Epididymal epithelium propels early sexual transmission of Zika virus in the absence of interferon signaling

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Recognition of Zika virus (ZIKV) sexual transmission (ST) among humans challenges our understanding of the maintenance of mosquito-borne viruses in nature. Here we dissected the relative contributions of the components of male reproductive system (MRS) during early male-to-female ZIKV transmission by utilizing mice with altered antiviral responses, in which ZIKV is provided an equal opportunity to be seeded in the MRS tissues. Using microRNA-targeted ZIKV clones engineered to abolish viral infectivity to different parts of the MRS or a library of ZIKV genomes with unique molecular identifiers, we pinpoint epithelial cells of the epididymis (rather than cells of the testis, vas deferens, prostate, or seminal vesicles) as a most likely source of the sexually transmitted ZIKV genomes during the early (most productive) phase of ZIKV shedding into the semen. Incorporation of this mechanistic knowledge into the development of a live-attenuated ZIKV vaccine restricts its ST potential.
The increasingly recognized ability of some arboviruses to utilize a non-vector-borne human-to-human mode of transmission via sexual contact\(^{1–6}\) presents new challenges to the understanding of virus maintenance in nature. To get ahead in developing preventive and mitigating measures to control viral outbreaks, extensive research to better understand such unusual viral tropism is needed. Zika virus (ZIKV) is a mosquito-borne flavivirus that recently emerged in outbreaks on the Pacific islands followed by a large-scale epidemic in the Central and South America (reviewed in ref. 7). Typically transmitted by infected *Aedes* mosquitoes, ZIKV has also been shown to disseminate among humans via male-to-female sexual contact\(^{8–10}\) (reviewed in refs. 5,11,12). The majority of infected men (>50%) shed ZIKV RNA in semen. The shedding peaks between 8 and 30 days after onset of the illness, followed by gradual viral clearance, which in some individuals takes up to 10 months\(^{13–15}\). Infectious ZIKV has also been recovered from a number of semen samples, although seminal clearance of the virus occurs faster than that of the viral RNA\(^{13,15,16}\).

Despite substantial research efforts, a clear understanding of the cellular and molecular mechanisms underlying ZIKV male-to-female ST is yet to emerge. A number of cell types within different parts of the male reproductive system (MRS) can support ZIKV replication, although susceptibility of these cells to ZIKV infection varies significantly among different MRS organs. In mice, ZIKV replication in testis and epididymis begins with viral infection of cells located in the interstitium of these organs, followed by robust replication in cells that constitute seminiferous tubules of the testis and in epididymal epithelium\(^{17–24}\). In the prostate, ZIKV targets the organ’s epithelial cells, although to a lesser extent than in the epithelium of the epididymis\(^{25,26}\). In mouse seminal vesicles, ZIKV replicates to a considerably lower titer than in the testis and epididymis\(^{20,21,26}\), however, specific cellular targets of ZIKV in this organ have not been characterized. ZIKV RNA and/or viral antigen have also been detected in many parts of the MRS of new-world and old-world nonhuman primates (NHPs)\(^{25,27–30}\). Studies in NHPs tend to indicate a higher load and longer persistence of ZIKV RNA in the testis and epididymis, as compared to other MRS organs\(^{28,30}\). This incriminates testis and/or epididymis as a possible source of sexually transmitted ZIKV, which is consistent with reports of a prolonged virus shedding (up to 60 days) in some infected NHPs\(^{31–33}\). However, a high variability of the results obtained in NHPs makes it challenging to identify the precise role that each individual MRS component plays in ZIKV infection. Moreover, ZIKV was also detected in the semen of vasectomized men\(^{34–36}\) and mice\(^{19}\), suggesting that infection of the accessory glandular organs of the MRS is sufficient to support ZIKV replication and its seminal shedding independently of testicular and epididymal infections.

Here, we aimed to elucidate the relative significance of different cell types within the MRS of mice with altered antiviral responses as a source of ZIKV viroins transmitted to the female sexual partner. For that, we used two independent yet complementary strategies that allowed us (i) to block ZIKV replication in various parts of the MRS by microRNA(miRNA)-targeting of viral genome and (ii) to trace the MRS tissue-dependent ZIKV progenies by the unique molecular identifiers (barcodes) incorporated into viral genome.

**Results**

**Organ-specific restriction of ZIKV replication in the MRS by miRNA targeting.** Presumably, all components of the MRS may be seeded by ZIKV from the blood, support virus replication, and contribute to the viral shedding into the final product of the MRS—the ejaculate. ZIKV replication can be selectively blocked in a tissue of interest by the modification of the viral genome with sequences complementary to a miRNA specific for (or highly enriched in) that tissue (i.e., miRNA-targeting)\(^{22}\). It was shown that the mir-202-5p (hereafter mir-202) is expressed in the seminiferous tubules of the testis (Sertoli and spermatogenic cells), but not in any other MRS tissue components (Fig. 1a)\(^{22,35–39}\). The precise role of mir-202 in testicular physiology has yet to be described. Conversely, the mir-141-3p (hereafter mir-141) is widely expressed in the epithidymal (epithelial cells), accessory glands (SV/P) (Fig. 1b), and the epididymis of the MRS ductal components such as vas deferens (Fig. S1), but not in the testis (Fig. 1b)\(^{22,35–42}\). Studies showed that mir-141 regulates maintenance of the epithelial cell phenotype by controlling the expression of E-cadherin\(^{35,43}\). This implies that mir-141 might be similarly expressed in all tissue components of the MRS that are comprised of the epithelial cells (i.e., epididymis, accessory glands, and ducts).

Previously, using 4–6 weeks old AG129 male mice that lack type I and type II interferon receptor (IFNR) genes, we showed that ZIKV genome targeting for mir-202 effectively restricts ZIKV replication in the cells located inside of seminiferous tubules, which blocks ZIKV migration from testis to epididymis\(^{22}\). Similarly, mir-141 targets restrict ZIKV infection of epididymal epithelium. Since most mouse strains reach sexual maturity after 6 weeks of age, in the present study, we used AG129 male mice of 10–20 weeks of age (hereafter, adult male mice). These mice were infected intraperitoneally (IP) with 10\(^6\) plaque forming units (pfu) of the mir-202-targeted [designated 2 × 202(T)] or mir-141-targeted [designated 2 × 141(T)] ZIKV constructs (Fig. S2), followed by analysis of viral replication in the MRS. We elected to dissect seminal vesicle and prostate as one specimen (designated as seminal vesicle/prostate or SV/P) since a precise anatomical separation of seminal vesicle from anterior prostate did not seem feasible and would be compromised by viral cross-contamination (Fig. S3). As a control, we infected mice with 2 × scr virus (contains scramble (scr) sequences at the sites of miRNA-targets), or with a parental ZIKV-NS3m virus (Fig. S2)\(^{22,45}\). Since miRNA-targeted viruses can accumulate escape mutants in the organs expressing a given miRNA, the regions containing scr sequences or miRNA targets were sequenced in all tissue-isolated viruses (with virus titers >10-fold over the limit of detection). If a deletion or a point mutation was detected in the region of miRNA target insertion, the titers for the stable (stb) or mutated (mut) viruses were presented separately.

Previously, we showed that in AG129 mice, the viremia with 2 × scr or ZIKV-NS3m virus peaks at 1 day post infection (dpi), and subsides rapidly thereafter\(^{22}\). At 1 dpi, mice inoculated with miRNA-targeted viruses developed a high level of viremia, which was comparable to that of 2 × scr-infected or ZIKV-NS3m-infected mice (Fig. 1c; p > 0.05, one-way ANOVA), suggesting that all viruses would have an equal opportunity to seed mouse tissues via a hematogenous route.

Seeding of ZIKV in the testis and epididymis begins with infection of the cells located in the interstitium of both organs\(^{17,22,24}\). Subsequently, infection progresses to the cells located inside the seminiferous tubules (expressing the mir-202) or epididymal epithelium (expressing the mir-141). It was shown that in the epididymis, progression of ZIKV infection from the interstitium to epithelium occurs ~2–4 days earlier than progression of infection to seminiferous tubules in the testis\(^{21,46–48}\). Replication of 2 × 202(T) and 2 × 141(T) viruses in the testis and epididymis (Fig. 1d, e) was consistent with the expression of mir-202 and mir-141 in these organs (Fig. 1a, b). Between 9 and 17 dpi, accumulation of 2 × 202(T)stb [but not 2 × 202(T)mut or 2 × 141(T)] was strongly inhibited in the testis as...
compared to $2 \times \text{scr}$. In contrast, accumulation of $2 \times 202(T)$ in the epididymis of mice was comparable to that of $2 \times \text{scr}$, and $2 \times 202(T)$ escape mutants were not detected in this organ. These results support the previous findings suggesting that ZIKV infection of the epididymis can occur via a hematogenous route independently of the testicular infection\textsuperscript{22}. The titer of $2 \times 141(T)$ stb in the epididymis was lowest at 6 dpi, but gradually increased from 9 to 17 dpi. Importantly, escape mutants of the $2 \times 141(T)$ virus [2 $\times 141(T)$mut in Fig. 1e] were detected only in the epididymis, but not in the testis of infected mice. The highest level
of 2 × scr replication in the epididymis occurred ~6 days earlier than in the testis (Fig. 1d, e). Since targeting of ZIKV genome for mir-141 does not block ZIKV replication in the testis (Fig. 1d), this suggests that accumulation of 2 × 141(T)stb in the epididymis between 9 and 17 dpi occurs due to the excurrent transport of the 2 × 141(T) from the testis (Fig. 1e)\(^\text{25}\). Escape mutations were not detected in the 2 × scr virus isolated from the testis and epididymis of any infected mice. Also, instability was not observed in the genome of 2 × 202(T) virus isolated from epididymis—the organ where mir-202 is not expressed (Fig. 1a). Similarly, mir-141 is poorly expressed in the testis (Fig. 1b), and all 2 × 141(T) genomes isolated form this organ remained stable (Fig. 1d). Together, these data demonstrate that escape mutations in the genome of 2 × 202(T) and 2 × 141(T) viruses are selected in a tissue-specific manner, suggesting that this phenomenon can be used to trace the tissue origin of ZIKV genomes transmitted from male to female mouse.

In the vas deferens, ZIKV can either be generated in situ or undergo transit with the flow of infected sperm from the 'upstream' organs (i.e. testis and epididymis, Fig. S3). Between 6 and 17 dpi, replication of only 2 × 141(T)stb in this organ was drastically inhibited. Escape mutants were detected only in mice infected with 2 × 141(T), but not with 2 × 202(T) virus (Fig. 1d), excluding testis as a likely source of the ZIKV in vas deferens. To identify the cell types supporting ZIKV replication in the epididymis and vas deferens, we compared distribution of ZIKV antigens by immunohistochemistry in mice infected with 2 × scr or 2 × 141(T) (Fig. 2). ZIKV-immunoreactivity (ZIKV-IR) was not detected in epithelial cells of vas deferens in any of the mice infected with either virus (Fig. 2f, i; n = 4 mice). In contrast, seven out of eight epididymides (87.5%) collected from four mice infected with 2 × scr virus were strongly ZIKV-positive (Fig. 2mb, m). This suggests that the high titer of 2 × scr in vas deferens (Fig. 1f) likely corresponds to the intraluminal virus derived from the epididymis. This is in agreement with sequencing results of 2 × 141(T) virus isolated from the different parts of the MRS at 17 dpi (Table S1). For instance, identical escape mutants were detected in the vas deferens and epididymis of two mice (mouse #3 and #4), whilst the virus isolated from the testis of these mice contained intact sequence of miRNA targets. ZIKV-IR was not detected in 87.5% of epididymides (7 out of 8 organs) collected from 4 mice infected with 2 × 141(T) at 9 dpi (Fig. 2c, m). In addition, growth of 2 × 141(T) virus in immortalized mouse distal caput epididymal epithelial cell line DC2 was significantly attenuated compared to 2 × scr virus (Fig. S4), confirming that mir-141 targeting strongly attenuates ZIKV infectivity for epididymal epithelium\(^\text{22}\). Detection of the ZIKV-IR unilaterally in the epididymis of one mouse (Fig. 2m, S5) likely corresponds to the emergence of an escape mutation in the mir-141-targeted virus under miRNA-mediated pressure.

Interestingly, miRNA targets in the 2 × 141(T) virus isolated from the testis, epididymis, and vas deferens of mouse #5 at 17 dpi remained stable (Table S1). This suggests that the ZIKV virus generated in the testis may also reach vas deferens with the effluent flow of the infected sperm. However, the escape mutations in the genome of the 2 × 202(T) virus, which were observed in the testis at 12 and 17 dpi [see 2 × 202(T)mut in Fig. 1d], were not detected in the epididymis and vas deferens of these mice (Fig. 1e, f). This implies that between 6 and 17 dpi trans-epididymal transport of ZIKV from the testis to vas deferens occurs quite inefficiently (as compared to the transport of the virus produced by epididymal epithelial cells), since this transport can be detected only when ZIKV infection of the epididymis is inhibited by mir-141 targeting of the viral genome.

Both 2 × scr and 2 × 202(T) replicated in the SV/P of AG129 mice, reaching peak titer at 9 dpi (Fig. 1g). We observed that 2 × scr virus primarily targets epithelial cells of the seminal vesicle (Fig. 2h, m); however, infection of the epithelial cells of the prostate occurred less frequently (Fig. 2k, m). ZIKV genome targeting for mir-141 effectively blocks infection of epithelial cells (Fig. 2i, l, m) and restricts virus replication in both organs (Fig. 1g). Escape mutations were detected in the SV/P at 17 dpi only in one out of five mice (see mouse #3) infected with 2 × 141(T) virus. The same escape mutants were also detected in the epididymis and vas deferens, but not in the testis of the mouse #3 (Table S1). This suggests that these escape mutants originated in the epididymis and were transported to the SV/P via vas deferens. Since the titer of the escape mutant in the epididymis of mouse #3 was considerably higher than that in the SV/P (mass of epididymis is ~0.1 g), a reverse spread of the mutant virus from the SV/P to the epididymis against the excurrent flow of sperm seems unlikely. This indicates that ZIKV escape mutant detected in the SV/P might be either of a local origin, or it could have been generated in the epididymis.

Together, these results indicate that with the exception of the testis, the epithelial cells expressing mir-141 are a common cell type capable of supporting productive ZIKV replication in the major MRS tissue components. Importantly, ZIKV infection of these cells can be restricted by mir-141 targeting of viral genome.
To establish the organ of origin of sexually transmitted virions within the MRS, we isolated viruses from the female serum (Fig. 3a) and compared viral sequences with those isolated from the MRS of the males involved in the mating. Scr sequences of $2 \times \text{scr}$ remained stable in all samples (female serum and MRS organs). All $2 \times 202(\text{T})$ viruses isolated from the serum of sexually infected females ($n = 9$) also contained intact miRNA targets (Fig. 3d). In contrast, only four male mating partners for those females ($n = 9$) contained stable $2 \times 202(\text{T})$ viruses in their testes at 17 dpi, and mean viral load in these testes

**Fig. 2** Genome targeting for mir-141 restricts ZIKV ability to infect epithelial cells of epididymis, seminal vesicles, and prostate. Adult AG129 mice were mock-inoculated (a, d, g, j; $n = 1$) or infected IP with $10^6$ pfu of $2 \times \text{scr}$ (b, e, h, k; $n = 4$) or $2 \times 141(\text{T})$ (c, f, i, l; $n = 4$) virus. Representative images of immunoreactivity (IR) for ZIKV antigen in the epididymis (a–c), vas deferens (d–f), seminal vesicles (g–i), and prostate (j–l) at 9 dpi (experiment was performed once). Scale bars: 50 µm.

**m** Summary of ZIKV-IR detection in the MRS of mice infected with $2 \times \text{scr}$ or $2 \times 141(\text{T})$ viruses as compared to mock. A paired mouse organs (epididymis, vas deferens, and seminal vesicles) were considered ZIKV positive (ZIKV+ mouse), if ZIKV+ cells were detected either unilaterally or bilaterally.
was significantly lower than that of 2 × scr virus (see 2 × 202(T) stb in Fig. 3e, p < 0.001, one-way ANOVA). In the tests obtained from the remaining five mice, the 2 × 202(T) virus acquired escape mutations (see 2 × 202(T)mut in Fig. 3e), which were associated with ~5000-fold increase in the virus titer in the tests as compared to the titer of 2 × 202(T)stb. However, these escape mutants were not detected in the female serum (Fig. 3d), which suggests that testicular infection of ZIKV does not contribute to the pool of the viruses transmitted from male to female mice.

At 17 dpi, the titers of 2 × 202(T)stb and 2 × scr viruses were very similar in the epididymis, vas deferens, and SV/P (Fig. 3f–h), suggesting that all these organs can be a source of 2 × 202(T)stb virus that was detected in the female serum (Fig. 3d). In contrast, the 2 × 141(T) virus was attenuated in these components of MRS (Fig. 1d–g). Only a single female (5%; n = 20) developed a detectable anti-ZIKV NA titer after mating with male infected with 2 × 141(T) virus (Fig. 3b, d). However, infectious 2 × 141(T) virus was not detected in the serum nor the brain of this animal, precluding subsequent analysis of the origin of the transmitted 2 × 141(T) virus in the MRS.

We also analyzed ST efficiency of the 2 × 202/141(T) virus (Fig. S2)22, which contains two target sequences for both mir-202 and...
mir-141. In contrast to the 2 × 202(T) or 2 × 141(T) constructs, presence of targets for both miRNAs in the 2 × 202/141(T) virus genome allows for the independent accumulation of escape mutants in the testis and in the mir-141-expressing epithelial cells within the MRS. Similar to the 2 × 141(T), transmission rate of the 2 × 202/141(T) virus was significantly attenuated (8.8%; n = 34) as compared to the 2 × scr (80%; n = 20; Fig. 3b, p < 0.001; Fishers’ exact test). Interestingly, 2 × 202/141(T) viruses isolated from the serum of all mating females contained deletions in the miRNA-targeting region (Fig. 3d, i). Analysis of viruses isolated from the MRS of the male mating partners indicated that it is unlikely that transmitted viruses were generated within the testis. The 2 × 202/141(T) virus isolated from the testis was either stable (see mating pair #3 in Fig. 3i) or the testicular virus titer was below the limit of virus detection (see mating pair #1 and #2 in Fig. 3i), and, therefore, their sequences were not assessed. In addition, an escape mutation in the 2 × 202/141(T) virus, which was isolated from serum of a female from mating pair #3 eliminated both targets for mir-141, but preserved one target for mir-202 (see Esc5 in Figs. 3i, S6). This suggests that replication of the ZIKV with such deletion in the 3’NCR likely remained restricted only in mouse testis, but not in the MRS cells expressing mir-141.

Escape mutants detected in the 2 × 202/141(T) virus isolated from females from mating pairs #2 and #3 matched exactly those found in the epididymis and vas deferens of their mating partners (Fig. 3i). Also, for the male from mating pair #3 the matching deletion was also found in the SV/P sample (see Esc5 in Figs. 3i, S6). Considering that (i) ZIKV infection of the cells of vas deferens occurs very inefficiently (Figs. 1 and 2) and that (ii) ZIKV generated in the epididymis can be transported to the SV/P with the flow of infected sperm through vas deferens (see mouse #3 in Table S1), the epididymis is the most likely source of the viruses transmitted from male to female mice (Fig. 3i).

The deletion in the 2 × 202/141(T) virus genome isolated from the female in mating pair #1 does not match the deletion found in the epididymis of their mating partner (Fig. 3i). It is possible that Esc1 could have been generated in the accessory glands or ducts of the MRS. Regrettably, these parts of the MRS of this animal were not preserved, precluding a more definitive analysis of the origin of the Esc1 mutant. It is also possible that Esc1 could have been generated in the epididymis but was subsequently washed out/replaced by another dominant escape mutant (Esc2) by 17 dpi.

Analysis of ZIKV shedding into the semen between 7 and 11 dpi. The low ST rate of viruses containing targets for mir-141 (Fig. 3b) may be attributed to an insufficient shedding of these viruses into the semen. Alternatively, inefficient ST of the 2 × 141(T) virus might be attributed to its attenuated vaginal infection and replication in the FRS (Fig. S7). To directly test the effect of mir-141 targeting on ZIKV shedding into the semen, we infected AG129 male mice with 2 × scr or 2 × 141(T) virus, and at 7 dpi each male was placed with two uninfected female CD-1 mice (to increase the probability of mating). Mice were allowed to mate for 4 days (Fig. 4a) instead of 10 days as was done in the study depicted in Fig. 3a (we noticed that most male mice used in that experiment copulated with females no later than 4–5 days of cohabitation). Female CD-1 mice that developed vaginal plugs were sacrificed to determine viral load in the FRS by titration in Vero cells. A mean ZIKV titer was reported if both CD-1 females from the same cage developed vaginal plugs. None of 2 × 141(T)-infected AG129 males (n = 15) deposited ZIKV+ semen into FRS of CD-1 females (Fig. 4b). In comparison, 70% of mating males (n = 10) infected with 2 × scr virus were able to transmit variable viral loads (p > 0.001, 2-tailed Fisher’s exact). This experiment reinforces the notion that the cells of epithelial origin in the MRS play a determining role in the sexual transmissibility of the ZIKV, since the blocking of ZIKV replication in such cells by targeting the viral genome for mir-141-mediated degradation efficiently inhibits viral shedding into semen and prevents the ST.

Tracing individual ZIKV genomes during ST of the barcoded ZIKVs from male to female mice. Mir-141 miRNA is not confined to the epithelial cells of the MRS, but it is also expressed in a variety of other tissues. This suggests that infectivity and titers of the mir-141-targeted ZIKV clones may become reduced before the seeding of the MRS. In turn, this may translate into a diminished shedding of the mir-141-targeted viruses into the ejaculate and result in inefficient ST. Therefore, we sought to apply a different methodology to verify our finding that the ZIKV shedding into the ejaculate and subsequent ST are defined by ZIKV replication in the cells of the epithelial origin in the MRS. One way of doing this is to analyze the genetic bottlenecks imposed onto ZIKV populations passing through the anatomical barriers and/or specific cell types of the MRS.

For that, we modified 2 × scr virus to generate a miRNA-target-free library of ZIKV genomes containing a stretch of 11 random nucleotides (designated n11). The n11 sequences partially substitute one of the two scr sequences in the 2 × scr genome (Fig. 5a), which can serve as ‘barcode’ tags for differentiating viral genomes. A plasmid library consisting of ~9.3 × 104 clones was transfected into Vero cells, generating ZIKV-lib/n11 virus library. Male AG129 mice were infected with ZIKV-lib/n11 viruses followed by a semen shedding experiment as depicted in Fig. 5b. Unlike the study depicted in Fig. 4a, in this experiment male mice
were sacrificed immediately after the vaginal plug was detected in one of the two mating partners. This modification allows to minimize the time interval between semen deposition into FRS and the MRS collection. The MRS was also collected from several males who did not deposit semen to female partners by 11 dpi.

We reasoned that similar to a simian immunodeficiency virus (SIV) infection of MRS in cynomolgus macaques\(^2\), the hematogenous dissemination of ZIKV from the infectious site should be followed by virus seeding and replication in the organs of the MRS\(^2\). This process is associated with genetic bottlenecks imposed by anatomical barriers and/or changes in the target cell types. This should translate into a reduction of a genome targeting for mir-141 restricts ZIKV shedding into the semen between 7 and 11 dpi. a Experimental design. Male AG129 mice were infected IP with 10^6 pfu of 2 * scr or 2 * 141(T) virus, and between 7 and 11 dpi mice were allowed to mate with two CD-1 females in the individual cages. b Mean virus titer ± SD in the ejaculate of AG129 mice that was deposited into FRS of CD-1 mice. Each dot represents an average ZIKV titer in the FRS of CD-1 females that developed vaginal plug (one or two females per cage) after mating with ZIKV-infected male partner. The dashed line indicates the limit of virus detection: 1.0 log_{10}(pfu/g of FRS). Differences between viral titers in the FRS were compared using two-tailed Mann-Whitney test.

Next, we tested whether ZIKV-lib/n11 viruses can be used to identify previously established ZIKV migration routes from the testis to epididymis\(^2\). For that, we identified all barcodes in reads_{testis} that are common between viruses in the testis and the organ in question, and calculated combined frequencies of common barcodes (CFCB) among all barcoded viruses which were found in these two particular mouse organs. There was almost no similarity among barcodes in reads_{testis} detected in the brain and testis (Fig. 5e), reflecting independent ZIKV infection of these organs from a pool of viruses with a high heterogeneity of n11 region. In contrast, mean CFCB value for the testis–epididymis pair was 58.2%, which was significantly higher than that of the brain–testis pair (Fig. 5e, \(p < 0.0001\)). This strongly suggests ZIKV migration between these two organs. Importantly, there were no significant differences between the CFCB values for viruses detected in the testis–vas deferens or testis–SV/P pairs, as compared to the brain–testis pair (Fig. 5e; \(p > 0.999\) and \(p = 0.622\), respectively). The absence of a high similarity in barcodes between the testis and vas deferens at 11 dpi suggests that ZIKV generated in the testis has not yet completed a full transition through the epididymis to be expelled into the vas deferens. These findings are in agreement with the kinetics of 2 * 141(T)stb accumulation in the MRS (Fig. 1d–f) showing that between 6 and 12 dpi, testis-derived 2 * 141(T)stb is present in the epididymis but not in the vas deferens.

Next, we analyzed whether ZIKV generated in the epididymis can be detected in the downstream MRS organs. Almost no similarity was detected among barcodes in reads_{vas} isolated from the epididymis and brain (Fig. 5f). In contrast, mean CFCB value for the epididymis–vas deferens pair was 51.1% (Fig. 5f), which was significantly higher compared to the CFCB values for the epididymis–brain or epididymis–SV/P pair (Fig. 5f). Perhaps not surprisingly, these results are in line with the MRS physiology and our earlier detection of the epididymis-generated miRNA-targeted ZIKV in the vas deferens (Fig. 3i, Table S1).

Next, we asked whether the barcode compositions of the viruses detected in the major anatomical component of the MRS would allow identification of the tissue/cellular sources of sexually transmitted ZIKV. The n11 region of ZIKV-lib/n11 viruses in the ejaculate that was recovered from the FRS of CD-1 mice \((n = 4)\) after mating (Fig. 5b) was sequenced and compared to the n11 region of ZIKV-lib/n11 viruses isolated from the MRS organs. None of the seven dominant barcodes present in the ejaculate could be traced back to the testis (not detected in the reads_{vas} and frequency < 0.1%) (Fig. 6). In contrast, all seven dominant
barcodes present in the ejaculate were detected in the epididymis (detected in the reads at a frequency of 0.7–6.8%). Some of these barcodes were also detected at high frequencies in the vas deferens and SV/P, most likely reflecting the excurrent transit and potential seeding of these downstream components of the MRS (see Fig. 5c for the anatomical/physiological schematic) with the sperm and/or epididymal secretions. Together, these findings identify the epididymis (specifically, the epididymal epithelial cells) as the main contributor of ZIKV output to the ejaculate and subsequent ST of the virus during the early course of infection (10 dpi).

Restrained male-to-female ST of live-attenuated ZIKV vaccine candidate. We previously reported the construction of a live-attenuated ZIKV vaccine candidate virus C/3’NCR-mir(T) that
**Fig. 5** Construction and application of a library of ZIKV genomes with unique molecular identifiers (barcodes). a Construction of a barcoded library of ZIKV genomes using genetic background of 2× scr virus. Green boxes and letters indicate scr sequences. Red letters highlight a stretch of 11 random nucleotides. b Experimental design of a semen shedding study using AG129 mice infected with a barcoded ZIKV library. c Hypothetical reduction of barcoded virus diversity during hematogenous dissemination of the ZIKV-lib/n11 and shedding of the viruses into FRS with ejaculate. d Mean number of barcodes in the reads90 ± SD of ZIKV-lib/n11 virus population isolated from: mouse serum at 1 dpi (n = 7); mouse organs at 10–11 dpi (n = 7); or from the inoculum that was used for mouse infection (n = 1). Difference between the number of barcodes in reads90 in the serum and mouse organs was compared using one-way ANOVA with multiple comparison adjustment (Dunnett’s test). e, f Mean combined frequencies of common barcodes (CFCB) among reads90 (%) ± SD for ZIKV-lib/n11 viruses recovered from the testis (e) or epididymis (f) and various organs of AG129 male mice (n = 7). Differences between common barcode frequencies among organ pairs were compared using one-way ANOVA with multiple comparison adjustment (Tukey’s test). *****p < 0.0001.

**Fig. 6** Tracing individual ZIKV genomes during male to female ST of ZIKV-lib/n11 viruses. Four male AG129 mice infected with ZIKV-lib/n11 deposited ZIKV+ ejaculate into FRS of CD-1 mice at 10 dpi (see Fig. 5b). For each mating pair, a top panel shows the frequency of barcodes that together constitute reads90 for the individual male or female organ. Each color represents a unique barcode. Only barcodes with frequencies of >1% in the FRS are shown as blue or red colors. Sequences and frequencies of these barcodes are presented in the middle panels. "Yes" or "No" denote whether the barcodes were among the reads90 for the given organ. Bottom panels for each mating pair show the titers of ZIKV-lib/n11 viruses in mouse organs collected at 10 dpi. Virus titers in the FRS (ejaculate), testis, SV/P, and brain of males are expressed as log10(pfu/g). Virus titers in the epididymis and vas deferens are expressed as log10(pfu/mouse).
contains multiple miRNA targets (Fig. 7a), including two targets for the testiculamir-202 miRNA, two targets for the epithelial mir-141 miRNA, and targets for mir-9 and mir-124 miRNAs that are highly expressed in the central nervous system (CNS). These targets are inserted into a duplicated C-gene region (dCGR) and 3′NCR of the parental ZIKV-NS3m virus genome. To assure that incorporation of these miRNA targets eliminates any potential of the vaccine candidate to be sexually transmitted, we compared the male-to-female transmission rate of the C/3′NCR-mir(T) with that of C/3′NCR-scr and 3′NCRΔ20 viruses (Fig. 7a) using the experimental design depicted in Fig. 3a. Both C/3′NCR-scr and 3′NCRΔ20 viruses (Fig. 7a) were constructed using the genetic background of ZIKV-NS3m22 and were used here as controls; C/3′NCR-scr contains scr and other random sequences inserted into the dCGR and 3′NCR, while 3′NCRΔ20 is attenuated by a 20 nt deletion in the 3′NCR22,52,53, a mechanism of attenuation that is different from the miRNA targeting.

We report a zero ST rate for the vaccine candidate C/3′NCR-mir(T) (n = 15 mating pairs), while the control viruses were sexually transmissible, although at the rate which was lower than that of 2×scr virus (Fig. 7b). Furthermore, the C/3′NCR-mir(T) virus was undetectable in the MRS tissues at 17 dpi (Fig. 7c–e). This is in contrast to the control viruses, both of which produced relatively high virus loads in the testis, epididymis, vas deferens, and less so in the accessory glands. These results support the strategy for using miRNA targeting to inhibit ZIKV tropism for the MRS, thereby eliminating any potential of the virus to be sexually transmitted. This reinforces the safety of the C/3′NCR-mir(T) vaccine candidate virus.

**Discussion**

To delineate the time frame when male mice would have the highest potential for ST of genetically modified ZIKV clones, we analyzed the infection events/kinetics occurring in the MRS of adult AG129 mice from 6 to 17 dpi. The control 2×scr virus reaches the peak of its replication in the testis at 12 dpi, in the epididymis at 6 dpi, and in the SV/P at 9 dpi, followed by a decline of viral titers in all these MRS components by 17 dpi (Fig. 1). This suggests that ZIKV virions with highest potential for ST are likely produced in the MRS prior to 17 dpi, justifies our decision to terminate ST studies at 17 dpi (Fig. 3a). Subsequently, we observed that 2×scr virus transmission to female AG129 mice occurs mostly before 12 days post male mouse infection. This allowed for a considerable reduction of the mating time for semen shedding studies [7–11 days post male mouse infection (Figs. 4a, 5b)]. This particular interval (7–11 dpi) coincides with the peak of virus shedding into the semen of immunodeficient mice [7–12 dpi19] and human semen [7–11 days post the onset of ZIKV symptoms observed for natural isolates of ZIKV49].

It is important to note that shedding of infectious ZIKV virions into mouse semen does not stop abruptly at 11 dpi, but continues up to 22 dpi, although at a much lower rate19. This suggests that additional studies may be needed to elucidate the source of infectious ZIKV that is sexually transmitted during the late phase of viral infection (12–21 dpi), as well as the source of non-infectious ZIKV RNA, which can persist in mouse and human semen for months after the onset of ZIKV symptoms13,19,49.

There are, however, several limitations of our experimental model...
(discussed below) that precluded us from addressing these questions in this study.

Role of the epididymal epithelium as a source of the ZIKV virions/genomes secreted into the ejaculate. The results of two independent yet complementary approaches used in this study identified the epithidymis as a leading source of the sexually transmitted ZIKV genomes during the early phase of infection (7–11 dpi). This conclusion is based on the several lines of evidence. First, the epithelial cells of vas deferens were refractory to ZIKV infection (Fig. 2e, m), making the vas deferens an unlikely source of the de-novo produced ZIKV virions shedded into the ejaculate. Second, at the time of virus shedding into the ejaculate and sexual transmission (9–11 dpi), most of ZIKV virions were likely generated by the epididymis, but not testis or SV/P. This is consistent with our observations that the peak titer of 2 × scr virus in the epididymis was ~300 fold higher than that in the SV/P, when normalized by mass (Fig. 1e, g). Furthermore, the peak of 2 × scr replication in the epididymis was reached at least 6 days earlier than that in the testis and 3 days earlier than that in the SV/P (Fig. 1). Third, the restriction of ZIKV replication in the epididymal epithelium by mir-141 targeting of viral genome (ref. 22 and Figs. 1e, 2c, 2m in this study) correlates with an attenuated transmission of 2 × 141(T) and 2 × 202/141(T) viruses from male to female AG129 mice (Fig. 3b), which is also in agreement with a reduced shedding of the 2 × 141(T) virus into the ejaculate (Fig. 4b). Finally, analysis of the 2 × 202/141(T) virus escape mutants isolated from the female of AG129 mice (Fig. 3i), as well as analysis of the barcode sequences of the viruses deposited into FR5 of CD-1 mice (Fig. 6), demonstrated that sexually transmitted ZIKV genomes could be traced only to viruses that were found in the epididymis of a mating male partner, but not in any other MRS organs. These findings are in concordance with earlier reports that suggested a temporal correlation between ZIKV infection of the epididymal epithelia and shedding of ZIKV into mouse semen19,21,26. Together, our results demonstrate that the ZIKV that was sexually transmitted between 7 and 11 dpi was primarily produced by the cells of epididymal epithelium. These types of cells may secrete Zika virions into the lumen of epididymal tubules in a cell-free form21, and these virions, in turn, are flushed out of the epididymis during the emission phase of the ejaculation (see Fig. 5c for schematics) and eventually expelled into the ejaculate and become sexually transmitted.

An important limitation of our immunodeficient AG129 mouse model of ZIKV infection is that it may not accurately recapitulate ZIKV pathogenesis in the MRS of humans or non-human primates (NHP) (reviewed in ref. 13). It is possible that the aberrant type I and II IFN responses in this model may render the epididymal epithelium more susceptible to ZIKV, leading to an overestimation of the contribution of these cell types to sexual transmission of ZIKV. Therefore, more studies using an immunocompetent mouse model34,35 or NHPs are needed to confirm the role of the epididymis in ZIKV shedding into semen during an acute infection.

Testicular ZIKV infection does not contribute to the early (prior to 12 dpi) shedding of ZIKV into the semen and ST of the virus. Between 7 and 17 dpi, natural ZIKV isolates replicate efficiently in the testis of both the immunodeficient and immunocompetent mice17–20,22,26,35. The genetically modified ZIKV clones used in this study (2 × scr, 2 × 141(T)) also attained high viral titers in the testis (Fig. 1d). However, we did not detect any evidence implicating testis as a source of sexually transmitted ZIKV genomes. For instance: (i) reduced accumulation of 2 × 202 (T)stb virus in the testis was not associated with a corresponding reduction in the ST rate of the virus from male to female AG129 mice (Fig. 3b, e); (ii) while escape mutants of the 2 × 202(T) virus that lost both mir-202 targets replicated efficiently in the testis, these mutants were not transmitted to the female mating partners (only viruses with intact miRNA targets were detected in the serum of these females) (Fig. 3d); (iii) barcode sequences of the ZIKV-lib/n11 viruses deposited into FR5 of CD-1 mice at 10 dpi did not match sequences found in the testis of their male mating partners (Fig. 6).

Previously, using miRNA-targeted viruses, we showed that ZIKV disseminates from the testis into the epididymis of young (4–6 weeks old) AG129 mice by 12 dpi22. Here, using older (>10 week old) mice infected with ZIKV-lib/n11 viruses, we demonstrated that efficient ZIKV migration between these parts of the MRS occurs as early as 10 dpi (Fig. 5e), confirming that ZIKV can exit the testis with the efferent flow of sperm. However, barcodes of the ZIKV-lib/n11 viruses that were abundant in the testes (testis-specific barcodes) were not detected among viruses isolated from the vas deferens (Fig. 5e). This suggests that migration of testis-specific ZIKV virions through epididymis is either blocked or requires a longer time to complete, attenuating the shedding of testis-specific ZIKV-lib/n11 virions into the semen prior to 12 dpi. This may be similar to other viruses. For example, seminal SIV has been shown to originate from multiple genital organs of NHPs and migrate through the testis–epididymis–vas deferens axis during a long-term chronic infection31,32.

In mice, under normal physiological conditions, it could take up to 9 days for maturing spermatozoa to pass through the entire epididymis50. Assuming that a secretion of ZIKV virions from the testis into epididymis begins at ~9 dpi (see virus 2 × 141(T)stb in Fig. 1d) and that migration rates of sperm cells and ZIKV virions through epididymis might be comparable, then the testis-produced ZIKV virions should not reach vas deferens prior to 18 dpi. Since the semen shedding and ST experiments in this study were terminated at 11 and 17 dpi, respectively, it is possible that there was not enough time for the testis-specific ZIKV virions to complete the passage through the epididymis to be emitted into the vas deferens. Alternatively, ZIKV infection may reduce the serum testosterone levels in mice18,20,46, which would enhance spermatozoa migration through the epididymis57,58, shortening the time for the testis-produced ZIKV virions to be expelled with the ejaculate. It also remains to be investigated whether the ZIKV-induced epididymal inflammation and infiltration of leukocytes into the lumen of epididymal tubules18,20,21 may affect the course of trans-epididymal migration of the testis-derived ZIKV virions.

Future studies are needed to elucidate the fate of the testis-derived ZIKV virions during their transition through the epididymis and the rest of the MRS, and whether these virions can be transmitted to the female partner after 11–17 dpi. However, unrestricted replication of the natural isolates of ZIKV19,59–61 and ZIKV clones that were utilized in this study (see Fig. 6 and ref. 22) in the CNS of AG129 mice results in a considerable mouse mortality after 14 dpi. This prevented us from analyzing the ZIKV pathogenesis in the MRS during the late phase of viral infection. Substantial alterations to experimental approaches would need to be made to increase mouse survival without affecting viral replication in the MRS and sexual behavior of infected animals. One possibility for reduction of ZIKV neurovirulence in AG129 mice would be to incorporate into ZIKV genome additional miRNA targets that are selectively expressed in the mouse CNS, but not in the MRS52,62. It would also be interesting to evaluate ZIKV pathogenesis in the MRS of an immunocompetent host (e.g., NHPs and immunocompetent...
mouse models, which do not develop neuroinvasive disease and may better recapitulate ZIKV infection in humans.

ZIKV infection of the MRS accessory glands may play a supplementary role in the ST of the virus. Natural ZIKV isolates can replicate in the SV/P of mice and non-human primates. However, our study suggests that these accessory glands of the MRS mostly play a supplementary role in the ST of the virus. They were able to support a relatively low-level (compared to the tests and epididymis) replication of our model ZIKV (2 × scr), mainly in the epithelial cells. As we anticipated, targeting of ZIKV genome for epithelial miRNA mir-141 effectively restricted ZIKV replication in the SV/P, confirming the epithelial tropism of ZIKV. However, these accessory glands, in addition to the epididymis, may also contribute ZIKV output to the ejaculate, albeit more variably. One limitation of our experiment with the bar-coded ZIKV is that we cannot exclude the possibility of contamination of the SV/P samples by the content of the adjacent vas deferens ampulla where the infected sperm could be stored downstream of the epididymis. These findings, together with the notion that the vasectomy attenuates shedding of infectious ZIKV virions and non-infectious viral RNA into seminal fluids of mice, suggest that MRS accessory glands may play only supplementary role in the ST of the virus under normal physiological conditions. Nevertheless, it is important to stress that this does not diminish the risk of ST of ZIKV in the vasectomized cases, which can potentially occur outside the acute phase of ZIKV infection.

Abolishment of the epithelial tropism of ZIKV reinforces the safety of a live attenuated vaccine candidate. A long-term persistence of ZIKV in the MRS and its propensity for horizontal transmission warrant the effort to eliminate these attributes in a live-attenuated ZIKV vaccine. Here, we demonstrate that ZIKV genome targeting for mir-141 restricts virus infection of the epithelial cells in the epididymis, seminal vesicles, and prostate. It also directly prevents ZIKV shedding into the semen, blocks ST, and attenuates to some extent ZIKV replication in the female reproductive system (Figs. 1–4, S7). Targets for this miRNA constitute an important attenuating component of our previously characterized ZIKV vaccine candidate C/3’NCR-mir(T) virus. Not surprisingly, male AG129 mice infected with this virus were not capable of transmitting the virus to the mating females, and the infectious virus was not detected in the epididymis and SV/P of male mice at 17 dpi (Fig. 7d, f). As discussed above, it remains unclear whether the natural isolates of ZIKV produced in the tests could be sexually transmitted by infected males after 17 dpi. However, targets for the tests-expressed mir-202 incorporated into the genome of the C/3’NCR-mir(T) vaccine candidate virus appear to be sufficient to completely abolish the testicular tropism of the virus (Fig. 7c)49, ensuring that transmissibility of vaccine candidate virus(es) containing targets for miRNAs mir-202 and mir-141 through sexual contact will remain completely restricted.

Methods

Statement of compliance. All experimental protocols were approved by the NIH Institutional Biosafety Committee. All animal study protocols were approved by the NIAID/NIH Institutional Animal Care and Use Committee (IACUC) and performed in compliance with the guidelines of the NIAID/NIH IACUC. The NIAID DIR Animal Care and Use Program acknowledges and accepts responsibility for the care and use of animals involved in activities covered by the NIH IACUC’s Animal Welfare Assurance D16-0062, last approved 6/10/2019.

Plasmids and viruses. Construction of infectious cDNA clones encoding ZIKV-NS3m, 2 × scr, 2 × 202(T), 2 × 141(T), 2 × 202/141(T), C/3’NCR-mir(T), C/3’NCR-scr, and 3’NCR2A20 viruses has been reported previously22,45. Complete sequences of these plasmids are available from the authors upon request. To construct ZIKV-lib/n1 plasmid library, we amplified 846 bp DNA fragment (Fig. 5a, BsaHII-NsiI fragment) corresponding to the 3’ terminal part of NS gene and 5’ end of the 3’ NCR of ZIKV viruses using Phusion® High-Fidelity DNA Polymerase (New England Biolabs [NEB], MA), a primer pair ZV-9574-F and Library-R [Table S2, Integrated DNA Technologies, IA]), and 2 × scr plasmid as a template. Degenerated primer Library-R contains a stretch of 11 randomized nucleotides, which partially substitute scr sequences located at the 5’ terminus of the NCR of the 2 × scr virus (Fig. 5a). The amplicon was digested with BsaHII and NsiI endonucleases, followed by transformation of E. coli cells, with the selection reaction. Colonies of E. coli (n ~ 93,000) containing recombiant DNA were propagated for 18 h at 37 °C in 30 agar plates (Falcone, 100 × 15-mm style) containing 50 µg/mL of ampicillin. To validate cloning efficiency, cells from eight randomly selected colonies of E. coli were amplified, and plasmid DNAs were analyzed by the sequencing of the 3’NCR of ZIKV cDNA (Fig. S8). Each agar plate containing E. coli colonies was washed twice with 5 mL of LB broth supplemented with 50 µg/mL ampicillin. Washing broth from all plates was combined into single flask, incubated in bacteriological shaker for 3 h at 37 °C to 200 rpm, followed by plasmid extraction using Endo Free Plasmid Maxi kit (Qiagen, Germany). Plasmid integrity was verified by restriction endonuclease digestion and Sanger sequencing.

Recovery of viruses from infectious cDNA clones. Vero cells (Cercopithecus aethiops kidney) were maintained at 37 °C and 5% CO₂ in Opti-Pro medium (HyClone, UT) supplemented with 4 mM L-glutamine. Recovery of ZIKV-NS3m, 2 × scr, 2 × 202(T), 2 × 141(T), 2 × 202/141(T), C/3’NCR-mir(T), C/3’NCR-scr, and 3’NCR2A20 viruses was performed using plasmid DNA transfection method22,45. Briefly, for each infectious clone 2.5 µg of plasmid DNA was transfected into 1.5 × 10⁶ Vero cells seeded into one 12.5-cm² flask using Lipofectamine 2000 transfection reagent (Invitrogen, CA). To achieve high ‘barcoding’ heterogeneity, ZIKV-lib/n1 plasmid library was transformed into four 25-cm² flasks of Vero cells using Lipofectamine 3000 reagent (Invitrogen, CA), which provides superior efficiency of plasmid DNA transfection as compared to Lipofectamine 2000 reagent. For each flask, we seeded 3 × 10⁶ Vero cells in DMEM supplemented with 10% FBS (HyClone laboratories, UT) and 1% penicillin–streptomycin–glutamine solution (Invitrogen, CA). Next morning DMEM was replaced with Opti MEM (Invitrogen, CA), and cells in each flask were transfected with 8 µg of ZIKV-lib/n1 plasmid DNA according to manufacturer’s instructions. At day 3 post transfection cell culture supernatants from all four of these flasks were combined, clarified by 5 min centrifugation at 2000 × g, then inoculated into 1× 10⁶ T7-infected Vero cells, which were grown in 100-cm² flasks using 1× SPG solution (218 mM sucrose, 6 mM L-glutamic acid, 3.8 mM KH₂PO₄, 7.2 mM K₂HPO₄, pH 7.2)48, aliquoted, and stored at −80 °C. Titers of recovered viruses was determined by plaque assay in Vero cells45. Viruses recovered after transfection of recombinant ZIKV clones into Vero cells were used in all animal experiments without additional propagation.

Mouse strains. A colony of AG129 mice was maintained at the NIAID/NIH animal facility at 20.6–23.9 °C, 30–70% relative humidity under a 14 h light and 10 h dark photoperiod. The colony was originally established from a breeding pair purchased from Marshall BioResources. Female CD-1 were purchased from Taconic Farms.

Growth kinetics of recombinant ZIKV in the tissues organs of adult male AG129 mice. L-15 media (Invitrogen, CA) supplemented with 1× SPG solution (L-15/1×SPG) was used to dilute ZIKV to a concentration of 10⁵ pfu/mL, and male AG129 mice (10–20-week-old) were inoculated IP with 0.1 mL of each virus (dose: 10⁶ pfu/mouse). High virus dose and IP route of inoculation were chosen to compensate for moderate attenuating effect associated with insertion of heterologous sequences (such as miRNA targets) into 3’NCR of ZIKV genome22,65. Mice were bled at 1 dpi and euthanized at 6, 9, 12, and 17 dpi. To minimize total number of animals used in this study, organs of MRS for time point 17 dpi were dissected from male mice that participated in the 5 dpi studies with female mice (see below). Mouse serum and MRS organs (a pair of testes, pair of epididymis, pair of vas deferens, and combined seminal vesicles and prostate (SV/P) specimen) were harvested and stored at −80 °C. The SV/P specimen consisted of a pair of seminal vesicles, an anterior prostate and a ventral prostate (Fig. S3). ZIKV titers in mouse tissues was determined by plaque assays in Vero cells using duplicate wells of 24-well plates as was described previously22,63. Light weight of vas deferens and significant variations in the amount of fat tissue collected during dissection of epididymis compelled us to perform normalization of ZIKV titer not to a pfu per gram of tissue, but rather to pfu per whole organ. For that epididymis and vas deferens were not weighted but homogenized in 1 mL of L-15/1×SPG solution. In contrast, testes and SV/P were weighed and triturated in 9 volumes of L-15/1×SPG solution (making 10% organ homogenate). Viral titers in these organs were normalized to pfu per gram or tissue. Serum was diluted in volumes of L-15/1×SPG, followed by Vero cells titration. At 5 dpi, virus-infected Vero cell monolayers were observed under light microscope for ZIKV-induced cytopathic effect (CPE). If the titrated sample induced CPE (plaque formation), the overlay medium (Opti-MEM containing 1%
methylcellulose (Invitrogen), 2% heat-inactivated FBS, 4 mM L-glutamine) was aspirated from duplicate wells of Vero cells infected with lowest dilution of titrated specimen. The number of ZIKV virions in the supernatant was determined by plaque assay in Vero cells with washed with 0.25 mL of Opti-Pro. Washing medium from duplicate wells was combined together and 0.14 mL of it was used for the QIAamp Viral RNA Mini kit (Qiagen). Subsequently, Vero cells in all wells of the 24-well plate were fixed with 100% methanol and stained with crystal violet.

**ST of ZIKV from male to female AG129 mouse.** Male AG129 mice (10–20 weeks-old) were infected IP with 10⁶ pfu of ZIKVs. At 7 dpi, each infected male was placed into separate cage containing a single non-infected AG129 female mouse. Mice were allowed to mate for 10 days. At 3, 5, and 10 days of mating females were bled, and virus titer in the serum was determined by plaque assay in Vero cells. At 17 dpi, male mice were sacrificed, and MRS tissues were collected and stored at −80°C. Female mice were returned to cages and monitored for signs of neurological paralytic for 8 days (28 days post mating). Brains from female mice that succumbed to paralysis were collected, and ZIKV titers in the brain homogenates were determined in Vero cells. Blood from the remaining (healthy) female mice was collected at 28 days post mating, and a titer of ZIKV-specific NA in the serum was determined using PRNT₅₀ assay described previously⁹⁸. The female was considered infected if: (i) ZIKV-specific NA were detected in the female serum and/or (ii) infectious ZIKV was detected in the serum or brain of the female by plaque assay in Vero cells.

The titer of 2 × 10⁵ pfu, 2 × 10⁴(T), 2 × 10³(T), and 2 × 10²/141(T) viruses in the organs of MRS was determined only for those male mice that transmitted the virus to females. In addition, if the male virus was also detected in the serum and/or the brain of this female by plaque assay in Vero cells. If serially diluted (titrated) male or female sample (organs or serum) induced CPE in Vero cells seeded in 24-well plate, at 5 dpi viral RNA was extracted from titration plate from each cell which was infected with the lowest dilution of titrated specimen (contains highest number of ZIKV plaques) as described above. The titer of the C/3′NCR-mir(T) in the organs of MRS at 17 dpi was determined for all male mice (n = 15) participating in the mating experiment, while the titer of C/3′NCR-scr and 3′NCR20 viruses in the MRS at 17 dpi was determined only for 10 and 5 randomly selected male mice, respectively.

**Replication of recombinant ZIKVs in female AG129 mice following intravaginal exposure.** Intravaginal infection of 10–20-week-old AG129 mice with ZIKVs was performed according to previously described protocol⁹. It was shown that mouse FR5 is the most susceptible to ZIKV infection during progesterone-high diestrus phase of estrous cycle⁹. To ensure synchronization of estrous cycle at diestrus phase, all females were subcutaneously injected with 0.1 ml (400 mg/mL) of Depo-provera 7 days prior to virus exposure (Pfizer, NY). Immediately prior to inoculation, mucus from mouse vaginal cavity was removed with sterile cotton swab moistened with PBS, followed by injection of 10 μl of diluted L-15/1×SPG solution as described above followed by plaque assay in Vero. In addition, if the male virus was also detected in the serum and/or the brain of this female by plaque assay in Vero cells. If serially diluted (titrated) male or female sample (organs or serum) induced CPE in Vero cells seeded in 24-well plate, at 5 dpi viral RNA was extracted from titration plate from each cell which was infected with the lowest dilution of titrated specimen (contains highest number of ZIKV plaques) as described above. The titer of the C/3′NCR-mir(T) in the organs of MRS at 17 dpi was determined for all male mice (n = 15) participating in the mating experiment, while the titer of C/3′NCR-scr and 3′NCR20 viruses in the MRS at 17 dpi was determined only for 10 and 5 randomly selected male mice, respectively.

**Studies of ZIKV shedding into the semen of AG129 mice.** Semen shedding experiments were conducted using CD-1 strain of female mice. In contrast to AG129 strain, CD-1 mice have intact IFN-signaling pathway and are resistant to ZIKV infection⁹. Inability of CD-1 to propagate ZIKV in their FR5 makes them a good source for ZIKV studies. In the experiment-1, 10–20-week-old male AG129 mice aged 10–20 weeks was infected IP with 10⁷ pfu of 2 × 10⁵(T) virus (n = 20 mice/virus). At 7 dpi each infected male was placed into separate cage containing two non-infected female CD-1 mice. Mice were allowed to mate for 4 days. Females were observed twice a day (morning and afternoon) for 8 days (28 days post mating). Brains from female mice that succumbed to paralysis were collected, and ZIKV titers were determined only for those male mice that transmitted the virus to females. In addition, if the male virus was also detected in the serum and/or the brain of this female by plaque assay in Vero cells. If serially diluted (titrated) male or female sample (organs or serum) induced CPE in Vero cells seeded in 24-well plate, at 5 dpi viral RNA was extracted from titration plate from each cell which was infected with the lowest dilution of titrated specimen (contains highest number of ZIKV plaques) as described above. The titer of the C/3′NCR-mir(T) in the organs of MRS at 17 dpi was determined for all male mice (n = 15) participating in the mating experiment, while the titer of C/3′NCR-scr and 3′NCR20 viruses in the MRS at 17 dpi was determined only for 10 and 5 randomly selected male mice, respectively.

**Detection of ZIKV immunoreactivity in the MRS of mice.** Male AG129 mice (aged 10–20 weeks) were mock inoculated (n = 1) or infected IP with 10⁶ pfu of 2 × 10⁵(T) virus (n = 10 mice/virus). At 10 dpi, mice were euthanized, and MRS tissues were collected and stored at −80°C. Immediately prior to tissue retrieval (H&E), tissues were fixed with 100% methanol and stained with hematoxylin–eosin (H&E) for examination by light microscopy. Immunohistochemical (IHC) staining was performed on a Leica Bond-RX automated system according to manufacturer recommended protocol. Tissue sections were heated to 72°C for 30 min in Bond Dewax Solution (Leica) then rehydrated with absolute alcohol washes and 1× ImmunoWash (ACR-024, StatLab). After that sections were heated to 100°C for 20 min in Bond Epitope Retrieval Solution 1 (Leica) for heat-induced antigen retrieval. After exposure to primary antibody block (Leica) for 5 min, tissues were incubated with the anti-ZIKV N52B antibody (1:500 dilution factor; GTX133308, GeneTex). The N52B is a non-structural protein that can be detected in the host cell during active virus replication. The tissues were then incubated with secondary antibody (anti-rabbit Poly–HRP-IgG, DS8900, Leica). Peroxidase activity was developed using Diaminobenzidine (DAB) chromogen using the Bond Polymer Reagent (DS8900, Leica). Sections were then counterstained with hematoxylin. ScanScope AT2 was used to acquire digital images of whole tissue sections at ×10 magnification. Digital slides were analyzed using Aperio Spectrum eSlide Manager and ImageScope software (Version 12.4).

**Detection of ZIKV-specific NA in mouse serum.** The titer of ZIKV-specific NA in mouse serum was determined by PRNT₅₀ assay as described previously⁹⁸. The serum was considered positive for ZIKV-specific NA if ≥1:10 dilution of serum caused ≥50% reduction in a number of ZIKV-N3Sm plaques in Vero cells.

**Stability of miRNA targets and scr sequences in viruses isolated from mouse organs and serum.** A pair of ZIKV-specific primers ZV-10044-F and ZV-10722-R (Table S2) was used to amplify a region containing miRNA target (or scr) sequences inserted into the 3′NCR of ZIKV. Viral RNA was extracted from supernatants of Vero cells, which were infected with a virus in the serum or homogenate of mouse organs (see above). The RT-PCR reaction (35 cycles) was carried out using the Transcripter One-Step RT-PCR Kit (Roche). Amplicons were purified by 1% agarose gel electrophoreses and sequenced by Sanger method using ZV-10722-R and/or ZV-10044-F primers, BigDye Terminator v3.1 (Applied Biosystems) and 3730 DNA Analyzer (Applied Biosystems).

**Deep-sequencing analysis of ZIKV-lib/n11 virus.** To reduce/remove residual amount of plasmid DNA, the sample containing inoculum for mouse infection was treated with micrococcal nuclease (NEB, MA) for 2 h at 37°C, followed by viral RNA extraction. Virus RNA from all other samples was extracted without micrococcal nuclease treatment (see above). The region in the ZIKV-lib/n11 virus genome containing barcode sequences was amplified for 35 cycles using the Transcripter One-Step RT-PCR Kit (Roche) and ZV-10722-R/ZV-10044-F primer pair, followed by additional 20 PCR cycles using inner pair of primers ZV-10239-F and ZV-10489R and LongAmp Tag 2x Master Mix (NEB, MA). RT-PCR products were purified using Agencourt AMPure XP Reagent (Beckman Coulter) and the purified DNA was eluted in 40 μl of DEPC-treated water. DNA concentration was measured with Qubit 2.0 fluorimeter (Invitrogen, Life Technologies), and the DNA froze at −20°C until further use. Illumina libraries were prepared using NEBNext Ultra II DNA Library Prep Kit (NEB, MA) and NEBNext Multiplex Oligos for Illumina (NEB, MA) with 1:10 volume of viral cDNA (quantity and quality) treated with micrococcal nuclease. The sample was then submitted to Digital RNA Sequencing using MiSeq instrument (Illumina). Deep sequencing was done using MiSeq instrument (Illumina) with MiSeq Reagent Kit v2, 300-cycles (Illumina). Bioinformatic analysis was performed using in-house SWARM software (Supplementary Software 1). Sequence reads were sorted by unique molecular identifiers (barcodes) introduced into the viral genome (see above). Since point nucleotide substitutions may have been introduced during sample processing and sequencing, barcodes differing by only one nucleotide were combined into a single group. To identify the source of virus in each sample, the diversity of viral barcodes was compared to each other by barcode profiles (quantitative distribution of unique barcodes, which is characteristic for each sample). To determine
number of barcodes in reads\textsubscript{rep} for each organ we counted minimal number of barcodes in the sample, frequencies of which together would be equal to or greater than 90% of all identified barcodes. The 90% cutoff was selected to eliminate non-
replicating genomes (background), which could have been seeded in the organ
during initial ZIKV-lib/n11 dissemination. In addition, it eliminates from
consideration most barcodes that differ from the most abundant barcodes in the
sample by two or three nucleotides. Genetic relatedness of such barcodes to
dominate barcode sequences (see Supplementary Data 1 for an example of barcode
distributions in the organs collected from mice in mating pair # 1 in Fig. 6) is a
clear indication that they represent an artifact of sample processing. To calculate a
combined frequency of common barcodes, we identified all barcodes which are simultaneously present among reads\textsubscript{rep} barcodes in both organs in question.
Individual frequencies of these barcodes were combined, and a resulting value was
divided by two for normalization.

**Reporting summary.** Further information on research design is available in the Nature
Research Reporting Summary linked to this article.

**Data availability**
The authors declare that all data supporting the findings of this study are available within
the paper and its Supplementary Information. Full-length sequences for all viruses and
for all cDNA infectious clones used in this study are available from the corresponding
author upon request. Microarray data used for construction of graphs in Fig. 1a, 1b were
obtained from https://static-content.springer.com/esm/art%3A10.1038%2Fdata/20145/5
MediaObjects/41597_2014_BFsdata20145_MOESM7_ESM.zip. Source data are
provided with this paper.

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A.G.P., K.C., and K.A.T. designed the experiments; A.G.P., O.A.M., I.M., K.C., and K.A.T. performed the research; A.G.P., O.A.M., G.L., H.K., B.M.N., T.Z., I.M., K.C., and K.A.T. analyzed the data; A.G.P., O.A.M., I.M., K.C., and K.A.T. wrote the paper. All authors reviewed the final draft of the manuscript.

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