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PNNL-38806

Development of high throughput and in vitro assays for analyzing RNA modifications

December 2025

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U.S. DEPARTMENT of ENERGY

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Abstract

Modifications on RNAs play major roles in their stability, translation, and enzymatic activity. Despite its importance, the current techniques are insufficient to study the structure and function of RNA modifications. Indeed, the National Academies of Science, Engineering and Medicine indicate that developing new tools and further study the function of RNA modifications is strategically a high priority for advancing science in the coming years (https://www.nationalacademies.org/our-work/toward-sequencing-and-mapping-of-rna-modifications). RNA modifications occur in all domains of life controlling processes such as RNA turnover, translation regulation, cellular defenses and bioproduction. Our preliminary data indicated that the insulin mRNA might get ADP-ribosylated by the ADP-ribosyltransferase PARP12. RNA ADP-ribosylation has been described in *Escherichia coli*. Combined to the fact that ADP-ribosyltransferase (PARP) genes are conserved throughout evolution we hypothesize that this modification might play essential roles in cells. Therefore, we proposed to develop sequencing techniques and *in vitro* enzymatic assays to identify and validate ADP-ribosylation motifs and sites. Here we report the development of RNA-seq and qPCR assays to identify ADP-ribosylated RNAs, in addition to a nicotinamide adenosine dinucleotide (NAD – ADP-ribosylation donor) consumption assay and an enzyme-linked immunosorbent assay (ELISA) to measure ADP-ribosyltransferase activity. Testing these assays with the insulin mRNA confirmed that this transcript is ADP-ribosylated. These assays will not only enable studying the function of ADP-ribosylation but can be easily adapted for studying other RNA modifications. This will open opportunities to study RNA modifications in different model systems from bacteria to viruses to plants, bringing insights into their cellular functions and the possibility of targeting them for biotechnological applications.

Summary

ADP-ribosylation was recently identified as a major modification of the cell transcriptome. There are only a few known functions known for this RNA modification due to the lack of assays to characterize it. In this project, we have developed a high throughput method to systematically identify RNAs that are ADP-ribosylated and in vitro assays to measure the ADP-ribosylation activity. Using the ADP-ribosylation binding domain AF1521, we captured ADP-ribosylated RNAs and submitted to RNA-seq. This analysis led to the identification of 1381 ADP-ribosylated RNAs, including 150 that are modified by the ADP-ribosyltransferase PARP12. This affinity capture allowed us also to perform qPCR analysis and validate the ADP-ribosylation of the insulin mRNA. *In vitro*, we measured the ADP-ribosylation of an oligoribonucleotide by measuring the consumption of the ADP-ribosylation donor, nicotinamide adenosine dinucleotide (NAD). Moreover, we partially developed an enzyme-linked immunosorbent assay (ELISA), consisting of ADP-ribosylating oligoribonucleotide immobilized into 96-well plates and measuring the modification levels using antibodies. Using the insulin mRNA, we have demonstrated that these assays allow identify and validate ADP-ribosylated RNAs. The *in vitro* assays also open opportunities to screen for ADP-ribosyltransferase inhibitors. In summary, we believe that the assays developed in this LDRD will contribute to characterizing the function of RNA ADP-ribosylation in a variety of model systems.

Acknowledgments

This research was supported by the Earth and Biological Sciences Directorate Mission Seed, under the Laboratory Directed Research and Development (LDRD) Program at Pacific Northwest National Laboratory (PNNL). PNNL is a multi-program national laboratory operated for the U.S. Department of Energy (DOE) by Battelle Memorial Institute under Contract No. DE-AC05-76RL01830.

Acronyms and Abbreviations

ADP – adenosine diphosphate
ELISA - enzyme-linked immunosorbent assay
NAD – nicotinamide adenosine dinucleotide
PARP – ADP-ribosyltransferase or poly(ADP-ribose)polymerase
PDB – Protein Data Bank
rPARP12 – recombinant PARP12 protein
WWE – conserved protein domain containing tryptophan-tryptophan-glutamate
ZnF – zinc finger domain

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1.0 Introduction

RNAs serve as intermediate templates from DNA to synthesize proteins. RNAs also regulate cellular splicing and translational machinery, and many have enzymatic activity (Crick 1970). RNAs can be modified by over 170 chemical post-transcriptional modifications, which regulate their stability, splicing, translation, cellular localization, and cellular responses (Roundtree et al. 2017; Cappannini et al. 2024). For instance, N6-methyladenosine impacts RNA metabolism, enhancing mRNA stability and translation efficiency (Meyer and Jaffrey 2014). Pseudouridine stabilizes rRNA and tRNA structures, while 2'-O-methylation ensures the fidelity of protein synthesis (Ge and Yu 2013; Dimitrova, Teysset, and Carre 2019; Li, Xiong, and Yi 2016). These modifications enable cells to swiftly adapt to changing environments and stressors by dynamically remodeling the transcriptome. The use of RNA modification for stabilizing and preventing excessive immune response enabled a fast development of mRNA immunotherapies. RNA modifications have been targeted in other fields too. RNA demethylation regulated for increasing the yield and biomass of rice and potato plants (Yu et al. 2021). Due to the importance of RNA modifications across health, biotechnology, and the environment, combined with a lack in analytical methods, the National Academies of Science, Medicine and Engineering as an area for high priority in investment for the next decade as the new frontier of Science.

Our preliminary data indicated that the insulin mRNA might be ADP-ribosylated. ADP-ribosylation is the addition of adenosine diphosphate (ADP) ribosyl from nicotinamide adenosine dinucleotide (NAD) to macromolecules by ADP-ribosyltransferases (ADRTs, more commonly known as PARPs – poly(ADP)ribosyl polymerases) (Dasovich and Leung 2023). ADP-ribosylation is a modification commonly found in proteins and DNA (Dasovich and Leung 2023). Thus far, only two RNAs from bacteriophages in *E. coli* have been shown to be ADP-ribosylated (Vassallo, Doering, and Laub 2024). As uncharacterized PARP genes are present in genomes from organisms across all domains of life (Perina et al. 2014), we hypothesize that many of these genes might be of RNA PARPs and that RNA ADP-ribosylation are evolutionarily conserved and have a broad impact. However, the main challenge to studying RNA ADP-ribosylation modification is the lack of high throughput technologies for identifying and quantifying RNA ADP-ribosylation sites. In this LDRD project, we addressed this challenge by capturing ADP-ribosylated RNAs using an affinity resin and sequencing the captured RNAs by RNA-seq or amplifying by PCR. We also developed NAD consumption assay and an enzyme-linked immunosorbent assay (ELISA) to measure ADP-ribosyltransferase activity. The methods developed in this project will open opportunities for exploring the functions of RNA ADP-ribosyltransferases in a variety of model systems.

2.0 Material and Methods

2.1 Prediction of RNA binding to PARP12

Binding of mouse insulin 2 pre-mRNA with PARP12 protein was predicted with RPISeq (http://pridb.gdcb.iastate.edu/RPISeq/index.html). Combinations of three zinc finger RNA-binding domains (ZnF1-3) were tested. Binding affinities were predicted for 50-nucleotide segments of mRNA with 10-nucleotide overlapping in both sides.

2.2 Prediction of PARP12 structure

The interaction strength of Ins2 pre-mRNA with PARP12 was calculated using the RPISeq program (http://pridb.gdcb.iastate.edu/RPISeq/index.html). For PARP12, we used sequences combinations of three zinc finger (ZnF1-3) RNA-binding domains. The interaction strength was predicted in 50-nucleotide segments of Ins2 pre-mRNA with 10-nucleotide overlapping in both ends.

2.3 Cell Culture

MIN6 cells were maintained in DMEM containing 4.5 g/L each of D-glucose and L-glutamine, 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 50 mM 2-mercaptoethanol under 5% CO_2 atmosphere at 37 ºC. Gene knockdown was performed on cells at 80% confluency with *Parp12* siRNA (Dharmacon, L-065127-01-0020) or non-targeting siRNA (Dharmacon, D-001810-10-20) with Lipofectamine RNAiMAX (Invitrogen, 13778150). Cells were treated simultaneously with 100 ng/mL IFN-γ (R&D, 485-MI-100), 10 ng/mL TNF-α (R&D, 410-MT-010), and 5 ng/mL IL-1β (R&D, 401-ML-005) for 24 h.

2.4 ADP-ribosylation RNA pulldown

RNAs from cells were extracted with Tri reagent (Zymo Research, R2050-1-200) and RNA Clean & Concentrator™-5 kit (Zymo Research, R1014). Aliquots of RNAs were treated with 2 nM SARS-CoV2 macrodomain MAC1 at 21 ºC for 1 h in 250 mM HEPES, 750 mM NaCl, 10 mM β-mercaptoethanol, 0.05 mM Triton X100, 5 mM MgCl2, 1X RNAsecure to remove ADP-ribosylation. The de-ADP-ribosylated aliquots were used to test the specificity of the RNA pulldown. RNA aliquots were incubated with 20 µL magnetic beads either conjugated to wild-type (Tulip Biolabs, 2305) or mutant (unable to bind to ADP-ribosylation) Af1521 macrodomain (Tulip Biolabs, 2306) in 600 µL 10 mM Tris-HCl, 0.6 M NaCl, 0.1% NP40, 1X RNA secure overnight at 4 ºC. Beads were then washed with same buffer three times and RNAs were extracted with Tri reagent and RNA Clean & Concentrator™-5 kit.

2.5 Quantitative RT-PCR analysis

RNAs were quantified with StepOnePlus RTPCR systems using QuantiNova™ SYBR Green RT-PCR reagent (Qiagen, 208154) using primers from IDT and Millipore sigma (KSPQ12012). Rlp13 and Nono were used as housekeeping genes and fold changes were calculated using the Livak's method (Sarkar et al. 2024; Livak and Schmittgen 2001).

2.6 RNA sequencing

RNAs were converted into cDNAs using SMARTer® Universal Low Input RNA Kit (Takara, 634938) and barcoded/made into a sequencing library using NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (New England Biolabs, E7805S). The library was sequenced on a NextSeq500/550 High Output v2.5 (20024907). The quality of the sequences was checked using FastQC (Version 0.12.0). Barcode and primer sequences were trimmed with BBDuk and sequences were aligned to the mouse genome GRCm39 using STAR (Dobin et al. 2013). ComBat-Seq (Zhang, Parmigiani, and Johnson 2020) was used for batch correction and differential expression analysis was done using DESeq2 (Love, Huber, and Anders 2014).

2.7 NAD consumption assay

This assay was designed to measure ADP-ribosylation by the consumption of its donor, NAD. In each well of a 96-well plate, 200 nM NAD (Sigma) was incubated with 200 nM oligoribonucleotide (IDT) and 4 ng of recombinant PARP12 protein (Cedarlane, CSB-EP875642HU) for 30 min at 30 °C in PARP reaction buffer (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 5 mM $MgCl_2$, 1X RNAsecure™ RNase Inactivation Reagent (Invitrogen) and QS RNAse/DNAse free Water). The remaining NAD was measured with NAD/NADH-Glo kit (Promega, G9071) with a plate reader (Synergy HT, BioTek).

2.8 ELISA assay

The streptavidin-coated 96-well plate (Thermo Scientific) was blocked overnight at 4 °C with blocking buffer (RNAse/DNase-free PBS and 3% BSA + 2 mM Ribonucleoside Vanadyl Complex, Millipore Sigma). One hundred nanomolar of biotinylated oligoribonucleotide suspended in RNase/DNase-free PBS were incubated on the pre-blocked streptavidin-coated plate for 1 h at room temperature. Unbound oligoribonucleotide was washed with wash buffer (1X RNAse/DNAse free PBST), and ADP-ribosylation reaction was carried out by incubating each well with 25 μM NAD and 0.5 μg of recombinant PARP12 protein in PARP reaction buffer for 30 mins at 30 °C. Plates were washed with wash buffer and incubated with anti-ADP-ribosylation rabbit monoclonal antibody (Cell Signaling Technology, 83732) diluted 500-fold in blocking buffer for 1 h at room temperature. Plates were washed with wash buffer and incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (Cell Signaling, 7074S) at a 3000-fold dilution in blocking buffer for 1 h at room temperature. Reactions were developed with SuperSignal™ ELISA Pico Chemiluminescent Substrate (Thermo Scientific) and measured on a plate reader (Synergy HT, BioTek).

3.0 Results

3.1 Insulin mRNA ADP-ribosylation

We examined if the PARP12 structure to determine what kind of substrate it would support. A crystallography-determined catalytic domain was available in Protein Data Bank (6V3W), while the remaining protein composed of two WWE (tryptophan-tryptophan-glutamate) domains, and four zinc finger domains (ZnF) were predicted by AlphaFold (AF-Q8BZ20-F1) (**Figure 1A**). The ZnFs can bind to either DNAs or RNAs (Cassandri et al. 2017), but PARP12 structure would not accommodate a nucleotide double helix, making RNA the best fit. Because PARP12 expression is highly induced by cytokines in β cells, we hypothesize it could target the insulin mRNA, as this is the most abundant transcript in this cell. We then aimed to identify regions of highest binding probability by dividing mouse insulin 2 mRNA into 27 oligonucleotide sequences of 50 nucleotides with 10 overlapping nucleotides on each side and submitted each to a binding prediction. Oligonucleotide 19 (INS19) had the highest binding prediction and corresponded to a region in intron 2 of insulin 2 mRNA (**Figure 1B**). We then positioned the oligonucleotide with the highest predicted binding affinity onto the PARP12 structure. This prediction analysis showed that the PARP12 structure can accommodate RNA molecules in proximity to its catalytic site (**Figure 1A**), indicating this enzyme may ADP-ribosylate transcripts such as insulin 2. To determine if the insulin 2 mRNA gets ADP-ribosylated, we extracted RNAs from MIN6 β cells treated or not with the cytokines IFNγ + IL-1β + TNFα to induce PARP12 expression. We then developed an ADP-ribosylation RNA enrichment method with AF1521 ADP-ribosylation binding macrodomain conjugated to magnetic beads. Extracted RNAs were incubated with the magnetic beads, which were then extensively washed and the enriched RNAs were eluted by extraction (**Figure 1C**). A qPCR analysis showed amplification of insulin 2 mRNA in both untreated and cytokine-treated cells, while the control beads only showed trace amplification (**Figure 1D**). We tested the pull down specificity by pre-treating RNA aliquots with SARS-CoV2 macrodomain MAC1 to remove ADP-ribosylation (**Figure 1C**), leading to 94% to 99% reduction in insulin 2 mRNA amplification (**Figure 1D**). Therefore, the SARS-CoV2 macrodomain MAC1 treatment indicates that the AF1521 magnet bead pull down is specific for ADP-ribosylated RNAs. Overall, these results indicate that (1) PARP12 is a possible RNA ADP-ribosyltransferase, (2) the insulin 2 mRNA is ADP-ribosylated, and (3) AF1521 conjugated magnetic beads are effective to pull down ADP-ribosylated RNAs.

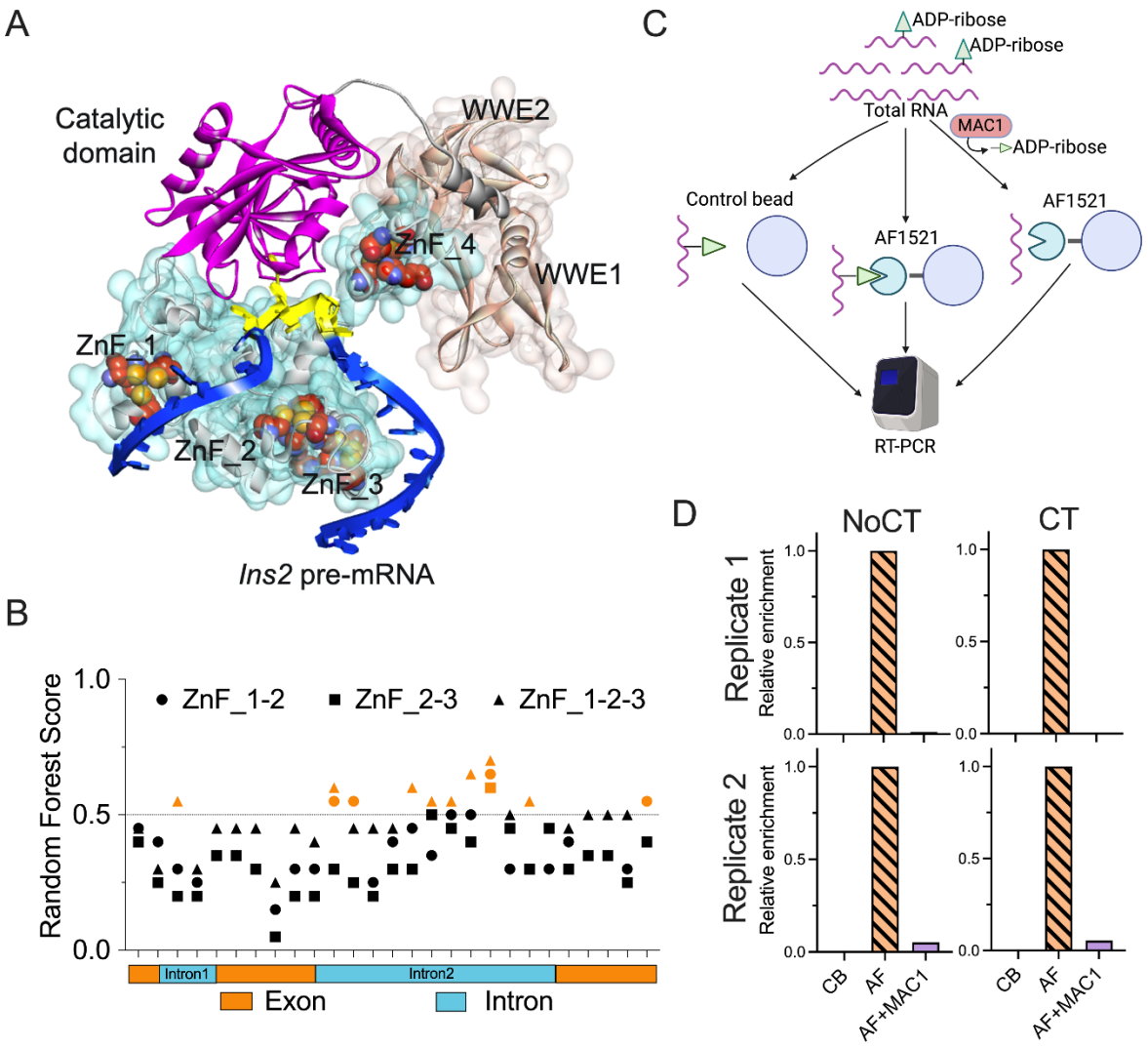

Figure 1. Insulin mRNA ADP-ribosylation. (A) Predictive complex structure of PARP12 and Ins2 mRNA. Four zinc finger motifs of PARP12 are highlighted by Corey–Pauling–Koltun representation. The yellow section of mRNA represents the potential ADP-ribosylation site as near the catalytic domain of PARP12. (B) Prediction of RNA-protein interactions was conducted using RPISeq. The random forest values for 27 segments of the insulin 2 mRNA were calculated against three different combinations of zinc finger domains of PARP12. Predictions probabilities greater than 0.5 were considered positive. (C) Scheme representing ADP-ribosylated RNA pulldown experimental design. (D) ADP-ribosylated RNA with/without MAC1 treatment were pulldown using AF1521 (AF) or naked-control beads (CB). Amplification was calculated against 0.3% Input and represented as relative enrichment to AF. CT- cytokines IFNγ + IL-1β + TNFα.

3.2 ADP-ribosylated RNA sequencing

To identify ADP-ribosylated RNAs we pulled them down with AF1521 beads and submitted them to RNAseq, leading to the identification of 1381 transcripts that were higher in AF1521 vs. controls beads, therefore, they were considered ADP-ribosylated RNAs. We also looked for RNAs that could be ADP-ribosylated by PARP12 by pulling down and sequencing RNAs from cells submitted

to Parp12 RNAi and treated with the cytokines IFNγ + IL-1β + TNFα. This analysis led to the identification of 150 RNAs that were significantly downregulated in PARP12 knockdown cells (**Figure 2A**), therefore, are potential PARP12 substrates. PARP12 knockdown led to a 63% reduction in insulin 2 ADP-ribosylation in cells treated with CT1 but no significant changes were observed in untreated cells (**Figure 2B**). These results show that ADP-ribosylation is a major RNA modification and that PARP12 is an RNA ADP-ribosyltransferase that modifies many transcripts, including the insulin 2 mRNA.

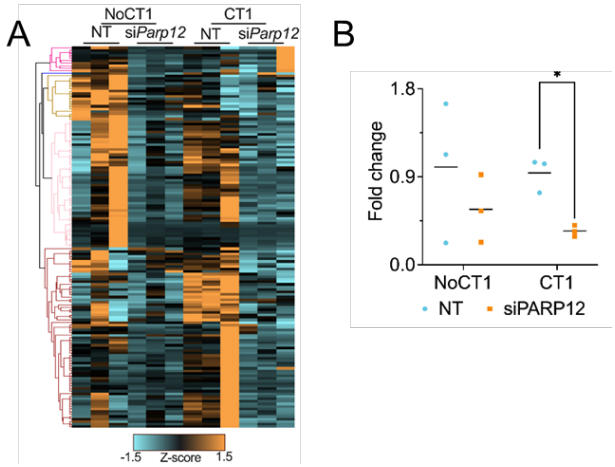

Figure 2. Identification of ADP-ribosylated RNAs in MIN6 cells (n=3). (A) Heatmap of RNA ADP-ribosylation potentially catalyzed by PARP12. (B) Quantification of the insulin 2 mRNA from the RNA-seq data. Abbreviations: CT1 – cytokine cocktail composed of IFNγ + IL-1β + TNFα, noCT – untreated control, siParp12 – small interference RNA knockdown of Parp12 gene.

3.3 Measuring PARP enzymatic activity by NAD consumption assay

We designed this assay based on the consumption of the ADP-ribosylation donor NAD (**Figure 3A**). In the assay, we incubated NAD with the oligoribonucleotide to be tested and a recombinant PARP12 protein. We tested two oligoribonucleotides from the insulin gene, the ones predicted to have the lowest (INS8) and the highest (INS19) binding to PARP12. As expected, INS19 led to a robust consumption of NAD, INS8 had a minor consumption that was not significantly different from the control without the oligoribonucleotide substrate (**Figure 3B**). This result shows a successful implementation of an in vitro ADP-ribosylation assay.

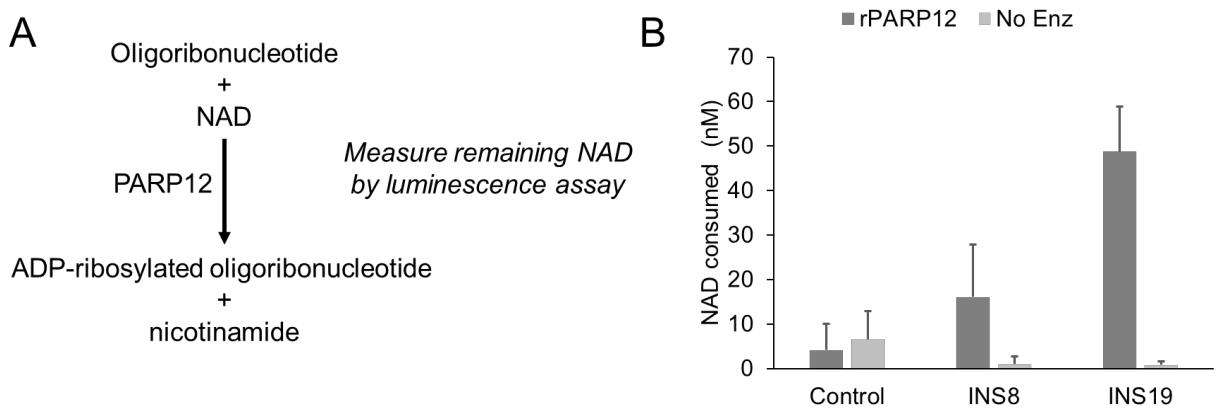

Figure 3. PARP12 enzymatic activity measurement by NAD consumption assay. (A) Reaction mechanism of the assay. (B) Testing the assay with oligoribonucleotides from the insulin gene sequence (n=2). Oligonucleotide INS8 was predicted to have a low binding affinity to PARP12, whereas INS19, high affinity (Figure 1B). Abbreviations: No Enz – reaction control without enzyme, rPARP12 – recombinant PARP12 protein.

3.4 Measuring PARP enzymatic activity by ELISA

We designed a second assay based on measuring the ADP-ribosylated oligoribonucleotides by ELISA. In this assay, biotinylated oligoribonucleotides are captured on 96-well plates coated with streptavidin. The reaction with NAD and recombinant PARP12 protein is done in plate and measured by antibodies against ADP-ribosylation (**Figure 4A**). In this assay as well, we observed an increased ADP-ribosylation with INS19 but no enzyme or no oligo controls (**Figure 4B**). However, the assay still presents a high background level that we were unable to solve until the end of the period of performance of this LDRD project. Therefore, despite promising initial results this assay requires further refinement.

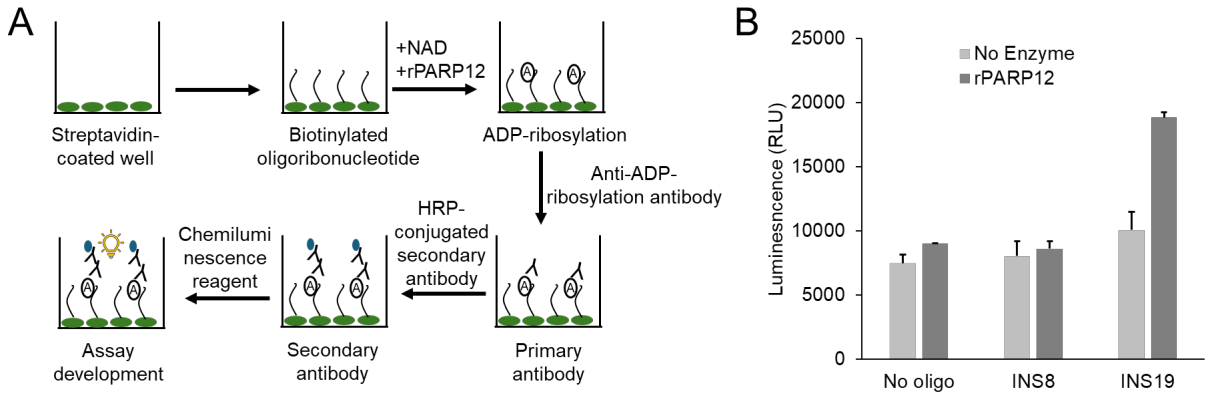

Figure 4. Enzyme-linked immunosorbent assay (ELISA) for measuring ADP-ribosyltransferase (PARP) activity. (A) Reaction mechanism of the assay. (B) Testing the assay with oligoribonucleotides from the insulin gene sequence (n=2). Oligonucleotide INS8 was predicted to have a low binding affinity to PARP12, whereas INS19, high affinity

(Figure 1B). Abbreviations: No Enzyme – reaction control without enzyme, rPARP12 – recombinant PARP12 protein.

4.0 Discussion

We developed a method to analyze ADP-ribosylated RNAs by sequencing and another by qPCR. In common both methods use pulldown of ADP-ribosylated RNAs with the AF1521 ADP-ribosylation-binding domain. While AF1521 has been used to pull down ADP-ribosylated proteins and DNA, the adaptation of the method for RNAs required several details. Unlike proteins and DNA that are more stable, RNAs are very easily degraded (Mathay et al. 2012). Therefore, the method required the use of RNAse-free materials, preparation of buffers in RNAse-free water, or even the addition of RNAse inhibitors. Once the ADP-ribosylated RNA is captured, it can be analyzed by conventional PCR and sequencing platforms. We validated both methods by analyzing the insulin 2 RNA. The sequencing analysis also revealed that cellular ADP-ribosylated transcriptomes are comprehensive and modify more than 1300 RNAs. Moreover, we used the platform to identify substrates of a specific RNA ADP-ribosyltransferase, PARP12. The analysis led to the identification of 150 potential PARP12 substrates, which indicates that there might be other ADP-ribosyltransferases that also modify RNAs.

We also developed two in vitro methods to measure ADP-ribosyltransferase activity. In the first method, the approach was to measure the amount of consumed NAD (the donor molecule of ADP-ribosylation) in the enzymatic reaction. This method is straightforward and performed on 96-well plates. Therefore, it can be used not only to study enzyme specificity but can also be adapted to high throughput screening of inhibitors, for example. An important variable of this method is to use the appropriate amounts of oligoribonucleotide, NAD and enzyme. The oligoribonucleotide is probably the least critical of the 3 molecules of the reaction. However, it should not be less than the NAD amounts. The used NAD needs to be in the linear range of the NAD assay kit. Therefore, we have used 200 nM, which is in middle point of the assay linear range. Similarly, the enzyme amount is critical. We recommend no more than 1% of the oligoribonucleotide amount as high concentrations of the enzyme can reduce its specificity. We also partially developed an ELISA assay to measure RNA ADP-ribosylation. Despite the promising initial results, the assay still needs optimization to become robust for routine analysis. The most critical part is to determine the right amounts of each reagent to be added to the reaction. Once the assay is optimized it can be applied to several applications, including validation of ADP-ribosylation sites, investigation of ADP-ribosyltransferase specificity and screening for inhibitors.

In summary, we developed assays to study RNA ADP-ribosylation. The assays allow to perform high throughput identification of ADP-ribosylation sites as well as validate those sites. We believe this will open opportunities for in-depth studying the roles of RNA ADP-ribosylation in cells. Moreover, these methods can be easily adapted to study other RNA modifications that have available affinity reagents.

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