Univerzita Karlova

1. lékařská fakulta

Autoreferát disertační práce

UNIVERZITA KARLOVA
1. lékařská fakulta

Studium funkce NAD-RNA v HIV-1 infekci

Studies of NAD-RNA and its function in HIV-1 infection

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Contents

1 Abstrakt

Viry se vyznačují jednoduchou strukturou a jsou dobře popsány ve vědecké literatuře. Na základě studií provedených na virech bylo učiněno mnoho objevů týkajících se RNA modifikací.

HIV-1 infekce snižuje množství buněčného nikotinamidadenindinukleotidu (NAD). Nedávné studie ukázaly, že NAD může sloužit jako 5' nekanonická čepička některých RNA u bakterií, kvasinek, rostlin a savců. NAD ovlivňuje RNA stabilitu a účinnost RNA translace. O funkci NAD čepičky se však ví stále málo. Tato práce se zabývá NAD čepičkou v souvislosti s HIV-1 infekcí, přičemž v rámci jiné práce jsme studovali m^1A modifikaci v HIV-1.

Zjistili jsme, že HIV-1 infekce ovlivňuje nejen buněčné hladiny NAD, ale také množství NAD čepiček na sRNA. Pomocí NAD captureSeq jsme identifikovali čtyři snRNA (U1, U4ATAC, U5E a U7) a čtyři snoRNA (SNORD3G, SNORD102, SNORA50A a SNORD3B), které po HIV-1 infekci ztrácejí NAD čepičky. Zvláště zajímavá je U1 snRNA, která má sekvenci komplementární s HIV-1 pre-mRNA a váže se na ni při sestřihu. Zjistili jsme, že NAD čepička destabilizuje komplex HIV-1 pre-mRNA a U1 snRNA.

DXO je enzym odstraňující NAD čepičku. Připravili jsme buňky se zvýšenou produkcí DXO a sledovali jsme množství NAD-RNA ve spojitosti s infektivitou HIV-1. Zvýšená produkce DXO způsobuje snížené množství NAD-RNA a zvýšenou infektivitu HIV-1. Naopak zvýšení množství buněčného NAD vede ke snížené infektivitě HIV-1.

Tato práce identifikuje nové sRNA s NAD čepičkou v lidských buňkách a navrhuje, že NAD-RNA snižuje infektivitu HIV-1 a může hrát roli v antivirové obraně.

Klíčová slova: RNA modifikace, NAD, U1 snRNA, RNA-seq, LC-MS, DXO, HIV-1

2 Abstract

Viruses have a simple structure and are well described in scientific literature. Based on studies conducted on viruses, many discoveries regarding RNA modifications have been made.

HIV-1 infection reduces the amount of cellular nicotinamide adenine dinucleotide (NAD). Recent studies have shown that NAD can serve as a 5' non-canonical cap for some RNAs in bacteria, yeast, plants, and mammals. NAD capping affects RNA stability and the efficiency of RNA translation. However, surprisingly little is known about the function of NAD cap. This work focuses on NAD capping in the context of HIV-1 infection, while in another study, we also investigated the m^1A modification in HIV-1.

We found that HIV-1 infection affects not only cellular levels of NAD but also the amount of NAD caps on sRNA. Using NAD captureSeq, we identified four snRNAs (U1, U4ATAC, U5E, and U7) and four snoRNAs (SNORD3G, SNORD102, SNORA50A, and SNORD3B) that lose their NAD caps after HIV-1 infection. Particularly interesting is U1 snRNA, which has a sequence complementary to HIV-1 pre-mRNA and binds to it during splicing. We discovered that the NAD cap destabilizes the complex between HIV-1 pre-mRNA and U1 snRNA.

DXO is an NAD decapping enzyme. We prepared cells with DXO overexpression and examined the amount of NAD-RNA in connection with HIV-1 infectivity. DXO overexpression causes reduced amounts of NAD-RNA and increased HIV-1 infectivity. In contrast, repletion of cellular NAD leads to decreased HIV-1 infectivity.

This work identifies new sRNAs with NAD caps in human cells and proposes that NAD-RNA decreases HIV-1 infectivity and may play a role in antiviral defense.

Key words: RNA modifications, NAD, U1 snRNA, RNA-seq, LC-MS, DXO, HIV-1

3 Introduction

To date, more than 170 different types of RNA modifications have been identified. These modifications are present in all types of RNA molecules, including tRNA, rRNA, mRNA, and various ncRNAs (Boccaletto et al., 2022). Several studies have shown that HIV-1 uses RNA modifications to regulate its gene expression during infection and replication (Fukuda et al., 2021; Ringeard et al., 2019). For example, m^1A is known to affect RNA stability and translation, contributing to the regulation of viral gene expression and replication (Burnett and McHenry, 1997).

HIV-1 infection causes reduced amounts of free NAD in human cells (Murray et al., 1995). The relatively recent discovery that NAD can serve as a 5' non-canonical RNA cap has opened a new and exciting area of RNA biology research (Chen et al., 2009). Despite extensive research on NAD-RNAs in various organisms, relatively little is known about the function of NAD on specific RNAs and in particular processes.

Since HIV-1 is a well-studied and characterized retrovirus, we focused our research on the physiological responses of human cells to HIV-1 infection. More specifically, our objective was to identify NAD-RNAs affected by the infection. As part of another project, we studied m^1A modification in HIV-1 particles.

3.1 snRNA

snRNAs are uridine-rich RNAs and about 150 nucleotides long. They are highly abundant in the nucleus and primarily involved in intron splicing (Karijolich and Yu, 2010).

3.1.1 U1 snRNA

U1 snRNA bears TMG cap and recognizes 5' SS of exons through complementary base-pairing. The 5'-terminal sequence of U1 snRNA is highly conserved across species (Zhuang and Weiner, 1986) (Fig. 1).

In HIV-1, U1 snRNP prevents polyadenylation at the 5' LTR, crucial for viral gene expression (Ashe et al., 1997). U1 snRNA also binds to the 5' splice site in env mRNA, protecting it from degradation and over-splicing. It is crucial for HIV-1 that most of its transcripts remain unspliced or partially spliced (Kammler et al., 2001; Lu et al., 1990).

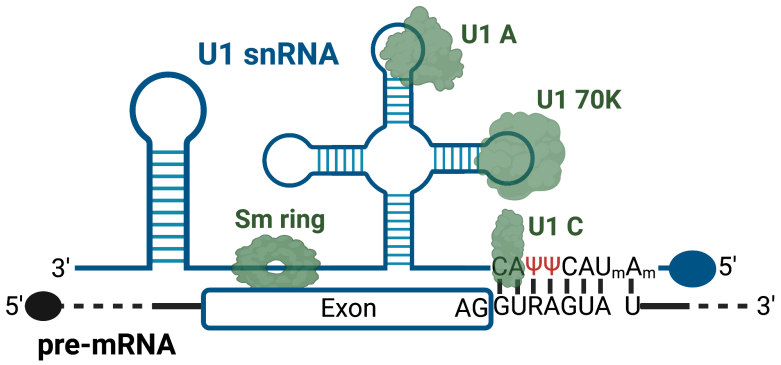

Fig. 1: Secondary structure model of human U1 snRNA with snRNA-pre-mRNA interactions shown.

3.1.2 snoRNA

snoRNAs are noncoding RNAs, 60-300 nucleotides long, typically transcribed from intronic regions by RNA pol II. There are two main classes: C/D box snoRNAs (SNORDs), involved in ribose methylation of rRNA, and H/ACA snoRNAs (SNORAs), responsible for pseudouridylation (Esteller, 2011).

3.2 RNA processing

RNA processing involves a complex of highly regulated steps that begins with the RNA transcription and continues until the production of mature RNA. This process includes several key stages such as capping, splicing, and polyadenylation of RNA.

3.2.1 RNA capping

The initial product of RNA pol II carries a 5' triphosphate end. The γ-phosphate is removed and GTase transfers GMP to the ppRNA, forming a reverse 5'-to-5' phosphate linkage and GpppNp-RNA. N7MTase adds a methyl group to form the Cap 0 structure. The 2'-hydroxyl of the first nucleoside can be further methylated by 2'OMTase to yield Cap 1 (Ramanathan et al., 2016).

In humans, some snRNAs, snoRNAs, and telomerase RNAs have Cap 0 which is further hypermethylated to $m^{2,2,7}G$ (TMG cap, Fig. 2). TMG cap serves as an import signal recognized by snurportin 1, allowing the mature snRNA to re-enter the nucleus (Huber et al., 1998).

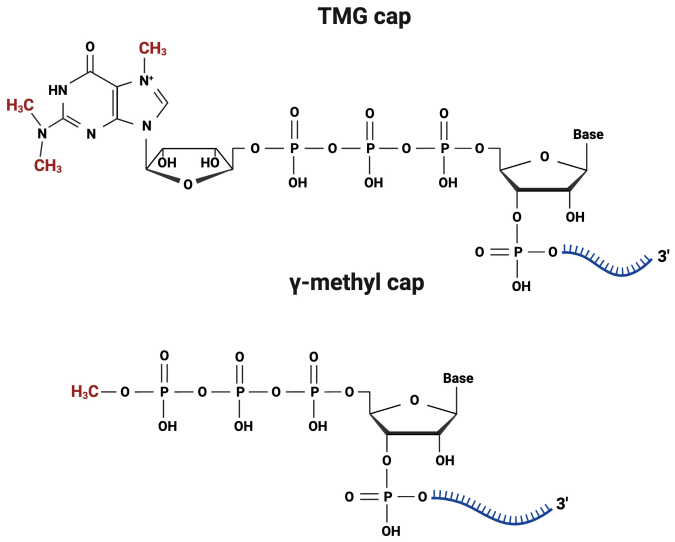

Fig. 2: Canonical caps of small RNAs in eukaryotes.

3.2.2 DXO decapping enzyme

DXO functions preferentially on incompletely capped RNAs and possesses both decapping and exoribonuclease activities (Jiao et al., 2013). DXO also decaps RNAs with non-canonical caps like NAD, FAD, and dpCoA (Doamekpor et al., 2020). DXO has a higher affinity for NAD-RNA compared to m^7G-capped RNA (Jiao et al., 2017).

3.3 Non-canonical RNA caps

Traditionally, RNAs in eukaryotic cells possess m^7G cap or TMG cap. However, recent research has revealed the existence of alternative cap structures known as non-canonical caps. The non-canonical caps originate from abundant coenzymes such as NAD, FAD, or dpCoA, and additionally from metabolites like dinucleoside polyphosphates (Np_nN).

3.3.1 NAD cap

NAD is a well-known coenzyme involved in cellular metabolism. It exists in oxidised (NAD+) or reduced (NADH) form (Huang, 2003). While NAD cap (Fig. 3) was detected *in vivo* across all organisms and many types of RNAs, NADH was only seen on mitochondrial RNAs.

NAD cap was first detected in RNA from *E. coli* and *S. venezuelae* using LC-MS. NAD cap was predominantly detected in sRNAs (Chen et al., 2009).

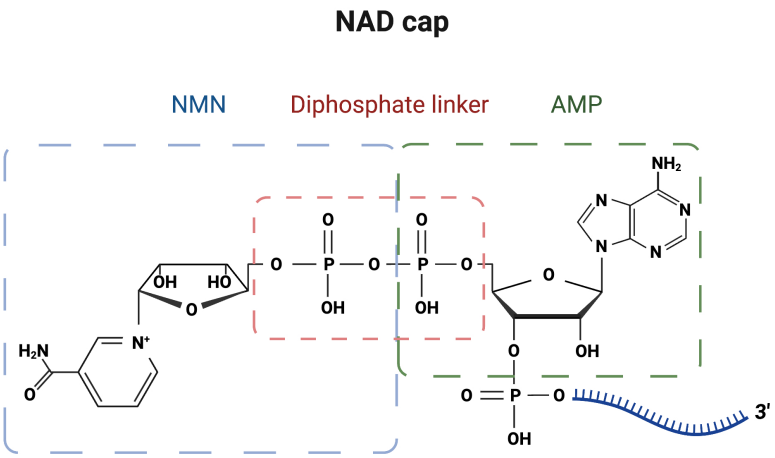

Fig. 3: Chemical structure of NAD non-canonical cap. NAD comprise a nicotinamide mononucleotide (NMN) and AMP moiety linked via two phosphate groups.

NAD captureSeq method was developed to identify NAD-RNAs (Cahová et al., 2015; Winz et al., 2017). It was first applied on total RNA from *E. coli* and revealed NAD caps on subsets of sRNAs (Cahová et al., 2015). NAD captureSeq also identified NAD caps in *Bacillus subtilis*, predominantly on mRNAs (Frindert et al., 2018).

NAD-RNAs are widespread in *A. thaliana*. NAD caps were found on mRNAs encoded by the nuclear and mitochondrial genomes but not the chloroplast genome. The NAD-RNAs from nuclear genes were spliced, polyadenylated, and associated with actively translating polysomes, indicating they can be translated in *A. thaliana* (Wang et al., 2019).

In human HEK293T cells, mRNAs, snoRNAs, and scaRNAs were found to possess NAD caps (Jiao et al., 2017). The ONE-seq method revealed that as mice age, cellular NAD and NAD-RNA levels decrease (Niu et al., 2023).

Biosynthesis of NAD-RNAs

NAD can serve as a non-canonical initiating nucleotide during transcription initiation for both bacterial RNA pol and eukaryotic RNA pol II *in vitro* and *in vivo*. snoRNAs and scaRNAs are intron-derived and exonucleolytically processed RNAs. Therefore, mammalian cells likely possess another, yet unknown, mechanism of NAD-capping that occurs after transcription initiation (Jiao et al., 2017).

NAD decapping

The primary deNADing enzyme in human cells is DXO, which decaps NAD-RNAs by hydrolyzing the phosphodiester bond between the first and second encoded nucleotides, producing pRNA (Fig. 4). DXO preferentially targets NAD-mRNAs, NAD-snoRNAs, and NAD-scaRNAs (Jiao et al., 2013, 2017).

Nudt12 hydrolyzes NAD-RNA to produce NMN and 5' pA-RNA. Comparison of NAD-RNA from Nudt12-KO cells and DXO-KO cells showed that Nudt12 and DXO have a pronounced preference for different sets of RNAs (Grudzien-Nogalska et al., 2019).

Nudt16 as an additional NAD decapping enzyme, hydrolyzing both free NAD and NAD-RNA (Sharma et al., 2020). Recently, human Xrn1 was also identified as a deNADing enzyme, releasing intact NAD and degrading the RNA *in vitro* (Sharma et al., 2022).

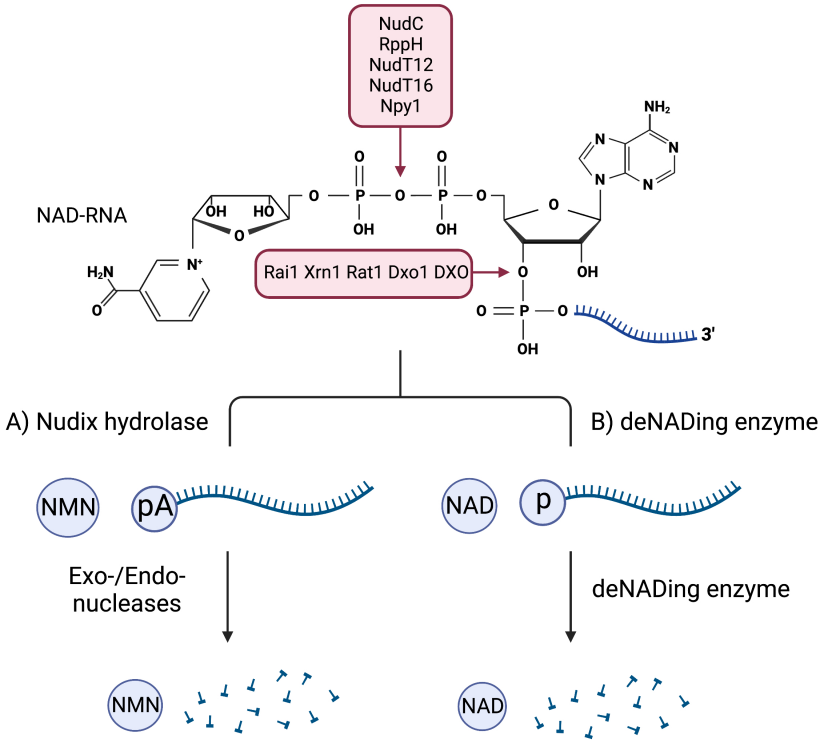

Fig. 4: Schematic overview of the known pathways of NAD cap removal.

3.4 HIV-1

HIV-1 belongs to the lentivirus subfamily of retroviruses. HIV-1 is an enveloped virus that contains two copies of single-stranded RNA bound to viral nucleocapsid protein, 7SL RNAs and tRNAs co-packed from the host, and integrase, protease and reverse

transcriptase enzymes (Fig. 5) (Zhao et al., 2013).

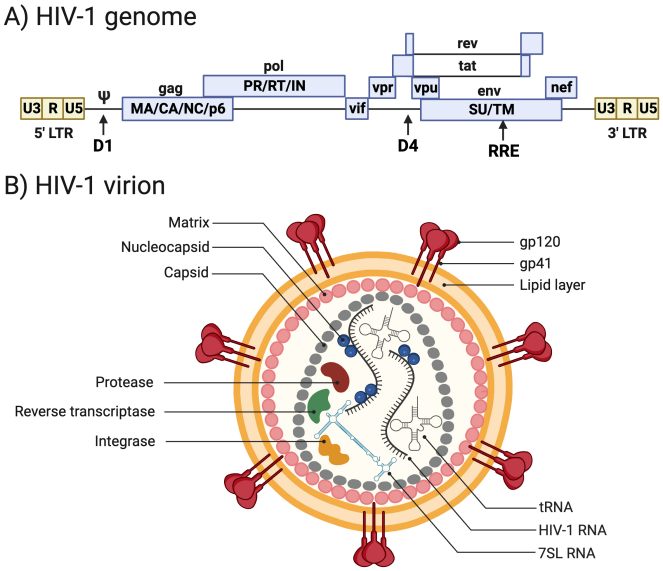

Fig. 5: Schematic representation of the HIV-1 genome and a mature HIV-1 particle. A) A diagram of the composition of the HIV-1 genome. B) Schematic representation of a mature HIV-1 particle.

3.4.1 HIV-1 life cycle

The key stages of the HIV-1 life cycle are viral entry, reverse transcription, integration, transcription, translation, assembly, and budding. The virion attaches to cells via gp120 binding to CD4 and capsid enters the cell (Chen, 2019).

The HIV-1 gRNA is converted into double-stranded complementary DNA (cDNA) by reverse transcriptase, which uses host $tRNA_3^{Lys}$ as a primer for DNA synthesis. HIV-1 co-packs around 770 copies of host tRNAs, among which Lys tRNAs are the most abundant (Šimonová et al., 2019). The viral cDNA is integrated into the host genome via integrase (Lesbats et al., 2016) and transcribed by host RNA pol II (Roebuck and Saifuddin, 1999).

Most viral pre-mRNAs remain unspliced to encode Gag and Gag-Pol polyproteins and serve as gRNA (Kuzembayeva et al., 2014; Purcell and Martin, 1993).

U1 snRNA binding is essential for HIV-1 env mRNA stability, independent of splicing (Kammler et al., 2001). Modifications in the sequence of U1 snRNA inhibit viral protein expression and lead to reduced HIV-1 replication (Knoepfel et al., 2012; Mandal et al., 2010; Sajic et al., 2007).

The newly synthesized viral proteins and RNAs assemble at the plasma membrane to form a new viral particle (Freed, 2015). After the release, the viral particle undergoes a

maturation step (Freed, 2015).

3.5 NAD role in HIV-1 infection

HIV-1 infection causes a decrease in cellular NAD and NADH levels. This decrease, called pellagra, varies with viral load and HIV strain. NAm supplement increases intracellular NAD levels in HIV-1 infected cells, so the depletion can be reversed (Murray et al., 1995). In addition, NAm supplement inhibits HIV-1 infection in cell culture in a dose-dependent manner (Murray and Srinivasan, 1995).

NAD acts as a cofactor for several essential enzymes, including cyclic ADP ribose hydrolases (CD38), sirtuins (Sirts), and Poly (ADP-ribose) polymerases (PARPs) (Savarino et al., 2003).

CD38 down-modulates the binding of HIV-1 gp120 to CD4 receptor by directly binding to gp120 and its expression is highly upregulated in response to viral infections (Savarino et al., 2003).

4 Aim of the work

To date, more than 170 different types of RNA modifications have been identified (Boccaletto et al., 2022). NAD as a cap structure was detected by LC-MS analysis in *E. coli* and *S. venezuelae* in 2009 (Chen et al., 2009). Since then, it has been found in bacteria, human cells, plants, and archaea (Cahová et al., 2015; Gomes-Filho et al., 2023; Jiao et al., 2017; Wang et al., 2019). However, little is known about the function of NAD cap in the context of host-pathogen interactions. Several studies have shown that HIV-1 uses RNA modifications to regulate its gene expression during infection and replication (Fukuda et al., 2021; Ringeard et al., 2019). Furthermore, it has been proven that HIV-1 infection reduces the total amount of cellular NAD (Murray et al., 1995). Therefore, we aimed to study the relationship between viral infection and NAD capping.

The objectives of this study:

1. Prepare sufficient quantities of HIV-1 virions for subsequent RNA isolation.
2. Generate cell lines with DXO overexpression (OE) and DXO knockdown (KD).
3. Prepare sufficient quantities of control MT-4 cells and MT-4 cells under all tested conditions for RNA isolation.
4. Employ NAD CaptureSeq method for the detection of NAD-capped RNAs in control and HIV-1 infected cells.
5. Assess the levels of free NAD in both control and HIV-1 infected MT-4 cells.
6. Verify the presence of NAD-capped RNA in HIV-1 virions via LC-MS analysis.
7. Measure the amount of NAD caps in control, HIV-1 infected, DXO OE, and DXO KD MT-4 cells using LC-MS.
8. Measure the NAD cap content on U1 snRNA using LC-MS.
9. Compare the stability of the complex formed by HIV-1 RNA with TMG-capped and NAD-capped U1 snRNA.
10. Evaluate the infectivity of HIV-1 in control, DXO OE, and DXO KD MT-4 cell.
11. Examine the splicing pattern in control and infected MT-4 cells under various conditions.

5 Experimental section

5.1 Cell culture

Experiments were conducted in the Biosafety Level 3 (BSL3) laboratory. The human CD4+ T-cell line MT-4 was cultured under standard conditions. MT-4 cells were infected with a cell-free HIV-1 strain NL4-3 at an MOI of 1. The infected cultures were subsequently expanded by co-cultivation and concentrated through a cushion of 20% (wt./wt.) sucrose. These samples were utilized for my research project and for the project analysing m^1A modification in HIV-1 (Šimonová et al., 2019).

5.1.1 HIV-1 RNA digestion and LC-MS analysis

HIV-1 RNA was digested by Nuclease P1 (1 U/µg of RNA) or mock treated with water. LC-MS was performed using a Waters Acquity UPLC SYNAPT G2 instrument 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 (pH 9), and the mobile phase B of 100% acetonitrile. The detector was operated in negative ion mode.

5.1.2 Preparation of DXO KD and DXO OE cells from lentiviral vectors

The lentiviral vectors were generated using HEK293T cells by a transient three-plasmid transfection as previously described (Soneoka et al., 1995).

5.1.3 NAD/NADH measurement

NAD total levels in control, HIV-1 infected, DXO OE, and HIV-1 infected DXO OE MT-4 cells (with or without NAm supply) were assessed using the NAD/NADH Quantification Kit according to the manufacturer's instructions (Sigma-Aldrich). The absorbance was measured at 450 nm (A_{450}).

5.1.4 U1 snRNA capture and quantification

Annealing of biotinylated U1 probe to sRNA was performed by slowly decreasing temperature (from 65 °C to 25 °C). Dynabeads™ MyOne™ Streptavidin C1 (ThermoFisher Scientific) were prepared based on manufacturer protocol. The concentration of released U1 snRNA was measured on NanoDrop, and the purity of samples was validated via 12.5% PAGE.

5.1.5 Sample preparation and LC-MS measurement

RNA was washed with urea to remove non-covalently bound NAD (Frindert et al., 2018; Zhang et al., 2020). Samples were separated with HPLC (Acquity H-class, Waters). Analytes were detected using Xevo G2–XS QTof mass spectrometer (Waters) equipped with an electrospray ionization source. This part was done by M.Sc. Jiří František Potužník, M.Sc. Anton Škríba, Ph.D.

5.1.6 NAD captureSeq library preparation

NAD captureSeq libraries from four biological replicates for each sample were performed similarly as described (Cahová et al., 2015). Only a short fraction of RNA from MT-4 cells infected by HIV-1 and MT-4 cells uninfected were used for the library preparation. The most critical step is the enzyme reaction catalysed by ADPRC, an enzyme specific mainly for NAD-RNAs. Samples not treated by ADPRC were used as a negative control for non-specifically bound RNAs.

Multiplexed samples were submitted to the IMG Genomics and Bioinformatics facility for library synthesis using the NextSeq® 500/550 High Output Kit v2, 75 cycles (Illumina) and sequencing on a NextSeq 500/550, Illumina.

5.1.7 Small RNA-seq library preparation

To measure the amounts of specific sRNAs in samples, three biological replicates of sRNA from MT-4 cells and MT-4 cells infected by HIV-1 were submitted for miRNA (LP-170) library preparation to SEQme.

5.1.8 Data processing and bioinformatic analysis

All data processing and bioinformatic analysis was done by M.Sc. Lenka Gahurová, Ph.D.

5.1.9 Deep sequencing data validation through RT-PCR

RT-PCR was performed to verify the NAD captureSeq data. Three biological replicates of sRNA from infected and uninfected MT-4 cells and cDNA samples after NAD captureSeq were measured in two technical repeats on LightCycler 480 II (Roche) by Luna Universal One-Step RT-qPCR Kit according to the manufacturer protocol. The Cp values were calculated by LightCycler 480 Software, and the reciprocal values were plotted.

5.1.10 U1 snRNA and HIV-1 mRNA complex stability assay

U1 snRNA with TMG or NAD cap, and HIV-1 mRNA were mixed with ResoLight dye and annealing buffer. The technical triplicates were prepared three times and measured on LightCycler 480 II. HRM curves were obtained by measuring the complex stability by temperature increase to 80 °C and then decrease to 20 °C with a ramp rate 0.01 °C/s in three cycles and thus obtaining a total of 27 melting curves per sample. HRM curve analysis was performed using the LightCycler 480 Software.

5.1.11 U1 snRNA decapping by DXO

32P-GTP labelled U1 snRNA with either TMG or NAD cap was incubated with different concentrations of DXO decapping enzyme in decapping buffer. Reactions were stopped by the addition of RNA loading dye and loaded into the gel wells.

5.1.12 HIV-1 infectivity determination in DXO-transduced cells

The HIV-1 infectivity in DXO transduced cells was determined by measuring the HIV-1-induced cytopathic effect by XTT cell proliferation assay. Control and DXO-transduced MT-4 cells were seeded with and without NAm, infected in triplicate by two-fold serially diluted HIV-1 (except for controls) and cultured. After 4 days XTT salt and PMS electron-coupling reagent were added. The formation of orange formazan dye was measured in the EnVision plate reader at 560 nm. HIV-1 titer was calculated by the Reed-Muench method and expressed as 50% tissue culture infectious dose ($TCID_{50}$).

6 Results

6.1 Changes in NAD concentration upon HIV-1 infection

HIV-1 infection causes a decrease in cellular NAD and NADH levels, a condition known as pellagra (Murray et al., 1995). We calculated that NAD levels dropped almost fourfold, from 4.64 pmol/1000 cells (SD = 0.138) in control MT-4 cells to 1.28 pmol/1000 cells (SD = 0.025) in HIV-1 infected cells (Fig. 6).

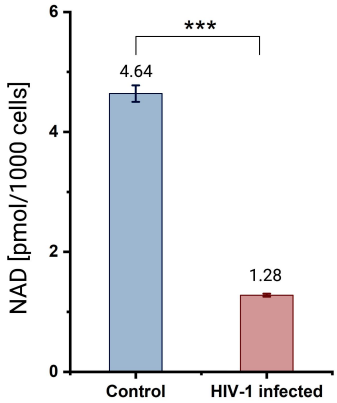

Fig. 6: Levels of free total NAD (NAD and NADH) in control and HIV-1 infected MT-4 cells. The difference between these samples is statistically significant (p-value <0.001).

6.2 Sequencing of NAD-RNAs from control and HIV-1 infected MT-4 cells

The bioinformatic analysis of NAD captureSeq libraries revealed four snoRNAs (SNORD3G, SNORD102, SNORA50A, and SNORD3B) and four snRNAs (U1, U4ATAC, U5E, and U7) which lose NAD cap upon HIV-1 infection (Fig. 7A). Using sRNA-seq we revealed that the amount of these sRNAs does not change with HIV-1 infection (Fig. 7B).

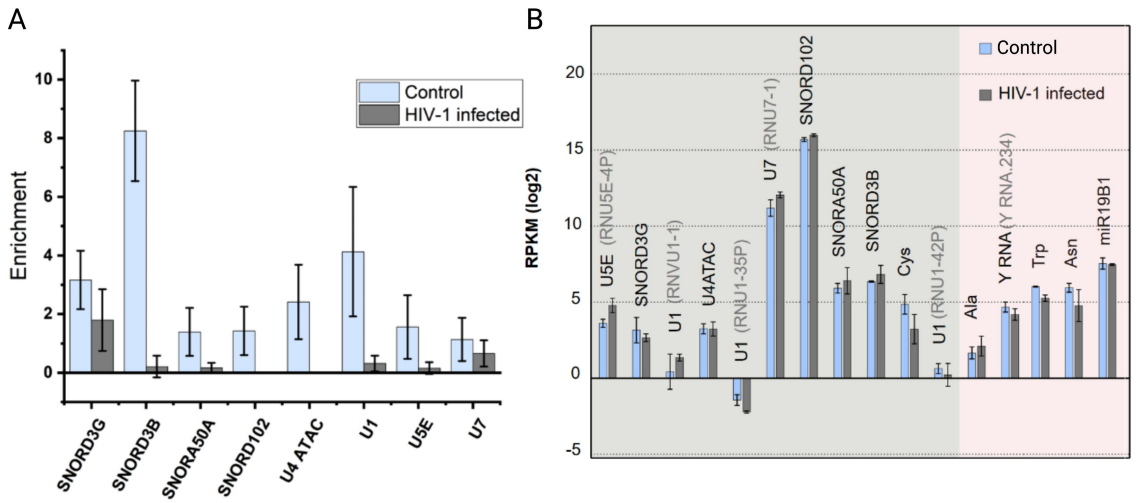

Fig. 7: Enrichment of particular sRNAs. A) Comparison of sRNAs enriched in control vs HIV-1 infected cells detected by NAD CaptureSeq. B) sRNA-seq analysis.

6.3 Evaluation of sRNAs levels by RT-PCR

We confirmed by RT-PCR that the number identified snoRNAs and snRNAs does not change with infection (Fig. 8).

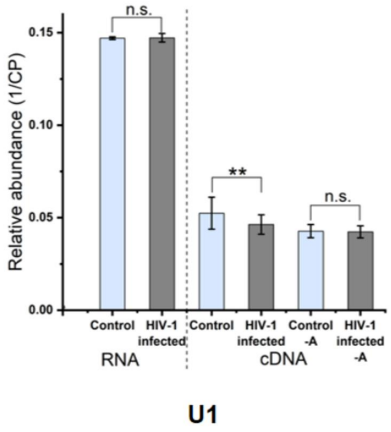

Fig. 8: Quantified RT-PCR measurements of U1 snRNAs. The first part compares sRNA abundance, the second part compares the relative abundance of cDNA obtained from NAD captureSeq control and infected samples. n.s. = not significant, * = p-value < 0.05, ** = p-value < 0.01.

6.4 Changes in NAD capping upon HIV-1 infection

We did not observe any statistical difference in NAD capping between infected and control cells in either sRNA or lRNA (Fig. 9).

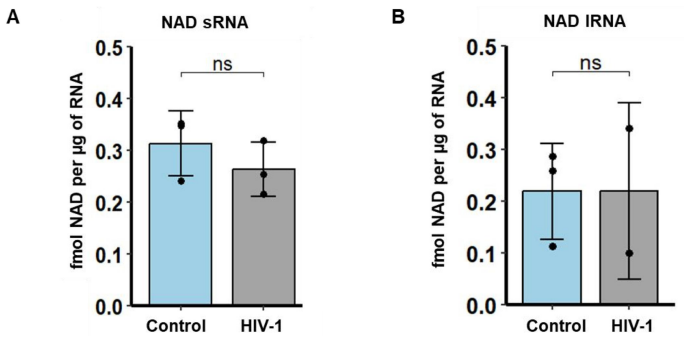

Fig. 9: Levels of NAD caps in fractions of sRNA and lRNA in control MT-4 cells and HIV-1 infected MT-4 cells.

We measured NAD-RNAs in HIV-1 virions. No peaks corresponding to NAD caps were observed suggesting that HIV-1 virions do not contain any NAD-RNAs (Fig. 10).

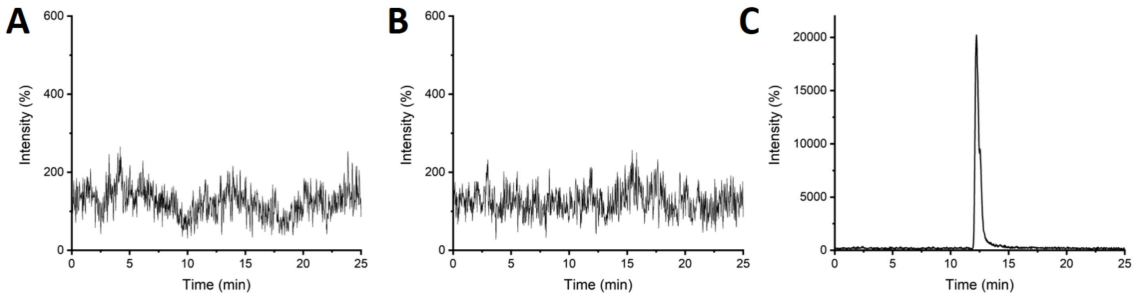

Fig. 10: EIC of NAD parent mass 664.11 from RNA isolated from A) HIV-1 virions, B) HIV-1 virions negative control (without NuP1 digestion) and C) HIV-1 virions with spiked NAD spiked.

6.5 LC-MS measurement of NAD caps on U1 snRNA

LC-MS analysis revealed a significant decrease in the NAD cap on U1 snRNA in HIV-1 infected cells (Fig. 11).

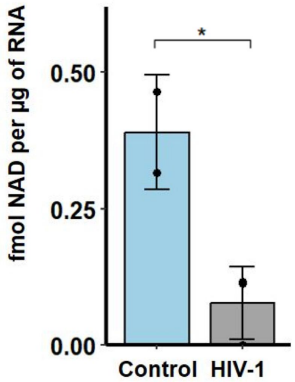

Fig. 11: Absolute quantification of NAD RNA cap in U1 pulled down from control and HIV-1 infected cells. * = p-value < 0.05.

6.6 Complex stability of U1 snRNA and HIV-1 pre-mRNA

U1 snRNA binds with a complementary region of 8 nucleotides at its 5' end to the 5' splice site of unspliced HIV-1 pre-mRNA. The HRM curve analysis revealed differences between the duplexes of HIV-1 pre-mRNA and NAD-U1 or TMG-U1. The NAD cap on U1 snRNA lowers the duplex stability (Fig. 12).

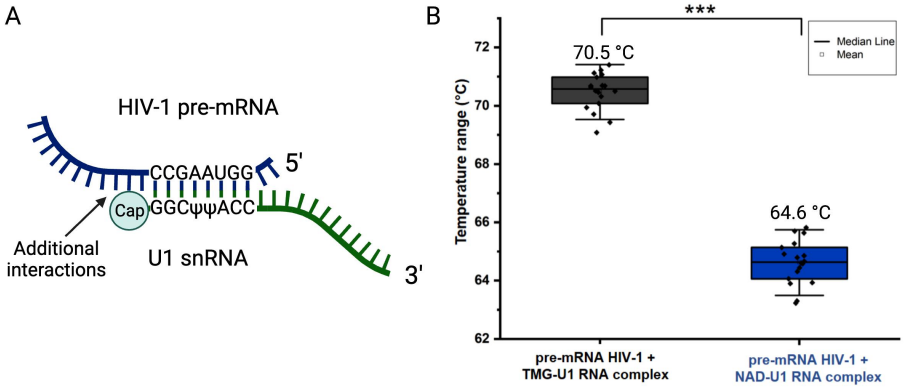

Fig. 12: Duplex stability of HIV-1 pre-mRNA and U1 snRNA (with TMG or NAD cap) measured by LightCycler. A) Schema of the prepared and annealed duplex with U1 snRNA pseudouridines at positions 4 and 5. B) Melting temperatures measured by LightCycler. *** = p-value < 0.001.

6.7 *In vitro* assessment of DXO decapping enzyme

We show that DXO preferentially decaps NAD-U1 snRNA (Fig. 13). Almost 50% of NAD-U1 snRNA was decapped by DXO after 15 minutes while only 5% of TMG-U1 snRNA was decapped.

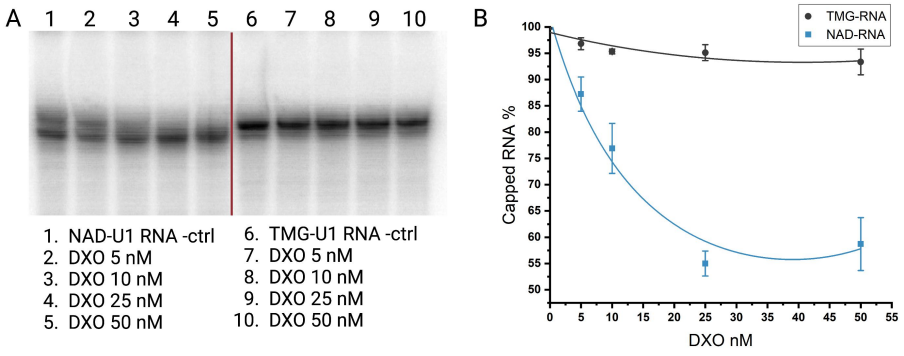

Fig. 13: Decapping of U1 snRNA by DXO enzyme. A) The scan of radioactive gel. B) Graph showing the analysed DXO cleavage of NAD-U1 and TMG-U1 at various DXO concentrations.

6.8 Comparison of DXO amounts in healthy and infected cells

By western blot analysis we revealed that the amounts of DXO in MT-4 infected cells are not elevated (Fig. 14).

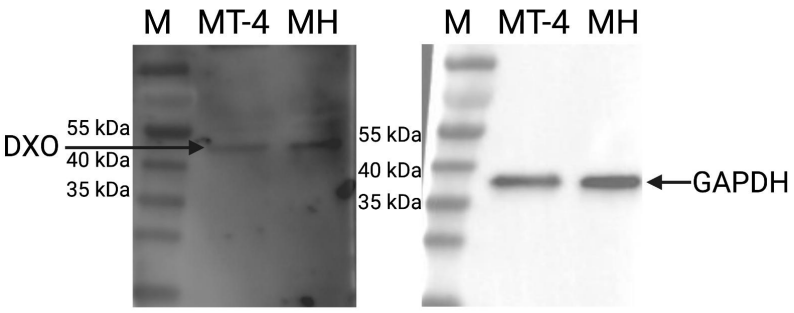

Fig. 14: Western blot analysis of DXO (44.9 kDa) and GADPH (36 kDa). The lanes were plotted, and the analysed areas of the bands were the following: DXO MT-4: 54960.170, DXO MH: 55908.877, GAPDH MT-4: 97662.437, GAPDH MH: 99765.487.

6.9 *In vivo* assessment of DXO decapping enzyme

Level of NAD capping in RNA isolated from DXO KD cells was decreased. However, the amount of NAD caps dropped drastically, more than 6-fold, in the DXO OE cells. This result confirmed that DXO functions as a deNADing enzyme in MT-4 cells, and its overexpression leads to increased NAD decapping (Fig. 15A).

DXO OE leads to statistically non-significant decrease in NAD capping of U1 snRNA, while DXO KD leads to non-significant increase in NAD capping of U1 snRNA (Fig. 15B).

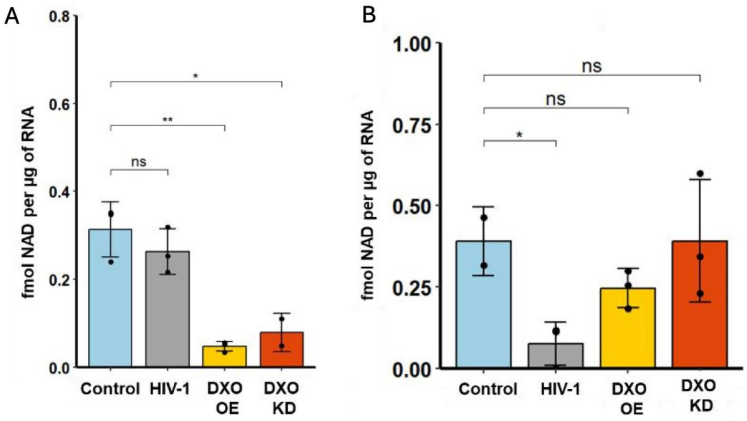

Fig. 15: A) Comparison of NAD cap levels in fraction of sRNA from control, HIV-1 infected, DXO OE, and DXO KD MT-4 cells. B) Comparison of NAD cap levels on U1 snRNA in control, HIV-1 infected, DXO OE, and DXO KD MT-4 cells. * = p-value < 0.05, ** = p-value < 0.01.

6.10 Effect of DXO decapping enzyme on HIV-1 infectivity

XTT assay was used. The viral production in DXO OE MT-4 cells was the highest, while in control MT-4 cells was the lowest. The viral production in DXO KD cells was slightly higher than in control cells but the difference was not statistically significant (Fig. 16).

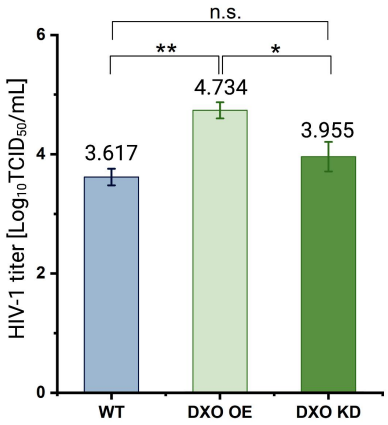

Fig. 16: Comparison of HIV-1 infectivity in WT, DXO OE, and DXO KD MT-4 cells. DXO OE significantly increased the propagation of the HIV-1 virus. n.s. = not significant, * = p-value < 0.05, ** = p-value < 0.01.

6.11 Effect of nicotinamide on HIV-1 infectivity

DXO OE, DXO OE HIV-1 infected, control, and HIV-1 infected MT-4 cells were cultured in normal and NAm supplemented media. The intracellular NAD levels were measured by NAD/NADH Quantification Kit. NAm repletion led to a significant rise of NAD levels (Fig. 17A).

The viral production in DXO OE MT-4 cells was the highest and it significantly decreased with NAm supply where it was comparable to the control cells. In wild type MT-4 cells the $TCID_{50}$ was also significantly lower after NAm supply (Fig. 17B).

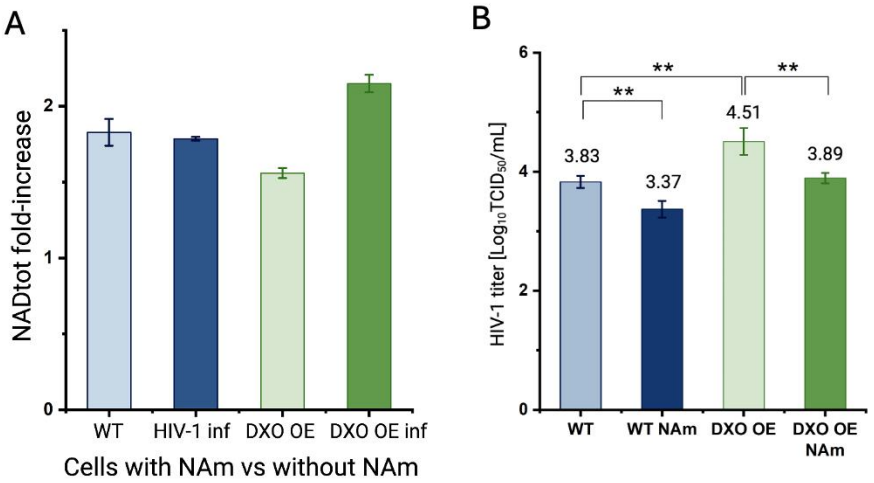

Fig. 17: A) NAD total levels. B) Comparison of HIV-1 infectivity in WT and DXO OE MT-4 cells with or without NAm supply. ** = p-value < 0.01.

7 Discussion

To date, more than 170 different types of RNA modifications have been identified across various RNA molecules (Boccaletto et al., 2022). HIV-1 uses RNA modifications, such as m^1A, to regulate its gene expression and replication (Burnett and McHenry, 1997). HIV-1 infection reduce free NAD levels in human cells (Murray et al., 1995). NAD was recently discovered to serve as a 5' non-canonical RNA cap, opening new research area in RNA biology (Chen et al., 2009). Several methods for capturing and sequencing NAD-RNAs have been developed, providing information on the types of modified RNAs (Cahová et al., 2015; Hu et al., 2021; Zhang et al., 2019). However, little is known about NAD cap role in host-pathogen interactions. Since HIV-1 is a well-studied and characterized retrovirus, we focused our research on NAD capping in the context of HIV-1 infection.

We confirmed previous findings by Murray et al. (1995) that HIV-1 infection leads to decreased NAD and NADH levels in human cells (Fig. 6). Using NAD captureSeq on sRNA isolated from control and HIV-1 infected cells, we identified four snoRNAs (SNORD3G, SNORD102, SNORA50A, and SNORD3B) and four snRNAs (U1, U4ATAC, U5E, and U7) whose NAD cap levels decreased significantly upon infection (Fig. 7).

NAD captureSeq was used in several studies. They revealed that copper, used in the library preparation, causes RNA fragmentation leading to a biased 5' end analyses and underestimating of the NAD-RNA number (Zhang et al., 2020). Furthermore, ADPRC is a promiscuous enzyme and can interact with m^7G-RNA to a certain degree (Sharma et al., 2023). Nevertheless, we studied the regulatory snRNAs and snoRNAs whose length is under 200 nt, and their fragmentation is much less probable than that of mRNA. Moreover, m^7G cap is on eukaryotic mRNAs while snRNAs have TMG as canonical cap and snoRNAs are intron-derived.

We provided evidence that the amounts of NAD caps are changing while the number of the identified NAD-RNAs are stable by two methods: sRNA-seq (Fig. 7) and RT-PCR (Fig. 8). Interestingly, the snoRNAs have processed 5' ends as they are intron-derived. It was initially thought that NAD cap could be only incorporated into RNA during transcription initiation by RNA polymerase (Bird et al., 2016). Our study brings additional proof that RNAs can be capped with NAD after transcription initiation and

that by yet unknown mechanism.

We investigated potential differences in NAD capping between control and HIV-1 infected cells by LC-MS. There was no statistical difference in NAD-lRNA levels from infected and control cells. We observed only statistically nonsignificant decrease in NAD-sRNA upon HIV-1 infection (Fig. 9). This could be due to similar quantities of sRNAs being enriched or depleted in the NAD captureSeq data. Other possibility is that HIV-1 modulates NAD capping only in a small subset of RNAs.

U1 snRNA was pulled down from control and HIV-1 infected cells and measured by LC-MS. The analysis revealed a significant decrease in the NAD cap in U1 snRNA upon HIV-1 infection (Fig. 11). These results support the findings of NAD captureSeq and formed the basis of a search for the NAD cap function. U1 snRNA binds to the 5' splice site to define the intron sequence of HIV-1 pre-mRNA (Knoepfel et al., 2012; Mandal et al., 2010; Sajic et al., 2007). Therefore, we studied the stability of the HIV-1 pre-mRNA and U1 snRNA duplex. We revealed that NAD cap in U1 snRNA causes lower stability of the complex with HIV-1 pre-mRNA (Fig. 12).

DXO is a deNADding enzyme that modulates cellular levels of NAD-RNAs (Jiao et al., 2017). We confirmed the ability of the DXO to remove the NAD cap from U1 snRNA, and we proved that the NAD cap is its preferred substrate in comparison to the TMG cap (Fig. 13). Furthermore, we showed that the amounts of DXO do not change upon HIV-1 infection (Fig. 14).

We measured the NAD capping in DXO transduced cells by LC-MS. Surprisingly, DXO KD led to lower number of NAD caps in comparison to the control (Fig. 15A). This discrepancy can be explained by the activity of other NAD decapping enzymes in human cells: Nudt12, Nudt16, Xrn1 or other unknown (Grudzien-Nogalska et al., 2019; Sharma et al., 2020, 2022), which could become more prominent in the absence of DXO. DXO OE led to significantly lower amounts of NAD caps in the sRNA fraction. We were interested in whether altering DXO levels influence NAD capping specifically of U1 snRNA. DXO KD led to statistically nonsignificant increase, while DXO OE to statistically nonsignificant decrease in NAD capping of U1 snRNA, suggesting that U1 snRNA is not its predominant

substrate (Fig. 15B).

We hypothesised that NAD caps are undesirable for HIV-1 replication and that excessive NAD cap removal might lead to increased proliferation of HIV-1 virus. Indeed, our data showed that DXO OE causes greater HIV-1 infectivity in MT-4 cells (Fig. 16). HIV-1 infectivity in DXO KD cells was slightly higher than in control cells, although not significantly. We proved that the addition of nicotinamide to the MT-4 cells increases the cellular levels of NAD (Fig. 17A). We demonstrated that increased NAD cellular levels decrease HIV-1 infectivity. In DXO OE cells, the addition of nicotinamide reduced HIV-1 infectivity to levels comparable to those observed in the control cells (Fig. 17B).

Overall, our findings imply that NAD cap might play a role in antiviral defence. Possible explanation could be that the NAD cap in U1 snRNA has a destabilising effect on the complex with HIV-1 pre-mRNA, which is then not protected and cannot be exported from the nucleus by Rev protein. Instead, such RNA is retained in the nucleus and further spliced or even degraded. This leads to reduced production of viral gRNA, and Gag and GagPol polyproteins, and decreased assembly and proliferation of HIV-1 virions. Nevertheless, this is a very simplified explanation, and further studies would be required to determine other factors involved in the decreased HIV-1 infectivity.

8 Conclusion

To date, the studies have focused on the effect of NAD cap on mRNA half-lives and translation efficiency. In our research, we were interested in more specialised roles that NAD cap may have. Therefore, we investigated the NAD capping in a more particular model system, human cells infected by HIV-1. We focused our attention on regulatory sRNAs that lose NAD cap upon HIV-1 infection.

We demonstrate that HIV-1 infection impacts both the total cellular NAD pool and NAD capping of specific snRNAs (U1, U4ATAC, U5E, and U7) and snoRNAs (SNORD3G, SNORD102, SNORA50A, and SNORD3B). U1 snRNA is particularly interesting as it has two roles in the HIV-1 replication cycle: defining the intron sequence for splicing and stabilising HIV-1 pre-mRNA retained in the nucleus. Both processes are dependent on the strength of the bond between U1 snRNA and HIV-1 pre-mRNA. We show that NAD-U1 snRNA has a destabilising effect on this interaction, implying that NAD cap may affect HIV-1 replication and overall HIV-1 infectivity.

We provide several lines of evidence which are consistent with this hypothesis. In our DXO transducing experiments, we show that DXO overexpression leads to decreased amounts of NAD caps on sRNAs and increased HIV-1 infectivity. We demonstrate that NAD repletion in the cytoplasm leads to decreased HIV-1 proliferation. Importantly, we prove that HIV-1 infection leads to reduced NAD capping of U1 snRNA, which might be part of the virus's strategy to overcome the unfavorable effect of NAD cap.

In conclusion, this study uncovered the role of NAD cap in U1 snRNA in HIV-1 infection: affecting the stability of the complex of HIV-1 mRNA and NAD-U1 snRNA, and decreasing HIV-1 infectivity.

Contribution of the author

In the study titled "LC/MS analysis and deep sequencing reveal the accurate RNA composition in the HIV-1 virion" (Šimonová et al., 2019) I contributed by preparing the HIV-1 material at the BSL 3 laboratory. This involved producing the HIV-1 virus through infection of MT4 cells, harvesting the supernatant from infected cells, and purifying the viral particles on a sucrose cushion. The material I prepared was then used for LC-MS analysis and deep sequencing, which helped uncovering the RNA composition and modifications within the HIV-1 virions.

In the study titled: "Honeybee Iflaviruses Pack Specific tRNA Fragments from Host Cells in Their Virions" (Šimonová et al., 2022) I contributed by isolating RNA from honeybee samples and conducting Northern blot analyses using this RNA. Through this process, I learned how to perform Northern blot techniques. In this work were identified specific tRNA fragments within the virions of sacbrood virus (SBV) and deformed wing virus (DWV). The Northern blot analyses revealed that while full-length tRNAs were present in honeybee RNA, only short fragments of these tRNAs were present in viral RNA. This finding demonstrated the selective packaging of tRNA fragments by these iflaviruses.

9 List of publications

9.1 Publications related to the dissertation

Benoni B., Potužník J. F., Škríba A., Benoni R., Trylcova J., Tulpa M., Spustová K., Grab K., Mititelu M.B., Pačes J., Weber J., Stanek D., Kowalska J., Bednarova L., Keckesova Z., Vopalensky P., Gahurova L. and Cahova H. "HIV-1 infection reduces NAD capping of host cell snRNA and snoRNA". ACS Chemical Biology 2024 19 (6), 1243-1249.

IF(2024) = 3,5

Šimonová A., **Svojanovská, B.**, Trylčová J., Hubálek M., Moravčík O., Zavřel M., Pávová M., Hodek J., Weber J., Cvačka J., Pačes J., Cahová H. "LC/MS analysis and deep sequencing reveal the accurate RNA composition in the HIV-1 virion." Scientific Reports 2019 9(1): 8697.

IF(2019) = 3,99

9.2 Other publications

Šimonová A., Romanská V., **Benoni, B.**, Škubník K., Šmerdová L., Procházková M., Spustová K., Moravčík O., Gahurova L., Pačes J., Plevka P., Cahová H. "Honeybee Iflaviruses Pack Specific tRNA Fragments from Host Cells in Their Virions." ChemBioChem 2022 23(17): e202200281.

IF(2022) = 3,2

Lobellova V., Entlerova M., **Svojanovská, B.**, Hatalova H., Prokopova I., Petrasek T., Vales K., Kubik S., Fajnerova I., Stuchlik A.. "Two learning tasks provide evidence for disrupted behavioural flexibility in an animal model of schizophrenia-like behaviour induced by acute MK-801: a dose-response study." Behavioural Brain Research 2013 Jun 1;246:55-62.

IF(2013) = 3,39

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