Report: Vincent P. Kelly
Date: 20th Sept 2021
PhD Title: Role of queuosine tRNA modification in the parasitic protist *Trypanosoma brucei*
Candidate: Sneha Kulkarni

General comments: This is a very interesting piece of work and the student has made very considerable progress in their studies. The work is all of a high quality and has contributed to significant publications in the queuine and tRNA modification field.

Thesis: In this thesis, the role of queuosine modification in tRNA was studied in *T. brucei*. Published and unpublished data is presented clarifying i. how Q-tRNA are affected by nuclear import/export (retrograde transport, function of Mex67, amino acid excess/deficiency), ii. how Q-tRNA are taken up and affect the function of the mitochondria (protein translation, codon bias, oxidative phosphorylation) and iii. the effect on proliferation *in vitro* and *in vivo* after modulation of protein levels (TbTGT1/TbTGT2/DUF2419 knockdown and TbTGT2 knockout) and reduction of the queuine metabolite in axenic animals.

The candidate has displayed a broad range of experimental capability in this thesis and is certainly a gifted experimentalist. The writeup is clear throughout and presented in a logical and informative manner.

The thesis is extremely good and fulfils the requirements for successful defence.

General questions: The questions approximately follow the flow of the PhD thesis but in related topics. It is expected this would facilitate a "walk through" of the thesis on the day of the *viva*.

i. *T. brucei* belongs to the order kinetoplastida. Their defining feature is the presence of mitochondrial DNA, in the form of an extensive, interlocked network of mini and maxi circles. Please could you describe in more detail the structure and genetic organisation of the kinetoplast and its DNA.
ii. The mitochondrion undergoes major transformation during the life cycle of *Trypanosoma*. Could you elaborate on current thinking of how this is controlled.
iii. Its mentioned that the mitochondria of kinetoplastids undergo a unique RNA editing mechanism that involves extensive U insertions or deletions, resulting in U-rich mRNAs. Could you explain more about this mechanism?
iv. In addition to proteins of the ETC and ribosomal RNA, the mitochondrial genome of T. brucei contains hundreds of guide RNAs required for RNA editing. Could you elaborate on the function and mechanism-of-action of these guide RNA?
v. Although mitochondria isolated from queuine depleted cells showed a 45% decrease in the activity of CIV, there was no pronounced growth defect or drop in mitochondrial oxidative phosphorylation. Do you have an explanation for this?
vi. The thesis describes how export-import from the nucleus and mitochondria are influenced by Q-modification. Do other examples exist for mRNA, small RNA species?

vii. RNA interference using the tetracycline-inducible p2T7-177 RNAi vector has been an important technique in this PhD. Could you describe in more detail how this system works?
viii. Page 76, Fig. 15: It is interesting that in RNAi studies TbTGT1 was found to be important for cell growth in the bloodstream form *T. brucei* but not TbTGT2. Indeed, even CRISPR knockout of

TbTGT2 did not result in a growth defect in cultured bloodstream cells despite Q-tRNA being absent. Ostensibly, TbTGT1 has a role beyond Q-modification of tRNA. Could you speculate on what these may be?

ix. It was observed that downregulation of TbTGT1 leads to a growth defect in the bloodstream form but not in the procyclic form of *T. brucei*. Do you believe this to be due to metabolic differences? Would you elaborate on why this is the case?

x. Related to above point, since effects of Q are not observed on the mitochondrial membrane potential or respiration do you think that this modification is only important as a stress response? i.e. not critical under normal conditions. Could you discuss?

xi. Page 78, Fig 19. The delay in growth of TbTGT2 knockout is pronounced on the first wave but the subsequent wave appears normal i.e. the timing and magnitude after the first wave. Could this indicate a delay in adaptation to the host?

xii. Changes in post-transcriptional modifications of tRNAs may be essential for the survival or virulence of the parasite. If queuine supply plays a role in this then the environments of bloodstream, procyclic, epimastigote trypanosome are the key to this control. What is know about these different environment with respect to the supply of other (micro)nutrients?

xiii. Related to the above question, do you have any ideas on how Q-modification of tRNA may be affected in the epimastigote stage of development?

xiv. In a previous study, it was shown that down-regulating the nuclear transporter TbMex67 causes longer nuclear retention of Q-tRNAs in *T. brucei*. What is known about the regulation of TbMex67 and TbMtr2 by amino acid supply?

xv. It is very curious that glutamine, glutamate, cysteine and tyrosine show changes in Q level that are inversely proportional to their concentration in the media. The effects of tyrosine could be explained by retrotransport to the nucleus. How do you think other amino acids are affecting Q-modification levels?

xvi. P147. Fig 8. Why do you think high tyrosine is causing a decrease in queuine levels in the media.

xvii. In the *T. brucei* genome, many genes are tightly clustered under a single promoter region and are transcribed as a polycistronic unit incl. tRNA. Its therefore assumed trypanosomes cannot exploit differences at the transcription level. Is this universally true? For example VSG switching. Could you give some examples where transcriptional control is used by *T. brucei*.

xviii. The observation that Q-modification affects codon usage suggests a role in protein synthesis. However, is it the modification of the tRNA that is the true control point or is it tRNA intracellular transport dynamics? Could you speculate?

xix. The concept of 'Modification Tunable Transcripts' as described by Pete Dedon and Thomas is interesting. If I was to ask you to speculate, how do you see this operating in *T. brucei*.

xx. *T. brucei* need to regulate the expression of several proteins such as nutrient transporters, surface proteases, metabolic enzymes and the differential expression of proteins across life cycle stages. What is the current explanations for how this occurs?

xxi. Fig 27, when axenic mice were infected with WT *T. brucei* they grew at the same rate in the presence and absence of Q. Could you discuss why you think this is the case.

xxii. Its shown that Q facilitates tRNA import into the mitochondria. Is there any situations where the import of tRNA unmodified by Q (i.e. G in the 34 position 34), is favoured?

Minor corrections:

i. *Statement correction:* The statement, "The mouse and human enzyme consists of queuine tRNA ribotransferase 1 (QTRT1), and its splice variant queuine tRNA ribotransferasae domain containing 1 (QTRTD1)" is not correct. QTRT1 and QTRT2 are separate genes.

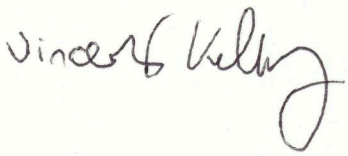
ii. *Spelling correction:* **N6-threonylcarbamoyladenosine** is the extended name for the t6A modification rather than N6-threonyladenosine, as stated.

iii. *Spelling correction:* Other advantages of *T. brucei* include easy cultivability, relatively shorter doubling time (6-8 hrs), and the availability of several forward and reverse genetics tools, along with fully sequenced genome. on of TbTGT enzymes as well as the role of Q tRNA modification in the **procuclic** stage of *T. brucei*.

iv. *Spelling correction:* Page 155: modification levels and could act as a possible **chorum**-sensing mechanism.

v. *Word omission:* Page 153. Being a eukaryote, *T. brucei* shares some basic aspects _ Q metabolism with other organisms

vi. *Clarification:* Page 86, It is speculated that other RNA molecules bearing structural similarity to the anticodon loop could be possible targets of the TbTGT enzyme. In a recent study from our group we attempted to answer this question in mammalian species; Nucleic Acids Research, Volume 49, Issue 9, 21 May 2021, Pages 4877–4890.



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Report of Sneha Kulkarni's PhD thesis:

"Role of queuosine tRNA modification in the parasitic protist Trypanosoma brucei"

The central goal of the doctoral work carried out by Ms Sneha Kulkarni was to characterize the tRNA guanine transglycosylase (TGT), the enzyme catalyzing the formation of the tRNA modification queuosine (Q), and the role of Q in the physiology of *Trypanosoma brucei*.

The outcome of gene expression, i.e. the transcription of DNA into RNA followed by the translation of RNA into proteins, was once thought to be solely dependent on the genome itself. However, today we know that variation can be introduced at many different levels. One prominent level is the addition of specific chemical modifications to DNA or RNA that can initiate a plethora of downstream signaling pathways affecting gene expression and other biological processes. Yet, while modifications of RNAs are ubiquities, especially on tRNA, for most modifications identifying the biological role has remained a great challenge.

To elucidate the role of queuosine (Q), one of the most complex tRNA modifications found in *T. brucei*, Ms Kulkarni combined a wide range of molecular biology and biochemical approaches. Her work led her to make several key findings including the observation that insect stage *T. brucei* cannot synthesize queuosine itself and relies on efficient salvage and the fact that the responsible enzyme TGT is localized to the nucleus, complicating the synthesis of functional Q-containing tRNA. In addition, her work demonstrated the importance of Q-modified tRNA for proper mitochondrial function. These findings were recently published in Nucleic Acids Research.

Subsequently, Ms Kulkarni studied the role of Q in the physiology of bloodstream form *T. brucei* cells leading her to observe interesting effects on parasite growth following deletion of different TGT enzymes.

In addition, Ms Kulkarni contributed to three additional projects (two published and one in revision) aiming to elucidate the interdependence of tRNA trafficking and modification. These studies describe the interdependence of Q and tRNA subcellular

trafficking and how these two factors regulate codon biased translation. The authors found that the effect of Q on translation has serious implications in the virulence of the parasite inside the mammalian host.

The submitted thesis, written in English, demonstrates a very profound understanding of tRNA research, epitranscriptomics and *T. brucei* biology. The introduction (chapter 1) contains a comprehensive and very well-researched overview focusing on tRNAs, queuosine and *T. brucei*.

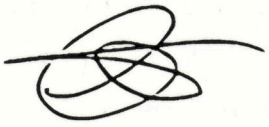
Chapter 2 is subdivided into three parts. It consists of published manuscripts and unpublished results and summarizes the key findings of Ms Kulkarni's doctoral work.

A well-written Concluding Remarks chapter (chapter 3) illustrates Ms. Kulkarni's ability to critically question her findings and to propose strategies to test her hypotheses.

In summary, the submitted thesis demonstrates that Ms. Kulkarni is able to address even complex biological questions and that she has acquired substantial knowledge of trypanosome biology, especially with regards to tRNA metabolism and gene expression. In my opinion, her achievements fully satisfy the requirement of a doctoral thesis.

I have the following questions for Ms Kulkarni to be answered/discussed at her defense:

- Could you speculate on the purpose of transporting tRNA out of the nucleus for splicing, back into the nucleus for modification and back out for translation. This seems to be unnecessarily complicated.
- The thesis states that with an excess of queuine in the culture, you observed almost 100% Q levels. Do you mean all guanosines in tRNAs or all guanosines in position 34 of tRNAs that contain a 5'-GUN-3' ? How was this determined and wouldn't this speak against any specific role of Q? or was there a strong growth defect?
- Does it really make sense to use Leishmania as a proxy to study the role of Q in bloodstream form *T. brucei*? The thesis lists several examples where *T. brucei* and Leishmania differ. For example, on page 20: In Leishmania tRNA is processed before important into Mitochondiria. 2su had opposing effect on import.



Tim Nicolai Siegel