Mitochondrial RNA capping: highly efficient 5'-RNA capping with NAD^+ and NADH by yeast and human mitochondrial RNA polymerase

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Summary

Bacterial and eukaryotic nuclear RNA polymerases (RNAPs) cap RNA with the oxidized and reduced forms of the metabolic effector nicotinamide adenine dinucleotide, NAD^+ and NADH, using NAD^+ and NADH as non-canonical initiating nucleotides for transcription initiation. Here, we show that mitochondrial RNAPs (mtRNAPs) cap RNA with NAD^+ and NADH, and do so more efficiently than nuclear RNAPs. Direct quantitation of NAD^+- and NADH-capped RNA demonstrates remarkably high levels of capping *in vivo*: up to ~50% NAD^+ and ~40% NADH capping of yeast mitochondrial transcripts, and up to ~10% NAD^+ capping of human mitochondrial transcripts. The capping efficiency is determined by promoter sequence at and upstream of the transcription start site and, in yeast and human cells, by intracellular NAD^+ and NADH levels. Our findings indicate mtRNAPs serve as both sensors and actuators in coupling cellular metabolism to mitochondrial gene expression, sensing NAD^+ and NADH levels and adjusting transcriptional outputs accordingly.

Introduction

Chemical modifications of the RNA 5'-end provide a layer of “epitranscriptomic” regulation, influencing RNA fate, including stability, processing, localization, and translation efficiency (Hofer and Jaschke, 2018; Jaschke et al., 2016; Ramanathan et al., 2016; Shuman, 2015). One well-characterized RNA 5'-end modification is the “cap” comprising 7-methylguanylate (m^7G) added to many eukaryotic messenger RNAs (Furuichi and Shatkin, 2000; Shatkin, 1976; Shuman, 1995; Wei et al., 1975). Recently, a new RNA 5'-end cap comprising the metabolic effector nicotinamide adenine dinucleotide (NAD) has been shown to be added to certain RNAs isolated from bacterial, yeast, and human cells (Cahova et al., 2015; Chen et al., 2009; Jiao et al., 2017; Walters et al., 2017).

In contrast to a m^7G cap, which is added to nascent RNA by a capping complex that associates with eukaryotic RNA polymerase II (RNAP II) (Ghosh and Lima, 2010; Martinez-Rucobo et al., 2015; Shuman, 1995, 2001, 2015), an NAD cap is added by RNAP itself during transcription initiation, by serving as a non-canonical initiating nucleotide (NCIN) (Bird et al., 2016) (Barvik et al., 2017; Julius et al., 2018). NCIN-mediated NAD capping has been demonstrated for bacterial RNAP (Bird et al., 2016; Julius and Yuzenkova, 2017; Vvedenskaya et al., 2018) and eukaryotic RNAP II (Bird et al., 2016). Thus, whereas m^7G capping occurs after transcription initiation, on formation of the ~20th RNA bond, and occurs only in organisms harboring specialized capping complexes, NAD capping occurs in transcription initiation, on formation of the first RNA

bond, and because it is performed by RNAP itself, is likely to occur in most, if not all, organisms.

NAD exists in oxidized and reduced forms: NAD^+ and NADH, respectively (Figure 1A). Capping with NAD^+ has been demonstrated both *in vitro* and *in vivo* (Bird et al., 2016; Julius and Yuzenkova, 2017; Vvedenskaya et al., 2018). Capping with NADH has been demonstrated *in vitro* (Bird et al., 2016; Julius and Yuzenkova, 2017).

Jäschke and co-workers developed a method that combines click-chemistry-mediated covalent capture and high-throughput sequencing, "NAD captureSeq," to detect NAD^+-capped RNA (Cahova et al., 2015; Winz et al., 2017). Jäschke and co-workers used this method to identify NAD^+-capped RNAs in bacterial cells (*Escherichia coli*). Parker, Kiledjian, and co-workers used the same method to identify NAD^+-capped RNAs in eukaryotic cells (*Saccharomyces cerevisiae* and human cell line HEK293T; (Jiao et al., 2017; Walters et al., 2017). Notably, the identified NAD^+-capped RNAs included not only RNAs produced by nuclear RNAPs, but also RNAs produced by mitochondrial RNAP (mtRNAP). The eukaryotic nuclear RNAPs--RNAP I, II, and III--are multi-subunit RNAPs closely related in sequence and structure to bacterial RNAP (Cramer, 2002; Darst, 2001; Ebright, 2000; Werner and Grohmann, 2011); in contrast, mtRNAPs are single-subunit RNAPs that are unrelated in sequence and structure to multi-subunit RNAPs and, instead, are related to DNA polymerases, reverse transcriptases, and DNA-dependent RNAPs from T7-like bacteriophages (Cermakian et al., 1996; Cheetham and Steitz, 2000; Masters et al., 1987; McAllister and Raskin, 1993; Ringel et al., 2011; Sousa, 1996).

The identification of NAD^+-capped mitochondrial RNAs in *S. cerevisiae* and human cells raises the question of whether the *S. cerevisiae* and human single-subunit mtRNAPs--like the structurally unrelated bacterial and eukaryotic nuclear multi-subunit RNAPs--can perform NCIN-mediated capping. A recent review discussed evidence supporting the hypothesis that human mtRNAP can perform NCIN capping (Julius et al., 2018). Here, we show that single-subunit *S. cerevisiae* mtRNAP and human mtRNAP perform NCIN-mediated capping with NAD^+ and NADH *in vitro*, and do so substantially more efficiently than bacterial and eukaryotic multi-subunit RNAPs. Further, we show that capping efficiency is determined by promoter sequence, we demonstrate very high levels of NAD^+ and NADH capping--up to ~50%--of mitochondrial transcripts *in vivo*, and we demonstrate that the extents of capping *in vivo*, and distributions of NAD^+ capping vs. NADH capping *in vivo* are influenced by intracellular levels of NAD^+ and NADH.

Results

S. cerevisiae and human mtRNAPs cap RNA with NAD^+ and NADH in vitro

To assess whether mtRNAP can cap RNA with NAD^+ and NADH, we performed *in vitro* transcription experiments (Figures 1 and S1). We analyzed *S. cerevisiae* mtRNAP and a DNA template carrying the *S. cerevisiae* mitochondrial 21S promoter (Deshpande and Patel, 2014), and, in parallel, human mtRNAP and a DNA template containing a derivative of the human mitochondrial light strand promoter, LSP_{AGU} (Sologub et al., 2009) (Figure 1C-D, top). We performed reactions using either ATP, NAD^+, or NADH as the initiating entity and using [α^{32}P]-GTP as the extending nucleotide (Figure 1C-D, middle). We observed efficient formation of an initial RNA product in all cases (Figure 1C-D, middle). The initial RNA products obtained with ATP, but not with NAD^+ or NADH, were processed by RppH, which previous work has shown to process 5'-triphosphate RNAs to 5'-monophosphate RNAs (Deana et al., 2008) (Figure 1B), whereas the initial RNA products obtained with NAD^+ or NADH, but not with ATP, were processed by NudC, which previous work has shown to process 5'-NAD^+- and 5'-NADH-capped RNAs to 5'-monophosphate RNAs (Cahova et al., 2015; Hofer et al., 2016) (Figure 1B). The results establish that *S. cerevisiae* mtRNAP and human mtRNAP are able to generate initial RNA products using NAD^+ and NADH as NCINs.

We next assessed whether the initial RNA products formed using NAD^+ and NADH as NCINs can be extended to yield full-length RNA products (Figure 1C-D, bottom). We performed parallel transcription experiments using either ATP, NAD^+, or

NADH as the initiating entity and using [α^{32}P]-GTP, ATP, UTP, and 3'-deoxy-CTP (Figure 1C, bottom) or [α^{32}P]-GTP, ATP, and UTP (Figure 1D, bottom) as extending nucleotides. We observed efficient formation of full-length RNA products in all cases, and we observed that full-length RNA products obtained with NAD^+ or NADH, but not with ATP, were sensitive to NudC treatment (Figure 1C-D, bottom). Similar results were obtained in transcription experiments using [α^{32}P]-ATP or [^{32}P]-NAD^+ as the initiating entity and using non-radiolabeled extending nucleotides (Figure S1). The results establish that *S. cerevisiae* mtRNAP and human mtRNAP not only generate initial RNA products, but also generate full-length RNA products, using NAD^+ and NADH as NCINs.

S. cerevisiae and human mtRNAPs cap RNA with NAD^+ and NADH more efficiently than bacterial and nuclear RNAPs

We next determined the relative efficiencies of NCIN-mediated initiation vs. ATP-mediated initiation, $(k_{cat}/K_M)_{NCIN}$ / $(k_{cat}/K_M)_{ATP}$, for mtRNAPs (Figures 2 and S2). We performed reactions with *S. cerevisiae* mtRNAP and DNA templates carrying the *S. cerevisiae* mitochondrial 21S promoter or 15S promoter (Figures 2A and S2A), and, in parallel, with human mtRNAP and DNA templates carrying the human mitochondrial LSP or heavy strand promoter (HSP1) (Figures 2B and S2B). We obtained values of $(k_{cat}/K_M)_{NCIN}$ / $(k_{cat}/K_M)_{ATP}$ of ~0.3 to ~0.4 for NCIN-mediated initiation with NAD^+ and NADH by *S. cerevisiae* mtRNAP and ~0.2 to ~0.6 for NCIN-mediated initiation with NAD^+ and NADH by human mtRNAP. These values imply that NCIN-mediated initiation

with NAD^+ or NADH is up to 40% as efficient as initiation with ATP for *S. cerevisiae* mtRNAP and up to 60% as efficient as initiation with ATP for human mtRNAP.

The observed efficiencies of NCIN-mediated initiation with NAD^+ or NADH by mtRNAPs are substantially higher than the highest previously reported efficiencies for NCIN-mediated with NAD^+ or NADH with cellular RNAPs (~15%) (Bird et al., 2017; Bird et al., 2016; Vvedenskaya et al., 2018). To enable direct comparison of efficiencies of NCIN capping by mtRNAPs vs. cellular RNAPs on the same templates under identical reaction conditions, we performed transcription assays using a "fork-junction" template that bypasses the requirement for sequence-specific RNAP-DNA interactions and transcription-initiation factor-DNA interactions for transcription initiation (Figure 2C, top). In these experiments, we observe efficiencies of NCIN-mediated initiation with NAD^+ and NADH by mtRNAP that are fully ~10- to ~40-fold higher than efficiencies of NCIN-mediated initiation with NAD^+ and NADH by *E. coli* RNAP and *S. cerevisiae* RNAP II (Figure 2C, bottom). We conclude that *S. cerevisiae* mtRNAP and human mtRNAP cap RNA with NAD^+ and NADH more efficiently than bacterial RNAP and eukaryotic nuclear RNAP II.

Promoter sequence determines efficiency of RNA capping by mtRNAP

In previous work, we have shown that NCIN capping with NAD^+ and NADH by bacterial RNAP is determined by promoter sequence, particularly at and immediately upstream of, the transcription start site (TSS) (Bird et al., 2016; Vvedenskaya et al., 2018). NCIN capping by bacterial RNAP occurs only at promoters where the base pair

(nontemplate-strand base:template-strand base) at the TSS is a T:A (+1A promoters), and occurs most efficiently at the subset of +1A promoters where the base pair immediately upstream of the TSS is purine:pyrimidine (-1R promoters). We have further shown that sequence determinants for NCIN capping by bacterial RNAP reside within the template strand of promoter DNA (i.e., the strand that templates incoming nucleotide substrates) (Vvedenskaya et al., 2018).

To determine whether the specificity for A:T at the TSS (position +1), observed with bacterial RNAP, also is observed with mtRNAP, we assessed NAD^+ capping by *S. cerevisiae* mtRNAP using promoter derivatives having A:T or G:C at position +1 (Figure 3A-B). We observed NAD^+ capping in reactions performed using the promoter derivative having A:T at position +1, but not in reactions performed using the promoter derivative having G:C at position +1 (Figure 3B), indicating specificity for A:T at position +1. To determine whether specificity resides in the template strand for A:T at position +1, we analyzed NAD^+ capping with *S. cerevisiae* mtRNAP using template derivatives having noncomplementary nontemplate- and template-strand-nucleotides (A/C or G/T) at position +1 (Figure 3B). We observed NAD^+ capping only with the promoter derivative having T as the template strand base at position +1, indicating that specificity at position +1 resides in the template strand.

To determine whether specificity for R:Y at position -1, observed with bacterial RNAP, also is observed with mtRNAP, we analyzed NAD^+ capping by *S. cerevisiae* mtRNAP using promoter derivatives having either R:Y (A:T or G:C) or Y:R (C:G or T:A) at position -1 (Figure 3C). We observed higher efficiencies of NAD^+ capping with

promoter derivatives having R:Y at position -1 than with promoter derivatives having Y:R (Figure 3C). To determine whether specificity at position -1 resides in the DNA template strand, we performed experiments using promoter derivatives having Y (C or T) or R (A or G) at position -1 of the template strand and having an abasic site (*) on the nontemplate strand (Figure 3D). We observed higher efficiencies of NAD^+ capping in reactions performed using promoter derivatives having Y at template-strand position -1 than with those having R. Furthermore, within error, the capping efficiencies for promoter derivatives having Y or R at template-strand position -1 matched the capping efficiencies for homoduplex promoter derivatives (Figure 3C-D), indicating that sequence information for NAD^+ capping with *S. cerevisiae* mtRNAP resides exclusively in the template strand.

We conclude that NCIN capping with NAD^+ by mtRNAP is determined by the sequence at, and immediately upstream of, the TSS (positions +1 and -1, respectively). We further conclude that the sequence and strand preferences at positions +1 and -1 for NCIN capping with NAD^+ by mtRNAP match the sequence and strand preferences observed for bacterial RNAP (Figure 3C-E) (Bird et al., 2016; Vvedenskaya et al., 2018), suggesting that these sequence and strand preferences may be universal determinants of NCIN capping with NAD^+ for all RNAPs. Consistent with this hypothesis, we find that sequence preferences for NCIN capping with NAD^+ by bacteriophage T7 RNAP, another member of the single-subunit RNAP family, match the sequence preferences observed for *S. cerevisiae* mtRNAP and bacterial RNAP (Figures 3D and S3). Further consistent with this hypothesis, structural modeling suggests the

basis for these sequence and strand preferences is universal: specifically, a requirement for template-strand +1T for base pairing to the NAD^+ adenine moiety, and a requirement for template strand -1Y for "pseudo" base pairing to the NAD^+ nicotinamide moiety (Bird et al., 2016; Vvedenskaya et al., 2018).

Detection and quantitation of NAD^+- and NADH-capped mitochondrial RNA in vivo: boronate affinity electrophoresis with processed RNA and synthetic standards

Kössel, Jäschke and co-workers have demonstrated that boronate affinity electrophoresis allows resolution of uncapped RNAs from capped RNAs--such as m^7G, NAD^+ and NADH--that contain a vicinal-diol moiety (Igloi and Kossel, 1985, 1987; Nubel et al., 2017). However, the procedures of Kössel, Jäschke and co-workers have two limitations: (i) boronate affinity electrophoresis does not allow resolution of RNAs longer than ~200 nt, and (ii) boronate affinity electrophoresis, by itself, is unable to distinguish between different vicinal-diol containing cap structures (m^7G, NAD^+, NADH, or others). Here, to overcome these limitations, we have combined boronate affinity electrophoresis with use of oligodeoxynucleotide-mediated RNA cleavage ("DNAzyme" cleavage) (Joyce, 2001) and use of synthetic NCIN-capped RNA standards generated using NCIN-mediated transcription initiation *in vitro* (Figure 4). Use of DNAzyme cleavage enables processing of long RNAs to yield defined, short, 5'-end-containing subfragments (Figure 4A). Use of synthetic NCIN-capped RNA standards enables distinction between capped species (Figure 4A,D).

To detect and quantify NCIN capping with NAD^+ and NADH in mitochondrial RNA isolated from cells, we employed the following steps: (i) DNAzyme cleavage of target RNAs to generate 5'-end-containing subfragments < 80 nt in length (Figure 4A, top); (ii) DNAzyme treatment of synthetic NAD^+- and NADH-capped RNA standards having sequences identical to RNAs of interest (Figure 4A, bottom); (iii) boronate affinity electrophoresis of DNAzyme-generated subfragments of mitochondrial RNA and DNAzyme-generated subfragments of synthetic NAD^+- and NADH-capped RNA standards having sequences identical to RNAs of interest (Figure 4B); and (iv) detection of DNAzyme-generated subfragments of mitochondrial RNAs and synthetic RNA standards by hybridization with a radiolabeled oligodeoxyribonucleotide probe (Figure 4C-D).

We selected for analysis two *S. cerevisiae* mitochondrial RNAs that previously had been detected as NAD^+-capped: COX2 and 21S (Walters et al., 2017). We isolated *S. cerevisiae* total RNA and analyzed COX2 and 21S RNAs using the procedure described in the preceding paragraph. The results are presented in Figures 4C-D and 5A (top). For both COX2 and 21S RNAs, we detect at least one RNA species with electrophoretic mobility retarded as compared to that of uncapped RNA, indicating the presence of capped RNA. Treatment with the decapping enzyme NudC eliminates these species, confirming the presence of capping. Comparison of the electrophoretic mobilities to those of synthetic NAD^+- and NADH-capped RNA standards indicates that one of the capped species is NAD^+-capped RNA, and the other capped species, present under these growth conditions only for COX2 RNA, is NADH-capped RNA. The

results show that both COX2 and 21S RNAs are present in NAD^+-capped forms, and that COX2 RNA also is present in an NADH-capped-form. The results confirm that *S. cerevisiae* mitochondrial RNAs undergo NAD^+ capping in cells, show that *S. cerevisiae* mitochondrial RNAs undergo NAD^+ capping at 5' ends generated by transcription initiation (as opposed 5' ends generated by RNA processing), and show that *S. cerevisiae* mitochondrial RNAs also undergo NADH capping in cells.

The observed levels of NAD^+- and NADH-capping of *S. cerevisiae* mitochondrial RNAs are remarkably high. For COX2 RNA, NAD^+-capped RNA comprises ~50% of the total COX2 RNA pool and NADH-capped RNA comprises ~10% of the total COX2 RNA pool (Figures 4C-D and 5A, top). For 21S RNA, NAD^+-capped RNA comprises ~30% of the total 21S RNA pool (Figure 5A, top). These levels of NCIN capping are ~2 to ~50 times higher than levels of NCIN-capping in exponentially growing *E. coli* (less than 1% to ~20% for NAD^+-capping; not previously detected for NADH-capping) (Bird et al., 2016; Cahova et al., 2015; Nubel et al., 2017; Vvedenskaya et al., 2018).

We performed analogous experiments analyzing RNAs produced by transcription from the human mitochondrial LSP promoter (Figure 5B, top). We isolated and analyzed total RNA from HEK293T cells. We observed an NAD^+-capped species comprising ~10% of the total LSP-derived RNA pool (Figure 5B, top). The results establish that human mitochondrial RNAs undergo NAD^+ capping in cells and show that human mitochondrial RNAs undergo NAD^+ capping at 5' ends generated by transcription initiation (as opposed 5' ends generated by RNA processing).

Detection and quantitation of NAD^+- and NADH-capped mitochondrial RNA in vivo: mtRNAPs serve as both sensors and actuators in coupling cellular metabolism to mitochondrial gene expression

Mitochondria are the primary locus of metabolism and energy transformation in the eukaryotic cell, serving as the venue for the TCA cycle and oxidative phosphorylation. The TCA cycle reduces NAD^+ to NADH and oxidative phosphorylation oxidizes NADH to NAD^+. Our finding that mtRNAPs perform NCIN capping with NAD^+ and NADH at efficiencies that vary in a simple mass-action-dependent fashion with [NAD^+] / [ATP] and [NADH] / [ATP] ratios *in vitro* (Figures 1, 2, S1, and S2), and our finding that mtRNAPs perform efficient NCIN capping to yield NAD^+- and NADH-capped mitochondrial RNAs *in vivo* (Figures 4 and 5), raise the possibility that mtRNAPs may serve as both sensors and actuators in coupling metabolism to mitochondrial gene expression *in vivo*.

As a first test of this hypothesis, we assessed whether changing intracellular [NAD^+] / [NADH] ratios results in changes in NAD^+ and NADH capping of mitochondrial RNAs. We isolated total RNA from *S. cerevisiae* grown under conditions that result either high or low [NAD^+] / [NADH] ratios (Bekers et al., 2015; Canelas et al., 2008): i.e., respiration (glycerol/ethanol; aerobic) or fermentation (glucose; anaerobic). We analyzed the same two mitochondrial RNAs as above: COX2 and 21S (Figure 5A). We observed marked changes in levels of NAD^+ and NADH capping for both analyzed mitochondrial RNAs. For COX2 RNA, on changing from the growth condition yielding a high [NAD^+] / [NADH] ratio to the growth condition yielding a low [NAD^+] / [NADH] ratio,

we observe a decrease in levels of NAD^+ capping (from ~50% to ~20%) and an anti-correlated increase in the level of NADH capping (from ~10% to ~40%). Notably, the total level of NAD^+ and NADH capping, NAD(H) capping, remains constant under the two conditions (~60%), indicating that the relative levels of NCIN-mediated initiation and ATP-mediated initiation do not change (Figure 5A). For 21S RNA, the same pattern is observed (Figure 5A): on changing from the growth condition yielding a high [NAD^+] / [NADH] ratio to the growth condition yielding a low [NAD^+] / [NADH] ratio, the level of NAD^+ capping decreases (from ~30% to ~10%), the level of NADH capping increases (from 0% to ~20%), and the total level of NAD^+ and NADH capping remains constant (~30%). The results indicate that changing the [NAD^+] / [NADH] ratio changes transcription outputs *in vivo*.

As a second test of this hypothesis, we assessed whether changing intracellular total NAD(H) levels results in changes in NCIN capping of mitochondrial RNAs (Figure 5B). We isolated RNA from human HEK293T cells grown under conditions yielding either high or low intracellular NAD(H) levels: standard growth media or growth media in the presence of the NAD(H)-biosynthesis inhibitor FK866 (Hasmann and Schemainda, 2003; Khan et al., 2006). We analyzed the same LSP-derived mitochondrial RNA as in the preceding section (Figure 5B). On changing from the growth condition yielding high intracellular NAD(H) levels to the growth condition yielding low NAD(H) levels, we observe a marked change in the total level of NCIN capping (from ~10% to 0%). The results indicate that changing NAD(H) levels changes levels of NCIN-capped mitochondrial RNAs *in vivo*.

Taken together, the results of the two experiments in Figure 5 indicate that mtRNAP serves as both sensor and actuator in coupling [NAD^+] / [NADH] ratios to relative levels of NAD^+- and NADH-capped mitochondrial RNAs (Figure 5A), and in coupling total NAD(H) levels to total levels of NCIN-capped mitochondrial RNAs (Figure 5B), thereby coupling cellular metabolism to mitochondrial transcription outputs. We suggest that mtRNAPs serve as sensors through their mass-action-dependence in selecting NAD^+ vs. NADH vs. ATP as initiating entity during transcription initiation, and serve as actuators by incorporating NAD^+ vs. NADH vs. ATP at the RNA 5' end during transcription initiation.

Discussion

Our results show that *S. cerevisiae* and human mtRNAPs cap RNA with NAD^+ and NADH (Figures 1 and S1), show that *S. cerevisiae* and human mtRNAPs cap RNA with NAD^+ and NADH more efficiently than bacterial and eukaryotic nuclear RNAPs (Figures 2 and S2), and show that capping efficiency by mtRNAPs is determined by promoter sequence (Figure 3). Our results further show that the proportions of mitochondrial RNAs that are capped with NAD^+ and NADH are remarkably high--up to ~50% and up to ~40%, respectively (Figures 4 and 5)--and that these proportions change in response to cellular NAD^+ and NADH levels (Figure 5).

We and others previously have shown that NCIN capping by cellular RNAPs has functional consequences (Bird et al., 2016; Cahova et al., 2015; Jiao et al., 2017). Our results here showing that *S. cerevisiae* and human mitochondrial RNAs are capped at substantially higher levels than non-mitochondrial RNAs--up to ~50% for analyzed *S. cerevisiae* mitochondrial RNAs and up to ~10% for analyzed human mitochondrial RNAs (Figures 4 and 5)--suggest that NCIN capping in mitochondria occurs at a higher efficiency, and has a higher importance, than NCIN capping in other cellular compartments. Four other considerations support this hypothesis. First, mtRNAPs are substantially more efficient at NAD^+ and NADH capping than bacterial and eukaryotic nuclear RNAPs (Figure 2C). Second, levels of NAD^+ and NADH in mitochondria are substantially higher than levels in other cellular compartments (Cambronne et al., 2016; Chen et al., 2016). Third, all *S. cerevisiae* and human mitochondrial promoters are +1A promoters (13 promoters in *S. cerevisiae* mitochondria; 2 promoters in human

mitochondria) (Chang and Clayton, 1984; Taanman, 1999; Turk et al., 2013), in contrast to bacterial and eukaryotic nuclear RNAP promoters, for which approximately half are +1A promoters (Chen et al., 2013; Haberle et al., 2014; Saito et al., 2013; Thomason et al., 2015; Tsuchihara et al., 2009). Fourth, we observe capping with both NAD^+ and NADH for mitochondrial RNAs *in vivo* (Figures 4 and 5), whereas, to date, capping has been observed with only NAD^+ for non-mitochondrial RNAs *in vivo*, raising the possibility that, in mitochondria, but not in other cellular compartments, NAD^+ and NADH caps dictate different RNA fates and, correspondingly, different transcription outputs.

Our results showing that levels of NAD^+ and NADH capping by mtRNAP correlate with changes in intracellular levels of NAD^+ and NADH (Figure 5) indicate that mtRNAP serves as a sensor, sensing [NAD^+] / [ATP] and [NADH] / [ATP] ratios, when selecting initiating entities and, simultaneously, serves as an actuator by altering RNA 5' ends when selecting initiating entities. Because all *S. cerevisiae* and human mitochondrial promoters are +1A promoters, this dual sensor/actuator activity of mtRNAP will occur at, and couple metabolism to gene expression at, all *S. cerevisiae* and human mitochondrial promoters. This dual sensor/actuator activity of mtRNAP obviates the need for a dedicated signal-processing machinery for coupling metabolism to gene expression, instead employing a pan-eukaryotic housekeeping enzyme for signal processing. As such, this dual sensor/actuator activity of mtRNAP provides a remarkably economical, parsimonious mechanism of coupling metabolism to gene expression.

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Declaration of Interests

The authors declare no competing interests.

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Figures

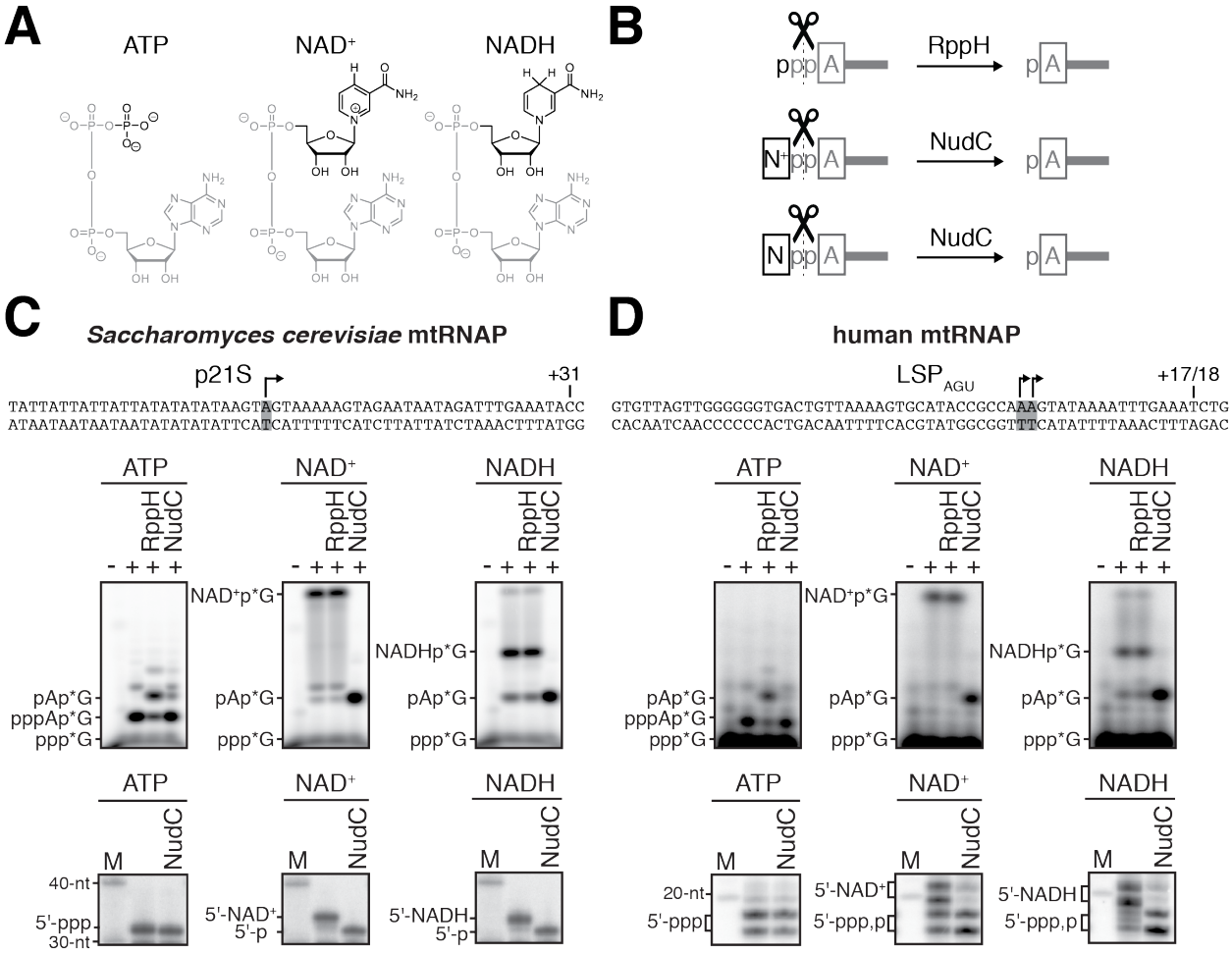


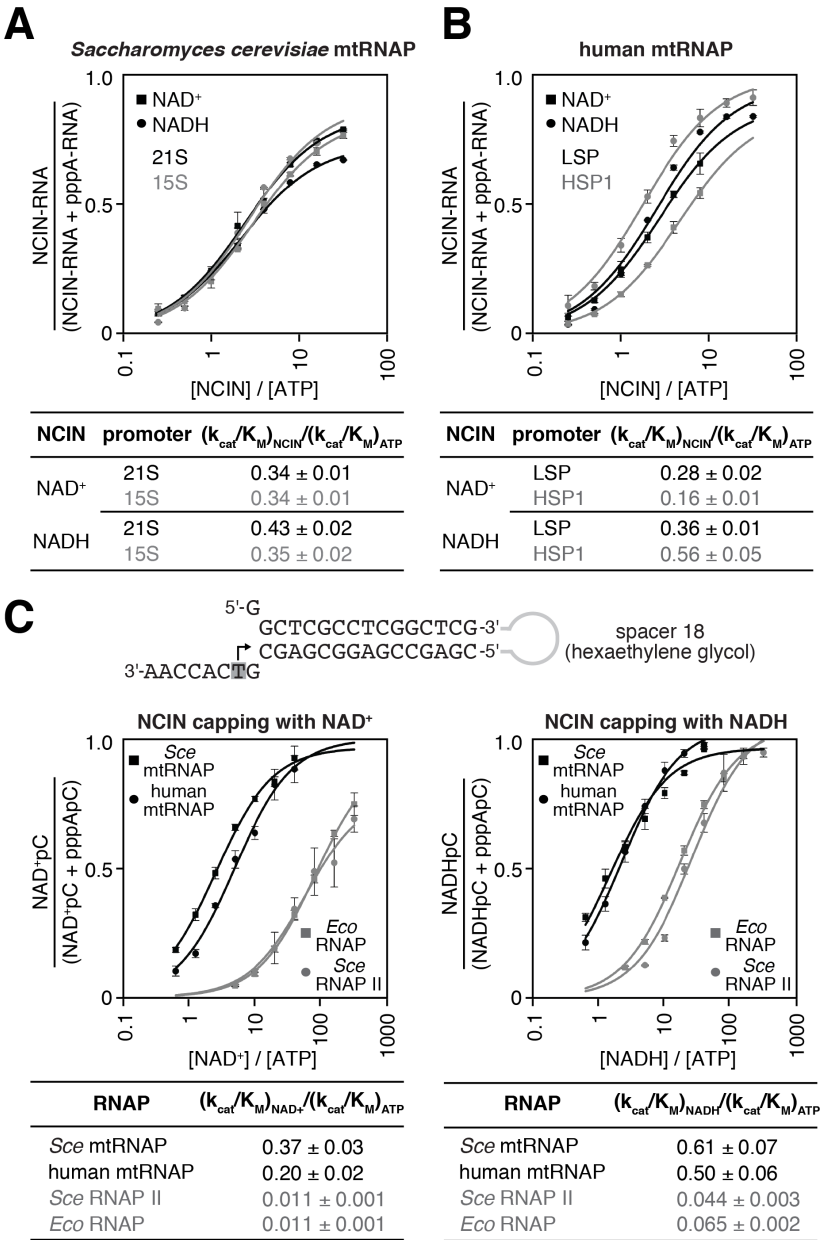
Figure 1. *S. cerevisiae* and human mtRNAPs cap RNA with NAD+ and NADH *in vitro*

A. Structures of ATP, NAD+, and NADH. Grey, identical atoms; black, distinct atoms.

B. Processing of RNA 5'-ends by RppH and NudC. A, adenosine; N+, NAD+ nicotinamide; N, NADH nicotinamide; p, phosphate.

C. and D. NCIN capping with NAD+ and NADH by *S. cerevisiae* mtRNAP (C) and human mtRNAP (D). Top, promoter derivatives. Middle, initial RNA products of *in vitro* transcription reactions with ATP, NAD+, or NADH as initiating nucleotide and [α^{32}P]-GTP as extending nucleotide. Bottom, full-length RNA products of *in vitro* transcription reactions with ATP, NAD+, or NADH as initiating nucleotide and [α^{32}P]-GTP, ATP, UTP, and 3'-deoxy-CTP (C), or [α^{32}P]-GTP, ATP, and UTP (D) as extending nucleotides. Products were treated with RppH or NudC as indicated. Grey box and arrow, transcription start site (TSS); +31 and +17/18, position of last NTP incorporated into RNA; M, 10-nt marker.

See also Figure S1.



NCIN	promoter	$(k_{cat}/K_M)_{NCIN}/(k_{cat}/K_M)_{ATP}$
NAD+	21S	0.34 ± 0.01
	15S	0.34 ± 0.01
NADH	21S	0.43 ± 0.02
	15S	0.35 ± 0.02

NCIN	promoter	$(k_{cat}/K_M)_{NCIN}/(k_{cat}/K_M)_{ATP}$
NAD+	LSP	0.28 ± 0.02
	HSP1	0.16 ± 0.01
NADH	LSP	0.36 ± 0.01
	HSP1	0.56 ± 0.05

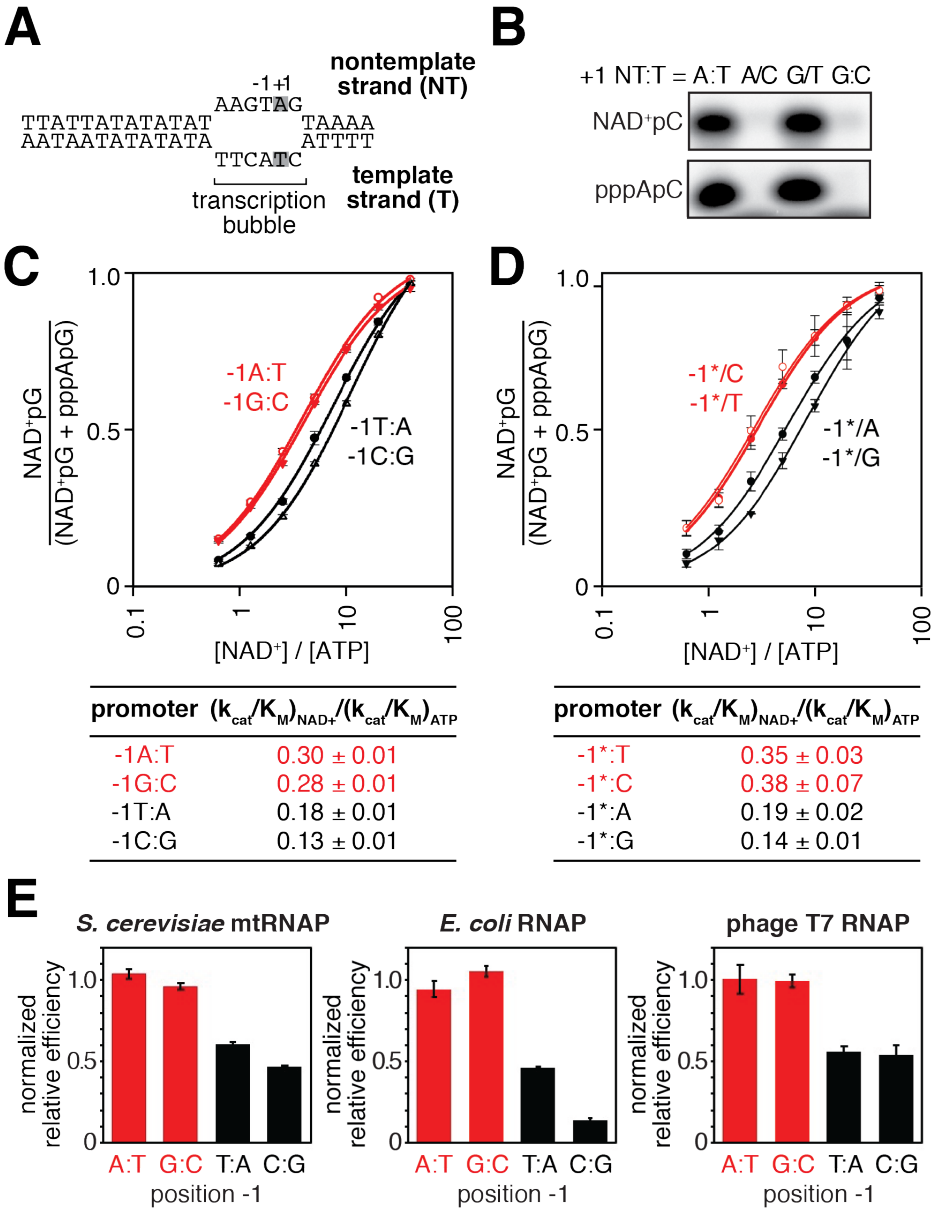
RNAP	$(k_{cat}/K_M)_{NAD+}/(k_{cat}/K_M)_{ATP}$
Sce mtRNAP	0.37 ± 0.03
human mtRNAP	0.20 ± 0.02
Sce RNAP II	0.011 ± 0.001
Eco RNAP	0.011 ± 0.001

RNAP	$(k_{cat}/K_M)_{NADH}/(k_{cat}/K_M)_{ATP}$
Sce mtRNAP	0.61 ± 0.07
human mtRNAP	0.50 ± 0.06
Sce RNAP II	0.044 ± 0.003
Eco RNAP	0.065 ± 0.002

Figure 2. *S. cerevisiae* and human mtRNAPs cap RNA with NAD+ and NADH more efficiently than bacterial and nuclear RNAPs

A. and B. Dependence of NCIN-mediated capping with NAD+ and NADH on [NCIN] / [ATP] ratio for *S. cerevisiae* mtRNAP (A) and human mtRNAP (B) (mean±SD; n=3). DNA templates and representative data are shown in Figure S2.

C. Dependence of NCIN-mediated capping with NAD+ and NADH on [NCIN] / [ATP] ratio for mtRNAPs vs. nuclear RNAPs. Top, Fork junction template. Grey box and arrow indicate TSS. Bottom, Dependence of NCIN-mediated capping with NAD+ and NADH on [NCIN] / [ATP] ratio for *S. cerevisiae* mtRNAP (*Sce* mtRNAP), human mtRNAP, *E. coli* RNAP (*Eco* RNAP) and *S. cerevisiae* RNAP II (*Sce* RNAP II) (mean±SD; n=3).
See also Figure S2.



promoter	$(k_{cat}/K_M)_{NAD+}/(k_{cat}/K_M)_{ATP}$
-1A:T	0.30 ± 0.01
-1G:C	0.28 ± 0.01
-1T:A	0.18 ± 0.01
-1C:G	0.13 ± 0.01

promoter	$(k_{cat}/K_M)_{NAD+}/(k_{cat}/K_M)_{ATP}$
-1*:T	0.35 ± 0.03
-1*:C	0.38 ± 0.07
-1*:A	0.19 ± 0.02
-1*:G	0.14 ± 0.01

Figure 3. Promoter sequence determines efficiency of RNA capping with NAD+ by mtRNAP

A. *S. cerevisiae* mitochondrial 21S promoter DNA depicted in the context of the mtRNAP-promoter open complex. DNA non-template strand (NT) on top; DNA template strand (T) on bottom; Unwound, non-base-paired DNA region, "transcription bubble," indicated by raised and lowered nucleotides; +1 and grey box, bases at the TSS; -1, bases immediately upstream of the TSS.

B. Products of transcription reactions with NAD+ as initiating nucleotide and [α^{32}P]-CTP as extending nucleotide for templates having complementary or non-complementary nucleotides at position +1.

C. Dependence of NAD+ capping on [NAD+] / [ATP] ratio for homoduplex templates having A:T, G:C, T:A, or C:G at position -1 relative to TSS (mean±SD; n=3). Red, -1R promoters; black, -1Y promoters.

D. Dependence of NAD^+ capping on [ATP] / [NAD^+] ratio for heteroduplex templates having an abasic site (*) on the DNA non-template strand (mean±SD; n=3). Red, promoters with a template-strand Y; black, promoters with a template-strand R.
E. Sequence preferences at position -1 for *S. cerevisiae* mtRNAP, *E. coli* RNAP, and T7 RNAP. Graphs show normalized values of $(k_{cat}/K_M)_{NAD+}$ / $(k_{cat}/K_M)_{ATP}$ determined for homoduplex templates having A:T, G:C, T:A, or C:G at position -1 (mean±SD; n=3). Normalized values were calculated by dividing the value for each individual promoter by the average value measured for -1R promoters. Data for *S. cerevisiae* mtRNAP is from panel C, data for *E. coli* RNAP is from (Vvedenskaya et al., 2018), and data for T7 RNAP is from Figure S3.
See also Figure S3.

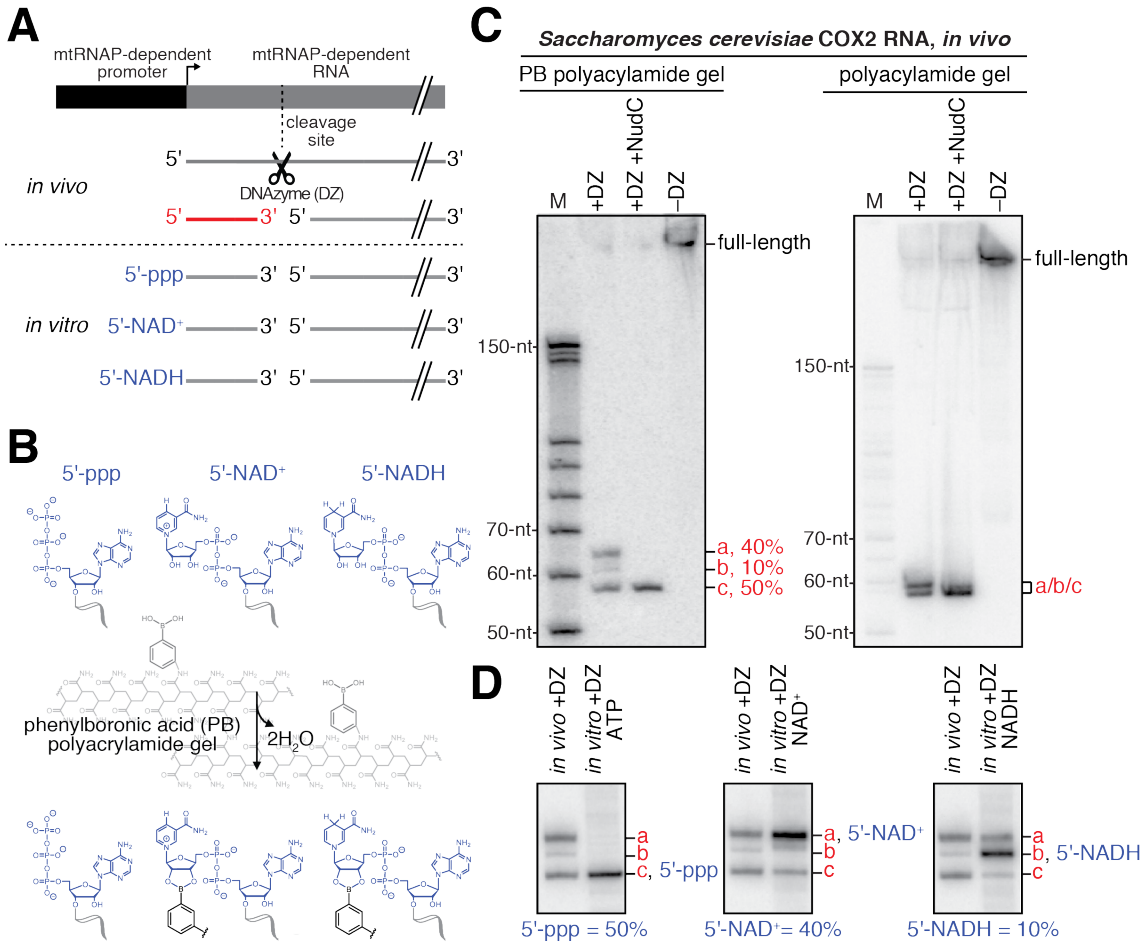


Figure 4. Detection and quantitation of NAD+- and NADH-capped mitochondrial RNA *in vivo*: boronate affinity electrophoresis with DNAzyme-cleaved cellular RNA and DNAzyme-cleaved synthetic NCIN-capped RNA standards

A. Use of DNAzyme (DZ) to process RNA to yield a defined, short 5'-end-containing subfragment, in parallel *in vivo* (red) and *in vitro* (blue). 5'-ppp, 5'-triphosphate end generated using ATP-mediated initiation; 5'-NAD+, NAD+-capped end generated using NAD+-mediated initiation; 5'-NADH, NADH-capped end generated using NADH -mediated initiation.

B. Use of boronate affinity electrophoresis to resolve 5'-ppp-, 5'-NAD+-, and 5'-NADH-containing RNAs. Grey, structure of phenylboronic acid (PB) polyacrylamide gel.

C. PB-polyacylamide gel (left) and polyacrylamide gel (right) analysis of DNAzyme-generated 5'-end-containing subfragments of *S. cerevisiae* mitochondrial RNA COX2. Red, observed 5'-end-containing RNA subfragments resolved by PB-polyacylamide-gel (left) or not resolved by polyacrylamide gel (right); identities of these subfragments are defined in Panel D.

D. Comparison of electrophoretic mobilities of observed 5'-end-containing subfragments of COX2 RNA generated *in vivo* to 5'-end-containing subfragments of synthetic RNA standards generated *in vitro*. a, NAD+-capped RNA; b, NADH-capped RNA; c, uncapped RNA.

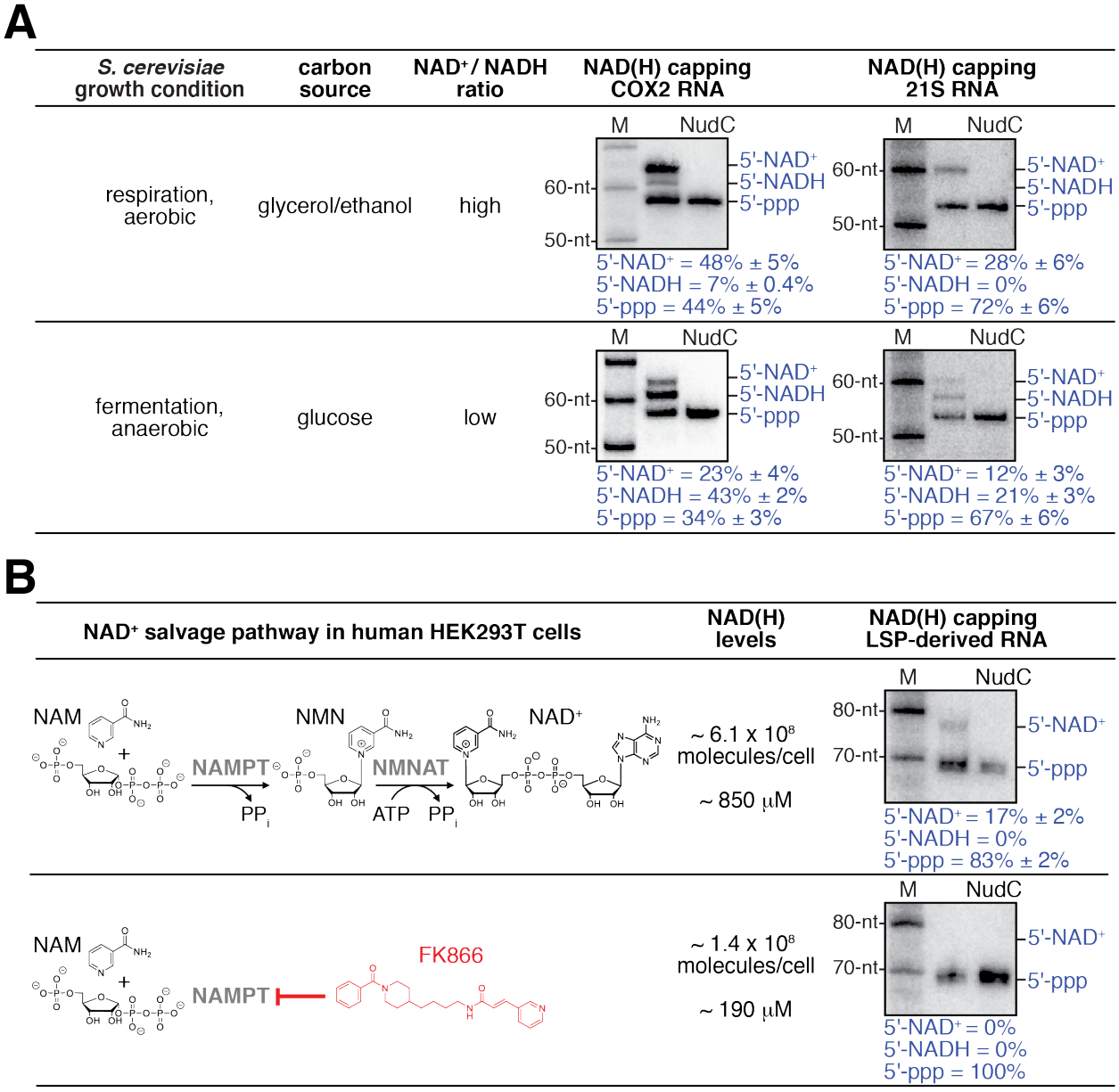


Figure 5. Detection and quantitation of NAD+- and NADH-capped mitochondrial RNA *in vivo*: effects of intracellular NAD+ and NADH levels in *S. cerevisiae* and human cells

A. Changes in intracellular NAD+ / NADH ratios result in changes in levels of NAD+- and NADH-capped mitochondrial RNA (mean±SD; n=3). Gel images show representative data for *S. cerevisiae* COX2 RNA (left) and 21S RNA (right). Blue annotations as in Figure 4.

B. Changes in intracellular NAD(H) levels result in changes in levels of NAD+- and NADH-capped mitochondrial RNA (mean±SD; n=3). Gel images show representative data for LSP-derived RNAs. Red, NAD(H) biosynthesis inhibitor FK866; NAMPT, Nicotinamide phosphoribosyltransferase; NMNAT, Nicotinamide mononucleotide adenylyltransferase.

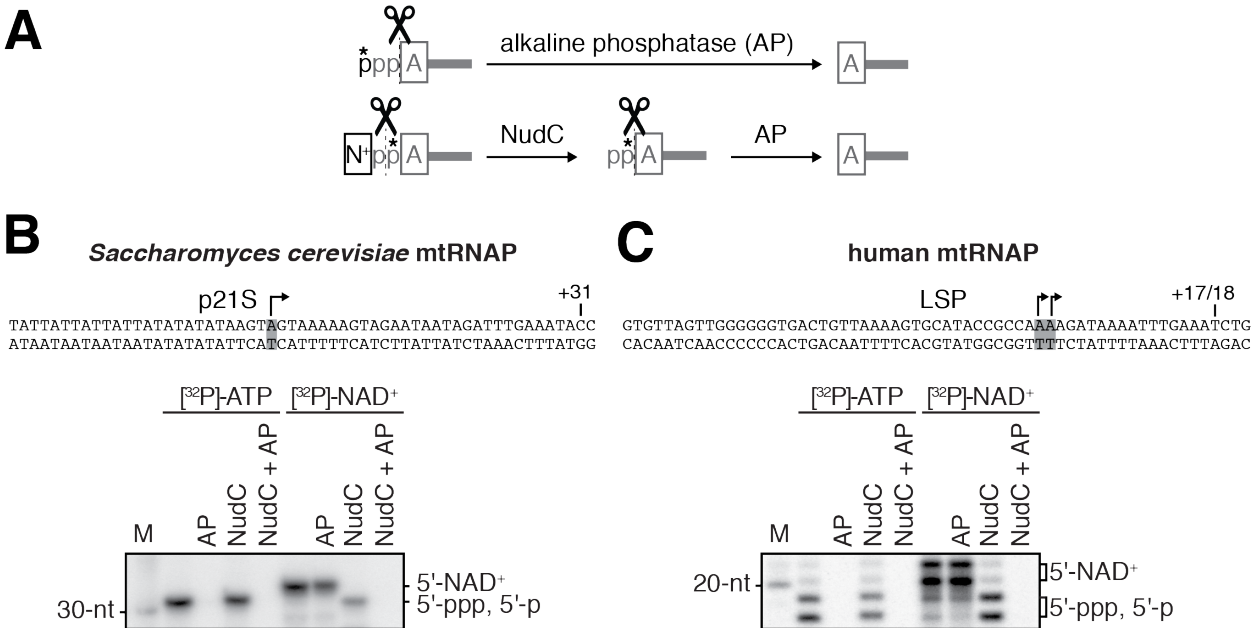


Figure S1 (Related to Figure 1). *S. cerevisiae* and human mtRNAPs cap RNA with NAD+ *in vitro*: additional data

A. Processing of radiolabeled RNA 5'-ends by alkaline phosphatase (AP) and NudC. A, adenosine; N+, NAD+ nicotinamide; p, phosphate. *, radiolabeled phosphate.

B. and C. NCIN capping with NAD+ by *S. cerevisiae* mtRNAP (B) and human mtRNAP (C). Top, promoter derivatives. Bottom, full-length RNA products of *in vitro* transcription reactions with [γ^{32}P]-ATP or [α^{32}P]-NAD+ as initiating nucleotide and GTP, ATP, UTP, and 3'-deoxy-CTP (C), or GTP, ATP, and UTP (D) as extending nucleotides. Products were treated with NudC alone, AP alone, or NudC and AP, as indicated. Grey box and arrow, TSS; +31 and +17/18, position of last NTP incorporated into RNA; M, 10-nt marker.

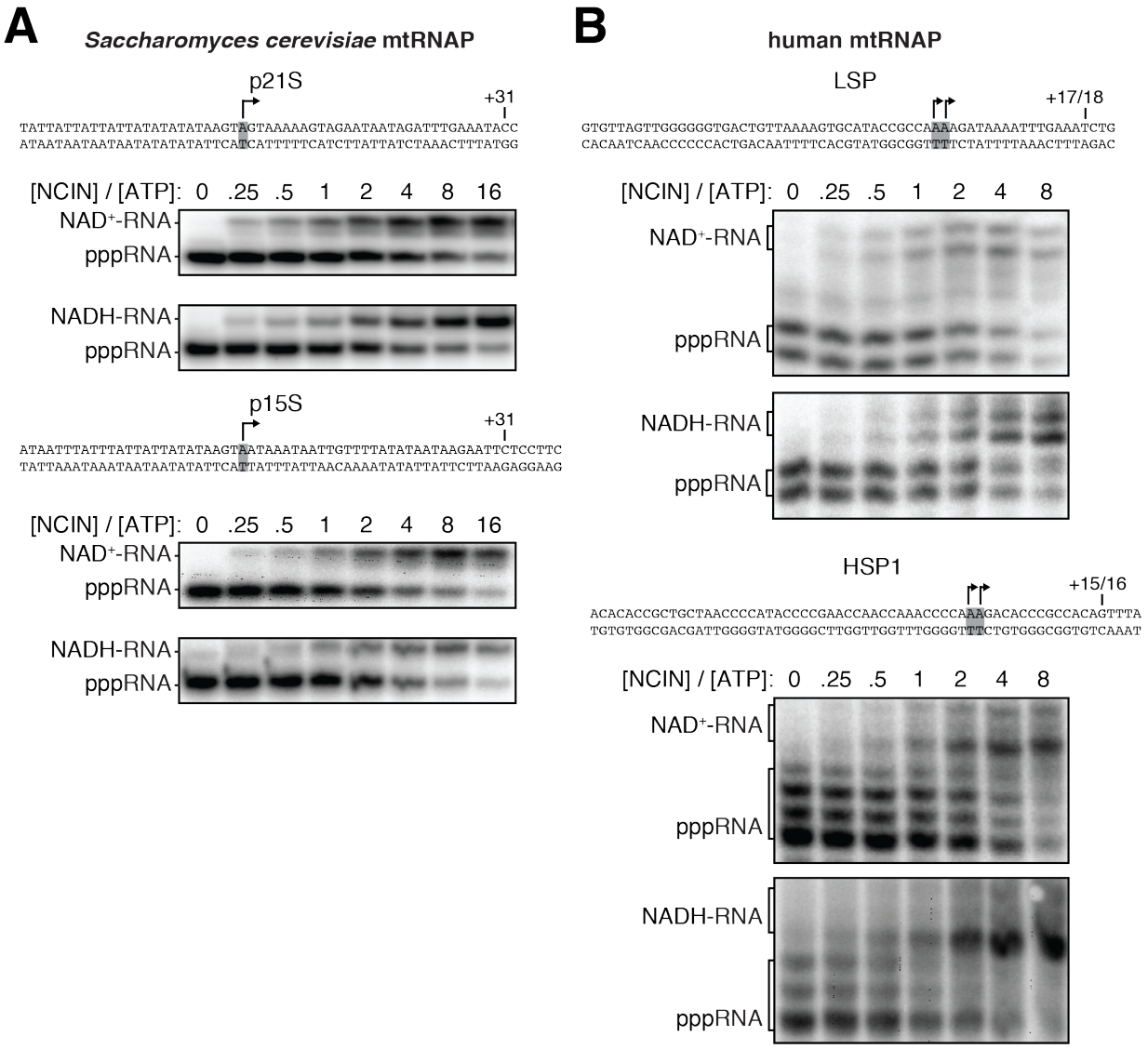


Figure S2 (Related to Figure 2). Dependence of NCIN-mediated capping with NAD+ and NADH on [NCIN] / [ATP] ratio for mtRNAPs: representative data

A. and B. Panels show DNA templates and full-length RNA products of *in vitro* transcription reactions performed with *S. cerevisiae* mtRNAP (A) and human mtRNAP (B) with the indicated [NCIN] / [ATP] ratio. Grey box and arrow, TSS; +31, +17/18, +15/16, position of last NTP incorporated into RNA; M, 10-nt marker.

A

bacteriophage T7 RNAP

-1 +1 ϕ2.5

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CAGTAATACGACTCACTATTAGCGAAGCGGGCATGCGGCCAGCCATAGCCGATCA -1A:T
GTCATTATGCTGAGTGATAATCGCTTCGCCCGTACGCCGGTCGGTATCGGCTAGT

CAGTAATACGACTCACTATTAGCGAAGCGGGCATGCGGCCAGCCATAGCCGATCA -1G:C
GTCATTATGCTGAGTGATAATCGCTTCGCCCGTACGCCGGTCGGTATCGGCTAGT

CAGTAATACGACTCACTATTAGCGAAGCGGGCATGCGGCCAGCCATAGCCGATCA -1T:A
GTCATTATGCTGAGTGATAATCGCTTCGCCCGTACGCCGGTCGGTATCGGCTAGT

CAGTAATACGACTCACTATTAGCGAAGCGGGCATGCGGCCAGCCATAGCCGATCA -1C:G
GTCATTATGCTGAGTGATAATCGCTTCGCCCGTACGCCGGTCGGTATCGGCTAGT
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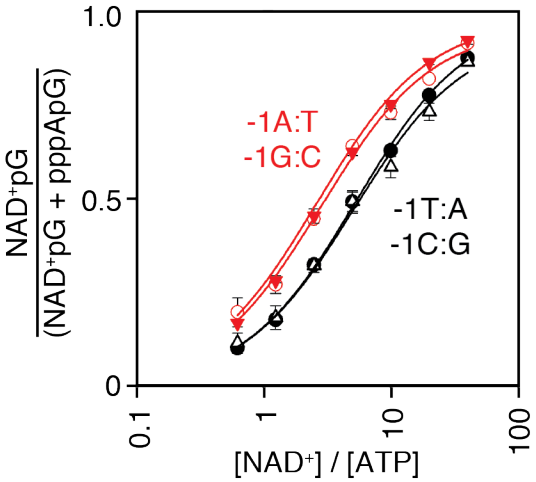
B



promoter	$(k_{cat}/K_M)_{NAD+} / (k_{cat}/K_M)_{ATP}$
-1A:T	0.32 ± 0.03
-1G:C	0.32 ± 0.01
-1T:A	0.18 ± 0.01
-1C:G	0.17 ± 0.01

Figure S3 (Related to Figure 3). Promoter sequence determines efficiency of RNA capping with NAD+: bacteriophage T7 RNAP

A. Bacteriophage T7 RNAP-dependent promoter derivatives analyzed. Red, -1R promoters; black, -1Y promoters; +1 and grey box, bases at the TSS; -1, bases immediately upstream of the TSS.

B. Dependence of NAD+ capping on [NAD+] / [ATP] ratio for homoduplex templates having A:T, G:C, T:A, or C:G at position -1 relative to TSS (mean±SD; n=3).

Table S1 (related to METHODS). Oligoribodeoxynucleotides

Name	Sequence (5' to 3')	Description
DK64	GGCTCGCCTCGGCTCG/iSp18/CGAGCCGAGGCGAGCGTCACCAA	fork-junction template with PEG6 linker
JB459	GTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAAGTATAAA ATTTGTGGGCC	LSP DNA template +1AGU variant non-template strand
JB460	GGCCCACAAATTTTATACTTTGGCGGTATGCACTTTTAACAGTCACCCC CCAACTAACAC	LSP DNA template +1AGU variant template strand
JB469	CAGTAATACGACTCACTATTAGCGAAGCGGGCATGCGGCCAGCCATAGC CGATCA	T7ϕ2.5-35n (-1T:A; WT) non-template strand
JB470	TGATCGGCTATGGCTGGCCGCATGCCCGCTTCGCTAATAGTGAGTCGTA TTACTG	T7ϕ2.5-35n (-1T:A; WT) template strand
JB471	CAGTAATACGACTCACTATAAGCGAAGCGGGCATGCGGCCAGCCATAGC CGATCA	T7ϕ2.5-35n (-1A:T) non-template strand
JB472	TGATCGGCTATGGCTGGCCGCATGCCCGCTTCGCTTATAGTGAGTCGTA TTACTG	T7ϕ2.5-35n (-1A:T) template strand
JB473	CAGTAATACGACTCACTATGAGCGAAGCGGGCATGCGGCCAGCCATAGC CGATCA	T7ϕ2.5-35n (-1G:C) non-template strand
JB474	TGATCGGCTATGGCTGGCCGCATGCCCGCTTCGCTCATAGTGAGTCGTA TTACTG	T7ϕ2.5-35n (-1G:C) template strand
JB475	CAGTAATACGACTCACTATCAGCGAAGCGGGCATGCGGCCAGCCATAGC CGATCA	T7ϕ2.5-35n (-1C:G) non-template strand
JB476	TGATCGGCTATGGCTGGCCGCATGCCCGCTTCGCTGATAGTGAGTCGTA TTACTG	T7ϕ2.5-35n (-1C:G) template strand
JB515	CACCAGCCTAACCAGATTTCAAATTTTATC	probe for LSP-generated RNA (complementary to positions +2 to +31)
JB525	CTATATAATAAATATTTCAAATCTATTATTCTAC	probe for *S. cerevisiae* 21S RNA (complementary to positions +9 to +42)
JB526	ACTCCATGATTAGGCTAGCTACAACGACTCTTTAAATCT	*S. cerevisiae* 21S RNA DNAzyme; cleaves transcript at position +53
JB555	ATCTTAACCTTTAGACTCTTTTGTCTATTTATAATATGT	probe for *S. cerevisiae* COX2 RNA (complementary to positions +8 to +46)
JB557	TCTTAATAAATCTAAGGCTAGCTACAACGAATTTTAATAAATCTT	*S. cerevisiae* COX2 DNAzyme; cleaves at position +57
JB559	GCACTTAAACAGGCTAGCTACAACGAATCTCTGCCA	LSP-generated RNA DNAzyme; cleaves at position +67
JB560	TATATAATAATAAATTATAAATAAATTTTAATTAAAAGTAGTATTAACA TATTATAAATAGACAAAAGAGTCTAAAGGTTAAGATTTATTAAAATGTT AGATTTATTAAGATTACAATTAACAAC	*S. cerevisiae* COX2 -40 to +125 non-template strand oligo (for generation of *in vitro* transcription template)
JB561	TATATAATAATAAATTATAAATAAATTTTAATTAAAAGTAGT	*S. cerevisiae* COX2 -40 to +3 forward primer (for generation of *in vitro* transcription template)

JB562	GTTGTTAATTGTAATCTTAATAAATCTAACATTTTAATAAATC	*S. cerevisiae* COX2 +83 to +125 reverse primer (for generation of *in vitro* transcription template)
UB1	ATGTGTTAGTTGGGGGGTGACTGTTAAAAGTGCATACCGCCAAAAGATA AAATTTGAAATCTG	LSP DNA template (-43 to +19) non-template strand
UB2	CAGATTTCAAATTTTATCTTTTGGCGGTATGCACTTTTAACAGTCACCC CCCAACTAACACAT	LSP DNA template (-43 to +19) template strand
UB3	ACACACCGCTGCTAACCCCATACCCCGAACCAACCAAACCCCAAAGACA CCCGCCACAGTTTA	HSP1 DNA template (-43 to +20) non-template strand
UB4	TAAACTGTGGCGGGTGTCTTTGGGGTTTGGTTGGTTCGGGGTATGGGGT TAGCAGCGGTGTGT	HSP1 DNA template (-43 to +20) template strand
UB5	ATAATTTATTTATTATTATATAAGTAATAAATAATTGTTTTATATAATA AGAATTCTCCTTC	*S. cerevisiae* 15S DNA template (-25 to +1; C-less cassette) non-template strand
UB6	GAAGGAGAATTCTTATTATATAAAACAATTATTTATTACTTATATAATA ATAAATAAATTAT	*S. cerevisiae* 15S DNA template (-25 to +1; C-less cassette) template strand
UB7	TATTATTATTATTATATATATAAGTAGTAAAAAGTAGAATAATAGATTT GAAATACC	S. cerevisiae 21S DNA template (-25 to +1; C-less cassette) non-template strand
UB8	GAAGGAGACCAACCACAAACACACAACAACCACCAACTACTTATATAAT AATAAATAAATTAT	*S. cerevisiae* 21S DNA template (-25 to +1; C-less cassette) template strand
UB9	ATAATTTATTTATTATTATATAAGTAGTTGGTGGTTGTTGTGTGTTTGT GGTTGGTCTCCTTC	*S. cerevisiae* 21S DNA template (-25 to +1; C-less and A-less cassette) non-template strand
UB10	GAAGGAGACCAACCACAAACACACAACAACCACCAACTACTTATATAAT AATAAATAAATTAT	*S. cerevisiae* 21S DNA template (-25 to +1; C-less and A-less cassette) template strand
UB11	ATAATTTATTTATTATTATATAAGAAGTTGGTGGTTGTTGTGTGTTTGT GGTTGGTCTCCTTC	*S. cerevisiae* 21S DNA template -1A:T non-template strand
UB12	GAAGGAGACCAACCACAAACACACAACAACCACCAACTTCTTATATAAT AATAAATAAATTAT	*S. cerevisiae* 21S DNA template -1A:T variant template strand
UB13	ATAATTTATTTATTATTATATAAGGAGTTGGTGGTTGTTGTGTGTTTGT GGTTGGTCTCCTTC	*S. cerevisiae* 21S DNA template -1G:C non-template strand
UB14	GAAGGAGACCAACCACAAACACACAACAACCACCAACTCCTTATATAAT AATAAATAAATTAT	*S. cerevisiae* 21S DNA template -1G:C template strand
UB15	ATAATTTATTTATTATTATATAAGCAGTTGGTGGTTGTTGTGTGTTTGT GGTTGGTCTCCTTC	*S. cerevisiae* 21S DNA template -1C:G non-template strand
UB16	GAAGGAGACCAACCACAAACACACAACAACCACCAACTGCTTATATAAT AATAAATAAATTAT	*S. cerevisiae* 21S DNA template -1C:G template strand

METHODS

Proteins

S. cerevisiae mtRNAP was prepared from *E. coli* strain BL21(DE3) transformed with pJJ1399 (gift of Judith A. Jaehning) using culture and induction procedures, polyethyleneimine treatment, ammonium sulfate precipitation, Ni-sepharose, DEAE sepharose and Heparin-sepharose chromatography as in (Tang et al., 2009). *S. cerevisiae* Mtf1 was prepared from *E. coli* strain BL21(DE3) transformed with pTrcHisC-Mtf1(Paratkar and Patel, 2010) and purified using culture and induction procedures, polyethyleneimine treatment, ammonium sulfate precipitation, and tandem DEAE sepharose and Ni-sepharose chromatography as in (Tang et al., 2009).

Human mtRNAP and TFAM were purified from *E. coli* strain BL21(DE3) transformed with pPROEXHTb-POLRMT(43-1230)-6xHis (Ramachandran et al., 2017) or pPROEXHTb-TFAM(43-245)-6xHis (Ramachandran et al., 2017), respectively, using culture and induction procedures, polyethyleneimine treatment, ammonium sulfate precipitation, and Ni-sepharose and heparin-sepharose chromatography as in (Ramachandran et al., 2017). Human TFB2 was purified from *E. coli* strain ArticExpress (DE3) (Stratagene) transformed with pT7TEV-HMBP4 (Yakubovskaya et al., 2014), using culture and induction procedures, polyethyleneimine treatment, ammonium sulfate precipitation, Ni-sepharose and heparin-sepharose chromatography, and size exclusion chromatography as in (Yakubovskaya et al., 2014).

T7 RNAP was prepared from *E. coli* strain BL21 transformed with pAR1219 using culture and inductions procedures, SP-Sephadex, CM-Sephadex and DEAE-Sephacel chromatography as described in (Jia et al., 1996).

E. coli RNAP core enzyme was prepared from *E. coli* strain NiCo21(DE3) transformed with plasmid pIA900 (Artsimovitch et al., 2003) using culture and induction procedures, immobilized-metal-ion affinity chromatography on Ni-NTA agarose, and affinity chromatography on Heparin HP as in (Artsimovitch et al., 2003).

S. cerevisiae RNA polymerase II core enzyme (gift of Craig Kaplan) was prepared as described in (Barnes et al., 2015).

E. coli NudC was prepared from *E. coli* strain NiCo21(DE3) transformed with plasmid pET NudC-His (Bird et al., 2016) using metal-ion chromatography and size-exclusion chromatography as in (Cahova et al., 2015).

RNA 5' pyrophosphohydrolase (RppH) and T4 polynucleotide kinase (PNK) were purchased from New England Biolabs (NEB). FastAP Thermosensitive Alkaline Phosphatase was purchased from Thermo Fisher Scientific. Molar concentrations of purified proteins were determined by light absorbance at 280 nm and the calculated respective molar extinction coefficients.

Oligodeoxyribonucleotides

Sequences of the oligodeoxyribonucleotides used in this work are provided in Table S1. All oligodeoxyribonucleotides were purchased from Integrated DNA Technologies (IDT) with standard desalting purification unless otherwise specified.

Linear *in vitro* transcription templates used for transcription assays shown in Figures 1, 2, 3, S1, S2, and S3 were generated by mixing complementary nontemplate- and template-strand DNA in a molar ratio of 1.1 to 1 in annealing buffer (10 mM Tris HCl pH 8.0), incubating the mixture at 95°C for 5 min, and cooling the mixture by 0.5°C per minute to 25°C.

Transcription templates used to generate *in vitro* RNA standards for Northern analysis (Figures 4 and 5) were generated by PCR. PCR reactions contained a mixture of 5 nM template oligo, 0.5 μM forward primer, 0.5 μM reverse primer, and Phusion HF Master Mix (Thermo Scientific). Reaction products were isolated using a Monarch PCR & DNA cleanup kit (NEB).

The radiolabeled 10-nt marker labeled "M" in gel images shown in Figures 1, 4, 5, and S1 was generated using the Decade Marker System (Thermo Fisher Scientific), PNK (NEB) and [γ^{32}P]-ATP (Perkin Elmer; 6,000 Ci/mmol).

In vitro transcription assays

Assays performed with *S. cerevisiae* mtRNAP were based on procedures described in (Deshpande and Patel, 2014) Assays performed with human mtRNAP were based on procedures described in (Ramachandran et al., 2017),

For initial RNA product assays in Figure 1C, 1 μM DNA template, 1 μM *S. cerevisiae* mtRNAP, and 1 μM Mtf1 were incubated at 25°C for 10 min in *Sce*-mtRNAP reaction buffer (50 mM Tris-acetate, pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 0.01% Tween-20, 1 mM DTT and 5% glycerol). A mixture containing the initiating nucleotide (200 μM ATP, 1 mM NAD^+, or 1mM NADH) and extending nucleotide (10 μM of non-radiolabeled GTP plus 6 mCi of [α^{32}P]-GTP at 3000 Ci/mmol) was added, and assays were incubated at 25°C for 30 min. Radiolabeled initial products were isolated using a Nanosep 3 kDa cutoff spin concentrator (Pall). For Figure 3B, reactions were stopped with 10 μl RNA loading dye (95% deionized formamide, 18 mM EDTA, 0.25% SDS, xylene cyanol, bromophenol blue, amaranth) and directly loaded onto 7.5 M urea, 1x TBE, 20% polyacrylamide gels (UreaGel System; National Diagnostics), followed by storage-phosphor imaging (Typhoon 9400 variable-mode imager; GE Life Science).

For the initial RNA product assays in Figure 1D, 1 μM DNA template, 1 μM human mtRNAP, 1 μM TFAM, and 1 μM TFB2M were incubated at 25°C for 10 min in human-mtRNAP reaction buffer (50 mM Tris-acetate, pH 7.5, 50 mM sodium glutamate, 10 mM magnesium acetate, 1 mM DTT and 0.05% Tween-20). A mixture containing the initiating nucleotide (200 μM ATP, 1 mM NAD^+, or 1 mM NADH) and extending nucleotide (10 μM of non-radiolabeled GTP plus 6 mCi of [α^{32}P]-GTP at 3000 Ci/mmol) was added, and assays were incubated at 25°C for 60 min. Radiolabeled initial RNA products were isolated using a Nanosep 3 kDa cutoff spin concentrator (Pall).

A portion of the recovered initial RNA products were mixed with either 10 U of RppH or 400 nM NudC and incubated at 37°C for 30 min. Reactions were stopped by addition of 10 μl RNA loading dye. Samples were analyzed by electrophoresis on 7.5 M urea, 1x TBE, 20% polyacrylamide gels (UreaGel System; National Diagnostics),

followed by storage-phosphor imaging (Typhoon 9400 variable-mode imager; GE Life Science).

For full-length product assays in Figures 1C and S1B, 1 μM DNA template, 1 μM *S. cerevisiae* mtRNAP, and 1 μM Mtf1 were incubated at 25°C for 10 min in *Sce*-mtRNAP reaction buffer. A mixture containing the initiating nucleotide (200 μM ATP, 1 mM NAD^+, or 1mM NADH for Figure 1C; 200 μM non-radiolabeled ATP plus 10 μCi [γ^{32}P]-ATP at 6,000 Ci/mmol, or 1 mM NAD^+ plus 20 μCi [α^{32}P]-NAD^+ at 800 Ci/mmol for Figure S1B) and extending nucleotides (200 μM GTP, 200 μM 3'-deoxy-CTP, 20 μM ATP, 200 μM non-radiolabeled UTP, and 6 mCi of [α^{32}P]-UTP at 3000 Ci/mmol for Figure 1C; 200 μM GTP, 200 μM 3'-deoxy-CTP, 20 μM ATP, 200 μM UTP for Figure 1D) was added, and assays were incubated at 25°C for 30 min. Reactions were stopped by addition of 1.5x stop solution (0.6 M Tris HCl pH 8.0, 18 mM EDTA, 0.1 mg/ml glycogen), samples were extracted with acid phenol:chloroform (pH 4.5; Thermo Fisher Scientific), and RNA products were recovered by ethanol precipitation and resuspended in NudC reaction buffer (10 mM Tris HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT).

For full-length product assays in Figures 1D and S1C, 1 μM DNA template, 1 μM human mtRNAP, 1 μM TFAM, and 1 μM TFB2M were incubated at 25°C for 10 min in human-mtRNAP reaction buffer. A mixture containing the initiating nucleotide (200 μM ATP, 1 mM NAD^+, or 1mM NADH for Figure 1C; 200 μM non-radiolabeled ATP plus 10 μCi [γ^{32}P]-ATP at 6,000 Ci/mmol or 1 mM NAD^+ plus 20 μCi [α^{32}P]-NAD^+ at 800 Ci/mmol for Figure S1B) and extending nucleotides (200 μM GTP, 20 μM ATP, 200 μM non-radiolabeled UTP, and 6 mCi of [α^{32}P]-UTP at 3000 Ci/mmol for Figure 1C; 200 μM GTP, 20 μM ATP, 200 μM UTP for Figure S2C) was added, and assays were incubated at 25°C for 60 min. Reactions were stopped by addition of 1.5x stop solution, samples were extracted with acid phenol:chloroform (pH 4.5), RNA products were recovered by ethanol precipitation and resuspended in NudC reaction buffer.

Full-length RNA products were incubated at 37°C for 30 min with 400 nM NudC alone (Figures 1C-D and S1B-C), 0.25 U FastAP Thermosensitive Alkaline Phosphatase alone (Figure S1B-C), or both NudC and FastAP (Figure S1B-C). Reactions were stopped by addition of an equal volume of 2x RNA loading dye (95% deionized formamide, 18 mM EDTA, 0.25% SDS, xylene cyanol, bromophenol blue, amaranth). Samples were analyzed by electrophoresis on 7.5 M urea, 1x TBE, 20% polyacrylamide gels (UreaGel System; National Diagnostics), followed by storage-phosphor imaging (Typhoon 9400 variable-mode imager; GE Life Science).

Determination of efficiency of NCIN-mediated initiation vs. ATP-mediated initiation, $(k_{cat}/K_M)_{NCIN}$ / $(k_{cat}/K_M)_{ATP}$, *in vitro*: full-length product assays

For experiments in Figures 2A-B and S2, 1 μM of template DNA, 1 μM of mtRNAP, and 1 μM transcription factor(s) (Mtf1 for *S. cerevisiae* mtRNAP; TFAM and TFB2M for human mtRNAP) were incubated at 25°C for 10 min in *Sce*-mtRNAP or human reaction buffer. A mixture containing 200 μM ATP, 200 μM UTP, 200 μM non-radiolabeled GTP, and 6 mCi [α^{32}P]-GTP at 3000 Ci/mmol and NCIN (0, 50, 100, 200, 400, 800, 1600, 3200, 6400 μM) was added, and assays were incubated at 25°C for 30

min. Reactions were stopped by addition of an equal volume of 2x RNA loading dye. Samples were analyzed by electrophoresis on 7.5 M urea, 1x TBE, 20% polyacrylamide gels (UreaGel System; National Diagnostics) supplemented with 0.2% 3-acrylamidophenylboronic acid (Boron Molecular), followed by storage-phosphor imaging (Typhoon 9400 variable-mode imager; GE Life Science).

Bands corresponding to uncapped (pppRNA) and NCIN-capped (NCIN-RNA) full-length products were quantified using ImageQuant software. The ratio of NCIN-RNA to total RNA [NCIN-RNA / (pppRNA + NCIN-RNA)] was plotted vs. the relative concentrations of NCIN vs. ATP ([NCIN] / [ATP]) on a semi-log plot (SigmaPlot) and non-linear regression was used to fit the data to the equation: y = (ax) / (b+x); where y is [NCIN-RNA / (pppRNA + NCIN-RNA)], x is ([NCIN] / [ATP]), and a and b are regression parameters. The resulting fit yields the value of x for which y = 0.5. The relative efficiency $(k_{cat}/K_M)_{NCIN}$ / $(k_{cat}/K_M)_{ATP}$ is equal to 1/x.

Determination of efficiency of NCIN-mediated initiation vs. ATP-mediated initiation, $(k_{cat}/K_M)_{NCIN}$ / $(k_{cat}/K_M)_{ATP}$, *in vitro*: initial product assays

For experiments in Figures 3C-D and S3, 1 μM of template DNA, 1 μM of mtRNAP, and 1 μM transcription factor(s) (Mtf1 for *S. cerevisiae* mtRNAP; TFAM and TFB2M for human mtRNAP; none for T7 RNAP) were incubated at 25°C for 10 min in *Sce*-mtRNAP, human mtRNAP reaction buffer, or T7 RNAP reaction buffer (40 mM Tris HCl pH 7.9, 6 mM $MgCl_2$, 2 mM DTT, 2 mM Spermidine). A mixture containing 1 mM NCIN, ATP (0, 25, 50, 100, 200, 400, 800, 1600 mM), 20 μM non-radiolabeled GTP, and 6 mCi [α^{32}P]-GTP at 3000 Ci/mmol was added, and assays were incubated at 25°C for 30 min. Reactions were stopped by addition of an equal volume of 2x RNA loading dye. Samples were analyzed by electrophoresis on 7.5 M urea, 1x TBE, 20% polyacrylamide gels (UreaGel System; National Diagnostics) supplemented with 0.2% 3-acrylamidophenylboronic acid (Boron Molecular), followed by storage-phosphor imaging (Typhoon 9400 variable-mode imager; GE Life Science).

For experiments in Figure 2C, 200 nM of fork-junction template and 500 nM RNAP (*S. cerevisiae* mtRNAP, human mtRNAP, *S. cerevisiae* RNAP II, or *E. coli* RNAP) were incubated at 25°C for 15 min in standardized transcription buffer (10 mM Tris pH 8.0, 50 mM potassium glutamate, 10 mM $MgCl_2$, 2 mM DTT, 50 ug/ml BSA). A mixture containing NCIN (1 mM NCIN for mtRNAPs and *E. coli* RNAP; 4 mM for *S. cerevisiae* RNAP II), ATP (0, 25, 50, 100, 200, 400, 800, 1600 μM for mtRNAPs and *S. cerevisiae* RNAP II; 0, 6.25, 12.5, 25, 50, 100, 200, 400 μM for *E. coli* RNAP), 10 μM non-radiolabeled CTP, and 6 mCi [α^{32}P]-CTP at 3000 Ci/mmol was added, and assays were incubated at 25°C for 1 h. Reactions were stopped by addition of an equal volume of 2x RNA loading dye. Samples were analyzed by electrophoresis on 7.5 M urea, 1x TBE, 20% polyacrylamide gels (UreaGel System; National Diagnostics), followed by storage-phosphor imaging (Typhoon 9400 variable-mode imager; GE Life Science).

Bands corresponding to uncapped (pppApC) and NCIN-capped (NCINpC) initial RNA products were quantified using ImageQuant software. The ratio of NCINpC to total RNA [NCINpC / (pppApC + NCINpC)] was plotted vs. the relative concentrations of NCIN vs. ATP ([NCIN] / [ATP]) on a semi-log plot (SigmaPlot) and non-linear regression

was used to fit the data to the equation: y = (ax) / (b+x); where y is [NCINpC / (pppApC + NCINpC)], x is ([NCIN] / [ATP]), and a and b are regression parameters. The resulting fit yields the value of x for which y = 0.5. The relative efficiency $(k_{cat}/K_M)_{NCIN}$ / $(k_{cat}/K_M)_{ATP}$ is equal to 1/x.

Detection and quantitation of NAD$^+$- and NADH-capped mitochondrial RNA *in vivo*: isolation of total cellular RNA from *S. cerevisiae*

For analysis of NAD$^+$ and NADH capping during respiration, *S. cerevisiae* strain AJ82 (*MATα ura3 trp1 leu2 his4*; gift of Andrew Vershon, Rutgers University) was grown at 30°C in 25 ml YPEG (24 g Bacto-tryptone, 20 g Bacto-yeast extract, 30 mL ethanol, 30% glycerol per liter) in 125 ml flasks (Bellco) shaken at 220 rpm. When cell density reached an OD600 ~1.8 (approximately 24 hours) the cell suspension was centrifuged to collect cells (5 min, 10,000 g at 4°C), supernatants were removed, and cell pellets were resuspended in 0.8 mL RNA extraction buffer (0.5 mM NaOAc pH 5.5, 10 mM EDTA, 0.5% SDS).

For analysis of NAD$^+$ and NADH capping during fermentation, *S. cerevisiae* strain AJ82 was grown at 30°C in 25 ml YPD [24 g Bacto-tryptone, 20 g Bacto-yeast extract, 2% (w/v) glucose] in 125 ml flasks (Bellco) with airlocks to prevent oxygenation without shaking for 46 h. The cell suspension was centrifuged to collect cells (5 min, 10,000 g at 4°C), supernatants were removed, and cell pellets were resuspended in 0.8 mL RNA extraction buffer (0.5 mM NaOAc pH 5.5, 10 mM EDTA, 0.5% SDS).

To extract RNA, an equal volume of 5:1 acid phenol:chloroform (pH 4.5) (Thermo Fisher Scientific) was added to cells in resuspension buffer and mixed by vortexing for 10 s. Samples were incubated at 65°C for 5 min, incubated on dry ice for 5 min, and centrifuged (15 min, 21,000 g, 4°C) to separate the aqueous and organic phases. The aqueous phase was removed and the extraction with acid phenol:chloroform was repeated two more times. RNA transcripts were recovered from the aqueous phase by ethanol precipitation and resuspended in RNase free H_2O.

Detection and quantitation of NAD$^+$- and NADH-capped mitochondrial RNA *in vivo*: isolation of total cellular RNA from human cells

Human embryonic kidney HEK293T cells (obtained from ATCC) were maintained under 5% CO2 at 37°C in DMEM medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Atlanta Biologics), 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293T cells were seeded and grown in 100 mm tissue-culture treated plates for 72 h without FK866 treatment or seeded in 100 mm tissue-culture treated plates, grown for 24 h, treated with 5 nM FK866 (Sigma Aldrich), and grown for an additional 48 h. Total cellular RNA was isolated with TRIzol Reagent according to the manufacture's protocol (Thermo Fisher Scientific).

Detection and quantitation of NAD$^+$- and NADH-capped mitochondrial RNA *in vivo*: DNAzyme cleavage

For analysis of NCIN capping of *S. cerevisiae* mitochondrial RNA, 40 μg of total cellular RNA was mixed with 1 μM DNAzyme (JB557 for *S. cerevisiae* COX2 RNA;

JB526 for *S. cerevisiae* 21S RNA) in buffer containing 10 mM Tris pH 8.0, 50 mM NaCl, 2 mM DTT, and 10 mM $MgCl_2$ (total volume 50 μl). When present, NudC was added to 400 nM. Reactions were incubated for 60 min at 37°C and 100 μl of 1.5x stop solution and 500 μl ethanol was added. Samples were centrifuged (30 min, 21,000 g, 4°C), the supernatant removed, and the pellet resuspended in 2x RNA loading dye.

For analysis of NCIN capping of human mitochondrial RNA, 40 μg of total cellular RNA was mixed with 1 μM DNAzyme (JB559 for human LSP-generated RNA) in buffer containing 10 mM Tris pH 8.0, 50 mM NaCl, 2 mM DTT (total volume 50 μl). Samples were heated to 85°C for 5 minutes, cooled to 37°C. $MgCl_2$ was added to a final concentration of 10 mM and, when present, NudC was added to 400 nM. Reactions were incubated for 60 min at 37°C and 100 μl of 1.5x stop solution and 500 μl ethanol was added. Samples were centrifuged (30 min, 21,000 g, 4°C), the supernatant removed, and the pellet resuspended in 2x RNA loading dye.

To prepare synthetic RNA standards non-radiolabeled full-length RNA products were generated by *in vitro* transcription reactions containing 1 μM of template DNA, 1 μM of mtRNAP, 1 μM transcription factor(s), 1 mM initiating nucleotide (ATP, NAD^+ or NADH), 200 μM GTP, 200 μM UTP, 200 μM CTP, and 20 μM ATP. Reactions were incubated for 60 min at 25°C, stopped by addition of 1.5x stop solution, extracted with acid phenol:chloroform (pH 4.5) and ethanol precipitated. Full-length RNAs were resuspended in buffer containing 10 mM Tris pH 8.0, 50 mM NaCl, 2 mM DTT, and 10 mM $MgCl_2$ and treated with DNAzyme as described above.

Detection and quantitation of NAD^+- and NADH-capped mitochondrial RNA *in vivo*: hybridization with a radiolabeled oligodeoxyribonucleotide probe

NCIN capping of DNAzyme-generated subfragments of mitochondrial RNA were analyzed by a procedure consisting of: (i) electrophoresis on 7.5 M urea, 1x TBE, 10% polyacrylamide gels supplemented with 0.2% 3-acrylamidophenylboronic acid; (ii) transfer of nucleic acids to a Nytran supercharge nylon membrane (GE Healthcare Life Sciences) using a semidry transfer apparatus (Bio-Rad); (iii) immobilization of nucleic acids by UV crosslinking; (iv) incubation with a ^{32}P-labelled oligodeoxyribonucleotide probe complementary to target RNAs (JB555 to COX2 RNA; JB525 to 21S RNA; JB515 to LSP-derived RNA; ^{32}P-labelled using T4 polynucleotide kinase and [$\gamma^{32}P$]-ATP (Perkin Elmer; 6,000 Ci/mmol); (v) high-stringency washing, procedures as in (Goldman et al., 2015); and (vi) storage-phosphor imaging.

Bands corresponding to uncapped and NCIN-capped DNAzyme-generated subfragments were quantified using ImageQuant software. The percentages of uncapped RNA (5'-ppp), NAD^+-capped RNA (5'-NAD^+), or NADH-capped RNA (5'-NADH) to total RNA were determined from three biological replicates. The average values of these measurements are reported in Figures 4 and 5.

Determination of NAD(H) levels in human HEK293T cells

Cells were lysed in 400 µl of NAD/H Extraction Buffer (NAD/H Quantitation Kit, Sigma-Aldrich) and total proteins were extracted with two freeze-thaw cycles (20 min on dry ice, 10 min at RT). Lysates were centrifuged (10 min, 13,000 g, 4°C), the

supernatant was isolated, and the protein concentration was determined. NAD(H) was measured using a NAD/H Quantitation Kit (Sigma) from a volume of supernatant containing 10 µg protein.

Molecules of NAD(H) per cell were calculated using a value of 300 pg total protein per cell. The cellular concentration of NAD(H) was then estimated by using a cellular volume of ~ 1200 μm^3 for HEK293T cells and assuming a homogenous distribution of NAD(H) in the cell. The volume of HEK293T cells was calculated using a value of 13 μm for the diameter of HEK293T cells, assuming trypsinized cells adopt a spherical shape.

QUANTIFICATION AND STATISTICAL ANALYSIS

Figure Legends indicate that values represent averages and standard deviations from three independent measurements.

ADDITIONAL RESOURCES

Table S1 (Related to Methods). List of oligoribodeoxynucleotides.