



**Supplementary Information for**

**Methylation of viral mRNA cap structures by PCIF1 attenuates the antiviral activity of interferon- $\beta$**

Michael A. Tartell<sup>1,2</sup>, Konstantinos Boulias<sup>3,4</sup>, Gabriela Brunsting Hoffmann<sup>4</sup>, Louis-Marie Bloyet<sup>1</sup>, Eric Lieberman Greer<sup>3,4\*</sup> and Sean P. J. Whelan<sup>1\*</sup>

<sup>1</sup>Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, USA, <sup>2</sup>Program in Virology, Harvard Medical School, Boston MA, USA, <sup>3</sup>Department of Pediatrics, Initiative for RNA Medicine, Harvard Medical School, Boston, MA, USA <sup>4</sup>Division of Newborn Medicine, Boston Children's Hospital, Boston, MA, USA

**\*Corresponding author:**

S. P. J. Whelan email: [spjwhelan@wustl.edu](mailto:spjwhelan@wustl.edu)

E. L. Greer email: [Eric.Greer@childrens.harvard.edu](mailto:Eric.Greer@childrens.harvard.edu)

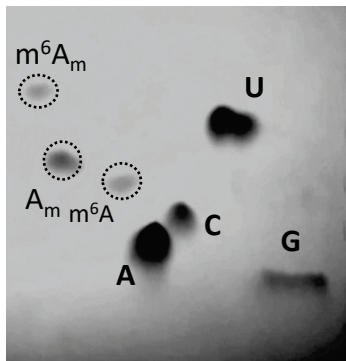
**This PDF file includes:**

Figures S1 to S11

Table S1

**A**

**Standards**



**B**

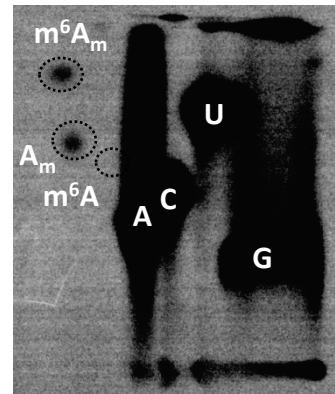
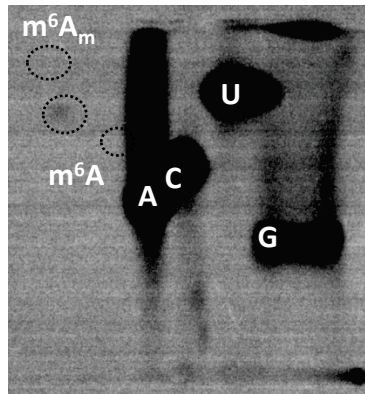
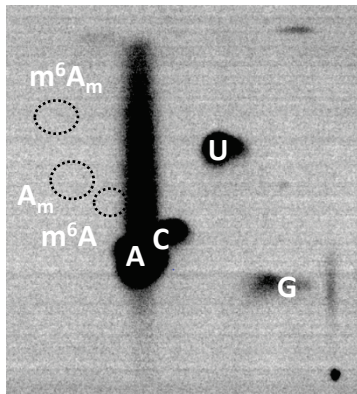
**Uninfected**

**VSV Infected**

**Cap-Clip + P1**

**P1**

**Cap-Clip + P1**

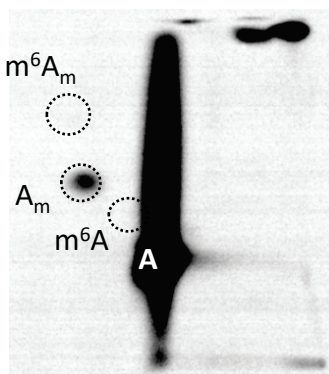
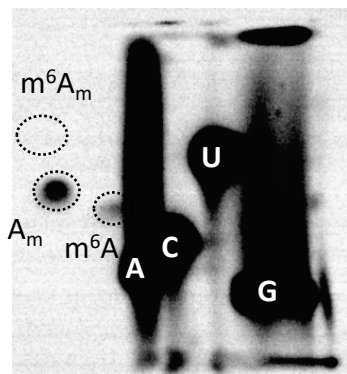


**C**

**PCIF1 KO: VSV Infected**

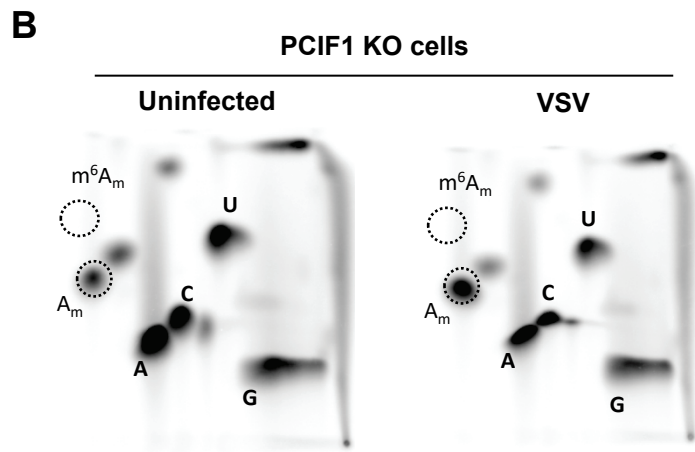
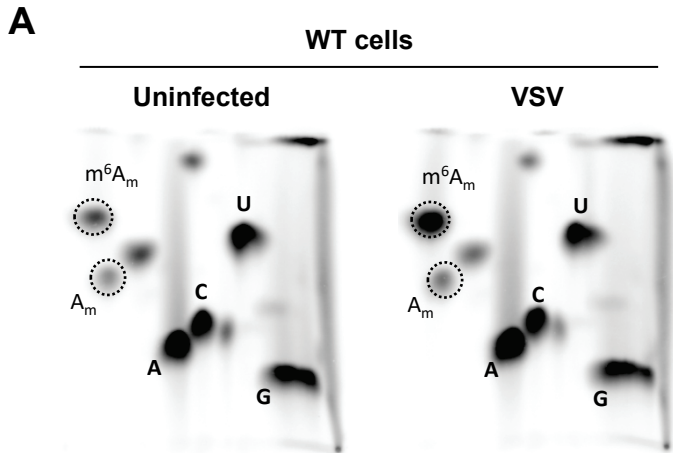
**D**

***In Vitro* Methylation Input**



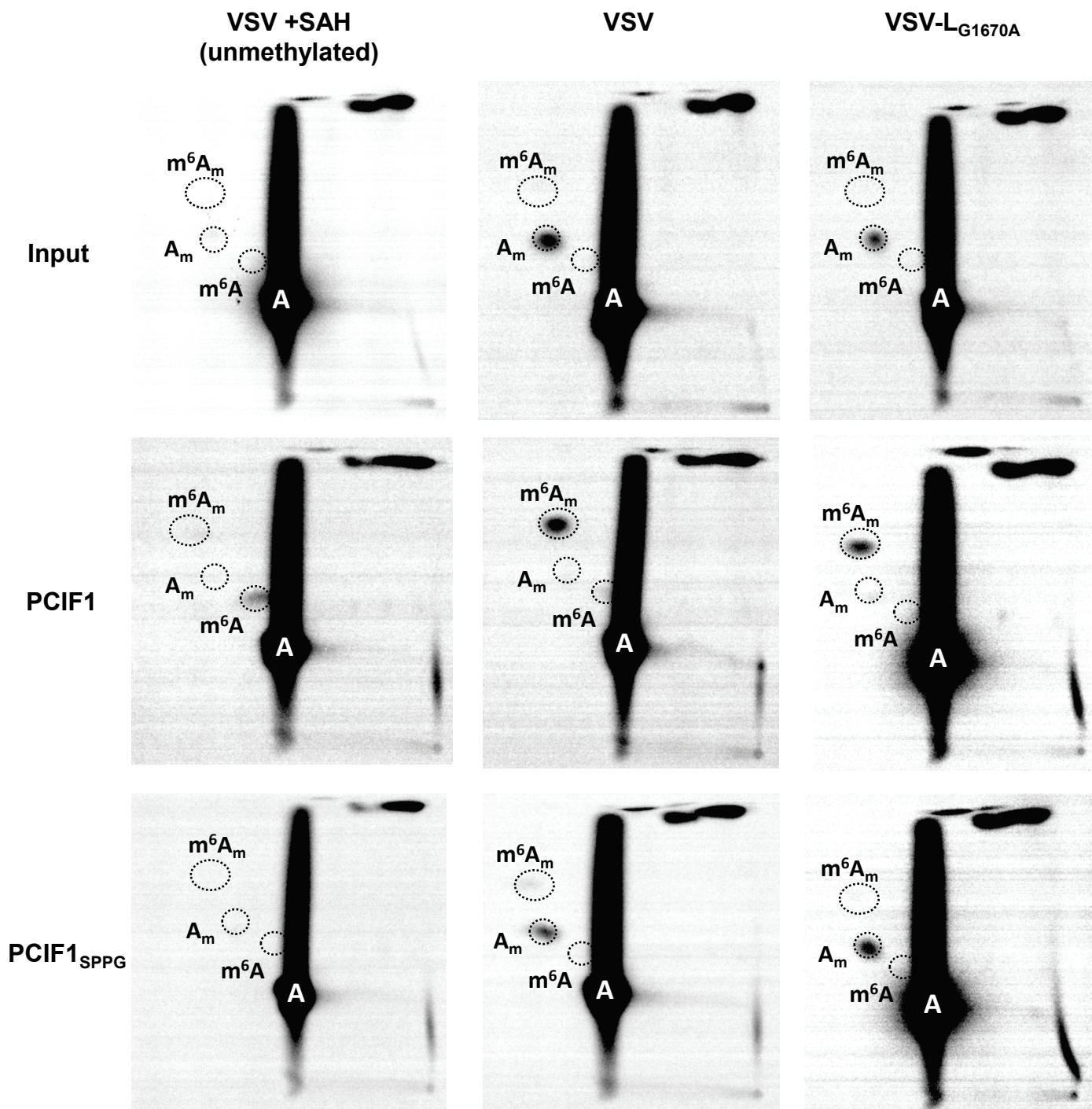
**Figure S1: VSV mRNAs in HeLa cells contain a 5' m<sup>7</sup>Gpppm<sup>6</sup>A<sub>m</sub> cap-structure.**

**(A):** Chemical standards used to identify nucleotide species visualized by UV shadowing (254 nm). **(B):** HeLa cells were infected with VSV at a MOI of 3, cellular transcription halted by adding 10 µg ml<sup>-1</sup> actinomycin D at 2.5 hpi, and viral RNA metabolically labeled with 100 µCi ml<sup>-1</sup> [<sup>32</sup>P] phosphoric acid from 3-7 hpi. RNA was extracted, poly(A) selected and incubated with the indicated nucleases and the products resolved by 2D-TLC and detected by phosphorimaging. Wild type HeLa cells (representative image; n=3). **(C):** 293T PCIF1 KO cells **(D):** In vitro transcribed VSV mRNA (input to Fig 2C).



**Figure S2: PCIF1 methylates viral mRNA.**

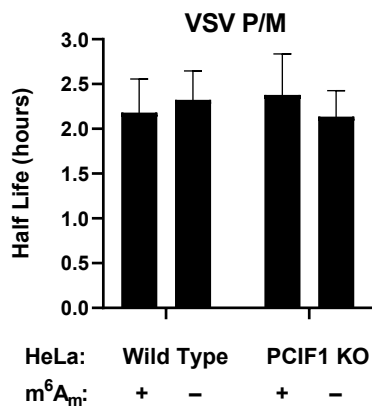
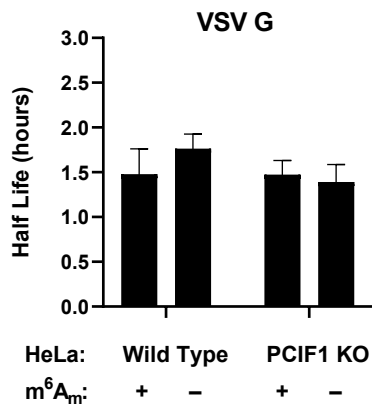
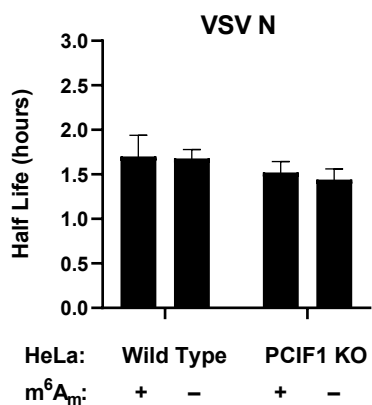
293T cells were infected with VSV at a MOI of 3, poly(A)+ RNA purified at 6 hpi, and cap-proximal nucleotide identity determined by selective radiolabeling. Poly(A)+ RNA was decapped with Cap-Clip, and the exposed 5' phosphate of the cap-proximal nucleotide was radiolabeled with [<sup>32</sup>P] γ-ATP by sequential treatment with shrimp alkaline phosphatase and polynucleotide kinase. Hydrolyzed nucleotide monophosphates were resolved by 2D-TLC and detected by phosphorimaging (representative images; n=3). **(A)**: Parental wild type 293T cells. **(B)**: PCIF1 knockout 293T cells.



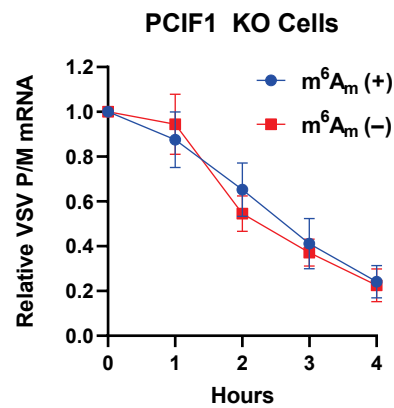
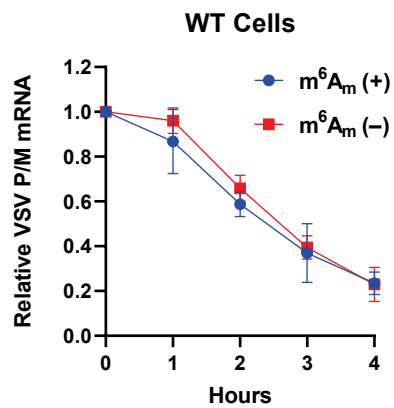
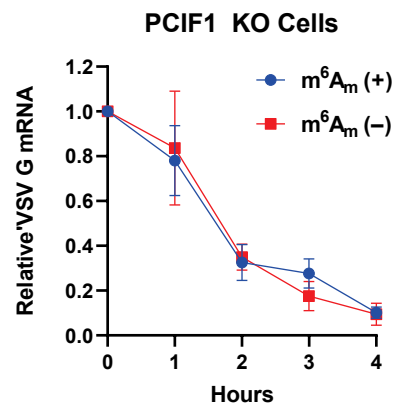
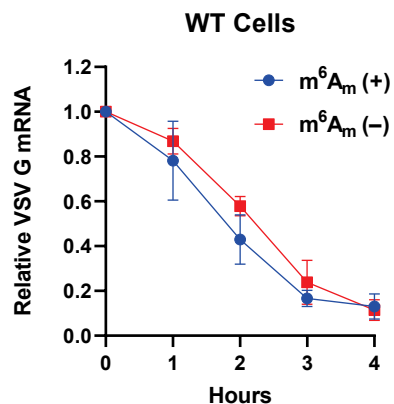
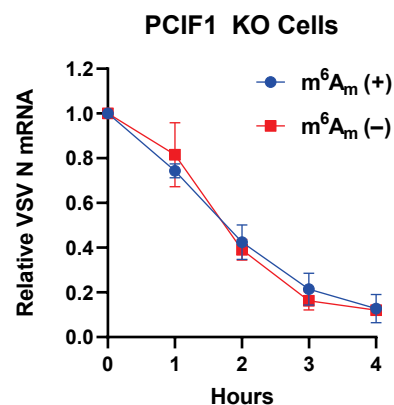
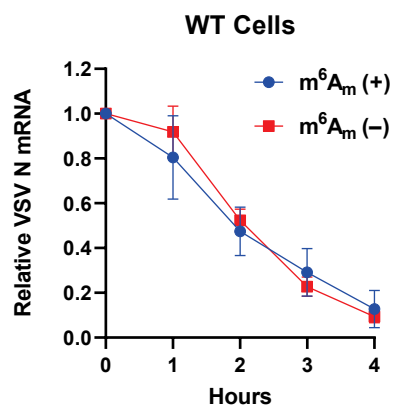
**Figure S3: Cap-methylation requirements for *in vitro* methylation of VSV mRNA.**

VSV mRNA was transcribed *in vitro* using purified virions from the indicated viruses used in Fig 3, and in the presence or absence of 200  $\mu$ M SAH (a methylation inhibitor) as noted, followed by *in vitro* methylation with no enzyme (“input”), purified PCIF1, or purified PCIF1<sub>SPPG</sub>. 2D-TLC was performed on the products to determine the relative amounts of m<sup>6</sup>A<sub>m</sub> and A<sub>m</sub> present (representative images; n=3).

**A**



**B**

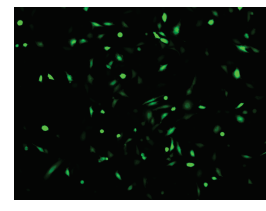




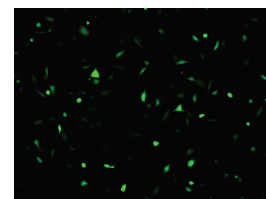
**Figure S4: Effect of m<sup>6</sup>A<sub>m</sub> on VSV mRNA stability.**

Purified stocks of VSV radiolabeled mRNA were generated by infecting 293T PCIF1 KO cells at a MOI of 10 with VSV as in Fig 1, followed by purification. Purified RNA (500 ng) was in vitro methylated with PCIF1, transfected into HeLa cells and RNA amounts assessed by re-extraction from cells at the indicated times followed by **(A)** electrophoresis on acid-agarose gels and phosphorimaging (see Fig 4) (n=3, +/- SD). **(B)**: Data from A was used to calculate half lives in (n=3, +/- SD). There is no significant difference between any decay curve (2 way ANOVA, 0.75>p>0.4), or calculated half life (student's t-test, 0.98 >p>0.06).

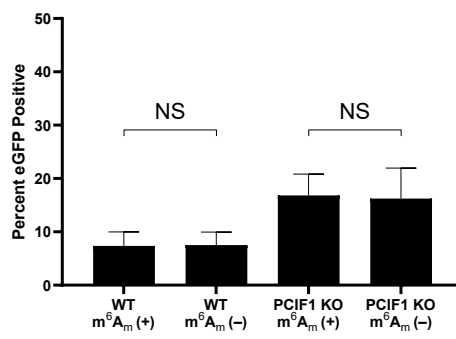
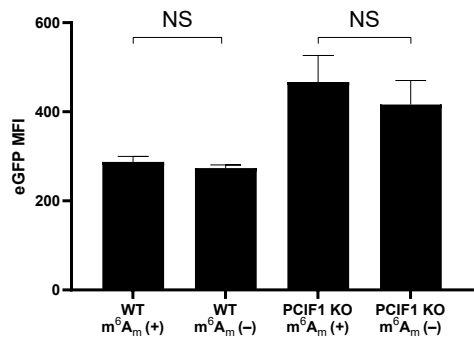
$m^6A_m$  (+)



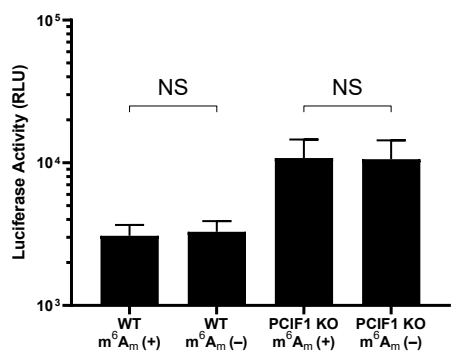
$m^6A_m$  (-)



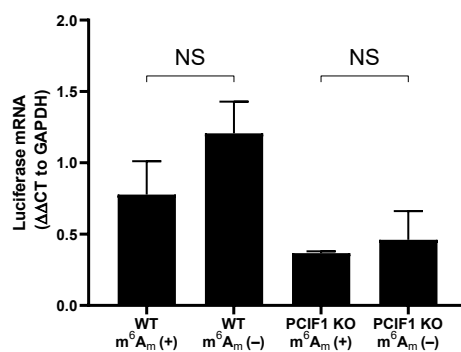
**A**



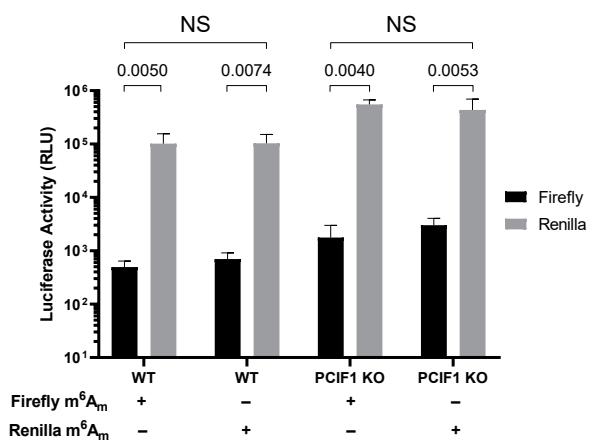
**B**



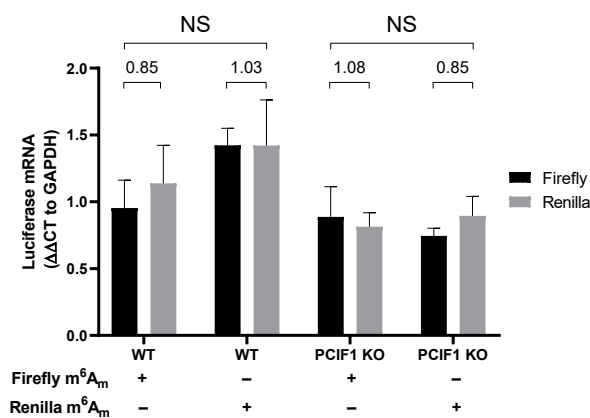
**C**



**D**



**E:**

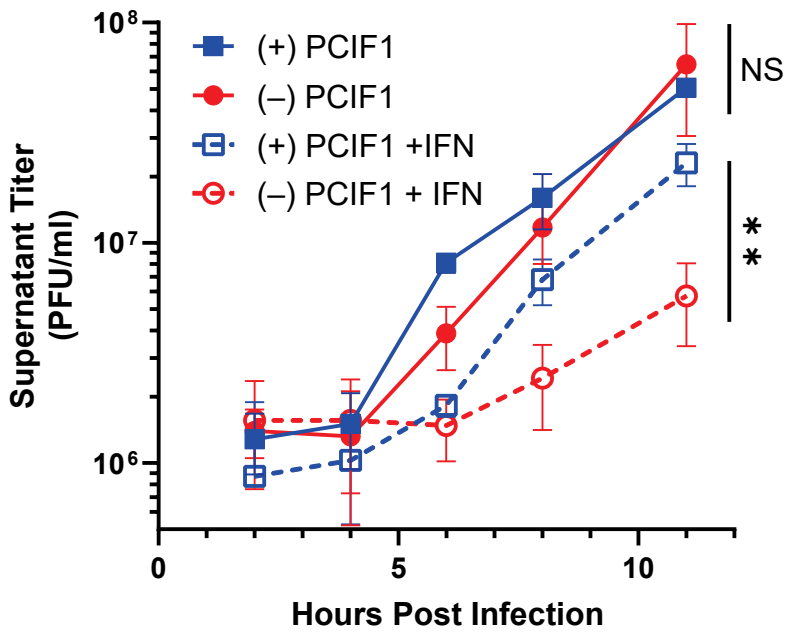


**Figure S5: Effect of m<sup>6</sup>A<sub>m</sub> on translation of VSV mRNA reporters.**

Purified stocks of VSV mRNA from the indicated virus were generated by infecting 293T PCIF1 KO cells at a MOI of 10 with VSV as in Fig 4, followed by purification of extracted RNA by poly(A) selection and a biotinylated oligonucleotide against the conserved VSV-stop sequence. 500 ng RNA was then mock or in vitro methylated with purified PCIF1, and transfected into HeLa wild type or PCIF1 knockout cells. **(A):** N6-methylation does not impact translation of a GFP reporter. VSV-eGFP mRNA was transfected into cells, and flow cytometry performed at 6 hpi. Mean fluorescence intensity (n=3, +/- SD, NS – p>0.18, student's t-test,) and percent GFP positive cells (n=3, +/- SD, NS – p>0.89, student's t-test) are shown, with a representative fluorescence microscopy image of transfected cells. **(B):** N6-methylation does not impact translation of a luciferase reporter. 500 ng VSV-luciferase mRNA with the indicated methylation was transfected into the indicated HeLa cells. Cells were lysed at 6 hpi, and luciferase levels were measured using a Promega Luciferase Assay kit (n=3, +/- SD, NS – p>0.70, student's t-test). **(C):** RNA was extracted from lysate from (B), RT-PCR performed with oligo-dT primers, and qPCR performed for luciferase RNA (n=3, normalized to GAPDH, +/- SD, NS – p>0.08, student's t-test). **(D):** Translation of an m<sup>6</sup>A<sub>m</sub> (+) reporter does not outcompete a co-transfected m<sup>6</sup>A<sub>m</sub> (-) reporter. 300 ng purified VSV-Luc (firefly) and VSV-RenP (renilla) mRNAs with opposing methylation status (m<sup>6</sup>A<sub>m</sub> (+) firefly with m<sup>6</sup>A<sub>m</sub> (-) renilla, and vice versa) were transfected into the indicated HeLa cells for 8 hours. Cells were lysed and luciferase levels of both reporters using a Promega Dual-Luciferase kit. Relative luminescence units (RLU) are shown (n=3, +/- SD, NS – 0.98 >p>0.11, student's t-test). Ratios of Firefly to Renilla are shown above each condition.

**(E):** No change in luciferase RNA levels from (D). RNA from (D) was extracted and qPCR performed as in C for Firefly and Renilla luciferase transcripts (n=3, normalized to GAPDH, +/- SD, 0.49>p >0.05, student's t-test).

Figure S6

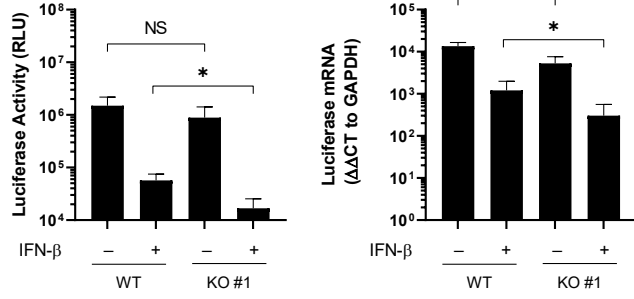


**Figure S6: Effect of IFN- $\beta$  pretreatment of cells on viral infection in a second *PCIF1*-addback clone**

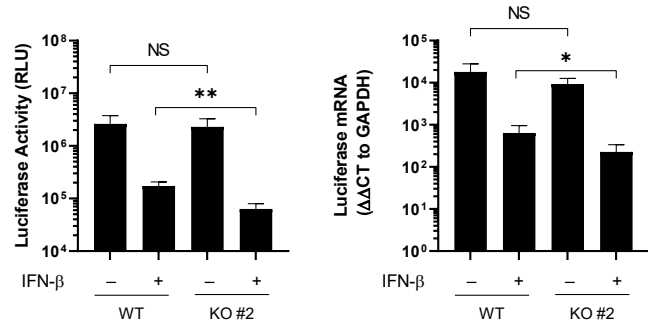
PCIF1 KO HeLa cells (different single cell clone from Fig 4C, 5A) reconstituted with PCIF1 or an empty vector were pretreated with vehicle (0.1% BSA) or 500 U ml<sup>-1</sup> interferon- $\beta$  for 5h. Treatment media was removed from the cells, followed by infection with VSV WT at a MOI of 3. After 1 hour, the inoculum was removed, cells washed, and initial treatment media added back to cells. At 2, 4, 6, 8, and 11 hpi, 1% of the supernatant was removed, and plaque assays performed on Vero cells to determine the titer of VSV in each sample. Growth curve of supernatant virus (n=3, +/-SD. NS – p>0.51, \*\* - p<0.01, student's t-test. Statistics shown are for the 11h timepoint).

**A**

## KO #1 Infection

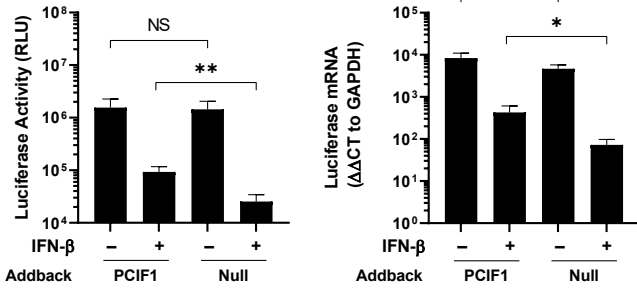


## KO #2 Infection



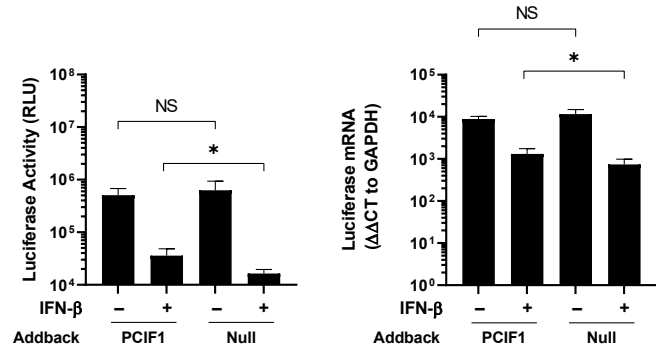
**B**

## Addback Infection



**C**

## Addback RNP Transfection



**Figure S7: Effect of IFN- $\beta$  pretreatment of cells on viral infection in multiple single-cell clones of PCIF1 KO cells.**

**(A):** PCIF1 KO HeLa cells reconstituted with PCIF1 or an empty vector were pretreated with IFN- $\beta$  for 5h, then infected with VSV expressing a luciferase reporter (VSV-Luc) at a MOI of 3. Cells were lysed at 6 hpi. Half the lysate was used to measure luciferase using a Promega Luciferase Assay kit (n=4, +/-SD, NS – p>0.80, \*\* – p<0.01, student's t-test). RNA was extracted from the other half in Trizol and RT-PCR performed using oligo-dT, followed by qPCR for luciferase mRNA (normalized to GAPDH, n=4, +/-SD, NS – p>0.05, \* – p<0.05, student's t-test). **(B):** As in A, except cells were transfected with 500 ng ribonucleoprotein cores of VSV-Luc. (n=4, +/-SD, NS – p>0.19, \* – p<0.05, student's t-test). **(C):** Wild type (parental) HeLa or PCIF1 KO cells (two independent clones) were pretreated with IFN- $\beta$  for 5h, then infected with VSV expressing a luciferase reporter (VSV-Luc) at a MOI of 3. Luciferase protein was measured as in A (n=4, NS – p>0.20, \* - p<0.05, \*\* - p<0.01, student's t-test). Luciferase mRNA was also measured as in A (normalized to GAPDH, n=4, NS – p>0.18, \* - p<0.05, \*\* - p<0.01, student's t-test).



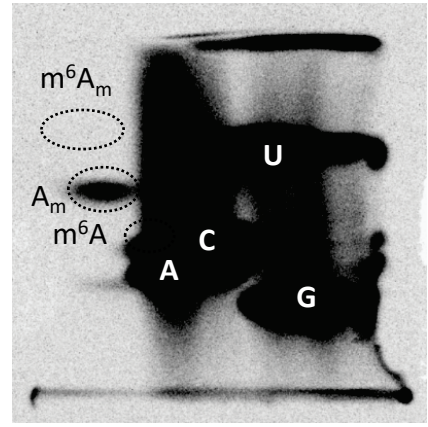
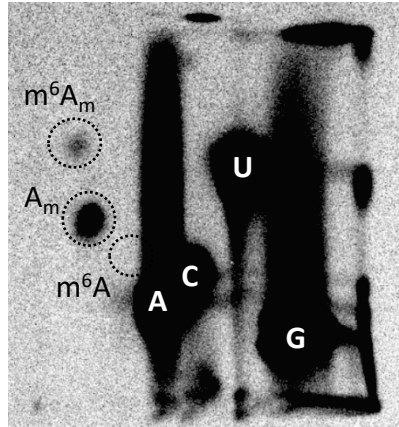
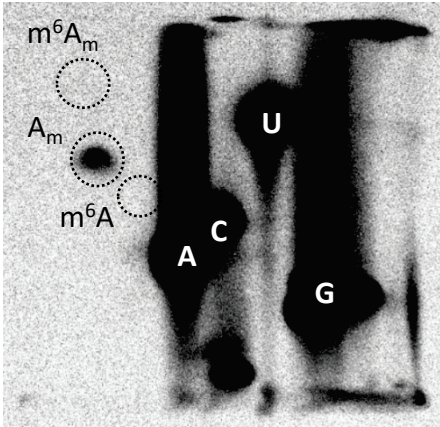
WT

PCIF1 KO

P1

Cap-Clip + P1

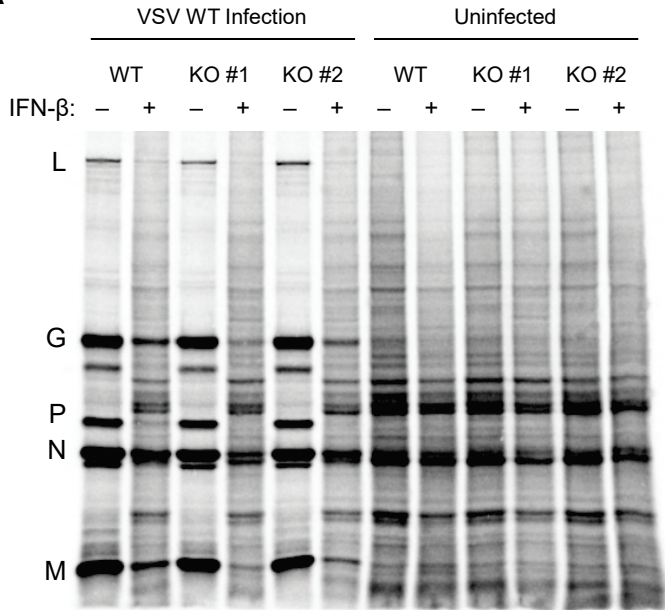
Cap-Clip + P1



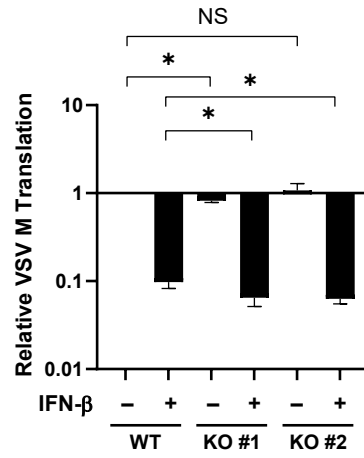
**Figure S8: VSV mRNA in A549 cells contain m<sup>6</sup>A<sub>m</sub>**

A549 WT or PCIF1 KO cells were infected with VSV at a MOI of 5, and viral mRNA specifically radiolabeled, extracted, and digested as in Fig 2A. Released nucleotide monophosphates were resolved by 2D-TLC and detected by phosphorimaging (representative images, n=3) **(A)** VSV mRNA from wild type cells digested with the indicated enzymes. **(B)** VSV mRNA from PCIF1 KO cells.

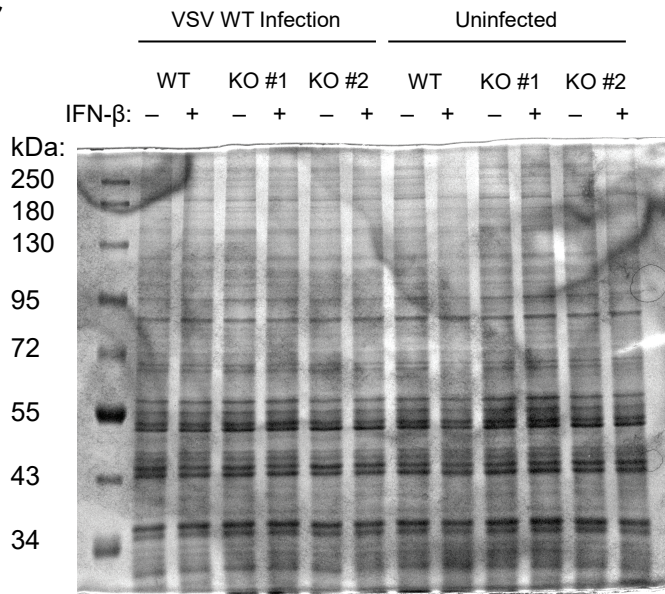
**A**



**B**

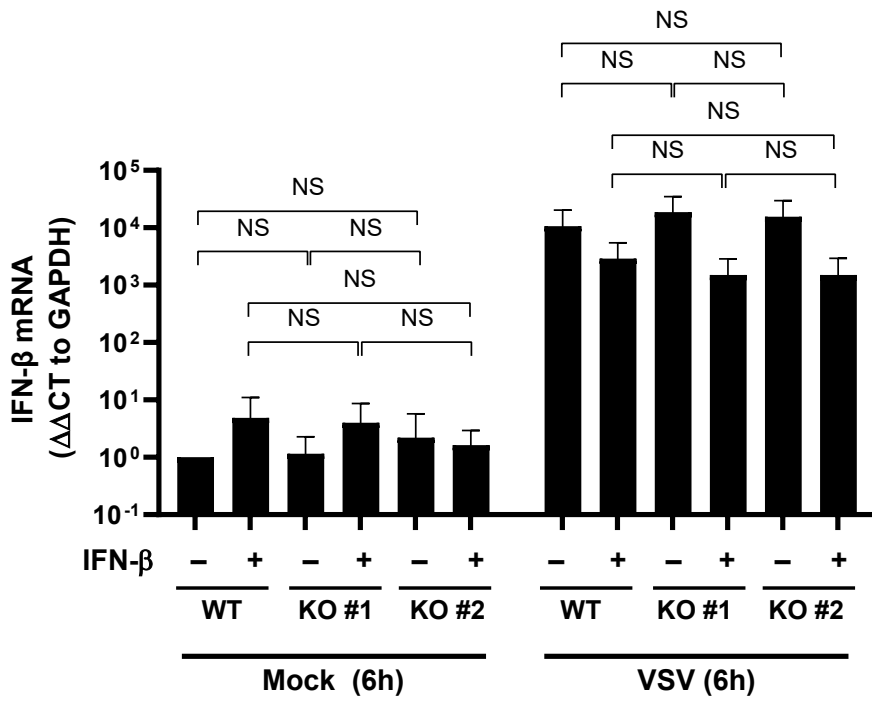


**C**



**Figure S9: Effect of IFN- $\beta$  pretreatment and PCIF1 knockout on rates of viral translation**

**(A):** The indicated A549 cells were pretreated with vehicle (0.1% BSA) or 500 U ml<sup>-1</sup> IFN- $\beta$  for 5 hours prior to infection with VSV at a MOI of 5 for 6 hours. Rates of viral translation were assessed by metabolic incorporation of [<sup>35</sup>S]-met and [<sup>35</sup>S]-cys into viral proteins for 20 minutes as described in methods. Proteins were analyzed by SDS-PAGE and visualized by phosphorimager (representative image, n=3). **(B):** Quantification of VSV M protein in A (n=3, +/- SD, \* – p < 0.05, NS – p >0.58). **(C):** Gels used for PAGE-analysis of radiolabeled products in A were stained with 0.25% Coomassie Brilliant Blue R-250 in 10% acetic acid, followed by destaining in 10% acetic acid. Gels were visualized by OD laser scanning on a GE Typhoon 5. Staining indicated even protein loading (representative image shown corresponding to autoradiogram in Fig 5D, n=3).

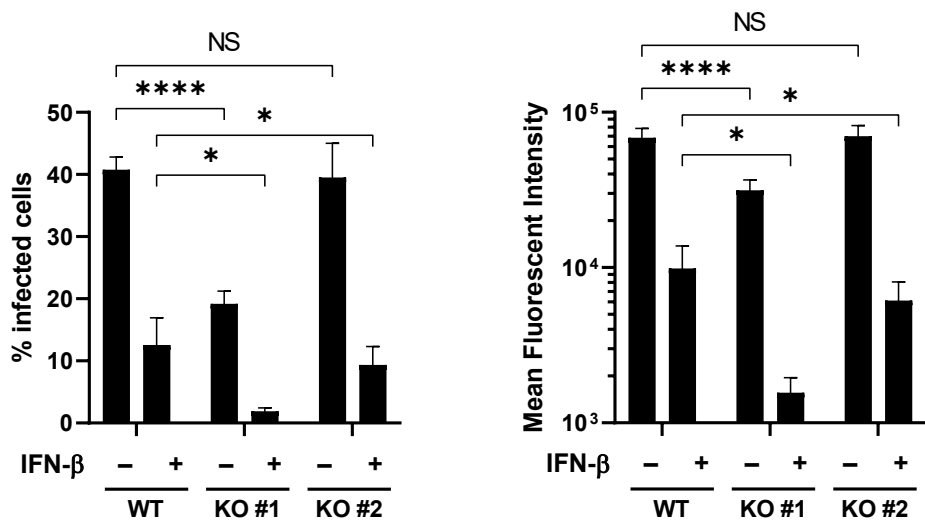


**Figure S10: PCIF1 does not regulate VSV mRNA sensing late in infection**

The indicated A549 cells were pretreated with vehicle (0.1% BSA) or 500 U ml<sup>-1</sup> IFN- $\beta$  for 5 hours prior to infection with VSV at a MOI of 5 for 6 hours. Cells were lysed and IFN- $\beta$  mRNA levels determined by qRT-PCR (n=4, +/- SD, NS – 0.08 < p < 0.80, student's t-test).

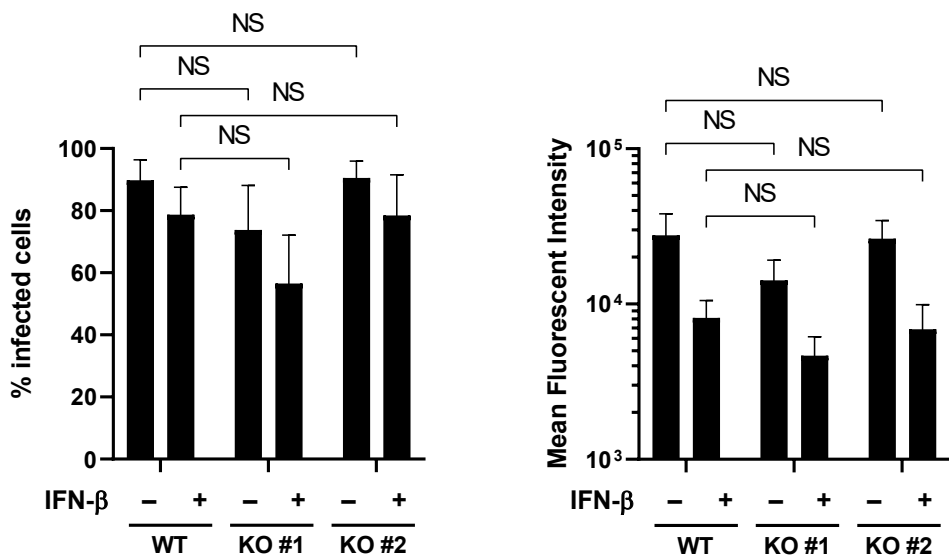
A

Rabies ΔG



B

Measles



### **Figure S11: Effect of PCIF1 on viral-encoded eGFP expression**

**(A):** The indicated A549 cells were pretreated with vehicle (0.1% BSA) or 500 U ml<sup>-1</sup> IFN-β concurrent with infection with RABV ΔG at a MOI of 1 for 24 hours. Cells were fixed in formaldehyde and flow cytometry performed for eGFP expression. Percent infected cells were defined as GFP positive, measured by gating on uninfected cells. Mean fluorescent intensity was measured based on the total population (n=3, +/- SD, NS – 0.56 < p < 0.58, \* – p < 0.05, \*\* – p < 0.01, \*\*\*\* – p < 0.0001, student's t-test). **(B):** The indicated A549 cells were pretreated with vehicle (0.1% BSA) or 500 U ml<sup>-1</sup> IFN-β for 5 hours prior to infection with measles virus at a MOI of 3 for 24 hours. Cells were fixed in formaldehyde and flow cytometry performed for eGFP expression. Percent infected cells were defined as GFP positive, measured by gating on uninfected cells. Mean fluorescent intensity was measured based on the total population (n=3, +/- SD, NS – 0.09 < p < 0.46, student's t-test).



**Table S1: Oligonucleotides used for qPCR or mRNA selection**

<b>Primer</b>	<b>Direction</b>	<b>Sequence</b>
Firefly Luciferase	Forward	CAACTGCATAAGGCTATGAAGAGA
Firefly Luciferase	Reverse	ATTTGTATTCAGCCCATATCGTTT
Renilla Luciferase	Forward	GAGCATCAAGATAAGATCAAAGCA
Renilla Luciferase	Reverse	CTTCACCTTTCTCTTTGAATGGTT
eGFP	Forward	GAACCGCATCGAGCTGAA
eGFP	Reverse	TGCTTGTGCGCCATGATATAG
IFIT1	Forward	AACTTAATGCAGGAACATGACAA
IFIT1	Reverse	CTGCCAGTCTGCCCATGTG
GAPDH	Forward	AGCCTCAAGATCATCAGCAAT
GAPDH	Reverse	ATGGACTGTGGTCATGAGTCCTT
Biotin-T(16)-VSVstop		5'biotin-TTTTTTTTTTTTTTTTTTCATA