Title: Collaborative Cross strains reveal that Irf3 deficiency results in uncontrolled Zika virus replication \textit{in vitro} but is insufficient to induce disease susceptibility in mice.

Running title: Irf3 deficiency promotes ZIKV replication

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Abstract

Zika virus (ZIKV) is a Flavivirus responsible for recent epidemics in Pacific Islands and in the Americas. In humans, the consequences of ZIKV infection range from asymptomatic infection to severe neurological disease such as Guillain-Barré syndrome or fetal neurodevelopmental defects, suggesting the influence of host genetic factors. We previously reported similar diverse outcomes of ZIKV infection in mice of the Collaborative Cross, a collection of inbred strains with large genetic diversity. CC071 was the most susceptible strain with severe symptoms and lethality. Here, we investigated viral replication in mouse embryonic fibroblasts from CC071 and two resistant strains. CC071 showed enhanced viral replication associated with delayed induction of type-I interferons (IFN-I). Using a combination of genetic approaches, we identified a loss of function (LOF) mutation in the Irf3 gene, specific to CC071, and demonstrated that it fully explains the defective IFN-I response and uncontrolled viral replication in CC071 MEFs. However, this mutation was not sufficient to induce the high plasma viral load and clinical severity observed in CC071 mice, indicating the involvement of other susceptibility genes in vivo. Considering the susceptibility of the CC071 strain to multiple viruses, our results provide new insight into the role of Irf3 in innate antiviral response.

Importance

Recent ZIKV outbreaks led to millions of infected people, with rare but severe complications such as Guillain-Barré syndrome and encephalitis in adults suggesting host genetic control. We previously reported the importance of host genes in ZIKV pathogenesis using a panel of genetically diverse mouse strains and identified CC071 as the most susceptible strain. Through a combination of functional and genetic approaches in a cellular model, we identified a mutation in the Irf3 gene which plays a key role in the expression of interferon beta. This mutation fully explains the high viral replication observed in CC071 cells. However, it was not sufficient to explain the symptoms displayed by CC071 ZIKV-infected mice, unraveling the implication of other host genes. Since CC071 has been reported as a valuable model for several viral infections, our results have implications beyond ZIKV infection and contribute to shedding light on host mechanisms fighting infectious diseases.
Introduction

Zika virus (ZIKV) is a mosquito-borne virus of the Flaviviridae family identified in 1947 in Uganda. The first noticeable human outbreaks occurred in Micronesia in 2007 and in French Polynesia and New Caledonia in 2013-2014. In 2015-2016, ZIKV caused an epidemic in Brazil which rapidly spread across the Americas and the Caribbean. To date, 89 countries have reported evidence of mosquito-transmitted Zika virus infection (https://www.who.int/health-topics/zika-virus-disease#tab=tab_1).

While most people infected with ZIKV remain asymptomatic, some develop non-specific symptoms including rash, fever, conjunctivitis, muscle and joint pain, malaise and headache. Neurological complications have been described in adults such as Guillain-Barré syndrome (1) and encephalitis (2). Infection of pregnant women was associated with congenital Zika syndrome in the fetus, which can lead to neurodevelopmental deficiencies, brain malformation (3) or in some cases to fetal loss (4).

Many factors may contribute to this variable severity, including the viral strain, the infection route and dose, and the host genetic background (5, 6). Indeed, mouse and human studies have shown that host genes influence flaviviral infections’ outcomes (7). While human genetic studies are hampered by the variability of these multiple factors, they can be controlled in mouse models which have proven very valuable to identify susceptibility variants (8, 9). Relevant ZIKV infection models have been developed in mice either using Ifnar1 knock-out (KO) mice in which the IFNAR receptor to IFN-I has been inactivated (10, 11), or by blocking this receptor using a monoclonal antibody targeting the IFNAR1 receptor subunit (MAR1-5A3 (12)).

We have previously explored the role of mouse natural genetic variants on ZIKV susceptibility in the Collaborative Cross (CC), a panel of recombinant inbred mice encompassing a genetic diversity similar to that of the human population and capturing approximately 90% of the mouse natural genetic variants (13, 14). We reported that the CC genetic diversity enabled large variations in the clinical severity of ZIKV disease, plasma viral load and intensity of brain pathology (15). We specifically identified CC071 mice as very susceptible, with high mortality and high peak plasma viral load. We also demonstrated that genetic background influenced the control of ZIKV replication in CC mouse embryonic fibroblasts (MEFs).

Here, we investigated the mechanisms driving high viral replication in CC071 MEFs. We found that, compared with the more resistant C57BL/6J (B6) and CC001 strains, ZIKV-infected CC071
MEFs displayed a delayed expression of the IFN-I response genes. Genetic and functional analyses identified a strain-specific, LOF variant in the *Irf3* gene, the main transcription factor involved in IFN-I expression. *In vivo* comparison of CC071 and B6-*Irf3* KO mice showed that *Irf3* deficit is not sufficient to explain the high susceptibility of CC071 mice to ZIKV infection, unraveling the plurality of genetic factors controlling susceptibility to viral infections. These findings provide new insights on the role of *Irf3* in viral diseases and exemplify how the study of CC strains allows deciphering the role of host genes in ZIKV and other viruses’ pathogenesis.
Results

CC071 MEFs show defective control of viral replication and delayed IFN-I expression, but normal response to IFN-I stimulation

We previously reported that, unlike CC001 MEFs, CC071 MEFs produced increasing quantities of viral particles during the first 72 hours post-infection (hpi) (15). Here, we confirmed and expanded this observation by infecting B6, CC001 and CC071 MEFs and by quantifying viral particles by FFA. After ZIKV infection, CC071 MEFs displayed high and increasing viral titers between 24 and 72 hpi, while CC001 and B6 MEFs showed stable and lower titers (Fig 1A).

To investigate the origin of the defective control of viral replication in CC071 MEFs, we measured the expression level of the Ifnb1 gene coding for IFNβ in ZIKV-infected CC071, CC001 and B6 MEFs. Ifnb1 expression is induced very rapidly after virus detection by sensors and triggers the innate antiviral response which is essential for limiting viral replication. In CC001 and B6 MEFs, Ifnb1 expression was significantly induced at 24 hpi and remained stable and high until at least 72 hpi (Fig 1B). In contrast, its expression in CC071 MEFs was low at 24 hpi and reached the level of CC001 only at 72 hpi. Similar results were obtained for Ifn4a which encodes one of the IFNα proteins (Sup Fig 1). Notably, Ifnb1 expression in CC071 MEFs at 72 hpi was significantly higher than in B6 MEFs, showing that CC071 MEFs were delayed but not intrinsically hampered in their ability to induce a strong IFN-I expression.

To test whether this defective induction of Ifnb1 expression was specific to ZIKV infection, MEFs were then transfected with the influenza A virus-derived 3-phosphate-hairpin-RNA (3p-hpRNA), an agonist of the RIG-I/MDA5-MAVS pathway, or treated with polyinosine-polycytidylic acid (poly (I:C)), that stimulates both Toll-like receptor 3 (TLR3) and the RIG-I/MDA5-MAVS pathway (16). Here again, CC071 MEFs showed a delayed expression of Ifnb1 by comparison with B6 and CC001 MEFs (Sup Fig 2), indicating that the defect in IFN-I genes expression in CC071 MEFs was not specific to ZIKV infection. This result suggested a defect in the molecular cascade between cellular sensors of pathogen-associated molecular patterns (PAMP) and the Ifnb1 gene transcription machinery.

To evaluate the capacity of CC071 MEFs to respond to IFN-I stimulation, they were treated with recombinant IFNα. The expression of IFN-stimulated genes (ISGs) such as Ifitm3 was induced with the same kinetics and level as in B6 and CC001 MEFs (Fig 1C), showing that CC071...
MEFs are able to respond normally to IFN-1 stimulation and that their defect is limited to the induction of Ifnb1 gene expression.

*CC071’s delayed Ifnb1 expression is strain-specific*

To gain insight into the mechanisms responsible for defective Ifnb1 induction, we investigated the expression levels of all genes involved in Ifnb1 expression on CC071, B6 and CC001 MEFs at 16, 24 and 32 hpi. Mock-infected MEFs were analyzed at 24 hours as controls. Expression levels were measured by RNA sequencing (RNAseq) which provided a comprehensive analysis of transcriptomic changes. In CC001 MEFs, the expression of many genes rapidly increased after infection (160 at 16h, 821 at 24h and 971 at 32h; log2 fold-change > 1, FDR = 0.05), reflecting a robust innate antiviral response (Sup Fig 3A). A similar pattern was observed in B6 MEFs. By contrast, the expression of only 38 genes was increased in CC071 MEFs at 32hpi (34 of which were also activated in CC001), consistent with the delayed induction of Ifnb1 expression. Among the genes that are involved in the pathway between PAMP sensors and Ifnb1 transcription, ISGs such as Tlr3, Ddx58 (coding for RIG-I sensor) or Irf7 were not activated upon infection in CC071, while constitutively expressed genes such as Mavs, Ticam1 (coding for the TRIF adaptor), Traf3 or Irf3, showed comparable levels of expression in the three strains (Sup Fig 3B). Therefore, this analysis did not provide new clues for identifying the gene responsible for the defect observed in CC071.

We then leveraged the genetic architecture of the CC which genomes are patchworks of haplotypes inherited from the eight founder strains (17). Although CC071 was the only strain with severe ZIKV disease, we hypothesized that, if the delayed activation of Ifnb1 resulting in uncontrolled viral replication observed in CC071 MEFs was due to an allele at one of the genes involved in the Ifnb1 induction pathway inherited from a parental strain, ZIKV-infected MEFs of CC strains carrying the same allele would present similarly high viral titers. We therefore derived MEFs from each available CC strain carrying the same ancestral haplotype as CC071 at one of the 13 genes of the pathway (Fig 2A). Upon ZIKV infection, none of these CC MEFs showed viral titer kinetics resembling that observed in CC071 MEFs (Fig 2B). These results suggested two alternative hypotheses. Either the delayed Ifnb1 activation involved two members of the pathway with a CC071-specific allelic combination resulting in non-functional interaction, or CC071 was carrying a strain-specific allele at one of these genes, resulting from a mutation that arose on an ancestral haplotype during the CC071 inbreeding. However, the
sequencing of one male of each CC strain reported in 2017 (18), did not identify such "private" variants with high predicted impact in CC071 for any of these genes. Whatever the molecular mechanism, our results indicated that it was specific to CC071.

Genetic analysis identifies Irf3 as a candidate gene in a haplotype shared between CC071 and CC001

We then turned to a genetic mapping approach. We first established that (CC001xCC071)F1 MEFs responded to infection with rapid induction of Ifnb1 expression, as CC001 (Sup Fig 4), suggesting that the CC071 trait was recessively inherited. F1 mice were therefore backcrossed with CC071. MEFs lines were produced from 51 backcross (BC) embryos, infected with ZIKV, and analyzed for viral titer and Ifnb1 expression as above. One CC001 and one CC071 MEF lines were included in each infection experiment as controls. BC MEFs displayed either high Ifnb1 expression and low viral titer (like CC001), or high viral titer and delayed Ifnb1 expression (like CC071, Fig 3A-B), showing that these two traits correlated across the BC diverse genetic backgrounds.

To confirm this apparently binary distribution, we conducted linear discriminant analysis (LDA) on CC001 and CC071 MEFs using Ifnb1 expression at the three time points. Applying the LDA coefficients to backcross MEFs data classified individuals either in a CC001-like group (n=31; 61%) or in a CC071-like group (n=20; 39%), with a mean probability of 0.975 and 0.991, respectively (Fig 3C). Quantitative trait locus (QTL) mapping was performed using LDA classification as a binary trait. Genome scan identified a peak on chromosome 7 with a LOD (logarithm of the odd) score of 9.138 (p < 0.001, Fig 4A) located in a region centered on the Irf3 gene which, given its main role in the regulation of Ifnb1 expression, appeared as an obvious candidate. However, both CC001 and CC071 inherited the CAST/EiJ haplotype in this region (Fig 4B), strongly suggesting that CC071’s defect was caused by a de novo mutation.

Abnormal splicing of Irf3 mRNA in CC071 leads to a loss of function

To identify the CC071-specific mutation, we re-analyzed the RNAseq data and investigated the splicing events between Irf3 exons. As shown in Fig 5A, no splicing was observed between exons 6 and 7 in CC071 MEFs, while a short cryptic exon was added to exon 6 (red in Fig 5A). This aberrant splicing resulted in an mRNA lacking the last two exons. Notably, exon 8 encodes the serin-rich region of the protein with the phosphorylation sites necessary for IRF3
activation and nuclear translocation leading to \textit{Ifnb1} transcription (Fig 5B). A combination of long-range PCRs and sequencing did not identify the exact nature of \textit{Irf3} genetic alteration in CC071 but suggested the insertion of a repeated sequence between exons 6 and 7. Nevertheless, the functional consequence of this mutation was confirmed by Western blot using a specific C-terminal IRF3 antibody which showed that full-length IRF3 protein was absent in CC071 MEFs (Fig 5C). Moreover, immunofluorescence using an antibody directed against phosphorylated IRF3 detected a positive signal in the nucleus of many ZIKV-infected CC001 and B6 MEFs, but not in CC071 MEFs (Fig 5D). Altogether, these results show that CC071 carries a mutation in \textit{Irf3} that prevents IRF3 dimerization and phosphorylation which are required to induce \textit{Ifnb1} expression. Whether the altered mRNA sequence prevents the production of the protein or alters its activity, this mutation results in IRF3 loss of function. 

\textit{Irf3 LOF is responsible for uncontrolled viral replication in CC071}

To test if the delayed \textit{Ifnb1} expression resulting in uncontrolled viral replication in CC071 MEFs was caused exclusively by \textit{Irf3} LOF, we performed a quantitative complementation test by producing compound heterozygous MEFs carrying a knockout \textit{Irf3} allele (\textit{Irf3}\textsuperscript{KO}) and the allele from CC071 (denoted \textit{Irf3}\textsuperscript{KO/CC071}). These MEFs were compared with CC071 MEFs and with heterozygous MEFs carrying a B6 wildtype allele and either an \textit{Irf3}\textsuperscript{KO} or an \textit{Irf3}\textsuperscript{71} allele (\textit{Irf3}\textsuperscript{+/KO} or \textit{Irf3}\textsuperscript{+/-71}, respectively, Fig 6A). While \textit{Irf3}\textsuperscript{+/KO} and \textit{Irf3}\textsuperscript{+/-71} ZIKV-infected MEFs showed the same pattern as CC001 or B6 MEFs (rapid induction of \textit{Ifnb1} expression and controlled viral replication, see Fig 1A for comparison), CC071 and \textit{Irf3}\textsuperscript{KO/71} MEFs carrying two defective alleles at \textit{Irf3} showed similar results (Fig 6B-C). These data demonstrate that, since the \textit{Irf3}\textsuperscript{KO} did not complement the \textit{Irf3}\textsuperscript{71} allele, the \textit{Irf3} mutation in CC071 contributes to the defects observed in ZIKV-infected MEFs. Moreover, since the data obtained on CC071 and on \textit{Irf3}\textsuperscript{KO/71} MEFs were identical, we conclude that the \textit{Irf3} mutation is sufficient to induce the defects observed in CC071 MEFs.

\textit{Irf3 LOF is not sufficient to explain CC071's susceptibility in vivo}

Lastly, we investigated whether the \textit{Irf3} LOF was also responsible for the high susceptibility to ZIKV of CC071 mice. We first compared clinical signs and plasma viral load in ZIKV-infected B6, B6-\textit{Irf3}\textsuperscript{KO/KO} and CC071 mice pre-treated with MAR1-5A3. While no differences were observed between B6 and B6-\textit{Irf3}\textsuperscript{KO/KO} (no clinical signs and moderate plasma viral loads), as expected
under conditions of neutralization of the IFN-I response, CC071 mice developed symptoms (ruffled fur, hunched posture and body weight loss) around 7 days p.i. (Fig 7A), and significantly higher viral loads at days 2 and 6 p.i. (Fig 7B), indicating that genes not associated to the IFN-I response play a major role in the susceptibility of CC071 mice to ZIKV infection. We then measured plasma viral load in mice of the same genotypes infected without MAR1-5A3 treatment. CC071 mice showed significantly higher viral loads at days 1 and 2 p.i., while similar values were again observed in B6 and B6-Irf3<sup>KO/KO</sup> mice (Fig 7C). These results show that, unlike at the cell level, Irf3 LOF does not result in elevated viral replication in infected mice (as measured by plasma viral load), either with or without IFNAR blockade, and therefore is not sufficient to explain the high susceptibility of CC071 mice, suggesting the involvement of other host genes.
Discussion

Among the CC strains we tested, CC071 mice pre-treated with anti-IFNAR antibody were the most susceptible to ZIKV infection, with disease severity and peak plasma viral load almost as high as those of B6-Ifnar1 KO mice, and higher clinical signs and mortality than 129-Ifnar1 KO mice (15). In this susceptible model, IFN-I response is not permanently and ubiquitously abrogated as in Ifnar1 deficient strains, but delayed, temporarily blocked and not in tissues unreach by the antibody (probably such as the brain). It is therefore particularly appropriate to identify host susceptibility genes. However, traits measured in vivo such as viremia, symptoms and death result from complex interactions between multiple pathways and cell types, from which distinct mechanisms are difficult to dissect. Cultured MEFs offer a lower level of complexity although they cannot provide the level of integration of a whole organism. To decipher the susceptibility of CC071 mice, we used MEFs as a convenient cellular model which can be easily derived from any mouse genetic background.

Starting from the confirmed observation of uncontrolled viral replication in CC071 MEFs, we identified a delayed activation of the Ifnb1 gene resulting in delayed stimulation of ISGs and used a combination of genetic approaches to find the causative gene defect. The observation that MEFs were normally responsive to IFN-I stimulation was consistent with the higher susceptibility of mice treated by the MAR1-5A3 antibody compared with untreated mice (15). These results pointed at the pathway from PAMP sensors to Ifnb1 transcription factors. Transcriptional analysis did not identify reduced expression of non-ISGs of this pathway. CC strains' genomic structure allows searching for haplotypes inherited from the same founder in CC strains showing similar phenotypes. However, we did not identify such haplotypes for genes of the Ifnb1 induction pathway, suggesting that the defects observed in CC071 MEFs were strain-specific. It is finally the analysis of MEFs derived from backcross embryos that established a monogenic inheritance, and genetic linkage unambiguously pointed at the causative Irf3 gene. The MEF experimental model was particularly appropriate since we could derive cell lines from every backcross embryo. Our RNAseq data informed on the RNA splicing defect in the Irf3 gene, which functional consequences could be validated in vitro. The formal proof that the CC071 Irf3 mutation was necessary and sufficient to cause Ifnb1 delayed activation and uncontrolled viral replication came from a quantitative complementation test using an Irf3 KO allele.
Notably, this is not the first example of a *de novo* mutation responsible for a CC-strain-specific trait. We (19) and others (20) previously reported the extreme susceptibility of CC042 to *Salmonella* Typhimurium and to *Mycobacterium tuberculosis*, respectively, as a consequence of a *de novo* 15-nucleotide deletion in the *Itgal1* gene. In the case of *Salmonella* Typhimurium, CC042 was standing out, with bacterial loads up to 1000 times higher than other CC and the susceptible B6 strains. In our previous study on ZIKV, CC071 was the most susceptible strain, but its peak viral load was just the highest in a continuous distribution of values. Additionally, this is not the first example of *Irf3* spontaneous polymorphism identified in mice modulating susceptibility to bacterial (21) or viral infections (22).

*Irf3* is the main transcription factor involved in the innate immune response. It is constitutively expressed and, at rest, inactive *Irf3* is present in the cytoplasm. Upon viral entry (or other stimuli that activate TLRs such as TLR3 and 4 or RIG-like receptors), signal transduction leads to the phosphorylation of *Irf3*, leading to its dimerization and translocation to the nucleus where it binds to the IFN-I genes promoters (23). This mechanism leads to the very fast production of IFNβ which is secreted by the cell and triggers the immediate response to viral infection through the activation of ISGs with diverse antiviral functions (24). In humans, LOF mutations in *Irf3* have been associated with increased susceptibility to West Nile virus (WNV) (25, 26), herpes simplex virus 1 (HSV-1) (27, 28) and more recently to SARS-CoV-2 (29).

In mice, many studies have used *Irf3*-deficient models. After infection with WNV, viral replication was increased in *Irf3*<sup>KO/KO</sup> bone marrow macrophages (BMMs) and primary neurons (30). Higher viral replication was observed in HSV-1-infected *Irf3*<sup>KO/KO</sup> bone marrow-derived dendritic cells (BMDCs) and BMMs, and IFNβ was reduced in BMDCs supernatants (31). Similarly, *Ifnb1* expression was reduced in HSV-1-infected (32) and CHIKV-infected (33) *Irf3*<sup>KO/KO</sup> MEFs. In line with these findings, our study provides, to our knowledge, the first evidence for a role of *Irf3* in the infection of murine cells by ZIKV.

*In vivo* studies led to contrasting results depending on the virus and infection route. *Irf3*<sup>KO/KO</sup> mice were reported to be resistant to ZIKV with no mortality nor body weight loss (10). Moreover, we found that plasma viral loads were similar in B6-*Irf3*<sup>KO/KO</sup> and B6 mice, whether mice were treated with anti-IFNAR antibody, or not. Likewise, *Irf3*<sup>KO/KO</sup> mice showed no mortality and low virus in the circulation following dengue virus (34) and CHIKV infection (33). Contrastingly, WNV infection was lethal in *Irf3*<sup>KO/KO</sup> mice while 65% of infected WT mice survived, suggesting that *Irf3* LOF may impact clinical parameters (survival, body weight loss)...
only in the case of highly pathogenic viruses. The route of infection may also be a critical parameter. After intravenous inoculation with HSV-1, all WT and Irf3KO/KO mice survived (32), while intranasal inoculation led to 30% and 90% mortality in WT and Irf3KO/KO mice, respectively (35).

Our in vivo data showing higher viral load in CC071 than in B6-Irf3KO/KO mice, with clinical signs, indicate that other genes control the susceptibility of CC071. The results of the complementation test on MEFs, with CC071 and Irf3KO/Irf3KO yielding very similar data, suggest that these genes may not act at the cell level, at least during the first 72 hpi. Our poor understanding of the severe disease developing in CC071 ZIKV-infected mice does not point at potential mechanisms. As in this study, appropriate crosses will be necessary to detect genomic regions from which candidate genes could be identified and validated. It will then be possible to assess the specific contribution of the Irf3 LOF mutation to the susceptibility of CC071 mice.

CC071 has been reported to be susceptible to other flaviviruses. High viral load was found after dengue infection (15). Lethality after infection with WNV (15) or Powassan virus (36) were also described, although there is likely contribution from the defective Oas1b allele that CC071 has inherited from 129S1/SvImJ. CC071 was also one of the most susceptible CC strains to Rift Valley Fever virus infection (37) and to hepacivirus, with long-term viral persistence (38). Because of its unique genetic background, this strain will be increasingly useful for infectious disease studies, which justifies deciphering its mechanisms of susceptibility.
Material and methods

Mice and crosses

C57BL/6J mice were purchased from Charles River Laboratories France. Collaborative Cross strains (CC001/Unc, CC071/TauUnc, CC005/TauUnc, CC011/Unc, CC026/GeniUnc, CC061/GeniUnc, CC021/Unc, CC006/TauUnc, CC025/GeniUnc, CC039/Unc, CC060/Unc) were purchased from the Systems Genetics Core Facility, University of North Carolina and bred at the Institut Pasteur. Irf3 Irf7 double KO mice (C57BL/6J-\textit{Bcl2l12}^-/-\textit{Irf3}^{	extit{tm1Ttg}}\textit{Irf7}^{	extit{tm1Ttg}}, (32, 39); B6-Irf3\textsuperscript{KO} and B6-Irf7\textsuperscript{KO} thereafter) were bred at the Institut Pasteur. All mice were maintained as described previously (15). (CC001 x CC071) x CC071 backcross embryos were obtained from reciprocal crosses (F1 female x CC071 male and vice-versa). For the quantitative complementation test, B6-Irf3\textsuperscript{+/KO} B6-Irf7\textsuperscript{+/KO} females were crossed with CC071 males and B6-Irf3\textsuperscript{+/KO} B6-Irf7\textsuperscript{+/KO} males were crossed with C57BL/6J females. Genotype at the Irf7 loci was not considered as only B6-Irf7\textsuperscript{+/KO} of B6-Irf7\textsuperscript{+/+} mice were used. For \textit{in vivo} infections, B6-Irf3 simple KO mice were obtained from an F2 between B6-Irf3 Irf7 double KO and B6 mice. Mouse experiments were approved by the Institut Pasteur Ethics Committee and authorized by the French Ministry of Research (project #19469), in compliance with French and European regulations.

ZIKA virus

The FG15 Asian Zika virus (ZIKV) strain, isolated from a patient during ZIKV outbreak in French Guiana in December 2015, was obtained from the Virology Laboratory of the Institut Pasteur of French Guiana. Viral stock (passage 5) was prepared from supernatant of infected C6/36 cells, clarified by centrifugation at 800g and titrated on Vero cells by focus-forming assay.

Mouse infection

All infection experiments were performed in a biosafety level 3 animal facility and mice were kept in isolators. Six- to 10-week-old, male or female mice were injected intraperitoneally with \(10^7\) PFU of ZIKV FG15. Mice received an IP injection of 2 mg of MAR1-5A3 anti-IFNAR antibody (Euromedex, Cat#BX-BE0241) one day prior infection. Mouse numbers are indicated in figure legends. Both males and females were used since no differences between sexes were detected in our previous and present experiments. Clinical signs and body weight loss were
recorded for up to seven days post infection. Blood samples were collected on EDTA at two- and six-days post infection from the retromandibular vein for plasma viral load assessment. Quantification of ZIKV viral copies by qPCR was previously described (15).

**MEFs isolation**

Pregnant females were euthanized at day 13.5-15.5 of gestation. For B6, CC001 and CC071, MEFs were isolated from individual fetuses to obtain biological replicates. For other CC strains, MEFs were derived from individual or pooled fetuses. Fetus bodies were chopped and digested with trypsin (Gibco Cat#25300054), then cultured at 37°C and 5% CO₂ in complete medium (DMEM Gibco Cat# 31966047, 10% fetal bovine serum PAA Laboratories Cat#A15-101, 1% penicillin/streptomycin Sigma Cat#P4333). MEFs were used until passage 2. For the backcross experiment, MEF lines were isolated from 52 backcross fetuses. Heads were used to prepare DNA for whole-genome genotyping.

**MEFs infection**

MEFs were seeded at 5.10⁴ cells per well in 24-well plates the day before infection. They were exposed to ZIKV FG15 strain at a MOI of 5 for 2 hours after which the inoculum was replaced with fresh complete medium and MEFs were incubated for up to 72 hours. For kinetics studies, different wells were used for each time point. Backcross MEFs were infected in 6 infection experiments, each of which included a CC001 and a CC071 MEF lines.

**MEFs IFNα stimulation**

MEFs were seeded at 5.10⁴ cells per well in 24-well plates the day before stimulation and treated with 300 IU/mL IFNα (Miltenyi Biotec Cat#130-093-131) and incubated for up to 24 hours.

**Focus forming assay**

Quantification of ZIKV particles was completed by focus forming assay on Vero cells (ATCC CRL-1586) as previously described (15).

**RNA extraction from cells**
MEFs were lysed in 350µL of RLT buffer (Qiagen) with 1% β-mercaptoethanol. RNA was extracted using RNeasy Mini Kit (Qiagen Cat#74104) according to the manufacturer’s instructions, with DNase I (Qiagen Cat# 79254) to prevent genomic DNA contamination.

Reverse transcription and qPCR

Reverse transcription was performed on 200ng of RNA using Superscript II polymerase (Invitrogen Cat#18064022) RNaseOUT ribonuclease inhibitor (Invitrogen Cat#10777019). qPCR was performed on 20ng of cDNAs using Power SYBR Green PCR Master Mix (Applied Biosystems Cat#4367659) and 6pmol of each primer, on a QuantStudio 12K Flex or a ViiA 7 (ThermoFisher Scientific). Primers (Eurofins) sequences are provided in Sup Table 1. Gene expression was expressed on a Log10 scale of relative expression to the reference Tbp gene.

Genotyping

Genomic DNA was prepared from backcross fetuses’ heads by proteinase K digestion, phenol-chloroform extraction and ethanol precipitation according to standard protocols. DNAs were sent to Neogen (Neogen/Geneseek, Inc, Lincoln, NE, USA) for whole-genome genotyping using the MiniMUGA array containing 11,125 SNP markers. For the quantitative complementation test, Irf3 and Irf7 genotyping was performed by Transnetyx (Cordova, TN) by real-time PCR on fetuses’ heads.

RNA splicing analysis from RNA sequencing data

RNA sequencing data were produced as described in the Supplementary methods. Splicing analysis was performed using Majiq 2.4 (40) with default parameters to investigate alternative transcripts between conditions, focusing on the Irf3 gene.

Immunofluorescence

MEFs were plated on glass coverslips before infection, fixed with 4% paraformaldehyde for 20 min and permeabilized with pure methanol for 10min at -20°C. Cells were incubated with blocking buffer (5% FBS 0.3% triton in PBS) for 1 hour, with primary antibodies diluted in antibody incubation buffer (AIB: 1% BSA 0.3% triton in PBS) overnight at 4°C and with secondary antibodies and Hoechst (dilution 1:1000) diluted in AIB for 1 hour. Coverslips were mounted on slides and imaged with a widefield microscope (Zeiss Axio Observer.Z1 with a
Plan-Apochromat 20x/0.8 M27 objective and a Hamamatsu sCMOS ORCA-Flash 4.0 v3 camera. ZEN blue 2012 software (ZEISS) imaging software was used for image capture and Image J software (National Institutes of Health) to adjust brightness and contrast. Primary and secondary antibodies are indicated in Sup Table 2.

**Western blot**

MEFs were trypsinized for 5 min, washed and lysed in a protein extraction buffer (10mM TrisHCl pH7.5, 5mM EDTA, 150mM NaCl, 30mM Na₂HPO₄, 50mM NaF, 10% glycerol, 1% NP40, 1X cOmplete (Roche #11873580001), 1X PhosSTOP (Roche #4906845001), 1/1000 benzonaze (Sigma Cat#E1014)) for 30 min at 4°C. Proteins diluted in Laemmli were resolved on 4-12% Bis-Tris gels (Invitrogen Cat#NP0323BOX) in MOPS buffer (Invitrogen Cat#NP0001) and transferred to nitrocellulose membranes (Bio-Rad Cat#1620112) in a 25mM Tris 200mM glycine 20% ethanol buffer. Blots were blocked in 5% milk in TBS-T (0.1% Tween20 in Tris Base Sodium), incubated with primary antibodies diluted in 3% milk in TBST overnight at 4°C, and incubated with secondary antibodies diluted in 3% milk in TBST for 90 min. Blots were revealed with ECL substrate (Thermo Scientific Cat#32132) and imaged with X-ray films. Primary and secondary antibodies are indicated in Sup Table 2.

**Statistical analysis**

Statistical analyses were performed using R (41) version 4.1.0. Viral titers, gene expression and genome copies were Log10-transformed for graphs and statistical tests. One way ANOVA followed by Tukey HSD were used for testing multiple comparisons. For *in vivo* studies, non-parametric Kruskal-Wallis followed by Wilcoxon tests with Bonferroni correction for multiple testing were used to handle values below the limit of detection. Linear discriminant analysis (LDA) was conducted using the MASS package (42). The LDA was trained on the phenotypes of the two parental CC001 and CC071 strains from each infection batch. LDA coefficients were applied to backcross mice for assignment to "CC071-like" or "CC001-like" groups.

**Genetic analysis**

Raw genotypes were curated using the stuart package (43). QTL mapping was performed using R/qtl (44). LDA prediction was used as a binary trait. Statistical significance thresholds were
computed by data permutation (n=1000). 95% confidence interval was estimated using the Bayesian method.
Acknowledgements

We thank Matthieu Prot, Maxime Chazal and Sandrine Vandormael-Pournin for technical advice and for providing reagents. We are grateful to Tommy Penel and Rachid Chennouf of the Institut Pasteur Central Animal Facility of the C2RA (Center for Animal Resources and Research) for the careful breeding of CC strains and for the maintenance of mice in the BSL-3 animal facility, respectively. We thank Etienne Simon-Lorier and Nolwenn Jouvenet for scientific advice and continuous support along this project.

Author contributions

X.M. conceived and supervised this study. M.B. and C.M. designed and performed the experiments and analyzed data. L.C. and C.R.P. performed experiments. E.K. analyzed RNAseq data. X.M. designed experiments and analyzed data. E.B. provided conceptual advice. M.B., C.M. and X.M. wrote the paper. All authors commented on and edited the manuscript.

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Availability of data and materials

RNAseq primary data are available from the European Nucleotide Archive (accession number E-MTAB-12765). All other experimental data that support the finding of this study are available from the corresponding authors upon reasonable request.

Competing interests

The authors declare that they have no competing interests.
Figure legends

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Figure 5. CC071 Irf3 mRNAs show abnormal splicing, resulting in defective IRF3 protein. (A) Schematic representation of the exons of the Irf3 gene with the number of reads spanning successive exons in the CC001 and CC071 RNAseq data (one sample of each strain). The red box between CC071's exons 6 and 7 depicts a novel exon resulting from abnormal splicing. (B) Schematic representation of the IRF3 protein structural domains (exon 1 is untranslated). Exon 8 encodes the serine rich region containing the phosphorylation sites for IRF3 activation. (C) Western blot using an anti-C-terminal IRF3 antibody from mock-infected and ZIKV-infected B6, CC001 and CC071 MEFs at 2 hpi, showing the absence of full-length IRF3 in CC071 MEFs. Vinculin was used as a loading control. (D) Immunofluorescence using an anti-phosphorylated IRF3 (pIRF3, green) in ZIKV-infected B6, CC001 and CC071 MEFs at 24 hpi, showing the absence of pIRF3 in the nucleus of CC071 MEFs upon infection. Red-labeled 4G2 antibody labels ZIKV-infected cells. Cell nuclear DNA labeled by Hoechst (blue). Quantification of the number of infected and pIRF3 positive cells is presented in the table. Proportions were established on 420, 428 and 551 cells for CC001, CC071 and B6, respectively.
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Supplemental information

Legend to supplemental figures

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Supplementary methods

**MEFs stimulation with poly(I :C) and 3p-hpRNA**

For intracellular stimulation with Poly(I:C) or with 3p-hpRNA, MEFs were seeded at $1.10^5$ cells per well in 12-well plates the day before stimulation, transfected with 1 μg/mL Poly(I:C) (InvivoGen Cat#vac-pic) or 0.5 μg/mL 3p-hpRNA (InvivoGen Cat#trl-hprna) using 5 μL Lipofectamine LTX and 1 μL for poly(I:C) stimulation or 0.5 μL for 3p-hpRNA stimulation of Plus Reagent (ThermoFischer Scientific Cat#15338100), according to the manufacturer's instructions. After stimulation, MEFs were incubated for 8 to 24 hours.

**RNA sequencing**

MEF RNA was prepared as described in the main text. RNA integrity and quantification were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). One microgram of high-quality RNA samples (RIN > 9.2) representing biological triplicates were submitted to Novogene for RNA-sequencing (Novogene Beijing, China). Poly-A selected RNA was used for paired-end library preparation and transcriptome sequencing. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's instructions. The library preparations were sequenced on an Illumina platform and paired-end reads were generated. The RNA-seq analysis was performed with Sequana 0.9.8 (1). In particular, we used the RNA-seq pipeline https://github.com/sequana/sequana_rnaseq) built on top of Snakemake 6.1.1 (2). Briefly, reads were trimmed from adapters using Cutadapt 2.7 then mapped to the Mus musculus genome assembly GCA_000001635.8 from NCBI using STAR 2.7.3a (3). FeatureCounts 1.6.4 (4) was used to produce the count matrix, assigning reads to features using corresponding annotation v92 from NCBI with strand-specificity information. Quality control statistics were summarized using MultiQC 1.6 (5). Clustering of transcriptomic profiles were controlled using a Principal Component Analysis (PCA). Differential expression testing was conducted using DESeq2 library 1.24.0 (6) scripts indicating the significance (Benjamini-Hochberg adjusted p-values, false discovery rate FDR < 0.05) and the effect size (fold-change) for each comparison.


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### Immunofluorescence antibodies

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### Western blot antibodies

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