

SUPPLEMENTARY INFORMATION

The Chromosome 19 miRNA Cluster Guards Trophoblasts Against Overacting Innate Immunity

Jean-Francois Mouillet, Yingshi Ouyang, Elena Sadovsky, Vinoth K. Kothnadan, Heather L. Sorenson, Logan J. Badeau, Saumendra N. Sarkar, Tianjiao Chu, Alexander Sorkin, Yoel Sadovsky

Original microscopy files, uncut gel image files, and numerical source data (Microsoft Excel files) are available for review in Figshare, <https://doi.org/10.6084/m9.figshare.28742777>

Supplementary Results Figures

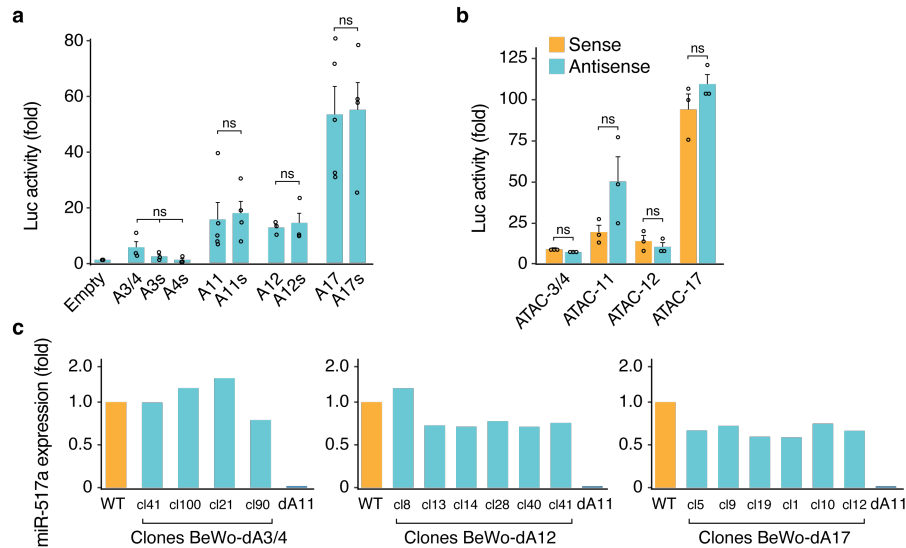


Figure S1. Analysis of the C19MC enhancer elements. **a)** Luciferase (Luc) activity of “short” ATAC elements versus “long” element in BeWo cells (Fig. 1). The short elements corresponded to sequences identified by ATAC-seq, ranging from 133 bp (ATAC-12) to 751 bp (ATAC-11). The long elements included these short sequences, with additional flanking regions, extending the total length to approximately 2 kb. These sequences were cloned in the pGL3-reporter plasmid and transiently transfected, along with a *Renilla* Luc plasmid, for normalization. Data are mean $\pm$ SD (n=5). ns, nonsignificant. **b)** Sequences corresponding to the four functional ATAC peaks were cloned in the pGL3-promoter reporter plasmid in sense and antisense orientation and transiently transfected as in (a). Luc activity for each ATAC region was compared to the empty pGL3-promoter plasmid. Data are mean $\pm$ SD. ns: nonsignificant. n=3. **c)** C19MC RNA output in the various ATAC-element deleted cells. RT-qPCR analysis of miR-517 expression in BeWo cells across different clones for each ATAC-3/4, ATAC-12, and ATAC-17 edited elements. Multiple independent clones for each deletion were tested for miR-517a expression and compared to wild-type (WT) cells and an dA11 clone. Each graph represents a single experiment.

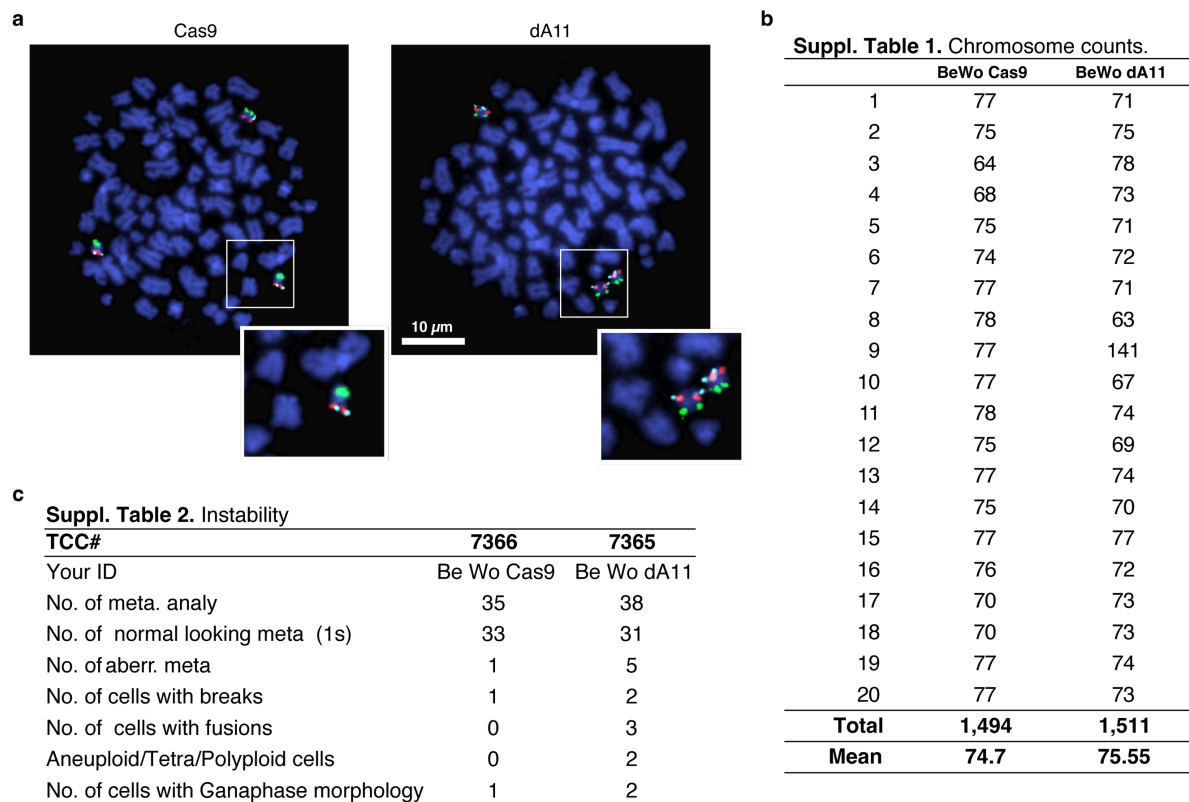


Figure S2. FISH validation of chromosome 19 copy number. **a)** FISH analysis of mitotic chromosomal spreads in BeWo-Cas9 and BeWo-dA11 cells, using a C19MC probe (aqua blue) and two control probes for c19q13 and c19p13 (green). Both cell lines exhibit triploidy for the C19MC locus. The lower insets represent an enlargement of the square box in the larger pictures. **b)** Chromosome counts from cytogenetic analysis performed on 20 chromosome spread samples in BeWo-Cas9 and BeWo-dA11 cells. **c)** The results of chromosome instability analysis conducted on 35 metaphase chromosome spreads for each cell line.

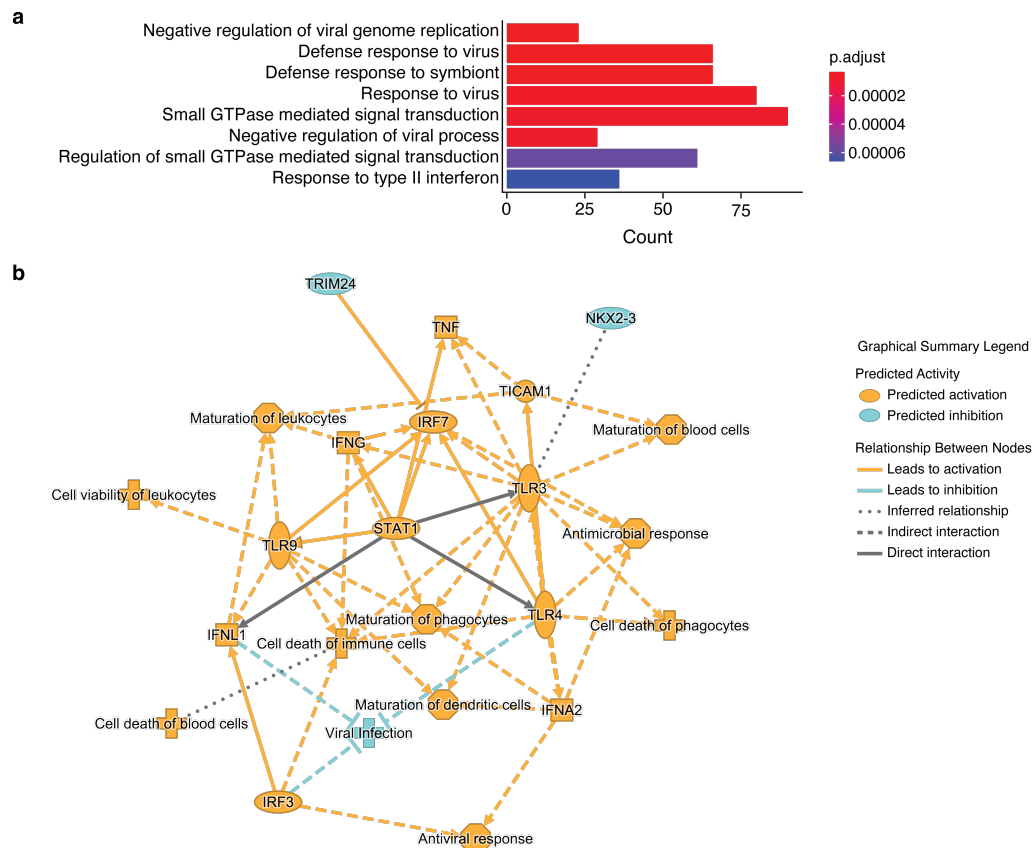


Figure S3: C19MC-altered signaling pathways. a) Enrichment analysis, illustrating GO biological processes that are associated with abrogated C19MC expression in trophoblasts. The figure shows the top eight significantly regulated GO biological processes. **b)** A graphical summary of IPA core analysis of differentially expressed genes in BeWo-Cas9 vs -dA11 cells.

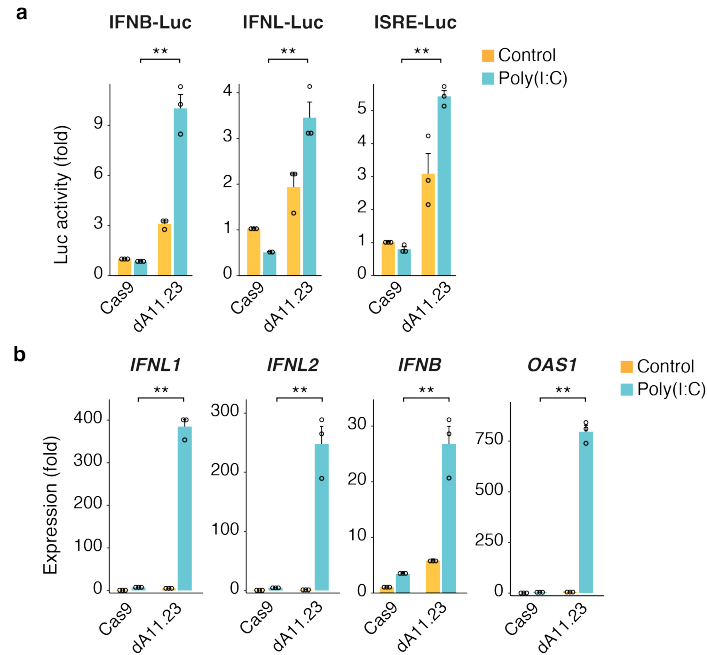


Figure S4. An additional clone of C19MC-silenced cells (BeWo-dA11.23) and innate immune response. The responses recapitulate the findings using the BeWo-dA11 line. **a)** Activation of various Luciferase (Luc) reporter genes by poly(I:C). The cells were transfected with a Luc reporter driven by the interferon- β promoter (IFNB), interferon- λ 1 promoter (IFNL1), interferon-stimulated response element (ISRE), along with a Renilla-Luc internal control. The next day, cells were exposed to poly(I:C) (10 μ g/ml) overnight before the Luc assay. The fold change was calculated relative to the mean of BeWo-WT cells without poly(I:C). Data are mean $\pm$ SD, n=3. ** p<0.001. **b)** RT-qPCR analysis of interferon- λ 1 (*IFNL1*), interferon- λ 2 (*IFNL2*), interferon- β (*IFNB*) and *OAS1* in BeWo-dA11 clone 23. 10 μ g/ml poly(I:C) was added overnight. Data are mean $\pm$ SD, n=3. ** p<0.001.

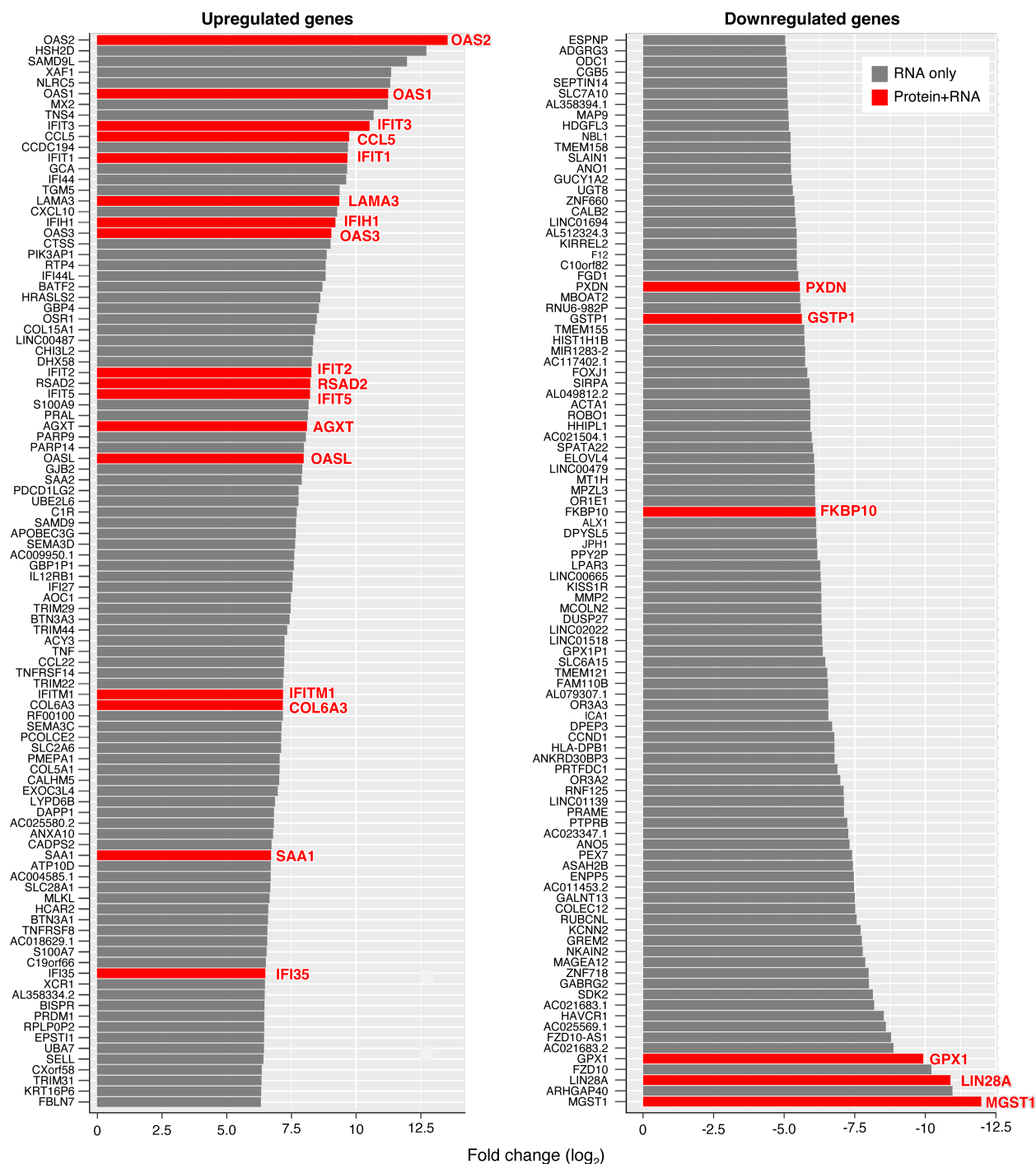


Figure S5: A proteomics analysis, focusing on expressed genes identified by transcriptomics. The graph displays the 100 most upregulated or downregulated transcripts in BeWo-dA11 cells vs BeWo-Cas9 cells, matched with the 100 most upregulated or downregulated proteins. Genes present in both analyses are red-shaded, while those found only by transcriptomics are grey-shaded.

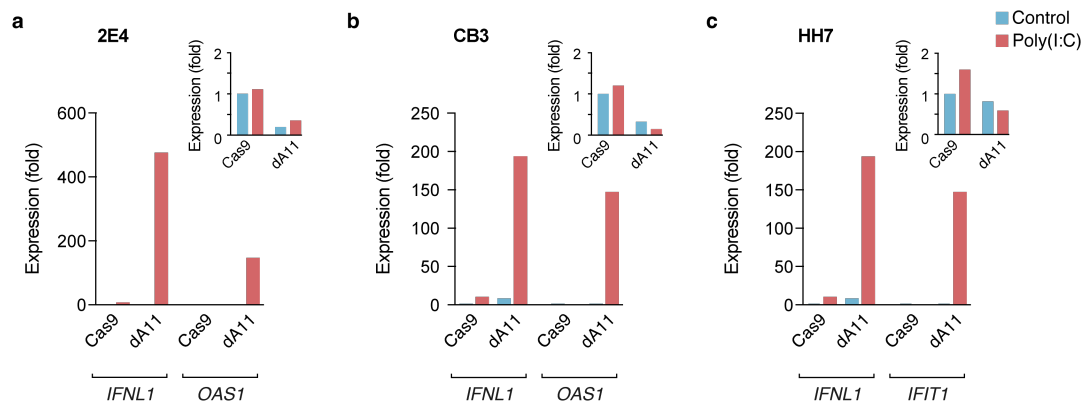


Figure S6: An innate immune response in the cloned lines of C19MC-dA11-deleted hTSCs. Each of the three panels represents the data from an independently edited clone that survived long enough in culture to yield sufficient RNA for RT-PCR analysis of a small set of genes. For each clone, the main panel shows *IFNL1* or *OAS1* expression in edited dA11 vs Cas9 control cells, after poly(I:C) stimulation. The inset panels show miR-517 expression in control vs dA11-edited cells exposed to 10 µg/ml poly(I:C) before RT-qPCR.

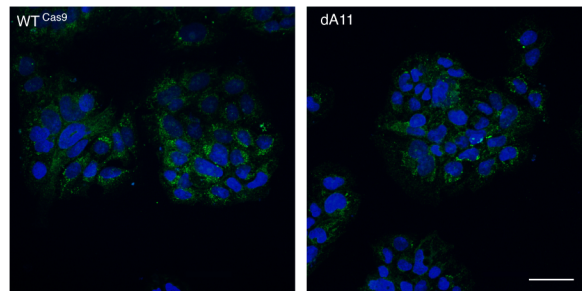


Figure S7: Immunofluorescence analysis of BeWo cells for the presence of dsRNA.

Detection of dsRNA in BeWo cells. BeWo-Ctr and BeWo-dA11 were stained with the anti-dsRNA antibody (J2, green signal) and DAPI (blue). Cells were then observed under a laser confocal microscope. Scale bar =50 μ m. The images are representative of at least 3 independent experiments.

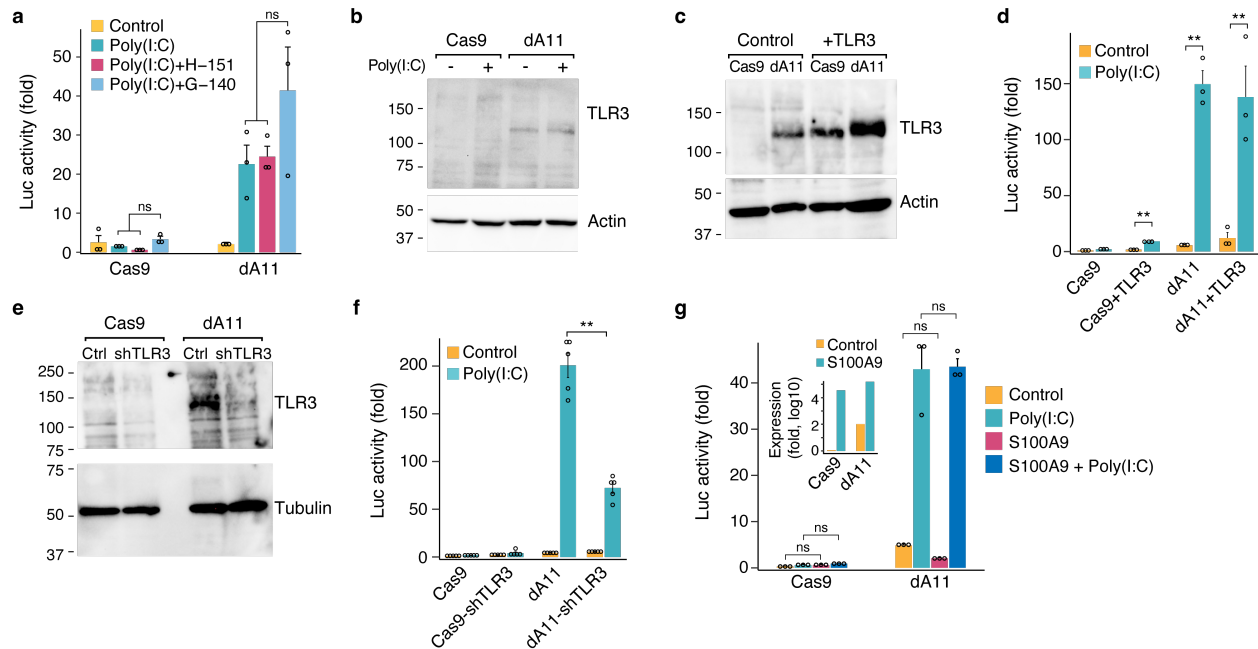


Figure S8: TLR3-mediated innate immune response in C19MC-deficient BeWo cells. a) Inhibitors of the cGAS-STING pathway do not alter the interferon response in BeWo cells. Cells were transfected with the IFNL1- luciferase (Luc) reporter and exposed to H-151 (10 μ M) or G-140 (10 μ M) for 2 h. Poly(I:C) 10 μ g/ml was added overnight. Data are mean $\pm$ SD, n=3. ns = not significant. **b)** Western blot analysis of TLR3 in BeWo-Cas9 and -dA11 cells. The cells were stimulated with poly(I:C) (10 μ g/ml) for 6 h and analyzed by western blot, as described in Methods, using an anti-TLR3 antibody. Actin was used as a loading control. **c)** Overexpression of TLR3. BeWo-Cas9 and -dA11 cells were transiently transfected with a TLR3 expression plasmid for 48 h, then analyzed by western blot, using a TLR3 antibody. Actin was used as a loading control. **d)** BeWo-Cas9 and -dA11 cells were transfected with IFNL1-Luc reporter and a plasmid expressing TLR3, as described in Methods. The next day, cells were incubated with 10 μ g/ml poly(I:C). Data are mean $\pm$ SD, **p<0.005. n=3. **e)** shRNA-mediated silencing of TLR3. Cells were transduced with a TLR3 shRNA lentivirus and selected for 10 days with puromycin. Western blot analysis of TLR3 protein from cell lysates of BeWo-Cas9 and BeWo-dA11. Tubulin was used as a loading control. **f)** BeWo-Cas9 and -dA11 cells expressing shTLR3 were transiently transfected with IFNL1-Luc reporter. The next day, cells were incubated with 10 μ g/ml poly(I:C) for 6 h. Data are mean $\pm$ SD, **p<0.001. n=5. **g)** BeWo-Cas9 and -dA11 cells were transiently transfected with IFNL1-Luc reporter and a construct expressing S100A9, as described in Methods. The next day, cells were incubated with 10 μ g/ml poly(I:C). Data are mean $\pm$ SD, ns, not significant. n=3. The inset RT-qPCR analysis (a single experiment) confirms S100A9 overexpression in cells transfected with the S100A9 construct or a control plasmid (pBluescript). The graph represents data from a single RT-qPCR experiment.

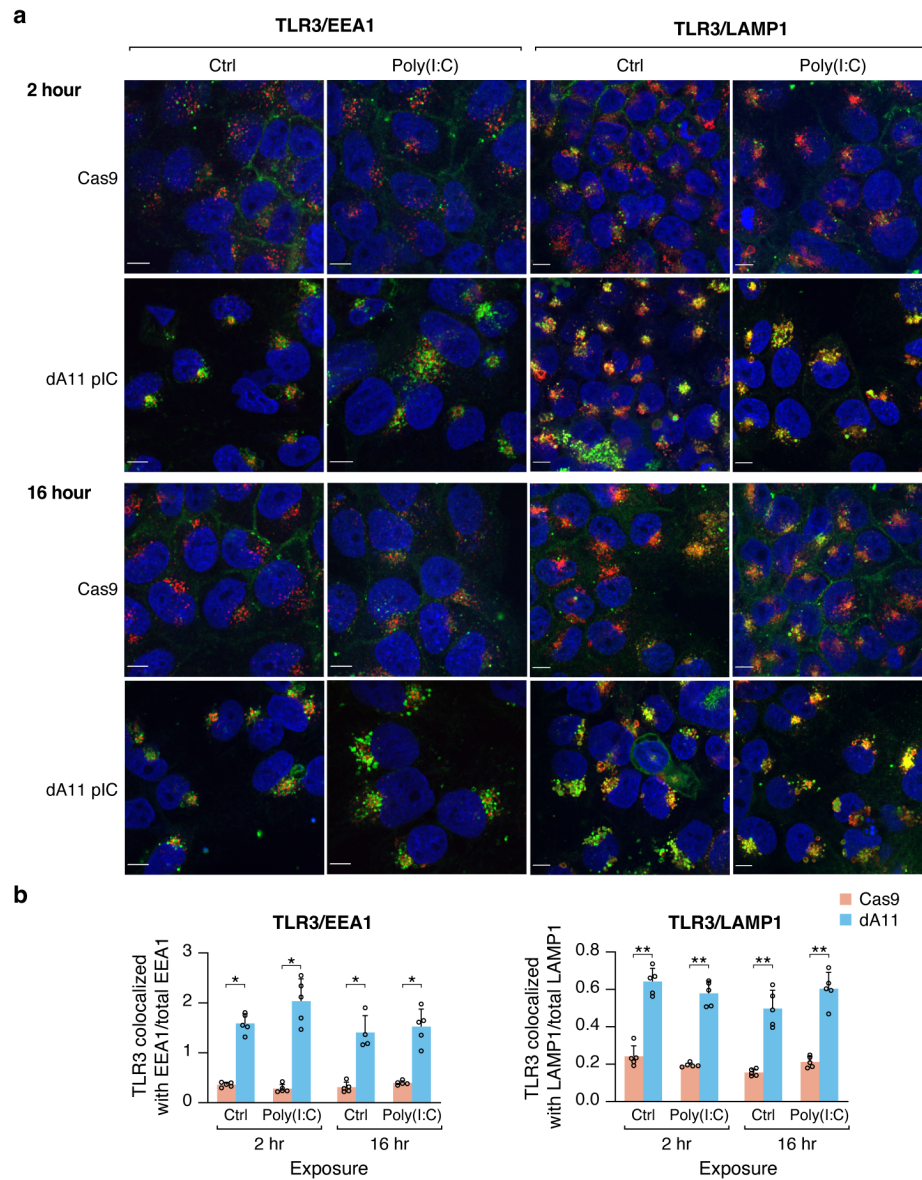


Figure S9. Localization of TLR3 in early and late endosomes. a) Single confocal sections of 3D images of BeWo-Cas9 and BeWo-dA11 exposed to poly(I:C) (10 $\mu\text{g/ml}$) for 2 h or 16 h, then fixed, immunolabeled and imaged for TLR3 (488 nm, green), EEA1 (640 nm, red) or LAMP1 (640 nm, red). DAPI staining (blue) was used for nuclei. Fluorescent intensity scales are identical in all images. Scale bars: 10 μm . **b)** Quantitative analysis of the amount of poly(I:C)-Rh colocalized with TLR3 in endosomes. Each bar represents ratio of the TLR3 fluorescence to EEA1 (early endosomes, left panel) or LAMP1 fluorescence (late endosomes and lysosomes, right panel) across multiple 3D images exemplified in **a**. Bars are mean $\pm$ SD. Images represent $n=5$. * $p<0.0005$ for the left panel, and ** $p<0.0001$ for the right panel.

Table S1: Primers used in the study.

Primer	Sequence	Purpose
ATAC-1F	GATCGGTACCGGTTTTATGGCAGGATTTGG	Cloning
ATAC-1R	GATCCTCGAGCCCTTCCTTCTAGTGCGTGA	Cloning
ATAC-2F	GATCGGTACCGGGGAGCCAACACCTTATCT	Cloning
ATAC-2R	GATCCTCGAGAGGCAAGTGGATCACCTGAG	Cloning
ATAC-3/4F	GATCGGTACCACAGTGCCCGGCTAATTTTT	Cloning
ATAC-3/4R	GATCCTCGAGCAGTGGCACAGTCTCAGCTC	Cloning
ATAC-5F	GATCGGTACCAACTCCTGGCCACAAGTGAT	Cloning
ATAC-5R	GATCCTCGAGTTATGCCCTGCACTCTAGC	Cloning
ATAC-6F	GATCGAGCTCCCCAGCCAAGATGTTTTTCT	Cloning
ATAC-6R	GATCCTCGAGCTGCAACTTCTCTGCCTCCT	Cloning
ATAC-7F	GATCGGTACCCAGTGGCTCACGCATGTAAT	Cloning
ATAC-7R	GATCCTCGAGTGTGTGTTGGGACTGAAAGAA	Cloning
ATAC-8F	GATCGGTACCGGAGGTTGGCAACACAAAAT	Cloning
ATAC-8R	GATCCTCGAGGGATGGGCACAAAACTAGG	Cloning
ATAC-9F	GATCGCTAGCCATGGTGAGATCCCTGACTA	Cloning
ATAC-9R	GATCCTCGAGGCACAATCTCGGCTCACTG	Cloning
ATAC-10F	GATCGGTACCCCTGGAAGTATCGCCACCT	Cloning
ATAC-10R	GATCCTCGAGTTGCAGTTCATCCACAAGGA	Cloning
ATAC-11F	GATCGGTACCGCACCAGTTAAGAGGGATGC	Cloning
ATAC-11R	GATCCTCGAGTTCCCAGAACCAGTCCAGTT	Cloning
ATAC-11F_s	GATCGGTACCGCCTGTAATCCCAGCACTTT	Cloning
ATAC-11R_s	GATCCTCGAGAGCGAGACTCCGTCTCAAAA	Cloning
ATAC-12F	GATCGGTACCCACATCGCTGGCTAATTTTT	Cloning
ATAC-12R	GATCCTCGAGTCACTCTGTCACCCAAGCTG	Cloning
ATAC-12F_s	GATCGGTACCGCAGCATGTCCATCCAAAG	Cloning
ATAC-12R_s	GATCCTCGAGTGGCAAGATTAGGGATGAGG	Cloning
ATAC-13/14F	GATCGGTACCGGCATTGCATTGACTCTGAA	Cloning
ATAC-13/14R	GATCCTCGAGACCCCGGGTATATCCAAAAG	Cloning
ATAC-15F	GATCGGTACCCAGCCTGTTTTCTGCATTT	Cloning
ATAC-15R	GATCCTCGAGCTGCTCTCGAACTCCTGACC	Cloning
ATAC-16F	GATCGGTACCTGTTTGTGTGTGCGACAATG	Cloning
ATAC-16R	GATCAAGCTTCTAGATGGCCACAGCCTGA	Cloning
ATAC-17F	GATCGAGCTCAGAGAGGCCAGTGTGGATGT	Cloning
ATAC-17R	GATCCTCGAGTGGAAAGGTTTTCATTGACGA	Cloning
ATAC-17F_s	GATCGGTACCTGTGCCAAGCAAGATACAGG	Cloning
ATAC-17R_s	GATCCTCGAGTGGTAGGAACCAACAAGGAAA	Cloning

Primer	Sequence	Purpose
ATAC-18F	GATCGGTACCTGGATCAAACCTGGTGCAACT	Cloning
ATAC-18R	GATCCTCGAGCCCCCGTCTCTACCAAAAAT	Cloning
ATAC-19F	GATCGGTACCCAGTTAATTTGCAGACCACCAA	Cloning
ATAC-19R	GATCCTCGAGATTGGCTGTGCGCCTGTAATC	Cloning
IFNL1-F	GATCGAGCTCACCCCCAGAGGTTCTCATCT	Cloning
INFL1-R	GATCGCTAGCGGCTAAATCGCAACTGCTTC	Cloning
hOAS1-F	TGTCCAAGGTGGTAAAGGGTG	RT-qPCR
hOAS1-R	CCGGCGATTCTAACTGATCCTG	RT-qPCR
hDDX60-F	CAGCTCCAATGAAATGGTGCC	RT-qPCR
hDDX60-R	CTCAGGGGTTTATGAGAATGCC	RT-qPCR
hOAS3-F	GAAGGAGTTCGTAGAGAAGGCG	RT-qPCR
hOAS3-R	CCCTTGACAGTTTTCAGCACC	RT-qPCR
hIFIH1-F	TCGAATGGGTATTCCACAGACG	RT-qPCR
hIFIH1-R	GTGGCGACTGTCCTCTGAA	RT-qPCR
hIFIT3-F	AAAAGCCCAACAACCCAGAAT	RT-qPCR
hIFIT3-R	CGTATTGGTTATCAGGACTCAGC	RT-qPCR
hIFIT3-F	AAAAGCCCAACAACCCAGAAT	RT-qPCR
hIFIT3-R	CGTATTGGTTATCAGGACTCAGC	RT-qPCR
hIFNL1-F3	AATTGGGACCTGAGGCTTCT	RT-qPCR
hIFNL1-R3	GTGAAGGGGCTGGTCTAGG	RT-qPCR
hIFNL2-F	AATTGTGTTGCCAGTGGGGA	RT-qPCR
hIFNL2-R	GCGACTGGGTGGCAATAAAT	RT-qPCR
hS100A9-F	GGTCATAGAACACATCATGGAGG	RT-qPCR
hS100A9-R	GGCCTGGCTTATGGTGGTG	RT-qPCR
hTLR3-F	GCCTTCTGCACGAATTTGAC	RT-qPCR
hTLR3-R	TCCAGCTGAACCTGAGTTCC	RT-qPCR
MCS-F	TCGAAGCTAGCTCTAGACTCGAGA	Cloning into pLV-hsp68
MCF-R	CGATCTCTCGAGTCTAGAGCTAGCT	Cloning into pLV-hsp68
VSV-F	TGCAAGGAAAGCATTGAACAA	RT-qPCR
VSV-R	GAGGAGTCACCTGGACAATCACT	RT-qPCR
GAPDH-F	GAAGGTCGGAGTCAACGGATTT	RT-qPCR
GAPDH-R	GAATTTGCCATGGGTGGAAT	RT-qPCR
YWHA-F	CTGAACTCCCCAGAGAAAGC	RT-qPCR
YWHA-R	CCGATGTCCACAATGTCAAG	RT-qPCR