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The function of 2'-O-methylated RNA in the context of viral infection

Funkce 2'-O-methylované RNA v kontextu virové infekce

Bachelor's thesis

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne: Podpis……

Jiří Potužník

Abstract:

RNA is subject to a wide array of post-transcriptional modifications. 2'-O-methylation is an essential intrinsic modification of RNA. It affects the structure and reactivity of the molecule as well as its function. 2'-O-methylation is highly conserved, present in all three domains of life. Viral RNA uses this modification to mimic the host and evade detection by the immune system. There are two main mechanisms, through which viral 2'-O-methylated RNA does this. The first is evading detection by a pattern recognition receptor form the RIG-I-like receptor family Mda5. Mda5 is capable of detecting unmethylated RNA and recognising it as non-self, thus initiating an immune response. The second mechanism the evasion and restriction of an effector molecule IFIT. IFIT proteins are capable of detecting the absence of 2'-O-methylation on viral RNAs and inhibiting their translation. They do this by interfering with the formation of the ternary complex, an essential member of ribosomal formation. Using viral 2'-O-methylation as a target for therapy, it is possible to develop attenuated vaccines.

Keywords: viral RNA, RNA modifications, 2'-O-methylation, Mda5, IFIT, RIG-I-like receptors, epitranscriptomics, WNV, JEV

Abstrakt:

RNA podléhá velkému množství post-transkripčních modifikací. 2'-O-metylace je přirozenou a nezbytnou modifikací RNA. Ovlivňuje její strukturu, reaktivitu a funkci. 2'-O-metylace je vysoce konzervovanou modifikací a je přítomná ve všech třech doménách života. Virová RNA využívá této modifikace k tomu, aby napodobila hostitelskou RNA a vyhnula se tak detekci hostitelským imunitním systémem. Existují dva hlavní mechanismy, pomocí kterých to 2'-O-metylovaná virová RNA dělá. Prvním způsobem je vyhnutí se rozpoznání pomocí pattern recognition receptoru Mda5. Mda5 dokáže rozpoznat nemetylovanou RNA jakožto nevlastní a následně spustit imunitní reakci. Druhým mechanismem je vyhýbání se a omezování efektorových molekul IFIT. Proteiny IFIT také dokáží detekovat nepřítomnost 2'-O-metylace na virové RNA a následně zabránit translaci virové RNA navázáním se na ternární komplex, který je nezbytný pro formaci ribozomu. Bylo dokázáno, že ovlivnění virové 2'-O-metylace může být využito pro tvorbu atenuovaných vakcín pro některá virová onemocnění.

Klíčová slova: virová RNA, RNA modifikace, 2'-O-metylace, Mda5, IFIT, RIG-I-like receptors, epitranskriptomika, WNV, JEV

List of Abbreviations:

ATP – adenosine triphosphate

CARD – caspase activation and recruitment domains

DENV – Dengue virus

DNA – deoxyribonucleic acid

dsRNA – double stranded RNA

hMPV – human metapneumovirus

IFIT – interferon-induced proteins with tetratricopeptide repeats

IFN – interferon

IRES – internal ribosome entry site

IRF – interferon regulatory factor

JEV – Japanese encephalitis virus

LGP2 – laboratory of genetics and physiology 2

LTA – lipoteichoic acid

m^6A – N^6-methyladenosine

MAVS – mitochondrial antiviral-signalling protein

MDA5 - melanoma differentiation-associated gene 5

mRNA – Messenger RNA

NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells

OED – oxidation, elimination and dephosphorylation

PAMP – pathogen-associated molecular pattern

Pre-mRNA – precursor messenger RNA

Pre-tRNA – precursor tRNA

PRR – pattern recognition receptor

RLR – RIG-I-like receptors

RNA – ribonucleic acid

rRNA – ribosomal RNA

snoRNA – small nucleolar RNA

snRNA – small nuclear RNA

ssRNA – single stranded RNA

TLR – toll like receptor

TNF – tumour necrosis factor

TRAF – TNF receptor-associated factor

tRNA – Transfer RNA

VP3 – viral protein 3

WNV – West Nile virus

WT – wild type

Contents

1. Introduction

This thesis aims to analyse and explain the function of 2'-O-methylation of RNA in a wider context. The general structures of nucleic acids are well known as are their properties and functions in many processes. They consist of one of four bases attached to a ribose (in RNA) or a deoxyribose (in DNA) and a phosphate (see fig. 1).

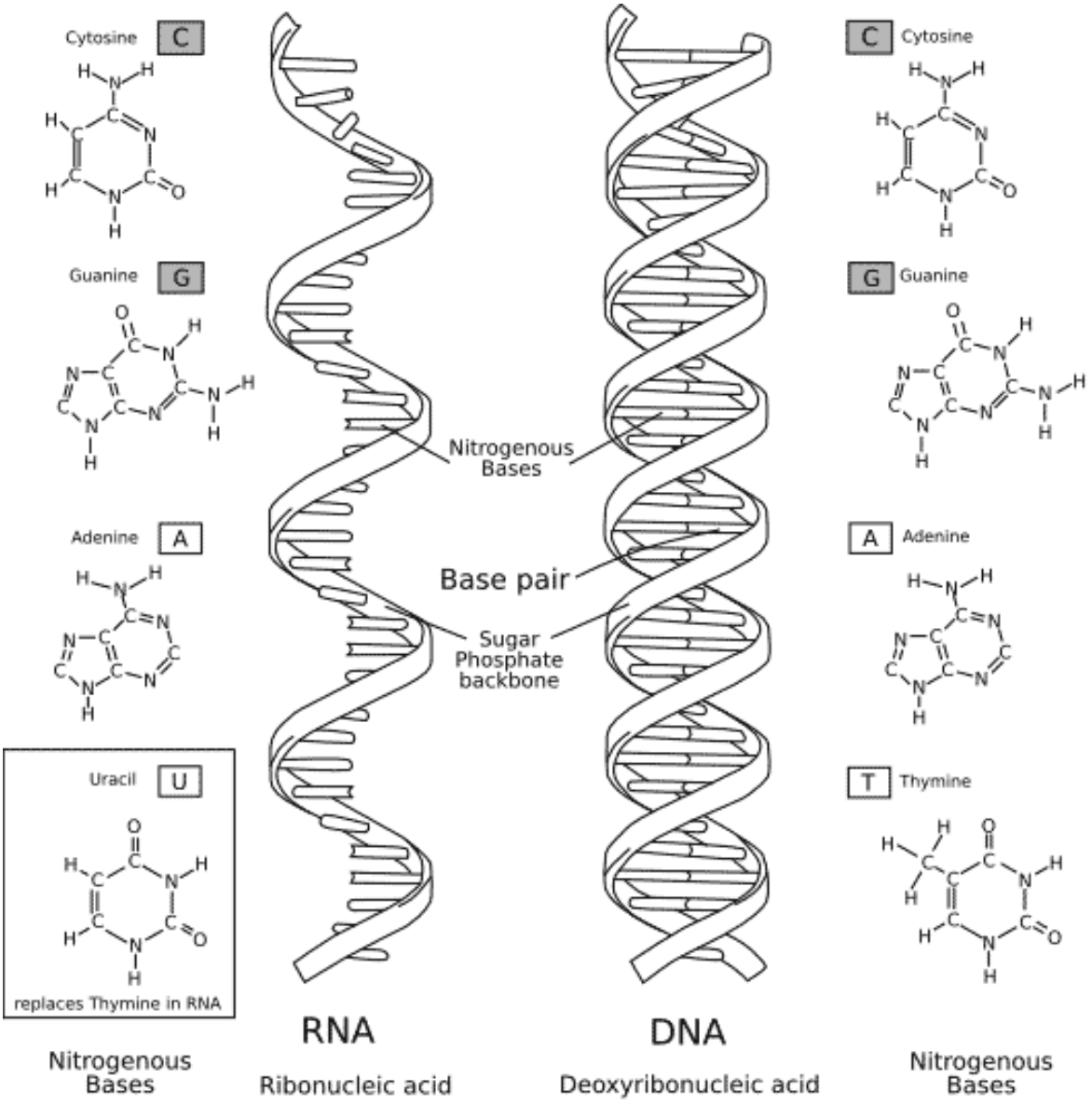

Figure 1. General structures of RNA and DNA with their corresponding bases. Original image at https://en.wikipedia.org/wiki/File:RNA-comparedto-DNA_thymineAndUracilCorrected.png and owned by multiple users, licensed CC-BY-SA

The functions include the storage of genetic information and its subsequent expression and regulation. There are still, however, many questions about other roles and functions these

nucleic acids might possess, as proven by the discovery of autocatalytic RNA in the early 1980s or the role of epigenetic modifications in the regulation of gene expression (Felsenfeld, 2014). DNA is the carrier genetic information (Houlihan et al., 2017). But its intrinsic ability to be modified is essentially limited to the actual bases with hydroxylations, deaminations and methylations. The most well-known of these modifications are the methylations of the position 5 of deoxycytidine and the position 6 of deoxyadenosine (Breiling and Lyko, 2015). Other modifications are restricted to histones, which then have a further regulatory role in gene expression (Handy et al., 2012).

Unlike DNA, RNA is subject to a wide variety of post-transcriptional modifications, which have been recognized and documented for over half a century (Roundtree et al., 2017). Currently over a 100 of these modifications have been discovered (Limbach, et al., 2017) and about two thirds of these modifications are methylations (Motorin and Helm, 2011). The fact, that RNA can be modified in so many ways may mean, that it possesses a plethora of functions other than just gene expression. In fact, the modifications also affect the molecular structure of RNA, effectively changing the charge, base-pairing potential and with it the secondary structure as well as numerous interactions with proteins (Roundtree et al., 2017). Recently a new field termed transcriptomics has emerged, which focuses on the dynamic modifications of mRNA and their function. Some of these modifications have received much more attention than others due to their easier detections and quintessential roles in gene regulation. These include RNA caps such as 7-methylguanosine also known as the 5'prime cap, responsible for eukaryotic mRNA stability, processing, nuclear export and translation initiation (Ramanathan et al., 2016) or the polyadenylation of mRNA to ensure a proper mRNA 3' end formation and nuclear export (Connelly et al., 1988). Some have been more recently discovered like the NAD cap of bacterial RNAs (Cahova et al., 2015; Jiao et al., 2017), whose functions are still somewhat unclear. These modifications can most aptly be summarized as cap and tail modifications (Roundtree et al., 2017) and their general discovery was quickly followed by the discovery of internal RNA modifications, who have only recently been brought to light thanks to advances in detection methods (Peer et al., 2017). Since the development of m6A-Seq, the most attention has been paid to m^6A in mRNA (Gilbert et al., 2016; Dominissini et al., 2012). The m^6A modification has been shown to drastically affect cell fate decisions, cellular pluripotency and is consequently linked to diseases such as cancer (Bertero et al., 2018).

These internal modifications are present all along the molecule, not just at the ends to stabilise and protect the molecule. Another important property of these internal RNA modifications is that they are not limited only to mRNA but are present in all types of RNAs both coding and non-coding (Xiong et al., 2017). In fact, some of these modifications are highly conserved from single cell organisms all the way to human cells, which yet again hints at the importance of their function (Erales et al., 2017). One of these modifications is the 2'-O- methylation of ribose (2'-O- Me), which is present in the RNAs of all the three domains of life as well as in viral RNAs. Although its functions are still largely unknown, its absence severely impairs the function of the nucleic acid and, in some cases, is lethal to the organism. This thesis is divided into three parts, the first of which will laconically attempt to explain the structure and role of 2'-O- Me in bacterial and eukaryotic RNAs. The second and main part will explain the role of this modification in viral RNA and the last part will provide a brief overview of the methods currently available for the molecular detection of 2'-O- methylations of RNA.

2. Chemical structure of RNA

DNA and RNA are chemically very much alike, as they are both linear polymers that comprise from 4 different nucleotides (Lodish et al., 2016). In turn, each nucleotide comprises of one of the four bases, adenine, cytosine, guanine for both DNA and RNA and thymine and uracil in DNA and RNA respectively. The base is attached to a ribose in RNA and a deoxyribose in DNA with a glycosidic bond and the sugar is connected to a phosphate group, which links one nucleotide to another with a phosphodiester bond (see fig. 2). The most obvious difference between the two nucleic acids is between the bases Uracil and Thymine. Chemically more significant, however, is the 2' position of the ribose, where DNA only has a hydrogen and RNA has a hydroxyl group.

This leads to a major difference in the reactivity. While RNA is capable of cleaving itself, using the hydroxyl as a nucleophile to cleave the phosphodiester bond between its own nucleotides, DNA is unable to do this (Ferré-D'Amaré and Scott, 2010). RNA 2' -O- methylation is the addition of a methyl (CH_3) group to the 2' hydroxyl. This essentially inactivates the 2' hydroxyl moiety. Through this inactivation, the modification determines which of the many riboses in the molecule remain catalytically active (Poole et al., 2000). The hydroxyl group of RNA is also an important factor in forming the tertiary structure of RNA and determining RNA to protein interactions. Both the structure and interactions are affected by the silencing of this -OH group (Roundtree et al., 2017). Overall, 2' -O- methylation of RNA is present in all forms

of life, but its abundance and function may differ greatly within one RNA molecule as well as in different types of RNAs from large ribosomal RNAs (rRNA) to small nuclear RNAs (snRNA) (Motorin and Helm, 2011).

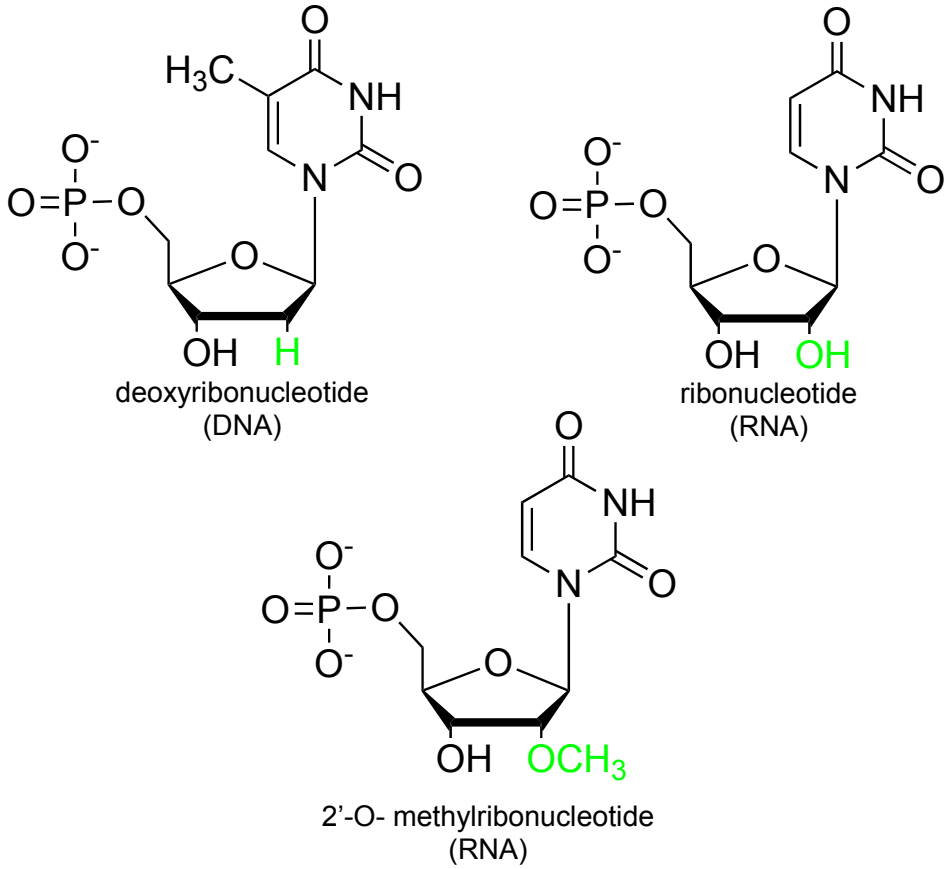

Figure 2. Structure of a DNA nucleotide (thymidine) with only a hydrogen at the 2' position, the same RNA nucleotide (uridine) with a hydroxyl group and a 2' -O- methylated RNA nucleotide (2' -O- methyluridine). All presented in their polymer chain form.

3. Prokaryotic RNA 2'-O- methylation

Compared to both archaeal and eukaryotic organisms, prokaryotic RNA 2'-O- methylation is fairly rare (Motorin and Helm, 2011) and the mechanism of ribose methylation differs as well. While eukaryotes and prokaryotes use ribonucleoprotein complexes, which will be discussed later, all mechanisms of ribose methylation in prokaryotes rely on site-specific or region-specific methyltransferases (Dennis et al., 2015). Although the effects of these methylations are numerous, they can be divided into three major categories based on the type of RNA they are in – bacterial messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA).

Although the presence of RNA 2'-O- methylation in bacterial mRNA has been reported more scarcely than in eukaryotes, its function in the translational apparatus has been tested both *in*

vitro and *in vivo* (Hoernes et al., 2016). Bacterial mRNA generally has a shorter half-life than eukaryotic mRNA (Dressaire et al., 2013) but the stabilising effect of 2'-O- methylation on RNA does not cause an increase in the rate of translation. It does the opposite. Based on the position of the 2'-O- methylated ribose within a codon, it can have varying effects and even hinder the translation of the full-length peptide. If the methylated nucleotide is placed at the second position of the specific codon CAA, it lowers the amount of peptide produced by up to 90%. This is probably due to the steric hindrance of the larger methyl group thus preventing the binding of the corresponding tRNA (Hoernes et al., 2016). This finding provides an interesting insight into the mechanism of stopping translation. It is an alternative to the stop codon and subsequent termination, thus providing more variability in translational processes.

The effect of RNA 2'-O- methylation of tRNA is twofold. The first is a relatively simple negative regulation of precursor tRNA (pre-tRNA) cleavage. Bacterial RNAse P cleaves the phosphodiester bonds between bases to create the 5' end of a mature tRNA (Gößringer et al., 2017). Due to the necessary presence of Mg^{2+} ions for this reaction and their interaction with the 2' hydroxyl of the ribose (Kazantsev and Pace, 2006), the methylation of this position significantly reduces the efficiency of cleavage. This is probably not only due to the changed chemical properties, but also due to the increased rigidity and different tertiary structure of the RNA (Kleineidam et al., 1993). The second effect can be observed in the methylations of some wobble positions in particular tRNAs, such as $tRNA^{Leu}$. The methylation effectively increases the recognition between the codon and anticodon and thus provides a minor advantage during peptide synthesis (Benítez-Páez et al., 2010).

Compared to mRNA and tRNA, rRNA has the most 2'-O- methylations and is methylated in conserved sites in all organisms as a part of rRNA maturation (Poole et al., 2000), this also hints at the importance of its role. The main sites of modification are at the 23S and 16S subunits and one of their functions is yet again the increased stability of the ribosomes as well as their heterogeneity (Dennis et al., 2015). The stability of the ribosome increases both in the sense of chemical reactivity and physical stability. It essentially refers to the dynamic stability of the molecule, where the ribosome has to react to factors like stress caused by heat. In strains of E. coli that had their methyltransferases inhibited, the cells experienced a difficulty of growth at normal conditions and an inability to respond to heat shock (Bügl et al., 2000). Another important aspect of rRNA 2'-O- methylation is interactions with antibiotics. The interactions between rRNA and antibiotics are very complex. The general function of the 2'-O- methyl

group in them can most aptly be characterised as mediation of the interactions. This affects the final functioning of the cellular translational apparatus for better or for worse. (Marchand et al., 2016). An entire group of rRNA methylations, including 2'-O- methylation, provides bacteria with resistance against antibiotics targeting the ribosome such as micrococcin or orthosomycin (Vester et al., 2013). An interesting aspect of these modifications is, that in some cases they may actually decrease the sensitivity of a bacterium to a certain kind of antibiotic. *Mycobacterium tuberculosis,* which causes tuberculosis, has 2'-O- methylations on both the 16S and 23S rRNA subunits. Without the methylations, the antibiotics viomycin and capreomycin lose their intended inhibitory effect (Monshupanee et al., 2012).

4. Archaeal RNA 2'-O- methylation

Although there are many similarities, the major difference between prokaryotic and archaeal RNA 2'-O- methylation is in the mechanism of the modification. As mentioned before, archaea mostly use a complex of proteins and sno-like RNAs which are the archaeal equivalent of eukaryotic small nucleolar RNAs (snoRNA) that guide methyltransferases to the designated position (Yip et al., 2013). Archaeal methylations are also more abundant, and their amount increases in thermophilic archaea (D' Orval et al., 2001). This is a due to the stabilising effect of 2'-O-methylations, which is especially important in increased temperatures, as some archaeal organisms have a growth temperature of up to 110 °C. In these temperatures, parts of the RNA may be bound to stabilizing proteins. The uncovered parts of the RNA, however, are highly methylated, which provides an alternate form of stabilization to the proteins (Dennis et al., 2015; Babski et al., 2014).

The other described function of archaeal RNA 2'-O- methylation is associated with translational fidelity and efficiency. A specific methylation on the tRNA anticodon loop was proved to protect against oxidative stress (Hori, 2017). Archaeal tRNA also has a similar ribose methylation as bacteria on the wobble residue of certain tRNAs (Joardar et al., 2011), facilitating an easier recognition between the codon and anticodon under stress conditions. Another similarity between archaeal and bacterial RNA 2'-O- methylation locations is the rRNA. Specifically, the area around the peptidyl transferase loop, which is densely methylated, shows the importance of these 2'-O- methylations in RNA-RNA interactions (Dennis et al., 2015). The 2'-O- methylations in the small ribosomal subunit help stabilize the interactions between its domains. This subunit is made up of 4 domains with specific secondary and tertiary structures necessary for their functioning. Changes in these structures can impair the function

and efficiency of the translational apparatus (Petrov et al., 2014). 2'-O-methylated nucleotides in specific locations enable better stability of the decoding centre along with tighter interactions between the 4 subunits, thus ensuring a stable ribosomal subunit and efficient translation (Dennis et al., 2015).

5. Eukaryotic RNA 2'-O- methylation

The conservation of RNA 2'-O- methylation and the similarity of its function mean, that even eukaryotic organisms employ this modification in their RNA. It is present across all types of RNAs. For example, in rRNA 2'-O- methylation is the most frequent chemical modification of the molecule (Erales et al., 2017). Although the function of this modification is in many ways similar to the previous types of organisms, there are some specificities in eukaryotes.

The first specifically and heavily 2'-O- methylated RNAs are small nuclear RNAs (snRNA) such as the U1, U2, U4, U5 and U6. These snRNAs are a part of the spliceosome and are responsible for pre-messenger RNA (pre-mRNA) processing – specifically for the excision of introns from it (Karijolich and Yu, 2010). Apart from a structural role, the methylations have a functional role as well. In the U2 snRNA they are an integral component in the formation of a spliceosomal intermediate complex known as the the E complex (Makarov et al., 2012). Due to their location, RNA 2'-O- methylations seem to play a role in the catalytic activity of the spliceosome. The 2'-O- methylations have the ability to affect the hydrogen bonding of the ribose and to change the hydration sphere around the modified nucleotide thus eliminating side interactions with the sugar (Karijolich and Yu, 2010). The ways in which each particular 2'-O-methylation affects each particular snRNA vary greatly. The resulting function of these modifications is, however, similar. They are essential to the snRNA and pre-mRNA interactions, which leads to correct, efficient pre-mRNA processing, and its maturation into translatable mRNA (Smietanski et al., 2013).

The splicing of pre-mRNA leads us to another type of RNA 2'-O- methylation in mRNA. The messenger RNAs of multicellular organisms possess a five-prime cap (5' cap), a 7-methyl guanosine connected through a 5'-5' triphosphate bond to the rest of the transcript (see fig. 3). The first and second nucleotides of the transcript often have a 2'-O- methylated ribose (Werner et al., 2011). This is essential for the processing of the mRNA, its efficient translation and of course the overall stability (Smietanski et al., 2013). The 2'-O- methylation of the 5' cap also complements the cap's ability to resist degradation, or rather complements the recognition of

the cap as functional. In the pre-mRNA state, the molecule is subject to a number of modifications that protect it from exonucleases and subsequent degradation. In fact, the enzymatic activities of some exoribonucleases are entirely stopped by the ribose methylation, whereas mRNAs without it are decapped and degraded (Picard-Jean et al., 2018).

Another eukaryotic RNA 2'-O- methylation is that of the rRNA. As mentioned before, it is the most common modification of the rRNA and it has been proven as essential in eukaryotic organisms. Without it, translation becomes inaccurate and also less efficient and less specific (Baudin-Baillieu et al., 2009). This is interesting as this type of modification would point to an intrinsic ability of the ribosome to regulate its own functions – something known as the specialised ribosome concept (Dinaman, 2016). This concept postulates, that the ribosome is not merely a ribozyme with a hardwired regulatory function. Instead it states that the ribosome is a highly heterogeneous complex, that can influence the way in which genes are translated into functional proteins.

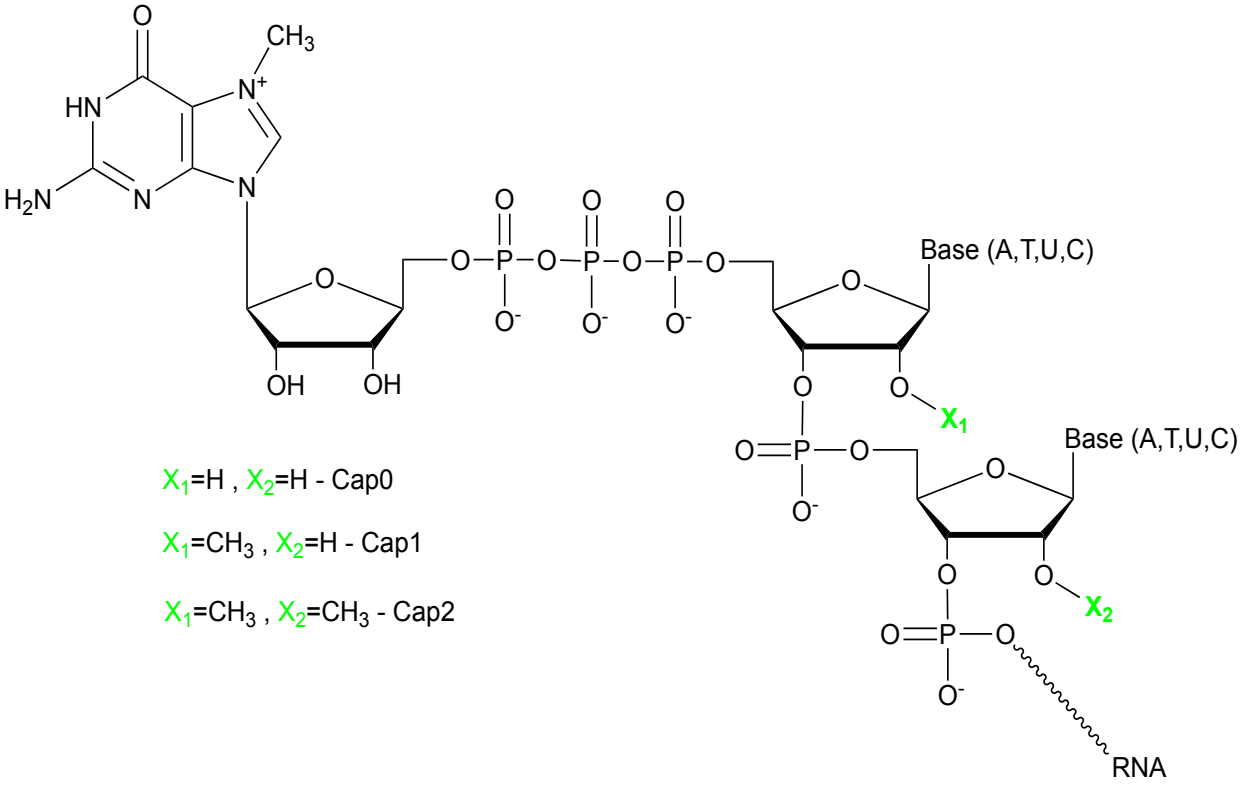

Figure 3. The structure of a 5'prime cap. The ribose after the triphosphate is 2'-O- methylated in cap1 and both riboses are 2'-O- methylated in cap2. In cap0 the CH_3 groups are replaced by H.

The ribosome does this through a wide array of functions, which include a number of post-translational modifications of proteins and post-transcriptional modifications of RNA (Xue and Barna, 2012). To illustrate the importance of these modifications not only in the context of their specific functions but as part of the functioning of the entire organism several inhibitory studies were done. In fact, the absence of a single 2'-O- methylation was enough to be lethal in a zebrafish embryo (Higa-Nakamine et al., 2012).

There is more proof of the functional aspects of ribose methylations. These include their importance in a cap independent translational mechanism and their location in internal ribosome entry sites (IRES). Clearly, the amount of 2'-O- methylation is also highly upregulated in cancer cells (Erales et al., 2017). It seems that the amount of processes 2'-O-methylation is essential for is higher than previously anticipated.

6. Viral 2'-O- methylation

RNA 2'-O- methylation is clearly conserved across all domains of life and viruses are no exception. Although there are numerous ways in which viral RNA can be 2'-O- methylated, all these modifications serve a similar function. A major evolutionary and selective force in viruses is driven by the induction and evasion of host immune response. The 2'-O-methylation of viral RNA is an evasive mechanism against a certain type of immune response (García-Sastre, 2011). The co-evolution of host organisms next to viruses has led to a plethora of methods, through which viruses evade or suppress the host immune system. The immune system, on the other hand, developed a method of recognizing pathogen-associated molecular patterns (PAMPs) and thus distinguishing between self and non-self (Chan and Gack, 2016). In order to understand the mechanisms of viral host system evasion, it is important to know how viral RNA is detected.

6.1 Immune detection of viral RNA

An intrinsic ability of the innate immune system is the ability to recognize foreign material based on the interaction between PAMPs and pattern recognition receptors (PRRs). PRRs are capable of detecting conserved molecular patterns and features of viruses (such as dsRNA or 5'-ppp-RNA). The detection of a pathogen sends a signal and starts expressing antiviral genes and antiviral cytokines (Chan and Gack, 2016). There are two main detection methods of viral RNAs. Specific membrane bound PRRs called Toll-like receptors (TLRs) detect extracellular PAMPs and those entering the cell through the endocytic pathway (Melchjorsen et al., 2013).

10 TLR genes are expressed in human organisms and each of them recognizes a specific type of ligand. The TLRs 1,2,4,5 and 6 are located on the plasma membrane and the TLRs 3,7,8 and 9 are located in the endosome. The TLR-1/TLR-2 heterodimer and the TLR-2/TLR-6 heterodimer detect bacterial and fungal PAMPs such as lipoteichoic acid (LTA), a component in the cell wall of bacteria or zymosan, a glucan found on the surface of fungi and many others. TLR-4 also detects bacterial liposaccharides and LTA, whereas TLR-5 detects flagellin a subunit of bacterial flagella. The TLR-7 and TLR-8 detect ssRNA viruses, TLR-3 detects dsRNA viruses and TLR-9 detects DNA with unmethylated CpG present in both bacteria and some types of viruses (Murphy et Weaver, 2017; Lester and Li, 2014).

Viral RNA, which is already inside the cell, is detected in a different manner by a specific protein family called RIG-I-like receptors (RLRs). These proteins have a helicase-like domain through which they bind to the viral RNA and set off a signal that starts the host antiviral response (Murphy et Weaver, 2017). The RLR protein family is made up of three homologous proteins called the RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation-associated gene 5) and LGP2 (laboratory of genetics and physiology 2). All three of them share the ability to detect and identify viral RNA and they are also capable of hydrolyzing ATP (Bruns and Horvath, 2015). However, their enzymatic activity, the method of detecting foreign RNA and the subsequent signaling to initiate the immune response is different for each of the three proteins (Ramos and Gale, 2011). RIG-I is mainly responsible for detecting double stranded RNA (dsRNA) or uncapped viral RNAs which possess a triphosphate or a diphosphate at the 5’ end (Goubau et al., 2014). LGP2 has an even larger binding affinity for viral dsRNAs and is able to recognize them even without the free phosphates at the 5’end (Bamming and Horvath, 2009). The protein MDA5 appears to detect atypical secondary structures of viral RNAs and it also uses RNA 2’-O- methylation to distinguish self RNA from viral ones (Bruns and Horvath, 2015). This hints at the aforementioned function of viral 2’-O- methylation as a camouflage from the immune system.

6.2 Viral RNA capping

As mentioned before, eukaryotic mRNA is methylated at the first and sometimes second nucleotide behind the 7-methyl guanosine cap (see fig. 3). These structures are called cap1 and cap2 respectively (Byszewska et al., 2014). There are several types of viruses that replicate in the cytoplasm and have adapted a mechanism in which they create alternative 5’ends, some of these examples include coronaviruses (5′-cap structures), flaviviruses (5′-cap structures),

picornaviruses (VPg and IRES), poxviruses (5′-cap structures). The alternative 5' end elements include small viral proteins sometimes termed Vp_g, which can directly interact with the host cell translational machinery. In addition, the RNA can have an internal ribosome entry site (IRES) facilitating a cap-independent translation (Decroly et al., 2011). Other viruses have evolved mechanisms in which they remove and use the cap of the host mRNA as a primer for the viral mRNA, this is called snatching (Stevaert at al., 2016). Viruses in the families *Retroviridae* and *Bornaviridae* use host RNA polymerase II as well as host machinery for RNA capping (Diamond, 2014). Lastly, some viral genomes carry with them encoded functional enzymes that create their own caps such as the RNA 5'-triphosphatase or RNA-guanine-N7-methyltransferase etc. An example of this is the Chikungunya virus, member of the togaviridae family, which uses a non-structural protein 1 (nsP1) as its capping enzyme. The inhibition of this capping mechanism appears as an ideal target for therapeutic development (Delang et al., 2016). All this is clear proof, that both the cap structure and the RNA 2'-O- methylation provides a molecular basis for the distinction of viral RNA (Züst et al., 2011).

6.3 Coronavirus and rotavirus 2'-O- methylation

Human and mouse coronaviruses are positive single strand RNA viruses (+ssRNA) that code their own N7 methyltransferases as well as 2'-O- methyltransferases in order to methylate their 5'caps (Chen et al., 2009, Decroly et al., 2008). Interestingly, the protein responsible for the 2'-O- methylation belongs to the same family as the human methyltransferase fibrillarin (Feder et al., 2003) which is guided by a snoRNA mechanism. Whether it employs the same mechanism as the viral methyltransferase remains to be elucidated but could eventually provide a new target for therapy. To fully confirm the function of the 2'-O- methylated RNA, an in vitro experiment with a mutant strain of human coronavirus was performed. The mutant had an inactivated 2'-O- methyltransferase and, compared to the wild type (WT) virus, induced a stronger immune response. Particularly the production of interferon – β (IFN-β) was significantly higher and the virus was completely restricted in cells pre-treated with interferon - α (IFN- α) (Züst et al., 2011). This provides conclusive proof of the biological role of the 2'-O- methyl moiety of mRNA in the induction of antiviral response – specifically the innate immune response with type I interferon (Schnierle et al., 1992). An in vivo version of the experiment with mouse hepatitis virus (MHV), a member of coronaviridae (+ssRNA), provided similar results. The strains with a mutated 2'-O- methyltransferase were not detected in the spleens and livers of infected mice after two days. The virus could, however, be detected in mice missing the Mda5.

Even in vivo, it is clear that viral RNA 2'-O- methylation is a mechanism through which the virus prevents the innate immune system from distinguishing it as non-self (Züst et al., 2011).

Another example of 2'-O- methylated virus is the rotavirus. Unlike the coronaviruses, rotaviruses are double stranded RNA (dsRNA) and thus can be recognized by RIG-I as well. The creation of the genomic dsRNA, however, doesn't take place freely in the cytoplasm, but happens in the viroplasm. The viroplasm protects the more easily detectable dsRNA against the host immune system. This makes it less likely to be detected by RIG-I. Before the dsRNA is formed however, the primary transcript is a ssRNA and could thus be detected by Mda5 (Silvestri et al., 2004). This stems from the fact, that the rotavirus VP3, the enzyme responsible for the 2'-O- methylation, is not a 100% efficient. At any given point then, there should be a set of viral RNAs lacking the 2'-O- methyl moiety, presenting themselves as non-self to the innate immune system (Morelli et al., 2015). When viral RNAs were taken from in vivo samples a control experiment was set up to elucidate and confirm the function of the 2'-O- methyl moiety. The incompletely methylated RNAs were incubated with 2'-O-methyltransferases in order to repair the unmethylated riboses. A phosphatase and a capping enzyme were also added as not to alert the immune system in some other way. Control reactions without the enzymes were set up to illustrate the difference.

The experiment results showed two major immunostimulatory effects. The first was an exposed triphosphate, the other was an incompletely 2'-O-methylated cap structure. The VP3 also carries a capping function in addition to the methyltransferase one. The interesting discovery was, that when presented with an excess of the methyl donor groups, the enzyme was more affective at methylating the RNA than capping it. This again points at the conservation and importance of viral RNA 2'-O- methylation. The in vivo experiment, however, clearly showed an increase in IFN-β production, when incompletely methylated (Uzri et Greenberg, 2013).

6.4 Viral 2'-O- Me detection with Mda5

The Mda5 and RIG-I are responsible for detecting complementary patterns of viral RNAs. They both detect dsRNA, but while RIG-I also detects 5'phosphorylated ends of viral RNAs, it does not detect 5'cap and ribose methylations. RIG-I also differ in the length of the RNA it binds to. It prefers short RNAs (about 300bp) with exposed diphosphate or triphosphate 5'ends. Mda5 binds internally to longer RNAs (more than 1000bp) and it is also capable of detecting RNAs

without 2'-O-methylations. Both RIG-I and Mda5 are mostly signalling proteins, that work best in conjunction with another RLR protein – the LGP2, which has much higher affinity for viral RNA (Reikne et al., 2014).

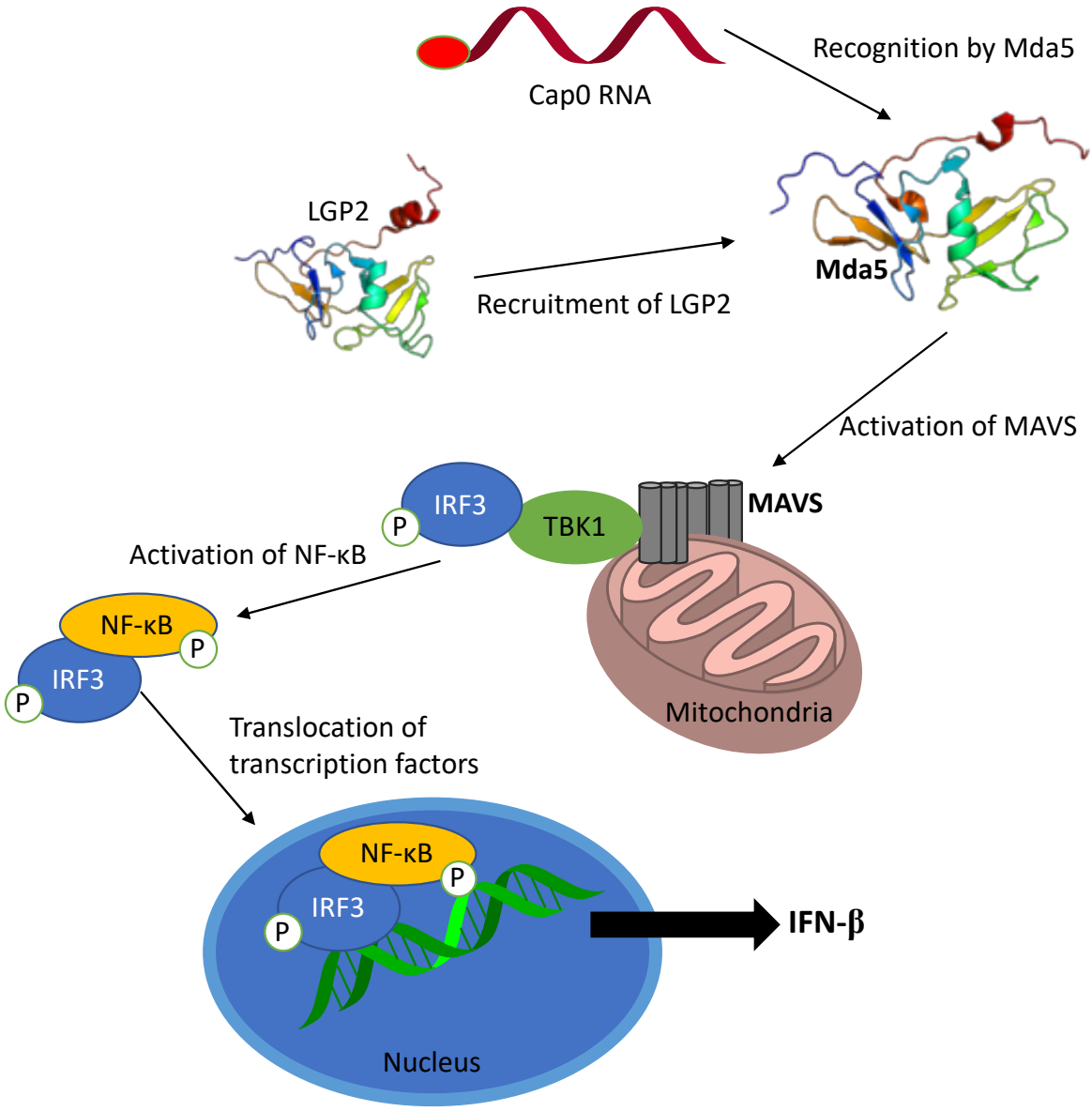

Figure 4 A scheme of the Mda5 signalling cascade leading to the production of IFN-β. Viral RNA lacking 2'-O- methylation is detected by Mda5 and LGP2. The CARD domain of Mda5 activates MAVS, which activate the kinase TBK. The kinase in turn activates IRF3 and NFκB. These transcription factors lead to the expression of IFN-β.

When Mda5 detects unmethylated viral RNA it binds to it. The binding results in a conformational change that exposes the caspase activation and recruitment domains (CARD) of the protein. CARD domains are capable of facilitating the formation of multiprotein

complexes. In the case of Mda5, the CARD domains recruit and activate mitochondrial antiviral-signalling protein (MAVS). The MAVS then assemble into filaments along the outer membrane of mitochondria and function as a signalling scaffold (Liu et al., 2013; Bruns et al., 2014). This scaffold then causes the assembly of several signalling proteins (TRAF2, TRAF5, and TRAF6 among others) and signalling kinases (TBK1 and IKK).
The kinases subsequently activate the two transcription factors IRF3 and NFκB which finally lead to the expression of type 1 IFN including IFN-β (see fig.4). Type I IFNs are the effector antiviral cytokines (Bruns and Horvath, 2015).

6.5IFIT detection of viral 2'-O- methylation

However, there is another family of molecules that is not only capable of detecting the absence of a 2'-O- methylation, but also of directly inhibiting its translation. It is the Interferon-induced protein with tetratricopeptide repeats 1 and 2 (IFIT 1, IFIT 2), molecules whose production is induced through interferon stimulation (Diamond, 2014). More specifically, the increase in the expression of the IFIT genes is due to the presence of IFN-stimulated response elements (ISRE) in the promoter regions of the IFIT genes (Levy et al., 1986). The expression of the IFIT genes is not, however, dependent only on type I IFN stimulation. It can also be directly caused by the ligation of PAMPs and PRRs such as Mda5 (Diamond, 2014).

West Nile virus (WNV) is a member of Flaviviruses, which are +ssRNA viruses and they possess a cap1 structure (see fig. 3). The methyltransferase responsible for the 2'-O-methylation is the non-structural protein 5 (NS5) (Dong et al., 2008). In order to test the effects of 2'-O- methylation, the methyltransferase was inhibited, and the mutant viral strain was produced. In mice, the original virus has a 40 % mortality rate whereas the mutant has 0%. However, the binding assays with RIG-I did not show any difference between the binding ability of RIG-1 to the methylated or unmethylated RNA. The absence of the 2'-O- methylation did not affect the distinction of non-self RNA and the IFN induced immune response through RIG-1 activation. This pointed to a different mechanism of action.

When the suppression of viral replication was tested against IFIT 1 and IFIT 2, it provided conclusive results, that IFITs are the molecules detecting the 2'-O- methylations. Viral replication in transgenic cells expressing the IFIT 2 was slowed up to 60x in unmethylated viral RNA compared to the methylated type. These results prove not only that IFIT 2 is an effector antiviral molecule, but also that its function can be inhibited by the presence of viral 2'-O-

methylation. Viral 2'-O- methylation stops the IFIT 2 from working properly (Daffis et al., 2010). An experiment, where Ifit 1 (a murine homolog of IFIT) deficient mice were infected, showed similar results. Compared to a WT virus, the virulence of the mutant virus lacking 2'-O- methylations was significantly lower. This again points to the importance of viral 2'-O-methylation in inhibiting the replication of viral RNA (Szretter et al., 2012).

The mechanism by which IFIT 1 inhibits viral replication is based on its high affinity for the cap0 structure (see fig. 3). Most types of viruses are dependent on exploiting the host translational system. They need it for the expression of their own viral proteins that can be assembled together and ultimately produce new viral particles (Abbas et al., 2017). The translation of mRNA, both cellular and viral, is dependent on the binding of initiation factors and the subsequent recruitment of a ribosome. Specifically, the eukaryotic initiation factor 4E (eIF4E), which is a part of the eukaryotic initiation factor 4F (eIF4F). The protein complex eIF4F is integral for the recruitment of the small ribosomal subunit to the mRNA. These factors bind to the 5' cap of mRNA as does the IFIT 1 protein (Kumar et al., 2014). The affinity of the initiation factors is higher for the cap1 and cap2 structures than that of IFIT 1, while the affinity of IFIT 1 is much higher for the cap0 structure. This means that IFIT 1 effectively outcompetes and preferentially binds to cap0 mRNA instead of these factors and abrogates the translation of viral proteins (Hyde et Diamond, 2015). In this manner the IFIT 1 may also be a part of an intrinsic complex that identifies and degrades nascent badly capped mRNAs, but this function remains to be elucidated (Kumar et al., 2014). This is not the only way that IFIT 1 interacts with viral RNA and inhibits its translation. It can supposedly block the recruitment of the ternary complex eIF2-GTP-$tRNA^{Met}$ with the 40S ribosomal subunit. The ternary complex is responsible for bringing the initiator $tRNA^{Met}$ to the P site of the ribosome. This enables the completion of the ribosome and effectively starts translation. If IFIT prevents the binding of the ternary complex to the 40S subunit, the subsequent recruitment of the 60S unit does not occur and translation does not start. The IFIT molecule is also capable of detecting an uncapped RNA with a triphosphate at the 5'end. However, these methods are independent on the 2'-O-methylation of viral mRNA (Diamond, 2014).

6.6 Internal viral RNA 2'-O- methylation

The functions of viral 2'-O- methylation discussed above are without question biologically important for the viral life cycle. They are however limited to the cap1 and cap2 structures. But

internal 2'-O- methylation of viral RNA has also been detected. Specifically, the internal 2'-O-methylation of adenosines in several flaviviruses including the WNV and Dengue virus. The methyltransferase NS5 methylates internal adenosines irrespectively of the surrounding sequence. The presence of internal methylations was proved experimentally by creating recombinant viral RNAs without cap structures and then confirming their presence. Even without a cap, NS5 was capable of methylating the RNA. The NS5 was also capable of methylating a prepared polyA oligonucleotide. Just like the cap1 and cap2 structures, the immunogenic effect of internally methylated viral RNA was lower than that of the unmethylated one (Dong et al., 2012). The effect of this internal 2'-O- methylation, however, also proved to be quite the opposite to the cap1 and cap2 methylations. The internally methylated adenosines of viral RNA attenuated both viral replication and viral translation by up to 22 %. Efficiency of viral RNA elongation was also reduced. The function of the attenuation of viral RNA production and replication needs to be further explored as well as the capability of the NS5 to methylate the internal adenosines of host RNA (Zhao et al., 2015, Dong et al., 2012).

6.7 Viral attenuation using 2'-O- methylation

2'-O- methylation plays an integral role in the virulence of a virus and in the effectivity of its replication cycle. As this modification increases the ability of the virus to evade the host immune system, 2'-O- methylation provides a perfect target for potential therapy. Several attempts were already made to use 2'-O- methylation deficient viral RNAs as a vaccine. Two viral families in particular, the flaviviruses (+ssRNA) and paramyxoviruses (-ssRNA,) have been explored in this manner (Li et al., 2013, Zhang et al., 2014). Historically speaking, live attenuated vaccines are one of the most effective protection against human viral diseases sometimes resulting in the eradication of a virus such as smallpox. They are essentially a viable, but less pathogenic and virulent version of the virus (Minor, 2015).

In order to test the functionality of this thesis the Japanese encephalitis virus (JEV) was used as a model flavivirus. A viral mutant was prepared lacking the 2'-O- methyltransferase ability but retaining the other enzymatic functions of the viral protein responsible. This mutant still remained replicative, but also became much more susceptible to the innate antiviral response. Indeed, in a mouse model, the mutant version of JEV proved to be less virulent than the wild type. When the immunized mice were presented with a lethal challenge of the virulent JEV

strain, they were completely protected (Li et al., 2013). This is concrete proof, that viral RNA lacking 2'-O- methylation works as an attenuated vaccine. A mathematical model of the kinetics of the attenuation managed to identify the determinants responsible for it. The determinant is the accelerated production of IFN. Mutant viral strains induce a faster IFN production, that manages to precede the resistance that infected cells have for IFN. Treatment of infected cells by IFN from a paracrine source is severely less effective, than autocrine IFN production (Schmid et al, 2015).

A similar experiment was performed with human metapneumovirus (hMPV), a negative sense ssRNA virus. This virus was first discovered in 2001 in the Netherlands and since then has been described worldwide and identified as one of the leaders in causing lower respiratory tract infections (Schildgen et al., 2011). Using cotton rats as a model, a similar mutant as the JEV was prepared. The mutant virus had a functional capping mechanism but was specifically inhibited in RNA 2'-O- methylation. In cellular cultures, the mutant strain had a lower plaque formation and slower growth. In cotton rats the mutants were attenuated, and no infectious viral RNA was found in the lower nor the upper respiratory tract. However, the rats produced a higher amount of viral antibodies in comparison with those infected by the normal (wild type) virulent strain. The immunized cotton rats also proved completely protected from any further viral replication even when presented with the virulent viral strain (Zhang et al., 2014).

The other benefits of these types of vaccines are twofold. An inactivated vaccine against hMPV had similar immunogenic effects as the attenuated one. However, the cotton rats suffered more lung damage upon virus reinfection (Yim et al., 2007). The attenuated vaccines prevent the enhancement of the lung damage not only in animal models but also in human clinical trials (Collins et al., 2002). More importantly, the fact that live attenuated vaccines mimic the effects of a natural wild type viral infection, means that they induce a more robust and longer lasting immune response (Zhang et al., 2014).

7. 2'-O- methylated RNA detection and isolation

Even though the 2'-O- methyl group is easily detectable in fragmented RNA using the LC/MS this method has one big drawback – it does not provide the location of the methylation on the RNA. That is why several other methods were developed in an attempt to pinpoint the exact location of the modified ribose. A fundamental problem that has made the localization of 2'-

O- methylation somewhat difficult is the intrinsic unreactivity of the methyl moiety, compared to the hydroxyl group.

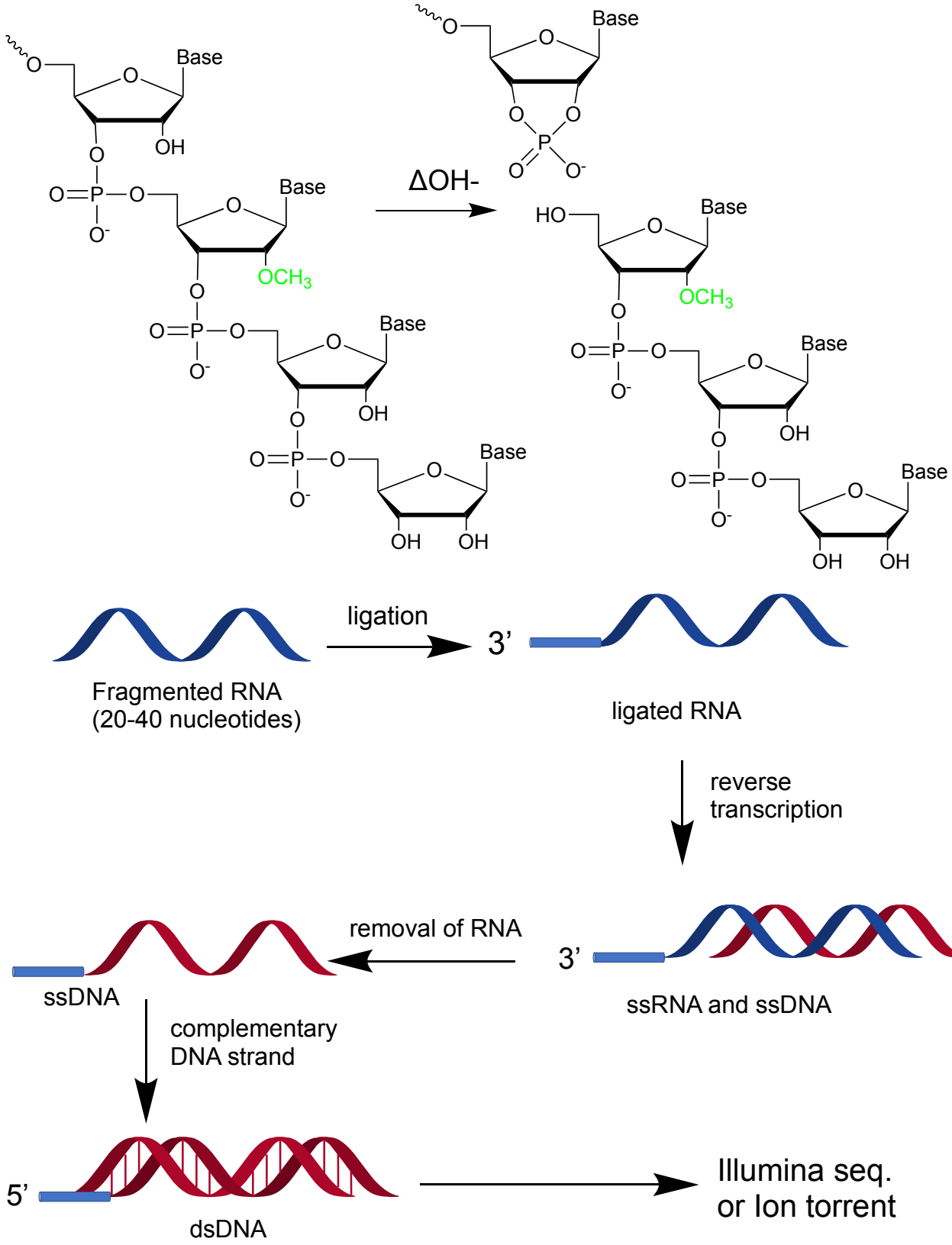

Figure 5. Alkaline cleavage of RNA and RiboMeth-seq.

The method used for the longest time was based on the ability of the methyl group to disrupt reverse transcription when presented with a limited amount of deoxynucleotides (Dai et al., 2017). This method is time consuming and only detects a limited number of nucleotides at a time. To increase efficiency, another method for high-throughput sequencing methods was developed. Even though this method has been further modified, the basis for all these modifications is the RiboMeth-seq. RiboMeth-seq is essentially a negative detection method. It takes advantage of the 2'-O- methyl resistance to alkaline cleavage (see fig. 5) and breaks the unmodified riboses by basic activation of the 2' hydroxyl group leading to a nucleophilic attack on the phosphodiester bond thus cleaving the RNA strand.

The RNA is degraded into shorter fragments a couple of tens of nucleotides long, which are ligated into RNA oligomers and subsequently reverse transcribed. The resulting cDNA is then analysed using ion semiconductor sequencing (Birkedal et al., 2015). The specificity and laborious preparation of the adapters for ligation etc. of this method led to the development of a method that changes Ion Torrent sequencing for a higher read next generation sequencing method – Illumina. This method requires a lower amount of starting material and uses commercially available materials in contrast to the original method, which required a mutated RNA ligase and homemade 3' end and 5'end ligating adapters (Marchand et al., 2016).

The last method is called the Nm-seq and is again based on the different reactivity of the 2'-OH and 2'-OMe groups. In this method, the methylated riboses are uncovered by series of chemical reactions repeated in cycles that remove the 2'-OH nucleotides in the 3' to 5'direction by subsequent oxidation, elimination and dephosphorylation. When the cycle reaches a methylated ribose, it stops. After the last cycle, the resulting mixture is ligated to a 3' adaptor and the methylated riboses are preferentially linked unlike the 3'end monophosphates left from the unmodified nucleotides (see fig, 6). The ligated molecules are then amplified by PCR and compared to the original sequence to identify the actual location of the RNA modification (Dai et al., 2017). This method remains to be more extensively tested with viral RNAs but could provide novel discoveries in viral RNA modifications.

All of the methods above have two major drawbacks – they need a library of sequences against which the results must be aligned, and they are actually analyses of DNA which is transcribed from the original RNA. This poses a number of problems, because there may be other factors or modifications that can affect the reverse transcription and thus provide false positives or

other clouded data. There is, however, a new emerging method capable of direct RNA sequencing and the detection and identification of RNA modifications Nanopore Sequencing (Garalde et al., 2018). This method is based on the running current in nanopores that are placed on a sensor microchip.

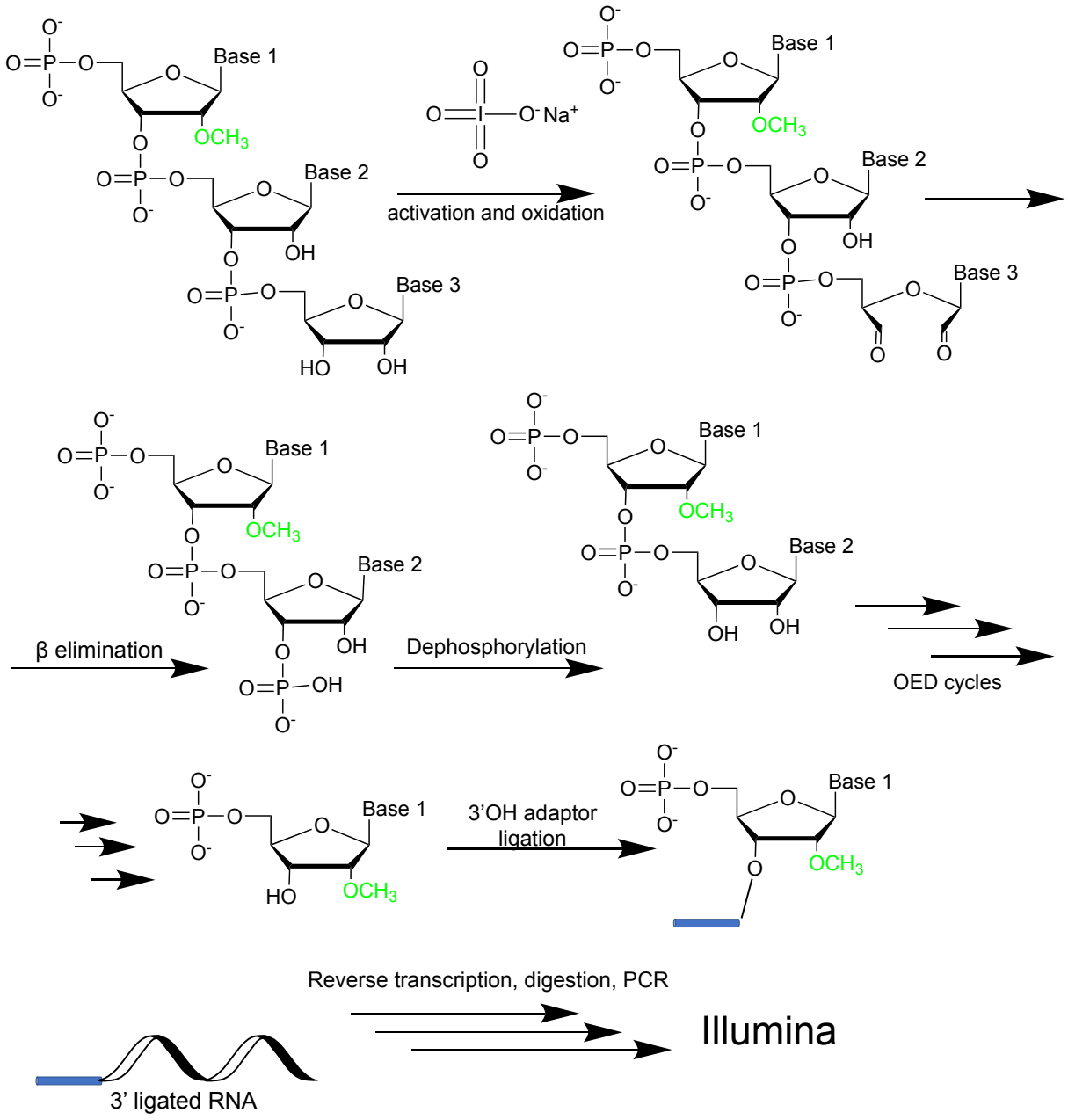

Figure 6. Nm-seq scheme

The change in current is specific to each nucleotide and modified nucleotides and the subsequent analysis provides the sequence as well as the location of the modification (Sauvage et al., 2018). The recent direct sequencing of the Influenza A genome is a confirmation of the effectivity of this method. Not only is it precise, with a perfect nucleotide coverage and near perfect consensus identity, but the authors also claim the method is capable of direct RNA modification detection, given the proper bioinformatics approach (Keller et al., 2018). A working method for using nanopores to detect RNA modifications was used to detect modifications of a wobble uridine in tRNA. Through this method, uridine was distinguished from 5-carbonylmethyluridine, 5-methoxycarbonylmethyl-2-thiouridine, 5-methoxycarbonylmethyluridine and several others (Onanuga et al., 2017). However, these modifications are significantly larger than the 2'-O- methyl moiety. Whether it is possible to detect all kinds of modifications remains to be elucidated.

8. Conclusion

Since their discovery, RNA modifications have played an increasing role in understanding the mechanics of RNA regulation and heterogeneity. Out of all these modifications, 2'-O-methylation is a fundamental and highly conserved one. From a chemical standpoint, it is a necessary stabilizing modification, affecting RNA reactivity and structure. The stabilizing impact is so paramount, that some claim 2'-O- methylated RNA to be an evolutionary intermediate between the RNA and DNA world (Poole et al., 2000). It is present in all domains of life and has various functions in each of the organisms.

Viral RNA modifications are probably acquired during the coevolution with their hosts. In viral RNA 2'-O- methylation is essential for viral virulence and replication. The host immune system is capable of detecting and differentiating between self and non-self. It does this through molecules called pattern recognition receptors, which detect pathogen associated molecular patterns or PAMPs. When a PAMP is detected it starts a cascade of signals that result in the production of effector molecules that eliminate the threat. 2'-O- methylation provides a pattern for the host organism to distinguish self RNA from viral RNA in the cytoplasm. Viral 2'-O-methylation thus serves an evasive function. By replicating the patterns found on the nascent RNA of the host, it hides itself from the immune system. The two types of molecules most responsible for detecting unmethylated viral RNA are the Mda5 and IFIT. Whereas Mda5 is only responsible for the detection of 2'-O- methylation and the subsequent activation of host immunity, IFIT molecules are also effector molecules capable of interfering with the translation

of viral proteins. They do this by blocking the assembly of the ribosome. This function of the ribose methylation makes it an ideal target for viral attenuation and vaccination.

Given the constant improvement in sequencing and isolation methods, it is probable that more intensive research into the function of 2'-O- methylation could provide us with a better understanding of viral host evasion as well as the host immune system.

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