Extended Data

Nucleolar stress generates ribosomal DNA–derived RNA–DNA hybrids that prime innate immunity

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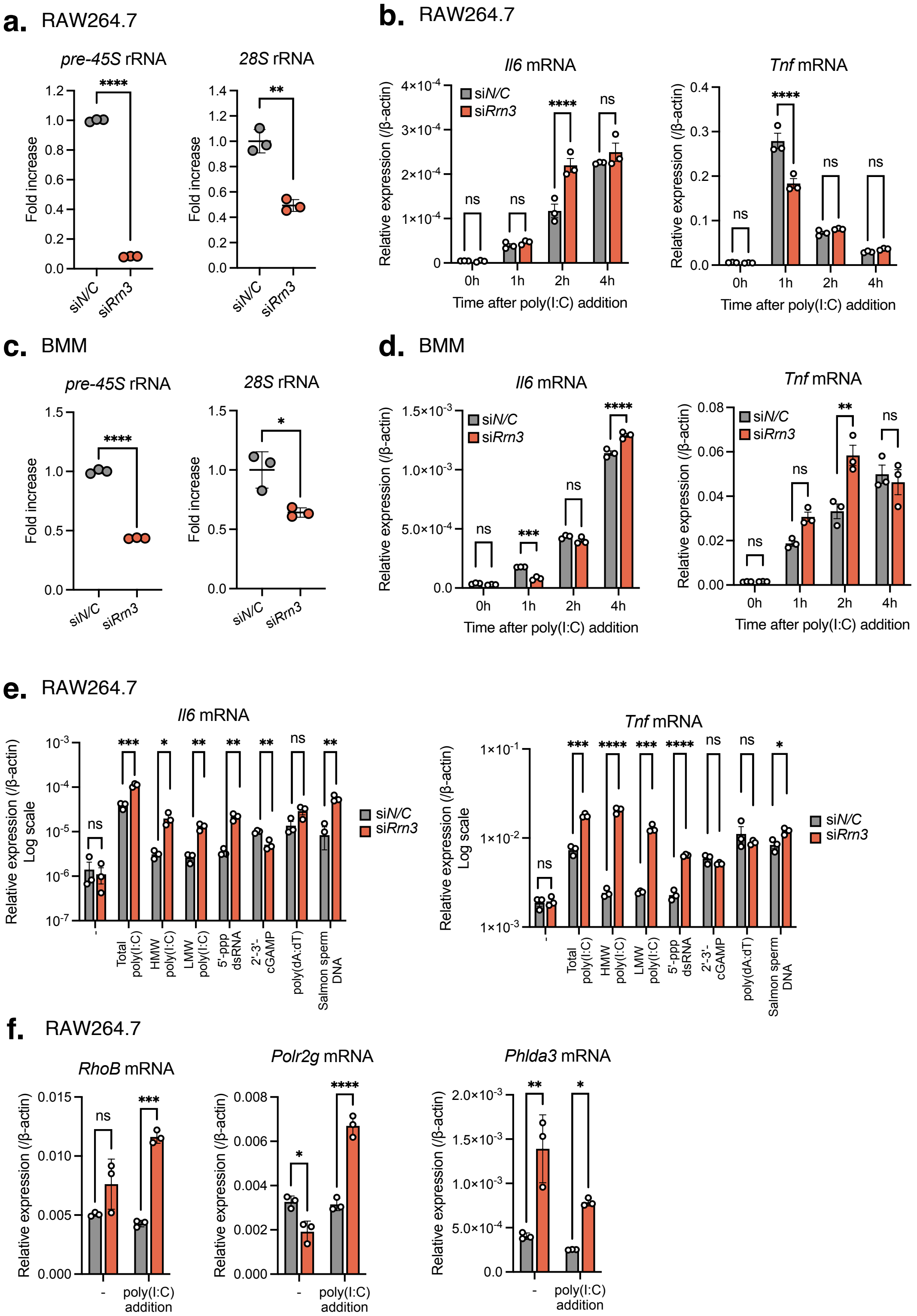
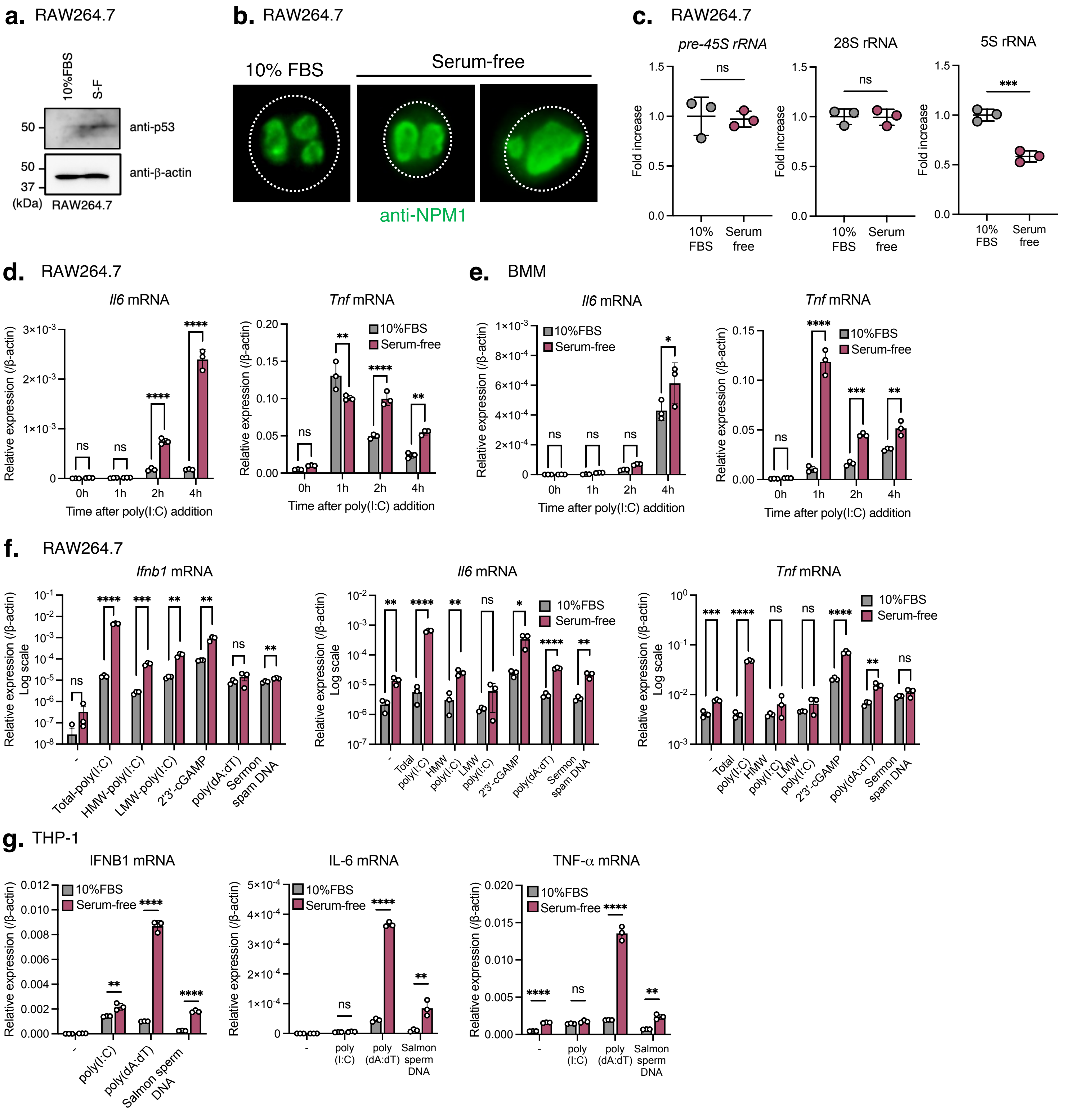
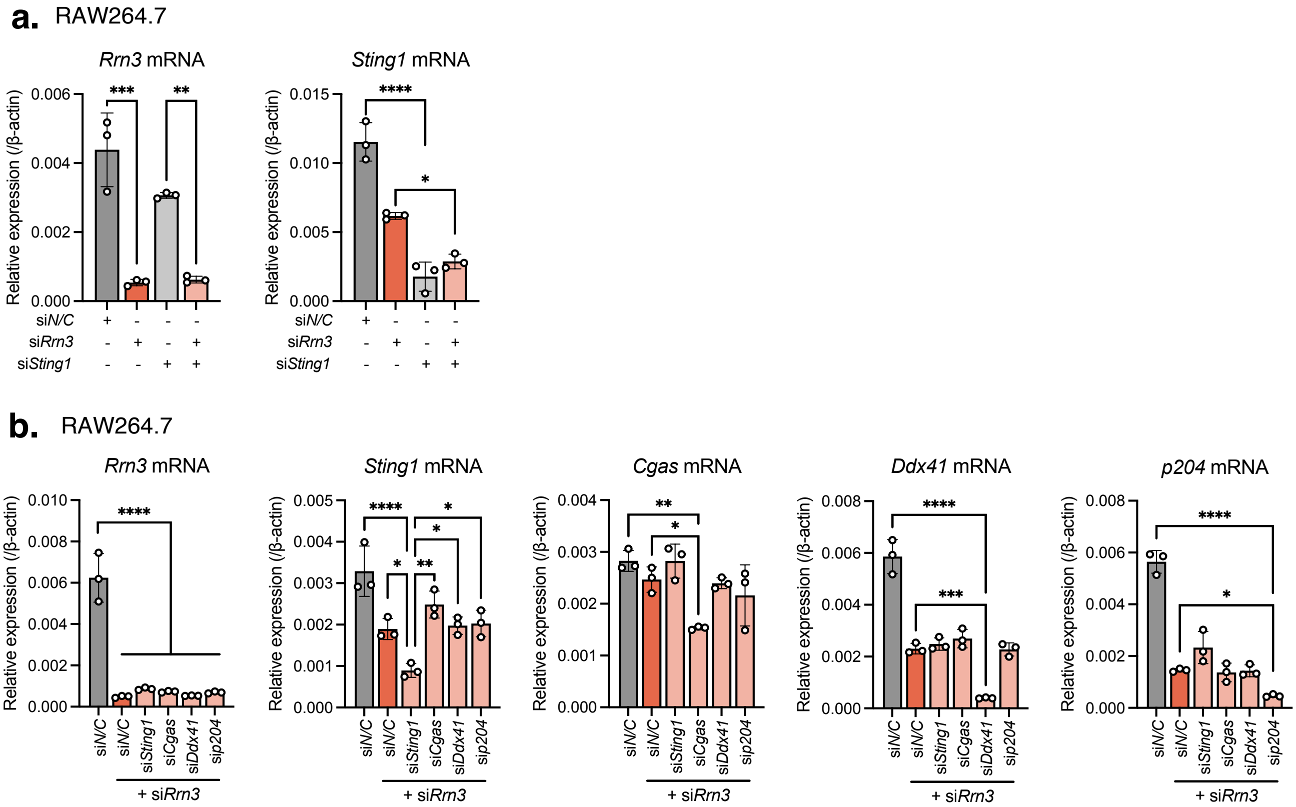
Extended Data Figure1.



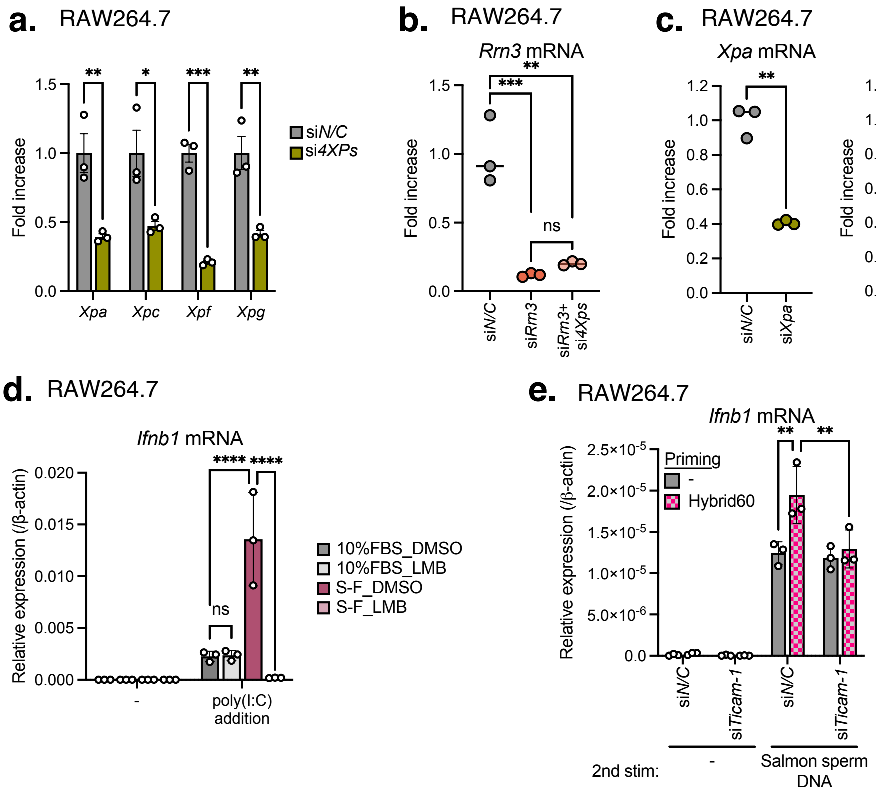
Extended Data Figure2.



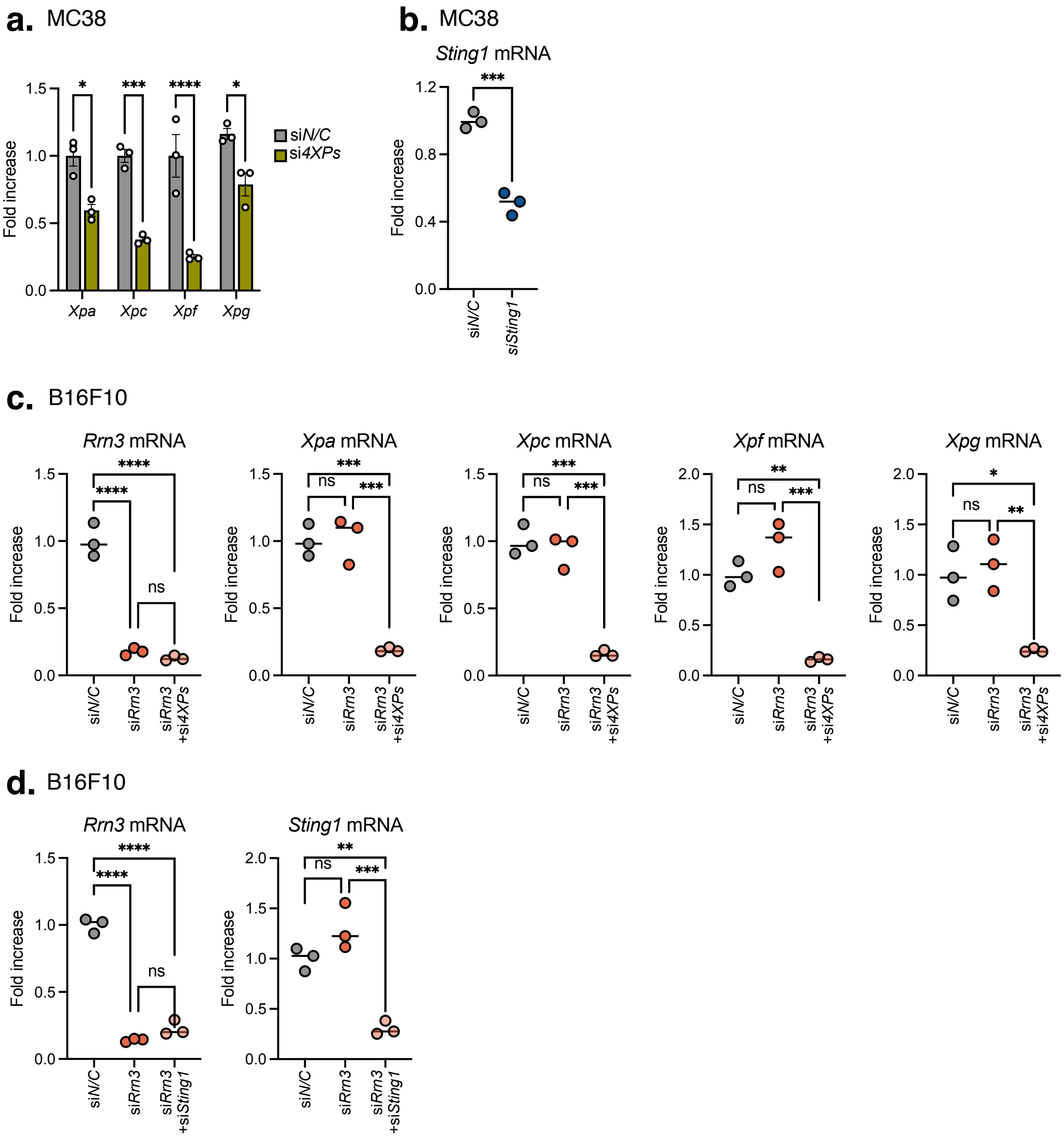
Extended Data Figure3.



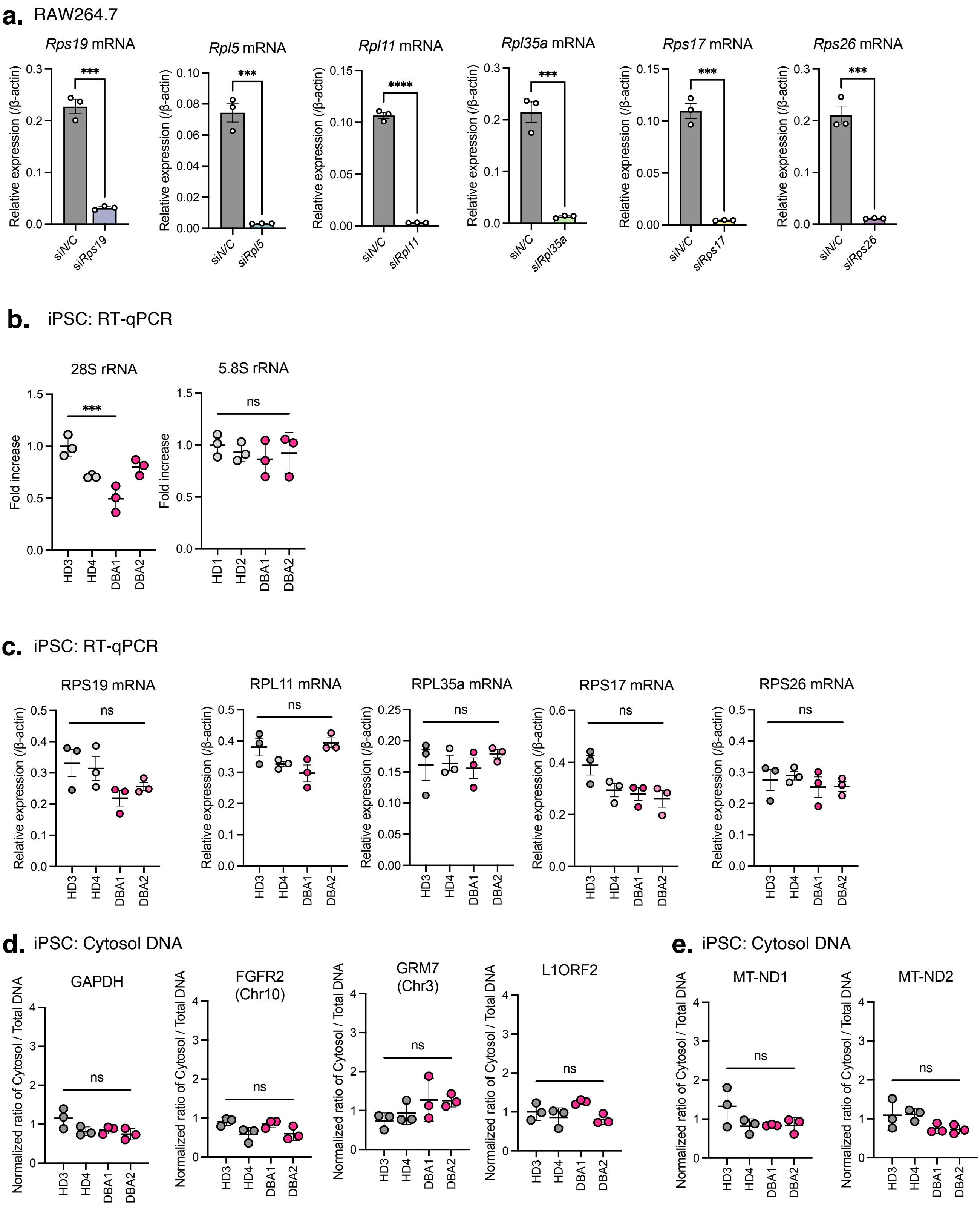
Extended Data Figure4.



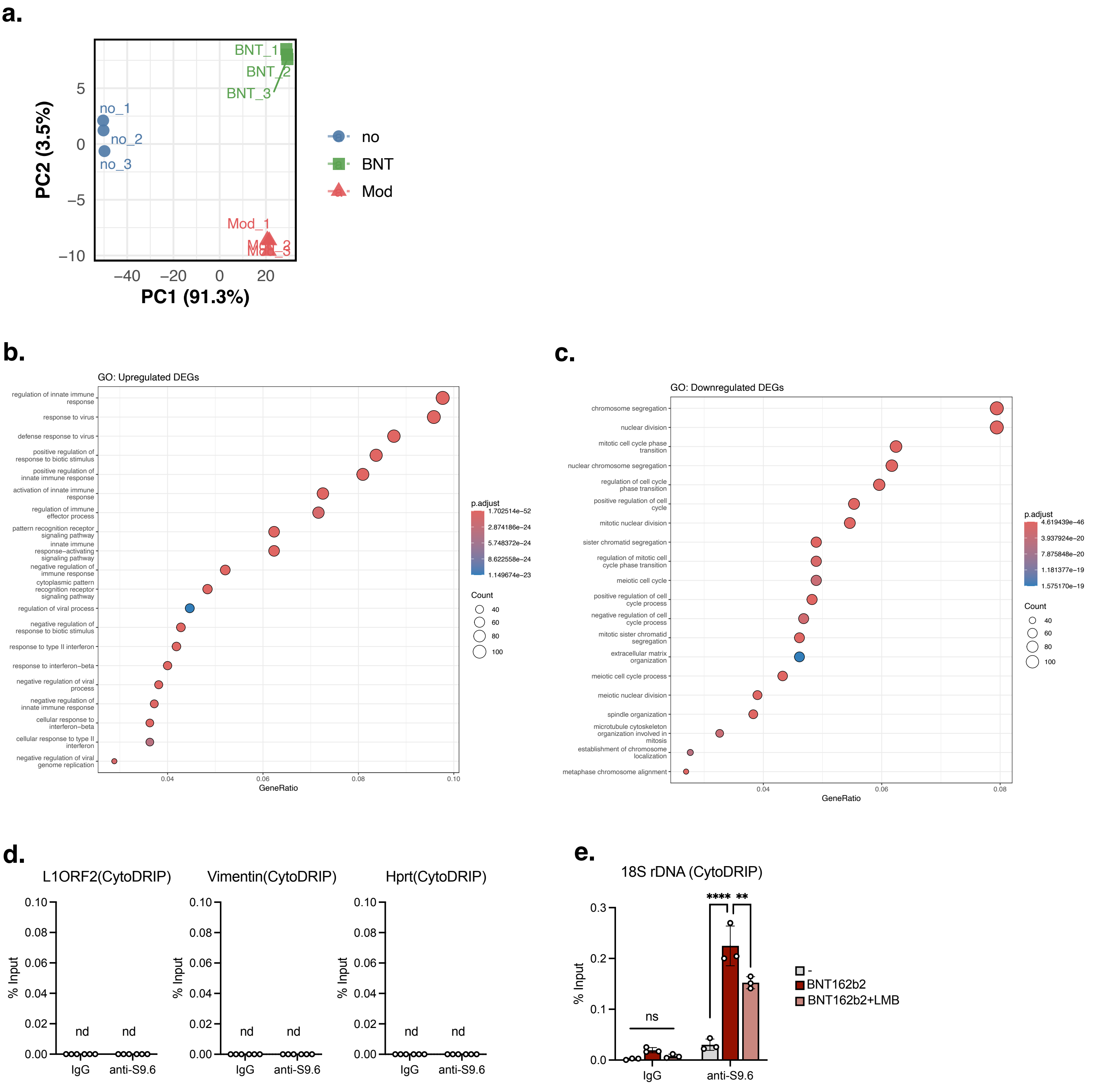
Extended Data Figure5.



Extended Data Figure6.



Extended Data Figure7.



Extended Data Figure legends

Extended Data Figure 1 | RRN3 knockdown primes type I IFN without broadly elevating proinflammatory cytokines (relates to Fig. 1)

(a) 45S pre-rRNA and 28S rRNA in control vs Rrn3-KD RAW264.7 cells.

(b) Time course of *Il6* and *Tnf* mRNA following poly(I:C) stimulation in control versus *Rrn3*-knockdown (KD) RAW264.7 cells.

(c) 45S pre-rRNA and 28S rRNA in control or *Rrn3*-KD BMMs.

(d) Time course of *Il6* and *Tnf* mRNA following poly(I:C) stimulation in control versus *Rrn3*-KD BMMs.

(e) *Il6* or *Tnf* mRNA 6 h after transfection with Total poly(I:C) (1 μg ml^{-1}), HMW-poly(I:C) (1 μg ml^{-1}), LMW-poly(I:C) (1 μg ml^{-1}), 5-ppp dsRNA (1 μg ml^{-1}), 2'3'-cGAMP (1 μg ml^{-1}), poly(dA:dT) (1 μg ml^{-1}) or Salmon sperm DNA (1 μg ml^{-1}) in control or *Rrn3*-KD RAW264.7 cells.

(f) *RhoB*, *Polr2g* and *Phlda3* mRNA 2 h after poly(I:C) addition in control or *Rrn3*-KD RAW264.7 cells.

Extended Data Figure 2 | Serum starvation induces nucleolar stress and amplifies the subsequent PAMP responses (relates to Fig. 1)

(a) Immunoblot of p53 and β-actin protein in RAW264.7 cells cultured with 10% FBS or serum-free medium.

(b) Immunofluorescence with anti-NPM1 antibody in RAW264.7 cells cultured in media with or without 10% FBS.

(c) Levels of 45S pre-rRNA, 28S rRNA and 5S rRNA in RAW264.7 cells cultured in media containing 10% FBS or not.

(d,e) Relative expression levels of *Ifnb1* (left) or *Il6* (right) mRNA at indicated time points after poly(I:C) addition (25 μg ml^{-1}) in RAW264.7 (d) or BMM cells (e) cultured with or without 10% FBS.

(f) Relative expression levels of *Ifnb1*, *Il6* or *Tnf* mRNA at 6 hours after Total poly(I:C) (1 μg ml^{-1}), HMW-poly(I:C) (1 μg ml^{-1}), or LMW-poly(I:C) (1 μg ml^{-1}) transfection in RAW 264.7 cells maintained with or without 10% FBS.

(g) Relative mRNA expression levels of *Ifnb1* in THP-1 cells cultured in RPMI media with or without 10%FBS at 6 hours after poly(I:C) (1 μg ml^{-1}), poly(dA:dT) (1 μg ml^{-1}), or Salmon sperm DNA (1 μg ml^{-1}) transfection.

Extended Data Figure 3 | Validation of DNA-sensing pathway knockdowns (relates to Fig. 2)

(a) *Rrn3* and *Sting1* mRNA in RAW264.7 with single or combined knockdown.

(b) *Rrn3*, *Sting1*, *Cgas*, *Ddx41* and *p204* mRNA under the indicated knockdowns.

Extended Data Figure 4 | XP proteins promote cytosolic ribo-RNA-DNA hybrid accumulation and priming of innate immunity (relates to Fig. 4)

(a) *Xpa*, *Xpc*, *Xpf*, *Xpg* mRNA in RAW264.7 transfected with control siRNA or pooled XP siRNAs (si4XPs); β-actin-normalized fold change vs siN/C.

(b) *Rrn3* mRNA in control, *Rrn3*-KD and/or si4XPs-transfected RAW264.7 cells.

(c) Single-gene XP KD validation (Xpa, Xpc, Xpf, Xpg).

(d) *Ifnb1* at 2 h after poly(I:C) in cells pretreated 24 h with DMSO or leptomycin B (LMB; 5 nM) under +FBS vs serum-free conditions.

(e) TICAM-1 dependence of hybrid60 priming: cells pre-transfected with hybrid60

(1,000 ng, 24 h) were stimulated with salmon sperm DNA (1 µg ml^{-1}, 6 h); *Ifnb1* mRNA expression in control vs *Ticam-1*-KD.

Extended Data Figure 5 | KD validation in tumor cell lines (relates to Fig. 5)

(a) *Xpa*, *Xpc*, *Xpf*, *Xpg* mRNA in MC38 with si4XPs; fold change vs siN/C.

(b) *Sting1* mRNA in MC38 ± *Sting1* siRNA.

(c) *Rrn3* and XP mRNAs in B16F10 with control, *Rrn3*-KD and/or si4XPs.

(d) *Rrn3* and *Sting1* mRNA in B16F10 with control, *Rrn3*-KD and/or *Sting1*-KD.

Extended Data Figure 6 | DBA models and patient-derived iPSCs: validation and additional readouts (relates to Fig. 6)

(a) KD validation in RAW264.7 for DBA genes (*Rps19, Rpl5, Rpl11, Rpl35a, Rps17, Rps26*).

(b) 28S and 5S rRNA in iPSCs from healthy donors (HD3, HD4) and DBA patients (DBA1, DBA2).

(c) RPS19, RPL11, RPL5, RPL35a, RPS17, RPS26 mRNA in iPSCs (healthy vs DBA).

(d,e) Cytosolic-to-total DNA ratios for nuclear loci (GAPDH, FGFR2, BRM7, L1ORF2) (d) and mitochondrial loci (ND1, ND2) (e).

Extended Data Figure 7 | Vaccine-stimulated macrophages: transcriptomics and hybrid controls (relates to Fig. 7)

(a) PCA of BMM RNA-seq: unstimulated (No), BNT162b2 (BNT), mRNA-1273 (Mod).

(b,c) GO enrichment for DEGs (No vs BNT): upregulated (b) and downregulated (c).

(d) cytoDRIP–qPCR for hybrids at L1ORF2, Vimentin, and Hprt.

(e) cytoDRIP–qPCR for ribo-RNA–DNA hybrids in BMMs treated with BNT162b2 (24 h) ± LMB.

[1] Antibodies

Reagents	Source	Identifier
Anti-β-actin	Sigma-Aldrich	Cat# AMAb91241; RRID: AB_2665860
Anti-p53	Cell Signaling Technology	Cat# 9282
Anti-TLR3	Novus Biologicals	Cat# NBP2-24875SS, Clone 40C1285.6
Anti-RIG-I	Cell Signaling Technology	Cat# 3743
Anti-MDA5	Cell Signaling Technology	Cat# 5321
Anti-TBK1	Cell Signaling Technology	Cat# 3504
Anti-phospho-TBK1	Cell Signaling Technology	Cat# 5483
Anti-NF-κB p65	Cell Signaling Technology	Cat# 8242
Anti-phospho-NF-kB p65	Cell Signaling Technology	Cat# 3033
Anti-p38 MAPK	Cell Signaling Technology	Cat# 8690
Anti-phospho-p38 MAPK	Cell Signaling Technology	Cat# 4511
Anti-IRF3	Cell Signaling Technology	Cat# 11904
Anti-phospho-IRF3	Cell Signaling Technology	Cat# 4947
Anti-IkB	Cell Signaling Technology	Cat# 4812
Anti-DNA-RNA Hybrid Antibody, clone S9.6	Sigma-Aldrich	MABE1095
Anti-NPM1	Thermo Fisher Scientific	FC-61991

[2] siRNA

Target	ID(Silencer select pre-designed siRNA)
Negative Contol(N/C)	Silencer Select™ Negative Control #1: 4390844
mRrn3	s98618
mUbft	s74871
mNcl	s70419
mSting1	s91057
mTicam-1	s98707
mMavs	s105943
mCgas	s103168
mIfi204	s68054
mDdx41	s91394
mXpf	s78344
mXpg	s233386
mRps19	s234433
mRpl5	s73095
mRpl11	s84307
mRpl35a	s81425
mRps17	s203528
mRps26	s233869
hRRN3	s29324
hSTING1	s50645
hTICAM-1	s45113
hXPG	s4803

[3] Primer for Real Time-qPCR(gene expression)

Mouse

Gene	F	R	Primer Bank ID/ Reference
β-actin	TTTGCGCTCCTTCGTTGC	TCGTCATCCATGGCGAACT	
Ifnb1	CCAGCTCCAAGAAAGGACGA	CGCCCTGTAGGTGAGGTTGAT	
Il6	GTCTCTGGGAAATCGTGGA	GGTACTCCAGAAGACCAGAGGA	
Tnf	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	
Rrn3	GTCTCAGCATGATTGCGTCTC	GGTGTCGATGGGACATATCTTG	21706613a1
Ubft	CCGCTGGTCCCAGGAAGATA	CGACTCTGTGGTTTTGAACTTGG	12836542a1
Ncl	AAAGGCAAAAAGGCTACCACA	GGAATGACTTTGGCTGGTGTAA	31543315a1
pre-45S rRNA	CTCTTAGATCGATGTGGTGCTC	GCCCGCTGGCAGAACGAGAAG	Watada E et al., MCB, 2020
28S rRNA	TGGGTTTTAAGCAGGAGGTG	GTGAATTCTGCTTCACAATG	Watada E et al., MCB, 2020
5S rRNA	TCTCGTCTGATCTCGGAAGC	AGCCTACAGCACCCGGTATT	
Ccl7	GCTGCTTTCAGCATCCAAGTG	CCAGGGACACCGACTACTG	7305463a1
Ccl5	GCTGCTTTGCCTACCTCTCC	TCGAGTGACAAACACGACTGC	7305461a1
Ccl9	CCCTCTCCTTCCTCATTCTTACA	AGTCTTGAAAGCCCATGTGAAA	6755434a1
Tnfaip2	AGGAGGAGTCTGCGAAGAAGA	GGCAGTGGACCATCTAACTCG	6678375a1
Nfkbia	TGAAGGACGAGGAGTACGAGC	TTCGTGGATGATTGCCAAGTG	6754840a1
Il1rn	GCTCATTGCTGGGTACTTACAA	CCAGACTTGGCACAAGACAGG	13624317a1
Phlda3	CCGTGGAGTGCGTAGAGAG	TCTGGATGGCCTGTTGATTCT	7305377a1
Tlr3	TTGCGTTGCGAAGTGAAGAA	ACTTGCCAATTGTCTGGAAACA	
Ticam-1	TGTTGGAAAGCAGTGGCCTAT	GATGACGTGGTGTTCTGCAGA	
Ddx58	GCCCTGTACCATGCAGGTTAC	AGTCCCAACTTTCGATGGCTT	
Ifih	GCTGCCCAGAAGACAACACAG	CGACAGCAGGCAGAAGACACT	
Mavs	AGCCCTCCAGAGAGCATCAA	GAGGCAACATTTGCTGCGT	
Cgas	ACCGGACAAGCTAAAGAAGGTGCT	GCAGCAGGCGTTCCACAACTTTAT	
Sting1	TGAAAGGCTCTTCATTGTCTCTT	TGGCATCTTCTGCTTCCTAGA	
Tbk1	ACTGGTGATCTCTATGCTGTCA	TTCTGGAAGTCCATACGCATTG	
Rps19	CAGCAGGAGTTCGTCAGAGC	CACCCATTCGGGGACTTTCA	12963511a1
Rpl5	GGCGGCGAGAGGGTAAAAC	GCACAGACGATCATATCCCCTTC	23956082a1
Rpl11	ATGGCGCAAGATCAAGGGG	GACTGTGCAGTGAACAGCAAT	13385408a1
Rpl35a	AGGCGTTTATGCCCGAGATG	TGTTTGGTTTACCTCCAGGAGT	194440705c2
Rps17	CCAAGACCGTGAAGAAGGCTG	GCTGGGGATAATGGCGATCT	6677801a1
Rps26	TCATTCGGAACATTGTAGAAGCC	ACAGCTCACGCAATAATGCAG	7305447a1
Rps21	GTCCATCCAGATGAACGTGG	CCATCAGCCTTAGCCAATCGG	21536222a1
Rps27a	GACCCTTACGGGGAAAACCAT	AGACAAAGTCCGGCCATCTTC	13195690a1
Xpf	CCATTCGACAAGACAATCCGC	ACACCTTCTCTGTTGCTCTCA	7657064a1
Xpg	TTCCTCCAGCAACGTACACAG	GTTCCTCTCCTTAGACTGCCT	6756023a1
Xpa	CCCAAAATGATTGACACCAAAGG	TGGTTCATAAGGTACGAGTCCA	239937474c1
Xpc	GGCCAGAAAACCGAGGACAA	TTCCTTGAGAGACTTTCGGGA	121247428c1
Tfam	ATTCCGAAGTGTTTTTCCAGCA	TCTGAAAGTTTTGCATCTGGGT	1575501a1
Ifi44	AACTGACTGCTCGCAATAATGT	GTAACACAGCAATGCCTCTTGT	19527086a1
Ifit1	GCCTATCGCCAAGATTTAGATGA	TTCTGGATTTAACCGGACAGC	145301610c1
Irf7	GAGACTGGCTATTGGGGGAG	GACCGAAATGCTTCCAGGG	8567364a1
Isg15	GGTGTCCGTGACTAACTCCAT	TGGAAAGGGTAAGACCGTCCT	7657240a1

Human

Gene	F	R	Primer Bank ID/ Reference
β-actin	CCTGGCACCCAGCACAAT	GCC GAT CCA CAC GGA GTA CT	
RRN3	GACCGTGTCTCAGCATGATTG	GGTGTCGATGGTACATATCTTGC	93102376c1
IFNB1	TGG GAG GAT TCT GCA TTA CC	CAG CAT CTG CTG GTT GAA GA	
IL6	AGGCACTGGCAGAAAACAAC	TTTTCACCAGGCAAGTCTCC	
TNF-α	TCT TCT CGA ACC CCG AGT GA	AGC TGC CCC TCA GCT TGA	
cGAS	GGGAGCCCTGCTGTAACACTTCTTAT	CCTTTGCATGCTTGGGTACAAGGT	
STING	AGCATTACAACAACCTGCTACG	GTTGGGGTCAGCCATACTCAG	
TLR3	AGTGCCGTCTATTTGCCACA	GCATCCCAAAGGGCAAAAGG	
TICAM-1	AGCGCCTTCGACATTCTAGGT	AGGAGAACCATGGCATGCA	
RIG-I	TGGCATATTGACTGGACGTG	TGCCTTCATCAGCAACTGAG	
MDA5	AGAGTGGCTGTTTACATTGCC	GCTGTTCAACTAGCAGTACCTT	
MAVS	AATGCCGTTTGCTGAAGACAA	CAGTCGATCCTGGTCTCTTGCT	
pre-45S rRNA	CCCACCCTCGGTGAGAAAAG	GGAAGCGGAGGAGGGTCCTC	Ma K et al., Cell Discovery, 2022
28S rRNA	CACGAGACCGATAGTCAACAAG	AACGGGGGGCGGGAAAGATC	Ma K et al., Cell Discovery, 2022
5S rRNA	GACTCTTAGCGGTGGATCAC	AAGCGACGCTCAGACAGGC	Ma K et al., Cell Discovery, 2022
RPS19	AAGCTGAAAGTCCCCGAATGG	AGTTCTCATCGTAGGGAGCAAG	48255921c1
RPL5	GCTTATGCCCGTATAGAGGGG	GCCAAACCTATTGAGAAGCCTG	71772259c2
RPL11	AAAGGTGCGGGAGTATGAGTT	TCCAGGCCGTAGATACCAATG	315221150c2
RPL35a	TTGAAGGTGTTTACGCCCGAG	TGCTTCGGAATTTGGCACGA	16117790c1
RPS17	GTTCGCACCAAAACCGTGAAG	CGCTTGTTCGTGTGGAAGT	71772428b1
RPS26	TAACTGTGCCCGATGCGTG	GCTCGCTTCAGAAATGTCCC	71559137c1

[4] Primer for Real Time-qPCR(Detection for cytosol DNA, CytoDRIP)

Mouse

Gene	F	R	Primer Bank ID/ Reference
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	Kinoshita K et al., Immunity, 1998
LINE1_ORF2(L1ORF2)	GAATAGGTGAGAGGGT	TCCAGAAGCTGTCAGGTTCTCTGGC	Newman et al., 2012
MT-Nd1	CAAACACTTATTACAACCCAAGAACA	TCATATTATGGCTATGGGTCAGG	White et al., 2014
18S rDNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	Chang et al., 2013
rDNA_Enhancer	TACTTCTGAGGCCGAGAGGA	GATCCAAAGCTCCAGCTGAC	Guzman-Ayala M et al., 2015
rDNA_Promoter	CCTTTGAGGTCCGGTTCTTT	TCCAGGTCCAATAGGAACAGAT	Guzman-Ayala M et al., 2015
rDNA_5'ETS	ACTGACACGCTGTCCTTTCC	CGACAGACCCAAGCCAGTA	Guzman-Ayala M et al., 2015
rDNA_5.8S	GACTCTTAGCGGTGGATCACTC	GACGCTCAGACAGGCGTAG	Guzman-Ayala M et al., 2015
rDNA_ITS	GTGTCGTTCCCGTGTTTTTC	ATCGGTATTTCGGGTGTGAG	Guzman-Ayala M et al., 2015
rDNA_28S	AAATGTGGCGTACGGAAGAC	CGTGCCGGTATTTAGCCTTA	Guzman-Ayala M et al., 2015
rDNA_3'ETS	CGTCTTCTCCTCCGTCTCC	GATCCCACCGTCGGTCAC	Guzman-Ayala M et al., 2015
rDNA_IGS1	TCTTCCGAAGGTGCAGAGTT	TCCTCCTCCTCCTCCTCTTC	Guzman-Ayala M et al., 2015
rDNA_IGS2	CTTCCCAAATGCTGGGATTA	AAGGCAGCTAGGGCTACACA	Guzman-Ayala M et al., 2015
rDNA_IGS3	CTTCCCAAATGCTGGGATTA	ACAAGGCAGCTAGGGCTACA	Guzman-Ayala M et al., 2015
rDNA_IGF4	CCATCTCGTGGGCTTATGTT	AGGCAGAGATGGGAGGATTT	Guzman-Ayala M et al., 2015

Human

Gene	F	R	Primer Bank ID/ Reference
GAPDH			
Vimentin	AGA GAA CTT TGC CGT TGA AGC T	GAA GGT GAC GAG CCA TTT CC	Son J et al., Front. Cell Dev. Biol., 2020
LINE1_ORF1	AGAACGCCACAAAGATACTCCTCG	CTCTCTTCTGGCTTGTAGGGTTTCTG	Papasotiriou et al., 2017
FGFR2	ACCTGGAAATGGCTGAAATG	AAGTCCTCGCAGAGGTTTCA	Takahashi A et al., Nat Commun, 2017
GRM7	TCAAGTGCCACATCCTATGC	ATTTTTCTAGCCAGGCACCA	Takahashi A et al., Nat Commun, 2017
MT-ND1	CTCTTCGTCTGATCCGTCCT	TGAGGTTGCGGTCTGTTAGT	White et al., 2014
MT-ND2	GTAGACAGTCCCACCCTCAC	TTGATCCCGTTTCGTGCAAG	White et al., 2014
hrDNA_H42.1	GCTTCTCGACTCACGGTTTC	CCGAGAGCACGATCTCAAA	Xu Y et al, Autophagy, 2016
hrDNA_H42.9	CCCGGGGGAGGTATATCTTT	CCAACCTCTCCGACGACA	Xu Y et al, Autophagy, 2016
hrDNA_H1	GGCGGTTTGAGTGAGACGAGA	ACGTGCGCTCACCGAGAGCAG	Xu Y et al, Autophagy, 2016
hrDNA_H4	CGACGACCCATTCGAACGTCT	CTCTCCGGAATCGAACCCTGA	Xu Y et al, Autophagy, 2016
hrDNA_H8	AGTCGGGTTGCTTGGGAATGC	CCCTTACGGTACTTGTTGACT	Xu Y et al, Autophagy, 2016
hrDNA_H13	ACCTGGCGCTAAACCATTCGT	GGACAAACCCTTGTGTCGAGG	Xu Y et al, Autophagy, 2016
hrDNA_H18	GTTGACGTACAGGGTGGACTG	GGAAGTTGTCTTCACGCCTGA	Xu Y et al, Autophagy, 2016
hrDNA_H27	CCTTCCACGAGAGTGAGAAGCG	CTCGACCTCCCGAAATCGTACA	Xu Y et al, Autophagy, 2016
hrDNA_H32	GGAGTGCGATGGTGTGATCT	TAAAGATTAGCTGGGCGTGG	Xu Y et al, Autophagy, 2016

[5] Oligo for RNA-DNA hybrid synthesis

Oligo

Gene	Sequence	Primer Bank ID/ Reference
RNA60	GUACCGGGAUCCUCUAGAGUCGAGCGUCGAUCCGAACUUGG CACUGGCCGUCGUUACAAC	Crossley MP et al., Nature, 2023
asRNA60	GUACCGGGAUCCUCUAGAGUCGAGCGUCGAUCCGAACUUGG CACUGGCCGUCGUUACAAC	Crossley MP et al., Nature, 2023
DNA60	GTACCGGGATCCTCTAGAGTCGAGCGTCGATCCGAACTTGGC ACTGGCCGTCGTTACAAC	Crossley MP et al., Nature, 2023
asDNA60	GTTGTAACGACGGCCAGTGCCAAGTTCGGATCGACGCTCGAC TCTAGAGGATCCCGGTAC	Crossley MP et al., Nature, 2023