Supplemental Information:

Table S1. Description of substrates. Related to Star Methods.

Plasmid Name	Plasmid Description	Amino acid sequence after titin-I27*
pAM91	GGG- titin-I27^{V15P}-23-K-9-C-25-intein-CBD	GGAGPPPYSAANDENYALAAHGGKHTFNNENVS**C**RLG GAASIAVQAPAQHTFNNENVSY
pAM92	GGG- titin-I27^{V15P}-21-C-2-K-35-intein-CBD	GGAGPPPYSAANDENYALAAH**C**GGKHTFNNENVSARL GGAASIAVQAPAQHTFNNENVSY
pAM93	GGG-titin-I27-23-K-9-C-25-intein-CBD	GGAGPPPYSAANDENYALAAHGGKHTFNNENVS**C**RLG GAASIAVQAPAQHTFNNENVSY
pAM94	GGG- titin-I27$^{V13P/V15P}$-23-K-9-C-25-intein-CBD	GGAGPPPYSAANDENYALAAHGGKHTFNNENVS**C**RLG GAASIAVQAPAQHTFNNENVSY
pAM95	GGG- titin-I27^{V15P}-43-K-8-C-25-intein-CBD	GGAGPPPYSAANDENYALAAHGGAHTFNNENVSAHTF NNENVSKHTFNNENV**C**RLGGAASIAVQAPAQHTFNNE NVSY
pAM96	GGG- titin-I27^{V15P}-33-K-9-K-8-C-25-intein-CBD	GGAGPPPYSAANDENYALAAHGGAHTFNNENVSKHTF NNENVSKHTFNNENV**C**RLGGAASIAVQAPAQHTFNNE NVSY
pAM97	GGG- titin-I27^{V15P}-23-K-9-K-9-K-8-C-25-intein-CBD	GGAGPPPYSAANDENYALAAHGGKHTFNNENVSKHTF NNENVSKHTFNNENV**C**RLGGAASIAVQAPAQHTFNNE NVSY
pAM98	titin-I27^{V15P}-23-K-9-C-15-intein-CBD	GGAGPPPYSAANDENYALAAHGGKHTFNNENVS**C**RLG GAASIAVQAPAY
pAM99	titin-I27^{V15P}-23-K-9-C-1-intein-CBD	GGAGPPPYSAANDENYALAAHGGKHTFNNENVS**C**Y
pAM100	titin-I27^{V15P}-21-C-2-K-1-intein-CBD	GGAGPPPYSAANDENYALAAH**C**GGKY
pAM101	titin-I27^{V15P}-23-K-9-C-15 serine rich-intein-CBD	GGAGPPPYSAANDENYALAAHGGKSSSSSSASS**C**SSG SSSSSSASSSSY
pAM104	titin-I27^{V15P}-43-K-11-intein-CBD	GGAGPPPYSAANDENYALAAHGGAHTFNNENVSAHTF NNENVSKHTFNNENVS**C**Y
pAM105	titin-I27^{V15P}-23-K-19-K-11-intein-CBD	GGAGPPPYSAANDENYALAAHGGKHTFNNENVSAHTF NNENVSKHTFNNENVS**C**Y

Table S2. Modification of Substrates. Related to Star Methods.

Plasmid Origin	Description	N-terminal modification	Tail modification	Figure
pAM91	titin-I27^{V15P}-23-K-35	-	-	1D, 2E-F, 4C-D, S2D, S3, Table S5, Table S7
pAM91	titin-I27^{V15P}-23-K-35	5-FAM-HHHHHHLPET	-	2B-C, 3A-B, 4D, S1, S2A/C, S5, S6, Table S3
pAM91	titin-I27^{V15P}-23-K-35	-	5-FAM	4A, S5B
pAM91	titin-I27^{V15P}-23-K-35	5-FAM-HHHHHHLPET	Cy5	Table S3
pAM91	titin-I27^{V15P}-23-K-35	-	Cy5	2D, 4B, S2B-C, Table 1, Table S4
pAM92	titin-I27^{V15P}-24-K-35 (DUB)	5-FAM-HHHHHHLPET	Cy5	2G, S2E, S2F, Table S6
pAM93	titin-I27-23-K-35	-	-	S3, Table S7
pAM93	titin-I27-23-K-35	5-FAM-HHHHHHLPET	-	3A, 3B, Table S3
pAM93	titin-I27-23-K-35	5-FAM-HHHHHHLPET	Cy5	Table S3
pAM94	titin-I27$^{V13P/V15P}$-23-K-35	-	-	S3, Table S7
pAM94	titin-I27$^{V13P/V15P}$-23-K-35	5-FAM-HHHHHHLPET	-	3A, 3B, Table S3
pAM95	titin-I27^{V15P}-43-K-34	5-FAM-HHHHHHLPET	-	3C, S1, S4, Table S3
pAM95	titin-I27^{V15P}-43-K-34	5-FAM-LPET	-	5, Table S8
pAM96	titin-I27^{V15P}-33-K-9-K-34	5-FAM-HHHHHHLPET	-	3C, S1, S4, Table S3
pAM97	titin-I27^{V15P}-23-K-9-K-9-K-34	5-FAM-HHHHHHLPET	-	3C, S1, S4, Table S3
pAM98	titin-I27^{V15P}-23-K-25	-	Cy5	4B, Table 1, Table S4
pAM98	titin-I27^{V15P}-23-K-25	-	5-FAM	Table S3, S5A
pAM99	titin-I27^{V15P}-23-K-11	-	Cy5	4B, Table 1, Table S4
pAM99	titin-I27^{V15P}-23-K-11	-	-	4C, 4D
pAM99	titin-I27^{V15P}-23-K-11	-	5-FAM	4A, S5B, S6
pAM100	titin-I27^{V15P} -23-K-1	-	Cy5	4B, Table 1, Table S4
pAM100	titin-I27^{V15P} -23-K-1	-	-	4C, 4D
pAM100	titin-I27^{V15P}-23-K-1	-	5-FAM	4A, S5B, S6
pAM101	titin-I27^{V15P}-23-K-25 (serine rich)	-	Cy5	4B, Table 1, Table S4
pAM101	titin-I27^{V15P}-23-K-25 (serine rich)	-	-	4C, 4D
pAM101	titin-I27^{V15P}-23-K-25 (serine rich)	-	5-FAM	4A, S5B, S6
pAM104	titin-I27^{V15P}-43-K-11	5-FAM-LPET	-	5, S6, Table S8
pAM105	titin-I27^{V15P}-23-K-19-K-11	5-FAM-LPET	-	5, S6, Table S8

Table S3. Single Turnover degradation fits. Related to Figure 2.

Substrate Description	A_1/A_2	τ_1+t_0 (s)	τ_2+t_0 (s)	t_0 (s)
FAM- titin-I27-23-K-35 (*N* = 3)	2.3 ± 0.3	47.2 ± 2.1	499 ± 98	11.0
FAM-titin-I27-23-K-35-Cy5 (*N* = 3)	4.2 ± 0.3	45.0 ± 1.8	223 ± 18	11.5
FAM-titin-I27^{V15P}-23-K-35 (*N* = 3)	2.1 ± 0.2	18.1 ± 0.4	89 ± 10	7.4
FAM-titin-I27^{V15P}-23-K-35-Cy5 (*N* = 5)	3.0 ± 0.6	20.0 ± 0.7	139 ± 44	7.5
FAM-titin-I27$^{V13P/V15P}$-23-K-35 (*N* = 3)	1.7 ± 0.1	14.7 ± 0.3	77 ± 5	6.0
titin-I27^{V15P}-23-K-25-FAM (*N* = 3)	5.4 ± 0.5	21.1 ± 0.5	157 ± 30	7.5
FAM-titin-I27^{V15P}-43-K-34 (*N* = 4)	2.6 ± 0.5	22.0 ± 1.8	111 ± 22	10.0
FAM-titin-I27^{V15P}-33-K-9-K-34 (*N* = 4)	3.1 ± 0.7	21.0 ± 0.8	108 ± 14	10.0
FAM-titin-I27^{V15P}-23-K-9-K-9-K-34 (*N* = 4)	3.2 ± 0.8	21.4 ± 0.2	126 ± 12	10.0

Table S4. Tail insertion fits. Related to Figure 2.

Substrate Description	A_1/A_2	τ_1 (s)	τ_2 (s)	A_1 (normalized to titin-I27^{V15P}-23-K-35)
titin-I27^{V15P}-23-K-35-Cy5 (*N* = 9)	2.0 ± 0.3	1.61 ± 0.32	17.3 ± 3.0	1.00 ± 0.03
titin-I27^{V15P}-23-K-25 (serine rich)-Cy5 (*N* = 5)	1.1 ± 0.1	7.15 ± 1.00	31.5 ± 4.3	0.51 ± 0.04
titin-I27^{V15P}-23-K-25-Cy5 (*N* = 3)	1.7 ± 0.1	1.73 ± 0.02	17.4 ± 0.9	0.87 ± 0.05
titin-I27^{V15P}-23-K-11-Cy5 (*N* = 3)	0.6 ± 0.2	0.70 ± 0.09	61.9 ± 27.3	0.24 ± 0.01
titin-I27^{V15P}-23-K-1-Cy5 (*N* = 3)	0.9 ± 0.1	0.46 ± 0.03	60.0 ± 12.6	0.16 ± 0.001

Table S5. Conformational change Fits. Related to Figure 2.

Substrate Description	A_1/A_2	τ_1+t_0 (s)	τ_2+t_0 (s)	t_0 (s)
titin-I27^{V15P}-23-K-35 (*N* = 6)	2.3 ± 0.2	2.22 ± 0.10	24.9 ± 1.8	0.4

Table S6. Deubiquitination Fits. Related to Figure 2.

Substrate Description	A_1/A_2	τ_1 (s)	τ_2 (s)
FAM-titin-I27^{V15P}-24-K-35(DUB)-Cy5 (*N* = 3)	0.9 ± 0.1	6.83 ± 0.17	35.6 ± 2.6

Table S7. ATPase rates. Related to Figure 3.

Sample	ATPase rate (ATP s^{-1} $enzyme^{-1}$)
26S alone (*N* = 3)	0.73 ± 0.02
26S + ub'd titin-I27$^{V13P/V15P}$-23-K-35 (*N* = 3)	1.12 ± 0.11
26S + ub'd titin-I27^{V15P}-23-K-35 (*N* = 3)	1.35 ± 0.12
26S + ub'd titin-I27-23-K-35 (*N* = 3)	1.47 ± 0.11

Table S8. Degradation velocities from SDS-PAGE analysis.* Related to Figure 5.

Substrate Description	A_1	τ_1 (s)	A_2	τ_2 (s)
FAM-titin-I27^{V15P}-43-K-34 (N = 5)†	0.87 ± 0.06	23.4 ± 5.6	N/A	N/A
FAM-titin-I27^{V15P}-43-K-11 (N = 5)	0.36 ± 0.06	45.8 ± 12.9	0.39 ± 0.05	609 ± 203
FAM-titin-I27^{V15P}-23-K-19-K-11 (N = 5)	0.38 ± 0.08	45.9 ± 13.3	0.37 ± 0.07	409 ± 118

*Because the reactions were performed with enzyme only slight about K_m these rates are likely underestimates.
†Fit with a single exponential curve.