Supporting Information

van Ginkel et al. 10.1073/pnas.1707207115

SI Methods

$ClpX_6$ Purification and Biotinylation. To ensure proper immobilization and hexamer formation of ClpX at low concentrations, $ClpX_6(\Delta N)$, a covalently linked hexamer containing a single biotinylation site, was used throughout the experiments. $ClpX_6$ (ΔN) was overexpressed and purified as described (1). In brief, $ClpX_6$ protein expression and biotinylation were induced in an *E. coli* BLR(DE3) strain at OD_{600} of ∼0.6 by adding 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 μM of biotin to increase BirA-mediated biotinylation efficiency. The culture was incubated overnight at 18 °C. Cells were pelleted and resuspended in lysis buffer (20 mM Hepes, pH 7.6, 400 mM NaCl, 100 mM KCl, 10% glycerol, 10 mM β-mercaptoethanol, 10 mM imidazole) in the presence of 1 mM PMSF and lysed by French press twice at 20 psi. $ClpX_6(\Delta N)$ was purified from the supernatant first with Ni^{2+}-NTA affinity resin, followed by size exclusion chromatography with a Prep Sephacryl S-300 16/60 high resolution column (GE Healthcare).

ClpP Mutations, Purification, and Labeling. Point mutations were constructed in ClpP by overlap extension PCR to produce the cysteine-free variant $ClpP_{C91S\text{-}C113S}$ and the subsequent variants $ClpP_{Q48C}$, $ClpP_{A139C}$, and $ClpP_{F31C}$. The variants were overexpressed in *E. coli* BL21(DE3)pLysS at OD_{600} of ∼0.6 by adding 0.5 mM IPTG and incubated for 3 h at 30 °C. Cells were pelleted and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 1 M NaCl, 10% glycerol, 5 mM imidazole) in the presence of Set III protease inhibitors (Calbiochem) and lysed by French press twice at 20 psi. ClpP was purified from the supernatant first with Ni^{2+}-NTA affinity resin, followed by size exclusion chromatography with a Prep Sephacryl S-300 16/60 high resolution column (GE Healthcare). ClpP was dialyzed overnight against PBS (pH 7.4) before labeling for 4 h at 4 °C with monoreactive maleimide donor dye [Cy3 (GE Healthcare) for two-color experiments, and Alexa488 (Invitrogen) for three-color experiments]. Then, 10× molar dye excess was used in PBS, pH 7.4 under nitrogen. Free dye was removed using PD Minitrap G-25 size exclusion columns (GE Healthcare). Labeling efficiency of 5.9, 1.1, and 1.7 dyes per tetradecameric $ClpP_{Q48C}$, $ClpP_{A139C}$, and $ClpP_{F31C}$, respectively, was measured by spectrophotometry (DeNovix DS-11 FX).

ClpP Inactivation. Purified $ClpP_{Q48C}$ was chemically inactivated as described previously (2). Briefly, $ClpP_{Q48C}$ (4 μM) was inactivated in PD buffer containing 10 mM diisopropyl fluorophosphate (DFP) (Sigma). The reaction was incubated for 6 h at 4 °C and then dialyzed twice: 1× 2 h and 1× overnight against 1× PBS (pH 7.4). $ClpP_{Q48C\_DFP}$ was labeled with monoreactive maleimide donor dye, Cy3, for 4 h at 4 °C. A 10× molar excess of dye was used in PBS, pH 7.4, under nitrogen. Free dye was removed using Pierce Dye Removal Columns (Thermo Fisher). A labeling efficiency of 1.8 dyes per tetradecameric $ClpP_{Q48C\_DFP}$ was measured by spectrophotometry (DeNovix DS-11 FX).

ClpXP Cleavage Reaction. To assess the enzymatic activity of donor-labeled ClpXP, 0.9 μM ClpX and 2.9 μM of ClpP (WT or variants) in PD buffer (25 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 40 mM KCl, 0.148% Nonidet P-40, 10% glycerol) were incubated at 30 °C in the presence of 10 μM titinV13P-ssrA and 5 mM ATP. Samples were taken at t = 0 min and 30 min and analyzed using 4 to 20% precast SDS/PAGE gels (Thermo Scientific) and Coomassie staining.

Substrate Preparation. Titin-I27 (WT, V13P and dimer) with the C-terminal ssrA tag was expressed in *E. coli* BL21AI at OD_{600} of ∼0.6 by adding 0.2% arabinose and incubated for 4 h at 37 °C. Cells were pelleted and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 10 mM imidazole) and then lysed by sonication. Titin was purified from the supernatant with Ni^{2+}-NTA affinity resin. Titin was dialyzed overnight against PBS (pH 7.4) before labeling for 4 h at 4 °C with 10× molar excess of monoreactive maleimide acceptor dye (Cy5; GE Healthcare) in the presence of 4 M GdnCl in PBS, pH 7.4, under nitrogen. Custom-designed polypeptides were obtained from Biomatik. Cysteine residues of the polypeptides were labeled with monoreactive maleimide-functionalized Cy5 as an acceptor for two-color measurements and with Cy3 and Cy5 as an acceptor for three-color measurements. Polypeptides were labeled in the presence of a 10× molar excess of dye overnight at 4 °C in PBS under nitrogen. For labeling with additional acceptors at the N terminus, monoreactive NHS-ester functionalized dyes (Cy3 or Cy5; GE Healthcare) were added to the reaction mixture described above, also in 10× molar excess. Free dye was removed using PD Minitrap G-25 size exclusion columns (GE Healthcare). Labeling efficiencies up to 95% were measured by spectrophotometry (DeNovix DS-11 FX) (see Table S1 for the full list of substrates).

1. Martin A, Baker TA, Sauer RT (2005) Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines. *Nature* 437:1115–1120.

2. Harper JW, Bennett EJ (2016) Proteome complexity and the forces that drive proteome imbalance. *Nature* 537:328–338.

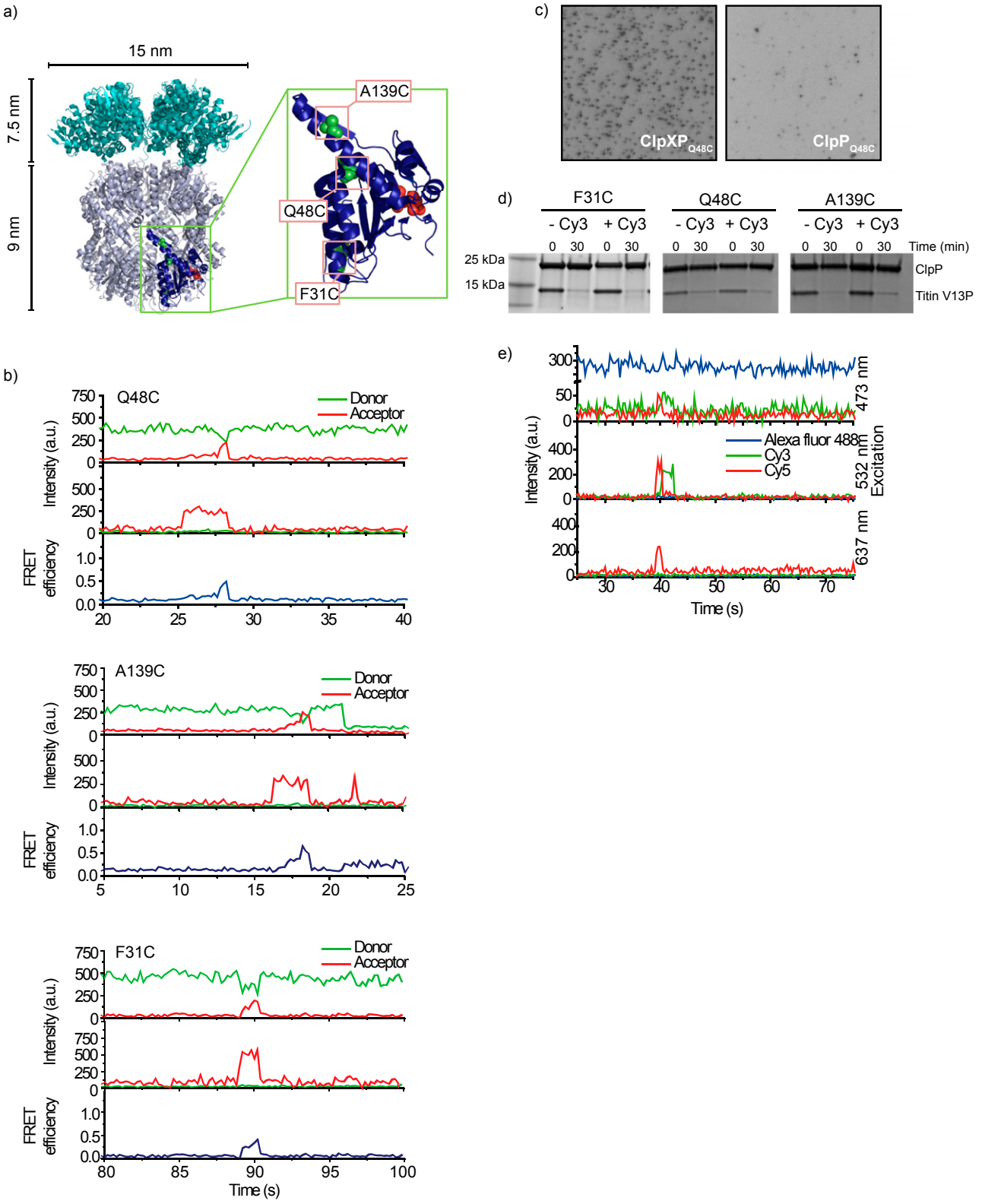

Fig. S1. ClpXP modifications. (*A*) Cocrystal structure of ClpXP, obtained by manually combining crystal structures from PDB:1YG6 ($ClpP_{14}$) and PDB: 3HTE ($ClpX_6$). (*Inset*) A structure of a monomeric ClpP. Highlighted in red are the two cysteine residues present in WT ClpP. Highlighted in green are cysteines introduced into three variants: $ClpP_{Q48C}$, $ClpP_{A139C}$, and $ClpP_{F31C}$. (*B*) Representative time trace from $ClpP_{Q48C}$ (*Top*), $ClpP_{A139C}$ (*Middle*), and $ClpP_{F31C}$ (*Bottom*). $ClpP_{Q48C}$ and $ClpP_{A139C}$ exhibit higher FRET efficiency than $ClpP_{F31C}$. (*C*) CCD images (donor channel) showing immobilization of donor-labeled $ClpP_{Q48C}$ in complex with $ClpX_6$ (*Left*) or in the absence of $ClpX_6$ (*Right*). Each spot represents a single donor-labeled $ClpP_{14}$ molecule. The CCD images are from over 25 × 25 μm^2 field of view. (*D*) Degradation of $titin_{V13P}$ by ClpXP. The degradation efficiency of unlabeled and labeled ClpP variants was compared at time 0 and 30 min. (*E*) Three-color time trace. The original time trace used in Fig. 2*B* to present a three-color FRET event. Note the original, not summed, levels of Cy3 and Cy5 signals in the *Middle*. Cy3 transfers its energy to Cy5 via FRET.

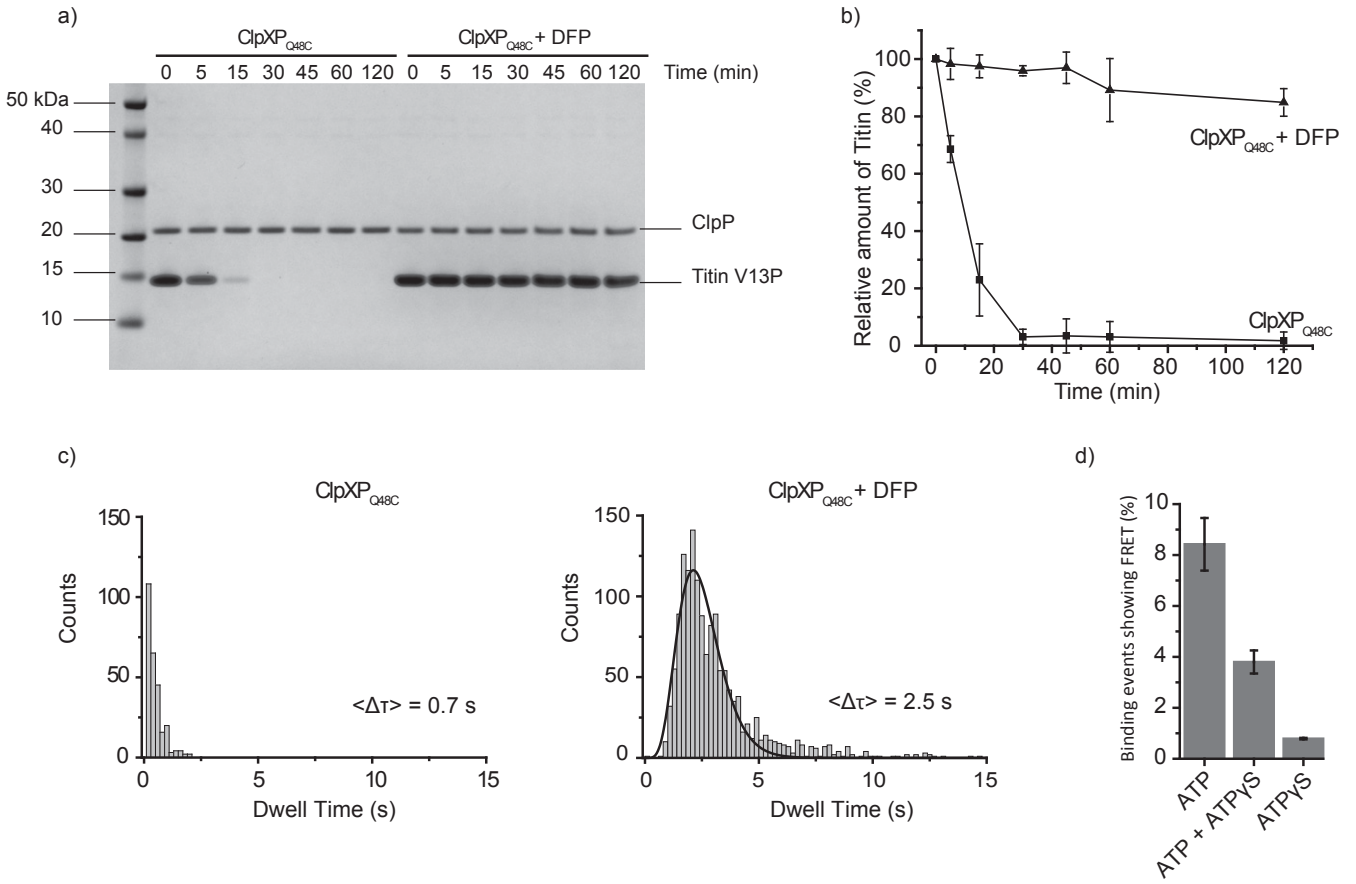

Fig. S2. Evaluation of chemically inactivated ClpX and ClpP. (*A*) Degradation of titin$_{V13P}$ by ClpP$_{Q48C}$ or chemically inactivated ClpP$_{Q48C}$ + DFP. (*B*) The change of the relative amount of titin substrate when incubated with ClpXP$_{Q48C}$ or chemically inactivated ClpXP$_{Q48C}$ plus DFP (bulk measurements from three independent experiments). (*C*) Dwell-time histograms of single-molecule experiments of K-38-C-ssrA with ClpXP$_{Q48C}$ (n = 239) or chemically inactivated ClpXP$_{Q48C}$ plus DFP (n = 1,470); the dwell-time of the high FRET increases 3.5-fold when ClpP$_{Q48C}$ plus DFP was used. (*D*) ClpX was inactivated by using an ATP analog ATPγS; increasing the ATPγS concentration reduces the number of binding events showing FRET. For ATP, n = 756; for ATP plus ATPγS, n = 446; for ATPγS, n = 554.

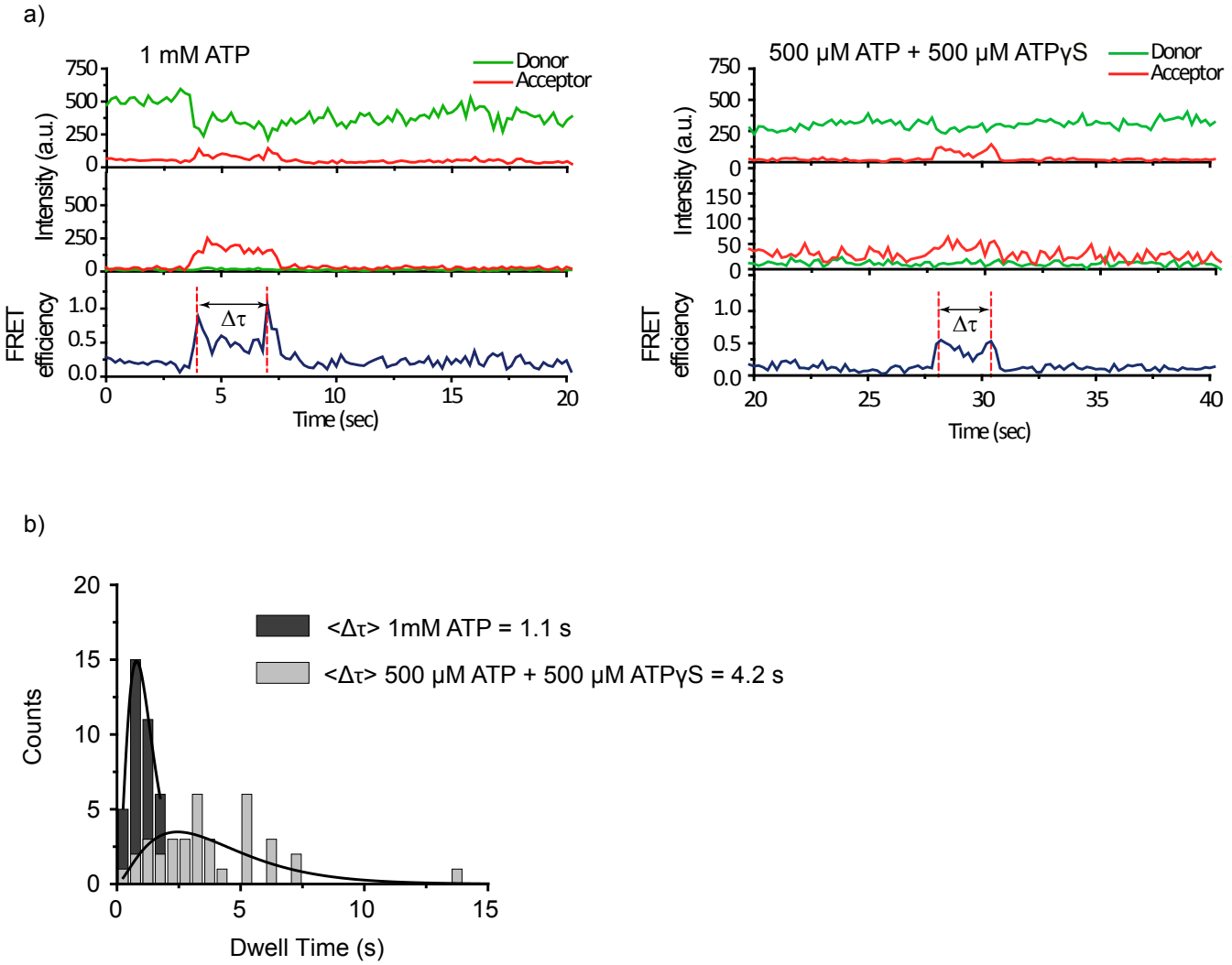

Fig. S3. Fingerprinting cysteine residues in titin. (*A*) Representative time traces of the titin substrate with both cysteines labeled with Cy5 (Cys64 and Cys80). A mixture of ATP and ATPγS (*Right* trace) was used to elongate the dwell time between two of the cysteines. (*B*) The dwell-time histograms of the elongated interval between the two peaks of 1 mM ATP (n = 37) and 500 μM ATP plus 500 μM ATPγS (n = 36).

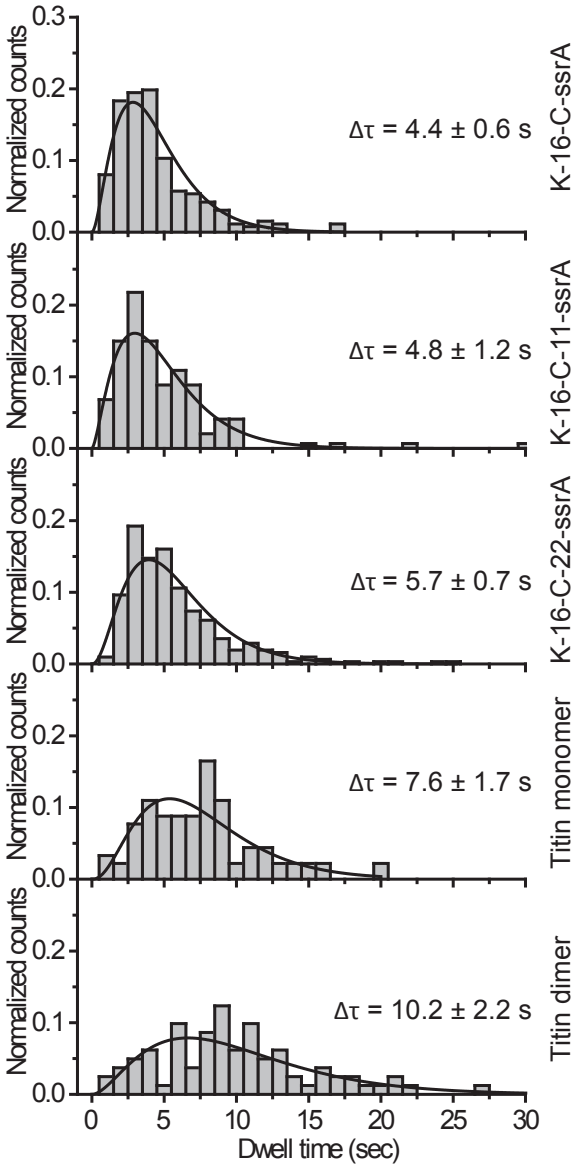

Fig. S4. Distribution of total dwell time. Total dwell times ($\Delta\tau$; see the definition in Fig. 3*A*) were determined for ssrA-tagged peptides of increasing lengths and monomeric and dimeric titin. Total dwell times for all substrates showed gamma-like distributions. Errors were obtained by bootstrapping with 1,000 resamples. For K-16-C-ssrA, $n = 262$; for K-16-C-11-ssrA, $n = 147$; for K-16-C-22-ssrA, $n = 312$; for titin monomer, $n = 91$; for titin dimer, $n = 82$.

Table S1. Overview of the substrates used in this study

Name	Sequence	Length, no. of amino acids	Molecular mass, kDa
NH_3-17-C-ssrA	ASGERDNFAPHMALVPV**C**AANDENYALAA	29	3.018
K-16-C-ssrA	KSGERDNFAPHMALVPV**C**AANDENYALAA	29	3.075
K-16-C-11-ssrA	KSGERDNFAPHMALVPV**C**AANDENYALAAAANDENYALAA	40	4.180
K-16-C-22-ssrA	KSGERDNFAPHMALVPV**C**AANDENYALAAAANDENYALAAAANDENYALAA	51	5.284
K-38-C-ssrA	KSGERDNFAPHMALVPVAANDENYALAAAANDENYALAA**C**AANDENYALAA	51	5.284
Titin-ssrA	MRGSHHHHHHGLVPRGSLIEVEKPLYGVEVFVGETAHFEIELSEPDVHGQWKLKGQPLAASPD**C**E-IIEDGKKHILILHN**C**QLGMTGEVSFQAANTKSAANLKVKELRSAANDENYALAA	119	13.050
$Titin_{V13P}$-ssrA	MRGSHHHHHHGLVPRGSLIEVEKPLYGVEPFVGETAHFEIELSEPDVHGQWKLKGQPLAASPD**C**E-IIEDGKKHILILHN**C**QLGMTGEVSFQAANTKSAANLKVKELRSAANDENYALAA	119	13.048
Titin-Titin-ssrA	MRGSHHHHHHGLVPRGSLIEVEKPLYGVEVFVGETAHFEIELSEPDVHGQWKLKGQPLAASPD**C**E-IIEDGKKHILILHN**C**QLGMTGEVSFQAANTKSAANLKVKELRSLIEVEKPLYGVEVFVGETAH-FEIELSEPDVHGQWKLKGQPLAASPD**C**EIIEDGKKHILILHN**C**QLGMTGEVSFQAANTKSAAN-LKVKELRSAANDENYALAA	210	23.056

All titin-based substrates used were derived from the WT titin-I27 domain. Dye-labeled Cystein residues are bold.