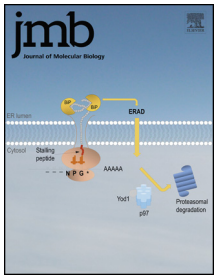

Quality Control across Compartments—Connecting ERAD with Ribosomal Quality Control

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Viral 2A peptides have become a valuable tool in the arsenal of molecular engineering because they have the unusual property of "self-cleavage" during translation in eukaryotic cells [1,2]. Therefore, they simplify simultaneous expression of polycistronic genes, such as subunits of complex proteins. First discovered and named after the 2A region of the foot-and-mouth disease virus genome, these 18- to 22-aa peptides share an NPGP C-terminal sequence, which allows the ribosome to skip the formation of the glycyl–prolyl peptide bond between G18 and P19 (underlined), but continue translation [3].

P19 is key to the function of 2A peptides. Interaction of 2A with the ribosome exit channel distorts the ribosomal structure and sterically constrains the C-terminus of 2A within the peptidyl-transferase center of the ribosome [4,5]. This steric constraint inhibits nucleophilic attack by the prolyl-tRNA (residue P19) in the A-site, stalling the translation. Amino acids that are stronger nucleophiles than P19 enable read-through of 2A, instead of release of the upstream nascent chain. The ribosomal stalling is resolved by the release factors eRF1 and eRF3 releasing the nascent protein with G18 at its C-terminus. Translation of the downstream protein then re-initiates, starting with P19 [4]. The process has been dubbed a "stop-carry on" or "StopGo" [6]. Remarkably, replacing P19 with a stop codon both stalls ribosome processivity and inhibits nascent chain release [5].

Cesaratto *et al.* [7] used this feature to investigate a "private case" of the 2A translational control–ER-targeted proteins whose translation is stalled by the 2A with a stop codon in position 19. When this modified 2A sequence (designated 2A-Gly-STOP) is included at the protein's C-terminus, it impairs expression of the upstream protein in mammalian cells as a consequence of ribosome stalling at the Gly-STOP-codon boundary, followed by proteasomal degradation of the nascent protein. Both ribosomes stalling and the reduced expression were abrogated when the boundary was changed to a 2A-Gly-Pro-STOP sequence, which re-creates the wild-type 2A sequence, allowing for the cleavage and release of the upstream protein before the ribosome decodes the stop codon. As is known for the chain termination by the wild-type 2A peptide, the observed ribosome stalling occurred on endoplasmic reticulum-bound as well as cytosolic ribosomes [7].

The polypeptides released from the ER-stalled ribosomes in this model were selectively degraded by proteasomes in a process that incorporates features of both ER-associated degradation of misfolded proteins (ERAD) and ribosomal quality control systems [8,9]. Like the classical ERAD substrates—completely translocated polypeptides that fail to fold in the ER lumen [10]—the products of the 2A-stalled translation were exposed to the glycosylation machinery in the ER and bound by the luminal peptide-binding chaperone BiP. Only then they were retro-translocated in a manner dependent on the activity of the AAA-ATPase VCP/p97, became accessible to a biotin ligase expressed in the cytosol, and targeted selectively to the ubiquitin/proteasome pathway [7].

However, unlike the usual ERAD substrates, the products of stalled translation in this 2A-Gly-STOP model also required the ribosome-associated ubiquitin ligase Listerin and the deubiquitinylase YOD1 for their degradation. These ribosomal quality control components normally operate in the disposal of defective cytosolic translation products and are not known to interact with ERAD substrates. They are, however, known to be recruited to ribosomes stalled on the ER

translocon [11]. Thus, two different modes of ribosome stalling on the ER membrane bring together two quality control mechanisms whose function is to prevent the production or accumulation of non-native proteins.

The involvement of Listerin and YOD1 in the triage of 2A-Gly-STOP substrates indicates that the 40S ribosomal subunit dissociated to allow Listerin access to the tRNA site on the 60S subunit [8,11] with the nascent protein still attached. The mechanism of ribosome splitting in this case is not clear and may be related to the conformational distortion of the ribosome by the 2A peptide. If the termination factor eRF1 binds at the stop codon within 2a sequence but cannot release the protein, the factor ABCE1 can possibly be recruited and cause an unusual ribosome splitting even before the peptidyl-tRNA has been hydrolyzed [12]. Alternatively, if eRF1 does not bind the stop codon of this nascent chain, the stalled ribosome may be split by recruitment of the rescue complex Pelota–Hbs1 [12,13]. After splitting of the stalled ribosomes and binding of Listerin, the ERAD machinery, including VCP/p97, is recruited. At first glance, binding of both Listerin and p97 is difficult to reconcile with the idea that the ribosome is still tethered to the ER translocon by the nascent chain because these proteins need access to the exit channel or the nascent protein, which are both "hidden" in the interface between the ribosome and the translocon. However, previous work did show that such binding is possible [13].

In addition to the classical ERAD substrates, the 2A-stalled translation products also differ from other co-translationally degraded secretory proteins, which are subject to ER pre-emptive quality control [14,15]. In that process, the nascent chains are rejected before they are fully translocated into the lumen and before their signal sequences are cleaved, and their targeting to proteasomal degradation depends on Bag6 [15]. Although in all three cases, the translation products targeted to proteasomes are secretory polypeptides and can be either glycosylated or not, the protein machinery associated with each process is distinct, though overlapping. The pre-emptive ER quality control involves Bag6 and the membrane proteins derlins; ERAD substrates are targeted for retro-translocation by luminal chaperones and, if glycosylated, by lectins; the 2A-Gly-STOP proteins associate with both luminal chaperones and with cytosolic ribosomal quality control components. However, all three types use the p97 ATPase and E3 ligases, albeit different ones in each case, for proteasomal targeting. All three also use different translational GTPases, which together with their binding partners monitor the state of the ribosome and its associated mRNA and initiate further reactions [9]. Thus, it would appear that while the recognition mechanisms are different in each of the three cases, they all lead to a similar process of extraction and degradation of the non-native protein.

The data of Cesaratto *et al*. [7] pose some interesting questions. First is the role of BiP in degradation of nascent proteins attached to stalled ribosomes. If the nascent protein has already been presented to the retro-translocation machinery is ubiquitinated on the cytosolic side, and if p97 is recruited by Listerin to extract it from the membrane, why is BiP still attached? One possibility is that persistent BiP association reflects the folding pathway of the translated polypeptides. Variable domains in the single chain antibody (used in Ref. [7]) are slow to fold [16,17], and ribosome stalling may slow the folding further, increasing the binding to chaperones like BiP. In contrast, the second nascent chain studied, the BioID enzyme targeted to the ER lumen, is active even when stalled [7], indicating that it is sufficiently folded, yet, it still binds BiP. The authors suggest that BiP binds to a region between the folded BioID domain and the ER membrane. An alternative explanation is that, due to the polysomal nature of translation, the nascent BioID protein attached to the leading (stalled) ribosome can be sufficiently folded and able to tag its neighboring proteome, while the nascent chains on the trailing ribosomes are still in various stages of incomplete folding and expose BiP binding sequences. This explanation would predict that Listerin and YOD1 will be found on the leading ribosomes, which would be split more readily, whereas interactors like BiP will be on the trailing ribosomes.

Another interesting question is whether ribosome stalling on 2A-Gly-STOP leads to fall-off of the trailing ribosomes [9]. Sizing of polysomes engaged with 2A-Gly-STOP *versus* 2A-Gly-Pro-STOP may resolve this question.

As pointed out by Cesaratto *et al*., their work is related to the recently described translational regulation of the macular degeneration-relevant mRNA *AMD1* [18], where read-through translation results in the leading ribosome stalling on the next in-frame stop codon within the 3′ untranslated region. The trailing ribosomes then queue along the mRNA and translation is halted. In contrast, ribosome stalling on the stop codon within 2A sequence triggers a post-translational mechanism—protein degradation. Future studies should clarify the role of 2A-Gly-STOP sequence in stalling *versus* splitting of ribosomes and thus in these different outcomes.

The self-cleaving property of viral 2A peptides is remarkable, so an obvious question is whether it is also used by higher eukaryotes. Cesaratto *et al*. show that at least two human proteins have C-terminal 2A-like sequences, the adhesion molecule CD99L2 and the ankyrin-rich protein POTE. Because 2A-like sequences in these proteins are followed by natural stop codons, these human 2A homologs confer 2A-Gly-STOP-like regulation onto reporter genes. These eukaryotic genes suggest that the 2A-like decoding of mRNA plays some role in the biology of higher organisms. One wonders whether a sequence that can potentially stall ribosomes at the end of nascent

chains only does so under some physiological or pathological conditions, when downregulation of expression, perhaps of an isoform, would be advantageous. This would add another option for quality control of the proteome.

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