Supplemental Information

Title: Lack of inflammatory gene expression in bats: a unique role for a transcription repressor.

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Supplemental Figure S1

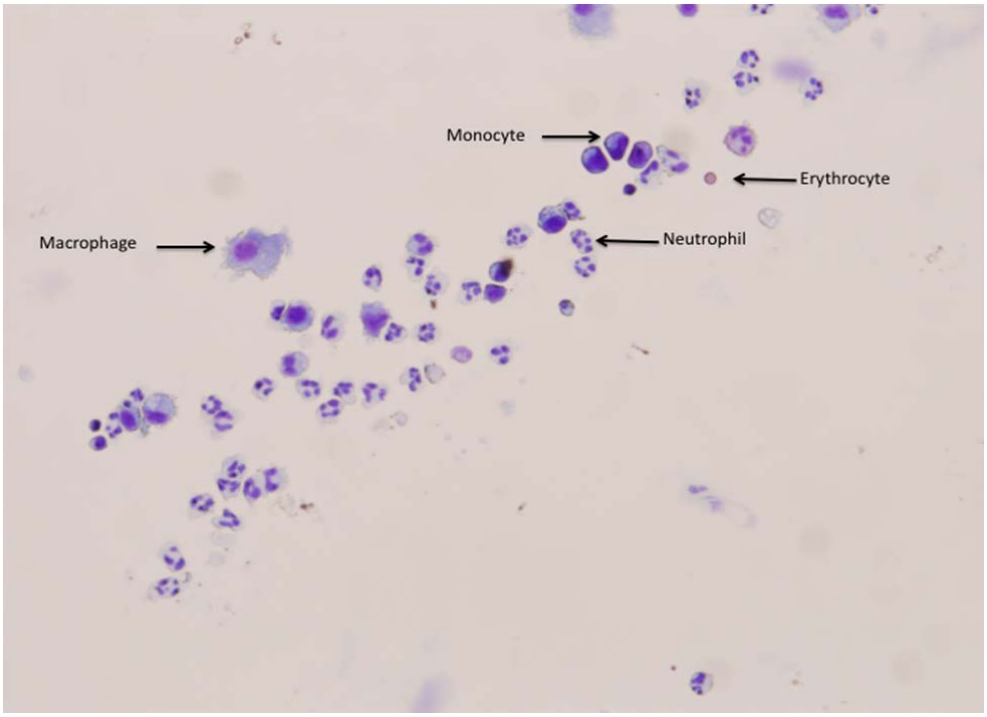


Figure S1. Big brown bat bone marrow derived myeloid cells were a mixed population. Bone marrow derived cells were deposited on a slide by centrifugation and differentially stained. Various populations of myeloid cells were observed. Cell types based on morphological features are labelled in the figure.

Supplemental Figure S2

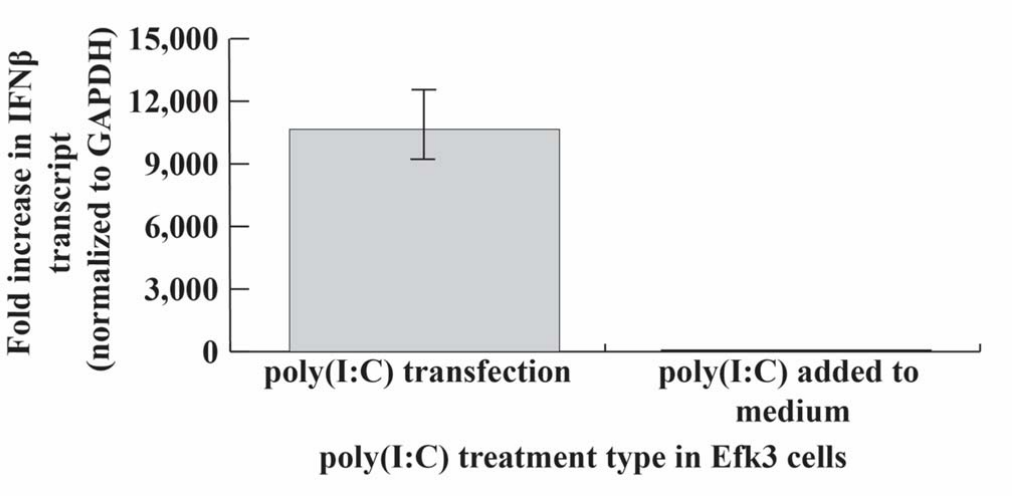
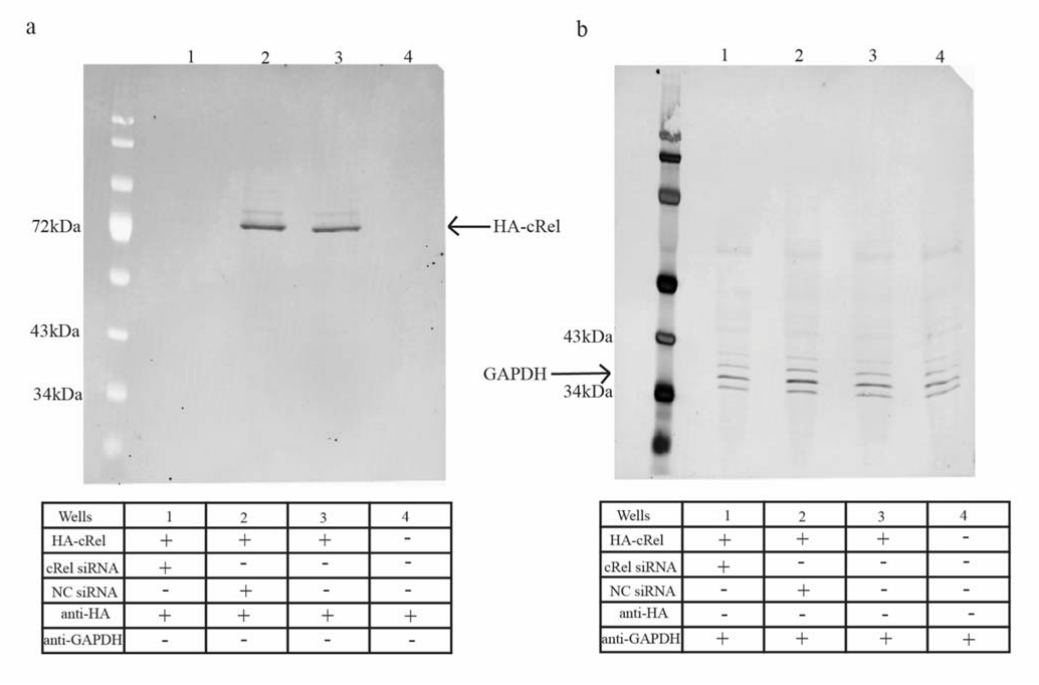


Figure S2. Transfection of poly(I:C) is essential to activation of the TLR3 pathway. To determine if poly(I:C) is recognized by a cell surface receptor or an internal receptor, such as TLR3, in bat cells, we quantified IFNβ transcripts in Efk3 cells after transfecting or adding poly(I:C) to culture medium. Adding poly(I:C) to the culture medium did not upregulate IFNβ transcripts in Efk3 cells, whereas transfecting poly(I:C) induced several thousand folds of IFNβ transcripts (Mean±SD, n=4).

Supplemental Figure S3



Wells	1	2	3	4
HA-cRel	+	+	+	-
cRel siRNA	+	-	-	-
NC siRNA	-	+	-	-
anti-HA	+	+	+	+
anti-GAPDH	-	-	-	-

Wells	1	2	3	4
HA-cRel	+	+	+	-
cRel siRNA	+	-	-	-
NC siRNA	-	+	-	-
anti-HA	-	-	-	-
anti-GAPDH	+	+	+	+

Figure S3. c-Rel siRNA shuts down expression of HA-tagged c-Rel. To study the knockdown potential of c-Rel siRNA at a protein level, we simultaneously transfected HEK293T cells with siRNA specific to c-Rel, non-specific scrambled siRNA (NC siRNA) or no siRNA and plasmids expressing HA-tagged c-Rel. (a) siRNA specific to c-Rel completely shuts down the expression of HA-c-Rel from the plasmid. In contrast, cells transfected with NC siRNA or no siRNA express HA-c-Rel. (b) There was no change in GAPDH expression between different treatments. NC = Negative control.

Supplemental Figure S4

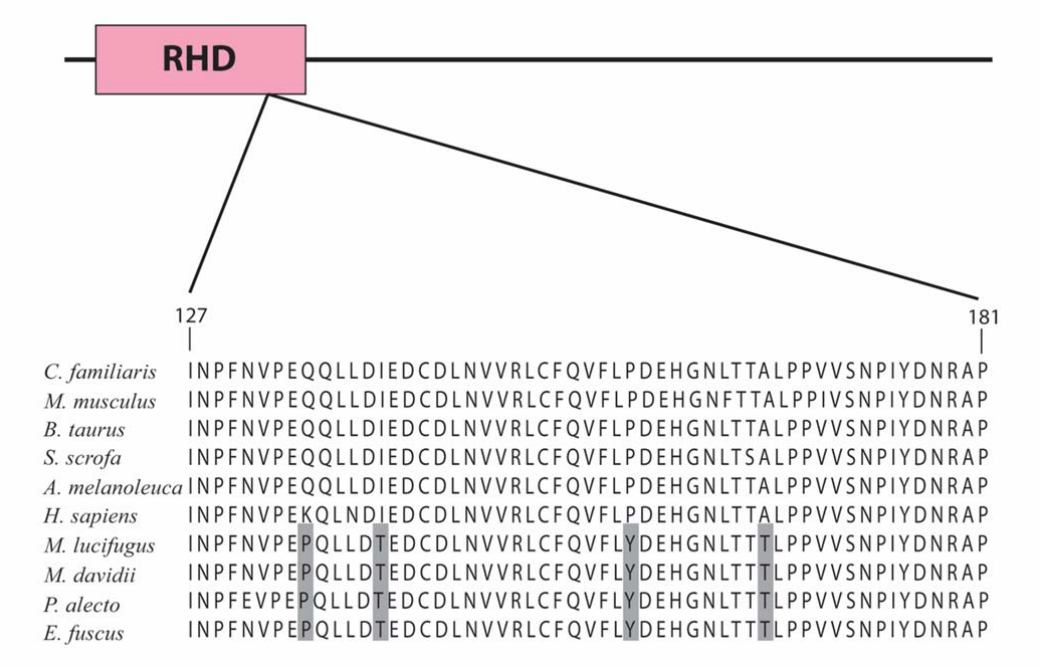


Figure S4. Mutations in the c-Rel Rel homology domain (RHD). Zhang *et al.* show that 4 mutations specific to bat c-Rel exist in the downstream region of the RHD [1]. IκB binding domain is located in the downstream region of the RHD and Zhang *et al.* propose mutations in this region can affect IκB binding. Differences in amino acids between bat RHD domains and those of other representative mammals are highlighted.

Supplemental Figure S5

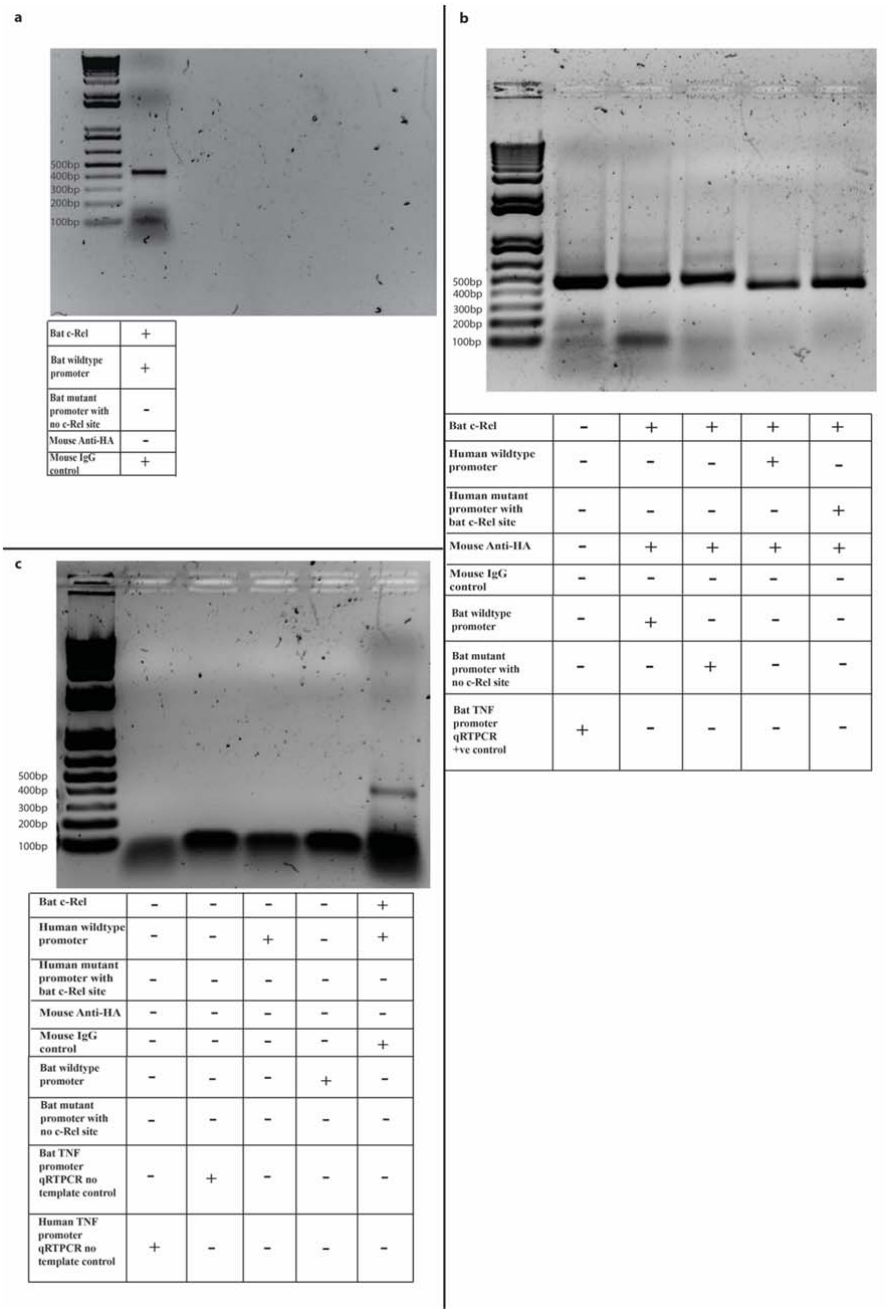


Figure S5. qRT-PCR products from ChIP assay were electrophoresed on separate gels. Images for figure 8 in the article file are shown above. Cropped images

were acquired from these images and scaled to fit under the qRT-PCR data in Fig. 8. ChIP and qRT-PCR controls are shown too.

Supplemental Table S1. PRR transcripts detected in human (MRC5) and bat (Efk3) cells by PCR.

PRR type	MRC5	Efk3
TLR2	+	+
TLR3	+	+
TLR7	+	+
TLR8	+	+
TLR9	+	+
RIG-I	+	+
MDA5	+	+

Supplemental Table S2. Fold changes observed for innate immune gene transcripts in MRC5 and Efk3 cells after different TLR ligand treatments. We transfected human fibroblasts and bat kidney (Efk3) cells with poly(I:C), a known TLR3 stimulant, and studied the expression of genes involved in the TLR3 pathway, relative to mock transfected cells. Experiments were carried out in replicates (n=3). Standard deviation values are reported along with mean fold changes.

TLR liga	**poly (I:C)**				**ssRNA 40**				**CpG ODN**			

nd												
Gene	**Mean fold upregulation - MRC5 cells**	**Standard deviation - MRC5 cells**	**Mean fold upregulation - Efk3 cells**	**Standard deviation - Efk3 cells**	**Mean fold upregulation - MRC5 cells**	**Standard deviation - MRC5 cells**	**Mean fold upregulation - Efk3 cells**	**Standard deviation - Efk3 cells**	**Mean fold upregulation - MRC5 cells**	**Standard deviation - MRC5 cells**	**Mean fold upregulation - Efk3 cells**	**Standard deviation - Efk3 cells**
IRF3	1.47	0.15	1.02	2.03	1.43	0.58	-1.09	0.44	0.53	1.42	0.17	0.05
STAT1	7.91	2.32	6.09	1.65	-1.66	0.37	-1.05	0.33	4.3	1.11	-1.35	0.09
STAT2	3.5	0.45	4.9	1.8	1.14	0.07	1.25	0.29	1.74	0.34	1.195	1.74
MDA5	58.97	14.54	50.21	18.8	-1	0.09	1.2	0.21	6.43	0.133	-1.35	0.33
IL12A	3.2	0.58	3.11	0.7	-1.2	0.152	1.31	0.49	1.53	0.32	1.995	0.03
IL12B	3.59	1.85	2.77	3.6	5.13	0.99	-2.14	1.26	-0.95	1.99	0.36	1.97
GBP1	17.13	1.87	32	3.33	-1.86	0.65	2.46	2.1	2.14	0.21	-1.43	0.28
TLR8	0.22	1.48	-1.11	0.38	-1.2	0.095	-1.08	0.5	-0.5	1.46	1.25	0.08
TLR7	-0.28	2.72	-1.11	1.54	1.42	0.59	2.36	0.99	1.23	0.375	1.22	1.85
IRF7	50.17	17.1	80.1	21.03	2.24	1.78	-1.01	0.24	6.72	0.71	0.615	0.19
IFNβ	**15511**	**10160**	**10594.5**	**2207.8**	8.3	1.29	3.01	0.38	35.4	9.84	5.65	3.15
NFκB1	3.85	0.74	-1.23	0.28	2.66	1.08	1.41	0.38	1.35	0.34	2.035	0.268
TLR9	1.78	0.468	-1.38	0.31	-1.22	0.11	-1.14	0.75	-0.43	1.72	-1.18	0.05
TNF	**315.16**	**208.4**	**2.4**	**0.09**	3.37	2.01	2.8	1.1	20.52	23.66	3.085	0.127
IFI6	84.58	33.08	43.26	20.95	4.7	0.45	1.07	0.22	15.99	9.58	2.885	1.56
IFI27	790.16	88.9	2.9	0.7	3.53	0.98	-1.07	0.36	34.93	5.91	1.23	1.44
IRF1	6	0.06	2.99	0.71	1.65	0.74	1.21	0.69	1.71	0.39	3.18	0.9

ISG 20	310.35	30.33	0.255	2.04	-1.08	0.036	-1.04	0.55	4.38	0.82	-1.12	0.4
OAS1	2949	2062.14	229.93	187.4	9.46	1.15	1.98	1.05	81.41	7.31	1.77	0.32
RSAD2	3637.4	3121.22	49.65	35.92	3.64	0.72	1.26	1.28	66.37	7.59	1.65	0.09
RIGI	86.34	17.71	5.92	0.1	2.51	1.55	1.51	0.41	7.36	2	-1.1	0.4
IL18	1.29	0.1	-1.9	0.7	-3.1	1.6	1.18	0.76	-0.45	1.28	-1.41	0.09
IL8	**565**	**100.26**	**-1.49**	**0.36**	2.89	1.11	1.84	0.19	27.25	8.07	1.39	0.36
IL1β	**112.3**	**14.31**	**3.16**	**2.95**	2.31	0.95	16.29	3.6	8.42	3.8	0.08	0.06
MX1	632.33	200.61	44.32	4.24	0.38	2.55	1.79	0.44	37.45	6.53	-1.65	7
TLR3	15.26	6.8	4.37	0.74	0.57	1.53	1.84	1.1	2.72	0.27	-1.065	0.04

Supplemental Table S3. c-Rel transcripts were detected in several big brown bat tissues by PCR.

Big brown bat organ	**c-Rel transcript**
Spleen	+
Gut	+
Ileum	+
Kidney	+
Lung	+
Liver	+

Efk3 cell line	+

Supplemental Table S4. Accession numbers, primer sequences and annealing temperatures for human and big brown bat genes and primers.

Gene	NCBI Accession - Human	NCBI accession *E. fuscus*	Primers - human (forward and reverse)	Annealing temperature	Primers– *E. fuscus* (forward and reverse)	Annealing temperature
IFNβ	NM_002176.3	XM_008145044.1	GCTTGGATTCCTACAAAGAAGCA ; ATAGATGGTCAATGCGGCGTC	54	GCTCCGATTCCGACAGAGAAGCA ; ATGCATGACCACCATGGCTTC	56
NFκB - 1	NM_003998.3	XM_008156644.1	GAAGCACGAATGACAGAGGC ; GCTTGGCGGATTAGCTCTTTT	54	GAAGCACGGATGACAGATGC ; GCCTGGCGGATGATCTCCTTT	56
IL12A	NM_000882.3	XM_008142364.1	CCTTGCACTTCTGAAGAGATTGA ; ACAGGGCCATCATAAAAGAGGT	53	TCCTGCACTTCTGAAGAGATTGA ; ACAGGGTCGTCATAAAAGAGGC	52
IL12B	NM_002187.2	XM_008147661.1	ACCCTGACCATCCAAGTCAAA ; TTGGCCTCGCATCTTAGAAAG	55	ACCTTGACCATCCAAGTCAAA ; TTTGCTTCACATTTTAGAAAG	52
IRF3	NM_001571.5	XM_008154348.1	AGAGGCTCGTGATGGTCAAG ; AGGTCCACAGTATTCTCCAGG	55	AGAAGCTAGTGATGGTCAAG ; AGGTCCACAGTGTTCTCCAGC	56
IL18	NM_001562.3	XM_008150175.1	TCTTCATTGACCAAGGAAATCGG ; TCCGGGGTGCATTATCTC	51	TCTTCGTTAACCAGGGAAGCCAA ; TCTGGGGTGCATTCTCTTC	52

			TAC		AC	
IRF7	NM_0015 72.3	XM_0081 59582.1	CCCACGCTATACCATCTA CCT ; GATGTCGTCATAGAGGC TGTTG	57	CCCGCACTGCACCATCTAC CT ; CAGGTCCTCGTACAGGCT GTTG	56
STAT1	NM_0073 15.3	XM_0081 46505.1	CAGCTTGACTCAAAATTC CTGGA ; TGAAGATTACGCTTGCTT TTCCT	54	CAGCTGGACGCCAAGTTC CTGGA ; TGCAGGTTGCGCTTGCTCT TCCT	56
STAT2	NM_0054 19.3	XM_0081 48645.1	GAGCCAGCAACATGAGA TTGA ; GCCTGGATCTTATATCGG AAGCA	53	GAGCCAGCAGCATGAGAT CGA ; GCCTGAGTCTTGTATCGGA AGAG	56
TLR3	NM_0032 65.2	XM_0081 51907.1	TCAACTCAGAAGATTAC CAGCCG ; AGTTCAGTCAAATTCGTG CAGAA	53	TCAGCTCAGAAAATTACC GCCTG ; AGTTCAGTCAAATTCCGG CAGAA	52
TLR7	NM_0165 62.3	XM_0081 56577.1	TCGTGGACTGCACAGAC AAG ; GGTATGTGGTTAATGGT GAGGGT	51	TTGTGGACTGCACGGACA AG ; GGTATGTGGTTGATGGTG AGGGT	52
TLR8	NM_1386 36.5	XM_0081 56576.1	GACTACAGGAAGTTCCC CAAAC ; ATACCGGGATTTCCGTTC TGG	51	GGCTGCAAGAAGTCCCCC AAAC ; TTGCAATAATTCTCACAGT AG	52
TLR9	NM_0174 42.3	XM_0081 55066.1	CTGCCACATGACCATCG AG ; GGACAGGGATATGAGGG ATTTGG	55	CTGCCACATGACCATCGA G ; GGCCAGGGTCCGGAGGGC GGGGG	56
TNF	NM_0005 94.3	XM_0081 57360.1	CAGCCTCTTCTCCTTCCT GA ; AGATGATCTGACTGCCT GGG	57	GCCCATGTTGTAGCAAAC C ; GCCCTTGAAGAGGACCTG GG	56
IL8	NM_0005	XM_0144	ACTGAGAGTGATTGAGA	51	AAACATGACTTCCAAGCT	52

	84.3	60599.1	GTGGAC ; AACCCTCTGCACCCAGTTTTC		GG ; TGTGGTCCACTCTCAATCAC	
IL1β	NM_000576.2	XM_008160259.1	ATGATGGCTTATTACAGTGGCAA ; GTCGGAGATTCGTAGCTGGA	54	ATGATGGCTTACTACAGTGACAA ; GTCGGAGATTTTCAGCTGGA	52
GBP1	NM_002053.2	XM_008157793.1	AGGAGTTCCTTCAAAGATGTGGA ; GCAACTGGACCCTGTCGTT	54	TTCAGCTGACTTTGTGAGCT ; ACTGCTGATGGCATTAACAT	52
IFI6	NM_002038.3	XM_008148008.1	GGTCTGCGATCCTGAATGGG ; TCACTATCGAGATACTTGTGGGT	57	GGTCGGCCATAGCGAACGGG ; TTGTTGTCTATCTGCCTGTGGAC	56
IFI27	NM_206949.2	XM_008159947.1	TGCTCTCACCTCATCAGCAGT ; CACAACTCCTCCAATCACAACT	56	GCCAAGATGATGTCATCAGC ; CACATCCAGGCCCCAATAG	56
IRF1	NM_002198.2	XM_008142488.1	ATGCCCATCACTCGGATGC ; CCCTGCTTTGTATCGGCCTG	56	ATGCCCATCACTCGGATGC ; CCCGACTTTGTACCGGCCTG	56
ISG20	NM_002201.5	XM_008155878.1	TCTACGACACGTCCACTGACA ; CTGTTCTGGATGCTCTTGTGC	56	CCCGAGACACCTCCCATATTC ; CCAGCCTGGATGTCCCGGTTC	56
MX1	NM_001144925.2	XM_008145691.1	AGGACTACGAGATTGAGAT ; TTATGCCAGGAAGGTCTA	51	GGACATGGAGATCAATCTT ; TGATGCCAGGAAGGTCTA	52
OAS1	NM_016816.3	XM_008142919.1	AGTTGACTGGCGGCTATAAAC ; GTGCTTGACTAGGCGGA	55	AGGTGACGGACGACTACAGAC ; GTGCTTGACCAGGCGGAT	56

			TGAG		GAG	
RSAD2	NM_080657.4	XM_008162430.1	TGGGTGCTTACACCTGCTG ; GAAGTGATAGTTGACGCTGGTT	53	CGAGTACCTGGGCCGCCTG ; GAAGCGGTTGATGACGGAGTTG	56
RIG-I	NM_014314.3	XM_008142005.1	CTGGACCCTACCTACATCCTG ; GGCATCCAAAAAGCCACGG	55	CTGGACCCCACCTACGTCCTC ; AGCATCCAAAAAGCCACGA	56
MDA5	NM_022168.3	XM_008138631.1	GCCCGCTACATGAACCCTG ; CAGCAATCCGGTTTCTGTCTT	55	GCCCGCTACATGGACCCCG ; CAGAAACCCGACTTCTGTCTT	56
GAPDH	NM_002046.5	XM_008144826.1	GGAGCGAGATCCCTCCAAAAT ; GGCTGTTGTCATACTTCTCATGG	57	GGAGCGAGATCCCGCCAACAT ; GGGAGTTGTCATACTTGTCATGG	56
β-actin	NM_001017992.3	XM_008156007.1	CTCGACACCAGGGCGTTATG ; CCACTCCATGCTCGATAGGAT	57	CCCGGCACCAGGGCGTGATG ; CGATGCCGTGCTCGATGGGGT	56
TLR2	NM_003264.4	XM_008144148.1	TTATCCAGCACACGAATACACAG ; AGGCATCTGGTAGAGTCATCAA	52	TTATCCAATACAAGAATACAGAG ; AGGCATCTGGTAGAGTCTTCAA	51
c-Rel	-	XM_008162099.1	-		GACGACTACTCTACCTCCTG ; ATCTTCACCGTCACTGGCTC	56
β2-microglobulin	NM_004048.2	-	GAGGCTATCCAGCGTACTCCA ; CGGCAGGCATACTCATCTTTT	55	-	

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102 **Supplemental Table S5**. siRNA sequences designed against *E. fuscus* (big brown

103 bat) TLR3, RIGI and MDA5. r = ribose sugar; BBB = big brown bat.

siRNA sequence	Duplex name	siRNA duplex sequences	Binding position on transcript
BBB TLR3 siRNA sequence	CD.Ri.26282.13.2	5' rCrUrArUrCrUrCrArArCrUrUrUrCrUrGrArCrArArArArCCT 3'	BBB TLR3 - position 337-362
BBB TLR3 siRNA sequence2		5' rArGrGrUrUrUrUrGrUrCrArGrArArArGrUrUrGrArGrArUrArGrCrU 3'	
BBB TLR3 siRNA sequence	CD.Ri.26282.13.1	5' rCrArArCrArUrGrArGrArUrArCrCrUrGrArArUrUrUrArAGA 3'	BBB TLR3 - position 972-997
BBB TLR3 siRNA sequence2		5' rUrCrUrUrArArArUrUrCrArGrGrUrArUrCrUrCrArUrGrUrUrGrArA 3'	
BBB RIGI siRNA sequence	CD.Ri.26280.13.2	5' rArGrCrUrUrUrCrUrArArArArCrCrUrGrArGrArUrArUrUGA 3'	BBB RIGI - position 1972-1997
BBB RIGI siRNA sequence2		5' rUrCrArArUrArUrCrUrCrArGrGrUrUrUrUrArGrArArArGrCrUrGrA 3'	
BBB RIGI siRNA sequence	CD.Ri.26280.13.10	5' rGrUrGrGrCrArArCrArGrUrCrArArArGrArCrArArCrUrUGG 3'	BBB RIGI - position 1312-1337
BBB RIGI siRNA sequence2		5' rCrCrArArGrUrUrGrUrCrUrUrUrGrArCrUrGrUrUrGrCrCrArCrCrA 3'	
BBB MDA5 siRNA sequence	CD.Ri.26279.13.2	5' rArCrUrUrGrGrArCrArArGrArArArArArArArGrCrArUrCrUGA 3'	BBB MDA5 - position 1256-1281
BBB MDA5 siRNA sequence2		5' rUrCrArGrArUrGrCrUrUrUrUrUrUrUrCrUrUrGrUrCrCrArArGrUrGrA 3'	
BBB MDA5 siRNA sequence	CD.Ri.26279.13.1	5' rGrArArGrArUrUrCrUrUrUrUrUrArArArArCrArUrUrGrUrCAA 3'	BBB MDA5 - position 3301-3326
BBB MDA5 siRNA		5'	

sequence2		rUrUrGrArCrArArUrGrUrUrUrUrArArArArGrArArUrCrUrUrCrArA 3'	

Supplementary Methods

c-Rel Western Blots: HEK293T cells were seeded at a concentration of 3x 10^5 cells/well in six well plates and simultaneously transfected with 100nM of 1:1 cocktail of two different siRNA specific to c-Rel (table 1), NC siRNA or no siRNA and plasmids expressing HA-tagged c-Rel. Cells were harvested in sample buffer for western blots 24 hrs post transfections. Western blots were carried out as previously mentioned [2]. Primary antibodies used were: 1: 1,000 mouse anti-HA (Clone HA7; Sigma) and 1: 5,000 rabbit anti-GAPDH (Cedarlane). Secondary antibodies used were: 1:10,000 goat anti-mouse Alexa 488 (Molecular Probes) and 1: 10,000 goat anti-rabbit Cy5 (GE Healthcare).

TLR3, RIGI and MDA5 knockdown: Dicer-ready siRNA (DsiRNA) specific to big brown bat TLR3, RIGI and MDA5 were designed and obtained through Integrated DNA Technologies (IDT). A 100nM final concentration of a 1:1 mixture of two DsiRNAs (see Supplementary Table S5) targeting separate regions on the respective transcripts was transfected into Efk3 cells using Lipofectamine 2000. Scrambled non-specific DsiRNA (NC DsiRNA; IDT) was used as a negative control. Transfections were carried out as previously mentioned for siRNA [3]. In brief, cells were transfected with the siRNA cocktail for 24 hours and then transfected with 750ng/ml poly(I:C) using Lipofectamine 2000. RNA was extracted 16h after poly(I:C) stimulation for cDNA preparation and qRT-PCR.

References:

1 Zhang, G. *et al.* Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. *Science* **339**, 456-460, doi:10.1126/science.1230835 (2013).

2 Bergeron, T. *et al.* The effect of Zhangfei on the unfolded protein response and growth of cells derived from canine and human osteosarcomas. *Vet Comp Oncol* **11**, 140-150, doi:10.1111/j.1476-5829.2011.00310.x (2013).

3 Nasirudeen, A. M. *et al.* RIG-I, MDA5 and TLR3 synergistically play an important role in restriction of dengue virus infection. *PLoS Negl Trop Dis* **5**, e926, doi:10.1371/journal.pntd.0000926 (2011).