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Supporting Information

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Supporting Information

for

Pathogenic Mutations Shift the Equilibria of α -Synuclein Single Molecules towards Structured Conformers

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Table S1. The following table records the χ^2 squares comparing the populations of the four sequence variants described in this work. For the sample size, see the caption of Figure 2 in the main text.

Comparison	χ^2	p
WT / A30P	46.64	< 0.001
WT / E46K	17.46	< 0.001
WT / A53T	112.97	< 0.001
A53T / A30P	3.14	0.2
A53T / E46K	17.10	< 0.001
A30P / E46K	20.28	< 0.001

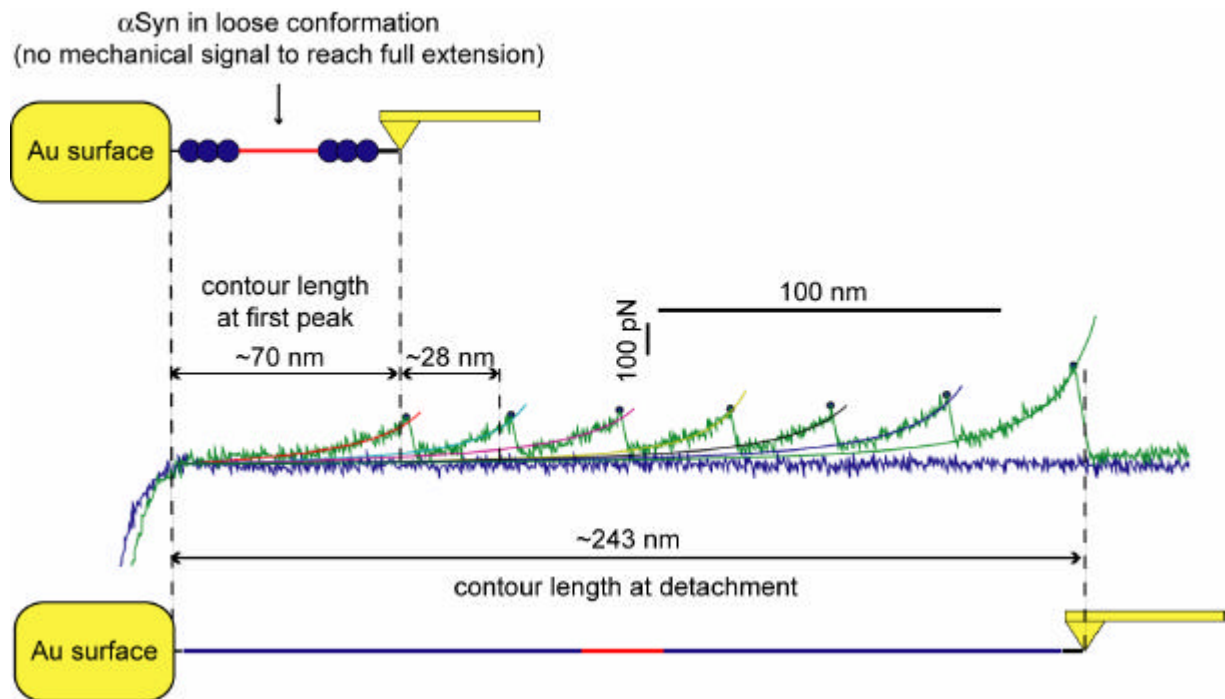


Figure S1. Single 3S3 molecule mechanical unfolding trace showing six mechanical events. Blue trace = approach. Green trace = retraction. The force curve shows six clean rupture peaks, separated by ~28 nm (see Figure S5). The average WLC contour length fitted on the first rupture event is ~70 nm (see Figure S7). This distance is compatible with the sum of six folded I27 modules (~ 4.5 nm each^[1] = $4.5 \times 6 = 27$ nm) and one fully unfolded α -Syn moiety (see cartoon at the top of the figure). The WLC contour length fitted on the last peak (detachment from surface) is ~243 nm, compatible with the expected length of a fully unfolded 3S3 construct (680 AA).

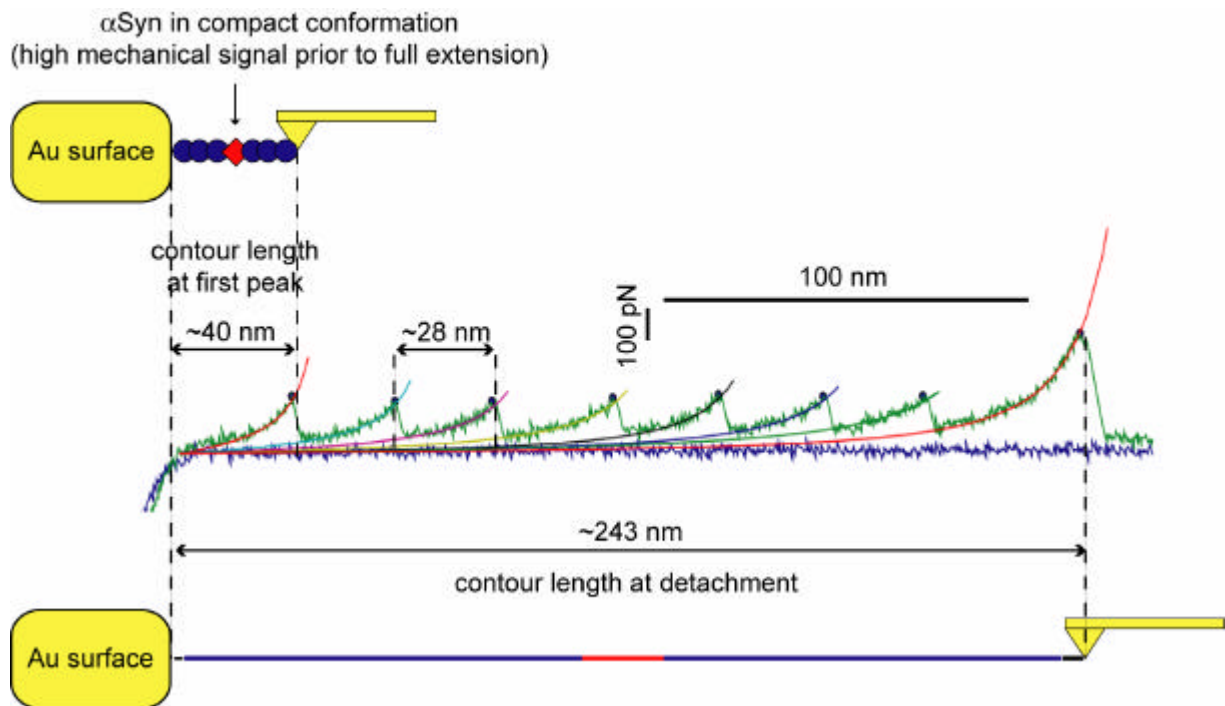


Figure S2. Example of a single 3S3 molecule mechanical unfolding traces showing seven mechanical events. Blue trace = approach. Green trace = retraction. The average WLC contour length fitted on the first rupture event is ~ 40 nm (see Figure S6). This distance is compatible with the sum of six folded I27 modules (~ 4.5 nm each = $4.5 \times 6 = 27$ nm) and one partially folded a-Syn moiety (see cartoon at the top of the figure). The folded portion of a-Syn has the same length of one I27 module (90AA, ~ 28 nm), which is compatible with the portion of a-Syn found to assume a beta conformation in fully formed amyloid fibrils. The WLC contour length fitted on the last peak (detachment from surface) is ~ 243 nm, compatible with the expected length of a fully unfolded 3S3 construct (680 AA).

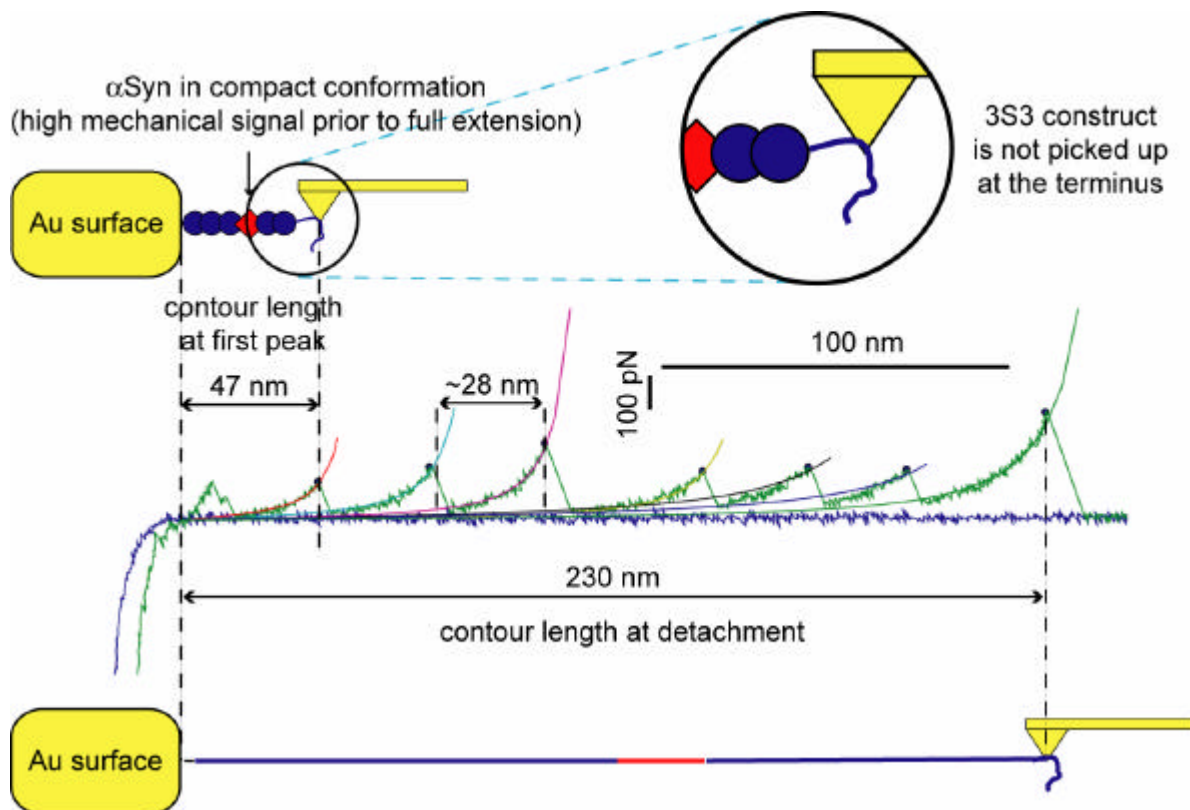


Figure S3. Example of one ambiguous force curve: single 3S3 molecule mechanical unfolding trace showing six mechanical unfolding events and a shorter-than-expected contour length for the first peak. As depicted in the cartoon at the top of the figure, an ambiguity is possible regarding force curves showing six peaks. The six mechanical unfolding peaks could arise from six I27 unfolding events (as in the class of curves described in Figure S1), or from five I27 and one a-Syn unfolding event. In the latter case, final contour length is lower than a full 3S3 construct (see the situation depicted in the cartoon at the top of the figure). Discerning between the two cases must rely on the contour length measurement of the first peak: the expected value for the first case is ~70 nm (see Figure S1), while the expected value in the second case is comprised 35-70 nm. The shorter of the two values is one I27 module less than a fully folded 3S3 ($\sim 40 - 4.5 = \sim 35$ nm, see Figure S2), while the longer is one fully unfolded I27 module plus the unfolded portion of a-Syn (~ 50 AA). Also due to intrinsic errors of WLC fitting and contact point determination, discerning between the two situations is impossible; this means that a portion of the curves classified in the same class described in Figure S1 could be due to 3S3 conformations in which a-Syn is in a compact conformation. This portion can be expected to be higher in situations in which a-Syn is more prone to assume compact conformations, thus reducing the average contour length at first peak of curves showing six peaks. This expectation is indeed confirmed by the measured contour lengths (see Figure S6), thus providing a further confirmation of the internal consistency of our data.

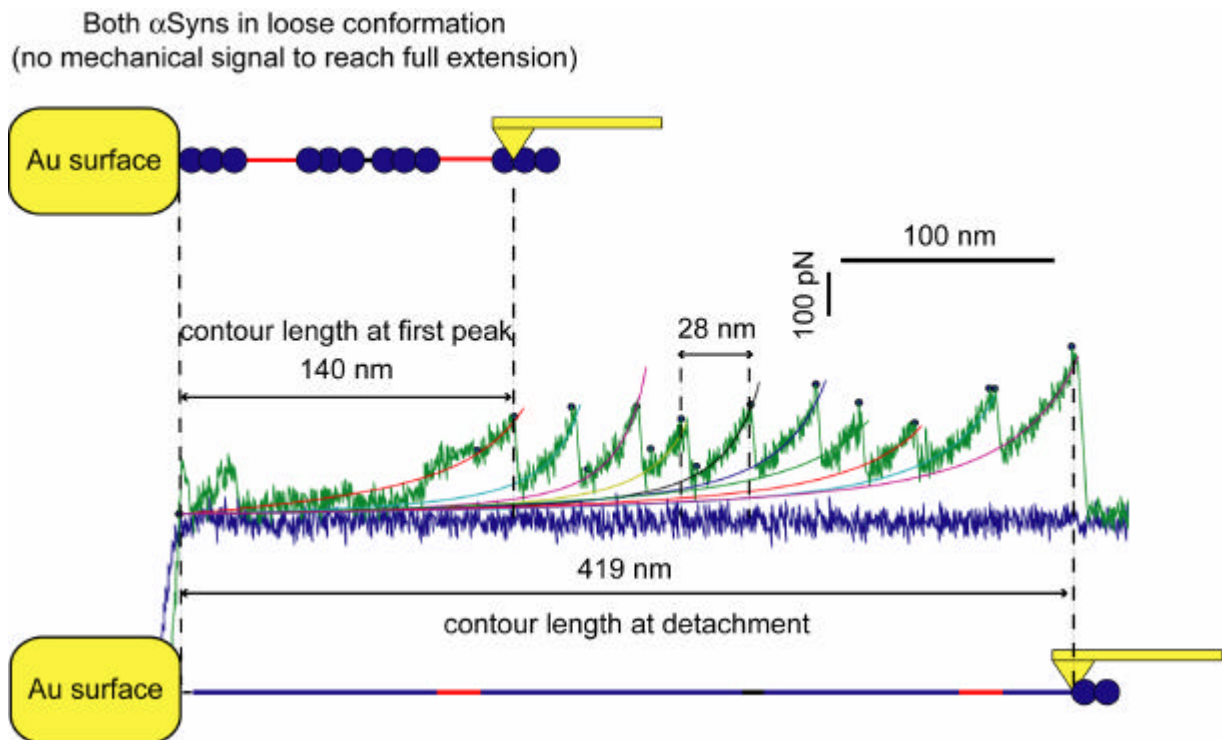
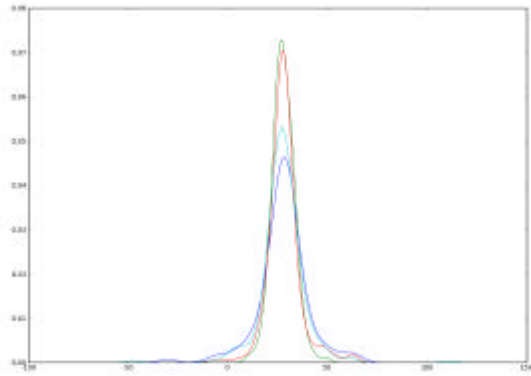


Figure S4. Example of one force curve resulting from the pulling of a 3S3-3S3 dimer. Dimers can form via oxidation of the C-terminal cysteines. While other thiol-functionalized proteins we studied in our lab are prone to dimerization via the formation of disulfide bonds, surprisingly low amounts of 3S3 dimers were observed in the present study (about 0.002% of the recorded curves were unambiguously attributed to dimerized 3S3-3S3 constructs). These traces could show up to twelve I27 mechanical rupture events, although we never observed curves with more than ten peaks. The curve reported here has ten I27 rupture events, and the contour lengths at first peak and at detachment are in accord with the situation depicted in the cartoon at the top of the figure. Curves with eight or more peaks were immediately recognized as dimers and discarded.

Dimerized 3S3 construct could also give mechanical traces showing seven peaks. These cannot in any case be mistaken for the seven-peaked curves described in Figure S2, because no pulling geometry of a 3S3-3S3 dimer can give the contour length at first peak observed in monomeric 3S3 seven-peaked curves (please refer to figure S2 and S6). This is due to the fact that, if seven I27 modules of a dimer are stretched, then the mechanical extension trace of at least one α -Syn moiety must be also included. Due to this, the minimum possible contour length at first peak for a seven-peaked curve generated by the stretching of a dimer is $(4.5 \times 7 = 31.5 \text{ nm for the I27 modules} + 49 \text{ nm for a fully stretched } \alpha\text{-Syn}) \sim 80 \text{ nm}$. Of course, also the detachment contour length is accordingly higher in this types of curves. Please refer to the distribution of contour lengths at first peak observed in our monomeric 3S3 seven-peaked curves (Figure S6).

A)



B)

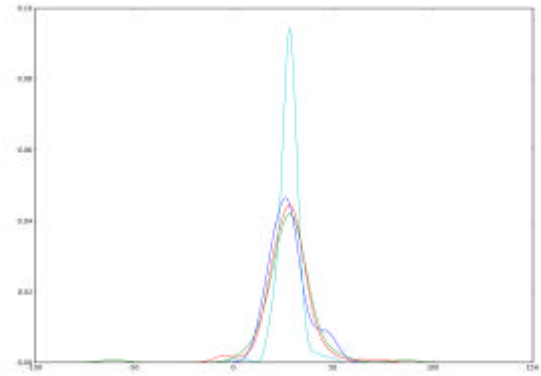


Figure S5. Kernel Density Estimation^[2] of the contour length increases (ΔL) observed after each strong mechanical unfolding event. Briefly, a Gaussian function (kernel) has been centered on each data point for each data set. The sum of the kernels, normalized to have unitary integral, is the KDE plot. Kernel bandwidth h (i.e. the Gaussian kernel standard deviation) was automatically calculated for each data set such that it minimizes the asymptotic mean integrated square error:

$$h = \sigma \left(\frac{4}{3n} \right)^{\frac{1}{5}}$$

where σ is the standard deviation of data and n is the size of the data set. The plot has been calculated using Statistics for Python (<http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/python/Statistics/>). A) Observed ΔL values in curves with six peaks (see Figure S1): Blue = WT ($N=126$ peaks), Green = A30P ($N=138$), Red = E46K ($N=114$), Cyan = A53T ($N=162$). B) Curves with seven peaks. Blue = WT ($N=21$ peaks), Green = A30P ($N=84$), Red = E46K ($N=70$), Cyan = A53T ($N=161$). No statistically significant differences between 3S3 variants are recognizable. No statistically significant differences between curves with six or seven peaks were observed.

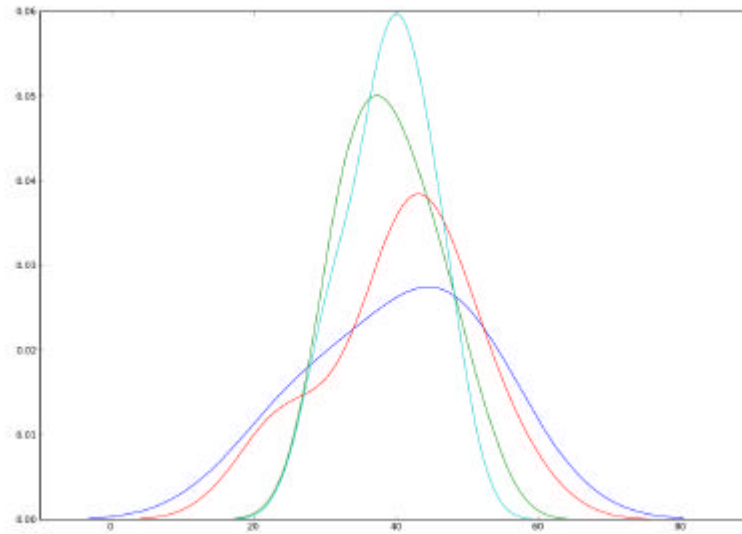
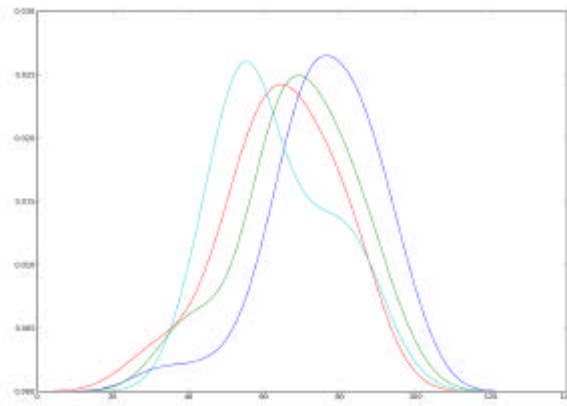


Figure S6. Contour length Kernel Density Estimation of the first peak in curves showing 7 mechanical events. Blue = WT (40 ± 12 nm, $N=3$), Green = A30P (39 ± 6 nm, $N=12$), Red = E46K (41 ± 10 nm, $N=10$), Cyan = A53T (39 ± 6 nm, $N=23$). No statistically significant differences between 3S3 variants are recognizable. Combined value for all 3S3 variants is 40 ± 7 nm ($N=48$). This value rules out the possibility that seven-peaked curves are artifacts due to 3S3-3S3 dimerization (see Figure S4)

A)



B)

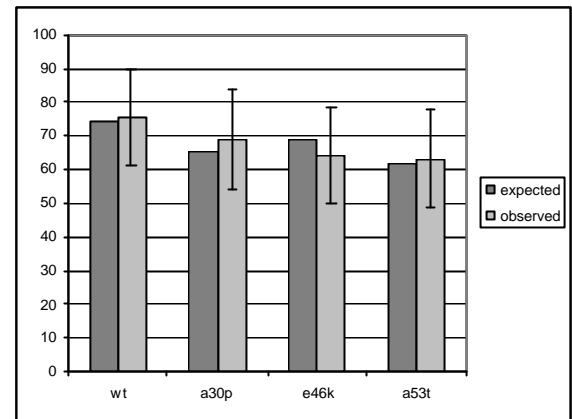


Figure S7. A) Contour length Kernel Density Estimation of the first peak in curves showing six mechanical events. Blue = WT ($76 \pm 14 \text{ nm}$, $N=21$), Green = A30P ($70 \pm 15 \text{ nm}$, $N=23$), Red = E46K ($64 \pm 14 \text{ nm}$, $N=19$), Cyan = A53T ($63 \pm 15 \text{ nm}$, $N=27$). Combined value for all 3S3 variants is $68 \pm 15 \text{ nm}$ ($N=90$). A trend is observed, which is briefly explained in Figure S3. B) Expected and measured contour lengths of the first peak in curves showing six mechanical events. The expected values are calculated following the model outlined in Figure S3: a portion of curves with six peaks are attributed to conformations in which the a-Syn moiety is in a compact conformation, and one less I27 module is not included in the portion of the molecule that is trapped between the surface and the AFM tip and, as such, its mechanical unfolding is not visible in the force trace. The portion of these traces included among the six peaked curves is proportional to the observed percentage of compact structures for each 3S3 variant. The expected length is $[(100 - \beta) \times L6] + (\beta \times L7) / 100$, where β is the percentage of observed compact conformations for the 3S3 variant, $L6$ is the geometrically calculated ideal value for the contour length of the first peak in six-peaked curves, and $L7$ is the geometrically calculated ideal value for the contour length of the first peak in seven-peaked curves.

Observed values are in good accord with this model, providing a further evidence of the internal consistence of our data.

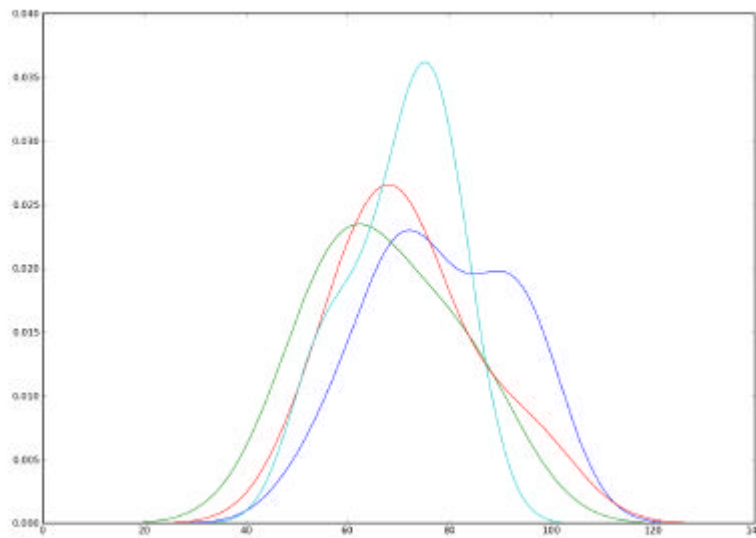


Figure S8. Contour length Kernel Density Estimation of the first peak in curves showing six strong mechanical events and one MWI rupture event. Blue = WT ($78 \pm 14 \text{ nm}$, $N=16$), Green = A30P ($67 \pm 14 \text{ nm}$, $N=5$), Red = E46K ($72 \pm 14 \text{ nm}$, $N=9$), Cyan = A53T ($70 \pm 10 \text{ nm}$, $N=14$). No statistically significant differences between 3S3 variants are recognizable. Combined value for all 3S3 variants is $73 \pm 13 \text{ nm}$ ($N=44$).

References

- [1] A. F. Oberhauser, P. E. Marszalek, M. Carrion-Vazquez, J. M. Fernandez, *Nature Structural Biology* **1999**, 6, 1025.
- [2] E. Parzen, *Ann of Math Stat* **1962**, 33, 1065.