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Neurodevelopmental protein Musashi 1 interacts with the Zika genome and promotes viral replication

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Abstract

A recent outbreak of Zika virus in Brazil has led to a simultaneous increase in reports of neonatal microcephaly. Zika targets cerebral neural precursors, a cell population essential for cortical development, but the cause of this neurotropism remains obscure. Here we report that the neural RNA-binding protein Musashi-1 (MSI1) interacts with the Zika genome and enables viral replication. Zika infection disrupts the binding of MSI1 to its endogenous targets, thereby deregulating expression of factors implicated in neural stem cell function. We further show that MSI1 is highly expressed in neural progenitors of the human embryonic brain, and is mutated in individuals with autosomal recessive primary microcephaly. Selective MSI1 expression in neural precursors could therefore explain the exceptional vulnerability of these cells to Zika infection.

Zika virus (ZIKV) recently emerged as a major public health risk because of its devastating effect on fetal neurodevelopment (1–3). ZIKV was first isolated in Uganda in 1947, and the virus subsequently spread through Asia, and from there to the Americas (4). A causal link between ZIKV infection and congenital brain malformations became apparent in 2016 following an outbreak in Brazil (1). Brazilian ZIKV is closely related to the Asian-lineage

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strain, which affected New Caledonia and French Polynesia, where cases of microcephaly were reported retrospectively (5).

Intrauterine infections can impair neurodevelopment (6), but ZIKV is highly neurotropic and interferes specifically with fetal brain development causing microcephaly, cortical malformations and intracranial calcifications (7–10). We hypothesized that the single-stranded RNA flavivirus ZIKV may hijack RNA-binding factors present in the developing central nervous system (11). Host RNA-binding proteins are known to interact with untranslated regions (UTRs) to regulate replication, translation and stabilization of viral genomes (11). In *silico* analysis of the genomic RNA of the Brazilian ZIKV strain, PE243, revealed three consensus binding sites in the 3'UTR for the highly conserved Musashi family of RNA binding proteins, Musashi-1 (MSI1) and Musashi-2 (MSI2), both important translational regulators in stem cells (12–15). Two sites were conserved between PE243 and the Ugandan MR766 strains (Sites 1, 2), whereas the third (Site 3) was found only in the Asian-lineage strains including PE243 (Fig. 1A; Fig. S1A, B). By mapping these sites onto a predicted secondary structure of ZIKV 3'UTR, we found all the three to be present on stem-loop structures, which are considered optimal for MSI binding (16, 17). Moreover, a recent study revealed nucleotide substitutions proximal to Sites 1 and 2 in the Asian-lineage strains, which could indicate positive selection for MSI1 binding during ZIKV evolution (18).

To address if the Musashi proteins interacted with ZIKV, we first tested their binding to ZIKV 3'UTR. RNA pull-downs identified binding of MSI1, but not MSI2, to the 3'UTR of PE243 (Fig. 1B) (15). Mutating the three consensus MSI1 sites in the 3'UTR of PE243 significantly weakened this interaction (Fig. 1C, D; Fig. S1C). We also confirmed binding between MSI1 and the 3'UTR of MR766 (Fig. 1C). To investigate whether MSI1 also binds ZIKV 3'UTR *in vivo*, ultraviolet (UV) crosslinking and RNA immunoprecipitation (CLIP) was performed from PE243-infected U-251 glioblastoma cells, revealing a robust direct interaction between MSI1 and PE243 ZIKV RNA (Fig. 1E). Consistently, in ZIKV-infected cells, MSI1 co-localised with double-stranded RNA, a viral replication intermediate, as visualised by confocal and STED super-resolution microscopy (Fig. 1F, G). These data confirm an interaction between MSI1 and ZIKV RNA, which is at least in part mediated by the 3'UTR of the virus.

To investigate whether MSI1 had an impact on the life cycle of ZIKV, we used RNA interference to deplete the protein in U-251 glioblastoma, SK-N-BE2c neuroblastoma and H9 derived neural stem cells (NSC) and performed PE243 viral infections. In all three cell types MSI1 depletion led to a marked reduction in viral RNA levels (Fig. 2A, B). We then generated MSI1 knockouts (KO) in U-251 cells by CRISPR/Cas9-mediated targeting of exons 8 or 6 of *MSI1* (KO1 and KO2, respectively; Fig. 2C; Fig. S2). Control cells were obtained through clonal expansion of cells transfected with Cas9 alone. By measuring viral RNA at different times following PE243 infection, a marked reduction of viral load was seen in KO1 and KO2 cells at 24 and 48 hours (Fig. 2D). Whereas extensive cell death precluded RNA analysis in the controls at 72 hours, viral RNA was comparable between the 48- and 72-hour timepoints in the KO cells. Consistently, levels of the viral dsRNA and flavivirus E protein as well as the infectious titre were reduced in the KOs (Fig. 2E; Fig S3). Because MSI2 levels were similar between control and KO cells (Fig. 2C), MSI1 and MSI2 are

unlikely to have complete functional redundancy in ZIKV replication. Replication of the MR766 strain was also impaired in the KO cells (Fig. 2F). In summary, we identify MSI1 as an important factor for ZIKV replication, both in primary and transformed neural cell lines.

Since there was no discernible difference between ZIKV binding and entry into control and KO cells (Fig. 2G, H), we asked if MSI1 could regulate translation through ZIKV UTRs. To this end, luciferase RNA flanked by the 5' and 3'UTRs of PE243, was transfected with increasing amounts of MSI1 into HEK293T cells, which do not normally express MSI1 (Fig. S4). We observed a modest MSI1-driven increase in luciferase expression. The ability of MSI1 to promote ZIKV UTR-driven translation *in vitro* raises the possibility that it performs a similar function *in vivo*. Alternatively, MSI1 might stabilise the viral RNA genome and/or regulate its cyclization or synthesis. In addition, given the pleiotropic roles of MSI1 in cellular pathways, it is plausible that MSI1-dependent regulation of gene expression contributes to the ZIKV life cycle (19). However, MSI1 is unlikely to act through general pro- or anti-viral pathways as infection with H1N1 influenza virus was unaffected by MSI1 expression levels (Fig. S5). In line with published work, we also find that MSI1 KO cells exhibited defective migration, increased doubling time and cell cycle delay (Fig. S6) (17, 20, 21). Because ZIKV replication requires cyclin-dependent kinase activity, such pro-proliferative effects exerted by MSI1 might contribute to virus production (22). Nevertheless, the direct interaction between MSI1 and the ZIKV genome is consistent with the hypothesis that the protein promotes some aspect of the viral life cycle.

ZIKV predominantly infects neural progenitors in human fetal brain. We find MSI1 to be highly enriched in neural precursors of the ventricular and subventricular zones of the human embryonic brain, but absent from mature neurons (Fig. 3A; Fig. S7). Owing to its high levels in neural progenitors, and its ability to stimulate ZIKV replication, MSI1 could be instrumental to ZIKV-induced cytopathicity in the fetal brain. In addition, MSI1 is required for neurodevelopment in both invertebrates and vertebrates, with MSI1-depleted zebrafish displaying microcephaly, and mutant mice exhibiting a thin cerebral cortex and reduced number of mature neural cell types among other morphological brain abnormalities (14, 19, 23–25). We have identified a consanguineous Turkish family in which two siblings displayed clinical features suggestive of autosomal primary microcephaly (MCPH), a condition associated with a significant reduction in cerebral cortex size, but a structurally normal brain (Fig. S8A, B) (26, 27). Exome sequencing uncovered potentially deleterious homozygous mutations in *MSI1*, *ACACB*, *DKK4* and *DTX3L* (Fig. S8C-F, Tables S1 and S2). Of these only MSI1 is known to have neural functions, but because mutations were present in four genes, the p. Ala184Val point mutation in MSI1 may not be the sole cause of MCPH in these individuals (referred to as MSI1^{A184V}). Nevertheless, we have three lines of evidence showing that A184V mutant MSI1 is functionally impaired. First, MSI1^{A184V} patient cells exhibit premature chromosome condensation (PCC), the same phenotype as MSI1-deficient glioblastoma cells. Second, we show that the A184V mutation impedes RNA binding of MSI1, leading to deregulated expression of its endogenous targets. Third, we find that the A184V mutant MSI1 is unable to support ZIKV replication.

The PCC phenotype seen in MSI1^{A184V} patient cells has been previously described in cells deficient of the MCPH-associated protein, Microcephalin (MCPH1) (Fig. S8G) (28, 29).

Since the *MCPH1* locus is unaffected in MSI1^{A184V} patients, we speculated that MSI1 could control chromosome condensation by regulating *MCPH1* expression. To determine if MCPH1 was a MSI1 target, we performed RNA immunoprecipitation under native conditions. In addition to its known targets *NUMB*, *p21^{WAF}* and the MCPH-associated gene, *CDK6/MCPH12*, MSI1 co-precipitated with two isoforms of *MCPH1* (*MCPH1_S* and *MCPH1_L*) despite their divergent 3' UTRs, but did not interact with other *MCPH* genes tested (Fig. S9A, B) (12, 30, 31). CLIP and RNA mobility shift assays suggest a direct interaction between MSI1 and *MCPH1_L* (Fig. 3B, C). MSI1 can act as a translational suppressor (i.e. for *NUMB* and *p21^{WAF}*) or activator (i.e. for *CDK6*) (12, 21, 30). Consistent with a role for MSI1 in translational activation of MCPH1, we observed low MCPH1 protein levels in MSI1^{A184V} patient and MSI1-deficient U-251 cells and a reduction in polysome-associated *MCPH1* transcripts in KO cells (Fig. 3D, E; Figs. S9C, S10). Given that MSI1 interacts with ZIKV RNA, and viral RNA is abundant in the infected cell, it could compete with endogenous targets for binding MSI1. Indeed, upon ZIKV infection of U-251 cells, we observed a marked reduction in the interaction between MSI1 and its target RNAs including *MCPH1* and *NUMB*, accompanied by changes in their protein levels that mirrored those of MSI1 KO cells (Fig. 3F,G).

MSI1 interacts with target transcripts via its two RNA recognition motifs (RRM) (19, 30, 32). NMR studies show that the conserved Ala184 within the RRM2 is an RNA-binding residue (33). Modeling based on the crystal structure of the RNA-binding protein HRP1 with RNA, indicates that the A184V mutation impairs the MSI1-RNA interaction (Fig. 4A). To evaluate the effect of A184V on MSI1 function in cells, transgenes encoding wild-type and A184V MSI1 (MsiWT and MsiAV, respectively) were randomly integrated into KO1 or KO2 U-251 cells and single clones were isolated (KO1/2-MsiWT or KO1/2-MsiAV). MSI1 protein expression in these clones was quantified with respect to endogenous protein levels in parental cells (Fig. 4B). When compared to KO2-MsiWT cells, MSI1 RIP recovered 2-4 fold fewer target transcripts from KO2-MsiAV, indicative of reduced RNA binding by the A184V mutant (Fig. 4C). *NUMB* and *MCPH1* protein levels changed accordingly (Fig. 4B). These results were recapitulated in HEK293T cells expressing Msi1WT or Msi1AV (Fig. S11). MSI1^{A184V}, MSI1 KO, KO1/2-MsiAV and MSI1-depleted cells all exhibited suboptimal MCPH1 protein levels and PCC, prompting us to investigate if a functional link existed between these phenotypes (Fig. S9C-F). MCPH1 overexpression reduced PCC frequency in MSI1-depleted cells, thereby confirming a role for MSI1 in chromosome condensation via translational control of MCPH1 (Fig. S8G, H). Therefore, the A184V mutation impairs binding of MSI1 to RNA, which leads to reduced MCPH1 expression and a concomitant increase in PCC frequency. Remarkably, defective chromosome condensation has been recently found to cause MCPH (34).

Given that the A184V mutation impedes binding of MSI1 to RNA, we next probed the effect of A184V mutant MSI1 on ZIKV replication. To this end, viral RNA levels and cell viability were assayed in KO1/2, KO1/2-MsiWT and KO1/2-MsiAV U-251 cells infected with PE243. Complementation of KO1/2 cells with MsiWT increased both ZIKV RNA levels and cell death (Fig. 4D, E). By contrast, MsiAV was unable to support ZIKV replication, and showed minimal impact on cell viability. Additionally, in HEK293T cells, expression of MsiWT, but not MsiAV, increased viral RNA and cell death upon infection (Fig. 4F, G; Fig.

S12). These findings also imply that MSI1 expression increases susceptibility of HEK293T cells to ZIKV infection (35). Furthermore, we noted an apparent dose-dependent effect of MSI1 on viral replication; those U-251 and HEK293T clones that express higher levels of MSI1 displayed greater viral RNA levels and increased cell death (Fig. 4B, D-G; Figs. S11 and S12).

Our study raises the question whether MSI1 could have functions in other flaviviruses. We have surveyed putative MSI1 binding sites in a number of flaviviruses by mapping the consensus (A/GU₍₁₋₃₎AG) onto predicted secondary structures of flaviviridae 3'UTRs obtained from a recent publication (Table S3) (16). Although several flaviviruses harbour consensus MSI1 sites within appropriate structural landscapes, whether MSI1 is relevant to the biology of these viruses remains to be established. Furthermore, while our data are consistent with a role for MSI1 in ZKV neurotropism and pathology, multiple factors must collude in ZIKV infection of the fetal brain, not least viral entry receptors that allow the virus to cross the placental barrier (36). Viruses such as human cytomegalovirus and Rubella can also access the developing fetal brain, but whether MSI1 contributes to their replication or pathogenesis is unknown and would require further study.

This work suggests that high MSI1 expression levels in neural precursors could be a key contributor to the fetal neurotropism exhibited by ZIKV (2, 10, 37) (Fig. S13). Intriguingly, MSI1 is also highly expressed in the retina and testis, other tissues deemed vulnerable to ZIKV infection (38–41). While our study provides new insight into the potential pathogenic mechanisms of ZIKV, further work will be required to determine if the modification or interference of the MSI1-ZIKV interaction results in neuronal attenuation of ZIKV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. MSI1 interacts directly with the ZIKV RNA genome.

A) Schematic diagram of PE243 ZIKV containing three putative MSI1 binding sites in its 3'UTR. Sites shared with MR766 are red, site unique to PE243 is blue.

B) RNA pull-down assays performed with the 3'UTR of PE243. Increasing concentrations of *in vitro* transcribed biotinylated PE243 RNA were incubated with U-251 cell extracts and RNA-protein complexes were captured on streptavidin beads. Representative western blots were probed with antibodies against MSI1 and the RNA binding proteins, Musashi-2 (MSI2) and hnRNP Q/R. Corresponding protein and RNA inputs are shown on right.

C) RNA pull-down assays performed with the wild type (WT) or triple mutant (Δ123) 3'UTR of PE243. Note that PE243-3'UTR_Δ123 lacks all three MSI1 binding sites depicted in Fig. 1A (see Fig. S1C for further details). Increasing concentrations of *in vitro* transcribed biotinylated RNA were incubated with U-251 cell extracts and RNA-protein complexes were captured on streptavidin beads. Representative western blots probed with antibody against MSI1 are shown together with corresponding protein and RNA inputs.

D) Densitometric analysis of MSI1 levels from western blots of RNA pull-down assays, an example for which is shown in Fig. 1C. The amount of MSI1 precipitated by PE243-3'UTR_Δ123 is expressed as a percentage of MSI1 precipitated by the same concentration of PE243-3'UTR_WT. n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * p<0.05, *** p<0.0005. Bar charts depict mean ±s.e.m.

E) CLIP analysis from mock- or ZIKV PE243-infected U-251 cells. Western blot shows immunoprecipitations (IP) by rabbit IgG and MSI1 antibodies from mock and PE243-infected U-251 cells following UV crosslinking. Input (5%) represents whole cell extract. Western blot was probed with MSI1 antibody. Graph below shows qPCR performed on bound RNA from IP. CLIP values are presented as a percentage of input following subtraction of the IgG background. *GAPDH* serves as negative control. n=3 biological replicates. Note that a primer pair against 9519-9681 bp of ZIKV genome was used in these qPCRs (Table S4).

F) Immunofluorescence of mock- or PE243-infected U-251 cells. MSI1 (green) and dsRNA (red) signals are detected by confocal microscopy. DNA is detected by Hoechst stain (blue). Framed area is shown at higher magnification below.

G) Immunofluorescence of a PE243-infected U-251 cell. MSI1 (green) and dsRNA (red) signals are detected by STED super resolution microscopy. Outlines of the cell and nucleus are indicated in white and blue, respectively. Framed area is shown at higher magnification.

Fig. 2. MSI1 is required for effective replication of ZIKV.

A) Graph on left depicts viral RNA copies in control siRNA- (siCon) and MSI1 siRNA (siMSI1)-treated SK-N-BE2c and U-251 cells following infection with PE243 (MOI: 1 FFU/cell, 72h). Graph on right shows viral RNA copies in MSI1 siRNA-treated H9-derived neural stem cells (NSC) following infection with PE243 (MOI: 1 FFU/cell, 48h). Note that in all viral replication assays ZIKV was quantified by TaqMan assay as described in Materials and Methods. n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * p<0.05, ** p<0.005, not significant (n.s.).

- B)** Representative western blots of cell lines treated with control and MSI1 siRNAs from Fig. 2A. Blots were probed with antibodies against MSI1 or p150 as loading control.
- C)** Location of the guide RNAs used for CRISPR/Cas9-mediated editing of the *MSI1* locus in U-251 cells. For further details see text and Fig. S2. Western blots of parental, control, KO1 and KO2 cell lines probed with antibodies against N- and C-termini of MSI1 (NT or CT) and MSI2. p150 serves as loading control.
- D)** Kinetics of PE243 viral RNA copies following infection in U-251 cells of different genotypes at the indicated time points. Note that cell death precluded collection of RNA from parental and control cells at 72h (MOI: 3 FFU/cell, 72h).
- E)** Confocal microscopy images of PE243-infected control and KO1 U-251 cells immunostained with antibodies against dsRNA (green) and Hoechst DNA stain (red) following mock or PE243 infection (MOI: 3 FFU/cell, 48h). Graph on top shows percentage of cells containing dsRNA signal, whereas box plot on bottom depicts total dsRNA staining volume per cell. Note that only cells with detectable dsRNA signal were included in the latter analysis. Boxes: 25th to 75th percentile; whiskers: 5-95% range; line: median. P-value represents Student's t-test, unpaired, two-tailed: **** p<0.0001.
- F)** Viral RNA copies in U-251 cells of different genotypes following infection with MR766 (MOI: 3 FFU/cell, 48 h). n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * p<0.05, ** p<0.005, not significant (n.s.).
- G)** Virus-binding assays performed under conditions that prevent internalisation. PE243 infection was performed in U-251 cells of different genotypes (MOI: 3 FFU/cell, 1h). n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * p<0.05, ** p<0.005, not significant (n.s.). Bar charts depict mean±s.e.m.
- H)** Pseudotyped particle infectivity assay in U-251 cells of different genotypes. HIV (pNL4-3.luc.R-E-) or MoMLV pseudotyped virus expressing a luciferase reporter, with either PE243 ZIKV, VSVg or a negative control envelope used to determine viral entry events. n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * p<0.05, ** p<0.005, not significant (n.s.). Bar charts depict mean±s.e.m.

Fig. 3. MSI1 is enriched in neural progenitors, regulates Microcephalin (MCPH1) expression and is mutated in MCPH patients.

A) Immunohistochemistry of human embryonic brain at post conception week (pcw) 10 and 12. Tissue sections stained with antibodies against MSI1 (red) combined with neuron-specific β -III tubulin (green), or the apical neural progenitor marker Nestin (green). DNA is detected by DAPI (blue). Note that MSI1 is enriched in neural progenitors at the ventricular and subventricular zones (VZ and SVZ), but is absent from the cortical plate (CP).

B) MSI1 CLIP from U-251 cells with genotypes as indicated. CLIP was performed with rabbit IgG or MSI1 antibodies. Graphs show qPCRs of bound transcripts. n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * $p<0.05$, ** $p<0.005$, not significant (n.s).

C) RNA EMSA analysis to detect binding between MCPH1_L 3'UTR and purified GST-MSI1 recombinant protein. Coomassie staining of corresponding purified proteins is shown below.

D) Western blots of parental, control, KO1 and KO2 cell lines. Blots were probed with antibodies as indicated. p150 serves as loading control.

E) Western blots of whole cell lysates from parent-of-patient- and patient-derived primary lymphocytes. Blots were probed with antibodies as indicated. MSI1 levels are unchanged.

F) MSI1 RIP from mock- and ZIKV-infected U-251 cells (MOI: 1 FFU/cell). Rabbit IgG or MSI1 antibodies were used for immunoprecipitation. Input corresponds to 10% of whole cell extract. Western blot was probed with MSI1 antibody. Graphs below show amounts of bound RNAs including ZIKV genome and endogenous target transcripts. RIP values are presented as a percentage of input following subtraction of the IgG background. MR766 and PE243 were quantitated by TaqMan assay, whereas endogenous transcripts with SYBR qPCR. Bar charts depict mean $\pm$ s.e.m. n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * $p<0.05$, ** $p<0.005$, not significant (n.s).

G) Western blots of PE243-infected U-251 cells. (MOI: 1 FFU/cell, 72h). Blots were probed with antibodies as indicated. α -tubulin serves as loading control. Note that MSI1 positively regulates MCPH1 and CDK6, and negatively regulates NUMB and p21 protein levels.

Fig. 4. The A184V MCPH mutation disrupts RNA binding by MSI1 and impairs the ability of MSI1 to drive ZIKV replication.

A) Structural model of MSI1 (blue) and HRP1/RNA complex (grey/orange) predicts that the A184V mutation impairs the interaction with RNA due to a steric clash.

B) Western blots of cell lines stably expressing WT or A184V MSI1 transgenes. Note that cell lines were derived from either KO1 or KO2 cells as specified. Blots were probed with antibodies as indicated. p150 serves as loading control. Graph shows signal intensities of each protein normalized to parental cells.

C) MSI1 RIP from U-251 cells with genotypes as indicated. Rabbit IgG or MSI1 antibodies were used for immunoprecipitation. Input corresponds to 10% of whole cell extract. Western blot was probed with MSI1 antibody. Graphs below show qPCRs of bound transcripts. n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * $p < 0.05$, ** $p < 0.005$, not significant (n.s).

D) Quantification of viral RNA copies in U-251 cells with the indicated genotypes following infection with PE243 (MOI: 3 FFU/cell, 48h). n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: ** $p < 0.005$, not significant (n.s).

E) Changes in survival of U-251 cells with different genotypes following infection with PE243 (MOI: 3 FFU/cell, 48h). n=3 biological replicates. P-values were obtained from Student's t-test, unpaired, two-tailed: * $p < 0.05$, ** $p < 0.005$, not significant (n.s).

F) ZIKV infection of HEK293T cells as measured by FACS analysis of flavivirus protein E. FACS was performed on control, MsiWT or MsiAV transgene-expressing cells following infection with PE243 (MOI: 1 FFU/cell, 48h). n=2 biological replicates. Bar charts depict mean $\pm$ s.e.m.

G) Changes in survival of control, MsiWT or MsiAV transgene-expressing HEK293T cells following infection with PE243 (MOI: 3 FFU/cell, 48h). n=3 biological replicates, and P-values were obtained from Student's t-test, unpaired, two-tailed: * $p < 0.05$, ** $p < 0.005$, not significant (n.s).