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Cell- and polymerase-selective metabolic labeling of cellular RNA with 2'-azidocytidine

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Supporting Information Placeholder

ABSTRACT: Metabolic labeling of cellular RNA is a powerful approach to investigate RNA biology. In addition to revealing whole transcriptome dynamics, targeted labeling strategies can be used to study individual RNA subpopulations within complex systems. Here, we describe a strategy for cell- and polymerase-selective RNA labeling with 2'-azidocytidine (2'-AzCyd), a modified nucleoside amenable to bioorthogonal labeling with SPAAC chemistry. In contrast to 2'-OH-containing pyrimidine ribonucleosides, which rely upon uridine-cytidine kinase 2 (UCK2) for activation, 2'-AzCyd is phosphorylated by deoxycytidine kinase (dCK), and we find that expression of dCK mediates cell-selective 2'-AzCyd labeling. Further, 2'-AzCyd is primarily incorporated into ribosomal RNA and displays low cytotoxicity and high labeling efficiency. We apply our system to analyze the turnover of ribosomal RNA during ribophagy induced by oxidative stress or mTOR inhibition to show that 28S and 18S rRNAs undergo accelerated degradation. Taken together, our work provides a general approach for studying dynamic RNA behavior with cell and polymerase specificity and reveals fundamental insights into nucleotide and nucleic acid metabolism.

The incorporation of modified nucleotides into cellular RNA is a powerful approach for probing RNA biology. In particular, nucleotides that serve as biorthogonal tags[1-3], photoaffinity labels[4], or generate characteristic mutational signatures[5-6] can be used as chemical probes for studying transcriptional dynamics and protein-RNA interactions in living cells. Several approaches have been developed for incorporating modifications into cellular RNA[7-10], but the most commonly used strategy rely on cellular uptake and activation of modified ribonucleosides. While labeling with ribonucleosides such as 4-thiouridine (4-SU)[4, 11] or 5-ethynyluridine (5-EU)[1] proceeds efficiently, the scope of modified nucleotides that can be accessed through nucleoside feeding has remained limited. Further, we lack approaches that can applied to cell-specific[10] or RNA polymerase-specific labeling *in vivo* with minimal cytotoxicity[12].

Previously, we applied protein engineering to UCK2 in order to facilitate cell-specific labeling with 5-azidomethyluridine[13]. Here, we explore metabolic labeling with 2'-azidocytidine (2'-AzCyd)[14-19], a modified nucleoside compatible with bioorthogonal SPAAC chemistry[20]. We show that in contrast to base-modified pyrimidine ribonucleosides, which rely on UCK2, incorporation of 2'-AzCyd is primarily mediated by deoxycytidine kinase (dCK)[21] and overexpression of this enzyme in diverse cell lines can facilitate cell-specific RNA labeling. Further, we find that 2'-AzCyd is well tolerated in cell culture and is mainly incorporated into ribosomal RNA in an RNA Pol I-dependent manner, providing a strategy for polymerase-selective RNA labeling which we exploit to study rRNA turnover during oxidative stress and drug treatment.

2'-azido RNA nucleotides have emerged as a promising scaffold for biorthogonal RNA labeling[22-23] and 2'-azidoadenosine (2'-AzAd) can be metabolically incorporated into mRNA primarily through polyA polymerases[3]. In contrast, less is known about the potential of 2'-azidopyridimine nucleosides for RNA labeling applications. While an early study by Åkerblom[15] found incorporation of 2'-

azidocytidine into cellular RNA, he concluded that only "minute" amounts were present. Further, recent work has indicated that 2'-azidouridine is a poor substrate for metabolic labeling but can be utilized for cell-specific labeling together with UCK2 overexpression[24]. To study metabolic 2'-azidonucleoside labeling further, we began by culturing HeLa cells with 1 mM 2'-AzAd or the related pyrimidines 2'-azidouridine (2'-AzUrd) or 2'-AzCyd (Figure 1a), and evaluated incorporation by CuAAC and fluorescence microscopy. Consistent with prior reports[3], we found efficient uptake of 2'-AzAd (Figure 1b). We could also detect uptake of 2'-AzCyd, as has been reported[15, 24], however labeling was less intense as compared to 2'-AzAd, and we were unable to observe incorporation by 2'-AzUrd (Figure 1b). The labeling pattern with 2'-AzCyd was both nuclear and cytosolic with strong staining in the nucleoli, consistent with incorporation into RNA. To confirm RNA labeling we performed feeding in the presence of actinomycin D, an RNA polymerase inhibitor, or hydroxyurea, an inhibitor of DNA synthesis. While 2'-AzCyd labeling was unaffected by hydroxyurea, we observed a strong reduction in signal upon treatment with actinomycin D, consistent with RNA incorporation (Figure 1c).

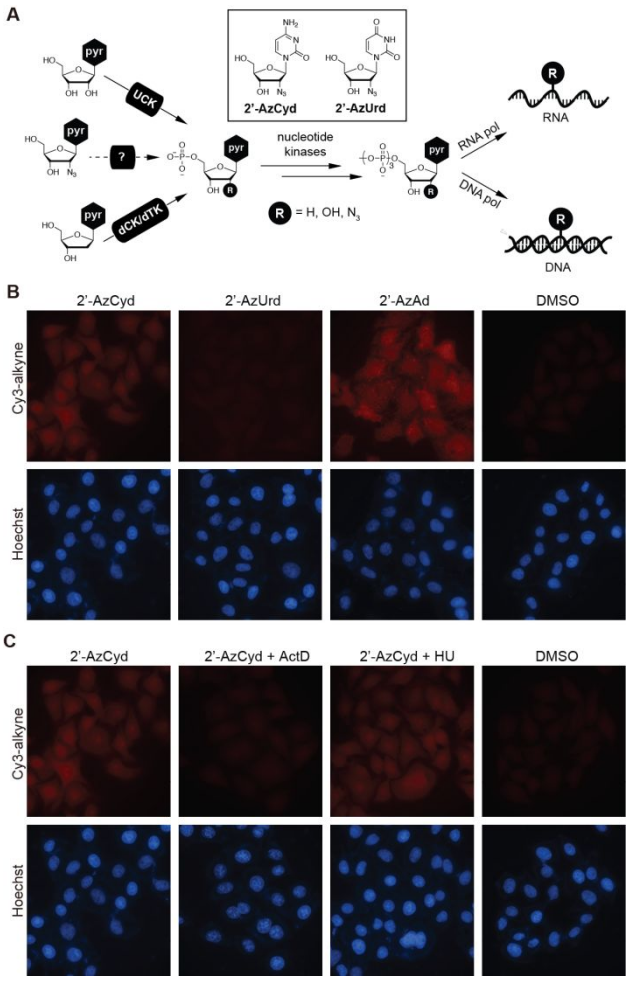

Figure 1. Metabolic incorporation of 2'azidopyrimidine nucleosides into cellular RNA. (A) Pyrimidine salvage pathway and 2'-azidopyrimidines. (B) Labeling of HeLa cells with 1 mM 2'-AzCyd or 2'-AzUrd for 6 hr. (C) 2'-AzCyd labeling in the presence of 2 μM ActD or 10 mM HU.

Next, we explored 2'-AzCyd metabolism (Figure 1a). Phosphorylation to NMPs has generally been proposed as rate limiting for nucleoside salvage therefore we focused on the known pyrimidine ribonucleoside and deoxyribonucleoside kinases UCK2 and dCK[21, 25]; indeed dCK has been proposed as the primary enzyme responsible for 2'-AzCyd phosphorylation although direct evidence is lacking[15]. We purified recombinant enzymes following literature precedent[25-26] and used an HPLC-based assay to analyze nucleoside phosphorylation[13]. Both kinases were active as judged by their ability to phosphorylate cytidine/deoxycytidine (Supplementary Figures 1 & 2). We next tested the ability of UCK2 and dCK to phosphorylate 2'-AzCyd and 2'-AzUrd. After 12 hr incubation of 2'-AzCyd with dCK and excess ATP, we

observed near complete consumption of starting material and the emergence of two new peaks – one consistent with ADP, and the other that we confirmed by mass spectrometric analysis as 2'-AzCyd monophosphate (Figures 2a and 2b). Incubation of 2'-AzCyd with UCK2 under the same conditions produced only 4% conversion to the monophosphate. To further characterize dCK and UCK2 activity on 2'-AzCyd, we performed a time course assay, which clearly demonstrates that dCK exhibits significantly higher activity on 2'-AzCyd than does UCK2 (Figure 2c). Interestingly, under our conditions we found that 2'-AzCyd is phosphorylated by dCK more rapidly than deoxycytidine, the native substrate of the enzyme (Figure 2c), and that cytidine is the best substrate out of all pyrimidine analogs tested with dCK. Analysis of dCK[26] and

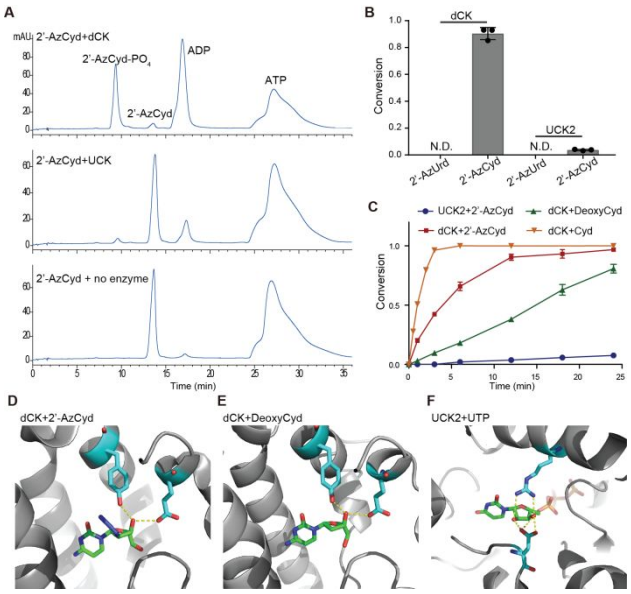

Figure 2. Phosphorylation of 2'-azidonucleosides by UCK2 or dCK. (A) HPLC chromatagrah of 2'-AzCyd phosphorylation by recombinant UCK2 or dCK enzymes. (B) Quantification of 2'-azidonucleoside phosphorylation. Data represent mean ± s.d. (n=3). (C) Kinetic analysis of cytidine, deoxycytidine, or 2'-AzCyd phosphorylation. (D) Structural model of 2'-AzCyd bound to dCK. (E) Crystal structure of dCK–deoxyCyd complex (PDB: 1P61) (F) Crystal structure of UCK2 with UTP (PDB: 1UEI).

UCK2[27] x-ray crystal structures suggests that 2'-azido or 2'-hydroxyl modifications can be readily accommodated in the dCK active site (where these functional groups could engage in additional polar interactions) (Figures 2d and 2e), while a 2'-azido group is likely unable to fully participate in the hydrogen bond network formed between UCK2 and the substrate 2'-hydroxyl group (Figure 2f). We also tested the activity of both enzymes on 2'-AzUrd and were unable to detect the formation of a new product or observe consumption of 2'-azidouridine during the course of the reaction (Figure 2b, Supplementary Figure 1). We did however measure a small amount of ADP production in both UCK2 and dCK reactions with 2'-AzUrd, suggesting that low amounts of phosphorylation may occur with this

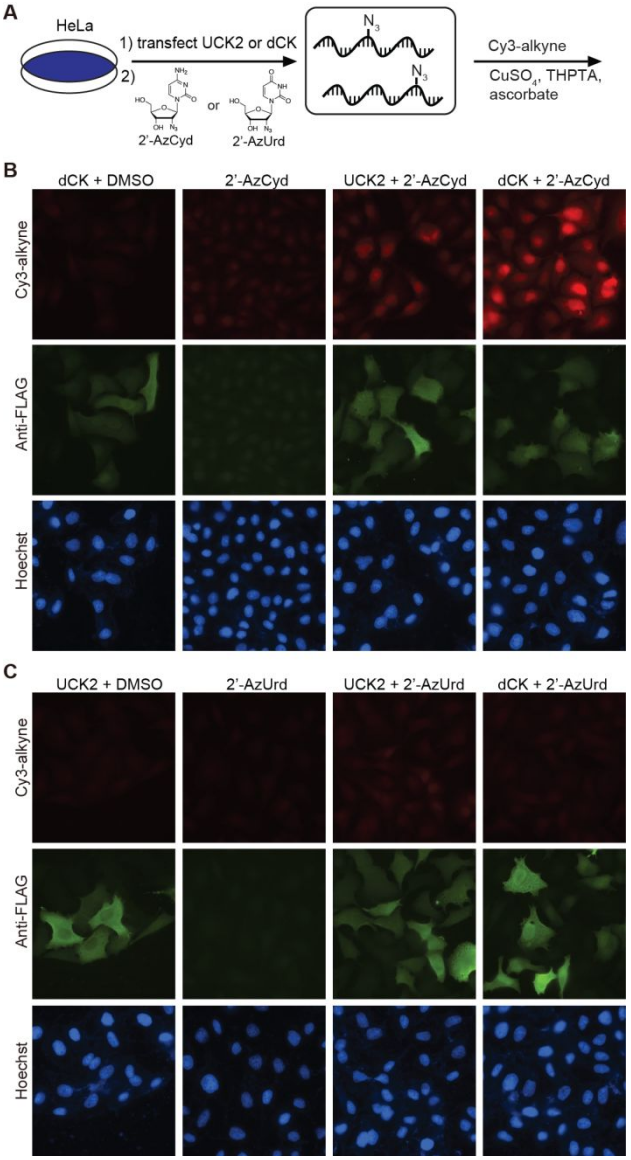

Figure 3. dCK mediates 2'-AzCyd incorporation. (A) Workflow for 2'-azidopyrimidine incorporation. (B) and (C) HeLa cells transfected with plasmids encoding FLAG-tagged UCK2 or dCK were treated with 1 mM 2'-AzCyd or 1 mM 2'-AzUrd for 6 hr.

substrate. Taken together, our results indicate that dCK can efficiently phosphorylate 2'-AzCyd and is likely to be the major cellular kinase mediating RNA incorporation of this modified nucleoside. Further, the activity of dCK on cytidine implies a potential role for this kinase in ribonucleoside salvage.

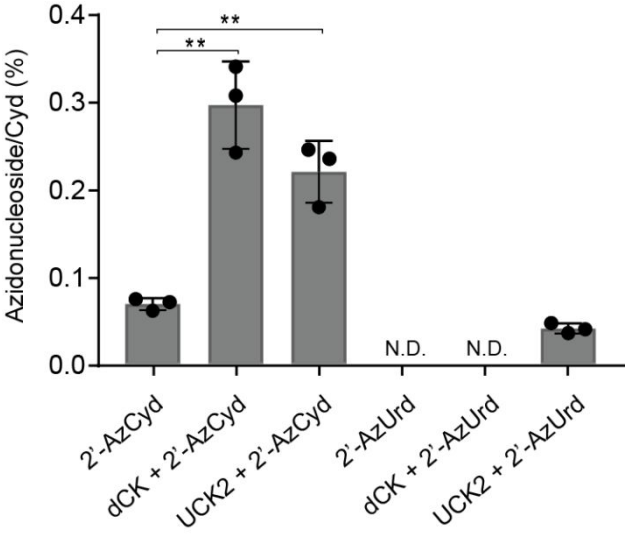

Figure 4. Incorporation of 2-'azidopyrimidines into HEK293T cellular RNA. Digested total RNA was analyzed by LC-MS/MS. Data represent mean ± s.d. (n= 3); $^{**}p < 0.01$; N.D. = not detected.

The ability of dCK to phosphorylate 2'-AzCyd led us to speculate that overexpression of this enzyme could increase incorporation and provide an avenue for cell-specific labeling. To probe this, we transfected HeLa cells with dCK plasmid and treated with 1 mM 2'-AzCyd. In parallel, we treated untransfected cells and cells transfected with UCK2 plasmid. After treatment, cells were imaged using CuAAC chemistry to detect the incorporation of 2'-AzCyd. Gratifyingly, overexpression of dCK produced a large increase in 2'-AzCyd labeling (Figure 3b). We were able to observe a similar effect using a lower concentration of 2'-AzCyd as well (Supplementary Figure 3). Interestingly, despite its poor ability to phosphorylate 2'-AzCyd *in vitro*, UCK2 overexpression also increased labeling relative to control cells, although by a smaller degree than dCK overexpression (Figure 3b) and only at the 1 mM treatment concentration (Supplementary Figure 3). Due to the finding that even low *in vitro* activity could enhance cellular accumulation (presumably due to high-level protein expression and low endogenous protein levels), we also investigated dCK and UCK2 overexpression with 2'-AzUrd. In this case, we observed no increase in 2'-AzUrd labeling upon dCK expression and a very minimal increase in 2'-AzUrd labeling with UCK2 (Figure 3c and Supplementary Figure 4), in-line with our inability to detect *in vitro* nucleoside phosphorylation.

To provide additional insight into 2'-azidopyrimidine incorporation, we measured modification levels in total RNA using quantitative nucleoside LC-MS/MS. In brief, cells were fed 2'-AzCyd or 2'-AzUrd for 12 hrs, total RNA was isolated and digested to nucleosides, and the individual levels were quantitated using LC-QQQ-MS with appropriate standard curves. Using this assay and consistent with our imaging results, 2'-AzCyd incorporation was dramatically increased by dCK overexpression (4.3-fold increase over control cells) resulting in a final 2'-AzCyd level of 0.3% relative to unmodified cytidine (Figure 4, Supplementary Table 3). UCK2 overexpression also increased 2'-AzCyd labeling but by a smaller magnitude compared to control cells. Additionally, we investigated 2'-AzUrd incorporation. In control cells and dCK-transfected cells, we were unable to detect 2'-AzUrd in total RNA. However, upon UCK2 transfection we could measure a small amount of cellular labeling resulting in 0.03% incorporation (Figure 4, Supplementary Table 4). While detectable, this is roughly 10-fold lower than 2'AzCyd labeling using the dCK overexpression system.

Having validated the dCK-2'-AzCyd pair as a robust metabolic labeling strategy, we further characterized 2'-AzCyd labeling in cellular RNA using a stable HeLa cell line containing inducible dCK (Supplementary Fig. 6). First we investigated the effect of long-term 2'-AzCyd treatment on cell viability. Notably, we observed a negligible change in viability compared to untreated cells after 3-day treatment with concentrations ranging from 10-200 μM 2'-AzCyd (Supplementary Fig. 11). In contrast, the commonly used ribonucleoside analog 4-SU demonstrated negative effects on HeLa cell viability at concentrations as low as 10 μM (Supplementary Fig. 11). Next we investigated the distribution of 2'-AzCyd in different cellular RNAs. We isolated total RNA and polyA RNA and analyzed the distribution of 2'-AzCyd containing RNA using SPAAC chemistry and gel electrophoresis. Interestingly, we observed 2'-AzCyd accumulation primarily in the 28S and 18S large rRNA species (Figure 5a, Supplementary Fig. 7 & 10) as well as in the small RNA fraction that likely consists of 5S RNA, tRNA, and other small non-coding RNAs (Figure 5a, Supplementary Fig. 7); minimal accumulation was observed in the polyA

RNA fraction (Supplementary Fig. 10). This is in stark contrast to the labeling pattern observed upon 2'-AzAd feeding (Figure 5a, Supplementary Fig. 10), which demonstrates a 'smear' of large transcripts consistent with its incorporation into cellular mRNA through polyA polymerases[3]. Previous studies with base-modified pyrimidine ribonucleosides have also observed accumulation primarily in mRNA[1, 13]. To further probe this finding, we investigated 2'-AzCyd labeling in the presence of small-molecule RNA polymerase inhibitors with different specificities. Consistent with our gel electrophoresis results, we found 2'-AzCyd labeling to be strongly inhibited by actinomycin D, an RNA pol I-specific inhibitor[28], but largely unchanged in the presence of α-amanitin, an RNA pol II-specific inhibitor[29], as measured by cellular microscopy and in-gel fluorescence of total RNA (Figure 5b and Supplementary Fig. 8).

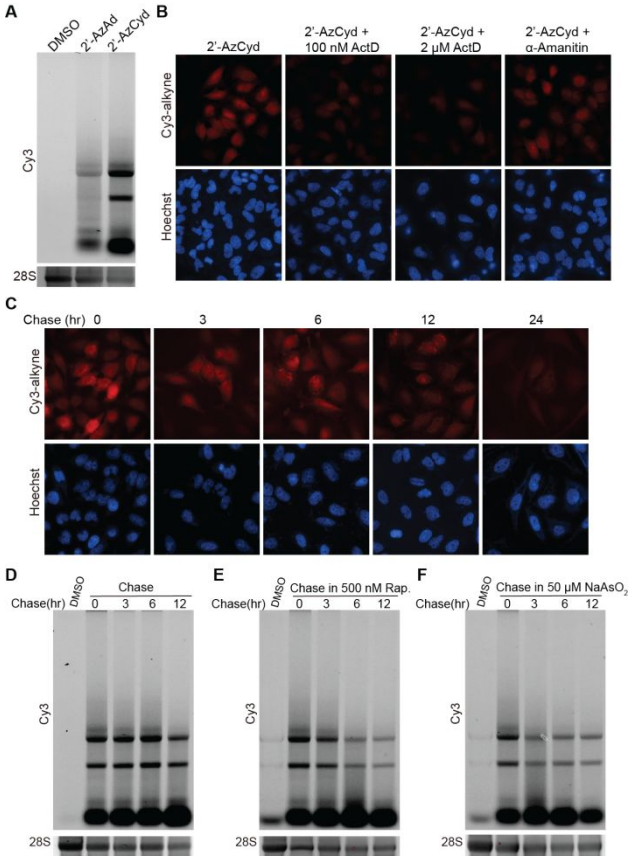

Figure 5. 2'-AzCyd predominantly labels rRNA. (A) Gel analysis of 2'-AzAd and 2'-AzCyd labeling. (B) Labeling of dCK-expressing HeLa cells with 2'AzCyd in the presence of ActD or α-amanitin. (C) Turnover of 2'-AzCyd labeled RNA in HeLa cells. Cells were pulsed with 1 mM 2'-AzCyd for 12 h and chased with complete medium. (D), (E) and (F) Turnover of 2'-AzCyd RNA during ribophagy: (D) untreated, (E) 500 nM rapamycin, and (F) 50 μM $NaAsO_2$ (EtBr stained samples can be found in Supplementary Fig. 12).

Finally, to demonstrate the utility of an RNA pol I-specific label, we performed pulse-chase experiments with 2'-AzCyd in our dCK overexpressing cell line to study rRNA turnover under different conditions. After pulse labeling, we observed a decrease of 2'-AzCyd labeling 12-24 hr into the chase (Figure 5c and 5d). As this time point is similar to the doubling time of HeLa cells we conclude that the decrease in signal is primarily due to dilution upon cell division rather than degradation of rRNA, consistent with prior reports of long rRNA half-life[30]. In contrast, when cells were treated with the mTOR inhibitor rapamycin or subjected to sodium arsenite, two conditions known to induce ribosomal protein degradation ('ribophagy')[31-33], we found a much more rapid decrease in signal starting around 3-6 hr (Figure 5e and 5f), indicating accelerated degradation of rRNA. Interestingly, rRNA degradation appeared to stop at the 6 hr time point into the chase suggesting that degradation during ribophagy only occurs on a subset of rRNAs.

In this work, we develop the 2'-AzCyd-dCK pair as a robust strategy for cell- and polymerase-specific RNA labeling. Compared to other nucleotides for RNA labeling, this system has several advantages. First, azido-nucleotides are amenable to biorthogonal chemistry using SPAAC, which does not result in RNA degradation. Second, 2'-AzCyd is less toxic than the commonly used ribonucleoside analog 4-SU[12], and can be incorporated at high levels into cellular RNA (0.3% of C) allowing us to perform functional assays on labeled RNA without enrichment. Notably, Spitale and co-workers recently reported cell-specific labeling using 2'-AzUrd and WT UCK2[24]. While we were able to observe low level cellular incorporation of 2'-AzUrd upon UCK2 overexpression (Figure 3 and Figure 4), we find the 2'-AzCyd-dCK pair to be superior as it facilitates RNA labeling with 10-fold higher efficiency (Figure 4). Additionally, we show that 2'-AzCyd is primarily utilized by RNA Pol I, providing a distinct labeling profile compared to other available probes. We exploit this property to develop a non-radioactivity-based rRNA turnover assay and study the lifetime of 28S and 18S rRNA during mTOR inhibition and arsenite treatment. These conditions

have been previously shown to promote ribophagic flux[32] and our work demonstrates concomitant degradation of rRNA, and provides a tool for dissecting the mechanism of this process in mammalian systems. Finally, while most approaches for functionalizing cellular RNA have focused on nucleobase modifications, our work demonstrates a unique synergy between deoxynucleoside salvage and RNA polymerases that can be exploited to incorporate modifications in place of the ribose 2'-OH, presenting new opportunities for labeling cellular RNA with diverse chemical groups for probing biological processes.

ASSOCIATED CONTENT

Supporting Information. Experimental methods, supplementary tables, and supplementary figures are available free of charge on the ACS Publications website.

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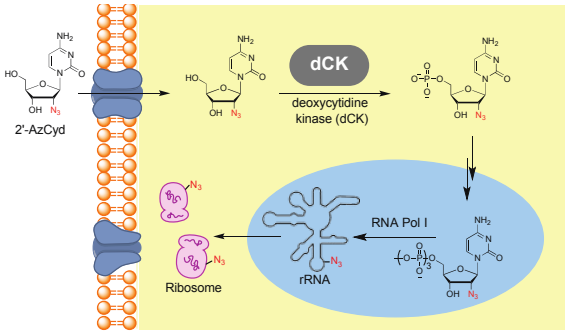
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