microRNA-122 amplifies hepatitis C virus translation by shaping the structure of the internal ribosomal entry site

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The liver-specific microRNA-122 (miR-122) recognizes two conserved sites at the 5′ end of the hepatitis C virus (HCV) genome and contributes to stability, translation, and replication of the viral RNA. We show that stimulation of the HCV internal ribosome entry site (IRES) by miR-122 is essential for efficient viral replication. The mechanism relies on a dual function of the 5′ terminal sequence in the complementary positive (translation) and negative strand (replication), requiring different secondary structures. Predictions and experimental evidence argue for several alternative folds involving the miR-binding region (MBR) adjacent to the IRES and interfering with its function. Mutations in the MBR, designed to suppress these dysfunctional structures indeed stimulate translation independently of miR-122. Conversely, MBR mutants favoring alternative folds show impaired IRES activity. Our results therefore suggest that miR-122 binding assists the folding of a functional IRES in an RNA chaperone-like manner by suppressing energetically favorable alternative secondary structures.
The hepatitis C Virus (HCV) is a positive strand RNA virus. The genome consists of ∼9600 nucleotides (nt) and contains a single open reading frame (ORF), flanked by 5′ and 3′ untranslated regions (UTR). The 5′ UTR contains a set of secondary structures that compose the internal ribosomal entry site (IRES), allowing a cap-independent translation of the viral genome. Cleavage of the resulting polyprotein by host and viral proteases gives rise to ten individual proteins, three structural proteins and seven non-structural (NS) proteins. The NS proteins are crucial for the formation of the membranous web, a vesicular structure containing the viral replication complexes. Genome amplification by the viral RNA polymerase initiates at the respective 5′ terminal structures of positive and negative strands.

The HCV 5′ UTR consists of four distinctly structured domains. Domain I (DI, nt 1–47, Fig. 1a) of the 5′ UTR is not essential for translation. Except for nt 5–20, which fold into a small G:C rich hairpin structure (stem-loop I, SLI), the current model suggests an extended single-stranded form of the remaining sequence. In contrast, the complementary 3′ region of the negative strand is engaged in an extensive stem-loop (SLIIz), which is crucial for RNA replication. Therefore, both strands contain a structural code in this region, which is necessary for either translation or replication.

The HCV IRES harbors three distinctly structured domains (DII-IV), which serve as a binding hub for the eukaryotic translation machinery. DII can adopt an L-shaped tertiary structure, which is essential for IRES function as it mediates the displacement of eukaryotic initiation factor 2 (eIF2) complex to facilitate attachment of the 60S ribosomal subunit. Furthermore, it contacts the 40S ribosomal subunit. DIII is mostly responsible for host factor recruitment, it binds eIF2 and competes for the association of eIF3 with the 40S ribosomal subunit. DIV includes a short segment of the core coding region and folds into a pseudoknot element, which positions the viral AUG start codon in the ribosomal P site.

MicroRNAs (miRNA or miR) are 20–24nt long non-coding RNAs, which fine tune the expression of the majority of human genes by inhibiting translation and accelerating mRNA decay. miRs are incorporated into an RNA-induced silencing complex (RISC), with one of four Argonaute (AGO 1–4) proteins as the central element. Target specificity is conferred by full complementarity of the seed region (5′ nucleotides 2–8), while the 3′ end is often imperfectly bound. The 5′ end of the HCV viral genomic RNA features two conserved microRNA-122 (mir-122) target sites. Mir-122 is liver-specific and highly abundant in hepatocytes, with over 60,000 copies per cell. It regulates lipogenesis, tumor suppression, and immune response. The impact of mir-122 binding on HCV genome, however, is in stark contrast to the canonical mode of action of miRNAs. Upon binding of mir-122, HCV genome translation and initiation of replication are increased. Mir-122 binding further masks the HCV RNA 5′ end, conferring resistance to the degradation by host nucleases Xrn1 and Xrn2 and thereby contributing to increased protein production.

In this study, we show that stimulation of IRES activity substantially contributes to the diverse functions of mir-122 in the HCV replication cycle. Our in silico analysis predicted that the mir-122-binding region (MBR) has a negative effect on the formation of SLII, due to its additional cis functions in the complementary negative strand sequence. We show that mutations in the MBR favoring or disfavoring SLII formation indeed stimulate or abrogate translation, independent of mir-122. Our data therefore suggest that mir-122 binding suppresses alternative folds of the 5′ UTR that interfere with IRES function.

Results

Stimulation of HCV translation is critical for replication. MiR-122 has been shown to be a vital host factor for HCV, as it enhances RNA stability, translation, and replication. We aimed to understand the contribution of translation enhancement to the miR-122 dependency of HCV, which is discussed controversially in literature, and to address the yet unknown mechanism.

To quantify the impact of miR-122 on HCV translation, we used full length (FL) viral genomes and subgenomic (SG) HCV replications, lacking the viral structural protein coding regions, both harboring luciferase reporter genes (Luc) as integral parts of the viral polyprotein (Fig. 1a). We generated two variants of these constructs: One version followed the regular architecture of the HCV genome, in which translation of the polyprotein is initiated at the HCV IRES (Luc-SG, Luc-FL), causing RNA stability, replication, and translation to be dependent on miR-122. In an alternative version, we inserted a heterologous poliovirus (PV) IRES element, rendering translation of the HCV genome independent from miR-122 (Fig. 1a, PI-luc-SG, PI-Luc-FL). Huh7 hepatoma cells and subclones (e.g. Huh7.5 cells16) are the standard cell culture model for studying HCV replication cycle and contain high copy numbers of miR-122.

To allow the most thorough analysis of miR-122 effects, we used Hep3B hepatoma cells lacking detectable miR-122 expression (Fig. 1b). Reconstitution of miR-122 by co-transfection of a miR-122 duplex (mirWT) rendered Hep3B cells permissive for HCV replication to similar levels as those measured in Huh7.5 cells (Fig. 1b, d), whereas no replication was observed upon transfection of a miR-122 mutant with a point mutation in the seed region (Fig. 1c, d, mirmut). To dissect the relative contribution of translation on miR-122 dependency of HCV, we co-transfected mirWT or mirmut with SG and FL replications capable of miR-122 dependent and independent translation (Fig. 1a) into Hep3B cells (Fig. 1e–h) and determined the impact of miR-122 on translation. Specifically, translation of SG and FL replications with the native HCV 5′ UTR and HCV IRES driving translation of the polyprotein was affected most by exogenous miR-122, leading to an increase in luciferase activity by ~4–6-fold, compared with mirmut (Fig. 1e, g, left panel). In contrast, those replications with insertion of a heterologous miR-independent PV IRES showed less stimulation than miR-122-dependent reporters (Fig. 1f, h, left panels). This remaining increase in luciferase activity mediated by mir-122 was likely due to increased stability of the HCV genome, which was comparable in the range of 1.7-fold for luc SG and PI-luc-SG, since both constructs shared the same 5′ end (Supplementary Fig. 1a–c).

Therefore, the net effect of miR-122 on translation efficiency of the HCV IRES was 2–3 fold, in line with previous reports10–12. Despite this apparently moderate additional impact of miR-122 on translation, the Luc-SG replicon did not replicate in absence of miR-122 (Fig. 1d, right panel), whereas insertion of the PV IRES allowed substantial replication levels under the same conditions (Fig. 1e, right panels). Similar results were obtained with full-length viral genomes (Fig. 1f, g). We validated these data in Huh7.5-based mir-122 knockout cells (Fig. 1b, d). Similarly, insertion of a miR-122 independent PV IRES element resulted in a significant increase in replication efficiency in the absence of miR-122 relative to a replication-deficient replicon (AGDD, Supplementary Fig. 1d–g). Due to the high reproducibility of data, we only used Hep3B cells in all subsequent experiments.

These results demonstrated that a moderate stimulation of HCV translation by miR-122 resulted in an overall substantial effect on viral RNA replication, indicating that translation significantly contributes to the miR-122 dependency of HCV.
Stability and translation are essential for HCV replication. To further disentangle the contribution of miR-122 on the processes of translation and/or stability/replication, a bicistronic luciferase reporter construct with two copies of the HCV 5′ UTR was designed (Bi-Luc-SG, Fig. 2a). The first cistron consisted of HCV 5′ UTR (UTR1) harboring the cis-acting elements essential for RNA synthesis fused to a PV IRES allowing miR-122-independent translation of the Nano luciferase (NLuc) gene. Translation of the second cistron comprising a firefly luciferase (FLuc) fused to the HCV NS proteins NS3-5B was initiated by the HCV IRES contained in the second copy of the HCV 5′ UTR (UTR2). Since translation of the replicase proteins NS3-5B is a prerequisite for RNA replication, UTR1 and UTR2 thereby allowed studying the relative contribution of miR-122 to genome

Fig. 1 Initial translation and its importance for the HCV life cycle. a Representation of replicons and full-length viruses used in this study (FLUC firefly luciferase, NL nano luciferase). 5′UTR/1RES elements are indicated. The box shows an enlarged schematic of the miR-122-binding sites in the 5′ UTR. b Quantification of mature miR-122 by stem-loop qRT-PCR in naïve Huh7.5 cells, a Huh7.5 miR-122 knock-out cell clone (Huh7.5ΔmiR), Hep3B cells before (Hep3B), and after (Hep3BmiR) electroporation of miR-122 mimic. All values were normalized to miR-23b levels, which is homogeneously expressed in hepatic cells. c Binding of wild type miR-122 (miRWT) to a target RNA (HCVWT) and influence of seed mutations (miRmut). d Comparison of HCV replication in different cell lines by luciferase assay after co-electroporation of reporter replicons (Luc-SG) and miRWT or miRmut into Hep3B cells. Reporter activity was measured at 4 and 48 h. e Luciferase assay of initial translation (left panel) and replication (right panel) of subgenomic HCV. f Luciferase assay to determine the effects of PV IRES-driven polyprotein translation on the viral life cycle (PI-Luc-SG). g, h Confirmation of results from (e) and (f), using full length reporter viruses (Luc-FL, PI-Luc-FL), n.d. not detectable. Mean values (±SD), n = 3, in technical duplicates. RLU relative light units. ΔGDD replication deficient mutant. For translation, statistical significance was determined for miRWT against miRmut; for replication HCV + miRWT was tested against ΔGDD. *P<0.05, **P<0.01, ***P<0.001
stability/replication and translation, respectively, on genome replication.

Northern blot analysis revealed that two identical copies of the 5'UTR yielded truncated fragments at late stages of the replication cycle, likely due to sequence recombination (Fig. 2b, band #2). Therefore, the stem sequence in SLI of UTR2 was mutated from C:G to U:A, inactivating replication functions of UTR2. This mutation prevented recombination without significantly affecting the replication efficiency of the bicistronic replicon (Fig. 2b).

To assess the individual contribution of miR-122 to HCV stability/replication/translation, we mutated UTR1 and UTR2 individually in the Bi-Luc-SG construct (Fig. 2a) by introducing a point mutation into the seed sequence of both miR-122-binding sites, complementary to the change in miR mut (Fig. 2c). Therefore, supplementation of miR WT or miR mut would only rescue miR-122-dependent functions in the respective matching UTR1 or 2 variants. Indeed, addition of either miR variant stimulated luciferase activities to varying extent early after transfection, either due to enhanced translation efficiency or RNA stability (Fig. 2d). For further control we deleted UTR1 and replaced UTR2 by an EMCV IRES to exclude crosstalk between UTR1 and UTR2 (Supplementary Fig. 2). These constructs revealed very similar results concerning miR-122 dependency as expected, apart from undetectable stimulation of translation by miR-122 upon deletion of UTR1, suggesting that in short-term experiments, the stabilizing effect of miR-122, preventing degradation by Xrn1, seems to dominate in these constructs, probably due to their excessive length. However, none of the replicons harboring a mutant UTR sequence replicated to significant levels in the presence of miR WT or miR mut (Fig. 2e), demonstrating that both functions of miR-122 in stability and translation substantially contributed to efficient HCV replication. We further could rescue replication of a Bi-Luc-SG replicon harboring two mutant UTRs with miR mut (Supplementary Fig. 3a), as well as constructs with one mutant UTR by simultaneous addition of miR WT and miR mut (Supplementary Fig. 3b), albeit with very low efficiency. This was likely due to a generally lower efficiency of miR mut even in the context of a monocistronic replicon (Supplementary Fig. 3c) and a reduced stimulation by miR WT upon co-application of miR mut in case of UTR1+2 WT Bi-Luc-SG (Supplementary Fig. 3d).

SLII is affected by alternative conformations. After establishing that miR122-driven translation stimulation is important to HCV replication, we investigated the underlying mechanism. Since the MBR itself is dispensable for IRES activity, the effect of miR-122 binding on the target sequence must be relayed from DI (nt1-47, Fig. 1a) to the downstream sequence to positively modulate translation. The current structural paradigm proposes a relaxed state of DI except for the stable SLI stem-loop. In contrast, the complementary region representing the 3′ end of the viral negative strand RNA (3′(-)) is engaged in a conserved stem-loop (SLIIz', Fig. 3b), which is a cis-acting replication element (CRE)
crucial for RNA amplification\(^3\). Since complementarity of sequence also favors similar structures (except for U-G rich sequences), a structure similar to SLII\(^z\) might also form in the positive strand 5' UTR, which would consequently prevent misfolding of the IRES. As the MBR is part of this alternative structure, miR-122 binding to this sequence could prevent misfolding of the IRES. Based on this hypothesis in vitro folding experiments of HCV RNA and in silico prediction of the conformation of DI and II were performed. Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) reactivity data was used as constraint to assist structure prediction by RNA structure (Fig. 3c). In line with our hypothesis, the algorithm predicted an alternative stem-loop, encompassing nucleotides 21–105 as energetically most favorable option, from here on referred to as SLII\(^{alt}\) (Fig. 3c), which partly resembled SLII\(^z\) in the negative strand RNA. Importantly, SLII\(^{alt}\) also included parts of SLII, preventing the formation of a functional IRES. Alternative labeling by DMS and NAz produced both a pattern matching the prediction obtained with NAI (Supplementary Fig. 4a, b).

To gain deeper insights into the impact of DI on the folding of SLII, we subjected fragments of DI + II (nt 1–120) or DII (nt 40–120) to 1D\(^1\)H NMR analysis. While the 1D NMR spectra of the DII imino protons were not significantly influenced by a decrease of experimental temperature (Fig. 3e), we observed line broadening of the imino proton resonances of the DI + II fragment at lower temperatures (Fig. 3d). This could indicate exchange between several conformers in solution at lower temperatures, or aggregates of RNA. To differentiate between these options, we performed size-exclusion chromatography combined with online detection by multi-angle laser light scattering (MALS) and refractometry. The DI + II fragment was present as a monomer or dimer, both binding a copy of miR-122, as expected (Supplementary Fig. 5c). However, native gel electrophoresis at 4°C with DI + II displayed only a single band, irrespective of the preincubation temperature (Supplementary Fig. 5d), further arguing against oligomerization. In addition, in silico folding analysis predicted six structures within the top 10%
of the minimal free energy structure for the DI + II fragment, in contrast to only three for the DII fragment (Fig. 3f), suggesting that the presence of DI increased the variety of alternative conformations of the 5′ terminus of the HCV RNA. Some of the energetically most favorable interfered with the formation of SLII.

We reasoned that binding of miR-122 would prevent the formation of alternative RNA structures in the positive strand RNA, and thereby support establishment of the IRES. To experimentally validate this assumption, we tried to assess the modulation of structures in the HCV 5′UTR by miR-122 using SHAPE. In vitro, miR-122 binding by annealing was efficient, indicated by an almost complete loss of SHAPE signal in the MBR, whereas, only moderate changes in reactivity were observed in the rest of the UTR (Supplementary Fig. 6a). Since in vitro binding of miR-122 might not reflect the situation in cellulo, we performed in cell SHAPE, which failed to detect any signal (Supplementary Fig. 4c). This result was in line with the previous data, identifying multiple secondary structures throughout the HCV genome, but not in the 5′UTR18. In addition, not many changes can be expected in the SHAPE signal, since most single stranded regions that are accessible by SHAPE in SLIIalt and SLII are fully or partially overlapping (e.g. nt 53–60, 71–73, 80–86, 93–97, 105–108, Fig. 3b), with only a few exceptions (e.g. 63–64). Moreover, phylogenic analysis suggests that, due the high conservation of the primary sequence across all HCV genotypes, the sequence of the 5′ UTR is compatible with SLIIalt folding. However, due to the few differences in primary sequence, which are also clustered within the bulge region of SLIIalt, we were not able to detect significant covariance to ascertain evolutionary conservation of this structure (Supplementary Fig. 4b).

Mutations in DI favoring SLII formation stimulate translation. The previous results suggested a negative influence of DI on the conformation of the IRES, which can be prevented by miR-122 binding. To test this assumption, we designed a panel of DI mutants, all localized outside SLII and the IRES, which should either support or prevent the formation of SLII, according to secondary structure predictions (Fig. 4a, b). SLII formation was favored either by deleting parts of DI (Δ10, Δ15, Δ20), by replacing 5 or 10 positions with A (pA5, pA10) or by introducing mutations engaging DI into compensatory stem-loop structures (C, SL). As control, one mutant was designed, which was not able to fold stably into either SLIIalt or SLII (M25), and another one was predicted to stabilize an SLIIz-like fold (inhibitory stem-loop, ISL), preventing the formation of SLII. As expected, all mutants were severely impaired in replication or did not replicate...
at all, most likely due to the destruction of essential parts of SLIIz' in the negative strand (Supplementary Fig. 7a, Fig. 3b), therefore we focused our analysis on translation efficiency and stability (Fig. 4c, S3b–d).

Indeed, translation efficiency as measured by firefly luciferase activity, was strongly dependent on miR-122 for the WT UTR, whereas none of the mutants was influenced by miR-122, due to the lack of binding sites (Fig. 4b, c; Supplementary Fig. 7b). Still, all mutants designed to favor the SLII formation translated comparable to WT in the presence of miR-122 (Δ10, pA5, pA10, C, SL) or even substantially more (Δ15, Δ20) at early time points (Fig. 4c). In contrast, translation of the M25 was comparable to WT in the absence of miR-122 and could not be induced by miR-122, due to the absence of binding sites. Interestingly, the ISL mutant exhibited strongly decreased IRES-mediated translation far below the basal WT levels, in line with the predicted secondary structure, favoring SLIIalt formation (Fig. 4c).

Subsequently, we assessed the impact of the mutations on RNA stability and structure for a selected set of mutants and chose Δ20 (strongest stimulation), C (stimulation comparable to WT with miRWT), and ISL (strong decrease in translation efficiency). We assessed short-term RNA decay kinetics by northern blot and found no correlation between stability and translation efficiency (Supplementary Fig. 7b–d). SHAPE analysis was performed to validate the successful generation of the desired structures by mutations in DI. Due to the limitations of SHAPE analysis discussed above, Δ20 and C did not show substantial differences compared to the WT. However, the ISL mutation, which was predicted to create a perfect mirror image of SLIIz', indeed substantially changed the SHAPE pattern (Supplementary Fig. 8a–c).

To exclude inadvertent effects related to the changes in primary sequence of the MBR, we aimed to introduce mutations in SLII affecting SLIIalt and chose residues that are not in contact with the ribosome19 (SLIIalt mut, C65G + G102C, Supplementary Fig. 9a). Indeed, SLIIalt destabilization lead to a miR-122 independent ~2-fold increase in early translation (Supplementary Fig. 9c, d). However, since these mutations were predicted to affect SLIIz' folding (Supplementary Fig. 9e), they consequently abrogated replication (Supplementary Fig. 9f). Importantly, introducing compensatory mutations predicted to restore SLIIalt formation (SLIIalt rev, C49G, Supplementary Fig. 9b) again reduced translation efficiency (Supplementary Fig. 9c, d), but partially restored replication (Supplementary Fig. 9f).

Conclusively, mutations preventing or favoring alternative folds within DI and II substantially increased or decreased translation efficiency of the HCV IRES, independent from miR-122 binding. Our results demonstrate that the primary sequence of DI indeed negatively influences the activity of the IRES element by disturbing the fold of SLII, due to the essential role of the complementary sequence in RNA replication.

miR-122-independent translation facilitates HCV replication.

To analyze how differences in translation efficiency exerted by DI mutations impacted overall replication efficiency, we used the Bi-Luc-SG construct (Fig. 5a). We compared a WT version of UTR1 and UTR2, keeping stability/replication and translation dependent on miR-122 with mutants harboring a UTR2 mutant either stimulating (Δ20, C) or reducing (ISL) translation efficiency independent from miR-122. NLuc activity at early time points after transfection was very similar and enhanced by miR-122 binding for all variants, reflecting increased RNA stability (Fig. 5b). FLuc activity was strongly stimulated by miR-122 binding in case of WT (Fig. 5c), whereas only a slight increase in FLuc activity was observed for the mutants due to the stabilizing effect of miR-122 upon binding to UTR1. Mutants Δ20 and C showed FLuc levels comparable to WT in the presence and absence of miR-122, respectively, whereas FLuc levels of ISL were strongly reduced, in line with the previously demonstrated impact of these mutations on the folding of SLII. At later time points, no replication was observed for any construct in the absence of functional miR-122 (Fig. 5d, miRmut), again demonstrating the important contribution of miR-122 to stability and replication of HCV genome. Yet, the mutants, all incapable of miR-122 binding, showed a divergent behavior: While the ISL mutant, impairing SLII formation, remained replication deficient even in the presence of miR-122, mutations Δ20 and C restored viral replication due to their capability to efficiently support SLII folding (Fig. 5c).

These data again emphasized the contribution of translation stimulation by miR-122 to HCV replication and provided further experimental evidence suggesting that miR-122 binding suppresses unfavorable folding of SLII.

DI interferes with 80S ribosome assembly. In the HCV IRES, SLII drives the assembly of the 48S pre-initiation complex with the 60S ribosome20 (Fig. 6a). Hence, we hypothesized that introduction of mutations like Δ20, as well as miR-binding should enhance 80S monosome complex assembly, if these conditions favor SLII formation. Conversely, the ISL mutant should impair this transition.

To address, whether changes in DI had the propensity to modulate SLII function, 80S ribosome assembly was analyzed by polysome profiling. In vitro transcripts of Luc SG replicons (WT, D20, and ISL) were translated in vitro using miR-122-deficient HeLa cell extracts. Replicon translation efficiency was assessed by measuring firefly luciferase activity and showed comparable levels with those obtained in Hep38 cells (Fig. 6b). In vitro translation extracts were analyzed by sucrose density centrifugation. Polysome profiles were continuously recorded and fractions collected based on the elution time (Supplementary Fig. 10a). Distributions of HCV RNA genomes were quantified in each fraction by qRT-PCR. Distribution of ribosomal RNAs was determined by agarose gel electrophoresis and allowed to distinguish fractions containing RNA associated with the 40S ribosome (18S rRNA), the 80S complex via abrogation of the peptidyltransferase reaction 21.

Accordingly, the assembly of 80S ribosomes, indicated by the presence of 28S rRNA in higher density fractions, was impaired by GMP-PNP, but not CHX (Fig. 6c).

As predicted, addition of miR-122 to the WT replicon resulted in less HCV RNA present in the 40/48S fraction, concomitant with a higher amount in the 80S fraction, indicating a more efficient association with the 80S ribosome (Fig. 6d). In line with this observation, a 40S ribosomal subunit marker (rps3), was detected in higher density fractions after addition of miR-122, corroborating the results of the RNA gels (Supplementary Fig. 10b). The shift in 28S/18S rRNA, 40/48S:80S ratio and rps3 recruitment by inhibition of eIF2 release, whereas CHX stalls the 80S complex via abrogation of the peptidyltransferase reaction 21. Additionally, the assembly of 80S ribosomes, indicated by the presence of 28S rRNA in higher density fractions, was impaired by GMP-PNP, but not CHX (Fig. 6c).
Natural DI variants stabilize SLII and increase translation. Several HCV variants with reduced miR-122 dependency have been described but their mechanism of action remained elusive so far. The U3 mutant was obtained by a cellular recombination process, functionally replacing the SLI with a stem-loop from the far. The U3 mutant was obtained by a cellular recombination process, functionally replacing the SLI with a stem-loop from the far. The U3 mutant was obtained by a cellular recombination process, functionally replacing the SLI with a stem-loop from the far. The U3 mutant was obtained by a cellular recombination process, functionally replacing the SLI with a stem-loop from the far. The U3 mutant was obtained by a cellular recombination process, functionally replacing the SLI with a stem-loop from the far.

Interestingly, in silico folding predicted native SLII formation of the U3 mutant in absence of miR-122 binding, which was reflected by strongly enhanced miR-122 independent translation (Supplementary Fig. 11a, b). Reintroduction of the missing miR-binding site was shown to restore miR-122 dependency (U3WTS1)\(^2\). In accordance with our hypothesis, mutant U3WTS1 was predicted to preferentially form SLII\(^{\Delta1}\) (Supplementary Fig. 11a). This resulted in early translation efficiency and miR-122 dependency comparable to the WT UTR (Supplementary Fig. 11a, b).

Two independent studies selected for resistance to miR-122 antagonists or for miR-122 independence using miR-122 knock-out cells\(^{24,25}\). Both identified a single G to A point mutation in the 5’ UTR at position 28 (Fig. 7a) and demonstrated that this variant enabled HCV to replicate in non-hepatic and miR-122-deficient cells. We therefore assessed whether this mutant could enhance translation in the absence of miR-122. Indeed, a ~2-fold increase in luciferase activity was observed, compared to the WT, with a further increase upon addition of miR-122 (Fig. 7b). Accordingly, we confirmed a partial rescue of replication in the absence of miR-122 for the G28A mutant using the monocistronic Luc-SG replicon in Hep3B cells (miR\(^{\text{mut}}\), Fig. 7c). RNA stability of mutant G28A was not significantly increased compared to the WT replicon (Supplementary Fig. 11c). To understand the mechanism underlying enhanced translation we performed in silico structure prediction of the mutant and WT (Fig. 7d, e). Interestingly, in silico folding predicted native SLII formation of the U3 mutant in absence of miR-122 binding, which was reflected by strongly enhanced miR-122 independent translation (Supplementary Fig. 11a, b). Reintroduction of the missing miR-binding site was shown to restore miR-122 dependency (U3WTS1)\(^2\). In accordance with our hypothesis, mutant U3WTS1 was predicted to preferentially form SLII\(^{\Delta1}\) (Supplementary Fig. 11a). This resulted in early translation efficiency and miR-122 dependency comparable to the WT UTR (Supplementary Fig. 11a, b). Two independent studies selected for resistance to miR-122 antagonists or for miR-122 independence using miR-122 knock-out cells\(^{24,25}\). 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Strikingly, only the G28A mutant but not the WT sequence was predicted to form the functional SLII (green) within the top 10% of energetically favorable secondary structures (Fig. 7e), suggesting that the G28A mutation destabilizes deleterious structures and favors SLII formation. Therefore, the miR-independent U3WTS1 was predicted to preferentially form SLII\(^{\Delta1}\) (Supplementary Fig. 11a). This resulted in early translation efficiency and miR-122 dependency comparable to the WT UTR (Supplementary Fig. 11a, b). Two independent studies selected for resistance to miR-122 antagonists or for miR-122 independence using miR-122 knock-out cells\(^{24,25}\). Both identified a single G to A point mutation in the 5’ UTR at position 28 (Fig. 7a) and demonstrated that this variant enabled HCV to replicate in non-hepatic and miR-122-deficient cells. We therefore assessed whether this mutant could enhance translation in the absence of miR-122. 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replication capacity of the G28A variant is likely the result of enhanced initial translation due to an increased occurrence of SLII in the absence of miR-122.

In conclusion, both HCV UTR variants reported to replicate independent from miR-122 in cell culture (U3 and G28A), showed an increased translation efficiency in the absence of miR-122 and they are predicted to increase the formation of a functional SLII structure relative to the WT sequence. These data further corroborate our central hypothesis that miR-122 binding to the HCV 5’ UTR stimulates translation by suppressing unfavorable alternative secondary structure interfering with IRES activity.

Discussion

In this study, we interrogated the mechanism by which the HCV IRES is activated in response to miR-122 binding. The process has been described extensively\textsuperscript{10–12}, but since the discovery of the RNA stabilizing effect\textsuperscript{14,26,27}, its relevance has been called into question. Our data now clearly demonstrate that translation stimulation by miR-122 can be functionally separated from RNA stabilizing effects by inserting heterologous, miR-122 RNA stabilizing effect\textsuperscript{14,26,27, its relevance has been called into question. Our data now clearly demonstrate that translation stimulation by miR-122 can be functionally separated from RNA stabilizing effects by inserting heterologous, miR-122

The sequence at 5’ end of the HCV genome serves two independent roles: IRES activity required for translation in the positive strand and essential replication signals in the complementary negative strand, both requiring very different secondary structures\textsuperscript{34}. This dual function of complementary sequences prompted us to investigate a potential impact of miR-122 on IRES conformation. Interestingly, the thermodynamically most
favorable structures predicted by folding algorithms involved sequences of DI (SLII\textsuperscript{Sl}), but not the active conformation of SLII required for IRES activity. Notably, a similar fold of this region was proposed earlier\textsuperscript{30}. The structural diversity of DI and II was corroborated by NMR studies and in vitro SHAPE analyses in absence of miR-122 supported the inclusion of the MBR in a stem-loop-structure, rather than the canonical SLII fold. However, we were yet not able to directly demonstrate a structural change induced by miR-122. This was on one hand due the significant overlap in the predictions regarding single stranded regions between the different models. On the other hand, an active structural change might require the additional action of proteins, e.g. AGO, available only in cellulo. However, our attempts to show the presence of SLII\textsuperscript{Sl} by in cell SHAPE failed, which is in line with literature\textsuperscript{18}, suggesting that the 5'-UTR of HCV is hardly accessible for unknown reasons. More elaborate in cell techniques will therefore be required in future studies to clarify whether miR-122 indeed prevents alternative RNA folds interfering with IRES activity.

We generated several mutants of DI predicted to either favor SLII formation or alternative structures interfering with IRES function. Alterations in DI that were designed to suppress deleterious structures, and in turn stabilize SLII, indeed activated early translation in absence of miR-122. Mechanistically, we could observe a shift towards 80S formation in ribosome profiling assays for the SLII stabilizing mutants \Delta20 and C, comparable to the addition of miR-122 to WT genomes. These data argue for a stabilized functional SLII, as this feature promotes eIF2 dissociation, and enables 60S recruitment\textsuperscript{20} and C, comparable to the addition of miR-122 to WT genomes. These data argue for a stabilized functional SLII, as this feature promotes eIF2 dissociation, and enables 60S recruitment\textsuperscript{20}. Accordingly, the ISL mutant, stabilizing SLII\textsuperscript{Sl} showed an inverse phenotype of abrogated translation and a block at the 40/48S stage. These results corroborate a previous report of Filbin and colleagues, observing a similar behavior of a \DeltaSLII construct\textsuperscript{29}. The data obtained with the DI mutants therefore are the strongest support for our hypothesis of a strong impact of DI on the folding of a functional SLII element. However, studies addressing HCV IRES structure often excluded DI, since it is not part of the IRES and thereby missed the negative impact on SLII formation\textsuperscript{30}.

Our data suggest that the predicted structural variation at the 5' terminus of the HCV genome could simply be the result of the reciprocal evolutionary pressure on the active conformations of each strand (SLII vs. SLII\textsuperscript{z}). Hence, miR-122 could assist the dual function of these complementary sequences and prevent deleterious folds. Currently it is not known whether miR-122 is already bound to the viral RNA within the virion or meets the 5' UTR upon release of the genome into the cytoplasm of a newly infected cell. In the latter case, an incorrectly folded IRES will abrogate the infection process at an early stage. Even a 2–3-fold increase in the population of a functional IRES element, as suggested by the stimulating effect of miR-122 on translation, might have a tremendous effect on the efficiency of viral spread in vivo.

The closest relatives to HCV have been identified only recently in cattle, horses, bats, and rodents\textsuperscript{31,32}. Interestingly, all examined specimens showed liver tropism and at least one conserved miR-122-binding site\textsuperscript{32,33}. It is interesting to consider how such an intricate relationship between microRNA and virus has evolved. In fact, miR-122 and its liver specificity is conserved throughout the vertebrates. Therefore, the use of miR-122 to facilitate the formation of alternative RNA structures serving different functions might already have evolved in ancestors of recent hepaciviruses and it may have been maintained throughout co-evolution of virus and host, enforcing liver tropism. In case of GBV-B, a mutant lacking the 5' terminus including the MBR is also attenuated, but viable\textsuperscript{33}.

Notably, a similar interplay between coding and non-coding RNA has been described in bacteria. Here, the ribosome-binding site of the RNA is occluded by an inhibitory structure, which is resolved by binding of an antisense RNA in complex with the bacterial AGO-analog Hfq\textsuperscript{34}. Additionally, over the last decade there have been several reports of cellular IRES elements\textsuperscript{35}, which
were experimentally validated. Some of them also need chaperones to attain their functional conformation, expose the ribosome-binding site and activate translation35. Moreover, increasing numbers of functional miR-binding sites in the 5′ UTR of eukaryotic genes have been discovered, and some of these interactions can increase translation activity by an unknown mechanism35. These reports and our data suggest the possibility that sequence-specific RNA chaperones based on microRNAs may have originally evolved for regulatory requirements in eukaryotic cells.

**Methods**

**Cells and cell culture.** Human Hep3B hepatoma cells (Prof. Ralf Bartenschlager, University of Heidelberg, Germany) were used as main model in this study. Huh7.5 cells were generated by CRISPR/Cas9 technology using a lentiviral vector system and clonal selection. The founder cells Huh7.5 were a gift from Prof. Charles M. Rice (Rockefeller University, New York City, USA).

Human fibroblast HEK293T (Prof. Ralf Bartenschlager, University of Heidelberg, Germany) cells were used for generation of lentiviruses. All cell lines were grown in Dulbecco’s modified Eagle medium (DMEM, Life Technologies) supplemented with 10% fetal calf serum (GE Healthcare/Sigma Aldrich), 100 μg/ml penicillin, 100 μg/ml streptomycin (Sigma Aldrich), and 1% non-essential amino acids (Life Technologies). Cells were maintained in a humidified incubator at 5% CO2 and 37 °C. Huh7.5 cells were cultured with 2 μg/ml puromycin (Sigma Aldrich).

**Generation of Huh7.5-miR cells by lentiviral transduction.** Lentiviruses were produced in HEK293T cells by CaPO4 transfection with pCMV-ΔR8.31 (HIV gag-pol), pMD2.G (VSV-G), and the pLenti-CRISPR-miR-122 vector in a 3:1:3 ratio, respectively. pcMV-ΔR8.31 and pMD2.G and were kindly provided by Prof. Didier Trono (University of Lausanne, Switzerland). Cell-free supernatants were harvested 48, 56 and 72 h post transfection and used for transduction of Huh7.5 cells. Transduced cells were selected by supplementing the culture medium with 2 μg/ml puromycin. Clones were produced by seeding single cells in a 96-well plate, using a limiting dilution approach.

**Reporter viruses and plasmids.** Luciferase reporter viruses (Luc-FL, PI-Luc-FL) were based on J22. To achieve better signal at early timepoints, the Renilla luciferase was exchanged for a smaller and brighter Nano luciferase (Promega). pCMV-ΔR8.31 (HIV gag-pol) and pMD2.G (VSV-G) were used as packaging plasmids for lentiviruses production. pLenti-CRISPR-miR-122 was produced by cloning a duplex of miR-122-specific sgRNA oligos (gRNA miR-122 sense oligo: CACCCAGTCTTCTTAGGAGACGC, gRNA miR-122 antisense oligo: AAACCCAGTCTGCTAAAGGAACTC), designed with CRISPR (www.e-crisp.de) into the pLenti-CRISPR plasmid (Addgene #52961) with BamBI (NEB). pFK-I341PILuc-ubi/NS3-3/JFH1 (Luc-SG) was described before33. All mutants within the HCV 5′ UTR were generated by PCR-based mutagenesis and inserted with Shfl and XbaI (NEB). pFK-I341PILuc-ubi/NS3-3/JFH1 (PI-Luc-SG) has been used in a previous study45. It was generated by replacing the HCV IRES of pFK-I341PILuc-ubi/NS3-3/JFH1 with the HCV 5′ UTR-Polio IRES cassette from pFK-I341PILuc/NS3-3/JFH1 (Luc-SG) using Shfl and XbaI (NEB). pL-CMV (GenBank AF025843) was used for in vitro transcription of Renilla luciferase mRNA, as spike-in template for translation assays. pUC18 vectors were used for transcription of HCV 5′ UTR truncations (1–120 nt, 40–120 nt) for NMR analysis. The fragments were amplified from pFK-I341PILuc-ubi/NS3-3/JFH1 plasmids with primers harboring Shfl and Smal sites.

**In vitro transcription.** Sub-genomic reporter plasmids: RNA for electroporation was generated from 5 μg of pFK-plasmid (linearized with MluI (NEB)) in a 100 μl reaction mixture containing 20 μl of XbaI restriction buffer (2000 U XbaI, Promega), and 200 ng XbaI-linearized pUC18 DNA, and 50 μl of H2O. The reaction was performed over night at 37 °C. The DNA was precipitated with 4 μl of ethanol and resuspended in RNase free water.

**Capped Renilla mRNA for translation experiments:** For capped Renilla transcripts, the pRL-CMV vector (Promega) was linearized with BamHI (NEB). The setup of the transcription reaction was as stated above, except 12.5 μM m7G cap analog (NEB) were added to the reaction, and the GTP concentration in the rNTP stock was reduced to 12.5 μM.

**Truncations of the 5′ UTR for NMR investigations.** 5′ UTR RNA constructs (1–120 nt (DI + II), 40–120 nt (DI)) for NMR studies were transcribed from pUC18 vectors, linearized using Smal (NEB). 100 μg of linearized DNA plasmids were used for 1 ml run-off transcription reaction. The reaction mixture contained 4 mM NTP, 16 mM MgCl2, 34 μg of BSA, 3.6 U of poly(A) polymerase from E. coli, 125 μg of T7 RNA polymerase, 5 mM DTT, 1 mM spermidine, 0.01% Triton X-100, 80 μg of PEG 8000, and 40 mM Tris at pH 8, which was incubated at 37 °C for 3 h in a Hybrigen HB-3D (Technon) at 20 rpm. Then, 50 units of RNase-free DNase (Euromedx) were added and incubated 30 min at 37 °C to stop the reaction. Reaction volumes were 1, 4, or 8 ml and RNA constructs were purified using Q-sepharose HiLoad column (GE Healthcare) and vacuum condition. Briefly, the reaction mixture was diluted 10-fold in 20 mM Tris buffer at pH 8, containing 10 mM EDTA and 8 M urea, and loaded on the column previously equilibrated with the same buffer. Then RNA was purified with a gradient from 0 to 1 M NaCl in the same buffer. Fractions containing RNA (identified using absorbance at 260 nm) were concentrated using desalting polyacrylamine (15%) gel electrophoresis. The fractions with the target transcript were pooled, dialyzed three times against 200 volumes of H2O, and lyophilized.

**SEC-MALLS—RI**. Size exclusion chromatography (SEC) combined with online detection by MALLS and refractometry (refractive index—RI) is a method allowing absolute determination of the absolute molecular mass of each component of a sample in solution, regardless of its dimensions and shape46. SEC was performed with a Superdex 200 column (GE Healthcare) equilibrated with 50 mM Heps buffer (pH 6.4, 100 mM NaCl, 5 mM MgCl2, and 5% D2O). The final RNA concentration for each NMR sample was 0.9 mM. All NMR experiments were recorded using a 950 MHz Bruker Avance III HD spectrometer equipped with a 5 mM cryogenically cooled pulsed-field-gradient triple-resonance probe.
and RNA concentration was measured online by measuring the differential RI using an Optilab T-rEX detector (Wyatt Technology Corp.) and an averaged RI background signal. Measurements were performed using a Mitras LB940 plate reader (Berthold).

Luciferase reporter assay. Activity of reporter replicons was measured after cell lysis in 350 μl luciferase lysis buffer (1% Triton X-100, 25 mM glycylglycine pH 7.8, 15 mM MgSO4, 4 mM EGTA, 10% glycerol) per well in a six-well plate. Plates were stored at −20 °C until measurement of luciferase activity.

Dual-luciferase assay. For the dual-luciferase replicons, the commercial Nano-Glo Dual-luciferase Reporter Assay System (Promega) was used. In brief, 40 μl of cell lysate were transferred from the six-well plate to a white walled 96-well plate (Falcon) in duplicates. Next, 40 μl of ONE-Glo EX Reagent (Promega) were added, and the reaction was incubated for 10 min at room temperature before measurement of the firefly luciferase signal. Then, firefly activity was quenched for 10 min using 40 μl of NanoLR StopGlo buffer (Promega) before determining the Nano luciferase signal. Non-transfected cells were used to determine the background signal. Firefly luciferase from IRES-dependent translation, was normalized to the renilla activity to account for transfection efficiency and cell numbers. Note that capped in vitro transcripts were degraded with NaOH and incubation at 95 °C, flash cooled on ice and re-holded in presence or absence of micr-1225-2p at 37 °C for 15 min in SHAPE buffer (5 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl2, 4 mM KCl, 15 mM MgSO4, 5 mM glutathione, 2 mM ATP) and electroporated (0.166 kV, 950 μF, 0.2 cm cuvettes) with 5 μg of reporter replicon RNA and 50 pmol wild type or mutant miR-122 mimic. For translation assays, 5 μg of a capped Renilla transcript was used as internal control for transfection efficiency. Electroporated cells were seeded onto six-well plates and incubated at 37 °C for the desired time.

Selective 2′-hydroxyl acylation analyzed by primer extension. SHAPE data were analyzed with SAFA 51. The data were normalized to the mean of the top 10% reactivities. Intermediate (0.2–0.8) is indicated in yellow, highly reactive nucleotides (>0.8) are colored in red. For differential analysis of miR-bound versus free RNA the normalized data were subtracted by subtracting all differences of at least 0.3 were considered as significant. Less reactive nucleotides are depicted in green, more reactive in red.

Translation assay in Hela cell lysates. 2.5 μg of reporter replicon RNA was added to 10 μl HeLa lystate (1-Step Human Coupled IVT Kit, Thermo Fisher Scientific) to generate a translation mix in a total volume of 15 μl, following the manufacturer’s instructions. When needed, mir-122 duplex RNA was incubated at 30 °C in the mix, 10 min prior to RNA addition. The translation reaction was performed for 30 min at 30 °C. Then, 235 μl of ice-cold lysis buffer, containing 200 μg/ml CHX was added to 10 μl HeLa lysate (1-Step Human Coupled IVT Kit, Thermo Fisher Scientific) to generate a translation mix in a total volume of 15 μl, following the manufacturer’s instructions.

In silico RNA structure calculation. The 5′ terminal truncations of HCV, the online versions of RNA structure 46, mfold 47 were used without constraints. RNA structure was also used for integration of SHAPE data. 10% polyacrylamide gels were pre-run for 10 min at running conditions. Native TBM (44 mM Tris–HCl, pH 8.3, 1 mM MgCl2) gels were run for 1 h at 100 V for 4 days, denaturing urea TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, 8 M urea) gels 1 h, 100 °C at room temperature. Gels were stained with a 1:10,000 dilution of diamond nucleic acid dye (Promega) in the respective running buffer for 10 min at room temperature and visualized on a UV transilluminator (INTAS).
Fractionation on sucrose gradients. Lysates were loaded onto a linear gradient of 17.5–30% in 15 mM Tris–HCl pH 7.4, 15 mM MgCl₂, 300 mM NaCl and subjected to ultracentrifugation at 165,000 g at 4 °C using a SW60 rotor (Beckman) for 2.5 h. Gradients were then eluted from the top using a Teledyne ISCO gradient elution system. Polysome profiles were obtained by measuring the absorbance at 254 nm. In parallel, fractions were collected every 15 s (equivalent to ~400 μl). Before RNA extraction from the fractions, 20 μg of glycogen, 350 μl urea buffer (10 mM Tris–HCl pH 7.5, 350 mM NaCl, 10 mM EDTA, 1% SDS, 7.5 M urea) were added. Moreover, 1 ng of in vitro transcribed Renilla luciferase RNA was supplemented as internal control. Following phenol-chloroform extraction and ethanol precipitation, the pellets were resuspended in 40 μl of RNase-free water. Samples were stored at –80 °C until further processing.

Detection of HCV RNA in fractions. HCV genomic RNA extracted from the fractions was analyzed by one-step qRT-PCR using qScript XLT one-step RT-qPCR ToughMix (Quanta Bio-science) according to the manufacturer’s instructions. Briefly, 15 μl of reaction mixture containing 7.5 μl of 2× RT mix, 1 μM of H71-specific and Renilla-specific primers (H71 S166 forward qPCR primer: TCTGGCGGACCCGGTAGTA, H71 A192 reverse qPCR primer: GGCTGATAGTTGGTTATTCGA, Renilla S767 forward qPCR primer: AATCCTGGACCCAGGATTC, Renilla A917 reverse qPCR primer: ACTCTGCTCAACAAGGGTT), 0.27 μM of the corresponding probes (HF1 TagMan probe: [FAM]AAAGGACCCAGTCTCCGCGCAATT[TAMRA], Renilla S859 TagMan probe: [HEX]TGGCAAAGATAGTCACCTGATAG[TAMRA]), 3 μl of template RNA, and RNase-free water. To generate a standard curve, serial dilutions of an RNA standard (10⁻⁹–10⁻⁶ copies of HCV RNA and Renilla in vitro transcript) was prepared for each plate. Reactions were performed using the following program: 50 °C for 10 min, 95 °C for 1 min, and 40 cycles of amplification: 95 °C for 10 s and 60 °C for 1 min. The amount of HCV RNA was normalized to the Renilla control and the input sample.

Immunoblotting. 10 μl of each fraction were substituted with 2 μl of 6× Lämmli buffer with 5% β-mercaptoethanol and denatured for 10 min at 95 °C. Samples were separated on a 10% SDS polyacrylamide gel and wet-blotted on a PVDF membrane for 1 h at 400 mA. Blots were blocked with low-fat milk in PBS-T (0.1% Tween) for 30 min at room temperature. Primary antibody (goat polyclonal eIF3 y, 15 μl of reaction mixture contained 7.5 μl of 2× RT mix, 1 μM of [FAM]AAAGGACCCAGTCTCCGCGCAATT[TAMRA], Renilla S859 TagMan probe: [HEX]TGGCAAAGATAGTCACCTGATAG[TAMRA], 3 μl of template RNA, and RNase-free water. To generate a standard curve, serial dilutions of an RNA standard (10⁻⁹–10⁻⁶ copies of HCV RNA and Renilla in vitro transcript) was prepared for each plate. Reactions were performed using the following program: 50 °C for 10 min, 95 °C for 1 min, and 40 cycles of amplification: 95 °C for 10 s and 60 °C for 1 min. The amount of HCV RNA was normalized to the Renilla control and the input sample.

Denaturing RNA gel electrophoresis. 18S and 28S ribosomal RNA was detected for each fraction using a denaturing TAE gel approach.25 The samples were heated to 65 °C for 5 min in loading buffer LDII (95% formamide, 0.05% SDS, 15 mM EDTA, xylene cyanole 0.025% w/v, bromophenol blue 0.025% w/v) and flash cooled on ice. The denatured samples were applied to a 1% TAE agarose gel, containing 10 μg/mL ethidium bromide. The gel was run for 30 min at 100 V in 1× TAE buffer and bands were visualized on a UV transilluminator (INTAS). Uncropped versions of the gels can be found in Supplementary Fig. 14.

Quantification and statistical analysis. Where appropriate, a paired, two-tailed Student’s t-test was performed using Prism 6 software (GraphPad). All data are presented as mean ± standard deviation. The sample size is specified in the corresponding figure legend. P values of <0.05 were considered statistically significant. In graphs, statistical significance is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

Conception of the study and writing of the paper was done by P.S. and V.L. P.S., H.R., C.O., R.L.A., L.I. and C.M. performed experiments. H.R., C.O., R.L.A., L.I., A.M.P., A.R. provided valuable intellectual input and assisted in editing the manuscript. V.L. acquired funding.

Additional information

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