments using
601 high-resolution optical tweezers. *Methods Mol Biol*. 2017;1486:357-90.
602 [30] Zorman S, Rebane AA, Ma L, Yang GC, Molski MA, Coleman J, et al. Common
603 intermediates and kinetics, but different energetics, in the assembly of SNARE proteins. *Elife*.
604 2014;3:e03348.
605 [31] Cecconi C, Shank EA, Bustamante C, Marqusee S. Direct observation of the three-state
606 folding of a single protein molecule. *Science*. 2005;309:2057-60.
607 [32] Bustamante C, Marko JF, Siggia ED, Smith S. Entropic elasticity of lambda-phage DNA.
608 *Science*. 1994;265:1599-600.
609 [33] Liphardt J, Onoa B, Smith SB, Tinoco I, Bustamante C. Reversible unfolding of single RNA
610 molecules by mechanical force. *Science*. 2001;292:733-7.
611 [34] Zhang YL, Jiao J, Rebane AA. Hidden Markov modeling with detailed balance and its
612 application to single protein folding *Biophys J*. 2016;111:2110-24.
613 [35] Rebane AA, Ma L, Zhang YL. Structure-based derivation of protein folding intermediates
614 and energies from optical tweezers. *Biophys J*. 2016;110:441-54.
615 [36] Jackson SE, Fersht AR. Folding of Chymotrypsin Inhibitor-2 .1. Evidence for a 2-State
616 Transition. *Biochemistry-U.S.* 1991;30:10428-35.
617 [37] Guinn EJ, Jagannathan B, Marqusee S. Single-molecule chemo-mechanical unfolding
618 reveals multiple transition state barriers in a small single-domain protein. *Nat Commun*. 2015;6.
619 [38] Zhang XM, Rebane AA, Ma L, Li F, Jiao J, Qu H, et al. Stability, folding dynamics, and
620 long-range conformational transition of the synaptic t-SNARE complex. *Proc Natl Acad Sci*
621 *USA*. 2016;113:E8031-E40.
622 [39] Mohrmann R, de Wit H, Verhage M, Neher E, Sorensen JB. Fast vesicle fusion in living
623 cells requires at least three SNARE complexes. *Science*. 2010;330:502-5.

624 [40] Shi L, Shen QT, Kiel A, Wang J, Wang HW, Melia TJ, et al. SNARE proteins: One to fuse
625 and three to keep the nascent fusion pore open. *Science*. 2012;335:1355-9.
626 [41] Gittes F, Schmidt CF. Interference model for back-focal-plane displacement detection in
627 optical tweezers. *Optics Letters*. 1998;23:7-9.
628 [42] Viterbi AJ. Error bounds for convolutional codes and an asymptotically optimum decoding
629 algorithm. *Ieee T Inform Theory*. 1967;13:260-9.
630 [43] Fernandez I, Ubach J, Dulubova I, Zhang XY, Sudhof TC, Rizo J. Three-dimensional
631 structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. *Cell*. 1998;94:841-9.
632 [44] Shen JS, Tareste DC, Paumet F, Rothman JE, Melia TJ. Selective activation of cognate
633 SNAREpins by Sec1/Munc18 proteins. *Cell*. 2007;128:183-95.

634

635

Habc

Syntaxin1A

||||| S-S

VAMP2

◆ Biotin

SNAP-25b

● Digoxigenin









