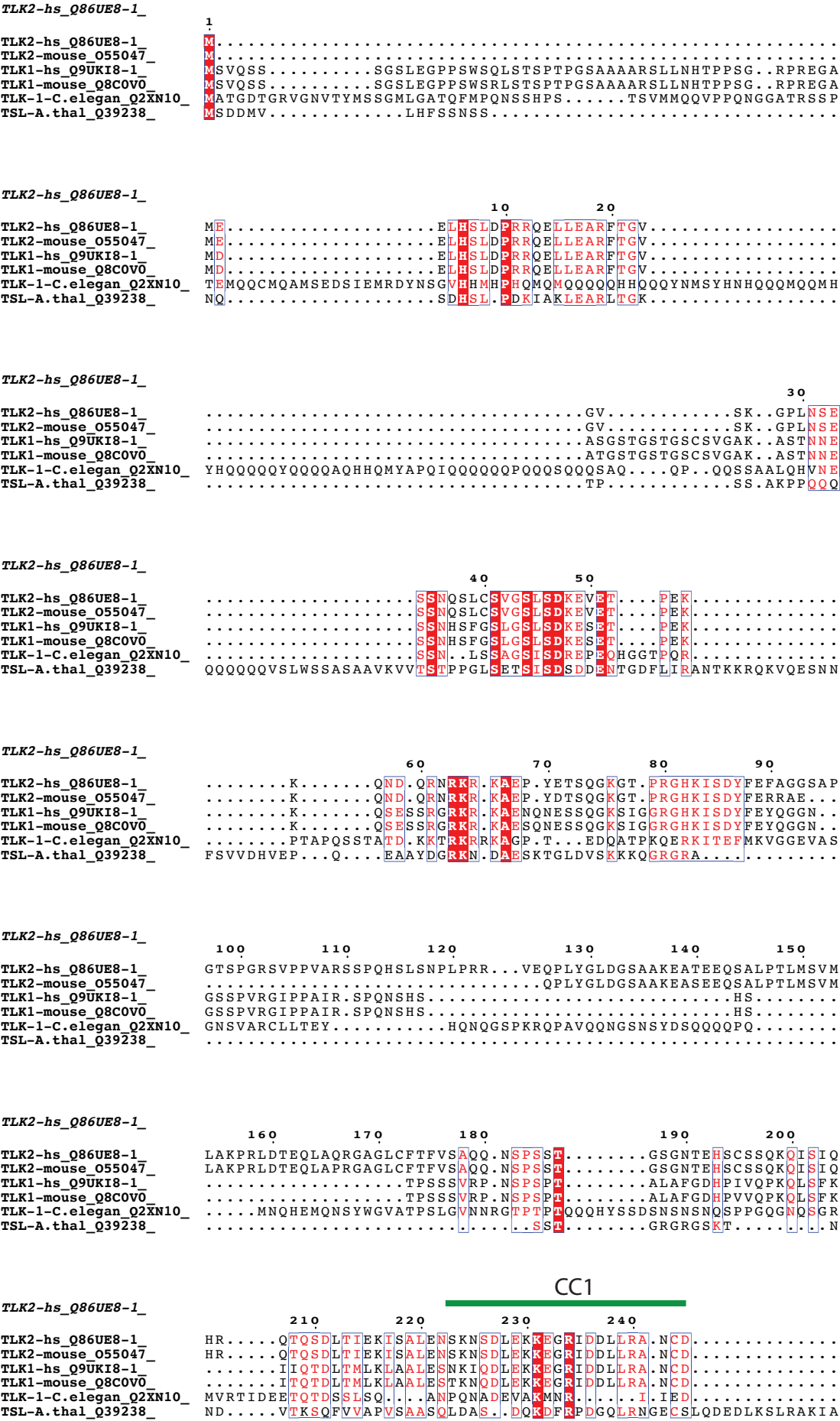
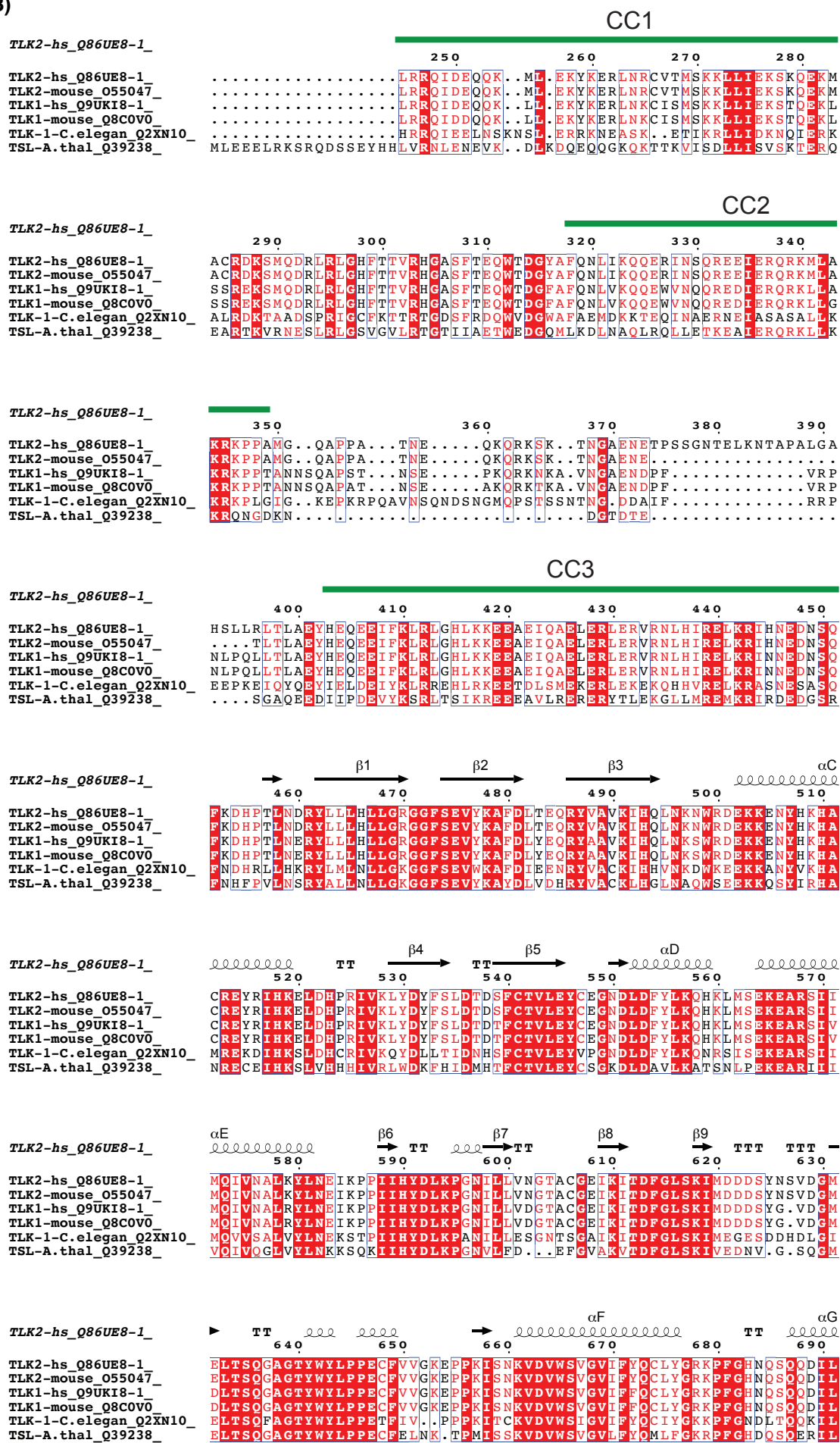


Molecular Basis of Tausled-like kinase 2 activation. Mortuza and Hermida et al.

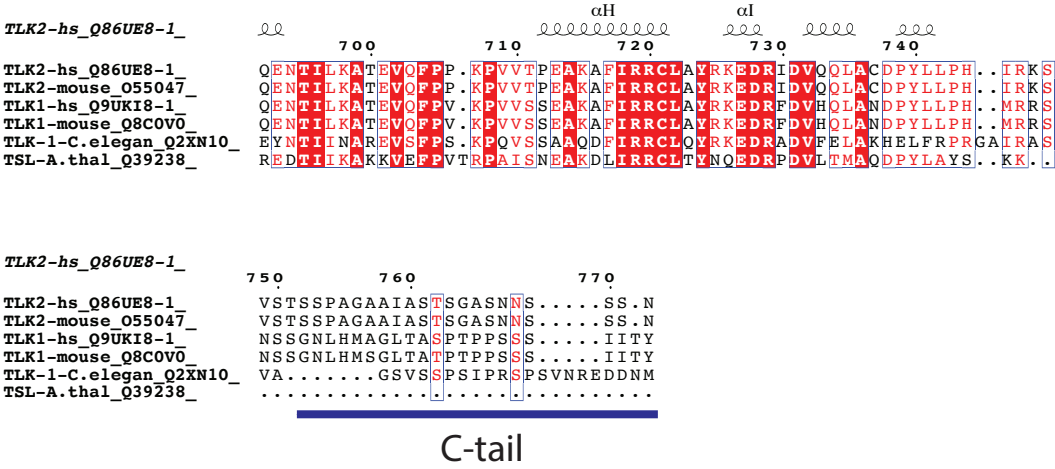
(Section A)



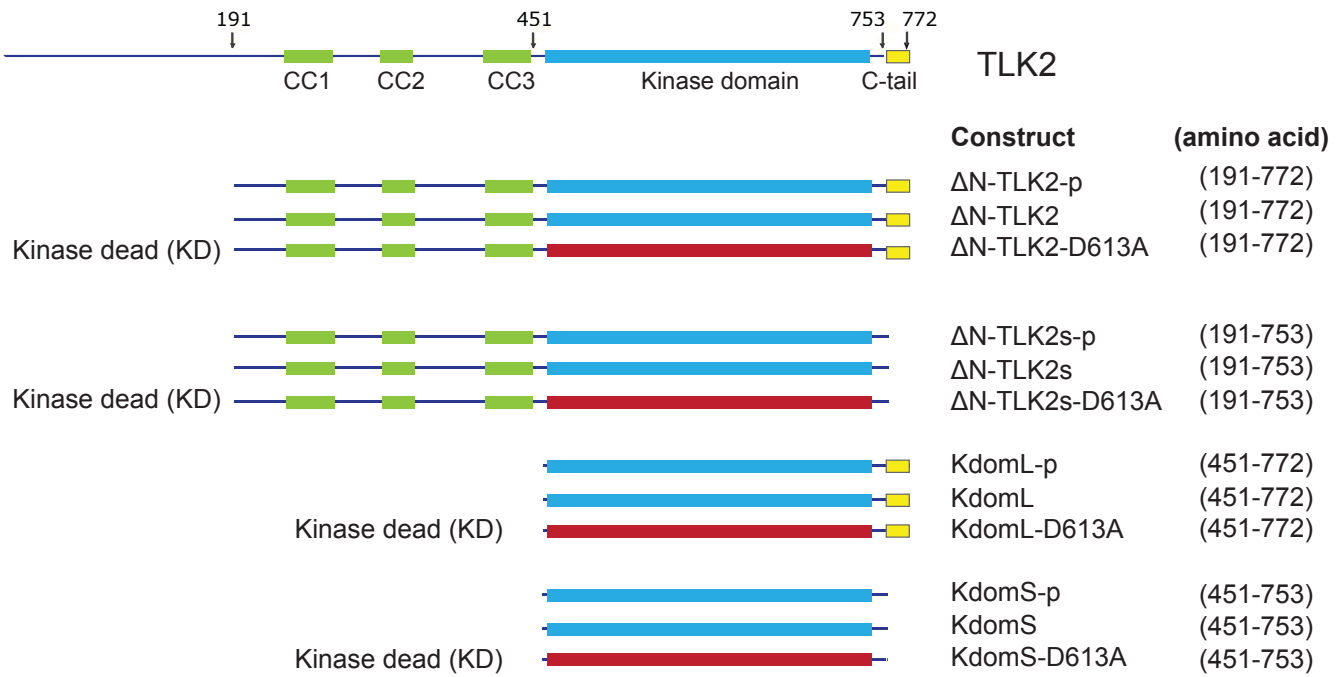
Supplementary Figure 1. TLKs sequence alignment. Sequence alignment of TLK2 from plants to human showing a highly-conserved protein sequence around the predicted coiled-coil regions and the kinase domain. Red box and white residue symbolise strict identity conservation. Red residue symbolises similar physicochemical properties. Blue frame symbolises similarity across the group.



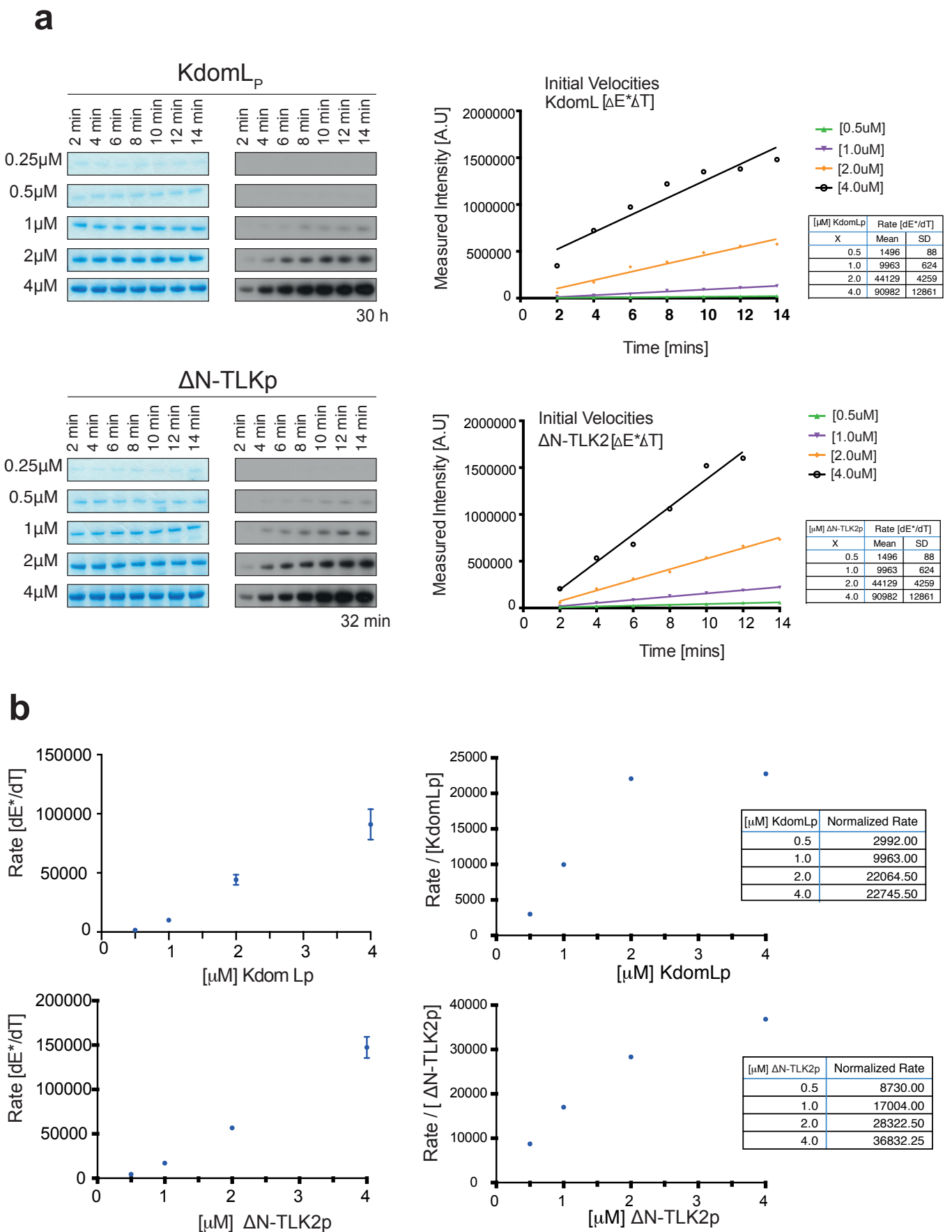
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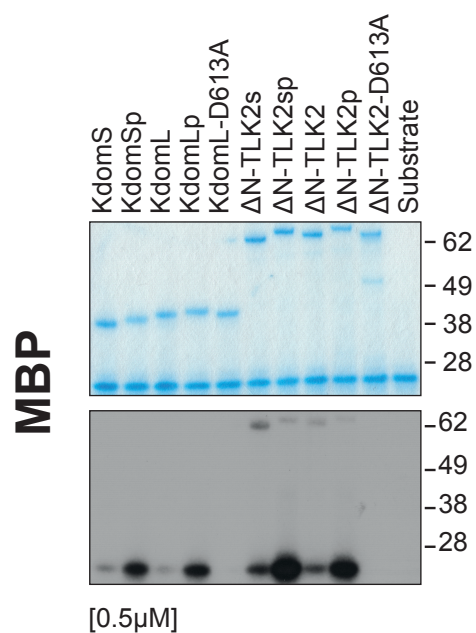
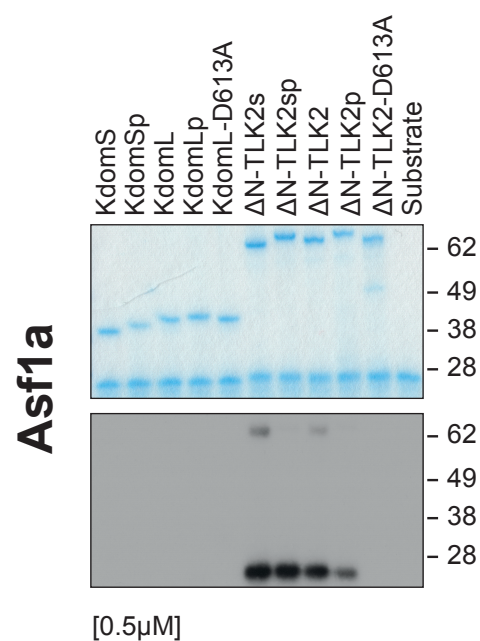
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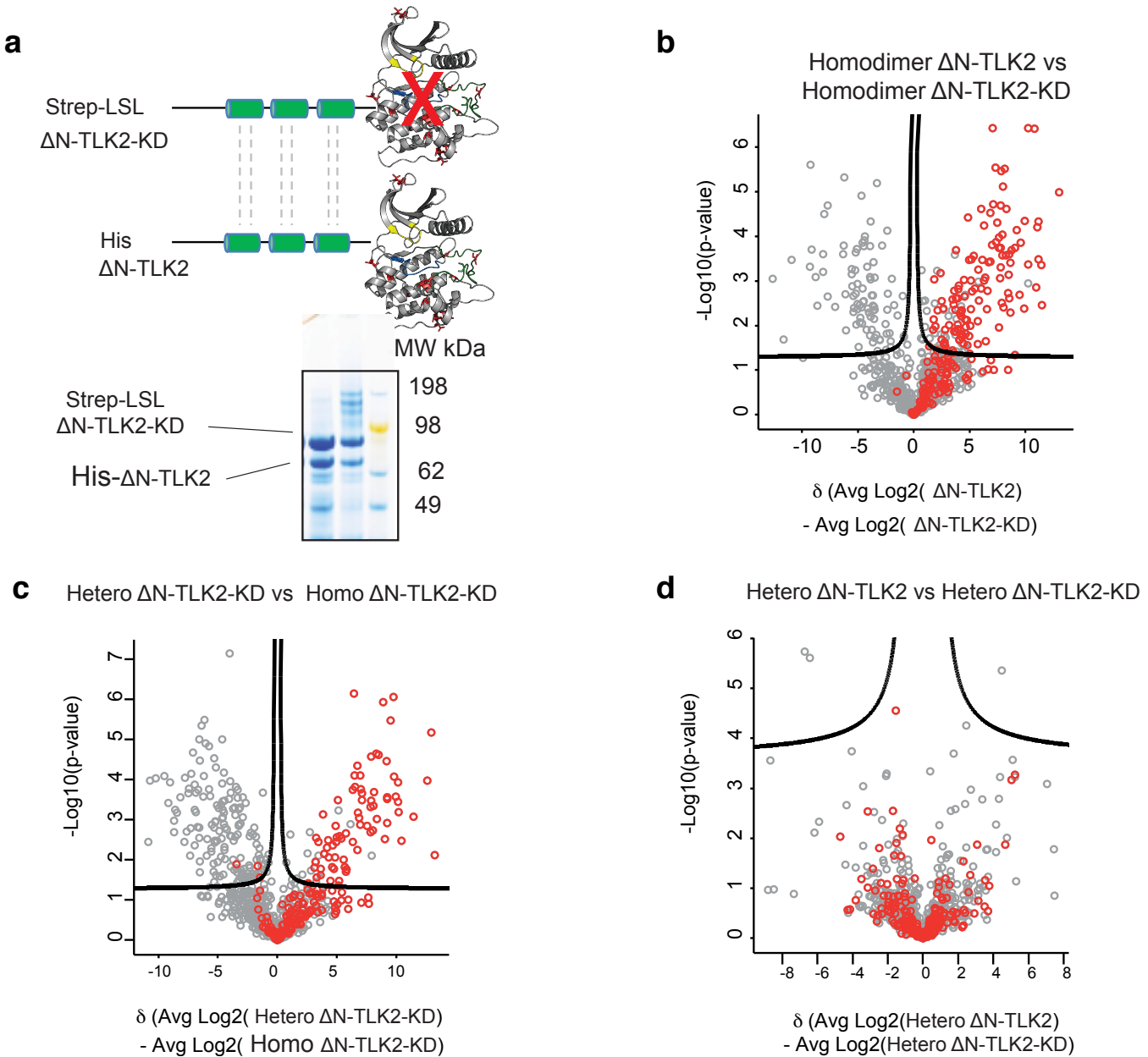
Supplementary Figure 2. Detailed scheme of the constructs used in this study. a) TLK2 Domain architecture and constructs. All the constructs were overexpressed, purified and validated by mass spectrometry. Coiled-coil domains (Green), wild-type kinase domain (Blue), kinase-dead domain D613A (Red), and C-tail (Yellow).



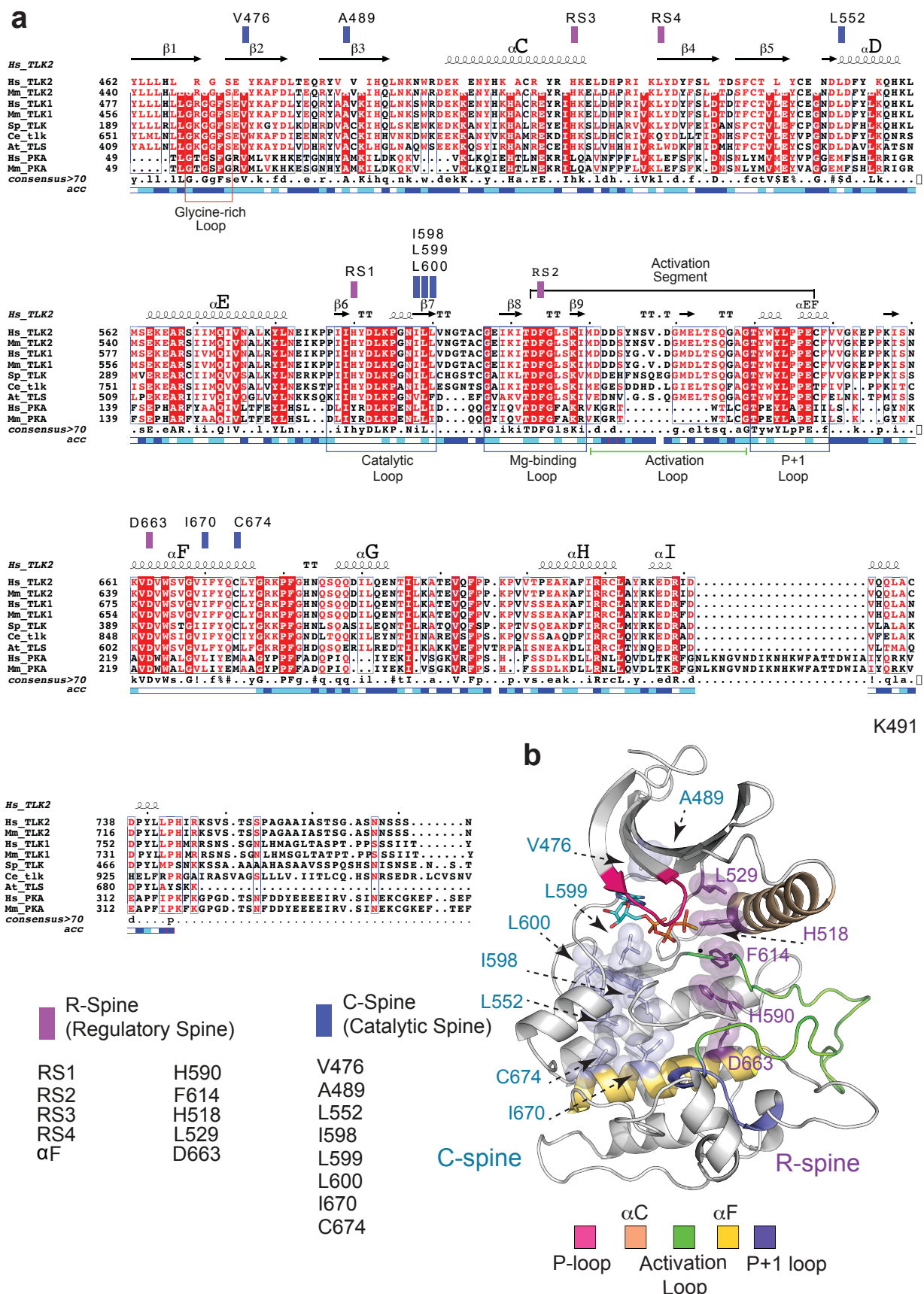
Supplementary Figure 3. ΔN-TLK2 and Kinase domain kinetics. a) SDS-PAGE and autoradiograms (left panels) together with the initial velocity for ΔN-TLK2-p and its respective kinase domain (KdomLp) calculated via densitometric measurements (right panels). b) Plots showing autophosphorylation rates. The tables show the autophosphorylation rates normalized with the protein concentration present in each measurement (mean ± s.d., $n = 3$ biological replicates).

a**b**

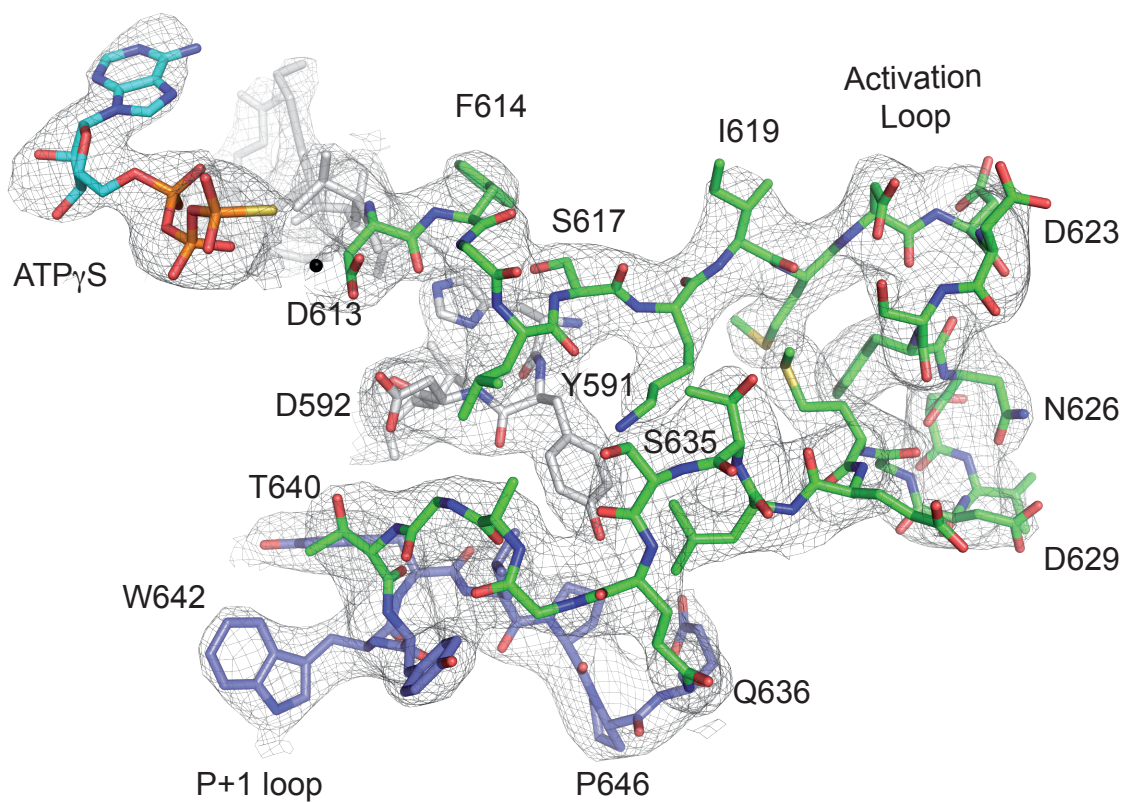
Supplementary Figure 4. Phosphorylation of TLK2 substrates. SDS-PAGE and autoradiograms of substrate phosphorylation by various TLK2 constructs showing a) MBP and b) ASF1a phosphorylation.



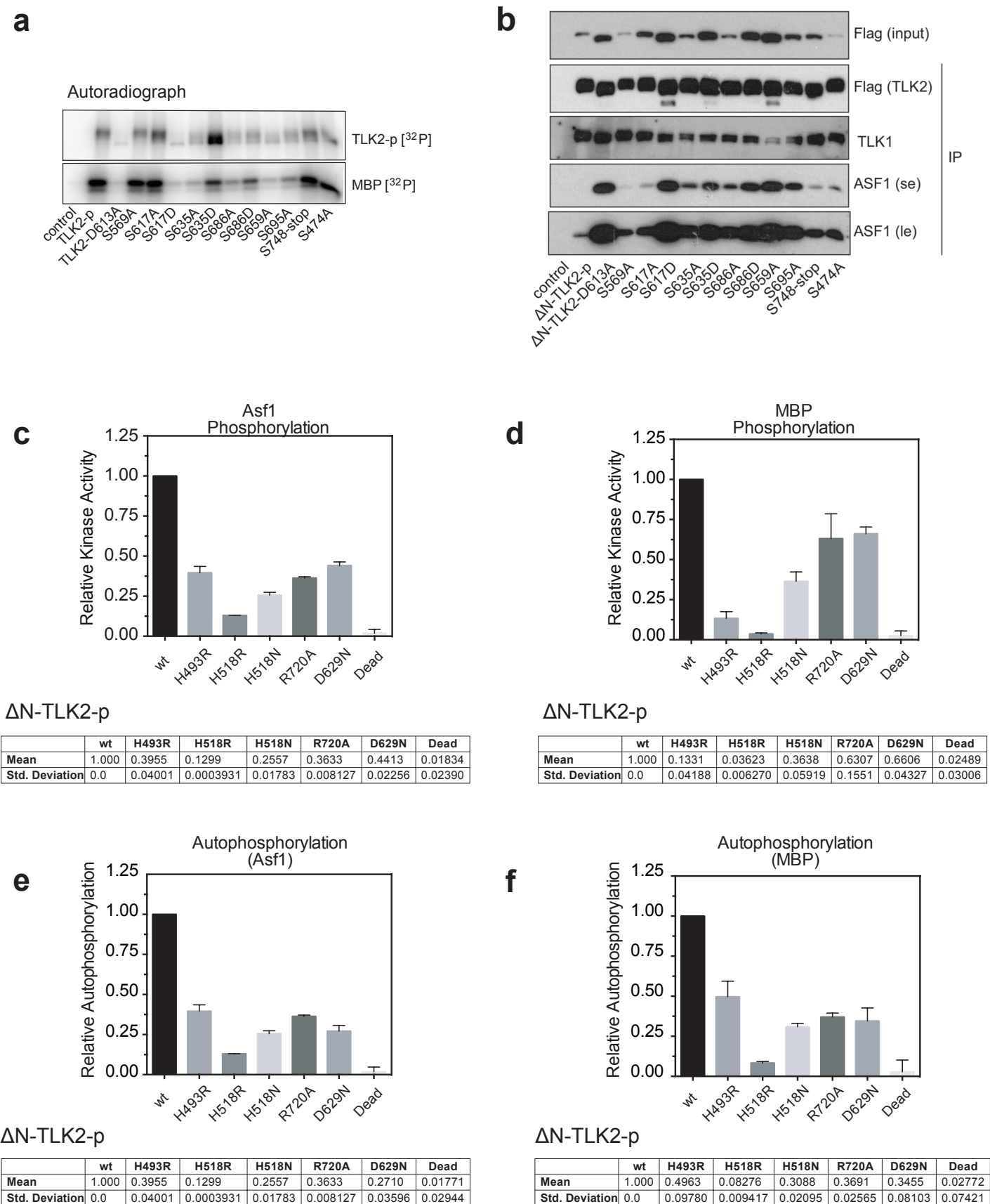
Supplementary Figure 6. Mass spectrometry analysis of TLK2, ΔN-TLK2 and ΔN-TLK2 heterodimer. a) Schematic representation of a fusion TLK2 construct with two different tags, Twin-Strep-LSL tag on the kinase-dead and a penta-His tag on a kinase active protein. SDS-PAGE showing the two proteins, Twin-Strep-LSL-ΔN-TLK2-KD dead and penta-His-ΔN-TLK2 that were separated and extracted for trypsin digest and MS analysis. b) Volcano plot based on t-test analysis showing a clear difference in phosphopeptides (marked in red) detected in the ΔN-TLK2 and ΔN-TLK2-KD homodimers. c) Volcano plot based on t-test analysis comparing the ΔN-TLK2-KD phosphopeptides in the context of the heterodimer and the homodimer. d) Volcano plot based on t-test analysis comparing the phosphopeptides of ΔN-TLK2 and ΔN-TLK2-KD in the context of the heterodimer.



Supplementary Figure 7. TLK2 kinase domain regulatory segments. a) Sequence alignment of TLK kinase domains from various species and the human PKA protein kinase. All the important elements in the kinase domain such as activation loop, activation segment, catalytic loop, glycine-rich region, Magnesium-binding loop, R-spine and C-spine are highlighted. Localization of the TLK2 residues presumably involved in the formation of the R-spine, and the C-spine was based on the localization of those residues in PKA. Localization of the important elements of the kinase domain according to Ref ⁴⁸. Red box and white residue symbolise strict identity conservation. Red residue symbolises similar physicochemical properties. Blue frame symbolises similarity across the group. b) Mapping of the R- and C- spines in the TLK2 kinase domain structure.

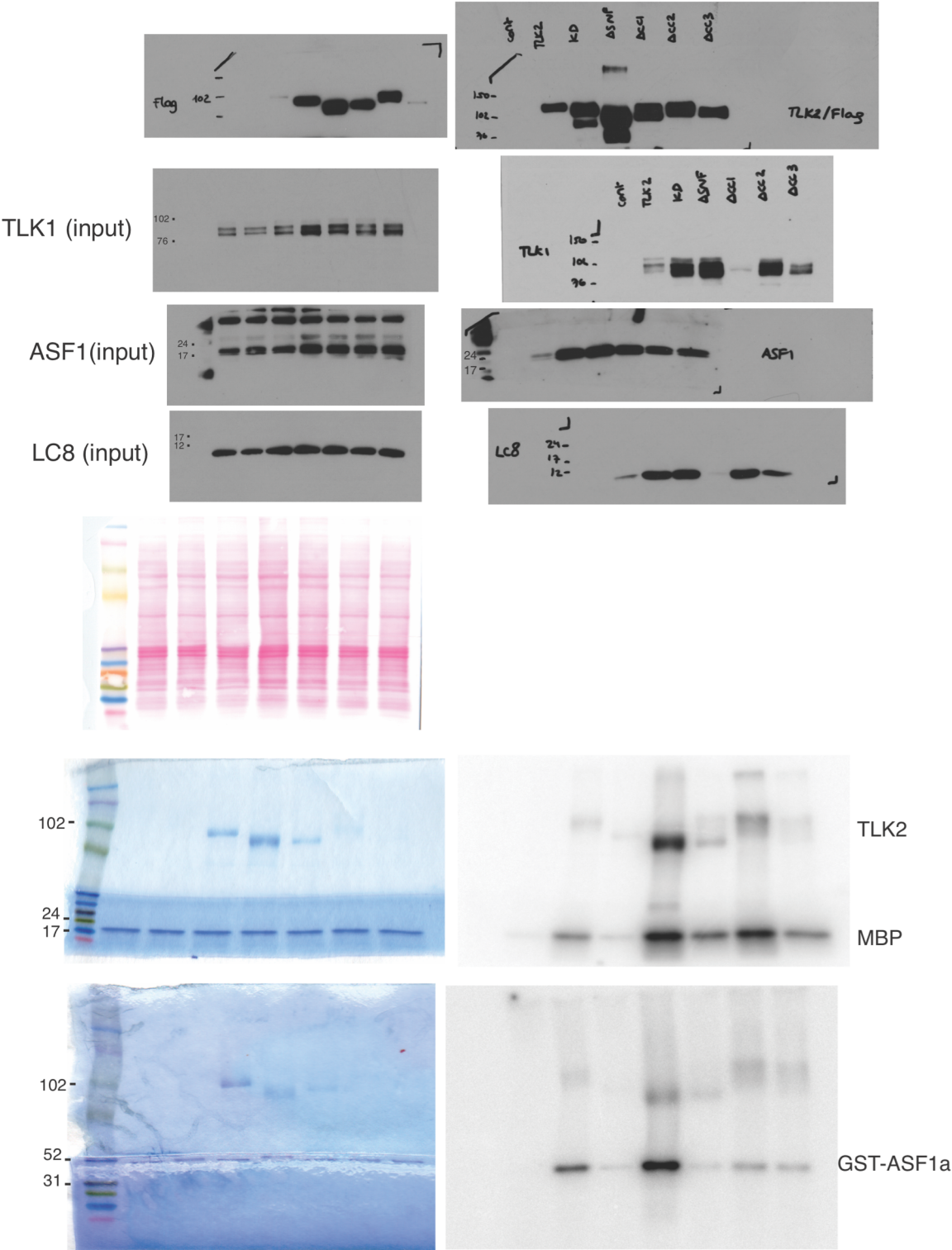


Supplementary Figure 8. Detailed view of the electron density map of the TLK2 kinase structure. The characteristic region of the TLK family containing the activation loop and a section of the P+1 loop is shown. The figure displays the 2mFo-DFc refined electron density map contoured at 1.2 σ .



Supplementary Figure 9. Activity assays for different TLK2 mutants. a) Representative autoradiograms of *in vitro* kinase assays of Strep-pulldowns from cells expressing Strep-FLAG tagged TLK2 phosphorylation site mutants (S>A) or mimics (S>D). Results are quantified in Fig. 5b. b) Western blotting of input or Strep-pulldowns from AD293 cells transiently expressed with Strep-FLAG tagged TLK2 mutants. Levels of co-purified ASF1 are shown. Short or long exposure of the same film is indicated as (se) or (le), respectively. Histograms showing ID mutants phosphorylation of c) ASF1a and d) MBP. Kinase autophosphorylation profiles of the ID mutants when using e) ASF1a and f) MBP as substrates. The data points indicate the relative kinase activity and the autophosphorylation activity of the TLK2-mutants normalized to the activity of the wild-type protein (mean $\pm$ s.d., $n = 3$ biological replicates).

Figure 1D-E: Western (D) and autoradiographs (E)



Supplementary Figure 10. Uncropped gels for figure 1D and 1E

Figure 2a

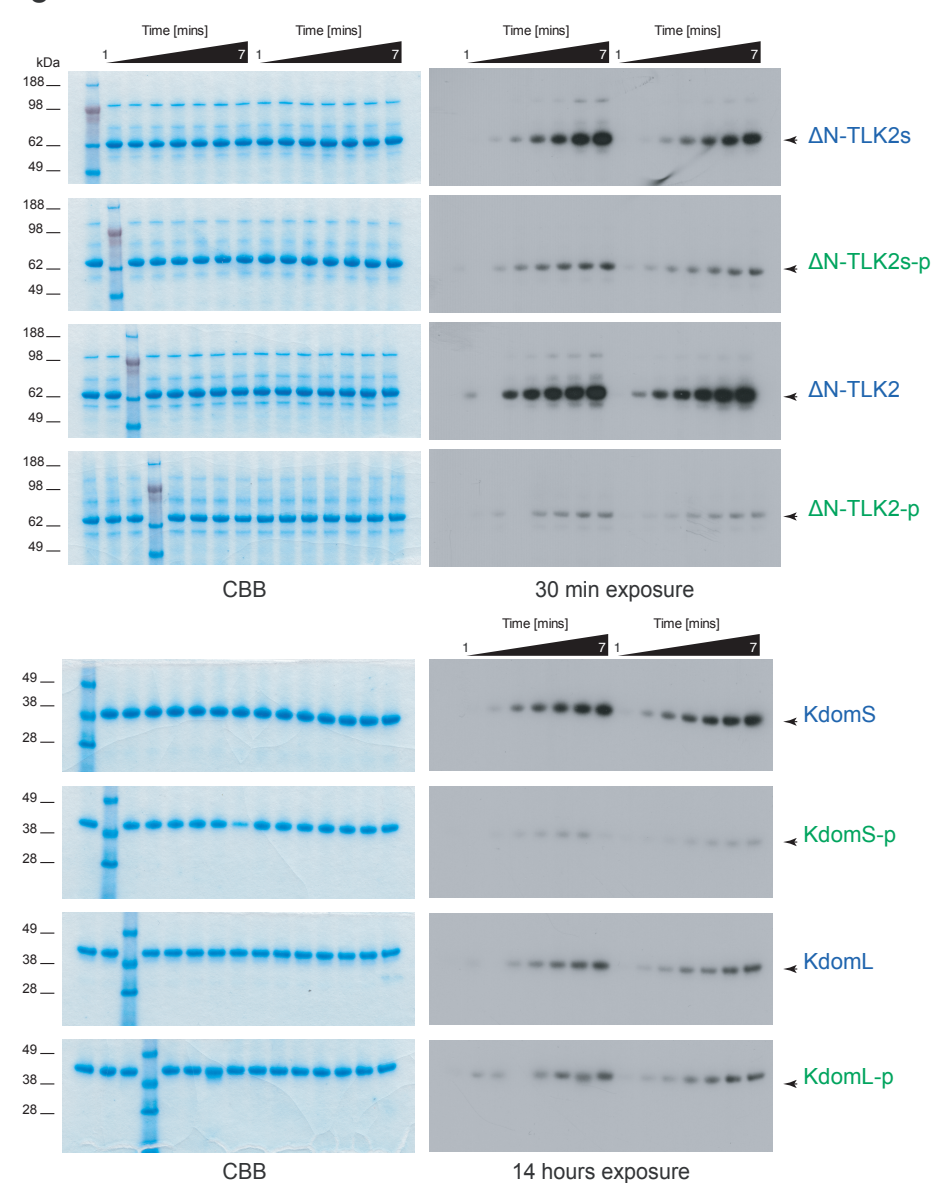
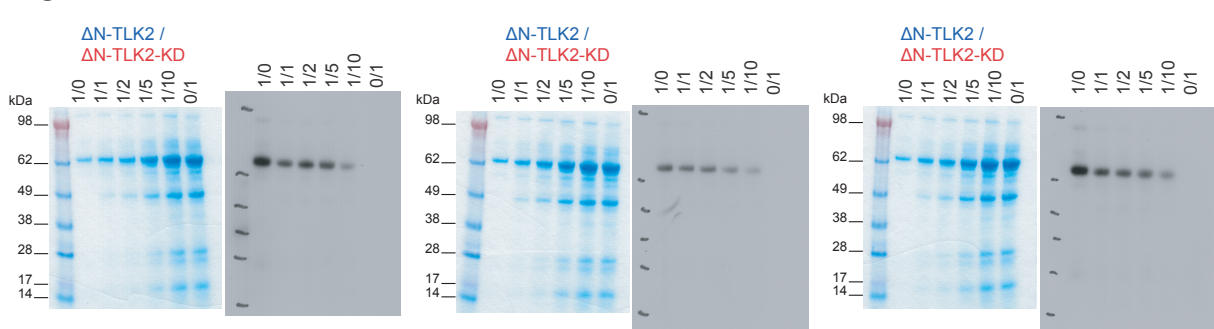
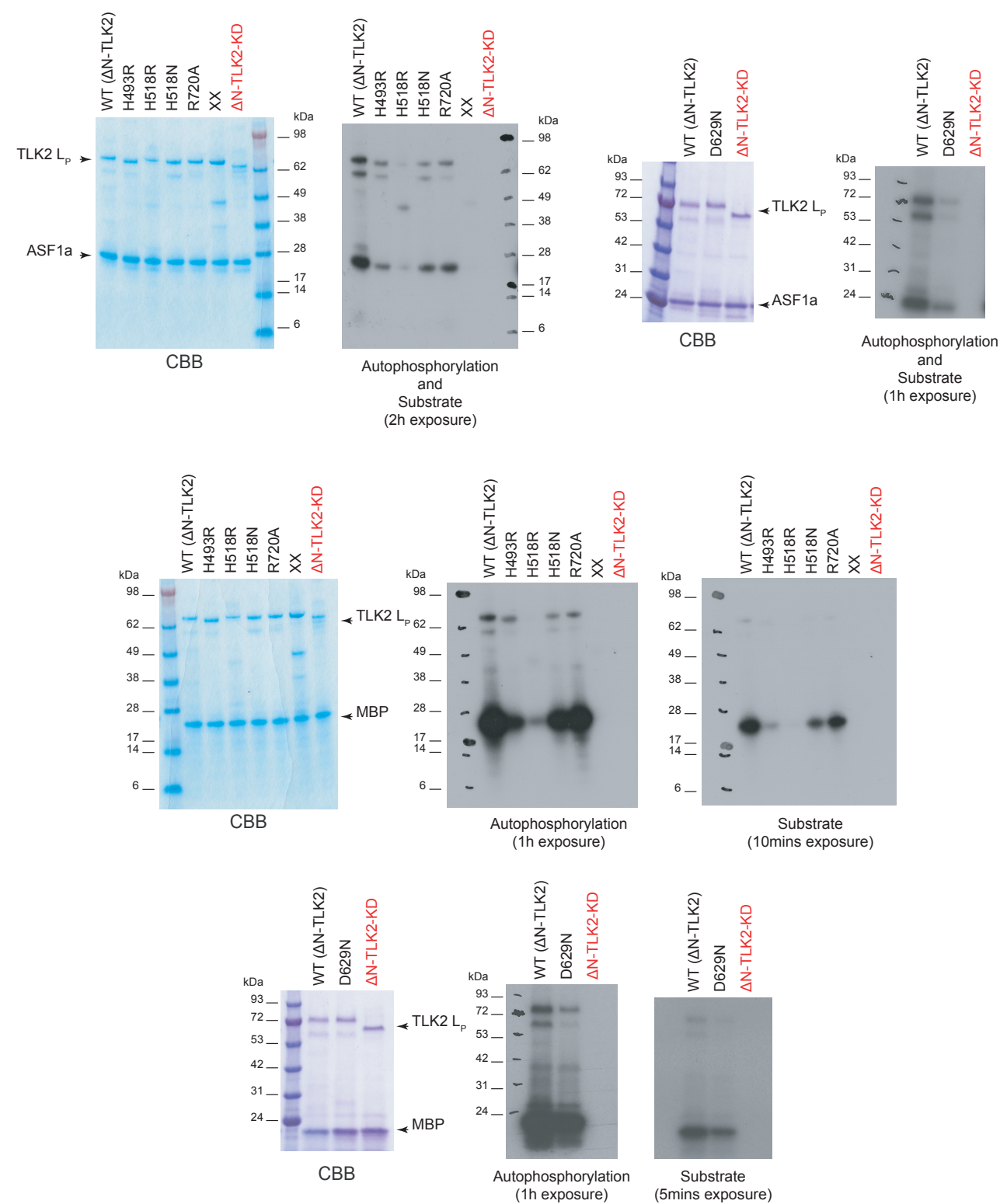


Figure 2c



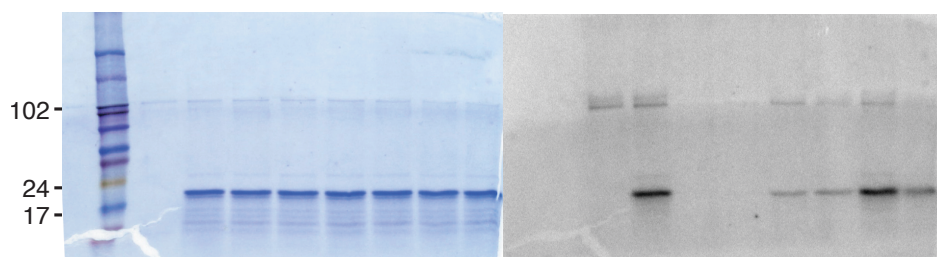
Supplementary Figure 11. Uncropped gels for figure 2a and 2c

Figure 5d



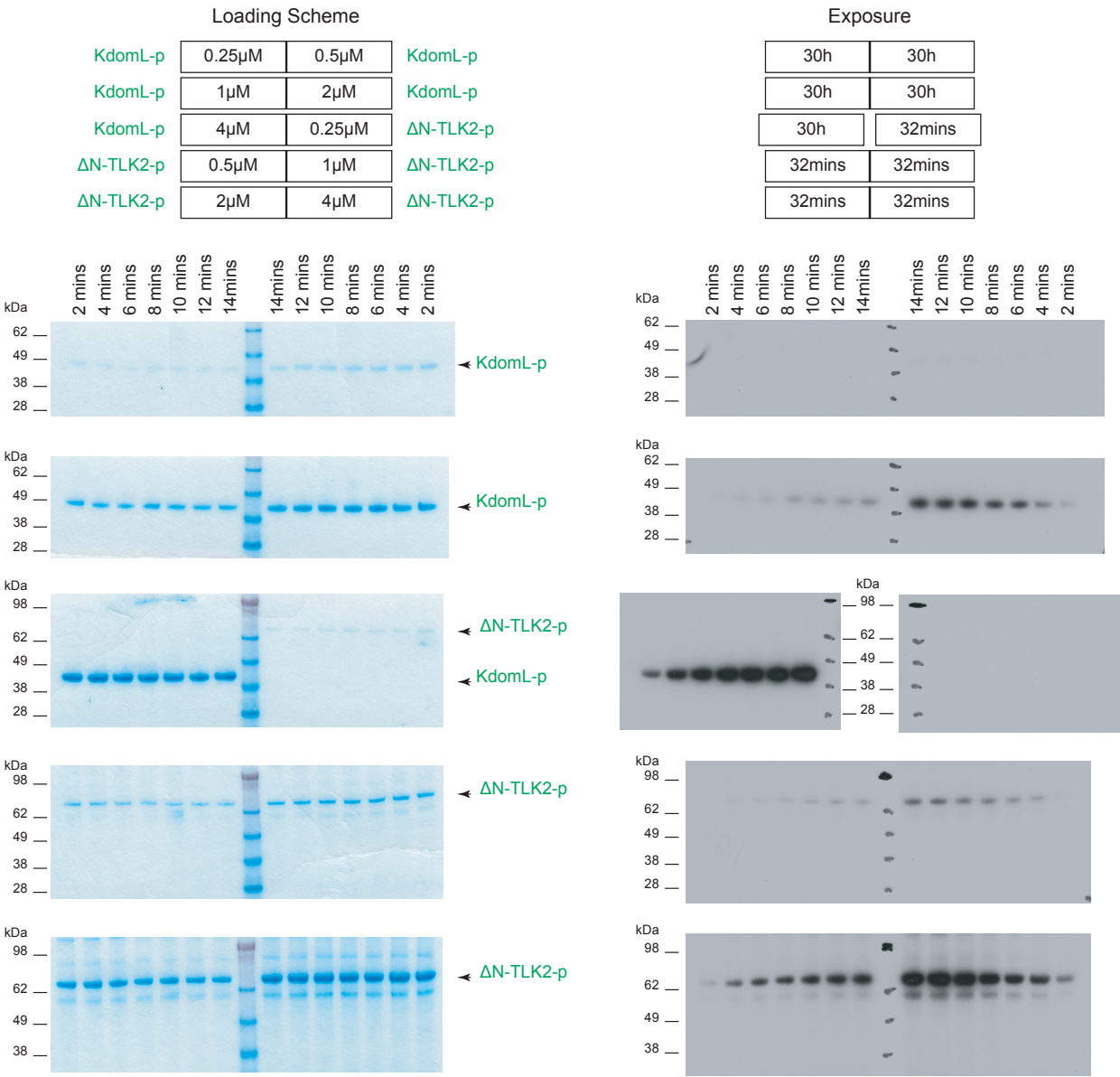
Supplementary Figure 12. Uncropped gels for figure 5d

Figure 6A: autoradiographs and Coomassie



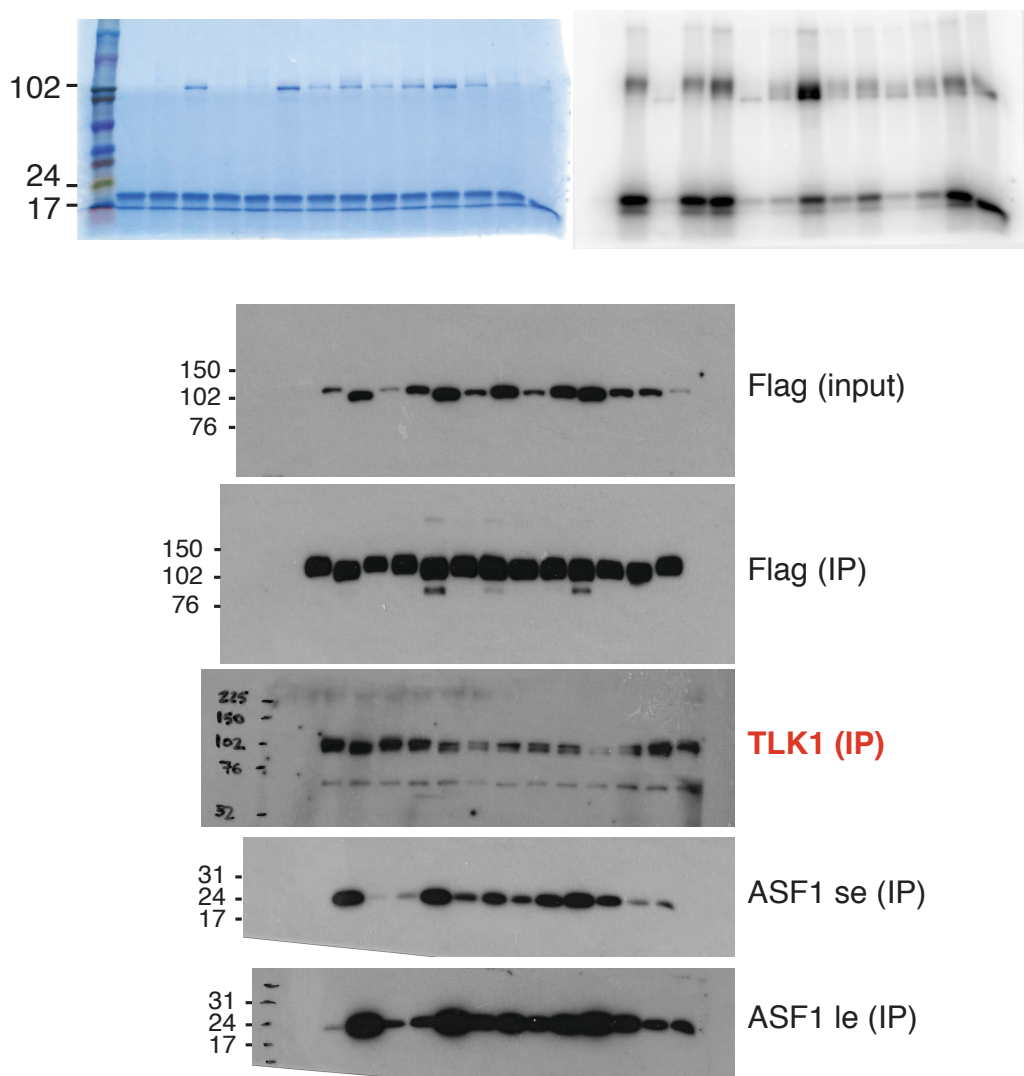
Supplementary Figure 13. Uncropped gels for Figure 6A autoradiograph and coomassie

Supplementary Figure 3a



Supplementary Figure 14. Uncropped gels for Supplementary figure 3a

Supplementary Figure S8A-B: autoradiographs (A) and Western (B)



Supplementary Figure 15. Uncropped gels for Supplementary figure 8a-b

Construct	TLK2 primer name	DNA sequence
	InFusion Cloning	
	Vector fwd	gcttgcgggccgcataatgcttaag
	Vector rev	ctggctgtggtgatgatggtgat
KdomS (451-753)	KdomS fwd	catcaccacagccagGATCCACAATTT
	KdomS rev	tatgcgggccgcaagctTTTAGCTACTT
KdomL (451-772)	KdomL fwd	catcaccacagccagCAATTTAAAGATCATCCAACGCTAAATGACAGA
	KdomL rev	tatgcgggccgcaagcTCAATTAGAAGAACTGTTATTGGACGCCC
Δ N_TLK2 (191-772)	Δ N_TLK2 fwd	catcaccacagccagACAGAGCATTCCTGCAGCTCCC
	Δ N_TLK2 rev	tatgcgggccgcaagcTCAATTAGAAGAACTGTTATTGGACGCCC
Δ N_TLK2s (191-753)	Δ N_TLK2s fwd	catcaccacagccagACAGAGCATTCCTGCAGCTCCC
	Δ N_TLK2s rev	tatgcgggccgcaagctTTTAGCTACTT
	SDM primer name	DNA sequence
D613A	D613A_Fwd	GTGGAGAGATAAAAAATTACAGcaTTTGGTCTTTTCGAAGATCATGGATGATG
	D613A_Rev	CATCATCCATGATCTTCGAAAGACCAAAtgCTGTAATTTTTATCTCTCCAC
H493R	H493R_Fwd	TGTGAAAATTcgcCAGTTAAATAAAAAAC
	H493R_Rev	GCTACGTATCTTTGCTCTG
H518R	H518R_Fwd	ATACCGGATTcgcAAAGAGCTGGATC
	H518R_Rev	TCCCTACATGCATGCTTG
H518N	H518N_Fwd	ATACCGGATTaacAAAGAGCTGGATC
	H518N_Rev	TCCCTACATGCATGCTTG
R720A	R720A_Fwd	GTTTATTTCGAgccTGCTTGGCCTACCGAAAG
	R720A_Rev	GCCTTTGCTTCAGGTGTT
D629N	D629N_Fwd	CAATTCAGTGaacGGCATGGAGC
	D629N_Rev	TAGCTATCATCATCCATG

Supplementary Table 1. TLK2 Primer sequences. All SDM were carried out using Q5 SDM Kit (NEB) except for D613A that was performed using QuickChange II Kit (Agilent). The lambda phosphatase was cloned into MCS2 of a pET_Duet vector using the traditional restriction enzymes cloning (BamHI and HindIII).

1st G-Block Codes for Twin-Strep_LSL and a part of TLK2

CATATGatggatagcgccttggagccacccgcagttcgagaaaggtggaggttccggaggtggatcgggaggtggatcg
tggagccacccgcagttcgaaaaagggccagcggtgtagatctgggtaccATGACCGACATCTACATCCCGCCGGAG
GGTCTCTACTTCCGCCCTCCTTGGCTTTGCCAGTCGGCAGGTGATCTTCGCGCGCAACTCTCCCTCTCCCGATGTTGGT
CTGTCTCCGGTCAACGACCAGGCTACCGACCAGTACTTCTCGCTCATCTACGGCACTGGAGAACACGCCGGTCTCTAC
GCGATAAAGAGCAAAGCGACGGGCAAGGTGCTCTTCTCGCGTAGGCCCTGCGGAACCGTATGTGGGCCAAATCGATGGC
GACGGGCGTTATCCCGACAAC'TGGT'TCAAGATTGAGCCAGGAAAGACCTATCTCTCCAAATATTTCCGGCTCGTTCAG
CCGTCGACTGGCACCGCGCTTGTCTCGCGCACGCATTTGCAGCCATACTTCTGGAATCACCTCAGACTGAAGTCTTC
GACGACCAATACTTTCACCTTCTCTTTCGAGGATgagaacctgtacttccaatccACAGAGCATTCCTGCAGCTCCCAA
AAACAGATCTCCATCCAGCACAGACAGACCCAGTCCGACCTCACAAATAGAAAAAATATCTGCACTAGAAAAACAGTAAG
AATTC'TGACTTAGAGAAGAAGGAGGGAAGAATAGATGATTTATTAAGAGCCAACTGTGATTTGAGACGGCAGATTGAT
GAACAGCAAAAAGATGCTAGAGAAAATACAAGGAACGATTAAATAGATGTGTGACAATGAGCAAGAAAACCTCTTATAGAA
AAGTCAAAAACAAGAGAAGATGGCGTGTAGAGATAAGAGCATGCAAGACCGCTTGAGACTGGGCCACTTTACTACTGTC
CGACACGGAGCCTCATTTACTGAACAGTGGACAGATGGTTATGCTTTTTCAGAATCTTATCAAGCAACAGGAAAGGATA
AATTCACAGAGGGAAGAGATAGAAAGACAACGGAAAATGTTAGCAAAGCGGAAACCTCCTGCCATGG

2nd G-Block code for the rest of the TLK2 protein (with the D613A mutation)

CCATGGGTCAGGCCCTCCTGCAACCAATGAGCAGAAACAGCGGAAAAGCAAGACCAATGGAGCTGAAAATGAAACGC
CCTCTTCTGGGAATACAGAGCTAAAGGATACAGCCCCAGCCTTAGGAGCCACAGTTTACTTAGGTTAACGTTAGCAG
AATACCATGAACAAGAAGAAATCTTCAAAC'TCAGATTAGGTCATCTTAAAAAGGAGGAAGCAGAGATCCAGGCAGAGC
TGGAGAGACTAGAAAGGGTTAGAAATCTACATATCAGGGAAC'TAAAAAGGATACATAATGAAGATAATTCACAATTTA
AAGATCATCCAACGCTAAATGACAGATATTTGTTGTTACATCTTTTGGGTAGAGGAGGTTTCAGTGAAGTTTACAAGG
CATTTGATCTAACAGAGCAAAGATACGTAGCTGTGAAAATTCACCAGTTAAATAAAAACTGGAGAGATGAGAAAAAGG
AGAATTACCACAAGCATGCATGTAGGGAATACCGGATTCATAAAGAGCTGGATCATCCAGAAATAGTTAAGCTGTATG
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AACAGCACAAATTAATGTGCGAGAAAGAGGCCCGGTCCATTATCATGCAGATTGTGAATGCTTTTAAAGTACTTAAATG
AAATAAAACCTCCCATCATACACTATGACCTCAAACCAGGTAATATTTCTTTTAGTAAATGGTACAGCGTGTGGAGAGA
TAAAAAT'TACAGCGTTTGGTCTTTCGAAGATCATGGATGATGATAGCTACAATTCAGTGGATGGCATGGAGCTAACAT
CACAAGGTGCTGGTACTTATTTGGTATTTACCACCAGAGTGT'TTTGTGGTTGGGAAAGAACCACCAAAGATCTCAAATA
AAGTTGATGTGTGGTGGTGGTGTGATCTTCTATCAGTGTCTTTATGGAAGGAAGCCTTTTGGCCATAACCAGTCTC
AGCAAGACATCCTACAAGAGAATACGATTCTTAAAGCTACTGAAGTGCAGTTCCTCGCCAAAGCCAGTAGTAACACCTG
AAGCAAAGGCGTTTATTCGACGATGCTTGGCCTACCGAAAGGAGGACCGCATTGATGTCCAGCAGCTGGCCTGTGATC
CCTACTTGT'TGCC'TCACATCCGAAAGTCAGTCTCTACAAGTAGCCCTGCTGGAGCTGCTATTGCATCAACCTCTGGGG
CGTCCAATAACAGTTCTTCTAATTGACTCGAG

Supplementary Table 2. Cloning strategy for heterodimer Δ TLK2- Δ TLK2 KD

The plasmid containing 6xHis_ Δ N_TLK2 was digested with NdeI and XhoI (MCS2 of pET_Duet)

Two inserts obtained from G-Blocks were then ligated after restriction enzyme digests:

1st G-Block was digested with NdeI / NcoI and the 2nd G-Block was digested with NcoI / XhoI to produce a heterodimer (6xHis_ Δ N_TLK2 and TwinStrepTAG_LSL_ Δ N_TLK2_KD).

Lysis Buffer	20mM Tris, 500mM NaCl, 20mM Imidazole, 0.2 mM TCEP, pH8.8, protease inhibitor (1/50ml)
HisTrap Chealting HP (5ml) Buffer A (Binding buffer)	20mM Tris, 500mM NaCl, 20mM Imidazole, 0.2 mM TCEP,
HisTrap Chealting HP (5ml) Buffer B (Elution buffer)	20mM Tris, 25mM NaCl, 500mM Imidazole, 0.2 mM TCEP, pH8.8
HiTrap Q HP (1ml) Buffer A (Binding buffer)	20mM Tris, 25mM NaCl, 0.2 mM TCEP, pH8.8
HiTrap Q HP (1ml) Buffer A (Elution buffer)	20mM Tris, 1.0 M NaCl, 0.2 mM TCEP, pH8.8
Superdex 75 16/60 SEC Buffer	20mM HEPES, 150 mM NaCl, 0.2 mM TCEP, pH7.5

Supplementary Table 3. TLK Purification buffers.