A Mammalian Pre-mRNA 5′ End Capping Quality Control Mechanism and an Unexpected Link of Capping to Pre-mRNA Processing

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SUMMARY

Recently, we reported that two homologous yeast proteins, Rai1 and Dxo1, function in a quality control mechanism to clear cells of incompletely 5′ end-capped messenger RNAs (mRNAs). Here, we report that their mammalian homolog, Dom3Z (referred to as DXO), possesses pyrophosphohydrolase, that their mammalian homolog, Dom3Z (referred to as DXO), possesses pyrophosphohydrolase, decapping activity on both methylated and unmethyl-capped RNAs. The identification of such catalytic activities suggests the hypothesis that mRNAs with incomplete 5′ end caps are produced in yeast and that Rai1 and Dxo1 function as quality control mechanisms to mediate the clearance of such defective mRNAs. In contrast, the primary transcripts, including the 5′ end triphosphate group, and other capping intermediates cannot serve as substrates for the classical decapping enzymes and would be protected from degradation by the 5′-to-3′ exoribonucleases. Therefore, it was generally believed that the capping process always proceeded to completion.

INTRODUCTION

The 5′ end 7-methylguanosine (m7G) cap of eukaryotic messenger RNAs (mRNAs) is the first modification of nascent transcripts (pre-mRNAs) shortly after the initiation of transcription, and it plays critical roles in mRNA biogenesis and stability (Furuichi and Shatkin, 2000; Ghosh and Lima, 2010; Liu and Kiledjian, 2006; Merrick, 2004; Meyer et al., 2004; Shatkin, 1976). The N7 methyl group on the cap is essential for recognition by the cap-binding proteins CBP20 and elf4E (Fischer, 2009; Gingras et al., 1999; Goodfellow and Roberts, 2008) and for efficient splicing, polyadenylation, mRNA export, and translation. Removal of the 5′ end cap is catalyzed by the Dcp2 (Dunkley and Parker, 1999; Lykke-Andersen, 2002; Wang et al., 2002) and Nudt16 (Li et al., 2011; Song et al., 2010) decapping enzymes, releasing m7 guanosine diphosphate (GDP) and 5′ monophosphate RNA. Decapping is associated with mRNA decay, turnover, and quality control because the 5′ monophosphorylated RNA is rapidly degraded by the cytoplasmic, processive 5′-to-3′ exoribonuclease Xrn1 (Decker and Parker, 1993; Hsu and Stevens, 1993). In contrast, the primary transcripts, including the 5′ end triphosphate group, and other capping intermediates cannot serve as substrates for the classical decapping enzymes and would be protected from degradation by the 5′-to-3′ exoribonucleases. Therefore, it was generally believed that the capping process always proceeded to completion.

Our recent studies on the yeast protein Rai1 and its homolog Dxo1 (Ydr370C) have demonstrated new catalytic activities that can remove incomplete caps from mRNAs, indicating that capping may be less efficient than initially thought. Specifically, Rai1 possesses RNA 5′ pyrophosphohydrolase (PPH) activity, hydrolyzing the 5′ end triphosphate to release pyrophosphates (Xiang et al., 2009). Rai1 also has a distinct “decapping” activity, hydrolyzing the entire cap structure from an unmethylated 5′ end-capped RNA to release GpppN (Jiao et al., 2010). In comparison, Dxo1 lacks PPH activity, but it does possess decapping activity on both methylated and unmethyl-capped RNAs (Chang et al., 2012).

The identification of such catalytic activities suggests the hypothesis that mRNAs with incomplete 5′ end caps are produced in yeast and that Rai1 and Dxo1 function as quality control mechanisms to mediate the clearance of such defective mRNAs. In fact, the catalytic activities of Rai1 and Dxo1 ultimately generate RNAs with 5′ end monophosphate, which can be readily degraded by 5′-to-3′ exoribonucleases. Dxo1 also possesses a distributive 5′-to-3′ exoribonuclease activity, enabling this enzyme to single-handedly decap and degrade incompletely capped mRNAs (Chang et al., 2012).

Studies in yeast cells have confirmed the existence of this mRNA 5′ end capping quality control. Unmethylated 5′ end-capped mRNAs were more stable in cells with a deleted Rai1 gene, and the exposure of these cells to nutrient stress...
generated mRNAs with incompletely capped 5’ ends (Jiao et al., 2010). More importantly, yeast cells in which both Rai1 and Dxo1 were disrupted produced mRNAs with incomplete caps, even under normal growth conditions (Chang et al., 2012). It appears that fungi possess two partially redundant proteins that can detect and degrade incompletely capped mRNAs, which are generated under both stress and nonstress conditions.

Rai1 and Dxo1 have a weak sequence homolog known as Dom3Z in mammals (Xue et al., 2000). Dom3Z has a similar three-dimensional structure (Xiang et al., 2009), but its biochemical activities and biological functions have not been characterized. Here, we report that Dom3Z possesses PPH, decapping, and exoribonuclease activities. Crystal structures of Dom3Z in complex with substrate mimic and products at a resolution of up to 1.5 Å provide elegant insights into the catalytic mechanism and the molecular basis for the three apparently distinct activities of these enzymes. Importantly, Dom3Z preferentially functions on incompletely capped pre-mRNAs. Our studies also reveal unexpected insights into the connection between 5’ end capping and splicing, showing that defective capping inhibits splicing at internal introns, whereas current data suggest that the cap affects the splicing of only the first intron.

RESULTS

Dom3Z/DXO Has Decapping, PPH, and Exonuclease Activities

Mammalian Dom3Z is a weak sequence homolog of yeast Rai1 and Dxo1 but has a strong structural similarity to both (Chang et al., 2012; Xiang et al., 2009). Therefore, we tested which biochemical activities Dom3Z shares with Rai1 and Dxo1. Mouse Dom3Z (Figure S1 available online) readily decapped unmethylated RNAs (25 nM) represented schematically with the P35 labeling indicated by the asterisk. The remaining RNAs were resolved by 5% denaturing PAGE. Catalytically inactive Dom3Z/DXO mutant (Dom3ZE234A) was used as a negative control.

(B) Wild-type (WT) and mutant Dom3Z/DXO proteins were incubated with 5’ end monophosphate 30 nt RNA or DNA substrates labeled at the 3’ end with the FAM (6-carboxyfluorescein) fluorophore. The remaining RNA or DNA fragments were resolved on PEI-TLC developed in 0.45 M (NH4)2SO4 (lanes 1–6) or 0.75 M KH2PO4 ([pH 3.4]; lanes 7 and 8). The migrations of cap analog markers are indicated.

(C) In vitro decay reactions were carried out with 100 nM recombinant His-tagged Dom3Z/DXO for the indicated times with methyl-capped RNAs (25 nM) represented schematically with the P35 labeling indicated by the asterisk. The remaining RNAs were resolved by 5% denaturing PAGE. Catalytically inactive Dom3Z/DXO mutant (Dom3ZE234A) was used as a negative control.

(D) The 5’ end substrate specificity of Dom3Z/DXO was tested as in (A) with RNAs containing distinct 5’ ends denoted schematically. RNAs with a 5’ hydroxyl were not degraded by Dom3Z/DXO.
monophosphate and a 3’ end FAM (6-carboxyfluorescein) fluorophore (Sinturel et al., 2009) was degraded by wild-type (WT) Dom3Z from the 5’ end, with clear intermediates being detected (Figure 1B; lanes 2–4), but not by a catalytically inactive Dom3Z (lane 5), suggesting that Dom3Z has distributive 5’-to-3’ exonuclease activity. The lack of detectable activity on a single-stranded DNA (ssDNA) substrate (lanes 6–9) demonstrated that Dom3Z exonuclease activity is RNA-specific.

To determine whether capped RNAs can be degraded by Dom3Z, we incubated 5’ end-labeled, methyl-capped, or unmethyl-capped RNA in vitro, and measured their decay over time in vitro. All the RNAs were efficiently degraded by Dom3Z (Figure 1C). Dom3Z activity was also tested on RNAs with different 5’ end modifications, which included an unmethylated cap, a triphosphate group, a monophosphate group, and a hydroxyl group. All the substrates except one with a 5’ end hydroxyl were degraded (Figure 1D), demonstrating that the Dom3Z 5’-to-3’ exonuclease activity requires a 5’ end monophosphate on the RNA substrate, which is analogous to the exonuclease activities of Xrn1 and Xrn2.

Overall, our data demonstrate that Dom3Z has three catalytic activities: decapping activity that removes the entire methylated cap-binding proteins, CBP20 and eIF4E (Figure S1), efficiently inhibited Dom3Z decapping of methyl-capped RNA in vitro but had no protective effect on unmethyl-capped RNA (Figure 2). These data indicate that Dom3Z should preferentially target defectively capped RNAs in cells.
Unexpectedly, the structure reveals that a second metal ion is bound in the active site in the presence of the pU5 oligo (Figure 3E). Both metal ions are located in an octahedral coordination sphere. The first metal ion (Mg1) is in the same position that was observed earlier in the free enzyme (Xiang et al., 2009), whereas binding of the second metal ion (Mg2) is possible only in the presence of the RNA, given that one of the terminal oxygen atoms on the 5′ end phosphate of the RNA is a bridging ligand to both metal ions. The side chain of Asp236 (motif III) makes a bidentate coordination of both metal ions, and the side chain of Glu192 (motif II) makes a hydrogen bond to one water ligand on each of the metal ions. Mg1 is also coordinated by the side chain of Glu253 (motif IV), the main-chain carbonyl of residue 254, as well as another terminal oxygen atom of the 5′ end phosphate group of the RNA. Therefore, this phosphate group replaces two of the water ligands of Mg1 in the free enzyme (Xiang et al., 2009). For Mg2, the side chain of Glu234 (the second acidic residue of motif III) and two additional water molecules complete the octahedral coordination.

The ribose of U2 is packed against the side chain of Tyr189 (motif II) (Figures 3C and S4). This residue is most often a phenthaline or tyrosine (Tyr) among these enzymes. In addition, the 2′ hydroxyl group of the ribose is hydrogen-bonded to the main-chain carbonyl of residue 185 (Figure S4). This residue is in the middle of helix aD, but a small kink in the helix in this region makes the carbonyl oxygen available for hydrogen bonding to the ribose.

Crystal Structure of DXO in Complex with an RNA Oligonucleotide Substrate Mimic

To observe the binding mode of the 5′ end nucleotide of a substrate RNA in the DXO active site, we used a hexanucleotide with a 5′ end monophosphate and with the phosphodiester group between nucleotides 1 and 2 and 2 and 3 replaced with a phosphorothioate group to inhibit the hydrolysis of this RNA. Therefore, this pU(S)6 oligonucleotide had the sequence pU1-SpU2-SpU3-U4-U5-U6. In addition, we replaced the Mg2 ion with a Ca2+ ion during crystallization to further block hydrolysis of the RNA. Our enzymatic assays showed that DXO is essentially inactive, Ca2+ being the divalent metal ion (data not shown).

We have determined the crystal structure of mouse DXO in complex with Ca2+ and the pU(S)6 oligonucleotide at a resolution of 1.7 Å (Table 1 and Figure S5). Clear electron density was observed for the first four nucleotides of the RNA (Figure 3F). Very weak electron density was observed for the bases of the last two nucleotides, and they were not included in the atomic model.

The RNA is bound in the active site with the phosphorothioate group between nucleotides 1 and 2 located next to the catalytic site (Figure 3G). The 5′ end phosphate group of the oligo interacts with the side chain of Arg132 (motif I), and it is also close to the side chain of Gln280 (Figure 3G). The base of the first nucleotide maintains π-stacking with that of the second nucleotide. The binding modes of nucleotides 2–4 are highly similar to those of their equivalents in the pU5 complex (Figure S5).

Only one Ca2+ ion was observed in the complex, at the same position as Mg1 in the pU5 complex (Figure 3G). The terminal oxygen atom of the phosphorothioate linkage between nucleotides 1 and 2 is a ligand to the metal ion. The conformation of this phosphorothioate group is different from that of the 5′ end phosphate group of the pU5 complex (Figure 3H), equivalent to a ~60° rotation around the O5′-P bond. The sulfur atom in pU(S)6 cannot have a strong interaction with the metal ion and may have (at least partly) driven this conformational change. In fact, this sulfur atom is positioned farthest away from the metal ion (Figure 3G). Besides the phosphorothioate group, a conformational change in the Tyr189 side chain is also observed in the pU(S)6 complex, resulting from the presence of the U1 base (Figure 3H). In addition, the side chain of Glu234 assumes a different conformation, resulting from the absence of the second metal ion.

Although the first nucleotide of the pU(S)6 RNA may mimic the 5′ end of the RNA substrate in terms of exonuclease activity, the phosphorothioate group between nucleotides 1 and 2 is not in the correct conformation for catalysis. The water molecule (or hydroxide ion) that would need to attack the phosphorus atom would not be in the correct position to be activated by any functional group in the active site (Figure 3G). Therefore, conformational changes are expected in this phosphate group, and possibly those atoms that are covalently attached to it, to make this a true substrate. The conformation of the 5′ end phosphate in the pU5 oligo might be a better mimic for the scissile phosphate group.

Crystal Structure of DXO in Complex with the m7GpppG Cap

To understand how the RNA cap is recognized by DXO, we determined the structure of the WT murine DXO in complex with m7GpppG at a resolution of 1.5 Å (Table 1 and Figure 4A). There are only a few conformational differences in the active site region between this structure and the one in complex with pU5, the most important of which is the side chain of Glu234. This side chain is somewhat disordered in the cap complex as well as in the free enzyme, and it becomes well-ordered as a ligand to Mg2 in the pU5 complex.

The m7Gpp group of the cap has good electron density (Figure 4B) and is bound in the active site region at a position similar to that of GDP observed earlier (Xiang et al., 2009) (Figure 4C). The 7-methyl group of guanine is not recognized specifically by the enzyme, which is consistent with our biochemical data showing that DXO is nonselective with regard to the methylation status of the cap (Figure 1A). In fact, residues that contact this guanine base are not well conserved among the enzymes (Figure S3). The ribose is packed against the side chain of Trp131, the residue just preceding motif I, which is conserved as an aromatic residue in most DXO and Rai1 homologs (Figure S3). One of the terminal oxygen atoms on the α-phosphate has ionic interactions with the side chain of Arg132 (motif I) and is hydrogen-bonded to the main-chain amide of Glu234, whereas the other terminal oxygen atom is hydrogen-bonded to the main chain amide of Arg132 at the N-terminal end of helix aB. Therefore, the α-phosphate also has favorable interactions with the dipole of this helix. The β-phosphate is located near the side chains of Arg132 and Gln280, and, in fact, this β-phosphate overlaps with the 5′ end phosphate of the pU(S)6 nucleotide (Figure 3H).
Figure 3. Crystal Structure of Wild-Type Murine DXO in Complex with pU5 and Two Mg\(^{2+}\) Ions

(A) A schematic drawing of the structure of DXO (in green for the large \(\beta\) sheet, cyan for the small \(\beta\) sheet, yellow for the helices, and magenta for the loops) in complex with a pU5 RNA oligonucleotide (in black for carbon atoms) and Mg\(^{2+}\) ions (orange).

(B) Simulated annealing omit Fo–Fc electron density at a resolution of 1.8 Å for pU5 contoured at 2.5σ.

(C) Interactions between the pU5 RNA with the DXO active site.

(D) The molecular surface of the active site region of DXO. The pU5 RNA is shown as sticks.

(E) The coordination spheres of the two Mg\(^{2+}\) ions and the detailed interactions between the 5’ end phosphate group of pU5 and the two Mg\(^{2+}\) ions.

(F) Simulated annealing omit F\(_o\)–F\(_c\) electron density at a resolution of 1.7 Å for pU(S)6 contoured at 2.2σ. Very weak electron density is observed for the last two nucleotides, and they are not included in the atomic model.

(legend continued on next page)
R145A (interacting with U6 at the opening of the active site nucleotide to the 3′ wall of the active site pocket, leaving no room to attach another structure figures were produced with PyMOL (www.pymol.org).

The second guanosine group of the cap is packed against the wall of the active site pocket, leaving no room to attach another nucleotide to the 3′ hydroxyl group of its ribose (Figure 4C).

Mutagenesis Studies Support the Structural Observations
To assess the importance of residues in the active site region of DXO, we created the following structure-based mutants (Figure S1): K273A, R294A, and K273A-R294A (interacting with the backbone phosphate; Figure 3C), Q280A (interacting with the 5′ end phosphate of the substrate; Figure 3G), Y189A (interacting with the ribose of U2; Figure 3C), H272A, and R145A (interacting with U6 at the opening of the active site pocket; Figure 3C). The Q173A mutant was created as a control, which is located far from the active site.

The K273A, R294A, and Y189A substitutions led to reduced exonuclease activity, whereas the K273A-R294A, E234A, and Q280A mutants ablated exonuclease activity (Figure 4E). The H272A and R145A mutants at the opening of the active site retain a majority of the WT exonuclease activity. The effects of these mutations on decapping activity mostly follow those for exonuclease activity; one exception being that the Y189A mutant had essentially normal decapping activity (Figure 4F). Overall, our structural observations provide the molecular framework for the decapping, PPH, and exonuclease activities of DXO.

**DXO Preferentially Functions on Defectively Capped Pre-mRNAs in Vivo**
Having established the biochemical activities and the molecular mechanism of DXO, we next characterized the functions of this protein in mammalian 293T cells with an shRNA-directed >95% reduction of DXO (DXO<sup>KD</sup>; Figure S6). Our previous studies with Rai1 and Dxo1 showed that the two proteins mediate the clearance of incompletely capped mRNA in yeast cells (Jiao et al., 2010, Chang et al., 2012). Using primer pairs that span two different exons to detect spliced mRNA from two randomly selected mRNAs, CamKl and Fhit, we observed only a modest 20% increase in the steady-state levels of these mRNAs between control and DXO<sup>KD</sup> cells (Figure 5A). However, analysis of the pre-mRNAs of the same genes revealed a more dramatic accumulation in the DXO<sup>KD</sup> cells. Using primers that span the exon 1-intron 1 junction to detect unspliced, intron 1-containing RNAs, we observed a 2-fold increase of both CamKl and Fhit pre-mRNAs under reduced DXO levels (Figure 5B). Because a link between capping and splicing of the first intron (but not subsequent introns) has been reported (Edery and Sonenberg, 1985; Izaurralde et al., 1994; Konarska et al., 1984), we next tested whether the increased level of pre-mRNA in the DXO<sup>KD</sup> cells was restricted to splicing of just the first intron. Surprisingly, the increase in CamKl and Fhit pre-mRNA levels were also detected when several different downstream unspliced introns were assessed (Figure 5B), demonstrating that unspliced pre-mRNAs accumulate in DXO<sup>KD</sup> cells. These findings demonstrate an unexpected link of the capping process to splicing beyond only the first intron. Moreover, analysis of whether these transcripts are properly polyadenylated by quanitative RT-PCR (qRT-PCR) amplification through the poly(A) addition site shows an increase in uncleaved 3′ ends for both genes in the DXO<sup>KD</sup> cells relative to control cells (Figure 5C), suggesting a defect in the cleavage reaction of 3′ end processing in DXO<sup>KD</sup> cells. Collectively, these data indicate a role for DXO in degrading unprocessed pre-mRNAs and demonstrate an important link of an endogenously produced, defective capped pre-mRNA with defective processing.

The preferential function of DXO on incompletely capped RNAs (Figures 1 and 2) and earlier in vitro demonstrations that suggested the polyadenylation cleavage step was facilitated by the mRNA cap (Cooke and Alwine, 1996; Flaherty et al., 1997; Gilmartin et al., 1988; Hart et al., 1985) indicate that the increase in pre-mRNA observed in Figures 5B and 5C would correspond to incompletely capped pre-mRNAs. To test this hypothesis, we resolved methyl-capped or incompletely capped RNA populations with anticap immunoprecipitation under conditions that retain methyl-capped, but not unmethyl-capped.
or uncapped, RNAs (Figure 5D) (Chang et al., 2012; Jiao et al., 2010). As expected, the level of methyl-capped mature CamKI and Fhit mRNAs were comparable between control and DXOKD cells (Figure 5E). In contrast, a significant decrease in the level of methyl-capped pre-mRNAs was detected in the DXOKD cells relative to the corresponding total pre-mRNA when compared to that observed in control cells (Figure 5F). The relative decrease in methyl-capped pre-mRNA as a proportion of the total pre-mRNA in the DXOKD cells indicates a corresponding increase of defectively capped pre-mRNAs relative to total pre-mRNA.

To determine whether the increased level of pre-mRNAs in the DXOKD cells could be attributed to pre-mRNA stability, we characterized the effect of DXO on a short-lived mRNA, c-fos. Similar to CamKI and Fhit, levels of c-fos pre-mRNA also increased in the DXOKD cells (Figure 6A), whereas methyl-capped pre-mRNA decreased (Figure 6B), corresponding to an increase in defectively capped pre-mRNAs. Moreover, as would be predicted from the above data, DXO selectively influenced the stability of the c-fos pre-mRNA, but not of the c-fos mRNA, after actinomycin D-directed transcriptional silencing (Figure 6C). The stability of the c-fos pre-mRNA increased >5-fold in DXOKD cells relative to control cells, with a t1/2 of 130 min versus 25 min in controls. A similar increase was not detected with the c-fos mRNA, indicating that DXO preferentially functions on pre-mRNAs lacking a normal m7G cap at the 5′ end.

**DISCUSSION**

Here, we report that the mammalian Dom3Z/DXO protein is a dual nuclease that preferentially functions on incompletely capped pre-mRNAs. DXO removes the entire cap structure to generate a 5′ end monophosphate that subsequently serves as a substrate for a second catalytic activity intrinsic to the same DXO active site, a 5′-to-3′ exoribonuclease activity, to degrade the RNA body, and defectively capped RNAs accumulate in DXOKD cells. These findings reinforce our recent demonstrations in S. cerevisiae (Chang et al., 2012; Jiao et al., 2010) that show
that cap addition is not a default process that always proceeds to completion. Importantly, our data also reveal that pre-mRNAs in mammalian cells with an aberrant 5’ end do not efficiently proceed into the normal RNA-processing pathways of splicing and polyadenylation. The lack of efficient processing of incompletely capped pre-mRNAs that are more apparent after DXO knockdown implicates DXO in a pre-mRNA quality control mechanism that detects and degrades defective pre-mRNAs (summarized in Figure 7).

Biochemical Activities of DXO
Thus far, we have identified two fungal proteins that possess decapping activity on incompletely capped mRNAs: the nuclear Rai1 protein (Jiao et al., 2010; Xiang et al., 2009) and the previously uncharacterized Ydr370C gene product, which encodes the predominantly cytoplasmic Dxo1 protein (Chang et al., 2012). On the basis of sequence homology, DXO was initially proposed to be the mammalian homolog of Rai1 (Xue et al., 2000); however, structural analysis reveals that all three proteins share extensive structural identity (Chang et al., 2012; Xiang et al., 2009), and biochemical studies suggest that DXO is a hybrid of both fungal proteins. In vitro, Rai1 functions on unmethyl-capped RNA (GpppRNA) and 5’ pppRNA (Jiao et al., 2010; Xiang et al., 2009). Dxo1 functions on methylated (m7GpppRNA) and unmethylated (GpppRNA) capped RNA but not on 5’ pppRNA, whereas DXO can function on all three substrates (Figure 1). Both Dxo1 and DXO possess intrinsic 5’-to-3’ exonuclease activity (Chang et al., 2012; Figure 1). One unanimous function for all three proteins is their preferential decapping of GpppRNA (summarized in Table S1). Collectively,
Molecular Basis of the Distinct DXO Catalytic Activities

The structures of the pU5, pU(S)6, and cap complexes provide elegant insights into the catalytic mechanism of DXO and related enzymes and indicate that the three distinct catalytic activities are mediated by the same active site machinery. The pU5 RNA is bound as a product, mimicking the RNA body. The 5’ end phosphate group of pU5 is the scissile phosphate, which is recognized by the enzyme through two metal ions. The catalytic nucleophile is a water molecule or hydroxide ion, bound and activated by one of the metal ions, most likely the sole water ligand of Mg1 in the pU5 complex, which is also activated by Glu192 (Figure 3E). This water molecule is located about 3.9 Å from the phosphorus atom and is at the correct position for an inline attack. The terminal oxygen atom opposite of this water molecule is then the leaving group, and the oxyanion can be stabilized by the side chain of Lys255 (motif IV) (Figure 4D). This two-metal-ion mechanism is similar to that employed by many other nucleases (Yang, 2011).

The structure shows that the cap (m7GpppG) is accommodated on the other side of the catalytic machinery from the RNA body (the pU5 oligo) (Figure 4D), thereby explaining the decapping activity that removes (m7)GpppG. Similarly, the structure of the pU(S)6 complex shows that the 5’ end nucleotide (pN1) or pyrophosphate group can also be accommodated across the active site (Figure 4D), giving rise to 5’-to-3’ exonuclease or PPH activity. Therefore, the three catalytic activities of these enzymes use the same catalytic machinery, and it is the distinct binding modes of the three different substrates that dictate the outcome of the reaction. Nevertheless, each of the DXO, Rai1, and Dxo1 enzymes also has its unique properties, such as the lack of PPH activity by Dxo1 and the selectivity between GpppRNA and m7GpppRNA by Rai1 and Dxo1 (Chang et al., 2012; Xiang et al., 2009). Additional studies will be needed in order to define the molecular mechanism for these unique properties.

DXO in Methyl-Capped mRNA Decapping

The ability of DXO to function on m7G capped RNA in vitro (Figure 1) indicates that DXO may also contribute to m7G-capped RNA decapping and decay. The finding that cap-binding proteins can efficiently inhibit DXO activity (Figure 2) suggests that any such activity is most likely regulated and would require the removal of cap-binding proteins. The modest 20% increase in mRNA levels observed in the DXO KD cells (Figure 3A) is consistent with a potential limited role on m7G-capped mRNAs. In addition, the nuclear localization of DXO (Zheng et al., 2011) and its function on m5,2,7G-capped RNA (Figure 1) also indicates that DXO may modulate trimethyl-capped uridylyl-rich small nuclear RNAs (UsnRNAs). Future studies will address this possibility.

m7G Cap and Pre-mRNA Processing

Our finding that incompletely capped pre-mRNAs are inefficiently spliced and polyadenylated demonstrates that the cap is more intimately linked to splicing and polyadenylation than previously perceived. Initial studies that established a link between the cap and first intron splicing (Edery and Sonenberg, 1985; Inoue et al., 1989; Izaurralde et al., 1995; Izaurralde et al., 1994; Konarska et al., 1984) either utilized m7G-capped mRNA with a depleted cap-binding protein or introduced unmethyl-capped pre-mRNA. In all cases, a requirement for the cap-binding protein was observed for first intron splicing, which was consistent with the exon definition of splicing (Colot et al., 1996; Fortes et al., 1999; Izaurralde et al., 1994; Lewis et al., 1996). An important distinction with the current study is that we are following nascent transcripts that are generated without a proper cap. An appealing model could be that cotranscriptional cap addition is required to facilitate subsequent pre-mRNA

Rai1, Dxo1, and DXO identify a class of proteins that function in a quality control mechanism to ensure proper mRNA 5’ end fidelity wherein DXO preferentially functions to contain incompletely capped pre-mRNAs (Figure 7).
processing and implies a cotranscriptional coordination of the capping process with splicing and polyadenylation factors to dictate pre-mRNA processing. One candidate that can coordinate these processes could be the cap-binding complex (CBC), which cotranscriptionally associates with the capped 5' end and indirectly affects the alternative splicing of a subset of genes in a transcript-specific manner (Lenasi et al., 2011). Whether the CBC is necessary and involved in recruiting splicing factors to intron-containing genes remains to be determined.

Our data reveal that incompletely capped pre-mRNAs do not efficiently proceed into the normal RNA processing pathways of splicing and polyadenylation and, instead, appear to be degraded by DXO. Importantly, such a quality control mechanism provides a dual layer of surveillance to prevent the accumulation of potentially deleterious defectively capped pre-mRNAs, whereby they are inefficiently processed into mature mRNA and selectively decapped and degraded by DXO from the 5' end. Future studies will begin to uncover the molecular mechanism underlying such a surveillance process.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Recombinant Protein Expression Mutagenesis**

Structure-based, site-specific mutations were created by PCR-based methods with the use of the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and sequenced for confirmation of correct incorporation of the mutations.

**Protein Expression, Purification, and Crystallization**

The protocols for the expression, purification, and crystallization of mouse DXO have been previously reported (Xiang et al., 2009). The mutant proteins were purified by Ni-NTA Superflow (QiAGEN) and gel filtration chromatography, through the same protocol as that used for the WT enzyme. Free enzyme crystals of DXO were obtained with the sitting-drop vapor diffusion method at 20°C with a reservoir solution containing 20% (w/v) PEG 3350. The pU5-Mg2+ complex was obtained by soaking the free enzyme crystals with 10 mM pU5 and 10 mM MgCl2 for 90 min in the presence of 15% ethylene glycol. The pU(S)6-Ca2+ complex was obtained by soaking DXO crystals with 10 mM pU(S)6 and 20 mM CaCl2 for 120 min in the presence of 15% ethylene glycol. The m7GpppG complex was obtained by soaking DXO crystals with 5 mM m7GpppG for 30 min in the presence of 15% ethylene glycol. Crystals were flash frozen in liquid nitrogen for diffraction analysis and data collection at 100 K.

**Data Collection and Structure Determination**

X-ray diffraction data were collected at the National Synchrotron Light Source (NSLS) beamline X29A. The diffraction images were processed and scaled with the HKL package (Otwinowski and Minor, 1997). The crystals belong to space group P212121, with cell parameters of a = 50.0 Å, b = 87.7 Å, c = 53.9 Å, and β = 112.2°. There is one molecule of DXO in the crystallographic asymmetric unit. The structure refinement was carried out with the Coot (Crystallographic Object-Oriented Toolkit) program (Emsley and Cowtan, 2004). The crystallographic information is summarized in Table 1.

**Cell Culture and Generation of Stably Transformed Knockdown Cell Line**

Human embryonic kidney 293T cells were obtained from ATCC and cultured according to the supplier’s protocol. DXO-specific small hairpin RNA (shRNA) plasmid and control nonspecific shRNA plasmids were obtained from Sigma-Aldrich, and transfections were carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Monoclonal lines of stably transformed DXO cells expressing DXO-specific shRNA (DXO(3b)) were selected with puromycin (3 μg/ml) and confirmed by western blotting.

**RNA Generation**

RNA corresponding to the pcDNA3 polylinker (pcP) region with a 3' end containing 16 guanosines was transcribed in vitro with T7 polymerase for the generation of pcP RNA with an N7-methylated or -unmethylated 5' cap or no cap at all, as previously described (Chang et al., 2012; Jiao et al., 2010). Tri-methylated 7mP-cap-labeled pcP RNA was generated in the capping reaction in the presence of human recombinant trimethyltransferase (Benaroch

**Figure 7. Model of 5' End Quality Control in Mammalian Cells**

Incompletely 5' end-capped pre-mRNA (unmethyl-capped and uncapped 5' triphosphate pre-mRNA) would be preferentially detected by DXO and subjected to 5' end cleavage and degradation. The RNA polymerase II (Pol II), the carboxyl terminal domain of RNAP II (CTD), the triphosphatase-guanylyltransferase capping enzyme (CE), the methyltransferase (MT), the nuclear CBC, and the cytoplasmic cap binding protein eIF4E are as indicated.
et al., 2010) and S-adenosyl methionine. We generated 3’ end 53P-labeled RNA with T4 RNA Ligase and [53P]pCp (Wang and Kiledjian, 2000a). In vitro transcriptions were carried out in the presence of [γ-32P]GTP for 5’ end 32P-labeled triphosphate RNA or with [α-32P]GTP to obtain 32P-uniform-labeled RNAs as described previously (Jiao et al., 2006). We generated 5’-monophosphate 32P-uniform-labeled RNAs by digesting methyl-capped 32P-uniform-labeled RNA with a human Dcp2 degrading enzyme. RNA lacking a phosphate at the 5’ end was generated by treating 32P-uniform-labeled triphosphate RNA with calf intestinal alkaline phosphatase. Fluorescently labeled RNA oligos (Chang et al., 2012) were purchased from Integrated DNA Technologies (IDT).

**RNA Decapping and In Vitro Decay Assay**

Decapping reactions used either His-tagged mouse Dxo WT or mutant recombinant protein (25 nM), and were carried out by incubating with 32P-cap-labeled or 32P-5’-end-labeled pcP RNAs in decapping buffer, as previously described (Jiao et al., 2010), at 37°C for 30 min. Exoribonuclease assays were carried out with 100 nM His-Dxo in the same buffer. The decapping products were resolved by polyethyleneimine-cellulose thin-layer chromatography (PEI-TLC) plates, and the decay reactions were resolve by 5% denaturing polyacrylamide gel electrophoresis.

**Exonuclease Assays with Fluorescently Labeled RNA**

The 3’-FAM-labeled 30-mer RNA with 5’ end monophosphate (Sinturel et al., 2009) and the equivalent ssDNA oligos were purchased from IDT. Exonuclease assays were performed at 37°C for 30 min with reaction mixtures containing 30 mM Tris (pH 8.0), 50 mM NH4Cl, 2 mM MgCl2, 0.5 mM dithiothreitol, 25 μM MgCl2, 2 mM 3’-FAM-labeled oligo, and the indicated amount of recombinant Dxo. The products were fractionated by 5% denaturing polyacrylamide gel and visualized on a UV illuminator. Assays were repeated at least three times to ensure reproducibility.

**RNA Isolation**

Total RNAs were isolated with Trizol reagent (Invitrogen) under the manufacturer’s protocol and treated with RNase-free DNase (Promega) for removal of the genomic DNA contamination. For mRNA in vivo stability assays, the transcriptions of 293T control or DxoKO cells were blocked by actinomycin D (5 μg/ml) or 18S rRNA or glyceraldehyde 3-phosphate dehydrogenase mRNA.

**Methyl-Capped RNA Immunoprecipitation**

Methyl-capped RNAs were immunoprecipitated with monoclonal antitrime-thylguanosine antibody column (Calbiochem), as previously described (Chang et al., 2012; Jiao et al., 2010), from 30 ng ribosomal RNA (rRNA) minus RNA with one round of immunoprecipitation. rRNA depletion was carried out from 0.5 μg total RNA with the RiboMinus Eukaryote Kit (Invitrogen).

**Real-Time qRT-PCR**

RNAs were reverse transcribed into complementary DNA with random primers and M-MLV Reverse Transcriptase (Promega) according to the manufacturer’s protocol. For detection of pre-mRNA, the reverse transcription was performed with gene-specific pre-mRNA primers. Real-time PCR was performed with Taq Supermix (Bio-Rad) on the ABI Prism 7900HT sequence detection system (Invitrogen) (Jiao et al., 2006; Jiao et al., 2010). Each gene was amplified with the appropriate specific primers (Table S2), mRNA levels were computed by the comparative Ct method and normalized to internal control 18S rRNA or glyceraldehyde 3-phosphate dehydrogenase mRNA.

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