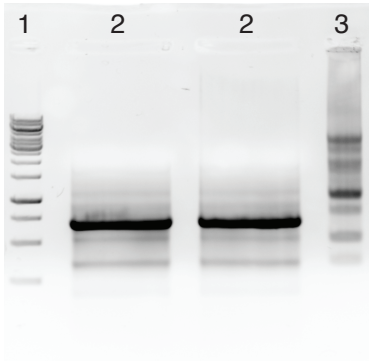
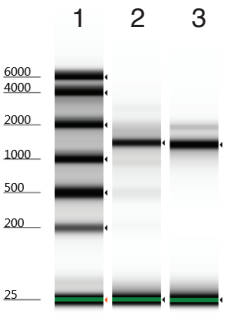
A

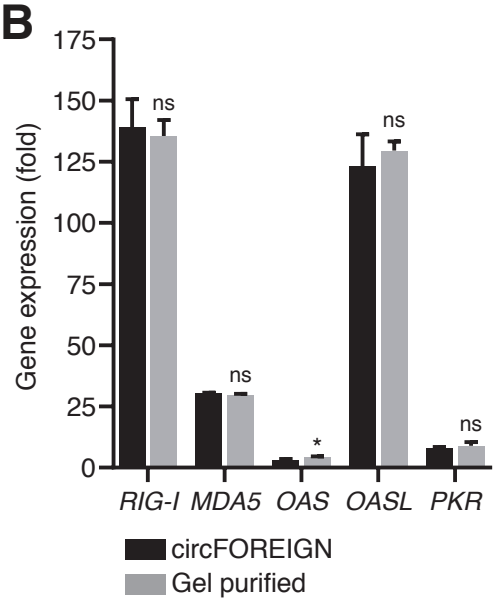


1. GeneRuler 1 kb DNA Ladder
2. circFOREIGN
3. RiboRuler low range RNA ladder



1. Tapestation ladder
2. circFOREIGN
3. Gel purified

B



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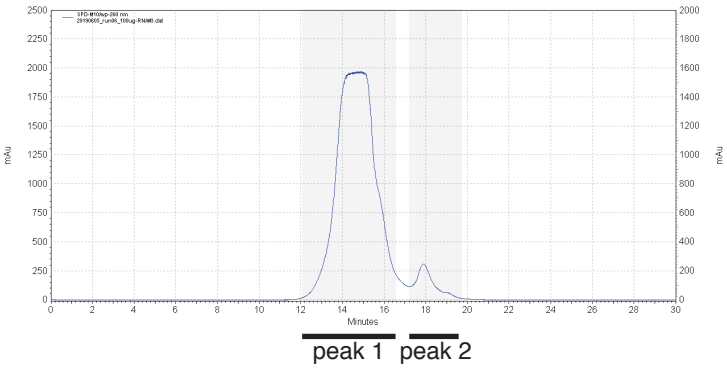
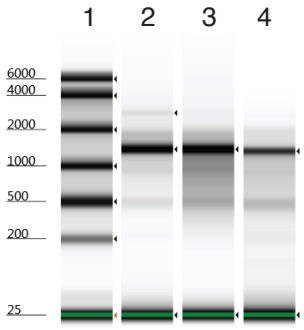




1. Tapestation ladder
2. circFOREIGN
3. HPLC peak 1
4. HPLC peak 2

D

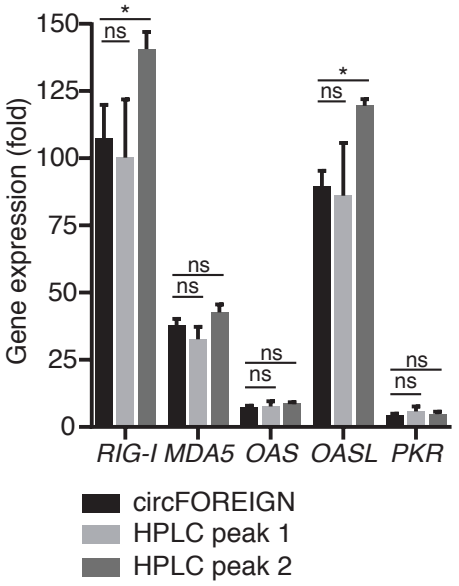


Figure S1. Gel and HPLC purification of circFOREIGN, related to STAR Methods.

A. Agarose gel electrophoresis of circFOREIGN prior to gel purification (left) and Tapestation analysis of resulting purified RNA (right).
B. Graph showing gene expression of innate immune genes 24 hours following RNA transfection into HeLa cells. Relative expression of the indicated mRNA and transfected RNA are measured by qRT-PCR, results normalized mock transfection. Means ± SEM are shown (n = 3).). *p<0.05, Student's t-test, comparing circFOREIGN to gel purified RNA transfection.
C. HPLC chromatogram of circFOREIGN purification. Collected fractions indicated on trace (left) and Tapestation analysis of purified RNA (right).
D. Graph showing gene expression of innate immune genes 24 hours following RNA transfection into HeLa cells. Relative expression of the indicated mRNA and transfected RNA are measured by qRT-PCR, results normalized to expression following mock transfection. Means ± SEM are shown (n = 3).). *p<0.05, Student's t-test, comparing circFOREIGN to indicated RNA transfection.

Figure S2, Chen et al. (CHANG)

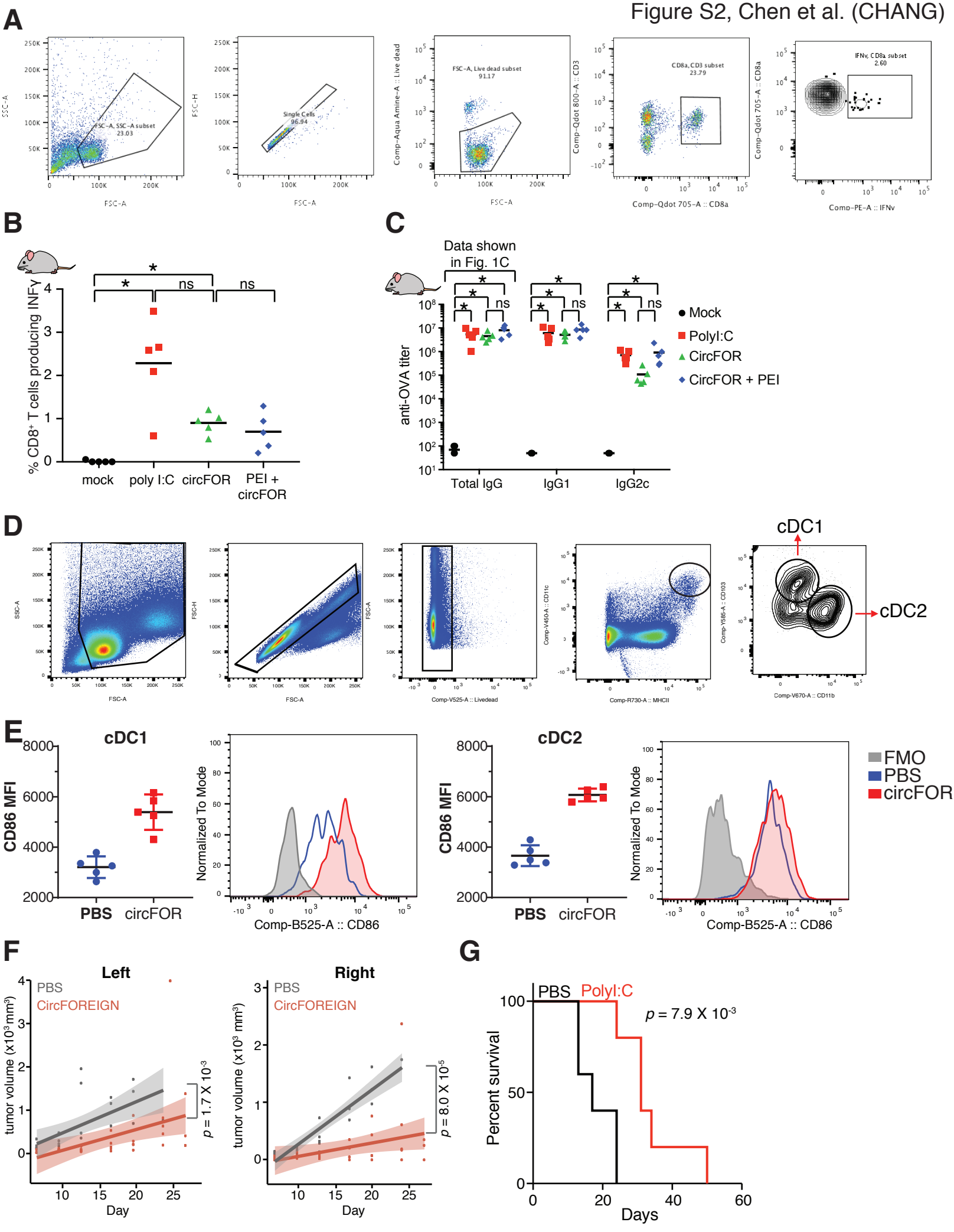
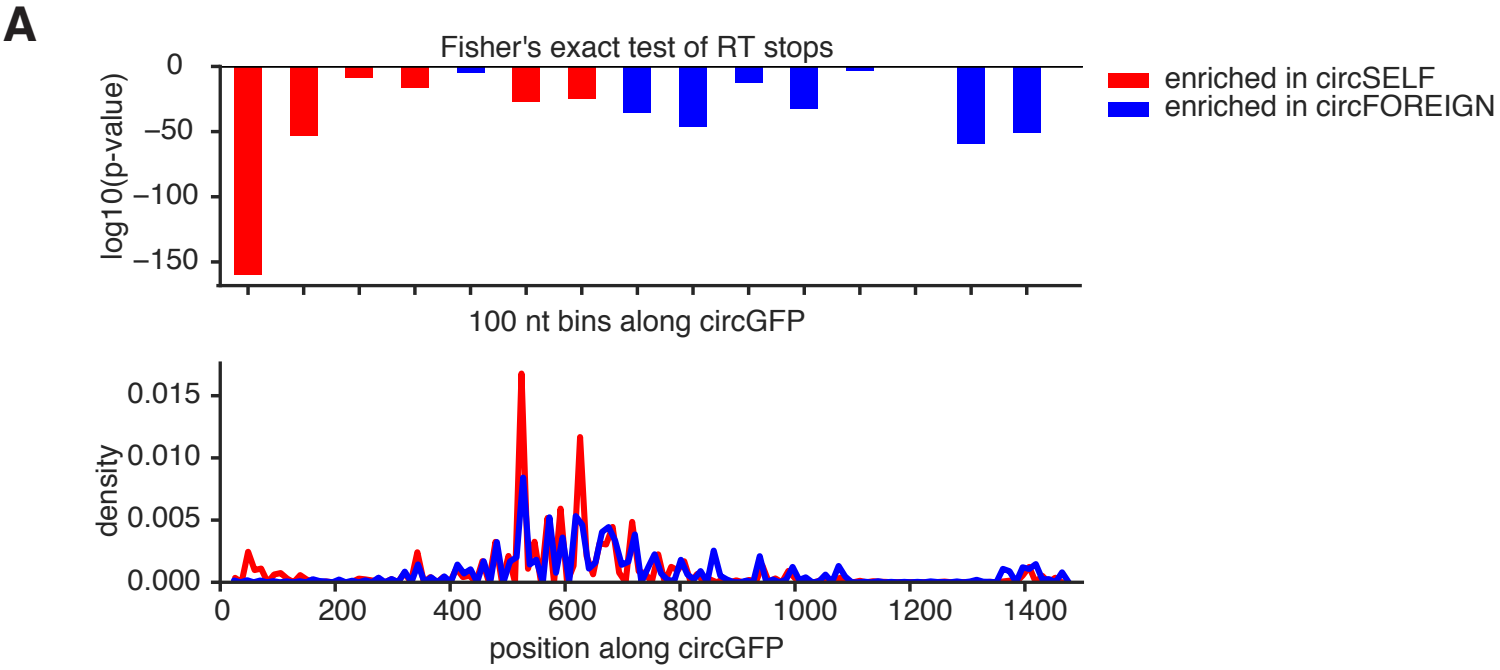
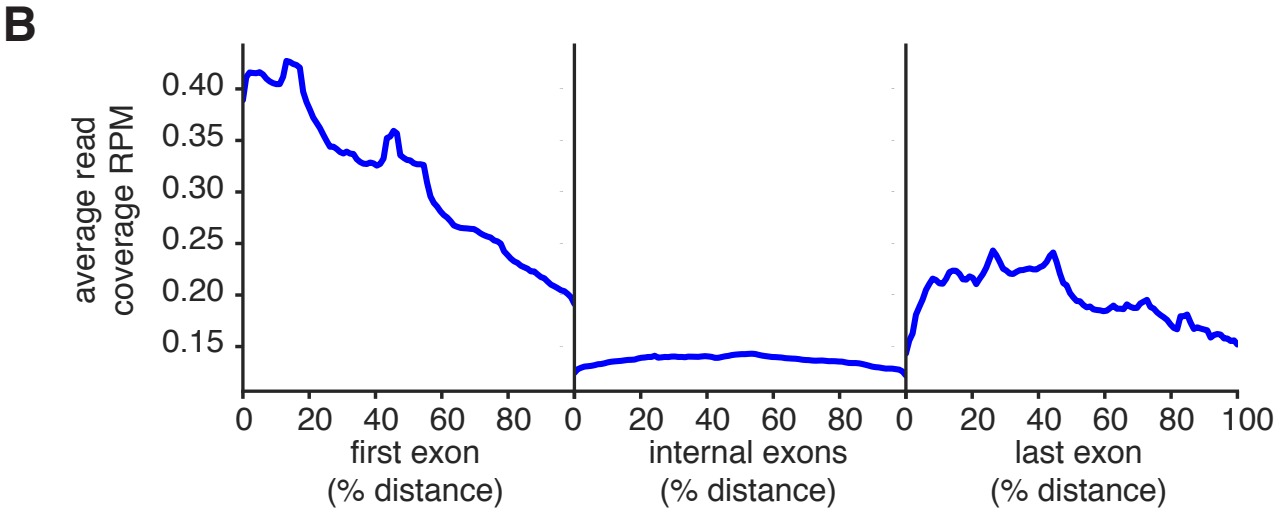


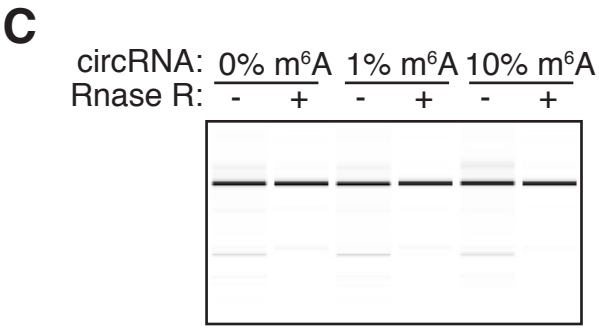
Figure S2. CircFOREIGN kinetics and vaccination effects in vivo, related to Figure 1.

A. Gating strategy for FACS analysis of IFNγ + CD8 + T cells.
B. CircFOREIGN stimulates anti-OVA specific T cell response independent of PEI after secondary immunization. Means are shown (n =5), *p<0.05, Anova-Tukey's test.
C. CircFOREIGN stimulates anti-OVA antibody titers independent of PEI after secondary immunization. Means are shown (n = 5), *p<0.05, Anova-Tukey's test.
D. Gating strategy for FACS analysis of cDC1 and cDC2 cells.
E. CircFOREIGN immunization activates DCs in mice.
F. Measurements of left and right tumor volumes in mice vaccinated with PBS or circFOREIGN. p value calculated by Wilcoxon signed-rank test.
G. Survival curve of mice vaccinated with PBS or positive control polyI:C. p value calculated by log-rank test.

Figure S3, Chen et al. (CHANG)







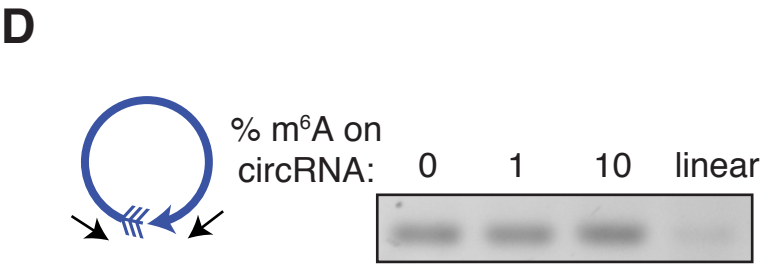


Figure S3. m^{6}A modification on endogenous RNAs and circFOREIGN, related to Figure 2.

A. m^{6}A-irCLIP identifies high confidence m^{6}A positions of circSELF or circFOREIGN. Fisher's exact test of RT stops enriched in circSELF (red) or circFOREIGN (blue). Density of m^{6}A-irCLIP reads normalized to reads per million.
B. Analysis determines m^{6}A frequency on endogenous linear RNA.
C. TapeStation analysis of in vitro transcribed circFOREIGN with the indicated levels of m^{6}A modification incorporated and with or without RNase R treatment.
D. qRT-PCR over splice junction confirms unmodified and m^{6}A-modied circRNA formation during in vitro transcription. Agarose gel of unmodified and m^{6}A-modified circRNA after qRT-PCR using "inverted" primers as indicated.

Figure S4, Chen et al. (CHANG)

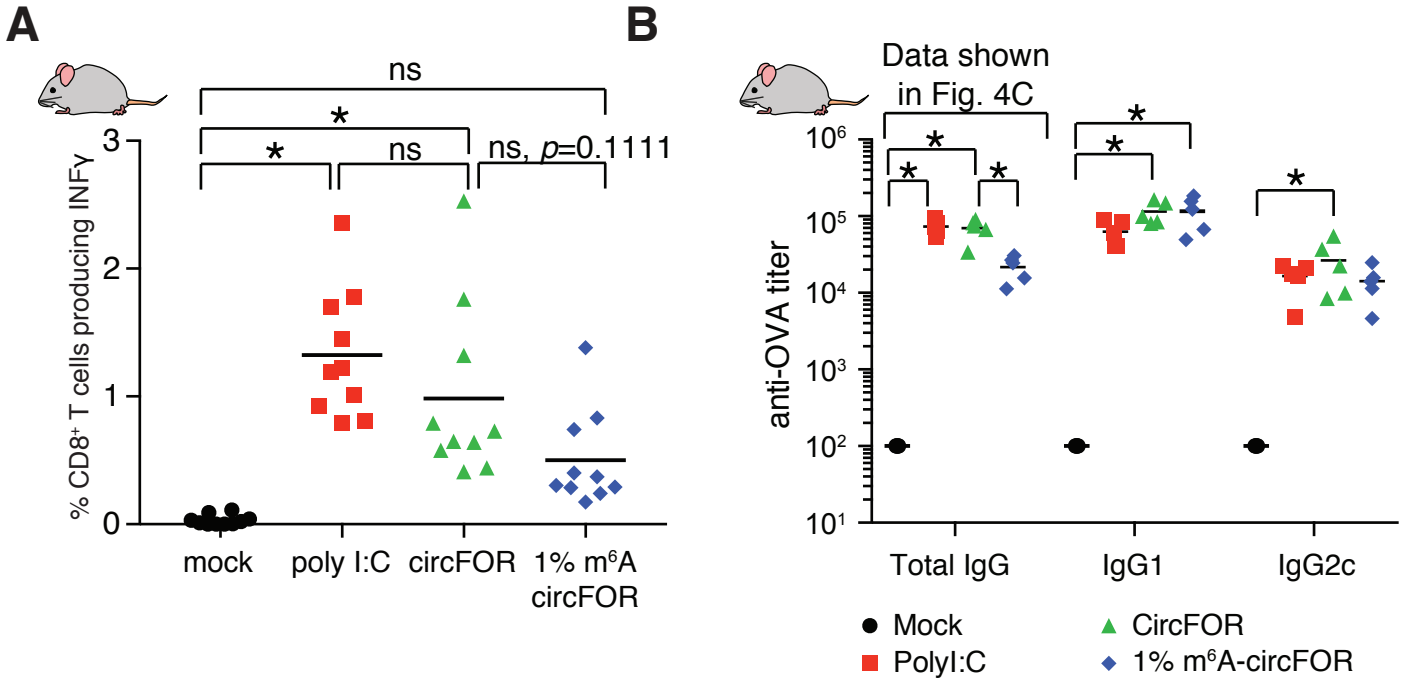


Figure S4. Effects of circFOREIGN immunization, related to Figure 3.

A. CircFOREIGN stimulates anti-OVA specific T cell response and 1% m^6A-modifed circFOREIGN attenuates immunity after secondary immunization. Means are shown (n = 10), **p*<0.05, Anova-Tukey's test.

B. CircFOREIGN stimulates anti-OVA antibody titers and 1% m^6A-modifed circRNA attenuates immunity after secondary immunization. Means are shown (n = 5), **p*<0.05, Anova-Tukey's test.

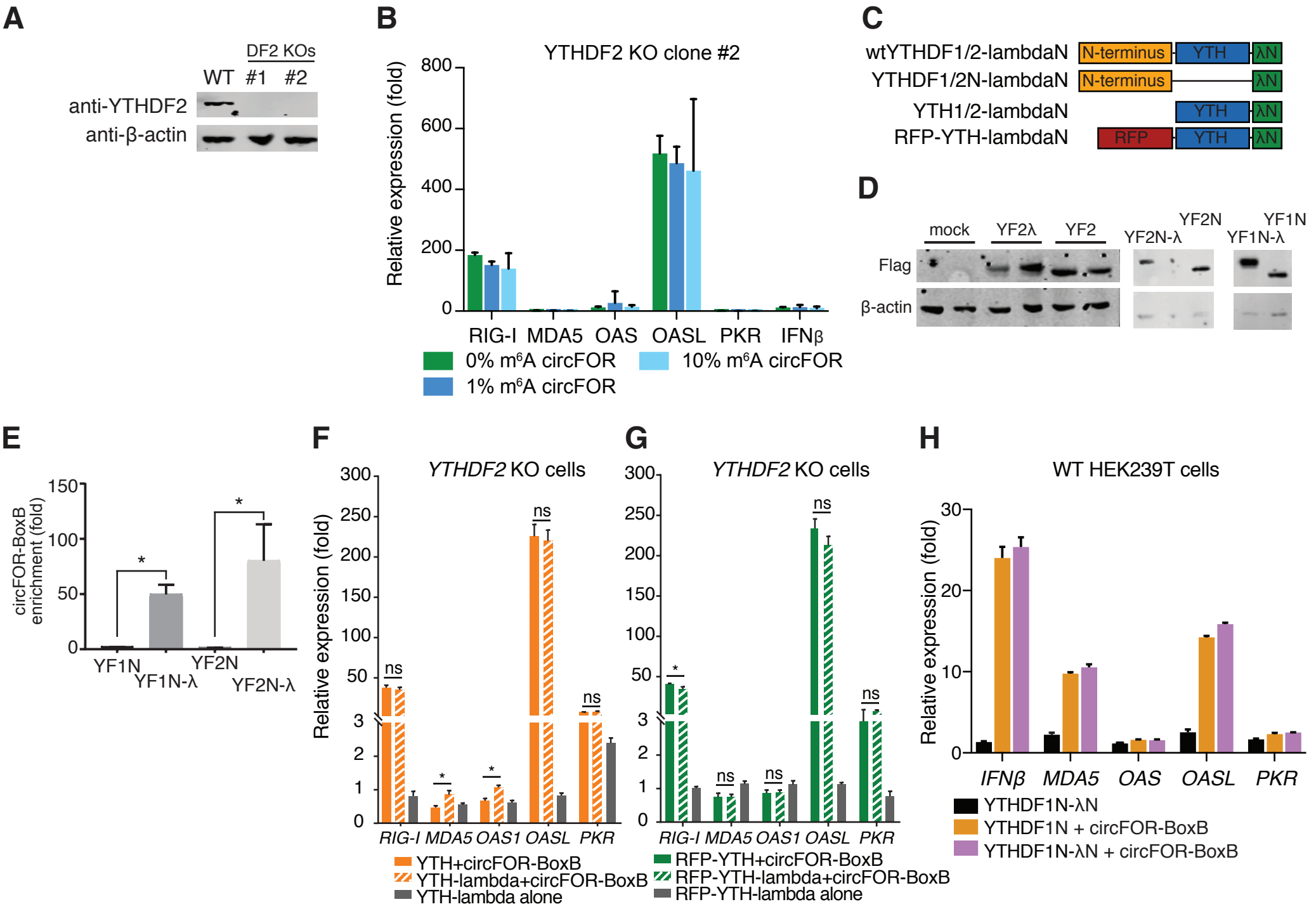
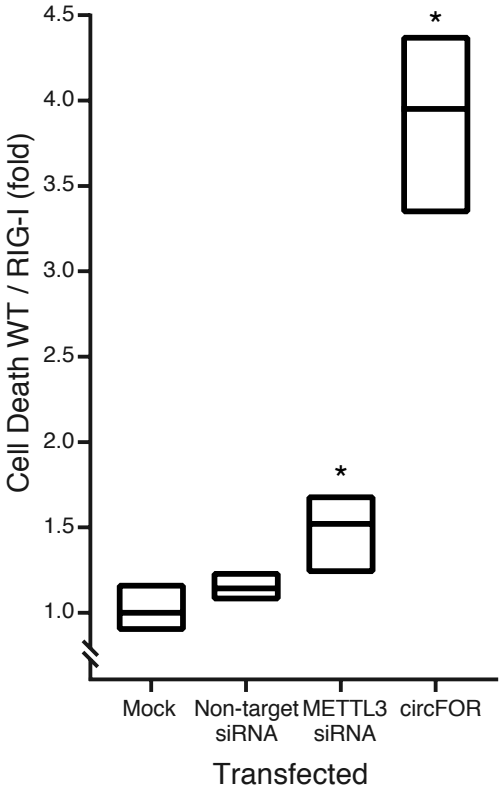

A
DF2 KOs
WT #1 #2
anti-YTHDF2
anti-β-actin
B
YTHDF2 KO clone #2
Relative expression (fold)
800
600
400
200
0
RIG-I MDA5 OAS OASL PKR IFNβ
0% m6A circFOR
10% m6A circFOR
1% m6A circFOR
C
wtYTHDF1/2-lambdaN
YTHDF1/2N-lambdaN
YTH1/2-lambdaN
RFP-YTH-lambdaN
N-terminus
YTH
λN
RFP
D
mock
YF2λ
YF2
YF2N-λ
YF2N
YF1N-λ
YF1N
Flag
β-actin
E
circFOR-BoxB enrichment (fold)
150
100
50
0
YF1N
YF1N-λ
YF2N
YF2N-λ
F
YTHDF2 KO cells
Relative expression (fold)
300
250
200
150
100
50
3
2
1
0
ns
RIG-I MDA5 OAS1 OASL PKR
YTH+circFOR-BoxB
YTH-lambda+circFOR-BoxB
YTH-lambda alone
G
YTHDF2 KO cells
RFP-YTH+circFOR-BoxB
RFP-YTH-lambda+circFOR-BoxB
RFP-YTH-lambda alone
H
WT HEK239T cells
Relative expression (fold)
30
20
10
0
IFNβ MDA5 OAS OASL PKR
YTHDF1N-λN
YTHDF1N + circFOR-BoxB
YTHDF1N-λN + circFOR-BoxB

Figure S5. m^{6}A reader proteins are not sufficient to block circRNA immunity, related to Figure 4.

A. Western blot of HeLa wild-type and two YTHDF2 KO clones.
B. Gene expression of innate immune genes 24 hours following RNA transfection into HeLa YTHDF2-/- clone #2. Relative expression of the indicated mRNA and transfected RNA are measured by qRT-PCR, results normalized to expression following mock transfection. Means ± SEM are shown (n = 3).
C. Schematic of YTHDF1/2 constructs used.
D. Western blots of YTHDF2-λ, YTHDF2, YTHDF2N, YTHDF2N- λ, YTHDF1N, and YTHDF1N-λ.
E. RIP-qPCR enrichment of indicated YTH protein followed by qRT-PCR of circRNA-BoxB or control actin RNA. Means ± SEM are shown (n = 3). *p<0.05, Student's t-test.
F. Transfection of unmodified circBoxB tethered to C-terminal YTH domain of YTHDF2 into YTHDF2 KO cells is insufficient to attenuate immune response. Relative expression of the indicated mRNA and transfected RNA are measured by qRT-PCR, results normalized to expression following mock transfection. Means ± SEM are shown (n = 3). *p < 0.05, Student's t-test, comparing cells receiving +/- YTHDF2 transfection.
G. Transfection of unmodified circBoxB tethered to RFP-YTH domain protein fusion into YTHDF2 KO cells is insufficient to attenuate immune response. Relative expression of the indicated mRNA and transfected RNA are measured by qRT-PCR, results normalized to expression following mock transfection. Means ± SEM are shown (n = 3). *p < 0.05, Student's t-test, comparing cells receiving +/- YTHDF2 transfection.
H. Transfection of unmodified circBoxB tethered to YTHDF1 is insufficient to attenuate immune response. Gene expression of innate immune genes 24 hours following RNA transfection into wild-type HEK 293T cells. Relative expression of the indicated mRNA and transfected RNA are measured by qRT-PCR, results normalized to expression following transfection of plasmid expressing YTHDF1N-λN. Means ± SEM are shown (n = 3).

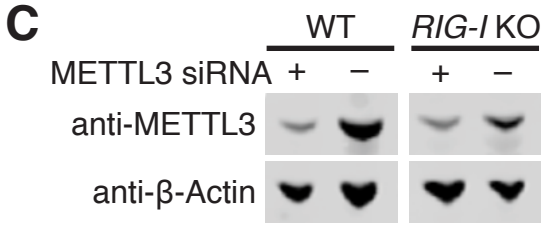
A



B

	Mock		Mock siRNA		METTL3 siRNA		circFOREIGN	
	Live	**Dead**	**Live**	**Dead**	**Live**	**Dead**	**Live**	**Dead**
Wild type	47958	226	48297	184	47447	266	8393	7033
	47801	180	48209	192	67073	282	9046	7153
	47931	172	47780	211	47469	274	8936	5705
RIG-I KO	39397	376	39124	316	48627	348	15429	5353
	39334	259	38775	218	48705	242	16622	6367
	39161	209	38876	206	48588	297	16574	4818

C



D

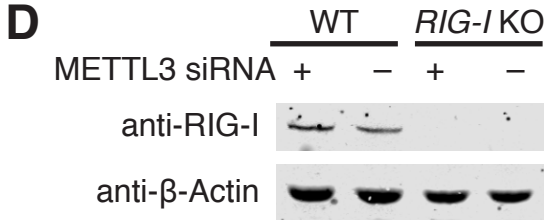


Figure S6. m^{6}A knockdown increases cell death, related to Figure 4.

A. RIG-I KO rescues cell death induced by depletion of m^{6}A writer METTL3. Graph showing the fold change of cell death in wild-type or RIG-I KO HeLa cells following transfection of indicated RNA. Means ± SEM are shown (n~50,000 cells analyzed). *p < 0.05, ***p<0.001, Student's t-test, comparing mock transfection to indicated RNA transfection.
B. Raw cell counts from FACS analysis in A.
C. Western blot validation of METTL3 knockdown efficiency in HeLa wild-type or RIG-I KO cells with METTL3 siRNA or non-targeting control siRNA transfection.
D. Western blot validation of RIG-I protein expression in HeLa wild-type and RIG-I KO cells. Cells were transfected with METTL3 siRNA or non-targeting siRNA under comparable conditions to FACS experiment.

Figure S7, Chen et al. (CHANG)

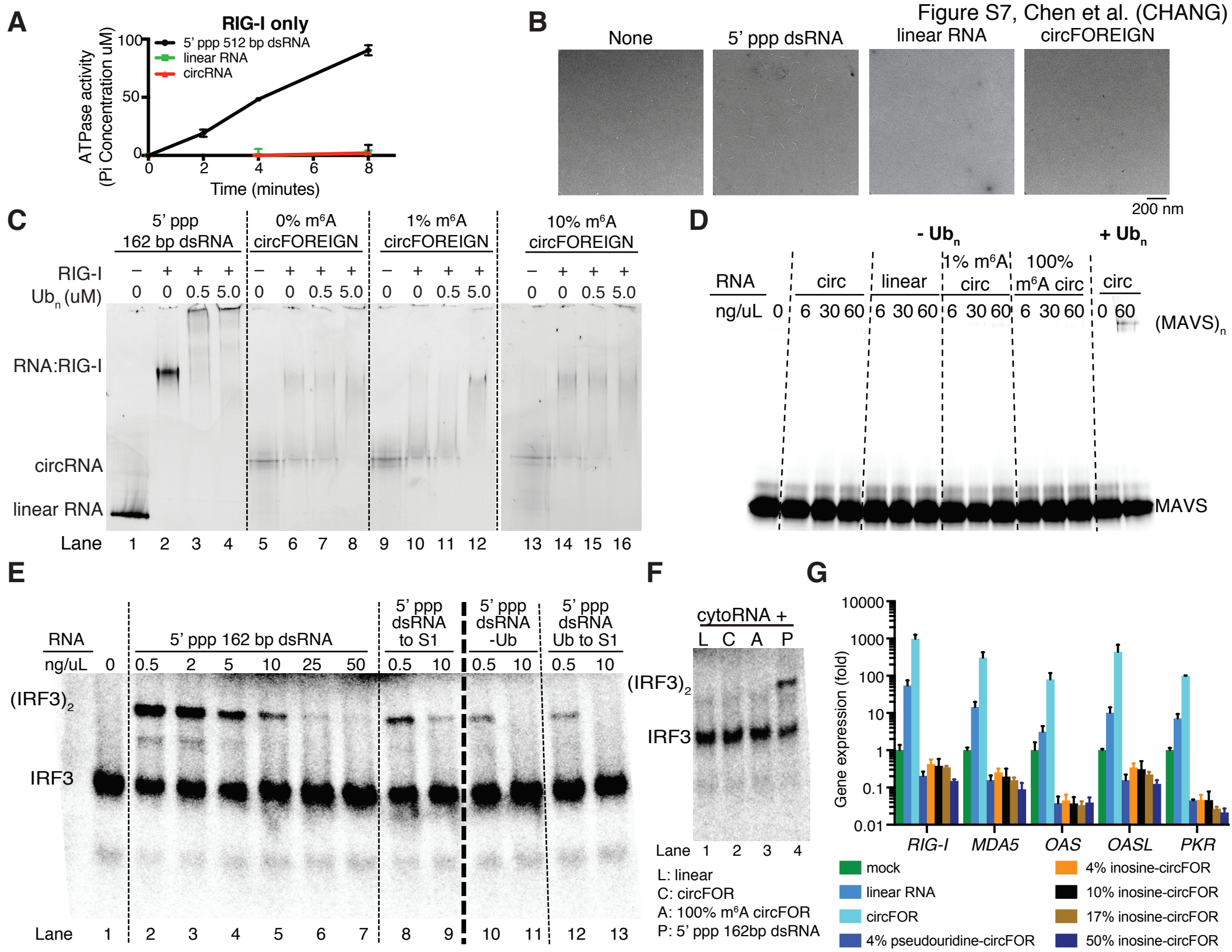


Figure S7. Regulation of circFOREIGN activation of innate immune signaling, related to Figure 5.

A. CircFOREIGN does not induce ATPase activity of RIG-I. RIG-I and RNA were incubated, and ATP added. Reaction quenched at indicated time points and Pi concentration measured. Means ± SEM are shown (n = 2).
B. Representative electron microscopy images of RIG-I filaments after RIG-I is incubated with indicated RNA.
C. *In vitro* RIG-I binding assay with purified RIG-I, K63-polyubiquitin the indicated RNA ligands. Native electrophoretic gel shift assay shows that RIG-I binding does not distinguish between unmodified and m^6A-modified circFOREIGN.
D. *In vitro* reconstitution with purified RIG-I, MAVS, the indicated RNA ligands, and the absence or presence of K63-polyubiquitin. Native gel of fluorescently-labeled MAVS 2CARD domain shows that circFOREIGN-initiated MAVS filamentation is dependent on K63-polyubiquitin.
E. In vitro reconstitution of the circRNA-mediated induction of IRF3 dimerization. RIG-I, IRF3, and the indicated RNA ligands were incubated. Native gel of radiolabeled-IRF3 with the indicated RNA ligands is shown.
F. Native gel of radiolabeled-IRF3 with the indicated RNA ligands is shown. Cytoplasmic RNA (cytoRNA) and indicated RNAs were each added at 0.5 ng/uL.
G. Transfection of unmodified circRNAs into wild-type HeLa cells stimulates immune response. Graph showing gene expression of innate immune genes 24 hours following RNA transfection. Relative expression of the indicated mRNA and transfected RNA are measured by qRT-PCR, results normalized to expression following mock transfection. Means ± SEM are shown (n = 3).