Sensing and sensibility: the role of non-coding RNAs in autoinflammation and viral infection

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General discussion

Main conclusions

RNA molecules are vital components of the cell. An increasing body of literature describing their function, expression, and regulation stresses that there is still much to learn about their roles and interactions with other cellular compartments. The studies performed in this thesis all revolve around the intricate interactions between endogenous non-coding (nc)RNAs and proteins. In **Chapter 3** and **4**, we studied immunostimulatory self-derived RNAs from a protein-centric point of view. In **Chapter 2** and **5**, we changed viewpoints and investigated the role and functions of ncRNA in regulating cellular pathways in homeostasis and viral infections. Altogether, this work led to the following conclusions:

1. LGP2 is essential for sensing unedited self RNAs in ADAR1 deficiency together with MDA5, both under conditions found in Aicardi-Goutières syndrome (AGS) and in therapies aiming to harness ADAR1 in antitumor treatment
2. LGP2 interacts with self RNAs in wild-type and ADAR1-deficient cells
3. Vault (vt)RNAs are upregulated during viral infection and aid viral replication by facilitating the nuclear export of the proviral host proteins ELAVL1 and hnRNP C

The findings in this thesis increase our understanding of the interactions between ncRNAs and cellular proteins, but also raise questions that will be discussed in this chapter.

The role of LGP2 in ADAR1 deficiency

MDA5 and LGP2 survey the cytoplasm for non-self dsRNAs. Since LGP2 supports, but is not essential for MDA5-mediated sensing of viral or synthetic dsRNAs, research has traditionally focused on the role of MDA5 in nucleic acid sensing. Additionally, LGP2 lacks CARD domains and thus needs other proteins to relay signaling, while MDA5 is signaling-proficient on its own. The role of LGP2 as an important co-receptor of MDA5 became evident in mice infected with the picornavirus EMCV. Genetic loss of LGP2 severely weakened the type I IFN response in mice infected with EMCV[1]. Later, this was demonstrated for other viruses as well, such as SARS-CoV-2[2]. **Chapter 3** and **4** reveal for the first time that LGP2 is essential in an autoinflammatory context. Specifically, we describe that LGP2 is required for sensing unedited RNA in the absence of the RNA-editing ADAR1. This was further strengthened by a study that showed that the type I IFN response resulting from mutations in the Z-RNA-binding domain of ADAR1 could be rescued by genetic loss of LGP2 in mice[3]. These findings show the important role of LGP2 in RNA sensing. However, the identity of ligands that bind and activate LGP2 is still unknown, as well as how these interactions subsequently activate MDA5.

What does LGP2 sense, and how does it work together with MDA5?

LGP2 binds to double-stranded (ds)RNAs with a wide variety of lengths, with a higher affinity compared to the other two RIG-I-like receptors[4–6]. Despite this, ligands for LGP2 have not been studied in detail and hence remain poorly characterized. Recent studies have shed light on how LGP2 engages dsRNA, showing that LGP2 can both bind internal sites of dsRNA, as well as interact with dsRNA termini[4,5,7–9]. Binding to dsRNA ends is independent of 5′ end modifications, like triphosphate, Cap0, or Cap1 moieties, and does not require ATP hydrolysis[8,9], while internal binding is achieved in an ATPase-dependent manner[4]. By docking onto dsRNA, LGP2 does not form filaments or oligomers on its own, but rather stimulates the formation of MDA5 filaments[4,8–10]. In the context of EMCV infection, for example, LGP2 strongly interacts

with EMCV antisense transcripts of the Leader region, which subsequently activate MDA5[11]. This finding led to our hypothesis in **Chapter 4** that we could use LGP2 as a proxy to identify unedited dsRNAs that stimulate MDA5 in ADAR1 deficiency.

In turn, MDA5 binds dsRNAs that are longer than 1000-2000 bp, which are typically found during the replication of RNA viruses, as they form perfectly base-paired duplexes[12–14]. MDA5 binds preferably to a 3' recessed end in dsRNA, and translocates to the 5' end[7,15]. Additionally, MDA5 can be activated by complex, branched dsRNA structures, which are likely formed by RNA overhangs that base-pair with adjacent RNA molecules[15,16]. During filament formation, MDA5 moieties cooperatively hydrolyze ATP. This process allows MDA5 to move on dsRNA, whilst also inducing monomers at the filament ends to dissociate[17,18]. The balance between dissociation and association in MDA5 filamentation forms the basis of self versus non-self-discrimination[17,18]. When LGP2 induces the nucleation of MDA5 oligomers, filaments become shorter, leading to exponentially less ATP hydrolysis[4,7,8,10]. This results in stable, short LGP2/MDA5 filaments that can form interactions with the downstream adapter MAVS to induce type I IFN signaling. Although most studies of MDA5 filament formation have been performed *in vitro*, our results in **Chapter 3** support that LGP2 and MDA5 form oligomers in ADAR1 KO cells. The activation and cooperation between LGP2 and MDA5 is also regulated by complex translocations to various subcellular compartments, such as the mitochondria-associated membranes, endoplasmic reticulum, and peroxisomes. Future research should investigate whether the formation of filaments happens in the context of their spatial regulation *in cellulo*[19].

Post-translational regulation of LGP2 and MDA5 in ADAR1 deficiency

Besides their physical location in the cell, MDA5 and LGP2 are tightly regulated by posttranslational modifications[20]. For example, MDA5 is modified with ubiquitin or ubiquitin-like modifiers such as interferon-stimulated gene (ISG)15 on different sites by various E3 ligases, such as TRIM65[21–23]. LGP2 is ubiquitinated by TRIM14 and HOIL[21,24]. These modifications are required for the oligomerization of MDA5's CARD domains or to help in other stages of its activation. However, there is a debate about which E3 ligases specifically target MDA5 and the precise impact of these modifications[20]. Similarly, the functions of LGP2 ubiquitination events are still poorly characterized. Phosphorylation and dephosphorylation events govern MDA5 activation as well[20]. Most notably, the protein phosphatase (PP)1α/γ dephosphorylates serine 88 in MDA5 upon virus or liposome-induced disturbances of the cytoskeleton, thus licensing its activation[25,26]. ADAR1-deficient HEK293 cell lines that are engineered to encode a doxycycline-inducible LGP2 gene, however, mount a type I IFN response following LGP2 induction without liposome-based transfection strategies (**Chapter 3** and **4**). It is tempting to speculate that LGP2's interaction with MDA5 eliminates the need for dephosphorylation of MDA5 at this site in contexts where LGP2 is required, such as is the case in ADAR1 deficiency. It is still unknown whether LGP2 is also regulated by phosphorylation and how these events impact its function. Addressing this outstanding question will not only provide information about the activation of LGP2, but may also shed light on its collaboration with MDA5.

The ligand of LGP2 in ADAR1 deficiency

The identification of RNA ligands that activate RLRs and the subsequent type I IFN response in ADAR1 deficiency remains a key focus in the field. Inverted-repeat Alu elements (IR-Alus), *cis*-naturally antisense transcripts (*cis*-NATs), and Z-RNAs have been proposed as ligands that induce MDA5 activation when no longer edited[27]. The affinity of LGP2 for short dsRNAs and

its ability to induce short MDA5 filaments are consistent with this idea, as IR-Alus and Z-RNAs are typically short sequences[28,29]. In **Chapter 3**, we showed that overexpression of an ATPase-deficient mutant of LGP2 (K30A) was unable to mount a type I IFN response in ADAR1 KO cells. Given that ATP hydrolysis in LGP2 is needed for internal, but not terminal, binding of dsRNA, we hypothesize that the ligand in ADAR1 deficiency is a sequence within a larger RNA molecule that forms internal dsRNA stretches. As such, unedited IR-Alus would be promising candidates to bind and activate LGP2, given their presence within mRNAs, their high level of editing by ADAR1p150, and their involvement in innate immune activation[4,27,30].

In contrast to our hypothesis about IR-Alus, *cis*-NATS, or Z-RNAs being a potential ligand for LGP2, we demonstrated in **Chapter 4** that exonic sequences of ISG mRNAs interact with LGP2 in ADAR1-deficient cells. Interestingly, Z-RNA-prone sequences are present in 3' UTRs of ISG mRNAs in mice, which suggests that LGP2 might interact with these RNAs via Z-RNA binding[28]. However, we could not precisely map the interaction site of LGP2 to the mRNAs due to limited sequencing depth, nor did we test whether these RNAs were immunostimulatory. Given the fact that some LGP2-interacting ISG mRNAs do not have A-to-I editing sites in their exonic sequences and that LGP2 can interact with single-stranded (ss)RNA, these mRNAs may present a *bona fide* pool of RNA ligands that are regulated by, but do not activate LGP2. Alternatively, as discussed in **Chapter 2**, these RNAs may have a riboregulatory influence on LGP2 function. Nevertheless, the retrieval of ISG mRNAs as ligands for LGP2 most likely derives from technical limitations in our iCLIP strategy, as elaborately discussed in **Chapter 4**. Importantly, formaldehyde crosslinking to study the RNA interactome of LGP2 in ADAR1 deficiency represents one of the possible solutions for unraveling the ligands that drive symptoms in AGS.

Additionally, careful consideration must be given to the cell lines in which these ligands are studied. The absence of functional LGP2 expression in HEK293 cells serendipitously provided us with a way to generate ADAR1 knockout cell lines, as shown in **Chapter 3**. Additionally, the manipulability of HEK293 cells makes them an ideal and flexible platform to study these basic phenotypes. However, AGS patients usually exhibit severe brain lesions, with calcification of basal ganglia, myelin defects, and neuronal cell death, as well as leukodystrophy and chilblain lesions[31]. This suggests that neurons, leukocytes, and skin cells may either be very sensitive to type I IFNs or produce unique or high amounts of ligands that activate PRRs. However, these possibilities are not mutually exclusive. Notably, both mouse and iPSC-derived human neuronal cells contain a relatively high burden of dsRNAs, due to the brain-specific protein ELAVL2, which lengthens 3' UTRs in mRNAs[32]. These sites often contain repetitive elements that need to be edited by ADAR1 to prevent immune activation[30]. Indeed, depletion of ADAR1 in these cells causes a toxic type I IFN response that leads to neuronal cell death[32]. This suggests that brain-specific RNAs trigger a unique type I IFN response in these cells. Moreover, as shown in **Chapter 4**, LGP2-proficient ADAR1 KO HEK293 cells experience growth arrest but do not undergo complete cell death. Hence, HEK293 cells may not be fully representative for studying LGP2/MDA5 ligands, and RNA interactome studies need to be validated in relevant primary human cells or neuronal cell lines. Of note, cell death in ADAR1-deficient cells can be attributed to the activation of either protein kinase R (PKR) or Z-binding protein 1 (ZBP1)[33,34]. Whether these PRRs recognize different ligands than LGP2 and MDA5 in the absence of ADAR1 remains to be determined.

Is LGP2 essential in sensing self dsRNAs beyond ADAR1 deficiency?

The importance of LGP2 for sensing unedited self RNAs in ADAR1 deficiency raises the

question of whether LGP2 is also essential for detecting dsRNAs in other contexts of dsRNA accumulation. For example, depletion of SETDB1 or components of the human silencing hub (HUSH) complex, which epigenetically suppress expression of transposable elements, results in dsRNA-driven type I IFN responses that are partially caused by MDA5 activation[35,36]. Determining the role of LGP2 in these contexts could provide valuable insights into the preference of LGP2 for self dsRNAs. Besides sensing endogenous RNA structures, LGP2 is also critical in MDA5-dependent sensing of viral RNA during hepatitis C and D virus infection, underscoring its importance despite lacking direct signaling capability[37,38]. Future research is needed to determine if LGP2 is also indispensable for detection of other viruses, and what structures in viral RNA are recognized by LGP2.

An interesting possibility is that the role of LGP2 extends beyond sensing viral or endogenous dsRNAs. Indeed, LGP2 can also bind single-stranded RNA and DNA:RNA hybrids[4]. Moreover, LGP2 can hydrolyze CTP and GTP in addition to ATP, which may enable LGP2 to recognize a wider range of ligands than previously anticipated. Intriguingly, MDA5 has been observed to form complexes on DNA:RNA hybrids, raising the possibility that MDA5 activation extends beyond its usual dsRNA ligand preference[4]. Whether LGP2 directs this interaction and whether MDA5 undergoes CARD oligomerization, activation, and subsequent interactions with MAVS after binding to DNA:RNA hybrids remains unclear. Nevertheless, it leads to exciting questions about the requirement of LGP2 to sense other structures than dsRNAs in concert with MDA5. For example, mutations in the genes *RNASEH2A*, *B*, or *C*, which encode subunits of the ribonuclease RNase H2, cause AGS[31]. This enzyme breaks down the RNA components of DNA:RNA hybrids formed during DNA transcription, telomere elongation or reverse transcription of retroviruses. RNase H2 deficiency induces accumulation of these structures, leading to DNA damage and a constitutive type I IFN response that is largely orchestrated by the DNA sensor cGAS, which detects dsDNA breaks[39,40]. However, the exact molecular mechanism that leads to constitutive type I IFN production remains incompletely understood[41]. RNase H2 deficiency is associated with increased expression of endogenous retroelements and accumulation of DNA:RNA hybrids in the cytoplasm, which may also contribute to the type I IFN response[39]. The potential of LGP2 to interact with a broad range of ligands raises the possibility that LGP2 senses these structures and induces a type I IFN response. In conclusion, the increasing knowledge about LGP2 and its ligands raises many new questions about the involvement of LGP2 in viral infection and autoinflammatory research. Future research will help to understand the role of LGP2 better, potentially even providing rationales to use or target LGP2 in therapeutic strategies.

Therapeutic potential of targeting LGP2 in autoinflammation and cancer

The essential nature of LGP2 in aiding MDA5 to unleash a type I IFN response in ADAR1-deficient cells presents opportunities in therapies that target ADAR1 in both autoinflammatory disease as well as in anti-cancer treatments.

Aicardi-Goutières syndrome is a type I interferonopathy caused by a monogenic defect in any one of several genes that are involved in cellular RNA metabolism. ADAR1 mutations represent approximately 7% of the pathogenic variants in these genes and are classified as mutations that cause AGS type 6 (AGS6)[31]. Current therapies are non-specific and mostly aim to alleviate symptoms like respiratory failure, although they do not target the cause of the condition[42]. Various studies in AGS patients with ADAR1 mutations tested inhibitors of Janus kinase 1 (Jak1), a protein that signals downstream of the type I IFN receptor. These treatments showed significant inhibition of the type I IFN response and improved symptoms such as

skin inflammation. However, neurological symptoms showed little to no improvements[43–45]. This may stem from the inhibitors being unable to cross the blood-brain barrier, or the need to target other pathways simultaneously to alleviate neurological symptoms. Additionally, severe bacterial infections occurred in some patients, likely a consequence of Jak1 signaling downstream of the IFN-γ and other cytokine receptors[45]. Hence, inhibiting type I IFNs with Jak1 inhibitors may lighten some symptoms, but will need to be complemented with other treatments to more specifically resolve neurological phenotypes. Our findings in **Chapter 3** highlight that LGP2 may serve as a selective target for patients with AGS6. In ADAR1-deficient cell lines and primary human macrophages, depletion of LGP2 diminished the type I IFN response, paired with a restoration of cell growth, as shown by cell growth assays in **Chapter 3** and **4**. This implies that PKR activation, a key driver of cell growth arrest, was also prevented[34]. Additionally, while mice with a genetic loss of LGP2 show decreased survival than expected according to Mendelian ratios, LGP2 knockout is tolerated overall[1]. Mice with ATPase-deficient LGP2, however, show no developmental abnormalities, although responses in both genetic models to various viral infections are weakened. Together, these findings indicate that depletion or inhibition of LGP2 could offer a focused approach to inhibit the hyperproduction of type I IFNs in ADAR1 deficiency[1].

In contrast to the role of ADAR1 in AGS, depletion of ADAR1 shows potential in anticancer therapies. Various studies have shown that loss of ADAR1 sensitizes tumor cells to PKR-mediated cell death, enhances the response to checkpoint blockade, and induces immune cell infiltration[46–48]. In **Chapter 3** and **4**, we confirmed these findings, showing that ADAR1 loss inhibits the growth of the colorectal cancer cell line CAL27 and LGP2-overexpressing HEK293 cells. In line with our results about the role of LGP2 in inducing the type I IFN response in ADAR1 deficiency, we showed that sensitivity to ADAR1 loss in tumor cells depends on LGP2 expression. We confirmed this relationship in bioinformatic analysis of patients with bladder cancer, breast cancer, and sarcoma. Patients with tumors expressing low ADAR1 and high LGP2 levels showed greater survival probability, whereas those with low LGP2 expression had poorer outcomes. These results suggest that assessing LGP2 levels may be critical for effective ADAR1-targeted therapies in tumors. Interestingly, not all tumor cell lines tested are sensitive to ADAR1 loss, such as the lung adenocarcinoma cell line A549[48]. It will be interesting to determine the level of LGP2 expression in these cells to explore whether the absence of functional LGP2 expression limits the type I IFN response. If this is the case, LGP2 expression may serve as a predictive marker for the expected efficiency of ADAR1-targeting therapies.

Targeting ADAR1 is also proposed as therapy in combination with DNA methyltransferase inhibitors (DNMTi) treatment. Many endogenous retroviruses and transposable elements are epigenetically silenced by DNA methylation of their promoters. Treatment with DNMTis such as 5-aza-2'-deoxycytidine (5-AZA-CdR) reduces promoter methylation, leading to transcription and accumulation of these endogenous retroviral elements, which can activate PRRs like MDA5 and TLR3[49,50]. Because these RNAs form dsRNAs, they are edited by ADAR1. Loss of ADAR1 expression in 5-AZA-CdR-treated cells leads to a synergistic type I IFN response[51,52]. Similar to the role of LGP2 in ADAR1-sensitive tumors, we showed in **Chapter 3** that the synergy between ADAR1 loss and 5-AZA-CdR treatment is fully dependent on LGP2 expression in two cancer cell lines. Likewise, when treatment with the Cdk4/6 inhibitor palbociclib, which also upregulates endogenous retroelements, is combined with ADAR1 depletion, a synergistic type I IFN response is elicited, which depends on LGP2 expression[53]. Altogether, these results indicate that ADAR1 targeting is a promising approach in antitumor therapy, but that successful treatment likely hinges on functional LGP2 expression.

In conclusion, the work presented in **Chapter 3** and **4** of this thesis demonstrates that LGP2 is not merely a regulator of RIG-I and MDA5, as frequently proposed in previous studies. LGP2 can have a crucial function in sensing ligands, ranging from unedited self RNAs to viral ligands. Though the exact ligand preference of LGP2 in these contexts should still be determined, recent biochemical advances show that LGP2 may sense other RNA structures as well. This also implies that LGP2 may be relevant in other autoinflammatory diseases or viral infections as well. With these advances, LGP2 has become an exciting new player in the arena of drug development, whether it is as a target against autoinflammation or as crucial factor in antitumor treatment.

The role of vtRNAs and viral infection

The role of noncoding (nc)RNAs in regulating processes in the cell is a fast-expanding field of research. In our study presented in **Chapter 5**, we investigated the function of the ncRNA family of vault (vt)RNAs in the context of viral infections. The expression of vtRNAs increases during viral replication. Using a proteomic approach, we showed that vtRNAs interact with the proteins ELAVL1 and hnRNP C, which are needed for viral replication. Our findings suggest that viruses exploit vtRNAs for their own benefit. These results raise further questions about the processes that govern vtRNA expression, their cellular function, and their potential use in therapies, which will be discussed below.

How are vtRNAs transcriptionally regulated?

Our work in **Chapter 5** builds on previous studies that showed that various viruses, such as EBV, KSHV, and IAV, induce increased vtRNA expression during infection[54–57]. However, one of the outstanding questions in the vtRNA field is how these RNAs are transcriptionally regulated on a molecular level, both in uninfected and infected cells. The finding that a wide variety of DNA and RNA viruses induce vtRNA upregulation, suggests that antiviral signaling pathways may play a role in regulating their expression.

In Chapter 5, we confirmed that activation of the type I IFN response, whether through stimulation of cells with synthetic RNA ligands or recombinant type I IFN, did not promote vtRNA expression, in line with a previous study[56]. Conversely, activation of the proinflammatory NF-κB pathway by the EBV membrane protein LMP1 in a B cell leukemia line induced the expression of vtRNA1-1 4-fold, without affecting the expression of vtRNA1-2 and vtRNA1-3[57]. Similarly, stimulation of a neuroendocrine tumor cell line with TNFα, a potent inducer of NF-κB signaling, resulted in a 2.5-fold increase in vtRNA1-1 expression[58]. While these findings implicate NF-κB signaling in vtRNA expression regulation, the amplitude of vtRNA induction in these studies is not comparable to the vtRNA expression levels we observed in **Chapter 5**, indicating that additional pathways are likely involved.

The promoter architecture of the four human vtRNA genes may shed light on the pathways that regulate vtRNA expression. The vtRNA promoters are similar to each other, with proximal cAMP-responsive-like (CRE-like) and tetradecanoylphorbol acetate-like (TRE-like) response elements and distal regulatory elements[59]. The proximal sequence elements can be bound by the transcription factors CREB and AP1, which induce activation of anti-apoptotic, proliferation, and anti-inflammatory pathways[60,61]. In line with this, vtRNA1-1 expression decreases under starvation conditions, as signals that stimulate proliferation are absent in nutrient-poor conditions[62]. Additionally, transcription factor profiling in the K562 chronic myelogenous leukemia cell line indicates that the transcription factors that bind all four vtRNA promoters, are functionally involved in viral infection- and cancer-related processes[63].

While the involvement of transcription factors associated with viral infection is in line with our findings in **Chapter 5**, it is still unclear what exact pathway governs the activation of such transcription factors. It is also likely that complex integration of multiple signaling pathways and/or epigenetic mechanisms control the expression of vtRNAs.

Indeed, promoter methylation strongly affects vtRNA expression. Increased methylation status of promoters is inversely correlated with vtRNA expression in various cancer cell lines[63–65]. The promoter of vtRNA1-1 is the least methylated, likely explaining its high baseline expression in various cell lines[63]. In line with this, treatment with the DNMTi 5-AZA-CdR, which induces genome-wide hypomethylation of promoters, strongly induces the expression of vtRNA1-2 and vtRNA1-3, while leaving vtRNA1-1 expression unchanged[65]. Interestingly, the magnitude of vtRNA1-2 and vtRNA1-3 upregulation in this study is similar to the inductions we observed in various viral infections in **Chapter 5**, suggesting that virus-induced vtRNA upregulation may be mediated through epigenetic modifications. While viruses like influenza A virus can indeed influence the epigenetic landscape in cells, it will be worthwhile to assess if epigenetic regulation is a universal mechanism that underlies the induction of vtRNAs across all viral infections presented in **Chapter 5** and other studies[54–57,66].

An alternative explanation for increased vtRNA expression in virally infected cells is enhanced stability rather than *de novo* transcription. Although we did not address this question in our studies, some preliminary insights can be distilled from **Chapter 5**. When we rescued expression of vtRNA1-1, vtRNA1-2, and vtRNA1-3 in vtRNA1-deficient HEK293 cells and assessed vtRNA expression levels after viral infection, we did not observe transcriptional upregulation of transgenic vtRNA1-1, vtRNA1-2 or vtRNA1-3. Transcription of ectopic vtRNAs is not controlled in a similar manner as their endogenous counterparts, but their stability and half-life should be comparable. Hence, the fact that ectopically-expressed vtRNAs do not accumulate during the course of viral infection argues against a role for RNA stability in the increased abundance of vtRNAs. However, future experiments, such as roadblock PCRs, are needed to determine whether increased vtRNA expression is caused by increased stability or de novo transcription[67].

The role of the vault complex in viral infection

The role of vtRNAs in the context of viral infection has been a subject of study, although a full understanding of their upregulation and their function is still missing. One possible mechanism for vtRNAs function during viral infection is through their interaction with vault complex, which was discovered in 1986[68]. The vault complex is a barrel-shaped, macromolecular structure composed of multiple copies of the proteins MVP, TEP1, and VPARP that is strongly conserved among eukaryotes[69]. Despite being known for almost 5 decades, the precise function of the vault remains unknown. Studies have shown that the vault complex is associated with chemotherapeutic resistance, or might serve as a scaffold for signaling. Additionally, the vault complex may play a role during viral infections. For example, MVP promotes the induction of type I IFNs and other proinflammatory cytokines such as interleukin 6, and enhances antibody responses against IAV[70,71]. MVP also contributes to resistance against *Pseudomonas aeruginosa* infection in epithelial cells, suggesting that the role of MVP and the vault extends beyond protection against viruses[72]. However, other studies indicate that MVP inhibits NF-κB activation and signaling downstream of the type I IFN receptor, indicating the complexity of the role of MVP in antiviral pathways[73,74]. Interestingly, despite its strong evolutionary conservation, genetic loss of MVP is well tolerated in mice, suggesting that the true function of vault particles has not been discovered yet[75]. One of the limitations of our work in **Chapter**

5 concerns the interactions of vtRNAs with the vault particle. In our interaction studies, we did not determine whether vtRNAs interacting with either hnRNP C or ELAVL1 still require MVP, TEP1, or VPARP expression. Future studies should assess whether components of the vault are needed for the interactions between vtRNAs and hnRNP C and ELAVL1, and how the interaction between vtRNAs and the vault influences viral replication.

The function of vtRNAs in viral infection and beyond

Our results on the function of vtRNAs during viral infection align with previous studies that showed that vtRNAs aid viral replication[56,57,76]. However, during KSHV and Dengue virus (DeV) infection, vtRNAs may also antagonize viral replication. Both viruses cause host translational shut-down, leading to lower expression of the nuclear RNA phosphatase DUSP11, which normally removes immunogenic 5′ triphosphate moieties from nascent RNAs[77]. Loss of DUSP11 causes accumulation of triphosphorylated vtRNAs, which subsequently bind and activate RIG-I[78,79]. This suggests that their 5′ phosphorylation status may determine whether vtRNAs have pro- or antiviral functions. Notably, small changes in the secondary structure of vtRNA2-1 ablate its inhibitory role on PKR[80]. It will be of interest to assess whether 5′ triphosphorylated vtRNAs have the same folding as monophosphorylated vtRNAs, whether they still interact with hnRNP C, ELAVL1, or the vault complex, and if so, whether their function remains the same.

Besides the presence of a triphosphate moiety, other posttranscriptional modifications could affect their function. All vtRNAs can be epigenetically modified by deposition of a 5-methylcytosine group. This modification enables interactions with the Dicer machinery and leads to the processing of vtRNAs into smaller fragments named small (s)vtRNAs that have miRNA-like abilities[64,81–84]. It is still unclear if cleavage products of vtRNAs have direct impact on viral replication. However, as the svtRNA fragment of vtRNA1-2 is mostly localized to the nucleus, it is to be expected that vtRNA processing profoundly changes their function[81]. Moreover, vtRNAs are likely further posttranscriptionally modified, all of which can impact the function of these ncRNAs. Hence, a solid understanding of the posttranscriptional modifications of vtRNAs and their effect on their function is required to predict their role during viral infection.

The interactions between vtRNAs and ELAVL1 and hnRNP C occur both during viral infection, as well as in homeostasis. While we have only assessed the function of this interaction during SINV or EMCV infection, these interactions may serve different functions in uninfected conditions. Both ELAVL1 and hnRNP C are proteins that fulfill a myriad of functions in homeostatic RNA metabolism. For example, ELAVL1 increases mRNA stability by binding to AU-rich elements in the 3′ UTR of mRNAs, while hnRNP C is strongly involved in alternative splicing of pre-mRNAs[85,86]. It is tempting to speculate that vtRNAs may compete with mRNAs for binding with these proteins to modulate mRNA stability or alternative splicing in homeostatic conditions.

An interesting point where the studies of **Chapter 3**, **4**, and **5** may converge, is in the activation of the PRRs PKR and OAS1. Like LGP2 and MDA5, these receptors can sense unedited self RNAs when ADAR1 expression is lost. PKR activation induces NF-κB-mediated inflammatory signaling and translational arrest, while activation of OAS1 leads to production of 2′-5′-oligoadenylate, which activates the endoribonuclease RNase L and leads to non-specific degradation of cellular RNA[34,87]. Interestingly, both receptors can interact with vtRNA2-1, which prevents their activation and inhibits downstream signaling[88,89]. It is still unclear whether this interaction occurs in ADAR1 deficiency, given the type I IFN-inducibility

of many dsRNAs and the lack of IFN-mediated induction of vtRNA 2-1 expression. However, the discovery of specific inhibitors like vtRNA2-1 could provide valuable tools to study the contribution of the various activated receptors to the phenotypes in ADAR1 deficiency. This knowledge could pave the way for targeted interventions to modulate PRR activation in this disease context or beyond.

The role of vtRNAs in diseases and their therapeutic potential

Mutations in ncRNAs can contribute to diseases, similar to mutations in proteins[90]. In the case of vtRNAs, however, it remains unknown whether mutations or full genetic loss are associated with specific diseases. Whole genome sequencing could be used to investigate whether mutations in either of the vtRNA genes are associated with diseases where no obvious disease-associated mutation in protein-coding sequences can be identified. Interestingly, mice with a genetic loss of the sole mouse vtRNA (mvtRNA) are fully viable, but exhibit decreased platelet numbers, hinting at a potential role in hematopoiesis[91]. This mouse model provides a valuable tool for studying the relationship between vtRNA deficiency and potential diseases.

The upregulation of vtRNAs by a broad range of unrelated viruses and their proviral role during EMCV, IAV, SINV, and EBV infections, suggest that vtRNAs may be targeted to inhibit viral replication. In other diseases attributed to ncRNA dysfunction, strategies to knock down ncRNA expression, such as antisense oligonucleotides (ASOs) and short interfering RNAs (siRNAs), have shown promising results[92]. These oligonucleotides, which are reverse complementary to their targets, induce their degradation through the RNA interference pathway. Clinical trials have successfully used ASOs and siRNAs to deplete ncRNA expression. For example, miR132, a miRNA that is upregulated during cardiomyocyte stress, contributes to the remodeling of cardiac tissue and, ultimately, heart failure. A recent clinical trial demonstrated that an ASO against miR-132 improved outcomes in patients with chronic ischemic heart failure[93]. Similarly, targeting vtRNAs during viral infection might counteract their proviral function, potentially slowing viral replication and tipping the balance in favor of the host's antiviral defense. As proof of principle, in **Chapter 5**, we used ASOs directed against vtRNA1-1, vtRNA1-2, and vtRNA1-3 to inhibit EMCV replication in the A549 cell line. However, further fundamental research is required to fully understand virus-induced vtRNA upregulation and their role in cellular mechanisms before pursuing them as therapeutic targets.

Our work in **Chapter 5** has focused on 4 ncRNAs, but the entire landscape of ncRNAs in and outside human cells in health and disease is only beginning to be uncovered. Hence, we anticipate that studies into ncRNAs, including vtRNAs, will have to progress to large-scale, omics-based approaches. These studies must simultaneously integrate ncRNA species, their posttranscriptional modifications, and their interactions with proteins and other molecules to fully understand their action on cellular pathways. Nevertheless, large-scale studies should always be complemented with fundamental research to precisely understand the functions of ncRNAs. With an advanced understanding of these ncRNAs, we will likely unravel a vast network of interactions, regulatory mechanisms, and functions that will not only bring us closer to understanding fundamental cellular pathways, but also provide new rationales to develop new therapies that target or exploit ncRNAs.

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