Zika virus causes supernumerary foci with centriolar proteins and impaired spindle positioning
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Zika virus (ZIKV) causes congenital microcephaly. Although ZIKV can impair cell cycle progression and provoke apoptosis, which probably contributes to disease aetiology through depletion of neural progenitor cells, additional cellular mechanisms may be important. Here, we investigated whether ZIKV infection alters centrosome number and spindle positioning, because such defects are thought to be at the root of inherited primary autosomal recessive microcephaly (MCPH). In addition to HeLa cells, in which centrosome number and spindle positioning can be well monitored, we analysed retinal epithelial cells (RPE-1), as well as brain-derived microglial (CHME-5) and neural progenitor (ReN) cells, using immunofluorescence. We established that ZIKV infection leads to supernumerary foci containing centriolar proteins that in some cases drive multipolar spindle assembly, as well as spindle positioning defects in HeLa, RPE-1 and CHME-5 cells, but not in ReN cells. We uncovered similar phenotypes in HeLa cells upon infection with dengue virus (DENV-2), another flavivirus that does not target brain cells and does not cause microcephaly. We conclude that infection with Flaviviridae can increase centrosome numbers and impair spindle positioning, thus potentially contributing to microcephaly in the case of Zika.

1. Background

Zika virus (ZIKV) is a member of the Flaviviridae family of viruses that can infect human beings [1]. Although adults infected by ZIKV usually suffer from mild clinical symptoms, numerous cases of congenital microcephaly, in Brazil in particular, transformed the Zika threat into a worldwide public health emergency [2,3]. Intrauterine infections with other viruses, including rubella virus, herpes simplex virus and cytomegalovirus, can also cause microcephaly, but these viruses impair the development of other organs in addition to that of the brain [4,5]. By contrast, the impact of ZIKV on the fetus is essentially limited to causing a small brain [6,7]. Intriguingly, such a brain-restricted impact is also observed in primary autosomal recessive microcephaly (MCPH), the most prevalent genetic cause of congenital microcephaly [8]. Whether ZIKV-mediated infection causes similar cellular phenotypes as those observed in MCPH is not clear.

Most genes mutated in MCPH encode centrosomal proteins [8–10]. Centrosomes are the primary microtubule organizing centre (MTOC) of animal cells and contain centrioles surrounded by pericentriolar material (PCM) [11,12]. The centrosome notably directs cell polarity during interphase and bipolar spindle assembly during mitosis. In tissue culture HeLa cells, depletion of MCPH gene products can lead to the assembly of a bipolar spindle that is mispositioned with respect to a reference substratum such as a fibronectin-coated surface [13,14]. In the developing mouse brain, such spindle mispositioning results in the
depletion of neural progenitor cells [15,16], raising the possibility that spindle positioning defects may contribute to disease in microcephaly patients. Furthermore, experimental induction of supernumerary centrioles in the developing mouse brain results in the assembly of multipolar spindles, leading to aneuploid daughter cells that undergo apoptosis and thereby cause microcephaly [17].

2. Results and discussion

We set out to investigate whether ZIKV infection of cultured human cells leads to abnormal centrosome numbers and spindle positioning defects. We conducted initial experiments in HeLa cells, which have been utilized previously to analyse these processes in a range of other experimental settings. We first tested whether HeLa cells could be infected by different strains of ZIKV. Phylogenetic analysis shows that ZIKV originated from Uganda, from where it followed two major lines of viral evolution, one in Africa and one in Asia, the latter then yielding the Brazilian strain causing the current epidemic outbreak [1]. Therefore, we included viral strains of both African (strains Arb 15076 and Hd 78788) and Asian (P1-25013-18) origins. As reported in electronic supplementary material, figure 1a, using immunofluorescence analysis of flavivirus envelopes (4G2) as a readout, we found that HeLa cells can indeed be infected by these three strains of ZIKV.

We restricted further analysis to 4G2 positive cells (electronic supplementary material, figure S1b), even though other cells exhibited clear signs of infection, such as cell membrane bags or cytoplasmic vacuoles (electronic supplementary material, figure S1c). Moreover, we excluded cells that were polypliod or that appeared compromised judging from their DNA (electronic supplementary material, figure S1c,d). Data from 24 and 48 h post-infection were pooled because the phenotypes of infected cells at the two time points were indistinguishable (see electronic supplementary material, table S1 for all experimental conditions and outcome).

To monitor centrosome numbers, we used immunofluorescence of cells stained with antibodies against 4G2, to ascertain infection status, as well as with antibodies against the centriolar markers POC5, CP110 or polyglutamylated tubulin (PolyE). Both POC5 and CP110 mark the distal part of centriolar cylinders [18,19]. Depending on the cell cycle stage, there are normally 2 or 4 foci of POC5 or CP110 per cell (figure 1a,b, ctrl) [11]. PolyE labels centrioles as they mature [20], such that two foci are present throughout the cell cycle (figure 1c, ctrl). We began our analysis with interphase cells. We found that infection of HeLa cells with ZIKV led to a significant augmentation of centriole numbers in ReN cells (figure 2a,b). Data from 24 and 48 h post-infection were pooled because the phenotypes of infected cells at the two time points were indistinguishable (see electronic supplementary material, table S1 for all experimental conditions and outcome). To cluster supernumerary centrioles into two spindle poles (figure 2c,d). We then assayed spindle positioning in infected cells that assembled a bipolar spindle. To this end, cells were plated on fibronectin-coated coverslips, which provide a planar substratum that normally directs spindle positioning parallel to it (figure 3a). Upon siRNA-mediated depletion of components critical for spindle positioning, such as LGN or β-integrin [27], the spindle tends not to be positioned parallel to the substratum, but instead at an angle from it (figure 3b). Importantly, measurements of the angle between the bipolar mitotic spindle and the substratum revealed striking defects in spindle positioning upon infection of HeLa cells with ZIKV (figure 3c,b). Moreover, we found that spindle-positioning defects were not correlated with increases in the number of foci with centriolar proteins, suggesting independent mechanisms (electronic supplementary material, figure S2b).

Next, we measured spindle positioning in RPE-1, CHME-5 and ReN cells. As shown in figure 3c–h, this analysis uncovered spindle-positioning defects in both RPE-1 and CHME-5 cells, but not in ReN cells. Overall, we conclude that ZIKV infection can cause impaired spindle positioning.
In order to test whether the above phenotypes are specific to ZIKV or instead represent more general characteristics of infection by Flaviviridae, we also tested the impact of dengue virus (DENV, strain DENV-2) on HeLa cells. Although the two viruses are closely related and can bind to similar cell surface receptors [28–30], DENV is not neurotrophic and therefore does not cause microcephaly [31,32]. After confirming that HeLa cells can be infected with DENV-2 virus (electronic supplementary material, figure S1a), we conducted analyses similar to those performed for ZIKV. As shown in figure 4a–d, we likewise found an increase in the number of centriolar foci in both interphase and mitotic HeLa cells upon DENV infection. As in the case of ZIKV, we also found an augmentation in foci of centriolar proteins irrespective of cell cycle stage upon DENV infection (electronic supplementary material, figure S2a). Moreover, DENV infection led to mitotic cells undergoing multipolar spindle assembly and impaired spindle positioning (figure 4e–g), independently of the number of centrioles (electronic supplementary material, figure S2d).

In conclusion, we have shown that ZIKV infection leads to the presence of supernumerary foci of centriolar proteins as well as to defects in spindle positioning in HeLa, RPE-1 and CHME-5 cells. DENV has similar effects in HeLa cells. Viral infection with ZIKV or DENV also decreased cell numbers in all cell lines (data not shown), in line with the notion that cases of viruses impacting centrosomes. For instance, mutated human T-cell leukemia virus type-1 (HTLV-1) causes abnormal centrosome fragmentation through the targeting of Run-binding protein c (RRM2), which is required for centrosome duplication and spindle assembly.

Figure 1. ZIKV infections lead to supernumerary foci of centriolar proteins and multipolar spindle assembly in HeLa cells. (a–c) Immunofluorescence images of mock-treated (ctrl) and ZIKV-infected HeLa cells in interphase, stained with antibodies against the centriolar proteins POC5 (a), CP110 (b) or PolyE (c) (all shown in purple), in combination with antibodies against the viral marker 4G2 (green). In this and other figures, DNA is shown in blue, scale bars correspond to 10 μm and insets are three times magnified views of select planes in the indicated regions from the lower magnification image (unless stated otherwise); the specific ZIKV strain is indicated in each case below the images. (d) Average percentage of cells (+ s.e.m. of three biological replicates for ctrl and two biological replicates for ZIKV) exhibiting supernumerary foci of centriolar proteins, pooling the data from CP110, POC5 and PolyE stainings (n: total number of scored cells; see electronic supplementary material, table S1 for details). Unpaired two-tailed Student’s t-test, comparing to control conditions: *p < 0.05. (e,f) Immunofluorescence images of mitotic HeLa cells infected by ZIKV and stained with antibodies against α-tubulin to mark spindle microtubules (green) and POC5 to mark centrioles (purple) (e), or against PolyE, which also label spindle microtubules close to the spindle poles (purple) and 4G2 (green) as an infection marker (f). Shown is a partial view of the 4G2 staining so as to not obliterate the DNA and centriolar signals. (g) Average percentage of mitotic cells (+ s.e.m. of three biological replicates for ctrl and two biological replicates for ZIKV) exhibiting supernumerary centriolar foci. Note that the average for both datasets with ZIKV (Arb 15076) is 50% of cells, thus explaining the lack of standard deviation (n: total number of scored cells, see electronic supplementary material, table S1 for details). (h) Average percentage of mitotic cells (+ s.e.m. of three biological replicates for ctrl and two biological replicates for ZIKV, data independent from that in figure 1g) exhibiting multipolar metaphase spindles. Unpaired two-tailed Student’s t-test, comparing to control conditions: *p < 0.05, **p < 0.01.
protein-1, leading to supernumerary centrioles [35]. Further work is needed in the case of the Flaviviridae to elucidate the mechanisms through which ZIKV and DENV alter the centriole duplication cycle. Besides the possibility that these phenotypes reflect a general non-specific cellular response, which appears unlikely given that they are not observed in ReN cells, potential mechanisms include the premature licensing of centriole formation, as observed in cells held in the

Figure 2. ZIKV infections lead to supernumerary foci of centriolar proteins and multipolar spindle assembly in CHME-5 cells and RPE-1 cells. (a,c,e) Immunofluorescence images of mitotic RPE-1 (a), CHME-5 (c) and ReN (e) cells either mock-treated (ctrl) or subjected to ZIKV infection (strain Pf-25013-18), and stained with antibodies against CP110 to mark centrioles (red) and 4G2 to mark viral infection (green). (b,d,f) Average percentage of mitotic RPE-1 (b), CHME-5 (d) and ReN (f) cells exhibiting supernumerary centriolar foci of CP110 or POC5 (+ s.d. of three coverslips—two stained for CP110 and one stained for POC5; see electronic supplementary material, table S1; n; total number of cells scored). Unpaired two-tailed Student’s t-test, comparing to control conditions for each cell line: *p < 0.05. (g) Immunofluorescence images of mitotic CHME-5 cells subjected to ZIKV infection and stained with antibodies against PolyE, which label also spindle microtubules close to the spindle poles (purple) and 4G2 as an infection marker (green). (h–j) Average percentage of mitotic RPE-1 (i), CHME-5 (h) and ReN (j) cells (+ s.d. of two technical replicates for each condition) exhibiting multipolar metaphase spindles. Unpaired two-tailed Student’s t-test, comparing to control conditions for each cell line: *p < 0.05, **p < 0.01. n: total number of metaphase cells scored.
Figure 3. ZIKV impairs spindle positioning. (a,b) Spindle positioning assay in HeLa cells. (a) Immunofluorescence images of mock-treated (ctrl) and ZIKV-infected HeLa cells stained with antibodies against POC5 (purple) and 4G2 (green). Z-stacks of 0.4 μm-high confocal sections were imaged between the two spindle poles; shown are projections of relevant planes (top, XY), as well as resliced sections (bottom, Z), which were used to determine the angle $\alpha$ of the spindle axis with respect to the substratum, as schematized below. (b) Average spindle positioning angle for indicated siRNA treatments (three left-most bars, two biological replicates) and indicated infection conditions (+ s.d. of three (mock) or two (ZIKV) biological replicates, four right-most bars; see electronic supplementary material, table S1 for details). Mann–Whitney U-test, comparing to control conditions: *** $p < 0.001$, ** $p < 0.01$. (c,g) Immunofluorescence images of mock-treated (ctrl) and ZIKV-infected RPE-1 (c), CHME-5 (e) and ReN (g) cells stained with antibodies against CP110 (purple) and 4G2 (green); spindle-positioning analysis was performed as described above for HeLa cells. (d,f,h) Average spindle positioning angle ($\pm$ s.d. of three technical replicates for each condition) of mock versus ZIKV-infected cells for different cell types. Mann–Whitney U-test, comparing to control conditions: *** $p < 0.001$, ** $p < 0.01$. See electronic supplementary material, table S1 for details.
G2 phase of the cell cycle [36], or overexpression of components driving centriole formation such as Plk4 or HsSAS-6 [37,38].

Together with previously established consequences of ZIKV on cell cycle progression and cell survival [39–41], the occurrence of multipolar and mispositioned spindles could conceivably contribute to intrauterine microcephaly caused by ZIKV infection. These findings offer a novel illustration of the intricate cellular relationships between viruses and their hosts, and should lead to a better understanding of viral pathological mechanisms.

3. Material and methods

3.1. Cells and viruses

HeLa cells were purchased from ECACC (ECACC 93021013), RPE-1 cells from ATCC (Manassas, VA; CRL-4000). CHME-5 cells were a kind gift of Ali Amara [42], ReN cells were a gift of Chiara Sartori, who originally purchased them from EMD Millipore (SCC008). Vero cells were used to titrate viruses. HeLa, RPE-1, CHME-5 and Vero cells were cultured in high-glucose DMEM medium with GlutaMAX (Invitrogen) or in DME-F12 (Vero cells, Invitrogen) medium, each supplemented with 10% FCS, in a humidified 5% CO2 incubator at 37°C. ReN cells were cultivated in DMEM F12 (Gibco) containing Glutamax (2 mM), B-27 stem cell supplement (Gibco) 1U ml⁻¹ Heparin (Sigma), 20 ng ml⁻¹ bFGF (Peprotech) and 20 ng ml⁻¹ EGF (Peprotech). ReN cells were grown on Laminin (0.7 ug cm⁻², Sigma) coated cell culture ware or coverslips.

Propagation of the ZIKV strains and DENV-2 was achieved using A. albopictus C6/36 cells having undergone limited passages. C6/36 cells were grown in DMEM supplemented with 10% FCS and cultivated at 28°C.

The following viruses were used: the clinical isolate Pf-25013-18 [30]; the Hd 78788 strain obtained from a patient in Senegal during routine surveillance in 1991 [43]; the Arb 15076 strain isolated from A. africanus in central African
3.2. Infection of cells

For infection, cells were seeded on glass- or fibronectin-coated coverslips in six-well culture plates at a density of 100 000 cells per well. Then, 24 h after seeding, cells were rinsed once with phosphate-buffered saline (PBS), and viruses, which were diluted to 5–10 multiplicity of infection (MOI), were added to the cells. Cells were then incubated for 2 h at 37°C with gentle agitation every 30 min. Next, the inoculum was removed and cells were washed twice with PBS. Culture medium was added to each well, and cells were incubated at 37°C and 5% CO₂ for the duration of the experiment. As a control, cells were incubated with the culture supernatant from uninfected C6/36 cells, referred to as mock treated - or control- cells.

3.3. Immunofluorescence, imaging and analysis

For immunofluorescence, cells were fixed in −20°C methanol for 7–10 min and washed in PBS-0.05% Triton X-100 (PBST). After blocking in 1% BSA in PBST for 1 h, cells were incubated with primary antibodies at room temperature for 4 h. After three washes in PBST for 5 min each, cells were incubated with secondary antibodies for 1 h at room temperature, stained with 1 μg mL⁻¹ Hoechst 33342 (Sigma-Aldrich), washed three times 5 min in PBST, and mounted. Primary antibodies were 1:200 mouse anti-4G2 (MAB10216, Millipore), 1:1000 rabbit anti-POC5 (127801-AP; ProteinTech Europe), 1:200 mouse anti-4G2 (MAB10216, Millipore), 1:1000 rabbit anti-α-tubulin (DM1α, Sigma-Aldrich). Secondary antibodies were Alexa Fluor 488-coupled antimouse and Alexa Fluor 568-coupled anti-rabbit, both used at 1:1000 (Life Technologies).

Images were acquired with a 63×, NA 1.0 oil objective on a confocal microscope (LSM 700; Carl Zeiss), then processed and analysed in IMAGEJ (National Institutes of Health). Relevant z-planes are shown as maximum intensity projections; in insets, z-planes, relevant for this particular centrosome are used for projections, while in overview images z-planes relevant for all centrosomes of the cell are used for projections. Data were analysed using EXCEL and statistics performed with GRAPHPAD.

For analysis of centriolar protein foci and of spindle positioning in HeLa cells, confocal images (using a 63× Plan-Apochromat, NA 1.4 objective) were taken of 154 control cells and 41 infected cells, with indicated numbers of metaphase cells (ctrl) independent coverslips originating from two biological replicates for ZIKV and three biological replicates for ctrl and DENV-2 with indicated number of metaphase cells (n) were analysed (n: control: 74, 101, 49, 52, 51; ZIKV (Hd 787788): 8, 10, 18, 3; ZIKV (Arb 15076): 54, 30, 20, 27; ZIKV (Pt-25013-18): 17, 15, 37, 14; DENV-2: 13, 26, 20, 21, 25). For RPE-1, CHME-5 and ReN cells, data from two technical replicates with indicated numbers of metaphase cells (n) were analysed (n; RPE-1 ctrl: 53, 25; RPE-1 ZIKV (Pt-25013-18): 19, 26; CHME-5 ctrl: 33, 31; CHME-5 ZIKV (Pt-25013-18): 39, 58; ReN ctrl: 50, 35; ReN ZIKV (Pt-25013-18): 23, 25).

Nuclear areas (in square micrometres) were measured from maximum intensity projections of confocal images using IMAGEJ.

We compared two conditions (ctrl versus infection) at a time using unpaired, two-tailed Student’s t-test for samples of unequal variances and sample sizes (also known as Welch’s test). If normality test failed for one of the groups within a comparison, Mann–Whitney-U test was applied as stated in figure legends for each case.

3.4. siRNA treatment

For siRNA experiments, approximately 100 000 cells were seeded on fibronectin-coated coverslips in six-well plates and transfected with 20 nM of validated stealth siRNAs (Qiagen) against β1-Integrin (accession number NM-033669, nucleotide 167–189), LGN (5′-GAACUAAACAGCAGCAGCUC-A-3′, 5′-CUUCAGGGAUUGAUAUAUCA-3′, 5′-ACAUGUGAAUUCUCUUGCUUA-3′, 5′-UGAAGGUGUUCUGACUCA-3′) or scrambled siRNA (SI03650318) as per the manufacturer’s instructions. In brief, 2.5 μl of 20 μM siRNA in 250 μl Optimem and 7.5 μl Lipofectamin RNAi max (Invitrogen) in 250 μl Optimem were incubated in parallel for 5 min, mixed for 20 min and then added to 2 ml of medium per well; cells were incubated for 48 h before fixation and analysis.

3.5. Spindle positioning assay

To monitor spindle positioning, cells were grown on coverslips uniformly coated with fibronectin (Neuvitro, GG-22-fibronectin).

After fixation and immunofluorescence, the angle of the metaphase spindle with respect to the fibronectin substratum was determined as described [27]. In brief, cells were stained with...


