Antisense inhibition of selenoprotein synthesis by Zika virus may contribute to neurological disorders and microcephaly by mimicking SePP1 knockout and the genetic disease PCCA

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Abstract:

Objective: Selenium status plays a major role in health impacts of various RNA viruses. We recently reported potential antisense interactions between viral mRNAs and host mRNAs of the antioxidant selenoprotein thioredoxin reductase (TR). Here, we examine possible targeting of selenoprotein mRNAs by Zika virus (ZIKV) as a pathogenic mechanism, because microcephaly is a key manifestation of Progressive Cerebello-Cerebral Atrophy (PCCA), a genetic disease of impaired selenoprotein synthesis. **Methods:** Potential antisense matches between ZIKV and human selenoprotein mRNAs were initially identified via nucleotide BLAST searches, using ZIKV genomic RNA as a probe. The strongest antisense matches of ZIKV regions, against human TR1 and selenoprotein P (SePP1), were validated by algorithms for prediction of RNA hybridization and microRNA/target duplexes. The ZIKV-SePP1 interaction was further assessed by gel shift assay. **Findings:** Computationally, ZIKV has regions of extensive (~30bp) and stable ($\Delta E < -50$kcal/mol) antisense interactions with mRNAs of both TR1 and SePP1, a selenium carrier protein essential for delivery of selenium to the brain. The ZIKV/SePP1 hybridization was experimentally confirmed at the DNA level using synthetic oligonucleotides. **Conclusion:** Antisense inhibition of TR may be a general RNA virus strategy to favor RNA synthesis over DNA. ZIKV-mediated antisense inhibition of SePP1 and TR1 in fetal brain could mimic SePP1 knockout in mice, contribute to neuronal cell death, and mimic the genetic disease PCCA, characterized by brain atrophy and microcephaly. When given to nursing mothers, sodium selenite can counteract neurological deficits in SePP1 knockout mice, suggesting that a similar approach might help reduce ZIKV-induced human fetal abnormalities.

Keywords: Zika virus | selenium | antisense inhibition | selenoprotein

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Antisense inhibition of selenoprotein synthesis by Zika virus may contribute to neurological disorders and microcephaly by mimicking SePP1 knockout and the genetic disease progressive cerebello-cerebral atrophy

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Methods: Potential antisense matches between ZIKV and human selenoprotein mRNAs were initially identified via nucleotide BLAST searches, using ZIKV genomic RNA as a probe. The strongest antisense matches of ZIKV regions, against human TR1 and selenoprotein P (SePP1), were validated by algorithms for prediction of RNA hybridization and microRNA/target duplexes. The ZIKV-SePP1 interaction was further assessed by gel shift assay.

Findings: Computationally, ZIKV has regions of extensive (~30bp) and stable ($\Delta E < -50$kcal/mol) antisense interactions with mRNAs of both TR1 and SePP1, a selenium carrier protein essential for delivery of selenium to the brain. The ZIKV/SePP1 hybridization was experimentally confirmed at the DNA level using synthetic oligonucleotides.

Conclusion: Antisense inhibition of TR may be a general RNA virus strategy to favor RNA synthesis over DNA. ZIKV-mediated antisense inhibition of SePP1 and TR1 in fetal brain could mimic SePP1 knockout in mice, contribute to neuronal cell death, and mimic the genetic disease PCCA, characterized by brain atrophy and microcephaly. When given to nursing mothers, sodium selenite can counteract neurological deficits in SePP1 knockout mice, suggesting that a similar approach might help reduce ZIKV-induced human fetal abnormalities.

Introduction

Among the many possible causes of microcephaly, one of the rarest is the genetic disease known as Progressive Cerebello-Cerebral Atrophy (PCCA)[a], which was first reported in 2003 by Ben-Zeev et al. as a previously undescribed autosomal recessive syndrome with an unknown genetic basis [1]. The syndrome was identified in children of Sephardic Jewish descent in Iraq and Morocco, and is characterized by progressive microcephaly, profound mental retardation, and severe quadriplegic spasticity. The underlying genetic basis was later identified by Agamy et al. [2] as a defect in selenium metabolism, disrupting the sole route to the biosynthesis of the 21st amino acid, selenocysteine (Sec), and thus making it impossible for affected individuals to synthesize selenoproteins. Specifically, the mutations responsible for this disease were found to be in the *SepSecS* gene, which encodes O-phosphoseryl-tRNA:selenocysteinyl-tRNA synthase, a selenium transferase that catalyzes the final step in the biosynthesis of Sec. Unique among the amino acids, Sec is synthesized while bound to its cognate tRNA, by conversion of O-phosphoseryl-tRNA^{Sec} to selenocysteinyl-tRNA^{Sec}. Homozygous individuals, lacking a functional copy of *SepSecS*, have therefore lost the unique catalytic benefits of Sec in selenoenzymes, at best having a serine hydroxyl group in place of the selenol of an active site Sec, leading to a drastic or total loss of catalytic activity in thioredoxin reductases, Se-dependent glutathione peroxidases, iodothyronine deiodinases, and other human selenoproteins.

The essential role of selenium and selenoproteins in mammalian brain function and development is well established (as briefly reviewed by Agamy et al. [2] and others [3-5]), and is critically dependent upon selenoprotein P (SePP1), a selenium carrier protein that is secreted by the liver and serves to transport selenium to other tissues [6]. Comparison of whole body knockout versus selective liver knockout of the *SePP1* gene shows that the brain is able to (and indeed *must*) synthesize its own version of the protein, in order to redistribute selenium within the brain [3, 7, 8]. After SePP1 uptake into cells of the blood brain barrier via its receptor ApoER2 [9, 10], selenium is cleaved from endocytosed SePP1 molecules by Sec lyase, so that selenium can then be dispersed within the brain, at least in part by incorporation into newly synthesized SePP1 that is in turn taken up by neurons [3, 5, 8].

This mechanism for SePP1-dependent transport of selenium into the brain is consistent with results showing that total knockout of SePP1 leads to a substantial reduction of selenoprotein synthesis within the brain [11], explaining the severe neurological and developmental deficits seen in *SePP1*$^{-/-}$ mice.

[a] PCCA is also known as pontocerebellar hypoplasia type 2D (PCH2D), Online Mendelian Inheritance in Man (OMIM) Phenotype MIM # 613811 Gene locus MIM # 613009.

Because biosynthesis of the entire selenoproteome within the brain is critically dependent on SePP1, it is not surprising that knockout of SePP1 in the brain produces a neurodegenerative phenotype under low selenium conditions that is similar to that observed by neuron-specific knockout of $tRNA^{Sec}$ (a condition analogous to the genetic defect of PCCA). Either SePP1 knockout under low selenium conditions [10], or $tRNA^{Sec}$ knockout alone [12], will result in total or near-abrogation of brain selenoprotein synthesis.

This raises the possibility that knockdown of SePP1 during fetal development by other mechanisms, such as antisense inhibition, might also be able to mimic the effects of SePP1 or $tRNA^{Sec}$ knockout, and produce neurodegenerative and neurodevelopmental symptoms similar to PCCA, including microcephaly, particularly if accompanied by selenium deficiency.

Significantly, we recently demonstrated potential virus-host RNA antisense interactions between Ebola and HIV-1 viral mRNAs and the mRNAs of several isoforms of human thioredoxin reductase (TR), which raises the possibility that this novel mechanism might contribute to the well-established role for selenium in the pathogenesis of a number of RNA virus infections [13]. Hence, we investigate here the possibility that ZIKV mRNA might target one or more human selenoprotein mRNAs by antisense, which could lead to knockdown of the target selenoprotein levels by either mRNA degradation, or, more likely, by inhibition of protein synthesis at the ribosomal level.

Although RNA viruses lacking a DNA stage generally replicate their RNA exclusively in the cytoplasm, it is well established that Flaviviruses in the same genus as ZIKV form replication complexes in the nucleus as well as in the cytosol of infected cells, with about 20% of viral RNA polymerase activity in the nucleus [14]. Thus, the possibility that ZIKV mRNA might be able to interact with unspliced cellular pre-mRNAs in the nucleus must be seriously considered. Formation of a virus-host antisense complex involving an intron of a cellular RNA transcript could lead to the inhibition of mRNA splicing and maturation, and thus serve as a mechanism for gene silencing.

In addition, Flaviviruses are known to produce a non-coding "subgenomic Flavivirus RNA" (sfRNA), corresponding more or less to the entire 3' untranslated region (3'UTR) of the mRNA [15], and which contains a number of highly conserved RNA secondary structures [16], some of which are quite dynamic and alter their conformation and interactions with different regions of the viral RNA during replication of the viral genome [17]. There is also some evidence that sfRNA may play various roles in the regulation and inhibition of cellular RNA processing [17-19]. Here we show that a region near the 5' end of the

predicted ZIKV sfRNA may target an intron in the TR1 primary transcript, potentially leading to knockdown of TR1 gene expression in infected cells. This is of particular interest in the case of ZIKV, because it has been shown that TR1 inhibition in neuronal cells leads to apoptotic cell death [20], which has also been demonstrated to be a consequence of ZIKV infection of cultured neurons [21].

Thus, virus-mediated antisense knockdown of host SePP1 and TR1 could together contribute to the most important ZIKV-associated pathologies.

Methods

Computational methods for identification of virus-host RNA antisense interactions: Potential antisense matches between regions of ZIKV and host selenoprotein mRNAs were initially identified via nucleotide BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A complete 2015 Brazilian ZIKV genomic mRNA sequence (see following section) was used as a probe for Nucleotide BLAST (blastn option) with default search parameters, initially against the Reference RNA sequence database (refseq.rna), restricted to Homo sapiens (taxid:9606) with selenoprotein as a search term. Because ZIKV is known to replicate in the nucleus, the search was later extended to include genomic sequences (corresponding to unspliced RNA; see below). The initial mRNA search lead to the identification of the core region of the antisense match to a coding region of SePP1 mRNA shown in Fig. 1. The RNAHybrid program (http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid) [22] was then used to assess the match using an algorithm and parameters designed for actual RNA:RNA interactions, and to investigate the possibility that, with some mismatches and bulges characteristic of dsRNA, the match at the RNA level might be extended in the 5′ and 3′ directions beyond the core region identified by BLAST. A more stringent method for accurate prediction of RNA-RNA interactions was then applied: the IntaRNA program (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) [22-24]. This algorithm factors in not only the hybridization energy of the interacting pair of RNAs, but also takes into consideration the "unfolding energy" required to overcome and outweigh the stability of any internal RNA secondary structures within the two individual RNA strands. The program will only identify a match if the net inter-strand binding energy is substantially lower (i.e., more stable) than the sum of the internal folding energies of the individual RNA strands. Using input fragments of up to 2000 bases in length (the maximum supported by the program) IntaRNA was in each case able to identify as the single most significant match an antisense interaction that was essentially identical to that found by the combined BLAST-RNAHybrid approach.

Reference sequences used in antisense sequence analysis and oligonucleotide design: The ZIKV Brazilian strain SPH2015, Genbank accession number KU321639, was used as a reference sequence for ZIKV, as it was the only complete Brazilian genomic sequence available at the time the study was initiated. Using methods described below, the most significant antisense match found to a region of a human selenoprotein was to selenoprotein P (SePP1). The initial SePP1 hit was to a region of mRNA common to all of its transcript variants; the sequence numbering used in the figures and text is from transcript variant 6 (the longest variant), Genbank # NM_005410.2. Because the antisense match to the SePP1 mRNA was at the very beginning of an exon, to explore the possibility that this match might extend into the upstream intron in unspliced RNA, that search was repeated using genomic DNA instead of the mRNA database, leading to the identification of an extended match spanning the intron-exon boundary in the SePP1 genomic sequence, Genbank DQ022288.1. An antisense match of a 3' noncoding region of ZIKV to an intron of thioredoxin reductase 1 (TR1) was also identified in the search vs. human genomic DNA; the TR1 sequence numbering used in the figures and text is from the complete TR1 gene sequence, Genbank # DQ157758.1.

Experimental confirmation of the predicted ZIKV-SePP1 antisense interaction at the DNA level: An electrophoretic mobility shift assay was used to confirm the predicted interaction shown in Fig. 1 via demonstration of *in vitro* DNA hybridization of the cognate ZIKV:SePP1 pair of sequences. The procedure was essentially as described previously [13]. Briefly, synthetic single stranded ssDNA oligomers (Integrated DNA Technologies, Inc., Coralville, IA) were obtained, corresponding precisely to the ZIKV and SePP1 sequence fragments shown in Fig. 1A. An additional oligo with a random sequence of identical base composition to the SePP1 fragment was used as a control. Prior to gel electrophoresis, oligos (~1 µg each in 10 µl PBS), either singly or in all three possible pairs, were incubated at 37°C for 15 hours, followed by cooling to room temperature over 1 hour. See legend to Fig. 1 for details about the arrangement of lanes on the gel. Bands were separated on a 4% agarose gel and visualized using ethidium bromide.

Results

The most significant BLAST-generated antisense match to a human selenoprotein identified using the entire ZIKV mRNA as a probe is to a coding region of SePP1 mRNA. The core match identified using BLAST involves ZIKV bases 9719-9740 (ATATGGGAAAAGTTAGGAAGGA) with 21/22 bases exactly matching bases in the minus sense of SePP1, with a single A base forming an unpaired bulge near the center of an extended double helix, observable in the computed hybridized RNA as the lower portion of

the more extended structure shown in Fig. 1A. By an iterative process with several slightly extended versions of this region of ZIKV as queries against the entire SePP1 mRNA, the RNAHybid program was able to identify the structure shown in Fig. 1A as a possible more extended antisense interaction between these two RNAs, as the minimum free energy result obtained when using the ZIKV 54mer shown as a query vs. the entire SePP1 mRNA.

Using fragments up to 2000 bases long from both ZIKV and SePP1 spanning the regions of interest as input, the IntaRNA program produced an essentially identical (but slightly truncated) interaction as the single most significant predicted RNA:RNA interaction (included as Fig. S1 in online Supplemental Materials). This result is important, as it indicates that even in the context of much larger lengths of RNA, these specific regions are potentially able to interact and form a hybrid virus-host dsRNA structure that is considerably more thermodynamically stable than the pair of individual mRNAs even when they are folded into their respective minimum energy states.

The potential of these two regions of the ZIKV and SePP1 mRNAs to interact was assessed at the DNA level via a gel shift assay, using synthetic oligos corresponding exactly in size and sequence to the regions shown in Fig. 1A, with a random oligo used as a control. Each of the three oligos (ZIKV, SePP1 and random) was run in an individual lane, and the three possible pairwise combinations were also run in three additional lanes (Fig 1B). Single-stranded DNA migrates to the bottom of the gel; only if the oligos interact to form dsDNA will a higher mass band be observed. Only the combination of ZIKV plus the native unscrambled SePP1 oligo form a band running at the expected mass for ~50bp dsDNA. The other pairs, ZIKV plus random control and SePP1 plus random control, still run as ssDNA, indicating a failure to hybridize, and demonstrating the specificity of the predicted ZIKV-SePP1 antisense interaction under these experimental conditions.

After these refinements of the extent of the region of strongest antisense interaction between the ZIKV and SePP1 mRNAs, yielding the interaction shown in Fig. 1A, a 52 base region of ZIKV was used as a BLAST query against the entire set of human mRNA (this region was identical to the 54 base ZIKV segment shown in fig 1A, minus the unpaired single bases at the 5' and 3' ends). The results of this BLAST search (included as Table S1 in online Supplemental Materials) show that the antisense match between this region of ZIKV and SePP1 is the very top hit in the entire database of human mRNA. Furthermore, most of the other highly ranked hits are not +/- strand antisense matches, but +/+ similarities, indicating possible sequence homology at the nucleic acid or protein level. This result

strongly suggests that the antisense pairing of these two mRNA regions of ZIKV vs. SePP1 could take place in vivo, as it would be stronger than any competing RNA:RNA interactions.

As mentioned in the introduction, the fact that Flaviviruses like ZIKV can form active replication complexes in the nucleus [14] raises the possibility that ZIKV mRNA might be able to interact with unspliced cellular pre-mRNAs. However, our initial search conducted vs. the human cellular mRNA database would fail to reveal potential virus-host antisense interactions targeting an intron of a cellular RNA transcript.

Consideration of this possibility led to the realization that the ZIKV/SePP1 interaction shown in Fig. 1A, which lies at the very beginning of an exon in SePP1, can be extended (after a short unpaired region) at the 3' end of the ZIKV sequence in question, into an upstream intron of SePP1. This expanded antisense interaction is shown as the RNAHybrid-generated structure in Fig. 2A, where an arrow indicates the intron/exon boundary in SePP1. This possibility was further validated using the IntaRNA program, which output an essentially identical RNA-RNA interaction to that shown in Fig. 2A when up to 2000 base long regions of ZIKV and genomic SePP1 were used as input. This result is shown as Fig. 3A; the full details and raw IntaRNA output are included as Fig. S2 in the online Supplemental Materials.

When the sequence of the entire ZIKV 3'UTR (corresponding more or less to the expected ZIKV subgenomic sfRNA sequence) was used as a BLAST query against the set of complete human selenoprotein genes (i.e., including introns), an antisense match vs. a TR1 intron forming the core of Fig 2B was identified. Following the same procedures detailed above for the ZIKV-SePP1 mRNA match (RNAHybrid followed by IntaRNA analysis), the result shown in Figs. 2B and 3B were obtained (i.e., the initial BLAST hit was slightly extended into a larger antisense interaction; the full details and IntaRNA output are included as Fig. S3 in the online Supplemental Materials). This finding suggests that the ZIKV 3' subgenomic sfRNA might target unspliced TR1 pre-mRNA in the nucleus, potentially resulting in knockdown of TR1 mRNA and protein synthesis.

Discussion

Selenium is an essential dietary trace element, having the lowest concentration in the earth's crust of any nutrient element, and whose bioavailability in the food chain varies widely by geographical location and agricultural practices [25]. Perhaps the most dramatic example of its critical importance for living organisms is evidence that periods of severe selenium depletion in Earth's oceans correlate closely with three major mass extinction events between 500 and 200 million years ago [26]. In regard to our results

involving ZIKV, it is significant that dietary selenium status has been associated with the pathogenesis and risk of disease progression for a number of viruses, almost all of which are RNA viruses (a point which we will revisit below). The most extensive evidence is related to HIV-1, for which a negative correlation between selenium status and disease progression or mortality has been firmly established (e.g., [27, 28]). Significant clinical benefits of selenium supplementation in HIV/AIDS cohorts have been demonstrated [29-32]. Other established cases of chemoprotective antiviral effects of dietary selenium include reduction in incidence of mammary tumors caused by MMTV, a retrovirus [33], the incidence of liver cancer and hepatitis in China linked to hepatitis B virus [34], and Keshan disease myocarditis linked to coxsackievirus, which becomes more virulent when combined with selenium deficiency [35]; similar effects have been reported for influenza virus [36, 37]. An underappreciated example of the pharmacological use of selenium vs. a viral infection is a reported case in which an Asian viral hemorrhagic fever was treated with oral sodium selenite (2 mg per day for 9 days), giving an overall 80% reduction in mortality [38].

The mechanisms underlying all the reported clinical impacts of selenium status on viral disease are probably complex and multifactorial, but selenium is certainly not an *antiviral* in the conventional sense of *a potent inhibitor of viral replication*. Typically, selenium seems to work by decreasing the harmful effects of viruses on the host, rather than blocking viral replication, because there are only a few reports of some direct inhibition of viral replication by selenium and other antioxidants (e.g., [39]), and some of that effect may be due to indirect mechanisms, such as NF-κB inhibition in the case of HIV-1.

The results reported here suggesting that ZIKV virus (like several other pathogenic RNA viruses [13]), may engage in antisense interactions with the mRNA or pre-mRNA of isoforms of TR, a ubiquitous selenoprotein oxidoreductase, may provide a new explanation for the observed interactions between dietary selenium and the pathogenesis of viruses with RNA genomes. Antisense targeting of host TR isoforms leading to decreased TR protein levels could be an effective strategy for many RNA viruses. DNA precursors can only be made from RNA precursors, and the key enzyme involved in DNA synthesis, ribonucleotide reductase (RR), uses thioredoxin (Trx) as the primary hydrogen donor for the reduction in mammalian cells [40]. Because they are required to regenerate reduced Trx, TR enzymes are important for sustaining the conversion of RNA precursors into DNA precursors. Note that, even with near-complete knockdown of TR1, an adequate basal level of RR activity (e.g. for DNA repair) could still be maintained in the cell by the glutaredoxin (Grx) system, as Grx can substitute for Trx; however, Trx is the favored S phase electron donor for RR, and the Grx system only produces about 10% as much

deoxyribonucleotide product as the Trx system (see Fig. 4 of Avval and Holmgren, 2009 [40]). Thus, viral antisense inhibition of TR protein synthesis is an ideal strategy for mammalian RNA viruses to slow down RR and to decrease the rate at which ribonucleotides are diverted for deoxyribonucleotide synthesis, thereby facilitating viral RNA synthesis.

In addition to our published results showing antisense targeting of TR isoforms by HIV-1 and Ebola [13], and the current results for ZIKV, we have found that a number of other RNA viruses show potential antisense anti-TR interactions, including avian influenza and mumps virus; some of these are shown in Fig. S4 in the online Supplemental Materials.

The potential virus-host "natural antisense" interactions that we have identified for ZIKV and other RNA viruses could have several possible biochemical outcomes, the most likely being inhibition of protein synthesis from the target mRNA (either via mRNA degradation, or via inhibition at the ribosomal level without mRNA degradation [41]). In the case of HIV-1 and Ebola, we have also proposed that, via host mRNA "antisense tethering interactions", viruses may capture functional RNA sequence motifs, e.g., to enable the expression of hidden viral selenoprotein genes, by recoding a conserved 3'-terminal UGA stop codon to be translated as Sec [13]. Thus, viral RNA antisense interactions with selenoprotein mRNAs could directly perturb host selenium biochemistry by various mechanisms.

For the current study, the most significant finding for ZIKV is its potential antisense targeting of SePP1, given the critical role of that selenoprotein in particular in brain function and development (as reviewed in the Introduction). Our results beg the question, could ZIKV-mediated perturbation of selenium biochemistry via downregulation of SePP1 and other selenoproteins like TR in the brain contribute to neuronal loss, cerebral atrophy and microcephaly?

We propose that ZIKV-mediated antisense inhibition of SePP1 and TR in fetal brain could mimic effects of SePP1 knockout in mice. Combined with low Se status, this could also mimic the genetic disease PCCA, via decreased brain selenoprotein synthesis. Selenoprotein downregulation could also contribute to other ZIKV-induced neurological symptoms; in addition to the evidence from SePP1 knockout studies reviewed in the Introduction, selenoprotein expression has been shown to be required for proper neuronal development and prevention of seizures and neurodegeneration [42]. Furthermore, SePP1 in particular has been shown to be a survival factor for embryonic neuronal cells [43].

In regard to the ZIKV-specific mechanism of neuronal pathogenesis, it has been reported that ZIKV infects neural progenitor cells and induces apoptosis and cell-cycle dysregulation [21]. This could be a

direct consequence of viral antisense-mediated TR downregulation, because inhibition of TR has been shown to induce apoptosis in neuronal cell lines [20].

If our proposed selenium-based mechanism for ZIKV pathogenesis proves to be valid, one reason for optimism is that Schweizer and coworkers found that supplementation of maternal drinking water with sodium selenite was able to prevent or reduce the neurological and growth deficits in newborn SePP1 knockout mice, presumably by supplying non-protein bound selenium directly to the brain via mother's milk, as a small molecule, either as selenite itself, or a metabolite [7]. This is consistent with evidence that *both* the absence of selenoprotein P and low dietary selenium have to be present for neuronal dysfunction to occur [10, 44]. It is important to note that selenite was found to be more effective than organic forms like selenomethionine in countering neurological defects and weight loss induced by SePP1 knockout in mice [44].

In the light of this mechanism, it is possible that selenium deficiency in pregnant mothers might be a risk factor for fetal microcephaly and related neurological birth defects. Although selenium deficiency per se may not be widespread in Brazil, it has been documented, e.g., in children [45]. However, there is another related environmental risk factor that has been associated with microcephaly in the past, and might be exacerbated by ZIKV-induced SePP1 knockdown: mercury toxicity. There is a well-documented antagonism between mercury and selenium [46, 47], with the latter able to mitigate mercury toxicity via an SePP1-related mechanism. SePP1 has been shown to decrease mercury toxicity and bioavailability via chelation of multiple mercury-selenium complexes by a single molecule of SePP1 [48]. ZIKV-induced SePP1 knockdown could impair this defense, and increase susceptibility to mercury-associated neonatal neuronal damage and microcephaly. High mercury levels exist in some Amazonian soils, rivers and fish, and ingestion of high levels of mercury may lead to a functional selenium deficiency, e.g., decreased selenoprotein expression [47]. This might help to explain why relatively higher rates of ZIKV-associated microcephaly have been observed in Brazil than in other some countries in the region.

Summary and Conclusions

We have presented evidence that several RNA viruses, including ZIKV (this work), HIV-1 and Ebola [13], may engage in antisense interactions with mRNAs of TR, an essential antioxidant selenoprotein. We propose here for the first time that TR inhibition may be a general RNA virus strategy to favor RNA synthesis over DNA, potentially shedding new light on the role of dietary selenium status in viral

pathogenesis that has been documented for many human and animal RNA viruses, including HIV-1, HCV and HBV, coxsackievirus, influenza, and viral hemorrhagic fever.

Most significantly, our results suggest that ZIKV may also target SePP1, a selenium carrier protein, which could disrupt selenoprotein synthesis in the brain. ZIKV-mediated antisense inhibition of SePP1 and TR in fetal brain could mimic the effects of SePP1 knockout in mice, contribute to neuronal cell death, and mimic the genetic disease PCCA, which has symptoms of brain atrophy and microcephaly. SePP1 knockdown could also increase susceptibility to mercury toxicity via a well-established mechanism. Lastly, when given to nursing mothers, the ability of sodium selenite to counter neurological deficits in SePP1 knockout mice suggests a similar approach with ZIKV-infected pregnant human mothers, particularly in the first trimester, might help to reduce the risk of ZIKV-induced fetal abnormalities.

Conflict of Interest

The authors have no conflicts of interest to declare.

Supplemental Material: The figures S1-S4 and Table S1 cited in the text can be obtained by email from the authors, or downloaded as a merged PDF file from: http://www.researchgate.net/profile/Ethan_Taylor2/publications

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References

1. Ben-Zeev B, Hoffman C, Lev D, Watemberg N, Malinger G, Brand N, et al. Progressive cerebellocerebral atrophy: a new syndrome with microcephaly, mental retardation, and spastic quadriplegia. J Med Genet. 2003 Aug;40(8):e96.
2. Agamy O, Ben Zeev B, Lev D, Marcus B, Fine D, Su D, et al. Mutations disrupting selenocysteine formation cause progressive cerebello-cerebral atrophy. Am J Hum Genet. 2010 Oct 8;87(4):538-44.
3. Richardson DR. More roles for selenoprotein P: local selenium storage and recycling protein in the brain. Biochem J. 2005 Mar 1;386(Pt 2):e5-7.
4. Schweizer U, Brauer AU, Kohrle J, Nitsch R, Savaskan NE. Selenium and brain function: a poorly recognized liaison. Brain Res Brain Res Rev. 2004 Jul;45(3):164-78.
5. Cardoso BR, Roberts BR, Bush AI, Hare DJ. Selenium, selenoproteins and neurodegenerative diseases. Metallomics. 2015 Aug;7(8):1213-28.
6. Hill KE, Wu S, Motley AK, Stevenson TD, Winfrey VP, Capecchi MR, et al. Production of selenoprotein P (Sepp1) by hepatocytes is central to selenium homeostasis. J Biol Chem. 2012 Nov 23;287(48):40414-24.
7. Schweizer U, Michaelis M, Kohrle J, Schomburg L. Efficient selenium transfer from mother to offspring in selenoprotein-P-deficient mice enables dose-dependent rescue of phenotypes associated with selenium deficiency. Biochem J. 2004 Feb 15;378(Pt 1):21-6.
8. Schweizer U, Streckfuss F, Pelt P, Carlson BA, Hatfield DL, Kohrle J, et al. Hepatically derived selenoprotein P is a key factor for kidney but not for brain selenium supply. Biochem J. 2005 Mar 1;386(Pt 2):221-6.
9. Burk RF, Hill KE, Olson GE, Weeber EJ, Motley AK, Winfrey VP, et al. Deletion of apolipoprotein E receptor-2 in mice lowers brain selenium and causes severe neurological dysfunction and death when a low-selenium diet is fed. J Neurosci. 2007 Jun 6;27(23):6207-11.
10. Burk RF, Hill KE, Motley AK, Winfrey VP, Kurokawa S, Mitchell SL, et al. Selenoprotein P and apolipoprotein E receptor-2 interact at the blood-brain barrier and also within the brain to maintain an essential selenium pool that protects against neurodegeneration. FASEB J. 2014 Aug;28(8):3579-88.
11. Hoffmann PR, Hoge SC, Li PA, Hoffmann FW, Hashimoto AC, Berry MJ. The selenoproteome exhibits widely varying, tissue-specific dependence on selenoprotein P for selenium supply. Nucleic Acids Res. 2007;35(12):3963-73.
12. Wirth EK, Bharathi BS, Hatfield D, Conrad M, Brielmeier M, Schweizer U. Cerebellar hypoplasia in mice lacking selenoprotein biosynthesis in neurons. Biol Trace Elem Res. 2014 May;158(2):203-10.
13. Taylor EW, Ruzicka JA, Premadasa L, Zhao L. Cellular Selenoprotein mRNA Tethering via Antisense Interactions with Ebola and HIV-1 mRNAs May Impact Host Selenium Biochemistry. Curr Top Med Chem. 2016;16(13):1530-5.
14. Uchil PD, Kumar AV, Satchidanandam V. Nuclear localization of flavivirus RNA synthesis in infected cells. J Virol. 2006 Jun;80(11):5451-64.
15. Pijlman GP, Funk A, Kondratieva N, Leung J, Torres S, van der Aa L, et al. A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. Cell Host Microbe. 2008 Dec 11;4(6):579-91.
16. Funk A, Truong K, Nagasaki T, Torres S, Floden N, Balmori Melian E, et al. RNA structures required for production of subgenomic flavivirus RNA. J Virol. 2010 Nov;84(21):11407-17.
17. Bidet K, Garcia-Blanco MA. Flaviviral RNAs: weapons and targets in the war between virus and host. Biochem J. 2014 Sep 1;462(2):215-30.
18. Clarke BD, Roby JA, Slonchak A, Khromykh AA. Functional non-coding RNAs derived from the flavivirus 3' untranslated region. Virus Res. 2015 Aug 3;206:53-61.

19. Schnettler E, Sterken MG, Leung JY, Metz SW, Geertsema C, Goldbach RW, et al. Noncoding flavivirus RNA displays RNA interference suppressor activity in insect and Mammalian cells. J Virol. 2012 Dec;86(24):13486-500.
20. Seyfried J, Wullner U. Inhibition of thioredoxin reductase induces apoptosis in neuronal cell lines: role of glutathione and the MKK4/JNK pathway. Biochem Biophys Res Commun. 2007 Aug 3;359(3):759-64.
21. Tang H, Hammack C, Ogden SC, Wen Z, Qian X, Li Y, et al. Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. Cell Stem Cell. 2016 May 5;18(5):587-90.
22. Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. RNA. 2004 Oct;10(10):1507-17.
23. Busch A, Richter AS, Backofen R. IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. Bioinformatics. 2008 Dec 15;24(24):2849-56.
24. Wright PR, Georg J, Mann M, Sorescu DA, Richter AS, Lott S, et al. CopraRNA and IntaRNA: predicting small RNA targets, networks and interaction domains. Nucleic Acids Res. 2014 Jul;42(Web Server issue):W119-23.
25. Haug A, Graham RD, Christophersen OA, Lyons GH. How to use the world's scarce selenium resources efficiently to increase the selenium concentration in food. Microb Ecol Health Dis. 2007 Dec;19(4):209-28.
26. Long JA, Large RR, Lee MSY, Benton MJ, Danyushevsky LV, Chiappe LM, et al. Severe selenium depletion in the Phanerozoic oceans as factor in three global mass extinction events. Gondwana Res. 2015;In Press.
27. Baum MK, Shor-Posner G, Lai S, Zhang G, Lai H, Fletcher MA, et al. High risk of HIV-related mortality is associated with selenium deficiency. J Acquir Immune Defic Syndr Hum Retrovirol. 1997 Aug 15;15(5):370-4.
28. Constans J, Pellegrin JL, Sergeant C, Simonoff M, Pellegrin I, Fleury H, et al. Serum selenium predicts outcome in HIV infection. J Acquir Immune Defic Syndr Hum Retrovirol. 1995 Nov 1;10(3):392.
29. Baum MK, Campa A, Lai S, Sales Martinez S, Tsalaile L, Burns P, et al. Effect of micronutrient supplementation on disease progression in asymptomatic, antiretroviral-naive, HIV-infected adults in Botswana: a randomized clinical trial. JAMA. 2013 Nov 27;310(20):2154-63.
30. Hurwitz BE, Klaus JR, Llabre MM, Gonzalez A, Lawrence PJ, Maher KJ, et al. Suppression of human immunodeficiency virus type 1 viral load with selenium supplementation: a randomized controlled trial. Arch Intern Med. 2007 Jan 22;167(2):148-54.
31. Jiamton S, Pepin J, Suttent R, Filteau S, Mahakkanukrauh B, Hanshaoworakul W, et al. A randomized trial of the impact of multiple micronutrient supplementation on mortality among HIV-infected individuals living in Bangkok. AIDS. 2003 Nov 21;17(17):2461-9.
32. Kamwesiga J, Mutabazi V, Kayumba J, Tayari JC, Uwimbabazi JC, Batanage G, et al. Effect of selenium supplementation on CD4+ T-cell recovery, viral suppression and morbidity of HIV-infected patients in Rwanda: a randomized controlled trial. AIDS. 2015 Jun 1;29(9):1045-52.
33. Schrauzer GN, Molenaar T, Kuehn K, Waller D. Effect of simulated American, Bulgarian, and Japanese human diets and of selenium supplementation on the incidence of virally induced mammary tumors in female mice. Biol Trace Elem Res. 1989 Apr-May;20(1-2):169-78.
34. Yu SY, Zhu YJ, Li WG. Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong. Biol Trace Elem Res. 1997 Jan;56(1):117-24.
35. Beck MA, Kolbeck PC, Shi Q, Rohr LH, Morris VC, Levander OA. Increased virulence of a human enterovirus (coxsackievirus B3) in selenium-deficient mice. J Infect Dis. 1994 Aug;170(2):351-7.
36. Beck MA, Nelson HK, Shi Q, Van Dael P, Schiffrin EJ, Blum S, et al. Selenium deficiency increases the pathology of an influenza virus infection. FASEB J. 2001 Jun;15(8):1481-3.

37. Yu L, Sun L, Nan Y, Zhu LY. Protection from H1N1 influenza virus infections in mice by supplementation with selenium: a comparison with selenium-deficient mice. Biol Trace Elem Res. 2011 Jun;141(1-3):254-61.
38. Hou JC. Inhibitory effect of selenite and other antioxidants on complement-mediated tissue injury in patients with epidemic hemorrhagic fever. Biol Trace Elem Res. 1997 Jan;56(1):125-30.
39. Hori K, Hatfield D, Maldarelli F, Lee BJ, Clouse KA. Selenium supplementation suppresses tumor necrosis factor alpha-induced human immunodeficiency virus type 1 replication in vitro. AIDS Res Hum Retroviruses. 1997 Oct 10;13(15):1325-32.
40. Zahedi Avval F, Holmgren A. Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for Mammalian s phase ribonucleotide reductase. J Biol Chem. 2009 Mar 27;284(13):8233-40.
41. Zeng Y, Yi R, Cullen BR. MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proc Natl Acad Sci U S A. 2003 Aug 19;100(17):9779-84.
42. Wirth EK, Conrad M, Winterer J, Wozny C, Carlson BA, Roth S, et al. Neuronal selenoprotein expression is required for interneuron development and prevents seizures and neurodegeneration. FASEB J. 2010 Mar;24(3):844-52.
43. Yan J, Barrett JN. Purification from bovine serum of a survival-promoting factor for cultured central neurons and its identification as selenoprotein-P. J Neurosci. 1998 Nov 1;18(21):8682-91.
44. Hill KE, Zhou J, McMahan WJ, Motley AK, Burk RF. Neurological dysfunction occurs in mice with targeted deletion of the selenoprotein P gene. J Nutr. 2004 Jan;134(1):157-61.
45. Vieira Rocha A, Cardoso BR, Cominetti C, Bueno RB, de Bortoli MC, Farias LA, et al. Selenium status and hair mercury levels in riverine children from Rondonia, Amazonia. Nutrition. 2014 Nov-Dec;30(11-12):1318-23.
46. Chen C, Yu H, Zhao J, Li B, Qu L, Liu S, et al. The roles of serum selenium and selenoproteins on mercury toxicity in environmental and occupational exposure. Environ Health Perspect. 2006 Feb;114(2):297-301.
47. Falnoga I, Tusek-Znidaric M. Selenium-mercury interactions in man and animals. Biol Trace Elem Res. 2007 Dec;119(3):212-20.
48. Suzuki KT, Sasakura C, Yoneda S. Binding sites for the (Hg-Se) complex on selenoprotein P. Biochim Biophys Acta. 1998 Dec 8;1429(1):102-12.

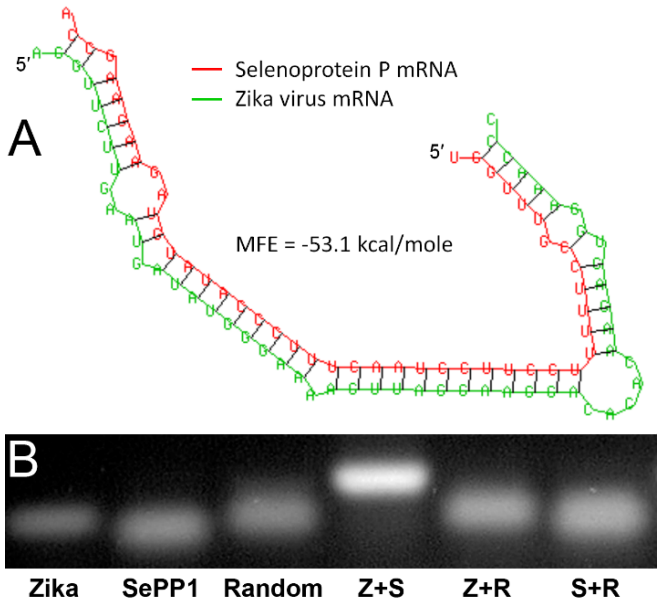


Figure 1. Predicted antisense interaction between complementary regions of human selenoprotein P (SePP1) mRNA and Zika virus mRNA.

A: The hybrid dsRNA secondary structure shown and computed interaction energy (as well as those in Fig. 2) were generated using the RNAHybrid 2.2 program. When used as a query vs. the entire SePP1 mRNA, the illustrated (green) 54 base region of Brazilian ZIKV strain SPH2015 (bases 9706-9759) yields the match shown as the minimum free energy (MFE) antisense interaction, to a (red) 47-base region of SePP1 (bases 541-587; these sequence ranges include the unpaired single base overhangs shown at each end).

B: Target-specific *in vitro* DNA hybridization of the predicted ZIKV-SePP1 antisense pairing was confirmed at the DNA level by gel shift assay, using DNA oligonucleotides corresponding exactly to the sequences shown in panel **A**. The left three lanes contain only a single (unpaired) ssDNA oligo, as follows: **Zika** = the 54 base fragment from panel A, **SePP1** = the 47 base fragment from panel A, **Random** = a 47 base randomly shuffled version of the **SePP1** fragment. The right 3 lanes are from incubations of pairwise combinations of those 3 oligos: Zika + SePP1 (**Z+S**), Zika + Random (**Z+R**) and SePP1 + Random (**S+R**). Of these, only the Zika + unshuffled SePP1 hybridize to form a slower moving dsDNA band that migrates as expected for ~50bp dsDNA (size markers not shown; for dsDNA ladder, see original uncropped gel photo, Fig. S5 in online Supplemental Materials). The **Z+R** and **S+R** combinations still migrate as single stranded DNA, demonstrating the specificity of the **Z+S** antisense interaction.

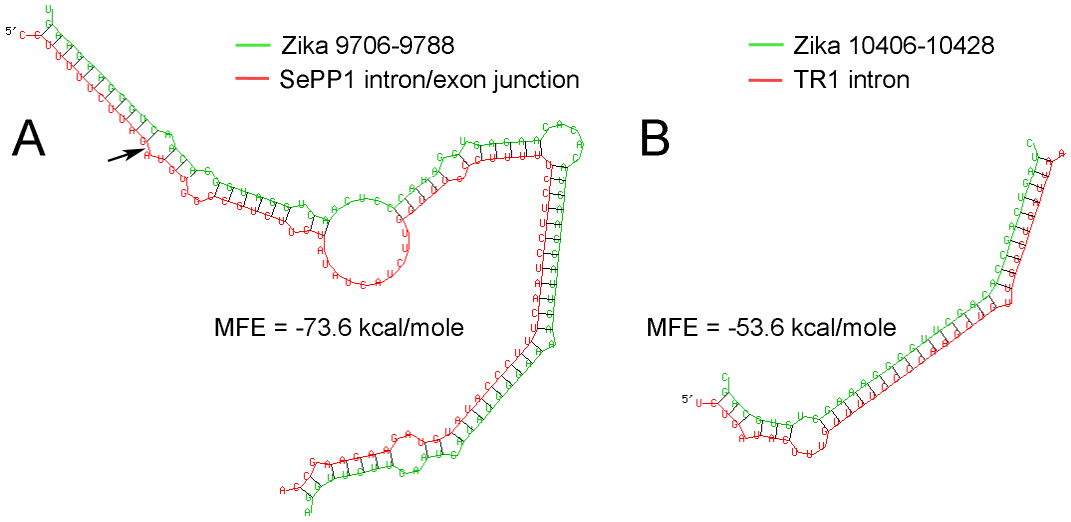
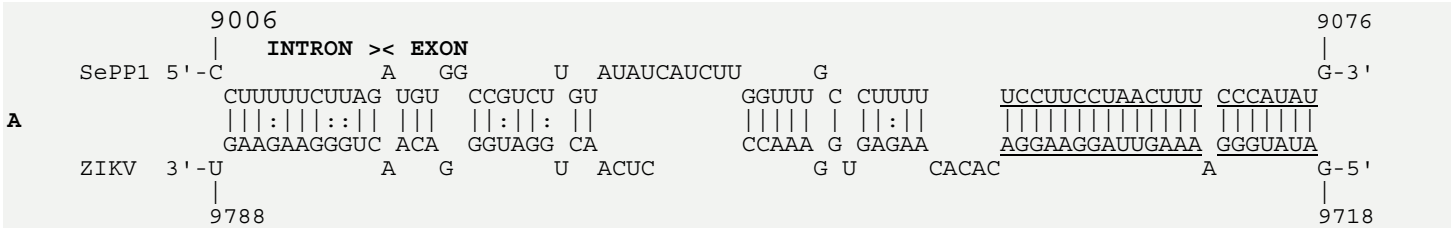


Figure 2. Predicted antisense interactions between Zika virus mRNA and regions either in introns or spanning RNA slice sites of human selenoprotein mRNAs.

A: Spanning an intron/exon junction of SePP1: Because Flaviviruses like ZIKV replicate in the nucleus as well as in the cytosol, ZIKV mRNA could also have the opportunity to interact with unspliced cellular pre-mRNAs. The ZIKV/SePP1 interaction shown in Fig. 1 can be extended by another ~30 bases at the 3' end of the ZIKV sequence, into an intron of SePP1, shown as the RNAHybrid-generated structure **A** above. The black arrow indicates the intron/exon boundary in SePP1. The right side of the structure pictured here is identical to that shown in Fig. 1A, but rotated counterclockwise. Formation of such a structure might inhibit the splicing and maturation of SePP1 mRNA. An essentially identical antisense complex is predicted using the IntaRNA progam, differing only in that the terminal 7 base pairs at the bottom of the structure shown above are not included in the IntaRNA prediction (see Fig. **3A**).

B: Targeting an intron of TR1: Both the RNAHybrid and IntaRNA programs predict the antisense interaction shown here between a 3' noncoding region of ZIKV mRNA (near the beginning of the well documented non-coding "subgenomic Flavivirus RNA" region) and an intron of TR1. An identical antisense complex is predicted using the IntaRNA progam (see Fig. **3B**).



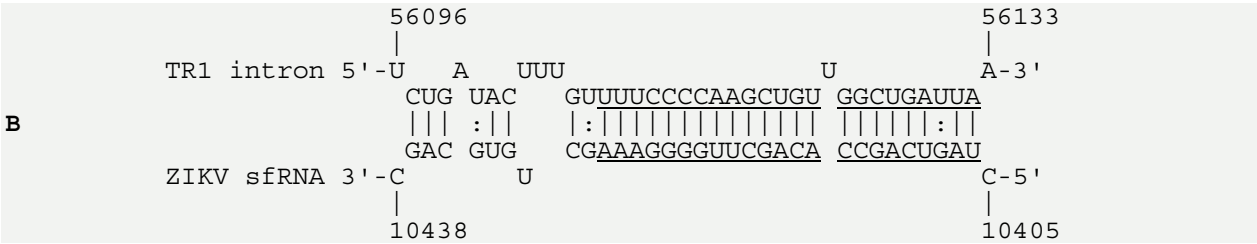


Figure 3. IntaRNA-predicted RNA-RNA interactions between Zika virus mRNA and regions of unspliced selenoprotein pre-mRNAs, confirming matches shown in Figure 2.

A. Predicted interaction between a 3' region of the ZIKV mRNA (polymerase coding region) and a region of SePP1 pre-mRNA, spanning an intron/exon boundary in SePP1. This is essentially the same structure predicted by RNAHybrid shown in Fig. 2A, but slightly truncated at the SePP1 3' end. The SePP1 intron/exon boundary is indicted by the **><** symbol above the sequence. For this analysis, a 500 base fragment of ZIKV (9,501-10,000) was scanned vs. a 2,000 base fragment (8,001-10,000) of the SePP1 genomic sequence. The computed hybridization energy for the structure shown is -59.6 kcal/mole, with a net energy of -25.7 kcal/mole, after subtraction of unfolding energies of 10.0 and 23.9 kcal/mole for the respective SePP1 and ZIKV RNA regions. The underlined region indicates the core of the antisense pairing of these two RNAs that was first identified by BLAST search as a 21/22 identity plus/minus sequence match, which is substantially extended by IntaRNA into the intron at left.

B. Predicted interaction between a region of the ZIKV non-coding "subgenomic flavivirus RNA" (sfRNA) and an intronic region of the human thioredoxin reductase 1 (TR1) pre-mRNA. For this analysis, the ZIKV ~300 base 3'UTR region was scanned vs. a 2000 base fragment (55001-57000) of the TXNRD1 genomic sequence. The computed hybridization energy for the structure shown is -48.0 kcal/mole, with a net energy of -24.8 kcal/mole, after subtraction of unfolding energies of 7.6 and 15.6 kcal/mole for the respective TR1 and ZIKV RNA regions. The underlined region indicates the core of the antisense pairing of these two RNAs that was first identified by BLAST search as a 22/24 identity plus/minus sequence match, which is only slightly extended by IntaRNA.