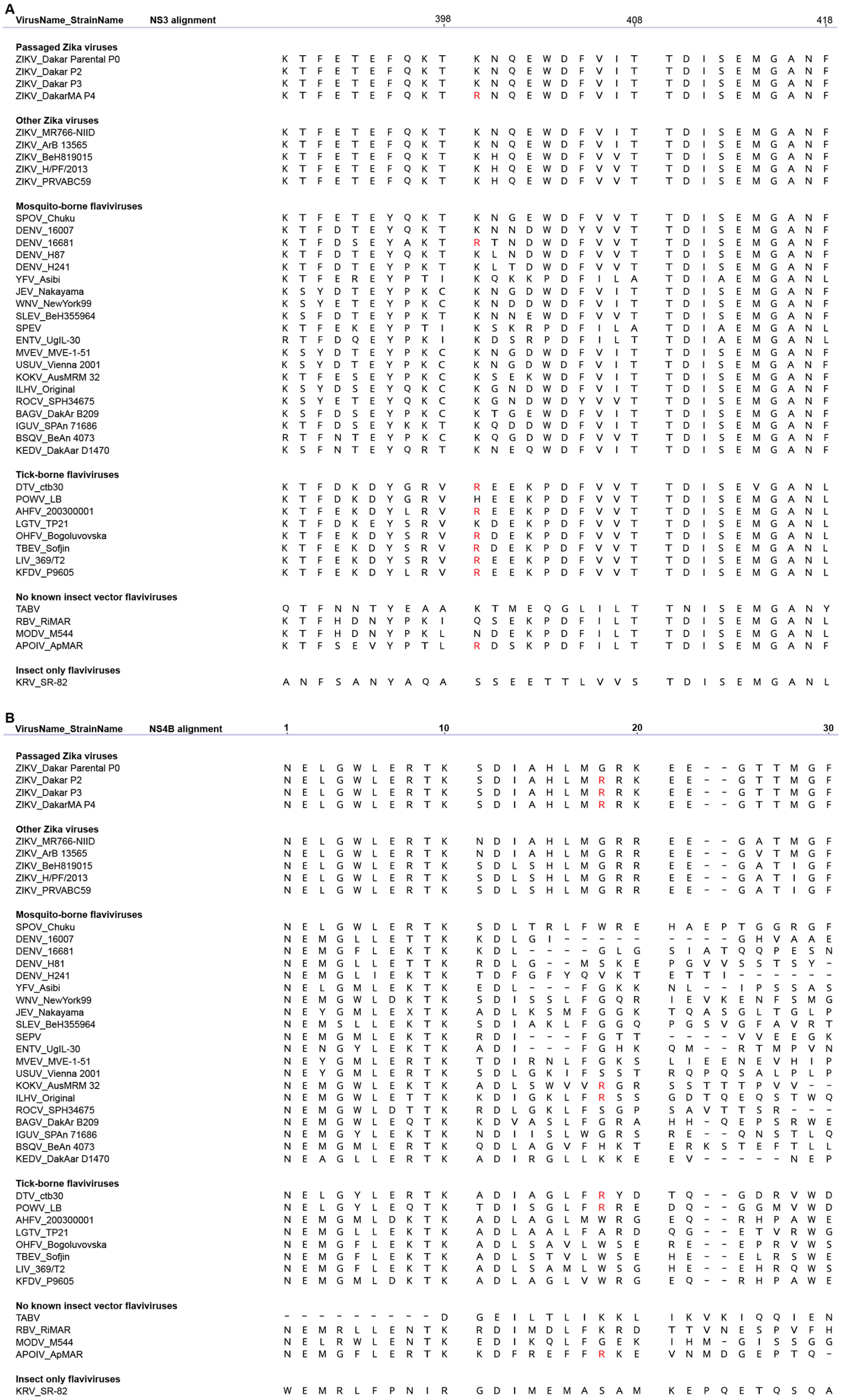
VirusName_StrainName	**Passage # in *Rag1*$^{-/-}$ mice**	**Amino acid at NS3 399**	**Amino acid at NS4B 18**	**Lethality in mice**
ZIKV_Dakar	**Parental**	**K**	**G**	**Low**
ZIKV_Dakar	**P1**	**N.D.**	**N.D.**	**N.D.**
ZIKV_Dakar	**P2**	**K**	**R**	**High**
ZIKV_Dakar	**P3**	**K**	**R**	**High**
ZIKV_Dakar	**P4**	**R**	**R**	**High**

Table S1, Related to Figure 1. Mutations acquired by ZIKV-Dak during serial passage in mice. Table listing the passage number of ZIKV-Dak in *Rag1*$^{-/-}$ mice, amino acid at position NS3 399 and NS4B G18R, and the observed lethality in 4-5 week-old C57BL/6 mice pretreated with anti-Ifnar1 antibody, of each passaged ZIKV-Dak isolate. (Lethality; Low = <75% lethality, High = > 75% lethality, N.D. = not determined).

Table S2. List of primers for Key Resources Table

Primer Name	**Primer Sequence (5' – 3')**
Titering ZIKV primers	
ZIKV RNA: Forward	5'-CCACCAATGTTCTCTTGCAGACATATTG-3'
ZIKV RNA: Reverse	5'- TTCGGACAGCCGTTGTCCAACACAAG-3'
ZIKV RNA: Probe	5'-/56-FAM/AGCCTACCT/ZEN/TGACAAGCAGTC/3IABkFQ/-3'
hSTAT2 genotyping primers	
hSTAT2 genotyping 5079-42	5'- CTGAGGTAGAATCACTTTGACTTCC -3'
hSTAT2 genotyping 5079-43	5'- GATGGCTCAGAGGTTAAGAGC -3'
hSTAT2 genotyping 5080-46	5'- GGCAAAGCCAAGACATAAACC -3'
hSTAT2 genotyping 5080-47	5'- ACAGGTTCCAGGCCATCAAG -3'
qRTPCR primers	
Oas1a Forward	5'-ATGGAGCACGGACTCAGGA-3'
Oas1a Reverse	5'-TCACACACGACATTGACGGC-3'
Ifnβ Forward	5'-CAGCTCCAAGAAAGGACGAAC-3'
Ifnβ Reverse	5'-GGCAGTGTAACTCTTCTGCAT-3'
Irf7 Forward	5'-GAGACTGGCTATTGGGGGAG-3'
Irf7 Reverse	5'-GACCGAAATGCTTCCAGGG-3'
18S rRNA Forward	5'- GTAACCCGTTGAACCCCATT-3'
18S rRNA Reverse	5'-CCATCCAATCGGTAGTAGCG-3'
Sequencing ZIKV primers	
ZIKV-Dakar 3500-7000 amplifying primer:	5'- AGA AAG GAA CCA GAG AGC AAC TTA-3'
ZIKV-Dakar 7100-3500 amplifying primer:	5'- GTGGTTACCGCATGTTGG-3'
ZIKV-Dakar 5500 sequencing primer:	5'-TGCACGAGGATATATATCAACAAGGG-3'
ZIKV-Dakar 6500 sequencing primer:	5'- TTG CCA GGA CAC ATG ACA GAG-3'
ZIKV amplicon primers	
ZIKV Conserved 1For.	AGTTGTTGATCTGTGTGAATCAGAC
ZIKV Conserved 1821Rev.	CTTTGCACCATCCATCTCAGCCTC
ZIKV Conserved 1663For.	GCAGACACCGGAACTCCACACT
ZIKV Conserved 3499Rev.	GCCTTATCTCCATTCCATACCA
ZIKV Conserved 3367For.	CWAGAGGACCATCTCTGAGATCAAC
ZIKV Conserved 5078Rev.	CACGACCCCATTGCCATAAAGTC
ZIKV Conserved 4665For.	GACCACAGATGGAGTGTACAGAGT
ZIKV Conserved 6086Rev.	CTTGCTTCAAGCCAGTGTGC
ZIKV African 6019For.	ATGAGTACATGTATGGAGGTGGGTG
ZIKV Sen 7580Rev.	GTGAGGTGGCTGTGGAGGAG
ZIKV Sen 7521For.	CAGCAGCAACCTCCACCTTATG
ZIKV Conserved 9037Rev.	CCCATCATGTTGTACACACAGCT
ZIKV Conserved 8989For.	ARCACCACCTGAGAGGAGAGTG
ZIKV Conserved 10140Rev.	GGTCGTTCTCCTCAATCCACACT
ZIKV Conserved 9857For.	CCGCCACCAAGATGAACTGATTG
ZIKV 3' UTR end Rev.	AGACCCATGGATTTCCCCACACC



A

VirusName_StrainName	NS3 alignment 398	408	418
Passaged Zika viruses			
ZIKV_Dakar Parental P0	K T F E T E F Q K T	K N Q E W D F V I T	T D I S E M G A N F
ZIKV_Dakar P2	K T F E T E F Q K T	K N Q E W D F V I T	T D I S E M G A N F
ZIKV_Dakar P3	K T F E T E F Q K T	K N Q E W D F V I T	T D I S E M G A N F
ZIKV_DakarMA P4	K T F E T E F Q K T	R N Q E W D F V I T	T D I S E M G A N F
Other Zika viruses			
ZIKV_MR766-NIID	K T F E T E F Q K T	K N Q E W D F V I T	T D I S E M G A N F
ZIKV_ArB 13565	K T F E T E F Q K T	K N Q E W D F V I T	T D I S E M G A N F
ZIKV_BeH819015	K T F E T E F Q K T	K H Q E W D F V V T	T D I S E M G A N F
ZIKV_H/PF/2013	K T F E T E F Q K T	K H Q E W D F V V T	T D I S E M G A N F
ZIKV_PRVABC59	K T F E T E F Q K T	K H Q E W D F V V T	T D I S E M G A N F
Mosquito-borne flaviviruses			
SPOV_Chuku	K T F E T E Y Q K T	K N G E W D F V V T	T D I S E M G A N F
DENV_16007	K T F D T E Y Q K T	K N N D W D Y V V T	T D I S E M G A N F
DENV_16681	K T F D S E Y A K T	R T N D W D F V V T	T D I S E M G A N F
DENV_H87	K T F D T E Y Q K T	K L N D W D F V V T	T D I S E M G A N F
DENV_H241	K T F D T E Y P K T	K L T D W D F V V T	T D I S E M G A N F
YFV_Asibi	K T F E R E Y P T I	K Q K K P D F I L A	T D I A E M G A N L
JEV_Nakayama	K S Y D T E Y P K C	K N G D W D F V I T	T D I S E M G A N F
WNV_NewYork99	K S Y E T E Y P K C	K N D D W D F V I T	T D I S E M G A N F
SLEV_BeH355964	K S F D T E Y P K T	K N N E W D F V V T	T D I S E M G A N F
SPEV	K T F E K E Y P T I	K S K R P D F I L A	T D I A E M G A N L
ENTV_UgIL-30	R T F D Q E Y P K I	K D S R P D F I L T	T D I A E M G A N L
MVEV_MVE-1-51	K S Y D T E Y P K C	K N G D W D F V I T	T D I S E M G A N F
USUV_Vienna 2001	K S Y D T E Y P K C	K N G D W D F V I T	T D I S E M G A N F
KOKV_AusMRM 32	K T F E S E Y P K C	K S E K W D F V I T	T D I S E M G A N F
ILHV_Original	K S Y D S E Y Q K C	K G N D W D F V I T	T D I S E M G A N F
ROCV_SPH34675	K S Y E T E Y Q K C	K G N D W D Y V V T	T D I S E M G A N F
BAGV_DakAr B209	K S F D S E Y P K C	K T G E W D F V I T	T D I S E M G A N F
IGUV_SPAn 71686	K T F D S E Y K K T	K Q D D W D F V I T	T D I S E M G A N F
BSQV_BeAn 4073	R T F N T E Y P K C	K Q G D W D F V V T	T D I S E M G A N F
KEDV_DakAar D1470	K S F N T E Y Q R T	K N E Q W D F V I T	T D I S E M G A N F
Tick-borne flaviviruses			
DTV_ctb30	K T F D K D Y G R V	R E E K P D F V V T	T D I S E V G A N L
POWV_LB	K T F D K D Y G R V	H E E K P D F V V T	T D I S E M G A N L
AHFV_200300001	K T F E K D Y L R V	R E E K P D F V V T	T D I S E M G A N L
LGTV_TP21	K T F D K E Y S R V	K D E K P D F V V T	T D I S E M G A N L
OHFV_Bogoluvovska	K T F E K D Y S R V	R D E K P D F V V T	T D I S E M G A N L
TBEV_Sofjin	K T F E K D Y S R V	R D E K P D F V V T	T D I S E M G A N L
LIV_369/T2	K T F E K D Y S R V	R E E K P D F V V T	T D I S E M G A N L
KFDV_P9605	K T F E K D Y L R V	R E E K P D F V V T	T D I S E M G A N L
No known insect vector flaviviruses			
TABV	Q T F N N T Y E A A	K T M E Q G L I L T	T N I S E M G A N Y
RBV_RiMAR	K T F H D N Y P K I	Q S E K P D F I L T	T D I S E M G A N L
MODV_M544	K T F H D N Y P K L	N D E K P D F I L T	T D I S E M G A N L
APOIV_ApMAR	K T F S E V Y P T L	R D S K P D F I L T	T D I S E M G A N F
Insect only flaviviruses			
KRV_SR-82	A N F S A N Y A Q A	S S E E T T L V V S	T D I S E M G A N L

B

VirusName_StrainName	NS4B alignment 1 10	20	30
Passaged Zika viruses			
ZIKV_Dakar Parental P0	N E L G W L E R T K	S D I A H L M G R K	E E – – G T T M G F
ZIKV_Dakar P2	N E L G W L E R T K	S D I A H L M R R K	E E – – G T T M G F
ZIKV_Dakar P3	N E L G W L E R T K	S D I A H L M R R K	E E – – G T T M G F
ZIKV_DakarMA P4	N E L G W L E R T K	S D I A H L M R R K	E E – – G T T M G F
Other Zika viruses			
ZIKV_MR766-NIID	N E L G W L E R T K	N D I A H L M G R R	E E – – G A T M G F
ZIKV_ArB 13565	N E L G W L E R T K	N D I A H L M G R R	E E – – G V T M G F
ZIKV_BeH819015	N E L G W L E R T K	S D L S H L M G R R	E E – – G A T I G F
ZIKV_H/PF/2013	N E L G W L E R T K	S D L S H L M G R R	E E – – G A T I G F
ZIKV_PRVABC59	N E L G W L E R T K	S D L S H L M G R R	E E – – G A T I G F
Mosquito-borne flaviviruses			
SPOV_Chuku	N E L G W L E R T K	S D L T R L F W R E	H A E P T G G R G F
DENV_16007	N E M G L L E T T K	K D L G I – – – – –	– – – – G H V A A E
DENV_16681	N E M G F L E K T K	K D L – – – – G L G	S I A T Q Q P E S N
DENV_H81	N E M G L L E T T K	R D L G – – M S K E	P G V V S S T S Y –
DENV_H241	N E M G L I E K T K	T D F G F Y Q V K T	E T T I – – – – – –
YFV_Asibi	N E L G M L E K T K	E D L – – – F G K K	N L – – I P S S A S
WNV_NewYork99	N E M G W L D K T K	S D I S S L F G Q R	I E V K E N F S M G
JEV_Nakayama	N E Y G M L E X T K	A D L K S M F G G K	T Q A S G L T G L P
SLEV_BeH355964	N E M S L L E K T K	S D I A K L F G G Q	P G S V G F A V R T
SEPV	N E M G M L E K T K	R D I – – – F G T T	– – – – V V E E G K
ENTV_UgIL-30	N E N G Y L E K T K	A D I – – – F G H K	Q M – – R T M P V N
MVEV_MVE-1-51	N E Y G M L E R T K	T D I R N L F G K S	L I E E N E V H I P
USUV_Vienna 2001	N E Y G M L E R T K	S D L G K I F S S T	R Q P Q S A L P L P
KOKV_AusMRM 32	N E M G W L E K T K	A D L S W V V R G R	S S T T T P V V – –
ILHV_Original	N E M G W L E T T K	K D I G K L F R S S	G D T Q E Q S T W Q
ROCV_SPH34675	N E M G W L D T T K	R D L G K L F S G P	S A V T T S R – – –
BAGV_DakAr B209	N E M G W L E Q T K	K D V A S L F G R A	H H – Q E P S R W E
IGUV_SPAn 71686	N E M G Y L E K T K	N D I I S L W G R S	R E – – Q N S T L Q
BSQV_BeAn 4073	N E M G M L E R T K	Q D L A G V F H K T	E R K S T E F T L L
KEDV_DakAar D1470	N E A G L L E R T K	A D I R G L L K K E	E V – – – – – N E P
Tick-borne flaviviruses			
DTV_ctb30	N E L G Y L E R T K	A D I A G L F R Y D	T Q – – G D R V W D
POWV_LB	N E L G Y L E Q T K	T D I S G L F R R E	D Q – – G G M V W D
AHFV_200300001	N E M G M L D K T K	A D L A G L M W R G	E Q – – R H P A W E
LGTV_TP21	N E M G L L E K T K	A D L A A L F A R D	Q G – – E T V R W G
OHFV_Bogoluvovska	N E M G F L E K T K	A D L S A V L W S E	R E – – E P R V W S
TBEV_Sofjin	N E M G F L E K T K	A D L S T V L W S E	H E – – E L R S W E
LIV_369/T2	N E M G F L E K T K	A D L S A M L W S G	H E – – E H R Q W S
KFDV_P9605	N E M G M L D K T K	A D L A G L V W R G	E Q – – R H P A W E
No known insect vector flaviviruses			
TABV	– – – – – – – – – D	G E I L T L I K K L	I K V K I Q Q I E N
RBV_RiMAR	N E M R L L E N T K	R D I M D L F K R D	T T V N E S P V F H
MODV_M544	N E L R W L E N T K	E D I K Q L F G E K	I H M – G I S S G G
APOIV_ApMAR	N E M G F L E R T K	K D F R E F F R K E	V N M D G E P T Q –
Insect only flaviviruses			
KRV_SR-82	W E M R L F P N I R	G D I M E M A S A M	K E P Q E T Q S Q A

Figure S1, Related to Figure 1. NS3 R399 and NS4B R18 is not observed in other ZIKV strains. A-B. MUSCLE sequence alignment examining the conservation of NS3 R399 (**A**) and

NS4B R18 (**B**) among ZIKV and related flaviviruses. Amino acid positions were determined using ZIKV-Dakar as the reference sequence. Virus abbreviation, virus name (accession number): AHFV, Alkurma hemorrhagic fever virus (JF416954); APOIV, Apoi virus (AF160193); BAGV, Bagaza virus (AY632545); BSQV, Bussuquara virus (AY632536); DENV, Dengue virus (AF180818, KU725663, KU050695, KR011349); DTV, Deer tick virus (AF311056); ENTV, Entebbe bat virus (AY632537); IGUV, Iguape virus (AY632538); ILHV, Ilheus virus (NC_009028); JEV, Japanese encephalitis virus (EF571853); KEDV, Kedougou virus (AY632540); KFDV, Kyanasur Forest disease virus (JF416958); KOKV, Kokobera virus (NC_009029); KRV, Kamiti River virus (NC_005064); LGTV, Langat virus (AF253419); LIV, Louping ill virus (Y07863); MODV, Modoc virus (AJ242984); MVEV, Murrary Valley encephalitis virus (AF161266); OHFV, Omsk Hemorrhagic fever virus (AY193805); POWV, Powassan virus (NC_003687); RBV, Rio Bravo virus (AF144692); ROCV, Rocio virus (AY632542); SEPV, Sepik virus (AY632543); SLEV, Saint Louis encephalitis virus (KM267635); SPOV, Spondweni virus (KX227369); TABV, Tamana bat virus (AF285080); USUV, Usutu virus (NC_006551); WNV, West Nile virus (HQ596519); YFV, Yellow fever virus (AY640589); ZIKV, Zika virus (KU365778, KJ776791, KU501215, LC002520, KF268948).

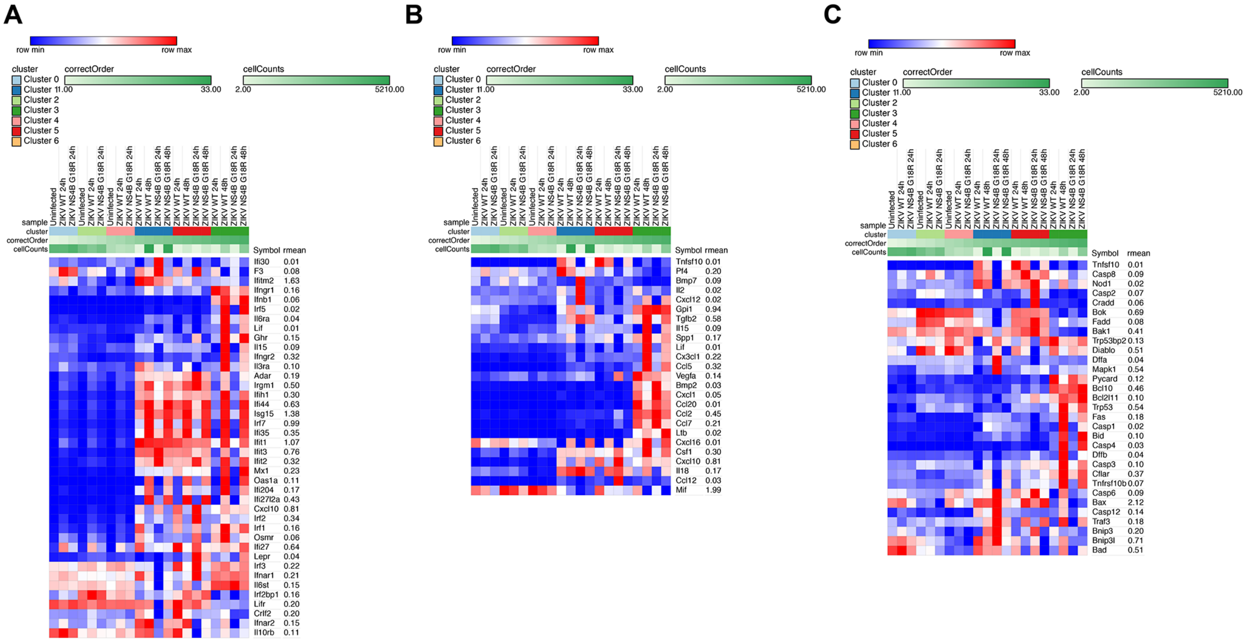


Figure S2, Related to Figure 5. Heatmap of genes between uninfected, ZIKV-Dak WT, and ZIKV-Dak NS4B G18R infected NSCs. Relative expression of genes representing interferon response (**A**), cytokine response (**B**), and apoptosis (**C**) related genes from the RNAseq experiment in **Fig 5** with NSCs infected with ZIKV-Dak WT and ZIKV-Dak NS4B G18R at 0 (uninfected), 24 and 48 h.

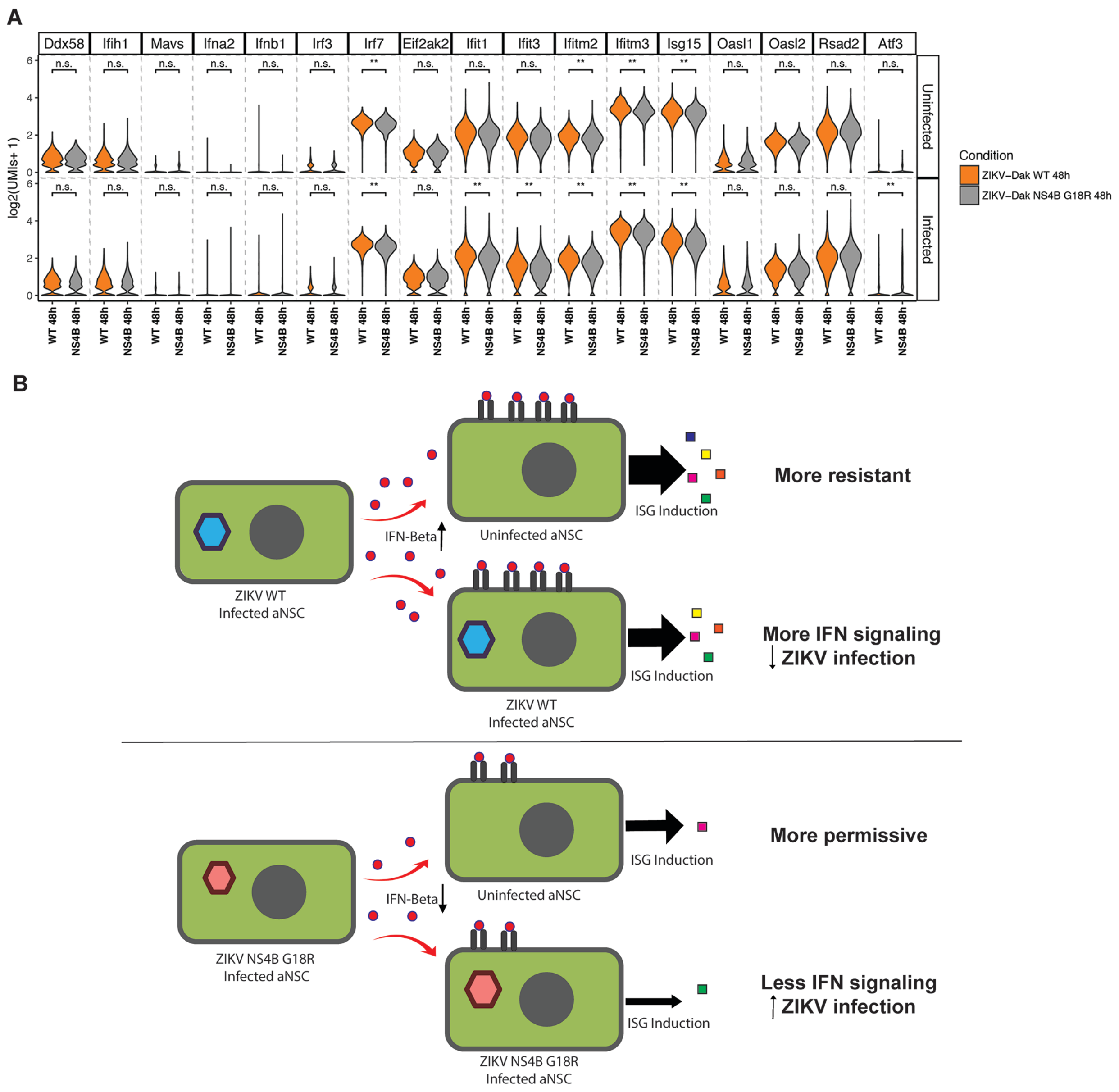


Figure S3, Related to Figure 5. Single-cell RNAseq analysis RLRs, IFNs, and ISG expression after ZIKV-Dak WT or ZIKV-Dak NS4B G18R infection of NSCs. (A) Violin plots showing expression of selected RLRs (*Ddx58*, *Ifih1*, and *Mavs*), type I IFNs (*Ifna2* and *Ifnb1*), and ISGs (*Eif2ak2*, *Ifit1*, *Ifit3*, *Ifitm2*, *Ifitm3*, *Isg15*, *Oasl1*, *Oasl2*, and *Rsad2*) in uninfected (ZIKV RNA-negative) and infected (ZIKV-RNA-positive) cells in the culture at 48 h after inoculation with ZIKV-Dak WT or ZIKV-Dak NS4B G18R. A MAST test was used to determine the statistical difference in expression of genes between different conditions. (** *P* adjusted < 0.01). (**B**) A model showing the differences in ISG production from ZIKV-Dak-infected, ZIKV-Dak NS4B G18R-infected, and uninfected cells.

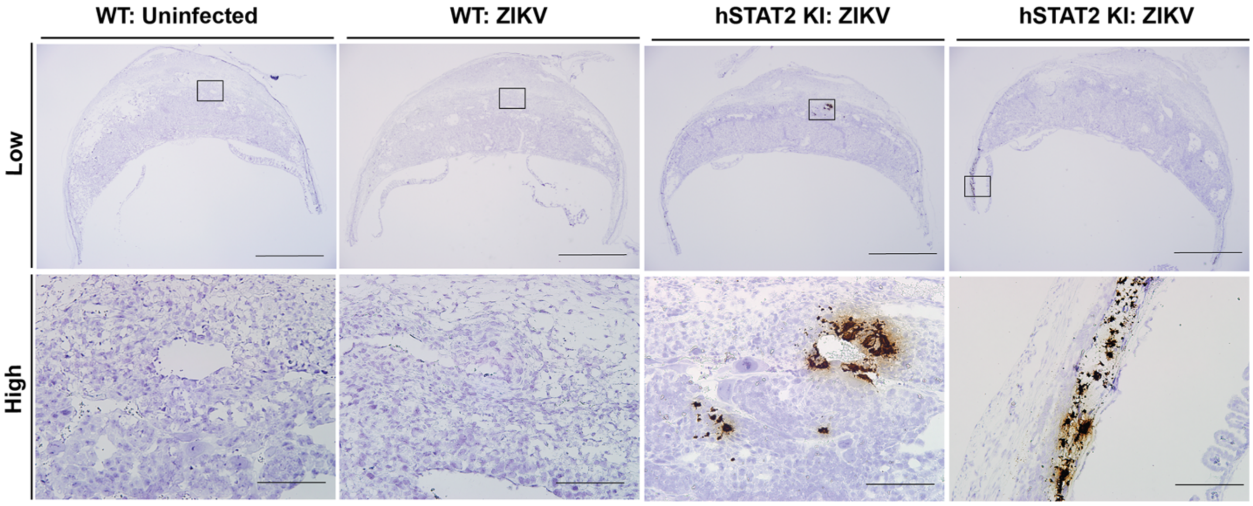


Figure S4, Related to Figure 6. ZIKV RNA in the placenta and uterine lining of hSTAT2 KI mice. WT or hSTAT2 KI mice were uninfected (column 1) or inoculated with ZIKV (columns 2-4) at E6.5. At E13.5, placentas were harvested and subjected to RNA *in situ* hybridization with a ZIKV-specific probe. Viral RNA was detected in the junctional zone of the placenta (third column) and uterine lining (fourth column) of hSTAT2 KI dams infected with ZIKV-Dak-MA. No viral RNA was detected in WT dams. Low (scale bar, 2 mm) and high (scale bar, 200 μm) magnification images are presented in sequence with the boxes indicating regions. The images are representative of several placentas from independent dams.

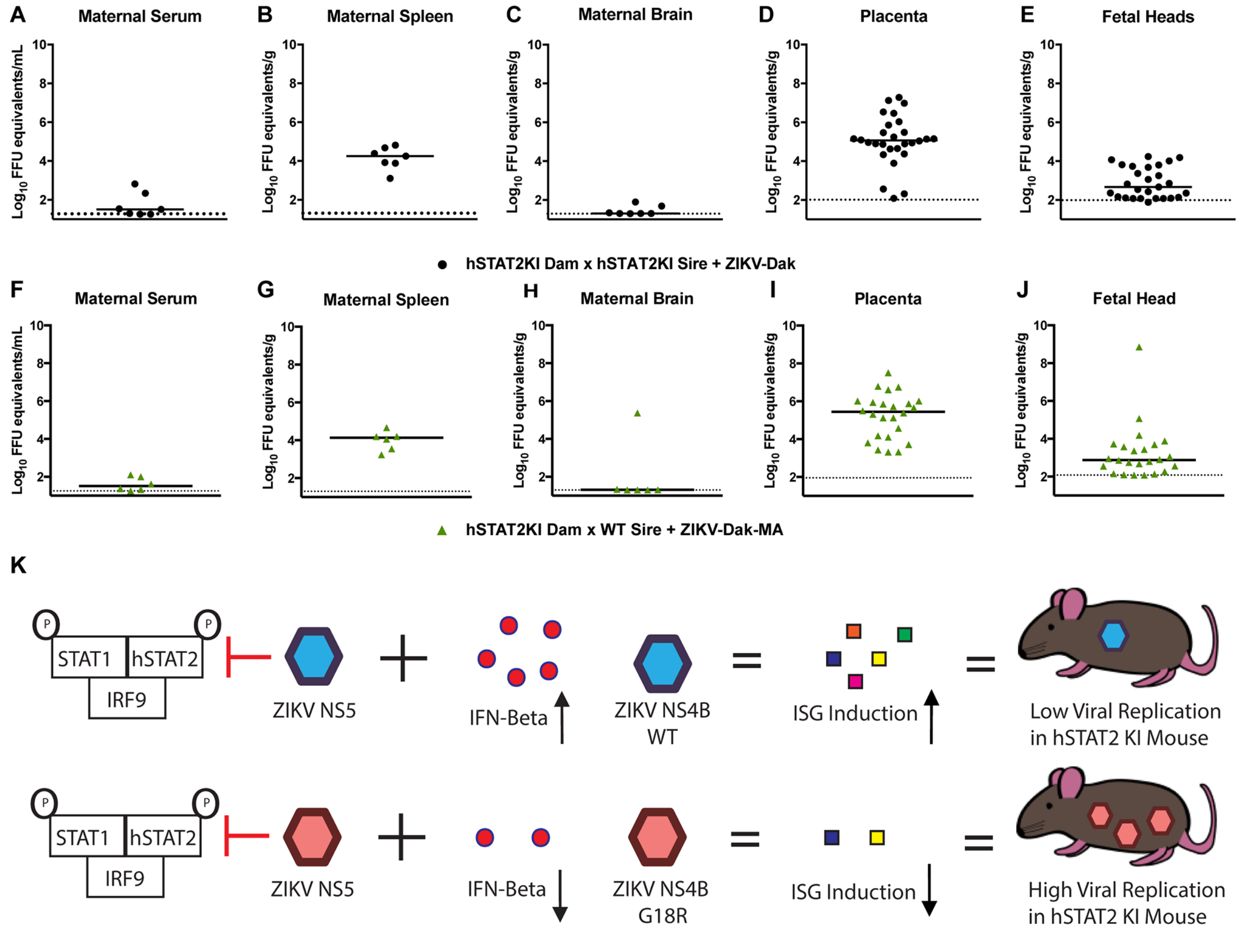


Figure S5, Related to Figure 6. Susceptibility of maternal and fetal tissues of hSTAT2 KI mice to ZIKV infection. Viral RNA burden from maternal serum (**A, F**), maternal spleen (**B, G**), maternal brain (**C, H**), placenta (**D, I**), and fetal head (**E, J**) of sexually mature (>8 weeks old) hSTAT2 KI dams mated to hSTAT2 KI sires and inoculated subcutaneously with 10^6 FFU of ZIKV-Dak (**A-E**) or WT sires and inoculated subcutaneously with 10^6 FFU ZIKV-Dak-MA (**F-J**). Dams were infected on E6.5 and tissues were harvested on E13.5. Data (**A-E**) are from 2 independent experiments (n = 7 dams, n = 28 placentas and fetal heads). Data (**F-J**) are from 2 independent experiments (n = 6 dams, n = 24 placentas and fetal heads). Solid lines represent the median values, and dotted lines denote the limit of detection of the assay. (**K**) A scheme depicting the ZIKV immunocompetent mouse model. The combined effects of the ZIKV NS5 inhibiting hSTAT2 and ZIKV NS4B G18R mutation decreasing amounts of IFN-β production results in lower levels of ISG that enable increased ZIKV replication and pathogenicity.