Supplemental Information

Antiviral activity of human oligoadenylate synthetases-like (OASL) is mediated by enhancing retinoic acid-inducible gene I (RIG-I) signaling.

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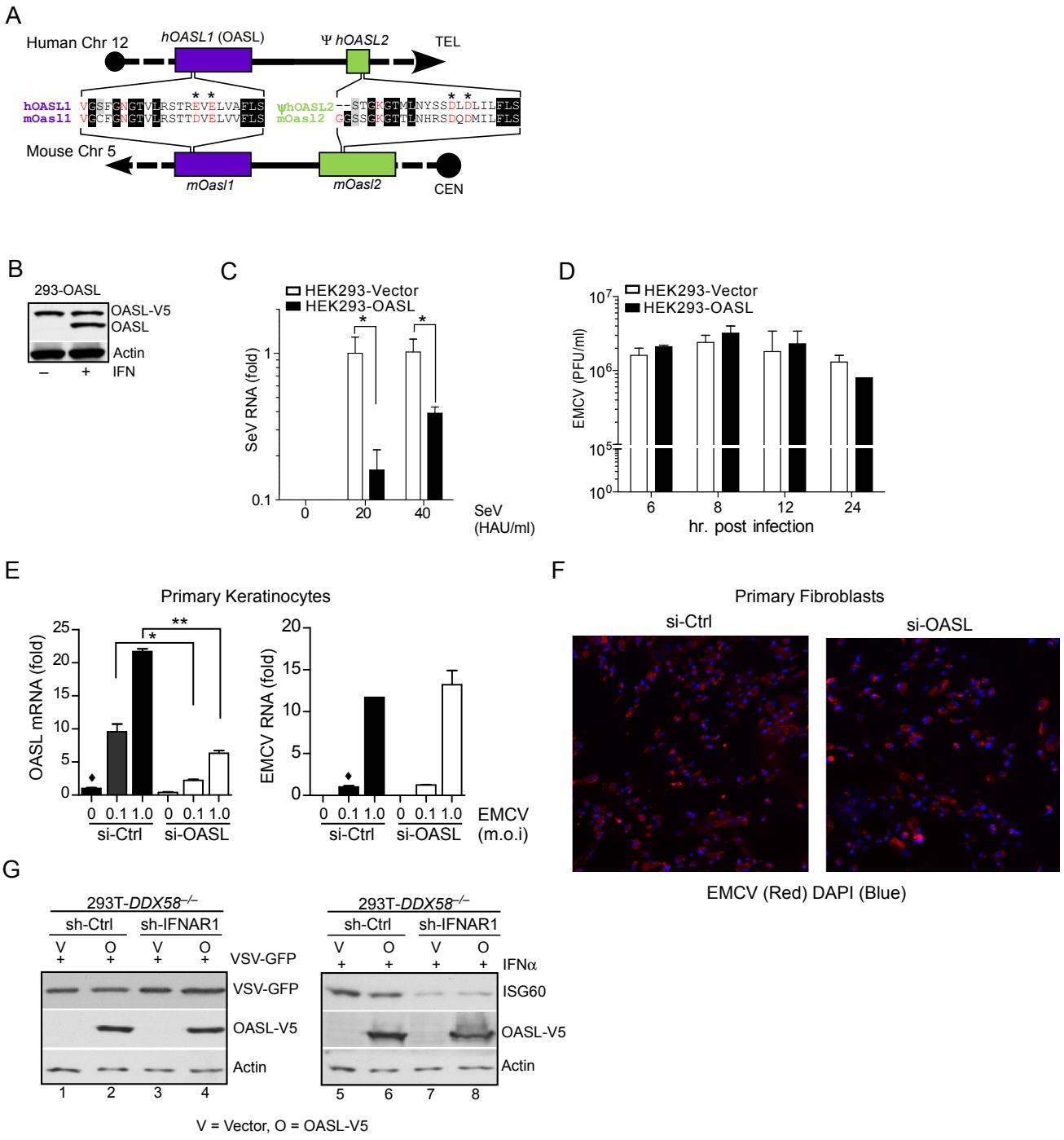


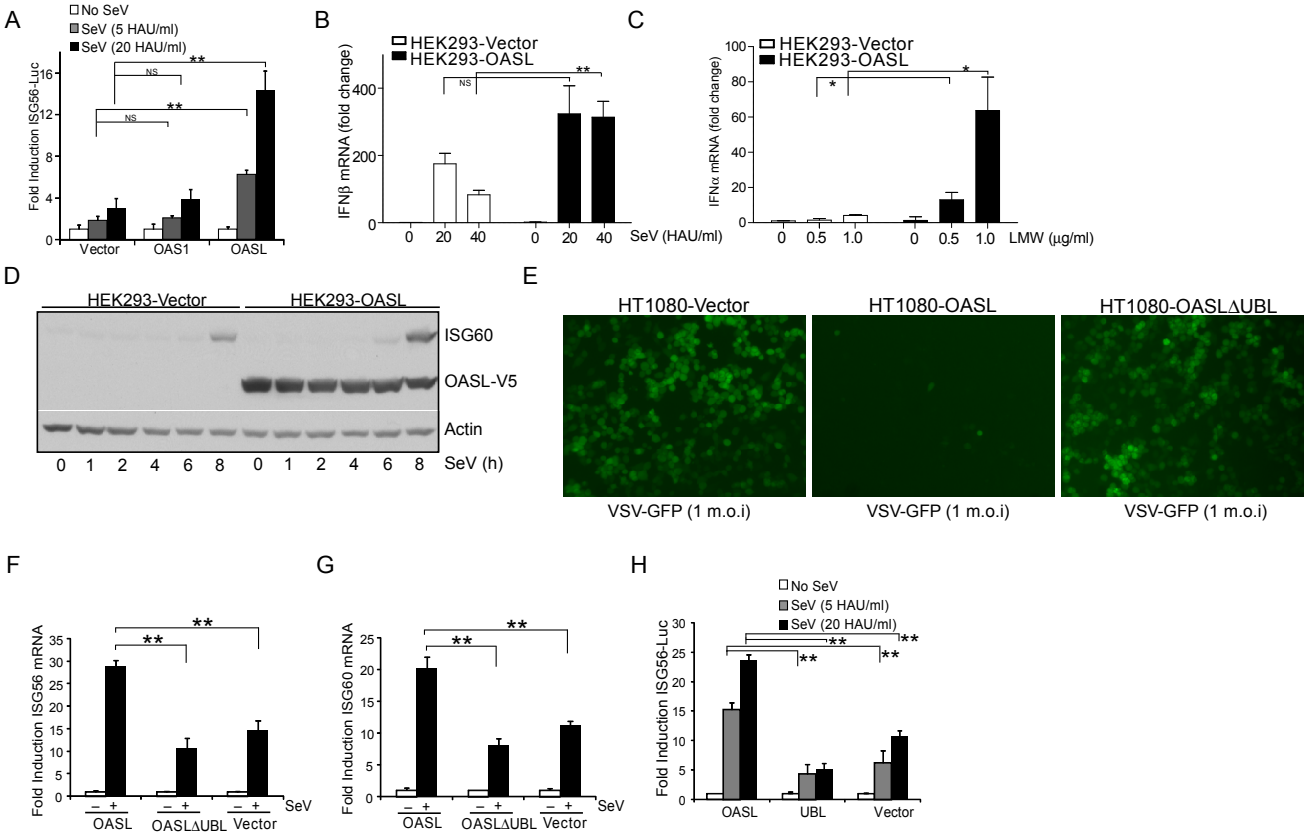
Supplementary Fig. S1

SUPPLEMENTATRY FIGURE LEGENDS:

Fig. S1: Loss of OASL enhances RNA virus replication in a RIG-I dependent manner. (Related to Fig. 1, 2 and 3)

(A) Schematic representation of human and mouse *OASL* locus and their respective active site amino acid sequence alignments. (B) Comparable expression of endogenous OASL protein in HEK293-OASL cells after IFN treatment. HEK293-OASL cells were treated with IFN (500U/ml, O/N) to induce expression of endogenous OASL followed by immunoblotting (IB) with OASL antibody. (C) Inhibition of SeV replication in OASL expressing cells. HEK293-OASL stable cells and HEK293-vector stable cells were infected with SeV for 24 h followed by detection of SeV RNA by qRT-PCR. (D) OASL expression has no effect in EMCV replication. HEK293-OASL stable cells and HEK293-vector stable cells were infected with EMCV at 5 m.o.i. Supernatants were harvested at the indicated time points and virus titers were determined by plaque assay on Vero cells. (E-F) OASL silencing does not affect EMCV replication in primary human cells. OASL expression was partially silenced in keratinocytes (E) or in primary fibroblasts (F) as described in Fig. 1G followed by EMCV infection at indicated doses. OASL expression and EMCV replication in keratinocytes were measured by qRT-PCR following 24h infection (E). EMCV replication in fibroblasts was detected 16 h post infection by indirect immunofluorescence using anti-dsRNA (J2) antibody (Weber et al., 2006) (F). (G) RIG-I dependent anti-VSV activity of OASL is independent of IFN signaling. 293T-*DDX58*$^{-/-}$ cells stably expressing OASL (O) or vector (V) control were transfected with IFNAR1 shRNA or control pLKO.1 vector control for 48 h (Umemura et al., 2012). Transfected cells were split into two parts: one part was infected with VSV (1 m.o.i) for 24h; another part was stimulated with

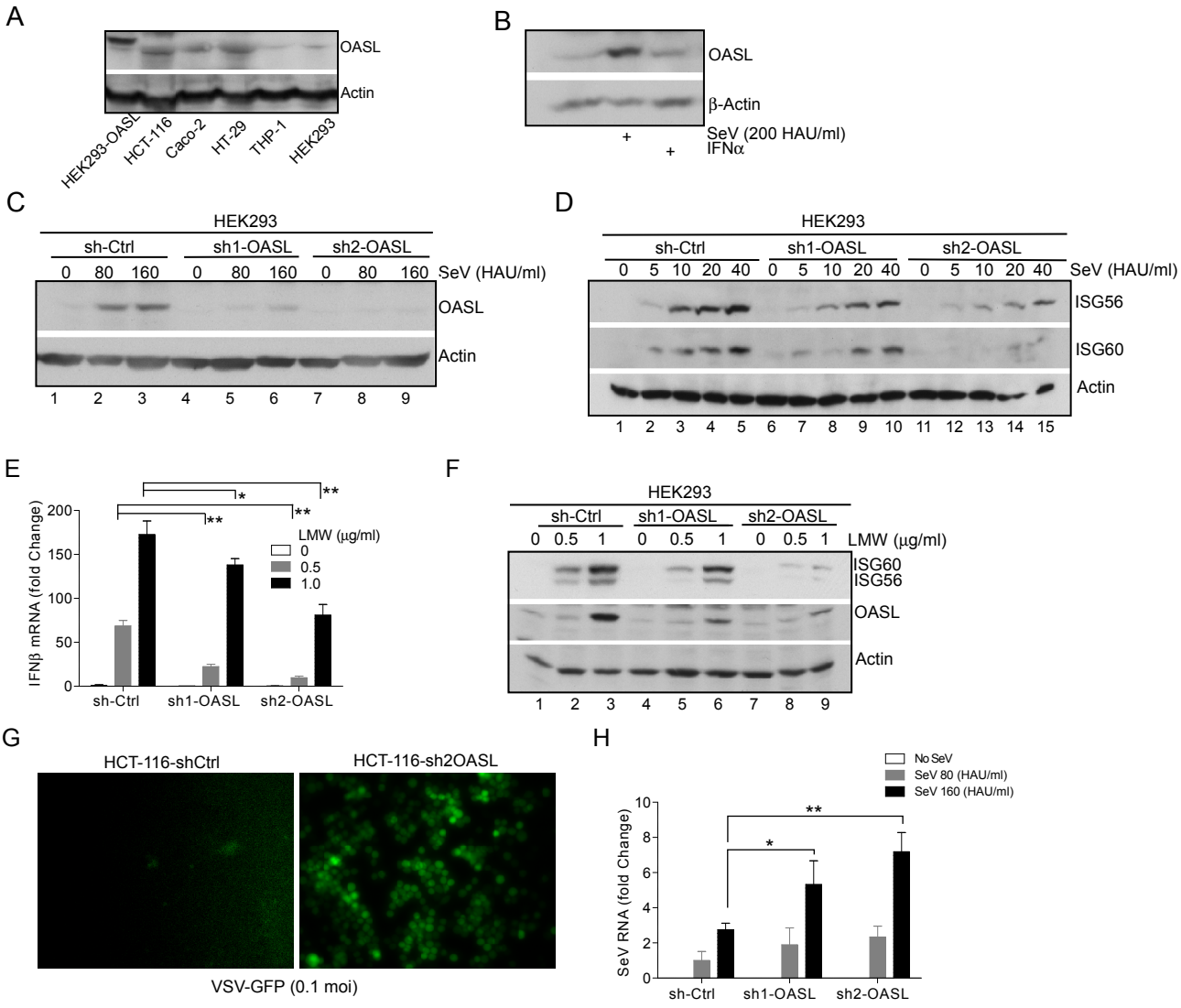
IFNα (1000 U/ml) for 14h. The cells lysates were analyzed by IB for GFP, V5-OASL, ISG60 and actin respectively. ♦ set as 1 for comparison. * $P < 0.05$ by two-tailed Student t test.



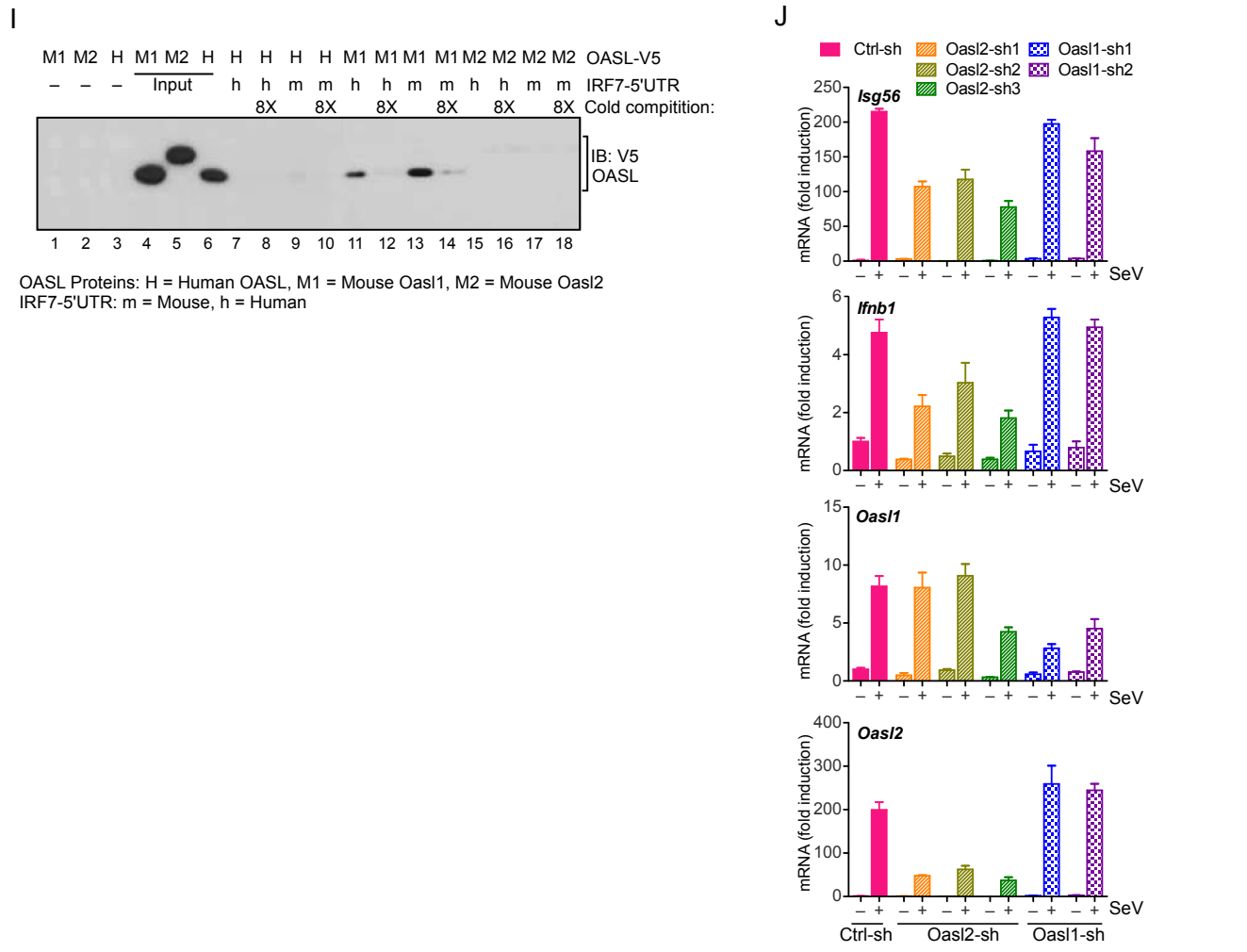
Supplementary Fig. S2

Fig. S2: OASL enhances RIG-I mediated IRF3 signaling in a UBL dependent manner. (Related to Fig. 4)

(A) HEK293 cells were co-transfected either with vector control, OASL or OAS1 along with ISG56-luciferase and β-actin *Renilla* luciferase reporter as described before. The cells were stimulated with SeV for 16 h as indicated, followed by luciferase activity measurement. (B) Induction of IFNβ mRNA by SeV infection is enhanced in OASL expressing cells. HEK293-OASL and HEK293-vector stable cells were infected with SeV at the indicated concentrations for 24 h followed by qRT-PCR using IFNβ primers. (C) Induction of IFNα mRNA by low molecular weight p(I):p(C) (LMW) transfection is enhanced in OASL expressing cells. Cells were transfected with LMW at the indicated concentrations for 24 h, followed by qRT-PCR using common IFNα primers. (D) Kinetics of ISG induction by SeV infection is not changed by OASL. HEK293-OASL and HEK293-vector stable cells were infected with SeV(20 HAU/ml) for various times as indicated followed by analysis of cell lysates by IB. (E-G) OASL UBL domain is required for its antiviral activity against VSV and enhancement of ISG induction by SeV. HT1080-OASL, HT1080-OASLΔUBL, and HT1080-vector stable cells were infected with VSV (1 m.o.i). GFP florescence was observed under florescence microscope at 8 h post-infection (E). HEK293-OASL, HEK293-OASLΔUBL, and HEK293-vector stable cells were infected with SeV (20 HAU/ml) or mock-infected for 16 h followed by total RNA extraction and quantitation by real-time RT-PCR using ISG56 (F) and ISG60 (G) primers. (H) OASL UBL domain alone does not enhance ISG induction by SeV. ISG56 reporter assays were performed in HEK293 cells as before in presence of OASL, UBL or control vector. * $P < 0.05$, ** $P < 0.01$, NS, not statistically significant.



Supplementary Fig. S3



Supplementary Fig. S3

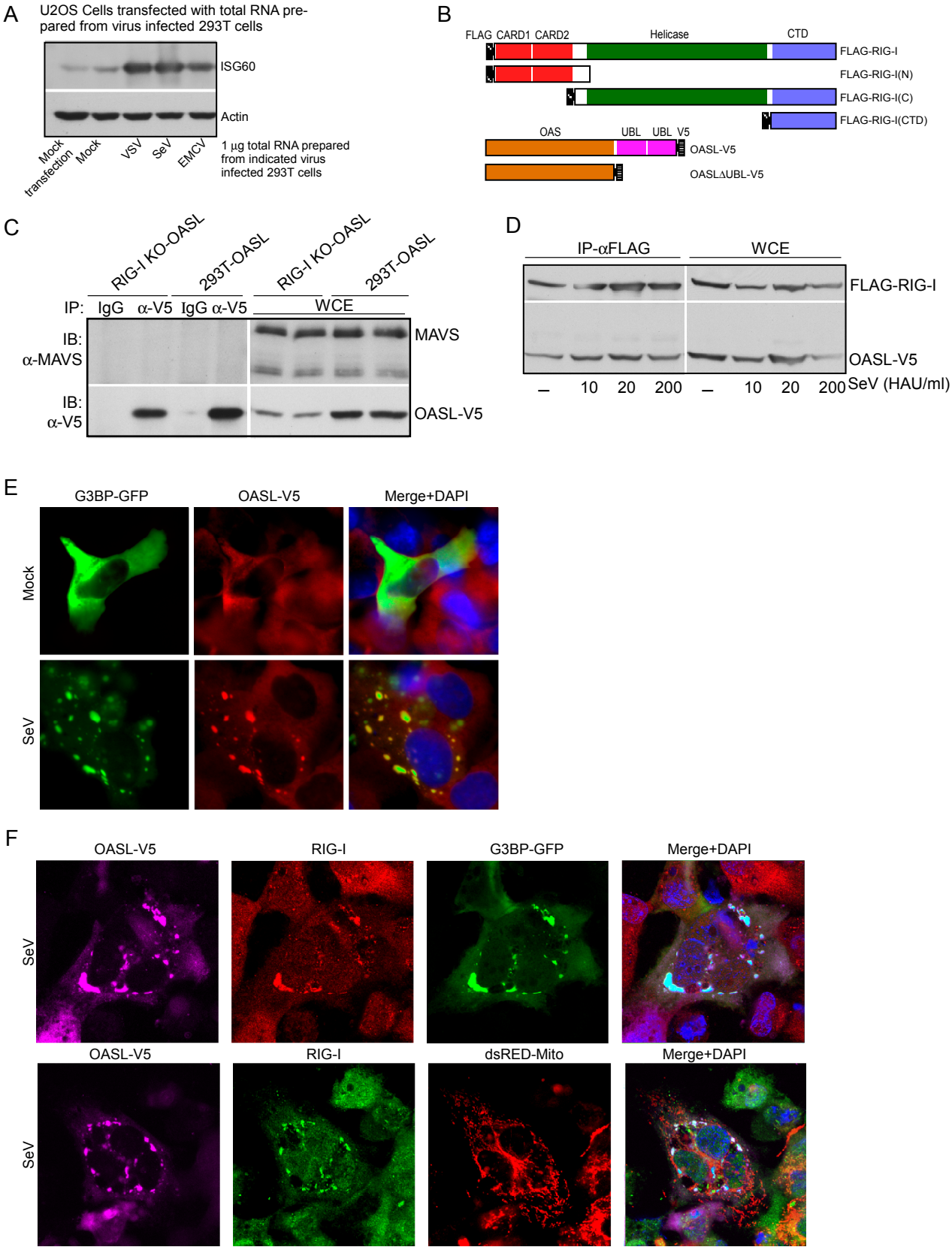
Fig. S3: Loss of OASL in different human cells and mouse macrophages decreased RIG-I signaling and enhanced virus replication. (Related to Fig. 5)

(A) OASL expression levels in different cell types were analyzed by IB with OASL antibody. (B) Analysis of OASL induction by SeV infection and IFNα stimulation in HCT-116 cells. (C) shRNA mediated silencing of OASL in HEK293 cells. HEK293 cells were transfected OASL shRNA or control vector, followed by puromycin selection. The selected cells were stimulated with SeV as indicated for 24h followed by IB analysis for OASL silencing with OASL antibody. (D) OASL silencing reduces RIG-I signaling in HEK293 cells. HEK293 cells stably expressing OASL shRNA were stimulated with SeV as indicated for 16 h followed by IB analysis. (E-F) HEK293 cells stably expressing OASL shRNA were transfected with low molecular weight p(I):p(C) (LMW) at the indicated concentrations for 24 h; the cells were harvested and analyzed by qRT-PCR for IFNβ mRNA induction (E), and immunoblotted for ISG56 and ISG60 protein induction (F). (G) Increased susceptibility of HCT-116 cells to VSV infection after OASL silencing. HCT-116 cells stably expressing OASL shRNA were infected with VSV (0.1 m.o.i) for 48 h, and GFP signal were visualized by fluorescence microscope. (H) RNA from Fig. 5 panel B were analyzed for SeV replication by qRT-PCR. (I) Unlike mouse Oasl1, human OASL or mouse Oasl2 does not bind to either mouse or human IRF7 5' UTR. RNA pull-down analysis of various partially purified human and mouse OASL proteins as done in Fig. 1A. (J) Silencing of mouse *Oasl2*, but not *Oasl1* leads to reduced RIG-I signaling. Wild type immortalized mouse embryonic fibroblasts (MEF) were transfected with various *Oasl1* or *Oasl2* or control shRNA (Sigma MISSION shRNAs in pLKO vector), and subjected to puromycin selection. The selected cells were stimulated with SeV (40 HAU/ml) as indicated for 24h followed by qRT-PCR analysis as indicated. (K) Schematic representation of modified *Oasl2* allele of the *Oasl2*$^{-/-}$ mice

(*Oasl2*$^{tm1a(EUCOMM)Wtsi}$,

http://www.mousephenotype.org/martsearch_ikmc_project/martsearch/ikmc_project/41225)

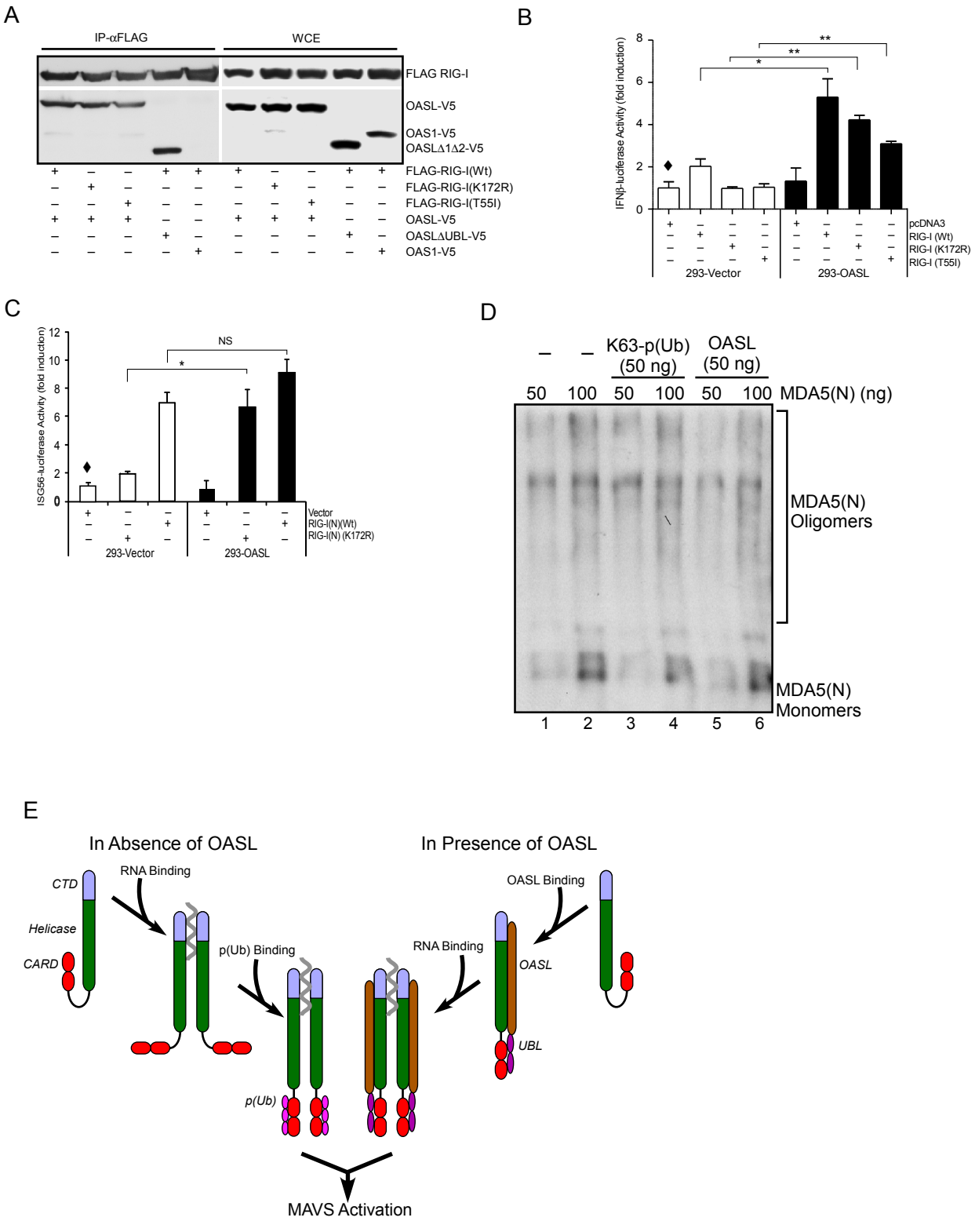
Location for genotyping and expression analysis PCR products are shown below. (L) Genotyping of *Oasl2*$^{-/-}$ mice. Genomic DNA obtained from the tail clippings of 1 month old *Oasl2*$^{-/-}$ and age matched wild type (Wt) (2 mice per group) were analyzed by PCR as indicated. (M) Loss of *Oasl2* mRNA expression in BMDM from *Oasl2*$^{-/-}$ mice. Quantitative RT-PCR analysis of *Oasl2* in BMDM from Wt or *Oasl2*$^{-/-}$ mice, infected with SeV as indicated (Fig. 5J) by. * $P < 0.05$, ** $P < 0.01$.



Supplementary Fig. S4

Fig. S4: Specific interaction of RIG-I and OASL, and their colocalization in stress granules. (Related to Fig. 6)

(A) ISG60 induction in U2OS cells following transfection of physiologically relevant RIG-I/MDA5 ligands. Total RNA from 293T cells infected with various viruses as indicated were transfected to U2OS cells (as in Fig. 6F) followed by IB with ISG60 antibody. (B) Schematic diagrams of various epitope- tagged RIG-I and OASL constructs used for RIG-I-OASL interaction studies. Three different domains of RIG-I – CARD, Helicase and C-terminal domain (CTD); and two different domains of OASL – OAS and UBL are represented in different colors. (C) OASL does not interact with MAVS either in presence or absence of RIG-I. Cell lysates from OASL-V5 stably expressing 293T or 293T-*DDX58*$^{-/-}$ cells were immunoprecipitated with anti-V5 or control IgG. The immunoprecipitates (IP) and whole cell extracts (WCE) controls were analyzed by IB with MAVS and V5 the antibodies. (D) SeV infection does not change coprecipitation of OASL and RIG-I. 293T cells were co-transfected for 24 h with FLAG-RIG-I and OASL-V5 followed by various doses of SeV infection for 8 h. Cell lysates were immunoprecipitated (IP) with FLAG antibody, followed by IB with V5 antibody. Whole cell extracts (WCE) from transfected cells were also similarly analyzed by IB to show expression levels. (E) OASL localizes to stress-granules upon SeV infection. HT1080-OASL cells were transfected with the stress granules marker G3BP-GFP for 18 h, infected with SeV (200 HAU/ml) for 8 h followed by immunofluorescence with anti-V5 antibodies. (F) OASL and RIG-I specifically co-localizes to stress granules and not in mitochondria upon virus infection. Same HT1080-OASL cells were either transfected with G3BP-GFP (top) or dsRED-Mito (Clontech) followed by SeV infection as above. Cells were imaged as described in the experimental procedures.



Supplementary Fig. S5

Fig. S5: Interaction and functional rescue of RIG-I pUb-binding mutants by OASL. (Related to Fig. 7)

(A) Interaction of RIG-I mutants with OASL. N-terminal FLAG-tagged wild type (Wt) or mutant RIG-I constructs were co-transfected with C-terminal V5-tagged OASL or OAS1 constructs as indicated. Cell lysates were analyzed by immunoprecipitation with FLAG antibody followed by IB with V5 antibody. Whole cell extracts were also similarly analyzed to show expression levels. (B-C) Rescue of the RIG-I pUb-binding mutants in the context of full length RIG-I (B), or RIG-I(N) (C) in presence of OASL as measured by IFNβ- or ISG56- reporter assay as described before. (D) MDA5 CARD oligomerization (MDA5(N)) is not affected by OASL or pUb. Purified GST-tagged MDA5(N) protein was incubated either with purified OASL or K63-linked pUb as described in Fig. 7(C) followed by native PAGE analysis of oligomer formation. (E) Proposed model of OASL mediated activation of RIG-I. ♦ set as 1 for comparison. * $P < 0.05$ and ** $P < 0.01$ by two-tailed Student t test analysis using GraphPad Prism.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of *DDX58*$^{-/-}$ and *OASL*$^{-/-}$ cells

For the generation of 293T-*DDX58*$^{-/-}$ cells, a pair of zinc finger nucleases targeting an early coding exon of the human RIG-I gene was designed using the ZiFit database (Sander et al., 2007). The genetic sequences encoding for zinc finger domain arrays targeting exon 1 of RIG-I TGACCACCGAGCAGCGACGCAGCCT (ZFN binding regions are underlined) were synthesized by ShineGene (Shanghai) and inserted into a backbone vector using Ligation Independent Cloning (LIC) as previously described (Schmid-Burgk et al., 2013). Plasmid sequences are available upon request. The resulting plasmid allowing expression of both zinc finger nucleases from a single promoter separated by a T2A peptide was transfected into HEK293T cells using GeneJuice according to the manufacturer's protocol. ZFN-targeted 293T cells were grown at limiting dilution conditions in 96 well plates (0.5 cells per well) to obtain single clones. After two weeks, cell clones were split in two replicates, one of which was subjected to PCR-based amplification of the targeted genomic region. Thus obtained amplicons were sequenced and a single cell clone harboring all-allelic frame shift mutations was chosen for further studies. For *OASL*$^{-/-}$ cells, a pair of TALE nucleases targeting exon 1 of OASL TCCTTCGTGGCTCAGTGGCTGCAGCCCCACCGGGAGTGGAAGGAAGAGGTGCTA (TALEN binding regions are underlined) was generated in 293T cells using GeneJuice and HCT-116 cells using X-tremeGENE 9 as previously described (Schmid-Burgk et al., 2013). The generation of cell clones with homozygous *OASL* loss was performed as described above for the RIG-I gene.

Virus infection and viral growth curve analyses

OASL and vector control stable cells in 24-well plates were infected with VSV expressing EGFP (VSV–GFP) at different m.o.i. After infection, the GFP signal was examined under fluorescence microscope and cells were harvested for IB analysis using anti-GFP antibody. OASL and vector control stable HEK293 cells were infected with SeV. Twenty-four hours post-infection, cells were lysed and subjected to IB using antibody against SeV C protein. One-step growth curve analyses were conducted with the different RNA and DNA viruses. OASL and control stable cells were infected with different viruses including VSV, HSV1, EMCV and RSV; cell-free medium were collected after infection, and virus titers determined by plaque assay using BHK21, Vero cells, and Hep2 cells, respectively, in 24-well plates. Each virus infection was performed in triplicate. The VSV, SeV, RSV and DenV replication were also measured using qRT-PCR with the following specific primers: VSV, forward, 5'-TGCAAGGAAAGCATTGAACAA-3', reverse, 5'-GAGGAGTCACCTGGACAATCACT-3'; SeV, forward, 5'-GCTGCCGACAAGGTGAGAGC-3', reverse, 5'-GCCCGCCATGCCTCTCTCTA-3'; RSV, forward, 5'-TGCAGGGCAAGTGATGTTAC-3', reverse, 5' TTCCATTTCTGCTTGCACAC-3'; DENV forward, 5'-AGTTGTTAGTCTACGTGGACCGA-3', reverse 5'-CGCGTTTCAGCATATTGAAAG-3'.

In vitro RNA pull-down assay

5'-UTR of human and mouse IRF7 were amplified by a composite T7 promoter primer, and the products were *in vitro* transcribed followed by 3'-biotin labeling. C-terminal V5 tagged Human OASL and mouse Oasl1 were purified from HEK293 cells stably expressing each protein by immunoprecipitation followed by elution with V5 peptide. Labeled 5'UTR RNA were incubated

in various combinations with human or mouse OASL and the complex pulled down with Streptavidin-agarose beads followed by SDS-PAGE analysis and IB with anti-V5 antibody.

Cloning of OASL and RIG-I mutants

The human OASL coding sequence, OASL UBL deletion mutant (OASLΔ1Δ2), OASL UBL domains were all amplified by PCR from pcDNA3-OASL-V5 (Hartmann et al., 1998). PCR products were cloned into pENTR/D-TOPO (Life Technologies) to obtain pENTR clones. The lentiviral expression vectors with C-terminus V5-tag were generated by Gateway cloning between pENTR clones and pLenti CMV-Puro-DEST (Addgene) using LR Clonase II enzyme mix (Life Technologies) according to manufacturer's guidelines. To make inducible OASL construct, the OASL-V5 was transferred from pENTR clone into pInducer 20 DEST (Meerbrey et al., 2011) by Gateway cloning. RIG-I expression vectors used here have been described before (Morosky et al., 2011). RIG-1CARDs (RIG-I(N)) and MDA5 CARDs (MDA5(N)) domains were also PCR amplified and cloned into pLenti CMV-Puro-DEST similarly as mentioned before. RIG-I K172R and T55I mutants were created by site directed mutagenesis using quick change site-directed mutagenesis kit (Agilent Technologies).

Establishment of different stable cells

HEK293, 293T or HT1080 cells were transfected with pLenti-CMV-OASL, pLenti CMV-OASLΔ1Δ2 or pLenti vector control, and selected with 1 μg/ml puromycin and collected as pool stable cells. The OASL expression in the above stable cells was confirmed by IB using anti-V5 antibody. HEK293 OASL and vector control stable cells were transfected with RIG-I shRNA plasmid (psiRNA-hRIG-I) and control plasmid (psiRNA-Ctrl) (Invivogen, San Diego, CA)), respectively, and then selected by 400 μg/ml Zeocin. HEK293 cells or HCT-116 cells were

transfected with OASL shRNA (shRNA-pLKO.1 constructs (Sigma-Aldrich)) or pLKO.1 vector control and selected with 1 μg/ml puromycin. Silencing of RIG-I and OASL in above stable cells were confirmed by real-time RT-PCR. HT1080 cells were transfected with pInducer 20 OASL and selected with 10 μg/ml puromycin. The puromycin resistant cells (HT1080-*i*OASL) were examined for the induction of OASL-V5 by adding various concentration of doxycycline.

IFNβ and ISG56 reporter assay

HEK293 (3×10^5 cells/well) in 24-well plate were transfected with OASL or relevant mutants or controls (1μg each) together with IFNβ or ISG56-firefly luciferase reporter (0.4 μg) and β-actin *Renilla* luciferase reporter (0.012 μg). Twenty-four hours later, the cells from each well were collected by trypsin digestion and seeded into the wells of white-wall 96-well plate. Forty-eight hours post-transfection, the cells were stimulated with SeV for 16 h, and luciferase activities were measured using the Dual-Glo luciferase assay system from Promega (Madison, WI). The results were expressed as fold induction of firefly luciferase relative to that of non-stimulated control-transfected cells after normalizing to *Renilla* luciferase.

Quantitative RT–PCR analysis

Total RNA was isolated from transfected and/or stimulated cells by Trizol (Life Technologies), and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). One part ($1/20^{th}$) of the cDNA synthesized from 1 μg RNA was subjected to real-time PCR using Fast EvaGreen Supermix in a CFX96 Real Time System (Bio-Rad) according to the manufacturer's instructions. All PCR amplification was normalized to ribosomal protein L32 (RPL32); the primers for human ISG56, ISG60, IFN-β and RPL32 have been described before (Zhu et al., 2010). All primers were custom synthesized by Integrated DNA Technologies (Coralville, IA).

Fluorescence microscopy

HT1080-OASL or vector cells were plated in 8-well chamber slides (4×10^4 cells/well). For RIG-I and OASL co-localization experiment, cells were infected with SeV (200 HAU/ml) for 8 h and fixed with paraformaldehyde (4% v/v). Following permeabilization with 1% Triton-X100, cells were incubated with anti-RIG-I antibody (Onomoto et al., 2012) for 48 h and anti-V5 antibody for 16 h at 4 ºC. Immunoflurescence detection was done with FITC conjugated anti-rabbit and Alexa Fluor 594 conjugated anti-mouse antibodies from Life Technologies. Cells were imaged as described before (Bozym et al., 2012). For the OASL and G3BP co-localization, cells in 8-well chamber slides were transfected with G3BP-GFP or dsRED-Mito plasmid (Tourriere et al., 2003) for 24 h followed by immunofluorescence as above.

Co-immunoprecipitation and Immunoblotting

One million cells in 6-well plate transfected and/or treated with SeV were lysed in lysis buffer (Triton-X 100 1%, HEPES (pH 7.4) 20mM, NaCl 150mM, $MgCl_2$ 1.5mM, EGTA 2mM, DTT 2mM, NaF 10mM, β-Glycerophosphate 12.5mM, Na_3VO_4 1mM, PMSF 1mM, and Protease Inhibitor). The cleared cell lysates were incubated at 4 ˚C with antibody plus protein A/G agarose beads or anti-FLAG beads overnight, washed five times with lysis buffer, and boiled in 2 × SDS–PAGE loading buffer for elution. Cell lysates boiled in 1 × SDS–PAGE loading buffer and immunoprecipitated samples were subjected to SDS-PAGE electrophoresis. Following transfer, the blots were incubated with target antibody followed by appropriate HRP-conjugated secondary antibody, and visualized by ECL detection (GE Healthcare).

Protein Purification

Purification of RIG-I(N) and MDA5(N) have been described before (Zeng et al., 2010). Recombinant C-terminally his-tagged OASL was expressed using the baculovirus expression system in Sf9 cells according to manufacturer's instructions (Oxford Expression Technologies). The Sf9 cells were lysed in 50 mM Tris-Cl (pH 9.0), 500 mM NaCl, 10 % glycerol, 0.5 % NP40, 1.5 mM $MgCl_2$, 2 M Urea, 20 mM Imidazole, 1mM β–ME and Protease Inhibitor Cocktail (Sigma) on ice for 30 minutes. Lysate was cleared by centrifugation at 200,000g. Supernatant was incubated with Ni-Sepharose Excel (GE Lifesciences) for 2 hrs at 4 °C. The nickel beads were washed in 50 mM Tris-Cl (pH 9.0), 500 mM NaCl, 10 % glycerol, 1.5 mM $MgCl_2$, 2 M Urea, 30 mM Imidazole and 1mM β–ME. Bound protein was eluted in 50 mM Tris-Cl (pH 9.0), 500 mM NaCl, 10 % glycerol, 1.5 mM $MgCl_2$, 2 M Urea, 500mM Imidazole and 1 mM β–ME. Recombinant C-terminally his-tagged OAS1 was expressed in *E. coli* BL21(DE3) and purified as previously described (Torralba et al., 2008).

SUPPLEMENTAL REFERENCES

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