

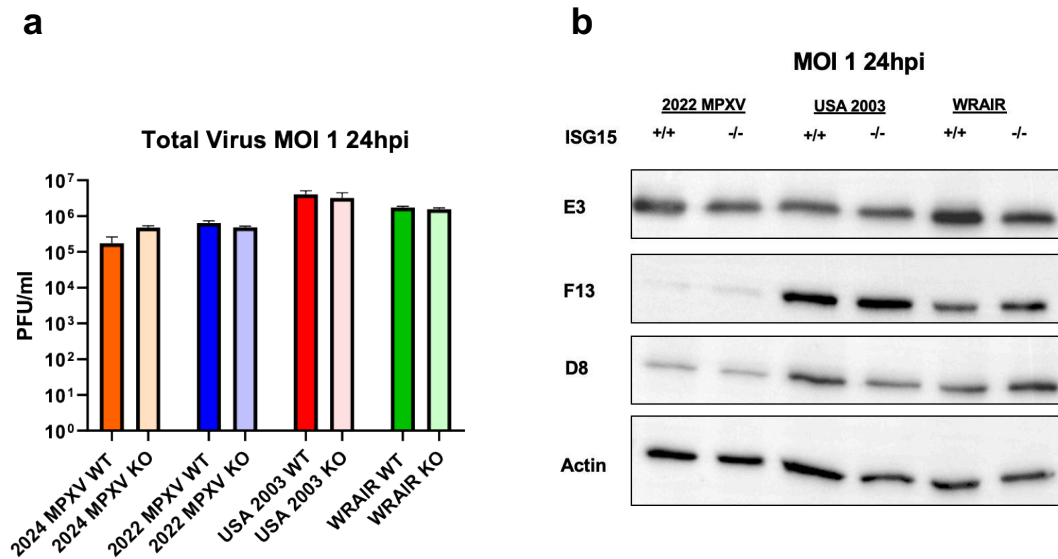
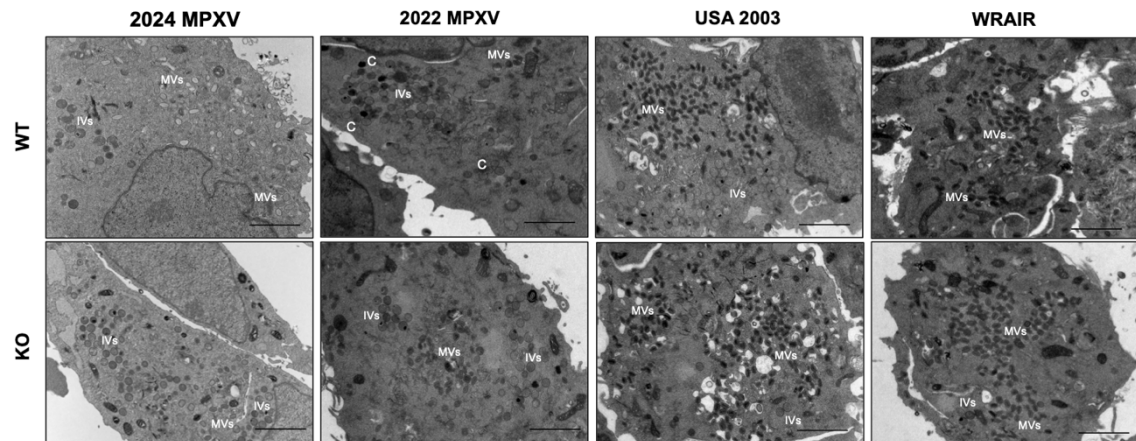
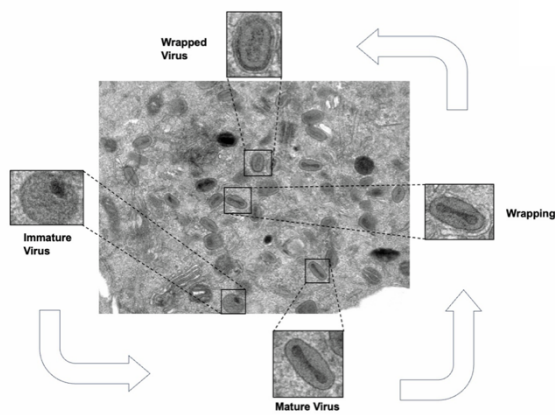


Figure S2. *High-dose MPXV infection overcomes the antiviral function of ISG15* (a) WT and ISG15-KO MEFs were infected with four MPXV strains at high MOI, and total virus was quantified by plaque assay at 24 hpi. Data represent mean $\pm$ SD from three independent experiments. (b) WT and ISG15-KO MEFs infected with Clade II MPXV strains (MOI 1, 24 hpi); equal amounts of cell lysates were analysed by immunoblotting with antibodies against VACV early protein E3, intermediate protein F13, and late protein D8.

a



b



c

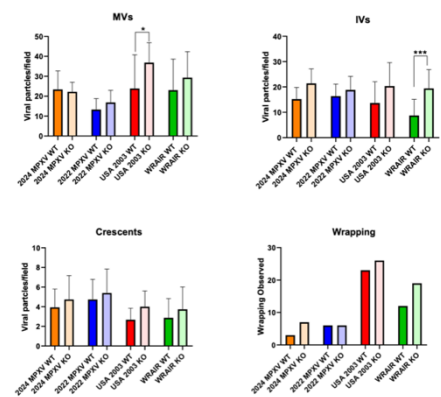


Figure S3. *ISG15* absence leads to accumulation of intracellular viral forms. (a) Representative TEM images of Clade Ib/II MPXV-infected WT and ISG15 KO MEFs (MOI 1, 21 hpi). Scale bar: 2 μ m. (b) TEM cell profile of a USA 2003 MPXV-infected ISG15 KO MEF showing individual viral forms: immature virus, mature virus, wrapping and wrapped virus. (c) Quantification of viral forms (mature virions (MV), immature virions (IV), crescents (C), Wrapping) in TEM cell profiles. Data from 15 cell profiles across three biological replicates.

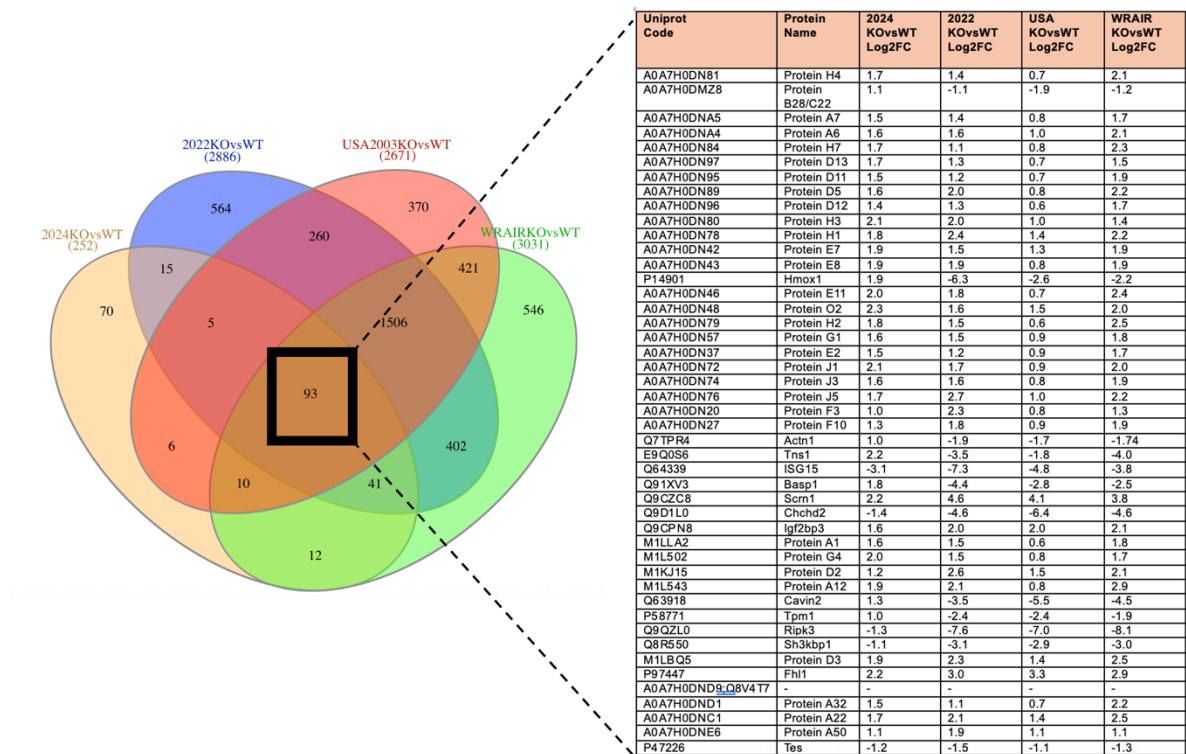
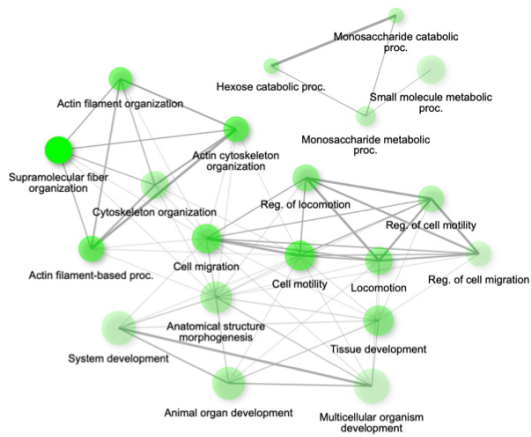
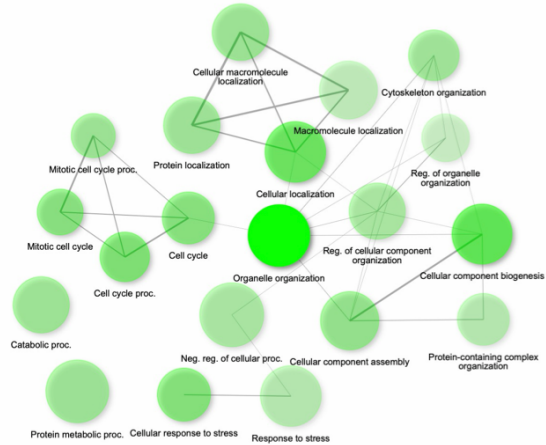


Figure S4. Venn Diagram displaying shared relevant differentially expressed proteins across all MPXVISG15 KO vs WT Infections. Table displays Uniprot code, Protein Name and FCs of every KO vs WT MPXV strain.

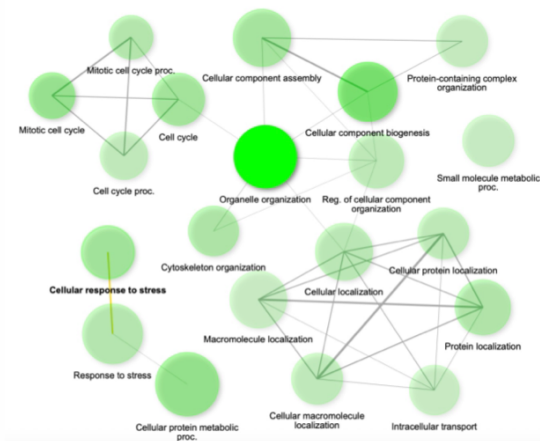
2024 MPXV



2022 MPXV



USA 2003



WRAIR

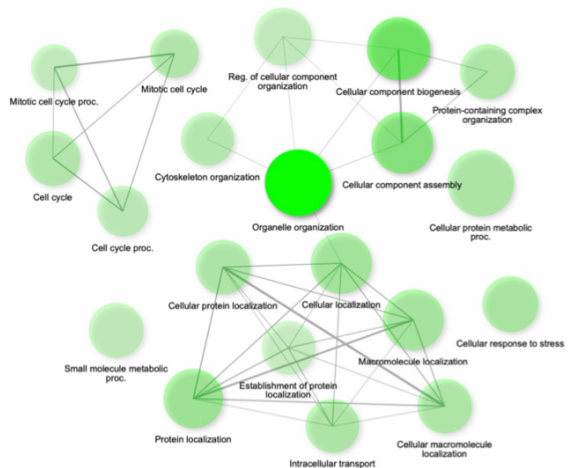


Figure S5 2024 MPXV presents reduced enrichment in biological processes during ISG15 absence compared to Clade II MPXV. Network graphs displaying the most enriched biological process pathways comparing ISG15 KO vs WT MPXV infected MEFs for each strain. n= 20 pathways. Data shown meet both $\log_2$ fold change and $-\log_{10}$ adjusted p-value cutoffs.

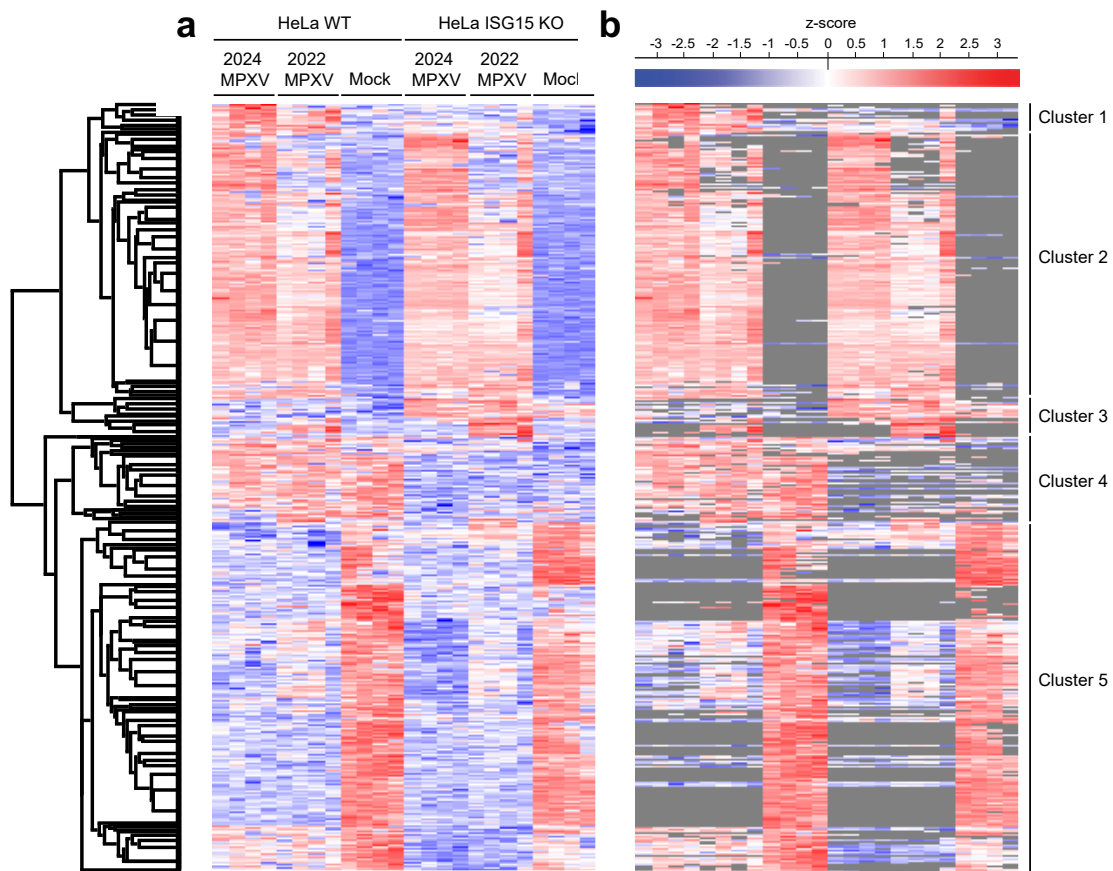


Figure S6. *Distinct ISGylation and ubiquitination signatures define the host response to MPXV infection across genotypes.* (a) Heatmap showing diGlyGly (K) sites after unsupervised hierarchical clustering (n=4 independent biological replicates). Different genotypes (WT and ISG15 KO) and infection (2024 MPXV, 2022 MPXV and Mock) are indicated. The colors represent upregulated (red) and downregulated (blue) sites. (b) the same heatmap is shown with the originally missing values depicted in gray. Five major clusters are indicated, ISG15 sites upregulated in MPXV infection (Cluster 1), UBQ sites upregulated in MPXV infection (Cluster 2), UBQ sites upregulated in KO (Cluster 3), ISG15 sites upregulated in mock and infection conditions (Cluster 4) and UBQ sites downregulated in infection (Cluster 5).

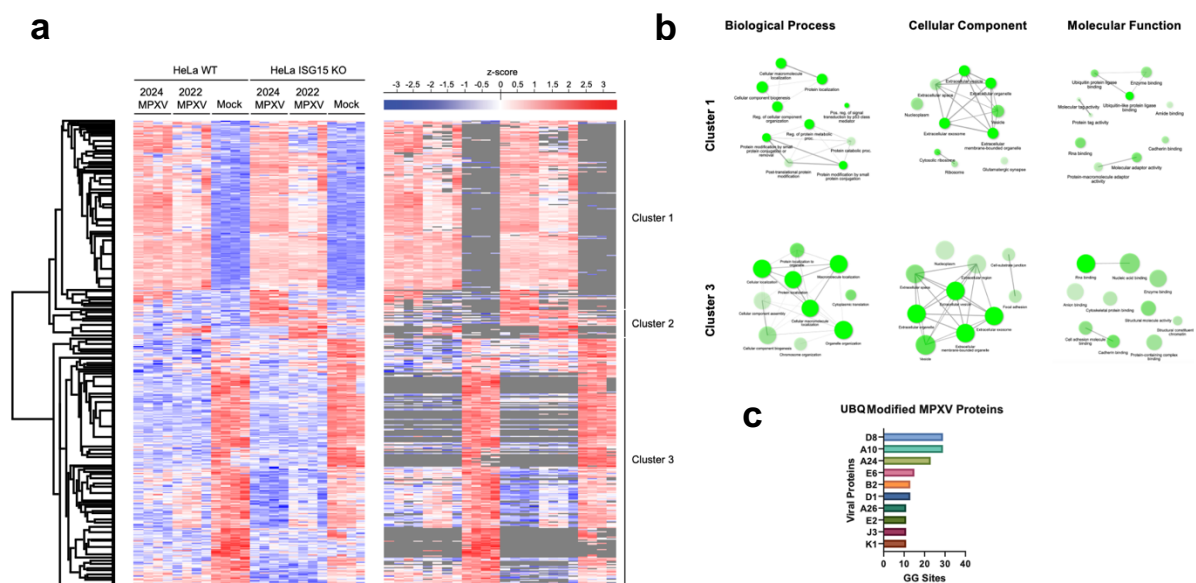


Figure S7. Gene ontology analysis of ubiquitin sites altered during MPXV infection. (a) Left, heatmap showing UBQ sites after unsupervised hierarchical clustering ($n=4$ independent biological replicates). Different genotypes (WT and ISG15 KO) and infection (2024 MPXV, 2022 MPXV and Mock) are indicated. The colors represent upregulated (red) and downregulated (blue) sites. Right, the same heatmap is shown with the originally missing values depicted in gray. Three major clusters are indicated, one that contains UBQ sites upregulated in MPXV infection (Cluster 1), UBQ sites upregulated in KO MPXV infection (Cluster 2) and UBQ downregulated sites in infection (Cluster 3). (b) Biological process, cellular component and molecular function pathway analysis of Cluster 1 and 3. (c) Identification of MPXV viral proteins with most UBQ modified sites.

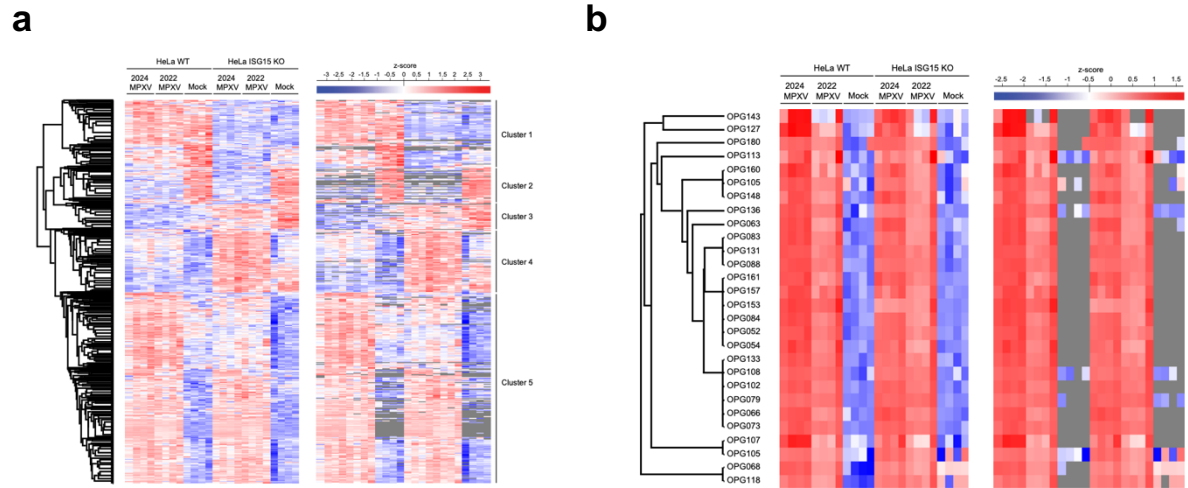


Figure S8. Verification of ISGylated viral proteins through shotgun analysis. (a) Heatmap showing significantly regulated proteins identified by shotgun proteomics in wild-type (WT) and ISG15 knockout (KO) HeLa cells. Unsupervised hierarchical clustering was performed across all samples and conditions. Different genotypes (WT and ISG15 KO) and infection (2024 MPXV, 2022 MPXV and Mock) are indicated. Protein expression changes are represented by color intensity (red:upregulated; blue: downregulated). The right panel displays the same heatmap with missing values shown in gray. Five main clusters are indicated, proteins increased in WT (Cluster 1), proteins decreased in infection (Cluster 2), proteins increased in KO (Cluster 3), proteins increased in KO infection (Cluster 4) and proteins increased in infection (Cluster 5). (b) Left, heatmap showing ISGylated MPXV proteins, selected from (c) to show only ISGylated viral proteins after unsupervised hierarchical clustering (n = 4 biologically independent replicates). The colors represent upregulated (red) and downregulated (blue) proteins. Right, the same heatmap is shown with the originally missing values depicted in gray.

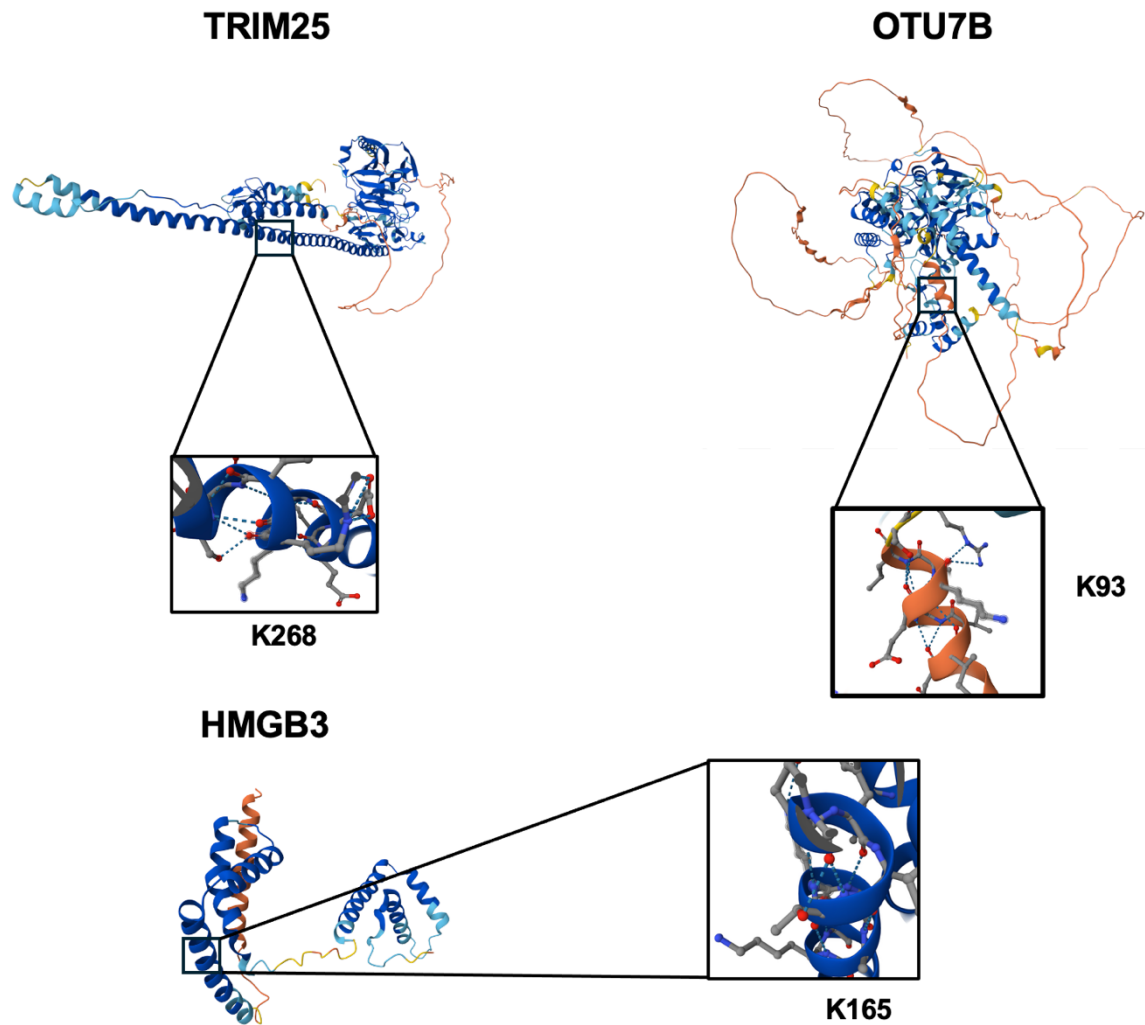


Fig S9. *Analysis of ISGylated cellular proteins.* Structure and sites of releant ISGylated cellular proteins during 2024 and 2022 MPXV infection using AlphaFold models. TRIM25 (AF-Q14258-F1-v6), OTUD7b (AF-Q6GQQ9-F1-v6), HMGB3 (AF-O15347-F1-v6).

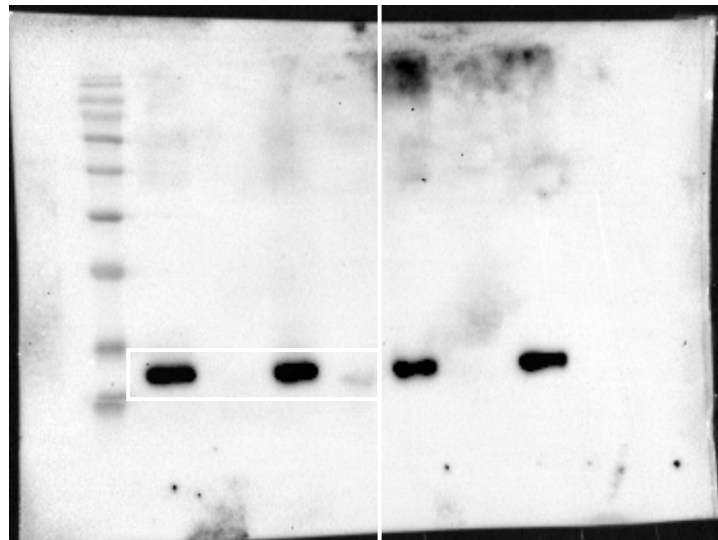
Original Western Blot Images

Protein ladder bands (Top to bottom, kDa): 180, 130, 100, 75 (Red band), 63, 48, 35, 28 (Green Band), 17, 10.

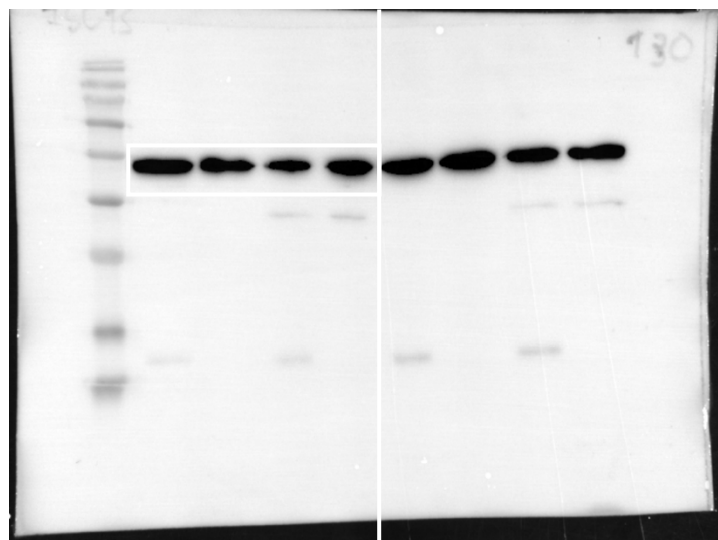
White line delimits lanes that were included in manuscript, and white rectangle indicates protein signal obtained with antibody.

Fig 1 a

Order (left to right lane): Protein Ladder, Mock WT MEFs, Mock ISG15 KO MEFs, 2022 MPXV WT MEFs, 2022 MPXV ISG15 KO MEFs, IFN Treated Mock WT MEFs, IFN Treated Mock ISG15 KO MEFs, IFN Treated 2022 MPXV WT MEFs, IFN Treated 2022 MPXV ISG15 KO MEFs.



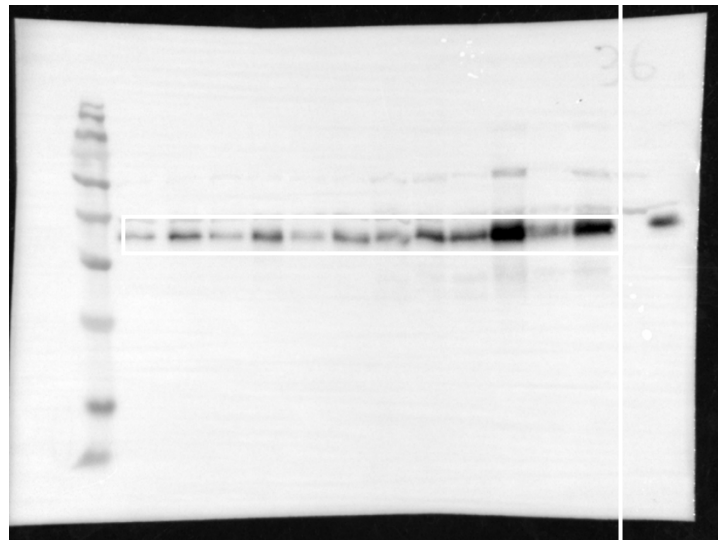
Murine ISG15 antibody (15 kDa)



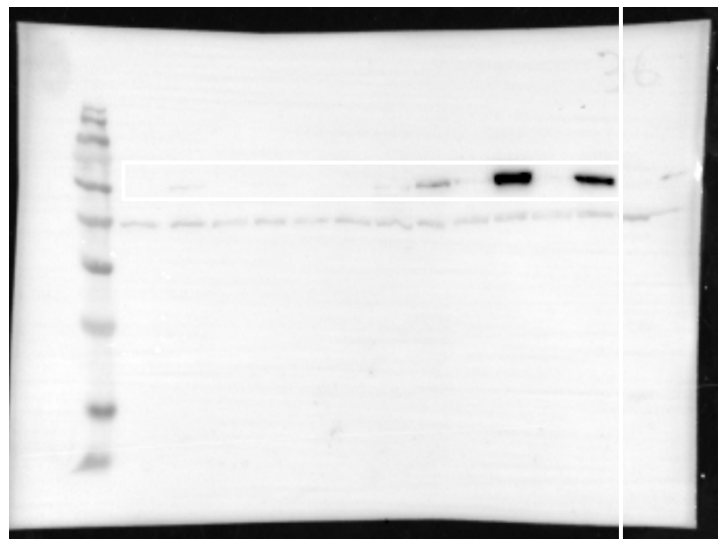
Actin antibody (45 kDa)

Fig 1 d

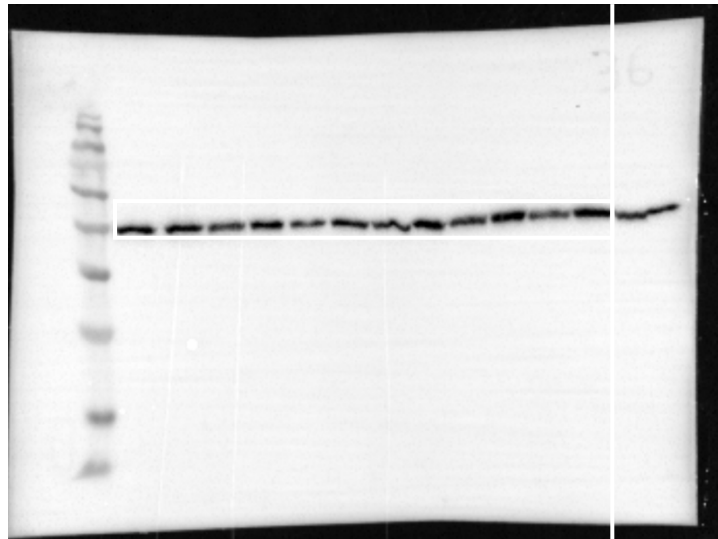
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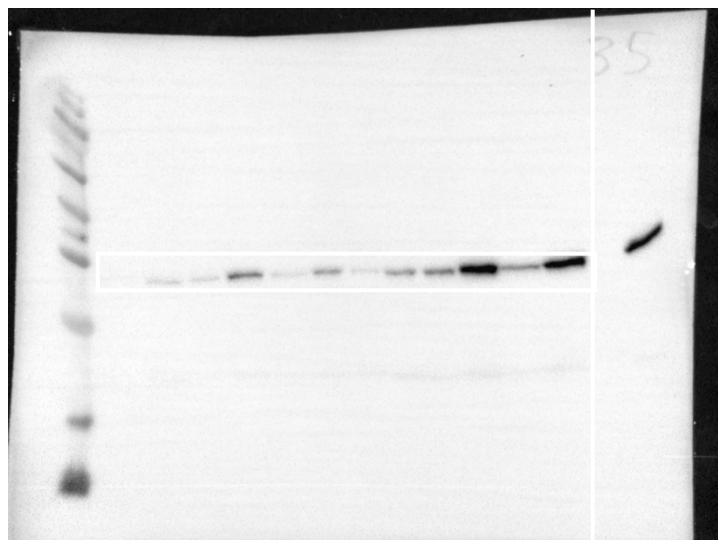
A4 antibody (39 kDa)



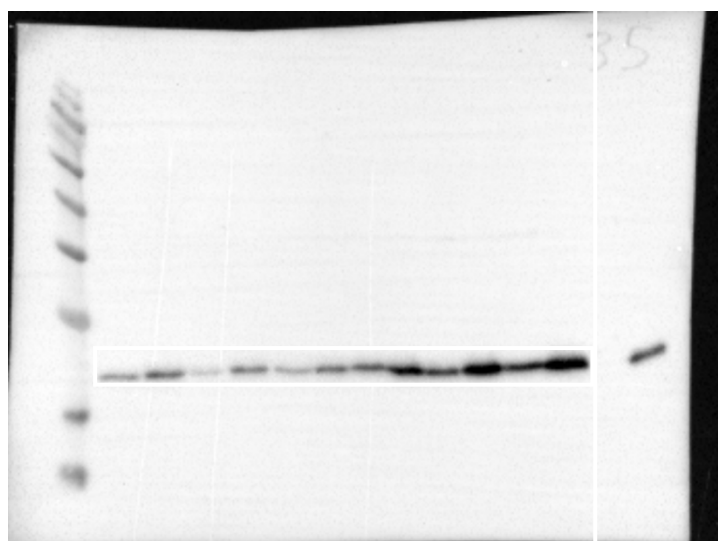
A26 antibody (58 kDa)



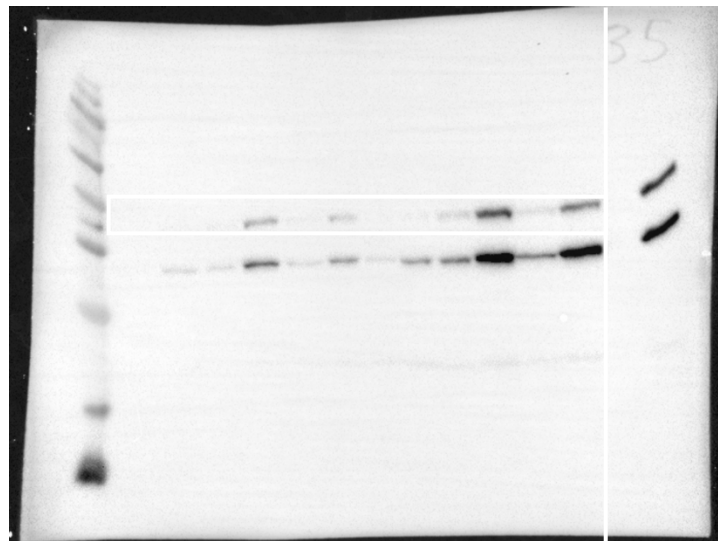
Actin antibody (45 kDa)



D8 antibody (32 kDa)



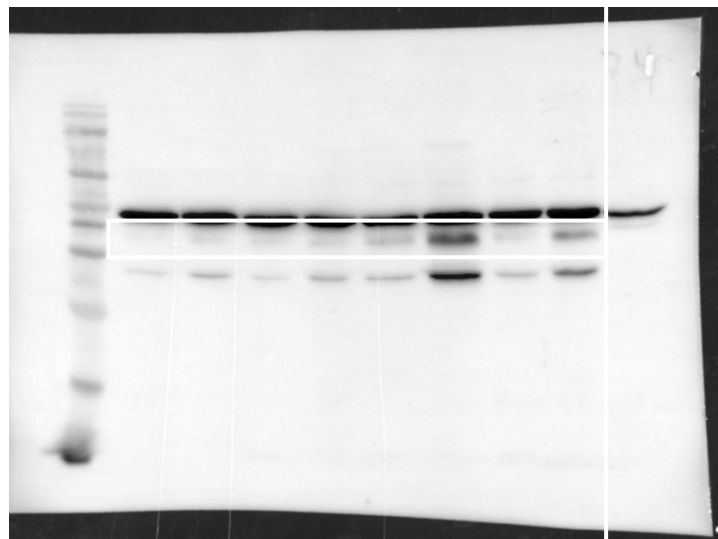
E3 antibody (17 kDa)



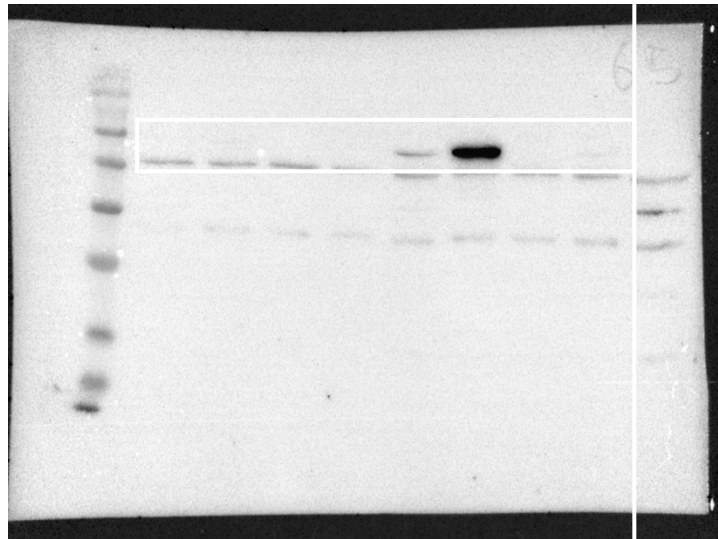
F13 antibody (42 kDa)

Fig 1 e

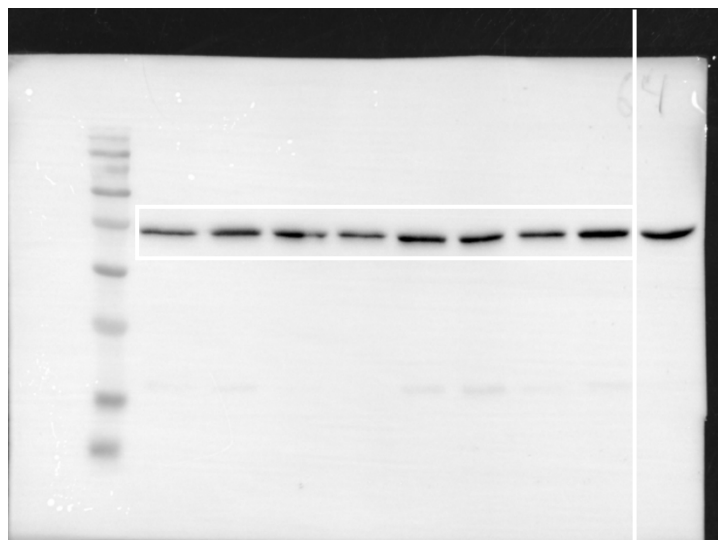
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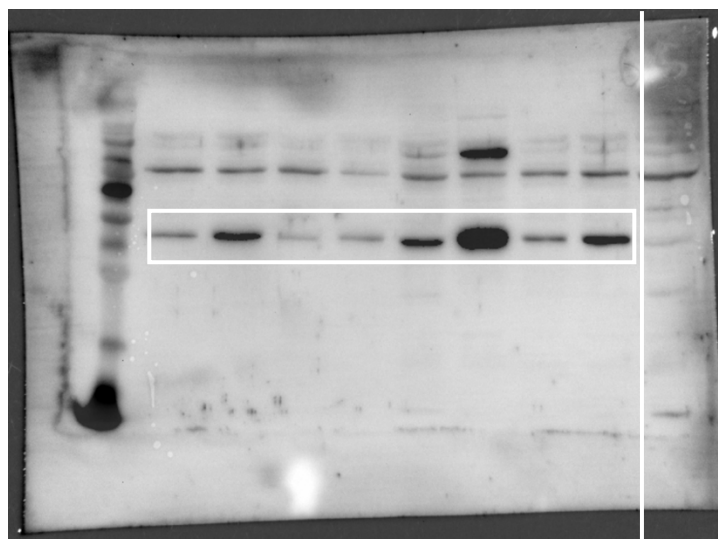
A4 antibody (39 kDa)



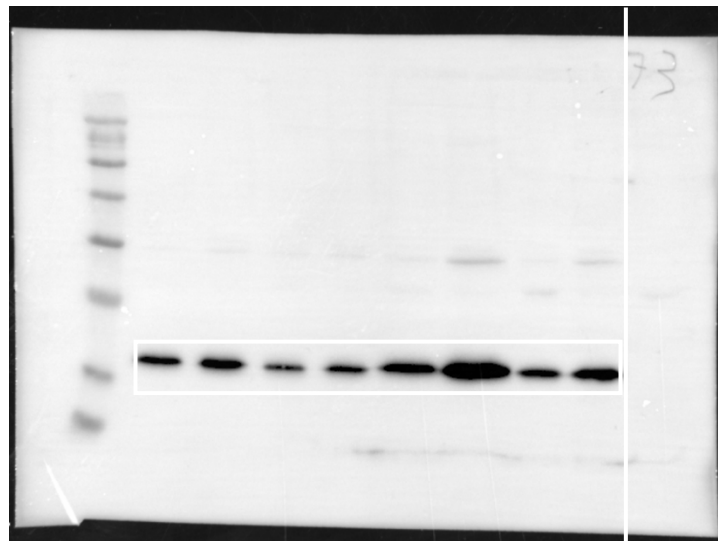
A26 antibody (58 kDa)



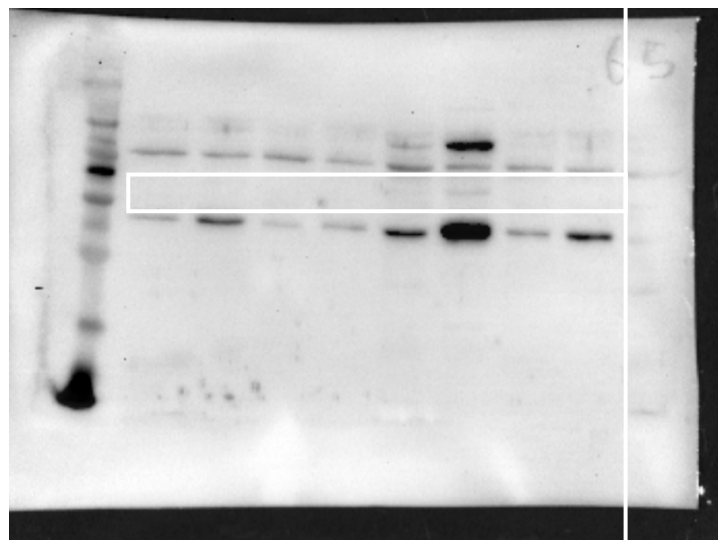
Actin antibody (45 kDa)



D8 antibody (32 kDa)



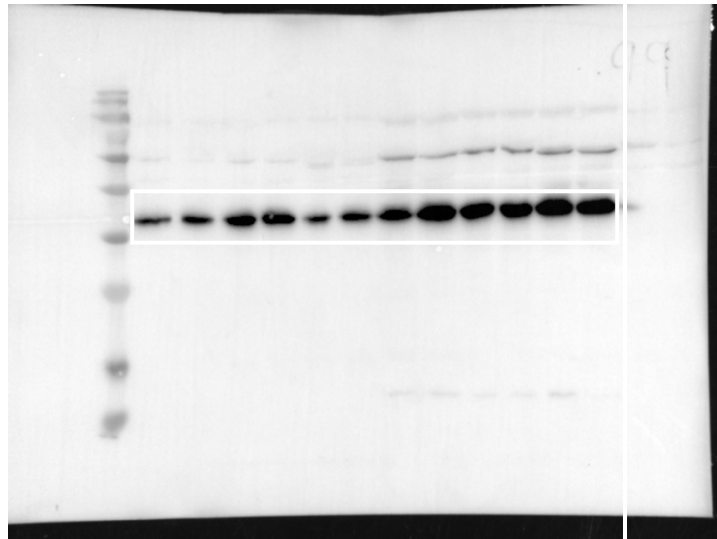
E3 antibody (17 kDa)



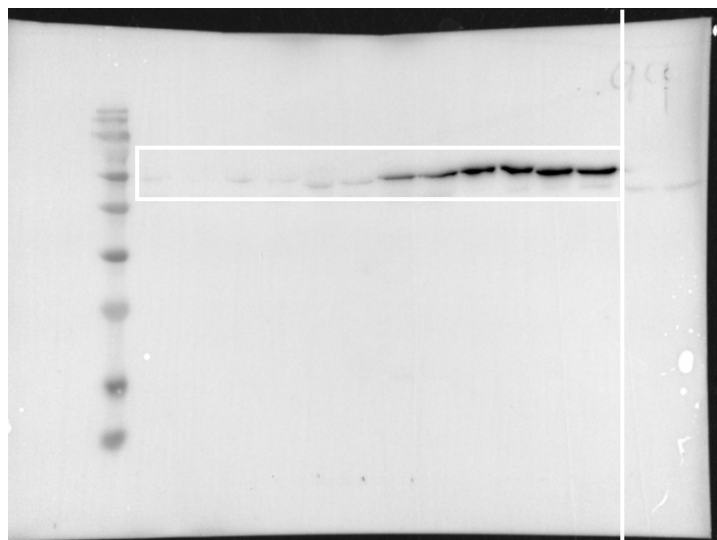
F13 antibody (42 kDa)

Fig 3 a

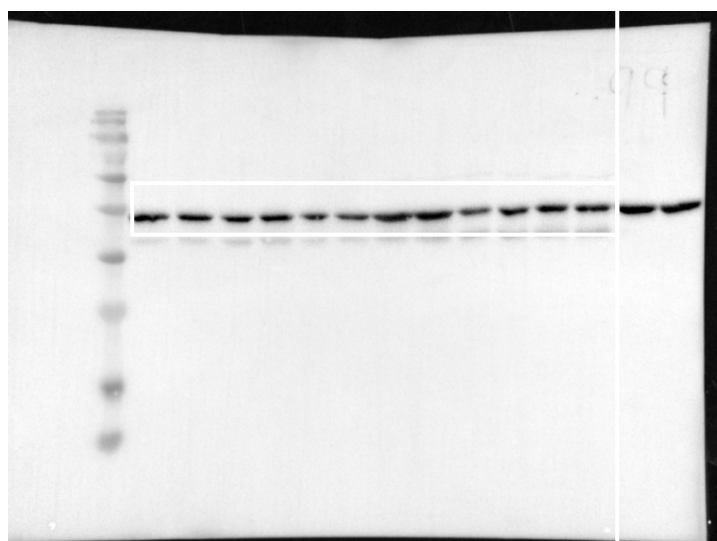
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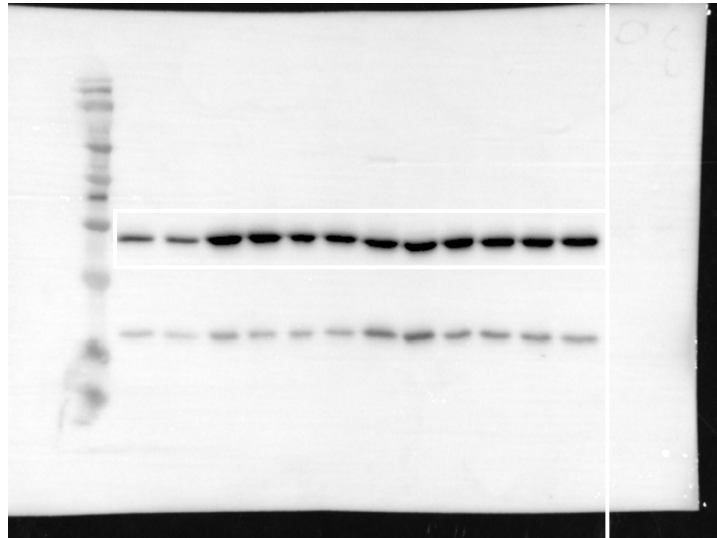
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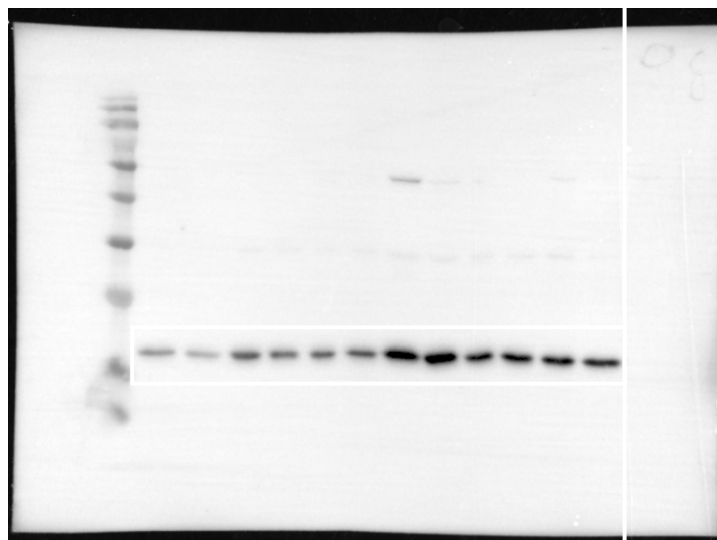
A26 antibody (58 kDa)



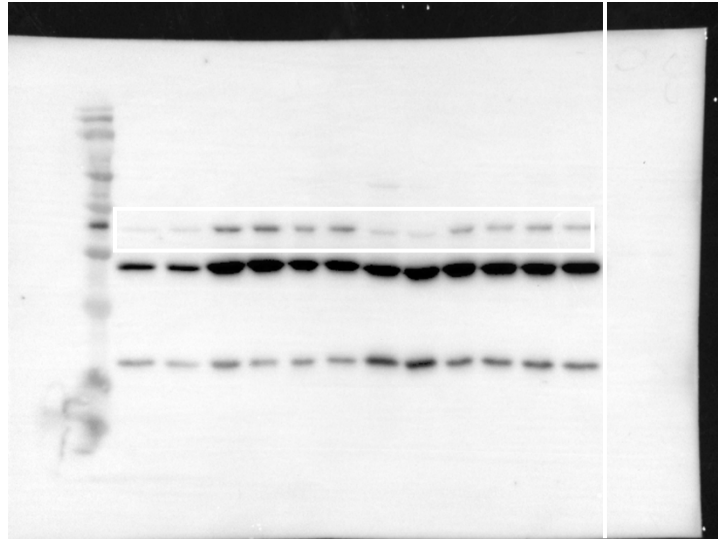
Actin antibody (45 kDa)



D8 antibody (32 kDa)



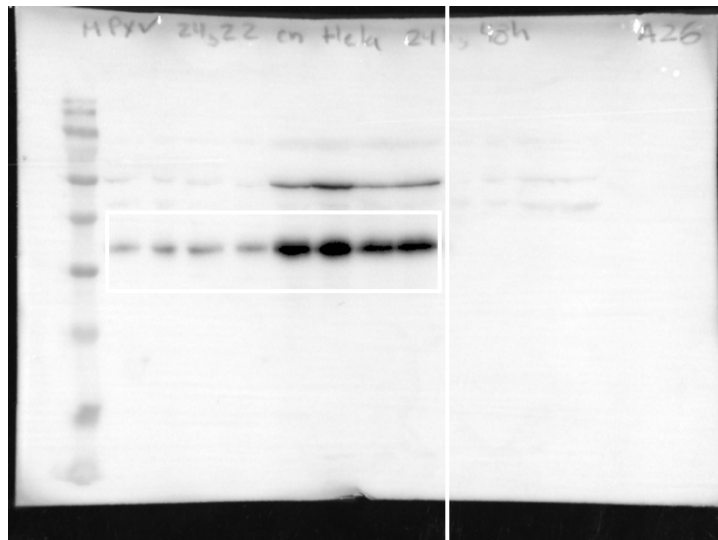
E3 antibody (17 kDa)



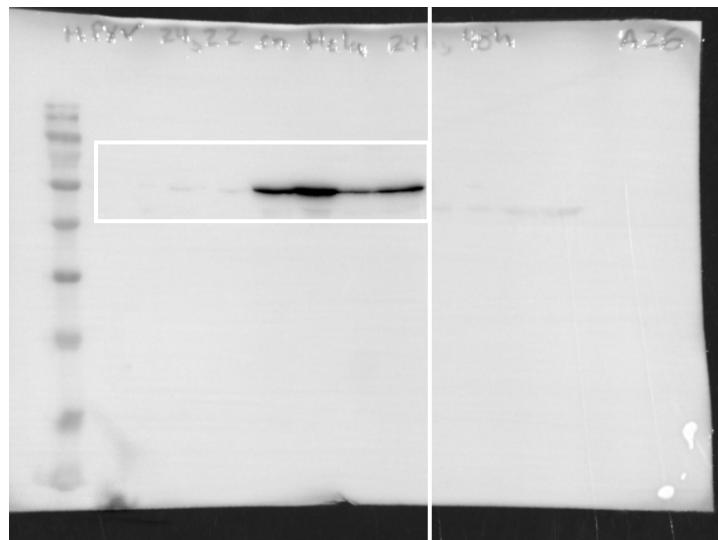
F13 antibody (42 kDa)

Fig 3 b

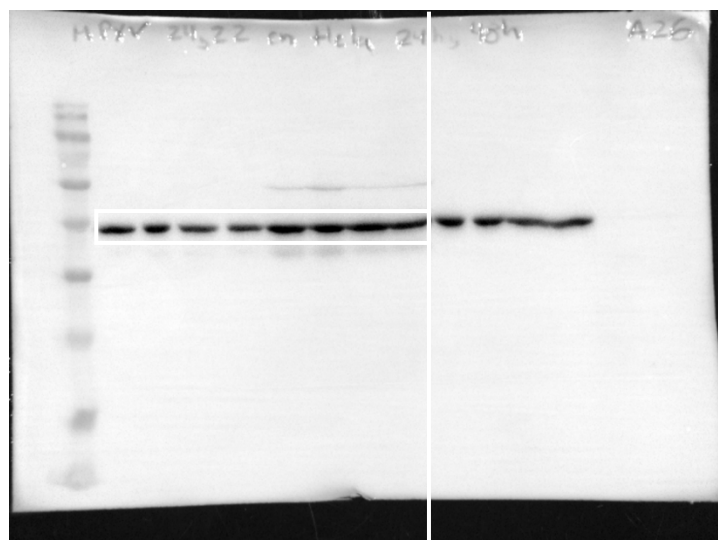
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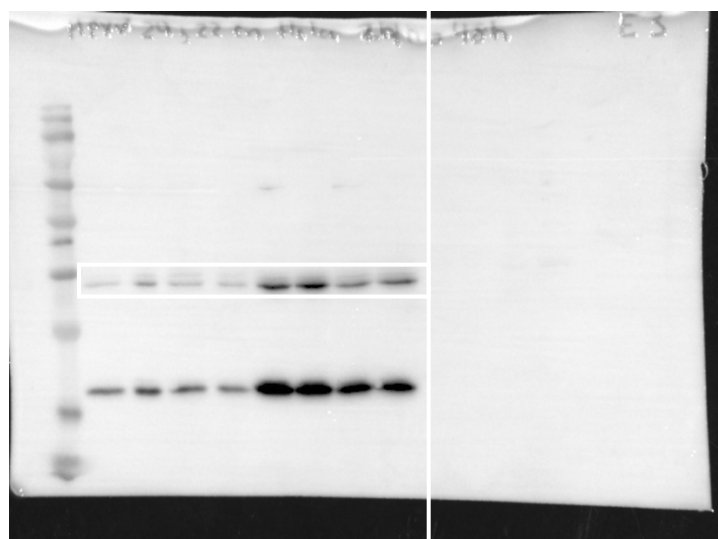
A4 antibody (39 kDa)



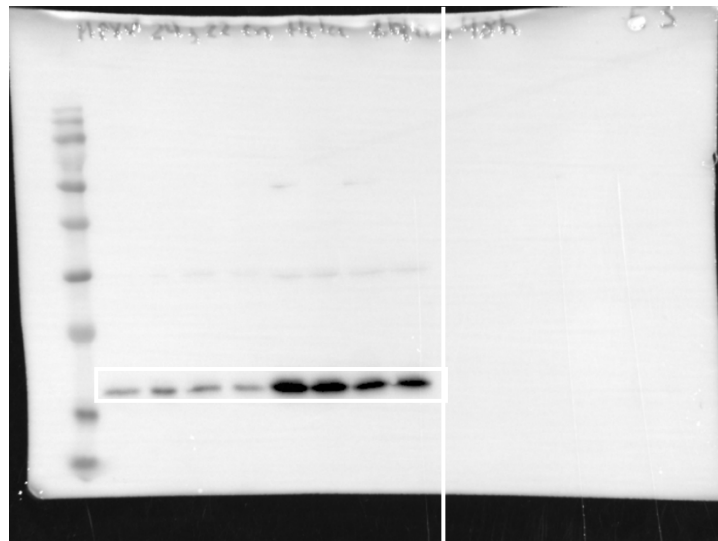
A26 antibody (58 kDa)



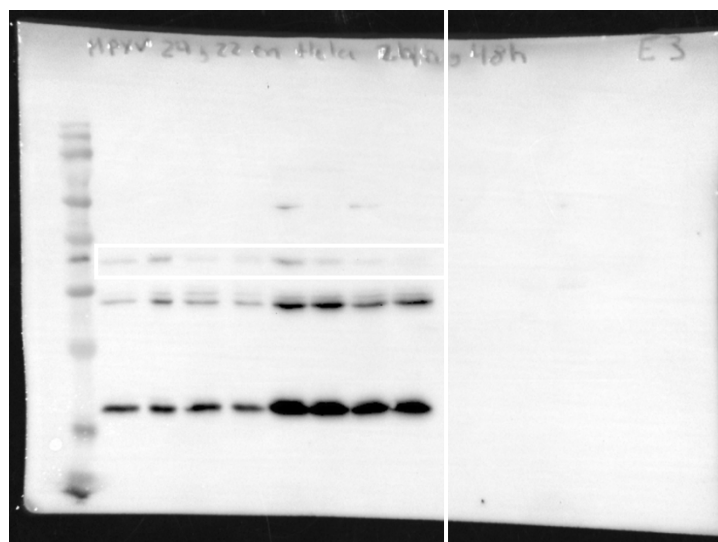
Actin antibody (45 kDa)



D8 antibody (32 kDa)



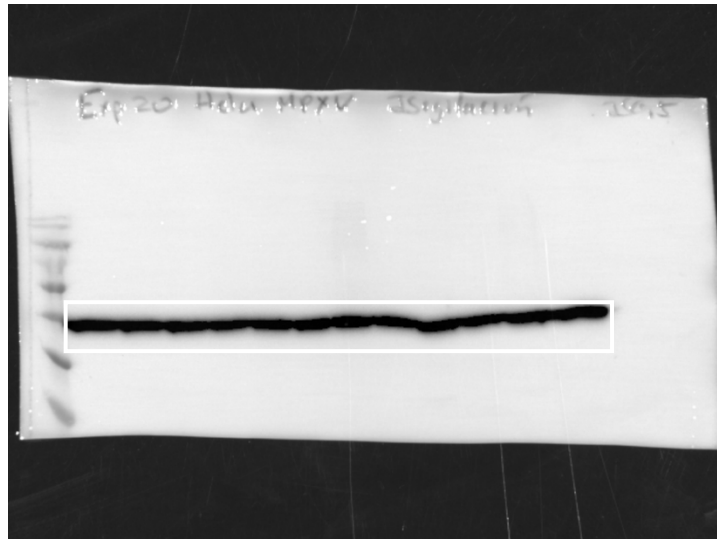
E3 antibody (17 kDa)



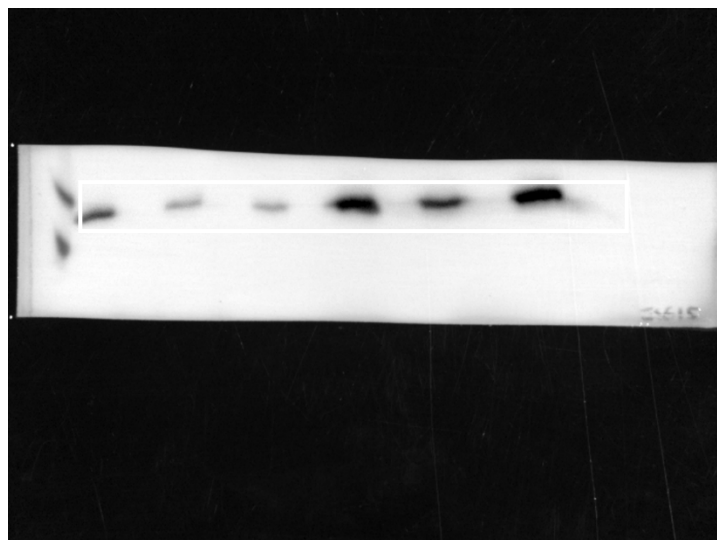
F13 antibody (42 kDa)

Fig 4 a

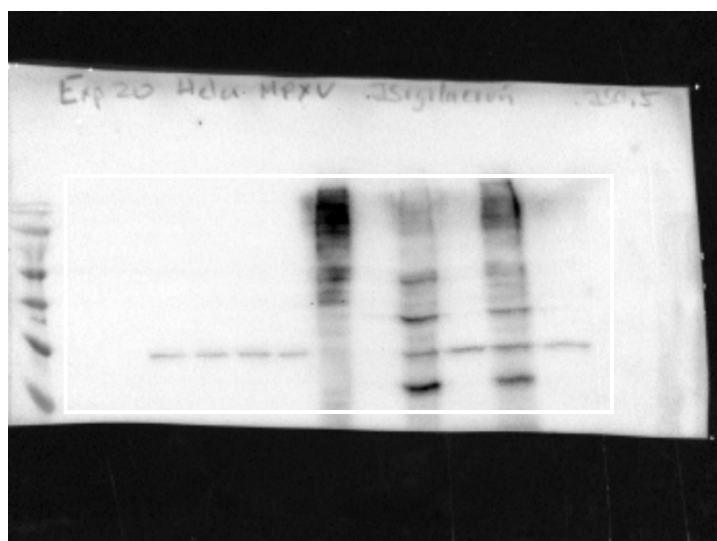
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Actin antibody (45 kDa)



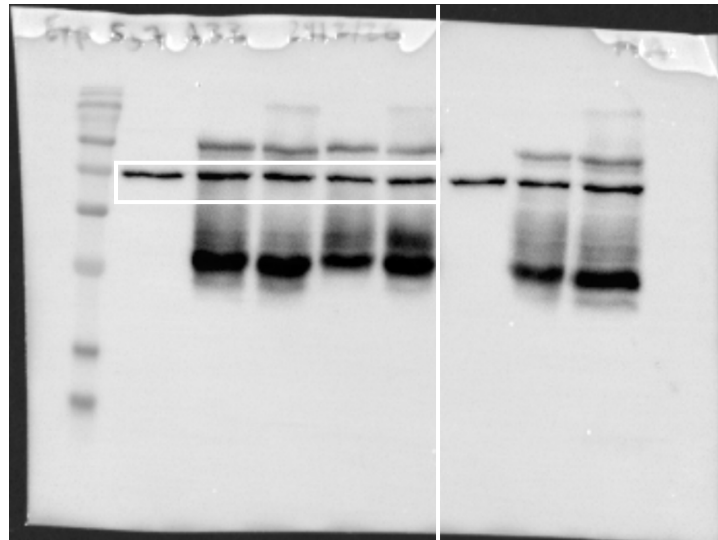
Human ISG15 antibody (15 kDa)



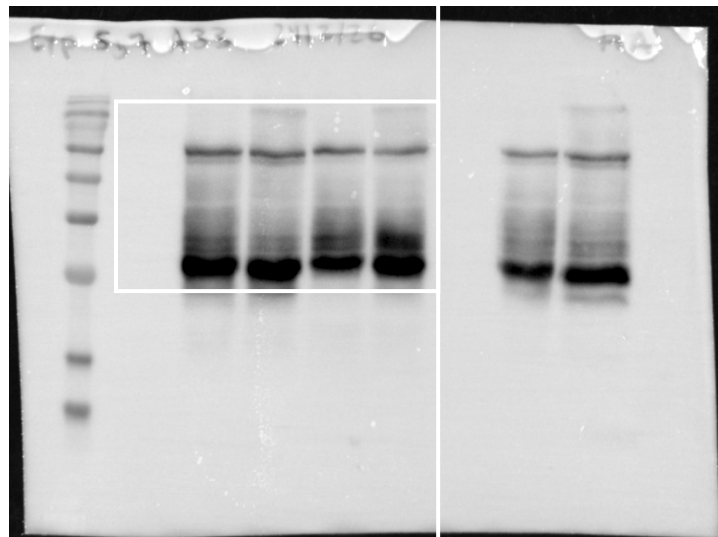
Human ISG15 antibody (ISGylation bands)

Fig 5 c

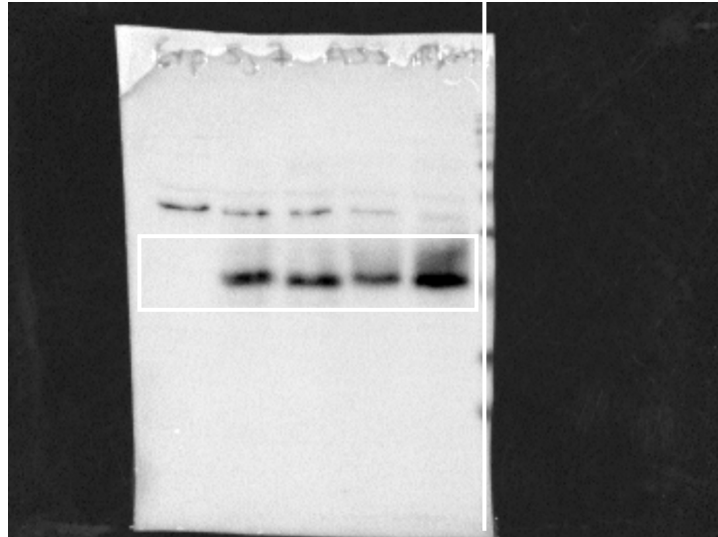
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Actin antibody (45 kDa)



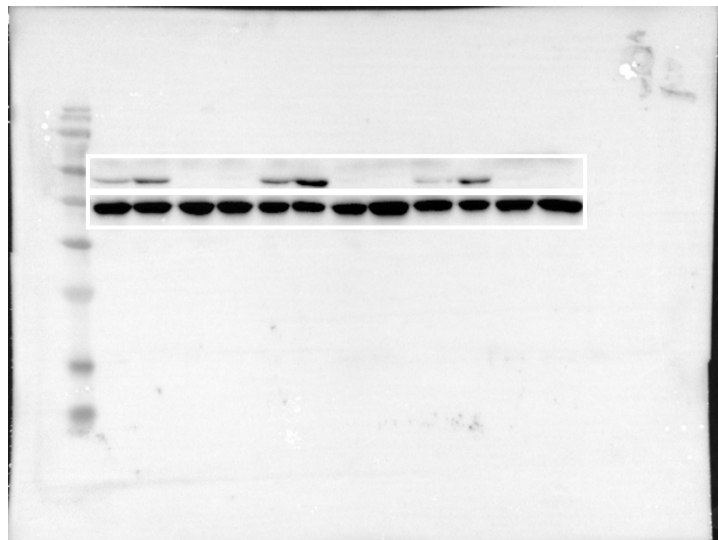
HA antibody



WR antibody

Fig S1c

Order (left to right lane): Protein ladder, MEFs WT IHD-J 24hpi, MEFs KO IHD-J 24hpi, MEFs WT Mock 24hpi, MEFs KO 24hpi, MEFs WT IHD-J 48hpi, MEFs KO IHD-J 48hpi, MEFs WT Mock 48hpi, MEFs KO Mock 48hpi, MEFs WT IHD-J 48hpi CX 24hpt, MEFs KO IHD-J 48hpi CX 24hpt, MEFs WT Mock 48hpi CX 24hpt, MEFs KO Mock 48hpi CX 24hpt.



Actin (45 kDa)/A26 (58 kDa)

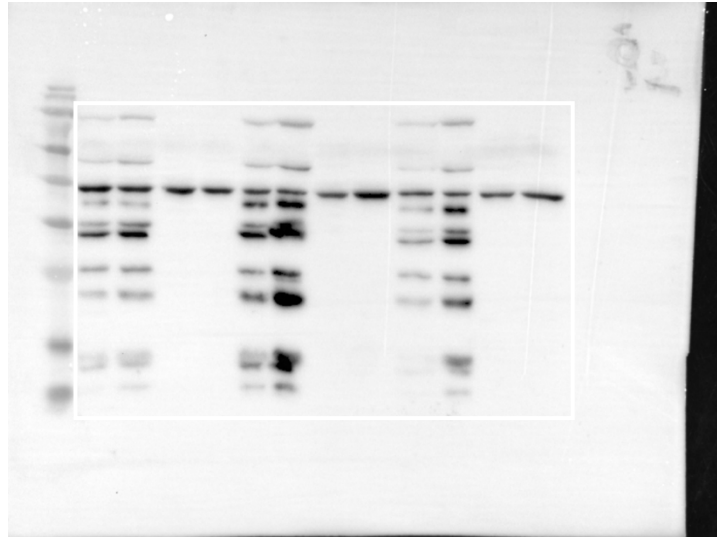
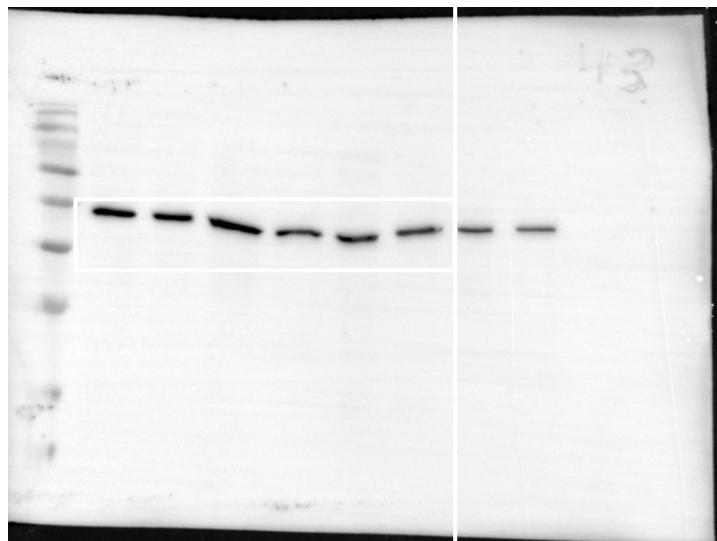
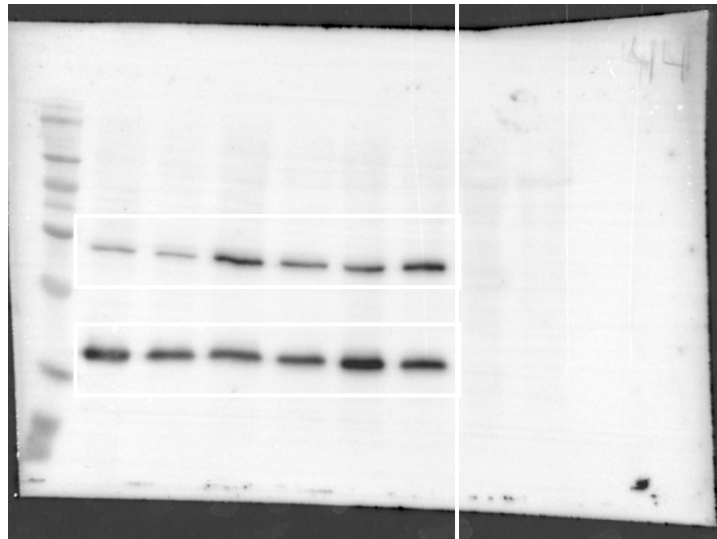


Fig S2b

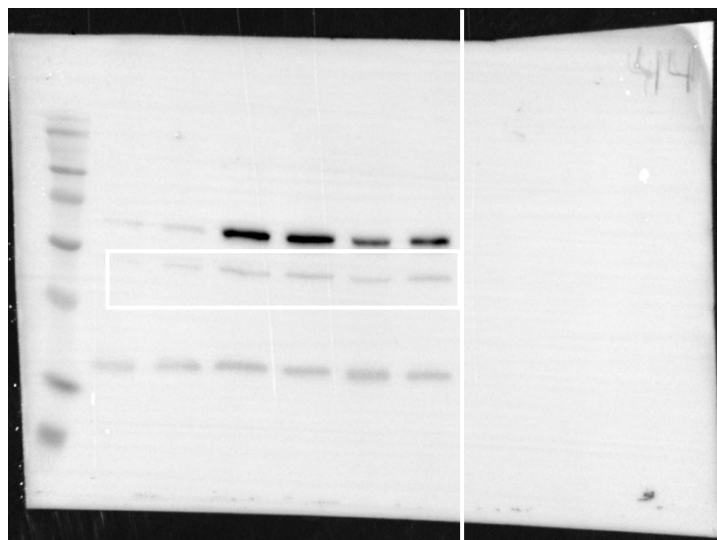
Order (left to right lane): Protein ladder, 2022 MPXV MEFs WT, 2022 MPXV MEFs KO, USA 2003 MEFs WT, USA 2003 MEFs KO, WRAIR MEFs WT, WRAIR MEFs KO (MOI 1), Mock MEFs WT, Mock MEFs KO.



Actin antibody



D8 (32 kDa)/ E3 (17 kDa) antibody



F13 (42 kDa) antibody