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Spotlight

# DXO gears mRNA with alternative NAD and m$^7$G caps

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**NAD is a noncanonical mRNA cap that challenges our traditional dogma of *N*7-methylguanosine (m$^7$G)-capped eukaryotic mRNAs. The relationship between NAD and m$^7$G caps has been elusive. Xiao *et al.* find that the deNADding enzyme DXO promotes maturation of m$^7$G caps, suggesting that DXO fine-tunes the dynamic balance between alternative RNA cap structures.**

## Mysterious RNA 5′-termini and coenzymes in the RNA world

RNA is the 'messenger' that connects DNA and protein in the central dogma, and eukaryotic mRNAs characteristically have an m$^7$G cap that acts as an identifier for appropriate biological functions. Recently, several coenzymes including NAD, coenzyme A (CoA), and flavin adenine dinucleotide (FAD) have been found to act as alternatives to the m$^7$G caps in various eukaryotic and prokaryotic organisms [1–3]. These coenzymes share a common adenine group, which is one of the four essential building monomers of RNA. It is proposed that these small molecules have actively interacted with RNAs since the origin of life [4]. The exciting discoveries of these noncanonical RNA caps raise the question of why RNAs harbor these different caps, and how.

Emerging methods for high-throughput sequencing of NAD-capped RNAs (NAD-RNAs) have allowed identification of some NAD-RNA genes, thus providing substantial understanding of the biological processes in which NAD-RNAs are involved (recently reviewed in [5]). However, it remains largely unknown how the cells tune the RNA 5'-terminal modification. DXO is an enzyme that robustly removes NAD caps of RNAs in mammals and plants [6–8]. Unexpectedly, the phenotypes of arabidopsis (*Arabidopsis thaliana*) *dxo1* mutants were reported to be rescued by catalytically inactive atDXO1 [with a single amino acid (aa) mutation in the NAD decapping activity site] by independent laboratories [6,7]. These unexpected findings raised a puzzle about the real roles of NAD-RNAs. Recently, Xiao *et al.* demonstrated a new role of DXO1 in regulating the maturation of RNA m$^7$G caps [9], thus bringing a new piece to the jigsaw puzzle by connecting NAD- and m$^7$G-capped RNAs.

## DXO plays a new role in tuning RNA 5′ termini

Plant DXO proteins have a specific extension of around 200 aa in their N termini relative to their mammal and yeast homologs [6,7]. Even in the absence of the additional N-terminal extension (NTE), plant DXO shows robust decapping activity towards NAD-RNAs [6,7]. What then is the function of the plant-specific NTE? Inspired by this question, Xiao *et al.* searched for proteins interacting with DXO in arabidopsis [9]. Surprisingly, a cap methyltransferase (At3g20650) was identified as a DXO1-interacting protein in their yeast two-hybrid (Y2H) screen. This cap methyltransferase is highly similar to the RNA guanosine-7-methyltransferase (RNMT) of yeast and animals and was thus named atRNMT1. RNMT is known to be a key player in RNA capping that converts the G cap to the m$^7$G cap in mammals [10]. Further investigation of the arabidopsis genome revealed that *RNMT1* is the sole RNMT gene because its closest homolog shares only 25% overall sequence identity with *RNMT1*, and the *S*-adenosylmethionine (AdoMet)-binding motif (that is crucial for RNA cap methyltransferase activity) is absent from the homolog. This finding provides new clues to possible functions of plant DXO in tuning the 5′ termini of RNAs.

Does the NTE of DXO1 play a role in mediating its interaction with RNMT1? Xiao *et al.* divided DXO1 into two parts (the NTE and the remaining 3′ fragment) and tested their interaction with RNMT1 via Y2H assays [9]. This revealed an interaction between RNMT1 and the NTE, but not with the 3′ fragment of DXO1. A similar set of Y2H assays suggested that the middle part of RNMT1 (aa 88–207) containing an AdoMet-binding motif is the key domain that mediates its interaction with DXO1. The interaction was shown to occur primarily in nuclei via a bimolecular fluorescence complement (BiFC) assay, and coimmunoprecipitation (co-IP) assays showed a direct interaction between DXO1 and RNMT1 *in planta*.

## DXO enhances the cap methyltransferase activity of RNMT

*In vitro* enzymatic assays suggested that RNMT1 methylates 15% of GpppG (an artificial analog of the unmethylated RNA cap) to m$^7$GpppG using AdoMet as the methyl donor [9]. When *in vitro* transcribed RNAs were used as the substrate, the methylation percentage was 50% for GpppG-RNAs and 20% for GpppA-RNAs [9]. The methylation percentages increased to ~90% for both GpppG- and GpppA-RNAs when DXO1 was added to the reaction [9]. DXO1 notably enhanced the methyltransferase activity of RNMT1, although it did not show methylation activity by itself. Strikingly, when the NTE domain (but not the 3′ fragment) of DXO1 was added together with RNMT1, similarly enhanced RNMT1 activity was observed. These results suggest that DXO1 enhances the cap methyltransferase activity of RNMT1 via its NTE domain.

To analyze the cap methylation activity of DXO1 and RNMT1 *in vivo*, Xiao *et al.* compared mRNA caps of four-week-old arabidopsis plants and mutants deficient in *DXO1* (a T-DNA insertion line) and *RNMT1* (two CRISPR/Cas9 lines) [9]. The mRNAs were digested to release the G

and $m^7G$ caps for quantification using mass spectrometry. It was found that the ratio of the $m^7G$ cap to total caps was decreased by ~25% and the unmethylated G caps were notably increased in the *dxo1* and *rnmt1* mutants relative to wild-type (WT) plants [9]. Thus, DXO1 functions as an RNMT1 activator *in vivo* in arabidopsis.

## DXO and RNMT act on common sets of mRNAs

The arabidopsis *rnmt1* mutants exhibited growth retardation and a pleiotropic developmental defect with pale-green leaves [9], which was similar to the phenotype of *dxo1* mutants [6,7]. Moreover, overexpression of RNMT1 partially complemented the phenotypes of the *dxo1* mutant [9]. It is highly likely that DXO1 cooperates with RNMT1 to tune the 5′-termini of specific RNAs during plant development. To test this hypothesis, Xiao *et al.* profiled the transcriptomes of WT, *dxo1*, *rnmt1*, and *RNMT1*-overexpressing *dxo1* (expression of RNMT1 under the control of the 35S promoter in the *dxo1* mutant) plants using RNA sequencing [9]. A notable similarity was found between the transcriptomes of the *dxo1* and *rnmt1* mutants. As many as 75% of the upregulated RNAs in *rnmt1* were also upregulated in *dxo1* relative to the WT, and the similarity was 60% for the downregulated RNAs. Overexpression of *RNMT1* in *dxo1* largely complemented the transcriptome, in accordance with the observations of phenotypic rescue. Thus, DXO1 cooperates with RNMT1 to regulate the expression of a set of essential genes via tuning RNA 5′ caps in arabidopsis.

It should be noted that transcriptomic differences were found between *rnmt1* and *dxo1*, in addition to the notable similarity. The *dxo1* mutant exhibited higher transcript levels of many stress response genes and lower transcript levels of numerous photosynthetic genes compared with the *rnmt1* mutant [9]. The disparity in the transcriptomes of the *dxo1* and *rnmt1* mutants highlighted an association between the deNADding activity of DXO1 and the fundamental biological processes of photosynthesis and stress responses in arabidopsis, which aligned with a previous report [7]. Thus, DXO may play dual roles in tuning both $m^7G$ and NAD caps in the regulation of gene expression in eukaryotic cells.

## Concluding remarks and future perspectives

Taken together, these findings provide substantial evidence that plant DXO tunes the $m^7G$ caps of mRNAs via the NTE-mediated interaction with RNMT [9], in addition to its known role as a decapper of NAD-RNAs [6,7]. Since NAD-RNAs do not support efficient translation [8], it is likely that the dynamic balance of NAD- and $m^7G$-capped RNAs, other than the NAD-RNA itself, functions in tuning biological processes in cells. Plant DXO might contribute to new machinery that tunes the $m^7G$ and NAD caps of RNAs during plant development and growth (Figure 1). Questions remain regarding how the RNA pools are tuned in other eukaryotes. Do mammalian and yeast DXOs play roles in tuning the cap balance of RNAs with multiple alternative 5′ termini? Considering that the NTE domain is key to mediating the DXO/RNMT interaction in plants, but is absent from mammal and yeast DXOs, it is also likely that there is another so far unidentified protein with a similar structure that takes over this role in mammals and yeast. The RNMT-activating mini-protein (RAM) is known to interact with RNMT to promote RNA cap methylation in mammals [11]. Are there any interactions between DXO and RAM that tune the RNA cap balance in mammals? Answers to these questions will greatly advance our knowledge of the RNA world and the evolution of life on Earth.

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## Declaration of interests

The authors declare no conflicts of interest.

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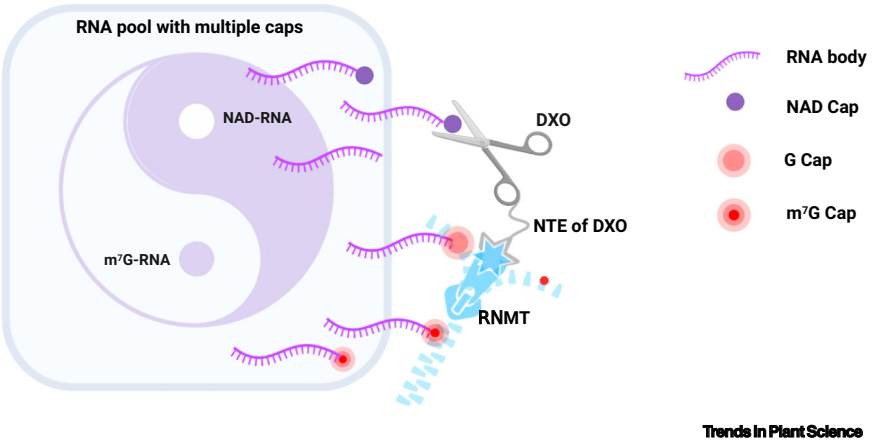


Figure 1. The DXO machinery tunes the dynamic balance of cellular NAD-RNAs and $m^7G$-RNAs. Plant DXO decaps NAD-RNAs and activates the RNA cap methyltransferase RNMT through a direct interaction via its plant-specific N-terminal extension (NTE) domain. RNMT converts unmethylated G caps of mRNAs into $m^7G$ caps. The DXO/RNMT machinery provides dynamic adjustment of the NAD-/$m^7G$-RNAs balance in the RNA pool, thus enabling fast responses to biological processes in plant cells. Abbreviations: $m^7G$, *N*7-methylguanosine; RNMT, RNA guanosine-7-methyltransferase. The figure was created using BioRender (https://biorender.com/).

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