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Review

Eukaryotic RNA 5′-End NAD$^+$ Capping and DeNADding

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A hallmark of eukaryotic mRNAs has long been the 5′-end m^{7}G cap. This paradigm was recently amended by recent reports that *Saccharomyces cerevisiae* and mammalian cells also contain mRNAs carrying a novel nicotinamide adenine dinucleotide (NAD$^+$) cap at their 5′-end. The presence of an NAD$^+$ cap on mRNA uncovers a previously unknown mechanism for controlling gene expression through nucleotide metabolite-directed mRNA turnover. In contrast to the m^{7}G cap that stabilizes mRNA, the NAD$^+$ cap targets RNA for rapid decay in mammalian cells through the DXO non-canonical decapping enzyme which removes intact NAD$^+$ from RNA in a process termed 'deNADding'. This review highlights the identification of NAD$^+$ caps, their mode of addition, and their functional significance in cells.

Highlights

Eukaryotic cells contain NAD$^+$-capped RNAs

The NAD$^+$ cap can be added by transcriptional initiation with NAD$^+$ in the place of ATP, as well as by a novel NAD$^+$ capping mechanism.

The 5′-end NAD$^+$ cap promotes rapid decay of the RNA mediated at least in part by the DXO family of proteins.

Overview

The triphosphorylated 5′ end of a nascent RNA polymerase II (RNAP II) transcript has long been known to undergo covalent modification to incorporate a 5′–5′ linked *N*^{7}-methyl guanosine (m^{7}G) cap [1,2]. This modification imparts a level of protection to the 5′ end of the mRNA against exonucleolytic attack [3,4]. The m^{7}G cap can further be modified in some RNAs. A class of small nuclear RNAs can be hypermethylated in the cytoplasm on the m^{7}G to generate the trimethylated m^{2,2,7}G cap, which is essential for the reimport of these RNAs into the nucleus [5,6]. Additional methylation can also occur on the m^{7}G capped mRNA on the ribose 2′-hydroxyl position of the first and second transcribed nucleotides to generate the 2′-*O*-methylated Cap1 and Cap2 mRNA forms, respectively [7,8]. These modifications facilitate mRNA translation and contribute to distinguishing host from foreign viral mRNAs [9,10]. Moreover, a modification identified over 40 years ago [8], consisting of methylations at the *N*^{6} and 2′-*O* positions (m^{6}A$_m$) when the first nucleotide following the m^{7}G cap is an adenosine, confers a key layer of epitranscriptomic regulatory information to the 5′ ends of m^{6}A$_m$ eukaryotic RNAs [11].

Recent findings in diverse organisms demonstrate that epitranscriptomic regulation at the 5′-end cap extends beyond m^{6}A$_m$. A key breakthrough was the observation that bacterial RNAs can carry the nucleotide-containing metabolite, NAD$^+$, at their 5′ ends [12–14]. These studies established the foundation for recent reports demonstrating that NAD$^+$ can also decorate the 5′ end of eukaryotic RNAs in place of an m^{7}G cap in *Saccharomyces cerevisiae* [15] and in human [16] cells. The NAD$^+$ cap can be introduced by RNA polymerase during transcription initiation with both bacterial RNAP and eukaryotic RNAP II *in vitro* [17–19], as well as *in vivo* in bacteria [17]. Thus NAD$^+$ cap addition (NADding) is likely to occur, at least in part, via the use of NAD$^+$ as an initiation nucleotide in eukaryotic cells [15,16]. An important distinction between the canonical m^{7}G cap and the NAD$^+$ cap is apparent in mRNA stability and translation. Whereas the m^{7}G cap promotes stability and translation, an NAD$^+$ cap in human cells targets the RNA for

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rapid decay and does not support translation. The decay is facilitated by the non-canonical decapping enzyme, DXO, which initiates degradation of the RNA by removing the NAD$^+$ cap (deNADding) [16]. Recent advances in our understanding of NADding and deNADding are highlighted in this review with an emphasis on eukaryotic systems.

Regulation of 5′-End Cap in Bacteria

The initial demonstration that bacterial cells contain RNAs with a 5′-end cap was provided in 2009 [13,14]. In particular, mass spectrometry of hydrolyzed *Escherichia coli* and *Streptomyces venezuelae* bacterial RNAs identified RNA species carrying 5′-end NAD$^+$ [13] or 5′-end dephospho-coenzyme A (dpCoA) [14]. Nevertheless, because these 5′ end nucleotide metabolites were generated by hydrolyses of total RNA, the nature of the RNAs was not clear. The presence of NAD$^+$ 'cap' at the 5′ end of bacterial RNA was further validated with the use of a chemoenzymatic modification of a terminal nicotinamide to an alkyne moiety amendable to 'click chemistry'-mediated biotinylation. Biotinylated RNAs are isolated by a streptavidin matrix and identified by high-throughput RNA sequencing in a process termed NAD-captureSeq [20] (Figure 1). NAD-captureSeq established the presence of NAD$^+$ caps at the 5′ ends of bacterial RNAs and enabled identification of a subset of small regulatory RNAs that carry an NAD$^+$-cap in *Escherichia coli* [12] (reviewed in [21]). Development of this approach proved an important tool to identify NAD$^+$ caps in other species.

NAD$^+$ Capping

Two models can be envisaged for NAD$^+$ incorporation at the 5′ end of RNA, one being a transcriptional process and the second being post-transcriptional. Addition of the NAD$^+$ cap in bacteria proceeds through the former mechanism, where NAD$^+$ serves as a non-canonical

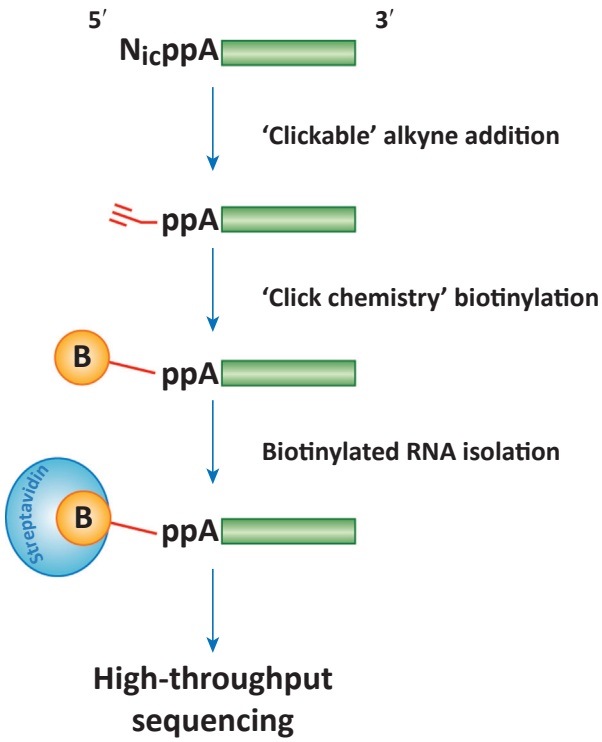

Figure 1. Schematic of the NAD-captureSeq Approach. An exchange of the nicotinamide moiety with an alkyne group is carried out with adenosine diphosphate-ribosylcyclase (ADPRC) and pentynol to attain the 'clickable' alkyne group which is biotinylated by a copper catalyzed azide-alkyne cycloaddition reaction (click chemistry) and retained on streptavidin beads. Identification of the captured RNAs is performed by high-throughput RNA sequencing ([20] for a detailed protocol). Nicotinamide is denoted as N_{ic}, the 'p' represents organic monophosphate, the green bar indicates the RNA, the red lines the alkyne group, 'B' denotes biotin, and streptavidin is indicated.

initiating nucleotide (NCIN) in place of ATP as the first nucleotide [17]. The NCIN model for NAD^+ capping is supported by evidence that bacterial RNAP can use NAD^+ as an initiating nucleotide in place of ATP *in vitro* [17–19]. Furthermore, promoter sequence determinants upstream of the transcription start-site can influence the efficiency by which NAD^+ can serve as an NCIN *in vitro* [17]. A direct correlation is observed between the promoter sequence determinants for use of NAD^+ as an NCIN *in vitro* and the amount of NAD^+-capped RNA detected in *E. coli* [17]. Correlations between the effects of promoter sequence on the efficiency of use of NAD^+ as an NCIN *in vitro* and the amounts of NAD^+-capped RNA generated *in vivo* provide strong support for the proposal that most (if not all) NAD^+-capped RNAs in bacterial cells are generated via the use of NAD^+ as an NCIN [17].

Interestingly, the capacity of bacterial RNAP to incorporate NCINs is not restricted to NAD^+. Transcription initiation through the adenosine residue of nucleoside metabolites (Figure 2) is also observed *in vitro*, including the reduced form of NAD^+ (NADH), flavin adenine diphosphate (FAD), and desphospho-coenzyme A (dpCoA), as well as uridine-containing metabolites including uridine diphosphate glucose (UDP-glucose) and uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) [17–19]. To date, however, dpCoA is the only nucleoside metabolite other than NAD^+ that has been detected at the 5′ end of bacterial RNA [14].

NudC Enzyme and DeNADding of Bacterial RNA

In mammals, the protective 5′ end m^7G cap is removed by a subclass of the Nudix (nucleoside diphosphate linked to another moiety X) hydrolase family of proteins, including Dcp2, Nudt3, and Nudt16, that are known to function as decapping enzymes in cells, and Nudt2, Nudt12, Nudt15, Nudt17, and Nudt19 that display *in vitro* decapping activity [22]. These decapping enzymes can remove the cap structure to generate a 5′-end monophosphorylated RNA substrate (Figure 3A) that is subjected to subsequent exonucleolytic decay. Similarly, in bacteria, the RppH Nudix protein can remove the first two phosphates from the 5′ end of triphosphorylated RNA to generate a monophosphorylated 5′-end RNA [23] that can serve as a substrate for RNase E-directed decay [24]. Another bacterial Nudix protein, NudC, that was previously shown to hydrolyze free NAD(H) into nicotinamide mononucleotide [NMN(H)] plus AMP [25], possesses deNADding activity that hydrolyzes NAD^+-capped RNA to NMN plus 5′-monophosphate RNA [12] (Figure 3B). The significance of NudC deNADding activity was demonstrated in *E. coli* harboring a deleted *NudC* gene, where the steady-state accumulation of NAD^+-capped small RNAs increased [12], as did their stability [17]. These findings indicate that the NAD^+ cap functions in a role analogous to the eukaryotic m^7G cap to stabilize RNA in bacteria, and NudC is a deNADding enzyme that removes the protective NAD^+ cap to promote decay. Furthermore, *in vitro* data show that NudC can remove dpCoA from the 5′ end of RNA [17], suggesting a potential role for NudC in the removal of dpCoA caps *in vivo*. It is likely now a question of when, rather than if, additional nucleoside metabolite 5′-end caps are identified, which in turn will raise new questions of whether the new caps are also removed by NudC or are removed by other so far uncharacterized enzymes.

Regulation of Non-Canonical 5′-End Capping in Mammalian Cells

The addition and removal of the m^7G cap has been extensively studied over the past four decades, with little emphasis on alternative caps. In eukaryotes, the first 5′ mRNA cap to be identified, the m^7G cap [1,2], functions to stabilize RNA and facilitate mRNA translation, as well as contributing to RNA splicing and RNA transport [26]. Additional caps have subsequently been described including the N^6- and 2′-*O*-methylated m^6A_m. The m^6A_m cap confers a key layer of regulatory information to 5′ ends of mRNA because this cap is less susceptible to decapping by the Dcp2 decapping enzyme [11]. Furthermore, capping does not always

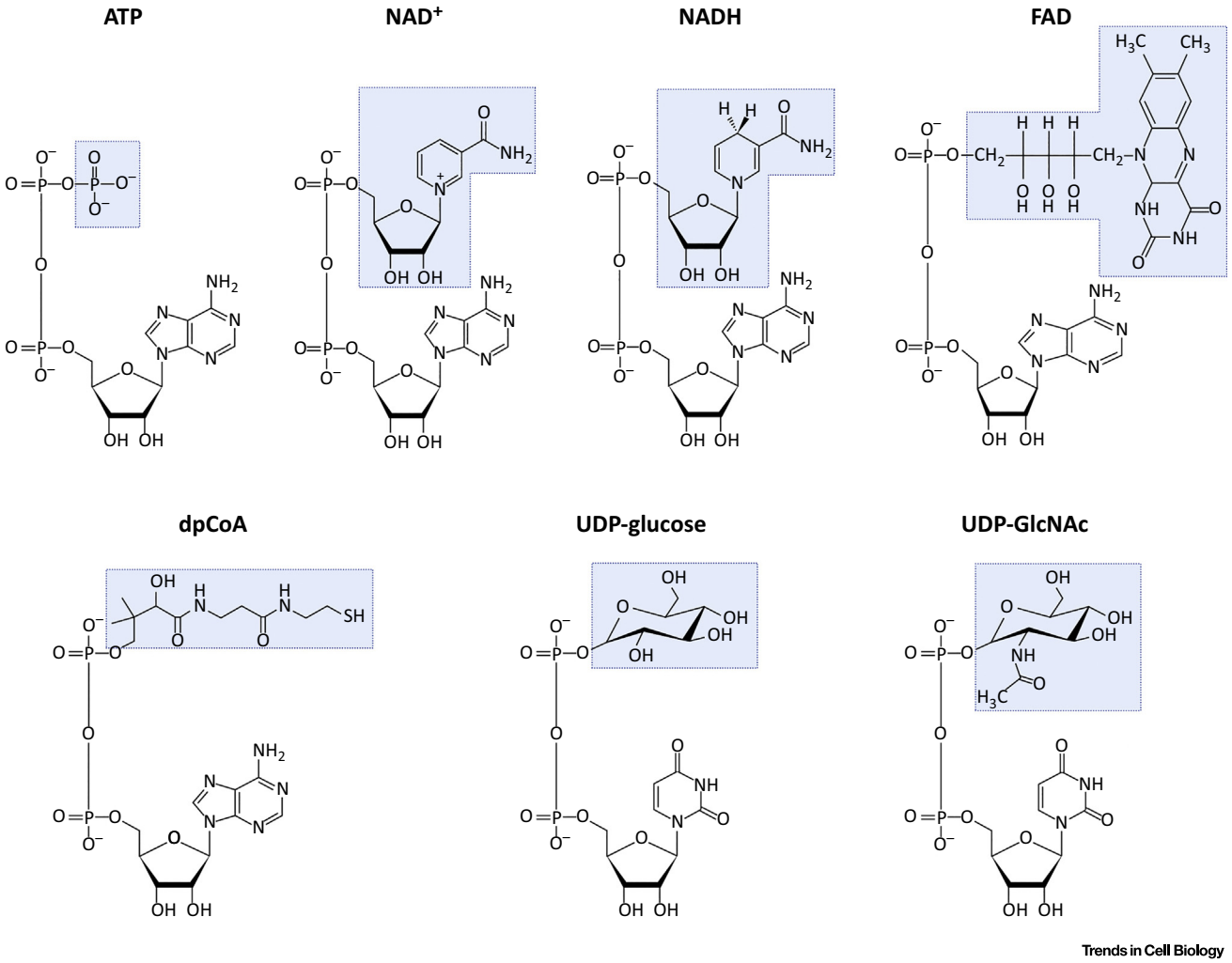

Figure 2. Adenosine-Containing Nucleotide Metabolites. The structure of each metabolite is shown. All structures have a common adenosine diphosphate. The unique features of each metabolite are shaded in blue. Abbreviations: dpCoA, dephospho-coenzyme A; GlcNac, *N*-acetylglucosamine.

proceed to completion in eukaryotic cells. Incomplete caps lacking the N^7-methyl moiety or lacking a cap altogether can also be generated. These incomplete caps are detected and cleared by a 5′-end quality control (5′QC) mechanism employing the DXO family of proteins [27–29] (Figure 3A). The existence of diverse mRNA caps underscores the significance of regulatory mechanisms that incorporate or remove the 5′-end cap and their influence on controlling gene expression.

NAD^+ Capping

Recently, the NAD-captureSeq approach was used to demonstrate that NAD^+ caps extend beyond bacterial RNA and are also present in human cells [16]. The majority of NAD^+-capped mRNAs overlap with canonical m^7G-capped mRNAs, indicating that mRNAs can be present as two distinct populations in cells that differ in the state of their 5′-end cap. One population carries the canonical m^7G-cap, while the other, estimated to comprise ~1–6% of the respective mRNA [16], harbors an NAD^+ cap. Interestingly, distinctions between the reads obtained from NAD-

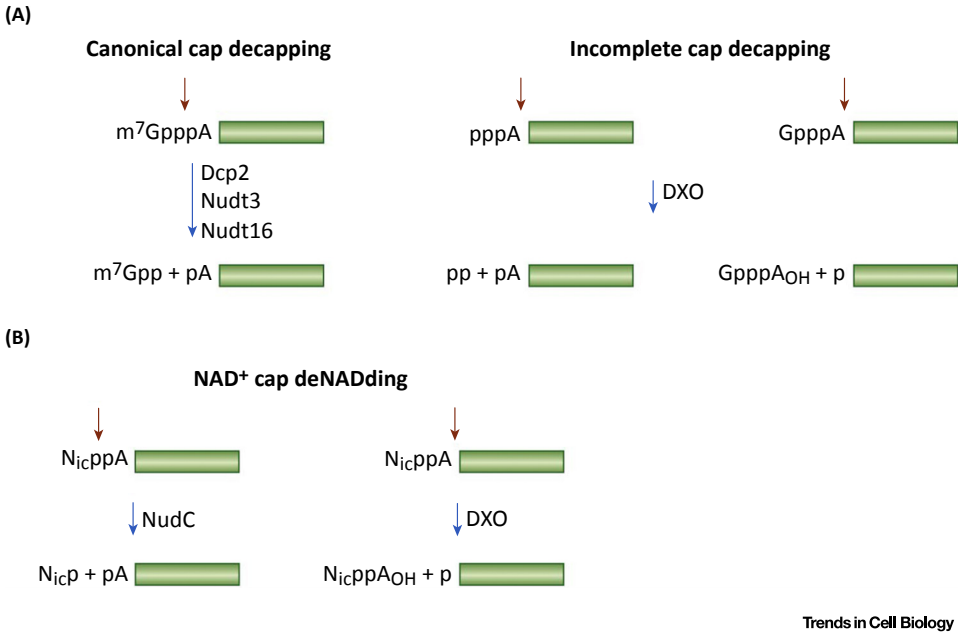

Figure 3. Decapping and DeNADding Activities. (A) Canonical and incomplete cap decapping. Canonical m^7G-cap decapping activity by Dcp2, Nudt3, and Nudt16 decapping enzymes releases m^7Gpp and 5′-monophosphate RNA (left). Non-canonical incomplete cap decapping activity by DXO removes either the diphosphate from a triphosphorylated RNA or the entire cap structure of an unmethylated capped RNA (right). (B) The deNADding activities of NudC and DXO. NudC cleaves the NAD^+ cap between the two phosphates to release nicotinamide mononucleotide ($N_{ic}p$) and 5′-monophosphate RNA (left). DXO hydrolyzes the phosphodiester linkage 3′ to the adenosine to remove the entire NAD^+ moiety and generate a 5′-monophosphate RNA (right). Labeling is as in the legend to Figure 1; the red arrow indicates the enzyme cleavage site.

captureSeq and standard RNA-seq are also evident. For example, while the proportion of small nuclear RNAs (snRNAs) are higher in NAD^+-capped RNA populations, the proportion of another class of NAD^+-capped RNAs consisting of antisense transcripts is significantly lower than that for m^7G-capped RNA [16], suggesting transcript preference in NAD^+ capping (see below).

Post-Transcriptional NAD^+ Capping in Mammalian Cells

The demonstration that eukaryotic RNAP II can use NAD^+ as an initiating nucleotide *in vitro* [17] suggests that NAD^+-capped mRNAs detected in yeast [15] and human cells [16] are also generated by use of NAD^+ as an NCIN by RNAP II. However, a similar transcriptional NCIN model cannot account for all mammalian NAD^+-capped RNAs. In particular, the existence of an NAD^+ cap on mammalian small nucleolar RNAs (snoRNAs) and the related small Cajal body RNAs (scaRNAs) indicates a post-transcriptional mechanism is also involved [16]. SnoRNAs and scaRNAs function as guide RNAs in the pseudouridylation and methylation of ribosomal RNAs and U-rich small nuclear RNAs, respectively [30,31]. They are predominantly encoded within host gene introns and are generated as exonuclease-resistant ribonucleoprotein complexes that lack a modification at their 5′ ends [30–32]. The presence of NAD^+ caps on intronic RNAs strongly suggests that NAD^+ caps can be added to the 5′ end of RNAs post-transcriptionally, and supports the existence of an NAD^+ capping mechanism in addition to the NCIN model in mammalian cells.

Addition of the canonical m^7G cap to the 5′ end of pre-mRNAs occurs cotranscriptionally by the combination of three enzymatic activities: a triphosphatase activity, a guanylyltransferase

activity, and a methyltransferase activity [33,34]. Three different proteins carry out the distinct activities in yeast, while the triphosphatase and guanylyltransferase activities are carried out by a single bifunctional capping enzyme in mammals [35]. A multistep cytoplasmic recapping of 5′-end monophosphorylated RNA has also been reported [36]. Although the mechanism for NAD^+ cap addition is unknown, at least two distinct modes of NADding can be envisaged. First, nicotinamide or a derivative could be linked onto an RNA containing a 5′ adenosine. Second, addition of an intact NAD^+ molecule onto the 5′ end of an RNA regardless of the first nucleotide of the RNA. Based on current identified RNA ligases [37], simple ligation of NAD^+ onto sno/scaRNA appears unlikely as the mechanism for NADding. Whether NADding occurs through a currently unknown RNA ligase or through a multiprotein sequential process is unknown. Identification of the NADding mechanism and the enzyme(s) involved will be an important step forward in our understanding of NAD^+-capping and will provide insight into potential modes of NADding regulation.

DXO and DeNADding of Mammalian RNA

The DXO family of proteins are non-canonical decapping enzymes that remove the entire cap structure of capped or incompletely capped RNAs [28] (Figure 3A). This activity is distinct from that of the 'traditional' decapping enzymes Dcp2 [38–40], Nudt3 [41], and Nudt16 [42] that cleave the cap within the triphosphate linkage (Figure 3A). The ability of DXO to function on incomplete cap structures suggested a potential role for this enzyme in the removal of NAD^+ caps. Consistent with this notion, DXO, but not Dcp2, exhibits deNADding activity on RNA transcripts *in vitro* [16]. The deNADding activity of DXO results in removal of the entire NAD^+ moiety to leave a 5′-end monophosphate RNA, which is mechanistically distinct from that of NudC-mediated deNADding, which results in the conversion of NAD^+-capped RNA to NMN plus 5′-monophosphate RNA (Figure 3B). Notably, the activity of DXO on NAD^+-capped RNA is ~sixfold more efficient than the activity on m^7G-capped RNA, suggesting that deNADding is likely to be a prominent activity for DXO in cells.

Two lines of evidence support a role for DXO in modulating the levels of NAD^+-capped RNAs in cells. The first is from transfected generic RNAs. NAD^+-capped RNAs transfected into HEK293T cells lacking DXO are more stable than NAD^+-capped RNAs transfected into cells lacking the Dcp2 decapping enzyme which does not cleave NAD^+-capped RNA [16]. Second, NAD^+-capped RNA levels are elevated in cells lacking DXO, the most prominent being the sno/scaRNAs, which can increase up to ninefold. Together, these findings indicate DXO is a deNADding enzyme that specifically targets a subset of NAD^+-capped RNAs.

The established role for the DXO family of proteins in 5′QC [43] raises an interesting question. Is NAD^+ capping the result of random aberrant incorporation of NAD^+ as an initiating nucleotide that is subjected to the DXO 5′QC mechanism? Although this remains a formal possibility, several observations argue against nonspecific NAD^+-capping as the predominant pathway. First, random incorporation of NAD^+ caps would be expected at all adenosine transcription initiation sites, resulting in a proportional random distribution of NAD^+ caps at the 5′ ends of RNAs. However, this is not the case because subsets of RNA classes contain either higher or lower proportions of NAD^+ caps relative to the same class of RNAs containing canonical caps [16]. The nonuniform distribution of NAD^+-capped RNAs supports the premise that NAD^+ capping is not a stochastic event that leads to uniform NAD^+ capping of all types of RNA. Studies on how the levels of different NAD^+-capped RNAs respond to treatments that increase or decrease cellular NAD^+ levels can begin to address this point. An outcome of 1:1 uniformity between cellular NAD^+ and NAD^+-caps would be predicted from random incorporation, while variances between the two would be observed with specific NAD^+-capping. Second, and

perhaps most significantly, identification of NAD^+ caps on a subset of sno/scaRNAs demonstrates that NAD^+-capping is an orchestrated and not a random process, at least for this subset of RNAs.

The NAD^+ Cap Promotes RNA Decay in Human Cells

In contrast to the m^7G cap, which stabilizes the 5′ end of mRNAs, the 5′ NAD^+ cap promotes decay of the RNAs it is attached to. NAD^+-capped and polyadenylated luciferase mRNAs transfected into HEK293T cells are less stable than the same mRNAs lacking a 5′-end cap [16]. Therefore, the NAD^+ cap does not serve as a simple passive marker at the 5′ ends of RNAs, but instead functions to actively recruit and confer deNADding and decay. Moreover, the decay of NAD^+-capped mRNAs is mediated through DXO, as indicated by the equivalent stability of an NAD^+-capped luciferase mRNA to that of an m^7G-capped luciferase mRNA in DXO-knockout cells. It appears the NAD^+ cap fulfills a function similar to mRNA 3′ terminal uridylation. The uridine tract on the 3′ end of an mRNA promotes its decay [44] via its susceptibility to Dis3L2 3′-exonuclease activity [45,46] as well as by recruiting the mRNA decapping complex [47,48]. By analogy, the NAD^+ cap serves as a 5′-end tag to recruit DXO and facilitate the demise of the mRNA (Figure 4). The deNADding activity of DXO hydrolyzes and releases NAD^+, leaving the 5′-monophosphorylated mRNA molecule which can further be degraded by the intrinsic 5′–3′ exonucleolytic activity of DXO [28].

An important question remains regarding the physiological function of the NAD^+ cap, to which the NAD^+-capped sno/scaRNAs may provide a clue. Intronic sno/scaRNAs are stabilized following release of the spliced intron because they are resistant to exonucleolytic degradation [49], as exemplified by snoRNA-associated long noncoding RNAs (sno-lncRNAs) which consist of stable intronic RNAs flanked on both termini by snoRNAs [50]. Addition of an NAD^+ cap could provide a mechanism to recruit the DXO exonuclease to promote their decay. It is also plausible

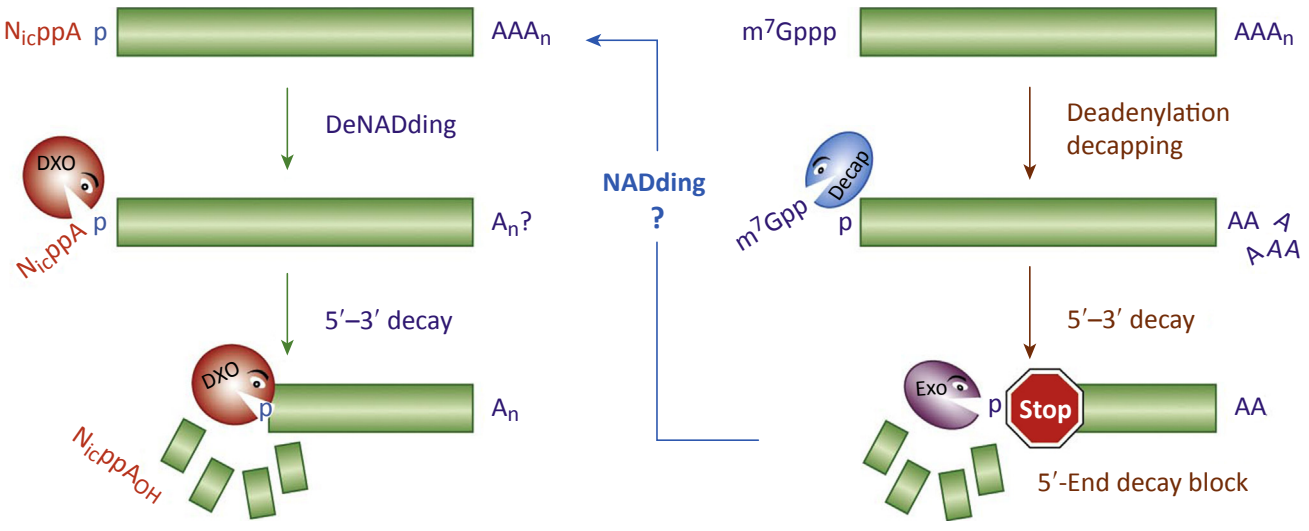

Figure 4. Model of DXO-mediated NAD^+-Capped mRNA decay. (Left) DXO can detect and remove the NAD^+ cap and degrade the mRNA by its intrinsic 5′ to 3′ exonucleolytic activity. The polyadenylated tail is shown as AAA_n with the 'A_n?' representing the unknown parameter of whether the tail is deadenylated to an oligoadenylated tail before deNADding. The DXO enzyme is as shown. Labeling is as in the legend to Figure 1. (Right) Decay of m^7G-capped RNA by decapping (decap, blue) and 5′-exonuclease (exo, purple) is shown. The STOP sign denotes a stable 5′-end decay intermediate refractory to exonucleolytic decay. The potential NADding of the stable intermediate to further promote decay is represented by the blue arrow and question mark.

that 5′-end stable decay intermediates of mRNAs that originally initiated as a m^7G-capped mRNA could potentially be NAD^+-capped to further promote their decay (Figure 4). However, it should be noted that this latter scenario is a model that requires experimental verification.

The NAD^+ Cap Does Not Support Translation in Human Cells

A prominent feature of the m^7G cap is its role in translation initiation via the recruitment of the initiation complex onto the mRNA [51]. Translation initiation complex assembly onto an mRNA can also occur by m^7G cap-independent mechanisms [52]. The ability of the NAD^+ cap to support translation was assessed by transfection of NAD^+-capped and polyadenylated luciferase mRNA into HEK293T cells where the level of translation from this mRNA was no more than the background level of translation from an identical uncapped RNA [16]. These findings show that the NAD^+ cap does not support translation of the exogenous mRNA used. However, these studies cannot rule out the possibility that the NAD^+ cap may promote alternative cap-independent mechanisms in the context of endogenous mRNAs. NAD-captureSeq of polysomal mRNA fractions will be necessary to begin addressing cap-alternative modes of potential NAD^+-capped mRNA translation.

Regulation of 5′-End Non-Canonical Capping in Yeast

NAD^+-capped mRNAs were also recently identified in the yeast *Saccharomyces cerevisiae* using the NAD-captureSeq approach [15], reinforcing the widespread utilization of NAD^+ caps in diverse organisms.

NAD^+ Capping

Addition of the NAD^+ cap in yeast appears to predominantly occur through the NCIN model of NADding [15], indicating that transcriptional incorporation of an NAD^+ cap is a prevalent theme across multiple kingdoms. Interestingly, the extent of NAD^+-capped mRNA is comparable between yeast and human cells. Estimates of NAD^+-capped mRNA in yeast range from 1–5% of a given mRNA population [15], which is similar to that in mammalian cells under normal growth conditions [16]. However, NAD^+-capped mRNAs increase in yeast cells grown in synthetic media [15], an indication that the levels of NAD^+-capped mRNA detected under optimal growth conditions may be an underestimate. The results also imply that cellular stress may contribute to the extent of NAD^+-capping of mRNA. Whether additional cellular or environmental conditions alter the levels of NAD^+-capped mRNA in yeast remains unknown. Beyond nucleus-encoded mRNAs, gene ontology analysis of NAD^+-capped transcripts in yeast uncovered an enrichment for mitochondrial mRNAs [15]. Mitochondrial transcription begins with an adenosine as the first nucleotide [53], suggesting that NAD^+ capping of mitochondrial mRNA through the NCIN mechanism may provide an avenue for coordinating the fate of RNA with cellular metabolism.

Removal of the NAD^+ Cap

At present the functional role of the NAD^+ cap in yeast has not been determined. Whether NAD^+ caps stabilize mRNAs as observed in bacteria [17], destabilize RNA as observed in mammals [16], and/or impact on translation remains unknown. Nevertheless, we have some insight into potential deNADding enzymes in yeast. Yeast contain two members of the DXO family of proteins, Rai1p [54] and Dxo1p [27]. Rai1p forms a heterodimer with the nuclear 5′–3′ exoribonuclease, Rat1p [54,55], which degrades RNAs carrying a 5′ monophosphate. Rai1p can generate 5′-monophosphorylated RNA substrates for Rat1p by catalyzing the removal of an incomplete cap lacking the N^- methyl moiety [29]. Dxo1p can remove an incomplete cap lacking the N^7-methyl moiety and, similarly to DXO, contains intrinsic 5′–3′ exoribonuclease activity to degrade the decapped mRNA [27]. Similarly to mammalian DXO, the yeast

Schizosaccharomyces pombe Rai1p and *Kluyveromyces lactis* Dxo1p exhibit deNADding activity *in vitro* [16]. Furthermore, the deNADding activity of both proteins is more robust than their respective incomplete cap-decapping activities. Whether one or both proteins function as deNADding enzymes in yeast cells and whether they target specific subclasses of NAD^+-capped RNAs will be important areas to address.

The yeast enhancer of decapping protein, Edc3, is an NAD(H)-binding protein and is reported to cleave free NAD^+ *in vitro* at excess protein concentrations [56]. These observations suggest that Edc3 may function as a deNADding enzyme. However, NAD(H) binding influences the cellular localization of Edc3 in yeast cells [56], implying that – similarly to the sirtuin family of DNA deacetylases that require NAD^+ binding for their function, [57,58] free NAD(H) may serve as an essential Edc3 cofactor rather than a deNADding substrate on the 5′ of an mRNA. Whether Edc3 utilizes NAD(H) as a cofactor or functions to deNAD NAD-capped mRNA remains to be determined.

Potential Link Between NAD^+ Capping and Cellular Metabolism

RNA-binding proteins have been known to bridge RNA metabolism and cellular metabolism since the 1990s, with the tricarboxylic acid (TCA) cycle enzyme, aconitase, being one of the best-studied examples [59]. In the absence of iron, the aconitase enzyme is endowed with RNA-binding properties to bind to a stem-loop structure and influence mRNA stability and translation [60,61]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) glycolytic enzyme is also an RNA-binding protein, and its binding activity is modulated by the presence of NAD^+. NAD^+ inhibits RNA binding by GAPDH, but is necessary for GAPDH enzymatic activity [62]. The identification of NAD^+-capped RNAs indicates that the connection between RNA fate and cellular metabolism may extend beyond RNA-binding proteins and may include the RNA itself through its 5′-end cap. The fact that DXO regenerates an intact NAD^+ from an mRNA is suggestive of an intimate link between these processes. Cellular NAD^+ levels are altered in response to energy stresses such as glucose deprivation [63], fasting [64,65], and caloric restriction [66], indicating that the nutritional status of a cell influences NAD^+ concentrations. It is highly probable that such changes would impact on NAD^+ capping. Because nutrient deprivation can manifest in altered mRNA stability [67–69], and NAD^+ caps promote mRNA decay [16], a tantalizing possibility exists that the energy status of a cell can impact on NAD^+-capping and in turn on mRNA turnover. Such a connection could position the NAD^+ cap as an important rheostat influencing the stability of transcripts involved in cellular energetics. The relatively higher levels of NAD^+-capped RNA observed in stationary phase bacteria [17] and yeast grown in synthetic media [15] support such a possibility.

Concluding Remarks

The discovery of the m^7G cap on mRNAs over four decades ago opened a new and exciting area of research for RNA biology and the control of mRNA fate through its addition and removal, that continues to date. Discovery of a new RNA cap, the NAD^+ cap, provides yet another layer of complexity and excitement to the field. We have only begun to delineate the role of the NAD^+ cap in mRNA turnover, and many of the molecular details of the functional role of the NAD^+ cap remain unknown. Other than transcriptional initiation, how is the NAD^+ cap added, and what are the enzyme(s) involved? Does the function of an NAD^+ cap extend beyond RNA turnover? Although an NAD^+-capped exogenous luciferase mRNA was not translated when transfected into cells [16], could endogenous NAD^+-capped mRNA be translated by cap-independent mechanisms [70], bypassing the need for a m^7G cap? Is there an equivalent to the eIF4E m^7G cap-binding protein for the NAD^+ cap? If so, would it modulate mRNA turnover, translation, or localization?

Outstanding Questions

What determines the proportion of a given mRNAs capacity to be capped with an NAD^+ cap, rather than a m^7G cap, and does the percentage of NAD^+ cap on any given mRNA population change with intracellular or extracellular stimuli?

Capping of intronic RNAs must proceed through a post-transcriptional NAD^+-capping mechanism. What are the enzymes and the pathway involved?

Do cells possess an NAD^+ cap-binding protein (NCBP), and is the status of the NAD^+ cap modulated by such a protein?

Do eukaryotic cells also contain additional 5′-end nucleotide metabolite caps other than NAD^+, and do they provide a conduit between RNA metabolism and cellular energetics?

Identification of the DXO family of proteins as deNADding enzymes is a major step towards understanding the physiological regulation of NAD^+ capping. However, based on the precedent in mammalian cells, where there are multiple m^7G-decapping enzymes [22], additional deNADding enzymes with selective specificities are expected. Moreover, all the studies thus far have focused on NAD^+ capping. RNAP can also incorporate the reduced form of NAD^+, NADH, as the first nucleotide [17]. Are RNAs capped with NADH and, considering the contrasting energy states of NAD^+ and NADH, do RNAs with these modifications have distinct functions? Beyond the NAD^+ cap, a pressing question is whether additional nucleoside metabolites are used as a 5′-end cap. Polymerases can incorporate NCINs *in vitro* [71], and *E. coli* and *S. venezuelae* RNAs can contain dpCoA at their 5′ ends [14]. It is highly likely that the NAD^+ cap is only the beginning of a new revolution in 5′-end capping (see Outstanding Questions). We are at an analogous position with nucleoside metabolite capping of mRNA as we were in the 1970s with the discovery of the m^7G cap. The road ahead will undoubtedly be equally exciting and full of surprising new discoveries to be uncovered.

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