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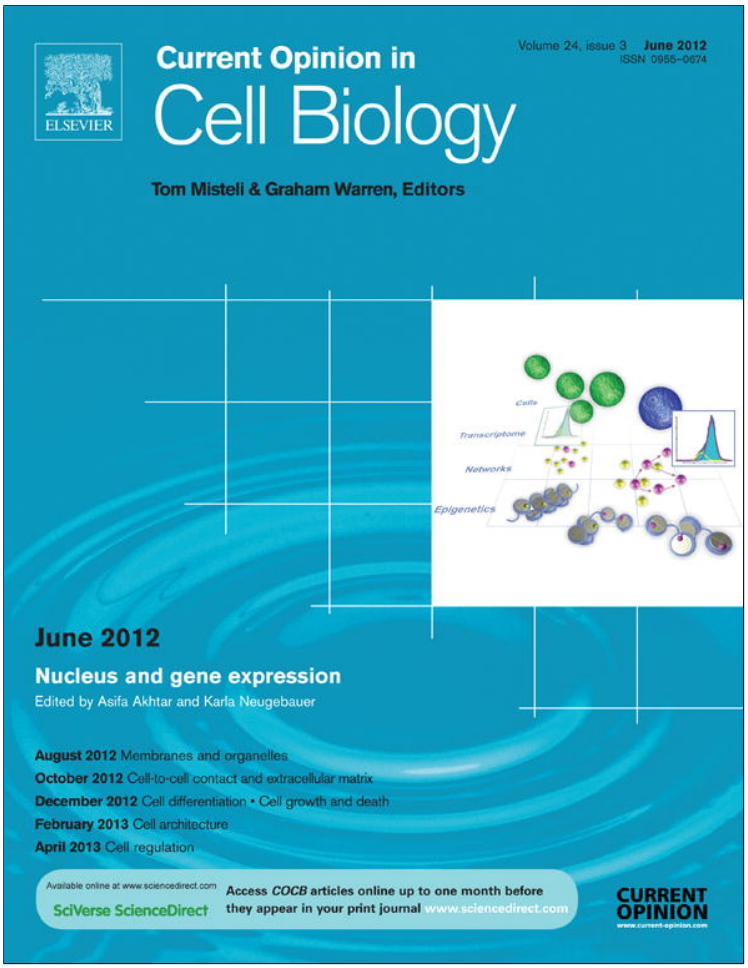

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Current Opinion in Cell Biology

Subcellular specialization of multifaceted 3′end modifying nucleotidyltransferases

Ryuji Minasaki and Christian R Eckmann

While canonical 3′end modifications of mRNAs or tRNAs are well established, recent technological advances in RNA analysis have given us a glimpse of how widespread other types of distinctive 3′end modifications appear to be. Next to alternative nuclear or cytoplasmic polyadenylation mechanisms, evidence accumulated for a variety of 3′end mono-nucleotide and oligo-nucleotide additions of primarily adenosines or uracils on a variety of RNA species. Enzymes responsible for such non-templated additions are non-canonical RNA nucleotidyltransferases, which possess surprising flexibility in RNA substrate selection and enzymatic activity. We will highlight recent findings supporting the view that RNA nucleotidyltransferase activity, RNA target selection and sub-compartimentalization are spatially, temporally and physiologically regulated by dedicated co-factors. Along with the diversification of non-coding RNA classes, the evolutionary conservation of these multifaceted RNA modifiers underscores the prevalence and importance of diverse 3′end formation mechanisms.

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Introduction

All classes of RNA molecules are subject to co-transcriptional or post-transcriptional processing steps. Importantly, many non-DNA-templated modifications occur at the 5′ends and/or 3′ends as part of stereotyped RNA biosynthesis pathways. Recently, numerous different types of regulatory 3′end additions have been identified. They greatly expand the repertoire of established modifications found on coding and non-coding RNAs, such as polyadenylation of mRNAs and CCA-addition of tRNAs. Although most novel 3′end modifications appear largely limited to the addition of adenine or uridine nucleotides, it is the number of nucleotides added that varies greatly, giving rise to mononucleotide/dinucleotide tags or oligonucleotide/polynucleotide tails. Consequently, different biological responses are elicited upon modification of specific RNA classes, affecting their functionalities, activities and half-lives. Therefore, post-transcriptional 3′end modifications represent key regulatory mechanisms that control the fate of all RNAs in the nucleus and the cytoplasm.

The majority of 3′end modifications is catalyzed by a diverse group of conserved protein subfamilies possessing nucleotidyltransferase activity [1]. Such nucleotidyltransferases (NTases) belong, together with canonical nuclear poly(A) polymerase and the CCA-adding enzyme, to the DNA polymerase β-like superfamily [2]. Each subfamily member contains a nucleotidyltransferase domain (NTD) with a highly conserved triad of aspartates that is essential for catalytic activity and a poly(A) polymerase-associated domain (PAD). However, significant divergences exist among the subfamily members in the areas outside these two domains and to a lesser degree within them, suggesting a high degree of functional specialization. The subject of this review is a distinct subclass of nucleotidyltransferases that based upon phylogeny comprises four evolutionary related subfamilies, whose members are collectively referred to as non-canonical NTases (ncNTase) (Figure 1). With the exception of a few group members that contain predicted or experimentally defined RNA-binding regions [3–7], most ncNTases lack predictable functional RNA-binding domains (summarized in Figure 1), resulting in the general assumption that associated co-factors are required for RNA-substrate specificity and the regulation of their enzymatic activity [1,8,9]. Depending on their nucleotide preference and the initially identified extent of 3′end modification, ncNTase family members were classified as a non-canonical poly(A) polymerase (PAP), poly(U) polymerase or terminal uridylyltransferase (TUTase) [8,10–12]. However, the recent discovery of novel RNA substrate classes for the very same ncNTases extends the capabilities of this multifaceted family of 3′end modifiers and strongly diversifies their biological roles. Consequently, numerous names were assigned to one gene (Figure 1).

By focusing mainly on three conserved ncNTase subfamilies, that is, the RBM21, ZCCHC and GLD-2 families, we highlight new findings that underscore their multifunctional abilities with respect to their activities in distinct subcellular compartments, choice of RNA substrates and regulatory capacity. The fourth subfamily of TRF4-like

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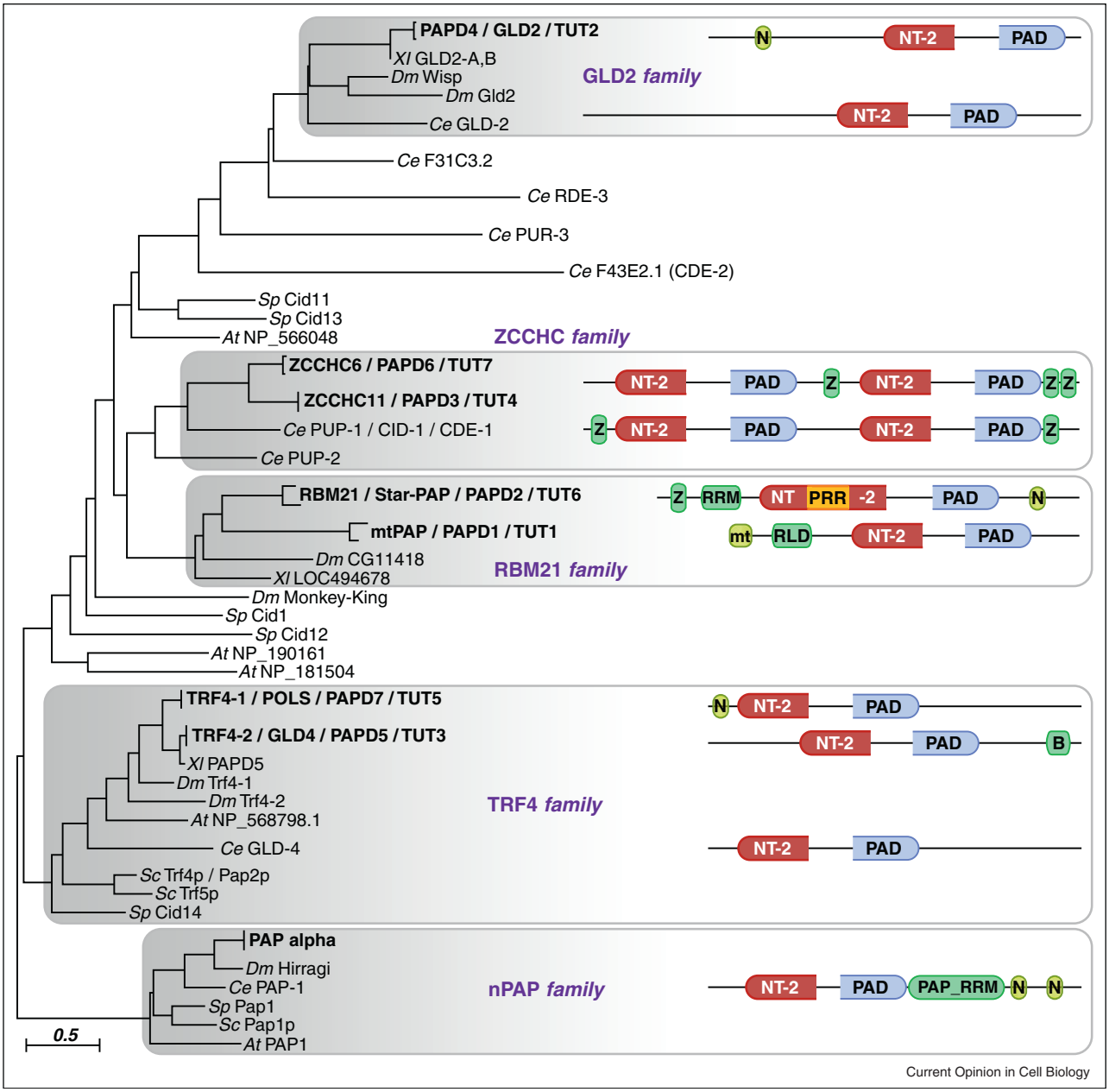
Figure 1



Non-canonical nucleotidyltransferases (ncNTases) throughout eukaryotes. Representative ncNTases from vertebrates, invertebrates, fungi and plants were chosen to generate a phylogenetic tree that is rooted with canonical nuclear poly(A) polymerases (nPAP). The evolutionary relationships are based only on an alignment of the TRF4 region (CDD34857, COG5260), which comprises the Nucleotidyltransferase_2 domain of poly(A) polymerases and terminal uridylyl transferases (NT-2, pfam01909) and the PAP/25A-associated domain (PAD, pfam03828). Five protein families are highlighted and named according to their founding members. Species-specific gene duplications indicate further subfunctionalization among orthologous proteins. For example, the RBM21 family consists of a predominantly nuclear Star-PAP and a mitochondrially localized PAP (mtPAP). Overall domain architectures are contrasted between mammalian and nematode family members: N, nuclear localization signal; Z, zinc-finger motifs; RRM, RNA recognition motif; PRR, proline-rich region; mt, mitochondrial import sequence; RLD, RRM-like domain; B, basic stretch for RNA association; PAP_RRM, an RNA-binding region specific to nPAPs with a three-dimensional fold of an RRM. For further explanations see text. Note that the relative domain positions are preserved, but their extent and protein sizes are not to scale. Homologs of human and mouse are labeled for clarity with their most commonly referred to names and lack a species designation. TUT, terminal uridyltransferase. For unnamed proteins NCBI Entrez accession numbers are given. Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*.

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enzymes is involved in nuclear RNA surveillance. Although a full display of its roles is beyond the scope of this review and the reader is referred to a more comprehensive review on RNA turnover [13], we like to highlight the emerging concept of subnuclear compartimentalization of TRF4-containing complexes [14]. As part of the nucleolar TRAMP complex, TRF4 ncNTases flag non-functional RNAs for exosome-mediated RNA turnover. In the nucleoplasm, TRF4-like ncNTases may deliver other RNAs to the nuclear exosome via the NEXT complex [15].

Regulated nuclear subspecialization of 3′end formation activities

In the nucleus, canonical poly(A) polymerase (nPAP) is chiefly responsible for 3′end formation of the majority of nascent mRNAs. Before the addition of ~250 adenosines to human mRNAs, nPAP also assists co-transcriptional RNA cleavage downstream of the hexa-nucleotide polyadenylation signal sequence (AAUAAA) to release the nascent mRNA from its site of synthesis. Cleavage site selection is determined with the help of cleavage and polyadenylation specificity factor CPSF and cleavage stimulation factor CstF, which recognize the AAUAAA signal sequence and a downstream U/GU-rich sequence, respectively, thereby flanking the site of cleavage (for a recent review see [16]). The newly made poly(A) tail enhances mRNA export, mRNA stability and the likelihood of serving as a template for protein synthesis in the cytoplasm. However, not all mRNAs require nPAP enzymatic activity for nuclear 3′end formation. A well-known alternative 3′end formation process has been described for non-polyadenylated replication-dependent histone pre-mRNAs, which depends on a shared stem-loop sequence in their 3′UTRs [17]. Recently, numerous stress-responsive genes were found to require a novel ncNTase that differs mechanistically from nPAP, termed nuclear speckle-targeted PIPKIα-regulated poly(A) polymerase (Star-PAP) [18••,19,20•].

To deal with changing physiological situations, cells use numerous signaling pathways to control global gene expression programs [21]. Changes in the 3′end formation mechanism appear to be no exception. As part of an oxidative stress signaling pathway in mammals, the nuclear phosphoinositide-stimulated ncNTase Star-PAP forms an alternative 3′end-processing complex on stress-responsive genes, including the cytoprotective enzyme, haem oxygenase-1 (HO-1) or the DNA damage-induced pro-apoptotic BCL-interacting killer protein (BIK) [18••,19,20•]. Unlike nPAP, Star-PAP has functional specificity for physiologically relevant messages to promote 3′end formation. Specific GC-rich RNA sequences upstream of the poly(A) signal of HO-1 and BIK mRNA are directly bound by the RRM and Zn-finger motifs of Star-PAP [19,20•], hinting at a rather complex binding sequence motif. A direct interaction with CPSF-160, the hexa-nucleotide recognition subunit of CPSF, may cooperatively assist target pre-mRNA identification and cleavage site selection. A minimal cleavage complex contains Star-PAP, CPSF-160 and CPSF-73, but lacks CPSF-100, the third CPSF subunit important for nPAP-mediated 3′end formation, which correlates with an observed depletion of U-rich sequences downstream of the poly(A) signal in Star-PAP mRNA targets [19,20•].

While other cleavage factors may also be important for efficient 3′end cleavage, the processivity of Star-PAP nucleotidyltransferase activity regarding poly(A) tail synthesis is greatly stimulated *in vitro* by the double phosphorylated lipid messenger phosphatidylinositol (PIP2) [18••]. In the nucleus, the second messenger PIP2 is generated by phosphatidylinositol 4-phosphate 5-kinase (PIPKIα), which functionally interacts with Star-PAP and co-localizes with it to nuclear speckles, where RNA processing and transcription factors are enriched [18••]. Further modulation of Star-PAP activity and selective target message regulation involves two redundantly acting isoforms of casein kinase I (CKI), and protein kinase C delta (PKCδ). Both CKI kinases promote the oxidative stress-induced expression of Star-PAP target messages and phosphorylate Star-PAP in a proline-rich loop region unique to its NTD, which in turn appears to regulate the polyadenylation activity of Star-PAP [22,23].

PKCδ regulation of Star-PAP activity is specific to the DNA damage-signaling pathway [20•]. Since PKCδ kinase activity is stimulated and CKI kinase activity is inhibited by PIPKIα-produced PIP2 [20•,22], the lipid-signaling molecule may therefore represent a molecular toggle for Star-PAP target messenger selection, in addition to stimulating its enzymatic activity. In summary, a Star-PAP-containing 3′end-processing complex may represent a platform where multiple signaling pathways converge to regulate the expression of distinct mRNA subsets at two interconnected processes, that is, mRNA cleavage and polyadenylation (Figure 2a, left column) [24].

Under steady-state conditions, it is assumed that nucleolar-localized Star-PAP stabilizes spliceosomal U6 snRNA [4]. U6 snRNA biogenesis is distinct from other non-coding spliceosomal RNAs and includes many modifications (for a review see [25]). Its gene-encoded four terminal uridines are post-transcriptionally maintained by non-templated re-addition [26,27]. The responsible uridyltransferase was identified, and is designated as U6 TUTase [4]. Although mammalian U6 TUTase is highly selective in its non-coding RNA substrate selection, nucleotide preference and added sequence length, it was also subsequently identified as the mRNA-specific Star-PAP [18••,26]. Obvious questions concerning the two distinctive activities of Star-PAP/U6 TUTase are how the enzyme switches between RNA substrates,

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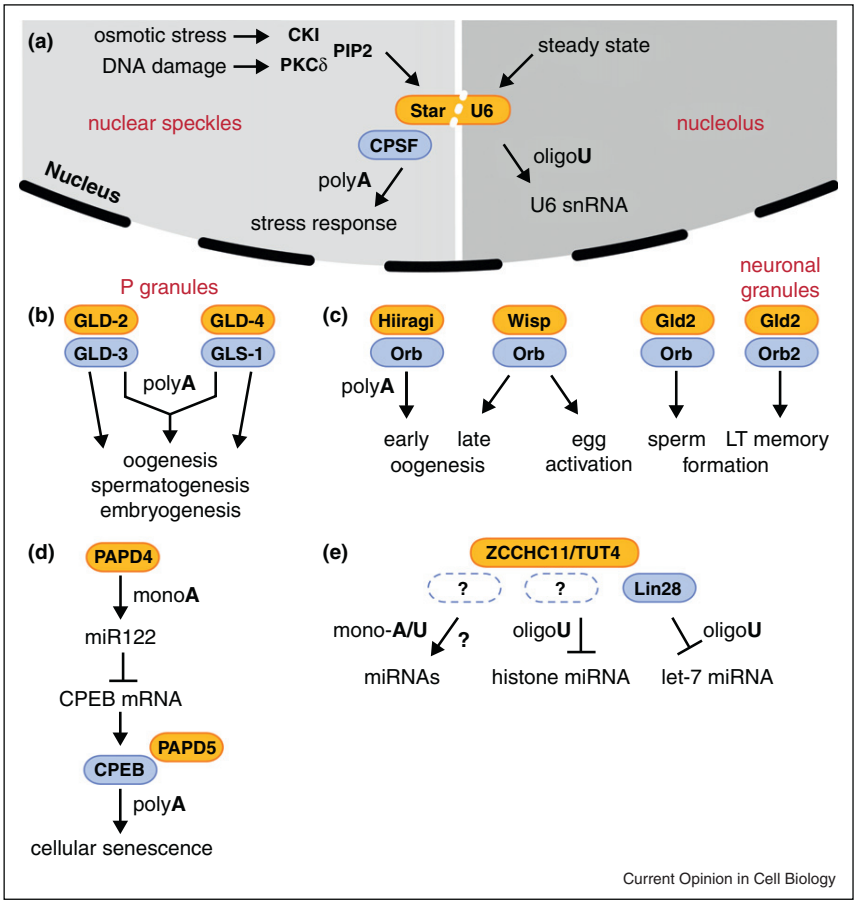
Figure 2



ncNTase-mediated RNA regulation in different subcellular regions. **(a)** Nuclear and **(b–e)** cytoplasmic activities of diverse ncNTases (in orange) are given in addition to their sites of enrichment, aside from being soluble in the nucleoplasm or cytoplasm. Dashed line indicates the nuclear envelope. Known regulators of enzymatic activity or protein co-factors (in blue) are combined with the respective ncNTase and the type of 3′end modification (nucleotide and length) carried out by the formed protein complex. Arrows mean activation/stabilization and repressive marks indicate RNA destabilization. Poly(A)-containing mRNAs are grouped into their biological function. Specific examples of different **(a)** human, **(b)** *C. elegans*, **(c)** *Drosophila*, **(d–e)** human ncNTases are in detail explained in the main text. LT, long-term.

correspondingly adjusts its nucleotide preference, and changes NTase elongation activities. The conversion between both ncNTase modes may be strictly correlated with the two distinct subnuclear localization patterns of Star-PAP/U6 TUTase, which manifest under different physiological conditions. While U6 TUTase is present throughout the nucleus with stronger accumulation in the nucleolus at steady-state conditions [4], Star-PAP accumulates in nuclear speckles upon oxidative stress [18••]. The re-localization event probably involves additional factors, which may promote the formation of distinct Star-PAP/U6 TUTase regulatory complexes. It is tempting to speculate that the small lipid-signaling molecule PIP2 may convert the enzymatic activity of U6 TUTase to a processive poly(A) Star-PAP. Therefore, under physiological circumstances, kinases and small signaling molecules appear to expand the functional capabilities of 3′end modifying enzymes to control their activities. It remains to be seen how broadly applicable this type of spatial and physiological regulation might be to other ncNTases.

Although the NTD of nuclear Star-PAP/U6 TUTase contains a proline-rich peptide insertion, its overall NTD and PAD sequence is most similar to mitochondrial mtPAP/PAPD1. Both proteins belong to the RBM21 family, which we have named after the initial founding member RBM21, which was later identified as Star-PAP/U6 TUTase (Figure 1). A recently published crystal structure of human mtPAP/PAPD1 gave first insights into structure/function information of ncNTases, which to date had exclusively relied on comparisons to nPAP. mtPAP/PAPD1 contains an amino-terminal RRM-like (RL) fold, which contributes together with the NTD

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to a dimerization of the enzyme that is essential for its function [28••]. As the RL-domain may be primarily involved in protein-protein interactions rather than in substrate binding and catalysis, it is functionally significantly different from the RRM-like fold in nPAP. It will be exiting to see how many more structural surprises are hidden in the remaining ncNTases.

Multifaceted 3′end formation via cytoplasmic ncNTases

The cytoplasmic fate of an mRNA is intimately linked with its poly(A) status; a long poly(A) tail enhances mRNA stability and translational engagement with ribosomes, whereas poly(A) tail shortening leads to translational inactivation and degradation. While miRNA-mediated mRNA regulation affects primarily RNA destabilization through enhanced deadenylation, developmentally important mRNAs are often subject to re-adenylation, improving mRNA stability and enhancing translational competency (for recent reviews see [9,29,30]). Hence, balancing the activities of deadenylases and cytoplasmic poly(A) polymerases (cytoPAPs) affects poly(A) tail-mediated mRNA fates. Dynamic poly(A) tail length control mechanisms are employed in stem cell maintenance, cellular senescence, gametogenesis, early embryogenesis, and neuronal activities, such as long-term memory formation [30–33].

Cytoplasmically localized ncNTases may act as translational activators of translationally dormant mRNAs by extending their poly(A) tail to presumably enhance the recruitment of numerous poly(A)-binding proteins. The first metazoan cytoplasmic ncNTase identified, possessing polyadenylation activity, was the *C. elegans* germline development regulator GLD-2 [34••]. In contrast to nPAP and Star-PAP, all members of the conserved GLD-2 family lack a recognizable RNA-binding domain (Figure 1). Hence, target mRNA selection appears to be provided by other RNA regulators, which either directly bind GLD-2 cytoPAP or are part of GLD-2-containing polyadenylation complexes (Figure 2b). In recent years, the list of such RNA selectors continuously increased and contains diverse members of conserved protein families, with KH, RRM and PUF RNA-binding motifs [35–37]. Importantly, RNA selectors and other GLD-2-associated co-factors stimulate GLD-2 cytoPAP enzymatic activity *in vitro* and influence GLD-2-mediated 3′end regulation *in vivo* [35–39]. However, it remains to be determined if the type of stimulation is merely enhancing its interaction with the RNA itself or directly influences its catalytic processivity, which may also depend on the type of RNA substrate.

Functional specialization is a common theme among closely related ncNTases. Although some members of RNA-binding protein families are associated with GLD-2-regulated mRNAs in disparate species, they do not appear to be part of deeply conserved GLD-2-containing polyadenylation complexes. For example, the association of the Bicaudal C-type proteins GLD-3 and Bic-C is either direct or RNA-mediated in *C. elegans* or *Drosophila*, respectively, suggesting great flexibility in assembling messenger target-specific cytoPAP complexes [35,40–43]. Nevertheless, numerous functional interactions among RNA-binding protein families with GLD-2 are preserved between frog, worm and flies [36,40••,44–46].

Temporal and spatial regulation of cytoPAPs is observed at multiple levels, including tissue-specific transcription, alternative transcript termination and post-transcriptional autoregulation. For example, sex-specific expression was described in *Drosophila* for two paralogous GLD-2-type cytoPAPs, Wisp and DmGld2 (Figure 1). While Wisp is only expressed in ovary and required for stage-specific poly(A) tail extension in the female germline and anteroposterior pattering of the early embryo, DmGLD2 is predominantly expressed in testis and required for post-meiotic spermatid development [40••,43,47]. Strikingly, aside from its general nuclear enrichment, canonical *Drosophila* nPAP/Hiiragi is also cytoplasmically localized in ovaries, consistent with its crucial role in cytoplasmic polyadenylation in developing oocytes [48]. Moreover, nPAP/Hiiragi and Wisp account for the sequential cytoplasmic mRNA polyadenylation waves that occur during oogenesis and egg activation (Figure 2c) [40••]. In frog oocytes, the two almost identical GLD-2 type ncNTases, XlGLD2-A and XlGLD2-B collectively referred to as XlGLD2, localize to both cellular compartments [46]. Although each protein is the product of a single genetic locus, XlGLD2-A is translated from two alternative mRNA transcripts that differ in 3′UTR length [49]. While a nuclear role of either XlGLD2 is unclear, XlGLD2 mediates oocyte maturation through cytoplasmic polyadenylation of meiotic mRNAs, including cyclin B1 mRNA and the longer 3′UTR-containing XlGLD2-A transcript [49,50].

Key regulators of spatial and temporal cytoPAP activities are members of the cytoplasmic polyadenylation element-binding protein (CPEB) family. The two *Drosophila* CPEBs, Orb and Orb2, are expressed in germ cells [51,52]. Notably, Orb2 is also present in the nervous system [52]. Orb partners with Wisp and nPAP/Hiiragi to guide egg formation; Orb2 is an interaction partner of DmGLD2 and instructive for neurogenesis and long-term memory formation (Figure 2c) [40••,52,53•,54•,55]. In *Xenopus* oocytes two CPEB proteins are sequentially required for egg formation, directing the activity of XlGLD2. Both CPEBs diversify the pool of GLD-2-dependent mRNA targets and incorporate developmental aspects of translational regulation as targets of cell cycle-dependent kinases [56•,57]. To control cytoplasmic polyadenylation, CPEB1 is part of a larger cytoPAP-containing complex that has striking similarity to the nuclear RNA

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3′end processing machineries, including CPSF-160 [50]. CPSF-160 may together with CPEB1 cooperatively identify messages for cytoplasmic polyadenylation, a function reminiscent with its nuclear role for nPAP or Star-PAP.

Combinatorial or sequential action of cytoPAP deployment may be a widespread phenomenon and highlights the complexity of cytoplasmic mRNA regulation. In *C. elegans*, two distinct cytoPAPs GLD-2 and GLD-4 act in parallel to promote gametogenesis [58••]. In contrast to *Drosophila* Wisp and DmGLD-2, GLD-4 is not a paralog of GLD-2; GLD-4 rather belongs to the subfamily of TRF4-type ncNTases (Figure 1). GLD-4 is co-expressed with GLD-2 in male and female germ cells. Both enzymes have at least one common mRNA target, the tumor suppressor *gld-1*, but also appear to have individual roles (Figure 2b) [58••,59,60]. Although GLD-4 NTase activity requires the co-activator GLS-1 *in vivo*, substrate selectivity and nucleotide specificity remains to be determined. Intriguingly, in addition to a diffuse cytosolic expression, members of the GLD2 family and GLD-4 cytoPAP enrich in cytoplasmic structures intimately linked with mRNA regulation, such as neuronal granules in *Drosophila* and P granules in *C. elegans* germ cells, suggesting subcellular compartimentalization and spatial regulation of cytoPAPs [34••,54•,58••,61]. In fibroblast, human GLD4/PAPD5 regulates cytoplasmic mRNA polyadenylation of the tumor suppressor p53 in a CPEB-dependent manner [62]. This action is indirectly antagonized by human GLD2/PAPD4-mediated miRNA-122 stabilization, which in turn suppresses CPEB expression (Figure 2d) [62,63•]. Thus, cytoplasmic poly(A)-tail metabolism via diverse ncNTases is a broadly observed phenomenon, affecting genes of similar function, for example, that of tumor suppressors. However, mechanistic diversification has occurred during evolution. Depending on the cellular, physiological and developmental context, the availability of co-factors appears to limit cytoPAPs target selection potential and enzymatic activities. Consistent with the view that ncNTases are intrinsically non-processive enzymes, no direct negative regulators of cytoPAPs have been reported to date.

ncNTase-mediated mononucleotide/ oligonucleotide addition affects RNA stability

miRNA biogenesis and turnover includes several post-transcriptional maturation steps (reviewed in [64]). In two successive endonucleolytic cleavages, miRNAs are first trimmed from pri-miRNA transcripts by nuclear Drosha activity and then liberated from pre-miRNA precursors by cytoplasmic Dicer activity. Recently, oligo-uridylation of pre-miRNA precursors has been correlated with enhanced miRNA turnover (for a detailed review see [65]). For let-7 pre-miRNA, the conserved cold-shock domain and Zn-finger-containing protein Lin28 stimulates ZCCHC11/TUTase4-mediated uridylation [6,7,66]. However, human let-7 miRNA regulation occurs at least at two steps. In humans, nuclear Lin28B represses pri-let-7 processing through a ZCCHC11/TUTase4-independent mechanism. On the contrary, Lin28A inhibits Dicer-mediated pre-let-7 processing by selectively stabilizing the interaction of ZCCHC11/TUTase4 with its substrate RNA, therefore, indirectly enhancing enzyme processivity (Figure 2e) [67•,68•]. Surprisingly, the very same ncNTase has been recently also proposed as an alternative to GLD4/PAPD5/TUTase3 in oligo-uridylating replication-dependent histone mRNAs (Figure 2e) [10,69]. Although ZCCHC11/TUTase4 possesses several Zn-fingers (Figure 1), it remains to be shown how it recognizes miRNAs and the special class of non-polyadenylated histone mRNAs as its substrates.

Previously, miRNA-122, which plays key roles in cholesterol and fatty acid metabolism in the mammalian liver, was found to be stabilized in mammals through GLD2/PAPD4-mediated 3′end mono-adenylation [63•]. Recent genome-wide analysis of animal miRNAs suggested a more widespread GLD2/PAPD4-mediated 3′adenylation adenylation of post-Dicer processed miRNAs [70•]. However, this work finds also that miRNA stability is not affected on a genome-wide level. Instead it is suggested that miRNA adenylation may reduce miRNA-targeting effectiveness, possibly through interfering with the formation of functional RNA-induced silencing complexes [70•]. On a global scale, many mammalian, fly and nematode microRNA variants carry non-templated 3′ mononucleotides, added by members of all four ncNTase subfamilies [71•]. While GLD2/PAPD4 and GLD-4/PAPD5 activities account for most mono-adenylations, Star-PAP and U6-TUTase mediate some mono-A/-U additions, consistent with its known role as a dual nucleotide ncNTase. While only certain miRNA species have a high frequency of 3′end addition, the extent of mono-adenylation and mono-uridylation differs among species, between tissues, disease state and developmental stage, indicating that 3′end additions is a physiologically regulated process [71•]. Whether all documented 3′end modifications of miRNAs are functionally relevant remains to be shown.

Conclusions

In parallel to the recent explosion of non-coding RNA classes, distinct 3′end modifications affecting the fate of RNAs have been reported and assigned to a multifaceted group of conserved non-canonical nucleotidyltransferases. Moreover, novel polyadenylases, different from canonical nPAP, are described to control nuclear and cytoplasmic 3′end formation of functionally related mRNAs. Although most ncNTase subfamily members are phylogenetically conserved across diverse species, their functional conservation is limited owing to species-specific diversification, such as the acquisition of novel RNA-regulatory potential, subcellular localization, or gene duplications. A surprising ability of ncNTases is that some enzymes appear capable of performing different 3′end modification reactions,

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matching nucleotide selectivity with RNA substrate specificity such as Star-PAP/U6 TUTase. As non-processive enzymes with limited RNA affinity, ncNTases require cofactors for RNA substrate specificity and/or enzymatic stimulation. Cellular signaling pathways and functional sub-compartimentalization emerge as common regulatory mechanisms directing their activities. Important tasks in the future are to decipher the biochemical and structural details of ncNTase complexes along with their cell biological regulation. Such future studies will pave the way to better understand the functional versatility of 3′end formation mechanisms, its impact on the complex life of RNA metabolism *per se*, and its role in modulating global gene expression programs at the cellular and organismal level.

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