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Critical Reviews and Perspectives

β-CASP proteins removing RNA polymerase from DNA: when a torpedo is needed to shoot a sitting duck

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ABSTRACT

During the first step of gene expression, RNA polymerase (RNAP) engages DNA to transcribe RNA, forming highly stable complexes. These complexes need to be dissociated at the end of transcription units or when RNAP stalls during elongation and becomes an obstacle ('sitting duck') to further transcription or replication. In this review, we first outline the mechanisms involved in these processes. Then, we explore in detail the torpedo mechanism whereby a 5′–3′ RNA exonuclease (torpedo) latches itself onto the 5′ end of RNA protruding from RNAP, degrades it and upon contact with RNAP, induces dissociation of the complex. This mechanism, originally described in Eukaryotes and executed by Xrn-type 5′–3′ exonucleases, was recently found in Bacteria and Archaea, mediated by β-CASP family exonucleases. We discuss the mechanistic aspects of this process across the three kingdoms of life and conclude that 5′–3′ exoribonucleases (β-CASP and Xrn families) involved in the ancient torpedo mechanism have emerged at least twice during evolution.

INTRODUCTION

Transcription is a process during which RNA polymerase (RNAP) uses DNA as a template to synthesize RNA. Transcription can be divided into initiation, elongation, and termination. During initiation RNAP recognizes promoter DNA, forms the transcription bubble, and commences RNA synthesis from nucleoside triphosphates (NTPs; (1)). During elongation, RNAP can stop due to regulatory processes or obstacles on/in DNA. This stalled RNAP is prone to backtracking, which positions the 3′ end outside of the active site (AS) of RNAP, pushing it into the secondary channel through which NTPs normally access the AS. In this stable but inactive complex the 3′ end of RNA is uncoupled from the DNA template strand. In all kingdoms of life, stalled and backtracked RNAPs can have deleterious consequences (2), hindering further transcription and translation of the same gene or adjacent genes (3), clashing with replication (4–6) or recycling of RNAP (7). Stalled and backtracked ECs can be reactivated or dismantled. However, this is not a trivial undertaking due to the high stability of the elongation complex (EC; RNAP–DNA–nascent RNA), which can resist salt up to 1 M NaCl or elevated temperature up to 65°C *in vitro* (8,9). Finally, transcription terminates in a defined manner at the ends of genes or operons, although in eukaryotes (RNA polymerase II [Pol II]) this process occurs within a relatively broad window and not in a defined place.

This review discusses recent advances in our understanding of processes that release RNAP from nucleic acids. Figure 1 provides an introductory overview of the bacterial proteins involved, and their categorization into classes according to how they act on RNAP. We start by briefly describing how ECs become stalled/backtracked, which mechanisms can reactivate/disassemble them, and how RNAP is released from DNA at the end of transcription units.

The main focus of this review is then on the torpedo mechanism that can dissociate terminating or stalled RNAPs. This mechanism is executed by 5′–3′ exoribonucleases that can attach onto the 5′end of RNA protruding from RNAP, degrade this RNA towards RNAP, and, upon contact, induce its dissociation (torpedo it) from nucleic acids. A special focus is on the involvement of the β-CASP family of metalloenzymes in this process. Other enzymes potentially functioning as torpedoes are also discussed,

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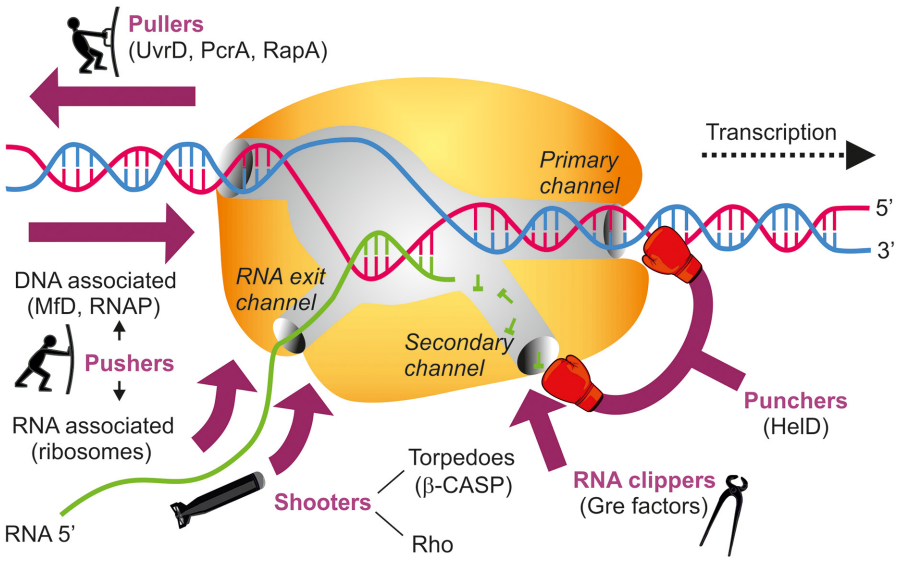

Figure 1. Overview of protein factors acting on stalled /terminating RNAPs in bacteria. The factors are divided into six groups according to their mechanistic modes of action they apply to RNAP (Pullers, DNA associated Pushers, RNA associated Pushers, Shooters, RNA clippers, Punchers). RNAP, orange; DNA, red (template) and blue (nontemplate) strands; RNA, green strand. See the main text for details.

and torpedoes are compared to other modes of RNAP release.

While the primary focus of this review is on Bacteria, comparisons between Bacteria, Eukaryotes and Archaea are made, revealing both homologous processes and processes that emerged by convergent evolution.

Formation of stalled and backtracked ECs

During transcription RNAP pauses every 100–200 bp due to sequence elements or obstacles, and these paused RNAPs may become stalled and even backtracked (10,11). In the stalled RNAPs, the enzyme displays an altered conformation whereby the 3′ end of nascent RNA strand is in the AS, but DNA is immobilized and cannot move into the reading site, stopping RNAP (12). Backtracked ECs can arise from these paused complexes throughout the whole transcribed region. Promoter proximal backtracking can be due to contacts between RNAP and transcription factors or promoter DNA (13). During elongation, backtracking can be DNA sequence-dependent (14) or induced by various roadblocks, such as DNA lesions (15,16), nucleoid-associated proteins in Bacteria or nucleosomes in Eukaryotes, as well as by a number of other DNA binding proteins (17,18). Backtracking can occur also at intrinsic terminators that consist of inverted repeats followed by a stretch of Ts. The 3′ proximal portion of the T-stretch induces RNAP pausing and even backtracking. This is important for folding of the termination hairpin and the efficiency of termination (for review see (19)).

Reactivation of backtracked ECs

Depending on the extent of backtracking, the backtracked elongation complexes can be reactivated by 1D diffusion of RNAP (20–22) or cleavage of backtracked RNA by protein factors. In Eukaryotes and Archaea, the process is mediated by TFIIS/TFS, which induces hydrolytic activity of Pol II (23–25), or by A12.2, a subunit of Pol I, which induces its hydrolytic activity (26,27). In Bacteria, the weak intrinsic RNA hydrolytic activity of RNAP can be augmented by elongation factors GreA(B) (28,29). The Gre factor-induced hydrolytic activity of RNAP removes (clips) the extruded 3′ portion of the transcript to generate a new RNA 3′ end in the AS, thereby reactivating the EC (19,30). A similar hydrolysis mechanism of 3′ ends of RNA is a built-in feature of cyanobacterial RNAPs as Cyanobacteria lack Gre factors (31). Transcription of backtracked ECs can also be reactivated by the Mfd factor (see further) or active pushing of translating ribosomes (32). The level of stalling/backtracking also depends on the number of RNAP molecules transcribing the gene. The trailing RNAP can push forward the stalled/backtracked leading RNAP, pushing it through the roadblock. Hence, the more heavily transcribed genes are less prone to contain stalled or backtracked ECs (33–35).

Non-torpedo protein factors inducing the release of ECs from DNA

When reactivation is not possible or desirable, then dissociation of stalled/backtracked ECs is mediated by various bacterial factors such as Mfd (36–38), Rep, UvrD (39), HelD (40) and RapA (41).

Transcription–repair coupling factor Mfd is an ATPase motor translocating along the double-stranded DNA template. When Mfd encounters backtracked RNAP, it pushes it forward, aligning the 3′ end of RNA in the active site and restarting transcription. If RNAP is stopped because of a roadblock, it pulls the DNA away from RNAP, causing the template and nontemplate strands of the transcription bubble to reanneal. Consequently, the RNA is released, and the complex is disassembled (38,42–44). A similar mode of

action was proposed for the archaeal transcription factor Eta (45).

In *Escherichia coli*, two replication fork accessory helicases, Rep and UvrD, facilitate efficient replication of double-stranded DNA and affect RNAP dissociation from DNA. Their homolog in *Bacillus subtilis*, PcrA was proposed to be also involved in suppression of R-loops, three-stranded nucleic acid structures where nascent RNA invades the DNA duplex with potentially inhibitory effects on transcription (46). The exact molecular basis of the action of these proteins is not known (39). However, *E. coli* UvrD and *B. subtilis* PcrA were previously shown to directly bind to RNAP and pull it back (i.e. induce backtracking). Such activity exposes DNA lesions, thereby allowing access to nucleotide excision repair enzymes (47,48).

Another *B. subtilis* putative helicase from the same family is HelD, an ATPase and GTPase which associates with RNAP, penetrating (punching into) both primary and secondary channels, clearing RNAP free of nucleic acids, and thereby stimulating RNAP recycling (40,49–51). Moreover, HelD from Streptomyces (termed HelR) was recently shown to induce dissociation of bound rifampicin, increasing the bacterium's resistance to this RNAP binding antibiotic (preprint by Surette *et al.*, doi: 10.1101/2021.05.10.443488). At least two classes of HelD proteins exist (one in Firmicutes and the other in Actinobacteria) that display different topology and possibly differ in the molecular details of the action. Interestingly, despite confirmed ATPase and GTPase activities, it is still unclear where the NTP hydrolysis by HelD is required in the transcriptional cycle.

A functional but not structural homolog of the above-discussed helicases is RapA, the RNAP binding partner identified originally in *E. coli* (41,52). Like other members of the SWI2/SNF2 protein family, RapA is an ATPase. Similar to HelD, it enhances RNAP recycling, and it was also suggested to induce backward translocation of RNAP (53). Whether RapA directly causes EC disassembly is not currently clear.

A number of conditions leading to persistent transcriptional stalling of eukaryotic Pol II molecules, that cannot be salvaged trigger the 'last resort' pathway: ubiquitylation and subsequent degradation of Pol II and the nascent transcript (54,55). Importantly, ubiquitylation of elongating Pol II in response to its stalling at DNA lesions (56–59) triggers also the transcription-coupled nucleotide excision repair (60). Ubiquitylation is also used when Pol II is stalled by a roadblock protein, such as Reb1 in yeast. This promotes Pol II termination to control pervasive transcription and prevents transcription through gene regulatory regions. The ubiquitylated Pol II is then degraded by the proteasome, ultimately terminating transcription (61,62).

Models of non-torpedo mediated transcription termination

Transcription is terminated at the 3′ end of transcription units. In Bacteria, this is mediated by two major pathways: (i) intrinsic termination (GC-rich RNA hairpin followed by a 7–9-nt U-rich tract) or (ii) Rho-dependent termination. The exact mechanisms of releasing RNAP from EC are still a subject of scientific discussions, even for well-studied intrinsic terminators (37,63).

Three different models describe putative movements of nucleic acids or proteins during the process of EC disassembly by intrinsic termination: (a) the hypertranslocation/forward translocation model where RNAP is moved forward, driven by the formation of the termination hairpin, and without further RNA synthesis; (b) the hybrid shearing model (also called slippage model) where the transcript is pulled out of the complex, and this is induced by hairpin formation or external protein action and (c) the allosteric model, where the terminator hairpin causes conformational changes in RNAP that result in melting of the RNA:DNA hybrid without translocation. Depending on the terminator, combinations of (a) and (b) appear to play roles in releasing RNAP from nucleic acids (37).

The Rho-dependent pathway requires an additional protein, Rho, terminating ∼50% of genes in *E. coli* (64,65). Rho is an ATP-dependent RNA–DNA helicase that forms hexamers. Traditionally it is supposed to bind to the 70–80 nt long, C-rich and G-poor Rho utilization site (rut) on the nascent RNA, and translocate along the RNA to catch up with RNAP. Alternatively, recent studies show that it can chronically associate with RNAP (66) or form pre-termination complexes with RNAP before interacting with the nascent RNA (67). Whether bound to RNAP or not, Rho requires RNAP pausing for efficient transcription termination (66–69). Recent cryoEM studies revealed details of Rho-dependent termination where Rho, together with transcription factors NusA and NusG, induces allosteric changes in RNAP (67,70). These changes lead to a partial opening of the β′ clamp domain, resulting in dislodging of RNA from the active site and its unwinding from DNA (70), effectively shooting down the transcription process. Rho also affects antisense-RNA production (68,71) and prevents R-loop formation (66,72).

In Eukaryotes, termination of RNA polymerase III (Pol III) is reminiscent of a combination of the bacterial intrinsic and Rho-dependent termination pathways as it involves RNA secondary structures of the nascent transcript and a Rho functional homolog, Sen1. Pol III recognizes poly-T termination signal, which is not causing termination in itself but causes catalytic inactivation and backtracking of Pol III, thus committing the enzyme to termination and transporting it to the nearest RNA secondary structure, which facilitates release (73,74). Additionally, the Sen1 helicase is essential for this process *in vivo* (75). Similarly to bacterial intrinsic terminators and Pol III termination mechanism, it was proposed that frequent Pol II stalling and backtracking within T- or AT-rich non-coding regions increases the chance of termination, suggesting a general propensity of RNA polymerases to terminate at such sequences (preprint by Vlaming *et al.*, doi: 10.1101/2021.06.01.446655) (76).

Torpedo mediated transcription termination of RNA polymerase II

The first described and most extensively studied type of the molecular torpedo acts at the ends of Pol II transcription units in Eukaryotes (77–79). Pol II transcription termination is complex. The main pathway conserved from yeast to metazoa targets poly(A) dependent transcription (Figure 2A). It requires cleavage of the nascent RNA at the poly(A)

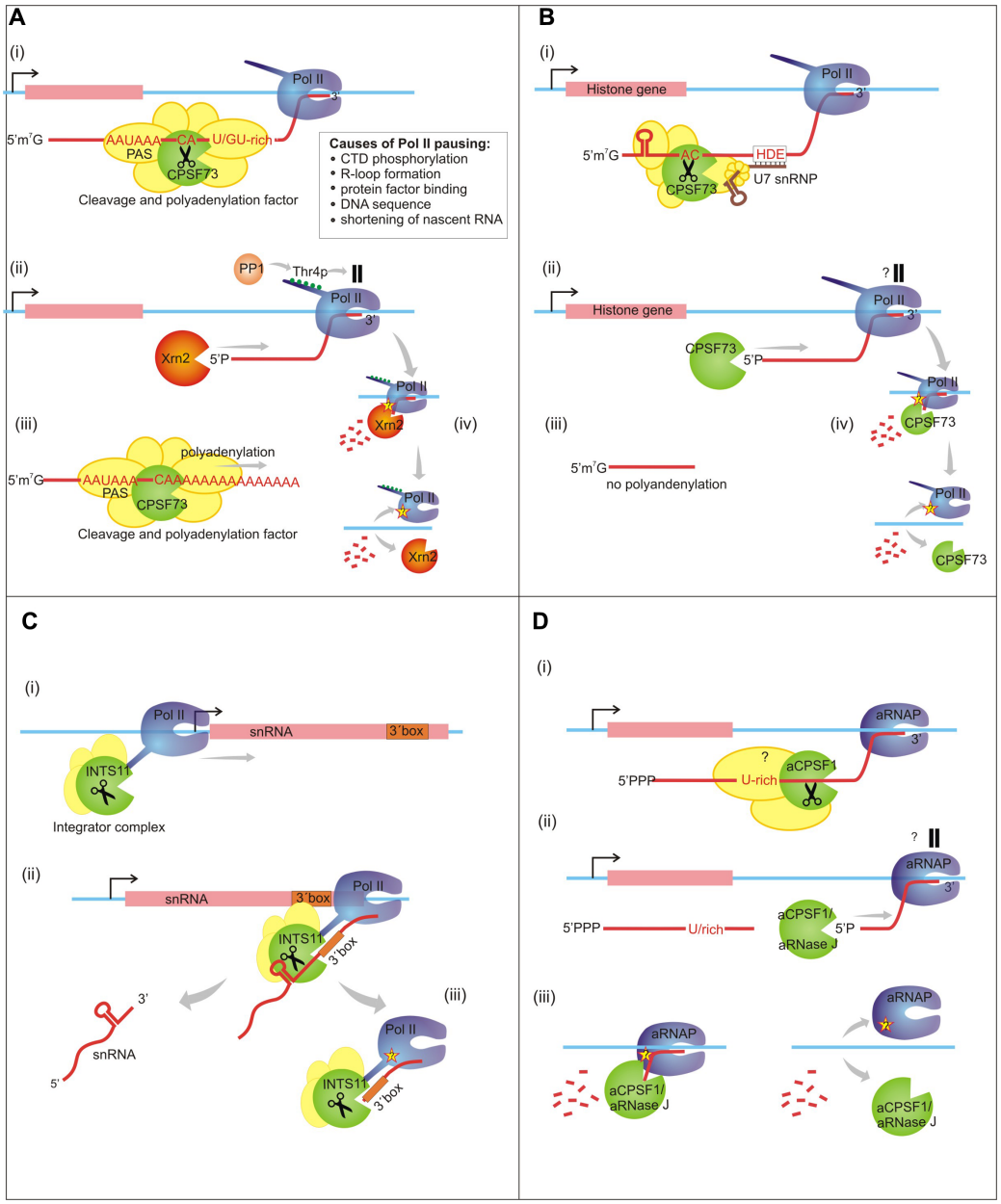

Figure 2. Mechanisms of termination by torpedo. Symbols as follows: DNA (blue), RNA (red), transcription unit (pink rectangular), promoter (black arrow), endonucleolytic cleavage (scissors), RNAP pausing (**II**), unknown mechanisms (?), proposed allosteric changes in RNAP (yellow asterisk). (**A**) Eukaryotic torpedo termination of polyadenylated transcripts. (i) Polyadenylation signal (PAS) is recognized in nascent RNA during transcription termination by the cleavage and polyadenylation factor (in yellow) and the RNA is endonucleolytically cleaved by CPSF73 in a sequence-dependent manner. (ii) Cleavage and polyadenylation complex is tethered to the upstream product of the cleavage (mRNA of the transcribed gene) and is subsequently needed for its polyadenylation (iii). Dephosphorylation of Thr4 of the C-terminal domain of Pol II and elongation factor SPT5 (not shown) by PP1 induces pausing. (iv) Torpedo 5′–3′ exonuclease (Xrn2 in human; Rat1 in yeast) is recruited to the monophoshorylated 5′ end of the downstream cleavage product, degrades RNA until it reaches RNAP and then releases RNAP from DNA. The mechanisms involved in pausing or slowing down RNAP to enable the torpedo nuclease to reach it are listed in the inset. (**B**) Eukaryotic torpedo termination of histone genes. (i) Histone cleavage complex (HCC; in yellow), including CPSF73, is recruited to the 3′ end of histone genes by pairing of U7 snRNA with histone downstream element (HDE) of the nascent RNA triggering its endonucleolytic cleavage (164,165). (ii) As histone mRNA is not polyadenylated (iii), CPSF73 can degrade the downstream cleavage product by its inherent 5′–3′ exonucleolytic activity. The exact mechanism(s) of Pol II pausing and dissociation from DNA are not known. (**C**) Termination of small nuclear RNAs (snRNAs) by the Integrator complex. (i) The Integrator complex is recruited to the Pol II CTD during transcription initiation. (ii) The Integrator complex travels with the polymerase up to the 3′ box (3′ stem-loop and a 13–16 nucleotide element located several nucleotides downstream of the mature 3′ end). Integrator interacts with the RNA stem-loop of the 3′ box and triggers endonucleolytic processing of the nascent RNA by INTS11. (iii) Termination occurs after the release of the Integrator complex and the cleaved snRNA (86). (**D**) Archaeal torpedo termination. (i) Uncoupling of archaeal translation from transcription disrupted by translation termination enables aCPSF1 (FttA) to perform endonucleolytic cleavage of the exposed nascent RNA downstream of the U-rich sequence at CA or CC dinucleotide sequences (94,95). (ii) The resulting monophosphorylated nascent RNA is exonucleolytically degraded by a member of β-CASP family (aCPSF1/aCPSF2/aRNase J). (iii) The cleavage and subsequent contact with aRNAP mediate transcription termination and release of aRNAP from DNA. The underlying mechanistic details are not known (unknown factors needed for cleavage site recognition are depicted in yellow).

signal (PAS), and an extensive array of factors is required for the termination process to be completed. Two key factors in mammals are CPSF73 and Xrn2. CPSF73 (cleavage and polyadenylation specificity factor, member of the β-CASP family of metallo-nucleases) is the endo- (as well as 5′–3′ exo-) nuclease that cleaves at PAS. Pol II, however, does not terminate at this point, continuing to transcribe downstream RNA, and needs to be stopped. This is mediated by Xrn2 (Rat1 in yeast), a 5′–3′ exonuclease that attacks the monophosporylated 5′ end, degrades the downstream RNA, and upon colliding with Pol II causes transcription to terminate by the torpedo effect (for mechanistic aspects see the section 'Mechanisms' below).

Recent studies indicate that CPSF73 is required not only for PAS cleavage but also together with protein phosphatase 1 (PP1) for slowing down Pol II, thereby allowing Xrn2 to catch up with the enzyme and terminate transcription (80,81). This combination of slowing down RNAP and its subsequent dissociation from DNA is currently termed as the unified allosteric/torpedo mechanism (81,82). In budding yeast, an alternative pathway that does not require cleavage-polyadenylation of mRNA depends on the Nrd1–Nab3–Sen1 complex (83).

Termination of histone mRNAs and small nuclear RNAs (snRNAs)

Whilst CPSF73 acts during termination of polyadenylated transcripts only endonucleolytically, it was proposed to use both its endo and exo activities during termination of histone mRNAs, the only eukaryotic mRNAs lacking a polyA tail (Figure 2B). Processing of histone pre-mRNA requires a single 3′ endonucleolytic cleavage by CPSF73 guided by the U7 snRNP that binds downstream of the cleavage site. Following the cleavage, the downstream cleavage product (DCP) is rapidly degraded by the 5′–3′ exonuclease activity of CPSF73 and is functionally linked to the release of Pol II from histone genes (84,85). Analogously, the processing of snRNAs utilizes the Integrator complex subunit 11 (IntS11; member of the β-CASP family) for the endonucleolytic cleavage of the nascent transcript (Figure 2C) (86) and the degradation of DCP was demonstrated to be independent of, or only modestly affected by Xrn2 (76,87). The exonucleolytic activity of CPSF73/IntS11 was previously proposed as one of the possible ways to terminate Pol II (84,85,88–90). Moreover, IntS11 is involved in promoter-proximal premature termination of hundreds of protein-coding genes, releasing paused/stalled RNAPs (90). The mechanism of Pol II termination involved in this process was not studied, but prior studies suggested roles for Integrator in termination (91,92). Hence, histone mRNA and snRNA termination may involve the torpedo mechanism by β-CASP proteins but direct termination of Pol II by these enzymes remains to be demonstrated.

The torpedo-like mechanism in transcription termination in Archaea

Archaea are known to combine bacterial and eukaryotic types of intrinsic termination, depending on short U-rich sequences but not requiring upstream secondary structures

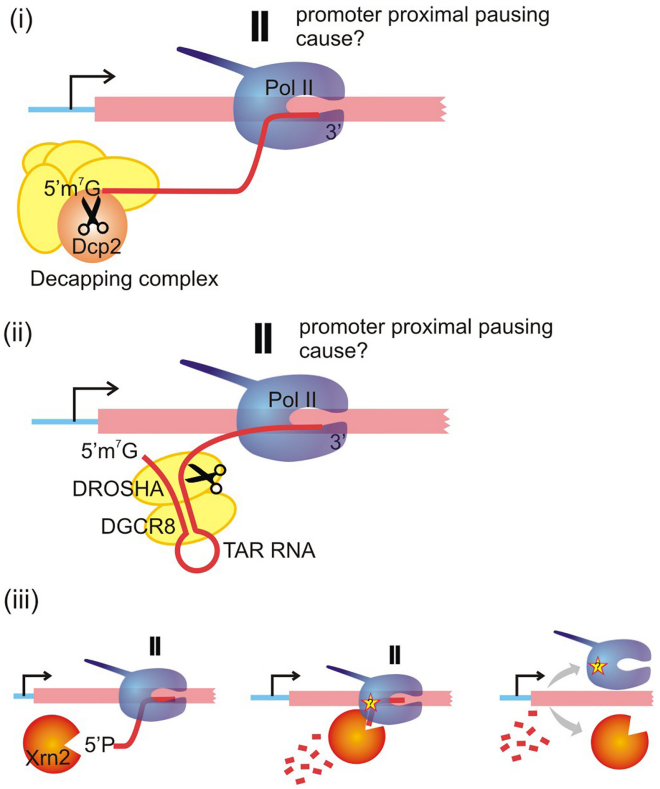

Figure 3. Two pathways of eukaryotic premature Torpedo termination: (i) 'decapping model' is initiated by removal of the 5′ cap structure of nascent transcripts by decapping factors (in yellow), Dcp1a and Dcp2 producing monophosphorylated RNA (97), (ii) 'Microprocessor model' of premature termination of HIV-1 transcription is initiated by an internal cleavage mediated by the nuclear endoribonuclease complex, Microprocessor, consisting of RNase III, Drosha, and RNA binding subunit, DGCR8 (96). (iii) Xrn2 is recruited to the 5′ monophosphate resulting from both models and exonucleolytically degrades the RNA up to RNAP, directly or indirectly causing its termination and release from DNA. RNA/DNA helicase SETX is involved in Pol II release in the 'decapping model'.

(93). Recently, a torpedo-like mechanism of transcription termination was reported in Archaea, mediated by the endonucleolytic activity of FttA/aCPSF1 followed by the 5′–3′ exonuclease activity of aCPSF1 (94) or possibly other β-CASP proteins aCPSF2/aRNase J1 (95) (Figure 2D).

Removal of stalled ECs by torpedo

Premature transcription termination by torpedo (Figure 3) was discovered when genome-wide studies had shown that Pol II pausing/accumulation near the promoters of many genes is a widespread, rate-limiting step in early elongation (96,97). Investigation of this phenomenon then revealed that these paused Pol II ECs are removed by the torpedo enzyme Xrn2 with the assistance of other termination factors such as TTF2 (shares homology with bacterial RapA) and SETX (96,97). *In vitro*, TTF2 induces both Pol I and Pol II termination (98,99). Moreover, the torpedo termination of Pol II by Xrn2 was recently utilized in experimental and therapeutical applications (100,101). The termination was triggered by antisense oligo-mediated cleavage of the targeted transcript by RNAse H. This nascent transcript

cleavage then induced premature transcription termination downstream of the cleavage site.

A process highly reminiscent of eukaryotic premature torpedo termination was recently identified in Bacteria (35) (Figure 4). Deletion of the *rnjA* gene, encoding *B. subtilis* RNase J1 (a member of the β-CASP protein family), decreased the levels of hundreds of transcripts, suggesting a positive role of RNase J1 in the expression of respective genes. At the same time, the occupancy of DNA by RNAPs on these genes increased, implying stalled, inactive ECs. Subsequent *in vitro* experiments then revealed the ability of RNase J1 to dissociate stalled ECs. This is similar to another eukaryotic 5′–3′ exonuclease, Xrn1. Yeast Xrn1 is present both in the cytoplasm and the nucleus, where it can bind directly to chromatin and stimulate transcription of most genes by an unknown mechanism (102). Deletion of Xrn1 decreased the levels of some mRNAs while increasing DNA occupancy by RNAP on these genes. Hence, it is tempting to speculate that the positive effect of Xrn1 on transcription might be, in part, through its torpedo effect on prematurely terminated/stalled ECs. We note, however, that Xrn1 involvement in transcription termination by Pol II at the 3′ ends of genes has not been observed (103).

Entry site for torpedo 5′–3′ exonucleases

For the 5′–3′ exonuclease activity to occur, the 5′ end must be devoid of protective structures. In Eukaryotes, CPSF73 forms the entry site at PAS (Figure 2A); in the case of premature termination, the 5′ m^7G cap is removed by decapping (decapping model) or as a consequence of endonucleolytic cleavage (microprocessor model) (96,97) (Figure 3). Consistently, decapping enzymes (Dcp1a and Dcp2) or respective endonucleolytic enzymes (Drosha and DGCR8) binding to the stem-loop structure in nascent RNA were reported to colocalize with Xrn2 in the promoter-proximal region (96,97).

In Archaea and Bacteria, transcripts do not contain the canonical m^7G cap but their 5′ ends are mostly triphosphorylated as the inherent result of RNAP initiating with nucleoside triphosphates (NTPs) (1). The triphosphate was previously also referred to as the prokaryotic cap as it increases the biological stability of RNA (104,105). It can be removed by RNA pyrophosphohydrolase (RppH in *B. subtilis*), which converts RNA 5′ triphosphates to 5′ monophosphates (106). This pathway is analogous to the mechanism of degradation of mRNA in Eukaryotes in which 3′-deadenylated transcripts are decapped by decapping enzymes and the monophosphorylated RNA is degraded by the 5′ exoribonuclease Xrn1 (107).

Moreover, noncanonical cap structures (NCIN caps) at the 5′ end of both prokaryotic and eukaryotic RNAs were identified. These caps arise from the incorporation of cofactors such as oxidized or reduced forms of nicotinamide adenine dinucleotide (NAD+/NADH), flavin adenine dinucleotide (FAD), uridine diphosphate glucose (UDPGlc), dephospoCoA, uridine diphosphate N-acetylglucosamine (UDPGlcNAc), dinucleoside polyphosphates (108–111). We note that this list is likely far from complete. These cap structures may form obstacles for the torpedo RNase effect in Bacteria as well as in Eukaryotes, should the degradation start from the natural 5′ end of the RNA. These caps can be removed by a large array of NUDIX enzymes that form 5′ ends (112,113) that are amenable to exonucleolytic cleavage. Bacterial RNase J1 and eukaryotic 5′–3′ exonucleases (Xrn proteins) efficiently process RNAs with 5′ monophosphates; RNAse J1 can process also 5′ hydroxyls (114). Despite the initial belief that the active sites of both protein families were incompatible with larger groups such as triphosphates, canonical m^7G caps or NCIN caps, Xrn1 was recently shown to degrade NAD-capped RNA (preprint by Sharma *et al.*, doi: 10.1101/2021.06.25.449970) (106). Therefore, capped transcripts can be degraded either directly by some exonucleases or after the NCIN cap removal by other enzymes or by endonucleolytic cleavage, which produces RNA fragments with 5′ ends that are susceptible to 5′–3′ degradation (114,115).

The endonucleolytic cleavage may be done by a different endonuclease, or by the exonuclease itself, provided it also has endonucleolytic activity (e.g. RNAse J1, CPSF73). In the case of RNase J1, it can also be done by its homolog and binding partner RNase J2 that displays strong endonucleolytic but only weak exonucleolytic activity. In complex, the two enzymes behave synergistically to alter cleavage site preference and increase cleavage efficiency at specific sites (116). This may be utilized in generating torpedo entry sites in RNAs. Whether specific sequences or secondary structures recruit the RNase J1/J2 complex is unknown. The RNase J1/J2 complex is then reminiscent of the eukaryotic CPSF73/100 complex, and IntS11/9 where the latter enzyme is catalytically inactive (117,118). Interestingly, pairs of active:inactive β-CASP proteins can also be found in other bacterial genomes, such as those of *Mycoplasma genitalium* (MG139/MG423) and *M. pneumoniae* (MPN280/MPN261), *Staphylococcus aureus* (SA0940/SA1118), *Lactobacillus lactis* (YciH/YqgA), *Deinococcus radiodurans* (DRA0069/DR2417m) and *Streptococcus pyogenes* (Spy1876/Spy1020) (119). Therefore, we speculate that a torpedo mechanism may also exist in these organisms.

An intriguing possibility for the torpedo-entry site formation for β-CASP proteins in Bacteria and Plants involves U7 snRNA homologs. U7 snRNA is required for histone pre-mRNA processing, guiding CPSF73 to the cleavage site (120). The unexpectedness of the discovery of U7 snRNA homologs in Bacteria and Plants even spurred a debate about the reliability of prediction algorithms as small regulatory RNAs similar to histone processing snRNA were not expected in organisms that lack the replication-dependent metazoan-style histone 3′ end processing machinery or in Bacteria which even lack histones (121). The hypothesis that the recruitment of prokaryotic and archaeal β-CASP proteins can be facilitated by small non-coding RNAs (possibly homologs of U7 snRNA), however, must be tested experimentally.

Torpedo entry sites in RNA could be also formed as a consequence of ribosome pausing. Arrested or paused ribosomes (which may also be caused by stalled RNAP as roadblocks) are rapidly removed by failsafe mechanisms resulting in mRNA cleavage possibly serving as a torpedo entry site. Such endonucleolytic cleavage can also be done by toxins such as RelE that cleaves mRNA in the tRNA-free

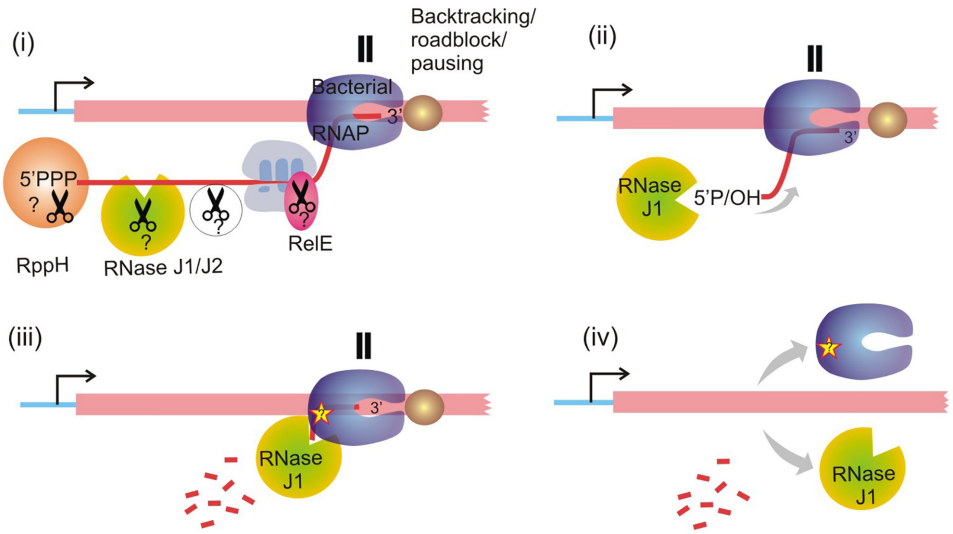

Figure 4. Bacterial premature torpedo termination. Symbols as in Figure 2. (i) Stalled RNAP (e.g. by the brown roadblock on DNA) needs to be removed. Possible ways to produce hydroxylated/monophosphorylated 5′ RNA ends, susceptible to torpedo exonuclease, are depicted and discussed in the text (pyrophosphate hydrolysis by RppH, endonucleolytic cleavage by RNAse J1 or J2 or by the J1/J2 heterodimer, cleavage of RNA by RelE toxin in the ribosomal aminoacyl site or cleavage by unknown enzyme). The exact mechanism is still unidentified. (ii-iii) Susceptible RNA is exonucleolytically degraded by RNase J1 up to the stalled RNAP. (iv) RNAP is released from DNA by an unknown mechanism.

A-site of ribosomes (122) or by MazF, a ribonuclease that cleaves single-stranded RNA (123,124). The torpedo acting on truncated nascent transcripts resulting from ribosome arrest would then stop downstream RNA synthesis and mediate RNAP release.

Finally, transcription-translation coupling where ribosomes closely follow RNAP is an obvious obstacle for the torpedo mechanism in Bacteria (125). Nevertheless, this coupling is not always tight as in e.g. *B. subtilis* RNAP outpaces the leading ribosome, creating alternative rules for global RNA surveillance (126).

Mechanisms of EC dissociation by torpedo

To start acting on RNAP, the torpedo must be able to catch up with it. However, elongating RNAP is typically too fast. A common requirement is to slow/pause/stall RNAP. Such immobilized RNAPs bound to DNA template were previously likened to 'sitting ducks' (127). The formation of these sitting ducks is induced by protein factors (128) or R-loops (129,130), and it can be sequence-specific (131–133) or induced by shortening of nascent RNA (134). Nevertheless, the nascent RNA must be sufficiently long to allow the exonuclease attachment (bacterial RNAP protects ca. 18 nt). This correlates with the finding that upon deletion of GreA from *B. subtilis*, accumulation of RNAP was identified only within promoter-proximal regions. These stalled RNAPs must be liberated by GreA as the nascent RNA is not yet accessible to RNase J1 (135).

Furthermore, the torpedo exonucleases need to be processive (84) and this may require accessory factors (*e.g.* Rai for Rat1 in yeast; (136)). The length of the extruding RNA is also important as RNase J1 and Rai/Rat1 were shown to act more processively with increasing length of RNA (137,138). In other words, it appears that sufficiently long RNA allows a smoother movement of the exonuclease, preventing its own pausing or dissociation from the RNA template, and this generates a driving force that contributes to the subsequent dissociation of the stalled EC.

The exact mechanism of RNAP release from EC by torpedo exoribonucleases is still a matter of debate. The original torpedo model presumes that a highly processive exonuclease, such as Rat1/Xrn2, pulls on the nascent RNA transcript when obstructed by RNAP from further progress (77–79,139). This mechanistic model corresponds with the hybrid shearing model of EC disassembly during intrinsic termination of transcription, causing the RNA:DNA hybrid in the active center to shorten, resulting in destabilization of the whole complex. It will be interesting to determine in future experiments whether the torpedo mechanism acts not only on stalled RNAP complexes but also on backtracked complexes (with low level of backtracking, e.g. by 1–2 bp) as backtracking is likely to impede this process.

More recently, allosteric models are being discussed. As for Rho, these new models presume contacts of RNAP with a particular DNA sequence or with the torpedo exonuclease, which changes the conformation of RNAP and subsequently causes the collapse of the transcription bubble and subsequent release of RNAP (103,140,141). Interestingly Miki *et al.* observed that the Xrn2 termination in *Caenorhabditis elegans* was affected by the promoter sequence (140). This is probably due to the recruitment of specific factors to EC or the interaction of EC with promoter during transcription initiation.

The mechanistic and allosteric models are not mutually exclusive; a combination of both models is possible. Nevertheless, the allosteric model is further supported by the fact that while RNAPs from prokaryotic and eukaryotic species are susceptible to torpedo termination, the involved exonucleases are not freely interchangeable within systems (see Table 1). While *B. subtilis* and *E. coli* RNAPs were torpedoed with similar efficiencies by *B. subtilis* RNase J1, eu-

Table 1. Ability of different 5′–3′ exoribonucleases and Rho termination factor to trigger transcription termination of prokaryotic versus eukaryotic RNAPs *in vitro* and *in vivo*.

	In vitro			*In vivo*	
	E. coli RNAP	*B. subtilis* RNAP	Eukaryotic Pol II	*B. subtilis* RNAP	Eukaryotic Pol II
Rat1	No (137)	Not tested	Yes (137,141)	Not tested	Yes (166)
Xrn1	Yes (35)	Yes (35)	Yes (137)	Not tested	No[a] (103,167)
Xrn2	Not tested	Not tested	Yes (137)	Not tested	Yes (87)
RNase J1	Yes (35)	Yes (35)	Not tested	Yes (35)	Not tested
Rho	Yes (168)	Yes (166)	Yes (142) not PolI or Pol III	Yes (7)	Not tested

[a]No *in vivo* effect of Xrn1 on termination at the ends of transcription units, but no data for premature termination.

karyotic Xrn1 was less efficient. So far, no one has tested prokaryotic RNase J1 in a eukaryotic system. Moreover, Rho was able to terminate Pol II *in vitro*, but not Pol I or Pol III (142). Taken together, these results suggest that the driving force resulting from RNA processing/translocation is not enough to enforce EC disintegration and that specific protein-protein interaction(s) between the termination factor and RNAP are critical to trigger the disassembly. Allosteric mechanisms of transcription termination were suggested to be likely universal in both prokaryotic and eukaryotic systems (82,143,144). Whether other interacting partners of exonucleases modulate the torpedo action is unknown [e.g. *B. subtilis* glycolytic enzyme GapA, interacting partner of RNase J1; (145)]. In Archaea, the mechanistic details are still unexplored.

Evolutionary considerations

Striking similarities can be found between the bacterial and archaeal RNase J1, aCPSF1 and the eukaryotic CPSF73 factor with respect to their endo- and exoribonucleolytic activities and involvement in the torpedo effect. All these enzymes are members of the β-CASP protein family (metallo-β-lactamase superfamily) which is represented by two separate evolutionary branches: one related to eukaryotic CPSF73 and the other to bacterial RNase J (117). In Bacteria, Archaea and histone processing in Eukaryotes, the members of the β-CASP family display both endo- as well as exoribonucleolytic activities needed for the torpedo.

While homologs of RNase J1 are found in Bacteria and Archaea, the only known eukaryotic homologs are in chloroplasts. RNase J from *Arabidopsis thaliana* chloroplasts (atRNAse J) was reported to prevent accumulation of the long antisense transcripts resulting from inefficient transcription termination, suggesting its role in 3′ end processing (146). While chloroplast transcription termination is not efficient (147), it still terminates and releases RNAP at some point. However, the mechanism remains elusive (148). Interestingly, atRNase J, similarly to other β-CASP proteins, displays both exo- and robust endonucleolytic activities (149) and is, therefore, the ideal candidate for termination of chloroplast RNAP by a torpedo mechanism.

CPSF homologs can also be found scattered throughout the bacterial kingdom (mainly in Clostridia and Proteobacteria), but information about their physiological function is missing (150).

The β-CASP ribonucleases have in common a core of 460 amino acid (aa) residues containing conserved sequence motifs involved in the tight binding of two catalytic zinc ions (117). The metallo-β-lactamase domain (MBL) is usually followed by β-CASP and RNA recognition domains (RRM - found in other RNA metabolism factors) (149). RNase Js of plant chloroplasts are longer – they contain a C-terminal region that displays high homology to the GT-1 DNA binding domain (149). In *Arabidopsis* an N-terminal extension was predicted to contain a sequence of 70 aa that was shown to be sufficient to confer chloroplast targeting (151). Archaeal CPSFs have an additional region called the archaeal CPSF-KH domain motif at the N-terminus. It belongs to a subfamily of type-II K homology (KH) RNA-binding motif (152). Structures reveal an unusual dimerization mode of the archaeal CPSF MBL domains, which suggests that RNA is bound across the dimer interface, recognized by the KH domains of one monomer, and cleaved at the active site of the other (153).

Although β-CASP and Xrn-like nucleases are structurally unrelated, it was previously noticed that they display striking parallels in the exonucleolytic mode of degradation in all aspects of enzymatic actions, including RNA binding, 5′-end recognition, catalysis, translocation and hydrolysis (154,155). Molecular details of the processive exoribonucleolytic mechanism employed by prokaryotic RNase J and eukaryotic Xrn1 can be found in Zheng *et al*. (154). We suggest that during evolution, the use of β-CASP in the cleavage at PAS tethered the β-CASP protein to the resulting mRNA and challenged the cell to evolve another mechanism to terminate the elongating RNAP by its exonucleolytic activity. This modern mechanism is fulfilled by Xrn-type nucleases, which are capable only of the exonucleolytic cleavage (103,140,141). In summary, the essential 5′–3′ exoribonucleases have evolved at least twice (β-CASP and Xrn families) (154) (Figure 5).

Missing 5′–3′ exoribonucleases in *E. coli*

β-CASP proteins are under-represented in Proteobacteria, namely in β and γ subdivisions (150,156). However, many gram-negative genera encode β-CASP proteins in their genomes (e.g. *Thermus*, *Agrobacterium, Brucella, Campylobacter, Caulobacter, Deinococcus, Helicobacter, Mesorhizobium, Mycobacterium, Mycoplasma, Nostoc, Pseudomonas, Rickettsia, Sinorhizobium, Thermotoga, Vibrio, Synechocystis*) (119,150).

Notably, until recently, neither β-CASP homologs nor 5’-3’ exonucleases have been found in *E. coli.* Nevertheless, in 2015, TrpH (subsequently renamed as RNase AM), which is unrelated to RNase J1 or CPSF73, was reported as the 5′–3′

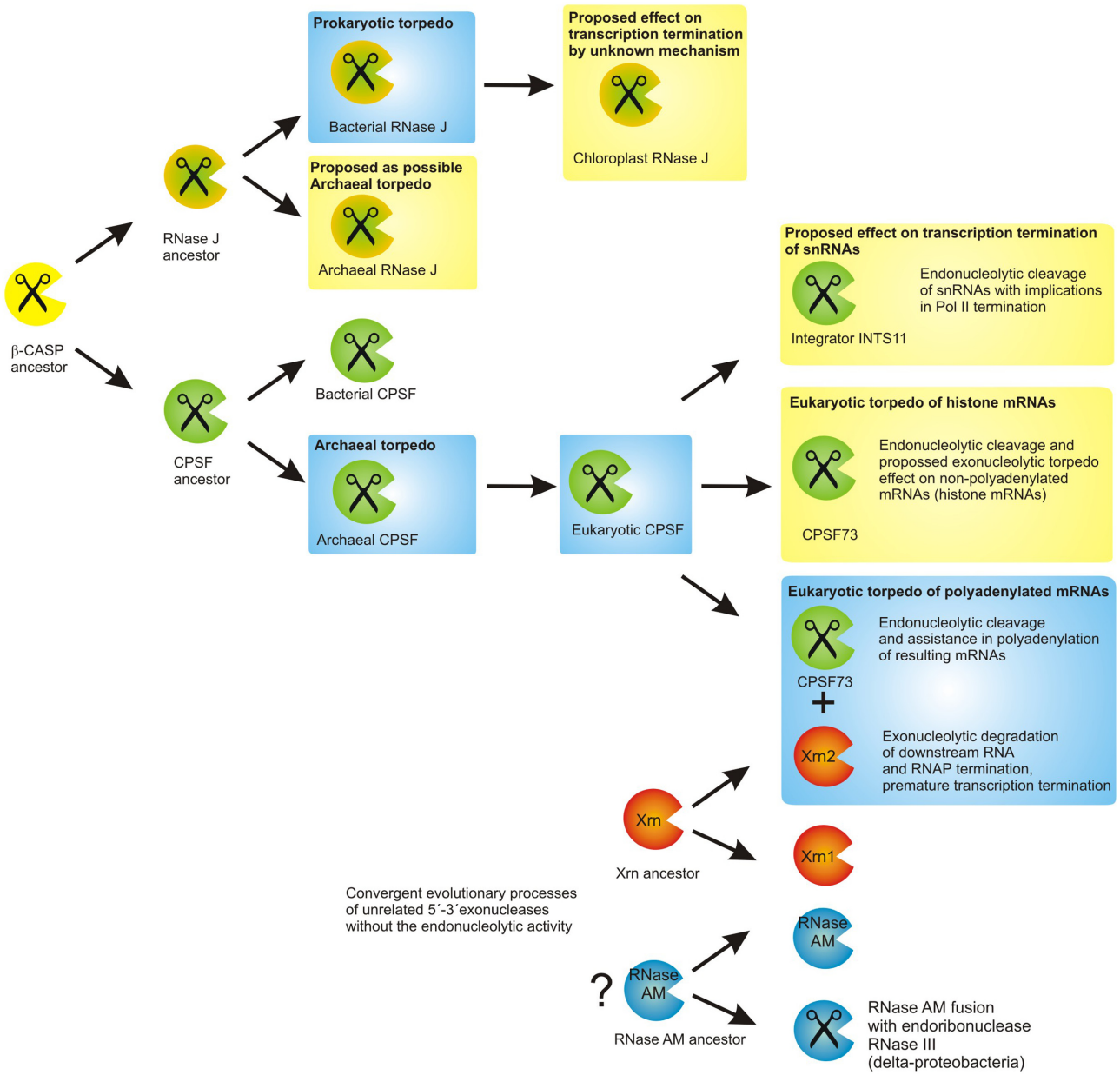

Figure 5. Possible evolution of 5′–3′ exonucleases involved in the torpedo termination mechanism of RNAP. Known torpedo mechanisms are in blue rectangles; proposed torpedo mechanisms are in yellow rectangles. Pacman icons symbolize exonucleolytic activity; scissors symbolize endonucleolytic activity. 5′–3′ exonuclease RNase AM is illustrated as a possible alternative mechanism in organisms missing β-CASP proteins but experimental data are missing.

exoribonuclease in *E. coli* (157). Interestingly in some members of delta-proteobacteria, paralogs of RNase AM were found as protein fusions with RNase III, providing the resulting protein with both exo- and endoribonucleolytic activities similarly to β-CASP enzymes (158).

Recently, the first physiological function of RNase AM was described, assigning it a role in 5S, 16S and 23S rRNA maturation in *E. coli* (158), similarly to the role of *B. subtilis* RNase J1 in the maturation of 16S rRNA and in some cases of 23S rRNA (114,159–161). Similarly, human Xrn2 as well as yeast homolog Rat1p also process the mature 5′ end of 5.8S and 28S/25S rRNA, respectively (reviewed in (162)). Thus, despite the significant differences in the structures and biochemical properties (XRN family/β-CASP family/polymerase and histidinol phosphatase (PHP) families of RNase AM), these proteins share common physiological functions: 5′–3′ exoribonuclease activity and involvement in rRNA maturation. Whether RNase AM can function to release stalled ECs in taxons lacking β-CASP proteins is yet to be tested.

Concluding remarks and outlook

β-CASP proteins are highly conserved and ubiquitous metalloenzymes involved in rRNA, mRNA maturation and degradation (119,150,163). However, recent studies demon-

strated that these enzymes not only degrade RNA but they also function in transcription termination. Studies in Bacteria and Archaea, as well as research of non-polyadenylated eukaryotic RNAs (histone RNAs) and snRNAs support a hypothesis whereby β-CASP proteins are generally involved in RNAP removal from DNA templates, representing the evolutionary old version of the torpedo mechanism. The evolutionarily advanced, convergent mechanism of Xrn-type nucleases terminating Pol II may have originated from the need to terminate transcription of polyadenylated RNAs. There, the β-CASP protein (e.g. CPSF73 in humans) remains associated with the polyadenylation complex after the endonucleolytic cleavage of RNA and cannot, therefore, degrade the downstream RNA. It is apparent that, the torpedo mechanism of transcription termination is a highly useful and efficient tool, which has evolved at least twice. RNase AM-like proteins, may then represent a third class of torpedoes (Figure 5). We envisage that β-CASP proteins may be involved in transcription termination and disassembly of RNAP from nucleic acids in systems where this process remains enigmatic (e.g. chloroplasts). Finally, the molecular details of the torpedo mechanism are still undefined, and future research will undoubtedly bring insights into the interaction between RNAPs and torpedo exonucleases in all kingdoms of life.

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