Upstream Open Reading Frames (uORFs) in the Apicomplexan Parasites

Plasmodium falciparum and Toxoplasma gondii: small yet powerful regulators of translation

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ABSTRACT

During their complex life cycles, the Apicomplexan parasites, Plasmodium falciparum and Toxoplasma gondii employ several genetic switches to regulate their gene expression. One such switch is mediated at the level of translation through upstream Open Reading Frames (uORFs). As uORFs are found in the upstream regions of a majority of genes in both the parasites, it is essential that their roles in translational regulation be appreciated to a greater extent. This review provides a comprehensive summary of studies that show uORF-mediated gene regulation in these parasites and highlights examples of clinically and physiologically relevant proteins that exhibit uORF-mediated regulation. In addition to these examples, several studies that use bioinformatics, transcriptomics, proteomics, and ribosome profiling also indicate the possibility of widespread translational regulation by uORFs. Further analysis of genome-wide datasets will reveal novel genes involved in key biological pathways such as cell-cycle progression, stress-response, and pathogenicity. The cumulative evidence from studies presented in this review suggests that uORFs will play crucial roles in regulating gene expression during clinical disease caused by these important human pathogens.

Key words: P. falciparum, T. gondii, upstream ORFs, translational regulation
INTRODUCTION

Eukaryotic translation initiation is a tightly regulated, multi-step process that involves scanning of messenger RNA (mRNA) by the preinitiation complex (Kozak, 1980). This complex, comprising of the small ribosomal subunit and numerous initiation factors, scans the mRNA for an authentic start codon (AUG) present in the coding sequence (CDS) (Kozak, 1991). The selection of the start codon is governed by the sequence surrounding the AUG codon i.e. the Kozak sequence, availability of initiation factors, molecules that provide energy, and methionyl-tRNAs (reviewed in Hinnebusch, 2011).

Other than these factors, the presence of start codon(s) that lie upstream of the start codon of the main CDS confers another layer of regulation. This is due to the scanning model of translation initiation where the ribosomes recognize the 5ʹ cap and move along the mRNA towards the 3ʹ end. During this process, the ribosomes encounter upstream start codons (uAUGs) before the main CDS and therefore, these uAUGs are capable of engaging the ribosome (Kozak, 2002). Similar to uAUGs, upstream open reading frames (uORFs), defined as an upstream start codon followed by an in-frame stop codon, also engage the scanning ribosome with varying capacities, which in turn alters the level of the protein encoded by the main CDS (reviewed in Morris and Geballe, 2000). The presence of these alternative initiation sites constitutes a “hurdle” for the ribosome and usually results in repression of translation of the main CDS. This repression can be relieved by the cellular translation machinery with a multitude of strategies, as and when required (Wang and Rothnagel, 2004; Iacono et al., 2005). Hence, uAUGs and uORFs act as regulatory elements in the 5ʹ leader sequences of eukaryotic mRNAs. Interestingly, as translation regulation allows the organism to respond more rapidly than transcriptional regulation, uORFs (rather than uAUGs) are used by cells to handle a wide range of environmental changes, affecting the survivability of the cell.

The earliest known evidence for uORF involvement in translational control was shown for
*Saccharomyces cerevisiae* General Control Non-depressible 4 (GCN4), a transcription factor that controls amino acid biosynthesis under conditions of starvation (reviewed in Hinnebusch, 1988). After these early reports, translation regulation by uORFs during stress conditions was shown in numerous organisms including *Homo sapiens, Mus musculus, Drosophila melanogaster, Neurospora crassa, Danio rerio, Arabidopsis thaliana, Zea mays*, and higher plants (Iacono *et al.*, 2005; Barbosa *et al.*, 2013; Chew *et al.*, 2013; von Arnim *et al.*, 2014; Lei *et al.*, 2015; Young and Wek, 2016; Zhang *et al.*, 2018, 2019; Silva *et al.*, 2019; Wu *et al.*, 2019; Chen and Tarn, 2019).

**Indicators of uORF-mediated gene regulation**

Regulation of translation is one mode of post-transcriptional gene regulation (PTGR). The presence of PTGR in cells can be inferred by several key cellular features. One such feature is a lack of correlation between peak mRNA levels and protein abundance for a given gene and/or temporal delay between the transcription and the translation of the gene (de Sousa Abreu *et al.*, 2009; Maier *et al.*, 2009; Liu *et al.*, 2016). These can be explained by multiple factors such as stability of mRNA, secondary structure of the transcript, post-translational modifications and cis-regulatory elements including uORFs (Araujo *et al.*, 2012; Carpenter *et al.*, 2014). Therefore, while studying regulation of a gene by uORFs, a delay between the transcription and translation of the gene is often used as a preliminary indicator of translational control (Figure 1).

Other indirect indicators that point towards uORF-mediated gene regulation are the proteins/factors that the translation machinery uses to overcome the challenge posed by the uORF. To cope with uORF-mediated translation repression, and allow protein synthesis to occur from the CDS, a cell adopts unconventional mechanisms of translation: reinitiation, leaky scanning, ribosome shunting, and the use of internal ribosome entry sites (IRES) (Reviewed in...
Morris and Geballe, 2000; Skabkin et al., 2013; Silva et al., 2019). In brief, reinitiation is a phenomenon where the ribosome, rather than dissociating after termination at a uORF, successfully re-initiates translation at the start codon of the main CDS. Leaky scanning occurs when the ribosome scans past and skips the start codon of the uORF, consequently initiating translation at the start codon of the main CDS (reviewed in Silva et al., 2019). Even though other unconventional translation mechanisms, such as ribosome shunting and IRES have been observed in eukaryotes, they are more prevalent in viruses (Yang and Wang, 2019) and hence, the discussion here will be limited to reinitiation and leaky scanning. Factors that promote reinitiation and leaky scanning are indicators of a dampening of global translation and up-regulation of certain genes. For some genes that are controlled by reinitiation/leaky scanning, there is an involvement of uORFs in regulating gene expression, particularly during a cellular stress response. Therefore, an indirect indicator of uORF-mediated translational control can be the up-regulation and/or modification of factors that regulate either reinitiation by employing reinitiation factors, or leaky scanning by phosphorylating the eukaryotic initiation factor 2 alpha (eIF2α) (Figure 1).

The phosphorylation status of eIF2α was demonstrated for the first time in the case of the GCN4 transcript, in which the translation of the CDS is regulated by uORFs. The choice of translating the CDS rather than the uORFs is driven by phosphorylation of eIF2α (reviewed in Hinnebusch, 2005). Similarly, during the integrated stress response (ISR), phosphorylated SceIF2α promotes translation of genes required for handling the stressor via reinitiation (Dever et al., 1992; Lu et al., 2004). Similar mechanisms that involve relieving uORF-mediated repression by phosphorylated eIF2α have been discovered for numerous genes (Vattem and Wek, 2004; Dang Do et al., 2009; Zhao et al., 2010; Palam et al., 2011; Baird et al., 2014; Zach et al., 2014; Aktas et al., 2015; Cnop et al., 2017; Guan et al., 2017; Asano, 2021). The phosphorylation of eIF2α is carried out by members of the eIF2α kinase family (Pakos-
Zebrucka et al., 2016; Wek, 2018; Costa-Mattioli and Walter, 2020) and leads to global inhibition of protein synthesis and up-regulation of genes involved in mediating the adaptive response. These studies indicate that the phosphorylation status of eIF2α is a global indicator for translational regulation of large numbers of genes, some of which could be controlled by uORFs.

A more definitive role for uORFs in translational regulation is provided by the presence of ribosomal footprints on the 5' leader of the transcripts undergoing PTGR (Schneider-Poetsch et al., 2010; Garreau de Loubresse et al., 2014). This provides a snapshot of the dynamics of translation on each transcript by determining the positions of the ribosomes engaged in elongating an ORF (Brar et al., 2012; Ingolia et al., 2014). Such studies in yeast and humans revealed that uORFs are the major contributors of ribosome occupancy in the 5' leaders of transcripts (Calvo et al., 2009; Brar et al., 2012; Ingolia et al., 2014; Johnstone et al., 2016), suggesting that the presence of ribosome footprints in the 5' leader of the transcript is a distinctive feature that indicates PTGR via uORFs. Ribosome footprints along the entire length of certain genes show that when the upstream regions are loaded with ribosomes, the CDS has lower ribosome occupancy (Ingolia et al., 2014). These data reinforce the notion that the presence of uORFs stalls the ribosome before it can reach the main CDS, resulting in repression of CDS translation. The conclusive proof of a uORF regulating the translation of a particular gene is provided when mutation of the start codon of the uORF results in a loss of repression/regulation of the gene (Harigai et al., 1996; Reynolds et al., 1996; Ruan et al., 1996; Schlüter et al., 2000; Sarrazin et al., 2000; Diba et al., 2001; Kwon et al., 2001; Jousse et al., 2001; Warnakulasuriyarachchi et al., 2003; Zhang and Dietrich, 2005; Lee et al., 2007; Song et al., 2007; Calvo et al., 2009; Devlin et al., 2010; Spevak et al., 2010; Qiao et al., 2011; Armata et al., 2013; Tennen et al., 2013; Bancells and Deitsch, 2013; Wu et al., 2014; Capell et al., 2014; Kumar et al., 2015; Guerrero-González et al., 2016). The direct and indirect
evidences that lead to the involvement of uORFs in mediating gene expression regulation have been shown in Figure 1.

Translational regulation mediated via uORFs in apicomplexan parasites, Plasmodium falciparum and Toxoplasma gondii

Apicomplexans belong to a large phylum of parasitic alveolates and due to their complex life cycles involving multiple hosts including humans, some members of the phylum cause widespread occurrence of diseases. For example, malaria and toxoplasmosis are caused by *P. falciparum* and *T. gondii* respectively (Jacobs, 1963; Sabin and Olitsky, 1937). These parasites exhibit many developmental stages in different hosts and so must regulate expression of their genes in a highly coordinated fashion for survival and transmission in order to complete their life cycles. Gene expression is regulated at multiple levels, including transcription and translation (White et al., 2014; Vembar et al., 2014, 2015, 2016; Holmes et al., 2017; Bennink and Pradel, 2019; Hollin and Le Roch, 2020; Sharma et al., 2020).

There is evidence for uORFs playing substantive roles in translational control in apicomplexan parasites; this evidence includes high frequencies and a widespread distribution of uORFs among large numbers of genes (Bunink et al., 2013; Caro et al., 2014; Kumar et al., 2015; Srinivas et al., 2016; Hassan et al., 2017; Holmes et al., 2019; Markus et al., 2021). Additionally, ribosome profiling studies in *P. falciparum* and *T. gondii* parasites reveal footprints in the 5' leader sequences of transcripts (Lacsina et al., 2011; Bunnik et al., 2013; Caro et al., 2014; Hassan et al., 2017; Holmes et al., 2019). Recent discoveries of clinically important genes, such as *var2csa* in *P. falciparum* (Chan et al., 2017) and *BFD1* in *T. gondii* (Waldman et al., 2020) that are regulated translationally by uORFs further reinforce the impact of these small, yet important features in translational regulation of gene expression. In the next sections, the major findings of these and other reports will be summarized and the need to
further understand the phenomenon of uORF-mediated PTGR in apicomplexan parasites will be highlighted in detail.

**UPSTREAM ORFS IN PLASMODIUM FALCIPARUM**

*A long uORF regulates translation of the var2csa gene*

The first example of uORF-mediated translational regulation was shown for a gene implicated in pregnancy-associated malaria (PAM), also termed malaria in pregnancy (MiP): *var2csa* (Lavstsen *et al.*, 2003; Amulic *et al.*, 2009; Bancells and Deitsch, 2013). This gene is a variant of the *var* gene family in *P. falciparum* that consists of ~60 *var* genes encoding Erythrocyte Membrane Protein 1 (PfEMP1). These proteins help the parasite evade clearance by the spleen of the host by binding to the endothelial lining of blood vessels (Kraemer and Smith, 2006). The *var* gene family has also been implicated in cerebral malaria, one of the major symptoms of severe malaria caused by *P. falciparum* that results due to sequestration of infected RBCs to capillaries in the brain (reviewed in van der Heyde *et al.*, 2006). This sequestration is due to binding of PfEMP1 proteins to receptors such as CD36, thrombospondin, and intercellular adhesion molecule 1 (ICAM1) found on the surface of different cell types (Baruch *et al.*, 1996; Smith *et al.*, 2000, 2013; Rowe *et al.*, 2009).

The transcription profile of members of this gene family is unusual, with only one of the *var* genes expressed at a given time (Scherf *et al.*, 1998) and a switch of gene expression between different *var* genes occurring at the rate of 0.03-2% (Gatton *et al.*, 2003). The mechanisms that regulate the switch are complex and regulation occurs at various levels including sub-nuclear organisation, epigenetic regulation, *cis*-acting DNA elements, transcriptional regulation as well as translational repression (reviewed in Deitsch and Dzikowski, 2017). Due to the importance of the *var* gene family in multiple clinical manifestations of severe malaria caused by *P. falciparum*, understanding the expression of the
members of this gene family has been of intense interest.

One variant of this gene family that has attracted clinical attention is \textit{var2csa}. Parasites expressing \textit{var2csa} adhere to chondroitin sulphate A (CSA) found in the placenta of pregnant women and block the supply of oxygen and nutrition to the foetus, thereby resulting in pregnancy-associated malaria (PAM) (Salanti \textit{et al.}, 2003, 2004). While mRNA from the \textit{var2csa} gene can be detected in asexual stages, the translation of the VAR2CSA protein is observed only in parasites that are selected for adherence to CSA in lab cultures (Mok \textit{et al.}, 2008; Chan \textit{et al.}, 2017). This lack of correlation between transcription and translation is a clear indicator of PTGR (Figure 2). Interestingly, this PTGR of \textit{var2csa} is regulated by a 360 nucleotide-long uORF in the 5' leader of the transcript (Dzikowski \textit{et al.}, 2007; Amulic \textit{et al.}, 2009; Bancells and Deitsch, 2013).

As expected, the 360 nucleotide-long uORF causes translational repression of the \textit{var2csa} gene, hence, it was of interest to understand how this repression is relieved to express the VAR2CSA protein when required. In cultured parasites, detailed molecular analysis showed that this switch depends on reinitiation of the \textit{var2csa} gene, after the uORF is translated (Bancells and Deitsch, 2013). In parasites derived from placental samples, this reinitiation was shown to occur due the presence of \textit{Plasmodium} Translation Enhancing Factor (PTEF). PTEF is highly up-regulated in parasites sequestered in the placenta and allows efficient reinitiation of translation at the \textit{var2csa} CDS when over-expressed in cultured parasites (Chan \textit{et al.}, 2017).

The molecular factors that lead to the expression of PTEF in a CSA-rich environment are still unknown and further studies need to be undertaken to understand the structure and the interacting partners of PTEF in the asexual stages to provide further clarity regarding its role. These detailed studies are particularly important, as recent reports have shown that the serum of non-pregnant individuals (men and children) contain antibodies recognizing the VAR2CSA protein (Reviewed in Gnidehou and Yanow, 2021). While the authors discuss technical issues
such as cross-reactivity to other proteins that cannot be ruled out, they also mention that deregulation of the uORF-mediated repression of the \textit{var2csa} gene might play a role in these clinical findings.

\textit{High prevalence of uORFs in the P. falciparum genome leads to repression of translation}

Reports establishing translational regulation of the \textit{var2csa} gene led to an interest in understanding whether this phenomenon was observed in other genes as well. Interestingly, subsequent studies showed that regulation by uORFs could be more prevalent in \textit{P. falciparum} than previously anticipated. For example, the \textit{P. falciparum} transcriptome displays widespread occurrence of uORFs (Caro \textit{et al}., 2014), with 99\% of the transcripts containing at least one uORF in their 5' leader (Kumar \textit{et al}., 2015). This number is extremely high when compared to human transcripts where only 49\% of the transcripts contain at least one uORF in their leader (Calvo \textit{et al}., 2009). With a prediction of an average of 11 uORFs per CDS (Kaur \textit{et al}., 2020), \textit{P. falciparum} exhibits the highest ever recorded number of uORFs in a transcript. These high frequencies of uORFs are a reflection of the distinctive genome of \textit{P. falciparum} whose composition is skewed heavily towards adenine (A) and thymine (T) nucleotides (Gardner \textit{et al}., 2002). This leads to a high probability of finding AT-rich start and stop codons in the 5' leader sequence, thereby giving rise to uAUGs and uORFs. The presence of multiple uAUGs and uORFs causes translational repression of the main CDS, posing a significant challenge to the parasite’s cytoplasmic translation machinery (Kumar \textit{et al}., 2015).

Each uORF has a very different ability to engage the scanning ribosome and experimental approaches have been used to study the features that contribute to the repressive capacity of uORFs. Such features are the Kozak sequence, codon composition, length of the uORF, and the distance between the uORF and the CDS (Kaur \textit{et al}., 2020). Further, bioinformatics was used to predict translatability of an ORF (therefore, the repressiveness of the uORF) by
calculating the probability of translation initiation and elongation of ORFs in the \( P. falciparum \) genome (Srinivas et al., 2016). The model utilizes positional features comprising of the Kozak sequence and compositional features comprising of the codon topography of the ORF to predict translation initiation and elongation probabilities of the ORF respectively.

The notion of translation repression by certain uORFs is reinforced by the presence of ribosome footprints on the 5' leaders of transcripts expressed in the intra-erythrocytic asexual stages of \( P. falciparum \), supporting the hypothesis that these huge numbers of uORFs are able to engage ribosomes. Ribosome profiling studies found rampant occurrence of ribosome density associated with 5' leaders of transcripts compared to the 3' UTRs (Bunnik et al., 2013; Caro et al., 2014). The data suggest active translation of uORFs present in the 5' leader sequence, with some instances where transcripts showed a higher abundance of ribosome footprints on the 5' leader than on the CDS, leading to low translational efficiency of the CDS (Caro et al., 2014). As the repressive capability of different uORFs has now been assessed by experimental/bioinformatics analysis and ribosome profiling, a holistic analysis of all these genome-wide datasets would shed light on specific uORFs regulating expression of classes of genes. Based on the evidence that uORF-mediated regulation is a strategy employed to handle stress responses, such classes of genes might be physiologically relevant in host-pathogen interactions and establishing the pathogenicity of this parasite.

Another indicator of translational control of gene expression, a delay between the peak of transcript abundance and translation of those transcripts, has also been observed in \( P. falciparum \). Early reports of a cascade of gene expression in synchrony with the asexual life cycle stages (Bozdech et al., 2003; Foth et al., 2011) suggested that transcription occurs only when the protein is required. Subsequent studies showed an absence of correlation between the peaks of transcripts and their encoded protein products for \(~30\%\) of the genes (Le Roch et al., 2004; Bunnik et al., 2013), indicative of PTGR for these genes. Interestingly, the var gene
family that is under multiple forms of regulation, has ~5 times more uAUGs and uORFs than other genes (Kumar et al., 2015).

Translation repression of the downstream gene in the presence of uORFs can be alleviated by unconventional translation mechanisms: reinitiation and/or other mechanisms including leaky scanning. The role of reinitiation in translation of VAR2CSA during PAM has been discussed in the previous section. However, the use of non-canonical translation mechanisms to circumvent translation repression caused by the uORFs is not limited to this gene. Indeed, it was demonstrated that reinitiation occurs in the case of the hsp70 gene in the presence of a native uORF and synthetic uORFs suggesting that there is widespread occurrence of reinitiation in the asexual stages of *P. falciparum* (Kaur et al., 2020).

More and more evidence points towards uORFs playing roles in translational regulation during the asexual stages of the intra-erythrocytic developmental cycle (IDC). As there may be parasite-specific proteins, such as PTEF, that are required for handling huge numbers of uORFs, a better understanding of PTGR by uORFs may lead to new targets of development of therapeutic interventions for the asexual stages of *P. falciparum* that are the major cause of the clinical symptoms of malaria.

*Upstream ORFs in stress conditions*

The role of uORFs in the stress response in yeast and mammals is well studied (Hinnebusch, 2005; Silva et al., 2019; Houston et al., 2020). However, this area of research requires more focus in *P. falciparum*, more so because of the widespread occurrence of uORFs. During its complicated life cycle, *P. falciparum* faces a variety of external conditions that are hostile to the parasite. As is the case with other parasites, *P. falciparum* has also evolved to use complex strategies to adapt to the changing environment (Camus et al., 1995). While the shift of host from mosquito to human is one of the major challenges faced by the parasite due to drastic
differences in the two hosts’ biology, understanding how the parasite responds to various stress conditions that it faces in the human erythrocytes holds importance from the clinical perspective of malaria treatment.

During the IDC, *P. falciparum* experiences a periodic rise in temperature every 48 hours due to the host inflammatory response (Brown, 1912). The temperature during these febrile episodes can elevate to 40–41°C (Kwiatkowski, 1989). The adaptive response to the cyclical heat stress experienced by intra-erythrocytic parasites has been studied at the level of the transcriptome (Oakley *et al*., 2007; Rawat *et al*., 2019). However, as translational responses afford a rapid adaptation mechanism, it would be informative to study whether uORFs play a role in heat stress by checking the phosphorylation status of PfeIF2α and differential ribosome occupancy during this stress condition.

Another stress faced by *P. falciparum* during its intra-erythrocytic cycle is the lack of essential amino acids, especially isoleucine. This stress arises from the fact that inside the red blood cell, the parasite salvages amino acids by degrading haemoglobin (Francis *et al*., 1997). However, of the twenty amino acids, isoleucine is completely absent in the α and β chains of haemoglobin (Sherman, 1977). Therefore, the parasite depends on an exogenous supply of isoleucine through the plasma of the host (Liu *et al*., 2006). Since isoleucine is an essential amino acid, the human host also depends on external sources of isoleucine to survive (Soeters *et al*., 2004) and in situations of malnourishment, isoleucine pools in the human host can drop significantly (Baertl *et al*., 1974).

Lack of an exogenous supply of isoleucine can lead to a delayed-growth phenotype, where the parasites enter a hibernatory state as a response (Babbitt *et al*., 2012). This response has been linked to phosphorylation of PfeIF2α via PfeIK1, an orthologue of GCN2 that is responsible for phosphorylation of eIF2α under nutrient starvation conditions in yeast (Hinnebusch, 2005; Fennell *et al*., 2009; Babbitt *et al*., 2012). A possible role of uORFs in
translation of the genes required for adaptive response to this nutritional stress faced by \textit{P. falciparum} can be illustrated by identifying genes having differential ribosome occupancy in parasites that are deprived of isoleucine. Further, ribosome profiling of PfeIK1 knock-out parasites would also reveal classes of genes that are under regulation by uORFs.

There is preliminary evidence to support the notion that translational regulation mediated by uORFs occurs during isoleucine starvation stress. The Maf1 protein is a part of the Target of Rapamycin Complex 1 (TORC1) pathway that responds to stress caused by nutrient deprivation in \textit{S. cerevisiae} and mammals (Loewith and Hall, 2011). Maf1 represses transcription of highly abundant tRNAs and ribosomal RNAs through its function as a regulator of RNA polymerase III (Upadhya \textit{et al.}, 2002; Boguta, 2013; Moir and Willis, 2015). In nutrient-rich conditions, Maf1 remains inactive due to phosphorylation (Pluta \textit{et al.}, 2001; Shor \textit{et al.}, 2010), while under starvation conditions, it is de-phosphorylated and the activated protein binds and inhibits RNA polymerase III (Vannini \textit{et al.}, 2010). Although the majority of proteins involved in the TORC1 pathway have been lost in the \textit{Plasmodium} genus during genome reduction, an orthologue of Maf1 has been identified in \textit{P. falciparum} that is essential for its recovery from the hibernatory state induced due to isoleucine starvation (Serfontein \textit{et al.}, 2010; McLean and Jacobs-Lorena, 2017). Ribosome profiling data show that Maf1 CDS is poorly translated despite being transcribed in all stages of IDC (Caro \textit{et al.}, 2014). However, a significant presence of ribosome footprints on the 5' leader sequence of the transcript points towards a possible uORF-mediated regulation that needs to be further investigated (Figure 2).

Another physiologically important stressor is treatment with anti-malarial drugs as they constitute a source of oxidative damage to the parasite. For example, chloroquine and artemisinin induce free radical production (Pandey \textit{et al.}, 2001; Haynes and Krishna, 2004; Zhang \textit{et al.}, 2010). Interestingly, parasites treated with dihydroartemisinin (DHA), a derivative of artemisinin show enhanced phosphorylation of eIF2\textalpha, a key regulator of stress
adaptation (Zhang et al., 2017), suggesting a possible role of PTGR in overcoming the drug-induced stress. Increased cases of resistance to anti-malarial drugs suggest that parasites have evolved to enhance their adaptive response to drug-induced stress, thus decreasing drug susceptibility (Rocamora et al., 2018). This has been shown in the case of artemisinin, where increased levels of phosphorylated PfIF2α induces latency in parasites, thereby causing them to re-emerge later when the drug pressure has subsided (Zhang et al., 2017). These studies could be extended by identifying genes that have repressive uORFs due to enhanced ribosome occupancy.

Clearly, there are gaps in our understanding of the adaptation responses mounted by *P. falciparum* during these stress conditions. Filling in these gaps by studying the role of uORFs in stress responses would be necessary to gain deeper insights into parasite biology, especially in conditions of clinical relevance.

**UPSTREAM ORFS IN TOXOPLASMA GONDII**

*Ribosome profiling in T. gondii points towards widespread translational regulation by uORFs*

Similar to *P. falciparum*, the transcripts of *T. gondii* also have widespread occurrence of uORFs. At least one uORF has been predicted in 90% of transcripts with annotated 5’ leader sequences (Markus et al., 2021). This number is 1.8 times higher than the reported number in human transcripts where only 49% of the transcripts contain at least one uORF in their leader sequence (Calvo et al., 2009). Evidence of translation occurring in the 5’ leaders of transcripts in *T. gondii* has been provided in two recent ribosome profiling studies that demonstrate a high prevalence of ribosome footprints on the long 5’ leaders of transcripts (Hassan et al., 2017; Holmes et al., 2019). Ribosomal occupancy on uORFs is indicative of the fact that ribosomes are engaged in translating uORFs rather than the CDS, thereby exerting translational control.
over the expression of the gene.

In an attempt to study translational control of genes that provide an adaptive advantage to the stress posed by the extracellular environment, comparative ribosome profiling of extracellular and intracellular tachyzoites was performed. This study identified more than a thousand genes that vary at the level of ribosome occupancy in intracellular and extracellular parasites, implying there is a widespread usage of translational regulation to cope with the stress imposed by the extracellular environment on *T. gondii*. Additionally, this study confirmed changes in gene expression between the two sets of parasites occurring at the translation level, involving regulation by uORFs and mRNA secondary structure (Hassan *et al.*, 2017). The widespread occurrence of uORFs warrants further examination of the role played by uORFs in mediating stress response.

*Translation regulation of the arginine transporter TgApiAT1*

*T. gondii* depends on nutrients derived from its host (Coppens, 2014; Zuzarte-Luís and Mota, 2018) and a nutrient for which the parasite is auxotrophic is the amino acid arginine (Fox *et al.*, 2004). Hence, there is a dedicated plasma membrane transporter (TgApiAT1) for the uptake of arginine (Rajendran *et al.*, 2017). Depletion of this amino acid results in the formation of bradyzoites, the latent forms of this parasite (Fox *et al.*, 2004; Butcher *et al.*, 2011). In order to maintain the virulent tachyzoite stages and cause infection, parasites need to sense the availability of arginine and respond accordingly to maintain the intracellular levels of arginine by regulating the expression of the transporter TgApiAT1.

The arginine-dependent expression of TgApiAT1 is mediated via an upstream ORF present in the 5′ leader sequence of the transcript (Rajendran *et al.*, 2019) (Figure 2). The uORF codes for a conserved peptide that is hypothesized to function in a similar manner to the arginine attenuator peptide found in *S. cerevisiae* (ScAAP) (Rajendran *et al.*, 2019). The
ScAAP stalls the ribosome and prevents it from reaching the downstream CDS in arginine-rich conditions. Conversely, in the conditions of arginine scarcity, ribosomes are able to reach and translate the downstream CDS (Wu et al., 2012; Wei et al., 2012). A similar switch is used by *T. gondii* for modulating the TgApiAT1-dependent uptake of arginine in varying arginine conditions (Rajendran et al., 2019). Given the extensive occurrence of uORFs in *T. gondii*, we believe that this might be among the first of many studies that unravel the existence of uORF-mediated translational regulation.

*Upstream ORFs play a crucial role in development of latent cysts in *T. gondii***

Inside the intermediate mammalian host, *T. gondii* parasites divide asexually to form tachyzoites, which develop into tissue cyst bradyzoites under certain conditions (reviewed in Cerutti et al., 2020). Bradyzoites are the latent stages of *T. gondii* that persist and cause reinfection when the immune system of the host lapses (Dubey, 1998; Montoya and Liesenfeld, 2004). While the host immune response can lead to stress that initiates bradyzoite formation *in vivo* (Bohne et al., 1993; Lüder et al., 1999), conversion of tachyzoites to bradyzoites *in vitro* can be induced under various stress conditions, such as pH change, heat shock, nutritional stress, stress to the endoplasmic reticulum (ER), mitochondrial inhibition, presence of nitric oxide, signalling through secondary messengers such as cAMP, and other *in vivo* factors (Bohne et al., 1993; Soete et al., 1993; Weiss et al., 1995, 1998; Dubey, 1998; Kirkman et al., 2001; Fox et al., 2004; Narasimhan et al., 2008). Stage conversion that can be triggered by a multitude of external stressors is highly reminiscent of an integrated stress response (ISR) that is controlled by uORFs in other eukaryotes (Reviewed in Young and Wek, 2016).

Another indicator of translational regulation, possibly through uORFs, is phosphorylation of eIF2α which has also been reported for bradyzoite conversion. TgIF2α is phosphorylated during alkaline stress when the developmental shift from tachyzoite to bradyzoite occurs
Disruption of this phosphorylation by either deleting TgIF2KB (Augusto et al., 2020) or inhibiting TgIF2KA (Augusto et al., 2018), both kinases responsible for phosphorylating TgIF2α, leads to significant loss of stage conversion.

The molecular factor responsible for the stage conversion was unidentified until the recent discovery of a master regulator, the Bradyzoite Formation Deficient 1 (BFD1) protein that encodes a transcription factor, which triggers the conversion of tachyzoites to the latent tissue cyst form (Waldman et al., 2020). Stress-dependent expression of BFD1 appears to be regulated at the translational level because although the transcript is detected both in tachyzoites and in bradyzoites (a marginal 1.5- to 3.6-fold upregulation in bradyzoites) the protein is expressed only in bradyzoites (Waldman et al., 2020) (Figure 2).

As bradyzoites can be formed in culture by a variety of stressors and their stage conversion coincides with the phosphorylation of TgIF2α, it would not be far-fetched to infer that uORFs play a role in the process. Most satisfyingly, evidence for the involvement of uORFs in translational regulation was provided by the observation that parasites expressing BFD1 without its 5’ leader can differentiate into bradyzoites even in the absence of any stress. This strongly alludes to the presence of regulatory cis-acting elements in the 5’ leader that act as a switch to turn on gene expression under stress conditions. The translational switch of the gene has been hypothesized to be under the control of four uORFs present in its 2.7 kb-long 5’ leader sequence (Waldman et al., 2020).

Understanding the control of BFD1 gene expression will have crucial implications from a clinical perspective, as it would allow development of drugs that inhibit the conversion of tachyzoites to persistent bradyzoites. Even though the number of parasites that transition from tachyzoites to bradyzoites can be suppressed by the small molecules tanshinone IIA and hydroxyzine (Murata et al., 2017), there are no known drugs that eradicate them. It would be possible to develop such drugs if light could be shed on the molecular mechanism that controls
the switch to bradyzoite formation.

**CONCLUDING REMARKS**

Given the sheer number of uORFs and wide prevalence of ribosomal footprints on the 5’ leader sequences in the Apicomplexan parasites, *P. falciparum* and *T. gondii*, their role in mediating translational regulation is certainly under-recognized. Efforts to understand translational regulation in these parasites is gradually gaining momentum (reviewed in Rao *et al.*, 2017) and in this review we highlight selected examples of genes that are regulated by uORFs giving rise to clinically relevant patho-physiology in the life cycles of these parasites. Due to the requirement of novel translation factors that promote non-canonical strategies of handling the “hurdles” created by uORFs, such as reinitiation and leaky scanning, further research in this area may lead to the identification of parasite-specific, essential proteins that might serve as drug targets for therapeutics. We conclude by predicting that, with transcriptome, proteome, ribosome profiling and bioinformatics analyses giving genome-wide pointers towards genes and pathways that might be subjected to uORF-mediated PTGR, the role of uORFs in regulating translation will surely be an area of intense research in the future.

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**CONFLICTS OF INTEREST**

The authors declare there are no conflicts of interest.

**ETHICAL STANDARDS**
Not applicable
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Figures

Figure 1: Evidence of uORF-mediated gene regulation

Evidence of uORF-mediated regulation of gene expression

Indirect evidence
- Lack of correlation between transcription and translation

Direct evidence
- Phosphorylated eIF2α
- Presence of reinitiation factor/s
- Presence of ribosome footprints on the 5' leader of the transcript
- Mutational analysis of start codon of uORF

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Figure 2: Examples of genes in *P. falciparum* and *T. gondii* that are regulated via uORFs

|                      | MAF1 | VAR2CSA | Evidence of uORF-mediated gene regulation | BFD1 | APIAT1 |
|----------------------|------|---------|------------------------------------------|------|--------|
| **Plasmodium falciparum** | ![image](image1.png) | ![image](image2.png) | ![image](image3.png) | ![image](image4.png) | ![image](image5.png) |
| VAR2CSA              | ![image](image6.png) | ![image](image7.png) | ![image](image8.png) | ![image](image9.png) | ![image](image10.png) |
| Placenta             | ![image](image11.png) | ![image](image12.png) | ![image](image13.png) | ![image](image14.png) | ![image](image15.png) |
| MAF1                 | ![image](image16.png) | ![image](image17.png) | ![image](image18.png) | ![image](image19.png) | ![image](image20.png) |
| Sensing amino acid deprivation | ![image](image21.png) | ![image](image22.png) | ![image](image23.png) | ![image](image24.png) | ![image](image25.png) |
| Aminoacyl-tRNA synthetase | ![image](image26.png) | ![image](image27.png) | ![image](image28.png) | ![image](image29.png) | ![image](image30.png) |
| **Toxoplasma gondii** | ![image](image31.png) | ![image](image32.png) | ![image](image33.png) | ![image](image34.png) | ![image](image35.png) |
| BFD1                 | ![image](image36.png) | ![image](image37.png) | ![image](image38.png) | ![image](image39.png) | ![image](image40.png) |
| Bradyzoite           | ![image](image41.png) | ![image](image42.png) | ![image](image43.png) | ![image](image44.png) | ![image](image45.png) |
| Tachyzoite           | ![image](image46.png) | ![image](image47.png) | ![image](image48.png) | ![image](image49.png) | ![image](image50.png) |
| APIAT1               | ![image](image51.png) | ![image](image52.png) | ![image](image53.png) | ![image](image54.png) | ![image](image55.png) |
| Arginine             | ![image](image56.png) | ![image](image57.png) | ![image](image58.png) | ![image](image59.png) | ![image](image60.png) |

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