A structured RNA motif locks Argonaute2:miR-122 onto the 5’ end of the HCV genome

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microRNAs (miRNAs) form regulatory networks in metazoans. Viruses engage miRNA networks in numerous ways, with Flaviviridae members exploiting direct interactions of their RNA genomes with host miRNAs. For hepatitis C virus (HCV), binding of liver-abundant miR-122 stabilizes the viral RNA and regulates viral translation. Here, we investigate the structural basis for these activities, taking into consideration that miRNAs function in complex with Argonaute (Ago) proteins. The crystal structure of the Ago2:miR-122:HCV complex reveals a structured RNA motif that traps Ago2 on the viral RNA, masking its 5’ end from enzymatic attack. The trapped Ago2 can recruit host factor PCBP2, implicated in viral translation, while binding of a second Ago2:miR-122 competes with PCBP2, creating a potential molecular switch for translational control. Combined results reveal a viral RNA structure that modulates Ago2:miR-122 dynamics and repurposes host proteins to generate a functional analog of the mRNA cap-binding complex.
Mammalian Argonaute (Ago) proteins use ~22 nt short RNA guides to target partially complementary sites generally found in the 3' UTRs of mRNAs, recruiting additional factors that promote post-transcriptional gene silencing through translational repression and induction of mRNA decay. miRNAs form a complex regulatory network, modulating diverse pathways in metazoan biology. Misregulation in miRNA activity is connected to developmental defects and cancer.

While the animal miRNA machinery appears to derive from a primordial antiviral RNA silencing mechanism, the relentless tides of viral evolution have carved out a multitude of ways in which host miRNA pathways are exploited to benefit viral fitness. Pro-viral adjustment of the host expressionome is achieved by viral-encoded miRNAs (e.g. KSHV1) or modulation of host miRNA activity (e.g. HPV5), including retargeting of host miRNA complexes to cellular mRNAs. The most direct, yet poorly understood, mechanisms involve binding of host miRNAs to genomes of single-stranded RNA viruses of the Flaviviridae family: HCV (miR-122)8,9, BVDV (miR-17)10, and ZIKV (miR-21)11 exploit host miRNAs to increase viral fitness through direct interactions with regions within their RNA genomes rich with secondary structure.

The interaction between the HCV genome and the liver-abundant miRNA miR-122 was the first direct viral RNA-host miRNA interaction to be discovered and is the best-studied to date. HCV is a positive-strand single-stranded RNA virus that infects approximately 71 million people worldwide. In most patients the acute infection progresses to chronic liver infection, negatively affecting liver function and increasing the risk for developing hepatocellular carcinoma. The virus' decade-long persistence in the host is maintained through a delicate balance of genome replication, translation, RNA decay, and maintenance of viral subcellular compartments.

Stabilization of HCV RNA was reported through two tandem miR-122 complementary sites at the very 5' end of the viral genome and the liver-abundant miRNA miR-122 was the first direct viral RNA-host miRNA interaction to be discovered and is the best-studied to date. HCV is a positive-strand single-stranded RNA virus that affects approximately 71 million people worldwide. In most patients the acute infection progresses to chronic liver infection, negatively affecting liver function and increasing the risk for developing hepatocellular carcinoma. The virus' decade-long persistence in the host is maintained through a delicate balance of genome replication, translation, RNA decay, and maintenance of viral subcellular compartments.

SL1 is required for full protection against XRN1. Site-1 contains a stem-loop structure (stem-loop 1, SL1) that physically separates predicted base-paired regions between miR-122 and the HCV RNA (Supplementary Fig. 1b). From a miRNA-targeting perspective, SL1 is highly unusual as miRNA binding sites are typically devoid of secondary structure. We wondered if this viral RNA structure contributes to protection from XRN1. We prepared a mutant 5' UTR RNA containing a 4-adenosyl linker in the place of SL1 (ΔSL1). Removal of SL1 led to an ~4-fold reduced half-life of the RNA in our nuclease assay compared WT (Fig. 1d). Surprisingly, combining the ΔSL1 mutation with a mutation that prevents Ago2 binding at Site-2 (MUT2) abolished protection from XRN1. The 5' terminus of HCV is required for replication and we observed that a replication-deficient mutant, with catalytic residues in NS5B mutated from GDD > AAG, exhibited a decline in viral RNA similar to the SL1, MUT1, and MUT2
mutants. To distinguish between replication and RNA stability effects, we prepared double mutant genomic RNAs, containing NS5B GDD > AAG in addition to MUT1 or ΔSL1. Viral RNA degradation immediately following electroporation is driven by multiple mechanisms, including exosome attack of the viral 3' end. Nonetheless, RT-PCR revealed a small but significant effect of the 5' terminal mutations on RNA stability (Supplementary Fig. 1g). The combined results demonstrate the SL motif at Site-1 is essential to HCV fitness in Huh-7.5.1 cells, and that SL1 is necessary for Ago2-mediated protection of the viral 5' UTR from XRN1, especially in the absence of Ago2 occupancy at Site-2.

Crystal structure of the Ago2:miR-122:HCV Site-1 complex. As features such as SL1 have not yet been recognized in cellular miRNA targets, we investigated the structure of the Ago2:miR-122:HCV Site-1 ternary complex. We crystallized recombinant
Ago2 loaded with miR-122 in complex with a synthetic oligo-nucleotide corresponding to a variation of nt 1-29 of genotype 1 HCV. Diffraction data were collected to a resolution of 3 Å (PDB ID: 7KI3, Table 1, and Supplementary Fig. 2). Ago2, with its four globular domains (N-terminal, PAZ, MID and PIWI, with N and PAZ connected by linker L1, and PAZ and MID connected by linker L2) is found in a wide-open conformation cradling the viral RNA, with SL1 prominently extending from between the PAZ and PIWI domains. The viral 5′-terminus is positioned on the surface of the complex, lying against the N-terminal domain (Fig. 1f).

As in previous Ago2-miRNA-target structures,31,32, miR-122 threads through the central cleft of Ago2, with the 5′ end bound to the MID domain and the 3′ end bound to the PAZ domain (Fig. 1f, g and Supplementary Fig. 3a). HCV nucleotides A22-C28 are paired to the miRNA seed region, and HCV nucleotide A29 is anchored to the target nucleotide 1-A-binding pocket in the Ago2 MID domain31,33. The central region of the miRNA threads underneath SL1, to rejoin pairing to the viral RNA in the miR-122 supplementary region (Fig. 2a, and Supplementary Fig. 3b, c). The viral RNA and miR-122 thus form a three-way junction (Fig. 2a), with the HCV-paired seed region, SL1, and HCV-paired supplementary region intersecting in the center of Ago2, cradled within a structure termed the central gate34. Intriguingly, the viral nucleotide opposite miR-122 nucleotide 9 (target 9, t9; viral nucleotide G21), positioned between the seed region and SL1, is flipped out from the seed-paired duplex and bound in a previously unrecognized nucleotide-binding pocket on the surface of Ago2 (Supplementary Fig. 3d).

We identified extensive interactions between the HCV RNA and Ago2, including the 5′ end of Site-1 (paired to miR-122 supplementary region), the region around SL1, the region around t9/G21, and the Site-1 3′ end (Fig. 2b). Sequence alignments reveal C2 and C3 in the supplementary-paired region, the seed-paired region, and a purine at position t9 are conserved across HCV genotypes (Fig. 2c). Sequences corresponding to SL1 appear less conserved at a first glance. The apical loop shows very poor conservation, and, indeed, is exposed to the solvent, with no specific contacts with Ago2 (Fig. 1g). However, all genotypes are predicted to form a hairpin structure similar to SL1 with an overall mean folding energy of −11.3 kcal/mol (Supplementary Fig. 4a). We conclude that the Ago2:miR122:HCV Site-1 structure presented here is likely representative of all known HCV strains.

Ago2:miR-122 masks the HCV 5′ end from exonucleolytic attack. The HCV RNA 5′ end is held to the Ago2:miR-122 complex through base-pairing interactions to the supplementary region of miR-122 (Fig. 2d). The supplementary pairing was previously shown to be important for HCV RNA accumulation in Huh-7 cells35. Indeed, a Site-1 mutant RNA, in which supplementary pairing was disrupted, was rapidly released from the Ago2:miR-122 complex (Fig. 2e) and, in the presence of excess Ago2:miR-122, degraded by XRN1 8-times faster than a WT Site-1 RNA (Fig. 2f). XRN1 resistance also decreased with shortened miR-122 forms (i.e. shorter 3′ isomiRs) (Fig. 2g), which promote weaker supplementary interactions36,37. These data demonstrate supplementary pairing is essential for protecting the HCV RNA from XRN1, and may explain why the accumulation of HCV RNA in cultured cells requires longer miR-122 isomirs38.
Fig. 2 Ago2 shields the HCV 5’ end from enzymatic attack. a 3D structures (left) and schematic representation (right) of the miR-122:Site-1 three-way junction, composed of the seed duplex, SL1, and the supplementary duplex. b Cartoon representation of Ago2 with major HCV RNA contacts highlighted (determined by PDB PISA50). c Multiple sequence alignment of HCV genotype 1 subtype sequences (top) and genotype 1-8 subtype sequences (bottom). The total height of the letters at each position corresponds to the conservation at that position, while the height of the letters within the stack corresponds to their relative frequency. Supplementary contacts, SL1, t9, and seed contacts are indicated. d Surface representation showing closeup of the HCV RNA (blue) 5’ end bound to miR-122 (red) and positioned against the N-terminal domain residues E64, K65, and C66 (orange). e Ago2:miR-122 off-rate experiment using Site-1 WT and Site-1 with supplementary-pairing region mutated (GCCA > GAAA). Data shown as means of 3 independent replicates with error bars representing SEM. f XRN1 mediated degradation of Site-1 WT and with mutated supplementary-pairing region RNAs in the presence of Ago2:miR-122. Data shown as means of 3 independent replicates with error bars representing SEM. g XRN1 mediated degradation of WT Site-1 in presence of Ago2 loaded with indicated miR-122 3’ isomiRs, or no Ago2. Data shown as means of 3 independent replicates with error bars representing SEM. h Polyacrylamide gels showing XRN1 mediated degradation of Site-1 RNA alone (top), Site-1 with Ago2:miR-122 (middle), and Site-1 containing a 5’ U10 extension with Ago2:miR-122. Images are representative gels of triplicate experiments. Ago2:miR-122:Site-1 structure with U10 extension modelled (right) to illustrate potential 5’ end exposure.
The HCV 5' end is pinned against a loop in the Ago2 N-terminal domain (residues E64, K65, and C66) (Fig. 2d). We, therefore, hypothesized supplementary interactions protect the viral RNA by holding the 5' end in a position inaccessible to enzymatic attack. Alternatively, we imagined the intact ternary complex may create a physical barrier impervious to XRN1. This mechanism would be reminiscent of XRN1-resistant pseudoknot structures in flavivirus RNAs known to stop the processive 5'-3' exonuclease.

To distinguish between these possibilities, we designed a Site-1 RNA variant with an extended 5' tail of 10 U residues (Fig. 2h). Our rationale was the extended 5' tail would ensure XRN1 has access to the viral RNA 5' end when Ago2:miR-122 is bound. We radiolabeled the WT and tailed Site-1 RNAs at the 3' ends so that degradation intermediates of the 5'-3' exonuclease could be visualized. Control experiments revealed Ago2:miR-122 strongly protected the WT Site-1 RNA from XRN1. In contrast, Ago2:miR-122 did not protect the 5'-tailed construct, which was rapidly degraded. The disappearance of the tailed RNA was not accompanied by the accumulation of degradation intermediates. Thus, the Site-1 motif cannot stop XRN1 once it engages the viral RNA.

Site-1 elements SL1 and t9 hold Ago2 in an open conformation. SL1 is wedged between the Ago2 PAZ and PIWI domains, holding open the central gate, a structure in Ago2 that discourages pairing to nucleotides in the miRNA central region (guide (g) nucleotides g9–g12) (Fig. 3a)36. On the PAZ side, the interface is primarily formed by two rows of positively charged residues: K260 and K263 of the PAZ domain, and K354 and K355, which reside on the PAZ side of the central gate along with I353, which stacks below the ribose of HCV RNA C6. On the PIWI side, the PIWI loop inserts into the middle of the HCV-miR-122 three-way junction, making contact with nucleobases at the base of SL1 (Fig. 3b).

Adjacent to SL1, HCV nucleotide t9/G21 binds a pocket formed by the PIWI loop of the central gate. The t9/G21 purine nucleobase inserts between P601 and the aliphatic portion of T599 and P609 (Fig. 3c). While all HCV genotypes possess a purine base at this position, the over-representation of a G (Fig. 2d) might be explained by hydrogen bonds between the G21 2-amino moiety and backbone carbonyls of T599 and P609 (Fig. 3c). PIWI domain residues forming the pocket are highly conserved among metazoan miRNA-loading Ago proteins (Supplementary Fig. 5).

To examine the contribution of SL1 and t9 to HCV RNA binding we measured release rates ($k_{off}$) of RNAs corresponding to wild type Site-1 (WT Site-1), Site-1 with SL1 replaced by an A4 bridge (Site-1 ΔSL1), and a Site-1 with an abasic nucleotide at position t9 (Site-1 t9 abasic) (Fig. 3e). Surprisingly, release of the ΔSL1 and t9 abasic RNAs from Ago2:miR-122 was ~6-fold and ~3-fold slower than $k_{off}$ of the WT Site-1 RNA, respectively (Fig. 3e). Thus, despite the numerous contacts described above,
SL1 and t9/G25 are both detrimental to Site-1 binding compared to a Site-1 mutant RNA with an unpaired A9 bridge between seed and supplementary paired regions. We suspect these differences may reflect tension in the Ago2:mir-122 complex, which must adopt an extended conformation to engage SL1 (Fig. 3a).

We also examined Ago2:mir-122-mediated XRN1 protection and found ΔSL1 and t9 abasic Site-1 constructs behaved almost identically to WT Site-1 (Fig. 3f). This observation was puzzling, considering the differences in $k_{\text{off}}$ described above (Fig. 3e) and earlier results showing removal of SL1 from the full HCV 5′ UTR reduced protection from XRN1 (Fig. 1e).

Extensions 3′ of Site-1 have a deprotective effect that is counteracted by SL1. Our experiments lead to an apparent contradiction: SL1 removal clearly reduces protection of the HCV 5′ UTR from XRN1 by Ago2:mir-122 (Fig. 1d), and yet SL1 removal does not inhibit binding to the 5′ UTR (Supplementary Fig. 1f) and, in fact, decreases $k_{\text{off}}$ of Ago2:mir-122 dissociating from an RNA corresponding to Site-1 alone (Fig. 3c).

The first clue for resolving this conundrum was the observation that SL1 removal had no effect on protection from XRN1 when using a short RNA construct limited to Site-1 (Fig. 3f), indicating RNA downstream of Site-1 influences our results. To explore this possibility, we made Site-1 RNA constructs with an extended 3′ tail (Fig. 4a). We chose a tail sequence corresponding to mutated HCV Site-2 (Supplementary Fig. 1b), which does not stably bind the wild type Ago2:mir-122 (Supplementary Fig. 1b, c). In contrast to results with RNAs limited to Site-1 (Fig. 3f), XRN1 degradation of the extended Site-1 ΔSL1 RNA was >16-fold faster than degradation of extended Site-1 WT RNA (Fig. 4b). Similarly, XRN1 degradation of the extended Site-1 t9 abasic was ~2.5 times faster than the extended Site-1 WT RNA (Fig. 4b). Thus, RNA downstream of Site-1 diminishes the ability of Ago2:mir-122 to protect the HCV 5′ end from attack by XRN1 in the absence of the SL1 motif.

The Site-1 motif contributes to nuclease protection by reducing Ago2 lateral diffusion.

To understand how SL1 contributes to protection from XRN1, we reexamined the structure of the Ago2:mir-122:Site-1 complex. We noticed that by propping open the Ago2 central gate (Fig. 4c), SL1 also prop up helix-7, a structural element that enables Ago2 to make and break base-pairing interactions in miRNA seed region, as well as a shuttle between binding sites on a target RNA without physically dissociating from the target RNA molecule, a process termed lateral diffusion.26,27. Compared with the structure of Ago2 engaged with a target with seed and supplementary pairing, helix-7 in the Site-1 structure makes fewer contacts to the seed-paired RNAs (Fig. 4d). We, therefore, hypothesized SL1 and t9/G21 might enhance the protective effect of Ago2:mir-122 by counteracting lateral diffusion.

To test this model, we first determined how 3′ extensions impact interactions with Ago2. In all cases, 3′ extended constructs exhibited a ~2-fold reduced $k_{\text{off}}$ compared with their unextended counterparts (Fig. 4e). Thus, the 3′ extensions moderately stabilize interactions with bound Ago2:mir-122 molecules. Importantly, the rate of dissociation from the 3′ extended ΔSL1 RNA was >20-times slower than the rate of degradation by XRN1 (Fig. 4b) under similar conditions, indicating that most degradation occurs while Ago2 is physically associated with the RNA.

To further test the model, we determined whether protection of the extended Site-1 ΔSL1 construct could be restored by occupying the RNA 3′ extension with a second Ago2 protein. Our rationale was that binding a second Ago2:miRNA should sterically prevent Ago2:mir-122 molecules at Site-1 from laterally diffusing onto the 3′ tail. Indeed, the addition of Ago2 loaded with a guide complementary to the 3′ extension completely restored protection from XRN1, increasing the RNA half-life 25-fold (Fig. 4f). These results are similar to our early observation that removal of SL1 in combination with mutation of Site-2 (ASL1,MUT2) abolished protection of the HCV 5′ UTR from XRN1 (Fig. 1d).

If we suppose XRN1 acts by attacking viral RNA molecules released from Ago2:mir-122, the rate of RNA degradation by XRN1 should not exceed Ago2 off-rates in our $k_{\text{off}}$ measurements. Indeed, this relationship appears true for RNA constructs restricted to the footprint of Site-1 (compare Fig. 2e, f, and Fig. 3e, f). In contrast, the extended Site-1 SL1 and extended Site-1 t9 abasic RNAs are degraded significantly more rapidly than the $k_{\text{off}}$ would seemingly allow, indicating the presence of an additional state in which the 5′ end is accessible but Ago2:mir-122 is still bound. We suggest this additional state arises from the transient movement of Ago2:mir-122 onto nearby regions of the RNA via lateral diffusion, which can be counteracted by SL1 physically locking Ago2 on the HCV RNA 5′ end.

SL1 directly protects the 5′ end from phosphatase. We also tested the effect of SL1 on degradation by an enzyme other than XRN1, commercial calf intestine alkaline phosphatase (CIP). Dephosphorylation experiments analogous to the XRN1 experiments described above showed the 5′ phosphate of extended Site-1 WT RNA was protected from CIP by Ago2:mir-122, with a half-life of >550 min (Supplementary Fig. 6a). For extended Site-1 ΔSL1 the half-life was reduced to 14 min, revealing SL1 is essential for robust protection from CIP. In contrast to results with XRN1, occupying the 3′ MUT2 extension with Ago2:mir-122–mut only moderately restored protection to the extended ΔSL1 RNA, increasing the half-life by 40% (to 20 min) (Supplementary Fig. 6a). Similarly, SL1 was necessary for full protection of the minimal Site-1 RNA constructs (lacking 3′ extensions) by Ago2:mir-122 (Supplementary Fig. 6b). We conclude that, even in the absence of downstream RNA, SL1 has a direct effect on protection by Ago2:mir-122 against attack by the phosphatase.

Ago2 and PCBP2 form a mixed complex on the HCV 5’ tail. The genomes of ssRNA viruses act as templates for both protein translation and RNA replication, each necessary to make new virions. As translation proceeds 5′ to 3′ and the RNA-dependent RNA polymerase replicates 3′ to 5′, regulation is required.13 For HCV, one proposed mechanism to alternate translation and replication involves Ago2:mir-122 competing for binding at the viral 5′ end with another host protein, PCBP2.13 We tried to replicate these findings in our reconstituted system, using the 5′–terminal two-site RNA construct from experiments described above. Analysis by EMSA revealed a PCBP2-induced shift (Fig. 5a, lanes 1 and 2), confirming PCBP2 recognizes the HCV 5′ tail. To our surprise, including increasing amounts of Ago2:mir-122 with PCBP2 (Fig. 5a, lanes 3 to 5) diminished the shifted band from PCBP2 alone, and created a super-shift, running between the two bands created by Ago2:mir-122 alone (Fig. 5a, lane 6). This result indicates PCBP2 and Ago2:mir-122 can simultaneously occupy the HCV 5′ tail, forming a mixed complex.

To determine how each miR-122 binding site contributes to the mixed complex, we repeated the experiment with constructs possessing miR-122 binding mutations at either Site-1 (MUT1) or Site-2 (MUT2). Both mutant RNAs bound PCBP2 comparably to the WT RNA (Fig. 5a, compare lanes 2, 8, and 14). For MUT1, a faint super-shift was visible when adding high amounts of Ago2:mir-122 to PCBP2, but the PCBP2-only shift remained the prominent band (Fig. 5a, lanes 9–11). In contrast, the MUT2 RNA formed a single super-shifted band as observed in WT for
Ago2 with PCPB2 (Fig. 5a, lanes 15–17). Testing short RNAs corresponding to the individual sites revealed no PCBP2 binding to Site-1, and apparent competition between PCBP2 and Ago2 at Site-2 (Supplementary Fig. 7a, b). These results suggest PCBP2 binding to the 5′ tail is compatible with Ago2 occupying Site-1, and competitive with Ago2 for binding Site-2.

Finally, we tested whether PCBP2 could take the place of Ago2 at Site-2 to prevent 5′ end attack by XRN1 in the absence of SL1.

Fig. 4 SL1 protects from XRN1 by attenuating Ago2 lateral diffusion. a Cartoons of extended Site-1 WT, ΔSL1 and t9 abasic RNA constructs (blue). Red box indicates Site-1. Model of the extended Site-1 WT (blue ribbon) in complex with Ago2:miR-122, to illustrate the relative size of the 3′ extension. b XRN1 mediated degradation of 3′ extended Site-1 WT, t9 abasic, and ΔSL1 in the presence of Ago2:miR-122 over time. Data are shown as means of 3 independent replicates with error bars representing SEM. c PAZ domain and helix-7 positions (relative to MID-PIWI lobe) in structures of Ago2:miR-122 bound to HCV Site-1 (coral), or seed plus supplementary paired RNA (dark blue, PDB code 6N4O). d Comparison of the Ago2 helix-7-target RNA contacts (spheres centred at Cα atoms in contacting residues) for the Site-1, and seed plus supplementary paired RNA. The miRNA strand is coloured red and the target strand blue (only seed base pairs shown). e Release of Site-1 WT, ΔSL1, and t9 abasic Site-1 RNAs, with and without 3′ extensions, from Ago2:miR-122 over time. Data are shown as means of 3 independent replicates with error bars representing SEM. f Cartoon representation of the proposed mechanism. Ago2:miR-122 transiently diffuses onto 3′ extensions in the absence of SL1, allowing XRN1 access to the HCV RNA 5′ end (left). The presence of a second Ago2:miRNA can prevent lateral diffusion, re-establishing protection (middle-left). Ago2:miR-122 also laterally diffuses from a Site-1 with a t9 abasic nucleotide, albeit at a reduced rate compared with the ΔSL1 construct, again enabling XRN1 (middle-right). The full motif at Site-1 prevents Ago2:miR-122 lateral diffusion, and the target RNA is protected (right).
Compared to Ago2:miR-122 at Site-1, ΔSL1 alone, including PCBP2 increased the half-life of the RNA significantly (from 13 min to 24 min) (Fig. 5b), but far less than the 326 min observed for two Ago2 molecules binding in tandem (Fig. 4f). These data provide additional evidence for the presence of a molecular switch between Ago2 and PCBP2, but compared to the previous model, we find that this switch occurs specifically at Site-2. Furthermore, Site-1 has the ability to remain occupied by Ago2.
independently of the state of Site-2 (Fig. 5a, c). Occupancy of Ago2:miR-122 at Site-1 may be beneficial for masking the viral 5′ end and maintaining the integrity of the genomic RNA. Notably, the mixed complex containing Ago2:miR-122 and PCBP2 cannot provide full nuclease protection in absence of SL1 (Fig. 5b), that may further explain the importance of the SL1 motif during the viral life cycle.

Discussion

Here we dissected the two HCV miR-122 sites with respect to protection from XRN1, finding that Site-1 is essential, and Site-2 has a synergistic effect. Importantly, the conserved SL1 within Site-1 is necessary for Ago2:miR-122-mediated protection of HCV RNA from XRN1 and CIP. The crystal structure of Site-1 in complex with Ago2:miR-122 revealed a particularly open conformation of Ago2, through the use of multiple RNA-protein interaction sites, including a previously unknown 99 purine-binding site in the Ago2 PIWI domain. We propose Site-1 represents a previously unrecognized RNA motif that combines sequence-specific pairing to the miRNA with the secondary structure of SL1 to lock Ago2 in an open conformation on the HCV RNA 5′ end.

Our findings highlight the modular architecture of the HCV 5′ UTR. We suggest Site-1 has a primary protective function, which in turn enables versatile roles for Site-2. While Site-1 remains fully protected through the miRNA-recognition site. Notably, SL1 protrudes beyond the Ago2:miR-122 complex were performed according a previously published protocol27. Pelleted cells were resuspended in lysis buffer (50 mM NaH2PO4 pH 8.0, 100 mM NaCl, and 0.5 mM TCEP) and lysed by three passages through an M-110P microfluidizer (Microfluidics). The lysate was clarified by centrifugation at 29000 g for 15 min and initial purification by affinity (Ni NTA agarose, Qiagen) was combined with Micrococcocal nuclease (Takara) treatment to remove bound RNA (buffer 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 5 mM CaCl2, and 0.5 mM TCEP). Ago2 was loaded with a synthetic miRNA bearing a 5′-terminal phosphate (IDT) and the His tag was removed using Tobacco Etch Virus protease. After removal of the His tag using TEV protease followed by reversed Ni affinity purification, Ago2 loaded with a specific microRNA was captured using an antisense oligonucleotide (IDT) and eluted with a DNA competitor (IDT). The protein was passed through Q Sepharose Fast Flow resin (Cytiva) to further remove nucleic acids, and purified using size exclusion on a Superdex 75 16 600 column (Cytiva) on an Akta Pure FPLC (Cytiva) using 50 mM Tris-HCl pH 8, 1 M NaCl, 0.5 mM TCEP. After final dialysis into 30 mM Tris-HCl pH 8, 100 mM NaCl, 0.5 mM TCEP, protein concentration was determined on NanoDrop OneC (Thermo Fisher Scientific) using a calculated extinction coefficient of 280 nm of 198370 M⁻¹·cm⁻¹. Aliquots were flash frozen in liquid nitrogen and stored at −80 °C until use.

Crystallization. Ago2:miR-122 was diluted to 2 g/l (~20 µM) and mixed with synthetic HCV site 1 RNA (IDT) at 1.2x molar equivalents. Initial crystallization experiments were set up manually using 24-well plates (Hampton) using the hanging drop vapour diffusion method and 24-well plates (Hampton). Crystals were grown by vapor diffusion against 10 µl of reservoir solution containing 150 mM NaCl, 10 mM Tris-HCl pH 8, yielding hexagonal crystals. Crystals were prepared for freezing by transferring to a solution of 7% w/v PEG8000, 50 mM BaCl2, 100 mM Tris-HCl pH 8, yielding elongated hexagons. The crystals were prepared for freezing by transferring to a solution of 7% w/v PEG8000, 50 mM BaCl2, 100 mM Tris-HCl pH 8, and 50% w/v sucrose as cryoprotectant.

X-ray diffraction data collection and processing. Diffraction experiments were performed at SSRRL 12-2, APS GMI 23-ID-B, and ALS 5.0.2, where the final data set was collected. Diffraction data were indexed using DIAlS40, then scaled and merged with Aimless41. Initial phases were generated by molecular replacement using Phaser tetrahedron (Amerham, GE Healthcare Life Sciences) and a Hybond N+ nylon membrane (Amerham, GE Healthcare Life Sciences) using a doblot apparatus (GE Healthcare Life Sciences). Initial (zero) time point was taken by applying 50 µl to the apparatus (with vacuum) and washing with 50 µl wash buffer (30 mM Tris-HCl pH 8.0, 100 mM KOAc, 2 mM Mg(OAc)2, 0.5 mM TCEP, 0.005% NP-40) with a total volume of 750 µl and incubated at room temperature for 45 min to reach equilibrium. Protein-bound and free RNAs were separated by passing samples through a Protran nitrocellulose membrane (Amerham, GE Healthcare Life Sciences) and a Hybond N+ nylon membrane (Amerham, GE Healthcare Life Sciences) using a doblot apparatus (GE Healthcare Life Sciences). Initial (zero) time point was taken by applying 50 µl to the apparatus (with vacuum) and washing with 50 µl wash buffer (30 mM Tris-HCl pH 8.0, 100 mM KOAc, 2 mM Mg(OAc)2, 0.5 mM TCEP). The reaction time course was then initiated by the addition of 7 µl of unlabeled target RNA (100 µM final concentration) to the sample. 50 µl aliquots were passed through the doblot apparatus at various times, with each time point followed by 50 µl wash buffer. After air drying, the membrane strips were exposed to phosphor screens (GE

Methods

Oligonucleotides. Short oligonucleotides and primers were ordered from IDT (Coralville, IA) either desalted (for biochemistry) or HPLC-purified (for crystallography) (Supplementary Table S1).
Healthcare Life Sciences) for visualization. Screens were imaged on a Typhoon phosphorimager (GE Healthcare Life Sciences). Data availability Atomic coordinates and diffraction data have been deposited in the Protein Data Bank under accession code 7K1J. The biochemical and cell data generated in this study are provided in the Source Data file. Source data are provided with this paper.

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PCBP2 expression and purification. The coding sequence for PCBP2 was amplified from HEK293T cDNA and cloned into a pET22a vector (Novagen) encoding an N-terminal His tag followed by a recognition site for Tobacco Etch Virus (TEV) protease. The recombinant protein was expressed in BL21 Escherichia coli, by inducing with IPTG (1 mM) overnight at 16 °C after cultures had reached an OD600 of 0.9. Pelleted cells were resuspended in lysis buffer (50 mM NaH2PO4 pH 8, 1 M NaCl, 0.5 mM TCEP, 0.005% NP-40, 0.05 g/l BSA, 0.02 U/µl RNase inhibitor (NEB)) in a total volume of 126 µl at room temperature for 45 min prior to starting the XRN-1 reaction. A time point 0 was taken by removing (9 µl) to a fresh tube containing 20 µl of formamide loading buffer. The reaction was started by the addition of 13 µl XRN-1 at 30 nM (final concentration of 3 nM), and 10 µl aliquots were removed and pipetted into formamide buffer to stop the reaction at the indicated time points. The aliquots were analyzed on a denaturing polyacrylamide gel (15–20% acrylamide, 8 M urea) run in 0.5x TBE. The gels were used to expose phosphor screens (GE Healthcare Life Sciences) for visualization. Screens were imaged on a Typhoon phosphorimagery (GE Healthcare Life Sciences) and signal was quantified using ImageQuant (GE Healthcare Life Sciences). Dissociation rates were calculated in Prism (GraphPad Software), using a one-phase decay model and decay constants were compared using the extra sum-of-squares F test25. PCR primers are listed in Supplemental Table S1.

Luciferase assays. Reporter plasmids were prepared by cloning the HCV Site-1 sequence and its mutants (ASL1, delta supplementary, ASL1 and delta supplementary) into the psiCHECK-2 vector (Promega). HEK293T cells were seeded into 96-well plates. 12 h after seeding, the cells were transfected with 40 ng/well of the indicated plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. miR-122 was annealed with its passenger strand and transfected 5 h later at the indicated concentrations with Lipofectamine 2000. After 48 h, the medium was removed from the cells, and luciferase activity was assayed using the Dual-Glo Luciferase Assay System (Promega) on a Synergy H1 Hybrid Reader (BioTek).

Clustal Omega multiple sequence alignment. Indicated sequences were aligned using the Clustal Omega tool from the website of the EMBL-EBI in Hixon, UK (www.ebi.ac.uk/Tools/msa/clustalo/). Genbank accession numbers for HCV genotypes were obtained from the website of the International Committee for the Taxonomy of Viruses (https://talk.icvonline.org). Sequences corresponding to miR-122 Site-1 were obtained from NCBI. The list was manually curated to include one sequence per genotype (using the more recent one in cases where two were published), and to remove any incomplete sequence. The sequences were aligned using the Clustal Omega tool from the website of the EMBL-EBI in Hixon, UK (www.ebi.ac.uk/Tools/msa/clustalo/). Alignments were performed for all remaining sequences, as well as for the sequences within each genotype. Minimum folding energy for each sequence was calculated using the Vienna RNA package29.

Sequence logo generation. Sequence logos were generated using the online tool from UC Berkeley (weblogo.berkeley.edu/) with the Clustal Omega multiple sequence alignments as input.

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

Data availability
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