Effects of lipid membranes on RNA catalytic activity and stability

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Abstract

RNA plays crucial roles in cellular organization and metabolism, and modulating its activity is essential for maintaining cellular functions. RNA activity, involving both catalytic (ribozymes) and translation processes, is controlled via myriad of different mechanisms involving different binding partners such as proteins and smaller polar solutes. We previously reported that lipid membranes can directly interact with the artificial R3C ribozyme changing its activity, however the effect of lipids on naturally occurring ribozymes remains unknown. Here, we report that both catalytic activity as well as RNA integrity can be controlled by the presence of different lipid membranes. Lipid gel membranes decreased the activity of hepatitis delta virus (HDV) and increased the hammerhead (HH) ribozyme reaction yield. The presence of lipid liquid membrane lattices triggered RNA degradation with greater degradation occurring in the single-stranded regions of RNA. The interplay between RNA activity and stability in the presence of different lipid membranes introduces multiple possibilities, where different combinations of ribozyme and lipid membrane composition could produce different effects on activity. Taken together, these observations support the hypothesis that the activity of both natural and artificial RNAs can be modulated by lipid membranes which, in turn, contribute to the development of novel riboswitch-like molecules, and lipid membrane-based RNA-biosensors.

Introduction

Ribozymes are catalytically active RNA molecules which play crucial role in the cellular as well as viral metabolism[1–4]. The activity of ribozymes is strictly dependent on correct RNA folding, and on the presence of interaction partners such as divalent cations. For example, hepatitis delta virus (HDV), a pathogen involved in liver cirrhosis development, contains the crucial ribozyme sequence involved in HDV replication in which activity is strictly dependent on a characteristic pseudoknot folding[5,6] and the presence of divalent cations. Similarly, another class of ribozyme, hammerhead (HH), involved, for example, in viroid and retrozyme replication, also requires specific structures in order to be catalytically active, however it does not necessarily require divalent cations[7,8]. Ribosome and RNAseP activity are modified by interaction with proteins[9–11]. These phenotypes offer a range of possibilities in which different natural ribozymes can be controlled in different ways using relatively simple changes in the ribozyme microenvironment.

Most small ribozymes are capable of maintaining their full catalytic activity without other macromolecules[2,12,13]. It was discovered, however, that RNA activity can be changed through the interaction with small solutes such as ATP, theophylline, and cyclic di-guanosine monophosphate in the special RNA classes such as aptazymes and riboswitches[14–17]. Moreover, recent research has shown that ribozyme activity can be also controlled through interactions with much larger binding partners such as single-component lipid membranes[18]. It is, however, unclear how different lipid membranes (various lipid composition, and membrane fluidity) can further tune the activity of ribozymes.

RNA-lipid interactions have been reported to involve electrostatic interactions[19–21], but also, hydrophobic interactions have been proposed (i.e. RNA penetrating lipid membrane lattice)[22–26]. RNA-lipid interplay can result in functional outcomes and regulatory mechanisms that influence RNA phenotype and activity. For instance, the interaction, which can be tuned both by the RNA structure and sequence, could change RNA conformation, which can potentially lead to changes in ribozyme activity[18,24,25,27]. Thus, it is potentially possible to use lipids to control the activity of ribozymes with different activity mechanisms, sequences, and structures.

The effect of lipid membranes on artificial R3C ligase[28] was previously demonstrated, showing that changes in the sequence of R3C ligase substrate significantly changed characteristics of the ribozyme activity. Namely, the presence of lipid gel membranes improved both the interaction between R3C ligase and its short RNA substrate via sequence-dependent RNA-lipid binding[18]. It is, however, unclear how the presence of lipid membranes can influence the non-artificial ribozyme activity directly by the binding of the ribozyme molecules.

Here, we show how different ribozymes can be affected by interactions with lipid gel membranes. The activities of hammerhead and HDV ribozymes were influenced differently. The HDV ribozyme showed inhibition, while the hammerhead ribozyme exhibited an increase in reaction rate. We further report lipid-membrane dependent RNA-degradation which depends on membrane fluidity. Taken together, both activity changes (R3C, HH, HDV) as well as lipid-triggered degradation create new level of control which might be employed in synthetic as well as molecular biology, and help to understand riboregulatory processes.

Methods

Materials

HEPES, magnesium chloride, calcium chloride, ammonium chloride were purchased in Carl Roth. Lead acetate, choline chloride, glycerophosphocholine, cholesterol were purchased in Sigma Aldrich. Dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), diarachidoylphosphatidylcholine (20:0 PC) were purchased in Avanti Polar Lipids. SybrGold was purchased in Thermo Fisher. All buffers were prepared in MilliQ water (Merck). All chemicals were used without further purification.

84 **RNA synthesis and purification**

85 HDV and HH constructs were obtained via standard in vitro T7 transcription (**Suppl.**
86 **Table 1**), overnight 37 °C. RNA was separated on denaturing polyacrylamide gel, post-stained
87 with SybrGold, and excised from the gel using the razor blade. Gel slices were then crush-and-
88 soaked in the Tris-borate-EDTA buffer, 90 °C, for 1 hour. Obtained RNA was ethanol
89 precipitated, washed with 70% ethanol and resuspended in MilliQ water. Concentration and
90 integrity of RNA were estimated using absorbance readout and denaturing PAGE, respectively.
91 6-FAM labelled hammerhead substrate was purchased at IDT and used without further
92 purification.

93

94 **Lipid vesicle preparation**

95 Lipid stocks dissolved in chloroform were pipetted into a glass vial and briefly
96 evaporated under a steady flow of nitrogen gas. To remove organic solvent residues, a lipid
97 film was dried under vacuum overnight. To obtain multi-layer vesicles (MLVs), lipid films were
98 hydrated in the reaction buffer (10 mM HEPES pH 7, 5 mM $CaCl_2$ and 5 mM $MgCl_2$). Liposomes
99 were shaken above the melting temperature of the lipids for one hour with brief vortexing
100 every 15 minutes. A cloudy liposome suspension was transferred to the Eppendorf SafeLock
101 tube and freeze-and-thawed 10x in liquid nitrogen/60 °C thermoshaker (1'/5', respectively),
102 to reduce multi-lamellarity of the vesicles. Liposome suspension was then extruded 17x
103 through 100 nm polycarbonate filters to achieve a consistent size distribution of vesicles.
104 Liposome stocks were kept at 4 °C.

105

106 **Activity assays**

107 All of the activity assays were performed in the 10 mM HEPES pH 7, 5 mM $CaCl_2$ and 5
108 mM $MgCl_2$. Hammerhead (67 nM ribozyme, 133 nM substrate, 30 uL reaction volume) and
109 HDV (14 nM, 30 uL reaction) incubations were conducted in the temperature cycling
110 conditions (15 cycles 24 °C -> 60 °C, 3' each) to enhance RNA refolding[5]. After the incubation,
111 RNA was ethanol precipitated and resolved on denaturing PAGE. Hammerhead activity was
112 measured using the normalized product intensity to sum of product and substrate intensities.
113 HDV activity readouts were based on the SybrGold-post stained gels using the ratio of un-
114 reacted HDV-tRNA band to the sum of tRNA and HDV-tRNA bands. Hammerhead kinetic
115 analysis in diluted conditions (5 nM ribozyme, 10 nM substrate, 400 uL total reaction volume)
116 was performed with 100 μM lipids (either DPPC or 20:0 PC). The reactions were stopped after
117 3 temperature cycles.

118

119 **Degradation readouts**

120 RNA was incubated with different additives (lipid membranes, parts of lipid
121 headgroup, lead acetate); after the incubation RNA was ethanol precipitated, separated on
122 denaturing PAGE, and post-stained with SybrGold.

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126 **Results**

127 **The catalytic activity of RNA is modified by the presence of lipid membranes**

128 We previously demonstrated an RNA-sequence dependent modification of ribozyme
129 catalytic activity based on the presence of lipid gel membranes[18]. The model system was based
130 on a trans-acting single-turnover R3C ligase and the activity changes were based rather on the
131 substrate-lipid membrane interaction and not direct ribozyme-membrane interactions. Here,
132 to diversify the potential modes of action, we chose different RNA with different mechanisms
133 of activity: trans and cis acting ribozymes which trigger trans- and cis-cleavage of RNA
134 substrates, hammerhead and HDV ribozyme, respectively. Both ribozymes bind to the lipid gel
135 membranes with relatively high partition coefficient values ($>1x10^6$)[18] and with lipid:nt ratios
136 around 1:1 (**Fig. 1a, b**).

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138 Activity assays of HDV and HH remarkably show different behaviors in the presence of
139 lipids. The hammerhead final reaction yield was not affected, whereas the yield for the HDV
140 ribozyme reaction decreased in the presence of the lipid gel membranes in a concentration-
141 dependent way. Interestingly, the overnight incubation of HDV construct did not give a 100%
142 reaction yield (**Suppl. Fig. 1**) which suggests that a fraction of HDV molecules bound to the
143 lipid membrane are unable to perform the cleavage reaction, most likely due to impaired
144 refolding. Indeed, the HDV ribozyme can fold into multiple different structures of which only
145 a subset are catalytically active[5]. Hammerhead ribozyme, on the other hand, as the shorter
146 molecule folds into one dominant structure. The presence of lipid gel membranes most likely
147 does not bias its folding, thus no differences in the final reaction yield were observed (**Fig. 1b**).
148 These differential lipid-dependent responses open a potential opportunity to create a system
149 in which RNA activity can be selectively influenced through interactions with lipid membranes.
150 As a proof of principle, we used hammerhead-HDV construct[29] in which both ribozymes act
151 together creating characteristic digestion pattern. As expected, the presence of lipid gel
152 membranes selectively inhibited the activity of HDV ribozyme whereas left hammerhead
153 functionality unbiased (**Fig. 1c**).

154

155 To estimate if gel membranes have any beneficial effect on hammerhead ribozyme
156 activity (e.g. through up-concentration of RNAs on the lipid surfaces, increased RNA-RNA
157 contacts) we performed a time-lapse experiment. We observed that the overall reaction rate
158 (yield over time, **Suppl. Fig. 2**) increased in the lipid gel membrane system (**Fig. 2**). A phase-
159 transition condition (lipid membrane coming through liquid-gel phase transition during the
160 reaction temperature cycling), which are closer to the biological systems than the solid gel
161 membranes, also showed significant increase of the hammerhead reaction yield proving that
162 the membrane does not necessarily have to be in the gel state over the course of the reaction;
163 moreover, when the membrane is in a liquid phase, RNA can possibly diffuse off from the
164 liquid membranes due to low binding affinity[18,21,30] allowing the RNA to freely interact with
165 other interaction partners.

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168 **The presence of lipid liquid membranes triggers RNA degradation**

169 It was previously shown that most RNA sequences do not bind well to biologically
170 relevant lipid liquid membranes. However, Suga et al. reported that RNA binds to lipid liquid
171 membranes which might affect its structure and catalytic activity[24,25,27]. Motivated by the
172 unclear role of liquid membranes in the RNA-lipid systems we performed an activity assay in
173 parallel with a binding assay of HDV ribozyme. We hypothesized that liquid membranes should
174 not affect RNA activity due to the lack of a stiff, RNA-structure-locking gel membrane. High
175 binding and inhibition of activity was observed for gel membranes which is in line with
176 previous observations. Additionally, no significant binding nor effect on HDV activity due to
177 the presence of liquid disordered membranes took place, and only slight HDV binding and
178 inhibition was observed for stiffer liquid ordered membranes (**Fig. 3a**), however, we noticed
179 that the HDV sequence undergoes degradation generating different RNA pattern than
180 expected (this process is dependent on the lipid membrane concentration, **Suppl. Fig. 3**). To
181 investigate which part of the lipid membrane is responsible for RNA degradation, RNA was
182 incubated with different chemical components of the phospholipid headgroup (**Fig. 3b**). None
183 of the components of the lipid headgroup can create stable lipid bilayers, and all are soluble
184 in the buffer solution. No enhanced RNA degradation was observed in the presence of choline,
185 ammonium ions, phosphate ions, and glycerophosphocholine, which proves that the presence
186 of the liquid membrane lattice is crucial for triggering enhanced RNA degradation. The RNA
187 degradation is dependent on lipid membrane fluidity with highest degradation observed for
188 lipid liquid membranes, followed by liquid ordered and gel lattices (**Fig. 3c**, left panel). We
189 noted that RNA degrades in a specific pattern rather than generating a smear of degradation
190 products which most likely is due to structure-dependent RNA cleavage: typically single
191 stranded parts of RNA are more prone to degrade[31,32].
192
193 To investigate if the observed RNA degradation pattern represents selective cleavage
194 of the single stranded segments we incubated the RNA in the presence of lead which
195 preferentially induces the cleavage at the single stranded regions. We observed that lead
196 generated a pattern similar to lipid-dependent cleavage, however it also generated more
197 degradation products. It is most likely that lead, as a metal ion, is smaller than lipid
198 membranes and has better access to all parts of RNA compared with bulky lipid membrane
199 lattice. We hypothesize that the presence of lipid liquid membranes allows single stranded
200 parts of RNA to penetrate the hydrophobic core of the membrane[22–26] which changes chemical
201 environment triggering specific RNA degradation.
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Discussion

RNA activity is involved in a diverse range of biologically relevant processes such as viral replication[3], retrotransposon migration[4], RNA maturation[2], and gene expression control[14]. RNA activity has been shown to be regulated through the interaction with small solutes, as well as by proteins[9,10,14,15]. Here, we demonstrate the potential to control the activity of naturally occurring RNAs with lipid membranes.

Here, we characterized how the activity of catalytic RNAs was affected by lipid membranes in a gel and liquid state, which significantly differ in their fluidity. Our results extend previous findings that RNA-lipid interactions are sensitive to several factors such as membrane fluidity, lipid composition, and RNA sequence and structure[18,21,30,33–35]. For example, the activity of R3C ligase ribozyme can be controlled through interactions with lipid gel membranes in a sequence and structure-dependent manner[18]. Moreover, the interaction with lipid membranes influenced RNA structure which could, in turn, also affect RNA activity. Here, we observed lipid-based RNA-activity changes for two different naturally occurring ribozymes, HDV and HH. The HDV ribozyme was partially inhibited, likely due to the refolding issues in the presence of stiff crystal-like lipid gel membrane[36], whereas HH remained active and even accelerated under similar conditions (**Fig. 1, 2**). Alternatively, steric hindrance from the bulky lipid membranes as well as direct nucleotide-lipid interactions, may have played a role[26,37]. Remarkably, the presence of biologically relevant lipid liquid membranes affected RNA integrity, resulting in partial cleavage with characteristic patterns that depended on the presence of the lipid liquid membrane lattice (**Fig. 3**).

Our results suggest that the viroid, HDV, and retrozyme replication could also potentially be affected by the presence of the lipid membranes. Our observations of simple RNA-lipid based systems create a framework to understand if and how lipid membranes can be involved in RNA metabolism. For instance, mRNA degradation might occur at specific membrane fluidities, either activating or inhibiting its translation activity. Additionally, lipid-sensitive riboswitches, might represent another level of gene expression in which RNA is activated by lipid membranes with specific composition or property. Lipid-membrane co-localized RNAs have been found *in vivo* in the form of mRNAs[38–40], lncRNAs[41,42], and glycoRNAs[43]. Recently it was also shown that cell-extracted RNA species can bind to lipid membranes[44]. In some cases, the mechanism of binding is not entirely clear, however it was proposed that some of the RNA sequences can have affinity towards the lipid membranes[33–35,45]. The lipid-driven RNA-activity changes might have several implications in cellular biology. The close proximity of catalytic RNAs, such as ribosomes, to different lipid membranes might have the influence on the RNA activity and integrity which further could influence cellular homeostasis. It was demonstrated that lipids forming the nuclear envelope increase RNA stability[46] which, for example, might improve the activity of RNAseP, a ribozyme involved in the small RNA processing.

Development of lipid-binding RNAs could potentially have applications for the artificial introduction of lipid-binding RNA species *in vivo*. For example, the mRNA translation process could be co-localized with the lipid membranes which would provide a means to control the localization of membrane protein translation[39,47]. Creating new aptazymes and bi-functional aptamers could also create diverse and controllable functional outcomes; it was reported that merging the lipid-binding sequences with tryptophan aptamers increased tryptophan lipid membrane permeability[48]. Modifying existing RNAs with lipid-binding motifs might play a role in the exosome packing processes[45,49–51]. Novel lipid-based aptazymes and riboswitches could generate a pool of lipid-dependent feedback loops controlling further the stability and activity of modified ribozymes and mRNAs. Lastly, a bioinformatic analysis of lipid-binding motifs in currently developed lipid-binding RNAs would help to find naturally existing lipid-binding RNAs.

Merging our observations of modified RNA activity (R3C ligase, hammerhead and HDV ribozymes) and altered RNA stability ((including RNA degradation and lipid-based cleavage of other RNA species) in the presence of lipid membranes provides a frame fork for developing functional systems based on the interactions between RNA and lipids. This framework lays the groundwork for understanding of RNA-lipid interplay in living cells, as well as in the synthetic and prebiotic systems.

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Author contributions

T.C. performed and analyzed experiments, conceptualized and wrote the manuscript. J.P.S acquired the funding, conceptualized, and edited the manuscript.

Conflict of interest

The authors declare no conflict of the interest.

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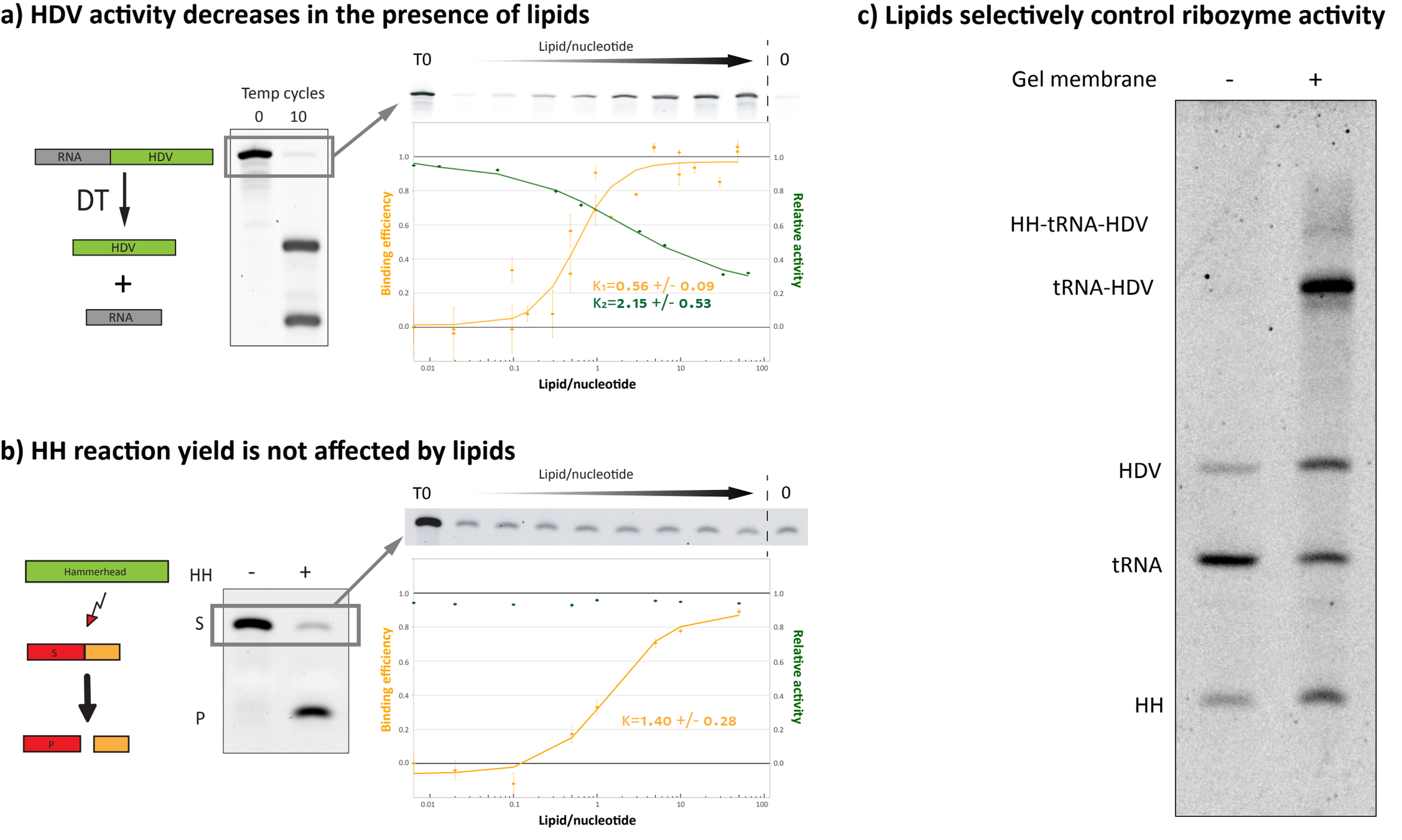

Fig. 1 Lipid gel membranes act differently on different ribozymes. a) HDV ribozyme self cleaves under temperature cycling conditions. The reaction yield (green curve) decreases with increasing lipid binding (orange curve). **b)** HH ribozyme cleaves its substrate which generates shorter RNA products. Despite significant lipid binding (orange curve) there is no change in the total reaction yield (green datapoints). **c)** HH-tRNA-HDV fusion construct (top band) self-cleaves in two separate spots generating three products: HH, HDV, and tRNA. The presence of lipid gel membranes selectively partially inhibits HDV activity generating half-cleaved intermediate. The obtained binding and activity fits are described by Hill's function.

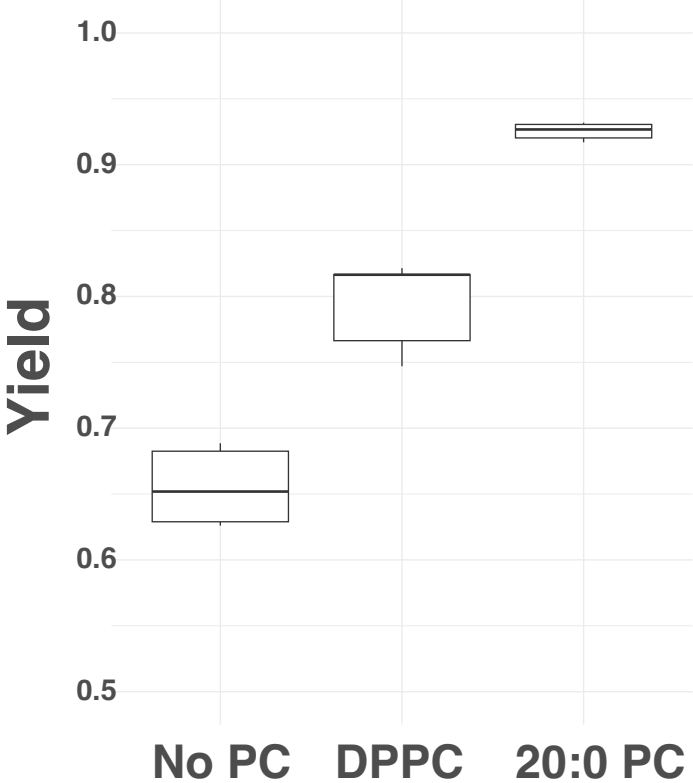

Fig. 2 Increased reaction rate of hammerhead ribozyme in the presence of lipid gel membranes. The hammerhead ribozyme reaction was stopped after 3 temperature cycles in which the reaction did not reach completion for lipid—free system (**Suppl. Fig. 2**). The reaction yield is significantly higher for the lipid-based systems.

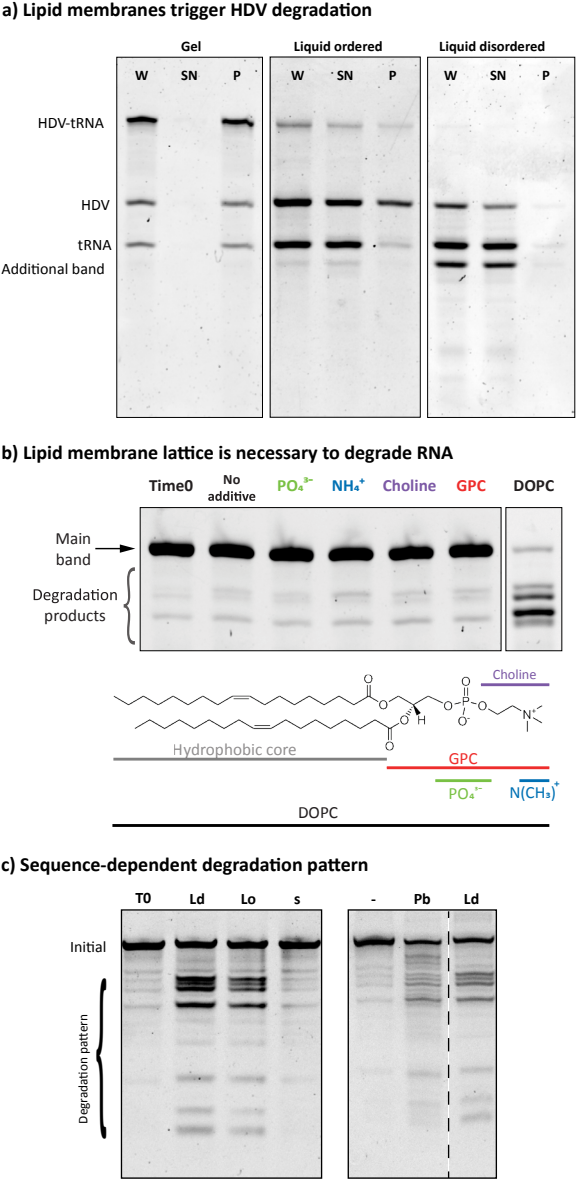

Fig. 3 Lipid membranes induce RNA degradation. a) HDV-tRNA construct was incubated in the presence of 1 mM lipids. RNA and lipids were then and analyzed on the denaturing PAGE. W – reaction before separation, SN – supernatant (lipid free fraction), P – pellet (lipid bound fraction). **b)** HDV RNA was incubated in the presence of 1 mM additives at 20 °C for 24 hours. The presence of water-soluble additives did not influence RNA integrity whereas liquid disordered lipid membranes (DOPC) induced RNA degradation. **c)** 50 ng of HH RNA was incubated for 3 hours at room temperature with 1 mM lipids with different membrane fluidities. Ld – liquid disordered (DOPC), Lo – liquid ordered (DPPC:Chol30%), s – gel phase (DPPC). Additionally, the RNA was incubated for 10' in the presence of 10 mM lead acetate (Pb) which preferentially triggers RNA cleavage at the single-stranded parts of RNA. Dashed line on the right gel symbolizes that a middle lane was removed from the original gel image.