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The emerging roles of CFIm25 (*NUDT21/CPSF5*) in human biology and disease

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Abstract

The mammalian cleavage factor I subunit CFIm25 (*NUDT21*) binds to the UGUA sequences of precursor RNAs. Traditionally, CFIm25 is known to facilitate 3'end formation of pre-mRNAs resulting in the formation of polyadenylated transcripts. Recent studies suggest that CFIm25 may be involved in the cyclization and hence generation of circular RNAs (circRNAs) that contain UGUA motifs. These circRNAs act as competing endogenous RNAs (ceRNAs) that disrupt the ceRNA-miRNA-mRNA axis. Other emerging roles of CFIm25 include regulating both alternative splicing and alternative polyadenylation (APA). APA generates different sized transcripts that may code for different proteins, or more commonly transcripts that code for the same protein but differ in the length and sequence content of their 3'UTRs (3'UTR-APA). CFIm25 mediated global changes in 3'UTR-APA affect human physiology including spermatogenesis and the determination of cell fate. Deregulation of CFIm25 and changes in 3'UTR-APA have been implicated in several human diseases including cancer. In many cancers, CFIm25 acts as a tumor suppressor. However, there are some cancers where CFIm25 has the opposite effect. Alterations in CFIm25-driven 3'UTR-APA may also play a role in neural dysfunction and fibrosis. CFIm25 mediated 3'UTR-APA changes can be used to generate specific signatures that can be used as potential biomarkers in development and disease. Due to the emerging role of CFIm25 as a regulator of the aforementioned RNA processing events, modulation of CFIm25 levels may be a novel viable therapeutic approach.

Graphical Abstract:

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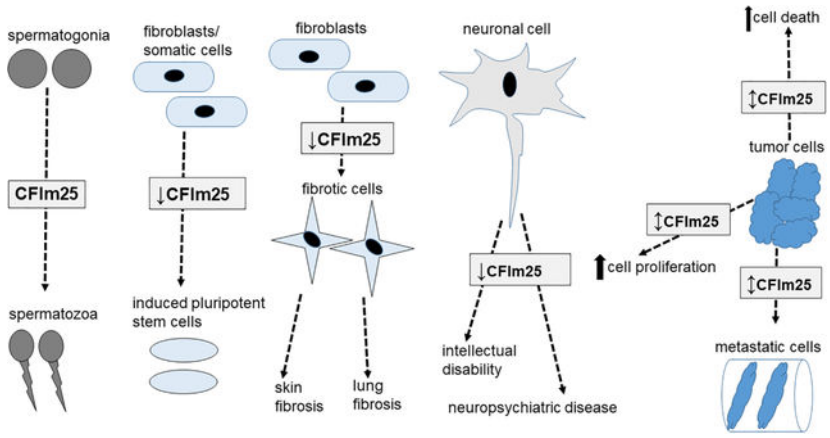
Conflict of Interest

The author declares no conflict of interest.



The impact of CFIm25 mediated 3'untranslated region alternative polyadenylation (3'UTR-APA) in human biology and disease

1. INTRODUCTION

The 25 kilodalton subunit of mammalian cleavage factor I, 25 (CFIm25) is a small protein that is encoded by the nudix hydrolase 21 gene (*NUDT21* also known as *CPSF5*). CFIm25 contains a Nudix hydrolase domain found in genes that mainly hydrolyze dinucleotides. However, the human CFIm25 core Nudix motif sequence lacks three core glutamate residues. The impact of this modification on its ability to bind and hydrolyze a substrate is still undetermined (McLennan, 2006). However, crystal structure comparisons of CFIm25 showed that unlike other Nudix hydrolases, CFIm25 has a distinctive α-helix loop motif before the Nudix fold, which blocks the enzymes catalytic site (Yang et al., 2010). Thus, in humans, CFIm25 does not have any hydrolase activity and can potentially play other roles. The best known role of CFIm25 is in 3'end processing of pre-mRNAs. Most human precursor mRNAs (pre-mRNAs) undergo capping at the 5'end, splicing, as well as cleavage and polyadenylation of the 3'end. The formation of the 3'end is directed by several *cis* elements found within the pre-mRNAs (Figure 1).

In general, the core *cis* elements in order consist of the polyadenylation signal (PAS), the cleavage site, and a GU/G/U rich downstream sequence element (Danckwardt et al., 2008; Mandel et al., 2008). In addition, there are two auxiliary sequences which consist of a U-rich sequence element upstream of the PAS and a poorly understood generally G-rich sequence element that is downstream of the cleavage site (Mandel et al., 2008). The *cis* elements are important because they are the binding sites for the 3' end processing factors, which make up the cleavage and polyadenylation complex. The key factors of the complex contain several multi-protein components: the cleavage and polyadenylation specificity factors (CPSFs), the cleavage stimulation factors (CstFs), and the mammalian cleavage factors I and II (CFIm and CFIIm) (Mandel et al., 2008). Binding of CFIm25 to the UGUA sequence on the pre-mRNA is one of the earliest steps in 3'end formation. CFIm25 is then believed to promote the recruitment and assembly of the rest of the 3'end processing machinery (Kim & Lee, 2001; Rüegsegger et al., 1996; Rüegsegger et al., 1998). UGUA sequences are often found about 40 nucleotides upstream of the polyadenylation signal

(Hu et al., 2005). CPSF30 and Wdr33 bind to the canonical PAS hexamer AAUAAA with CPSF160 (which was previously believed to bind to the PAS (Murthy & Manley, 1995; Zhao et al., 1999)) acting as a scaffold. The CstF64 subunit binds to the G/U rich downstream sequence element on the pre-mRNA (Clerici et al., 2018; Sun et al., 2018). The recruitment of the 3'end processing factors brings the pre-mRNA cleavage site into close proximity with the endoribonuclease CPSF73, which then cleaves the pre-mRNA ~15 nucleotides downstream of the PAS. This cleavage is followed by the addition of untemplated adenines by poly (A) polymerase to generate the poly (A) tail (Danckwardt et al., 2008; Mandel et al., 2008; Millevoi & Vagner).

Although the UGUA *cis* element bound by CFIm25 is not considered essential for 3'end processing, CFIm25 is considered an enhancer-dependent activator of 3'end cleavage and polyadenylation (Zhu et al., 2018). Structural studies show that when CFIm25 binds, it functions as a hetero-tetramer consisting of two CFIm25 subunits which each bind to two distinct UGUA sequences on the RNA (Yang et al., 2010). The CFIm25 subunits simultaneously bind to two subunits of one of its binding partners CFIm59 (*CPSF7*) or CFIm68 (*CPSF6*) or CFIm72 (a splice variant of CFIm68) (Rüegsegger et al., 1996; Yang et al., 2010). The CFIm68/CFIm59/CFIm72 subunits interact with CFIm25 via their N-terminal RNA recognition motifs (RRM) (Yang et al., 2010).

2. REGULATION OF ALTERNATIVE RNA PROCESSING BY CFIm25

Besides 3'end formation, CFIm25 has been implicated in pre-mRNA processing including both alternative splicing and alternative polyadenylation. In addition CFIm25 is also involved in cyclization of circular RNAs. The presence of multiple UGUA binding motifs within the precursor RNAs may facilitate for binding of multiple CFIm25 dimers. This potentially allows CFIm25 to play a regulatory role in different types of RNA processing.

2.1 Alternative splicing and CFIm25

Analysis of the 3'end processing complex by mass spectrometry identified ~85 proteins including factors involved in splicing (Shi et al., 2009). Hence, it is not surprising that CFIm25's binding partners CFIm59 and CFIm68 have long been implicated in splicing. CFIm59 interacts with the splicing factor U2 snRNP Auxiliary Factor 65 (U2AF65) (Millevoi et al., 2006). Sequence alignments of the CFIm68 RRM domain also identified potential interactions with U2AF65 (Yang, Gilmartin, et al., 2011). In addition, CFIm68 subunits have been detected in spliceosomes (Zhou et al., 2002). The links between its binding partners and splicing thus indirectly links CFIm25 to the process. Other 3'end processing factors, including CPSFs and Symplekin, have also been shown to play a role in splicing by globally increasing usage of alternative internal exons (Misra et al., 2015).

RNA Sequencing (RNA-seq) data showed that knockdown of CFIm25 in HeLa cells resulted in decreased splicing to a mutually exclusive glutaminase exon 15, giving rise to the long kidney-type (*KGA*) isoform which consists of 18 exons. This was accompanied by reduced splicing to the shorter glutaminase C (*GAC*) isoform consisting of only 15 exons. These CFIm25 driven splicing changes were confirmed by both qRT-PCR and western blot analysis (Masamha et al., 2016). Mechanistic studies showed that in the presence

of CFIm25, the noncoding RNA *CCAT2* (Colon Cancer Associated Transcript 2) binds to the *GAC* terminal exon and then recruits CFIm25 and CFIm68 to activate usage of the alternative exon resulting in the *GAC* isoform (Redis et al., 2016). This suggests that CFIm25 may determine terminal exon definition in select genes. Another role of CFIm25 in splicing was shown for methionine adenosyltransferase 2A (*MAT2A)*, where depletion of CFIm25 resulted in increased intron retention (Scarborough et al., 2021). A proposed model for splicing may involve recruitment of the splicing factors which then interact with CFIm25 and other members of the 3'end processing machinery allowing for both splicing and 3'end formation to occur (Martinson, 2011). Interestingly, CFIm25 depletion not only results in increased splicing to generate the longer KGA isoform, but this is also accompanied by shortening the 3'UTR of the *KGA* transcript (Masamha et al., 2016). On a global scale, it is becoming increasingly clear that there is reciprocal regulation between both splicing and 3'end formation, particularly within the terminal exon (Kaida, 2016). However, the role of this reciprocity in regulating alternative polyadenylation still remains unknown.

2.2 3'UTR-alternative polyadenylation (3'UTR-APA) and CFIm25

An estimated 70% of human genes contain multiple polyadenylation signals (PASs). Hence 3'end cleavage and polyadenylation can occur at multiple sites through a process called alternative polyadenylation (Derti et al., 2012; Shi, 2012; Tian et al., 2005). The different types of alternative polyadenylation have recently been extensively reviewed in detail elsewhere (Mohanan et al., 2022; Zhang et al., 2021). The simplest form of splicing alternative polyadenylation involve use of alternative exons through alternative splicing giving different protein products (Figure 2). When alternative polyadenylation occurs within the same terminal exon (3'UTR-APA), it results in transcripts that contain the same protein coding region but have 3'UTRs of different length. These differently sized 3'UTRs contain different sequence elements including miRNA binding and RNA binding protein (RBP) motifs (Plass et al., 2017; Sandberg et al., 2008). The different RBPs and miRNA motifs may affect the stability of the differently sized transcripts as well as the translation efficiency of the mRNA (Geisberg et al., 2014; Spies et al., 2013). The length of the 3'UTR may also affect sub-cellular localization of different transcripts and proteins (Berkovits & Mayr, 2015; Ciolli Mattioli et al., 2019). Global differences in 3'UTR length have been observed during development, in different tissues, during stress and in different biological and disease states (Derti et al., 2012; Hu et al., 2017; Sadek et al., 2019; R. Wang et al., 2018). One of the earliest applications of differences in 3'UTR lengths was in distinguishing between clinically similar tumor subtypes with different prognosis (Singh et al., 2009). Hence, signature changes in the 3'UTR length through 3'UTR-APA can potentially be used as biomarkers for disease.

2.2.1 Factors driving 3'UTR-APA—

In 3'UTR-APA, there are several factors that may drive usage of a particular PAS including the sequence content of the PAS. In almost three quarters of the annotated genome, the PAS consists of the hexameric A(A/U)UAAA sequence (Mandel et al., 2008). The canonical PAS sequence is AAUAAA and early studies showed that it is the most prevalent hexamer occurring in ~58% of all PAS sequences and is followed by AUUAAA which occurs in ~15% of all human PAS sequences (Beaudoing et al., 2000). In cases where more than one PAS exists, the most distal PAS (dPAS) typically

contains the optimal hexameric sequence AAUAAA, and 'weaker' variants of the sequence are typically located more proximal (pPAS) to the stop codon (Mangone et al., 2010). Based on the PAS sequence itself, the implication of this trend is that the default choice is to utilize the dPAS giving rise to transcripts with longer 3'UTRs. Alternatively, the non-canonical "weaker" pPAS may be the one that is intrinsically more susceptible to regulation. Hence the dPAS is the default choice and specific factors including changes in CFIm25 levels may increase or decrease usage of the pPAS. Some pre-mRNAs do not have a canonical AAUAAA sequence, as either the dPAS or pPAS, and in these cases the presence of UGUA binding motifs facilitates CFIm25 mediated non-canonical PAS driven 3'end formation (Venkataraman et al., 2005). Hence, CFIm25 mediated 3'end formation based on the recognition of UGUA motifs in the absence of an optimal PAS gives credence that CFIm25 itself can regulate 3'UTR-APA where there are multiple PASs and in cases where the pPAS is non-canonical.

Another driver of PAS choice in 3'UTR-APA are the levels of different proteins including 3'end processing factors as well as other RNA binding proteins. Regulation of 3'UTR-APA by these aforementioned proteins in human biology and disease has been extensively reviewed elsewhere including in WIRES RNA series (MacDonald, 2019; Mohanan et al., 2022) and elsewhere (Masamha & Wagner, 2018). Although other 3'end factors can regulate 3'UTR-APA, the impact of CFIm25 mediated 3'UTR-APA has so far been the most widely studied with far reaching potential impacts in human biology and disease, hence the focus of this article.

2.2.2 Models of CFIm25 mediated 3'UTR-APA—

According to one model when there are multiple PASs, CFIm25 binds to two UGUA sequences located upstream and downstream of the pPAS (Figure 3). CFIm25 forms a dimer and recruits CFIm59/CFIm68 which bind to CFIm25 forming a tetrameric complex (Rüegsegger et al., 1996). This then results in looping of the pre-mRNA which hides the pPAS, and facilitates in the skipping of the pPAS. The end result is increased usage of a more dPAS (Yang, Coseno, et al., 2011; Yang, Gilmartin, et al., 2011). Loss of CFIm25 would be expected to favor usage of the pPAS and generation of shorter 3'UTRs. Although the UGUA sequence is traditionally believed to be upstream of the PAS, many genes contain several of these sequences both upstream and downstream of the PAS (Masamha et al., 2014; Yang, Gilmartin, et al., 2011).

Another model suggests that when there are multiple UGUA sequences, these are often concentrated at the dPAS sites. CFIm25 and CFIm68/CFIm59 are then recruited to activate usage of the dPAS. Hence the presence of CFIm25 favors preferential dPAS usage which generates transcripts with longer 3'UTRs. Loss of CFIm25 in this case also results in decreased usage of the dPAS. Loss of CFIm68 has a similar effect whereas depletion of the alternative CFIm25 binding partner CFIm59 has a lesser effect on 3'UTR-APA (Zhu et al., 2018).

2.3 Regulation of other RNAs by CFIm25

Apart from regulating pre-mRNA processing, CFIm25 levels can directly or indirectly affect levels of non-coding RNAs. One study showed that, depletion of CFIm25 does not have

a global effect on the biogenesis of microRNAs (miRNAs). However, CFIm25 had an effect on competing endogenous RNA (ceRNAs) (Park et al., 2018). CeRNAs work in the ceRNA-miRNA-mRNA axis, where the ceRNA acts as a molecular sponge for miRNAs allowing de-repression of mRNAs, resulting in increased mRNA translation (Salmena et al., 2011).

2.3.1 CFIm25 and 3'UTRs acting as competing endogenous RNAs (ceRNAs)

—Knockdown of CFIm25 resulted in increased association of tumor suppressive competing endogenous RNA (ceRNAs) with argonaute RISC catalytic component 2 (AGO2) (Park et al., 2018). Depletion of CFIm25 resulted in 3'UTR shortening of YOD1 deubiquitinase (*YOD1)* transcript resulting in increased protein expression. The shortening of the *YOD1* 3'UTR released miR-3187–3p and miR-549 which subsequently targeted and bound the tumor suppressor PHD finger protein 6 (*PHF6)* transcript and reduced its expression. The same effect was observed for the tumor suppressor La ribonucleoprotein 1, translational regulator (*LARP1*) transcript. Based on this model of CFIm25 regulation, the long 3'UTR of *YOD1* acts in the long *YOD1* 3'UTR (ceRNA)-miRNA-*PHF6*/*LARP1* (mRNA) network (Park et al., 2018). This suggests that CFIm25 driven regulation of the 3'UTR length through 3'UTR-APA may disrupt ceRNA-miRNA-mRNA networks. This adds credence to the ceRNA hypothesis unifying different types of RNAs (Salmena et al., 2011).

2.3.2 CFIm25 and UGUA circRNAs—

Another type of ceRNA that binds miRNAs and acts as a molecular sponge are circular RNAs (circRNAs) (Hansen et al., 2013; Wilusz & Sharp, 2013). A study showed an association between global downregulation of circRNAs and low levels of CFIm25. CFIm25 overexpression and CFIm25 depletion resulted in changes in the expression of several circRNAs showing their dependence on CFIm25 levels. Further analysis revealed that all the circRNAs with altered expression had UGUA CFIm25 binding motifs within 200 nucleotides of the circRNAs circularization site (Li et al., 2020). Hence, the UGUA element was determined to be crucial in the formation of CFIm25 mediated cyclization of circRNAs with the UGUA sequences but not for the non-UGUA containing circRNAs (Figure 4). Consequently, depletion of CFIm25 decreased biogenesis of UGUA containing circRNAs resulting in the repression of circRNAs ability to act as miRNA sponges. As a result, reduction in circRNAs resulted in miRNAs that are normally absorbed by the circRNAs being redirected to tumor suppressor mRNAs (Li et al., 2020). Thus, in this model, CFIm25 indirectly regulates gene expression by modulating the circRNAs-miRNA-mRNA axis.

3. IMPACT OF CFIm25 MEDIATED 3'UTR-APA ON MAMMALIAN BIOLOGY

Although the main focus of the work presented in this review is on human biology, a lot of work has been done in rodents and we will talk about this research and explain the implications in humans where no human data exists.

3.1 CFIm25 and determination of cell fate

There is a general increase in 3'UTR lengths during differentiation of myoblasts to myotubes. This increase in 3'UTR length was also observed during embryonic development

in mice (Ji et al., 2009). The 3'end processing factor Fip1 activates rat embryonic stem cells (ESCs) 3'UTR-APA promoting expression of essential genes that promote ESC self renewal and somatic cell reprogramming (Lackford et al., 2014). Work by Brumbaugh et al found that CFIm25 (encoded by *Nudt21* gene in mice) was a powerful barrier to cellular reprogramming in mice. The group used a lentiviral library containing hairpins targeting over 18,000 genes and found that shRNA against *Nudt21* had the strongest effect on the generation of induced pluripotent stem cells (iPSCs) from mouse embryonic fibroblasts. CFIm25 knockdown resulted in 3'UTR-APA changes in over 1,500 transcripts in cells developing pluripotency and favored dedifferentiation into stem/progenitor cells. A fraction of these changes in 3'UTR-APA were accompanied by alterations in proteins which were enriched for chromatin regulators some of which had already been connected to iPSC. Furthermore, CFIm25 depletion also inhibited differentiation of ESCs and myeloid precursors (Brumbaugh et al., 2018).

In a different study, bioinformatic analysis of microarray data from both mouse and human samples showed that iPSCs produced by reprogramming somatic cells into an ESC-like state had transcripts with truncated 3'UTRs. However, reprogramming of spermatogonial cells into ESC-like state resulted in transcripts with longer 3'UTRs (Ji & Tian, 2009).

3.2. CFIm25 and spermatogenesis

Research conducted in mice showed that many male germ cell specific mRNAs use non-canonical PASs for 3'end formation (Liu et al., 2007; MacDonald & Redondo, 2002). As previously discussed, selection of these non-canonical PASs is directed by CFIm25 binding to UGUA sequence elements and the recruitment of its binding partner CFIm68 (Venkataraman et al., 2005). Interestingly, male germ cells expressed the highest levels of both CFIm25 and CFIm68 compared to all other tissues. Hence, in addition to regulating 3'UTR-APA of other transcripts, it was posited that the two factors may also play a role in their own post-transcriptional regulation in mouse male germ cells. Levels of both factors were also shown to fluctuate during spermatogenesis suggesting that they may play a role in 3'UTR-APA of non-canonical PASs during spermatogenesis (Sartini et al., 2008).

Although the work above was done in mice, work in humans found that CFIm25 upregulates the mRNA expression of spindle associated receptor for hyaluronan mediated motility (RHAMM) (encoded by *HMMR*). RHAMM regulates planar cell division of male germ cells in the testis. Dysregulation of CFIm25 expression resulted in decreased levels of RHAMM expression leading to testicular atrophy and decreased fertility. Although the effects of CFIm25 induced 3'UTR-APA of RHAMM were not determined, loss of RHAMM is associated with the development of testicular germ cell tumors (Li et al., 2016).

4. IMPACT OF CFIm25 MEDIATED 3'UTR-APA ON HUMAN DISEASE

4.1 The role of CFIm25 regulated 3'UTR-APA in cancer CFIm25

In cancer, 3'UTR-APA can activate proto-oncogenes by shortening the 3'UTR and eliminating miRNA target sites located within the longer 3'UTR (Mayr & Bartel, 2009). Although the impact is not as clear cut, redirection of miRNAs from transcripts with

truncated 3'UTR to the 3'UTRs of tumor suppressor genes can result in inactivation of tumor suppressors (Park et al., 2018). Previous studies have shown that during both de-differentiation and increased cell proliferation, cells preferentially use pPAS sites generating transcripts with short 3'UTRs (Elkon et al., 2012; Ji & Tian, 2009; Sandberg et al., 2008). In contrast during differentiation, cells preferentially use the more dPAS generating transcripts with longer 3'UTRs (Ji et al., 2009; Ji & Tian, 2009). A recent study using patient samples revealed global shortening of the 3'UTRs of genes involved in blocking differentiation of acute myeloid leukemia cells which was mediated by the presence of the 3'end processing factor hFip1 (Sommerkamp & Trumpp, 2022). Changes in levels of other core 3'end processing factors have also been implicated in driving 3'UTR-APA patterns seen in cancer (Aragaki et al., 2011; Ogorodnikov et al., 2018; Singh et al., 2009; Turner et al., 2020; Xia et al., 2014; Ye et al., 2019). An early study showed that knockdown of CFIm25 in the cervical cancer cell line HeLa, induced shortening of the 3'UTRs of tissue inhibitor of metalloproteinases 2 (*TIMP2)*, excision repair cross-complementation group 6 (*ERCC6)* and syndecan 2 (*SDC2)* transcripts (Kubo et al., 2006). RNA-seq data analysis revealed global shortening of 3'UTRs after CFIm25 knockdown in 293T cells (Gruber et al., 2012; Martin et al., 2012). However, the biological impact of CFIm25 knockdown in these cells was not determined in these studies.

4.1.1 CFIm25 mediated 3'UTR-APA in cervical cancer—

A study that systematically knocked down of each of the core 3'end processing factors (with the exception of WDR33) in Hela cells resulted in differential 3'UTR-APA changes of three genes, cyclin D1 (*CCND1*), *TIMP2* and double stranded RNA specific endoribonuclease (*DICER)* as measured by qRT-PCR. However, depletion of CFIm25 resulted in the most drastic and consistent decrease in dPAS usage for all three genes (Masamha et al., 2014). Global analysis using RNA-seq identified 1,450 genes that underwent 3'UTR shortening and three genes that underwent 3'UTR lengthening after knockdown of CFIm25 in HeLa cells. The results were validated using qRT-PCR for the following genes: glycogen synthase kinase 3 beta (*GSK3β*), secreted modular calcium-binding protein 1 (*SMOC1*), alkaline ceramidase 3 (*ACER3*), transmembrane protein 48 (*TMEM48*), methionine sulfoxide reductase B3 (*MSRB3*) and vacuolar ATPase assembly factor (*VMA21*). There was a corresponding increase in the protein levels of glutaminase (*GLS*), methyl CpG binding protein 2 (*MECP2*), *CCND1*, *VMA21*, *GSK-3β* and *SMOC1* as shown by Western blot analysis (Masamha et al., 2014). This supports previous findings that for oncogenes, shortening the 3'UTR results in increased protein expression of the oncogene (Mayr & Bartel, 2009). Furthermore, in the HeLa cell line, depletion of CFIm25 resulted in increased cell proliferation, suggesting for the first time that CFIm25 has an anti-proliferative role and is a potential tumor suppressor (Masamha et al., 2014).

A more recent study of seven cervical cancer cell lines showed that tumor cells express lower levels of CFIm25 compared to normal cervical squamous cells. Furthermore, tissue samples from cervical cancer patients showed lower levels of CFIm25 in tumor tissue and this was associated with reduced survival. Depletion of CFIm25 in both Hela and SiHa cell lines resulted in increased cell proliferation, increased tumor growth, increased metastasis and decreased survival in mouse xenograft models. CFIm25 overexpression had

the opposite effects (Xing et al., 2021). Analysis of RNA-seq data after ectopic expression of CFIm25 in cervical cancer cell (presumably the SiHa cell line) showed that CFIm25 overexpression induced 3'UTR shortening of 348 genes and 3'UTR lengthening of 457 genes. In contrast, knockdown of CFIm25 resulted in 3'UTR lengthening of 1,458 genes and 3'UTR shortening of 1,503 genes (Xing et al., 2021). The changes in the 3'UTR length of different genes may be due to the actual levels (dosage) of CFIm25 resulting from the two different expression models as well as the cell line used suggesting cell/tissue specific 3'UTR-APA. Verification of 3'UTR-APA changes was done for interleukin 1 alpha (*IL-1A*) and Wnt family member 10B (*WNT10B*) which mediate Wnt signaling, as well as high mobility group box 1 (*HMGB1*) which facilitates the nuclear factor kappa B (NF-κB) signaling. Furthermore, CFIm25 depletion resulted in increased pPAS usage of acetyl coA acetyltransferase 2 (*ACAT2*) which is important in fatty acid metabolism (Xing et al., 2021). Hence, the two different studies confirm the role of CFIm25 as a tumor suppressor that regulates 3'UTR-APA in cervical cancer.

4.1.2 CFIm25 mediated 3'UTR-APA in glioblastoma—The effects of CFIm25 are not as clear cut in glioblastoma. Analysis of glioblastoma RNA-seq data from the Cancer Genome Atlas (TCGA) showed that when stratified according to CFIm25 levels, 59 genes showed 3'UTR truncations in samples of patients who expressing lower levels of CFIm25. Of these, 24 overlapped with genes that also showed 3'UTR shortening in HeLa cells (Masamha et al., 2014). Depletion of CFIm25 in LN229, a glioblastoma cell line that expresses high levels of CFIm25, resulted in increased cell proliferation, increased cell invasion as well as increased colony formation. Subsequent injection of CFIm25 depleted LN229 cells in a mouse xenograft model of glioblastoma resulted in increased tumor growth. Overexpression of CFIm25 in U251 (a glioblastoma cell line that expresses lower levels of CFIm25 when compared to LN229) had the opposite effect both *in vitro* and *in vivo*. (Masamha et al., 2014). These results strongly suggested that CFIm25 plays a significant tumor suppressive role in glioblastoma through 3'UTR-APA of select genes. Furthermore, analysis of TCGA data found that reduced expression of CFIm25 was associated with reduced survival in patients with low grade and in two out of the four high grade glioma subtypes. Analysis of 3'UTR-APA in LN229 cells after CFIm25 knockdown identified a subset of 350 genes whose 3'UTRs underwent shortening and 24 genes whose 3'UTRs became longer. Several of the genes including p21 activated kinase 1 (*PAK1*) and *PAK2* are involved in oncogenic Ras signaling (Chu et al., 2019). Knockdown of CFIm25 in LN229 also resulted in increased cell proliferation and migration as shown in a previous study (Chu et al., 2019; Masamha et al., 2014).

However, a different study showed conflicting results. Depletion of CFIm25 in two glioblastoma cell lines, U87 and U251, resulted in decreased cell viability and cell proliferation *in vitro*, and decreased tumor volume in nude mice. This was accompanied by increased apoptosis in the CFIm25 depleted cells (Lou et al., 2017). The previous study did not knockdown CFIm25 but overexpressed it instead due to lower levels of CFIm25 in the U251 cell line (Masamha et al., 2014). The differences in cell survival in the two experiments may be due to the actual levels of CFIm25 in the U251 cell line. It is possible that if the levels of CFIm25 are below a certain currently unknown threshold level,

cells undergo apoptosis. On a molecular level, CFIm25 knockdown was shown to increase expression of the *NF-κB* inhibitor subunit zeta, (*NFKBIZ*) through an unknown mechanism. (Lou et al., 2017). The results suggest that CFIm25 acts upstream and regulates the *NF-κB* signaling pathway in glioblastoma. The impact of CFIm25 on 3'UTR-APA in glioblastoma was not determined in this study. These conflicting results suggest that under different conditions, CFIm25 may play a tumor suppressive or oncogenic role in glioblastoma which may allow fine tuning to regulate specific effects in cancer. The source of these differences is still unknown. The dual role of CFIm25 as a tumor suppressor and an oncogene would be in line with studies which suggest that the tumor protein (*TP53*), which is traditionally considered a tumor suppressor, may also have oncogenic properties (Soussi & Wiman, 2015).

4.1.3 CFIm25 mediated 3'UTR-APA in bladder cancer—Another tumor type where CFIm25 has been shown to act as a tumor suppressor is bladder cancer. Xiong and colleagues analyzed mRNA expression data from two different cohorts i.e. Oncomine data and publicly available data from a gene expression study (GEO accession number GSE13507) (Xiong et al., 2019). They found that CFIm25 was downregulated in bladder cancer when compared to normal bladder mucosa and this was associated with reduced survival. Low levels of CFIm25 expression were also observed at the mRNA and protein levels in bladder cancer cell lines. Knockdown of CFIm25 in the bladder cancer cell lines EJ and T24 resulted in increased cell proliferation, increased cell migration and invasiveness *in vitro*. Furthermore, injection of cells in a mouse xenograft model resulted in increased tumor size after CFIm25 knockdown. In contrast, overexpression of CFIm25 had the opposite effects. Several genes whose 3'UTR-APA was potentially regulated by CFIm25 were identified. From these oncogenic annexin A2 (*ANXA2)* and LIM domain kinase 2 (*LIMK2*) transcripts were shown to undergo 3'UTR lengthening after CFIm25 overexpression resulting in reduced protein expression. Lengthening of the 3'UTRs of these two genes was correlated with reduced Wnt/β-catenin and NF-κB signaling resulting in decreased tumorigenicity. CFIm25 knockdown in the same cell lines resulted in the generation of shorter 3'UTRs of both *ANXA2* and *LIMK2* and increase oncogenic Wnt/β-catenin and NF-κB signaling (Xiong et al., 2019).

4.1.4 CFIm25 mediated 3'UTR-APA in hepatocellular carcinoma—In hepatocellular carcinoma (HCC), several studies showed that CFIm25 levels were lower in tumor tissues compared to normal/adjacent tissue samples (Li et al., 2020; Tan et al., 2018; Y. Wang et al., 2018). In one study, qRT-PCR analysis of levels of different 3'end processing factors identified CFIm25 as the factor which was expressed at low levels in HCC tissue compared to adjacent normal tissue (Tan et al., 2018). Knockdown of CFIm25 in HCC cell lines HL-7702 and PLC, which express high levels of CFIm25, resulted in increased cell numbers, increased cell viability, increased colony formation and increased tumor volume in a mouse xenograft model. Overexpression of CFIm25 in Hep3B and SMMC-7721 HCC cell lines, which express lower levels of CFIm25, resulted in the opposite effects and was non-tumorigenic. Depletion of CFIm25 in HL-7702 cells resulted in 3'UTR shortening of ~80 genes and lengthening of ~55 genes. Ectopic overexpression of CFIm25 in Hep3B cells resulted in 3'UTR shortening of 1,328 genes. Of these, 22 genes were found in common

between the two different CFIm25 expression conditions. Validation of CXXC finger protein 5 (*CXXC5)* and proteasome 20S subunit beta 2 (*PSMB2)* 3'UTR-APA was done using qRT-PCR and 3'RACE where forced expression of CFIm25 resulted in a slight increase in dPAS usage. Depletion of CFIm25 resulted in increased protein levels of both *CXXC5* and *PSMB2* while overexpression of CFIm25 had the opposite effect (Tan et al., 2018).

In a different study, lower expression of CFIm25 in HCC was also associated with metastasis in paired HCC tumor and normal tissue samples. Lower levels of CFIm25 correlated with decreased survival in HCC patients (Y. Wang et al., 2018). Overexpression of CFIm25 in Sk-hep-1 and MHCC-LM3 HCC cells resulted in reduced cell migration and invasion whereas knockout of CFIm25 in SMMC-7721 and Hep-G2 cells had the opposite effect. Ectopic expression of CFIm25 inhibited intrahepatic and lung metastasis in mouse xenograft models of HCC. Although 3'UTR-APA was not investigated in the study, CFIm25 depletion resulted in increased phosphorylation of JNK/c-Jun and p38 signaling pathways thus activating E-cadherin and metastasis (Y. Wang et al., 2018). This proposed role of CFIm25 in metastasis further supports the tumor suppressive role of CFIm25 in HCC. Another study showed that CFIm25 knockdown was associated with increased cell proliferation (Li et al., 2020). As detailed above (Section 2.3.2), knockdown of CFIm25 in HCC cells prevented formation of circRNAs with a UGUA motifs. These circRNAs are known to play a role in HCC oncogenesis and metastasis. The reduction in circRNAs biogenesis resulted in retargeting of miRNAs to tumor suppressor genes which resulted in decreased tumor suppressor levels and increased cell proliferation (Li et al., 2020).

4.1.5 CFIm25 mediated 3'UTR-APA in lung cancer—Knockdown of CFIm25 in the lung cancer cell line A549 resulted in increased 3'UTR shortening of insulin like growth factor 1 receptor (*IGF1R)* and *CCND1* transcripts, resulting in an increase in both protein expression and cell proliferation. Analysis of RNA-seq data from lung adenocarcinoma and lung squamous cell carcinoma patient tissue samples from TCGA RNA-seq data validated that the 3'UTR of *IGF1R* was truncated in cancer patients but not in normal tissue. However, there were no differences in CFIm25 levels between lung cancer and normal tissue samples (Huang et al., 2018). The discrepancy between in vitro knockdown and tissue expression of CFIm25 suggests that regulation of 3'UTR-APA of *IGF1R* by CFIm25 in lung cancer *in vivo* may be more complex and involve more factors than CFIm25 alone.

4.1.6 CFIm25 and other cancers—Although the impact of CFIm25 on 3'UTR-APA was not studied in other cancers, loss of CFIm25 has been shown to affect tumorigenesis in several other tumor models which are briefly discussed below. A study in osteosarcoma found that there was a correlation between low levels of CFIm25 and high levels of mir-181a. The 3'UTR of CFIm25 was shown to be targeted by mir-181a resulting in the downregulation of CFIm25 protein expression and increased cell proliferation while inhibiting apoptosis (Zhu et al., 2016). Tissue microarray analysis of colorectal cancer patient tissues showed that low levels of CFIm25 were associated with poor prognosis (Cai et al., 2021). In contrast, gastric cancer patients whose tumors expressed higher levels of CFIm25 had reduced overall survival. Patients with metastatic tumors expressed higher levels of CFIm25 than those with non-metastatic gastric cancer. Ectopic expression of

CFIm25 in gastric cancer cell lines resulted in increased cell proliferation, increased tumor growth and invasion (Zhu et al., 2021). High levels of CFIm25 mRNA were detected by qRT-PCR in samples of patients with the hematological malignancy chronic myeloid leukemia (CML) compared to healthy control samples. Knockdown of CFIm25 in CML cell lines inhibited cell proliferation and induced apoptosis. In addition, CFIm25 depletion was shown to inhibit ERK/MAPK signaling and induce phosphatase and tensin homolog (*PTEN)* expression in the CML cell line, K562 (Zhang & Zhang, 2018). This further confirms that CFIm25 plays a major role in oncogenesis and metastasis in cancer.

4.2 The role of CFIm25 driven 3'UTR-APA in fibrosis

In addition to cancer, the impact of CFIm25 mediated 3'UTR-APA on other disease states is also under investigation.

4.2.1 CFIm25 and pulmonary fibrosis—A study showed that CFIm25 (with CFIm59 and CFIm68) was downregulated in the lungs of patients with idiopathic pulmonary fibrosis (IPF) and mice with pulmonary fibrosis. RNA-seq data analysis showed that depletion of CFIm25 in human lung fibroblast cells resulted in global 3'UTR shortening of 808 genes while 29 genes shifted to using the dPAS. Some of these genes were involved in the pro-fibrotic transforming growth factor beta (*TGF-β),* and hypoxia inducible factor 1 subunit alpha (*HIF1α*) signaling pathways. Validation of the RNA- Seq. data by qRT-PCR of CFIm25 depleted fibroblasts showed 3'UTR shortening of the pro-fibrotic genes, collagen type 1 alpha (*COL1A),* Wnt family member 5A (*WNT5A),* frizzled class receptor 2 (*FZD2)* and transforming growth factor beta receptor 1 (*TGFβR1).* These 3'UTR-APA changes resulted in increased protein expression. There was decreased proliferation of human fibroblast cells after CFIm25 knockdown. Overexpression of CFIm25 had the opposite effects on 3'UTR-APA. Hence CFIm25 depletion may play an important role in increased expression of pro-fibrotic genes and this may facilitate pulmonary fibrosis (T. Weng et al., 2019).

4.2.2 CFm25 and dermal fibrosis—In addition to IPF, CFIm25 has also been implicated in dermal fibrosis. Analysis of gene expression data from skin arm biopsies of patients with the multisystem fibrotic disease, systemic sclerosis/sclerodema, found that these patients expressed lower levels of CFIm25 than matched controls. Knockdown of CFIm25 in human dermal fibroblasts resulted in 3'UTR shortening of 971 genes and 3'UTR lengthening of 93 genes. Of the genes with truncated 3'UTRs, 97 of them were in the *TGFβ* signaling pathway. Subsequent qRT-PCR analysis validated 3'UTR shortening of *COL1A1, TGFβR1, COL11A1* and secreted protein acidic and rich in cysteine (*SPARC)* (Weng et al., 2019). *COL1A1* and TGFβR1 were previously shown to also undergo 3'UTR-APA in pulmonary fibrosis (Weng et al., 2019). Hence, 3'UTR shortening of genes involved in the *TGF-β* signaling pathway resulting from CFIm25 depletion is important for different types of fibrosis.

4.3 Role of CFIm25 driven 3'UTR-APA in neural dysfunction

Gennarino *et al* found copy number variations of the CFIm25 gene *NUDT21* in several individuals with neuropsychiatric syndromes. This was linked to higher levels of CFIm25

in lymphoblastic cells derived from these individuals resulting in reduced protein expression of methyl CpG-binding protein 2 (*MECP2*) (Gennarino et al., 2015). The *MECP2* 3'UTR is approximately 8.5kb long and it contains a dPAS and a pPAS. Usage of each PAS results in transcripts about 10kb and 1.9kb long respectively. The two transcripts have similar half-lives (Reichwald et al., 2000). In the presence of high levels of CFIm25, *MECP2* transcripts with longer 3'UTRs were made but these longer transcripts were inefficiently translated resulting in a slight decrease in protein levels. In this model, deregulation of CFIm25 is posited as a driver of intellectual disability and neuropsychiatric disease (Gennarino et al., 2015). Further work done to mimic CFIm25 loss in patients by *NUDT21* gene deletion in *Nudt21*$^{+/-}$ mice was associated with changes in alternative polyadenylation of 129 genes with a loss of function intolerance. These changes in 3'UTR-APA were accompanied by proteomic deregulation. Twenty-six of those genes are known to result in intellectual disability if mutated. Thus, their deregulation by 3'UTR-APA could potentially contribute to pathogenesis. The *Nudt21*$^{+/-}$ mice had learning defects and cortical hyperexcitability suggesting that decreased levels of CFIm25 in neurons results in intellectual disability (Alcott et al., 2020).

5. REGULATION OF CFIm25 LEVELS

A major unanswered question in the field is how CFIm25 is normally regulated and how it is dysregulated in different disease states.

5.1 CFIm25 levels in normal biology

Not much is known about changes in CFIm25 levels during normal biology. During spermatogenesis in mice, *Nudt21* mRNAs were significantly elevated in the later spermatid and meiotic developmental stages than in the earlier stages. However, high levels of *Nudt21* transcripts were not associated with an increase in protein levels of CFIm25 (Sartini et al., 2008). This suggests that there may be issues with translational efficiency of the *Nudt21* mRNAs, as has been reported for other transcripts involved in spermatogenesis (Persengiev et al., 1996; Sartini et al., 2008). However, the mechanism driving this is not fully understood.

5.2 Insights on CFIm25 expression from the brain

A study determined that a rare number of individuals contain copy number variations of *NUDT21*. Even though four subjects had *NUDT21* duplications and one had a deletion, both types of changes were associated with some form of intellectual impairment (Gennarino et al., 2015). A follow up study found that a neuronal *Nudt21*$^{-/-}$ homozygous knockout mouse model was embryonic lethal suggesting that complete CFIm25 loss in the brain is incompatible with life. Interestingly, heterozygous *Nudt21*$^{+/-}$mice had a 50% reduction in *Nudt21* mRNA with only a 30% reduction at the protein level. The authors concluded that this was due to partial post-transcriptional compensation that incrementally restored CFIm25 protein levels (Alcott et al., 2020). The implication from this is that a certain level of CFIm25 protein is needed to maintain cell viability. This would offer an alternative hypothesis in cancer that partial depletion of CFIm25 or overexpression in tumor cells may

result in increased tumorigenicity whereas complete depletion of CFIm25 in these same tumor cells is incompatible with survival.

5.3 The role of 3'UTR-APA and miRNAs in regulating CFIm25 levels

Interestingly, CFIm25 transcripts also undergo 3'UTR-APA, generating three transcripts of different sizes (Kubo et al., 2006). These transcripts also contain UGUA binding motifs and fluctuating CFIm25 levels during spermatogenesis suggest that CFIm25 might self-regulate 3'UTR-APA of its own transcripts by binding to its own pre-mRNAs (Sartini et al., 2008). In addition to CFIm25, other RNA binding proteins may also bind to CFIm25 transcripts and affect their stability as well as translational efficiency. Another form of post-transcriptional regulation of CFIm25 may include miRNAs binding to the differently sized 3'UTRs. One study identified miR-23, miR-24, miR-27, miR-135, miR -182 and miR-374 as miRNAs that can bind the *NUDT21* full length 3'UTR and thus potentially regulate CFIm25 expression (Tamaddon et al., 2020). As with other genes, truncation of the 3'UTR would eliminate some of these miRNA binding sites, as well as RNA protein binding sites.

5.4 Post-translational regulation of CFIm25

CFIm25 can also potentially undergo post-translational modification. CFIm25 was one of several non-histone proteins that was acetylated by CREB binding protein (CBP). In direct binding assays, CBP alone was unable to acetylate CFIm25. The acetylation of CFIm25 was dependent on CFIm68 recruiting CBP to the CFIm25/CFIm68 complex. CFIm25 acetylation reduced its interaction with polyA polymerase suggesting that CFIm25 acetylation and deacetylation may affect 3'end formation (Shimazu et al., 2007). As identified by mass-spectrometry from the PhosphoSitePlus® database, in addition to having several lysine acetylation sites, CFIm25 also contains several potential phosphorylation sites (Hornbeck et al., 2015). The impact of phosphorylation and dephosphorylation of the different serine, tyrosine and threonine residues on regulating CFIm25 activity still remains undetermined. More work needs to be done to determine the effects of post-translational modifications on CFIm25 function.

6. CONCLUSION-CFIm25 MEDIATED 3'UTR-APA AND IMPLICATIONS FOR THE FUTURE

Work done so far shows that 3'UTR-APA is pervasive in both human biology and in human disease. With the increasing impact of 3'UTR-APA in regulating gene expression, determining what drives changes in 3'UTR-APA is crucial. CFIm25 is so far one of the most studied core 3'end processing factor that has also been shown to extensively regulate 3'UTR-APA resulting in a wide range of effects on cell biology. In addition, CFIm25 also regulates splicing of pre-mRNAs as well as cyclization of circRNAs. At a mechanistic level, a minimum of two CFIm25 subunits binds to two separate UGUA sequence elements on RNA-precursors and in turn recruit CFIm59/CFIm68 to give a heterotetrameric complex. While this may suffice for 3'end formation, different levels of CFIm25 and the spatial location of the bound UGUA motifs together with other factors regulate alternative splicing and 3'UTR-APA. This may involve looping of the precursor RNAs optimizing selection of

one splicing or polyadenylation signal over another. Further mechanistic studies need to be done to determine the intricacies of these CFIm25 mediated RNA processing events.

There are few documented changes in CFIm25 levels in normal human biology. Tissues such as the brain provide some insight into the impact of CFIm25 regulated 3-UTR-APA in human disease. Slight changes in the levels of some proteins are associated with neurological disease, thus expression levels need to be tightly regulated (Chao & Zoghbi, 2012). Hence in this case, CFIm25 driven 3'UTR-APA may provide a mechanism to fine-tune protein levels in the brain and other tissues. This could be through slight alterations of CFIm25 expression levels. More drastic changes in CFIm25 levels may result in disease. So far, decreased levels of CFIm25 has been mainly associated with increased tumorigenicity in cancer, increased skin and lung fibrosis, as well as neurological dysfunction. The role of transcriptional and post-transcriptional regulation on CFIm25 expression still remains unresolved. Post-translational modifications may affect CFIm25 activity in 3'end processing and 3'UTR-APA regulation.

Most studies have focused on CFIm25 knockdown or overexpression to determine the impact of CFIm25 on 3'UTR-APA as well as different cellular processes. Changes in CFIm25 levels regulate 3'UTR-APA of genes involved in human biology and human disease. Hence, different levels of CFIm25 in different biological states may act as a potential biomarkers of these states. In many cancers, CFIm25 depletion and its accompanying 3'UTR-APA changes suggest that CFIm25 has tumor suppressive effects. However, in some cancers, CFIm25 can also act as an oncogene. These differences may be due to differences in tumor type, tumor source, tumor stage, cell type, cell state as well as levels of CFIm25 knockdown and/or post-translational modifications. Since CFIm25 is an enhancer-dependent activator of 3'end formation, there may be a threshold level at which CFIm25 knockdown may interfere with normal 3'end processing of certain transcripts needed for survival in some cells. While the emerging role of CFIm25 mediated 3'UTR-APA changes during fibrosis and neural disease give credence in modulation of CFIm25 for targeted therapies, the effects of modulating levels of CFIm25 for cancer therapy will need to be done with care. Future studies are needed to determine how levels of CFIm25 and CFIm25 mediated activities are regulated *in vivo* in different biological and disease states.

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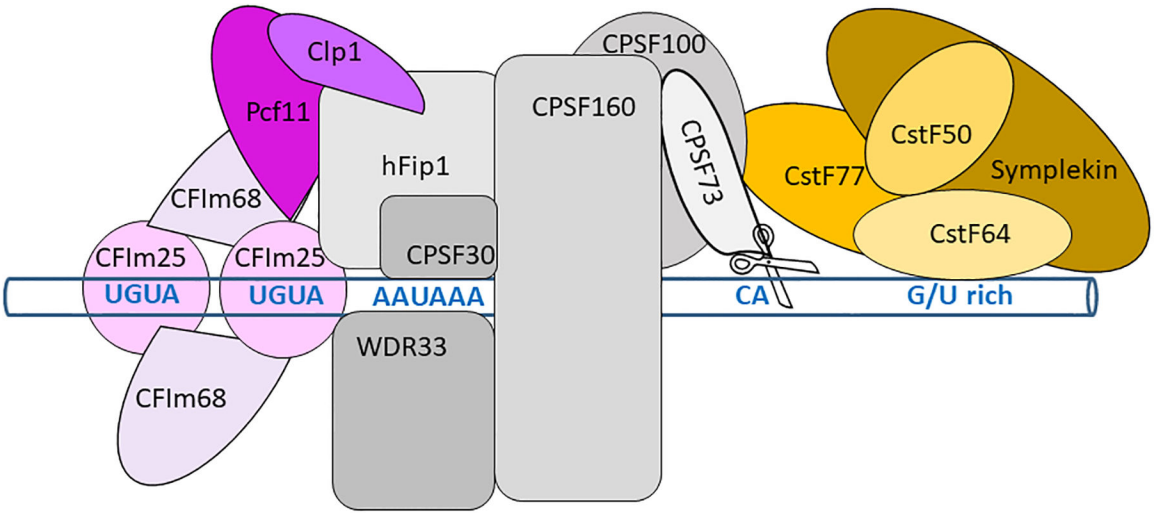

FIGURE 1.

Cis elements and core proteins involved in 3'end formation. The *cis* elements found in pre-mRNAs include at a minimum, two UGUA sequences that are each bound by a CFIm25 subunit and the polyadenylation signal AAUAAA which interacts with CPSF30 and WDR33. CstF64 recognizes and binds the GU/G/U rich element. Cleavage is done by the CPSF73 endoribonuclease with optimal cleavage occurring after the CA dinucleotide. Also shown are other members of the 3'end processing complex, CPSF160, CPSF100, hFip1, Pcf11, Clp1, CFIm68, CstF77, CstF50 and symplekin.

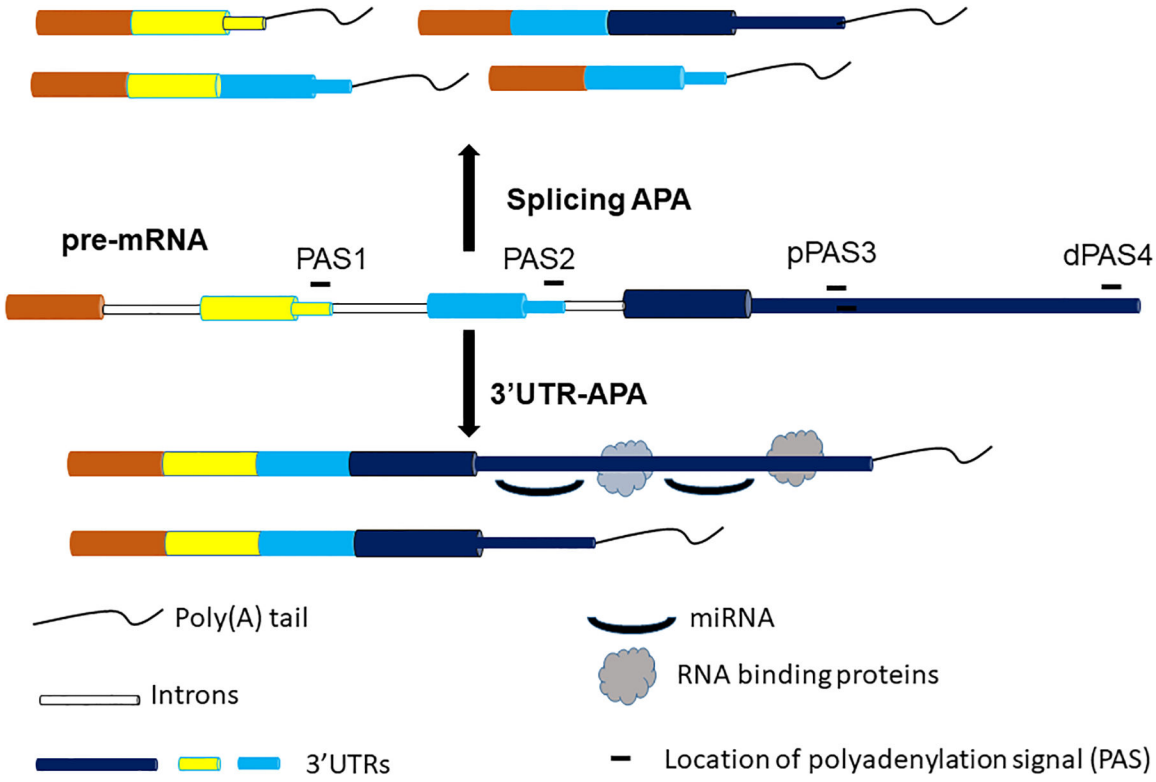

FIGURE 2.

Different types of alternative polyadenylation (APA). Splicing APA. Splicing APA also known as alternative terminal exon APA occurs when APA occurs concurrently with splicing. In it's simplest form, splicing APA involves splicing to different terminal exons. Transcripts with different protein coding regions and different 3'UTRs are generated using different polyadenylation signals (PASs), shown here as PAS1, PAS2 and pPAS3, located on different exons. 3'UTR-APA. In 3'UTR-APA (also known as Tandem/Terminal exon APA) occurs when different PASs located within the same terminal exon are used. The simplest example of 3'UTR-APA is when there is a PAS proximal to the stop codon (pPAS3) and one that is more distal (dPAS4). Usage of either the dPAS or pPAS within the same terminal exon generates transcripts with the same protein coding region but with 3'UTRs of different lengths. The longer 3'UTR contain more binding sites for miRNAs and RNA binding proteins which may be eliminated in the smaller 3'UTR.

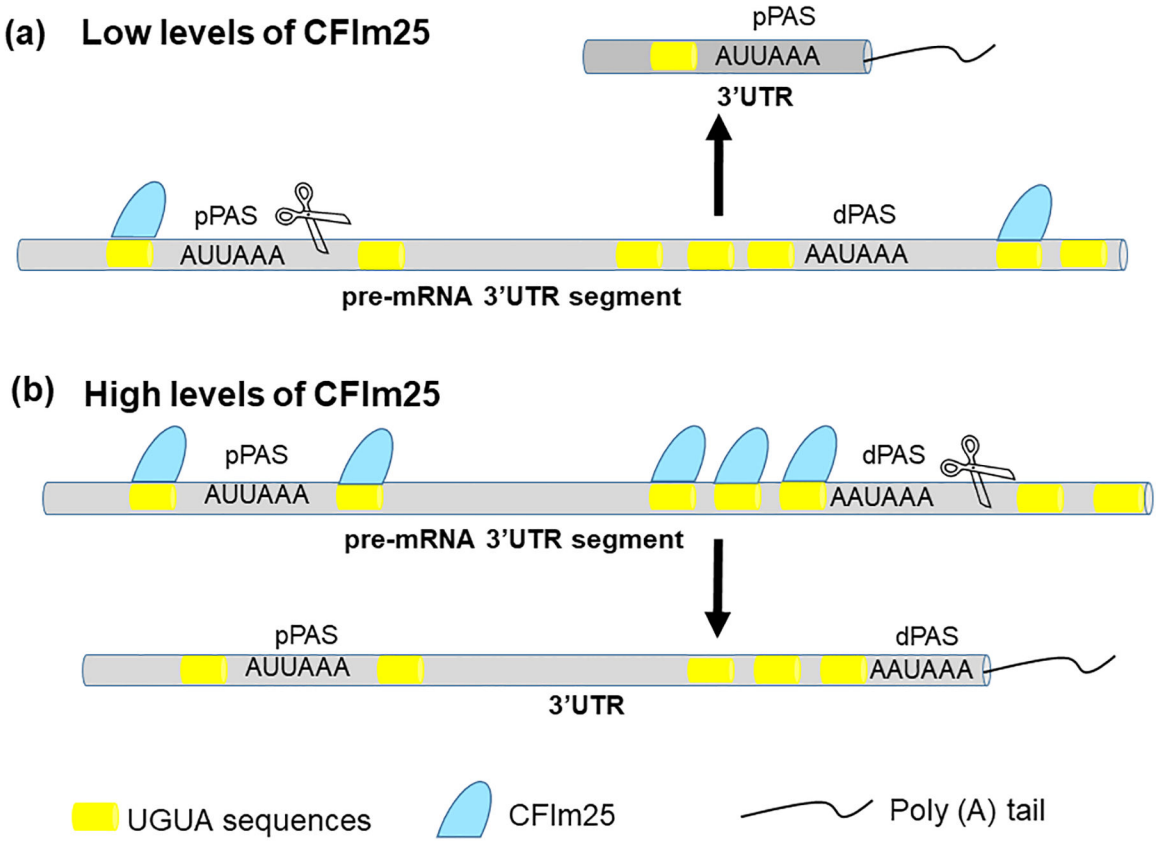

FIGURE 3.

Model showing effects of different levels of CFIm25 on 3'UTR-APA. A. Low levels of CFIm25 favor usage of the proximal polyadenylation signal (pPAS) generating transcripts with short 3'UTRs. B. High levels of CFIm25 favor usage of the distal polyadenylation signal (dPAS) resulting in transcripts with long 3'UTRs.

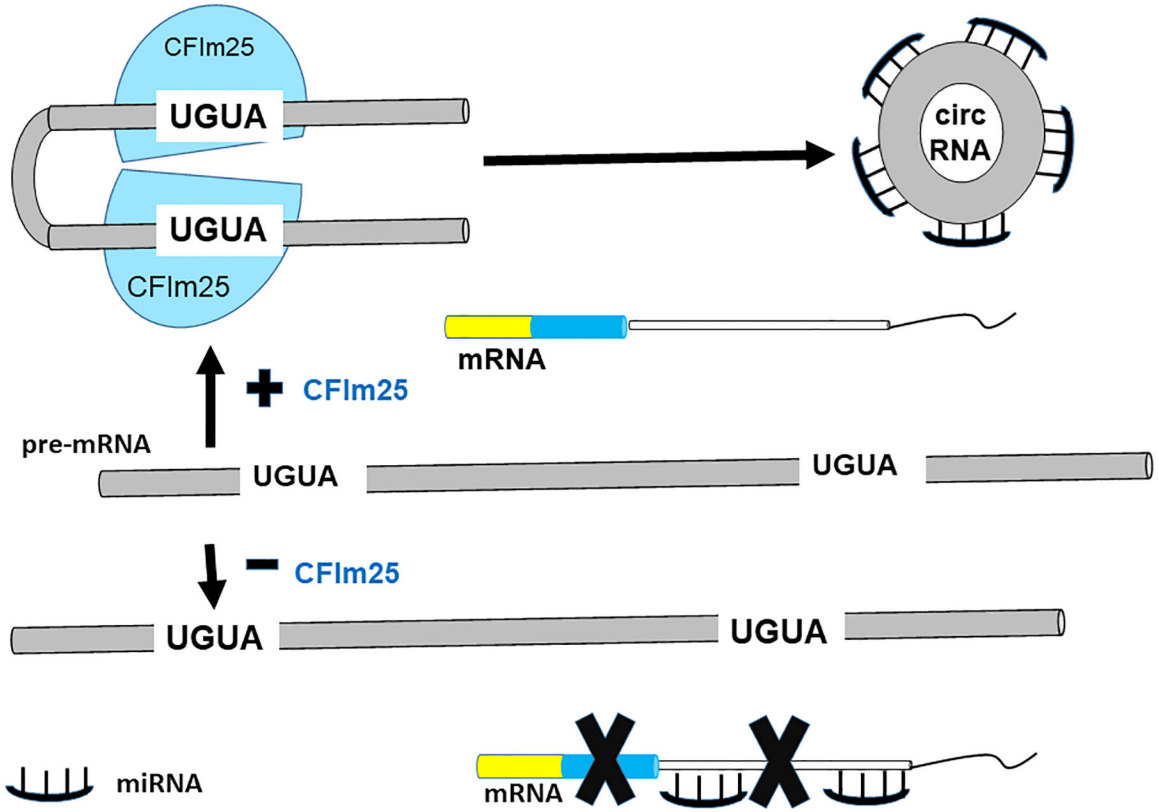

FIGURE 4.

Schematic diagram showing effects of CFIm25 on circRNAs and mRNAs. High levels of CFIm25 facilitate the cyclization of UGUA containing precursor RNAs resulting in circRNA biogenesis. These circRNAs act as molecular sponges for miRNAs redirecting miRNAs from target mRNAs. In the absence of CFIm25, there is reduced circRNAs formation and the miRNAs target mRNAs and reduce translation of target transcripts.