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## Supplemental information

# The RNA helicase DHX16 recognizes specific viral RNA to trigger RIG-I-dependent innate antiviral immunity

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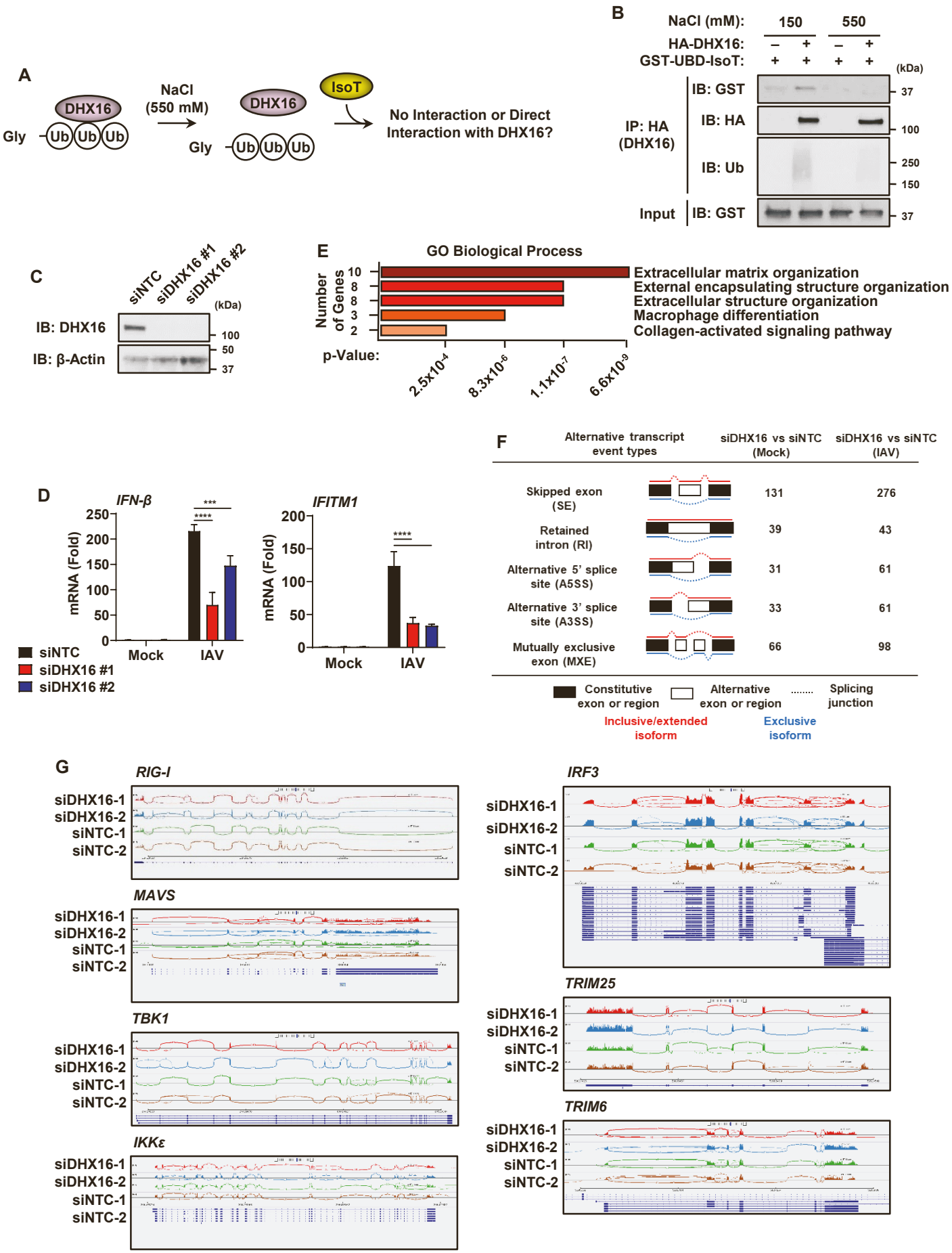
A
DHX16
Gly
Ub
NaCl
(550 mM)
IsoT
No Interaction or Direct
Interaction with DHX16?
B
NaCl (mM):
150
550
HA-DHX16:
GST-UBD-IsoT:
(kDa)
IP: HA
(DHX16)
IB: GST
IB: HA
IB: Ub
Input
C
siNTC
siDHX16 #1
siDHX16 #2
IB: DHX16
IB: β-Actin
D
IFN-β
IFITM1
mRNA (Fold)
siNTC
siDHX16 #1
siDHX16 #2
Mock
IAV
E
GO Biological Process
Number of Genes
p-Value:
Extracellular matrix organization
External encapsulating structure organization
Extracellular structure organization
Macrophage differentiation
Collagen-activated signaling pathway
F
Alternative transcript event types
siDHX16 vs siNTC (Mock)
siDHX16 vs siNTC (IAV)
Skipped exon (SE)
131
276
Retained intron (RI)
39
43
Alternative 5' splice site (A5SS)
31
61
Alternative 3' splice site (A3SS)
33
61
Mutually exclusive exon (MXE)
66
98
Constitutive exon or region
Alternative exon or region
Splicing junction
Inclusive/extended isoform
Exclusive isoform
G
RIG-I
MAVS
TBK1
IKKε
IRF3
TRIM25
TRIM6
siDHX16-1
siDHX16-2
siNTC-1
siNTC-2

**Figure S1. DHX16 knockdown does not alter splicing of IFN-I pathway genes. Related to Figures 1 and 2.** (A) Representation of the denaturing Co-IP assay used to determine if IsoT interacts with DHX16 directly or indirectly via unanchored Ub. (B) GST-UBD-IsoT interacts with DHX16 *in vitro* via unanchored Ub after normal ionic strength (150 mM NaCl), but not high ionic strength (550 mM NaCl), RIPA washes (denaturing Co-IP). (C) Individual siRNA silencing efficiency of DHX16 protein expression (immunoblot). (D) *IFN-β* and *IFITM1* expression from siNTC or individual siDHX16 treated A549s stimulated with IAV for 24 hrs (PR8 MOI=0.1) (qRT-PCR). (E) Enriched GO terms and pathways (Enrichr) of upregulated transcripts identified in the NGS heat map. (F) Number of alternative exons between siNTC and siDHX16 treated mock or IAV-infected A549s. (G) RNA-seq reads of human *RIG-I*, *MAVS*, *TBK1*, *IKKε*, *IRF3*, *TRIM25*, and *TRIM6* genes. Data are expressed as means (n=3) SD ***p < 0.001; ****p < 0.0001 (Two-way ANOVA with Dunnett's multiple comparisons). Data are representative of 2-3 independent experiments.

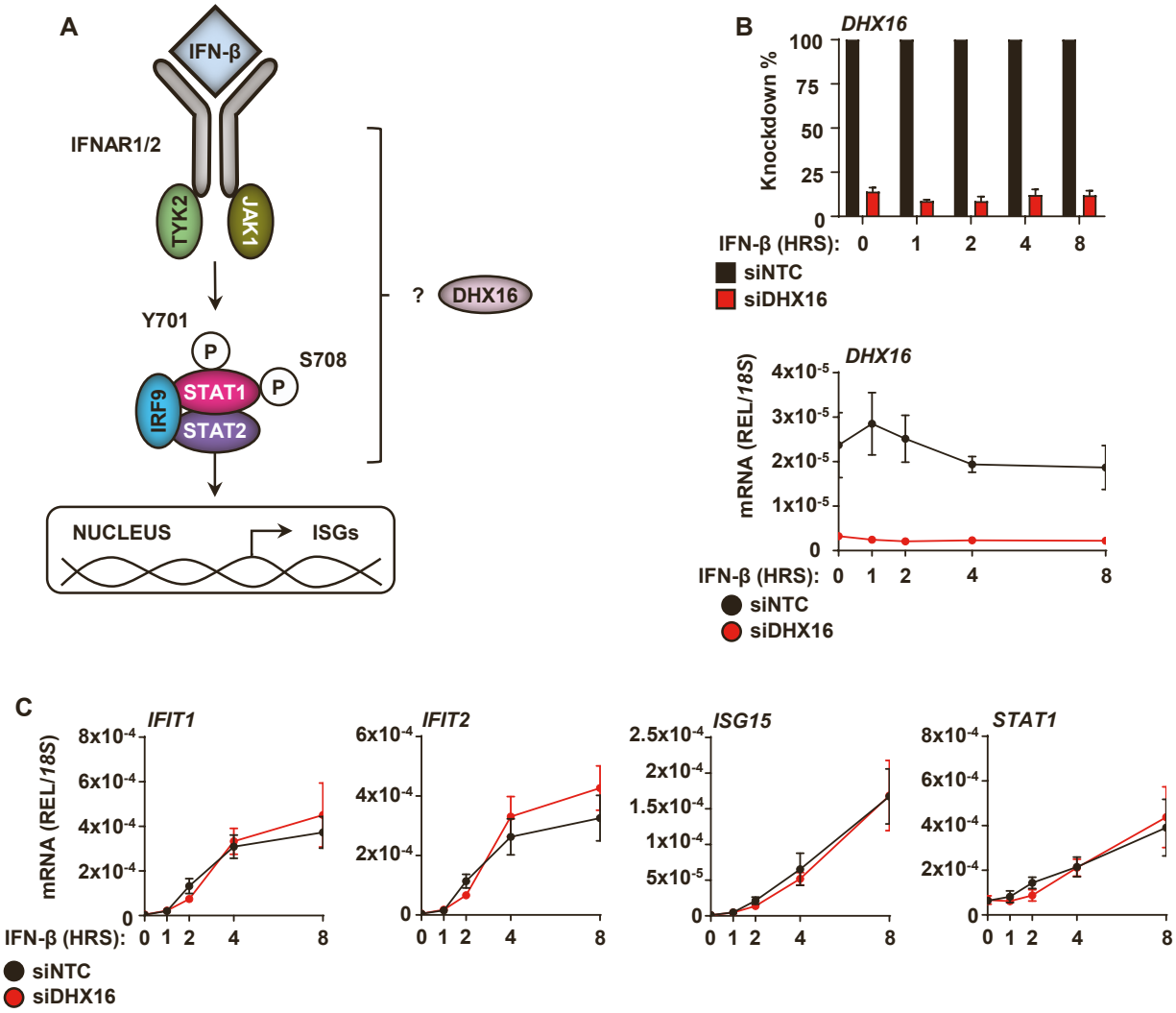


**Figure S2. DHX16 is not an IFN-stimulated gene. Related to Figure 3.** (A) Diagram of IFN-signaling pathway. (B) *DHX16* expression from siNTC or siDHX16 treated A549s stimulated with IFN-β (1000 IU/mL) (qRT-PCR). (C) ISG expression from siNTC or siDHX16 treated A549s stimulated with IFN-β (1000 IU/mL) (qRT-PCR). Data are expressed as means (n=3)  SD (Student's t-test). Data are representative of 2-3 independent experiments.

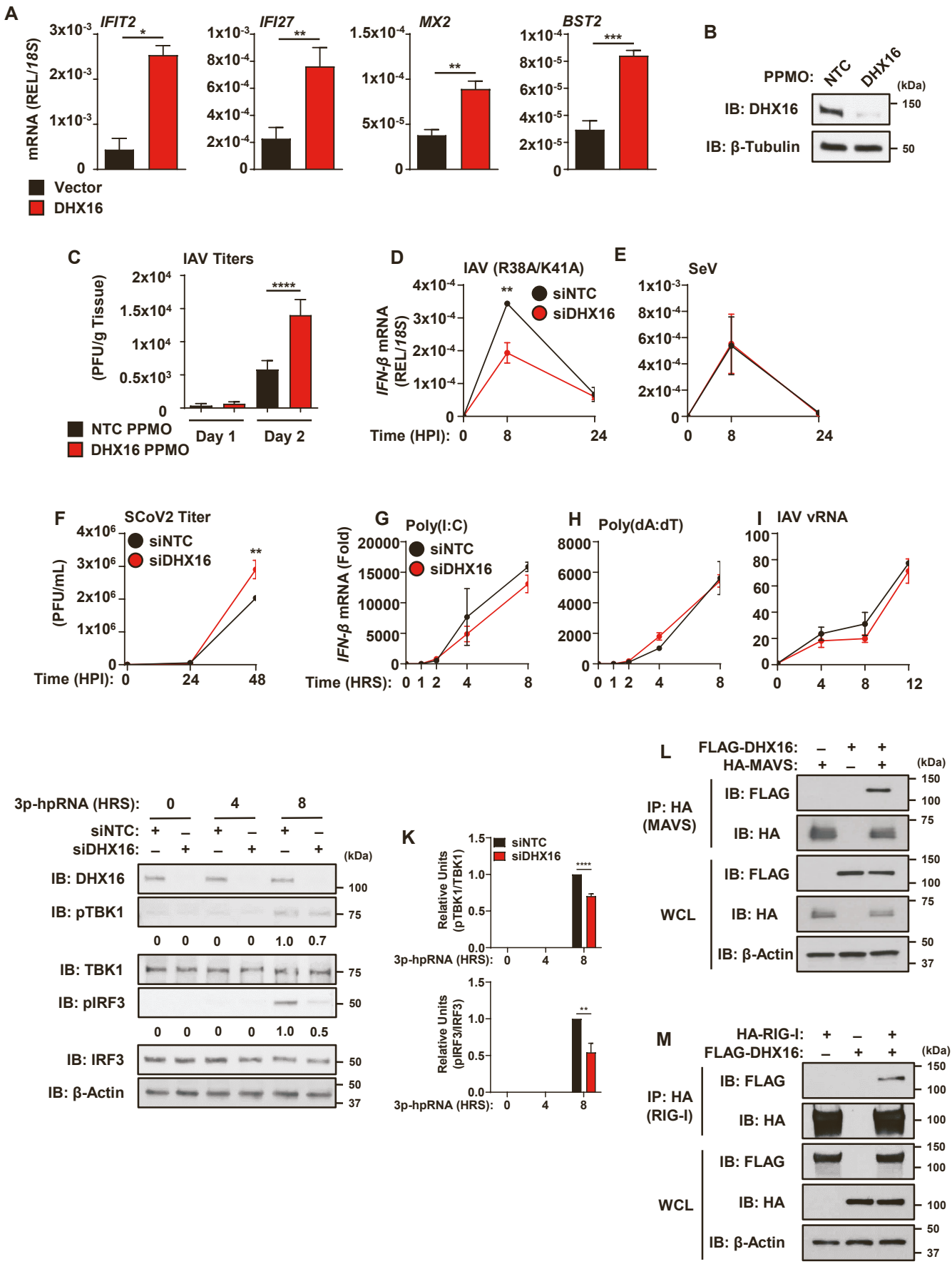
A
IFIT2
IFI27
MX2
BST2
mRNA (REL/18S)
Vector
DHX16
B
PPMO: NTC DHX16
(kDa)
IB: DHX16
IB: β-Tubulin
150
50
C
IAV Titers
(PFU/g Tissue)
NTC PPMO
DHX16 PPMO
Day 1
Day 2
D
IAV (R38A/K41A)
IFN-β mRNA (REL/18S)
siNTC
siDHX16
Time (HPI): 0 8 24
E
SeV
F
SCoV2 Titer
(PFU/mL)
Time (HPI): 0 24 48
G
Poly(I:C)
IFN-β mRNA (Fold)
Time (HRS): 0 1 2 4 8
H
Poly(dA:dT)
I
IAV vRNA
J
3p-hpRNA (HRS): 0 4 8
siNTC:
siDHX16:
IB: DHX16
IB: pTBK1
IB: TBK1
IB: pIRF3
IB: IRF3
IB: β-Actin
0 0 0 0 1.0 0.7
0 0 0 0 1.0 0.5
K
Relative Units (pTBK1/TBK1)
Relative Units (pIRF3/IRF3)
3p-hpRNA (HRS): 0 4 8
L
FLAG-DHX16:
HA-MAVS:
IP: HA (MAVS)
IB: FLAG
IB: HA
WCL
IB: β-Actin
M
HA-RIG-I:
FLAG-DHX16:
IP: HA (RIG-I)

**Figure S3. DHX16 promotes the IFN-I production pathway. Related to Figure 3 and 4.** (A) *IFIT2*, *IFI27*, *Mx2*, and *BST2* expression from A549s transfected with DHX16 for 24 hrs (qRT-PCR). (B) Validation of PPMO-mediated silencing efficiency of DHX16 in MEFs (immunoblot). (C) Viral titers from NTC or DHX16 PPMO treated C57BL/6 mice infected with IAV (PR8 100 PFU) (n=3-4/group). (D and E) *IFN-β* expression from siNTC or siDHX16 treated A549s infected with IAV (PR8 R38A/K41A MOI=0.1) (D), or SeV (Cantell 100 HAU/mL) (E) (qRT-PCR). (F) Viral titers from siNTC or siDHX16 treated Calu-3s infected with SCoV2 (icSARS-CoV-2-mNG MOI=1). (G-I) *IFN-β* expression from siNTC or siDHX16 treated A549s transfected with Poly(I:C) HMW (10 μg/mL) (G), Poly(dA:dT) (10 μg/mL) (H), or IAV vRNA (100 ng/mL) (I) (qRT-PCR). (J) IFN-I production pathway activation from siNTC or siDHX16 treated A549s stimulated with 3p-hpRNA (100 ng/mL) (immunoblot). (K) Western blot quantification for pTBK1 and pIRF3 in (J) (densitometry). (L) Interaction between DHX16 and MAVS following co-expression in HEK293Ts (Co-IP). (M) Reciprocal interaction between DHX16 and RIG-I following co-expression in HEK293Ts (Co-IP). Data are expressed as means (n=3) SD *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (Student's t-test or one-way ANOVA with Tukey's multiple comparisons). Data are representative of 2-3 independent experiments.

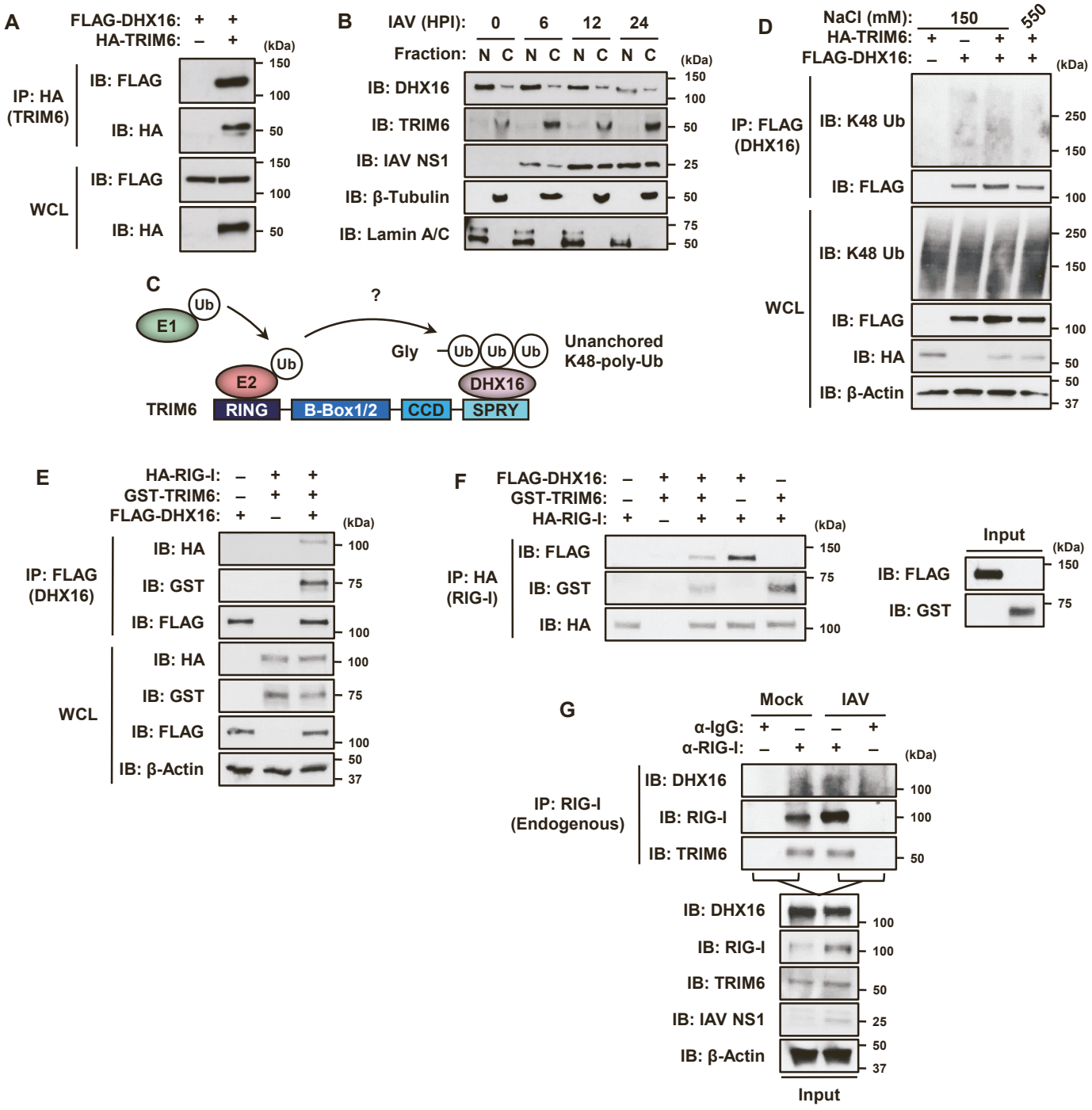


**Figure S4. DHX16 interacts with TRIM6. Related to Figure 6.** (A) Reciprocal interaction between DHX16 and TRIM6 following co-expression in HEK293Ts (Co-IP). (B) Nuclear and cytoplasmic distribution of DHX16 in mock and IAV infected A549s (PR8 MOI=1) (immunoblot). (C) Representation of RING and SPRY domain involvement in the TRIM6-mediated association of poly-Ub to targets. (D) Non-covalent interactions between DHX16 and K48-poly-Ub following co-expression with WT TRIM6 in low, but not high, salt washes in HEK293Ts (denaturing Co-IP). (E) Interaction between DHX16, RIG-I, and TRIM6 following co-expression in HEK293Ts (Co-IP). (F) Interaction between purified DHX16, RIG-I, and TRIM6 protein *in vitro* (Co-IP). (G) Interaction between endogenous DHX16, RIG-I, and TRIM6 in A549s infected with IAV for 24 hrs (PR8 MOI=1) (Co-IP). Data are representative of 2-3 independent experiments.

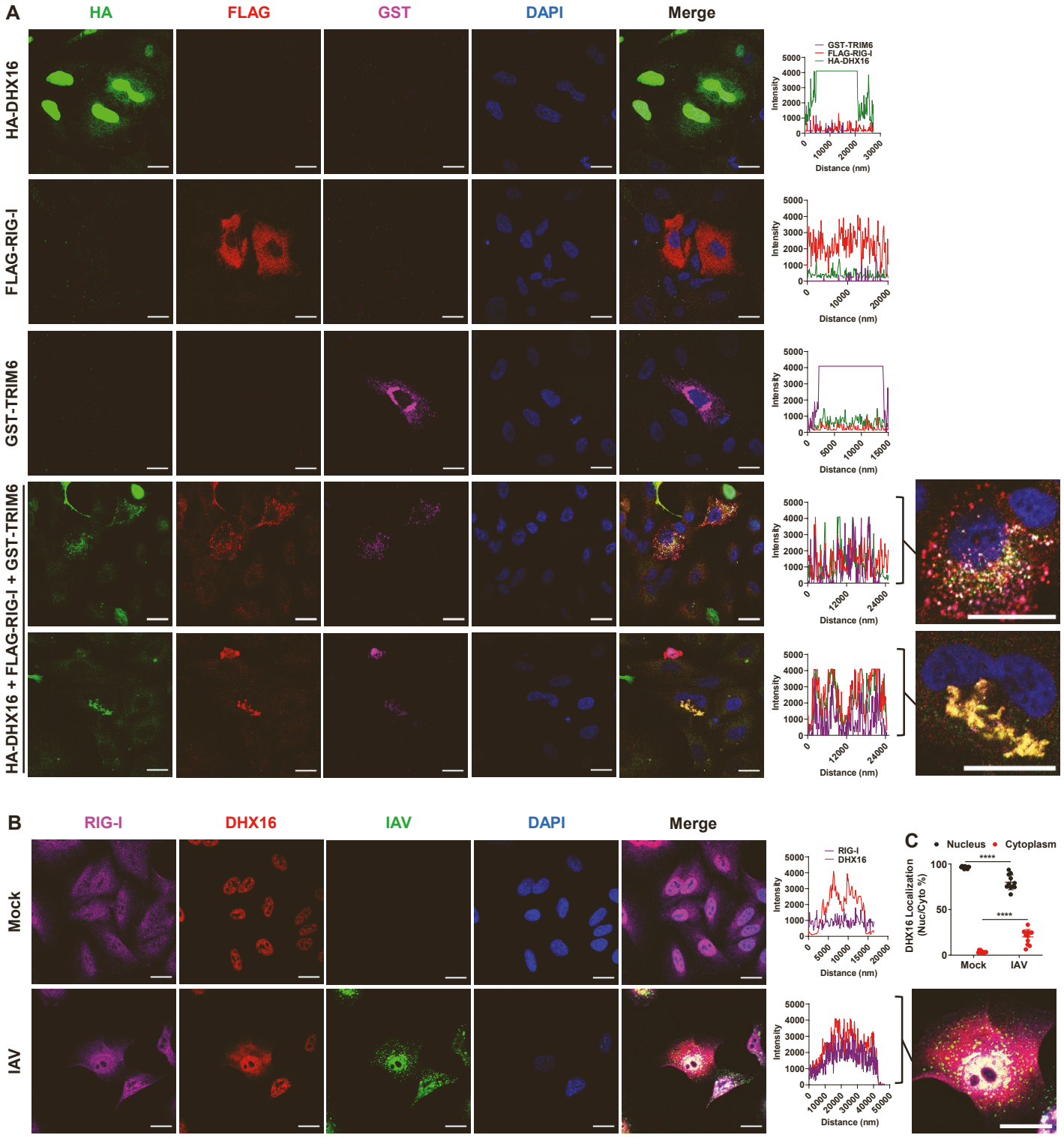


**Figure S5. DHX16 interacts with RIG-I and TRIM6 in the cytoplasm. Related to Figure 6.** (A) Co-localization between DHX16, RIG-I, and TRIM6 following co-expression in A549s. (B) Co-localization between DHX16 and RIG-I following IAV infection for 24 hrs in A549s (PR8 MOI=1). Scale bar=20 μm (confocal microscopy). (C) Nuclear/cytoplasmic distribution of DHX16 following IAV infection for 24 hrs in A549s (PR8 MOI=1) (n=11). Data are expressed as means SD ****$p < 0.0001$ (Two-way ANOVA with Tukey's multiple comparisons). Data are representative of 2-3 independent experiments.

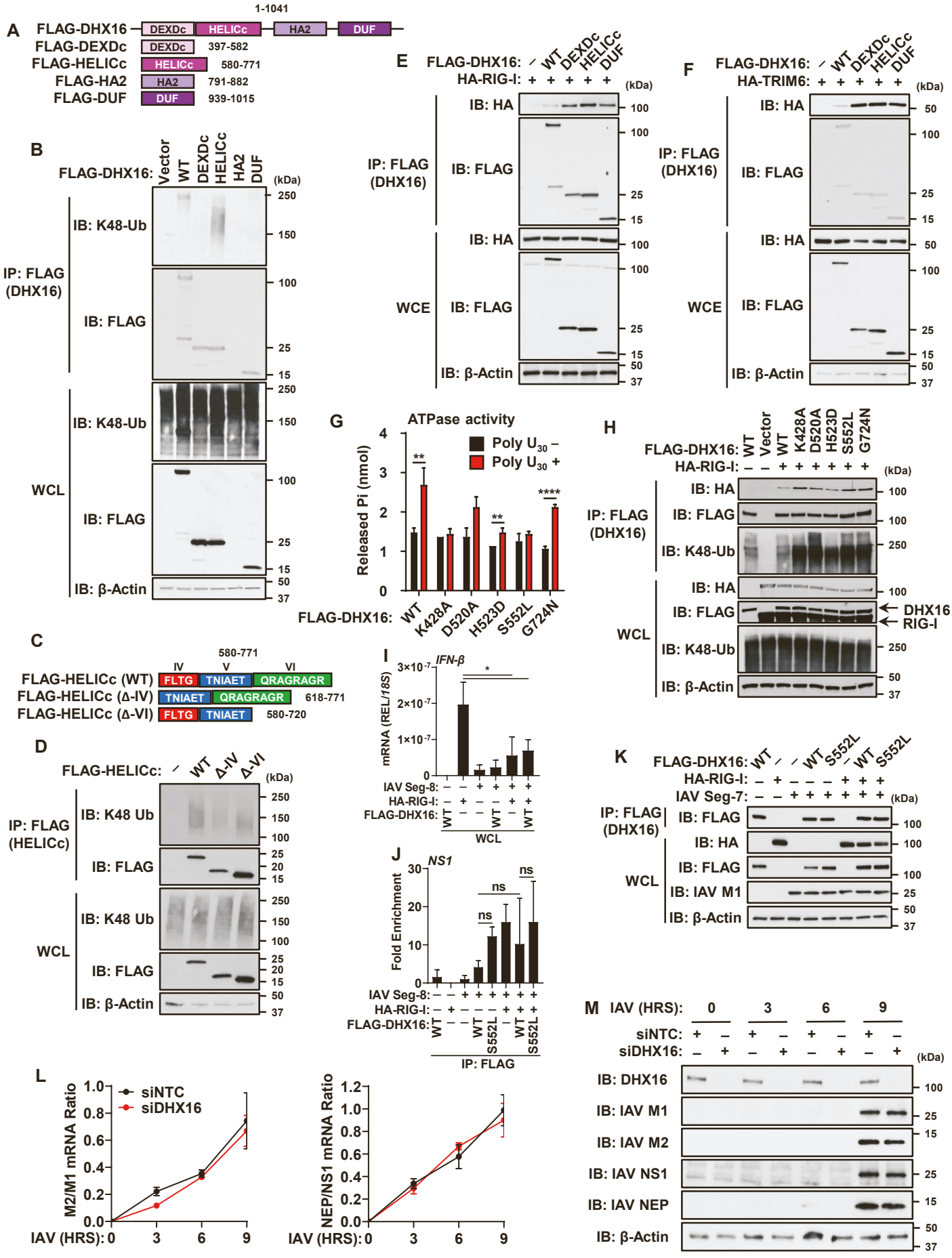
A
1-1041
FLAG-DHX16
DEXDc
HELICc
HA2
DUF
FLAG-DEXDc
397-582
FLAG-HELICc
580-771
FLAG-HA2
791-882
FLAG-DUF
939-1015
B
FLAG-DHX16:
Vector
WT
DEXDc
HELICc
HA2
DUF
(kDa)
IP: FLAG (DHX16)
IB: K48-Ub
IB: FLAG
WCL
IB: β-Actin
C
580-771
IV
V
VI
FLAG-HELICc (WT)
FLTG
TNIAET
QRAGRAGR
FLAG-HELICc (Δ-IV)
618-771
FLAG-HELICc (Δ-VI)
580-720
D
FLAG-HELICc:
Δ-IV
Δ-VI
IP: FLAG (HELICc)
IB: K48 Ub
E
HA-RIG-I:
IB: HA
F
HA-TRIM6:
G
ATPase activity
Poly U30 –
Poly U30 +
Released Pi (nmol)
H
K428A
D520A
H523D
S552L
G724N
DHX16
RIG-I
I
IFN-β
mRNA (REL/18S)
IAV Seg-8:
J
NS1
Fold Enrichment
ns
IP: FLAG
K
IB: IAV M1
L
siNTC
siDHX16
M2/M1 mRNA Ratio
NEP/NS1 mRNA Ratio
IAV (HRS):
M
IAV (HRS):
siNTC:
siDHX16:
IB: DHX16
IB: IAV M1
IB: IAV M2
IB: IAV NS1
IB: IAV NEP
IB: β-Actin

**Figure S6. DHX16 knockdown does not alter splicing of IAV genes. Related to Figure 7.** (A) Diagram of DHX16 domains mutants used. (B) Interactions between DHX16 (WT and domain mutants) and K48-poly-Ub following co-expression in HEK293Ts (Co-IP). (C) Diagram of DHX16-HELICc motif mutants used. (D) Interactions between DHX16-HELICc (WT and motif mutants) and K48-poly-Ub following co-expression in HEK293Ts (Co-IP). (E and F) Interactions between DHX16 (WT and domain mutants) and RIG-I (E) or TRIM6 (F) following co-expression in HEK293Ts (Co-IP). (G) ATPase activity of DHX16 (WT or mutants) in an *in vitro* ATPase assay. (H) Interactions between DHX16 (WT and mutants) and RIG-I following co-expression in HEK293Ts (Co-IP). (I) Input *IFN-β* expression from IAV segment 8 RNA-IP (qRT-PCR). (J) Interaction between DHX16 (WT or S552L) and segment 8 of IAV in the presence or absence of RIG-I (fold enrichment over segment 8 alone) (RNA-IP). (K) Input protein expression from RNA-IP using IAV segments (immunoblot). (L) *M2*/*M1* and *NEP*/*NS1* expression from siNTC or siDHX16 treated A549s infected with IAV (PR8 MOI=1) (qRT-PCR). (M) Expression of IAV proteins from siNTC or siDHX16 treated A549s infected with IAV (PR8 MOI=1) (immunoblot). Data are expressed as means (n=3) SD *p < 0.05; **p < 0.01; ****p < 0.0001 (Student's t-test or one-way ANOVA with Tukey's multiple comparisons). Data are representative of 2-3 independent experiments.

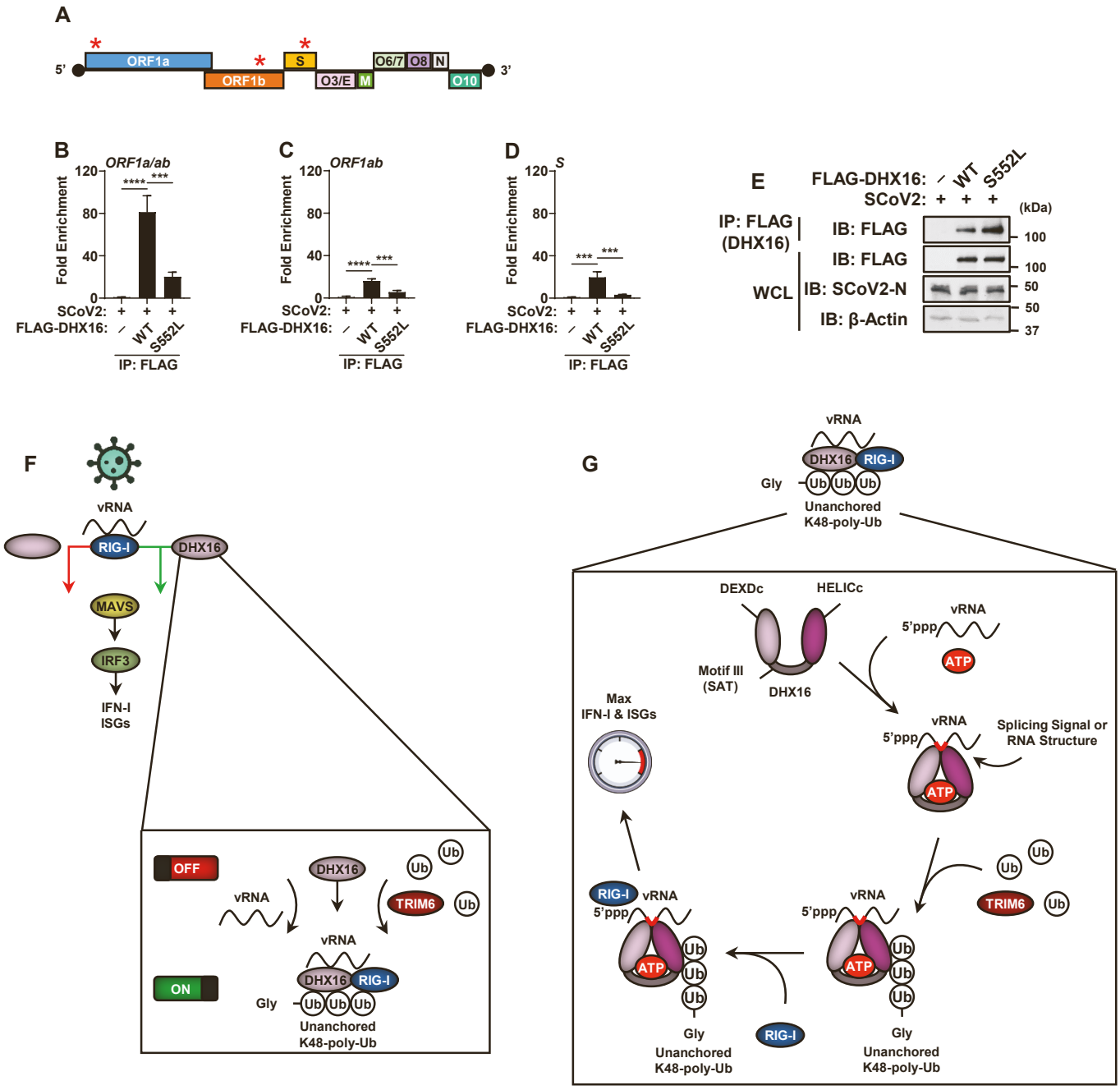


**Figure S7. DHX16 enhances IFN-I production in response to virus infection. Related to Figure 7.** (A) Diagram of SCoV2 genome. Stars indicate amplification regions of qRT-PCR primers used. (B-D) Interaction between DHX16 (WT or S552L) and SCoV2 vRNAs from HEK293T-hACE2s infected for 24 hrs (USA-WA1/2020 MOI=0.1) (fold enrichment over SCoV2 alone) (RNA-IP). (E) Input protein expression from RNA-IP using SCoV2 (immunoblot). (F and G) Model of DHX16-dependant enhancement of antiviral innate immunity. (F) DHX16 enhances RIG-I-mediated IFN-I production in response to virus infection. Activation of DHX16 requires recognition of vRNA and interactions with unanchored K48-poly-Ub chains synthesized by TRIM6. These interactions promote the recruitment of RIG-I. (G) Sensing of vRNAs by DHX16 requires specific PAMPs (splicing signals in IAV and potentially RNA structures or Poly U sequences for other viruses). This recognition requires an intact motif III (SAT) which utilizes the binding energy of ATP to alter the conformation of the helicase domain into a high-affinity RNA clamp. Upon engagement with an RNA substrate, K48-poly-Ub chains synthesized by TRIM6 associate with DHX16's HELICc domain. The presence of these unanchored Ub chains facilitate interactions with RIG-I, which is required for the DHX16-dependent enhancement of downstream IFN-I signaling and optimal antiviral innate immunity. Data are expressed as means (n=3) SD ***$p < 0.01$; ****$p < 0.0001$ (One-way ANOVA with Tukey's multiple comparisons). Data are representative of 2-3 independent experiments.

**SUPPLEMENTAL TABLES**

**Table S1: Primer sequences to generate mutations and fusion constructs for DHX16, related to STAR Methods.**

| CONSTRUCT | FORWARD | REVERSE |
|---|---|---|
| Oligonucleotides for cloning constructs | | |
| HA-DHX16 | 5'-ATAATAGAATTCATGGCGACGCCGGCGGGTCTGGAGCGCTGGGTTCAGGACGAGCTGCACTCGGTGTTG-3' | 5'-TAATATCTCGAGTTAGTAATCTGGAACATCGTATGGGTACATTGCTGCTGCCCCTAGCTCTTCTCGTGTTTTG-3' |
| FLAG-DHX16 (K428A) | 5'-AGACAGGCTCAGGGGCGACCACCCAGAT-3' | 5'-ATCTGGGTGGTCGCCCCTGAGCCTGTCT-3' |
| FLAG-DHX16 (D520A) | 5'-CGTGGTGATGGTGGCAGAGGCACACGAAA-3' | 5'-TTTCGTGTGCCTCTGCCACCATCACCACG-3' |
| FLAG-DHX16 (H523D) | 5'-TGGTGGATGAGGCAGATGAAAGGACCCTACACACAGA-3' | 5'-TCTGTGTGTAGGGTCCTTTCATCTGCCTCATCCACCA-3' |
| FLAG-DHX16 (S552L) | 5'-TCAAGGTCCTGGTGGCTTTAGCCACAATGGACACT-3' | 5'-AGTGTCCATTGTGGCTAAAGCCACCAGGACCTTGA-3' |
| FLAG-DHX16 (G724N) | 5'-ATTAGAATTCGTCGACTGGATCCGGTACCGAGGAGATCT-3' | 5'-TAATGTATACAGGCGGAAGCACTTCCCTGCAGCCACCCGACCTGCCCTGTTAGCTCGCTGAT-3' |
| FLAG-DHX16 (DEXDc) | 5'-ATTAGCGATCGCCATGAGCCTCCCGGTGTTC-3' | 5'-TAATACGCGTGAAGATGTCCACAGGAAACCT-3' |
| FLAG-DHX16 (HELICc) | 5'-ATTAGCGATCGCCATGGACATCTTCTACA-3' | 5'-TAATACGCGTCCCTAAGCTCTTGAGCAGCAACA-3' |
| FLAG-DHX16 (HA2) | 5'-ATTAGCGATCGCCATGCTGGCTTTGGAGCAGCTGTAT-3' | 5'-TAATACGCGTTAGCAGAACCAGGTGGTCA-3' |

| | | |
|---|---|---|
| FLAG-DHX16 (DUF) | 5’-ATTAGCGATCGCCATGGTACGCAAGGCCATCACT-3’ | 5’-TAATACGCGTATAATGGGGAGCCACCTCCAGAA-3’ |
| FLAG-DHX16 (HELICc Δ-IV) | 5’-ATTAGCGATCGCCATGCCCATTTATGCCAATCTGCCCTCT-3’ | 5’-TAATACGCGTCCCTAAGCTCTTGAGCAGCAACA-3’ |
| FLAG-DHX16 (HELICc Δ-VI) | 5’-ATTAGCGATCGCCATGGACATCTTCTACA-3’ | 5’-TAATACGCGTGCTCTTCTGCTTACAGAACCCT-3’ |

**Table S2: Primer sequences to determine mRNA transcript levels, related to STAR Methods.**

| REAGENT or RESOURCE | FORWARD | REVERSE |
|---|---|---|
| Oligonucleotides for qRT-PCR | | |
| *h18S rRNA* | 5'-GTAACCCGTTGAACCCCATT-3' | 5'-CCATCCAATCGGTAGTAGCG-3' |
| *hDHX16* | 5’-CCAACTGCTCTCAATCTCCA-3’ | 5’-ATCCCAACTCCTCCCTCTTT-3’ |
| *hIFN-β* | 5'-TCTGGCACAACAGGTAGTAGGC-3' | 5'-GAGAAGCACAACAGGAGAGCAA-3' |
| *hIFIT1* | 5’-CAAAGGGCAAAACGAGGCAG-3’ | 5’-CCCAGGCATAGTTTCCCCAG-3’ |
| *hIFITM1* | 5’-TGACCATTGGATTCATCCTG-3’ | 5’-TGCACAGTGGAGTGCAAAG-3’ |
| *hIFIT2* | 5'-ATGTGCAACCTACTGGCCTAT-3' | 5'-TGAGAGTCGGCCCATGTGATA-3' |
| *hIFI27* | 5’-ACTGGGAGCAACTGGACTCT-3’ | 5’-TAGAACCTCGCAATGACAGC-3’ |
| *hMX2* | 5’-AGGTTCCAGACCTGACCATC-3’ | 5’-GTCTGCTGCCTCTGGATGTA-3’ |
| *hBST2* | 5’-GAAAGTGGAGGAGCTTGAGG-3’ | 5’-ACTTCTTGTCCGCGATTCTC-3’ |
| *hISG15* | 5'-TCCTGGTGAGGAATAACAAGGG-3' | 5'-GTCAGCCAGAACAGGTCGTC-3' |

| | | |
|---|---|---|
| *hSTAT1* | 5'-ACAGCAGAGCGCCTGTATTG-3' | 5'-CAGCTGATCCAAGCAAGCAT-3' |
| *mβ-Actin* | 5'-CGGTTCCGATGCCCTGAGGCTCTT-3' | 5'-CGTCACACTTCATGATGGAATTGA-3' |
| *mIFN-β* | 5'-CAGCTCCAAGAAAGGACGAAC-3' | 5'-GGCAGTGTAACTCTTCTGCAT-3' |
| *mIFITM1* | OriGene Cat #MP206689 | OriGene Cat #MP206689 |
| *IAV NP* | 5'-GCCTGCCTGCCTGTGTGTATGGAT-3' | 5'-GGCATGCCATCCACACCAGTTGAC-3' |
| *IAV M1* | 5'-AGATGAGTCTTCTAACCGAGTCG-3' | 5'-TGCAAAAACATCTTCAAGTCTCT-3' |
| *IAV M2* | 5'-CCGAGGTCGAAACGCCTATCAG-3' | 5'-GCAATAGTGAGAGGATCACTTGAAC-3' |
| *IAV NS1* | 5'-GATCCAAACACTGTGTCAAGCTTTC-3' | 5'-ATCCGCTCCACTATCTGCTT-3' |
| *IAV NEP* | 5'-GGGTGACAAAGACATAATGG-3' | 5'-TCTCCCATTCTCATTACTGC-3' |
| *SCoV2 ORF1a/ab* | 5'-AGTTACGGCGCCGATCTAAAGTCAT-3' | 5'-TAGCCATCAGGGCCACAGAAGTT-3' |
| *SCoV2 ORF1ab* | 5'-TACGTGCATGGATTGGCTTCGAT-3' | 5'-GTTTAAATTGATCTCCAGGCGGTGGT-3' |
| *SCoV2 S* | 5'-GCCTTACTGTTTTGCCACCT-3' | 5'-TGATTGTACCCGCTAACAGTGC-3' |