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From structure to function: Computational insights into Musashi-RNA complexes in the context of viral pathogenesis and beyond

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ABSTRACT: The Musashi (MSI) family of RNA-binding proteins, including MSI1 and MSI2, plays a crucial role in the post-transcriptional regulation of gene expression. This review delves into the involvement of Musashi proteins in the life cycle of RNA viruses, particularly their interaction with viral genomes. Emerging research highlights how Musashi proteins, by binding to specific motifs such as UAG in viral RNA, modulate viral replication and influence pathogenesis. Understanding these interactions is critical, as it reveals how viruses exploit host RNA-binding proteins to enhance their replication and to open potential avenues for therapeutic intervention. We explore the structural and computational insights into Musashi-RNA interactions using molecular dynamics simulations, docking studies, and other computational approaches to provide a detailed understanding of Musashi's role in viral pathogenesis. Furthermore, we highlight potential therapeutic strategies targeting Musashi-RNA interactions to mitigate viral diseases.

KEYWORDS: RNA-binding protein, exoribonuclease-resistant RNA, molecular dynamics simulation, Musashi protein family, viral infection

INTRODUCTION

RNA-binding proteins (RBPs) are essential regulators of gene expression, playing crucial roles in various cellular processes by interacting with RNA molecules [1]. These versatile proteins influence multiple aspects of RNA metabolism, including splicing, polyadenylation, nuclear export, localization, translation, and decay. By binding to specific RNA sequences or structures, RBPs can modulate the stability, translation efficiency, and localization of their target RNAs, thereby contributing to the precise control of gene expression. The diversity and specificity of RBPs allow cells to rapidly respond to environmental changes, developmental cues, and stress signals, ensuring the proper regulation of cellular function and homeostasis [2–7].

One example of RBPs that have attracted considerable research interest over the last years are the Musashi proteins, a family comprising the two paralogs Musashi-1 (MSI1) and Musashi-2 (MSI2), which are known for their roles in post-transcriptional gene regulation [8, 9]. By binding to their target messenger RNA (mRNA), Musashi proteins regulate translation, either repressing or activating mRNA expression depending on the cellular context [10].

The Musashi protein family demonstrates remarkable evolutionary conservation across vertebrates and invertebrates. In vertebrates, Musashi proteins have been identified and studied in various species, including human [11], mouse [12–14], frog (*Xenopus*) [15], and zebrafish [16]. The amino acid sequences at the epitope sites of MSI1 are conserved among human, mouse, and *Xenopus* proteins, allowing for common antibodies across these species [17]. In invertebrates, Musashi proteins have been identified in organisms such as fruit fly (*Drosophila*) [18], where they are essential for regulating asymmetric cell division of sensory organ precursor cells [19], as well as in acidians [20] and nematodes [21]. Musashi proteins are predominantly expressed in stem and progenitor cells [17, 22–24], where they play a vital role in neural development, stem cell maintenance, and cell fate determination [11, 25].

Dysregulation of Musashi proteins has been associated with various pathological conditions, most notably cancer, where they often play oncogenic roles [26, 27]. Their involvement is characterized by overexpression and dysregulation of key cellular processes [28]. In hematological malignancies, MSI2 is a critical regulator in acute myeloid leukemia and chronic myeloid leukemia, where its overexpression is associ-

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ated with blast crisis and poor prognosis [29]. In solid tumors, Musashi proteins are overexpressed in colorectal, lung, and pancreatic cancers and glioblastoma. This oncogenic potential is largely due to their ability to promote the translation of oncogenic mRNAs while repressing tumor suppressor mRNAs, contributing to tumor progression and resistance to therapy [30–32].

STRUCTURAL BIOLOGY OF MUSASHI PROTEIN RNA RECOGNITION

Musashi proteins comprise two highly structured RNA recognition motifs (RRMs), which are among the most abundant and evolutionarily conserved protein domains in eukaryotes. The tandem arrangement of RRMs in Musashi proteins is a common feature among many RRM-containing proteins and allows for cooperative binding, enabling recognition of longer RNA sequences with higher specificity than single RRMs alone [33]. While both domains recognize similar sequence motifs, they exhibit subtle differences in their binding preferences, allowing for fine-tuned regulation of diverse target mRNAs [34].

The Musashi RRMs, also referred to as RNA binding domains 1 and 2 (RBD1 and RBD2), each comprise approximately 90 amino acids that adopt a compact β1 – α1 – β2 – β3 – α2 – β4 secondary structure, which fold into a characteristic four-stranded anti-parallel β-sheet packed against the two α-helices [35, 36]. They bind with high specificity and affinity to single-stranded RNA targets containing the sequence (G/A)UnAGU ($n = 13$) [37, 38], typically located in the 3′ untranslated regions (UTRs) of target mRNAs. The central UAG trinucleotide has been identified as the preferred binding motifs of the Musashi RBDs [39], with additional flanking nucleotides further modulating the binding affinity, adding another layer of specificity to these interactions [40].

Structurally, the Musashi RBDs feature two highly conserved sequence motifs, RNP1 (octamer) and RNP2 (hexamer), located in the β3 and β1 strands, respectively. These motifs each contain three solvent-exposed phenylalanine residues which are significantly essential for the RNA recognition through base-stacking interactions [37, 41].

MUSASHI PROTEINS AND THEIR ROLE IN VIRAL INFECTIONS

In addition to their role as endogenous translational regulators and being linked to various cancers, Musashi proteins have become increasingly recognized for their involvement in viral infections, underscoring their broader biological relevance. RNA viruses, which rely on the host cellular machinery, frequently exploit host RNA-binding proteins to facilitate their replication, translation, and overall survival within the host. With their ability to bind specific RNA motifs, Musashi proteins can modulate various processes such as RNA stability, translation, and degradation. These regulatory effects may alter viral replication rates and the synthesis of viral proteins, collectively driving viral pathogenesis.

Zika virus (ZIKV), a mosquito-borne flavivirus (family Flaviviridae, genus *Orthoflavivirus*), has gained significant attention due to its association with severe neurological sequelae, including microcephaly in neonates and Guillain-Barré syndrome in adults [42, 43]. The identification of Musashi binding elements (MBEs), i.e. potential RNA targets for MSI binding, in the 3′-UTR of the ZIKV genome, coupled with *in vivo* data demonstrating that Musashi proteins not only interact with the ZIKV RNA but also enhance viral replication, has led to the understanding that Musashi proteins are involved in ZIKV-induced neurotropism [44]. ZIKV predominantly targets neural progenitor cells in the developing human fetal brain, where MSI1 is highly expressed. ZIKV infection disrupts the binding of MSI1 to its endogenous mRNA targets, leading to the dysregulation of genes involved in neural stem cell function and cell cycle regulation. This disruption interferes with neurogenesis and contributes to the developmental brain defects observed in congenital Zika syndrome [44].

To further investigate whether ZIKV is unique among flaviviruses in presenting accessible targets for Musashi proteins, we examined whether other flaviviruses share a similar neurotropic potential in an earlier study [45]. Our investigation focused on potential MBEs in the 3′-UTR and their presence in a single-stranded structural context, which is crucial for effective Musashi-RNA interactions. Using a thermodynamic model for RNA secondary structure prediction built on the ViennaRNA package [46], we calculated the average opening energies required to keep a stretch of RNA in a single-stranded structural context for each of the 64 possible trinucleotides in the 3′-UTRs of ZIKV and other flaviviruses. This approach provides a high-throughput, systematic alternative to experimental methods, enabling precise, large-scale mapping of RNA accessibility. Our data indicates that the canonical MBE, i.e. the UAG trinucleotide, is highly accessible in ZIKV, suggesting that it represents a bona fidae target for potential Musashi RRM-RNA interaction.

The 3′-UTR of the ZIKV genome harbors evolutionarily conserved RNA elements critical for viral replication and pathogenesis [47–49]. These conserved RNA elements are critical regulators of ZIKV's ability to evade host immune responses and promote viral replication, thereby contributing to its pathogenicity. A recent study examined the biophysical properties of the interaction between MSI1 and the ZIKV 3′-UTR, revealing that MSI1 binds not only to the canonical UAG motif but also to a non-canonical AGAA motif [50]. This motif is located within an evolutionarily conserved,

functional RNA structure known as exoribonuclease-resistant RNA (xrRNA), which protects parts of the viral genome from degradation by host exonucleases such as Xrn1 [51]. ZIKV has two xrRNAs in its 3′-UTR, xrRNA1 and xrRNA2 (Fig. 1), where only xrRNA2 exposes the non-canonical MBE. Notably, the AGAA motif is present multiple times in the ZIKV 3′-UTR, however, only one of these non-canonical motifs has been reported to bind MSI1 [50].

xrRNAs in flaviviruses fold into a distinctive three-way junction structure, consisting of stems P1, P2, and P3, along with two pseudoknots (PK1 and PK2). This configuration forms a ring-like structure that encircles the 5′-end of the xrRNA, providing a physical barrier against degradation by exoribonucleases [52]. When an exoribonuclease processes the viral genome from the 5′ to 3′ direction, it encounters this ring structure, which effectively blocks its progression. The mechanical stability of the xrRNA fold prevents the enzyme from extracting the 5′ end or disrupting the ring, thereby protecting downstream RNA sequences from degradation [53].

Research indicates that xrRNAs exhibit resistance against different exoribonucleases, suggesting that their protective mechanism is primarily based on the structural characteristics of the ring-fold rather than specific interactions with Xrn1 [54]. This is further supported by the observation that RNA-dependent RNA polymerases can process xrRNAs from the 3′ end, which lacks the protective ring-like structure [55]. Additionally, xrRNAs have not demonstrated any protective capacity against exonucleases operating in the 3′ to 5′ direction. These findings support the idea that the xrRNA resistance mechanism is predominantly structural and directionally specific.

xrRNAs are critical regulators of ZIKV's ability to evade the host immune response. Incomplete degradation products of the viral genome after dissociation of Xrn1, termed short flavivirus RNA (sfRNA), accumulate in the host cells and undertake regulatory functions. sfRNA interferes with several antiviral strategies of the host cells. By dysregulating mRNA turnover and inhibiting Xrn1 activity, the balance of mRNA within the host cell, and therefore also its ability to efficiently form antiviral proteins, is disrupted. Additionally, sfRNA has been observed to be an antagonist to the interferon pathway in vertebrate cells which drives the expression of proinflammatory cytokines and consequently a plethora of antiviral genes. These mechanisms, along with further regulatory effects such as interference with siRNA and miRNA generation, are vital contributions to the life cycle of flaviviruses. Flavivirus mutants without the ability to block Xrn1 and form sfRNAs have therefore been shown to exhibit a drastically reduced pathogenicity compared to wild-type viruses [56].

COMPUTATIONAL STUDIES OF MUSASHI RRM-RNA COMPLEXES

To obtain a detailed understanding of the interaction between Musashi proteins and their RNA targets, we employed theoretical and computational approaches [34, 57]. Initially, the apo-form of Musashi's RNA-binding domains, RBD1 and RBD2, were compared with their experimentally determined RNA-bound structures. The structures were predicted from the sequence using the AI-based method AlphaFold2 [58, 59], which produces highly reliable models in good agreement with experimental data, as indicated by high per-residue measure of local confidence (pLDDT) scores. These predictions produced highly reliable structural models. Our analysis revealed that both RBD1 and RBD2 undergo minimal conformational changes upon RNA binding, suggesting that the Musashi proteins' RNA recognition is pre-determined by their native structure, rather than induced by the binding event.

In a recent study, all-atom Gaussian accelerated MD (GaMD) simulations were performed to investigate dynamic interactions between the Numb RNA and MSI1 protein [63]. In continuation of our earlier work, molecular dynamics (MD) simulations were performed starting from AlphaFold-predicted structures of Musashi proteins [34]. Each system was simulated for 100 ns at 310 K using explicit solvent conditions, following MD procedure as previously outlined [32]. These simulations offered atomistic insights into the binding of the canonical UAG motif to Musashi's RBDs, highlighting the structural and energetic contributions to RNA recognition (Fig. 3).

To quantify the binding affinities of Musashi-RNA interactions, the Solvated Interaction Energy (SIE) method [64] was employed. The SIE method calculates binding affinities by combining molecular energies and solvation effects, adjusted by empirical scaling. Our results showed that both RBD1 and RBD2 exhibit comparable binding free energies of approximately −16.5 kcal/mol when interacting with the canonical UAG motif. Altering the UAG sequence significantly reduced the binding affinity, further confirming that UAG is the preferred RNA target of Musashi proteins [39].

In a subsequent study, the interactions between Musashi's RNA-binding domains and RNA were investigated using parallel cascade selection molecular dynamics (PaCS-MD) [57], starting from the AlphaFold predicted structures of MSI1 and the NMR-determined structures of RNA binding motifs (PDB IDs: 2RS2 and 5X3Z). This analysis highlighted that MSI1 consists of two well-structured RNA binding domains, while the unstructured linker region connecting them remains challenging to predict using AlphaFold. The PaCS-MD simulations provided further insight into how these RNA binding domains interact with target RNA se-

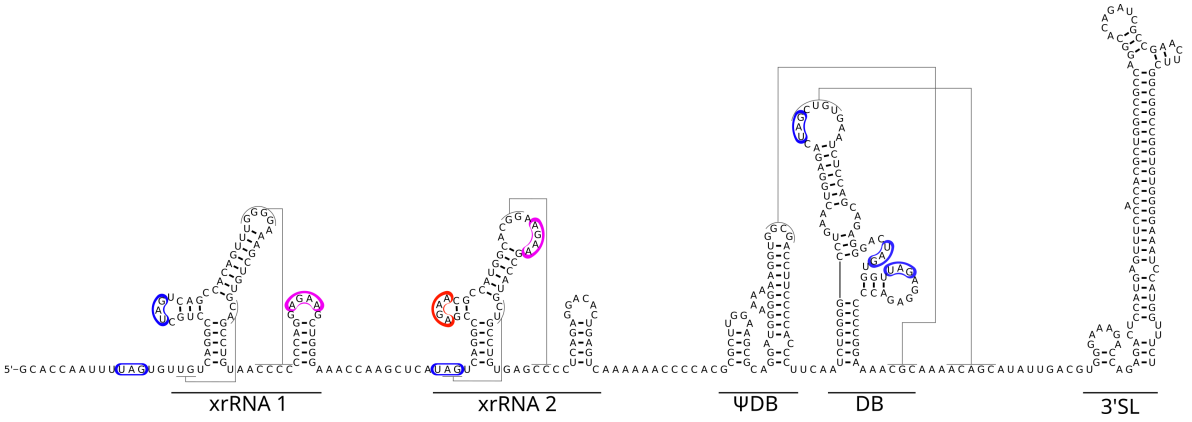

Fig. 1 Secondary structure of the ZIKV 3′-UTR, encompassing evolutionarily conserved, functional RNA elements. These include two xrRNAs (xrRNA1 and xrRNA2), a pseudo-dumbbell (ΨDB) element as well as a canonical dumbbell (DB) element, and a terminal 3′-stem loop element (3′SL). Pseudoknot interactions are indicated by canonical and newly described non-canonical binding motifs depicted in blue and red, respectively. Alternative non-canonical motifs exhibiting the same AGAA sequence are located in a hairpin downstream of xrRNA1 and an apical hairpin loop of xrRNA2 (highlighted in purple).

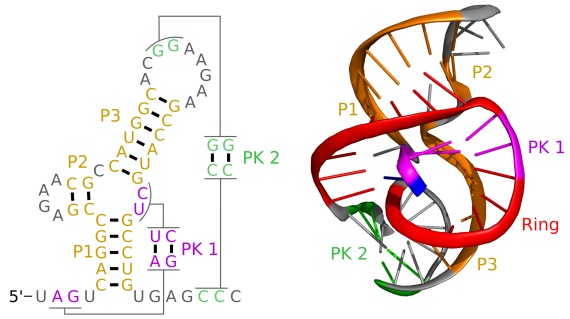

Fig. 2 Secondary (left) and tertiary (right) structures of the ZIKV xrRNA2 element. The non-canonical binding motif AGAA reported by Chen et al [50] is present in a single-stranded structural context in the hairpin loop of helix P2. The three-dimensional fold exhibits a ring-like architecture (depicted in red), through which the 5′-end of the xrRNA (highlighted in blue) threads.

quences, reinforcing the notion that MSI1's recognition of the UAG motif is highly specific. Fig. 4 displays the AlphaFold-predicted structure of MSI1 (panel a) and the RNA-bound complex (panel b). These findings contribute to a more comprehensive understanding of the mechanistic basis for Musashi-RNA interactions, particularly the protein's specificity toward the UAG motif.

Simulating MSI-RNA complexes with PACS-MD was demanding, particularly when the RNA binding motifs were separated by considerable distances. Nevertheless, experimental data supports that Musashi proteins can bind to RNA sequences where the motifs are separated by several nucleotides, sometimes extending to dozens [33]. One potential explanation for this simulation challenge could be attributed to the inherent structural complexity of RNA molecules, which are known to fold back onto themselves, thereby forming stable secondary and tertiary structures, such as helical regions, through intramolecular base pairing. Accurate RNA 3D structure prediction remains a significant burden due to the computational demands associated with modeling these large and intricate folding patterns [65].

MUSASHI BINDING OF ALTERNATIVE RNA MOTIFS

The recent discovery that MSI1 also binds non-canonical RNA targets motivated us to follow up on our earlier studies and explore the nature of these interactions in more detail. To this end, we performed simulations to study the binding of the MSI1 RBDs to sequence motifs present in the ZIKV xrRNA2, as postulated [66]. We were particularly interested in the association complexes of MSI1 RBD1 and RBD2 with the RNA pentamers AGAAC and GAGAA, which are found in the ZIKV xrRNA2. Fig. 5 depicts the structure of MSI1 RBD1 and RBD2 in complex with the non-canonical RNA motif GAGAA, as predicted using AlphaFold3 [67]. Of particular interest is the thermodynamics of the non-canonical MSI1-RNA interactions. In continuation of our earlier work [34], we computed the binding free energies of MSI1 RBD1 and RBD2 with non-canonical targets AGAAC and GAGAA, addressing the question of whether canonical motifs confer higher specificity and affinity for Musashi binding (Fig. 6). For RBD1, these interactions exhibited lower binding affinities compared to the classical GUAGU motif. For RBD2, however, our data suggest binding free energies for the non-canonical GAGAA motif in the same range as for the canonical GUAGU motif. This exhibits the possibility that RBD1 binds the canonical GUAGU

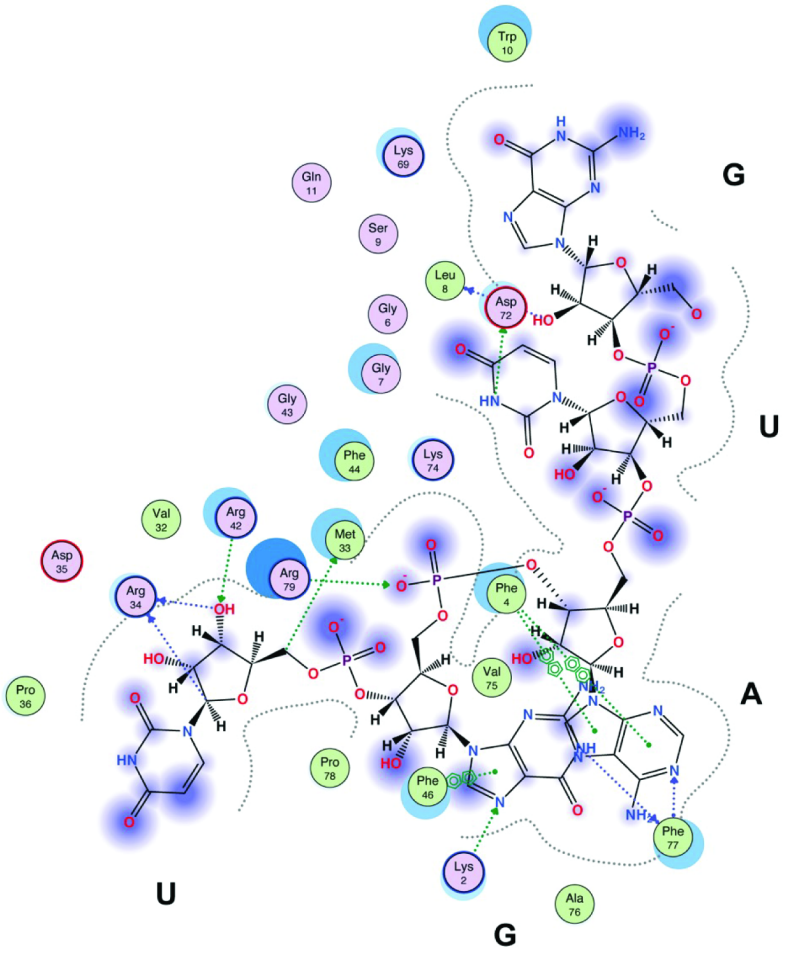

Fig. 3 RNA-protein interaction scheme of Musashi1 RBD1 bound to the GUAGU RNA pentamer, which includes the canonical UAG binding site. The initial structure has been calculated by AlphaFold2 using Colabfold [60], followed by MD 100 ns simulation using the AMBER16 program package [61]. The plot has been generated with the Molecular Operating Environment (MOE) program package [62].

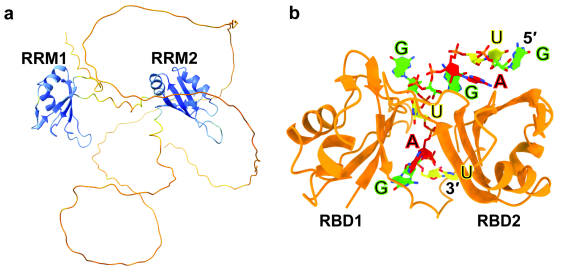

Fig. 4 (a) Structure of the human MSI1 protein calculated by AlphaFold (ID AF-O43347-F1-v4). The two RRMs are well-defined, while regions outside the RMMs are unstructured. (b) Interaction complex of the MSI1 RBDs with the target RNA (sequence GUAGGUAGU) computed by PACS-MD56.

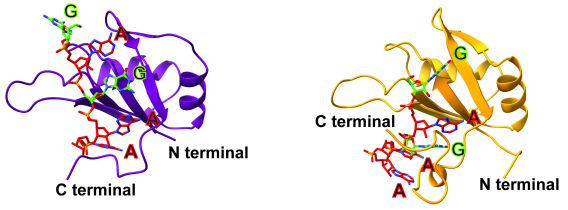

Fig. 5 MSI1 RBD1 (left) and RBD2 (right) in complex with the non-canonical RNA sequence GAGAA, predicted by AlphaFold3 [67].

motif upstream of the ZIKV xrRNA, while RBD2 may interact with the non-canonical motif within the AGAA tetraloop in stem-loop P2 of the ZIKV xrRNA2 (Fig. 1).

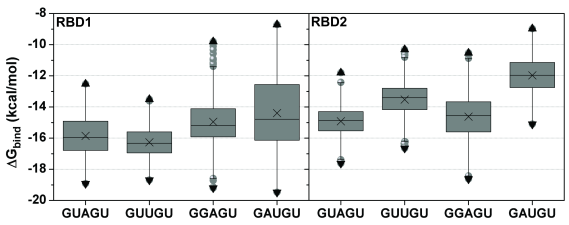

Fig. 6 Binding free energies of various pentanucleotides with MSI1 RBD1 and RBD2 calculated by the SIE method, following the computational procedures described in [34]. Dark grey boxes represent interquartile ranges (IQR) containing energy values between 25% and 75% of the distribution. Whiskers denote the 1.5 IQR rang values. Lines within the boxes represent the median, while cross signs represent mean values. Grey balls represent outliers.

INTERPRETATION AND SIGNIFICANCE

The study of Musashi proteins reveals how RNA-binding proteins influence cell fate and contribute to disease progression. Understanding the structural characteristics of RNA-protein interactions becomes crucial for achieving deeper insights into these mechanisms. Through computational approaches, we have gained insights into how these proteins interact with their target RNA sequences, shedding light on their involvement in both oncogenesis and viral pathogenesis.

CONTEXT-DEPENDENT ROLES AND MECHANISTIC INSIGHTS INTO MUSASHI-RNA INTERACTIONS

Musashi proteins exhibit remarkable functional plasticity, acting as either translational repressors or activators depending on the cellular context and specific RNA targets. In stem and progenitor cells, Musashi proteins help regulate the temporal and spatial expression of proteins necessary for stem cell maintenance and differentiation. This same regulatory flexibility becomes pathological when Musashi is dysregulated, contributing to the development and progression of various cancers.

In the context of viral infections, Musashi proteins are emerging as key regulators of RNA virus replication. In particular, ZIKV's interaction with MSI1 in neural progenitor cells reveals how viral genomes can hijack host RNA-binding proteins to enhance viral replication. Musashi binding elements identified in the 3′-UTR of the ZIKV genome are accessible to MSI1, promoting viral replication and contributing to ZIKV-induced neurotropism. The disruption of MSI1's normal function during ZIKV infection interferes with neural development, leading to the neurological defects seen in congenital Zika syndrome.

The two RNA recognition motifs of Musashi proteins are critical for their RNA-binding specificity and function. Structurally, the tandem arrangement of the RRMs allows for coordinated binding to target RNAs, enhancing specificity for the canonical UAG motif. Our computational data, based on AlphaFold [68] predictions and molecular dynamics simulations, show minimal conformational changes in the RRMs upon RNA binding [34, 57].

Further insights into the nature of Musashi-RNA interactions have been revealed by simulating association complexes with both canonical and non-canonical RNA motifs. Notably, Musashi proteins exhibit flexibility in binding alternative motifs, as evidenced in their interaction with the AGAA motif found in the evolutionarily conserved xrRNA2 element in the ZIKV 3′-UTR. This adaptability underscores the protein's wider regulatory potential.

THERAPEUTIC IMPLICATIONS OF TARGETING MUSASHI-RNA COMPLEXES

The multifaceted role of Musashi proteins in cancer and viral infections presents both challenges and opportunities for therapeutic intervention. Musashi proteins have emerged as promising targets for cancer therapy [69, 70] due to their significant roles in promoting cancer stem cell properties, tumor growth, and therapy resistance [30]. Their overexpression in various cancers and association with poor prognosis make Musashi proteins attractive targets for cancer therapy. Inhibition of these proteins could potentially suppress tumor growth, invasion, and metastasis by disrupting their regulation of oncogenic signaling pathways such as NUMB/Notch, PTEN/mTOR, and MYC2 [30, 71]. Promising results have been observed in preclinical studies with several small-molecule inhibitors, including (-)-gossypol and gossypolone, which exhibit anti-cancer effects [72, 73], largazole, which shows inhibitory effects on malignant cells [74], and natural product secondary metabolites [75]. Additionally, targeting MSI's oncogenic activity with small molecules has also shown therapeutic potential [76, 77]. Furthermore, targeting Musashi proteins may help overcome chemotherapy resistance, as their inhibition has been shown to enhance the response to paclitaxel in ovarian cancer [78–80].

However, the widespread expression of Musashi proteins in normal stem cells and their involvement in essential physiological processes necessitate careful consideration of potential side effects [81]. Given their regulatory roles in both healthy and pathological cells, it is important to understand how these proteins function in different biological contexts. Notably, their role in viral mRNA translation indicates that therapies targeting Musashi proteins could have broader applications beyond cancer treatment, potentially extending to antiviral strategies. As research advances, developing highly specific inhibitors or utilizing targeted delivery methods may help mitigate off-target effects while harnessing the therapeutic potential of modulating Musashi protein activity in cancer and other

diseases.

In addition to cancer, the role of Musashi proteins in viral infections opens new avenues for antiviral strategies. Disrupting the interaction between Musashi proteins and viral RNAs could reduce viral replication rates, as demonstrated in studies on ZIKV [44]. Developing selective inhibitors that specifically target Musashi's interaction with viral RNAs could lead to novel antiviral therapies with minimal off-target effects on the host's cellular processes.

EXPANDING THE POTENTIAL OF MUSASHI PROTEINS IN SYNTHETIC BIOLOGY

The evolutionary conservation of Musashi proteins across vertebrate and invertebrate species underscores their fundamental role in RNA metabolism, highlighting a possible way for leveraging Musashi proteins in synthetic biology applications. However, while direct applications of Musashi proteins in synthetic systems are limited, their well-characterized role as translational repressors make them promising candidates for regulatory modules in engineered gene circuits. For instance, truncated versions of MSI1 have been investigated as tools for regulating gene expression in prokaryotes, highlighting the versatility of Musashi proteins as regulators in synthetic biology. A recent study specifically explored the use of a truncated MSI1 variant, containing both RNA recognition motifs, as a translational repressor in *Escherichia coli* [82, 83].

The unique RNA-binding properties of Musashi proteins offer intriguing possibilities for other synthetic biology applications. For example, by engineering specific RNA sequences that interact with Musashi proteins, one could create modular components for complex genetic circuits [84] which could be used to control gene expression with high specificity. Alternatively, the specific RNA-binding properties of Musashi proteins could be harnessed to develop novel biosensors. By fusing Musashi's RNA-binding domains with catalytic domains of RNA-modifying enzymes, one could create chimeric proteins capable of site-specific RNA modifications. This approach could be used to edit RNA sequences, opening up new avenues for manipulating gene expression and RNA processing.

FUTURE DIRECTIONS

Despite the advances made in understanding Musashi-RNA interactions, several questions remain. Future research should explore the role of non-canonical binding motifs in greater detail, particularly in the context of viral infections. The discovery of non-canonical MBEs in ZIKV raises the possibility that other RNA viruses could also exploit Musashi proteins for their replication [45]. Comparative studies across different RNA viruses could reveal conserved mechanisms of RNA-protein interaction, providing new targets for therapeutic intervention.

Additionally, while computational approaches have significantly advanced our understanding of Musashi's structural and energetic contributions to RNA binding, experimental validation is highly essential in comparison with computational considerations. Integrating such computational predictions with high-resolution structural studies, like cryo-electron microscopy (cryo-EM) and NMR spectroscopy could provide deeper insights into the mechanistic basis of Musashi-RNA interactions and help refine models for drug design. Further molecular simulations of MSI1, in particular of the binding sites RBD1 and RBD2 in complex with various RNAs, based on AlphaFold3 and molecular docking using HADDOCK [85] should lead to some progress in the understanding of the selectivity of the MSI1-RNA interaction.

In conclusion, the Musashi family of RNA-binding proteins represents a critical node in the regulation of mRNA translation, with significant implications for stem cell biology, cancer, and viral infections. As our understanding of these interactions deepens, it will pave the way for the development of targeted therapies that exploit the unique properties of Musashi proteins to treat a range of diseases.

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