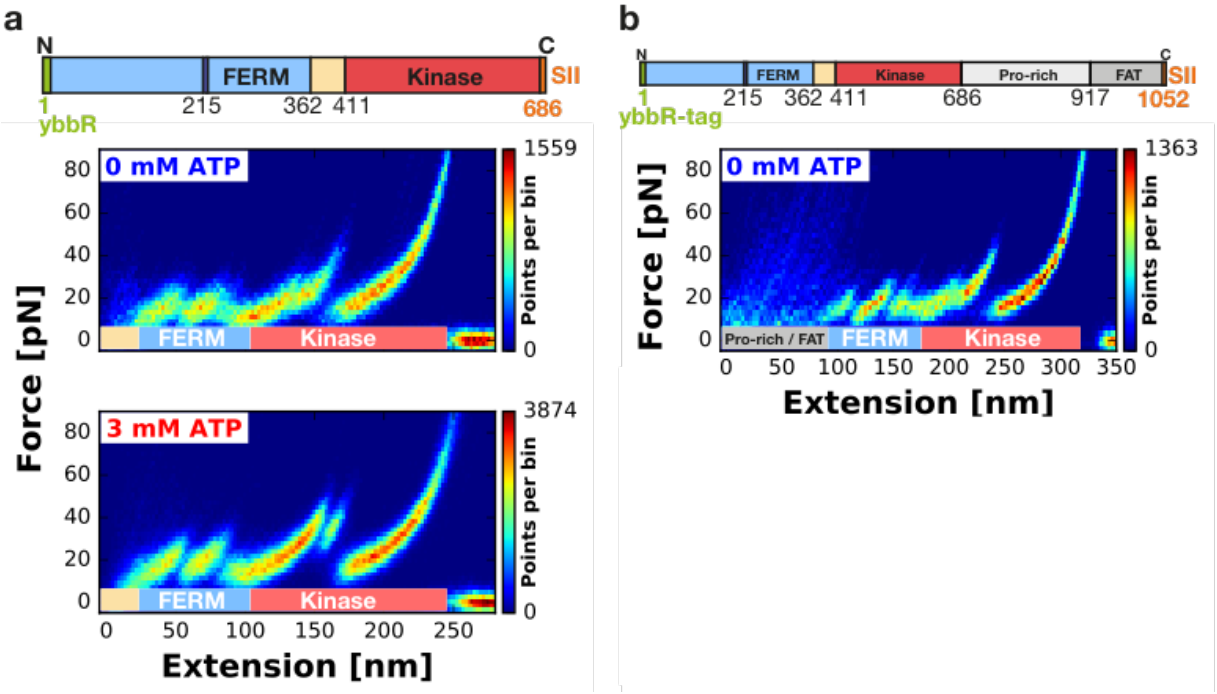
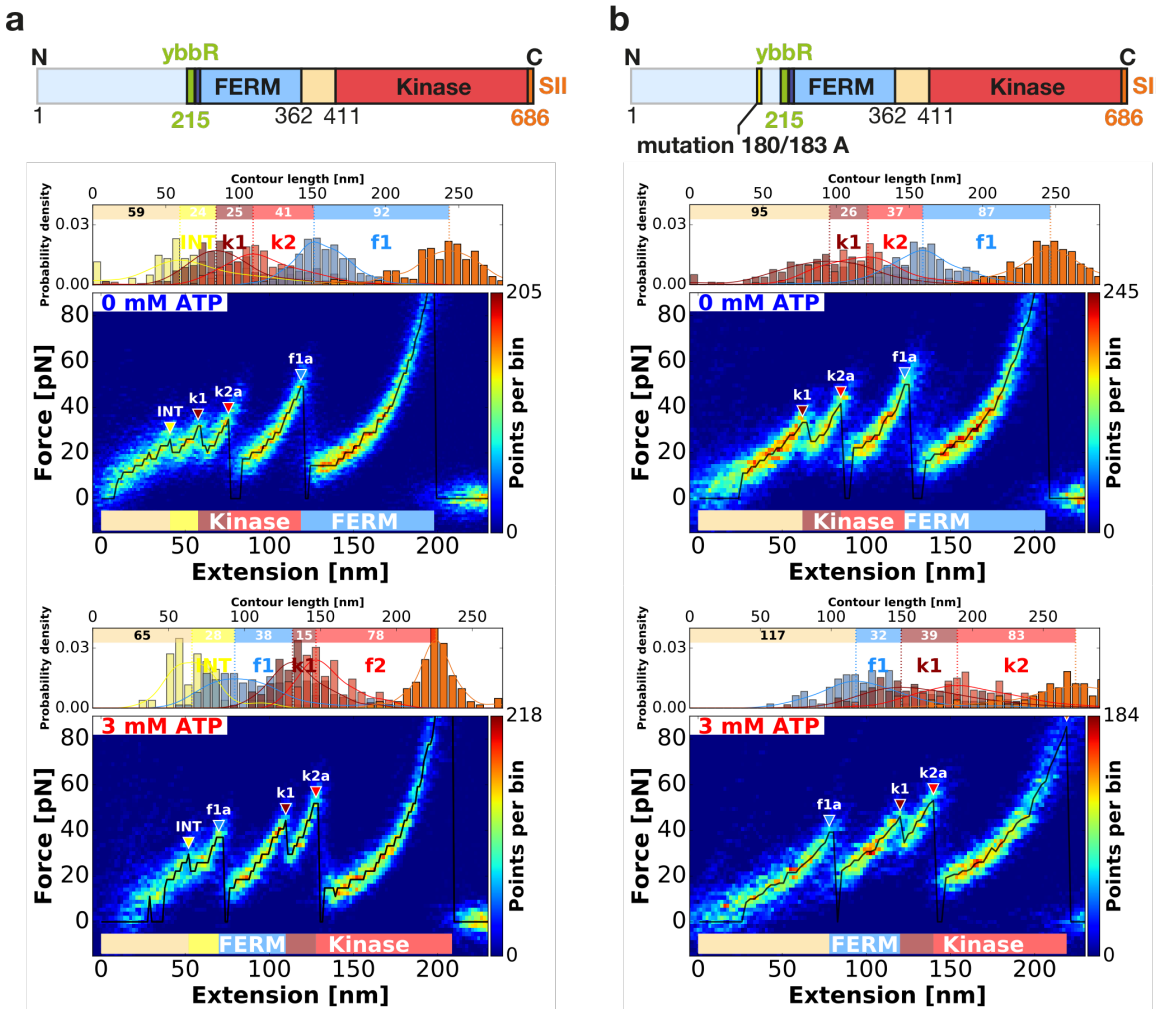
Supplementary Information

N-C terminal pulling of the FAK construct and full-length unfoldings



Supplementary Figure S1: *NC-terminal pulling of the FAK construct.* ***(a)*** *In initial experiments we attached FAK with tags placed at the N- and C-termini and retracted the cantilever at a speed of 800 nm/s with 5,000 Da PEG linkers. The top panel shows the unfolding without ATP present and the bottom with 3 mM ATP in the measurement buffer.* ***(b)*** *Probing of full-length FAK molecules (1-1052 amino acids) with 800 nm/s with 425.39 Da PEG resulted a longer unfolding pattern accounting for the longer total length. However, the pattern occurring at 100 nm is the same as in* ***(a)*** *as indicated by the labels below. This leads to the conclusion that the proline-rich region and FAT domain do not significantly contribute to unfolding of the autoinhibitory structure from (1-686 amino acids). This supports the findings of Goni et al.*

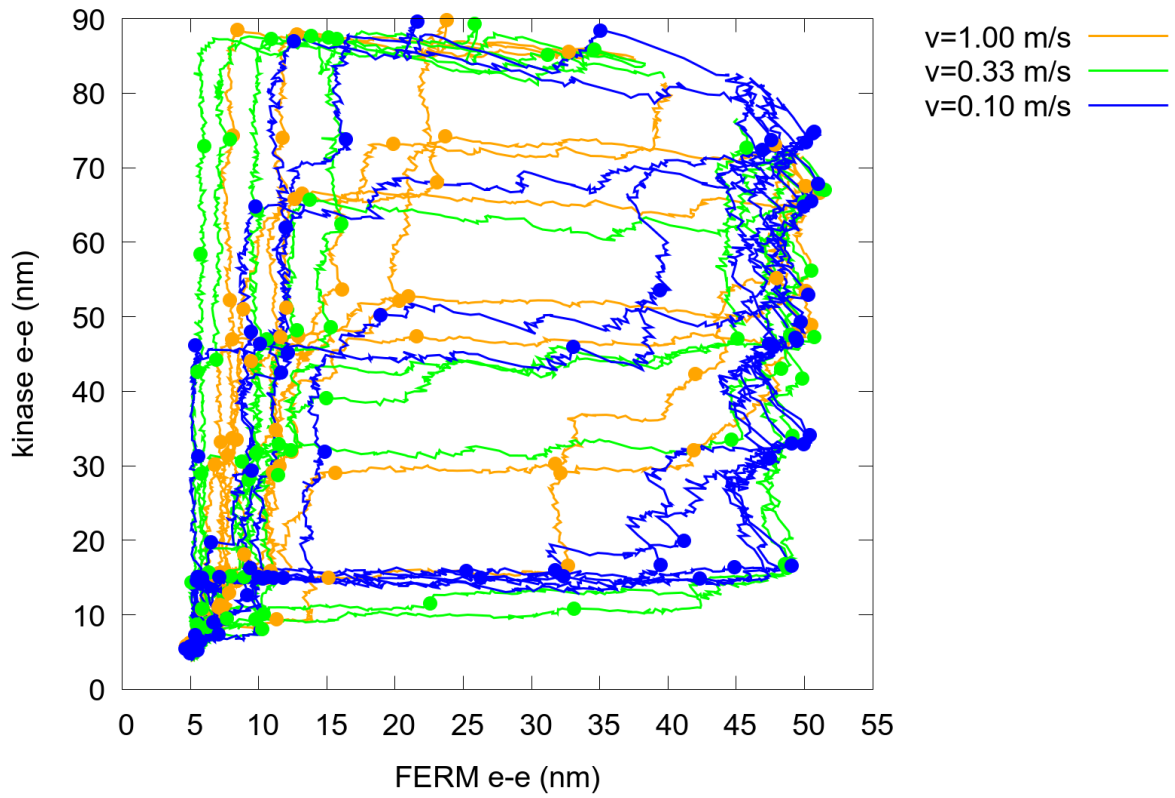
Contour length histograms for Figure 5



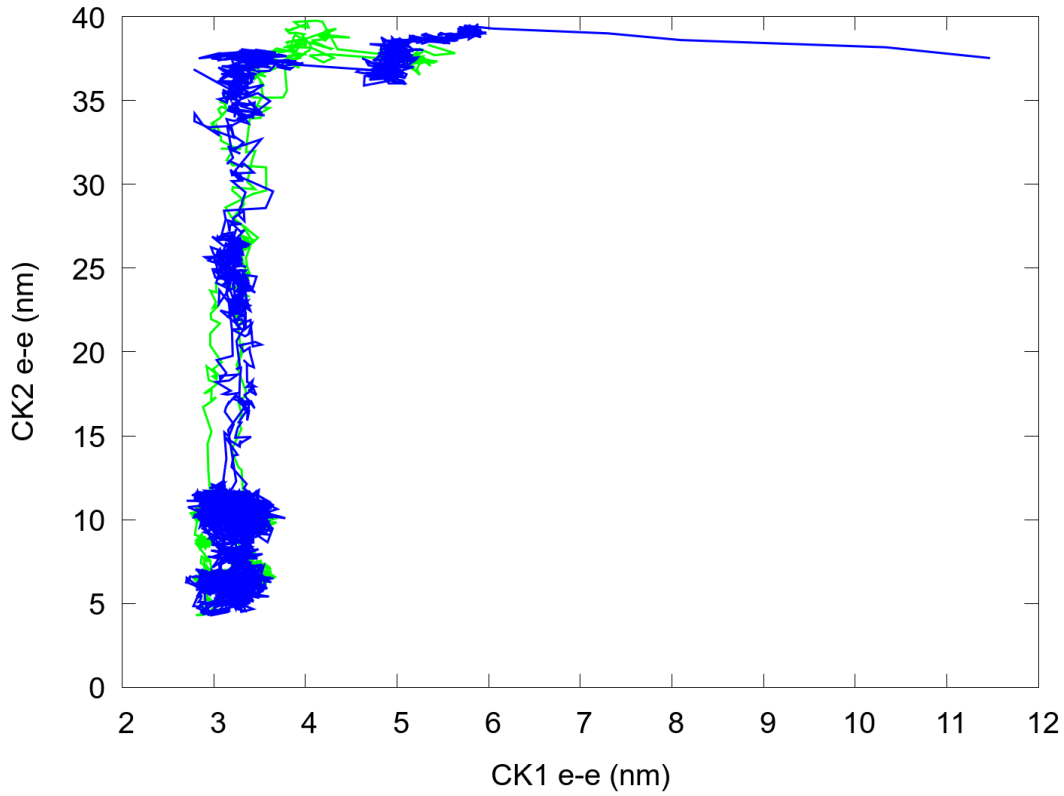
Supplementary Figure S2*: Figure 5 with analysis of contour length histograms.* *The Figure shows the same heatmaps as in Figure 5 for pullings with PEG 5,000 Da and 12,800 nm/s with added contour length histograms. Since the persistence length is changing too much over the course of the whole unfolding length, the increments are not very reliable. This is due to the long PEG linker (low persistence length) that is dominating the persistence length in the beginning of the curve and the increase in persistence length once parts of the protein get unfolded. It is possible to conduct WLC fits however the persistence length and contour length as fit parameters are not stable enough to produce comparable contour length increments. This is in contrast to the measurements with the short PEG 425.39 Da (Fig. 2, 3, 4) where only the first unfolding is dominated by the persistence length of the PEG and therefore yields comparable increments for further unfoldings.*

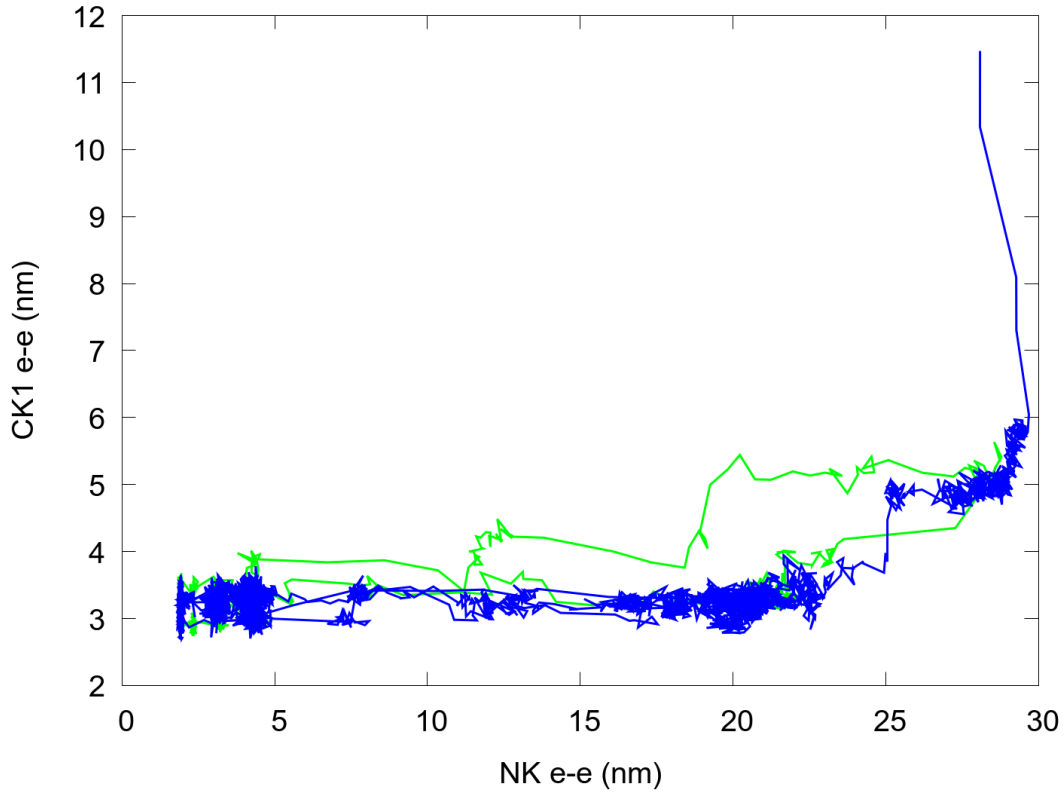
Kinase unfolding in molecular dynamics simulations

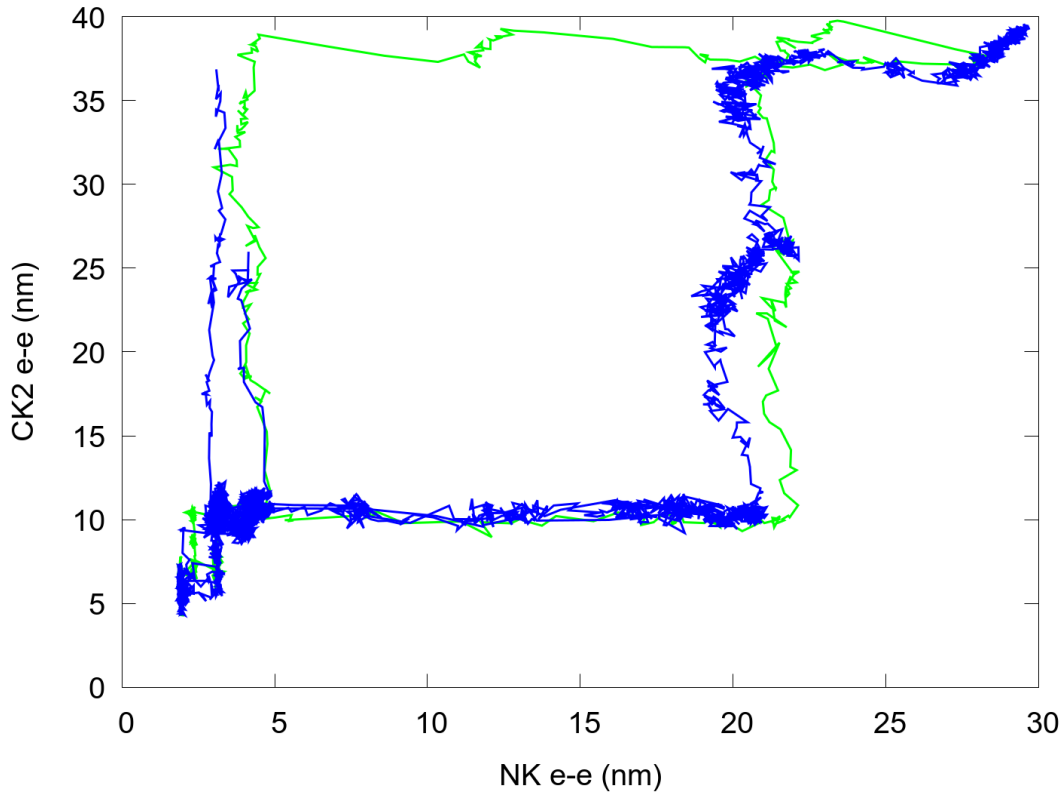
The kinase domain, in the presence of ATP, has been observed in AFM experiments to unfold in two stages. The first step amounts to 13 nm and the second one to 66 nm. In the MD simulations, the kinase domain stretches by 10 nm during or before FERM unfolding. 7 nm out of this stretching is due to a partial unfolding of the C-terminal region of the C-lobe, and another 3 nm is due to the lobes rearranging. While experimentally, kinase unfolding happens fully after the FERM domain unfolds, this partially unfolded state is observed in all of our simulations, and we consider it the most likely explanation to this first jump. The further unfolding of the kinase domain happens through numerous pathways, but we can see that the last part of the kinase that unfolds is the part of the C-lobe that is before the activation loop (cf. SI, where this subdomain is dubbed “CK1”). We note that kinase domain unfolding leads to FAK deactivation independent from the detailed sequence of events.



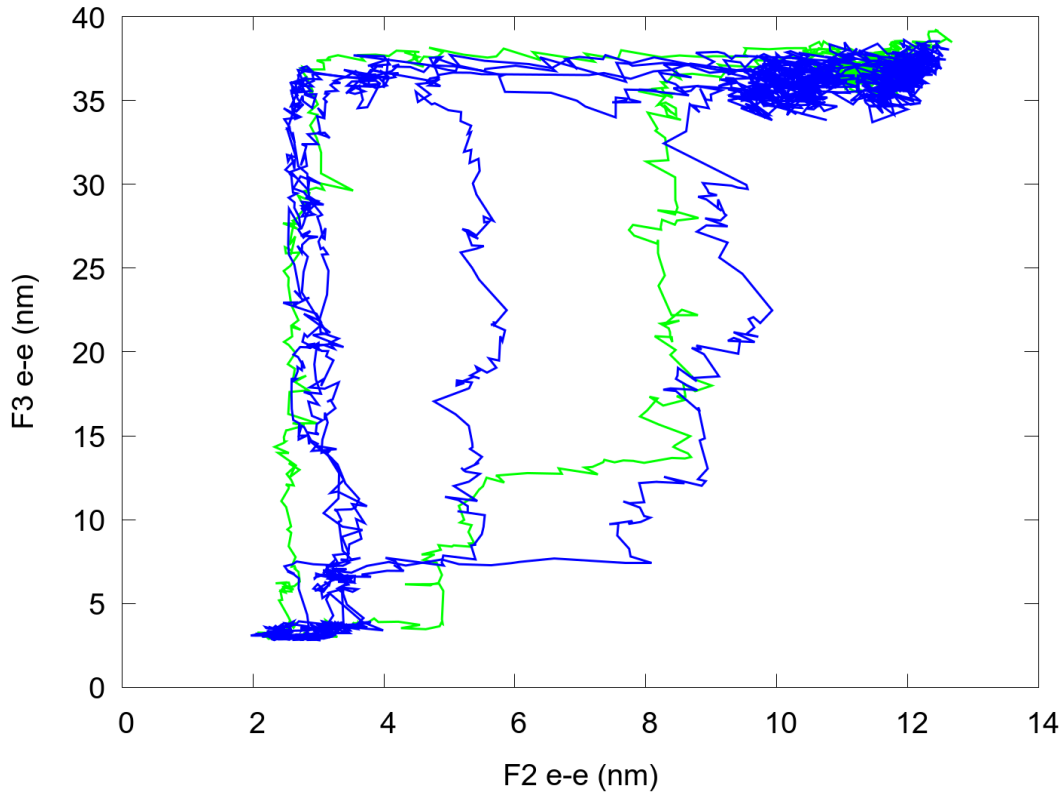
Supplementary Figure S3: *unfolding trajectories visualized in the "phase space" of FERM end-to-end distance and kinase end-to-end distance. Points are rupture peaks.*


CK2 e-e (nm)
CK1 e-e (nm)
0
5
10
15
20
25
30
35
40
2
3
4
5
6
7
8
9
10
11
12

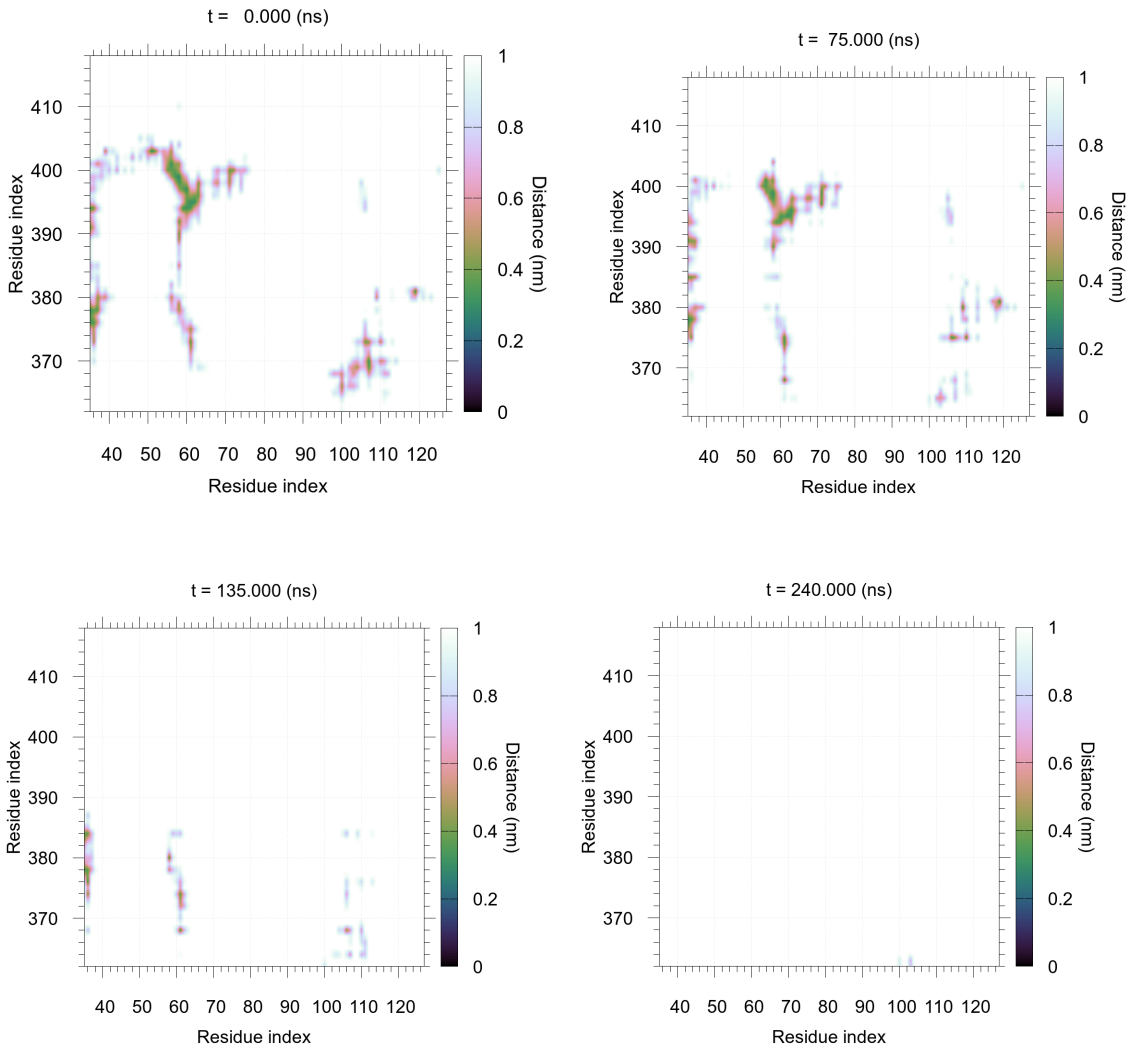

CK1 e-e (nm)
NK e-e (nm)
2
3
4
5
6
7
8
9
10
11
12
0
5
10
15
20
25
30



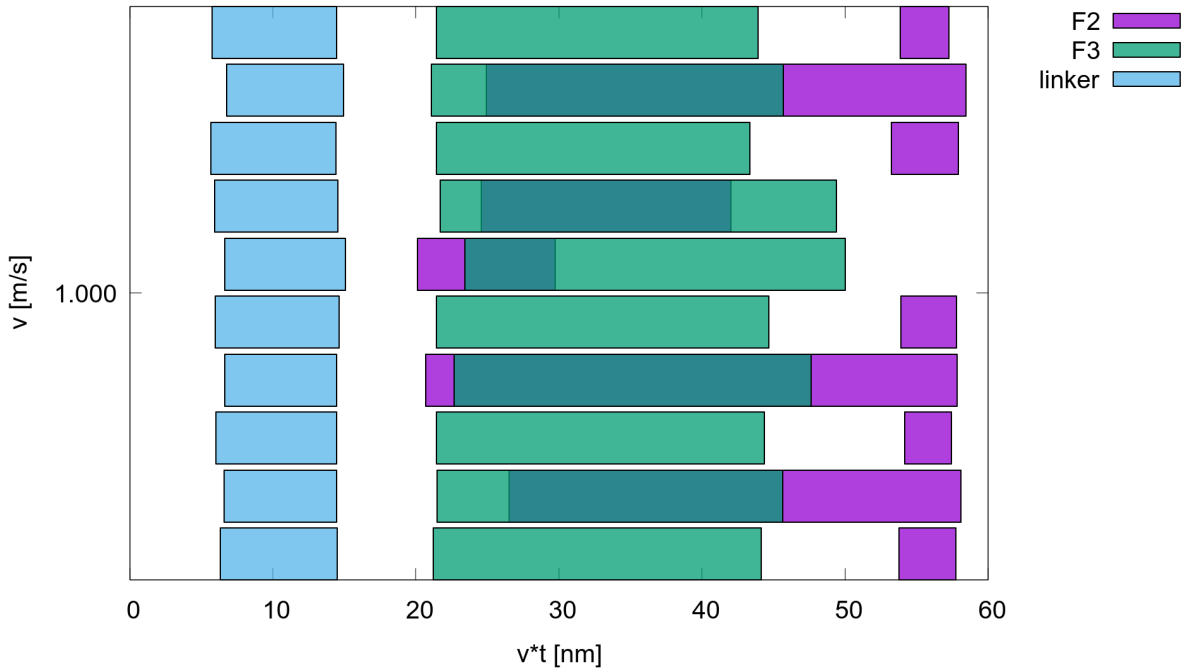
Supplementary Figures S4-6: *Order of unfolding between the three constitutive parts of the kinase domain. Only the 7 simulations consistent with the experiments are considered.*



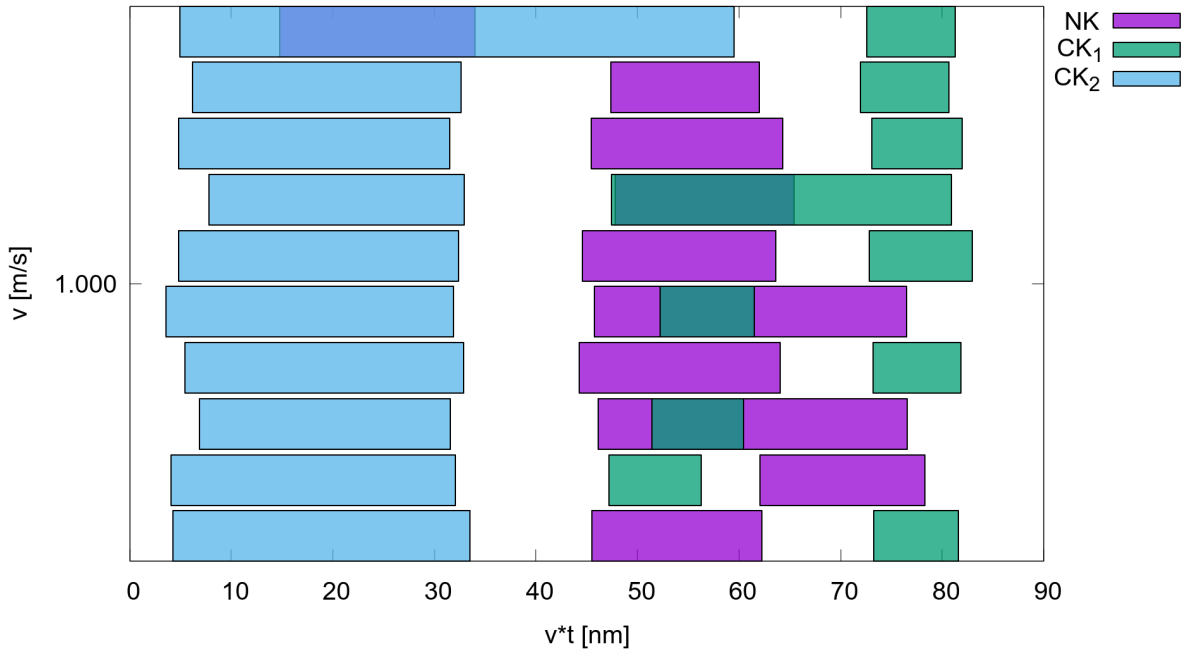
Supplementary Figure S7: *Order of unfolding between the two constitutive parts of the FERM domain. Only the 7 simulations consistent with the experiments are considered.*



Supplementary Figure S8: *contact maps of the linker-F1 interaction corresponding to the poses from Fig 5.*



Supplementary Figure S9: *the FERM-only unfolding simulations follow the same hierarchy of unfolding events as the unfoldings in the main text: The linker loses contact with lobe F1 first, then F3 unfolds in 8/10 cases, then F2 stretches.*

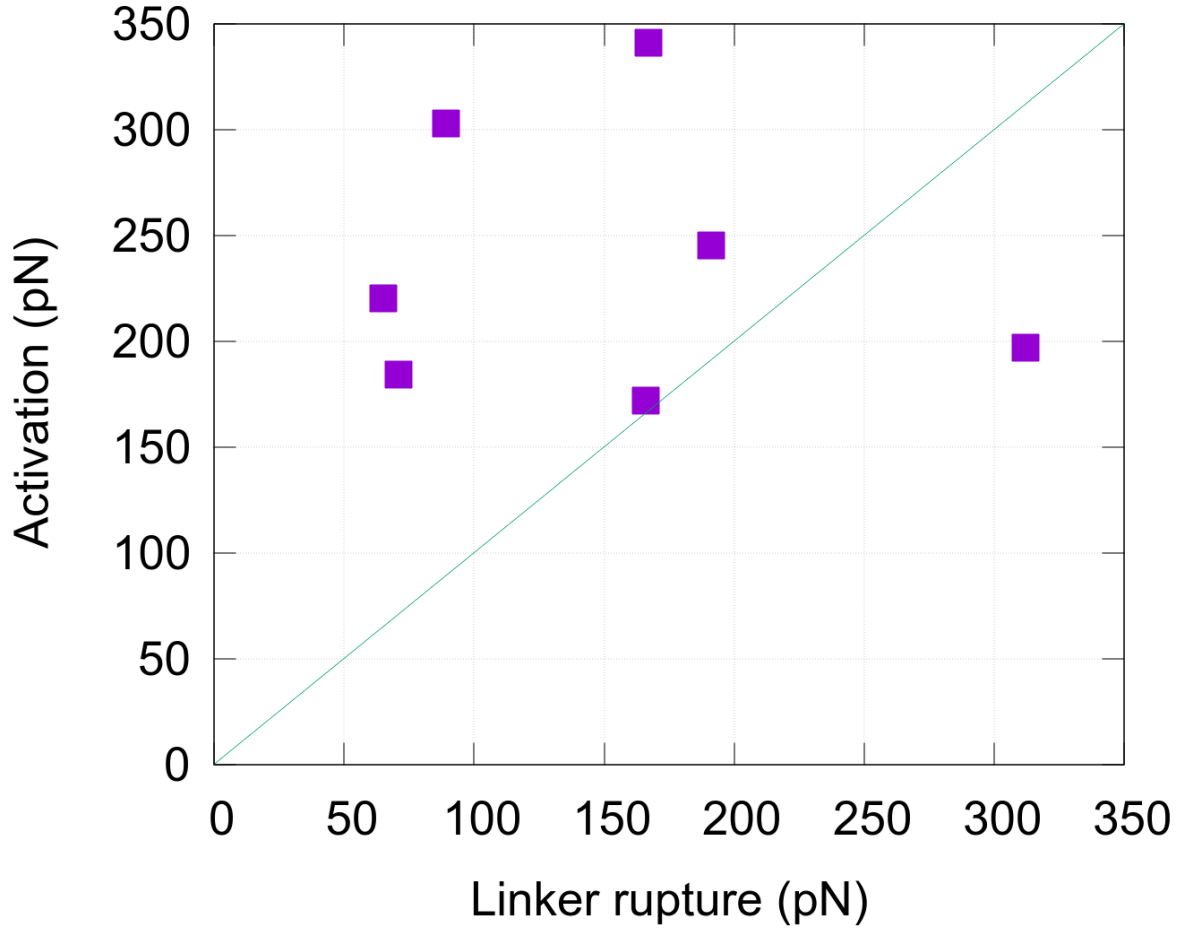


Supplementary Figure S10: *the kinase-only unfolding simulations follow the same hierarchy of unfolding events as the unfoldings in the main text: CK2 usually unfolds first, followed by NK and CK1.*

Event	MD (nm)	AFM (nm)	Assignment in AFM plots
Domain separation	10		first extension (prior to first unfolding)
Linker-F1 separation	12		first extension
F3 unfolding	30	29-32	f1a+f1b
F2 unfolding	13*	14-19	f1c
CK partial	10	13	k1
Kinase rest	70	68	k2

** 9 nm increase in the simulations and an estimated 4 nm from a loop region of the ybbR-tag included in the experimental construct*

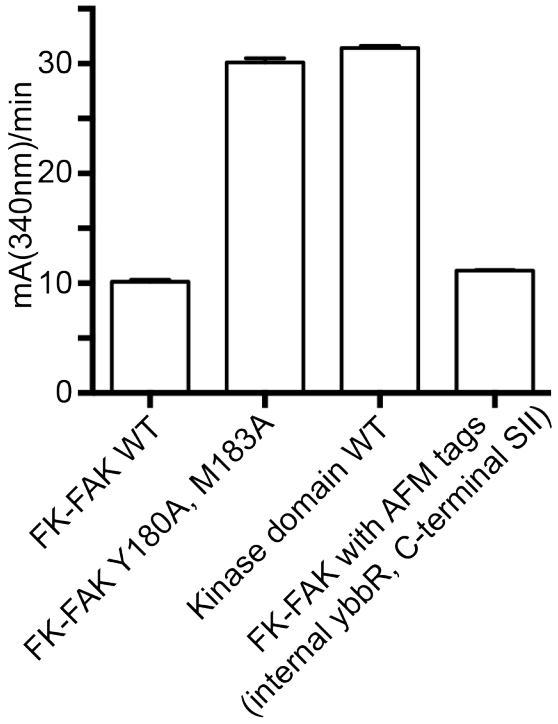
***Supplementary Table S1: Summary of the length changes observed in MD simulations (end-to-end distance changes) and AFM experiments (contour length increments)**. Due to the relatively high pulling speed in experiments (0.1m/s or higher), the MD increments can within the error of the two methods be assumed to be similar to the AFM increments.*



Supplementary Figure S11: Conformational activation requires more force than linker release. *We compare the rupture forces corresponding to conformational activation (y-axis) with those corresponding to the linker release (x-axis). Since 6/7 of our simulations required more force for activation than linker release, we conclude that linker rupture was not observed in experiments.*

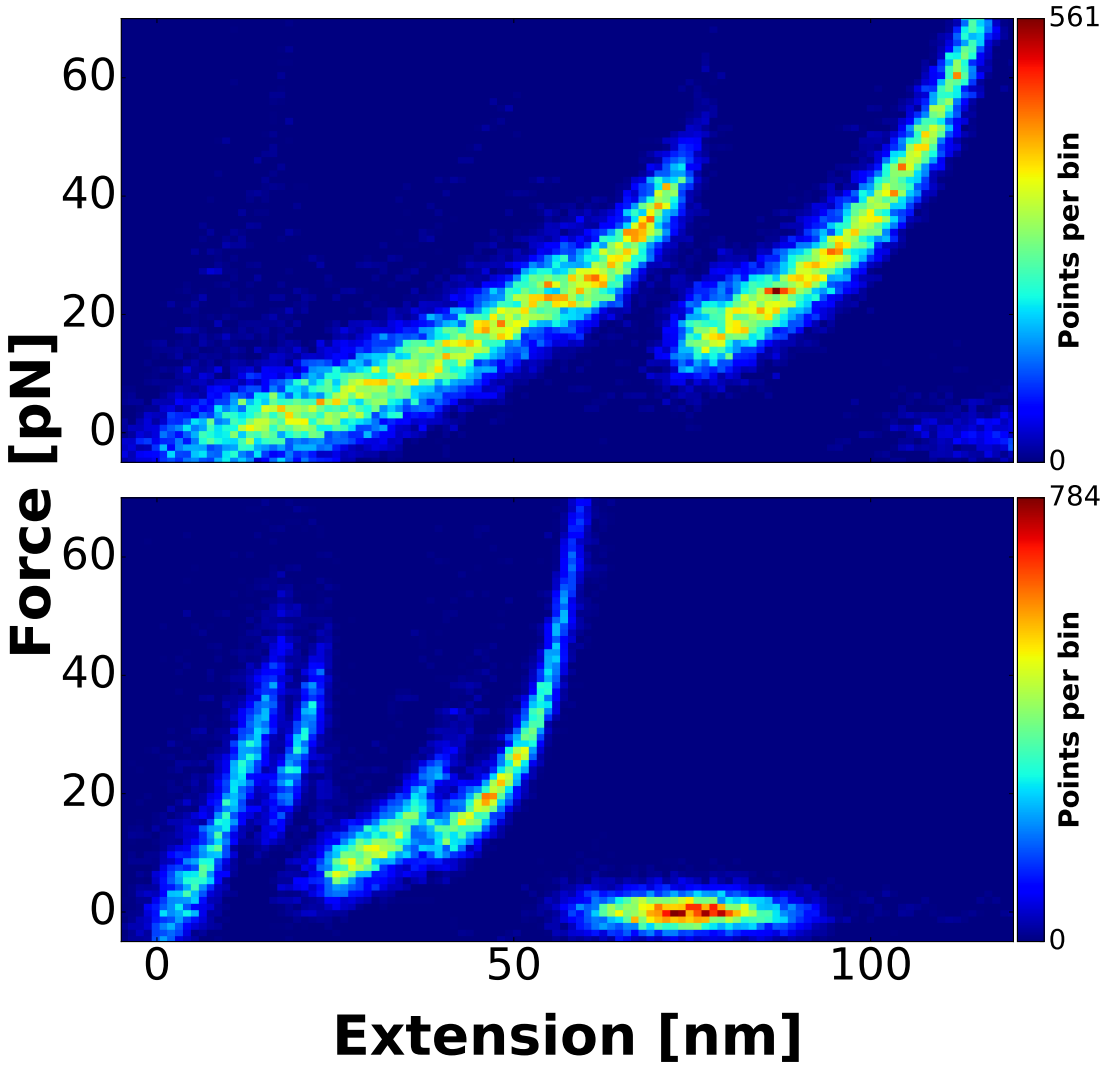
Kinase activity

An enzyme-coupled spectrophotometric assay was used to determine ATP turnover of FAK proteins as described by [2]. In brief, reactions were performed with 1 μM FAK, 2 mM MgCl2, 1 mM phosphoenolpyruvate, 0.25 mM NADH, 0.08 units/ L pyruvate kinase, 0.1 units/ L lactate dehydrogenase, and 100 μM E4Y (as polyGlu-Tyr, 4:1 Glu/Tyr; Sigma). Reactions were initiated with 1 mM ATP and NADH depletion was monitored by UV absorption at 340 nm.



Supplementary Figure S12: *Activity of FAK constructs was measured using a coupled kinase assay and readout of NADH consumption at 340 nm. Y180, M183 mutations disrupt autoinhibitory interactions between FERM and kinase domain [1]. Introduction of tags for AFM experiments do not affect FAK activity or autoinhibition.*

Resolution increase with shorter PEGs

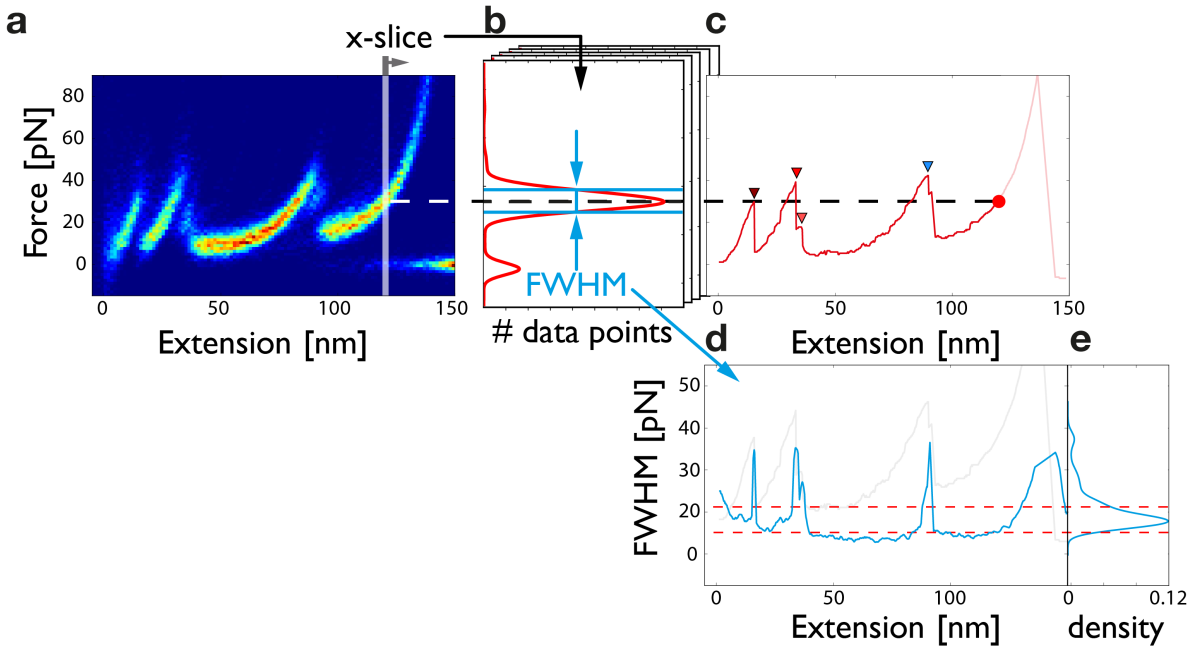


Supplementary Figure S13*: Comparison of the FERM construct with different linkers.* *Depicted is a comparison of the same FERM only construct (1-405 amino acids) between PEG 5,000 Da on top and PEG 425.39 Da on the bottom showing a much detailed unfolding pattern at 800 nm/s. This way it is possible to gain information on contour length increments not possible with the curves measured with the long PEG on top.*

In all previous experiments we used PEG with an average of 5,000 Da (long PEG), which has been used as a standard linker length in previous similar experiments.[3] Reducing the linker length to dimeric PEG (425.39 Da – short PEG), we indeed obtained greatly improved plots with reduced noise levels and increased force

signals. The increase in force signals we attribute to an increase in the average loading rates due to the the WLC behavior, resulting in higher force peaks. To even further boost the height of the force drops the loading rates where increased by using higher pulling velocities, 12,800 nm/s (fast pulling) instead of 800 nm/s (slow pulling) in some experiments. The experimental conditions indicated above (shorter linker, faster pulling) were applied accordingly in order to get enhanced results.

Most probable unfolding curve assembly and peak detection



Supplementary Figure S14*: Depiction of the most probable unfolding curve assembly and peak detection.* *For assembly of the most probable unfolding curve, the denoised data (Savitzky–Golay) in force-distance space are sliced in distance-axis slices (2.5 nm) with a moving slice window of 0.2 nm (a) and their densities (b) on the force axis (y-axis) were estimated by a kernel density estimate (KDE, bandwidth: 0.2 pN) (b). The most probable value is then plotted in c (red curve) to assemble the most probable unfolding curve. The FWHM of the most probable values of the KDEs in b are then plotted in d. Afterwards the KDE over the FWHMs of the distance-slices in d are shown in e, together with their FWHM (red dashed lines). This FWHM value describes the noise level of the most probable unfolding curve with points deviating showing unfolding events. The most probable unfolding curve can thereby be analyzed for most probable unfolding peaks (colored triangles on top of the red curve). A first selection is done by selecting peaks based on its first order difference. Then, the peaks are evaluated concerning their FWHM value in d. Only peaks above the FWHM of the noise level are accepted as peaks.*

1. Goni, G.M. et al. Phosphatidylinositol 4,5-bisphosphate triggers activation of focal adhesion kinase by inducing clustering and conformational changes. *Proc Natl Acad Sci U S A* **111**, E3177-86 (2014).
2. Lietha, D. et al. Structural basis for the autoinhibition of focal adhesion kinase. *Cell* **129**, 1177-87 (2007).
3. Baumann, F. et al. Increasing evidence of mechanical force as a functional regulator in smooth muscle myosin light chain kinase. *eLife* **6**, 621 (2017).