SUPPLEMENTARY INFORMATION

Polyubiquitination of Transforming Growth Factor β-activated Kinase 1 (TAK1) at Lysine 562 Residue Regulates TLR4-mediated JNK and p38 MAPK Activation

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Supplementary Figure legends

Supplementary Figure 1. TAK1 is required for IKK and MAPK signaling. *A*, TAK1 is required for IKK and MAPK activation. TAK1 in human THP-1 cells was knocked down with lentiviral-based shRNA. After treatment with LPS (100 ng/mL) for the indicated times, cells were lysed and subjected to immunoblot analysis. *B* and *C*, TAK1 is required for TLR4-mediated TNF and IL-6 production. Control and TAK1-silenced THP-1 cells were stimulated with LPS for indicated times, and total RNA was extracted. After reverse transcription, TNF and IL-6 mRNA were analyzed with Q-PCR. Results of Q-PCR are averages ± SD of three separate experiments (*, *significant difference*).

Supplementary Figure 2. IKK and MAPK activation is blocked by TAK1 inhibition. *A*, after treatment with TAK1 inhibitor 5Z-7-oxozeanenol (100 nM) for 30 min, RAW264.7 cells were stimulated with LPS for indicated times. The cells were lysed and kinase activation was analyzed by immunoblotting. *B* and *C*, TAK1 inhibition decreased TLR4-mediated TNF and IL-6 mRNA production in RAW264.7 cells. RAW264.7 cells were pretreated with TAK1 inhibitor 5Z-7-oxozeanenol (100 nM) for 30 min, and stimulated with LPS for indicated times. Total RNA was extracted, and TNF and IL-6 mRNA were analyzed with Q-PCR. *D* and *E*, TAK1 inhibition decreased TLR4-mediated TNF and IL-6 mRNA production in THP-1 cells. THP-1 cells were pretreated with TAK1 inhibitor and stimulated with LPS for indicated times. Total RNA was extracted, and TNF and IL-6 mRNA were analyzed with Q-PCR. Results of Q-PCR are averages ± SD of three separate experiments (*, *significant difference*).

Supplementary Figure 3. Lys63-linked polyubiquitination of TAK1 is enhanced by blocking TAK1 phosphorylation. In response to LPS, TAK1 polyubiquitnation is enhanced by inhibiting TAK1 activation. *A*, RAW264.7 cells and *B*, THP-1 cells were pretreated with TAK1 inhibitor 5Z-7-oxozeanenol and incubated with LPS for the indicated times. After immunoprecipitation, polyubiquitination of TAK1 was detected with anti-Lys63-linked Ub antibodies.

Supplementary Figure 4. Phosphorylation of TAK1 at Thr184 and Thr187 residues is critical for the activation of MAPKs. Flag-tagged TAK1 wild-type (WT), T(184,187)A or T(184,187)D mutant, Renilla-Luc plasmid and AP-1 reporter were co-expressed in HEK293T cells for 24 h. Relative luciferase activity was measured and normalized with the Renilla activity. Results are averages ± SD of three separate experiments (*, *significant difference*).

Supplementary Figure 5. Lys562 residue of TAK1 is a Lys63-linked ubiquitination site. *A*, in response to LPS, Lys562 or Lys563 residue of TAK1 undergoes Lys63-linked polyubiquitination. TAK1-silenced THP-1 cells were reconstituted with Flag-tagged TAK1 wild-type (WT) or K(562,563)R mutant. The cells were incubated with LPS for the indicated times, lysed, immunoprecipitated, and detected polyubiquitination with anti-Lys63-linked Ub antibodies. *B* and *C*, Lys562 or Lys563 residue of TAK1 is critical for Lys63-linked polyubiquitination with in vitro ubiquitination assay. Flag-tagged TAK1 WT, K562R, or K(562,563)R mutants was incubated with Myc-TRAF6 immunoprecipitated beads in the present of ATP, UBE1, E2 UbcH enzymes, and either

ubiquitin K63 only or ubiquitin K48R mutant for 1 hour. The supernatant containing TAK1 protein and the beads containing TRAF6 were isolated and subjected for immunoblotting analysis. *D*, in response to Pam3CSK4, Lys562 or Lys563 residue of TAK1 undergoes Lys63-linked polyubiquitination. TAK1-silenced RAW264.7 cells were reconstituted with Flag-tagged TAK1 wild-type (WT) or K(562,563)R mutant. The cells were incubated with Pam3CSK4 for the indicated times, lysed, immunoprecipitated, and detected polyubiquitination with anti-Lys63-linked Ub antibodies.

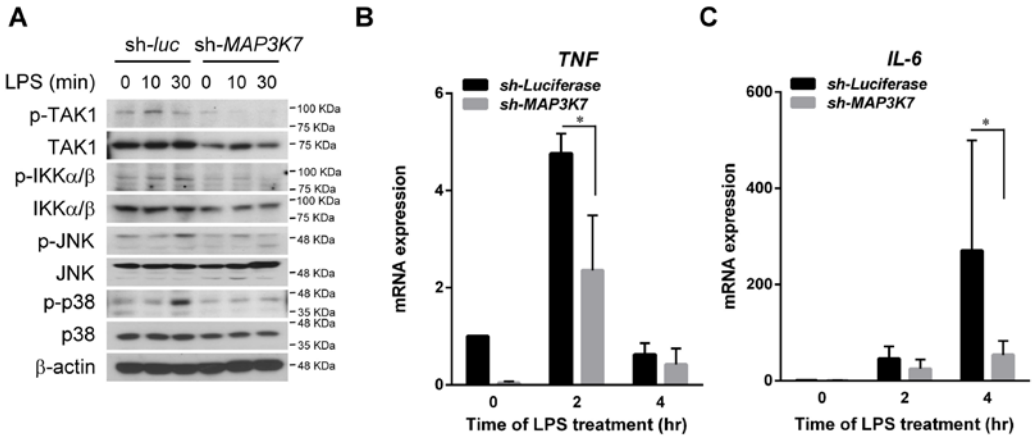
Supplementary Figure 6. Polyubiquitination of TAK1 at Lys562 is required for the activation of TAK1 and MAPK, but not IKK. *A*, mutation in Lys562 residue of TAK1 inhibits the activation of TAK1 and MAPK, but not IKK, in response to LPS. TAK1-silenced THP-1 cells were reconstituted with Flag-tagged TAK1 wild-type (WT) or K(562,563)R mutant. The cells were incubated with LPS for the indicated times, and phosphorylated proteins were analyzed with immunoblotting. *B* and *C*, mutation in Lys562 residue of TAK1 inhibits the activation of TAK1 and MAPK, but not IKK, with stimulation of IL-1beta and TNF. RAW264.7 cells were overexpressed with Flag-tagged TAK1 wild-type (WT) or K(562,563)R mutant. The cells were stimulated with *B*, IL-1beta and *C*, TNF for the indicated times, lysed and phosphorylated proteins were analyzed with immunoblotting. *D*, mutation in Lys562 residue of TAK1 inhibits the activation of MAPK in response to Pam3CSK4. Flag-tagged TAK1 wild-type (WT), K562R or K(562,563)R mutant, Renilla-Luc plasmid and AP-1 reporter were co-expressed with or without TLR2 in HEK293T cells for 24 h. Relative luciferase activity

was measured and normalized with the Renilla activity. Results are averages ± SD of three separate experiments (*, *significant difference*).

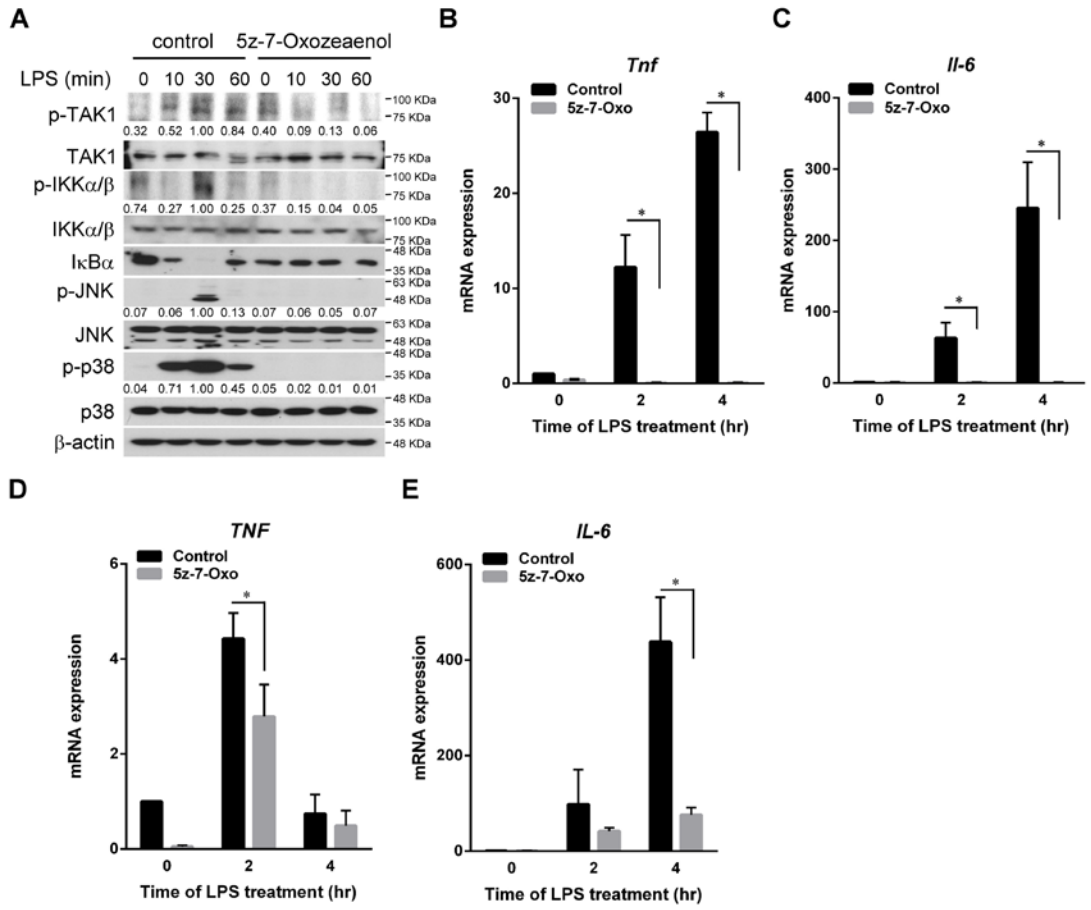
Supplementary Figure 7. Mutation in Thr184 and 187 residues of TAK1 does not disrupt the interaction of TAK1 with TRAF6. Flag-tagged TAK1 wild-type (WT), or T(184,187)A mutant were co-expressed in HEK293T cells with myc-tagged TRAF6. The cells were lysed and immunoprecipitated with anti-Flag antibody. After gel separation, TAK1-containing complex was detected with the indicated antibodies.

Supplementary Figure 8. *A* and *B*, TAK1 is required for TLR4-mediated induction of TNF and IL-6 mRNA. TAK1-silenced THP-1 cells were reconstituted with Flag-tagged TAK1 wild-type (WT), or K(562,563)R mutant. After treatment with LPS for indicated times, the cells were collected and the total RNA was extracted, reverse transcribed, and analyzed for TNF and IL-6 mRNA with Q-PCR. *C*, TAK1 is required for TLR2-mediated induction of IL-6 mRNA. TAK1-silenced RAW264.7 cells were reconstituted with Flag-tagged TAK1 wild-type (WT), T(184,187)A or K(562,563)R mutants. After treatment with Pam3CSK4 for indicated times, the cells were collected and the total RNA was extracted, reverse transcribed, and analyzed for IL-6 mRNA with Q-PCR. Results are averages ± SD of three separate experiments (*, *significant difference*).

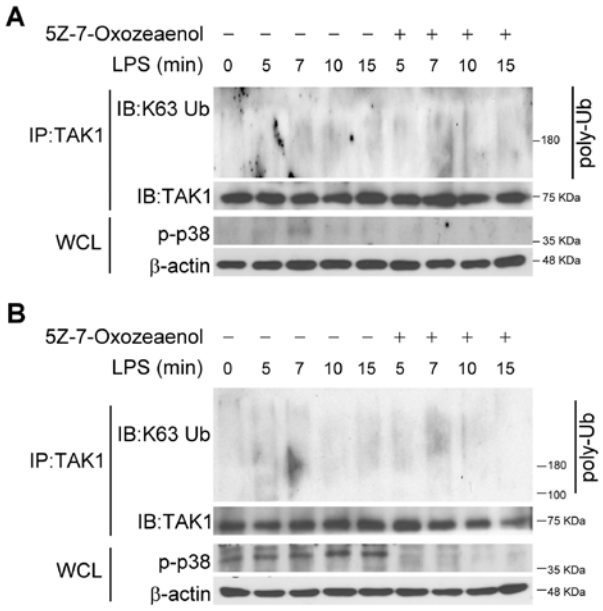
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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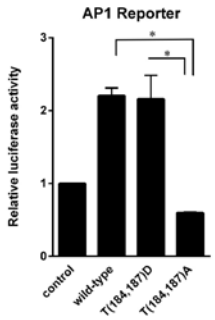

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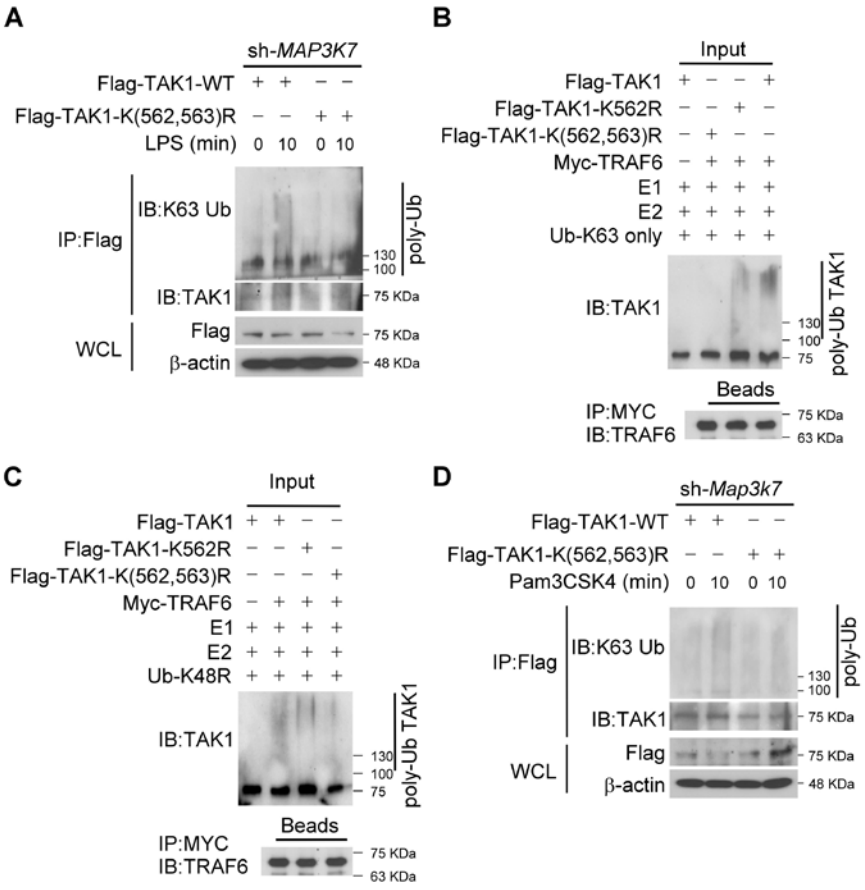

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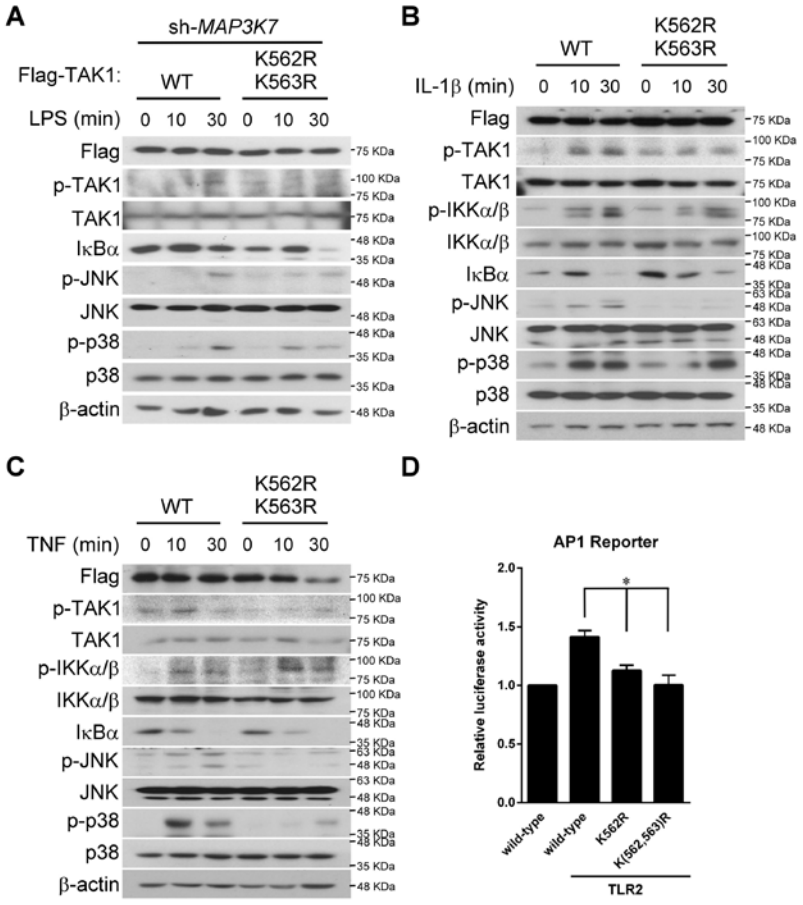

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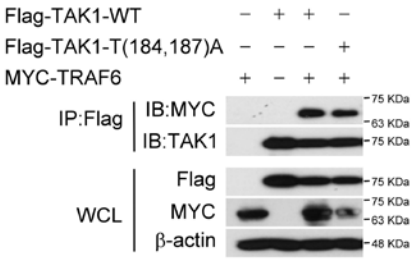

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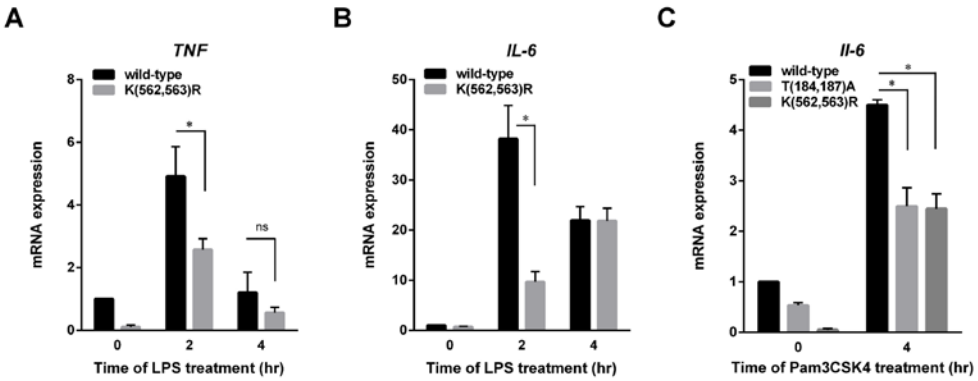


Original blots before cropping for main figures

Fig 1A

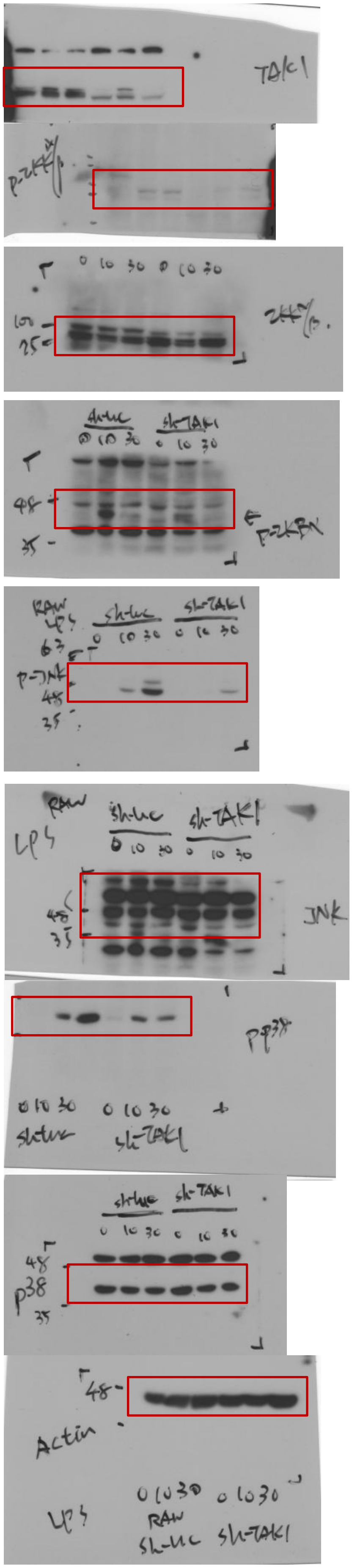


Fig 1E

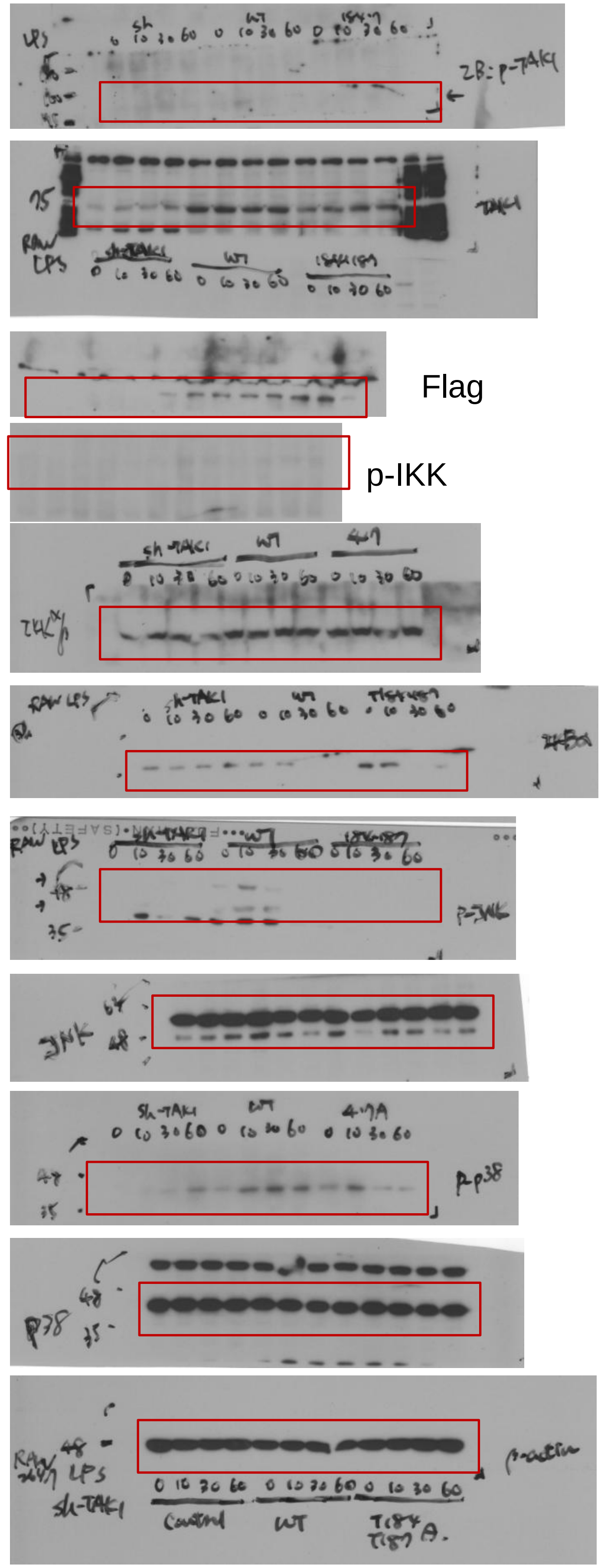
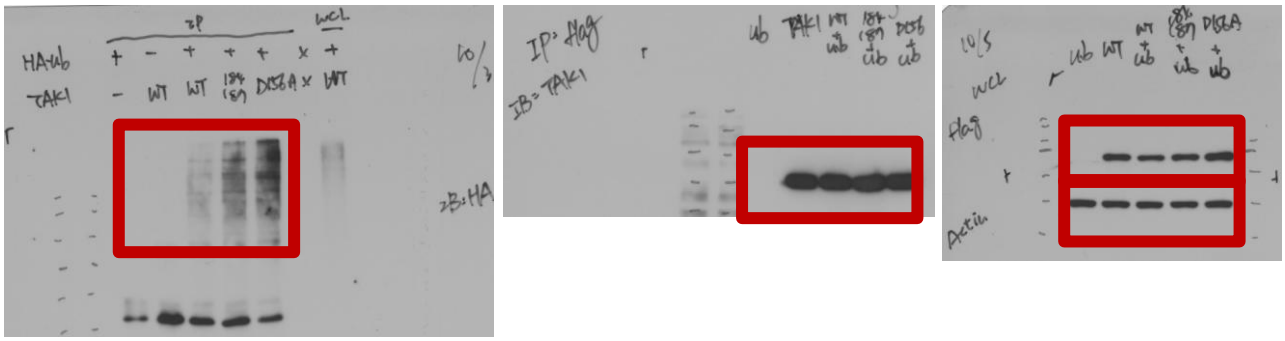
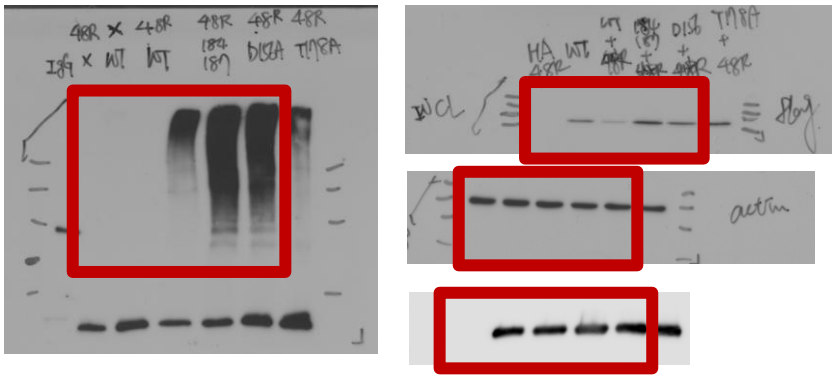


Fig 2A

WT-Ub



K48R-Ub



K63R-Ub

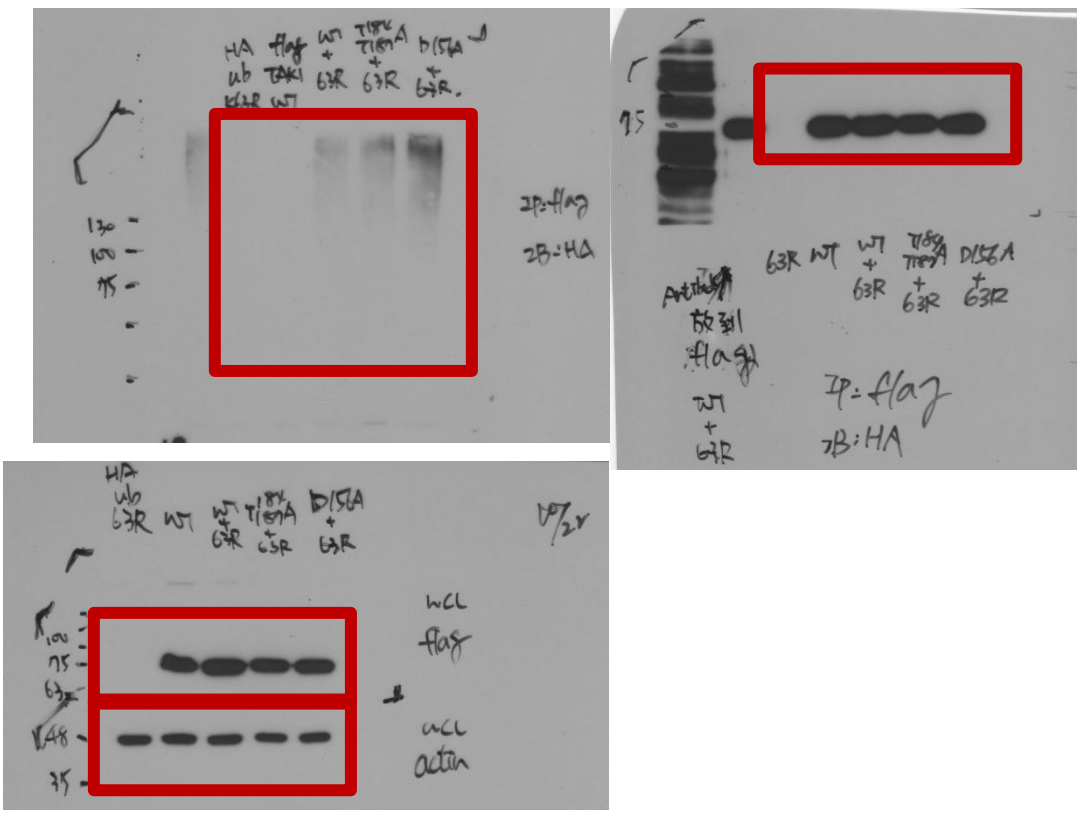
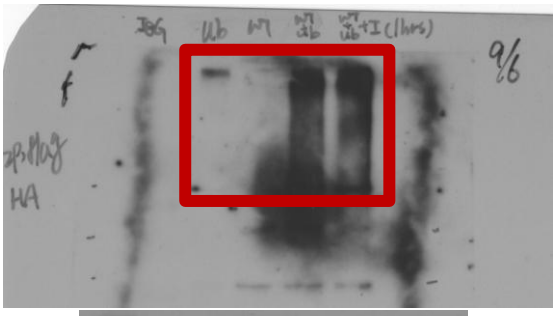
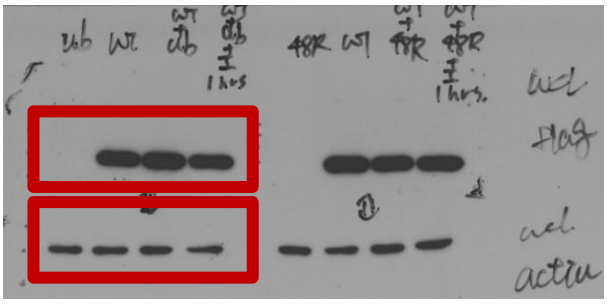
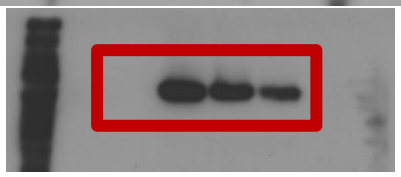


Fig 2B

WT-Ub







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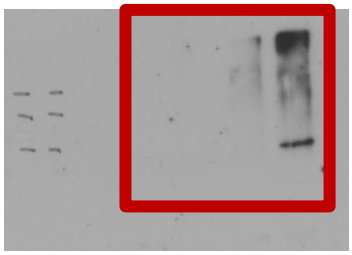
IB: TAK1

K48R-Ub



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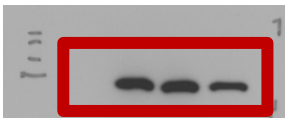

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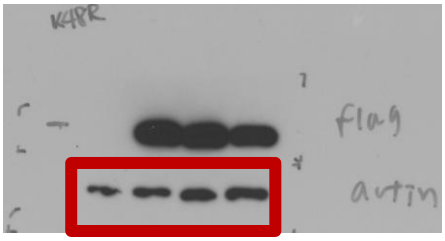

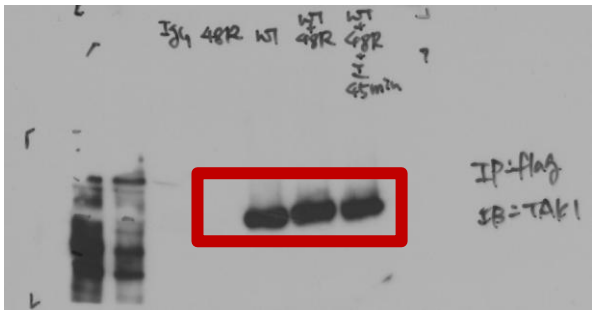

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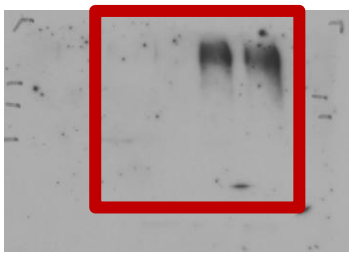

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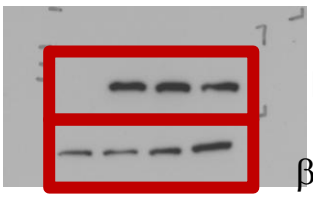

Flag

β-actin

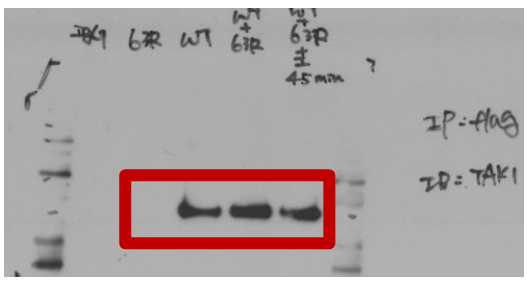

Fig 2C

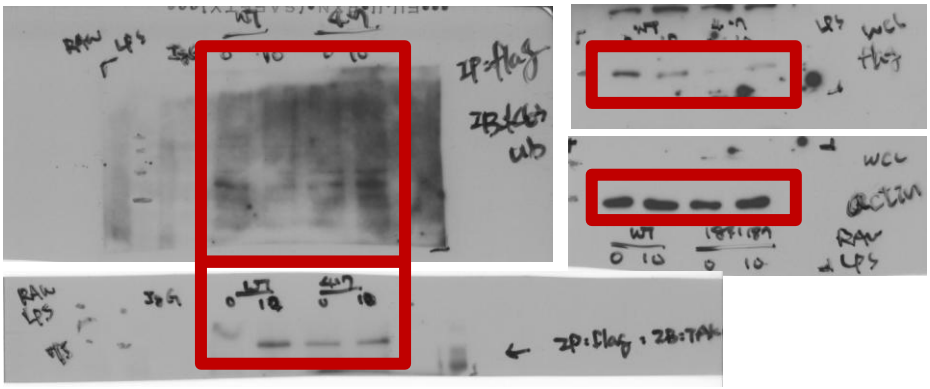

Fig 2D

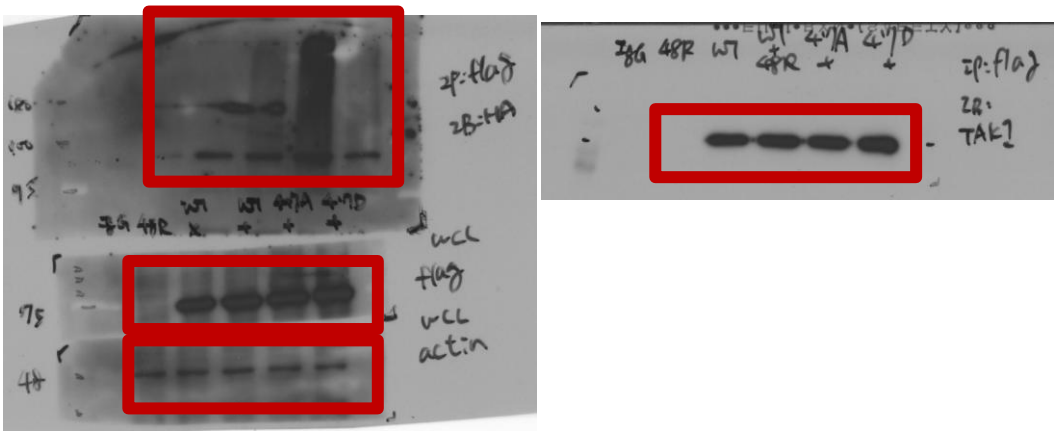

Fig 2E

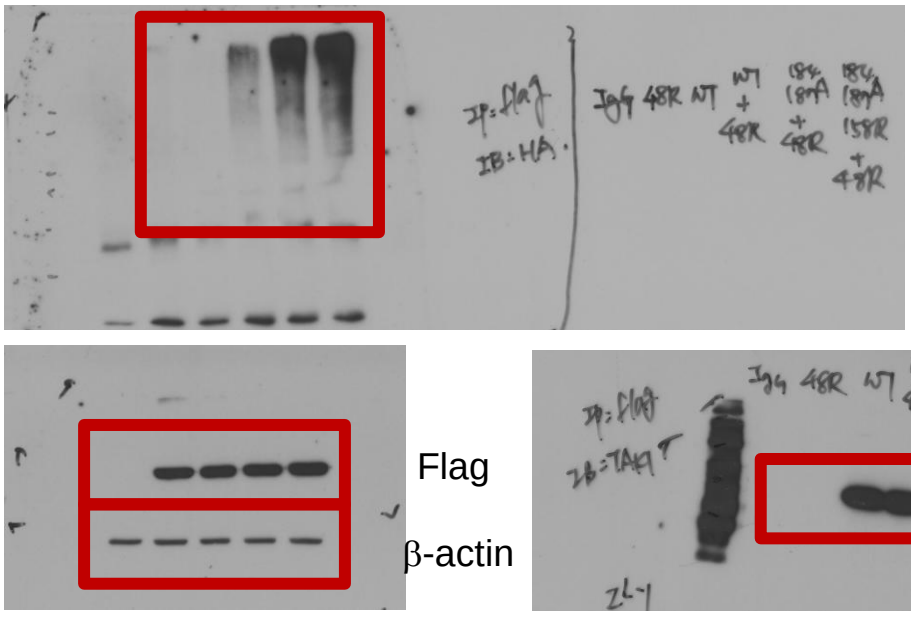

Fig 4A

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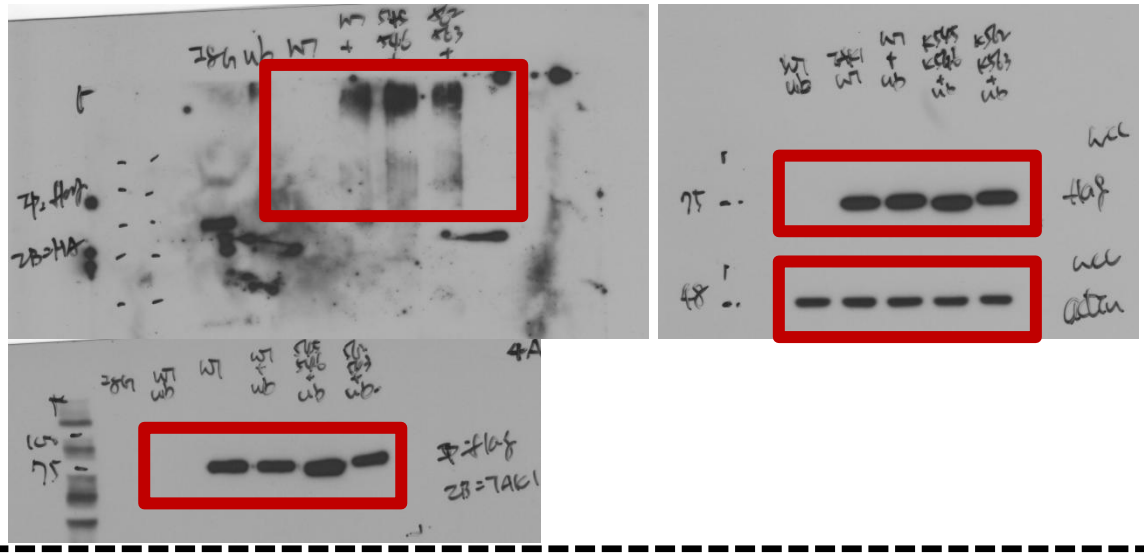

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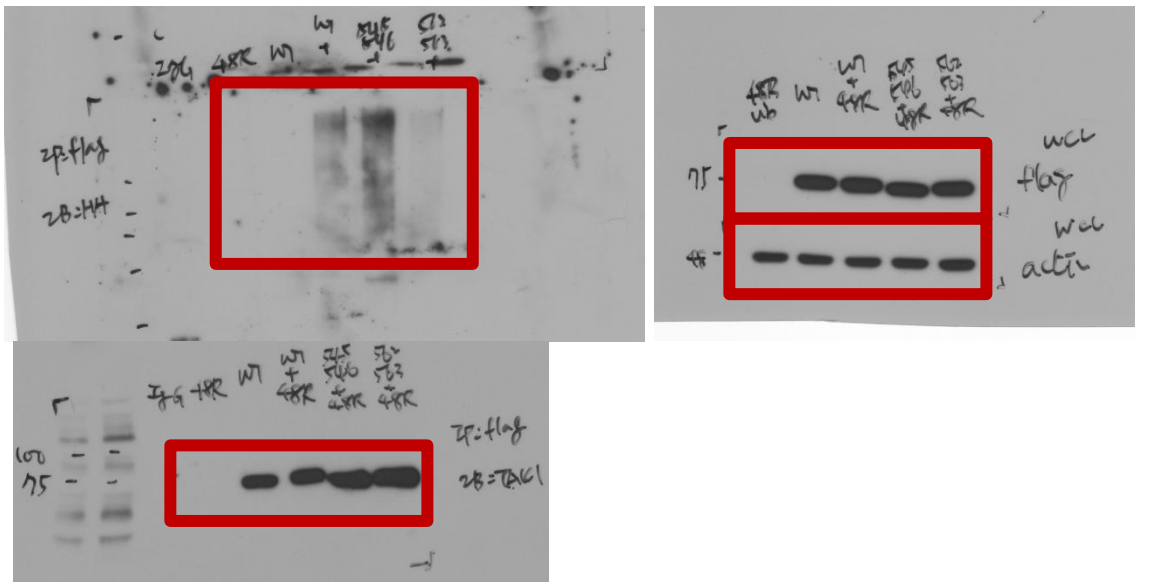

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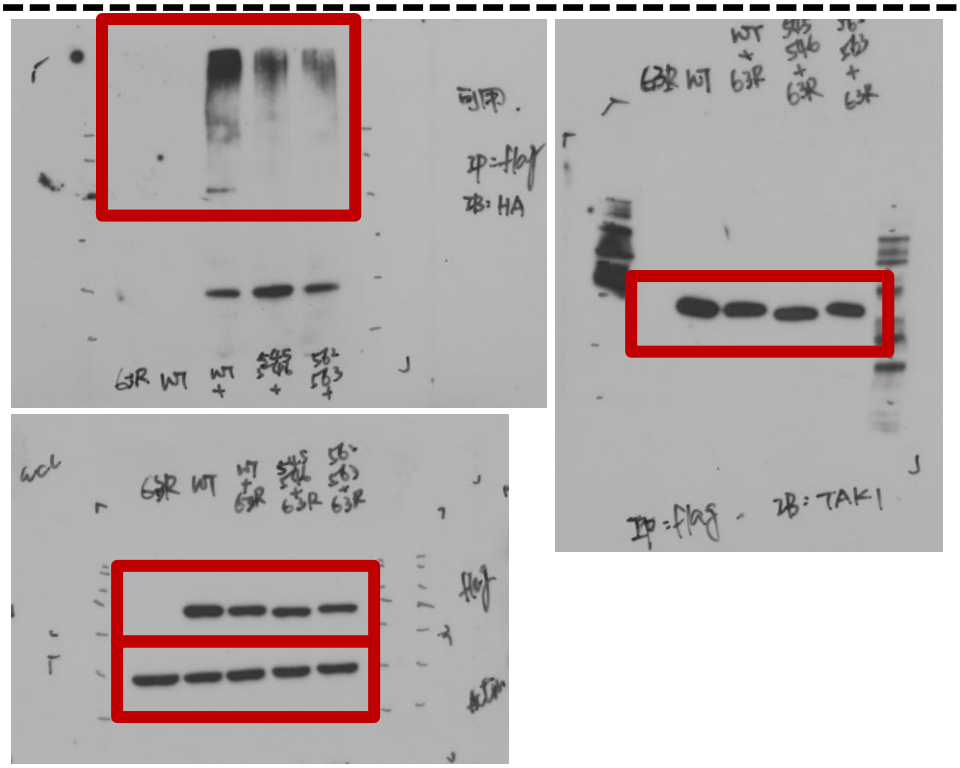

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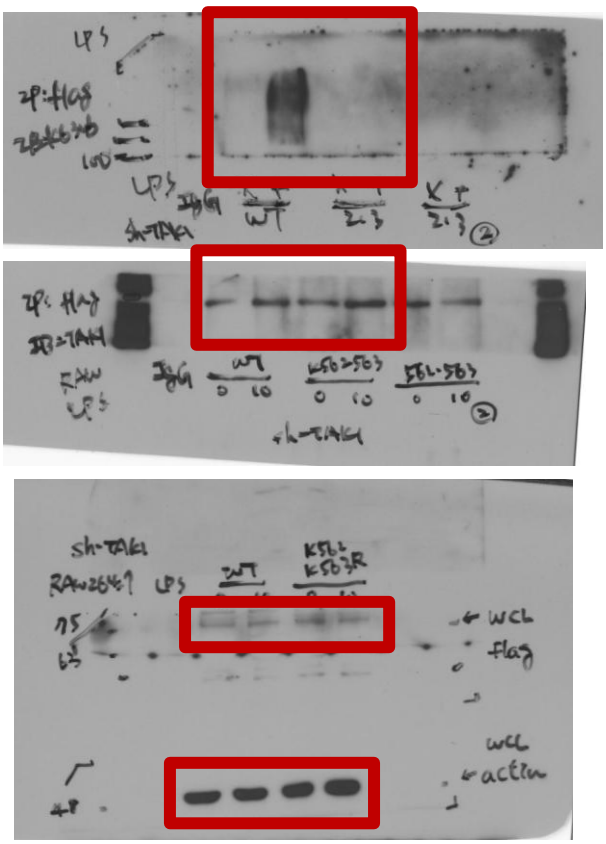

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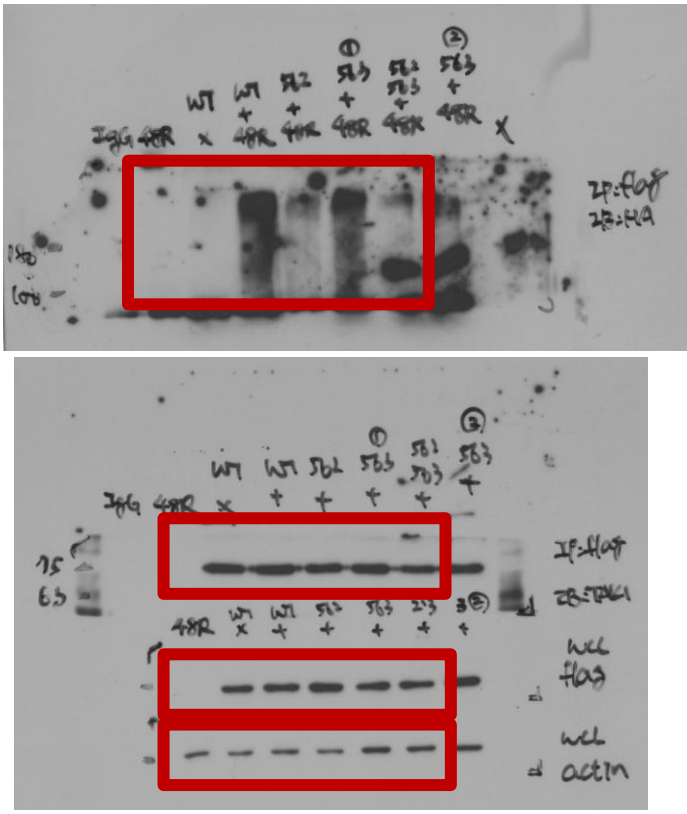
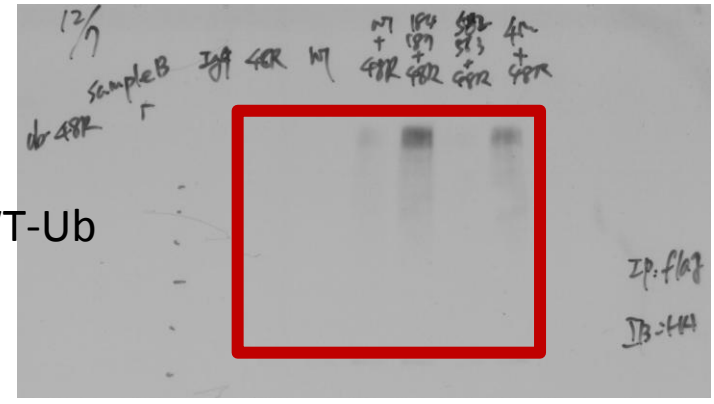
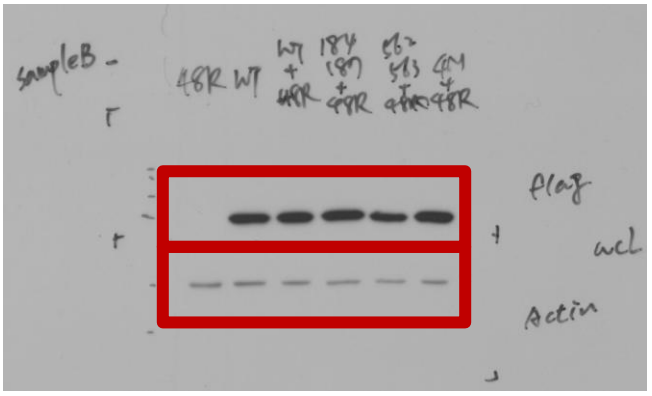
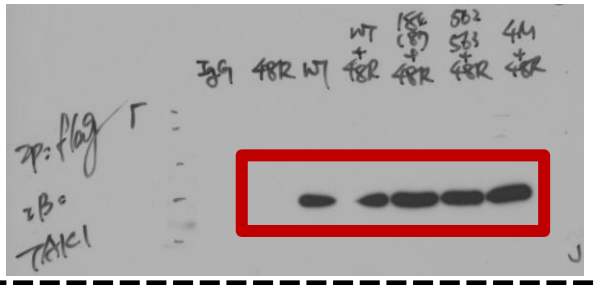


Fig 4D

WT-Ub







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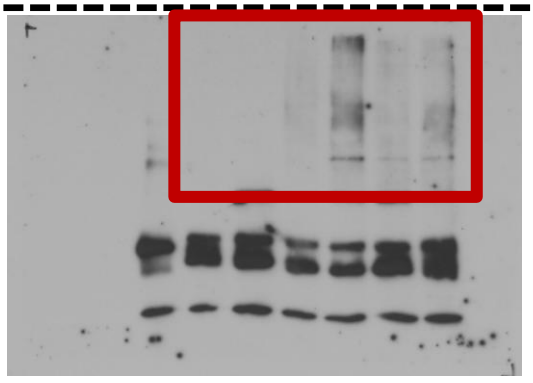

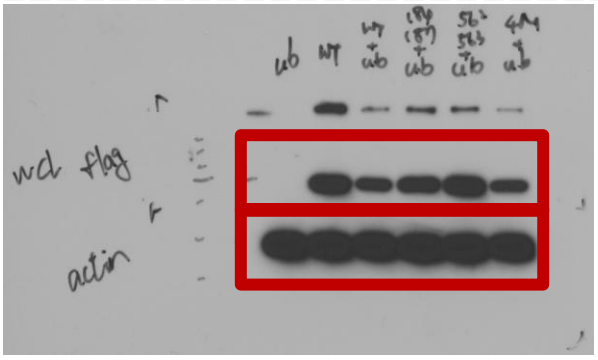

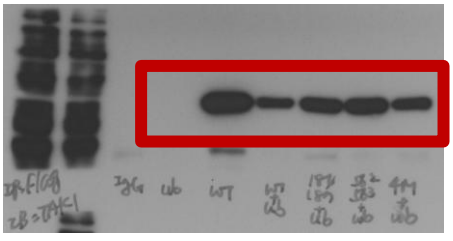

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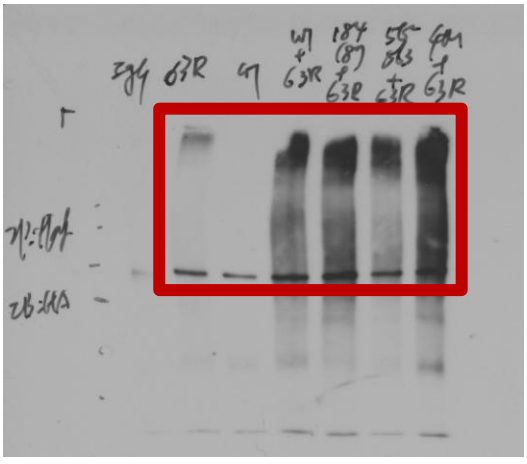
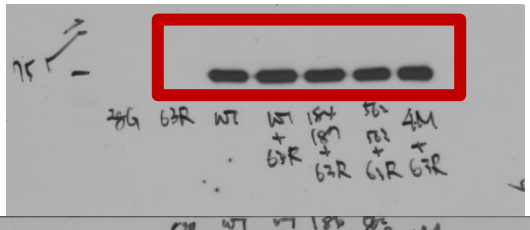




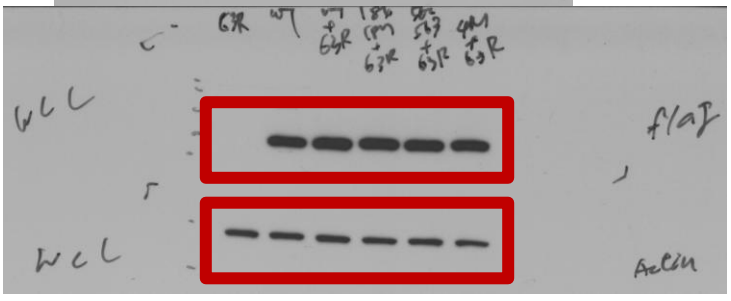


Fig 4E

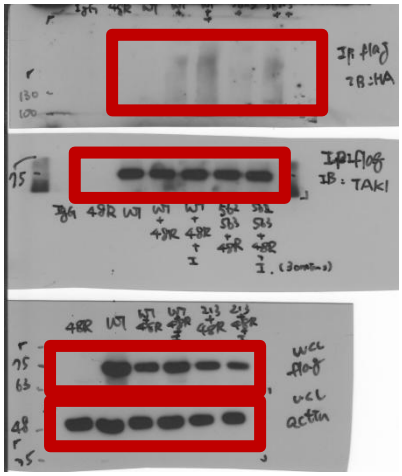


Fig 4F

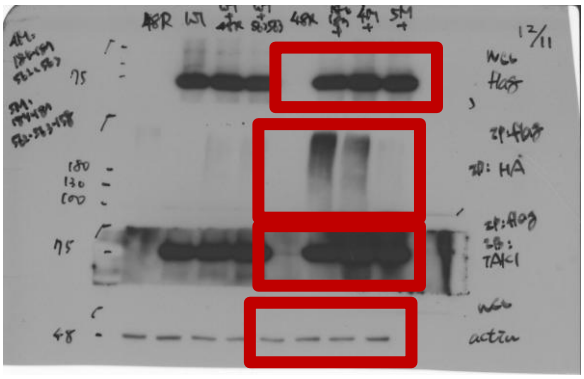
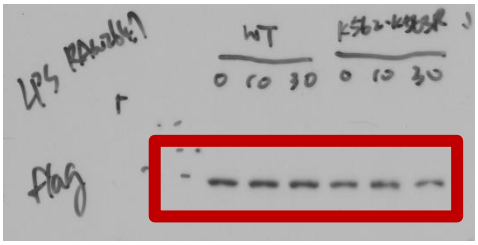
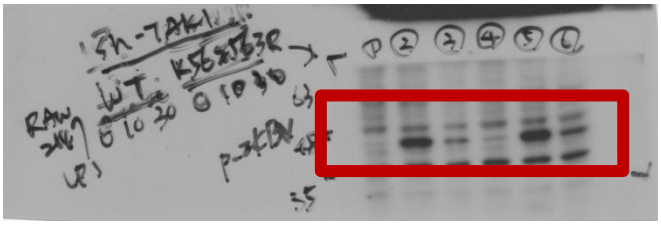
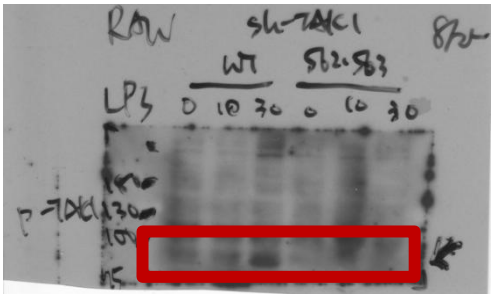
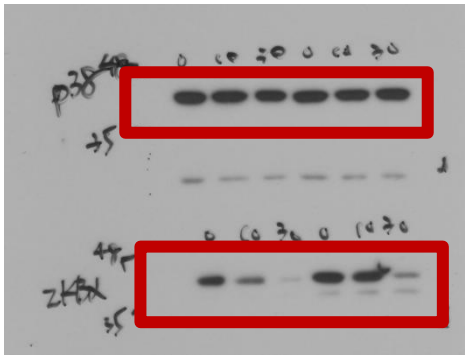


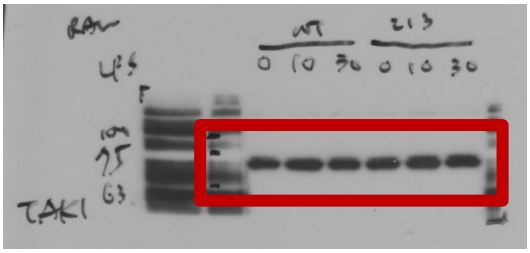
Fig 5A

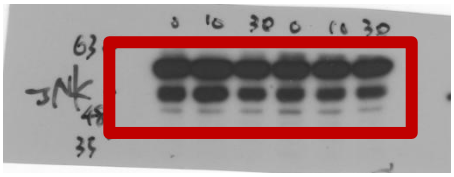


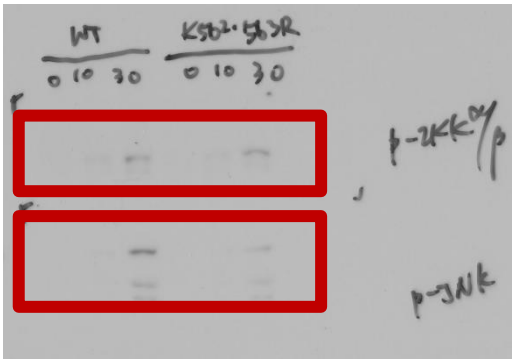


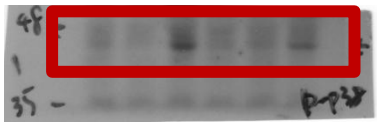


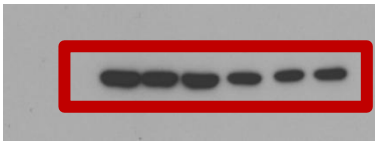












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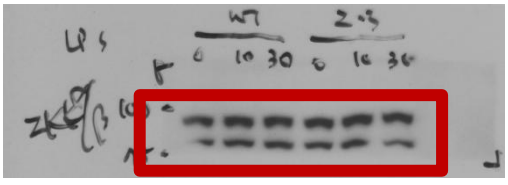


Fig 6A

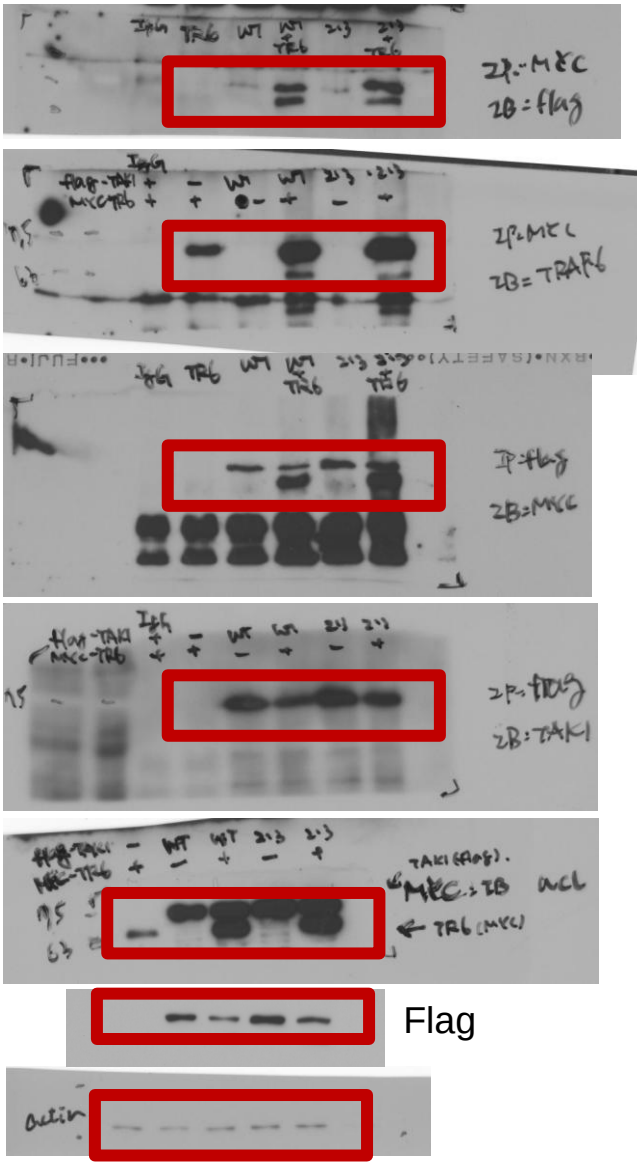


Fig 6B

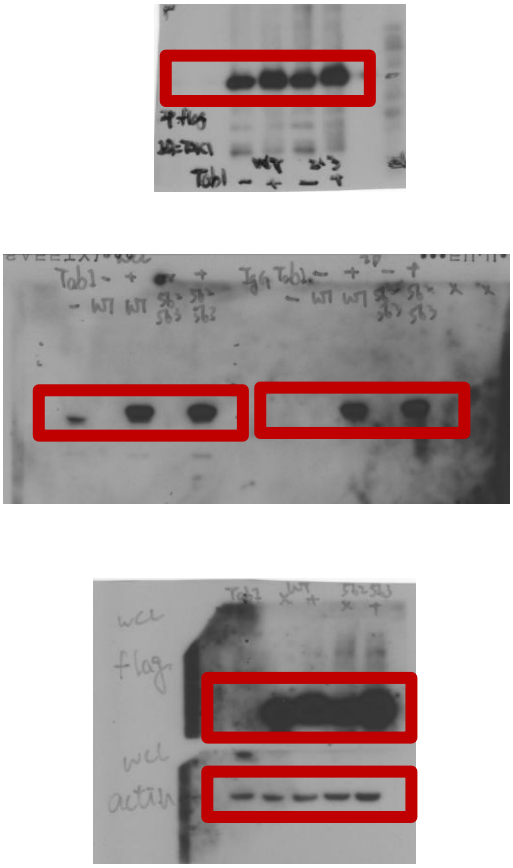
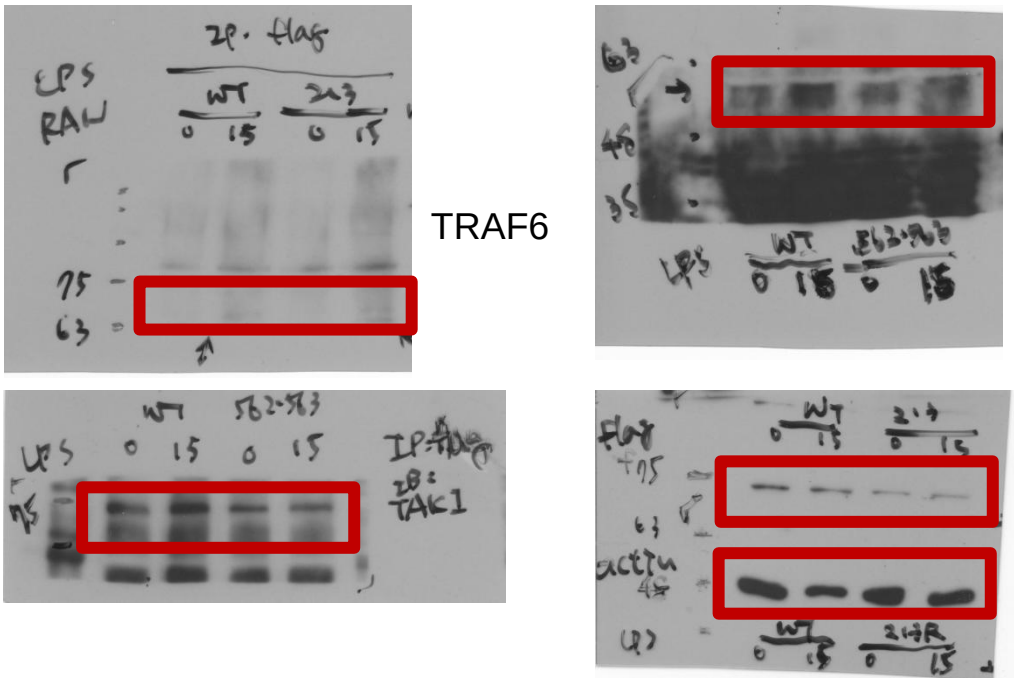
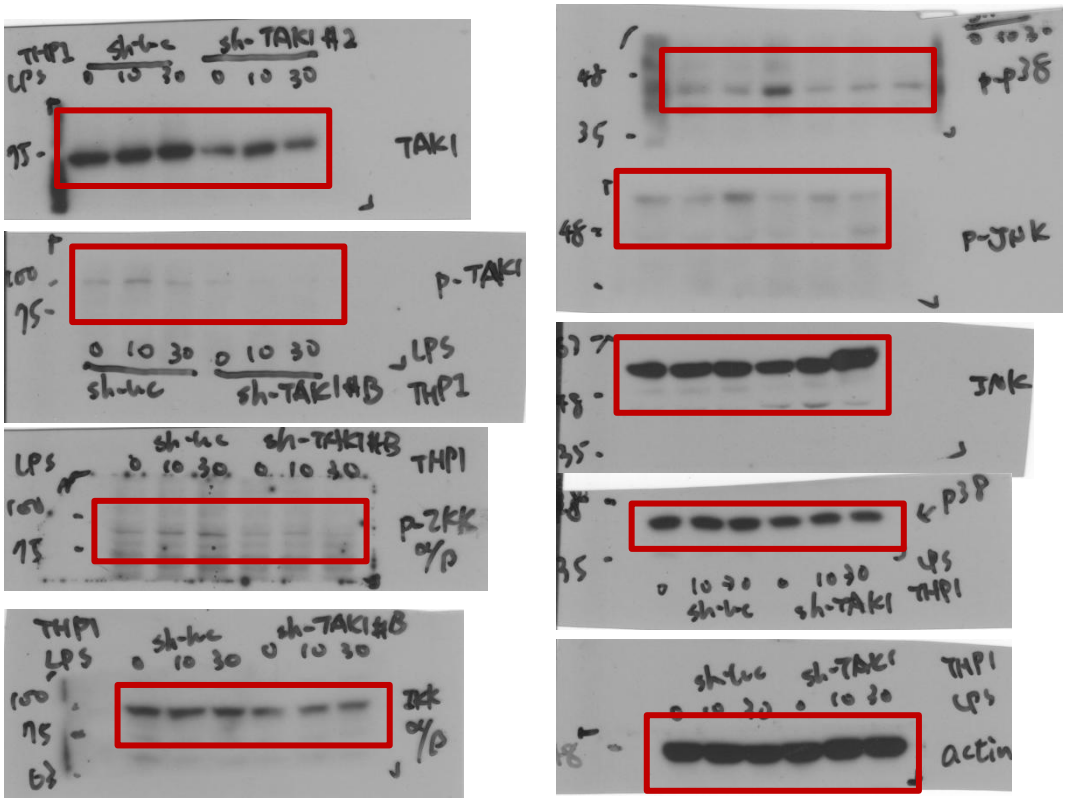


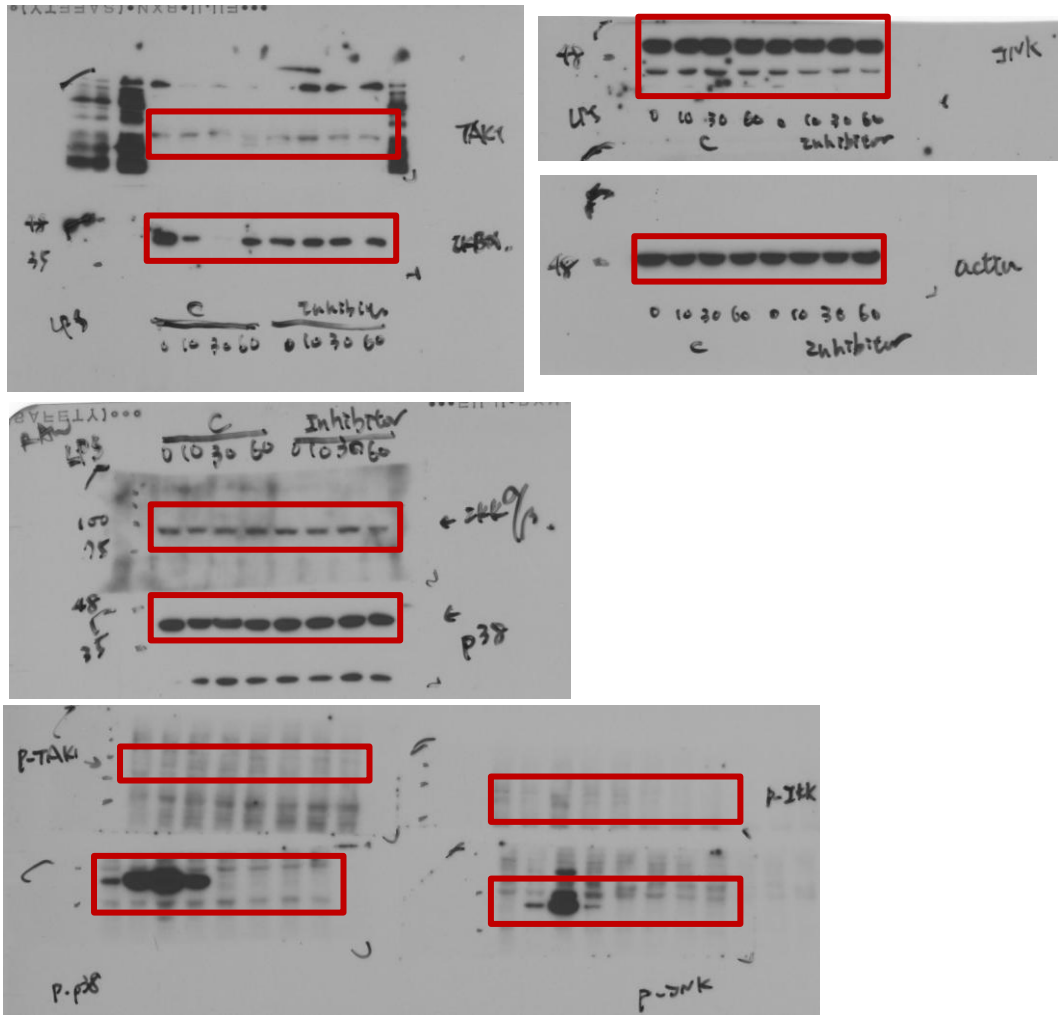
Fig 6C



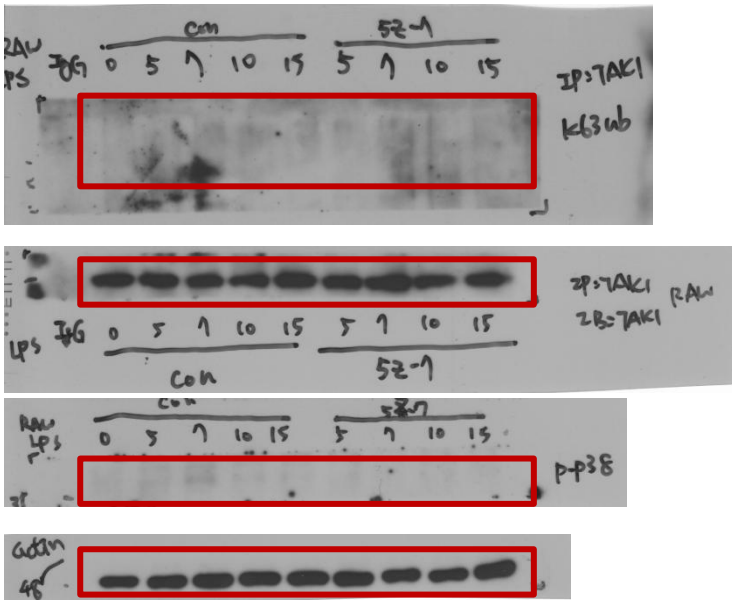
Suppl. Fig1A



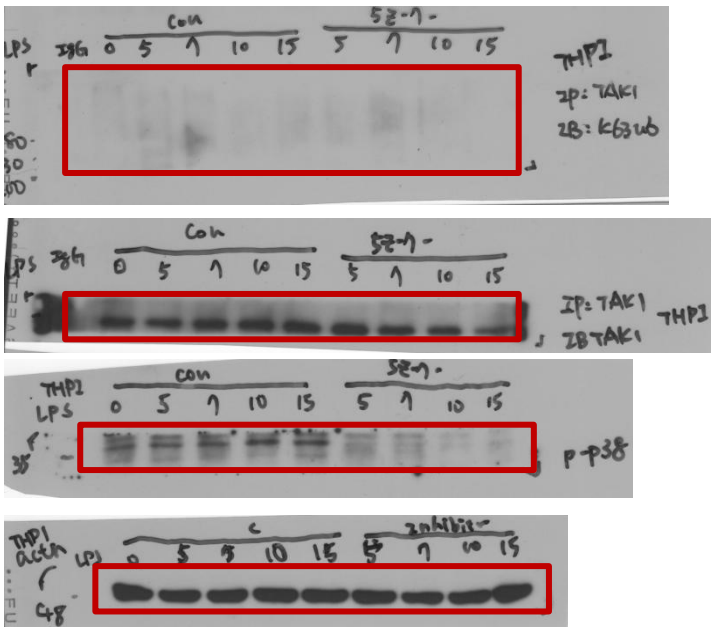
Suppl. Fig2A



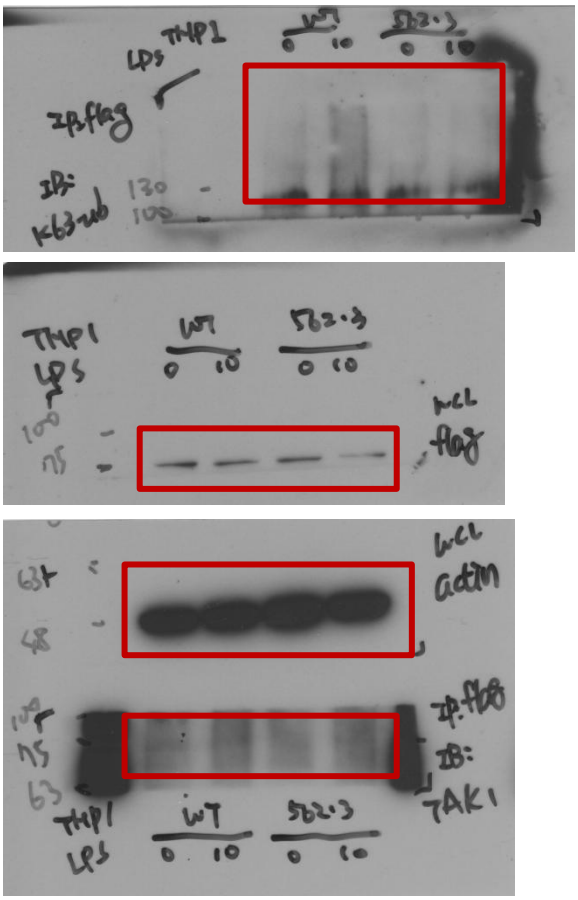
Suppl. Fig3A



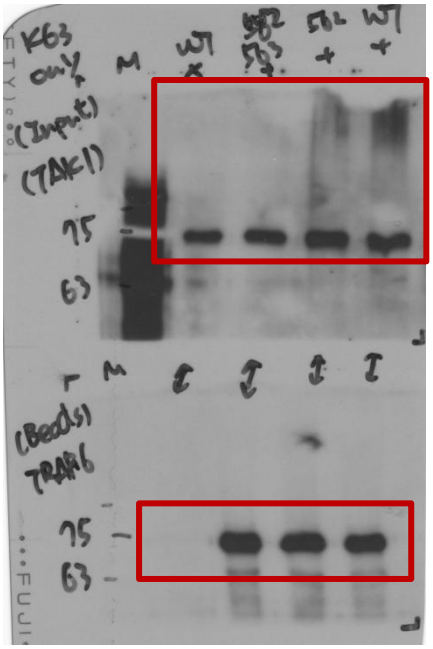
Suppl. Fig3B



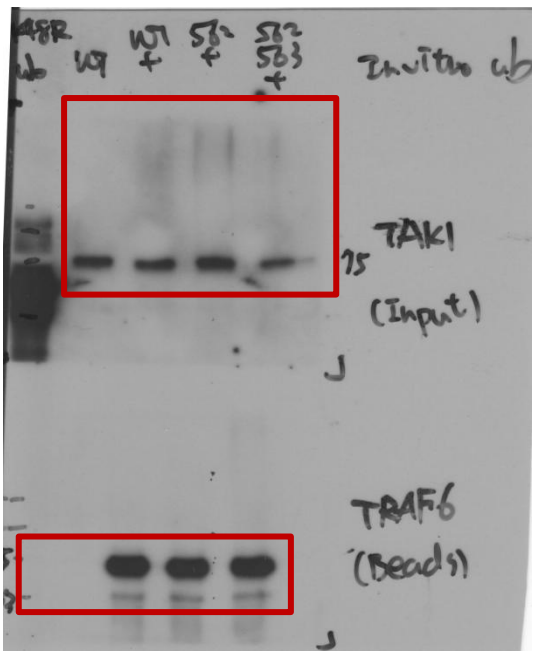
Suppl. Fig5A



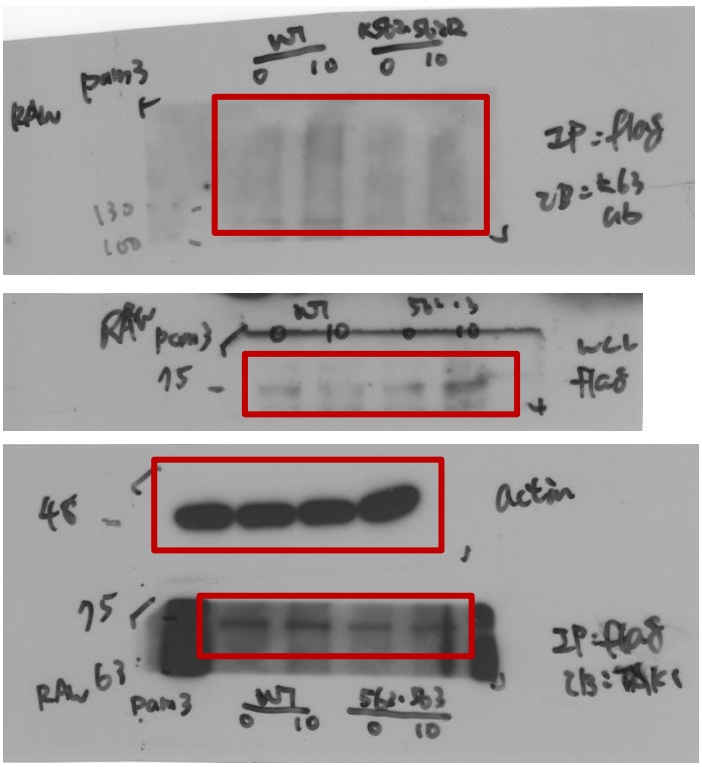
Suppl. Fig5B



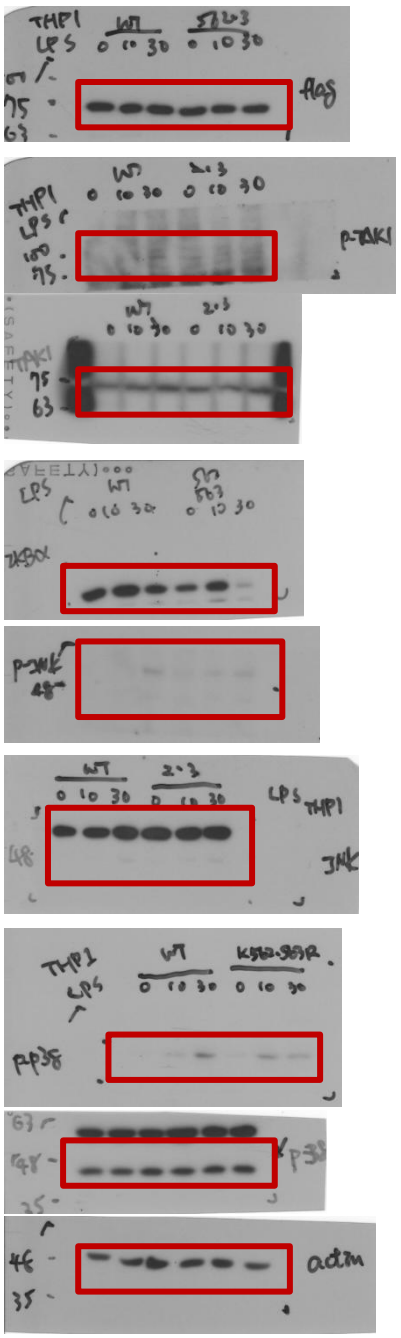
Suppl. Fig5C



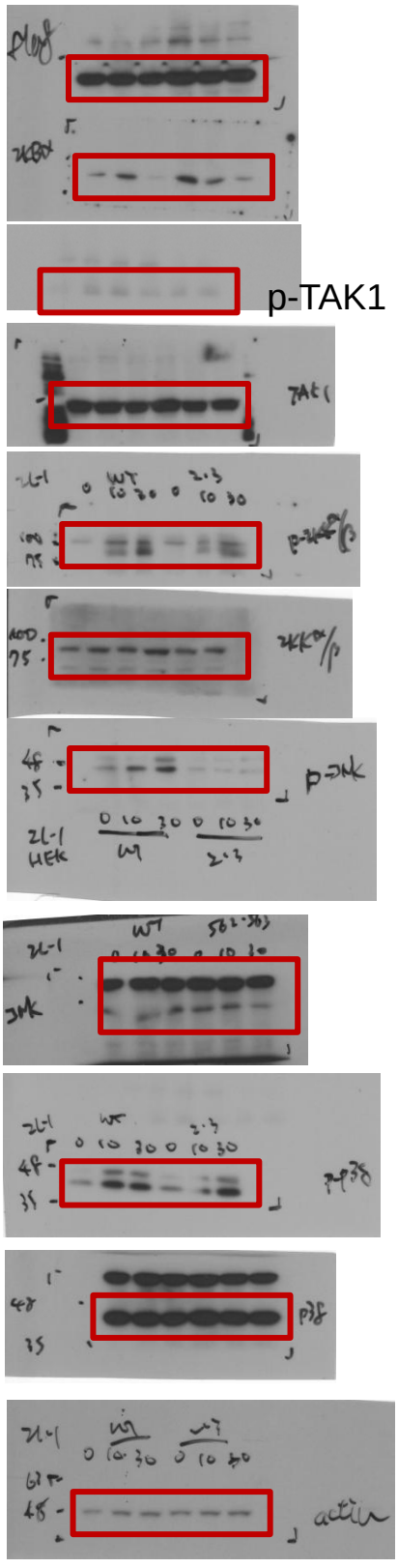
Suppl. Fig5D



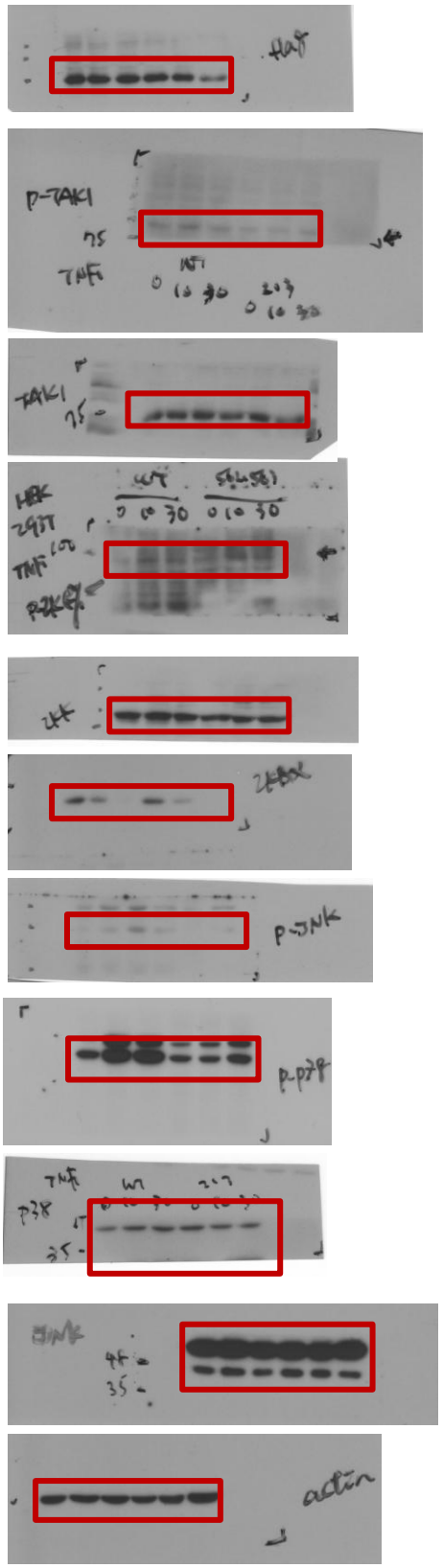
Suppl. Fig6A



Suppl. Fig6B



Suppl. Fig6C



Suppl. Fig7