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Micro**Commentary**

Metabolite-dependent regulation of gene expression in *Trypanosoma brucei*

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Summary

Mechanisms regulating gene expression in trypanosomatid protozoa differ significantly from those in other eukaryotes. Transcription of the genome appears to be more or less constitutive with the polyadenylation and trans-splicing of large polycistronic RNAs producing monocistronic RNAs whose translation may then depend upon information within their 3' untranslated regions (3'UTRs). Various 3'UTR sequences involved in life-cycle stage-dependent differential gene expression have been described. Moreover, several RNA-binding proteins have been implicated in regulating expression of these transcripts through altering either their stability or their ability to interact with ribosomes. In this issue of *Molecular Microbiology* Xiao *et al.* report on a regulatory element within the 3'UTR of the transcript that encodes the polyamine pathway regulatory protein called prozyme. It appears that the RNA element controls translation of the prozyme RNA causing expression to be upregulated when levels of decarboxylated *S*-adenosylmethionine (dcAdoMet) are depleted. Since prozyme activates the enzyme *S*-adenosylmethionine decarboxylase (AdoMetDC), which is responsible for the production of dcAdoMet, losing this metabolite leads to upregulation of prozyme, activation of AdoMetDC and restoration of optimal levels of dcAdomet. The system thus represents a novel metabolite-sensing regulatory circuit that maintains polyamine homeostasis in these cells.

The Nobel Prize winning work of Francois Jacob and Jacques Monod on regulation of bacterial gene expression in response to the environment, broadened thinking about how cellular metabolism can be regulated as a function of nutrient availability (Jacob and Monod, 1961). In the case of the *Escherichia coli lac* operon, regulation of a set of genes controls lactose utilization. When lactose is absent a repressor binds at a site within the bacterial chromosome known as the *lac* operator that is proximal to the promoter sequence that drives expression of the *lac* operon. When present, lactose stimulates production of allolactose which binds to the repressor protein causing it to disassociate from the operator site thus allowing transcription. Conversely, the *E. coli trp* operon contains a series of genes involved in the production of tryptophan. In this case a repressor protein binds to the *trp* operator when tryptophan is present thus switching off unnecessary synthesis of that amino acid. We now know that many metabolic systems are controlled by a variety of switch-based mechanisms, which can occur at different points in the flow of information from gene, through RNA to proteins, their modification and function.

Parasitic protozoa of the phylogenetic order Kinetoplastida are responsible for a range of important neglected tropical diseases including human African trypanosomiasis, Chagas disease and the leishmaniases (Stuart *et al.*, 2008). These organisms have gene regulatory processes that differ from those of classical eukaryotes (Clayton, 2002; Martinez-Calvillo *et al.*, 2010). Promoter sequences for RNA polymerase II are largely absent. Instead it appears that transcription of the entire genome occurs in a constitutive fashion, with large polycistronic transcripts processed by the combined action of polyadenylation at the 3' end of individual genes and addition of a spliced leader sequence at the 5' end.

In spite of the lack of transcriptional control it is clear that many genes in these organisms are regulated (Jensen *et al.*, 2009; Kabani *et al.*, 2009; Veitch *et al.*, 2010; Archer *et al.*, 2011). For example different genes are switched on as parasites pass through their life cycle stages, which include both mammalian and insect hosts that offer radically different environments with respect to available nutri-

ents. It is generally believed that the control of expression of these genes is through the ability to translate RNA molecules into protein and that regulatory elements within the 3' untranslated region (3'UTR) of these transcripts are fundamental to this process. The 3'UTRs can determine stability of the message or otherwise affect the ability of translation, for example through interactions with ribosomes.

Scores of RNA-binding proteins have been predicted to exist in trypanosomes, covering the major classes of eukaryotic RNA-binding proteins (RRM, CCCH and puf families) (Hendriks and Matthews, 2005; De Gaudenzi *et al.*, 2011). Several have been functionally characterized, including RBP10 that has a key role in controlling bloodstream for specific gene expression in *Trypanosoma brucei* (Wurst *et al.*, 2009).

The differentiation pathways of trypanosomes have provided a relatively straightforward context in which to study the processes that govern the regulation of gene expression. Less work has focused on specific adaptations to metabolic signals. It is clear that a number of metabolic pathways are regulated in response to the differentiation pathway and several examples have been shown where changes in availability of metabolic substrates appear to underpin metabolic switches. For example, when procyclic trypanosomes are grown in the presence of proline alone they consume significant quantities of this amino acid to provide cellular carbon and energy (Lamour *et al.*, 2005). When glucose is available, however, they consume less proline and alter the flow of its metabolic products through the system, while using glucose as the principal energy and carbon source (Besteiro *et al.*, 2005; Lamour *et al.*, 2005; Coustou *et al.*, 2008; Ebikeme *et al.*, 2010). In Leishmania, purine transporters are regulated as a function of available substrates (Carter *et al.*, 2010) and the P2 aminopurine transporter in *T. brucei* has been shown to decrease in abundance as cells move from blood to *in vitro* culture medium (Ward *et al.*, 2011). Mechanistically, however, the processes that govern these switches are not known.

The polyamine pathway in trypanosomes has been the focus of a significant research effort (Birkholtz *et al.*, 2011; Willert and Phillips, 2012). This is partly because the only enzyme that is known to be targeted by a drug used against human African trypanosomiasis today, eflornithine, is ornithine decarboxylase, a key enzyme in this pathway (Vincent *et al.*, 2012). The pathway converts ornithine to spermidine via putrescine. Spermidine plays multiple roles within trypanosomes including its conjugation to two molecules of glutathione to generate the trypanosomatid-specific metabolite N^1,N^8 bis-glutathionylspermidine [or simply trypanothione (Fairlamb *et al.*, 1985)]. The aminopropyl group essential to convert putrescine to spermidine

is provided by decarboxylated *S*-adenosylmethionine (dcAdoMet), itself a product of *S*-adenosylmethionine (AdoMet). AdoMet is also a key methyl donor to an array of reactions within the cell. It seems that the sole role of dcAdoMet is in the synthesis of polyamines, since depletion of the AdoMet decarboxylase (AdoMetDC) enzyme either genetically using RNAi, gene knockout or pharmacologically using specific inhibitors such as MDL73811, is lethal to trypanosomes, unless they are provided with excess spermidine in the medium (Willert and Phillips, 2008).

In mammalian cells, several key enzymes of the polyamine pathway are subject to tight regulation (Persson, 2009). Ornithine decarboxylase (ODC), for example, is regulated by transcription, by translation and also by the stability of the protein itself, through binding of a regulatory protein known as antizyme that marks ODC for degradation (Perez-Leal and Merali, 2012). Antizyme levels rise when polyamine levels rise, thus reducing ODC levels causing synthesis of new polyamines to diminish. Further control is provided by a catalytically dead paralogue of ODC, an antizyme inhibitor that binds and neutralizes antizyme (Mangold, 2006). AdoMetDC is also regulated at the transcriptional and translational level. Trypanosomes do not regulate ODC or AdoMetDC using the mechanisms outlined for mammals. This fact may contribute to the selective effect of eflornithine which is an equally good inhibitor of the trypanosomal and mammalian ODC enzymes (Phillips *et al.*, 1988). In trypanosomes, however, the irreversible inhibition of ODC is long lasting as the enzyme is not turned over quickly. In mammals, where the protein is turned over quickly and fresh enzyme constantly produced, however, ODC activity is replenished as drug is removed from the system.

A series of elegant papers from the Phillips group have shown that regulation of the polyamine biosynthetic pathway in trypanosomes occurs using novel mechanisms. Crucially, a paralogue of the AdoMetDC gene that resides next to the actual gene in the genome encodes a protein that lacks key residues within the active site that are required for activity (Willert *et al.*, 2007). However, mixing this catalytically dead version with the true enzyme stimulated a huge (10^2- to 10^3-fold) increase in catalytic activity which pointed to the second protein acting as a positive regulator of the enzyme through heterodimerization (Willert *et al.*, 2007). Recently it has been shown that another enzyme in the polyamine pathway, deoxyhypusine synthase, which is responsible for the spermidine-dependent hypusine modification to elongation factor eIF5A is similarly regulated by dimerization with a catalytically dead orthologue of the enzyme (Nguyen *et al.*, 2013). Using either pharmacological or genetic methods to reduce the abundance of AdoMetDC in cells revealed a loss of spermidine production and a concomitant increase

in the protein abundance of prozyme (but not the RNA levels encoding this regulator) (Xiao *et al.*, 2009). Diminishing spermidine levels by reduction of ornithine decarboxylase, or spermidine synthetase, however, did not lead to the increase in prozyme. This suggested, therefore, that the increase in prozyme was not due to decreases in spermidine itself but something more directly related to AdoMetDC, i.e. its metabolic product – dcAdoMet.

Given the lack of regulation of gene expression in trypanosomes at the level of transcription, Xiao *et al.* (2013) went on to analyse the 3'UTR of prozyme for clues as to how expression might be regulated. They identified three transcripts associated with the gene, one of which was probably an RNA processing product. The other two, which contained the prozyme open reading frame were of 4.7 kb and 2.2 kb, respectively, the difference in size attributable to different lengths of the 3'UTR. To determine whether the 3'UTR regions of these transcripts might contribute to the expression of prozyme, a series of constructs containing either the intact 3'UTR, or various deletion derivatives, were placed behind the chloramphenicol acetyl transferase (CAT) reporter gene and expressed in trypanosomes. By adding MDL73811 to medium (and thus diminishing the level of dcAdoMet through inhibition of the enzyme that produces it) it was possible to upregulate CAT activity with some of the constructs. In this way, the 1.2 kb region immediately downstream of the prozyme ORF was identified as being necessary to respond to MDL73811 and thus termed the prozyme regulatory element.

RNA sequence analysis also indicated that a region of the 3'UTR, within the 1.2 kb element, was lost during the reverse transcriptase processing step of RNA for sequence analysis and hairpin forming flanking regions to the lost segment indicated a secondary structure forming region of the RNA. This secondary structure forming RNA was hypothesized to be responsible for the cellular response to MDL73811. Since inhibition of the enzyme leads to a decrease in dcAdoMet (although levels of this metabolite in wild-type cells were below detection levels using LC-MS/MS) it appears that a reduction in cellular dcAdoMet levels leads to an increase in the translational efficiency of the prozyme RNA through the regulatory element.

To rule out a role for the AdoMetDC protein itself in regulating its own expression [in a manner analogous to that of human and *Plasmodium* dihydrofolate reductases which bind to their own mRNAs preventing translation when abundant (Zhang and Rathod, 2002)] a human version of AdoMetDC was expressed in the parasites. Overexpression enabled detection of dcAdoMet, proving the enzyme to be functional, and RNAi was then used to deplete the trypanosomal AdoMetDC levels. No impact on prozyme levels were seen, ruling out a role for the protein itself in the regulation. However, using MDL73811 to deplete dcAdoMet (the inhibitor also being active against mammalian AdoMetDC), caused prozyme levels to rise. This showed that it is a response to decreasing levels of dcAdoMet that induces upregulation of prozyme. Unfortunately, exogenously added dcAdoMet was incapable of eliciting a response which may be due to its inability to enter cells due to either its chemical lability or lack of transport.

Whether dcAdoMet binds directly to the RNA regulatory element or whether it acts indirectly through binding to other regulatory effectors remains to be determined. In the case of the RBP10 RNA-binding protein, the initial hypothesis that it worked by binding directly to a cohort of mRNAs was dismissed on the grounds the protein could not be localized to the large polysomal fraction nor could it be found associated with specific RNAs (Wurst *et al.*, 2012). Instead the protein affects expression of a range of genes through yet to be determined effectors.

It is tempting to speculate that the dcAdoMet itself binds directly to the RNA regulatory element. In other systems, primarily bacteria, AdoMet and its demethylated product *S*-adenosylhomocysteine, have been shown to bind directly to RNA structures known as riboswitches to regulate methionine and cysteine metabolism (Batey, 2011). Riboswitches are RNA structures that change conformation upon binding to metabolite ligands, and this in turn alters the ability of these RNA molecules to bind to ribosomes for translation to protein. It has been hypothesized that AdoMet may be an ancient metabolite that played key roles in methyl transfer reactions in the 'RNA world' prior to proteins reaching ascendency in biological catalysis (Breaker, 2012).

It will be of great interest to learn more about whether and how the RNA response element of prozyme interacts with dcAdoMet and whether the system could be manipulated in the design of novel reagents to treat human African trypanosomiasis (and other diseases caused by Kinetoplastids should the regulatory system be conserved). Moreover, the system could also be adapted for use in experimental regulation of gene expression in trypanosomatids, offering a potential new tool to titrate levels of expressed proteins in these cells. Whether it would be superior to current regulatable systems, such as the tetracycline-inducible transcription vectors already available (Wirtz and Clayton, 1995), will require detailed analysis on the fine-tuning response to levels of MDL73811, or other regulators of dcAdoMet abundance.

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