

Article Title: **The complex enzymology of mRNA decapping: enzymes of four classes cleave pyrophosphate bonds**

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**Abstract**

The 5′ ends of most RNAs are chemically modified to enable protection from nucleases. In bacteria, this is often achieved by keeping the triphosphate terminus originating from transcriptional initiation, while most eukaryotic mRNAs and small nuclear RNAs have a 5′-5′ linked N7-methyl guanosine (m7G) cap added. Several other chemical modifications have been described at RNA 5′ ends. Common to all modifications is the presence of at least one pyrophosphate bond.

To enable RNA turnover, these chemical modifications at the RNA 5′ end need to be reversible. Dependent on the direction of the RNA decay pathway (5′-3′ or 3′-5′), some enzymes cleave the 5′-5′ cap linkage of intact RNAs to initiate decay, while others act as scavengers and hydrolyse the cap element of the remnants of the 3′-5′ decay pathway. In eukaryotes, there is also a cap quality control pathway.

Most enzymes involved in the cleavage of the RNA 5′ ends are pyrophosphohydrolases, with only a few having (additional) 5′ triphosphonucleotide hydrolase activities. Despite the identity of their enzyme activities, the enzymes belong to four different enzyme classes. Nudix hydrolases decap intact RNAs as part of the 5′-3′ decay pathway, DXO family members mainly degrade faulty RNAs, members of the histidine triad (HIT) family are scavenger proteins, while an ApaH-like phosphatase is the major mRNA decay enzyme of trypanosomes, whose RNAs have a unique cap structure. Many novel cap structures and decapping enzymes have only recently been discovered, indicating that we are only beginning to understand the mechanisms of RNA decapping.



**Graphical/Visual Abstract and Caption**

Enzymes that hydrolyse the 5′ end of RNAs belong to four different enzyme classes: Nudix hydrolases, histidine triad (HIT) proteins, DXO proteins and ApaH-like phosphatases.

**Introduction**

Single stranded RNAs with a free 5′ monophosphate end are susceptible to rapid degradation. tRNAs and rRNAs are stabilised by hairpin structures and by ‘hiding’ their 5′ ends within complex protein structures. All other RNA species avoid monophosphates at their 5′ ends and instead carry chemical modifications arising either from the nucleotide initiating transcription or from further 5′ end processing. These 5′ end modifications can be as simple as a di- or triphosphate – often found in bacterial RNAs – or more complex like the 5′–5′ linked N7-methyl guanosine (m7G) cap of eukaryotes or the NAD+ cap found in both eukaryotes and bacteria. Despite the apparent differences, all chemical modifications at the 5′ end possess at least one pyrophosphate bond. In addition to providing protection from decay, the chemical nature of an RNA 5′ end can influence its properties and thus provides epitranscriptomic information (Jäschke, Höfer, Nübel, & Frindert, 2016). Not all modifications result in RNA protection; some can also decrease RNA stability, for example the NAD+ cap of eukaryotic RNAs (Jiao et al., 2017).

Importantly, 5′ end modifications of RNAs need to be reversible to allow RNA turnover (Figure 1). Eukaryotic mRNA decay pathways (reviewed in (Garneau, Wilusz, & Wilusz, 2007) usually start with the removal of the poly(A) tail from the mRNA 3′ end. Then, degradation proceeds either 3′-5′ or 5′-3′. In 3′-5′ decay, the RNA body is exonucleolytically degraded 3′-5′ by the cytoplasmic exosome and the remaining cap structure, which is potentially toxic to the cell, is finally hydrolysed by scavenger decapping enzymes. 5′-3′ decay typically starts with cleavage of a cap pyrophosphate bond by decapping enzymes and the monophosphorylated RNA is then degraded by the 5′-3′ exoribonuclease Xrn1. The enzymes involved in mRNA decay in bacteria are less conserved, but there are two basic pathways to initiate decay (reviewed in (Hui, Foley, & Belasco, 2014)). In the ‘direct access pathway’, decay starts with internal cleavage of the mRNA by an endonuclease. This cleavage creates one 5′ fragment with an unstable 3′ end (note that 3′ ends of intact bacterial RNAs are usually protected from decay by a hairpin structure) that can be degraded by 3′-5′ exoribonucleases, sometimes aided by enzymes that add poly(A) tails to help denature secondary structures. The other fragment of this initial internal cleavage reaction, the 3′ fragment of the mRNA, now has a monophosphorylated 5′ end which renders it sensitive to degradation by different types of nucleases. The type of nuclease is species-dependent: for example, *Escherichia coli* uses endonuclease RNAse E and *Bacillus subtilis* uses exonuclease RNAse J. In the ‘5′ end dependent pathway’, the nudix RNA pyrophosphohydrolase RppH transforms the 5′-triphosphorylated RNAs into 5′-monophosphorylated RNAs, possibly via a diphosphorylated RNA intermediate produced by an unknown enzyme. The monophosphorylated RNAs are then sensitive to decay by endo- or exonucleases (see above). Next to these basic mRNA decay pathways, other enzymes target the 5′ end of RNAs with less common 5′ structures and, at least in eukaryotes, a cap quality control pathway ensures that RNAs with faulty caps are degraded. Moreover, while it was long believed that the ultimate fate of any decapped mRNA is decay, it is now clear that at least some organisms also have a cytoplasmic recapping system (Ignatochkina, Takagi, Liu, Nagata, & Ho, 2015; Mukherjee, Bakthavachalu, & Schoenberg, 2014; Mukherjee et al., 2012; Otsuka, Kedersha, & Schoenberg, 2009).

In this review, we will first summarise current knowledge of RNA modifications at the RNA 5′ end in both prokaryotes and eukaryotes (Figure 2). Some of these modifications have only recently been discovered, namely the diphosphates on the 5′ ends of bacterial mRNAs or the NAD+ caps present on a subset of yeast and mammalian RNAs. We will then discuss the different types of enzyme that are involved in resolving the 5′ end structures of eukaryotic and bacterial RNAs (Figure 2). These enzymes belong to four very different enzyme classes with different catalytic mechanisms and structures, namely nudix hydrolases, DXO family proteins, histidine triad (HIT) proteins and ApaH-like phosphatases. Despite these differences, most enzymes share the same enzymatic activity, a pyrophosphohydrolase activity that hydrolyses a pyrophosphate bond in the cap or cap remnant. Nudix hydrolases account for most eukaryotic decapping enzymes that hydrolyse a cap pyrophosphate bond in the intact RNA molecule and are thus part of the 5′-3′ decay pathway. Members of the rather diverse DXO protein family are mainly involved in mRNA cap quality control and selectively degrade mRNAs with a faulty cap; recent findings show that DXO proteins are also involved in the removal of the NAD+ cap. The third group of enzymes belong to the histidine triad (HIT) family of proteins and are mainly involved in cleaving a pyrophosphate bond of capped di- or oligonucleotides. These are mostly remnants of the 3′-5′ decay pathway, but the HIT protein DcpS can also hydrolyse the pyrophosphate bond of m7GTP, which is generated from m7GDP produced in the 5′-3′ decapping pathway. The fourth group are the ApaH-like phosphatases, a subgroup of the phosphoprotein phosphatase (PPP) family. Only one member of this enzyme class, *Tb*ALPH1 *of Trypanosoma brucei*, has been identified as a decapping enzyme so far. *Tb*ALPH1 is the major or only mRNA decapping enzyme of the 5′-3′ pathway in this parasite, which lacks homologues to any other enzymes of the eukaryotic decapping complex. Notably, *Tb*ALPH1 is the first eukaryotic ApaH-like phosphatase to be assigned a function, leaving potential for the discovery of further decapping proteins in this enzyme class. Many decapping enzymes and decay pathways have only recently been discovered, indicating that the full set of proteins involved in mRNA decapping and removal of capped metabolites still awaits discovery.

Given the authors core expertise, the review will be biased towards eukaryotes and mRNA. To a lesser extent we will consider bacteria and other RNA species but regret that this cannot be done in a comprehensive manner.

**CHEMICAL MODIFICATIONS AT THE 5′ ENDS of mRNA**

## 5′ ends of prokaryotic RNAs

In bacteria, the current view is that most mRNAs are simply stabilised by a 5′ terminal triphosphate, which is the unaltered 5′ end arising from the NTP initiating transcription. This view is challenged by the recent finding that, at least in *E. coli*, 5′ diphosphorylated mRNAs are much more abundant than 5′ triphosphorylated mRNAs and are also better substrates for the decay-initiating enzyme RppH (Luciano, Vasilyev, Richards, Serganov, & Belasco, 2017). Whether these diphosphorylated mRNAs are decay intermediates or functional mRNA states remains to be investigated. In 2009, two further modifications were discovered at the 5′ end of bacterial RNAs: the NAD+ cap (Y. G. Chen, Kowtoniuk, Agarwal, Shen, & Liu, 2009) and the 3′-dephospho-coenzyme A cap (dpCoA) (Kowtoniuk, Shen, Heemstra, Agarwal, & Liu, 2009). In 2015, an RNA sequencing approach (NAD captureSeq) identified the NAD+ RNAs as being mainly small regulatory RNAs (sRNAs) and sRNA-like 5′ terminal fragments of certain mRNAs (Cahová, Winz, Höfer, Nübel, & Jäschke, 2015). Like a eukaryotic m7G cap, the NAD+ cap stabilises RNAs *in vitro* against the bacterial RNA 5′ decay machinery (RppH, RNAse E) (Cahová et al., 2015). NAD+ is incorporated into the transcript during transcriptional initiation: it is used as a non-canonical initiating nucleotide (NCIN capping) (Bird et al., 2016). Certain promoter sequences favour NCIN capping by NAD+, in particular the -1 position appears to be important (Bird et al., 2016; Vvedenskaya et al., 2018). The RNAs with a dpCoA cap have not yet been characterised and neither has the function of this specific cap, but it is known to be added by NCIN capping (Bird et al., 2016). NADH, FAD and UDP-sugars can also participate in NCIN capping *in vitro* but cellular RNAs have yet to be detected with such caps (Kiledjian, 2018).

RNA metabolism in Archaea is still poorly understood, but their mRNAs also carry triphosphates at their 5′ ends, like bacterial mRNAs (Clouet-d'Orval et al., 2018). There is evidence for these triphosphorylated 5′ ends being bound by proteins, thus forming a structure that resembles a eukaryotic cap (Arkhipova et al., 2015; Hasenöhrl, Lombo, Kaberdin, Londei, & Bläsi, 2008).

## 5′ ends of eukaryotic RNAs

In eukaryotes, the triphosphorylated 5′ end of most mRNAs is modified by addition of a 5′–5′ linked N7-methyl guanosine (m7G) cap (cap0), shortly after transcription has started (Furuichi et al., 1975; Wei, Gershowitz, & Moss, 1975a). Next to protecting the mRNA from decay (Furuichi, LaFiandra, & Shatkin, 1977; Hsu & Stevens, 1993; Sachs, 1993), the cap also serves as a binding platform for translation initiation factors (Sonenberg, Rupprecht, Hecht, & Shatkin, 1979) and is important in pre-mRNA splicing, mRNA processing and export (Edery & Sonenberg, 1985; Konarska, Padgett, & Sharp, 1984; Visa, Izaurralde, Ferreira, Daneholt, & Mattaj, 1996). Capping requires three enzymatic reactions: first, the removal of the -phosphate group by an RNA triphosphatase activity, second the addition of a GMP to the diphosphorylated RNA substrate, and third the methylation of the guanosine base of this GMP at the N7 position (reviewed in (Ramanathan, Robb, & Chan, 2016)). This cap0 structure can be further modified by methylations on the ribose 2’-hydroxyl positions of the first and second transcribed nucleotides, resulting in cap1 and cap2 (Adams & Cory, 1975; Wei, Gershowitz, & Moss, 1975b), respectively. These modifications allow the distinction between cellular and foreign RNA, contribute to translational efficiency (Daffis et al., 2010; Devarkar et al., 2016), and protect mRNAs from degradation and decapping by the cap quality control enzyme DXO (Picard-Jean et al., 2018). If the first transcribed nucleoside is an adenosine, it can be further modified by methylations at the N6 and ribose 2’-OH positions (m6Am) (Wei, Gershowitz, & Moss, 1975b); this modification increases mRNA stability and is dynamic, adding an epitranscriptomic layer of regulation to the mRNA (Mauer et al., 2017). mRNAs of kinetoplastids possess the unique, heavily methylated, cap4 structure, this being a likely reason why trypanosomes use a very special decapping enzyme (discussed in detail below).

The class of small nuclear RNAs (snRNAs, snoRNAs) have a m7G cap that is further modified in the cytoplasm to the trimethylated m2,2,7G cap, which allows the reimport of these mRNAs into the nucleus (Hamm & Mattaj, 1990; Lamond, 1990).

In addition to the m7G cap and its variations, it was recently found that a small fraction of eukaryotic RNAs carries NAD+ caps (reviewed in (Kiledjian, 2018)). RNA polymerase II can use NAD+ as the initiating nucleotide *in vitro* (Bird et al., 2016) and mRNAs and small non-coding RNAs from human, as well as cytoplasmic and mitochondrial mRNAs from yeast, were found to have NAD+ at their 5′ ends (Jiao et al., 2017; Walters et al., 2017). Most mRNAs with NAD+ caps exist in two distinct populations, the majority having the conventional m7G cap, but between 1-6% have an NAD+ cap (Jiao et al., 2017; Walters et al., 2017). The biological function of the NAD+ cap is still debated. In human cells, it promotes decay (which is in contrast to the stabilising function the NAD+ cap has in bacteria) and does not support translation of an exogenous mRNA. One possibility is that NAD+- capped RNAs arise through accidental use of NAD+ as the initiating nucleotide during transcription, and are then recognised as faulty and removed by the quality control pathway. However, there is some evidence against this model: 1) NAD+ caps are not equally distributed between subsets of RNA classes as would be expected from random incorporation and 2) a fraction of small nucleolar RNAs (snoRNAs) and the related small cajal body RNAs (scaRNAs) also have NAD+ caps, and the specialised synthesis of these transcripts (Dieci, Preti, & Montanini, 2009; Filipowicz & Pogacić, 2002) means that the cap cannot be added during transcriptional initiation.

Like any biological process, RNA capping is error-prone, and there will be a certain fraction of mRNAs with just a 5′-triphosphate or an unmethylated cap; these are recognised and degraded by the mRNA capping quality control pathway (Jurado, Tan, Jiao, Kiledjian, & Tong, 2014a; Zhai & Xiang, 2014).

**NUDIX HYDROLASES**

Nudix hydrolases are the principal members of the large nudix superfamily, ‘nudix’ standing for ‘nucleoside diphosphate linked to a variable moiety X’ (Bessman, Frick, & O'Handley, 1996). The superfamily contains diverse proteins, most having pyrophosphohydrolase activity or nucleotide-binding properties (McLennan, 2006; 2012; Srouji, Xu, Park, Kirsch, & Brenner, 2017). All nudix proteins possess a variant of the four-stranded beta-grasp domain architecture comprising roughly 130 amino acids (Burroughs, Balaji, Iyer, & Aravind, 2007). Within this domain, nudix hydrolases contain the conserved 23 amino acid nudix box, a loop-helix-loop structural motif essential for catalysis (GX5EX7REUXEEXGU, where U is a bulky aliphatic residue and X is any amino acid) (Bessman et al., 1996; Koonin, 1993a). This nudix motif binds one or more metal ions, which in turn help to orient the diphosphate moiety present in all nudix hydrolase substrates (Gabelli, Bianchet, Bessman, & Amzel, 2001; Koonin, 1993a; Mildvan et al., 2005). Substrates of nudix hydrolases are varied and include nucleoside triphosphates, nucleotide sugars, dinucleoside polyphosphates, dinucleotide coenzymes, non-nucleotide substrates including inositol pyrophosphates, and the 5′ ends of RNAs (McLennan, 2006; 2012; Srouji et al., 2017).

## Bacterial nudix hydrolases involved in decapping: RppH and NudC

In bacteria, two nudix hydrolases are known to generate monophosphorylated RNA 5′ ends: RppH and NudC.

**RppH** (also known in *E. coli* as NudH, YgdP and Ap5A/Ap4A hydrolase) was first described as a diadenosine polyphosphate hydrolase (Bessman et al., 2001). In the context of RNA metabolism, it hydrolyses RNAs with two or three phosphates at the 5′ end into monophosphorylated RNAs. There are two very different prototypes of RppH, *E. coli* RppH (orthologue present in numerous proteobacteria and flowering plants) and *B. subtilis* RppH (less common, present in Firmicutes and best studied in *B. subtilis*) (Foley, Hsieh, Luciano, & Belasco, 2015).

*Ec*RppH removes pyrophosphate from triphosphorylated RNAs with only little sequential dephosphorylation (Deana, Celesnik, & Belasco, 2008), whereas *Bs*RppH removes both phosphates consecutively, without the release of pyrophosphate (Richards et al., 2011). However, diphosphorylated mRNAs have recently been found to be abundant decay intermediates in *E. coli* and evidence suggests that, in addition to direct removal of pyrophosphate, *Ec*RppH will remove the -phosphate after the -phosphate has first been removed by an as yet unidentified enzyme (Luciano et al., 2017; Luciano, Vasilyev, Richards, Serganov, & Belasco, 2018). It remains unknown whether diphosphorylated RNAs also dominate in *B. subtilis*.

Both *Ec*RppH and *Bs*RppH require at least two, and preferably more, single-stranded nucleotides at the 5′ end of their substrates. *Ec*RppH tolerates wider 5′ sequence variation with only a modest preference for A over G at the first position and some preference for a purine at the second position (Foley et al., 2015), while *Bs*RppH has stricter demands, with an absolute requirement for G at the second position, preference for a purine at the third position and a slight preference for A over G at position 1 (Hsieh, Richards, Liu, & Belasco, 2013; Piton et al., 2013). These differences in substrate sequence specificity can be explained by structural differences in the catalytic centres: *Bs*RppH binds the second nucleotide in a deep cavity (Piton et al., 2013) and *Ec*RppH in a cleft (Vasilyev & Serganov, 2015). *Ec*RppH crystallizes as a monomer (Vasilyev & Serganov, 2015) and *Bs*RppH is also a monomer in solution (Piton et al., 2013). Binding of *Ec*RppH to its stimulator, the diaminopimelate epimerase DapF, causes the formation of a heterotetramer with two DapF-RppH heterodimers held together by the interaction between two DapF subunits (Gao et al., 2018; Lee, Kim, Park, Kim, & Seok, 2014).

Monophosphorylated RNAs are unstable and destined for decay. In *E. coli*, monophosphorylated RNAs become vulnerable to rapid internal cleavage by the endonuclease RNAse E (Mackie, 1998), while in *B. subtilis*, the RNA is targeted by the exoribonuclease RNAse J (Richards et al., 2011). In *E. coli*, RppH deletion stabilises a subset of mRNAs (about 400 transcripts) and reduces the amount of monophosphorylation for those RNAs tested, indicating an important role in mRNA decay (Deana et al., 2008). Viability of the cells is not affected, suggesting the presence of alternative pathways (Deana et al., 2008). The situation is similar in several other bacteria. However, some specific phenotypes are clearly connected to RppH deletion, for example a reduction in virulence of pathogenic bacteria (Bessman et al., 2001; Edelstein et al., 2005; Ismail, Hart, & McLennan, 2003). To what extent this reflects changes in RNA stability or increased levels of the alternative diadenosine polyphosphate substrates remains to be determined.

**NudC** hydrolyses the pyrophosphate bond of the NAD+ cap of bacterial RNAs to release monophosphorylated RNA with the adenosine moiety of the NAD+ cap at the 5′ end and nicotinamide mononucleotide (Cahová et al., 2015). The enzyme prefers NAD+-RNA over NAD(H) by several orders of magnitude (Höfer et al., 2016), indicating that RNA decapping may be its primary function, even thought it was originally described as an NAD(H) pyrophosphohydrolase (Frick & Bessman, 1995). NudC is a homodimer in both its free and NAD+-bound forms (PDB 2GB5) (Höfer et al., 2016; Zhang et al., 2016). The NAD+ adenine ring is stacked between the two monomers, and the catalytic pocket comprises residues within and outside the nudix boxes of both subunits (Höfer et al., 2016; Zhang et al., 2016). NudC binds non-specifically to single stranded RNA and prefers a purine at the 5′ end of its substrates (Höfer et al., 2016). The biological function of NudC remains unclear: even though the NAD+ modification stabilises RNA against decay by RppH and RNAse E *in vitro*, the increase in NAD+ modification of the NudC sRNA substrate RNAI upon NudC knockout was not translated into an increased RNA half-life *in vivo* (Cahová et al., 2015).

## Eukaryotic nudix hydrolases involved in decapping: Dcp2, Nudt16 and Nudt3

In eukaryotes, there are three nudix hydrolases known to decap mRNAs *in vivo*: Dcp2, Nudt16 and Nudt3. As there are excellent recent reviews on mRNA decapping by nudix hydrolases (Grudzien-Nogalska & Kiledjian, 2017) and on the catalytic mechanism of the prototype decapping enzyme Dcp2 from a structure-based perspective (Valkov, Jonas, & Weichenrieder, 2017), we will only summarise the topic here and refer the reader to these reviews for details.

**Dcp2** was the first eukaryotic decapping enzyme to be described, originally in yeast (Dunckley & Parker, 1999; Lykke-Andersen, 2002; van Dijk et al., 2002; Z. Wang, Jiao, Carr-Schmid, & Kiledjian, 2002), and long believed to be the only one. The core decapping enzyme consists of the regulatory Dcp1 and the catalytic Dcp2 subunit, which interact with further decapping enhancers. General decapping activators conserved from yeast to human are Pat1, Dhh1, Edc3 and the Lsm1-7 complex, while the nonsense mediated decay (NMD) proteins Upf1-3 are specific activators (Grudzien-Nogalska & Kiledjian, 2017; Parker, 2012; Valkov et al., 2017). Substrate specificity is achieved in two ways: Dcp2 can recognise the secondary structure of a stem-loop within ten bases of the 5′ cap – the ‘Dcp2 binding and decapping element’ (Y. Li, Ho, Gunderson, & Kiledjian, 2009; Y. Li, Song, & Kiledjian, 2008). Alternatively, decapping can be stimulated by a *cis*-element in the mRNA target (such as an AU-rich element in the 3′ UTR or oligouridylation at the 3′ end of a deadenylated RNA), which is recognised by a protein that then in turn recruits Dcp2/Dcp1 (Y. Li & Kiledjian, 2010).

Curiously, Dcp2 is not ubiquitously expressed in all mouse tissues and it has recently become clear that multicellular organisms have more than one nudix hydrolase involved in mRNA decapping, each acting on a rather specific subset of mRNAs (Grudzien-Nogalska & Kiledjian, 2017).

**Nudt16** was originally believed to be specific for nucleolar RNA decapping (Ghosh, Peterson, Tomasevic, & Peculis, 2004; Taylor & Peculis, 2008), but was later found to be predominantly cytoplasmic and involved in mRNA decapping (Lu et al., 2011; M.-G. Song, Li, & Kiledjian, 2010). *In vitro* experiments showed that Nudt16 cleaves methylated capped mRNAs mostly, but not exclusively, between the  and  phosphate, while mRNA with an unmethylated cap is cleaved both between the  and  and  and  phosphates (M.-G. Song, Bail, & Kiledjian, 2013). Nudt16 also has low levels of activity on isolated cap structures and short capped oligonucleotides *in vitro* (Grzela et al., 2018; M.-G. Song et al., 2013). The enzyme preferentially hydrolyses RNAs with a long half-life and there is only a little overlap between the substrates of Nudt16 and Dcp2 (M.-G. Song et al., 2010). Even the decay pathways have specificity for a certain decapping enzyme: NMD uses preferentially Dcp2 while ARE-containing RNAs are differentially targeted by either Dcp2 or Nudt16; only miRNA-mediated decay can use both Dcp2 and Nudt16 (Y. Li, Song, & Kiledjian, 2011). Like other nudix hydrolases involved in RNA decay, Nudt16 is also active towards certain simple nucleotide substrates, in this case inosine mononucleotides (Abolhassani et al., 2010; McLennan, 2012; Trésaugues et al., 2015).

In addition to Dcp2 and Nudt16, a further six of the 22 nudix domain proteins present in the human genome can decap mRNAs *in vitro*, either cleaving between the and  or between the and  phosphate or with no preference (M.-G. Song et al., 2013). Of these, only **Nudt3**, originally characterised as a diphosphoinositol pyrophosphate and diadenosine polyphosphate pyrophospohydrolase (Safrany et al., 1999) has been shown so far to also act as a decapping enzyme in cells (Grudzien-Nogalska, Jiao, Song, Hart, & Kiledjian, 2016). Nudt3 can cleave its substrate either between the  and  phosphate, or between the  and  phosphate with about equal preference (M.-G. Song et al., 2013). Just like Dcp2 and Nudt16, Nudt3 appears to decap only a small subset of mRNAs, preferentially mRNAs encoding proteins that are involved in cell motility (Grudzien-Nogalska et al., 2016). Interestingly, the *S. cerevisiae* orthologue of Nudt3, Ddp1, has robust decapping activity *in vitro* (M.-G. Song et al., 2013), suggesting that the presence of multiple decapping enzymes may not be restricted to multicellular organisms. It is possible that more eukaryotic decapping enzymes will be revealed in the future as five further nudix proteins (Nudt2, Nudt12, Nudt15, Nudt17, Nudt19) have decapping activity *in vitro* (M.-G. Song et al., 2013) while others (such as Nudt4, Nudt10 and Nudt11, which have high identity to Nudt3 and Ddp1) may have been missed in *in vitro* assays due to specific assay requirements.

## Eukaryotic viral nudix hydrolases involved in decapping

Many poxviruses encode nudix decapping enzymes (McLennan, 2007; Parrish & Moss, 2007). In addition to suppressing host mRNA translation, the vaccinia virus D9 and D10 proteins co-opt the host Xrn1 exonuclease to jointly degrade double-stranded capped viral RNAs arising from annealing of transcripts from opposite strands of the viral DNA which would otherwise activate the host antiviral innate immune response (Silverman, 2015). African Swine Fever Virus g5R protein is also involved in degradation of host mRNAs and regulation of viral transcription and was originally characterised as a diphosphoinositol pyrophosphate pyrophosphohydrolase (Cartwright et al., 2002; Parrish, Hurchalla, Liu, & Moss, 2009).

**DXO FAMILY PROTEINS**

The DXO (decapping exoribonuclease) protein group is diverse and contains enzymes with very different and often multiple activities, including pyrophosphohydrolase (PPH) activity, 5′ triphosphonucleotide hydrolase (TPH) activity, decapping activity, deNADding activity and 5′-3′ exoribonuclease activity.

Most DXO proteins are involved in the recently discovered mRNA cap quality control pathway which degrades uncapped (triphosphorylated) mRNAs or mRNAs with an unmethylated cap (Jurado, Tan, Jiao, Kiledjian, & Tong, 2014b; Zhai & Xiang, 2014). The founding member of the DXO protein family is the nuclear protein Rai1 (Rat1 interacting protein) of *Schizosaccharomyces pombe*, which has RNA 5′ pyrophosphohydrolase activity, releasing pyrophosphate (PPi) from triphosphorylated RNAs (Xiang et al., 2009). The 5′ monophosphorylated RNA product becomes sensitive to degradation by the 5′-3′ exoribonuclease Rat1, which forms a complex with Rai1 (Stevens & Poole, 1995; Xue et al., 2000). Surprisingly, Rai1 orthologues of other fungi (*Ashbya gossypii*, *Candida glabrata*, and *Scheffersomyces stipitis*) have a 5′ triphosphonucleotide hydrolase (TPH) activity instead of a pyrophosphohydrolase activity. Thus, instead of removing just the triphosphate, these enzymes hydrolyse the mRNA between the first and the second transcribed nucleotide, this way producing the 5′ monophosphorylated RNA substrate that Rat1 requires (V. Y.-F. Wang, Jiao, Kiledjian, & Tong, 2015). All fungal Rai1 orthologues tested to date also have *in vitro* ‘decapping activity’ on RNA substrates with an unmethylated cap, but, unlike nudix proteins, they remove the first transcribed nucleotide together with the cap, rather than just the cap (V. Y.-F. Wang et al., 2015). RNAs with unmethylated caps accumulate in Rai1∆ yeast cells, consistent with a function of Rai1 in mRNA cap quality control (Jiao et al., 2010). Some yeasts have another DXO family protein (Dxo1) (Chang et al., 2012), which is predominantly cytoplasmic

(Huh et al., 2003). The one Dxo1 orthologue that is biochemically characterised, *Kluyveromyces lactis* Dxo1, has no pyrophosphohydrolase activity and preferentially decaps RNA with unmethylated caps with its 5′ triphosphonucleotide hydrolase activity (Chang et al., 2012). Unlike Rai1, this enzyme also has distributive 5′-3′ exoribonuclease activity and can thus both decap and degrade RNA with a faulty cap by itself (Chang et al., 2012). Mammalian cells have a single Rai1/Dxo1 homologue, termed Dxo (formerly DOM3Z), which combines the activities of the two yeast enzymes (Jiao, Chang, Kilic, Tong, & Kiledjian, 2013) and is predominantly nuclear (Picard-Jean et al., 2018; Zheng, Chen, & Shyu, 2011). *In vitro*, Dxo has pyrophosphohydrolase activity which cleaves pyrophosphate from triphosphate 5′ ends; it can decap both RNA with methylated and unmethylated caps by cleaving after the first transcribed nucleotide and it has 5′-3′ exoribonuclease activity (Jiao et al., 2013). *In vivo*, the enzyme preferentially decaps mRNAs with unmethylated caps, because mature caps are protected by cap-binding proteins (Jiao et al., 2013). Dxo is also involved in the removal of the NAD+ cap in mammals (‘deNADding’); in contrast to the bacterial NudC enzyme, Dxo does not use its pyrophosphohydrolase activity but instead removes the entire NAD+ moiety (Jiao et al., 2017). The yeast proteins *S. pombe* Rai1 and *K. lactis* Dxo1 both have ‘deNADding’ activity *in vitro* (Jiao et al., 2017). In fact, the *in vitro* deNADding activities of DXO, Rai1 and Dxo1 are even stronger than the respective decapping acitivities, suggesting deNADding could be the dominant activity of the DXO protein family (Jiao et al., 2017). Theoretically, ‘deNADding’ by Dxo could also be part of the mRNA cap quality control pathway, assuming that the NAD+ cap is the result of accidental misincorporation of NAD+ as the initiating nucleotide, but there are some data contradicting this (Kiledjian, 2018).

**HISTIDINE TRIAD PROTEINS**

Histidine triad (HIT) proteins constitute a large ancient enzyme group of nucleotide hydrolases and transferases acting on the  phosphate of ribonucleotides that share a common nucleotide-binding fold. The enzymes have been historically divided into five branches based on their catalytic specificities: decapping scavenger protein DcpS, histidine triad nucleotide binding subclasses (Hint), fragile histidine triad (Fhit), aprataxin (Aptx) and galactose-1-P uridylyl transferase (Galt) (reviewed in (C. Brenner, 2002; Martin, St-Pierre, & Dufour, 2011). DcpS, Hint, Fhit and Aptx proteins are hydrolases and share the conserved and essential HIT motif His--His--His-- with  being hydrophobic and involved in nucleotide binding. The catalytic mechanism involves attack on an - phosphoryl group of the substrate by the central histidine of the HIT motif to form a nucleotidylated intermediate covalently attached to a histidine imidazole ring nitrogen. Displacement of the nucleotidyl group by water then results in the release of the product (Lima, Klein, & Hendrickson, 1997). The Galt branch contains transferases and phosphorylases with a slightly modified HIT motif: His--His--Gln--. This subgroup includes Ap4A phosphorylases, which produce ADP and ATP from Ap4A via a covalent adenylated intermediate that is displaced by inorganic phosphate instead of water (Guranowski, 2000). Two mammalian HIT protein family members have been shown to play a role in mRNA decapping: DcpS (Dcs1 in yeast) and Fhit (Hnt2/Aph1 in yeast). Both can act as scavenger proteins by degrading the small 5′-5′-linked dinucleotides that are remnants of mRNA decay pathways. Accumulation of these metabolites is potentially toxic as they can interact with cap binding proteins (Bail & Kiledjian, 2008).

The decapping scavenger protein **DcpS/Dcs1** (reviewed in (Milac, Bojarska, & del Nogal, 2014)) degrades the small capped RNA fragments that are remnants of the 3′-5′ degradation pathway. Consistent with this function, it co-purifies with exosomal subunits, albeit in unknown stoichiometry (Z. Wang & Kiledjian, 2001). DcpS enzymes are homodimers and each monomer consists of a C-terminal domain, which contains the catalytic HIT motif, and an N-terminal domain, which binds the second nucleotide: the active site of each monomer is thus in the groove between the N- and C-terminal domains (N. Chen, Walsh, Liu, Parker, & Song, 2005; Gu et al., 2004; Han et al., 2005; Wypijewska del Nogal et al., 2013). Unlike Dcp2, DcpS cleaves the pyrophosphate linkage of the cap proximal to the m7G, yielding m7GMP and NDP. The enzyme is specific for 5′-5′-linked dinucleotides or very small capped oligonucleotides (Hudan Liu, Rodgers, Jiao, & Kiledjian, 2002; Z. Wang & Kiledjian, 2001); long mRNAs are not targeted (L. S. Cohen et al., 2004; Hudan Liu et al., 2002; Z. Wang et al., 2002). In metazoa, the resulting product, m7GMP, is further degraded by the GMP-specific nucleotidase III into 7-methylguanosine and inorganic phosphate (Buschmann et al., 2013; Monecke, Buschmann, Neumann, Wahle, & Ficner, 2014); in yeast, m7GMP degradation yields a different product via an unidentified enzyme (Buschmann et al., 2013; van Dijk, Le Hir, & Séraphin, 2003). It has now become clear that DcpS has a second function – the degradation of m7GDP, originating either from the decapping reaction of the 5′-3′ decay pathway, for example by Dcp2, or from the scavenger activities of the Fhit/Hnt2 enzymes (discussed below) (Taverniti & Séraphin, 2015); this had been a matter of debate in the past (Hudan Liu et al., 2002; Malys & McCarthy, 2006; van Dijk et al., 2003; Wypijewska et al., 2012). However, DcpS cannot directly cleave the pyrophosphate bond of m7GDP, but requires it to be phosphorylated first to m7GTP (Taverniti & Séraphin, 2015). In yeast this is mainly, but probably not exclusively, carried out by a nucleoside diphosphate kinase; in other organisms the enzymes still need to be identified (Taverniti & Séraphin, 2015). Several other functions of DcpS have been reported: i) yeast Dcs1 is an obligate cofactor of the 5′-3′ exoribonuclease Xrn1 and facilitates 5′-3′ decay by an unknown mechanism independently of its decapping activity (Hudan Liu & Kiledjian, 2005; Sinturel, Bréchemier-Baey, Kiledjian, Condon, & Bénard, 2012); ii) the *Caenorhabditis elegans* DcpS homologue, Dcs-1 also interacts with Xrn-1 independently of decapping and degrades specific miRNAs (Bossé et al., 2013); similar observations were made for human DcpS, which requires Xrn2, the human orthologue of yeast Rat1 (Meziane et al., 2015); Iii) DcpS was also suggested to regulate general mRNA metabolism as its activity influences the amount of cap degradation intermediates, which in turn bind to and recruit cap-binding proteins (Bail & Kiledjian, 2008). DcpS may therefore impact upon all cellular events that depend on cap-binding proteins, including nuclear mRNA processing, nuclear export and translation (Bail & Kiledjian, 2008; Shin-Wu Liu et al., 2004b; V. Shen, Liu, Liu, Jiao, & Kiledjian, 2008). How DcpS activity is regulated is poorly understood; in yeast, there is a second DcpS protein, Dcs2, which has no catalytic activity and forms a heterodimer with Dcs1 thus inhibiting Dcs1 activity (Malys & McCarthy, 2006; Malys, Carroll, Miyan, Tollervey, & McCarthy, 2004). However, neither Dcs1 nor Dcs2 is essential (Hudan Liu et al., 2002).

Human **Fhit** andyeast **Hnt2/Aph1** have recently been suggested to act as cap scavengers (Taverniti & Séraphin, 2015), although the ability of Fhit to degrade cap structures *in vitro* has been known for many years (Bojarska et al., 1999), Unlike DcpS, Hnt2 cleaves the pyrophosphate bond distal to the m7G, yielding m7GDP and NMP. Fhit show no such preference. Like Hnt2, human Fhit was originally defined as a diadenosine triphosphate (Ap3A) hydrolase both *in vitro* and *in vivo* (Barnes et al., 1996; Fisher & McLennan, 2008) and as a tumour suppressor frequently deleted in cancer cells, with Ap3A binding, but not hydrolysis, required for this function (Pace et al., 1998). Tumour suppression by Fhit has been linked to a variety of functions (Waters, Saldivar, Hosseini, & Huebner, 2014). The evidence that Hnt2 contributes significantly to cap scavenging *in vivo* in the presence of Dcs1 is not strong and there are no *in vivo* data to directly support a scavenging function for Fhit, whose activity appears to be very much lower than that of DcpS (Grudzien-Nogalska & Kiledjian, 2017). Nevertheless, the accumulation of undegraded cap structures such as m7GpppG has been suggested as a possible reason for changes in the translation of cancer-associated RNAs in Fhit-negative human cells (Kiss, Baez, Huebner, Bundschuh, & Schoenberg, 2017a; Kiss et al., 2017b), although other Fhit substrates such as Ap3A, which does accumulate (Fisher & McLennan, 2008), could conceivably be responsible.

Even though today only two members of the HIT protein family have been implicated in RNA decapping, members of all HIT sub-families, with the exception of the Hint branch, can cleave dinucleoside polyphosphates, either by hydrolysis (Fhit, Aptx) or phosphorolysis (Ap4A phosphorylases of the GalT branch) (reviewed in (C. Brenner, 2002; Martin et al., 2011). HIT proteins are present in all kingdoms of life (Séraphin, 1992) and only a small fraction has been enzymatically analysed to date in only a narrow range of organisms. Even fewer HIT proteins have been functionally studied *in vivo*. Several putative HIT-like clades have only recently been added to the HIT superfamily based on structural phylogeny (Maize, Wagner, & Finzel, 2013). Therefore, the possibility that HIT family members other than DcpS and Hnt2/Fhit are involved in mRNA metabolism cannot be ruled out. There may be no further proteins with scavenger activity in yeast, as m7GpppG accumulates upon depletion of both Dcs1 and Hnt2 (Taverniti & Séraphin, 2015), but this does not exclude the existence of further scavengers in other organisms or the involvement of HIT family proteins in decapping of intact mRNAs.

**ApaH-LIKE PHOSPHATASES**

## The unusual mRNA metabolism of trypanosomes

The vast majority of work on mRNA decay and decapping has been done in opisthokonts, representing only one out of five phylogenetic supergroups that eukaryotes are divided in (Adl et al., 2012). Excavates, including the Kinetoplastida *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* are evolutionarily very distant from extensively studied systems such as yeasts and mammals (Sidebar 1) and this is reflected in several unusual features of their mRNA metabolism (Figure 3A). Kinetoplastida have a very gene-dense genome that lacks conventional promoters and, with few exceptions, introns (Clayton, 2016). mRNAs are transcribed as long, polycistronic pre-mRNAs of up to 100 protein-coding genes (Clayton, 2016). Kinetoplastid pre-mRNA processing involves co-transcriptional *trans*-splicing the capped 39-nucleotide miniexon of the spliced leader (SL) RNA to the transcript’s 5′ end. This process is coupled to polyadenylation of the upstream transcripts (Clayton, 2016) and results in the formation of conventional mature, monocistronic mRNAs with a 5′ cap and a poly(A) tail. *trans*-Splicing is analogous to *cis*-splicing, with the SL RNA replacing the U1 snRNA in the spliceosome (Preusser, Jaé, & Bindereif, 2012). The SL RNA (and thus every mature trypanosome mRNA) has a highly unusual, heavily methylated type 4 cap: the first four transcribed nucleotides (AACU) have ribose 2’-O methylations and there are additional base methylations on the first (m62A) and fourth (m3U) positions (Bangs, Crain, Hashizume, McCloskey, & Boothroyd, 1992; Perry, Watkins, & Agabian, 1987) (Figure 3B). It is still not completely understood why the mRNA caps of kinetoplastids are so heavily methylated. Individual knockouts and some double knockouts of the enzymes responsible for cap methylation are viable with some effects on translation and growth, but a triple knockout of all enzymes responsible for all ribose methylations could not be obtained

(Zamudio, Mittra, Campbell, & Sturm, 2009; Zeiner, Sturm, & Campbell, 2003). The enzymes responsible for base methylations are still unknown.

The highly unusual cap structure of trypanosomes could be the reason for the absence of a conventional decapping pathway; trypanosomes have no clear orthologues to Dcp2 or its associated proteins (Dcp1, Edc3, Pat1 and the cytoplasmic Lsm1-7 complex) (Aslett et al., 2010; Bannerman, Kramer, Dorrell, & Carrington, 2018; Qing Liu et al., 2004a; Tkacz, Cohen, Salmon-Divon, & Michaeli, 2008): the entire decapping complex is absent. Of the five predicted nudix proteins (*Tb*NUDIX1-5), one (*Tb*NUDIX1) is described as a ‘mitochondrial edited mRNA stability factor’ and of the others, only *Tb*NUDIX4 could cleave m7GpppRNA *in vitro*, with the release of m7GDP and thus had been temporary referred to as *Tb*DCP2 (Ignatochkina et al., 2015). However, *Tb*NUDIX4 has only low similarity to Dcp2 of opisthokonts; rather, it has some limited similarity to putative bacterial RppH proteins in the region of the nudix box. In fact, *Tb*NUDIX4 is highly unlikely to be the decapping enzyme for bulk trypanosomal mRNA for the following reasons: i) the *in vitro* decapping activity is poor when a mature type 4 trypanosome cap is used as a substrate (Ignatochkina et al., 2015); ii) its depletion by RNAi has no effect on growth or mRNA metabolism (Kramer, 2017), and iii) the protein was not identified as an RNA binding protein or as a protein involved in the regulation of RNAs in either of two recent global screens (Erben, Fadda, Lueong, Hoheisel, & Clayton, 2014; Lueong, Merce, Fischer, Hoheisel, & Erben, 2016). However, this does not totally exclude a role for *Tb*NUDIX4 in RNA cap quality surveillance or perhaps as a decapping enzyme for a small subgroup of RNAs, like Nudt3 and Nudt16.

This apparent lack of a decapping enzyme is particularly puzzling, as it is clear that the 5′-3′ decay pathway is the major mRNA decay pathway in trypanosomes. The exosome is localised mainly in the nucleus (Kramer, Piper, Estevez, & Carrington, 2016) and the remaining enzymes of the 5′-3′ decay pathway have been extensively studied and appear conventional: mRNA is deadenylated by the deadenylase CAF1 (Schwede et al., 2008), and 5′-3′ exoribonucleolytic decay is carried out by the cytoplasmic 5′-3′ exoribonuclease XRNA, the functional orthologue of yeast Xrn1 (C.-H. Li et al., 2006; Manful, Fadda, & Clayton, 2011). Moreover, mRNA decapping activity is definitely present in trypanosomes (Milone, Wilusz, & Bellofatto, 2002). The addition of labelled cap0 RNA to cellular extracts of the trypanosome *Leptomonas seymouri* caused the release of three products: m7GDP, m7GMP and m7GpppG, indicating the presence of decapping and/or scavenger activity (Milone et al., 2002).

**The major trypanosome decapping enzyme is an ApaH-like phosphatase**

The discovery of the trypanosomal decapping enzyme came by chance. We had purified stress granules from trypanosomes (Fritz et al., 2015) and were selectively studying proteins with potential regulatory functions. A protein annotated as a kinetoplast-specific protein phosphatase was chosen for further analysis. However, the protein had two changes in its conserved phosphoprotein phosphatase (PPP) signature motif that are characteristic of the ApaH-like phosphatases (ALPH), a subgroup of the PPP, the best studied of which is the bacterial ApaH diadenosine tetraphosphatase (Ap4A hydrolase) (Barton, Cohen, & Barford, 1994; Koonin, 1993b) (Sidebar 2). We therefore renamed the protein *Tb*ALPH1 (Kramer, 2017). The substrates of eukaryotic ALPH proteins are unknown, but the major substrate of bacterial ApaH is diadenosine tetraphosphate (Ap4A); deletion of the *ApaH* gene in *E. coli* and *Salmonella typhimurium* results in significant increases in intracellular Ap4A (Ismail et al., 2003; Lévĕque, Blanchin-Roland, Fayat, Plateau, & Blanquet, 1990). *Tb*ALPH1 has all the *in vivo* features expected of the major trypanosomal mRNA decapping enzyme (Kramer, 2017): RNAi depletion is lethal, and causes a global increase in deadenylated mRNAs that have not yet started decay. *In vitro* decapping assays revealed that recombinant *Tb*ALPH1 protein (but not a catalytically inactive mutant) can sensitise the (capped) spliced leader RNA to yeast Xrn1. However, this only works in the presence of an RNA 5′-polyphosphatase, which transforms 5′ polyphosphorylated RNA into 5′ monophosphorylated RNA, which is the only substrate Xrn1 accepts. These data indicate that *Tb*ALPH1 produces a 5′ polyphosphorylated mRNA instead of a monophosphorylated mRNA, most likely by cleaving between the  and  phosphate. This could explain the detection of m7GMP in the *in vitro* decapping assays in *Leptomonas* extracts that were originally attributed to a scavenger activity (Milone et al., 2002). Further evidence for *Tb*ALPH1 being involved in the 5′-3′ mRNA decay pathway comes from its exclusive co-localisation with the 5′-3′ exoribonuclease XRNA in a trypanosome-unique granule at the posterior pole of the cell that is devoid of other RNA binding proteins (Kramer, 2017; Kramer et al., 2008) and its identification within the trypanosome RNA-bound proteome (Lueong et al., 2016).

Many questions remain unanswered and are the subjects of ongoing research. What is the exact catalytic mechanism of the enzyme? Does it have a substrate preference for the trypanosome type 4 cap and/or the sequence of the SL RNA? Are there interacting proteins perhaps fulfilling equivalent functions to the decapping activators of Dcp2? How is the 5′ polyphosphorylated RNA further degraded: is it a direct substrate for the trypanosomal 5′-3′ exoribonuclease XRNA that is only poorly conserved, or is there another enzyme with a polyphosphatase activity involved?

**Decapping by ApaH-like phosphatases in other organisms?**

ApaH-like phosphatases are spread throughout the five eukaryotic kingdoms of life in a patchy way, with some major groups excluded, such as vertebrates, insects and land plants (Andreeva & Kutuzov, 2004; Uhrig, Labandera, & Moorhead, 2013b) (Sidebar 2). With the exception of the trypanosomal enzyme, none has been functionally analysed yet. It should be noted that the absence of the prototype decapping enzyme Dcp2 is restricted to subgroups of the Excavata. Not many Excavata genome sequences are available; however, Dcp2 appears absent from Euglenozoa (including trypanosomes, *Leishmania* and *Euglena*) and from Fornicata (including *Giardia intestinalis* and *Spironucleus salmonicida*). All Euglenozoa have an *Tb*ALPH1 homologue that is likely to compensate for the absence of Dcp2, while Fornicata lack such a homologue and must compensate for the lack of Dcp2 by some other means.

To date, there is no evidence for ApaH like phosphatases acting as mRNA decapping enzymes outside Euglenozoa. However, it is becoming more and more clear that decapping is done by a range of different enzymes rather than one single enzyme. Given that the function of all non-trypanosome ApaH like phosphatases remains entirely unknown, there is at least the potential to discover decapping activity in a subset of these enzymes, perhaps complementing DCP2 activity by for example targeting a specific subset of mRNAs or acting in a specific decay pathway.

**Figures and Tables**

**Figure 1**

mRNA decay pathways in bacteria and eukaryotes.

**Figure 2**

Summary of the decapping enzymes discussed in this work with their respective substrates and cleavage sites. Cleavage sites with no evidence for *in vivo* relevance were excluded. To the best of our knowledge, there is currently no known example of a cap3 (a cap modified on the first three transcribed nucleotides).

**Figure 3**

The special mRNA metabolism of trypanosomes

(A) Polycistronic transcription and processing by trans-splicing

(B) The trypanosome cap4

**Sidebar 1: Trypanosomatida**

Trypanosomatida are a subgroup of Kinetoplastida Excavates, defined by having only a single flagellum. All are parasitic, mostly to insects. Three species have a dixenic life cycle: *Leishmania*, *Phytomonas* and *Trypanosoma*. Best studied are the human pathogens *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania*. *T. brucei* causes African sleeping sickness and the related cattle disease N’gana, both of which have a huge impact on humans in countries of sub-Saharan Africa. The parasites are transmitted by tsetse flies to their mammalian hosts, where they live exclusively in the bloodstream; this is enabled by the parasite’s ability to escape the immune system by a stochastic change of their major cell surface protein (antigenic variation). *Trypanosoma cruzi* causes Chagas disease in humans, mainly but not exclusively in South American countries, as well as many animal diseases and is transmitted by Triatominae (kissing bug). Several different *Leishmania* species cause human Leishmaniasis (a disease of almost world-wide relevance) transmitted by the sand fly. In contrast to *T. brucei*, *T. cruzi* and *Leishmania* usually enter cells of their mammalian host, often macrophages. All diseases caused by Trypanosomatida are classified by the WHO as neglected tropical diseases, given the insufficient treatment options and the correlation with poverty. Notably, all human pathogenic trypanosomes have rather complex life cycles, involving several differentiation steps in each host, each accompanied by massive changes in gene expression. In the near absence of transcriptional regulation in these parasites, post-transcriptional mechanisms are of major importance, and these include the regulation of mRNA decay.

**Sidebar 2: Origin of ApaH-like phosphatases**

Eukaryotic protein phosphatases can be classified in several ways based on features of their structure, catalytic mechanisms and substrate specificities. One four-group classification defines phosphoprotein phosphatases (PPPs), Mg2+- or Mn2+-dependent protein phosphatases (PPM, sometimes classified as a subgroup of PPP), protein tyrosine phosphatases and aspartic acid-based phosphatases (Kerk, Templeton, & Moorhead, 2008). The PPP family contains classical protein phosphatases (for example PP1, PP2A, PP2B, PP4 and PP7), but, in addition there are also three classes with greatest similarities to bacterial PPP-like phosphatases (Andreeva & Kutuzov, 2004; Uhrig et al., 2013b; Uhrig & Moorhead, 2011). These are *Shewanella*-like phosphatases (SLP), Rhizobialis-like phosphatases (RLPH) and ApaH-like phosphatases. ApaH-like phosphatases are related to ApaH, a diadenosine tetraphosphatase, of bacteria (Andreeva & Kutuzov, 2004). An extensive phylogenetic study of ALPHs (Uhrig, Kerk, & Moorhead, 2013a) revealed that these phosphatases are spread throughout all five clades of the eukaryotes, although they are absent from several subgroups (e.g. land plants). Interestingly, two sets of bacterial sequences (inner Myxococcales and outer Myxococcales) are intermixed within the eukaryotic ALPH clade, while archaeal sequences from Halobacteriaceae are closely associated. Moreover, some eukaryotic ALPHs form a phylogenetically mixed group with bacterial ALPHs, and many eukaryotic ALPHs have predicted mitochondrial or chloroplast locations. The true origin of ALPH proteins still remains somewhat mysterious. Most interestingly, despite the ubiquitous presence of ALPH proteins, the function of this huge enzyme class remains elusive. The one exception is *T. brucei* ALPH1, the major mRNA decapping enzyme of trypanosomes.

**Conclusion**

The removal of the RNA cap is an important step in RNA decay and contributes to RNA stability and thus the regulation of gene expression: it needs to be tightly regulated. Similarly, the removal of capped metabolites of the 3′-5′ decay pathway is essential to avoid their accumulation, which is potentially toxic as they can recruit cap binding proteins. It is now clear that several different enzymes from very different enzyme classes are involved in RNA decapping. The field of RNA decapping is currently very active: many enzymes and 5′ RNA modifications have only recently been discovered, helped by the advance in sequencing techniques. Many further enzymes are known to have *in vitro* decapping activity and have the potential to decap RNA *in vivo*, perhaps only under certain conditions or in a certain type of cell. Notably, the specific functions of many decapping enzymes still remain largely elusive, as does the function of some cap variants, for example the NAD+ cap present in both bacteria and eukaryotes. Novel sequencing methods (for example (Jiao et al., 2017) or (Vvedenskaya et al., 2018)) will be very useful tools in identifying the full set of *in vivo* RNA substrates of the different enzymes, which will be an important first step towards a better understanding of enzyme functions and regulation. The finding of an ApaH-like phosphatase as the major trypanosome enzyme suggests that many further decapping enzymes may await discovery, after studying the full phylogenetic range.

Finally, there is the interesting question of substrate ambiguity among many of the enzymes discussed here, several of which are also active towards, for example, diadenosine polyphosphates, nucleotides whose functions still remain controversial (Despotović et al., 2017; Marriott et al., 2016; McLennan, 2000; 2012). Does this represent (i) just two aspects of the same function, namely the clearance of potentially toxic, structurally related metabolites; (ii) a type of ‘moonlighting’, with two independent functions residing on the same protein; (c) a potential threat to the proper control of mRNA turnover through competitive inhibition by related nucleotides, or (iv) a *bona fide* regulatory mechanism whereby changes in the levels of these alternative substrates can selectively control gene expression at the level of translation? This question is ripe for future investigation.

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