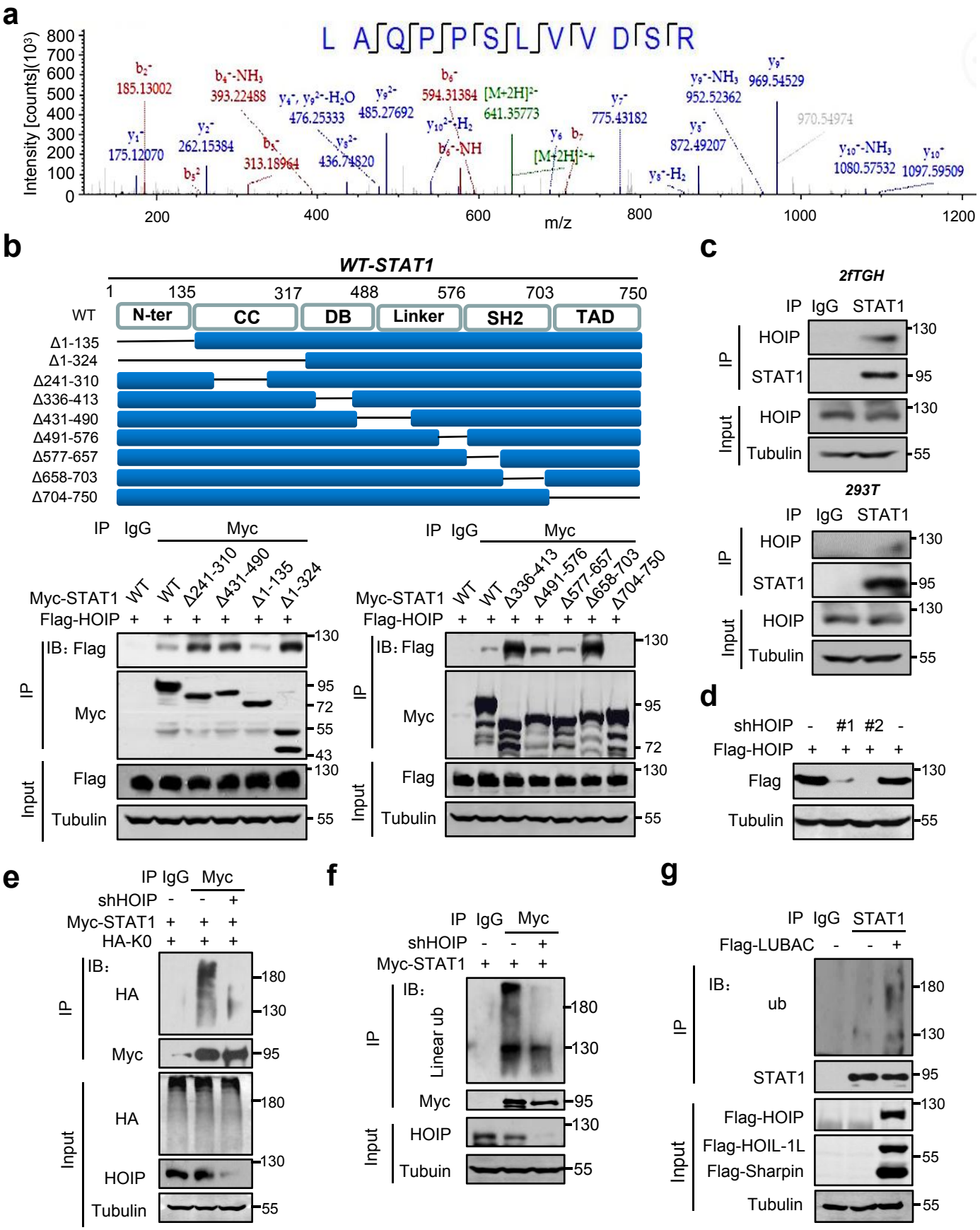
**Supplementary Information**

# Regulation of the linear ubiquitination of STAT1 controls antiviral interferon signaling

Zuo et al.

# Supplementary Figure 1

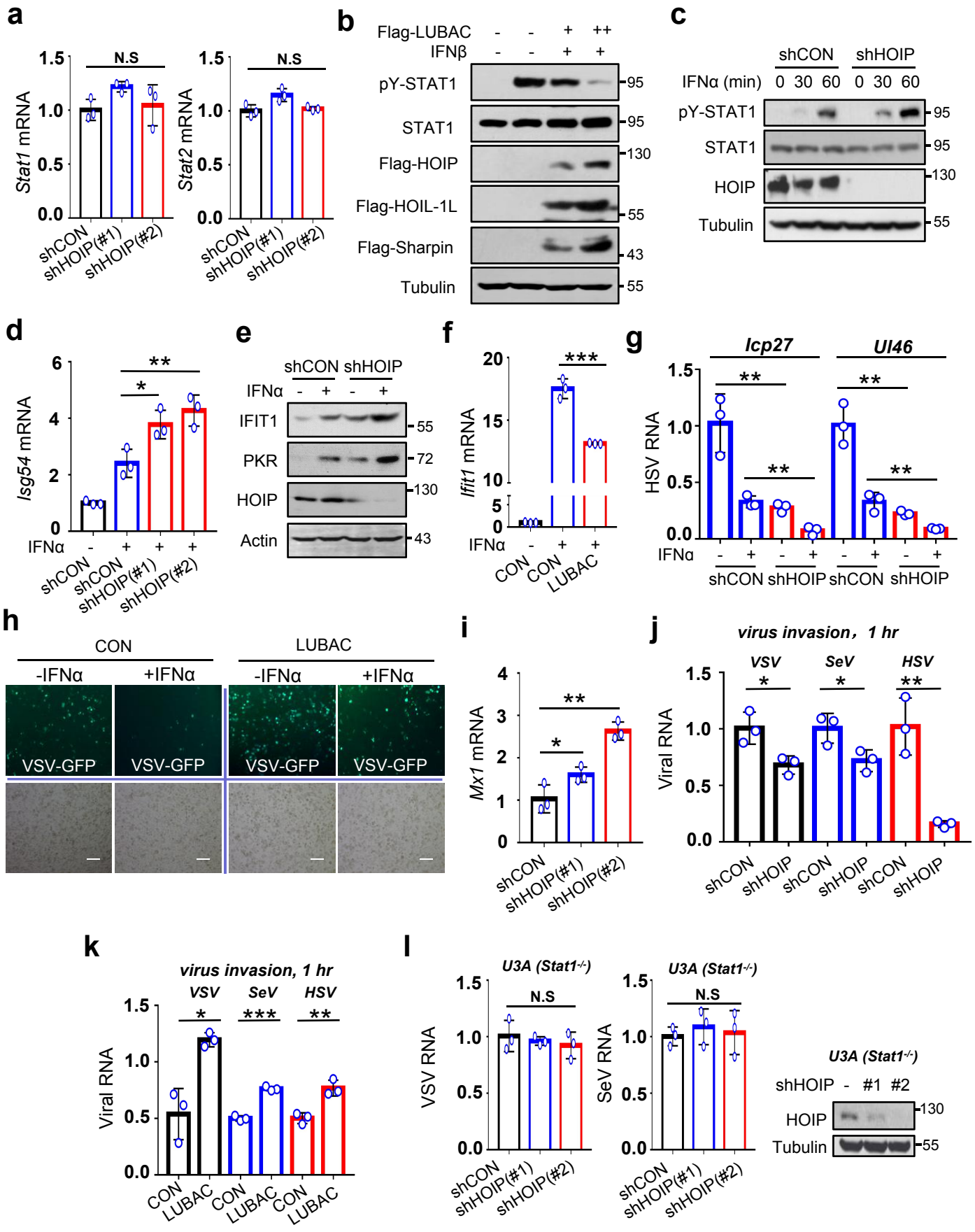


**Supplementary Figure 1**

**STAT1 has linear ubiquitination mediated by HOIP.**

**(a)** Mass spectrometry analysis of the HOIP protein interacting with STAT1. **(b)** Immunoprecipitation (IP) and immunoblotting (IB) analysis of the interaction between Flag-HOIP and Myc-STAT1(WT) or its different deletion mutants (Δ1-135, Δ1-324, Δ241-310, Δ336-413, Δ431-490, Δ491-576, Δ577-657, Δ658-703, Δ704-750) in HEK293T cells. **(c)** Immunoprecipitation analysis of the interaction between endogenous HOIP and STAT1 in 2fTGH and HEK293T cells. **(d)** Western blot analysis of Flag-HOIP protein levels in HEK293T cells cotransfected with shHOIP (#1, #2) and Flag-HOIP. **(e)** Immunoprecipitation analysis of ubiquitination of STAT1 in HEK293T cells cotransfected with Myc-STAT1, shHOIP and HA-Ub-K0 (HA-K0, all lysines on Ub are mutated to arginine). **(f)** Immunoprecipitation analysis of linear ubiquitination of STAT1 in HEK293T cells cotransfected with shHOIP and Myc-STAT1. **(g)** Immunoprecipitation analysis of ubiquitination of STAT1 in HEK293T cells transfected with or without Flag-LUBAC. Data are representative of three independent experiments **(b-g)**.

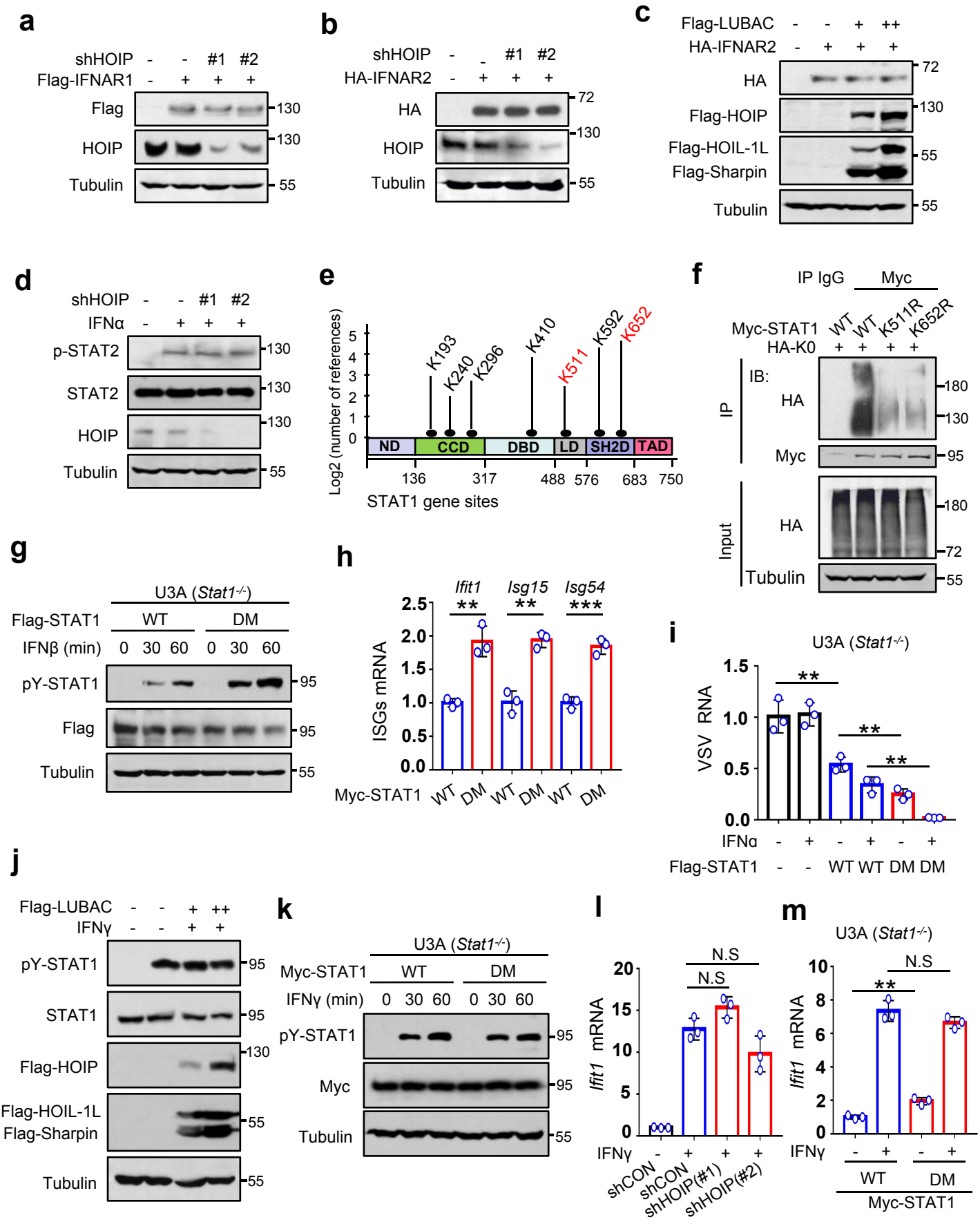
# Supplementary Figure 2



**Supplementary Figure 2**

**Linear ubiquitination sustains STAT1 signaling homeostasis.**
**(a)** RT-qPCR analysis of *Stat1* and *Stat2* mRNA in HEK293T cells transfected with control shRNAs (shCON) or shHOIP (#1, #2). **(b)** Western blot analysis of pY701-STAT1 (pY-STAT1) levels in HEK293T cells transfected with increasing amounts of Flag-LUBAC, and then treated with IFNβ (1,000 IU/ml) for 30 min. **(C)** Western blot analysis of pY-STAT1 in HeLa cells transfected with shHOIP and then treated with IFNα (1,000 IU/ml) for the indicated times. **(d)** RT-qPCR analysis of *Isg54* mRNA in HEK293T cells transfected with either shCON or shHOIP (#1, #2) and then stimulated with IFNα (1,000 IU/ml) for 4 hrs. **(e)** Western blot analysis of the representative ISGs proteins (IFIT1 and PKR) in HEK293T cells transfected with shHOIP and then treated with IFNα (500 IU/ml) for 24 hrs. **(f)** RT-qPCR analysis of *Ifit1* mRNA in HEK293T cells transfected with vectors (CON) or LUBAC-expressing constructs and then stimulated with IFNα (1,000 IU/ml) for 4 hrs. **(g)** RT-qPCR analysis of HSV viral *Icp27* and *Ul46* RNA in 2fTGH cells transfected with shHOIP and then treated with IFNα (60 IU/ml) for 20 hrs, followed by infection with HSV (MOI=1.0) for 24 hrs. **(h)** Fluorescence microscopy of VSV-GFP in A549 cells transfected with empty vectors (CON) or Flag-LUBAC and then infected by VSV-GFP (MOI=0.1) for 24 hrs. Scale bar: 100 μm. **(i)** RT-qPCR analysis of a representative ISG (*Mx1*) mRNA in HEK293T cells transfected with shHOIP (#1, #2). **(j, k)** RT-qPCR analysis of viral RNA in 2fTGH cells transfected with either shHOIP **(j)** or Flag-LUBAC **(k)** and then infected by VSV (MOI=0.1), SeV (MOI=1.0) or HSV (MOI=1.0) for 1 hr to observe virus invasion. **(l)** RT-qPCR analysis of viral RNA in U3A cells transfected with shHOIP (#1, #2) and then infected by VSV (MOI=0.1) or SeV (MOI=1.0) for 1 hr. HOIP knockdown was analyzed by Western blot. N.S, not significant ($p>0.05$) and *$p<0.05$, **$p<0.01$, ***$p<0.001$ (two-tailed unpaired Student's *t*-test). Data are shown as mean and s.d. of three biological replicates **(a, d, f, g, i-l)**, or are representative of three independent experiments **(b, c, e, h)**.

# Supplementary Figure 3

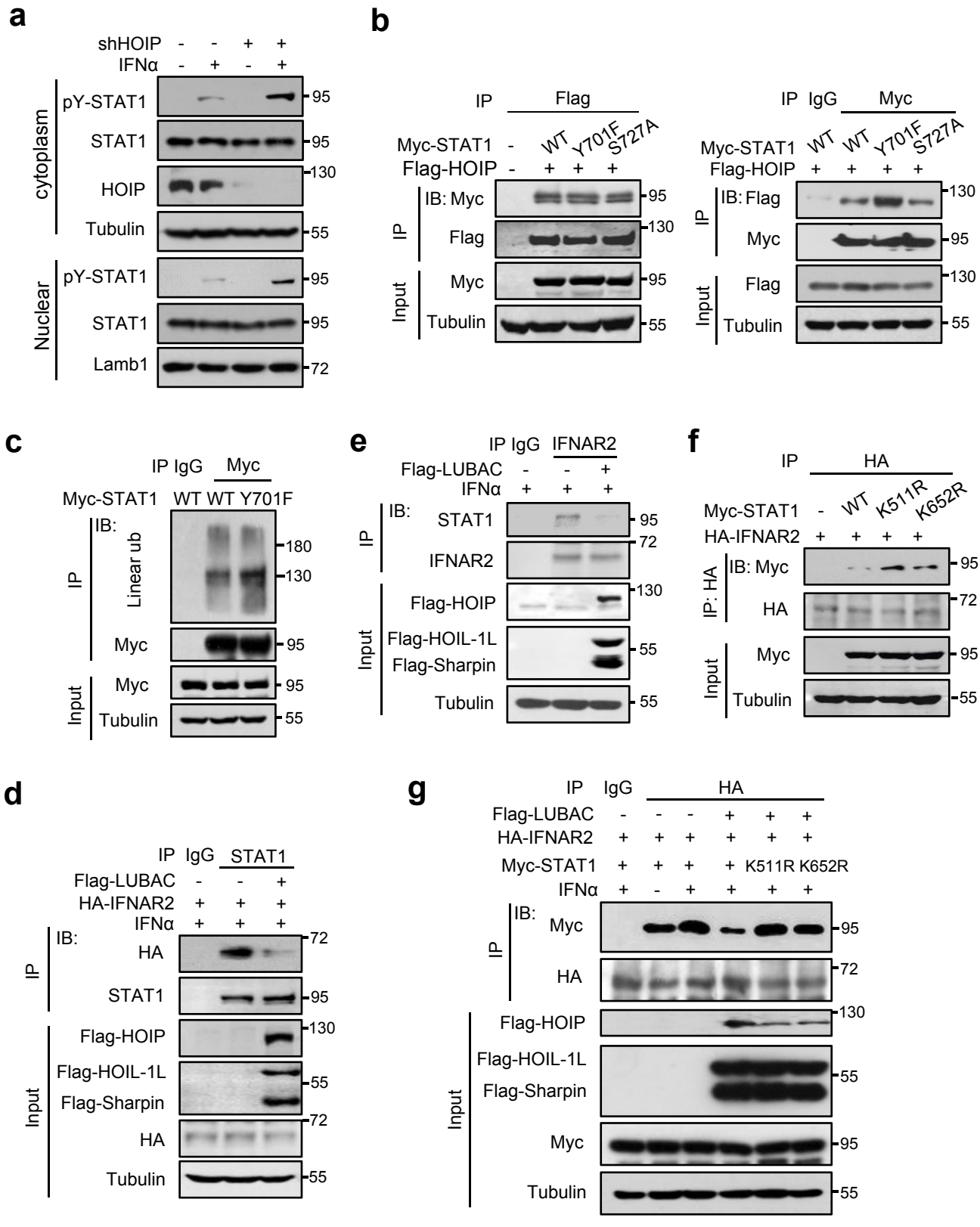


**Supplementary Figure 3**

**LUBAC induces STAT1 linear ubiquitination at Lys511/652.**

**(a)** Western blot analysis of Flag-IFNAR1 levels in HEK293T cells cotransfected with Flag-IFNAR1, together with shCON (-) or shHOIP (#1, #2). **(b,c)** Western blot analysis of HA-IFNAR2 levels in HEK293T cells cotransfected with HA-IFNAR2 and either shHOIP (#1, #2) **(b)** or increasing amounts of Flag-LUBAC **(c)**. **(d)** Western blot analysis of p-STAT2 in HEK293T cells transfected with shHOIP (#1, #2) and then treated with IFNα (1,000 IU/ml) for 30 min. **(e)** Putative ubiquitination sites of STAT1 obtained from the PhosphoSitePlus database. **(f)** Immunoprecipitation analysis of ubiquitination of Myc-STAT1 (WT, K511R, or K652R) in HeLa cells cotransfected with Myc-STAT1 (WT, K511R or K652R) and HA-K0 using a HA antibody. **(g)** Western blot analysis of pY-STAT1 in U3A cells transfected Flag-STAT1 (WT or DM) and then treated with IFNβ (1,000 IU/ml) as indicated. **(h)** RT-qPCR analysis of the representative ISGs (*Ifit1*, *Isg15* and *Isg54*) mRNA in U3A cells transfected with Myc-STAT1 (WT or DM). **(i)** RT-qPCR analysis of VSV viral RNA in U3A cells transfected with Flag-STAT1 (WT or DM) and then treated with IFNα (60 IU/ml) for 20 hrs, followed by infection with VSV (MOI=0.1) for 24 hrs. **(j)** Western blot analysis of pY-STAT1 levels in HEK293T cells transfected with increasing amounts of Flag-LUBAC, and then treated with IFNγ (1,000 IU/ml) for 30 min. **(k)** Western blot analysis of pY-STAT1 in U3A cells transfected Myc-STAT1 (WT or DM) and then treated with IFNγ (1,000 IU/ml) as indicated. **(l)** RT-qPCR analysis of the representative ISG (*Ifit1*) mRNA in HEK293T cells transfected with shHOIP (#1, #2) and then stimulated with IFNγ (1,000 IU/ml) for 4 hrs. **(m)** RT-qPCR analysis of the representative ISG (*Ifit1*) mRNA in U3A cells transfected with Myc-STAT1 (WT or DM) and then treated with IFNγ (1,000 IU/ml) for 4 hrs. N.S, not significant ($p>0.05$), **$p<0.01$ and ***$p<0.001$ (two-tailed unpaired Student's *t*-test). Data are shown as mean and s.d. of three biological replicates **(h, i, l, m)**, or are representative of three independent experiments **(a-d, f, g, j, k)**.

# Supplementary Figure 4

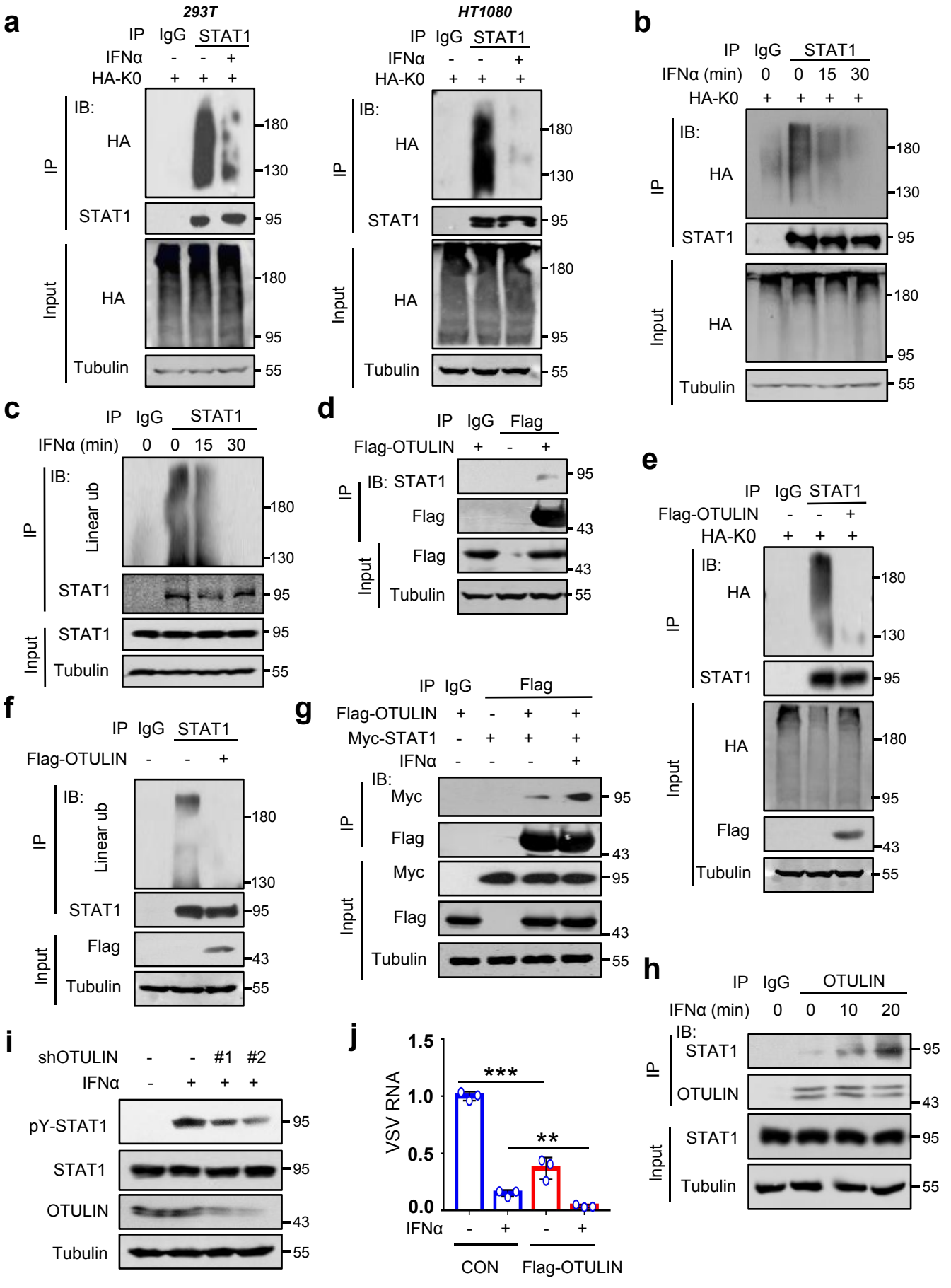


**Supplementary Figure 4**

**Linear ubiquitination blocks the binding of STAT1 to IFNAR2.**

**(a)** Western blot analysis of pY701-STAT1 (pY-STAT1) in the cytoplasm and nucleus of HEK293T cells transfected with shHOIP and then treated with IFNα (1,000 IU/ml) for 30 min. **(b)** Immunoprecipitation analysis of the interaction between Flag-HOIP and Myc-STAT1 (WT, Y701F or S727A) in HEK293T cells. **(c)** Immunoprecipitation analysis of linear ubiquitination of Myc-STAT1 (WT or Y701F) in HEK293T cells transfected with Myc-STAT1 (WT or Y701F). **(d)** Immunoprecipitation analysis of the interaction between HA-IFNAR2 and STAT1 in HEK293T cells transfected with Flag-LUBAC and then treated with IFNα (1,000 IU/ml) for 15 min. **(e)** Immunoprecipitation analysis of the interaction between endogenous IFNAR2 and STAT1 in HEK293T cells treated as **(d)**. **(f)** Immunoprecipitation analysis of the interaction between HA-IFNAR2 and Myc-STAT1 (WT or its mutants) in HEK293T cells cotransfected with HA-IFNAR2 and Myc-STAT1 (WT, K511R or K652R). **(g)** Immunoprecipitation analysis of the interaction between HA-IFNAR2 and Myc-STAT1 (WT and its mutants) in HEK293T cells cotransfected with HA-IFNAR2, Flag-LUBAC and Myc-STAT1 (WT, K511R or K652R) and then treated with IFNα (1,000 IU/ml) for 15 min. Data are representative of three independent experiments.

# Supplementary Figure 5

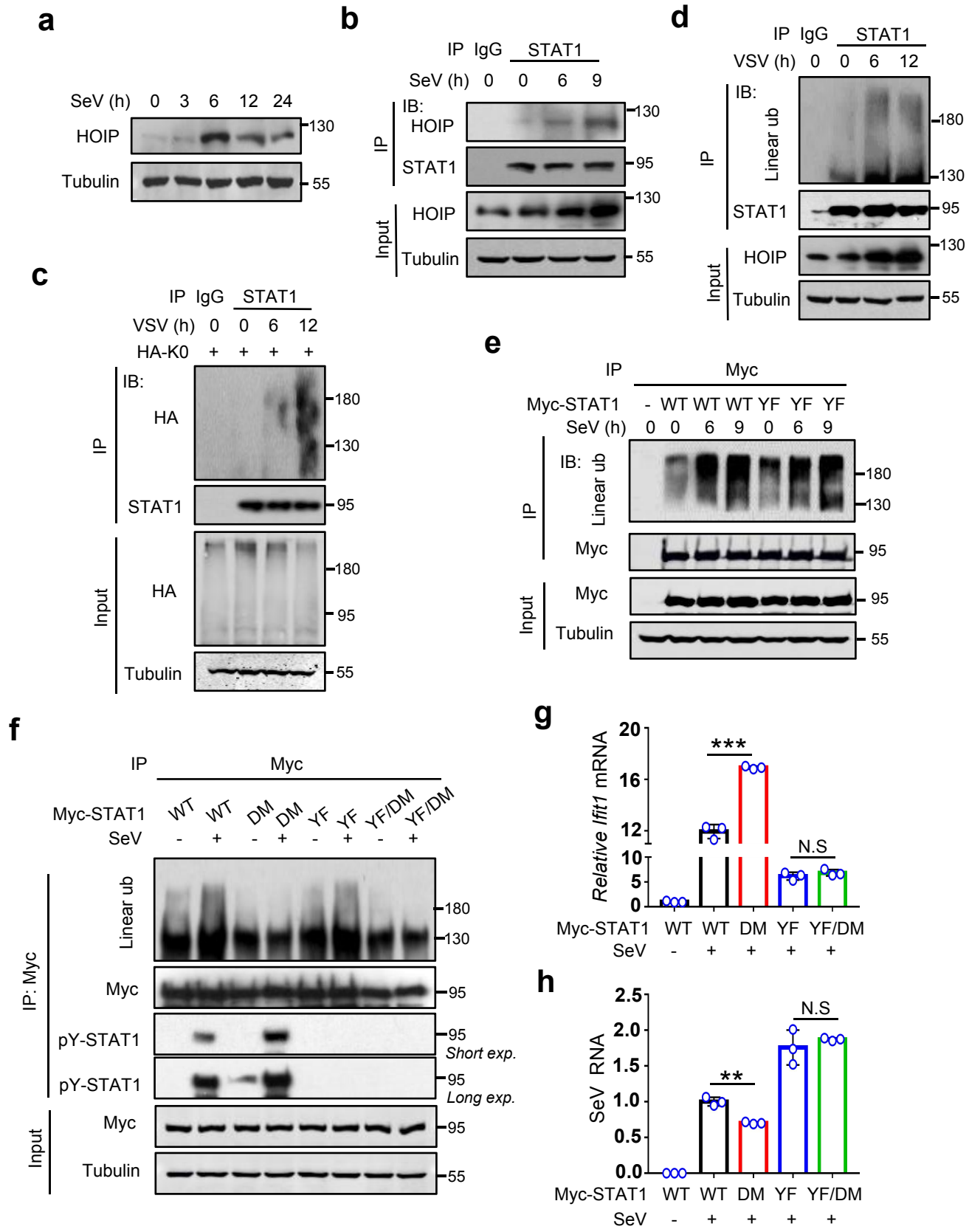


**Supplementary Figure 5**

**IFN-I removes STAT1 linear ubiquitination via OTULIN.**

**(a)** Immunoprecipitation analysis of ubiquitination of STAT1 in HEK293T (left) or HT1080 (right) cells transfected with HA-K0 and then treated with IFNα (1,000 IU/ml) for 30 min. **(b)** Immunoprecipitation analysis of ubiquitination of STAT1 in 2fTGH cells transfected with HA-K0 and then treated with IFNα (1,000 IU/ml) for the indicated times. **(c)** Immunoprecipitation analysis of linear ubiquitination of STAT1 in HEK293T cells treated with IFNα (1,000 IU/ml) as indicated. **(d)** Immunoprecipitation analysis of the interaction between Flag-OTULIN and STAT1 in HEK293T cells transfected with Flag-OTULIN. **(e)** Immunoprecipitation analysis of ubiquitination of STAT1 in HEK293T cells cotransfected with HA-K0 and Flag-OTULIN using a HA antibody. **(f)** Immunoprecipitation analysis of linear ubiquitination of STAT1 transfected with Flag-OTULIN using a linear ubiquitination antibody. **(g)** Immunoprecipitation analysis of the interaction between Flag-OTULIN and Myc-STAT1 in HEK293T cells transfected with Flag-OTULIN and Myc-STAT1 and then treated with IFNα (1,000 IU/ml) for 15 min. **(h)** Immunoprecipitation analysis of the interaction between endogenous OTULIN and STAT1 in HEK293T cells treated with IFNα (1,000 IU/ml) for the indicated times. **(i)** Western blot analysis of pY-STAT1 in HEK293T cells transfected with shOTULIN (#1, #2) and then treated with IFNα (1,000 IU/ml) for 30 min. **(j)** RT-qPCR analysis of VSV viral RNA in 2fTGH cells transfected with Flag-OTULIN and then stimulated with IFNα (60 IU/ml) for 20 hrs, followed by infection with VSV (MOI=0.1) for 24 hrs. $^{**}p<0.01$ and $^{***}p<0.001$ (two-tailed unpaired Student's *t*-test). Data are shown as mean and s.d. of three biological replicates **(j)**, or are representative of three independent experiments **(a-i)**.

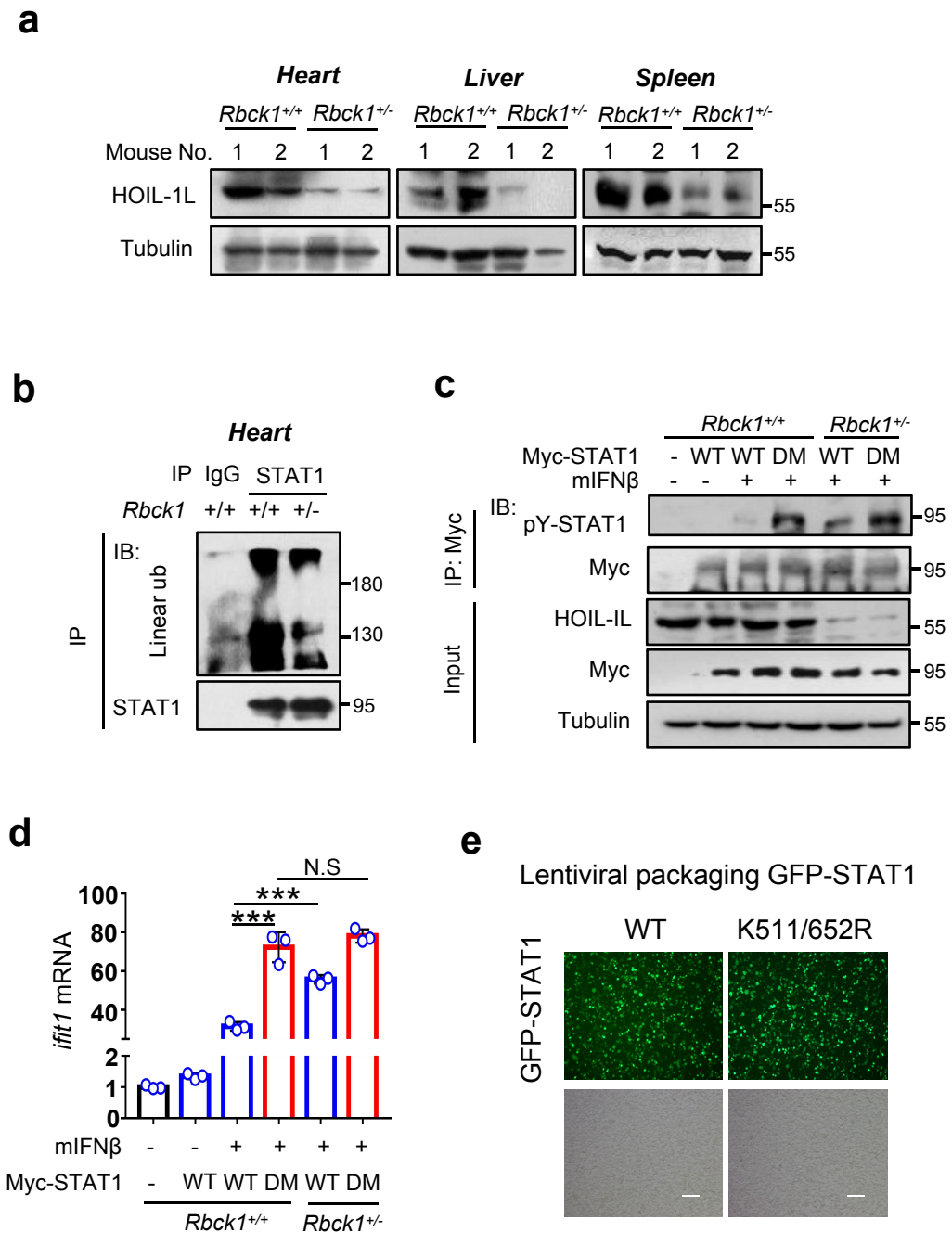
# Supplementary Figure 6



**Supplementary Figure 6**

**Viruses upregulate HOIP and STAT1 linear ubiquitination.**
**(a)** Western blot analysis of HOIP proteins in A549 cells infected with SeV (MOI=1.0) for the indicated times. **(b)** Immunoprecipitation analysis of the interaction between HOIP and STAT1 in HEK293T cells infected with SeV (MOI=1.0) for the indicated times. **(c)** Immunoprecipitation analysis of ubiquitination of STAT1 in HT1080 cells infected with HA-K0 and then infected with VSV (MOI=0.5) as indicated. **(d)** Immunoprecipitation analysis of linear ubiquitination of STAT1 in A549 cells infected with VSV (MOI=0.5) for the indicated times. **(e)** Immunoprecipitation analysis of linear ubiquitination of STAT1 in HEK293T cells transfected with Myc-STAT1 (WT or YF) and then infected with SeV (MOI=1.0) as indicated. **(f)** Immunoprecipitation analysis of linear ubiquitination of STAT1 in U3A cells transfected with Myc-STAT1 (WT, DM, YF or YF/DM) and then infected with SeV (MOI=1.0) for 12 hrs. **(g)** RT-qPCR analysis of the representative ISG (*Ifit1*) mRNA in U3A cells transfected with Myc-STAT1 (WT, DM, YF or YF/DM) and then infected with SeV (MOI=1.0) for 12 hrs. **(h)** RT-qPCR analysis of SeV viral RNA in U3A cells transfected with Myc-STAT1 (WT, DM, YF or YF/DM) and then infected with SeV (MOI=1) for 12 hrs. N.S, not significant ($p>0.05$), $^{**}p<0.01$ and $^{***}p<0.001$ (two-tailed unpaired Student's *t*-test). Data are representative of three independent experiments **(a-f)**, or are shown as mean and s.d. of three biological replicates **(g, h)**.

# Supplementary Figure 7



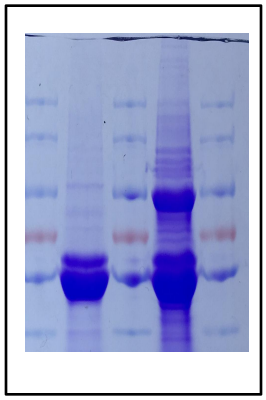
**Supplementary Figure 7**

**HOIL-1L deficiency enhances IFN-I antiviral activity.**
**(a)** Western blot analysis of HOIL-1L proteins in the heart, liver and spleen tissues from *Rbck1*$^{+/+}$ or *Rbck1*$^{+/-}$ mice. **(b)** Immunoprecipitation analysis of linear ubiquitination of STAT1 in the heart tissues from *Rbck1*$^{+/+}$ or *Rbck1*$^{+/-}$ mice. **(c)** Immunoprecipitation analysis of pY-STAT1 in *Rbck1*$^{+/+}$ or *Rbck1*$^{+/-}$ MEF cells transfected with Myc-STAT1 (WT or DM) and then treated with mIFNβ (1,000 IU/ml) as indicated. **(d)** RT-qPCR analysis of the representative ISG (*Ifit1*) mRNA in *Rbck1*$^{+/+}$ or *Rbck1*$^{+/-}$ MEF cells transfected with Myc-STAT1 (WT or DM) and then treated with mIFNβ (1,000 IU/ml) for 4 hrs. **(e)** The lentiviruses containing GFP-STAT1 (WT or K511/652R) were made in HEK293T cells. GFP signaling in cells was observed by the fluorescence microscopy. Scale bar: 100 μm. N.S, not significant ($p>0.05$) and ***$p<0.001$ (two-tailed unpaired Student's *t*-test). Data are shown as mean and s.d. of three biological replicates **(d)**, or are representative of three independent experiments **(a-c, e)**.

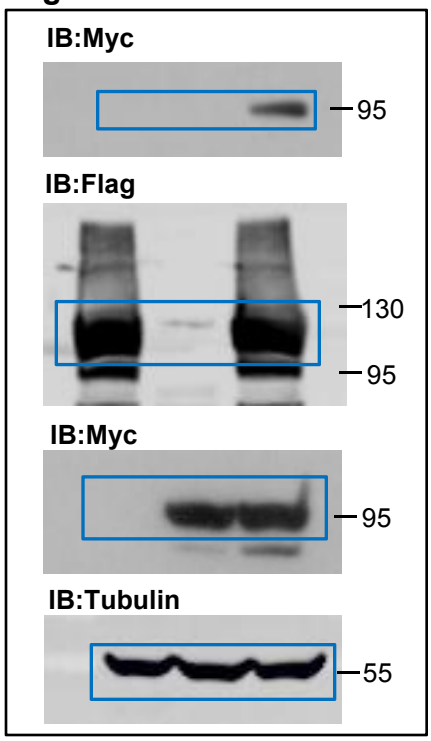
**Supplementary Figure 8: Original scans of immunblots.**

## For Figure 1

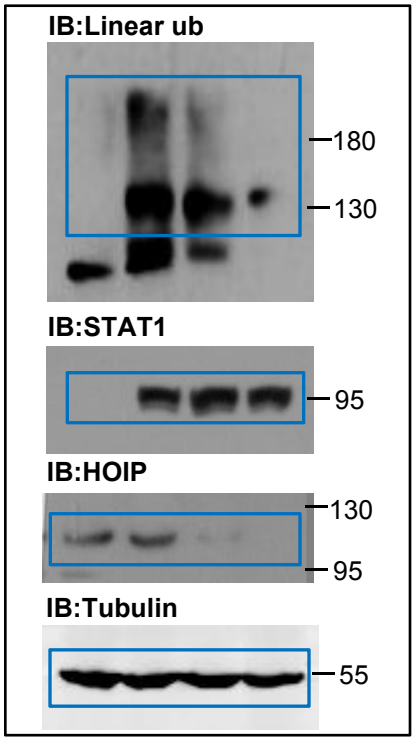
### Figure 1a



### Figure 1b




### Figure 1e

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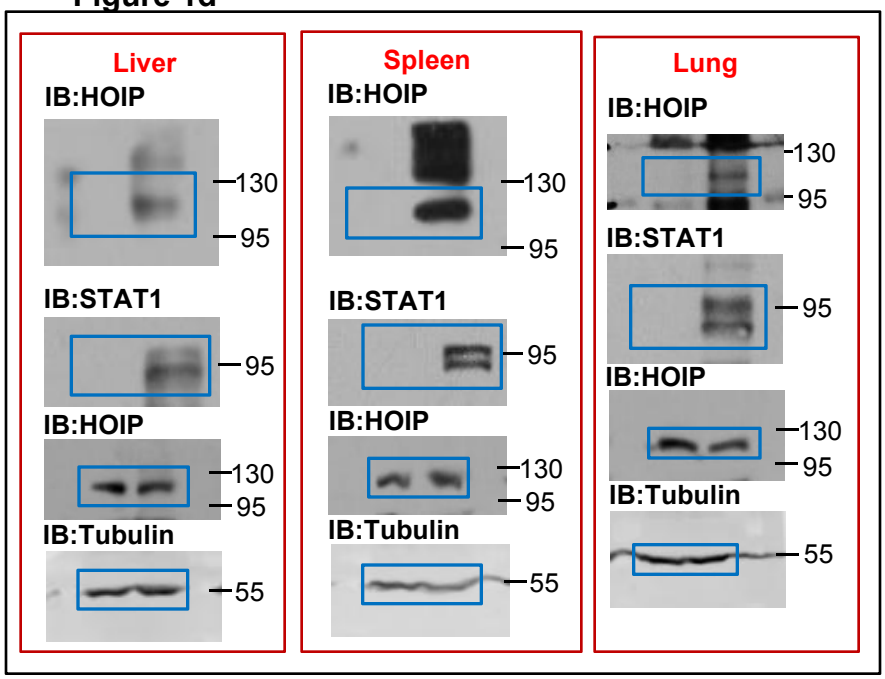
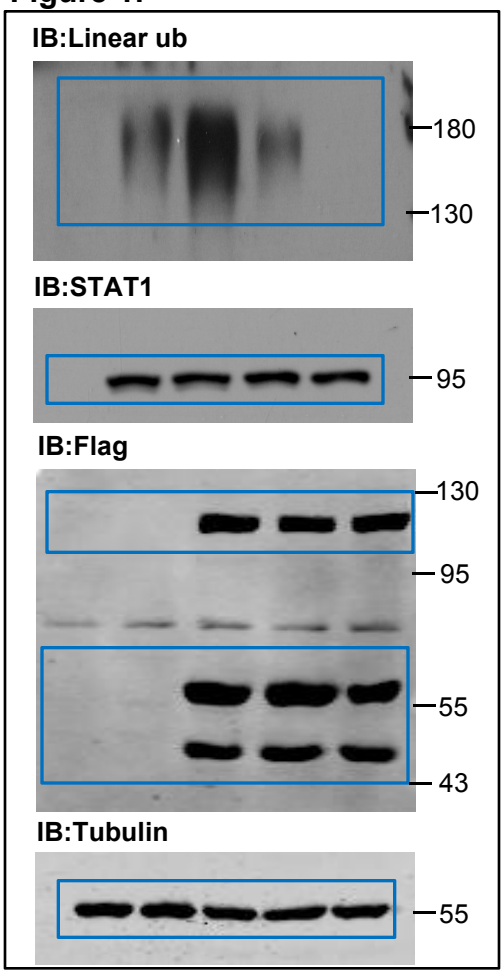
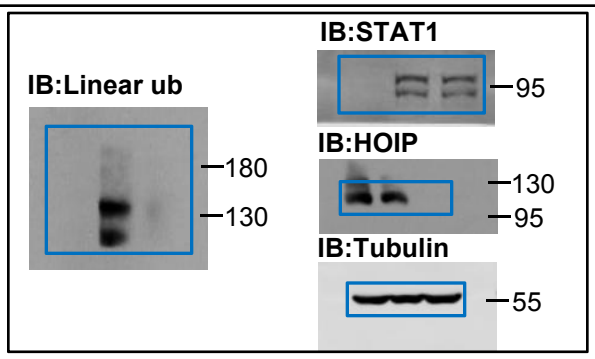



### Figure 1f

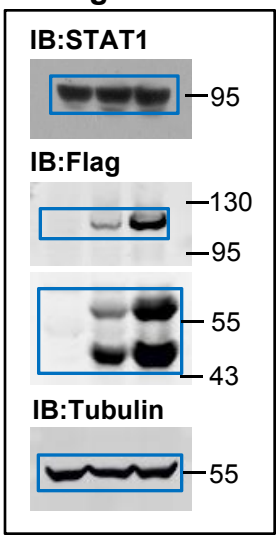



### Figure 1g



# For Figure 2

## Figure 2a

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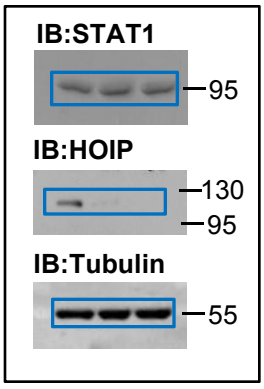
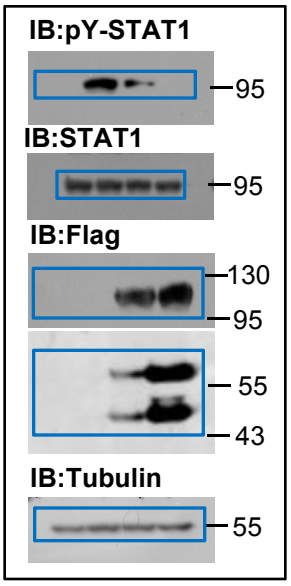



## Figure 2c

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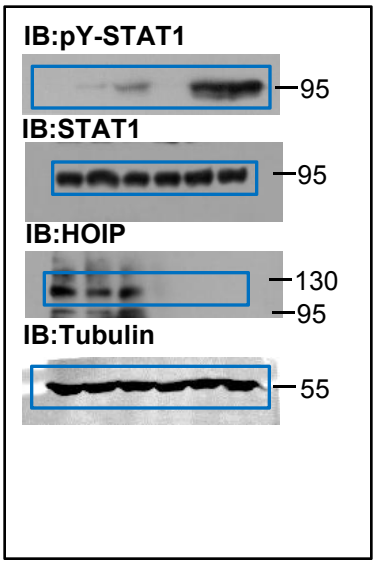


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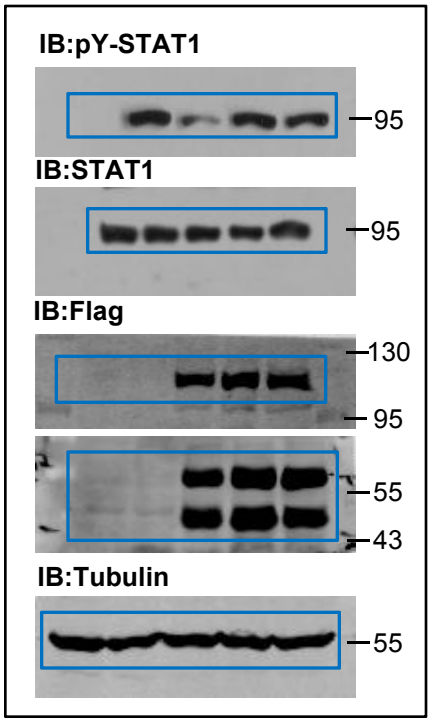


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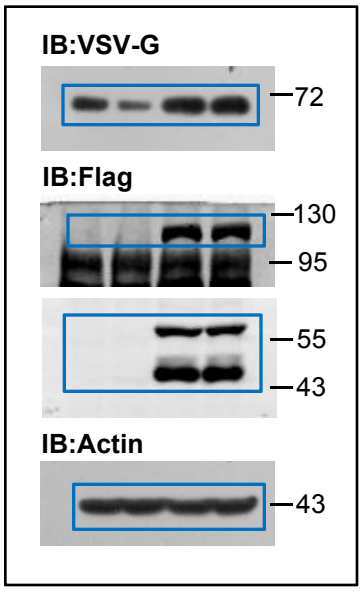
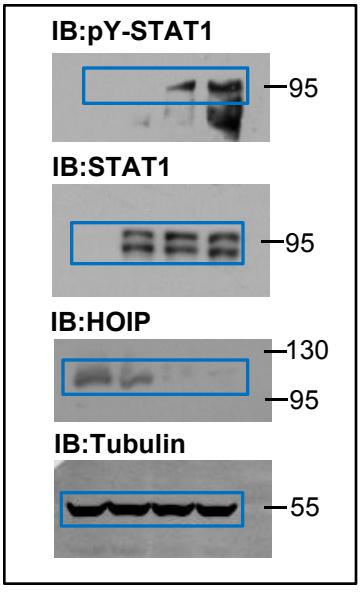
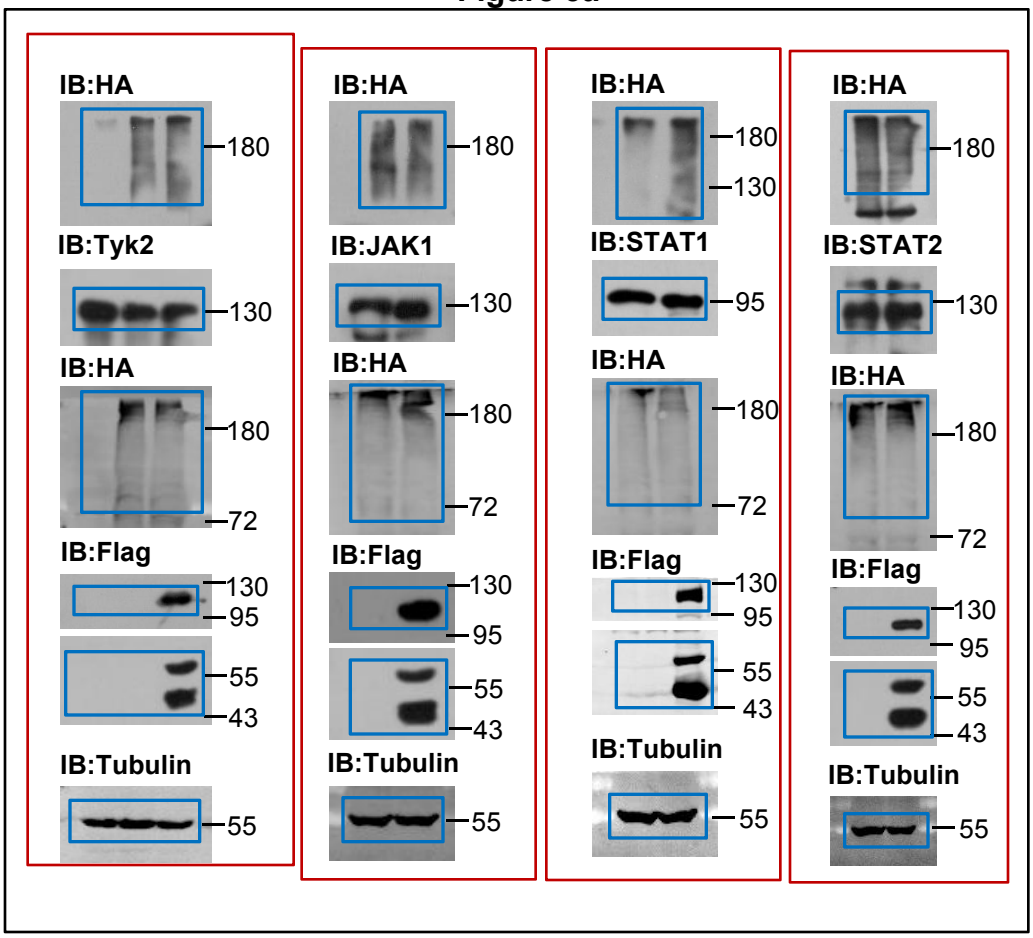



## Figure 2l



# For Figure 3

## Figure 3a

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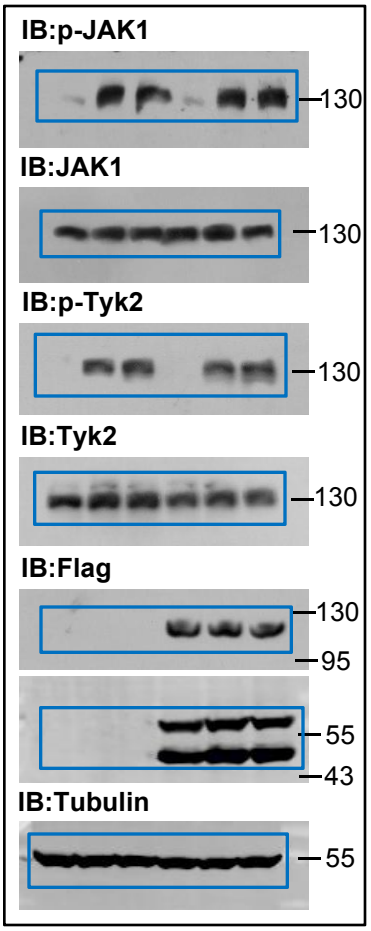


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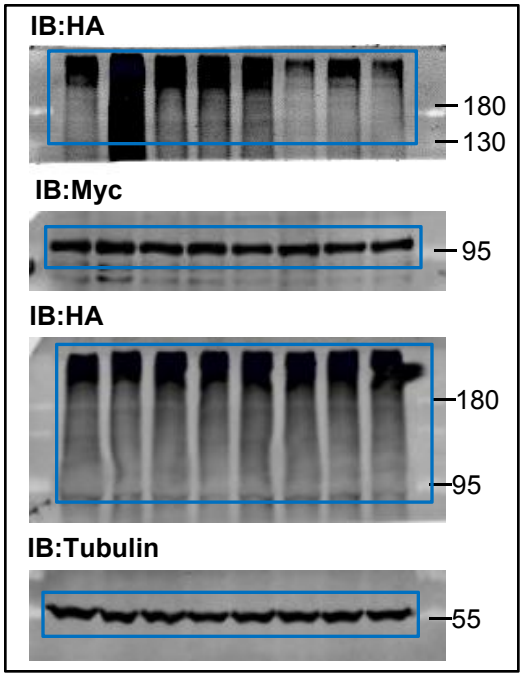


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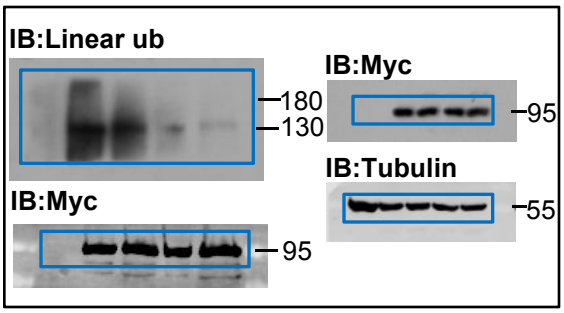


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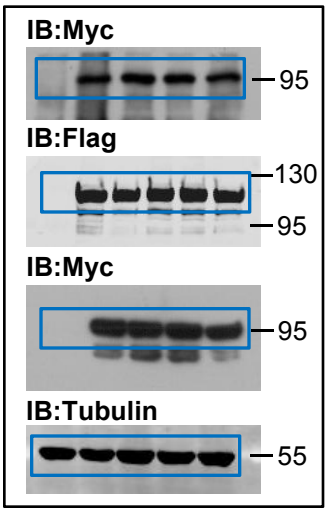


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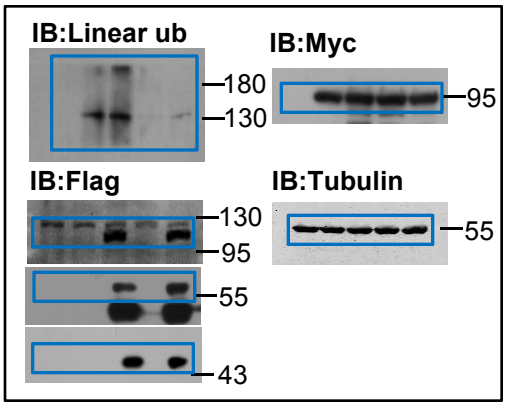


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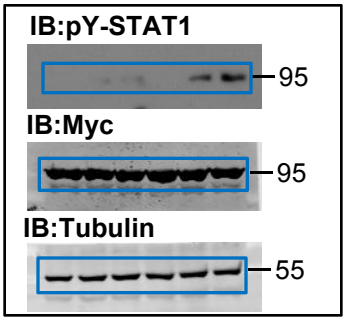
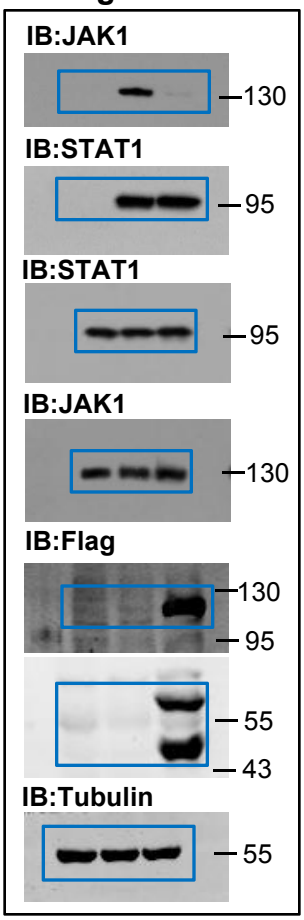


# For Figure 4

## Figure 4a

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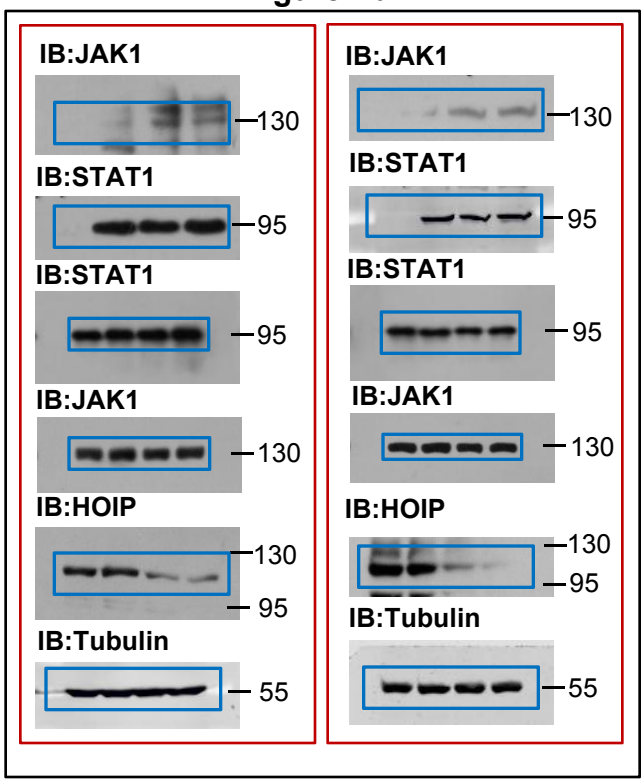


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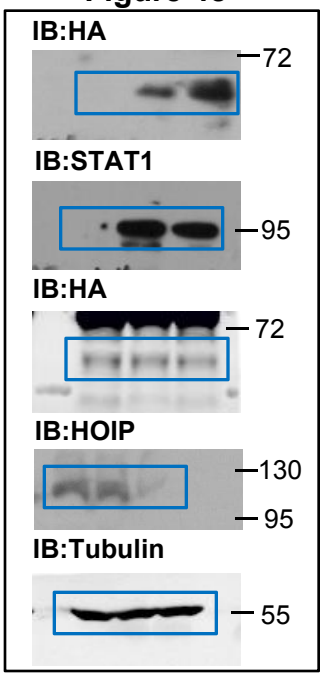


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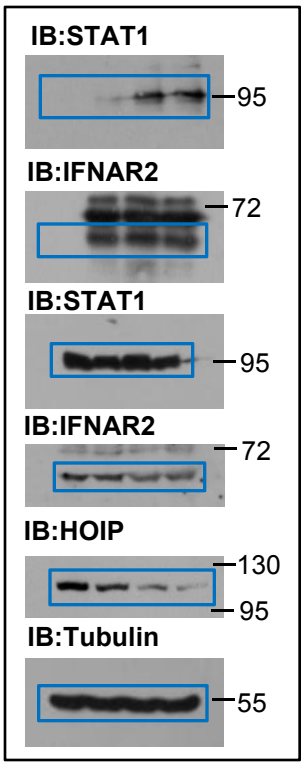


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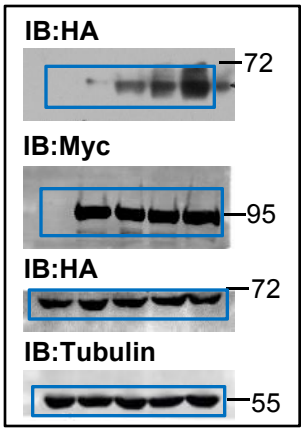


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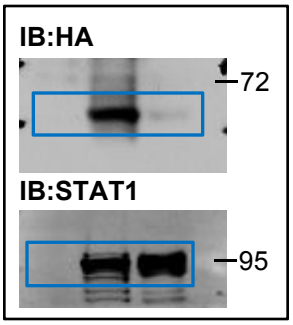
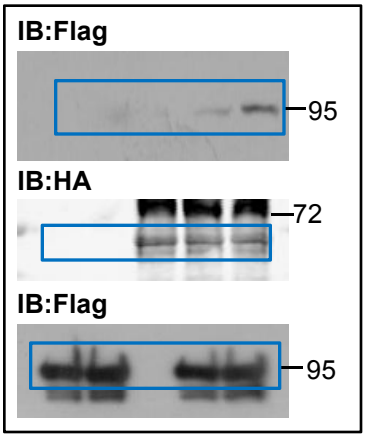
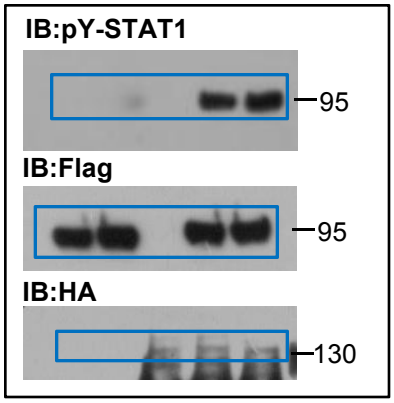


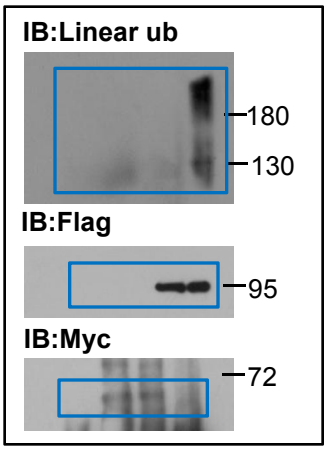

## Figure 4g




## Figure 4h

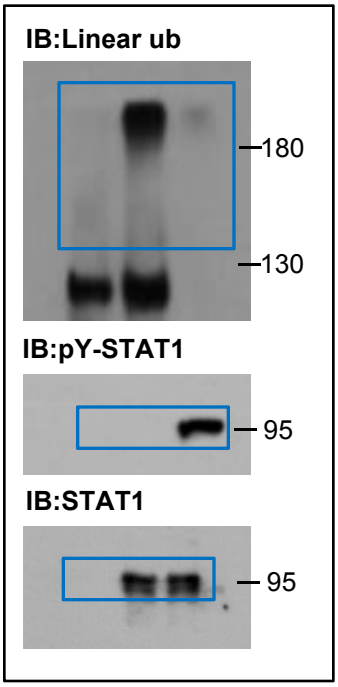



## Figure 4i

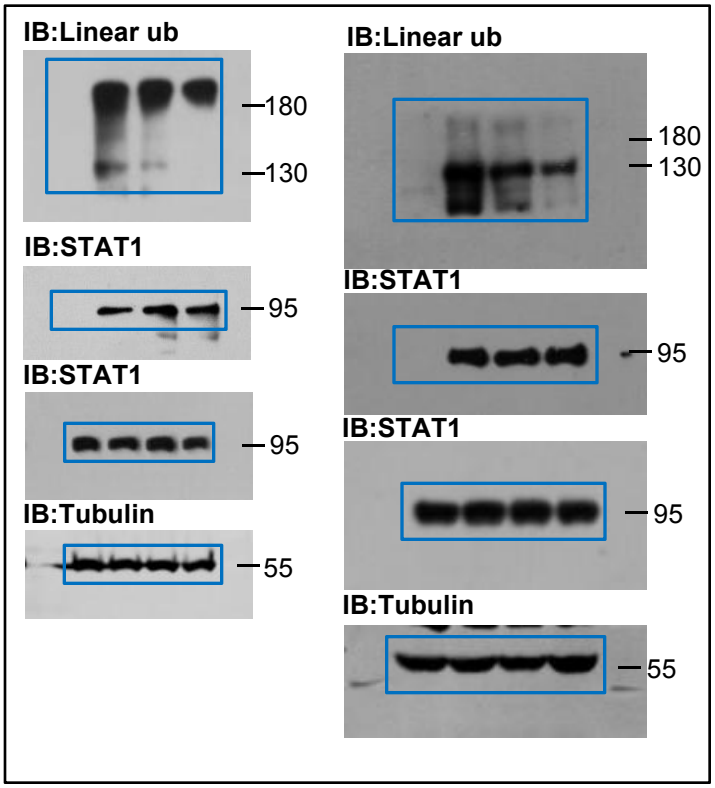


# For Figure 5

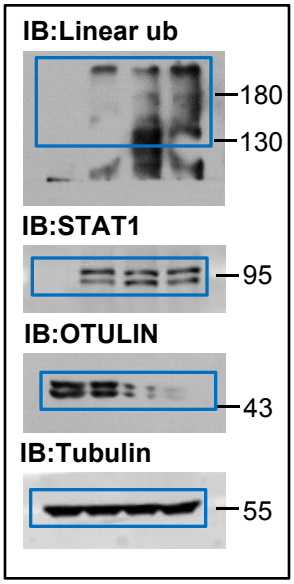
## Figure 5a



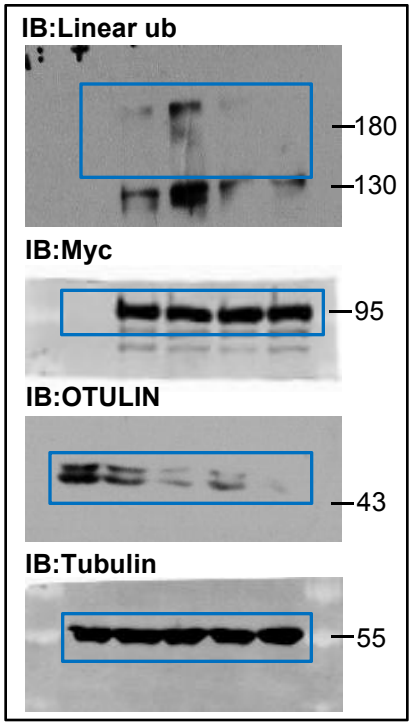

## Figure 5b



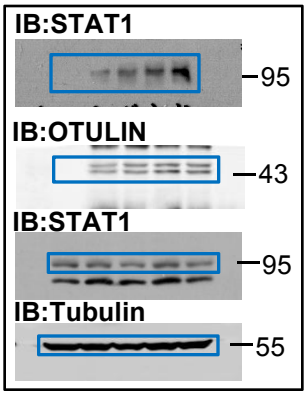

## Figure 5c



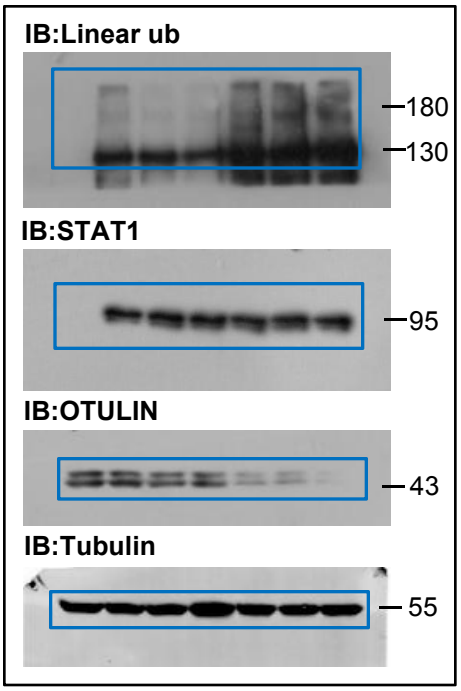

## Figure 5d




## Figure 5e

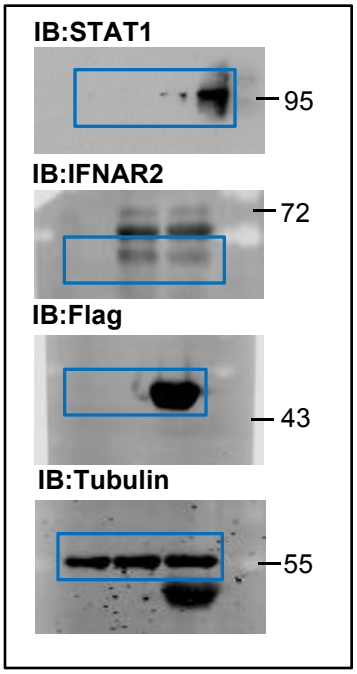



## Figure 5f

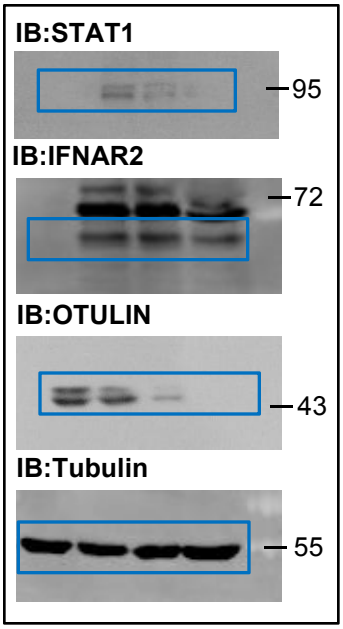


# For Figure 5

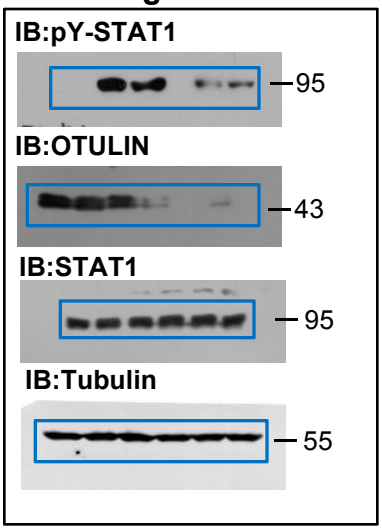
## Figure 5g




## Figure 5h

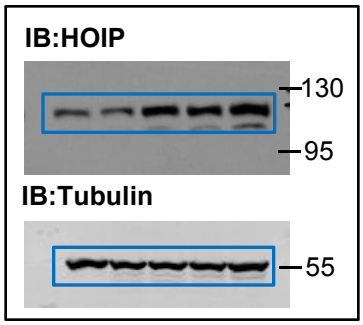



## Figure 5i

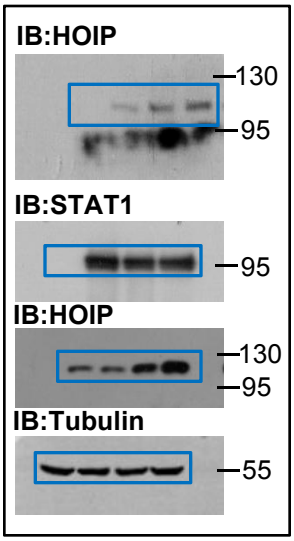


# For Figure 6

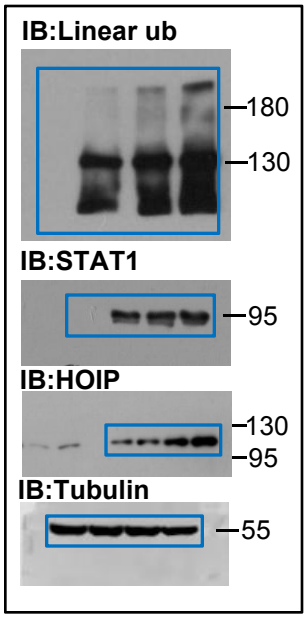
## Figure 6d



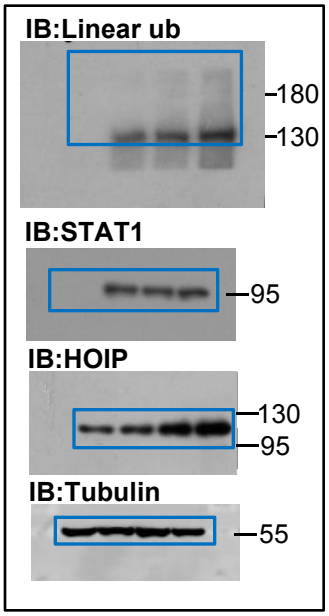

## Figure 6e



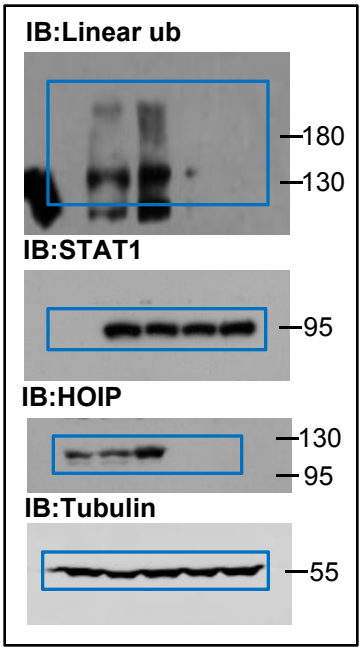

## Figure 6f



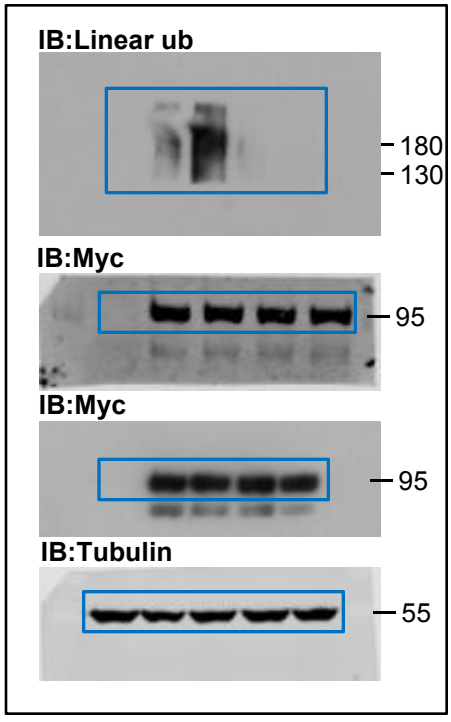

## Figure 6g



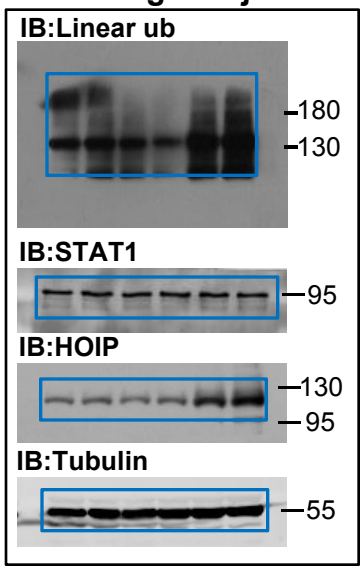

## Figure 6h



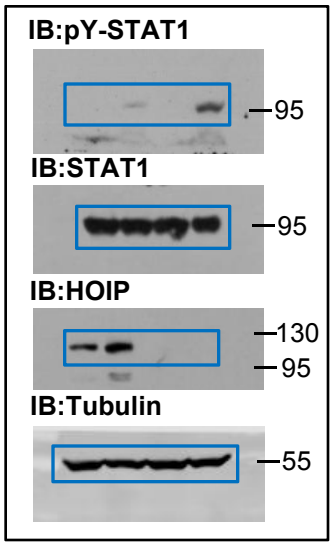

## Figure 6i




## Figure 6j




## Figure 6k



# For Figure 7

Figure 7a

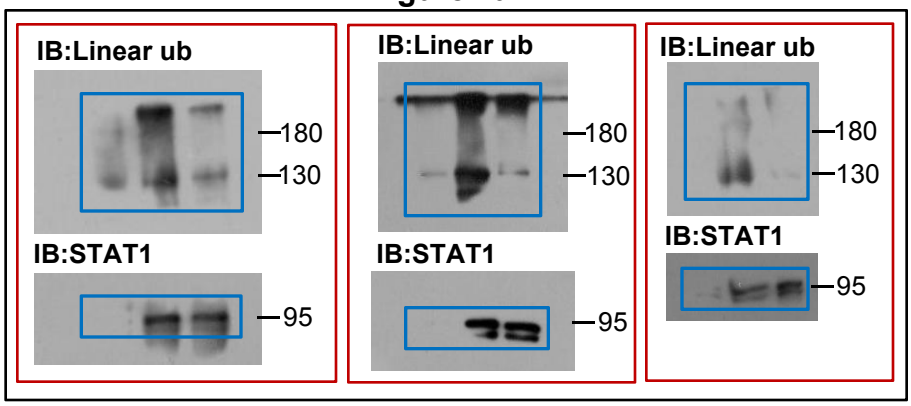
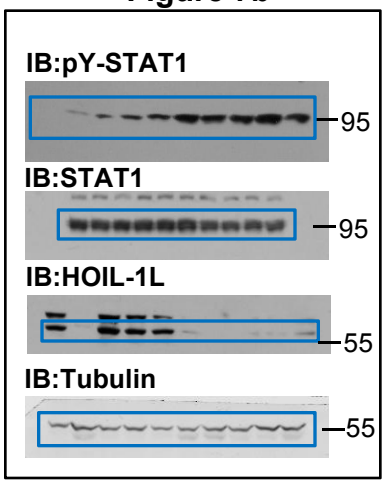
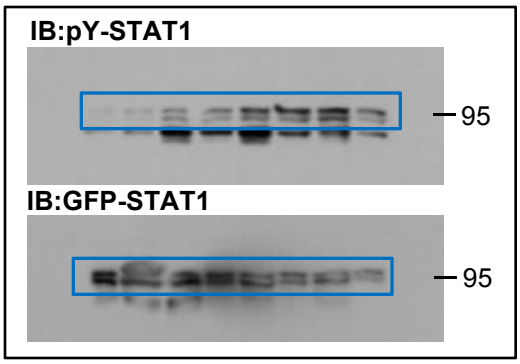



Figure 7b



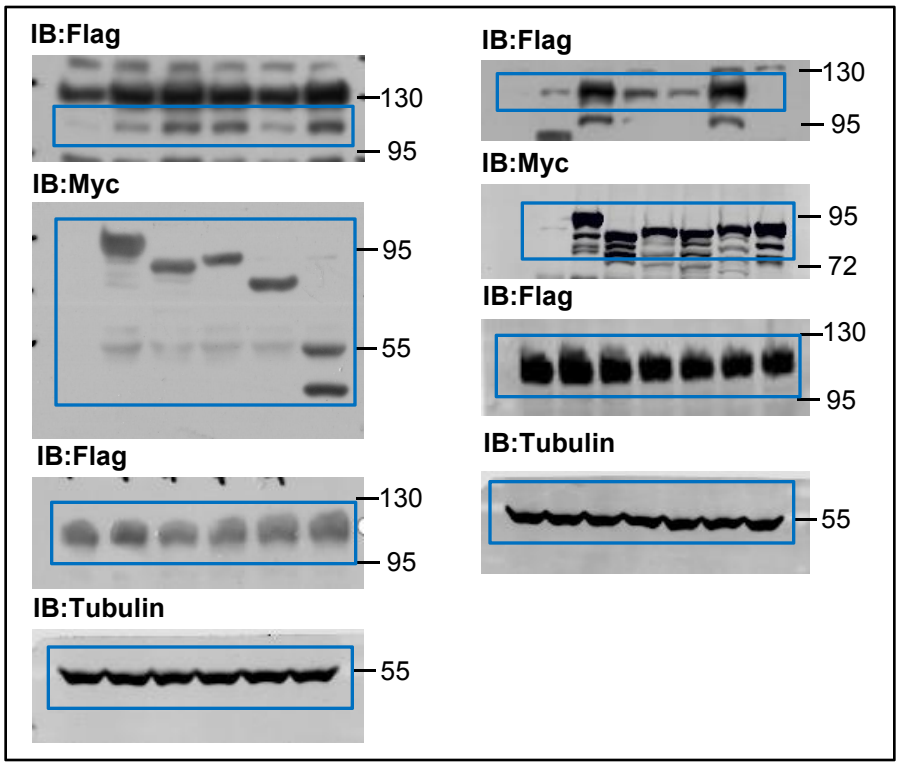

# For Figure 8

Figure 8b

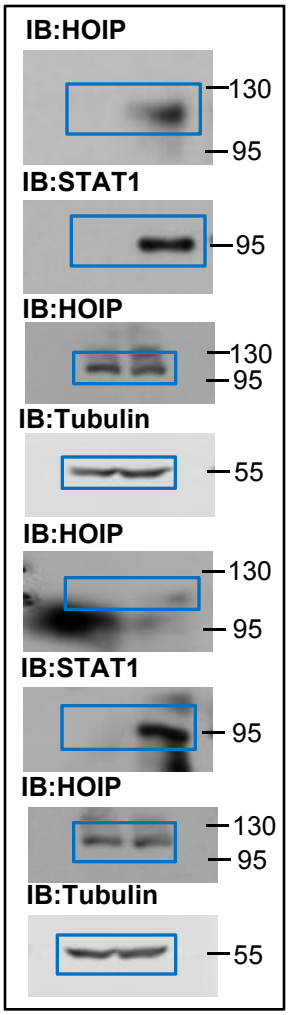


# For Supplementary Figure 1

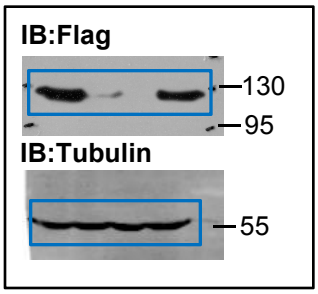
## Figure S1b



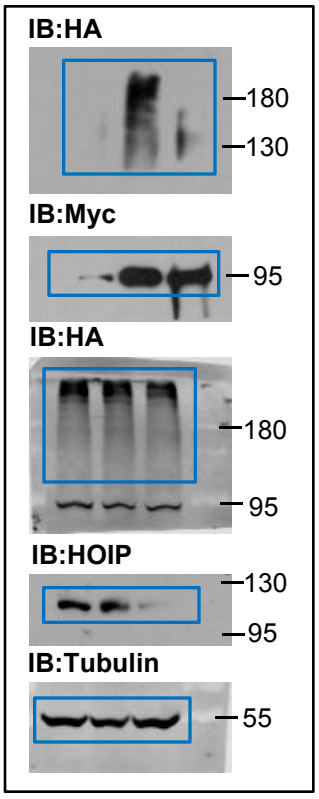

## Figure S1c



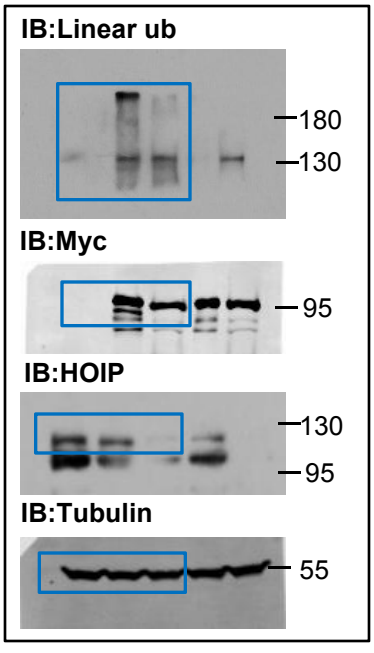

## Figure S1d



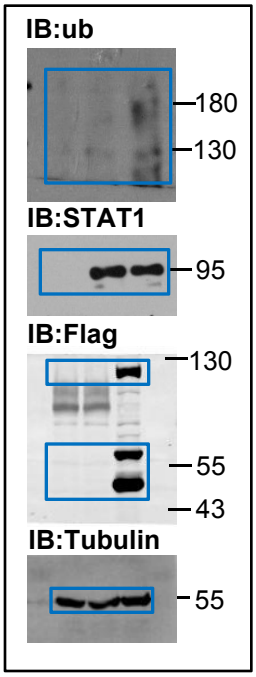

## Figure S1e




## Figure S1f

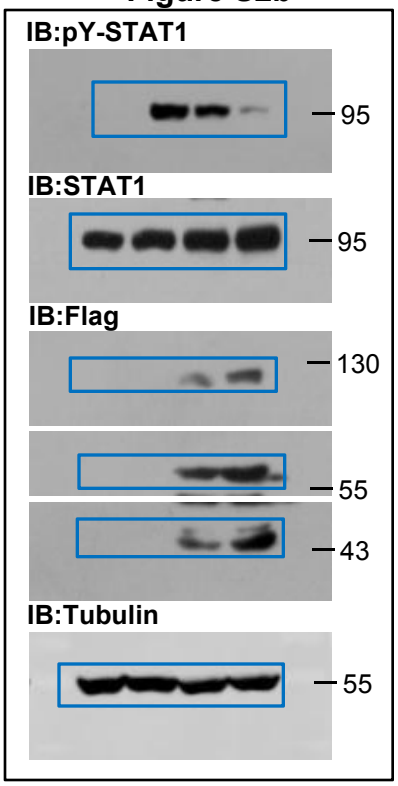



## Figure S1g

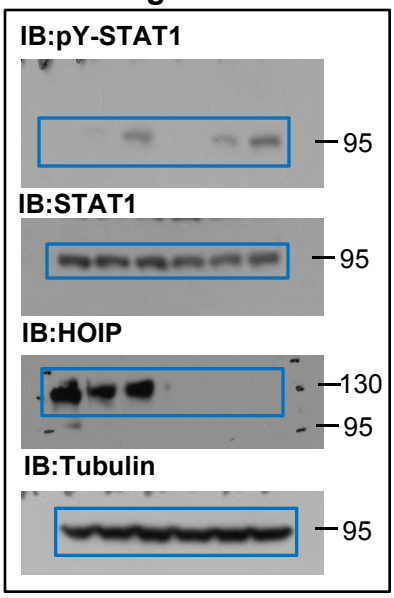


# For Supplementary Figure 2

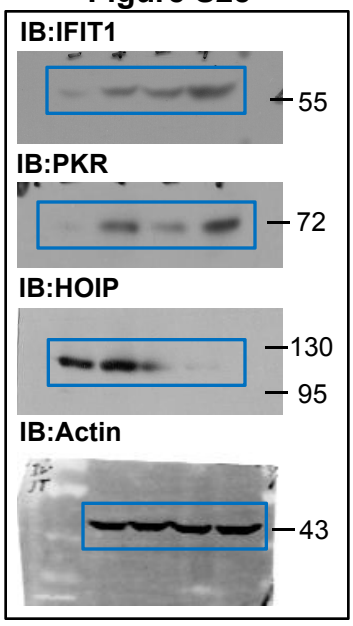
## Figure S2b



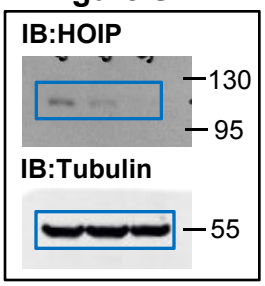

## Figure S2c




## Figure S2e

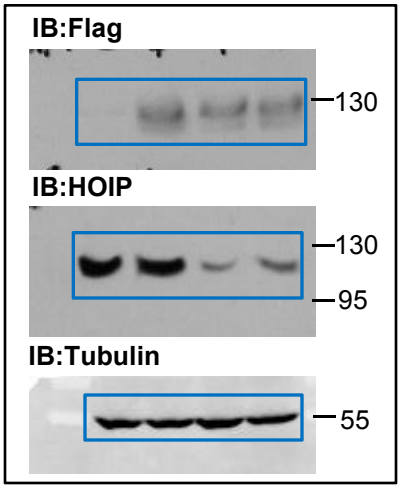



## Figure S2l

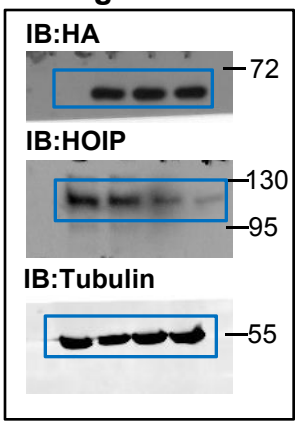


# For Supplementary Figure 3

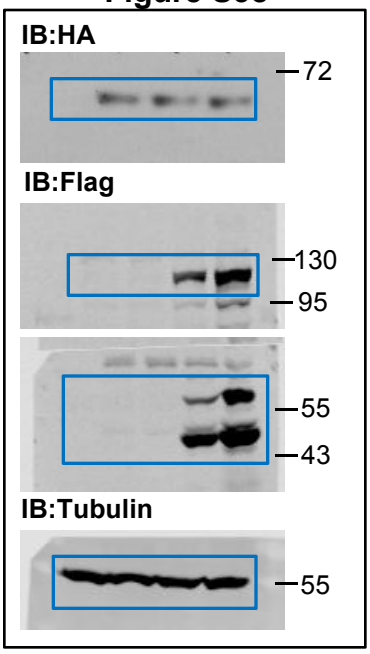
## Figure S3a



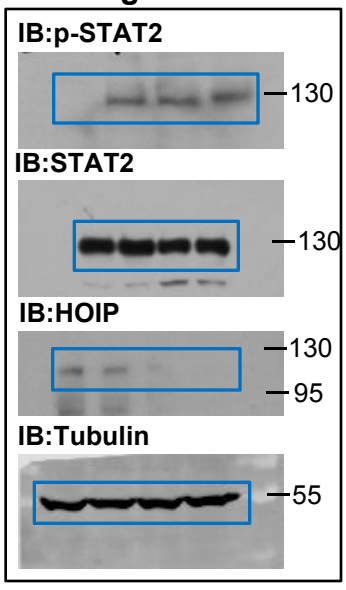

## Figure S3b



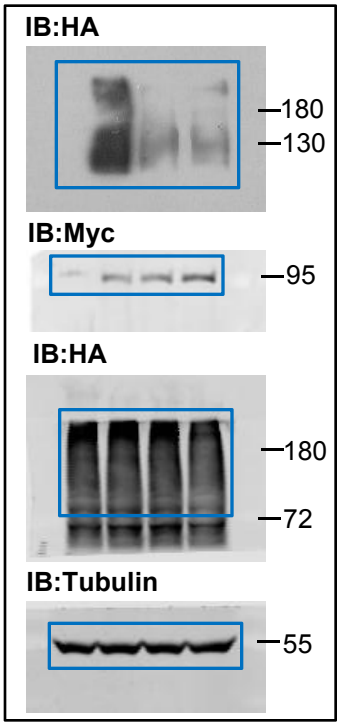

## Figure S3c



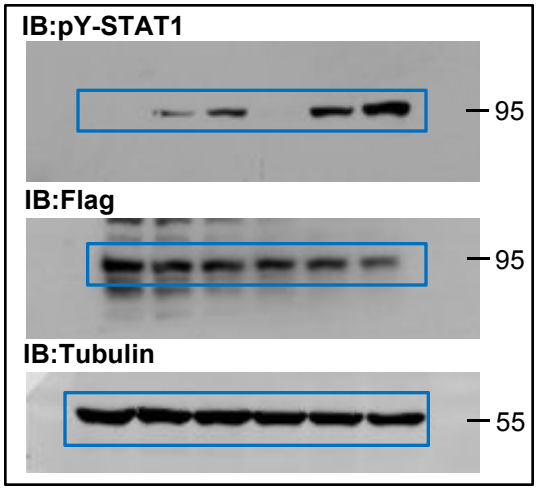

## Figure S3d



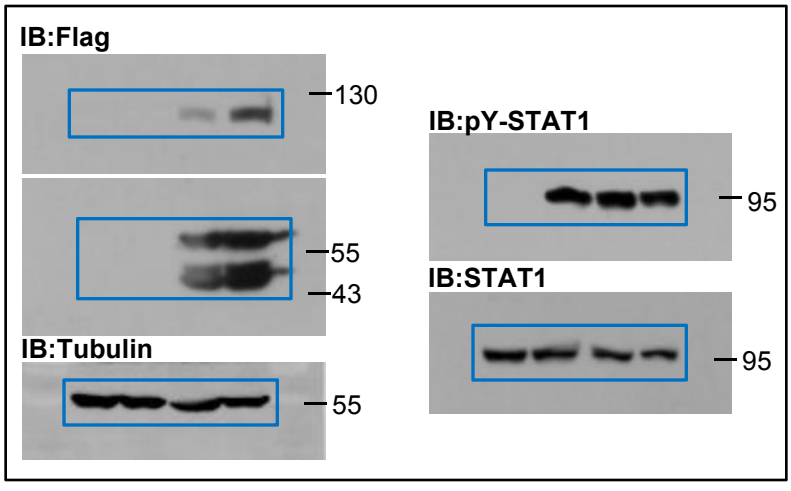

## Figure S3f



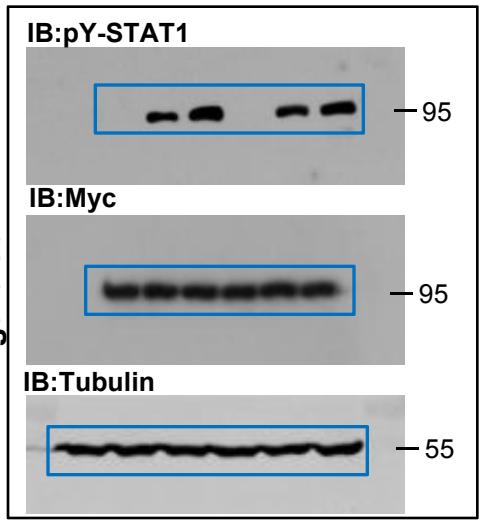

## Figure S3g




## Figure S3j

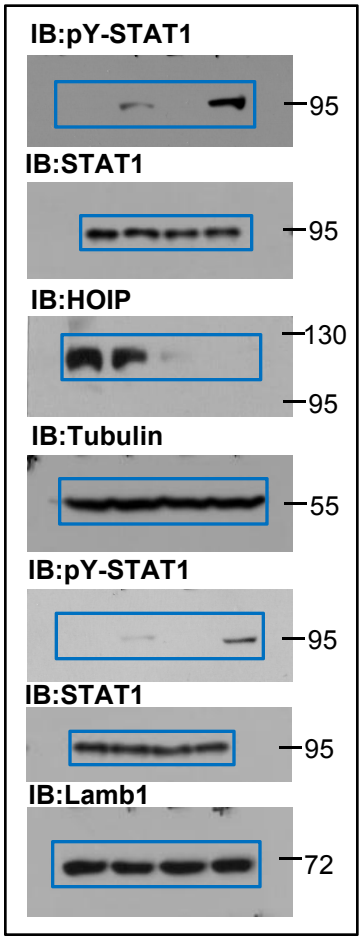



## Figure S3k

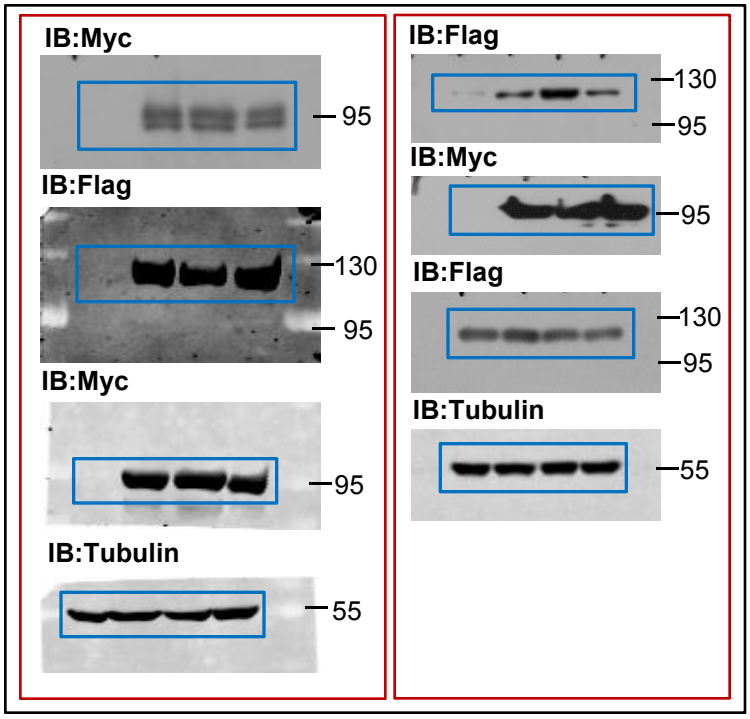


# For Supplementary Figure 4

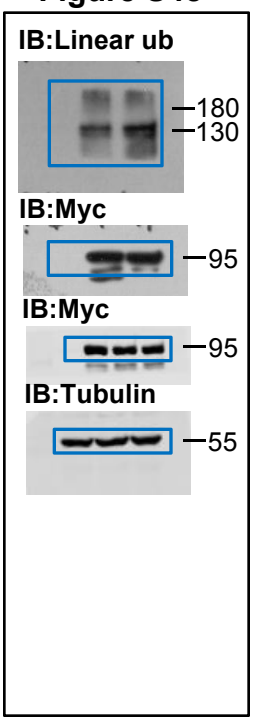
## Figure S4a



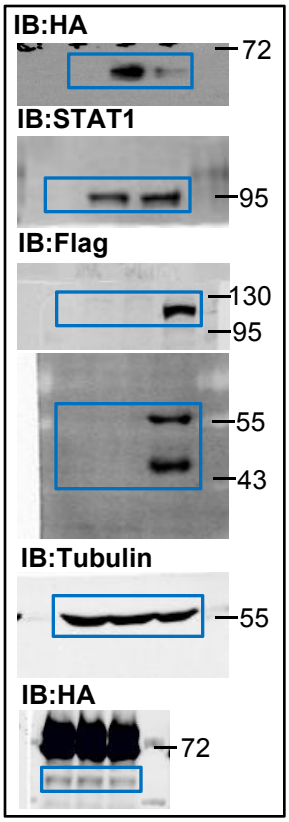

## Figure S4b



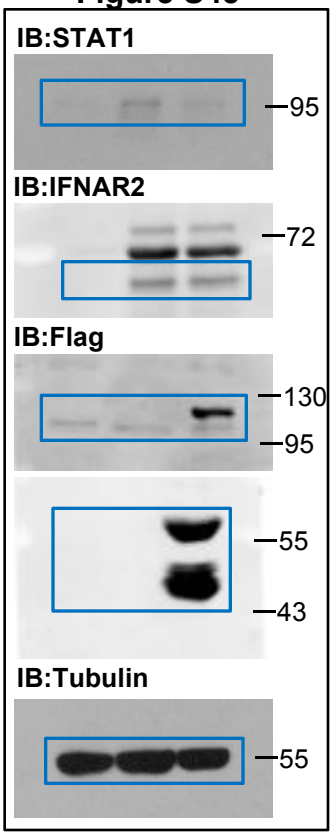

## Figure S4c



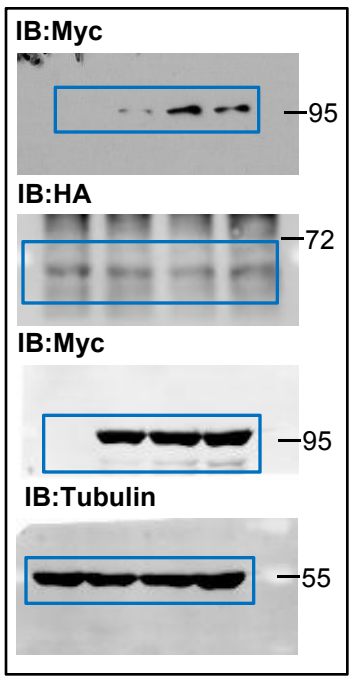

## Figure S4d



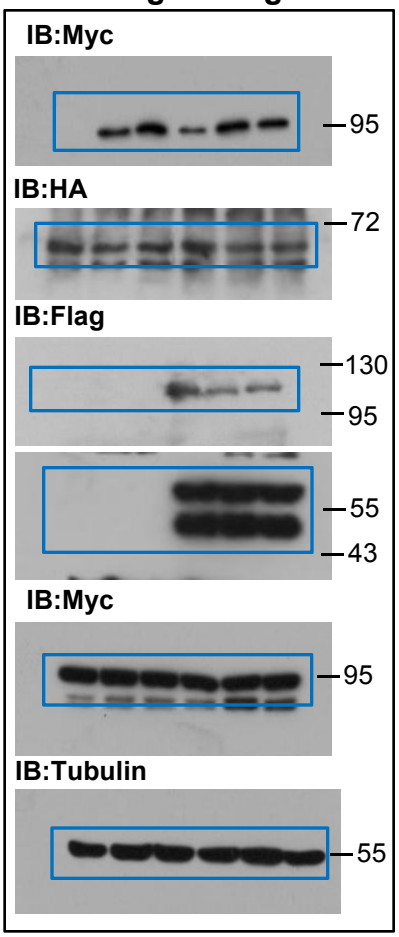

## Figure S4e




## Figure S4f

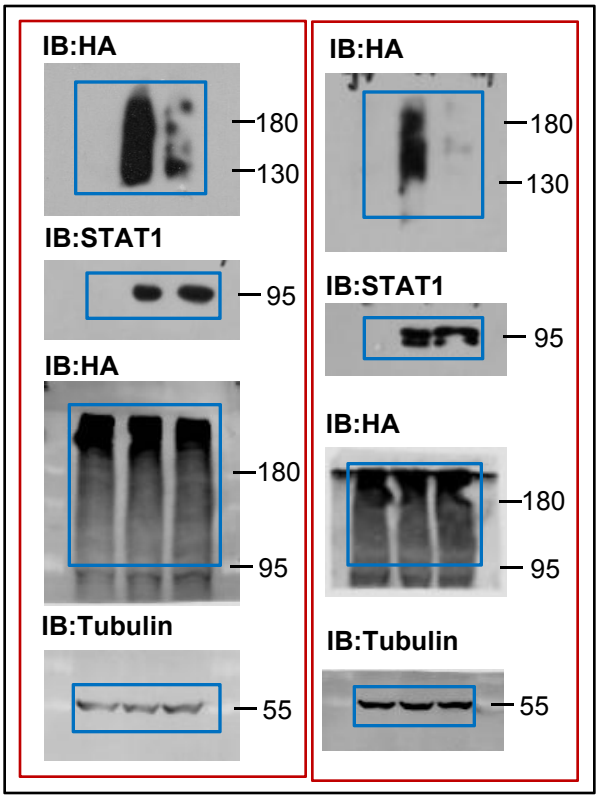



## Figure S4g

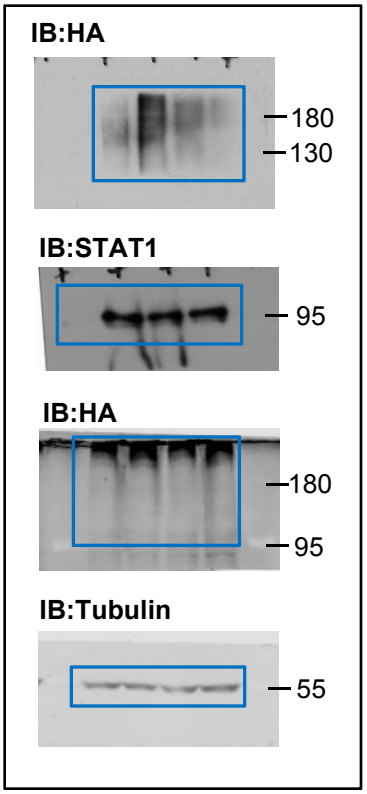


# For Supplementary Figure 5

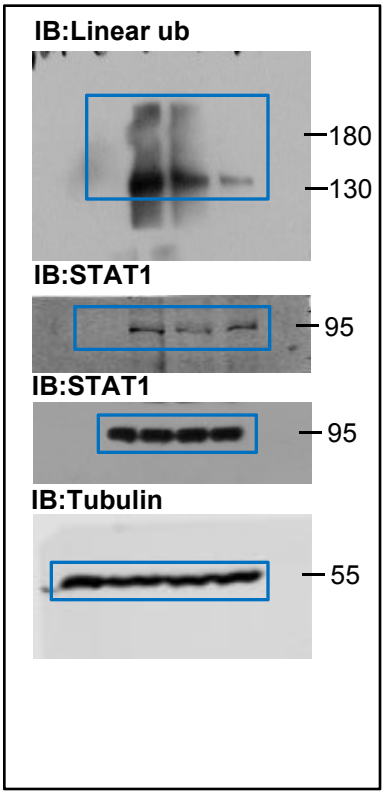
## Figure S5a



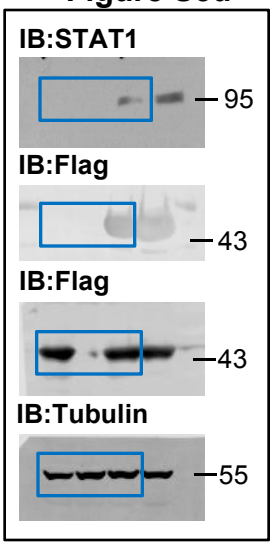

## Figure S5b



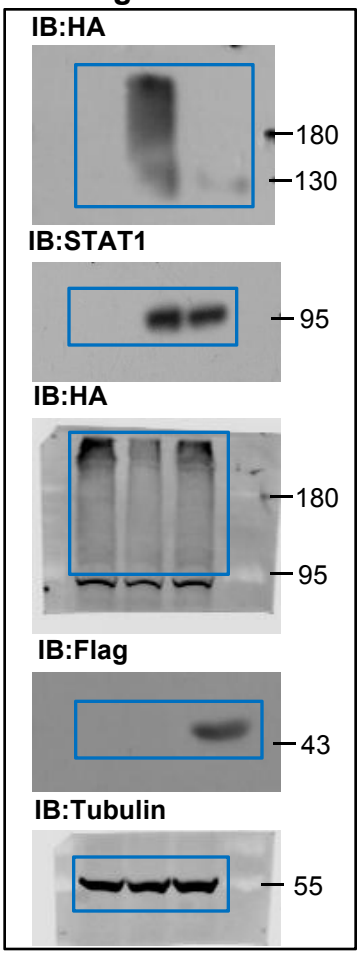

## Figure S5c



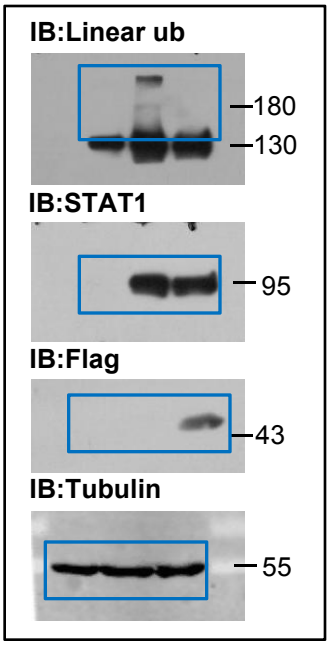

## Figure S5d



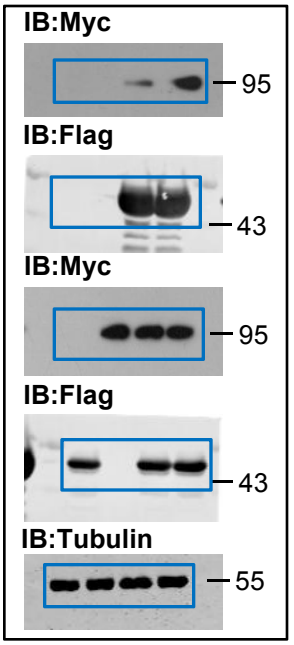

## Figure S5e




## Figure S5f




## Figure S5g



# For Supplementary Figure 5

Figure S5h

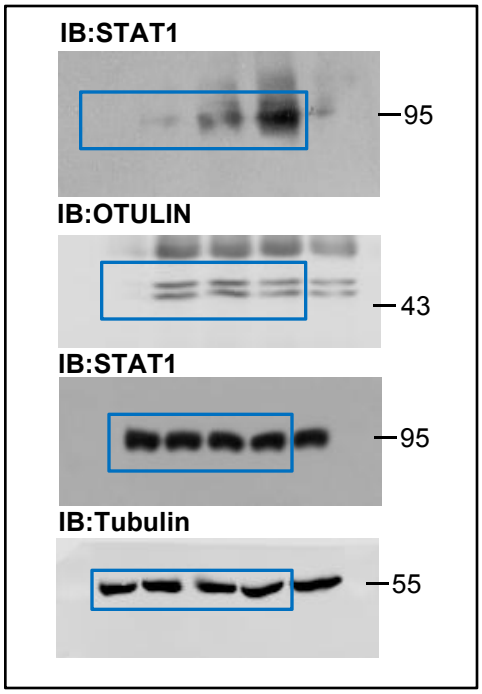
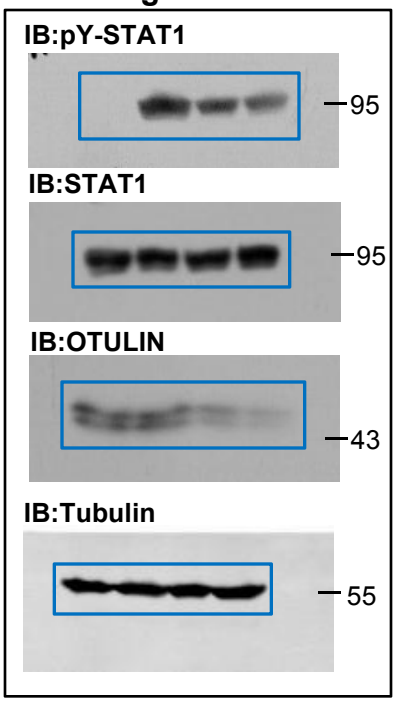
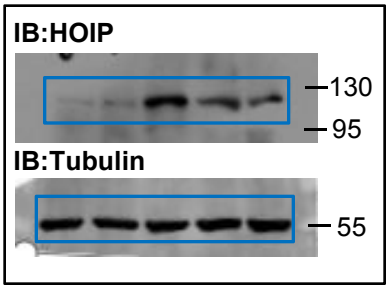



Figure S5i

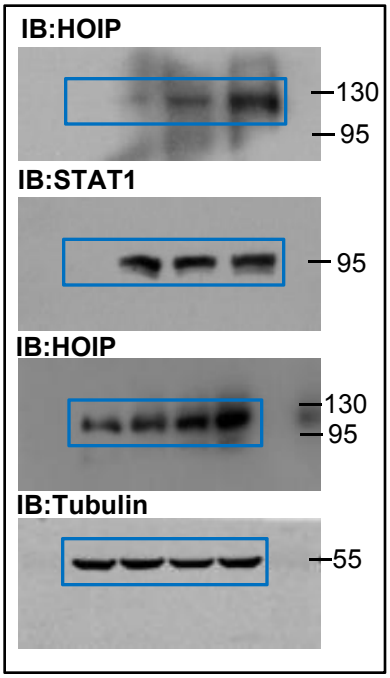


# For Supplementary Figure 6

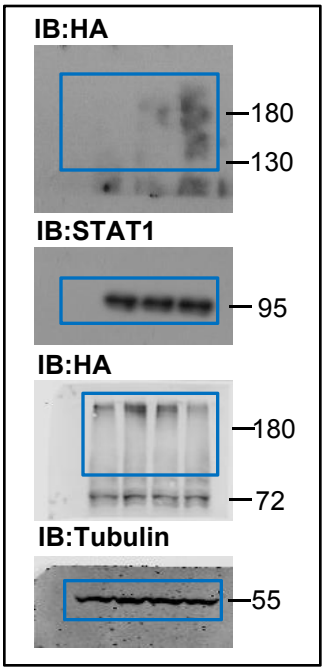
## Figure S6a



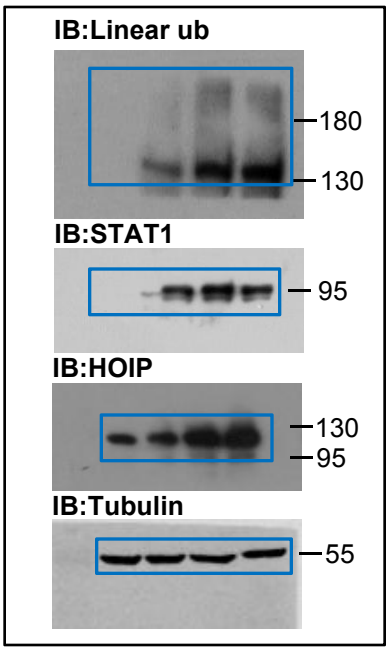

## Figure S6b



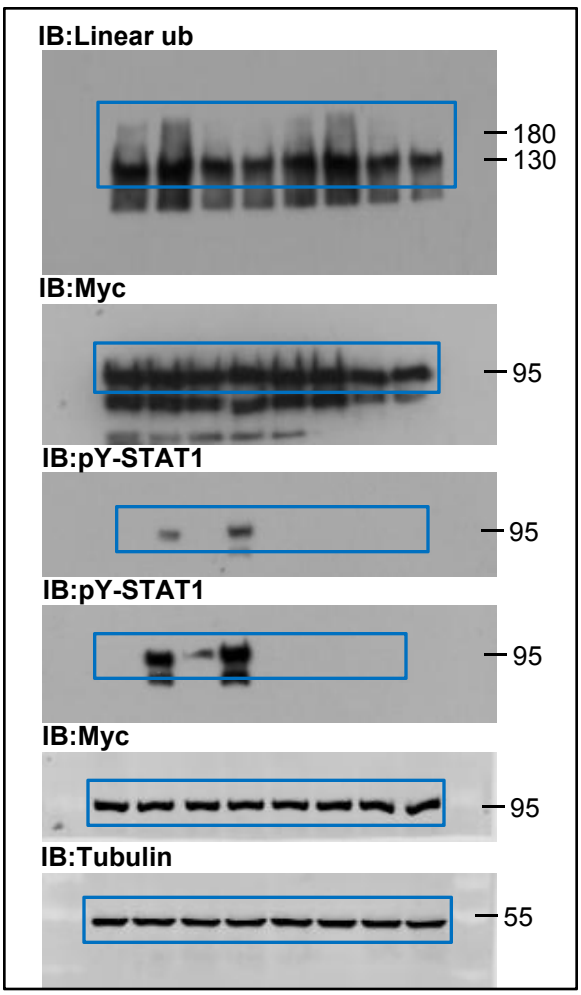

## Figure S6c



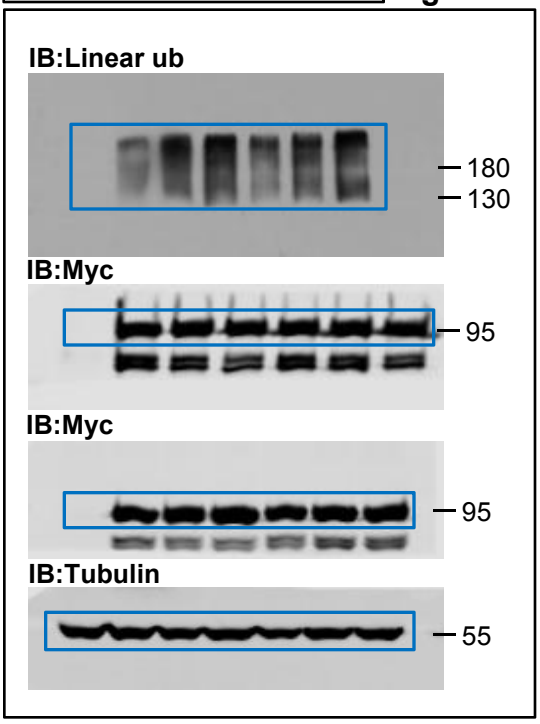

## Figure S6d




## Figure S6f

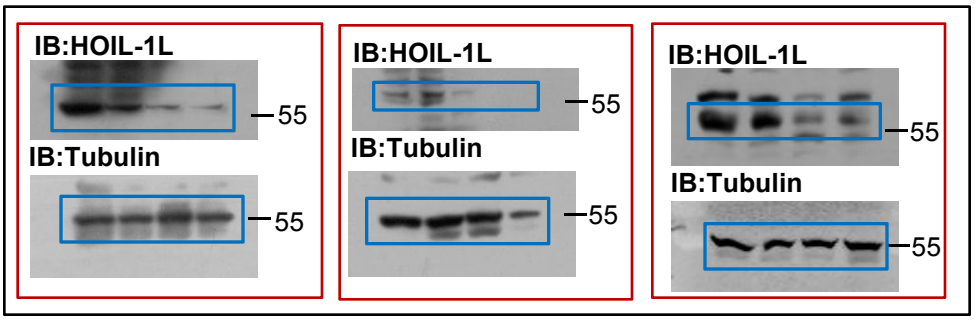



## Figure S6e

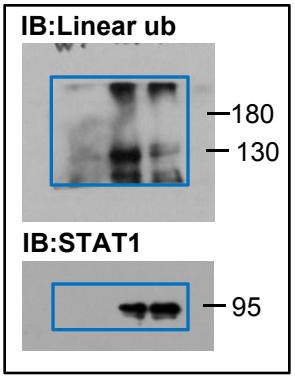


# For Supplementary Figure 7

## Figure S7a




## Figure S7b




## Figure S7c