Fluorescent probe displacement assays reveal unique nucleic acid binding properties of human nudix enzymes

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1. Introduction

This study reports the development of new fluorescence based nucleic acid binding assays designed to analyze the interaction between Nudix enzymes and their ligands. The E. coli genome encodes 13 Nudix proteins [1,2], and the human genome encodes 22 Nudix proteins [3]. A subset of these Nudix proteins have been shown to bind nucleic acids, and some of these are thought to remove caps from RNA 5’ ends [4]. However, how these relatively small proteins identify certain nucleic acid targets is still unclear. This report details methods using fluorescently labeled oligonucleotide probes to measure the affinity of Nudix hydrolases for DNA and RNA. The assays are used to demonstrate a clear preference of the NudC-class of Nudix hydrolases for RNA molecules capped with nicotinamide adenine dinucleotide (NAD) on their 5’ ends.

The name “Nudix” was coined to describe enzymes that were discovered because they share an amino acid signature sequence with the E. coli antimutator protein MutT [1]. MutT prevents mutations by degrading damaged dGTP [5] before it forms mismatches during DNA replication [6]. The first two enzymes sharing the MutT-like motif that were analyzed also cleave nucleoside triphosphates [7,8], but the third, which is now called “NudC,” does not. Instead, NudC degrades NAD, with a curious preference for the more valuable reduced form (NADH) over the oxidized form (NAD+) [9]. The NudC discovery led to the idea that MutT-like proteins might cleave nucleoside diphosphates linked to other compounds (q). This hypothesis was later tested by analyzing MutT-like proteins, most of which were found to hydrolyze phosphate bonds in nucleoside triphosphates, coenzymes, dinucleotide polyphosphates, RNA 5’ capping nucleotides, and nucleotide sugars [10,11]. Most of these proteins share a “Nudix-box” signature with the sequence GX₆EX₄REUXEEGU, where U is I, L, or V, and X is any amino acid [12,13]. However, similar proteins were later discovered that possess incomplete Nudix-box sequences yet still adopt three dimensional structures similar to MutT [14]. Some of these proteins lack hydrolytic activities and are therefore often referred to as possessing a “Nudix homology domain (NHD)” [11]. Examples of NHDs in noteworthy proteins are the 8-oxoguanine binding domain of the MutY DNA glycosylase [15], NHDs of inositol polyphosphate hydrolyses [16], and an NAD binding pocket on the protein DCBl (deleted in breast cancer 1) [17].

About 10 years ago, some cellular RNA molecules were found to contain NAD at their 5’ ends [18]. Since a pyrophosphate links the adenine and nicotinamide nucleotides in NAD, such a structure resembles the 7-methylguanylate (m’G) cap found on the 5’ end of most eukaryotic messenger RNAs. The main functions that have been attributed to this unique NAD “cap” have been analogous to those of the...
canonical m^7G caps seen on the vast majority of eukaryotic messenger RNAs [19]. For example, have shown NAD capping effects translation [20], RNA lifetime and stability [19,21–28]. Shortly after NAD-capped RNA was discovered, NudC was found to remove nicotinamide mononucleotide (NMN) from the 5′ ends of bacterial NAD-capped RNA [21], and more recently a NudC homolog in humans called Nudt12 was found to perform the same function in human cells [29].

The purpose of this project was to determine if NudC and Nudt12 differentiate between RNAs with m^G and non-canonical 5′ caps. To this end, they were compared to human Nudix proteins that remove canonical m^G caps (Nudt3, Nudt16 and Nudt20) and the prototype human MutT homolog that does not remove caps, but instead hydrolyzes 8-oxo-dGTP (Nudt1 aka MTH1). Nudt20, which more commonly is referred to as mRNA-decapping enzyme 2 (Dcp2), was the first human mRNA decapping enzyme to be discovered [30]. Nudt3 (aka DIPP1), was first found to be a diphosphoinositol polyphosphate phosphohydrolase [31], but was later also demonstrated to be an mRNA decapping enzyme [32,33]. Nudt16, which cleaves protein-bound ADP-ribose [47] [48], also removes m^7G caps [49]. However, unlike Nudt20 and Nudt3, Nudt16 has a preference for the U5 small nuclear RNA [34]. Other work suggests that another biological role for Nudt16 might be to cleave the cell of potential harmful inosine triphosphate [35].

Below we report new assays that reveal the affinity and specificity of this collection of Nudix enzymes for a variety of DNA and RNA oligonucleotides. The assays were designed to determine the concentration of enzyme bound to a fluorescent oligonucleotide at equilibrium by monitoring changes in fluorescence intensity or polarization. Not surprisingly, all enzymes tested, except Nudt1, bound nucleic acids with dissociation constants in the low nanomolar range. However, most enzymes did not appear to distinguish between DNA and RNA, with the notable exception of Nudt12, which only bound RNA oligonucleotides. Nudt3, Nudt16, and Nudt20 all bound RNA capped with m^G tighter than other RNAs, but NudC and Nudt12 preferred NAD-capped RNA. NudC did not differentiate between the oxidized and reduced form, but Nudt12 did, binding NAD^+ -capped RNA with far higher affinity than NADH-capped RNA.

2. Materials and methods

DNA and RNA oligonucleotides were obtained from Integrated DNA Technologies, Coralville, IA). All fluorescent probes were HiPLC purif ied.

**RNA transcription and capping** - The HiScribe T7 ARCA mRNA Kit (New England Biolabs) was used to synthesise capped RNA by substituting the kit's “ARCA mix” with individual NTPs. Reactions (20 μL) contained final concentrations of 1 mM GTP, 1.25 mM CTP, 1.25 mM UTP, 1.25 mM ATP, 4 mM 3′-O-Me m^GpppA, NAD^−, or NADH, 1 μg DNA template, and 0.1X T7 RNA Polymerase Mix. Gel extracted HindIII digested pET24d-NudC was used as the DNA template for in vitro RNA transcription (846 nt long product). The mixture was incubated at 37 °C for 30 min followed by DNase I treatment, after which it was incubated at 37 °C for another 15 min. Newly synthesized RNA was precipitated using a LiCl solution after incubation at −20 °C for 30 min and centrifuged at 4 °C for 15 min. The RNA was rinsed with cold ethanol, and dissolved in 50 μl 0.1 mM EDTA, to which 6X loading dye was added. The RNA was then subjected to electrophoreses on a 1% TAE gel in RNAasy-free buffers, excised, and purified with the E.Z.N.A. Gel Extraction Kit (Omega, Biotek).

Plasmids expressing NUDT1 (Muth1), NUDT3A, NUDT16A and NUDT20 (Dcp2) were obtained from Addgene. NUDT1 was a gift from Nicola Burgess-Brown (Addgene plasmid # 74660; RRID:Addgene74660). NUDT3A was a gift from Nicola Burgess-Brown (Addgene plasmid # 42348; RRID: Addgene42348). NUDT16A was a gift from Nicola Burgess-Brown (Addgene plasmid # 42404; RRID: Addgene42404). pET28a-hDcp2 was a gift from Megerditch Kiledjian (Addgene plasmid # 72214; http://n2t.net/ addgene:72214; RRID: Addgene72214) [30].

Plasmid Vectors expressing NudC and Nudt12 were constructed as follows. The E. coli nudc gene was PCR amplified from E. coli DH5α, with forward and reverse primers encoding Nudol and BamHI restriction sites (5′-GGG CCGCCT GGA TCG TAT AAT TGA AAA ATT AGA TC-3′ and 5′-GGC CGG CGG ATC CTC ACT CAT ACT CTG CCC GAC-3′). The PCR product was then cut with NcoI and BamHI and ligated into a similarly treated pET24d plasmid (EMD Millipore). DNA sequencing revealed a single nucleotide difference between the Nudc gene studied here and the one studied previously from E. coli strain MG1655 [9] at the 33rd codon. The Nudc protein here (from DH5α) has an Ala at position 33, whereas the Nudc encoded by MG1655 has an Arg. This amino acid is far from the conserved Nudix box and the difference does not affect the ability of Nudc to cleave NAD^− or NADH. The NUDT12 gene was amplified using PCR from pCMV-Nudt12 (Origene Technologies Inc, Rockville, MD), with primers containing BamHI and NdeI restriction sites. The PCR product was then cut with NolI and ligated into a similarly digested pET33 plasmid (Novagen).

**Proteins Purification** - All Nudix proteins studied here, except NudC, possess an N-terminal six-residue HIS-tag, which was used to capture each protein from crude cell lysates. After immobilized metal affinity chromatography (5 ml Ni-NTA, GE Healthcare), each protein was further purified using gel filtration column chromatography (300 ml Sephacryl S-300 HR, GE Healthcare), and ultrafiltration. Nudc was captured from lysates based on its affinity for nucleic acids and purified as described before [9]. Briefly, colonies of BL21 (DE3) cells harboring each plasmid were inoculated into 3 ml of LB medium containing 100 μg/ml kanamycin. After the cells grew to an OD600 of 0.6, they were transferred to 1 L of fresh medium containing kanamycin. After the cells reached an OD600 of 0.6 again, they were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG). For His-tagged proteins, the induced cell pellet was suspended in IMAC buffer (20 mM Tris pH 8, 0.5 M NaCl, 5 mM imidazole), sonicated for 5 min and cell debris removed by centrifugation (15,000 g, 30 min). crude extract was loaded onto a 1 ml Ni-NTA column and the fractions were eluted over a 5–500 mM imidazole gradient. Fractions containing each Nudix protein were loaded on a 250 ml Sephacryl S300 gel filtration column and eluted with 50 mM Tris, 1 mM EDTA, 0.1 mM dithiothreitol, and 50 mM NaCl. The fractions containing the Nudix proteins were identified using SDS-PAGE, combined and concentrated by ultrafiltration, and stored at > 1 mg/ml in GF buffer containing 30% glycerol.

Concentrations of the purified proteins were determined by measuring absorbance at 260 nm using the following molar extinction coefficients that were calculated with the ProtParam tool (https://web.expasy.org/protparam/): Nudc (UniProtKB - P32664), 61,880 M^−1 cm^−1; Nudt1 (UniProtKB - P36639), 33,460 M^−1 cm^−1; Nudt3 (UniProtKB - O95989), 28,420 M^−1 cm^−1; Nudt12 (UniProtKB - Q9BBQ2), 66,640 M^−1 cm^−1; Nudt16 (UniProtKB - Q96DE0), 12,950 M^−1 cm^−1; Nudt20 (UniProtKB - Q8IU60), 49,390 M^−1 cm^−1.

**Fluorescence Intensity Binding Assay** - Assays (100 μL) were performed in 384 well black plates (Thermo Scientific, catalog #9502867) such that final reagent concentrations were 50 mM Tris pH 7.5, 5 mM MgCl2, 0.11 mM DTT, 0.1 mg/ml BSA, 0.01% Tween 5–40 mM DNA/RNA oligonucleotides, and indicated amounts of Nudix proteins. Fluorescence of fluorescein-labeled oligonucleotides (Ex. Wavelength filter: 485 ± 10 nm, Em. Wavelength filter: 520 ± 10 nm) and Cy5-labeled oligonucleotides (Ex. Wavelength filter: 640 nm, Em. Wavelength filter: 670 nm) was measured on a FLUOSTar Omega (BMG LABTECH) at 25 °C, with a gain of 100. The observed fluorescence (Fobs) was fit to Eq. (1).

\[
F_{obs} = F_{\ast}(L_{T} - \text{EL}) + F_{\ast}\text{EL}
\]

(1)

Where:
EL = \( \left( K_d + \frac{E_T}{n + L_T} \right) - \sqrt{\left( K_d + \frac{E_T}{n + L_T} \right)^2 - \left( 4^*\frac{E_T}{n^*E_T} \right)^2} \)

and \( E_T \) is the concentration of Nudix hydrolase with \( L_1 \) being the total oligonucleotide ligand (20 nM or 40 nM or 80 nM); \( n \) is the number of nucleotides bound per enzyme, \( K_d \) is the dissociation constant, \( P_i \) is a coefficient relating probe concentration (i.e. oligo alone) to \( P_{obs} \), \( F_i \) is a similar coefficient relating \( P_{obs} \) to the concentration of an Enzyme-NA complex (EL).

Fluorescence Polarization Binding Assay. Polarization assays were performed as described above except that a G-factor reference well was set with 1 nM fluorescein in 0.01 M NaOH, while a blank well was set with 50 mM Tris pH 7.5. Polarization (Ex. wavelength = 470 nm, Em. wavelength = 520 nm) was measured on a TECAN infinite M1000 at 25 °C, with ex/em slit widths 5 nm/10 nm. Fluorescence polarization (P) is defined from fluorescence intensities of both parallel (\( F_{||} \)) and perpendicular (\( F_{\perp} \)) light with respect to the plane of linearly polarized excitation light (Eq. (2)).

\[
P = \left( \frac{F_{||} - F_{\perp}}{F_{||} + F_{\perp}} \right)
\]

(Eq. 2)

Since polarization is independent of probe concentration, data were fit to Eq. (3).

\[
P_{obs} = P_i (L_T - EL) / L_T + P_c * EL / L_T
\]

(Eq. 3)

Where

\[
EL = \left( K_d + \frac{E_T}{n + L_T} \right) - \sqrt{\left( K_d + \frac{E_T}{n + L_T} \right)^2 - \left( 4^*\frac{E_T}{n^*E_T} \right)^2} \]

and \( E_T \) is the concentration of Nudix hydrolase with \( L_1 \) being the total oligonucleotide (20 nM, 40 nM or 80 nM); \( n \) is the number of nucleotides bound per enzyme, \( K_d \) is the dissociation constant, \( P_i \) is a coefficient relating probe concentration (i.e. oligo alone) to \( P_{obs} \), \( P_c \) is a similar coefficient relating \( P_{obs} \) to the concentration of an enzyme-probe complex (EL).

Probe Displacement Assay. The probe displacement assay was designed to estimate binding of unlabeled RNA or DNA ligands. Unlabeled oligonucleotides were added to the above described binding assays to displace the labeled oligonucleotide probe from the Nudix protein. The fluorescence intensities were fit to an inhibition dose response equation:

\[
P_{obs} = P_i + \frac{(F_i - F_c)}{1 + E_T / (K_d + E_T)}
\]

(Eq. 4)

In Eq. (4), IC50 is the concentration of nucleic acid needed to displace 50% of the probe, “x” is the concentration of unlabeled “competitor” oligonucleotide, both the maximum fluorescence \( F_i \) and \( F_c \) were as defined in Eq. (1).

\( K_i \) values were determined by assuming unlabeled probe (or capped RNA) bound like a competitive inhibitor:

\[
K_i = \frac{IC_{50}}{\left( \frac{L_T}{E_T} + 1 \right)}
\]

(Eq. 5)

When titrations were performed in the presence of more than one probe concentration (e.g. Fig. 3D), a dissociation constant (\( K_i \)) for unlabeled DNA/RNA was determined by assuming unlabeled oligos act as competitive inhibitors and fitting datasets an explicit for competitive inhibition of a tight binding ligand:

\[
P_{obs} = P_i (L_T - EL) + F_c * EL
\]

(Eq. 6)

Where:

\[
EL = \left( K_d (1 + I/K_d) + E_T + L_T \right) - \sqrt{\left( K_d (1 + I/K_d + E_T / L_T) \right)^2 - \left( 4^*E_T / n^*L_T \right)^2}
\]

In Eq. (6), “I” is the concentration of unlabeled DNA/RNA, \( EL \) is the concentration of Nudix hydrolase and \( L_T \) is the total labeled oligonucleotide probe concentration, \( K_d \) is the dissociation constant for enzyme: probe complex (calculated from Eq. (1), values in Fig. 3C), \( F_i \) is a coefficient relating probe concentration to \( P_{obs} \) (i.e. probe alone), \( F_c \) is a similar coefficient relating \( P_{obs} \) to the concentration of the Enzyme-probe complex (EL).

3. Results

Initial attempts to analyze Nudix binding to nucleic acids were made using electrophoretic mobility shift assays (EMAS). In such experiments, shifts of DNA oligonucleotides were observed with Nudt3, Nudt16, Nudt20 and NudC, but not with Nudt1 or Nudt12. Unfortunately, however, the “shifts” seen in such assays were never clear bands, but rather smears, which were difficult to quantify. The same results have been reported by others studying nucleic acid binding by the same proteins (e.g. see Fig. S10 of Reference [36]). Such results might be explained by the ability of Nudix hydrolases to form dimers [9], or oligomers, on nucleic acid that might not be stable enough to survive electrophoresis. In support of this idea, others have needed to resort to crosslinking to demonstrate Nudix-nucleic acid interactions [37]. Regardless, electrophoretic techniques do not prove the affinity of Nudix proteins for nucleic acids in aqueous solution under equilibrium conditions.

Nudix enzymes bind fluorescently labeled DNA and RNA. To better estimate affinities under equilibrium conditions, we instead used fluorescent probes that were used in other studies to examine protein: nucleic acid interactions [38]. A variety of probes were tested with various fluorophores and nucleic acid sequences, four of which are shown (Table 1), two composed of DNA and two of RNA (Fig. I). Assays were performed in well plates in the presence of blocking agents designed to prevent proteins or nucleic acids from absorbing to the well’s surface (BSA and non-ionic detergent). In each case, probe fluorescence intensity was dependent on the concentration of Nudix protein in a manner which fit a model describing a stoichiometric DNA: protein interaction (Eq. (1)).

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<td>OLIGONUCLEOTIDE</td>
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<td>(CYS)DPT15</td>
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<td>FAMJDNA18</td>
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an deoxythymidine a oligomer attached to Cy5 ([Cy5]dT15). The increase in fluorescence intensity suggests that [Cy5]dT15 binds Nudt3, Nudt16, Nudt20 (Dcp2) and NudC. However, even with the addition of Nudt1 and Nudt12, the fluorescence intensity remained unchanged, suggesting that there was no interaction between the oligonucleotide and those proteins. The fluorescence intensity data were fit to Eq. (1) to estimate the $K_d$. Similar best-fit dissociation constants were obtained in triplicate repeat experiments (11 ± 4 nM for Nudt3; 2 ± 1 nM for Nudt16, 6 ± 2 nM for Nudt20, 7 ± 3 nM for NudC).

Different results were obtained however with RNA oligonucleotides. For example the RNA counterpart of [Cy5]dT15, a Cy5-labeled uridylylate oligomer ([Cy5]rU15) likewise did not bind Nudt1, but it bound all other proteins, including Nudt12 (Fig. 1B). When [Cy5]rU15 was titrated with each Nudix proteins, and data fit to Eq. (1), best-fit values for $K_d$ were: 17 ± 14 nM for Nudt3, 10 ± 4 nM for Nudt12, 27 ± 12 nM for Nudt16, 17 ± 5 nM for Nudt20, 9 ± 6 nM for NudC. Uncertainties above reflect standard deviations seen in triplicate repeat titrations.

The unique preference of Nudt12 for RNA was also observed with oligo. The above results suggest that Nudix hydrolases bind nucleic acid probes to enhance their fluorescence intensity. If that is the case, then the presence of Nudix proteins should also affect probe anisotropy (or polarization). We therefore designed a fluorescence-based polarization assay to further examine the binding between DNA or RNA and Nudix hydrolases. Since polarization is independent of probe concentration (Eq. (2)), this assay was used to estimate the relative affinity and stoichiometry of binding of each Nudix protein and [FAM]RNA36, which was the probe that appeared to bind the proteins with highest affinity. Assays were performed at three different probe concentrations and data globally fit to Eq. (3) (Methods). With enzymes that bound more weakly, similar isotherms were obtained with each probe concentration and data fit to Eq. (3) (Methods).

![Figure 1](image1.png)

**Fig. 1. Interactions of various Nudix proteins with fluorescently labeled oligonucleotides.** Oligonucleotide sequences are shown in Table 1. (A) 20 nM [Cy5]dT15 was titrated with various concentrations of Nudix proteins and fluorescence emission was observed at 670 nm when excited at 649 nm. (B) 20 nM [Cy5]rU15 was titrated with various concentrations of Nudix proteins and fluorescence emission was observed at 670 nm when excited at 649 nm. (C) 20 nM [FAM]DNA18 was titrated with various concentrations of Nudix proteins and fluorescence emission was observed at 520 nm when excited at 485 nm. (D) 20 nM [FAM]RNA36 was titrated with various concentrations of Nudix proteins and fluorescence emission was observed at 520 nm when excited at 485 nm. In each panel, data were fit to Eq. (1) (Methods).

![Figure 2](image2.png)

**Fig. 2. Effect of Nudix hydrolases on the polarization of different concentrations of [FAM]RNA36.** (A) 20 nM circles), 40 nM (squares) and 80 nM (triangles) of [FAM]RNA36 were titrated with various concentrations of Nudt1, Nudt3, Nudt12, Nudt16, or Nudt20 and data fit to Eq. (2) (A) Data with Nudt3. (B) Data with Nudt16. In (A) and (B) data are fit to Eq. (2) (Methods).
Kₐ values and stoichiometries (nucleotides/protein) are shown for each enzyme on Fig. 2C. The stoichiometries should be interpreted cautiously, however, because our use of extinction coefficients to determine binding site concentrations might overestimate binding sites. For example, several Nudix proteins form dimers and higher order oligomers, and not all protein might be active (i.e. some may have denatured during purification).

A probe-displacement assay to monitor ligand binding to Nudix proteins.

To study the binding of a wider variety of unlabeled nucleic acids to each Nudix protein, a probe displacement assay was designed in which 20 nM [FAM]RNA36 was 90% saturated with Nudix proteins (5 nM each) and bound proteins displaced with unlabeled oligonucleotides. RNA29 and its deoxynucleotide counterpart, DNA29 (Table 1) were chosen to disrupt the binding between [FAM]RNA36 and Nudix proteins. This experiment confirmed that irrespective of having the same sequence, the deoxynucleotide version (DNA29) failed to interact with the human Nudt12 protein (Fig. 3B), while its RNA counterpart, RNA29 displaced the existing interaction between [FAM]RNA36 and Nudix proteins. In other words, as would be expected if all proteins except Nudt12 bound RNA, and all except Nudt12 bound DNA, [FAM]RNA36 fluorescence decreased upon unlabeled RNA oligonucleotide addition to Nudt12, but not upon addition of DNA oligonucleotides to a Nudt12: [FAM]RNA36 complex (Fig. 3A and B).

To further test the idea that Nudt12 exclusively binds RNA and not DNA, the enhanced fluorescence intensity of [FAM]RNA36 when bound with Nudt12 was examined in the presence and absence of unlabeled DNA oligonucleotides G9, C18 and RNA oligonucleotides RNA18 and RNA36 (Table 1). In each case, only oligonucleotides made of RNA lowered fluorescence, and the length of the competing unlabeled oligonucleotide did not affect the results, as similar results were obtained with RNA36 and RNA18 (Fig. 4C). When more DNA probe was present in an assay, more unlabeled oligonucleotide was needed to decrease fluorescence to similar extents, and data fit a model for competitive inhibition (Eq. (6)), as shown with RNA36 (Fig. 3D). The best fit value for the Kᵢ of RNA36 (0.7 nM, Fig. 3D) was similar to the Kᵢ of Nudt12 for [FAM]RNA36 (1 nM, Fig. 2C). Since the two oligonucleotides have the same sequence and composition, except for the 5′ fluoroscein label, these data suggest that the fluorescence modification does not affect the binding of RNA to Nudt12.

**Nudix proteins differentiate between RNA Caps.** To test how various Nudix proteins interact with RNA possessing m7G and non-canonical caps, T7 RNA polymerase was used to synthesize 846 nucleotide long RNA initiating with either m⁷GpppA, NAD⁺, or NADH. The DNA template used for each RNA transcription was a linearized HindIII-digested pET24-NudC vector, which encodes an 846 product assuming transcription run-off (Methods). The transcription products were gel purified, and concentrations determined from absorbance at 260 nm (Fig. 4A).

Each purified RNA product was used to titrate complexes of each Nudix protein bound to [FAM]RNA36 (Fig. 4B–F). Data were fit to a dose-response equation (Eq. (4)) to estimate the concentration of each RNA needed to displace 50% of the probe (IC₅₀). Kᵢ values for each RNA were then calculated using the Cheng-Prusoff relationship (Eq. (5)) assuming competitive inhibition (Fig. 4G). Remarkably, all of the enzymes previously thought to remove m⁷G caps (Nud3, Nud16, and Nud20) bound m⁷G RNA more tightly than either NADH or NAD⁺ capped RNA. Those suspected to remove non-canonical caps, NudC and Nudt12, bound more tightly to NAD⁺ or NADH capped RNA. NudC bound NAD⁺ and NADH capped RNA with a similar affinity, while Nudt12 preferred NAD⁺ caps over NADH-caps. Since the above experiments were all performed in the presence of MgCl₂, which is needed for Nudix protein to hydrolyze phosphoanhydride bonds, the titrations were repeated in the absence of MgCl₂, i.e. under condition where the proteins would not hydrolyze the caps. Similar RNA preferences were observed in the absence of metal (Fig. 4H), although all Kᵢ values were notably higher in the absence of metal, indicative of somewhat weaker interactions.

**4. Discussion.**

This study started as an attempt to understand whether the human NudC homolog Nudt12 also targets NAD-capped RNA. Although clear evidence was found that Nudt12 removes NMN from NAD-capped RNA to yield RNA with a 5′ monophosphate, those results are not presented here because they were essentially the same as those that were recently reported by the Kiledjian Lab [29]. The two new insights here regarding Nudt12 that were not reported yet involve the unique nucleic acid binding specificity of Nudt12. Unlike related proteins, Nudt12 differentiates between RNA and DNA (Figs. 1–3) and shows a clear preference for NAD⁺-capped RNA rather than NADH-capped RNA (Fig. 4). Our new data with Nud3, Nud16, and Nud20, which shows their preference for m⁷G capped RNA, are also important because they support the notion that the biological role of those proteins is to remove m⁷G caps.

Besides being the first MutT-like enzyme that was shown to hydrolyze substrates other than NTPs, NudC was also the first MutT-like enzyme that co-purified with nucleic acids [9]. Since MutT is so intimately involved in DNA replication, we initially thought NudC might bind DNA, but the subsequent finding that NudC and other prokaryotic and eukaryotic Nudix hydrolases remove RNA caps has revealed that
RNA is more likely the biologically relevant NudC ligand. However, the data presented above show that none of the Nudix proteins tested differentiate between DNA and RNA. With the notable exception of Nudt12, which binds RNA but not DNA.

The collection of proteins that were chosen to study help provide insights into which part of the Nudt12 protein might determine RNA specificity. All enzymes analyzed here have NHDs (blue, Fig. 5A). All also possess the Nudix signature (GX 5Ex7REUxEExGU), except for Nudt16, which has an Asp instead of the first Glu and three additional amino acids inserted within the Nudix box (Fig. 5B). Nudt1, Nudt3, and Nudt16 are all small proteins (< 20 kDa) that consist mostly of an NHD, yet two of the three bind nucleic acids, suggesting residues on the NHDs are responsible for nucleic acid binding. Nudt20, Nud12 and NudC are larger proteins with additional regions N-terminal to the NHD. The N-terminal domain of human Nudt20 has not yet been visualized on the atomic level, but it has been visualized for yeast homologs [40]. In a crystal structure of *S. pombe* Dcp2 bound to its partner Dcp2, a positively charged ion channel was observed between the N-terminal domain and C-terminal NHD [40]. NMR studies and site-directed mutagenesis studies of *S. cerevisiae* DCP2 support the idea that RNA wraps around the protein in this cleft [36]. A similar cleft is formed between the NHDs and the domains on the N-terminal side of the NHDs of NudC [41,42] and Nud12 [29], which might also accommodate longer nucleic acids (Fig. 5C). Interestingly, these domains also contain zinc-fingers, which are conserved in all NudC-like proteins, although there is no clear evidence that either interacts with nucleic acids. Instead, the zinc fingers seem to stabilize the dimeric structures formed by NudC and Nudt12 [42]. Outside the zinc ligands, there is notably less homology between the zinc-finger domains of Nut12 and NudC than there is between the NHDs of each protein (Fig. 5C), indicating that amino acids responsible for the differences seen between the two proteins might reside there, or in the ankyrin repeats found in Nudt12 but not NudC (Fig. 5A).

Prior studies have suggested that in cells up to 60% of yeast mitochondrial transcripts and up to 15% of human mitochondrial RNAs are NAD capped [43]. However, whether NAD is placed at the 5’ end of RNA during or after transcription is still debated. NMN could, in theory, react with RNAs that retain a 5’-triphosphate to form the 5’ to 5’ linkage.

**Fig. 4. Interaction of various enzymes with capped RNA.** (A) Structure of canonical mRNA cap (m7G) compared to non-canonical NAD+ and NADH caps. (B–H) The fluorescence emission of a 20 nM [FAM]RNA36: 5 nM Nudix complex when titrated with m7G capped (circles), NAD+–capped (squares) and NADH-capped (triangles) RNA. Concentrations of 846 nucleotide-long gel-purified capped RNA were determined from A260 values and expressed as concentration of 5′ ends. Data in panel B–F are fit to equation (4) to calculate IC50 values, which in turn were used with the Kd in Fig. 3C to calculate K values with Eq. 5. (G) Resulting K values plotted for each Nudix protein. (H) Titrations in panels B–H were repeated in the absence of MgCl2, and the resulting K values are shown.
Fig. 5. Possible Nucleic acid Binding Regions in Nudix Proteins. (A) Domain structure and evolutionary relationships between the proteins analyzed. Proteins are shown as cartoons with key domains colored. Numbers designate the amino acid at the beginning and end of each domain, and the total amino acids in each protein. Relationships are shown by a circular cladogram based on an alignment of the full sequences of each protein (generated by CLC Sequence Viewer, V8). (B), (C) Crystal structures of each protein analyzed were structurally aligned along the NHDs using the program UCPF Chimera (v1.13.1). Panel B shows PDB file sequences with highlighted conserved residues. Letters are colored based on amino acid type, dots are gaps, numbers are based on peptide sequences of each Nudix protein, and histograms designate degree of consequence conservation and structural overlap (RMSD). Panel C shows a ribbon diagram of each protein colored based on sequence conservation. Cyan is not conserved, white is moderately conserved, and magenta is well conserved. The NAD from the NudC structure (PDB51W4 is shown as sticks, a possible path for RNA extending from the 3' OH of the AMP of NAD⁺ is highlighted with an arrow.

seen in NAD. Ribozymes that catalyze such reactions have been isolated [44]. Alternatively, NAD could act as a “non-canonical initiating nucleotide” to prime RNA synthesis by RNA polymerase [45]. Although some initial experiments suggested such reactions might be restricted to those catalyzed by T7 RNA polymerase [18], others have now observed the ability of bacterial, mitochondrial, and eukaryotic RNA polymerase II to initiate RNA with NAD [43,46]. Regardless of how it originates in nature, a NAD cap does not appear to facilitate mRNA translation like the canonical m7G cap [20]. The assays reported above could be used to help explain why some RNAs are NAD capped. For example, the displacement assay could be used to screen for RNA sequences that bind tightly to Nud12 or find small molecules that could be used as molecular probes to inhibit removal of NAD caps.

CRediT author statement

Atreyei Ray: Methodology, Investigation, Data Curation, Writing - Original Draft. David N Frick: Conceptualization, Funding acquisition, Resources, Supervision, Writing - Review & Editing.

Acknowledgement

This work was supported, in part, by National Institutes of Health Grant R01 AI088001 (to D.N.F.).

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