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Ap_4A-RNA in IgE activated mast cells

Ap_4A-RNA v IgE aktivovaných žírných buňkách

Diploma 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, 23.04.2021

Podpis……

Jiří František Potužník

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ABSTRACT:

Mast cells are tissue resident members of the immune system. They have a wide range of functions and receptors including the FcεRI receptor, which gets activated by binding to IgE bound to an antigen. When the cells are activated in this manner, a process termed the LysRS-Ap4A-MITF signalling pathway occurs, resulting in the translocation of the Lys tRNA synthetase into the nucleus and an activation of its moonlighting activity – the production of diadenosine tetraphosphate (Ap_4A). Ap_4A is a dinucleoside polyphosphate, a type of ubiquitous molecule present in all domains of life. They are made up of two nucleosides joined together by a 5' to 5' phosphodiester bridge of variable lengths. Recently, these molecules have been shown to serve as non-canonical initiating nucleotides during bacterial transcription, where they function as 5' RNA caps, similar to the well-known 7-methylguanosine eukaryotic mRNA cap. In this thesis, I present proof of existence of Ap_4A capped RNA in mast cells, a previously unknown 5' RNA structure in eukaryotic cells, and I attempt to pinpoint its role in the activation of these cells and in the wider context of mast cell mediated immune response.

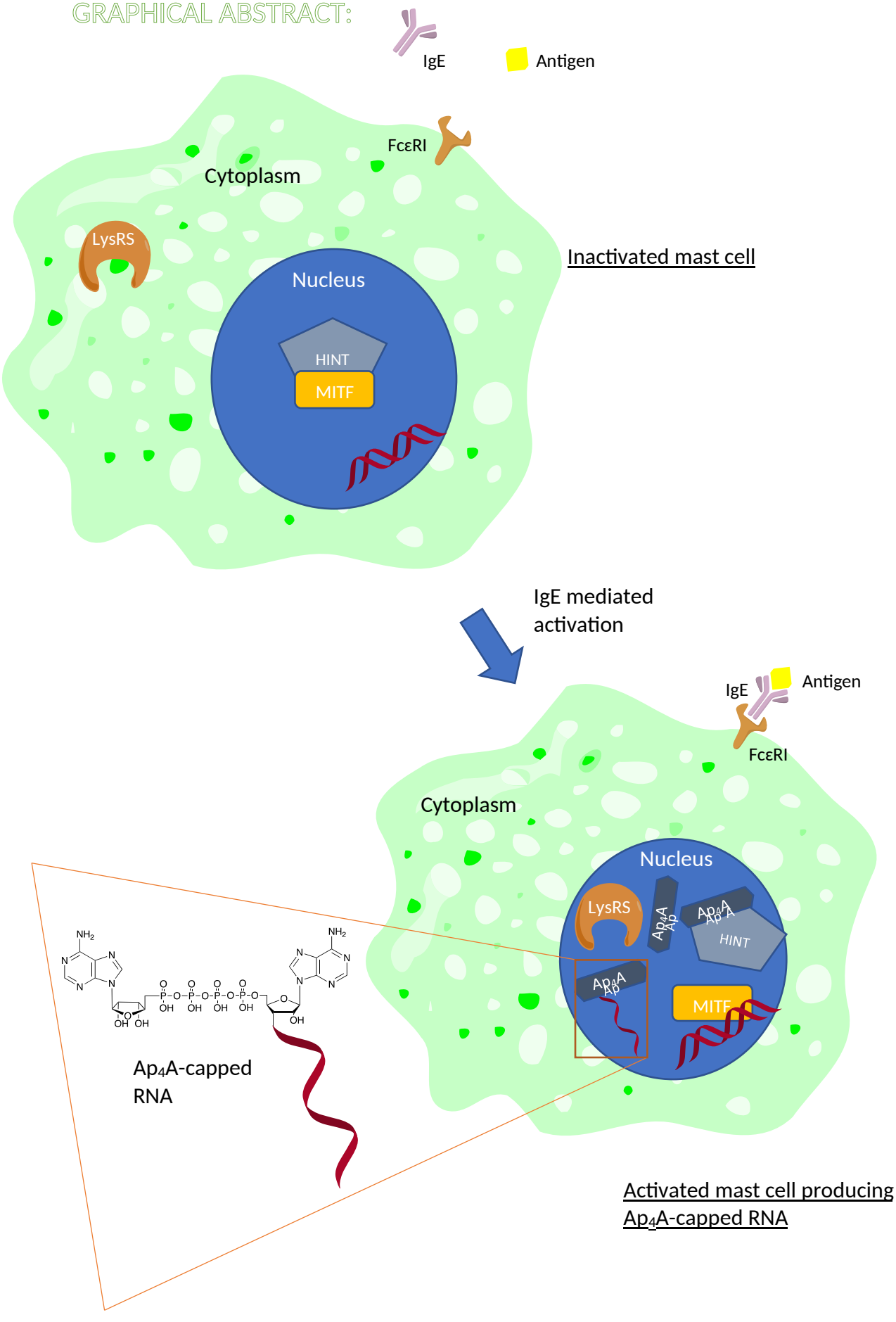
Keywords: mast cells, RNA caps, Dinucleoside polyphosphates, Ap_4A, RNA modification, IgE, FcεRI receptor, Lysine tRNA synthetase

ABSTRACT (CZ):

Žírné buňky jsou buňkami imunitního systému přítomné ve tkáních. Mají širokou škálu funkcí a receptorů, včetně receptoru FcεRI, který se aktivuje vazbou na IgE navázaný na antigen. Když jsou buňky aktivovány tímto způsobem, jeden z procesů, který nastává je tzv. signální dráha LysRS-Ap4A-MITF, která vede k translokaci Lys tRNA syntetázy do buněčného jádra a aktivaci její nekanonické aktivity – tvorbě diadenosin tetrafosfátu (Ap_4A). Ap_4A je dinukleosid polyfosfát, typ molekul přítomných ve všech doménách života. Dinukleosid polyfosfáty se skládají ze dvou nukleosidů spojených dohromady 5 '- 5' fosfodiesterovým můstkem o různých délkách. Nedávno bylo prokázáno, že tyto molekuly slouží jako nekanonické iniciační nukleotidy transkripce u bakterií, kde fungují jako 5 'RNA čepičky, podobně jako dobře známá 7-methylguanosinová čepička eukaryotické mRNA. V této práci předkládám důkaz o existenci Ap_4A čepičkované RNA v žírných buňkách, 5’ RNA struktuře, jejíž existence zatím nebyla popsána v eukaryotických buňkách, a pokouším se určit její roli při aktivaci těchto buněk a v širším kontextu imunitní odpovědi zprostředkované žírnými buňkami.

Klíčová slova: žírné buňky, RNA čepičky, dinukleosid polyfosfáty, Ap_4A, IgE, FcεRI receptor, Lys tRNA syntetáza

GRAPHICAL ABSTRACT:



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LIST OF ABBREVIATIONS:

Ap_4A	diadenosine tetraphosphate
RNA	ribonucleic acid
CTMC	connective tissue mast cell
TC mast cell	tryptase positive/chymase positive
T mast cell	tryptase positive/chymase negative
MMC	mucosal mast cell
TNF	tumour necrosis factor
UV	ultraviolet
LPS	lipopolysaccharide
IL-	interleukin
HSC	hematopoietic stem cell
MCP	mast cell progenitor
MPP	multipotent progenitors
CLP	common lymphoid progenitor
GMP	granulocyte/macrophage progenitors
MEP	megakaryocyte/erythrocyte progenitors
SCF	stem cell factor
VCAM-1	vascular cell adhesion molecule-1
MAdCAM-1	mucosal addressin cell adhesion molecule-1
cPLA2	cytoplasmic phospholipase A2
LT	leukotrienes
PG	prostaglandin
IgE	immunoglobulin E
$Np_{(n)}N$	dinucleoside polyphosphate
A	adenine
G	guanine
ATP	adenosine triphosphate
KATP	ATP sensitive potassium channels
MHC	major histocompatibility complex
ROS	reactive oxygen species

CHS	Béguez-Chédiak–Higashi syndrome
APC	antigen presenting cell
LysRS	lysyl tRNA synthetase
tRNA	transfer RNA
AlaRS	alanyl tRNA synthetase
MCM	multimeric complex
AIMP	aminoacyl tRNA synthase complex-interacting multifunctional proteins
MAPK	mitogen-activated protein kinase
HIV	human immunodeficiency virus
HINT	histidine triad nucleotide binding protein
PKCI	protein kinase C inhibitor
AMP	adenosine monophosphate
GMP	guanosine monophosphate
MITF	microphthalmia transcription factor
FHIT	fragile histidine triad protein
PTK	protein tyrosine kinase
ITAM	immunoreceptor tyrosine-based activation motifs
LAT	linker for activation of T cells
MEK	MAPK/ERK kinase
CTP	cytidine triphosphate
UTP	uridine triphosphate
mRNA	messenger RNA
CoA	coenzyme A
MDA5	melanoma differentiation-associated protein 5
IFIT	interferon-induced protein with tetratricopeptide repeats
m^7G	7-methylguanosine
NAD	nicotinamide adenine dinucleotide
RppH	RNA 5′-pyrophosphohydrolase
ApaH	bis(5′-nucleosyl)-tetraphosphatase
NTP	nucleoside triphosphate
LC	liquid chromatography

MS	mass spectrometry
PAGE	polyacrylamide gel electrophoresis
TOF	time of flight
TNP	trinitrophenol
DNP	dinitrophenol
E. Coli	Escherichia coli

1 INTRODUCTION:

Mast cells play a key role in both the adaptive and innate immune system. They are tissue resident cells that are often amongst the first at the site of injury or infection. There, they either directly participate in the defence by producing various proteases disabling the threat, or by producing cytokines, chemokines, and growth factors that attract other members of the immune system, promote cellular growth, and aid in wound healing. Several surface receptors for antibodies are expressed on the surface of mast cells including the mast cell high affinity IgE receptor FcεRI. Once bound to the mast cell receptor, IgE also binds an antigen, which causes the antibody to crosslink with the FcεRI receptor, and a cascade of reactions follows. One of these reactions is the phosphorylation of the Lysine tRNA synthetase. Phosphorylation triggers the detachment of Lysine tRNA synthetase from the multisynthetase complex. Once detached, the Lysine tRNA synthetase translocates into the nucleus, where it abandons its canonical function of charging tRNAs and starts producing a dinucleoside polyphosphate - diadenosine tetraphosphate (Ap_4A), a second messenger with a number of different roles.

Dinucleoside polyphosphates (including Ap_4A) are a group of molecules made up of two nucleosides joined by a 5'to 5'phosphodiester linkage. The length of the phosphodiester chain can be anywhere from two to seven phosphates long and they are often regulated/cleaved by enzymes from the NudiX family. This regulation is important as the intracellular concentration of these molecules greatly increases when the cells are stressed – that is why dinucleoside polyphosphates are often called alarmones. They have also been shown to interact with a number of cellular receptors that can respond to the dinucleosides in different ways based on the phosphate chain length of the molecule bound. Dinucleoside polyphosphates affect proliferation, apoptosis, angiogenesis and other various processes in the cells.

The basic chemical structure of dinucleosides polyphosphates is very similar to the structure of the 7-methylguanosine cap present on eukaryotic mRNA. The fairly recent discovery of alternative RNA caps such as the nicotinamide adenine dinucleotide cap or the coenzyme A cap has led several groups to entertain the possibility that these molecules can also be

incorporated at the 5'end of RNA. This notion proved to be correct and the presence of dinucleoside polyphosphate capped RNA was confirmed in bacteria.

Given that activated mast cells produce large amounts of diadenosine tetraphosphate in the nucleus and the possibility of this molecule being incorporated into the RNA, this thesis presents the hypothesis that Ap $_4$A-RNA is formed and plays a role in the immune response upon the activation of mast cells. The main goal of this thesis is to reveal the presence of a previously unknown 5' RNA cap structure and attempt to identify the types of RNA bearing the Ap$_4$A cap.

2 THEORETICAL PART:

2.1 Mast cells and their development

Mast cells are an integral component of the immune system. They are derived from haematopoietic stem cells and participate in both adaptive and innate immunity (1). Nearly all vascular tissue has resident mast cells and their roles in chronic or immediate allergy response, parasite and venom resistance, wound healing, blood clotting, and angiogenesis are non-replaceable (2–5). There can be great variability in the receptors that mast cells express on their surface and the proteases that they produce, but they are generally separated into two broad phenotypic groups (6). This division of mast cells is based on the content of their secretory granules and their location either in mucosal or epithelial tissues, as they have different biochemical, functional and immunological properties (7). One type is the connective tissue mast cell (CTMC) which produces carboxypeptidases along with the serine proteases chymase and tryptase and is sometimes termed the TC mast cell (tryptase positive/chymase positive). The other type is the mucosal mast cell (MMC) which only produces tryptase and is sometimes called the T mast cell (tryptase positive/chymase negative) (8, 9). CTMCs are commonly found in connective tissues such as the skin, tongue, trachea etc. around nerve endings and venules, whereas the MMCs are present in respiratory mucosa and gut epithelia (10). Another difference is in the longevity of the cells, as the transient and inducible MMCs only live for 10 to 14 days, while the constitutive CTMCs are much more stable and can survive for months once in the designated tissue (11, 12).

Mast cells produce a plethora of cytokines, chemokines and growth factors based on the specific type of activation and their location. Tumour necrosis factor (TNF) for example, is produced by mast cells in the skin upon degranulation which can be caused by antigen and antibody activation as well as physical factors such as ultraviolet (UV) light irradiation (13, 14). Interleukin 8 (IL-8) also known as CXCL8 is also produced by skin resident mast cells, in response to lipopolysaccharide (LPS) stimulation or the same activation by the antibody-antigen complex. IL-8 was found both directly secreted from the cell via the cytoplasmatic membrane as well as inside the mast cell granules(15, 16). Mast cells derived from peripheral blood are capable of Interleukin 5 (IL-5) production upon stimulation by Interleukin 33 (IL-33) (17). Interestingly enough, some of the cytokines and growth factors are produced by mast

cells in an autocrine manner and promote their own survival, maturation and receptor expression (18). In the same manner, the proteases produced by mast cells are able to degrade their own products; the mast cell protease 4 degrades TNF and another mast cell chymase can degrade IL-33 (19, 20). The cytokines and growth factors secreted by the mast exceed the scope of this thesis and have been the topic of a number of articles and reviews (21).

While mast cells originate from bone marrow, they mature and differentiate in peripheral tissues, where they arrive as mast cell progenitors (MCP). This is a big difference between mast cells and other cells from the myeloid lineage which leave the bone marrow as circulating differentiated cells (22). After hematopoietic stem cells (HSC) stop renewing themselves and develop through multipotent progenitors (MPP) into the common myeloid progenitor (CMP) or the common lymphoid progenitor (CLP) (23). The CMPs then further differentiate into either granulocyte/macrophage progenitors (GMP) or megakaryocyte/erythrocyte progenitors (MEP) (see ***Figure 1.***) (24).

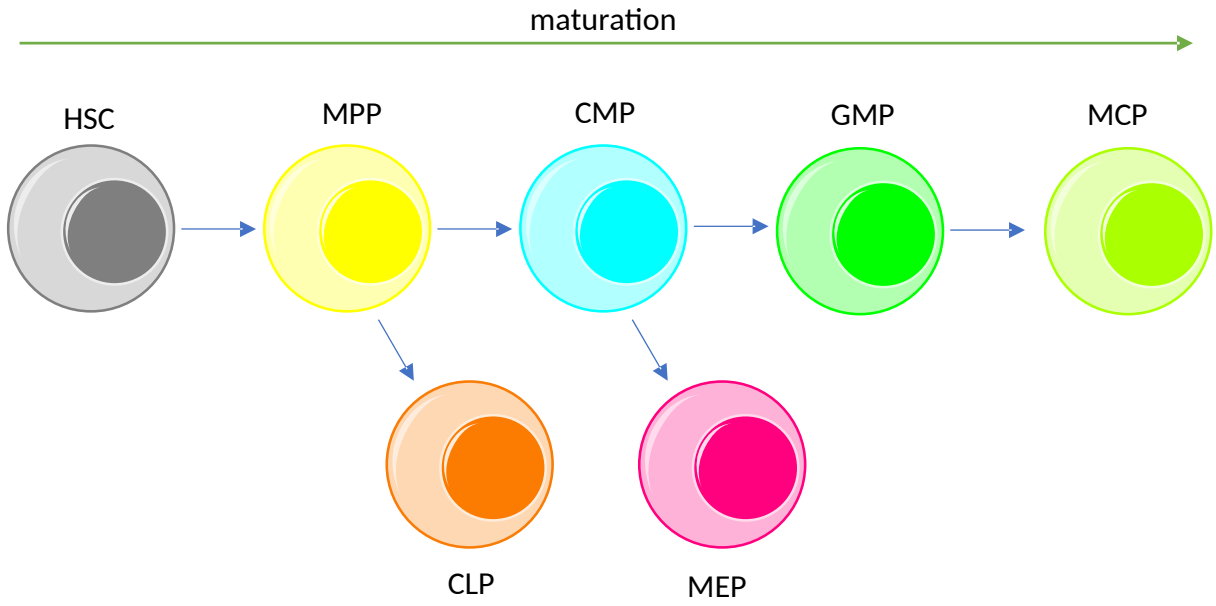


Figure 1: Development from hematopoietic stem cells into mast cell progenitors

Hematopoietic stem cells (HSC) develop into multipotent progenitors (MPP) and either into the common lymphoid (CLP) or myeloid (CMP) progenitors. The myeloid progenitor gives rise to megakaryocyte/erythrocyte progenitors (MEP) or to granulocyte/macrophage progenitors (GMP). The development of the mast cell progenitors (MCP) from GMP is accompanied by the expression of the FcεRI receptor. Adapted from (7).

It was shown, that mast cells can differentiate from single GMP cells that have the potential to turn into neutrophils and basophils (25). Actually, an analysis of mouse spleens revealed a bipotent type of cell able to either develop into the mast cell or into a basophil hinting at a close relationship between these two cellular lineages (26).

The survival and maturation of mast cells is decided by a plethora of cytokines and transcription factors. Interleukin 3 (IL-3), also known as the multi colony stimulating factor, is considered one of the most important for the differentiation and growth of mast cells (27). Together with Interleukin 6 (IL-6), it has been shown to enable the survival of mast cell progenitors and to continue their proliferation and subsequent differentiation up to the point of pre-mast cells (28). IL-3 also stimulates the production of TNF in the bone marrow and TNF subsequently affects the production of mast cell antibody receptors and even the total number of peritoneal mast cells present (29).

Stem cell factor (SCF) is another cytokine responsible for mast cell development. It binds to the tyrosine kinase growth factor receptor c-Kit and has a number of functions in mast cell growth and differentiation. It prevents the apoptosis of the cells by activating the phosphoinositide 3-kinase – protein kinase B cascade (PI3K–AKT), a key pathway in cell cycle regulation. The reactions of this cascade lead to the inactivation of the pro-apoptotic proteins Bim and Bad. This effect is compounded with an increased expression of pro-survival proteins such as Bcl-2 and Bcl-XL. Thus, without the effects of SCF, the mast cell would die a programmed cellular death (30, 31). The presence of SCF also affects the migration of mast cells, their adhesion, interleukin secretion, degranulation, and activation via the FcεRI receptor, which is a key step in the production of dinucleoside polyphosphates by the cell (32–35).

The transcription factors involved in mast cell development include the protein PU.1, which also affects the development of both T and B lymphocytes, neutrophils, and macrophages (36). PU.1 works in tandem with the transcription factors GATA, named for their ability to bind to a GATA sequence of the DNA. Specifically, it cooperates with GATA-2 to facilitate the early differentiation from MPPs into immature mast cells. It is PU.1, however, that is responsible for the biogenesis of granules in the mast cells and more importantly, for the

expression of the high affinity IgE receptor FcεRI (37). Another important transcription factor that helps to seal the progenitor cell fate, as that of a mast cell, is the microphthalmia transcription factor (discussed more later) and its antagonistic partner C/EBPα, which turns the progenitors into basophils (26).

As mentioned before, migration is another key factor in the development of mast cells – it starts in the bone marrow, where the progenitors migrate to the sinusoids, then through them into the sinusoidal endothelium and into tissues through venules (38). MCPs can be recruited to their designated tissues by a number of different means or an interplay thereof. One of the ways is tissue specific homing by surface adhesion receptors and their downregulation or upregulation coordinated to the changes in the cellular cytoskeleton. For example, a large amount of mast cells are present in the gut, where the integrin $\alpha_4\beta_7$ serves as the homing signal by interacting with the endothelial vascular cell adhesion molecule-1 (VCAM-1) or with the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (39). The same integrin $\alpha_4\beta_7$ in tandem with the integrin $\alpha_4\beta_1$ are responsible for mast cell recruitment to the lungs and airways by interacting with VCAM-1 and regulated by CXCR2 (sometimes known as Interleukin 8 receptor β), which is expressed by mast cell progenitors (39, 40). The integrin $\alpha_m\beta_2$ localizes mast cells to the peritoneal cavity as does the integrin $\alpha_{IIb}\beta_3$ and other integrins affect mast cell localization even after maturation, implying that integrins specifically recruit mast cells and their progenitors to the designated locations, though the changes in receptor expression and the signals surrounding them need more research (41–43).

There is also a number of chemoattractants that can direct the migration of both mast cell progenitors and fully differentiated mast cells, the aforementioned SCF being one of them. SCF causes the dimerization of c-Kit upon binding to it, which in turn activates its kinase activity, the subsequent phosphorylation of other signalling molecules and the initiation of various signalling pathways such as the Jak–Stat pathway, Ras–MAPK cascade and more (44, 45). Mature mast cells can also produce chemoattractants for mast cell progenitors, these include members of the eicosanoid family such as prostaglandins and the inflammatory mediators leukotrienes (46). They are produced by mast cells upon the ERK dependent phosphorylation of cytoplasmic phospholipase A2 (cPLA2) resulting in the release of

arachidonic acid from the cytoplasmic membrane and its subsequent metabolization into either leukotrienes or prostaglandins (47). Leukotrienes B4 (LTB_4), D4 (LTD_4) and C4 (LTC_4) all function as chemoattractants through their own specific receptors, without being redundant (48–50). The same applies for the prostaglandins D2 (PGD_2) and E2 (PGE_2) (51, 52). The amount and mechanisms of action of all the chemoattractants exceed the scope of this thesis, but have been the topic of at least one review (53).

The high affinity IgE receptor (FcεRI) can also play a role in mast cell chemotaxis as well as their activation and immunological responses. In mature mast cells, antigen induced crosslinking between FcεRI and the immunoglobulin E (IgE) cause the release of a number of mediators with immunomodulatory and proinflammatory roles and/or cellular degranulation (54). It is also a key component in the reactions leading to the production of dinucleoside polyphosphates.

2.2 Dinucleoside polyphosphates

Dinucleoside polyphosphates are a specific type of nucleotides present across all domains of life. Though their functions and targets are greatly varied, a lot about them is till shrouded in mystery (55, 56). They are made up of two ribosylated nitrogenous bases joined together by a 5' to 5'phosphodiester bridge which has between two to seven phosphate groups ($Np_{(n)}N$, where N is the nucleoside and n is the number of phosphates in the polyphosphate chain) (see ***Figure 1.***). They are structurally very similar to canonical mononucleosides. The best known and most thoroughly studied $Np_{(n)}N$ is diadenosine tetraphosphate, two adenosines linked together by four phosphodiester bonds (Ap_4A), which was also the first dinucleoside polyphosphate discovered in mammals and even in humans, specifically in aggregated platelets (57).

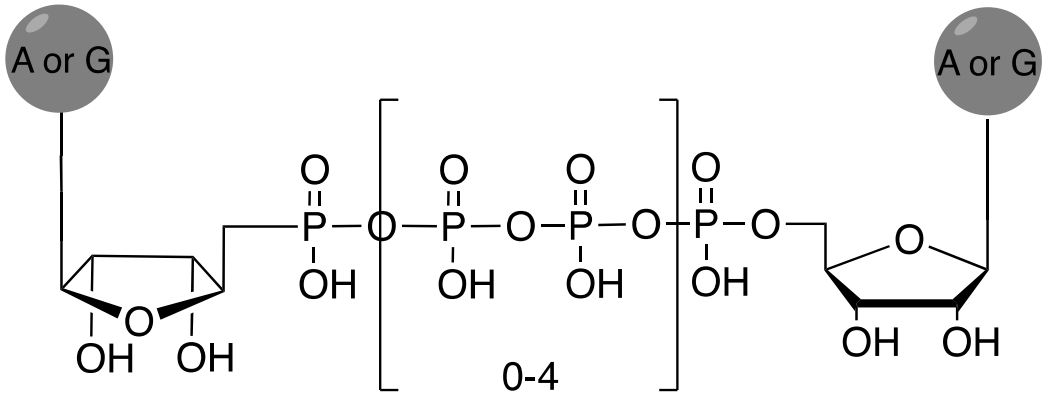


Figure 2. Dinucleoside polyphosphate

The general structure of dinucleoside polyphosphates. The grey circles represent either Adenine (A) or Guanine (G). The phosphate chain length can be between 2 and 7 phosphates long.

Under normal conditions, the intracellular concentrations of dinucleoside polyphosphates remain relatively low at around 3 µM or less, but their concentrations can rapidly increase more than a hundred-fold after certain types of stress, such as oxidative stress, cadmium poisoning, or in cells suffering from heat shock (58, 59). This phenomenon is present in bacterial cells as well cultured eukaryotic cells, where a sever-fold increase is observable after only hour (60, 61). Because of the apparent increase in concentration upon cellular stress, the alarmone hypothesis was formulated; it states that these molecules were a sign of stress in cells and a key factor in signalling and the subsequent survival of the damaged cells (62).

It is important to note that Np $_{(n)}$Ns can have two different types of activity. They can either interact with extracellular receptors or function intracellularly. At the time of Np$_{(n)}$N discovery, purine mononucleoside polyphosphates such as adenosine triphosphate (ATP) were commonly accepted as signalling molecules within the extracellular purinergic receptor system (63). Given the already mentioned similar structure of Np$_{(n)}$Ns, their effects on human physiological conditions via stimulating this type of receptors became the focus of study. They were implicated in a number of processes such as vascular regulation and cellular division (64–66). Today, it is well established that dinucleoside polyphosphates ranging from shorter ones such as Ap $_2$A or Ap $_2$G in the myocardium or the placenta, to the longer chained ones such as Gp$_6$G or Ap$_7$A in platelets are agonists of specific purinergic receptors and affect vasoconstriction, smooth muscle cell proliferation, and more (67–69). Though the effects of

$Np_{(n)}Ns$ on the purinergic system is not the topic of this thesis, it is an important component of the hypothesis and has been thoroughly described in a review in the British Journal of Pharmacology (70).

The scope of $Np_{(n)}N$ function, however, extends beyond the vascular effects mentioned above as their presence has been detected across a wide range of tissues and cells. When artificially added to cultured cells penetrated by cold shock, some dinucleoside polyphosphates promoted cellular apoptosis while others participated in the induction of cellular differentiation suggesting a role within the cell cycle (71). They have also been shown to act as an inhibitory intracellular ligand to different ion channels. Dinucleoside polyphosphates prevent spontaneous openings of ATP sensitive potassium channels (KATP) in ventricular myocytes as well as regulating KATPs in glucose stimulated Pancreatic β-Cell (72, 73). In liver cells, $Np_{(n)}Ns$ can interact with calcium channels (74). They also play a role in activating the release channels of Ca^{2+} ryanodine receptors in a number of cell types, increasing their sensitivity to Ca^{2+} and thus function as a stimulator of the channel (75). Dinucleoside polyphosphates are also present in nerve and neuroendocrine cells (76). $Np_{(n)}Ns$ are released in the secretory granules of chromaffin cells. They also bind to several high affinity binding sites on the chromaffin cells, such as the extracellular P2Y purinergic receptors (77, 78). In these sites in the hippocampus, they can cause a block in the excitation of synapses and stop the signal transmission (79). On the other hand, the intracellular functions of $Np_{(n)}Ns$ remain more enigmatic.

Dinucleoside polyphosphates are also present in a number of immune cells. T-lymphocytes, for example, have been shown to increase their intracellular concentration of dinucleoside polyphosphates upon Interleukin-2 (IL-2) stimulation, before new DNA is synthetised. The cellular potential for DNA synthesis decreases upon the removal of IL-2. At the same time, the concentration of $Np_{(n)}Ns$ also decreases pointing to a role as a second messenger or an initiator of DNA replication (80, 81). This was further proven, when the molecule showed a high affinity for binding to DNA polymerase alpha (82). Immune cells from the myeloid lineage have also respond to dinucleoside polyphosphates of variable chain lengths. Induced monocytes and granulocytes increase their production of reactive oxygen species (ROS) upon stimulation by dinucleosides with 4 and 5 phosphate groups which shows

a pro-inflammatory role of $Np_{(n)}Ns$ (83). Antigen presentation on the major histocompatibility complex class II (MHC) is also affected by dinucleoside polyphosphates. Béguez-Chédiak–Higashi syndrome (CHS) results in an impaired immune response due to a worsened presentation of peptides on the MHC class II, and this is accompanied by a decrease in intracellular $Np_{(n)}N$ concentration (84, 85).

Dendritic cells (DCs) are one of types of antigen presenting cells (APCs) possessing MHC II. They are highly mobile cells able to bring the captured antigen to the lymph nodes and present it to the naïve lymphocytes there in order to elicit an adaptive immune response (86). When the amount of $Np_{(n)}Ns$, specifically diadenosine tetraphosphate, is increased by knocking out the enzyme responsible for the hydrolysis of Ap_4A, the cells exhibit increased motility as well as an enhancement of cross-presentation of antigens. The signalling cascade from LysRS through Ap_4A and Hint1 to MITF is a process that was previously observed only in mast cells, and it has been implicated as a key component of this dendritic cell process (87).

2.3 The LysRS-Ap4A-Hint1-MITF pathway

Lysyl tRNA synthetase (LysRS) is one of the aminoacyl-tRNA synthetases, old, conserved proteins whose canonical role is an integral component of the central dogma of molecular biology. tRNA synthetases are responsible for the aminoacylation of transfer RNA (tRNA), i.e., taking an amino acid and covalently linking it to its corresponding tRNA, creating a “charged” tRNA. This is the fundamental principle of the genetic code, a mechanism of translating the genetic information into proteins (88). Aminoacyl-tRNA synthetases can be divided into two categories based on the structure of the catalytic domains in their active sites (89). Class I synthetases share a typical Rossman fold in the active site and tend to be monomeric, while the class II synthetases have a number of highly conserved regions and are commonly multimeric (89, 90). In the early 1980s, it was discovered that aminoacyl-tRNA synthetases may also have alternate functions, such as binding to their own upstream sequence and autoregulating their transcription in the case of alanyl tRNA synthetase (AlaRS) (91). Another mminoacyl-tRNA synthetase alternate function is that of Tyrosyl tRNA synthetase, which can be cleaved to produce two different cytokines that are essentially hidden until cleaved. The amino-terminal part has a similar function to interleukin 8 and binds to the same receptor,

while the carboxy-terminal domain is a chemoattractant for both leukocytes and monocytes (92). This places the aminoacyl tRNA synthetases and their moonlighting functions among the many components of the immune system.

Aminoacyl-tRNA synthetases often reside in multimeric complexes (MCM) that may also include other proteins such as the aminoacyl tRNA synthase complex-interacting multifunctional proteins (AIMPs). These complexes are present across all domains of life, though their complexity increases with the complexity of the organism (93). This led to the formulation of the depot hypothesis in the context of these multimeric complexes. The main point of the hypothesis is that inside the complexes, proteins are close enough to efficiently facilitate the processes where their proximity to each other is necessary. However, they can also be released from the complex and once they are released, they can function as smaller complexes or as monomeric proteins. These may easily have different roles than those they had within the multimeric complex (94).

Lysyl tRNA synthetase belongs to the second class of aminoacyl-tRNA synthetases and as such, it can exist in a number of multimeric forms. It mainly resides in the multisynthetase complex, in which a minimum of nine aminoacyl-tRNA synthetases and three supplemental proteins are joined together (95). Once released, LysRS can perform a versatile set of functions and bind to a number of different ligands. One of the ligands is the mutated superoxide dismutase which has been implicated in the onset of amyotrophic lateral sclerosis (also known as Lou Gehrig's disease). When bound together, the proteins are highly likely to misfold and further affect the disease progression (96). LysRS by itself has also been isolated as one of the autoantigens after cardiac transplantation and it serves as a chemoattractant (97). In fact, tumour necrosis factor alpha (TNF-α) induces the secretion of LysRS, which binds to peripheral blood mononuclear cells and macrophages further enhancing the production of TNF-α and stimulating their migration with the signal being transduced in a Mitogen-Activated Protein Kinase (MAPK) dependent manner (98).

Other non-canonical binding partners of LysRS include viral proteins. tRNA3Lys is the primer for the reverse transcription of the human immunodeficiency virus (HIV). However, it is not just the tRNA that interacts with the virus. It was shown that the LysRS itself can bind to the

viral Gag protein and can also get incorporated into the viral particles along with the tRNA. The sequestered enzyme bound to the Gag protein then enhances HIV-1 replication (99–101). Another way in which LysRS modifies its canonical role, is changing its conformation in order to stop charging tRNA and create 2 types of new molecules. One is adenylated lysine residues, which are very short lived, dependent on ion concentration and have only been detected *in vitro*. They do, however, serve as a substrate for the histidine triad nucleotide binding proteins (HINT), which binds to it (102). The other molecule produced by LysRS is the dinucleoside polyphosphate Ap_4A, also a substrate for HINT (103).

Originally thought to be a member of the protein kinase C inhibitor (PKCI-1) gene family, HINT-1 is a transcriptional regulator (104). It belongs to the superfamily of histidine triad proteins (HIT) with the HIT sequence motif which is His – X – His – X – His – X – X, where X stands for a hydrophobic amino acid (105). HIT proteins mainly function as nucleotide hydrolases and transferases (106, 107). HINT-1 is known to bind to guanosine monophosphate (GMP) or adenosine monophosphate (AMP) as well as the modified monophosphate AMP-NH_2 (107). HINT-1 also binds to the microphthalmia transcription factor (MITF) – hence its own role as a regulator of transcription. Apart from that, it also binds to Ap_4A. Given the size of the mononucleotides, their interaction with the enzyme does not cause a large conformational change. Ap_4A, however, is larger and causes such a change in the conformation of the enzyme, that it changes its ability to bind to MITF. The interaction with the dinucleoside causes MITF and HINT-1 to dissociate, making Ap_4A an important player in the regulation of the transcription of MITF activated genes (108).

In the text above, $Np_{(n)}Ns$ were repeatedly referred to as second messengers, in order to be able to define Ap_4A as a second messenger, however, it needs to fulfil a number of different criteria. One of the criteria is that Ap_4A needs to have an enzyme capable of metabolizing it and thus control its intracellular levels during activation, signalling etc. (109). A number of enzymes that are able to hydrolyse $Np_{(n)}Ns$ and Ap_4A in particular belong to the Nudix family. Substrates for the Nudix enzymes are nucleoside diphosphates linked to x, hence their name. The enzymes have a conserved consensus motif Gly – X – 5Glu – X - 5[U, Ala] – X – Arg – Glu – X – 2Glu – Glu – X – Gly - U, with U representing an aliphatic residue and X an amino acid of any kind and they are present across all domains of life (110). They can hydrolyse a wide range

of targets from mononucleoside diphosphates and triphosphates, through dinucleoside polyphosphates and diphosphoinositol polyphosphates, and different types of RNA caps (111). Given that some of the metabolites can be toxic when they accumulate in the cell, the Nudix enzymes have been called housecleaning genes due to their function of removing them (112). Other enzymes hydrolysing Ap_4A include some members of the ecto-nucleotide pyrophosphatase and phosphodiesterase family and another member of the histidine triad family - fragile histidine triad protein (FHIT) (113, 114).

Nudix type 2 or NUDT2 is an Ap_4A hydrolase that has been implicated in several processes including cellular proliferation associated with human breast cancer (115). It is also responsible for keeping the correct levels of Ap_4A in immunologically activated mast cells (116). In fact, NUDT2 has been shown to directly participate in the changes to transcription in mast cells following their activation by IgE binding an antigen. Cells that are depleted of NUDT2 using siRNA were shown to produce almost double the amount of some MITF transcriptional targets compared to the control cells (117).

The transcription factor MITF mentioned above, is in itself an important aspect of the immune response of mast cells. It has a basic helix-loop-helix leucine zipper structure and it is produced from several different promoters in several isoforms, each of which can have a varying effect on mast cell differentiation and morphology (118). MITF also regulates the transcription and hence the expression of enzymes and proteases such as Granzyme B, Chymase 1, and even the tyrosine-protein kinase c-Kit, which is an integral cytokine receptor in the development and survival of mast cells (119, 120).

When put together, all of the aforementioned enzymes form a picture of very precise immune response called the LysRS-Ap_4A-MITF signalling pathway based around the molecule Ap_4A (see ***Figure 1.***). Once this pathway starts, it results in the production of a number of different factors that have an effect on the cell as well as its surroundings. It changes the expression patterns of various genes, such as the proteases produced by the mast cells and released in their secretory granules (121). The sequence of reactions leading to these changes starts when IgE binds to the mast cell high affinity IgE receptor FcεRI and to an antigen. This activates a member of Src-family protein tyrosine kinase (PTK) Lyn. Lyn is responsible for the

phosphorylation of tyrosines on the immunoreceptor tyrosine-based activation motifs (ITAMs), which are present in the γ and β chain subunits of the FcεRI receptor.

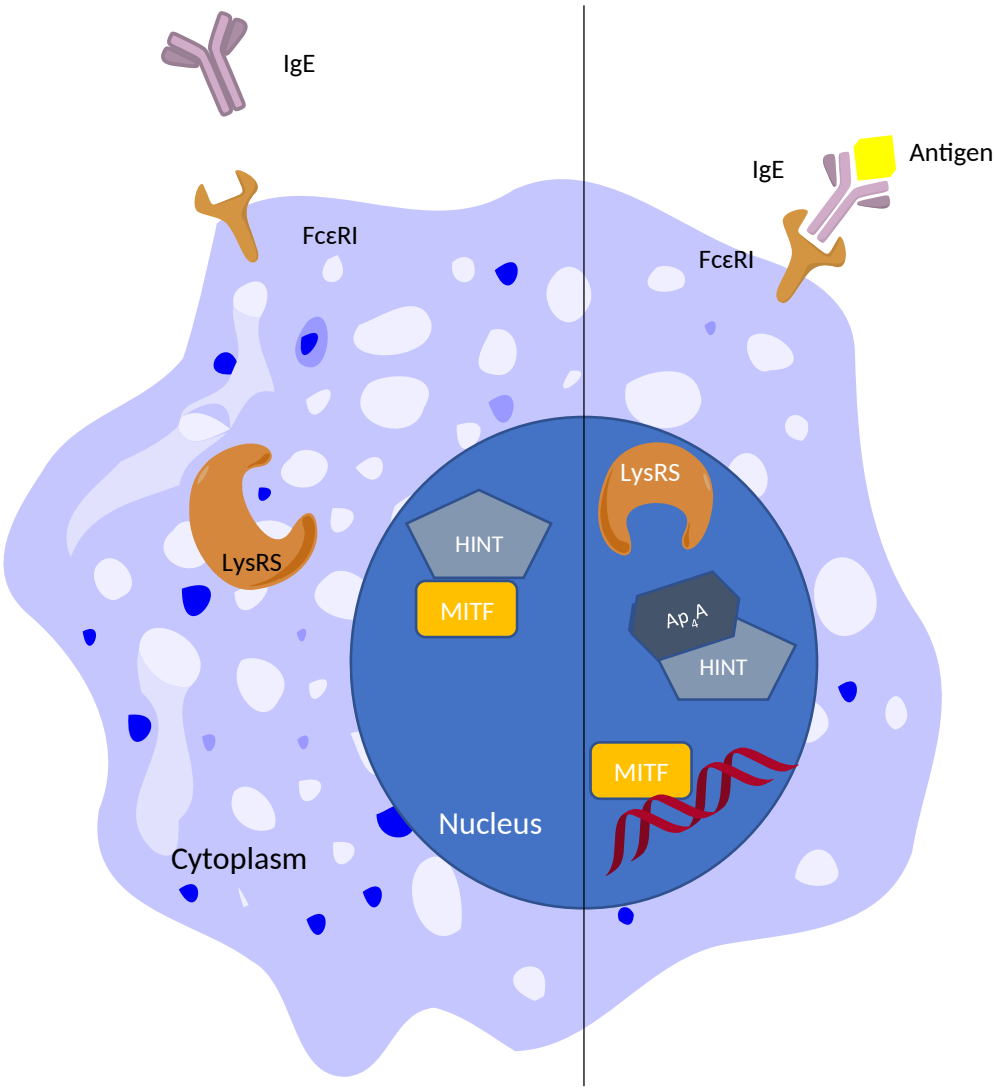


Figure 3. LysRS-AP$_4$A-MITF pathway

A visual representation of the LysRS-Ap$_4$A-MITF pathway. The left side of the cell shows the state of the cell before activation. On the right side, IgE is bound to an antigen and to the FcεRI receptor. This causes the LysRS to translocate into the nucleus and produce Ap $_4$A. Ap$_4$A binds to HINT and releases MITF, which starts the transcription of the target genes

The phosphorylated subunits are then bound by the PTK Syk (122, 123). The linker protein tyrosine kinase called Linker for activation of T cells (LAT) gets phosphorylated by the bound Syk and in turn, the phosphorylated LAT serves as a scaffold for the construction of

multimolecular signalling complexes. The complexes built around phosphorylated LAT then facilitate the activation of small GTPases and the activation of the MAPK/ERK pathway (124). LAT deficient mutants have decreased activation of mitogen-activated protein kinase (MAPK). Given that MAPK is integral for the detachment of the LysRS, this prevents the complete activation of the cell (125, 126). Once the MAPK/ERK pathway is initiated, MAPK/ERK kinase (MEK) phosphorylates LysRS on the serine 207 and causes a conformational change.

In this changed state, the LysRS captures the tRNA and prevents it from getting to the active site, where it would be charged. This means that lysyl-AMP cannot be transferred to the tRNA, and the tRNA is kept it in an inactive state, effectively stoping the canonical function of the LysRS. The LysRS also translocates from the cytoplasm to the nucleus and starts producing Ap_4A (127). The concentrations of Ap_4A inside the nucleus of activated mast cells reaches up to 700 µM, rivalling the concentration of normal nucleotide triphosphates such as uridine triphosphate (UTP) or cytidine triphosphate (CTP) (108, 128). MITF is also bound to and inhibited by HINT-1. When HINT-1 binds Ap_4A, it polymerizes and releases MITF enabling the transcription of the MITF activated genes (129).

2.4 RNA modifications and non-canonical RNA caps

Ribonucleic acid (RNA) is a versatile molecule with a multitude of functions in cells. These include messenger RNA (mRNA), tRNA, ribosomal RNA (rRNA), small nuclear RNA (snRNA), and many more (130). They are polymeric molecules made up of the purine nucleotides adenosine, guanosine, and the pyrimidine nucleotides cytidine and uridine (131). They are also subject to a wide array of chemical modifications; there are currently over 170 of known modifications, across all domains of life, ranging from the smaller ones such as methylations and acetylations to large covalently linked groups such as succinyl coenzyme A (CoA) (132, 133). A number of these modifications play important roles in the activation of the immune system. Either by providing molecular patterns that distinguish foreign RNA from the one produced in the cell, or by increasing the transcription of the modified RNA that can have beneficial effects or lead to pathologies such as cancer.

One example of the chemical modifications is the 2′-*O*-methylation (Nm), which is the addition of a single methyl group to the 2′-OH of the ribose. It is usually found on the first or second nucleotides behind the 7-Methylguanosine (m^7G) cap on the 5' terminus of eukaryotic mRNA (134, 135). When an unmethylated RNA enters the cell, it is immediately recognized by reader proteins such as the melanoma differentiation-associated protein 5 (MDA5) and marked as foreign (136). This recognition causes a cascade of responses resulting in a strong immune response from the cell such as an increase in the production of interferon genes IFN-α and IFN-β (137). The unmethylated RNA can also be directly recognized by interferon-induced proteins with tetratricopeptide repeats (IFITs), old and conserved members of the immune system designed to inhibit the replication of viral RNA (138). The presence of Nm has also been shown to play a role in cellular development and cancer (139, 140).

N^6-methyladenosine (m^6A) is another chemical modification of RNA. It is one of the most common and best studied modifications and as such, it has been linked to a number of functions and pathologies (141). m^6A is a very dynamic modification installed by "writer" proteins and functioning through "reader" proteins, so its effects can be reversed or modified and directly impacts the immune system (142, 143). One of the roles of m^6A is promoting the activation of dendritic cells. If the modification is erased, dendritic cells show a worsened expression of co-stimulatory molecules such CD40 or CD80, and their ability to stimulate the response of T-cells also gets impaired (144). m^6A also affects the translational efficiency of modified mRNA and as such, plays a role in several cancers; lung cancer, hepatocellular carcinoma, and breast cancer being just 3 examples (145–147).

A recently discovered type of RNA modifications is non-canonical RNA caps. They are present at the 5' termini of RNA and bear a structural resemblance to the canonical 7-methylguanosine (m^7G) cap present on eukaryotic mRNA (134) (see ***Figure 2.***). The canonical cap is responsible for mRNA stability, translation, degradation, protein binding, transport from the nucleus, and more (148). The first types of non-canonical caps discovered were covalently attached cofactors: CoA and nicotinamide adenine dinucleotide (NAD). Both of these RNA caps were discovered in bacterial RNA shorter than 200 nucleotides (149).

NAD was detected at the 5’ ends of some bacterial transcripts, where it affects their processing and stability (150). It prevents the RNA from being degraded by enzymes such as the bacterial RNA 5′-pyrophosphohydrolase (RppH) and RNase E. Thus, the small regulatory RNA with this cap is protected and able to perform its designated function (150). Since its discovery, NAD capped RNA has also been found in mammalian cells (including human). In eukaryotes, the NAD cap is incorporated in a similar way to bacteria, as a non-canonical initiating nucleotide. This mechanism, however, does not account for all of the capped RNA present. mRNA could be capped in such a manner, but the presence of the NAD cap on processed RNA such as small nuclear RNAs points to a post-transcriptional means of capping (151, 152).

The canonical m^7G cap, for example, is added co-transcriptionally after the first 25 to 30 nucleotides of the transcript in a series of sequential steps. It starts with the removal of the γ-phosphate from the 5’ end triphosphate of the RNA by an RNA triphosphatase. The enzyme guanylyltransferase interacts with GTP to form a guanylyltransferase-GMP complex. The enzyme then adds the GMP to the 5’ diphosphate of the RNA. This structure is subsequently methylated at the *N*7 position and on the first couple of riboses after the cap (153, 154). It is still unknown, whether the NAD cap can be added in a similar manner. The importance of the non-canonical caps is further implied by the ability of the cells to specifically decap this type of RNA. In fact, some of the enzymes decapping NAD-RNA in eukaryotes belong to the Nudix family, just like RppH in bacteria, hinting at a conserved origin of this modification as a means of regulation (155).

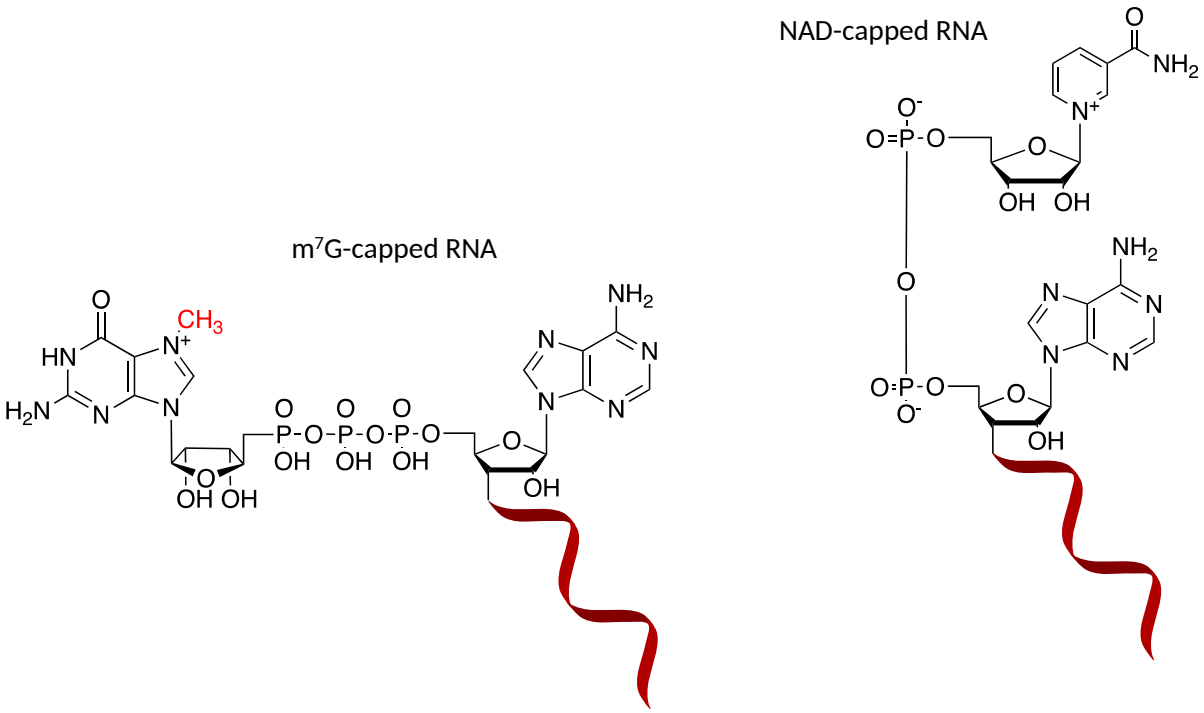


Figure 4. RNA caps

A structure of the canonical m^{7}G cap and the NAD cap linked to an RNA molecule.

The aforementioned dinucleoside polyphosphates have a very similar structure to the canonical and non-canonical 5' caps. In fact, dinucleoside polyphosphate capped RNA was recently discovered in bacteria by our research group using a combination of LC-MS analysis and PAGE experiments. The results were confirmed by boronate gel electrophoretic analysis from independent group at New York University (156, 157).

Np$_{(n)}$Ns can be incorporated into the RNA by the bacterial RNA polymerases or T7 bacteriophage RNA polymerase as a non-canonical initiating nucleotide just like NAD or CoA. The cellular concentration of Np$_{(n)}$N-capped RNA greatly increases when the bacteria are exposed to different types of stress such as starvation, heat shock or heavy metal toxicity. The bacteria have also been shown to methylate the Np$_{(n)}$N-capped RNA in order to further stabilize it and protect it from decapping (156, 158). The unmethylated Np$_{(n)}$N-capped RNA can be decapped by the same enzyme as the NAD cap – RppH. The methylated versions, however, are resistant to cleavage by RppH and have to be cleaved by bis(5′-nucleosyl)-tetraphosphatase (ApaH). This means that cells are not only able to cap their RNA in this manner, but are also able to stabilize the capped RNA against unwanted digestion (156, 157). While the concentration of capped RNA *in vivo* changes, *in vitro* experiments showed that

when there is an equimolar concentration of NAD or Np$_{(n)}$Ns as that of normal nucleoside triphosphates (NTPs), from 27% to 46% of the RNA was capped with them (156).

Given that IgE activated mast cells have the ability to produce Ap$_4$A in a similar amount to canonical NTPs and given the ability of different polymerases to use Np$_{(n)}$Ns as RNA caps, it is possible for Ap$_4$A-capped RNA to exist and to play an important role in the regulation of mast cell immune response. This led to the formulation of the hypothesis:

Mast cells create Ap$_4$A-capped RNA in response to activation by IgE.

While the theoretical part of this thesis attempted to provide general information on mast cells and dinucleoside polyphosphates and explain why Ap$_4$A capped RNA could play an important immunological role. The next part of this thesis is the experimental description of the discovery that mast cells use Ap$_4$A in order to cap their RNA and not just as a second messenger or a signalling molecule. It also describes how the resulting data was used to calculate the amount of capped RNA present in the activated and non-activated cells.

3 AIMS OF THE THESIS

1) Preparation of two fractions of RNA (long and short) from activated and non-activated mast cells

2) Detection of Ap_4A capped RNA using LC-MS methods

3) A relative quantification of the amount of capped RNA between the activated and non-activated mast cells using LC-MS methods

4) Identification of the specific capped transcript

4 MATERIALS AND METHODS

Materials:

All chemicals were purchased from Merck or Jena Biosciences…

4.1 Cells:

Rat basophilic leukaemia cells – 2H3-RBL; the cells are a line that was established in the early 1980s and has since been used as a model for mast cells(159). The cells were kindly given to us by Dr. Petr Dráber from the Institute of Molecular Genetics AS CR.

The electrocompetent *Escherichia coli* (*E. Coli*) strain used for the transfection and protein expression was BL21(DE3).

4.2 Antibodies:

Monoclonal anti-dinitrophenyl antibody produced in mouse, IgE isotype. Produced by Sigma-Aldrich, catalogue number D8406-2MG.

Monoclonal anti-trinitrophenyl antibody produced in mouse, IgE isotype. Given to us by Dr. Petr Dráber from the Institute of Molecular Genetics AS CR.

4.3 DNA oligomer sequences:

Table 1. Oligomer sequences

The names and sequences of used oligomers. The A Φ 2.5 promoter is underlined, and the highlighted letter is the first transcribed nucleotide.

Oligomer name	**Sequence**
Chymase 1 forward template	5'- CAG TAA TAC GAC TCA CTA TTA GGG ATG AAG ACA ACT TAA AGA AGA CGC CTT GAC CAC ACA GTT CCT GTG ATA ACA GCT CTG CCA CTC CCC – 3'
Chymase 1 reverse template	5' – GGG GAG TGG CAG AGC TGT TAT CAC AGG AAC TGT GTG GTC AAG GCG TCT TCT TTA AGT TGT CTT CAT CCC TAA TAG TGA GTC GTA TTA CTG – 3'
Chymase 1 20 nucleotide 5'end complementary probe	5' – CTG TTG AAT TTC TTC TGC GG – 3'
RNase H probe	5'– TCA CAG GAA CTG TGT GGT CA – 3'

4.4 Methods:

1) 2H3-RBL growth
2) RNA isolation
3) Mass spectrometry analysis
4) *In vitro* RNA preparation
5) RNAse H experiments
6) Northern blotting

4.5 2H3-RBL growth and activation

The frozen cells were thawed at 37 °C in a water bath, transferred to a 50 mL falcon tube containing 9 mL of complete growth medium made on the premises. The medium composition was 43 % Roswell Park Memorial Institute medium (RPMI), 43 % Dulbecco's Modified Eagle's Medium (DMEM) High Glucose, 1.2 % $NaHCO_3$ (7.5%), 1 % Penicillin-Streptomycin, 1.2 % Glutamine (200 mM), 0.9 % Glucose, and 9.7 % of foetal bovine serum (inactivated at 65 °C for 30 minutes). The cells were centrifuged for 5 minutes at 4 °C and 1200 RPM in a Thermo Scientific™ Megafuge™. After aspirating the supernatant, the cells were resuspended in 15 mL of the complete growth medium and transferred to a sterile 150 mm VWR tissue culture dish with a treated surface for them to adhere to. They were grown at 37 °C in 5% CO_2.

Based on their growth, the cells were passaged every 3-4 days. After that time, the medium was aspirated, and the cells were washed with Phosphate-Buffered Saline. They were treated with 4 mL of 1x Trypsin Ethylenediaminetetraacetic acid (EDTA) solution, removed and resuspended in an equal amount of complete growth medium. The cells were subsequently split into new culture dishes at 1/15 cell suspension to growth medium volume ratio.

Upon activation, the cells were removed from the dishes in the same manner as during subculturing. Their number and viability were counted in a mixture of 10 µL of cell suspension and 10 µL trypan blue in Invitrogen Countess™ II Automated Cell Counter. The cells were split into two groups and resuspended in 50 mL falcon tubes in complete growth

medium. One group was sensitized by adding IgE at 1000 ng mL^{-1} for 90 minutes at 37 °C. The suspension was then centrifuged for 5 minutes at 4 °C and 1200 RPM and washed with a BSS activation buffer. The buffer composition is 20 mM HEPES, 135 mM NaCl, 5mM KCl, 1.8 mM $CaCl_2$, 5.6 mM Glucose, 1 mg·mL^{-1} BSA, 1 mM $MgCl_2$ and adjusted to a pH 7.4. After a second wash, the cells were resuspended in BSS buffer at 10^7 per mL and the activated cells were treated with trinitrophenol (TNP) antigen at 2 µg ·mL^{-1} and shaken at 37 °C for 30 minutes. A small amount of cells was taken for the activation assay and the rest was used for RNA extraction.

The degranulation of the cells was then measured using a β-Glucuronidase Activity Assay. 200 µL aliquots of activated and non-activated cells were taken, centrifugated at 1200 RPM for 3 minutes at 4 °C using Eppendorf® Centrifuge 5810R. 30 µL of the supernatant were mixed with 50 µL of 12.5 mM 4-methylumbelliferyl β-D-glucuronide in DMSO diluted 100x in acetate buffer adjusted to pH 4.4 in a transparent 96-well plate. The plate was shaken at 37 °C for 60 minutes at which point 120 µL of a solution with 350 mM glycine and 450 nM Na_2CO_3 was added. Cells treated with 10x diluted 5 % Triton X 100 served as a positive control. The results were measured on Tecan Infinite 200 Pro, excitation 355 nm, emission 460 nm, integration time 20 µs, 15 flashes and optimal gain.

4.6 RNA Extraction and isolation

The cells were centrifuged in the 50 mL falcon tubes, the supernatant was aspirated, and the pellet was immediately dissolved in RNAzol® RT from Sigma-Aldrich. The volume of RNAzol depended on the number of cells and their lysis. Once the cells were completely homogenized in the RNAzol®, they were stored at -80 °C. The next day, the cells were slowly thawed on ice and 0.4 mL of nuclease-free water for molecular biology was added. The mixture was thoroughly vortexed for 20 s and left on ice for 15 minutes. The sample was then centrifuged for 45 minutes at 6000 g and -2 °C. The supernatant with the RNA was transferred to new 50 mL falcon tubes and the pellet was discarded. After that, 0.4 mL of 75 % ethanol (EtOH) per 1 mL of supernatant was added, vortexed as before, and left on ice again. The RNA was then centrifuged at 6000 g and -2 °C again, for 16 minutes. The short RNA (sRNA) in the supernatant was transferred to new 50 mL falcon tubes and the pellet of

long RNA (lRNA) was washed twice with 5 mL of 75 % EtOH at 6400 G for 30 minutes. The sRNA was vortexed with 0.8 mL of pure isopropanol and kept on ice for 35 minutes. After that, it was centrifuged at 6400 g and -2 °C for 60 minutes, and washed twice with 5 mL of 70 % isopropanol at 6400 G for 30 minutes. The resulting pellets of both long and short RNA were dissolved in 250 µL of nuclease-free water and stored at -80 °C.

4.7 RNA preparation for Mass Spectrometry

The concentration of the isolated RNA was measured using the microvolume UV-Vis spectrophotometer NanoDrop™ One from Thermo Scientific or the Qubit fluorometer from Invitrogen. The purity and correct separation of the RNA was measured using electrophoresis and the Agilent 4200 TapeStation System. The sRNA was also purified from unincorporated nucleosides that are retained in the sRNA fraction. Amicon ultra 0.5 mL 3 kDa filter was rinsed with nuclease free water, the entire volume of sRNA was added, the filter was filled to the brim with nuclease free water and centrifuged at 14000 g, 4 °C for 20 minutes. This process was repeated 4 times (for a total of 5). The filter was then turned over, and centrifuged for 1 minute at 1000 g. Both the cleaned sRNA and the lRNA were then prepared into 500 µg samples and digested with 10 U of Nuclease P1 in a total reaction volume of 200 µL. The digested RNA was then filtered using Microcon 10 centrifugal filters at 14100 g for 15 minutes. The liquid was then evaporated in Savant SpeedVac SPD131DDA Concentrator at 35 °C. The dry digested RNA nucleoside monophosphates were then resuspended in 8 µL of 10 mM NH_4OAc with 0.2 µL and analysed using SYNAPT G2 quadrupole-ion mobility-TOF hybrid MS and SCIEX QTRAP 6500+. The software used for the subsequent data analysis was Analyst for the QTRAP and MassLynx for the SYNAPT.

4.8 LC-MS analysis

4.8.1 Q-TOF analysis:

The Waters Acquity UPLC SYNAPT G2 machine fitted with an Acquity UPLC BEH Amide column (1.7 µm, 2.1 mm × 150 mm, Waters). The mobile phase A consisted of 10 mM ammonium acetate, adjusted to pH 9, and the mobile phase B was 100 % acetonitrile. The flow rate was set at 0.25 mL/min and the composition gradient of the mobile phase was as follows: 80 % of B for 2 min; a linear decrease down to 68.7 % B over 13 min; a linear

decrease down to 5 % B over 3 min; steady at 5 % B for 2 min; a linear return up to 80 % of B over 2 min. The analysis was done using electrospray ionization (ESI) with a capillary voltage of 1.80 kV, a sampling cone voltage of 20.0 V, and an extraction cone voltage of 4.0 V. The source temperature was 120 °C and the desolvation temperature 550 °C, the cone gas flow rate was 50 L/h and the desolvation gas flow rate 250 L/h. The detector was operated in negative ion mode. 10 µL of the digested sample was dissolved and 8 µL was injected per sample.

4.8.2 Multiple Reaction Monitoring (MRM) analysis:

The studies of the fragmentation were done using SCIEX QTRAP 6500+ machine with an Acquity UPLC BEH Amide column (1.7 µm, 2.1 mm × 150 mm, Waters). Mobile phase A was 10 mM ammonium acetate adjusted to pH 9, and mobile phase B was 100 % acetonitrile. The flow rate was set at a constant speed of 0.2 mL/min and the mobile phase composition was as follows: 80 % of B for 2 min; decreasing linearly over 12 min down to 50 % of B; and maintaining at 50 % B for 1 min before a linear return up to 80 % B over 2 min. ESI was used with curtain gas of 35 (arbitrary units), Ionspray voltage of -4.5 kV. The ion source gas was 50 (arbitrary units), and the drying gas temperature was 400 °C. The declustering potential was -180 V, the entrance potential -10 V, average collision energy -40 V. The detector was operated in negative ion mode. For the quantification of Ap_4A, the first precursor ion was [M - H]- at m/z 834.9 and the second ions selected were [M - H]- at m/z 487.9 and 407.9. and 505.9 For each sample, 10µL of the material was dissolved and 8µL was injected per sample.

4.9 *In vitro* RNA preparation:

All the artificially made RNA was prepared using T7 RNA Polymerase (T7 RNAP) from New England BioLabs (NEB) and had the promoter A φ 2.5. using a standardised protocol(160). The total reaction volume was 50 µL, containing: 80 ng/µL of the DNA template, 1 mM ATP, 1 mM UTP, 1 mM GTP, or 1mM CTP. In the case of α-^{32}P labelling, 0.2 µL of GTP (activity: 9.25 MBq) was added. 1.6 mM Ap_4A (Sigma Aldrich)was only added to create the capped RNA. 1× reaction buffer for T7 RNAP and 125 units of T7 RNAP, 0.12 % triton X-100, 4.8 mM $MgCl_2$, 5 % dimethyl sulfoxide (DMSO), 12 mM dithiothreitol (DTT) The mixture was

incubated for 180 minutes at 37 °C. After 120 minutes, an equivalent amount of T7 RNAP was added to increase the yield.

The DNA template was digasted using DNase I from NEB. A total reaction volume of 60 µL, with 1x DNase I reaction buffer 10 mM Tris-HCl, 0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, adjusted to a pH of 7.6 and 8 units of the enzyme were incubated at 37 °C for 60 minutes and then for 10 minutes at 75 °C in order to inactivate the enzyme. The mixture was then purified using RNA mini–Quick Spin Columns (Merck) before subsequent analysis and enzymatic treatments.

4.10 RNase H treatment

RNase H is an enzyme that cleaves DNA and RNA hybrids. 5 µg of the RNA prepared from the Chymase I template (sequence in oligomer sequence table) was heated at 85 °C for 10 minutes and an equimolar amount of the Chymase 1 20 nucleotide 5'end complementary probe was added. The mixture was left to cool for 5 hours until it reached 30 °C in order for the probe and the RNA to hybridize. 1x RNase H reaction buffer 75 mM KCl, 50 mM Tris-HCl, 10 mM DTT and 3 mM $MgCl_2$ adjusted to a pH of 8.3 and 2 units of the enzyme were then added to a total reaction volume of 30 µL and incubated at 37 °C for 10 minutes. The enzyme was then heat inactivated at 75 °C for 10 minutes. 20 µL of the samples was mixed with 20 µL of 2× RNA loading dye and analysed using 12 % PAGE (pre run at 600 V for 30 minutes, then 4.5 hours at 600 V – until the dye reaches about 90 % of the gel length) .

4.11 Northern blotting RNA detection

The gel was cut based on the location of the RNA visible under a UV light lamp. The RNA from the cut gel was then transferred to a nylon charged membrane Amersham Hybond-N+ from GE Healthcare by blotting. This took place in 20x SSC buffer 0.3 M tri-sodium citrate and 3 M NaCl adjusted to a pH of 7 for 18 hours. The transferred RNA was then twice crosslinked to the membrane using UltraLum CEX-1500 UV CrossLinker at 120 mJ for 30 s. The membrane was then pre-hybridized in 20 mL of Church buffer (180 mM Na_2HPO_4, 70 mM NaH_2PO_4, 7 % Sodium dodecyl sulfate (SDS), 1 % bovine serum albumin (BSA), 1 mM EDTA, adjusted to a pH of 7.2). Using a Labnet ProBlot hybridization oven, the membrane was kept at 45 °C for 90 minutes while turning on a setting of 3. Using γ-^{32}P ATP (activity:

9.25 MBq), 5 µL of 100 µM 20 nt DNA probe was labelled by using 20 units of T4 polynucleotide kinase (Thermo Scientific™) with 1x buffer in a total reaction volume of 20 µL. The enzyme was inactivated at 75 °C for 10 minutes and the probe was purified using RNA mini–Quick Spin Columns (Merck). The Church buffer was changed, and the probe was added to the fresh 20 mL of it. The probe was left at 45 °C overnight to hybridize. Before analysis, the membrane was washed with 2x SSC, 0.1 % SDS, low stringency buffer twice and once with 0.2x SSC, 0.1 % SDS high stringency buffer for 10 minutes each wash. A phosphor imaging plate (GE healthcare) was irradiated using the membrane sealed in foil and analysed on a Typhoon FLA 9500 (GE Healthcare).

5 RESULTS:

The existence of dinucleoside polyphosphate capped RNA has been an unknown molecular phenomenon until recently. Their existence in bacteria was reported by two independent groups and confirmed by several independent techniques(156, 157). Subsequent findings of our group, about the mechanism of their incorporation into the RNA molecule was then further explored(158). Given that these molecules served as excellent substrates for both the bacteriophage T7 RNA polymerase and the Escherichia coli (*E. Coli*) RNA polymerase, we postulated that they could serve a similar function inside eukaryotic cells. Ap_4A, a dinucleoside polyphosphate, has been reported as a key component of the immune activation of mast cells through the binding of IgE to the cellular receptor FcεRI and to an antigen, as was described in the theoretical part of this thesis. This activation causes a significant increase in the concentration of Ap_4A in the nucleus of the cell, where RNA transcription takes place(161). The objective of this thesis was to isolate the RNA from activated and non-activated mast cells and analyse it, in order to study the presence of Ap_4A capped RNA, a previously undescribed molecule.

All the experiments were done in biological triplicates, except for the SCIEX QTRAP 6500+ spike measurements, which is a duplicate due to the large amount of RNA necessary.

The measurements done on the LC-MS were done under the direct supervision and with the help of Roberto Benoni as was the final result interpretation.

5.1 RNA preparation:

RBL-2H3 cells were used for RNA isolation (see Methods and Materials for detailed information). An electrophoretic analysis of the RNA was performed using isolated RNA diluted to a concentration of 10 ng·µL^{-1}. As can be seen in *Figure 5.*, the results of the Agilent High Sensitivity RNA ScreenTape System aligned with an electronic ladder showed good separation of the RNA into the short fraction, shorter than 200 nucleotides, which mainly contains tRNA, microRNA, and small ribosomal RNA. The long fraction is then composed of mRNA and ribosomal RNA.

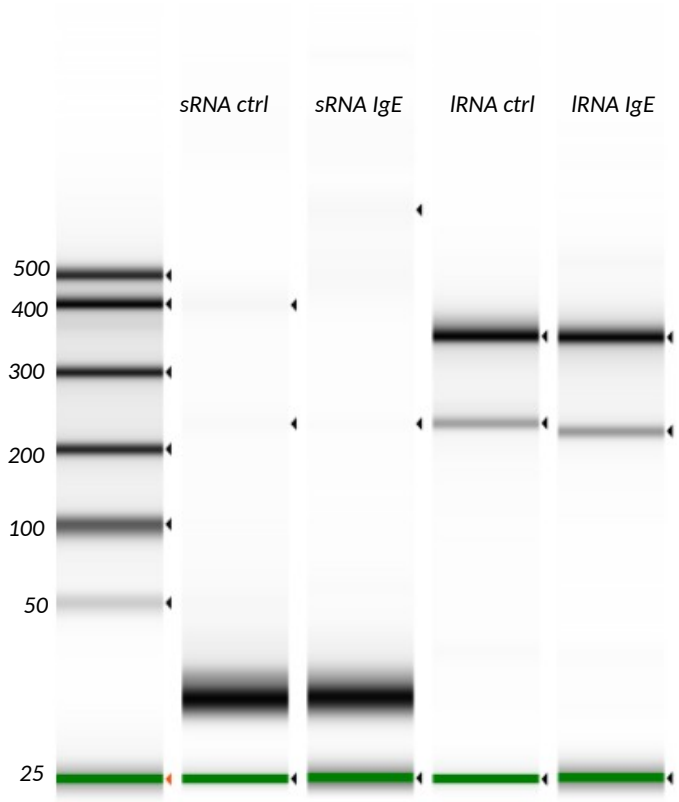


Figure 5. Tape station analysis of isolated RNA

A PAGE analysis showing the purity and separation of long and short RNA fractions from activated and non-activated RBL-2H3 cells. Short RNA (sRNA) is in the first lanes next to the ladder and is shorther than 200 nucleotides and the long RNA (lRNA) is longer than 200, which is the RNAzol separation cut-off.

Table 2. Nanodrop analysis of isolated RNA

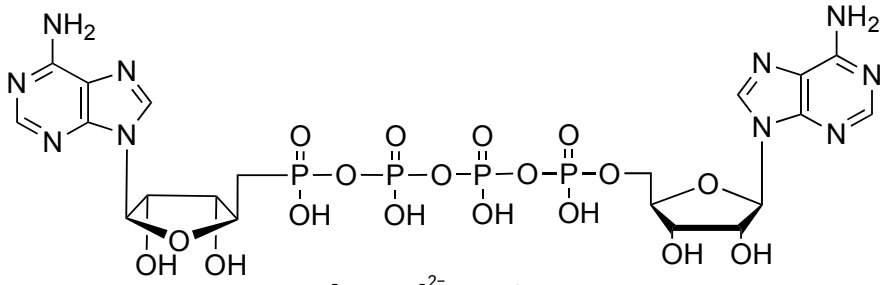
Example of Nanodrop analysis of fractions of isolated RNA from activated and inactivated mast cells. The total volume of long RNA fractions was 250µL and the total volume of short RNA was 200µL. The absorbance A260 unit is the amount of RNA in 1 mL with an optical density (OD) of 1. The ratio between A260 and A280 of 2 corresponds to 100% nucleic acid purity for the long fraction and 1.97 reflects 90% purity of the short fraction.

Sample Name	Nucleic Acid(ng/µL)	A260/A280	A260/A230	A260	A280	Nucleic Acid Factor	Baseline Correction (nm)	Baseline Absorbance	Total RNA Isolated (µg)
Long RNA ctrl 1	11907.092	2.040	2.265	297.677	145.908	40.00	340	1.120	2976.773
Long RNA IgE 1	9297.699	2.027	2.241	232.442	114.649	40.00	340	1.093	2324.425
Short RNA ctrl 1	4287.740	1.966	1.808	107.194	54.535	40.00	340	-0.282	857.548
Short RNA IgE 1	4501.657	1.970	1.798	112.541	57.126	40.00	340	-0.163	900.331
Long RNA ctrl 2	17748.989	2.037	2.202	443.725	217.832	40.00	340	0.251	4437.247
Long RNA IgE 2	9842.154	2.021	2.215	246.054	121.752	40.00	340	0.862	2460.539
Short RNA ctrl 2	4390.265	1.884	1.311	109.757	58.258	40.00	340	0.238	878.053
Short RNA IgE 2	2602.865	1.913	1.377	65.072	34.009	40.00	340	-0.223	520.573
Long RNA ctrl 3	14187.555	2.007	2.100	354.689	176.682	40.00	340	0.307	3546.889
Long RNA IgE 3	21637.443	2.035	2.230	540.936	265.766	40.00	340	1.142	5409.361
Short RNA ctrl 3	2982.212	1.982	1.851	74.555	37.622	40.00	340	-0.185	596.442
Short RNA IgE 3	3198.844	1.989	1.883	79.971	40.198	40.00	340	-0.042	639.769

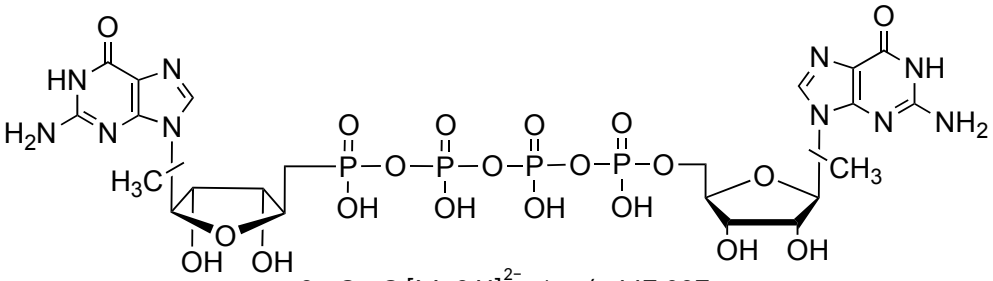
This confirms the successful isolation and separation of cellular RNA. Based on the concentration and total volume of isolated RNA, several milligrams of long RNA and over 500 micrograms of short RNA were isolated from one batch of activated and non-activated cells.

5.2 LC-MS Analysis

Once isolated and purified, the short RNA was cleaned from unincorporated or free-floating mononucleotides using a 3 kDa cut-off filter (see Methods and Materials for detailed information). The long RNA was already rid of these molecules from the RNAzol extraction process, as they get isolated in the short fraction due to their size. After the filtration, 500 µg of RNA per sample were digested by Nuclease P1 into nucleoside monophosphates and evaporated using Savant SpeedVac. The dried digested RNA was then dissolved in 10 µL of 10 mM NH_4OAc and 8 µL of the sample were analysed using the SYNAPT G2 quadrupole-ion mobility-TOF hybrid MS using a method previously developed in our laboratory(156). Samples prepared as a negative control, made without the addition of Nuclease P1 in the digestion step, were analysed for comparison. We did not observe any signals of nucleotides or $N_{(n)}$Ns. This eliminates the possibility of contaminants and non-covalently bound molecules to the RNA.



Ap_4A $[M-2\,H]^{2-}$ at m/z 417.016.



$2mGp_4G$ $[M-2\,H]^{2-}$ at m/z 447.027

Figure 6. Structure of Ap_4A

A chemical structure of Ap_4A and $2mGp_4G$ with the calculated masses. Given the presence of the 4 phosphate groups, the molecules get charged twice in the negative mode, which is why the calculated mass is $[M-2\,H]^{2-}$ at m/z 417.016 and $[M-2\,H]^{2-}$ at m/z 447.027. It is also why the isotopic shift in the results is only +0.5 to the mass.

The first analysed sample was a mixture of the RNA with an added Ap $_4$A standard to a total concentration of 0.5 µM in order to reflect the sample matrix effect and to identify the retention time of the molecule. The extracted ion chromatogram showed a signal in both the activated and non-activated cells and the subsequent analysis of the spectra confirmed the presence of Ap4A - $[M-2H]^{2-}$ at m/z of 417.021 with an isotopic shift to 417.521. It is this shift, that confirms the presence of the desired molecule and not a different molecular fragment of the same m/z. The analysis also revealed the presence of dimethylated diguanosine tetraphosphate-capped RNA (2mGp $_4$G) $[M-2H]^{2-}$ at m/z 447.027 in the short fraction. The existence of this molecule was also confirmed in bacteria and the bacterial enzymes responsible for its cleavage were identified.

The same analysis of the long RNA did not lead to detection of Ap$_4$A. Nevertheless, we cannot exclude the presence of this cap in the fraction of long RNA as the signal can be lost in the background signal produced by the abundance of RNA nucleotides coming from long RNA (ratio of cap/nucleotides in long RNA is much lower in comparison with short RNA).

The background noise of the other modified RNA nucleotide from ribosomal RNA in the long fraction did not allow for proper Ap $_4$A detection in the long RNA fraction. However, the LC-MS analysis clearly showed the presence of two types of capped RNA (Ap $_4$A and 2mGp$_4$G) in the short fraction from both activated and non-activated RBL-2H3 cells. 2mGp $_4$G, which was described as one of the caps present in bacterial RNA. The methylation conferred an extra layer of stability on the RNA in bacteria, protecting it from decapping by certain enzymes (156). We can speculate that the molecule may have the same role here, though its decapping partners and function remain to be elucidated. We further optimized the ionization and chromatographic conditions for the detection of Ap$_4$A capped RNA (changes in the pressure, temperature, ionization), but we were unable to detect even traces of Ap$_4$A in fraction of long RNA.

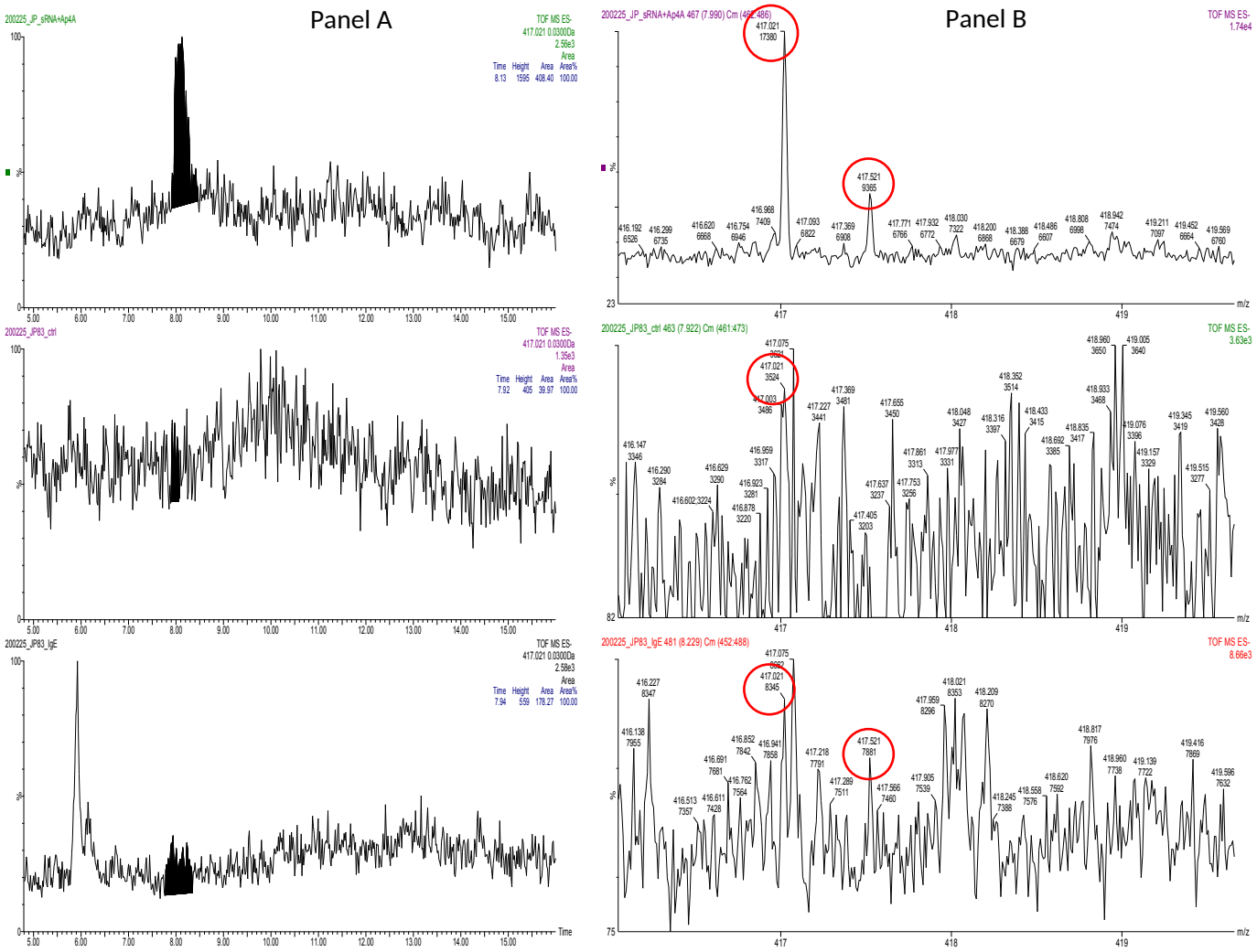


Figure 7. An extracted ion chromatogram and the analysed spectrum of Ap_4A

Panel A of the image shows three extracted ion chromatograms at a calculated mass of 417.021 and a detected mass within range. The uppermost sample was spiked with a 0.5 µM Ap_4A standard, the middle sample is from non-activated short RNA and the bottom is from IgE and antigen activated RBL-2H3 cells. Panel B shows the analysed spectra of the highlighted ions from the right. The $[M-2H]^{2-}$ *at m/z of 417.021 and the associated isotope shift to m/z 417.521 confirm the presence of Ap_4A.*

5.3 Multiple Reaction Monitoring (MRM) analysis:

When using the SYNAPT G2 quadrupole-ion mobility-TOF hybrid MS, the signal to noise ratio in the sample did not allow for proper identification of the cap. The SCIEX QTRAP 6500+ provides higher sensitivity, which is why the method and the analysis was changed to this device. A method for the specific detection of only Ap_4A was developed for the SCIEX QTRAP 6500+ by Roberto Benoni, a postdoc from our lab (see Methods and Materials for more detailed information). This method specifically targets the ion of interest in the mixture - Ap_4A, and then follows the fragmentation of this specific ion into "daughter" ions, which can then be quantified. Only the ions that fulfil both of the criteria i.e., they correspond to the original ion and the daughter ions, are isolated. The other ions that produced too much background noise during the SYNAPT measurements before are ignored by the QTRAP giving a clearer analysis of the desired molecule.

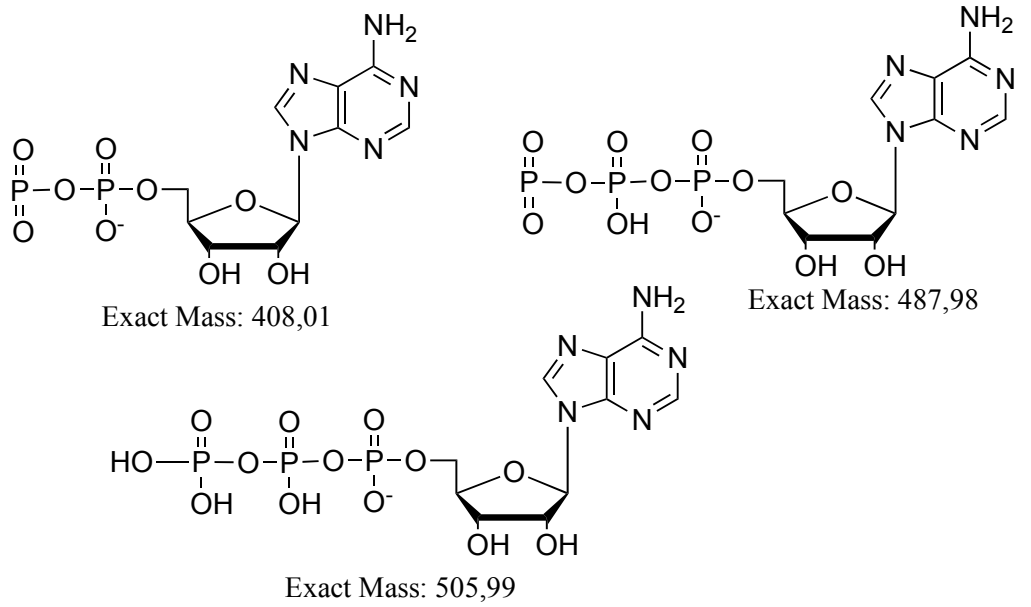


Figure 8. Ap_4A fragmentation

The fragments coming from Ap_4A and their calculated masses in Da.

The presence of Ap_4A was confirmed in the short fraction of the RNA, but the low amount did not enable proper quantification. We tried analysing the long fraction of the RNA, where the same biological sample provides enough material for the addition of different concentrations of the standard and to create a calibration curve.

A series of 5 samples of the same long RNA was prepared and 1 µL of various concentrations of Ap_4A was added to them (final concentrations and peak areas in ***Table 2***). This series was measured first in order to ascertain the retention time and the fragmentation properties of Ap_4A. The fragments of the molecule (see ***Figure 8.***) were clearly visible at the time of 10 minutes (see ***Figures 4. And 5.)*** with the decreasing peak area corresponding to the decreased Ap_4A spike concentration. Based on the addition of the individual peak areas of each fragment, a linear equation was extrapolated and used to quantify the concentration of Ap_4A-capped RNA in both activated and not activated cells.

The measurements from the SCIEX QTRAP 6500+ showed a higher relative amount of Ap_4A in the short RNA fraction than in the long one, which confirmed the previous measurements from the SYNAPT G2 quadrupole-ion mobility-TOF hybrid MS. The results from the long fragment analysis enabled the quantification of the capped RNA in the long fraction (see **Table 3.**). The isolated amount of short RNA was never sufficient to create enough spiked samples in order to calculate the exact amount of the capped RNA present. However, based on the signal intensity and relative amount of detected Ap_4A-capped RNA, we can estimate that the concentration in the short fraction is about 30 times higher than in the long fraction. An important discovery was that the amount of Ap_4A-capped RNA does not increase with the IgE mediated activation of the mast cell and with an intracellular increase in the concentration of Ap_4A. This would suggest a carefully regulated amount of the capped RNA, rather than arbitrary RNA capping.

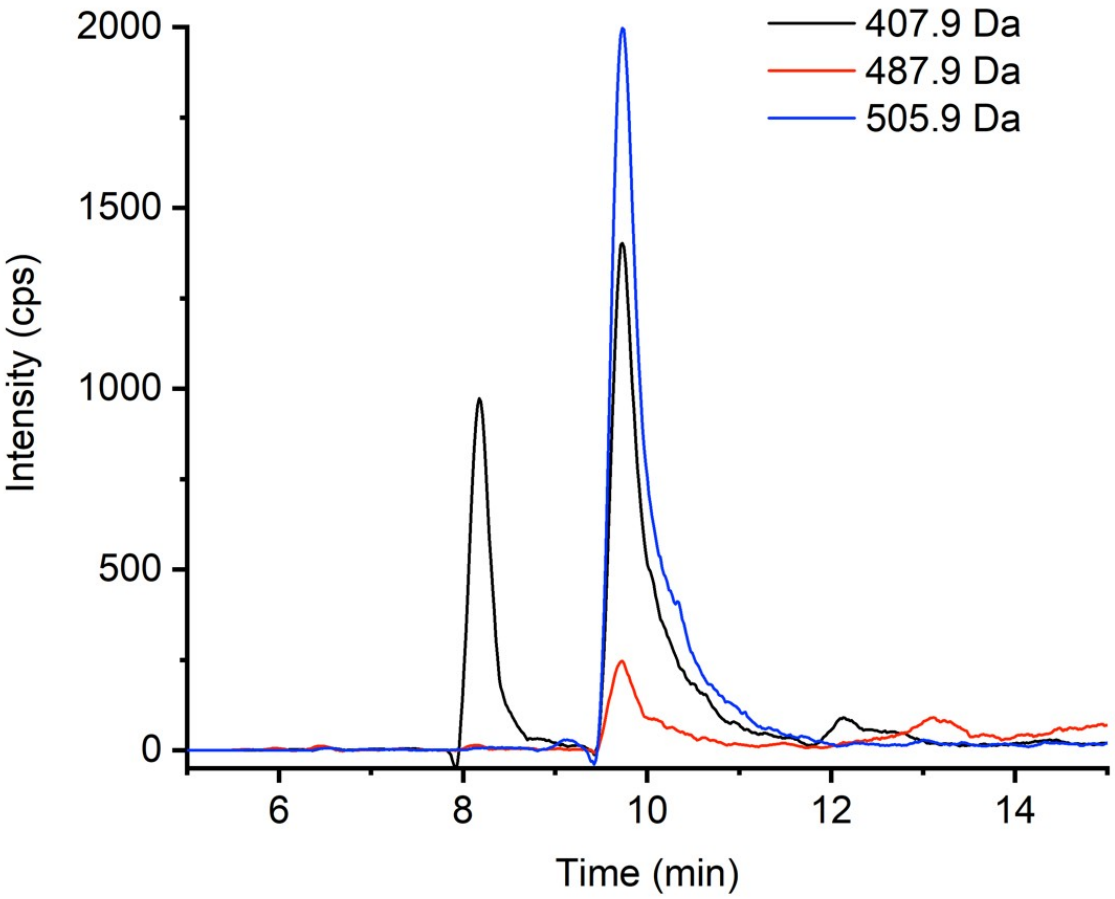


Figure 9. Graph of fragment intensity in time of 0.5 µM Ap_4A spiked RNA

*The data from the SCIEX QTRAP 6500+ measurements transformed into a linear graph in Origin software. The fragments of Ap_4A - 407.9 Da, 487.9 Da and 505.9 Da (see **Figure 8.**) are visible in black, red and blue respectively. The peak intensities and areas were calculated using the Analyst software (see **table 3.**) The highest intensity of all three peaks is clearly visible at the time of 10 minutes.*

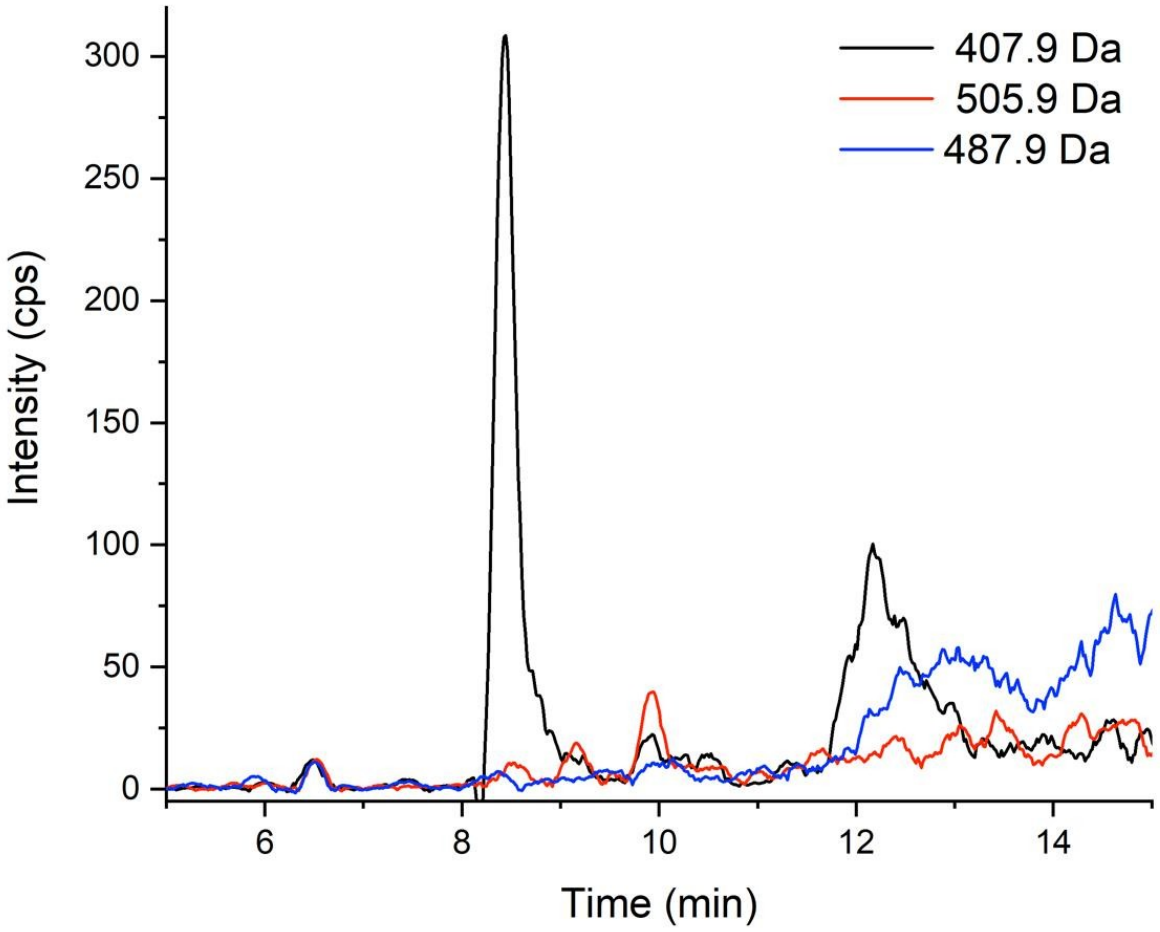


Figure 10. Graph of fragment intensity in time of Ap$_4$A-capped RNA without spike

The data from the SCIEX QTRAP 6500+ measurements transformed into a linear graph in Origin software. The fragments of Ap $_4$A - 407.9 Da, 487.9 Da and 505.9 Da are visible in black, red and blue respectively. The intensity of the peaks at 10 minutes is much lower than in the spiked samples (See ***Figure 9.****), but the presence of all the 3 fragments (see* ***Figure 8****) is clearly visible confirming the presence of Ap$_4$A and enabling quantification.*

Table 3. Fragment peak areas

The table shows the added peak areas of the 407.9 Da, 487.9 Da and 505.9 Da fragments in the spiked control sample, and then the measurements of RNA from IgE activated cells in biological duplicate. The RNA was measured in the same run, in order to use the spiked sample for quantification without changing the technical conditions. Interestingly, the peak areas in the samples from the IgE activated cells are both lower than the controls. This is in contrast to our predictions. The cellular concentration of long Ap_4A capped RNA was calculated at around the 8 pM range.

Final Ap_4A spike concetration (µM)	Long RNA (ctrl) fragment peak area	Long RNA (IgE) fragment peak area	Long RNA (ctrl) fragment peak area	A Long RNA (IgE) fragment peak area
0	514	469	513	457
0.02	2575	x	x	x
0.1	14259	x	x	x
0.2	25415	x	x	x
Cellular concentration (pM)	8.9	8.1	8.9	7.9

5.4 RNA identification

The presence of an Ap_4A on a specific RNA is possible using MS analysis for more abundant RNA. Some transcripts, however, are present in such low numbers that the LC-MS could overlook them in the background signal. That is why we developed the alternative method of radiolabelling particular transcripts that could be capped in this manner. Given the increase in transcription of a number of mRNAs upon mast cell activation and the ability of RNA polymerases to readily incorporate Ap_4A into their structure, a candidate RNA was chosen to test for the presence of the cap – Chymase 1 mRNA. Chymase 1 mRNA is expressed 7 times more after mast cell activation. The first transcribed nucleotide in the sequence is an A, providing the potential conditions for Ap_4A incorporation (120).

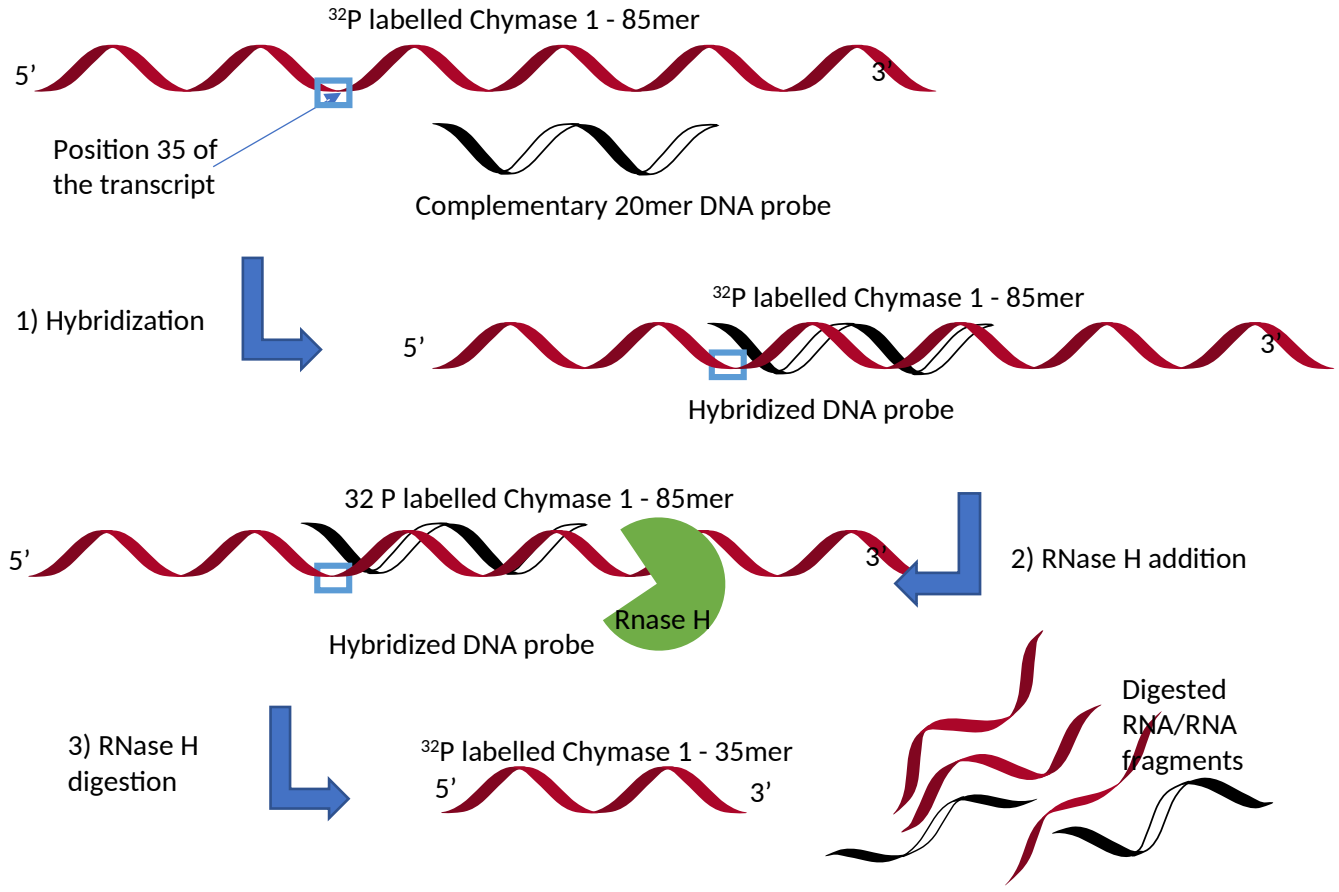


Figure 11. Experimental design of RNase H treatment

The in vitro prepared Chymase 1 85mer is hybridized with a 20 nt long ssDNA probe from the 35th nucleotide (1). RNase H is added, and it binds to the RNA/DNA hybrid (2). RNase H then digests the DNA/RNA hybrid, leaving the first 35 nucleotides intact for PAGE analysis.

Therefore, we prepared model (see ***Figure 11.***) RNA starting with sequence of Chymase mRNA to develop specific detection technique of Ap4A cap. A 35-mer RNA was prepared by T7 *in vitro* transcription with the corresponding sequence to the chymase 5' end nucleotides (see Methods and Materials for sequence and detailed information). The RNA was ^{32}P radiolabelled, purified and analysed using PAGE, which enabled us to distinguish Ap4A-capped RNA and uncapped RNA.

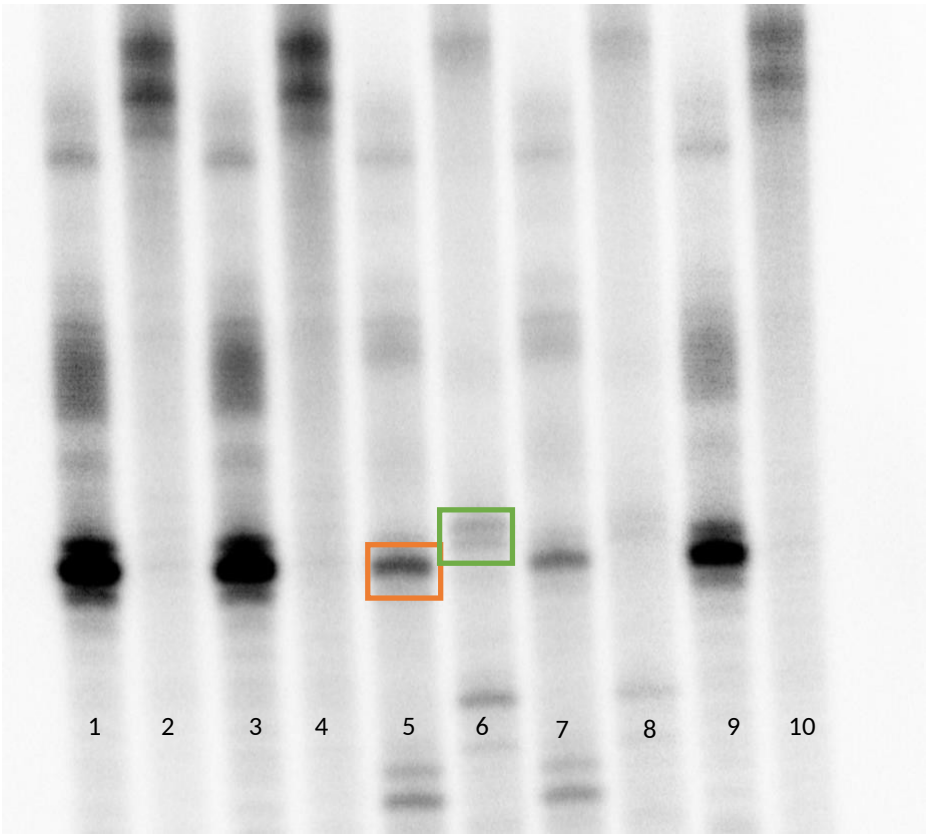


Figure 12. Radioactive PAGE analysis of RNase H activity

A PAGE analysis of ^{32}P radioactively labelled RNA after RNase H treatment. The lanes 1 and 2 are length controls for the 35 and 85 nucleotide long RNAs respectively. Lanes 4 and 5 are the same samples, hybridized with the DNA probe. Lanes 5 and 6 are after 10 minutes of RNase H treatment of uncapped and 5' capped RNA respectively. Lanes 7 and 8 are the same RNA samples as the two previous lanes, but after 20 minutes of treatment. Lanes 9 and 10 are the 35 and 85 nucleotide long RNAs with the RNase buffer, without the added enzyme as a control. From the gel, we can clearly see the model Chymase I RNA cleaved to a length of 35 nucleotides with a visible difference in uncapped (orange) and capped (green) molecules. Though the treatment of 20 minutes is too long and results in nonspecific RNA cleavage, the method is optimal at 10 minutes of incubation.

The *in vitro* part was designed to optimize the method before wasting precious mast cell RNA. The transcribed RNA was hybridized with a 20 nucleotide long single stranded DNA probe. The probe sits from the 35th nucleotide of the RNA downstream. When treated with RNase H (see materials and methods for detailed information), the RNA/DNA hybrid gets cleaved leaving just the top 35 nucleotides of the RNA with the cap.

The northern blot protocol is still being optimized and has not yet produced any conclusive data.

These results combined conclusively prove the existence of two previously undiscovered types of molecules in mast cells - Ap_4A-capped RNA and $2mGp_4G$-capped RNA. This is the first time, that these types of molecule were detected in eukaryotic cells. When the mast cells undergo IgE mediated activation, the intracellular concentrations of Ap_4A rise up to 700 µM. Based on this increase, the amount of Ap_4A-capped RNA should also increase. However, upon immune activation of the cells, the concentration of the capped transcripts decreases. This would suggest that mast cells are able to carefully regulate the amount of capped RNA, and that the molecule itself is not a by-product of other reactions, though the actual capped RNA evades identification. These results will be discussed in the next chapter.

6 Discussion

The activation of mast cells, when the FcεRI receptor is bound by IgE which then binds an antigen is one of the key modes to induce their immune response. While the molecular mechanisms of this process have been described, there are still aspects that need more research. Several articles describe a large spike in intracellular Ap_4A concentration during this IgE mediated activation of mast cells (1, 2). Based on the recent discovery from our group, that dinucleoside polyphosphates act as 5' caps in bacterial RNA, we postulated that the eukaryotic RNA polymerase inside mast cells will readily accept Ap_4A and incorporate it into the RNA as a non-canonical 5'cap. This chapter puts the results presented in this thesis within a wider context of the current knowledge about the given topic and presents the future direction of the research.

The first goal set forth by the thesis was the isolation of total RNA from activated and non-activated RBL-2H3 cells in order to analyse it for the presence of Ap_4A-capped transcripts. I successfully identified Ap_4A-capped RNA within the cellular transcriptome. The total RNA of a cell is a very complex mixture of molecules. While the three most common types of RNA are messenger, transfer, and ribosomal, there are also small regulatory RNAs with different functions, nuclear organelles made up of RNA and proteins such as Cajal bodies, and more (3). The largest constituents of total cellular RNA are rRNA at about 80%, tRNA at 15% and mRNA at 4% (4, 5). The rest of the cellular RNA only constitutes about 1% in spite of its huge variability. The 1% of RNA was shown to harbour other non-canonical caps in eukaryotic cells. Specifically in subsets of small regulatory RNAs, which makes the identification of a new type of RNA chemical modifications in the transcriptome particularly difficult, even when using highly sensitive methods such as the LC-MS/MS. Without proper sample preparation and results analysis, such experiments have led to wild unsubstantiated claims, such as previously reported? presence of over 30 modifications on the viral RNAs of positive single stranded RNA viruses. The mentioned modifications in the article included modifications exclusive to tRNA etc. and were probably the result of poor experimental design, as their presence was linked to packaged cellular tRNA in the viral article(6–8). Both tRNA and rRNA are very heavily modified, which presents another potential complication in the search for new modifications and their roles (9, 10). The RNAzol protocol enables the separation of the isolated total RNA

into two fractions based on their different solubility in ethanol and isopropanol. One is longer and the other shorter than 200 nucleotides. The composition of the extracted RNA is largely rRNA in the long fraction and tRNA in the short fraction. Nonetheless, if the capped RNA stays within a specific fraction, it helps to lower the noise from other modifications present and facilitates easier target molecule recognition. This is helpful, because the peak signal intensity of the newly discovered caps is often at the level of background noise.

A key factor in the confirmation that the molecule detected by MS is really Ap_4A is the isotopic shift from naturally present isotopes, mainly C^{13}. Absolute recognition of the cap can only be achieved based on the measurements of a standard. . Based on the behaviour of the standards and other dinucleoside tetraphosphates during ionization by electrospray, we know that Ap_4A is double charged. As such, the m/z of the isotopic peaks is shifted by 0.5 Da (compared to single charged ions shifted by 1 Da) (see the circled peaks in ***Figure 7.***). Seeing this shift in the raw data indicates, that we are, in fact, seeing a double charged ion that could come from the molecule of interest and not just a single charged ion of that same mass.

While $Np_{(n)}N$-capped RNA was never before discovered in eukaryotic cells, mast cells are known to produce copious amounts of Ap_4A when undergoing IgE mediated activation(11). In accordance with the main premise of the thesis, I actually discovered the Ap_4A-capped RNA present inside the mast cells. Using two separate mass spectrometry techniques, its presence was confirmed in both the short and long fractions of the RNA. I also discovered $2mGp_4G$

Various non-canonical RNA caps were discovered in the past several years. It started with the co-factors CoA and NAD. These caps were originally found in bacteria, where they cap a group of regulatory RNAs. They protect the RNA from degradation by a number of enzymes but can also be decapped in a controlled manner and show a new layer of RNA regulation in bacteria(12). It did not take long for the NAD-capped RNA to be discovered in eukaryotic cells. The mechanisms of incorporation of the NAD cap in eukaryotes are unclear, while NAD can be incorporated as an initiating nucleotide, the cap was found on processed RNA where it had to be installed after the transcription(13). The function of the NAD capped RNA also needs more research. Currently, it is known that the capped RNA interacts with the decapping and exoribonuclease protein DXO and that this interaction leads to the degradation of the RNA. It

is unknown, however, if such RNA can be translated before it is degraded or if it functions as some sort of localization signal(14).

Dinucleoside polyphosphates were also found to function as non-canonical RNA caps in bacteria. They bear a structural resemblance to the canonical m ^{7}G cap or the NAD cap and are incorporated into the RNA in a similar manner to the NAD or CoA caps – as a non-canonical initiating nucleotide(15). While the specific RNAs with this cap have not yet been identified, it is known, that the amount of Np$_{(n)}$N-capped RNA increases as a cellular response to various types of stress. They can be selectively decapped by enzymes from the NudiX family and they can also be stabilised by methylation, which protects them against some of the decapping enzymes. This capping process presents previously undiscovered potential to control RNA metabolism and localization(16).

The Ap$_4$A-capped RNA I discovered in the short fraction was more abundant relative to the mass of the sample than in the long fraction. The amount of total short RNA that was isolated from the mast cells did not allow for absolute quantification though. The 5S rRNA and tRNA (the main component of the short RNA fraction) have been thoroughly searched for modifications and Ap$_4$A was not found(10, 17). This would leave the modification on some small non-coding regulatory RNAs. If it were present there, it could potentially have similar roles to that of the NAD capped RNA. An extra added layer of protection and function affecting the longevity of the capped molecule. If that were the case, there a mechanism removing these caps must exist. In fact, a number of mammalian NudiX enzymes were shown to be very adept at cleaving some of the non-canonical caps(18). It is fathomable, that some members of the NudiX family could also cleave the Np$_{(n)}$N-capped RNA. Free Ap$_4$A is cleaved by the NudiX enzyme NUDT2 – which points to a potential well-orchestrated capping and decapping regulation inside the cell.

The Ap$_4$A-capped RNA in the long RNA fraction was quantified at a cellular concentration of around 9 pM in the non-activated and 8 pM in the activated cells, which is very similar. Relative quantification of the RNA is difficult due to the dynamics of the cellular transcriptome. Some rarer mRNAs have been shown to exist in as little as 5 copies per cell and have great impact on cellular fate(19). While it is pure conjecture at this point, it is

possible that some mRNAs might be capped with Ap_4A. The cap could then function as a localisation signal or even present the RNA for some sort of internal ribosomal entry site translation, as was suggested for the NAD capped eukaryotic RNA(13).

Interestingly, the results presented show, that the concentration of Ap_4A-capped RNA did not increase with the IgE mediated activation of the mast cells. This activation is supposed to increase the concentration of free Ap_4A inside the mast cells several fold (1). The contrast between our results and hypothesis may stem from an alternative activation of the mast cells. The classical activation pathway in this case was the treatment of the cells with IgE against either trinitrophenol (TNP) or dinitrophenol (DNP) and then adding the specific antigen. This resulted in the degranulation of the cells, used as a control for their activation. The non-activated cells, however, were also exposed to certain types of stress. RBL-2H3 cells are adherent and as such, they had to be treated with trypsin and then mechanically removed from the petri dishes. A number of studies has shown that mast cells can be activated by a plethora of factors, one of which is through mechanical stimuli(20, 21). The activation of the cells was checked by measuring their degranulation. However, if there was any activation of the control cells leading to the LysRS translocation without the degranulation, it would be reflected in the results. A more likely explanation is a carefully regulated level of the Ap_4A capped RNA controlled by the decapping enzymes, again similarly to the NAD capped RNA in human cells.

Our hypothesis was also supported by what we observed during the bacterial response, where, as mentioned before, the amount of capped RNA increased with stress(16). It can be because the process of bacterial transcription is simpler than the eukaryotic one, the mechanisms are incomparable.

The next steps in elucidating the role of Ap_4A-capped RNA will undoubtedly focus on identifying and sequencing the capped transcripts. Identifying the RNA in this manner could conclusively show, if Ap_4A-capped RNA directly participates in the IgE mediated immune response of mast cells. This could be achieved by developing a capturing technique based on the methods used to analyse other non-canonical caps or RNA modifications. For example, the method used for capturing the NAD-capped RNA was based on the reactivity of the NAD

cap and the attachment of a “clickable” alkyne group to it. The alkyne was then biotinylated using click chemistry and captured using streptavidin beads(22). Antibody based capturing techniques could theoretically also be used, as antibodies have been used in the labelling of chemical RNA modifications such as m^{6}A(23). The last option would be to use enzymatic capture of the capped RNA, perhaps by a specific decapping enzyme with a mutated active site. Nevertheless, the experiments in this thesis led to the discovery of a new type of RNA modifications in mast cells.

7 Conclusion

The focus of this thesis was the identification of Ap_4A capped RNA in the total RNA extracted from mast cells before and after IgE mediated activation. This type of transcript was only recently discovered in prokaryotic organisms, but it has been shown to play a role in response to various types of stress. Given the specific goals set forth in this thesis (see Aims of the thesis), the results are as follows:

1) ***Used IgE to activate model RBL-2H3 cells and isolated total RNA from both the activated and non-activated cells.***

   The RNA was successfully split into two fractions – longer and shorter than 200 nucleotides. The activation of the cells was confirmed by confirming their degranulation using a β-Glucuronidase Activity Assay.

2) ***Ap_4A-capped RNA was detected inside both the activated and non-activated cells.***

   Two new dinucleoside polyphosphate capped RNAs were found. The Ap_4A-capped RNA and $2mGp_4G$-capped RNA was found in the short RNA fraction of both activated and non-activated cells using the LC-MS technique. Ap_4A-capped RNA was also discovered in the long fraction of the RNA using the more sensitive LC-MRM analysis. With the supervision of and help of a postdoc from our group Roberto Benoni, I was able to optimize a new LC-MS method sensitive to the Ap_4A-cap. I was also able to prepare samples for an LC-MRM method developed by him. We are currently using the method to quantify the Ap_4A-capped RNA in the short RNA fraction from mast cells.

3) ***The concentration of Ap_4A-capped RNA inside the activated and non-activated cells is 8.9 pM and 8 pM respectively.***

   Using the results obtained from the MRM measurements, the intracellular concentration of Ap_4A-capped was calculated at an average of 8.9 pM for the non-activated cells and 8 pM for the activated ones. This would point to the capping of a specific transcript with a relatively low abundance.

4) ***Identification of the Ap_4A-capped transcript***

A specific mRNA was identified as a potential capping target but has not yet been identified. The experiment was designed, but has not produced any data and is currently being optimized.

8 References

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