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Spatially-Restricting Bioorthogonal Nucleoside Biosynthesis Enables Selective Metabolic Labeling of the Mitochondrial Transcriptome

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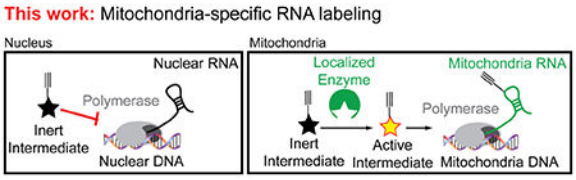
Abstract

The cellular RNA pool in animals arises from two separate genomes stored in the nucleus and multiple mitochondria. Chemical methods to track nascent RNA synthesis are unable to distinguish between these two with stringency. Herein we report that spatially restricting bioorthogonal nucleoside biosynthesis enables, for the first time, selective metabolic labeling of the RNA transcribed in the mitochondria. We envision this approach could open the door for heretofore-impossible analyses of mitochondrial RNA. Beyond our results revealed herein, our approach provides a roadmap for researchers to begin to design strategies to examine biomolecules within subcellular compartments.

Graphical Abstract



Understanding the synthesis and lifetime of RNA molecules is key to elucidating how cells behave and control their biology[1, 2] Recent approaches toward understanding the dynamics of transcription have relied on metabolic labeling of RNA.[3–5] For example, alkynyl-[6–8] and azido-containing[9] modified nucleoside metabolic intermediates can be introduced into cells

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ASSOCIATED CONTENT

Supporting Information. Experimental methods, synthetic schemes and spectra, for all compounds are available free of charge via the Internet at http://pubs.acs.org.

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and nascent RNA can be tracked through bioorthogonal chemical reactions. Both Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) and strain-promoted alkyne-azide cycloaddition (SPAAC) can be utilized for imaging or biotinylation and streptavidin enrichment of labeled RNA.

RNAs are transcribed from two DNA repositories inside cells: the nucleus and the mitochondria. Both organelles have distinct genome sequences and thus can give rise to distinct RNA pools that perform diverse biological functions. A critical missing component in metabolic labeling of RNA (as well as other biomolecules) is the ability to distinguish with high stringency between RNA that arises from the two genomes. This represents a major challenge to overcome and may present a unique opportunity to expand the capabilities of chemical methods to study RNA molecules in living systems.

Mitochondria are the powerhouses of the cell and are vitally important for the normal physiology of every cell type. Changes in their function have been linked to many cancer types and neurological disorders.[10, 11] Characterizing the molecular components of the mitochondria is critical for understanding its function. For mitochondrial analysis the traditional method is fractionation and enrichment.[12–17] These approaches are extremely laborious and suffer from a very high false-positive rate.[18] This is mostly attributed to the reportedly high RNA content on the outer membrane of the mitochondria.[12, 13, 19, 20] Furthermore, fractionation methods suffer from the inability to track nascent RNA synthesis, due to mitochondria still being viable during centrifugation protocols.[21–23] The canonical animal mitochondrial transcriptome consists of 13 mRNAs, 22 tRNAs, and 2 rRNAs, whose expression can be profiled by conventional RT-PCR;[24] however, recent reports have suggested that the types of RNAs originating from the mitochondrial genome are much more complex than previously thought, surprisingly comprising miRNAs, antisense RNAs, and long non-coding RNAs.[12, 13, 25–27] Moving beyond such a narrow view of mitochondrial RNA and identifying novel RNAs that arise from the mitochondrial genome is a major challenge in the field.[12, 13] The lack of tools to stringently purify and identify new RNAs solely transcribed from the mitochondrial genome (while removing labeling of RNAs from the nuclear genome) prevents such analyses and discovery.

Within this Letter we report a novel approach toward addressing the many weaknesses described above. We demonstrate that spatially-restricting modified nucleotide biosynthesis within mitochondria, permits selective metabolic labeling of RNA that arises from the mitochondrial transcriptome (Figure 1**)**. We also show that this approach can be used to specifically track mitochondrial RNA synthesis.

We recently demonstrated that nascent RNA synthesis can be tracked using 5-ethynyluracil (Inert Intermediate) with the enzyme uracil phosphoribosyltranferase (UPRT).[28] UPRT converts uracil and phosphoribosyl pyrophosphate (PRPP) to 5’-phosphoro-5-ethynyluridine (Active Intermediate, 5’P-5EU), which is eventually incorporated into nascent RNA. Our original hypothesis was that we could spatially restrict UPRT to enable nascent RNA labeling within the mitochondria.

Consistent with this hypothesis PRPP has been observed within chloroplasts, which are plant organelles with their own genomes akin to mitochondria.[29] Mitochondria are also known to have nucleoside kinase enzymes localized within their inner matrix, which would permit the eventual biosynthesis of a modified nucleoside triphosphate.[30, 31] As such, we rationalized that localizing UPRT in the mitochondria may permit specific synthesis of 5'P-5EU (Figure 2, A) and eventual RNA labeling.

To localize UPRT into the mitochondria we constructed a Mito-GFP-UPRT fusion construct (Figure 2, B). Cellular imaging of the fusion demonstrated precise localization into the mitochondria, with strong overlap with a mitochondrial localized red fluorescent protein (Figure 2, C) and mitotracker fluorescent dye, which localizes into the mitochondrial matrix (Figure S1).

To demonstrate that the Mito-GFP-UPRT fusion converts 5EU to 5'P-5EU, with eventual incorporation into RNA, we performed a time-course dot blot analysis. 5EU was fed to cell media and RNA incorporation was assayed using dot blot analysis as shown in Figure 3, A and Figure S2. Comparison to whole-cell UPRT, there was markedly less RNA signal, which may be attributed to either weak activity or the extremely low percentage of total RNA that is comprised of mitochondrial RNA. Normal fractionation of mitochondria (inner matrix, outer membrane, and cytosolic RNA) demonstrated that the majority of signal is in mitochondrial-enriched RNA, with undetectable labeling of cytoplasmic RNA (Figure S3). Furthermore, to test for potential toxicity, we isolated mitochondria and performed a Cytochrome c oxidase assay, which reports on the activity and concentration of Cytochrome c at the mitochondrial membrane. As shown in Figure S4, there was no observed difference in Cytochrome c activity in the presence of 5EU or not, even after 8 hours of 5EU incubation. Overall, these data suggest that our approach can be utilized to metabolically label RNA in the mitochondrial matrix.

To identify RNA synthesis within the mitochondria with higher stringency we used confocal imaging. After treatment with 5EU, cells were fixed, permeabilized, and stringently washed. RNA was imaged using CuAAC-mediated reaction to append Cy5-azide at 5'ethynyl uracil residues. As shown in Figure 3, B, cells expressing Mito-GFP-UPRT had RNA signal (Cy5-N_3) that overlapped with Mito-GFP-UPRT localization. In contrast, cells expressing spatially-unrestricted UPRT had the large majority of their signal in the nucleus, which is consistent with our previous results with significant metabolic labeling of rRNA within the nucleolus (Figure 3, C).[32] RNase treatment of labeled Mito-GFP-UPRT cells or Mito-GFP-UPRT cells lacking 5EU showed loss of signal in mitochondria, further demonstrating that CuAAC-mediated staining is specific for RNA (Figure S5).

To explore the selectivity of RNA incorporation we assayed RNA enrichment by RT-PCR. We chose five RNAs to compare: (1) GAPDH mRNA, which is one of the most highly abundant RNAs transcribed from the nuclear genome, (2) 18S rRNA a highly abundant RNA from the cytoplasmic ribosome, (3) U6 snRNA, an abundant splicing RNA that is transcribed in the nucleus, (4) 12S mitochondrial rRNA, and (5) MT-CO2 mRNA (cytochrome-c oxidase 2), mitochondrial mRNA.

Briefly, Mito-GFP-UPRT-containing cells were incubated with 5EU for 5 hours, and total RNA isolated. CuAAC-mediated reaction appended biotin to total RNA. Biotin-appended RNA was enriched and profiled by RT-PCR (Supplementary Information). As shown in Figure 4, A, enrichment RT-PCR clearly demonstrates enrichment of all assayed RNAs in cells expressing UPRT. RNAs transcribed from the mitochondrial genome are selectively enriched and those from the nuclear genome are not in Mito-GFP-UPRT expressing cells. This result is especially impressive because the raw copy numbers of the RNAs coming from the nuclear genome (such as 18S rRNA) are much higher than those of mitochondria, and as such any incorporation of 5EU into these RNAs would be robust. These results nicely demonstrate that mitochondrial RNA is selectively labeled over RNA transcribed from the nuclear genome.

Nascent tracking of RNA synthesis is an important experimental approach toward characterizing RNA expression dynamics. Although there has been considerable analysis of nuclear RNA expression through pulse-labeling of nucleoside analogs, the approach has yet to be demonstrated in an approach only labeling mitochondrial RNA. We sought to test if our strategy could be used to track RNA synthesis for mitochondrial RNA. For this, we tracked several mitochondrial RNAs: MT-CO1/MT-CO2 mRNAs (cytochrome c oxidase subunit I & II enzymes, also known as COX1 and COX2), CYB mRNA (cytochrome B), ND2/ND3/ND5 mRNAs (NADH dehydrogenase subunits 2, 3 and 5), [33, 34] and along with nuclear transcribed RNA such as GAPDH and U6. Mito-GFP-UPRT-containing cells were treated with 5EU and total RNA isolated at different time points. RNA was enriched and quantified using RT-qPCR. As shown in Figure 4, B, we were able to enrich and track the nascent transcription of the mitochondrial RNAs, but not RNAs from the nuclear genome. Overall, these results demonstrate the ability to stringently label mitochondrial RNA (and not nuclear RNA), they also show the power of our approach in being able to track nascent RNA synthesis and dynamics.

Herein we have developed a method for spatially-restricted tracking of RNA biosynthesis in cells, through the controlled localization of an enzyme controlling bioorthogonal nucleotide biosynthesis. We have demonstrated, through imaging, that our Mito-specific UPRT-mediated analog and nascent RNA labeling with a bioorthogonal handle co-localize inside cells. Furthermore, we have demonstrated distinct populations of the controlled labeling of mitochondrial RNA, and undetectable labeling of RNA arising from the nuclear genome. This approach is well-positioned to enable controlled and high-stringency analysis of the dynamics of mitochondrial RNA synthesis, as demonstrated in Figure 4. This approach can be married with more unbiased analyses, such as RNA sequencing, to preferentially uncover novel RNA molecules produced from the mitochondrial genome. Furthermore, as the analogs and enzymes used herein have already been demonstrated to work in animals we anticipate our approach may also be used to study the dynamics of mitochondrial RNA expression in living animals,[7] even perhaps in complex environments such as the central nervous system.[35] Future efforts will be focused on extending these findings into complex cellular environments, *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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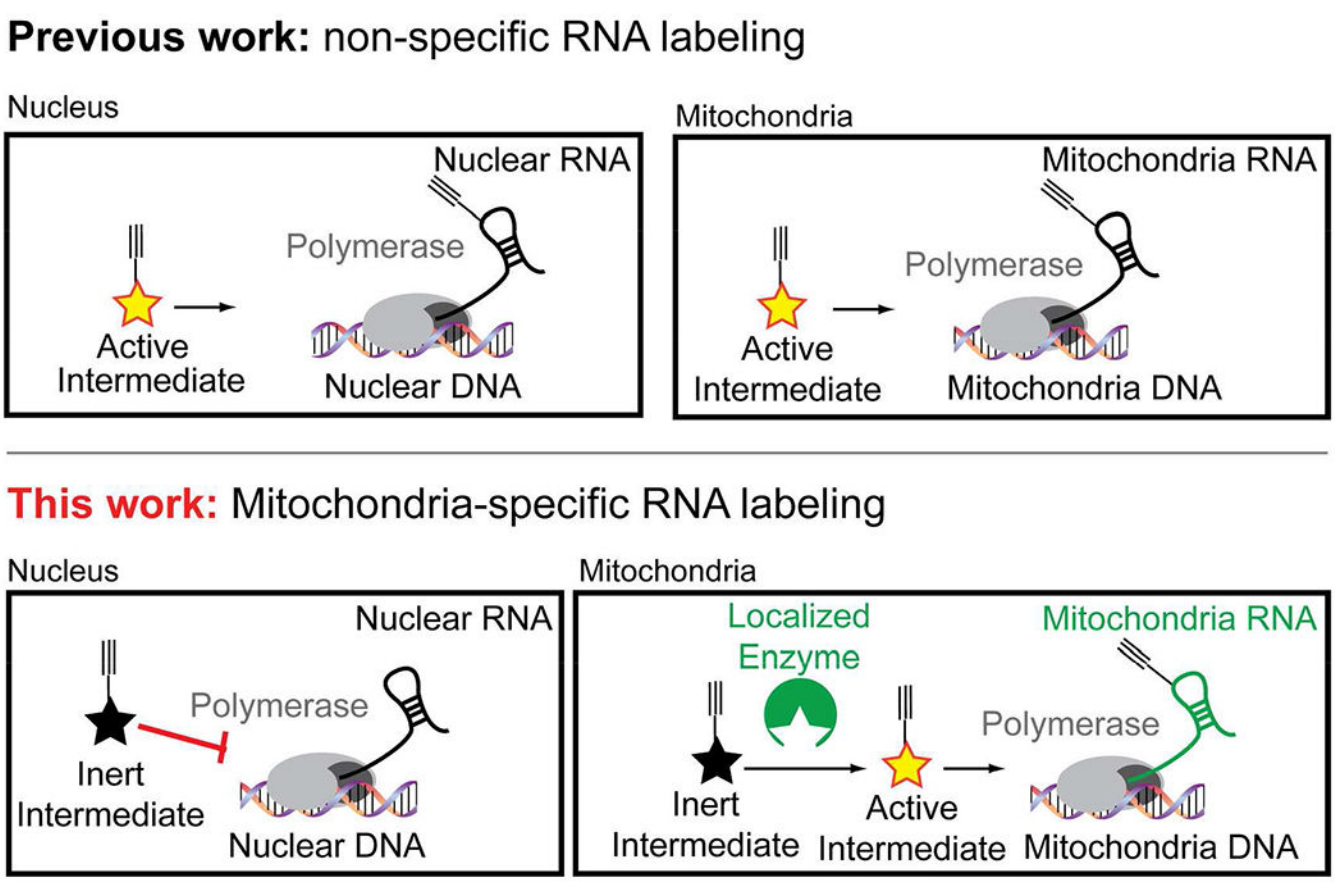

Figure 1.
Outline of mitochondria-specific metabolic labeling of RNA.

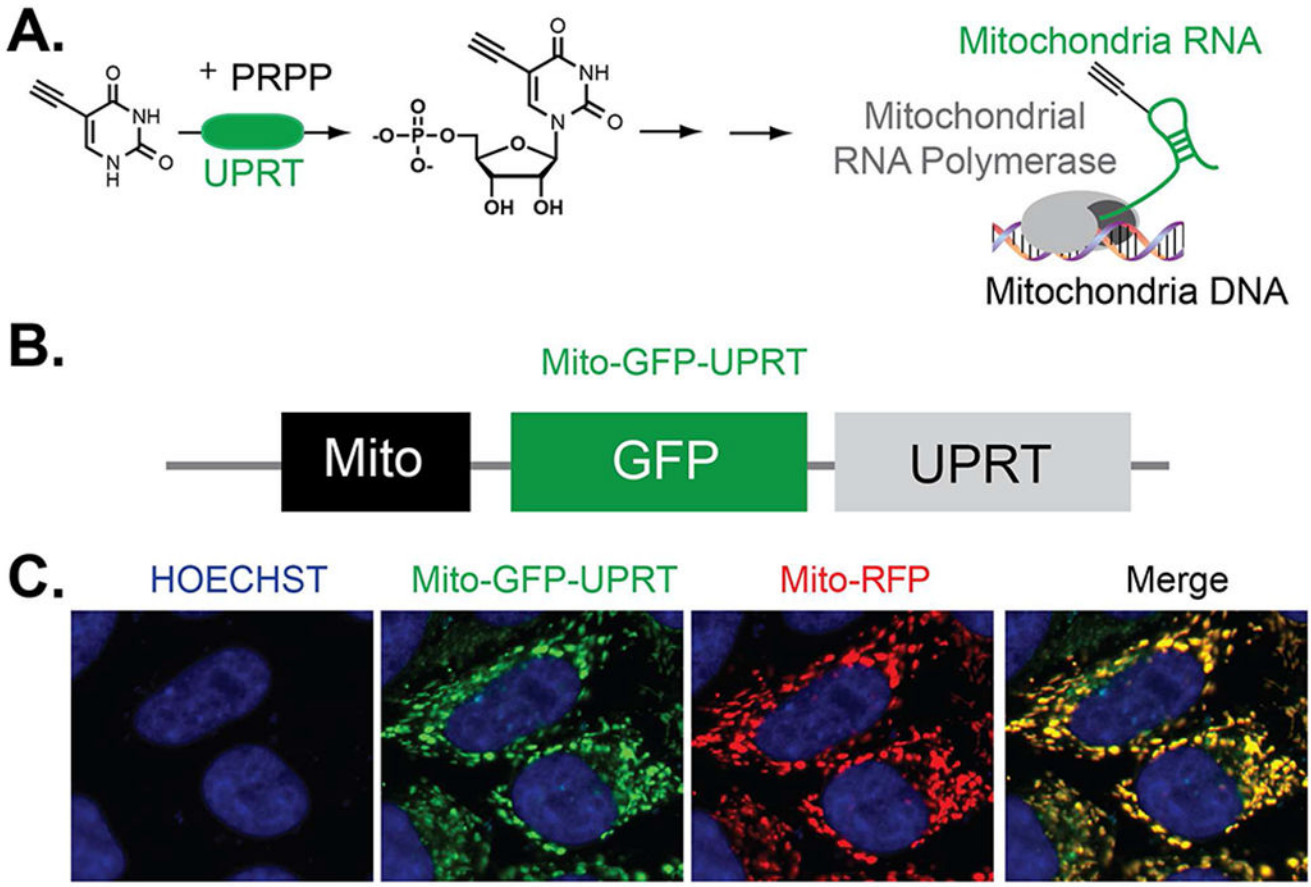

Figure 2. Design and localization of a UPRT enzyme construct to mitochondria.

A. Schematic of proposed UPRT-mediated RNA synthesis in the mitochondria. B. Design of a mitochondria-targeted UPRT construct. C. Imaging of the mitochondria-targeted UPRT construct displays overlap with a Mito-RFP construct. Hoechst stains nuclear DNA.

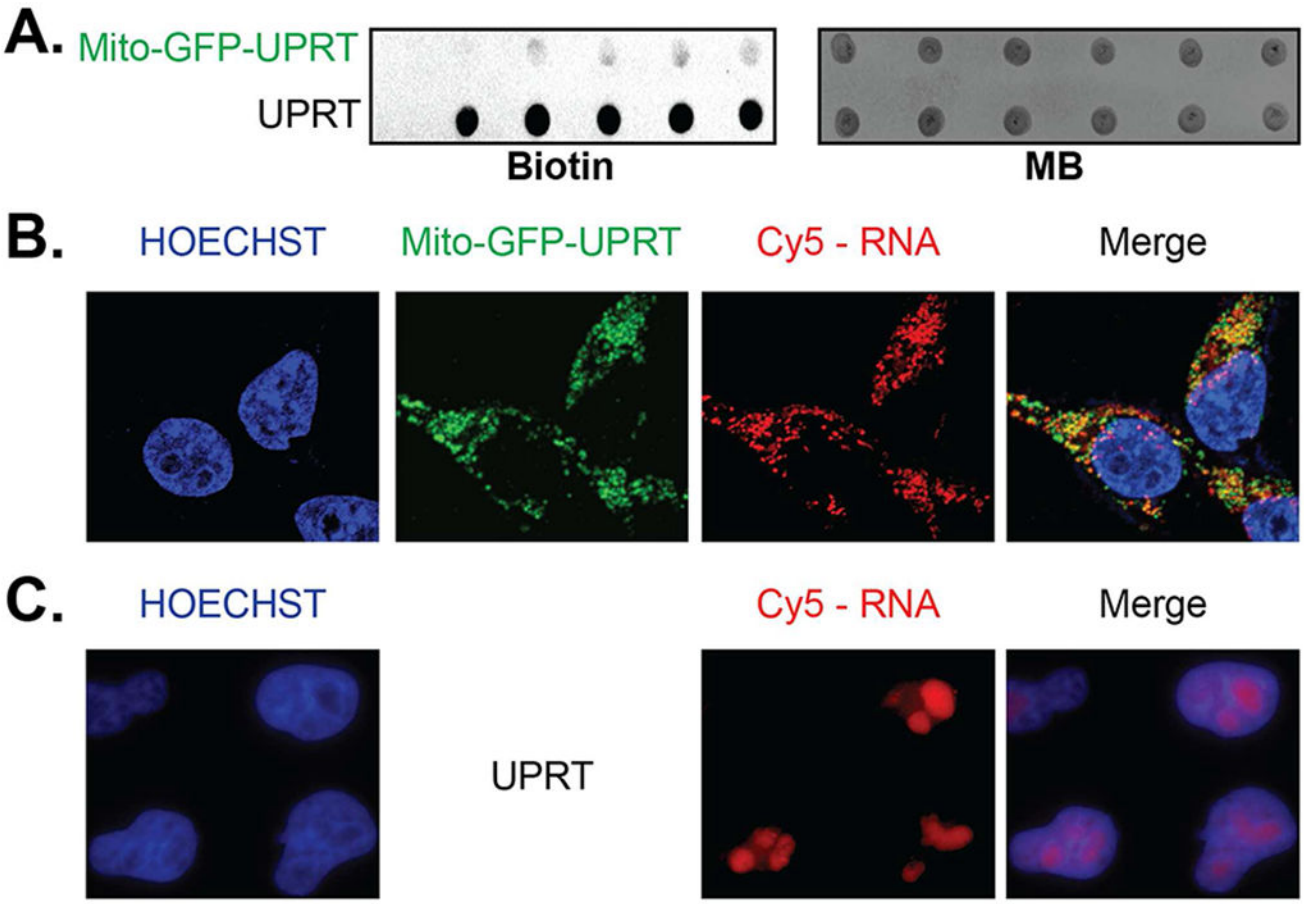

Figure 3. Mitochondria-targeted UPRT enables metabolic incorporation of 5EU into mitochondrial RNA.

A. Dot blot analysis of equal amount of RNA derived from Mito-GFP-UPRT and spatially-unrestricted UPRT expressing cells in a time titration of 5EU treatment (left to right): 0, 0.5, 1, 2, 3 and 5h. MB: methylene blue stain-served as loading control B. Imaging of Mito-GFP-UPRT-transiently expressed cells and metabolically labeled RNA (Cy5-azide) show overlap in mitochondria. C. Imaging of spatially-unrestricted UPRT cells show RNA labeling in nucleolus, indicative of whole-cell RNA labeling. Hoechst stains nuclear DNA.

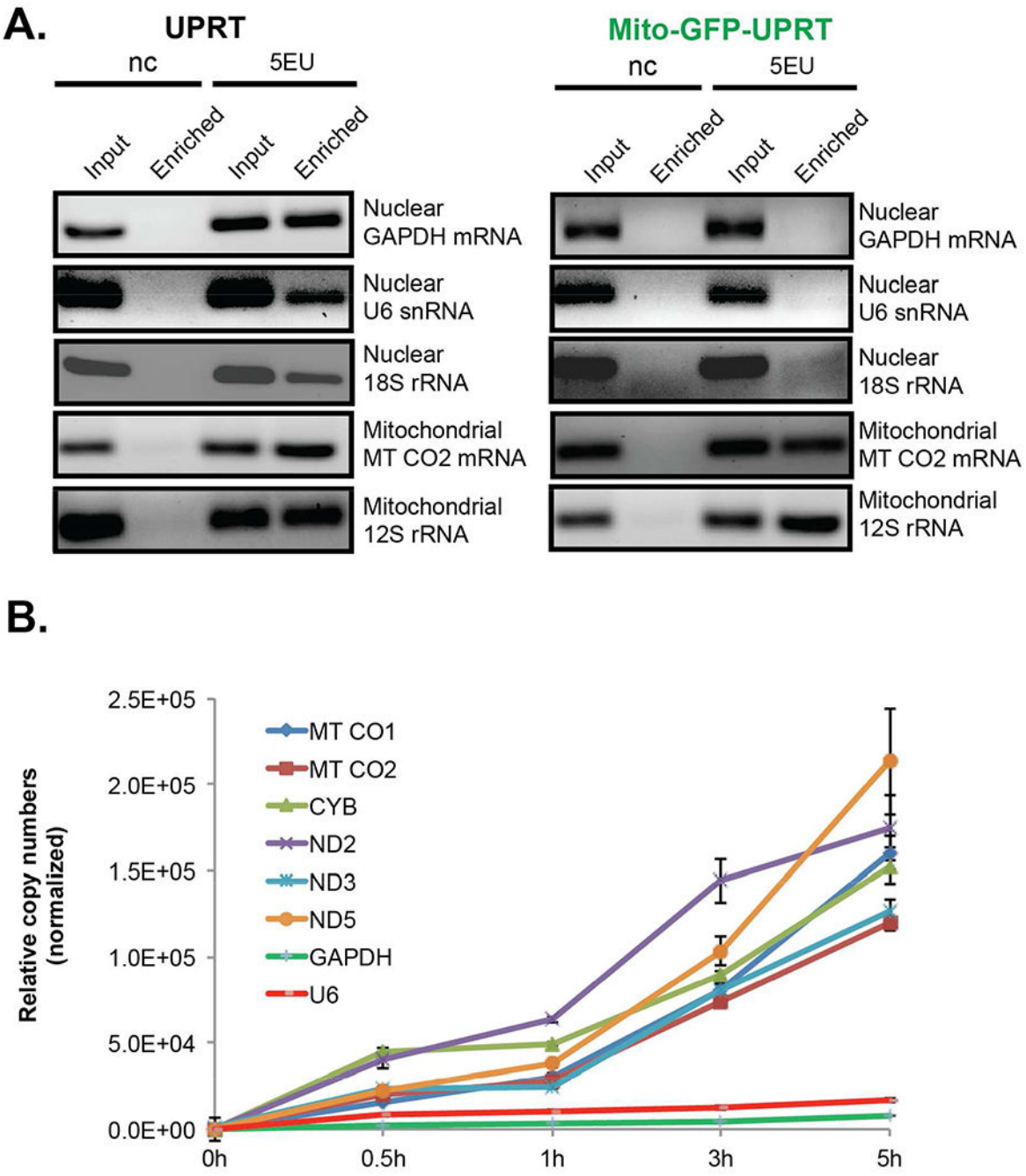

Figure 4. Selectively labeled mitochondrial transcripts in Mito-GFP-UPRT expressing cells.

A. Enriched cDNA templates derived from RNA of spatially-unrestricted UPRT vs. Mito-GFP-UPRT expressing cells were assayed by PCR with specific primer pairs for nuclear or mitochondrial transcripts (nc: negative control = DMSO; 5EU = 5'Ethynyl uracil). B Assessment of six mitochondrial and two nuclear transcripts (served as negative controls) from RNA of transiently transfected Mito-GFP-UPRT cells exposed to 5EU in a time course by SYBR Green RT-qPCR analysis (n = two technical duplicates)